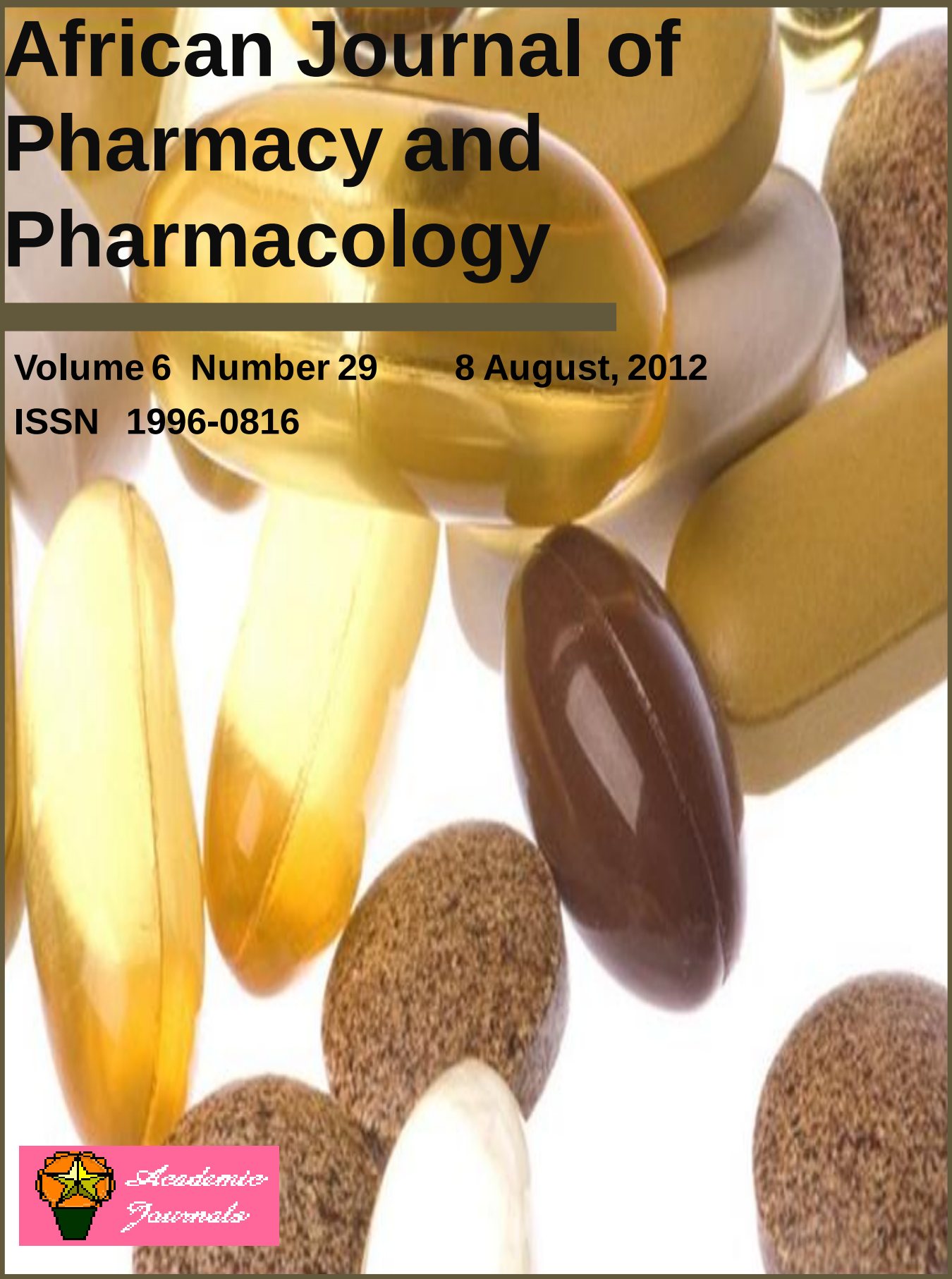
African Journal of Pharmacy and Pharmacology

Volume 6 Number 29 8 August, 2012
ISSN 1996-0816



Academic Journals

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: ajpp@academicjournals.org, ajpp.academicjournals@gmail.com. A manuscript number will be mailed to the corresponding author shortly after submission.

For all other correspondence that cannot be sent by e-mail, please contact the editorial office (at ajpp@academicjournals.org, ajpp.academicjournals@gmail.com).

The African Journal of Pharmacy and Pharmacology will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Sharmilah Pamela Seetulsingh- Goorah
Associate Professor,
Department of Health Sciences
Faculty of Science,
University of Mauritius,
Reduit,
Mauritius

Himanshu Gupta
University of Colorado- Anschutz Medical Campus,
Department of Pharmaceutical Sciences, School of Pharmacy Aurora, CO 80045,
USA

Dr. Shreesh Kumar Ojha
Molecular Cardiovascular Research Program
College of Medicine
Arizona Health Sciences Center
University of Arizona
Tucson 85719, Arizona,
USA

Dr.Victor Valenti Engracia
University of São Paulo.
Av Dr. Arnaldo, 455
Cerqueira Cesar
01246-903 - Sao Paulo, SP - Brazil

Prof. Sutiak Vaclav
Rovníková 7, 040 20 Košice,
The Slovak Republic,
The Central Europe,
European Union
Slovak Republic
Slovakia

Dr.B.RAVISHANKAR
Director and Professor of Experimental Medicine
SDM Centre for Ayurveda and Allied Sciences,
SDM College of Ayurveda Campus,
Kuthpady, Udupi- 574118
Karnataka (INDIA)

Dr. Manal Moustafa Zaki
Department of Veterinary Hygiene and Management
Faculty of Veterinary Medicine, Cairo University
Giza, 11221 Egypt

Prof. George G. Nomikos
Scientific Medical Director
Clinical Science
Neuroscience
TAKEDA GLOBAL RESEARCH & DEVELOPMENT CENTER, INC. 675 North Field Drive Lake Forest, IL 60045
USA

Prof. Mahmoud Mohamed El-Mas
Department of Pharmacology,
Faculty of Pharmacy
University of Alexandria,
Alexandria,
Egypt.

Dr. Caroline Wagner
Universidade Federal do Pampa
Avenida Pedro Anunciação, s/n
Vila Batista, Caçapava do Sul, RS - Brazil

Editorial Board

Prof. Fen Jicai
School of life science, Xinjiang University,
China.

Dr. Ana Laura Nicoletti Carvalho
Av. Dr. Arnaldo, 455, São Paulo, SP.
Brazil.

Dr. Ming-hui Zhao
Professor of Medicine
Director of Renal Division, Department of Medicine
Peking University First Hospital
Beijing 100034
PR. China.

Prof. Ji Junjun
Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences,
China.

Prof. Yan Zhang
Faculty of Engineering and Applied Science,
Memorial University of Newfoundland,
Canada.

Dr. Naoufel Madani
Medical Intensive Care Unit
University hospital Ibn Sina, Univesity Mohamed V Souissi, Rabat,
Morocco.

Dr. Dong Hui
Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University,
China.

Prof. Ma Hui
School of Medicine, Lanzhou University,
China.

Prof. Gu HuiJun
School of Medicine, Taizhou university,
China.

Dr. Chan Kim Wei
Research Officer
Laboratory of Molecular Biomedicine,
Institute of Bioscience, Universiti Putra,
Malaysia.

Dr. Fen Cun
Professor, Department of Pharmacology, Xinjiang University,
China.

Dr. Sirajunnisa Razack
Department of Chemical Engineering, Annamalai University,
Annamalai Nagar, Tamilnadu,
India.

Prof. Ehab S. EL Desoky
Professor of pharmacology, Faculty of Medicine
Assiut University, Assiut,
Egypt.

Dr. Yakisich, J. Sebastian
Assistant Professor, Department of Clinical Neuroscience
R54
Karolinska University Hospital, Huddinge
141 86 Stockholm ,
Sweden.

Prof. Dr. Andrei N. Tchernitchin
Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA
University of Chile Medical School,
Chile.

Dr. Sirajunnisa Razack
Department of Chemical Engineering,
Annamalai University, Annamalai Nagar, Tamilnadu,
India.

Dr. Yasar Tatar
Marmara Unıversıty,
Turkey.

Dr Nafisa Hassan Ali
Assistant Professor, Dow institude of medical technology
Dow University of Health Sciences,Chand bbi Road, Karachi,
Pakistan.

Dr. Krishnan Namboori P. K.
Computational Chemistry Group, Computational Engineering and Networking,
Amrita Vishwa Vidyapeetham, Amritanagar, Coimbatore-641 112
India.

Prof. Osman Ghani
University of Sargodha,
Pakistan.

Dr. Liu Xiaoji
School of Medicine, Shihezi University,
China.

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJPP to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b; Tristan, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $600 handling fee. Publication of an article in the African Journal of Pharmacy and Pharmacology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Copyright: © 2012, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJPP, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication.
While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 6 Number 29 8 August, 2012

ARTICLES

Research Articles

Table of Contents: Volume 6 Number 29 8 August, 2012

ARTICLES

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2176-2179, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP10.275
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Effect of extract of *Urtica dioica* on insulin and C-peptide secretion from rats (RIN5F) pancreatic beta cells

Akbar Aliasgarzadeh[1], Majid Mobasseri[1]*, Amir Bahrami[1], Nosratollah Zargami[2] and Ali Tabrizi[3]

[1]Department of Endocrinology, Bone Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[2]Department of Clinical Biochemistry, Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
[3]Students' Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.

Accepted 2 April, 2012

Plants are being used in the treatment of diabetes in traditional system of medicine. *Urtica dioica* (UD) has a variety of uses in traditional medicine. There are few reports about hypoglycemic mechanisms of *U. dioica*. The present study was designed to determine the possible mechanisms of hypoglycemic effects of UD on RIN5F rat pancreatic beta cells *in vitro* models. Beta cells were prepared in multiple flasks containing culture medium. Alcoholic extract of UD at concentrations of 50, 100 and 200 µg/ml was added to flasks. Insulin and C-peptide level were measured at 0, 60, 120 and 80 min. Insulin level in pancreatic cells media before and after addition of UD extract at different concentrations and in different times did not changed significantly (p > 0.2). Also, C-peptide (µg/ml) levels in these media with dose of 50, 100 and 200 µg/ml UD, did not change significantly. The results of the present study demonstrated that alcoholic extract of UD was unable to increase insulin and C-peptide secretion from RIN5F pancreatic beta cells. Hence, the hypoglycemic effects of UD are not based on enhancement of insulin secretion and needs more study.

Key words: *Urtica dioica*, insulin secretion, hypoglycemic activity.

INTRODUCTION

Diabetes is a chronic disorder in metabolism of carbohydrates, proteins, and fat, due to absolute or relative deficiency of insulin secretion or varying degree of insulin resistance (American Diabetes Association, 2011; Farmer and Fox, 2011). The number of adults with diabetes in the world is anticipated to rise from 285 million in 2010 to 439 million in the year 2030 (Shaw et al., 2010).

Patients with diabetes experience significant morbidity and mortality from microvascular (retinopathy, neuropathy and nephropathy) and macrovascular complications (heart attack, stroke and peripheral vascular disease). Microvascular disease leads to retinopathy, neuropathy and nephropathy (nephropathy leads to uremia) (Halder et al., 2003; Merlin et al., 2005). Macrovascular disease leads to cardiovascular disease, mainly by accelerating atherosclerosis. These disorders include: coronary artery disease, leading to myocardial infarction (heart attack) or angina, Stroke (mainly ischemic type), peripheral vascular disease, which contributes to intermittent claudication (exertion-related foot pain) as well as diabetic foot (The Advance Collaborative Group, 2008).

The use of herbal remedies has been on the rise worldwide (Naito et al., 2005; Azaizeh et al., 2003; Guarrera, 1999). Plants are being used in the treatment of diabetes in traditional system of medicine (Luke, 2000). *Urtica dioica* (stinging nettle) and *Urtica urens* (dwarf nettle) are members of the Urticaceae family native to Eurasia, and are considered therapeutically

*Corresponding author. E-mail: mobasserimajid@yahoo.com. Tel: +98 411 3357850 / +98 9143135856.

interchangeable (Blumenthal et al., 1998). We hypothesized that the extracts *U. dioica* can increased insulin and C-peptide secretion from (RIN5F) pancreatic B cell.

METHODS AND MATERIALS

Preparation of extract

Dried UD was purchased from the market and identified by a pharmacognosist. Using an electric mill, plants were crushed into a fine powder. Obtained powder was extracted repeatedly by 70% methanol as solvent (for 5 days) by soak method (maceration). In second stage, this hydro-alcoholic extract were dried completely by rotary evaporator in temperature of 45°C and pressure below 100 mmHg. The dried extract was stored in a refrigerator at temperatures below zero degrees of centigrade for later stages.

Cell culture

RIN-5F (code NCBI: C526) cells type obtained from National Cell bank of Iran (NCBI) affiliated to Pasteur Institute of Iran. Cells were prepared in DMEM [Dulbecco/Vogt modified Eagle's (Harry Eagle) minimal essential medium] and RPMI (Roswell Park Memorial Institute) 1640 medium and were cultured in 5% CO_2 and 10% fetal calf albumin with penicillin G 80 mg and 50 mg streptomycin in sterile conditions. After reaching the required number of specific cells and confluence state, insulin and C-peptide were measured as base line values.

Cell viability

Trypan Blue color was used to assess the live cells proportion in the flask. This color only painted the living cells but could not enter dead cell. In this way, after painting, with NEUBAUER slides and a light microscope determination of percentage of viable and dead cells were become possible. We found that more than 85% of the cells were alive, which technically and according to references were acceptable (Jones and Senft, 1985).

Study protocol

Pancreatic beta cell in Cell Culture Laboratory of Drug Applied Research Center was prepared in DMEMI medium in six flasks. Cells were prepared in the same size case and control flasks. The suspensions of 50, 100 and 200 micrograms of the UD extract in 1ml normal Saline were added to the case flasks. In control flask, beta cells were primed only with 1 ml of normal saline. Insulin and C-peptide levels were measured in before and 60, 120 and 180 min after priming in all case and control samples. Concentration of insulin is determined by Chemiluminescence (CLIA) method according to DiaSorin (LIASONInsulin, 310360, with measuring range of 0.2 to 500 µIU/ml, with less than 10% coefficient of variation). C-Peptide measurement was performed based on Imminoenzymometric method and according to Monobind kite protocol (AccuBind ELISA Microwells, 2725-300, measuring range of 0.01 to 30 ng/ml, with less than 10% coefficient of variation).

Statistical analysis

Statistical analysis was done by means of the statistical package SPSS 16. Values are presented as mean and standard deviation, and 95% confidence interval. Comparison between groups in different time was performed by Repeat Measure Model. A 0.05 level of significant was set.

RESULTS

Table 1 shows concentrations of insulin in medium containing pancreatic beta cells in the control, and case groups before and after adding of UD extract in doses 50, 100, 200 µg/ml and at times before 60, 120 and 180 min. There were no significant changes in insulin levels in all groups with and without UD in different times ($P > 0.05$).

In Table 2, C-peptide concentration in pancreatic beta cells containing media before and after treating of alcoholic extract of UD with concentrations of 50, 100 and 200 µg/ml at times before 60, 120 and 180 min were shown. Between C-peptide concentration at different time and different UD extract concentration in each group, no statistically significant difference was found ($P > 0.05$).

DISCUSSION

Despite all the marvelous advancements in modem medicine, traditional herbal medicine has always been practiced. Alternative therapies have been used by people in our region (as in other regions) who have the faith in spiritual healers. More than 800 plants are reported to have antidiabetic properties (Eddouks and Maghrani, 2004), for example today up to 600 traditional plant medicine has been reported in India for diabetes (Jarald et al., 2008). Ethnopharmacological surveys show that more than 1200 plants are used in traditional medicine for their alleged hypoglycemic activity (Kesari et al., 2007). Like all green vegetables, UD leaf densely contains several micronutrients (Wagner et al., 1994). Despite abundance of reports about antidiabetic properties of UD, there is little scientific explanation of these effects. The present study was designed to determine the possible mechanisms of hypoglycemic effects of UD on RIN5F rat pancreatic beta cells.

Insulin discovery in 1921 was the major breakthrough in the treatment of diabetes mellitus (Swanston-Flatt et al., 1991a). Insulin is known as an anabolic hormone that plays an important role in maintenance of body growth and regulation of overall body metabolism (Clark and Wallis, 2003). Before the introduction of insulin, the treatment of diabetes mellitus mainly relied on dietary measures, which included the use of traditional herbal therapies. Many traditional plants have been introduced for treatments of diabetes (Swanston-Flatt et al., 1991a; Gray and Flatt, 1997a). There is data that UD can reduce blood glucose levels, and studies have different results with together (Fawzi and Kamal, 2009). Oral administration of hydroalcoholic extract UD in doses of 100 mg/kg showed a strong glucose lowering effects on streptozocin (STZ) induced diabetes in rats. It has the protective effect on pancreatic cells in animal models

Table 1. Concentration of insulin (µIU/ml) in medium containing beta cells in the control (before) and case (after) groups after addition of UD extract with doses 50 and 100 and 200 µg/ml at different times.

Time (min)	Concentration of insulin in the control group	Concentrations of insulin in media containing 50 µg/ml UD	Concentrations of insulin in media containing 100 µg/ml UD	Concentrations of insulin in media containing 200 µg/ml UD
Before adding	0.16 ± 0.00	1 ± 0.02	0.18 ± 0.00	0.20 ± 0.00
60 min after adding	0.16 ± 0.00	0.19 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
120 min after adding	0.16 ± 0.00	0.18 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
180 min after adding	0.17 ± 0.00	0.19 ± 0.00	0.20 ± 0.00	0.20 ± 0.00

Table 2. Concentration of C-peptide (ng/ml) in medium containing beta cells in the control (before) and case (after) groups after addition of UD extract with doses 50 and 100 and 200 µg/ml at different times.

Time (min)	Concentration of C-peptide in the control group	Concentration of C-peptide in media containing 50 µg/ml UD	Concentration of C-peptide in media containing 100 µg/ml UD	Concentration of C-peptide in media containing 200 µg/ml UD	P value¶
Before adding	0.76 ± 0.03	0.31 ± 0.01	0.7 ± 0.02	0.32 ± 0.01	NS
60 min after adding	0.50 ± 0.01	0.33 ± 0.01	0.2 ± 0.02	0.33 ± 0.02	NS
120 min after adding	0.60 ± 0.01	0.86 ± 0.03	0.40 ± 0.01	0.93 ± 0.04	NS
180 min after adding	0.36 ± 0.01	0.80 ± 0.03	0.39 ± 0.01	0.77 ± 0.04	NS
P-value*	NS	NS	NS	NS	

¶ Comparison of different concentration of UD; * Comparison fixed concentration UD in different time; NS: non SIGNIFICANT.

(Kavalal et al., 2003).

In an animal study, diabetic Rats treated with a Methanol extract derived from UD showed a significant decreased in blood glucose level (Fathi Azad et al., 2005). Golalipour and Khori (2007) showed hydroalcoholic extract of UD has hypoglycemic effects in hyperglycemic Rats. According to this study, animals that received hydroalcoholic extract of UD 100 mg/kg for five days had been beneficially affected. On the other hand, in another study by Golalipour et al (2006), chronic administration of UD did not showed hypoglycemic effect or induction of regeneration of beta cells of pancreas in rats. Durdi et al. recently showed that alcoholic extract of *U. dioica* leaves can reduce glucose level and increase insulin secretion, acetyl coenzyme A carboxylase, and nucleoside diphosphate kinase activity in the alloxan diabetic animals (Durdi et al., 2011). Other researchers found more than glycemic effect for UD (Alisi et al., 2008). Bnouham et al. (2003) demonstrated that when UD administered 30 min before glucose loading, a strong glucose lowering effect appeared. However, the aqueous extract of UD (500 mg/kg) did not modify the blood glucose level. They showed that UD has a significant antihyperglycemic effect in oral glucose tolerance test (OGTT) model. They attributed this effect in part to the reduction of intestinal glucose absorption.

In vivo study by Bijan et al. (2003) about the blood glucose lowering effect of the extract of UD showed an enhancement of insulin secretion by Langerhance islets. Results of our study are challenge that study, due to inability of UD extract in enhancing insulin and C-peptide secretion from RIN5F pancreatic beta cells in our study. Based on these results, it seems that hypoglycemic effect of UD, if any, is not the anticipated mechanisms mentioned in the objectives of this study. Probably similar to Bnouham et al.'s study hypoglycemic effects of hydroalcoholic extract of UD may be exerted by reduction of intestinal glucose absorption.

ACKNOWLEDGEMENT

This study was financially supported by Drug Applied Research Centre of Tabriz University of medical sciences.

REFERENCES

Alisi CS, Emejulu AA, Alisi PNC, Nwaogu LA, Onyema OO (2008). Decreased cardiovascular risk and resistance to hyperlipemia-induced hepatic damage in rats by aqueous extract of *Urtica dioica*. Afr. J. Biochem. Res. 2(4):102-106.

American Diabetes Association (2011). Diagnosis and classification of diabetes mellitus. Diabetes Care 34(1):S62-69.

Azaizeh H, Fulder S, Khalil K, Said O (2003). Ethnobotanical knowledge of local Arab practitioners in the Middle Eastern region: The status of traditional Arabic medicine. Fitoterapia 74(1-2): 98-108.

Bijan F, Ahmadvand D, Vardasbi S, Majin FJ, Khaghani S (2003). Induction of insulin secretion by a component of *Urtica dioica* leave extract in perifused Islets of Langerhans and its *in vivo* effects in normal and streptozotocin diabetic rats. J. Ethnopharmacol. 89(1):47-53.

Blumenthal M, Busse WR, Goldberg A, Gruenwald J, Hall T, Riggins CW , Rister RS (eds) (1998). The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines. Austin: American Botanical Council and Boston. Integr. Med. Commun., p. 216.

Bnouham M, Merhfour F-Z, Ziyyat A, Mekhfi H, Aziz M, Legssyer A (2003). Antihyperglycemic activity of the aqueous extract of *Urtica dioica*. Fitoterapia 7(4):677-681.

Clark MG. Wallis MG (2003). Blood flow and muscle metabolism: A focuse on insulin action. Am. J. Physiol. Endocrinol. Metab. 284(2):241-258.

Durdi Q, Saied D, Zoleika M, Soleiman M (2011). Effect of *Urtica dioica* leaf extract on activities of nucleoside diphosphate kinase and acetyl coenzyme, a carboxylase, in normal and hyperglycemic rats. Afr. J. Pharm. Pharmacol. 5(6):792-796.

Eddouks M, Maghrani M (2004). Phlorizin-like effect of Fraxinus excelsior in normal and diabetic rats. J Ethnopharmacol. 94(1):149-154.

Farmer A, Fox R (2011). Diagnosis, classification, and treatment of diabetes. BMJ 9(1):342.

Fathi AF,Garjani AR, Maleki N, Ranj Doust S (2005). Study of the hypoglaemic activity of the hydroalcoholic extract of *Urtica dioica* in normal and diabetic rats. Pharmaceut. Sci. 2:65-69.

Fawzi I, Kamal M (2009). The effects of the methanol extract which was derived from the *Urtica pilulifera* leaves on some hematological and biochemical parameters of diabeticrats. Res. J. Biol. Sci. 4(6):657-81

Golalipour MJ, Khori V (2007). The Protective Activity of *Urtica dioica* Leaves on Blood Glucose Concentration and *ƒ*½,²-cells in Streptozotocin-Diabetic Rats, Pakistan. J. Biol. Sci. 8(10):1200-1204.

Golalipour MJ, Khori V, Ghafari S, Gharravi AM (2006). Chronic Effect of the Hydroalcholic Extract of *Urtica dioica* Leaves on Regeneration of ك-cells of Hyperglycemic Rats. Pakistan. J. Biol. Sci. 8(9):1482-1485.

Gray AM, Flatt PR (1997a). Nature's own pharmacy: the diabetes perspective, Proc. Nutr. Soc. 56:507-517.

Guarrera PM (1999). Traditional antihelminthic, antiparasitic and repellent uses of plants in central Italy. J. Ethnopharmacol. 15:183-192.

Halder N, Joshi S, Gupta SK (2003). Lens aldose reductase inhibiting potential of some indigenous plants. J Ethnopharmacol. 86:113-116.

Jarald E, Joshi SB, Jain DC (2008). Diabetes and Herbal Medicines. Iranian J. Pharmacol. Therapeut., 7(1):97-106.

Jones KH, Senft JA (1985). An Improved Method to Determine Cell Viability by Simultaneous Staining with Fluorescein Diacetate-Propidium Iodide. J. Histochem. Cytochem. 33(1):77-79.

Kavalal G, Tuncelb H, Gksel S, Hatemid HH (2003). Hypoglycemic activity of *Urtica pilulifera* in streptozotocin-diabetic rats, 84(2-3): 241-245.

Kesari AN, Kesari S, Santosh KS, Rajesh KG, Geeta W (2007). Studies on the glycemic and lipidemic effect of *Murraya koenigii* in experimental animals. J. Ethnopharmacol. 112(2):305-311.

Luke RB (2000). Selected herbals and human exercise performance. Am. J. Clin. Nutr. 72:624S-636S.

Merlin T, Con T, Richard M, George J (2005). Anaemia in Diabetes: An Emerging Complication of Microvascular Disease. Curr. Diab. Rev. 1(1):107-126.

Naito Y, Akagiri S, Uchiyama K, Kokura S, Yoshida N, Hasegawa G, Nakamura N, Ichikawa H, Toyokuni S, Ijichi T, Yoshikawa T (2005). Reduction of diabetes-induced renal oxidative stress by a cantaloupe melon extract/gliadin biopolymers, oxykine, in mice. Bio. Factors 23(2):85-95.

Shaw JE, Sicree RA, Zimmet PZ (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes. Res. Clin. Pract. 87(1):4-14.

Swanston-Flatt SK, Day C, Flatt PR, Bailey CJ (1991a). Evaluation of the antihyperglycaemic properties of traditional plant treatment for diabetes. In Frontiers in Diabetes Research: Lessons from Animal Diabetes. Ed E Shafrir. London, UK: Smith-Gordon and Company, pp. 286-293.

The ADVANCE Collaborative Group (2008). Intensive Blood Glucose Control and Vascular Outcomes in Patients with Type 2 Diabetes. N. Engl. J. Med. 358(24):2560-3572.

Wagner H, Willer F, Samtleben R, Boos G (1994). Search for the antiprostatic principle of stinging nettle (*Urtica dioica*) roots. Phytomedicine 1:213-224.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2180- 2187, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.857
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

A meta-analysis of the efficacy and safety of lacidipine in Chinese patients with mild to moderate essential hypertension

Xu Guo-liang, Du bing, Cao Hong-yan, Wu Hai-di and Qin Ling*

Department of Cardiology, 1st Bethune Hospital, Jilin University, 71 xinmin Avenue, Changchun 130021, Jilin Province, P. R. China.

Accepted 1 February, 2012

The purpose of this study was to evaluate the efficacy and safety of lacidipine tablets to treating Chinese patients with mild to moderate essential hypertension. We systematically searched the Cochrane central register of controlled trials (Issue 2, 2011), Medline (1966 to June 2011), EMbase (1966 to June 2011), CNKI (1993 to June 2011), WANFANG (1981 to June 2011), VIP (1989 to June 2011) and CBM (1991 to June 2011) through the computer and manually retrieved the relevant literature with pre-specified criteria. Then, we evaluated the quality of selected articles, extracted data and used Revman 5.1 software to do a meta-analysis. A total of 819 articles were found and 13 articles finally included were all randomized clinical trials that examined the efficacy and safety of using lacidipine tablets to treat mild to moderate essential hypertension among Chinese people. For the heterogeneity test, the efficacy analysis (Q statistic = 12.02, I^2 = 0%, Z = 3.43, *P* = 0.0006) and safety analysis (Q statistic = 15.77, p = 0.20, I^2 = 24%, Z = 3.58, P= 0.0003) showed that lacidipine was more effective and safer than other currently available antihypertensive agents. Meta-analysis showed that there was a significant difference between the total efficiency and adverse effect of lacidipine and other antihypertensive drugs. The evidence currently available shows that lacidipine tablets have a better efficacy and safety compared with other active antihypertensive agents used to treat mild to moderate essential hypertension.

Key words: Lacidipine, essential hypertension, systematic review, meta-analysis.

INTRODUCTION

Hypertension is the most important risk factor of cardiovascular, cerebrovascular and kidney diseases occurrence and death (Zhao et al., 2012). It is the most common chronic non-infectious diseases worldwide. The prevalence rate of hypertension continues to increase in China. It is estimated that the total number of patients nationwide with hypertension had reached 200 million (Liu et al., 2010), which accounted for one-fifth of the world's population with hypertension. But the awareness rate, treatment rate, and control rate of the Chinese population is only 30.2, 24.8 and 6.1%, respectively (Law et al., 2009). For decades, despite that anti-hypertension, drugs of different mechanisms all achieve positive effect however (Du et al., 2012), calcium antagonists have been the main antihypertensive agent, they act through dilating blood vessels to reduce peripheral vascular resistance. And the calcium antagonist was included to the list of first-line antihypertensive agents by the domestic and foreign numerous guidelines (Committee of guidebook on prevention and treatment of hypertension, 2011). Lacidipine is the third-generation dihydropyridine calcium antagonist, first produced by Italy GSK, and put into market in 1991. It works with voltage-dependent L-type calcium channel blocking effects to reduce the transmembrane Ca^{2+}, cause vasodilation thus leading blood pressure to decrease (Zhang, 2004). Lacidipine was used for many years in Europe and the United States because of its unique high lipophilicity, high vascular selectivity, good tolerability and longer half-life,

*Corresponding author. E-mail: qinling1958@yahoo.com.cn. Tel: +86 15843073203. Fax: +86 0431 84841049.

so it achieved a desired antihypertensive effect. Domestic lacidipine was approved in China in 1995, gradually, to become one of the agents for treatment of mild to moderate essential hypertension (Wu, 2005). Although domestic lacidipine has made a positive efficacy and there are several studies about lacidipine tablets for the treatment of hypertension, the studies lack strong conclusions because the quality of research is without systematic evaluation. For this reason, the goal of the current study was to perform a meta-analysis on clinical random control trials (RCTs) that focused on using lacidipine tablets to treat mild to moderate essential hypertension in Chinese patients, in order to obtain evidence on the efficacy and safety.

METHODOLOGY

Search strategy

The search strategy was devised according to the working handbook 5.1 from the Cochrane collaboration. We systematically searched the Cochrane central register of controlled trials (Issue 2, 2011), Medline (1966 to June 2011), EMbase (1966 to June 2011), CNKI (1993 to June 2011), Wangfang (1981 to June 2011), VIP (1989 to June 2011), and CBM (1991 to June 2011) for randomized clinical trials that examined the efficacy and safety of using lacidipine tablets to treat mild to moderate essential hypertension among Chinese people. In addition, we conducted a manual search of abstracts from selected references and we also searched by hand the bibliographies of all relevant trials. The following search criterion was used: hypertension, essential hypertension and lacidipine, and the language was limited to peer-reviewed articles written in English or Chinese.

Study selection

Two reviewers independently conducted the literature searches and extracted the relevant articles. The flow chart for article selection is shown in Figure 1. The title and abstract of potentially relevant studies were screened for appropriateness before retrieval of the full articles. The following selection criteria were used to identify published studies for inclusion in this meta-analysis: (a) the study design was a randomized clinical trial; (b) the population - was Chinese patients with mild to moderate essential hypertension (WHO-ISH Hypertension Guidelines Committee, 1999; Committee of guidebook on prevention and treatment of hypertension, 2000); (c) the intervention - was lacidipine tablets versus other active antihypertensive agents as monotherapy; (d) the outcome variables — was the overall response rate and adverse reaction rate; and (e) the efficacy criteria — was the Guiding Principles for Clinical Research of New Drugs developed by the Chinese Ministry of Health in 1993 (Liu et al., 1988). Subjects were excluded if they had severe heart, brain, lung, liver, kidney organ dysfunction and diabetes, combination therapy and those who cannot complete the follow-up.

Data extraction

From each study, the following information was extracted: author, year of publication, study design, characteristics of the population, simple size, treatment proposal, time of the therapy, overall response rate, and adverse reaction rate. The total effective rate and adverse effect incidence rate of used drugs was considered the ultimate goal of observation.

Assessment of study quality

The Jadad score was used to assess the quality of the trials methodology, and this assessment was independently performed by each of the two reviewers (Sackett et al., 2002). Articles given 1 to 2 points were regarded as low quality and the ones given 3 to 5 points were regarded as high quality through Jadad scale method (Jadad et al., 1996).

Statistical methods

For dichotomous outcomes, we calculated a pooled odds ratio (OR) and 95% confidence interval (CI). The OR was defined as the odds of an outcome in those who received lacidipine compared with the odds in those who received other active hypertensive agents. The ORs of different randomized clinical trials were combined by using the random-effects model of Der Simonian and Laird, if between-study heterogeneity existed. The Mantel and Haenszel fixed-effects were used if there was no between-study heterogeneity. Intertribal statistical heterogeneity was explored using the Cochrane Q test with the calculated I^2, indicating the percentage of the total variability in effect estimates among trials that is, due to heterogeneity rather than to chance. The I^2 values of 50% or more indicated a substantial level of heterogeneity. We evaluated the presence of publication bias by means of visual inspection of the funnel plot (whether it was symmetrical or not). To exclude the possibility that any one study was exerting excessive influence on the results, we conducted a sensitivity analysis by excluding those studies with low quality and then rerunning the analysis to assess the change in the odds ratios. All p-values were two-sided with statistical significance set at a level of 0.05. All the statistical analysis was carried out by the Cochrane collaboration's RevMan 5.1 software.

RESULTS

Characteristics of the included trials

There were 819 articles relevant to the search terms and a total of 13 articles matched inclusion criteria (Sun et al., 1995; Zhu, 1997; Zhang et al., 1999; Wu and Fu, 2001; Yi et al., 2001; Zhou et al., 2001; Xue 2001; Fang et al., 2001; Jiang et al., 2002; Duan et al., 2003; Xu et al., 2004; Li et al., 2005; Wu and Xiong, 2007). The 13 researches included 1348 Chinese patients with mild to moderate essential hypertension, 688 patients used Lacidipine tablets and 660 patients used Nitrendipine (229 patients), Captopril (108 patients), Nifedipine (85 patients), Lisinopril (51 patients), Amlodipine (85 patients), and Benidipine (102 patients) in controls. The 13 articles included in this meta-analysis were all randomized controlled trials. There were 11 high quality articles, in which 9 researches got 4 points and 2 researches got 3 points, the remaining 2 researches got 2 scores which were considered low quality through Jadad scale method. The characteristics of the included trials are shown in Table 1.

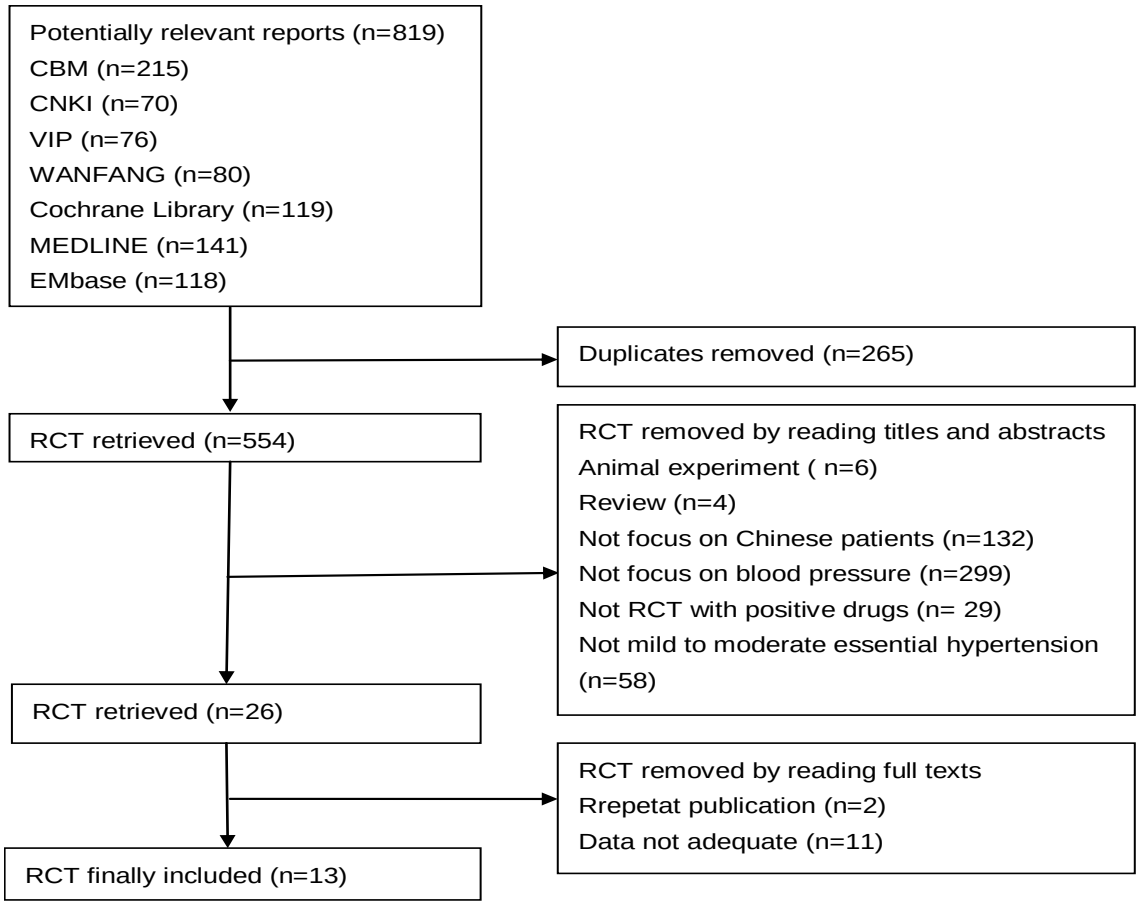


Figure 1. Flow chart of article selection.

Heterogeneity test

We chose the fixed-effect model to perform our meta-analysis, because there were no significant heterogeneities among the-studies, in both efficacy analysis (Q statistic = 12.02, p = 0.44, I^2 = 0%) and safety analysis (Q statistic = 15.77, p = 0.20, I^2 = 24%).

Meta-analysis of efficacy

The overall response rates were 89.2% for lacidipine and 82.7% for the control group. From the meta-analysis, there were significant differences in efficacy between lacidipine group and control group in treating Chinese patients with mild to moderate essential hypertension (Figure 2).

Meta-analysis of safety

The major adverse reactions of lacidipine tablets were mild headache (1.3%), dizziness (0.9%), facial flushing (3.9%), palpitations (0.5%), and mild edema (2.0%). The major adverse reactions of control group were cough (2.4%), headache (1.5%), dizziness (0.6%), facial flushing (4.8%), mild edema (3.4%), and gastrointestinal symptoms (1.1%). The results of meta-analysis showed that the incidence of the adverse reactions in lacidipine tablets were lower than the controlled groups, there were significant differences in treating Chinese patients with mild to moderate essential hypertension (Figure 3).

Publication bias

An analysis of publication bias was conducted. The funnel plots were symmetrical based on visual analysis, indicating that there was no evidence for publication bias (Figure 4).

Sensitivity analyses

In an analysis excluding the 2 low quality trials, our results were consistent with those found in our main

Table 1. Characteristics of important studies admitted.

Studies	Group	Treatment proposal (mg/d)	Times of therapy (weeks)	sample size	Overall response rate (%)	Adverse reaction rate (%)	SBP baseline (mmHg)	SBP after medicine end (mmHg)	DBP baseline (mmHg)	DBP after medicine end (mmHg)	Jadad score
Sun (1995)[11]	Lacidipine	2-6	6	61	98.4	5	164 ± 17	139 ± 12	100 ± 6	82 ± 6	4
	Nitrendipine	20-40	6	60	88.3	30	166 ± 16	140 ± 17	100 ± 5	83 ± 7	
Zhu (1997)[12]	Lacidipine	5	8	40	92.5	16.5	151 ± 13	127 ± 13	99 ± 4	85 ± 9	4
	Amlodipine	5	8	40	95.0	18.2	148 ± 13	130 ± 13	99 ± 4	84 ± 8	
Zhang (1999)[13]	Lacidipine	2-6	4	32	93.7	6.3	160 ± 12	133 ± 9	101 ± 7	84 ± 8	4
	Nitrendipine	10-20	4	28	85.7	17.9	159 ± 9	136 ± 6	102 ± 7	84 ± 8	
Wu (2001)[14]	Lacidipine	4	4	30	96.7	0	162 ± 19	147 ± 10	101 ± 7	86 ± 6	4
	Captopril	50	4	26	95.0	19.2	163 ± 19	142 ± 13	100 ± 6	91 ± 6	
YI (2001)[15]	Lacidipine	2-8	4	60	96.7	20.0	160 ± 10	1127 ± 9	98 ± 7	80 ± 8	2
	Nifedipine	30-60	4	60	95.0	21.7	162 ± 8	123 ± 4	96 ± 7	83 ± 7	
Zhou (2001)[16]	Lacidipine	2-8	4	60	96.7	20.0	169 ± 13	134 ± 18	105 ± 7	82 ± 6	3
	Nitrendipine	30	4	50	84.0	24.0	168 ± 12	142 ± 15	102 ± 6	89 ± 6	
Fang (2001)[17]	Lacidipine	5	4	30	73.3	13.3	169 ± 13	134 ± 18	105 ± 7	82 ± 6	4
	Nitrendipine	10	4	30	73.0	13.3	154 ± 11	140 ± 14	105 ± 8	87 ± 6	
Xue (2001)[18]	Lacidipine	4-8	8	51	72.5	5.9	156 ±14	139 ± 7	105 ± 7	89 ± 8	4
	Lisinopril	10-20	8	51	70.6	2.0	156 ± 12	142 ± 16	102 ± 5	90 ± 8	
Jiang (2002)[19]	Lacidipine	4-8	8	68	93.0	1.5	160 ± 10	127 ± 9	98 ± 7	80 ± 8	4
	Nitrendipine	20	8	61	83.0	8.2	162 ± 8	123 ± 14	96 ± 7	83 ± 7	
Duan (2003)[20]	Lacidipine	4	6	45	92.0	13.3	160 ± 12	136 ± 6	102 ± 7	84 ± 8	2
	Amlodipine	5	6	45	93.0	11.1	159 ± 9	127 ± 10	97 ± 3	82 ± 6	
Xu (2004)[21]	Lacidipine	4	4	25	88.0	8.0	155 ± 15	135 ± 19	104 ± 6	90 ± 12	4
	Nifedipine	30	4	25	76.0	16.0	154 ± 13	134 ± 14	98 ± 4	85 ± 8	
Li (2005)[22]	Lacidipine	4-8	8	104	78.4	11.5	152 ± 12	137 ± 16	98 ± 4	87 ± 10	4

Table 1. Contd.

	Benidipine	2	8	102	74.4	10.8	150 ± 11	134 ± 7	98 ± 2	84 ± 5	
Wu (2007)[23]	Lacidipine	4-6	8	82	91.6	4.9	175 ± 3	135 ± 5	105 ± 2	81 ± 2	3
	Captopril	50	8	82	80.7	9.8	162 ± 5	149 ± 6	111 ± 6	89 ± 6	

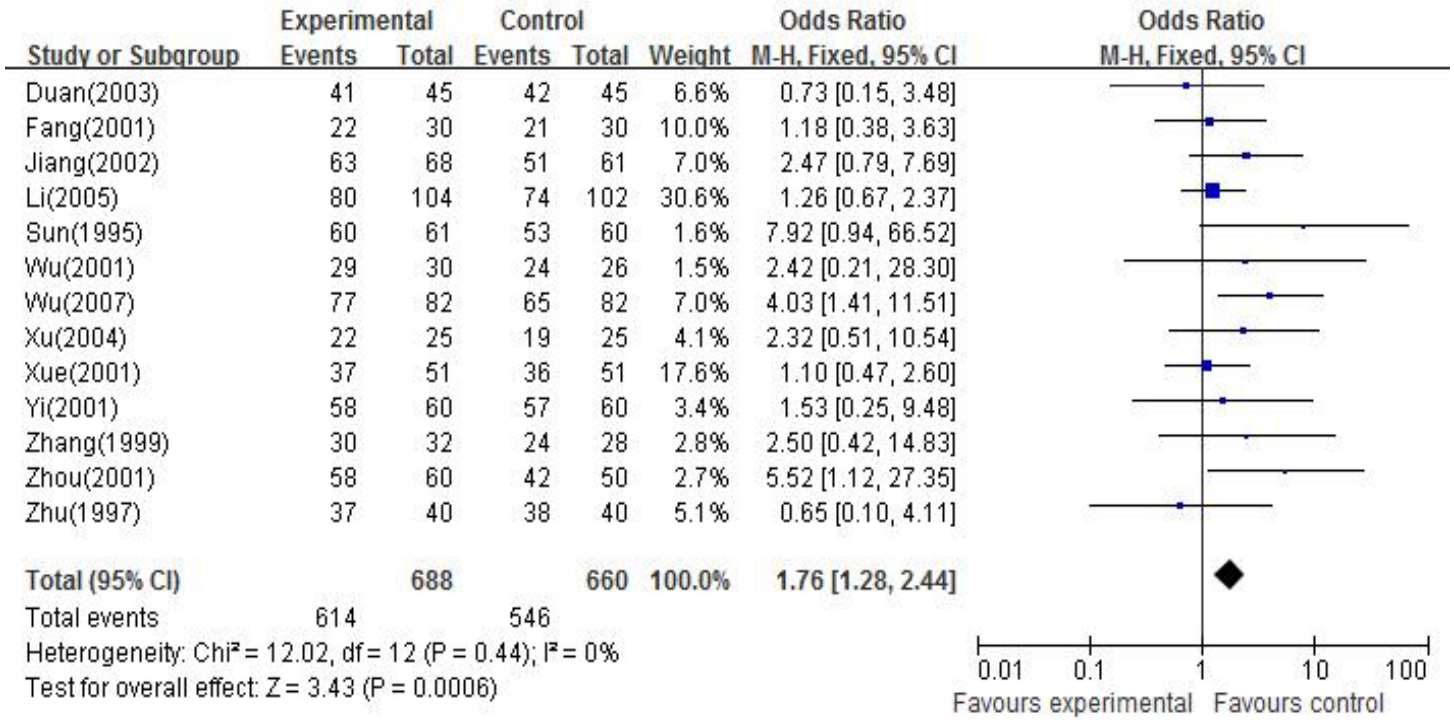


Figure 2. OR estimates with the corresponding 95% CI for the efficacy. The OR estimate of each study is marked with a ■. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond; this line might be contained within the diamond if the confidence interval is narrow.

analysis described earlier: in the efficacy analysis, there was a significant difference in overall response rates between lacidipine group and control group [Z = 3.43 (p = 0.0006), OR = 1.76, 95% CI (1.28 to 2.44)], furthermore, a significant difference was found in adverse reaction rates

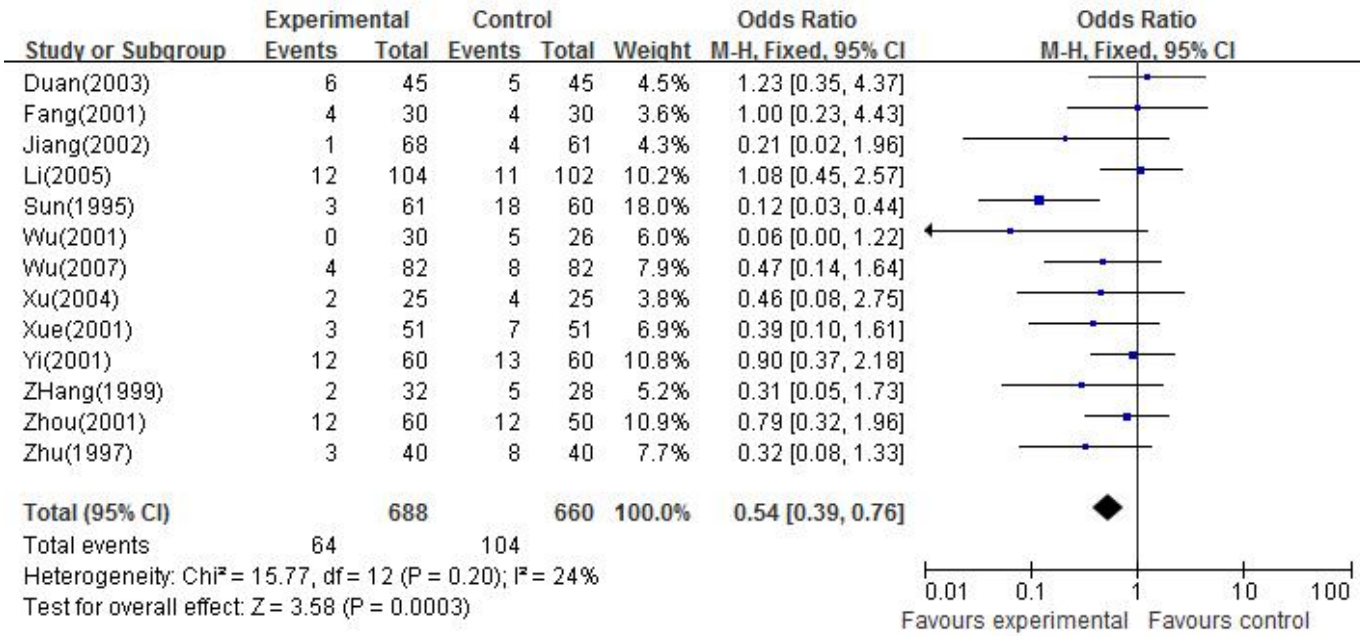


Figure 3. OR estimates with the corresponding 95% CI for the safety. The OR estimate of each study is marked with a ■. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond; this line might be contained within the diamond if the confidence interval is narrow.

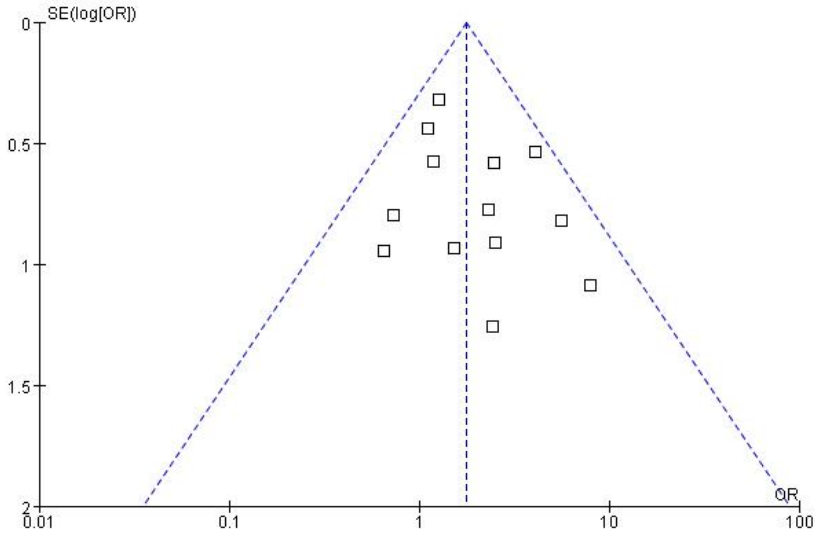


Figure 4. Funnel plot to examine publication bias.

between lacidipine group and control group in the safety analysis [Z = 3.58 (p = 0.003), OR = 0.54, 95% CI (0.39 to 0.76)].

DISCUSSION

Summary of the literature quality

A total of 13 articles were included in this systematic review. From these articles, we included a total sample size of 1348 patients for the meta-analysis. The Jadad score was at least two points for each of the 13 articles.

Moreover, no evidence of publication bias was found and there were no significant heterogeneities between studies in both efficacy analysis and safety analysis. Combined, this suggests that the overall quality of the systematic review was high. However, there were still a few methodological insufficiencies. These included: (a) the randomization method for the individual trials may not be rigorous, because the specific randomization schemes

were inadequately described in all except one article; (b) a selection bias may exist, as the allocation concealment was not described in any of the articles; and (c) a measuring bias and implementation bias may exist, because 4 studies did not describe whether the trial was a double blind design.

Analysis of efficacy and safety

According to the drug core molecular structure and the role of L-type calcium channels in different sub-units, calcium channel blockers are divided into dihydropyridine and non-dihydropyridine. The long-acting and high vascular selective dihydropyridine calcium have been recognized at home and abroad, its antihypertensive effect acts by blocking extracellular calcium through voltage-dependent L-type calcium channels into vascular smooth muscle cells, then reduce excition-contraction coupling, lowering the contractile response of resistance vessels. Its advantages include rapid effect, relative stronger antihypertensive effect and magnitude, and do not affect glucose and lipid metabolism.

As a long-lasting and high vascular selective calcium antagonist, the fat-soluble of lacidipine is significantly higher than other calcium antagonist; it can accumulate in the lipid bilayer of the cell membrane, continually released in the cleanup phase (Sidorenko et al., 2002). It has a complete oral absorption, drug concentrations peak-at 3 to 5 h, has a nearly 99% binding rate with plasma protein. The half-life is 14 h, but has a long-lasting anti-hypertension effect. Its metabolism is in the liver, especially for patients with renal insufficiency. As lacidipine has a high selectivity on vascular smooth muscle, thus rarely case reflex tachycardia (McCormack and Wagstaff 2003).

This study included a meta-analysis of 13 articles, in which the trial designs are all clinical randomized controlled, the total sample is 1348 cases. 11 articles had Jadad scores of more than 2 points, and the overall quality is acceptable. The study showed that lacidipine had a significant efficacy on treatment of mild to moderate essential hypertension compared with the controlled group. The results demonstrate that the main adverse reactions of lacidipine are mild headache, face flushing, palpitations, mild edema, but it had a low incidence and a lesser extent, and all can be tolerated without stopping halfway. The incidence of adverse reactions was significantly compared with the controlled group, which suggests that it had a better security.

Conclusion

The study also showed that lacidipine had a significant hypertension compared with the controlled group, which efficacy on treatment of mild to moderate essential indicated that lacidipine had a better antihypertensive effect compare with other first-line antihypertensive agents. In this study, there were so many types in the controlled group. Although the doses, the period of treatment were basically the same, some studies were not described in detail for the random method. These factors may affect the credibility of the results of meta-analysis. Therefore, more and more double-blind randomized controlled trials are needed to get better clinical evidence.

REFERENCES

Committee of guidebook on prevention and treatment of hypertension (2011). Guidebook on prevention and treatment of hypertension. Chinese J. Cardiol. 39(7):579-615.

Committee of guidebook on prevention and treatment of hypertension (2000). Guidebook on prevention and treatment of hypertension. Chinese J. Hypertens. 8(2):103-112.

Du B, Cui WP, Xu GL (2012). A meta-analysis of the efficacy and safety of arotinolol in the treatment of Chinese patients with essential hypertension. Afr. J. Pharm. Pharmacol. 6(1):36-42.

Duan YJ, Zhang C, Ji YJ (2003). Clinical study of Lacidipine in treatment of essential hypertension. China Prescription Drug, 12(12):70-72.

Fang GQ, Yan WG, Shen LL (2001). The treatment of lacidipine for essential hypertension. Capital Med. 8(4):44.

Jadad AR, Moore A, Carroll D (1996). Assessing the quality of reports of randomized clinical trials: is blinding necessary? Control Clin. Trials 17(1):1-12.

Jiang B, Chen JM, Wang JX (2002). The comparition of efficacy on treatment of hypertension between Lacidipine and Nitrendipine, Captopril. Strait Pharm. J. 14(1):46.

Law MR, Morris JK, Wald HJ (2009). Use of blood pressure lowering drugs in the prevention of cardiovascular disease: meta-analysis of 147 randomized trials in the context of expectations from prospective epidemiological studies. BMJ 338:1665.

Li XF, Ke YN, Jiang H (2005).Study of the anti-hypertensive efficacy and safety of Benidipine in patients with essential hypertension. Pharm. J. Chinese People's Libetation Army 21(6):429-432.

Liu GZ, Hu DY, Hu P (1998). Recommendations on evaluation of clinical trials of cardiovascular drugs. Chinese J. Cardiol., 26(1): 7-9.

Liu LS, Wang W, Yao CH (2010). Hypertension prevention guide (2009 grass-roots version). Chinese J. Hypertens. 18(1):11-30.

McCormack PL, Wagstaff AJ (2003). Lacidipine: a review of its use in the management of hypertension. Drugs, 63(21): 2327-2356.

Sackett DL, Clarke M, Oxman AD (2002). Cochrane Reviewers, Handbook 4.2, In Renew Manager. Oxford: The Cochrane Collaboration. pp.13-36.

Sidorenko BA, Stetsenko TM, Preobrazhenskii DV (2002). Third generation of calcium antagonists: focus on lacidipine. Kardiologiia 42(12):81-90.

Sun XC, Tao P, Xu CB (1995). Clinical effects of Lacidipine in the treatment with essential hypertension. Chinese J. Clin. Pharmacol., 11(2): 78-83.

WHO-ISH Hypertension Guidelines Committee (1999). 1999 World Health Organization- International Society of Hypertension Guidelines for the Management of Hypertension Hypertens. 17(2):151-183.

Wu GP (2005). Research progress of Lacidipine. Chinese J. Cardiovascular Rev. 3(6):468-470.

Wu SG, Fu YQ (2001).Evaluate the efficacy of treatment of hypertension with Captopril and lacidipine China Pharmaceuticals 10(10):49.

Wu SM, Xiong GH (2007).Clinical study of 82 cases of Lacidipine in patients with essential hypertension.. China Practical Med. 2(32):34-36.

Xu CY, Zheng CS, Wang ZC (2004).The efficacy of Lacidipine in treatment of mild to moderate hypertension. Chongqing Med. J.

33(12):1911-1912.
Xue ZL (2001).Efficacy and safety of Lacidipine in treatment of essential hypertension. Chinese J. Modern Appl. Pharm. 18(2):150-152.
Yi M, Li GM, Huang R (2001). The comparition of efficacy on treatment of hypertension between Lacidipine and Nifedipine Controlled Release Tablets. Chinese J. Clin. Pharmacol. 8(10):2-3.
Zhang SH, Cui WD, Zhang RH (1999). Clinical study of Lacidipine in treatment of hypertension. J. Shanghai Tiedao Univ. 20(9):42-44.
Zhang WZ (2004).The antihypertensive mechanism and application of calcium antagonist. Chinese J. Cardiol. 32(sup2):43-44.
Zhao PX, Wang C, Qin L (2012). Effect of clinical pharmacist's pharmaceutical care intervention to control hypertensive outpatients in China. Afr. J. Pharm. Pharmacol. 6(1):48-56.
Zhou SG, Hu LL, Shi TF (2001).Lacidipine treatment of hypertension in 60 cases. China Pharm. 10(6):46-47.
Zhu JH (1997). Clinial efficacy of Lacidipine in treatment of essential hypertension J. Nantong Medical College 17(4):527-528.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2188-2193, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.076
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Impact of gender, weight and CYP2B6 genotype on efavirenz exposure in patients on HIV/AIDS and TB treatment: Implications for individualising therapy

Tafireyi Nemaura[1,2], Charles Nhachi[1] and Collen Masimirembwa[2]*

[1]Department of Clinical Pharmacology, College of Health Sciences, University of Zimbabwe, Zimbabwe.
[2]Department of DMPK/PD-BAC, African Institute of Biomedical Science and Technology, Zimbabwe.

Accepted 10 March, 2012

Gender, weight and cytochrome P450 2B6 (CYP2B6) 516G>T genetic data of 61 patients on efavirenz containing highly active anti-retroviral therapy (HAART) was collated and analysed. Multivariate data analysis and correlations between variables were done to determine the relative contributions of gender, weight and CYP2B6 genetic polymorphism. Models were derived to guide dose adjustment in patients predicted to have unsafe drug exposure. The data showed that 44% of patients had concentrations above the minimum safe concentrations. Gender, weight and genetics explain 22% of the variation of therapeutic levels efavirenz drug exposure in the model. The model generated indicates that all patients homozygous for the 516G>T variant, irrespective of gender or weight required dose adjustment to 200 mg/day, whilst patients of the G/G genotype should be given the standard 600 mg/day. Patients of the G/T genotype showed mixed outcomes. Analysis of this group showed that females of weight less than 62 kg need dose adjustment to 400 mg/day whereas their male counterparts did not need dose adjustment.

Key words: Efavirenz, CYP2B6 516G>T, weight, gender, dose adjustment, partial least squares.

INTRODUCTION

Work on the exposure levels of efavirenz has received particular attention because this drug is used as a substitute of nevirapine in patients undergoing both HIV/AIDS and TB treatment. Efavirenz is metabolised more by cytochrome P450 2B6 (CYP2B6) than by cytochrome P450 3A4 (CYP3A4) (Ward et al., 2003). In the use of efavirenz, the CYP2B6 516G>T genotype has been associated with high plasma levels of the drug (Leger et al., 2009; Rotger et al., 2005) and also susceptibility to CNS adverse reactions (Gatanaga and Oka, 2009; Haas et al., 2004). Many studies have shown this and other variants of CYP2B6 result in increased exposure of efavirenz in many populations (Gatanaga et al., 2007; Jamshidi et al., 2010; Mukonzo et al., 2009).

Population studies have shown the frequency of 516G>T variant to be relatively high across populations with African populations, 28 to 49%, compared to Caucasian 22 to 29% and oriental populations 14 to 43% (Gatanaga et al., 2007; Nyakutira et al., 2008; Xu et al., 2007; Matimba et al., 2008).

Based on these recent observations, we re-evaluate our data (Nyakutira et al., 2008) for the relative contribution of gender, weight and genetic polymorphism of CYP2B6 on efavirenz exposure levels towards the derivation of a clinical dose adjustment algorithm in the use of the drug. A new analysis of the data using partial least squares (PLS) method was used in order to capture the multivariate nature of the effect of several variables on the efavirenz exposure levels.

MATERIALS AND METHODS

Patient data

Patient data was obtained from our previous work (Nyakutira et al., 2008) and only 61 patients were considered based on their

*Corresponding author. E-mail: collen.masimirembwa@aibst.com, collenmasimirembwa@yahoo.com.

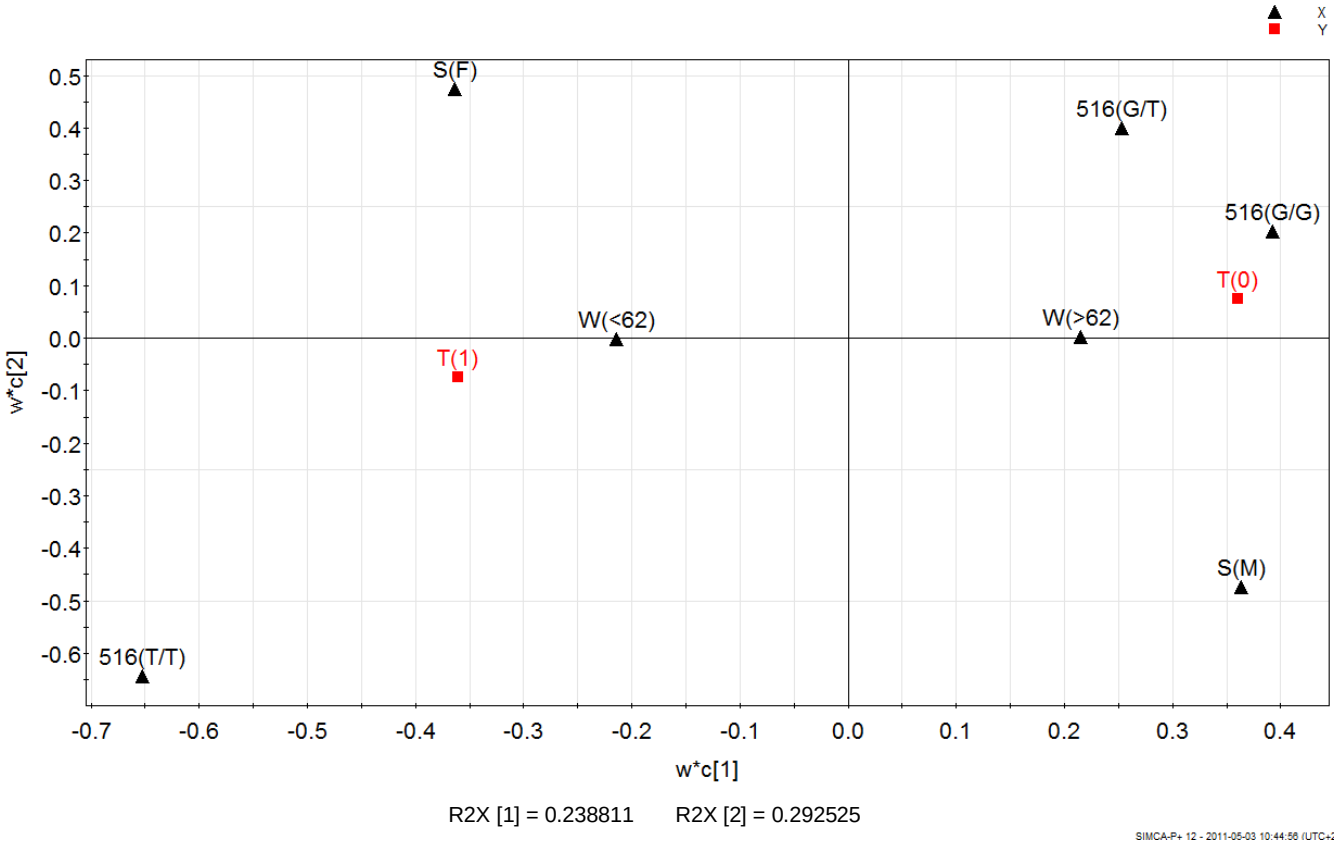


Figure 1. A PLS loading plot of gender weight and genotype and therapeutic margins indicating a positive correlation of 516G>T, T/T, female and weight of below 62 with efavirenz concentrations above 4 µg/ml, T (1), and positive correlation of male, 516G>T, G/G, G/T and weight greater or equal to 62 kg with efavirenz concentration within the 1 to 4 µg/ml range, T (0). The variance explained in this model for efavirenz plasma concentration is 22% (n = 61).

complete data with respect to efavirenz plasma concentration, 516G>T genotype, gender, age and weight. The patients were on TB treatment with regimen containing Rifampcin and Isoniazid, and also on Stavudine and Lamivudine as part of their highly active anti-retroviral therapy (HAART).

Statistical analysis of patient data

Multivariate analysis method of PLS on gender, weight and genotype data against therapeutic exposure levels was done using the Soft Independent Modelling of Class Analogy (SIMCA 12+) software. Principal components 1, 2, and 3 were considered for overall modelling, but only data from PC1 was further considered for PLS because it was the statistically significant component on all the models at 5% level of significance. Age was dropped because of its low contribution to variability in explaining efavirenz levels (R^2 = 0.003). The median age (year) and interquartile range was 39 (36 to 44).

In the analyses, gender, weight and genotype were coded as follows: Gender(S): Males represented by M, Females represented by F; Genotypes for CYP2B6 516G>T(516): G/G,G/T and T/T; Weight(W): less than 62 kg (<62), greater or equal to 62 kg (≥ 62); Therapeutic range(T): within the target therapeutics window (1 to 4 µg/ml (Katsounas et al., 2007) represented by 0; >4 µg/ml -1) and below the maximum effective concentration (MEC) (<1 µg/ml represented by -1). Since there was no patient with concentration below the MEC in this study, only codes T (0) and T (1) were used in the analysis. The weight category cut-off of 62 kg was arrived at after plotting an estimated linear regression line of efavirenz concentration against weight, which gave the equation:

y = -0.403x + 63.183 (y = weight in kg, x = drug concentration in µg/ml).

There is evidence of negative correlation between weight and plasma concentration (r = -0.144, P = 0.269). Taking the efavirenz cut-off of 4 µg/ml, to divide the patients into those within the therapeutic range (1 to 4 µg/ml), T (0) and those above 4 µg/ml (T (1)), the weight cut-off was derived as 62 kg.

Dosing algorithm generation

From the PLS global model (Figure 1), patients in various combinations of gender, weight and genotype were evaluated for closeness to either the T (0) or T (1) concentration range. This closeness was captured by values between 0 and 1 with values associated with T (0) being between 0 and 0.5 and those associated with T (1) being between 0.5 and 1. These values are weighted predictions for each group generated by PLS model. For each variable combination of gender, weight and genotype, patients falling in each group had the median of their plasma concentrations

Table 1. Gender, weight, and CYP2B6 516G>T genotype and efavirenz exposure level data of 61 patients on HIV and TB treatment.

Demographics and genetics		Number of patients	Median efavirenz (IQR) µg/ml	Patients with concentrations above 4 µg/ml (%)	VIP for 1st PC for global model
Gender	Male	25	3.44 (2.72,7.23)	8/25 (32)	0.96622
	Female	36	4.53 (2.80,9.06)	19/36 (53)	0.96622
Weight	≥62 kg	29	3.32 (2.23,9.03)	11/29 (38)	0.5676
	<62 kg	32	4.17 (3.09,7.12)	16/32 (50)	0.5676
Genotype (CYP2B6 516G>T)	G/G	13	3.32 (1.62,3.64)	3/13 (23)	1.0382
	G/T	32	3.49 (2.81,6.14)	12/32 (38)	0.6689
	T/T	16	8.70 (5.01,11.40)	12/16 (75)	1.7259

VIP: Very important variable.

calculated. The median drug concentration (*y*) of each category was plotted against the predicted weighted value (*x*), between 0.0 and 1.0, of the fitting of the variable clusters. The data was used to generate the standard curve ($y = 8.9517x + 1.1601$, $R^2 = 0.89761$) from which, efavirenz concentrations associated with various drug doses could be estimated (a direct linear proportional relationship was assumed).

RESULTS

From Figure 1, the plot on the relationship of gender, genotype, weight and 516G>T genotype to efavirenz concentration above 4 µg/ml, coded T(1) showed that, in the first component, PC1, the T/T genotype, female status and weight below 62 kg positively correlated with concentrations above 4 µg/ml. The plot also showed that the G/G and G/T genotype, the male status and weight above or equal to 62 kg correlated with concentration within the therapeutic window [code T(0)] (Figure 1). The variables gender, weight and CYP2B6 genotype were shown to explain 22% of the variation in efavirenz concentration. Following the correlations revealed by PLS (Figure 1), statistical measures of the association of gender, weight categories and 516G>T genotypes were calculated. The order of importance of the variables in determining how the model is formed is as shown in Table 1. The T/T genotype and G/G genotype were shown to be the most important followed by gender. Weight was shown to have the least separation capabilities into the therapeutic window and above 4 µg/ml efavirenz concentration. Furthermore, T/T and G/G genotypes were shown to contribute significantly in how they distinctly relate to plasma efavirenz levels and are very significant along the 1st principal component (Table 1). Separation of efavirenz plasma concentration is relatively significant according to gender and relatively weak according to G/T genotype.

Prediction of dose adjustments

Using the standard curve for 600 mg/day based on a plot of the level of fitting of variables to the T(1) and T(0), efavirenz concentrations were plotted against the median plasma concentration of efavirenz in patients of different variable clusters, after which predictions of plasma concentration of efavirenz when given at different doses were then made. Predictions of efavirenz concentrations for the various variable combination patients were predicted for 200, 400, 600 and 800 mg/day doses (Table 2). The choice of 800 mg/day is based on some recommendations to increase efavirenz concentration to this dose when co-administered with rifampcin (Stohr et al., 2008). The choice of 200 and 400 mg was based on previous modelling and clinical dose adjustment that have been associated with the use of efavirenz (Gatanaga and Oka, 2009; Nyakutira et al., 2008; Gatanaga et al., 2007). The data showed that if given 800 mg, 53/61 patients were predicted to have plasma concentrations above 4 µg/ml. If given 200 mg/day, 8/61 patients (all males of weight >62 kg and of the GG or GT genotype) were predicted to have concentration below the MEC. If given 400 mg/day, 16/61 patients (all of the T/T genotype and of mixed gender and weight categories) are predicted to have concentration above 4 µg/ml. The results we obtained on dose proposition are comparable to the ones from NONMEM (Cabrera et al., 2009; Nyakutira et al., 2008).

Model validation

Using $PE_i = ODV_i - PDV_i$ where PE_i is the prediction error of the *ith* individual, ODV_i is the observed dependent variable in the *ith* individual and PDV_i is the predicted dependent variable in the *ith* individual. The 95% confidence interval for the mean prediction error for the

Table 2. Predicted 12 h post plasma efavirenz concentration in relation to dose taken.

Weighted prediction for the clusters PLS[1]	n	Patient description	Median (µg/ml)	200 mg/day predicted plasma efavirenz conc. (µg/ml)	400 mg/day predicted plasma efavirenz conc. (µg/ml)	600 mg/day predicted plasma efavirenz conc. (µg/ml)	800 mg/day predicted plasma efavirenz conc. (µg/ml)
0.0583139	3	M,GG,W≥62	2.53	0.56	1.12	1.68	2.24
0.153393	5	M,GT,W≥62	3.27	0.84	1.69	2.53	3.38
0.232465	5	M,GG,W<62	3.52	1.08	2.16	3.24	4.32
0.236611	2	F,GG,W≥62	3.18	1.09	2.19	3.28	4.37
0.327544	5	M,GT,W<62	3.31	1.36	2.73	4.09	5.46
0.33169	9	F,GT,W≥62	3.20	1.38	2.75	4.13	5.51
0.410762	3	F,GG,W<62	3.64	1.61	3.22	4.84	6.45
0.505841	13	F,GT,W<62	4.90	1.90	3.79	5.69	7.58
0.583863	6	M,TT,W≥62	7.23	2.13	4.26	6.39	8.52
0.758015	1	M,TT,W<62	9.25	2.65	5.30	7.95	10.59
0.762161	4	F,TT,W≥62	8.17	2.66	5.32	7.98	10.64
0.936312	5	F,TT,W<62	9.14	3.18	6.36	9.54	12.72

[1]A value close to zero for the values in the first column indicates a gravitation towards the therapeutic window, T(0). As the prediction move toward 1 it indicates weighting towards above 4 µg/ml [T(1)].

model (Figure 1) is given by (-0.1135802, 0.1135802). Since the confidence interval (CI) contain 0, the model has adequate predictability and without significant error (Ette and Williams, 2007). Cross-validation [where one data point is left out (leave one out approach)] showed statistically significant results with the cumulative fraction of the total variation of therapeutic concentrations ($Q^2_{cumulative}$) that could be predicted by components as 0.08 for the model. This was significant since it is greater than 0.05 (n<100).

The percentage positive (correct) predictions for individuals in the model, 74%, for all individuals global are as shown in Figure 1.

DISCUSSION

In this study, we have shown that gender, weight, and 516G>T genotype are important determinants on whether a patient will have efavirenz concentrations within the therapeutic range, T (0), or above 4 µg/ml, T (1). Based on data from the PLS and dose predictions, a clinical dosing algorithm was proposed (Figure 2). This proposes that all patients of the T/T genotype have their dose adjusted to 200 mg/day. Those of the G/G genotype should be given the standard dose of 600 mg/day. Patients of the G/T genotype given mixed results, males generally tolerating the standard dose of 600 mg, whilst their female counterparts of weight <62 kg requires a dose adjustment to 400 mg/day.

Our results agree with observations by a number of research groups. Manosuthi et al. (2009) showed that higher weights were associated with lower efavirenz concentrations. Several works showed that gender affected efavirenz plasma concentration (Burger et al., 2005; Mukonzo et al., 2009; Nyakutira et al., 2008). Without taking 516G>T variant into account, Burger et al. (2005) observed that women were at risk of higher efavirenz concentrations as compared to men and hinted weight difference as a possible cause. Females had mean body weight of 65.3 kg as compared to 75.1 kg of males.

Our findings however differ on conclusions reached by others, who discounted weight and gender as important determinants of plasma concentrations using multiple linear regressions (Cohen et al., 2009; Ramachandran et al., 2009). The use of multiple linear/logistic regressions in these studies, might have failed to identify covariances of these variables as we did by using PLS. The conclusion of Cohen et al. (2009) and Ramachandran et al. (2009) could also have been affected by the fact that their samples were predominantly male. This group is not significantly affected by weight.

We do not discount the importance of CYP2A6 and UGT2B7 as highlighted by Kwara et al. (2009). Other variants commonly found in African populations and associated with reduced enzyme activity include CYP2B6*16 and CYP2B6*18 (Jamshidi et al., 2010). The relative high frequency of 516G>T in African populations as compared to Caucasian and Oriental populations make it a promising biomarker for efavirenz exposure levels in Africa. Follow up studies are important, given the fact that the variables considered in this study account for only 22% of the variability in efavirenz concentration, implying that there are other environmental and/or genetic factors that can improve our proposed algorithm. With our sample size of 61 patients, the mean C_{min} (±SD) was 5.8 (±4.2) µg/ml, with a coefficient of variance (CV)

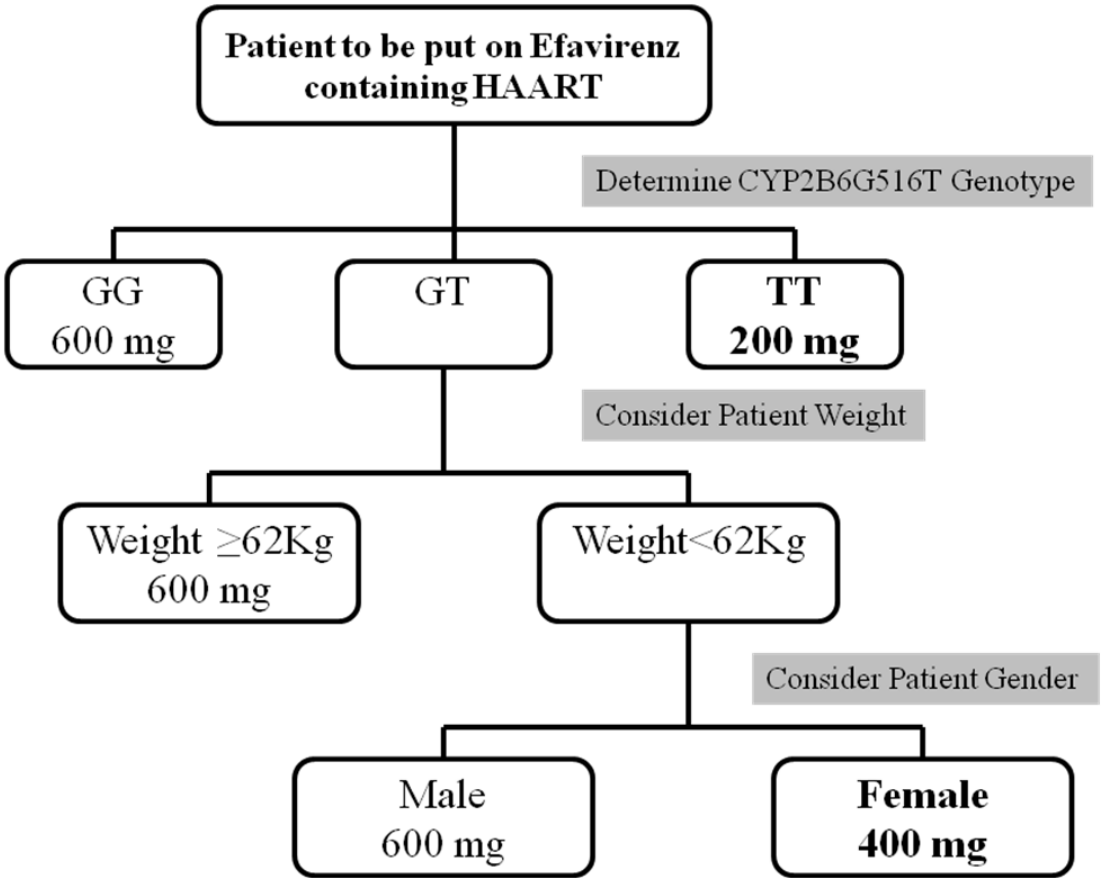


Figure 2. Proposed efavirenz dose adjustment algorithm. CYP2B6 516G>T genotype has the greatest effect on efavirenz concentration with all patients with the poor metaboliser status, T/T, requiring dose adjustment downwards to 200 mg. Female patients of the G/T genotype and of weight lower than 62 kg, also require dose adjustment to 400 mg/day.

of 73%. This level of variation is lower than the 121% observed in 94 patients (Kwara et al., 2009). To capture the large variation of efavirenz exposure levels, a larger patient's sample size would be desirable. A large sample size will also ensure that we have more patients in the various cluster groups we have identified in this study.

ACKNOWLEDGEMENTS

We acknowledge funding from ISP, WHO-TDR and EDCTP.

REFERENCES

Burger D, van der Heiden I, la Porte C, lan der Ende M, Groeneveld P, Richter C, Koopmans P, Kroon F, Sprenger H, Lindermans J, Schenk P, van Schaik R (2005). Interpatient variability in the pharmacokinetics of the HIV non-nucleoside reverse transcriptase inhibitor efavirenz: the effect of gender, race, and CYP2B6 polymorphism. Br. J. Clin. Pharmacol., 61: 148-154.

Cabrera SE, Santos D, Valverde MP, Domínguez-Gil A, González F, Luna G, García MJ(2009). Population Pharmacokinetics of Efavirenz in HIV patients: Influences of CYP2B6 Genotype. Antimicrobial Agents and Chemother., 53: 2791-98.

Cohen K, Grant A, Dandara C, McIlleron H, Pemba L, Fielding K, Charalombous S, Churchyard G, Smith P, Maartens G (2009). Effect of rifampcin-based antitubercular therapy and cytochrome P450 2B6 516G>T polymorphism on efavirenz concentrations in Adults in South Africa. Antiviral Therapy, 14:687-695.

Ette EI, Williams PJ (2007). Pharmacometrics: The Science of Quantitative Pharmacology; Wiley: Hoboken, New Jersey, USA, pp. 237-239.

Gatanaga H, Hayashida T, Tsuchiya K, Yoshino M, Kuwahara T, Tsukada H, Fujimoto K, Sato I, Ueda M, Horiba M, Hamaguchi M, Yamamoto M, Takata N, Kimura A, Koike T, Gejyo F, Matsushita S, Shirasaka T, Kimura S, Oka S (2007). Successful Efavirenz Dose Reduction in HIV Type 1-Infected individuals with Cytochrome P4502B6 *6 and *26. Clin. Inf. Dis., 45: 1230-1237.

Gatanaga H, Oka S (2009). Successful genotype-tailored treatment with small-dose efavirenz. AIDS., 23: 433-435.

Haas DW, Ribaudo HJ, Kim RB, Tierney C, Wilkinson GR, Gulick RM, Clifford DB, Hulgan T, Marzolini C, Acosta EP (2004). Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS, 18: 2391-2400.

Jamshidi Y, Moreton M, McKeown DA, Andrews S, Nithiyananthan T, Tinworth L, Holt DW, Sadiq ST (2010). Tribal ethnicity and CYP2B6 genetics in Ugandan and Zimbabwean populations in the UK: implications for efavirenz dosing in HIV infection. J. Ant.

Chemotherapy, 65: 2614-2619.
Katsounas A, Frank A, Klinker H, Langmann P (2007). Efavirenz-therapy in HIV-patients with underlying liver disease: importance of continuous TDM of EFV. Eur. J. Med. Res., 12: 331-336.
Kwara A, Lartey M, Sagoe KWC, Kenu E, Court MH (2009). CYP2B6, CYP2A6 and UGT2B7 Genetic Polymorphisms are Predictors of Efavirenz Mid-dose Concentration in HIV-infected Patients. AIDS, 23: 2101-2106.
Leger P, Dillingham R, Beauharnais CA, Kashuba ADM, Rezk NL, Fitzgerald DW, Pape JW, Haas DW (2009). CYP2B6 Variants and Plasma Efavirenz concentrations during Antiretroviral Therapy in Port-au-Prince, Haiti. J. Inf. Dis., 200: 955-964.
Manosuthi W, Sungakanuparph S, Tantanathip P, Mankatitham W, Luenganiyomkul A, Thongyen S, Eampokarap B, Uttayamakul S, Suwanvattana P, Kaewsaard S, Ruxrungtham K (2009). Body Weight Cut off for Daily Dosage of Efavirenz-Based Regimen in Human Immunodeficiency Virus and Tuberculosis Coinfected Patients Receiving Rifampin. Antimicrobial Agents and Chemother, 53: 4545-4548.
Matimba A, Oluka MN, Ebeshi BU, Sayi J, Bolaji OO, Guantai AN, Masimirembwa C (2008). Establishment of a biobank and Pharmacogenetics database of African populations. Eur. J. Hum. Gen., 16: 780-783.
Mukonzo JK, Roshammar D, Waako P, Andersson M, Fukasawa T, Milani L, Svensson JO, Ogwal-Okeng J, Gustafsson LL, Aklillu L (2009). A novel polymorphism in ABCB1 gene, CYP2B6*6 and sex predict single-dose efavirenz population pharmacokinetics in Ugandans. Br. J. Clin. Pharmacol., 68: 690-699.
Nyakutira C, Roshammar D, Chigutsa E, Chonzi P, Ashton M, Nhachi C, Masimirembwa C (2008). High prevalence of the CYP2B6 516G->T (*6) variant and effect on the pharmacokinetics of Efavirenz in HIV/AIDS outpatients in Zimbabwe. Eur. J. Clin. Pharmacol., 64: 357-365.
Ramachandran G, Kumar AKH, Rajasekeran S, Kumar P, Ramesh K, Anitha S, Narendran G, Menon P, Gomathi C, Swaminathan S (2009). CYP2B6 G516T Polymorphism but Not Rifampin Co administration Influences Steady-State Pharmacokinetics of Efavirenz in Human Immunodeficiency Virus-Infected Patients in South India. Antimicrob. Agents Chemother., 53: 863-868.
Rotger M, Colombo S, Furrer H, Bleiber G, Budin T, Lee BL, Keiser O, Biollaz J, Décostered L, Telenti A, Swiss HIV Cohort Study (2005). Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. Pharmacogenet. Genomics, 15: 1-5.
Stohr W, Back D, Dunn D, Sabin C, Winston A, Gilson R, Pillay D, Hill T, Ainsworth J, Pozniak A, Leen C, Bansi L, Fisher M, Orkin C, Anderson J, Johnson M, Easterbrook P, Gibbons S, Khoo S (2008). Factors influencing efavirenz and nevirapine plasma concentration: effect of ethnicity, weight and co-medication. Antivir. Ther.: 13: 675-685.
Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z (2003). The Cytochrome P450 2B6 (CYP2B6) Is the Main Catalyst of Efavirenz Primary and Secondary Metabolism: Implication for HIV/AIDS Therapy and Utility of Efavirenz as a Substrate Marker of CYP2B6 Catalytic Activity. J. Pharmacol. Exp. Ther., 306: 287-300.
Xu BY, Guo LP, Lee SS, Dong QM, Tan Y, Yao H, Li LH, Lin CK, Kung HF, He ML (2007). Genetic Variability of CYP2B6 polymorphisms in four southern Chinese populations. World J. Gastroenterol., 13: 2100-2103.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2194-2204, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.296
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Comparative therapeutic potentials of acarbose and a formulated herbal extract on type 2 diabetic rats

Hulyam Kurt[1], Cansu Ozbayer[1]*, Irfan Degirmenci[1], Mehmet Cengiz Ustuner[1], Hilmi Ozden[2], Kismet Civi[3] and Hasan Veysi Gunes[1]

[1]Department of Medical Biology, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey.
[2]Department of Anatomy, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey.
[3]Pathology Laboratory, Inegol State Hospital, Bursa, Turkey.

Accepted 20 July, 2012

Oxidative stress plays an important role in chronic complications of diabetes. Acarbose is an alpha-glucosidase inhibitor used for the treatment of diabetes. A commercial herbal formulated extract, which consists of 13 herbal extract (F13), is also described to have a potential antidiabetic action. The aim of this study was to determine the comparative effects of acarbose and F13 on type 2 diabetic rats. Three to five weeks after induction of diabetes by single dose systemic administration of streptozotocin and nicotinamide (STZ-NA), diabetic rats were treated with acarbose and F13 for two weeks. After the treatment period, the blood glucose, hemoglobin A1c (HbA_1c), triglyceride, cholesterol and nitric oxide synthases (NOS) levels, as well as liver and erythrocyte superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and glutathione peroxidase (GPx) levels were determined. Renal filtration changes were determined by measuring urine creatinine, plasma creatinine and creatinine clearance. Histological analyses were also performed in liver and kidney. The rats in diabetic groups had significantly higher blood glucose levels than control groups. Induction of diabetes was confirmed by histological analyses of liver and kidney tissues. High blood glucose level in diabetic rats results in peroxidative reactions in lipids, thus MDA levels were increased in diabetic control while acarbose and F13 treatment reduced MDA production. Also, increased SOD levels were found in STZ-NA diabetic rat liver. Both acarbose and F13 treatment, however, showed similar improving effects on diabetic complication in diabetes. Our results, therefore, support the validity of this herbal extract on the management of diabetes as well as diabetes-induced liver and renal complications.

Key words: Acarbose, type 2 diabetes mellitus, free radicals, herbal preparation, histology, rats.

INTRODUCTION

Diabetes mellitus is a syndrome characterized by abnormal insulin secretion, derangement in carbohydrate and lipid metabolism, and is diagnosed by the presence of hyperglycemia. Diabetes is also a risk factor for chronic renal disease. It is a major worldwide health problem and once it occurs, chronic renal failure and end-stage renal disease increases the mortality in type 2 diabetic patients (Atkins, 2005; Atalay and Laaksonen,2002; Ritz and Orth, 1999, Akyuz et al., 2012). According to previous studies, oxidative stress playing an important role in chronic complications of diabetes is postulate to be associated with increased lipid peroxidation. In addition, enhanced oxidative stress and lower antioxidant capacity might be related to etiology of diabetic complications (Pitkanen et al., 1992; Elangovan et al., 2000; Bukan et al., 2003).

Experimental diabetic models could be developed by using certain genetic, chemical and surgical methods. In the new rat model characterized with reduction of 40%

*Corresponding author. E-mail: c.ozbayer@gmail.com. Tel: +90-222-2392979/4598. Fax: +90-222-2393772.

beta islets in pancreas; 290 mg/kg of nicotinamide (NA) is injected intraperitoneally (i.p) 15 min before the administration of 60 mg/kg of streptozotocin (STZ) (i.p). The main basis of this model is to supply nicotinamide stores consumed by the body and to protect beta cells via nicotinamide.

As a result, this model imitates the characteristics of human type 2 diabetes both histologically and metabolically (stable mild hyperglycemia and glucose intolerance, etc.) (Novelli et al., 2001, 2004; Akyuz et al., 2012).

Acarbose is an alpha-glycosidase inhibitor and an antidiabetic drug used to treatment of diabetes. Acarbose shows its effect by inhibiting intestinal enzymes (alpha-glycosidases), thereby interfering the catabolism of disaccharides, oligosaccharides and polysaccharides in intestines.

Thus, digestion of carbohydrate is delayed dependent on dose, and more importantly, liberation of glucose and its presence in the blood slows down. Moreover, both fluctuations in daily blood sugar and average blood sugar level decrease as a result of this delayed glucose intake through intestines with acarbose. Acarbose also reduces abnormal high concentrations of glycosylated hemoglobin (Wright et al., 1998; Kawamura et al., 1998; Hwua et al., 2003).

Nowadays, drugs are expensive and have many side effects during the treatment of any disorders. Therefore, many herbs have been used for a long time for claimed health benefits, and the potential of the health promoting and disease preventing properties of plant-derived compounds has received increased attention from researchers in recent years (Nasri et al., 2012; Asgarpanah and Ramezanloo, 2012; Alam et al., 2012). Different plants have been used alone or in formulations for the treatment of diabetes and its complications. An example of such herbal formulation under trade name is F13 that is obtained from Karkim (F13®, Karkim Co., Turkey) and traditionally used for the treatment of diabetes in Turkey.

This formulation has the potential to act as antidiabetic. The herbal extract F13 consists of 13 plant parts (*Thymbra spicata*, *Folium myrti, Folium eucalypti globuli, Folium olivarum, Folium sorbi domesticae, Folium juglandis, Lavandula stoechas, Semen foenugraeci, Herba fumariae, Alchemilla vulgaris, Herba millefolii, Folium salviae* and *Herba chamaedrys*) and it is prepared with steam distillation and extraction method by the manufacturer. Most of the herbs in this formulation are used in alternative medicine as antidiabetic, anti-cholesterolemic, antioxidant, diuretic, free radicals cleaner, antiseptic, antihypertensive, antimicrobial and antiviral both in Turkey and in other parts of the world (Buckle, 2001; Inanc et al., 2007; Gunes et al., 1999; Sabu and Kuttan, 2002; Modak et al., 2007; Seeff et al., 2001). The aim of this study was to evaluate the antihyperglycemic, antioxidant and histoprotective effects of acarbose and herbal formulation (F13) on STZ-NA induced type 2 diabetic rats.

MATERIALS AND METHODS

Experimental animals

In this study, three-month-old Sprague-Dawley breed male rats weighing 200 to 250 g and grown in the Department of Medical Biology, Eskisehir Osmangazi University, Medical Faculty, were used. The animals were housed in individual cages at room temperature and left for 1 week for acclimatation before the start of experiment. This study was approved by the local ethical committee of Eskisehir Osmangazi University (affirmation number: 09/21/122).

Treatment

Glucose levels were measured from tail vein at the beginning of the experiment and also once a week during the experiment. The rats were divided into six groups as shown in Table 1. Diabetes was induced by a single intraperitoneal injection of 290 mg/kg body weight NA (Sigma Chemical, St. Louis, Mo., USA) dissolved in saline 15 min before a single intraperitoneal injection of 60 mg/kg STZ (Sigma Chemical, St. Louis, Mo., USA) dissolved in saline immediately before use. Subsequently, the animals were treated with the extract of the herbal formulation (F13®, Karkim Co., Turkey) during two weeks to test possible antidiabetic and antioxidative effects. Moreover, to determine whether the inhibition of alpha-glycosidase has any effect on free radical formation in the type 2 diabetes, acarbose was administrated (Sigma Chemical, St. Louis, Mo., USA) 5 to 8 weeks after the induction of the diabetes, and the effects of administration of F13 were determined by comparing with acarbose. Doses of the given substrates are summarized in Table 1. Subsequently, the rats were sacrificed after the treatment period, and blood, urine, liver and kidney tissue samples were collected under ether anesthesia.

Biochemical analysis

Fasting and postprandial blood glucose was measured every week during the experiments with an Accu-Chek® Go Glucometer (Roche Diagnostics, Mannheim, Germany) in all animals. After determining the volume and pH (pH meter; inoLab® pH 720, WTW Laboratory, Germany) of urine samples collected, urine creatinine and plasma creatinine activity were measured spectrophotometrically by Jaffe's reaction. Creatinine clearance was also calculated from urine creatinine, plasma creatinine and the 24-h urinary excretion volume as described by Gunes et al. (1999). All spectrophotometric measurements were performed by Shimadzu UV-1601 digital spectrophotometer (Schimadzu Corp., Kyoto, Japan). Hemoglobin A1c (HbA_1c) levels of groups were measured by Boehringer-Mannheim 911 Hitachi Automatic Analyzer and triglyceride and cholesterol levels were determined by Roche Diagnostic Modular System.

Antioxidant and free radical assays

The method described by Sun et al. (1988) was used to prepare erythrocyte hemolysate. Superoxide dismutase (SOD) activity was determined by SOD determination kit (FLUKA, St. Louis, MO, Cat. No: 19160) based on water-soluble tetrazolium salt (WST) reaction. Malondialdehyde (MDA) reaction was applied based on color reaction of MDA, one of the final products from lipid peroxidation,

Table 1. The substrates given to control and experimental groups.

Groups		n	Administrated substrates
	I	7	(Control) Water + CSD
Control	II	7	Acarbose [5 mg/kg per day for 14 days, i.g.] + Water + CSD
	III	7	F13 [5 ml/kg per day for 14 days, i.g.] + Water + CSD
	IV	7	(Diabetic Control) [60 mg/kg STZ + 290 mg/kg NA i.p.] + Water + CSD
Diabetic	V	7	(60 mg/kg STZ + 290 mg/kg NA i.p.)+ Acarbose (5 mg/kg per day for 14 days, i.g.)+ Water + CSD
	VI	7	(60 mg/kg STZ + 290 mg/kg NA i.p.) + F13 (5 ml/kg per day for 14 days, i.g.) + Water + CSD

CSD, Commercial standard diet; i.g, intragastric.

with thiobarbituric acid (TBA) (Uchiyama and Mihara, 1978). Nitric oxide synthase (NOS) activity, catalase (CAT) activity and glutathione peroxidase (GPx) activity were determined by using nitric oxide synthase assay kit (Bioxytech®, Oxis International Inc, Portland, OR, USA, Cat No: 22113), ammonium molybdate-hydrogen peroxide reaction with manual assay (Goth, 1991) and glutathione peroxidase assay kit (Calbiochem®, EMD Biosciences,. Inc., San Diego, CA, cat. No: 354104), respectively.

Histopathological examination

All tissues were collected from rats, immediately fixed in 10% neutral formalin solution, embedded in paraffin, and then stained with haematoxylin and eosin.

Statistical evaluation

The obtained data were expressed as mean ± standard deviation (S.D.) and analyzed using analysis of variance (ANOVA). Tukey's test was used to test for differences among means when ANOVA indicated a significant difference. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Effects acarbose and F13 treatment on blood glucose levels

The blood glucose levels were determined every week during the experiment. The levels of fasting and post-prandial blood glucose at the beginning of the experiment, and at 5th and 7th weeks are given in Tables 2 and 3, respectively. The diabetic animals exhibited gradually increased hyperglycemia. Acarbose and F13 treatment caused a decrease in the elevated blood glucose levels in STZ-NA diabetic rats. The fasting blood glucose levels were similar in controls and experiment groups before STZ+NA injection (P>0.05). Meanwhile, the glucose levels increased gradually in STZ+NA treated groups (IV, V and VI). The blood glucose levels of groups IV and V were higher in the 5th week when compared to controls (P< 0.05, P< 0.001, respectively). Moreover, at the 7th week, fasting blood glucose levels of acarbose and F13 treated groups decreased significantly (P<0.001) when compared to diabetic control (Tables 2 and 3). The postprandial blood glucose levels were similar in controls and experimental groups before STZ+NA injection (P>0.05). The glucose levels increased gradually in STZ+NA treated groups (IV, V and VI). At the 7th week, after the acarbose and F13 treatment, postprandial blood glucose levels of only acarbose treated group decreased significantly (P< 0.05) when compared to diabetic control (group IV) (Tables 2 and 3). Postprandial blood glucose levels of diabetic control (group IV) and F13 treated diabetic group (group VI) were significantly higher (P<0.01, P<0.001, respectively) when compared to control. At the 7th week, fasting blood glucose levels of acarbose treated group decreased significantly (P< 0.05) when compared to diabetic control.

Biochemical analysis

Triglyceride levels were significantly higher in the acarbose and F13 treated diabetic groups (P<0.05, P<0.001); meanwhile there was no significant difference in HbA_1c and cholesterol levels between controls and other experimental groups (P>0.05) (Table 4).

Urine and plasma creatinine and creatinine clearance

We found significantly higher urine creatinine levels in control groups treated with acarbose and F13 when compared to the control (P<0.01) (Table 5). However, no significant difference in plasma creatinine was found between the control and experimental groups (P>0.05) (Table 5). In addition, creatinine clearance was different in the F13 treated control and diabetic control groups compared to the control group (P<0.01 and P<0.05, respectively) (Table 5). When groups II and III with group I were compared; creatinine clearance level of F13 treated control group (III) was significantly higher (P < 0.05). Further, when groups V and VI with group IV were compared, creatinine clearance levels of acarbose and F13 treated diabetic groups was reduced (P<0.05, P<0.01). No statistical differences were found when

Table 2. The fasting blood glucose levels of control and diabetic groups.

Groups		n	Before injection	5th week (beginning of the treatment)	7th week (final of the treatment)
	I	7	82.42 ± 2.4	75.42 ± 3.0	81.14 ± 2.3
Control	II	7	81.00 ± 1.3	83.42 ± 1.4	84.85 ± 1.9
	III	7	81.42 ± 1.7	84.28 ± 2.1	80.28 ± 1.8
	IV	7	78.00 ± 1.5	89.28 ± 4.1*	103.57 ± 4.1***
Diabetic	V	7	75.42 ± 1.3	93.42 ± 2.6**	78.42 ± 1.83^{+++}
	VI	7	78.85 ± 1.7	87.71 ± 4.1	77.85 ± 4.0^{+++}

* P<0.05 ** P<0.01 *** P<0.001 (Compared to the control) $^{+++}$ P<0.001 (Significance between the diabetic groups when compared to group IV).

Table 3. The postprandial blood glucose levels of control and diabetic groups.

Groups		n	Before injection	5th week (beginning of the treatment)	7th week (final of the treatment)
	I	7	107.85 ± 2.7	102.00 ± 2.7	109.57 ± 3.8
Control	II	7	98.14 ± 3.5	110.00 ± 2.6	111.00 ± 4.5
	III	7	98.71 ± 3.9	111.57 ± 1.7	114.85 ± 5.9
	IV	7	113.14 ± 3.2	154.00 ± 12.4	169.28 ± 6.0***
Diabetic	V	7	102.14 ± 3.8	134.42 ± 7.5	137.28 ± 4.2^{+}
	VI	7	100.85 ± 4.0	162.00 ± 29.8*	149.42 ± 14.3**

* P<0.05, ** P<0.01, *** P<0.001 (compared to the control); $^{+}$P<0.05 (significance between the diabetic groups when compared to group IV).

Table 4. The HbA_1c, triglyceride and cholesterol levels of controls and experimental groups (mg/dl).

Groups		n	HbA_1c (mg/dl)	Triglyceride (mg/dl)	Cholesterol (mg/dl)
	I	7	4.58 ± 0.30	24.14 ± 4.05	46.14 ± 3.23
Control	II	7	4.92 ± 0.77	29.14 ± 6.22	33.28 ± 4.64
	III	7	4.31 ± 0.13	27.14 ± 3.71	43,00 ± 5.85
	IV	7	4.85 ± 0.58	28.85 ± 6.91	44.42 ± 11.19
Diabetic	V	7	4,58 ± 0.51	46.14 ± 15.74**	44.57 ± 16.52
	VI	7	4.25 ± 0.12	60.71 ± 10.35***	42.71 ± 6.82

** P<0.01, *** P<0.001 (compared to the control).

Table 5. The Urine pH, urine volume, urine creatinine, plasma creatinine and creatinine clearance levels of controls and experimental groups.

Groups		n	Urine pH	Urine volume (ml)	Urine creatinine (mg/dl)	Plasma creatinine (mg/dl)	Creatinine clearance (ml/min)
	I	7	8.0 ± 0.3	3.4 ± 0.5	139 ± 6	1.90 ± 0.05	0.168 ± 0.02
Control	II	7	7.7 ± 0.2	3.9 ± 0.3	345 ± 35**	1.91 ± 0.02	0.475 ± 0.04
	III	7	7.6 ± 0.3	4.0 ± 0.3	344 ± 7**	1.84 ± 0.01	0.528 ± 0.04*
	IV	7	8.3 ± 0.3	7.0 ± 0.4**	236 ± 53	1.86 ± 0.03	0.637 ± 0.15**
Diabetic	V	7	8.8 ± 0.7	2.6 ± 1.0	191 ± 38	1.84 ± 0.02	0.149 ± 0.06^{++}
	VI	7	8.6 ± 0.7	3.7 ± 0.7	240 ± 7	2.03 ± 0.09	0.286 ± 0.05^{+}

* P<0.05, ** P<0.01 (compared to the control); $^{+}$P<0.05, $^{++}$P<0.01 (significance between the diabetic groups when compared to group IV).

groups V and VI were compared with groups II and III.

Effects on antioxidant and free radical levels

Erythrocyte MDA, SOD, CAT, GPx and serum NOS levels are presented in Table 6. There were no significant differences in the SOD, CAT and GPx activities between control and diabetic groups. Erythrocyte MDA levels significantly decreased in the F13 treated diabetic groups ($P<0.001$). On the other hand, increased serum NOS levels in acarbose treated diabetic groups were obtained. MDA, SOD, CAT and GPx levels of liver homogenates were presented are Table 7. The results obtained indicated no significant differences in the CAT and GPx activities between control and diabetic groups. In addition, SOD and MDA levels of the untreated diabetic groups (diabetic control) were significantly increased ($P<0.001$).

Histopathological findings

In this study, binuclear hepatocytes, sinusoidal dilatations, nuclear hypertrophy, necrotic cells with pyknotic nucleus and eosinophilic cytoplasm were observed in diabetic control groups (Figures 1 and 2). In the acarbose and F13 treated diabetic group, hepatocytes and sinusoidal structures showed nearly normal histology and binuclear hepatocytes were observed in some areas (Figures 3 and 4). In addition, normal liver histology was seen in control groups. Moreover, the kidney samples of control groups were histologically normal (Figure 5), whereas glomerular basement membrane thickening, the distal tubular epithelium, cytoplasmic clear cell change and medial thickening of small arteries were observed in diabetic control (Figure 6). The kidney samples of acarbose treated diabetic groups showed almost normal histological structure, although glomerular basement membrane thickening were observed in some areas (Figure 7). In F13 treated diabetic groups, glomerular basement membrane thickening was also observed in some areas (Figure 8).

DISCUSSION

In the present study, antihyperglycemic, antidiabetic and antioxidative potential effects of the formulated herbal extract were evaluated in a nicotinamide and streptozotocin -induced diabetic rat model. The herbal extract (F13) consisted of *T. spicata*, *F. myrti, F. eucalypti globuli, F. olivarum, F. sorbi domesticae, F. juglandis, L. stoechas, S. foenugraeci, H. fumariae, A. vulgaris, H. millefolii, F. salviae* and *Herba chamaedrys* extracts. The antidiabetic, anti-cholesterolemic, antioxidant, diuretic, free radicals cleaner, antiseptic, antihypertensive, anti-microbial and antiviral effects of these plants have been reported (Buckle, 2001; Inanc et al., 2007; Gunes et al., 1999; Sabu et al., 2002; Modak et al., 2007; Seeff et al., 2001). Likewise, in our study, clear reduction in blood glucose levels was observed in STZ-NA induced diabetic rats treated with F13.

Hemoglobin A1c (HbA1c) is the most common measurement for the determination of glycemic control for patients with diabetes. There is a concern that the measurement of HbA1c may be affected by the severity of kidney dysfunction or the hematological complications of kidney disease (Cavanaugh, 2007). It has been reported that HbA1c values, triglyceride and cholesterol levels in diabetic subject were significantly higher (Kuppusamy et al., 2010). In our study, however, there were no significant differences in HbA_1c and cholesterol levels between control and treated diabetic groups. STZ-NA induced type 2 diabetic model imitates the characteristics of human type 2 diabetes and it is characterized by stable mild hyperglycemia and glucose intolerance (Novelli et al., 2001; Novelli et al., 2004). The stable HbA1c values in our study may be due to stable mild hyperglycemia or short retention time of the experiment (lack of glucose toxicity). Ordinarily, insulin activates the lipoprotein lipase, which hydrolyses triglycerides. However in diabetic condition, lipoprotein lipase is not activated due to insulin deficiency, thus resulting in hypertriglyceridemia (Kuppusamy et al., 2010). Similarly, we found increased triglyceride levels in diabetic acarbose and diabetic F13 groups when compared to control.

The kidneys are important target organs of diabetes and kidney failure often leads to death in diabetes. Diabetes causes glomerular lesions, atherosclerosis of renal veins, pyelonephritis and nephropathy (Prakash et al., 2007; Chen et al., 2007). Increased urine volume and creatinine clearance can also be observed in diabetes (Gunes et al., 1999; Murali et al., 2003). Glomerular filtration rate is determined by measuring creatinine clearance, and decrease in creatinine clearance indicates glomerular degeneration (Murali et al., 2003). In contrast, we found increased creatinine clearance levels in diabetic control. Also, creatinine clearance levels of acarbose and F13 treated diabetic groups were decreased when compared to diabetic control. The assay for creatinine in plasma and urine were defined by Jaffé (1886). However, difficulties with the reaction with respect to its lack of specificity and sensitivity have been discussed. Thus, plasma creatinine based measurements remain the most widely used method to assess renal function in animals (Dunn et al., 2004). In this study, there were no statistical differences in plasma creatinine levels of groups when compared to control, and this may be because STZ-NA diabetes shows mild renal insufficiency depending on the duration of experiment.

Diabetes causes increased oxidative damage through generation of reactive oxygen species (ROS), and free

Table 6. Erythrocyte SOD, MDA, CAT, GPx, and serum NOS levels.

Groups		n	SOD (% inhibition)	MDA (U/gHb)	CAT (kU/L)	Gpx (nmol/min/ml)	NOS (nmol/ml/s)
Control	I	7	53.53 ± 10.2	13.10 ± 2.0	213.13 ± 25.58	4.00 ± 2.00	0.23 ± 0.19
	II	7	46.65 ± 5.30	25.50 ± 4.5	208.57 ± 21.29	5.09 ± 2.07	0.21 ± 0.10
	III	7	52.97 ± 4.26	19.11 ± 4.6	207.30 ± 30.33	3.63 ± 1.36	0.27 ± 0.04
Diabetic	IV	7	48.58 ± 1.87	35.27 ± 12.6***	209.48 ± 26.05	5.45 ± 3.09	0.17 ± 0.06
	V	7	58.01 ± 14.52	23.27 ± 12.2	215.56 ± 45.17	3.42 ± 1.20	0.38 ± 0.09^{++}
	VI	7	44.79 ± 3.76	15.47 ± 1.9^{+++}	218.50 ± 20.64	3.74 ± 1.23	0.10 ± 0.03

*P<0.05, ** P<0.01, *** P<0.001 (compared to the control); $^{+}$P<0.05 ,$^{+++}$ P<0.001 (significance between the diabetic groups when compared to group IV).

Table 7. Liver SOD, MDA, CAT and GPx levels.

Groups		n	SOD (%inhibition)	MDA (U/wet tissue)	CAT (kU/ml protein)	Gpx (nmol/min/ml)
Control	I	7	50.14 ± 3.80	2.57 ± 0.12	3.25 ± 0.20	11.31 ± 1.98
	II	7	50.57 ± 3.50	2.57 ± 0.18	3.21 ± 0.40	12.01 ± 1.01
	III	7	48.85 ± 7.81	2.49 ± 0.05	3.28 ± 0.14	11.56 ± 0.96
Diabetic	IV	7	63.28 ± 3.90***	3.27 ± 0.38***	3.43 ± 0.14	11.64 ± 1.27
	V	7	56.71 ± 4.68	2.79 ± 0.10^{++}	3.42 ± 0.14	10.40 ± 1.73
	VI	7	51.28 ± 3.59	2.69 ± 0.19^{+++}	3.19 ± 0.12	12.88 ± 2.35

*** P<0.001 (compared to the control); $^{++}$P<0.01, $^{+++}$P<0.001 (significance between the diabetic groups when compared to group IV).

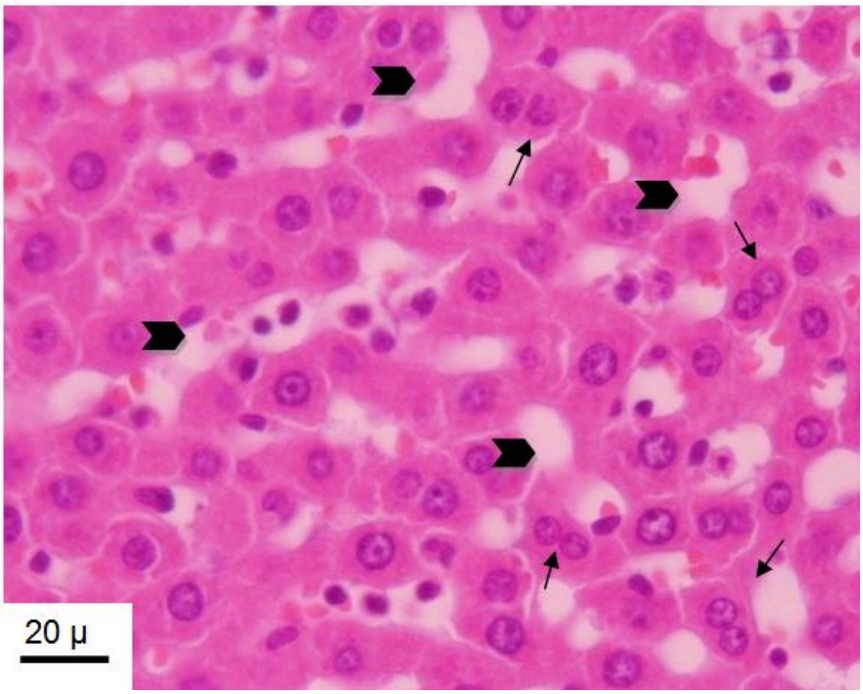


Figure 1. Diabetic groups: binuclear hepatocytes (→) and sinusoidal dilatations ( ), H and E × 100.

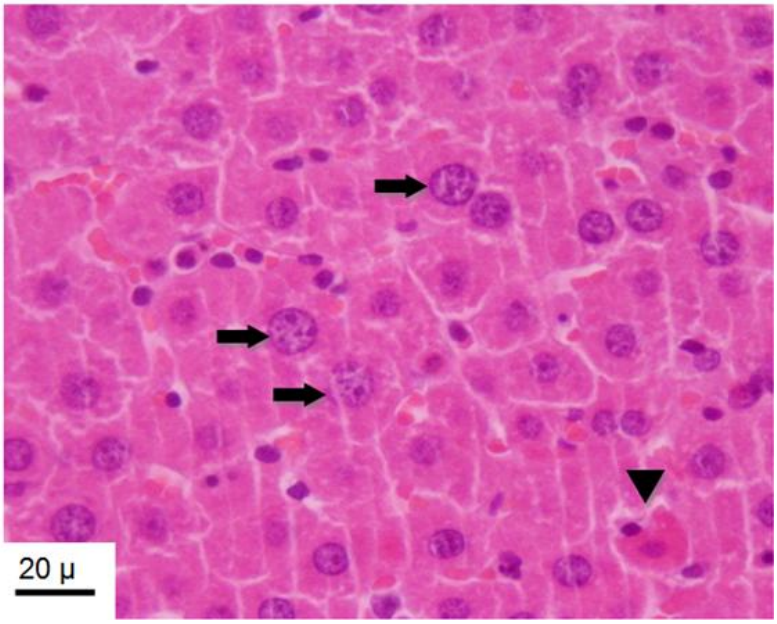


Figure 2. Diabetic group showing nuclear hypertrophy (▬►) and the necrotic cells with pyknotic nucleus and eosinophilic cytoplasm (▼), H and E × 100.

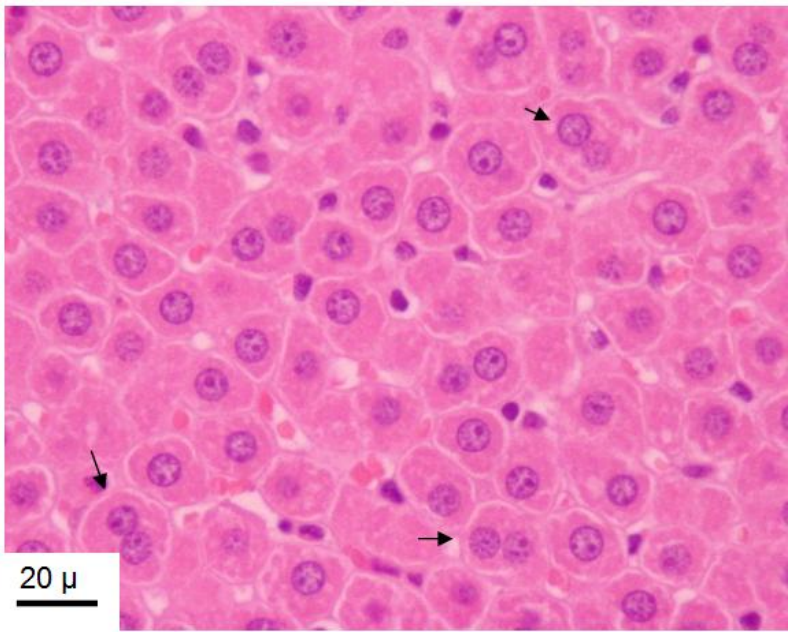


Figure 3. Acarbose treated diabetic group: Hepatocytes and sinusoidal structures in diabetic-acarbose group showed nearly normal histology, while binuclear hepatocytes were observed in some areas (→). H and E × 100.

radical-mediated oxidative stress has an important role in the pathogenesis of various diabetic complications. Antioxidant defense system allows a balance between the generation of oxidants and antioxidants. The range of antioxidant defenses should be adequate to protect against oxidative damage (Pitkanen et al., 1992; Elangovan et al., 2000; Kuppusamy et al., 2010). Overproduction of superoxide takes place when cellular

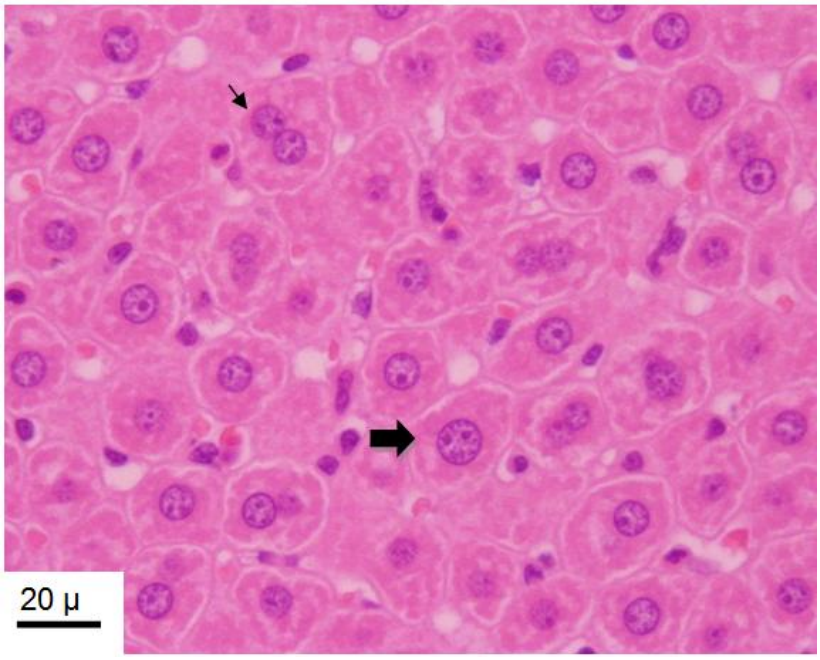


Figure 4. F13 treated diabetic group: Hepatocytes and sinusoidal structures in diabetic-F13 group showed nearly normal histology, binuclear hepatocytes (→), while nuclear hypertrophy (▬►) were observed in some areas, H and E × 100.

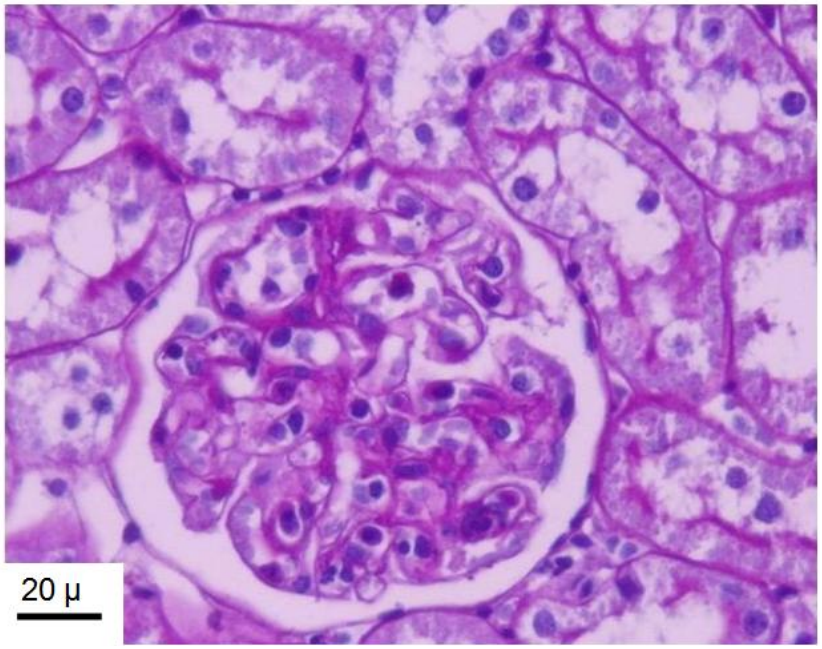


Figure 5. Control group showing histologically normal kidney samples. H and E × 100.

metabolism is destroyed by overproduced glucose, and these results in diabetes complications. Superoxide dismutase is an important antioxidant enzyme and it reduces the increased superoxide by converting it into peroxide and oxygen (Ceriello, 2010; Kuppusamy et al., 2010). Hyperglycemia as demonstrated in this study

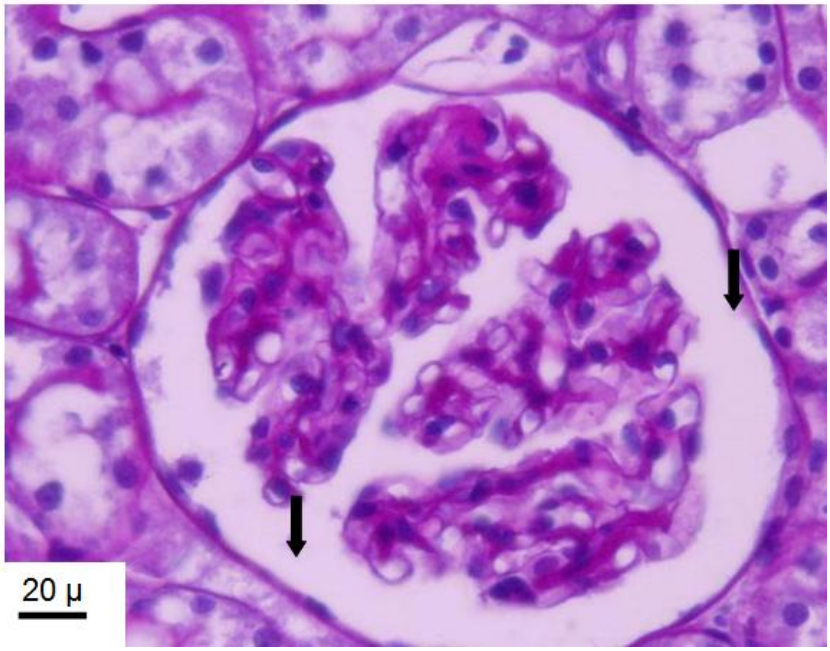


Figure 6. Diabetic control group showing glomerular basement membrane thickening (→). H and E × 100.

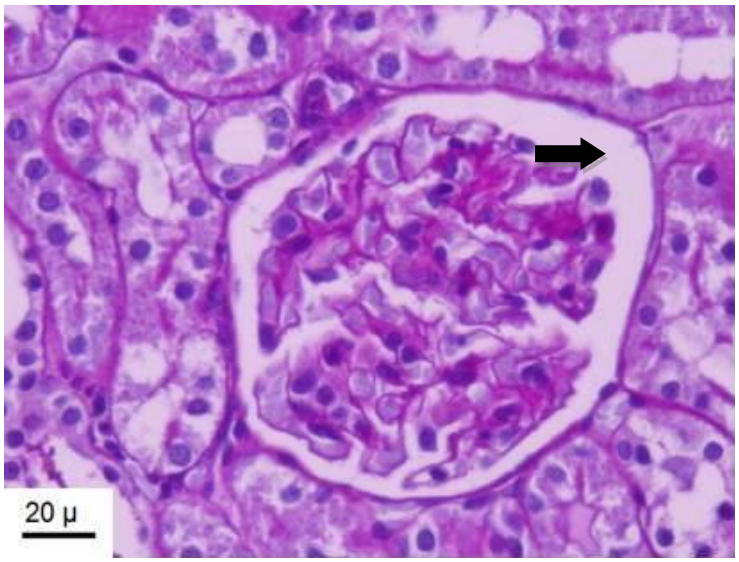


Figure 7. Diabetic acarbose group: nearly normal histological structure, glomerular basement membrane thickening (→) was observed in some areas. H and E × 100.

might be associated with the oxidative stress. In several studies, contradictory SOD activities were reported as decreased, unchanged or increased in type 2 diabetes (Tas et al., 2007). In our study, no difference was found in erythrocyte SOD activities, whereas liver SOD activities significantly increased in diabetic control group. Decreased superoxide dismutase in liver tissue indicated an increased oxidative stress in diabetic control.

Furthermore, high blood glucose level in diabetic patients leads to formation of reactive oxidants causing oxidative damage (Atalay, 2002). In diabetes mellitus, hyperglycaemia induces the peroxidative reactions in lipids,

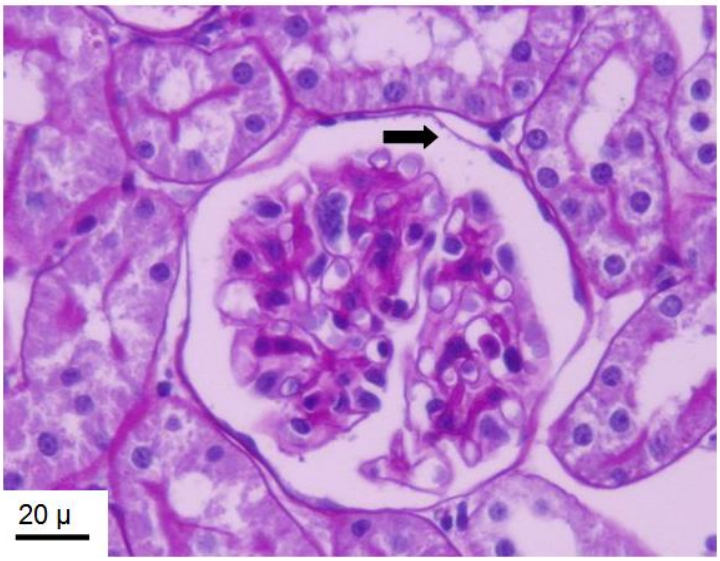


Figure 8. Diabetic F13 group showing glomerular basement membrane thickening (→) as observed. H and E × 100.

thus the product of lipid peroxidation (MDA) increases in diabetes. It was also reported that diabetes causes increased oxidative stress in many organs, especially in liver (Yilmaz et al., 2004). Similarly, we found increased erythrocyte and liver MDA levels in diabetic control group, whereas erythrocyte MDA levels reduced in diabetic group treated with F13. Also, liver MDA levels of acarbose and F13 treated diabetic groups were significantly decreased when compared to diabetic control. However, acarbose and F13 treatment resulted in normalization of MDA levels which may contribute to the beneficial effect on liver lipid peroxidation.

Considering the NOS activities of the groups, no statistically significant differences were found between the control and the other groups. In addition, significantly lower NOS levels were observed in diabetic group treated with acarbose compared to diabetic control group. In diabetic patients, oxidative stress produced in tissues as a result of hyperglycemia leads to overproduction of NO. This overproduction results in various complications in a number of organs such as eye, kidney and cardiovascular system in type 1 and type 2 diabetes (Kawamura et al., 1998; Hwua et al., 2003). In our study, we found that acarbose, an antidiabetic agent, affects diabetes in a positive way by reducing NOS levels and decreasing NO production. Meanwhile, no significant differences were found between control and experimental groups regarding GPx and catalase activities. It has been reported that the levels of antioxidant enzymes such as erythrocyte GPx and catalase of type 1 and type 2 diabetic patients were decreased (Atalay, 2002).

In various studies, it has been reported that diabetes developed by STZ causes histological changes in liver (Gunes et al., 1999). In our study, nuclear hypertrophy and binuclear hepatocytes were observed in liver tissues of diabetic control groups, whereas normal formations were seen in liver histology of control groups and treated diabetic group. As a result, we observed that the herbal formulation (F13) obtained from Karkim shows antidiabetic and antioxidative properties in diabetes complications, and acarbose cured partially the defects of antioxidant enzymes and histological structure caused by diabetes by reducing blood sugar. Hence, there is need for conducting clinical research in herbal drugs and formulation, developing simple bioassays for biological standardization, pharmacological and toxicological evaluation, and developing various animal models for toxicity and safety evaluation. It is also important to establish the active components from these plant extracts.

ACKNOWLEDGEMENT

This study was supported by a grant of the Research Foundation of University of Eskisehir Osmangazi, Turkey (Grant No.200411017).

REFERENCES

Akyuz F, Tekin N, Aydın O, Temel HE, Isıklı B (2012). The effect of metformin and exercise on serum lipids, nitric oxide synthase and liver nitric oxide levels in streptozotocin-nicotinamide induced diabetic rats. Afr. J. Pharm. Pharmacol. 6(5):336-342.

Alam M, Ghiasuddin, Sadat A, muhammd N, Khan AA, Siddiqui BS (2012). Evaluation of *Viburnum grandiflorum* for its *in-vitro* pharmacological screening. Afr. J. Pharm. Pharmacol. 6(22):1606-1610.

Asgarpanah J, Ramezanloo F (2012). Chemistry, pharmacology and

medicinal properties of *Peganum harmala* L. Afr. J. Pharmacy and Pharmacol. 6(22):1573-1580.
Atalay M, Laaksonen DE (2002). Diabetes, oxidative stress and physical exercise. J. Sports Sci. Med. 1:1-14.
Atkins RC (2005). The changing patterns of chronic kidney disease: The need to develop strategies for prevention relevant to different regions and countries. Kidney Int. 68:83-85.
Buckle J (2001). Aromatherapy and diabetes. Diabetes Spectrum 14 (3):124-126.
Bukan N, Sancak B, Yavuz O, Koca C, Tutkun F, Ozcelikay TA, Altan N (2003). Lipid peroxidation and scavenging enzyme levels in the liver of streptozotocin-induced diabetes rats. Indian J. Biochem. Biophysics 40(6):447-450.
Cavanaugh KL (2007). Diabetes management issues for patients with chronic kidney disease. Clin. Diab. 25(3):90-97
Ceriello A (2010). Hyperglycaemia and the vessel wall: The pathophysiological aspects on the atherosclerotic burden in patients with diabetes. European J. Preventive Cardiol. 17(1):15-19.
Chen G, Adeyemo AA, Zhou J, Chen Y, Doumatey A, Lashley K, Huang H, Amoah A, Agyenim-Boateng K, Eghan BA Jr, Okafor G, Acheampong J, Oli J, Fasanmade O, Johnson T, Rotimi C (2007). A Genome-Wide Search for Linkage to Renal Function Phenotypes in West Africans With Type 2 Diabetes. Am. J. Kidney Dis. 49:394-400.
Dunn SR, Qi Z, Bottinger EP, Breyer MD, Sharma K (2004). Utility of endogenous creatinine clearance as a measure of renal function in mice. Kidney Int. 65:1959-1967.
Elangovan V, Shohami E, Ghati I, Kohea R (2000). Increased hepatic lipid soluble antioxidant capacity as compared to other organs of streptozotocin-induced diabetic rats: A cyclic voltammetry study. Free. Rad. Res. 32:125-134.
Goth L (1991). A Simple method for determination of serum catalase activity and revision of reference range. Clinica Chimica Acta 196: 143-152.
Gunes HV, Degirmenci I, Aydin M, Bozan B, Aral E, Tunalier Z, Ustuner C, Ercakar M, Baser KHC, Basaran A (1999). The effects of *Rumex patientia* L. and *Urtica dioica* L. on some blood and urine parameters, and liver and kidney histology in diabetic rats. Tr. J. Med. Sci. 29: 227-232.
Hwua C, Hob L, Fuh MMT., Chung Siu S, Sutanegara D, Piliang S, Chan JCN (2003). Acarbose improves glycemic control in insulin-treated Asian type 2 diabetic patients: Results from a multinational, placebo controlled study. Diabetes Res. Clin. Practice 60:111-118.
Inanc N, Cicek B, Sahin H, Bayat M, Tasci S (2007). Use of herbs by patients with diabetes in Kayseri, Turkey. Pak. J. Nutr. 6 (4):310-312.
Kawamura T, Egusa G, Fujikawa R, Watanabe T, Oda K, Kataoka S, Takayama S, Kubo. K, Yamamoto S, Noma K, Orita R, Yamakido M (1998). Effect of acarbose on glycemic control and lipid metabolism in patients with non-insulin-dependent diabetes mellitus. Current Therapeutic Res. 59(2):97-106.
Kuppusamy AK, Muthusamy U, Shanmugam SS, Thirumalaisamy SA, Varadharajan S, Ramanathan S (2010). Antidiabetic, hypolipidemic and antioxidant properties of *Asystasia gangetica* in streptozotocin - nicotinamide-induced type 2 diabetes mellitus (NIDDM) in rats. J. Pharmacy Res. 3(10):2516-2520.
Modak M, Dixit P, Londhe J, Ghaskadbi S, Paul A Devasagayam T (2007). Indian herbs and herbal drugs used for the treatment of diabetes. J Clin. Biochem. Nutr. 40(3):163-173.
Murali B, Umrani DN, Goyal RK (2003). Effect of chronic treatment with losartan on streptozotocin-induced renal dysfunction. Mol. Cell Biochem. 249(1-2):85-90.
Nasri S, Anoush M, Khatami N (2012). Evaluation of analgesic and anti-inflammatory effects of fresh onion juice in experimental animals. Afr. J. Pharm. Pharmacol. 6(23):1679-1684.
Novelli M, Fabregat ME, Fernandez-Alvarez J, Gomis R, Masiello P (2001). Metabolic and functional studies on ısolated ıslets in new rat model of type 2 diabetes. Mol. Cellular Endocrinol. 175:57-66.
Novelli M, Pocai A, Lajoix AD, Beffy P, Bezzi D, Marchetti P(2004). Alterations of β-cell constitutive NO synthase activity is involved in the abnormal insulin response to arginine in new rat model of type II diabetes. Mol. Cellular Endocrinol. 219(1-2):77-82.
Pitkanen OM, Martin JM, Hallman M, Akerblom HK, Sariola H, Andersson SM (1992). Free radical activity during development of insulin dependent diabetes mellitus in the rat. Life Sci. 50 (5):335-339.
Prakash J, Lodha M, Singh SK, Vohra R, Raja R, Usha (2007). Diabetic retinopathy is a poor predictor of type of nephropathy in proteinuric type 2 diabetic patients. J. Assoc. Physicians India. 55:412-6.
Ritz E, Orth SR (1999). Nephropathy in patients with type 2 diabetes mellitus. N. Engl. J Med. 341:1127-1133.
Sabu MC, Kuttan R (2002). Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. J. Ethnopharmacol. 81(2):155-160.
Seeff LB, Lindsay KL, Bacon BR, Kresina TF, Hoofnagle JH (2001). Complementary and alternative medicine in chronic liver disease. Hepatol. 34(3):595-603.
Sun Y, Oberley LW, Li Y (1988). A simple method for clinical assay of superoxide dismutase. Clin. Chem. 3413:497-500.
Tas S, Sarandol E, Ayvalik SZ, Serdar Z, Dirican M (2007). Vanadyl sulfate, taurine, and combined vanadyl sulfate and taurine treatments in diabetic rats: Effects on the oxidative and antioxidative systems. Arch. Med. Res. 38(3):276-283.
Uchiyama M, Mihara M (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical Biochem. 86:279-286.
Wright BE, Vasselli JR, Katovich MJ (1998). Positive effects of acarbose in the diabetic rat are not altered by feeding schedule. Physiol. Behavior 63(5):867-874.
Yilmaz HR, Uz E, Yucel N, Altuntas I, Ozcelik N (2004). Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver. J. Biochem. Mol. Toxicol. 18(4):234-238.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2205-2210, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.331
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Ferriprotoporphyrin IX-*Combretum imberbe* crude extracts interactions: Implication for malaria treatment

P. Dzomba[1]*, T. Chayamiti[1], S. Nyoni[1], P. Munosiyei[2] and I. Gwizangwe[1]

[1]Chemistry Department, Faculty of Science Education, Bindura University of Science Education, Bindura, Zimbabwe.
[2]Biological Science Department, Faculty of Science Education, Bindura University of Science Education, Bindura, Zimbabwe.

Accepted 26 April, 2012

Leaves from *Combretum imberbe* traditionally used to treat symptoms of malaria in most parts of Southern Africa were studied for interaction of its crude methanolic, ethyl acetate and hexane extracts with hemin a product of hemoglobin degradation in 40% water-dimethylsulphoxide at pH 9, 7.4 and 5 using a spectrophotometric method. It has been established that hemin is the target of antimalaria quinolines thus, interaction of extracts with hemin may represent a crucial initial screening test to define efficacy. Observations were compared to results of interaction of hemin with quinine and artemisia as standards. The present results indicate that hemin complexed more strongly with methanolic extracts than with ethyl acetate and hexane extracts. The binding constants were pH-dependent. The present results are interesting because the crude extracts share a similar mode of action with quinine and artemisia with an isosbetic point at 352 nm. Methanolic extract showed better affinity for hemin than artemisia with complexation constants (K) of 1.011×10^4 and 0.612×10^4 M, respectively. However, quinine showed better affinity than methanolic extract with K of 1.101×10^4 M.

Key words: Ferriprotoporphyrin IX, *Combretum imberbe,* crude extracts, hemin.

INTRODUCTION

Malaria is becoming very difficult to treat everyday due to drug resistance of plasmodium (WHO, 2005; Egan, 2004), therefore calling for studies in alternative medication. Medicinal plants from folk remedies offer attractive options. It has been established that hemin is primarily involved in the anti-malarial activity of drugs (Tekwan and Walker, 2005; Dechy et al., 2002, 2003; Gong et al., 2001; Egan, 2002, 2004; Cointeaux et al., 2003). Thus, the interaction of herbal extracts with hemin may represent an initial crucial screening test to define efficacy. In the blood, *Plasmodium falciparum* use host's hemoglobin as a food source. This takes place in an acidic environment within the parasite called a food vacuole that has a pH in the range 5.0 to 5.6 (Spiller et al., 2002). Its proteolytic enzymes degrade hemoglobin and it use the amino acids derived from digestion for its biosynthetic needs. Hemoglobin degradation is an ordered process, which involves a number of proteases (Eggleson et al, 1999; Rosenthal et al., 2002). Denatured globin formed by plasmepsins is further degraded into small peptides by other proteases. A cysteine protease, falcipain, has been characterized from *P. falciparum (*Wu et al., 2003). It degrades denatured globin (Mpiana et al., 2007). Large quantities of free nonpoisonous heme groups are released from hemoglobin degradation (Tekwan and Walker, 2005; Macreadie et al., 2000). The formed heme is autoxidized into ferric form (hematin; aquaferriprotoporphyrin IX), which is highly toxic. It inhibits vacuolar proteases and damage parasite membranes (Berman and Adams, 1997). Detoxification of heme is therefore important for the survival and growth of malaria parasite (Meshnick, 2002). In the host, detoxification of heme is achieved by an enzyme heme

*Corresponding author. E-mail: pdzomba@gmail.com. or pdzomba@buse.ac.zw. Tel: +263773474525. Fax: +263 7536.

oxygenase. It breaks heme to form biliverdin.

A second enzyme biliverdin reductase, converts biliverdin into bilirubin, which is further converted into a water-soluble conjugate and excreted through urine. Malaria parasite does not use this pathway for heme metabolism. In the food vacuole, heme is changed into hemozoin, a malaria pigment, which is a dimer of heme units linked through an iron-carboxylate bond (Pagola et al., 2000). Digestion of hemoglobin releases heme into the food vacuole, where it is oxidized to hematin. Heme is becoming another possible drug target and has been implicated in the mode of action of endoperoxide antimalarials, such as artemisinin and its derivatives (Tekwan and Walker, 2005; Robert et al., 2002). Hematin is said to be the target of chloroquine and other quinoline antimalarials. It has been shown that histidine-rich protein 2, (a histidine- and alanine-rich protein produced by *Plasmodium falciparum*) has been implicated as an enzyme or, more likely an initiator in the process of formation of hemozoin. Drugs like chloroquine and quinine mode of action has been shown to inhibit hemozoin formation through direct interaction with hematin (Kaschula et al., 2002), by displacing hematin from histidine-rich protein 2 (Pandey et al., 2006), or by preventing its binding to this protein. In this study a rapid screening method for investigating the ability of *Combretum imberbe* extracts to prevent hemozoin formation is followed.

C. imberbe Warwa*,* family Combretaceae grows widely in Southern African forests. In Zimbabwe, Zambia, Namibia and Mozambique it is used in folk medicine for treating various diseases such as malaria, diarrhea and bilharzia (Dan et al., 2010). Chewing *Combretum imberbe* leaves is regarded as a remedy for coughing or a bad cold (Ribeiro et al., 2010). Its ashes are used as toothpaste (Ribeiro et al., 2010). Angeh et al. (2007) reported antibacterial and anti-inflammatory activity of a triterpenoid extracted from *Combretum imberbe*. The interactions of hemin with selected herbal extracts obtained from *Combretum imberbe* leaves were investigated in water-dimethylsulphoxide mixture at pH 5, 7.4 and 9 using spectrophotometric method.

MATERIALS AND METHODS

All of the solvents used were of analytical grade purchased from Merck Co. (Germany). Hemin chlorides, Artemisinin, Tris (hydroxymethyl)-methylamine (Tris), and hydrochloric acid were purchased from Sigma Chemical Co. (St Louis, Germany). Quinine sulfate dihydrate was purchased from Fluka.

Plant collection

Plant leaves were collected from Chindunduma Mountains, Mashonaland Central, Zimbabwe, in December 2011. Selection was based on interviews with local communities. The plant specimens were identified by a taxonomist at Harare Botanical Garden and voucher specimen 2011/6 was deposited in the Chemistry Department of Bindura University (natural product section). Leaves were air-dried in the shade for two weeks and then ground into a fine powder using a wooden mortar and pestle.

Extraction

Ground Leaves of about 100 g were subjected to sequential cold extractions for 5 h using hexane, ethyl acetate and methanol. The extracts were decanted and filtered through Whatman No. 1 filter paper and the resulting filtrates were dried under reduced pressure at 40°C on a Bucchi rotary evaporator. The percentage yield for each sample was determined and the crude extracts were stored in a freezer.

Preparation of buffer solutions

Tris-HCl buffer solutions were prepared by mixing different amounts of 0.2 $mol.dm^{-3}$ Tris and 0.2 $mol.dm^{-3}$ HCl to give the required pH. For 100 ml of Tris-HCl buffer, 25 ml of Tris was mixed with 20.7 ml of HCl and diluted with distilled water to achieve a pH of 7.4. For pH 9, 25 ml of Tris was mixed with 2.5 ml of HCl and diluting it with distilled water to 100 ml. 25 ml of Tris was mixed with 30.6 ml of HCl and diluted with distilled water to achieve a pH of 5.

Water- Dimethylsulphoxide (DMSO) mixture

Water-dimethylsulphoxide (DMSO) mixture was adopted as a solvent of choice because it does not present some limitations with regard to the solubility of the reacting parties and dimerization process of hemin can be well controlled (Mpiana et al., 2007).Forty percent aqueous DMSO solutions (v/v) were prepared by mixing 40 ml of DMSO with 60 ml of corresponding buffer so that the final pH of the mixture was 5, 7.4 or 9.

Hemin solutions

Stock solutions, 306 µmol L^{-1} in concentration were prepared by first dissolving 10 mg of hemin in 20 ml of DMSO, followed by addition of 30 ml of buffer solutions. Stock solutions of hemin were refrigerated at 4°C and stored in the dark.

Quinine

Stock solutions (0.002 $mol.L^{-1}$) were prepared by dissolving 16.2 mg with 25 ml of acidic distilled water and then 10 ml of DMSO, followed by 15 ml of buffer.

Artemisinin solutions

Artemisinin stock solutions, (0.002 mol L^{-1}) were prepared by dissolving 14.1 mg with 10 ml of DMSO, followed by 15 ml of buffer.

Extract solutions

Extract stock solutions were prepared by dissolving 14.1 mg with 10 ml of DMSO, followed by 15 ml of buffer.

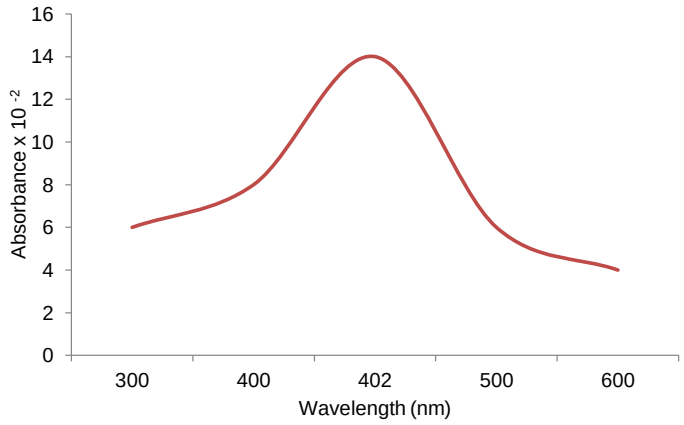


Figure 1. Spectra of hemin solutions in 40 % dimethylsulphoxide pH 5, temperature 36°C.

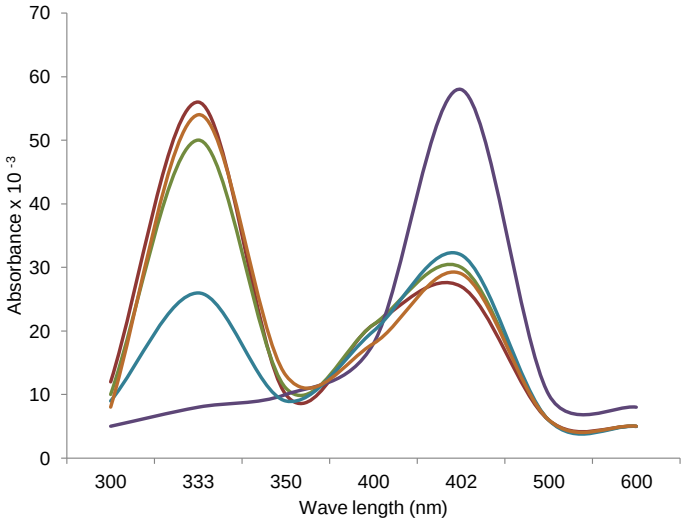


Figure 2. Hemin-drugs absorption spectrum after 10 h incubation pH 5, temperature 36°.

Hemin- drugs spectrophotometric titrations.

This was investigated in water-DMSO mixture at pH 5, 7.4 and 9 using spectrophotometric method. Titrations were carried out at the hemin characteristic Soret band at 402 nm by mixing a constant volume (0.3 ml) of hemin solution with various concentrations of quinine/ artemisinin / extract solutions. Before each measurement of absorbance, working solutions were incubated at 36°C for 10 h.

RESULTS AND DISCUSSION

Hemin was dissolved in 40% dimethylsulphoxide and it showed maximum wavelength at 402 nm (Figure 1). This was adopted as the working wave length.

The absorption wavelength at 333 nm is for derivatives of quinine, artemisia and extracts except for hexane extract. It can be seen that addition of quinine, artemisia and extracts modifies the hemin spectrum; however the peak maximums of hemin are still at about 402 nm. This reveals that the complexation does not involve significant changes in the structure of the porphyrin ring of the ferriprotoporphyrin IX. The results also show that quinine, artemisia and extracts produces an isosbetic point at 352 nm Figure 2. Titration of hemin by increasing amount of quinine, artemisia and extracts in mixed water-dimethylsulphoxide solution produced spectral changes shown in Figure 3 to 5. It can be seen that the hemin peak decreases with increasing total quinine, artemisia and extracts concentration except that for hexane extract. The results are similar to those observed on deuterohemin-quinine and hemin-chloroquine interactions in other mediums (Mpiana et al 2007; Tekwan and Walker, 2005; Gushimana et al., 1993).

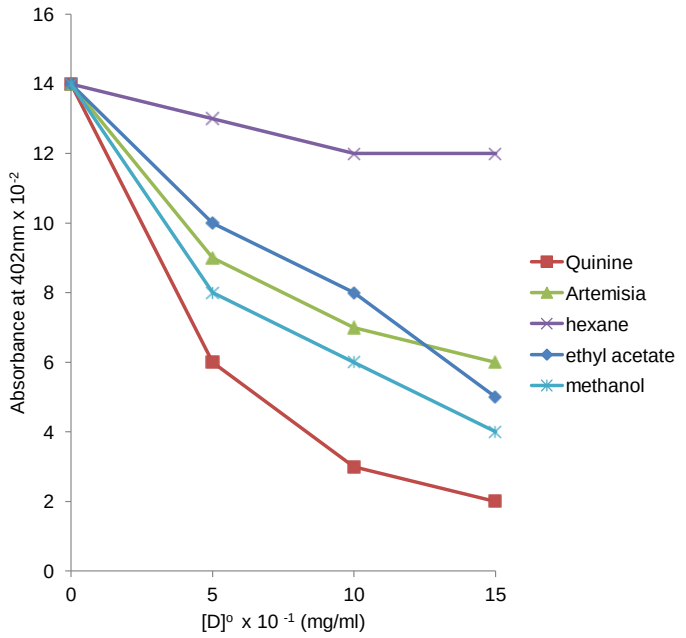


Figure 3. Absorbance of hemin at 402 nm at different concentrations of quinine, artemisia and extracts after 10 h incubation pH 5, temperature 36°C.

Binding constant of hemin-drug complexes

The interaction between hemin (H) and antimalarial drug (D) can be described according to the equilibrium;

$$H + D \rightleftharpoons HD \quad (1)$$

If solutions are dilute, the association constant K of complex *HD* can be written as:

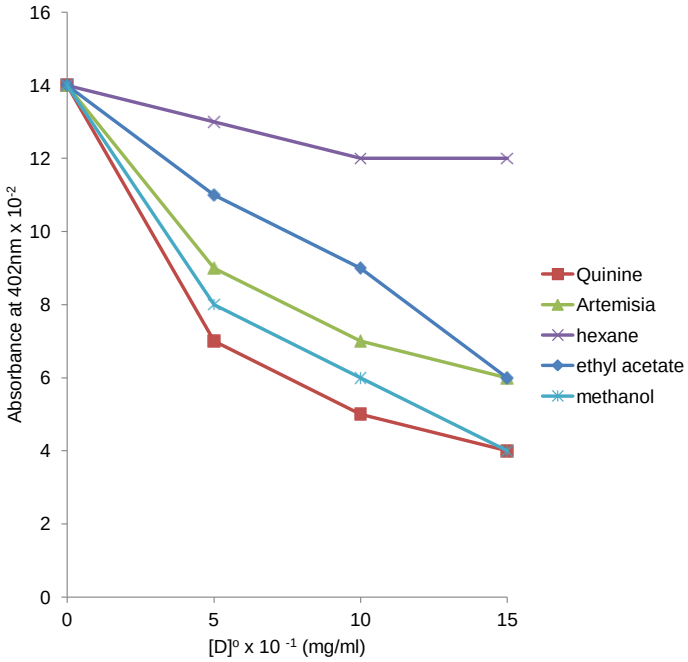


Figure 4. Absorbance of hemin at 402 nm at different concentrations of quinine, Artemisia and extracts after 10 h incubation pH 7.4, temperature 36°C.

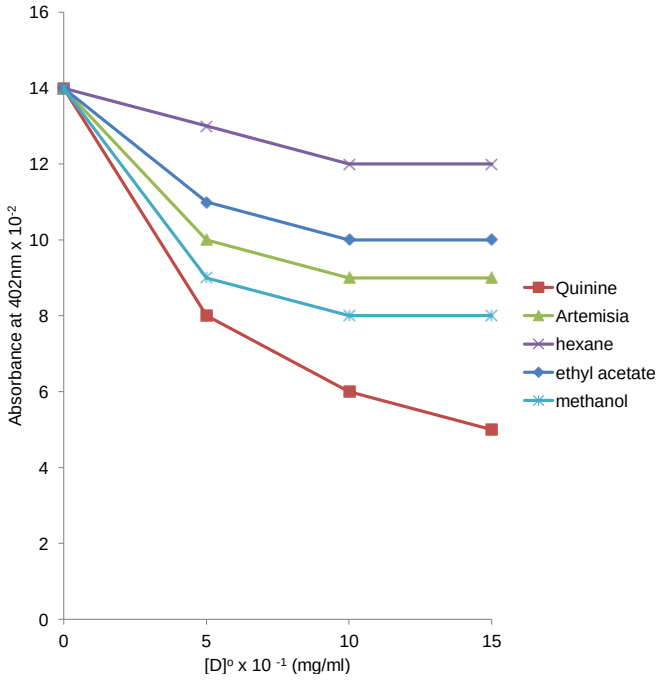


Figure 5. Absorbance of hemin at 402 nm at different concentrations of quinine, Artemisia and extracts after 10 hour incubation pH 9, temperature 36°C.

$$K = \frac{[HD]}{[H]_i[D]_i} \quad (2)$$

where [HD] is the concentration of complex and $[H]_i$ and $[D]_i$ are initial concentration of hemin and drug respectively.

It can also be seen that;

$$[H]_i = [H]_t + [HD] \quad (3)$$

where $[H]_t$ is the concentration of free hemin after time t Similarly for the drug the expression becomes

$$[D]_i = [D]_t + [HD] \quad (4)$$

where $[D]_t$ is the concentration of free drug after time t Combining equations 1 to 4 affords a quadratic equation:

$$[HD]^2 - ([H]_i + [D]_i + \frac{1}{K})([HD] + [H]_i[D]_i = 0 \quad (5)$$

One root of Equation 5 is given by;

$$[HD] = \frac{1}{2}[H]_i + [D]_i + \frac{1}{K} - \sqrt{([H]_i + [D]_i + \frac{1}{K})^2 - 4[H]_i [D]_i} \quad (6)$$

The optical density of the hemin-quinine, artemisia or extracts can be expressed as;

$$\frac{A}{d} = [H]\acute{\epsilon}_H + [HD]\ \acute{\epsilon}_{HD} \quad (7)$$

where *A* and *d* are the optical density and the light path, respectively, and $\acute{\epsilon}_H$ and $\acute{\epsilon}_{HD}$ are the molar extinction coefficients of hemin and its quinine, artemisia or extracts complex.

