# African Journal of Pharmacy and Pharmacology

Volume 7 Number 31 22 August, 2013

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. 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**
*Department of Speech-Language and*
*Hearing Therapy Faculty of Philosophy*
*and Sciences, UNESP*
*Marilia-SP, Brazil.l*

**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,*

**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: © 2013, 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 7 Number 31 22 August, 2013

## ARTICLES

### Research Articles

**Survey of Brazilian community pharmacist knowledge about combined oral contraceptives** 2214
Paulo Roque Obreli-Neto, Leonardo Régis Leira Pereira, Camilo Molino Guidoni, André de Oliveira Baldoni, Renata Cristine Martins, Juliane Aparecida de Abreu, Carolina Blasco Aquino, Nathaly da Silva Faria, Edmarlon Girotto and Roberto Kenji Nakamura Cuman

**Impact of artemether on some histological and histochemical parameters in *Biomphalaria alexandrina*** 2220
Hanan S. Mossalem, Hoda Abdel-Hamid and Nashwa A. El-Shinnawy

**Immunologic effects of *Moringa oleifera* methanolic leaf extract in chickens infected with Newcastle disease virus (kudu 113) strain** 2231
Didacus C. Eze, Emmanuel C. Okwor, John O. A. Okoye and Denis N. Onah

**Cytotoxic and anticoagulant peptide from *Scolopendra subspinipes* mutilans venom** 2238
Yi Kong, Jing Hui, Yu Shao, Shilong Huang, Hao Chen and Ji-Fu Wei

**Preparation of curcumin ethosomes** 2246
CHEN Jin-guang, LAI Wei and JIANG Yu

**Antioxidant markers in guinea pig exposed to obidoxime and HI-acetylcholinesterase oxime reactivators containing oxime moiety** 2252
Lucie Drtinova and Miroslav Pohanka

Table of Contents: Volume 7 Number 31 22 August, 2013

## ARTICLES

### Research Articles

**Anti-*Plasmodium falciparum* activity of Aloe dawei and *Justicia betonica* with the brand** 2258
Godfrey S. Bbosa, David B. Kyegombe, Aloysius Lubega, Nathan Musisi, Jasper Ogwal-Okeng and Olwa Odyek

**Monotherapy versus combinations of nucleos(t)ide in treatment-naive hepatitis B decompensated cirrhotic patients: A nested case-control study** 2264
Lei Li, Wei Liu, Yuhan Chen, Peng Li,Feili Wei, Chunlei Fan, Peiling Dong, Bing Li Dexi Chen and Huiguo Ding

**Social stratification predicts the risk of ischemic heart disease: A cross sectional study In Pakistani population above 40 years** 2273
Mahtab Ahmad Khan, Maqsood Ahmad, Sohail Safdar, Ahmed Subhani, Muhammad Usman Minhas, Saba Sajjad, Muhammad Asadullah Madni and Sajjad Muhammad

academicJournals

Vol. 7(31), pp. 2214-2219, 22 August, 2013
DOI 10.5897/AJPP2013.3675
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Survey of Brazilian community pharmacist knowledge about combined oral contraceptives

**Paulo Roque Obreli-Neto[1]*, Leonardo Régis Leira Pereira[2], Camilo Molino Guidoni[3], André de Oliveira Baldoni[2], Renata Cristine Martins[4], Juliane Aparecida de Abreu[4], Carolina Blasco Aquino, Nathaly da Silva Faria[4], Edmarlon Girotto[3] and Roberto Kenji Nakamura Cuman[1]**

[1]Department of Pharmacology and Therapeutics, State University of Maringa, Maringa, Brazil.
[2]Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Sao Paulo, Brazil.
[3]Department of Pharmaceutical Sciences, State University of Londrina, Londrina, Parana, Brazil.
[4]Department of Pharmacy, Faculdades Integradas de Ourinhos, Sao Paulo, Brazil.

Accepted 5 August, 2013

**The present study evaluated community pharmacist (CP) knowledge about combined oral contraceptives (COCs) in a developing country. A cross-sectional study was conducted in community pharmacies of the Assis and Ourinhos micro-regions, Brazil, between June 1, 2012 and October 30, 2012. A structured questionnaire developed by the research team was applied to evaluate CP knowledge about COCs. The structured questionnaire consisted of seven multiple-choice questions about the mechanism of action, method of initiation, drug dosage, recommendations for missed COC doses, contraindications, adverse effects, and drug interactions. The percentage of CPs who answered the questions correctly was determined. Of the 185 CPs contacted, 41 (22.2%) agreed to participate in the study and finished the study protocol. A total of 13 (31.7%), 19 (46.3%), 19 (46.3%), and 5 (12.2%) CPs correctly answered questions about the mechanism of action, method of initiation, drug dosage, and recommendations for missed COC doses, respectively. With regards to contraindications, adverse effects, and drug interactions, the number of CPs who answered these questions correctly was 23 (56.1%), 29 (70.1%), and 23 (56.1%), respectively. The CPs evaluated in the present study had several gaps in their knowledge about COCs, with approximately half of the individuals inadequately answering the questions asked.**

**Key words:** Pharmacy practice, pharmacy education, combined oral contraceptive.

## INTRODUCTION

Combined oral contraceptives (COCs) are the most commonly used reversible method of birth control in developed and developing countries. Nearly 100 million women worldwide use this method. It has a very high efficacy rate, and the appropriate use of COCs can yield contraceptive efficacy greater than 99% (Nelson, 2007). COCs also provide several noncontraceptive benefits, such as a reduction of the risk of ovarian and endometrial cancers and improved menstrual regulation (Dayal and Barnhart, 2001; Jensen and Speroff, 2000). Despite the contraceptive and noncontraceptive benefits, COCs are associated with a high self-reported 12-month discontinuation rate (approximately 50%), and COC discontinuation has been reported to lead to unintended

*Corresponding author. E-mail: paulorobreli@yahoo.com.br. Tel: (+55 44) 3011-4867.

pregnancies in more than 50% of the cases (Nelson, 2007; Westhoff et al., 2007). Unintended pregnancies carry huge humanistic and economic burdens for both individuals and society, with an estimated cost of USD$11.1 billion each year in the United States (Sonfield et al., 2011). The adoption of strategies that improve patient adherence to COC use is needed to decrease these high rates of unintended pregnancies caused by the inappropriate use of COC.

Community pharmacists (CPs) are in a strategic position to improve COC use, because they are the last healthcare professionals who patients see before initiating drug administration. Pharmacy-based counseling on the correct use, benefits, and adverse effects of COCs can improve patient adherence (Gardner et al., 2008). Some requirements are necessary to provide patient counseling appropriately, such as learning how to establish a therapeutic relationship with the patient and acquiring a unique and specific pharmacotherapy knowledge base (Cipolle et al., 2012). However, pharmacy education curricula in several developing countries are not focused on patient care, with only few disciplines that focus on developing communication skills and pharmacotherapy knowledge (de Castro and Correr, 2007; Ghilzai and Dutta, 2007; Volmer et al., 2008). This gap in pharmacy graduation curricula in developing countries can result in inappropriate CP knowledge, skills, and competency to provide patient counseling.

Studies that evaluate CP knowledge about COCs may help identify areas where educational interventions are needed. To the best of our knowledge, despite the importance of evaluating CP knowledge about COCs, studies that have evaluated these aspects are scarce, especially in developing countries. Previous studies were conducted in developed countries (Bennett et al., 2003; Hellerstedt and Van Riper, 2005) and evaluated knowledge about other less frequently used contraceptive methods (e.g. emergency contraceptives) (Blanchard et al., 2005; Ehrle and Sarker, 2011). The aim of this study was to evaluate CP knowledge about COCs in a developing country.

## METHODOLOGY

### Study design and setting

This study was approved by the Research Ethics Committee of the State University of Maringa, Brazil. This was a cross-sectional study conducted from June 1, 2012, to October, 30, 2012, in community pharmacies in the Assis and Ourinhos micro-regions in Brazil. The Assis and Ourinhos micro-regions comprise 29 municipalities and have an estimated population of 544,000, with a total of 185 community pharmacies. Brazilian community pharmacies are private health establishments where commercial drugs, medical products, and other products (e.g. toiletries and beauty products) are available without a prescription (depending on the specific legislation concerning the commercial product).

Brazilian legislation requires the presence of at least one pharmacist in the community pharmacy during all times that the pharmacy is open to the public, and the establishment must have on it frame of employees how many pharmacists is necessary to follow-up this legislation. To assume the responsibility for a community pharmacy, the professional must have graduated from pharmacy school at an institution recognized by the Brazilian Ministry of Education and registered in the Regional Board of Pharmacy of the state where he or she works (Brasil, 1973).

### Inclusion and exclusion criteria

Community pharmacist employees of a participating community pharmacy during the study period who were registered in the Regional Board of Pharmacy of Sao Paulo State and worked Monday through Friday, 8:00 AM to 5:00 PM, were eligible for inclusion. Community pharmacists who worked in two or more participating community pharmacies (that is, to avoid evaluating the same CP more than once) or had difficulty speaking (which would interfere with their participation in the study) were excluded.

### Identifying eligible community pharmacists

All of the community pharmacies in the aforementioned micro-regions were visited by eight researchers during working hours. The researchers contacted the CPs present in the establishments and invited them to participate in the study. The researchers explained to the CPs that the objective of the study was to evaluate their knowledge about COCs using a structured questionnaire. Information about the content of the questionnaire and study protocol was also provided. The CPs were advised that no identifying information about the CP would be noted on the questionnaire to guarantee anonymity. Community pharmacists who agreed to participate in the study were evaluated with regard to the eligibility criteria, and the general characteristics of the CPs were noted on our research form, including age, sex, function in the pharmacy, length of time working as a CP, and hours worked per week.

### Structured questionnaire

Five research specialists in COC pharmacotherapy developed a structured questionnaire for this study. The questionnaire intended to evaluate CP knowledge about COC use (that is, mechanism of action, method of initiation, drug dosage, and recommendations for missed COC doses) and safety aspects (that is, contraindications, adverse effects, and drug interactions). The structured questionnaire consisted of seven multiple-choice questions. Two questions had just one correct answer (e.g. What is the COC mechanism of action? What is the COC drug dosage?), whereas other questions had more than one correct answer (e.g. How to initiate COC use? What are the recommendations for missed COC doses? What are COC contraindications? What are COC adverse effects? What are COC drug interactions?).

### Study protocol

After evaluating the CPS with regard to the eligibility criteria, the eligible CPs received the structured questionnaire. They had to answer the structured questionnaire in the presence of the researcher, without consulting books, websites, manuscripts, or package inserts. The CPs had no time limit to answer the structured questionnaire.

The answers of each CPs were corrected independently by three researchers to avoid mistakes in the correction process. If different

**Table 1.** General characteristics of the community pharmacists who participated in the study (June-October, 2012; *n* = 41).

| Characteristic | Value |
|---|---|
| **Age groups, years** | |
| 20-24 | 1 (2.4) |
| 25-29 | 12 (29.3) |
| 30-34 | 19 (46.3) |
| 35-39 | 9 (22.0) |
| | |
| **Gender, *n* (%)** | |
| Female | 29 (70.7) |
| Male | 12 (29.3) |
| | |
| **Function in the pharmacy, *n* (%)** | |
| Employee of the pharmacy, without administrative functions | 27 (65.9) |
| Employee of the pharmacy, with administrative functions (manager) | 8 (19.5) |
| Owner of the pharmacy | 6 (14.6) |
| | |
| **Length of time as community pharmacist, years** | |
| 0-4 | 5 (12.2) |
| 5-9 | 23 (56.1) |
| 10-14 | 13 (31.7) |
| | |
| **Hours worked per week** | |
| 20-29 | 1 (2.4) |
| 30-39 | 5 (12.2) |
| 40-49 | 35 (85.4) |

corrections occurred, then the researchers checked the answers again to identify any questions that were corrected inadequately. After reaching a consensus on the corrections, the answers where entered in an electronic database to perform the data analysis.

#### Data analysis

The following general CP characteristics were described: age, sex, function in the pharmacy, length of time working as a CP, and hours worked per week. Descriptive statistics were used to analyze most of the variables. The results are expressed as medians and interquartile ranges, means ± standard deviations (SDs), or proportions. Numerical variables were tested for a normal distribution using the Kolmogorov-Smirnov and Shapiro-Wilt tests. The analyses were performed using Statistica version 8.0 software (StatSoft, Sao Caetano do Sul, SP, Brazil).