Combining Equations 3, 6 and 7 yield the following equation;

$$A = A_i + \frac{1}{2}d\Delta\acute{\epsilon}[H]_i + [D]_i + \frac{1}{K} - \sqrt{([H]_i + [D]_i + \frac{1}{K})^2 - 4[H]_i [D]_i} \quad (8)$$

where A_i is the molar extinction of hemin solution at $[D]_i$ =0, and ($\Delta\acute{\epsilon} = \acute{\epsilon}_{HD} - \acute{\epsilon}_H$) is the difference of the molar extinction coefficients between hemin complex and free hemin. The basic data are initial concentrations of hemin $[H]_i$ and quinine, artemisia or extracts $[D]_i$ and the corresponding optical absorption of hemin (A). Given this data (parameters) more importantly the equilibrium constant *K*, can be obtained according to Equation (8). Microsoft Origin 6.1 package computed K values for quinine, artemisia or extracts at different pH as shown in Table 1.

The complexation of ferriprotoporphyrin IX with the drugs plays the role of bringing back the hemin into solution such that it is prevented from polymerization.

Table 1. Binding constant of hemin- quinine, artemisia or extracts complexes at various pH.

pH	*K* (10^4 M)				
	Hemin-quinine	**Hemin- artemisia**	**Hemin- methanol extract**	**Hemin- ethyl acetate extract**	**Hemin- hexane extract**
5	1.101	0.612	1.011	0.410	0.010
7.4	1.103	0.611	1.010	0.401	0.010
9	0.501	0.610	0.408	0.311	0.010

Accumulation of hemin in the food vacuole will result in the death of the parasite (Mpiana et al., 2007; Meshnick, 2002). The capability of extracts to complex with hemin will inhibit the formation of hemozoin (hematin). The extract that has a greater affinity with hemin maintains more hemin in solution and is thus more effective. This means that methanolic extracts (K = 1,011 x 10^4M at pH 5) show the highest efficiency, followed by ethyl acetate extract (K = 0.410 × 10^4M at pH 5). Hexane extract revealed no affinity for hemin hence less effective. It can also be seen that values *of K* are pH-dependent. The dependence may be due to acidic-basic equilibrium influence on electrostatic interactions between hemin and the drugs (Steele et al., 2002; Bienvenu, 2007).

The decrease in absorbance of the hemin band can be as a result of two possible processes, either addition of micro molar concentrations of drug inducing aggregation of hemin or the changes may show drug association with hemin (Bienvenu, 2007). A large decrease in the absorbance of the band may indicate aggregation; equally large decreases may be caused by formation of p-p (donor-acceptor) complexes (Egan et al., 2000, Egan and Marques 1997). Generally, spectral changes of iron porphyrins in the visible region vary depending on the conditions of solvents and pH and the nature of interacting species. The decrease of hemin absorbance is dependent on the drug concentration. For this study dilution experiments showed that Beer's law is strictly adhered to in the concentration range studied thus providing no evidence of hemin aggregation within this concentration range (Mpiana et al., 2007)

The most plausible explanation for these spectral changes therefore, is the presence of drug-hemin association. Another feature on the titration curves Figure 2 is an appearance of an isosbetic point around 352 nm. Such behaviour indicates an equilibrium establishment between two species (Bilia et al., 2002). These spectral changes reveals progressive disruption of delocalized -electron system of the hemin tetrapyrrole ring. Similar results have been observed with *Momordica foetida* extracts (Froelich et al., 2007) and medicinal plant extracts (Steele et al., 2002).

Conclusion

In the light of the present results it has been shown that hemin, a product of hemoglobin degradation complex with crude extract of *Combretum imberbe*. It was observed that hemin complex more strongly with methanolic extract than with ethyl acetate and hexane extract, and binding constants were pH-dependent. The present results show that *Combretum imberbe* has a potential of becoming a future medication for malaria treatment. It shares a simillar mode of action as the highly successful quinolines drugs such as quinine. Future studies may focus on isolation identification and toxicity studies of the bioactive compounds.

REFERENCES

Angeh JE, Huang X, Sattler I, Swan GE, Dahse H, Härtl A, Eloff JN (2007). Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from *Combretum imberbe* (Combretaceae). Ethnopharmacy 1:56-60.

Bienvenu MK (2007). Interaction of quinolines and artemisinin based antimalarials drugs with ferriprotoporphyrin IX" (http://www.memoireonline.com)

Berman PA, Adams PA (1997). Artemisinin enhances Heme-catalyse oxidation of lipid membranes. Free Radical Bio. Med., 22: 1283-1288.

Bilia AR, Lazaria D, Messori L, Taglioli V, Temperini C, Vinceri FF (2002). Simple and rapid physico-chemical methods to examine action of antimalarial drugs with hemin: Its application to *Artemisia annua* constituents. Life Sci. 70:769-778.

Cointeaux L, Berrien JF, Peyrou V , Provot O, Ciceron L, Danis M, Robert A, Meunier B, Mayrargue J. (2003). Synthesis and antimalarial activity of 2-methoxyprop-2-yl peroxides derivatives. Bioorg. Med. Chem. Lett. 13:75 -77.

Dan V, Mchombu K, Mosimane A (2010). Indigenous medicinal knowledge of the San people: the case of Farm Six, Northern Namibia. Inf. Dev. 26(2):129-140.

Dechy-Cabaret O, Benoit-Vical F, Robert A, Magnaval JF, Seguela JP, Meunier B (2003). Synthesis and biological evaluation of a new trioxaquine containing a trioxane moiety obtained by halogenocyclisation of a hemiperoxyacetal. C. R. Chimie 6:153-160.

Dechy-Cabaret O, Robert A, Meunier B (2002). Synthesis and stereochemical study of a trioxaquine prepared from *cis*-bicyclo[3.3.0]octane-3,7-dione. C. R. Chimie 5:297-302.

Egan TJ (2004). Haemozoin formation as a target for the rational design of new antimalarials. Drug Des. Rev. 1:93-110.

Egan TJ, Marques HM (1997). The role of haem in the activity of chloroquine and related antimalarial drugs. Coordination Chem. Rev. 190:493-517.

Egan TJ, Hunter R, Kaschula CH, Marques HM, Misplon A, Walden J (2000). Structure-Function relationships in aminoquinolines: effects of amino and chloro groups on quinoline-hematin complex formation, inhibition of beta-hematin formation and, antiplasmodial activity. J. Med. Chem. 43:283-291.

Egan TJ (2002). Discovering Antimalarials: A New Strategy. Chem. Biol. 9:852-853.

Eggleson KK, Duffin KL, Goldberg DE (1999). Identification and

characterization of falcilysin, a metallopeptidase invovled in hemoglobin catabolism within the malaria parasite *Plasmodium falciparum*. J. Biol. Chem. 274:32411-32417.
Froelich S, Onegi B, Kakooko A, Siems K, Schubert C, Siems KJ (2007). Plants traditionally used against malaria: phytochemical and pharmacological investigation of *Momordica foetida*. Brazilian J. Pharmacol. 17(1):1-7.
Gong DH, Li JF, Yuan CY (2001). New and Facile Synthesis of 6-Methyl-2-trifluoromethy1-4- (O, O-dialkyl)phosphoryl-quinoline. Chin. J. Chem., 19: 1263.
Gushimana Y, Doepner B, Martinez-Hackert E, G. Ilgenfritz (1993). Kinetics of quinine-deuterohemin binding. Biophys. Chem. 47:153-162.
Kaschula CH, Egan TJ, Hunter R, Basilico N, Parapini S, Taramelli D, Pasini E, Monti D (2002). Structure-activity relationships in 4-aminoquinoline antiplasmodials. The role of the group at the 7-position. J. Med. Chem. 45:3531-3539.
Macreadie IG, Hagai G, Worachart S, Leann Tilley (2000). Antimalarial drug development and new drug targets. Parasitol. Today 16:438-444.
Mpiana PT, Mavakala BK, Yu Zhi-Wu (2007). Interaction of Artemisinin Based Antimalarial Drugs with Hemin in Water-DMSO Mixture. Int. J. Pharm. 3:302-310.
Meshnick SR (2002). Artemisinin: mechanisms of action, resistance and toxicity. Int. J. Parasitol. 32:165-1960.
Pagola S, Stephens PW, Bohle DS, Kosar AD, Madsen SK (2000). The structure of malaria beta-haematin. Nature 404:307-310.
Pandey KC, Singh N, Arastu-Kapur S, Bogyo M, Rosenthal PJ (2006). Falstatin, a cysteine protease inhibitor of *Plasmodium falciparum*, facilitates erythrocyte invasion. PLoS Pathog. 2(11):117.
Ribeiro A, Romeiras MM, Tavares J, Faria MT (2010). Ethnobotanical survey in Canhane village, district of Massingir, Mozambique: medicinal plants and traditional knowledge. J. Ethnobiol. Ethnomed. 6:33.
Robert A, Yannick C, Meunier B (2002). NMR characterization of covalent adducts obtained by alkylation of heme with the antimalarial drug artemisinin. Inorg. Chimica Acta 339:488-496.
Rosenthal PJ, Sijwali PS, Singh A, Shenai BR (2002). Cysteine proteases of malaria parasites: targets for chemotherapy. Curr. Pharm. Des. (8):1659-1672.
Spiller DG, Bray PG, Hughes RH, Ward SA, White MRH (2002). The pH of the *Plasmodium falciparum* digestive vacuole: holy grail or dead-end trail? Trends Parasitol. 18:441-444.
Steele JCP, Phelps RJ, Simmonds MSJ, Warhurst DC, Meyer DJ (2002). Two novel assays for the detection of haemin-binding properties of antimalarials evaluated with compounds isolated from medicinal plants. J. Antimicrob. Chemother. 50:25-31.
Tekwan BL, Walker LA (2005). Targeting the hemozoin synthesis pathway for new antimalarial drug discovery: Technologies for *in vitro* β-hematin formation assay. Comb. Chem. High Throughput Screen 8:63-79.
WHO (2005). The world health report 2005: Make every mother and child count. World Health Organization, Geneva. http://www.who.int/whr/2005/en/index.html.
Wu Y, Wang X, Liu X, Wang Y (2003). Data-mining approaches reveal hidden families of proteases in the genome of malaria parasite. Genom. Res. 13:601-616.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2211-2223, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.381
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Effect of vitamin E and α-lipoic acid on nano zinc oxide induced renal cytotoxicity in Rats

Nouf Al Rasheed[1], Nayira A. Abdel Baky[1,2]*, Nawal Al Rasheed[1], Wedad Shebly[1], Aly Mohamed Ahmed[3] and L. M. Faddah[1]

[1]Department of Pharmacology, Faculty of Pharmacy, King Saud University, Riyadh, Saudi Arabia.
[2]Department of Pharmacology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.
[3]Department of Anatomy and Stem Cell Unit, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia.

Accepted 4 June, 2012

The objective of this study is to detect toxic effects of two different doses of nano zinc oxide (ZnO-NP) particles. Moreover, a comparative study was conducted to modulate this toxic effect by the use of two natural antioxidants vitamin E (Vit E) and α-lipoic acid (α-Lip). The results of the current study revealed that ZnO-NP treatment produced hazard effects which were confirmed by the elevation of vascular endothelium growth factor (VEGF) as well as nitric oxide levels in rat serum. Inflammatory markers, including tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), C-reactive protein (CRP) and IgG were also elevated in rat serum compared to control normal group. Additionally, blood glucose level, as well as serum urea, and creatinine levels were significantly increased in rats intoxicated with ZnO-NP compared to normal control group. On the other hand, reduced glutathione (GSH) level was decreased in renal tissue. These biochemical findings were supported by the histopathological examination of renal tissue and the hazardous effects were dose dependant. Treatment of rats with Vit E or α–Lip along with ZnO-NP administration significantly alleviates most of the elevated previous biochemical parameters. Histopathological examination revealed that animals that received α-Lip or Vit E along with ZnO-NP showed moderate histopathological changes in the form of shrinkage and fragmentation of moderate number of glomeruli with exfoliation of tubular epithelial cells and tubular casts in moderate number of renal tubules. It was concluded that treatment with either α-Lip or Vit E has a beneficial effect against ZnO-NP oxidative stress and related vascular complications.

Key words: C-reactive protein (CRP), interleukin-6 (IL-6), nano zinc oxide, tumor necrosis factor alpha (TNF-α), vascular endothelium growth factor (VEGF).

INTRODUCTION

Nanoparticles are potential hazardous compound that can stick to cell membrane and penetrate into specific cells in the body. The surface of the nanoparticles could be modified and adapted to the environmental change so as to avoid the recognition and elimination by the human body (Salata, 2004). Nanoparticles could translocate from the lumen of the intestinal tract via aggregations of intestinal lymphatic tissue (Peyer's patches [PP]) containing M-cells (specialized phagocytic enterocytes) (Delie, 1998). Accidental or involuntary contact during production or use is most likely to happen via the lungs from where a rapid translocation through the blood stream is possible to other vital organs (Brook et al., 2004). Due to their small size, nanoparticles can translocate from these entry portals into the circulatory and lymphatic systems, and ultimately to body tissues and organs. Some nanoparticles, depending on their

*Corresponding author. E-mail: nayiraabdelbaky@yahoo.com. Tel: +9661 2913728. Fax: +9661 2913728.

composition and size, can produce irreversible damage to cells by oxidative stress or/and organelle injury (Nam et al., 2003).

The distribution of these NP to other organs, such as liver, spleen, brain, heart and kidney may lead to dysfunction of these organs as well. Exposure to some nanoparticles is associated to the occurrence of autoimmune diseases such as systemic lupus erythematous, scleroderma a, and rheumatoid arthritis (Buzea et al., 2007). It has also been proposed that the size of NP surface area greatly increases their ability to produce reactive oxygen species (ROS) (Moller et al., 2010). Published data indicated that numerous metallic elements are selective nephrotoxins that preferentially accumulate and produce cellular injury in the kidney which permits unrestricted use, distribution and reproduction in any medium (Igor Pujalté et al., 2011).

Alteration in total reduced glutathione (GSH) (tGSH) level content in cells can be considered as an indication of adaptive response of the cell to oxidative damage. Nanoparticles of ZnO at high concentrations significantly decreased the tGSH level compared with control values, indicating functional damage to kidney tissues (Moron, 1979). GSH is essential for maintaining cellular integrity. Thus, it protects cells from oxidative damage. GSH also has a major role in restoring other free radical scavengers and antioxidants such as Vit E and L-ascorbic acid (Ankush Gupta et al., 2011). GSH is able to conjugate with endogenous or exogenous substances and prepare them to eventual excretion; this occurs enzymatically by series of enzymes called glutathione transferase. This detoxification of compounds by conjugation with GSH occurs mainly in kidney and liver (Lomaestro and Malone, 1995). Tumor necrosis factor alpha (TNF-α) is an important upstream regulator of various cytokines induced in response to diverse stimuli as well as ROS. Recently, *in vivo* cell experiments showed that exposure to ZnO-NP resulted in oxidative damage and inflammation response in vascular endothelial cells (Gojova et al., 2007). Considering the hazards of treatment failure, drug resistance and heavy costs associated with current drug therapy, natural products and medicinal plants have attracted interest of many researchers in this field (Innsan et al., 2011; Gavanji et al., 2011; Lin et al., 2011).

α–Lipoic acid is a natural cofactor for pyruvate dehydrogenase complex that occurs in the mitochondria of different tissues as liver, kidney and heart tissues (Biewenga et al., 1997). α-Lipoic (α–Lip) acid is a potent antioxidant. Three distinct antioxidant actions of α-Lip and its reduced form, dihydrolipoic acid, have been observed to posses reactive oxygen species scavenging activity; capacity to regenerate endogenous antioxidants such as glutathione and vitamins C and E and metal-chelating activity (Biewenga et al., 1997; Packer et al., 1995). A-Lipoic acid administration to obese Zucker rats improves insulin-stimulated glucose uptake in muscle (Jacob et al., 1996; Streeper et al., 1997). α–Lipoic acid was reported to cause acute hypoglycemia by decreasing hepatic glucose output (Randle et al., 1988), lowered systolic blood pressure, glucose level and tissue aldehyde conjugates and attenuated adverse renal vascular changes (Vasdev et al., 2000).

Vitamin E is also essential for maintaining the integrity, function and flexibility of cell membranes, Vit E is also an important fat soluble antioxidant. Tocopherol serves to detoxify and remove reactive nitrogen species (RNS) from the body. Tocotrienols work best as a team to quench the lipid and nitrogen free radicals known to cause injury to cells and tissues. Aside from its antioxidant properties, Vit E may support normal cell division and immune health, influence blood coagulation speed and provide protection to neural tissues (Skrzydlewska et al., 2001; Coulter et al., 2006). The objective of this study was to assess renal cell responses to ZnO-NPs so as to show their potential toxic biological responses and investigate the renoprotective effect of α-Lip and Vit E.

MATERIALS AND METHODS

Chemicals

The 50-nm ZnO powders were purchased from Sigma Co. (USA). All chemicals used were of high analytical grade, product of Sigma and Merck companies.

Animals and treatments

Fifty Wistar albino rats weighing 180 to 200 g were used. The rats were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University. Animals have been kept in special cages and maintained on a constant 12-h light/12-h dark cycle with air conditioning and temperature ranging 20 to 22°C and humidity (60%). Rats were fed with standard rat pellet chow with free access to tap water *ad libitum* for one week before the experiment for acclimatization. Animal experimental protocols were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Ethic Committee in Research of the King Saud University, College of Pharmacy.

After one week acclimation, the rats were kept fasting over night before treatment and randomly divided into two classes according to the dose of ZnO-NP administered to rats. Class I consisted of four groups (ten rats per group): G1: normal healthy animals; G2 - G4: animals orally administered 600 mg/kg body weight/day ZnO-NP for 5 days (Wang et al. 2008a) and divided as follows: G2: ZnO-intoxicated animals with a low oral dose (600 mg/kg/day) daily for 5 days; G3: ZnO-intoxicated animals co-administered α-Lip (200 mg/kg) daily (Sharma and Gupta, 2003); G4: ZnO-intoxicated animals co-administered Vit E (100 mg/kg) daily (Ishrat et al., 2009). On the other hand, Class II consisted of three groups (G5-G7; ten rats per group) orally administered 1 g/kg body weight/day for 5 days ZnO-NP (Wang et al. 2008a), and divided as follows: G5: ZnO-intoxicated animals with a high dose (1 g/kg/day) daily for 5 days; G6: ZnO-intoxicated animals co-administered α-Lip (200 mg/kg) daily; G7: ZnO-intoxicated animals co-administered Vit E (100 mg/kg) daily.

A-Lipoic acid and Vit E were orally administered daily for three

constitutive weeks from the beginning of the experiment. The body weights of rats were recorded before and after the administration period. Three weeks later and after 24 h of the last dose administration, rats were fasted overnight then sacrificed and the blood was collected. Serum was separated and kept at -80°C for different biochemical estimations. Both the kidneys were harvested through a midline incision, rinsed in cold isotonic saline, homogenized, and frozen at -80°C for estimations of GSH content. Another three kidneys from each group were kept in 4% formalin for histopathological examination.

Serum biochemical analysis

Determination of TNF-α level

TNF-α in serum was determined using commercially available enzyme-linked immunosorbent assays (ELISA) following the instructions supplied by the manufacturer (Duo Set kits, R and D Systems; Minneapolis, MN, USA). The results are shown as pg of cytokine per ml.

Determination of C-reactive protein (CRP) level

CRP was measured with latex-enhanced immunonephelometry on a Behring BN II nephelometer (Dade Behring). In this assay, polystyrene beads coated with rat monoclonal antibodies bind CRP present in the serum sample and form aggregates. The intensity of scattered light is proportional to the size of the aggregates and thus concentration of CRP present in the sample. The intra-assay and inter-assay coefficients of variation for CRP were 3.3 and 3.2%, respectively. The lower detection limit of the assay was 0.15 mg/L (Kim et al., 2010).

Determination of vascular endothelium growth factor (VEGF) level

The level of VEGF in serum was determined at 492 nm by quantitative colorimetric sandwich ELISA (R and D systems, UK) in accordance with the manufacturer's instructions (Yao et al., 2005). Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer.

Determination of IgG level

The IgG level was measured in serum using a sandwich ELISA. The capture antibody was goat anti-rat IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Standards were prepared from rat IgG (Sigma Chemical Co., St. Louis, MO). Goat anti-rat IgG peroxidase conjugates were diluted at 1:250 in phosphate buffered saline/bovine serum albumin (PBS/BSA) (from Kirkegaard and Perry Laboratories, Inc.) and used as detecting antibodies. The chromogenic substrate used was 2,2′-azino-di[3-ethylbenzthiazoline sulfonate] (ABTS; Kirkegaard and Perry Laboratories, Inc.). Color development was detected via optical density at 405 nm using an automated ELISA plate reader (Bio-tek Instruments, Inc., Winooski, VT) and immunoglobulin concentrations were determined by comparing sample color development to standard curves (Kineticalc, Bio-tek Instruments Inc.).

Determination of interleukin-6 (IL-6) level

IL-6 was measured by ultra-sensitive ELISA (Quantikine HS Human IL-6 Immunoassay; R and D Systems, Minneapolis, MN) with an analytical CV of 6.3% and a detection level of 0.04 pg/ml (Kaden, 2007).

Determination of nitrite level

Nitrite level concentration (an indirect measurement of NO synthesis) was assayed using Griess reagent (sulfanilamide and N-1-naphthylethylenediamine dihydrochloride) in acidic medium (Moshage et al, 1995).

Determination of glucose level, serum urea, creatinine and uric acid level

Glucose level was estimated using the method of Trinder (1969). Moreover, serum was assayed for urea, creatinine, and uric acid by using standard diagnostic kits.

Determination of GSH level in renal tissue

Renal content of GSH was estimated according to the method of Moron et al. (1979).

Histopathological technique

Samples of kidney tissues were collected to be fixed in 4% formaldehyde for 24 h, and then they were dehydrated in ascending grades of ethyl alcohol, then cleared in xylene and embedded in paraffin. Paraffin blocks were cut by microtone at 4 µM, and then fixed on glass slides to be ready for staining. Subsequently, sections were stained with hematoxylin and eosin (H and E), hydrated in descending grades of alcohol, stained with hematoxylin to stain the nuclei, and then stained with eosin to stain the cytoplasm. Another set of unstained slides were stained with Masson's trichrome stain to visualize deposition of collagen fibers which usually takes the green coloration (Smith and Bruton1978).

Statistical analysis

Data are presented as the mean ± S.D. Statistical analysis was performed using Instat-3 computer program (Graph pad software Inc, San Diego, CA, USA). One way analysis of variance (ANOVA) followed by Bonferroni multiple tests was used to determine the differences between means of different groups. The level of significance was set at $p \leq 0.05$.

RESULTS

The current investigation revealed that ZnO-NP treatments either in low or high dose did not affect neither body weight nor kidney weight compared to control group. Also, administration of the two antioxidants had no effect on body or kidney weights (Tables 1 and 2). Oral administration of the two doses of ZnO-NP significantly elevated NO, glucose and IgG serum levels, whereas the elevation in VEGF level was dose-dependent compared to normal control values (Figures 1 and 3). Administration of either α-Lip or Vit E markedly down regulated the previous elevated biomarkers. Moreover TNF- α, IL6 and CRP serum levels were significantly elevated in both low

Table 1. Effect of α –Lip or Vit E treatments on body weight, kidney weight, and kidney/body weight % in intoxicated rats with small dose of ZnO-NP particles.

Groups	Body Weight (g)		Kidney Weight (g)	Kidney /Body Weight %
	Initial	Final		
Control	240.6 ± 8.39	266 ± 24.73	1.23 ± 0.154	0.443 ± 0.190
ZnO-NP	254.4 ± 15.15	279.6 ± 9.50	1.32 ± 0.214	0.415 ± 0.044
α –Lip	249.4 ± 14.84	277 ± 20.30	1.31 ± 0.262	0.493 ± 0.088
Vit E	248.2 ± 12.27	271.8 ± 19.66	1.22 ± 0.092	0.499 ± 0.064

Data are presented as mean ± SD of 10 rats.

Table 2. Effect of α –Lip or Vit E treatment on body weight, kidney weight and Kidney/body weight % in intoxicated rats with high dose of ZnO-NP particles.

Groups	Body Weight (g)		Kidney Weight (g)	Kidney/Body Weight %
	Initial	Final		
Control	256 .9 ± 8.38	281.8 ± 20.82	1.23 ± 0.130	0.485 ± 0.088
ZnO-NP	235.5 ± 18.66	266.6 ± 25.83	0.93 ± 0.26	0.366 ± 0.151
α –Lip	250.4 ± 20.30	282.8 ± 38.85	1.33 ± 0.236	0.477 ± 0.088
Vit E	245 ± 20.58	270.6 ± 18.57	1.17 ± 0.333	0.428 ± 0.044

Data are presented as mean ± SD of 10 rats.

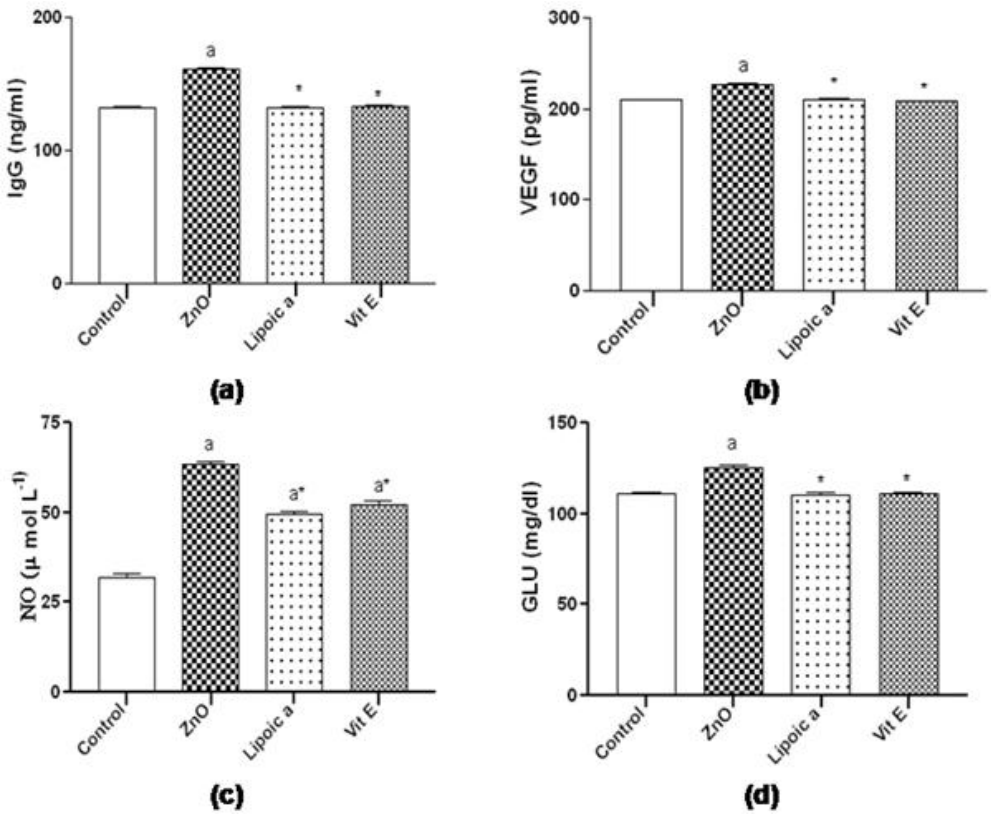


Figure 1. Effect of α-Lip or Vit E treatments on serum IgG (a), VEGF (b), total nitrate/nitrite (c) and glucose (d) levels in intoxicated rats with small dose o ZnO-NP particles. Values are expressed as mean ± S.E. $^{a}P < 0.001$ compared to normal control group, *P < 0.001 compared to ZnO-NP intoxicated group respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

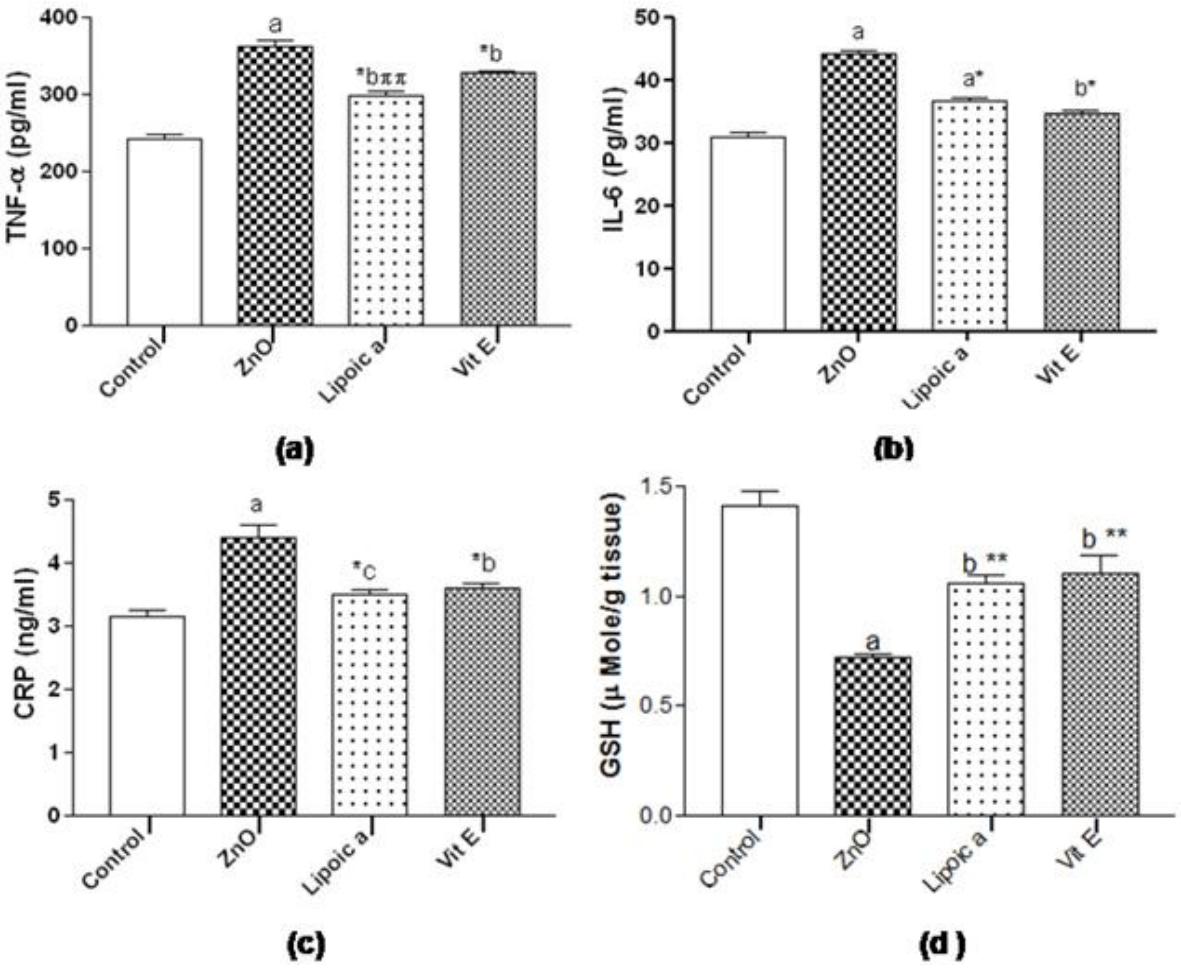


Figure 2. Effect of α-Lip or Vit E treatment on serum TNF-α (a), IL-6 (b), CRP (c), and GSH (d) levels in intoxicated rats with small dose of ZnO-NP. Values are expressed as mean ± S.E. $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$ compared to normal control group, *P<0.001 ,**P<0.01 compared to ZnO-NP intoxicated group, $^{\pi\pi}P < 0.001$ compared with vitamin E group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

and high doses ZnO-NP treatments compared to untreated group. A-Lipoic acid or Vit E treatments reduced these elevated parameters (Figures 2 and 4). On the other hand, renal GSH level decreased upon ZnO-NP treatments. Administration of either α-Lip or Vit E ameliorated the reduced GSH level (Figures 2 and 4). There was also a significant increase in urea and creatinine levels in serum of ZnO-NP intoxicated rats compared to normal control group (p<0.05) (Tables 3 and 4). Vitamin E significantly decreased serum creatinine level compared to rats intoxicated with high dose of ZnO-NP ($p < 0.05$).

In general, it is obvious that α-Lip and Vit E have nearly the same antioxidant effect. The previous biochemical parameters were supported by the histopathological examination which revealed that kidney sections stained with H and E treated with high dose of ZnO-NP showed massive atrophy and fragmentation of numerous glomeruli. In addition the renal tubules showed epithelial exfoliation, degeneration and necrosis. Some of renal tubules showed casts in their lumina. Severe congestion was observed in renal interstitium (Figure 5B). Treatment with α-Lip showed moderate histopathological changes in the form of shrinkage and fragmentation of few glomeruli with exfoliation of tubular epithelial cells and tubular casts in few renal tubules (Figure 5C). Animals that received ZnO-NP and Vit E showed histopathological changes in the form of marked hyperplasia of glomerular mesangial cells in many glomeruli, obliteration of many capsular spaces and necrosis and exfoliation of tubular epithelial cell lining of few renal tubules (Figure 5D).

On the other hand, Kidney sections stained with Masson's trichrome, of control group showed minimum amount of collagen fibers in the form of thin rim in between the renal tubules in the interstitial tissue (Figure 6A). While animals receiving high dose of ZnO-NP showed marked increase of deposition of collagen fibers

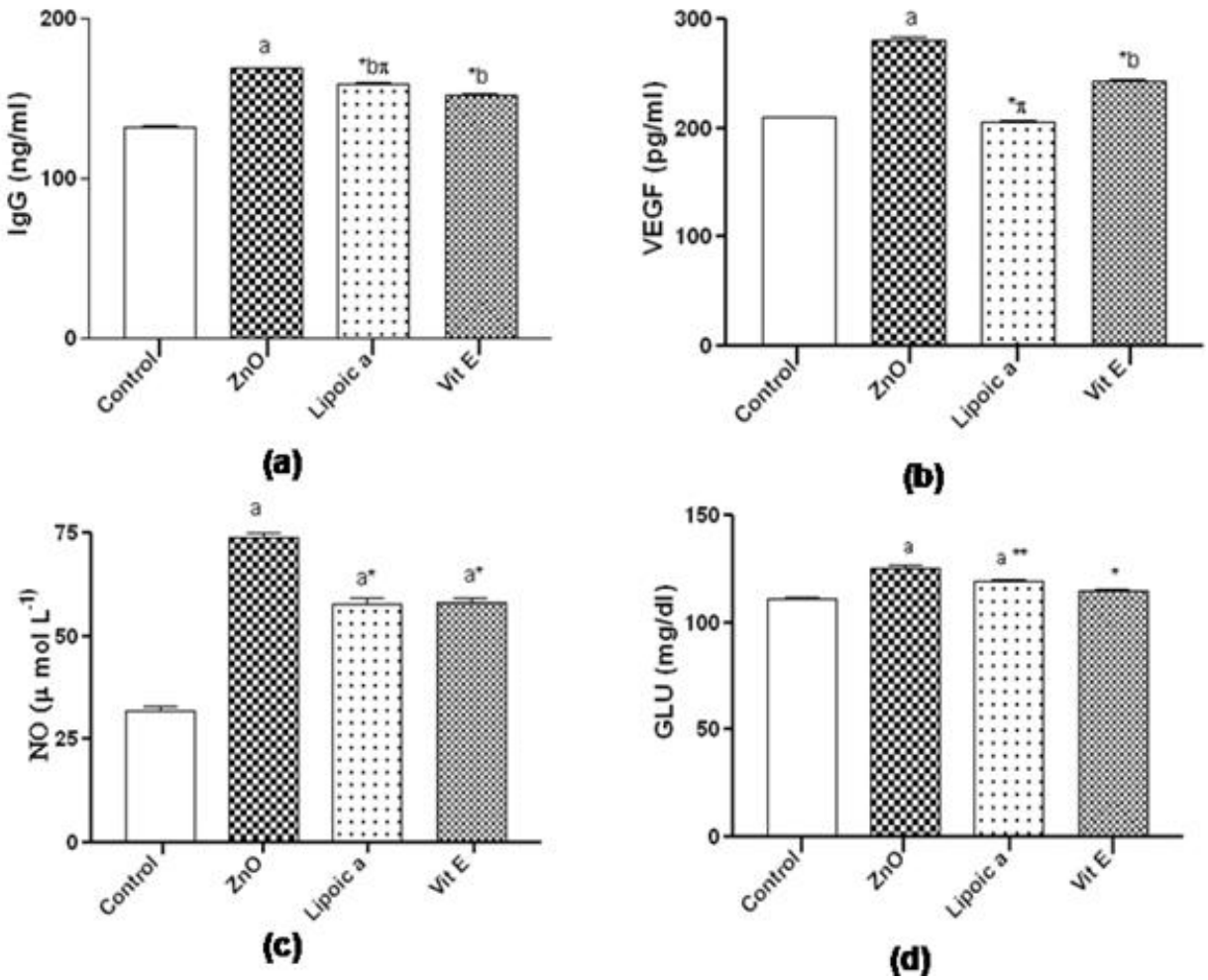


Figure 3. Effect of α-Lip or Vit E treatment on serum IgG (a), VEGF (b), total nitrate/nitrite (c), and glucose (d) levels in intoxicated rats with large dose of ZnO-NP particles. Values are expressed as mean ± S.E. $^{a}P < 0.001$, $^{b}P < 0.01$ compared to normal control group, *P < 0.001, **P < 0.01 compared to ZnO-NP intoxicated group, $^{\pi}P \leq 0.05$ compared with vitamin E group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

in the interstitial tissue (Figure 6B), administration of α-Lip along with ZnO-NP showed mild increase of collagen deposition (Figure 6C). Vitamin E with ZnO-NP showed moderate increase of collagen deposition (Figure 6D). Animals that received low dose of ZnO-NP showed either atrophy and fragmentation or mesangial hyperplasia of few glomeruli. However, many renal tubules showed epithelial exfoliation, degeneration and necrosis. In addition, few renal tubules showed casts in their lumina. Severe congestion was observed in renal interstitium (Figure 7B). Co-administration of ZnO-NP and α-Lip produced a shrinkage and fragmentation of few glomeruli with necrosis and exfoliation of tubular epithelial cells and tubular casts in few renal tubules (Figure 7C). In addition, animals that received ZnO-NP and Vit E showed moderate mesangial hyperplasia in many glomeruli with necrosis and exfoliation of tubular epithelial cells and tubular casts in few renal tubules (Figure 7D). Animals that received low dose of ZnO-NP showed marked increase of collagen fibers in the interstitial tissue (Figure 8B). ZnO-NP and α-Lip and or Vit E administration showed mild increase of collagen deposition (Figure 8C and 8D), respectively. Pathological changes of low-dose group are less than those of high dose group; also collagen deposition in low-dose group is much less than high dose group.

DISCUSSION

Nanoparticles are known to disseminate to several organs such as liver, spleen, kidneys, brain or heart (Oberdorster et al., 2005; Jain et al., 2008). Kidneys play an important role in eliminating xenobiotics from the body, and thus NPs absorbed in the systemic circulation can be excreted by renal clearance (Schipper et al., 2009). Until now, little attention has been paid to renal cells as a target for NP toxicity. This study aimed to

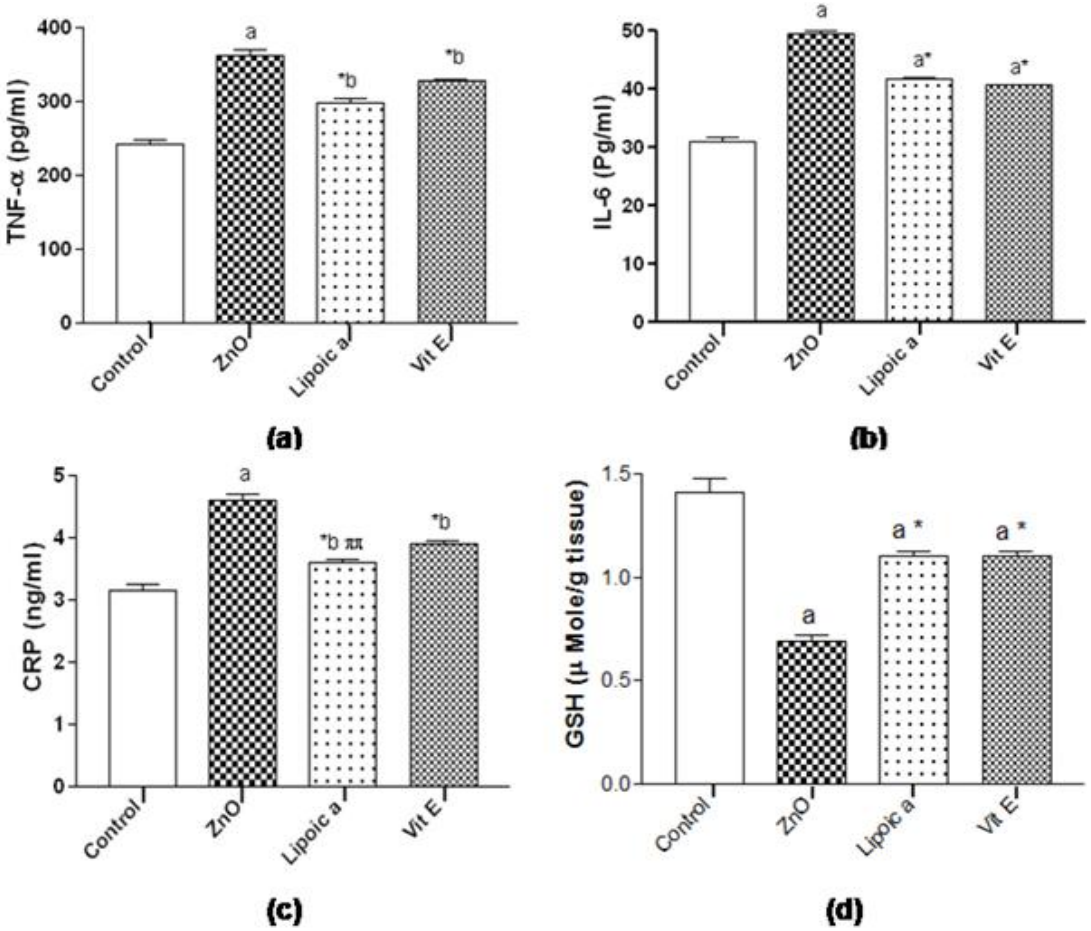


Figure 4. Effect of α-Lip or Vit E treatments on serum TNF-α (a), IL-6 (b), CRP (c), and GSH (d) levels in intoxicated rats with large dose of ZnO-NP particles. Values are expressed as mean ± S.E. [a]P<0.001, [b]P<0.01 compared to normal control group, *P<0.001 compared to ZnO-NP intoxicated group, ππP < 0.001 compared with Vit E group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

Table 3. Effect of α–Lip or Vit E treatment on serum creatinine, urea and uric acid levels in intoxicated rats with low dose of ZnO-NP.

Parameters	Control	ZnO-NP	α –Lip	Vit E
Creatinine(mg/dl)	0.5 ± 0.077	0.61 ± 0.087	0.57 ± 0.079	0.53 ± 0.026
Urea(mg/dl)	22.4 ± 5.01	33.3 ± 7.03 [a]	29.6 ± 5.77	28.8 ± 2.82
Uric acid(mg/dl)	2.2 ± 0.35	2.72 ± 0.60	2.54 ± 0.66	2.34 ± 0.51

Data are presented as mean ± S.D. of 10 rats, [a] $P \leq 0.05$ compared with normal group using ANOVA followed by Bonferroni as a post-ANOVA test.

Table 4. Effect of α–Lip or Vit E treatment on serum creatinine, urea and uric acid level in intoxicated rats with high dose of ZnO-NP particles.

Parameters	Control	ZnO-NP	α –Lip	Vit E
Creatinine (mg/dl)	0.5 ± 0.073	0.73 ± 0.037 [a]	0.61 ± 0.077	0.60 ± 0.044 [*]
Urea (mg/dl)	20.4 ± 5.01	34.3 ± 5.77 [b]	28.9 ± 4.66	27.97 ± 2.05
Uric acid (mg/dl)	2.2 ± 0.56	3.12 ± 0.56	2.94 ± 0.88	2.99 ± 0.60

Data are presented as mean ± S.D. of 10 rats, [a] $P \leq 0.001$, [b] $P \leq 0.01$, [c] $P \leq 0.05$ compared with normal group, [*] $P \leq 0.05$ compared with ZnO-NP intoxicated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test

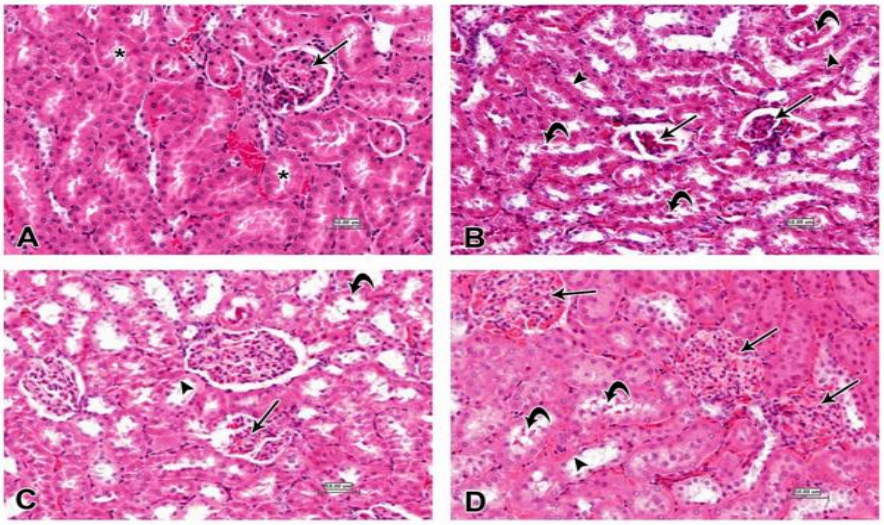


Figure 5. Photomicrographs of kidney sections stained with H and E from animals received high dose of nZnO. Scale bar= 50 µM. (A) Kidney from control animal showing normal renal corpuscle (arrow) and renal tubules (asterisk). (B) Kidney from animal that received high dose of ZnO-NP showing marked shrinkage and fragmentation of glomeruli (arrow) and necrosis (arrow head) and exfoliation (curved arrow) of epithelial cell lining of many renal tubules. (C) Kidney from animal that received high dose of ZnO-NP and α-Lip acid showing shrinkage and fragmentation of few glomeruli (arrow) and necrosis (arrow head) and exfoliation (curved arrow) of epithelial cell lining of few renal tubules. (D) Kidney from animal that received high dose of ZnO-NP and Vit E showing marked hyperplasia of glomerular mesangial cells in many glomeruli (arrow) with obliteration of capsular spaces. Few renal tubules show necrosis (arrow head) and exfoliation (curved arrow) of their epithelial cell lining.

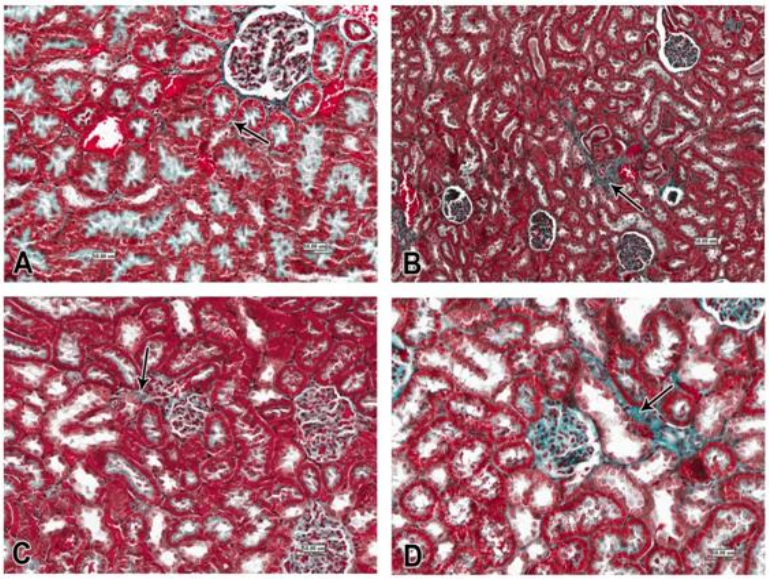


Figure 6. Photomicrographs of kidney sections stained with Masson's trichrome from animals received high dose of nZnO. Scale bar=50 µM. (A) Kidney from control animal showing thin rim of collagen fibers (arrow) in between renal tubules. (B) Kidney from animal that received high dose of ZnO-NP showing marked increase of collagen fibers deposition (arrow). (C) Kidney from animal that received high dose of ZnO-NP and α-Lip showing mild increase of collagen fibers deposition (arrow). (D) Kidney from animal that received high dose of ZnO-NP and Vit E showing moderate increase of collagen fibers deposition (arrow).

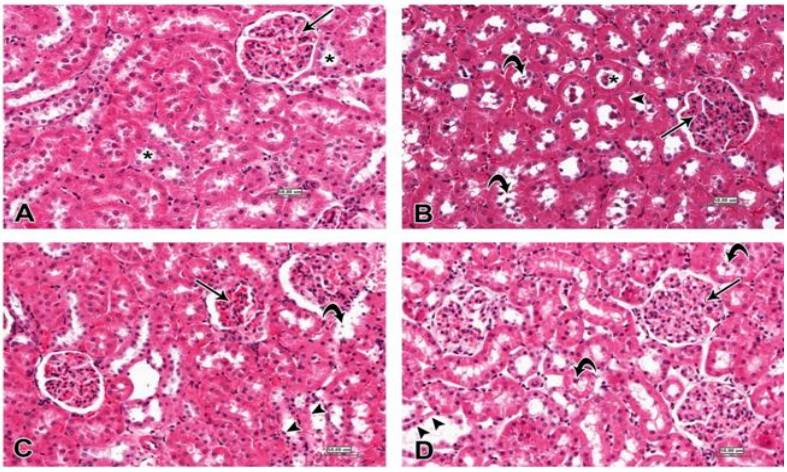


Figure 7. Photomicrographs of kidney sections stained with H and E from animals received low dose of nZnO. Scale bar = 50 µM. (A) Kidney from control animal showing normal renal corpuscle (arrow) and renal tubules (asterisk). (B) Kidney from animal that received low dose of ZnO-NP, showing mesangial hyperplasia in few glomeruli (arrow) and necrosis (arrow head) and exfoliation (curved arrow) of epithelial cell lining of many renal tubules. Few tubules show casts (asterisks). (C) Kidney from animal that received low dose of ZnO-NP and α–Lip showing shrinkage and fragmentation of few glomeruli (arrow) and necrosis (arrow head) of epithelial cell lining of few renal tubules. (D) Kidney from animal that received low dose of ZnO-NP and Vit E showing moderate hyperplasia of glomerular mesangial cells in many glomeruli (arrow). Few renal tubules show necrosis (arrow head) and exfoliation (curved arrow) of their epithelial cell lining.

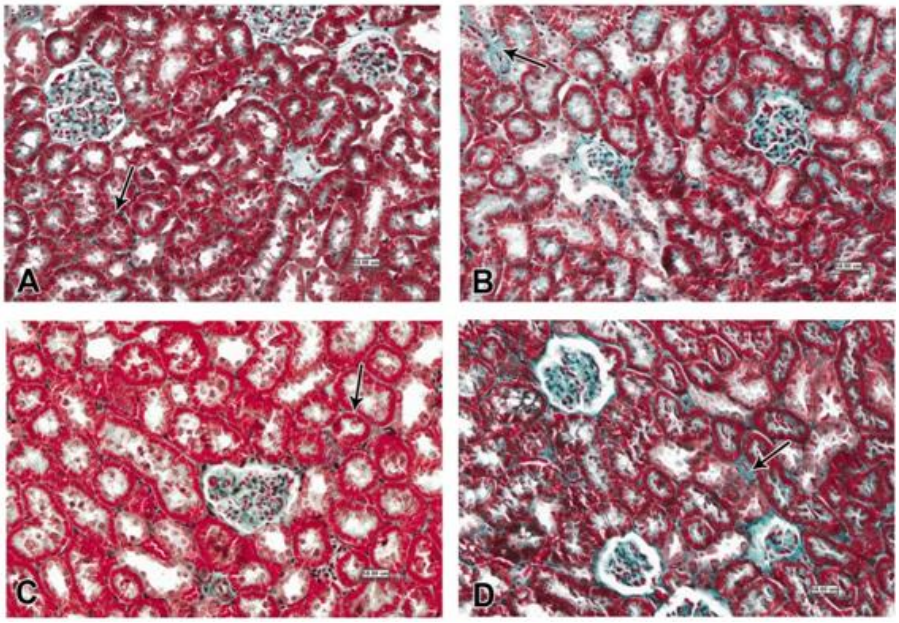


Figure 8. Photomicrographs of kidney sections stained with Masson's trichrome from animals received low dose of nZnO. Scale bar=50 µM. (A) Kidney from control animal showing thin rim of collagen fibers (arrow) in between renal tubules. (B) Kidney from animal that received low dose of ZnO-NP showing marked increase of collagen fibers deposition (arrow). (C) Kidney from animal that received low dose of ZnO-NP and α-Lip showing mild increase of collagen fibers deposition (arrow). (D) Kidney from animal that received low dose of ZnO-NP and Vit E showing mild increase of collagen fibers deposition (arrow).

investigate human renal cell responses to manufactured NPs in order to highlight their potential toxicity and/or biological responses, and investigate the effect of Vit E or α –Lip treatment on ZnO-NPS induced renal cytotoxicity.

In the present work, ZnO-NP significantly elevated the levels of TNF-α, IL-6 and VEGF compared to normal control group. This is in agreement with the study of Tsuo et al. (2010) who clarified the inflammatory effects of ZnO-NP particles on vascular endothelial cells, revealing that ZnO-NP particles induced a dose-dependent increase in the expression of intercellular adhesion molecule-1 (ICAM-1), an indicator of vascular endothelium inflammation, protein expression and marked increases in NF-κB reporter activity. Additionally, TNF-α, a typical inflammatory cytokine, induced ICAM-1 expression in an NF-κB-dependent manner, and ZnO-NP synergistically enhanced TNF-α-induced ICAM-1 expression of vascular disease. In the present study, the level of TNF-α was reduced post α–Lip treatment as compared to ZnO-NP treated group. These results were in accordance with a previous study in which α-Lip strongly inhibited TNF-α and induced mRNA expression of monocyte chemo attractant protein-1 (Packer et al, 1995). Furthermore, α-Lip dose-dependently inhibited TNF- α -induced I kappa B kinase activation**,** subsequent degradation of I kappa B.

Nitric oxide (NO) is a chemical mediator involved in the maintenance of physiological homeostasis due to its regulatory and protective functions. Besides its known antioxidant property, NO which is produced by inducible nitric oxide synthase (iNOS) can be cytotoxic, especially at higher local concentrations. Also, it can react with reactive oxygen species (ROS) or oxygen yielding reactive nitrogen species (RNS), which causes damage on biological molecules such as enzymes, lipids and DNA by nitrosation, oxidation and nitration. Mesangial and invading immune cells are capable of expressing iNOS upon stimulation with TNF-α, IL-1b and bacterial lipopolysaccharide (LPS), and thus are likely to be responsible for the release of large amounts of NO during TNF α, IL-1b and LPS-triggered inflammatory conditions in the glomerulus cells (Aiello et al., 1998). In the present study, ZnO-NP produced an elevation of IL-6 and nitric oxide levels, and such elevation may be due to increased expression of neuronal NOS (nNOS) mRNA and NOS activity. This was confirmed with a previous study which revealed that exposure to low concentrations of ZnO-NP elevated circulating levels of IL-6, and that could account for the symptoms of the metal fume fever syndrome (Fine et al., 1997). Administration of either Vit E, or α-Lip along with ZnO-NP produces a significant decrease in IL-6 as well as NO levels. In accordance with our results, Kielstein et al (2002) reported a reduction in NO level post Vit E treatment in chronic kidney disease patients.

Vascular endothelial growth factor (VEGF), which is a potent mitogen for endothelial cells, has been reported to be expressed in several tissues, including kidney.

Besides its mitogenic properties, VEGF is able to promote angiogenesis-induce proteases (Drexler, 1994) and increase vascular leakage. In the present study the level of VEGF was elevated post ZnO-NP treatment compared to normal group. The reduction in VEGF level in ZnO-NP along with α-Lip treatment compared to ZnO-NP treated group was confirmed with the study of Moore et al. (2009), who observed that α-lip effectively prevented Ang II-induced glomerular and vascular damage in the kidneys and completely prevented the development of albuminuria through its anti-inflammatory/antioxidative mechanisms. The effects are associated with decreased nuclear factor (kappa) B (NF-κB) and activator protein-1 (AP-1) activation, as well as improved thiol homeostasis. Ang II–induced leukocyte infiltration and cell proliferation in the kidney were attenuated. The redox-sensitive transcription factors NF-κB and AP-1 in the kidneys were increased, and were effectively reduced post α–Lip administration.

Renal oxidized GSH levels were much higher, while the opposite was true for cysteine levels. These results suggested increased renal glutathione oxidation, leading to cysteine shortage. α-Lipoic acid partly prevented renal cysteine depletion and increased hepatic cysteine and glutathione concentrations. This effect was accompanied by increased hepatic gamma-glutamyl cysteine synthetase mRNA expression, with decreased NF-κB and AP-1 activation. α–Lipoic acid can regenerate vitamin C from its oxidized form, dehydroascorbic acid and regenerate other antioxidants, as well as chelates transition metal ions (e.g. iron and copper). it can enhance the synthesis of glutathione, the main antioxidant within our cells (Randle et al., 1988; Vasdev et al., 2000). Glutathione effectively mops up all types of toxins and free radicals. It can even pitch in and help when the body is lacking Vit E. Previous results have explained the modulatory effect in GSH level in animals treated with either α–Lip or Vit E post ZnO-NP administration. Vitamin E allows free radicals to abstract a hydrogen atom from the antioxidant molecule rather than from polyunsaturated fatty acids, thus breaking the chain of free radical reactions, the resulting antioxidant radicals being a relatively unreactive species (Ramos et al., 2011). In many studies Vit E neutralizes lipid peroxidation and unsaturated membrane lipids because of its oxygen scavenging effect (Kalender et al., 2004; Suna et al., 2004). It is concluded that Vit E is an essential component of the kidney for the protection of this tissue against peroxidative damage (Al-Attar, 2011).

In the present study, it was found that the level of pro-inflammatory biomarkers including CRP was elevated markedly in rat sera intoxicated with either two doses of ZnO-NP in relation to normal group implying immune disorder. Elevation of CRP post ZnO-NP exposure compared to normal control group was confirmed with the study of Kim et al. (2010) that clarify the increased CRP level with inflammation. In the present study, CRP level

was reduced post Vit E treatment compared to ZnO-NP exposed groups which was coincide with previous studies which revealed that Vit E plays a major role in reducing inflammation as well as cleansing the body of free radicals. As vitamin E supplements lowered CRP and IL6 concentrations dramatically in diabetic people (Singh et al, 2005; Upritchard et al., 2000). Oxidative stress and acute phase inflammation are now recognized to be highly prevalent in both the chronic kidney disease (CKD; pre-dialysis) and end stage renal disease (ESRD); on hemodialysis populations and several lines of evidence point to their contribution in the development of atherosclerosis. Biomarkers of the inflammatory state such as CRP and IL-6 are robust predictors of cardiovascular events and death in these two populations. The uremic state is characterized by retention of oxidized solutes including reactive aldehyde groups and oxidized thiol groups. It has recently been demonstrated that administration of antioxidant therapy such as Vit E and or α-Lip will decrease biomarkers of acute phase inflammation and oxidative stress in these patients (Suna et al., 2004).

The marked increase in circulating IgG in rat sera intoxicated with both doses of ZnO-NP is another response to immune disorder induced by this NPs toxicity in the current work. It was suggested that the increase in the circulating antibody production is the result of production of different inflammatory cytokines including TNF-α with potential impact on immunoglobulin production during inflammation. These results may indicate that ZnO-NP induced inflammatory kidney injury through production of the inflammatory mediators (Davis et al., 1998). IgG level was reduced by either Vit E or α-Lip treatments and this may explain the role of these agents to suppress the release of inflammatory mediators. There was also a significant increase in urea and creatinine levels in serum of ZnO-NP intoxicated rats confirmed by its nepherotoxic effect, compared to normal control group ($p < 0.05$) (Tables 3 and 4). Vitamin E significantly decreased serum creatinine level compared to rats intoxicated with high dose of ZnO-NP ($p < 0.05$). Serum glucose level was downregulated by the administration of either Vit E or α-Lip along with ZnO-NP compared to ZnO-NP treated group, and this coincide with Wang et al. (2008a) who observed that nano particles affects the pancreas. Randle et al. (1988) also reported that α-Lip causes acute hypoglycemia by decreasing hepatic glucose output.

In the present study, renal histopathological examination revealed that there was alteration of proteinaceous casts in the tubules and renal tubular dilatation in the ZnO-NP treated rats. High dose of ZnO-NP showed massive atrophy and fragmentation of numerous glomeruli, the renal tubules showed epithelial exfoliation, degeneration and necrosis. Some of renal tubules showed casts in their lumina. Severe congestion was observed in renal interstitium (Figure 5B). Treatment with α-Lip showed moderate histopathological changes in the form of shrinkage and fragmentation of few glomeruli with exfoliation of tubular epithelial cells and tubular casts in few renal tubules (Figure 5C). Animals that received ZnO-NP and Vit E showed histopathological changes in the form of marked hyperplasia of glomerular mesangial cells in many glomeruli, obliteration of many capsular spaces and necrosis and exfoliation of tubular epithelial cell lining of few renal tubules (Figure 5D). Animals that received high dose of ZnO-NP showed marked increase of deposition of collagen fibers in the interstitial tissue (Figure 6B). This histopathological change coincided with the results of Wang et al. (2006) study. Administration of α-Lip along with ZnO-NP showed mild increase of collagen deposition (Figure 6C). Moreover, vitamin E with ZnO-NP showed moderate increase of collagen deposition (Figure 6D). This can be attributed the role of Vit E or α-Lip to suppress the release of inflammatory mediators.

Conclusion

This study highlights the nepherotoxic effect of low and high doses of ZnO-NP. This was confirmed by the significant increase in urea and creatinine levels in serum of ZnO-NP intoxicated rats. Moreover, TNF-α, IL-6 and CRP levels were significantly increased, and that contributed to the nephrotoxic potential of ZnO-NP. Treatment with either Vit E or α-Lip successively alleviated the alterations in TNF-α, IL-6 and VEGF, as well as effectively ameliorated the histopathological changes of ZnO-NP intoxicated rats. Moreover, these antioxidants markedly reduced inflammatory cytokines levels. This may be related to their ability to attenuate the extent of NO synthesis. Our data demonstrated that α–Lip and Vit E are potent antioxidants that protect renal cells from injury caused by ROS oxidative stress and related vascular complications induced by ZnO-NP. Further studies are needed to evaluate the synergistic combination of Vitamin E and α-Lip, which is known to have an additional potential concern in ameliorating ZnO-NP nepherotoxic effect.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the Strategic Technique Program of the National Plane for Science, Technology, and Innovation (NPST) in Riyadh, Kingdom of Saudi Arabia for the financial support of this work in the form of Research Fellowship.

REFERENCES

Aiello S, Remuzzi G, Noris M (1998). Nitric oxide/endothelin balance after nephron reduction. Kidney Int. Suppl. 65:S63-67.

Al-Attar AM (2011). Antioxidant effect of Vit E treatment on some heavy metals induced renal and testicular injuries in male mice. Saudi J. Biol. Sci. 18(1):63-72.
Biewenga GP, Haenen GR, Bast A (1997). The pharmacology of the antioxidant lipoic acid. Gen. Pharmacol. 29:315-331.
Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Mittleman M, Samet J, Smith SC Jr, Tager I (2004). Expert Panel on Population and Prevention Science of the American Heart Association, Air pollution and cardiovascular disease: a statement for healthcare professionals from the expert panel on population and prevention science of the american heart association. Circulation 109:2655-2671.
Buzea II, Blandino P, Robbie K (2007). Nanomaterials and nanoparticles, Sources and toxicity. Biointerphases 2(4):172-179.
Coulter ID, Hardy ML, Morton SC, Hilton LG, Tu W, Valentine D, Shekelle PG (2006). Antioxidants vitamin C and Vit E for the prevention and treatment of cancer. J. Gen. Int. Med. 21:735-744.
Davis GS, Pfeiffer LM, Hemenway DR (1998). Persistent overexpression of interleukin-1 beta and tumor necrosis factor-alpha in murine silicosis, briefly describes possible therapeutic interventions. J. Environ. Pathol. Toxicol. Oncol. b17: 99-114.
Delie F (1998). Evaluation of nano-and microparticle uptake by the gastrointestinal tract. Adv. Drug Deliv. Rev. 34:221-233.
Drexler H (1997). Endothelial dysfunction: clinical implications. Prog. Cardiovasc. Dis. 39(4):287-324.
Fine JM, Gordon T, Chen LC, Kinney P, Falcone G, Beckett WS (1997). "Metal Fume Fever: Characterization of Clinical and Plasma IL 6 Responses in Controlled Human Exposures to Zinc Oxide Fume at and Below the Threshold Limit Value". J. Occup. Environ. Med. 39(8):22-26.
Gavanji S, Asgari MJ, Vaezi R, Larki B (2011). Antifungal effect of the extract of propolis on the growth of three species of Epidermophyton flucosum, Trichophyton violaseum and Trichophyton tonsorans in laboratory environment. AJPP 5(24):2642-2646.
Gojova A, Guo B, Kota RS, Rutledge JC, Kennedy IM, Barakat AI (2007). Induction of Inflammation in Vascular Endothelial Cells by Metal Oxide Nanoparticles: Effect of Particle Composition. Environ. Health Persp. 115:3.
Gupta A, Singh S, Jamal F, Nath, Mehrotra S, Sharma B (2011). Synergistic Effects of Glutathione and Vit E on ROS Mediated Ethanol Toxicity in Isolated Rat Hepatocytes. Asian J. Biol. 6:347-356.
Innsan MF, Shahril MH, Samihah MS, Asma OS, Radzi SM, Abd JAK, Hanina MN (2011). Pharmacodynamic properties of essential oils from Cymbopogon species. AJPP 5(24):2676-2679.
Ishrat T, Parveen K, Hoda MN, Khan MB, Yousuf S, Ansari MA, Saleem S, Islam F (2009). Effects of Pycnogenol and Vit E on cognitive deficits and oxidative damage induced by intracerebroventricular streptozotocin in rats. Behav. Pharmacol. 20/7(10):567-575.
Jacob S, Streeper RS, Fogt DL, Hokama JY, Tritschler HJ, Dietze GJ (1996). H stimulated glucose metabolism in insulin-resistant rat skeletal muscle. Diabetes 45:1024-1029.
Jain TK, Reddy MK, Morales MA, Leslie-Pelecky DL, Labhasetwar V (2008). Biodistribution, clearance, and biocompatibility of iron oxide magnetic nanoparticles in rats. Mol. Pharm. 5(2):316-327.
Kaden J (2007). IL-6 Determination in Serum of Kidney Graft Recipients by a New Bedside Test: Its Diagnostic Relevance 39(2):511-513.
Kalender S, Kalender Y, Ogutcu A, Uzunhisarcikli M, Durak D, Açikgoz F (2004). Endosulfan-induced cardiotoxicity and free radical metabolism in rats: the protective effect of Vit E. Toxicology 202(3):227-235.
Kielstein JT, Boger RH, Bode-Boger SM, Jurgen CF, Haller H, Ritz E, Fliser D (2002). Marked increase of asymmetric dimethylarginine in patients with incipient primary chronic renal disease. J. Am. Soc. Nephrol. 13:170-176.
Kim N, Kim YJ, Cho DK (2010). Gold nanoparticle-based signal augmentation of quartz crystal microbalance immunosensor measuring C-reactive protein. Curr. Appl. Phys. 10(4):1227-1230.
Lin W, Zheng L, Zhao J, Zhuang Q, Hong Z, Xu W, Chen Y, Sferra TJ, Peng J (2011). Anti-angiogenic effect of Spica prunellae extract *in vivo* and *in vitro*. AJPP 5(24):2647-2654.
Lomaestro BM, Malone M (1995). Glutathione in health and disease: pharmacotherapeutic issues. Ann. Pharmacother. 29: 1263-1273.
Moller P, Jacobsen NR, Folkmann JK, Danielsen PH, Mikkelsen L, Hemmingsen JG, Vesterdal LK, Forchhammer L, Wallin H, Loft S (2010). Role of oxidative damage in toxicity of particulates. Free Radical Res. 44(1):1-46.
Moore RM, Novak JB, Kumar D, Mansour JM, Mercer BM, Moore JJ (2009). Alpha-lipoic acid inhibits tumor necrosis factor-induced remodeling and weakening of human fetal membranes. Biol. Reprod. 80(4):781-787.
Moron MS, Depierre J, Mannervik B (1979). Levels of glutathione, glutathione reductase and glutathione –S- transferase activities in rat lung and liver. Biochem. Biophys. Acta 582:67-78.
Moshage H, Kok B, Uuizenga JR, Jansen P (1995). Nitrite and Nitrate Determination in Plasma: A Critical Evaluation. Clin. Chim. 41:892-896.
Nam JM, Thaxton CS, Mirkin CA (2003). Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. Science 301: 1884-1886.
Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warheit D, Yang H (2005). Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol. 6(10):2-8.
Packer L, Witt E, Tritschler HJ (1995). Alpha-lipoic acid as a biological antioxidant. Free Rad. Biol. Med. 19:227-250.
Pujalté I, Passagne I, Brouillaud B, Tréguer M, Durand E, Ohayon-Courtès C, L'Azou B (2011). Cytotoxicity and oxidative stress induced by different metallic nanoparticles on human kidney cells. Part Fibre Toxicol. 8:10.
Ramos LF, Kane J, McMonagle E, Le P, Wu P, Shintani A, Ikizler TA, Himmelfarb J (2011). Effects of combination tocopherols and alpha lipoic acid therapy on oxidative stress and inflammatory biomarkers in chronic kidney disease. J. Ren. Nutr. 21(3):211-218.
Randle PJ, Kerbey AL, Espinal J (1988). Mechanisms decreasing glucose oxidation in diabetes and starvation:role of lipid fuels and hormones. Diabetes Metab. Rev. 4:623-638.
Salata OV (2004). Application of nanoparticles in biology and medicine. J. Nanobiotechnol. 2:3.
Schipper ML, Iyer G, Koh AL, Cheng Z, Ebenstein Y, Aharoni A, Keren S, Bentolila LA, Li J, Rao J, Chen X, Banin U, Wu AM, Sinclair R, Weiss S, Gambhir SS (2009). Particle size, surface coating, and PE Gylation influence the biodistribution of quantum dots in living mice. Small 5(1):126-134.
Sharma M, Gupta YK (2003). Effect of alpha lipoic acid on intracerebroventricular streptozotocin model of cognitive impairment in rats. Eur. Neuropsychopharmacol. 13(4): 241-247.
Singh U, Devaraj S, Jialal I (2005). Vit E, oxidative stress and inflammation. Ann. Rev. Nutr. 25(7):151-174.
Skrzydlewska EI, Dobrzyńska IKI, Figaszewski Z (2001). Effect of Vit E Derivative (U83836E) on Membranes of Rat Liver Cells after Methanol Intoxication. Pol. J. Environ. Stud. 10(2):95-100.
Smith A, Bruton J (1978). A colour Atlas of histological staining techniques. J. Clin. Pathol. 31(3): 298.
Streeper RS, Henriksen EJ, Jacob S, Hokama JY, Fogt DL, Tritschler HJ (1997). Differential effects of lipoic acid stereoisomers on glucose metabolism in insulin resistant skeletal muscle. Am. J. Physiol. 36:185-191.
Trinder P (1969). Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor. Ann. Clin. Biochem. 6: 24-25.
Tsou TC, Yeh CF, Tsai YF, Lin JH, Cheng TJ, Chao HR, Tai LA (2010). Zinc oxide particles induce inflammatory responses in vascular endothelial cells via NF-κB signaling. J. Hazard Mater. 183(1-3):182-188.
Upritchard JE, Sutherland WHF, Mann JI (2000). Effect of supplementation with tomato juice, Vit E, and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. Diabetes Care 23:733-738.
Vasdev S, Ford CA, Parai S, Longerrich L, Gadag V (2000). Dietary alpha-lipoic acid supplementation lowers blood pressure in spontaneously hypertensive rats. J. Hypertens. 18:567-573.
Wang B, Feng WY, Wang T, Jia G, Wang M, Shi JW, Zhang F, Zhao L,

Fang Z (2006). Acute toxicity of nano- and micro-scale zinc powder in healthy adult mice. Toxicol. Lett. 161:115-123.
Wang BWY, Feng W, Wang M, Wang T, Gu Y, Zhu M, Ouyang H, Shi J, Zhang F, Zhao Y (2008a). Acute toxicological impact of nanao-and submicro-scaled zinc oxide powder on healthy adult mice. J. Nano Particles Res. 10(2):263-276.
Yao DF, Wu XH, Zhu Y, Shi GS, Dong ZZ, Yao DB, Wu W, Qiu LW, Meng XY (2005). Quantitative analysis of vascular endothelial growth factor, microvascular density and their clinicopathologic features in human hepatocellular carcinoma. Hepatobiliary Pancreat. Dis. Int. 4(2):220-226.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2224-2228, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.396
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Protective effect of tyrosol on apoptosis in PC12 cell induced by paraquat

Zhenxing Zhang[1]*, Xiaofeng Song[2], Xiaoyuan Du[3], Yu Cui[1] and Jishou Dai[1]

[1]Department of Neurosurgery, The First Affiliated Hospital of Liaoning Medical University, Liaoning Medical University, Jinzhou 121001, China.
[2]Department of Histology and Embryology, Liaoning Medical University, Jinzhou 121001, China.
[3]Department of Pathology, The First Affiliated Hospital of Liaoning Medical University, Liaoning Medical University, Jinzhou 121001, China.

Accepted 11 June, 2012

The protective effect of tyrosol on apoptosis in PC12 cell induced by paraquat (PQ) was studied. The PC12 cell was cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a 5% CO_2 incubator. 800 µm of PQ solution was added to establish an oxidative-stress injury model *in vitro*. The protective effect of tyrosol was evaluated by using this model. We compared the lactate dehydrogenase (LDH) release rate and malondialdehyde (MDA) content of PC12 cells in different treatment group. The cell apoptosis was detected by using flow cytometry (FCM) and the cell proliferation by using 5-diphenyltetrazolium (MTT) assay. The results showed that pretreatment with tyrosol could improve cell growth and proliferation in a concentration dependent manner, reduce the release of LDH and MDA content, suppress the PQ-induced apoptosis of PC12 cells. Therefore, these results suggested that tyrosol possessed protective activity of PC12 cell's injury by PQ i*n vitro*.

Key words: Tyrosol, PC12 cell, paraquat, protective effect.

INTRODUCTION

Paraquat (PQ) is a widely used herbicide that possesses a similar structure to MPP^+ and is toxic to mesencephalic dopaminergic neurons. PQ has been demonstrated to induce oxidative stress, cause toxicity to human and animals, and produce Parkinson disease (PD) symptoms (Houze et al., 1990; Liou et al., 1996, 1997; Morano et al., 1994). It has been reported that PQ selectively kills nigrostriatal dopaminergic neurons in the experimental animals and causes dopaminergic neurons damage *in vitro* midbrain culture (Corasaniti et al., 1992, 1998; Keiko et al., 2003; McCormack et al., 2002; Thiruchelvam et al., 2003). Apoptosis is thought to play an important role in the neuronal loss in PQ-induced neurological disorders. However, the mechanism of PQ neurotoxicity is still unclear. Growing studies suggest that PQ-induced apoptosis is mediated by the oxidative stress (Cappelletti et al., 1998; Fabisiak et al., 1998; McCarthya et al., 2004; Mollace et al., 2003). Therefore, developing the novel agents for anti-oxidative damage to improve the processes of these diseases, and then exploring their protection of cellular mechanisms have become an important research topic in the medicinal fields.

Rhodiola crenulata (Crassulaceae, Hongjingtian in Chinese) has been known as a medicinal plant for a long time. This precious perennial herbaceous plant is distributed at high altitudes in the Polar Arctic and Alpine regions throughout Europe and Asia (Qu et al., 2009). It was reported that *R. crenulata* has multiple pharmacological activities such as, antioxidant, antihypoxia, antifatigue, antiapoptosis, anticancer and enhancement in learning and memory (Jung et al., 2002; Kwon et al., 2008; Ming, 1986; Panossian et al., 2010; Petkov et al., 1986; Shevtsov et al., 2003). Tyrosol (its structure is as shown in Figure 1) is one of the major active constituents in *R. crenulata*. It has been reported that tyrosol has some interesting biological properties including anticancer, anti-depressant, stress-protective, cardioprotection, anti-osteoporosis, and antioxidative effects (Ahn et al., 2008; Chernyshov et al., 2007; Dinnella et al., 2007; Panossian et al., 2008, 2009).

*Corresponding author. E-mail: tangf66@126.com.

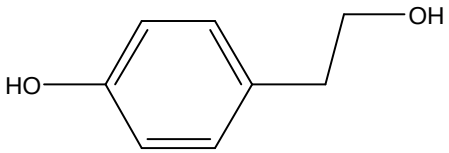


Figure 1. The chemical structure of tyrosol.

Meanwhile, several Chinese scholars also found that tyrosol could protect PC cells from apoptosis induced by different ways, such as NaCN, PQ, glucose deprivation and MPP^+ (Chen et al., 2007; Wang et al. , 2007). However, the effects of tyrosol on PC12 cells apoptosis induced by PQ are still unclear.

In this present study, we investigate the effect of tyrosol on apoptosis induced by PQ in PC12 cell lines. The lactate dehydrogenase (LDH) release rate, malondialdehyde (MDA) content, cell apoptosis and proliferation of PC12 cells in different treatment group were evaluated by using LDH release assay, thiobarbituric acid assay, flow cytometry (FCM) and 5-diphenyltetrazolium (MTT) assay, respectively.

MATERIALS AND METHODS

Reagents

Tyrosol was obtained from China pharmaceutical and biological products inspection (Lot: 111676-200602). PQ, MTT, Tris and Australia phenol blue were purchased from Sigma (USA). RPMI-1640 medium and Dulbecco's minimum essential medium (DMEM) were obtained from Gibco (USA). Newborn calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Company (Hangzhou, China). Trypsin, Tween20, and dimethyl sulfoxide (DMSO) were obtained from Amresco (USA). LDH and MDA kits were purchased from Nanjing Jiancheng Bioengineering Research Institute (China).

Cell culture and treatment

PC12 cells (rat adrenal pheochromocytoma cells) originated from Experimental Animal Center of Fudan University (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a 5% CO_2 incubator. When cells were near 80% confluence, new media with newborn calf serum were added before the compounds treatment. We used 800 μm of PQ to establish an oxidative-stress injury model *in vitro*, and evaluate the effect of tyrosol by using this model. When needed, cells were incubated for 30 min with different concentrations of tyrosol and then exposed to 800 μm of PQ for 24 h.

Detection of LDH release of PC12 cells

When PC12 cells were near 80% confluence, new media with newborn calf serum was added before the compounds treatment. Cells were treated with tyrosol for 30 min followed by the addition of PQ to a final concentration of 800 μm, and incubated for 24 h. The medium was collected to a 1 ml Eppendorf (EP) tube. At the end of treatments, PC12 cells were treated with 10% Triton X-100, and the media which contained detached cells were collected and centrifuged at 800 g at 4℃ for 2 min. The supernatant was used for the assay of LDH release. The enzyme was determined by using an assay kit according to the manufacturer's protocol. The absorbanceof the samples were read at 440 nm. The LDH release was in proportional to the number of damaged PC12 cells. Reagent blanks were subtracted.

Detection of MDA content of PC12 cells

The PC12 cells were cultured in six-well plates and pretreated with varying concentrations of tyrosol for 1 h prior to exposure to PQ. After 24 h, cells were collected in 1.5 ml of tubes in 0.5 ml phosphate-buffered solution (PBS). Cell lysis was performed by means of three cycles of freezing and thawing. MDA content was measured using a thiobarbituric acid assay according to the manufacturer's instructions.

FCM with propidium iodide (PI) staining

The PC12 cells were treated in the same way as described earlier. Cells were collected, digested with 0.25% trypsin and made into a single cell suspension by RPMI-1640 medium supplemented with 10% newborn calf serum. The single cell suspension was centrifuged at 1000 rpm for 5 min at 4℃. Then, the supernatant was removed, washed with cold PBS, centrifuged at 1000 rpm for 5 min at 4℃. The cell pellets were resuspended in 1 ml binding buffer (10 mm of Hepes/NaOH, pH 7.4; 140 mm of NaCl; 2.5 mm of $CaCl_2$) and were incubated for 15 min in the dark with Annexin V-FITC (20 μg/ml) and PI (50 μg/ml) at 4℃. Fluorescence was analyzed with an FCM.

MTT assay

Logarithmic growth phase cells were seeded in 96-well plates at a density of 5×10^3 ml^{-1}. Cells were cultured for 24 h at 37℃ with 5% CO_2, treated in the same way as described earlier. Each group was set up in three parallel holes. Cells were cultured for 24 h, followed by incubation with 0.5 mg/ml MTT, 200 μl serum-free medium for 4 h. Finally, 100 μl of DMSO was added and absorbance at 570 nm wavelength (A_{570}) was measured by means of enzyme-linked immunosorbent instrument. Relative cell proliferation inhibition rate (IR) = (1 - average A_{570} of the experimental group/average A_{570} of the control group) × 100%.

Statistical analysis

The database was set up with the Statistical Package for Social Sciences (SPSS) 16.0 software package for analysis. Data were represented as mean ± standard deviation (SD). The means of multiple groups were compared with one-way analysis of variance (ANOVA), after the equal check of variance, and the two-two comparisons among the means were performed by Student's *t*-test. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of tyrosol on LDH release from PQ-induced PC l2 cells

After PC12 cells were treated with 800 μm of PQ for 24 h, the release of LDH was significantly increased from 29.32

Table 1. The LDH release in different groups from PQ-induced PC l2 cells.

Group	LDH release (U/ml)
Medium	29.32 ± 1.45**
PQ	98.23 ± 4.68
0.1 μm tyrosol	78.22 ± 4.23*
1 μm tyrosol	68.45 ± 3.68*
2 μm tyrosol	63.42 ± 3.35**
5 μm tyrosol	59.57 ± 3.21**
10 μm tyrosol	52.18 ± 2.68**
20 μm tyrosol	38.53 ± 2.16**

PC12 cells were seeded and treated as described in the materials and methods. The culture medium from each treatment was collected and the LDH release was analyzed. Data shown here represent the average of three experiments. **P < 0.001 versus PQ injury group , *P < 0. 01 versus PQ injury group.

Table 2. The MDA content in different groups from PQ-induced PC l2 cells.

Group	MDA content (nmol/mg)
Medium	3.32 ± 0.25**
PQ	9.26 ± 0.98
0.1 μm tyrosol	7.32 ± 0.42*
1 μm tyrosol	6.95 ± 0.48*
2 μm tyrosol	6.44 ± 0.39**
5 μm tyrosol	5.77 ± 0.21**
10 μm tyrosol	5.28 ± 0.38**
20 μm tyrosol	4.58 ± 0.26**

PC12 cells were seeded and treated as described in the materials and methods. The culture medium from each treatment was collected and the MDA content was analyzed. Data shown here represent the average of three experiments. **P < 0.001 versus PQ injury group , *P < 0. 01 versus PQ injury group.

Table 3. The apoptosis rate in different groups from PQ-induced PC l2 cells**.**

Group	Apoptosis rate (%)
Medium	1.38 ± 0.23**
PQ	24.36 ± 3.28
0.1 μm tyrosol	19.44 ± 2.69*
1 μm tyrosol	17.35 ± 1.96*
2 μm tyrosol	15.48 ± 1.78**
5 μm tyrosol	13.47 ± 1.42**
10 μm tyrosol	9.65 ± 1.14**
20 μm tyrosol	7.49 ± 0.97**

FCM showed that all genistein derivatives can decrease apoptosis rate of PQ-induced PC12 cells. Data shown here represent the average of three experiments. **P < 0.001 versus PQ injury group , *P < 0. 01 versus PQ injury group.

± 1.45 to 98.23 ± 4.68 U/ml. When 0.1, 1.0, 2, 5, 10, and 20 μm of tyrosol were added to the assay, the release of LDH were reduced to 78.22 ± 4.23, 68.45 ± 3.68, 63.42 ± 3.35, 59.57 ± 3.21, 52.18 ± 2.68, and 38.53 ± 2.16 U/ml in a concentration dependent manner. The results showed that tyrosol could effectively reduce the LDH release of PQ-induced PC12 cells in a concentration dependent manner (Table 1).

Effects of tyrosol on MDA content from PQ-induced PC l2 cells

After PC12 cells were treated with 800 μm of PQ for 24 h, the MDA content was significantly increased from 3.32 ± 0.25 to 9.26 ± 0.98 nmol/mg. When 0.1, 1.0, 2, 5, 10, and 20 μm of tyrosol were added to the assay, the MDA content was reduced to 7.32 ± 0.42, 6.95 ± 0.48, 6.44 ± 0.39, 5.77 ± 0.21, 5.28 ± 0.38, and 4.58 ± 0.26 nmol/mg in a concentration dependent manner. The results showed that tyrosol could effectively reduce the MDA content of PQ-induced PC12 cells in a concentration dependent manner (Table 2).

Effects of tyrosol on apoptosis rate of PQ-induced PC l2 cells

FCM results showed that the apoptosis rate of PC12 cell line treated with 40 μm PQ for 24 h was 24.36 ± 3.28%, which was significantly higher than that of medium group (1.38 ± 0.23%). When 0.1, 1.0, 2, 5, 10, and 20 μm of tyrosol was added to the assay, cell apoptosis rate was reduced to 19.44 ± 2.69, 17.35 ± 1.96, 15.48 ± 1.78, 13.47 ± 1.42, 9.65 ± 1.14, and 7.49 ± 0.97% in a concentration dependent manner. This indicated that tyrosol could effectively reduce the PQ-induced apoptosis of PC12 cells (Table 3).

Effects of tyrosol on proliferation inhibition rate of PQ-induced PC l2 cells

The MTT assay demonstrated that the inhibitory rate of cells was significantly increased to 88.34 ± 2.67% after PC12 cells were treated with PQ. When PC12 cells were treated with 0.1, 1.0, 2, 5, 10, and 20 μm of tyrosol, the apoptosis rate of cells were reduced to 57.36 ± 3.26, 71.25 ± 3.56, 73.48 ± 4.21, 78.82 ± 4.02, 81.21 ± 3.48, and 84.33 ± 4.26%. These results indicated that tyrosol could significantly decrease PQ-induced inhibition of PC12 cells in a concentration dependent manner (Table 4).

DISCUSSION

PC12 cells, a rat adrenal pheochromocytoma cell line,

Table 4. The cell viability in different groups from PQ-induced PC l2 cells.

Group	Cell viability (%)
Medium	88.34 ± 2.67**
PQ	45.32 ± 3.79
0.1 μm tyrosol	57.36 ± 3.26*
1 μm tyrosol	71.25 ± 3.56*
2 μm tyrosol	73.48 ± 4.21**
5 μm tyrosol	78.82 ± 4.02**
10 μm tyrosol	81.21 ± 3.48**
20 μm tyrosol	84.33 ± 4.26**

MTT assay showed that all genistein derivatives can increase proliferation rate of PQ-induced PC12 cells. Data shown here represent the average of three experiments. **P < 0.001 versus PQ injury group , *P < 0. 01 versus PQ injury group.

can extend processes similar to those produced by sympathetic neurons when exposed to nerve growth factor, and these cells exhibit a single phenotype, with stable features that can be sub-cultured (Cheng et al., 2008; McLaurin et al., 2000). PC12 cells are extremely similar to neurons in cell morphology, structure and function. Therefore, it has been widely used as a cell model for study of neuron cells (Saito et al., 2003).

Numerous central nervous system diseases are closely linked to oxygen free radicals excess, such as cerebral ischemia, Alzheimer's disease (AD), Parkinson's disease and multiple sclerosis (Weber, 1994; Burton, 1995; Irani et al., 1997). Growing studies showed that oxygen free radicals and their derivatives are also closely related to cell apoptosis (Wang et al., 2005). For example, some concentration of PQ can induce certain types of cells apoptosis including PC12 cells, vascular endothelial cells and HL-60 cells. Oxidized LDL can induce lymphocyte apoptosis, peroxynitrite radicals can induce apoptosis of thymocytes, etc.

LDH is a stable cytoplasmic enzyme that is present in all cells and is rapidly released into the culture supernatant when the plasma membrane is damaged; thus, it can be used as a reliable biochemical index for damage of the plasma membrane (Zhao et al., 2002). Our results showed that tyrosol could decrease LDH release of PC12 cells injured by PQ. MDA is the end product of free radical-initiated lipid peroxidation and thus reflects the level of lipid peroxidation. Our results also showed that PQ may reduce the MDA content in PC12 cells, thus suggesting that its neuroprotective effects are potentially due to its antioxidant property (Xu et al., 2008). MTT assay results confirmed that cell growth and proliferation were suppressed when PC12 cells were treated with PQ for 24 h, while tyrosol could effectively decrease the suppression. FCM with PI staining results showed that tyrosol could reduce the PQ-induced apoptosis of PC12 cells in a concentration dependent manner.

Conclusion

In this present work, the model of injury and apoptosis induced by PQ to evaluate different concentration of tyrosol against oxidative stress injury in PC12 cells lines was established. Tyrosol could decrease LDH release, the MDA content, the suppression of cell proliferation, and the PQ-induced apoptosis of PC12 cells in a concentration dependent manner. The findings suggest that tyrosol might have the protective effect against neurotoxicity induced by PQ in PC12 cells.

REFERENCES

Ahn EY, Jiang Y, Zhang Y (2008). Cytotoxicity of *p*-tyrosol and its derivatives may correlate with the inhibition of DNA replication initiation. Oncol. Rep. 19:527-534.

Burton RH (1995). Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Rad. Biol. Med. 18:775-794.

Cappelletti G, Maggioni MG, Maci R (1998). Apoptosis in human lung epithelial cells: triggering by paraquat and modulation by antioxidants. Cell Biol. Int. 22 671-678.

Chen H, Chen JZ, Hou RR, Yang AL, Kang XG, Zhang Y, Li Yl, Li Hl (2007). Inhibition of s alidroside on apoptosis of PC12 cells i nduced by paraquat and its related mechanisms. Chin. J. Pharmacol. Toxicol. 21(3):190-196.

Cheng L, Zhang CD (2008). Cyclophilin A affects Bcl-2 and Bax expression following beta-amyloid fragment 25-35-induced injury to C12 cells. Neural. Regen. Res. 3(6):585-588.

Chernyshov GA, Plotnikov MB, Smol'iakova VI, Krasnov EA (2007). Therapeutic effect of *p*-tyrosol on myocardial electric instability induced by coronary occlusion. Eksp. Klin. Farmakol. 70:23-25.

Corasaniti MT, Bagetta G, Rodino P, Gratteri S, Nistico G (1992). Neurotoxic effects induced by intracerebral and systemic injection of paraquat in rats. Hum. Exp. Toxicol. 11:535-539.

Corasoniti MT, Strongoli MC, Roteroti D, Bagetta G, Nistico G (1998). Paraquat: a useful tool for the *in vivo* study of mechanisms of neuronal cell death. Pharmacol. Toxicol. 83:1-7.

Dinnella C, Minichino PD, Andrea AM, Monteleone E (2007). Bioaccessibility and antioxidant activity stability of phenolic compounds from extra-virgin olive oils during *in vitro* digestion. J. Agric. Food Chem. 55:8423-8429.

Fabisiak JP, Kagan VE, Tyurina YY, Tyurin VA, Lazo JS (1998). Paraquat-induced phosphatidylserine oxidation and apoptosis are independent of activation of PLA2. Am. J. Physiol., 274: 793-L802.

Houze PFJ, Baud R, Mouy C, Bismuth R, Bourdon R, Scherrmann JM (1990). Toxicokinetics of paraquat in humans. Hum. Exp. Toxicol. 9: 5-12.

Irani K, Xia Y, Zweier JL (1997). Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 275:1649-1652.

Jung IM, Kim H, Fan WZ, Tezuka Y, Kadota S, Nishijo H, Jung MW (2002). Neuroprotective effect of constituents of the oriental crude drugs, *Rhodiola sacra*, *R. Sachalinensis* and Tokaku-joki-to, against beta-amyloid toxicity, oxidative stress and apoptosis. Biol. Pharm. Bull. 25(8):1101-1104.

Keiko S, Kazuo M, Ko-ichi O, Hiroshi S (2003). Paraquat leads to dopaminergic neural vulnerability in organotypic midbrain culture. Neurosci. Res. 46:523-532.

Kwon M, Qadir SA, Han J, Jeong H, Lee H (2008). Effect of high pressure on extracts of *Rhodiola sachalinensis* in cytotoxicity and anticancer activity. J. Biotechnol. 136S:S460-495.

Liou HH, Chen RC, Tsai YF, Chem WP, Chang YC, Tsai MC (1996). Effects of paraquat on the substantia nigra of the Wistar rats: neurochemical, histological and behavioral studies. Toxicol. Appl. Pharmacol. 137:34-41.

Liou HH, Tsai MC, Chen CJ, Jeng JS, Chang YC, Chen SY, Chen RC (1997). Environmental risk factors and Parkinson's disease: a case control study in Taiwan. J. Neurol. 48:1583-1588.
McCarthya S, Somayajulua M, Sikorskab M, Borowy-Borowskib H, Pandey S (2004). Paraquat induces oxidative stress and neuronal cell death; neuroprotection by water-soluble Coenzyme Q10. Toxicol. Appl. Pharmacol. 201:21-31.
McCormack AL, Thiruchelvam M, Manning-Bog AB (2002). Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neurobiol. Dis. 10:119-127.
McLaurin J, Golomb R, Jurewicz A (2000). Inositol stereoisomers stabilize an oligomeric aggregate of Alzheimer amyloid beta peptide and inhibit abeta-induced toxicity. J. Biol. Chem. 275(24):18495-18502.
Ming HQ (1986). The synthesis of salidroside and its pharmacological properties. Chin. J. Pharm. Bull. 21:373-375.
Mollace V, Iannone M, Muscoli C, Palma E, Granato T, Rispoli V, Nistico R, Rotiroti D, Salvemini D (2003). The role of oxidative stress in paraquat-induced neurotoxicity in rats: protection by nonpeptidyl superoxide dismutase mimetic. Neurosci. Lett. 335:163-166.
Morano A, Jimenez-Jimenez FJ, Molina JA, Antolin MA (1994). Risk factors for Parkinson's disease: case control study in the province Caceres, Spain. Acta Neurol. Scand. 89:164-179.
Panossian A, Nikoyan N, Ohanyan N (2008). Comparative study of *Rhodiola* preparations on behavioral despair of rats. J. Phytomed. 15: 84-91.
Panossian A, Wikman G, Sarris J (2010) Rosen root *Rhodiola rosea*): Traditional use, chemical composition, pharmacology and clinical efficacy. Phytomedicine 17(7):481-493.
Panossian A, Wikman G, Kaur P, Asea A (2009). Adaptogens exert a stress-protective effect by modulation of expression of molecular chaperones. Phytomedical 16:617-622.
Petkov VD, Yonkov D, Mosharoff A (1986). Effects of alcohol aqueous extract from *Rhodiola rosea L.* roots on learning and memory. Acta Physiol. Pharmacol. Bulg. 12(1):3-16.
Qu ZQ, Zhou Y, Zeng YS, Li Y, Chung P (2009). Pretreatment with Rhodiola Rosea extract reduces cognitive impairment induced by intracerebroventricular streptozotocin in rats: implication of anti-oxidative and neuroprotective effects. Biomed. Enviroment. Sci. 22: 318-326.
Saito Y, Tsuzuki K,Yamada N (2003). Transfer of NMDAR2 cDNAs increases endogenous NMDARI1 protein and induces expression of functional NMDA receptors in PC12 cells. Mol. Brain Res. 110:159-168.
Shevtsov VA, Zholus BI, Shervarly VI (2003). A randomized trial of two different doses of a SHR-5 *Rhodiola rosea* extract versus placebo and control of capacity for mental work. Phytomedicine 10(2-3):95-105.
Thiruchelvam M, McCormack A, Richfield EK, Baggs RB, Tank AW, DiMonte DA, Cory-Slechta DA (2003). Age-related irreversible progressive nigostriatal dopaminergic neurotoxicity in the PQ and maneb model of the Parkinson's disease phenotype. Eur. J. Neurosci. 18(3):589-600.
Wang H, Yao WB, Qian YF, Gao XD (2007). Protective effect of salidroside on apoptosis in PC12 cells induced by sodium cyanide and gluco se deprivation. J. China Pharmaceut. University 38(3): 273-276.
Wang LM (2005). Adhesion molecules and atherosclerosis. Cardiovascular Dis. Study Progress 5:494-497.
Weber GF (1994). The pathophysiology of reactive oxygen inter mediates in the central nervous system. Med. Hypoth. 43:223-230.
Xu DH, Zhou CH, Xu BL, Luo SY (2008). Protective effect of paeonol on beta-amyloid 25-35-induced toxicity in PC12 cells. Neural. Regen. Res. 3(8):863-866.
Zhao L, Chen Q, Brinton RD (2002). Neuroprotective and neurotrophic efficacy of phytoestrogens in cultured hippocampal neurons. Exp. Biol. Med. 227(7):509-519.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2229-2234, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.469
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

The effects of caffeine and carvedilol on skeletal system of rat embryos in prenatal period

Rashidi, F[1], Mahmood Khaksary Mahabady[2]*, Ranjbar R[2] and Najafzadeh Varzi H [3]

[1]Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[2]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
[3]Department of Pharmacology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

Accepted 9 July, 2012

Caffeine at high doses is a known rodent teratogen and induces limb malformations along with cleft palate in various strains of rats and mice. The teratogenic effects of some drugs can be prevented by the application of antioxidant drugs and stimulation of the maternal immune system. Also, there are some evidence that carvedilol is antioxidant. Therefore, in this study, the prophylactic effect of carvedilol on teratogenic effects of caffeine was evaluated. This study was performed on 24 pregnant rats that were divided into four groups. Control group received normal saline and test groups received caffeine (80 mg/kg), caffeine (80 mg/kg) plus carvedilol (5 mg/kg) and carvedilol (5 mg/kg), intraperitoneally at 9 to 11th days of gestation, respectively. Fetuses were collected at 20th day of gestation and after determination of weight and length, they were stained by Alizarin red - Alcian blue method. Cleft palate incidence was 33.33% in fetuses of rats that received only caffeine, while it was 2.85% in group which received caffeine plus carvedilol (5 mg/kg). The means of weight and length of fetuses from rats that received carvedilol were significantly greater than those that received only caffeine. It is concluded that carvedilol decreased cleft palate induced by caffeine, but this subject needs more detailed evaluation.

Key words: Caffeine, carvedilol, gestation, cleft palate, teratogenicity, fetus, rat.

INTRODUCTION

Caffeine, or 1, 3, 7-trimethylxanthine, is a widely used substance present in habitual beverages and chocolate-based foods (Olcina et al., 2006). Caffeine represents one of the most common pharmacologically active substances used by pregnant women. Exposure of the conceptus to this drug occurs primarily as a result of maternal consumption of caffeine containing beverages, especially coffee (Nash and Persaud, 1988).

Caffeine at high doses is a known rodent teratogen and induces limb malformations along with cleft palate in various strains of rats and mice (Moriguchi and Scott, 1986). Several studies have demonstrated the teratogenicity of caffeine in laboratory animals (Fujii and Nishimura, 1969; Fujii et al., 1969; Palm et al., 1978), but the experimental results cannot be applied to humans due to the variability of caffeine dose, exposure time and species differences.

The sensitivity of different animal species is variable. Malformations have been demonstrated in mice at 50 to 75 mg/kg of caffeine (Nehlig and Debry, 1994), whereas the lowest dose usually needed to induce malformations is 80 mg/kg in rats (Nehlig and Debry, 1994). However, when caffeine is administered in fractioned amounts during the day, 330 mg/kg/day are necessary to reach teratogenicity in rats (Nehlig and Debry, 1994). In rodents, the most frequently observed malformations are those of the limbs and digits, ectrodactyly, craniofacial malformations (labial and palatal clefts) and delays in ossification of limbs, jaw and sternum (Nehlig and Debry, 1994).

*Corresponding author. E-mail: mkhaksary@scu.ac.ir or mkhaksarymahabady@yahoo.com. Tel: +986113330073 or +989131619252. Fax: +986113360807.

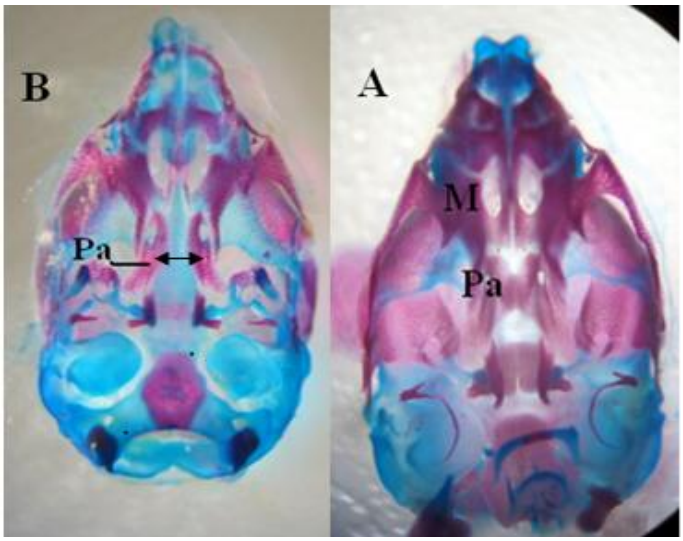


Figure 1. Ventral view of skull of rat fetuses of GD 20, stained with alizarin red S- alcian blue. A, Normal palatine bone; B, cleft palate induced by caffeine (arrow). M, maxilla; Pa, palatine.

Some of these caffeine-derived effects could favour the production of free radicals and a subsequent increase of oxidative stress such as the metabolic inactivation of catecholamines Jewett et al., 1989) and the increase of oxidative metabolism (Shigenaga et al., 1994) including its own hepatic metabolism (Vistisen et al., 1992). There are also reports suggesting that caffeine is capable of including certain forms of oxidative damage by increasing lipid peroxidation (Dianzani et al., 1991).

One of the free radical scavengers currency used in clinical setting is carvedilol which is a blocker of α1- and β- adrenoreceptors (Yue et al., 1992). Because of its unique ability to interact with free radicals, carvedilol has been proposed to be a useful adrenergic antagonist in the treatment of hypertention and cardiac diseases in which oxidant stress prevails (Feuertein et al., 1998). In physicochemical, biochemical and cellular assays, carvedilol inhibited the formation of reactive oxygen radicals and lipid peroxidation, scavenged oxygen free radicals, and prevented the depletion of endogenous anti-oxidants. It has been suggested that carvedilol may provide greater benefit than traditional β-blockers in chronic heart failure because of its antioxidant actions that synergize with its nonspecific β- and α-blocking effects (Noguchi et al., 2000).

In the present study, the preventive effect of carvedilol on caffeine-induced cleft palate in rats was evaluated.

MATERIALS AND METHODS

Caffeine powder (Merck, Germany) and carvedilol (Abidi Co, Iran) were purchased**.** Male and female healthy rats of Wistar strain, 3 to 4 months old, weighing 200 to 250 g were purchased (Joundishapour Jaboratory Animal Center, Ahvaz, Iran) and housed individually (males) or at 10 per polycarbonate cage (female) for a 2-week acclimation period. Rats were fed *ad libitum* by standard laboratory pellet (Pars khurakdam, Tehran, Iran.) and tap water. Rats were maintained in animal rooms controlled for temperature (23 ± 2°C), relative humidity of 45 to 55% and light (12/12 h light/ dark cycle).

Females were mated overnight with males. Pregnancy was ascertained the next morning by presence of a vaginal plug, and this time was designated as gestational day (GD) 0. Pregnant rats (n = 24) were randomly divided into four groups (16 pregnant rats in treatment groups, 8 pregnant rats in control group) and treated as follows;

Group 1 (Control group): Normal saline in equal volume of caffeine was injected to pregnant rats for inducing similar condition (injection and handling) to other groups.

Group 2 (Caffeine group): Caffeine (80 mg/kg) was administrated intraperitoneally at 9 to 11th day of gestation.

Group 3 (Carvedilol group): Carvedilol (5 mg/kg) was administrated intraperitoneally at 9 to 11th day of gestation.

Group 4 (Caffeine + carvedilol group): Caffeine (80 mg/kg) plus carvedilol (5 mg/kg) was administrated intraperitoneally at 9 to 11th day of gestation.

The animals were sacrificed by cervical dislocation at 20th day of gestation. Following laparotomy, the uterus was exteriorized and the number and location of fetuses and resorption were noted, then their weight and length (crown- rump length) were measured. Individual fetuses were examined carefully for external anomalies then were stained in a mixture of 0.14% Alcian blue and 0.12% alizarin red S in ethanol and glacial acetic acid. Fetuses are then macerated in 2% KOH, cleared and hardened in 1:1 glycerin and distilled water, and stored in pure glycerin (Kimmel and Trammekl, 1981) and investigated by stereomicroscope (Nikon, SMZ200, Japan) for skeletal malformations. The incidence of skeletal malformations was determined and was compared in the groups.

Statistical significance between groups was determined using SPSS program and compared by one way analysis of variance (ANOVA) and Post hoc least significant difference (LSD). The minimum level of significance was $p< 0.05$.

RESULTS

Sixty-two (62) fetuses were obtained from 8 rats of control group. There were not observed macroscopic anomalies in the control animals. In the control group, palatal closures of fetuses were normal at GD 20 (that is, palatal shelves had grown vertically on the sides of the tongue, then horizontally to meet and fuse) (Figure 1A). No maternal death or abortion occurred in any experimental groups. There were not any aborted fetuses from total groups but percentage of resorbed fetuses were 0, 3.38, 2.77 and 0% in groups that received normal saline, caffeine, caffeine plus carvedilol and carvedilol, respectively; so carvedilol decreased resorption rate (Table 1).

Caffeine induced cleft palate (Figure 1B) at 33.33% incidence. Caffeine plus carvedilol (5 mg/kg) significantly reduced incidence of cleft palate to range of 2.85%.

The mean of weight of animals' fetuses that received caffeine (80 mg/kg) in 9 to 11th days was significantly

Table 1. Incidence of anomalies in rat fetuses of groups.

Group	No. of litters	Implantations	Resorbed fetuses	Live fetuses	Fetal length (mm): (mean ± SEM	Fetal weight (g): (mean ± SEM)	%fetuses with cleft palate
Control	8	62	0 (0)	61	38.01 ± 0.26*	4.93 ± 0.08*	0 (0)
Caffeine	6	59	2 (3.38)	57	28.92 ± 0.81**	3.03 ± 0.17**	19 (33.33)#
Caffeine + carvedilol	5	36	1 (2.77)	35	35.68 ± 0.57	4.30 ± 0.13	1 (2.85)
Carvedilol	5	38	0 (0)	38	36.31 ± 0.40	4.49 ± 0.11	0 (0)

Numerals in parentheses are percentages; *, Significant difference when compared with other groups ($p < 0.05$); **, significant difference when compared with other groups; #, significant difference when compared with other groups ($p < 0.05$), Incidence of cleft palate was significantly difference at groups which received caffeine with control and carvedilol group ($p = 0.0001$).

decreased in comparison with other groups (Table 1). The mean of weight and length of animals' fetuses that received normal saline in 9 to 11th days was significantly increased in comparison with other groups (Table 1).

DISCUSSION

Since data are not available on the effect of carvedilol on the teratogenicity of caffeine in rat embryos this study was initiated. Several studies have reported that the maternal immune stimulation can reduce teratogenic anomalies (Ivnitsky et al., 2001). Mechanisms of this effect remain unclear, but it is thought that the fetal gene expression has been modulated (Holladay et al., 2002).

The enhancing antioxidative effects can protect fetuses against drugs teratogenicity (Winn and Wells, 1999). Sharova et al. (2002) showed that interferon-gamma and Freund's complete adjuvant reduced severity of the urethane-induced cleft palate in mice (Sharova et al., 2002).

In the present study for first time, the effect of carvedilol on teratogenicity of caffeine in rat embryos was evaluated.

According to a recent report by Lelo et al. (1986), the average daily human caffeine intake of moderate to heavy consumers ranges from approximately 300 to 600 mg/kg/day, or from 3 to 6 cups of coffee (assuming 100 mg/cup). The dosage level therefore in a person weighing 70 kg ranges from approximately 4.3 to 8.6 mg/kg/day. In comparison, caffeine doses administrated to laboratory animals ranged from 30 mg/kg (Palm et al., 1978) to 250 mg/kg (Fujii and Nishimura, 1969). Even when species variation is taken into account, the practical application of the results obtained from many of these animal experiments to the human condition is unrealistic due to the excessive dose levels administered (Nash and Persaud, 1988).

A moderate dosage level of 80 mg/kg caffeine was administered as a three intraperitoneal injection on GDs 9 to 11. Fujii et al. (1969) demonstrated that in mice, whereas embryo-lethality is related to the duration of caffeine exposure, teratogenic effects are more dependent on a sufficiently high concentration of the drug. Though intraperitoneal injections dose not stimulate human caffeine consumption the method of caffeine administration in present study was the most expedient and in accordance with that utilized by others.

Fujii and Nishimura (1974) postulated that caffeine was teratogenic by virtue of catecholamine release from maternal or embryonic tissue. They reported that administering 175 mg/kg of caffeine intraperitoneally on days 11 and 12 of pregnancy in mice induced malformation that is initiated by release of catecholamines from the maternal adrenal gland.

Ross and Persaud (1989) reported neural tube defects in early rat embryos following maternal treatment with caffeine. Kimmel et al. (1984) reported a significant in resorptions following oral administration of caffeine to pregnant rats at a dose level of 120 mg/kg on the day 12 of gestation. Even though epidemiological studies have found no real association between coffee consumption during pregnancy and adverse fetal outcome (Linn et al., 1982), the United State Food and Drug Administration still advised pregnant women to avoid caffeine-containing foods and methylxanthine which resembles the purines found in genetic material. Thus, caffeine possesses the potential to derange the processes involved in cell proliferation. Because it has been known for some time that caffeine readily crosses

the placenta and reaches the fetus (Goldestein and Warren, 1962), the warning of the Food and Drug Administration merits serious consideration. In the present study, embryo from mothers treated with caffeine revealed a significant reduction in crown-rump length. It is believed that maternal treatment with caffeine alters utero-placental circulation to such an extent that normal embryonic development is impaired (Adamson et al., 1971).

Burdan (2003) reported that the mixture of paracetamol and caffeine decreased fetal length and body weight, and placental weight. Nishimura and Nakai (1960) reported increased cleft palate and digital defects in mice offspring exposed to caffeine at a dose of 250 mg/kg.

In one study, Colomina et al. (2001) reported a single oral dosage of caffeine or aspirin (ASA) on p.c.d 9 was given to mice orally exposed to toxic levels of caffeine (30 mg/kg/day), ASA (250 mg/kg), or a combination of caffeine and ASA (30 and 250 mg/kg, respectively). Three additional groups were given the same doses and restrained for 14 h. The pregnant mice were restrained 2 h/day on p.c.ds 0 to 18 by placing them in methacrylate cylindrical holders and keeping them in a prone position with the paws immobilized with elastic adhesive tape, a procedure the authors previously reported to produce stress in pregnant mice (Colomina et al., 1995; 1999). Other mice were given toxic dosages of caffeine by gavage at 30, 60, and 120 mg/kg/day on GDs 0 to 18, and another group was administered the same dosages of caffeine immediately followed by restraint stress for 2 h/day on the same days (Colomina et al., 1999). No caffeine levels were recorded. Although the authors do not identify maternal toxicity, it is noteworthy that the weekly intervals measured for body weights are inappropriate (drug treatments and restraint occurred on one day; the intervals are evaluated for 3 or 4 days). Maternal toxicity was evident, with reductions or frank weight losses in body weight and feed consumption measurements. Regarding caffeine, these effects were most severe for the three groups of interest (restraint, 30 mg/kg caffeine and combined 30 mg/kg of caffeine and 14 h of restraint), on p.c.ds 9 to 11. Of these three groups, the effects were most severe for the combined caffeine and stress group. The 30 mg/kg plus restraint group also had an increase in post-implantation loss, including dead fetuses and late resorptions. An increase in early resorptions was seen in the restraint alone group, but the group with both restraint and 30 mg/kg of caffeine were increased compared with the restraint alone group. As would be expected, there was an increase in reduced ossification in the restraint group alone, the 30 mg/kg caffeine alone, and the combined caffeine and stress group. There was no increase in malformations in any group. The authors considered there to be some clinical relevance for the data because real life involves multiple simultaneous exposure to many chemicals. However, the duration of oral exposure to ASA and caffeine on GD 9 in this study is not analogous to the type of stress experienced by pregnant women who drink coffee and take ASA. Interspecies differences and pharmacokinetics and bioavailability are both important considerations (Brent et al., 2011).

Colomina et al. (2001) exposed mice to caffeine (30 mg/kg) and ASA (250 mg/kg by gavage on the 9th post conception day.) There was no significant maternal or developmental toxicity in this group of animals and offspring. The studies also included stressful restraint. However, the exposure and the stress in the mouse studies cannot be utilized to determine human developmental risks, especially since the developmental results were minimal and the exposure equivalency in the human is unknown.

Differences in outcome after intrauterine caffeine exposure dependent on dose and rout of administration were also seen in rats. A lack of embryo or fetotoxicity or teratogenicity was observed when caffeine was administrated for whole gestational period at doses 16 to 17 and 25 to 33 per day (Aeschbacher et al., 1980). A reduction in fetal weight was found after maternal pregnancy exposure to 62 mg/kg per day. In contrast, Nolen (1981) reported that daily, long-term caffeine exposure at doses up to 80 mg/kg per day in drinking water did not affect fetal development. They also showed that such administration caused no differences in body weight gain or feed consumption. Aeschbacher et al. (1980) reported that caffeine dietary concentration of 0.25 and 0.5 g/kg throughout gestation and lactation had no significant effect on birth weight, litter size or development. At 1 g/kg, there was a slight reduction of birth weight. In animal studies, fetal loss, decreased fetal weight and size, and major skeletal defects have been reported when dosages of more than 80 mg/kg of caffeine were used (McKim, 1991).

A number of observations suggest that detoxification of a xenobiotic free radical intermediate with antioxidants may provide important embryo protection (Wells et al., 1997).

In one study, carvedilol protected oxidative stress induced by okadaic acid in N1E-115 cells. It seems that protective effect of carvedilol, as well as its ability to modify cell response to okadaic acid, involving like cytoprotective mechanism is its antioxidative properties (Tunez et al., 2006). Huang et al. (2006) reported carvedilol treatment increased activities of antioxidant enzymes and expression of Bcl-2 in healthy rats as well as diabetic rats. These results indicated that carvedilol partly improves cardiac function via its antioxidant properties in diabetic rats (Haung et al., 2006).

Prakash and Kumar (2009) reported the effectiveness of carvedilol in preventing cognitive deficits as well as the oxidative stress caused by intracerbroventicular administration of streptozotocin in rats (Prakash and Kumar, 2009). Carvedilol has been shown to preserve the endogenous antioxidant system, that is, vitamin E

and reduced glutathione (GSH), which are normally consumed when tissues or cells are exposed to oxidative stress (Feuerstein et al., 1997).

Antelava et al. (2009) reported that antioxidant and positive treatment effects of carvedilol could be explained by its wide range of pharmacological ability: as non-selective beta-adrenergic blocker (via inhibition of adenylatecyclase and decreasing cyclic adenosinemonophosphate), alpha 1-adrenoblocker [decreasing activation of phospholipase C and concentration of inositoltriphosphate, diacylglicerole and Ca (++)] and antioxidant (Antelava et al., 2009). Tasset et al. (2009) reported that carvedilol and melatonin prevented the increases in lipid peroxidation and total lactate dehydrogenase (LDH) activity, as well as the depletion of reduced GSH and the reduction of antioxidative enzymes activities in N1E-115 cells incubated with 100 mM 3NP (Tasset et al., 2009).

In conclusion, the present study showed the effects of carvedilol for the first time on cleft palate induced caffeine in rat fetuses. The present results indicate that exposure 80 mg/kg of caffeine in 9 to 11th days of gestation of rat decreases weight and length of embryos and did influence on skeletal system. It is probable that caffeine influences antioxidant system that produces teratogenic effects including cleft palate. Effects of caffeine immune suppression are mediated indirectly by inducing oxidative stress. The protective effect of carvedilol against caffeine-induced cleft palate in rat may, at least in part, be due to its antioxidant activity, which we believe deserves further investigation.

ACKNOWLEDGEMENT

The authors wish to express his gratitude to the research council of Shahid Chamran University for his financial supports.

REFERENCES

Antelava N, Gabunia L, Gambashidze K, Petriashvili SH, Bejitashvili N (2009). Effects of carvedilol, losartan and trimetazidin on functional parameters of isolated heart of rats at oxidative stress. Georgian Med. News 167:81-84.

Adamson K, Muller-Heubach E, Meyers RE (1971). Production of asphyxia in the Rhesus monkey by administration of catecholamines to the mother. Am. J. Obested. Gynecol. 109:248-262.

Aeschbacher HU, Milon H, Foot A, Würzner HP (1980). Effect of caffeine on rat offspring from treated dams. Toxicol. Lett. 7(1):71-77.

Brent RL, Christian MS, Diener RM (2011). Evaluation of the Reproductive and Developmental Risks of Caffeine. Birth Defects Res. B Dev. Reprod. Toxicol. 92(2):152-187.

Burdan F (2003). Intrauterine growth retardation and lack of teratogenic effects of prenatal exposure to the combination of paracetamol and caffeine in Wistar rats. Reprod. Toxicol. 17:51-58.

Colomina MT, Albina ML, Domingo JL, Corbell J (1995). Effects of maternal stress on methylmercury-induced developmental toxicity in mice. Physiol. Behav. 58:979-983.

Colomina Mt, Sanchez DJ, Esparaza JL, Domingo JL (1999). Prenatal effects of caffeine and restraint stress in mice. Proc. Soc. Exp. Biol. Med. 220:106-111.

Colomina MT, Albina ML, Sanchez DJ, Domingo JL (2001). Interactions in developmental toxicology: combined action of restraint stress, caffeine, and aspirin in pregnant mice. Teratology 63(3):144-151.

Dianzani MU, Muzio G, Biocca ME, Canuto RA (1991). Lipid peroxidation in fatty liver induced by caffeine in rats. Int. J. Tissue Reactions 13(2):79-85.

Feuerstein GZ, Shusterman NH, Ruffolo RRJR (1997). Carvedilol update IV: prevention of oxidative stress, cardiac remodeling and progression of congestive heart failure. Drugs Today 33:453-457.

Feuertein G, Yue TL, Ma X, Ruffolo RR (1998). Novel mechanisms in the treatment of heart failure: inhibition of oxygen radicals and apoptosis by carvedilol. Prog. Cardiovasc. Disc. 41(suppl. 1):17-24.

Fujii T, Sasaki H, Nishimura H (1969). Teratogenicity of caffeine in mice related to its mode of administration. Jpn. J. Pharmacol. 19:134-138.

Fujii T, Nishimura H (1969). Teratogenic actions of some methylated xanthines in mice. Okajimas Folia Anat. Jap. 46:167-175.

Fujii T, Nishimura H (1974). Prevention of embryonic effects of caffeine in mice by pretreatment with propranolol. Jpn J. Pharmacol. 24:44

Goldestein A, Warren R (1962). Passage of caffeine into human gonadal and fetal tissue. Biochem. Pharmacol. 11:166-168.

Holladay SD, Sharova LV, Punareewattana K, Hrubec TC, Gogal RM, Prater MR, Sharov AA (2002). Maternal immune stimulation in mice decreases fetal malformations caused by teratogens. Int. Immunopharmacol. 2:25- 332.

Huang H, Shan J, Pan XH, Wang H, Qian L (2006). Carvedilol protected diabetic rat hearts via reducing oxidative stress. J. Zhejiang. Univ. Sci. B 7(9):725-731.

Ivnitsky I, Torchinsky A, Savion S, Shepshelovich J, Orenstein H, Toder V, Fein A (2001). TGFbeta2 in embryos with inborn anomalies: effect of maternal immunopotentiation. Am. J. Reprod. Immunol. 45(1):41-51.

Jewett SL, Eddy LJ, Hochstein P (1989). Is the autoxidation of catecholamines involved in ischemia-reperfusion injury? Free Radic. Biol. Med. 6:185-188.

Kimmel CA, Kimmel GL, White CG, Grafton TF, Young JF Nelson, CJ (1984). Bloodflow changes and conceptal development in pregnant rats in response to caffeine. Fundam. Appl. Toxicol. 4(2):240-247.

Kimmel CA, Trammekl CA (1981). A rapid procedure for routine double staining of cartilage and bone in fetal and adult animals. Stain Technol. 56:271-273.

Lelo A, Miners JO, Robson R, Birkett DJ (1986). Quantitative assessment of caffeine partial clearances in man. Br. J. Clin. Pharmacol. 22(2):183-186.

Linn S, Schoenbaum SC, Monson RR, Rosner B, Stubblefield PG, Ryan KJ (1982). No association between coffee consumption and adverse outcomes of pregnancy. N. Engl. J. Med. 21:306(3):141-145.

McKim EM (1991). Caffeine and its effects on pregnancy and the neonate. J. Nurse Midwifery 36(4):226-231.

Moriguchi M, Scott G Jr (1986). Prevention of caffeine-induced limb malformations by maternal adrenalectomy. Teratology 33(3):319-322.

Nash JE, Persaud TVN (1988). Influence of nicotine and caffeine on rat embryonic development. Histol. Histopath. 3:377-388.

Nehlig A, Debry G (1994). Potential teratogenic and neuro developmental consequences of coffee and caffeine exposure: a review on human and animal data. Neurotoxicol. Teratol. 16(6):531-543.

Nishimura H, Nakai K (1960). Congenital malformation in offspring of mice treated with caffeine. Proc. Soc. Exp. Biol. 104:140-142.

Nolen GA (1981). The effect of brewed and instant coffee on reproduction and teratogenesis in the rats. Toxicol. Appl. Pharmacol. 58:171-183.

Noguchi N, Nishino K, Niki E (2000). Antioxidant action of the antihypertensive drug, carvedilol, against lipid peroxidation. Biochem. Pharmacol. 59(9):1069-1076.

Olcina GJ, Muŏz D, Timń R (2006). Effect of caffeine of on oxidative stress during maximum incremental exercise. J. Sport Sci. Med. 5:621-628.

Palm PE, Arnold EP, Rachwall PC (1978). Evaluation of the teratogenic potential of fresh-brewed coffee and caffeine in the rat. Toxicol. Appl. Pharmacol. 44:1-16.

Prakash AK, Kumar A (2009). Effect of chronic treatment of carvedilol

on oxidative stress in an intracerebroventricular streptozotocin induced model of dementia in rats. J. Pharm. Pharmacol. 61(12):1665-1672.
Ross CP, Persaud TV (1989). Neural tube defects in early rat embryos following maternal treatment with ethanol and caffeine. Anat. Anz. 169(4):247-252.
Tasset I, Espínola C , Medina FJ , Feijó M , Rui, C, Moreno E , Gḿez MM, Collado JA, Muǒz C , Muntané J , Montilla P , Tȉez I (2009). Neuroprotective effect of carvedilol and melatonin on 3-nitropropionic acid-induced neurotoxicity in neuroblastoma. J. Physiol. Biochem. 65(3):291-296.
Sharova LV, Gogal RM, Sharov AA, Crisman MV, Holladay SD (2002). Stimulation in urethane- exposed pregnant mice increase expression level of spleen leukocyte genes for TGF beta 3 GM- CSF and other cytokines that may play a role reduced chemical - induced birth defects. Int. Immunopharmacol. 10:1477-1489.
Tunez I, Collado JA, Medina FJ, Muǒz MC, Gordillo R, Sampedro C, Moyano MJ, Feijó M, Muntané J, Montilla P (2006). Protective effect of carvedilol on oxidative stress induced by okadaic acid in N1E-115 cells. Pharmacol. Res. 54(3):241-246.
Vistisen K, Poulsen HE, Loft S (1992). Foreign compound metabolism capacity in man measured from metabolites of diatery caffeine. Carcinogenesis 13(9):1561-1568.
Winn LM, Wells PG (1999). Maternal administration of superoxide dismutase and catalase in phenytoin teratogenicity. Free. Radic. Biol. Med. 26:266-274.
Yue TL, Cheng HY, Lysko PG, Mckenna PJ, Feuerstein R, Gu JL, Lysko KA, Davis LL, Feuerstein G (1992). Carvedilol, a new vasodilator and β-adrenoreceptor antagonist, is an antioxiadant and free radical scavenger. J. Pharmacol. Exp. Ther. 263:92-98.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2235-2240, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.580
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Significance of the distribution of platelet-derived growth factor B chain in arteriosclerosis plaque in type-2 diabetic patients with arteriosclerosis obliterans of lower extremity

Xu Li *, Jian-Jun Liu and De-Chun Xu

Department of Vascular Surgery, Qingpu Branch of Zhongshan Hospital, Fudan University, Shanghai 201700, China.

Accepted 13 July, 2012

This study aims to explore the expression of platelet-derived growth factor (PDGF) in arteriosclerosis plaque on type 2 diabetes mellitus (DM) with arteriosclerosis obliterans (ASO) of the lower extremity. A total of 24 samples from 12 patients with lower-extremity ASO who underwent surgical treatment were collected in this study. Among these samples were 12 iliofemoral arterial plaques and 12 popliteal or distal arterial plaques. Immunohistochemistry staining was performed on the plaque intima to determine the expression level of platelet-derived growth factor receptors α and β. Reverse-transcription polymerase chain reaction was carried out to detect the mRNA level of the PDGF A and B chains. PDGFR-α and PDGFR-β were excessively expressed in lower-extremity arteriosclerosis plaques. The mean PDGF B chain mRNA level in the popliteal arterial plaques of ASO patients with type-2 DM was increased when compared with that in the iliofemoral arterial plaques. The PDGF B chain mRNA level was higher in the popliteal arterial plaques in type-2 DM with ASO than in iliofemoral artery plaques. This finding confirmed that the PDGF B chain has a key function in arteriosclerosis development.

Key words: Arteriosclerosis obliterans, platelet-derived growth factor, type-2 diabetes.

INTRODUCTION

Arteriosclerosis obliterans (ASO) refers to the arteriostenosis and occlusion caused by the accumulation of atherosclerotic material, which results in thrombosis. Type-2 diabetes mellitus (DM) patients are at risk for ASO pathogenesis 11 times higher than the general population. Type-2 DM patients also show more severe pathological symptoms, poorer prognoses, higher amputation rates, and higher death rates (Mutirangura et al., 2008).

In the ASO pathogenic progression, a large amount of platelet-derived growth factor (PDGF) can be secreted via autocrine and paracrine secretions by damaged epithelial cells, activated platelets, mononuclear macrophages, and the phenotypically transformed smooth muscle cells. Once PDGF binds to its receptor, the proliferation of mononuclear cells is enhanced, cell attachment to the intima is promoted, and cells migrate toward the subintima. The growth and proliferation of epithelial cells, fibroblasts, and vascular smooth muscle cells (VSMCs) are also PDGF dependent. The formation of foam cells from epithelial cells, fibroblasts, and VSMCs due to excessive lipid endocytosis has a key function in the occurrence and progression of arteriosclerotic plaques (Edelberg et al., 2002). The high serum glucose levels in diabetic patients may induce glycosylation of hemoglobin, causing hypoxia and lipid hypermetabolism which may further damage vascular endothelial cells (van den Oever et al., 2010; Bakker et al., 2009). Platelets form a major defense system that can be triggered by subepithelial components such as collagen fiber and von Willebrand factor. Activated platelets induce vasculitis, arteriosclerosis, and thrombus via P-selectin glycoprotein

*Corresponding author. E-mail: longhuafan@yeah.net. Tel: 86-21-69719190.

-1-mediated neutrophil and monocyte adhesion (Furie and Furie, 2004). Activated platelets also enhance the formation and progression of atherosclerotic plaques by facilitating the release of mitogen as well as chemokines such as PDGF and TGF-β (Karvinen et al., 2009; Brown et al., 2010; Doran et al., 2008). It has been demonstrated that celastrol inhibits atherogenesis in celastrol-treated apoE-mice fed an atherogenic diet by inhibiting inflammation in the arterial wall without improving the lipid profile (Cheng et al., 2011), and avocado fruit pulp (AFP) possesses hypocholesterolemic and antioxidant properties due to its phytoconstituents contents and substantiates its use in folkloric practices to control dyslipidemia (Mohammed and Al-Dosari, 2011). In addition, Mahbubeh et al. (2011) demonstrated that verjuice could reduce some atherosclerotic risk factors in long term treatment.

DM patients with lower extremity ASO typically contract vascular occlusions at remote ends of the cardiovascular circulation, which may further aggravate the ischemia symptom. Reports on the pathogenic correlation of the expression level of PDGF and its receptors with the pathogenic location and severity are limited, which necessitates the present study and related ones. In this study, we explored the expression of PDGF in arteriosclerosis plaque on type-2 DM with ASO of the lower extremity.

MATERIALS AND METHODS

Samples

A total of 24 arteriosclerosis samples from 12 patients (8 males and 4 females) were acquired in our hospital from January, 2007 to December, 2010. The average age of the patients was 76 years (range = 68 to 86 years). The patients suffered from many concomitant diseases such as high blood pressure, coronary heart disease, carotid artery stenosis, and cerebral lacunar infarction. All patients received clopidogrel (75 mg/day) and novolin therapy (Table 1). Patients (9) underwent femoral-popliteal artery bypass surgery and 3 patients underwent above-knee amputation. Among the samples were 12 iliofemoral arterial plaques and 12 popliteal or distal arterial plaques. All patients gave written informed consent, and the local ethics committee approved the study.

Assay methods

All operations were carried out under general anesthesia or continuous epidural anesthesia. During the femoral-popliteal artery bypass surgery (vascular prosthesis or great saphenous vein), proximal and distal samples were taken from the endarterectomy. Samples from the above-knee amputation patients were acquired by collecting 2 cm of the proximal superficial femoral artery and the distal popliteal artery. All samples were immediately split after acquisition. One part was stored at 4% formalin solution; the other part was mounted in a cryovial for liquid nitrogen freezing, and subsequently stored in a -70°C freezer.

Immunohistochemistry

Formalin samples were washed and desiccated before wrapping in paraffin wax. After hematoxylin and eosin (HE) staining, microscopic morphological recognition of VSMCs was carried out by identifying the nuclei as blue, cytoplasm as magenta or pink, and collagen as pink.

Expressed PDGF receptors α and β were immunostained by the following procedures. Samples were dewaxed and rehydrated before washing three times in phosphate-buffered saline (PBS, pH 7.4) for 3 min each time. Antigen was retrieved by microwave incubation at 90°C in 10 M citric acid buffer for 20 min. After washing, the samples with distilled water, endogenous peroxidase, was quenched by adding one drop of 0.3% hydrogen peroxide and was incubated at 37°C for 30 min. The samples were again washed with PBS and incubated at room temperature with 10% horse antiserum for 30 min. After incubating the samples with primary antibody (1:50 dilution of anti-PDGFR-α, 1:25 dilution of anti-PDGFR-β, rabbit anti-human monoclonal antibody; Santa Cruz BIO, USA) at room temperature for 60 min and then at 4°C overnight, they were washed again with PBS three times. The slides were then incubated with 50 µl of MaxVision^{TM} (KIT-5004 HRP-polymer anti-rabbit IHC Kit, Maixin BIO, Fujian, China) at room temperature for 45 min (9) and were washed with PBS three times. Microscopic examination was performed after adding 3,3'-diaminobenzidine (DAB) reagent (Biomiga, USA). The slides were HE stained, washed with PBS, desiccated with ethanol, and then sealed with neutral gum. The stained images were verified by examining the staining colors, that is, blue for VSMC nuclei, light pink for cytoplasms, and brown granular particle deposition for PDGFR.

Reverse transcription-polymerase chain reaction (RT-PCR)

Ribonucleic acid (RNA) was extracted by the standard Trizol method (Trizol Reagent, TakaRa) from the samples stored at -70°C. RNA was quantified before being reverse transcribed into cDNA. The reaction system (20 µl total volume) contained the following: 0.1 M dithiothreitol (DTT; Invitrogen, USA), 2 µl; 40 U/µl RNasin (Promega; USA), 0.5 µl; 10 mM 2'-deoxynucleoside 5'-triphosphate (dNTP; TakaRa, Japan), 1.0 µl; Random Primer (TakaRa, Japan), 1.0 µl; 5 × first-strand buffer, 4.0 µl; 5 U/µl DnaseI (TakaRa; Japan), 1.0 µl; and RNA, 10.5 µl. The reaction was carried out at 37°C for 30 min and 75°C for 10 min. The reaction system for reverse transcription contained the following: 0.5 µl of 40 U/µl RNasin and 0.5 µl of 200 U/µl reverse transcriptase SSII (Invitrogen, USA). The reaction was carried out at 25°C for 10 min, 42°C for 1 h, 52°C for 15 min, and 70°C for 15 min. The reaction was then stored at 4°C.

cDNA (1.0 µl) was collected from each sample for fluorescent qPCR assay. The reaction system contained the following: 5 × R-PCR buffer (TakaRa, Japan), 5.0 µl; 250 mM Mg^{2+}, 0.3 µl; 10 mM dNTP (TakaRa; Japan), 0.75 µl; 10 µM forward primer (CASarray), 0.5 µl; 10 µM reverse primer (CASarray), 0.5µl; 25 × SYBR Green I (Bio-Rad; USA), 1.0 µl; 10^{-3} calibration (Bio-Rad), 1.0 µl; 5 U/µl HS-ExTaq (TakaRa; Japan), 0.25 µl; ddH_2O, 14.7 µl; and cDNA, 1.0 µl. The reaction program was as follows: 95°C for 90 s, 95°C for 5 s + 58°C for 30 s (40 cycles), 95°C for 1 min, 58°C for 1 min, and 58°C for 10 s (+0.5°C cycle^{-1} × 68 cycles). The reaction was stored at 4°C. The primer sequences were as follows: PDGF A chain forward, 5'-CTACGGTGACCTGGTGGACT-3'; PDGF A chain reverse, 5'-ACTCGTCCTTGCTCATGTCC-3' (product size: 188); PDGF B chain forward, 5'-TACGGCAATGGCTTT ATCAC-3'; PDGF B chain reverse, 5'-CCCTCCTGCAACTTCTCAAT-3' (product size: 209 bp); and GAPDH forward, 5'-AAGGTCGGAGTCAACGGATT-3'; GAPDH reverse, 5'-CTGGAAGAT GGTGATGGGATT-3' (product size: 222 bp).

Statistics analysis

The mRNA expression level was summarized into target mRNA

Table 1. Patients' data.

Patient	Sex	Age	Preoperative fasting plasma glucose levels (mmol/L)	Symptom	Operation	Medications
1	M	86	7.1	Rest pain (R)	Bypass	Clopidogrel and Novolin50R
2	M	73	11.0	Foot (L) gangrene	Amputation	Clopidogrel and Novolin30R
3	M	77	8.5	Intermittent claudication (R)	Bypass	Clopidogrel and Novolin30R
4	M	73	8.4	Rest pain (L)	Bypass	Clopidogrel and NovolinR
5	M	82	9.8	Foot (R) gangrene	Amputation	Clopidogrel and Novolin50R
6	M	71	10.1	Rest pain (L)	Bypass	Clopidogrel and NovolinR
7	M	70	6.9	Intermittent claudication (L)	Bypass	Clopidogrel and NovolinR+N
8	M	68	8.6	Rest pain (R)	Bypass	Clopidogrel and Novolin30R
9	F	76	9.2	Intermittent claudication (L)	Bypass	Clopidogrel and Novolin50R
10	F	79	7.1	Rest pain (R)	Bypass	Clopidogrel and Novolin30R
11	F	76	13.5	Foot (R) gangrene	Amputation	Clopidogrel and Novolin30R
12	F	81	7.9	Rest pain (R)	Bypass	Clopidogrel and Novolin30R

copies against every 106 copies of GAPDH. Statistical Package for Social Sciences (SPSS) 10.0 software was used for the related analysis.

RESULTS

HE staining

In patients with type-2 DM plus ASO, HE staining showed significant VSMC hyperplasia and disordered cell alignment with some inflammatory cell penetration, as well as considerable lipid penetration around iliofemoral vascular plaques (Figure 1a). Among the popliteal arteriosclerotic samples, HE staining showed disordered VSMC alignment, with similar lipid penetration as the iliofemoral samples (Figure 1b).

Expression levels of PDGFR-α and -β

Immunostaining revealed that PDGFR-α accumulated in the shuttle-shaped stripe. In patients with type-2 DM plus ASO patients, PDGFR-α was highly expressed in plaques at the iliofemoral section (Figure 1c), with increased expression in plaques at the popliteal section (Figure 1d). PDGFR-β demonstrated granular accumulation in plaques. The expression in plaques at the iliofemoral and popliteal sections was significantly increased (Figure 1e and f).

PDGF A and B chains mRNA expression

The average PDGF A chain mRNA level in type-2 diabetes combined with ASO in iliofemoral arteriosclerosis was 26 408 ± 3447 copies per 106 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) copies. The corresponding average in popliteal obliterans patients was 27 725 ± 5127 copies per 106 GAPDH copies. The difference in the two averages is statistically insignificant ($P > 0.05$). The average PDGF B chain expression level in iliofemoral arteriosclerosis samples was 102 633 ± 65267 per 106 GAPDH copies, which is far lower than that in the popliteal samples at 135 541 ± 96196 per 106 GAPDH copies. The difference is significant ($P < 0.05$) (Table 2).

DISCUSSION

PDGF is a group of mitogen and chemokine in serum with a molecular weight range of 28 to 35 kDa. The currently identified peptides in the PDGF family include four, EST ID PDGF-A, -B, -C, and -D. Functional PDGF molecules are normally homodimers or heterodimers constituted by any two of the four PDGF peptide types. Hyperplasia VSMC has been shown to express PDGF A chain and PDGF receptor gene, as well as secrete bioactive PDGF-like molecules. Thus, the expression of PDGF aggravates arteriosclerosis and further leads to obliterans (Doran et al., 2008).

PDGF receptor is a single-chain cross-membrane tyrosine kinase receptor with a molecular weight range of 170 to 180 kDa. The receptor includes two types of subunits, namely, α and β, and is functional as either a monomer or an instable dimer. PDGFR-α binds PDGF-A, -B, and -C, whereas PDGFR-β binds PDGF-B and -D. PDGF homodimer CC and DD can both bind PDGF receptor heterodimer αβ. PDGFR-α has an important function in the regulation of neural ridge cell development and metamere development in the embryonic period (Tallquist and Sodano, 2003), whereas PDGFR-β is involved in vascular wall development (Winkler et al., 2010). The expression of both PDGFR-α and -β has been

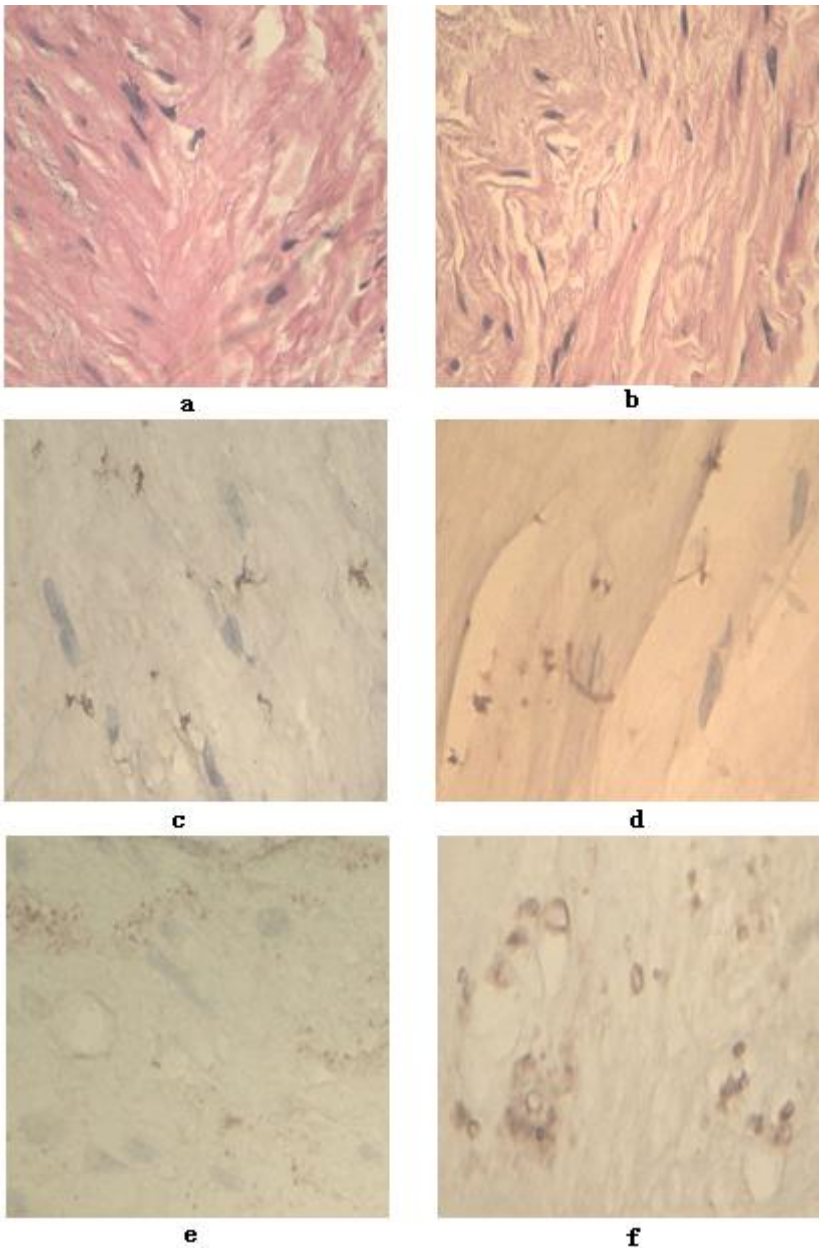


Figure 1. Hematoxylin and eosin staining. Significant VSMC hyperplasia and disordered cell alignment with some inflammatory cell penetration as well as considerable lipid penetration around iliofemoral vascular plagues (a). Popliteal arteriosclerotic samples disordered VSMC alignment, with similar lipid penetration as iliofemoral samples (b). PDGFR-α was accumulated in shuttle shaped stripe. Among type-2 diabetes induced ASO patients, PDGFR-α was intensively expressed in plagues at iliofemoral section (c), with increased expression in plagues at popliteal section (d). PDGFR-β demonstrated granular accumulation in plagues at femoral artery, with significant expression increase in plagues at iliac and popliteal section (e, f).

shown to increase in the vessels of atherosclerosis patients, and the mechanical stimulation of smooth muscle cells induces increased expression of receptors to PDGFR-α (Zhang and Khachigian, 2010).

Clinical studies revealed that arteriosclerosis is one of the major causes of amputation due to its rapid pathogenic progression, accompanied with iliofemoral obsterans, popliteal obsterans, tissue ischemia, ulcer, and necrosis. Related investigations on arteriosclerosis pathogenic mechanism, particularly, distal

Table 2. PDGF A chain and B chain mRNA expression level comparison between type-2 diabetes iliofemoral arteriosclerosis samples and popliteal artery samples.

S/N	PDGF A chain		PDGF B chain	
	Iliofemoral	Popliteal	Iliofemoral	Popliteal
1	1.96×10^4	2.35×10^4	2.65×10^4	3.29×10^4
2	3.09×10^4	3.38×10^4	2.10×10^5	2.86×10^5
3	2.67×10^4	2.24×10^4	8.01×10^4	1.22×10^5
4	2.65×10^4	3.02×10^4	6.07×10^4	6.48×10^4
5	2.67×10^4	2.15×10^4	8.44×10^4	9.14×10^4
6	2.44×10^4	2.63×10^4	7.33×10^4	9.25×10^4
7	2.15×10^4	2.71×10^4	8.12×10^4	1.05×10^5
8	3.02×10^4	3.35×10^4	1.96×10^5	2.97×10^5
9	2.97×10^4	3.58×10^4	2.16×10^5	2.89×10^5
10	2.73×10^4	2.59×10^4	6.75×10^4	7.84×10^4
11	2.45×10^4	2.12×10^4	5.37×10^4	6.88×10^4
12	2.89×10^4	3.15×10^4	8.22×10^4	9.87×10^4
M	26408 ± 3447	27725 ± 5127	102633 ± 65267	135541 ± 96196
P	P > 0.05		P < 0.05	

arteriosclerosis, may improve the rate of limb salvation. The tissue-specific pathogenic mechanism of diabetic arteriosclerosis is not yet clear. Genetics, immunological specificity, environment, and living pattern may all be effective pathogenic elements, and the related research is also sociologically importance (Boulton, 2004; Vuorisalo et al., 2009; Edmonds, 2006).

The key function of PDGF in general arteriosclerosis pathogenesis is the major consideration of the present study. The specific functionality of PDGF in lower-extremity ASO featured by distal-end vascular obliterans and severe ischemia is under debate.

Conclusion

Conclusively, the results showed that the mean PDGF B chain mRNA level in the popliteal arterial plaques of ASO patients with type-2 DM was increased when compared with that in the iliofemoral arterial plaques. The PDGF B chain mRNA level was higher in the popliteal arterial plaques in type-2 DM with ASO than in iliofemoral artery plaques. The PDGF B chain mRNA level was higher in the popliteal arterial plaques in type-2 DM with ASO than in iliofemoral artery plaques. The increased PDGF B chain expression shows that the PDGF B chain may be closely involved in the pathogenesis of distal end obstruction.

REFERENCES

Bakker W, Eringa EC, Sipkema P, van Hinsbergh VW (2009). Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. Cell Tiss. Res. 335:165-189.

Boulton AJ (2004). Pressure and the diabetic foot: clinical science and offloading techniques. Am. J. Surg. 187:17S-24S.

Brown XQ, Bartolak-Suki E, Williams C, Walker ML, Weaver VM, Wong JY (2010). Effect of substrate stiffness and PDGF on the behavior of vascular smooth muscle cells: implications for atherosclerosis. J. Cell. Physiol. 225:115-122.

Cheng J, Tian Z, Li J, Hu H (2011). Celastrol attenuates atherosclerosis in Apolipoprotein E (apoE) knockout mice fed an atherogenic diet. Afr. J. Pharm. Pharmacol. 5:1247-1251.

Doran AC, Meller N, McNamara CA (2008). Role of smooth muscle cells in the initiation and early progression of atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 28:812-819.

Edelberg JM, Lee SH, Kaur M, Tang L, Feirt NM, McCabe S, Bramwell O, Wong SC, Hong MK (2002). Platelet-derived growth factor-AB limits the extent of myocardial infarction in a rat model: feasibility of restoring impaired angiogenic capacity in the aging heart. Circulation 105:608-613.

Edmonds M (2006). Diabetic foot ulcers: practical treatment recommendations. Drugs 66:913-929.

Furie B, Furie BC (2004). Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. Trends Mol. Med. 10:171-178.

Karvinen H, Rutanen J, Leppanen O, Lach R, Levonen AL, Eriksson U, Ylä-Herttuala S (2009). PDGF-C and -D and their receptors PDGFR-alpha and PDGFR-beta in atherosclerotic human arteries. Eur. J. Clin. Invest. 39:320-327.

Mahbubeh S, Bahar N, Sedighe Asgary, Leila Azadbakht, Mahmoud Rafieian-Kopaei (2011). Anti atherosclerotic effects of verjuice on hypocholesterolemic rabbits. Afr. J. Pharm. Pharmaco. 5:1038-1045.

Mohammed S, Al-Dosari (2011). Hypolipidemic and antioxidant activities of avocado fruit pulp on high cholesterol fed diet in rats. Afr. J. Pharm. Pharmacol. 5:1475-1483.

Mutirangura P, Ruangsetakit C, Wongwanit C, Sermsathanasawadi N, Chinsakchai K (2008). Comparative study of the management of diabetic versus nondiabetic patients with atherosclerosis obliterans of the lower extremities. Vascular 16:333-339.

Tallquist MD, Sodano P (2003). Cell autonomous requirement for PDGFR alpha in populations of cranial and cardiac neural crest cells. Development 130:507-518.

van den Oever IA, Raterman HG, Nurmohamed MT, Simsek S (2010). Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus. Mediators Inflamm. 2010:792393.

Vuorisalo S, Venermo M, Lepntalo M (2009). Treatment of diabetic foot ulcers. J. Cardiovasc. Surg. (Torino) 50:275-291.

Winkler EA, Bell RD, Zlokovic BV (2010). Pericyte-specific expression of PDGF beta receptor in mouse models with normal and deficient PDGF beta receptor signaling. Mol. Neurodegener 5:32.
Zhang N, Khachigian LM (2010). Injury-induced platelet-derived growth factor receptor-alpha expression mediated by interleukin-1beta (IL-1beta) release and cooperative transactivation by NF-kappaB and ATF-4: IL-1beta facilitates HDAC-1/2 dissociation from promoter. J. Biol. Chem. 285:21902.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2241-2246, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.628
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Renoprotective effect of silymarin on gentamicin-induced nephropathy

Mohammad Mashayekhi

Department of Clinical Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran.
E-mail: mashayekhi79@yahoo.com, mashayekhi@iaut.ac.ir

Accepted 13 July, 2012

Gentamicin (GM) is an aminoglycoside antibiotic which is used in clinical practice to treat severe gram-negative infections. *Silybum marianum*, also known as milk thistle, is a member of Asteraceae family and is well recognized as a hepatoprotective herbal medicine. The main objective of this present study was to evaluate the renoprotective effect of silymarin on GM-induced nephropathy. Eighteen healthy Ghezel sheep were randomly divided into three groups of control and treatments. All sheep of two groups received 20 mg/kg/body weight of GM every 8 h by intramuscular injection for 10 days. Treatment group also receive silymarin at the dose of 80 mg/case three-times a day for 20 days intramuscular injection for 14 days. This study showed that the GM-induced renal toxicity, as measured by multiple functional, structural and enzymatic factors is significantly reduced by co-supplementation of silymarin. The results of this study suggest the potential of silymarin to protect against GM-induced nephrotoxicity.

Key words: Renoprotection, silymarin, gentamicin, nephropathy.

INTRODUCTION

Gentamicin (GM) is an aminoglycoside antibiotic which is used in clinical practice to treat severe gram-negative infections. However, its nephrotoxic action has limited the extent of its use (Mingeot-Leclercq and Tulkens, 1999). Routine therapeutic use of aminoglycoside, GM (80 mg/kg/body weight) for more than 7 days, has long been the commonest cause of nephrotoxicity in approximately 30% of patients (Moore et al., 1984; Barclay and Begg, 1994; Pedraza-Chaverrı et al., 2003). The specificity of GM for renal toxicity is apparently related to its ability to increasingly facilitate the generation of radical species, including superoxide anions, hydrogen peroxides and hydroxyl radicals in mitochondria, a few of which appears to be a crucial part of the antioxidant deficiency-associated oxidative stresses in the renal proximal convoluted tubules (Maldonado et al., 2003; Yanagida et al., 2004). Recently, a number of studies demonstrating reduced plasma concentration of endogenous chain-breaking antioxidant, like vitamin E, in nephrotoxicity and interactions between this vitamin and biochemical reactions such as cortical lipid peroxidation, synthesis of radical-driven metabolites and electron-transferring pathway, suggest that disturbed metabolism of vitamin E may be important in the pathogenesis of nephrotoxicity with GM (Ademuyiwa et al., 1990; Abdel-Naim et al., 1999; Kadkhodaee et al., 2004).

Silybum marianum, also known as milk thistle, is a member of Asteraceae family and is well recognized as a hepatoprotective herbal medicine. Silymarin is a lipophilic extract of the milk thistle seeds. It is composed of three isomers of flavonolignans (silybin, silydianin and silychristin), and two flavonoids (taxifolin and quercetin) (Kren and Walterova, 2005; Abenavoli et al., 2010).

Silymarin is commonly prescribed in cases of cirrhosis, viral hepatitis and *Amanita phaloides* poisoning (Loguercio and Festi, 2011; Abenavoli et al., 2010; Saller et al., 2001). Two major mechanisms have been proposed to account for the hepatoprotective effects of silymarin. The first mechanism is due to its dose-dependent antioxidant effect. This effect is mediated by scavenging of free radicals, decreasing formation of reactive oxygen species (ROS) and inhibition of fatty acid peroxidation. The second mechanism involves anti-inflammatory and anti-apoptotic actions through interference with nuclear factor kappa-B (NF-κB), modulation of inducible nitric oxide and decreases in

Table 1. Biochemical parameters analysis on day 0 of study.