## RESULTS

### General characteristics of the community pharmacists

Of the 185 CPs contacted, a total of 41 individuals (22.2%) agreed to participate in the study and finished the study protocol. Most of the CPs were young female adults (youngest CP, 24 years old; oldest CP, 39 years old). More than 65% of these professionals did not have administrative functions in the community pharmacies. The time of CP experience varied between 3 and 13 years, with the majority of CPs with less than 10 years of experience. Thirty-five CPs worked ≥ 40 h per week (range, 20 to 46 h) (Table 1).

### Knowledge about use of combined oral contraceptives

Fewer than 50% of the CPs knew the mechanism of action and drug dosage of conventional COC regimens. With regard to knowledge about how to initiate COC use, a total of 46.3% CPs answered the question correctly, but all of the CPs only knew that COC use should start at the beginning of the next menstrual cycle (that is, none of the CPs knew about the “quick start” and “first-day start” methods). Only 5 (12.2%) of the CPs knew the recommendations for missed COC doses (Table 2).

### Knowledge about safety aspects of combined oral contraceptives

Table 3 shows the number of CPs who knew information

**Table 2.** Community pharmacist knowledge about information related to the use of combined oral contraceptives (June-October, 2012; *n* = 41).

| Variable | *n* (%) | p value |
|---|---|---|
| Mechanism of action of COCs | 13 (31.7) | 0.001 |
| How to initiate COC use[a] | 19 (46.3) | 0.001 |
| Drug dosage of COCs[b] | 19 (46.3) | 0.001 |
| Recommendations for missed COC doses | 5 (12.2) | < 0.001 |

COC: Combined oral contraceptive. [a] The questions asked how to initiate COC use in women who were not currently using any contraceptive method. [b] The questions asked about the drug dosage of conventional regimens of COC (21 days of active pills, 7 days of placebo).

**Table 3.** Community pharmacist knowledge about safety aspects of combined oral contraceptives (June-October, 2012; *n* = 41).

| Variable | *n* (%) | p value |
|---|---|---|
| COC contraindications[a] | 23 (56.1) | < 0.001 |
| COC adverse effects[b] | 29 (70.7) | < 0.001 |
| COC drug interactions[c] | 23 (56.1) | < 0.001 |

[a]The community pharmacist must know at least one COC contraindication. [b] The community pharmacist must know at least one COC adverse effect. [c] The community pharmacist must know at least one COC drug interaction.

about the safety aspects of COCs. The COC contraindications reported by the CPs were of age ≥ 35 years and smoking ≥ 15 cigarettes/day (*n* = 7), vascular disease (*n* = 5), systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 100 mmHg (*n* = 5), current breast cancer (*n* = 4), a history of deep venous thrombosis/pulmonary embolism (*n* = 3), and migraine with aura (*n* = 3). The adverse effects reported by the CPs were headache (*n* = 24), nausea (*n* = 19), weight gain (*n* = 18), breast tenderness (*n* = 10), dysmenorrhea (*n* = 6), and menorrhhagia (*n* = 4). The unique drug interaction reported by the CPs was COC + antibiotics (that is, tetracyclines and penicillin derivatives) (Table 3).

## DISCUSSION

To our knowledge, this is the first study that evaluated CP knowledge about COCs in a considerable number of cities in a developing country using a structured questionnaire. The awareness of gaps in the knowledge about COCs may assist in the development of educational interventions that address this potential public health problem and reformulation of pharmacy school curricula. In the present study, the CPs had several gaps in their knowledge about COCs, with approximately half of the CPs inadequately answering the questions asked. The results of this study suggest the need to develop educational interventions for CP knowledge about COCs to improve their counseling practice.

Studies of community pharmacies conducted in developing countries, such as Mexico (Becker et al., 2004), Jamaica (Chin-Quee et al., 2006), and Thailand (Ratanajamit and Chongsuvivatwong, 2001), found that COCs are sold with little or no patient counseling. A lack of counseling is an important cause for the incorrect use of COCs (e.g. discontinuation and wrong attitude when missing pills) (Nelson, 2007; Sonfield et al., 2011), and most patients in developing countries do not have appropriate knowledge about COCs (Espejo et al., 2003; Martins et al., 2007). This lack of counseling could be attributable to gaps in knowledge about COCs.

Previous studies conducted in developed countries verified that CP knowledge about emergency contraceptive pills was low. One study performed in the state of South Dakota in the United States found that only 5% of CPs correctly answered all of the questions about emergency pills (Hellerstedt and Van Riper, 2005). Only 3% of New York CPs correctly provided all of the key facts about emergency contraceptive pills (Draut, 1999). These results suggest that CP education about oral contraceptives must be strengthened to ensure that women receive appropriate counseling on these contraceptive methods.

The lack of CP knowledge about COCs identified in Brazil and emergency contraceptive pills in other countries requires us to analyze, reflect on, and revise pharmacy school curricula and CP continuing education. In recent decades, the education and training of pharmacists and health professionals in general has been the subject of extensive discussions (Brasil, 2002; Harden et al., 1997; Storpirts, 2012).

The literature shows that these discussions first started in developed countries. Harden et al. (1997) discussed the necessity of developing new curricula and educational strategies for medical education. Among these changes, they highlighted elements of problem-based and community-based learning. Problem-based learning and community-based learning stimulate students

students to enhance their skills in problem-solving, critical thinking, clinical reasoning, and self-directed learning, which are indicated to be a more effective methodology to prepare students to provide appropriate patient care (Cisneros et al., 2002). These strategies can encourage students to take more responsibility for their own learning.

Another important change required is integration among disciplines (Harden et al., 1997). According to Frenk et al. (2010), the education of health professionals must eliminate fragmented, outdated, and static curricula. From this study point of view, professors should have new ideas and teaching strategies that better integrate university and health service to promote integration among disciplines. For example, the undergraduate pharmacy curriculum in Brazil has undergone substantial changes during the past two decades, and this issue has been widely discussed among researchers, policy-makers, and practitioners. This change was marked with the implementation of general curricula for pharmacy courses by the National Council of Education/Ministry of Education and Culture in 2002 (Storpirtis, 2012).

Although this change in pharmacy undergraduate curricula was implemented in Brazil more than a decade ago, universities have failed to implement general, humanistic, critical, reflexive, and contextualized integration with the health system. In this context, many CPs in this study were not educated using the new general curriculum.

This study has some limitations that should be addressed. It only involved CPs in two Brazilian micro-regions, and the generalization of these findings to other locations may be limited. Only 22.2% of the CPs responded to the structured questionnaire, and ascertaining the impact of not having data from the other 77.8% of the CPs is difficult. Knowledge about COCs from CPs who did not participate in this study may be different from these results because Brazil has many different universities that offer pharmacy undergraduate courses, with high variability in the methodologies applied in these universities.

## Conclusion

This study found that CPs had several gaps in their knowledge about COCs, with approximately half of the individuals inadequately answering all of the questions. These results suggest the need to develop educational interventions for CP knowledge about COCs to improve their counseling practice. Changes in undergraduate pharmacy curricula and teaching methodologies are important to train professionals who are more able to provide appropriate patient care.

### REFERENCES


Becker D, Garcia SG, Ellertson C (2004). Do Mexico City pharmacy workers screen women for health risks when they sell oral contraceptive pills over-the-counter? Contraception 69:295-299.

Bennett W, Petraitis C, D'Anella A, Marcella S (2003). Pharmacists' knowledge and the difficulty of obtaining emergency contraception. Contraception 68:261-267.

Blanchard K, Harrison T, Sello M (2005). Pharmacists' knowledge and perceptions of emergency contraceptive pills in Soweto and the Johannesburg Central Business District, South Africa. Int. Fam. Plan Perspect. 31:172-178.

Brasil (1973) Lei nº 5.991, de 17 de dezembro de 1973. Dispõe sobre o controle sanitário do comércio de drogas, medicamentos, insumos farmacêuticos e correlatos, e dá outras providências, Diário Oficial da União, Brasil.

Brasil (2002). Conselho Nacional de Educação/Câmara de Educação Superior Resolução CNE/CES 2, de 19 de fevereiro de 2002. Institui Diretrizes Curriculares Nacionais do Curso de Graduação em Farmácia, Diário Oficial da União, Brasil.

Cipolle RJ, Strand LM, Morley PC (2012). Pharmaceutical care practice: The Patient-Centered Approach to Medication Management. McGraw-Hill Medical, United States of America.

Chin-Quee DS, Cuthbertson C, Janowitz B (2006). Over-the-counter pill provision: evidence from Jamaica. Stud. Fam. Plann 37: 99-110.

Cisneros RM, Salisbury-Glennon JD, Anderson-Harper HM (2002). A Review of Problem-Based Learning Research in Pharmacy: A Call for Future Research. Am. J. Pharm. Educ. 66:19-26.

Dayal M, Barnhart KT (2001). Noncontraceptive benefits and therapeutic uses of the oral contraceptive pill. Semin Reprod. Med. 19:295-303.

de Castro MS, Correr CJ (2007). Pharmaceutical care in community pharmacies: practice and research in Brazil. Ann. Pharmacother. 41:1486-1493.

Draut T (1999). Emergency contraception: do pharmacists know about this important method to prevent pregnancy? Planned Parenthood of New York City, United States of America.

Ehrle N, Sarker M (2011). Emergency contraceptive pills: knowledge and attitudes of pharmacy personnel in Managua, Nicaragua. Int. Perspect. Sex Reprod. Health 37:67-74.

Espejo X, Tsunechiro MA, Osis MJ, Duarte GA, Bahamondese L, de Sousa MH (2003). Knowledge adequacy on contraceptives among women in Campinas, Brazil. Rev. Saude Publica. 37:583-590.

Frenk J, Chen L, Bhutta ZA, Cohen J, Crisp N, Evans T, Fineberg H, García PJ, Ke Y, Kelley P, Kistnasamy B, Meleis A, Naylor D, Pablos-Mendez A, Reddy S, Scrimshaw S, Sepulveda J, Serwadda D, Zurayk H (2010). Health professionals for a new century: transforming education to strengthen health systems in an interdependent world. The Lancet 376(9756):1923-1958.

Gardner JS, Miller L, Downing DF, Le S, Blough D, Shotorbani S (2008). Pharmacist prescribing of hormonal contraceptives: results of the Direct Access study. J. Am. Pharm. Assoc. (2003) 48:212-221.

Ghilzai NM, Dutta AP (2007). India to introduce five-year doctor of pharmacy program. Am. J. Pharm. Educ. 71:38.

Harden RM, Davis MH, Crosby JR (1997). The new Dundee medical curriculum: a whole that is greater than the sum of the parts. Med. Educ. 31:264-271.

Hellerstedt WL, Van Riper KK (2005). Emergency contraceptive pills: dispensing practices, knowledge and attitudes of South Dakota pharmacists. Perspect. Sex. Reprod. 37:19-24.

Jensen JT, Speroff L (2000). Health benefits of oral contraceptives. Obstet. Gynecol. Clin. North Am. 27:705-721.

Martins LB, Costa-Paiva L, Osis MJ, Sousa MH, Pinto Neto AM, Tadini V (2006). Knowledge of contraceptive methods among adolescent students. Rev. Saude Publica. 40:57-64.

Nelson A (2007). Combined oral contraceptives. In: Hatcher RA, Trussel J, Nelson AL (eds) Contraceptive Technology, Ardent Media, United States of America pp. 193-270.

Ratanajamit C, Chongsuvivatwong V (2001). Survey of knowledge and practice on oral contraceptive and emergency contraceptive pills of drugstore personnel in Hat Yai, Thailand. Pharmacoepidemiol. Drug Saf. 10:149-156.

Sonfield A, Kost K, Gold RB, Finer LB (2011). The public costs of births resulting from unintended pregnancies: national and state-level estimates. Perspect. Sex. Reprod. Health 43:94-102.