Parameter / No.	Creatinine (mg/dl)	Uric acid (mg/dl)	BUN (mg/dl)	Urea (mg/dl)	GGT (IU/L)	Urine casts	Dipstick testing
1	1.11	0.48	18.45	39.4	16	Negative	Normal
2	1.12	0.45	19.11	40.8	18	Negative	Normal
3	1.11	0.46	19.15	40.9	20	Negative	Normal
4	1.09	0.51	18.85	40.3	18	Negative	Normal
5	1.14	0.53	18.90	40.4	16	Negative	Normal
6	1.11	0.46	19.50	41.7	15	Negative	Normal
7	1.08	0.5	19.35	41.4	19	Negative	Normal
8	1.12	0.51	18.45	39.4	18	Negative	Normal
9	1.11	0.45	18.85	40.3	17	Negative	Normal
10	1.07	0.51	19.22	41.1	18	Negative	Normal
11	1.13	0.46	20.31	43.4	19	Negative	Normal
12	1.11	0.47	18.45	39.4	21	Negative	Normal
13	1.12	0.47	18.85	39.8	17	Negative	Normal
14	1.14	0.45	19.20	40.9	18	Negative	Normal
15	1.14	0.49	19.55	41.8	19	Negative	Normal
16	1.07	0.46	19.82	42.4	19	Negative	Normal
17	1.09	0.48	18.25	39	18	Negative	Normal
18	1.11	0.51	20.68	44.2	19	Negative	Normal
Total	1.04	0.48	19.16	40.92	18.05	-	-

cyclooxygenase-2 expression. Silymarin also possesses antiviral and anti-fibrotic effects (Saller et al., 2001; Vaid and Katiyar, 2010).

As noted, silymarin has shown promising hepatoprotective effects, both experimentally and clinically. Antioxidant and anti-inflammatory properties of silymarin may also have protective role against photocarcinogens (Vaid and Katiyar, 2010) and nephropathic processes (Kren and Walterova, 2005). Silybin has been found to stimulate kidney cells in a similar manner to that seen in liver cells. Silybin and silychristin have been shown to increase proliferation rate, protein and DNA biosynthesis and lactate dehydrogenase (LDH) activity in kidney cells that have been damaged *in vitro* by paracetamol, cisplatin or vincristine. Administration of silybin prior to or following the chemical-induced injury has prevented or reduced nephrotoxic effects (Sonnenbichler et al., 1999). Silymarin therefore, appears to have the potential as a renoprotective agent against nephrotoxic medications due to its antioxidant, anti-inflammatory and anti-apoptotic actions. The main objective of present study was to evaluate the renoprotective effect of silymarin on GM-induced nephropathy.

MATERIALS AND METHODS

Experimental protocol

This study was conducted in Tabriz during summer, 2011. Vials of both, injectable (i.m.) GM sulphate and silymarin assigned for medical applications were purchased from Drugstore Co. (Tabriz, Iran).

In present study, after induction of nephropathy, we used silymarin as renoprotective at the dose of 80 mg/case three-times a day for 20 days.

Eighteen healthy Ghezel sheep divided into three groups including (1) control, (2) treatment with vitamin E and (3) treatment with vitamin C.

Biochemical assay

Urine and blood samples were obtained on days 0, 7, 10, 14 and 17 from sheep of each group and values of creatinine, uric acid, blood **urea** nitrogen (BUN), urea and glutamyltransferase (GGT) were measured by special kits.

Statistical analysis

The statistical package for social sciences (SPSS Inc., Chicago, IL, USA), version 13.0, was used for statistical analysis. All data are presented as mean ± SEM. Before statistical analysis, all variables were checked for normality and homogeneity of variance by using the Kolmogorov-Smirnoff and Levene tests, respectively. The data obtained were tested by analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test. $P<0.05$ was considered statistically significant.

RESULTS

Data associated with biochemical parameters on day 0 are shown in Table 1. Data associated with biochemical

Table 2. Biochemical parameters analysis of normal control group on days 7, 10, 14 and 17.

Parameter / No.	Creatinine (mg/dl)	Uric acid (mg/dl)	BUN (mg/dl)	Urea (mg/dl)	GGT (IU/L)	Urine casts	Dipstick testing
			Day 7				
1	3.4	1.1	35	74.9	31	Negative	Normal
2	3.3	1.2	32	68.4	35	Epithelial cells	Normal
3	2.8	1	28	59.9	27	Negative	Normal
4	2.8	1.2	30	64.2	30	Epithelial cells	Normal
Total	3.07	1.12	31.25	66.85	30.75	-	-
			Day 10				
1	3.9	1.8	51	109.1	40	Normal	Normal
2	4	1.9	46	98.4	32	Epithelial cells	Normal
3	4	1.7	48	102.7	37	Normal	Normal
4	3.7	1.7	49	104.8	43	Epithelial cells	Normal
Total	3.90	1.77	48.50	103.75	38.00	-	-
			Day 14				
1	6.3	2.2	54	115.5	46	Epithelial cells	Normal
2	6.4	2.3	53	113.4	45	Epithelial cells	Normal
3	6	2.3	51	109.1	42	Normal	Normal
4	6.1	2	51	109.1	50	RBC	Hb
Total	6.20	2.20	52.25	111.77	45.75	-	-
			Day 17				
1	6.4	2.3	60	128.4	52	Epithelial cells	Normal
2	6.3	2.2	58	124.1	48	Epithelial cells	Normal
3	6.4	2.3	59	126.2	45	Normal	Normal
4	6.2	2.3	59	126.2	53	RBC	Hb
Total	6.32	2.27	59.00	126.22	49.50	-	-

parameters on days 7, 10, 14 and 17 in the normal control group are shown in Table 2. Data associated with biochemical parameters on days 7, 10, 14 and 17 in the treated group with silymarin are shown in Table 3.

As shown in Table 1, the mean value of creatinine, uric acid, BUN, urea and GGT on day 0 is 1.04, 0.48, 19.16, 40.92 and 18.05, respectively, while, these values are 3.07, 1.12, 31.25, 66.85 and 30.75, respectively on day 7 in toxic controls compared with 1.90, 0.65, 22.00, 47.02 and 20.75 in the silymarin group on same day; so there is significant difference among two groups.

On day 10, the mean value of creatinine, uric acid, BUN, urea and GGT were 3.90, 1.77, 48.50, 103.75 and 38.00, respectively in the GM toxic group and were 2.07, 0.97, 27.25, 58.25 and 20.30 in silymarin group; so there is significant difference among two groups.

On day 14, the mean value of creatinine, uric acid, BUN, urea and GGT were 6.20, 2.20, 52.25, 111.77 and 45.75, respectively in the GM toxic group and were 3.82, 1.02, 29.00, 62.02 and 23.00 in silymarin group; so there is significant difference among two groups.

Thus, it reveals that silymarin reduced the renal damage makers, so it can be stated that it protects the kidney against GM toxic effects.

DISCUSSION

Aminoglycosides are generally known as most potent nephrotoxic drugs. Aminoglycoside uptake occurs via internalization through megalin transporters. After internalization, aminoglycosides initiate a cascade of reactions that finally result in cell death. To date, increases in intracellular sodium concentration, ROS and proinflammatory cytokines and in apoptosis, decreases in glucose, and depletion of adenosine triphosphate (ATP) storage have been proposed as being responsible for aminoglycoside-induced tubular, glomerular and vascular damage. In the renal tubules, aminoglycosides may interfere with mitochondrial function and compromise ATP production. Induction of apoptosis and ultimately necrosis of tubular epithelial cells have been implicated in tubular toxicity. In the glomerulus, aminoglycosides may decrease glomerular filtration rate through mesangial

Table 3. Biochemical parameters analysis of treated group with silymarin on days 7, 10, 14 and 17.

Parameter / No.	Creatinine (mg/dl)	Uric acid (mg/dl)	BUN (mg/dl)	Urea (mg/dl)	GGT (IU/L)	Urine casts	Dipstick testing
			Day 7				
1	1.9	0.7	22	47	21	Normal	Normal
2	1.8	0.6	23	49.2	25	Normal	Normal
3	1.9	0.8	22	47	18	Normal	Normal
4	2	0.5	21	44.9	19	Normal	Normal
Total	1.90	0.65	22.00	47.02	20.75	-	-
			Day 10				
1	1.9	0.9	28	59.9	22	Normal	Normal
2	2.2	1	27	57.7	20	Normal	Normal
3	2.3	1.1	27	57.7	18	Normal	Normal
4	1.9	0.9	27	57.7	21.2	Normal	Normal
Total	2.07	0.97	27.25	58.25	20.30	-	-
			Day 14				
1	3.9	1	30	64.2	20	Normal	Normal
2	3.8	1.1	29	62	26	Epithelial cells	Normal
3	3.9	1	29	62	25	Normal	Normal
4	3.7	1	28	59.9	21	Normal	Normal
Total	3.82	1.02	29.00	62.02	23.00	-	-
			Day 17				
1	3.5	0.9	27	57.7	20	Normal	Normal
2	3.6	0.8	26	55.6	23	Epithelial cells	Normal
3	3.4	0.8	24	51.3	24	Epithelial cells	Normal
4	3.5	0.7	24	51.3	20	Normal	Normal
Total	3.50	0.80	25.25	53.97	21.75	-	-

contraction, stimulation of mesangial proliferation and neutralization of negative charges of the glomerular filtration barrier. An increase in vasoconstrictor mediators, including increases in angiotensin-II, endothelin-I, thromboxane A2 and ROS, and decreases in vasodilator prostaglandins, have been proposed to explain aminoglycoside induced vascular dysfunction. The net effects of aminoglycosides on kidney are therefore apparently mediated by vasoconstrictors, ROS, inflammatory cytokines and apoptosis. Aminoglycoside-induced nephrotoxicity may be non-oliguric or polyuric and its clinical presentations include increased serum creatinine and urea concentrations, proteinuria, enzymuria, glycosuria, hypomagnesemia and hypocalcemia (Lopez-Novoa et al., 2011).

Varzi et al. (2007) evaluated silymarin effects on GM-induced nephrotoxicity in a study of five groups of dogs. Group 1 was injected with saline as control group. Groups 2 to 5 received intramuscular injection of 20 mg/kg of GM sulfate once daily for 9 days. Group 3 was administered vitamin E orally at dosage of 25 mg/kg once daily for 9 days. Group 4 received silymarin 20 mg/kg daily for 9 days. Group 5 was administered both vitamin E and silymarin at the same doses as groups 3 and 4 for 9 days. Rises in serum creatinine and urea levels and decreases in glomerular filtration rate were considered as markers of deteriorating renal function. Total serum antioxidant activity was also assessed as a marker of antioxidant defense capacity. The dogs that received silymarin concomitant with GM had lower rises in serum creatinine, urea concentrations and higher glomerular filtration rates compared to the group that was administered GM alone. Serum levels of malondialdehyde (MDA), a marker of lipid peroxidation, were also significantly lower and total serum antioxidant activity was higher in silymarin treated dogs. Interestingly, in this study, silymarin showed greater nephroprotective and antioxidant effects than did with vitamin E (Varzi et al., 2007).

About 60% of hospital-acquired acute kidney injury can be accounted for by drug-induced nephrotoxicity, which is a main cause of mortality and morbidity. Several options, such as dose adjustment based on renal function, hydration and avoidance of nephrotoxic agents, have

been proposed to prevent or ameliorate drug-induced nephrotoxicity (DIN) (Dolin and Himmelfarb, 2008; Cynthia, 2008). Nevertheless, DIN remains a major problem for health care professionals. Silymarin is widely used as a hepatoprotective remedy. Antioxidant and anti-inflammatory actions; protein synthesis induction; inhibition of lipid peroxidation, leukotriene and prostaglandin synthesis; and neutrophil migration are among the pharmacologically described effects of silymarin bioflavonoids (Loguercio and Festi, 2011; Saller et al., 2001; Dixit et al., 2007). Silymarin may exert positive effects in the management of patients with renal insufficiency. Recently, Roozbeh et al. (2011) reported that treatment of hemodialysis patients with silymarin, alone or in combination with vitamin E, significantly decreased plasma MDA concentration and increased red blood cell glutathione peroxidase and hemoglobin levels.

Silymarin also reduced kidney damage and restored activities of superoxide dismutase, glutathione peroxidase and catalase enzymes in rats with alloxan-induced diabetes (Soto et al., 2010). In streptozotocin-induced diabetes rats, milk thistle extract attenuated diabetic nephropathy, probably by increasing catalase and glutathione peroxidase activity and reducing lipid peroxidation in renal tissue (Vessal et al., 2010). Renoprotective effects of silymarin against some chemical nephrotoxins other than drugs have also been reported. An animal model of chloroform-induced nephrotoxicity was designed in Sprague-Dawley male rats by administering 20% chloroform in olive oil at dose of 2 ml/kg every other day. When silymarin was intragastrically administered at a dose of 50 mg/kg, 24 h after intraperitoneal injection (IP) administration for 2 weeks, a significantly restored renal function was observed based on the urine and serum markers of kidney function (urea, creatinine, creatinine clearance, protein, albumin, urobilinogen and nitrite). Silymarin treatment also restored losses in body weight and rises in kidney weight that had been induced by chloroform (Khan and Siddique, 2012).

Female Swiss albino mice fed with silymarin and ferric nitrilotriacetate, a potent nephrotoxic agent and a renal carcinogen, showed lower rates of lipid peroxidation, kidney cell hyper proliferation, and expression of proinflammatory mediators compared with the group that received ferric nitrilotriacetate alone (Kaur et al., 2010). Conversely, silymarin exacerbated renal impairment in an animal model of glycerol-induced acute kidney injury. In that study, silymarin administration resulted in persistence of oxidative stress and inflammatory processes, tubular necrosis and apoptosis in rats with glycerolinduced AKI (Homsi et al., 2010).

Another renoprotective impact of silymarin includes preventive effects against ischemia/reperfusion (I/R) renal injury. Silymarin dose-dependently prevented I/R-induced renal morphology changes in Sprague-Dawley rats, including tubular dilatation and vacuolization, pelvic inflammation, interstitial inflammation, perirenal adipose infiltration and tubular and glomerular necrosis (Senturk et al., 2008). Silymarin prevented I/R-induced renal damage in Wistar rats based on other kidney markers such as serum creatinine, urea, and cystatin C concentrations, serum enzymatic activity of gluthathione peroxidase, serum and tissue MDA and nitrogen oxides (NO) levels (Turgut et al., 2008).

Silymarin has also shown anti-cancer activities against renal cell carcinoma in some studies (Cheung et al., 2010; Chang et al., 2011; Li et al., 2008). Possible mechanisms of silymarin anti-cancer effects include inhibition of cell proliferation, enhancement of apoptosis, decreases in angiogenesis, blockage of cell cycle regulators and increases in the expression of cell cycle inhibitors (Cheung et al., 2010). Silymarin is rapidly absorbed following oral administration and undergoes both phase I and especially phase II metabolism in the liver. Although silymarin reduces activities of some cytochrome P-450 isoenzymes, UDP-glucuronosyl transferase and P-glycoprotein, no clinically significant drug interaction has yet been reported with usual dose of silymarin (Wu et al., 2009; Han et al., 2009; Deng et al., 2008). Milk thistle extract has a good safety profile and most associated adverse events are mild in nature and include mild dyspepsia, allergic reactions, urticarial and nausea. Pruritus, headache, exanthema, malaise, asthenia and vertigo have been reported less frequently following silymarin administration (Ghosh et al., 2010; Karimi et al., 2011).

Conclusion

Most of the studies of silymarin are suggestive of promising positive effects on drug-, chemical-, and to some extent, disease-induced nephropathy (DIN). Due to the high burden that DIN places with respect to patient morbidity, mortality and health-related costs, silymarin may be recommended as a renoprotective agent to attenuate toxicity of some valuable drugs such as cisplatin and aminoglycosides that currently have a high likelihood of inducing nephrotoxicity.

ACKNOWLEDEGMENTS

This study was Adapted from a research plan which was supported financially by Islamic Azad University, Tabriz branch. So, author declare own thankful from grant staff of research deputy of Islamic Azad University.

REFERENCES

Abdel-Naim AB, Abdel-Wahab MH, Attia FF (1999). Protective effects of vitamin E and probucol against gentamicin-induced nephrotoxicity in rats. Pharmacol. Res. 40:183-187.

Abenavoli L, Capasso R, Milic N, Capasso F (2010). Milk thistle in liver disease: past, present, future. Phytother. Res. 24(10):1423-1432.

Ademuyiwa O, Ngaha EO, Ubah FO (1990). Vitamin E and selenium in gentamicin nephrotoxicity. Hum Exp Toxicol. 9:281-288.
Barclay ML, Begg EJ (1994). Aminoglycoside toxicity and relation to dose regimen. Adv. Drug React. Toxicol. Rev. 13:207-234.
Chang HR, Chen PN, Yang SF, Sun YS, Wu SW, Hung TW, Lian JD, Chu SC, Hsieh YS (2011). Silibinin inhibits the invasion and migration of renal carcinoma 786-O cells in vitro, inhibits the growth of xenografts in vivo and enhances chemosensitivity to 5-fluorouracil and paclitaxel. Mol. Carcinog. 50(10):811-823.
Cheung CW, Gibbons N, Johnson DW, Nicol DL (2010). Silibinin-A promising new treatment for cancer. Anticancer Agents Med. Chem. 10(3): 186-195.
Cynthia A (2008). Naughton. Drug-Induced Nephrotoxicity. Am Fam Physician 78:743-750.
Deng JW, Shon JH, Shin HJ, Park SJ, Yeo CW, Zhou HH, Song IS, Shin JG (2008). Effect of silymarin supplement on the pharmacokinetic of rosuvastatin. Pharm. Res. 25:1807-1814.
Dixit N, Baboota S, Kohli K, Ahmad S, Ali J (2007). Silymarin: A review of pharmacological aspects and bioavailability enhancement approaches. Indian J. Pharmacol. 39:172-179.
Dolin TD, Himmelfarb J (2008). Drug-induced kidney disease, in Dipiro JT (eds), Pharmacotherapy, a pathophysiologic approach, 7th ed., Mc Graw Hill companies Inc, New York pp. 795-810.
Ghosh A, Ghosh T, Jain S (2010). Silymarin-areview on pharmacodynamics and bioavailability enhancement approaches. J Pharm. Sci. Technol. 2(10):348-355.
Han Y, Guo D, Chen Y, Chen Y, Tan ZR, Zhou HH (2009). Effect of silymarin on the pharmacokinetic of losartan and its active metabolite E-3174 in healthy Chinese volunteers. Eur. J. Clin. Pharmacol. 65: 585-591.
Homsi E, de Brito SM, Janino P (2010). Silymarin exacerbates p53-mediated tubular apoptosis in glycerol-induced acute kidney injury. Ren. Fail. 32:623-632.
Kadkhodaee M, Aryamanesh S, Faghihi M, Zahmatkesh M (2004). Protection of rat renal vitamin E levels by ischemicpreconditioning. B.M.C. Nephrol. 5:6-12.
Karimi G, Vahabzadeh M, Lari P, Rashedinia M, Moshiri M (2011). “Silymarin”, a promising pharmacological agent for treatment of diseases. Iranian J. Basic Med. Sci. 14(4):308-317.
Kaur G, Athar M, Alam MS (2010). Dietary supplementation of silymarin protects against chemically induced nephrotoxicity, inflammation and renal tumor promotion response. Invest New Drugs 28:703-713.
Khan MR, Siddique F (2012). Antioxidant effectsof Citharexylum spinosum in CCl4 induced nephrotoxicity in rat. Exp. Toxicol. Pathol. Exp. Toxicol. Pathol. 64(4):349-55.
Kren V, Walterova D (2005). Silybin and silymarin- new effects and applications. Biomed. Papers 149(1):29-41.
Li L, Gao Y, Zhang L, Zeng J, He D, Sun Y (2008). Silibinin inhibits cell growth and induces apoptosis by caspase activation, down-regulating survivin and blocking EGFR-ERK activation in renal cell carcinoma. Cancer Lett. 272:61-69.
Loguercio C, Festi D (2011). Silybin and the liver: from basic research to clinical practice. World J Gastroenterol. 17(18):2288-2301.
Lopez-Novoa JM, Quiros Y, Vicente L, Morales AI, Lopez-Hernandez FJ (2011). New insight into the mechanism of aminoglycoside nephrotoxicity: an integrative point of view. Kidney Int. 79:33-45.
Maldonado PD, Barrera D, Rivero I, Mata R, Medina-Campos ON, Hernandez-Pando R, Pedraza-Chaverri J (2003). Antioxidant S-allylcysteine prevents gentamicin-induced oxidative stress and renal damage. Free Radic. Biol. Med. 35(3):317-24.
Mingeot-Leclercq MP, Tulkens PM (1999). Aminoglycosides: nephrotoxicity, Antimicrob Agents. Chemotherapy 43:1003-1012.
Moore RD, Smith CR, Lipsky JJ, Mellits ED, Lietman PS (1984). Risk factors for nephrotoxicity in patients treated with aminoglycosides. Ann. Intern. Med. 100:352-357.
Pedraza-Chaverrı J, Gonzalez-Orozco AE, Maldonadoa PD, Barreraa D, Medina-Camposa NO, Hernandez-Pandob R (2003). Diallyl disulfide ameliorates gentamicin-induced oxidative stress and nephropathy in rats. Eur. J. Pharm. 473:71-78.
Roozbeh J, Shahriyari B, Akmali M, Vessal G, Pakfetrat M, Raees Jalali GA, Afshariani R, Hasheminasab M, Ghahramani N (2011). Comparative effects of silymarin and vitamin E supplementation on oxidative stress markers, and hemoglobin levels among patients on hemodialysis. Ren Fail. 33(2):118-123.
Saller R, Meier R, Brignoli R (2001). The use of silymarin in the treatment of liver diseases. Drugs 61(14): 2035-2063.
Senturk H, Kabay S, Bayramoglu G, Ozden H, Yaylak F, Yucel M, Gurlek Olgun E, Kutlu A (2008). Silymarin attenuates the renal ischemia/reperfusion injury-related morphological changes in the rat kidney. World J. Urol. 26:401-407.
Sonnenbichler J, Scalera F, Sonnenbichler I, Weyhenmeyer R (1999). Stimulating effects of silibinin and silicristin from the milk thistle Silybum marianum on kidney cells. J. Pharm. Exp. Ther. 290:1375-1383.
Soto C, Perez J, Garcia V, Uria E, Vadillo M, Raya L (2010). Effects of silymarin on kidneys of rats suffering from alloxan-induced diabetes mellitus. Phytomed. 17:1090-1094.
Turgut F, Bayrak O, Catal F, Bayrak R, Atmaca AF, Koc A, Akbas A, Akcay A, Unal D (2008). Antioxidant and protective effects of silymarin on ischemia and reperfusion injury in the kidney tissues of rats. Int. Urol. Nephrol. 40:453-460.
Vaid M, Katiyar SK (2010). Molecular mechanisms of inhibition of photocarcinogenesis by silymarin, a phytochemical from milk thistle (Silybum marianum L. Gaertn)(Review). Int. J. Oncol. 36(5):1053-1060.
Varzi HN, Esmailzadeh S, Morovvati H, Avizeh R, Shahriari A, Givi ME (2007). Effect of silymarin and vitamin E on gentamicin-induced nephrotoxicity. J. Vet. Pharmacol. Therap. 30:477-481.
Vessal G, Akmali M, Najafi P, Moein MR, Sagheb MM (2010). Silymarin and milk thistle may prevent the progression of dibatic nephropathy in streptozotocin-induced diabetic rats. Ren. Fail. 32:733-739.
Wu JW, Lin LC, Tsai TH (2009). Drugdrug interactions of silymarin on the perspective of pharmacokinetics. J. Ethnopharmacol. 121:185-193.
Yanagida C, Kousei I, Izumi K, Toshiharu H (2004). Protective effect of fosfomycin on gentamicin-induced lipid peroxidation of rat renal tissue. Chem. Biol. Interact. 148:139-147.

African Journal of Pharmacy and Pharmacology Vol. 6(29), pp. 2247-2254, 8 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.638
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Unsupervised data mining technology based on research of stroke medication rules and discovery of prescription

Xinjie Li[1,2], Mingqi Wang[3], Xujie Yu[3], Xiaoyan Li[3], Jingwei Liu[3], Zhenhua Jia[1,4*] and WEI Cong[5]

[1]Hebei Yiling Medicine Research Institute, Shijiazhuang 050035, China.
[2]Key Laboratory of collateral disease of Hebei Province, Shijiazhuang 050035, China.
[3]Beijing University of Chinese Medicine, Beijing 100029, China.
[4]Hebei Yiling Hospital Cardiovascular Division, Shijiazhuang 050091, China.
[5]Key Research Centre of State Administration of Traditional Chinese Medicine (Collateral Disease of Cardiovascular/TCM Collateral Disease Theory Key discipline of State Administration of TCM of China), Shijiazhuang 050035, China.

Accepted 1 June, 2012

For collecting and sorting literature on treatment of ancient and modern stroke at the entry point, frequency and charts analysis methods were applied. Modern computer technology and statistics were used to do in-depth study of stroke literature data, explore stroke differentiation of symptoms and signs and regular treatment by ancient physicians. On this basis, the unsupervised data mining technology was applied to obtain each syndrome commonly used for drug pair and combination of core. In assembling empirically drug pair and combination of core, 3 new prescriptions were obtained for each syndrome.

Key words: Unsupervised data mining, stroke, medication rules, new prescription.

INTRODUCTION

Traditional Chinese medicine has a long history of stroke awareness, and has experienced thousands of years of transmutation for its etiology, pathogenesis and treatment medication. From the "outside wind theory'' to the "internal wind theory"; from dispelling wind and releasing the exterior to nourish the blood and sinew, resolve phlegm, dispel wind, tonify qi, activate blood, calm the liver and extinguish wind; from houshiheisan, daxiaoxumingtang to dihuangyinzi, sanhuatang, buyanghuanwutang and zhenganxifengtang, almost every period, representative physicians had unique insights of stroke disease and accumulated very rich experience in its diagnosis and treatment.

The evolution of the ancient physicians treating stroke reflected the innovation and advancement of the theory and methods of stroke treatment in a particular historical period. The systematic analysis of the compatibility law of prescription is an important way to accurately grasp the pathogenesis of stroke evolution. Close attention has been paid to how to use modern scientific research methods, and how to carry out high efficient and relevant research on stroke.

Research purposes

In this study, we gathered literatures on treatment of ancient and modern stroke, and with the methods of modern computer technology combined with statistics; we established the disease prescription database. We used frequency, charts and other mathematical analysis methods to summarize and sum up the ancient physicians' knowledge of stroke and the general awareness of the law of the treatment and characteristics

*Corresponding author. E-mail: yijianxilai534@hotmail.com.

of the medicine on the basis of the analysis of stroke prescription drug law. We then applied the unsupervised data mining technology to excavate new prescriptions of stroke common syndromes and also used the experiment to verify the validity, and opened new avenues for the discovery of new stroke compound medicines. Through the study, we establish a compound Chinese medicine research and development mode about combination of data mining and the Chinese Medical Literature Analysis.

RESEARCH CONTENTS AND METHODS

The study on ancient and modern literature of stroke medication rules

Establishing the stroke literature database

We collected literature widely on treatment of ancient and modern stroke, sorted out in line with the literature included in the standard literature records of 855 cases. We normalized their symptoms, such as traditional Chinese medicine name, collated and entered data in Epidata 3.1 software and transformed them into the SPSS18.0 database with requirements of data mining and statistical analysis; and then proceeded to the next step of database analysis.

The study on each historical period of stroke medication rules

We used the computer statistical software, SPSS18.0 with frequency analysis method to do comparative analysis on the frequency of stroke drug use and percentage of drug genus. And thus, add up the high frequency of drug and high utilization of percentage of drug genus. From there, we then inferred the disease medication rules and dominant trend in the different historical periods.

The horizontal bar chart analysis method was used to compare lateral number of various drugs flavor and the percentage of all drugs in the same dynasty, analyze briefly stroke medication rules in the same period and compare vertical commonly used class of drugs of different dynasties to grasp stroke medication rules in history.

Unsupervised data mining technology on stroke for new discovery of prescription

From 855 prescriptions of total database of stroke literature, the followings were screened respectively: syndrome of qi deficiency and blood stasis, syndrome of obstruction of collateral due to wind-phlegm, syndrome of internal block of phlegm-heat, syndrome of yin deficiency liver, and four wind syndrome prescription. Each syndrome prescription was entered respectively into SPSS18.0 software and data were processed by the Institute of Automation, Chinese Academy of Sciences, Chen (2008) designed unsupervised pattern discovery analysis software. Analyzed Drug correlation was analyzed by improved mutual information and entropy clustering method of complex systems, respectively. The unsupervised data mining technology was applied to obtain each syndrome commonly used for drug pair and combination of core. In assembling empirically drug pair and combination of core, 3 new discovered prescriptions were obtained for each syndrome, and through research and discussion, Traditional Chinese Medicine experts identified new prescription, in line with the clinical treatment of stroke based on each syndrome compatible law.

RESULTS

The study on each historical period stroke medication rules

On the basis of the stroke literature database using frequency analysis method, the following were derived: For Tang and Song dynasties, 67 flavors with high-frequency of drugs were derived; among them, exterior-releasing medicine, tonifying and replenishing medicine, and wind-dampness-dispelling medicine were used most frequently. For Jin and Yuan dynasties, 52 flavors with high-frequency of drugs were derived; among them, tonifying and replenishing medicine, resolving phlegm drug, exterior-releasing medicine, liver-pacifying and wind-extinguishing medicine were used most frequently. For Ming and Qing dynasties, 70 flavors with high-frequency of drugs were derived; among them, tonifying and replenishing medicine, exterior-releasing medicine, resolving phlegm drug, liver-pacifying and wind-extinguishing medicine were used most frequently. Near the modern era, 47 flavors with high-frequency of drugs were derived; among them, tonifying and replenishing medicine, liver-pacifying and wind-extinguishing medicine, and resolving phlegm drug were used most frequently. In the total database, 78 flavors with high-frequency of drugs were derived; among them, tonifying and replenishing medicine, exterior-releasing medicine, resolving phlegm drug, liver-pacifying and wind-extinguishing medicine, wind-dampness-dispelling medicine, blood-activating and stasis-resolving medicine, warming the interior medicine, heat-clearing formula, qi-regulating medicine, orifice-opening medicine, etc., were used most frequently.

Horizontal bar chart analysis method was used to compare the dynasties' various types of drugs, flavor number and the percentage of all the medicines. Results were analyzed with the frequency analysis method. We also longitudinally compared the different dynasties' commonly used drug classes, and found out exterior-releasing medicines were used most frequently, followed by Tang and Song dynasties. On the contrary, in near the modern era, frequent use of wind-dampness-dispelling medicine led to its descending on time; tonifying and replenishing medicines were used frequently more than 10% of all drugs used in this period. The highest in Ming and Qing dynasties were liver-pacifying and wind-extinguishing medicines; frequency of resolving phlegm drug rose with time. Blood-activating and stasis-resolving medicines and orifice-opening medicines were used frequently with little changes in the treatment of stroke during each period.

Unsupervised data mining technology on stroke for new discovery of prescription [1-2]

From the total database of ancient and modern stroke

Table 1. 78 flavors high-frequency drugs of syndrome of qi deficiency and blood stasis.

S/N	Drug	Frequency	No	Drug	Frequency
1	Chuan Xiong	151	40	Bing Pian	24
2	Fang Feng	147	41	Ru Xiang	23
3	Dang Gui	133	42	Chi Shao	21
4	Ren Shen	115	43	Gui Zhi	20
5	Bai Shu	110	44	Yuan Zhi	20
6	Qiang Huo	80	45	Ling Yangjiao	20
7	Fu Ling	78	46	Ju Huang	19
8	Bai Shao	78	47	Cang Shu	19
9	Ma Huang	77	48	Mo Yao	19
10	Rou Gui	76	49	Wei Lingxian	18
11	Tian Ma	68	50	Bai Huashe	18
12	Du Huo	64	51	Wu Yao	18
13	Fu Zi	64	52	Du Zhong	18
14	Bai Zhi	56	53	He Shaowu	18
15	Niu Xi	54	54	Mai Dong	18
16	Huang Qi	52	55	Ge Gen	17
17	Huang Qin	50	56	Zhi Qiao	17
18	Chen Pi	47	57	Gao Ben	16
19	Xi Xin	42	58	Huang Xiang	16
20	Ban Xia	42	59	Niu Huang	16
21	Quan Xie	40	60	Xi Jiao	15
22	Jiang Can	38	61	Suan Zaoren	15
23	Tian Nanxing	37	62	Sheng Ma	14
24	Chuan Wu	36	63	Wu Qiaoshe	14
25	She Xiang	35	64	Chen Xiang	14
26	Mu Xiang	34	65	Xiang Fu	14
27	Gan Jiang	32	66	Hong Hua	14
28	Sheng Dihuang	31	67	Cao Xie	13
29	Shi Gao	30	68	Man Jingzi	12
30	Qin Jiu	30	69	Huang Lian	12
31	Fang Ji	30	70	Cao Wu	12
32	Ku Xingren	30	71	Di Long	12
33	Bai Fuzi	29	72	Shi Changpu	12
34	Jie Geng	27	73	Yi Yiren	11
35	Zhu Sha	27	74	Zhi Shi	11
36	Shu Dihuang	27	75	Da Zao	11
37	Jing Jie	26	76	Fu Shen	10
38	Bo He	26	77	Tao Ren	10
39	Sheng Jiang	25	78	Zhu Li	10

literature, the followings were screened respectively: syndrome of qi deficiency and blood stasis, syndrome of obstruction of collateral due to wind-phlegm, syndrome of internal block of phlegm-heat, syndrome of yin deficiency liver wind four syndrome prescription. By analyzing 277 cases drugs of syndrome of qi deficiency and blood stasis, obtaining 78 flavors high-frequency drugs (Table 1),applied respectively improved mutual information method, entropy clustering method of complex systems, obtaining 44 drug pair (Table 2)and 61 combination of core(Table 3),assembled empirically drug pair and combination of core, obtained 3 new prescription the syndrome(Table 4) (Shenlonghuoxuefang was inside, as depicted in Figure 1);By analyzing 213 cases drugs of syndrome of obstruction of collateral due to wind-phlegm, 53 flavors with high-frequency of drugs were obtained. By applying respectively the above method, 26 drug pairs, 19 combinations of core and 3 new prescriptions for the syndrome were obtained. By analyzing 128 cases of drugs for syndrome of internal block of phlegm-heat, 43

Table 2. 44 drug pairs of syndrome of qi deficiency and blood stasis.

S/N	Drug Pair		S/N	Drug Pair	
1	Chuan Xiong	Ma Huang	23	Huang Qi	Tian Nanxing
2	Fang Feng	Jing Jie	24	Huang Qi	Bo He
3	Dang Gui	Huang Qi	25	Huang Qin	Jie Geng
4	Dang Gui	Sheng Dihuang	26	Xi Xin	Xiang Fu
5	Ren Shen	Fu Zi	27	Jiang Can	She Xiang
6	Bai Shu	Xi Xin	28	Tian Nanxing	Suan Zaoren
7	Bai Shu	Yi Yiren	29	Chuan Wu	Jie Geng
8	Qiang Huo	Bo He	30	She Xiang	Man Jingzi
9	Bai Shao	Huang Qin	31	Mu Xiang	Du Zhong
10	Ma Huang	Huang Qi	32	Gan Jiang	Chi Shao
11	Ma Huang	Quan Xie	33	Sheng Di	Mai Dong
12	Rou Gui	Du Huo	34	Shi Gao	Ge Gen
13	Rou Gui	Fu Zi	35	Jie Geng	Bo He
14	Rou Gui	Gui Zhi	36	Zhu Sha	Bai Huashe
15	Rou Gui	Ge Gen	37	Jing Jie	Bo He
16	Tian Ma	Jing Jie	38	Sheng Jiang	Ge Gen
17	Du Huo	Xi Xin	39	Sheng Jiang	Zhu Li
18	Du Huo	Shi Gao	40	Ling Yangjiao	Xi Jiao
19	Fu Zi	Cao Wu	41	Ling Yangjiao	Fu Shen
20	Bai Zhi	Wu Yao	42	Niu Huang	Xi Jiao
21	Niu Xi	Xi Xin	43	Niu Huang	Wu Qiaoshe
22	Huang Qi	Xi Xin	44	Sheng Ma	Zhu Li

flavors with high-frequency of drugs were obtained. By applying respectively the above method, 26 drug pairs, 19 combinations of core and 3 new prescriptions for the syndrome were obtained. By analyzing 64 cases of drugs for syndrome of yin deficiency liver wind, 28 flavors with high-frequency of drugs were obtained. By applying respectively the above method, 20 drug pairs,19 combinations of core and 3 new prescriptions for the syndrome were obtained.

DISCUSSION

Application of mathematical statistics methods

Using TCM to carry out research on stroke has past two thousand years ago. the ancient physicians made further exploration and played with stroke etiology and pathogenesis of the law governing its prescription. Their expanded ideas and accumulated experience were conducive to further improve the prevention and treatment of this disease. However, these researches were scattered, disordered, low-level and very repetitive. But, with the rapid development and popularization of computer and software technology, the application of certain mathematical statistical methods can lead to a more comprehensive, systematic, and in-depth study of the incidence of the law and principles of treatment of stroke. The frequency and bar chart analysis methods are simple, intuitive, practical, and more appropriate for this study.

In this study, we comparatively analyzed the frequency of stroke drug use, the percentage of drug genus though frequency analysis; and thus obtained high frequency of drug and high utilization of drug genus. We then inferred the laws of drug and the dominant trend of the disease in different historical periods. Horizontal bar chart analysis method was used to compare lateral number of various drugs flavor and the percentage of all drugs in the same dynasty. There were more user-friendly analyzed stroke medication rules in the same period, compared to the vertical commonly used class of drugs of different dynasties, making it more conducive to grasp stroke medication rules in history.

The application based on unsupervised data mining technology

Data mining technology of the present situation in the TCM Syndrome drug law

Along with the research of the Chinese literature system, TCM Syndrome drug law research also made some progress, but still did not find appropriate ways and means. Jianxin et al. (2007) by means of artificial

Table 3. 61 combinations of core for syndrome of qi deficiency and blood stasis.

S/N	Combinations of drugs			
1	Chuan Xiong	Fang Feng	Huang Qin	
2	Fang Feng	Qiang Huo	Bai Zhi	
3	Fang Feng	Rou Gui	Fang Ji	
4	Fang Feng	Huang Qin	Fang Ji	
5	Fang Feng	Shi Gao	Fang Ji	
6	Dang Gui	Bai Shu	Ban Xia	
7	Ren Shen	Hong Hua	Tao Ren	
8	Bai Shu	Fu Ling	Ban Xia	
9	Bai Shu	Fu Ling	Shu Dihuang	
10	Bai Shu	Shu Dihuang	Suan Zaoren	
11	Qiang Huo	Tian Ma	Tian Nanxing	
12	Qiang Huo	Niu Xi	Du Zhong	
13	Qiang Huo	Shu Dihuang	Du Zhong	
14	Fu Ling	Bai Shao	Sheng Dihuang	
15	Fu Ling	Chen Pi	Sheng Dihuang	
16	Fu Ling	Sheng Dihuang	Shu Dihuang	
17	Bai Shao	She Xiang	Zhu Sha	
18	Bai Shao	Zhu Sha	She Xiang	
19	Tian Ma	Jiang Can	Tian Nanxing	
20	Du Huo	Hong Hua	Tao Ren	
21	Bai Zhi	Bai Fuzi	Cao Wu	
22	Niu Xi	Sheng Dihuang	Suan Zaoren	
23	Niu Xi	Du Zhong	Bei Jie	
24	Niu Xi	Du Zhong	Yi Yiren	
25	Niu Xi	Suan Zaoren	Hong Hua	
26	Huang Qin	Fang Ji	Xing Ren	
27	Chen Pi	Ban Xia	Tian Nanxing	
28	Chen Pi	Wu Yao	Zhi Qiao	
29	Chen Pi	Wu Yao	Huang Lian	
30	Quan Xie	Jiang Can	Huo Xiang	
31	Chuan Wu	Jing Jie	Cao Wu	
32	She Xiang	Zhu Sha	Bing Pian	
33	Mu Xiang	Zhu Sha	Chen Xiang	
34	Mu Xiang	Cang Shu	Wei Ling Xian	
35	Mu Xiang	Wei Lingxiang	Man Jingzi	
36	Gan Jiang	Sheng Jiang	Da Zao	
37	Sheng Dihuang	Shu Dihuang	Suan Zaoren	
38	Jie Geng	Wu Yao	Zhi Qiao	
39	Jie Geng	Wu Yao	Huang Lian	
40	Zhu Sha	Bing Pian	Huo Xiang	
41	Zhu Sha	Bing Pian	Chen Xiang	
42	Shu Dihuang	Suan Zaoren	Hong Hua	
43	Jing Jie	He Shouwu	Cao Wu	
44	Bing Pian	Ru Xiang	Wei Lingxian	
45	Bing Pian	Huo Xiang	Xiang Fu	
46	Ru Xiang	Mo Yao	Di Long	
47	Ru Xiang	Xiang Fu	Di Long	
48	Chi Shao	Di Long	Tao Ren	
49	Yuan Zhi	Mai Dong	Shi Changpu	
50	Ju Hua	Gao Ben	Man Jingzi	
51	Dang Gui	Ban Xia	Huang Lian	Zhi Shi

Table 3. Contd.

52	Ma Huang	Rou Gui	Fang Ji	Xing Ren	
53	Bai Zhi	Cang Shu	Wei Lingxian	He Shouwu	
54	Bai Zhi	Cang Shu	He Shouwu	Wu Cao	
55	Quan Xie	Bai Fuzi	Bai Huashe	Gao Ben	
56	She Xiang	Bing Pian	Ru Xiang	Xiang Fu	
57	She Xiang	Bing Pian	Niu Huang	Xiang Fu	
58	Bing Pian	Ru Xiang	Chen Xiang	Xiang Fu	
59	Ren Shen	Ma Huang	Shi Gao	Fang Ji	Xing Ren
60	Fu Ling	Chen Pi	Ban Xia	Huang Lian	Zhi Shi
61	Tian Ma	Quan Xie	Jiang Can	Bai Fuzi	Bai Huashe WuQiaoshe

Table 4. 3 new prescriptions for syndrome of qi deficiency and blood stasis.

S/N	3 New Prescriptions				
1	Ren Shen	Hong Hua	Tao Ren	Chi Shao	Di Long
2	Dang Gui	Huang Qi	Ru Xiang	Mo Yao	Di Long
3	Dang Gui	Huang Qi	Chi Shao	Di Long	Tao Ren

intelligence, data mining, clustering analysis techniques, initially revealed a unique insight of the Xin'an physicians on stroke pathogenesis, diagnosis and treatment; they dug out the idea of their clinical treatment, to provide a reference for the prevention and treatment of stroke. The cluster analysis had its limitations; it required a specific attribution of symptoms in the study of Chinese medicine symptom. There was no objective criterion for evaluating whether clustering results are good or bad in order to determine the usefulness of the results (Xiaoyu, 2010).

Guo (2011) conducted a statistical analysis on stroke hemiplegia medical case through the use of association rules of data mining technology. They summarized the characteristics of the type of medicine for each syndrome and commonly used drug pair. But association rules in the application process may exist such as the higher rules of support and confidence when there was no significance of application. This required the operation to ensure that there are high quality data for rule validation, as well as, a number of feedback correction (Tong et al., 2009).

Liang et al. (2009) used the covariance matrix of factor analysis method for 343 prescriptions, from Han to Yuan Dynasty. They summed up the 22 kinds of factors representing different drugs compositions. Regarding outside treatment of stroke in this period, before the Yuan Dynasty, an understanding of the etiology of stroke was obtained. There are still many arguments to the "outside wind". The majorities of physicians still disperse wind and eliminate the pathogenic factors as the main treatment, combined with resolving phlegm, freeing the collateral vessels and activating blood. The modern studies showed that it may be related to the study of Jianxin et al. (2011, 2012b), but factor analysis was only normalized in the same category to the more closely associated with several variables; each type of variables became a (public) factor, reflecting most of the information of the original variables to the few factors (Wang, 2010).

These were more or less the drawbacks in the aforementioned commonly used data mining techniques in TCM Syndrome drug law research. Another method can take effects in quality of medicine (Jianxin, 2010).

The advantages of unsupervised data mining techniques in TCM Syndrome drug law

Compared with cluster analysis, association rules analysis, factor analysis and the more commonly used data mining methods; unsupervised data mining technique is more suitable for the present needs of TCM Syndrome drug law. Many scholars, who applied the unsupervised data mining analysis method in their studies, made some valuable experience. An example is Huang et al. (2010) who researched on proprietary Chinese medicine prescription law of combating influenza and tuberculosis prescription drug law by using complex systems entropy clustering method of unsupervised data mining method. He obtained commonly used drugs, drug combinations and provided a reference and a basis for further prevention and control of influenza A (H1N1), tuberculosis and related drug screening.

The unsupervised data mining method applies not only dealing with linear data, but also to dealing with non-linear data. Because this method did not do a rigid linear segmentation with data, it was especially suitable for processing complex relationship data of existing multivariate and multi-level on TCM. Its advantages according to Tang et al. (2009) and Jianxin et al. (2007) were: 1) It was a very good and important method which portrayed correlation of complex system, and it did not require data consistency, data normalization, and required less calculation; 2) It objectively reflected the situation of the data, gathered out of class elements

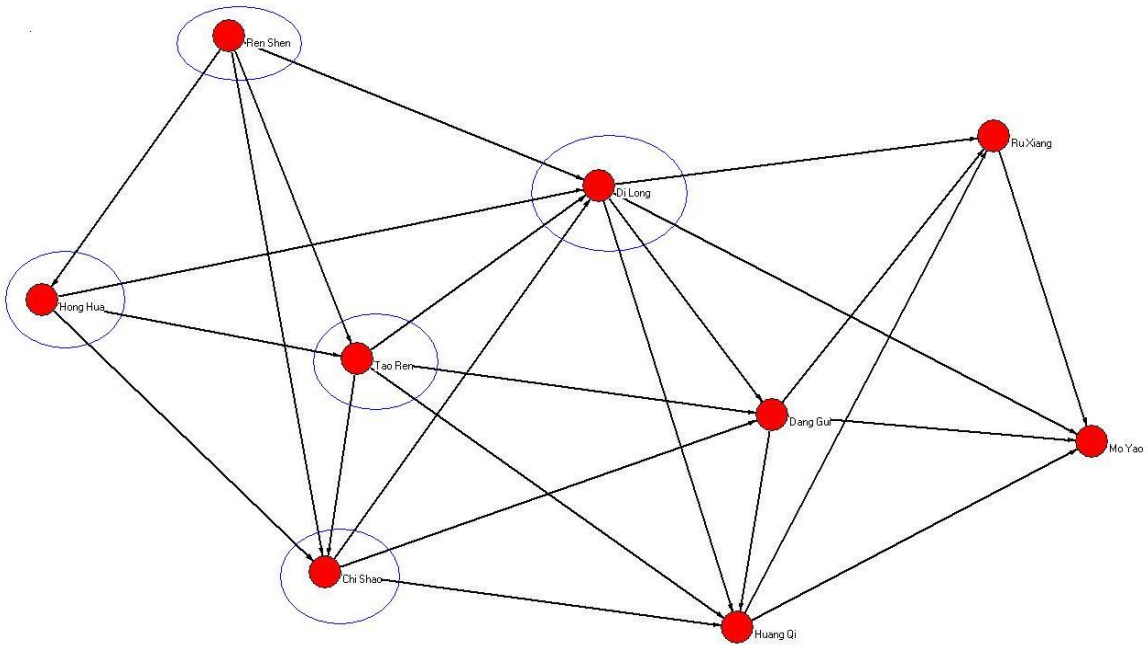


Figure 1. The three new prescriptions are discovered. Drugs of Shenlonghuoxuefang are in blue circle.

correlation, which was particularly large; 3) Convergence speed was fast for processing large amounts of data; 4) Could assign a drug to different class. This was consistent with the TCM theory, and paid special attention to the characteristics and properties of these drugs.

So, what is unsupervised data mining method? It was compared to the method of supervision. These two methods were the two strategies of machine learning methods. Supervision mining method achieved the classification and judgment of unknown samples by learning the training samples of known classes. In this case of no expert's knowledge of pre-participation, unsupervised mining method proceeded from the characteristics of the samples (variables), researched through some kind of algorithm for collecting the sample (variable) of similar characteristics together, and to achieve the purpose of distinguishing different characteristics of the samples (Qiu et al., 2004).

The improved mutual information and complexity of the system entropy clustering method with unsupervised data mining methods are more in line with the TCM theory. The latter, as an improved clustering method could not only lead to fast clustering, achieving a variable which appears in a different class, it also improved the shortcomings of traditional clustering methods "rigid" division in order to achieve a "soft" partition. The working principle proposed the concept of the variable "friends group" based on improved mutual information. It took a relative approach, selected a specific variable m, removed the value in the collection of m before the largest Z1≤Z≤ (N-1) variables, and formed a congregation of Z elements, denoted by D (m). Generally, Z was small compared to N, so this collection could be called "friends group" of the variable m. This is because each element of them is very related to m. Obtaining the "friends group" of each variable could lead to getting the clustering through the entropy clustering algorithm. For each variable, we all took their respective "friends group". If two variables were each in their respective friends and relatives, we think that these two variables were strongly correlated, for only strong related could be together. By analogy, the three variables could be together in a class if and only if any two variables were related. Z was finite, so this algorithm was certainly convergence. The class numbers were the algorithm automatically determined and a function of the number of variables N and the "friends group" of the number of Z (Tang et al., 2010).

This study, researched by using Epidata database platform, applied frequency statistics method and improved mutual information (Yang et al., 2005) entropy clustering method of complex systems (Chen, 2008) such as unsupervised data mining methods that primarily excavated the prescription drug law from the four levels implied in the database behind. The first level was a single herb, mainly by frequency method; the second level was the analysis of drug pair, mainly through improved mutual information method; it investigated commonly used drug pair for each syndrome and the treatment of stroke, and initially revealed the prescription drug law. One study showed that the way of taking drug may have effects (Jianxin et al., 2012a).

The third level involved rule of combination between

multiple drugs through entropy clustering method of complex systems; commonly combination of core obtained for each syndrome. The last level was that by assembling empirically drug pair and combination of core, new prescriptions were obtained for each syndrome and TCM experts by researching and discussing identified a new prescription clinical efficacy.

Conclusion

In the study, we have established stroke literature database, excavated the law of the treatment and characteristics of medicine of each historical period of stroke, and expanded its connotation, thus, providing a theoretical and clinical basis for diagnosis and treatment of stroke. Complex systems analysis methods were applied to stroke literature mining, by excavating the potential law of drugs combination, and discovering new prescription in line with the clinical treatment of stroke different syndrome.

ACKNOWLEDGEMENT

This work was supported by the National Basic Research Program of China (973 Program) under grant No. 2012CB518606 and 2005CB523301 and Science and Technology project of Administration of Traditional Chinese Medicine of Hebei Province under grant no. 2009165.

REFERENCES

Chen J (2008). Study on complex system modeling of syndrome in Traditional Chinese Medicine and its association with disease [D]. Chinese Academy of Sciences Graduate School p. 7.

Guo L (2011). Study on the Law of Stroke Hemiplegia's Syndromes [D].Hunan University of Traditional Chinese Medicine Doctoral Dissertation.

Huang L, Yang H, Tang S, Chen J (2010). Research on regulations of medicine usage in epidemic prevention based on complex system entropy method [J].The twelfth China Association for Science and Technology 22 sub-venues-"The forum Chinese medicine the status and role in the event of a major public health". Proceedings pp. 246-253.

Jianxin C, Guangcheng X, JING C (2007). An unsupervised pattern (syndrome in traditional Chinese medicine) discovery algorithm based on association delineated by revised mutual information in chronic renal failure data. J. Biol. Syst. 15(4):435–451.

Jianxin C, Huihui Z, Liangtao L, Chenglong Z, Fugang W, Qi S, Bing L, Wei W (2012a). Pharmacokinetics of oral administration of 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-d-glucoside from *Polygonum multiflorum* in beagle dogs. Afr. J. Pharm. Pharmacol. 6(14):1022–1025.

Jianxin C, Huihui Z, Xueling M, Xiao H, Liangtao L, Luya W, Jing H, Bing L, Wei W (2012b). The Effects of Jiang-Zhi-Ning and ItsMain Components on Cholesterol Metabolism. Evid-based. Compl. Alt. Volume 2012, Article ID 928234, p.10

Jianxin C, Huihui Z, Ying Y, Bing L, Jian N, Wei W (2011). Lipid-lowering and antioxidant activities of Jiang-Zhi-Ning in Traditional Chinese Medicine. J. Ethnopharmacol. 134:919–930.

Jianxin C, Xueling M, Huihui Z, Ying Y, Jing H, Shuzhen G, Bing L, Jianxin C, Zhenhua J, Xiangchun W, Guoqiang Y, Cong W, Chenglong Z, Jianqiang Y, Yiling W (2010). Selecting biomarkers for primary hyperlipidemia and unstable angina in the context of neuro-endocrine-immune network by feature selection methods. J. Biol. Syst. 18(3):605–619.

Liang R, Huang J, Zhao W, Li J, Zhang F (2009). Research on the documents about the rules of the external treatment to Stroke before JIN and YUAN dynasty [J]. China J. Trad. Chin. Med. Pharm. 24(10):1332-1334.

Qiu D, Chen C (2004). Construction of Multi-classification Decision Tree Combining Unsupervised and Supervised Learning Strategy [J]. Mini-Micro Comput. Syst. 25(4):555-559.

Tang S, CHEN J, Wang B (2009). Designing New TCM Prescriptions Based on Complex System Entropy Cluster [J]. World Science and Technology – Modern. Trad. Chin. Med. 11(2):225-228.

Tang S, Chen J, Yang H, Huang L (2010). The New Research Field of Chinese Medicine: Syndrome-Entropy-Formula [J]. Chin. J. Basic Med. Trad. Chin. Med. 16(4):267-270.

Tong Y, Zhao Y, Yu J, Hu Y, Pan Y (2009). The application and research association rules in the TCM field [J]. Chin. J. Inform. Trad. Chin. Med. 16(7):95-96.

Wang B (2010). Study on syndrome differentiation and treatment rules of water-qi disease based on data analysis of ancient and morden medical records [D]. Heilongjiang University of Chinese Medicine Doctoral Dissertation pp. 34-35.

Xiaoyu SU (2010).The using Data Mining cluster analysis method in TCM clinical [J]. Pract. Clin. J. Integr. Trad. Chin. West. Med. 10(6):90-92.

Yang H, Zhao Y, Tang S, Huang L, Liu Y, Wang Y (2005). Study on the Relation of the Chinese herbs in the Prescription of Stroke Based on the Method of Entropy [J]. Chin. J. Basic Med. Trad. Chin. Med. 11(9):706.

American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

Conferences and Advert

October 2012
American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

December 2012
International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

- ***Journal of Medicinal Plant Research***
- ***Journal of Dentistry and Oral Hygiene***
- ***Journal of Parasitology and Vector Biology***
- ***Journal of Pharmacognosy and Phytotherapy***
- ***Journal of Veterinary Medicine and Animal Health***

academicJournals

African Journal of Pharmacy and Pharmacology

Volume 6 Number 30 15 August, 2012
ISSN 1996-0816

Academic Journals

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: ajpp@acadjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The African Journal of Pharmacy and Pharmacology will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Sharmilah Pamela Seetulsingh- Goorah
Associate Professor,
Department of Health Sciences
Faculty of Science,
University of Mauritius,
Reduit,
Mauritius

Himanshu Gupta
University of Colorado- Anschutz Medical Campus,
Department of Pharmaceutical Sciences, School of Pharmacy Aurora, CO 80045,
USA

Dr. Shreesh Kumar Ojha
Molecular Cardiovascular Research Program
College of Medicine
Arizona Health Sciences Center
University of Arizona
Tucson 85719, Arizona,
USA

Dr.Victor Valenti Engracia
University of São Paulo.
Av Dr. Arnaldo, 455
Cerqueira Cesar
01246-903 - Sao Paulo, SP - Brazil

Prof. Sutiak Vaclav
Rovníková 7, 040 20 Košice,
The Slovak Republic,
The Central Europe,
European Union
Slovak Republic
Slovakia

Dr.B.RAVISHANKAR
Director and Professor of Experimental Medicine
SDM Centre for Ayurveda and Allied Sciences,
SDM College of Ayurveda Campus,
Kuthpady, Udupi- 574118
Karnataka (INDIA)

Dr. Manal Moustafa Zaki
Department of Veterinary Hygiene and Management
Faculty of Veterinary Medicine, Cairo University
Giza, 11221 Egypt

Prof. George G. Nomikos
Scientific Medical Director
Clinical Science
Neuroscience
TAKEDA GLOBAL RESEARCH & DEVELOPMENT CENTER, INC. 675 North Field Drive Lake Forest, IL 60045
USA

Prof. Mahmoud Mohamed El-Mas
Department of Pharmacology,
Faculty of Pharmacy
University of Alexandria,
Alexandria,
Egypt.

Dr. Caroline Wagner
Universidade Federal do Pampa
Avenida Pedro Anunciação, s/n
Vila Batista, Caçapava do Sul, RS - Brazil

Editorial Board

Prof. Fen Jicai
School of life science, Xinjiang University,
China.

Dr. Ana Laura Nicoletti Carvalho
Av. Dr. Arnaldo, 455, São Paulo, SP.
Brazil.

Dr. Ming-hui Zhao
Professor of Medicine
Director of Renal Division, Department of Medicine
Peking University First Hospital
Beijing 100034
PR. China.

Prof. Ji Junjun
Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences,
China.

Prof. Yan Zhang
Faculty of Engineering and Applied Science,
Memorial University of Newfoundland,
Canada.

Dr. Naoufel Madani
Medical Intensive Care Unit
University hospital Ibn Sina, Univesity Mohamed V Souissi, Rabat,
Morocco.

Dr. Dong Hui
Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University,
China.

Prof. Ma Hui
School of Medicine, Lanzhou University,
China.

Prof. Gu HuiJun
School of Medicine, Taizhou university,
China.

Dr. Chan Kim Wei
Research Officer
Laboratory of Molecular Biomedicine,
Institute of Bioscience, Universiti Putra,
Malaysia.

Dr. Fen Cun
Professor, Department of Pharmacology, Xinjiang University,
China.

Dr. Sirajunnisa Razack
Department of Chemical Engineering, Annamalai University,
Annamalai Nagar, Tamilnadu,
India.

Prof. Ehab S. EL Desoky
Professor of pharmacology, Faculty of Medicine
Assiut University, Assiut,
Egypt.

Dr. Yakisich, J. Sebastian
Assistant Professor, Department of Clinical Neuroscience
R54
Karolinska University Hospital, Huddinge
141 86 Stockholm ,
Sweden.

Prof. Dr. Andrei N. Tchernitchin
Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA
University of Chile Medical School,
Chile.

Dr. Sirajunnisa Razack
Department of Chemical Engineering,
Annamalai University, Annamalai Nagar, Tamilnadu,
India.

Dr. Yasar Tatar
Marmara Unıversıty,
Turkey.

Dr Nafisa Hassan Ali
Assistant Professor, Dow institude of medical technology
Dow University of Health Sciences,Chand bbi Road, Karachi,
Pakistan.

Dr. Krishnan Namboori P. K.
Computational Chemistry Group, Computational Engineering and Networking,
Amrita Vishwa Vidyapeetham, Amritanagar, Coimbatore-641 112
India.

Prof. Osman Ghani
University of Sargodha,
Pakistan.

Dr. Liu Xiaoji
School of Medicine, Shihezi University,
China.

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJPP to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b; Tristan, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $600 handling fee. Publication of an article in the African Journal of Pharmacy and Pharmacology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Copyright: © 2012, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJPP, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication.
While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 6 Number 30 15 August, 2012

ARTICLES

Research Articles

Table of Contents: Volume 6 Number 30 15 August, 2012

ARTICLES

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2255-2267, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.370
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Antioxidant and hepatoprotective effects of *Oxalis corniculata* against carbon tetrachloride (CCl_4) induced injuries in rat

Muhammad Rashid Khan*, Afifa Marium, Maria Shabbir, Naima Saeed and Jasia Bokhari

Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan.

Accepted 3 April, 2012

Many human diseases are caused due to oxidative stress involving excessive production of free radicals that can be ameliorated by the antioxidant activities of plant extracts. Present study was designed to characterize the chemical composition of *Oxalis corniculata* methanol extract (OCME) and its various fractions; *O. corniculata* n-hexane (OCHE), *O. corniculata* ethyl acetate (OCEE), *O. corniculata* chloroform (OCCE) and *O. corniculata* aqueous (OCAE); and to determine the antioxidant potential by different *in vitro* assays. OCME was also evaluated for its antioxidant capacity against hepatotoxicity induced with carbon tetrachloride (CCl_4: 1 ml/kg b.w., 20% in olive oil, seven doses) in rat. The results showed the presence of flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, phlobatannins and steroids in OCME while tannins were absent. Total amount of phenolic and flavonoids was affected by the solvents and the sequence of solvents for phenolic contents was OCME > OCAE > OCCE > OCEE > OCHE while for flavonoids was OCME > OCCE > OCAE > OCEE > OCHE. Free radicals were scavenged by the extract/fraction in a dose response curve in all models. Biochemical parameters of serum; aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase (γ-GT), total bilirubin, cholesterol and triglycerides were significantly increased while total protein and albumin were decreased by CCl_4. Treatment of CCl_4 significantly decreased the liver contents of reduced glutathione (GSH) and activities of antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), glutathione reductase (GSR) and quinone reductase (QR) whereas elevated the thiobarbituric acid reactive substances (TBARS) contents, and hepatic lesions. All the parameters were brought back to control levels by the supplement of OCME. The results of the present study suggest the antioxidant potential of OCME and its fractions as evidenced by scavenging of free radicals and hepatoprotective capacity.

Key words: *Oxalis corniculata*, carbon tetrachloride (CCl_4), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), diphenylpicrylhydrazyl (DPPH), hepatoprotective, lipid peroxidation.

INTRODUCTION

Carbon tetrachloride (CCl_4) is an archetype of hepatotoxin used commonly in experimental models to induce oxidative stress in liver (Shyu et al., 2008). CCl_4 is also known to be involved in inducing injuries to other organs (Khan and Ahmed, 2009; Khan et al., 2009; Khan and Zehra, 2011). In its first step of metabolism, CCl_4 is converted by cytochrome P450 to a carbon centered radical, the trichloromethyl ($\bullet CCl_3$) that can dismutate to chloroform, cause lipid peroxidation by attacking polyenoic fatty acids (Halliwell and Whiteman, 2004). The trichloromethyl radical can also react with oxygen to form the peroxy trichloromethyl free radical ($\bullet CCl_3O_2$), which is more reactive than the 3, 2 trichloromethyl radical with the corresponding health disturbances (Rechnagel et al*.,* 1989; He et al., 2006).

*Corresponding author. E-mail: mrkhanqau@yahoo.com. Tel: +92 51 90643086. Fax: +92 51 9205753.

Multiple processes are involved in the progress of liver diseases. Experimental and clinical results indicate that oxidative stress may be the link, connecting different types of chronic liver injuries (Parola and Robino, 2001). The use of effective antioxidants may be the major therapeutic strategy to reduce the oxidative stress, which leads to progression of liver injuries.

Previous studies have shown that antioxidants like silymarin, vitamins C and E decrease lipid peroxidation and partially ameliorate liver injuries. Over the years, many researchers have reported that plants containing phenolics and flavonoids exhibit a large array of biological activities like hepatoprotection and reversal of fibrosis. These phytochemicals are widely found in fruits, vegetables and herbal plants and are found as reliable hepatoprotective against liver damage, as well as curtailing process in the progression to liver fibrosis (Wang et al., 2008). A number of findings have indicated the scavenging effects of flavonoids and polyphenols *in vivo* and *in vitro* conditions (Khan et al., 2009; Sahreen et al., 2010; Khan and Siddique, 2012).

Oxalis corniculata (Oxalidaceae) is locally used in various ailments. It is rich in niacin, vitamin C and β-carotene (Manandhar, 2002). The juice of the plant is given in jaundice and in stomach troubles (Hussain et al., 2008). The juice of the plant, mixed with butter, is applied to muscular swellings, boils and pimples (Manandhar, 2002). *O. corniculata* is also used as antiseptic, refrigerant, diaphoretic, diuretic and anti diabetic (Hussain et al., 2008). It is used as complementary medicine in wound healing, anemia, dyspepsia, cancer, piles, dementia and convulsions (Taranalli et al., 2004; Madhavachetty et al., 2008). Other alternative uses are; anthelmintic, anti-inflammatory, astringent, depurative, diuretic, emmenagogue, febrifuge, lithontripic, stomachic and styptic. It is also used in the treatment of influenza, fever, urinary tract infections, enteritis, diarrhea, traumatic injuries and sprains (Chopra et al., 1986). It was also reported that *O. corniculata* have hypoglycemic, antihypertensive, antipsychotic, nervous system stimulant and have chronotropic and inotropic effect (Achola et al., 1995; Raghavendra et al., 2006). Chemical characterization of *O. corniculata* showed the presence of glyoxylic acid, oxalic acid, pyruvic acid, vitexin and isovitexin, vitexin-2-O-beta-D- glucopyranoside, neutral lipids, glycolipids; vitamin C; phospholipids; fatty acids, 18:2, 18:3, 16:0; saturated (C10-C14) acids; alpha and beta tocopherols (Raghavendra et al., 2006).

The plant based therapeutics against oxidative stress induced diseases is the research of medicament of these days. Therefore, the present study was conducted to find out the protective effect of *O. corniculata* methanol extract (OCME) against CCl_4-induced oxidative stress and liver injuries in Sprague-Dawley rats. The protective proceedings of OCME are compared with silymarin, which has been used for over 20 years in clinical practice for the treatment of toxic liver diseases. OCME was fractionated with various solvents to determine the chemical composition and *in vitro* antioxidant assays in this study.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, gamma-glutamyl p-nitroanilide, glycylglycine, bovine serum albumin (BSA), 1,2-dithio-bis nitro benzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), CCl_4, flavine adenine dinucleotide (FAD), glucose-6-phosphate, Tween-20, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA), picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) and perchloric acid (PCA) were purchased from Sigma Chemicals Co. USA.

Plant material

At maturity, plants of *O. corniculata* were collected in August, 2009 from the campus of Quaid-i-Azam University, Islamabad Pakistan, identified by Dr. Mir Ajab Khan of Department of Plant Sciences. A voucher specimen was deposited at the Herbarium of Pakistan, Quaid-i-Azam University Islamabad.

Shade dried aerial parts of the plant was chopped and grinded mechanically in 1 mm mesh size. 2 kg powder was extracted with methanol (5 L) for 4 days with occasional shaking and the process repeated twice. The extract was concentrated under reduced pressure using rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) at 40°C until a semi-solid sticky mass of OCME was obtained. In order to resolve the compounds contributing to antioxidant capacity of extract, the extract was further subjected to liquid–liquid partition (suspended in 50 ml distilled water) with *O. corniculata* n-hexane fraction (OCHE), *O. corniculata* ethyl acetate fraction (OCEE), *O. corniculata* chloroform fraction (OCCE) while the remaining portion was used as *O. corniculata* aqueous fraction (OCAE). The solvent of the fractions was also removed using a rotary evaporator after partition.

Phytochemical studies

Chemical composition of different extracts for the presence of flavonoids, alkaloids, terpenoids, steroids and saponins were carried out according to Harborne (1973), tannins (Sofowara, 1993) while cardiac glycosides and phlobatannins by Trease and Evans (1989).

Determination of total flavonoid contents

Concentration of flavonoids was measured according to the method of Singleton and Rosi (1996). Different extracts were dissolved in 5% $NaNO_2$, 10% $AlCl_3.6H_2O$ and 1 M NaOH and concentration was measured at 510 nm with a known rutin concentration as a standard (in triplicate). The results were expressed as milligrams of rutin equivalents (RE) per gram of dry weight of various fractions.

Total phenolic contents

The total phenolic content of various fractions was determined using the method of Singleton and Rossi (1996). Methanolic solution of gallic acid (GA) (1 ml; 0.025 to 0.400 mg/ml) with 5 ml

Folin-Ciocalteu reagent (diluted 10-fold) was used for the calibration curve and mixture was incubated for 5 min before the addition of sodium carbonate (4 ml, 0.115 mg/ml). Absorbance was measured at 765 nm. 1 ml of different extracts (0.5 to 5.0 mg/ml) was also mixed with the stated reagents and after 2 h, the absorbance was measured to determine total plant phenolic contents. All determinations were carried out in triplicate. The total content of phenolic compounds in the extract was expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

In vitro antioxidant activity

Diphenylpicrylhydrazyl (DPPH) radical scavenging activity

Antioxidant capacity of different samples, GA and ascorbic acid (ASA) was measured with the stable radical DPPH in terms of hydrogen-donating or radical-scavenging activity at 517 nm, according to the described procedure of Gyamfi et al. (1999).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

ABTS assay was carried out by using the protocol of Re et al. (1999) using ABTS and potassium persulfate solution to generate $ABTS^{\bullet+}$ radicals. ASA and GA were used as positive controls and absorbance was determined at 715 nm.

Determination of superoxide radical scavenging activity

Superoxide scavenging for each fraction and for GA and ASA was determined by the nitroblue tetrazolium reduction method (Nishikimi et al., 1972). The tubes were uniformly illuminated with an incandescent visible light for 15 min, and the optical density was measured at 530 nm before and after the illumination.

Hydrogen peroxide scavenging activity

The scavenging capacity for hydrogen peroxide was measured according to the method of Ruch et al. (1989). Hydrogen peroxide scavenging ability of each extract and standards (GA and ASA) was determined at 230 nm against a blank having phosphate buffer.

Phosphomolybdate assay

Phosphomolybdate assay was performed to assess the antioxidant activity of samples and standard compounds; GA and ASA according to the procedure of Umamaheswari and Chatterjee (2008) by using sulphuric acid, sodium phosphate and ammonium molybdate. After incubation at 95°C for 90 min, absorbance of each reaction mixture was measured at 765 nm against a blank.

Chelating power

The iron (II) chelating ability of the extracts was estimated according to the method of Dastmalchi et al. (2008) by using ferrozine. To determine the chelating activity, the absorbance of each assay was recorded at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as control.

Reducing power

The reducing powers of the extracts were determined according to the method of Chung et al. (1998) by using potassium ferricyanide $[K_3Fe(CN)_6]$ and $FeCl_3$ and absorbance was measured at 700 nm in spectrophotometer.

In vivo studies

Animals and treatment

Male Sprague-Dawley rats (190 ± 10 g) were housed in large spacious cages. Feed and water *ad libitum* were available to the rats. The animals used in this study were treated and cared for their well being in accordance with the guidelines recommended by the Ethical Committee of the Quaid-i-Azam University Islamabad, Pakistan for the care and use of laboratory animals. Animals were divided into seven equal groups with 6 rats in each group. The experimental protocol was approved by the institutional ethics committee.

Group 1 received only raw water and free access to food materials. Group II received olive oil intraperitoneally and dimethyl sulfoxide (DMSO) intragastric for 7 days at a dose of 1 ml/kg body weight. Group III received CCl_4 1 ml/kg body weight (20% in olive oil) intraperitoneally for 7 days. Groups IV and V received CCl_4 and OCME (100 or 200 mg/kg body weight) intragastric for 7 days. Group VI was given OCME (200 mg/kg body weight) intragastric for 7 days. Group VII was treated with CCl_4 and silymarin (50 mg/kg body weight) intragastric for 7 days. After 24 h of the last treatment, all the animals were weighted, sacrificed; collected their blood, weighted and perfuse liver in ice-cold saline solution. Half of liver tissues were treated with liquid nitrogen for further enzymatic analysis while the other portion was processed for histology.

Serum analysis for liver marker enzymes

Serum samples were assayed for aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transpeptidase (γ-GT) by using standard diagnostic kits standard AMP diagnostic kits (Stattogger Strasse 31b 8045 Graz, Austria).

Serum analysis for biochemical studies

Total bilirubin, cholesterol, triglycerides, total protein and albumin were estimated by using standard diagnostic kits (Stattogger Strasse 31b 8045 Graz, Austria).

Hepatic biochemical studies

Hepatic tissue was homogenized in 10 volume of 100 mM KH_2PO_4 buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected and used for various biochemical studies. Protein concentration of the supernatant of liver tissue was determined by the method of Lowry et al. (1951) using crystalline BSA as standard.

Catalase (CAT) assay

CAT activities were determined by the method of Chance and Maehly (1955) by using H_2O_2 as substrate. Changes in absorbance

of the reaction solution at 240 nm were determined for 1 min. One unit of CAT activity was defined as an absorbance change of 0.01 as units/min.

Peroxidase (POD) assay

Activities of POD were determined by the method of Chance and Maehly (1955) with guaiacol and H_2O_2 as substrates. Changes in absorbance of the reaction solution at 470 nm were determined for 1 min. One unit of POD activity was defined as an absorbance change of 0.01 as units/min.

Superoxide dismutase (SOD) assay

SOD activity of thyroid was estimated by the method of Kakkar et al. (1984). Reaction mixture of this method contained phenazine methosulphate and sodium pyrophosphate buffer. Amount of chromogen formed was measured by recording color intensity at 560 nm. Results are expressed in units/mg protein.

Glutathione-S-transferase (GST) assay

GST activity was assayed by the method of Habig et al. (1974). The reaction mixture consisted of GSH and CDNB. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as nM CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3\ M^{-1}cm^{-1}$.

Glutathione peroxidase (GSH-Px) assay

GSH-Px activity was assayed by the method of Mohandas et al. (1984). The disappearance of NADPH at 340 nm was recorded at 25°C. Enzyme activity was calculated as nM NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^3\ M^{-1}cm^{-1}$.

Glutathione reductase (GSR) assay

This assay was conducted according to the protocol of Carlberg and Mannervik (1975). The reaction solution contained EDTA, oxidized glutathione, and NADPH. After mixing, the absorbance was measured at 340 nm. GST activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^3\ M^{-1}cm^{-1}$.

Reduced glutathione (GSH) assay

GSH was estimated by the method of Jollow et al. (1974) using DTNB. The yellow color developed was read immediately at 412 nm on a SmartSpecTM plus Spectrophotometer. It was expressed as µM GSH/g tissue.

Estimation of lipid peroxidation [thiobarbituric acid reactive substances (TBARS)] assay

The assay for lipid peroxidation was carried out following the method of Iqbal et al. (1996) by using TBA as the substrate. The amount of TBARS formed in each of the samples was assessed by measuring optical density of the supernatant at 535 nm using spectrophotometer against a reagent blank. The results were expressed as nM TBARS/min/mg tissue at 37°C using molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

Histopathalogical studies

For microscopic evaluation, hepatic tissues were fixed in a fixative (absolute alcohol 60%, formaldehyde 30%, glacial acetic acid 10%) and embedded in paraffin, sectioned at 4 µm and subsequently stained with hematoxylin/eosin (Fischer et al., 2006). Sections were studied under light microscope (DIALUX 20 EB) at 20X magnifications. Slides of all the treated groups were studied and photographed.

Statistical analysis

Parametric data, expressed as mean and standard deviation (SD), were analyzed through one way analysis of variance (ANOVA), followed by the *post hoc* Fisher least significant difference (LSD) for comparison of various treatments using the SPSS 13.0. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Plant composition studies

The aerial part of *O. corniculata* was extracted with methanol and fractionated with various solvent of increasing polarity such as n-hexane, ethyl acetate, choloroform and water. The results obtained in this study indicated the presence of flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, phlobatannins and steroids in OCME and OCAE fraction. Cardiac glycosides and phlobatannins were absent in OCCE. Alkaloids and cardiac glycosides were not present in OCEE while alkaloids, cardiac glycosides, phlobatannins and steroids were absent in OCHE. Presence of tannins was not confirmed in OCME and in other fractions; OCAE, OCCE, OCEE and OCHE (Table 1).

According to the data shown in Table 2, the significant difference in the amount of the total phenolic or total flavonoid was observed among these extracts, respectively ($P < 0.05$). The richest amount of total flavonoid was found in the OCME (6.92 mg RE/g extract), followed by OCCE (5.32 mg RE/g extract), OCAE (4.68 mg RE/g extract), OCEE (5.95 mg RE/g extract) and OCHE (2.52 mg RE/g extract). Similarly, the highest phenolic content (7.76 mg GAE/g extract) was observed in OCME, while OCHE had the lowest content (4.41 mg GAE/g extract). The amounts of total phenolics were affected by the extraction solvents with the following order: OCME > OCAE > OCCE > OCEE > OCHE ($P < 0.05$ among the extracts).

In vitro antioxidant studies

Scavenging activity methanol extract and various fractions on DPPH radical

It is shown in Figure 1A that the tested samples dose-

Table 1. Composition of various fractions of *O. corniculata*.

Chemical	Methanol extract	Chloroform fraction	Ethyl acetate fraction	n-hexane fraction	Aqueous fraction
Flavonoids	++	++	++	+	++
Alkaloids	+	+	-	-	+
Terpenoids	++	+	++	+	++
Saponins	++	+	+	+	++
Tannins	-	-	-	-	-
Cardiac glycosides	+	-	-	-	++
Phlobatannins	++	-	+	-	++
Steroids	++	++	+	-	+

++, + and -, strong presence, presence and absence respectively.

Table 2. Total phenolic and flavonoid contents in *O. corniculata*.

Plant extract	Total phenolics (mg GAE/g extract)	Total flavonoids (mg RE/g extract)
Methanol extract	7.76 ± 0.36^{a}	6.92 ± 0.52^{a}
Ethyl acetate fraction	4.76 ± 0.14^{c}	2.86 ± 0.35^{d}
Chloroform fraction	6.06 ± 0.27^{b}	5.32 ± 0.41^{b}
n-Hexane fraction	4.41 ± 0.17^{c}	2.52 ± 0.23^{d}
Aqueous fraction	6.78 ± 0.34^{ab}	4.68 ± 0.26^{bc}

n, 03 ±SD; $^{a\ to\ d}$, Means with different superscript letters indicate significance at $P < 0.05$.

Table 3. Scavenging activity of OCME and its various fractions.

Plant extract	IC_{50} (µg/ml)					
	Scavenging of DPPH radicals	Scavenging of ABTS radicals	Scavenging of hydrogen peroxide	Scavenging of superoxides	Phosphomolybdate assay	Iron chelating ability
Methanol extract	68.5 ± 0.96^{b}	269.2 ± 4.2^{c}	26.7 ± 1.5^{b}	29.7 ± 2.4^{g}	213.2 ± 2.7^{a}	52.8 ± 2.4^{e}
Ethyl acetate fraction	34.4 ± 1.17^{d}	340.5 ± 4.4^{b}	11.6 ± 0.9^{c}	173.0 ± 3.0^{a}	211.1 ± 2.9^{a}	201.7 ± 3.5^{a}
Chloroform fraction	52.4 ± 1.03^{c}	112.9 ± 3.8^{e}	9.6 ± 0.8^{c}	34.7 ± 1.4f	49.0 ± 3.3^{c}	100.9 ± 2.6^{c}
n-Hexane fraction	32.0 ± 1.42^{e}	268.9 ± 2.6^{c}	10.5 ± 0.7^{c}	115.4 ± 2.0^{b}	108.6 ± 1.6^{b}	150.7 ± 3.2^{b}
Aqueous fraction	78.5 ± 1.65^{a}	$>500^{a}$	26.0 ± 1.4^{b}	105.7 ± 2.4^{c}	105.3 ± 2.3^{b}	57.8 ± 2.2^{d}
Gallic acid	7.6 ± 0.88^{f}	170.1 ± 3.8^{d}	32.0 ± 3.3^{a}	52.5 ± 2.7^{d}	34.5 ± 2.5^{d}	-
Ascorbic acid	1.1 ± 0.14^{g}	55.7 ± 1.0^{f}	7.9 ± 0.2^{d}	32.7 ± 1.8^{e}	31.0 ± 2.3^{d}	-
EDTA	-	-	-	-	-	51.2 ± 2.1^{e}

-, Not determined; Mean ± SD (n = 03); $^{a\ to\ d}$, Means with different superscript letters indicate significance at $P < 0.05$.

dependently exhibited a DPPH• -scavenging ability at all the investigated concentrations. OCHE exhibited maximum scavenging ability (IC_{50}, 32.0 ± 1.42), while OCAE was found the least effective (IC_{50}, 78.5 ± 1.65) for DPPH radical scavenging. IC_{50} values indicated significant differences among all the extracts ($P < 0.05$), where the IC_{50} values can be ranked as OCAE > OCME > OCCE > OCEE > OCHE (Table 3). It is also observed that none of the extract was as effective as the positive controls ($P < 0.05$, GA, ASA).

Scavenging activity of ABTS and various fractions extracts on superoxide radical

Characterization of scavenging activity of OCME and its different fractions against ABTS radical revealed that all the tested samples exerted scavenging effects in a concentration dependent manner (Figure 1B). As shown in Table 3, the IC_{50} values was found to be 269.2 ± 4.2, 340.5 ± 4.4, 112.9 ± 3.8, 268.9 ± 2.6, > 500, 170.1 ± 3.8, 55.7 ± 1.0 µg/ml for OCME, OCEE, OCCE, OCHE,

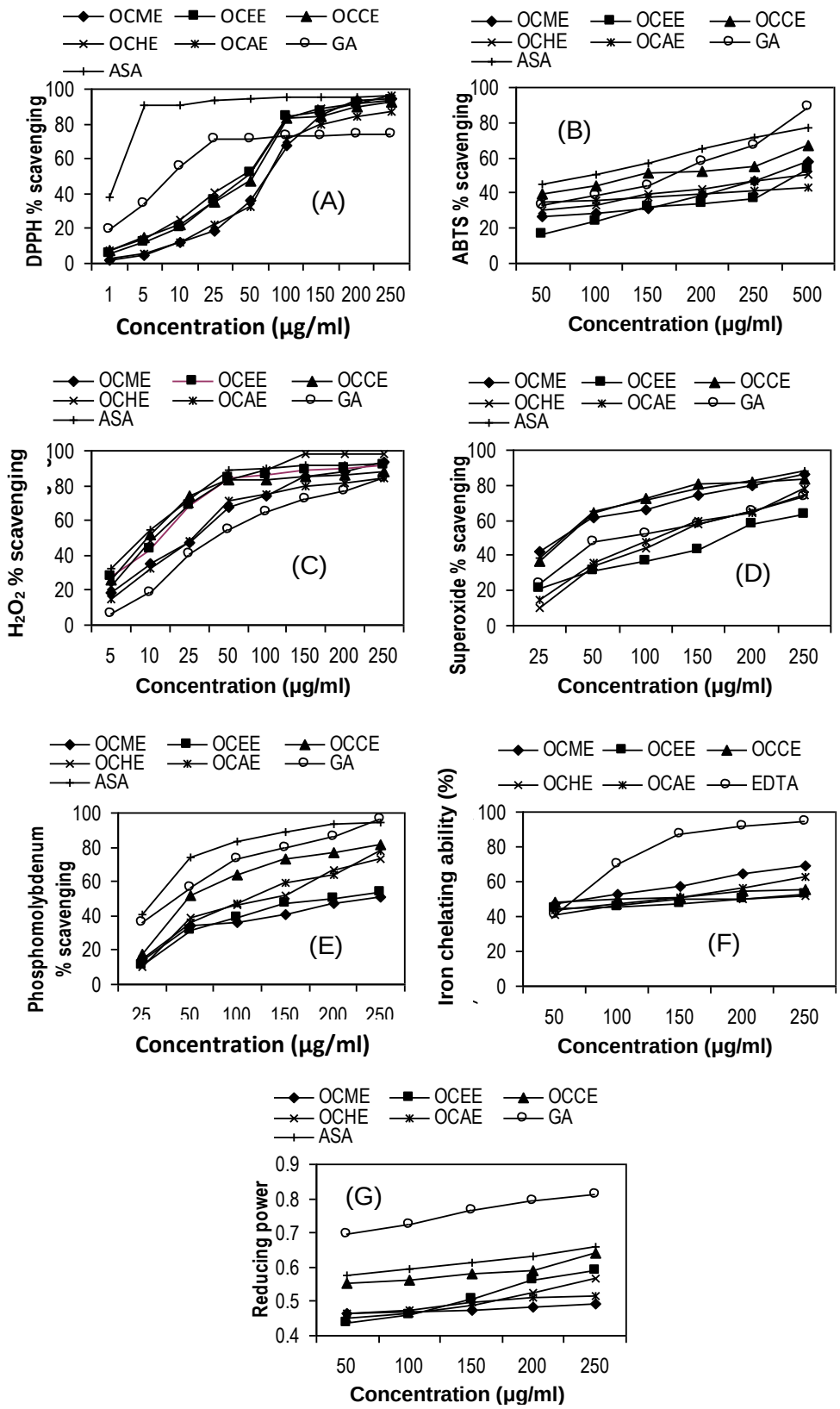


Figure 1. Antioxidant effects of *O. corniculata* in different *in vitro* assays. OCME, *O. corniculata* methanol extract; OCEE, *O. corniculata* ethyl acetate fraction; OCCE, *O. corniculata* chloroform fraction; OCHE, *O. corniculata* n-hexane fraction; OCAE, *O. corniculata* aqueous fraction; GA, gallic acid; ASA, ascorbic acid; EDTA, ethylene diamine tetracetate.

OCAE, GA and ASA, respectively. The order of IC_{50} values for different samples was OCAE > OCEE > OCME > OCHE > GA > OCCE > ASA where the OCME and OCHE were statistically similar to each other ($P > 0.05$).

Scavenging activity of OCME and its various fractions on hydroxyl radical

Scavenging of hydroxyl radical with the different test samples showed a dose response curve (Figure 1C). All the test samples showed strong scavenging effects against hydroxyl radical with the IC_{50} values, 26.7 ± 1.5, 11.6 ± 0.9, 9.6 ± 0.8, 10.5 ± 0.7 and 26.0±1.4 for OCME, OCEE, OCCE, OCHE and OCAE, respectively. Evidently, the order of scavenging activity for hydroxyl radical was OCME > OCAE > OCEE > OCHE > OCCE where the IC_{50} value of OCME and OCAE did not reach the level of statistical significance ($P > 0.05$). However, the least and the maximum IC_{50} value for the positive controls ASA (7.9 ± 0.2) and GA (32.0 ± 3.3) were obtained in this study.

Scavenging activity of OCME and its various fractions on superoxide radical

Figure 1D shows the dose response curve for superoxide radical scavenging ability for OCME and different fractions. All the test samples exerted strong scavenging activity against the superoxide radicals with IC_{50} values of 29.7 ± 2.4, 173.0 ± 3.0, 34.7 ± 1.4, 115.4 ± 2.0, 105.7 ± 2.4 for OCME, OCEE, OCCE, OCHE and OCAE, respectively. The IC_{50} values obtained for standard compounds GA and ASA were 52.5 ± 2.7 and 32.7 ± 1.8, respectively. All the test samples and positive control were statistically different from each other ($P < 0.05$). However, OCME exhibited significantly ($P < 0.05$) less IC_{50} value as compared to the GA and ASA.

Scavenging activity of OCME and its various fractions on phosphomolybdate assay

The data obtained indicated the marked scavenging potency of OCME and its various fractions for phosphomolybdate assay in a dose-dependent way (Figure 1E). The IC_{50} values obtained were 213.2 ± 2.7, 211.1 ± 2.9, 49.0 ± 3.3, 108.6 ± 1.6, 105.3 ± 2.3, 34.5 ± 2.5, 31.0 ± 2.3 for OCME, OCEE, OCCE, OCHE, OCAE, GA and ASA, respectively. The IC_{50} values of OCME, OCEE and OCHE, OCAE did not reach the level of significance with each other ($P < 0.05$). However, inferior IC_{50} values were obtained for all the test samples with respect to the positive controls GA and ASA.

Scavenging activity of OCME and its various fractions on Fe^{2+} chelating potency

As shown in Figure 1F, all the samples displayed different magnitudes of Fe^{2+} chelating potency in a dose-dependent manner. From the estimated IC_{50} values in Table 3, it can be seen that the most effective Fe^{2+} chelating extract was OCME (52.8 ± 2.4) and then followed by OCAE (57.8 ± 2.2), OCCE (100.9 ± 2.6), OCHE (150.7 ± 3.2) and OCEE (201.7 ± 3.5). However, the lowest Fe^{2+} chelating IC_{50} value was obtained for positive control EDTA (51.2 ± 2.1). The sequence of IC_{50} value for Fe^{2+} chelating activity for different samples was EDTA > OCME > OCAE > OCCE > OCHE > OCEE ($P < 0.05$).

Scavenging activity of OCME and its various fractions on reducing power

As shown in Figure 1G, OCME and its different fractions exhibited varied degrees of reducing power. Among all the extracts, at 250 µg/ml OCCE exhibited the strongest reducing power with absorbance values of (0.641 ± 0.004) followed by OCEE (0.595 ± 0.014), OCHE (0.565 ± 0.013) and OCAE (0.515 ± 0.006). At a concentration 250 µg/ml, the absorbance recorded for positive controls GA and ASA was (0.809 ± 0.018) and (0.655 ± 0.007), respectively (Table 4).

Effect of OCME on liver marker enzymes

Ameliorative effects of OCME on liver marker enzymes such as AST, ALT, ALP, LDH and γ-GT in serum are shown in Table 5. The treatment of CCl_4 to rats significantly elevated the serum level of AST, ALT, ALP, LDH and γ-GT as compared to the control group. Administration of both CCl_4 and OCME (100 and 200 mg/kg) alleviated the toxicity of CCl_4 and the changed serum level of AST, ALT, ALP, LDH and γ-GT, reverted towards the control group with significance difference from the CCl_4 group. Similarly, treatment of CCl_4 alleviated the toxicity of CCl_4 and serum level of AST, ALT, ALP, LDH and γ-GT was observed significantly less than that of CCl_4 only. However, treatment of OCME alone did not statistically change the level of AST, ALT, ALP, LDH and γ-GT in serum compared with controls.

OCME on serum biochemical profile

In the CCl_4 treated group the serum level of total bilirubin, cholesterol, triglycerides were significantly increased while total protein and albumin were decreased compared with the control group (Table 6). The groups treated with both CCl_4 and OCME (100 and 200 mg/kg) showed a significant change in the level of serum total bilirubin, cholesterol, triglycerides, total protein and albumin compared with the CCl_4 group. Higher dose of OCME (200 mg/kg) produce more appreciable change in the level of serum biochemical profile with respect to the

Table 4. Reducing activity of OCME and its various fractions.

Plant extract	Reducing power absorbance at 700 nm (250 µg/ml)
Methanol extract	0.498 ± 0.020^{e}
Ethyl acetate fraction	0.595 ± 0.014^{c}
Chloroform fraction	0.641 ± 0.004^{b}
n-Hexane fraction	0.565 ± 0.013^{d}
Aqueous fraction	0.515 ± 0.006^{e}
Gallic acid	0.809 ± 0.018^{a}
Ascorbic acid	0.655 ± 0.007^{b}

n, 03 ± SD; $^{a\ to\ d}$, Means with different superscript letters indicate significance at $P < 0.05$.

Table 5. Protective effect of OCME on liver marker enzymes.

Group	Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	LDH (U/L)	γ-GT (U/L)
I	Control	42.2 ± 3.7^{A}	135.3 ± 9.7^{A}	227.8 ± 12.4^{A}	57.5 ± 11.9^{A}	36.6 ± 7.7^{A}
II	DMSO + olive oil	40.9 ± 4.2^{A}	132.7 ± 5.4^{A}	226.12 ± 10.3^{A}	55.3 ± 7.9^{A}	38.9 ± 5.7^{A}
III	CCl_4 (1 ml/kg)	$78.8 \pm 6.^{a}$	298.5 ± 9.6^{a}	331.4 ± 15.3^{a}	101.3 ± 12.8^{a}	176.6 ± 7.0^{a}
IV	CCl_4 + OCME (100 mg/kg)	68.8 ± 2.7^{Aa}	217.2 ± 6.3^{Aa}	293.4 ± 11.7^{Aa}	69.2 ± 6.8^{Aa}	85.9 ± 8.5^{Aa}
V	CCl_4 + OCME (200 mg/kg)	48.5 ± 2.5^{A}	145.7 ± 7.1^{A}	238.2 ± 12.8^{A}	56.1 ± 4.8^{A}	42.4 ± 8.0^{A}
VI	OCME (200 mg/kg)	43.2 ± 3.9^{A}	128.3 ± 7.2^{A}	231.5 ± 8.08^{A}	54.7 ± 9.6^{A}	32.1 ± 7.5^{A}
VII	CCl_4 + silymarin (100 mg/kg)	44.2 ± 2.6^{A}	147.8 ± 6.5^{A}	240.3 ± 9.84^{A}	57.4 ± 8.1^{A}	46.7 ± 7.3^{A}

Mean ± SD (n=6 number). ^{a}, Significance at $P < 0.01$ from control group; ^{A}, significance at $P < 0.01$from CCl_4 group; OCME, *Oxalis corniculata* methanol extract.

Table 6. Protective effect of OCME on serum biochemical profile.

Group	Treatment	Total bilirubin (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Total protein (g/dl)	Albumin (g/dl)
I	Control	10.1 ± 1.5^{B}	56.3 ± 5.1^{B}	26.7 ± 5.1^{B}	52.9 ± 3.5^{B}	32.8 ± 3.1^{B}
II	DMSO+ Olive oil	10.4 ± 1.7^{B}	55.7 ± 4.4^{B}	23.3 ± 2.4^{B}	52.0 ± 5.2^{B}	30.0 ± 5.2^{B}
III	CCl_4 (1 ml/kg)	20.6 ± 1.4^{b}	71.9 ± 5.0^{b}	51.0 ± 5.8^{b}	41.0 ± 3.6^{b}	21.0 ± 2.6^{b}
IV	CCl_4 + OCME (100 mg/kg)	16.3 ± 1.9^{Bb}	66.2 ± 5.9^{Ab}	43.2 ± 5.9^{Bb}	44.8 ± 2.8^{Ab}	26.8 ± 3.8^{Ab}
V	CCl_4 + OCME (200 mg/kg)	13.4 ± 1.0^{Ba}	59.4 ± 7.6^{B}	29.4 ± 7.1^{B}	49.3 ± 3.8^{B}	29.1 ± 3.0^{B}
VI	OCME (200 mg/kg)	9.3 ± 1.5^{B}	57.6 ± 4.6^{B}	27.2 ± 1.6^{B}	51.4 ± 2.2^{B}	31.4 ± 3.2^{B}
VII	CCl_4 + silymarin (100 mg/kg)	11.3 ± 1.2^{B}	58.1 ± 2.0^{B}	28.1 ± 2.5^{B}	48.2 ± 3.8^{B}	30.5 ± 2.8^{B}

Mean ±SD (n=6 number). $^{a\ and\ b}$, Significance at $P < 0.05$ and $P < 0.01$ from control group; $^{A\ and\ B}$, significance at $P < 0.05$ and $P < 0.01$ from CCl_4 group; OCME, *Oxalis corniculata* methanol extract.

lower dose of OCME (100 mg/kg). Treatment of silymarin (50 mg/kg) in combination with CCl_4 significantly restored the level of serum total bilirubin, cholesterol, triglycerides, total protein and albumin as against the CCl_4 group. Ameliorating effects of OCME at higher dose (200 mg/kg) were determined similar to silymarin for the stated parameters. However, treatment of OCME (200 mg/kg) alone did not cause any significant change in the level of total bilirubin, cholesterol, triglycerides, total protein and albumin compared with the control group.

Effect of OCME on hepatic antioxidant enzymes

In the CCl_4 treated group, the activity level of antioxidant enzymes, CAT, POD and SOD in hepatic samples (Table 7) showed a significant ($P < 0.01$) decrease as compared with the control group. The groups treated with both CCl_4 and OCME (100 and 200 mg/kg b.w.) showed a significant ($P < 0.01$) increase in the activity level of CAT, POD and SOD compared with the CCl_4 group. However, the activity levels of these antioxidant enzymes were

Table 7. Effect of OCME on antioxidant enzymes of liver in rat.

Group	Treatment	CAT (U/min)	POD (U/min)	SOD (U/mg protein)	GSH-Px (nM/mg protein)	GSR (nM/min/mg protein)	GST (nmol/min/mg protein)	QR (µM/mg protein)
I	Control	25.4 ± 1.2^{A}	13.5 ± 0.89^{A}	5.8 ± 0.06^{A}	63.5 ± 5.5^{A}	117.2 ± 4.5^{A}	14.5 ± 1.2^{A}	25.1 ± 0.2^{A}
II	DMSO + olive oil	24.3 ± 2.3^{A}	12.9 ± 0.45^{A}	5.4 ± 0.78^{A}	62.4 ± 4.2^{A}	114.5 ± 4.7^{A}	15.4 ± 1.0^{A}	24.2 ± 0.3^{A}
III	CCl_4 (1 ml/kg)	12.8 ± 1.8^{b}	7.3 ± 0.57^{b}	3.1 ± 0.23^{b}	35.6 ± 3.6^{b}	220.3 ± 5.7^{b}	7.8 ± 1.3^{b}	10.6 ± 1.0^{b}
IV	CCl_4 + OCME (100 mg/kg)	16.8 ± 2.0^{Ab}	9.3 ± 0.67^{Ab}	4.7 ± 0.2^{Ab}	43.0 ± 5.3^{Ab}	176.9 ± 8.2^{Ab}	10.9 ± 1.0^{Ab}	17.2 ± 2.4^{Ab}
V	CCl_4 + OCME (200 mg/kg)	22.8 ± 1.6^{A}	11.4 ± 0.34^{Aa}	5.5 ± 0.32^{A}	54.2 ± 3.4^{Aa}	126.5 ± 3.9^{Aa}	12.4 ± 2.1^{A}	20.3 ± 1.5^{Aa}
VI	OCME (200 mg/kg)	26.4 ± 2.9^{A}	13.0 ± 0.56^{A}	6.0 ± 0.12^{A}	60.2 ± 4.5^{A}	115.3 ± 4.9^{A}	15.2 ± 2.1^{A}	25.2 ± 1.5^{A}
VII	CCl_4 + silymarin (100 mg/kg)	21.3 ± 1.0^{Aa}	12.6 ± 0.76^{A}	4.9 ± 0.12^{Aa}	52.2 ± 3.6^{Aa}	134.8 ± 5.7^{Aa}	12.8 ± 1.2^{A}	21.2 ± 0.9^{Aa}

Mean ±SD (n=6 number). [a] and [b], Significance at $P < 0.01$ from control group; [A], significance at $P < 0.01$ from CCl_4 group; OCME, *Oxalis corniculata* methanol extract.

more pronounced at the higher dose (200 mg/kg b.w.) and activity level of CAT and SOD was statistically similar to the control group while POD activity was significantly ($P < 0.05$) less as compared with the control group. Group treated with CCl_4 and silymarin showed statistical increase ($P < 0.01$) in CAT, POD and SOD activity level with respect to the CCl_4 group whereas CAT and SOD showed significantly ($P < 0.05$) lower activity compared with the control group. The OCME treated group (200 mg/kg b.w.) did not exhibit antioxidant activity of CAT, POD and SOD that was different from the control group.

As shown in Table 7 the activity levels of GSH-Px, GST, GSR and quinone reductase (QR) were significantly ($P < 0.01$) lowered in CCl_4 treated group in hepatic samples compared with controls. The groups treated with both CCl_4 and different dosages of OCME (100 and 200 mg/kg b.w.) had significantly ($P < 0.01$) elevated the activity levels of GSH-Px, GST, GSR and QR in liver samples compared with the group given CCl_4 only. However, the increase in the activity level was more pronounced at higher dose of OCME (200 mg/kg b.w.). Activity level of GSH-Px, GSR and QR at higher dose of OCME (200 mg/kg b.w.) was obtained statistically lower ($P < 0.05$) as against the control group. Treatment of silymarin (50 mg/kg b.w.) effectively ameliorated the toxicity of CCl_4 and activity level of GSH-Px, GST, GSR and QR activity was restored towards the control group. However, activity level of GSH-Px, GSR and QR was found statistically lower ($P < 0.05$) compared with the control group. OCME (200 mg/kg b.w.) administration alone did not significantly change the activity levels of GSH-Px, GST, GSR and QR in hepatic samples when compared with those of the control group.

Effect of OCME on lipid peroxidation and glutathione

Treatment of CCl_4 to rats significantly ($P < 0.01$) elevated the TBARS level an indicator of lipid peroxidation while the contents of GSH, an endogenous antioxidant were significantly ($P < 0.01$) decreased in hepatic samples compared with the control group (Table 8). The groups administered both with CCl_4 and OCME (100 and 200 mg/kg b.w.) had significantly ($P < 0.01$) decreased TBARS while increased GSH levels ($P < 0.01$) in liver samples compared with the group given CCl_4 only. Higher dosage of OCME (200 mg/kg b.w.) was found to be more potent in the reversal of TBARS and GSH level in liver. Treatment of silymarin (50 mg/kg b.w.) in combination with CCl_4 was able to restore the TBARS and GSH level towards the control group. OCME administration alone did not statistically change the TBARS and GSH levels in the hepatic samples when compared with the controls.

Effect of OCME on histopathology of liver

Hematoxylin and eosin stained section (Figure 2) indicated that administration of CCl_4 markedly increasedthe fatty changes with white and yellow areas, cellular hypertrophy, and necrotic foci, degeneration of the lobular architecture and the formation of septa and congested blood vessels with disturbed epithelium. Co-administration of various doses of OCME attenuated the hepatic injuries with very less or no fatty changes, dilation of blood vessel, uniform morphology of hepatocytes near to control group was found. Co-treatment with silymarin showed near to normal

Table 8. Effect of OCME on lipid peroxidation and glutathione in hepatic of rat.

Group	Treatment	Lipid peroxidation TBARS (nM/min/mg protein)	GSH (µM/g tissue)
I	Control	19.1 ± 1.2^{A}	1.69 ± 0.002^{A}
II	DMSO + olive oil	18.4 ± 2.1^{A}	1.73 ± 0.004^{A}
III	CCl_4 (1 ml/kg)	39.8 ± 1.6^{b}	0.51 ± 0.006^{b}
IV	CCl_4 + OCME (100 mg/kg)	30.7 ± 2.0^{Ab}	0.96 ± 0.006^{Ab}
V	CCl_4 + OCME (200 mg/kg)	22.2 ± 1.5^{A}	1.54 ± 0.003^{A}
VI	OCME (200 mg/kg) alone	19.4 ± 1.3^{A}	1.71 ± 0.001^{A}
VII	CCl_4 + silymarin (50 mg/kg)	22.8 ± 1.3^{A}	1.44 ± 0.001^{Aa}

Mean ±SD (n = 6 number). [a] and [b], Significance at P < 0.05 and *P* < 0.01from control group; [A], significance at *P*<0.01from CCl_4 group; OCME, *Oxalis corniculata* methanol extract.

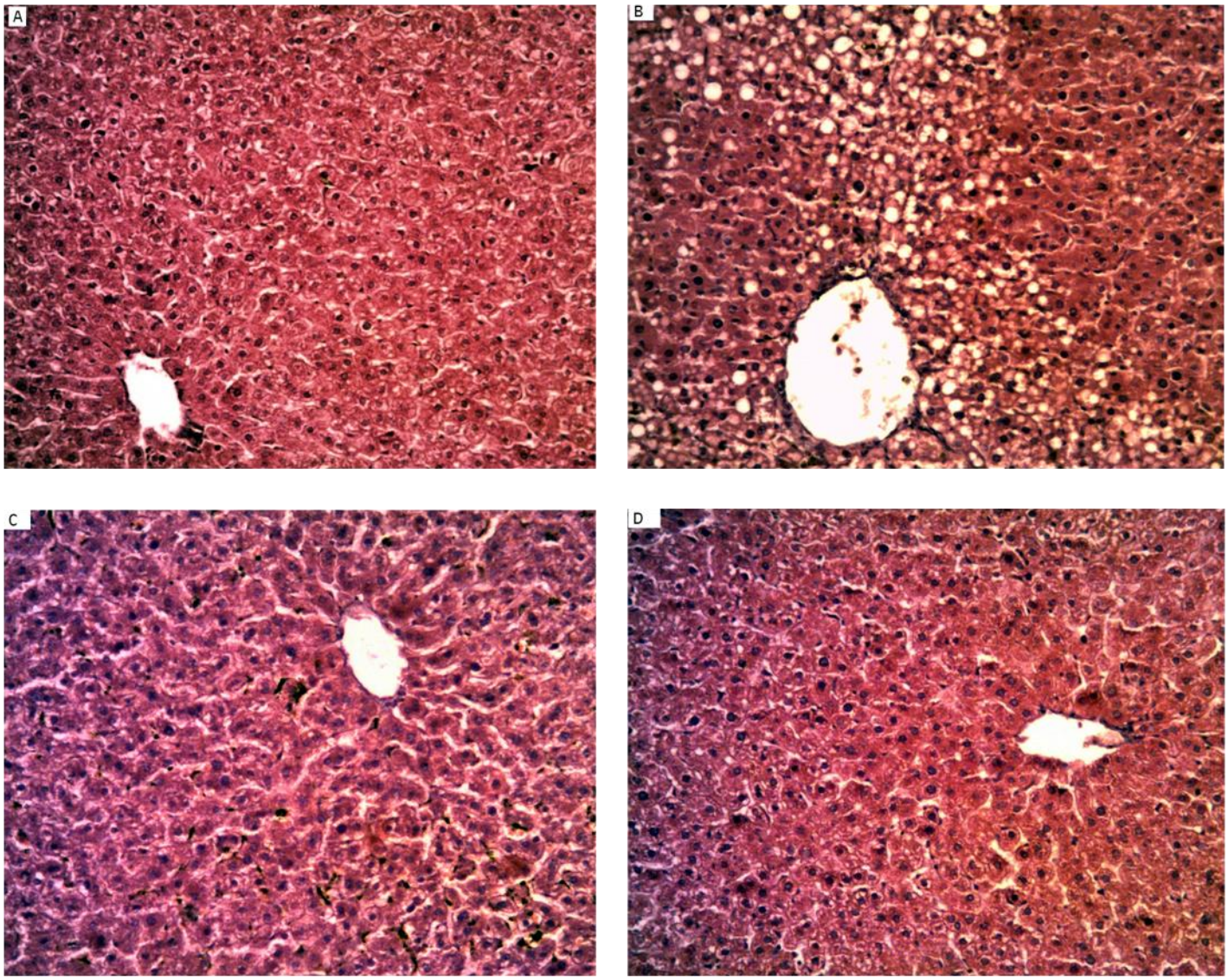


Figure 2. H & E stain (20x). Effects of OCME on histopathology of liver in rat. (A) Control group; CL centrilobule; HP, hepatocytes. (B) CCl_4 (1 ml/kg b.w., 20% in olive oil) group; ICL, injured centrilobule; MC, macrosteatosis. (C) CCl_4 + OCME (200 mg/kg b.w.) group; CL, centrilobule; MIS, microsteatosis. (D) CCl_4 + silymarin (50 mg/kg b.w.) group; CL, centrilobule; HP, hepatocytes..

morphology. No abnormal changes was found in the morphology of control group was found.

DISCUSSION

In recent years, plant extracts have been widely used as natural antioxidants because of the presence of polyphenolics (Nuengchamnong et al., 2009). Flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, phlobatannins and steroids have been determined in OCME. In the present investigation, highest quantity of total phenolics and flavonoid components was found in OCME. The presence of these phenolic and flavonoid compounds, contribute diverse biological activities such as anti-carcinogenic, anti-inflammatory, and anti-atherosclerotic. These activities might be related to their antioxidant activity (Nuengchamnong et al., 2009). The polar solvents such as methanol and water are the best solvent in extracting the flavonoid from *O. corniculata*, indicating that most of the flavonoid exist in a conjugated form through their hydroxyl groups with glycosides, lead to the increasing polarity and solubility in methanol and water (Mohsen and Ammar, 2009).

The antioxidant ability of OCME and its fractions was measured through the DPPH and ABTS radical scavenging potential. The present results indicated that OCME and OCAE have high IC_{50} values as compared to the non polar solvents (Table 3). However, reverse order was obtained for the iron chelating ability. The antioxidant activity of phenolic is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers and some of the pharmacological effects might be due to these valuable compounds. In addition, they have a metal chelating potential (Rice-Evans et al., 1995). Chelating agents may act as secondary antioxidants because of their ability to reduce the redox potential to stabilize the oxidized form of the metal ions. Therefore it is of importance to screen the iron (II) chelating ability of extracts. Accordingly it is suggested that the iron (II) chelating properties of these fractions may be attributed to the nature of endogenous chelating agents like phenolics and flavonoids. Some phenolic compounds have properly oriented functional groups, which can chelate metal ions to protect against oxidative damage.

It is evident from these results that the chemical constituents found in OCME and its fractions are potent scavengers of free radicals such as $O^{\bullet-}$ and hydrogen peroxide, at very low concentrations (Sahreen et al., 2010). Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell. Among different reactive oxygen substance (ROS), $O_2^{\bullet-}$ is one of the precursors of $HO^{\bullet}$ or singlet oxygen, and also can produce other kinds of oxidizing agents, where it indirectly initiates lipid peroxidation and magnifies cellular damage. Antioxidants presents in the OCME and other fractions are able to scavenge the $O_2^{\bullet-}$ and H_2O_2 to encounter the deleterious chain reactions.

The reducing capacity is a significant reflection of the antioxidant activity in assessing potential antioxidants (Sahreen et al., 2010). In this assay system, the presence of antioxidants causes the reduction of the $Fe^{3+}/K_3Fe(CN)_6$ complex to the ferrous form (Fe^{2+}), and consequently, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. It is suggested that there is a direct correlation between antioxidant activities and reducing power of components of some plants (Sahreen et al., 2010). The results obtained in this study indicate that OCME and its different fractions have a remarkable potency to donate electron to reactive free radicals, converting them into more stable non-reactive species and terminating the free radical chain reaction.

In this study, elevated levels of liver marker enzymes such as AST, ALT, ALP, LDH and γ-GT in serum are obtained with CCl_4 treatment indicating the pathophysiology of liver. Generally, CCl_4 is metabolized by the liver in to highly reactive metabolites which either directly or indirectly cause lipid peroxidation of the hepatocytes. Different cytosolic liver marker enzymes would then leaked out from these swollen and necrotic hepatocytes in to blood circulation and evidently elevated levels are obtained that is associated with the massive centrilobular necrosis, ballooning, degeneration and cellular infiltration of the liver (Singh et al., 2008). Treatment of rats by both CCl_4 and the OCME/silymarin reversed the increase in liver marker enzymes towards the control group and hepatic lesions are also minimized (Figure 2).

It is well known that the liver play a pivotal role in the regulation of various chemicals. Administration of CCl_4 causes hepathopathy as indicated by elevation in serum level of total bilirubin, total cholesterol and triglycerides whereas decreases total protein and albumin in rat. These pathological changes signify the potential damage in hepatic induced with CCl_4 treatment. Treatment of rat with CCl_4 and with either OCME or silymarin ameliorated the toxic effects of CCl_4 and restored the level of above biochemicals towards the control group in accordance with other findings (Lin et al., 2008).

Treatment of rats with CCl_4 decreases the level of endogenous glutathione due to its increased utilization in hepatocytes in scavenging of CCl_4 metabolites. GSH is catalyzed by GST for conjugation to different xenobiotics and their metabolites and converting them in to more soluble compounds. GSH is oxidized to GSSH by GSH-Px and then GSSH is reverted back to GSH by GSR. On account of excessive oxidative stress induced by CCl_4 lead to decline in GSH contents and in the activity of GSH-Px, GSR and GST of liver (Hayes et al., 2005).

Activity level of CAT, POD, SOD and QR are reduced in CCl_4 group. Among them CAT is actively engaged in

the catalytic breakdown of H_2O_2 into H_2O and oxygen. Superoxides are highly reactive and the precursor of singlet oxygen and hydroxyl radicals, are dismutated by SOD to H_2O_2 and oxygen (Reiter et al., 2000). Administration of OCME/silymarin along with CCl_4 scavenge the toxic radicals of CCl_4 thereby maintained the GSH level towards the control, increased the capacity or synthesis that confers enhanced protection against oxidative injuries, consequently increase the hepatic level of CAT, POD, SOD, GSH-Px, GST, GSR and QR (Benson et al., 1980).

Measurement of TBARS serves as an indirect indicator of lipid peroxidation of polyunsaturated fatty acids of hepatocyte membrane. Elevation in TBARS level by CCl_4 treatment in this study exhibits the liver damage involving series of chain reactions (Ohkawa et al., 1979). Treatment of OCME/silymarin alleviated the toxicity of CCl_4 and restored the level of TBARS near to control group in conformity to other studies.

It was apparent from the results that administration of CCl_4 induces extensive fatty change with white and yellow areas due to lipid peroxidation, congestion in blood vessels, cellular hypertrophy, and necrotic foci, destruction of the lobular architecture, the formation of septa and congested blood vessels with disturbed epithelium and nuclear degeneration in some areas occur which was significantly recovered by the various concentrations of OCME. The study revealed that the OCME was comprised of polyphenol and terpenoids which show significant protective effect against hepatotoxicity induced by CCl_4. Similar histological observation was found by various investigators (Lin et al., 2008; Khan et al., 2011) while evaluating the protective effect of medicinal plant against CCl_4 and other drugs induced hepatotoxicity in rats.

Conclusion

OCME and various fractions possess efficient scavenging potential as evident in different *in vitro* antioxidant assays. Present findings indicate the protective potential of OCME against CCl_4 induced oxidative stress, elevated the GSH and activity level of various enzyme defenses, decrease lipid peroxidation, restore serum level of liver marker enzymes and hepatic lesions possibly through polyphenolic and other active constituents and accredit its local use in liver disorders.

REFERENCES

Achola KJ, Mwangi JW, Munenge RW (1995). Pharmacological activity of Oxalis corniculata. Pharm. Biol. 33:247-249.

Benson AM, Hunkeler MJ, Talalay P (1980) not cited. Increase of NADPH, quinone reductase activity by dietary antioxidant: Possible role in protection against carcinogenesis and toxicity. Proc. Nat. Acad. Sci. 77:5216-5220.

Carlberg I, Mannervik EB (1975). Glutathione level in rat brain. J. Biol. Chem. 250:4475-4480.

Chance B, Maehly AC (1955). Assay of catalase and peroxidases. Method. Enzymol. 11:764-775.

Chopra RN, Nayar SL, Chopra IC (1986). Glossary of Indian medicinal plants (Including the Supplement). Council of Scientific and Industrial Research, New Delhi.

Chung KT, Wong TY, Huang YW, Lin Y (1998). Tannins and human health: A review. Critical Rev. Food Sci. Nutr. 38:421-464.

Dastmalchi KD, Doeman HJD, Oinonen PP, Darwis Y, Laaso I, Hiltunen R (2008). Chemical composition and *in vitro* antioxidative activity of a lemon balm (*Melissa officinalis* L.) extract. LWT- Food Sci. Technol. 41:391-400.

Fischer AH, Jacobson KA, Rose J, Zeller R (2006). Hematoxylin and Eosin Staining of Tissue and Cell Sections**.** "Preparation of Cells and Tissues for Fluorescence Microscopy," *In* Basic Methods in Microscopy (eds. Spector and Goldman). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Gyamfi MA, Yonamine M, Aniya Y (1999). Free radical scavenging activity of medicinal herb of Ghana: *Thonningia sanguinea* on experimentally induced liver injuries. Gen. Pharmacol. 32:61-67.

Habig WH, Pabst MJ, Jakoby WB (1974). Glutathione-*S*-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249:7130-7139.

Halliwell B, Whiteman M (2004). Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Br. J. Pharmacol., 42: 231-255.

Harborne JB (1973). Phytochemical methods. Chapman and Hall, Ltd. London. pp. 49-188.

Hayes JD, Flanagan JU, Jowsey IR (2005). Glutathione transferases. Ann. Rev. Pharmacol. Toxicol. 45:51-88.

He SX, Luo JY, Wang YP, Wang YL, Fu H, Xu JL, Zhao G, Liu EQ (2006). Effects of extract from *Ginkgo biloba* on carbon tetrachloride-induced liver injury in rats. World J. Gastroenterol. 12(24):3924-3928.

Hussain K, Shahazad A, Hussnain ZU (2008). An ethnobotanical survey of important wild medicinal plants of Hattar, District Haripur, Pakistan. Ethnobotanical Leaflets 12:29-35.

Iqbal M, Sharma SD, Zadeh HR, Hasan N, Abdulla M, Athar M (1996). Glutathione metabolizing enzymes and oxidative stress in ferric nitrilotriacetate (Fe-NTA) mediated hepatic injury. Redox Rep. 2:385-391.

Jollow DJ, Mitchell JR, Zampaglione N, Gillete JR (1974). Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as a hepatotoxic metabolite. Pharmacology 1:151-169.

Kakkar P, Das B, Viswanathan PN (1984). A modified spectrophotomateric assay of superoxide dismutase. Ind. J. Biochem. Biophy. 21:130-132.

Khan MR, Ahmed D (2009). Protective effects of *Digera muricata* (L.) Mart. on testis against oxidative stress of carbon tetrachloride in rat. Food Chem. Toxicol. 47:1393-1399.

Khan MR, Rizvi W, Khan GN, Khan RA, Shaheen S (2009). Carbon tetrachloride induced nephrotoxicity in rats: Protective role of *Digera muricata*. J. Ethnopharmacol., 122:91-99.

Khan MR, Siddique F (2012). Antioxidant effects of *Citharexylum spinosum* in CCl_4 induced nephrotoxicity in rat. Exp. Toxicol. Pathol., http://dx.doi.org/10.1016/j.etp.2010.09.009.

Khan MR, Badar I, Siddiquah A (2011). Prevention of hepatorenal toxicity with *Sonchus asper* in gentamicin treated rats. BMC Comp. Alter Med. 11:113.

Khan MR, Zehra H (2011). Amelioration of CCl_4-induced nephrotoxicity by *Oxalis corniculata* in rat. Exp. Toxicol. Pathol., http://dx.doi.org/10.1016/j.etp.2011.11.007

Lin HM, Tseng HC, Wang CJ, Lin JJ, Lo CW, Chou FP (2008). Hepatoprotective effects of *Solanum nigrum* Linn. extract against CCl_4-induced oxidative damage in rats. J. Chemico-Biol. Interac. 171:283-293.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Madhavachetty K, Sivaj JK, Tulasi RK (2008). Flowering plants of Chittoor district, Andhra Pradesh, India, Student offset printers, Tirupati, 1st edition. pp. 54-55.

Manandhar NP (2002). Plants and People of Nepal Timber Press.

Oregon. ISBN 0-88192-527-6. p. 599.
Mohandas J, Marshal JJ, Duggin GG, Horvath JS, Tiller DJ (1984). Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. Biochem. Pharmacol. 33:1801-1807.
Mohsen SM, Ammar ASM (2009). Total phenolic contents and antioxidant activity of corn tassel extracts. Food Chem., 112: 595-598.
Nuengchamnong N, Krittasilp K, Ingkaninan K (2009). Rapid screening and identification of antioxidants in aqueous extracts of *Houttuynia cordata* using LC-ESI-MS coupled with DPPH assay. Food Chem. 117:750-756.
Nishikimi M, Rao NA, Yagi K (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem. Biophy. Res. Comm. 46:849-854.
Ohkawa H, Ohishi N, Yagi K (1979). Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction. Ann. Biochem., 95: 351-358.
Parola M, Robino G (2001). Oxidative stress-related molecules and liver fibrosis. J. Hepatol. 35:297-306.
Raghavendra MP, Satish S, Raveesha A (2006). Phytochemical analysis and antibacterial activity of *Oxalis corniculata*, a known medicinal plant. My. Sci. 1:72-78.
Re R, Pellegrini N, Proteggente A, Pannala A, Yong M, Rice-Evas C (1999). Antioxidant activity applying an improved ABTS radical cation decoloursation assay. Free Rad. Biol. Med. 26:1231-1237.
Rechnagel RO, Glende EA, Dolak JA, Waller RL (1989). Mechanisms of carbon tetrachloride toxicity. J. Pharmacol. Exp. Ther. 43:139-154.
Reiter RJ, Tan D, Osuna C, Gitto E (2000). Actions of melatonin in the reduction of oxidative stress. J. Biomed. Sci. 7: 444-458.
Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Rad. Res. 22:375-383.
Ruch RJ, Cheng SJ, Klaunig JE (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechin isolated from Chinese green tea. Carcinogen 10:1003-1008.
Sahreen S, Khan MR, Khan RA (2010). Evaluation of antioxidant activities of various solvent extracts of *Carissa opaca* fruits. Food Chem. 122(4):1205-1211.
Shyu MH, Kao TC, Yen GC (2008). Hsian-tsao (*Mesona procumbens* Heml.) prevents rat liver fibrosis induced by CCl_4 via inhibition of hepatic stellate cells activation. Food Chem. Toxicol. 46:3707-3713.
Singh N, Kamath V, Narasimhamurthy K, Rajini PS (2008). Protective effects of potato peel extract against carbon tetrachloride-induced liver injury in rats. Environ. Toxicol. Pharmacol. 26:241-146.
Singleton VL, Rossi JA (1996). Colorimetry of total phenolics with phosphomolybdic_phosphotungstic acid reagents. Am. J. Enol. Viticul. 16:144-153.
Sofowara A (1993). Medicinal plants and traditional medicine in Africa. John Wiley and Sons Ltd. Spectrum Books Ibadan, Nigeria, p. 289.
Taranalli AD, Tipare SV, Kumar S, Torgal SS (2004). Wound healing activity of *Oxalis corniculata* whole plant extract in rats. Ind. J. Pharma. Sci. 66(4):444-446.
Trease GE, Evans WC (1989). Pharmacognosy. 13th Edition, University Press Cambridge, p. 107-148.
Umamaheswari M, Chatterjee TK (2008). In vitro antioxidant activities of the fractions of *Coccinnia grandis* L. leaf extract. Afr. J. Trad. Comp. Alt. Med. 5(1):61-73.
Wang MY, Anderson G, Nowicki D, Jensen J (2008). Hepatic Protection by Noni Fruit Juice against CCl4-Induced Chronic Liver Damage in Female SD Rats. Plant Foods Human Nut. 63:141-145.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2268-2276, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.805
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Efficacy and safety of flurbiprofen axetil on preemptive analgesia for Chinese surgical patients: A meta-analysis

Huifen Li[1]*, Chunyan Zhang[2] and Jintai Jia[1]

[1]Department of Anesthesia, Heping Hospital of Changzhi Medical College, Changzhi 046000, Shanxi Province, China.
[2]Department of Anesthesiology, General Hospital of Linfen City, Linfen 041000, Shanxi Province, China.

Accepted 31 January, 2012

This study was performed to evaluate the efficacy and safety of flurbiprofen axetil (FA) on preemptive analgesia for Chinese surgical patients. Medicinal databases and review articles were screened with prespecified criteria for randomized controlled trials that reported the effects of adverse reactions to FA and other analgesics for preemptive analgesia. The qualities of included studies were critically evaluated. A total of 848 articles were found and 17 articles were finally included. Heterogeneity test: Analysis of visual analog scale (VAS) at 4 h after operation (Qstatistic = 97.37, P < 0.00001, I^2 = 87%), analysis of VAS at 8 h after operation (Qstatistic = 128.95, P < 0.00001, I^2 = 90%), analysis of VAS at 12 h after operation (Qstatistic = 20511.23, P = 0.13, I^2 = 100%), analysis of VAS at 24 h after operation (Qstatistic = 188.15, P < 0.00001, I^2 = 91%), and safety analysis (Q statistic = 17.61, P = 0.05, I^2 = 38%). The results of meta-analysis showed that compared with the control group, FA was more effective in VAS at 4 h after operation (mean difference (MD) = -1.23, 95% confidence interval (CI): -1.54 to -0.92), 12 h after operation (MD = -4.42, 95%CI: -10.06 to 1.23), and 24 h after operation (MD = -0.87, 95%CI: -1.24 to -0.51). There were no significances between FA treatment and control group in VAS at 8 h after operation. Moreover, FA was safer than the control group (OR = 0.70, 95%CI: 0.49 to 0.99). Funnel-plot displayed some unsymmetrical figures, indicating that there were publication biases in each analysis. The evidence currently available shows that FA was effective and safe on preemptive analgesia for Chinese surgical patients.

Key words: Flurbiprofen axetil, preemptive analgesia, systemic review, meta-analysis.

INTRODUCTION

Flurbiprofen axetil (FA) is a member of the phenylalkanoic acid derivative family of nonsteroidal anti-inflammatory drugs (NSAIDs), and it exerts potent function of anti-inflammation and antinociception after intravenous (iv) injection (Buritova and Besson, 1998; Roszkowski et al., 1997). It has been reported that FA, an injectable prodrug of flurbiprofen, when administered intravenously could reduce the pain on injection of propofol (Fujii and Shiga, 2006, 2009). Recently, some studies also reported that FA could reduce postoperative pains (Lin, 2010; Mikawa et al., 1997; Nakayama et al., 2001; Nishiike et al., 2007; Takada et al., 2007; Wang et al., 2008, 2009). However, the conclusions of which are not credible, because of small sample size and lacks of systemic evaluation of methodologic quality.

In order to demonstrate its efficacy and safety, we make a systemic review about clinical random control trials (RCTs) focused on FA in preemptive analgesia for Chinese surgical patients.

METHODOLOGY

Search sources and strategy

The search strategy was made according to working handbook 4.2.7 from the Cochrane collaboration (Sackett et al., 2002). We systematically searched Medline (1991 to November, 2011), EMbase (1991 to November, 2011), CBMdisc (1991 to November,

*Corresponding author. E-mail: li46000@126.com.

2011), and CNKI (1994 to November, 2011) for randomized trials examining the efficacy and safety of FA on preemptive analgesia for Chinese surgical patients. In addition, we conducted a manual search of abstracts from selected conferences and also searched by hand the bibliographies of all relevant trials. The following search criterion was used: ("preemptive analgesia" or "analgesia") and ("flurbiprofen axetil" or "flurbiprofen"), and language was limited to English or Chinese.

Study selection

Two reviewers independently conducted the literature search and extraction of relevant articles. The title and abstract of potentially relevant studies were screened for appropriateness before retrieval of the full articles. The following selection criterions were used to identify published studies for inclusion in this meta-analysis: (a) study design: RCTs; (b) population: Chinese surgical patients; (c) intervention: FA versus other active analgesics, such as tramadol and fentanyl; (d) outcome variable: VAS at 4 h after operation, VAS at 8 h after operation, VAS at 12 h after operation, VAS at 24 h after operation and adverse reaction rate.

Data extraction

From each study, the following information was abstracted: author, year of publication, study design, characteristics of the population, simple size, treatment proposal, types of surgical operation, VAS at 4 h after operation, VAS at 8 h after operation, VAS at 12 h after operation, VAS at 24 h after operation and adverse reaction rate.

Assessment of study quality

The quality of the included studies was assessed based on a well-established, validated scale developed by Jadad et al. (1996). A Jadad score was calculated using the following 7 items: (1) Was the study described as a random trial? (2) Was the randomization scheme described and appropriate? (3) Was the study described as double-blind? (4) Was the method of double blinding appropriate? That is, were both the patient and the assessor appropriately blinded? (5) Was there a description of dropouts and withdrawals? (6) Deduct one point if the method used to generate the sequence of randomization was described and was inappropriate. (7) Deduct one point if the study was described as double blind, but the method of blinding was inappropriate.

The first five items were indications of good quality, and each was counted as one point towards an overall quality score. The final two items indicated poor quality, and a point was subtracted for each if its criteria were met. The range of possible scores was 0 to 5 (0 being weakest and 5 being strongest). Any study with a Jadad score < 3/5 was considered to be of poor quality, and excluded.

Statistical analysis

For dichotomous outcomes, we calculated a pooled mean difference (MD), odds ratio (OR) and 95% confidence interval (CI). The MDs and ORs of different RCTs were combined by using the random effects model as previous described (Der et al., 1986), if true, between-study heterogeneity exists or else use Mantel and Haenszel fixed-effects model instead (Mantel et al., 1959). Intertrial statistical heterogeneity was explored using the Cochran Q test with calculated I^2, indicating the percentage of the total variability in effect estimates among trials, that is, due to heterogeneity rather than to chance (Higgins et al., 2003). I^2 values of 50% or more indicate a substantial level of heterogeneity. We evaluated the presence of publication bias by means of visual inspection of the funnel plot (whether it was symmetrical or not). All P values were two-sided with statistical significance set at an α level of 0.05. We followed the "quality of reporting meta-analysis guidelines" for reporting and discussing these meta-analytical results (Moher et al., 1999). All the statistical analysis was carried out by the Cochrane collaboration's RevMan 5.0 software.

RESULTS

Study characteristics

There were 848 articles relevant to the search term and 17 articles (Chen et al., 2011, 2008; Ding et al., 2007; Fan, 2009; He et al., 2008; Li and Xie 2007; Li and Lin 2010, Li et al, 2009; Luo et al., 2009; Song et al., 2010; Sun et al., 2007; Wang et al., 2007, 2008; Xie et al., 2006; Zeng et al., 2008; Zhao et al., 2009) involving 848 Chinese surgical patients (FA treatment group: 425 patients; control group: 423 patients) were included in this meta-analysis finally. Ages and sex ratio were similar in each group, respectively. The flow chart for the selection of RCTs to be included in our analysis is as shown in Figure 1. The characteristics of the included trials are showed in Table 1.

Methodologic quality assessment

All the trials included in this meta-analysis mentioned the term "random", but the detail method was illuminated in 1 article only. There were 17 trials that mentioned the term "double blind", but only 7 articles explained the detail method. All the 17 trials described the data of the patients who withdrew during the treatment. According to the Jadad score, 10 articles and 7 articles were regarded as high quality literature and low quality literature, respectively (Table 1).

Heterogeneity test

We choose fixed-effect model to make meta-analysis, because there was no significant heterogeneities in safety analysis (Q statistic = 17.61, P = 0.05, I^2 = 38%). Because of heterogeneity, random-effect model was used to make analysis for VAS at 4 h after operation (Qstatistic = 97.37, P < 0.00001, I^2 = 87%), VAS at 8 h after operation (Qstatistic = 128.95, P < 0.00001, I^2 = 90%), VAS at 12 h after operation (Qstatistic = 20511.23, P = 0.13, I^2 = 100%), and VAS at 24 h after operation (Qstatistic = 188.15, P < 0.00001, I^2 = 91%).

Meta-analysis of VAS at 4 h after operation

FA treatment group and control group were recorded in

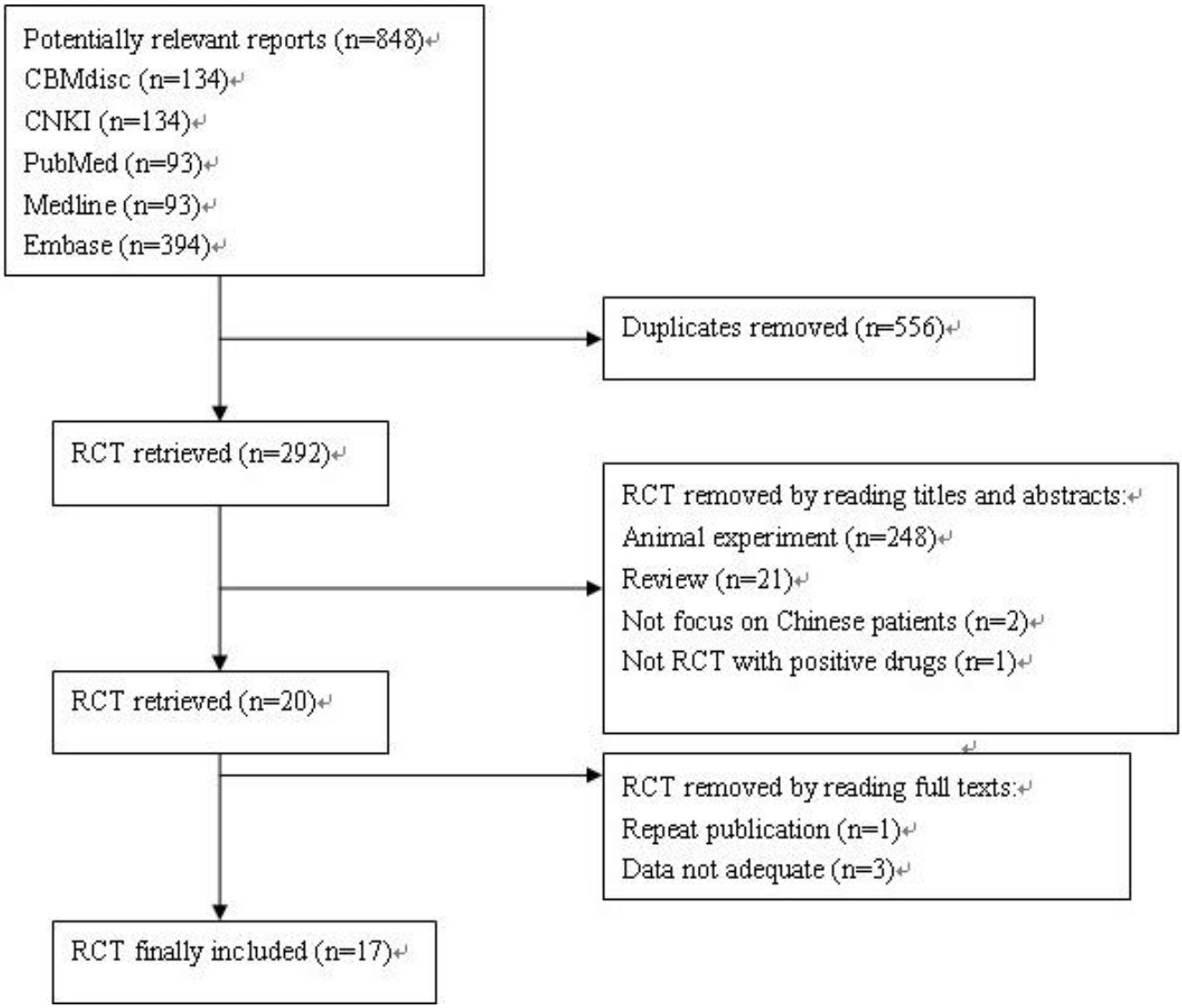


Figure 1. Chat for the search result and trials screen.

the 14 trials finally included. Active analgesics involved in this analysis were tramadol or fentanyl. The results of meta-analysis (MD = -1.23, 95%CI: -1.54 to -0.92) confessed that VAS at 4 h after operation in FA treatment group is less than in control group of Chinese surgical patients (Figure 2).

Meta-analysis of VAS at 8 h after operation

FA treatment group and control group were recorded in the 14 trials finally included. Active analgesics involved in this analysis were tramadol or fentanyl. The results of meta-analysis (MD = -1.13, 95%CI: -1.54 to -0.73) confessed that VAS at 8 h after operation in FA treatment group is less than in control group of Chinese surgical patients (Figure 3).

Meta-analysis of VAS at 12 h after operation

FA treatment group and control group were recorded in the 12 trials finally included. Active analgesics involved in this analysis were tramadol or fentanyl. The results of meta-analysis (MD = -4.42, 95%CI: -10.06 to 1.23) confessed that VAS at 4 h after operation in FA treatment group is less than in control group of Chinese surgical patients (Figure 4).

Meta-analysis of VAS at 24 h after operation

FA treatment and control groups were recorded in all the

Table 1. Characteristics of the 17 randomized clinical studies included in this meta-analysis.

Author	Jaded score	Treatment protocol (T/C)	Sample size (T/C)	VAS at 4 h after operation	VAS at 8 h after operation	VAS at 12 h after operation	VAS at 24 h after operation	Adverse reaction rate (%)
Chen et al. (2008)	3	T: FA(pre)+GA C:GA	T: 40 C: 40	NA	NA	1.10±0.90 2.60±0.90	1.00±0.90 2.00±0.90	20.00 32.50
Chen et al. (2011)	3	T: FA(pre) C:FA (pro)	T: 20 C: 20	1.20±0.20 1.80±0.50	2.10±0.70 3.40±0.80	2.90±0.50 4.2±0.70	3.10±0.70 4.60±0.90	NA
Ding et al. (2007)	3	T: FA(pre)+GA C: GA	T: 32 C: 32	1.10±1.00 2.40±1.90	1.10±1.50 1.50±1.90	NA	0.90±1.20 1.20±1.60	31.25 50.00
Fan et al. (2009)	2	T: FA(pre)+GA C: GA	T: 24 C: 24	2.05±0.77 2.83±0.74	2.27±0.81 3.01±0.85	2.23±0.51 1.88±0.69	1.15±0.35 1.25 0.25	NA
He et al. (2008)	3	T: FA(pre)+GA C: GA	T: 15 C: 15	1.20±0.90 2.70±1.00	2.70±1.30 3.90±1.30	3.00±1.30 3.10±1.50	1.70±1.50 1.80±1.40	53.30 60.00
Li and Xie (2007)	2	T: FA(pre)+GA C: GA	T: 20 C: 20	NA	NA	NA	4.8±0.8 6.8±0.9	15 10
Li et al. (2009)	3	T: FA(pre)+GA C: GA	T: 10 C: 10	2.50±1.52 3.83±0.75	2.33±1.37 4.17±1.6	2.00±1.41 3.67±0.51	2.50±1.52 5.17±1.52	NA
Li and Lin (2010)	3	T: FA(pre)+GA C: GA	T: 30 C: 30	3.30±0.80 4.50±1.30	2.80±1.00 3.80±1.10	2.20±1.00 3.40±0.90	2.00±1.00 2.30±0.70	10.00 40.00
Luo et al. (2009)	2	T: FA(pre)+GA C: GA	T: 20 C: 20	3.66±0.18 4.36±0.64	3.47±0.24 3.84±0.17	3.11±0.17 3.42±0.10	2.82±0.32 3.14±0.56	70.00 40.00
Song et al. (2010)	2	T: FA(pre)+GA C: GA	T: 30 C: 30	1.60±1.40 2.50±1.60	1.30±1.40 1.90±1.40	NA	0.80±1.40 1.30±0.90	20.00 46.70
Sun et al. (2007)	4	T: FA(pre)+GA C: GA	T: 30 C: 30	2.00±0.80 4.80±1.10	1.90±0.70 4.10±0.80	1.70±0.80 3.80±1.10	0.90±0.50 2.70±1.10	3.33 6.67
Wang et al. (2007)	3	T: FA(pre)+GA C: GA	T: 23 C: 21	2.00±0.74 2.76±0.77	2.34±0.71 3.05±0.59	NA	2.48±0.59 2.90±0.62	10.30 10.50
Wang et al. (2008)	2	T: FA(pre)+GA C: GA	T: 21 C: 21	1.85±0.71 3.50±1.81	1.71±0.67 3.30±1.51	NA	1.46±0.58 1.48±0.53	NA
Xie et al. (2006)	2	T: FA(pre)+GA C: GA	T: 40 C: 40	NA	NA	3.05±2.93 8.11±2.47	3.67±1.72 6.22±1.73	NA
Zeng et al., (2008)	3	T: FA(pre)+GA C: GA	T: 25 C: 25	3.10±0.80 4.60±1.10	2.00±0.90 4.00±1.20	1.90±0.70 3.50±1.40	1.00±0.50 2.30±1.10	24.00 16.00
Zeng and Li (2008)	2	T: FA(pre)+GA C: GA	T: 20 C: 20	2.50±0.90 3.40±1.20	3.20±1.10 3.40±1.30	3.60±1.40 3.80±1.30	2.70±0.90 2.60±1.10	NA
Zhao et al. (2009)	3	T: FA(pre)+GA C: GA	T: 25 C: 25	2.50±1.20 4.30±1.50	2.70±1.40 4.70±1.30	2.20±1.40 3.10±1.30	2.20±1.50 2.50±1.60	16.00 20.00

FA: Flurbiprofen axetil; FA(pre): flurbiprofen axetil of preemptive analgesia; FA(pro): flurbiprofen axetil of preemptive analgesia; GA: general anesthesia.

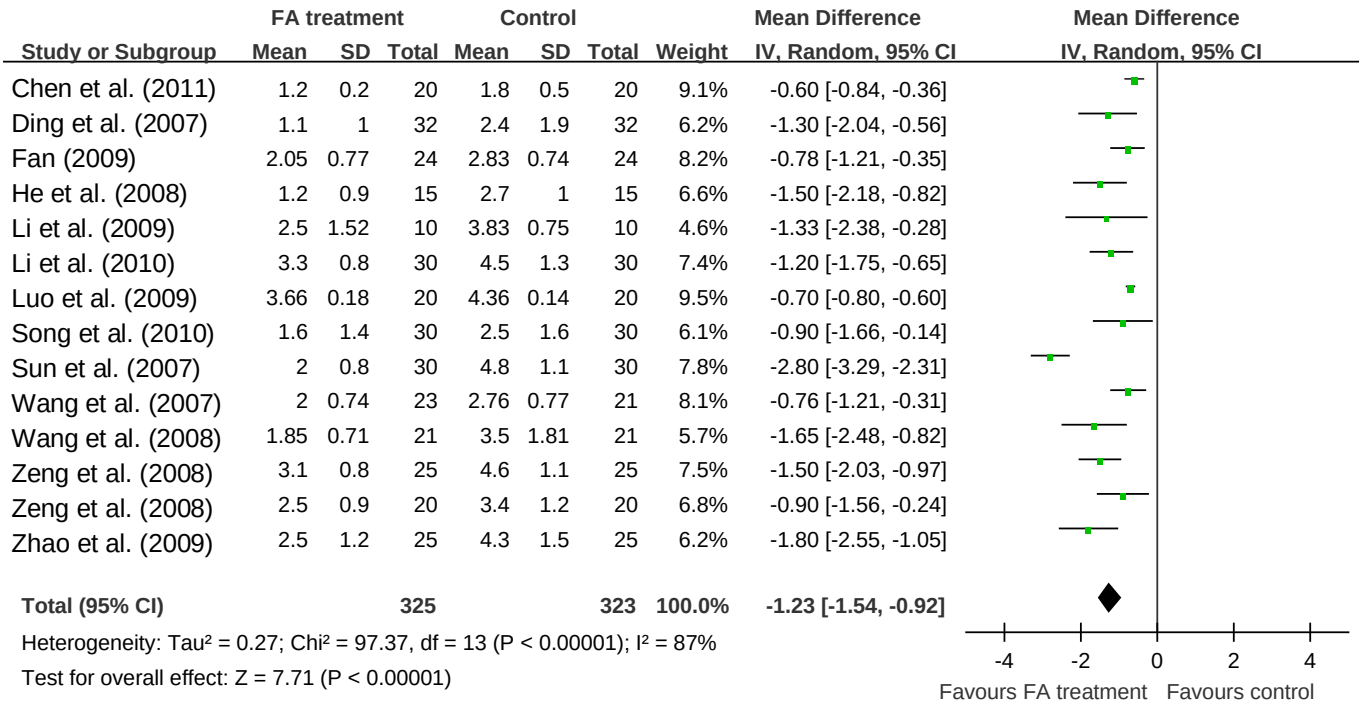


Figure 2. VAS at 4 h after operation between the FA treatment group and the control group.

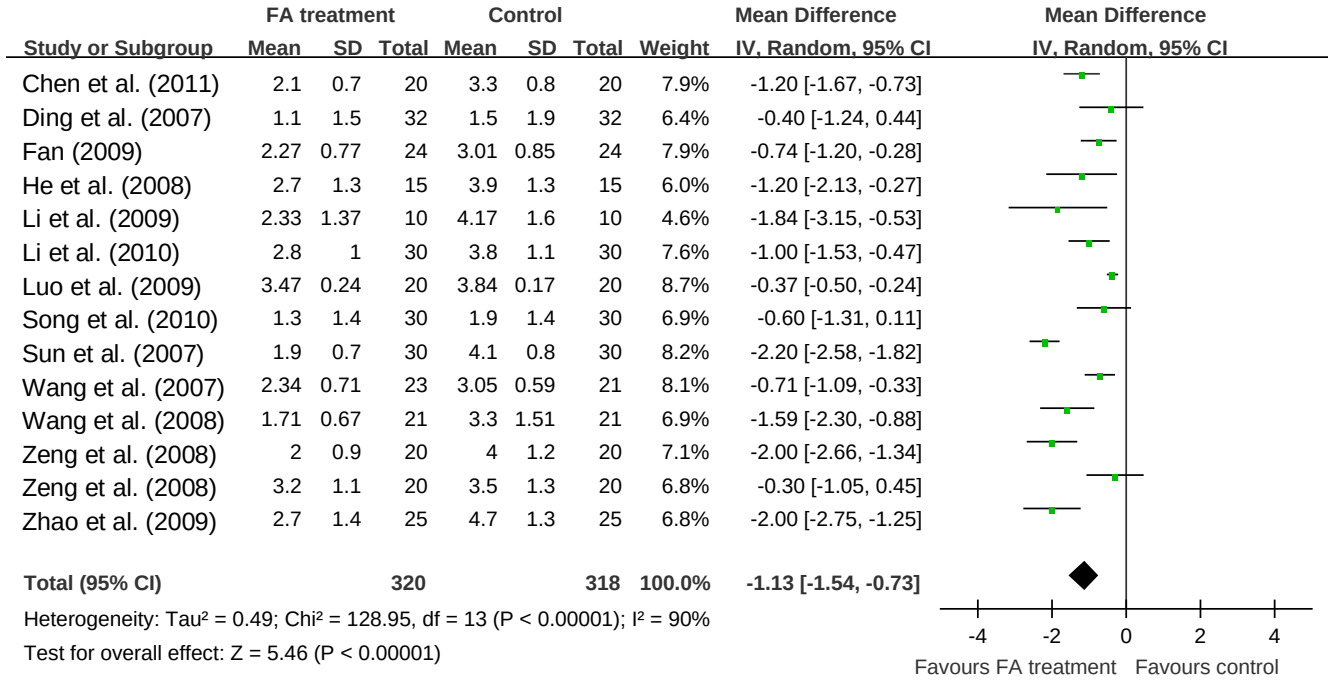


Figure 3. VAS at 8 h after operation between the FA treatment group and the control group.

17 trials finally included. Active analgesics involved in this analysis were tramadol or fentanyl. The results of meta-analysis (MD = -0.87, 95%CI: -1.24 to -0.51) confessed that VAS at 24 h after operation in FA treatment group is less than in control group of Chinese surgical patients (Figure 5).

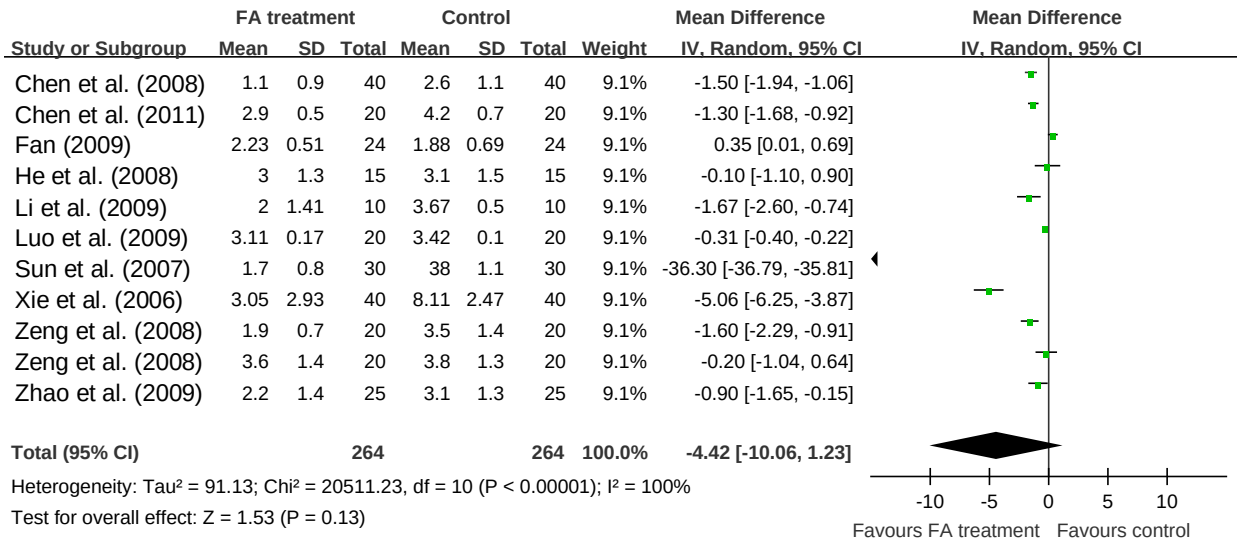


Figure 4. VAS at 12 h after operation between the FA treatment group and the control group.

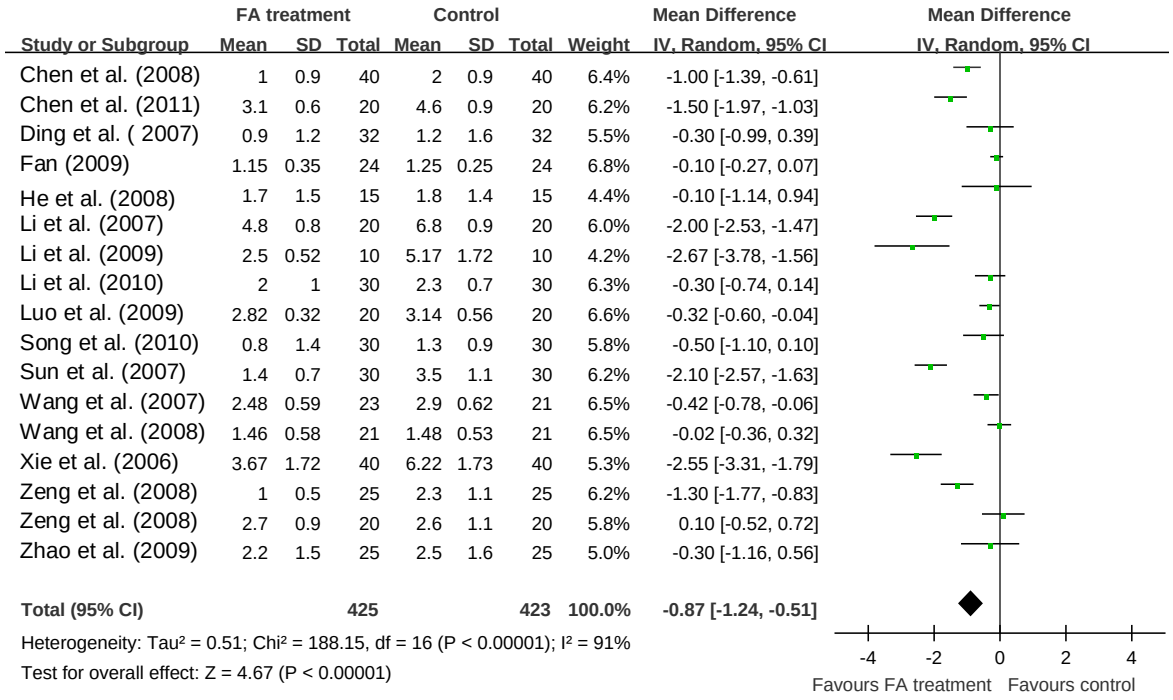


Figure 5. VAS at 24 h after operation between the FA treatment group and the control group.

Meta-analysis of safety

Adverse reaction rates of both FA and control group were recorded in the 12 trials finally included. The results of meta-analysis (OR = 0.70 95%CI: 0.49 to 0.99) confessed that FA is safer than the control group in Chinese surgical patients (Figure 6).

Publication bias

An analysis of publication bias was conducted. The funnel plots, to assess publication bias, are as shown in Figure 7. The shape of the funnel plots show some unasymmetries in all studies included in the meta-analysis. There exist some publication biases since the

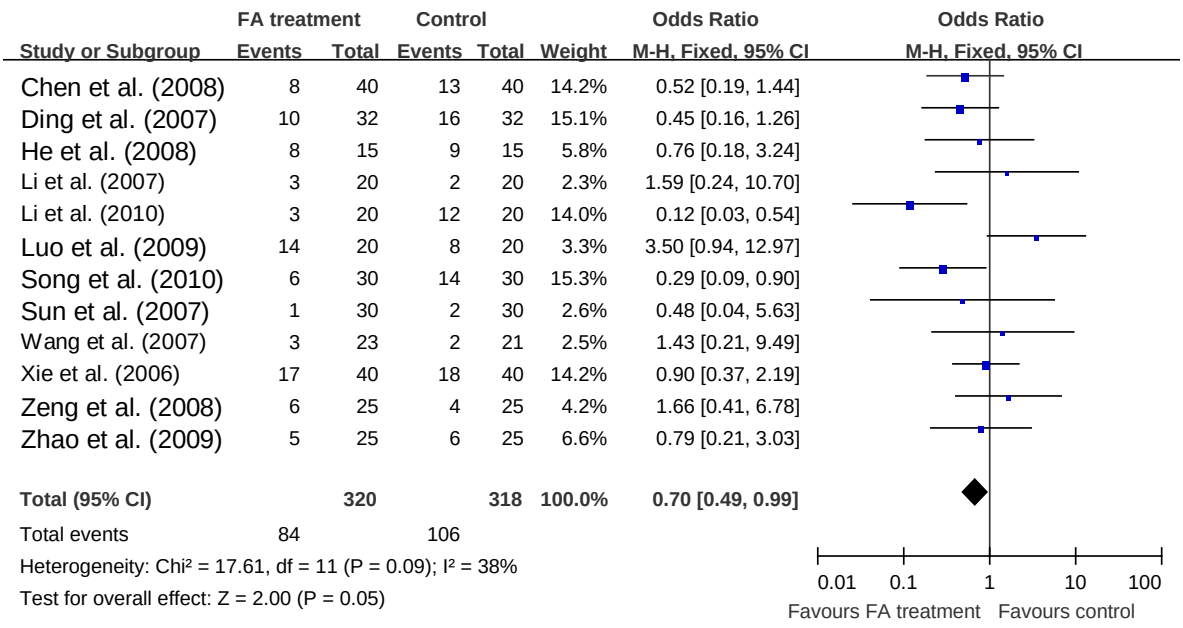


Figure 6. Adverse reactions rate between the FA treatment group and the control group.

funnel plots were unsymmetrical based on a visual analysis (Figure 7).