Storpirts S (2012). Perspectives and challenges in Pharmaceutical Care. Braz. J. Pharm. Sci. 48.
Volmer D, Vendla K, Vetka A, Bell JS, Hamilton D (2008). Pharmaceutical care in community pharmacies: practice and research in Estonia. Ann. Pharmacother. 42:1104-1111.
Westhoff CL, Heartwell S, Edwards S, Zieman M, Stuart G, Cwiak C, Davis A, Robilotto T, Cushman L, Kalmuss D (2007). Oral contraceptive discontinuation: do side effects matter? Am. J. Obstet. Gynecol. 196:412.e1–7.

academicJournals

Vol. 7(31), pp. 2220-2230, 22 August, 2013
DOI 10.5897/AJPP2013.3762
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Impact of artemether on some histological and histochemical parameters in *Biomphalaria alexandrina*

**Hanan S. Mossalem[1]*, Hoda Abdel-Hamid[1] and Nashwa A. El-Shinnawy[2]**

[1]Environmental Researches and Medical Malacology Department, Theodor Bilharz Research Instiute, Egypt.
[2]Histology and Histochemistry Department, Girls College for Arts, Science and Education, Ain Shams University, Egypt.

Accepted 12 August, 2013

**The present study elucidated the molluscicidal properties of the antihelminthic plant derivative, dihydro-artemisinin methyl ether (Artemether) against some histological and histochemical parameters of *Biomphalaria alexandrina* snails. Results indicated that Artemether has a toxic effect against *B. alexandrina* snails expressed by $LC_{90}$ of 32.69 ppm, after 24 h exposure. The rate of infection with *Schistosoma mansoni* in snails exposed to sublethal concentrations ($LC_0$ and $LC_{10}$) of Artemether either for 1 or 7 successive days pre-miracidial exposure was significantly lower than that of control group ($P < 0.001$). The reduction rates of this parameter ranged from 76.9 to 87.3%. Moreover, snails treated with $LC_{25}$ for 7 days were free from infection (0% infection rate). Moreover, the survival rate in snails exposed to $LC_{25}$ on 1 and 7 days pre-miracidial exposure were 23.3 and 10%, respectively compared to the 93.3% in control group. Histological investigation of the exposed snails' gland showed that the mature ova lost their normal shape, disappearance of most of the oocytes, spermatogenesis and connective tissues. Moreover, complete destruction of gametogenic cells and severe damage of hermaphrodite gland tissues were clear when the exposure period increased. Haemocytes' morphology showed obvious abnormalities as decrease in their number, activation, and irregular aggregation in exposed snails were recorded. Also, snails' exposure to $LC_{25}$ of Artemether demonstrated a decreased glycogen content in hermaphrodite gland tissues while it increased in the haemolymph. In conclusion, the use of sublethal concentration of Artemether showed remarkable molluscicidal activities against *B. alexandrina* snails which could be a promising and safe biological agent to control schistosomiasis disease.**

**Key words:** *Biomphalaria alexandrina* snails, *Schistosoma mansoni,* artemether, glycogen.

## INTRODUCTION

In the tropics and subtropics, schistosomiasis is the second most important parasitic disease after malaria in terms of prevalence, public health and socio-economic importance (Chitsulo et al., 2004; Steinmann et al., 2006). Freshwater snails of the genus Biomphalaria plays a major role as intermediate hosts of *Schistosoma mansoni* is the etiological agent of intestinal schistosomiasis while *Biomphalaria* spp. controled by molluscicides is one of the main strategies to reduce the snail population in infected areas. There are few effective molluscicides commercially available. Natural products are considered as potentially useful and safe molluscicides (Miyasato et al., 2012).

There is a great interest in the use of molluscicides of plant origin by local communities in self-supporting system of schistosomiasis control program. Such molluscicides seem to be less expensive, readily available, rapidly biodegradable and probably easily

*Corresponding author. E-mail: gibriel2003@gmail.com

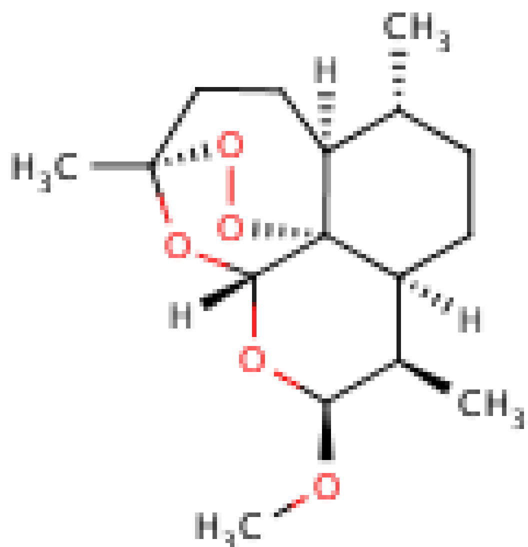


**Figure 1.** Artemether structure.

applicable with simple techniques appropriate to developing countries (Ibrahim et al., 2004). Plant molluscicides have been regarded as possible alternatives to the costly and environmentally hazardous molluscicides currently available (Oliveira-Filho et al., 2010). Also, there is a continuous need to search for new plant species with ideal molluscicidal properties (Tantawy et al., 2004; Bakry and Hamdi, 2007). Many investigators have studied the molluscicidal activity of certain plants and their extracts (El-Emam et al., 1990; Shalaby and Abdel-Hamid, 1999; Mossalem, 2003; Bakry et al., 2007; Abdel-Hamid, 2008; Hasheesh et al., 2011).

A more successful approach to chemoprophylaxis has been to exploit a methyl ether plant derivative; Artemether which was first synthesized and used as an antimalarial drug in China (Hien and White, 1993). The anti-malarial drug Artemether was derived and developed from the plant Artemisia which was known since ancient time for its medicinal importance. Artemether was discovered to also have anti-schistosomal properties and had no drug-related adverse effects, and significantly reduced the incidence and intensity of schistosome infections, including those of *S. mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium* (Xiao et al., 2000a, 2002; Hou et al., 2008). Because Artemether selectively targets the larval migratory stages of the parasite, known as schistosomulae, it blocks the development of ovipositing adult schistosome worm pairs in the vasculature (Xiao et al., 2000). The plant Artemisia was proved to have a molluscicidal and miracidicidal activity (Mansour et al., 2002; Hafez et al., 2007).

Gastropods have an effective internal defense system consisting of cellular defense factor (Boehmler et al., 1996) and humeral defense factors (Johnston and Yoshino, 1996; Kofta, 1997). Exposure of snails to sub-lethal concentrations of molluscicidal plants resulted in reduction in total number of haemocytes (Sharaf El-Din, 2003; Martin et al., 2006; Souza and Andrade, 2006; El Sayed, 2006; Kamel et al., 2006, 2007; Gawish et al., 2008) and complete damage in their hermaphrodite gland tissues (Henry et al., 1989; Brackenbury, 1999; Al-Qormuti, 2008; Bakry, 2009; Hasheesh et al., 2011).

In order to promote energy production gastropods categorize primarily carbohydrates, which are stored in certain tissues as glycogen and transported in the haemolymph as glucose (Livingstone and Zwaan, 1983). The molluscicides greatly affect the metabolic activities of the snail intermediate hosts (Rawi et al., 1995).

The aim of this work is to study the molluscicidal effect of the antihelminthic plant derivative Artemether on some histological and histochemical parameters of *Biomphalaria alexandrina* and its susceptibility to infection with *S. mansoni*, as a possible safe method of schistosomiasis control.

## MATERIALS AND METHODS

### Snails

*B. alexandrina* snails were collected from different water courses at Giza Governorate, Egypt, during spring, 2012, and transferred in plastic bags to the laboratory. Snails were reared in de-chlorinated water (25°C ± 1) (Liang et al., 1987) at Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, Egypt. Healthy snails free from trematode infections were used in the experimental testes.

### Artemether

Artemether is an antimalarial agent for the treatment of multi-drug resistant strains of *falciparum* malaria. It is a methyl ether derivative of artemisinin, which is a peroxide lactone isolated from the Chinese antimalarial plant, *Artemisia annua*. Its chemical nomenclature is (+)-(3-alpha, 5a-beta, 6-beta, 8a-beta, 9-alpha, 12-beta, 12aR) -decahydro- 10-methoxy-3, 6, 9-trimethyl-3, 12-epoxy-12H-pyrano (4, 3-j)-1, 2-benzodioxepin (Figure 1). It is a relatively lipophilic and unstable drug (De Spiegeleer et al., 2012). Artemether tablets (50 mg with documented purity of 99.6%, Kunming Pharmaceutical Corporation, Kunming, China) were used in this study. The actual concentration was calculated as the percentage of the active material in the used weight. Artemether was applied to snails as aqueous solution of tablets.

### Molluscicidal activity

A stock solution of 1000 ppm was prepared from Artemether powder on the basis of w/v using de-chlorinated water (pH 7.0 to 7.5). A series of concentrations was prepared on the basis of volume/volume (World Health Organization, 1965). Three replicates were used, each of ten snails (6 to 8 mm/L, for each concentration. Exposure and recovery periods were 24 h each; at 25 ± 1°C. For each test, 3 replicates of control snails were maintained under the same experimental conditions in de-chlorinated water. The effectiveness of Artemether has been expressed as $LC_{50}$ and $LC_{90}$ (Litchfield and Wilcoxon, 1949). The sublethal concentrations were calculated through a computer program (IPM SPSS Statistics pro-

**Table 1.** Molluscicidal activity of Artemether against adult *B. alexandrina* snails after 24 h exposure.

| $LC_0$ ppm | $LC_{10}$ ppm | $LC_{25}$ ppm | $LC_{50}$ ppm | $LC_{90}$ ppm | Slope ppm |
|---|---|---|---|---|---|
| 2.11 | 9.43 | 14.94 | 21.06 | 32.69 | 1.63 |

gram, version 20 for Windows), employing the probit analysis (Finney, 1971).

### Effect of Artemether on infection of *B. alexandrina* snails with *S. mansoni*

*Biomphalaria* snails (6 to 8 mm) were exposed to miracidia after one and seven successive days of exposure to sublethal concentrations ($LC_0$, $LC_{10}$ and $LC_{25}$) of Artemether. Snails were exposed to miracidia in mass (10 fresh hatched miracidia/snail) for 24 h under ceiling illumination. For each concentration of each group, three replicates (each replicate of 10 snails/L in glass container) were prepared. After that, snails were transferred to clean de-chlorinated water (25 ± 1°C) and daily fed with oven dried lettuce leaves throughout the pre-patent and patent periods (Massoud et al., 1973). A control group of three replicates, each 10 snails/L was exposed to miracidia concurrently with the experimental snails and treated similarly till cercarial emergence. Dead snails were removed daily and surviving snails were individually examined once weekly for cercarial shedding 24 days post-miracidial exposure. The number of snails survived at the first shedding and the number of infected snails were calculated (Youssef, 2010). The survival and infection rates were compared with that of control using CHITEST of EXCEL program, Microsoft Office 2007 for Windows.

### Histological and histochemical tests

For each group, three replicates (each of twenty *B. alexandrina* snails, 6 to 8 mm) were exposed to $LC_{25}$ of Artemether continuously for 1, 7, 14 and 21 days. Another group of snails was maintained in de-chlorinated water under the same laboratory conditions as control. The haemolymph of about 10 snails of each group was collected according to Michelson (1966). Three snails from each group were crushed gently and the hermaphrodite gland was removed, fixed in Bouin's fluid for 5 h and then transferred to 70% alcohol. Further procedures were followed including dehydration in 100% alcohol, clearing in xylol and paraffin embedding. Five µm paraffin-embedded sections were stained with hematoxylin and eosin for general histological examination (Mohamed and Saad, 1990). Blood film slides were also stained with hematoxylin and eosin. To demonstrate the presence of glycogen, other sections of the hermaphrodite gland and blood film slides were stained with periodic Schiff technique (PAS) (Sheehan and Hrapchak, 1980). Stained slides were examined under light microscope and photos were taken and investigated.

### Statistical analysis

Student's *t-test* and chi-square test (Petrie and Sabin, 2000) were used in comparing the means and rates of experimental and control groups statistically.

## RESULTS

This experiment was planned to elucidate the molluscicidal properties of Artemether against adult *B. alexandrina* snails after 24 h of exposure followed by another of recovery. It was noticed that $LC_{50}$ and $LC_{90}$ values of Artemether were 21.06 and 32.69 ppm, respectively (Table 1). Table 2 indicated that survival rate, at first cercarial shedding, of *Biomphalaria* snails exposed for 1 and 7 days to sublethal concentrations ($LC_{0,}$ $LC_{10}$ and $LC_{25}$) of Artemether decreased compared to that of control. Thus, the survival rates of 7 days groups were 60, 33.3 and 10% compared to that of control one 93.3%, ($P < 0.0001$).

The infection rates of survived snails in the groups exposed to $LC_0$, $LC_{10}$ and $LC_{25}$ for 1 or 7 days pre-miracidial exposure were significantly ($P < 0.001$) less than that of control group (Table 2). Thus, infection rates of 1 day groups were 18.2, 17.6 and 14.3%, respectively, compared to 78.6% for control group. It is worthy mention that no snail was infected in the group exposed to $LC_{25}$ for 7 days pre- miracidial exposure (0%). The Light photomicrograph of the hermaphrodite gland complex of *B. alexandrina* exposed continuously to $LC_{25}$ of Artemether 1, 7, 14 and 21 days was shown in Figure 2. Figure 2A showed that *B. alexandrina* snails exposed to $LC_{25}$ of Artemether 1 day resulted in loose of connective tissues, short and irregular sperms, a slight decomposition in ova and a partial damage in some cell walls of hermaphrodite gland. In Figure 2B, snails exposed to Artemether, 7 days, showed lyses of cells and matrix' components. Also, the mature ova appeared dense and irregular in shape with congeal clear and atrophy. A reduction in the number of sperms was noticed. With the increase of exposure period to 14 days, ova lost their normal shape, the damage increases showing discharge, evacuation and disappearance of most of the components of the gonad cells (Figure 2C). Moreover, complete destruction of gametogenic cells and sever damage of ovotestis gland were clear in Figure 2D when the exposure period increased to 21 days. Also, disappearance of the oocytes, spermatogenesis and connective tissue was noticed. Figure 2E showed transverse section in the hermaphrodite gland of unexposed control snails showing the gonadal components, mature ova and oocytes and spermato-genesis occurs in centripetal form toward the lumen of the acinus expressed as sperms and spermatocys.

It is seen also that the photomicrographs in Figure 3 showed PAS positive reaction for glycogen (blue color) in the gonads' cells in *Biomphalaria* snails exposed continuously to $LC_{25}$ of Artemether compared to unexposed control. Glycogen contents aggregated between hermaphrodite cells in their connective tissue condensed in a large amount in case of groups exposed to Artemether 1 and 7 days (Figure 3A and B) compared to control group in which the glycogen is distributed at the

**Table 2.** The survival and infection rates of *B. alexandrina* snails with *S. mansoni* after exposure to Artemether for one and seven days.

| Exposure period (days) (pre-miracidial exposure) | Survival rate (%) | | | | Infection rate (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | $LC_0$ (2.11) ppm | $LC_{10}$ (9.43) ppm | $LC_{25}$ (14.94) ppm | Control | $LC_0$ (2.11) ppm | $LC_{10}$ (9.43) ppm | $LC_{25}$ (14.94) ppm | Control |
| 1 | 73.3*** | 56.7*** | 23.3*** | 93.3 | 18.2*** | 17.6*** | 14.3*** | 78.6 |
| 7 | 60.0*** | 33.3*** | 10.0*** | | 11.1*** | 10.0*** | 0*** | |

*** = Significantly lower than control at $p < 0.001$ (CHITEST).

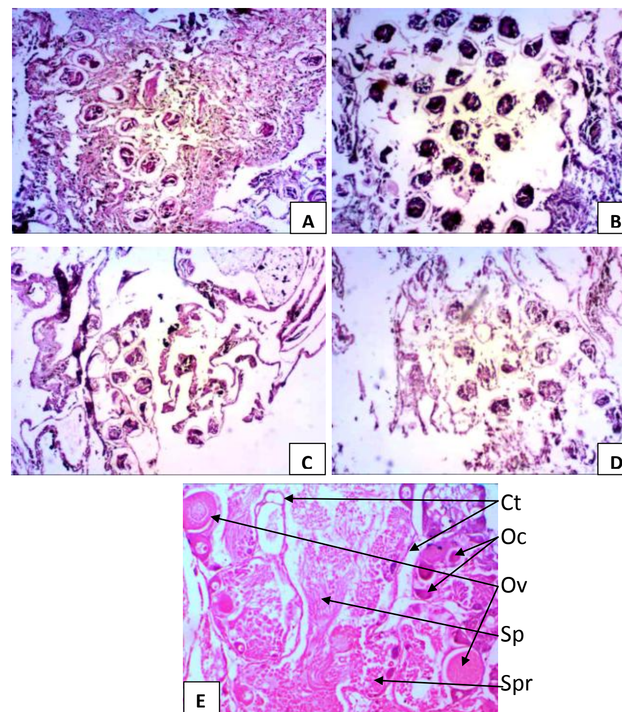


**Figure 1.** Light photomicrographs of T.S sections in the hermaphrodite gland of *Biomphalaria alexandrina* exposed continuously to $LC_{25}$ of Artemether 1, 7, 14 and 21 days (A, B, C &D). E: T.S section in the hermaphrodite gland of unexposed control snails. (Hematoxylin and eosin) ×400. Ct: Connective tissue; Ov: mature ova; Oc: Oocytes; Sp: Sperms; Spr: Spermatocytes

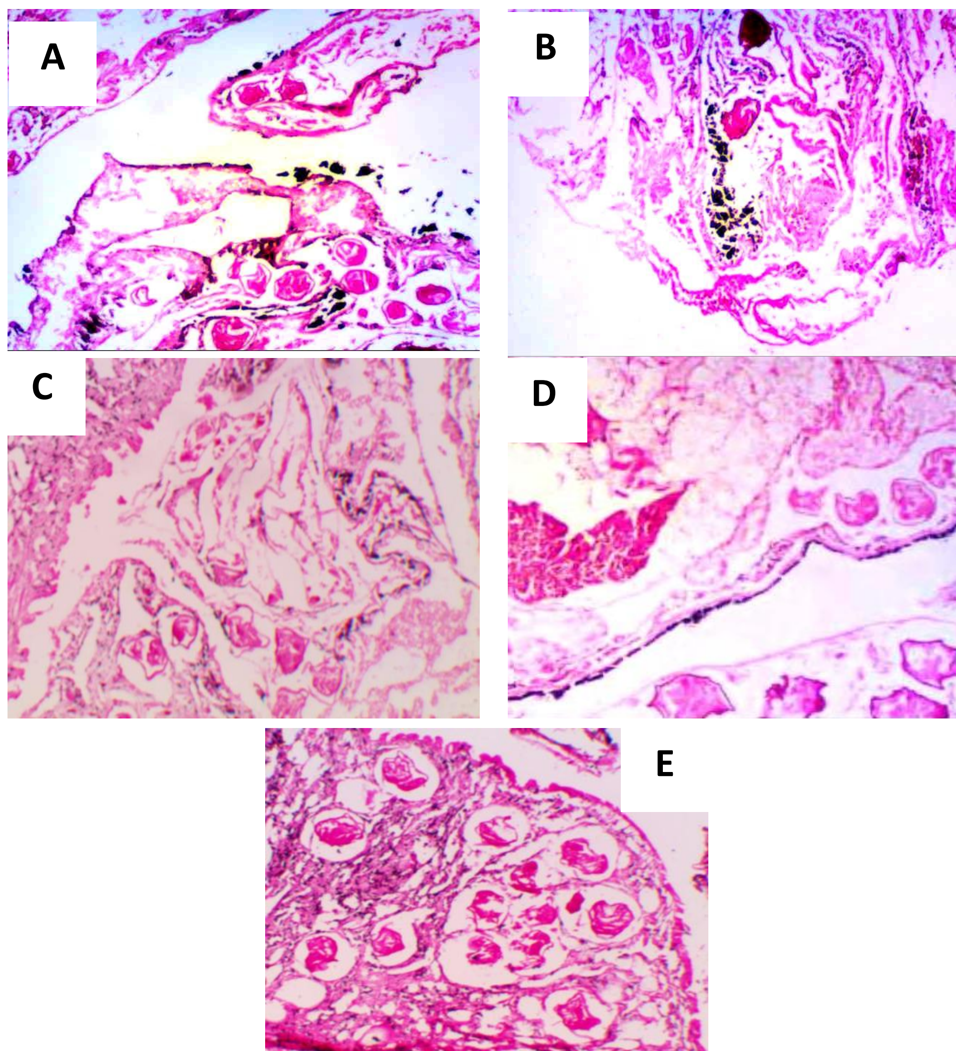


**Figure 2.** Light photomicrographs of T.S (Transverse section) sections in the hermaphrodite gland of *Biomphalaria alexandrina* exposed continuously to $LC_{25}$ of Artemether 1, 7, 14 and 21 days (A, B, C &D). E: T.S section in the hermaphrodite gland of unexposed control snails. (Hematoxylin and eosin) ×400. Ct: Connective tissue; Ov: mature ova; Oc: Oocytes; Sp: Sperms; Spr: Spermatocytes

at the epithelium of the cells and in connective tissue (Figure 2E). Elongation of exposure period to 2 and 3 weeks resulted in reduction and elevation in localization of glycogen at the peripheral edges of the gonadal tubules tissue compared to control (Figure 3C and D).

Concerning the effect of Artemether on *Biomphalaria* snails' haemocytes, it was shown obviously in Figure 4 that the three types of haemocytes distributed in the haemolymph of *B. alexandrina* (granulocytes, hyalinocytes and amoebocyte) were influenced by snail continuous exposure to Artemether. The number of cells was decreased by the increase of the exposure time. Also haemocytes' morphology showed clear abnormalities and irregular aggregation especially in snails exposed 21 days continuously to $LC_{25}$ Artemether. It was observed that the present results (Figure 5) showed glycogen organelles scattered, increasing the number of haemocytes in the haemolymph of the exposed snails with Artemether (a, b, c, d) than control (e). Increasing of the glycogen amount distributed inside haemocytes caused increase in their size by the elongation of exposure time showing irregular big cells (d) compared to control ones (e).

## DISCUSSION

Artemisinin (qinghaosu) is the active principle from the leaves of *Artemisia annua* L., a plant which is widespread throughout China, and also grows naturally in central

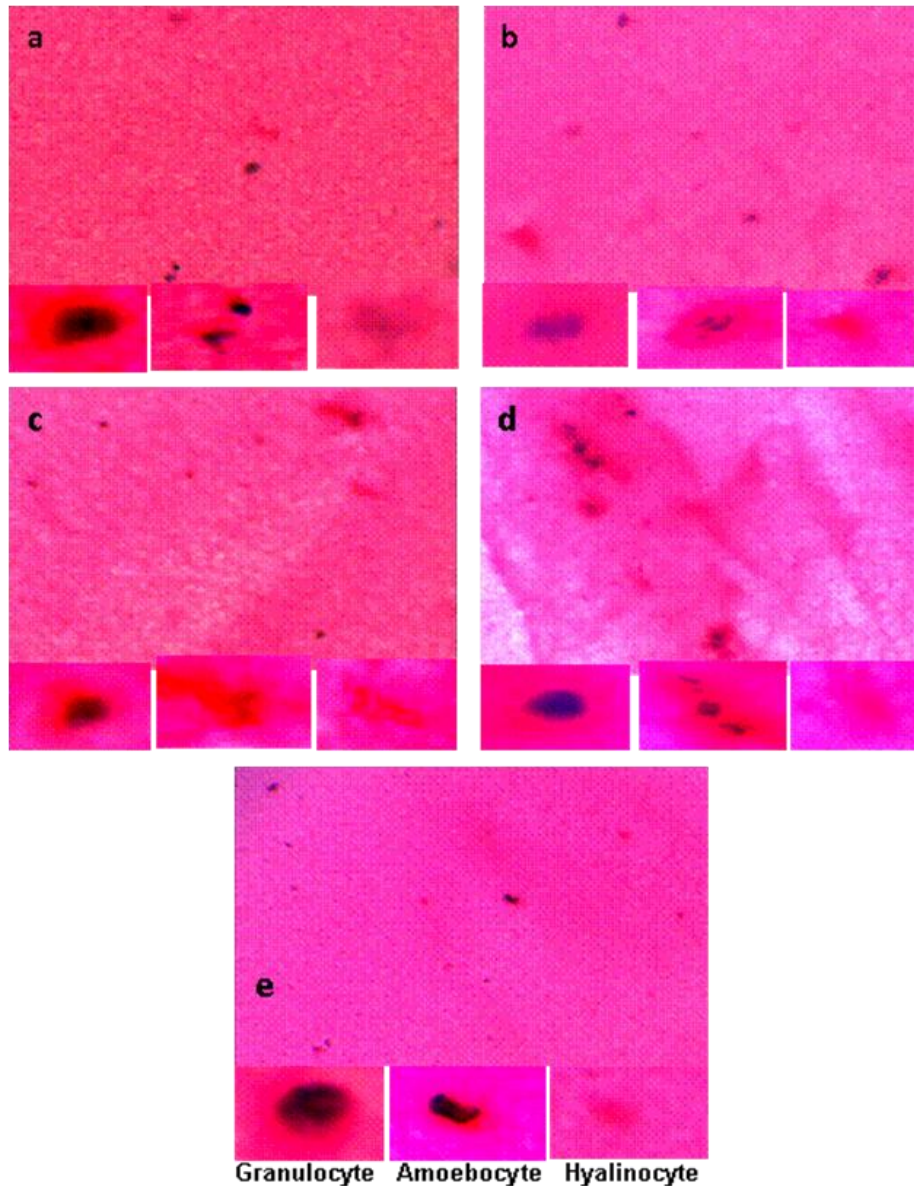


**Figure 3.** Light photomicrographs of T.S (Transverse section) in the hermaphrodite gland of *Biomphalaria alexandrina* exposed continuously to $LC_{25}$ of Artemether 1, 7, 14 and 21 days (A, B, C and D) showing reaction of glycogen contents. E: T.S section in the hermaphrodite gland of unexposed control snails. (PAS stain) ×400.