DISCUSSION

FA is prepared by esterification of flurbiprofen which makes the compound lipophilic and soluble in soybean oil within an intralipid based emulsion formulation for less irritation after iv injection (Simon and Benita, 1998). Flurbiprofen acts through inhibiting the cyclooxygenase reversibly, resulting in corresponding anti-inflammatory effect and causing the peripheral inhibition of prostaglandin (PG) synthesis (Basselin et al., 2007). In addition, flurbiprofen could also inhibit the migration of leukocytes into sites of inflammation and prevent the formation of thromboxane A2 by the platelets (Bolla et al., 2004; Van Ryan-McKenna and Buchanan, 1989). Thus, the anti-inflammatory and antinociceptive effects of flurbiprofen on postoperative pain was recommended for preemptive administration to suppress the synthesis of prostaglandin prophylactically at the area of the surgical injuries, which in return relieve the pain from the surgical wound (Nakayama et al., 2001).

A total of 17 literatures were finally included in this systemic review. All these articles, including a sample size of 848 totally were RCTs. Jadad score in 10 out of the 17 articles were more than three points. All the trials included in this meta-analysis mentioned the term "random", but the detail method was illuminated in 1 article only. Obviously, the included trials were lack of well-designed randomizations. A well-designed randomized controlled trial requires a thorough understanding of randomization, so that better results could be achieved. Randomization includes three important steps, namely, sequence generation, allocation concealment, and randomization implementation. Sequence generation is a method used to generate the random allocation sequence, including details of any restriction. Allocation concealment is to implement the random allocation sequence. Randomization implementtation is to generate the allocation sequence. Well-men designed randomized controlled trials are required to evaluate FA treatment versus routine treatment in Chinese surgical patients. It was suggested that we should be careful for randomization.

Moreover, there exist some publication biases in each analysis. The publication biases might be relevant to some methodological insufficiencies: (1) Randomization method may not be rigorous because the specific program of randomization was inferred in only one literature. (2) Selection bias may exist, for allocation concealment was not described in all of these articles included. (3) Selection bias, measuring bias, and implementation bias may exist because some studies did not describe whether blind method was used or not.

The results of this systemic review showed that FA was more effective than control group on preemptive analgesia for Chinese surgical patients. Thus, we can conclude that FA has stronger analgesic effect when compared with other active analgesics. The adverse reactions (ADRs), mainly gastrointestinal symptoms of FA referred to in this study were less likely to happen. The results of this systemic review showed that FA has less

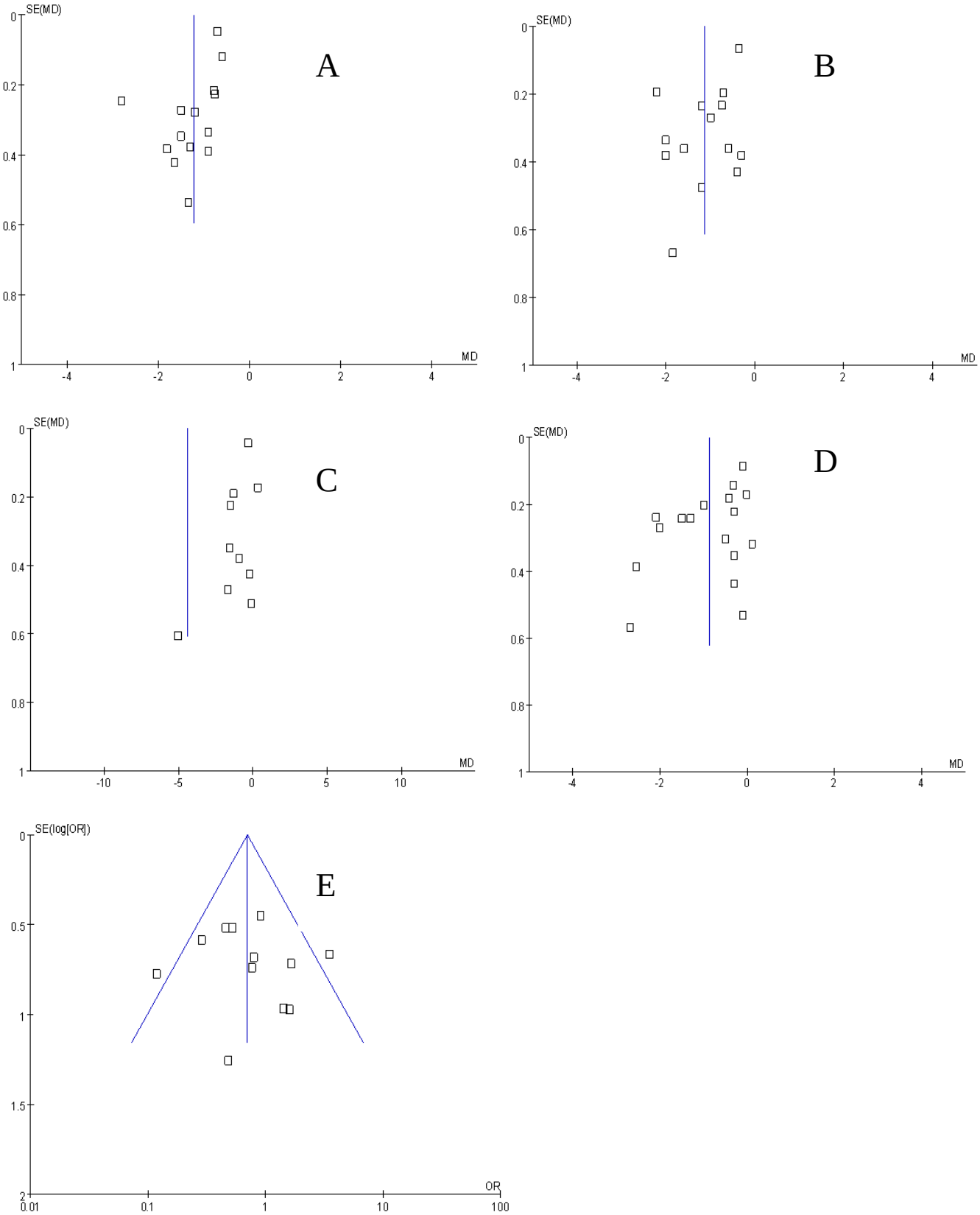


Figure 7. Funnel plots of sputum negative conversion tuberculosis cavity changes focus absorption and adverse effect. A: Funnel plot for VAS at 4 h after operation; B: Funnel plot for VAS at 8 h after operation; C: Funnel plot for VAS at 12 h after operation; D: Funnel plot for VAS at 24 h after operation; E: Funnel plot for ADRs rate.

ADRs than the control group in Chinese surgical patients. Therefore, we could conclude that FA was safer than other active analgesics.

Conclusion

In summary, our systemic review initially demonstrated the analgesic effects of FA in Chinese surgical patients, such as decreasing pain induced by various surgical operations. However, all the clinical trials involved were of small samples without blind methods, their results may show some uncertainties. We urgently hope the high-quality, double-blinded, and multi-centered RCTs will be carried out in the future to further confirm its efficacy and safety.

ACKNOWLEDGEMENT

We appreciate Dr. Jintai Jia, who rechecked the paper for the language.

REFERENCES

Basselin M, Villacreses NE, Lee HJ, Bell JM, Rapoport SI (2007). Flurbiprofen, a cyclooxygenase inhibitor, reduces the brain arachidonic acid signal in response to the cholinergic muscarinic agonist, are coline, in awake rats. Neurochem. Res. 32:1857-1867.

Bolla M, You D, Loufrani L, Levy BI, Levy-Toledano S, Habib A (2004). Cyclooxygenase involvement in thromboxanedependent contraction in rat mesenteric resistance arteries. Hypertens 43:1264-1269.

Buritova J, Besson JM (1998). Peripheral and/or central effects of racemic - S (+)- and R (-) -flurbiprofen on inflammatory nociceptive processes: ac-Fos protein study in the rat spina cord. Brit. J. Pharmacol. 125:87-101.

Chen XY, Cao HZ, Sun ZH (2011). Clinical effects of flurbiprofen axetil on preemptive analgesia during radical operation of thyroid cancer. Modern Oncolo. 19(3):565-566.

Chen Z, Li L, Ou YW (2008). Flurbiprofen used for preemptive analgesia in patients undergo gynecological laparoscopic surgery. J. China-Japan Friendship Hosp. 22(2):95-97.

Ding YH, Gu CY, Lou Y (2007). Application of pre- emptive analgesia with flurbiprofen axetil in patients undergoing laparoscopic cholecystectomy. Clin. Med. l27(11):29-31.

Fan ML (2009). Preemptive analgesia effects of flurbiprofen axetil injection in patients undergoing gastrectomy operation. Chin. J. Ethnomed. Ethnopharm. 18(7):23.

Fujii Y, Shiga Y (2005). Flurbiprofen axetil preceded by venous occlusion in the prevention of pain on propofol injection in the hand: A prospective, randomized, double-blind, vehicle-controlled, dose-finding study in Japanese adult surgical patients. Clin. Ther. 27(8):588-593.

Fujii Y, Shiga Y (2009). Pretreatment with flurbiprofen axetil, flurbiprofen axetil preceded by venous occlusion, and a mixture of flurbiprofen axetil and propofol in reducing pain on Injection of propofo in adult Japanese surgical patients: A prospective, randomized, double-blind, placebo-controlled study. Clin. Ther. 31(4):721-727.

He GW, Zhou ZC, Yuan SY (2008). Effect of preemptive analgesia with flurbiprofen axetil on serum gastrin in patients undergoing the lower extremity fracture surgery. Cent. Chin. Med. J. 32(1):35-37.

Li AB, Xie H (2007). Flurbiprofen used as preemptive analgesia of patients with laparoscopic cholecystectomy. Pract. Clin. Med. 8(12):55-57.

Li ZP, Lin YF (2010). Antinociceptive effects of flurbiprofen axetil on patients received upper abdominal surgery: the role of preemptive analgesia. Qinghai Med. Pharm. J. 40(4):6-8.

Li YH, Han M, Ma HC (2009). Preemptive analgesia effects of flurbiprofen axetil on respiratory function in patients undergoing thoracotomy. J. Jilin Univ. (Medicine Edition) 35(3):515-518.

Lin Q (2010). Preemptive analgesia with flurbiprofen axetil or tramadol before gynecologic hysteroscopic surgery: a comparison of outcome. Fujian Med. J. 32(5):123-125.

Luo H, Li J, Huang ZL (2009). Postoperative analgesic effects of flurbiprofen axetil applied on patients with arthroscopic surgery of knee. Prac. Med. J. 25(10):1666-1667.

Mikawa K, Nishina K, Maekawa N, Shiga M, Obara H (1997). Dose-response of flurbiprofen on postoperative pain and emesis after paediatric strabismus surgery. Can. J. Anaesth., 44: 95-98.

Nakayama M, Ichinose H, Yamamoto S (2001). Perioperative intravenous flurbiprofen reduces postoperative pain after abdominal hysterectomy. Can. J. Anesth., 48: 234-237.

Nishiike S, Kato T, Nagai M (2007). Preoperative flurbiprofen for pain prevention after tonsillectomy in adults. J. Clin. Anesth., 19: 596-600.

Roszkowski MT, Swift JQ, Hargreaves KM (1997). Effect of NSAID administration on tissue levels of immunoreactive prostaglandin E2, leukotriene B4, and (S)-flurbiprofen following extraction of impacted third molars. Pain 73:339-345.

Simon, Benita (1998). Submicron emulsions in drug targeting and delivery. CRC Press. pp. 135.

Song WJ, Zhai XD, Dong JL (2010). Postoperative analgesic effects of flurbiprofen axetil injection applied on patients with naigated knee arthroplasty. Hebei Med. J., 32(20): 2877-2878.

Sun ZT, Han XP, Miao LJ (2007). Preemptive analgesia for postoperative pain relief of flurbiprofen axeti injection on children. Chin. J. Pain. Med. 13(4):213-217.

Takada M, Fukusaki M, Terao Y, Yamashita K, Inadomi C (2007). Preadministration of flurbiprofen suppresses prostaglandin production and postoperative pain in orthopedic patients undergoing tourniquet inflation. J. Clin. Anesth. 19:97-100.

Xie YH, Fang C, Zhou L (2006). Flurbiprofen used for preemptive analgesia of patients wi th abdomi nal cholecystectomy. Anhui Med. Pharmaceut. J., 10(8): 571-572.

Van Ryan-McKenna J, Buchanan MR (1989). Relative effects of flurbiprofen on platelet 12-hydroxy-eicosatetraenoic acid and thromboxane A2 production: influence on collagen-induced platelet aggregation and adhesion. Prostaglandins Leukot. Essent. Fatty Acids 36:171-174.

Wang FZ, Shen XF, Xu SQ (2008). Preemptive combined preventive delivery of flurbiprofen axetil produced effective analgesia after lumpectomy. Acute Pain 10:65-71.

Wang FZ, Shen XF, Guo XR (2009). Analgesic effectiveness of flurbiprofen axetil after uterine curettage on abortion: A randomized controlled trial. Acute Pain 11:43-50.

Wang Q, Wang ZC, Liu HQ (2007). Postoperative analgesic effects of flurbiprofen axetil applied preoperatively on patients with spinal cord injury. Chin. J. Rehabil. Theory Pract. 13(11):1019-1020.

Zeng RF, Ni YF, Wang JG (2008). Postoperative analgesic effects of flurbiprofen axetil on postoperative analgesia in patient undergoing surgery of vocal cord under suspension laryngoscope. Clin. Med. 28(9):68-69.

Zeng ZD, Li HM (2008). Postoperative analgesic effects of flurbiprofen axetil on postoperative analgesia in patient undergoing laparoscopic cholecystectomy. Chin. J. Med. Guide 10(5):723-724.

Zhao B, Xia XQ, Luo HL (2009). Postoperative analgesic effects of flurbiprofen axetil applied on patients with laparoscopic cholecystectomy. Anhui Med. J. 30(9):1114-1115.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2277-2280, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.143
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

The effect of TGF-β_1 on the Bax in rats with hypoxic brain damage after surgical treatment of gliomatosis cerebri (HBD/GC)

XianZhen Chen, ZhaoLi Shen, Fei Xue, Rui Shen and Jia Yin*

Department of Neurosurgery, Shanghai Tenth People's Hospital, Tongji University School of Medcine, Shanghai 200072, P.R. China.

Accepted 26 March, 2012

The effect of transforming growth factor beta 1 (TGF-β_1) on Bax in rats with hypoxic brain damage after surgical treatment of gliomatosis cerebri (HBD/GC) were investigated. The SD rats were randomly divided into three groups: Sham control group, HBD/GC model group, HBD/GC + TGF-β_1 injection treatment (E) group. The SD rats were treated in hypoxic environment after a surgical treatment of gliomatosis cerebri to establishe HBD/GC models. The expressions relative rate of Bax in CA1 region of hippocampus were detected by Reverse transcription-Polymerase chain reaction (RT-PCR) and Immunohistochemistry (ICH). After 24 h of operation, there was a significant decrease in Bax expression in E groups, while an increase in HBD/GC model group. TGF-β_1 could comparatively increase the Bax mRNA and protein expressions in the hippocampal CA1 region of HBD/GC rats. These results suggested that TGF-β_1 might develop its improvement by increasing Bax mRNA and protein expression in the CA1 region of hippocampal of HBD/GC rats.

Key words: Transforming growth factor beta 1, HBD/GC, Bax.

INTRODUCTION

Gliomatosis cerebri (GC) is a rate tumor of the central nervous system. It is characterized by diffuse neoplastic infiltration of glial cells in varying stages of differentiation, with the preservation of anatomical architecture the sparing of neurones (Zhao et al., 2008). There has been considerable progress in the initiation of clinical trials to establish treatment regimes specifically designed for GC an optimal therapeutic strategy is still not well established. (Zhang et al., 2005) and (Zheng et al., 2003) propose that surgery is an optional therapeutic tool for GC, and the degree of operational incision is an important factor affecting the prognosis. However, the hypoxic brain damage (HBD) after surgical treatment significantly reduced the therapeutic effect of the GC surgery.

Up to now, there are no effective therapeutics tools for this case. Growing studies showed that transforming growth factor beta 1 (TGF-β_1) has several pharmacological activities such as anti-inflammation, adjusting differentiation and proliferation of immunologic cells and non-immunologic cells, and enhancing epithelial recovery (Spurgeon et al., 2005). Nevertheless, the effect of TGF-β_1 in rats with hypoxic brain damage after surgical treatment of gliomatosis cerebri (HBD/GC) and its mechanism are all still unclear. Bax, as a proapoptosis gene of the Bcl-2 family, has extensive amino acid homology with Bcl-2, may contribute to cell death, which is the key regulators of apoptosis (Zhang et al., 2000). Therefore, we assumed that TGF-β_1 might have some effects on the Bax expressions in rats with HBD/GC.

In the present work, we establish HBD/GC model by putting the SD rats to hypoxic environment after a surgical treatment of gliomatosis cerebri. We also detected the expressions relative rate of Bax in CA1 region of hippocampus by Reverse transcription-polymerase chain reaction (RT-PCR) and Immunohisto-

*Corresponding author: E-mail: yi200072@126.com.

Table 1. Primers for the various genes investigated in this study.

Gene	Direction	Primer sequence (5'-3')	Product size (bp)
β-actin	Forward	CCTCTATGCCAACACAGTGC	211
	Reverse	GTACTCCTGCTTGCTGATCC	
Bax	Forward	AAGCTGAGCGAGTGTCTCCGGCG	284
	Reverse	GCCACAAAGATGGTCACTGTCTGCC	

chemistry (ICH) methods.

MATERIALS AND METHODS

Reagents and apparatus

TGF-β_1 was purchased from Sunshine Pharmaceutical (Shenyang, China). Trizol and Bax (rabbit polyaclonal) were obtained from Wuhan Biostor (Wuhan, China); Y-maze (type MG -3) was obtained from Zhangjiagang Biomedical Instrument Factory (Zhangjiagang, China).

Laboratory animals

Fifty-four SD rats, aged >16 months, weighing (300 ± 41) g, of either gender, were provided by the Experimental Animal Center of the Second Military Medical University of Chinese PLA between July 2009 and December 2011. The rats were housed under controlled conditions (room temperature, 22 ± 2°C).

Establishment of HBD/GC rats models

Following the rats were intraperitoneally anesthetized by chloral hydrate, a median incision was made at the cervical part. Muscle was carefully bluntly dissected to expose bilateral common carotid artery. A No.7 surgical suture was embedded under common carotid artery. Vagus nerve was avoided to be stimulated.

Grouping and drug intervention

Fifty-four Wistar rats were randomly divided into three groups: Sham-operation group (n = 18): Surgical suture embedding, then surgical suture was drawn out and incision was sutured, while without hypoxic treatment. HBD/GC model group (n = 18): rat models of HBD/GC were developed according to the previous method. TGF-β_1 treatment group (n = 18): following a cannula was buried in lateral cerebral ventricle for one week, rat models of HBD/GC were developed and injected with 20 ng of TGF-β_1 via lateral cerebral ventricle. The experimental temperature was kept at 25℃. Rats in the Sham operation group and HBD/GC group were intraperitoneally injected with the same amount of normal saline.

Reverse transcription-polymerase chain reaction (RT-PCR)

Rats were anaesthetized with 10% of urethane and then sacrificed by transcardiac perfusion with PBS followed by separate their hippocampus immediately, cryoprotected by immersion in 30% sucrose for 24 h at 4 to 8℃ and frozen in a tissue-freezing medium. The total RNA was extracted with Trizol reagent according to the manufacturer's protocol. A_{260} readings were taken on the extracted samples to quantify the amount of RNA present, and A_{260}/A_{280} ratios were calculated to determine the purity. The reverse transcription reaction was carried out with 1 mM of dNTP, 40 U/μl of Rnase innhibitor, 5 U/μl of AMV-RT and 5 U/μl of AMV- Optimized Taq in the provided reaction buffer at 50℃ for 30 min. The primers were designed and synthesized based on the published gene sequence as shown in Table 1. The PCR reaction was done in a thermal cycler (Model 7500, Beckman) with an initial denaturation step at 94℃ for 5 min, followed by a variable number of cycles of denaturation 94℃ for 45 s, annealing for 45 s, elongation 72℃ for 1 min and a final elongation step at 72℃ for 7 min. The numbers of cycles was 30 for Bax, and were defined after titration between 20 and 45 cycles and were within the logarithmic phase of amplification. The amplified products were run on a 1.5% agarose gel with ethidium bromide. Images were documented with a digital CCD camera in a BX60, and the intensity of the product bands was measured with Flous-SmutiImage Spectrum software (Bio-Rad, USA).

Immunohistochemistry (ICH)

Rats were anaesthetized with 10% of urethane and then sacrificed by transcardiac perfusion with PBS followed by separate their hippocampus immediately, cryoprotected by immersion in 30% sucrose for 24 h at 4 to 8℃ and frozen in a tissue-freezing medium. The brains were cut on a freezing microtome at the level of the anterior hippocampus, into six adjacent series of 4-μm-thick coronal sections.

The sections were dehydrated through an alcohol series. Prior to immunohistochemical processing, sections were rinsed in 2% PBS-Triton X-100 and mounted onto gelatine-coated slides. Immunohistochemistry was performed on slide-mounted sections utilizing the following antibodies: Bax (dilution 1:100). The sections were incubated overnight at room temperature with the primary antibody diluted in PBS-bovine serum albumin (BSA). After rinsing, sections were incubated for 1 h at room temperature in biotinylated goat antimouse serum (1:500), sections were incubated for 1 h in avidin–biotin–horseradish peroxidase complex (1:200). Following rinses, sections were placed for 30 min in chromagen solution consisting of 0.05% diaminobenzidine and 0.01% H_2O_2. The reaction was monitored visually and stopped by rinses of 0.1 M PBS.

In order to minimize variability, sections from all animals were stained simultaneously. Cell counts were performed blindly in all sections using a Nikon Eclipse E800 microscope. Counts were made in six randomly selected optical fields under 400× magnification by individuals who were blinded to diagnosis. Bax immunoreactivity was assessed semi-quantitatively using Image Pro Plus software Version 4.5.129 (Media Cybernetics). The percentage area covered by immunoreactivity was measured and the mean value taken.

Table 2. Expression relative rate of Bax mRNA in the hippocampal CA1 region.

Group	24 h after operation
Sham	0.17 ± 0.007
HBD/GC	0.987 ± 0.047△
E	0.583 ± .017△*

(VS Sham: △P < 0.05; VS HBD/GC: *P < 0.05).

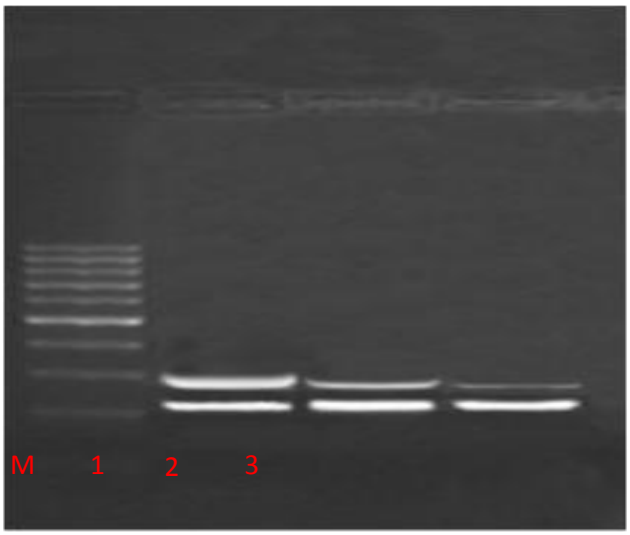


Figure 1. Comparison of the RT-PCR of Bax in different groups. (M:Mark; 1: Bax mRNA expression in CA1 region of hippocampus in HBD/GC model group at postoperative 24 h; 2: Bax mRNA expression in CA1 region of hippocampus in TGF-β_1 treatment group at postoperative 24 h; 3: Bax mRNA expression in CA1 region of hippocampus in Sham operation group at postoperative 24 h).

Table 3. The IOD of Bax immunoreactivity in the hippocampal CA1 region.

Group	24 h after operation
Sham	9.17 ± 1.73
HBD/GC	28.33 ± 5.17△
E	16.67 ± 3.47△*

(VS Sham: △P < 0.05; VS HBD/GC: *P < 0.05).

Statistical analysis

The database was set up with the SPSS 16.0 software package for analysis. Data were represented as mean ± S.D. The means of multiple groups were compared with one-way ANOVA, after the equal check of variance, and the two-two comparisons among the means were performed by Student's t-test. P < 0.05 was considered as statistically significant.

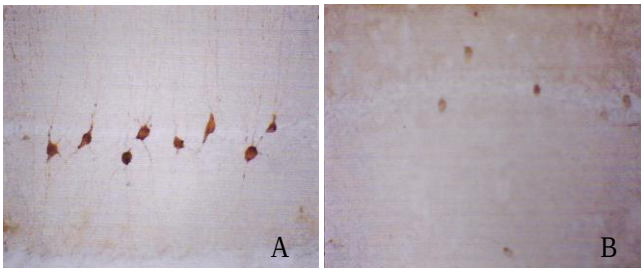


Figure 2. Bax positive cells of CA1 region of hippocampus in different groups (×400). (Figure 2A: Bax immunoreactivity in CA1 region of hippocampus in HBD/GC model group at postoperative 24 h; Figure 2B: Bax immunoreactivity in CA1 region of hippocampus in TGF-β_1 treatment group at postoperative 24 h).

RESULTS

Effects of TGF-β_1 on the expression of Bax mRNA in CA1 region of hippocampal of rats

After 24 h of administration, Bax mRNA in the HBD/GC group and TGF-β_1 treatment group were significantly more than those in the Sham-operation group (P < 0.05), while in TGF-β_1 treatment group was significantly less than those in the HBD/GC group (P < 0.05) (Table 2, Figure 1). These results suggested that TGF-β_1 could significantly down-regulating the decreased expressions of Bax mRNA of HBD/GC rats.

Effects of TGF-β_1 on the expressions of Bax protein in CA1 region of hippocampal of rats

After 24 h administration, Bax protein in the HBD/GC group and TGF-β_1 treatment group were significantly much more than those in the sham-operation group (P < 0.05), while in TGF-β_1 treatment group was significantly less than those in the HBD/GC group (P < 0.05) (Table 3, Figure 2). These results suggested that TGF-β_1 could significantly down-regulating the decreased expressions of Bax protein of HBD/GC rats.

DISCUSSION AND CONCLUSION

TGF-β_1 is a family of related proteins that regulate many cellular processes including growth, differentiation, extracellular matrix formation, and immunosuppression (Katabami et al., 2005 and TenDijke et al., 2002). Every cell in the body produces TGF-β and has receptors for it. TGF-β_1 is one of the isoforms (TGF-β1-5), and arrests the cell cycle in the G1 phase, thereby inhibiting cell proliferation and triggering apoptosis (Damdinsuren et al., 2006). Growing studies showed that TGF-β_1 plays important roles in growth, differentiation and repair of neuron cells. When neuron cells were treated with hypoxia, the expression of TGF-β_1 significantly increased,

indicating that TGF-β_1 could repair the injury induced by hypoxia (Lesen et al., 2002; Magy et al., 2002). Meanwhile, HenrichNoack et al. (1996) also found that TGF-β_1 could inhibit the apoptosis of neuron cell damage induced by hypoxia, however, its real mechanism are still unclear.

In our present work, we establish HBD/GC model by putting the SD rats to hypoxic environment after a surgical treatment of GC. We then detected the expressions relative rate of Bax in CA1 region of hippocampus by RT-PCR and ICH methods. These results suggested that TGF-β_1 could down-regulating the decreased expressions of Bax mRNA and protein of HBD/GC rats. However, the precise mechanism for the down-regulation of TGF-β_1 requires further investigation.

REFERENCES

Damdinsuren B, Nagano H, Kondo M, Natsag J, Hanada H, Nakamura M, Monden M (2006). TGF-beta1-induced cell growth arrest and partial differentiation is related to the suppression of Id1 in human hepatoma cells. Oncol. Rep. 15:401-408.

HenrichNoack P, Prehn JH, Kriglstein J (1996). TGF-beta1 protects hippocampal neurons against degeneration caused by transient global ischemia Dosese relationship and potential neuroprotective mechanisms, Stroke 27(9):1609-1614.

Lesen SB (2002). Transforming growth factor beta1 modulated cerebral gene expression, Int. Soci. Cerebr. Blood Flow, Metabol. pp.1114 - 1112.

Katabami K, Mizuno H, Sano R, Saito Y, Ogura M, Itoh S (2005). Transforming growth factor-beta1 upregulates transcription of alpha3 integrin gene in hepatocellular carcinoma cells via Ets-transcription factor-binding motif in the promoter region, Clin. Exp. Metastasis. 22: 539-548.

Magy Z, Simonm L, Bori Z (2002). Regulatory mechanisms in focal cerebral ischemia: New Possibilities in neuroprotective therapy, Ideggyogy Sz, 55(324): 73 -85.

Spurgeon KR, Donohoe DL, Basile DP (2005).Transforming growth factor-β_1 in acute renal failure: receptor expression, effects on proliferation, cellularity, and vascularization after recovery from injury, Am. J. Physiol. Renal. Physiol. 288(3):F568-F577.

TenDijke P, Goumans MJ, ItohF, Itoh S (2002). Regulation of cell proliferation by Smad proteins. J. Cell. Physiol. 191:1-16.

Zhang L, Yu J, Park BH, Kinzler KW and Vogelstein B (2000). Role of Bax in the apototic response to anticaner agents, Sci., 290: 989-992.

Zhao XL, Tian Y, Li ZH, Ji W, Du C (2008). Glopmatosis Crebri. Chin. J. Clin. Oncol. 5:242-250.

Zheng ZC, Wang SS, Wang RM (2003). Diagonsis and treatment of neurogliomatosis cerebri, Neur. Ren. Res., 2: 16-17.

Zheng HQ, Shu K, Ye F (2005). Diagonsis and treatment of gliomatosis cerebral. Neur. Ren. Res. 4:582-584.

African Journal of Pharmacy and Pharmacology Vol. 3(30), pp. 2281-2284, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.201
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

The role of intralesional Pingyangmycin in the treatment of nasal polyp in aged population

Zhenghui Wang, Xianghong Zhang*, Baoshu Li, Fang Quan and Miaoli Yang

Department of Otolaryngology-Head and Neck Surgery, the Second Hospital, Xi'an JiaoTong University, Xi'an 710004, China.

Accepted 3 April, 2012

This study is to investigate the effect of intralesional Pingyangmycin injection in nasal polyp. 82 patients with nasal polyp were treated with intralesional Pingyangmycin injection from September 2001 to September 2006. After a minimum follow-up of 2 years, 67 patients (81.7%) developed 76 to 100% regression in their lesions; 17 patients (18.3%) had 51 to 75% regression in their lesions. No changes in renal function or cytopenias were encountered and no changes were found with chest X-ray during the course of therapy. Thus, intralesional injection of Pingyangmycin seemed to be an effective, safe and inexpensive method to treat nasal polyp, especially in patients not suitable for surgeries.

Key words: Pingyangmycin, nasal polyp, intralesional, aged.

INTRODUCTION

Nasal polyp is a common disorder that occurs in the nasosinus region and can be treated with different surgical methods. However, there were many patients with recurrence after surgery, or they are intolerant to the surgery. Agents like bleomycin have now been used for percutaneous sclerotherapy for venous malformation in the head and neck (Zheng et al., 2010). Pingyangmycin (PYM) was extracted from one type of fungi at Pingyang (South-East Zhejiang Province of China), which is a subbranch of bleomycin A5. The therapeutic effect is derived from its endothelial toxicity. Because of the low cost, safety and ease of availability, we have used PYM for over 20 years in the treatment of venous malformation and nasal polyp in Xi'an Jiaotong University Hospital. The aim of this study therefore is to evaluate the safety and efficacy in treating nasal poly using PYM.

MATERIALS AND METHODS

Fifty four (54) patients (male 37, female 17, range from 49 to 84 years old, mean age 63.1±5.3 years old) referred with nasal polyp were treated with intralesional Pingyangmycin injection from September 2001 to September 2006 as shown in Table 1. Informed consent was obtained from the patient and this study has been approved by Institutional Review Board of our hospital.

Treatments could be given to the patients with normal examination results from X-ray in lungs, routine blood tests and urine test before surgery. 8 mg PYM dissolved in 5 ml 0.9% sodium chloride and 3 ml 2% lidocaine was injected from the front part of polyp through a long tiny injector advancing with slow injection. During injection, the polyp became pale and swelling. The injection was given once a week and the period of treatment was 4 to 6 times. Antibiotics were used only in patients with infected lesions. All patients underwent monthly hemogram, renal function tests and chest X-ray prior to each injection to evaluate for toxicity.

RESULTS

Fifty four (54) patients presented an esthetic complaint, 17 with pain and 3 with bleeding. 3 days after the first injection of Pingyangmycin, 5 cases experienced some inflammatory symptoms consisting of a febrile response and 5 cases complained of a degree of swelling. These were the only side effects. The fever lasted 1 day after injection and disappeared next day without any treatment. Five cases complained the local swelling, which was not obvious after injection and resolved after 1 week without any therapy. All patients stated improved ventilation after 5 days.

*Corresponding author. E-mail: zhangxianghong_PYM@163.com, ehui4298@163.com. Tel: +86-29-87283769. Fax: +86-29-87678421.

Table 1. Patient characteristics.

Characteristics	Patients no.
Sex	
Male	47
Female	32
Mean age (years)	63.1
Accompanying disease	
Hypertension	46
Cardiac disease	41
Diabetes	38
Asthma	51
Rheumatoid arthritis	16
Stroke	11
Recurrent polyp	
One surgery	24
Two surgeries	13
Three surgeries	9
Four surgeries	5
Accompanying symptoms	
Nose obstruction	65
Dizzy	46
Loss of olfaction	27
Headache	18
Pus snivelling	32
Blood sniveling	24
Refusing surgery	46
Intolerance of surgery	33

Table 2. The effects after treatment.

Symptom	Effective (total cases)	Percentage (%)	Length of time
Nose obstruction	45 (65)	100	4.3
Dizzy	1 (46)	30.4	29
Loss of olfaction	5 (27)	18.5	87
Headache	5 (18)	27.8	46
Pus snivelling	16 (32)	50	13
Blood sniveling	17 (24)	70.8	9

No changes in renal function nor cytopenias were encountered. No patients developed chest X-ray changes during the course of therapy. There were no mortalities. After the second injection, the lesions began to regress and the swelling disappeared. Significant regression was apparent after an average of 3 weeks of treatment. The longest time of treatment was 2 months and about 10 injections were administered; the results of these treatments are shown in Table 2. Sixty-seven patients (81.7%) developed 76 to 100% regression in their lesions (Figures 1 to 3); 15 patients (14.6%) had 51 to 75% regression in their lesions. The patients were followed for at least 1 year and no enlargement in the lesion or recurrence was observed during the period.

DISCUSSION

The common treatment for nasal polyp is excision by Surgery (DeMarcantonio and Han, 2011). However, this

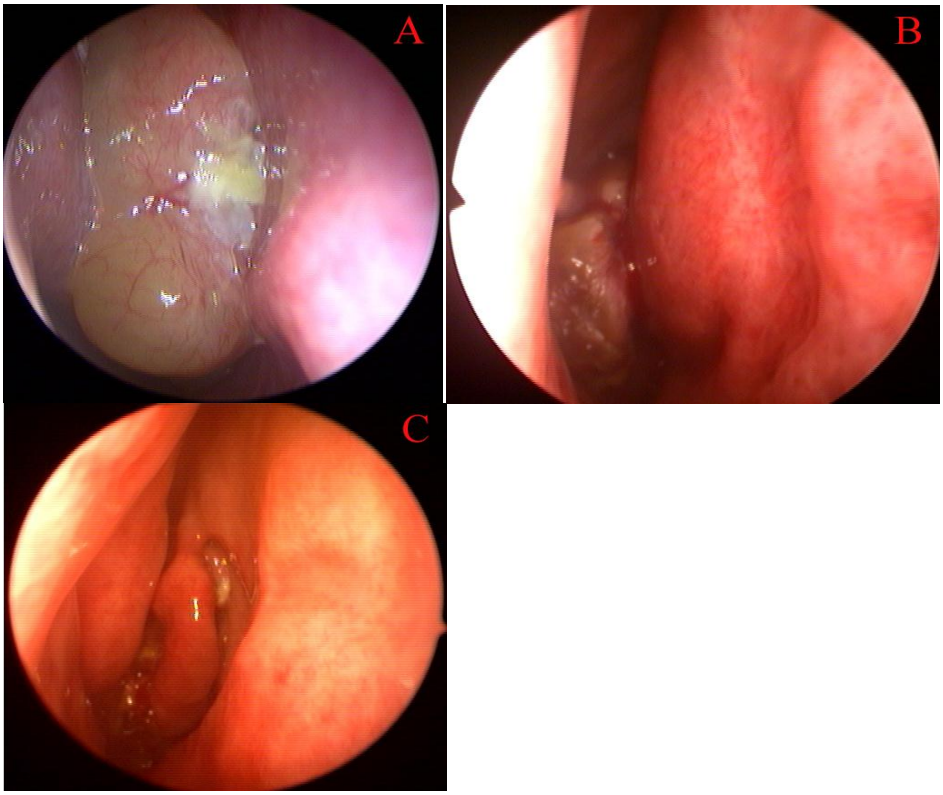


Figure 1. Patient 1; A: Before treatment; B: After one dose of PYM; C: After 3 doses of PYM.

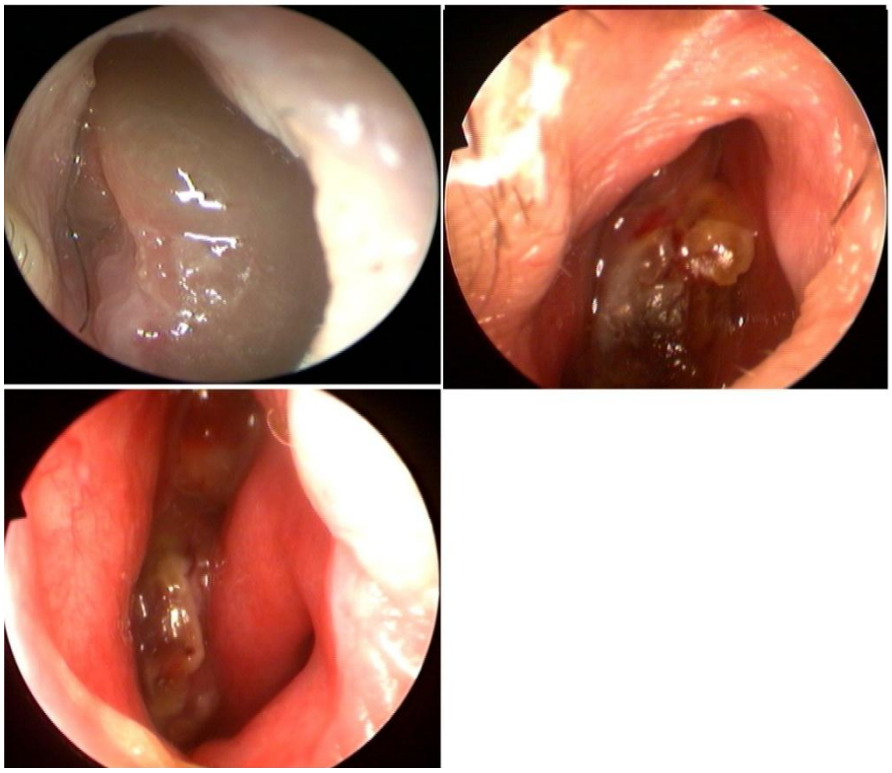


Figure 2. Patient 2; A: Before treatment; B: After 2 doses of PYM; C: After 3 doses of PYM.

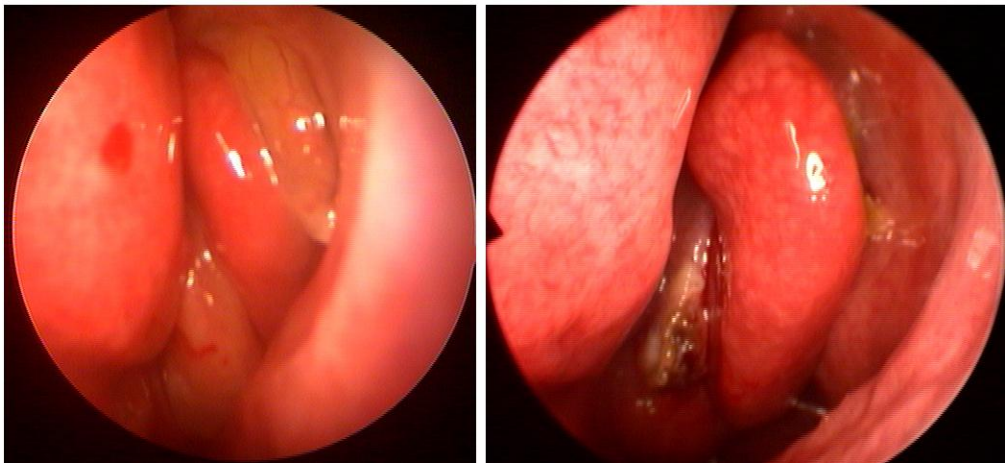


Figure 3. Patient 3; A: Before treatment; B: After one dose of PYM.

leads to incidences of hemorrhage, with a high recurrence rate or even a tendency to deterioration. The percentage of old patients is dramatically increasing in recent years, which also limited the implement of surgery especially several times of surgeries.

PYM is consisted of monoconstitution of Bleomycin A5, which could kill and prohibit the proliferating cells. Previous studies have been extensively applied in the cures of some benign tumors and heoplasms. Satisfying results can be got in the treatment of pedolymphangioma, hemangioma of maxillofacial regions and pterygium etc. In view of the pathogeny of the formation of polyp may had some relation to allergy, steroid hormone were adopted by Hartwig (1988), etc. in part or whole of body to decrease the recurrent rate. However in a long term perspective, this kind of medicine should be avoided for the patients suffered from hypertension, diabetes, ulcer of digestive treat and glaucoma. In present study, different levels of improve-ments were obtained in the 82 cases of nasal polyp group after one period of treatment.

The allergic reaction (fever) is among the common harmful reactions which occur in clinical application of PYM, with incidence of about 6.7% according to Shou Boquan (2008). We did not find this side effect in our handling. This might be because we used the dose of 40 mg in all period of treatment (>160 mg could cause lung fibrosis). Additionally, PYM can be used by intravenous injection, intra-artiricl perfusion and intramuscular injection without hemolysis and the damage of the adjacent tissues. A few amount of lidocaine is added to dramatically reduce the pain during injection which is able to be accepted by the aged patients.

In the current study, intralesional injection of Pingyangmycin seemed to be an effective, safe and inexpensive method. This treatment has no serious side effects and does not produce any scars. We now favor Pingyangmycin sclerotherapy for nasal polyp in all cases for those who cannot bear surgery in our hospital.

REFERENCES

DeMarcantonio MA, Han JK (2011). Nasal polyps: pathogenesis and treatment implications. Otolaryngol Clin. North Am. 44(3):685-95.

Hartwig S, Lindén M, Laurent C, Vargö AK(1988). Lindqvist N udesonide nasal spray as prophylactic treatment after polypectomy (a double blind clinical trial). J. Laryngol Otol. 102(2): 148-151.

Shou BQ, Shou WD, YANG Z(2008). Analysis of the side effects of intralesional injection of pingyangmycin in hemangiomas and vascular malformations. China J. Oral Maxillo Surg. 6(1):34-37.

Zheng JW, Zhou Q, Yang XJ, Wang YA, Fan Xd, Zhou GY, Zhang ZY, Suen JY(2010). Treatment guideline for hemangiomas and vascular malformations of the head and neck. Head. Neck. 32:1088-1098.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2285-2288, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.410
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Ephrin B3 (EphB3) receptors expressed in spinal tissue of rats with spinal cord injury

Xiaodong Zhi[1], Mingyan Dong[1], Desui Yu[1], Yansong Wang[1], Da Huang[2] and Gang Lv[1]*

[1]Liaoning Medical University, Jinzhou 121001, People's Republic of China.
[2]CNPG Central Hospital, Jinzhou 121002, People's Republic of China.

Accepted 26 July, 2012

Ephrin (Eph) receptors are the largest family of receptor tyrosine kinases (RTKs) and their membrane bound ligands, The Eph plays an important role in formation of spines with normal morpholog and synaptic plasticity. However, the Eph receptors expression in spinal tissue with spinal cord injury (SCI) was unclear to have little regarding molecular studies; thus, the present study aims to investigate changes from EphB3 receptors in spinal tissue of rats with injured spinal cord on mRNA level and protein level by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and western blot, respectively. It was found that EphB3 receptors were significantly up-regulated in SCI group in comparison to the control group on mRNA level. On protein level, EphB3 receptors were also highly increased in SCI group compared with control group. These results suggested that decreased EphB3 expression might also become a potential target in the treatment of SCI in future.

Key word: Spinal cord injury, Ephrin B3 (EphB3) receptors, qRT-PCR, Ephrin B (EphB), western blot.

INTRODUCTION

Traumatic spinal cord injury (SCI) is still a major clinical problem with permanent neurological deficits and secondary complications (Emine et al., 2012). It is characterized by a total or partial loss of motor and sensory functions due to the inability of neurons to regenerate. Inhibitory molecular cues of Eph/ephrins, was the best known and most intensively studied neurite outgrowth inhibitors (Irizarry-Ramírez et al., 2005; Silver and Miller, 2004). Previous studies suggest that Eph/ephrin molecules of both subclasses regulate bone homeostasis (Zhao et al., 2006; Allan et al., 2008; Irie et al., 2009). These membranes bound receptor tyrosine kinases (RTKs) predominantly function by inhibitory or repulsive cellular responses via cell-cell contact; where the responses can be mediated through the receptor (Cooke and Moens 2006; Mellitzer et al., 1999). Therefore, it is necessary to study Eph/ephrins and its receptors in neural regeneration field for control and treatment SCI.

The Eph RTK family is the largest RTK family known. Their main characteristic is their ability to mediate cell-cell repulsion after binding their ligand to an adjacent cell surface (Pasquale, 2008). They play prominent and well-described roles in the formation and function of excitatory synapses (Dalva et al., 2000; Henkemeyer et al., 2003; Kayser et al., 2006; Kayser et al., 2008; Murai et al., 2003).

The EphB3 receptor subtype is expressed during embryonic development and in discrete areas of the adult brain, including the cerebellum and hippocampus (Lixin et al., 2009). It co-localizes to brain regions with high levels of ephrin B (EphB) ligand expression (Klein, 2009), and its expression also increases following central nervous system injury (Liu et al., 2006), However, EphB3 receptor expression change was little report after SCI; thus, in this study, we evaluated the EphB3 receptor expression on protein and transcript level in spinal tissue of rats with injured spinal cord to clarify a reasonable value of EphB3 receptor in SCI.

*Corresponding author. E-mail:ganglv514@yahoo.com.cn. Tel: +86 0416 467 3886. Fax: +86 0416 467 3528.

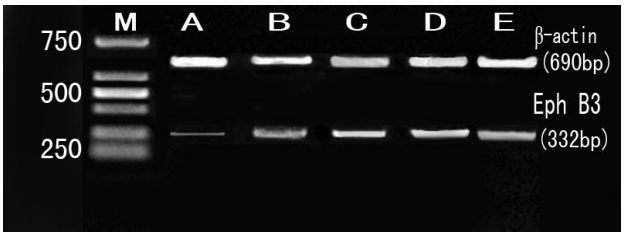


Figure 1. The mRNA expression of EphB3 receptors at different time points in rats from control group and SCI group. M, Marker; A, control group; B to E, experiment group after SCI at 1, 2, 4 and 8 weeks, respectively.

MATERIALS AND METHODS

Spinal cord injury

Adult female Sprague-Dawley (SD) rats (200 to 240 g) were obtained from Liaoning Medical University Laboratory (Liaoning, China). SD rats were randomly divided into control group (n = 8) and experiment group (n = 32) after observation of 2 weeks. Experiment group were anesthetized with a cocktail of 40 mg/kg ketamine, 4 mg/kg xylazine and 0.9 mg/kg acepromazine administered by intraperitoneal injection. A dorsal incision was made to expose T10 vertebra and a laminectomy was performed, leaving the spinal segment exposed. After exposure of the T10 segment by laminectomy, animals received a moderate contusion using the NYU impactor that provides a contusion of 12.5 g cm as previously described (Gruner, 1992; Miranda et al., 1999; Irizarry-Ramírez et al., 2005). Experiment group were randomly divided into 1, 2, 4 and 8 weeks group after SCI (n = 8 in each group). At the same time, control group was divided into four groups (n = 2 in each group) according to experiment group. Spine tissues were collected from rats in different time. All animal procedures were performed in accordance with the National Institutes of Health Animal Protection Guidelines and were approved by the Liaoning Medical University Committee on Animal Research.

RNA preparation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from frozen spinal tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol and as described in the online supplement. In short, the spinal tissue was ground with mortar and pestle cooled by liquid nitrogen of the ground tissue, 100 mg was incubated with 1 ml TRIzol for 5 min at room temperature (RT). Cell debris was removed by centrifugation (12,000 × g at 4°C for 10 min) and 0.4 ml chloroform was added. After vortexing, the mixture was incubated for 5 min at RT. The phases were separated by centrifugation (12,000 × g at 4°C for 15 min) and the aqueous phase was transferred to a new tube. 0.6 × volume of isopropyl alcohol and a 0.1 × volume of 3 M sodium acetate were added to this aqueous phase and incubated for 10 min at 4°C. The precipitated RNA was pelleted by centrifugation (12,000 × g at 4°C for 15 min) and after the removal of the supernatant, the RNA pellet was washed twice with 70% ethanol. After drying, the RNA was re-suspended in 30 µl diethylpyrocarbonate (DEPC)-treated water. The quality and quantity of the RNA was verified by the presence of two discrete electropherogram peaks corresponding to the 28S and 18S rRNA at a ratio approaching 2:1. Using mRNA as template, single-stranded cDNAs were generated by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's directions. The EphB3 receptor primer sequences were as follows: Sense prime: 5′- ACTCCTTTCTACGGCTCAAT-3′; Anti-sense prime: 5′- CTCCCACATGACAATCCC -3′. β-actin (Applied Biosystems) served as the internal control. The β-actin primer sequences were as follows: Sense prime: 5′-CTCCATCCTGGCCTCGCTGT-3′; Anti-sense prime: 5′-GCTGTCACCTTCACCGTTCC-3′. The PCR conditions were 94°C for 3 min, followed by 30 cycles of DNA amplification (30 s at 94°C, 30 s at 65°C, and 1 min at 72°C) and 5 min incubation at 72°C. PCR products were separated by electrophoresis at a constant voltage (2 V/cm) in a 1.2% (w/v) agarose gel. Images were captured using a Gel Print 2000i/VGA (Bio Image), and the integrated densities value (IDV) was analyzed with computerized image analysis system (Motic Images Advanced 3.2). All DNA manipulations were performed as described by Sambrook and Russell (2001).

Western blot

In order to detect protein expression level of EphB3 receptor, western blot was performed. The spinal cord tissues were obtained from the peri-lesion region at 1, 2, 4 and 8 weeks after SCI. Total protein was extracted from fresh spinal tissue using Tissue or Cell total protein extraction Kit (Shengong., Shanghai, China) according to the manufacturer's protocol and as described in the online supplement. Bradford protein assay method was used to determine the protein concentrations. Total protein extracted (20 µg) was boiled at 100°C with loading buffer for 5 min, and then subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were briefly equilibrated in transfer buffer, and then transferred onto nitrocellullose membranes. Transfer was performed at 70 V for 2 h at 4°C. After blocking in 5% Nonfat milk for 1 h, membranes were incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-EphB3 receptor, Santa Cruz, USA) in 0.5% blocking solution. After washing with phosphate-buffered saline (PBS), the membranes were incubated with secondary antibody (rabbit anti-goat, Qiangen, Germany) for 1 h at room temperature, washed again with PBS, took photos with film exposures for analysis. The purities of cytosolic fraction were confirmed by western blotting using anti-β-actin antibody as a loading control. Protein levels were quantitated by densitometry using Alphalmager Series 2200 software.

Statistics analysis

To calculate the statistical differences between the control and SCI group, the statistical package SPSS13.0 (SPSS Incorporated, Chicago) was used for all analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test were utilized to determine the significant difference among multiple groups. Student's *t*-test was used to determine the significance of differences between the groups. All values were expressed as mean ± SD. In general, p-values less than 0.05 were considered statistically significant.

RESULTS

Semi-quantitative RT-PCR analysis of EphB3 receptor expression

In order to detect the mRNA expression of EphB3 receptor in spinal tissue of rats with injured spinal cord, RT-PCR was conducted. As shown in Figure 1 and

Table 1. The integrated density value of EphB3 receptors on mRNA level and protein level at different time points in rats from different group after transplantation.

Group	mRNA (PCR)	Protein (Western)
Control	0.21 ± 0.02	1.01 ± 0.03
1 Week	1.22 ± 0.04$^{\Delta}$	4.96 ± 0.04$^{\Delta}$
2 Week	1.19 ± 0.04$^{\Delta}$	4.86 ± 0.06$^{\Delta}$
4 Week	1.25 ± 0.03$^{\Delta}$	4.98 ± 0.04$^{\Delta}$
8 Week	1.20 ± 0.04$^{\Delta}$	5.02 ± 0.05$^{\Delta}$

Different mark represent the significant difference at $p < 0.05$.

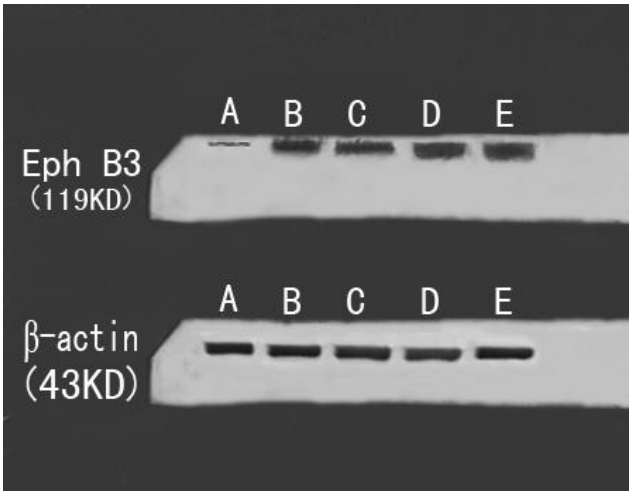


Figure 2. The protein expression of EphB3 receptors at different time points in rats from control group and SCI group. A, Control group; B to E, experiment group after SCI at 1, 2, 4 and 8 weeks, respectively.

Table 1, EphB3 receptor mRNA expression levels in SCI group were significantly increased at 1, 2, 4 and 8 weeks after post injury compared to control group ($P < 0.05$). However, there was no significant difference among the group after post injury in different time. These results showed that EphB3 receptor expression up-regulate on mRNA expression after SCI.

Western bolt analysis of EphB3 receptor expression

The western blot was performed to detect protein expression of EphB3 receptor after SCI. The result showed that EphB3 receptor expression was on a low level in the normal group rats (Figure 2). EphB3 receptor protein expression levels in each SCI group were significantly increased compared to control group ($P < 0.05$). However, there was no significant difference among the SCI groups (Table 1), which showed that EphB3 receptor had high expression on protein level for long time after SCI. These results comply with the results that EphB3 receptor expression increase on mRNA expression.

DISCUSSION

Eph RTKs and their ligands, Eph, have been implicated in the establishment of functional circuits, cell migration, and excitatory synapse regulation, actions mediated mainly by repulsive activity (Klein, 2009). The pattern of expression of Eph and its receptor is complex and reflects the diverse functions these proteins play in the developing and mature brain (Flenniken et al., 1996; Liebl et al., 2003; Migani et al., 2009; Mori et al., 1995). However, Eph RTKs and their ligands expression in spinal have little report; for these reason, we detect EphB3 receptor expression on mRNA level and protein level in spinal of normal rats and rats with SCI, and found that that EphB3 receptors was significantly up-regulated in SCI group in comparison to the control group on mRNA level and protein level, which demonstrated that EphB3 receptors might play a key role in SCI.

Previous studies showed that Eph RTKs and their ligands are important for the formation of spines with normal morphology (Irie and Yamaguchi, 2002; Margolis et al., 2010; Penzes et al., 2003; Tolias et al., 2007) and synaptic plasticity (Grunwald et al., 2001; Henderson et al., 2001). In present study, we found that EphB3 receptor had high expression on protein level and mRNA level for long time after SCI; therefore, we reckon that EphB3 receptor might involve in inhibition axis cylinder regenerates after SCI, which will depend on further study to prove hypothesis.

In conclusion, we have demonstrated that EphB3 receptors was significantly up-regulated in SCI in comparison to the normal spine tissue of rats, and EphB3 receptor had high expression on protein level and mRNA level for long time after SCI. The current study provides a new approach for studying the mechanism underlying the pathogenesis of SCI. This study suggests that decreased EphB3 receptor expression may be a potential target in the treatment of SCI.

ACKNOWLEDGEMENTS

This work was supported by Science and Technology Research and Innovation Team Fund of Liaoning provincial (LT2010062).

REFERENCES

Allan EH, Hausler KD, Wei T, Gooi JH, Quinn JM, Crimeen-Irwin B (2008). EphrinB2 regulation by PTH and PTHrP revealed by

molecular profiling in differentiating osteoblasts. J. Bone Miner. Res. 23:1170-1181.
Cooke JE, Moens CB (2002). Boundary formation in the hindbrain: Eph only it were simple. Trends Neurosci. 25:260-267.
Dalva MB, Takasu MA, Lin MZ, Greenberg ME(2000). EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. Cell, 103: 945-956.
Emine AK, Bulent B, Sebnem K, Kamer K (2012). Neuroprotective effects of racemic ketamine and (S)-ketamine on spinal cord injury in rat. Injury. (In press).
Flenniken AM, Gale NW, Yancopoulos GD, Wilkinson DG (1996). Distinct and overlapping expression patterns of ligands for Eph-related receptor tyrosine kinases during mouse embryogenesis. Dev. Biol. 179:382-401.
Grunwald IC, Korte M, Wolfer D, Klein R(2001). Kinase-independent requirement of EphB2 receptors in hippocampal synaptic plasticity. Neuron 32:1027-1040.
Henderson JT, Georgiou J, Jia Z (2001).The receptor tyrosine kinase EphB2 regulates NMDA dependent synaptic function. Neuron, 32: 1041-1056.
Henkemeyer M, Itkis OS, Ngo M (2003). Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus. J. Cell Biol. 163:1313-1326.
Irie F, Yamaguchi Y (2002). EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. Nat. Neurosci. 5: 1117-1118.
Irie N, Takada Y, Watanabe Y, Matsuzaki Y, Naruse C, Asano M (2009). Bidirectional signaling through ephrinA2-EphA2 enhances osteoclastogenesis and suppresses osteoblastogenesis. J. Biol. Chem. 284:14637-14644.
Irizarry-Ramírez M, Willson CA, Cruz-Orengo L, Figueroa J (2005).Upregulation of EphA3 receptor after spinal cord injury. J. Neurotrauma 22(8): 929-935.
Kayser MS, Nolt MJ, Dalva MB (2008). EphB receptors couple dendritic filopodia motility to synapse formation. Neuron. 59:56-69.
Kayser MS, McClelland AC, Hughes EG (2006). Intracellular and trans-synaptic regulation of glutamatergic synaptogenesis by EphB receptors. J. Neurosci. 26:12152-12164.
Klein R (2009). Bidirectional modulation of synaptic functions by Eph/ephrin signaling. Nat. Neurosci. 12(1):15-20.
Liebl DJ, Morris CJ, Henkemeyer M, Parada LF (2003). mRNA expression of ephrins and Eph receptor tyrosine kinases in the neonatal and adult mouse central nervous system. J. Neurosci. Res. 71:7-22.
Liu X, Hawkes E, Ishimaru T, Tran T (2006). EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. J. Neurosci. 26 (12):3087-3101.
Lixin Q, Sungwoon C, April C (2009).Structure–activity relationship study of EphB3 receptor tyrosine kinase inhibitors. Bioorg. Med. Chem. Lett. 19:6122-6126.
Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP Greenberg ME (2010). EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. Cell 143:442-455.
Mellitzer G, Xu Q, Wilkinson DG (1999). Eph receptors and ephrins restrict cell intermingling and communication. Nature 400:77-81.
Migani P, Bartlett C, Dunlop S, Beazley L, Rodger J (2009). Regional and cellular distribution of ephrin-B1 in adult mouse brain. Brain Res. 1247:50-61.
Mori T, Wanaka A, Taguchi A, Matsumoto K (1995).Differential expressions of the eph family of receptor tyrosine kinase genes (sek,elk, eck) in the developing nervous system of the mouse. Brain Res. 29:325-335
Murai KK, Nguyen LN, Irie F, Pasquale EB (2003). Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. Nat. Neurosci. 6:153-160.
Pasquale EB (2008). Eph-ephrin bidirectional signaling in physiology and disease. Cell, 133: 38-52.
Penzes P, Beeser A, Chernoff J, Schiller MR (2003). Rapid induction of dendritic spine morphogenesis by transsynaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. Neuron 37:263-274.
Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, pp.132-150.
Silver J, Miller JH (2004). Regeneration beyond the glial scar. Nat. Rev. Neurosci. 5:146-156.
Tolias KF, Bikoff JB, Kane CG, Tolias CS, Hu L, Greenberg ME (2007).The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptordependent dendritic spine development. Proc. Natl. Acad. Sci. U.S.A. 104:7265-7270.
Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T(2006). Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. Cell. Metab. 4:111-121.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2289-2298, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.430
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Service development in community pharmacies in Taiwan

Yafang Tsai[1, 5], Chien-Ying Lee[2, 6], Shih-Wang Wu[3*] and Shwn-Huey Shieh[4]

[1]Department of Health Policy and Management, Chung Shan Medical University, Taiwan.
[2]Institute of Medicine, Chung Shan Medical University, Taiwan.
[3]Department of Hospital and Health Care Administration, Chia Nan University of Pharmacy & Science, Taiwan.
[4]Department of Health Services Administration, China Medical University, Taiwan.
[5]Department of Medical Management, Chung Shan Medical University Hospital, Taiwan
[6]Department of pharmacy, Chung Shan Medical University Hospital, Taiwan.

Accepted 23 July, 2012

Facing changes in the medical environment, community pharmacies are seeking new strategies and developing new services in order to survive. In this two-phase study, we explored certain aspects of service development in community pharmacies. The first phase involved collection of qualitative data through interviews. In the second phase, a quantitative study was designed on the basis of the results of interviews conducted in the first phase, and sampling was conducted using a questionnaire. The results of the qualitative study showed that development of community pharmacies could enhance public health services and the pharmacy profession and facilitate a redesign of the service environment. In the quantitative study, over 64% of the respondents agreed that development of community pharmacies would enhance the pharmacy professional environment and provide flexible and diversified services. Another 95% of the respondents were able to accept the concept of self-care management systems that are based on public health services provided by community pharmacies. Regarding the redesign of the service environment of community pharmacies, 42% of the respondents reported that this is 'important', and 53% indicated that this is 'very important'.

Key words: Community pharmacy, pharmacy, healthcare policy, healthcare service, service science.

INTRODUCTION

The role of community pharmacy is focused on dispensing professional health advice and other services provided by community pharmacists and their assistants as they give out prescriptions (Goel et al., 1996). Pharmacies have a long history of serving as effective components of healthcare teams and have served an important function in the entire healthcare system. Pharmacies in both hospitals and communities have become important safeguards for the health and safety of the public (Smith, 2011; Paudyal et al., 2010). Members of the public find pharmacy to be a convenient setting and feel that pharmacists should provide public health services beyond dispensing medicine. Those that had experienced public health services in a community pharmacy, such as self-management interventions, were largely satisfied with their experience with these services (Bell, 2009).

This study evaluated the service development in community pharmacies in Taiwan. In recent years, the healthcare environment in Taiwan has undergone significant changes, including implementation of a national health insurance policy, delineation of medicine and pharmacy practices, and changes in pharmacy marketing orientation. These changes in the healthcare environment have led pharmacies in Taiwan to progress from traditional drug dispensaries to valued, integrated links in the healthcare industry chain.

Facing the changes in the healthcare environment, community pharmacies have sought new strategies and developed new markets in order to survive (Paudyal et

*Corresponding author. E-mail: avon611@gmail.com or scottwu101@gmail.com. Tel: 886-24730022/12127. Fax: 886-24730022/11714.

al., 2010). Development and invention are the two important concepts of innovation (De Bie et al., 2011). Recent breakthroughs in community pharmacy practices include health enhancement services provided to the customer. These health enhancement services include advice on healthy living and self-care, and involvement in health promotion campaigns (Bell, 2009). The changing requirements that lead pharmacists to provide health-enhancement services should be formalized and integrated into standard practice.

Community pharmacists have the opportunity to improve the healthcare of the population, particularly, for those in disadvantaged sectors of the society (both poor and rural populations) that do not have the resources to visit clinics (Basak et al., 2009). Community pharmacies must utilize the existing internal resource systems, update their knowledge, and enhance their competence to restructure their business models (Basak et al., 2009). In other words, it is imperative to implement the concept of innovation in healthcare services to improve the service quality of pharmaceutical care.

The promotion of healthy lifestyles is one of the core roles of pharmacists. Although, pharmacists have always had some involvement in health improvement; the focus on this aspect has greatly increased over recent years. Community pharmacy has a number of benefits as a setting for public health activities. In recent years, studies in the United Kingdom (McDonald et al., 2010; Silcock et al., 2004; Benson et al., 2009), South Africa (Williams, 2007), and Tanzania (Kamat and Nyato, 2010) emphasized that community pharmacists should improve their role within the larger team of healthcare professionals, moving away from the traditional view that the main role of a community pharmacist is in the sale of drugs. Nevertheless, in Asian countries, there is a lack of research on the development of community pharmacies. This study examined pharmacists in Taiwan in an effort to supplement the existing studies on Asian pharmacy practice.

Aim of the study

The purpose of this study was to explore service development in community pharmacies in Taiwan.

METHODOLOGY

Study subject

The operational format of community pharmacies in Taiwan can be classified into two types. From the perspective of the operator's characteristics, there are either pharmacies run by the pharmacists themselves (self-employed) or chain pharmacies. In Taiwan, a chain pharmacy system is usually operated by a firm that also allows individual pharmacists to join the system as a franchisee and operate a community pharmacy.

The pharmacy in the system can be either a chain operated by the franchiser or a franchised pharmacy operated by the pharmacist, with the pharmacist paying a franchise fee. From the perspective of the products they handle, there are pharmacies that handle only medicines approved by the Department of Health, Executive Yuan, Taiwan, community pharmacies that handle medicines and maternal and infant products (e.g. diapers, baby formula, etc.), and community pharmacies that handle medicines and cosmetic products (e.g. skin care products). As stipulated in the national health regulations, all pharmacists in Taiwan are professionals with a national license and their services focus on handling medicines approved by the Department of Health and filling doctors' prescriptions.

Research design

This research was divided into two phases. The first phase consisted of a qualitative study, which involved the collection of data through interviews. As a principle, the targeted sampling covered pharmacists from all types of pharmacies found in Taiwan, and their willingness to participate in the survey was considered. The sampling approach used is known as 'snowballing'. Using this approach, researchers first found a participating community pharmacist, and through his recommendations, obtained a list of other community pharmacists as potential study participants. The subjects, including two pharmacists employed by chain pharmacies, one self-employed pharmacist from a community pharmacy, a pharmacist employed at a community pharmacy, a pharmacy director from a medical centre, and a pharmacy director from a regional hospital, were interviewed in an effort to understand the opinions of pharmacists regarding service development in community pharmacies. The interviews were conducted from October 15 to October 23, 2009. Each interview lasted one to two hours and the interview questions included: 'What kind of problems do you think community pharmacy operations currently face'?, 'What innovative services do you think community pharmacies can develop'?, 'What other professional services do you think community pharmacists can provide other than filling prescriptions'?, and 'How can community pharmacies integrate themselves with healthcare service'?

In the second phase, a quantitative study was designed based on the results of the interviews conducted in the previous phase, and data were obtained using a structured questionnaire (Appendix). The questionnaire content was reviewed by two scholars from the Department of Health Service Administration and two community pharmacists. Questionnaires were distributed to the customers of the two community pharmacies described in the aforementioned qualitative survey. Thus, the goal was to understand public opinion of service development in community pharmacies from the viewpoint of pharmaceutical service recipients. The survey lasted from October 27, to December 9, 2009. Two hundred and sixty questionnaires were distributed, and 240 valid questionnaires were returned. The respondents were all residents of Taiwan visiting the case community pharmacies in this study, and participated in the survey voluntarily. In the process of conducting the survey, the respondents were first advised that the information they provided was to be used strictly for academic purposes, the research data would remain confidential, and it would be anonymized. Then, the respondents were asked if they would voluntarily take the survey and whether they had reached legal age. If they were willing to volunteer and had reached legal age, the questionnaire was distributed. A research assistant was available on site should the respondent have any questions about the survey.

Data analysis

Content analysis was employed for qualitative data analysis. With the consent of the interviewees, the interviews were recorded and \

the recordings were converted into verbatim transcripts. Content analysis was subsequently conducted on these verbatim transcripts. The first step was to identify and confirm specific keywords from among the following words: the service development and service innovation in community pharmacies. Then, three regularly paid research assistants from the Graduate Program of the Department of Health Service Administration, who were well trained in qualitative data analysis, coded the data on the basis of the verbatim transcript. Next, the coding results were compared, discussed, and modified for discrepancies. The coding process is complete only after the results received unanimous approval.

In the second phase, quantitative data were analysed using Statistical Package for Social Sciences (SPSS) 17.0, with descriptive statistics indicating the demographics of the sample. The items of the quantitative questionnaire included the following: 'I agree that the "self-care management" system is helpful in personal health management'; 'I agree to pay for the "self-care management" system'; 'the willingness to support hospital healthcare services referred by the community pharmacy'; and 'the design of the service venue' (Appendix). Likert-type five-point scales were used (1 = not very important and 5 = very important), and the Cronbach's α value of the design of the service venue scale was 0.821.

Ethical considerations

This survey was performed after obtaining agreement of the volunteering respondents. All responses were anonymous, and the respondents were not acquainted with the researchers. The researchers invited the respondents to participate in this research when distributing the questionnaires. They were also informed that all information was to be provided on a voluntary basis and would be used for research purposes only. Because names were not required on the data collection forms, privacy and anonymity were ensured. To guarantee the privacy of the respondents, the questionnaires were sealed after they were retrieved to keep their contents secure and anonymous.

RESULTS

Phase 1: Interview used in the qualitative study

The comprehensive study of community pharmacies through qualitative data analysis can lead to opportunities for service development. The development of community pharmacies can begin with an emphasis on professional service, enhanced public health services, a redesign of the service environment, and enhanced management of their relationship with the public.

Enhancement of professional services offered by pharmacies

Emphasis on the professional consulting services offered by pharmacists: In the traditional public view, the main role of a community pharmacist is to sell drugs. However, the professional image of the pharmacist has not typically been emphasized. Farris and Schopflocher (1999) proposed that pharmaceutical care should be based upon reviewing information in medication profiles and patient counselling. The future development of pharmacies should involve further enhancement of the professionalism of pharmacists, as indicated by a pharmacist who resigned from a community pharmacy (Pharmacist A) who said the following:

'To build the image of a professional pharmacy, we hope to put more effort in the field of medicines. We have not handled many cosmetics as we are not familiar with them'.

Provide convenient and flexible services to the public: Modern community pharmacies should provide the public with more convenient access to services, focusing on the variety of products offered. For example, community pharmacies should provide a chronic prescription medicine blending service and deliver medicine to customers in need via courier to eliminate the need for customers to travel. The service should be integrated to provide convenient methods for customers to pick up and receive products. A pharmacist in a chain pharmacy system (Pharmacist C) said the following:

'Our pharmacy is quite flexible, and we can even deliver the medicines to the public one week earlier'.

Enhancement of public health services

Cooperate with other healthcare professionals to promote public health and home care services: To implement the concept of community, and to comply with the public health policies of the National Health Department, community pharmacies can cooperate with other healthcare professionals (like public health nurses and family doctors) to promote home care services. For example, a community pharmacist can assist home care patients when they self-medicate, that is, when the patients have a question about the medication, they can consult the community pharmacist nearby. A pharmacist from a chain pharmacy (Pharmacist B) said the following:

'The government requires pharmacists to practice in hospitals; so pharmacists and students in pharmaceutical science will come to our community pharmacy for practical training every semester. Therefore, with this extra temporary manpower, I can provide a two-month home care service to the public in the community, and the students can learn while I conduct pharmaceutical consulting and instruction'.

Cooperate with other sectors to provide diversified services: Community pharmacists in the United Kingdom work in both professional and commercial settings. Their professional role involves dispensing prescribed medicines as well as advice about medicines to the public. The commercial role of pharmacists revolves around the sale of over-the-counter medicines and other

Table 1. Respondents' demographics.

Variable	Number	%
Gender (n = 240)		
Female	135	56.3
Male	104	43.3
Missing data	1	0.4
Occupation (n = 240)		
Self-employment	31	12.9
Agriculture	2	0.8
Commerce	45	18.8
Industry	22	9.2
Public servant	6	2.5
Academia	3	1.3
Student	72	30.0
Homemaker	19	7.9
Others	34	14.2
Missing data	6	2.5
Age (years, n = 240)		
18 - 20	14	5.8
21 - 30	134	55.8
31 - 40	48	20.0
41 - 50	32	13.3
51 - 60	8	3.3
>61	4	1.7

non-medical, but often health-related merchandise (Anderson, 2000). Just like the community pharmacists in the United Kingdom, some community pharmacists in Taiwan, in addition to providing consultation for the use of medicines, also provide additional services, such as beauty services. A pharmacist from a chain pharmacy (Pharmacist B) said the following:

'We have in-house beauticians to provide beauty services, which is one of our secondary services'.

Redesign of the service venue

Open shelf design to alleviate shopping pressure on the customers: A pharmacist believes that open shelves facilitate buying convenience for the customers, and that these open shelves can alleviate consumer pressure and make customers feel more at ease during over-the-counter (OTC) medicine shopping. The manager of a chain pharmacy (Pharmacist C) said the following:

'The open shelves in pharmacies will lessen pressure as the public can freely choose the products they need'.

Emphasis on window dressing and colour choices in the shop: Similar to department stores, the window dressing of a community pharmacy will influence the public's first impression of the pharmacy. A pharmacist of community pharmacy (Pharmacist A) said the following:

'A large shop window can attract more attention. The shopping windows of many pharmacies are filled with various advertisements, which appear quite messy. The window dressing of my pharmacy is an important part of my business strategy'.

Phase 2 (Quantitative study): Questionnaire investigation

Based on the qualitative data obtained from the interviews conducted in the first phase, we designed a quantitative study. Once the content of the questionnaire was determined, we invited pharmacists from the community pharmacies that were interviewed during the first phase to review the questionnaire. Table 1 shows the distribution of the respondents' demographics.

Enhancement of professional services

Williams (2007) proposed that pharmacies could benefit from a service-based philosophy, rather than the current product-based approach, which has a fee-for-product focus. This study found that besides having their prescriptions filled, there are 3 reasons for people to seek help from community pharmacies: nutrition consultation, health education, and simple health examinations. From investigation and analysis of the results of our questionnaire, and the qualitative data generated in the first phase of our study, we found that the subjects generally recognized that the development of community pharmacies should move towards professional consulting services, while the traditional service image that is limited to the sale of drugs should be phased out. Moreover, the service development should provide more flexible and diversified services, such as integration with public healthcare services in providing self-care management.

Enhancement of public health service

In the qualitative analysis of the data obtained in the first phase, we found that the subjects believed that the community pharmacy services can be developed, such as those related to public health (e.g. self-care management systems), by working in cooperation with other healthcare professionals, such as public health nurses or family doctors. For example, a community pharmacy could develop a 'Self-care Management Card', which includes a customer's health condition and medication records for the eyes, nose, oral cavity, ears,

Table 2. The frequency of agreement to the "self-care management" system.

Items/Number (%)	Yes	No	Missing data
I agree that the "self-care management" system is helpful in personal health management.	228 (95.0)	11 (4.6)	1(0.4)
I am interested in the "self-care management" system.	208 (86.7)	30 (12.5)	2(0.8)
I would recommend the "self-care management" system to my family.	210 (87.5)	28 (11.7)	2(0.8)

and skin. It would also be linked to a customer's family medication history, preference in medicine, and purchase record of health products. When a community pharmacist accesses this system, it can provide the pharmacist a comprehensive record of the customer's personal and family health record, which is accessible from other domestic community pharmacies or community pharmacies abroad.

In the quantitative study performed in the second phase, we investigated the opinions of the respondents on the combination of community pharmacies and public health to expand the concept of a self-care management system. In general, the respondents accepted the concept of self-care management systems proposed by community pharmacies. Among the respondents, 95.0% believed that this system would be helpful in personal health management, 86.7% were interested in this system, and 87.5% said that they would recommend this system to their family (Table 2).

Since Taiwan's state health insurance program does not cover the expenses associated with self-care management, this study examined the willingness of the respondents to bear the expenses of implementing a self-care management system. We found that, while 54.6% of the respondents were willing to pay for this system, 45.0% were unwilling. Furthermore, 52.5% of the respondents were willing to pay for it if the cost would be less than 1,000 NT dollars/month, while only 4.6% were willing to pay for it if the cost was between 1,001 to 1,500 NT dollars/month. In terms of the method of payment, 36.3% of the respondents agreed to make a one-time lump sum payment, 8.8% agreed to make a monthly payment, and 12.5% of the respondents agreed to make an annual payment (Table 3).

We asked the respondents regarding their support for hospital healthcare services referred by community pharmacies, and 87.9% of them were willing to avail from such services. In addition, we further investigated the customer's opinions on the medical care referral proposals provided by pharmacists from community pharmacies, and 84.6% of the respondents replied that they would accept these recommendations. These findings suggest that the respondents clearly trust the healthcare advice of pharmacists working in community pharmacies. In addition, we also asked respondents regarding a free hospital online registry service provided by community pharmacies, and 81.7% replied that they would accept such a service (Table 4). The free hospital online registry service allows community pharmacists to make appointments for their customers with hospital physicians through the internet, based on the respective conditions of their customers.

The aforementioned findings indicate the respondents' high rate of acceptance of supporting health care services provided by community pharmacies. Therefore, community pharmacies and hospitals should cooperate with each other in the future to become primary referral providers for health care services.

Redesign of the service venue

We also examined the respondent's opinions regarding eight features related to the design of services provided by community pharmacies. A rating of 5 indicated that a feature was very important to the respondent, and a rating of 1 indicated that it was not very important. The Cronbach's α value for this part of the questionnaire was 0.814. A Cronbach's α value higher than 0.6 is accepted as credible (Onwuegbuzie and Leech, 2009).

The respondents' opinions of the redesign of the service venue of community pharmacies showed that 53% of the respondents believed that this is very important, while 42% believed that this is important. With regard to the professional image of pharmacists (including wearing of pharmacist uniforms and badges), 48% of the respondents agreed that this is very important, and 41% agreed that this is important.

With regard to an independent consulting area for pharmacists, 45% of the respondents agreed that this is very important, and 43% agreed that this is important. Regarding a special medication record for individual customers in the community pharmacy, 46% of the respondents agreed that this is very important, and 41% agreed that it is important. With regard to the notion that a pharmacist should sit instead of stand during a consultation, 31% of the respondents agreed that this is very important, and 34% agreed that it is important.

When asked whether the consulting desk of the pharmacist should have a computer to allow rapid searching for medical information, 39% of the respondents agreed that this is very important, and 50% agreed that it is important. With regard to the notion that the lighting in the pharmacy should be appropriate, 32% of the respondents agreed that this is very important, and 44% agreed that this is important (Table 5).

Table 3. The payment willingness for the "self-care management system".

Item	Number (%)
I agree to pay for the "self-care management" system.	
Yes	131 (54.6)
No	108 (45.0)
Missing data	1 (0.4)
How much are you willing to pay for the "self-care management system"?	
<1000 NT dollars/month	126 (52.5)
1001 - 1500 NT dollars/month	11 (4.6)
1501 - 2000 NT dollars/month	0 (0.0)
>2000 NT dollars/month	0 (0.0)
Others	3 (1.3)
Missing data	100 (41.7)
How to pay for it?	
One lump sum payment	87 (36.3)
Once a month	21 (8.8)
Once a year	30 (12.5)
Missing data	102 (42.5)

Table 4. The willingness to support hospital health care services referred by the community pharmacy.

Item/Number (%)	Yes	No	Missing data
I would use hospital health care services referred by community pharmacies.	211 (87.9)	28 (11.7)	1 (0.4)
I would follow the referring suggestions by community pharmacies.	203 (84.6)	35 (14.6)	2 (0.8)
I wish there were a free hospital online registry service provided by community pharmacies.	196 (81.7)	44 (18.3)	0 (0)

According to the results of our study of the design of the service venue, the pharmaceutical service provider and the pharmaceutical service recipient had the same point of view, in that they both accepted that the medicines should be displayed on open-style shelves and that the lighting in the sales area should be appropriate to create a comfortable shopping environment. The attire of the pharmacist should exhibit a professional image, and the pharmacist should have a computer during consulting services to quickly obtain relevant medical information and the medication data of the customer.

DISCUSSION

As Taiwan's economy has grown rapidly over the past 30 years, its healthcare system has also shown an enormous transition from a centralized state-controlled system to a more market-based system (Ho and Gostin, 2009). To avoid the high cost of healthcare, many people, especially the uninsured, chose self-medication as their first option when they encounter a need for health care (Zhao et al., 2008; Peng et al., 2010). The principles of self-care, which can be applied to prevent and manage illness, are known to have arisen from a number of theoretical models such as the theory of self-regulation. Self-regulation models emphasize the importance of self-efficacy, which relates to an individual's belief in their ability to learn and perform specific behaviours, and self-management (Lorig and Holman, 2003), which relates to the practical adoption of such behaviours.

This study found that 95% of respondents agreed that future community pharmacies could link with public health practices and provide healthcare services like a 'self-care management' system. Silcock et al. (2004) proposed that community pharmacists mainly provide prescription-dispensing services and give OTC healthcare advice, thereby, becoming the primary channel of public access to medication. A community pharmacy is not only the frontline for the community to access public health care services, but is also the final perimeter for medication safety and quality, which prevents the incorrect use and misuse of drugs and prevents other medication hazards. The community pharmacy offers the general public access to healthcare professionals and medicines without appointment, and thus, it is a key focus area for promoting self-care of minor ailments (Paudyal et al., 2010). Bell (2009) suggested that the community

Table 4. The willingness to support hospital health care services referred by the community pharmacy.

Item/Number (%)	Yes	No	Missing data
I would use hospital health care services referred by community pharmacies.	211 (87.9)	28 (11.7)	1 (0.4)
I would follow the referring suggestions by community pharmacies.	203 (84.6)	35 (14.6)	2 (0.8)
I wish there were a free hospital online registry service provided by community pharmacies.	196 (81.7)	44 (18.3)	0 (0)

pharmacist must also provide healthcare services (Brahm et al., 2003) in addition to performing the professional duties of a pharmacist. He believed that future development for community pharmacies should focus on (1) public hygiene and health, and (2) the promotion of a community self-healthcare system. Bush et al. (2009) proposed the following examples of public health roles for community pharmacists: providing advice on self-care, supporting customers with chronic illness, maintaining customer medication records, promoting public medication awareness, and providing after-hours services, etc.

Although, there is a clear potential for pharmacies to contribute in a unique way to public health; changes in the behaviour of both pharmacists and the public are likely to be required for innovative services to be successful. Community pharmacists must accept their role in public health and make the necessary behavioural changes to carry out the service more effectively. Similarly, the general public must accept pharmacists as providers of public health services and be willing to seek advice on some health issues from pharmacists rather than other sources (Eades et al., 2011). Even though consumers are generally satisfied with the health advice given by pharmacists, they primarily use pharmacies for dispensing prescriptions and buying OTC medication. As a consequence, to facilitate the necessary behavioural changes among consumers, pharmacists need to continuously enhance their professional knowledge through further education and training. Training has been found to positively affect pharmacists' attitudes and behaviours in relation to health promotion (Eades et al., 2011). For example, some studies reported that pharmacists felt that lack of training or lack of knowledge and skills was a barrier to their ability to provide smoking cessation services (Hudmon et al., 2006). Similarly, over 70% of pharmacists who responded to a survey in Scotland reported that they would like to receive further training on drug misuse (Iversen et al., 2001). Over half of the pharmacists in another study conducted in Scotland reported that attaining additional pharmaceutical knowledge in public health was a priority for their practice now, and two-thirds thought it would be a priority in the future (Pfleger et al., 2008). To facilitate the public's identification and reliance on the health services provided by community pharmacists, it is imperative to strengthen community pharmacists' knowledge and training in public health issues.

This study found that 84.6% of the respondents agreed that community pharmacists should develop a consulting service that provides public healthcare-related knowledge. Pharmacy, like other healthcare professions, is both a knowledge-based and a value-based profession (Benson et al., 2009). Pharmacists can contribute their therapeutic knowledge and medication process expertise to create collaborative and innovative solutions to address medication use and safety problems (Smith, 2011). The healthcare team in a hospital is comparatively more comprehensive than that in a community pharmacy. A community pharmacist does not enjoy the education and training opportunities provided by the hospital to its healthcare teams. To ensure that community pharmacists maintain adequate and sufficient professional knowledge, it is recommended that the FDA, Taiwan Pharmacists Group, or Taiwan Pharmacists Society should provide pharmacists' healthcare knowledge.

Another finding of this study was the fact that 87.9% of respondents were willing to make use of the integrated healthcare services provided by hospitals and community pharmacies. Sultan (2009) believed that the success of community pharmacies in England is due to the following reasons: (1) community pharmacies were integrated into the community care team; (2) community pharmacies maintained their core advantages while providing other services and healthcare assistance; (3) community pharmacies provided customer-oriented services; (4) community pharmacies were willing to take on new challenges; and (5) the promotion of the role of community pharmacies in primary health care enabled them to become healthy living centres in communities, which allowed the residents in communities to learn correct and proper self-healthcare practices. Both the public and scholars from Taiwan and the United Kingdom agreed that community pharmacists could provide healthcare consultation services. We can infer then that the professionalism of community pharmacists with regard to healthcare advice and consultation is recognized by the public.

In our study, 81.7% of respondents believed that community pharmacists could assist the public by providing online hospital registration. This service can help the public make hospital appointments more efficiently, and thus, reduce the waiting time at the hospital. A few small-scale studies have demonstrated cost savings resulting from the health care-related services provided by pharmacists. These economic

Table 5. Design of the service venue.

Item/Number (%)	Very important	Important	General	Not very important	Not important	Missing data
The product display location in community pharmacies should be simple and clean.	126 (53)	100 (42)	13 (5)	1 (0)	0 (0)	0 (0)
The professional image of the pharmacist (including pharmacist uniforms and badges).	114 (48)	99 (41)	23 (10)	3 (1)	1 (0)	0 (0)
There is an independent consulting area for the pharmacist.	109 (45)	102 (43)	26 (11)	1 (0)	1 (0)	1 (0)
There is a special medication record for public.	111 (46)	98 (41)	29 (12)	1 (0)	1 (0)	0 (0)
A pharmacist should sit instead of stand during the consulting service.	74 (31)	81 (34)	67 (28)	15 (6)	3 (1)	0 (0)
The consulting desk of the pharmacist should have a computer to allow the pharmacist to quickly search for medical information.	94 (39)	121 (50)	23 (10)	2 (1)	0 (0)	0 (0)
The lighting in the pharmacy should be appropriate.	76 (32)	106 (44)	48 (20)	10 (4)	0 (0)	0 (0)

Cronbach's

savings largely result from the lower operating cost of pharmacies as opposed to general practitioner (GP) consultations (Bojke et al., 2004; Sewak, 2010). In addition, such services have also shown to be effective in reducing GP workload, especially in treating minor ailments (Bojke et al., 2004). However, overall GP workload was shown to be unaffected, mainly due to the reallocation of GP time from minor ailments to other illnesses (Bojke et al., 2004). Pharmacy management would reduce patient waiting time at the GP's office and increase patient access to their services.

Conclusion

Whether considering the perspectives of service providers or those of consumers of different countries or cultures, the future service development in community pharmacies should undoubtedly focus not just on strengthening professional medical care services, but also on integration with hospitals in promoting public healthcare. In addition to committing to strengthening the professional image of community pharmacists, community health wellness should be integrated with public healthcare to increase pharmacists' service value to the public (Benson et al., 2009). Appropriate training and support is needed in order to increase pharmacists' confidence in providing public health services. Future research should explore the effectiveness of strategies that increase pharmacists' confidence and enhance their public health practice.

This study's objective was to determine the positions of both pharmaceutical service providers and consumers in Taiwan, and to understand their views regarding service development in community pharmacies. Our results were identical to those obtained in the United Kigdom and South Africa, with the conclusion that future developments for community pharmacists should focus on strengthening their professional pharmacy services.

Furthermore, they should also play a role in promoting community health and wellness. In other words, community pharmacies' promotion of health and wellness must be integrated with the public health system. In order to continue to improve the public health service provided in community pharmacies, up-to-date information regarding the beliefs and attitudes of pharmacists and consumers towards pharmaceutical public health is needed. Community pharmacists opportunities for continuous education or training to enrich and update community.

LIMITATIONS AND SUGGESTIONS FOR FUTURE STUDIES

For qualitative data collection in this study, we employed a snowballing sampling method. The researchers first located a pharmacist in a community pharmacy, and through his recommendations, obtained a list of potential pharmacists for participation in the survey. Though, the surveyors selected community pharmacists from various types of operations to obtain more extensive perspectives, only six pharmacists were interviewed. As a consequence, the extrapolation of the research results may be limited. In the quantitative data collection, the survey subjects were the customers of the two community pharmacies mentioned in the qualitative study. The total number of valid

samples was only 240, which also limits the extrapolation of the results. Considering the interviewees' willingness in participating in the survey, the questionnaire was designed to be very concise and did not pursue in-depth questions like the public's need for innovation in community pharmacy service. It is suggested that in future studies, besides improving sampling design, the depth of the questionnaire content should be increased. This study found that both the public and community pharmacists agreed that the future development of a pharmacy could be integrated with public health systems. Thus, it is suggested that future studies look into the linking of these two issues.

ACKNOWLEDGEMENT

The authors would like to thank Orchid Mei-Hwa Chen who helped edit the manuscript.

REFERENCES

Anderson C (2000). Health promotion in community pharmacy: the UK situation. Patient Educ. Couns. 39:285-291.

Basak SC, van Mil JW, Sathyanarayana D (2009). The changing roles of pharmacists in community pharmacies: perception of reality in India. Pharm. World Sci. 31:612-618.

Bell JA (2009). Promoting community pharmacy through public health initiatives. International Conference on Community Pharmacy Service-The Role of the Pharmacist in Improving Patient Care and Public Health, Taiwan.

Benson A, Cribb A, Barber N (2009). Understanding pharmacists' values: a qualitative study of ideals and dilemmas in UK pharmacy practice. Social Sci. Med. 68:2223-2230.

Bojke C, Gravelle H, Hassell K, Whittington Z (2004). Increasing patient choice in primary care: the management of minor ailments. Health Econ. 13:73-86.

Brahm NC, Palmer T, Williams T, Clancy G (2003). Bedlam Community Health Clinic: a collaborative interdisciplinary health care service for the medically indigent. J. Am. Pharm. Assoc. 47:398-403.

Bush J, Langley CA, Wilson KA (2009). The corporatization of community pharmacy: implications for service provision, the public health function, and pharmacy's claims to professional status in the United Kingdom. Res. Social Adm. Pharm. 5:305-318.

De Bie J, Kijlstra NB, Daemen BJ, Bouvy ML (2011). The development of quality indicators for community pharmacy care. BMJ Qual Saf. 20:666-671.

Eades CE, Ferguson JS, O'Carroll RE (2011) Public health in community pharmacy: a systematic review of pharmacist and consumer views. BMC Public Health 11:582.

Farris KB, Schopflocher DP (1999). Between intention and behavior: an application of community pharmacists' assessment of pharmaceutical care. Soc. Sci. Med. 49:55-66.

Goel P, Ross-Degnan D, Berman P, Soumerai S (1996). Retail pharmacies in developing countries: a behavior and intervention framework. Soc Sci Med. 42:1155-1161.

Ho CS, Gostin LO (2009). The social face of economic growth: China's health system in transition. JAMA. 301:1809-1811.

Hudmon KS, Prokhorov AV, Corelli RL (2006). Tobacco cessation counseling: pharmacists' opinions and practices. Patient Educ. Couns. 61:152-160.

Iversen L, Mollison J, MacLeod TN (2001). Attitudes of the general public to the expanding role of community pharmacists: a pilot study. Fam Pract. 18:534-536.

Kamat VR, Nyato DJ (2010). Soft targets or partners in health? Retail pharmacies and their role in Tanzania's malaria control program. Soc Sci Med. 71:626-633.

Lorig KR, Holman H (2003). Self-management education: history, definition, outcomes, and mechanisms. Ann. Behav. Med. 26:1-7.

McDonald R, Cheraghi-Sohi S, Sanders C, Ashcroft D (2010). Professional status in a changing world: The case of medicines use reviews in English community pharmacy. Soc. Sci. Med. 71:451-458.

Onwuegbuzie AJ, Leech NL (2009). Taking the "Q" out of research: Teaching Research methodology courses without the divide between quantitative and qualitative paradigms. Qual. Quant. 39:267- 296.

Paudyal V, Hansford D, Scott Cunningham IT, Stewart D (2010). Cross-sectional survey of community pharmacists' views of the electronic Minor Ailment Service in Scotland. Int. J. Pharm. Pract. 18:194-201.

Peng Y, Chang W, Zhou H, Hu H, Liang W (2010). Factors associated with health-seeking behavior among migrant workers in Beijing, China. BMC Health Serv. Res. 10:69.

Pfleger DE, McHattie LW, Diack HL, McCaig DJ, Stewart DC (2008). Views, attitudes and self-assessed training needs of Scottish community pharmacists to public health practice and competence. Pharm. World Sci. 30:801- 809.

Sewak NS (2010). Community pharmacy. Costs of minor ailment schemes. BMJ. 340: c2865.

Silcock J, Raynor DK, Petty D (2004). The organisation and development of primary care pharmacy in the United Kingdom. Health Policy. 67:207-214.

Smith MA (2011). Innovation in health care: a call to action. Ann Pharmacother. 45:1157-1159.

Sultan D (2009). Pharmaceutical care and clinical services in UK community pharmacies. International conference on community pharmacy service: The role of the pharmacist in improving patient care and public health, Taiwan.

Williams KF (2007). Re-examining 'professionalism' in pharmacy: a South African perspective. Soc. Sci. Med. 64:1285-1296.

Zhao G, Detels R, Gu F, Li D, Li X, Li Y (2008). The distribution of people seeking STD services in the various types of health care facilities in Chao Yang District, Beijing, China. Sex Transm. Dis. 35:65- 67.

APPENDIX

1. Imagine that your community pharmacy develops a 'Self-care Management Card', which includes your records of your health condition and medication for eyes, nose, oral cavity, ears, and skin. It also links to your family medication history, preference in medicine, and purchase record of health products. The database provides your community pharmacist access to a comprehensive record of you and your family's health, which is accessible from other domestic community pharmacies or overseas community pharmacies. Please answer the following questions:

(a) Do you agree that the 'self-care management' system is helpful for personal health management?
□Yes □No
(b) Are you interested in the 'self-care management' system?
□Yes □No
(c) Would you recommend the 'self-care management' system to your family?
□Yes □No
2. Would you pay for the 'self-care management' system?
□No □Yes (If yes, go to item 3.)
3. How much are you willing to pay for the 'self-care management'?
□<1000 NT dollars/month □1001–1500 NT dollars/month □1501–2000 NT dollars/month □>2000 NT dollars/month
4. How would you pay for it?
□Lump sum payment □Once a month □Once a year
5. The following questions pertain to your willingness to support hospital health care services referred by the community pharmacy:

(a) Would you use the hospital health care services referred by community pharmacies?
□Yes □No
(b) Would you follow the referral suggestions given by community pharmacies?
□Yes □No
(c) Should community pharmacies provide a free hospital online registry service?
□Yes □No
6. The following statements pertain to the design of the service venue. Please rank their importance.

(a) The product display locations in community pharmacies should be simple and clean.
□Very important □Important □General □Not very important □Not important
(b) Pharmacists' attire should portray a professional image (i.e. pharmacist uniforms, badges, etc.).
□Very important □Important □General □Not very important □Not important
(c) There should be an independent consulting area for the pharmacist.
□Very important □Important □General □Not very important □Not important
(d) There should be special medication records for all customers.
□Very important □Important □General □Not very important □Not important
(e) The pharmacist should be sitting instead of standing during a consultation.
□Very important □Important □General □Not very important □Not important
(f) The consulting desk of the pharmacist should have a computer to allow the pharmacist to quickly search for medical information.
□Very important □Important □General □Not very important □Not important
(g) The lighting in the pharmacy should be appropriate.
□Very important □Important □General □Not very important □Not important

Demographics

1. Gender: □Female □Male
2. Occupation: □Self-employment □Agriculture □Commerce □Industry
□Public servant □Academia □Student □Homemaker □Others
3. Age: □18–20 years □21–30 years □31–40 years □41–50 years □51–60 years □>61 years

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2299-2308, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.446
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Authentication of herbal drug Senna (*Cassia angustifolia* Vahl.): A village pharmacy for Indo-Pak Subcontinent

Shazia Sultana[1]*, Mushtaq Ahmad[1], Muhammad Zafar[1], Mir Ajab Khan[1] and Muhammad Arshad[2]

[1]Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan.
[2]Department of Botany, PMAS-Arid Agriculture University, Rawalpindi, Pakistan.

Accepted 30 July, 2012

The present study deals with the taxonomic and pharmacognostic authentication of the herbal medicine Senna (*Cassia angustifolia* Vahl.). This plant of considerable pharmaceutical importance, has been recommended for constipation, piles, epilepsy, respiratory diseases, skin infections, migraine and heart diseases. No studies are available on the micro, Scanning Electron Microscopy (SEM), pharmacognostic and taxonomic analysis of traded parts of *C. angustifolia* and its substitute *Cassia obtusifolia*. Hence, the present study was undertaken to authenticate genuine source of herbal drug Senna based on microscopic palynomorph, anatomical features, behavior of powdered drug with different chemical reagents and fluorescence analysis. The *C. angustifolia* is a perennial herb up to 80 cm tall with tricolporate pollen, foveolate sculpturing, paracytic stomata and single celled non-glandular trichome. It is differentiated from its substitute, *C. obtusifolia*, which is a perennial shrub up to 3.5 m tall having pollen with widely minute holes on surface, anomocytic stomata and elongated trichomes with pointed tips. The powdered drug of *C. angustifolia* is pale greenish in color, while its adulterant *C. obtusifolia* is dark green. It was concluded from this study that the knowledge of morpho-palynological, anatomical and pharmacognostical analysis may lead to authentication of herbal drugs like Senna for the purpose of employment in quality assurance of pharmaceutical products globally.

Key words: Senna, authentication, herbal drug, pharmacognosy, scanning electron microscopy (SEM).

INTRODUCTON

It is evident from the literature that 80% of the world's population is dependent on herbal drugs. Meanwhile, the limitation encountered in the use and research of herbal medicine is the lack of authentication, standardization, quality and purity of raw material. The need for in-depth and systematic investigations into indigenous drugs use cannot be overemphasized. Authenticity, purity and assay are important aspects of standardization and quality control. Adulteration of botanical medicine is an important hindrance to quality control and standardization (Shinde et al., 2009). Due to over exploitation of certain plants, habitat loss and collection for medicinal purposes, plants species are endangered or rare. These and many other factors like cost of the raw material, may cause the problem of availability of genuine drug, which encourages the adulteration of plant by substitution with inferior commercial varieties; cheaper plant are closely related vegetative parts (Sultana et al., 2011). In spite of botanical authentication, there is confusion with respect to some traditional herbal drugs like Senna, which is intentionally adulterated by its closely related species, *Cassia obtusifolia.*

*Corresponding author. E-mail: awancancer@yahoo.com.

Senna is widely used for its numerous benefits. Scientifically, it is known as *Cassia angustifolia,* and belongs to family Leguminosae. There are a number of species of Senna used throughout the world for medicinal purposes. Linnaeus classified these under single species of *Cassia senna* L. Since then, there have been a number of studies which indicate that there are different variants of senna. However, the two most widely used species for medicinal purposes are *Cassia acutifolia* Delile., also known as *Alexandrian senna* and *C. angustifolia* Vabl., also known as Tinnevelly senna or Arabian senna (Khan et al., 2011). Herbal practitioners in Indo-Pak subcontinent, Arabian countries and oriental physicians prefer the use of *C. angustifolia* as herbal drug Senna, while at herbal shops in Arabian countries, India, Pakistan and other South Asian countries, there is an adulteration of broken aerial parts of closely related species *C. obtusifolia.*

C. angustifolia was first discovered growing wild in and around the ancient and blessed city of Makkah, in the heart of the old province Hijaz. The plant grows in abundance and was first used as herbal medicine by the Holy Prophet Muhammad (Peace Be Upon Him) (Ahmad et al, 2010). At herbal shops in India, Pakistan and Arabian countries, it is traded under the name of Senna or sana makkahi, and is considered to be a remedy as a cleanser of the digestive system and tonic for the entire body. The Holy Prophet Muhammad (Peace Be Upon Him) said; “If there is any remedy against death, it is Sana, the gladdened, the graceful one” (Al-Suyui, 1985). Nowadays, Senna (*C. angustifolia*) is distributed worldwide, especially in Pakistan, India, Arabian countries, Sudan, China, Kenya, Europe, Britain, etc. Senna is widely used in traditional medicine of China, Indo-Pakistan, Africa, and is also employed in Western Allopathic System of medicine (Dymock, 1972). *C. angustifolia* is commonly used for digestive disorders, constipation, tonic, depression, asthma, eczema and other skin diseases. However, due to its extensive use and trade, this herbal drug is intensively adulterated by *C. obtusifolia* and other allied species.

The ultimate objective of the present study was to use taxonomic and pharmacognostic techniques for the authentication of genuine crude drug Senna from its adulterant. Hence, the present attempt has been undertaken to authenticate genuine source of herbal drug Senna (*C. angustifolia*) based on microscopic palynomorph, anatomical features, behavior of powdered drug with different chemical reagents and fluorescence analysis.

METHODOLOGY

The traded part of Senna was procured from different herbal shops of Indo-Pak subcontinent. The fresh plant specimens of both *C. angustifolia* and *C. obtusifolia* were collected from different parts of the country and authenticated by a plant taxonomist, Prof. Dr. Mir Ajab Khan, at the herbarium of Pakistan (ISL), Quaid-i-Azam University Islamabad.

Morpho-palynological Study

Morphological investigation was based on macro and microscopic features of plant, habit, root, stem, leaf, flower and seed using binocular light microscope (Model SZF Kyowa, Japan). Morphological description was further reconfirmed through various Floras (Saldanha and Nicolson, 1976; Nasir and Ali, 1974, 1975; Hooker, 1875; Tutin and Heywood, 1972; Hooker, 1885a, 1894). For palynological studies, standard procedure of acetolysis (Erdtman, 1960; Ahmad et al., 2011) was used. For scanning electron micrographs, the methodology of Zafar et al. (2011) was adapted. Palynological description was characterized using the terminology of various authors (Barthlott, 1984; Erdtman, 1960; Ronald, 2000).

Leaf epidermal anatomy (LM and SEM)

For leaf epidermal anatomy, the modified method of Ahmad et al. (2011) was followed. The peelings of leaves were prepared for qualitative and quantitative features of adaxial and abaxial surfaces by using light microscope (Meiji-Japan). The SEM and LM microphotographs were taken using JEOL-JSM (5910) and Leica (DM-1000) light microscopes, respectively. The qualitative characteristics of the leaf epidermal anatomy was described according to Prat (1932) and Metcalfe (1960) terminology.

Pharmacognostic studies

Different pharmacognostic tests such as the fluorescence and solubility test (cold and hot) were carried out for crude herbal parts of *C. angustifolia* and its adulterant *C. obtusifolia.* For the cold method, 2 g of powdered drug was mixed in 10 ml of solvent at room temperature (25 - 30°C), while for hot method the same solution was slightly heated on a burner in a test tube. The methods of Harborne (1973), Trease and Evans (1989) and Sofowora (1993) were followed. All the reagents were of analytical grade and of Merck (Germany). For solubility and fluorescence analysis, standard procedures were adopted (Afaq et al., 1998; Abid et al., 2005). Crude herbal parts, powdered drugs and the extracts were studied under visible light ultraviolet (UV; long and short wavelength) following the procedure of Ahmad et al. (2010). For color analysis, a paint chip card from Indigo Company (Pakistan) was used for comparison.

RESULTS AND DISCUSSION

C. angustifolia sold at herbal shops under the trade name of senna or sana makki is a branched erect perennial shrub used medicinally throughout the world (Anonymous, 1992). It is a small under shrub up to 1 or 1.5 m height with variable branches and with compound pinnate leaves (Figure 1A) This species can be distinguished morphologically from its adulterant allied species *C. obtusifolia*, which is a tall shrub up to 2 or 2.5 m in height with obtuse or elliptic leaves (Figure 2A). Srivastava et al. (2006) distinguished *C. angustifolia* by

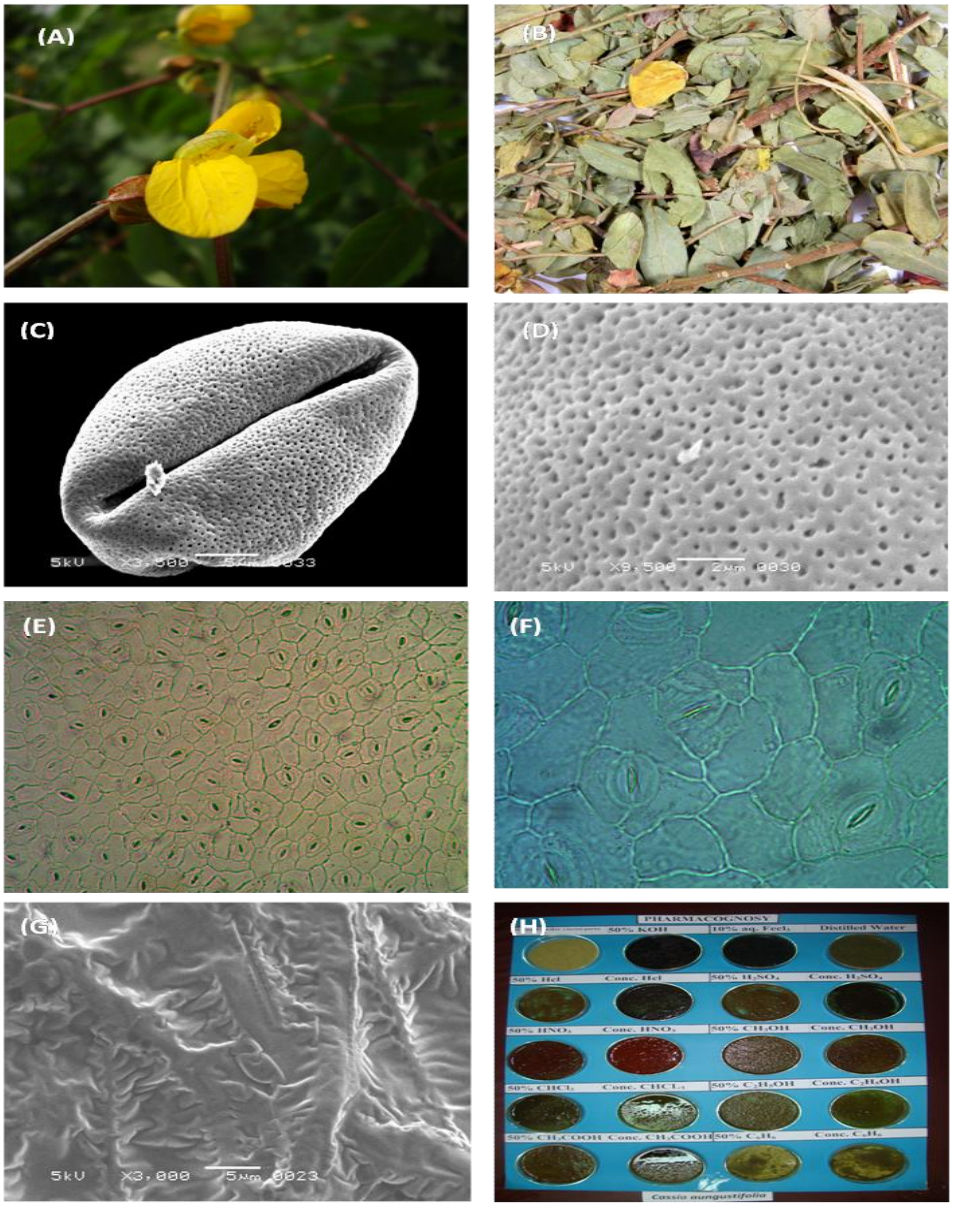


Figure 1. A *Cassia angustifolia;* (B) dried aerial parts; (C) equatorial view of pollen (SEM); (D) pollen sculpturing (SEM); (E) stomata and epidermal cells (abaxial : LM 40X); (F) stomata and epidermal cells (adaxial : LM-40X); (G) trichome and epidermal cells (SEM); (H) pharmacognostic flow chart.

the presence of greenish brown or dark brown pod of 3 – 9 cm in size, while its adulterant *C. obtusifolia* have greenish pod, which is 10 - 15 cm long. From palynological point, pollen of the *C. angustifolia* are tricolporate, which are spherical to subprolate in shape and having polar diameter 30 µM and equatorial 27.5 µM with faveolate sculpturing (Figure 1C and D) and can be distinguished from its adulterant *C. obtusifolia* by the presence of minute holes on pollen. While in *C. obtusifolia*, the pollen are subangular to spheroidal shaped, having polar and equatorial diameter of 29.5 and 15.25 µM, respectively, with widely distributed minute holes on pollen surface (Figure 2C and D).

Similarly based on foliar epidermal anatomy at microscopic level, the *C. angustifolia* can be distinguished from *C. obtusifolia* by the presence of smooth walled polyhedral epidermal cells with paracytic stomata and conical unicellular trichomes (Figure 1E to G), while *C. obtusifolia* have irregular epidermal cells with diacytic stomata and flat trichome (Figure 2E to G). The

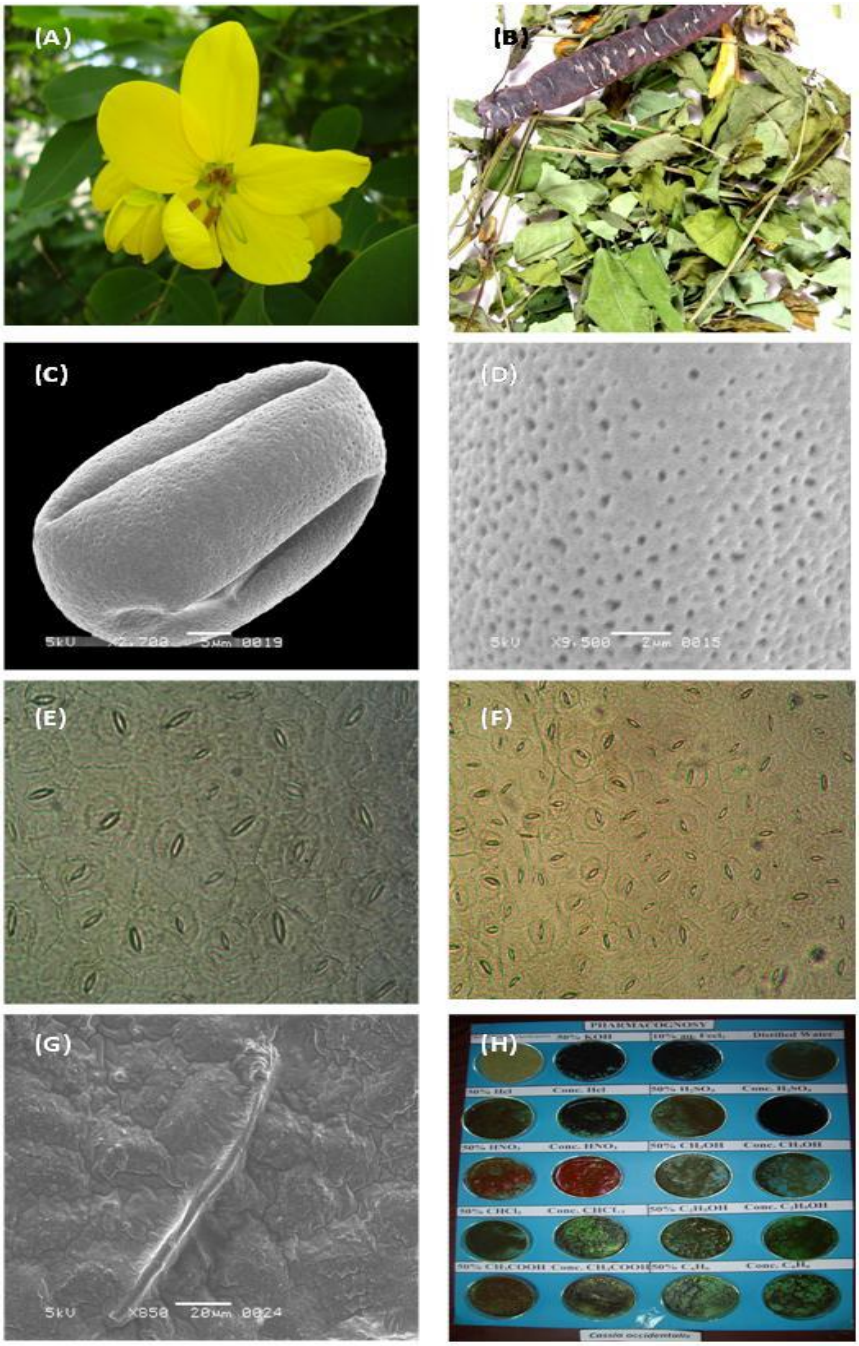


Figure 2. (A) *Cassia obtusifolia*; (B) dried aerial parts; (C) equatorial view of pollen (SEM); (D) pollen sculpturing (SEM); (E) stomata and epidermal cells (abaxial: LM 40X); (F) stomata and epidermal cells (adaxial: LM-40X); (G) trichome (SEM); (H) pharmacognostic flow chart.

results of preliminary phytochemical tests for the presence or absence of active constituents is reported herein. The behavior of powdered drug on treatment with different chemical reagents and the fluorescence analysis under ultraviolet and visible day light are shown in Tables 1 to 5. Powder drug analysis with various chemical reagents also showed positive results for secondary metabolites. Drug powder of *C. angustifolia* is pale

Table 1. Comparative characterization for differentiation of *Cassia angustifolia* and *Cassia obtusifolia*.

No.	Characters	***Cassia angustifolia* Vahl**.	***Cassia obtusifolia* L.**
01	Nomenclature	English Names : Tinne velly Senna, Indian Senna Local Names : Senna, Sana, Sanna Makki Trade Names: Senna, Sanna Makki	English Name : Coffee Weed, Sicklepod Local Name : Chota Amaltas Trade Name : None
02	Geographic distribution	In Pakistan; Punjab and Sindh province. In World; Pakistan (Punjab, Sind); India (Bombay, Madras); Algeria; Libya; Egypt, Sudan, Saudi Arabia. It has also been found in Kenya and Mozambique.	In Pakistan; Chakwal, Gujrat, Salt Range. In World; Central and South America.
03	Occurrence and habitat	In Pakistan, it is cultivated but very rare.	It is commonly cultivated along roads, garden and house lawns.
04	Morphology	Perennial, 60-80 cm tall, branches glabrous to subglabrous, stipules lateral, 1.6 mm long, leaf paripinnate, 5.5-10.5 cm long, Leaflets 5-9 pairs, lamina 1.2-4.5 cm long, 3.5-10 mm wide, glabrous hairy on both sides, lanceolate to ovate, tip acute. Inflorescence terminal or axillary raceme, up to 15 cm long. Young flowers with 8-9 mm long cup-shaped bracts. Pedicel 3-4 cm long. Sepals 5, subequal, 10-13 mm long, 6-9 mm broad, spoon shaped or cup shaped, light yellow in color. Petals 5, subequal, 14-17 mm long, 7-10 mm wide, obviate, stamens 10, rest perfect, 2 lower largest, ovary hairy, stipitate. Fruit 5-6 cm long, 17-23 mm broad, sparsely hairy, turning black at maturity, generally 5-10 seeded, flowering April – June (Fig. 20: A1).	An erect shrub, 0.5-3.5 m tall. Leaf stipulate, stipule 3-.8 mm long leaflets 3 pairs, 1.7-7 cm long, obovate, glabrous, rounded at the top, apex minutely acute. Racemes short, peduncle 2 mm long, bearing 1-2 flowers. Bracts linear-acute, glabrous except the edges, 3-9 mm long, pedicel 1.2-3.5 cm long, hairy. Sepals membranous, ovate, 6-7 mm long. Petals unequal in size, more or less ovate, obtuse or rounded, 2-15 mm long; Stamens 10, 3 lower longest, 4 lateral smaller, 3 upper stamens staminoidal. Ovary glabrous, stigma truncate. Pods, glabrous to subglabrous, indehiscent, 10-25 cm long, 4-8 mm wide, septate, 20-50 seeded, 5-10 mm long, albumen copious. Flowering March - September (Fig. 21: A2).
05	Palynology	Pollen monad, tricolporate, shape in polar circular view, polar diameter 30 µM (27.5-32.5 µM), polar length 35 µM (32.5-37.5 µM), shape in equatorial view subprolate, equatorial diameter 27.5 µM (25-30 µM), equatorial length 38.75 µM (37.5-40 µM), P/E ratio 1.09, exine thickness 2.13 µM (1.25-5 µM), length of colpi 4.25 µM (2.5-6.25 µM) and width 8.75 µM (7.5-10 µM), sculpturing faveolate with holes or depressions which are evenly distributed over the surface. These holes are minute and variable in size (Fig. 20: C1 & D1).	Pollen monad, tricolporate, shape in polar view subangular, polar diameter 30 µM (27.5-32.5 µM), polar length 33.75 µM (32.5-35 µM), shape in equatorial view spheroidal, diameter 15.25 µM (10-30 µM), equatorial length 32.5 µM (30-35 µM), P/E ratio 1.9, exine thickness 3.12 µM (2.5-3.75 µM), length colpi 5.62 µM (5-6.25 µM) and width of colpi 6.87 µM (6.25-7.5 µM) (Fig. 21: C2 & D2).
06	Leaf epidermal	**Abaxial surface:** Ordinary epidermal cells of various shapes, length of ordinary epidermal cell 30 µM (22.5-37.5 µM), width 17.5 µM (15-20 µM), stomata irregularly oriented, paracytic, length 16.87 µM (16.25-17.5 µM), width 8.75 µM (7.5-10 µM), length of guard cell 21.25 µM (20-22.5 µM), width 15 µM (12.5-17.5 µM), stomatal complex: 21.5 µM (19.5-23.5 µM) long, 22.75 µM (20-23.5 µM) wide. Subsidary cell 28.5 µM (22-35 µM) long, and 19.5 µM (15-24 µM) wide. Trichomes unicellular and pointed at the tip, length 121.25 µM (87.5-155 µM), width 17.5µM (15-20µM) (Fig. 20: E1).	**Abaxial surface:** Epidermal cells variously shaped, length 29µM (22.5-35.5 µM), width 18.5 µM (16-21 µM), stomata length 18.25 µM (17-19.5 µM), width 14 µM (12-16 µM), length of guard cells 17 µM (16-18 µM), width of guard cells 3.25 µM (3-5.5 µM), stomatal complex: 20.8 µM (20-21.5 µM) long, and 23.8 µM (23-25 µM) wide, subsidary cells: 20.5 µM (16-25.5 µM) long, 15.5 µM (12-18 µM) wide (Fig. 21: E2). **Adaxial surface:** Epidermis cells narrowly elongated and tetragonal or variously shaped. Ordinary epidermal cell, length 33.33 µM (27.5-42.5

Table 1. Contd.

	anatomy	**Adaxial surface:** Ordinary epidermal cells pentagonal, hexagonal or various shaped, mostly narrow elongated cells with very thin smooth walls. Length of ordinary epidermal cells 41.25 µM (32.5-50 µM), width 30 µM (25-35 µM), stomata irregularly oriented and much abundant, paracytic, length 16.25 µM (15-17.5 µM), width 7.5 µM (6.25-8.75 µM), length of guard cell 21.25 µM (20-22.5 µM), width 14.37 µM (12.5-17.5 µM), stomatal complex: 22.33 µM (21.5-23.5 µM) long, 24.3 µM (23.5-25 µM) wide, subsidary cell 32.75 µM (25-40.5 µM) long, 24.5 µM (19.5-29 µM) wide, glands surrounded by six cells and six spikes are protruding out. Trichome single celled, non glandular, hair like structures attached at base, tapering at the tip, by 6-7 cells, length 343.5 µM (325-362 µM), width of larger trichomes 23.75 µM (20-27.5 µM) (Fig. 20: F1).	µM), width 22.5 µM (20-25 µM), anomocytic type of stomata, uniformly distributed over the surface, length 20.9 µM (20-22.5 µM), width 17.5 µM (15-20 µM), length 20 µM (19-21 µM), width 5.83 µM (5-7.5 µM), stomatal complex: 20.8 µM (20-21.5 µM) long, and 23.8 µM (23-25 µM) wide, subsidary cell 23.5 µM (18-28 µM) long, 18 µM (14-20.5 µM) wide. Long trichomes pointed at tip. Length of trichome 122.6 µM (95.5-141.8 µM) and width 15.6 µM (8.9-20.5 µM) (Fig. 21: F2).
07	Trade part and Status	Leaves and aerial parts are traded commonly at herbal shops throughout the country, under the name of Sana makki.	Leaves and aerial parts are generally mixed in genuine drug Sana Makki.
08	Organoleptgraphy	In dried herbal drug leaves, branches and flowers are mixed. Aerial parts odorless and good in taste. Branches brown in color, size 0.6-1.5 cm, branches hard, narrow, pointed and have rough surface. Leaves green in color, irregular margins and have rough texture. The size of leaves ranges from 0.8-2.5 cm. Flowers yellowish brown in color. Flowers have delicate surface and 0.5-0.7cm in length (Fig. 20: B1).	Flowers, branches and leaves mixed in dried herbal drug. The aerial parts odorless and has herbal taste. Branches cylindrical and greenish brown in color. The length of branches 12.2 cm-22 cm. Branches hard and narrow, diameter of branches is 0.8-1.9 cm. Leaves are green in color and broad. The size of leaves 1-1.5 cm in length. Pods also mixed with aerial parts. Pods black brown in color, segmented 7cm-11cm in size(length). Flowers yellow in color (Fig. 21: B2).
09	Part use	Leaves and aerial parts	Leaves, pods, flowers, aerial parts
10	Medicinal uses	Constipation, digestive disorder, piles, migraine, epilepsy, heart diseases, respiratory diseases, skin diseases.	Constipation, digestive disorder
11	Indigenous herbal recipes	Dried leaves are ground to obtain powder. 2 g of powdered leaves are mixed with equal amount of quality powdered rose petals. 5-10 g of this mixed powder is given with water to patients for 5 days regularly at night to cure constipation, digestive disorders and respiratory diseases. In 2-3 g powdered leaves, mix with equal amount of *Plantago ovata* husk. Take 5-8 g of this powder with glass of milk at night for 10-20 days to cure piles. In 2-5 g powdered leaves of Senna, mix equal amount of pure honey to make tablets. Take one tablet with glass of fresh water at night for a month to treat migraine, heart diseases and epilepsy.	Dried leaves and flowers are used medicinally for constipation and digestive disorder. According to women at Khyber Pukhton Khawa and Punjab Provinces. Dried leaves and pods are ground to obtain powder. 1-2 g of this powder is given with water to patients suffering from constipation and stomach disorders.
12	Toxicity	Non toxic	Non toxic

Table 2. Fluorescence analysis and solubility tests (Cold method) of powdered drug of *Cassia angustifolia* in various solvents.

S/N	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wave length (UV) 365 nm	On filter paper (under short wavelength UV)	On filter paper (under long wavelength UV)	Solubility Analysis
1	Dried plant Powdered	Dull muddy green	Green	Green	-	-	-
2	Powdered drug+50% KOH	Pine forest	Black	Brownish black	Yellow	Yellow	Partially soluble
3	Powdered drug+10% aq. $Fecl_3$	Pine forest	Dark green	Dark green	Dark brown	Chocolate brown	Partially soluble
4	Powdered drug + Distilled H_2O	Leaf green	Greenish brown	Greenish brown	White	Whitish pink	Partially soluble
5	Powdered drug + HCL Conc.	Golden glimmer	Black	Greenish black	Pink	Pinkish brown	Partially soluble
6	Powdered drug + HCL 50%	Golden glimmer	Reddish brown	Red oxide	Yellow	Pinkish yellow	Partially soluble
7	Powdered drug+H_2SO_4 Conc.	Dark red	Reddish brown	Brown	Brownish white	Whitish brown	Partially soluble
8	Powdered drug + H_2SO_4 50%	Red oxide	Black	Reddish black	Brown	Brown	Partially soluble
9	Powdered drug+HNO_3 Conc.	Red oxide	Black	Reddish black	Brown	Chocolate brown	Soluble
10	Powdered drug+HNO_3 50%	Golden brown	Reddish brown	Reddish brown	Yellow	Yellowish brown	Partially soluble
11	Powdered drug + Conc. CH_3OH	Leaf green	Dark green	Reddish brown	Yellow	Yellow	Soluble
12	Powdered drug + CH_3OH 50%	Golden glimmer	Pine forest	Leaf green	Pink	Pink	Partially soluble
13	Powdered drug + Conc. $CHCl_3$	Golden glimmer	Leaf green	Spring green	Pink	Pinkish white	Soluble
14	Powdered drug + $CHCl_3$ 50%	Copper	Dark brown	Reddish brown	Pinkish yellow	Pinkish yellow	Soluble
15	Powdered drug + Conc. C_2H_5OH	Golden glimmer	Fresh green	Spring green	Glowing white	Shiny white	Soluble
16	Powdered drug + C_2H_5OH 50%	Dull green	Leaf green	Spring green	Yellow	Yellow	Partially soluble
17	Powdered drug + Conc. CH_3COOH	Golden glimmer	Dark green	Leaf green	Pink	Pink	Partially soluble
18	Powdered drug+CH_3COOH 50%	Golden glimmer	Green	Dark green	Yellow	Yellow	Partially soluble
19	Powdered drug + Conc. C_6H_6	Golden glimmer	Leaf green	Reddish green	Pink	Pink	Soluble
20	Powdered drug+C_6H_6 50%	Golden glimmer	Leaf green	Black	White	Whitish yellow	Partially soluble

Table 3. Fluorescence analysis and solubility tests (Hot method) of powdered drug of Cassia angustifolia in various solvents.

S/N	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wave length (UV) 365 nm	Solubility analysis
1	Powdered drug + 50% KOH	Brownish black	Reddish brown	Reddish brown	Soluble
2	Powdered drug+10% aq. $Fecl_3$	Brownish green	Dark green	Dark green	Partially soluble
3	Powdered drug + Distilled H_2O	Redo green	Dark green	Pine forest	Partially soluble
4	Powdered drug + HCL Conc.	Brownish black	Black	Black	Partially soluble
5	Powdered drug + HCL 50%	Golden glimmer	Dark brown	Chocolate brown	Partially soluble
6	Powdered drug + H_2SO_4 Conc.	Reddish black	Black	Black	Partially soluble
7	Powdered drug + H_2SO_4 50%	Blackish brown	Black	Black	Partially soluble
8	Powdered drug + HNO_3 Conc.	Red orange	Dark red	Dark red	Soluble
9	Powdered drug + HNO_3 50%	Orange	Dark brown	Dark brown	Partially soluble

Table 3. Continued.

10	Powdered drug + Conc. CH_3OH	Pine forest	Dark green	Dark green	Partially soluble
11	Powdered drug + CH_3OH 50%	Mustard	Leaf green	Dark green	Soluble
12	Powdered drug + Conc. $CHCl_3$	Gold dust	Leaf green	Spring green	Soluble
13	Powdered drug + $CHCl_3$ 50%	Red oxide	Dark brown	Reddish brown	Partially soluble
14	Powdered drug + Conc. C_2H_5OH	Golden glimmer	Dark green	Reddish green	Soluble
15	Powdered drug + C_2H_5OH 50%	Lemon color	Leaf green	Light green	Insoluble
16	Powdered drug + Conc. CH_3COOH	Golden glimmer	Reddish green	Reddish green	Partially soluble
17	Powdered drug + CH_3COOH 50%	Fresh orange	Dark green	Dark green	Partially soluble
18	Powdered drug + Conc. C_6H_6	Gold dust	Leaf green	Red	Partially soluble
19	Powdered drug+C_6H_6 50%	Golden glimmer	Leaf green	Dark brown	Soluble

Table 4. Fluorescence analysis and solubility tests (Cold method) of powdered drug of Cassia obtusifolia in various solvents.

S/N	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wave length (UV) 365 nm	On filter paper (under short wavelength UV)	On filter paper (under long wavelength UV)	Solubility Analysis
1	Dried Plant Powdered	Fresh green	Green	Buckingham green	-	-	-
2	Powdered drug + 50% KOH	Mustard green	Black	Blackish brown	Pink	Purple blue	Partially soluble
3	Powdered drug + 10% aq. $FeCl_3$	Dark brown	Black	Black	Pale brown	Dark brown	Partially soluble
4	Powdered drug + Distilled H_2O	Light green	Light grey	Grayish white	Light blue	Pink with white outline	Insoluble
5	Powdered drug + HCL Conc.	Leaf green	Dark green	Dark green	Pink	Light purple	Partially soluble
6	Powdered drug + HCL 50%	Golden glimmer	Pine forest	Pine forest	Purple	Light pink	Partially soluble
7	Powdered drug+ H_2SO_4 Conc.	Pink	Dark green	Buckingham green	Black	Blackish brown	Partially soluble
8	Powdered drug + H_2SO_4 50%	Reddish black	Black	Blackish brown	Black	Chocolate brown	Insoluble
9	Powdered drug + HNO_3 Conc.	Reddish brown	Dark brown	Dark brown	Light brown	Brownish purple	Partially soluble
10	Powdered drug+ HNO_3 50%	Golden glimmer	Leaf green	Golden glimmer	Light brown	Woody brown	Partially soluble
11	Powdered drug + Conc. CH_3OH	Spring green	Brown green	Reddish green	Light purple	Fresh pink	Soluble
12	Powdered drug+CH_3OH 50%	Pale green	Dark green	Leaf green	Brownish green	Pinkish green	Partially soluble
13	Powdered drug + Conc. $CHCl_3$	Pine forest	Dark green	Reddish brown	Bluish brown	Brown	Soluble
14	Powdered drug + $CHCl_3$ 50%	Spring green	Algal green	Dark green	Dark green	Green	Soluble
15	Powdered drug + Conc. C_2H_5OH	Spring green	Fresh green	Reddish green	Dull brown	Soil brown	Soluble
16	Powdered drug + C_2H_5OH 50%	Pale green	Leaf green	Lemon green	Pink	Purple	Partially soluble
17	Powdered drug + Conc. CH_3COOH	Leaf green	Reddish brown	Reddish brown	Bluish brown	Pink flash	Partially soluble
18	Powdered drug + CH_3COOH 50%	Leaf green	Leaf green	Redo green	Light blue	Pinkish brown	Partially soluble
19	Powdered drug + Conc. C_6H_6	Pine forest	Leaf green	Reddish green	Pinkish blue	Light pink	Soluble
20	Powdered drug + C_6H_6 50%	Light green	Leaf green	Spring green	Brown with blue shade	Pinkish brown	Partially soluble

Table 5. Fluorescence analysis and solubility tests (Hot method) of powdered drug of Cassia obtusifolia in various solvents.

S/N	Treatments	Under visible light	Under short wavelength (UV) 254 nm	Under long wave length (UV) 365 nm	Solubility analysis
1	Powdered drug + 50% KOH	Greenish black	Black redo green	Fresh green Reddish black	Soluble
2	Powdered drug + 10% aq. $FeCl_3$	Orange brown	Black	Greenish black	Soluble
3	Powdered drug + Distilled H_2O	Light green	Golden glimmer	Golden glimmer	Partially soluble
4	Powdered drug + HCL Conc.	Brownish green	Brown	Blackish brown	Partially soluble
5	Powdered drug + HCL 50%	Brownish green	Black	Dark brown	Partially soluble
6	Powdered drug + H_2SO_4 Conc.	Black	Black	Black	Insoluble
7	Powdered drug + H_2SO_4 50%	Black	Black	Black	Insoluble
8	Powdered drug + HNO_3 Conc.	Orange	Dark brown	Dark brown	Soluble
9	Powdered drug + HNO_3 50%	Light brown	Dark green	Golden glimmer	Soluble
10	Powdered drug + Conc. CH_3OH	Buckingham green	Spinach green	Red	Partially soluble
11	Powdered drug + CH_3OH 50%	Light green	Brownish green	Golden green	Soluble
12	Powdered drug + Conc. $CHCl_3$	Redo green	Fresh green	Red green	Soluble
13	Powdered drug + $CHCl_3$ 50%	Leaf green	Dull green	Brown green	Partially soluble
14	Powdered drug + Conc. C_2H_5OH	Fresh green	Spring green	Reddish green	Soluble
15	Powdered drug + C_2H_5OH 50%	Leaf green	Algal green	Pine forest	Insoluble
16	Powdered drug + Conc. CH_3COOH	Leaf green	Reddish brown	Reddish brown	Partially soluble
17	Powdered drug + CH_3COOH 50%	Redo green	Pine forest	Pine forest	Partially soluble
18	Powdered drug + Conc. C_6H_6	Spring green	Dark green	Red	Partially soluble
19	Powdered drug+C_6H_6 50%	Leaf green	Brown	Brownish green	Soluble

greenish in day light and showed various shades under UV light.

This is the first report of the pharmacognostic standardization of powder drug *C. angustifolia* in comparison with its adulterant *C. obtusifolia.* Similar phytochemical standardization of herbal drug such as *Mimosa pudica* L. and *Biophytum sensitivum* DC. was carried out by Saritha and Brindha (2008). From such type of studies, it is clear that the knowledge of morpho-palynological, anatomical and preliminary phytochemical characters used in pharmacognostic investigation is important in the identification and delimitation of taxa like *C. angustifolia* for the purpose of employment in pharmaceutical preparations.

Conclusion

The present work focuses on the taxonomic and pharmacognostic authentication of the herbal drug Senna marketed in Indo-Pak subcontinent and other parts of the world, with its common adulterant *C. obtusifolia* which is a medico botanically different species. Since there is no such authentication screening on record for this much valued Senna drug, the present work was aimed at establishing standards that could be used in deciding the genuineness of the aforementioned drug from its closely related adulterant. The colored macro and micro photographs of plant, drug part, pollen, foliar epidermis and pharmacognostic tests might facilitate the researchers for correct identification and pharmaceutical industries in providing safe and authentic drug of genuine source.

ACKNOWLEDGEMENT

The authors are thankful to the Higher Education Commission of Pakistan for financial support through indigenous PhD scholarships scheme.

REFERENCES

Abid M, Ahmad M, Jabeen A, Zafar M, Nadeem S (2005).

Pharmacognostic studies of some indigenous medicinal plants of Pakistan. http://www.siu.edu/~ebl/leaflets. J. Ethnobotanical Leaflets. 9:1.
Afaq SH (1998). A comparative introduction of the Unani and Tibetan medical traditions, AyurVijnana, (http://www.ittm.org/publications/AyurVijnana/Vol_06/AV_V06_5.htm) . p. 6.
Ahmad M, Khan MA, Zafar M, Arshad M, Sultana S, Abbasi BH, Din SU (2010). Use of chemotaxonomic markers for misidentified medicinal plants used in traditional medicines. J. Med. Plant Res. 4(13):1244-1252.
Ahmad FM, Khan A, Ahmad M, Zafar M, Khan A, Iqbal Z (2011). Palynological studies in tribe Chlorideae (Poaceae) from salt range of Pakistan. Afr. J. Biotechnol. 10(44):8909-8913.
Al-Suyuti J (1985). Tibb-ul-Nabbi. Osiris 4:92.
Anonymous (1992). The wealth of India II-Raw materials. New Delhi, Publication and information Directorate, CSIR, pp. 354-363.
Barthlott W (1984). Microstructural features of seed surfaces. In: V.H. Heywood, D.M. Moore, Editors, Current Concepts in Plant Taxonomy, Academic Press, London pp. 95-105.
Dymock W (1972). Pharmacographia Indica. Hamdard Press, Karachi 1:528.
Erdtman G (1960). The acetolysis method. A revised description. Sven. Bot. Tidskr. 54:561-564.
Hooker JD (1875). Flora of British India, Rananculaceae to Sapindaceae. Utarpradesh Press India 1:5-15.
Hooker JD (1885a). Flora of British India. Asclepideae to Amarantaceae p. 4.
Hooker JD (1894). Flora of British India, Orchideae to Cyperaceae p. 4.
Khan MA, Ahmad M, Zafar M, Sultana S, Shaheen S, Leghari MK, Jan G, Ahmad F, Nazir A (2011). Medico-botanical and chemical standardization of pharmaceutically important plant of Tricholepis chaetolepis (Boiss.) J. Med. Plants Res. 5(8):1471-1477.
Metcalfe CR (1960). Anatomy of the Monocotyledons. I. Gramineae. Clarendon Press, Oxford London.
Nasir E, Ali SI (1974). Flora of West Pakistan (Verbenaceae). Karachi University Press Pak. 77:4-10.
Nasir E, Ali SI (1975). Flora of West Pakistan. (Lythraceae). Karachi University Press Pak. 78:6-7.
Prat H (1932). L'Épiderme des graminées. Étude anatomique et systématique. Ann. Sci. Nat., Bot. 10e(14):117-324.
Ronald OK (2000). Pollen and spores. 2nd Ed. Am. Assoc. Stratographic Palynologists pp. 13-21.
Saldanha CJ, Nicolson DH (1976). Flora of Hassan District Karnataka, India. Amerind Publishing Co. Pvt. Ltd. New York.
Shinde VM, Dhalwal K, Potdar M, Mahadik KR (2009). Application of quality control principles to herbal drugs. Int. J. Phytomed. 1(4-8):1-11.
Sofowora A (1993). Medicinal Plants and Traditional Medicines in Africa. John Wiley & Sons New York pp. 97-145.
Srivastava M, Srivastava S, Khatoon S, Rawat AKS, Mehrotra S, Puhpangadan P (2006). Pharmacognostical evaluation of Cassia angustifolia seeds. Pharmaceut. Biol. 44(3):202-207.
Subrahmanyam NS (1996). Laboratory manual of plant taxonomy. Vikas Publishing House (Pvt) Ltd., New Dehli , pp. 153–156.
Sultana S, Khan M A, Ahmad M, Bano A, Zafar M, Shinwari ZK (2011). Authentication of herbal medicine neem (Azadirachta indica A. Juss.) by using taxonomic and pharmacognostic techniques. Pak. J. Bot. 43:141-150.
Trease GE, Evans WC (1989). Pharmacognosy 12th Ed. Bailliere Tindal, Oxford, U.K. p. 685.
Tutin TG, Heywood VH (1972). Flora of Europe. Cambridge University Press. London p. 3.
Zafar M, Ahmad M, Khan MA, Sultana S, Jan G, Ahmad F, Jabeen A, Shah GM, Shaheen S, Shah A, Abdul N, Sarfaraz KM (2011). Chemotaxonomic clarification of pharmaceutically important species of Cyperus L. Afr. J. Pharm. Pharmacol. 51(1):67-75.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2309-2317, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.524
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Some *in vitro* and pharmacodynamic evaluation of indomethacin solid lipid microparticles

N. C. Obitte[1]*, S. A. Chime[1], A. A. Magaret[1], A. A. Attama[2], I. V. Onyishi[1] and S. A. Brown[3]

[1]Department of Pharmaceutical Technology and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria.
[2]Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria.
[3]Department of Pharmaceutics and Pharmaceutical Technology, University of Port Harcourt, Rivers State, Nigeria.

Accepted 29 June, 2012

The purpose of this study was to investigate some *in vitro* and pharmacodynamic properties of indomethacin-loaded solid lipid microparticles (SLMs). A blend of homolipid from *Capra hircus* and Phospholipon® 90G comprised of the lipid matrix. The SLMs were prepared by the hot homogenisation technique. The effects of Carbosil® and sodium chloride on the release profile and particle size of the SLMs were investigated. The anti-inflammatory and gastro-protective characteristics of the SLMs were also studied. The particle size ranged from 5.0 to 20 µm. The encapsulation efficiency ranged from 46 to 72%; with sodium chloride-containing batches recording highest values. Maximum drug release was within 80 min. Significant (P < 0.05) anti-inflammatory effect was exerted by the SLM. Relative high ulcer index associated with unformulated indomethacin powder and the absence of gastric lesions in rats that received oral administration of SLM affirmed the gastro-protective potential of the SLM. In conclusion, improved anti-inflammatory and gastro-protective effects were achieved with indomethacin-loaded SMLs.

Key words: Anti-inflammatory, gastro-protective, solid lipid microparticles (SLMs).

INTRODUCTION

The oral delivery of lipophilic drugs presents a major challenge, because of their low aqueous solubility and possible unpredictable bioavailability. Although, lipophilicity is a prerequisite for most drug permeation and absorption through the lipid-rich biological membranes, the feasibility of absorption is contingent upon aqueous solubility, without which absorption may be impaired (Arun et al., 2008).

Unionized species as postulated by Henderson-Hasselback are characteristically lipidic in nature. It then follows that an enabled dosage form capable of enhancing the solubility of poorly soluble drugs in the gastrointestinal tract (GIT) may produce soluble unionized species with chemical suitability for absorption, especially when nonionic excipients are utilized.

*Corresponding author. E-mail: obittenick@yahoo.com. Tel: + 234 8060532739.

Lipid-based formulations are typically reputed to improve the solubility and bioavailability of per orally-administered poorly soluble drugs (Hou et al., 2003; Pouton, 2000). Fundamentally these formulation techniques promote wetting or solubilization of drug and enhance permeability or further undergo intraluminal processing to solubilize the drug (Fricker et al., 2010). Intraluminal processing is facilitated by bile secretion which creates a pool of cholesterol/phospholipid/bile salt complex with surfactant property which facilitates dissolution of poorly soluble drugs and lipophilic drug formulations. Examples of lipid formulations include emulsions, micellar solutions, liposomes, lipid nanoparticles, structured lipid carriers, self-emulsifying oil formulations, solid dispersions, solid-lipid compacts, and drug lipid conjugates.

Apart from synthetic forms, plant-based lipids have also been explored as potential excipients in lipid-based formulations. Although, chemical specificity and purity may not be optimal plant lipids, may be superior to

synthetic forms as regards toxicity and biocompatibility. Solid lipid microparticles (SLMs) are a simple lipid-based delivery system comprising of drug dispersion in a solid lipid matrix.

They are emulsion systems, differing from conventional emulsions by their particulate nature, and can be lyophilized into discrete microparticles (Jasper et al., 2007). If the post-homogenization product yields particles of spherical micrometric dimensions ranging from 1 to 250 µm and having a hydrophobic solid drug core in a phospholipid matrix, they are called lipospheres (Domb and Maniar, 2007). The formulation principle is similar to oil-in-water (O/W) emulsions. Instead of using a liquid lipid, a solid lipid whose solid state is maintained at both room and body temperature is used for this case. Several authors have adopted the melt dispersion cum homogenization technique to prepare lipospheres as an alternative method of microencapsulation to avoid the use of toxic organic solvents and monomers associated with polymeric microparticles (El-Gibaly and Abdel-Ghaffer, 2005; Tewes et al., 2006). In addition to biocompatibility, other advantages include large-scalability, flexible dosage form applicability, controlled drug release, taste-masking, and solubility-enhancement of poorly soluble drugs (EL-Kamel et al., 2007; Shivakumar et al., 2007).

Indomethacin, a weak acidic drug with a PK_a of 4.5 belongs to a class of drugs called non-steroidal anti-inflammatory drugs (NSAIDs) and class 11 biopharmaceutic classification system (De Filippis et al., 1991; Tirkkonen and Paronen, 1992; Yuce and Canefe, 2008). NSAIDs (non-selective) are drugs with analgesic, antipyretic and anti-inflammatory properties (Domb and Maniar, 1996; Martindale, 2009) which inhibit cyclo-oxygenase (COX) enzyme and consequently inhibit the synthesis of prostaglandins and thromboxanes from arachidonic acid. They therapeutically inhibit COX-2, the inflammatory agent at inflamed tissues and also COX-1 with attendant toxicities, such as gastrointestinal problems (Bhupinderjit et al., 1999; Martindale, 2009).

The poor solubility and GIT irritation effect of indomethacin are core limitations to its oral use. A formulation strategy therefore that is capable of addressing these two concerns will be highly beneficial. In this work, therefore, the objective was to formulate indomethacin-loaded SLMs using a matrix blend of synthetic and animal-based fat, and to follow up *in vitro* evaluation with pharmacodynamic (anti-inflammatory and ulcerogenic) studies. The potential usefulness of this formulation strategy to improve the poor solubility and reduce or prevent the gastric irritation effect of indomethacin is being evaluated in this work.

MATERIALS AND METHODS

The following materials were used as procured from their local suppliers without further purification: hydrochloric acid, sodium hydroxide, potassium dihydrogen phosphate, Tween 80 (Sigma Aldrich, Seelze, Germany), sorbitol, indomethacin (Merck, Germany), Phospholipon® 90G (GmbH, Köln, Germany), activated charcoal (Bio–Lab. UK ltd, London), and thiomersal (Synochem, Germany). Goat *(Capra hircus)* fat was obtained from a batch processed in our laboratory. All other reagents and solvents were of analytical grade.

Extraction and purification of fat from *C. hircus*

The fat was extracted by grating the adipose tissue prior to boiling with half its weight of water on a water bath for 45 min. Molten fat was separated from the aqueous phase using a muslin cloth. Further purification was carried out by heating a 2% w/w suspension of a 1:9 ratio blend of activated charcoal and bentonite in the lipid at 80 to 90°C for 1 h. Thereafter, the suspension was vacuum-filtered using buchner funnel.

Preparation of lipid matrix (LM)

Hot melt (80°C) of Phospholipon® 90G and *C. hircus* fat were mixed together at a ratio of 30:70. The mixture was continuously stirred with a stirrer until it attained homogeneity and cooled into a solid mass.

Preparation of SLMs

The method of previous workers (Jaspert et al., 2007; Cortesi et al., 2003) was adopted with modification, using the hot homogenization technique. Indomethacin and Carbosil® were consecutively dispersed in a hot melt (80°C) of lipid matrix and were stirred to constitute the lipid phase. The latter was then introduced into a hot (70°C) aqueous solution of sorbitol, Tween 80, thiomersal, with or without sodium chloride and were homogenized (Ultra-turrax, T25 Basic digital, Ika/Staufen, Germany) for 10 min at 5000 rpm. The final volume of the emulsion was maintained at 100 ml. Table 1 shows the formulation excipients and their w/v % concentrations.

Particle size determination (microscopy)

The particle size of the SLMs was determined by introducing few drops of aqueous dispersion of the SLM on a slide and was imaged under a Hund® binocular microscope (Weltzlar, Germany) with a Motic image analyser (Multicam, China) at X100 magnification.

Encapsulation/Loading efficiency

SLMs from each batch were centrifuged at 3000 rpm for 30 min and 0.5 g quantity was triturated with 20 ml of phosphate buffer prior to subsequent transference to a 100 ml volumetric flask. The flask was made up to volume, stirred, and filtered (Whatman No. 1 filter paper). Indomethacin content of appropriate dilutions were spectrophotometrically (Model SP6 - 450 UV/Vis Pye Unicam) determined at 278 nm. Duplicate determinations were made for all the batches.

The encapsulation efficiency (EE) (the percentage fraction of the theoretical quantity of drug entrapped in the lipid matrix post-homogenization) was therefore calculated from the following equation:

$$EE = \frac{\text{Final quantity of drug encapsulated}}{\text{Initial quantity of drug incorporated}} \times 100 \quad (1)$$

Table 1. Formulae for different batches of SLMs.

Batch	LM (w/v %)	Indomethacin (w/v %)	Tween 80 (w/v %)	Sorbitol (w/v %)	Thiomersal (w/v %)	Sodium chloride (w/v %)	Carbosil® (w/v %)
X1	5.0	0.50	2.5	4.0	0.0025	-	0.1
X2	5.0	0.75	2.5	4.0	0.0025	-	0.2
X3	5.0	1.00	2.5	4.0	0.0025	-	0.5
X4	5.0	1.50	2.5	4.0	0.0025	-	1.0
Y1	5.0	0.50	2.5	4.0	0.0025	0.9	0.1
Y2	5.0	0.75	2.5	4.0	0.0025	1.2	0.2
Y3	5.0	1.00	2.5	4.0	0.0025	1.5	0.5
Y4	5.0	1.50	2.5	4.0	0.0025	2.0	1.0
Z1	5.0	-	2.5	4.0	0.0025	-	0.1
Z2	5.0	-	2.5	4.0	0.0025	0.9	0.1

X and Y: various formulation codes of indomethacin-loaded SLMs; Z: control; LM: lipid matrix.

Dissolution studies

The release of indomethacin from the SLMs was studied using the USP paddle method. The dissolution medium consisted of 900 ml of freshly prepared phosphate buffer (pH 7.4) maintained at 37 ± 0.5°C. Appropriate amount of SLM in 2 ml of the buffer solution was introduced into a polycarbonate dialysis membrane (previously macerated in the medium for 24 h) and securely tied with a non-reactive thermo-resistant thread. It was immersed and firmly suspended in the dissolution medium. The equipment was operated at an agitation speed of 50 rpm, while 5 ml sampling at predetermined time intervals was followed by fresh equivalent volume replacement. Samples were filtered and spectrophotometrically assayed for indomethacin content as described earlier (under encapsulation efficiency).

Anti-inflammatory studies

Egg albumin-induced rat paw oedema method was adopted in this study. All experimental protocols were in compliance with and approved by the animal ethics committee of the University of Nigeria, Nsukka and in compliance with the Federation of European Laboratory Animal Science Association and the European Community Council Directive of November, 1986 (86/609/EEC). Acute inflammation induced by sub plantar injection of egg-albumin was measured in terms of change in the volume of the rat hind paw (Ekpendu et al., 1994). Wistar rats (150 to 200 g) of both sexes were divided into 5 rats per group. The rats were fasted for 6 h with no access to water during the experiment. Water deprivation was to ascertain uniform hydration and to reduce unwanted differences in oedematous response (Winter et al., 1963). Dose of SLM (Y1) equivalent to 1 mg/kg of indomethacin was administered orally to the rats. The control group received normal saline (P2), while the reference group received pure sample of 1 mg/kg amount of indomethacin (P1). After 30 min post-treatment, oedema was induced by injecting 0.1 ml of fresh undiluted egg albumin into the sub planter region of the right hind paw of the rats. Subsequently, the volume of distilled water displaced by the paw was measured with the aid of a plethysmometer before and at 1, 2, 3, 4, and 5 h post induction of oedema. The anti-inflammatory activity was calculated at each time as percent inhibition of oedema using the following equation:

$$\text{Inhibition (\%)} = \frac{Vo - Vt}{Vo} \times 100 \quad (2)$$

where Vt is the volume of oedema in reference group at time t and Vo is the volume of oedema in control rats at the same time (Perez, 1996).

Ulcerogenic properties of SLMs

The method described by some workers (Cashin et al., 1979) was employed in this study. Experimental protocols were in compliance with and approved by the University of Nigeria, Nsukka animal ethics committee and compliant with the Federation of European Laboratory Animal Science Association and the European Community Council Directive. Healthy Wistar rats of both sexes (150 to 215 g) of five rats per group were fasted for 12 h. SLM (Y1) equivalent to 10 mg/kg of indomethacin was administered orally to the rats. The control group received normal saline (P2), while the reference group received indomethacin powder (P1) sample (10 mg/kg). After 5 h post treatment, the animals were sacrificed by ether anesthesia. Gastric mucosa was removed, cut along the lesser curvature and opened up to expose the mucosal surface (Ajali and Okoye, 2009). The mucosa was washed with normal saline and observed with an X10 magnifying glass. The number of observed ulcers was counted and the ulcer index determined as described previously (Main and Whittle, 1975).

Statistics

All statistical analysis was carried out using Statistical Packages for Social Sciences (SPSS) version 13 at $P < 0.05$.

RESULTS

Figures 1 to 4 show the photomicrographs of a few indomethacin-loaded SLMs. Student t-test showed that X1 and X2 without sodium chloride (NaCl) significantly ($P < 0.05$) had higher particle sizes than Y1. In most cases, the two control batches (Z1 and Z2), without entrapped drug, significantly ($P < 0.05$) recorded higher particle sizes than the drug-loaded batches. Furthermore, the control (Z1, without NaCl) indicated significantly higher particle size than the NaCl-containing batch (Z2). Apparently, drug entrapment and the inclusion of NaCl in

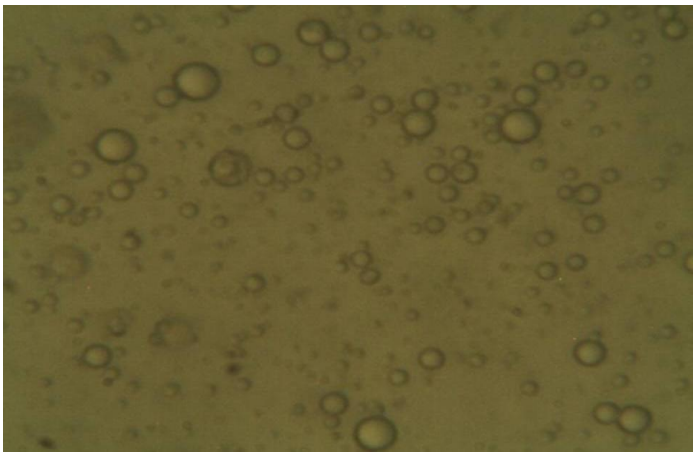


Figure 1. Photomicrograph of batch X1 indomethacin-loaded SLM: X1 contains 0.5% drug without NaCl.