Europe. Several derivatives of artemisinin showed improved solubility, chemical stability and enhanced antimalarial activity, one of the most important of which are Artemether. The biological activity of artemisinin and its derivatives is based on its endoperoxide bridge which break down releasing carbon-centered free radicals (Wu et al., 2003). The resulting free radical intermediate may then kill by alkylating and poisoning one or more essential proteins (Meshnick, 2002) in the snail.

The present results cleared that Artemether has a toxic effect against *B. alexandrina* snails with $LC_{90}$ of 32.69 ppm, after 24 h exposure according to WHO (1965) recommendations on plant molluscicides. These findings are supported by those of Marston et al. (1993) and Hafez et al. (2007) who reported that *Artemisia* spp. had a molluscicidal activity against *Biomphalaria* snails. Also, El Sayed et al. (2011) recorded that $LC_{90}$ values for latex of *Cryptostegia grandiflora* were 8.65 and 14.03 ppm for *B. alexandrina* and *Biomphalaria galabrata*, respectively. Similar results were obtained by Sharaf El Din (2006) who verified that $LC_{90}$ values for latex aqueous solutions of *C. grandiflora* were 8.48 and 7.55 ppm for *B. alexandrina* and *Lymnaea natalensis*, respectively.

The current data showed severe decrease in the survival

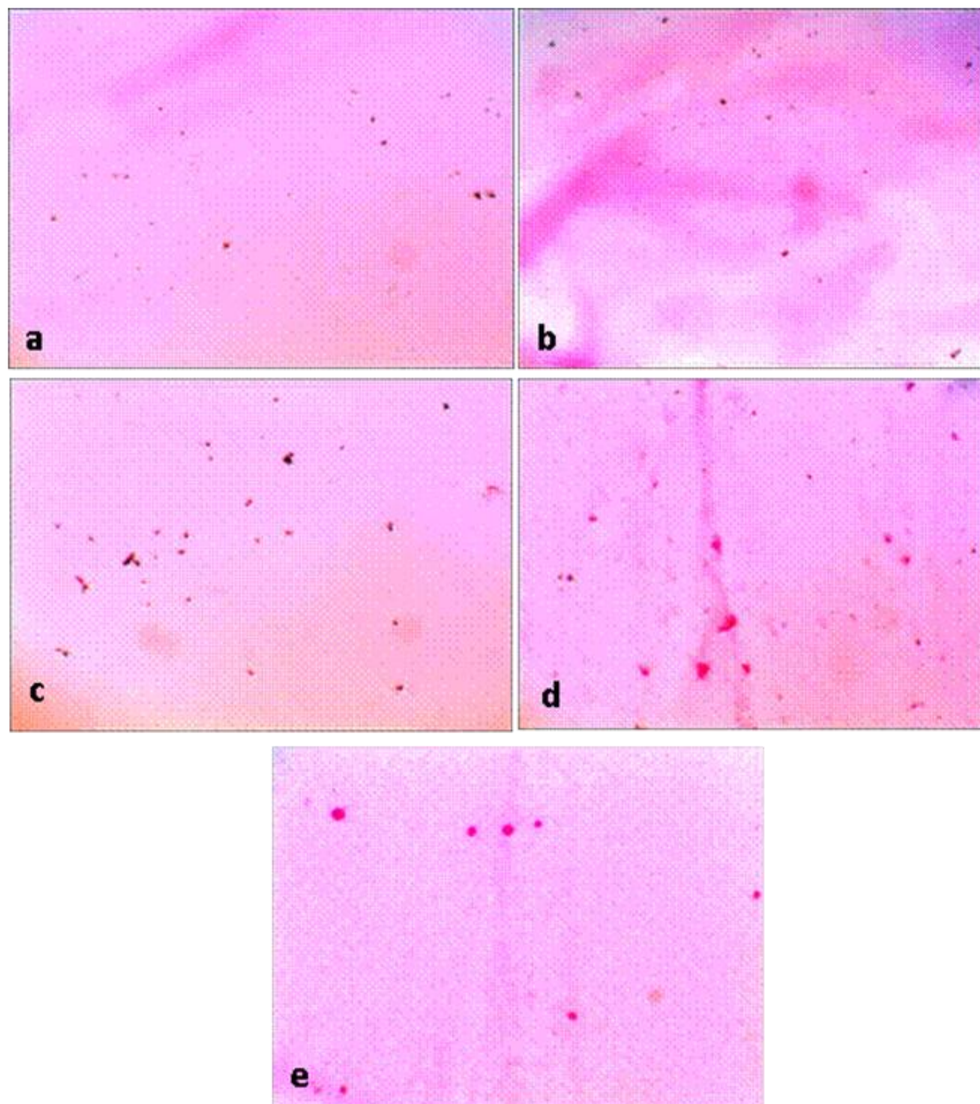


**Figure 4.** Blood films of *Biomphalaria alexandrina* exposed continuously to $LC_{25}$ of Artemether 1, 7, 14 and 21 days (a, b, c, d). E: Blood films of unexposed control snails. (Hematoxylin and eosin) ×400, showing types of hemocytes.

survival rate of snails continuously exposed to $LC_{25}$ Artemether for 1 and 7 days pre-miracidial exposure (23.3 and 10%, respectively) compared to that in control group (93.3%). This finding agrees with the reduction in the survival rate of snails exposed to plant molluscicides recorded by Rawi et al. (1994, 1996), Bakry and Sharaf El-Din (2000), Sharaf El-Din et al. (2001), Mostafa and Tantawy (2000), Gawish et al. (2008), Mostafa et al. (2005), Bakry et al. (2007), Abdel-Hamid (2008) and Hasheesh et al. (2011).

Exposure of *B. alexandrina* snails to sublethal concentrations ($LC_0$, $LC_{10}$ and $LC_{25}$) of Artemether either for 1 or 7 days pre-miracidial exposure significantly reduced their infection rates with *S. mansoni*. These results agree with that of El Sayed et al. (2011) who found that exposure of *B. alexandrina* and *B. glabrata* snails to sublethal concentration of *C. grandiflora* 3 days pre-miracidial exposure led to a significant reduction in the infection rate with *S. mansoni* by 55.47 and 58.9%, respectively. Also, Mahmoud et al. (2011) recorded a reduction in infection rate of *B. alexandrina* snails exposed to $LC_{10}$ of *Datura stramonium* and *Sesbania sesban* during *S. mansoni* miracidial exposure by 41.7 and 52.2%, respectively compared to control group. This may be explained by the deteriorations of physiological parameters of snails making them unsuitable for the parasite development (Gawish et al., 2008). These results accord mostly with many authors working on various chemical and plant molluscicides (Warren and Weisberger, 1966; Mohamed et al., 1981; Vianant et al.,

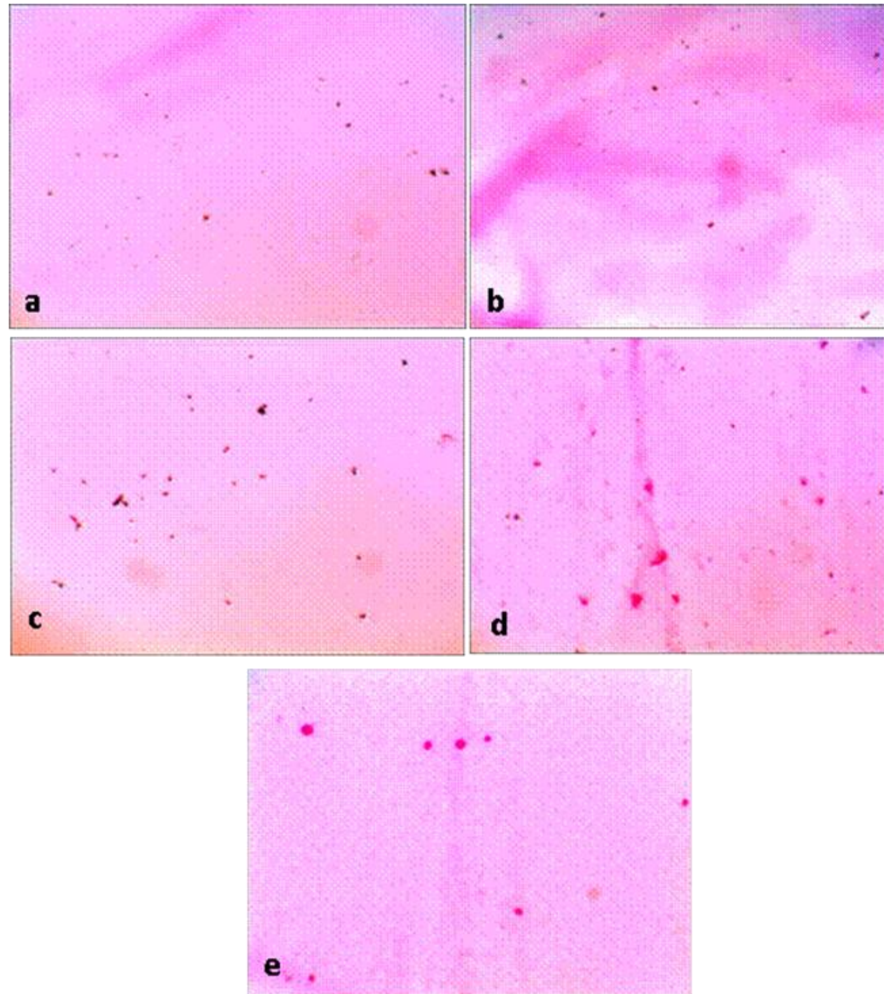


**Figure 5.** Blood films of *Biomphalaria alexandrina* exposed continuously to $LC_{25}$ of Artemether 1, 7, 14 and 21 days (a, b, c, d) showing reaction of glycogen content. E: Blood films of unexposed control snails (PAS stain) ×100.

1982; El-Emam et al., 1986; Mahmoud, 1993; Rizk, 1995; Rawi et al., 1995; Gawish, 1997; El-Ansary et al., 2000; Tantawy et al., 2000; Bakry and Abdel-Monem, 2005; Bakry et al., 2007; Hasheesh et al., 2011).

Regarding the histological study, the hermaphrodite gland cells of snails exposed to $LC_{25}$ Artemether showed loose of connective tissues, short and irregular sperms and the mature ova appeared dense and irregular in shape with congeal clear and atrophy. Elongation of Artemether exposure period resulted in ova lost their normal shape and the damage increased showing discharge, evacuation and complete destruction of gametogenic cells. This may be attributed to the molluscicidal properties of Artemether as it selectively kills the ova; it precludes their development and thus prevents their normal formation (Xiao et al., 2000). These findings were supported by the study of Mohamed et al. (2004) who concluded that *B. alexandrina* exposed to Mepiquat chloride (plant growth regulator) caused noticeable changes in the histological architecture of the digestive and ovotestis glands. Hasheesh et al. (2011) attributed the reduction in egg laying of *Bulinus truncatus* treated with *Sesbania sesban* to severe histological damages to the snail's hermaphrodite gland cells and evacuations of some of its tubules from various gametogenetic stages. Also, Al-Qormuti (2008) found that exposure of *B. alexandrina* snails to sublethal concentrations of the herbicides Roundup or Topik, showed complete destruction of gametogenic cells. This also agrees with Henry et al. (1989), Brackenbury (1999) and Bakry (2009) who recorded degeneration and evacuation in the gametogenic stages of the exposed snails.

In similar studies, Rosès et al. (1999) noticed that kidney cells of *Physa acuta* displayed cell lyses when snails were exposed to atrazine (herbicide) for 10 days,

and this effect was not reversed after a decontamination process. Also, Langiano and Martinez (2008) reported that short-term exposure to sub-lethal concentration of Roundup induced several histological alterations in Neotropical fish, *Prochilodus lineatus.*

In the present study, light microscopic investigation revealed that the three types of haemocytes distributed in the haemolymph of *B. alexandrina* (granulocytes, hyalinocytes and amoebocyte) were influenced by snail continuous exposure to Artemether. The number of cells decreased by the increase of the exposure time. Furthermore, haemocytes' morphology showed obvious abnormalities and irregular aggregation especially in snails exposed for 21 days continuously to $LC_{25}$ Artemether. These results confirmed with the findings of Sharaf El-Din (2003), Martin et al. (2006), Souza and Andrade (2006), Kamel et al. (2006, 2007) and Gawish et al. (2008). Also, Kamel et al. (2006, 2007) evaluated that exposure of *B. alexandrina* snails to sublethal concentration of *Anagallis arvensis* and *Calendula micrantha* caused a significant reduction in total number of haemocytes in comparison with the control. El Sayed (2006) stated that exposure of *B. alexandrina* snails to sublethal concentrations ($LC_0$, $LC_{10}$, $LC_{25}$) of the dry powder of the plant *Cupressus macrocorpa* for three weeks significantly decreased the number of circulating haemocytes.

Elongation of the exposure period to 2 and 3 weeks resulted in reduction in localization of glycogen at the peripheral edges of the gonad tubules in the hermaphrodite gland tissue compared to control in which the glycogen appeared distributed at the epithelium of the gonadal cells and in their connective tissues. On the other hand, the continuous exposure to sublethal concentrations of Artemether increased the glycogen amount distributed inside haemocytes causing increase in their size by increasing the time of exposure, showing irregular big cells compared to control ones. This agrees with Hasheesh et al. (2011) who found that the glycogen in haemolymph of *Bulinus truncatus* snails exposed to methanol extract of *Sesbania sesban* plant increased, while it decreased in soft tissues when compared with the control group. The reduction in glycogen content might be explained by an increase in the activated conformation of phosporylase, and to a lesser extent the decreased glucose uptake (Xiao et al., 2000). This may also be attributed to the activity of the tested plants that impedes oxygen consumption of snails, thus inducing anaerobic respiration. To restore its energy requirements, the snail has to increase the rate of glycolysis, thus, bringing out a reduction of the glycogen content and increase glucose level in the haemolymph. This finding was reported by Bakry et al. (2001) using *Agave franzosini* plant against *B. alexandrina*, and Bakry (2009) using *Furcraea gigantea* and *Lampranthus spectabilis* plants. Other similar studies proved also the elevation in glycogen content in snails exposed to molluscicidal plants (for example, Mantawy and Mahmoud, 2002; Bakry et al., 2002, 2004, 2012; Sun et al., 2012).

It can be concluded that the exposure of *B. alexandrina* snails to the sublethal concentrations of Artemether resulted in remarkable influence on the hermaphrodite gland and glycogen amount and distribution in snails' tissues that may deteriorate their physiological and biological activities. Moreover, the obvious role of Artemether in reduction of snails' susceptibility to infection with *S. mansoni* may suggest it as a promising and safe agent in schistosomiasis control.

## REFERENCES


Abdel-Hamid H (2008). Effect of ethanol extracts from the plants, *Anagallis arvensis* and *Zingiber officinale* and their mixtures against *Lymnaea natalensis,* the snail vector of *Fasciola gigantica*. New Egypt. J. Med. 38(2):109-116.

Al-Qormuti SAA (2008). Effect of certain herbicides on the biology of *Biomphalaria alexandrina* intermediate host of *Schistosoma mansoni*. MSc Thesis, Faculty of Science, Menufiya University.

Bakry FA (2009). Use of some plant extracts to control *Biomphalaria alexandrina* snails with emphasis on some biological effects. Pestic. Biochem. Physiol. 95:159-165.

Bakry FA, Abd-El-Monem S (2005). Effect of water plants and non-target snails on the infectivity of *Bulinus truncatus* with *Schistosoma haematobium*. J. Egypt. Soc. Parasitol. 35(3):859-874.

Bakry FA, Hamdi SAH (2007). Molluscicidal activity of latex aqueous solution of *Euphorbia acetonitril* and *Euphorbia granulate* against the intermediate hosts of Schistosomiasis and Fascioliasis. J. Union Arab Biol. 27:101-126.

Bakry FA, Sharaf El-Din AT (2000). Effect of sublethal concentrations of Bayluscide on *Biomphalaria alexandrina*. J. Egypt. Ger. Soc. Zool. 31:15-25.

Bakry FA, El-Hommossany K, Mossalem HS (2012). Immunological and physiological parameters of *Biomphalaria alexandrina* snails exposed to *Azadirachta indica* plant. Eur. Rev. Med. Pharmacol. Sci. 16(3):133-143.

Bakry FA, Ismail SM, Abd EL-Monem S (2004). Effect of two plant-extracts on some biological and enzymatic activities of *Bulinus truncatus* with *Schistosoma haematobium*. J. Aqual. Biol. Fish 8:313-446.

Bakry FA, Ragab FMA, Sakran AMA (2002). Effect of some plant extracts with molluscicidal properties on some biological and physiological parameters of *Biomphalaria alexandrina* snails. J. Geriatr. Soc. Zool. 38:101-111.

Bakry FA, Tantawy AA, Ragab FMA, Abdel-Kader A (2001). Effect of sublethal concentrations of *Agave franzosini* plant (Agavaceae) on the infectivity of *Schistosoma mansoni* to *Biomphalaria alexandrina* snails. J. Egypt Geriatr. Soc. Zool. 15:129-141.

Bakry FA, Abdel-Hamid H, Abu El Einin HM (2007). Effect of neem plant (*Azadirachta indica*) on some biological and histological parameters of non-infected *Biomphalaria alexandrina* and infected with *Schistosoma mansoni.* J. Egypt. Geriatr. Soc. Zool. 54(D):51-68.

.Boehmler AM, Fryer SE, Bayne CJ (1996). Killing of *Schistosoma mansoni* sporocysts by *Biomphalaria glabrata* haemolymph *in vitro*: Alteration of haemocyte behavior after poly-L-lysine treatment of plastic and the kinetics of killing by different host strains. J. Parasitol. 82(2):332-335.

Brackenbury TD (1999). Gross histopathological effects of an extract of *Agave attenuate* on the epithelium of the digestive tract of *Bulinus africanus*. Ann. Trop. Med. Parasitol. 93:519-526.

Chitsulo L, Loverde R, Engels D, Barakat R, Colley D, Cioli D, Engels D, Feldmeier HM, Loverde P, Olds J, Ourna GR, Rabello A, Savioli L, Traore MB, Ennerwald V (2004). Schistosomiasis. Nat. Rev. Microbiol. 2:12-13.

De Spiegeleer BMJ, Hondt MD, Vangheluwe E, Vandercruyssen K, De

Spiegeleer BGI, Jansen H, Koijen I, Van Gompel J (2012). Relative response factor determination of Artemether degradants with a dry heat stress approach. J. Pharm Biomed. Anal. 70:111-116.

El-Ansary A, El-Shaikh K, El-Sherbini M (2000). SDS-PAGE separated tissue protein of *Biomphalaria alexandrina* snails in the presence and absence of *Schistosoma mansoni*. J. Egypt. Soc. Parasitol. 30(1):125-136.

El-Emam MA, El-Amin SM, Ahmed WS (1990). Molluscicidal properties of plant Cestrum parqui and Headra canariensis. Egypt J. Bilharz. 12:185.

El-Emam MA, Shoeb HA, Ebid FA, Refai LA (1986). Snails control by *Calendula micrantha officinalis*. J. Egypt. Soc. Parasitol. 16(2):563-571.

El Sayed KA (2006). Effect of the plant *Cupressus macrocarpa* (Cupressacea) on some haematological and biochemical parameters of *Biomphalaria alexandrina* snails. J. Egypt. Soc. Parasitol. 36(3):911-924.

El Sayed KA, Mahmoud MB, Mossalem HS (2011). Cryptostegia Grandiflora Affecting Compatibility of *Biomphalaria Alexandrina* and *Biomphalaria Galabrata* to Infection With *Schistosoma Mansoni* With Emphasis on Some Hematological Effects. Aust. J. Basic Appl. Sci. 5(12):3357-3365.

Gawish FM (1997). Evaluation of combination of certain molluscicides against *Biomphalaria alexandrina* and the free living stages of *Schistosoma mansoni*. Ph.D. Thesis. Zoology Department, Girls College For Arts and Science, Ain Shams University.

Gawish F, Mossalem H, Abou El-Einin H (2008). Bioassay of the plant *Callistemon citriun* against *Bulinus truncatus* snails and their infection with S*chistosoma haematobium*. Egypt J. Schisto. Infect. Endem. Dis. 30:33-41.

Hafez SM, Hussein MI, Hassan SI, El-Shennawy AM, El-Komy WM, Amer NM (2007). Evaluation of molluscicidal and schistosomicidal activity of two species of Egyptian *Artemisia* plant in schistosomiasis *mansoni*. New Egypt. J. Med. 36(5):256-264.

Hasheesh W, Mohamed R, El-Monem S (2011). Biological and physiological parameters of *Bulinus truncatus* snails exposed to methanol extract of the plant *Sesbania sesban* plant. Adv. Biol. Chem. 1:65-73.

Hien TT, White NJ (1993). Qinghaosu. Lancet 341:603-608.

Hou XY, McManus DP, Gray DJ, Balen J, Luo XS, He YK, Ellis M, Williams GM, Li YS (2008). "A randomized, double-blind, placebo-controlled trial of safety and efficacy of combined praziquantel and artemether treatment for acute schistosomiasis japonica in China". Bull. World Health Organ. 86(10):788-795.

Ibrahim AM, El-Emam MA, El-Dafrawy SM, Mossalem HS (2004). Impact of certain plant species on *Schistosoma mansoni Biomphalaria alexandrina* system. Proceeding 3rd International Conference Science 3:390-413.

Johnston LA, Yoshino TP (1996). Analysis of lectin and snail plasma-binding glycopeptides associated with the tegumental surface of the primary sporocysts of *Schistosoma mansoni*. Parasitolology 112(5):469-479.

Kamel EG, Refaat SW, El-Dafrawy SM, Mohamed AH, Mossalem HS (2006). The effect of *Schistosoma mansoni* infection on *Biomphalaria alexandrina* haematocytes at ultra structural level. Proceeding of the 4th International Conference of Biological Science (Zoology) p. 219.

Kamel EG, Refaat SW, El-Dafrawy SM, Mohamed AH, Mossalem HS (2007). Toxicological effect of certain plants and synthetic molluscicides on ultra structural changes in haemocytes of *Biomphalaria alexandrina*. Egypt. J. Exper. Biol. (Zoology) 3:135.

Kofta W (1997). Inwazje przywr a uklad obronny slimaka. [Fluke invasion and the immune system of the snail]. Wiadomosci Parazytologiczne 43(1): 27-38.

Langiano VC, Martinez CB (2008). Toxicity and effects of a glyphosate-based herbicide on the Neotropical fish *Prochilodus lineatus*. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 147(2): 222-231.