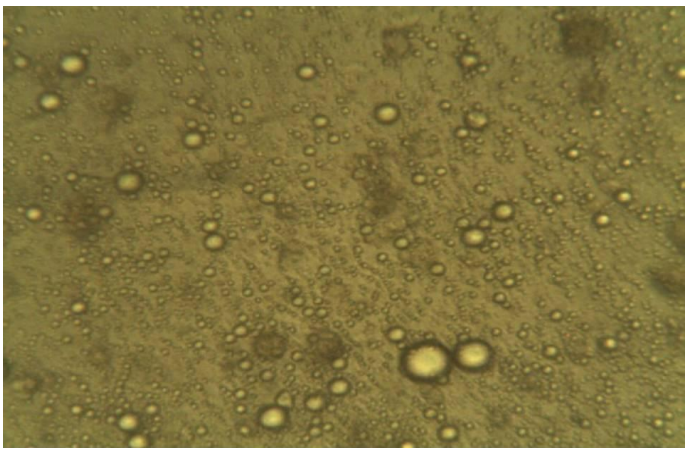


Figure 2. Photomicrograph of indomethacin-loaded batch Y1 SLM: Y1 contains 0.5% of drug and 0.1% NaCl.

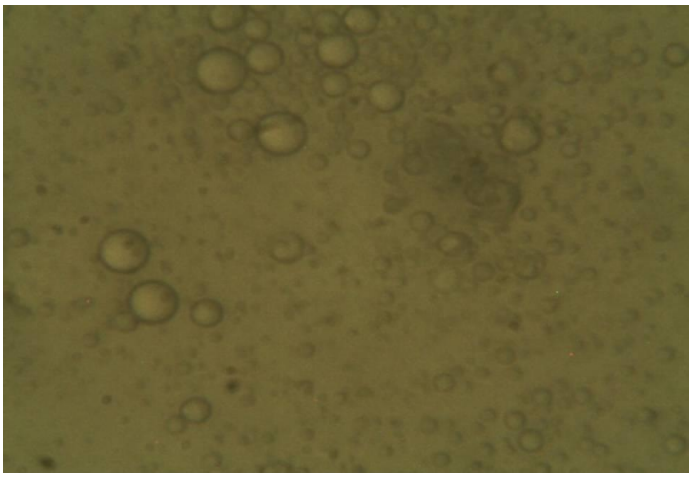


Figure 3. Photomicrograph of batch X2 indomethacin-loaded SLM: X2 contains 0.75% of drug, without NaCl.

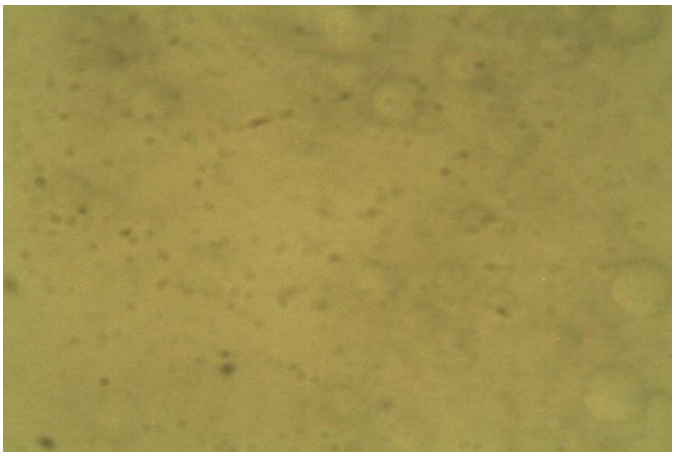


Figure 4. Photomicrograph of batch Z2 indomethacin-loaded SLM: Z2 contains 0.9% NaCl and no drug.

the continuous aqueous phase contributed to reduced particle size in some cases.

The EE was derived as percent fraction of the incorporated drug that was ultimately encapsulated. Student t-test ($P < 0.05$) evaluation indicated that NaCl significantly ($P < 0.05$) promoted higher EE in most cases. Drug load inversely affected EE significantly ($P < 0.05$) (Figure 5). Theoretical drug:Carbosil® (DC) ratio calculated from Table 1, for X1 to X4 or Y1 to Y4 was 5, 3.75, 2.0, and 1.5 respectively. X1 and Y1 with DC value of 5 recorded highest EE, thus projecting them as optimum formulations.

The drug release profile in Figure 6 indicates a biphasic pattern, while Figure 7 shows the T50 and T85 values of the various batches, with maximum drug release taking place between 45 and 75 min. X1 to X4 batches witnessed an initial slow release for about 40 min prior to a faster release phase. On the other hand, the initial release phase for Y1 to Y4 was between 10 to 30 min. Generally, batches containing NaCl witnessed minimal but distinct faster drug release than those without it, with the curves of the later (X1 to X4) having a cluster effect, and similar T50 or T85 values (Figures 6 and 7).

The result of anti-inflammatory effect (AIE) of the SLMs in Table 2 is indicative that indomethacin-loaded SLMs demonstrated significantly ($P < 0.05$) better AIE than the unformulated indomethacin. The time (T50) for 50% AIE to be achieved was 2 h for Y1, while dissolution studies profiled 42 min as its dissolution T50. It is interesting to note that sharpest AIE rise (30 to 45.5%) occurred between 0.5 and 1 h (30 to 60 min). Subsequent increments were rather gradual. This seemed to approximate the dissolution T50 of 42 min.

Furthermore, drug release profile in Figure 3 evidenced a biphasic release that delineated T50 as part of the second and sharp phase. Remarkable absence of mucosal lesions in the GIT depicted in the indomethacin SLM-treated animals (Table3) was indicative of gastro-

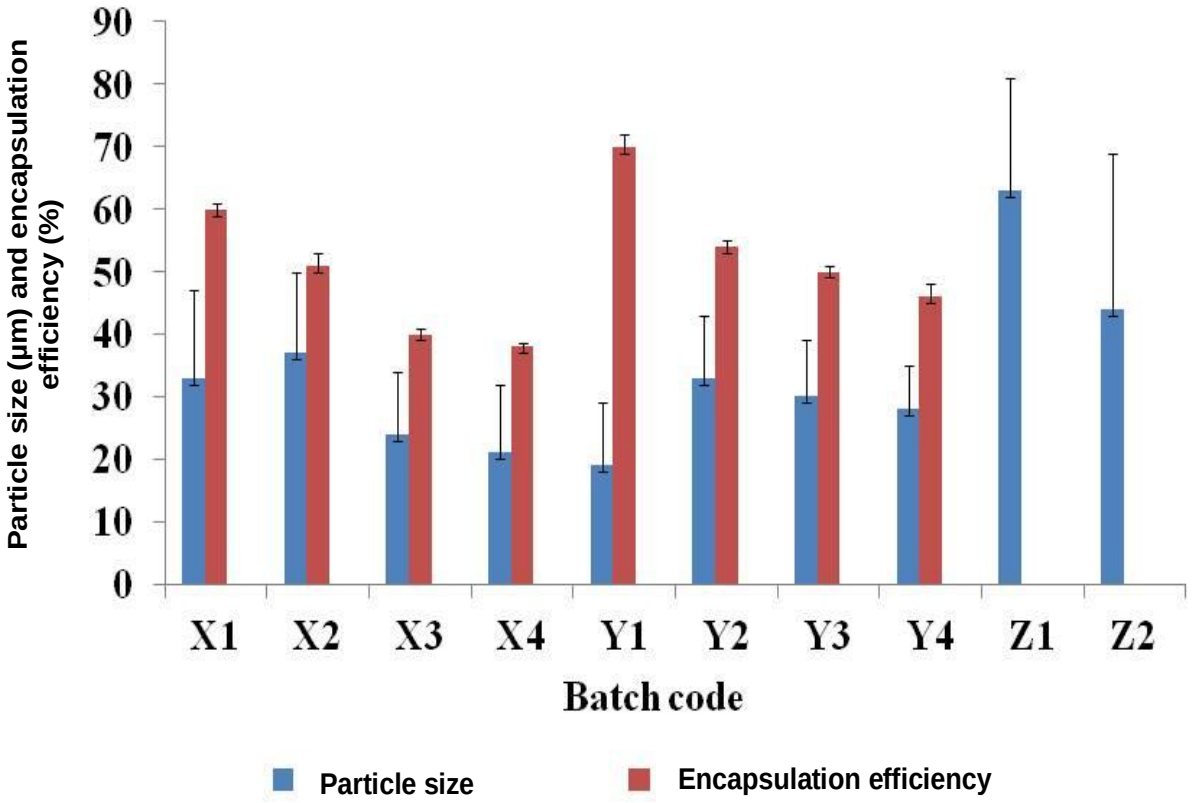


Figure 5. Chart representation of the mean particle size and encapsulation efficiency values of the SLMs. X – Y: various formulations of indomethacin-loaded SLMs; X1 – X4: 0.5 to 1.5% indomethacin and 0.1 to 1% carbosil® without sodium chloride; Y1 – Y4: 0.5 to 1.5% indomethacin, 0.1 to 1% carbosil® and 0.9 to 2% sodium chloride; Z1 – Z2: contain no drug.

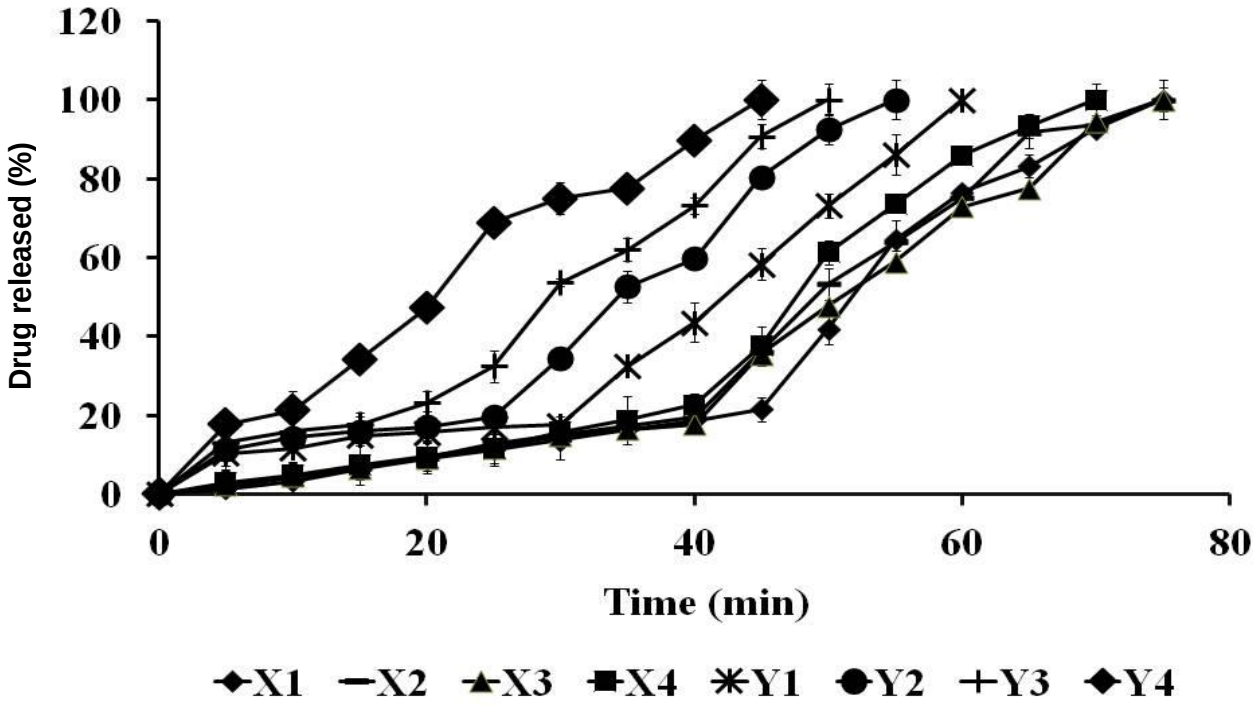


Figure 6. The release profile of indomethacin SLMs in phosphate buffer, pH 7.4.

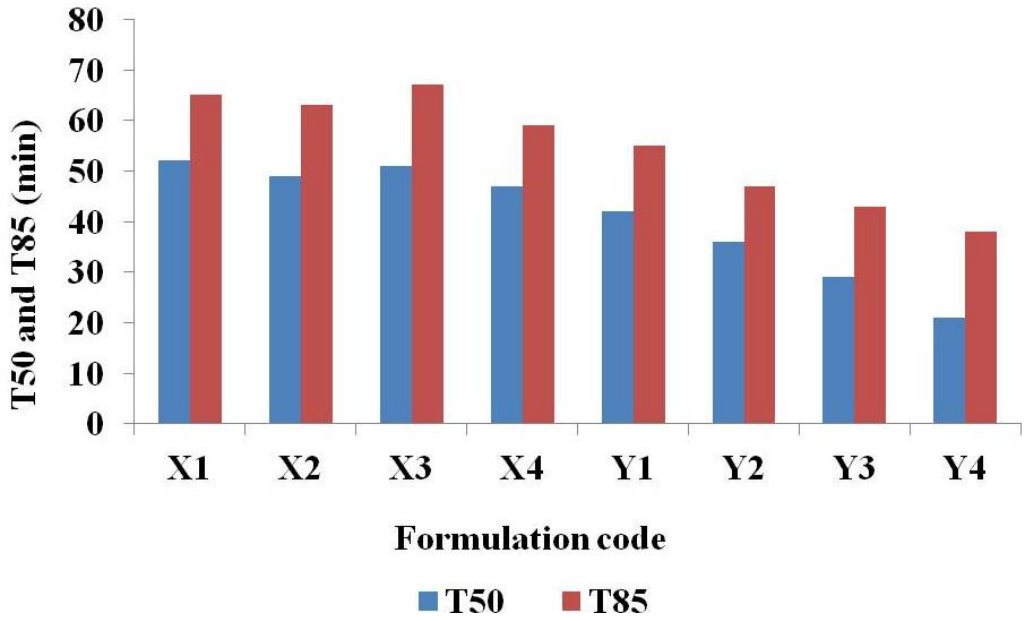


Figure 7. Chart representation of the T50 and T85 values of indomethacin SLMs.

protective potential of the lipid formulation. In contrast, the unformulated indomethacin powder induced multiple ulcers in the animals' gastric mucosa, to the index tune of 14.

DISCUSSION

The incorporation of materials into lipid matrices constitutes external stress on the membrane which imposes elastic deformation that varies with the properties of the material. Three elastic modes of membranes that have been identified include, bending (curvature), stretching and tilting deformations (May et al., 2004). Bending has been reported as prominent during self-emulsification of self-emulsifying drug delivery system (SEDDS)/self-microemulsifying drug delivery system (SMEDDS) (Bagwe et al., 2001). Tilting, on the other hand, involves hydrophobic (hydrocarbon chain) tail tilting with respect to the monolayer plane. Drug entrapment which significantly reduced particle size, in comparison to drug-free SLMs, may have predisposed the phospholipid membranes to tilting and bending since the hydrophobic characteristic of indomethacin warranted concentration and entrapment within the hydrocarbon tail of the amphiphile already consolidated by the animal lipid constituent. Since Z1 and Z2 were not drug-loaded, obvious absence of significant external stress would be anticipated within their particles. This was probably responsible for the observed larger particle sizes. On the other hand, particle size reduction in some of the batches containing NaCl may be attributed to a possible interaction between the hydrophilic polar heads of the phospholipid and the sodium chloride. Increased membrane potential and permeability by NaCl (Nwafor and Coakley, 2003) probably facilitated more aqueous diffusion, thus making the lipid particles of Y1 and Z2 (amidst NaCl) relatively more susceptible to particle disruption than X1 and Z1, and consequently, yielding comparatively smaller particles.

In a previous unpublished research work, we reported higher encapsulation efficiency (89 to 91%) at 0.5% drug load, for a Carbosil® containing lipid matrix as compared to a control without Carbosil®. This actually motivated the incorporation of Carbosil® in all the batches in our present investigation. Carbosil® forms a colloidal dispersion in water, but a smooth viscous dispersion in oil. Its inclusion was intended to generally enhance drug entrapment efficiency. However, there was an interplay between drug load and Carbosil® content as interpreted by drug:carbosil® ratio. This interplay was most significant in X1 and Y1 at a value of 5, followed by X2 and Y2. High value was indicative of higher drug concentration relative to Carbosil®. Below this value palpability waned or was lost, as depicted in the EE values in Figure 5. Furthermore, the hydrophilicity of Carbosil® is attributed to the presence of silanol (Si-OH) group on its surface which potentially interacts with non-polar moieties via hydrogen bonding that exists between the silanol group and other Carbosil® particles to form a three dimensional gel structure (Obitte et al., 2009, 2010; Patil et al., 2004). It may be that the three dimensional gel structures promoted drug entrapment within them. The presence of NaCl in some of the formulations also had effect on the EE. Batch Y1 that contained NaCl entrapped 72% of the drug, whereas X1 without it entrapped 60%. The

Table 2. Anti-inflammatory property of indomethacin SLM.

Batch	Paw volume oedema (ml ± SD)[a] and percent inhibition of oedema (%)					
	0.5 h	1 h	2 h	3 h	4 h	5 h
Y1	0.70 ± 0.18(30.0)	0.60 ± 0.34(45.5)	0.50 ± 0.27*(50.0)	0.45 ± 0.19*(55.0)	0.40 ± 0.27*(57.9)	0.30 ± 0.37*(68.4)
P1 (Reference)	0.80 ± 0.17(20.0)	0.85 ± 0.11(22.7)	0.70 ± 0.23*(30.0)	0.60 ± 0.12*(40.0)	0.55 ± 0.22*(42.0)	0.40 ± 0.29*(1.5)
P2 (Control)	1.00 ± 0.13	1.10 ± 0.16	1.00 ± 0.19	1.00 ± 0.11	0.95 ± 0.16	0.95 ± 0.12

*Reduction in oedema significant at $P < 0.05$ compared to control. Values of oedema shown are mean ± SD, [a]n = 5; values in parenthesis are percent inhibition of oedema calculated relative to control; Y1: indomethacin-loaded SLM; P2: normal saline; P1: unformulated indomethacin powder.

presence of the electrolyte in the aqueous phase may have provided an ionized aqueous milieu that interacted strongly with the polar groups through pore development caused by membrane potential increase (Nwafor and Coakley, 2003), thus minimizing escape of entrapped drugs. Pore-opening may have additionally impelled re-entrapment of drug lost to the aqueous phase during agitation.

Some workers reported faster drug release caused by 0.9% NaCl in the dissolution medium (Yue et al., 2008). Others also attributed increased dissolution rate to the osmotically induced microcapsule pore-opening occasioned by NaCl (Tirrkonnen et al., 1995) probably due to the membrane potential-increasing ability of sodium chloride (Nwafor and Koakley, 2003). Although, in this work, NaCl was only incorporated into the aqueous phase and not the lipid matrix; however, during the homogenization process, some interaction may have taken place between the SLM and aqueous-borne NaCl, especially at such a high agitation speed. This may have promoted pore-opening of the matrix; consequently, enhancing drug release during dissolution studies. Another factor that had across-the-board effect on dissolution rate was the alkaline dissolution medium that enhanced the release of the acidic drug, indomethacin.

Drug absorption enhancement was the basis for the observed anti-inflammatory activity. Phosphatidylcholine has been reported to enhance the lymphatic transport of some drugs(Koo and Noh, 2001; Trevaskis et al., 2006). Hydrolyses of endogenous and formulation-based phospholipids by phospholipase A2 and the stimulation of the release of bile salts, phospholipid, and cholesterol by exogenous lipid, probably associated to produce mixed micelles which ultimately provided a solubilizing platform for the poorly soluble drug (Fricker et al., 2010; Porter et al., 2007; Carey et al.,1983; Tirkkonen et al., 1995).

The aforementioned mechanisms may have been responsible for the superior anti-inflammatory effect of indomethacin SLMs as compared to the unformulated indomethacin, ultimately occasioning absorption-enhancement and possible consistent bioavailability. NSAIDS associated with phosphatidylcholine have previously been reported to improve therapeutic effect of the NSAIDS relative to the NSAID alone (Bhupinderjit et al., 1999).

It should be recalled that indomethacin belongs to class 11 biopharmaceutics classification system, characterized by low aqueous solubility and high permeability. High and consistent permeability can only be guaranteed if solubility, which is the rate-limiting step is improved. In this work, the lipid formulation carrier has demonstrated the capacity to enhance drug absorption by pharmacodynamically providing significantly ($P < 0.05$) higher anti-inflammatory activity over unformulated indomethacin. NSAIDs pharmacologically inhibit COX-2, the inflammation promoting agent at inflamed tissues and also COX-1 with attendant toxicities, such as gastrointestinal problems (Bhupinderjit et al., 1999; Martindale, 2009). The assiduous search for selective NSAIDs gave birth to such hope-raising drug alternatives as celecoxib, rofecoxib, and valdecoxib. Unfortunately, cardiovascular risks truncated the fate of rofecoxib and valdecoxib. Hitherto, in some countries, the acceptability and continued use of celecoxib is contingent upon label warnings of potential cardiovascular risks. In the light of this, recourse to traditional non-selective, inexpensive NSAIDs (indomethacin, acetylsalicylic acid, etc) with pharmaceutically enabled functional capability to mitigate or preclude gastrointestinal disorders without compromising therapeutic efficacy would be a welcome development. This is the reason for our choice of a lipid- based delivery system for the delivery of indomethacin. The application of phospholipids in the oral delivery of NSAIDs is premised upon the gastroprotective potential of both the endogenous and exogenous forms (Fricker et al., 2010; Parnham and Leyek, 1988). Phospholipid (phosphatidylcholine) maintains the

Table 3. Ulcerogenic property of Indomethacin SLM.

Batch	Ulcer index (Mean ± SD)[a]
Y1	0.00 ± 0.00
P1 (Reference)	14.00 ± 1.12
P2 (Control)	0.00 ± 0.00

[a]n = 5; Y1: indomethacin-loaded SLM; P1: unformulated indomethacin powder; P2: normal saline.

hydrophobic integrity of the mucosal surface of the GIT which NSAIDs may erode (Katare et al., 1991). Gastric surface mucus produces surfactant-like phospholipid which is subsequently mobilized to the luminal interface of the mucus gel layer to institute hydrophobicity (Bhupinderjit et al., 1999; Lichtenberger, 1985; Kao and Lichtenberger, 1991). Phospholipids stimulate COX-1 to synthesize prostaglandin, whereas some NSAIDs including indomethacin inhibit COX-1. Synthesis of prostaglandin by the phospholipid appeared to have superseded its inhibition by indomethacin, hence, the observed gastro protection. It is pertinent to mention that *C. hircus* lipid which constituted 70% of the entire lipid matrix could be sourced at a very little cost. Therefore, the economic and pharmaceutical significance of a blend of Phospholipon 90 G and the animal lipid was underscored by obvious synergism provided by the retention of the unique biological function of the phospholipid and the impartation of firm structural discreteness by the animal lipid.

Conclusion

In this study, particle size, encapsulation efficiency, and drug release were affected by sodium chloride, drug load, and the lipid matrix. Secondly, the formulation strategy employed in this work provided barrier fortification against gastric ulceration without compromising the anti-inflammatory activity of indomethacin. We therefore conclude that lipid microparticles involving a phospholipid and lipid derived from *C. hircus* were robustly effective in ensuring acceptable pharmacodynamic characteristics.

REFERENCES

Ajali U, Okoye FBC (2009). Antimicrobial and anti-inflammatory activities of *Olax viridis* root bark extracts and fractions. Int. J. Appl. Res. Nat. Prod. 2(1):27-32.

Arun R, Ashok KCK, Sravanthi V (2008). Cyclodextrins as drug carrier molecule: A Review. Sci. Pharm. 76:567–598.

Bagwe RP, Kanicky JR, Palla BJ, Patanjali PK, Shah DO (2001). Improved drug delivery using microemulsions: Rationale, recent progress and new horizons. Crit. Rev. Ther. Drg. Carr. Syst. 18:177-140.

Bhupinderjit SA, Jim JR, Sudershan KS, Lenard ML (1999). Phospholipid Association Reduces the Gastric Mucosal Toxicity of Aspirin in Human Subjects.The AMER. J. Gastroent. 94: 1818-1822.

Carey MC, Small DM, Bliss CM (1983). Lipid digestion and absorption. Ass Rev Physiol. 45: 651-677.

Cashin CH, Dawson W, Kitchen EA (1979). The pharmacology of benoxaprofen (2, 4 chloropheny-methyl-5-benzoxazole acetic acid) LRC.L3694, a new compound with anti-inflammatory activity apparently unrelated to inhibition of prostaglandin synthesis. J. Pharm. Pharmacol. 29:330-336.

Cortesi R, Esposita E, Luca G, Nastruzzi C (2003). Production of lipospheres as carries for bioactive compounds. Biomaterials 23:2283-2294.

De Filippis P, Boscolo M, Gibellini M, Rupena P, Rubessa F, Moneghini M (1991). The release rate of indomethacin from solid dispersions with Eudragit E. Drug. Dev. Ind. Pharm. 17:2017-2028.

Domb AJ, Maniar M (1996). Lipospheres for controlled delivery of substances. European Patent, EP0 502119.

Ekpendu JO, Akah PA. Adesomoju AA, Okogun JI (1994). Anti-inflammatory and Anti-microbial activities of *Mitracarpus scaber* extracts. Int. J. Pharmacog. 32:991-2196.

El-Gibaly I, Abdel-Ghaffer SK (2005). Effect of hexacosanol on the characteristics of novel sustained release allupurinol solid lipospheres: factorial design application and product evaluation. Int. J. Pharm. 294:33-51.

EL–Kamel HA, AL–fagih MI, Alsarra AI (2007). Testosterone solid lipid microparticles for transdemal drug delivery formulation and physicochemical characterisation. J. Microencapsul. 24(5):457-475.

Fricker G, Kromp T, Wendel A, Blume A, Zirkel J, Rebmann H, Setzer C, Quinkert R, Martin F, Müller-Goymann C (2010). Phospholipids and lipid-based formulations in oral drug delivery. Pharm. Res. 27:1469-1486.

Hou DZ, Xie CS, Huang K, Zhu CH (2003). The production and characterisatics of solid lipid nanoparticles (SLN). Biomaterials 24:1781-1785.

Jaspert S, Bertholet P, Piel G, Dogne JM, Delattre L, Evrard B (2007). Solid lipid microparticles as a sustained release system for pulmonary drug delivery. Eur. J. Pharm. Biopharm. 65:47-56.

Kao YCJ, Lichtenberger LM (1991). Phospholipid- and neutral-lipid organelles of the rat gastroduodenal mucus cells. Gastroenterology 101:7-21.

Katare OP, Vyas SP, Dixit VK (1991). Preparation and performance evaluation of plain proliposomal systems for cytoprotection. J. Microencaps 8:295-300.

Koo SI, Noh SK (2001). Phosphatidylcholine inhibits and lysophosphatidylcholine enhances the lymphatic absorption of alphatocopherol in adult rats. J. Nutr. 131:717-22.

Lichtenberger LM (1995). The hydrophobic barrier properties of gastrointestinal mucus. Annu. Rev. Physiol. 57:565-583.

Main IHM, Whittle NB (Jnr) (1975). Investigation of vasodilator and antisecretory role of prostagladin in the rat mucosa by use of NSAIDs. Brit. J. Pharmacol. 53:217-224.

Martindale (2009).The Pharmaceutical Press, London, UK, 36 edn., Sweetman, S.C.(ed).

May S, Kozlovsky Y, Ben-Shaul A, Kozlov MM (2004). Tilt modulus of a lipid monolayer. Eur. Phys. J. E. 14:299-308.

Nwafor A, Coakley WT (2003). The effect of membrane diffusion potential change on anionic drugs indomethacin and barbitone induced human red blood cell shape change and on cellular uptake of drugs. Afri. J. Biomed. Res. 6:95-100.

Obitte NC, Chukwu A, Onyishi VI, Obitte BCN (2009/2010). The physicochemical evaluation and applicability of Landolphia owariensis latex as a release modulating agent in its admixture with carbosil® in ibuprofen-loaded self-emulsifying oil formulations. Int. J. Appl. Res. Nat. Prod. 2(4):27-43.

Parnham MJ, Leyck S (1988). Phospholipon 100, New use. Drugs. Fut. 13:324-325.

Patil P, Joshi J, Paradkar P (2004). Effect of formulation variables on preparation and evaluation of gelled self-emulsifying drug delivery system (SEDDS) of Ketoprofen. AAPS Pharm. Sci. Tech. 5(3):42.

Perez GRM (1996). Anti-inflammatory activity of *Ambrosia artemisiaefolia* and *Rheo spathaceae*. Phytomedicine 3:163-164.

Porter CJH, Trevaskis NL, Charman WN (2007). Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. Nat. Rev. Drug. Discov. 6(3):231-248.

Pouton CW (2000). Lipid formulations for oral administration of drugs: non-emulsifying, self emulsifying and self microemulsifying drug delivery systems Eur. J. Pharm. Sci. Suppl. 2:S93-S98.
Shivakumar HN, Patel PB, Desai BG, Ashok P, Arulmozhi S (2007).
Design and statistical optimization of glipizide loaded lipospheres using response surface methodology. Acta. Pharm. 57:269-285.
Tewes F, Boury F, Benoit J (2006). Biodegradable Microspheres: Advances in production technology. In: Benita S., editor. *Microencapsulation*: Methods and industrial applications. 2nd ed. New York: Taylor and Francis Group. pp. 1-20.
Tirkkonen S, Paronen P (1992). Enhancement of drug release from ethylcellulose microcapsules using solid sodium chloride in the wall. Int. J. Pharm. 88:39-51.
Tirkkonen S, Urtti A, Paronen P (1995). Buffer controlled release of indomethacin from ethylcellulose microcapsules. Int. J. Pharm. 124:219-229.
Trevaskis NL, Porter CJH, Charman WN (2006). The lymph lipid precursor pool is a key determinant of intestinal lymphatic drug transport. J. Pharmacol. Exp. Ther. 316:881-91.
Winter EA, Risley EA, Nuss GU (1963). Anti-inflammatory and antipyretic activities of indomethacin. J. Pharm. Exp. Ther. 141:367-376.
Yuce M, Canefe K (2008). Indomethacin-loaded microspheres: Preparation, characterization and *in vitro* valuation regarding ethylcellulose matrix material. Turk. J. Pharm. Sci. 5(3):129-142.
Yue C, Yu Z, Xing T (2008). *In vitro* and *in vivo* evaluation of ofloxacin sustained release pellets. Int. J. Pharm. 360:47-52.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2318-2321, 15 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.711
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Combination therapy with simvastatin and xuezhikang improves the lipid-lowering efficacy in hyperlipidemic rats

Yang Lin, Ming Cai, Yanru Lin and Hong Teng*

Department of Gynaecology and Obstetrics, the Second Hospital of Jilin University, Jilin, 130021, China.

Accepted 7 August, 2012

The objective of the present study was to investigate the effects of combination therapy with simvastatin and xuezhikang on the lipid-lowering efficacy and liver functions in hyperlipidemic rats. Forty (40) hyperlipidemic rats induced by feeding with high-fat diet for 6 weeks were randomly divided into four groups (n = 10), and treated with saline (10 ml/kg/d), simvastatin (12 mg/kg/d), xuezhikang (300 mg/kg/d) and xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d), respectively. Six (6) weeks later, all the rats were killed to collect plasma for determination of blood lipid, liver enzymes and total bilirubin (TB). The results showed that plasma levels of total cholesterol (TC), total carbohydrates (TCHO) and low-density lipoprotein cholesterol (LDL-C) of hyperlipidemic rats treated with Xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d) were significantly lower than those with 12 mg/kg/d of simvastatin and 300 mg/kg/d of Xuezhikang ($p < 0.05$). The results also showed that treating with xuezhikang (300 mg/kg/d) plus Simvastatin (6 mg/kg/d) could significantly decrease the level of glutamate pyruvate transaminase (ALT) and glutamate oxaloacetate transaminase (AST), while treating with simvastatin (12 mg/kg/d) only significantly decreased plasma ALT concentration ($p < 0.05$). The results suggested that combination therapy with xuezhikang (300 mg/kg/d) and simvastatin (6 mg/kg/d) not only could significantly improve the lipid-lowering efficacy, but also significantly decrease the level of liver enzymes, compared to simvastatin monotherapy.

Key words: Simvastatin, xuezhikang, combination therapy, hyperlipaemia.

INTRODUCTION

Hyperlipidemia refers to a disorder of lipid metabolism which causes abnormally elevated levels of cholesterol, triglycerides (TG) and lipoproteins in the blood. It is widely accepted that hyperlipidemia is a key risk factor for atherosclerosis (AS) and coronary heart disease (CHD) and peripheral vascular disease (PVD) (Poss et al., 2011; Vaziri and Norris, 2011). Therefore, it is of great importance to treat hyperlipidemia. Statins, the 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors, have shown to be the most efficacious drug therapy for hyperlipidemia. Statins not only can significantly reduce the plasma low-density lipoprotein cholesterol (LDL-C) concentration, but also reduce cardiovascular morbidity and mortality in patients with dyslipidemia (Moghadasian, 2002; Shurraw and Tonelli, 2006). In clinical practice, intensive lipid-lowering was often needed in order to reduce cardiovascular events (Murphy et al., 2007; Nissen et al., 2004). Statin monotherapy cannot fully meet this demand for two reasons. First, studies have shown that most of the LDL-C lowering effect of statins occurs at the starting dose; doubling the dose of statin results in only a 6% incremental reduction in LDL-C levels (Jones et al., 2003). Second, high-dose of statins therapy may cause liver enzyme abnormalities which make some patients cannot effectively accomplish the treatment (Bhardwaj and Chalasani, 2007; Tolman, 2002). This suggests that combination therapy of a moderate dose of statins with

*Corresponding author. E-mail: tenghong126@yahoo.cn.

Table 1. Level of plasma lipid of rats in control and high-fat diet group (mmol/L).

Diet	TCHO	LDL-C	TG	HDL-C
Normal diet	1.84 ± 0.32^{B}	0.25 ± 0.12^{B}	0.40 ± 0.12	0.42 ± 0.13
High-fat diet	9.46 ± 2.18^{A}	4.22 ± 0.52^{A}	0.59 ± 0.19	0.48 ± 0.18

Values in the same column with different superscript capital letters (A, B) indicate significant differences at $p < 0.01$.

other drugs rather than only high dose of statins may be more effective in intensive lipid-lowering goals. Xuezhikang, an extract from red yeast rice, contains a lot of natural statins, unsaturated fatty acids and other substances. Xuezhikang has been proved to markedly lower fasting total cholesterol and triglyceride concentrations in patients with hyperlipidemia (Kou et al., 1997; Liu et al., 2003). To our knowledge, there is lack of information of combination therapy of simvastatin and xuezhikang in hyperlipidemia. The aim of the present study was to compare the therapeutic effect of combination therapy of simvastatin and xuezhikang with that of using high dose of simvastatin intensive lipid-lowering in hyperlipidemic rats induced by high lipid diet.

MATERIALS AND METHODS

Drugs

Xuezhikang (300 mg of cholestin per capsule, WBL Peking University Biotech Co., Ltd); Simvastatin (Yichang Changjiang Pharmaceuticals Co., Ltd).

Acute toxicity test

Twenty (20) Wistar rats (10 of each sex), weighing190 ± 10 g, were divided into two groups (each with 5 females and 5 males). Standard diet was provided and water was available *ad libitum*. For the two groups, saline (10 ml/kg/d) and xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d) were administrated to stomach of the rats, respectively. All animals were treated once daily for 14 days and were observed for the changing behavior, mortality and poisoning. After treatments, all sacrificed animals were dissected for their livers, kidneys for histopathological examination.

Lipid-lowering test

Animals and diets

Sixty male Wistar strain rats, weighing 190 ± 10 g, were used in this study. All the rats were maintained in an air conditioned room under lighting conditions (12 h-light and 12 h-dark cycle) and fed with standard diet and water available *ad libitum*. After 1 week's accommodation, control group (n = 10) was fed with standard diet, and the treatment group (n = 50) was fed with high-fat diet containing 10% yolk powder, 5% lard, 0.2% propylthiouracil and 84.5% basal diet to establish hyperlipidemic rat (Wang et al., 2003). Six (6) weeks later, 10 rats were randomly selected from each group and treated with intraperitoneal injection of 5% pentobarbital (0.1 ml/100 g) for collecting arterial blood. Blood samples mixed with heparin (20 IU/ml) were centrifuged at 3000 rpm for 5 min, and plasma was separated and then stored at -20℃ until b lood lipid analysis .

The remainder rats in high-fat diet group (n = 40) were randomly divided into four groups (n = 10), and saline (10 ml/kg/d), simvastatin (12 mg/kg/d), xuezhikang (300 mg/kg/d), xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d) were administrated to stomach of the rats in four groups, respectively. Simvastatin and xuezhikang were both administrated to stomach for 6 weeks. All the rats were fed with high-fat diet during the experiment. Six (6) weeks later, all the rats were killed to collect plasma for determination of blood lipid, liver enzymes and total bilirubin (TB).

Biochemical determination of plasma

Serum LDL-C, triglycerides (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels were determined as described by Kong et al. (2005). Glutamate oxalocetate transaminase (AST) and glutamate pyruvate transaminase (ALT) were determined by method of Wilkinson et al (1972). TB was measured by the 2,5-dichlorophenyldiazonium (DPD) method (Wahlefeld et al., 1972).

Statistical analysis

Data were analyzed using the SPSS 13.0 for Windows statistical software. Differences in LDL-C, TG, TC, HDL-C, AST, ALT and TB were analyzed using one-way analysis of variance (ANOVA) and Newman-Keuls multiple comparison test. In general, p-values less than 0.05 were considered statistically significant.

RESULTS

During the acute toxicity test period, no poisoning symptoms, abnormal behaviour and death were observed. There was no abnormal condition for the pathologic examination of livers and kidneys observed with the naked eye. Effects of high-fat diet on level of blood lipid in rats are shown in Table 1. The results showed that plasma levels of total carbohydrates (TCHO) and LDL-C of rats fed with high-fat diet for 6 weeks were significantly increased, compared to control group ($p < 0.01$). There were no significant differences in plasma level of TG and HDL-C in rats of high-fat diet groups in comparison to normal control group. The plasma levels of lipid in hyperlipidemic rats treated with different treatment protocols are shown in Table 2. The results showed that simvastatin (12 mg/kg/d), xuezhikang (300 mg/kg/d) and xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d)

Table 2. Level of plasma lipid of rats in different treatment groups (mmol/L).

Group	TC	LDL-C	TG	HDL-C
Saline	13.24 ± 1.17[a]	6.48 ± 1.32[a]	0.58 ± 0.15[a]	0.58 ± 0.10[a]
Simvastatin	7.86 ± 1.21[b]	3.95 ± 0.96[b]	0.21 ± 0.12[b]	1.21 ± 0.25[b]
Simvastatin plus xuezhikang	4.28 ± 1.12[c]	1.28 ± 0.47[c]	0.19 ± 0.13[b]	1.15 ± 0.34[b]
Xuezhikang	7.58 ± 1.08[b]	3.71 ± 1.15[b]	0.28 ± 0.23[b]	1.33 ± 0.14[b]

[a–c], Means within a column without a common superscript letter significantly different ($P < 0.05$).

Table 3. Level of liver enzymes and TB of rats in different treatment groups.

Group	AST (U/L)	ALT (U/L)	TB (umol/L)
Saline	210.32 ± 54.21[a]	59.74 ± 18.34[a]	3.64 ± 2.55[a]
Simvastatin	148.53 ± 65.89[a]	26.28 ± 10.49[b]	3.99 ± 2.48[a]
Simvastatin plus xuezhikang	114.28 ± 23.46[b]	22.13 ± 9.26[b]	4.01 ± 2.82[a]
Xuezhikang	117.46 ± 25.66[b]	28.30 ± 8.19[b]	3.82 ± 2.14[a]

[a–b], Means within a column without a common superscript letter significantly difference ($P < 0.05$).

could significantly decrease plasma levels of TCHO, LDL-C and TG of hyperlipidemic rats ($p < 0.05$); however, plasma level of HDL-C of hyperlipidemic rats was significantly increased ($p < 0.05$). Plasma level of TC, TCHO and LDL-C of hyperlipidemic rats treated with xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d) were significantly lower than those with simvastatin and xuezhikang monotherapy ($p < 0.05$). Effects of different remedy protocols on plasma levels of AST, ALT and TB are shown in Table 3. The results showed that treatment with xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d) and xuezhikang monotherapy could significantly decrease the level of ALT and AST, while treatment only with simvastatin significantly decreased only plasma ALT concentration. There was no significant difference in plasma TB for the four groups ($P > 0.05$).

DISCUSSION

In order to investigate the combination therapy of simvastatin and xuezhikang on hyperlipidemic rats, the present study first successfully established hyperlipidemic rats by feeding with high-fat diet for 6 weeks and then compared the treatment effect of saline, simvastatin alone and in combination with xuezhikang on hyperlipidemic rats. The results demonstrated that treatment with xuezhikang (300 mg/kg/d) plus simvastatin (6 mg/kg/d) not only could significantly improve the antilipemic effects, but also significantly decreased the level of liver enzymes, compared to simvastatin and Xuezhikang monotherapy.

Hyperlipidemia is involved in CHD process, and effective lipid-lowering remedy is important for CHD prevention (Dembowski and Davidson, 2009). Statins are considered first-line therapy in lipid-lowering according to large clinical outcome trials which have consistently demonstrated efficacy in LDL-C reduction and prevention of cardiovascular events. However, statins alone in many patients are insufficient to achieve current guideline-recommended LDL-C goals and the safety of statins at high doses was also concerned (Jones et al., 2003; Tolman, 2002). For this reason, combination therapy of a moderate dose of statins with other drugs has been studied and applied in clinical treatments. For example, combination therapy with a statin and fibrate offers significant therapeutic advantage for the treatment of severe or refractory mixed hyperlipidemia (Shek and Ferrill, 2001). The rationale, effectiveness, and safety of the combination therapy for hyperlipidemia using berberine and simvastatin were displayed and the results showed that therapeutic effect was significantly increased compared with that in simvastatin monotherapy (Kong et al., 2008). Combination therapy of ezetimibe with statin results in an additional 15 to 20% reduction in LDL-C in primary hypercholesterolemia patients (Davidson et al., 2004). In addition, studies have demonstrated that combination therapy of ezetimibe with the lowest statin dose was as effective as statin monotherapy at the highest dose (Dembowski and Davidson, 2009).

Xuezhikang, an extract of red yeast rice, has been shown to significantly reduce serum TC levels, TG levels and LDL-C levels in hyperlipidemic rat model and patients, and the lipid modification effects appeared to be similar to pravastatin, simvastatin, lovastatin and atorvastatin (Liu et al., 2003; Wu et al., 2009). Study has demonstrated that treatment with xuezhikang is safe and effective for the secondary prevention of CHD in older

people (Ye et al., 2007). To our knowledge, there is no information on combination therapy of xuezhikang and statins in hyperlipidemic rats. The results showed that combination therapy with xuezhikang and simvastatin could significantly improve the effect of lipid-lowering hyperlipidemic rats.

The present study also investigates the effect of combination therapy on liver enzymes. The results showed that combination therapy could significantly decrease the level of ALT and AST, while high dose of simvastatin monotherapy only decreased level of ALT. Studies in humans have suggested that elevated liver enzymes are often caused by fatty liver and statins can treat this disease (Argo et al., 2008). Recently, it has been shown that statins treatment could improve the liver function and decreases the liver enzymes concentrations for patients with non-alcoholic fatty liver disease. The exact mechanisms of the beneficial effects of statins on liver function are not clear. Statins have been shown to decrease serum concentrations of advanced glycation end products in non-alcoholic steatohepatitis patients with dyslipidaemia, which have a detrimental effect on the liver (Kimura et al., 2010). For the chickens with non-alcoholic fatty liver disease induced by feeding a hyperlipidaemic diet, treating with atorvastatin could decrease steatosis and inflammation, and reduce hepatocellular damage compared with non-treated animals (Martín-Castillo et al., 2010). The limitation of this study is shortage of long-term observation, which cannot show the effects of long-term treatment on lipid-lowering efficacy and liver function in hyperlipidemic rats.

In conclusion, the present study showed that combination therapy of xuezhikang and 6 mg/kg/d of simvastatin could significantly improve the effect of lipid-lowering and decrease the level of liver enzymes, compared to high dose of simvastatin monotherapy.

REFERENCES

Argo CK, Loria P, Caldwell SH, Lonardo A (2008). Statins in liver disease: a molehill, an iceberg, or neither? Hepatology 48(2):662-669.

Bhardwaj SS, Chalasani N (2007). Lipid-lowering agents that cause drug-induced hepatotoxicity. Clinics liver dis. 11(3): 597-613.

Davidson M, Ballantyne C, Kerzner B, Melani L, Veltri E (2004). Efficacy and safety of ezetimibe coadministered with statins: randomised, placebo-controlled, blinded experience in 2382 patients with primary hypercholesterolemia. Int. J. Clin. practice 58(8):746-755.

Dembowski E, Davidson MH (2009). Statin and ezetimibe combination therapy in cardiovascular disease. Current Opinion in Endocrinology. Diab. Obesity 16(2):183-188.

Jones PH, DavidsonM H, Stein EA, Bays HE, Blasetto JW (2003). Comparison of the efficacy and safety of rosuvastatin versus atorvastatin, simvastatin, and pravastatin across doses (STELLAR* Trial). Am. J. Cardiol. 92(2):152-160.

Kimura Y, Hyogo H, Yamagishi S, Chayama K (2010). Atorvastatin decreases serum levels of advanced glycation endproducts (AGEs) in nonalcoholic steatohepatitis (NASH) patients with dyslipidemia: clinical usefulness of AGEs as a biomarker for the attenuation of NASH. J. Gastroenterol. 45(7):750-757.

Kong WJ, Wei J, Zuo ZY, Jiang JD (2008). Combination of simvastatin with berberine improves the lipid-lowering efficacy. Metabolism 57(8):1029-1037.

Kong W, Abidi P, Kraemer FB, Liu J (2005). In vivo activities of cytokine oncostatin M in the regulation of plasma lipid levels. J. Lipid Res. 46(6): 1163-1171.

Kou W, Lu Z, Guo J (1997). Effect of xuezhikang on the treatment of primary hyperlipidemia. Zhonghua nei ke za zhi 36(8):529-531.

Liu L, Zhao S P, Cheng YC, Li YL (2003). Xuezhikang decreases serum lipoprotein (a) and C-reactive protein concentrations in patients with coronary heart disease. Clin. Chem. 49(8):1347-1352.

Martín-Castillo A, Castells MT, Ayala I (2010). Effect of atorvastatin and diet on non-alcoholic fatty liver disease activity score in hyperlipidemic chickens. Biomed. Pharmacother. 64(4):275-281.

Moghadasian MH (2002). A safety look at currently available statins. Expert opinion on drug safety 1(3):269-274.

Murphy SA, Cannon CP, Braunwald E (2007). Effect of intensive lipid-lowering therapy on mortality after acute coronary syndrome (a patient-level analysis of the Aggrastat to Zocor and Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction 22 trials). The Am. J. Cardiol. 100(7):1047-1051.

Nissen SE, Tuzcu EM, Brodie B (2004). Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis. JAMA 291(9):1071-1080.

Poss J, Custodis F, Werner C, Laufs U (2011). Cardiovascular disease and dyslipidemia: Beyond LDL. Current Pharmaceutical Design 17(9): 861-870.

Shek A, Ferril MJ (2001). Statin-fibrate combination therapy. Annals pharmacother. 35(7/8):908-917.

Shurraw S, Tonelli M (2006). Statins for treatment of dyslipidemia in chronic kidney disease. Peritoneal Dialysis Int. 26(5): 523-539.

Tolman K G (2002). The liver and lovastatin. Am. J. Cardiol. 89(12): 1374-1380.

Vaziri ND, Norris K (2011). Lipid disorders and their relevance to outcomes in chronic kidney disease. Blood Purif.31(1-3):189-196.

Wahlefeld A, Herz G, Bernt E (1972). Modification of the Malloy-Evelyn method for a simple, reliable determination of total bilirubin in serum. Scand. J. Clin. Lab. Invest. 29(26):11-12.

Wilkinson J, Baron D, Moss D,Walker P (1972). Standardization of clinical enzyme assays: a reference method for aspartate and alanine transaminases. J. Clinical Pathol. 25(11):940-940.

Wu H, Bei J, Guo J (2009). Chinese herbal medicine for treatment of dislipidemia. J. Geriatric Cardiol. 6(2):119-125.

Ye P, Yu X H, Zhao Y C (2007). Effect of xuezhikang on cardiovascular events and mortality in elderly patients with a history of myocardial infarction: a subgroup analysis of elderly subjects from the China Coronary Secondary Prevention Study. J. Am. Geriatrics Soc. 55(7): 1015-1022.

African Journal of Pharmacy and Pharmacology Vol. 6(30), pp. 2322-2331, 15 August, 2012
Available online http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.1048
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Ab initio studies of single-wall carbon nanotube for drug delivery of (N-acetyl-L-cysteinato-O, S) diphenyl tin (IV) anticancer drug

Amir Sobhanmanesh[1]*, Majid Monajjemi[1], Karim Zare[1] and Golnaz shams[2]

[1]Department of Chemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Entomology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Accepted 23 April, 2012

Carbon nanotubes (CNT) have emerged as a new alternative and efficient tool for transporting and translocating therapeutic molecules. CNT can be attached to drugs, and used to deliver their cargos to cells and organs. In this work, the interaction behavior of the anticancer drug $Sn(C_6H_5)_2$(N-acetyl-L-cysteinate) that is called (N-acetyl-Lcysteinato-O,S) diphenyl tin (IV) when binding single-walled carbon nanotubes was studied based on the Quantum chemical *ab initio* calculations by using the HF/ (LanL2DZ+STO-3G) and HF/ (LanL2DZ+6-31G) levels in both gas phase and solution. Thermodynamical analysis (large negative values of the ΔG and high positive values of ΔS) confirmed the structural stability of the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT in both gas phase and in solution. Also, The thermodynamic analysis show that $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT has maximum stability in methanol at 313K. Moreover, nuclear magnetic resonance (NMR) parameters such as chemical shift tensor (δ), total atomic charge and asymmetry parameter (η) have been calculated using the Gauge Independent Atomic Orbital (GIAO) method, results being compared with CGST data. From the NMR calculations, it can be seen that the NMR parameters (δ, η) at the sites of nitrogen and oxygen are significantly influenced by intermolecular hydrogen- bonding interactions but the quantity at the site of S-27 is influenced by nonspecific solute-solvent interaction such as polarisability/polarity.

Key words: $Sn(C_6H_5)_2$(N-acetyl-L-cysteinate), *ab initio*, single wall carbon nanotubes (SWCNT), drug delivery.

INTRODUCTION

The chemistry of organotin (IV) has witnessed an increased interest during the last fifty years, owing to their potential biological and industrial applications. However, some organotin (IV) compounds, which were originally modeled on the first tumor-active platinum compound, cisplatin (Barnard, 1989), have also found their place among a class of non-platinum chemotherapeutic metallopharmaceuticals exhibiting good antitumor activity (Clarke et al., 1999; Nath et al., 2001). In this context, Diorganotin (IV) derivatives and mainly those of dialkyltin (IV) complexes from amino acids ligand are known to possess antimicrobial (Plesch et al., 1988), antimalarial (Goldberg et al., 1997), antiproliferative (K♀pf-Maier and K♀pf, 1987), chemotherapeutic (Wang et al., 2005), radiopharmaceutical (Wang and Meng, 2006), insulin-mimetic (Pessoa et al., 1999) and fungicidal (Eng et al., 1996) activities. Further, tin (IV) complexes characterized by the presence of amino acid ligands have proved to be cytotoxic against the breast adenocarcinoma tumor MCF-7, the colon carcinoma (Gielen, 1996) and hepatocarcinoma HCC Hep G2 cancer, (Pellerito et al., 2010). In 2010 Girasolo et al. reported the antitumor activity of organotin(IV) complexes containing L-Arginine, Nα-t-Boc-L-Arginine and L-Alanyl-L-Arginine against the Human colon-rectal carcinoma HT29, observing that for all these complexes, cytotoxic activity was higher than that exerted by cisplatin (Girasolo et al., 2010).

In 2010 Tzimopoulos et al. reported the results of a screening on wide range of triorganotin aminobenzoates in the K562 myelogenous leukaemia, HeLa

*Corresponding author. E-mail: amirsobhanmanesh@yahoo.com. Tel: +989179113508. Fax: +982144865464.

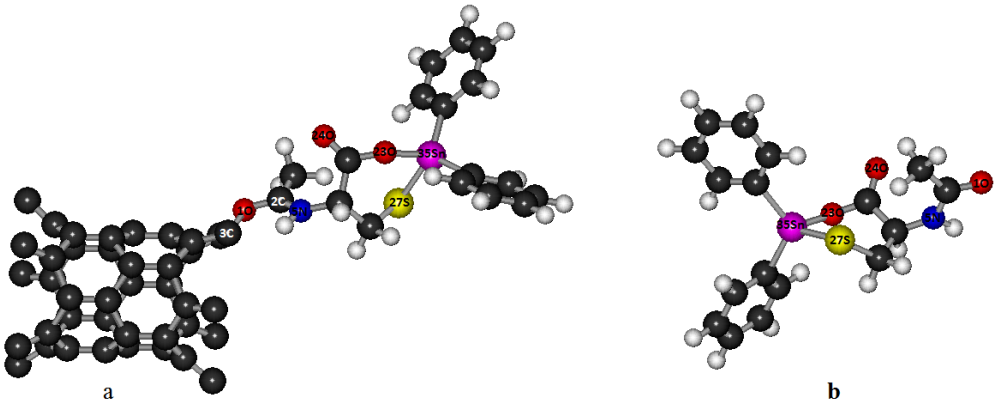


Figure 1. Optimized geometry of the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex (a) and $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) (b), obtained at the (LanL2DZ+6-31G) level.

cervical cancer and HepG2 hepatocellular carcinoma cells, observing that for triorganotin complexes containing aminobenzoates, cytotoxic activity was better than cisplatin and some triorganotin carboxylates drugs (Tzimopoulos et al., 2010). Furthermore, the cytotoxic activity of Diorganotin (IV) N-acetyl-L-cysteinate complexes towards Human hepatocarcinoma HCC Hep G2 cells were studied by Lorenzo Pellerito and Cristina Prinzivalli in 2010. As result Sn(C6H3)2(N-acetyl-L-cysteine) has been proposed as a therapeutic against the human hepatocarcinoma HCC Hep G2 cells (Pellerito et al., 2010). In the case at hand, since the discovery of carbon nanotubes (CNTs) (Iijima, 1991), they have been considered as the ideal material for a variety of applications owing to their unique properties. These properties include their potential biocompatibility in pharmaceutical drug delivery systems and their excellent role as drug carriers with a highly site-selective delivery and sensitivity (Bianco et al., 2005; Mollaamin et al., 2010a; Popov et al., 2007; Banerjee et al., 2005; Pastorin et al., 2006; Klumpp et al., 2006; Kostarelos et al., 2007; Raffa et al., 2008; Zhang et al., 2009; Monajjemi et al., 2010b).

To accelerate the optimal development of CNT as a new effective drug transporter, it is required to better understand the structural properties of the drug–CNT complex. In this paper we reported a computational study of the interaction between Diorganotin (IV) complexes of N-acetyl-L-cysteine (H2NAC; (R)-2-acetamido-3-sulfanylpropanoic acid) whit CNT. We perform a full geometrical, energetical, nuclear magnetic resonase, and vibrational analysis of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT with different basis set to elucidate the effect of site-specific of these systems. The aim of this study was to investigate the stability of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT at physiological conditions (temperature, solvent, etc.) and examine the effect of dielectric constant on stability of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex.

COMPUTATIONAL METHODS

The armchair carbon nanotube (5,5) that simulated in this study containing 60 carbon atoms, with both ends opened. In our model, $Sn(C_6H_5)_2$(NCA)was attached covalently to carbon nanotube (CNT). All calculations for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT were carried out by employing the GAUSSIAN 98 suite of programs (Frisch et al., 1998). Full geometry optimization and further frequency analysis were performed using Hartree-Fock (HF) level of theory in conjunction with the LanL2DZ basis set for Sn atom only (Mollaamin et al., 2010b; Monajjemi et al., 2010a; Hay and Wadt, 1985; Wadt and Hay, 1985) and STO-3G and 6-31G basis sets for other atoms. This was done for calculating the Gibbs free energy (ΔG) as well as entropy changes (ΔS) in both gas phase and in solution. The NMR parameters (such as Chemical shifts (δ), asymmetry parameter (η) and total atomic charges) were also evaluated on the optimized geometries employing the Gauge Independent Atomic Orbital (GIAO) and Continuous set of gauge transformations (CSGT) methods (Wrackmeyer 1985; Ditchfield 1972; Pulay et al., 1990; Keith and Bader, 1993). Moreover, the solvent effect is taken into account via the self-consistent reaction field (SCRF) approach (Miertus et al., 1981; Miertus and Tomasi, 1982; Monajjemi and Chahkandi, 2005).

RESULTS AND DISCUSSION

Molecular geometry

Figure 1 denotes the optimized structures of the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) and $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT whit active sites labeling. Also, some of selected parameters for the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT such as relative energy (kcal/mol) and dipole moments (Debye) are listed in

Table 1. Calculated relative energy (kcal/mol) and dipole moments (μ in Debye) of the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex obtained at the (LanL2DZ+STO-3G) and (LanL2DZ+6-31G) levels.

Phase/solvent	E (RHF)		E (Kcal/mol)		Dipole moment (Debye)	
Basis set	HF/Sto-3G	HF/6-31G	HF/Sto-3G	HF/6-31G	HF/Sto-3G	HF/6-31G
Gas phase	-2811.97925162	-2845.69296503	-1764520.9452822947842	-1785676.3479834056923	9.4891	23.2198
In water	-2811.98223377	-2845.71912981	-1764522.8165856246157	-1785692.7664197480321	11.3497	41.1363
In methanol	-2811.98211572	-2845.71790385	-1764522.7425090831652	-1785691.9971281194285	11.1915	40.2412
In ethanol	-2811.98205148	-2845.71724484	-1764522.7021983925868	-1785691.5835984152244	11.1059	39.7634

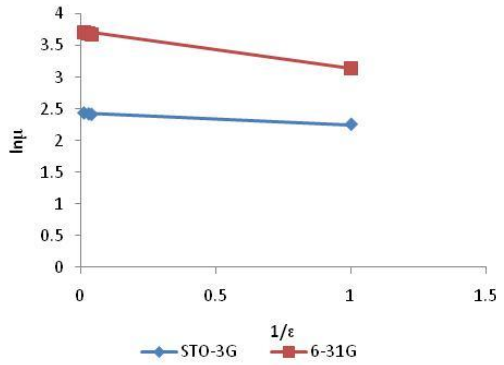


Figure 2. Plot of the lnμ (Debye) versus the 1/ε, obtain from the (LanL2DZ+STO-3G) and (LanL2DZ+6-31G) calculations for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT Complex.

Table 1. The results show that the calculated energy and dipole moment of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT are increased by increasing dielectric constant (ε) of solvent. The double reciprocal plot of lnμ versus 1/ε in gas phase and various solvents are shown in Figure 2.

Calculated NMR parameters

The calculated NMR parameters (such as Chemical shift (δ), asymmetry parameter (η) and total atomic charges) for active sites of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT, are given in Table 2. Also, the graphs of calculated Chemical shifts (δ), asymmetry parameter (η) and total atomic charges versus the number of atomic centers for selected atoms of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT system are displayed in Figures 3a to c respectively. As shown in Figure 3c, the Sn-35 nucleus has maximum total atomic charge and low δ values in both gas phase and in solution, meaning the relative chemical shift at the site of Sn-35 is predominantly governed by local diamagnetic shielding term (σ^d). Further, Tin atom has large amounts of asymmetry parameter (η) in both gas phase and in solution (sees Figure 3b). The results show that the calculated chemical shift tensor and asymmetry parameter at the site of Sn-35 are reduced by increasing dielectric constant (ε) of solvent (Figure 3a). The observed changes can be due to presence of the solvent molecule in the Tin inner coordination sphere. Since S-27 is more negative than Sn-35 (Figure 3c), this difference in the charges leads to a smaller chemical shift tensor (δ) for the sulfur atom (Figure 3a). The results in Figure 3a show that, with increase of dielectric constant from gas phase to water, the δ values at the site of sulfur atom increases. The observed effect is probably due to the nonspecific solute-solvent interaction (such as polarisability/polarity) at the site of S-27 nucleus. Since N has a lone pair of electrons in the valance shell, the electronic environment at the site of N is completely different from that of Sn, therefore, different behaviors are expected. This leads to the lower chemical shift values for the nitrogen atom (Figure 3a). The results in solution indicate that, the chemical shift tensor (δ) at the site of N-5 decreases in the order Gas Phase > Water > Ethanol > Methanol. In this regard, it seems that the chemical shift tensor at the site of N-5 nucleus is significantly influenced by intermolecular hydrogen- bonding interactions. Since carbon atoms (C-2 and C-3) are more positive than N-5 (Figure 3c), the δ values at the sites of C-2 and C-3 are greater than the N-5 nucleus (Figure 3a). The results show that the calculated chemical shift tensor and asymmetry parameter at the site of C-2 are reduced by increasing dielectric constant (ε) of solvent (Figure 3a and b). Furthermore, the calculated chemical shift tensor at the site of C-3 is reduced by increasing dielectric constant (ε) of solvent (Figure 3a). As shown in Figure 3b, C-3 has maximum asymmetry parameter in both water and Methanol, but the quantity in gas phase is minimum. Moreover, the chemical shift constants for the C-3 are larger than chemical shifts values for the C-2. This observation indicates that the C-2 is more shielded than the C-3 nucleus. It should be noted that C-2 nucleus has the greater η

Table 2. Calculated NMR parameters of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex in gas phase and various solvents at GIAO and CSGT method.

Atoms	Δ				H				Charge			
	GIAO		CSGT		GIAO		CSGT		GIAO		CSGT	
	HF/STO-3G	HF/6-31G	HF/STO-3G	HF/6-31G	HF/STO-3G	HF/6-31G	HF/STO-3G	HF/6-31G	HF/STO-3G	HF/6-31G	HF/STO-3G	HF/6-31G
						Gas phase						
1O	149.0242	270.3139	130.8648	257.8294	0.138392	0.087549	0.72994	0.065623	-0.19367	-0.76844	-0.19367	-0.76844
5N	137.0314	-140.103	-123.962	-140.451	0.767358	0.752471	0.576403	0.395483	-0.3738	-0.96905	-0.3738	-0.96905
23O	593.6829	506.8701	457.5637	438.5751	0.442454	0.378132	0.264253	0.424838	-0.23417	-0.5324	-0.23417	-0.5324
24O	37.5792	-126.16	-34.3105	-145.771	0.769042	0.626849	0.138742	0.517359	-0.33101	-0.90003	-0.33101	-0.90003
27S	203.8254	139.4606	-151.63	-139.746	0.280375	0.88155	0.818837	0.824889	0.049421	-0.27264	0.049421	-0.27264
35Sn	-4.5841	-3.3452	-4.5042	3.2571	0.522633	0.985801	0.78953	0.895981	0.526593	1.783351	0.526593	1.783351
2C	-47.177	121.4287	-32.0628	123.4359	0.289531	0.569121	0.46796	0.471298	0.245502	0.927947	0.245502	0.927947
3C	86.4499	-274.304	75.3443	-267.147	0.193959	0.024732	0.496049	0.087031	0.079401	0.24033	0.079401	0.24033
						Water						
1O	151.7131	272.34	132.8003	261.849	0.126644	0.193906	0.753713	0.079657	-0.19389	-0.76788	-0.19389	-0.76788
5N	136.1049	-142.382	-123.797	-141.149	0.746149	0.872626	0.567151	0.506131	-0.37362	-0.96513	-0.37362	-0.96513
23O	592.2061	496.0106	456.837	426.8305	0.443363	0.398766	0.264554	0.471952	-0.23424	-0.54585	-0.23424	-0.54585
24O	37.5217	-127.113	-34.9522	-147.984	0.75209	0.691053	0.13471	0.538829	-0.33389	-0.87797	-0.33389	-0.87797
27S	202.2831	-123.306	-151.151	-135.515	0.295098	0.999492	0.813059	0.696511	0.048433	-0.27743	0.048433	-0.27743
35Sn	-4.551	-3.5516	-4.4467	3.2057	0.527466	0.864371	0.762251	0.933774	0.527578	1.794255	0.527578	1.794255
2C	-48.4571	118.7468	-33.0871	121.2403	0.243265	0.703378	0.390291	0.631234	0.249783	0.926535	0.249783	0.926535
3C	85.7514	-342.939	75.2883	-330.226	0.221726	0.124264	0.512995	0.06738	0.079324	0.282279	0.079324	0.282279
						Methanol						
1O	151.9503	271.7766	132.9426	261.289	0.124139	0.181824	0.756078	0.065711	-0.194	-0.76811	-0.194	-0.76811
5N	136.1414	-142.284	-123.775	-141.119	0.746452	0.86511	0.570508	0.499591	-0.37363	-0.96561	-0.37363	-0.96561
23O	593.0419	496.2566	457.5026	427.1057	0.442207	0.397422	0.263622	0.469237	-0.23429	-0.54484	-0.23429	-0.54484
24O	37.4594	-127.329	-34.9035	-148.112	0.753095	0.68847	0.135571	0.538483	-0.33344	-0.87901	-0.33344	-0.87901
27S	202.4667	124.2449	-150.969	-135.884	0.295853	0.993235	0.812421	0.704528	0.04852	-0.27726	0.04852	-0.27726
35Sn	-4.5517	-3.5395	-4.4504	3.2086	0.526463	0.870801	0.761707	0.930063	0.527589	1.793847	0.527589	1.793847
2C	-48.382	119.0312	-33.0418	121.5143	0.242857	0.695786	0.389824	0.623234	0.249552	0.926554	0.249552	0.926554
3C	85.9541	-338.234	75.4805	-325.813	0.222186	0.120747	0.508269	0.063366	0.079171	0.279923	0.079171	0.279923
						Ethanol						
1O	151.6237	272.1817	132.808	261.4736	0.130365	0.179243	0.748562	0.062492	-0.1939	-0.76773	-0.1939	-0.76773
5N	136.197	-142.031	-123.666	-140.868	0.745975	0.865021	0.56683	0.499491	-0.37359	-0.96555	-0.37359	-0.96555
23O	592.01	496.286	456.6419	427.1608	0.442882	0.39728	0.263612	0.468624	-0.23426	-0.54458	-0.23426	-0.54458

Table 2. Contd.

24O	37.5154	-127.326	-34.9162	-148.035	0.753587	0.681847	0.134379	0.53522	-0.33361	-0.87974	-0.33361	-0.87974
27S	202.7531	124.6111	-150.798	-136.098	0.295062	0.992988	0.815267	0.705183	0.048393	-0.27741	0.048393	-0.27741
35Sn	-4.5531	-3.5293	-4.4522	3.2027	0.525598	0.872496	0.761017	0.929497	0.527108	1.793564	0.527108	1.793564
2C	-48.4346	119.2185	-33.0688	121.6555	0.248657	0.690011	0.396788	0.615337	0.249455	0.926916	0.249455	0.926916
3C	85.8151	-335.378	75.3244	-323.209	0.217786	0.114918	0.51115	0.057308	0.079446	0.278123	0.079446	0.278123

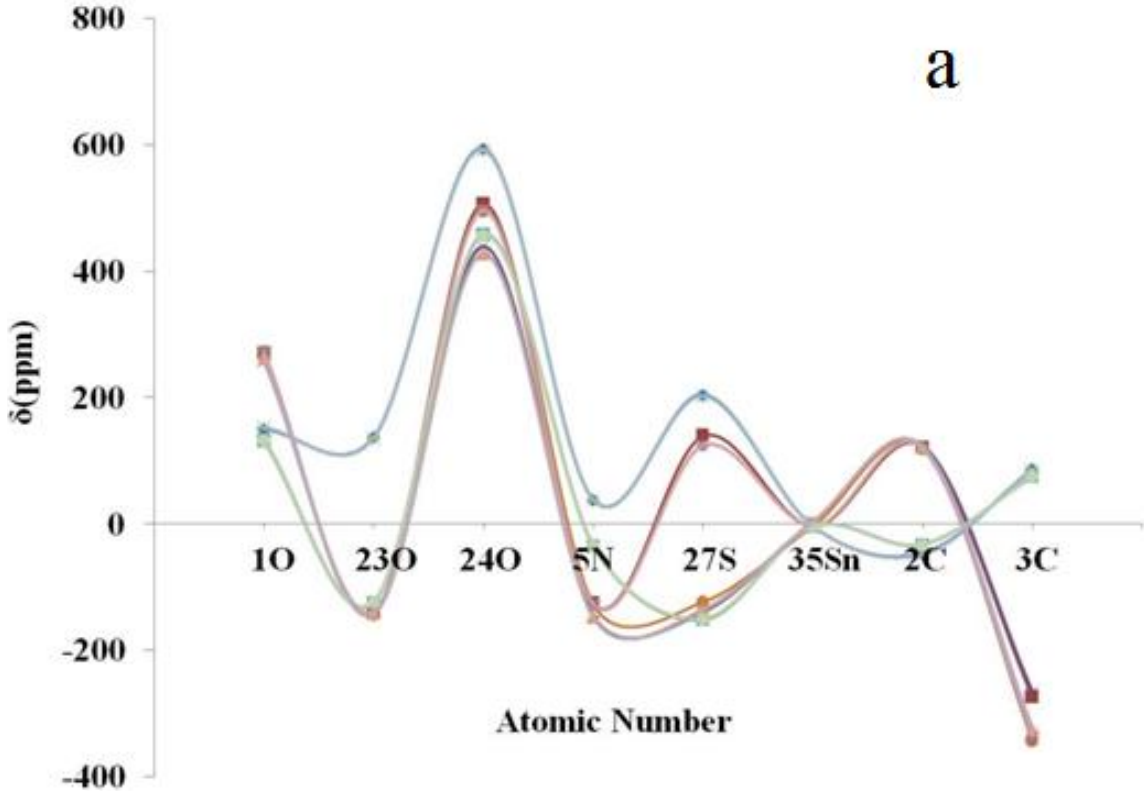


Figure 3. Graphs of the δ(ppm) versus atomic number (a), η versus atomic number (b), total atomic charge (a.u) versus atomic number (c), for selected atoms of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex in both gas phase and in solution obtain from the GIAO and CSGT methods.

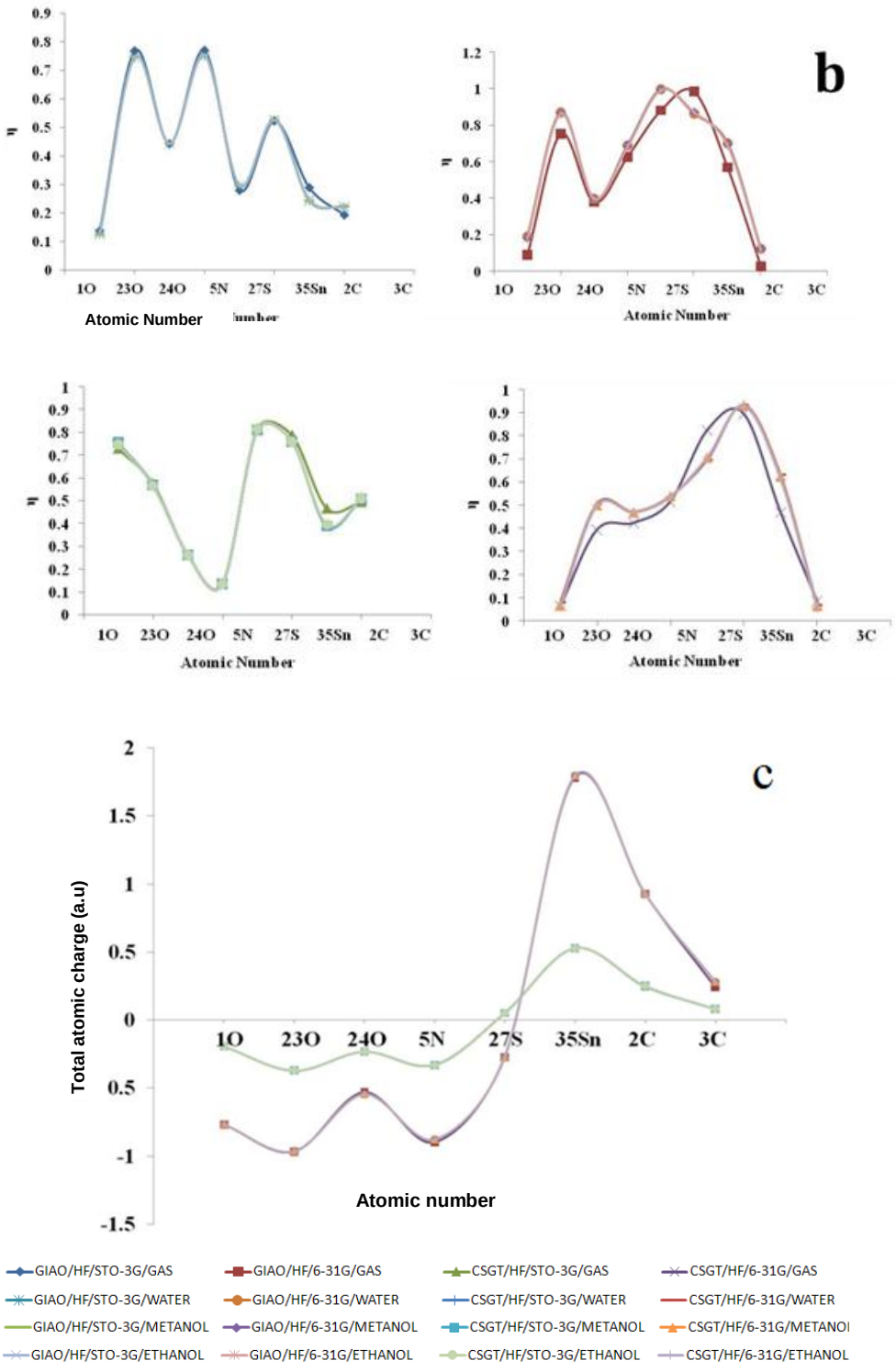


Figure 3. Contd.

Table 3. Calculated free Gibbs energies (kcal/mol) and entropies (kcal/molK) in both gas phase and in solution, for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex obtained at the level of HF/ (LanL2DZ+STO-3G).

Temperature (K)	Gas phase	Water ΔG(Kcal/mol)	Ethanol	Methanol
298.15	-1764193.05971898	-1764193.1099191	-1764191.510418	-1764193.14443167
300.15	-1764193.59748769	-1764193.64894281	-1764192.03438168	-1764193.68345539
302.15	-1764194.13776641	-1764194.19047652	-1764192.56148286	-1764194.2249891
304.15	-1764194.67992762	-1764194.73452025	-1764193.09046655	-1764194.76903282
306.15	-1764195.22522635	-1764195.28107397	-1764193.62133275	-1764195.31558655
308.15	-1764195.77240758	-1764195.82951021	-1764194.15533645	-1764195.86402278
310.15	-1764196.32147131	-1764196.38045645	-1764194.69122265	-1764196.41496902
312.15	-1764196.87367255	-1764196.93328519	-1764195.22899136	-1764196.96842527
313.15	-1764197.15040068	-1764197.21064081	-1764195.49944447	-1764197.24578089
298.15	0.268331	0.269013	0.261607	0.269043
300.15	0.2695	0.270183	0.262764	0.270213
302.15	0.270668	0.271352	0.263919	0.271382
304.15	0.271834	0.272519	0.265074	0.272549
306.15	0.272999	0.273685	0.266227	0.273715
308.15	0.274163	0.27485	0.267378	0.274879
310.15	0.275326	0.276013	0.268529	0.276043
312.15	0.276487	0.277176	0.269678	0.277205
313.15	0.277068	0.277756	0.270253	0.277785

values than the C-3 nucleus (Figure 3b). Since the electrostatic properties are mainly dependent on the electronic densities at the sites of nuclei, Oxygen plays a significantly different role among the other nuclei (S, C, Sn and N atoms). Total atomic charge for O-23 nucleus is minimum meaning O-23 nucleus has maximum electron shielding (Figure 3c). This leads to a minimum chemical shift tensor (δ) at the site of O-23 atom (Figure 3a). Also, Figure 3b shows that O-23 has large amounts of η in both gas phase and solution. The results in Figure 3a indicate that, the calculated chemical shift tensor at the site of O-23 is reduced by increasing dielectric constant (ε) of solvent. Also, with increase of dielectric constant from gas phase to water, the asymmetry parameter value at the site of O-23 atom increases. In this regard, it seems that the NMR parameters at the site of O-23 are significantly influenced by intermolecular hydrogen- bonding interactions. On the other hand, Figure 3c shows that O-23 posses more negative than O-24 nucleus. This difference in the total atomic charge values lead to a maximum chemical shift tensor (δ) at the site of O-24 atom (Figure 3a). As shown in Figure 3c, O-24 has maximum asymmetry parameter in water, but the quantity in Methanol is minimum. Furthermore, the chemical shift tensor (δ) at the site of O-24 decreases in the order Gas Phase > Ethanol > Methanol > Water (Figure 3a). It can be said that NMR parameters at the site of O-24 nucleus are significantly influenced by intermolecular hydrogen- bonding interactions. Moreover, the chemical shift tensor (δ) at the site of O-1 decreases in the order Water > Ethanol > Methanol > Gas Phase (Figure 3a). The observed effect is probably due to the intermolecular hydrogen- bonding interactions at the site of O-1 nucleus. Finally, the chemical shift tensor (δ) at the site of Oxygen decreases in the order O-24 > O-1 > O-23. It can be said that O-23 has maximum electron shielding but the quantity in the

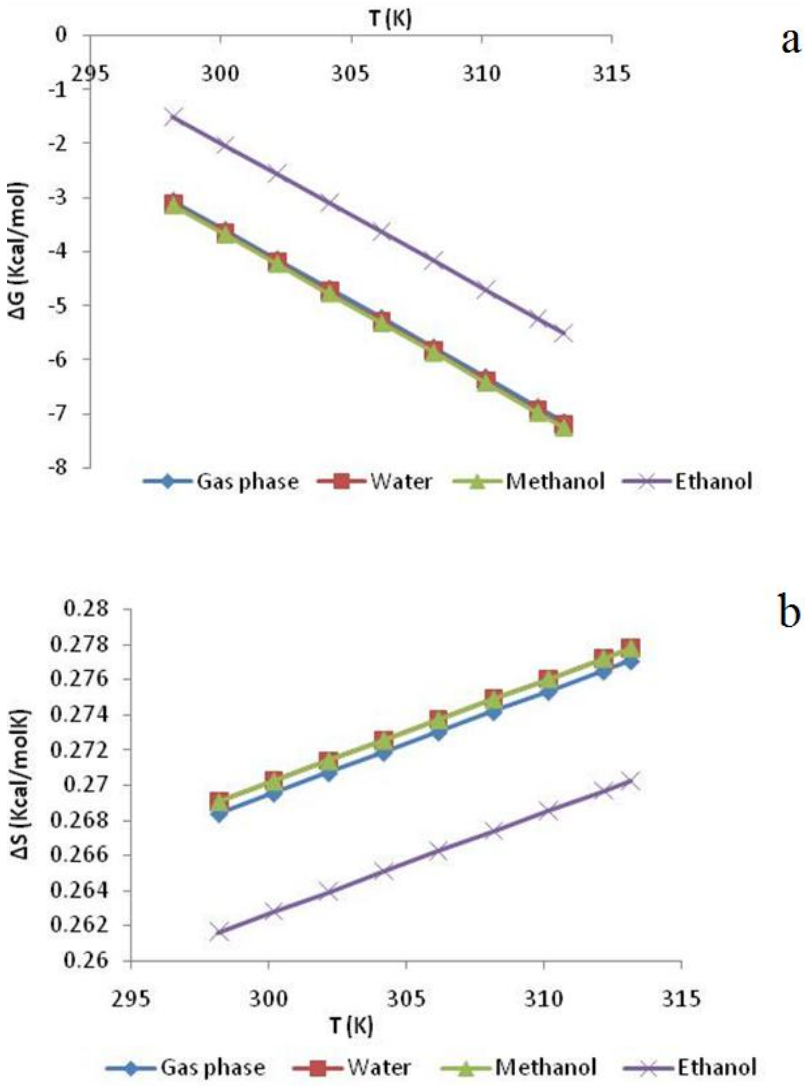


Figure 4. Plots of the ΔG (Kcal/mol) versus the T (K) (d) and ΔS (Kcal/molK) versus the T (K) (c) for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT Complex in both gas phase and in solution, obtain from the (LanL2DZ+STO-3G) calculations.

O-24 nuclei is minimum.

Thermodynamic analysis

The computed free Gibbs energies (ΔG) and entropy changes (ΔS) in both gas phase and in solution, for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex at the different temperature are given in Table 3. Based on these results, the plots of calculated free Gibbs energies (ΔG) and entropies (ΔS) versus the physiological temperature are displayed in Figures 4a and b respectively. As shown in Table 3 and Figure 4 it can be said $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex has negative values of free Gibbs energies (ΔG) in both gas phase and in solution. Also, the calculated entropies (ΔS) for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT system have positive values (Table 3 and Figure 4c). These results can be related to the structural stability of the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT in both gas phase and in solution.

The results in Figure 4 show that, the calculatedfree Gibbs energies (ΔG) of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT system decrease in the order Ethanole > Gas phase > Water > Methanol. These results indicate that $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT has maximum stability in Methanol at 313K. And also, the calculated entropies (ΔS) decrease in the order Methanol > Water > Gas phase > Ethanol. It can be said that $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT has maxi

mum entropies (ΔS) in Methanol but the quantity in Ethanol is minimum.

Conclusion

1. As the dielectric constant of the solvent increases, the dipole moment (μ) of $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT increased.
2. The calculated chemical shift tensor (δ) at the site of Oxygen decreases in the order O-24 > O-1 > O-23.
3. O-23 has minimum total atomic charge but the quantity in the Sn-35 nucleus is maximum.
4. The NMR parameters (δ, η) at the site of S-27 is influenced by nonspecific solute-solvent interaction such as polarisability/polarity but the quantity at the sites of nitrogen and oxygen as are significantly influenced by intermolecular hydrogen- bonding interactions.
5. The thermodynamic analysis show that $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT has maximum stability in Methanol at 313K.
6. Carbon nanotube that used in this study can act as a good carrier for $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) in physiological temperature.
7. Since the $Sn(C_6H_5)_2$(N-acetyl-L-cysteinato-O,S) – CNT complex has little stability in H_2O, pure water cannot act as a good solvent for drug delivery of drug-cnt system.

ACKNOWLEDGMENT

My sincere thanks to Dr. Shahram Aramideh for his critical comments on the manuscript.

REFERENCES

Banerjee S, Hemraj-Benny T, Wong SS (2005). Covalent surface chemistry of singlewalled carbon nanotubes. Adv. Mater. 17:17–29.
Bianco A, Kostarelos K, Partidos CD, Prato M (2005). Biomedical applications of functionalised carbon nanotubes. Chem. Commun. pp. 571–577.
Clarke MJ, Zhu F, Frasca DR (1999). Non-platinum chemotherapeutic metallopharmaceuticals. Chem. Rev. 99:2511–2534.
Ditchfield R (1972). Molecular-orbital theory of magnetic shielding and magnetic susceptibility. J. Chem. Phys. 56:5688–5692.
Eng G, Whalen D, Zhang YZ, Kirksey A, Otieno M, Khoo LE, James BD (1996). Appl. Organomet. Chem. 10(7):501–503.
Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Zakrzewski VG, Montgomery JA Jr, Stratmann RE, Burant JC, Dapprich S, Millam JM, Daniels AD, Kudin KN, Strain MC, Farkas O, Tomasi J, Barone V, Cossi M, Cammi R, Mennucci B, Pomelli C, Adamo C, Clifford S, Ochterski J, Petersson GA, Ayala PY, Cui Q, Morokuma K, Malick DK, Rabuck AD, Raghavachari K, Raghavachari JB, Cioslowski J, Ortiz JV, Baboul AG, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Gomperts R, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Gonzalez C, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Andres JL , Gonzalez C, Head-Gordon M, Replogle ES, Pople JA (1998). Gaussian 98 Revision A.7. Gaussian, Inc., Pittsburgh.
Gielen M (1996). Tin-Based Antitumor Drugs. Coord. Chem. Rev. 151:41–51.
Girasolo MA, Rubino S, Portanova P, Calvaruso G, Stocco G, Ruisi G (2010). Novel diorganotin(IV) derivatives of l-Arginine (HArg), Na-(tert-Butoxycarbonyl)-l. J. Organom. Chem. 695:609–618.
Goldberg DE, Sharma V, Oksman A, Gluzman I, Wellems TE, Piwnica-Worms D (1997). Probing the metal (III) coordination complexes. J. Biol. Chem. 272(10):6567–6572.
Hay PJ, Wadt WR (1985). Ab initio effective core Potentials for the transition metal atoms Sc to Hg. J. Chem. Phys. 82:270-270.
Iijima S (1991). Helical microtubules of graphitic carbon, Nature, 354:56–58.
Keith TA, Bader RFW (1993). Calculation of Magnetic Response. Chem. Phys. Lett. 210:223-223.
Klumpp C, Kostarelos K, Prato M, Bianco A (2006). Functionalized carbon nanotubes as emerging nanovectors for the delivery of therapeutics, Biochim. Biophys. Acta: Biomembr. 1758:404–412.
Kostarelos K, Lacerda L, Pastorin G (2007). Cellular uptake of functionalized carbon nanotubes is independent of functional group and cell type. Nat. Nanotechnol. 2:108–113.
K♀pf-Maier P, K♀pf H (1987). Anti-HIV Effects of Gramicidin in vitro. Chem. Rev. 87(5):1137–1152.
Miertus M, Scrocco E, Tomasi J (1981). From Theozymes to Artificial Enzymes: Enzyme-Like Receptors. Chem. Phys. 55:117–129.
Miertus S, Tomasi J (1982). Approximate evaluations of the electrostatic free energy and internal changes in solution. Chem. Phys. 65:239–252.
Mollaamin F, Layali I, Ilkhani A R, Monajjemi M (2010a). Nanomolecular simulation of the voltage–gated potassium channel protein by gyration radius study. Afr. J. Microbiol. Res. 4(24):2795-2803.
Mollaamin F, Shahani poor K, Nejadsattari T, Monajjemi M (2010b). Bio-nanomodeling of active site in oxidized azurin using by computational methods. Afr. J. Microbiol. Res. 4(20):2098-2108.
Mollaamin F, Varmaghani Z, Monajjemi M (2011). Dielectric effect on thermodynamic properties in vinblastine by DFT/Onsager modeling. Phys. Chem. Liquids 49(3):318-336.
Monajjemi M, Baie M T, Mollaamin F (2010a). Interaction between threonine and cadmium cation in [Cd(Thr)n]2+ (n = 1—3) complexes: density functional calculations. Rus. Chem. Bull. 59(5):886-889.
Monajjemi M, Lee V S, Khaleghian M, Honarparvar B, Mollaamin F (2010b). Theoretical Description of Electromagnetic Nonbonded Interactions of Radical, Cationic and Anionic NH2 BHNBHNH 2 Inside of the B 18 N18Nanoring. J. Phys. Chem. C. 114:15315-15330.
Monajjemi M. Chahkandi B (2005). Theoretical investigation of hydrogen bonding in Watson-Crick, Hoogestein and their reversed and other models: comparison and analysis for configurations of adenine-thymine base pairs in 9 models. J. Molecular Structure-Theochem. 714(1):43-60 JAN 31.
Nath M, Pokharia S, Yadav R (2001). Calix[4]arenes as Molecular Platforms in Magnetic Resonance Imagery. Coord. Chem. Rev. 215:99–149 and references therein.
Pastorin G, Wu W, Wieckowski SB, Briand J-P, Kostarelos K, Prato M, (2006). Double functionalisation of carbon nanotubes for multimodal drug delivery. Chem. Commun. pp. 1182–1184.
Pellerito L, Prinzivalli C, Casella G, Fiore T, Pellerito O, Giuliano M, Scopelliti M, Pellerito C (2010). Diorganotin(IV) N-acetyl-L-cysteinate complexes: synthesis. J. Inorg. Biochem. 104:750–758.
Pessoa JC, Covaco I, Correia I, Duarte MT, Gillard R D, Henriques RT, Higes FJ, Madeira C, Tomaz I (1999). Preparation and derived from amino acids and aromatic o-hydroxyaldehydes. Inorg. Chim. Acta, 293(1):1–11.
Plesch G, Friebel C, Svajlenova O, Kratsmar-Smogrovic J, Mlynarcik D (1988). Copper (II) complexes of the general composition Cu(ligand)2X2 (where X=Cl−, Br. Inorg. Chim. Acta 151(2):139–143.
Popov AM, Lozovik YE, Fiorito S, Yahia LH (2007). Biocompatibility and applications of carbon nanotubes in medical nanorobots. Int. J. Nanomed., 2: 361–372.
Pulay P, Wolinski K, Hinton JF (1990). The 3–21+G basis set for first row elements. J. Am. Chem. Soc. 112:8251–8260.
Raffa V, Ciofani G, Nitodas S, Karachalios T, D'Alessandro D, Masini M (2008). Can the properties of carbon nanotubes influence their internalization by living cells? Carbon 46:1600–1610.
Tzimopoulos D, Sanidas I, Varvogli AC, Czapik A, Gdaniec M, Nikolakaki E, Akrivos P D (2010). Detection of elevated antibodies

against SR. J. Inorg. Biochem. 104:423–430.
Wadt WR, Hay PJ (1985). DeMon2k. J. Chem. Phys. 82:284-284.
Wang MZ, Meng Z (2006). Determine the effects of acupuncture on specific clinical symptoms in. Acupuncture for the Prevention of Radiation-Induced Xerostomia. J. Fu, Appl. Radiat. Isot. 64(2):235–240.
Wang MZ, Meng Z, Liu B, Cai G, Zhang C, Wang X (2005). Effects of extrusion and a Magnesium Lattice. Inorg. Chem. Commun. 8(4):368–371.
Wrackmeyer B (1985). A multinuclear NMR study unambiguously confirmed that metal tetraolates retain their polynuclear structure in solution. Annu. Rep. NMR Spectrosc. 16:73–186.
Zhang X, Meng L, Lu Q, Fei Z, Dyson PJ (2009). Targeted delivery and controlled release of doxorubicin to cancer cells using modified single wall carbon nanotubes. Biomaterials 30:6041–6047.

American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

Conferences and Advert

October 2012
American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

December 2012
International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

- ***Journal of Medicinal Plant Research***
- ***Journal of Dentistry and Oral Hygiene***
- ***Journal of Parasitology and Vector Biology***
- ***Journal of Pharmacognosy and Phytotherapy***
- ***Journal of Veterinary Medicine and Animal Health***

academicJournals

African Journal of Pharmacy and Pharmacology

Volume 6 Number 31 22 August, 2012

ISSN 1996-0816

Academic Journals

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: ajpp@acadjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The African Journal of Pharmacy and Pharmacology will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Sharmilah Pamela Seetulsingh- Goorah
Associate Professor,
Department of Health Sciences
Faculty of Science,
University of Mauritius,
Reduit,
Mauritius

Himanshu Gupta
University of Colorado- Anschutz Medical Campus,
Department of Pharmaceutical Sciences, School of Pharmacy Aurora, CO 80045,
USA

Dr. Shreesh Kumar Ojha
Molecular Cardiovascular Research Program
College of Medicine
Arizona Health Sciences Center
University of Arizona
Tucson 85719, Arizona,
USA

Dr.Victor Valenti Engracia
University of São Paulo.
Av Dr. Arnaldo, 455
Cerqueira Cesar
01246-903 - Sao Paulo, SP - Brazil

Prof. Sutiak Vaclav
Rovníková 7, 040 20 Košice,
The Slovak Republic,
The Central Europe,
European Union
Slovak Republic
Slovakia

Dr.B.RAVISHANKAR
Director and Professor of Experimental Medicine
SDM Centre for Ayurveda and Allied Sciences,
SDM College of Ayurveda Campus,
Kuthpady, Udupi- 574118
Karnataka (INDIA)

Dr. Manal Moustafa Zaki
Department of Veterinary Hygiene and Management
Faculty of Veterinary Medicine, Cairo University
Giza, 11221 Egypt

Prof. George G. Nomikos
Scientific Medical Director
Clinical Science
Neuroscience
TAKEDA GLOBAL RESEARCH & DEVELOPMENT CENTER, INC. 675 North Field Drive Lake Forest, IL 60045
USA

Prof. Mahmoud Mohamed El-Mas
Department of Pharmacology,
Faculty of Pharmacy
University of Alexandria,
Alexandria,
Egypt.

Dr. Caroline Wagner
Universidade Federal do Pampa
Avenida Pedro Anunciação, s/n
Vila Batista, Caçapava do Sul, RS - Brazil

Editorial Board

Prof. Fen Jicai
School of life science, Xinjiang University,
China.

Dr. Ana Laura Nicoletti Carvalho
Av. Dr. Arnaldo, 455, São Paulo, SP.
Brazil.

Dr. Ming-hui Zhao
Professor of Medicine
Director of Renal Division, Department of Medicine
Peking University First Hospital
Beijing 100034
PR. China.

Prof. Ji Junjun
Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences,
China.

Prof. Yan Zhang
Faculty of Engineering and Applied Science,
Memorial University of Newfoundland,
Canada.

Dr. Naoufel Madani
Medical Intensive Care Unit
University hospital Ibn Sina, Univesity Mohamed V Souissi, Rabat,
Morocco.

Dr. Dong Hui
Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University,
China.

Prof. Ma Hui
School of Medicine, Lanzhou University,
China.

Prof. Gu HuiJun
School of Medicine, Taizhou university,
China.

Dr. Chan Kim Wei
Research Officer
Laboratory of Molecular Biomedicine,
Institute of Bioscience, Universiti Putra,
Malaysia.

Dr. Fen Cun
Professor, Department of Pharmacology, Xinjiang University,
China.

Dr. Sirajunnisa Razack
Department of Chemical Engineering, Annamalai University,
Annamalai Nagar, Tamilnadu,
India.

Prof. Ehab S. EL Desoky
Professor of pharmacology, Faculty of Medicine
Assiut University, Assiut,
Egypt.

Dr. Yakisich, J. Sebastian
Assistant Professor, Department of Clinical Neuroscience
R54
Karolinska University Hospital, Huddinge
141 86 Stockholm ,
Sweden.

Prof. Dr. Andrei N. Tchernitchin
Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA
University of Chile Medical School,
Chile.

Dr. Sirajunnisa Razack
Department of Chemical Engineering,
Annamalai University, Annamalai Nagar, Tamilnadu,
India.

Dr. Yasar Tatar
Marmara Unıversıty,
Turkey.

Dr Nafisa Hassan Ali
Assistant Professor, Dow institude of medical technology
Dow University of Health Sciences,Chand bbi Road, Karachi,
Pakistan.

Dr. Krishnan Namboori P. K.
Computational Chemistry Group, Computational Engineering and Networking,
Amrita Vishwa Vidyapeetham, Amritanagar, Coimbatore-641 112
India.

Prof. Osman Ghani
University of Sargodha,
Pakistan.

Dr. Liu Xiaoji
School of Medicine, Shihezi University,
China.

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJPP to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b; Tristan, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $600 handling fee. Publication of an article in the African Journal of Pharmacy and Pharmacology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Copyright: © 2012, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJPP, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication.
While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 6 Number 31 22 August, 2012

ARTICLES

Review

Research Articles

Table of Contents: Volume 6 Number 31 22 August, 2012

ARTICLES

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2332-2339, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.509
ISSN 1996-0816 ©2012 Academic Journals

Review

Phytochemistry and pharmacologic properties of *Ziziphus spina christi* (L.) Willd.

Jinous Asgarpanah* and Elaheh Haghighat

Department of Pharmacognosy, Pharmaceutical Sciences Branch, Islamic Azad University (IAU), Tehran, Iran.

Accepted 12 June, 2012

Ziziphus spina-christi **known as Christ's Thorn Jujube, is a native plant that grows in tropical and subtropical regions especially in Middle East. Its extracts are important in drug development with pharmacological activities in the Middle East and South and East of Asia including Iran. For a long time,** ***Z. spina-christi*** **has been used in alternative medicine for the treatment of fever, pain, dandruff, wounds and ulcers, inflammatory conditions, asthma and to cure eye diseases.** ***Z. spina-christi*** **has recently been shown to have antibacterial, antifungal, antioxidant, anti-hyperglycemic, and antinociceptive activities. Flavonoids, alkaloids and saponins are the main phytochemicals that are reported from this plant. Geranyl acetone, methyl hexadecanoate, methyl octadecanoate, farnesyl acetone C, hexadecanol and ethyl octadecanoate are characterized as the major components of the leaves' volatile oil. Due to the easy collection of the plant materials, its being cheap and widespread in many countries and also remarkable biological activities, this plant has become both medicine and food in some parts of the world, especially throughout the Middle East including Iran. This paper presents comprehensive analyzed information on the botanical, chemical and pharmacological aspects of** ***Z. spina-christi.***

Key words: *Ziziphus spina-christi*, rhamnaceae, pharmacology, phytochemistry.

INTRODUCTION

Ziziphus spina-christi commonly known as Christ's Thorn Jujube, is a deciduous tree and native to the warm-temperate and subtropical regions, including North Africa, South Europe, Mediterranean, Australia, tropical America, South and East of Asia and Middle East (Yossef et al., 2011). It belongs to the Rhamnaceae family in the order of Rosales that contains about 60 genera and more than 850 species. The genus *Ziziphus* consists of about 100 species of deciduous or evergreen trees and shrubs throughout the world (Abalaka et al., 2010). *Z. spina-christi* has been among the key plants of the Iranian traditional medicine since ancient times and is indigenous and naturalized throughout Iran (Solati and Soleimani, 2010). It has been known as "Sedr" in Iran and wildly distributed in East, South, North-East and central parts of Iran (Salehi, 2010).

Z. spina-christi is a shrub, sometimes a tall tree, reaching a height of 20 m and a diameter of 60 cm; its bark is light-grey, very cracked, scaly; trunk twisted; very branched, crown thick; shoots whitish, flexible, drooping; thorns in pairs, one straight, the other curved (Figure 1). Its leaves are glabrous on upper surface, finely pubescent below, ovate-lanceolate or ellipsoid, apex acute or obtuse, margins almost entire, lateral veins conspicuous (Figure 2). Flowers in cymes, subsessile, peduncle 1 to 3 mm (Figure 3). Fruit about 1 cm in diameter (Figure 4) (Zargari, 1988). *Z. spina-christi* has very nutritious fruits that are usually eaten fresh. The flowers are important source for honey in Yemen and Eritrea (Adzu and Haruna, 2007). The fruits are applied on cuts and ulcers. They are also used to treat pulmonary ailments and fevers and to promote the healing of fresh wounds, for dysentery (Abalaka et al., 2010).

For a long time, in folklore medicine, *Z. vulgaris* has been used for the treatment of some diseases, such as

*Corresponding author. E-mail: asgarpanah@iaups.ac.ir. Tel: 22640051. Fax: 22602059.

Figure 1. *Ziziphus spina-christi* L.

Figure 2. The leaves of *Z. spina-christi*.

digestive disorders, weakness, liver complaints, obesity, urinary troubles, diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anemia, diarrhea, and insomnia (Han and Park, 1986; Kirtikar and Basu, 1984). The leaves are applied locally to sores, and the roots are used to cure and prevent skin diseases (Adzu et al., 2001). The seeds are sedative and are taken sometime with buttermilk to halt nausea, vomiting and abdominal pains associated with pregnancy (Kaaria, 1998). The leaves are applied as poultices and are

Figure 3. *Z. spina-christi* flower.

Figure 4. The fruits of *Z. spina-christi*.

helpful in liver troubles, asthma and fever (Michel, 2002). *Z. spina-christi* extract has also been reported to possess protective effect against aflatoxicosis (Abdel-Wahhab et al., 2007) and anti-conceptive properties in the rat and have a calming effect on the central nervous system. Flavonoids, alkaloids, triterpenoids, saponins, lipids,

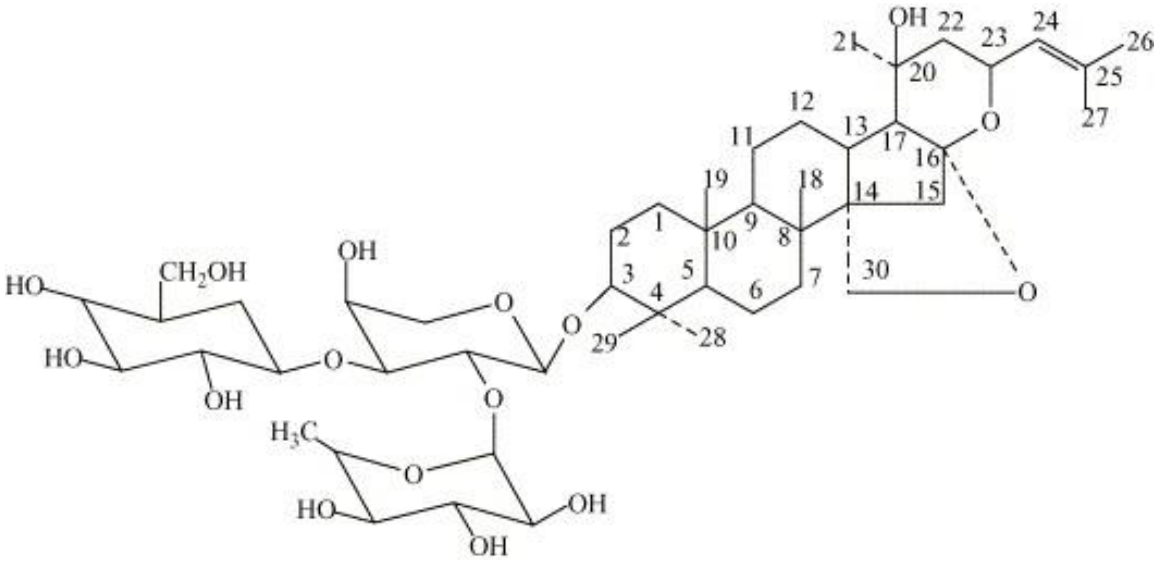


Figure 5. Molecular structure of christinin-A, the major components of *Z. spina-christi* leaves.

proteins, free sugar and mucilage are the main important compounds characterized in this plant (Adzu et al., 2003). Plant materials are cheap and significantly contribute to the improvement of human health in terms of cure and prevention of diseases (Okoko and Oruambo, 2008). Plants have been useful as food and medicine and a few have been studied especially African medicinal plants (Abalaka et al., 2010). They contain vitamins needed by human body for healthy living (Szeto et al., 2002; Jimoh et al., 2008). From current pharmaceutical studies, additional pharmaceutical applications of *Z. spina-christi* have revealed antifungal, antibacterial, antinociceptive, antioxidant, antidiabetic, antiplasmodial, anti-schistosomiasis, analgesic and anticonvulsant activities among others (Adamu et al., 2006; El-Kamali and Mahjoub, 2009; Adzu et al., 2001, 2011; Abalaka et al., 2011; Abdel-Zaher et al., 2005; El-Rigal et al., 2006; Adzu and Haruna, 2007; Waggas and Al-Hasani 2010).

Since a review and systemic analysis of chemistry, pharmacology and clinical properties of *Z. spina-christi* have not been reported, we prompted to provide the currently available information on traditional and local knowledge, ethnobiological and ethnomedicinal issues, identification of pharmacologically important molecules and pharmacological studies on this useful plant. The aim of this paper is to introduce *Z. spina-christi* as a potent medicinal plant by highlighting its traditional applications, as well as the recent findings for novel pharmacological and clinical applications.

CHEMICAL COMPOSITION

A survey of the literature revealed that a number of cyclopeptide and isoquinoline alkaloids, flavonoids, terpenoids and their glycosides have been found to occur in various amounts in most *Ziziphus* species. The leaves of these plants contain betulinic and ceanothic acids, various flavonoids, saponins, erols, tannins and triterpenes (Ali and Hamed, 2006; Glombitza et al., 1994). The extract of *Z. spina-christi* was shown to contain butic acid and ceanothic acid (a ring-A homologue of betulinic acid), cyclopeptides, as well as saponin glycoside and flavonoids, lipids, protein, free sugar and mucilage (Adzu et al., 2003). Cardiac glycosides and polyphenols (such as tannins) are also reported from the leaves (Abalaka et al., 2010).

Geranyl acetate (14.0%), methyl hexadecanoate (10.0%), methyl octadecanoate (9.9%), farnesyl acetone C (9.9%), hexadecanol (9.7%) and ethyl octadecanoate (8.0%) were characterized as the main components of *Z. spina-christi* leaves essential oil (Ghannadi et al., 2002). Zizyphine-F, jubanine-A and amphibine-H and a new peptide alkaloid spinanine-A have been isolated from the stem bark of *Z. spina-christi*. Spinanine-A is a 14-membered cyclopeptide alkaloid of the amphibine-B type (Fathy et al., 1990). Christinin-A is the major saponin of the leaves (Patel et al., 2012) (Figure 5). Dodecaacetylprodelphinidin B3 has been also isolated from the leaves (Weinges and Schick, 1995). New flavonoid, quercetin 3-xylosyl(1→2)rhamnoside-4′-rhamnoside (Pawlowska et al., 2008) accompanying with rutin, hyperin, quercetin, apigenin-7-O-glucoide, isovitexin and quercetin-3-O lucoside-7-O-rhamnoside were characterized from *Z. spina-christi* fruits. A flavonoid, C-glycoside, 3′,5′-di-C-β-d-glucosylphloretin, was also identified in *Z. spina-christi* leaves (Nawwar et al., 1983). In addition, 4-hydroxymethyl-1-methyl pyrrolidine-2-carboxylic acid and 4-hydroxy-4-hydroxymethyl-1-methyl pyrrolidine-2-carboxylic acid were characterized as two

new cyclic amino acids from *Z. spina-christi* seeds (Said et al., 2010).

POTENTIALS OF *Z. SPINA-CHRISTI*

A tropical evergreen tree of many parts of Iran, it is cultivated mainly as a dry crop for its mucilage nutritious fruits, honey production and landscaping purposes. It serves the ecosystem by controlling erosion, acting as wind break and it improves soil quality by increasing available phosphorus. Traditionally, it is used in Iran as a medicinal plant; the fruits are used for the treatment of fever, pain, dandruff, wounds and ulcers, in inflammatory conditions, asthma and to cure eye diseases, while the seeds are used as a tonic (Shah et al., 1989; Adzu and Haruna, 2007). Extracts from the plant could be useful in the treatment of nosocomial infections, opportunistic infection of the unary tract, infantile gastroenteritis, traveler's diarrhea, wound infection, meningitis, and wounds infection which are diseases caused by some of these organisms (Adzu and Haruna, 2007). Additionally, *Z. spina-christi* fruit extract causes neurotransmitters release, which is probably related to presence of ascorbic acid and the leaves may potentially be safe for use as sedative drug (Waggas and Al-Hasani 2010). A variable activity of the plant extract is against *Staphylococcus aureus* which highly infects various burns (Alsaimary, 2009).

Moreover, the methanol extract of Sidir could be used not only as a safe potential natural functional food ingredient or as therapeutic drug in the treatment of diabetes, but also it is effective in reducing both hyperlipidemia and oxidative stress accompanying diabetes (Hussein et al., 2006; Sudhersan and Hussain, 2003). It easily domesticated and can be grown commercially for the benefit of pharmaceutical industry and vegetation purposes.

Antibacterial and antifungal properties

The aqueous extract of *Z. spina-christi* stem bark has shown significant antibacterial activity against *S. aureus, Bacillus subtilis, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella para typhi B* and *Klebsiella pneumonia* (El-Kamali and Mahjoub, 2009). *Z. spina-christi* stem bark aqueous extract has shown highly significant antibacterial effect activity on some Gram negative bacterial growth including *Brucella abortus, Brucella melitensis, Proteus spp., Klebsiella spp., P. aeruginosa, E. coli and Enterobacter spp.* in comparison with eight antibiotics (Korji, 2012). Alcoholic extract of the leaves has also shown good antibacterial activity against *S. aureus* isolated from eye infections (conjunctivitis). An inhibition zone of 20 mm was recorded for 1 mg/ml of the extract (Alsaimary, 2012). Additionally, the leaves were active against *Salmonella typhi, Proteus mirabilis, Shigella dysenteriae, E. coli, K. pneumonia, B. melitensis, Bordetella bronchiseptica* and *P. aeruginosa*. The highest activity (20 mm) was against *B. bronchiseptica* byconcentration of 100 mg/ml (Motamedi et al., 2009)The pulp aqueous extract of *Z. spina-christi* also showed inhibitory activity on *E. coli, P. aeruginosa and Candida albicans in vivo*. The extract showed MIC of 6.25 mg/ml against *E. coli* and *C. albicans*. The minimum bactericidal concentration of the pulp aqueous extract was 12.5mg/ml for *Streptococcus pyogenes* (Tom et al., 2009). Methanol extract of *Z. spina-christi* roots showed antifungal activity against dermatophytes, including *Trichophyton rubrum, T. mentagaphytes, Microsporum canis* and *Aspergillus fumigatus* (Adamu et al., 2006). The fruits were also active against *C. albicans* (Ghasemi Pirbalouti et al., 2009).

As shown by these results, the extracts from *Z. spina-christi* could be useful in the treatment of nosocomial infections, opportunistic infection of the unary tract (UTI), infantile gastroenteritis, travelers' diarrhea, wound infection, meningitis and wounds infection which are diseases caused by some of these organisms.

Antinociceptive effects

The antinociceptive effect of the aqueous extract of *Z. spina-christi* root bark was shown in mice and rats by acetic acid-induced writhing, formalin and thermal (hot plate) tests. The extract (50 and 100 mg/kg, i.p.) demonstrated a dose-dependent analgesic effect in all the tests used. Its i.p. LD_{50} in mice was 2236.07 mg/kg (Adzu et al., 2001). The aqueous extract of the leaves were also active. The extract (250-1000 mg kg^{-1}) in a dose-dependent fashion significantly reduced the number of writhes induced by 0.6% aqueous solution of acetic acid in Wistar rats. At a dose of 250 mg/kg, the extract produced comparable effect to that of 10 mg/kg of pethidine hydrochloride in suppressing the number of writhing induced by acetic acid (Effraim et al., 1998).

Antioxidant activity

An antioxidant is defined as 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Rhee et al., 2009; Wiseman et al., 1997; Mates et al., 1999). Antioxidants are of interest to biologists and clinicians because they help to protect the human body against damages induced by reactive free radicals generated in atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease and even in aging process (Aruoma, 2003; Hemati et al., 2010). There are many evidences that natural products and their derivatives have efficient

anti-oxidative characteristics, consequently linked to anti-cancer, hypolipidemic, anti-aging and anti-inflammatory activities (Rhee et al., 2009; Wiseman et al., 1997; Hogg, 1998; Mates et al., 1999; Aruoma, 2003; Cho et al., 2006).

The anti-oxidative capacities of ethanol and petroleum ether extracts of *Z. spina-christi* leaves were evaluated by hydroxyl radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, lipid peroxidation and superoxide radical standardization methods (Abalaka et al., 2011). The EC_{50} values for hydroxyl radical with ethanol and hexane extract of *Z. spina-christi* were found to be 198.34 and 234.11 µg, while that of ascorbic acid was found to be 219.31 µg. The EC_{50} values for the two plant extracts were ethanol 101.02 µg and hexane 124.21 µg. These results compare favorably with that of standard ascorbic acid which had the EC_{50} value of 78.12. Moreover, the EC_{50} values for lipid peroxidation with ethanol extract and hexane extract of *Z. spina-christi* were 298.65 and 376.35 µg, while that of ascorbic acid was 191.42 µg. The EC_{50} value for superoxide radical scavenging with ethanol and hexane extract of *Z. spina-christi* were 156.45 and 265.22 µg, while that of ascorbic acid was 138.26 µg (Abalaka et al., 2011). These activities indicate that the extracts from *Z. spina-christi* are good antioxidants.

It was also indicated that the fruits contained high level of total phenolic compounds (7.55mg /g as gallic acid) (Yossef et al., 2011). The fruit administration inhibited lipid peroxidation at higher level after CCL4 treatment. Interestingly, the methanolic extract of these fruits with dose of 200 mg/kg was able to increase the activities of endogenous antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)) and levels of GSH in hepatic tissue. The fruits extract pretreatment demonstrated to inhibit malondialdehyde (MDA) of the reactive oxygen radical production (Xiangchun et al., 2009).

Antidiabetic properties

Pretreatment either with 100 mg/kg butanol extracts of the leaves or christinin-A, the major saponin glycoside of the leaves, potentiated glucose-induced insulin release in non-diabetic control rats. In type-II but not in type-I diabetic rats, pretreatment with the butanol extract or christinin-A improved the oral glucose tolerance and potentiated glucose-induced insulin release. Treatment either with 100 mg/kg butanol extract or christinin-A reduced the serum glucose level and increased the serum insulin level of non-diabetic control and type-II diabetic rats but not of type-I diabetic rats. Pretreatment of non-diabetic control and type-II diabetic rats either with 100 mg/kg butanol extract or christinin-A also enhanced the glucose lowering and insulinotropic effect of 5 g/kg glibenclamide. The hyperglycemic and hypoinsulinemic effects of 30 mg/kg diazoxide in non-diabetic control and type-II diabetic rats were inhibited and antagonized, respectively by pretreatment with the butanol extract or christinin-A. Treatment of rats with 100 mg/kg butanol extract for 3 months produced no functional or structural disturbances in liver and kidney and no haematological changes. In addition, the oral LD50 of the butanol extract in mice was 3820 mg/kg, while that of glibenclamide was 3160 mg/kg. Thus, *Z. spina-christi* leaves appear to be a safe alternative to lower blood glucose. The safe insulinotropic and subsequent hypoglycemic effects of *Z. spina-christi* leaves may be due to a sulfonylurea-like activity (Abdel-Zaher et al., 2005).

Oral administration of *Z. spina-christi* leaf extract, plain and formulated for 28 days, reduced blood glucose level with significant increase in serum insulin and C-peptide levels. Marked elevation in total antioxidant capacity with normalization of percentage of glycated hemoglobin (HbA1C%) was reported. Moreover, they succeeded in reducing the elevated blood lactate level and to elevate the reduced blood pyruvate content of diabetic rats. In line with amelioration of the diabetic state, the extract, plain and formulated, restored liver and muscle glycogen content together with significant decrease of hepatic glucose-6-phosphatase and increase in glucose-6-phosphate dehydrogenase activities. *In vitro* experiments showed a dose-dependent inhibitory activity of the extract against α-amylase enzyme with IC_{50} at 0.3 mg/ml. Such finding has been supported by the *in vivo* suppression of starch digestion and absorption by the extract in normal rats. The results revealed that *Z. spina-christi* leaf extract improved glucose utilization in diabetic rats by increasing insulin secretion, which may be due to both saponin and polyphenols content, and controlling hyperglycemia through attenuation of meal-derived glucose absorption that might be attributed to the total polyphenols (Michel et al., 2011).

CONCLUSION

The objective of this paper has been to show the recent advances in the exploration of *Z. spina-christi* as phytotherapy and to illustrate its potential as a therapeutic agent. With the current information, it is evident that *Z. spina-christi* has pharmacological functions, including antihyperglycemic, antibacterial, antifungal, antioxidant and antinociceptive activities, among others. As the current information shows, it is also possible that various flavonoids and saponin glycosides such as christinin-A might be useful in the development of new drugs to treat various diseases. It must be kept in mind that clinicians should remain cautious until more definitive studies demonstrate the safety, quality and efficacy of *Z. spina-christi*. For these reasons, extensive pharmacological and chemical experiments, together with human metabolism will be a focus for future studies. Last but not the least, this article emphasizes the potential of

Z. spina-christi to be employed in new therapeutic drugs and provide the basis for future research on the application of transitional medicinal plants.

REFERENCES

Abalaka ME, Daniyan SY, Mann A (2010). Evaluation of the antimicrobial activities of two *Ziziphus* species (*Ziziphus mauritiana* L. and *Ziziphus spina-christi* L.) on some microbial pathogens. Afr. J. Pharm. Pharmacol. 4(4):135-139.

Abalaka ME, Mann A, Adeyemo SO (2011). Studies on *in-vitro* antioxidant and free radical scavenging potential and phytochemical screening of leaves of *Ziziphus mauritiana* L. and *Ziziphus spina-christi* L. compared with Ascorbic acid. J. Med. Gen. Geno. 3(2):28-34.

Abdel-Wahhab MA, Omara EA, Abdel-Galil MM, Hassan NS, Nada SA, Saeed A, ElSayed MM (2007). *Ziziphus spina-christi* extract protects against Aflatoxin B1-intitiated hepatic carcinogenicity. Afr. J. Trad. CAM. 4(3): 248-256.

Abdel-Zaher AO, Salim SY, Assaf MH, Abdel-Hady RH (2005). Antidiabetic activity and toxicity of *Zizyphus spina-christi* leaves. J. Ethnopharmacol. 101(1-3):129-138.

Adamu HM, Abayeh OJ, Ibok NU, Kafu SE (2006). Antifungal activity of extracts of some *Cassia*, *Detarium* and *Ziziphus* species against dermatophytes. Nat. Prod. Radiance 5(5):357-360.

Adzu B, Amos S, Amizan MB, Gamaniel K (2003). Evaluation of the antidiarrhoeal effects of *Ziziphus spina-christi* stem bark in rats. Acta. Trop. 87(2):245-250.

Adzu B, Amos S, Wambebe C, Gamaniel K (2001). Antinociceptive activity of *Ziziphus spinachristi* root bark extract. Fitoterapia 72:334-350.

Adzu B, Haruna AK (2007). Studied on the use of *Zizyphus spina-christi* against pain in rats and mice. Afr. J. Biotechnol. 6(11):1317-1324.

Adzu B, Haruna AK, Ilyas M, Pateh UU, Tarfa FD, Chindo BA, Gamaniel KS (2011). Structural characterization of ZS – 2A: An antiplasmodial compound isolated from *Zizyphus spina-christi* root bark. J. Pharm. Nut. Sci. 1:48-53.

Ali SA, Hamed MA (2006). Effect of Ailanthus altissima and *Zizyphus spina-christi* on Bilharzial infestation in mice: histological and histopathological studies. J. Appl. Sci. 6:1437-1446.

Alsaimary IE (2009). Efficacy of some antibacterial agents on *Staphylococcus aureus* isolated from various burn cases. Int. J. Med. Med. Sci. 1(4):110-114.

Alsaimary IE (2012). A study of antibacterial activity of plant extracts on bacterial pathogens isolated from Eye infections (conjunctivitis). Int. J. Microbiol. 4(1):1-5.

Aruoma OI (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat. Res. 523-524:9-20.

Cho JY, Prak SC, Kim TW, Kim KS, Song JC, Kim SK, Lee HM, Sung HJ, Park HJ, Song YB, Yoo ES, Lee CH, Rhee MH (2006). Radical scavenging and anti-inflammatory activity of extracts from *Opuntia humifusa*. Raf. J. Pharm. Pharmacol. 58:113-119.

Effraim KD, Osunkwo UA, Onyeyilli P, Ngulde A (1998). Preliminary investigation of the possible antinociceptive activity of aqueous leaf extract of *Ziziphus spina-christi* (LINN) Desf. Indian J. Pharmacol. 30(4):271-272.

El-Kamali HH, Mahjoub SA (2009). Antibacterial activity of *Francoeuria crispa*, *Pulicaria undulata*, *Ziziphus spina-christi* and *Cucurbita pepo* against seven standard pathogenic bacteria. Ethnobot. Leaflets 13:722-733.

El-Rigal NS, Aly SA, Rizk M, Said A (2006). Use of *Ailanthus altissima* and *Ziziphus spina-christi* extracts as folk medicine for treatment of some hepatic disorders in *Schistosoma mansoni* infected mice. Trends. Med. Res. 1(2):100-112.

Fathy M, Galil A, Mervat A, El-Jissry (1990). Cyclopeptide alkaloids from *Zizyphus spina-christi*. Phytochemistry 30(4):1348-1349.

Ghannadi A, Tavakoli N, Mehri-Ardestani M (2002). Volatile constituents of the leaves of *Ziziphus spina-christi* (L.) Willd. from Bushehr, Iran. Biodiversity Biomol. Asp. Biodiv. Innov. Util. 15(3): 191-192.

Ghasemi Pirbalouti A, Bahmani M, Avijgan M (2009). Anti-Candida Activity of Some of the Iranian Medicinal Plants. Electron. J. Biol. 5(4):85-88.

Glombitza K, Mahran G, Mirhom Y, Michel K, Motawi T (1994). Hypoglycemic and antihyperglycemic effects of *Zizyphus spinachristi* in rats. Planta Med. 60:244.

Han BH, Park MH (1986). Folk Medicine: The Art and Science. The American Chemical Society, Washington DC, p. 205.

Hemati A, Azarnia M, Angaji AH (2010). Medicinal effects of *Heracleum persicum* (Golpar). Middle-East J. Sci. Res. 5(3):174-176.

Hogg N (1998). Free radicals in disease. Seminars in reproductive Endocrinol. 16:241-248.

Hussein HM, El-Sayed EM, Said AA (2006). Antihyperglycemic, antihyperlipidemic and antioxidant effects of *Zizyphus spina christi* and *Zizyphus jujube* in Alloxan diabetic rats. Int. J. Pharmacol. 2(5):563-570.

Jimoh FO, Adebayo AA, Aliero AA, Afolayan AJ (2008). Polyphenol contents and Biological activities of *Rumex ecklonianus*. Pharm. Biol. 45(5):333-340.

Kaaria I (1998). Seed production, dispersal and germination in *Cryptostegia grandifolia* and *Ziziphus mauritiana*, two invasive shrubs in tropical woodlands of Northern Australia. Aust. J. Ecol. 21:324-331.

Kirtikar KR, Basu BD (1984). Indian Medicinal Plants, Lalit Mohan Basu, Allahabad p. 593.

Korji SHA (2012). Inhibition of nitrate reductase production from Gram-negative bacteria using *Ziziphus spina-christi* extract and comparing with some antibiotics. Iraqi J. Agric. Sci. 43(2):144-150.

Mates JM, Perez-Gomez C, Nunez de Castro I (1999). Antioxidant enzymes and human diseases. Clin. Biochem. 32:595-603.

Michel A (2002). Tree, Shrub and Liana of West African Zone. Margraf Publishers GMBH, Paris p. 440.

Michel CG, Nesseem DI, Ismail MF (2011). Anti-diabetic activity and stability study of the formulated leaf extract of *Zizyphus spina-christi* (L.) Willd with the influence of seasonal variation. J. Ethnopharmacol. 133(1):53-62.

Motamedi H, Safary A, Maleki S, Seyyednejad SM (2009). *Ziziphus spina-christi*, a native plant from Khuzestan, Iran, as a potential source for discovery new antimicrobial agents. Asian J. Plant Sci. 8(2):187-190.

Nawwar MAM, Ishak MS, Michael HN, Buddrust J (1983). Leaf flavonoids of *Ziziphus spina-christi.* Phytochemistry 23(9):2110-2111.

Okoko T, Oruambo IF (2008). The effects of *Hibiscus sabdariffa* calyx on cisplatin-induced tissues damaged in rats. Biokem. 20(2):47-52.

Patel DK, Prasad SK, Kumar R, Hemalatha S (2012). An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pacific J. Trop. Biomed. pp. 320-330.

Pawlowska AM, Camangi F, Bader A, Braca A (2008). Flavonoids of *Zizyphus jujuba* L. and *Zizyphus spina-christi* (L.) Willd (Rhamnaceae) fruits. Food Chem. 112(4):858-862.

Rhee MH, Park HJ, Cho JY (2009). Salicornia herbaceae: Botanical, Chemical and pharmacological review of halophyte marsh plant. J. Med. Plants Res. 3(8):548-555.

Said A, Huefner A, Abu Tabl ESA, Fawzy G (2010). Isolation and identification of two new cyclic amino acids from the seeds of *Ziziphus spina-christi* L. (Willd) by means of 1H-NMR, 13C-NMR, HSQC, HMBC and GC-MS. IUFS J. Biol. 69(1):13-23.

Salehi MH (2010). Medicinal plants and phytotherapy. Vol. 3, Donya e Taghzieh Publications., Tehran, p. 292.

Shah AH, Qureshi S, Tariq M, Ageel AM (1989). Toxicity studies on six plants used in the traditional Arab system of medicine. Phytother. Res. 3(1):25-29.

Solati J, Soleimani N (2010). Antihyperglycemic and antihyperlipidemic effects of *Ziziphus vulgaris* L. onreptozocin-induced diabetic adult male Wistar rats. Acta Diabetol. 47(1):219-223.

Sudhersan C, Hussain J (2003). *In vitro* clonal propagation of a multipurpose tree, *Ziziphus spina-christi* (L.) Desf. Turk. J. Bot. 27: 167-171.

Szeto YP, Tomlinson B, Benzie IFF (2002). Total antioxidant and ascorbic acid content of fresh fruits and vegetables: Implications for dietary planning and food preservation. Br. J. Nutri. 87:55-59.

Tom GM, Yesufu HB, Abdulrahman FI (2009).Antimicrobial screening and effect of the pulp extracts of *Zizyphus spina-christi* (Linnaeus

Desf) on some biochemical parameters in rats. J. Pharm. Biores. 6(2):58-64.
Waggas AM, Al-Hasani RH (2010). Neurophysiological study on possible protective and therapeutic effects of Sidr (*Zizyphus spina-christi* L.) leaf extract in male albino rats treated with pentylenetetrazol. Saudi J. Biol. Sci. 5(3):1-6.
Weinges K, Schick H (1995). Dodecaacetylprodelphinidin B3 from the dried leaves of *Ziziphus spina-christi*. Phytochemtry 38(2):505-507.
Wiseman SA, Balentine DA, Frei B (1997). Antioxidants in tea. Crit. Rev. Food Sci. Nutr. 37:705-718.
Xiangchun S, Yuping T, Ruihui Y, Li Y, Taihui F, Jin-ao D (2009). The protective effect of *Zizyphus jujube* fruit on carbon tetrachloride-induced hepatic injury in mice by anti-oxidative activities. J. Ethnopharmacol. 122:555-560.
Yossef HE, Khedr AA, Mahran MZ (2011). Hepatoprotective activity and antioxidant effects of El Nabka (*Zizyphus spina-christi*) fruits on rats hepatotoxicity induced by carbon tetrachloride. Nat. Sci. 9(2):1-7.
Zargari A (1988). Medicinal plants. Tehran Univ. Press, Iran 2:42.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2340-2345, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.901
ISSN 1996-0816 © 2012 Academic Journals

Review

Phytochemistry, pharmacology and medicinal properties of *Coriandrum sativum* L.

Jinous Asgarpanah* and Nastaran Kazemivash

Department of Pharmacognosy, Pharmaceutical Sciences Branch, Islamic Azad University (IAU), Tehran, Iran.

Accepted 6 August, 2012

***Coriandrum sativum* L. commonly known as "Coriander" is an annual herb, indicated for a number of medical properties in traditional medicine. For a long time, *C. sativum* has been used in traditional medicines as an anti-inflammatory, analgesic, and antibacterial agent. Its essential oil is also used as a natural fragrance with some medicinal properties. *C. sativum* has recently been shown to have antioxidant, antidiabetic, hepatoprotective, antibacterial, and antifungal activities. Volatile components, flavonoids, and isocoumarins are the main constituents of *C. sativum*. 2-decenoic acid, E-11-tetradecenoic acid, and capric acid were identified as the major components for *C. sativum* leaves essential oil. The seed oil contained linalool and geranyl acetate. Due to the easy collection of the plant and being widespread and also remarkable biological activities, this plant has become both food and medicine in many parts of the world. This review presents comprehensive analyzed information on the botanical, chemical, and pharmacological aspects of *C. sativum*.**

Key words: *Coriandrum sativum*, apiaceae, phytochemistry, pharmacology.

INTRODUCTION

Coriandrum sativum L. commonly known as "Coriander" is an annual small plant like parsley which dates back to around 1550 BC, and is one of the oldest spice crops in the world (Coskuner and Karababa, 2007). It belongs to Apiaceae family in the order of Apiales that contains about 300 genera and more than 3000 species (Asgarpanah et al., 2012).

C. sativum probably originated from Eastern Mediterranean and it is spread as a spice plant to India, China, Russia, Central Europe, and Morocco, and has been cultivated since human antiquity (Small, 1997). India is the largest producer of coriander which is used extensively in curry powder (Coskuner and Karababa, 2007). Coriander has been known as "Geshniz" in Iran.

C. sativum is an annual, herbaceous plant that grows 25 to 60 cm in height. It has thin, spindle-shaped roots, erect stalk, alternate leaves (Figure 1), and small, pinkish-white flowers. The plant flowers from June to July and yields round fruits consisting of two pericarps (Burdock and Carabin, 2009). These fruits are almost ovate globular and there are many longitudinal ridges on the surface. The length of this fruit is 3 to 5 mm and the color, when dried, is usually brown, but may be green, straw-colored or off white (Figure 2) (Coskuner and Karababa, 2007).

The plant is grown widely all over the world for seed, as a spice, or for essential oil production (Bhuiyan et al., 2009). The whole or ground seed (fruit) is an ingredient of pickling spices also used to flavor various commercial foods, particularly, to prepare some instant soups and dishes, in many cakes, breads and other pastries, alcoholic beverages, frozen dairy desserts, candy, and puddings. The fruit's essential oil is a common ingredient in creams, detergents, surfactants, emulsifiers, lotions, and perfumes (Coskuner and Karababa, 2007). There are two varieties of *C. sativum*: *vulgare* Alef. and *microcarpum* DC. These varieties differ in the fruit size and oil yield: *vulgare* has fruits of 3 to 5 mm diameter and yields 0.1 to 0.35% essential oil, while *microcarpum* fruits are 1.5 to 3 mm and yield 0.8 to 1.8% essential oil (Small, 1997).

The green leaves of coriander are known as "cilantro" in the United States, and are consumed as fresh herb in

*Corresponding author. E-mail: asgarpanah@iaups.ac.ir. Tel: 22640051. Fax: 22602059.

Figure 1. *C. sativum* L. (Coriander).

Figure 2. *C. sativum* fruits (seeds).

preparing chutneys, sauces, in flavoring curries and soups. The fruits are mainly responsible for the medical use of coriander and have been used as a drug for indigestion, against worms, rheumatism, and pain in the joints (Wangensteen et al., 2004). The fruit extract is used in lotions and shampoos as an antibacterial agent

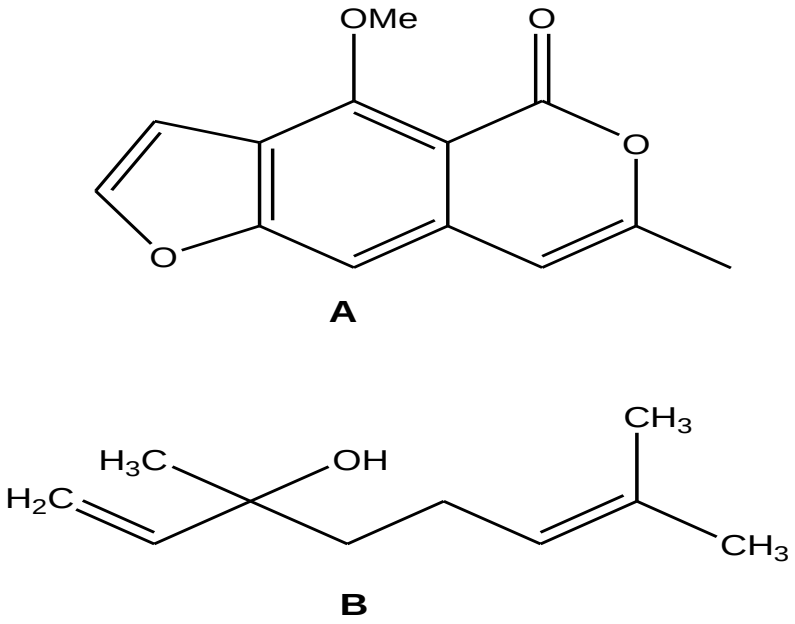


Figure 3. Structures of (A) coriandrin and (B) linalool from *C. sativum*.

(Bhuiyan et al., 2009). There are records that it is effective for relief of insomnia, anxiety, and convulsion (Emamghoreishi and Heidari-Hamedani, 2008). It is also used for sub-acid gastritis, diarrhea, and dyspepsia of various origins as well as for its digestive stimulation, stomachic, and antibilious properties (Platel and Srinivasan, 2004). In folk medicine, coriander is used against intestinal parasites (Wichtl, 1994). Coriander has been reported to possess strong lipolytic activity (Leung and Foster, 1996), and, as a member of Apiaceae family, its use has been suggested with caution, because of potential allergic reactions from furanocoumarins (Burdock and Carabin, 2009). Coriander leaves are widely used as folk medicine as carminative, spasmolytic, digestive, and galactagogue. It has the advantage of being more stable and of retaining its agreeable odor longer than any other oil of its class (Eikani et al., 2007).

A number of chemical constituents such as volatile constituents, flavonoids, isocoumarins, and coriandrones have been isolated from different parts of the plant (Taniguchi et al., 1996). From current pharmaceutical studies, additional pharmaceutical applications of *C. sativum* have revealed antibacterial (Silva et al., 2011a), antifungal (Silva et al., 2011b), antioxidant (Wangensteen et al., 2004), hepatoprotective (Sreelatha et al., 2009), antihelmintic (Eguale et al., 2007), anticonvulsant (Emamghoreishi and Heidari-Hamedani, 2008), protection of gastric mucosal damage (Al-Mofleh et al., 2006), hypocholestrolemia (Dhanapakiam et al., 2008) and antileishmania (Rondon et al., 2011), gut modulatory, blood pressure lowering, and diuretic (Jabeen et al., 2009) activities among others.

Since review and systemic analysis of chemistry, pharmacology, and clinical properties of *C. sativum* have not been reported, we were prompted to provide the currently available information on the traditional and local knowledge, ethno biological and ethno medicinal issues, identification of pharmacologically important molecules, and pharmacological studies on this useful plant. The aim of this paper is to introduce *C. sativum* as a potent medicinal plant by highlighting its traditional applications as well as the recent findings for novel pharmacological and clinical applications.

CHEMICAL COMPOSITION

The odor and flavor of mature fruits and fresh herbage are completely different. While aliphatic aldehydes (mainly C10 to C16 aldehydes) with fetid-like aroma are predominant in the fresh herb oil (Potter, 1996), major components in the oil isolated from coriander fruit include oxygenated monoterpenes and monoterpene hydrocarbons (Bhuiyan et al., 2009).

The most important constituents of coriander fruits are the essential oil and fatty oil. The essential oil content of dried coriander fruits varies between 0.03 and 2.6%, while the fatty oil content varies between 9.9 and 27.7%. Other constituents including crude protein, fat, crude fiber, and ash contents vary from 11.5 to 21.3%, 17.8 to 19.15%, 28.4 to 29.1%, and 4.9 to 6.0%, respectively (Coskuner and Karababa, 2007).

The essential oil content of the dried coriander fruits varies from 0.1 to 0.36%. Linalool (40.9 to 79.9%) (Figure 3), neryl acetate (2.3 to 14.2%), γ-terpinene (0.1 to 13.6%), and α-pinene (1.2 to 7.1%) were identified as the

main components in the oil of the coriander fruits cultivated in Iran (Nejad et al., 2010), while linalool (37.7%), geranyl acetate (17.6%), and γ-terpinene (14.4%) were characterized as the main ones in Bangladesh coriander cultivars (Bhuiyan et al., 2009). The leaf oil contained mostly aromatic acids, including 2-decenoic acid (30.8%), E-11-tetradecenoic acid (13.4%), capric acid (12.7%), undecyl alcohol (6.4%), tridecanoic acid (5.5%), and undecanoic acid (7.1%) as major constituents (Bhuiyan et al., 2009). Analysis of Kenya coriander leaves essential oil showed the presence of 2E-decenal (15.9%), decanal (14.3%), 2E-decen-1-ol (14.2%), and n-decanol (13.6%) as the main ones (Matasyoh et al., 2009). The commonly known phytochemicals from *C. sativum* are volatile components, flavonoids, isocoumarins, fatty acids, sterols, and coriandrones, coumarins, catechins, polyphenolic compounds (Taniguchi et al., 1996; Sriti et al., 2009; Al-Mofleh et al., 2006).

Two new isocoumarins, coriandrone A and B were isolated from the aerial parts of *C. sativum* together with two known isocoumarins, coriandrin and dihydrocoriandrin (Baba et al., 1991) (Figure 3). Three new isocoumarins, coriandrones C, D, and E were also isolated from *C. sativum* whole plants (Taniguchi et al., 1996).

Caffeic acid, protocatechinic acid, and glycitin were characterized as the major polyphenolics of coriander aerial parts (Melo et al., 2005).

POTENTIAL OF *C. SATIVUM* IN PHYTOTHERAPIES

Antibacterial and antifungal properties

C. sativum essential oil has been reported to inhibit a broad spectrum of micro-organisms (Silva et al., 2011b). The effective antibacterial activity of *C. sativum* essential oil against *Staphylococcus aureus* and Gram-negative bacterial strains including *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* and two clinical multidrug-resistant *Acinetobacter baumannii* isolates has been shown. The primary mechanism of action of coriander oil is membrane damage, which leads to cell death (Silva et al., 2011b). Aliphatic (2E)-alkenals and alkanals characterized from the fresh leaves of *C. sativum* were found to possess bactericidal activity against the food-borne bacterium, *Salmonella choleraesuis* subsp. *choleraesuis* with the minimum bactericidal concentration (MBC) of 6.25 µg/ml (34 µM) and 12.5 µg/ml (74 µM), respectively (Kubo et al., 2004).

Coriander essential oil has a fungicidal activity against the *Candida* strains tested with minimal lethal concentrations (MLC) values equal to the MIC value and ranging from 0.05 to 0.4% (v/v). The fungicidal effect is as a result of cytoplasmic membrane damage and subsequent leakage of intracellular components such as DNA (Silva et al., 2011a). The efficacy of *C. sativum* essential oil has also been shown against *Candida* species isolates from the oral cavity of patients with periodontal disease. 2-hexen-1-ol, 3-hexen-1-ol and cyclodecane were characterized as the active constituents in the oil (Furletti et al., 2011).

Antioxidant activity

An antioxidant is defined as ‘any substance that, when present at low concentrations as compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate’ (Rhee et al., 2009; Halliwell and Gutteridge, 1995; Wiseman et al., 1997; Mates et al., 1999). Antioxidants are of interest to biologists and clinicians, because they help to protect the human body against damages induced by reactive free radicals generated in atherosclerosis, ischemic heart disease, cancer, Alzheimer's disease, Parkinson's disease, and even in aging process (Aruoma, 2003; Hemati et al., 2010). There are many evidences that natural products and their derivatives have efficient anti-oxidative characteristics, consequently linked to anti-cancer, hypolipidemic, anti aging, and anti-inflammatory activities (Rhee et al., 2009; Halliwell and Gutteridge, 1995; Wiseman et al., 1997; Hogg, 1998; Mates et al., 1999; Aruoma, 2003; Cho et al., 2006).

Anti-oxidative capacities of different parts of *C. sativum* were evaluated by three methods, including determining its effect on scavenging the diphenylpicrylhydrazyl (DPPH) radical, inhibition of 15-lipoxygenase (15-LO), and inhibition of Fe^{2+} induced porcine brain phospholipid peroxidation. The leaves showed stronger antioxidant activity than the fruits. Positive correlations were found between total phenolic content in the extracts and antioxidant activity (Wangensteen et al., 2004).

Polyphenolic compounds are present in *C. sativum*, and are known to be excellent antioxidants. They have the capacity to reduce free-radical formation by scavenging free radicals and protecting antioxidant defenses. The antioxidant potencies of polyphenolic compounds from *C. sativum* against hydrogen peroxide-induced oxidative damage in human lymphocytes have also been shown. H_2O_2 treatment significantly decreased the activities of antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, and caused decreased glutathione content and increased thiobarbituric acid-reacting substances (TBARS). Treatment with polyphenolic fractions (50 µg/ml) increased the activities of antioxidant enzymes and glutathione content and reduced the levels of TBARS significantly. Polyphenolic compounds are effectively responsible for suppression of hydrogen peroxide-induced oxidative stress (Hashim et al., 2005).

Analyses also showed that caffeic acid, protocatechinic acid, and glycitin were present in high concentration

(6.98, 6.43, and 3.27 µg/ml) in coriander aerial parts. They are principal components responsible for the antioxidant activity of the aqueous coriander extract (Melo et al., 2005).

Hepatoprotective activity

C. sativum extract protects liver from oxidative stress induced by carbon-tetrachloride (CCl_4) and thus helps in evaluation of traditional claim on this plant. Pretreatment of rats with different doses of plant extract (100 and 200 mg/kg) significantly lowered serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and TBARS levels against CCl_4 treated rats. Hepatic enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were significantly increased by treatment with plant extract, against CCl_4 treated rats. Oral administration of the leaf extract at a dose of 200 mg/kg significantly reduced the toxic effects of CCl_4. The activity of leaf extract at this dose was comparable to the standard drug, silymarin (Sreelatha et al., 2009).

Antidiabetic effects

Sub-chronic oral administration of *C. sativum* extract (20 mg/kg) in obese-hyperglycemic and hyperlipidemic animal model normalized glycemia and decreased the elevated levels of insulin, insulin resistance (IR), total cholesterol (TC), low density lipoprotein (LDL)-cholesterol, and triglycerides (TG). Since *C. sativum* extract decreased several components of the metabolic syndrome and decreased atherosclerotic and increased cardioprotective indices, its extract may have cardiovascular protective effect (Aissaoui et al., 2011).

It has been demonstrated that *C. sativum* extract was able to decrease hyperglycemia and increase glucose uptake and metabolism, and insulin secretion (Gray and Flatt, 1999; Swanston-Flatt et al., 1990).

Safety of *C. sativum* essential oil

Coriander essential oil is obtained by steam distillation of the dried fully ripe fruits (seeds). Based on the results of a 28 day oral gavage study in rats, a no-observed effect-level (NOEL) for coriander oil is approximately 160 mg/kg/day. In a developmental toxicity study, the maternal no-observed adverse effect level (NOAEL) of coriander oil was 250 mg/kg/day and the developmental NOAEL was 500 mg/kg/day. Coriander oil is not clastogenic, but results of mutagenicity studies for the spice and some extracts are mixed. The major component of the essential oil, linalool, is non-mutagenic. Coriander oil has broad-spectrum, antimicrobial activity. Coriander oil is irritating to rabbits, but not to humans; it is not a sensitizer, although, the whole spice may be. Based on the history of consumption of coriander oil without reported adverse effects, lack of its toxicity in limited studies and lack of toxicity of its major constituent, linalool, the use of coriander oil as an added food ingredient is considered safe at present levels of use (Burdock and Carabin, 2009). The median lethal dose (LD_{50}) of *C. sativum* essential oil was determined as 2.257 ml/kg (Özbek et al., 2006).

C. sativum as an oilseed crop grown in Italy was investigated regarding anti-nutritive compounds such as glucosinolates, sinapine, inositol phosphates, and condensed tannins, which can adversely affect the nutritional value of residues from the oilseed processing. All these compounds were found in *C. sativum* fruits in different amounts (Matthäus and Angelini, 2005).

CONCLUSION

The objective of this review has been to show the recent advances in the exploration of *C. sativum* as phytotherapy and to illustrate its potential as a therapeutic agent. With this present information, it is evident that *C. sativum* has pharmacological functions including antioxidant, antibacterial, antifungal, antidiabetic, hepatoprotective, and antihyperlipidemic activities, among others. As this present information shows, it is also possible that the fruit's essential oil or the whole plant extract might be useful in the development of new drugs to treat various diseases. However, the present results suggest a possibility that volatile components and polyphenolics can be further developed as a potential disease-curing remedy. It must be kept in mind that clinicians should remain cautious until more definitive studies demonstrate the quality and efficacy of *C. sativum*. For these reasons, extensive pharmacological and chemical experiments, together with human metabolism will be a focus for future studies. Finally, this review emphasizes the potential of *C. sativum* to be employed in new therapeutic drugs and provides the basis for future research on the application of transitional medicinal plants.

REFERENCES

Aissaoui A, Zizi S, Israili ZH, Lyoussi B (2011). Hypoglycemic and hypolipidemic effects of *Coriandrum sativum* L. in Meriones shawi rats. J. Ethnopharmacol. 137(1):652-661.

Al-Mofleh IA, Alhaider AA, Mossa JS, Al-Sohaibani MO, Rafatullah S, Qureshi S (2006). Protection of gastric mucosal damage by *Coriandrum sativum* L. pretreatment in Wistar albino rats. Environ. Toxicol. Pharmacol. 22(1):64-69.

Aruoma OI (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat. Res. 523-524:9-20.

Asgarpanah J, Dadashzadeh Mehrabani G, Ahmadi M, Ranjbar R, Safialdin-Ardebily M (2012). Chemistry, pharmacology and medicinal properties of *Heracleum persicum* Desf. Ex Fischer: A review. J. Med. Plants Res. 6(10):1813-1820.

Baba K, Xiao YQ, Taniguchi M, Ohishi H, Kozawa M (1991). Isocoumarins from *Coriandrum sativum*. Phytochemistry 30(12): 4143–4146.

Bhuiyan NI, Begum J, Sultana M (2009). Chemical composition of leaf and seed essential oil of *Coriandrum sativum* L. from Bangladesh. Bangladesh J. Pharmacol. 4:150-153.

Burdock GA, Carabin IG (2009). Safety assessment of coriander (*Coriandrum sativum* L.) essential oil as a food ingredient. Food Chem. Toxicol. 47:22-34.

Cho JY, Prak SC, Kim TW, Kim KS, Song JC, Kim SK, Lee HM, Sung HJ, Park HJ, Song YB, Yoo ES, Lee CH, Rhee MH (2006). Radical scavenging and anti-inflammatory activity of extracts from *Opuntia humifusa*. Raf. J. Pharm. Pharmacol. 58:113-119.

Coskuner Y, Karababa E (2007). Physical properties of coriander seeds (*Coriandrum sativum* L.). J. Food Engin. 80(2):408-416.

Dhanapakiam P, Joseph JM, Ramaswamy VK, Moorthi M, Kumar AS (2008). The cholesterol lowering property of coriander seeds (*Coriandrum sativum*): Mechanism of action. J. Environ. Biol. 29(1):53-56.

Eguale T, Tilahun G, Debella A, Feleke A, Makonnen E (2007). *In vitro* and *in vivo* anthelmintic activity of crude extracts of *Coriandrum sativum* against *Haemonchus contortus*, J. Ethnopharmacol. 110:428-433.

Eikani M, Golmohammad F, Rowshanzamir S (2007). Subcritical water extraction of essential oils from coriander seeds (*Coriandrum sativum* L.). J. Food Eng. 80(2):735-740.

Emamghoreishi M, Heidari-Hamedani GH (2008). Effect of extract and essential oil of *Coriandrum sativum* seed against pentylenetetrazole-induced seizure. Pharm. Sci. 7(2):1-10.

Furletti VF, Teixeira P, Obando-Pereda G, Mardegan RC, Sartoratto A, Figueira GM, Duarte RMT, Rehder VLG, Duarte MCT, Hofling JF (2011). Action of *Coriandrum sativum* L. essential oil upon oral *Candida albicans* Biofilm formation. Evidence-Based Comp. Alter. Med. 20(11):1-9.

Gray AM, Flatt PR (1999). Insulin-releasing and insulin-like activity of the traditional anti-diabetic plant *Coriandrum sativum* (coriander). Br. J. Nutr. 81:203-209.

Halliwell B, Gutteridge JMC (1995). Role of free radicals and catalytic metal ions in human disease: An overview. Method. Enzymol. 186:1-85.

Hashim MS, Lincy S, Remya V, Teena M, Anila L (2005). Effect of polyphenolic compounds from *Coriandrum sativum* on H_2O_2-induced oxidative stress in human lymphocytes. Food Chem. 92(4):653–660.

Hemati A, Azarnia M, Angaji AH (2010). Medicinal effects of *Heracleum persicum* (Golpar). Middle-East J. Sci. Res. 5(3):174-176.

Hogg N (1998). Free radicals in disease. Seminars in Reproductive Endocrinol. 16:241-248.

Jabeen Q, Bashir S, Lyoussi B, Gilani AH (2009). Coriander fruit exhibits gut modulatory, blood pressure lowering and diuretic activities. J. Ethnopharmacol. 122(1):123-130.

Kubo I, Fujita KI, Kubo A, Nihei KI, Ogura T (2004). Antibacterial activity of coriander volatile compounds against *Salmonella choleraesuis.* J. Agri. Food Chem. 52(11):3329-3332.

Leung AY, Foster S (1996). Coriander. Encyclopedia of Common Natural Ingredients. New York. John Wiley and Sons Inc., p.193-194.

Matasyoh JC, Maiyo ZC, Ngure RM, Chepkorir R (2009). Chemical composition and antimicrobial activity of the essential oil of *Coriandrum sativum*. Food Chem. 113(2):526-529.

Mates JM, Perez-Gomez C, Nunez de Castro I (1999). Antioxidant enzymes and human diseases. Clin. Biochem. 32:595-603.

Matthäus B, Angelini LG (2005). Anti-nutritive constituents in oilseed crops from Italy. Indust. Crops Prod. 21(1):89-99.

Melo EA, Filho JM, Guerra NB (2005). Characterization of antioxidant compounds in aqueous coriander extract (*Coriandrum sativum* L.). Food Sci. Technol. 38(1):15-19.

Nejad Ebrahimi S, Hadian J, Ranjbar H (2010). Essential oil compositions of different accessions of *Coriandrum sativum* L. from Iran. Nat. Prod. Res. 24(14):1287-1294.

Özbek H, Him A, Turkozu D (2006). The levels of lethal dose and anti-inflammatory effect of *Coriandrum sativum* L. essential oil extract. Ege J. Med. 45(3):163-167.

Platel K, Srinivasan K (2004). Digestive stimulant actions of spices: a myth or reality? Indian J. Med. Res. 119:167-179.

Rhee MH, Park HJ, Cho JY (2009). Salicornia herbaceae: Botanical, Chemical and pharmacological review of halophyte marsh plant. J. Med. Plants Res. 3(8): 548-555.

Rondon FCM, Bevilaqua CML, Accioly MP, Morais SM, Andrade-Junior, Machado LKA, Cardoso RPA, Almeida CA, Queiroz-Juniora EM, Rodrigues ACM (2011). In vitro effect *of Aloe vera, Coriandrum sativum* and *Ricinus communis* fractions on *Leishmania infantum* and on murine monocytic cells. Vet. Parasitol. 178(3-4):235-240.

Silva F, Ferreira S, Duarte A, Mendonça DI, Domingues FC (2011a). Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B. Phytomed. 19(1):42-47.

Silva F, Ferreira S, Queiroz JA, Domingues FC (2011b). Coriander (*Coriandrum sativum* L.) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. J. Med. Microbiol. 60(10):1479-1486.

Small E (1997). Culinary herbs. Ottawa. NRC Research Press, pp 219-225.

Sreelatha S, Padma PR, Umadevi M (2009). Protective effects of *Coriandrum sativum* extracts on carbon tetrachloride-induced hepatotoxicity in rats. Food Chem. Toxicol. 47(4):702-708.

Sriti J, Talou T, Wannes WA, Cerny M, Marzouk B (2009). Essential oil, fatty acid and sterol composition of Tunisian coriander fruit different parts. J. Sci. Food Agric. 89(10):1659-1664.

Swanston-Flatt SK, Day C, Bailey CJ, Flatt PR (1990). Traditional plant treatments for diabetes: studies in normal and streptozotocin diabetic mice. Diabetologia 33:462-464.

Taniguchi M, Yanai M, Xiao YQ, Kido T, Baba K (1996). Three isocoumarins from *Coriandrum sativum*. Phytochemistry 42(3):843-846.

Wangensteen H, Samuelsen AB, Malterud KE (2004). Antioxidant activity in extracts from coriander. Food Chem. 88:293-297.

Wichtl M (1994). Coriandri fructus. Herbal Drugs and Phytopharmaceuticals. CRC Press, Boca Raton, FL: 159-160.

Wiseman SA, Balentine DA, Frei B (1997). Antioxidants in tea. Crit. Rev. Food Sci. Nutr. 37:705-718.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2346-2353, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.927
ISSN 1996-0816 © 2012 Academic Journals

Review

Berberis lycium Royle: A review of its traditional uses, phytochemistry and pharmacology

Arham Shabbir[1,2]*, Muhammad Shahzad[2], Yasir Arfat[2], Liaqat Ali[2], Rao Salman Aziz[2], Ghulam Murtaza[1], Sobia Anwar Waqar[3] and Alamgeer[4]

[1]Department of Pharmaceutical Sciences, COMSATS Institute of Information Technology, Abbottabad-22060, Pakistan.
[2]Department of Pharmacology, University of Health Sciences, Lahore, Pakistan.
[3]Wah Medical College, Wahcantt, Pakistan.
[4]Faculty of Pharmacy, University of Sargodha, Sargodha, Pakistan.