Litchfield JT, WiLcoxon F (1949). A simplified method of evaluating dose effect experiments. J. Pharmacol. Exp. Therap. 96:99-113.

Mahmoud MB (1993). Effect of certain pesticides on *Biomphalaria alexandrina* and the intramolluscan larval stages of *Schistosoma mansoni*. MSc Thesis, Faculty of Science, Cairo University.

Mansour SA, Ebrahim AM, Abdel-Hamid HF (2002). Botanical biocides: 8. Impact of some plant extracts on *Biomphalaria alexandrina* snails and on *Schistosoma mansoni* miracidia and cercariae. Egypt. J. Schist. Infect. Endem. Dis. 24:81-99.

Mantawy MM, Mahmoud AH (2002). Effect of *Allium cepa* and *Allium sativum* feeding on glucose, glycogen, protein bands profile and phenol oxidase activity in *Biomphalaria alexandrina*. J. Egypt Soc. Parasitol**.** 32(1):271-83.

Meshnick SR (2002). Artemisinin: mechanism of action, resistance and toxicity. Int. J. Parasitol. 32:1655–60.

Miyasato PA, Kawano T, Freitas JC, Berlinck RG, Nakano E, Tallarico LF (2012). Molluscicidal activity of some marine substances against the snail *Biomphalaria glabrata* (Mollusca, Planorbidae). Parasitol. Res. 110(5):1873-9.

Mohamed AH (2011). Sublethal toxicity of Roundup to immunological and molecular aspects of *Biomphalaria alexandrina* to *Schistosoma mansoni* infection. Ecotoxicol. Environ. Saf. 74:754-780.

Mohamed SH, Saad AA (1990). Histological studies on the hermaphrodite gland of *Lymnaea caillaudi* and *Biomphalaria alexandrina* upon infection with certain larval trematodes. Egypt. J. Histol. 13:47-53.

Mossalem HS (2003). Studies on the molluscicidal and miracidicidal effects of certain wild plants on *Schistosoma mansoni* – *Biomphalaria alexandrina* system. Msc. Thesis. Dep. of Zoology, Faculty of Science, Ain Shams University.

Mostafa BB, Tantawy AA (2000). Bioactivity of *Anagallis arvensis* and *Calendula micrantha* plants, treated with ammonium nitrate, superphosphate and potassium sulphate fertilizers, on *Biomphalaria alexandrina*. J. Egypt. Soc. Parasitol. 30(3):929-42.

Mostafa BB, El-Khayat HM, Ragab FM, Tantawy AA (2005). Semi field trials to control *Biomphalaria alexandrina* by different modes of exposure to certain plant and chemical molluscicides. J. Egypt. Soc. Parasitol. 35(3):925-40.

Oliveira-Filho EC, Geraldino BR, Coelho DR, De-Carvalho RR, Paumgartten FJ (2010). Comparative toxicity of *Euphorbia milii* latex and synthetic molluscicides to *Biomphalaria glabrata* embryos. Chemosphere 81(2):218-27.

Petrie A, Sabin C (2000). Medical statistics at a Glance. Blackwell Science Ltd, Oxford.

Rawi SM, El-Gindy HI, Abdel-Kader A (1994). The effect of some fresh water pollutants on the survival and egg production of the snail *B. alexandrina*. J. Egypt Geriatr. Soc. Zool. 13:273-288.

Rawi SM, El-Gindy HI, Abdel-Kader A (1996). New possible molluscicides from *Calendula micrantha* and *Ammi majus*. II. Molluscicidal, physiological and Egg laying effects against *Biomphalaria alexandrina* and *Bulinus truncatus*. Ecotoxicol. Environ. Saf. 35:261-267.

Rawi SM, El-Gindy H, Haggag AM, Elhassan AA, Abdel-Kader A (1995). New possible molluscicides from *calendula micrantha officinalis* and *Ammi majus* plants. 1. Physiological effect on *Biomphalaria alexandrina* and *Bulinus truncatus*. J. Egypt. Geriatr. Soc. Zool. 16:69-75.

Rizk ET (1995). Studies on the effect of certain molluscicidal agents on the snail intermediate host of *Schistosoma mansoni*. Tanta University Egyptian, Tanta p 120.

Rosès N, Poquet M, Muñoz I (1999). Behavioural and histological effects of atrazine on freshwater molluscs (*Physa acuta* Drap. and *Ancylus fluviatilis* Müll. Gastropoda). J. Appl. Toxicol. 19(5):351-356.

Shalaby NM, Abdel-Hamid AZ (1999). Ammimjus extracts as potent molluscicides and antimicrobial agents. Bull. Natl. Res. Cent. 24:153-170.

Sharaf El-Din AT (2003). Effects of double infection with *Echinostoma liei* and *Schistosoma mansoni* on hemoglobin content and circulating haemocytes in the haemolymph of the host snail *Biomphalaria alexandrina*. Egypt. J. Schist. Infect. Endem. Dis. 25:41-52.

Sharaf El-Din AT, Bakry FA, Tantawy A (2001). Molluscicidal activity of *Zygophyllum simplex* (Family; Zygophyllaceae) against *Biomphalaria alexandrina* and *Bulinus truncates*. Egypt. J. Aqua. Biol. Fish. 4:131-143.

Shoeb HA, El-Sayed MM, Khalifa AM (1990). Some Egyptian plants with molluscicidal activity. Egypt J. Bilharz. 12:35.

Souza S, Andrade ZA (2006). On the origin of the *Biomphalaria glabrata* haemocytes. Mem Inst Oswaldo Cruz. 101(1):213-218.

Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect. Dis. 6:411-425.

Sun Q Yang Y, Zhang Q (2012). Effect and Mechanism of *Sapium sebiferum* Against Snail (*Oncomelania hupensis*) and Control of Schistosomiasis. International Conference of Biomedical Engineering and Biotechnology (ICBEB), 28-30 May 2012, China. Conference Publications pp 190-193.

Tantawy A, Mostafa BB, Sharaf El-Din AT (2004). Molluscicidal activity of *Synadenium grantii* (Euphorbiaceae) against *Biomphalaria alexandrina* and *Bulinus truncatus* the intermediate host snails of schistosomiasis in Egypt and their infectivity with the parasite. Egypt J. Sci. 14:183–196.

Wu WM, Chen YL, Zhai Z, Xiao SH, Yu-Lin Wu YL. (2003). Study on the Mechanism of Action of Artemether against Schistosomes: The Identification of Cysteine Adducts of Both Carbon-Centred Free Radicals Derived from Artemether. Bioorg Med. Chem. Lett. 13:1645-1647.

Xiao S, Peter JH, Marcel T (2000). Artemether, an effective new agent for Chemoprophylaxis against shistosomiasis in china: Its *in vivo* effect on the biochemical Metabolism of the Asian schistosome. Southeast Asian J. Trop. Med. Public Health 31(4):724-732.

Xiao S, Tanner M, N'Goran EK, Utzinger J, Chollet J, Bergquist R, Chen M, Zheng J (2002). "Recent investigations of artemether, a novel agent for the prevention of schistosomiasis *japonica*, *mansoni* and haematobia". Acta Trop. 82(2):175-181.

Youssef AA (2010). Studies on the impact of some pesticides and Egyptian plants on some biological and physiological parameters of *Biomphalaria alexandrina* snails and their susceptibility to infection with *Schistosoma mansoni* miracidia. MSc Thesis, Faculty of Science, Al-Azhar University, Egypt.

academicJournals

Vol. 7(31), pp. 2231-2237, 22 August, 2013
DOI 10.5897/AJPP2013.3471
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Immunologic effects of *Moringa oleifera* methanolic leaf extract in chickens infected with Newcastle disease virus (kudu 113) strain

**Didacus C. Eze[1]*, Emmanuel C. Okwor[1], John O. A. Okoye[1] and Denis N. Onah[2]**

[1]Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Accepted 28 June, 2013

**This study was aimed at evaluating the immune boosting potential of crude methanolic extract of *Moringa oleifera* in chickens experimentally challenged with Newcatle disease (ND) virus. One hundred and twenty forty two (42) day old chicks were randomly divided into four equal groups. Groups I and II were given daily oral treatment of methanolic extract of *M. oleifera* at 200 mg/kg body weight until day 56 of age. Groups II and III were vaccinated with the La Sota strain of ND vaccine. Group I was not vaccinated while group IV was left as untreated/unvaccinated control. All the groups were challenged with the velogenic strain of ND virus (VNDV) on day 56. Following challenge, the birds were assessed for cellular and humoral immune responses. Data on cellular and humoral immune responses were analysed using the statistical package for social sciences (SPSS). Increases in total and differential cell numbers and haemagglutination inhibition (HI) titre in the extract-treated groups did not correlate with total protection against ND. *M. oleifera* extract increased ND HI titre and the total and differential leukocyte counts in the treated and unvaccinated group I birds much more than those of treated and vaccinated group II birds, hence it could be recommended as a prophylactic treatment against ND in non vaccinated birds.**

**Key words:** Velogenic Newcastle disease, chickens, *Moringa oleifera*, immunity.

## INTRODUCTION

Newcastle disease (ND) is a serious threat to aviculturists and poultry industry worldwide. ND belongs to OIE listed diseases and is characterized as "a transmissible disease that has the potential for very rapid spread irrespective of national borders; a disease of serious socio-economic or public health consequence, and of major importance in the international trade of animals and animal products (OIE 2005; Facon et al., 2005)". Thus outbreaks of velogenic ND are characterized by high mortality, condemnation of other infected flocks, trade restrictions associated with quarantine and surveillance of affected areas within individual states where outbreaks have been detected (Talebi, 2006; Yongola et al., 2006). ND is caused by ND virus (NDV) which is an Avian Paramyxovirus type 1 (APMV-1) that belongs to the family Paramyxoviridae and genus *Avulavirus* (Aldous and Alexander, 2001; Alexander, 2003). The only effective means of preventing NDV is through vaccination commonly given by the oral, ocular and intranasal routes (Wambura, 2009). *Moringa oleifera* is the most widely

*Corresponding author. E-mail: didacus.eze@unn.edu.ng. Tel: +2348037292020

cultivated species of the family Moringaceae. It is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. This rapidly growing tree (also known as the horseradish tree, drumstick tree, benzolive tree, or Ben oil tree), was utilized by the ancient Romans, Greeks and Egyptians. It is now widely cultivated and has become naturalized in many locations in the tropics. It is a perennial softwood tree, producing low quality, but which for centuries has been advocated for traditional medicinal and industrial uses (Anwar et al., 2007). All parts of the *M. oleifera* tree are edible and have long been consumed by humans. *M. oleifera* is said to be a natural anthelmintic, mild antibiotic, detoxifier and an outstanding immune builder and is used in many countries to treat malnutrition and malaria (Khesorn, 2009). It is also regarded by water purification experts as one of the best hopes for reducing the incidence of waterborne diseases. Moreover, there has been recently an increased interest in the utilization of *M. oleifera*, as a protein source for livestock (Sarwatt et al., 2002; Kakengi et al., 2007). It is therefore a multipurpose tree of significant economic importance with industrial and medicinal uses (Umar, 1998; Anwar et al., 2007). However, there is paucity of information on the use of the leaves as an immunomodulator, especially in reducing the mortality rate in chickens infected by NDV, and also as an adjuvant to vaccination. In this project, the effects of the leaf extract of *M. oleifera* on antibody responses to ND were evaluated. This study was therefore designed to investigate the effects of crude methanolic extract of *M. oleifera* in chickens experimentally challenged with velogenic Newcastle disease virus.

## MATERIALS AND METHODS

### Plant material

The green leaves of *M. oleifera* were collected during the months of March, and April at Ibagwa-Aka, Nsukka, Enugu State, Nigeria. The plant was identified at the Bioresources Development and Conservation Programme, Nsukka. Extraction of the dried leaves was performed by soaking in absolute methanol (98%) for 24 h at room temperature (28°C). The resulting extract was concentrated *in vacuo* and subsequently air dried in a shade. The extract was solubilized in 5% Tween 80. Phytochemical analyses of the extraxts were performed using standard methods (Evans, 2002).