Accepted 16 July, 2012

Berberis lycium **Royle (family: Berberidaceae), a native to Pakistan, India and whole region to Himalyas is widely used like food and in folk medicine. A wide range of medicinally and nutritionally important phytochemical constituents have been isolated from plant such as alkaloids, cardioactive glycosides, saponins, tannins, anthocyanins, vitamins, carbohydrates, proteins, lipids, fiber content, β carotein, cellulose, phytic acid and phytate phosphorous. Plant possesses minerals such as Sodium, Calcium, Sulphur, Iron, Zinc, Copper, Lead, Manganese, Potassium and Phosphorus, which contribute to broad variety of biological processes and are valuable in the treatment of various disorders. Traditionally, the plant has been used against diarrhea, intestinal colic, piles, jaundice, internal wounds, rheumatism, diabetes, ophthalmia, gingivitis, throat pain, backache, scabies, bone fractures, sun blindness, pustules, manorrhagia, fever and as diuretic, expectorant and diaphoretic.** ***B. lycium*** **is known to possess antidiabetic, antihyperlipidemic, hepatoprotective, antibacterial, antifungal, anticoccidial, pesticidal, antimutagenic and wound healing properties, supporting its traditional uses. In this review, a comprehensive account of phytochemical constituents and pharmacological activities is presented along with traditional uses in a view of many recent findings and its potential for future research. To what extent, the findings about pharmacological activities are of potential clinical relevance and are unclear due to lack of clinical data.**

Key words: Antidiabetic, antihyperlipidemic, antimicrobial, antimutagenic, *Berberis lycium,* hepatoprotective.

INTRODUCTION

Nowadays, food is not only considered as the source of nutrients but also a powerful medicine. Information about the new food sources and exploitation of large number of less familiar plant resources which are present in nature is present in today's necessity (Kaur et al., 2001). *Berberis lycium* Royle (family: Berberidaceae), a lesser-known plant is named in English as barberry (Anwar et al., 1979), whereas, its fruit is called as "kashmal" (Usmanghani et al., 1997; Baquar, 1989) and roots are known as "Darhald" (Nadkarni, 1980). It is native to the whole region of Himalyas Mountains and is widely distributed in temperature and semi temperature areas of Pakistan, India, Afghanistan, Nepal and Bangladesh. In Pakistan, it grows in Baluchistan, NWFP, Punjab and Azad Kashmir at elevation of 900 to 2900 m (Fluck, 1971; Ali and Khan, 1978). According to International Union for Conservation of Nature (IUCN), categories *B. lycium* species are vulnerable (Waseem et al., 2006; Hamayun et al, 2006). Fruits (Kashmal) have been used by

*Corresponding author. E-mail: charham007@hotmail.com. Tel: (+92) 313 6220766.

primitive societies living in Himalyan range of Jammu and Kashmir and Himachal Pradesh since ancient times as a food source (Kaur et al., 2001), and also made into sauce (Tiwari et al., 2010).

B. lycium contains alkaloids like berberine (Ali and Khan, 1978; Chandra and Purohit, 1980; Gosh et al., 1990; Gulfraz et al., 2004), plamitine (Gosh et al., 1990; Gulfraz et al., 2004), berbamine (Khare, 2004), baluchistanamine, karakoramine, gilgitine, jhelumine, punjabine, sindamine, chinabine (Manske, 1998) and umbellatine (Baquar, 1989). Plant is documented to posses proteins, carbohydrates, lipids, vitamin C (Gulfraz et al, 2004; Sood et al., 2010), hydrolysable tannins, cardioactive glycosides and saponins (Ahmad et al., 2009). Especially, fruits have moisture, vitamin A, fibre content, cellulose, hemicelluloses, β carotein, anthocyanins, phytic acid and phytate phosphorous (Sood et al., 2010). A wide variety of minerals are also documented such as Sodium, Calcium, Sulphur, Iron, Zinc (Gulfraz et al., 2004), copper, lead, manganese (Srivastava et al., 2006), Potassium and Phosphorus (Sood et al., 2010).

Plant is reported to possess antibacterial, antifungal (Singh et al., 2007), anticoccidial, immunostimulant (Nidaullah et al., 2010), pesticidal (Tewary et al., 2005), antimutagenic (Khan et al., 2010), hypoglycemic (Gulfraz et al., 2007; Gulfraz et al., 2008; Ahmad et al., 2009a), antihyperlipidemic (Chand et al., 2007; Ahmed et al., 2009b), hepatoprotective (Khan et al., 2008) and wound healing properties (Asif et al., 2007). Berberine, an alkaloid from *B. lycium* is also known to posses antidiarrheal (Zhang and Shang, 1989), antinocioceptive, antipyretic (Küpeli et al., 2002), anti inflammatory (Kuo et al., 2004), anti hypercholesterolemia (Doggrell, 2005), antitumor (Issat et al., 2006) and antidiabetic properties (Steriti, 2010). It also reduces brain ischemic-hypoxic injury (Benaissa et al., 2009) and can ameliorate spatial memory impairment (Zhu and Qian, 2006).

In this review, a comprehensive description of its traditional uses, pharmacological activities and phytochemical constituents is presented in a view of various recent findings and its potential for future research. Information on *B. lycium* Royle was gathered via the internet (using Sciencedirect, PubMed, Google Scholar, Google patent, and Endnote software) and libraries.

BOTANICAL ASPECTS

Plant is a semi deciduous shrub, 2 to 3 m high; twigs yellowish, glabrous or minutely pubescent; leaves 2.5 to 7.5 by 8.18 mm, sessile lanceolate or narrowly obovate-oblong, cariaceous, entire or with a few large spinous teeth, dull green above; racemes shortly stalked, drooping, longer than leaves, often with a few long-stalked, flowers at the base; pedicles slender, 1 to 3 cm long. Berries are globose ovoid, 8 mm long, blue, covered with a bloom (Baquar, 1989). The cork is 5 to 10 cell thick, cambium, xylem with spiral, pitted and reticulate tracheae (Mahmood et al., 2005). Investigation on pollen morphology was carried out through light and scanning electron microscope. Pollen diameter in pollen type *Berberis kunawurensis* in µm is 38.71 (41.5 ± 0.57) 45.5 and exine thickness in µm is 2.25 (2.42 ± 0.34) 2.59 (Perveen and Qaiser, 2010). It is also visited by honey bees for nector (Zabihullah et al., 2006).

USE IN TRADITIONAL MEDICINE

B. lycium Royle has also been used in folk medicine against diarrhea (Zaman and Khan, 1970; Baquar, 1989; Usmanghani et al., 1997; Kaur et al., 2001; Hamayun et al., 2005; Hamayun et al., 2006; Afzal et al., 2009), intestinal colic (Kaur et al., 2001; Hamayun et al., 2006), jaundice (Baquar, 1989; Hamayun et al., 2005; Zabihullah et al., 2006; Hamayun et al., 2006), internal wounds (Ahmed et al., 2004; Hamayun et al., 2005; Hamayun et al., 2006), piles (Zaman and Khan, 1970; Baquar, 1989; Gosh et al., 1990; Hamayun et al., 2006; Afzal et al., 2009), ophthalmia (Zaman and Khan, 1970; Khan et al., 1979; Baquar, 1989; Kaur et al., 2001; Uniyal et al., 2006), diabetes (Ahmed et al., 2004; Waseem et al., 2006; Tiwari et al., 2010), rheumatism (Kaur et al., 2001; Zabihullah et al., 2006), backache (Zabihullah et al., 2006), gingivitis (Hamayun et al., 2005), throat pain, scabies, pustules, bone fractures (Ahmed et al., 2004), sun blindness, intermittent fever, remittent fever (Kaur et al., 2001) and manorrhagia (Baquar, 1989). Plant is also expectorant, diuretic (Hamayun et al., 2006), diaphoretic (Kaur et al., 2001) and febrifuge (Baquar, 1989; Gosh et al., 1990; Shamsa et al., 1999; Mahmood et al., 2005).

Different parts of plant have been used in different preparations traditionally. Dried powdered plant bark is used for dysentery, internal wounds and throat pain. Root bark water extract is used against diabetes, pustules and scabies while root powder paste has been used in bone fracture (Ahmed et al., 2004). A decoction is prepared by drying root in shade and then boiling in water. This decoction is then concentrated and finally dried at low temperature. The end product is called “Rasaunt” and is used to cure eye infections. New vegetable atypical shoots are crushed and sap is also used for the same purpose (Uniyal et al., 2006). Decoction of rhizome is also utilized for jaundice by traditional practitioners (Zabihullah et al., 2006).

Traditional systems of medicine have produced many beneficial leads in the development of medications for various diseases. Several studies have proposed that plants and their constituents mediate their effects by modulating several of these recently recognized

therapeutic targets. However, traditional medicine needs rediscovery in light of our existing understanding of allopathic (modern) medicine (Aggarwal et al., 2006). *B. lycium* Royle has been reported by various studies for its use in traditional system of medicine to treat diabetes. Pharmacological studies in recent years showed that plant reduces hyperglycemia, and insulin like effect is suggested as possible mechanism of antidiabetic activity of plant. Similarly, this plant is well documented for its folkric use to treat rheumatism, and gingivitis. Berberine, a major constituent of this plant is now well known to possess anti-inflammatory property. Traditional practitioners have also used this plant to treat wounds. Recent experiments revealed that plant causes healing of wound by increasing the area of epithelialization and collagen deposition. Useful leads which are provided by practitioners of traditional system of medicine continue to help us in development of modern medicine and novel therapeutic targets.

PHYTOCHEMISTRY

There are more than 20 chemical elements (often called minerals) necessary for humans. Deficiency in such essential nutrients leads to a wide range of symptoms depending upon the deficient mineral (Campbell, 1996). Roots and fruits were analyzed for mineral contents through chemical analysis. Roots contain 0.2% Sulphur and 0.2% Zinc, while in fruits, amount of Sulphur and Zinc present is 0.1 and 0.8%, respectively (Gulfraz et al., 2004). Analysis of roots and stem for mineral contents was done by using Atomic absorption spectrophotmeter. Copper (Cu) detected in the roots and stem of *B. lycium* were 4.360 ± 0.176 and 5.031 ± 0.176 ppm, respectively. Amount of Lead (Pb) found in the roots and stem of plant was 4.360 ± 0.176 and 7.992 ± 0.106 ppm, respectively. Manganese (Mn) present in the roots and stem of plant is 15.500 ± 0.212 and 18.272 ± 0.212 ppm, respectively (Srivastava et al., 2006). Atomic absorption spectrophotometer was used to analyze Sodium, Potassium and Iron, while Calcium by flame photometer. Calcium (18.272 ± 0.212 mg/100 g), Sodium (14.5± 0.11 mg/100 g), Potassium (161.42 ± 0.41 mg/100 g), Phosphorus (38.0 ± 0.24 mg/100 g) and Iron (2.61 ± 0.06 mg/100 g) content of the fruit are in appreciable amount (Sood et al., 2010). Leaves are abundant in Zinc (37.71 ± 0.02 μgg^{-1}), Mn (136.12 ± 0.01μgg^{-1}), Iron (528.47 ± 0.02 μgg^{-1}), Cu (53.41 ± 0.09 μgg^{-1}), Phosphorous (1315.00 ± 0.01 μgg^{-1}), Potassium (4077.00 ± 0.58 μgg^{-1}), Sodium (79.00 ± 0.01 μgg^{-1}) and Calcium (2389.00 ± 0.04 μgg^{-1}). It was revealed that Zinc, Cu and Sodium were maximum in root, while Mn, Phosphorous, Calcium in leaves, whereas, Potassium in shoot (Shah et al., 2003).

There is hardly a food, beverage, pharmaceutical, or cosmetic preparation which does not contain essential oils, glycosides, enzymes, resins, mucilages, tannins, gums, fibers and other botanical ingredients (Said, 1996). Roots of *B. lycium* possess dry matter (61.2%), moisture (20.5%), protein (4.5%), fat (2.6%), sugar (3.5%), fiber (2.5%) and vitamin C (0.3%). Fruits also contain dry matter (62.5%), moisture (12.5%), protein (2.5%), fat (1.8%), sugar (4.5%), fiber (1.5%) and vitamin C (0.8%) in considerable amount (Gulfraz et al., 2004). It was revealed that leaves have maximum amount of moisture followed by shoot and root, respectively. Maximum amount of fat and fiber was observed in roots, while highest amount of crude proteins was found in leaves (Shah et al., 2003). Alkaloids, saponins, cardioactive glycosides and hydrolysable tannins were found to be present in water extract of plant (Ahmad et al., 2009). β-Carotene (343.0 ± 0.89 µg/100 g), vitamin A (85.65 ± 0.17 µg/100 g), tannins (8.9 ± 0.15 mg/100g), phytic acid (2.5 ± 0.04 mg/100 g), phytate phosphorus (0.78 ± 0.06 mg/100 g), anthocyanin (82.47 ± 0.29 mg/100 ml juice), cellulose (7.94 ± 0.60 %) and hemicellulose (6.01 ± 0.41 %) were detected in fruits. Diameter, weight and length were detected as 1.5 cm, 17.15 g/100 fruits and 0.80 cm, respectively in physiochemical evaluation (Sood et al., 2010). Berberine and palmitine are found in roots of *B. lycium* in a concentration of 4.5 and 3.1%, respectively, while 2.9% berberine is present in fruits (Gulfraz et al., 2004). Berbamine (Khare, 2004), baluchistanamine, karakoramine, gilgitine, jhelumine, punjabine, sindamine, chinabine (Manske, 1998) and umbellatine (Baquar, 1989) are also documented to be present in plant. Structures of the major constituents of *B. lycium* Royle are showb in Figure 1.

PHARMACOLOGICAL PROPERTIES

Antidiabetic activity

Effect of *B. lycium* Royle was evaluated in alloxan-induced diabetic rabbits and plant was collected by native author Alamgeer from Gilgit district, Pakistan. Crude powder of *B. lycium* reduced the blood glucose levels of both diabetic and normal rabbits. Water, methanolic, aqueous methanolic, n-hexane and chloroform extracts of plant were prepared for screening their antidiabetic activity in alloxanized rabbits. Results indicated that among the extracts, orally administered 500 mg/kg of water extract produced maximum hypoglycemic activity for up to 6 h. Same doses of methanolic, aqueous methanolic and n-hexane extracts decreased blood glucose level up to 4 h. Chloroform extract did not show any significant antidiabetic activity. Water extract was further compared in combination with insulin. The results of 500 mg/kg of water extract with 2 units of insulin were comparable with 6 units of insulin (Ahmad et al., 2009a). Ethanolic and aqueous extracts of plant roots were

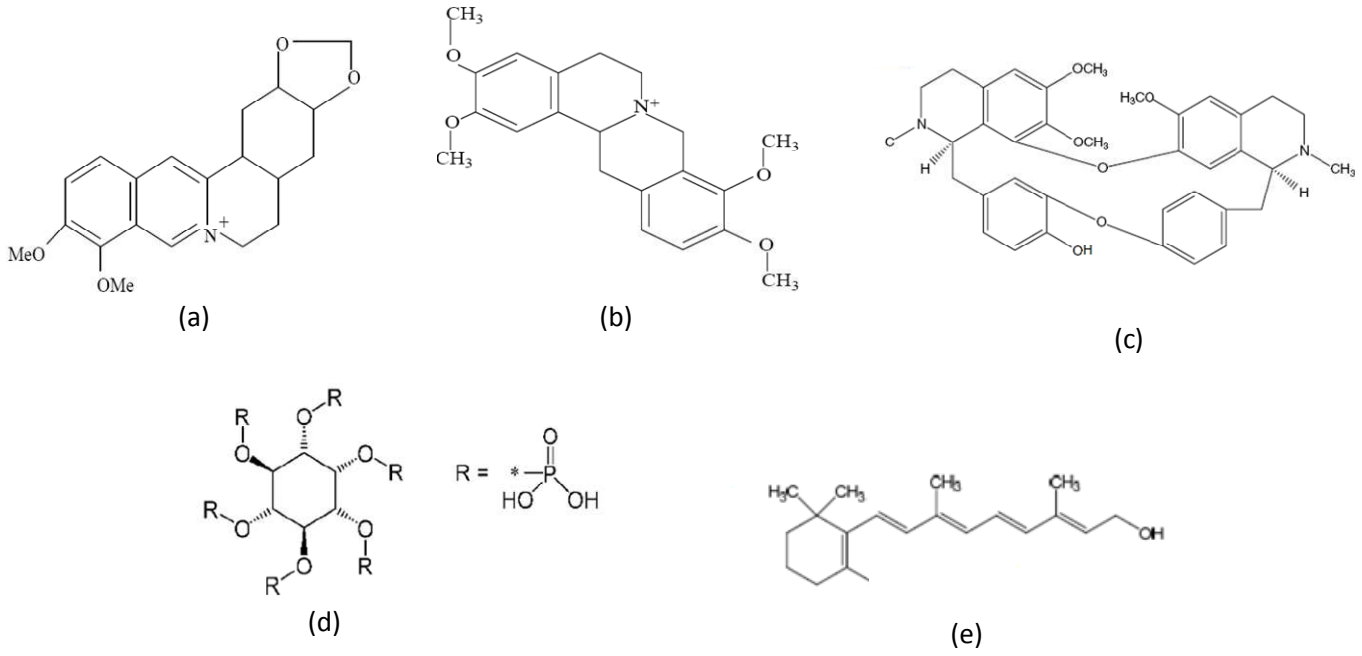


Figure 1. Structures of the major constituents of *B. lycium* Royle. a) Berberine (Singh et al., 2010); b) Palmitine (Singh et al., 2010); c) Berbamine (Wen-Ying et al., 2006); d) Phytic acid (Ali et al., 2010); and e) Vitamin A (Huang et al., 2009).

administered in normal and alloxanized rats and 20 mg/kg glibenclamide was used as reference drug. Serum was utilized to evaluate blood glucose level by the glucose oxidase method. The doses of 50 and 100 mg/kg decreased hyperglycemia after 3 to 5 h of treatment but the effect of later dose was more pronounced. Oral glucose tolerance test showed that plant extracts reduced serum glucose level in a dose-dependent manner. The observed mechanism involved in hypoglycemia is insulin-like effect, possibly through the peripheral glucose consumption. The applied doses were devoid of any behavioural changes or acute toxicity in experimental animals (Gulfraz et al., 2007). Antidiabetic activity of pure berberine was compared with ethanolic root extract of *B. lycium* in normal and alloxan-induced diabetic rats using similar doses (50 mg/kg) of each. Plant extract and berberine reduced blood glucose level significantly and demonstrated significant effects on glycosylated haemoglobin, glucose tolerance, serum lipid profiles and body weight. Plant extract was comparable in efficacy with berberine (Gulfraz et al., 2008).

Antihyperlipidemic property

Antihyperlipidemic property was investigated and roots of *B. lycium* Royle were collected for this purpose. Results indicated that oral administration of 250 and 500 mg/kg crude powder for 4 weeks resulted in significant decline in triglyceride, low density lipids (LDLs) and total cholesterol levels in male albino rabbits, while high density lipids (HDLs) were enhanced. Furthermore, same doses stabilized the weight of diabetic rabbits (Ahmad et al., 2009b). In another study, *B. lycium* root bark powder was studied for hypolipidemic effect in broilers. The powder was added to commercial broiler feed at the rate of 2.0%. *B. lycium* crude powder significantly decreased the total cholesterol, triglycerides and LDL, while HDL was significantly increased dose dependently (Chand et al., 2007).

Hepatoprotective property

To evaluate hepatoprotective effect, crude powder and methanolic extract of *B. lycium* Royle was used. Paracetamol was used to induce hepatotoxicity in rabbits. Results showed that plant significantly reduced the raised levels of alkaline phosphatase, serum glutamic pyruvic transaminase and serum glutamic oxaloacetic transaminase enzymes in treated hepatotoxic rabbits (ahmad et al., 2008). In another study, six poly herbal formulations including Livokin (Herbo-med, Kolkata) which also contains *B. lycium* were studied in mice. This formulation was found to have hepatoprotective effect in paracetamol induced hepatotoxic mice (Girish et al., 2009). Powder of *B. lycium* bark along with *Pistacia integerrima* and *Gallium aparine* were mixed in distilled

water (2, 1 and 1 mg/ml, respectively) and shaken vigorously. Carbon tetrachloride was used to induce hepatotoxicity in male Sprague-Dawley rats. Study indicated that these medicinal plants have more effect as curative agents rather than preventive agents (Khan et al., 2008).

Antimicrobial property

Hydroalcoholic (50%) extract of air dried root and stem of *B. lycium* were used to determine antibacterial activity through microdilution method. Plant extract demonstrated antibacterial activity against *Micrococcus luteum, Bacillus subtilis, Bacillus cereus, Enterobactor aerogenus, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium* and *Streptococcus pneumonia.* Minimum inhibitory concentration (MIC) showed by root extract against each test organism was 1.25, 0.62, 2.50, 2.50, 0.31, 1.25, 1.25, 0.62, 0.62, 2.5 and 0.62 µg/ml, respectively, while values for stem extract were; 0.31, 0.31, 2.50, 1.25, 0.62, 0.31, 0.31, 0.31, 0.62, 0.62, and 1.25 µg/ml, respectively. Root and stem extracts also inhibited fungal strains of *Aspergillus terreus* at MIC 0.31 and 0.62 µg/ml respectively. Root extract was also found active against *Aspergillus spinulosus* and *Aspergillus flavus* at MIC 0.62 and 1.25 µg/ml, respectively (Singh et al., 2007). The hydroalcoholic extract exhibited stronger and broader spectrum against bacterial strains as compared to fungal strains (Singh et al., 2009). *B. lycium* was mixed in drinking water of broilers along with other medicinal plants. It showed better immune performance against newcastle disease, infectious bursal disease and infectious bronchitis. A significant reduction in coccidial oocysts per gram of feces was also noticed (Nidaullah et al., 2010).

Pesticidal property

Petroleum ether and aqueous methanol extracts of *B. lycium* root was prepared using Soxhlet apparatus and dried under vaccum. The activity of plant extracts were tested at two higher doses (5000 and 10000 ppm) against pests. Petroleum ether extract showed 25% mortality rate against *Helicoverpa armigera* Hub and 92% mortatlity rate against *Aphis craccivora* Koch at the dose of 5000 ppm. Extract also exhibited 26% mortality rate against *Tetranychus urticae* Koch, 98% mortality rate against *A. craccivora* Koch, while 28% mortality rate against *H. armigera* Hub and *Plutella xylostella* L. each at the dose of 10,000 ppm. Petroleum ether extract inhibited *A. craccivora* Koch at 458.65 ppm lethal concentration at 50% (LC_{50}) after 24 h contact time and 57.79 ppm LC_{50} after 48 h contact time. The LC_{50} at 48 h exposure was nearly comparable with that of Dimethoate (a chemical insecticide) at 24 h exposure. Aqueous methanolic extract demonstrated 26% mortality rate against *A. craccivora* Koch at the dose of 5000 ppm. Extract also showed 44% mortality rate against *H. armigera* Hub, 41% against *P. xylostella* L., 43% against *T. urticae* Koch and 68% against *A. craccivora* Koch (Tewary et al., 2005).

Antimutagenic property

Anti-neoplastic activities of *B. lycium* root extracts were evaluated using p53 deficient HL-60 cells along with berberine and palmatine. The n-butanol extract demonstrated highest toxicity against HL-60 cells (IC_{50} 2.3 µg extract / ml medium after 48 h of treatment), followed by the ethanol extract (23.5 µg/ml) and the water extract (110 µg/ml). Berberine showed IC_{50} 1.2 µg/ml after 48 h of treatment, while palmatine did not exhibit inhibitory effect on cell growth. HL-60 cells were exposed to 5.5 µg butanol extract/ml and 0.6 µg berberine/ml for 48 h to evaluate the cell cycle distribution which resulted in reduction of G1 cells and accumulation of cells in the S-phase by both plant extract and berberine. All three types of extracts induced apoptosis and the butanol extract was found to be superior in activity followed by the ethyl acetate and the water extracts. Berberine produced a similar pro-apoptotic effect as the extract in a comparable concentration as contained in the butanol extract. Both induced apoptosis in HL-60 cells without concomitant induction of γH2AX, which shows that the anti-neoplastic effects have not been stimulated by berberine-caused genotoxicity. Comet assay revealed that both plant extract and the pure compound cause no DNA damage. Both demonstrated ~2-fold transient phosphorylation of p38-MAPK compared to untreated control. Chk2 also became activated and pattern was found correlated with the accumulation of cells in S-phase. Cdc25A proto-oncogene inactivation was the earliest event exhibited by the berberine and butanol extract, followed by the acetylation of α-tubulin, activation of Chk2 and p38, and the down-regulation of cyclin (Khan et al., 2010).

Wound healing property

Root extracts of *B. lycium* was studied in Swiss Wistar rats for wound healing activity**.** Aqueous and methanol extracts of the plant root were examined using, excision, incision and dead wound space models of wound repair. Both extracts increased the area of epithelialization and also showed increase in breaking strength. In aqueous extract treated group moderate collagen deposition, macrophages and fibroblasts were found, whereas a significant rise in collagen deposition with lesser

macrophages and fibroblasts were observed in methanol extract treated group. A noteworthy increase in dry weight and hydroxyproline content of granulation tissue was also noticed. It was revealed that methanolic extract was more efficient than the aqueous extract (Asif et al., 2007).

CONCLUSION

Over the years, scientists have investigated and verified many of the traditional uses of *B. lycium* that continue to be an important natural remedy for various diseases. From medicinal point of view this rare plant possesses important pharmacological properties such as antidiabetic, antihyperlipidemic, hepatoprotective, antibacterial, antifungal, anticoccidial, pesticidal, antimutagenic and wound healing properties. From nutritional point of view, fruits have been used by primitive societies living in Himalayan range since centuries when in winter temperature falls down to -40℃ and the intensity of ultraviolet radiations are high. Plant possesses Sodium, Calcium, Sulphur, Iron, Zinc, Cu, Lead, Mn, Potassium, Phosphorus, Vitamin A and Vitamin C. Human and animal, studies originally demonstrated that optimal intakes of minerals such as, Potassium, Cu, Zinc, Calcium and Mn could decrease individual risk factors, including those associated with cardiovascular disorders (Mertz, 1982; Brody, 1994; Sanchez-Castillo et al., 1998). Calcium is helpful in teeth growth and is considered as major element present in bone (Anke et al., 1984). Sodium and Potassium are involved in membrane functions and are principal cations of extracellular and intracellular fluids, respectively (Bender and Mayes, 2003a). Iron plays important role in oxygen and electron transfer and is useful in the formation of hemoglobin (Dalziel, 1936; Kaya and Incekara, 2006). Zinc and Cu are required in our diet because they perform a broad range of biological functions such as parts of enzymatic and redox systems (McLaughlin et al., 1999). Dietary Vitamin A has important role in vision, its deficiency can lead to night blindness and xerophthalmia, Vitamin A and Provitamin A (β-carotene) are also antioxidants and have possible role in atherosclerosis and cancer prevention. Vitamin C functions as enzyme cofactor and its deficiency leads to scurvy (Bender and Mayes, 2003b).

A few patents have been granted by United States Patent and Trademark Office (USPTO). A herbal formulation for the treatment of diabetes and associated complications (Krishnan: 8163312), and a process for the preparation of herbal wines from ripe Himalayan berries (Singh et al: 6793957) are present in USPTO gazette.

B. lycium has considerable potential for future research. Plant is known to possess tannins and anthocyanins. Both have antioxidant property (Okamura et al., 1993; Yang and Zhai, 2010), but fewer research works have been done in this direction. So future research should be undertaken using plant extracts and different antioxidant models. Plant has been used traditionally in diarrhea and in intestinal colic since centuries. Berberine, a plant constituent is documented to possess antidiarrheal property but the exact mechanism is still uncertain. Therefore, *in vitro* spasmolytic activities and *in vivo* antidiarrheal activities of different plant extracts should be evaluated in a view to find the mechanism. Some other *Berberis* species have also been evaluated for various potential pharmacological properties. *Berberis aristata* has been examined for anti-inflammatory and cardiotonic properties (Potdar et al., 2012). *Berberis vulgaris* is known to possess antioxidant (Zovko Končić et al, 2010), anti-histaminic, anti-cholinergic (Shamsa et al., 1999), and anti-inflammatory property (Ivanovska and Philipov, 1996). These results indicate that *B. lycium* Royle might also possess similar activities. Therefore, research should be directed to evaluate *B. lycium* Royle for these pharmacological properties in future. Critical evaluation revealed that pharmacological studies are deficient in the identification of active constituents responsible for pharmacological activities, therefore, more emphasis towards identification and isolation of active constituents in future studies is suggested. A serious limitation in our knowledge is the lack of clinical data and it is not yet apparent to what extent the findings about pharmacological activities are of potential clinical relevance.

REFERENCES

Afzal S, Afzal N, Awan MR, Khan TS, Gilani A, Khanum R, Tariq S (2009). Ethno-botanical studies from northern Pakistan. J. Ayub. Med. Col. Abbott. 21(1):52-57.

Aggarwal BB, Ichikawa H, Garodia P, Weerasinghe P, Sethi G, Bhatt ID, Pandey MK, Shishodia S, Nair MG (2006). Traditional Ayurvedic medicine to modern medicine: identification of targets for suppression of inflammation and cancer. Expert. Opin. Ther. Targets 10(1):87-118.

Ahmad M, Alamgeer, Chaudhary MZ, Nadeem M, Sharif T, Ahmad B (2008). Hepatoprotective effect of *Berberis lycium* (Royle) in hepatotoxic rabbits. Gomal. Uni. J. Res. 24:24.

Ahmad M, Alamgeer, Sharif T (2009). A potential adjunct to insulin *Berberis lyceum* Royle. Diabet. croatic 38(1):13-18.

Ahmed E, Arshad M, Ahmad M, Saeed M, Ishaque M (2004). Ethnopharmacological survey of some medicinally important plants of Galliyat areas of NWFP, Pakistan. Asian. J. Pl. Sci. 3(4):410-415.

Ahmed M, Alamgeer, Sharif T, Zabta CHM, Akbar A (2009). Effect of *Berberis lycium* Royle on Lipid Profile in Alloxan Induced Diabetic Rabbits. Ethnobot. Leaflets 13:702-708.

Ali M, Shuja MN, Zahoor M, Qadri I (2010). Phytic acid: How far have we come?. Afr. J. Biotechnol. 9(11):1551-1554.

Ali MN, Khan AA (1978). Pharmacognostic studies of *Berberis lycium* Royle and its importance as a source of raw material for the manufacture of berberine in Pakistan. Pak. J. For. p.26.

Anke M, Groppel B, Kronemann H (1984) Significance of newer essential trace elements (like Si, Ni, As, Li, V,. . .) for the nutrition of man and animals. In: Bratter, P. and Schramel, P. (eds), Trace element-analytical chemistry in medicine and biology (Berlin) p. 421.

Anwar AK, Ashfaq M, Nasveen MA (1979). Pharmacognostic studies of selected indigenous splants of Pakistan, (Peshawar, NWFP, Pakistan: Pakistan Forest Institute).
Asif A, Kakub G, Mehmood S, Khunum R, Gulfraz M (2007). Wound Healing Activity of Root Extracts of *Berberis lyceum* Royle in Rats. Phytother. Res. 21:589-591.
Baquar SR (1989). Medicinal and Poisonous plants of Pakisan, (Karachi, Pakistan: Printas) p.55.
Benaissa F, Mohseni-Red, Rahimi-Moghaddam M, Mahmoudian M (2009). Berberine reduces the hypoxic-ischemic insult in rat pup brain. Acta Physio. Hung 96(2):213-220.
Bender DA, Mayes PA (2003a). Vitamins and Minerals. In: Murray, R.K., Granner, D.K., Mayes, PA.and Rodwell, VW (eds), Harper's Illustrated Biochemistry (New York: Lange Medical Books/McGraw-Hill Medical Publishing Division) p.496.
Bender DA, Mayes PA (2003b). Vitamins & Minerals. In: Murray, R.K., Granner DK, Mayes PA. and Rodwell VW (eds), Harper's Illustrated Biochemistry (New York: Lange Medical Books/McGraw-Hill Medical Publishing Division) p.481.
Brody T (1994) Nutritional Biochemistry, (San Diego, CA: Academic Press).
Campbell NA (1996) Biology, (California: The Benjamin/Cummings Publishing Company, Inc) p.812.
Chand N, Durrani FR, Qureshi MS, Durrani Z (2007). Role of Berberis lycium in reducing serum cholesterol in broilers. Asian-Australasian. J. Ani. Sci. 20(4):563-568.
Chandra P, Purohit AN (1980). Berberine contents and alkaloid profile of Berberis species from different altitudes. Biochem. Sys. Eco. 8(4):379-380.
Dalziel JM (1936). The useful plants of west tropical Africa, (London, The Crown Agents for the Overseas Governments and Administrations).
Doggrell SA (2005). Berberine-a novel approach to cholesterol lowering. Exp. Opi. Inv. Drug. 14(5):683-685.
Fluck H (1971) Medicinal Plants (New York, USA: W. Foul Sham and Co. Ltd), pp.149-165.
Girish C, Koner BC, Jayanthi S, Rao KR, Rajesh B (2009). Hepatoprotective activity of six polyherbal formulations in paracetamol induced liver toxicity in mice Pradhan. Indian. J. Med. Res. 129:569.
Gosh AK, Bhattacharyya FK, Ghosh DK (1990). Leishmania donovani: A mastigote Inhibition and mode of action of berberine. Exp. Para. 60(3):404-413.
Gulfraz M, Arshad M, Nayyer N, Kanwal N, Nisar U (2004). Investigation for bioactive compounds of Berberis lyceum Royle and Justicia adhatoda L. Ethnobot. Leaflets 1:51-62.
Gulfraz M, Mehmood S, Ahmad A, Fatima N, Parveen Z, Williamson EM (2008). Comparison of the antidiabetic activity of *Berberis lyceum* root extract and berberine in alloxan induced diabetic rats. Phytother. Res. 22(9):1208-1212.
Gulfraz M, Qadir G, Nosheen F, Parveen Z (2007). Antihyperglycemic effects of Berberis lyceum Royle in alloxan induced diabetic rats, Diabet. Croatic. 36(3):49-54.
Hamayun M, Khan A, Khan MA (2005). Common Medicinal Folk Recipes of District Buner, NWFP, Pakistan. Ethnobot. Leaflets 1:45.
SIUC. USA. http://opensiuc.lib.siu.edu/ebl/vol2005/iss1/45/
Hamayun M, Khan SA, Sohn EY, In-jung L (2006). Folk medicinal knowledge and conservation status of some economically valued medicinal plants of District Swat Pakistan. Lyonia 11(2):101-113.
Issat T, Jakóbisiak M, Golab J (2006). Berberine, a natural cholesterol reducing product, exerts antitumor cytostatic/cytotoxic effects independently from the mevalonate pathway. Onco. Rep. 16:1273-1276.
Ivanovska N, Philipov S (1996). Study on the anti-inflammatory action of *Berberis vulgaris* root extract, alkaloid fractions and pure alkaloids. Int. J. Immunopharmacol. 18(10):553-561.
Kaur C, Miani SB (2001). Fruits and vegetables health foods for new millennium. Indian Hort. 45(4):29-32.
Kaya I, Incekara N (2006). Contents of some wild plants species consumed as food in Aegean region. J. Tur. Weed. Sci. 3:56.
Khan AA, Ashfaq A, Ali MN (1979). Pharmacognostic Studies of Selected Indigenous Plants of Pakistan. (Peshawar: Spinezer Printers, Pakistan Forest Institute).
Khan M, Giessrgl B, Vonach C, Madlener S, Prinz S, Herbaceck I, Hölzl C, Bauer S, Viola K, Mikulits W, Quereshi RA, Knasmüller S, Grusch M, Kopp B, Krupitza G (2010). Berberine and a Berberis lycium extract inactivate Cdc25A and induce α tubulin acetylation that correlate with HL-60 cell cycle inhibition and apoptosis. Mut. Res. 683:123-130.
Khan MA, Jehanzeb, Shafiullah, Malik SA, Shafi M (2008). Hepatoprotective effects of *Berberis lycium*, *Galium aparine* and *Pistacia integerrima* in carbon tetrachloride (CCL4) treated rats. J. Post Grad. Med. Inst. 22(2):91-94.
Khare CP (2004) Indian Herbal Remedies. In: Rational Western Therapy, Ayurvedic and Other Traditional Usage, Botany. (Springer) p.98.
Kuo C, Chi C, Liu T (2004). The anti-inflammatory potential of berberine *in vitro* and *in vivo*. Can. Lett. 203:127-137.
Küpeli E, Koşar M, Yeşilda E, Başer KHC, Başer C (2002). A comparative study on the anti inflammatory, antinociceptive and antipyretic effects of isoquinoline alkaloids from the roots of Turkish *Berberis* species. Life Sci. 72:645-657.
Mahmood A, Ahmad M, Jabeen A, Zafar M, Nadeem S (2005). Pharmacognostic Studies of Some Indigenous Medicinal Plants of Pakistan. Ethnobot. Leaflets 9:20.
Manske RHF (1998). The alkaloids: Chemistry and Pharmacology, (San Diego, California: Academic press, INC) p.287.
McLaughlin MMJ, Parker DR, Clarke JM (1999). Metals and micronutrients-food safety issues. Field. Crop. Res. 60:143.
Mertz W (1982). Trace minerals and atherosclerosis. Food Process. 41:2807-2812.
Nadkarni KM (1980) Indian Material Medica, (Bombay, India: Popular Parakshan Depot) pp.180-190.
Nidaullah H, Durrani FR, Ahmad S, Jan IU, Gul, S (2010). Aqueous extract from different medicinal plants as anticoccidial, growth promotive and immunostimulant in broilers. ARPN. J. Agric. Biol. Sci. 5(1):53-59.
Okamura H, Mimura A, Yakao Y, Niwano M, Takahara Y (1993). Antioxidant activity of tannins and flavonoids in *Eucalyptus rostrata*. Phytochem. 33(3):557-561.
Perveen A, Qaiser M (2010). Pollen flora of Pakistan–Lxv. Berberidaceae. Pak. J. Bot. 42(1):1
Potdar D, Hirwani RR, Dhulap S (2012). Phyto-chemical and pharmacological applications of *Berberis aristata*. Fitoterap 83(5):817-830.
Said HM (1996). Medicinal herbal-a text book for medical students and doctors, (Nazimabad, Karachi-74600: Sindh, Pakistan: Humdard Foundation).
Sanchez-Castillo CP, Dewey PJS, Aguirre A, Lara JJ, Vaca R, Leon de la Barra P, Ortiz M, Escamilla I, James WPT (1998). The mineral content of mexican fruits and vegetables. J. Food. Comp. Anal. 11:340-356.
Shah H, Shad AA, Perveen S, Khattak S, Khattak KF (2003). Physiochemical composition of wild medicinal plant *Berberis lycium*. Pak. J. App. Sci. 3(6):370-375.
Shamsa F, Ahamadiani A, Khosrokhavar R (1999). Antithistaminic and anticholinergic activity of Berbery fruit (*Berberis vulgaris*) in the guinea pig ileum. Ethnopharmacol. 64:161-166.
Singh A, Duggal S, Kaur N, Singh J (2010). Berberine: Alkaloid with wide spectrum of pharmacological activities. J. Nat. Prod. 3: 64-75.
Singh M, Srivastava S, Rawat AKS (2007). Antimicrobial activities of Indian Berberis species. Fitoterap. 78: 574-576.
Singh M, Srivastava S, Rawat AKS (2009). Antimicrobial Studies of Stem of Different Berberis Species. Nat. Prod. Sci. 15(2):60-65.
Sood P, Modgil R, Sood M (2010). Physico-chemical and nutritional evaluation of indigenous wild fruit Kasmal, *Berberis lycium* Royle. Indian. J. Nat. Prod.Res. 1(3):362-366.
Srivastava S, Vartika R, Srivatava M, Rawat AKS, Shanta M (2006). Estimation of heavy metals in different *Berberis* species and its market samples. Env.Monit. Assess. 116:315-320.

Steriti R (2010). Berberine for Diabetes Mellitus Type 2. Nat. Med. J. 2(10):5-6.
Tewary DK, Bhardwaj A, Shanker A (2005). Pesticidal activit ies in five medicinal plants collected from mid hills of western Himalayas. Indus. Crop. Prod. 22:241-247.
Tiwari JK, Dangwal LR, Rana CS, Tiwari P, Ballabha R (2010). Indigenous uses of plant species in Nanda Devi Biosphere Reserve, Uttarakhand, India. Repo. Opin. 2(2):58-61.
Uniyal SR, Singh KN, Jamwal P, Lal B (2006). Traditional use of medicinal plants among the tribal communities of Chhota Bhangal, Western Himalaya. J. Ethnobiol. Ethnomed. 2:14.
Usmanghani K, Saeed A, Alam MT (1997) Indusyunic medicine, (Karachi, Pakistan: University of Karachi press) p.120.
Waseem M, Shah MA, Qureshi RA, Iqbal M, Afza R, Yousaf S (2006). Ethnopharmacological Survey of Plants Used for the Treatment of Stomach, Diabetes, and Ophthalmic Diseases in Sudhan Gali, Kashmir, Pakistan. Acta. Bot. Yunn. 28(5):535.
Wen-Ying H, Cheng-Hung S, Shueen-Jyi S (2006). Separation and Identification of the Constituents in Fangchi Radix of Different Origins. J. Food. Drug. Anal. 14(4):357-367.
Yang Z, Zhai W (2010). Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (Zea mays L.). Inn. Food. Sci. Emerg. Tech. 11:169-176.
Zabihullah Q, Rashid A, Akhtar N (2006). Ethnobotanical survey in kot Manzaray Baba valley Malakand agency, Pakistan. Pak. J. Plant Sci. 12(2):115-121.
Zaman MB, Khan S (1970) Hundred Drug Plants of West Pakistan. (Peshawar, NWFP, Pakistan: Pakistan Forest Institute).
Zhang MF, Shen, YQ (1989). Antidiarrheal and anti-inflammatory effects of berberine. Acta. Pharmacol. Sin. 10(2):174-176.
Zhu F, Qian C (2006). Berberine chloride can ameliorate the spatial memory impairment and increase the expression of interleukin-1beta and inducible nitric oxide synthase in thrat model of Alzheimer's disease. BMC Neurosci. 7:78.
Huang ZR, Lin YK, Fang JY (2009). Biological and Pharmacological Activities of Squalene and Related Compounds: Potential Uses in Cosmetic Dermatology. Molecules 14:540-554.
Zovko Končić M, Kremer D, Karlović K, Kosalec I (2010). Evaluation of antioxidant activities and phenolic content of *Berberis vulgaris* L. and *Berberis croatica* Horvat. Food. Chem. Toxic. 48(8-9):2176-2180.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2354-2358, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.360
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

The protective effect of *Acanthopanax senticosus* B on apoptosis in rat adrenal pheochromocytoma cell line (PC12) cell induced by sodium cyanide (NaCN)/glucose deprivation

Yi Ran[1], Zhang Yaguang[1], Qi Yan[2], Zhan Xiaorong[1] and Dong Qi[2]*

[1]Department of Endocrinology and Metabolism, the First Affiliated Hospital of Harbin Medical University, Harbin 150001, PR China.
[2]Department of Neurology, the First Affiliated Hospital of Harbin Medical University, Harbin 150001, PR China.

Accepted 28 May, 2012

The protective effect of *Acanthopanax senticosus* B (ASB) on apoptosis in Rat adrenal pheochromocytoma cell line (PC12) cell induced by sodium cyanide (NaCN)/glucose deprivation was studied. The PC12 cell was cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a 5% CO_2 incubator. NaCN/glucose deprivation was conducted to establish an oxidative-stress injury model *in vitro*. The protective effect of ASB was evaluated by using this model. We compared the lactate dehydrogenase (LDH) release rate and malondialdehyde (MDA) content of PC12 cells in different treatment group. The cell apoptosis was detected by using flow cytometry (FCM) and the cell proliferation by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. These results showed that pretreatment with ASB could improve cell growth and proliferation in a concentration dependent manner, reduce the release of LDH and MDA content, and inhibit the NaCN/glucose deprivation-induced apoptosis of PC12 cells. Therefore, these results suggested that ASB possessed the protective activity of PC12 cells injury by NaCN/glucose deprivation *in vitro*.

Key words: *Acanthopanax senticosus* B, PC12 cell, sodium cyanide, glucose deprivation, protective effect.

INTRODUCTION

Acanthopanax senticosus (Rupret Maxim) Harms is a shrub of the family Araliaceae, which is commonly grown in the forests in South-East Russia, North-East China, Korea, South-East Asia and Japan (Deyama et al., 2001; Jiang et al., 2006). Since 1971, the triterpenoid saponins in the leaves of this plant have been well known (Frolova et al., 1971; Shao et al., 1988). Up to now, more than 25 species of *A. senticosus* saponins have been isolated from the leaves of *A. senticosus* (Shao et al., 1989; Melek et al., 2002; Jin et al., 2004). These saponins have been used widely in traditional Chinese medicine (TCM) for treatment of many diseases. *A. senticosus* saponins can inhibit the production of nitric oxide and reactive oxygen species in murine macrophages and show antioxidant and antiglycation activity in diabetes mellitus (Lin et al., 2008; Lin et al., 2007; Xi et al., 2008). Moreover, *A. senticosus* saponins can also resist serum malondialdehyde (MDA) in postmenopausal women (Young et al., 2008), In addition, *A; senticosus* has been reported to treat the following diseases, such as brain ischemia, chronic renal failure, diabetes mellitus, chronic bronchitis, hypertension, gastric ulcers, anti-rheumatic, hypertension, anti-stress, gastric ulcer and insulin resistance (Bu et al., 2005; Tsang-Pai et al., 2005; Nishibe et al., 1990; Fujikawa et al., 1996; Davydov and Krikorian, 2000; Cha et al., 2003).

A. senticosides B (ASB) is a monomer of *A. senticosus* saponins. It was reported that ASB was a mitochondrial

*Corresponding author. E-mail: qidong15@126.com.

adenosine triphosphate (ATP)-sensitive potassium channels agonist ventricular myocytes (Zhou et al., 2005). Meanwhile, several Chinese scholars also found that ASB could protect PC cells from apoptosis induced by different ways, such as hypoxia and MPP^+ (Ji et al., 2006; Wang et al., 2007). However, the effects of ASB on rat adrenal pheochromocytoma cell line (PC12) cells apoptosis induced by sodium cyanide (NaCN)/glucose deprivation are still unclear.

In our present study, we studied the effect of ASB on apoptosis induced by NaCN/glucose deprivation in PC12 cell lines. The lactate dehydrogenase (LDH) release rate, MDA content, cell apoptosis and proliferation of PC12 cells in different treatment group was evaluated by using LDH release assay, thiobarbituric acid assay, flow cytometry (FCM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, respectively.

MATERIALS AND METHODS

Reagents

ASB was obtained from China pharmaceutical and biological products inspection (Lot: 111616-201002). NaCN, glucose, MTT, Tris and Australia phenol blue were purchased from Sigma (USA). RPMI-1640 mediu and Dulbecco's minimum essential medium (DMEM) were obtained from Gibco (USA). Newborn calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Company (Hangzhou, China). Trypsin, Tween20, and dimethyl sulfoxide (DMSO) were obtained from Amresco (USA). LDH and MDA kits were purchased from Nanjing Jiancheng Bioengineering Research Institute (China).

Cell culture and treatment

PC12 cells (rat adrenal pheochromocytoma cells) were taken from Experimental Animal Center of Harbin Medical University (Harbin, China). Cells were cultured in RPMI-1640 medium supplemented with 10% newborn calf serum at 37℃ in a 5% CO_2 incubator. When cells were closed to 80% confluence, new media with newborn calf serum were added before the compounds treatment. We used 10 mM of glucose-free medium with NaCN to establish an oxidative-stress injury model *in vitro*, and evaluate the effect of ASB by using this model. When needed, cells were incubated for 30 min with different concentrations of ASB (0.1, 1, 2, 5, 10 and 20 M) and then exposed to 10 mM of glucose-free medium with NaCN for 24 h.

Detection of LDH release of PC12 cells

When PC12 cells were closed to 80% confluence, new media with newborn calf serum was added before the compounds treatment. Cells were treated with ASB for 30 min followed by the addition of glucose-free medium with NaCN to a final concentration of 20 μM, and incubated for 24 h. The medium was collected to a 1 ml EP tube. At the end of treatments, PC12 cells were treated with 10% Triton X-100, and the media which contained detached cells were collected and centrifuged at 800 g at 4℃ for 2 min. The supernatant was used for the assay of LDH release. The enzyme was determined by using an assay kit according to the manufacturer's protocol. The absorbance of the samples was read at 440 nm. The LDH release was in proportional to the number of damaged PC12 cells. Reagent blanks were subtracted.

Detection of MDA content of PC12 cells

The PC12 cells were cultured in 6-well plates and pretreated with varying concentrations of ASB for 1 h prior to exposure to glucose-free medium with NaCN. After 24 h, cells were collected in 1.5 ml of tubes in 0.5 ml phosphate-buffered solution (PBS). Cell lysis was performed by means of three cycles of freezing and thawing. MDA content was measured using a thiobarbituric acid assay according to the manufacturer's instructions.

FCM with propidium iodide (PI) staining

The PC12 cells were treated in the same way as previously described. Cells were collected, digested with 0.25% trypsin and made into a single cell suspension by RPMI-1640 medium supplemented with 10% newborn calf serum. The single cell suspension was centrifuged at 1000 rpm for 5 min at 4℃. Then the supernatant was removed, washed with cold PBS, centrifuged at 1000 rpm for 5 min at 4℃. The cell pellets were resu spended in 1 ml binding buffer (10 mM of Hepes/NaOH, pH 7.4; 140 mM of NaCl; 2.5 mM of $CaCl_2$) and incubated for 15 min in the dark with Annexin V-FITC (20 μg/ml) and PI (50 μg/ml) at 4℃. Fluorescence was analyzed with an FCM.

MTT assay

Logarithmic growth phase cells were seeded in 96-well plates at a density of 5×10^3/ ml. Cells were cultured for 24 h at 37℃ with 5% CO_2, treated in the same way as previously described. Each group was set up three parallel holes. Cells were cultured for 24 h, followed by incubation with 0.5 mg/ml MTT, 200 μl serum-free medium for 4 h. Finally, 100 μl of DMSO was added and absorbance at 570 nm wavelength (A_{570}) was measured by means of enzyme-linked immunosorbent instrument. Relative cell proliferation inhibition rate (IR) = (1 - average A_{570} of the experimental group/average A_{570} of the control group) $\times$ 100%.

Statistical analysis

The database was set up with the SPSS 16.0 software package for analysis. Data were represented as mean ± SD. The means of multiple groups were compared with One-way analysis of variance (ANOVA), after the equal check of variance, and the two-two comparisons among the means were performed by Student's *t*-test. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of ASB on LDH release from NaCN/glucose deprivation-induced PCl2 cells

After PC12 cells were treated with 10 mM of glucose-free medium with NaCN for 24 h, the release of LDH significantly increased from (26.45 ± 1.37 U/ml) to (88.23 ± 4.32 U/ml). When 0.1, 1.0, 2, 5, 10 and 20 μM of ASB were added to the assay, the release of LDH reduced to (78.42 ± 3.92 U/ml), (68.47 ± 3 .56 U/ml), (63.89 ± 3.39

Table 1. Collection of culture medium and analysis of LDH release.

Group	LDH release (U/ml)
Medium	26.45± 1.37**
NaCN/glucose deprivation injury	88.43 ± 4.32
0.1 M ASB	78.22 ± 3.92*
1 M ASB	68.47 ± 3.56*
2 M ASB	63.89 ± 3.39**
5 M ASB	53.45 ± 3.51**
10 M ASB	42.89 ± 2.78**
20 M ASB	35.54 ± 1.98**

Data shown represent the average of three experiments. ***P*<0.001 versus NaCN/glucose deprivation injury group, **P*<0.01 versus NaCN/glucose deprivation injury group.

Table 2. Collection of culture medium and analysis of MDA content.

Group	MDA content (nmol/mg)
Medium	3.91 ± 0.34**
NaCN/glucose deprivation injury	10.32 ± 1.18
0.1 M ASB	8.38 ± 0.49*
1 M ASB	6.75 ± 0.34*
2 M ASB	6.24 ± 0.29**
5 M ASB	5.88 ± 0.22**
10 M ASB	4.65 ± 0.28**
20 M ASB	4.02 ± 0.21**

Data shown represent the average of three experiments. ***P*<0.001 versus NaCN/glucose deprivation injury group, **P*<0.01 versus NaCN/glucose deprivation injury group.

U/ml), (53.45 ± 3.51 U/ml), (42.89 ± 2.78 U/ml) and (35.54 ± 1.98 U/ml) in a concentration dependent manner. The results showed that ASB could significantly inhibit the LDH release of NaCN/glucose deprivation-induced PC12 cells in a concentration dependent manner (Table 1).

Effects of ASB on MDA content from NaCN/glucose deprivation deprivation-induced PCl2 cells

After PC12 cells were treated with 10 mM of glucose-free medium with NaCN for 24 h, the MDA content significantly increased from (3.91 ± 0.34 nmol/mg) to (10.32 ± 1.18 nmol/mg). When 0.1, 1.0, 2, 5, 10 and 20 μM of ASB were added to the assay, the MDA content reduced to (8.38 ± 0.49 nmol/mg), (6.75 ± 0.34 nmol/mg), (6.24 ± 0.29 nmol/mg), (5.88 ± 0.22 nmol/mg), (4.65 ± 0.28 nmol/mg) and (4.02 ± 0.21 nmol/mg) in a concentration dependent manner. The results showed that ASB could significantly inhibit the MDA content of NaCN/glucose deprivation-induced PC12 cells in a concentration dependent manner (Table 2).

Effects of ASB on apoptosis rate of NaCN/glucose deprivation deprivation-induced PCl2 cells

FCM results showed that the apoptosis rate of PC12 cell line treated with 10 mM of glucose-free medium with NaCN for 24 h was 34.39 ± 4.25%, which was significantly higher than that of medium group (2.14 ± 0.24%). When 0.1, 1.0, 2, 5, 10 and 20 μM of ASB were added to the assay, cell apoptosis rate was reduced to 25.48 ± 3.67%, 19.45 ± 2.86%, 15.88 ± 2.27%, 13.42 ± 1.82%, 10.54 ± 1.76% and 8.49 ± 1.21% in a concentration dependent manner. This indicated that ASB could significantly inhibit the NaCN/glucose deprivation-induced apoptosis of PC12 cells (Table 3).

Effects of ASB on proliferation IR of NaCN/glucose deprivation deprivation-induced PCl2 cells

The MTT assay demonstrated that the IR of cells significantly increased to 92.14 ± 7.27% after PC12 cells were treated with glucose-free medium with NaCN. When PC12 cells were treated with 0.1, 1.0, 2, 5, 10 and 20 μMof ASB, the apoptosis rate of cells reduced to (57.35 ± 4.25%), (67.24± 3.96%), (73.58 ± 5.21%), (81.82 ± 4.02%), (86.59 ± 8.38%) and (92.33 ± 7.25%), respectively. These results indicated that ASB could significantly inhibit NaCN/glucose deprivation-induced inhibition of PC12 cells in a concentration dependent manner (Table 4).

DISCUSSION

NaCN is known to be a blocker in electron transport chain of various biological functions. Under such conditions, a series of metabolic stress responses can be triggered within the injured cells, which include the elecation of extracellular glutamate, LDH activity. The accumulation of glutamate causes toxic effects to cells. Meanwhile, glutamate activated NMDA receptor and opens its ion channel in cell membrane. The large influx of calcium into cells could cause cell death. This NaCN/glucose deprivation injury mimics the oxidative stress-related neurodegenerative disorder such as Alzheimer's disease, Down syndrome (Shimohama, 2000; Yuan et al., 2000).

PC12 cells, a rat adrenal pheochromocytoma cell line, can extend processes similar to those produced by sympathetic neurons when exposed to nerve growth factor and these cells exhibit a single phenotype, with stable features that can be sub-cultured (Cheng and Zhang, 2008; McLaurin et al., 2000). PC12 cells are extremely similar to neurons in cell morphology, structure

Table 3. FCM showing that all genistein derivatives can decrease apoptosis rate of NaCN/glucose deprivation-induced PC12 cells.

Group	Apoptosis rate (%)
Medium	2.14 ± 0.24**
NaCN/glucose deprivation injury	34.39 ± 4.25
0.1 M ASB	25.48 ± 3.67*
1 M ASB	19.45 ± 2.86*
2 M ASB	15.88 ± 2.27**
5 M ASB	13.42 ± 1.82**
10 M ASB	10.54 ± 1.76**
20 M ASB	8.49 ± 1.21**

Data shown represent the average of three experiments. **P<0.001 versus NaCN/glucose deprivation injury group, *P<0.01 versus NaCN/glucose deprivation injury group.

Table 4. MTT assay showing that all genistein derivatives can increase proliferation rate of NaCN/glucose deprivation-induced PC12 cells.

Group	Cell viability (%)
Medium	92.14 ± 7.27**
NaCN/glucose deprivation injury	25.44 ± 4.78
0.1 M ASB	57.35 ± 4.25*
1 M ASB	67.24± 3.96*
2 M ASB	73.58 ± 5.21**
5 M ASB	81.82 ± 4.02**
10 M ASB	86.59 ± 8.38**
20 M ASB	92.33 ± 7.25**

Data shown represent the average of three experiments. **P<0.001 versus NaCN/glucose deprivation injury group, *P<0.01 versus NaCN/glucose deprivation injury group.

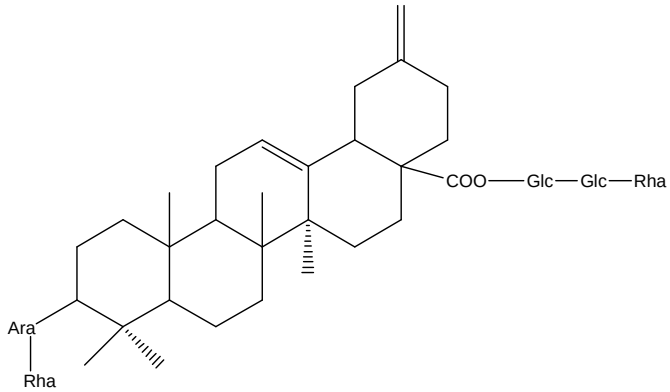


Figure 1. The chemical structure of ASB.

and function. Therefore, it has been widely used as a cell model for study of neuron cells (Saito et al., 2003).

LDH is a stable cytoplasmic enzyme that is present in all cells and is rapidly released into the culture supernatant when the plasma membrane is damaged; thus, it can be used as a reliable biochemical index for damage of the plasma membrane (Zhao et al., 2002). Our results showed that ASB could decrease LDH release of PC12 cells injured by NaCN/glucose deprivation. MDA is the end product of free radical- initiated lipid peroxidation and thus, reflects the level of lipid peroxidation. Our results also showed that NaCN/glucose deprivation may reduce the MDA content in PC12 cells, thus suggesting that its neuroprotective effects are potentially due to its antioxidant property (Xu et al., 2008; Mills et al., 1996). MTT assay results con-firmed that cell growth and proliferation were suppressed when PC12 cells were treated with glucose-free medium with NaCN for 24 h, while ASB could effectively decrease the suppression. FCM with PI staining results showed that ASB could reduce the NaCN/glucose deprivation-induced apoptosis of PC12 cells in a concentration dependent manner (Figure 1).

Conclusion

In our present work, we established the model of injury and apoptosis induced by NaCN/glucose deprivation to evaluate different concentration of ASB against oxidative stress injury in PC12 cells lines. ASB could inhibit LDH release, the MDA content, the suppression of cell proliferation and NaCN/glucose deprivation-induced apoptosis of PC12 cells in a concentration dependent manner. These findings suggest that ASB might have the protective effect against oxidative stress injury induced by NaCN/glucose deprivation in PC12 cells. However, further study in this field is needed.

REFERENCES

Bu YM, Jin ZH, Park SY, Baek SK, Rho SJ, Ha N, Park SK, Kim SY, Kim H (2005). Siberian ginseng reduces infarct volume in transient focal cerebral ischaemia in Sprague-Dawley rats. Phytother. Res. 19:167-169.

Cha YS, Soh JR, Kim JW (2003). Acanthopanax senticosus extract prepared from cultured cells improves lipid parameters in rats fed with a high fat diet. J. Korean Soc. Nutraceut. Food 8:40-45.

Cheng L, Zhang CD (2008). Cyclophilin A affects Bcl-2 and Bax expression following beta-amyloid fragment 25-35-induced injury to PC12 cells. Neural. Regen. Res. 3(6):585-588.

Davydov M, Krikorian AD (2000). *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Araliaceae) as an adaptogen: a closer look. J. Ethnopharmacol. 72:345-393.

Deyama T, Nishibe S, Nakazawa Y (2001). Constitutents and pharmacologic effects of Eucommia and Siberian ginseng. Acta Pharmacol. Sin. 22:1057-1070.

Frolova GM, Ovodov YS, Suprunov NI (1971). Tri terpene glycoside s from the leaves of eleutherococcus-senticosus part1 isolation and general characteristics. Khim. Prir. Soedin. (Tashk) 7:614-618.

Fujikawa T, Yamaguchi A, Morita I, Takeda H, Nishibe S (1996). Protective effects of Acanthopanax senticosus Harms from Hokkaido and its components on gastric ulcer in restrained coldwater stressed

rats. Biol. Pharm. Bull. 19:1227-1230.
Ji QH, Gu YJ, Zhu L (2006). Protective effect of acanthopanax senticosuson saponins PC 12 cell activity and the expression of hypoxia inducible factor-1 alpha. Chin. J. Clin. Rehab. 10(43):92-96.
Jiang W, Li W, Han L, Liu L, Zhang Q, Zhang S, Nikaido T, Koike K (2006). Biologically active triterpenoid saponins from *Acanthopanax senticosus*. J. Nat. Prod. 69:1577-1581.
Jin J, Lee S, Lee Y, Kim J, Heo J (2004). Platelet anti-aggregating triterpenoids from the leaves of *Acanthopanax senticosus* and the fruits of A. sessili florus. Hye, S. Planta Med. 70:564-566.
Lin QY, Jin LJ, Cao ZH, Lu YN, Xu YP (2008). *Acanthopanax senticosus* suppresses reactive oxygen species production by mouse peritoneal macrophages in *vitro* and in *vivo*. Phytother. Res. 22:740-745.
Lin QY, Jin LJ, Ma YS, Shi M, Xu YP (2007). *Acanthopanax senticosus* inhib its nitric oxide produc tion in murine macrophages in *vitro* and in *vivo*. Phytother. Res., 21, 879-883.
Liu TP, Lee CS, Liou SS, Liu IM, Cheng JT (2005). Improvement of insulin resistance by acanthopanaxsenticosus root in fructose-rich chow-fed rats. Clin. Exp. Pharmacol. 32:649-654.
McLaurin J, Golomb R, Jurewicz A (2000). Inositol stereoisomers stabilize an oligomeric aggregate of Alzheimer amyloid beta peptide and inhibit abeta-induced toxicity. J. Biol. Chem. 275(24):18495-18502.
Melek FR, Toshio, Miyase, Abdel-Khalik SM, Hetta MH, Mahmoud II (2002). Trit erpenoid sapon ins from Oreopana x guatemalensis. Phytochemistry 60:185-195.
Mills EM, Gunasekar PG, Pavlakovic G, Isom GE (1996). Cyanide-induced apoptosis and oxidative stress in differentiated PC12. J. Neurochem. 67:1039-1046.
Nishibe S, Kinoshita H, Takeda H, Okano G (1990). Phenolic compounds from stembark of *Acanthopanax senticosus* and their pharmacological effect in chronic swimming stressed rats. Chem. Pharm. Bull. 38:1763-1765.
Saito Y, Tsuzuki K, Yamada N (2003). Transfer of NMDAR2 cDNAs increases endofenous NMDARI1 protein and induces expression of functional NMDA receptors in PC12 cells. Mol. Brain Res. 110:159-168.
Shao CJ, Kasai R, Xu JD, Tanaka O (1988). Saponins from leaves of acanthopanax senticosus harms, ciwujia. Structures of ciwujia nosides B, C1, C2, C3, C4, D1, D2 and E. Chem. Pharm. Bull. 36: 601-608.
Shao CJ, Kasai R, Xu JD, Tanaka O (1989). Saponins from leaves of Acanthopanax senticosus Harms, ciwujia. Structures of ciwujia nosides B, A1, A2, A3, A4, and D3. Chem. Pharm. Bull. 37:42-45.
Shimohama S (2000). Apoptosis in the Alzheimer's disease- an update. Apoptosis 5:9-16.
Wang H, Yao WB, Qian YF, Gao XD (2007). Protective effect of *Acanthopanax senticosus* extract on apoptosis in PC12 cells induced by MMP^+. J. China Pharmaceut. Uni. 38(3):273-276.
Xi MM, Hai CX, Tang HF, Chen MS, Fang KQ, Liang X (2008). Antioxidant and antiglycation properties of total saponins extracted from traditional chinese medicine used to treat diabetes mellitus. Phytother. Res. 22:228-237.
Xu DH, Zhou CH, Xu BL, Luo SY (2008). Protective effect of paeonol on beta-amyloid 25-35-induced toxicity in PC12 cells. Neural. Regen. Res. 3(8):863-866.
Yuan JY, Yankner BA (2000). Apoptosis in the nervous system. Nature 407: 802-809.
Zhou Y, Tang QZ, Shi XT, Wang X, Fu JR (2005). Effect of acanthopanax senticosides B on sarcolemmal ATP sensitive potassium channel of ventricular myocytes. Chin. J. Cardiac Arrhythm. 9:378.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2359-2367, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.459
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Effect of the type of disintegrant on the characteristics of orally disintegrating tablets manufactured using new ready-to-use excipients (Ludiflash® or Parteck®) by direct compression method

Aleksandra Amelian and Katarzyna Winnicka*

Department of Pharmaceutical Technology, Faculty of Pharmacy, Medical University of Bialystok, Bialystok, Poland.

Accepted 18 June, 2012

The challenge in orally disintegrating tablets (ODTs) production encompasses the compromise between instantaneous disintegration and sufficient physico-mechanical properties, therefore the aim of this study was to evaluate the influence of selected disintegrants on the characteristics of ODTs manufactured using novel ready-to-use excipients (Ludiflash® or Parteck®) by direct compression method. Effect of selected disintegrants (croscarmellose sodium, microcrystalline cellulose and four types of crospovidone) on hardness, friability, wetting time, *in vitro* and *in vivo* disintegration time, roughness and stability parameters has been studied. The prepared tablets were also evaluated for surface morphology and pore structure. 3% w/w addition of all examined disintegrants enabled obtaining tablets showing physico-mechanical properties that are suitable for ODTs, but disintegration times < 30 s were observed only in formulations with crospovidones. The surface of Ludiflash® and Parteck® ODTs was relatively homogenous dispersion with pores, cracks and fissures. Formulations with Ludiflash® showed both less porous textures with low specific surface area (BET values up to 0.77 m^2/g) and shorter wetting and disintegration times. The optimized formulation containing superfine crospovidone and Ludiflash® (L2) or Parteck® (P2) was found to be stable, had a pleasant mouth feel and disintegrated in the oral cavity within only 10 and 26 s, respectively.

Key words: Orodispersible tablet, Ludiflash®, Parteck®, direct compression, disintegrant.

INTRODUCTION

Recently, there has been the increasing interest in development of orally disintegrating tablets (ODTs), which are a convenient solid oral dosage form for patients who have difficulty with swallowing conventional tablets or capsules. ODTs are a new generation of formulations which combine the advantages of both liquid and traditional tablet formulations. Several technologies are available to manufacture this type of tablets (Shukla et al., 2009a). The most common preparation methods are moulding, lyophilisation, direct compression, sublimation, mass extrusion and spray drying. Direct compression is the simplest and the most cost effective technique for manufacturing ODTs, as conventional tablet machines and conventional packaging machinery can be used (Shukla et al., 2009a).

Excipients for ODTs have to be selected based on material characteristics and desired functionalities like defined particle size distribution, good flowability, enhanced compactability or fast disintegration. Mannitol is often used as the excipient for ODTs. However, when it is used as untreated powder, it is characterized by poor flowability, insufficient binding properties and compactability; therefore co-processed excipients with mannitol are used (Stoltenberg and Breitkreutz, 2011). Co-processing means the interacting of two or more excipients at the subparticle level, due to co-spray-drying, co-spray-agglomerating or co-granulating, which led to an improved functionality (Nachaegari and Bansal, 2004; Gohel and Jogani, 2005; Saha and Shahiwala, 2009).

*Corresponding author. E-mail: kwin@umb.edu.pl. Tel: +48 85 748 5615. Fax: + 48 85 748 5616.

Co-processed mannitol could be useful because of its sweet taste, good disintegration properties and low hygroscopicity, which enables stability comparable with conventional tablets without the need of sophisticated packaging (Stoltenberg and Breitkreutz, 2011). Ludiflash® and Parteck® are new commercially available ready-to-use tableting excipients based on co-processed mannitol. Ludiflash® is a combination of D-mannitol, crospovidone and polyvinyl acetate, whereas Parteck® contains two components: D-mannitol and croscarmellose sodium (Ludiflash® Technical Information- BASF, 2011; Parteck® Technical Information- Merck Chemicals, 2011).

The challenge in ODTs manufacturing encompasses the compromise between instantaneous disintegration and sufficient hardness. Direct compression is one of the techniques that requires the incorporation of disintegrants into the formulation, which mainly affects the rate of disintegration (Shukla et al., 2009a). To our knowledge, little experimental data on the preparation of ODTs using Ludiflash® and Parteck® have been reported thus far. Therefore, the aim of this study was to compare the disintegrants' efficiency of six disintegrants (croscarmellose sodium, microcrystalline cellulose and four types of polyvinylpyrrolidone) in ODTs manufactured using Ludiflash® or Parteck® by direct compression method. The influence of selected disintegrants on physico-mechanical properties of prepared ODTs was also examined. Tablets were evaluated for weight variation, hardness, friability, thickness, wetting time, disintegration time, stability, surface morphology and pore structure. To demonstrate the basic properties of the excipients, and because of consideration of the ethical aspects involved in testing ODTs in humans (Mimura et al., 2011), only placebo formulations that lacked inclusion of drugs were manufactured and examined.

MATERIALS AND METHODS

The ready-to-use tableting excipient Ludiflash®, Kollidon® CL (crosslinked PVP), Kollidon® CL-F (standard fine), Kollidon® CL-SF (superfine), Kollidon® CL-M (micronized), were obtained from BASF, Ludwigshafen, Germany. Parteck® ODT was purchased from Merck, Darmstadt, Germany. Magnesium stearate was a product of POCH, Piekary Slaskie, Poland. Avicel® PH-102 and Ac-Di-Sol® were purchased from FMC Biopolymer, Brussels, Belgium.

Characteristics of the powder flowability

Bulk density (Db) is the ratio of total mass of powder to the bulk volume of powder. The weighed powder was poured into a measuring cylinder and initial weight was noted. This initial volume was called the bulk volume. The bulk density was calculated according to the formula:

$$Db = M / Vb$$

where M and Vb are mass of powder and bulk volume of the powder, respectively (EP, 2008).

Tapped density (Dt) is the ratio of total mass of the powder to the tapped volume of the powder.

Volume was measured using a tapping density analyzer (Electrolab ETD-1020, Mumbai, India) by tapping the powder for 750 times, and the tapped volume was noted if the difference between these two volumes is less than 2%. If it is more than 2%, tapping is continued for 1,250 times and tapped volume was noted (EP, 2008). It is expressed in g/ml and is given by:

$$Dt = M / Vt$$

where M and Vt are mass of the powder and tapped volume of the powder, respectively.

For determination of angle of repose (θ), the blend were poured through the walls of a funnel, which was fixed at a position such that its lower tip was at a height of exactly 2.0 cm above hard surface. The blends were poured till the time when the upper tip of the pile surface touched the lower tip of the funnel. Angle of repose was calculated using following equation:

$$\tan \theta = h / r,$$

where h is height and r is radius of pile.

Carr's index (or % compressibility) is expressed in percentage and is given by:

$I = Dt - Db / Dt \times 100$, where Dt and Db are tapped and bulk density, respectively.

Hausner ratio is an indirect index of ease of powder flow and is calculated by the following formula:

Hausner ratio = Dt / Db, where Dt and Db are tapped and bulk density, respectively (EP, 2008).

All the procedures was repeated three times per batch.

Preparation of ODTs

ODTs were prepared by direct compression method according to the formulae given in Table 1. Ludiflash® or Parteck® and various disintegrants were mixed for 20 min in porcelain mortar and passed through a 0.8 mm sieve. This blend was mixed with magnesium stearate for 5 min and processed for direct compression by using 8 mm round flat-faced single punch tablet press (EP1 Erweka, Heusenstamm, Germany). Different adjustments of the machine settings were tried. The adjustment giving the highest possible hardness value with the highest accepted disintegration time was selected and applied to all tablet formulations.

Evaluation of tablets

Weight variation and thickness

Twenty tablets were selected randomly to determine the tablets weight variation and thickness. Tablets were weighed individually using an electronic balance and compared with an average weight. Thickness of the tablets was assessed using digital calliper (Beta 1651 DGT, Sovico, Italy).

Hardness

The tablet crushing load, which is the force required to break a tablet by compression in the radial direction, was measured using a tablet hardness tester (5Y, Pharmatron AG, Thun, Switzerland). The test was performed in 10 runs, and the average was calculated.

A piece of tissue paper folded twice was placed

Table 1. Composition of manufactured ODTs formulations.

Ingredient (mg/tablet)	L0	L1	L2	L3	L4	L5	L6	P0	P1	P2	P3	P4	P5	P6
Ludiflash®	178.2	172.8	172.8	172.8	172.8	172.8	172.8	-	-	-	-	-	-	-
Parteck®	-	-	-	-	-	-	-	178.2	172.8	172.8	172.8	172.8	172.8	172.8
Kollidon® CL	-	5.4	-	-	-	-	-	-	5.4	-	-	-	-	-
Kollidon® CL-SF	-	-	5.4	-	-	-	-	-	-	5.4	-	-	-	-
Kollidon® CL-M	-	-	-	5.4	-	-	-	-	-	-	5.4	-	-	-
Kollidon® CL-F	-	-	-	-	5.4	-	-	-	-	-	-	5.4	-	-
Avicel® PH 102	-	-	-	-	-	5.4	-	-	-	-	-	-	5.4	-
AcDiSol®	-	-	-	-	-	-	5.4	-	-	-	-	-	-	5.4
Magnesium stearate	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8

in a Petri dish Ø 7 cm containing 7 ml of water. A tablet was put on the paper and the time for complete wetting was measured. The wetted tablet was again weighed. The water absorption ratio (R) was calculated using the formula: $R = 100 (W_a - W_b) / W_b$, where W_a and W_b are the weight before and after water absorption (Shukla et al., 2009b; Bi et al., 1999). The results were the average of six measurements.

Measurement of disintegration time

Disintegration test in conventional disintegration apparatus

Disintegration time was determined using tablet disintegration test apparatus (Erweka ED-2L, Heusenstamm, Germany). The tablet was placed in 900 ml distilled water maintained at 37°C and agitation speed of 30 shakes per min. Only one tablet at a time was tested. The tablet was considered disintegrated completely when all the particles passed through the screen (EP, 2008). The disintegration time of 6 individual tablets were recorded, and the average was reported.

Disintegration test on wire cloth

Disintegration time was determined according to Motohiro et al. (2001) by placing ODT on a wire cloth (Ø 2 mm) and water was dropped on it at a rate of 4 ml/min. The time required by the tablet to completely pass through the wire cloth was noted as disintegration time.

Disintegration test on a Petri dish

Disintegration time was measured using a modified disintegration test method (Gohel et al., 2004; Giri and Sa, 2009). Water (10 ml) was placed in a Petri dish Ø 7 cm and a tablet was carefully placed in the centre and agitated mildly. Time required for complete disintegration of the tablet into fine particles was noted.

Disintegration time in the mouth

Measurements of disintegration time in the mouth were carried out in 6 volunteers. After the mouth was rinsed with purified water, one tablet was held in the mouth until the tablet disintegrated without chewing and then spat out, and the mouth was rinsed again. The time required for complete disintegration of the tablet was measured.

Moisture uptake studies

The test was performed by keeping ten tablets in a desiccator (containing calcium chloride) for 24 h at 37°C to assure complete drying. The tablets were then weighed and stored for 2 weeks at 75% humidity. Tablets were re-weighed and the percentage increase in the weight was recorded (Shukla et al., 2009b).

Sensory evaluation of roughness of tablets

Sensory test of roughness of tablets was carried out in 6 volunteers. After the mouth was rinsed with purified water, one tablet was held in the mouth for 60 s and then spat out, and the mouth was rinsed again. The roughness level was recorded. A numerical scale was used with the following values: 0, no roughness; 1, slight roughness; and 2, roughness.

Determination of the specific surface area

The specific surface area of the co-processed excipients was measured by nitrogen adsorption (EP, 2008) using nitrogen porosimeter Gemini VII (Micromeritrics, Norcross, USA). About 2 g of each excipient was weighed in a sample tube, and was then degassed for 24 h at a temperature of 80°C using helium as purge gas. Evaluation of the specific surface was made in an adsorbing device (Tristar 3000, Micromeritrics, Norcross, USA), where a mixture of nitrogen and helium flowed over the powder. The adsorbed amount of nitrogen was calculated using the equation according to Brunauer, Emmet and Teller, to determine the specific surface (BET). The average pore radius and cumulative volume of pores were calculated

using Micromeritrics Gemini VII software (Micromeritrics, Norcross, USA). The measurements were performed in triplicate per batch.

Surface morphology

Powders and ODTs were studied for surface morphology by scanning electron microscope (Hitachi S-3000N, Ibaraki, Japan). Prior to examination, samples were mounted on an aluminium stub using a double sided adhesive tape and thereafter making it electrically conductive by coating with a thin layer of gold (approximately 20 nm) in vacuum. The scanning electron microscope was operated at an acceleration voltage of 15 kV.

Stability studies

The obtained tablets were stored in desiccators at 40, 60 and 80% relative humidity (RH) for a period of six months at 25°C. After 1, 3 and 6 months, samples were withdrawn and evaluated for appearance, weight variation, thickness, hardness, friability, water absorption, wetting and disintegration times.

Statistical analysis

The differences in average of data were compared by simple analysis of variance (one-way ANOVA) at $p < 0.05$ level using the software SPSS (SPSS Inc., Chicago, USA). The significance of the difference was determined at 95% confidence limit.

RESULTS AND DISCUSSION

In the present study ODTs were prepared by direct compression method. For direct compression, the flowability of the powder blends is very important. Flow properties of the powder can be judged from the angle of repose. This measurement gives qualitive and quantitative assessment of internal cohesive and frictional force. The angle of repose < 30° indicates free flowing material and > 40° with poor flow properties (Lachman et al., 1991). Values for angle of repose for Ludiflash® or Parteck® was 29° and 30° (Table 2) showing that the base was free flowing. The spray dried excipients showed lower angle of repose (better flow properties) than the excipients alone (Table 2), which is probably due to increased sphericity of spray dried excipients base. The Hausner ratio and the Carr's index refer to the packing characteristics of the materials, and are also used as indicators for flowability of the powders. The values for Ludiflash® or Parteck® indicate a passable flowability (Table 2), whereas the flow properties of different disintegrants according to European Pharmacopoeia (EP, 2008) were fair (Kollidon® CL), poor (Kollidon® CL-F, Avicel® PH-102, Ac-Di-Sol®) or very poor (Kollidon® CL-SF, Kollidon® CL-M). Poor fluidity of excipients is improved by the use of lubricants. However, lubricants may reduce the tablet strength and has a negative effect on the wettability of the tablet, which could prolong the disintegration time. Preliminary experiments with powder blends containing Ludiflash® or Parteck® were performed to determine a suitable amount of lubricant. The comparative study between ODTs containing various concentrations (between 0.5 to 5%) of magnesium stearate was performed. Hence, a concentration 1% w/w was used as lubricant for the investigated formulations to balance lubrication and disintegration properties.

ODTs were prepared using either Ludiflash® or Parteck® by direct compression method using single punch tablet press type EP1 Erweka, Heusenstamm, Germany. All the tablets were prepared under similar conditions. To examine the influence of different types of disintegrants, formulations L0 to L6 and P0 to P6 were prepared (Table 1). Disintegrants are the class of compounds which primarily aid the rapid disintegration of ODTs in the oral cavity. This class of disintegrants have been shown to be effective at excipients concentrations as low as 2 to 10% when compared to traditional disintegrant starches, which may need concentrations as high as 20% (Augsburger et al., 2007; Zhao and Augsburger, 2006). Croscarmellose sodium and crospovidone are well known disintegrants used in ODTs. They have very strong disintegrating ability. Croscarmellose sodium swells to a large extent when it comes in contact with water. Also, it has a fibrous nature that allows intraparticulate as well as extraparticulate wicking of water even at a low concentration (Augsburger et al., 2007; Zhao and Augsburger, 2005). Crospovidone has an excellent wicking nature though it swells only minimally (Augsburger et al., 2007; Thibert and Hancock, 1996). Microcrystalline cellulose is widely used in oral pharmaceutical formulations as a binder, diluent, antiadherent and disintegrant (Watanabe et al., 1995). It has a good compressibility, compactibility and lubricity, and is generally regarded as relatively nontoxic and nonirritant (Rowe et al., 2009; Rojas et al., 2011). To examine the most suitable ratio of disintegrants, various formulation of ODTs were prepared. The disintegration time of tablets were decreased significantly with respect to increase in the concentration of disintegrants from 1 to 3 and 5%. However, formulations containing 5% of disintegrant did not meet the hardness and friability requirements. Therefore, the concentration of disintegrants was estimated as 3%, because using disintegrants in lower or higher concentration did not enable obtaining ODTs with satisfactory disintegration time and physico-mechanical properties (data not shown).

The manufactured formulations L0 to L6 and P0 to P6 exhibited white colour, odourless, flat in shape, with smooth surface, and were evaluated for physical parameters such as weight and thickness variation, hardness and friability. The average weight and thickness of tablets for all the formulations was found to be in the range of 180.6 to 181.8 mg and 4.01 to 4.07 mm, respectively (Table 3). All the formulations exhibited low weight variation which lies within the pharmacopoeial limits of ± 7.5% of the average weight (EP, 2008; USP 35: United States Pharmacopeial Convention, 2012). The

Table 2. Flow properties of the powders used for ODTs formulations.

Formulation	Density (g/ml)		Flow property			
	Bulk	Tapped	Hausner ratio	Carr's index	Angle of repose (°)	According to EP
Ludiflash®	0.51±0.01	0.65±0.00	1.28±0.01	22.0±0.30	29.0±0.90	Passable
Parteck®	0.54±0.01	0.67±0.01	1.25±0.02	20.0±0.20	30.0±0.60	Fair
K*_CL	0.43±0.01	0.52±0.01	1.21±0.02	17.0±0.95	32.0±0.60	Fair
K*_CL-SF	0.28±0.01	0.44±0.01	1.57±0.02	36.6±0.56	-	Very poor
K*_CL-M	0.19±0.00	0.28±0.00	1.48±0.01	32.4±0.32	-	Very poor
K*_CL-F	0.28±0.01	0.39±0.00	1.39±0.01	28.0±0.93	-	Poor
Avicel®	0.43±0.01	0.60±0.01	1.40±0.01	29.0±0.97	31.0±0.60	Poor
AcDiSol®	0.61±0.01	0.85±0.01	1.41±0.01	28.0±0.92	-	Poor

*K_ - Kollidon®; – not determinable. All values are expressed as mean ± SD.

Table 3. Physical parameters of prepared ODTs.

Formulation	Weight (mg)	Thickness (mm)	Hardness (N)	Friability (%)	Wetting time (s)
L0	181.8±1.16	4.07±0.09	60±2.02	0.70	18±0.57
L1	181.3±1.21	4.06±0.08	77±1.92	0.60	15±0.57
L2	180.8±0.98	4.04±0.06	56±1.85	0.82	14±1.15
L3	181.3±0.89	4.05±0.07	74±1.89	0.81	23±0.00
L4	180.8±0.75	4.01±0.03	72±1.20	0.72	21±2.00
L5	181.3±1.51	4.03±0.05	75±2.51	0.75	29±1.15
L6	180.6±0.81	4.02±0.04	73±1.79	0.73	26±0.57
P0	181.1±1.16	4.01±0.03	59±2.04	0.70	35±0.00
P1	180.9±0.98	4.04±0.06	70±2.07	0.51	38±1.15
P2	181.2±0.87	4.01±0.05	45±2.13	0.85	22±0.00
P3	180.9±1.14	4.01±0.05	45±2.13	0.85	22±0.00
P4	181.6±1.51	4.04±0.07	72±1.74	0.78	48±0.00
P5	181.8±1.15	4.01±0.04	75±1.56	0.60	45±1.15
P6	181.8±1.15	4.03±0.05	75±2.44	0.57	45±0.00

All values are expressed as mean ± SD.

pharmacopoeial limit of friability using tablet friability apparatus, carried out at 25 rpm for 4 min, is not more than 1%. However, it becomes a great challenge to achieve friability within this limit, keeping hardness at its lowest possible level in order to achieve a minimum possible determination time. The hardness of the obtained tablets was maintained in the range of 45 to 77 N and the friability results were found to be < 1%, which suggest that all the manufactured formulations posses the sufficient mechanical integrity (Table 3) and are able to withstand abrasion in handling, packaging and shipment.

Wetting time is related to the inner structure of the tablets and hydrophobicity of the components. The results of wetting time are shown in Table 3. It was observed that formulations L2 and P2 (containing crospovidone Kollidon® CL-SF) had the shortest wetting time. The most important parameter that needs to be optimized in the development of ODTs is the disintegration time. Focusing on a tri-regional Pharmacopeia, that is the Japanese Pharmacopeia (JP), United States Pharmacopeia (USP) and European Pharmacopeia (EP), the EP has categorized ODTs as tablets which disintegrate in less than 3 min, using a conventional test (EP, 2008). More recently, in Food and Drugs Administration (FDA) Guidance for Industry, ODTs are considered as solid oral preparations that disintegrate rapidly in the oral cavity, with *in vitro* disintegration time of approximately 30 s or less, when based on the USP disintegration test method for conventional tablets (Food and Drug Administration, 2008). However, no special apparatus is mentioned in the pharmacopoeias for disintegration test of ODTs, and the conventional method available seems to be inappropriate for this type of tablets. The conventional test employs a relative huge volume of test solution (900 ml) compared to the volume of saliva in human buccal cavity, which is less than 6 ml. Therefore, the results obtained from the conventional disintegration test do not reflect the actual disintegration time in the human mouth. In this study, disintegration times of manufactured ODTs were measured using four

Table 4. Disintegration times of manufactured ODTs evaluated by four independent methods.

Formulation	Disintegration time (s)			
	In vivo	Conventional apparatus	Petri dish	Metal mesh
L0	35±0.57	37±0.57	34±0.57	37±0.62
L1	37±0.64	59±0.57	45±0.74	30±0.57
L2	10±0.97	23±0.57	15±1.15	18±0.57
L3	33±0.42	32±1.00	28±1.47	33±0.57
L4	27±0.57	36±0.57	30±0.57	33±1.15
L5	63±0.57	98±1.15	80±0.57	80±1.15
L6	46±0.61	38±1.15	34±0.57	35±1.00
P0	56±1.15	57±0.57	56±0.00	52±0.57
P1	59±1.00	76±0.57	70±0.57	64±0.57
P2	26±1.15	21±1.15	23±1.15	25±0.57
P3	39±0.00	35±0.00	37±1.15	35±1.00
P4	41±0.57	29±1.15	28±1.15	30±1.15
P5	120±0.57	120±0.57	90±1.52	90±0.57
P6	42±0.57	30±1.00	30±1.15	33±1.15

All values are expressed as mean ± SD.

independent methods, both *in vitro* and *in vivo* (Table 4). Although the different types of disintegrants used in the examined formulations enabled obtaining acceptable hardness and friability of ODTs (Table 3), they significantly influenced the disintegration time of the tablets. The results shown in Table 4 indicate that crospovidone is the strongest among other disintegrants, which results in the fastest *in vitro* and *in vivo* disintegration times, followed by croscarmellose sodium, then microcrystalline cellulose. Disintegration times < 30 s measured by four independent methods was observed only in formulations with Kollidon® CL-SF (L2 and P2). The faster disintegration of ODTs with crospovidone may be attributed to the strong wicking action of this disintegrant, its rapid capillary activity and hydration with little tendency to gel formation (Rowe et al., 2009). Based on these results, superfine crospovidone seems to be the most suitable disintegrant for ODTs manufactured both with Ludiflash® and Parteck®.

The relationship between tablet hardness and disintegration time is shown in Figure 1. Generally, disintegration time increases with increased hardness. Our results have demonstrated that an increase in tablet hardness has a different extent of impacts on disintegration time, depending on the type of disintegrant used. Hardness of tablets containing Avicel® PH 102 as a disintegrant (formulation L5 and P5) was the most responsive to increases in disintegration time, while that of tablets containing Kollidon® CL-SF (formulation L2 and P2) was the least responsive. It was shown, that Kollidon® CL-SF can be suitable disintegrant for the production of ODTs because it enables it to obtain tables with relatively high hardness and short disintegration time. Tablets containing Avicel® PH 102 are the most difficult and slowest to disintegrate.

Using a scanning electron microscope (SEM), Ludiflash® and Parteck® particles before and after compression were analyzed for surface morphology. It has been shown that Parteck® particles have more porous and filamentous structure, which remains unchanged after mixing and compression (Figure 2D and F). The surface of Ludiflash® and Parteck® ODTs was relatively homogenous dispersion with pores, cracks and fissures (Figure 2). The porous and filamentous Parteck® structure is a measure of its large specific surface area and contributes to nitrogen adsorption (BET) of the tablet matrix of up to 4.00 m^2/g (Table 5), which enhances the water uptake and disintegration time of the matrix. However, the differences in the wetting time and disintegration time values between Ludiflash® and Parteck® formulations (L0 to L6 and P0 to P6) cannot be easily justified by the scanning electron microscope images and pore structure examinations. Formulations with Ludiflash® showed both less porous textures, with low specific surface area (BET values up to 0.77 m^2/g) (Table 5) and shorter wetting and disintegration times (Tables 3 and 4). Although pores form channels, which are assumed to facilitate water ingress and support rapid tablet disintegration, the main role in disintegration time is played by the presence of disintegrants.

Due to high content of hydrophilic excipients, ODTs have increased chance of moisture uptake which greatly affects stability of products, so there is a need for special attention towards storage and packaging. Therefore, moisture uptake studies are strongly recommended for ODTs (Shukla et al., 2009b). The moisture uptake study indicates no significant uptake of moisture by the prepared batches during the 14 days test period (data not shown). Obtained formulations of ODTs exhibited low hygroscopicity, mainly driven by the specific character of

Table 5. Parameters of the pore structure of manufactured ODTs.

Formulation	Total surface area (m^2/g)	Pore radius (Å)	Cumulative volume of pores (cm^3/g)
L0	0.38±0.02	16.80±0.1	0.001
L1	0.66±0.04	43.01±0.2	0.001
L2	0.38±0.01	46.02±0.2	0.001
L3	0.77±0.02	40.00±0.3	0.001
L4	0.56±0.04	74.02±0.8	0.001
L5	0.59±0.05	47.00±0.4	0.001
L6	0.66±0.03	48.04±0.8	0.001
P0	3.60±0.04	109.00±0.6	0.021
P1	3.98±0.07	137.01±0.5	0.013
P2	3.20±0.04	94.05±0.9	0.014
P3	3.70±0.08	122.00±0.9	0.022
P4	3.00±0.06	79.05±0.7	0.012
P5	3.12±0.04	12.00±0.8	0.017
P6	4.00±0.05	84.02±0.8	0.021

All values are expressed as mean ± SD.

Table 6. Sensory evaluation of manufactured ODTs.

Volunteer	Score[a]													
	L0	L1	L2	L3	L4	L5	L6	P0	P1	P2	P3	P4	P5	P6
A	0	0	0	0	0	1	1	0	1	0	0	0	0	1
B	0	0	0	0	0	1	1	0	0	0	0	0	1	1
C	0	0	0	0	0	0	1	1	0	0	0	0	1	0
D	1	0	0	0	0	0	0	0	0	0	1	0	1	0
E	0	0	0	0	0	0	0	0	0	1	1	0	0	0
F	0	0	0	0	1	0	0	0	0	0	0	1	0	1
G	0	0	1	0	0	0	0	0	0	0	0	1	0	1

[a]Scored as follows: 0 = not rough; 1 = slightly rough; 2 = markedly rough.

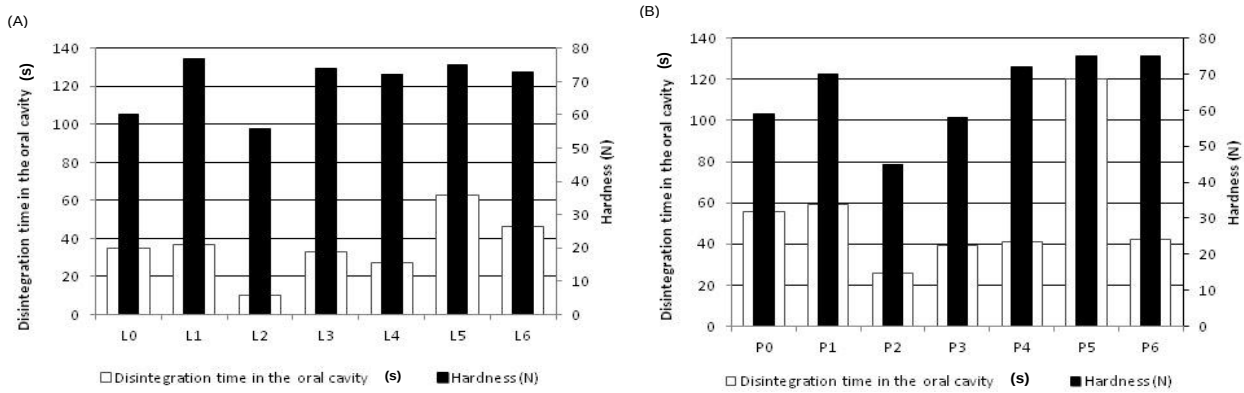


Figure 1. Effect of hardness on disintegration time in: (A) formulations L0 to L6 and (B) P0 to P6.

D-mannitol as the main component.

Another important feature of ODTs is taste and roughness. In general, polyols are known to exhibit a sweet taste and a pleasant mouth feeling, with mannitol being one of the most commonly used of this class (Rowe et al., 2009). In this study, microcrystalline cellulose, crospovidones and croscarmellose sodium were used as disintegrants.These water-insoluble excipients have a very rough texture. Watanabe et al. (1995) reported that tablets pre-pared with

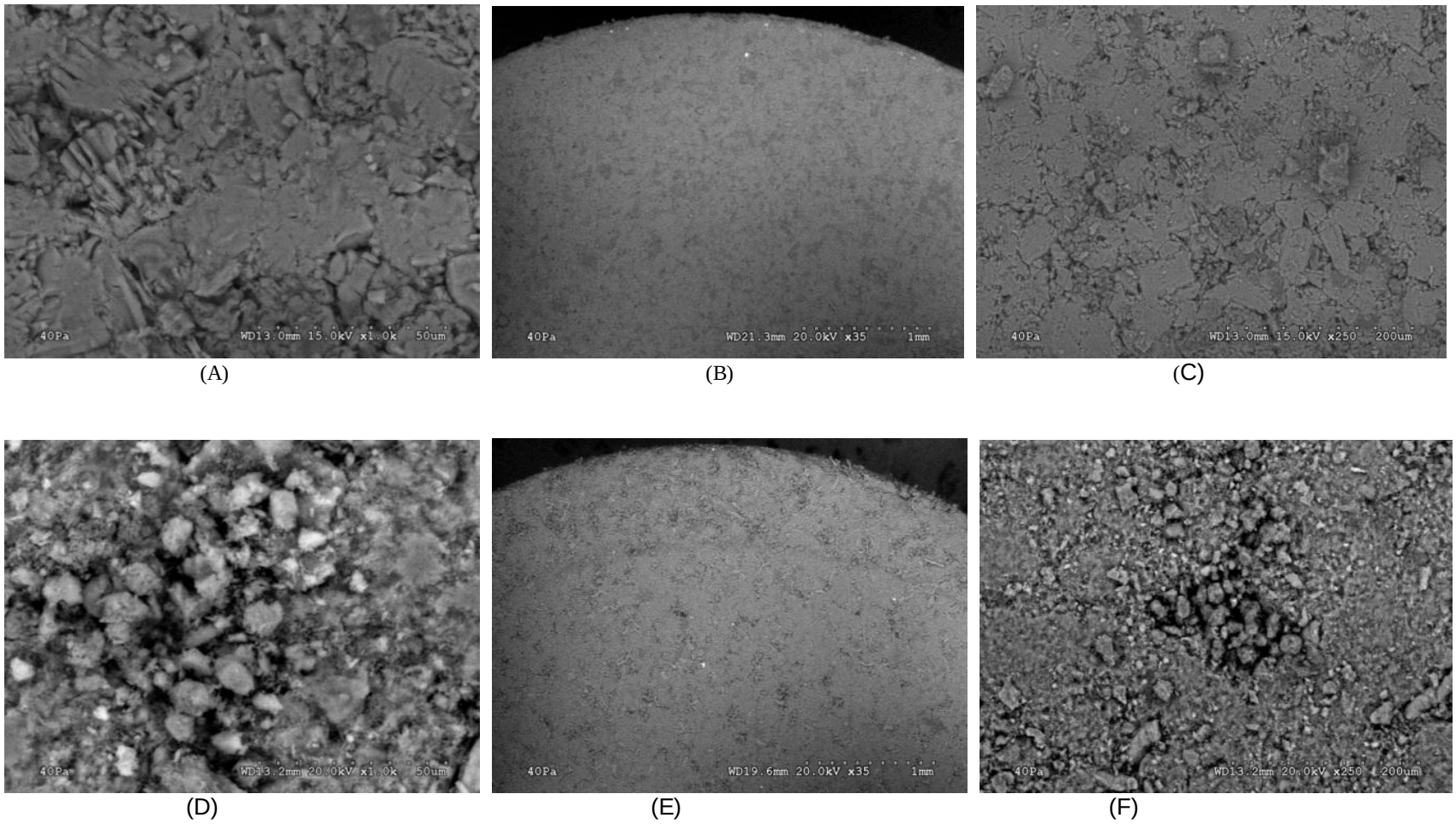


Figure 2. Scanning electron micrographs of: (A) Ludiflash® particle before compression (original magnification ×1000); (B), (C) ODT formulation L0 (original magnification ×35 and ×250); (D) Parteck® particle before compression (original magnification ×1000); (E), (F) ODT formulation P0 (original magnification ×35 and ×250).

microcrystalline cellulose rapidly disintegrated with saliva, but it was indicated that patients sometimes sensed roughness in the mouth due to the incomplete solubili-zation (Ishikawa et al., 2001). However, water- insolubleexcipients with small particle size are smoother. To elucidate the effect of disintegrant type on the feeling of roughness when prepared, tablets were taken orally, and a sensory evaluation of ODTs

containing different disintegrants was performed. The results of evaluation by volunteers are presented in Table 6. The rough texture was evaluated as more unpleasant in the order: ODTs with croscarmellose sodium > ODTs withmicrocrystalline cellulose > ODTs with crospovidones. Short-term stability studies (40, 60 and 80% relative humidity for a period of three months at 25°C) indicated that there were no significant changes in appearance of ODTs, weight variation, thickness, hardness, friability, wetting and disintegration times of the prepared formulations (data not shown). After six months of storage, all obtained ODTs were characterized by slightly more hardness and friability (but still within pharmacopoeial limits), whereas disintegration times were without significant changes.

Conclusion

It has been provided that selecting an appropriate disintegrant is extremely important in designing ODTs. The results from this study suggest that disintegration of ODTs is dependent on the nature of disintegrant, and the most effective disintegrant in ODTs manufactured using new ready-to-use excipient Ludiflash® or Parteck® is superfine crospovidone, which enables obtaining pleasant-tasting and pleasant feeling in the mouth tablets that disintegrate rapidly and posses satisfactory physico-mechanical properties.

ACKNOWLEDGEMENT

This research was supported by Medical University of Bialystok grant (123-15877 F).

REFERENCES

Augsburger LI, Brzeczko AW, Shah U, Hahm HA (2007). Super Disintegrants: characterization and function. In: Swarbrick J (ed.), Encyclopedia of Pharmaceutical Technology 3rd ed. Informa Healthcare USA Inc., New York. pp. 3553-3567.

BASF- The Chemical Company (2011). Ludiflash® Technical Information. available on: http://www.basf.com/group/corporate/en/brand/LUDIFLASH.

Bi YX, Sunada H, Yonezawa Y, Danjo K (1999). Evaluation of rapidly disintegrating tablets prepared by a direct compression method. Drug Dev. Ind. Pharm. 25:571-581.

European Pharmacopoeia EP (2008). European Pharmacopoeia, 6th Edition. Strasbourg, Council of Europe.

Food and Drug Administration (2008). Guidance for Industry: Orally Disintegrating Tablets. US Department of Health and Human Services. FDA Center for Drug Evaluation and Research.

Giri TK, Sa B (2009). Statistical evaluation of influence o polymers concentration on disintegration time and diazepam release from quick disintegrating rapid release tablet. Yakugaku Zasshi, 129:1069-1075.

Gohel M, Patel M, Amin A, Agarwal R, Dave R, Bariya N (2004). Formulation design and optimization of mouth dissolve tablets of nimesulide using vacuum drying technique. AAPS Pharm. Sci. Tech. 5:10-15.

Gohel MC, Jogani PD (2005). A review of coprocessed directly compressible excipients. J. Pharm. Pharm. Sci. 8:76-93.

Ishikawa T, Koizumi N, Mukai B, Utoguchi N, Fujii M, Matsumoto M, Endo H, Shirotake S, Watanabe Y (2001). Pharmacokinetics of acetaminophen from rapidly disintegrating compressed tablet prepared using microcrystalline cellulose (PH-M-06) and spherical sugar granules. Chem. Pharm. Bull. 49:230-232.

Lachman L, Liberman J, Kaing JL (1991). Tablets, 3rd ed. Varghese Publishing House, Bombay.

Merck Chemicals (2011). Parteck® Technical Information. Available on: http://www.merck-chemicals.pl/pharmaceutical-ingredients/parteck-odt/c_bnyb.s1OES0AAAEkLKdk10iV.

Mimura K, Kanada K, Uchida S, Hamada M, Namiki N (2011). Formulation study for orally disintegrating tablet using partly pregelatinized starch binder. Chem. Pharm. Bull. 59:959-964.

Motohiro O, Hayakawa E, Ito K, Tokuno M, Morimoto K, Watanabe K (2001). Intrabuccally rapidly disintegrating tablet. US Patent Application 20010014340.

Nachaegari SK, Bansal AK (2004). Coprocessed excipients for solid dosage forms. Pharm. Technol. 28:52-64.

Rojas J, Lopez A, Gamboa Y, Gonzalez C, Montoya F (2011). Assessment of processing and polymorphic form effect on the powder and tableting properties of microcrystalline celluloses I and II. Chem. Pharm. Bull. 59:603-607.

Rowe RC, Sheskey PJ, Quinn ME (2009). Handbook of pharmaceutical excipients, 6th ed. The Pharmaceutical Press, London and Washington DC. pp. 129-133.

Saha S, Shahiwala AF (2009). Multifunctional coprocessed excipients for improved tabletting performance. Export. Opin. Drug Deliv. 6:197-208.

Shukla D, Chakraborty S, Singh S, Mishra B (2009 a). Mouth dissolving tablets I: An overview of formulation technology. Sci. Pharm. 76:309-326.

Shukla D, Chakraborty S, Singh S, Mishra B (2009 b). Mouth dissolving tablets II: An overview of evaluation techniques. Sci. Pharm. 77:327-341.

Stoltenberg I, Breitkreutz J (2011). Orally disintegrating mini tablets – a novel solid oral dosage form for paediatric use. Eur. J. Pharm. Biopharm. 78:462-469.

Thibert R, Hancock BC (1996). Direct visualition of superdisintegrant hydration using environmental scanning electron microscopy. J. Pharm. Sci. 85:1255-1258.

USP 35: United States Pharmacopeial Convention (2012). United States Pharmacopeia and the National Formulary. The United States Pharmacopeial Convention, Rockville.

Watanabe Y, Koizumi K, Zama Y, Kiriyama M, Matsumoto T, Matsumoto M (1995). New compressed tablet rapidly disintegrating in saliva in the mouth using crystalline cellulose and a disintegrant. Biol. Pharm. Bull. 18:1308-1310.

Zhao N, Augsburger LI (2005). The influence of swelling capacity of superdisintegrants in different pH media on the dissolution of hydrochlorthiazide from directly compressed tablets. AAPS Pharm. Sci. Tech. 6:E120-E126.

Zhao N, Augsburger LI (2006). The influence of product brand-to-brand variability on superdisintegrants performance. A case study with croscarmellose sodium. Pharm. Dev. Technol. 11:179-185.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2368-2373, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.534
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Pharmacokinetics of vitexin in rats after intravenous and oral administration

Yunjiao Wang[2], Chunhui Han[1], Aijing Leng[1]*, Wenjie Zhang[2], Hefei Xue[2], Yinghui Chen[2], Jingjing Yin[2], Dongrui Lu[2] and Xixiang Ying[2]

[1]First Affiliated Hospital of Dalian Medical University, Dalian, 116011, China.
[2]School of Pharmacy, Liaoning University of Traditional Chinese Medicine, Dalian 116600, China.

Accepted 13 August, 2012

Vitexin was isolated from the leaves of *Crataegus pinnatifida* Bge. var *major*, and its pharmacokinetics and bioavailability were carried out *via* validated high-performance liquid chromatography (HPLC) method using hesperidin as internal standard in healthy rats after intravenous and oral administration at a dose of 10 mg/kg and 30 mg/kg, respectively. The pharmacokinetic parameters were calculated by both compartmental and non-compartmental approach. When intravenous administration was used, the elimination half-life ($t_{1/2\beta}$), the mean residence ($MRT_{0\rightarrow t}$), the total body clearance (CL) were 46.01 ± 0.810 min, 26.23 ± 1.51 min and 0.031 ± 0.035 L/kg·min. When oral administration was used, the t_{max} and C_{max} were 15.82 ± 0.172 min and 0.51 ± 0.015 µg/ml, the $MRT_{0\rightarrow t}$ and CL were 60.41 ± 5.41 min and 0.71 ± 0.056 L/kg·min. The result showed that vitexin was rapidly eliminated and presented a low absolute bioavailability (F), 4.91 ± 0.761%.

Key words: Bioavailability, high-performance liquid chromatography (HPLC), pharmacokinetics, rat plasma, vitexin.

INTRODUCTION

Flavonoids are polyphenolic compounds occurring in many plants, which have shown many biological and pharmacological activities, such as the prevention of coronary heart disease (Gambelunghe et al., 2003), inhibition of tumor promotion (Bomser et al., 1999), anti-oxidation(Hollman and Katan, 1999), and anti-inflammation (Rahman et al., 2006). Vitexin being a flavonoid component was found with many pharmacological actions, such as anti-hypotensive, anti-spasmodic, anti-inflammatory properties (Prabhakar et al., 1981), and antithyroid effect (Gaitan et al., 1995).

Recently, Tong and Liu, 2007 reported the pharmacokinetics in rats after multi-dose intravenous administration of vitexin (Tong and Liu, 2007). However, the pharmacokinetics and bioavailability after intravenous and oral administration of pure vitexin have never been reported until now, and therefore an HPLC method herein will be developed and validated for the determination of vitexin in rat plasma to investigate the pharmacokinetics of pure vitexin. To the best of our knowledge, this is the first report on the development, validation and application of HPLC method with internal standard for the determination of vitexin in rat plasma and its pharmacokinetic study after intravenous and oral administration, and its bioavailability can also be calculated.

MATERIALS AND METHODS

Chemicals

Vitexin (Figure 1a) was isolated from the hawthorn leaves in our laboratory, and its purity was over 99% by HPLC analysis. The internal standard, hesperidin (Figure 1b) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC grade) were obtained from Xinxing (Chemical Reagent Plant, Shanghai, China), and the water used in all experiments was purified by a Milli-Q® Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). All other chemicals were of analytical reagent grade

*Corresponding author. Email: aijingleng@hotmail.com. Tel: +86 (0)411 87586005. Fax: +86 (0)411 87586078.

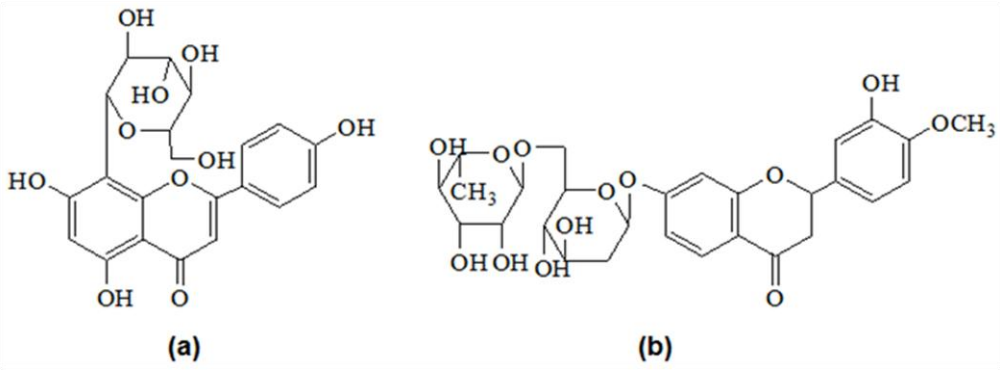


Figure 1. Chemical structures of vitexin (a) and hesperidin (b).

purchased from Sinopharm Chemical Reagent Co., Ltd.

Chromatographic equipment and conditions

The analyses were carried out on an Agilent 1100 series HPLC system (Agilent technology, Palo Alto, CA, USA) which consisted of a quaternary Pump (G1310A), a vacuum degasser (G1322A), a UV-VIS spectrophotometric detector (G1314A) and Chemstation software (Agilent). The analytical column was a Kromasil C18 column (150 mm × 4.6 mm i.d., 5 µm, Dalian Sanjie, China) protected by a KR C18 guard column (35 mm × 8.0 mm, i.d., 5 µm, Dalian Create Science and Technology Co., Ltd., China). The mobile phase for HPLC analysis consisted of a mixture of methanol-acetonitrile-0.3% formic acid (3:1:6, v/v/v), was passed under vacuum through a 0.45 µm membrane filter and degassed before use. All the chromatographic measurements were performed at room temperature and a flow rate of 1 ml/min with the detection wavelength of 330 nm.

Animals

Male Wistar 300-330 g were obtained from the Laboratory Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). They were kept in an environmentally controlled breeding room for 1 week before the experiments and fed with standard laboratory food and water ad libitum and fasted overnight before the experiment. All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Liaoning University of Traditional Chinese Medicine.

Drug administration and sample collection

To the pharmacokinetic study, 10 rats were randomly assigned to 2 groups. Vitexin was prepared with 20% propylene glycol-water (v/v) to the concentration of 2 mg/ml for intravenous injection and 3 mg/ml for oral administration. Vitexin solution was administered intravenously at a dosage of 10 mg/kg, orally at a dosage of 30 mg/kg. Blood samples (0.3 ml) were collected into heparinized tubes from the vena orbitalis at times of 2, 5, 8, 11, 15, 20, 30, 45, 60, 90 and 120 min for intravenous administration, and at times of 3, 5, 10, 15, 20, 30, 45, 60, 80, 120 and 180 min for oral administration, and then centrifuged at 3500 r/min. The obtained plasma was stored at -20°C until analysis.

Extraction of analyte from plasma

To 200 µl plasma, 20 µl acetic acid, 30 µl hesperidin, and 1 ml acetonitrile were added, followed by vortex mixing for 1 min and centrifuged at 3500 r/min for 15 min. The supernatant was collected and evaporated to dryness at 50°C under a gentle stream of nitrogen. The dried residue was then reconstituted in 200 µl of mobile phase, and centrifuged at 10000 r/min for 10 min, and an aliquot (20 µl) of the supernatant was injected into the HPLC system for analysis.

Preparation of calibration standards and quality control samples

Stock standard solutions of vitexin and hesperidin were prepared with methanol, and the concentrations of them were 237 and 248 µg/ml, respectively. All solutions were stored at 4°C and were found to be stable for at least 1 month. Seven calibrators (0.1, 0.2, 0.5, 1, 2.5, 5 and 20 µg/ml) of vitexin and hesperidin (124 µg/ml) were prepared by dilution of stock solutions followed by spiking with drug free plasma. Quality control (QC) samples were prepared at three concentrations (low, middle and high) representing the whole range of the calibration curve. The low concentration (0.3 µg/ml) was 3 times of lower limit of quantitation, high concentration (16 µg/ml) was 80% of the upper limit of quantitation, and middle concentration (1.5 µg/ml) was near the geometric mean of low and high concentration. All of the QC samples were stored at -20°C until analysis.

Method validation

Selectivity was investigated by comparing chromatograms of blank plasma obtained from rats prior to dosing with those of corresponding standard plasma sample spiked with vitexin and hesperidin, and plasma sample from rats after intravenous doses of them.

The linearity was evaluated over the concentration range of 0.1-20 µg/ml at seven levels of vitexin. The calibration curves for vitexin in plasma were generated by plotting the peak area ratio of vitexin to hesperidin versus the nominal concentrations in the standard plasma samples. The regression equation was obtained by weighted ($1/c^2$) least square linear regression. The limit of detection (LOD) was determined by a signal-to-noise ratio (S/N) of 3. The lower limit of quantification (LLOQ) was defined as the lowest concentration of vitexin in the calibration curves, giving an acceptabl accuracy (RE) within ±20% and a precision (RSD) that

did not exceed 20%.

The accuracy and precision of the method were evaluated with QC samples at three concentrations by five replicates on three consecutive days. The intra- and inter-assay precisions were assessed by determining the quality control samples at three concentration levels of vitexin (0.3, 1.5 and 16 µg/ml). For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day. For the inter-day validation, five replicates of the QC plasma samples were analyzed on three different days. The precision was expressed as the RSD which should be less than 15%, except at the LLOQ where it should not exceed 20%, and the accuracy of the assay was determined by comparing the means of the determined vitexin concentrations with the nominal concentrations. The mean percentage deviation from the nominal values expressed as the RE which should be within ±15% of the nominal value, except at the lower limit of quantification where it should not exceed ±20%.

The extraction recoveries of vitexin were determined by comparing the peak area of QC samples that were at low, middle and high three concentrations to that of the unextracted standard solutions containing the equivalent amount of analyte (n = 5).

Five aliquots of QC samples at low, middle and high concentration unextracted QC samples were subjected to the conditions below. Short-term stability was determined by analyzing QC plasma samples kept at ambient temperature (25°C) for 4 h. Long-term stability was assessed by analyzing the extracted QC plasma samples stored at -20°C for 1 month. Freeze-thaw stability was investigated after three freeze (-20°C) -thaw (room temperature) cycles. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples.

Pharmacokinetics and bioavailability study

Pharmacokinetic analysis of the data was performed using both compartmental and non-compartmental methods, with the help of the practical program (3P97) of the Chinese Society of Mathematical Pharmacology (Beijing, China). The compartmental parameters were generated, such as volume of distribution of the central compartment (V_c), α half-life ($t_{1/2\alpha}$), β half-life ($t_{1/2\beta}$), clearance rate (CL). The area under the plasma concentration-time curve ($AUC_{0\rightarrow t}$) was calculated by the trapezoidal method. The area under the plasma concentration-time curve from zero to infinity ($AUC_{0\rightarrow\infty}$) was calculated by means of the trapezoidal rule with extrapolation to infinity with terminal elimination rate constant. The peak plasma concentration (C_{max}) and the peak time were directly obtained from the drug plasma concentration-time profiles. The noncompartmental based on statistical moment theory, the parameters such as; the terminal elimination rate (λ_z) was estimated by the least-square regression analysis of at least last four time points of the semilogarithmic plasma concentration-time curves. The mean residence time (MRT) was determined as (AUMC)/AUC. The absolute bioavailability (F) was calculated as $(AUC_{oral}/AUC_{i.v})\times(dose_{i.v}/dose_{oral})$.

The data were statistically analyzed (ANOVA). The differences were considered significant when $P<0.05$. All data are presented as means ± standard errors.

RESULTS

Method validation

Typical chromatograms obtained from blank plasma, blank plasma spiked with vitexin standard analytes and hesperidin, plasma samples from rats after intravenous and oral administration of vitexin in rats have been shown in Figure 2. The chromatograms showed that there were no interfering peaks in the region of the peaks of the vitexin and hesperidin, which present a complete baseline resolution of analyte and internal standard with adjacent peaks. The evaluation of the linearity was performed with a seven-point calibration curve over the concentration range of 0.1-20 µg/ml The slope and intercept of the calibration graphs were calculated by weighted ($1/c^2$) least squares linear regression. The regression equation of the calibration curves was typically: $y = 0.2031x-0.0158$, and r was 0.9993, where y is the peak area ratio of vitexin to hesperidin, and x is the plasma concentration of vitexin. The limit of detection (LOD) was 0.035 µg/ml determined by a signal-to-noise ratio (S/N=3). The lower limit of quantification (LLOQ) was 0.1 µg/ml (S/N=10). The RSD of three concentrations ranged from 4.8 to 7.4% for intra-day assay and from 6.3 to 7.3% for inter-day assay, respectively. The REs of intra- and inter-day accuracy were within -6.9 to 6.9%. The RSD determined at each concentration level is required not exceeding 15% and RE within ±15% of the actual value which conforms to the criteria for the analysis of biological sample according to guidance of USFDA (USFDA, 2001). These results in Table 1 suggested that the procedures described as above were satisfactory with respect to both accuracy and precision, and the extraction recoveries of vitexin at three concentrations (0.3, 1.5 and 16 µg/ml) were no less than 83.83 ± 0.157% (Table 1), and that of hesperidin was 98.81 ± 0.165%, of which the precision and accuracy of this method were acceptable. The results of short-term stability, long-term stability and freeze-thaw stability indicated that no significant degradation occurred during chromatography, extraction and sample storage processes for analytes plasma samples.

Pharmacokinetic studies

The validated method was successfully applied to monitor the concentrations and pharmacokinetics of vitexin after intravenous and oral administration at a dose of 10 mg/kg and 30 mg/kg, respectively. The plasma concentration-time profile of vitexin was shown in Figure 3. The main pharmacokinetic parameters of vitexin were listed in Table 2.

DISCUSSION

Method development

To obtain solution for intravenous injection and oral administration, some auxiliary solvent, such as dimethyl sulfoxide (DMSO) and propylene glycol were tried to add

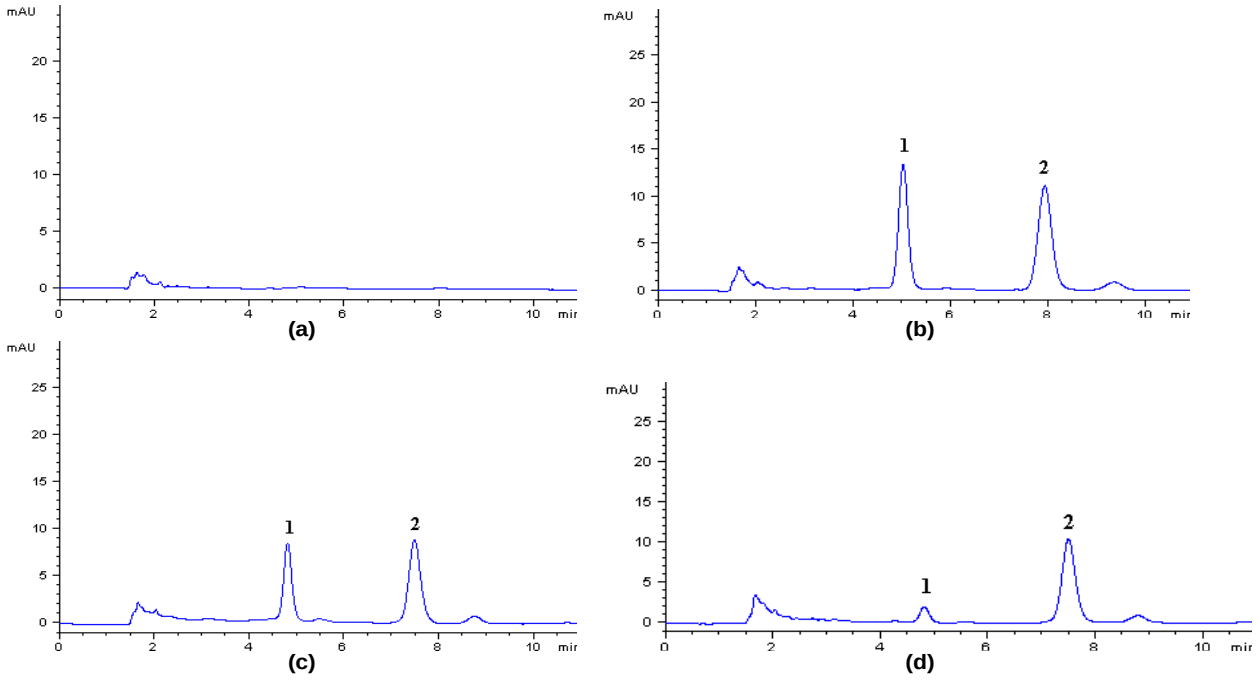


Figure 2. Representative chromatograms of blank plasma (a), plasma spiked with vitexin and hesperidin (b), plasma sample at 10min after intravenous administration of vitexin at dose of 10 mg/kg (c) and plasma sample at 20 min after oral administration of vitexin at dose of 30 mg/kg (d). Peak 1:vitexin; Peak 2: hesperidin.

Table 1. Precision and accuracy of vitexin determination in rat plasma (intra-day: n = 5; inter-day: n = 3 days with 5 replicates per day).

Added Conc. (μg/ml)	Intra-day			Inter-day			Recovery (%)	RSD (%)
	Conc. (μg/ml) (mean ± SD)	RSD (%)	RE (%)	Conc. (μg/ml) (mean ± SD)	RSD (%)	RE (%)		
0.3	0.32 ± 0.124	7.4	6.9	0.32 ± 0.119	6.3	5.9	94.68±0.132	3.3
1.5	1.40 ± 0.110	7.9	-6.9	1.46 ± 0.107	7.3	-2.6	83.83±0.157	6.8
16	15.11 ± 0.720	4.8	-5.9	15.41 ± 0.781	5.1	-3.8	90.45±0.320	3.5

in because of the poor solubility of vitexin in water. When DMSO > 1% (Ma et al., 2010), it presents toxicity for animals, and different concentration of propylene glycol were therefore selected, 20% of propylene glycol with good solubility for vitexin was finally chosen as the auxiliary solvent.

To obtain suitable retention time and good separation for the analysis, the mobile phase was chosen after several trials in various proportions with methanol-water (30:70-45:55), methanol-acetonitrile-water (15:15:70-30:10:60). In addition, to improve the peak shape, 0.1-0.5% formic acid were added. however, only when 0.3% formic acid was applied to the mobile phase, a good peak shape and good separation were obtained, and thus the mobile phase consisting of methanol-acetonitrile-water containing 0.3% formic acid (30:10:60, v/v/v) was chosen in our study.

The UV absorption spectrum of vitexin has two maximum absorptions at 269 and 331 nm, and hesperidin at 204 and 284 nm. The interferences from endogenous substances in the plasma were observed when the wavelength was set at 269 nm, especially for low concentration samples, and the peak area of hesperidin was weak at 269 nm, which is not conducive to the determination of vitexin. Considering the above factors, 330 nm was chosen as the detection wavelength and suitable for the analysis of both of them.

To simultaneously acquire high extraction recovery and precision of vitexin and hesperidin, acetonitrile was selected as the precipitant after several extraction solvents including methanol, acetonitrile, in different ratios being tried to precipitate the protein. In addition, 10, 20, 30 μl of acetic acid were respectively tried and added in the plasma to avoid the dissociation of polyphenols.

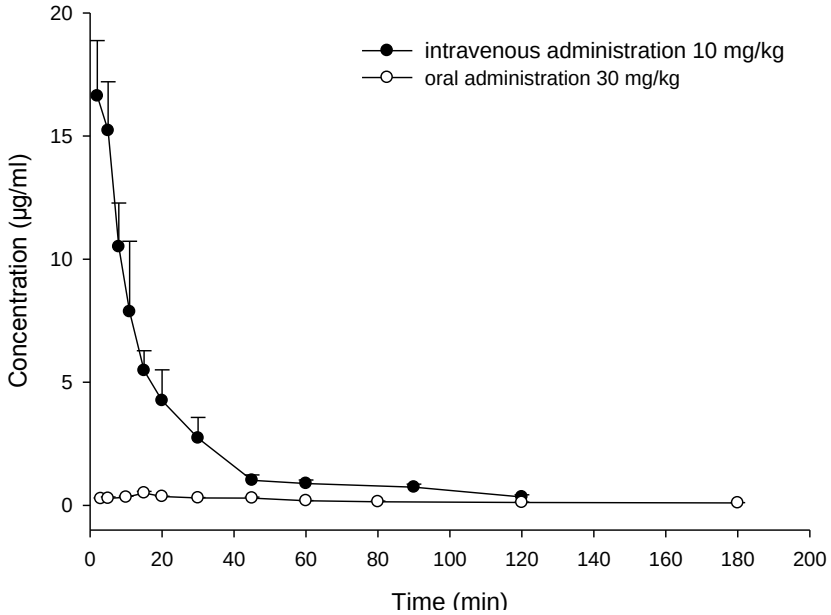


Figure 3. Plasma concentration-time profile of vitexin after intravenous (10 mg/kg) and oral administration (30 mg/kg).

Table 2. Pharmacokinetic parameters of vitexin in rats (mean ± SD, n = 5) after intravenous administration and oral administration at a dose of 10 and 30 mg/kg, respectively.

Parameters	Route of administration	
	Intravenous (10 mg/kg)	Oral (30 mg/kg)
C_{max}	16.61 ± 2.32	0.51 ± 0.015
T_{max}(min)	—	15.82 ± 0.172
V_c (L/kg)	0.47 ± 0.106	0.62 ± 0.16
$t_{1/2\alpha}$ (min)	6.78 ± 0.771	—
$t_{1/2\beta}$ (min)	46.01 ± 0.810	—
$t_{1/2}(k_a)$ (min)	—	3.68 ± 0.085
$t_{1/2}(k_e)$ (min)	—	59.81 ± 2.31
[a]$AUC_{0\rightarrow\infty}$(μg·min/ml)	327.11 ± 26.6	42.70 ± 6.35
[a] CL(L/kg· min)	0.031 ± 0.035	0.71 ± 0.16
$MRT_{0\rightarrow t}$ (min)	26.23 ± 1.51	60.42 ± 5.41
$MRT_{0\rightarrow\infty}$ (min)	32.30 ± 2.92	127.3 ± 6.59
[b]$AUC_{0\rightarrow t}$ (μg·min/ml)	324.21 ± 26.1	35.38 ± 3.56
[b]$AUC_{0\rightarrow\infty}$ (μg·min/ml)	335.61 ± 32.4	49.34 ± 3.32
F(%)	—	4.91 ± 0.761

[a] and [b] were the compartmental and non-compartmental approach, respectively.

Eventually, 20 μl of acetic acid was added in the plasma.

Pharmacokinetics and bioavailability of vitexin in rats

The pharmacokinetics relative parameters were calculated by both compartmental and non-compartmental approach. Comparing the parameters of AIC and R^2, a two-compartment open model (weight=1/C^2) is best fit to intravenous administration and a one-compartment open model (weight=1) is best fit to oral administration, respectively.

VIT is rapidly removed from the blood by the obtained values for the total body clearance (0.0298 ± 0.23 $L \cdot kg^{-1} \cdot min^{-1}$), the $MRT_{0\rightarrow t}$ (26.17 ± 1.53 min) after intravenous administered to rats. And the pharmacokinetic parameters $t_{1/2\beta}$ (46.0 ± 0.81 min), V_c (0.47 ± 0.06 $L \cdot kg^{-1}$) and CL (0.0306 ± 0.035 $L \cdot kg^{-1} \cdot min^{-1}$) is similar from the previous reference, $t_{1/2\beta}$ (43.53 ± 4.23 min), V_c (0.62 ± 0.16 $L \cdot kg^{-1}$) and CL (0.011 ± 0.005 $L \cdot kg^{-1} \cdot min^{-1}$) (Tong and Liu, 2007). When administered orally to the rats, the plasma concentration was very low, C_{max} (0.413 ± 0.015 μg/ml). Vitexin was rapidly absorbed with peak concentrations occurring at around 15.82 ± 0.172 min, and rapidly eliminated from plasma with $t_{1/2}$ (59.81 ± 2.31 min), $MRT_{0\rightarrow t}$ (60.42 ± 5.41 min), CL (0.71 ±0.156 L/kg· min).

The absolute bioavailability calculated *via* the formula of $(AUC_{oral}/AUC_{i.v})\times(dose_{i.v}/dose_{oral})$ was very low, only 4.91%, indicating that vitexin exibitts a prominent first-pass effect, corresponding to the report of the flavonoids having poor bioavailability and the native structure of flavonoids, many common metabolites or biotransformation products (the methyl, sulfate or glucuronide conjugates) appeared by the action of enzyme in liver and small intestine (Hu, 2007; Chen et al., 2005; Zhang et al.; 2006). Taken together, the results of the pharmacokinetics and bioavailability of pure vitexin in rats were first investigated, which will provide the reliable scientific data for its clinic application, and the reason leading to the lower bioavailability of vitexin will be investigated in further studies.

ACKNOWLEDGEMENT

This study was supported by the Natural Science Foundation of Liaoning Province (20072073), China.

REFERENCES

Bomser JA, Singletary KW, Wallig MA, Smith MA (1999). Inhibition of TPA-induced tumor promotion in CD-1 mouse epidermis by a polyphenolic fraction from grape seeds. Cancer Lett. 135:151-157.

Chen J, Lin H, Hu M (2005).Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model. Cancer Chemother. Pharmacol. 55(2):159-169.

Gaitan E, Cooksey RC, Legan J, Lindsay RH (1995). Antithyroid effects *in vivo* and *in vitro* of vitexin: a C-glucosylflavone in millet. J. Clin. Endocrinol. Metab. 80(4):1144-1147.

Gambelunghe C, Rossi R, Sommavilla M, Ferranti C, Rossi R, Ciculi C, Gizzi S, Micheletti A, Rufini S (2003). Effects of chrysin on urinary testosterone levels in human males. J. Med. Food 6:387-390.

Hollman PC, Katan MB (1999). Health effects and bioavailability of dietary flavonols. Free Radic. Res. 31 Suppl: 75-80.

Hu M (2007). Commentary: bioavailability of flavonoids and polyphenols: call to arms. Mol. Pharm. 4(6):803-806.

Ma N, Wang JF, Xu F (2010). Studies on absorptive characters of resveratrol derivative(E)-3,5,4'-trimethoxystilbene in rat intestine. Chin. J. Pharm. Anal. 30(4):586-591.

Prabhakar MC, Bano H, Kumar I, Shamsi MA, Khan MS (1981) Pharmacological investigations on vitexin. Planta. Med. 43(4):396-403.

Rahman I, Biswas SK, Kirkham PA (2006). Regulation of inflammation and redox signaling by dietary polyphenols. Biochem. Pharmacol. 72:1439-1452.

Tong CL, Liu XD (2007). Pharmacokinitics of vitexin in rats. Journal of China Pharmaceutical University. 38(1):65-68.

USFDA (2001). Food and Drug Administration Center for Drug Evaluation and Research (CDER): Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services. Retrieved from http://www.fda.gov/cder/guidance/index.htm.

Zhang L, Lin G, Zuo Z (2006）.Position preference on glucuronidation of mono-hydroxylflavones in human intestine. Life sci. 78(24):2772-2780.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2374-2379, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.616
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

The role of two natural flavonoids on human amylin aggregation

Mohammad-Hossein Aarabi and Seyyed Mehdi Mirhashemi*

Research Center for Biochemistry and Nutrition in Metabolic Diseases, Kashan University of Medical Sciences, Kashan, I.R. Iran.

Accepted 26 July, 2012

Misfolded amylin, the human polypeptide hormone, forms amyloid deposits in pancreatic islets. These amyloid deposits contribute to the dysfunction of beta-cells and the loss of beta-cell mass in type 2 diabetes mellitus (T2DM). Inhibition of amylin fibrillization has been regarded as a potential therapeutic approach for T2DM. Using fluorescence spectroscopic analysis with thioflavin T, the role of two naturally occurring flavonoids named myricetin and epigallocatechin gallate on human amylin hormone fibrillization and destabilization of fibrillar aggregates were examined under near physiological conditions. The results showed that after 168 h incubation by shaker incubator in 37°C. Myricetin at 10 and 40 µM repressed amylin amyloid formation by 25.3 and 22.4%, respectively ($p<0.05$), and the similar values of epigallocatechin gallate inhibited the formation of β-sheet structure by 18.1 and 16.7%, respectively ($p<0.05$). The obtained data also confirmed that amyloidal sheet opening was induced by myricetin and epigallocatechin gallate significantly ($p<0.05$). Therefore, it was concluded that islet amyloid cytotoxicity to β-cells may be reduced by these two flavonoids, and these compounds should be key molecules for the development of the therapeutics for diabetes mellitus.

Key words: Human islet amyloid polypeptide, hyperglycemia, myricetin, epigallocatechin gallate, flavonoids.

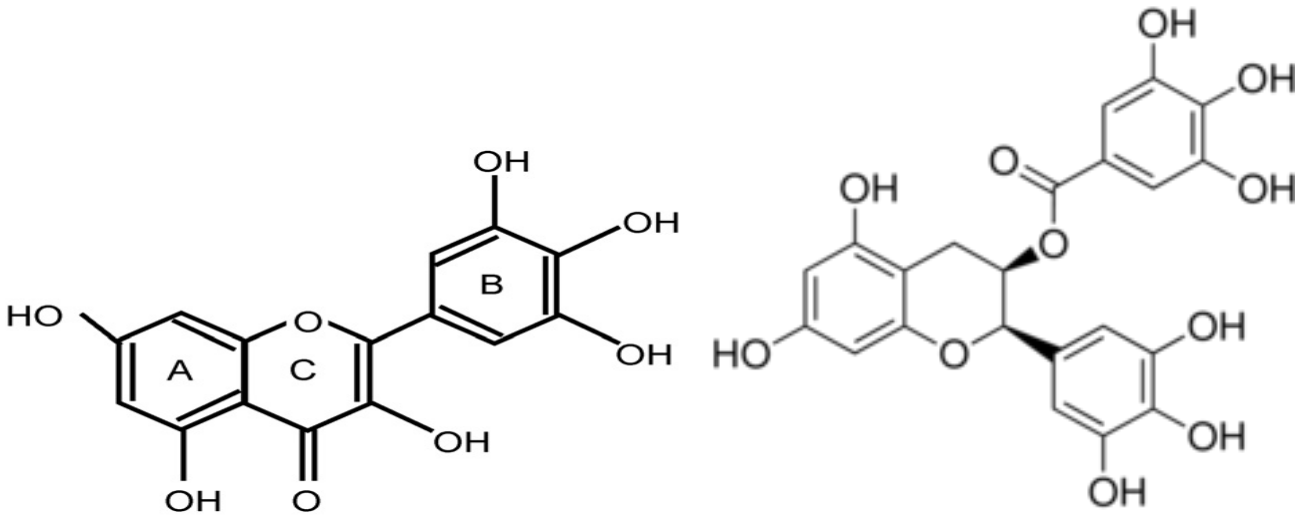
INTRODUCTION

Protein misfolding plays an important role in more than twenty destructive human diseases, including Alzheimer's disease, Parkinson's disease and type 2 diabetes mellitus. A key factor in the development of type 2 diabetes is the loss of insulin producing beta-cells. Amylin, a peptide hormone co-secreted with insulin in the pancreatic beta-cells, is suggested to play a crucial role in this process since amyloid deposits of this peptide are found in the islets of Langerhans in the vast majority of type 2 diabetes patients (Young, 2005; Reddy Nanga et al., 2011; Cheng et al., 2012). Owing to the increasing prevalence of diabetes, multidisciplinary study aimed at prevention and treatment is one of the world-wide research priorities. Considering the high cost of medication and side effects of synthetic medicine, as well as lack of full recovery of diabetic patients treated with chemical agents, has encouraged the researchers to use herbal medications (Akbarzadeh et al., 2012).

*Corresponding author. E-mail: mirhashemism@gmail.com.

Flavonoids are ubiquitous group of polyphenolic substances present in a variety of plants, such as onion (Nasri et al., 2012). Myricetin (Figure 1) is a natural flavonoid that is commonly found in tea, berries, fruits, vegetables and the medicinal herb *Abelmoschus moschatus* (Harnly et al., 2006). Myricetin is reported to have many therapeutic applications, such as anticarcinogenic action (Ko et al., 2005), antioxidative and cytoprotective properties, and ability to lower plasma glucose in diabetic rats (Liu et al., 2006, 2007). Epigallocatechin gallate (Figure 1), the major component of polyphenols in green tea, has also attracted considerable attention for its antioxidative, anti-inflammatory, anti-mutagenic, anti-thrombotic and neuroprotective properties (Higdon and Frei, 2003; Sheng et al., 2011; Mandel et al., 2008; Koh et al., 2006: Sachdeva et al., 2011).

The improvement of efficient inhibitors against the toxic formation of amylin amyloids has been enormously challenging and would be attractive therapeutic targets for the treatment of diabetes mellitus. Hence, this present study was carried out to assess the potential effects of



Myricetin **Epigallocatechin gallate**

Figure 1. Structures of myricetin and epigallocatechin gallate.

two flavonoids, myricetin and epigallocatechin gallate, on amylin aggregation and destabilization of preformed amyloid under near physiologic circumstances.

MATERIALS AND METHODS

Preparation of amylin stock solution

Human amylin full length peptide and other materials were purchased from Sigma-Aldrich Company.The human amylin used in this study had the following characteristics: (1-37)(Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH2, intra-molecular disulfide bridge: between Cys2 and Cys7). Its purity was 97% and the lyophilized salt included 70% peptide by weight. Amylin stock solution was prepared by adding 1.0 ml dimethylsulfoxide (DMSO) to dry purified peptide, and then sonicating at room temperature for 15 min. The experiments were performed in the two different phases as follows:

The first series of experiment: In order to assay the effects of different concentrations of myricetin and epigallocatechin gallate on amylin aggregation and amyloidogenesis, control and treated groups were considered.

The peptide stock solution was diluted by PBS 50 mM at pH: 7.5, to the final concentration of 10 µM. Different concentrations of myricetin (10 and 40 µM) and epigallocatechin gallate (10 and 40 µM) were prepared in PBS buffer containing 10 µM amylin as treated groups, separately. The samples without myricetin and epigallocatechin gallate were selected as the control group. All studied groups were incubated at 37°C for 168 h with shaking by a shaker incubator (GFL 3031, Germany).

The second series of experiments: These series of experiments were carried out to elucidate the destabilizing effect of the two flavonoids on preformed amyloid sheet of amylin. For this purpose, the amyloid prepared from the previous step was used. Amyloid was incubated with different concentrations of each of the agents for 6 h in 37°C.

Amyloid formation and destabilization assay

To determine the level of amyloid beta-pleated sheets at the end of the two series of experiments, thioflavin T (ThT) fluorescent assay was used. Thioflavin T assay was performed by adding 40 µL of each incubated solution to 700 µL of 10 µM ThT solution. Fluorescence measurements were recorded in a Perkin-Elmer LS55 fluorescence spectrometer (Perkin-Elmer LS55, USA) at room temperature using a 1-cm path length quartz cell. The ThT signal was quantified by averaging the fluorescence emission at 485 nm (slit width = 10 nm) when excited at 440 nm (slit width = 5 nm).

Immunofluorescence (IF) assay

The intrinsic fluorescence of the peptide tyrosine residue was measured for the studied groups after 96 h by averaging the fluorescence emission at 304 nm when excited at 270 nm.

Statistical analysis

Descriptive statistics was accomplished to obtain means and standard error of mean. Between groups, comparisons were performed with independent sample t-tests. Statistic significance level was established at $p<0.05$. Analysis of data was performed using SPSS statistical software package.

RESULTS AND DISCUSSION

The first run of experiments showed that amylin itself readily aggregated and formed a ThT-Positive material in control group. The results indicated that at zero time, ThT-fluorescence mean value for control group was 30.35, which at 168 h had increased to mean value of 49.1 ($p<0.05$). In myricetin treated groups, ThT fluorescence assay indicated that 10 and 40 µM of myricetin inhibited amyloid formation by 25.3 and 22.4%,

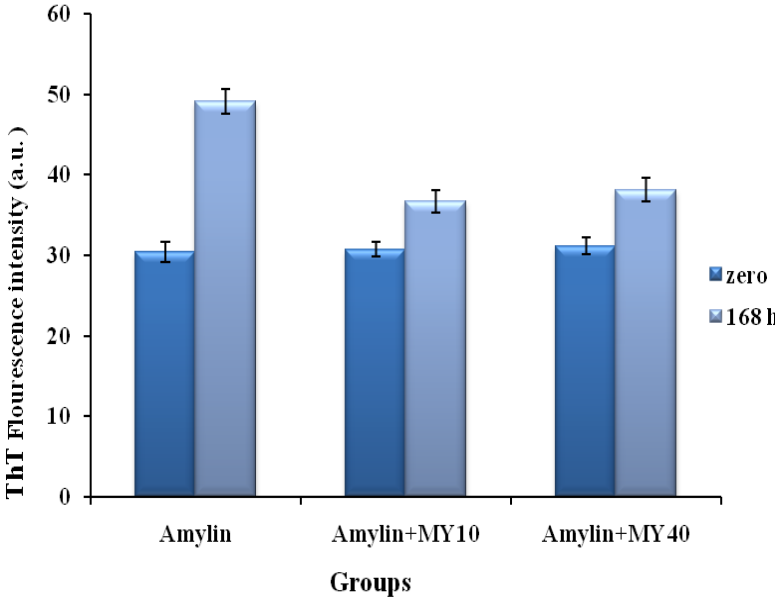


Figure 2. Thioflavin T fluorescence assay of inhibitory effect of Myricetin on amylin aggregation. All groups were incubated at 37°C for 168 h with shaking by a shaker incubator. At zero time (before incubation), there were no significant differences between the three groups: amylin, amylin+MY10 and amylin+MY40 ($p>0.05$). However, myricetin (MY) inhibited amylin aggregation significantly ($p<0.05$) at the end of incubation time. Data are shown as mean ± SEM, n = 5.

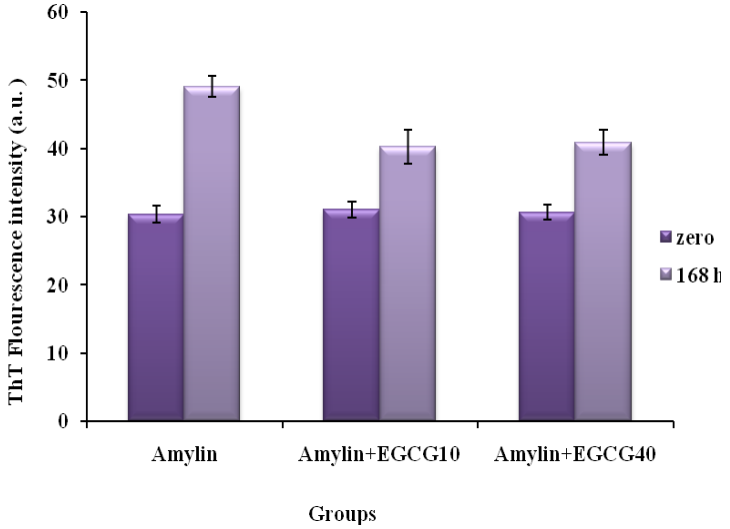


Figure 3. Thioflavin T fluorescence assay of inhibitory effect of epigallocatechin gallate on amylin aggregation. All groups were incubated at 37°C for 168 h with shaking by a shaker incubator. At zero time (before incubation), there were no significant differences between the three groups: amylin, amylin+EGCG10 and amylin+EGCG40 ($p>0.05$). However, epigallocatechin gallate (EGCG) inhibited amylin aggregation significantly ($p<0.05$) at the end of incubation time. Data are shown as mean ± SEM, n = 5.

respectively after 168 h incubation at 37°C ($p<0.05$) (Figure 2). Different concentrations effects of epigallocatechin gallate on amylin aggregation were demonstrated in Figure 3. These data indicated that

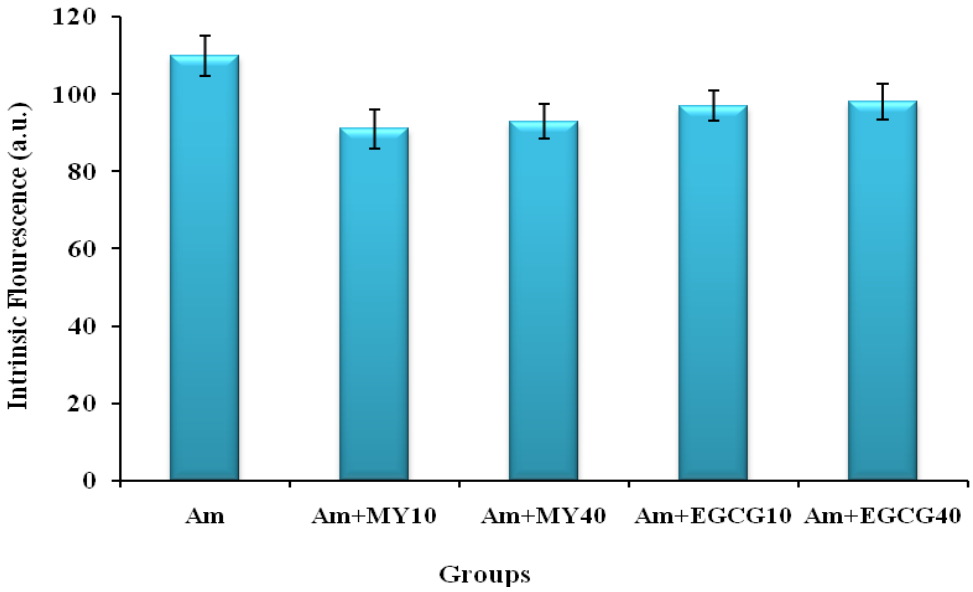


Figure 4. Intrinsic fluorescence of the control and treated groups. Tyrosine intrinsic fluorescence of amylin solutions in the absence and presence of the agents was measured after 96 h incubation at 37°C. Data are shown as mean ± SEM, n = 5.

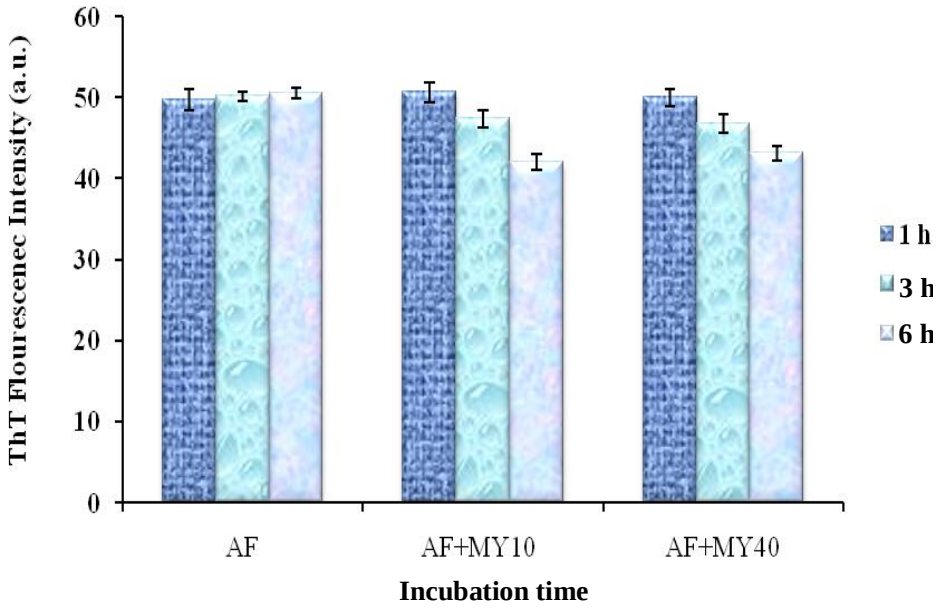


Figure 5. Effect of Myricetin on amylin fibril destabilization. Myricetin (MY) with two different concentrations destabilized amylin fibril (AF) significantly ($p<0.05$) after 6 h incubation.

compared to control group, at the end of incubation time, ThT-fluorescence decreased significantly in the presence of 10 and 40 µM of epigallocatechin gallate by 18.1 and 16.7%, respectively ($p<0.05$). The data also showed that the inhibitory effect of these flavonoids versus amyloid formation were not dose-dependent ($p>0.05$). Figure 4 indicates that the addition of myricetin and epigallocatechin gallate significantly ($P<0.05$) reduced the intrinsic fluorescence (IF) of amylin relative to the control (Figure 4). In addition, amyloid destabilizing effects of these components were shown in Figures 5 and 6. The obtained data from the 2nd run of experiments confirmed that both compounds were able to open the amyloid sheet significantly ($p<0.05$).

Diabetes mellitus is a group of metabolic diseases characterized by abnormally high concentrations of

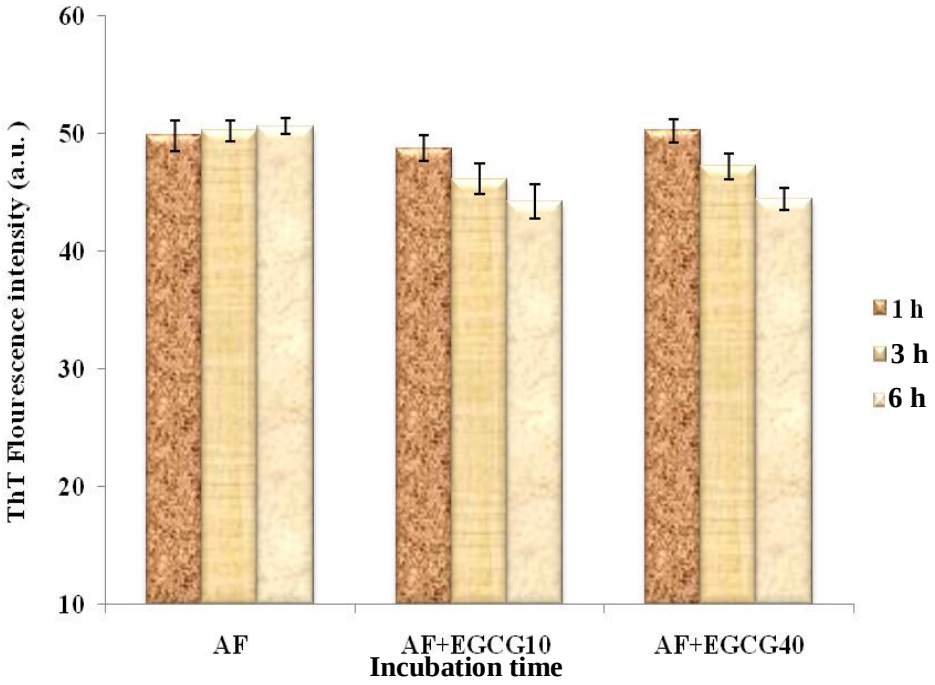


Figure 6. Effect of epigallocatechin gallate on amylin fibril destabilization. Epigallocatechin-gallate (EGCG) with two different concentrations destabilized amylin fibril (AF) significantly ($p<0.05$) after 6 h incubation.

glucose in blood and/or urine. In addition to neurological complications and premature death, consequences of these disorders include vascular complications such as coronary artery disease, cerebrovascular disorders, renal failure, blindness, and limb amputation (Liu et al., 2006). It is implicated that human amylin is a small fibrillogenic protein that is the major constituent of pancreatic islet amyloid, which occurs in most subjects with type 2 diabetes (Konarkowska et al., 2006; Zheng et al., 2010; Wang et al., 2011). Although the amyloid contribution to islet β-cell dysfunction is well clarified in the diabetic patients, the factors affecting this process remain elusive. We previously reported that some herbal compounds influence the aggregation of amylin (Mirhashemi et al., 2012) *in vitro*. Since the existing documents regarding effects of myricetin and epigallocatechin gallate on amylin depositions are very little (Meng et al., 2010; Noor et al., 2012) the present study was designed.

This study showed significant inhibitory role of myricetin and epigallocatechin gallate on amylin amyloidogenesis and thus demonstrated the β-sheet opening ability for these compounds. The formation of amyloid fibrils via self-assembly of peptide is assumed to be a crucial step in the pathogenesis of many amyloid diseases, including type 2 diabetes mellitus (Tabner et al, 2001). Previous investigations have shown that fibrillization of several polypeptides such as amylin is accompanied by formation of free radicals. In turn, reactive oxygen species (ROS), mainly free radicals, accelerate fibril formation, possibly via oxidation reactions so that the free radicals formed during amyloid fibrillization enhance fibrillization (Schoneich, 2005; Shoval et al., 2007). ROS may impact disulfide bond formation (Cummings et al., 2004) and subsequently influence the development of amylin misfolding. Disulfide bonds are important for proper protein structure, biological activity, and stability of many secreted and membrane proteins (Kopito and Ron, 2000; Anelli et al., 2002; Fassio and Sitia, 2002). Although the exact mechanism by which myricetin and epigallocatechin gallate inhibit amyloid formation and destabilize preformed amyloid *in vitro* remains unclear, it may be suggested that the inhibitory power of these compounds in amyloid fiber formation may be due to their potent antioxidant and free radical scavenging properties (Abdel-Raheem et al., 2009; Zhang et al., 2009; Piao et al., 2008). Flavonoids can provide both short and long-term protection against oxidative stress via a variety of mechanisms, including directly neutralizing toxic ROS through the donation of hydrogen ions (Wanga et al., 2010). Further study is required to elucidate the exact mechanism.

Conclusion

The improvement of efficient inhibitors against the toxic formation of amylin amyloids has been enormously challenging, since amylin is one of the most amyloidogenic polypeptides. Flavonoids are structurally heterogeneous polyphenolic compounds that are widely

distributed in plant foods, and which may exert beneficial effects. The antioxidant activity of myricetin and epigallocatechin gallate was examined on the formation and destabilization of amylin amyloid fibril *in vitro*. Our re showed that these two compounds inhibited amylin amyloid formation significantly. In addition, they destabilized preformed amylin fibrils. It may be concluded that these compounds should be key molecules for the development of the therapeutics for diabetic patients.

ACKNOWLEDGEMENT

The financial support from the Research Vice Chancellor of Kashan University of Medical Sciences was highly appreciated.

REFERENCES

Abdel-Raheem IT, Abdel-Ghany AA, Mohamed GA (2009). Protective effect of quercetin against gentamicin-induced nephrotoxicity in rats. Biol. Pharm. Bull. 32:61-67.

Akbarzadeh S, Bazzi P, Daneshi A, Nabipour I, Pourkhalili K, Mohebbi G, Sartavi K, Abdi M, Mirzaei M, Bargahi A (2012). Anti-diabetic effect of *Otostegia persica* extract on diabetic rats. J. Med. Plants Res. 6(16): 3176-3180.

Anelli T, Alessio M, Mezghrani A, Simmen T, Talamo F, Bachi A, Sitia R (2002). ERp44, a novel endoplasmic reticulum folding assistant of the thioredoxin family. EMBO. J. 21:835-44.

Cheng B, Gong H, Li X, Sun Y, Zhang X, Chen H, Liu X, Zheng L, Huang K (2012). Silibinin inhibits the toxic aggregation of human islet amyloid polypeptide. Biochemical and Biophysical Res. Communications 419: 495-499.

Cummings DE, Overduin J (2004). Gastrointestinal regulation of food intake. J. Clin. Invest. 117:13-22.

Fassio A, Sitia R (2002). Formation, isomerization and reduction of disulphide bonds during protein quality control in the endoplasmic reticulum. Histochem. Cell. Biol. 117:151-7.

Harnly JM, Doherty RF, Beecher GR, Holden JM, Haytowitz DB, Bhagwat S, Gebhardt S (2006). Flavonoid content of U.S. fruits, vegetables, and nuts. J. Agric. Food Chem. 54 (26):9966-9977.

Higdon JV, Frei B (2003). Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Crit. Rev. Food. Sci. Nutr. 43:89-143.

Ko CH, Shen SC, Lee TJ, Chen YC (2005). Myricetin inhibits matrix metalloproteinase 2 protein expression and enzyme activity in colorectal carcinoma cells. Molecular Cancer Therapeutics 4 (2): 281-290.

Koh SH, Lee SM, Kim HY, Lee KY, Lee YJ, Kim HT, Kim J, Kim MH, Hwang MS, Song C, Yang KW, Lee KW, Kim SH, Kim OH (2006). The effect of epigallocatechin gallate on suppressing disease progression of ALS model mice, Neurosci. Lett. 395: 103-107.

Konarkowska B, Aitken JF, Kistler J, Zhang S, Cooper GJ (2006). The aggregation potential of human amylin determines its cytotoxicity towards islet beta-cells. FEBS J. 273(15):3614-24.

Kopito RR, Ron D (2000). Conformational disease. Nat. Cell. Biol. 2(11): E207-9.

Liu IM, Liou SS, Lan TW, Hsu FL, Cheng JT (2006). Mediation of β-endorphin by myricetin to lower plasma glucose in streptozotocin-induced diabetic rats. Journal of Ethnopharmacol. 104 (1-2):199-206.

Liu IM, Tzeng TF, Liou SS, Lan TW (2007). Myricetin, a naturally occurring flavonol, ameliorates insulin resistance induced by a high-fructose diet in rats. Life Sci. 81:1479–1488.

Mandel SA, Amit T, Weinreb O, Reznichenko L, Youdim MB (2008). Simultaneous manipulation of multiple brain targets by green tea catechins: a potential neuroprotective strategy for Alzheimer and Parkinson diseases. CNS Neurosci. Ther. 4:352-365

Meng F, Abedini A, Plesner A, Verchere CB, Raleigh DP (2010).The flavanol (-)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against IAPP-induced toxicity. Biochem. 9(37):8127-33.

Mirhashemi SM, Aarabi MH (2012). To evaluate likely antiamyloidogenic property of ferulic acid and baicalein against human islet amyloid polypeptide aggregation, in vitro Study. Afr. J. Pharm Pharmacol. 6(9):671-676.

Nasri S, Anoush M, Khatami N (2012). Evaluation of analgesic and anti-inflammatory effects of fresh onion juice in experimental animals. Afr. J. Pharm. Pharmacol. 6(23):1679-1684.

Noor H, Cao P, Raleigh DP (2012). Morin hydrate inhibits amyloid formation by islet amyloid polypeptide and disaggregates amyloid fibers. Protein Sci. 21(3):373-382.

Piao MJ, Kang KA, Zhang R, Ko DO, Wang ZH, You HJ, Kim HS, Kim JS, Kang SS, Hyun JW (2008). Hyperoside prevents oxidative damage induced by hydrogen peroxide in lung fibroblast cells via an antioxidant effect. Biochim. Biophys. Acta. 1780:1448-1457.

Reddy Nanga, Brender JR, Vivekanandan S, Ramamoorthy A (2011). Structure and membrane orientation of IAPP in its natively amidated form at physiol. pH in a membrane environment. Biochim. Biophys. Acta. 1808:2337-2342.

Sachdeva AK, Kuhad A, Chopra K (2011). Epigallocatechin gallate ameliorates behavioral and biochemical deficits in rat model of load-induced chronic fatigue syndrome. Brain Res. Bull. 86:165-172.

Schoneich C (2005). Methionine oxidation by reactive oxygen species: reaction mechanisms and relevance to Alzheimer's disease, Biochim. Biophys. Acta 1703:111-119.

Sheng R, Gu ZL, Xie ML (2011). Epigallocatechin gallate, the major component of polyphenols in green tea, inhibits telomere attrition mediated cardiomyocyte apoptosis in cardiac hypertrophy. International J. Cardiol. (2011), in press.

Shoval H, Lichtenberg D, Gazit E (2007). The molecular mechanisms of the anti-amyloid effects of phenols. Amyloid. 14:73-87.

Tabner BJ, Turnbull S, El Agnaf O, Allsop D (2001). Production of reactive oxygen species from aggregating proteins implicated in Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. Curr. Top. Med. Chem. pp.507-517.

Wanga ZH, Kanga KA, Zhanga R, Piaoa MJ, Jo SH, Kimc JS, Kang SS, Leed JS, Parkd DH, Hyuna JW (2010). Myricetin suppresses oxidative stress-induced cell damage via both direct and indirect antioxidant action. Environmental Toxicol. Pharmacol. 29:12-18.

Wang L, Middleton CT, Singh S, Reddy AS, Woys AM, Strasfeld DB, Marek P, Raleigh DP, de Pablo JJ, Zanni MT, Skinner JL (2011).2DIR spectroscopy of human amylin fibrils reflects stable β-sheet structure. J. Am. Chem. Soc. 133(40):16062-16071.

Young A (2005). Effects on plasma glucose and lactate. Adv. Pharmacol. 52:193-208.

Zhang R, Kang KA, Piao MJ, Ko DO, Wang ZH, Chang WY, You HJ, Lee IK, Kim BJ, Kang SS, Hyun JW (2009). Preventive effect of 7,8-dihydroxyflavone against oxidative stress induced genotoxicity. Biol. Pharm. Bull. 32:166-171.

Zheng X, Ren W, Zhang S, Liu J, Li S (2010). Serum levels of proamylin and amylin in normal subjects and patients with impaired glucose regulation and type 2 diabetes mellitus. Acta. Diabetol. 47(3):265-70.

African Journal of Pharmacy and Pharmacology Vol. 6(31), pp. 2380-2388, 22 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.617
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Cardioprotective effects of aerobic regular exercise against doxorubicin-induced oxidative stress in rat

Javad Ashrafi[1], Valiollah Dabidi Roshan[1]* and Soleiman Mahjoub[2]

[1]Department of sport physiology, College of Physical Education and Sport Sciences, University of Mazandaran, Babolsar, Iran.
[2]Department of Biochemistry and Biophysics, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran.

Accepted 10 August, 2012

Doxorubicin (DOX) is an anthracycline antibiotic that is widely used as an anticancer agent. However, the clinical use of DOX is limited due to its cardiotoxic side effects. Few studies have assessed pretreatment effects of chronically exercise against doxorubicin-induced cardiotoxicity. The aim of this study was to determine cardioprotective effects of aerobic regular exercise against doxorubicin-induced oxidative stress in rat. Forty-eight Wistar male rats were randomly assigned to sedentary and trained groups. Training program included treadmill running between 25 to 54 min/day and 15 to 20 m/min, 5 days/week for 6 weeks. The biomarkers related to oxidative stress were assessed in heart tissue after administration of the saline solution (0.9% NaCl i.p) and/or DOX 20 mg/kg and DOX 10 mg/kg. Doxorubicin administration (10 and 20 mg.kg^{-1}) causes an imbalance in the oxidant/antioxidant markers in heart. Six weeks of the aerobic training led to a significant increase of apelin, nitric oxide(NO), superoxide dismutase(SOD) and an insignificant decrease of malondialdehyde(MDA), as compared to sedentary+placebo group. However, after six weeks of aerobic training and DOX treatment with 10 and 20 mg.kg^{-1}, a significant increase in apelin and SOD, and a significant decrease in MDA were detected in comparison to sedentary+DOX 10 and/ or sedentary+DOX 20 groups. However, there was a significant difference between DOX 10 mg.kg^{-1} and DOX 20 mg.kg^{-1} treatments in NO and SOD levels, only. Our study suggests that cardioprotection induced by chronically exercise in DOX treated rats was associated with inhibition of oxidative stress and the up-regulation of antioxidant enzymes.

Key words: Cardiotoxicity, doxorubicin, antioxidant, endurance training, rat.

INTRODUCTION

Cancer is one of the leading causes of death worldwide, accounting for 13% of all deaths, equivalent to 7.4 million people per year (Barbaric et al., 2010). It is treated with various procedures such as: surgery, chemotherapy and radiation (Minghua and Zhi-Gang, 2011). Doxorubicin (Dox) is a powerful and highly efficacious drug and shows a broad range of antitumor activity in many kinds of cancers (Vishwanatha et al., 2012). However, the clinical use of DOX is often limited because of its undesirable serious cardiotoxic side effects on cardiac tissues (Evert et al., 2001; Chatterjee et al., 2010; In Duk et al., 2002; Raschi et al., 2010; Andreadou et al., 2007). Thus, drug-induced cardiotoxicity is emerging as an important issue among cancer survivors and unfortunately, its major adverse effect may limit its use.

Several mechanisms have been proposed to account for the DOX-induced cardiotoxic side effects including free radical induced myocardial injury, lipid peroxidation, mitochondrial damage, vasoactive amine release and cellular toxicity (Evert et al., 2001; Chatterjeeet al., 2010; Vishwanathaet al., 2012, 2011). Oxygen free radicals are apparently involved in all mechanisms proposed (Abdel-Moneim et al., 2009) and increased oxidative stress and release of free radicals as well as endogenous

*Corresponding author. E-mail: v.dabidi@umz.ac.ir, vdabidiroshan@yahoo.com. Tel: +98 (0) 11252 32091-95. Fax: +98 (0) 1125342202.

antioxidant deficits have been suggested to play a major role in Dox-induced cardiotoxicity and heart damage (Vishwanatha et al., 2012; Andreadou et al., 2007; Hitesh et al., 2011). The heart is particularly vulnerable to injury from free radicals because it has a lower level of protective enzymes such as superoxide dismutase than other tissues (Cecen et al., 2011). Ascensão (2005) reported that the weakness of the heart to oxidative damage may be in part explained by the fact that heart demonstrates a slow turnover and relatively lower levels of antioxidant enzyme activity when compared to most other tissues. Apelin was recently found to be an inotropic polypeptide in isolated rat hearts, and directly activated the vascular L-Arg/NOS/NO pathway, which could be one of the important mechanisms of apelin-regulated vascular function (Jia et al., 2007). Furthermore, Duparc et al. (2011) reported peripheral administration of apelin stimulates glucose utilization and insulin sensitivity through a nitric oxide (NO) pathway. On the other hand, Hitesh et al. (2011) reported that high concentrations of NO participate in cardiomyocyte oxidative damage, apoptosis, and/or necrosis through peroxynitrite formation. DOX promotes the synthesis of NO and ROS, such as the superoxide anion. In contrast, Chatterjee et al. (2010) reported that lack of nitric oxide was associated with enhanced cardiac injury, and mitochondrial injury was attenuated by an increase in manganese superoxide dismutase.

In recent years, by understanding the free radical mechanism of DOX-induced cardiotoxicity, it has become possible to develop effective strategies to prevent or modify expected damage. To date, a number of pharmaceutical agents have been tested to assess their potential to reduce the risk of doxorubicin cardiotoxicity (Raschi et al., 2010; Vishwanatha et al., 2012, 2011; Hitesh et al., 2011; Abdel-Moneim et al., 2009; Cecen et al., 2011). On the other hand, there is a growing interest in the usage of aerobic regular training as a non-drug therapeutics protective strategy against problems related to cardiovascular health such as Dox-induced cardiotoxicity (Ascensão et al., 2012). Although, there is evidence that acute exercise resulted in oxidative stress and cardiac damage (Teixeira et al., 2011), it seems probable that regular endurance exercise training could constitute an excellent tool either to prevent and/or to treat several diseases. Moreover, it provides myocardial protection against many cardiac insults (Ascensão et al., 2006, 2005). The exact mechanisms responsible for this protection continue to be debated. However, it has been argued that they are in part, associated with the decreased free radical production (ROS) and with increased response of the several antioxidant defense systems (Ascensão et al., 2006; Ascensão et al., 2005).

To our knowledge, there are few studies dealing with the preventive effect of moderate-term endurance training on Dox-induced cardiotoxicity and oxidative stress in rats. It was hypothesized that regular exercise attenuated the oxidant/antioxidant imbalance caused by various dosages (10 and 20 mg/kg) of the DOX drug in heart tissue. These new insights would consist in the recognition of regular training as a non-drug therapeutics protective strategy against DOX treatment. Thus, the main purpose of this study was to determine the preventive effects of 6 weeks of aerobic training on biomarkers related to the cardiac oxidative damage including; apelin, malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) in rats that have been acutely exposed to DOX-induced cardiotoxicity.

MATERIALS AND METHODS

Experimental design and laboratory environment

The experimental protocol of the current study approved by department of physiology, university of Mazandaran were performed according to guiding procedures in the care and use of animals, prepared by the Council of the American Physiological Society. The experiments were carried out with forty-eight Wistar male rats, (8-weeks-old, initially weighing 269 ± 4 g), which were obtained from the laboratory of animal bearing and multiplying at the Pasture institute of Iran.

Rats were housed in standard cages of polycarbonate (20 × 15 × 15 cm), made at the Pasture institute of Iran, in a large air-conditioned room with a controlled temperature of 22 ± 2°C, light-dark cycles of 12 : 12 h and humidity of 50 ± 5%. The pollutant standard index (PSI) was in the acceptable range as determined by the Iranian meteorological organization. Rats were fed with a standard rat chow provided by Pars Institute for animals and poultry with a daily regimen of 10 g per 100 g body weight for each rat. Water was available *ad libitum*.

Familiarization and exercise training protocols

Rats in all groups were adapted to the treadmill by running for 5 days. The familiarization protocol was designed as once a day for 10 min/session at a speed of 10 m/min at a slope of 0 degree. Because rats are more active in darkness, the front portion of the treadmill lines was covered with a dark thick paper to darken this area. At the rear of the lines, an electric grid provided a stimulus for running. An electric stimulus (30 V and 0.5 A) was manually turned on for less than 2 s when the animals stayed on the electric grid for longer than 10 s. Rats quickly learned to stay on the belt and avoid shock, except for one rat, which would not stay on the moving belt, and thus was quickly removed from familiarization process. Following this familiarization period, they were randomly assigned into sedentary and trained groups. Exercise training protocol was performed on treadmill with zero slopes between 25 to 54 min/session and 15 to 20 m/min, 5 days/week for 6 weeks (Table 1). We replicated the aforesaid exercise training protocol that was previously reported by Roshan et al. (2011).

Subjects classification

At the end of the exercise training protocol, rats from the sedentary and trained groups were again randomly separated into subgroups; the DOX (10, 20 mg/kg) and placebo treatment. Thus, the sedentary rats were distributed into sedentary + placebo (S + P, n = 8), sedentary + DOX (S + $DOX_{10\ mg/kg}$, n = 8) and sedentary + DOX (S + $DOX_{20\ mg/kg}$, n = 8) groups and the trained rats into trained + placebo (T + P, n = 8), trained + DOX (T + $DOX_{10\ mg/kg}$, n = 8) and trained + DOX (T + $DOX_{20\ mg/kg}$, n = 8) groups.

Table 1. Exercise training protocol in the current study.

Training sessions and variables		Weeks of training					
		1	2	3	4	5	6
1	Speed*	15	16	17	18	19	20
	duration#	25	30	35	40	45	50
2	Speed	15	16	17	18	19	20
	duration	26	31	36	41	46	51
3	Speed	15	16	17	18	19	20
	duration	27	32	37	42	47	52
4	Speed	15	16	17	18	19	20
	duration	28	33	38	43	48	53
5	Speed	15	16	17	18	19	20
	duration	29	34	39	44	49	54

*Meter/min; # min/session.

Doxorubicin treatment

Doxorubicin hydrochloride (EBEWE Pharma Ges.m.b.H.Nfg.KG) was dissolved in saline and administered by i.p injection at two dosages of 10 mg/kg (Karen et al., 2009) and 20 mg/kg (Ascensão et al., 2006), and control animals received saline with comparable volume. Both treatments were carried out at 24 h after the last exercise bout, and animals were sacrificed 24 h after DOX and placebo injections.

Heart tissue collection and preparation

All groups were anesthetized with ketamine and xylozine and decapitated after 10 to 12 h overnight fasting. The Thoracic cavity was opened and the heart was quickly excised from the aortic root. Heart tissues were weighed and it was placed into Petri dishes containing cold isolation medium (0.1 mol/L K_2HPO_4, 0.15 mol/L NaCl, pH 7.4) to remove the blood and were frozen immediately in liquid nitrogen and stored at -80ºC for subsequent analysis of apelin, NO, SOD and MDA. Heart tissue was squashed in liquid nitrogen, homogenized in a lysis buffer (5 ml/g of tissue) with a protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma-Aldrich, St. Louis, U.S.A) 100 ul/1 ml, and 10 m Mtris base (Sigma-Aldrich, St. Louis, U.S.A), pH 7.4 and centrifuged at 1500 g at 4ºC for 15 min. Heart supernatant was diluted 1:30. Plasma was diluted 1:10 and the fluids were used in an Apelin-13 ELISA kits (Phoenix peptides, Burlingame, California, USA), following the manufacturer's instructions.

Biochemical analysis

The assay kit was very specific and detects apelin-13 with 100% cross reactivity. It has an inter-assay variation less than 14% and intra assay coefficient of variation less than 10%. Apelin-13 in the mentioned sample was measured using ELISA kits too (Rat Apelin, ELISA, USCN LIFE Science Inc., Wuhan, P. R. China, USCN, Life Science Inc, Sensitivity 0.128 ng/ml and IntraCV: 5%). Lipid peroxidation (MDA) levels, as important marker in oxidative stress in the heart tissue, were measured with the thiobarbituric-acid reaction using the method of Daniel (Daniel et al., 2004). The quantification of thiobarbituric acid reactive substances was determined at 532 nm by comparing the absorption to a standard curve of MDA equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetramethoxypropane. The values of MDA in heart tissue were expressed as nmol/g tissue. The NO concentration was determined by first reducing the nitrate to nitrite using nitrate reductase (Sigma). Superoxide dismutase (SOD) activity was determined spectrophotometrically using the method described by Roshan et al. (2011). In brief, for total SOD (tSOD) activity, the adequate amount of protein (2 mg tissue wet weight) was incubated at 25ºC with 1 m MN, Nbis (2-(bis(carboxymethyl)amino)-ethyl) glycine (DTPA) in 50 m MTris_HCl, pH 8.2, in 1 ml final volume. Reaction was started with 0.3 m mpyrogallol, in which the auto-oxidation rate was recorded at 420 nm.

Statistical analysis

All data have been expressed as mean ± SD. Statistical analysis was performed using a commercial software package (SPSS version 16.0 for Windows). Data of the biomarkers related to the cardiac oxidative damage including; apelin, malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) were normally distributed after log-transformation. A one-way analysis of variance (Statistics software, Stat Soft, Inc., Tulsa, OK) was used to detect statistical differences between groups. A post-hoc test (Tukey test) was performed to determine differences in the various biomarkers between groups. Differences were considered statistically significant at p-value < 0.05.

RESULTS

Mean values of body weight, heart weight and heart-body weight ratio for the six groups are shown in Table 2. At first, no differences existed in the aforesaid characteristic between groups (Table 2). Table 3 shows changes in apelin, NO, MDA and SOD levels following doxorubicin treatment in the various groups. After Doxorubicin administration (10 and 20 mg kg^{-1}), a significant increase in MDA (51 and 96%, respectively), an increase in apelin (41 and 49%, respectively), a significant decrease in SOD (9 and 18%, respectively) and an insignificant increase in NO (8 and 12%) were detected, as compared to S + P group ($P < 0.05$). Although, there was no

Table 2. Effect of aerobic training and DOX treatment on body weight, heart weight and heart-body weight ratio for each group.

Groups	Markers of weight		
	Body weight(g)	Heart weight(g)	Heart-body weight ratio
S+P	333±22.6	1.2±0.1	0.004±0.03
S+DOX_{10}	331±8.7	1.1±0.03	0.003±0.01
S+DOX_{20}	325±14.3	1.1±0.03	0.003±0.01
T+P	339±31.5	1.2±0.1	0.004±0.02
T+DOX_{10}	330±36.8	1.2±0.1	0.004±0.02
T+DOX_{20}	328±34.4	1.1±0.1	0.004±0.01

Data are presented as the Mean ± SD for 8 rats.

Table 3. Effect of aerobic training and DOX treatment on apelin, NO and MDA levels in the various groups.

Markers	Groups					
	S+P	S+DOX_{10}	S+DOX_{20}	T+P	T+DOX_{10}	T+DOX_{20}
Apelin (pg/mg protein)	3.6±0.46	5.1±0.33	5.4±0.44	8.2±0.9	7.1±0.6	6.8±0.8
NO (nmol/mg protein)	0.24±0.02	0.3±0.02	0.3±0.03	0.3±0.03	0.3±0.03	0.4±0.03
MDA (nmol/g protein)	29.6±1.9	44.7±3.7	58.7±8	20.3±4	31.4±7	37.5±6.4
SOD (u/mg protein)	92.3±2.9	84.3±4.4	75.3±6	125.6±2.6	113.4±3	105.7±4.1

Data are presented as the Mean ± SD for 8 Rats, Abbreviations: nitric oxide (NO), malondialdehyde (MDA), Superoxide dismutase (SOD). S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg.kg^{-1}), S + DOX_{20} (sedentary+ doxorubicin 20 mg.kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}).

significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg\text{-}1}$ treatments in apelin and NO levels, there was a significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg}^{-1}$ treatments in MDA and SOD levels.

Six weeks of the aerobic training led to a significant increase of heart apelin, NO and SOD levels (126, 25 and 36%, respectively), and an insignificant decrease in MDA, as compared to S + P group ($P < 0.05$) (Table 3). However, after six weeks of aerobic training and DOX treatment with 10 mg kg^{-1}, a significant increase in apelin and SOD (38 and 34%, respectively), and an insignificant increase in NO (10%), and a significant decrease in MDA (42%) were detected in comparison to S + DOX_{10} group ($P < 0.05$). In contrast, six weeks of aerobic training and DOX treatment with 20 mg kg^{-1} resulted in a significant increase in apelin, NO and SOD (25, 29 and 40%, respectively), and a significant decrease in MDA (36%), in comparison to S + DOX_{20} group ($P < 0.05$).

Data in Figures 1, 2, 3 and 4 show changes in the heart tissue apelin, MDA, NO and SOD levels, respectively, in the six groups. After six weeks of aerobic training and doxorubicin treatment, both 10 and 20 mg kg^{-1}, a significant decrease in apelin (13 and 17%, respectively), a significant decrease in SOD (10 and 16%, respectively), and a significant increase in MDA (54 and 85%, respectively) were detected, as compared to T + P group ($P < 0.05$). Moreover, treatment with $DOX_{20\ mg\ kg}^{-1}$ after six weeks of an aerobic training caused a significant increase in NO level (17%), as compared to T + P group ($P < 0.05$). However, there was no significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg}^{-1}$ treatments in apelin and MDA levels. Furthermore, there was a significant difference between $DOX_{10\ mg\ kg}^{-1}$ and $DOX_{20\ mg\ kg}^{-1}$ treatments in NO and SOD levels.

DISCUSSION

Doxorubicin, a very potent and often used anti-cancer drug, has a wide spectrum of biological activity (Vishwanatha et al., 2012). The heart is particularly susceptible to free radical injury, because it contains less free radical detoxifying substances, superoxide dismutase, glutathione, and catalase than do metabolic organs such as liver or kidney. Moreover, doxorubicin is known to have a high affinity for cardiolipin, a major phospholipid component of the mitochondrial membrane in heart cells, resulting in selective accumulation of doxorubicin inside cardiac cells (Evert et al., 2001). Several studies have shown that doxorubicin induced cardiotoxicity (Evert et al., 2001; Chatterjeeet al., 2010; In Duk et al., 2002; Raschiet al., 2010; Andreadou et al., 2007; Khositseth et al., 2011). This study was designed to determine the preventive effects of aerobic regular

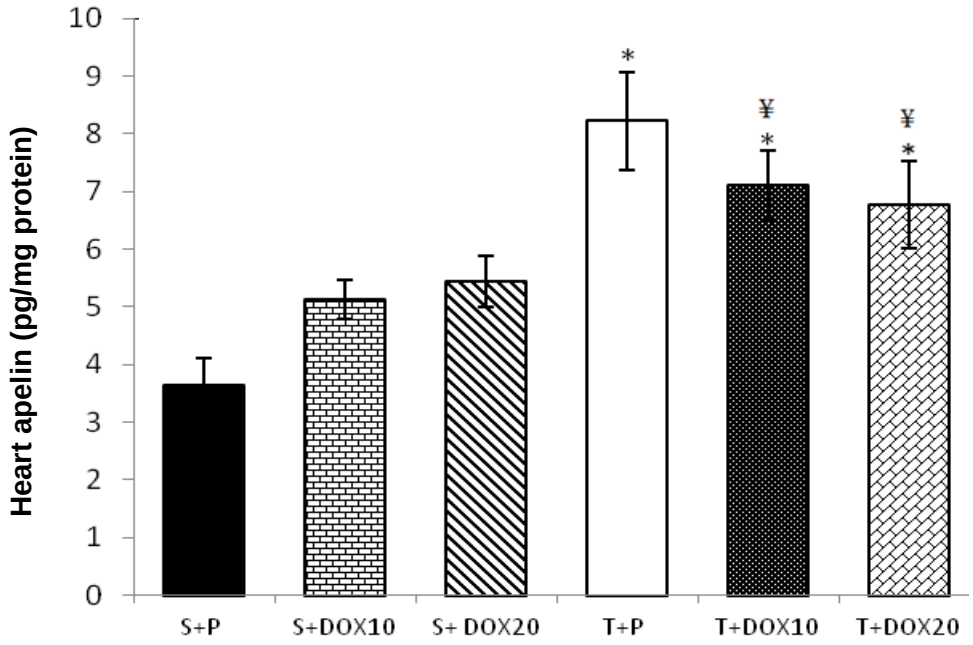


Figure 1. Apelin level after six weeks of aerobic training and DOX treatment. Abbreviations; S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg kg^{-1}), S + DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}). Data are presented as the mean ± SD for 8 Rats. *Significantly different to similar sedentary group (P<0.05), ¥ significantly different to T+P group (P < 0.05).

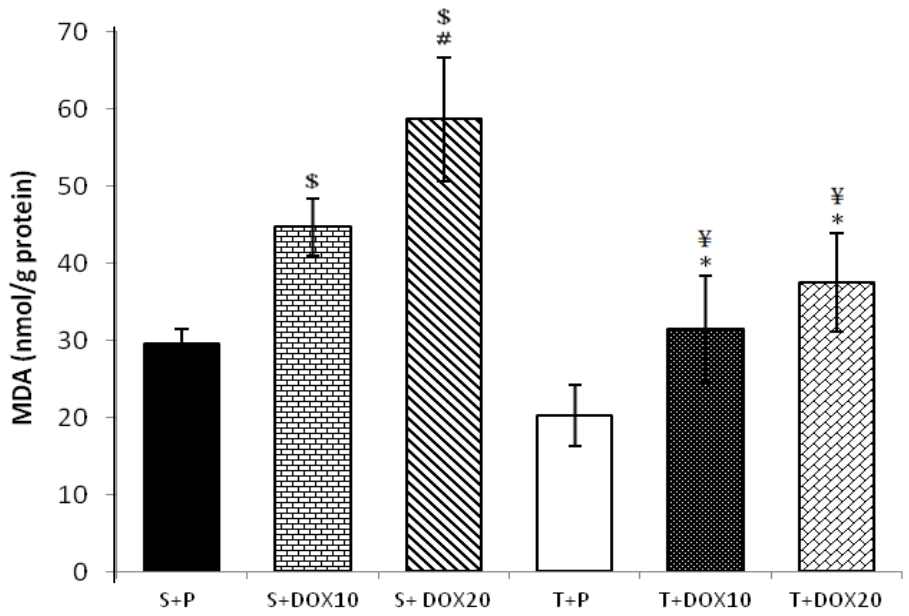


Figure 2. Malondialdehyde (MDA) level after six weeks of aerobic training and DOX treatment. Abbreviations; S+P (sedentary + placebo), S+DOX_{10} (sedentary + doxorubicin 10 mg.kg^{-1}), S+DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T+P (training + placebo), T +DOX_{10} (training + doxorubicin 10 mg.kg^{-1}), T +DOX_{20} (training + doxorubicin 20 mg.kg^{-1}). Data are presented as the mean ± SD for 8 Rats.*significantly different to similar sedentary group (P<0.05), $ significantly different to the S+P group (P<0.05), # significantly different to dose 10 mg kg^{-1}in it's group (P < 0.05), ¥ significantly different to the T + P group (P < 0.05).

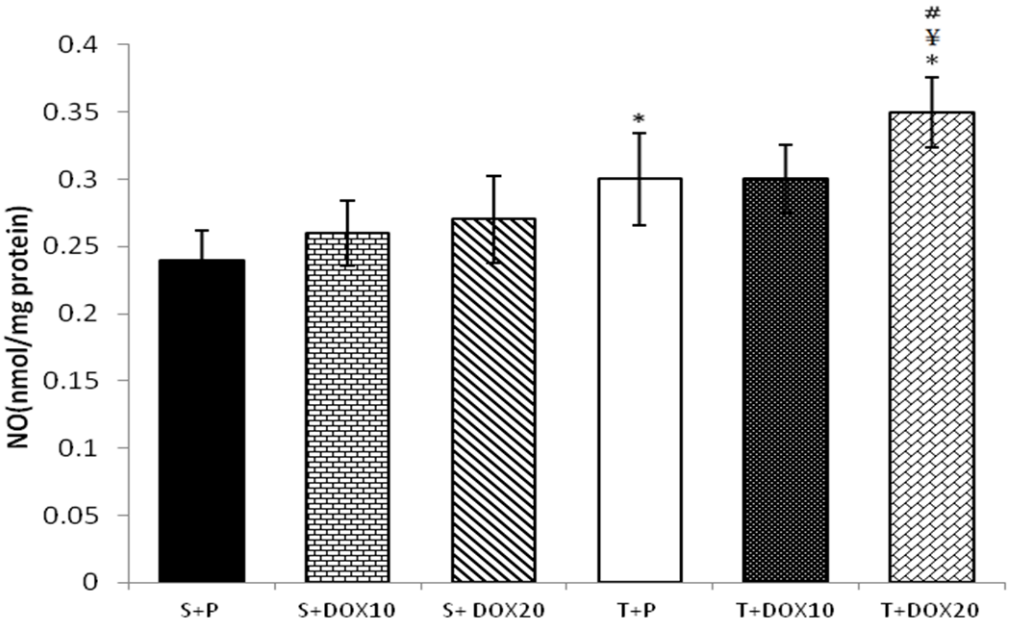


Figure 3. Nitric oxide (NO) level after six weeks of aerobic training and DOX treatment. Abbreviations; S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg kg^{-1}), S + DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}). Data are presented as the mean ± SD for 8 Rats. * significantly different to similar sedentary group ($P < 0.05$), # significantly different to dose 10 mg kg^{-1}in it's group ($P < 0.05$), ¥ significantly different to the T + P group ($P < 0.05$).

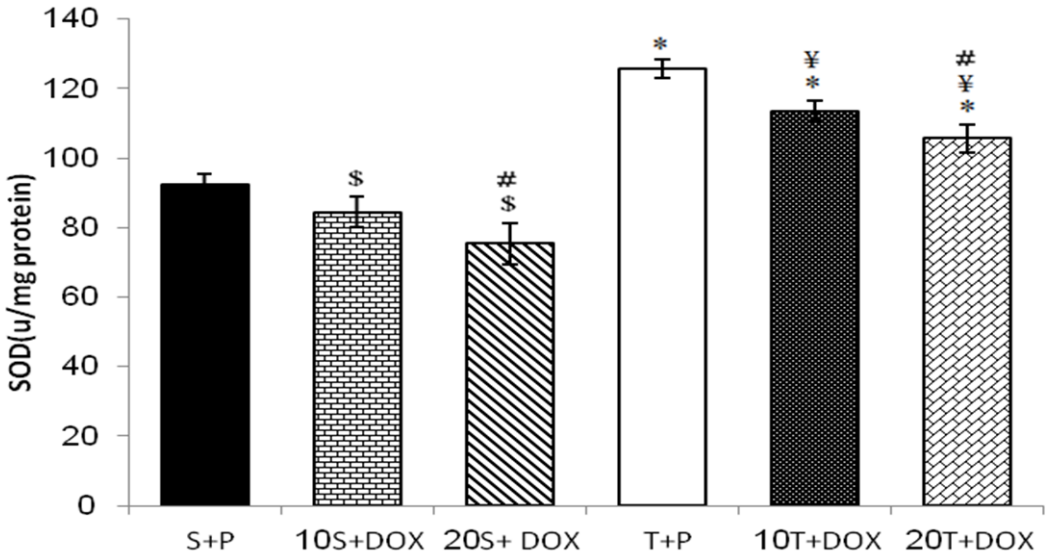


Figure 4. Superoxide dismutase (SOD) level after six weeks of aerobic training and DOX treatment. Abbreviations; S + P (sedentary + placebo), S + DOX_{10} (sedentary + doxorubicin 10 mg kg^{-1}), S + DOX_{20} (sedentary + doxorubicin 20 mg kg^{-1}), T + P (training + placebo), T + DOX_{10} (training + doxorubicin 10 mg kg^{-1}), T + DOX_{20} (training + doxorubicin 20 mg kg^{-1}). Data are presented as the mean ± SD for 8 Rats. *Significantly different to similar sedentary group ($P < 0.05$), $ significantly different to the S + P group ($P < 0.05$), # significantly different to dose 10 mg kg^{-1}in it's group ($P < 0.05$), ¥ significantly different to the T + P group ($P < 0.05$).

exercise on Doxorubicin-induced oxidative stress in rats. The primary finding in the present study was that after doxorubicin administration (10 and 20 mg kg^{-1}), a significant increase in MDA, increase in apelin, a significant decrease in SOD and an insignificant increase in NO were detected, as compared to sedentary + placebo (S + P) group.

In the past years, several mechanisms have been suggested to explain the pathogenesis of DOX-induced cardiotoxicity. Chatterjee reported that the mechanisms of therapeutic effects of doxorubicin on tumor cells are different from those of the mechanisms of its cardiotoxicity (Chatterjee et al., 2010). Oxidative stress, ion dysregulation, and alterations of the cardiac specific gene expression program cooperate at inducing cardiotoxicity and oxidative stress (Evert et al., 2001; Raschi et al., 2010; Vishwanatha et al., 2012; Viswanatha et al., 2011). Moreover, decreased levels of antioxidants and sulfhydryl groups, inhibition of nucleic acid and protein synthesis, release of vasoactive amines, altered adrenergic function and decreased expression of cardiac-specific genes are other proposed mechanisms (Chatterjee et al., 2010). However, the proposed principal mechanisms of doxorubicin cardiotoxicity are increased oxidative stress, as evident from increased levels of reactive oxygen species and lipid peroxidation (Chatterjee et al., 2010; Chen et al., 2007).

Raschi et al. (2010) reported that the high reactivity of ROS against cell constituents and the lack of effect of antioxidants against chronic oxidative stress suggest a role of ROS confined to acute cardiac dysfunction. Recent findings also suggest that cellular stress activates a host of kinase pathways that appear important in determining cell fate, and these pathways modulate the response of the heart to DOX exposure (Raschi et al., 2010). Although DOX can readily generate oxygen radicals in several ways, only few free radical scavengers have been reported to protect the heart from doxorubicin induced toxicity. Its mechanism of action appears to be the prevention of free radical formation by doxorubicin, probably through binding of iron. Furthermore, it has been reported that free radical scavengers such as vitamin E and N-acetylcysteine decrease both lethality and severity of cardiac histological lesions in rodents injected with doxorubicin. In contrast, other studies have shown that vitamin E and N-acetylcysteine failed to attenuate doxorubicin-induced cardiotoxicity in rats and dogs. Possible explanations could be the different experimental setups, timing of measurement, etc. Also, the reserve in antioxidant defense plays an important role. However, from the point of view of the free radical hypothesis, the study of Arai et al is noteworthy because this study showed how the formation of hydrogen peroxide and the down-regulation of the sarcoplasmic reticulum calcium pump are connected; explaining the pathway whereby the two could be related (Arai et al., 2000).

In this study, we found out that six weeks of aerobic training leads to a significant increase in apelin and SOD, an insignificant decrease in MDA, as compared to sedentary + placebo (S + P) group. While, treatment with $DOX_{10\ mg\ kg^{-1}}$ after six weeks of aerobic training caused a significant increase in apelin and SOD, an insignificant increase in NO levels and also, a significant decrease in MDA levels, as compared to sedentary + doxorubicin 10 mg kg^{-1} (S + DOX_{10}) group, treatment with $DOX_{20\ mg\ kg^{-1}}$ resulted in a significant increase in apelin, SOD and NO level and also, a significant decrease in MDA level, as compared to sedentary + doxorubicin 20 mg kg^{-1} (S + DOX_{20}). Moreover, a significant difference was detected between $DOX_{10\ mg\ kg^{-1}}$ and $DOX_{20\ mg\ kg^{-1}}$ treatments in apelin, NO, SOD and MDA levels. These data suggest that the increased oxidative stress production by DOX could be blocked by the pretreatment with aerobic regular exercise, with improve antioxidants and dysfunction markers. Data of the current study provided additional support to understand how regular physical exercise, particularly treadmill running training, could contribute to augmentation of cardiac muscle resistance against oxidative stress-based cardiotoxicity induced by DOX administration. Two lines of evidence can be emphasized from the present study. First, and considering cardiac stress marker, namely MDA, aerobic regular training decreased the rise of cardiac disturbances induced by acute single doses of DOX administration, particularly, in dose of 10 mg kg^{-1}. Second, and according to changes observed in cardiac apelin and SOD responses in both sedentary and trained rats hearts treated with DOX, it is likely that these markers might be considered as essential cellular defense against free radical-based cardiotoxicity caused by DOX, providing enhanced tolerance to trained myocardium at least in the first 48 h after the end of training period.

Physical exercise in its various forms has been shown to be an effective intervention that can provide a protective effect against acute and chronic deleterious insults for the myocardium. Moreover, although current data demonstrate that exercise training protects the heart against Dox-induced damage (Kavazis et al., 2010; Chicco et al., 2006), the mechanism(s) by which exercise training protects cardiotoxicity remain unclear. There were three possible pathways to explain the protective effects of regular endurance exercise against DOX-induced cardiotoxicity. At present, the principal mechanism of Dox-induced cardiotoxicity is believed to be increased oxidant production by the mitochondria (Kavazis et al., 2010; Chicco et al., 2006; Ascensão et al., 2005; Chicco et al., 2005). Mitochondria are also the major sites for the production of ROS. Exercise seems to increase the oxygen consumption rate by 10 to 20 folds as reported earlier, and might have released the above factors and thereby induced heart-SOD activity (Husain, 2002). In addition, researchers reported among other cell sources, heart mitochondria electron transport chain,

which has been referred to as one of the major sites of ROS production, through the so-called electron leakage. Whereas, the activity of mammalian cytochrome C oxidase is O_2-saturated at very low O_2 tensions, the rate of electron leakage by mitochondria increases at high O_2 concentrations during certain conditions such as exercise, favoring enhanced ROS production.

Nevertheless, when moderate and systematic, exercise could constitute an excellent tool either to prevent and/or to treat several diseases, providing enhanced parallel resistance to the cardiac muscle tissue. This phenomenon usually referred as cross-tolerance has been demonstrated by several studies in which endurance training seems to up-regulate heart antioxidant systems and mitochondrial function, to reduce the formation of lipid peroxidation by-products and to induce antioxidant defenses such as SOD and heat shock protein after certain stress stimuli (Ascensão et al., 2005). There is now considerable number of studies that suggests daily exercise antagonizes the harmful consequences of *in vivo* and *vitro* DOX treatment on rodent heart, either by preventing, attenuating or reverting the toxicity (Ascensão et al., 2005, 2006, 2012; Teixeira et al., 2011; Kavazis et al., 2010). Considering that DOX-induced cardiac toxicity has a marked oxidative etiology and that chronic exercise ameliorates the cardiac capacity of antioxidant systems to counteract with deleterious ROS effects, it can be suggested that the cardioprotection induced by aerobic exercise training against DOX was attributed at least in some extent to the up-regulation of antioxidant enzymes (Ascensão et al., 2005). Our results also support the concept that oxidant/antioxidant imbalance could be the primary event in DOX-induced cardiotoxicity (Ascensão et al., 2006). The authors reported that aerobic exercise results in many types of physiological (vascular, metabolic and functional) adaptations in the heart. Indeed, several studies have proposed that the increased cardiac function induced by regular exercise can be attributed to the enhanced cell defenses against oxidant production in the re-establishment of redox status.

According to the fact that DOX-induced cardiotoxicity is in part, due to increased free radical generation and hence, to oxidative stress, it is possible that the cardiac redox adaptations induced by aerobic training can contribute to the previously referred tolerance of myocardium to DOX. This up-regulation of glutathione system was expected to be accompanied by an increased activity of antioxidant enzymes. In our study, aerobic regular training induced significant increases in the apelin, NO and SOD levels. Although a significant increase in cardiac glutathione reductase (GR) and glutathione peroxidase (GPx) had been reported elsewhere after endurance swimming training (Childs et al.,2002), other studies demonstrated that swimming training was as such ineffective in raising these and other gene-modulated antioxidant enzymes like SOD and CAT (Ascensão et al., 2006). Some studies reported that exercise related to little but significant increases in myocardial SOD activity may be critical for protection against myocardial injury. The biological rationale for this adaptive response is unclear, but could be associated with the fact that regular exercise induced increases in myocardium ability to eliminate hydrogen peroxide and other organic peroxides resulting from DOX toxicity (Ascensão et al., 2006). In fact, the significant increased activity of SOD in DOX exposed hearts by aerobic regular training (training + placebo group versus training + doxorubicin 10 mg kg^{-1} group and training + doxorubicin 20 mg kg^{-1} group) may be possibly attributed to DOX-induced activation of enzyme activity through protein synthesis, indicating that the adaptations induced by aerobic regular training can be important for increasing the activity of this antioxidant enzyme in the presence of DOX.

Nitric oxide (NO) is a short lived free radical, synthesized from arginine, with extremely high reactivity and a variety of physiological activities. NO cause damage to DNA and is a potential endogenous carcinogen, and its increased production may increase angiogenesis and contribute to tumor progression (In Duk et al., 2002). It has been reported that doxorubicin increases superoxide formation by increasing endothelial nitric oxide synthase, which promotes intracellular hydrogen peroxide formation and recent evidence supports the significant role of nitric oxide synthase (NOS) (Chatterjee et al., 2010; Andreadou et al., 2007). Although, the basal production of NO through constitutive NOS isoforms in cardiomyocytes modulates ventricular contractility and blood flow distribution, higher NO production through iNOS is associated with severe cardiac lesions such as dilated cardiomyopathy and congestive heart failure (In Duk et al., 2002; Andreadou et al., 2007). It has been reported taht high concentrations of NO participate in cardiomyocyte oxidative damage, apoptosis, and/or necrosis through peroxynitrite formation (Evert et al., 2001). Furthermore, overproduction of NO is cytotoxic, and suppresses tumor growth, whereas low NO production may protect cells from apoptosis and promote tumor growth. However, the effects of NO on tumor cells are apparently production dependent, and cell type specific (In Duk et al., 2002). It has also been reported that NO contributes to DOX's antitumor effect (Liu et al., 2008). NO may influence several aspects of tumor biology including cell growth, apoptosis, differentiation, metastatic capability and tumor induced immune suppression (Kavazis et al., 2010).

More recent data support that nitric oxide is involved in DOX-induced toxicity in cardiac (Ascensão et al., 2005) as well as cerebral (Gruber et al., 2010) tissues. Doxorubicin promotes the synthesis of NO and reactive oxygen species, such as the superoxide anion. The reaction of NO and superoxide anion leads to the synthesis of peroxynitrite which is a potent cellular

oxidant (Evert et al., 2001). The effects of DOX on NOS are well established, and show that an increased expression of NOS is associated with DOX-induced apoptosis. However, the effects of DOX on the production of NO by iNOS from studies on rats are not well identified (In Duk et al., 2002).

In summary, our study suggests that the cardiotoxicity induced by DOX may be attributed to oxidant/peroxidant imbalance in heart. Moreover, in this study, we found that cardioprotection induced by aerobic regular exercise in hearts from DOX treated rats was associated with inhibition of oxidative stress and the up-regulation of antioxidant enzymes. Therefore, our study suggests that aerobic regular exercise before administration of DOX may be considered as a potentially useful candidate to limit cardiotoxicity during and after DOX therapy.

REFERENCES

Abdel-Moneim M, Osman, Marwa MN, Amany AAB, Omimah AN, Mohamed TK (2009). Effect of methimazole treatment on doxorubicin-induced cardiotoxicity in mice. Food Chem. Toxicol. 47:2425–2430.

Andreadou I, Sigala F, Iliodromitis EK, Papaefthimiou M, Sigalas C, Aligiannis N, Savvari P, Gorgoulis V, Papalabros E, Kremastinos DT (2007). Acute doxorubicin cardiotoxicity is successfully treated with the phytochemical oleuropein through suppression of oxidative and nitrosative stress. J.Mol.Cell Cardiol. 42:549–558.

Arai M, Yoguchi A, Takizawa T, Yokoyama T, Kanda T, Kurabayashi M, Nagai R (2000). Mechanism of doxorubicin-induced inhibition of sarcoplasmic reticulum Ca2q-ATPase gene transcription. Circ. Res. 86: 8–14.

Ascensão A, Magalhães J, Soares J, Ferreira R, Neuparth M, Marques F, Oliveira J, Duarte J (2005). Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. Int. J. Cardiol. 100:451-460.

Ascensão A, Magalhães J, Soares J, Ferreira R, Neuparth M, Marques F, Duarte J (2006). Endurance exercise training attenuates morphological signs of cardiac muscle damage induced by doxorubicin in male mice. Basic Appl. Myol. 16 (1):27-35.

Ascensão A, Oliveira, Paulo J., Magalhães J (2012). Exercise as a beneficial adjunct therapy during Doxorubicin treatment—Role of mitochondria in cardioprotection. Int. J. Cardiol.156:4-10.

Barbaric M, Brooks E, Moore L, Cheifetz O (2010). Effects of physical activity on cancer survival: a systematic review. Physiother .Can. 62:25–34.

Cecen E, Dost T, Culhacı N, Karul, A. Ergur B, Birincioglu M (2011). Protective Effects of Silymarin against Doxorubicin-induced Toxicity. Asian Pacific. J. Cancer. Prev, 12:2697-2704.

Chatterjee K, Zhang J, Honbo N, Joel SK (2010). Doxorubicin Cardiomyopathy. Cardiology. 115:155–162

Chen B, Peng X, Pentassuglia L, Lim CC, Sawyer DB (2007). Molecular and cellular mechanisms of anthracyclinecardiotoxicity. Cardiovasci. Toxicol. 7:114-121.

Chicco AJ, Schneider CM, Hayward R (2006). Exercise training attenuates acute doxorubicin-induced cardiac dysfunction. J. Cardiovasc. Pharmacol. 47:182– 189.

Chicco AJ, Schneider CM, Hayward R (2005). Voluntary exercise protects against acute doxorubicin cardiotoxicity in the isolated perfused rat heart. Am. J. Physiol.Regul. Integr. Comp. Physiol. 289: R424-R43.

Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C (2002). Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl- 2: Bax ratio. Cancer .Res. 16:4592-4598.

Roshan DV, Assali M, Hajizadeh Moghaddam Ak, Hosseinzadeh M, Myers J (2011). Exercise Training and Antioxidants: Effects on Rat Heart Tissue Exposed to Lead Acetate. Int. J. Toxicol. 30(2):190-196.

Daniel S, Limson JL, Dairam A, Watkins GM, Daya S (2004). Through mental binding, curcumin protects against lead- and cadmium-induced lipid peroxidation in rat brain homogenates and against lead-induced tissue damage in rat brain. J. Inorg. Biochem. 98:266-275.

Duparc T, Colom A, Cani PD, Massaly N, Rastrelli S, Drougard A, Le Gonidec S, Moulédous L, Frances B, Leclercq I, Llorens-Cortes C, Pospisilik JA, Delzenne NM, Valet P, Castan-Laurell I, Knauf C (2011). Central apelin controls glucose homeostasis via a nitric oxide-dependent pathway in mice. Antioxid Redox Signal 15: 15(6):1477-1496.

Evert L. De Beer, Antonio E. Bottone, Emile EV (2001). Doxorubicin and mechanical performance of cardiac trabeculae after acute and chronic treatment: a review. Eur J. Pharmacol. 415Ž:1–11

Gruber BM, Krzyszton-Russjan J, Bubko I, Anuszewska EL (2010). Possible role of HSP60 in synergistic action of anthracyclines and sulindac in HeLacells. Acta. Pol. Pharm. 6:620–624.

Hitesh S, Gaurav P, Praful P, Aviseka AC, Mukul J, Anita AM (2011). Beneficial effects of carbon monoxide-releasing molecule-2 (CORM-2) on acute doxorubicin cardiotoxicity in mice: Role of oxidative stress and apoptosis. Toxicol. Appl. Pharmacol. 253:70–80.

Husain K (2002). Exercise conditioning attenuates the hypertensive effects of nitric oxide synthase inhibitor in rat. Mol. Cell Biochem. 231:129–137.

In Duk J, Jang-Soon L, Seong YY, Chang GP, Jeung WH, Hyang W L, Hoi YL (2002). Doxorubicin Inhibits the Production of Nitric Oxide by Colorectal Cancer Cells. Arch. Pharm. Res. 25(5):691-696.

Jia YX, Lu ZF, Zhang J, Pan CS, Yang JH, Zhao J, Yu F, Duan XH, Tang CS, Qi YF (2007). Apelin activates L-arginine/nitric oxide synthase/nitric oxide pathway in rat aortas. Peptides 28(10):2023-2029.

Karen Y. Wonders, David SH, Stephanie G, Carole MS, Reid H (2009). Endurance exercise training preserves cardiac function in rats receiving doxorubicin and the HER-2 inhibitor GW2974. Cancer. Chemother. Pharmacol. 64:1105–1113.

Kavazis AN, Smuder AJ, Min K, Tumer N, Powers SK (2010). Short-term exercise training protects against doxorubicin-induced cardiac mitochondrial damage independent of HSP72. Am. J. Physiol. Heart Circ. Physiol. 5:H1515-1524.

Khositseth A, Jirasakpisarn S, Pakakasama S, Choubtuym L, Wattanasirichaigoon D (2011). Carnitine levels and cardiac functions in children with solid malignancies receiving doxorubicin therapy. Indian. J. Med. Paediatr. Oncol. 32:38-43.

Liu TJ, Yeh YC, Ting CT, Lee WL, Wang LC, Lee HW, Wang KY, Lai HC (2008). Ginkgo biloba extract 761 reduces doxorubicin-induced apoptotic damage in rat hearts and neonatal cardiomyocytes. Cardiovasc. Res. 2:227-235.

Minghua Li, Zhi-Gang X (2011). Ion channels as targets for cancer therapy. Int. J. Physiol. Pathophysiol. Pharmacol. 3(2):156-166.

Raschi E, Vasina V, Ursino MG, Boriani G, Martoni A, De Ponti F (2010). Anticancer drugs and cardiotoxicity: Insights and perspectives in the era of targeted therapy. Pharmacol. Ther. 125:196–218.

Teixeira de Lemos E, Pinto R, Oliveira J, Garrido P, Sereno J, Mascarenhas-Melo F, Páscoa-Pinheiro J, Teixeira F, Reis F (2011). Differential effects of acute (extenuating) and chronic (training) exercise on inflammation and oxidative stress status in an animal model of type 2 diabetes mellitus. Mediators Inflamm p.253061.

Vishwanatha SA, GulliayaS, Thippeswamy A, Basavaraj CK, Donnahalli VM (2012). Cardioprotective effect of curcumin against doxorubicin-induced myocardial toxicity in albino rats. Indian. J. Pharmacol. 44(1):73–77.

Viswanatha Swamy AHM, Wangikar UBC, Koti AHM, Thippe swamy PM, Ronad Manjula D V(2011).Cardioprotective effect of ascorbic acid on doxorubicin-induced myocardial toxicity in rats. Indian. J. Pharmacol. 43(5):507–511.

American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

Conferences and Advert

October 2012
American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

December 2012
International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

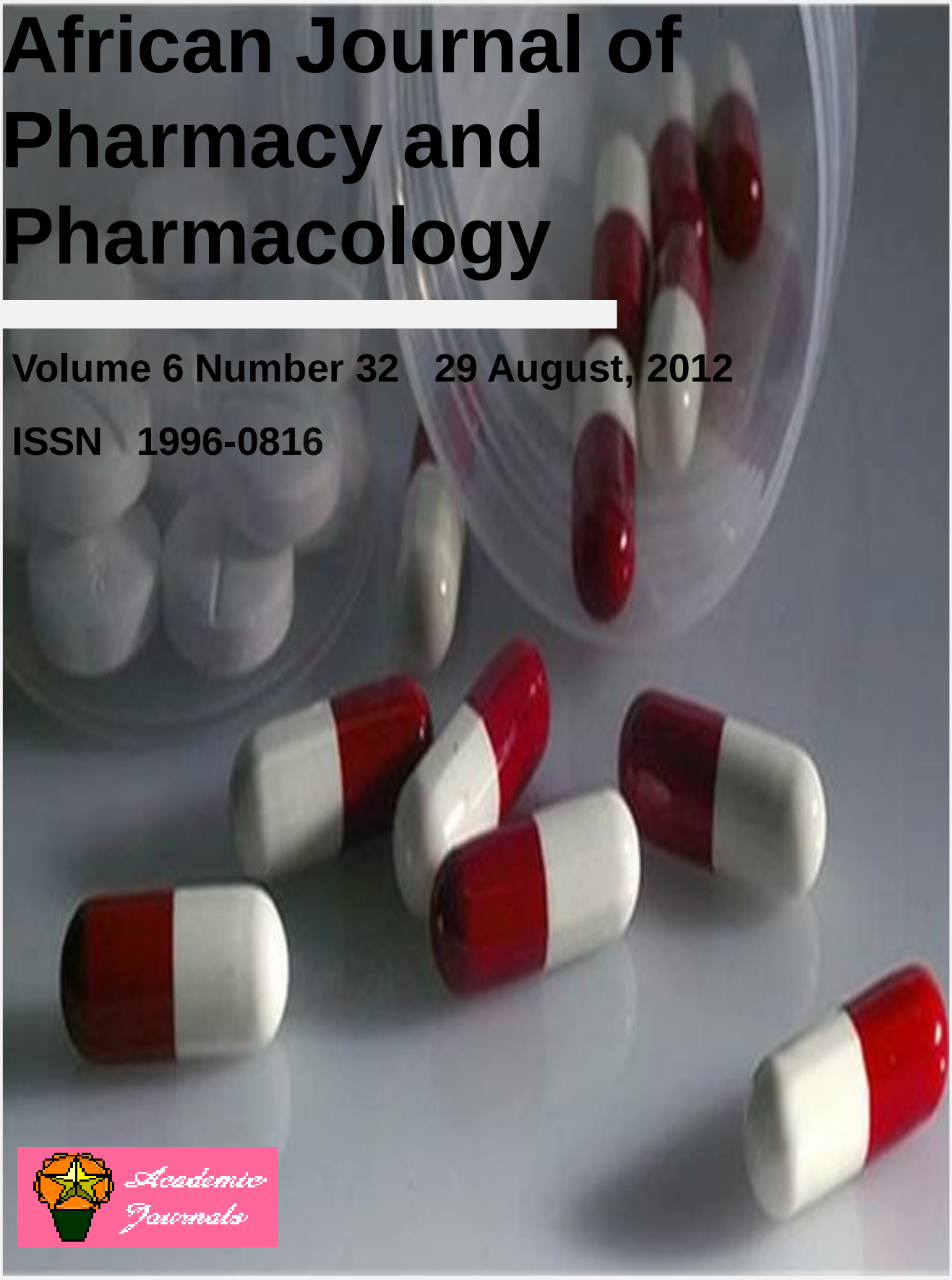
- *Journal of Medicinal Plant Research*
- *Journal of Dentistry and Oral Hygiene*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Veterinary Medicine and Animal Health*

academicJournals

African Journal of Pharmacy and Pharmacology

Volume 6 Number 32 29 August, 2012

ISSN 1996-0816



Academic Journals

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: ajpp@acadjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The African Journal of Pharmacy and Pharmacology will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Sharmilah Pamela Seetulsingh- Goorah
Associate Professor,
Department of Health Sciences
Faculty of Science,
University of Mauritius,
Reduit,
Mauritius

Himanshu Gupta
University of Colorado- Anschutz Medical Campus,
Department of Pharmaceutical Sciences, School of Pharmacy Aurora, CO 80045,
USA

Dr. Shreesh Kumar Ojha
Molecular Cardiovascular Research Program
College of Medicine
Arizona Health Sciences Center
University of Arizona
Tucson 85719, Arizona,
USA

Dr.Victor Valenti Engracia
University of São Paulo.
Av Dr. Arnaldo, 455
Cerqueira Cesar
01246-903 - Sao Paulo, SP - Brazil

Prof. Sutiak Vaclav
Rovníková 7, 040 20 Košice,
The Slovak Republic,
The Central Europe,
European Union
Slovak Republic
Slovakia

Dr.B.RAVISHANKAR
Director and Professor of Experimental Medicine
SDM Centre for Ayurveda and Allied Sciences,
SDM College of Ayurveda Campus,
Kuthpady, Udupi- 574118
Karnataka (INDIA)

Dr. Manal Moustafa Zaki
Department of Veterinary Hygiene and Management
Faculty of Veterinary Medicine, Cairo University
Giza, 11221 Egypt

Prof. George G. Nomikos
Scientific Medical Director
Clinical Science
Neuroscience
TAKEDA GLOBAL RESEARCH & DEVELOPMENT CENTER, INC. 675 North Field Drive Lake Forest, IL 60045
USA

Prof. Mahmoud Mohamed El-Mas
Department of Pharmacology,
Faculty of Pharmacy
University of Alexandria,
Alexandria,
Egypt.

Dr. Caroline Wagner
Universidade Federal do Pampa
Avenida Pedro Anunciação, s/n
Vila Batista, Caçapava do Sul, RS - Brazil

Editorial Board

Prof. Fen Jicai
School of life science, Xinjiang University,
China.

Dr. Ana Laura Nicoletti Carvalho
Av. Dr. Arnaldo, 455, São Paulo, SP.
Brazil.

Dr. Ming-hui Zhao
Professor of Medicine
Director of Renal Division, Department of Medicine
Peking University First Hospital
Beijing 100034
PR. China.

Prof. Ji Junjun
Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences,
China.

Prof. Yan Zhang
Faculty of Engineering and Applied Science,
Memorial University of Newfoundland,
Canada.

Dr. Naoufel Madani
Medical Intensive Care Unit
University hospital Ibn Sina, Univesity Mohamed V Souissi, Rabat,
Morocco.

Dr. Dong Hui
Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University,
China.

Prof. Ma Hui
School of Medicine, Lanzhou University,
China.

Prof. Gu HuiJun
School of Medicine, Taizhou university,
China.

Dr. Chan Kim Wei
Research Officer
Laboratory of Molecular Biomedicine,
Institute of Bioscience, Universiti Putra,
Malaysia.

Dr. Fen Cun
Professor, Department of Pharmacology, Xinjiang University,
China.

Dr. Sirajunnisa Razack
Department of Chemical Engineering, Annamalai University,
Annamalai Nagar, Tamilnadu,
India.

Prof. Ehab S. EL Desoky
Professor of pharmacology, Faculty of Medicine
Assiut University, Assiut,
Egypt.

Dr. Yakisich, J. Sebastian
Assistant Professor, Department of Clinical Neuroscience
R54
Karolinska University Hospital, Huddinge
141 86 Stockholm ,
Sweden.

Prof. Dr. Andrei N. Tchernitchin
Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA
University of Chile Medical School,
Chile.

Dr. Sirajunnisa Razack
Department of Chemical Engineering,
Annamalai University, Annamalai Nagar, Tamilnadu,
India.

Dr. Yasar Tatar
Marmara Unıversıty,
Turkey.

Dr Nafisa Hassan Ali
Assistant Professor, Dow institude of medical technology
Dow University of Health Sciences,Chand bbi Road, Karachi,
Pakistan.

Dr. Krishnan Namboori P. K.
Computational Chemistry Group, Computational Engineering and Networking,
Amrita Vishwa Vidyapeetham, Amritanagar, Coimbatore-641 112
India.

Prof. Osman Ghani
University of Sargodha,
Pakistan.

Dr. Liu Xiaoji
School of Medicine, Shihezi University,
China.

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJPP to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b; Tristan, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $600 handling fee. Publication of an article in the African Journal of Pharmacy and Pharmacology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

Copyright: © 2012, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJPP, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication.
While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 6 Number 32 29 August, 2012

ARTICLES

Research Articles

Table of Contents: Volume 6 Number 32 29 August, 2012

ARTICLES

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2389-2392, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.892
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

The preliminary study of the mechanism and efficacy of non-steroidal anti-inflammatory drug treatment on nerve root type cervical spondylosis

Haibin Lin*, Xuanhuang Chen, Guodong Zhang and Xianwei Wu

Department of Orthopedics, Affiliated Hospital of Putian University, Putian 351100, Fujian Province, PR China.

Accepted 9 March, 2012

This study aims to evaluate the cyclooxygenase-2 (COX-2) protein expression in spinal cord dorsal horn of rats with nerve root type cervical spondylosis (NRCS) and the clinical efficacy of different non-steroidal anti-inflammatory drugs (NSAIDs) treatment on Chinese patients with NRCS. 24 Sprague-Dawley (SD) rats were randomly divided into normal health group and NRCS model group, The COX-2 protein expression in spinal cord dorsal horn of rats was detected by immunohistochemical staining after administration. 52 NRCS patients were divided into celecoxib treatment group and diclofenac treatment group to compare the efficacy of different NSAIDs. The efficacy of NSAIDs on NRCS was assessed by visual analog scale (VAS) test. Compared with normal health group, the expressions of COX-2 protein in spinal cord dorsal horn increased significantly than in the NRCS model group. According to the results of VAS test, we found out that celecoxib is more effective than diclofenac sodium. Clinical drug therapy for NRCS can give priority to specific COX-2 inhibitors. Over expression of COX-2 might be a potential pathological mechanism of NRCS.

Key words: Nerve root type cervical spondylosis, non-steroidal anti-inflammatory drugs, clinical observation, cyclooxygenase-2.

INTRODUCTION

Nerve root type cervical spondylosis (NRCS) is usually caused by stimulation and oppression of the cervical nerve root due to retrograde degeneration of the small intervertebral joints and Luschkas joints (uncinate vertebral joints). It is a common disease in clinic. NRCS patients may have symptoms of cervical stiffness, rest rained movement, arm pain and numbness of the fingers which may affect patients' normal work and daily life (Allan and Binder, 2007). In recent years, conservative treatment for NRCS has been adopted by modern medicine for most patients, while surgical treatment has also been used for a small number of patients who were conservatively treated with unsatisfactory effect or lingered by obstinate disease with operation indications. It was reported that extradural injection of non-steroidal anti-inflammatory drugs (NSAIDs) is a conservative treatment to relieve inflammation and edema of the nerve root. However, the therapic mechanism of the NSAIDs and pathological mechanism of NRCS are still unclear.

In the present work, we detected the cyclooxygenase-2 (COX-2) protein expression in spinal cord dorsal horn of NRCS rats by immunohistochemical staining. Moreover, we also compared the efficacy of celecoxib and diclofenac on 52 NRCS patients.

MATERIALS AND METHODS

The general kit of immunohistochemistry (ICH) and monoclonal antibody of COX-2 were obtained from SANTA CRUZER Biotechnology Company in USA.

Laboratory animals

Twenty-four Sprague-Dawley (SD) rats, aged>16 months, weighing

*Corresponding authors. E-mail: linhb3511@126.com.

(225 ± 25) g, of either gender, were provided by the Experimental Animal Center of the Putian University between July 2010 and November 2011. The rats were housed under controlled conditions (room temperature, 22 ± 2°C).

Establishment of NRCS rats model

The rats in model group were anesthetized by injecting intraperitoneally 40 mg/kg of pentobarbital sodium. Dorsal neck was shaved and a longitudinal incision about 1.5 cm was followed. The dorsal muscles were separated and reserved. Spinal processes, inter spinal ligaments, part of superior and inferior articular processes between C6 and 7 levels were cut off. When the movement degree between the neighboring superior and inferior laminae was increased obviously after operation, the incision was then closed. The successful models were confirmed by evaluating X-ray films and the motion function with oblique board test 3, 4 and 5 mo, respectively, after the operation. For rats in the normal health group, they were closed after opening without further operation.

Detection of COX-2 protein expression

Rats were anaesthetized with 10% of urethane and then sacrificed by transcardiac perfusion with phosphate-buffered saline (PBS) followed by separating their lumbar spinal cord immediately, cryoprotected by immersion in 30% sucrose for 24 h at 4 to 8℃ and frozen in a tissue-freezing medium. The brains were cut on a freezing microtome, into six adjacent series of 30-µm-thick coronal sections. The paraffin section was general deparaffinated to water, and the section was placed in 10% of hydrogen dioxide at room temperature for 5 to 10 min to blockade effects of endogenous peroxidase. Then, PBS was used to washout the section for 3 times and one time was for 2 min. The pamcreatin fluid was added on the section and incubated at 37°C for 20 min. Then, the PBS was used to washout for 3 times.

The blood serum that was found in the section was incubated for 15 min at ambient temperature. The blood serum was poured and added 50 µl of antibody of COX-2, then stored at 4°C overnight. The PBS was used to washout the section for 3 times and general antibody of 2 was added for 15 min at ambient temperature. The PBS was used to washout the section and dropped wise the fluid labeled by horse-radish enzyme for 15 min at room temperature. Then, the PBS was used to washout the section for three times. Dimethylaminoazobenzene (DAB) fluid was added on the section and control colouration under the microscope. The distilled water was used to poach, and the campeachy was used to stain and mount.

Patients

56 NRCS patients were taken as the objects of study. Among 56 patients who volunteered to participate in the intervention treatment, there were 24 men and 32 women with a proportion of 1: 1.33. The age ranks and average age was (52 to 71) and (61.80 ± 4.20), respectively. 56 NRCS patients were averagely randomized into Celecoxib group and Diclofenac Sodium group. Their height, weight, gender, age and average course of disease and X-ray staging among groups are comparable.

Drug intervention

Random, open and self control test were adopted in drug intervention treatment, in which 26 patients in celecoxib group took celecoxib tablet at the dosage of 100 mg, 1#bid , course of treatment: 3 weeks. 30 patients in diclofenac sodium group took diclofenac sodium tablets at the dosage of 50 mg, 1#tid, course of treatment: 3 months. Visual analog scale (VAS) records were made before and after administration. Patients with severe adverse reactions discontinued the administration.

Evaluation of efficacy

VAS (Majani et al., 2003) scoring was used for evaluation of the therapeutic effects before and after the therapy. Among the score, 0 to 10 (0 was no pain, 10 was acute pain), the value indicated the painful intensity and mental assault degree. Less than 3 scores indicated good, 3 to 4 basically satisfied and ≥ 5 poor. The VAS scores of the patients were evaluated before and after the therapy.

Statistical analysis

The database was set up with the Statistical Package for Social Sciences (SPSS) 16.0 software package for analysis. Data were represented as mean ± standard deviation (SD). The means of different groups were compared with student's *t*-test. $P<0.05$ was considered as statistically significant.

RESULTS

ICH results

Compared with normal health control group, the COX-2 protein expression increased significantly in the NRCS group ($t=7.0716$, $P<0.001$) (Table 1 and Figure 1). These results suggested that increased expressions of COX-2 protein might play an important role in the mechanism of NRCS generation.

VAS score results

After three months treatment of the 56 cases, the therapeutic results are as shown in Table 2. Compared with the VAS scores before administration, the VAS scores after administration decreased significantly in each group. Meanwhile, the VAS scores after administration in the celecoxib treatment group were less than that in the diclofenac treatment group ($t=-14.0602$, $P<0.0001$).

DISCUSSION

In rat cerebral cortex, a selective expression of COX-2 was shown. COX-2 immunoreactivity was found especially in a subpopulation of excitatory neurones in allocortices, hippocampus and amygdala. Immunoreactivity was compartmentalised to dendritic arborisations. Moreover, COX-2 protein is present in dendritic spines, which are specialized structures in synaptic signaling. The developmental profile of COX-2 expression coincided with the critical period for activity-

Table 1. The comparison of COX-2 positive cells between NRCS model group and Normal health control group.

Group	n	COX-2 positive cells
NRCS model	12	0.2604 ± 0.0353
Normal health control	12	0.1728 ± 0.0244

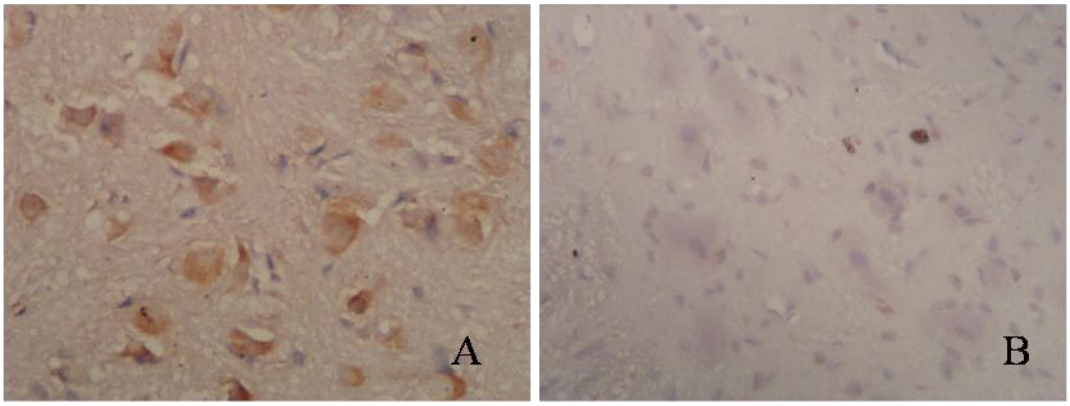


Figure 1. COX-2 positive cells of NRCS model group and normal health control group. A: COX-2 immunoreactivity in NRCS model rat; B: COX-2 immunoreactivity in normal health control rat.

Table 2. The comparison of VAS score between celecoxib treatment group and diclofenac treatment group.

Group	Cases	VAS (before administration)	VAS (after administration)	*t* value	P value
Celecoxib	26	8.6 ± 1.2	1.4 ± 0.3	29.6807	<0.01
Diclofenac	26	8.4 ± 1.3	3.5 ± 0.7	16.9221	<0.01

dependent synaptic remodeling. Results of Kaufmann et al. (1996) indicated that COX-2 and its diffusible prostanoid products, may play a role in postsynaptic signaling of excitatory neurones in cortex and associated structures. In contrast to COX-1, COX-2 is admittedly constitutively expressed in brain and spinal cord, but at the same time highly regulated by different influencing factors, like ischaemia, immunomodulators and cytokines, structural brain damages, toxic agents and during maturational processes (Hoffmann et al., 2000; Kuslich et al., 1991). In our present work, we found out that the COX-2 protein expression was higher in the NRCS rats than normal healthy rats. The results indicated that COX-2 might play an important role in the process of NRCS.

NSAIDs inhibit cyclooxygenase (COX) activity, thereby suppressing the synthesis of proinflammatory prostaglandins. The identification and molecular-biological characterization of an inducible COX isoform (COX-2) in inflammatory cells lead to the hypothesis that a selective inhibition of COX-2 would result in relief of inflammation and pain without causing the COX-1-dependent side effects (gastrointestinal ulceration, platelet dysfunction and kidney damage) of conventional NSAIDs (Bensen et al., 2000; Goldstein et al., 2001; Slivcrstein et al., 2000).

NRCS can be treated by selected NSAIDs in the light of the overall analysis of symptoms and signs. The therapeutic effects have been shown to be satisfactory. However, since the samples size is small, it is difficult to exactly identify the therapeutic effect of the NSAIDs treatment. Therefore, it is highly necessary that strict clinical observation indexes and a universally used standard for the therapeutic effect should be worked out in the future.

Conclusion

In the present work, animal results showed that COX-2 protein expression was higher in the NRCS rats than normal healthy rats. The over expression of COX-2 protein might be a potential mechanism of NRCS generation. Clinical results with the selective COX-2

inhibitors show a better safety profile than non-selective COX inhibitors. Clinical use after drug registration will be decided on further role of this new class of drugs in NRCS therapy and on new fields of clinical use.

REFERENCES

Allan I, Binder (2007). Cervical spondyloeis and neck pain. BMJ 334(10): 527-531.

Bensen WG, Zhao SZ, Burke TA, Zabinski RA, Makuch RW, Maurath CJ, Agrawal NM and Geis GS (2000). Upper gastrointestinal tolerability of celecoxib, a COX-2 specific inhibitor, compared to naproxen and placebo. J. Rheumatol. 27(8):1876-1883.

Goldstein JL, Correa P, Zhao WW, Burr AM, Hubbard RC, Verburg KM, Geis GS (2001). Reduced incidence of gastroduodenal ulcers with celecoxib, a novel cyclooxygenase-2 inhibitor, compared to naproxen in patients with arthritis, Am. J. Gastroenterol. 96(4):1019-1027.

Hoffmann C. (2000). COX-2 in brain and spinal cord implications for therapeutic use, Curr. Med. Chem. 7(11):1113-1120.

Kaufmann WE, Worley PF, Pegg J, Bremer M, Isakson P (1996). COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex, Proc. Natl. Acad. Sci. 93(6):2317-2321.

Kuslich SD, Ulstrom CL, Michael CJ (1991). The tissue origin of low back pain and sciatica: a report of pain response to tissue stimulation during operations on the lumbar spine using local anesthesia, Orthop. Clin. North. Am. 22(2):181-187.

Majani G, Tiengo M, Giardini A, Calori G, De Micheli P, Battaglia A (2003). Relationship between MPQ and VAS in 962 patients: a rationale for their use, Minerva. Anestesiol. 69:67-73.

Slivcrstein FE, Faith G, Cioldstein JI, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM, Zhao WW, Kent JD, Lefkowith JB, Verburg KM, Geis GS (2000). Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study, A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. JAMA 284(10):1247-1255.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2393-2399, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.028
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

A meta-analysis of the efficacy and safety of cilnidipine in Chinese patients with mild to moderate essential hypertension

Xu Guo-liang, Xu Hui, Wu Hai-di and Qin Ling*

Department of Cardiology, First Bethune Hospital, Jilin University. 71 xinmin Avenue, Changchun 130021, Jilin Province, P.R. China.

Accepted 10 August, 2012

The purpose of this study was to evaluate the efficacy and safety of cilnidipine tablets to treat Chinese patients with mild to moderate essential hypertension, and to examine the ability of cilnidipine to lower blood pressure without eliciting unfavorable side effects. Medical databases and review articles were screened for randomized controlled trials (RCTs) that reported the effects of adverse reactions to cilnidipine and amlodipine in treating Chinese patients with mild to moderate essential hypertension. The quality of the included studies was critically evaluated. A total of 547 articles were found, from which 11 articles met the inclusion criteria. The heterogeneity test, the efficacy analysis (Q statistic = 4.62, p = 0.91, I^2 = 0%) and safety analysis (Q statistic = 3.73, p = 0.93, I^2 = 0%) showed that cilnidipine was equally effective and safe compared to amlodipine. The funnel-plot displayed a symmetrical figure, indicating there was no publication bias, and all articles included described high quality trials. In conclusion, cilnidipine is a useful agent to treat mild to moderate essential hypertension.

Key words: Cilnidipine, hypertension, review, meta-analysis.

INTRODUCTION

Hypertension is one of the most common cardiovascular diseases, and prevalence of hypertension continues to increase in China. Each year, it is estimated that an additional 10 million patients will be diagnosed with hypertension, and the current total number of patients nationwide with hypertension surpasses 200 million. Studies completed by the National Health and Nutritional Examination Survey and the World Health Organization estimate that fewer than 30% of hypertensive patients worldwide are adequately controlled and achieve an acceptable lowering of their blood pressure (Papadopoulos and Papademetriou, 2009). The awareness rate, treatment rate, and control rate for the Chinese population are only 30.2, 24.8 and 6.1%, respectively (Liu et al., 2010; Law et al., 2009).

Pharmaceutical intervention is the best way to control hypertensive outpatients in China (Pei-Xi et al., 2012). There are many kinds of antihypertensive drugs nowadays (Du et al., 2012), but calcium antagonist is the most widely used one. Calcium antagonists dilate blood vessels to reduce peripheral vascular resistance to reduce blood pressure. Cilnidipine is a new dihydropyridine calcium antagonist with both L- and N-type calcium channel blocking effects, and has recently been included in the list of first-line antihypertensive agents by the Chinese Guideline for the Prevention and Treatment of Patients with Hypertension in 2009. The antihypertensive effects of cilnidipine are significant, and main features include good oral absorption and a long time of action. After oral administration, drug concentrations peak at 1.8 to 2.2 h and show a long half-life of 7.5 h. Importantly, cilnidipine inhibits sympathetic activation to effectively prevent the reflex tachycardia often reported in similar antihypertensive agents, particularly amlodipine. Cilnidipine, therefore, has the

*Corresponding author. E-mail: qinling1958@yahoo.com.-cn. Tel: +86 15843073203. Fax: +86 0431 84841049.

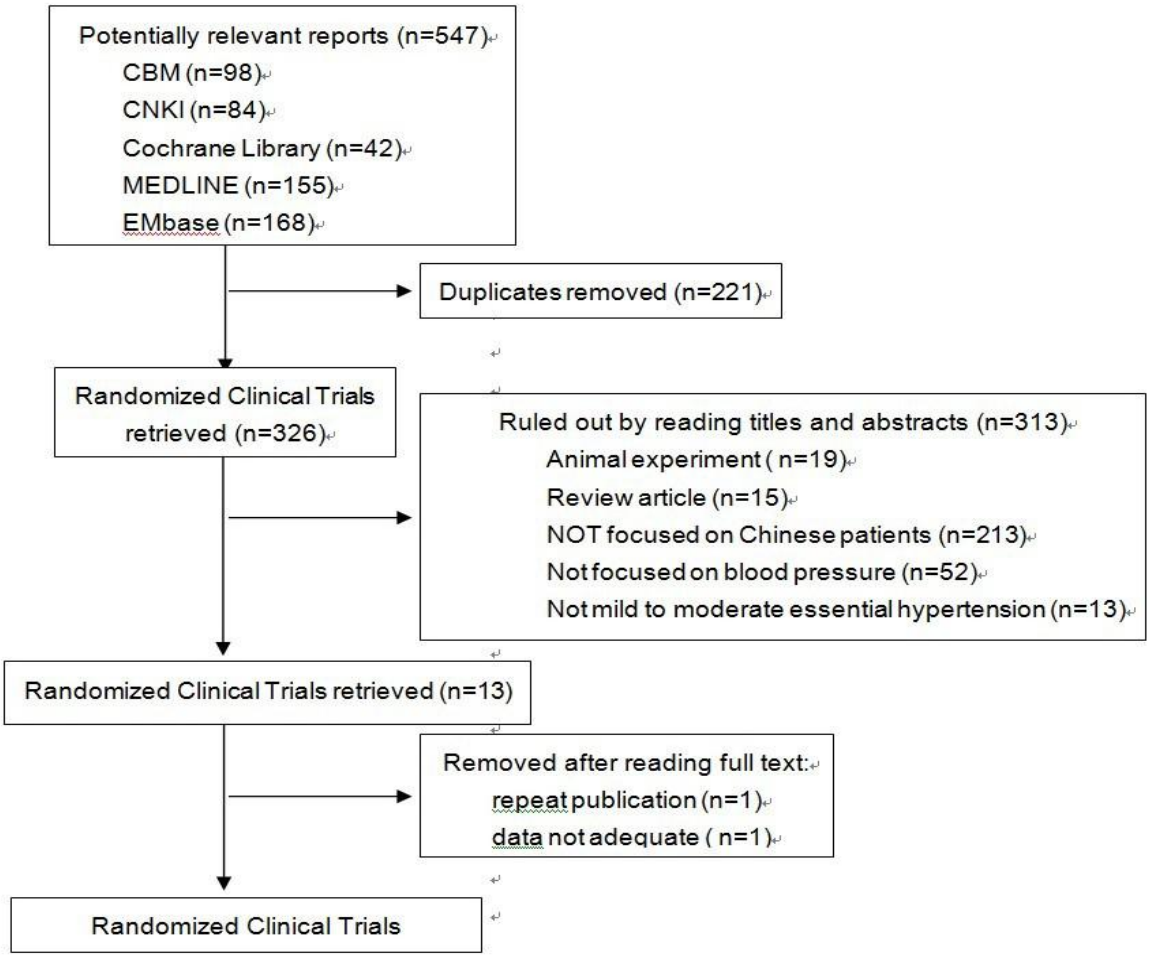


Figure 1. Decision flow chart for article inclusion.

potential to improve patient compliance (Zhang and Zhao, 2003). Cilnidipine tablets first went on the market in Japan in 1995 and were subsequently approved by other countries (for example, China in 2002) to become the primary antihypertensive drug used today.

Although, there have been several small clinical studies that report the use of cilnidipine tablets for the treatment of hypertension, each individual study lacks the power to make strong conclusions because of the individually small sample sizes. For this reason, the goal of the current study was to perform a meta-analysis on clinical randomized controlled trials (RCTs) that focused on using cilnidipine tablets to treat mild to moderate essential hypertension in Chinese patients, in order to better understand the efficacy and safety profiles of cilnidipine.

METHODOLOGY

Search strategy

The search strategy was devised according to the guideline 4.2.7 from the Cochrane collaboration (Sackett et al., 2002). We systematically searched the Cochrane central register of controlled trials (Issue 2, 2011), MEDLINE (1991 to June, 2011), EMbase (1991 to June, 2011), CBM (1991 to June, 2011), and CNKI (1979 to June, 2011) for RCTs that examined the efficacy and safety of using cilnidipine tablets to treat mild to moderate essential hypertension among Chinese people. In addition, we conducted a manual research of abstracts from selected references and searched manually the bibliographies of all relevant trials. The following search criteria were used: ("hypertension" or "essential hypertension") and ("cilnidipine"). The language was limited to peer-reviewed articles written in English or Chinese.

Study selection

Two reviewers independently conducted the literature searches and extracted the relevant articles. The flow chart for article selection is shown in Figure 1. The title and abstract of potentially relevant studies were screened for appropriateness before retrieval of the full articles.

The following selection criteria were used to identify published studies for inclusion in this meta-analysis: (a) the study design was a RCT; (b) the population was Chinese patients with mild to moderate essential hypertension (WHO-ISH Hypertension

Guidelines Committee, 1999 (WHO-ISH Hypertension Guidelines Committee, 1999); Committee of guidebook on prevention and treatment of hypertension, 2000); (c) the intervention was cilnidipine tablets compared to other active anti-hypertensive agents that were being used in a monotherapy strategy; (d) the outcome variables were the overall response rate and adverse reaction rate; (e) the efficacy criteria followed the Guiding Principles for Clinical Research of New Drugs developed by the Chinese Ministry of Health in 1993 (Liu et al., 1998).

Data extraction

From each study, the following information was extracted: author, year of publication, study design, characteristics of the population, sample size, treatment scheme, time of therapy, overall response rate and adverse reaction rate.

Assessment of study quality

The Jadad score was used to assess the quality of the trial methodology, and this assessment was independently performed by each of the two reviewers (Jadad et al., 1996). Articles given 1 to 2 points were regarded as low quality and articles given 3 to 5 points were regarded as high quality. The pre-determined criterion was to exclude articles whose study quality scored below 2 points mark.

Statistical methods

For dichotomous outcomes, we calculated a pooled odds ratio (OR) and 95% confidence interval (CI). The OR was defined as the odds of an outcome in those who received cilnidipine compared with the odds in those who received another active hypertensive agent. The ORs of different randomized clinical trials were combined by using the random-effects model of Der Simonian and Laird if between-study heterogeneity existed. The Mantel and Haenszel fixed-effects model was used if there was no between-study heterogeneity.

Intertribal statistical heterogeneity was explored using the Cochrane Q test with calculated I^2, indicating the percentage of the total variability in effect estimates among trials, due to heterogeneity rather than to chance. The I^2 values of 50% or more indicated a substantial level of heterogeneity. We evaluated the presence of publication bias by means of visual inspection of the funnel plot (whether it was symmetrical or not). To exclude the possibility that any one study was exerting excessive influence on the results, we conducted a sensitivity analysis by excluding those studies with low quality and then rerunning the analysis to assess the change in the ORs.

All p-values were two-sided and statistical significance was set at a level of 0.05. We followed the Quality of Reporting Meta-analysis Guidelines. All the statistical analysis was carried out by the Cochrane collaboration's RevMan 4.2 software (Moher et al., 1999).

RESULTS

Characteristics of the included trials

There were 547 articles relevant to the search terms, from which a total of 11 articles matched inclusion criteria. The most common reason for excluding an article were that, the patient population was not Chinese, the focus of the study was not hypertension, or the patients did not have mild to moderate hypertension. The 11 articles included 790 Chinese patients with mild to moderate essential hypertension (n = 396 on Cilnidipine and n = 394 controls), which were included in this meta-analysis (Zhang and Liu, 2003; Chen et al., 2003; Liang et al., 2003; Ma et al., 2004; Chen et al., 2004; Zhao et al., 2005; Jing et al., 2005; Huang et al., 2006; Huang et al., 2007; Zhao et al., 2008; Zhou, 2011). The antihypertensive agent used for the control group for all of the studies was amlodipine. The mean values for age, gender and initial blood pressure were similar between the two groups ($p > 0.05$). The characteristics of the included trials are shown in Table 1.

Heterogeneity test

We chose the fixed-effect model to perform our meta-analysis because there were no significant heterogeneities among the studies, in both the efficacy analysis (Q statistic =4.62, $p = 1.00$, $I^2 = 0\%$) and the safety analysis (Q statistic =3.73, $p = 0.93$, $I^2 = 0\%$).

Meta-analysis of efficacy

There was 396 of 456 persons in cilnidipine group that is efficacious, the overall response rates were 86.8% (with an average blood pressure lowering of 21 mmHg) and there was 394 of 453 persons in control group that is efficacious, the overall response rates were 87.0% (with an average blood pressure lowering of 21 mmHg). 95% CI [0.68,1.48]. From the meta-analysis, there were no significant differences in efficacy between cilnidipine and amlodipine in treating Chinese patients with mild to moderate essential hypertension (Figure 2).

Meta-analysis of safety

Adverse reaction rates for clnidipine tablets and the amlodipine control group were recorded in all 11 trials. The major adverse reactions for cilnidipine included headache (3.29%), dizziness (4.61%), and facial flushing (5.04%). The major adverse reactions of the control amlodipine group were headache (3.10%), dizziness (6.65%), cough (0.66%) and gastrointestinal symptoms (5.76%). The results of meta-analysis showed that there were no significant differences in safety between cilnidipine and the control amlodipine group in treating Chinese patients with mild to moderate essential hypertension (Figure 3).

Publication bias

An analysis of publication bias was conducted. The

Table 1. Characteristics of the included studies.

Study	Group	Treatment proposal (mg/d)	Times of theraphy (Weeks)	Sample size	Overall response rate (%)	Adverse reaction rate (%)	SBP baseline (mmHg)	SBP after medicine end (mmHg)	DBP baseline (mmHg)	DBP after medicine end (mmHg)	Jadad score
Zhang (2003)	Cilnidipine	5	8	22	86.4	0	149 ± 11	127 ± 13	101 ± 3	84 ± 7	4
	Amlodipine	5	8	24	95.8	0	150 ± 13	118 ± 11	101 ± 4	82 ± 7	
Chen (2003)	Cilnidipine	5	8	109	79.2	16.5	151 ± 13	127 ± 13	99 ± 4	85 ± 9	4
	Amlodipine	5	8	110	83.5	18.2	148 ± 13	130 ± 13	99 ± 4	84 ± 8	
Li (2003)	Cilnidipine	5	8	23	77.3	52.4	165 ± 10	140 ± 18	101 ± 4	86 ± 16	4
	Amlodipine	5	8	20	77.8	40.9	163 ± 12	130 ± 20	102 ± 5	86 ± 13	
Ma (2004)	Cilnidipine	5	8	24	83.3	16.7	151 ± 11	126 ± 8	99 ± 2	86 ± 6	4
	Amlodipine	5	8	24	75.0	12.5	147 ± 11	134 ± 12	100 ± 2	89 ± 6	
Chen (2004)	Cilnidipine	5	8	27	85.2	11.1	144 ± 10	126 ± 7	98 ± 4	86 ± 5	4
	Cilnidipine	5	8	27	88.9	14.8	146 ± 13	126 ± 11	98 ± 3	84 ± 7	
Zhao (2005)	Amlodipine	5	8	19	84.2	0	146 ± 13	126 ± 11	98 ± 3	84 ± 7	4
	Cilnidipine	5	8	21	90.5	9.5	144 ± 10	126 ± 7	98 ± 4	86 ± 5	
Jing (2005)	Amlodipine	5	8	32	90.6	3.1	150 ± 12	128 ± 11	99 ± 3	83 ± 6	4
	Cilnidipine	5	8	32	90.6	0	150 ± 13	133 ± 14	100 ± 5	87 ± 8	
Huang (2006)	Amlodipine	5	8	25	88.0	4.0	146 ± 10	128 ± 8	99 ± 4	86 ± 7	4
	Cilnidipine	5	8	25	88.0	8.0	146 ± 12	127 ± 10	97 ± 3	82 ± 6	
Huang (2007)	Amlodipine	5	8	117	92.5	9.7	148 ± 10	125 ± 11	99 ± 3	77 ± 9	4
	Cilnidipine	5	8	117	87.2	8.6	148 ± 9	127 ± 12	99 ± 3	76 ± 7	
Zhao (2008)	Amlodipine	5	8	24	91.3	21.7	151 ± 10	140 ± 9	97 ± 2	86 ± 4	4
	Cilnidipine	5	8	24	90.5	23.8	150 ± 11	134 ± 7	98 ± 2	84 ± 5	
Zhou (2011)	Amlodipine	5	8	49	93.9	12.2	151 ± 11	132 ± 12	98 ± 4	85 ± 8	3
	Cilnidipine	5	8	49	91.8	16.3	148 ± 3	127 ± 10	98 ± 3	82 ± 5	

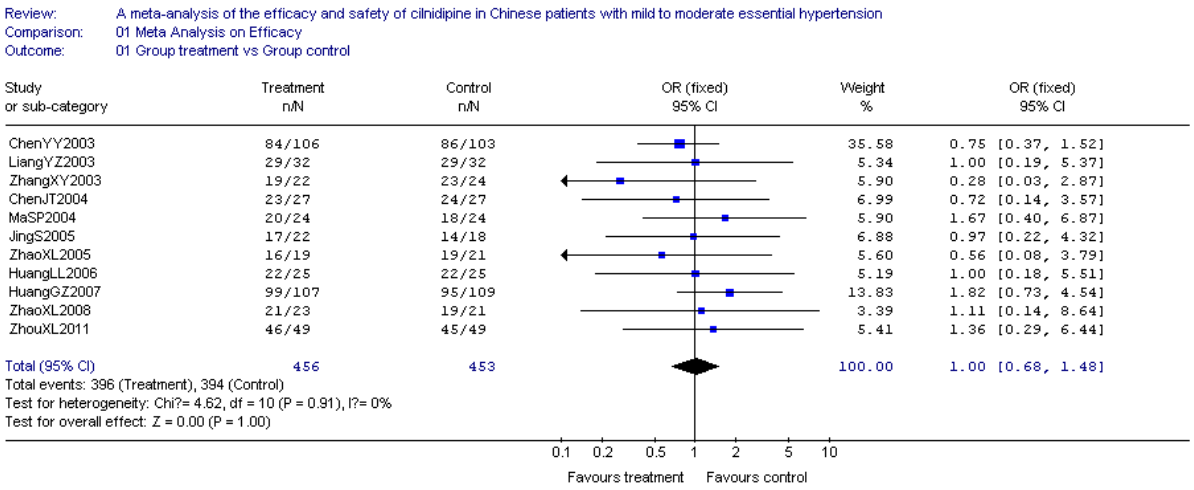


Figure 2. The OR estimates, with the corresponding 95% CI for efficacy. The OR estimates for each study is denoted by a box. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond. Note that this line might be contained within the diamond, if the CI is narrow.

funnel plots were symmetrical based on visual analysis, indicating that there was no evidence of publication bias (Figure 4).

Sensitivity analyses

In the efficacy analysis, there was no difference in the overall response rates between cilnidipine and the control amlodipine group [Z = 0.00 (p = 1.00), OR =1.00, 95% CI (0.68, 1.48)]. Further, no difference was found in the adverse reaction rates between cilnidipine and the control amlodipine group in the safety analysis [Z = 0.26 (p = 0.80), OR = 0.95, 95% CI (0.64, 1.41)].

Summary of the literature quality

In an analysis of the articles, we found that all trials that were included in the meta-analysis were of high quality. The Jadad score was at least 2 points for each of the 11 articles. Moreover, there was no evidence of publication bias found and there were no significant heterogeneities between studies in both the efficacy analysis and the safety analysis. Combined, this suggests that the overall quality of the systematic review was high.

There were, however, still a few methodological insufficiencies that should be mentioned. These included: (a) the randomization method for the individual trials may not be rigorous because the specific randomization schemes were inadequately described in all except one article; (b) a selection bias may exist, as the allocation concealment was not described in any of the articles; (c) a measuring bias and implementation bias may exist because one study did not describe whether the trial was a double blind design.

DISCUSSION

Hypertension is a leading cause of cardiovascular morbidity and mortality. In particular, hypertension is a leading cause of congestive heart failure due to the increased pressure overload placed on the myocardium. Cilnidipine is a calcium channel blocker that is widely prescribed in China for the treatment of hypertension. The goal of this study was to use a meta-analysis to examine the efficacy and safety of cilnidipine compared to amlodipine.

The significant finding of this study was that, cilnidipine was equally effective as amlodipine in lowering blood pressure. In addition, cilnidipine shared a similar safety profile with amlodipine. These results indicate that cilnidipine is an effective antihypertensive agent to treat mild to moderate essential hypertension.

Summary of quality of included studies

A total of 11 studies were included in this systematic review, of which all were RCTs. Combined, the 11 studies yielded a total sample size of 790. The Jadad scores of the 11 articles were at least 2 points and the overall quality of this meta-analysis was high.

Meta-analysis of efficacy and safety

In general, calcium channel blockers have a very reliable and stable antihypertensive effect and do not affect

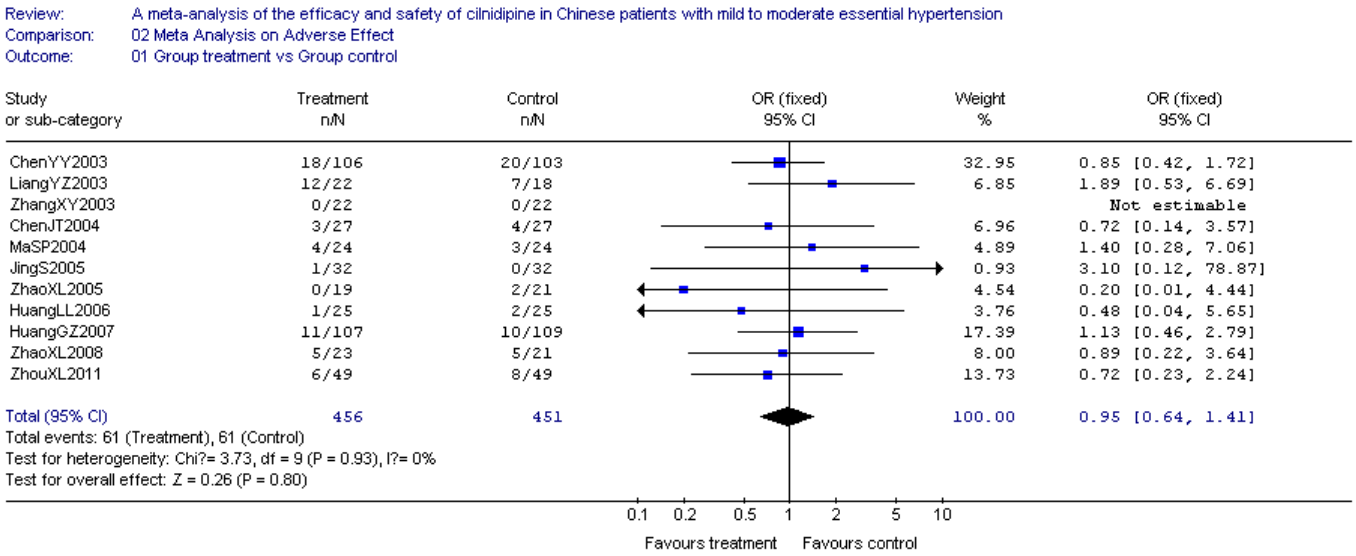


Figure 3. OR estimates, with the corresponding 95% CI for safety. The OR estimate of each study is marked with a box. The size of the square represents the weight that the corresponding study exerts in the meta-analysis. The CIs of pooled estimates are displayed as a horizontal line through the diamond. Note that this line might be contained within the diamond, if the CI is narrow.

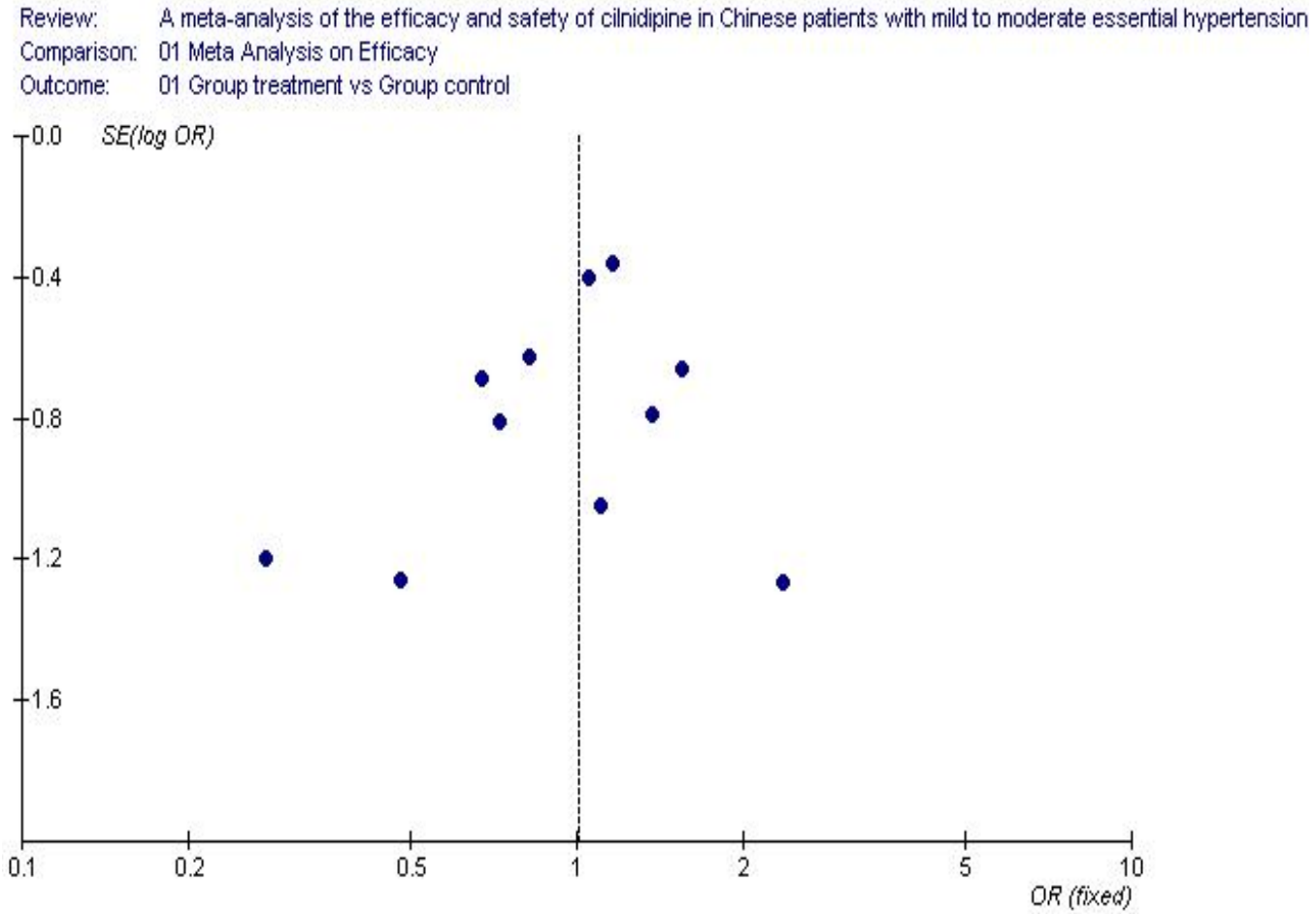


Figure 4. Funnel plot to examine publication bias. Based on the symmetrical shape, there was no publication bias.

glucose and lipid metabolism. As such, calcium channel blockers are useful drugs to control blood pressure and reduce the complications of cardiovascular disease (Wang et al., 2010). In addition to blocking the L-type Ca^{++} channel, cilnidipine has been shown to inhibit the N-type Ca^{++} channel current in sympathetic neurons (Fujil et al., 1997; Hosono et al., 1995). Cilnidipine reduces arterial blood pressure and lowers total peripheral resistance, but does not affect heart rate, cardiac index or cardiovascular structure (Jasmina et al., 2002).

There were several limitations of this study. This study included a meta-analysis of 11 trials, in which the test group had similar treatment doses and times, and all trials used amlodipine as the comparison control. For most of the articles, however, the randomization method was not well-described, which may affect the strength of the meta-analysis. In order to obtain more rigorous and objective clinical evidence, this study should be followed up with a prospective clinical trial with more randomization methods, including a blinding allocation scheme and longer-term follow-up.

Although amlodipine has already been shown to be efficacious, cilnidipine may be selected over amlodipine for the treatment of hypertension. In particular, cilnidipine may be a suitable alternative for patients who experience cough or gastrointestinal symptoms when given amlodipine.

The results of this systematic review revealed that there were no significant differences in efficacy in treating Chinese patients with mild to moderate essential hypertension between cilnidipine and the control amlodipine group.

We can conclude, therefore, that cilnidipine has the same antihypertensive effects compared with a first-line antihypertensive drug.

REFERENCES

Chen JT, Sun NL, Chen YY (2004). The comparition of efficacy and safety on treatment of essential hypertension between cilnidipine and amlodipine. Clin. Med. China. 20:197-199.

Chen YY, Sun NL, Zhao XL (2003). The efficacy and safety study of domestic cilnidipine treatment of mild to moderate hypertension. Chin. J. Clin. Pharmacol. 19:334-337.

Committee of guidebook on prevention and treatment of hypertension (2000). Guidebook on prevention and treatment of hypertension. Chinese J. Hypertens. 8:103-112.

Du B, Cui Wen-peng, Xu Guo-liang (2012). A meta-analysis of the efficacy and safety of arotinolol in the treatment of Chinese patients with essential hypertension. Afr. J. Pharm. Pharmacol. 6:36-42.

Fujil S, Kameyama K, Husone M (1997). Effect of cilnidipine, a novel dihydropyridine Ca^{++} channel in rat dorsal root ganglion neurons . J. Pharmacol. Exp. T. Her. 280:1184-1191.

Hosono M, Fujii S, Hiruma T (1995). Inhibitory effect of cilnidipine on vascular sympathetic neurotransmission and subsequent vasoconstriction in spontaneously hypertensive rats. Jpn J. Pharmacol. 69:127-134.

Huang GZ, Wu ZG, LU GP (2007). Domestic cilnidipine treatment of mild to moderate essential hypertension. Chin. J. Hypertens. 15:124-127.

Huang LL, Li GY, Bai Y (2006). Cilnidipine treatment of mild to moderate essential hypertension in 25 cases. Herald of Med. 25:919-920.

Jadad AR, Moore A, Carroll D (1996). Assessing the quality of reports of randomized clinical trials: is blinding necessary? Control Clin. Trials. 17:1-12.

Jasmina J, Dinko S, Edward F (2002). Cilnidipine improves spontaneously hypertensive rat coronary hemodynamics without alterring cardiovascular mass and collagen. J. Hypertens. 20:317-322.

Jing Shan, Sun NL, Wang HP (2005). The efficacy and safety of Domestic cilnidipine treatment of mild to moderate hypertension. Chin. J. New Drugs. 14:473-475.

Law MR, Morris JK, Wald HJ (2009). Use of blood pressure lowering drugs in the prevention of cardiovascular disease: meta-analysis of 147 randomised trials in the context of expectations from prospective epidemiological studies. BMJ. 338:1665.

Liang YZ, Wang L, Huang TG (2003). The clinical observation of cilnidipine treatment of mild to moderate hypertension. Tianjin Med. J. 31:365-367.

Liu GZ, Hu DY, Tao P (1998). Recommendations on evaluation of chinical trials of cardiovascular drugs. Chin. J. Cardiol. 26:7-9.

Liu LS, Wang W, Yao CH (2010). Hypertension prevention guide (2009 grass-roots version). Chin J Hypertens. 18:11-30.

Ma SP,Guo XM, Li CaiRu (2004). The efficacy and safety of cilnidipine treatment of mild to moderate hypertension. Chin. J. New Drugs Clin. Rem. 23:873-875.

Moher D, Cook DJ, Eastwood S (1999). Improving the quality of reports of meta-analyses of randomised controlled trials: the QUOROM statement. Quality of Reporting of Meta-analyses. Lancet. 354:1896-1900.

Papadopoulos DP, Papademetriou V (2009). Low-dose fixed combination of bisoprolol/ hydrochlorothiazide as first line for hypertension: a review of the rationale and clinical evidence. Angiol. 60:601-607.

Pei-Xi Zhao, Chao Wang, Li Qin (2012). Effect of clinical pharmacist's pharmaceutical care intervention to control hypertensive outpatients in China. Afr. J. Pharm. Pharmacol. 6:48-56.

Sackett DL, Clarke M, Oxman AD (2002). Cochrane Reviewers, Handbook 4.2, In Renew Manager.Versions 4.2.Oxford, England: The cochrane collaboration, pp.13-36.

Wang JY, Miu EY, Hang CX (2010). Internal Medicine. Beijing : People's Medical Publishing House, pp.266-267.

WHO-ISH Hypertension Guidelines Committee (1999). 1999 World Health Organization- International Society of Hypertension Guidelines for the Management of Hypertension. J. Hypertens. 17:15l-183.

Zhang H, Zhao XL (2003). New calcium channel blocker-cilnidipine. Evaluation and analysis of drug use in hospitals of China 3:126-128 .

Zhang XY, Liu GS (2003). The comparition of efficacy and safety on treatment of essential hypertension between domestic cilnidipine and Amlodipine. Med. J. Chin. PLA. 28:851.

Zhao XL, Zhang H, Hu DY (2005). The efficacy and safety of cilnidipine treatment of mild to moderate hypertension. Chin. J. Clin. Pharmacol. 21:8-10.

Zhao XL, Zhou YM, Li Jie (2008). The efficacy and safety of cilnidipine treatment of mild to moderate hypertension. Chinese Journal of New Drugs 17:157-159.

Zhou XL (2011). The efficient observation of cilnidipine treatment of mild to moderate hypertension. Chin. J. Clin. Rational Drug Use 4:49-50.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2400-2407, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.204
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Sliencing of secretory clusterin blocks tumor growth and metastasis of hepatocellular carcinoma HCCLM3 cells

Bing LU[1#], [2]Fuchun Wang[2#], Xinhui LI[3], Zimin LIU[2], Bo MIN[3] and Jun LIANG[3]*

[1]Hepatobiliary and pancreatic surgery, Henan Province People's Hospital, Zhengzhou, Henan, 450003. R.P. China.
[2]Department of Surgery, the second hospital of Cao County, Heze, Shangdong, 266245. R.P. China.
[3]Department of Surgery, The Affiliated Hospital of medical college, QingDao University, QingDao, Shan Dong, 266003. R.P. China.

Accepted 8 June, 2012

The secretory clusterin (sClu) protein is highly expressed in many types of cancer, including human hepatocellular carcinoma (HCC), and is involved in tumor progression. The current study has investigated whether sClu silencing affects tumor cell behavior in HCC cells. HCCLM3 cells were transfected with an antisense oligonucleotide (ASO) against clusterin (OGX-011). Apoptosis was determined by TUNEL staining and flow cytometric (FCM) assay. Cell survival was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The matrigel invasion assay was used for invasion assays. Xenografting was used to demonstrate HCCLM3 growth and metastasis. OGX-011 specifically attenuated the expression of sClu. Inhibition of sClu strongly induced significant reduction in cellular growth and higher rates of spontaneous endogenous apoptosis. Invasion assays indicated that OGX-011 significantly inhibits HCCLM3 cell migration and invasion *in vitro*. HCCLM3 xenografts treated with OGX-011 showed growth delay beyond 14 days. Moreover, OGX-011 treatment led to a significant decrease in lung metastasis by HCCLM3 cells. Thus, OGX-011, in potentially silence *CLU* gene expression, may prove to be of value during HCC therapy.

Key words: Hepatocellular carcinoma, clusterin, antisense oligonucleotide, gene treatment.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality (Blaschuk et al., 1983). It is typically aggressive and intrinsically resistant to standard chemotherapeutic agents, underscoring the need for developing more effective therapies for HCC patients (Bi et al., 2010; Cochrane et al., 2007).

Clusterin (Clu), also known as apolipoprotein J (ApoJ), sulfated glycoprotein 2 (SGP-2), testosterone-repressed prostate message 2 (TRPM-2), glycoprotein III, secreted protein 40,40 (SP-40,40) and complement lysis inhibitor (CLI), is a heterodimeric glycoprotein present in most animal tissues and body fluids (Chi et al., 2005; Chuan et al., 2012). Clu is a pro- or anti-apoptotic in different cell types or systems (Chuan et al., 2012). The discovery of Clu isoforms with distinct functions can explain some of the apparent controversies. Although Clu is encoded by a single gene, different isoforms exist as a result of alternative splicing and post-translational modifications

*Corresponding author. E-mail: qyfyliang@163.com. Tel: +86 532 82910257.

[#]These authors contributed equally to this work.

(Gleave and Miyake, 2005). One major isoform is the 76 to 80 kD secretory Clu (sClu) that appears in Western analysis as 2 protein bands, that is, a ~60 kD full-length uncleaved glycosylated cytoplasmic precursor (cClu) and a ~40 kD band consisting of Clu α-chain and β-chain from cleavage of the mature sClu (Garden et al., 1991). Another major isoform is the 50 to 55 kD cytoplasmic/nuclear nClu, which is translated from a different AUG codon, does not undergo α/βcleavage, and is not extensively glycosylated (Gleave and Chi, 2005). sClu is pro-survival and plays a role in conferring chemoresistance in cancer cells (Hazzaa et al., 2010), while nClu is a pro-death factor whose over expression inhibits cell growth and survival (Harborth et al., 2001). Accumulating evidence indicates sClu upregulation occurs in various human malignancies, including HCC (Humphreys et al., 1999), cervical neoplasia (Hayashi and Di Bisceglie, 2006), gastric cancer (Hasan et al., 2003), ovarian cancer (July et al., 2004), bladder cancer (Jones and Jomary, 2002), pancreatic cancer (Kang et al., 2004), prostate cancer (Leskov et al., 2003) and breast cancer (Lokamani et al., 2011). Furthermore, it plays a significant role in the tumorigenesis and metastasis of several human cancers (Hasan et al., 2003; Lau et al., 2006).

sClu is not a traditional druggable target and can only be targeted at the mRNA level. An antisense inhibitor targeting the translation initiation site of human exon II Clu (OGX-011) was developed at the University of British Columbia and out-licensed to OncoGeneX Pharmaceuticals Inc. OGX-011 is a second-generation antisense oligonucleotide (ASO) with a long tissue half-life of ~7 days, which potently suppresses sClu levels *in vitro* and *in vivo*. OGX-011 improves the efficacy of chemotherapy, radiation, and hormone withdrawal by inhibiting expression of sClu and enhancing apoptotic rates in preclinical xenograft models of prostate, lung, renal cell, breast, and other cancers (Li et al., 2004; Liangpu et al., 2011; Miyake et al., 2005). To date, >300 patients have been treated with custirsen in 6 phase I and II clinical trials. The first-in-human phase I study with custirsen used a novel neoadjuvant design to identify effective dosing of custirsen to inhibit sCLU expression in human cancer (Miyake et al., 2002).

In the present study, we have investigated the effect of OGX-011 on the sClu protein form in the rich-sClu-expressed HCCLM3 cell line by OGX-011, and provide evidence to show that sClu silencing induces growth retardation and suppresses invasion and metastasis *in vivo* and *in vitro*.

MATERIALS AND METHODS

Tumor cell lines

Human HCC cell lines with high metastatic capacity (HCCLM3 and MHCC97-H) were kindly gifted from Prof Liu (Liver Cancer Research Center, Zhong Shan Hospital, Shanghai, China), and low metastatic capacity (SMCC7721 and HepG2) were from the ATCC cell bank. They were grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 µg/ml streptomycin. The cells were cultured at 37°C in 5% CO_2/95% air environment in humidified incubators.

Clusterin antisense oligonucleotide (OGX-011)

OGX-011 was purchased from OncoGenex Technologies. ASO sequences were manufactured by ISIS Pharmaceuticals (Carlsbad, CA) and supplied by OncoGenex Technologies (Vancouver, British Columbia, Canada). The antisense oligonucleotides were second-generation 21-mer antisense oligonucleotides with a 2′-O-(2-methoxy)ethyl modification. The ASO Clu sequence corresponding to the human Clu initiation site was 5′-CAGCAGCAGAGTCTTCATCAT-3′ and designated OGX-011 (OncoGenex Technologies).

Protein levels detected by Western blotting

Total protein levels of sClu in HCC cells were measured by Western blotting. About 30 µg protein was extracted, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride membranes, and reacted with primary rabbit antibodies against sClu (1:200; Bioworld Tech, Minneapolis, MN, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After being extensively washed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody for 30 min at room temperature. The bands were visualized using 1-step™ NBT/BCIP reagents (Thermo Fisher Scientific, Rockford, IL, USA) and detected by the Alpha Imager (Alpha Innotech, San Leandro, CA, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays

The antiproliferative effect of OGX-011 on HCCLM3 cells was measured by MTT assay as describe previously (Miyake et al., 2000). HCCLM3 cells were seeded at 2×10^4 cells per well in 96-well plates 1 day prior to addition of OGX-011. After addition, cells were incubated overnight with OGX-011 (5 to 50 µg/ml). CellTiter assay was used according to the manufacturer's instructions. At the end of the incubation, medium was aspirated and replaced with 100 µl of diluted MTT dye solution, and cells were incubated in a 5% CO_2/95% air atmosphere at 37°C for 4 h. Solubilization–stop solution was added to each well (100 µl) to stop the reaction, followed by incubation at 37°C for 1 h. The contents of wells were mixed by gentle pipetting prior to spectrophotometric analyses at 550 nm with a reference wavelength of 770 nm. MTT assays were performed in triplicate, and data-points were calculated as a percentage of viable cells over untreated control ± standard deviation (SD).

Flow cytometric (FCM) assay

CellTiter-Glo® luminescent cell viability assay (Promega, Madison, WI) was used to measure the proliferation of cells in response to different concentrations of OGX-011 as the method reported

previously (Miyake et al., 2001). Briefly, 2×10^3 HCCLM3 cells with rich-sClu expression were plated in the 96-well opaque-walled multi-well plates in culture medium. Control wells containing medium without cells were set up to obtain a value for background luminescence. After adherent culture, OGX-011 (final concentrations of 5 to 50 µg/ml) was added to each well and incubated for 48 h. Cells were collected and analyzed for the presence of apoptotic cells using Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA), following the manufacturer's instruction. FCM analysis used a FACS Calibur cytometer (R&D Systems, Minneapolis, MN), with replicate assays made on each sample.

TUNEL detection of apoptosis

To detect the effect of OGX-011 treatment *in vitro*, TUNEL was used involving the immunohistochemical detection of apoptosis with an Apoptag *in situ* Apoptosis Detection Kit (Chemicon International, Int. Beijing China), in accordance with manufacturer's recommendations. Briefly, equal numbers of cells were seeded in duplicates in 6-well plates, allowed to attach overnight on cover slips, and treated with 5 to 50 µg/ml for 48 h. Subsequently, cells were allowed to grow for an additional 24 h in drug-free medium before the TUNEL reaction. Positive apoptotic nuclei were recorded by microscopy.

To follow the effect of OGX-011 *in vivo*, 3 core tissue biopsies (4 mm in diameter) were obtained from each individual paraffin-embedded tissue sample using a trephine apparatus. Each tissue array block contained samples from all the animals. Sections (4 µm) were cut from each of the triplicate tissue array blocks, deparaffinized, and dehydrated. TUNEL staining was carried out as previously stated.

Tumor cell migration and invasion assay

Transwell chamber inserts (Corning Inc, Corning, NY) with filter membrane pore size 8 µm were coated with 80 µl Matrigel (0.8 mg/ml, BD Bioscience, Mountain View, CA). Tumor cell HCCLM3 at 3×10^5/ml serum-free DMEM were incubated with 50 µg/ml OGX-011 or vehicle at the upper chamber. DMEM containing 10% fetal bovine serum (FBS) was added to the lower compartment. 72 h later, cells that migrated through the permeable membrane were fixed in paraformaldehyde, stained with Giemsa, and counted under an inverted light microscope at 100× magnification, each assay being done at least in triplicate. Migration assay were applied similarly without coating the upper chamber with Matrigel, and the cells migrated through the membrane were counted at 48 h.

Effects of OGX-011 on HCCLM3 tumor growth and metastasis *in vivo*

Female athymic nude mice 4 to 6 weeks old (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai) were housed in laminar-flow cabinets under specific pathogen-free conditions. The mice were cared for and handled according to the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. The experimental protocol was approved by the Qingdao Medical Experimental Animal Care Committee.

For subcutaneous xenograft growth study, HCCLM3 cells (1×10^6) were inoculated s.c. with 0.1 ml Matrigel (Shenggong, Shanghai, China) in the flank region of the mice via a 27-gauge needle. When HCCLM3 tumors had reached 1,000 mm^3, usually 3 to 4 weeks after injection, mice were intraperitoneally injected with OGX-011 (n = 8) or BPS (n = 8) at 10 mg/kg once daily for 42 consecutive days. Tumor volumes were measured once weekly and calculated by the formula: tumor volume V (mm^3) = $width^2$ (mm^2) × length (mm)/2. Data points were expressed as average tumor volume levels ± SE. Mice tumors were excised on day 42. Western blot analysis was done on the total cell lysate to assess total Clu levels. Protein (30 µg) was separated by 10% PAGE, transferred to 0.45 µm Immobilon-P transfer membranes, and analyzed by Western blotting for Clu. To determine effect of treatment on apoptotic rates, TUNEL staining of the tissue microarray was scored as a percentage of the total number of cells by 2 pathologists.

For orthotopic xenograft growth and metastasis study, when HCCLM3 tumors reached 1,000 mm^3, they were removed, cut into 1 mm^3 pieces, and implanted into livers of another 16 mice to establish orthotopic xenograft models, as previously described (O'Bryan et al., 1993). Mice were intraperitoneally injected with OGX-011 (n = 8) or BPS (n = 8) at 10 mg/kg once daily for 21 consecutive days. On day 22, all mice were killed and the lungs fixed in Bouin's solution. Lung tumors were analyzed: first by obtaining a surface tumor count, and second, by assessing tumor area in histologic sections from multiple depths throughout the lungs. All animal procedures were done according to the guidelines of the Qingdao University on Animal Care and with appropriate institutional certification.

Statistical analysis

Results were expressed as the mean ± SE. Statistical analysis was done by t-test. $P \leq 0.05$ was considered significant.

RESULTS

Effect of OGX-011 treatment on sClu expression *in vitro*

The expression of sClu in high metastatic capacity (HCCLM3 and MHCC97-H) and low metastatic capacity (SMCC7721 and HepG2) cells by Western blot method was examined. The highest levels of sClu occurred in HCCLM3 and SMCC7721 cells; moderate expression in MHCC97-H cells, with no detectable sClu in HepG2 cells (Figure 1A).

HCCLM3, SMCC7721 and MHCC97-H cells were treated with 0, 5, 25, 50 and 100 µg/ml OGX-011 for 48 h before sClu protein was analyzed by Western blot. sClu was completely inhibited after treatment of OGX-011 at 50 and 100 µg/ml (Figure 1B). HCCLM3 cells treated with 5 to 50 µg/ml OGX-011 for 48 h were used for further investigation.

Inhibitory effects of OGX-011 on HCCLM3 proliferation *in vitro*

To explore on proliferation, HCCLM3 cells were treated with OGX-011 at from 0 to 50 µg/ml. OGX-011 dramatically decreased cell numbers of all tested cell

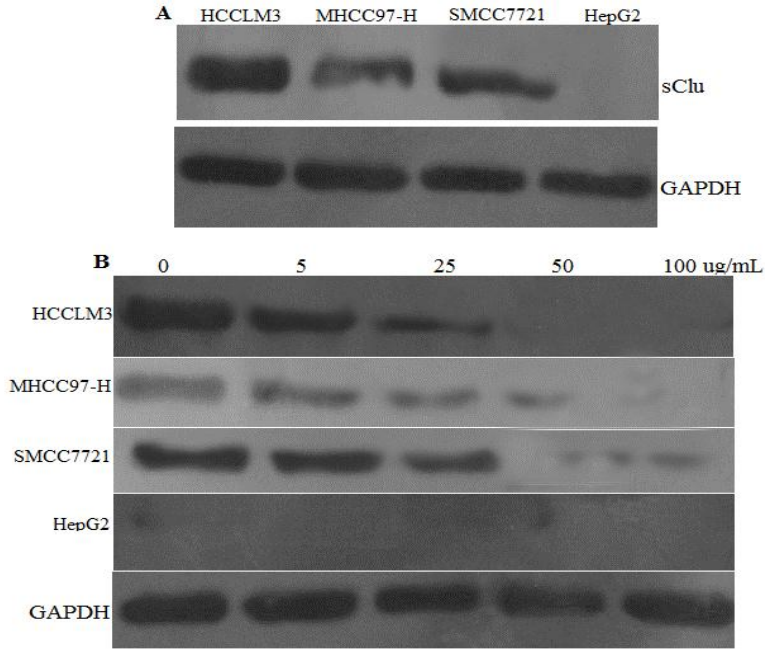


Figure 1. Western blot assay. **A**, Basal levels of sClu in HCCLM3, SMCC7721, HepG2 and MHCC97 cells. Highest levels of sClu were in HCCLM3 and SMCC7721 cells, moderate sClu expression in MHCC97-H cells, and no detectable in HepG2 cells; **B**, OGX-011 treatment for 48 h inhibits sClu in HCCLM3, SMCC7721 and MHCC97 cells *in vitro* in a dose-dependent manner.

lines in a dose-dependent manner, especially when exposed to 50 µg/ml (Figure 2A). The inhibitory ratio of OGX-011 was significantly increased from 14.3 ± 1.2% to 91.4 ± 5.3% during a 5-day treatment (Figure 2B). Thus, OGX-011 has robust suppressive effects on HCCLM3 proliferation.

Inhibitory effects of OGX-011 on HCCLM3 apoptosis *in vitro*

To see whether cell apoptosis cold account for reduced growth due to sClu, cells stained with annexin V and PI. The percent of apoptosis cells significantly increased in HCCLM3 cells after ≥5 µg/ml OGX-011 (Figure 3 A). To analyze the extent of the sClu silencing by OGX-011 on cell death, we scored apoptosis by TUNEL. A representative analysis in HCCLM3 cells is shown in Figure 3B, which was in agreement with the results obtained by FCM.

Inhibitory effects of OGX-011 on HCCLM3 migration and invasiveness *in vitro*

To detect antitumor activity of OGX-011, HCCLM3 cells treated with OGX-011 at 0, 5, 25 and 50 µg/ml were assay by the transwell method. Migration was significantly suppressed in a dose- dependent manner (Figure 4). In addition, the numbers of transmembrane cells after OGX-011 treatment with doses of 0, 5, 25 and 50 µg/ml were 152.4 ± 4.9, 120.3 ± 5.2, 74.0 ± 10.8, and 21.4 ± 4.9, respectively. The data show that OGX-011 is a potent inhibitor of cell migration of HCCLM3 cells.

OGX-011 Treatment *in vivo*

The effects of OGX-011 treatment on the growth of HCCLM3 subcutaneous tumors *in vivo* are shown in Figure 5A. Female nude mice bearing s.c HCCLM3 tumors (100 mm^3) were treated with OGX-011 or PBS controls as previously described. The xenografts treated with OGX-011 had delayed growth beyond 14 days (from 7 to 21 days).

After 21 days, HCCLM3 xenografts grew a little faster than before. Figure 5A shows that OGX-011 treatment, compared with PBS control, significantly reduced mean HCCLM3 tumor volume by >60 % after 6 weeks of treatment ($P \leq 0.05$). No adverse effects were observed under the experimental conditions described.

TUNEL assay was carried out on 16 HCCLM3

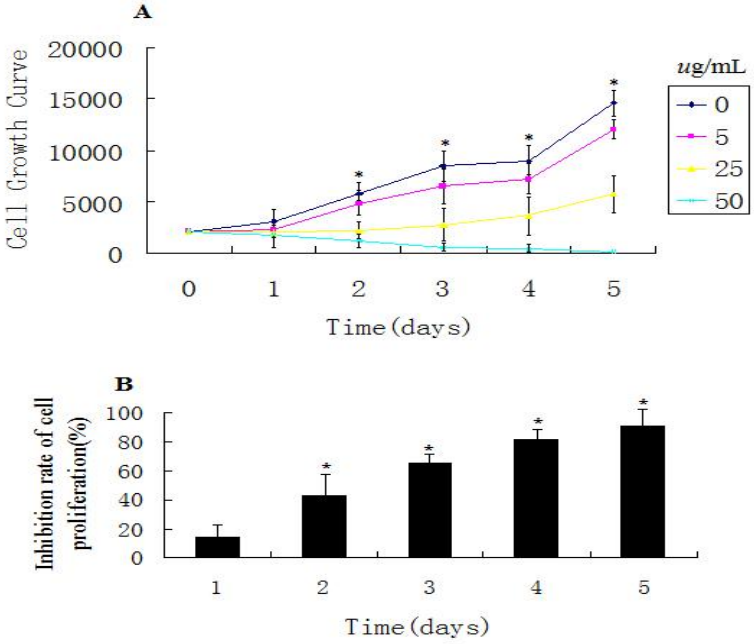


Figure 2. Effects of OGX-011 on proliferation of HCCLM3 cells. Proliferation of HCCLM3 cells was significantly inhibited by OGX-011 in a dose-dependent manner, especially when exposed to 50 µg/ml OGX-011. **A,** Growth curve of HCCLM3 cells treated with OGX-011 at from 0 to 50 µg/ml for 5 consecutive days. **B,** Inhibition ratio of proliferation of HCCLM cells was markedly increased in HCCLM3 cells treated with OGX-011 at 50 µg/ml for 5 days (*P<0.01).

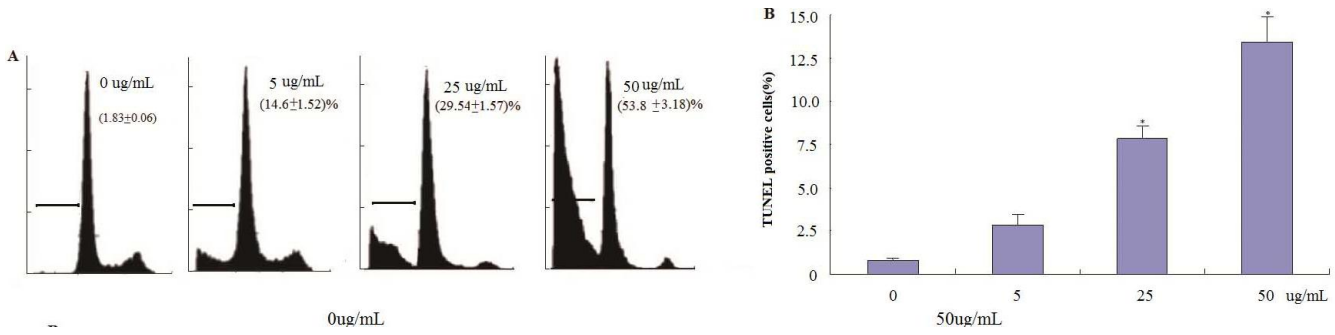


Figure 3. Inhibitory effects of OGX-011 on apoptosis of HCCLM3 cells. **A,** After exposure to 5, 25, and 50 µg/mL OGX-011 for 48 h, apoptosis was detect by FCM .The number of apoptotic cells was significantly increased compared with control cells; **B**, TUNEL was used to detect apoptosis. After treatment for 48 h, OGX-011 significantly increased apoptotic death. A representative picture of apoptotic cells (right hand panel) after exposure 50 µg/ml OGX-011 for 48 h is shown.

subcutaneous xenografts from each treatment group to assess whether OGX-011 induced suppression of sClu with enhanced apoptotic rates *in vivo*. OGX-011-treated tumors had higher levels of apoptosis when compared with tumors treated with PBS controls (Figure 5B), suggesting that the delay in tumor progression in the OGX-011 resulted from increased apoptosis induced by Clu suppression.

Western blot analysis (Figure 5C) of s.c. xenograft tissues shows that sClu expression was significantly reduced in mice treated with OGX-011. The data confirm that systemically given OGX-011 can suppress target clusterin expression *in vivo*, consistent with a recent report of a phase I pre-surgery trial in men with localized

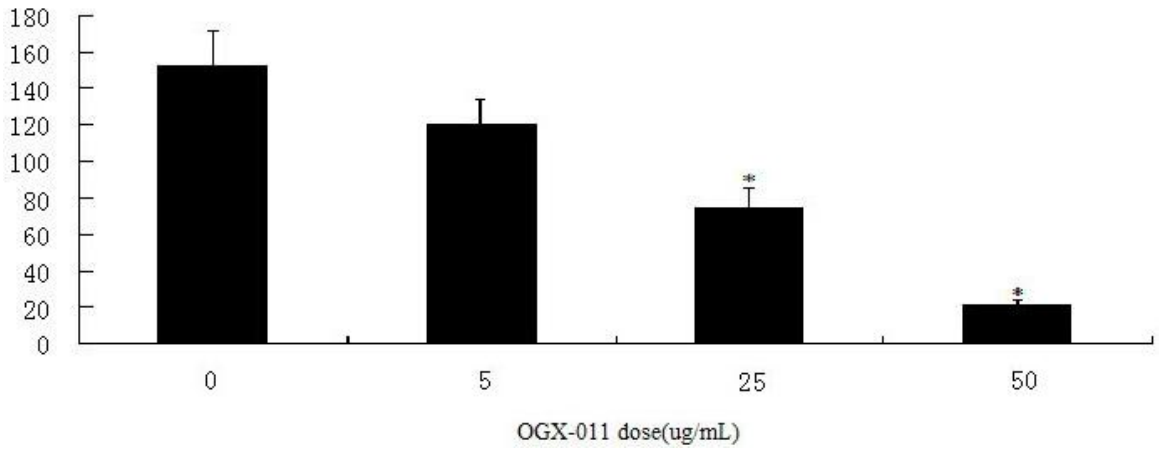


Figure 4. Inhibitory effects of OGX-011 on HCCLM3 cell migration and invasiveness. transmembrane cells (×200) after treatment with 0, 5, 25, and 50 µg/ml OGX-011; invasiveness of HCCLM3 was inhibited by OGX-011 in a dose-dependent manner (*P<0.01).

prostate cancer (Li et al., 2004).

In accord with the results *in vitro*, OGX-011 inhibited orthotopic tumor growth (data not shown) and metastasis of HCCLM3. OGX-011 treatment had a significant effect on the development of lung metastasis, with a 55% reduction in surface tumor number (Figure 5D). To examine the metastatic lesions, the lungs were sectioned to obtain representative tissue from multiple depths. These were assessed morphometrically to determine the percent area of lung parenchyma occupied by tumor lesions. Similarly to the gross surface counts, tumor burden was significantly reduced after OGX-011 treatment compared with the BPS controls. A 60% reduction in total tumor area in the lungs correlated well with the 55% reduction in the number of surface tumor lesions (Figure 5E).

DISCUSSION

Clu is an enigmatic molecule, and despite extensive studies, it remains unclear whether its functions reflect multifunctionality or alternatively they mask a common function. In particular, sClu's implication in cell death is still debatable because, according to the relatively few studies where the effects of experimental manipulation of sClu expression on cell death and survival were analyzed, sClu reportedly is associated with both a proapoptotic and an anti-apoptotic function. The ability of 21-nt duplexes of siRNA to direct sequence-specific degradation of mRNA (Parkin, 2001; Poon et al., 2002) and thereby induce specific gene silencing in mammalian cells has raised the possibility that siRNA can be used to investigate gene function in a high-throughput fashion or modulate gene expression in human diseases.

OGX-011 is an antisense inhibitor targeting the translation initiation site of human exon II Clu. Inhibition of Clu with OGX-011 significantly increases apoptosis, and reduces survival and invasion of HCCLM3 liver cancer cell, resulting in significant growth delay and lung metastasis regression of xenografts. OGX-011 attenuated Clu expression at the protein level in HCCLM3 liver cancer cells. This finding is consistent with previous studies that demonstrated Clu expression attenuation by an ASO (Poon et al., 2000; Rizzi and Bettuzzi, 2009; Redondo et al., 2000).

Antiapoptotic effects of the secreted form of Clu have been suggested in several studies (Rizzi and Bettuzzi, 2009; Redondo et al., 2000; Sowery et al., 2008). Other groups have also suggested that sClu may act as an extracellular scavenger for misfolded proteins after injury induced by cellular stress (Trougakos and Gonos, 2002; Trougakos et al., 2004). Clu may promote the phagocytosis and clearance of cellular debris that results from cytotoxic stress (Sowery et al., 2008).

Our results clearly demonstrate that sClu mRNA is amenable to OGX-011-induced degradation *in vitro* and that the secreted sClu protein form is an essential molecule for the cellular homeostasis of the HCCLM3 cells. Moreover, it is evident that the primary function of sClu in HCCLM3 cells is antiapoptotic. In the *in vitro* study, a significantly increased number of apoptotic cells were detected when HCCLM3 cells were treated with OGX-011, as determined by TUNEL analysis (p<0.01). Consistent with these *in vitro* results, the effects of OGX-011 was also observed *in vivo*. The effects of sClu gene expression silencing are related to the endogenous sClu protein levels expressed by HCCLM3 cells that express the highest sClu endogenous amounts. Other studies that used siRNA against Clu expression showed similar

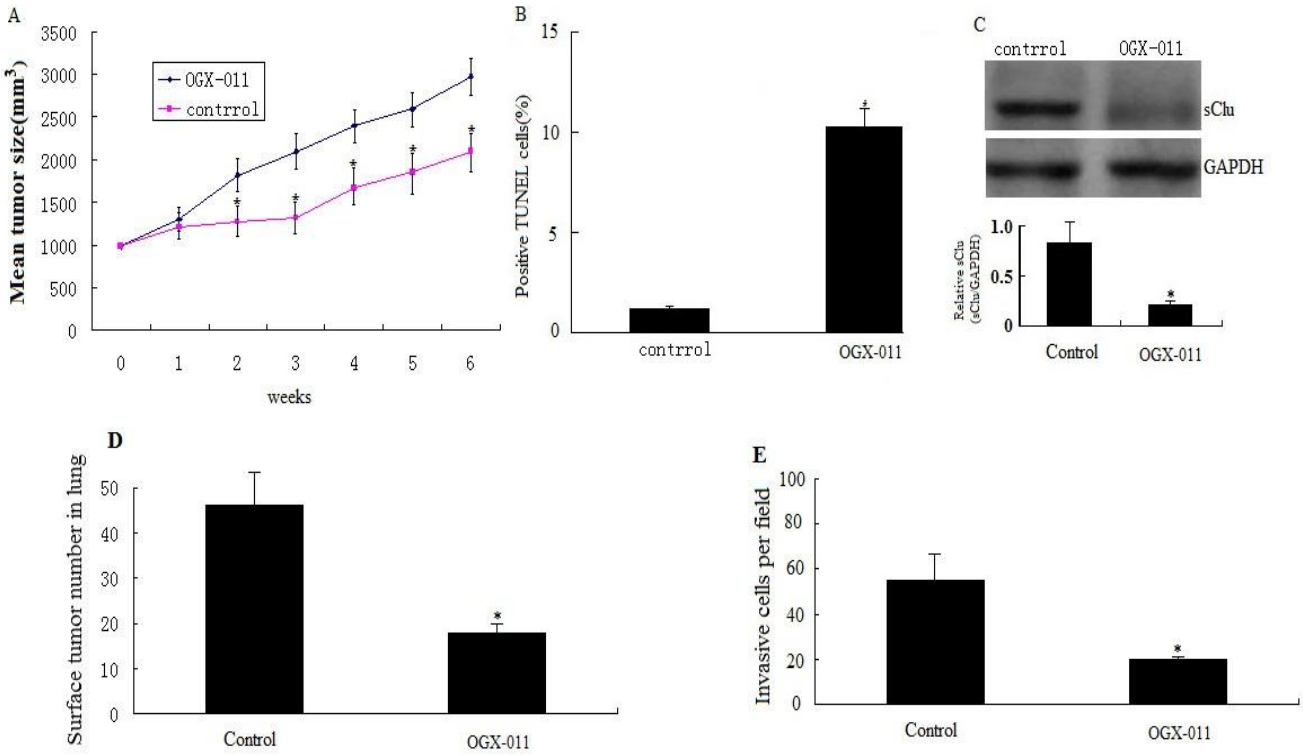


Figure 5. OGX-011 treatment on growth and metastasis *in vivo*. **A,** OGX-011 inhibits growth of HCCLM3 xenografts in vivo. When OGX-011 tumors had reached 1,000 mm^3, usually 3-4 wks after injection, 8 mice were randomly selected for treatment with 10 mg OGX-011 or PBS controls once daily by i.p. injection for 42 d. Tumor volume was measured weekly. *, $P \leq 0.01$; **B**, apoptosis was measured in paraffin-embedded xenograft tumors by TUNEL immunohistochemistry. Apoptotic index increased in HCCLM3 xenografts treated with OGX-011; **C**, OGX-011 suppresses clusterin levels in HCCLM3 xenografts *in vivo*. HCCLM3 xenografts were collected at day 42 and Western blot analysis was done to quantify Clu levels. Loading levels were normalized by blotting with anti-GAPDH and densitometric analysis. Three representative samples are shown in each group; **D**, representative histogram showing the surface tumor number in OGX-011-treated groups; **E**, Representative histogram showing the total tumor area in the lungs in OGX-011-treated groups. Each bar represents mean ± SE; *, $P < 0.01$. All experiments were repeated 3 times with similar results.

findings with induction of apoptosis (Xie et al., 2002).

sClu over-expression into human renal cell carcinoma cells enhances their metastatic potential (Yang et al., 2000).The proposed essential role of sClu for cellular homeostasis is clearly supported by the fact that sClu is associated with cells surviving programmed cell death during development (Xie et al., 2002; Yang et al., 2000). Our *in vitro* and *in vivo* studies suggest OGX-011 reduces invasion and lung metastasis. Hasan et al. (2003) reported that glucocorticoids can induce capillary growth inhibition in hemangioma cells *in vitro*, an effect associated with an increased transcription of clusterin. Whether angiogenesis is associated with Clu needs further investigation.

In summary, considering the sClu gene is differentially expressed during carcinogenesis in tumor progression, we hypothesize that sClu is a target for therapeutic inhibition in HCC. OGX-011 is a potent agent for modulating Clu gene expression and they may ultimately develop into an attractive antitumor therapy.

ACKNOWLEDGEMENTS

This work was supported by the Henan Institutes of Health Grant (No: HK2011-021-C5). We thank Dr Zhang and Chen for their technical help.

REFERENCES

Blaschuk O, Burdzy K, Fritz IB (1983). Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. J. Biol. Chem. 258:7714-7720.

Bi J, Guo AL, Lai YR, Li B, Zhong JM, Wu HQ, Xie Z, He YL, Lv ZL, Lau SH, Wang Q, Huang XH, Zhang LJ, Wen JM, Guan XY (2010). Overexpression of clusterin correlates with tumor progression, metastasis in gastric cancer: a study on tissue microarrays.

Neoplasma 57:191-197.
Cochrane DR, Wang Z, Muramaki M, Gleave ME, Nelson CC (2007).Differential regulation of clusterin and its isoforms by androgens in prostate cells. J. Biol. Chem. 282:2278-2287.
Chi KN, Eisenhauer E, Fazli L (2005). A phase I pharmacokinetic and pharmacodynamic study of OGX-011, a 2′-methoxyethyl antisense oligonucleotide to clusterin, in patients with localized prostate cancer. J. Natl. Cancer Inst. 97:1287-1296.
Chuan D, Muhammad K, Bin Z, Jingbo Y, Lili Z, Tonghui M (2012). Casticin induces apoptosis and mitotic arrest in pancreatic carcinoma PANC-1 cellsAfrican J. Pharm. Pharmacol. 6(6):412-418.
Garden GA, Bothwell M, Rubel EW (1991). Lack of correspondence between mRNA expression for a putative cell death molecule (SGP-2) and neuronal cell death in the central nervous system. J. Neurobiol. 22:590-604.
Gleave M, Chi KN (2005). Knock-down of the cytoprotective gene, clusterin, to enhance hormone and chemosensitivity in prostate and other cancers. Ann. NY. Acad. Sci. 1058:1-15.
Gleave M, Miyake H (2005). Use of antisense oligonucleotides targeting the cytoprotective gene, clusterin, to enhance androgen- and chemo-sensitivity in prostate cancer. World J. Urol. 23:38-46
Hazzaa SM, Elashry OM, Afifi IK (2010). Clusterin as a diagnostic and prognostic marker for transitional cell carcinoma of the bladder. Pathol. Oncol. Res. 16:101-109
Harborth J, Elbashir SM, Bechert K, Tuschl T, Weber K (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. J. Cell Sci.114:4557-4565.
Humphreys DT, Carver JA, Easterbrook-Smith SB (1999). Clusterin has chaperone-like activity similar to that of small heat shock proteins. J. Biol. Chem. 274:6875-6881.
Hayashi PH, Di Bisceglie AM (2006). The progression of hepatitis B- and C-infections to chronic liver disease and hepatocellular carcinoma: presentation,diagnosis, screening, prevention, and treatment of hepatocellular carcinoma. Infect. Dis. Clin. North. Am. 20:1–25.
Hasan Q, Tan ST, Xu B (2003). Effects of five commonly used glucocorticoids on haemangioma in vitro. Clin. Exp. Pharmacol. Physiol. 30:140 –144.
July LV, Beraldi E, So A (2004). Nucleotide-based therapies targeting clusterin chemosensitize human lung adenocarcinoma cells both in vitro and in vivo. Mol. Cancer Ther. 3:223–232.
Jones SE, Jomary C (2002). Clusterin. Int. J. Biochem. Cell. Biol. 34:427-431.
Kang YK, Hong SW, Lee H, Kim WH (2004). Overexpression of clusterin in human hepatocellular carcinoma. Hum. Pathol. 35:1340-1346.
Leskov KS, Klokov DY, Li J, Kinsella TJ, Boothman DA (2003).Synthesis and functional analyses of nuclear clusterin, a cell death protein. J. Biol. Chem. 278:11590-600.
Lokamani I, Looi ML, Ali SA, Dali AZ, Jamal R (2011). Clusterin as a potential marker in distinguishing cervical neoplasia. Anal. Quant. Cytol. Histol. 33:223-228
Lau SH, Sham JS, Xie D, Tzang CH, Tang D, Ma N, Hu L, Wang Y, Wen JM, Xiao G, Zhang WM, Lau GK, Yang M, Guan XY (2006). Clusterin plays an important role in hepatocellular carcinoma metastasis.Oncogene. 25:1242-1250
Li Y, Tian B, Yang J, Zhao L, Wu X, Ye SL, Liu YK, Tang ZY (2004). Stepwise metastatic human hepatocellular carcinoma cell model system with multiple metastatic potentials established through consecutive in vivo selection and studies on metastatic characteristics. J. Cancer Res. Clin. Oncol. 130:460-468.
Liangpu Z, Youqin C, Wei L, Qunchuan Z, Xuzheng C, Wei X, Xianxiang L, Jun P, Thomas J (2011). Sferra.Spica Prunellae extract promotes mitochondrion dependent apoptosis in human colon carcinoma cell line. Afri. J. Pharm. Pharmacol. 5(3):327-335
Miyake H, Hara I, Gleave ME (2005). Antisense oligodeoxynucleotide therapy targeting clusterin gene for prostate cancer: Vancouver experience from discovery to clinic. Int. J. Urol. 12:785-794.
Miyake H, Gleave ME, Arakawa S, Kamidono S, Hara I (2002). Introducing the clusterin gene into human renal cell carcinoma cells enhances their metastatic potential. J. Urol.167:2203-2208.
Miyake H, Chi KN, Gleave ME (2000). Antisense TRPM-2 oligodeoxynucleotides chemosensitize human androgen-independent PC-3 prostate cancer cells both in vitro and in vivo. Clin. Cancer Res. 6:1655-1663.
Miyake H, Hara I, Kamidono S (2001). Synergistic chemsensitization and inhibition of tumor growth and metastasis by the antisense oligodeoxynucleotide targeting clusterin gene in a human bladder cancer model. Clin. Cancer Res. 7:4245–4252.
O'Bryan MK, Cheema SS, Bartlett PF, Murphy BF, Pearse MJ (1993). Clusterin levels increase during neuronal development. J. Neurobiol. 24:421-432.
Parkin DMN (2001). Global cancer statistics in the year 2000. Lancet Oncol. 2:533-543.
Poon S, Treweek TM, Wilson MR (2002). Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. FEBS Lett. 513:259-266.
Poon RT, Fan ST, Lo CM, Ng IO, Liu CL, Lam CM, Wong J (2000).Improving survival results after resection of hepatocellular carcinoma: a prospective study of 377 patients over 10 years. Ann. Surg. 234:63–70.
Rizzi F, Bettuzzi S (2009). Clusterin (CLU) and prostate cancer. Adv. Cancer Res.105:1-19
Redondo M, Villar E, Torres-Muñoz J, Tellez T, Morell M, Petito CK (2000). Overexpression of clusterin in human breast carcinoma. Am. J. Pathol. 157:393-399.
Sowery RD, Hadaschik BA, So AI (2008). Clusterin knockdown using the antisense oligonucleotide OGX-011 re-sensitizes docetaxel-refractory prostate cancer PC-3 cells to chemotherapy. BJU Int. 102:389–97.
Trougakos IP, Gonos ES (2002). Clusterin/apolipoprotein J in human aging and cancer. Int. J. Biochem. Cell Biol. 34:1430-1448.
Trougakos IP, So A, Jansen B (2004). Silencing expression of the clusterin/apolipoprotein j gene in human cancer cells using small interfering RNA induces spontaneous apoptosis, reduced growth ability, and cell sensitization to genotoxic and oxidative stress. Cancer Res. 64:1834-1842.
Xie MJ, Motoo Y, Su SB, Mouri H, Ohtsubo K, Matsubara F, Sawabu N (2002). Expression of clusterin in human pancreatic cancer. Pancreas. 25:234-8.
Yang CR, Leskov K, Hosley-Eberlein K, Criswell T, Pink JJ, Kinsella TJ, Boothman DA (2000). Nuclear clusterin/XIP8, an x-ray-induced Ku70-binding protein that signals cell death. Proc. Natl. Acad. Sci. U. S. A. 97:5907-5912.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2408-2412, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.263
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Comparative effects of propolis of honey bee on pathogenic bacteria

Shahin Gavanji[1]*, Behrouz Larki[2], Alireza jalali Zand[2], Elmira Mohammadi[2], Mohammad Mehrasa[3] and Amir Hosein Taraghian[2]

[1]Young Researchers Club, Khorasgan Branch, Islamic Azad University, Khorasgan, Isfahan, Iran.
[2]Department of Plant Protection, Khorasgan (Isfahan) Branch, Islamic Azad University, Isfahan, Iran.
[3]Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran.

Accepted 4 June, 2012

Propolis is one of the useful products in the hive. This substance is a natural product derived from plant resins gathered by honey bees. Its protective properties are because of the components that contribute in propolis production. So far, more than 300 different combinations, such as polyphenols, aldehydes, phenolic, monoterpene, amino acids, steroids and other inorganic compounds are found in the structure of propolis. In this study, the power of inhibitory effect of propolis prepared from 3 provinces of Iran was compared. As a result, it was revealed that the propolis which was prepared from Lorestan Province showed a better inhibitory effect compared with 2 other provinces on species of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus* bacterial.

Key words: Propolis, *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneumonia, Staphylococcus aureus*.

INTRODUCTION

Propolis (bee glue) is one of the useful products in the hive. This substance is a natural product derived from plant resins gathered by honey bees. This material is normally solid, pasty and sticky, greenish brown to dark brown that depends on the origin of the resin composition and the duration and condition of storage. Date and time of collection by the worker bees is also effective in its colour. Propolis has bitter and acrid taste and will numb the gums and oral mucosa. This material is made by bees and used in the inner wall of the hive to protect the hive and the bees. So far, more than 300 different combinations, such as polyphenols, aldehydes, phenolic, monoterpene, and amino acids, steroids and other inorganic compounds are found in the structure of propolis (Khalil, 2006). In addition to the benefits of propolis to hive that mentioned earlier, it has been proved that this compound is also important for various medical properties. For example, in traditional medicine and in the treatment of malignant tumors, calluses, ulcers and gangrene, as well as conditions such as a local anesthetic (Miyataka et al., 1997; Lee et al., 2000; Choi et al., 1999; Buono et al., 2001). The high percentage of flavonoids in this matter has caused anti-bacterial and anti-inflammatory properties (Grunberger et al., 1988; Kimato et al., 1998). Chromatographic methods have proved the existence of the antibacterial substance in propolis (Eley, 1999; Burdock, 1998). Propolis and its extract is used in the treatment of diseases such as rheumatism, diabetes, tumors, allergies, asthma, heart diseases, pneumonia, influenza, stomach ulcers and chronic wounds of vaginitis.

Conducted researches on propolis have shown antibacterial, anti-protozoa, antifungal and antiviral properties. Inhibitory effect of propolis on at least 21 bacterial species of protozoa, 9 of yeasts and viruses such as herpes and influenza range had been shown

*Corresponding author. E-mail: shahin.gavanji@yahoo.com. Tel: (+98) 311 4427155, (+98) 09138072585, (+98) 09389365450.

(Marquele et al., 2006). One of the challenges in controlling infectious diseases is antimicrobial drug resistance. Formation of multiple drug resistance in bacteria over the past several decades has created many problems in the treatment of bacterial diseases. Antibiotic resistance in bacteria is the inherent product genetics [include the stable attainment of new genetic information, such as mutation (Hancock, 1998) and physiology (such as outer membrane of Gram-negative bacteria, the expression of innate efflux pumps and natural mutations in antibiotic targets), which is vertically transmitted through species (Wright and Sutherland, 2007). The main mechanisms of drug resistance consists of enzymatic transformation, modification of the molecular target, sequestration of the drug, active efflux from the cell interior and reversely prohibition of entry of the compound into the cell (Wright, 2005; Walsh, 2003; Levy and Marshall, 2004). Organisms such as *Escherichia coli* and *Staphyloccocus aureus* simply obtain antibiotic resistance gene from plasmid and mobile genetic elements. These bacteria are attendant with infections in the community and hospitals (Wright and Sutherland, 2007). *S. aureus* is an facultative anaerobic Gram-positive coccobasil which is naturally found in part of skin and nasal passage (Kluytmans et al., 1997). If the barriers such as skin and mucosal lining have been damaged, these bacteria can infect other tissue and cause pimples and acnes (Curran and Al-Salihi, 1980). Drug resistance is common among pathogenic staphylococci. Due to the inherent virulence of *S. aureus* as well as its ability to create a diverse array to life-threatening infections and its capacity to adapt to different environmental conditions, this pathogene is a main concern (Lowy, 1998; Waldvogel, 2000). *E. coli* is Gram-negative basil that is generally found in lower intestine in endotherm organisms. However, most strains are part of the normal gut flora, some serotype of *E. coli* are pathogenic in human and can cause food poisoning and bloody diarrhea (Vogt and Dippold, 2005). *E. coli* is the most common cause of urinary tract infection in approximately 90% of urinary tract infections in young women for the first time (Brooks et al., 1991). *E. coli* carries multiple drug resistance plasmids, under stress conditions and transfers them to other species easily. *E. coli* strain can contribute in biofilm formation and this phenomena cause these strains and other Entrobactericea which are remarkable reservoirs of transferable antibiotic resistance (Salyers et al., 2004). *Klebsiella pneumonia* is another genus of Entrobactericea that is a Gram-negative, facultative anaerobic basilus, non-mobile and encapsulated which is found in normal flora of intestines (Brooks et al., 1991). These bacteria cause infections in hospital and community (Jayavanth et al., 2011). In hospital, the majority of infections caused by *K .pneumonia* are urinary tract infections, nosocomical pneumonia, diarrhea and introabdominal infections (Podschun and Ullmann, 1998). *K. pneumonia* is existed in respiratory tract and faces of about 5% of normal people. It can produce considerable hemorrhagic necrotizing consolidation of the lung (Brooks et al., 1991). *K. pneumoniae* may also be attributable to multi-drug efflux systems (Deguchi et al., 1997, Martinez et al., 1998). *Pseudomonas aeruginosa* is Gram-negative aerobic motile basil (Brooks et al., 1991). This bacteria is widely distributed in nature and commonly present in most environment in hospitals. *P. aeruginosa* is frequently existed in small numbers in the normal intestinal flora and also on the skin of humans. It becomes pathogenic only when introduced into areas without normal defenses. For instance, when mucous membranes or skin disrupted by direct tissue damage, this bacterium attaches to colonize the mucous membranes or skin, invades locally and causes systemic diseases (Brooks et al., 1991). These bacteria can express variety of efficient efflux pump and antibiotic inactivating enzymes. These protection systems are difficult to overcome *P. aeruginosa* when it is associated with infections.

MATERIALS AND METHODS

Preparing extract

It has been revealed that the biological activities of a sample depend on a methodology which is used for preparing the extract of the sample. The most common materials which are used for the preparation of the extract in biological method are ethanol, methanol and water (De Vecchi and Drago, 2007). In this study, the samples of propolis were grinned and to prepare the extract, 10 g of them were weighed carefully. Then they were poured in 250 ml balloon and by adding ethanol 96%, the volume of sample was reached to 100 mm. The mixture then was stirred very well. This practice was repeated once or twice every day for 3 days. For 1 to 2 weeks, the mixture was kept in a warm and dark place. After this period of time, the mixture was filtered and was placed in a refrigerator at the temperature of 1 to 4°C for 1 day so that the solution was filtered again and the resulting extract was kept in a dark and impenetrable container. The remaining alcohol in suspension was completely separated by Soxhlet apparatus and this pure alcoholic extract was obtained. Hence, this solvent of alcohol evaporates in a temperature below the temperature for water evaporation. Within a few hours, it was completely isolated from the solution and alcoholic extract of propolis was collected in the case (Gavanji et al., 2011).

Bacterial strains tested

For bacterial strains, the species are as follows: *S. aureus, E. coli, P. aeruginosa* and *K. pneumonia* were prepared from Traditional Medicine Institute of Esfahan.

Culture preparation and treatment of the extracts

Initially, Muller-Hinton agar (MHA) medium was prepared and was poured in sterilized Petri dishes at 5 cm thick. In aseptic condition, the samples of bacteria were taken from basal culture with applicator and inoculated in medium. To examine the antimicrobial effect of ethanol extracts, disc-diffusion assay method was used.

This method is the most common method and known as Kirby-Bauer which is the most common form of antimicrobial assay (Khosravi and Behzadi, 2006). The first disk is placed in extract after soaking. In this test, concentrations of 0.1, 0.312, 0.625, 1.2, 2.5, 5, 10, 20 and 30% of the alcoholic extract of bee propolis were used. Blanch disks with a diameter of 6.5 mm (manufactured by Diagnostic Mass, England) for half an hour in soluble extracts was soaked in sterile conditions. With using the prepared disks (manufactured by Diagnostic Mass, England), Tetracycline (concentration of 30 μg), penicillin (concentration of 10 μg) and ampicilin (concentration of 10 μg) were used as positive control. Petri dishes were incubated for 24 h at 37°C. Finally, after 24 h microbial growth inhibition zone diameter around was measured by Colis.

RESULTS

The results of propolis experiments prepared from West Azarbayjan Province showed no inhibitory effect in concentrations of 0.01, 0.312 and 0.625%. But in concentration of 1.25, inhibitory effect on 3 species of *E. coli*, *S. auerus* and *K. pneumonia* was observed (Table 1) where maximum inhibitory effect was on *K. pneumonia* with 1.81 mm. In this concentration, there was no effect on *P. aeroginosa.* In concentrations of 2.5, 5, 10, 20 and 30% of propolis, the maximum inhibitory effect was related to *K. pneumonia* and the minimum inhibitory effect was related to *P. aeroginosa*. The antimicrobial property of propolis prepared from West Azarbayjan Province in comparison with tetracycline, penicillin and ampiciline had been determined. It revealed that the concentration of 5% of propolis has more inhibitory effect compared with penicillin and tetracycline on *E. coli*. Also, the concentration of 20% of propolis has more inhibitory effect compared with ampiciline disk on *E. coli*. In *S. auerus*, the concentration of 20% of propolis has a better inhibitory effect compared with penicillin and ampiciline disks and the concentration of 30% of propolis has a better inhibitory effect compared with tetracycline disk too. In *K. pneumonia*, the concentration of 5% of propolis has more inhibitory effect compared with ampiciline, penicillin and tetracycline disks. In the next step, the propolis which was prepared from Lorestan Province was tested on 4 species of bacteria. The effect of this propolis similar to propolis of West Azarbayjan Province had no inhibitory effect in concentrations of 0.1, 0.312 and 0.625. But in concentration of 1.25, the inhibitory effect was observed on all 4 species. In this concentration, maximum inhibitory effect was on *S. auerus* (Table 2). Also in concentration of 2.5, maximum inhibitory effect was observed on *S. auerus* too and the minimum inhibitory effect was on *P. aeroginosa*. But in concentration of 5% of propolis, the maximum inhibitory effect was observed on *K. pneumonia* and the minimum inhibitory effect was on *P. aeroginosa*. In concentrations of 10, 20 and 30% of propolis, the maximum inhibitory effect was observed on *S. auerus* and the minimum inhibitory effect like the previous concentrations was on *P. aeroginosa*. Comparison of antimicrobial property of propolis prepared from Lorestan Province with antibiotic disks showed that the concentration of 5% of propolis has a better inhibitory effect compared with penicillin and tetracycline disks on *E. coli* and concentration of 10% showed a better inhibitory effect compared with ampiciline disk on *E. coli* too. At last the effect of propolis prepared from Chaharmahal Province was determined (Table 3). In this test like previous tests, concentrations of 0.1, 0.312 and 0.625% showed no inhibitory effect on 4 species. Also, in concentration of 1.25% no inhibitory effect was observed on *P. aeroginosa*. In concentrations of 2.5, 5 and 10%, the maximum inhibitory effect was observed on *S. aureus* and the minimum inhibitory effect was on *P. aeroginosa*. In 20% of concentration, the maximum inhibitory effect was observed on *K. pneumonia* and in 30% of concentration; the maximum inhibitory effect was on *S. aureus*. Finally, the power of inhibitory effect of propolis prepared from 3 provinces was compared. As a result, it was revealed that the propolis which was prepared from Lorestan Province showed a better inhibitory effect compared with 2 other provinces on 4 bacterial species.

DISCUSSION

The word propolis is derived from Greek words; pro (‘in front of’) and polis (‘city’ or ‘community’), which means that this natural product associates to hive defense. Its protective properties are because of components that contribute in propolis production. Many investigations prove the antibacterial activity of this substance (Marcucci, 1995; Burdock, 1998; Kujumgiev et al., 1999; Banskota et al., 2001; Farooqui and Farooqui, 2010). The propolis of bees or plant resins is the product of worker bees. The bees gathered the plant resins in the hive and with making some changes to it, use it as sealant, polisher, disinfectant and to mummify the dead insects in hives. The propolis consists of resins, wax, volatile and pollens of flowers and by using biochemical analysis, it consists of alcohols, aldehydes, phenolics, amino acids esters and fatty acids which are valuable in pharmaceutic industry (Gavanji et al., 2011). Researches showed that the propolis possess antimicrobial, anti unicellular, anti viruses and anti fungal properties (Fernandes et al., 2007; Liu et al., 2002). Considering to broad therapeutic spectrum of propolis, it can be used to heal some diseases. One of the most important compounds in propolis is phenolic which give a considerable antimicrobial property to it. According to experiments all around the world, the propolis can control a broad spectrum of Gram-negative and Gram-positive bacteria. In relation to bacterial diseases, it is revealed that although propolis has a considerable effect on Gram-positive bacteria, it has a few effect on Gram-negative bacteria (Liu et al., 2002). The antimicrobial properties of

Table 1. Different concentrations of alcoholic extract of propolis prepared from West Azarbayjan Province.

	Concentration											
Bacteria	**0.1**	**0.312**	**0.625**	**1.25**	**2.5**	**5**	**10**	**20**	**30**	**Ampiciline**	**Penicillin**	**Tetracycline**
E. coli	-	-	-	0.75	4.5	9.25	13.75	16.45	20.70	14.33	6.5	6.5
S. aureus	-	-	-	1.23	5.1	11.14	16.65	20.35	22.80	17.3	15.8	20.4
P. aeroginosa	-	-	-	-	2.24	6.13	9.10	13.49	17.43	6.5	6.5	13.25
K. pneumoniae	-	-	-	1.81	5.33	12.2	15.70	19.60	23.61	6.5	6.5	6.5

The data are the average of 5 replicates.

Table 2. Different concentrations of alcoholic extract of propolis prepared from Lorestan Province.

	Concentration											
Bacteria	**0.1**	**0.312**	**0.625**	**1.25**	**2.5**	**5**	**10**	**20**	**30**	**Ampiciline**	**Penicillin**	**Tetracycline**
E. coli	-	-	-	2.63	5.20	10.88	15.75	20.16	25.42	14.33	6.5	6.5
S. aureus	-	-	-	3.17	6.48	13.5	19.26	24.71	28.95	17.3	15.8	20.4
P. aeroginosa	-	-	-	1.08	3.29	7.32	10.67	15.19	19.33	6.5	6.5	13.25
K. pneumoniae	-	-	-	2.25	4.73	14.51	17.26	21.55	24.52	6.5	6.5	6.5

The data are the average of 5 replicates.

Table 3. Different concentrations of alcoholic extract of propolis prepared from Chaharmahal Province.

	Concentration											
Bacteria	**0.1**	**0.312**	**0.625**	**1.25**	**2.5**	**5**	**10**	**20**	**30**	**Ampiciline**	**Penicillin**	**Tetracycline**
E. coli	-	-	-	0.5	3.09	8.32	11.82	14.02	17.88	14.33	6.5	6.5
S. aureus	-	-	-	1.45	5.42	10.75	15.49	17.90	23.30	17.3	15.8	20.4
P. aeroginosa	-	-	-	-	2.13	5.91	7.16	10.66	15.20	6.5	6.5	13.25
K. pneumoniae	-	-	-	1.91	3.96	8.19	13.41	18.33	21.95	6.5	6.5	6.5

The data are the average of 5 replicates.

propolis may occur by direct action on microorganism or non-direct action by stimulation of immune system resulting in killing more microorganisms. The *in vivo* and *in vitro* methods proved that propolis may activate macrophages, increase their antimicrobial activities and also stimulate the production of antibodies (De Vecchi el al., 2007). The antimicrobial property of propolis is related to numerous quantities of phenolics in it which can be referred to some derivatives such as pinobanksin and pinocembrin. In addition to high percentage of phenolics, the antimicrobial property of propolis has also been referred to an activated part called caffeic acid phenethyl ester (Grunberger et al., 1988; Na et al., 2000). The fulfilled experiments about propolis in different places showed special phenolic compounds with different percentage in samples (Greenaway et al., 1990). In an experiment, the effect of propolis prepared from 4 regions of Turkey and also a propolis from Brazil were tested on 9 bacteria of pathogen in mouth. The results showed that the propolis prepared from different regions is effective on all 9 species of bacteria, but the propolis prepared from Kaza in Ankara City possess a higher inhibitory effect compared with other propolis from different regions in Turkey and Brazil (Koru et al., 2007). Hence, the compounds of propolis depend on geographical positions; so in this study, we tested the effect of propolis from 3 regions of West Azarbayjan, Lorestan and Chaharmahal Provinces. Different concentrations of alcoholic extract of propolis prepared from different regions were tested on 4 bacterial species. The propolis prepared from Lorestan Province showed a better inhibitory effect compared with 2 other provinces on 4 bacterial species. Comparison of antimicrobial property of propolis which was prepared from Lorestan Province with antibiotic disks revealed that the concentration of 5% of propolis shows a better inhibitory effect compared with penicillin and tetracycline disks on *E. coli.*

Conclusion

From the obtained results, it is clear that the alcoholic

extract of propolis can be used against the examining bacteria in different concentrations. With regard to the fact that the minimum inhibitory concentration (MIC) of the 3 species has equal values, it is necessary to carry out studies on laboratory creatures *in vivo* conditions, so that the advantages and the side effects of this substance can be examined carefully.

REFERENCES

Banskota AH, Tezuka Y, Kadota S (2001). Recent progress in pharmacological research of propolis. Phytother. Res. 15:561-571.

Brooks GF, Butel JB, Ornston LN (1991). Jawets,Melnick & Adelberg's Medical Microbiology. New Yourk. Appleton & Lange.

Buono MD, Urciuoli O, Marrietta P, Padoani W, De LD (2001). Alternative medicine in a sample of 655 community-dwelling eldery. J. Psychosom. Res. 50:147-154.

Burdock GA (1998). Review of the biological properties and toxicity of bee propolis (Propolis). Food Chem. Toxicol. 36:347-363.

Choi YH, Lee WY, Nam SY, Choi KC, Park YE (1999). Apoptosis induced propolis in human hepatocellular carcinoma cell. Int. J. Mol. Med. 4:29-32.

Curran JP, Al-Salihi FL (1980). Neonatal staphylococcal scalded skin syndrome: massive outbreak due to an unusual phage type. Pediatrics, 66:285.

Deguchi T, Kawamura T, Yasuda M, Nakano M, Fukuda H, Kato H, Kato N, Okano Y, Kawada Y (1997). *In vivo* Selection of *Klebsiella pneumoniae* strains with enhanced quinolone resistance during fluoroquinolone treatment of urinary tract infections. Antimicrob. Agents. Ch. 41:1609-1611.

Eley BM (1999) Antibacterial agent in the control of supragingival plaque, a review. Br. Dent. J. 186: 286-296.

Farooqui T, Farooqui A (2010). Molecular Mechanism Underlying the Therapeutic Activities of Propolis: A Critical Review. Curr. Nutr. Food Sci. 6:188-199.

Fernandes FF, Dias AL, Ramos CL, Ikegaki M, de Siqueira AM, Franco MC (2007). The "*in vitro*" antifungal activity evaluation of propolis G12 ethanol extract on Cryptococcus neoformans. Rev. Inst. Med. Trop. Sao Paulo 49(2):93-95.

Gavanji S, Asgari MJ, Vaezi R, Larki B (2011). Antifungal effect of the extract of propolis on the growth of three species of Epidermophyton flucosum, Trichophyton violaseum and Trichophyton tonsorans in laboratory environment. Afr. J. Pharm. Pharm. 5(24):2642-2646.

Grunberger D, Banerjee R, Elsinger K, Oltz EM, Efros L, Caldwell M (1988) Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. Experientia 44:230-232.

Hancock REW (1998). Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative Gram-negative bacteria. Clin. Infect. Dis. 27:S93-S99.

Jayavanth P, Kaur K, Junainah AH (2011). Antibacterial Efficacy of Chitosan, Manuka Honey and Chlorophyll against Klebsiella Pneumoniae. J. Nat. Prod., 4: 94-99.

Khalil ML (2006) Biological activity of bee propolis in health and disease. Asian Pac. J. Cancer Prev. 7(1):22-31.

Khosravi AD, Behzadi A (2006) Evaluation of the Antibacterial Activity of The Seed Hull of Quercus Brantii on some Gram Negative Bacteria. Pak. J. Med. Sci., 22(4): 429-432.

Kimato T, Arai S, Kohguchi M, Aga M, Nomura Y, Micallef MJ, Kurimoto M, Mito K (1998). Apoptosis and suppression of tumor growth by artepillin C extracted from Brazilian propolis. Cancer Detect. Prev. 22: 506-515.

Kluytmans J, Van Belkum A, Verbrugh H (1997). Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin. Microbial. Rev. 10:505.

Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova VS, Christov R, Popov S (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol., 64: 235-240.

Lee YJ, Liao PH, Chen WK, Yang CC (2000). Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells. Cancer Lett. 153:51-56.

Levy SB, Marshall B (2004). Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10:S122-S129.

Lowy FD (1998) *Staphylococcus aureus* infections. New Engl. J. Med. 339:520-532.

Marcucci MC (1995) Propolis: chemical composition, biological properties and therapeutic activity. Apidologie 26:83-99.

Marquele FD, Stracieri KM, Fonseca MJ, Freitas LA (2006) Spray-dried propolis extract. I: physicochemical and antioxidant properties. Pharmazie 61(4):325-330.

Martinez LM, Garcia I, Ballesta S,Benedi VJ, Alles SH, Pascula A (1998). Energy-Dependent Accumulation of Fluoroquinolones in Quinolone-Resistant *Klebsiella pneumoniae*Strains. Antimicrob. Agents Ch. 42: 1850–1852.

Miyataka H, Nishiki M, Matsumoto H, Fujimoto T, Matsuka M, Satoh T (1997). Evaluation of propolis. I. Evaluation of Brazilian and Chinese propolis by enzymatic and physicochemical methods. Biol. Pharm. Bull. 20:496-501.

Podschun R, Ullmann U (1998). *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 11:589-603.

Salyers AA, Gupta A, Wang Y (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. TRENDS. Microbiol. 12:412-416.

Vogt RL, Dippold L (2005). *Escherichia coli* O157: H7 outbreak associated with consumption of ground beef, June-July 2002. Public Health Rep. 120:174-178.

Waldvogel FA (2000). *Staphylococcus aureus* (including staphylococcal toxic shock). In Principles and practice of infectious diseases. Philadelphia: Churchill Livingstone.

Walsh C (2003). Antibiotics: actions, origins, resistance. Washington, DC: ASM Press.

Wright GD (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. Adv. Drug Deliv. Rev. 57:1451–1470.

Wright GD, Sutherland AD (2007). New strategies for combating multidrug-resistant bacteria. Trends. Mol. Med. 13:260-267.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2413-2423, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.549
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

Investigation of temperature-induced physical instability of preserved coenzyme Q10-loaded (NLC): A comparative study at different temperatures

Wasfy M. Obeidat

Department of Pharmaceutical Technology, Faculty of Pharmacy, Jordan University of Science and Technology, P. O. Box 3030, Irbid, Jordan. Email: obeidatw@just.edu.jo. Tel: +962-2-7201000. Fax: +962-2-7201075.

Accepted 7 August, 2012

In this work, physical stability of coenzyme Q10 nanostructured lipid carriers (NLC) with and without selected preservatives was investigated at 4 and 40°C and compared to those obtained at 25°C. The NLC dispersions were produced using the hot high pressure homogenization, and physical stability was evaluated in terms of particle size and zeta potential analysis. At 40°C, six preserved coenzyme Q10 NLC were stable only on short term storage. The short term stability results obtained at 4°C were similar with those obtained at 25°C where seven preserved NLC were stable at both temperatures. However, six preserved dispersions were apparently more stable on long term storage at 4°C compared to those at 25°C. Thus, in addition to pentylene glycol and a mixture of pentylene and propylene glycol which exhibited stabilizing effects effect at 25°C, propylene glycol, phenonip and Euxyl PE 90 were found to have a stabilizing effect at 4°C. Results indicated that temperature is a major triggering factor of destabilization process at 40°C. Generally, stabilities were in the order 4°C > 25°C > 40°C. In addition, leakage of coenzyme Q10 occurred at 40°C only.

Key words: Nanostructured lipid, nanoparticle, coenzyme Q10, stability, temperature.

INTRODUCTION

Nowadays, nanostructured lipid carriers (NLC) are most commonly used for dermal application (Müller et al., 2007; Pardeike et al., 2009) and thus must be preserved due to the presence of water and frequent exposure to environmental contaminants after production. However, preservatives can impair NLC physical stability (Lippacher et al., 2002). A previous study (Obeidat et al., 2010) conducted at room temperature (25°C) using eleven preservatives different in molecular structure and hydrophobicity showed that the interaction and stabilization/destabilization mechanisms for coenzyme Q10 NLC were different from those affecting nanosuspensions of hesperidin with same preservatives (Al Shaal et al., 2010). The effect of preservatives varied from pronouncedly impairing the short and long term NLC stabilities such as those exhibited by Euxyl K700, Euxyl K702, MultiEx Naturotics and ethanol, to those that provide a stabilizing effects such as pentylene glycol and a mixture of pentylene and propylene glycol. On the other hand, preservatives such as propylene glycol, Rokonsal PB5, Euxyl PE9010, caprylyl glycol and phenonip exhibited no or minor impairment of the physical stability. Based on these findings, a classification system was suggested and a mechanistic model describing six key parameters affecting the physical stability of NLC was developed.

The present study was aimed at investigating the effect of refrigeration (4°C) and elevated (40°C) storage temperatures on the physical stabilities of preserved and non-preserved coenzyme Q10 NLC dispersions in terms of particle size and particle charge, comparing the results to those obtained at 25°C, and then identifying preservatives that showed stabilizing effects under these storage temperatures.

Selection of preservatives

Preservatives that vary in their properties and their use

Table 1. List of preservatives investigated, chemical composition, source, recommended concentration range and used concentration.

Name/trade name	Chemical composition	Source	Concentration (%w/w)	
			Effective range	Used
Hydrolite® S	Pentylene glycol (1.3-pentanediol)	COSNADERM GmbH (Ladenburg, Germany)	1.5 - 5.0	3.25
Dermosoft® Octiol	Caprylyl glycol (1,2-octanediol)	Brenntag GmbH (Mühlheim a.d. Ruhr,Germany)	0.5 - 1.0	0.75
Rokonsal®PBS	Phenoxyethanol 71 - 73%, methylparaben 14 - 15%, ethylparaben 5.5-6.1%, butylparaben 3.4 - 3.8%, propylparaben 2.2 - 2.6%, isobutylparaben 1.6 - 2%.	ISP Techn. Deutschland GmbH (Köln, Germany)	0.3 - 1.2	0.75
Phenonip®	Phenoxyethanol 70 - 75%, Methylparaben 14.5 - 16.5%, ethylparaben 3.3 - 4.3%, propylparaben 1.7-2.3%, butylparaben 3.4 - 4.3%, isobutylparaben 1.7 - 2.3%.	NIPA Laboratories Limited (Pontypridd, Great Britain)	0.5 - 1.0	0.75
Euxyl®PE9010	Phenoxyethanol 90%, ethylhexylglycerin (1,2-propanediol, 3-(2-ethylhexyloxy) 10%.	Schülke & Mayr GmbH (Norderstedt, Germany)	1.0	1.0
Propylene glycol®	Propylene glycol	CG Chemikalien GmbH (Laatzen, Germany)	10.0	10.0
Ethanol 96%®	Ethanol 96% (v/v)	CG Chemikalien GmbH (Laatzen, Germany)	>20.0 v/v%	20.0
Euxyl®k700	Phenoxyethanol 30%, benzyl alcohol 30%, potassium sorbate 15%, tocopherol 15%, water 15%.	Schülke & Mayr GmbH (Norderstedt, Germany)	0.5 - 1.5	1.0
Euxyl®k702	Phenoxyethanol 77%, benzoic acid 13%, dehydroacetic acid 6%, polyaminopropyl bigunide 2%, ethylhexylglycerin 1%, water 2%.	Schülke & Mayr GmbH (Norderstedt, Germany)	0.2 - 1.0	0.6
MultiEx Naturotics™	*Magnolia biondii* bark extract 44%, *Salix alba* (Willow) bark extract 15%, butylene glycol 15%, *Citrus grandis* (Grapefruit) fruit extract 6.0%, *Camellia sinensis* leaf extract 6.0%, Propolis extract 1.5%, *Thujopsis dolabrata* extract 0.15%, *Chamomilla recutita* (Matricaria) extract 0.16%, alcohol 0.1%, water 0.1%.	MAWI Industrieagentur OHG (Limburgerhof, Germany)	1.0 - 3.0	2.0

(pharma, cosmetic and nutrition) were chosen. In addition, mixtures of preservatives that possess synergistic action and more favourably preservatives that can provide skin caring action in addition to their antimicrobial activities were chosen. Table 1 gives an overview of the presser-vatives selected, their chemical composition, their concentration range typically used and the concentration selected for the study.

Preservatives concentrations were selected being in the middle of the typical suggested concentration range (Obeidat et al., 2010).

MATERIALS AND METHODS

Reagent composition

The composition was 14.45% Precifac® ATO (cetyl palmitate, kindly provided by Gattefosse, France), 0.75% Miglyol® (Caelo GmbH, Germany), 1.80% TegoCare®450 (polyglyceryl-3-methylglucose distearate, Goldschmidt AG, Germany), 4.50% coenzyme Q10 (Q10, BIK Internationaler Handel GmbH, Switzerland) and ultra-purified water up to 100.0% (MilliQ, Millipore, USA). The preservatives, source and used concentrations are shown in Table 1.

Preparation of the Q10-loaded NLC and addition of preservatives

The Q10-loaded NLC dispersions were produced as per (Umbach, 2004) using high pressure homogenizer (Micron LAB40, APV Deutschland GmbH, Unna, Germany) applying two cycles at 800 bars at 85°C.

The preservatives (and if necessary, water up to 100.0%) were added to NLC concentrate at room temperature by gentle stirring. The samples were stored in dark places at 4, and 40°C for one year to compare results to those at 25°C.

Particle size characterization

Particle size analysis (PCS) and laser diffraction (LD) were performed using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK) and a Malvern Mastersizer 2000 (Malvern instruments, Malvern, UK), respectively. The LD data obtained were evaluated using volume distribution as diameter (d) values of 50, 90, 95 and 99%. The results were analyzed using Mie theory with optical parameters 1.456 (real refractive index) and 0.01 (imaginary refractive index).

Light microscopy

Light microscopy was used as an additional characterization method to detect possible large particles (Keck, 2006; Keck and Müller, 2008) using an Orthoplan (Leitz, Germany) equipped with a digital camera and an Olympus CX41 (Olympus Deutschland GmbH, Germany). The potential presence of drug crystals was monitored using polarized light.

Zeta potential measurements

The zeta potential (ZP) was measured by determining the electrophoretic mobility using the Malvern Zetasizer ZS (Malvern Instruments, UK) first in purified water after adjusting the conductivity to 50 µS/cm using 0.9% (w/w) sodium chloride (NaCl) solution.

Secondly the ZP was measured in the original dispersion medium of each sample.

RESULTS AND DISCUSSION

Production of Q10-loaded NLC

High pressure homogenization led to the production of homogeneous and small sized Q10-loaded NLC with a mean particle size of 196 nm and a Hein plaque index (PI) of 0.12. The zeta potential (ZP) in water was -46mV and was -37mV in the original dispersion medium. The difference in measured ZP between the two media is attributed to desorption of some non-ionic stabilizer molecules from the surface of the particles in water (Obeidat et al., 2010; Müller, 1996; Malvern, 2011).

Zeta potential measurements

After storage at all studied temperatures for one month, no significant changes in ZP were detected for most of the preservatives when ZP was assessed in water (practically identical to Stern potential) (Figure 1). All values were > I30I mV even for visually unstable dispersions. Generally, ZP was reduced upon the addition of preservatives except for propylene glycol at 25°C, caprylyl glycol, Euxyl PE9010, ethanol and Euxyl K 700 at 4°C and phenonip, Euxyl PE9010, ethanol and Euxyl K 700 at 40°C. In this study the same NLC dispersion was used for all preserved samples. Therefore in theory, independent on the added preservative, in water a similar Stern potential should be measured for all samples. This was the case for most of the NLC dispersions. However deviations can occur if preservatives are tightly adsorbed on the particle surface and do not desorbed upon the dilution with water (e.g. MultiEx).

Some preservatives appeared to reduce ZP of NLC dispersions significantly to values below the critical value of I30I mV defined by Riddick (Riddick, 1968) while others only slightly affected it. The extent of stability impairment depends on the extent of interference of the preservative with the stabilizer film behavior. We found that at all storage temperature after one month, both pentylene glycol and MultiEx reduce ZP when added to NLC and analyzed in water (Figure 1) indicating the adsorption of the preservative onto the surface of particles. Whereas MultiEx strongly impaired the stability of the NLC (ZP =-35), pentylene glycol (ZP = -37) was stable. Thus, even though both systems had ZP above I30I mV in water, MultiEx seems to disrupt the stabilizer film and reduce ZP since molecules of MultiEx are tightly adsorbed on the particle surface and did not desorb upon dilution with water. Moreover, pentylene glycol was less potentially adsorbed, thus it did not disturb the stabilizer film; and what practically adsorbs to NLC surface could be desorbed easily with water dilution. In other words, a dispersion with a value > I30I mV does not guarantee its physical stability. However, for long term stability, the

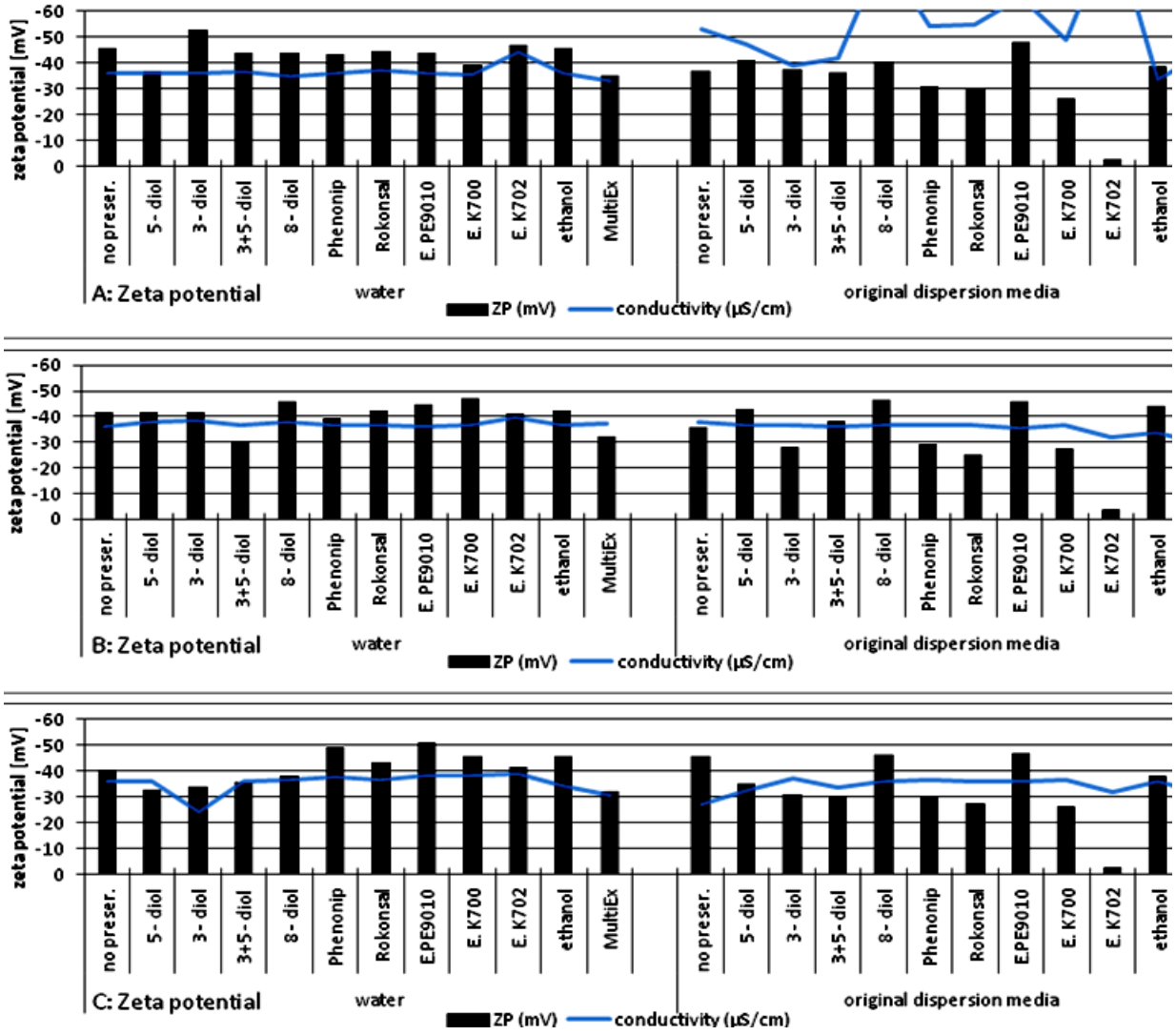


Figure 1. Zeta potential of non-preserved NLC and preserved NLC measured in conductivity water (50 µS/cm, left) and in the original dispersion media (right) one month after adding the preservatives at 25°C (A), 4°C (B) and 40°C (C).

dispersion should have high ZP (> I30I mV) along with other factors to attain stability.

Measuring ZP in the original dispersion medium is a measure of the thickness of the diffuse layer (Müller, 1996). The lower the zeta potential, the thinner the diffuse layer and the less stable is the particle dispersion. ZP values larger than I30I mV were found for the non-preserved NLC dispersion, and for the seven stable preserved NLC dispersions at 25°C (ZP =-37.1), 4°C (ZP =-35.9) and at 40°C (ZP =-45.6). In contrast, ZP values for NLC dispersion which were preserved with Euxyl K700 and MultiEx Naturotics were close to zero (-2.4, -2.9 mV) at 25°C, (-3.8, -2.9 mV) at 4°C, and (-3, -2.1 mV) at 40°C, respectively. Euxyl K700 exhibited a higher ZP of (-26.4 mV, -27.5 mV and -26.4 mV at 25, 4 and 40°C, respectively) which indicated slower aggregation and was fully confirmed by PCS and LD data.

Physical short and long term stability of NLC

Physical short term stability

During the first month of storage period, PCS analysis of the seven best preservatives at day 1 did not show any significant increase in PCS diameter at all storage temperatures (Figure 2). Ethanol preserved NLC continued to grow in particle size at 4 and 25°C to 210 and 233 nm, respectively. However, at 40°C, ethanol preserved systems had a very slight decline in the size to 210 nm starting at 215 at day 1. Although NLC dispersion preserved with Euxyl K700 showed a slight increase in the particle size at 25 and 40°C, the largest increase in particle size occurred at 4°C, reaching 457 nm at day 30. The PCS diameters of MultiEx Naturotics and Euxyl K702 were very pronounced at all storage temperatures. In

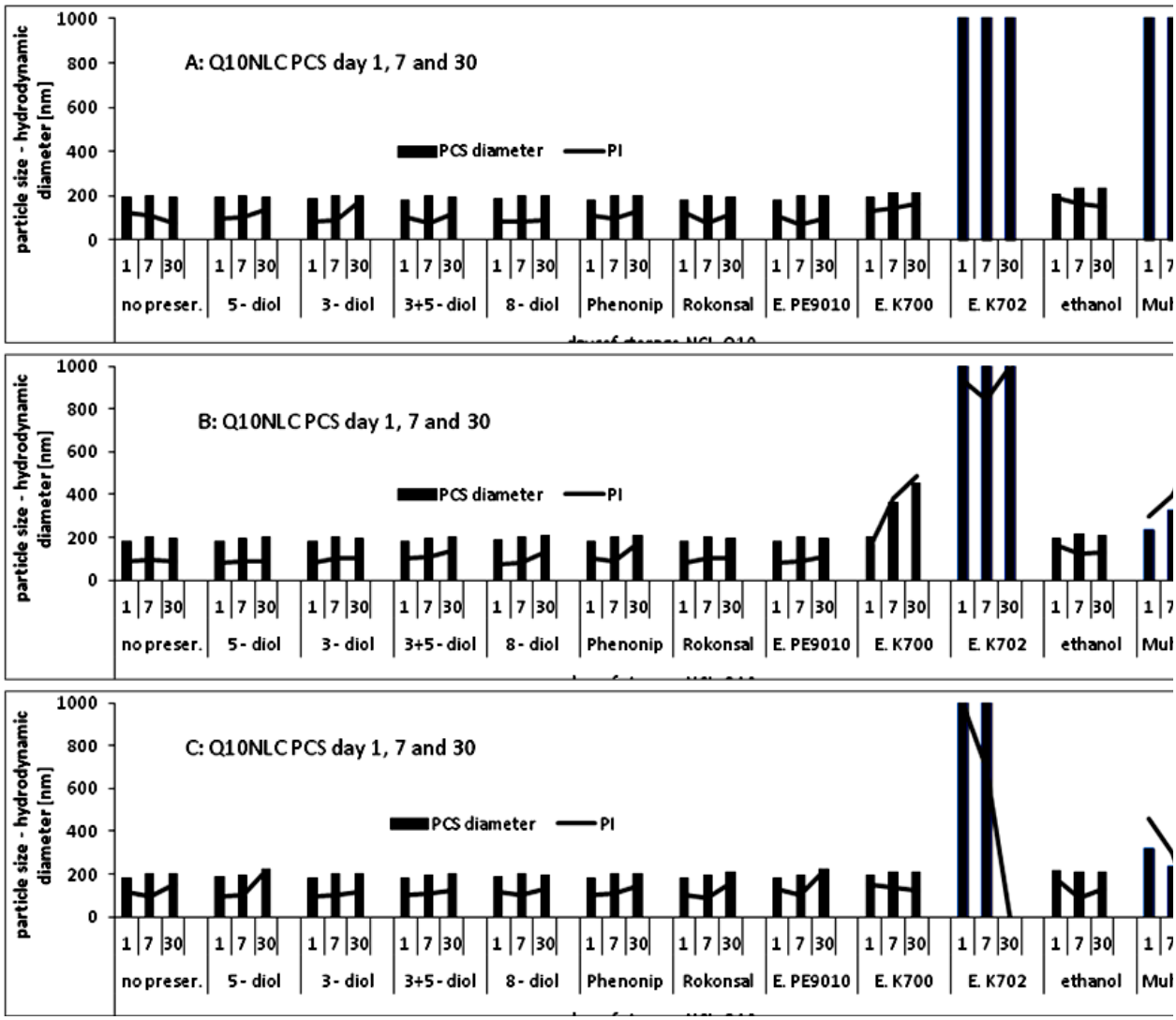


Figure 2. PCS mean particle size (nm) and polydispersity index (PI) of preserved versus non-preserved Q10-loaded NLC dispersions (NLC Q10), after 1 day, 7 days and 1 month of adding the preservatives at 25°C (A), 4°C (B) and 40°C (C).

addition, it should be noted that NLC formulations preserved with Euxyl K700 and K702 showed an apparent increase in their viscosities; they became slightly semi-solid (paste-like) at all storage temperatures during the first month. Similarly, systems containing MultiEx Naturotics showed a remarkable increase in viscosity at 4 and 25°C, while they were solids at 40°C, a matter that imposes difficulties in sampling by the end of the first month.

Furthermore, LD analysis confirmed the stability of the best preservatives identified by PCS. As shown in Figure 3, little insignificant changes in D99% were observed during one month storage period at 4 and 25°C. Similar results were noticed at 40°C except for NLC dispersions containing caprylyl glycol where D99% of 152 µM was recorded at day 30. The instability of NLC dispersions preserved with ethanol, Euxyl K700, Euxyl K702 and MultiEx Naturotics became very clearly visible.

Physical long term stability

For the seven preservatives which resulted in stable NLC dispersions on the short term study, PCS data again led to no changes for the non-preserved NLC and for the systems preserved with propylene glycol, pentylene glycol, the mixture of these preservatives, Euxyl 9010 and Rokonsal PB5 after one year of storage at both 4 and 25°C as shown in Figure 4. However, NLC preserved with Phenonip showed a small increase in size over this period at 25°C and an increase in size in both NLC dispersions preserved with Phenonip and caprylyl glycol

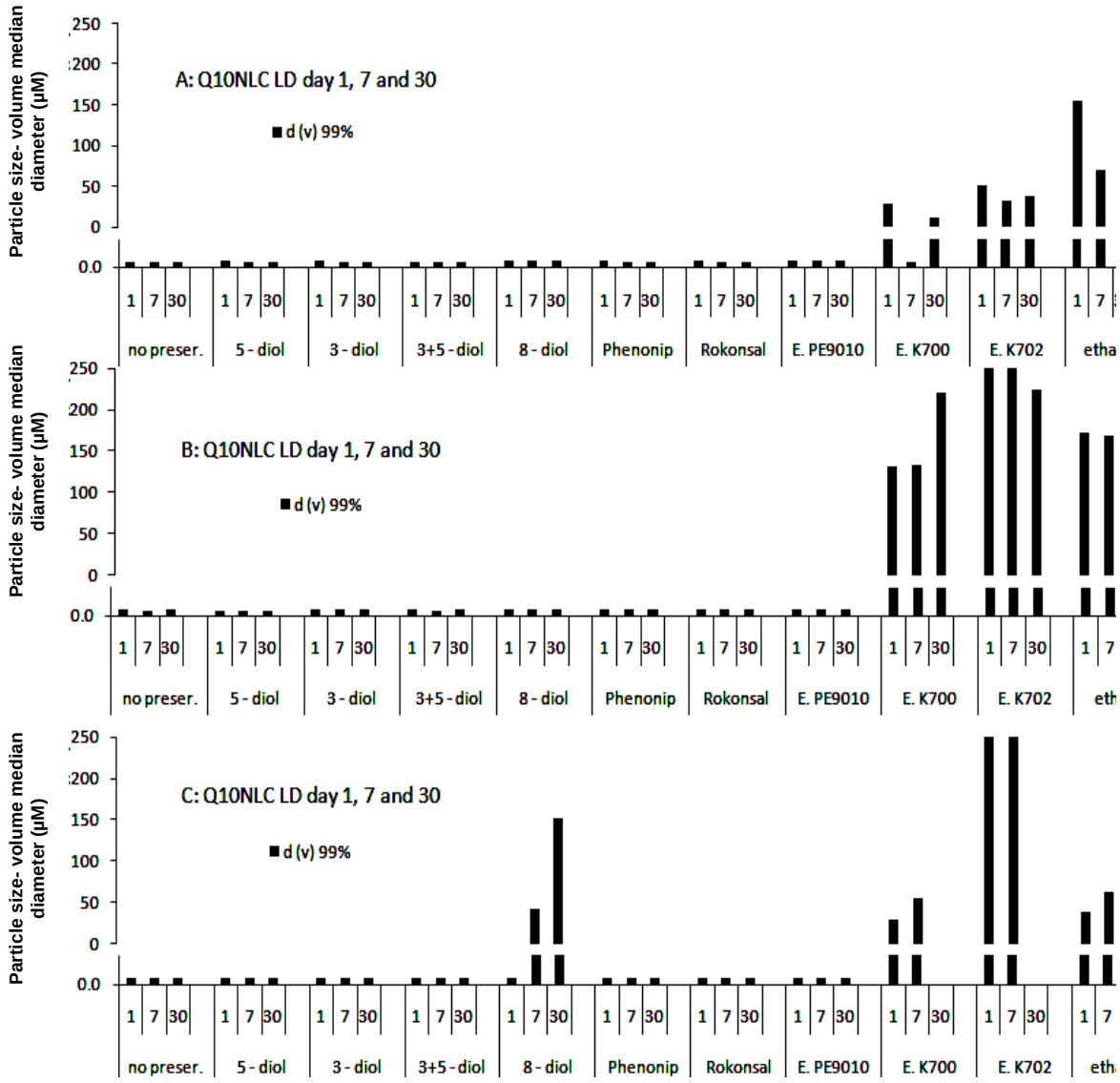


Figure 3. LD 99% diameters (µM) of preserved versus non-preserved Q10-loaded NLC dispersions (NLC Q10) stored at 25°C (A), 4°C (B) and 40°C (C) after one month of adding the preservatives.

stored at 4°C. At 40°C, all NLC systems, including those containing the seven preservatives, showed continuous increase in size after 6 months of storage and by the end of one year all systems showed separations and were impractical for particle size measurements. Results were confirmed by statistical analysis using non-parameteric test. Wilcoxon test was used to compare between samples at the same temperature while Friedman test was used to compare means for the sample at different temperatures (level of signicance 0.05). Analysis of PCS data confirmed the observed aforementioned results. Test statistics at 4 and 25°C after 12 months were: 0.202, 0.202, 0.207, 0.20, 0.182, and 0.368 for non-preserved NLC (control), systems containing pentylene glycol, propylene glycol, mixture of these preservatives, Rokonsal, and EPE which indicates insignificant difference in particle size for those systems with test stistic being more than 0.05.

LD analysis represented by D50% and D95% is shown in Figure 5. Primarily, unlike at 4°C, certain instability of the non-preserved NLC concentrates after six months of storage at 25 and 40°C was noticed. This was more pronounced after 12 months of storage. Generally, all systems preserved by the seven preservatives mentioned earlier were stable when stored at 4°C according to LD analysis throughout the one year period. In storage at 40°C, all NLC dispersions were unstable and separated by the end of the year including non-preserved ones. However, few systems such as those preserved with propylene glycol, mixture of propylene glycol and

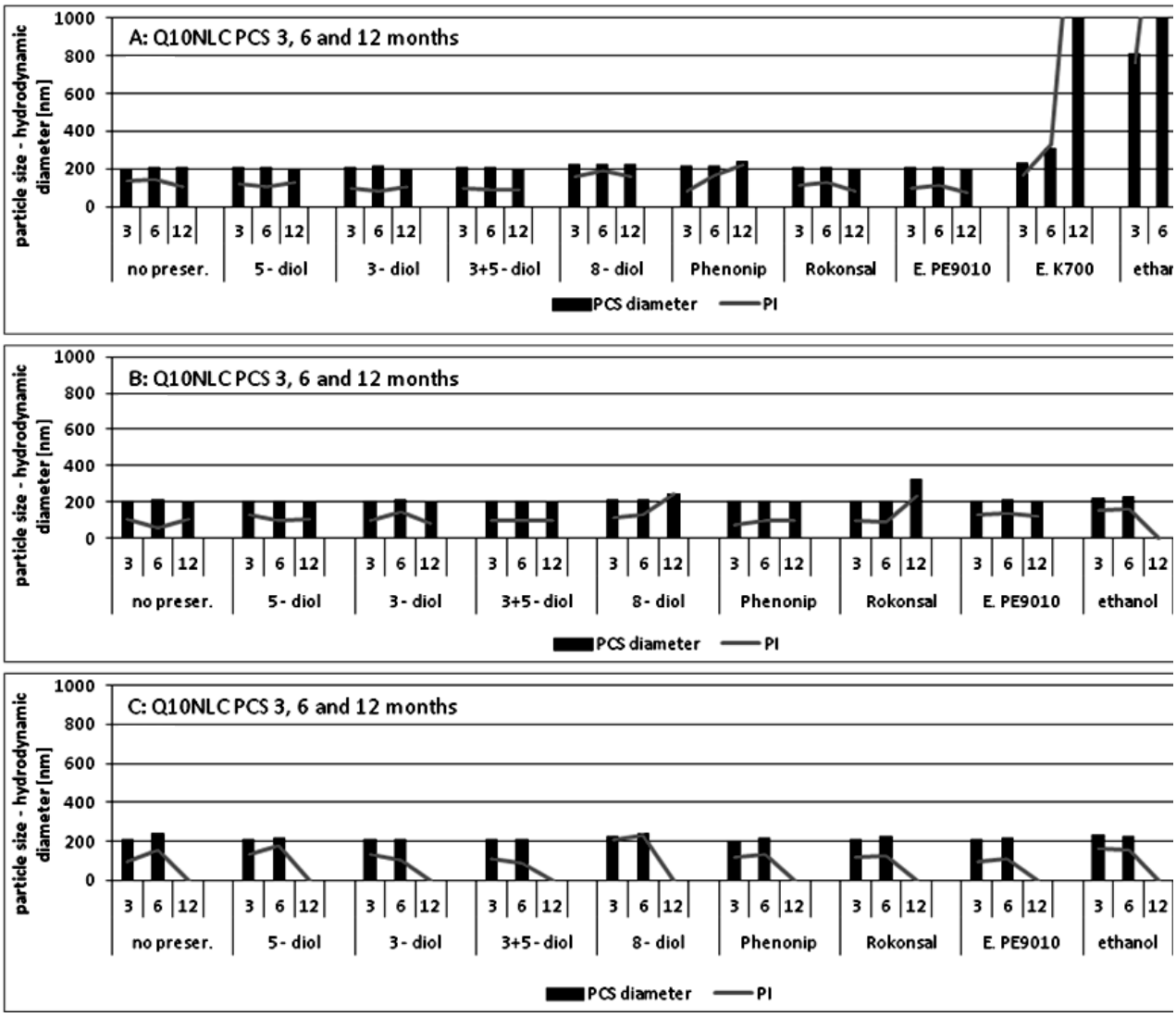


Figure 4. PCS mean particle size (nm) and polydispersity index (PI) of preserved versus non-preserved Q10-loaded NLC dispersions (NLC Q10), after 3, 6, and 12 months of adding the preservatives at 25°C (A), 4°C (B) and 40°C (C).

pentylene glycol, Phenonip and Euxyl 9010 showed stability for 6 months at this temperature. On the other hand, test statistics obtained from using Wilcoxon's and Friedman's non-parametric tests (level of signicance 0.05) after 6 months were all below 0.05 except for mixture of propylene glycol and pentylene glycol (test statistic 0.218), indicating that means are significantly different for the same sample at different tempertures except for NLC systems containing this mixture.

Macroscopic observations and effect of temperature

The obtained results revealed that temperature had a strong effect on the stability of Q10-loaded NLC. Heat, as well as light, is known to initiate the reaction leading to the formation of free radical. This free radical combining with the oxygen molecule causes the propagation step and finally the oxidation progresses to the termination step (chain reactions) (Alexander and David, 2006). Fortunately, all NLC dispersions stored at 4 and 25°C were considerably stable for at least one year in this regard; no Q10 crystals were apparent in all systems. This proves that Q10 was firmly enclosed in the particle matrix, no expulsion of the active occurred. Storage of Q10 NLC at low temperature can therefore minimize chain reactions and oxidation reaction can then be retarded.

However, this was not the case for NLC dispersions stored at 40°C; Q10 crystals started to show up after the short term study at about three months of storage, which became more obvious after six months of storage. These

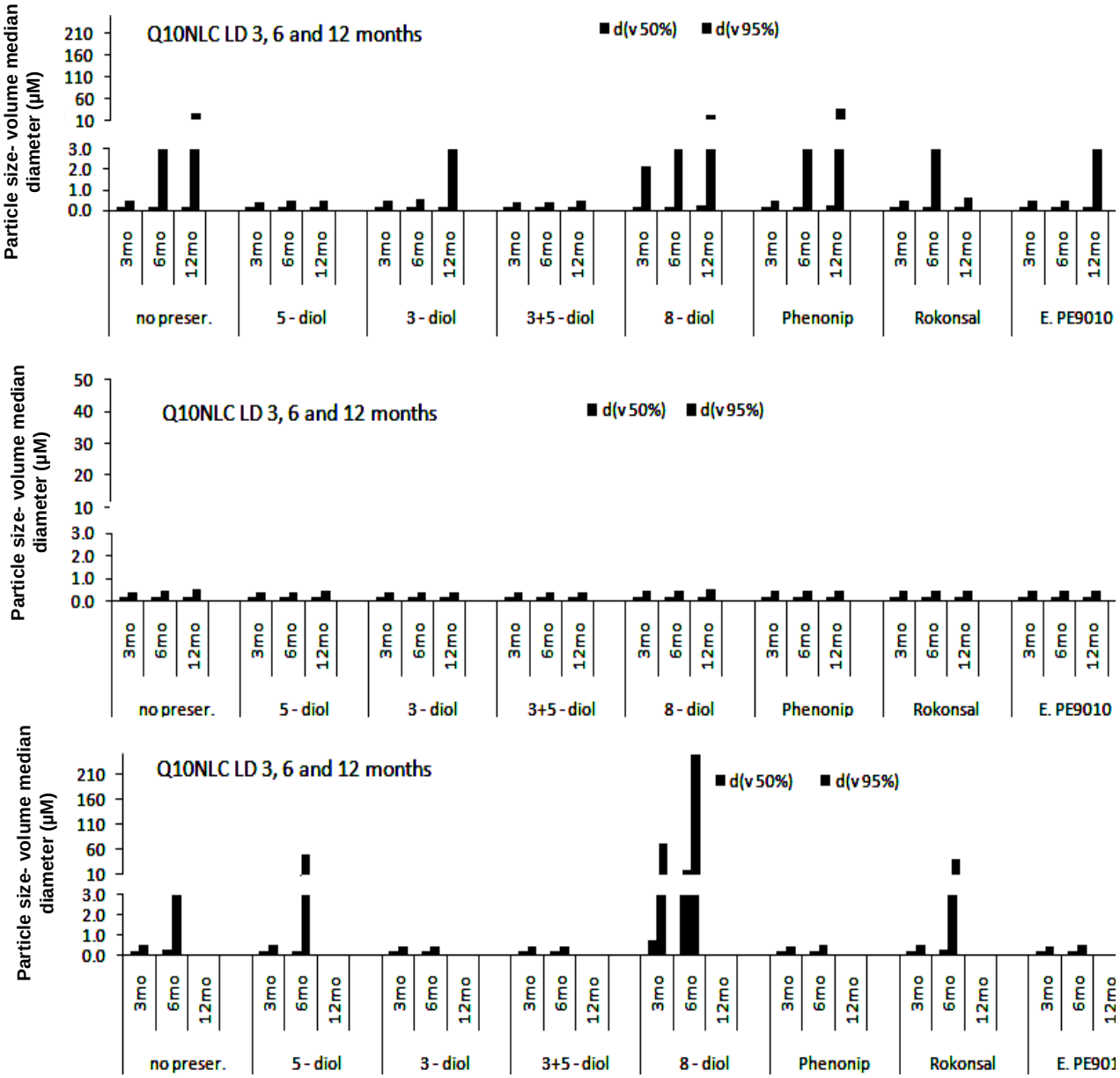


Figure 5. LD 50% and 99% diameters (µM) of preserved versus non-preserved Q10-loaded NLC dispersions (NLC Q10) stored at 25°C (A), 4°C (B) and 40°C (C) after 3, 6, 12 months of adding the preservatives.

dispersions end up in separation into two layers as shown in Figure 6 where NLC dispersions stored at different temperatures are also shown for comparison. This indicates that Q10 has been pushed out of the NLC matrix during long term storage at 40°C. Previous studies indicated that this kind of active ingredient expulsion could occur when the lipid transfers to the more ordered beta confirmation (crystallization) (Westesen and Siekmann, 1997). Such a transition in crystallization can happen statistically during storage, but also can be triggered by the incorporated active itself (Souto and Müller, 2006), light irradiation (Freitas and Müller, 1998) or excipients in the formulation. In this study, it was concluded based on the absence of Q10 crystals in NLC dispersions stored at 4 and 25°C that none of the preservatives affected the particle matrix structure to cause any expulsion of active. Thus, all preservatives were excluded as being triggering factors for drug expulsion.

The effects of temperature on the stability of SLN suspensions was also studied by Freitas (Freitas and Müller, 1999), where Compritol solid lipid nanoparticles (SLN) was investigated at 8, 20 and 50°C under exclusion of light. Storage of Compritol SLN at

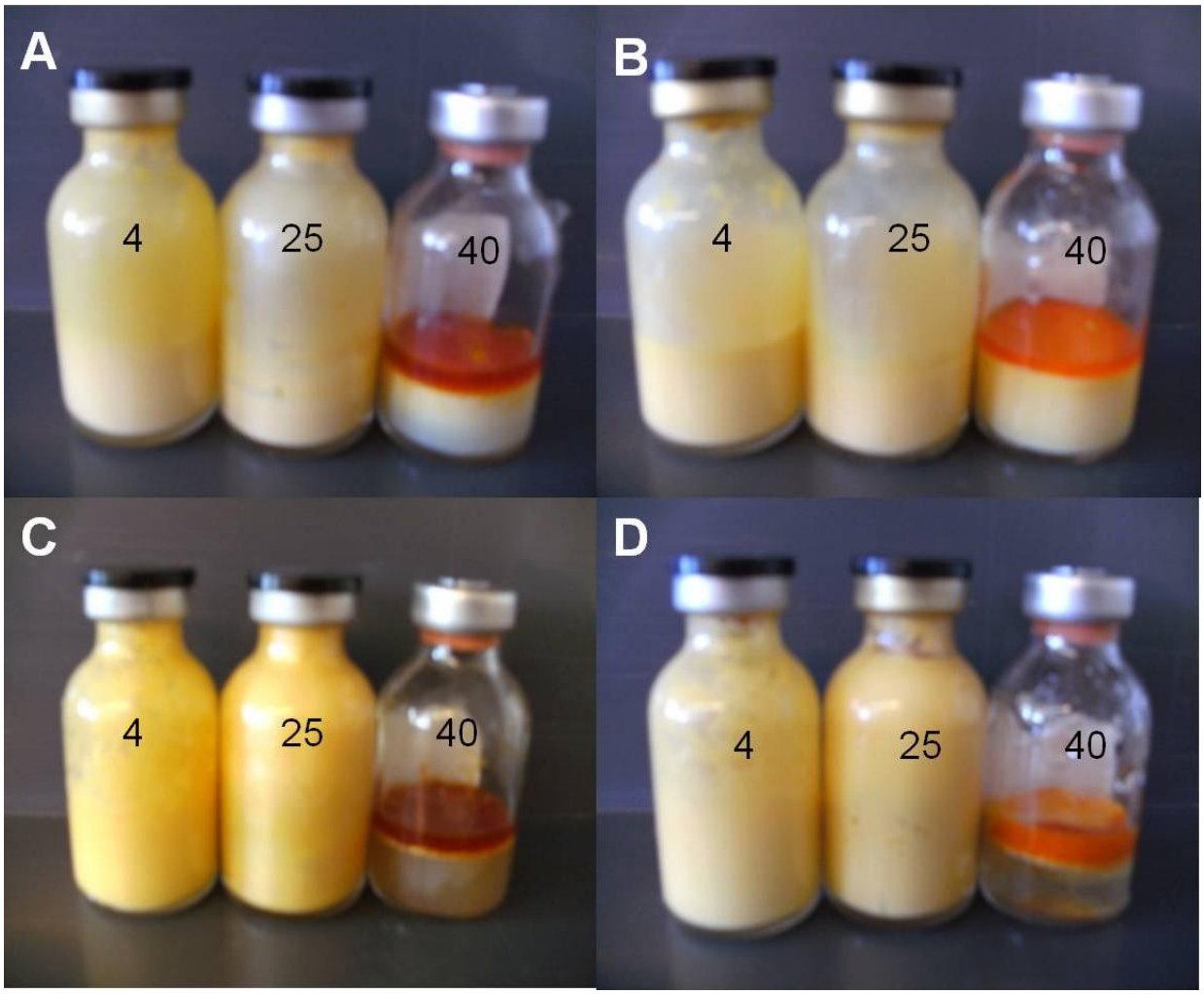


Figure 6. Macrographs showing the long term physical stability of A: Non-preserved Q10 NLC, B: Q10 NLC preserved with 5 diol + 3 diol, C: Q10 NLC preserved with Euxyl K702, and D: Q10 NLC preserved with MultiEx Naturotics.

50°C was found to induce rapid particle growth within 3 days and all samples were solid after 7 days and input of kinetic energy (temperature) causes a transformation to β' accompanied by gel formation. Schuhmann (1995) suggested that an increase in temperature reduces the micro viscosity of film of emulsifier around dispersed particles leading to destabilization. This could be one explanation for the separation and expulsion of drug out of Precifac® ATO/Miglyol® particles stabilized by TegoCare®450 in this study. Another factor is surely the presence of the oil as an essential constituent in the formation of Q10 NLC.

Oils become more fluid at higher temperatures and could easily leak out when its viscosity decreases. It is also suggested that the dominant influence of temperature in NLC dispersions is the properties of the lipid and oil. The breakage and separation may be also due in part to the decrease in the viscosity of the continuous phase with rising temperature and also to the increase aqueous solubility of the active ingredient at elevated temperatures. It is apparent from the six key parameters revealed in a previous study which described the physical stability at 25°C that stabilizer anchoring and the integrity of the film around the nanoparticles are compromised at 40°C, thereby resulting in separation and breakage of the NLC dispersion. However, this did not occur at 4°C indicating that stabilizer film and its anchoring were maintained at this temperature. The zeta potential data shown in Figure 1 did not reveal significant changes at 40°C compared to 4 and 25°C at the short term which might indicate that the aforementioned mechanisms of destabilization at 40°C might require quite some time to occur, ending up with complete drug expulsion and emulsion separation. In summary, although refrigeration assured the stability of Q10 NLC dispersions for long times, storage at room temperature (25°C) restores the NLC stability for long time also with more convenient and economically favoured way.

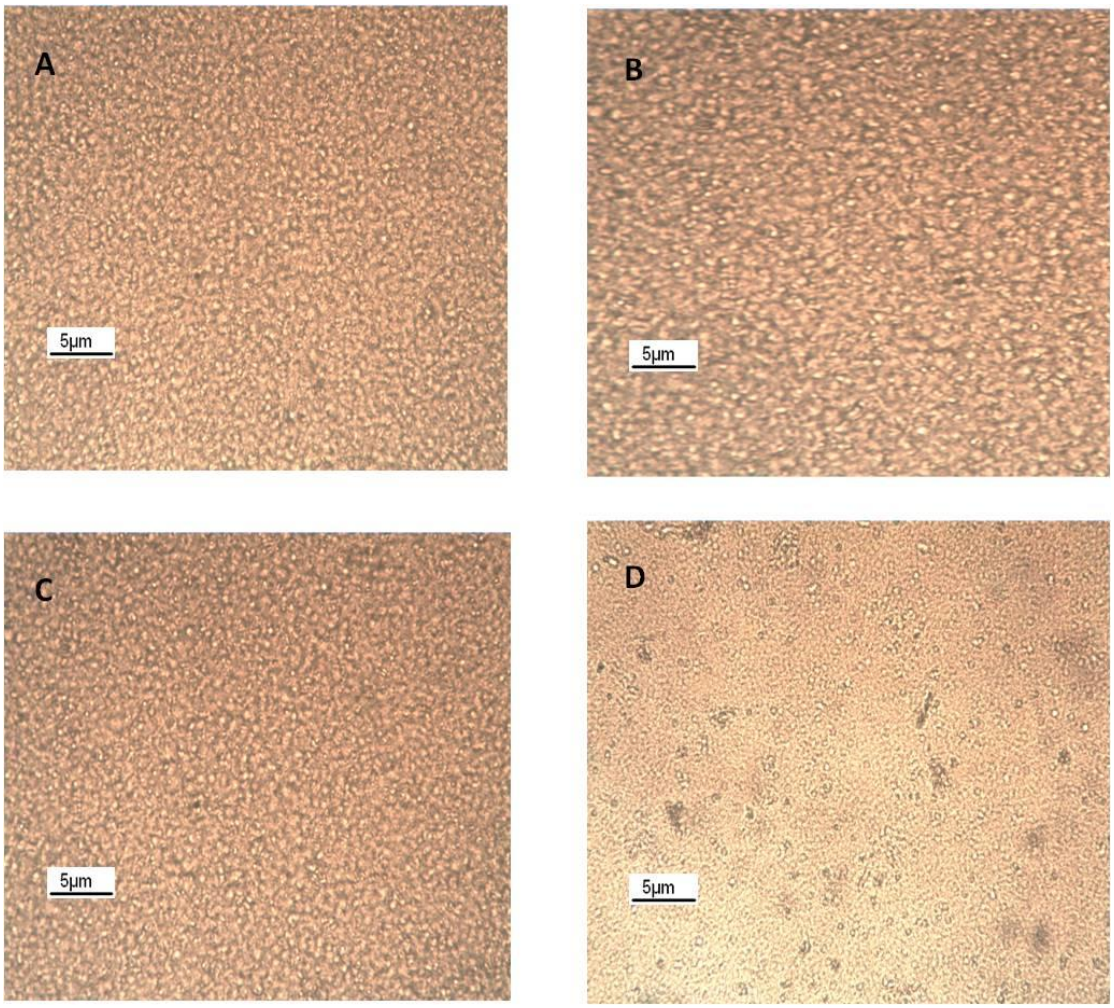


Figure 7. Light microscopy images (magnification 630 folds) of Q10-loaded NLC non-preserved dispersions (A), stable dispersions preserved with Euxyl PE 9010 (B) and with a combination of pentylene glycol and propylene glycol at 5:3 ratio (C), and an instable dispersion stabilized with MultiEx Naturotics (D), after one month of storage at room temperature (bar size: 5 µM).

Microscopic evaluation

Microscopy cannot analyze the sizes of nanoparticles below 500 nm, however, it is a very useful tool to screen for the absence of aggregates above 1 µM. To increase the likelihood of detecting even a very low amount of aggregates, undiluted dispersions need to be analyzed (Müller and Heinemann, 1991). In case of a very low number of large particles, very often they cannot be detected by LD, hence the microscope is more sensitive (Keck and Müller, 2005). Microscopic analysis was performed directly after production of the NLC dispersion, and one month of storage at 4, 25 and 40°C after the addition of preservatives. Microscopic analysis was well in agreement with PCS and LD data. In the systems identified as stable, no increase in the number of particles above 1 µM was found. The seven best preservatives not impairing the physical stability that were identified are: Rokonsal, Phenonip, Euxyl PE 9010, propylene glycol, pentylene glycol (Hydrolite 5) and a mixture of propylene glycol and pentylene glycol at all storage temperatures. A clear increase in aggregates was detected for the instable NLC systems, preserved with ethanol, Euxyl K700, Euxyl K702 and MultiEx Naturotics at all storage temperatures and for caprylyl glycol at storage temperature of 40°C. Microscopic graphs at 25°C various systems are shown in Figure 7.

Conclusions

A surprisingly high number of seven different preservatives could be identified as suitable for long term preservation of NLC concentrated dispersions at 4 and 25°C. Unlike storage at 25°C, refrigeration at 4°C proved to be superior in maintaining the integrity and the long

term stability of these dispersions preserved using these seven preservatives. However, storage of Q10 NLC at 40°C resulted in long term destabilization and separation of NLC dispersions. The six key parameters earlier described as affecting the physical stability at 25°C will still be valid for the other storage temperatures with stabilizer film integrity and stabilizer anchoring disrupted at 40°C. This is believed to be the major factor in drug expulsion and dispersion breakage, and separation in long term storage at 40°C. It is, therefore, recommended that stabilizers that can compensate as much as possible for destabilization by the preservative should be used; that is stabilizers adsorbed in a dense packaging and having a thick sterically stabilizing layer of polymer tails.

ACKNOWLEDGEMENTS

The author would like to thank the DFG (Germany) and JUST (Jordan) for their support during this study. Materials and equipment were provided by PharmaSol GmbH Berlin. The author also thanks Professor Rainer. H. Müller and Cornelia M. Keck for their kindness and the extraordinary effort and help in finishing the first paper, and Ms. Corinna Schmidt for her assistance.

REFERENCES

Al Shaal L, Müller RH, Keck CM (2010). Preserving hesperetin nanosuspensions for dermal application. Pharmazie 65 (2): 86-92.

Alexander TF, David A (2006). Drug stability. In: Alexander TF, David A (Eds.), Physicochemical Principles of Pharmacy. Pharmaceutical Press, London pp. 93–138.

Freitas C, Müller RH (1998). Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN™) dispersions. Int. J. Pharm. 168:221–229.

Freitas C, Müller RH (1999). Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase. Eur. J. Pharm. Biopharm. 47 (2):125–132.

Keck CM (2006). Cyclosporine nanosuspensions: optimised size characterisation and oral formulations. PhD thesis. Freie Universita¨t Berlin, Berlin. p.42.

Keck CM, Müller RH (2005). Particle Size Analysis with Laser Diffractometry is not Sensitive Enough to Detect Changes in a Lipid Carrier System. AAPS, Nashville.

Keck CM, Müller RH (2008). Size analysis of submicron particles by laser diffractometry – 90% of the published measurements are false. Int. J. Pharm. 355 (1-2):150–163.

Lippacher A, Müller RH, Mäder K (2002). Semisolid SLN dispersions for topical application: influence of formulation and production parameters on viscoelastic properties. Eur. J. Pharm. Biopharm. 53 (2):155–160.

Malvern (2011). Zeta Potential: An Introduction in 30 min, in: Application Note <http://www.malvern.co.uk>, MRK654 01.

Müller RH (1996). Zeta potential und Partikeladung in der Laborpraxis, Band 37, in: A. Paperback (ed.), Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, pp. 37

Müller RH, Heinemann S (1991). Photon correlation spectroscopy and zeta potential characterization of model particles and colloidal drug carriers – essential information for the interpretation of cell culture studies. Biochem. Soc. Trans. 19 (2):502.

Müller RH, Petersen RD, Hommoss A, Pardeike J (2007). Nanostructured lipid carriers (NLC) in cosmetic dermal products. Adv. Drug Deliv. Rev. 59(6):522–530.

Obeidat WM, Kay S, Müller RH, Keck CM (2010). Preservation of Nanostructured Lipid Carriers (NLC). Eur. J. Pharm. Biopharm.., 76 (1):56–67.

Pardeike J, Hommoss A, Müller RH (2009). Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products. Int. J. Pharm., 366 (1-2): 170–184.

Riddick TM (1968). Control of Colloid Stability through Zeta Potential, Zeta-Meter Inc. via Livingston Publishing Company, Wynnewood. p.91.

Schuhmann R (1995). Physikalische Stabilita¨t parenteraler Fettemulsionen—Entwicklung eines Untersuchungsschemas unter besonderem Aspekt analytischer Mo¨ glichkeiten. Ph.D. thesis. Freie Universita¨t Berlin, Berlin pp. 262-264.

Souto EB, Müller RH (2006). Investigation of the factors influencing the incorporation of clotrimazole in SLN and NLC prepared by hot high-pressure homogenization. J. Microencapsulation 23 (4):1–12.

Umbach W (2004). Kosmetik und Hygiene: von Kopf bis Fuß, Wiley VHC, Weinheim. pp. 123-128.

Westesen K, Siekmann B (1997). Investigation of the gel formation of phospholipidstabilized solid lipid nanoparticles. Int. J. Pharm. 151:35-45.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2424-2428, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.700
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Development and validation of high-performance liquid chromatographic-ultraviolet (HPLC-UV) method for the bioequivalence study of atorvastatin

Muhammad Sher[1], Ghulam Hussain[1], Muhammad A. Hussain[1], Taslim Akhtar[1], Muhammad R. Akram[2], Rizwan N. Paracha[1] and Ghulam Murtaza[3]*

[1]Department of Chemistry, University of Sargodha, Sargodha 40100, Pakistan.
[2]Department of Pharmacy, University of Sargodha, Sargodha 40100, Pakistan.
[3]Department of Pharmaceutical Sciences, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan.

Accepted 18 July, 2012

A sensitive and rapid high-performance liquid chromatographic method with ultraviolet detection was developed and validated for the determination of atorvastatin in human plasma. After separation of drug from plasma samples and following protein precipitation, the chromatographic separation was accomplished using C_{18} analytical column. The mobile phase consisted of methanol, acetonitrile, and sodium phosphate buffer (0.01 M, pH 4.5) in the ratio of 40:30:30 (v:v:v). The ultraviolet detection was done at 247 nm. The average recovery of drug was 98.7%, with a limit of detection of 7.82 ng/ml and limit of quantification of 22.86 ng/ml. The calibration curve was found to be linear in concentration range of 5 to 160 ng/ml of atorvastatin, both in mobile phase as well as in plasma. After studying the performance parameters, the method was applied in a randomized, cross-over bioequivalence study of two different tablet preparations of atorvastatin (Lipitor, the brand leader and Lipirex, the generic product) in twelve healthy male volunteers. The values for C_{max}, T_{max}, and AUC_{0-t} for Lipitor and Lipirex were found to be 50.5 ng/ml, 3 h and 882.5 h.ng/ml and 49.5 ng/ml, 3.2 h, and 845.5 h.ng/ml, respectively. The pharmacokinetic parameters of both preparations were found to be comparable; hence, can be regarded as bioequivalent.

Key words: Atorvastatin, bioequivalence, high-performance liquid chromatographic-ultraviolet (HPLC-UV).

INTRODUCTION

Atorvastatin (AT), [R-(R*,R*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4[phenylamino) carbonyl]-1H-pyrrole-1-heptanoic acid, is a synthetic member of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, better known as statins (Mason, 2004). Statins are the most commonly used lipid-lowering drugs that help in treating dyslipidemia (one or more disorders caused by lipids) (Nirogi et al., 2007; Sassano and Platanias, 2008). Dyslipidemia plays a central role in the pathogenesis of atherosclerosis, which in turn produces coronary heart disease (Koda, 2005; Marks et al., 1996; Wu et al., 2005). HMG-CoA reductase is the enzyme responsible for catalyzing the reduction of HMG-CoA to mevalonate, the major rate limiting step in cholesterogenesis (Marks et al., 1996). Statins possess a side group similar in the structure to HMG-CoA, the natural substrate of HMG-CoA reductase, and hence act as reversible, competitive inhibitor of this enzyme. Consequently, blood cholesterol level is reduced by statins. In fact, statins cause up-regulation of low-density lipoprotein (LDL)-receptors which results in increased removal of the LDL from the blood, thereby, reducing LDL-cholesterol level (Goodman et al., 2001). It shows rapid absorption after oral administration, with an absolute bioavailability of about 14%. Presystemic clearance takes place in gastrointestinal mucosa, and/or the first-pass metabolism in liver to which low systemic

*Corresponding author. E-mail: gmdogar356@gmail.com. Tel: 0092-314-2082826. Fax: 0092-922-383441.

availability can be attributed (Mason, 2004; Goodman et al., 2001).

Due to such a low bioavailability, a sensitive and specific method of analysis is required for the quantification of atorvastatin in its pharmacokinetic and bioequivalence studies (Bahrami et al., 2005). Nearly all the assays that are being used for the separation of statins rely on gas-chromatography (GC) or high performance liquid chromatography (HPLC); the latter being more popular, and based on reverse phase separation for all the statins. The detector mostly used is the UV-detector; however, a mass spectrometer and rarely a fluorescence detector have also been employed in some methods.

Before injection of the samples to the chromatographic system, some purification steps have also been used, like solid-phase extraction, liquid-liquid extraction, etc. Most of the assays involve the use of one statin as the internal standard on account of the analogous structures of the substances (Erturk et al., 2003). The existing analytical methods for atorvastatin include an enzyme inhibition assay (Velesky et al., 2008), HPLC-mass spectrometry (HPLC-MS) (Bullen et al., 1999), HPLC-tandem mass spectrometry (HPLC-MSMS) (Hermann et al., 2005), high performance thin layer chromatography (HPTLC) (Jamshidi et al., 2007), liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) (Miao and Metcalfe, 2003; Ma et al., 2007), HPLC with ultra-violet detection (HPLC-UV) for determination of atorvastatin in human serum with a complicated method of extractions (Bahrami et al., 2005), and HPLC-UV for its determination in the bulk drug (Erturk et al., 2003; Khan et al., 2011).

Bioavailability and pharmacokinetics of drugs allow the manipulation of their clinical, therapeutic, and toxic effects. Additionally, these facilitate in monitoring the route of therapy. The difference in the bioavailability of various formulations of the same drug, having same strength and in the same dosage form causes a special challenge to health care expert. Atorvastatin is available in the market in various dosage forms. Bioavailability and pharmacokinetics parameters can be greatly affected by the way of manufacturing and formulation of drug.

The aim of this study was to assess the bioequivalence of a locally produced atorvastatin tablet brand (Lipirex) and the original commercially available product (Lipitor) in healthy male Pakistani volunteers. As the most preferable mode for the assays of different drugs in pharmaceutical industries is HPLC coupled with ultra-violet/visible (UV) detector, therefore, this study is focused to develop a novel and simple method for the determination of atorvastatin.

EXPERIMENTAL

Chemicals and reagents

Analytical reagent grade chemicals (acetic acid, methanol, acetonitrile, and sodium phosphate) are used for HPLC analysis, all from E. Merck, Germany. Standard atorvastatin was gifted by Highnoon laboratories (Pvt.) Ltd., Lahore, Pakistan. Lipitor (Pfizer Pharmaceuticals, Pakistan) and Lipirex (High-Noon Laboratories, Pakistan) were purchased from the market.

Study participants

Two brands of atorvastatin commercially available in Pakistan were selected; one of which is the generic leading brand available with name Lipitor (Pfizer Pharmaceuticals, USA) and second one is Lipirex (High-Noon Laboratories Pakistan). The study was carried out on 12 healthy male volunteers. There mean age was 21.5 ± 2 years with a range of 18 to 27 years and mean body weight was 58.1 ± 5.4 kg with a range of 50 to60 kg. The contributors were well-informed about the type of study: the protection of the medicine and possible adverse effects, etc., and permission was obtained. All the procedures followed were in harmony with the present revision of the Declaration of Helsinki (World Medical Association Declaration of Helsinki, 2008), and the entire participants gave consent permission for this study. The ethics committee of University of Sargodha has approved this study. Affiliated hospital (District Head Quarter) was used to conduct the study (Usman et al., 2012).

Study design

A single dose study was planned as, open label, randomized, two-stage, and cross over check using fasting states. The contributors were set aside to fast for a period of at least 12 h. They were asked to stop the fast by taking water 1 h before medicine administration. The washout period between the administrations of each of the compared products was 10 days. Along with 250 ml of water, a single dose of 40 mg of the test product (Lipirex) and reference standard product (Lipitor) was administered to the participants. After administration of the drug, the participants were kept fasting for 5 h. Blood samples (3 to 5 ml) were collected from each participant by using syringes (disposable), canulas, and butterflies under aseptic environment. Blood was collected from antecubital vein. As an anticoagulant, Heparin (Leo, Denmark) was used. Blood samples were collected just before (blank) and after 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, and 36.0 h of drug administration. The samples were centrifuged at 3000 ×g for 10 min. By using micropipette with sucker, the plasma was separated and stored in specially capped test tubes.

Preparation of standard

Preparation of atorvastatin standard solution was done by dissolving 100 mg of reference standard material in the mobile phase (100 ml). Ten milliliter of the aforementioned solution was diluted into 100 ml with mobile phase. The solution was filtered through 0.45 µm filter and was degassed before use.

Plasma sample preparation

A plasma sample (1 ml) was transferred to a 2 ml polypropylene vial (Shifa glass, Pakistan) to which 1 ml of acetic acid and acetonitrile was added. The sample was centrifuged at 3000 ×g for 10 min and at room temperature (31°C). The vials were then frozen and the upper layer separated. The collected layer was transferred to 2 ml glass vials and the solvent was evaporated to dryness at 40°C under a stream of nitrogen. The residue was redissolved in a 250 µl mobile phase, of which 200 µl was transferred into 250 µl glass

Table 1. Validation parameters of HPLC analysis of plasma.

Parameter	Atorvastatin (Means ± SD)
Precision (CV, within day/between days)	0.2/0.7 ± 0.02 at 10 ng/ml 0.5/0.8 ± 0.01 at 50 ng/ml 0.3/0.8 ± 0.03 at 160 ng/ml
Accuracy (% recovery)	98.5 ± 0.01 at 10 ng/ml 99.4 ± 0.02 at 50 ng/ml 99.05 ± 0.1 at 160 ng/ml
LOD (ng/ml)	7.82 ± 0.02
Tailing factor, As	1.01 ± 0.03
LOQ (ng/ml)	22.86 ± 0.7
Theoretical plates, N	15542 ± 0.05
Resolution, Rs[a]	1.2 ± 0.01
Capacity factor, k	2.0 ± 0.01

[a]Resolution between the adjacent peaks.

vials and was placed in the autosampler for analysis. The injection volume was 20 µl. Sample vials were wrapped in aluminium foil to protect atorvastatin from light exposure.

HPLC method

An HPLC of Shimadzu, Japan having LC-10AT VP pump, SPD-10A VP UV-Vis detector, and SCL-10A VP system controller were used. The column used was Shim-Pak ODS 5 µm (4.6 × 250 mm). The mixture of methanol, acetonitrile, and sodium phosphate buffer (0.01 M, pH 4.5) in ratio 40:30:30 were used as mobile phase. The flow rate of mobile phase was 1.0 ml/min with 247 nm detection wavelength and injection volume was 20 µl.

Method validation

Validation of method was carried out in the plasma according to a reported method (Vinod et al., 2000). Performance parameters of newly developed and validated method are given in Table 1. The accuracy and precision were estimated by the use of quality control sample prepared by adding to the mobile phase in the known amount (lying in the middle range of entire standard curve) of standard. Measurements were completed as ten replicates at each concentration and mean and coefficient of variance (CV) were carefully calculated. Ten different concentrations of Atorvastatin in the mobile phase were used to determine the linearity. The injection volume of each concentration was 20 µl (Usman et al., 2012).

The solutions of standard atorvastatin in the mobile phase were used to calculate limit of detection (LOD) and limit of quantification (LOQ) and were diluted to known concentrations to a final response equal to three times of the signal-to-noise ratio. LOQ was taken to be ten times that of signal-to-noise ratio. By using six different samples, specificity of the method was established. Similarly, the calibration curve in the mobile phase was generated by using six concentrations.

The quality control samples were used to accept or reject the run. The replicate measurements were made at three concentrations, one at lower LOQ, one in the mid range, and another approaching high end of the range (Usman et al., 2012).

Specimen analysis

The earlier validated HPLC method was used for the analysis of plasma samples, and concentration of atorvastatin was determined.

Pharmacokinetics and bioequivalence evaluation

A model independent method was used for the pharmacokinetic analysis employing software, PKSolver (menu-driven software written in Microsoft Excel). The maximum atorvastatin concentration (C_{max}) and the corresponding peak times (T_{max}) were determined by the inspection of the individual drug plasma concentration-time profiles. The elimination half life ($T_{1/2}$) was calculated as $0.693/K_e$. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/K_e$, where C_t is the last measurable concentration.

Statistical analysis

For the purpose of bioequivalence analysis, AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were considered as primary variables and were transformed to ln AUC_{0-t}, ln $AUC_{0-\infty}$, and ln C_{max}. Bioequivalence was assessed by means of an analysis of variance (ANOVA) for cross-over design and calculating the standard 90% confidence intervals of the ratio test/reference. The products were considered bioequivalent if the difference between two compared parameters was found to be statistically insignificant ($P > 0.05$) and 90% confidence intervals for these parameters fell within 80 to 120%.

RESULTS AND DISCUSSION

Atorvastatin determination

Validated HPLC-UV method was used for the determination of atorvastatin. LOD and LOQ values for atorvastatin were 7.82 and 22.86 ng/ml, respectively.

Table 2. Result of F-test applied on pharmacokinetic data of lipirex and lipitor.

Parameter	Lipitor	Lipirex	F-test result
t_{max} (h)	3.0 ± 0.2	3.2 ± 0.3	0.409
C_{max} (ng/ml)	50.5 ± 0.9	49.5 ± 0.7	0.930
$t_{1/2}$ (h)	36.7 ± 1.1	38.1 ± 0.8	0.769
$AUC_{0-t\,h}$ (h.ng/ml)	882.5 ± 2.7	845.2 ± 1.4	0.866
$AUC_{0-\infty h}$ (h.ng/ml)	1452.7 ± 4.8	1546.6 ± 3.2	0.964

Between the days precision near LOD in terms of coefficient of variation (CV) ranged from 0.2 to 0.8 and accuracy in terms of percent recovery was found to be greater than 98.77% for atorvastatin. All performance parameters clearly established the validity of the HPLC method for this study. The specificity and selectivity of the HPLC system were determined by a separate chromatographic analysis of the excipients mixtures without atorvastatin; no interfering peaks at the retention times of atorvastatin were observed. All results are summarized in Table 1. The method developed was found to posses better performance parameters as compared to the available methods in literature (Bullen et al., 1999; Hermann et al., 2005).

Pharmacokinetics and bioequivalence evaluation

For bioequivalence evaluation, various statistical modules were applied to $AUC_{0-t,}$ $AUC_{0-\infty}$, and C_{max}. Table 2 shows the results of the statistical analysis for $AUC_{0-t,}$ $AUC_{0-\infty}$, and C_{max}. According to the mean plasma levels of the 12 subjects completing the study, the relative bioavailability was found to be 108.4, 109.0, and 110.5% on the basis of mean $AUC_{0-t,}$ $AUC_{0-\infty}$, and C_{max}, respectively.

Area under the curve (AUC_{0-t})

The mean AUC_{0-t} was 882.5 and 845.2 h.ng/ml for the test and reference products, respectively. ANOVA did not show any significant differences for period's effects and treatments (Formulations). The 90% confidence interval also fell within the bioequivalence acceptance criteria. Two one-sided t-tests were also performed on the ratio of the mean AUC_{0-t} of the test to the mean AUC_{0-t} of the reference product. These tests showed that P ($r < 0.8$) = 0.001 and P ($r < 1.2$) = 0.007; so, both tests were rejected and it was accepted that the probability for the ratio (T/R) to lie within 0.8 and 1.2 was 0.99.

Area under the curve ($AUC_{0-\infty}$)

The mean $AUC_{0-\infty}$ was 1452.7 and 1546.5 h.ng/ml for the test and reference products, respectively. ANOVA did not show any significant differences for periods effects and treatments (formulations). The 90% confidence interval ranges also fall within the bioequivalence acceptance criteria. Two one-sided t-tests were also performed on the ratio of the mean $AUC_{0-\infty}$ of the test to the mean $AUC_{0-\infty}$ of the reference product. These tests showed that P ($r < 0.8$) = 0.001 and P ($r < 1.2$) = 0.009; so, both tests were rejected and it was accepted that the probability for the ratio (T/R) to lie within 0.8 and 1.2 was 0.99.

Peak plasma concentration (C_{max})

The C_{max} was 49.5 and 50.5 ng/ml for the test and reference product, respectively. ANOVA did not show any significant differences; 90% confidence interval ranges among the reference and test products also fall within the bioequivalence acceptance criteria for C_{max} (70 to 143%). Two one-sided t-tests were also performed on the ratio of the mean C_{max} of the test to the mean C_{max} of the reference product. These tests showed that P ($r < 0.8$) = 0.001 and P ($r < 1.2$) = 0.001; so, both tests were rejected and it was accepted that the probability for the ratio (T/R) to lie within 0.8 and 1.2 was 0.999. For T_{max}, the tests were carried out using original scaled values of T_{max}. The non-parametric test of Wilcoxon on T_{max} did not show any statistically significant difference between the test (3.0 h) and reference product (3.1 h). Both formulations were well tolerated. No clinical adverse events were encountered in the subjects.

The plasma concentration-time curves are as shown in Figure 1 for Lipitor and Lipirex, respectively. All pharmacokinetics parameters: t_{max}, h; C_{max}, ng/ml; $t_{1/2}$, h; $AUC_{0-\,th,}$ h.ng/ml, and $AUC_{0-\,\infty h}$, h.ng/ml of both formulation were found to be closely related. For the comparison of two sets of data, F test was applied and it was found that F < F-critical, which clearly indicates that there is no significant difference between the two sets of data. The results are given in Table 2.

Conclusion

It was described here that the development of a new, selective, precise, and accurate method for the quantification of atorvastatin in human plasma using HPLC with UV detection and liquid-liquid sample extraction was applied to a bioequivalence study. The

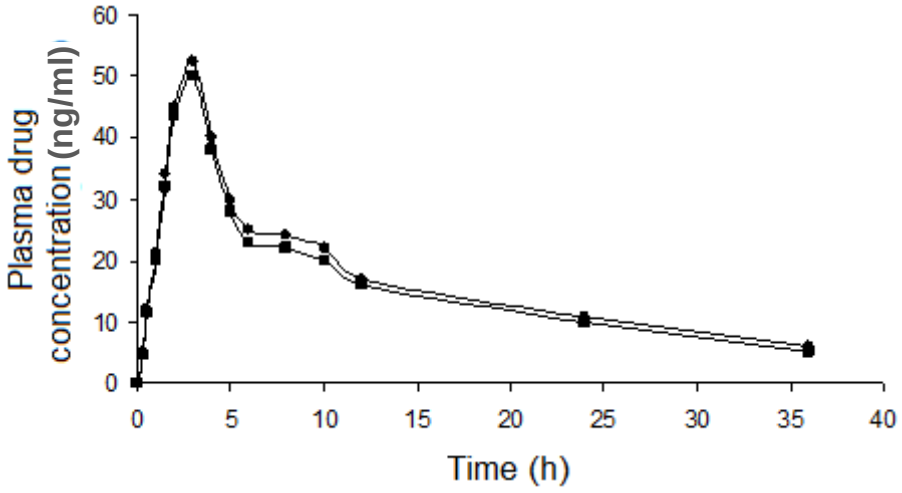


Figure 1. Plasma atorvastatin concentration versus time curve after a single 40 mg dose of lipirex and lipitor.

method reported here uses a simple and effective extraction technique with good and reproducible recovery and a LOQ of 7.82 ng/ml. The developed method is suitable for pharmacokinetic studies of atorvastatin as well. This study was able to demonstrate the bioequivalence between the two formulations with a 90% confidence interval. Ahead of the clinical observations carried through during and after the study, no adverse effect was observed, showing a good tolerability to both formulations.

REFERENCES

Bahrami G, Mohammadi B, Mirzaeei S, Kiani A (2005). Determination of atorvastatin in human serum by reversed-phase high-performance liquid chromatography with UV detection. J. Chromatogr. B. 826:41-45.

Bullen WW, Miller RA, Hayes RN (1999). Development and validation of a high-performance liquid chromatography tandem mass spectrometry assay for atorvastatin, ortho-hydroxy atorvastatin, and para-hydroxy atorvastatin in human, dog, and rat plasma. J. Am. Soc. Mass Spectrom 10:55-66.

Erturk S, Aktas ES, Ersoy L, Ficicioglur S (2003). HPLC method for the determination of atorvastatin and its impurities in bulk drug and tablets. J. Pharm. Biomed. Anal. 33:1017-1023.

Erturk S, Onal A, Cetine SM (2003). Analytical methods for the quantitative determination of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in biological samples. J. Chromatogr. B. 793:193-205.

Goodman GA, Hardman JG, Limbird LE (2001). The Pharmacological Basis of Therapeutics. 10th edition; McGraw-Hill companies Ind, USA. pp. 984-995.

Hermann M, Christensen EH, Reubsaet E (2005). Determination of atorvastatin and metabolites in human plasma with solid-phase extraction followed by LC-tandem MS. Anal. Bioanal. Chem. 382:1242-1249.

Jamshidi A, Nateghi AR (2007). HPTLC analysis of tamoxifen citrate in drug-release media during development of an in-situ-cross-linking delivery system. Chromatogr. 65:763-766.

Koda MA (2005). Applied Therapeutics - The Clinical Use of Drugs. 8th edition, Vol. 1, Lipponcot Williams and Wilkins -A Wolters Kluwer Company, Philadelphia pp. 13-89.

Ma L, Dong J, Chen XJ, Wang GJ (2007). Development and validation of atorvastatin by LC–ESI–MS and application in bioequivalence research in healthy chinese volunteers. Chromatographia 65:737-741.

Marks BD, Marks AD, Smith CM (1996). Basic Medical Biochemistry. Lippincott Williams and Wilkins-A Wolters Kluwer Company, Philadelphia pp. 525-526.

Mason P (2004). Update on statins. J. Vasc. Nurs. 22:49-50.

Miao XS, Metcalfe CD (2003). Determination of cholesterol-lowering statin drugs in aqueous samples using electrospray liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. 998:133-141.

Nirogi R, Mudigonda K, Kandikere V (2007). Chromatography-mass spectrometry methods for the quantitation of statins in biological samples. J. Pharm. Biomed. Anal. 44:379-387.

Sassano A, Platanias LC (2008). Statins in tumor suppression. Cancer Let. 260:11-19.

Usman M, Ahmad M, Dayo A, Madni A, Ali L, Yousuf M, Khan MA, Munir A, Sohail M, Mahmood M (2012). Effect of β-Glucuronidase on Extraction Efficiency of Silymarin from Human Plasma Samples Using Validated HPLC-UV Analysis. Trop. J. Pharm. Res. 11:84-90.

Velesky RJ, Liu L, Musson DG, Zhao JJ (2008). Automated enzyme inhibition assay method for the determination of atorvastatin-derived HMG-CoA reductase inhibitors in human plasma using radioactivity detection. J. Pharm. Toxicol. Methods 57:61-69.

Vinod P, Kamal K, John WA, Howard M, James D, Iain J (2000). Bioanalytical method validation - A revisit with a decade of progress. Pharm. Res. 17:1551-1557.

World Medical Association Declaration of Helsinki (2008). Ethical principles of medical research involving human subjects. http://www.wma.net/en/30publications/10policies /b3/index.html.

Wu SC, Shiang JC, Lin SL, Wu T, Huang WC, Chiou KR, Liu CP (2005). Efficacy and safety of statins in hypercholesterolemia with emphasis on lipoproteins. Heart Vessels 20:217-223.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2429-2432, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.862
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

ERCC1 and ERCC2 polymorphisms predict the clinical outcomes of oxaliplatin-based adjuvant chemotherapy in colorectal cancer

Lei Shi[1], Ran Wang[2], Wen-ming Wu[3] and Yi-fei Huang[1]*

[1]Department of Ophthalmology, Chinese People's Liberation Army General Hospital, China.
[2]Department of Hematology, the First Affiliated Hospital of Xinxiang Medical University, China.
[3]Department of Gastroenterology and Hepatology, Chinese People's Liberation Army General Hospital, China.

Accepted 17 August, 2012

Platinum agents have shown to be effective in the treatment of colorectal cancer. We conducted a prospective study to investigate the effect of excision repair cross-complementation group 1 (*ERCC1 rs11615C>T*) and Excision repair cross-complementation group 2 (*ERCC2 rs13181T>G*) on the efficacy of oxaliplatin-based adjuvant chemotherapy in colorectal cancer patients. A total of 335 cases newly diagnosed by histologically procedure as primary advanced colorectal cancer were collected. The genotypes of *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* genotyping was conducted with TaqMan Gene Expression assays using the ABI PRISM®7900HT Sequence Detection System. All the patients were followed up until death or the end of November, 2011. The median follow-up period was 34.6 months, and 195 patients died during the follow-up. Compared with carrying *ERCC1 T/T* genotype, patients with a homozygous *ERCC1 C/C* genotype had a longer survival time. Similarly, the *ERCC2 G/G* genotype carriers had a lower risk of death from colorectal cancer compared with T/T genotype carriers. Our study suggested that the *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* single-nucleotide polymorphisms (SNPs) could be a predictive marker for the prognosis of colorectal cancer. Further studies are needed to validate the results of our study in Chinese population.

Key words: Excision repair cross-complementation group 1 (*ERCC1 rs11615C>T*), excision repair cross-complementation group 2 (*ERCC2 rs13181T>G*), colorectal cancer, chemotherapy.

INTRODUCTION

Colorectal cancer is the fifth leading cause of death in Chinese population, and there is an increasing trend of colorectal cancer morbidity in recent years (Steward et al., 2003). Despite the development of treatment in terms of colorectal cancer, the prognosis of colorectal cancer is poor. In recent years, chemotherapy either in the adjuvant or palliative setting is the standard treatment of choice for advanced colorectal cancer. However, interindividual variation was shown in colorectal cancer patients with similar clinical characteristics. Cisplatin or oxalipatin is commonly used with 5-fluorouracil (5-FU) as chemotherapy doublets in the treatment of colorectal cancer. The major feature of oxaliplatin-DNA adducts is to block the DNA replication of cancer cell and thus, to induce death of cell (Faivre et al., 2003; Reed, 2005) and the DNA damage, and repair was modified by the nucleotide excision repair (NER) pathways. Excision repair cross-complementation group 1 (*ERCC1 rs11615C>T*) and Excision repair cross-complementation group 2 (*ERCC2 rs13181T>G*) are the two important proteins in NER pathway. Previous studies showed that the polymorphisms of the two genes have an important role in influencing the response to chemotherapy and chemotherapeutic sensitivity (Ishibashi et al., 2011;

*Corresponding author. E-mail: wujunhui198005@163.com.

Kuwabara et al., 2011; Noda et al., 2012). Therefore, the *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* might be two effective prognostic markers in colorectal cancer patients with chemotherapy treatment.

Previous reports on the association between the two gene polymorphisms and chemotherapy are inconsistent (Ishibashi et al., 2011; Kuwabara et al., 2011; Noda et al., 2012). We therefore, conducted a prospective study to investigate the effect of *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* on the efficacy of oxaliplatin-based adjuvant chemotherapy in colorectal cancer patients.

MATERIALS AND METHODS

Patients

A total of 335 cases newly diagnosed by histologically procedure as primary advanced colorectal cancer were collected. All these patients were treated by 5-FU/oxaliplatin regimen (FOLFOX6) chemotherapy. The clinical data of all patients were obtained from medical record. Exclusion criteria included a history of other malignancies and not a metastatic colorectal cancer.

All patients were treated with six cycles of chemotherapy. The regimen included intravenous leucovorin (400 mg/m^2) on the first day, intravenous 5-FU (400 mg/m^2) on the first day, followed by an intravenous dose of 2,400 mg/m^2 over 46 h, and intravenous oxaliplatin (85 mg/m^2) on the first day.

The potential lifestyle factors were included by a uniform questionnaire including sociodemographic characteristics, alcohol, smoking and family history of colorectal cancer.

DNA extraction and genotyping

The peripheral blood samples were collected from all participants and they were stored at -20°C until DNA extraction. Genomic DNA was extracted by using a Qiagen Blood Kit (Qiagen, Chastworth, CA) according to the manufacturer's protocol.

For each single-nucleotide polymorphism (SNP), a pair of amplification primers and an extension primer was designed by using Assay design 3.1 software. The forward and reverse primers of *ERCC1 rs11615C>T* were 5'-AAGCTGGAAAAGACCCTGCC-3' and 5'-CTCACCTGAGGAACAGGGCA-3', respectively. The forward and reverse primers of *ERCC2 rs13181T>G* were 5'-CCCCCTCTCCCTTTCCTCTG-3' and 5'-AACCAGGGCCAGGCAAGAC-3', respectively. The genotypes of *ERCC1* rs11*615C>T* and *ERCC2 rs13181T>G* genotyping was conducted with TaqMan Gene Expression assays using the ABI PRISM®7900HT Sequence Detection System (Applied Biosystems, Poster City, CA). 20% of all the samples were genotyped again for quality control.

Statistical analysis

All analyses were performed with SPSS Version 16.0 software (SPSS Inc., Chicago, IL, USA). The overall survival was the time from study entry until death regardless of cause. All statistical tests are two-sided. The Kaplan-Meier method was adopted to estimate survival curves, and the log-rank test was used to compare patients' survival time between genotype groups. Cox's proportional hazards model was used to estimate hazard ratios (HRs) and their 95% confidence intervals (CIs), representing the overall relative risk of relapse or death associated with polymorphisms of *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G*. Primary death from ovarian cancer was defined as the failure event, and the time of survival as the time between diagnosis and death. All surviving patients were censored at the date of last follow-up. Statistical significance was defined as a two-sided P-value of less than 0.05.

RESULTS

Among 335 patients, 307 enrolled, with a participation rate of 91.6%. All the patients were followed up until death or the end of November, 2011. The median follow-up period was 34.6 months, and 195 patients died during the follow-up. The mean age was 61.5 ± 6.9 years when diagnosed. About 64.7% of the patients were males. Almost all of the patients were adenocarcinoma. People who had a family history of colorectal cancer has short survival rate, and the HR (95% CI) was 1.65 (0.86 - 3.18) (Table 1).

Table 2 showed the genotype distributions for *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* among patients with colorectal cancer. Compared with carrying *ERCC1 T/T* genotype, patients with a homozygous *ERCC1 C/C* genotype had a longer survival time, and the median survival time was 43.2 months. The adjusted HR ((95% CI) of *ERCC1 C/C* genotype was 0.20 (0.10 - 0.79) compared with T/T genotype. Similarly, the *ERCC2 G/G* genotype carriers had a lower risk of death from colorectal cancer compared with T/T genotype carriers, and the median overall survival was 41.4 months. The adjusted HR (95% CI) of *ERCC2 G/G* genotype was 0.48 (0.19 - 0.97).

DISCUSSION

In this present study, we provided evidence of an association between *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* SNPs and clinical outcomes of colorectal cancer in Chinese population. Our study showed that *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* SNPs could play an important role in the efficacy of chemotherapy for colorectal cancer, and thus to be associated with the survival of patients.

The oxaliplatin-based adjuvant chemotherapy is a very common chemotherapy for the treatment of patients with advanced colorectal cancer. However, there are few studies on the predictive factors of patients' response to the chemotherapy. Previous studies suggested that *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G*, the two key enzymes of the NER pathway, play an important role in the prognosis of various cancers, such as bladder cancer, lung cancer, esophageal cancer and breast cancer as well as colorectal cancer (Kawashima et al., 2012; Das et al., 2012; Leichman et al., 2011; Goyal et al., 2010; Kuwabara et al., 2011). The proteins of NER pathway are thought to be repair DNA damage caused by platinum agents, and the polymorphisms of the enzyme in NER pathway could influence the DNA repair capability

Table 1. Survival of colorectal patients according to the demographic and clinical characteristics.

Variable	Cases (%) N = 307	Patient deaths (%) N = 195	Five-year survival rate (%)	HR (95% CI)
Age (mean ± SD, years)	61.5 ± 6.9			
Gender (%)				
Female	108 (35.3)	67 (34.5)	36.7	-
Male	199 (64.7)	128 (65.5)	34.4	0.97 (0.67 - 1.41)
Smoking				
No	142 (46.4)	85 (43.4)	40.6	-
Yes	165 (53.6)	110 (56.6)	32.9	1.11 (0.76 - 1.63)
Drinking				
No	207 (67.3)	126 (64.8)	38.8	-
Yes	100 (32.7)	69 (35.2)	31.6	1.13 (0.76 - 1.68)
Family history of colorectal cancer				
No	284 (92.4)	172 (45.7)	39.4	-
Yes	23 (7.6)	23 (11.9)	0.5	1.65 (0.86 - 3.18)
TNM stage				
III	165 (53.6)	89 (45.7)	44.8	-
IV	142 (46.4)	106 (54.3)	24.2	1.38 (0.97 - 1.98)
Histological subtype				
Other	140 (45.7)	80 (41.2)	41.6	-
Adenocarcinoma	167 (54.3)	115 (58.8)	29.9	1.21 (0.84 - 1.73)
Location				
Colon	161 (52.5)	88 (45.3)	44.1	-
Rectal	146 (47.5)	107 (54.7)	25.4	1.34 (0.94 - 1.92)

Table 2. Cox proportional regression analysis for the overall survival of *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* gene polymorphisms among colorectal cancer patients.

Genotype	No. of patients (%) N = 307	Patient deaths N = 195	Median overall survival (Months)	HR (95% CI)	HR (95% CI)[1]
ERCC1 rs11615C>T					
T/T	146 (47.6)	104 (53.2)	29.3	-	-
C/T	128 (41.7)	79 (40.5)	31.7	0.86 (0.58 - 1.28)	0.82(0.49 - 1.07)
C/C	33 (10.7)	12 (6.3)	36.8	0.51 (0.22 - 1.07)	0.43(0.18 - 0.96)
ERCC2 rs13181T>G					
T/T	143 (46.6)	102 (52.4)	28.9	-	-
T/G	130 (42.5)	81 (41.5)	30.8	0.87 (0.59 - 1.29)	0.75(0.49 - 1.13)
G/G	33 (10.9)	12 (6.1)	35.1	0.51 (0.22 - 1.07)	0.43(0.15 - 0.94)

[1], Adjusted for sex, age, TNM stage, histological subtype and location.

and efficacy of chemotherapy. The inactive of *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* had low activity of DNA repair and could induce the better efficacy of chemotherapy and longer survival of patients. Our study showed that the inactive genotypes of the two genes had longer survival of colorectal cancer. However, the results on the polymorphisms of two genotypes in colorectal cancer are inconsistent. Two studies conducted in Japan stated that *ERCC1* was not related to unresectable colorectal cancer (Ishibashi et al., 2010; Kuwabara et al., 2011). However, a meta-analysis showed that *ERCC1 rs11615C>T* polymorphisms are useful prognostic factors in oxaliplatin-based treatment of colorectal cancer. The inconsistency of these studies may be explained by differences in population background, source of subjects, sample size and by chance.

This study has several major limitations. Firstly, there was a certain risk of selection bias since they were not a random sample of the general population and may not fully represent the underlying base population. Secondly, due to selecting patients in one hospital, we only included 307 patients in our study. The sample size could limit the statistic power to find the difference, especially for the slightly rate of variant alleles. Further large sample study is still needed. Finally, we could only find few evidence for the role of the two gene polymorphisms in Chinese population. Future studies on the two gene polymorphisms are still needed.

Therefore, our study suggested that the *ERCC1 rs11615C>T* and *ERCC2 rs13181T>G* SNPs could be a predictive marker for the prognosis of colorectal cancer. Further studies are needed to validate the results of our study in Chinese population.

REFERENCES

Das M, Riess JW, Frankel P, Schwartz E, Bennis R, Hsieh HB, Liu X, Ly JC, Zhou L, Nieva JJ, Wakelee HA, Bruce RH (2012). ERCC1 expression in circulating tumor cells (CTCs) using a novel detection platform correlates with progression-free survival (PFS) in patients with metastatic non-small-cell lung cancer (NSCLC) receiving platinum chemotherapy.Lung Cancer. 77(2):421-426.

Faivre S, Chan D, Salinas R, et al (2003). DNA strand breaks and apoptosis induced by oxaliplatin in cancer cells. Biochem. Pharmacol. 66:225-237.

Goyal S, Parikh RR, Green C, Schiff D, Moran MS, Yang Q, Haffty BG (2010). Clinicopathologic significance of excision repair cross-complementation 1 expression in patients treated with breast-conserving surgery and radiation therapy. Int. J. Radiat. Oncol. Biol. Phys. 76(3):679-684.

Ishibashi K, Okada N, Tajima Y, Ishiguro T, Kuwabara K, Ohsawa T, Kumamoto K, Tsuji Y, Haga N, Iwama T, Ishida H, Onouchi T, Yakabi K (2011). Prediction of the efficacy of modified FOLFOX6 therapy according to the mRNA levels of thymidylate synthase (TS), excision repair cross-complementing-1 and -2(ERCC-1 and ERCC-2) and methylenetetrahydrofolate dehydrogenase(MTHFD) in the primary lesion of colorectal cancer. Gan To Kagaku Ryoho. 38(12):2220-2223.

Kawashima A, Takayama H, Kawamura N, Doi N, Sato M, Hatano K, Nagahara A, Uemura M, Nakai Y, Nishimura K, Miyoshi S, Kawano K, Nishimura K, Nonomura N, Tsujimura A (2012). Co-expression of ERCC1 and Snail is a prognostic but not predictive factor of cisplatin-based neoadjuvant chemotherapy for bladder cancer. Oncol. Lett. 4(1):15-21.

Kuwabara K, Kumamoto K, Ishibashi K, Okada N, Ishiguro T, Ohsawa T, Haga N, Miura I, Ishida H (2011). The Relationship between the efficacy of mFOLFOX6 treatment and the expression of TS, DPD, TP, and ERCC-1 in unresectable colorectal cancer. Gan To Kagaku Ryoho. 38(12):2224-2227.

Leichman LP, Goldman BH, Bohanes PO, Lenz HJ, Thomas CR, Billingsley KG, Corless CL, Iqbal S, Gold PJ, Benedetti JK, Danenberg KD, Blanke CD (2011). S0356: a phase II clinical and prospective molecular trial with oxaliplatin, fluorouracil, and external-beam radiation therapy before surgery for patients with esophageal adenocarcinoma.J Clin Oncol. 29(34):4555-4560.

Noda E, Maeda K, Inoue T, Fukunaga S, Nagahara H, Shibutani M, Amano R, Nakata B, Tanaka H, Muguruma K, Yamada N, Yashiro M, Ohira M, Ishikawa T, Hirakawa K (2012). Predictive value of expression of ERCC 1 and GST-p for 5-fluorouracil/oxaliplatin chemotherapy in advanced colorectal cancer. Hepatogastroenterology 59:130-133.

Reed E (2005). ERCC1 and clinical resistance to platinum-based therapy. Clin Cancer Res. 11:6100-6102.

Steward BW, Kleihues P (2003). World Cancer Report. Lyon: IARC Press pp. 198-202.

African Journal of Pharmacy and Pharmacology Vol. 6(32), pp. 2433-2440, 29 August, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.866
ISSN 1996-0816 ©2012 Academic Journals

Full Length Research Paper

Dihydroartemisinin suppresses cell proliferation, invasion, and angiogenesis in human glioma U87 cells

Xiangrong Chen[1, 2], Hong Lin[2], Jun Wen[2], Qingshan Rong[2], Weiwei Xu[3], Xiangyu Wang[3]*

[1]Department of Neurosurgery, the Second Affiliated Hospital, Fujian Medical University, Quanzhou 362000, Fujian Province, China.
[2]Guangdong Neuroscience Institute, Key Laboratory of Brain Function and Regeneration, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, Guangdong Province, China.
[3]Department of Neurosurgery, the First Affiliated Hospital, Jinan University, Guangzhou 510630, Guangdong Province, China.

Accepted 13 August, 2012

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, which is a well tolerated and effective drug for malaria treatment, has shown potent antitumor ability. This study is explored to evaluate whether DHA can inhibit glioma cell proliferation, invasion, and glioma cell mediated angiogenesis. We determined the glioma U87 cell proliferation by Cell Counting Kit-8 (CCK-8) assay, fluorescence, and flow cytometry (FCM). The invasion and migration of U87 cell were tested by wound-scratch assay and martrigel-transwell methods, while its angiogenesis tube formation assay was tested with reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Our data suggested that DHA inhibited cell viability in a dose- and time-dependent manner, triggered a stringent G1 cell cycle arrest, and induced cell apoptosis of glioma U87 cells. Tube formation assays showed that DHA significantly decreased glioma cell tube formation in human umbilical vein endothelial cell (HUVC), and suppressed vascular endothelial growth factor (VEGF) mRNA expression and its release in U87 cells. In addition, wound-scratch assay and martrigel-transwell showed that DHA decreased U87 cell invasion and migration *in vitro*. These results indicated that DHA might be a valuable candidate for treatment of human glioma.

Key words: Dihydroartemisinin, glioma, proliferation, invasion, angiogenesis.

INTRODUCTION

Malignant glioma is one of the most malignant tumors with a very poor prognosis. Survival for patients with glioblastoma multiforme (GBM), the most malignant glioma (World Health Organization (WHO) grade IV), is only on average 14 months after diagnosis, despite aggressive surgery, radiation, and chemotherapies. Due to their insidious invasion and extensive neovascularization, glioblastomas are characterized by recurrence (Claes et al., 2007; Mentlein et al., 2011). The diffusing glioma cells invade into the normal brain adjacent to the tumor, which may cause treatment failure by conventional therapy. Therefore, there is an urgent need to develop effective chemotherapeutic agents for glioma.

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin (Figure 1), isolated from the traditional Chinese medicine, *Artemisia annua*, is approved for the treatment of multidrug-resistant malaria and has an excellent safety profile. More recently, growing studies suggested that artemisinin derivatives including DHA also have profound effect against human tumors, such as leukemia cells (Zhou et al., 2007), prostate cancer (He et al., 2010), ovarian cells (Jiao et al., 2007, pancreatic cancer (Du et al., 2010), lung adenocarcinoma and non-

*Corresponding author. E-mail: wang__xy123@126.com.

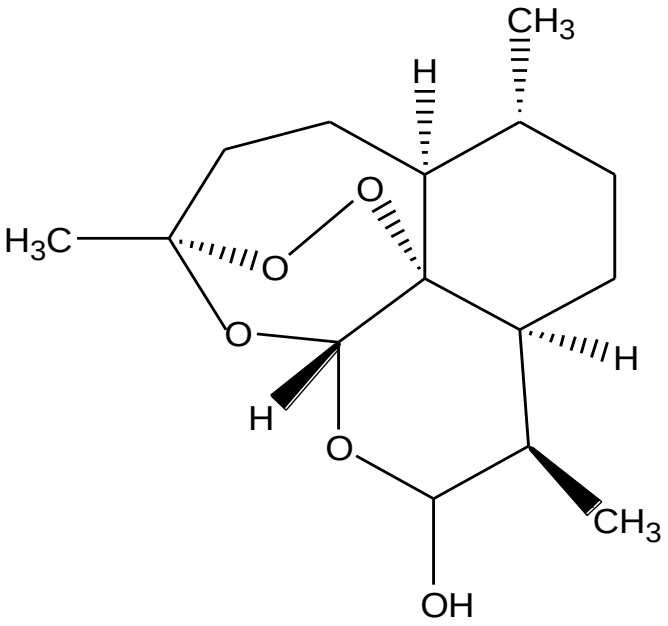


Figure 1. Chemical structure of dihydroartemisinin (DHA).

small cell lung cancer (Lu et al., 2009; Zhao et al., 2011). However, the effect of DHA on glioma is unclear. Therefore, there is an urgent need to study the effect of DHA on glioma.

In this study, we evaluated the therapeutic potential of DHA against human glioma U87 cells *in vitro*, and demonstrated that DHA is a novel potential drug for the treatment of glioma via the induction of apoptosis, inhibiting tumor angiogenesis, and blocking of the cell cycle progression. We determined the glioma U87 cell proliferation by Cell Counting Kit-8 (CCK-8) assay, fluorescence, and flow cytometry (FCM). The invasion and migration of U87 cell were tested by wound-scratch assay and martrigel-transwell methods, while its angiogenesis tube formation assay was tested with reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively.

MATERIALS AND METHODS

DHA was purchased from Shanghai Yi & Yuan Ye Biotech Company in China. 284 mg of DHA was dissolved in 1 ml of dimethyl sulfoxide (DMSO) as stock solution. This stock solution of DHA (1 mmol/L) was further diluted to appropriate concentrations with cell culture medium immediately before use. Control experiments contained DMSO without DHA.

Cell culture

Human glioblastoma U87 and human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the Central Laboratory of Zhujiang Hospital of Southern Medical University. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cell cultures were maintained in 25 cm^2 flasks and were kept in a humidified atmosphere with 5% CO_2 at 37°C.

Cell proliferation assay

To measure cell viability, cells were seeded into 96-well plates at a density of 1×10^4 per well. After overnight incubation, cells were rinsed with phosphate buffered saline (PBS) and incubated with different concentrations of DHA (0, 5, 10, 20, 40, 80, and 160 µmol/L, respectively) in complete medium. Then, CCK-8 assay was performed to test the proliferation. The absorption of solubilized formazan was measured at the wavelength of 450 nm by an ELISA plate reader (EL340 microplate reader; Bio-Tek Instruments, Winooske, VT). The cells were incubated for 6, 12, 24, 48, and 72 h after treated with different concentrations of DHA to measure the dose- and time-dependent effects, CCK-8 assay was performed at each time point in triplicate.

Determination of apoptosis

Cells were plated in triplicate and treated with 40 µmol/L DHA for 48 h. Apoptotic cell death was examined by two parameters: cell and nuclear morphology by fluorescence microscope (Axiovert-200M microscope, Zeiss) after intravital staining with 1 µmol/ml Hoechst 33258 for 20 min at room temperature (RT) in the dark.

Cell cycle analysis

Cells (1×10^6) were treated with DHA as described earlier for 48 h. The harvested cells were resuspended in 200 µl of cold PBS, to which cold ethanol (600 µl) was added, and the mixture was then incubated for 2 h at 4°C. After centrifugation, the pellet was washed

with cold PBS, suspended in 500 µl PBS, and incubated with 50 µl RNase (20 µg/ml final concentration) for 30 min. The cells were incubated with propidium iodide (50 µg/ml final concentration) for 30 min in the dark. The cell cycle distribution was then elaborated by the Cell Quest software (Becton Dickinson (BD)), determined by using a FACS Aria instrument (BD Biosciences). The experiment was repeated thrice under the same conditions (Riganti et al., 2009).

Cell invasion and migration assays

BD BioCoat Matrigel invasion chambers were used to examine the ability of U87 cells to penetrate the extracellular matrix (ECM). Cells (5×10^4 ml^{-1}), after being treated with DHA for 24 h, were resuspended in 100 µl of serum-free medium and added to the upper chamber, while the lower chamber was filled with 600 µl of complete medium containing FBS, which served as a chmo-attractant. Cells were then incubated for 24 h at 37°C. After removal of cells on the upper surface of the membrane, cells on the lower surface of the membrane were fixed in 4% of formaldehyde and then stained with 0.1% of crystal violet for five min. Four fields of cells were counted randomly in each well under a fluorescent microscope at 200× magnification. Data was expressed as the percentage of invasive cells as compared to the control.

Wound scratch assay was also used to evaluate cell migration ability. In brief, cells were seeded at a density of 2×10^5 per well in 6-well plates in complete medium. After cell growth to about 80% confluence, the medium was changed into a serum-free medium for 6 h. Then, the monolayers were scratched with a 10 µl plastic pipette tip to create a uniform wound and 3 ml of complete medium containing 10% of FBS with various concentrations of DHA (25, 50, 75, and 100 µmol/L) were added to the wells. The wound area was then examined after 24 h of incubation under a phase-contrast microscope at 100× magnification. Photographs of three random fields were taken and the cell migration ability was expressed by the closure of gap distance.

Tube formation assay

Matrigel (Becton Dickinson) was added to each well of a 24-well plate and then incubated for 30 min at 37°C to allow the Matrigel solution to form a gel. After treatment with indicated concentrations of DHA for 24 h, 2×10^5 HUVEs were resuspended in 1 ml of complete medium, seeded onto the solidified Matrigel gel, and incubated for 8 h. The endothelial tubes of five random fields were examined under a phase-contrast microscope, and the extent of tube formation was estimated by inspecting the overall tube length per area.

ELISA for vascular endothelial growth factor (VEGF) in cell culture supernatants

U87 cells pretreated with DHA were grown in serum-free medium for 24 h. After treatment with indicated concentrations of DHA for 24 h, VEGF concentration in supernatants of the cultured U87 cells was determined by using commercial human VEGF ELISA kit according to the manufacturer's instructions. The results were presented as VEGF (pg/ml) concentration per 10^5 cells.

RT-PCR for VEGF mRNA expression

RT-PCR was performed with the SYBR Green reporter. Cell cultures were washed in PBS and RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). Optical density (OD) 260/280 ratio was used to determine RNA purity and 260 nm was used to determine RNA yield. RNA was subsequently reverse transcribed to cDNA with SuperScript First-strand Synthesis System (Invitrogen). RT-PCR was performed afterward. Primer (Invitrogen) concentrations (10 nM) were optimized before use. SYBR Green PCR master kit was used with the appropriate concentrations (10 nM) of forward and reverse primers in a total volume of 20 µl. PCR reactions contained 1 µl cDNA. Optimization was performed of each gene-specific primer prior to the experiment to confirm that 10 nM primer concentrations did not produce non-specific primer-dimer amplification signals in no-template control wells. RT-PCR was performed on as ABI 7000 PCR instrument (PE Applied Biosystems, Foster City, CA) by using three-stage program parameters provided by the manufacturer as follows; 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during RT-PCR. Each sample was tested in triplicate with RT-PCR, and samples obtained from three independent experiments were used for analysis of relative gene expression. The following primers for RT-PCR were designed using Premier 5 software. VEGF: forward 5'- CAGCTACTGCCATCCAATC-3'; reverse 5'-CAAATGCTTTCTCCGCTCTG-3', product size: 313 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-TCCACCAC CCTGTTGCTGTA-3′, reverse 5′-ACCACAGTCCATGCCATCAC-3′, product size: 450 bp, GAPDH was used as the internal control.

Statistical analysis

The Statistical Program for the Social Sciences software for Windows 16.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. All experiments were performed in triplicate unless otherwise noted; results were expressed as mean ± standard deviation (SD). Significance between the two groups was examined by a two tailed. When two groups were compared, the unpaired Student's *t*-test was used. When multiple groups were evaluated, one-way analysis of variance (ANOVA) was used. $P \leq 0.05$ was considered statistically significant.

RESULTS

DHA inhibits proliferation of glioma cells

CCK-8 was used to determine anti-proliferative effect of DHA on glioma U87 cells. The proliferation of the cells was inhibited in a dose-dependent manner at 48 h after treatment with different concentration (0, 10, 20, 40, 80, and 160 µmol/L) of DHA (Figure 2A). Treatment with 40 µmol/L of DHA for different times (0, 6, 12, 24, 48, and 72 h) demonstrated that the cells growth was inhibited in a time-dependent manner (Figure 2B). These results showed that the proliferation of the cells could be significantly inhibited by DHA, even at a very low treating-dose (5 µmol/L) and a very short treating-time (6 h) after treatment with indicated concentrations of DHA.

DHA reduces glioma cell migration and invasion

Gliomas are characterized by insidious invasion to

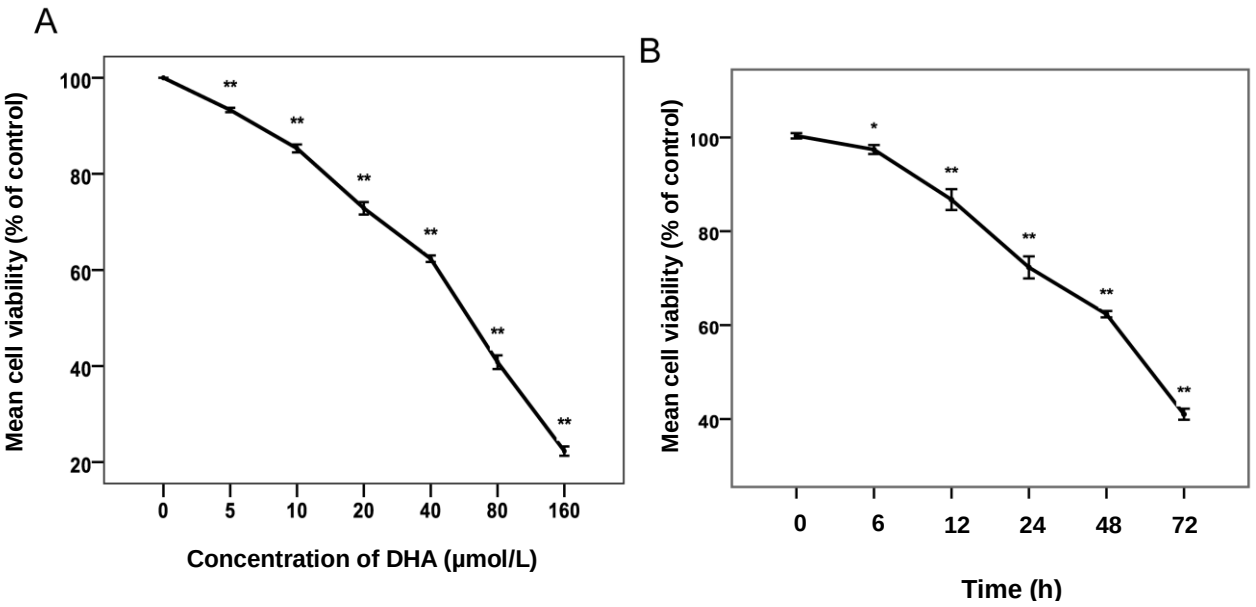


Figure 2. Cell viability was measured by CCK-8 assay via concentration-and time-dependent manners *in vitro* (n = 3). (A) U87 cells were incubated with various concentrations of DHA for 48 h. (B) Cells were incubated with 40 μmol/L of DHA for the indicated times. *P < 0.05, **P < 0.01, compared with control group.

adjacent normal brain tissue (Claes et al., 2007; Mentlein et al., 2011). To investigate the effect of DHA on glioma cell U87 migration and invasion ability, a wound-scratch assay and martrigel-transwell assay were used. As compared to the control group, the gap distance was significantly reduced in U87 cells by DHA at the concentration of 20 and 40 μmol/L for 24 h (P < 0.01 or P < 0.05). DHA dramatically inhibited cell migration to 71.82 and 36.12% of the control in U87 cells, respectively (P < 0.01 or P < 0.05) (Figure 3A). Meanwhile, as shown in Figure 3B, DHA caused a significant reduction of invasiveness to approximately 76.09 and 39.80% of the control of U87 at a concentration of 20 and 40 μmol/L (P < 0.01 or P < 0.05).

DHA induce G1-phase cell cycle arrest in human glioma cells

As there is a significant growth-inhibitory effect of DHA on glioma cells, we investigated whether DHA had any inhibitory effect on cell cycle progression. After incubation for 48 h in the presence of DHA, cells were permeabilized and assessed for the cell cycle phases by fluorescence activated cell sorting (FACS) analysis. DHA (40 μmol/L) lowered the percentage of cells entering the S-phase (39.30%), relative to the untreated cells (20.90%, P < 0.05). G1-phase arrest was also observed when the effects of DHA on cell cycle progression of U87 were analyzed (P < 0.01). The percentage of cells entering the G1-phase increased from 26.50% in untreated cells to 67.19%. These results suggest that DHA showed stronger inhibitory effects on cell cycle progression and was associated with the induction of G1 arrest (Figure 4).

Morphological changes of DHA-induced apoptosis

After exposure to 40 umol/L DHA for 48 h, cells underwent typical morphologic changes of apoptosis such as cell shrinkage, membrane frill, blebbing, and ovalisation, while the cell membrane remained well defined (Figure 5B). The Hoechst 33258, a sensitive fluorochrome to DNA, was used to assess changes in nuclear morphology following DHA treatment. The nuclei in normal cells exhibited diffused staining of the chromatin (Figure 5C). However, DHA significantly induced chromatin condensation, margination and the boundary between nucleus and cytoplasm became blurred (Figure 5D).

DHA inhibits tube formation

Glioma induces extensive neovascularization in adjacent tissue (Kesari, 2011; Corsini et al., 2012). Therefore, we investigated whether DHA had the ability to inhibit tube formation in HUVECs treated for 24 h. Control groups treated with medium only were composed of multiple cells gathered together and that adhered to each other. However, DHA significantly decreased tube formation by 69.50 and 73.97% of the control group, at all concentrations of 20 and 40 μmol/L, respectively (Figure 6A and B).

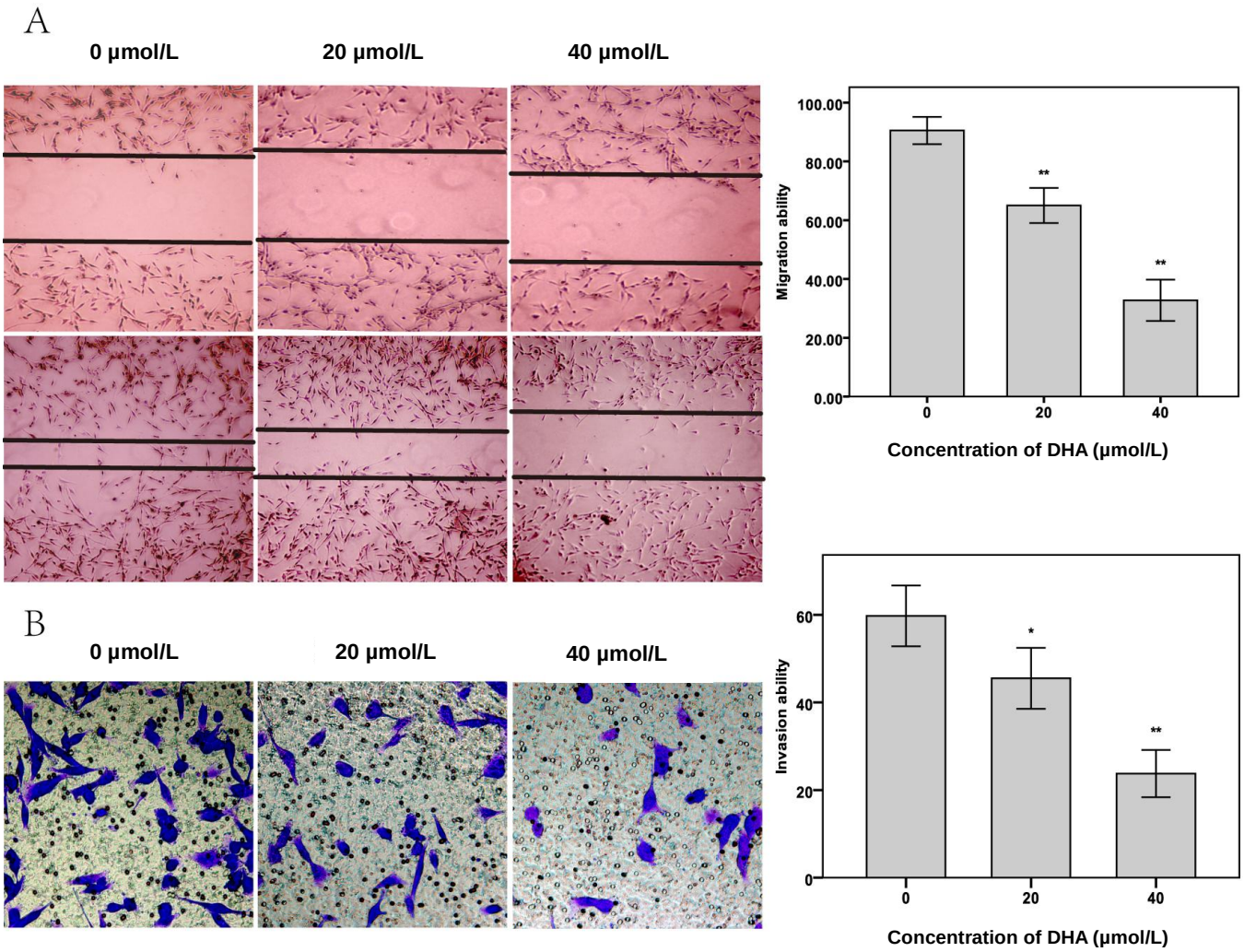


Figure 3. DHA inhibits U87 cells migration and invasiveness. (A) The effect of DHA on migration was measured by wound healing assay. The wound line was prepared using the tip of 10 μl pipett, and the cells were then treated with various concentration of DHA. Photographs were taken and the width of the wound line was measured at 24 h. Results were representative of three independent experiments (magnification, ×50). Bar graphs revealed that DHA dramatically inhibited cell migration to 71.82 and 36.12% of the control in U87 cells at the concentrations of 20 and 40 μmol/L, respectively. (B) DHA inhibits U87 cells invasiveness using martrigel-transwell assay. Cells were starved for 6 h and suspended in serum free DMEM containing various concentration of DHA. 5×10^4 cells were added to the upper chamber. Twenty-four hours *in vitro* invasion assay revealed that the invasiveness of U87 cells were inhibited by DHA. Bar graphs showed that DHA inhibited cell invasion to 76.09 and 39.80% of the control at the concentrations of 20 and 40 μmol/L, respectively. *P < 0.05, **P < 0.01, compared with control group.

DHA decreases VEGF mRNA expression and its release in U87 cells

The level of VEGF mRNA expression was tested by using a RT-PCR assay. U87 cells were exposed to different concentrations of DHA for 48 h, then total RNA was isolated and RT-PCR was performed to measure the cells. DHA significantly decreased the level of VEGF mRNA in U87 cells from 68.3 to 45.0% and from 68.3 to 28.7%, as compared to GAPDH (Figure 6C).

VEGF protein showed the same trend as mRNA level in all experimental groups. ELISA analysis was performed to determine the amount of secreted VEGF protein. Cells pretreated with DHA were grown in serum-free medium for 24 h, and the secreted protein of VEGF in culture media was determined by ELISA. The levels of VEGF in U87 cells decreased by 21.4 and 43.1% after being treated with 20 and 40 μmol/L of DHA for 48 h, when compared with the control group (P < 0.05) (Figure 6D).

DISCUSSION

Lack of effective long term treatments for glioma highlights the necessity to identify new potent anti-cancer

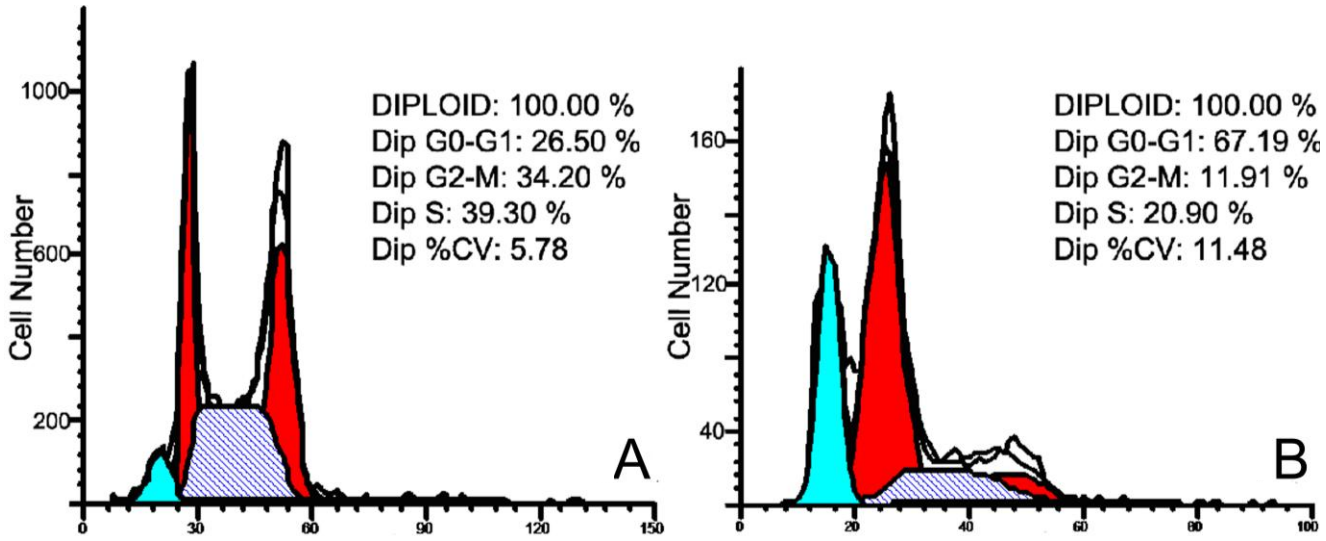


Figure 4. Effects of on glioma cells cycle progression of human glioma cells U87. Cells were exposed to 40 µmol/L DHA for 48 h followed by cell cycle distribution assay. All assays were done in triplicate. DHA lowered the percentage of cells entering the S-phase (A, 39.30%), relative to the untreated cells (B, 20.90%, $P < 0.05$). However, the percentage of cells entering the G1-phase increased from 26.50% in untreated cells to 67.19% ($P < 0.01$).

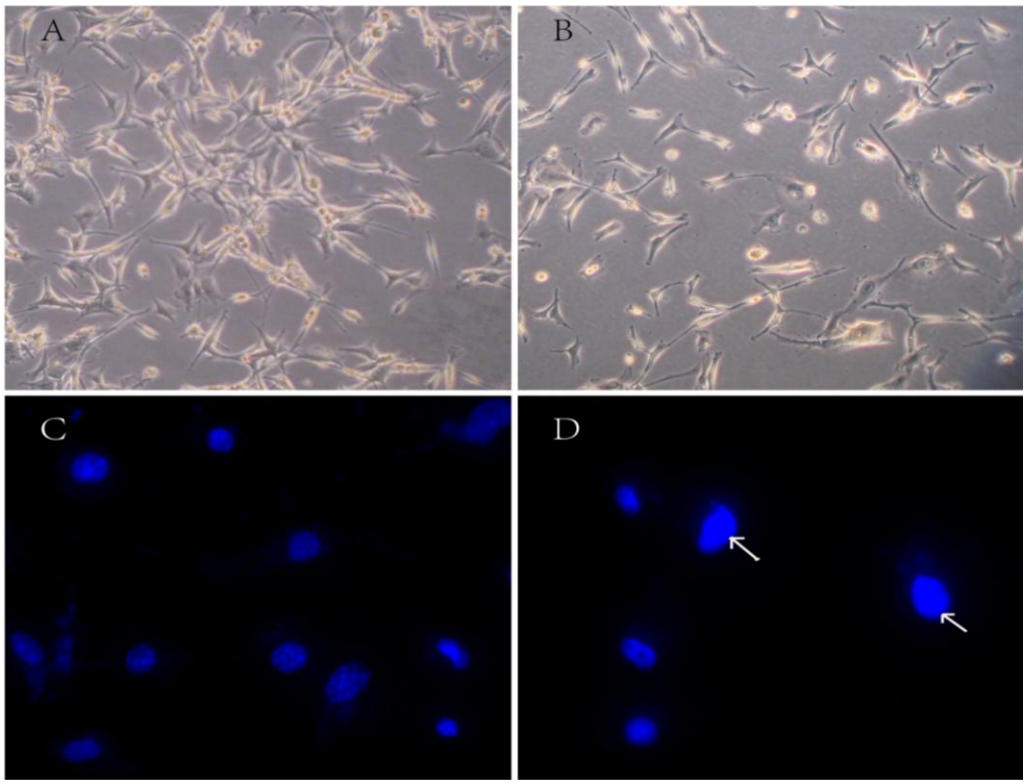


Figure 5. Apoptosis induced by DHA in U87 cells. The cells were treated with 40 µmol/L of DHA for 48 h. (A) Control cells; (B) treatment of U87 cells with 40 µmol/l DHA, cell shrinkage, membrane frill, blebbing, and ovalisation, while the cell membrane remained well defined (magnification, ×200). Nuclear morphology was detected by Hoechst 33258 staining and examined by fluorescence microscope. (C) Control cells with diffused staining of the chromatin in normal cells. (D) With 40 µmol/L of DHA treated, chromatin condensation, margination and the boundary between nucleus and cytoplasm became blurred (the arrows point to cells displaying nuclear condensation) (magnification, ×200).

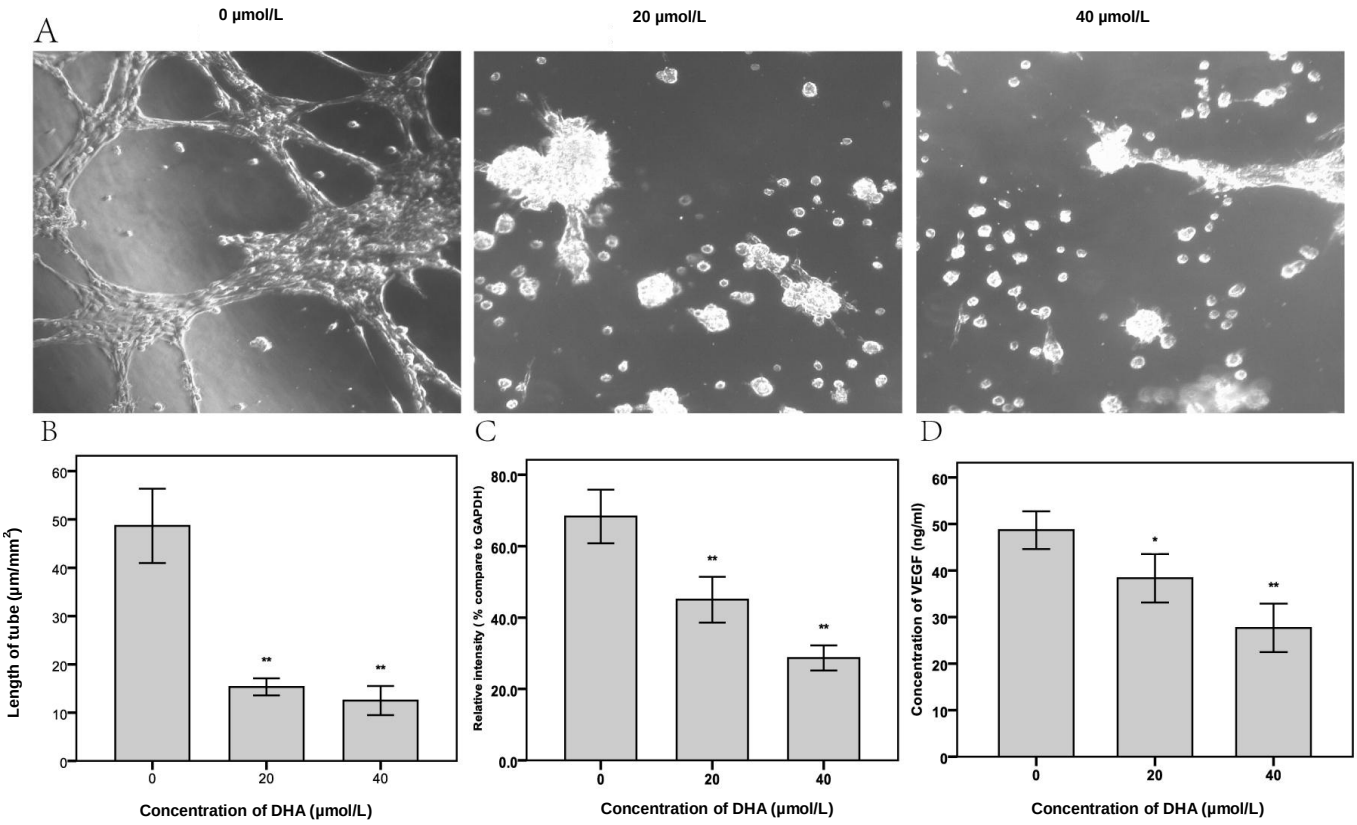


Figure 6. DHA inhibits tube formation in human umbilical vein endothelial cells. (A) Representative photomicrographs during the tube formation of human umbilical vein endothelial cells pretreated with indicated concentrations of DHA for 24 h. (B) The ability to form tubes was expressed as ratios of length of formed tubes per picture field. DHA significantly decreased tube formation by 69.50 and 73.97% of the control group, at all concentrations of 20 and 40 μmol/L, respectively when compared to the control group. (C) DHA significantly decreased VEGF mRNA expression from 68.3 to 45.0% and 68.3 to 28.7%, respectively after 48 h treatment. (D) Secreted VEGF release was also significantly inhibited by DHA in cell culture supernatants. With 20 and 40 μmol/L of DHA treated, the secreted protein of VEGF in culture media was decreased by 21.4 and 43.1% when compared with the control group, *P < 0.05, **P < 0.01, compared with control group.

compounds. Naturally occurring plant compounds represent a possible source of molecules that may have anti-proliferative effects on a variety of cancers. Although, it has been reported that artemisinin and several of its derivatives inhibit the growth of various cancer cell types, it is still little known whether artemisinin and its derivatives work as growth inhibitors in glioma cells. Our results in this study suggest that DHA inhibits growth of human glioma U87 cells by doses at micromolar levels (Figure 2), and these doses of DHA exhibit little or no significant cytotoxicity and neuropathologic toxicity in normal neuronal cells (Meshnick, 2002; Houle, 2011).

Cell proliferation is highly regulated in the G1 phase of the cell cycle in most normal and tumorigenic mammalian systems, because cancer cells are highly proliferative (Willoughby et al., 2009). Cellular apoptosis is characterized morphologically by cell shrinkage, nuclear pyknosis, chromatin condensation, and blebbing of the plasma membrane (Lu et al., 2009). DHA results from dose-dependent G2 arrest, although, the involved mechanisms is unclear. Thus, cell cycle arrest and apoptosis might be responsible, at least in part, to DHA inhibition of cell growth in glioma.

Angiogenesis, the growth of new capillaries in response to pro-angiogenic factors secreted by glioma cells due to a lack of oxygen and nutrients, plays a crucial role in tumor growth (Kesari, 2011; Corsini et al., 2012). Among various activators of angiogenesis, VEGF is considered as one of the most potent angiogenic factors (Arbab, 2012). The expression of VEGF was closely associated with angiogenesis, growth, metastasis, and poor outcome in solid tumors (Holmes et al., 2007; Kaiser et al., 2011). In order to analyze the effect of DHA on VEGF secretion and expression in U87 cells, we detected the VEGF mRNA expression by RT-PCR and the secreted VEGF level in supernatant by ELISA assay. These results suggested that DHA could inhibit VEGF expression and secretion effectively in U87 cells, similar to previous studies (Chen et al., 2004; Lee et al., 2006). Moreover, we selected an *in vitro* tube formation assay for the

angiogenesis study (Figure 6). DHA dramatically decreases the tube formation rate in HUVECs, which means DHA might be useful for targeting against angiogenesis.

Invasiveness and migration are important characteristics of glioma cells and targets of anti-cancer agent development (Claes et al., 2007; Mentlein et al., 2011). In this study, we used the wound scratch assay to assess the motility of U87 cells and the Matrigel invasion assay to assess the ability of U87 cells to penetrate the ECM. The data show that the motility as well as the invasion potential of U87 cells were significantly reduced by DHA, but further clarification of the underlying mechanisms is required.

Conclusion

Conclusively, our results demonstrate that DHA significantly inhibits human glioma U87 cells viability, proliferation, invasion, and tube formation. Furthermore, our results provide little evidence of potential implications for the rational application of DHA as a potential anticancer drug against human glioma, although, much studies are needed.

REFERENCES

Arbab AS (2012). Activation of alternative pathways of angiogenesis and involvement of stem cells following anti-angiogenesis treatment in glioma. Histol. Histopathol. 27:549-557.

Chen HH, Zhou HJ, Wang WQ, Wu GD (2004) Antimalarial dihydroartemisinin also inhibits angiogenesis. Cancer Chemother Pharmacol. 53:423-432.

Claes A, Idema AJ, Wesseling P (2007). Diffuse glioma growth: a guerilla war. Acta Neuropathol. 114:443-458.

Corsini E, Ciusani E, Gaviani P, Silvani A, Canazza A, Bernardi G, Calatozzolo C, Meco FD, Salmaggi A (2012). Decrease in circulating endothelial progenitor cells in treated glioma patients. J. Neurooncol. 108(1):123-129.

Du JH, Zhang HD, Ma ZJ, Ji KM (2010) Artesunate induces oncosis-like cell death in vitro and has antitumor activity against pancreatic cancer xenografts in vivo. Cancer Chemother Pharmacol. 65:895-902.

He Q, Shi J, Shen XL, An J, Sun H, Wang L, Hu YJ, Sun Q, Fu LC, Sheikh MS, Huang Y (2010). Dihydroartemisinin upregulates death receptor 5 expression and cooperates with TRAIL to induce apoptosis in human prostate cancer cells. Cancer Biol. Ther. 9:819-824.

Holmes K, Roberts OL, Thomas AM, Cross MJ (2007). Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. Cell Signal 19:2003-2012.

Houle CD (2011). Neuropathology standards: what constitutes an optimal histomorphologic evaluation of the nervous system in general toxicity studies. Toxicol. Pathol. 39:1010-1012.

Jiao Y, Ge CM, Meng QH, Cao JP, Tong J, Fan SJ (2007). Dihydroartemisinin is an inhibitor of ovarian cancer cell growth. Acta Pharmacol. Sin 28:1045-1056

Kaiser R, Dubovy P, Haninec P (2011). (Vascular endothelial growth factor). Cesk Fysiol. 60:48-51.

Kesari S (2011) Understanding glioblastoma tumor biology: the potential to improve current diagnosis and treatments. Semin. Oncol. 38 Suppl 4:S2-10

Lee J, Zhou HJ, Wu XH (2006). Dihydroartemisinin downregulates vascular endothelial growth factor expression and induces apoptosis in chronic myeloid leukemia K562 cells. Cancer Chemother. Pharmacol. 57:213-220.

Lu YY, Chen TS, Qu JL, Pan WL, Sun L, Wei XB (2009). Dihydroartemisinin (DHA) induces caspase-3-dependent apoptosis in human lung adenocarcinoma ASTC-a-1 cells. J. Biomed. Sci. 16:16

Mentlein R, Hattermann K, Held-Feindt J (2011). Lost in disruption: Role of proteases in glioma invasion and progression. Biochim. Biophys. Acta 1825:178-185.

Meshnick SR (2002). Artemisinin: mechanisms of action, resistance and toxicity. Int. J. Parasitol. 32:1655-1660.

Riganti C, Doublier S, Viarisio D, Miraglia E, Pescarmona G, Ghigo D, Bosia A (2009) Artemisinin induces doxorubicin resistance in human colon cancer cells via calcium-dependent activation of HIF-1alpha and P-glycoprotein overexpression. Br. J. Pharmacol. 156:1054-66.

Willoughby JS, Sundar SN, Cheung M, Tin AS, Modiano J, Firestone GL (2009). Artemisinin blocks prostate cancer growth and cell cycle progression by disrupting Sp1 interactions with the cyclin-dependent kinase-4 (CDK4) promoter and inhibiting CDK4 gene expression. J. Biol. Chem. 284:2203-2213.

Zhao Y, Jiang W, Li B, Yao Q, Dong J, Cen Y, Pan X, Li J, Zheng J, Pang X, Zhou H (2011). Artesunate enhances radiosensitivity of human non-small cell lung cancer A549 cells via increasing NO production to induce cell cycle arrest at G2/M phase. Int. Immunopharmacol. 11:2039-2046.

Zhou HJ, Wang WQ, Wu GD, Lee J, Li A (2007). Artesunate inhibits angiogenesis and downregulates vascular endothelial growth factor expression in chronic myeloid leukemia K562 cells. Vascul. Pharmacol. 47:131-138.

American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

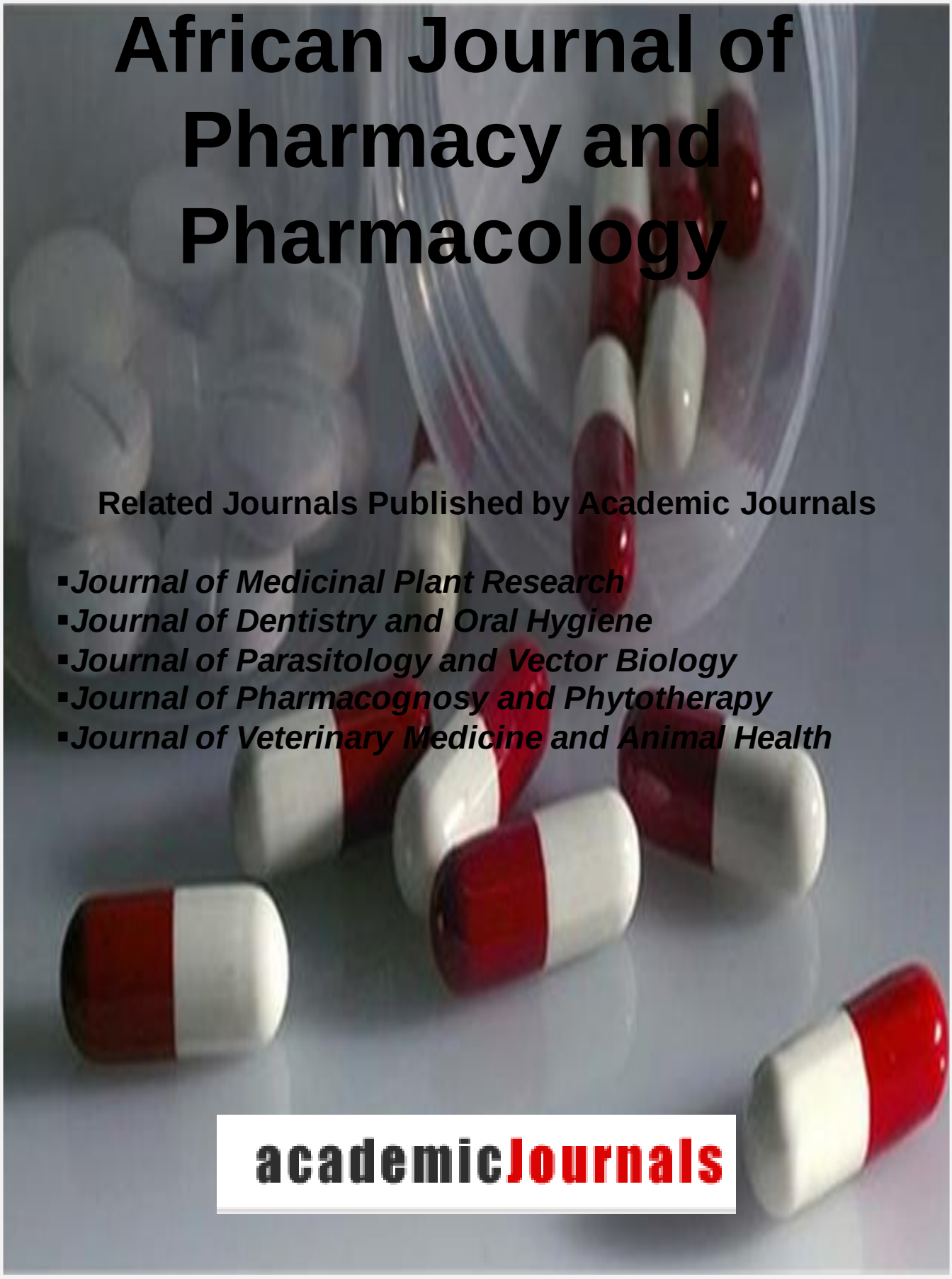
International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

Conferences and Advert

October 2012
American Association of Pharmaceutical Scientists Annual meeting and Exposition (AAPS), Chicago, USA, 14 Oct 2012

December 2012
International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 26 Dec 2012

African Journal of Pharmacy and Pharmacology



Related Journals Published by Academic Journals

- *Journal of Medicinal Plant Research*
- *Journal of Dentistry and Oral Hygiene*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Veterinary Medicine and Animal Health*

academicJournals