### Experimental animals

One hundred and twenty day-old White Harco cockerels procured from Zartec Ltd Ibadan, South west Nigeria were used for the study. The chicks were not vaccinated against any disease. The birds were housed in an isolated pen at the Poultry Disease Research Unit of the Department of Veterinary Pathology and Microbiology, University of Nigeria, Nsukka. They were fed with commercial poultry feed *ad-libitum* and provided with drinking water.

### Experimental procedure

The birds were randomly divided into four equal groups of 30 chicks each per group at 42 days of age. Group IV was isolated from the other groups. Groups I and II were treated orally with 200 mg/kg body weight of the extract daily for two weeks while other groups were not treated. Groups II and III chicks were vaccinated with La Sota® vaccine. Two weeks post treatment; birds in all the groups were inoculated intramuscularly with 0.2 ml challenge dose of VNDV strain (Kudu 113) with titre $10^{9.5}$ $EID_{50}$ per milliliter of the inoculum. On days 42, 49, 56, 63, 70 and 77 of age, blood samples were collected from each group for serology and haematology. Sera from the blood samples were stored at -20°C until used.

### *Haemagglutination (HA) and heamagglutination inhibition (HI) tests*

Two milliter of blood was collected from each of three adult birds in a test tube containing EDTA as anticoagulant. The blood was washed in phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 5 min. This was repeated until a clear supernatant was obtained. The packed red blood cells (RBC) were re-suspended in a measured volume of PBS solution to make 0.5% RBC suspension (Beard, 1989).

The antigen titre for running the HI test was determined by standard HA technique using La Sota ND vaccine as antigen (Alexander, 2003). The reciprocal of the highest dilution of the La Sota ND antigen causing 100% agglutination of an equal volume of standardized RBCs was taken as the HA titre of the antigen. The HI titres were determined, also by the method of Beard (1989). The HI titers were reciprocal of the highest dilutions of the sera at which 100% RBC HI occurred. The geometric mean titre (GMT) was calculated using the Tube Number Method and Table (Villegas and Purchase, 1989).

### *Haematology*

An assessment of the cellular response was made by determining the total and differential counts of white blood cells (WBC). Total white cell counts were obtained standard methods using improved Neubeur heamocytometer while the differential counts were done in stained thin blood smears (Coles, 1986).

### Statistical analyses

The antibody titres were transformed to base 2 logarithms while the total and differential WBC counts were subjected to Analysis of Variance (ANOVA) using the Statistical Package for Social Sciences (SPSS). Significant means were separated using the Duncan's New Multiple Range Test and tests were considered significant at a probability of $P < 0.05$ (Duncan, 1955).

## RESULTS

### Serology

The titres on week 1 of the experiment indicated low levels of antibody in the range of 1.3 to 2.8 (Figure 1). The GMT post treatment and vaccination indicated obvious differences between the vaccinated Groups II (294.1) and III (274.4) and the unvaccinated Groups I (73.3) and IV (nil). Also the GMT of the treated and vaccinated Group II and untreated and vaccinated Group III showed steady increase until the end of the experiment

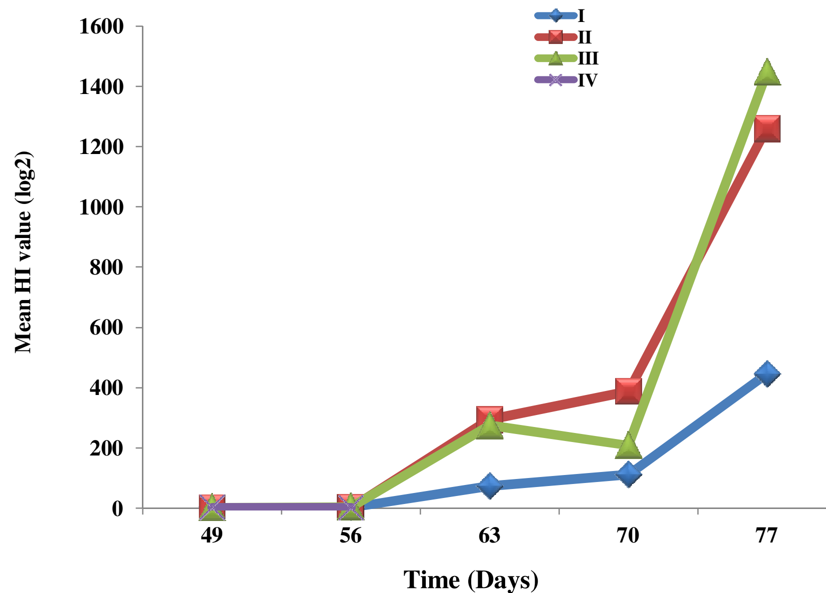


**Figure 1.** The HI titre for Newcastle disease antigen of the different groups of the birds treated with *M. oleifera* and or NDV vaccination. Group I = treated, unvaccinated, challenged; Group II = treated, vaccinated, challenged; Group III = untreated, vaccinated, challenged; Group IV = untreated, unvaccinated, challenged.

**Table 1.** The mean leukocyte counts of the different groups of the birdstreated with *M. oleifera* and or NDV vaccination.

| Age (Weeks) | Mean leukocyte counts ($10^3$/µl) ± S.D. | | | |
|---|---|---|---|---|
| | **Group I** | **Group II** | **Group III** | **Group IV** |
| 0 (Vaccination and commencement of treatment with the extract 0 | 17.18±0.22$^{a}$ | 15.76±0.28$^{bc}$ | 15.16±0.48$^{c}$ | 17.42±0.36$^{a}$ |
| 1 | 42.68±0.27$^{a}$ | 32.14±1.02$^{b}$ | 25.36±1.17$^{c}$ | 22.38±2.09$^{d}$ |
| 2 challenge and end of treatment | 58.82±2.93$^{a}$ | 30.46±1.37$^{b}$ | 43.94±1.44$^{c}$ | 28.22±0.89$^{b}$ |
| 3 | 66.30±2.04$^{a}$ | 48.20±0.90$^{b}$ | 232.90±8.68$^{c}$ | - |
| 4 | 57.18±1.32$^{a}$ | 44.32±230.40$^{b}$ | 36.58±1.48$^{c}$ | - |
| 5 | 33.16±0.72$^{a}$ | 33.48±1.53$^{a}$ | 39.90±1.44$^{b}$ | - |

[a,b,c]Different alphabetical superscripts in a row indicate significant differences between the means: $p < 0.05$. Group I = treated, unvaccinated, challenged; Group II = treated, vaccinated, challenged; Group III = untreated, vaccinated, challenged; Group IV = untreated, unvaccinated, challenged.

on week 5.

## Total leukocyte counts (TLC)

On week 1 and 2 of the experiment there were significant ($P < 0.05$) differences in the mean TLC across the groups (Table 1). The mean TLC of the treated and unvaccinated Group I was significantly ($P < 0.05$) higher than the mean TLC of the treated and vaccinated Group II on weeks 1, 2, 3 and 4 while the mean TLC of the treated and vaccinated group II was significantly ($P < 0.05$) lower than the values of the untreated but vaccinated Group III throughout the study. On week 1 and 2 of the experiment, the mean TLC of the untreated and vaccinated Group III was significantly ($P < 0.05$) higher than those of the untreated and unvaccinated Group IV (Table 1).

## Differential leukocyte counts (DLC)

The mean absolute lymphocyte counts (ALC) of the treated and unvaccinated Group I was significantly ($P<0.05$), higher than those of treated and vaccinated Group II and untreated and vaccinated Group III, on weeks 1, 2, 3 and 4; and higher than those of untreated and unvaccinated Group IV on weeks 1 and 2. The ALC of the treated and vaccinated Group II was also

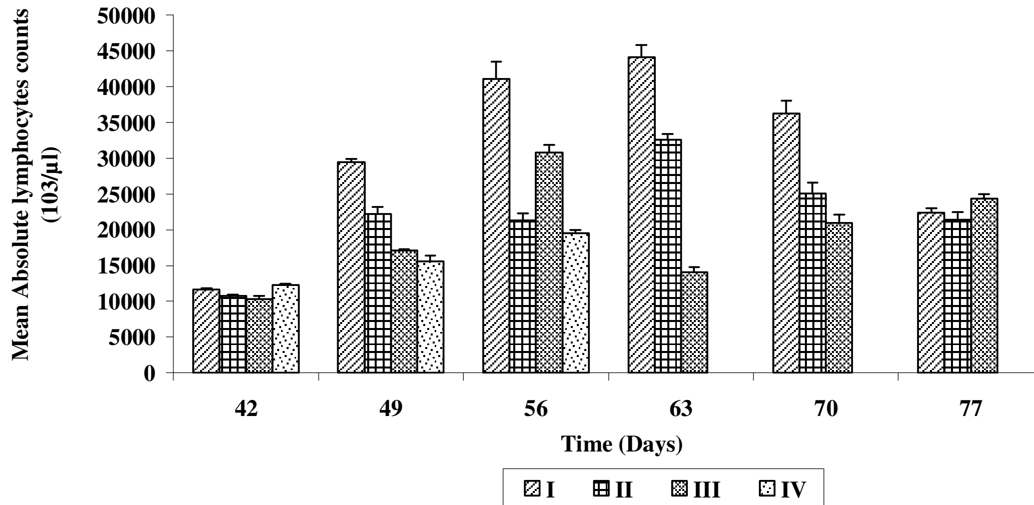


**Figure 2.** The absolute lymphocytes counts of the different groups of the birds treated with *M. oleifera* and or NDV vaccination. Group I = treated, unvaccinated, challenged; Group II = treated, vaccinated, challenged; Group III = untreated, vaccinated, challenged; Group IV = untreated, unvaccinated, challenged.

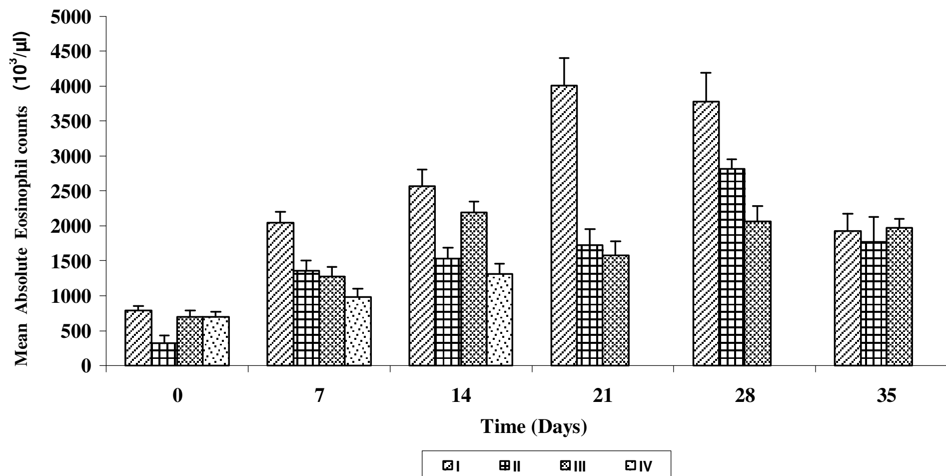


**Figure 3.** Absolute eosinophils counts of the different groups of the birds treated with *M. oleifera* and or NDV vaccination. Group I = treated, unvaccinated, challenged; Group II = treated, vaccinated, challenged; Group III = untreated, vaccinated, challenged; Group IV = untreated, unvaccinated, challenged.

significantly ($P< 0.05$) higher than the untreated and vaccinated Group III on weeks 2, 3, and 4 (Figure 2). On weeks 1, 3 and 4, the mean absolute eosinophils count AEC in treated and unvaccinated Group I was significantly ($P< 0.05$) higher than those of the Groups I to IV while the AEC of the treat and vaccinated Group II was significantly ($P< 0.05$) higher than those of untreated and vaccinated group III on weeks 2 and 4 (Figure 3). On weeks 1, 2, and 3, the mean absolute heterophils count (AHC) of the treated and unvaccinated Group I was significantly ($P < 0.05$) higher than those of treated and vaccinated Group II and untreated and vaccinated Group III (Figure 4). On week 3 of the study, the treated and unvaccinated group I had significantly ($P< 0.05$) higher mean absolute basophil counts (ABC) per chick than treated and vaccinated group II and untreated and vaccinated Group III (Figure 5).

## DISCUSSION

The sera collected immediately before vaccination of chicks had very low maternal antibody titres, suggesting that the birds had not been exposed to NDV, and that the

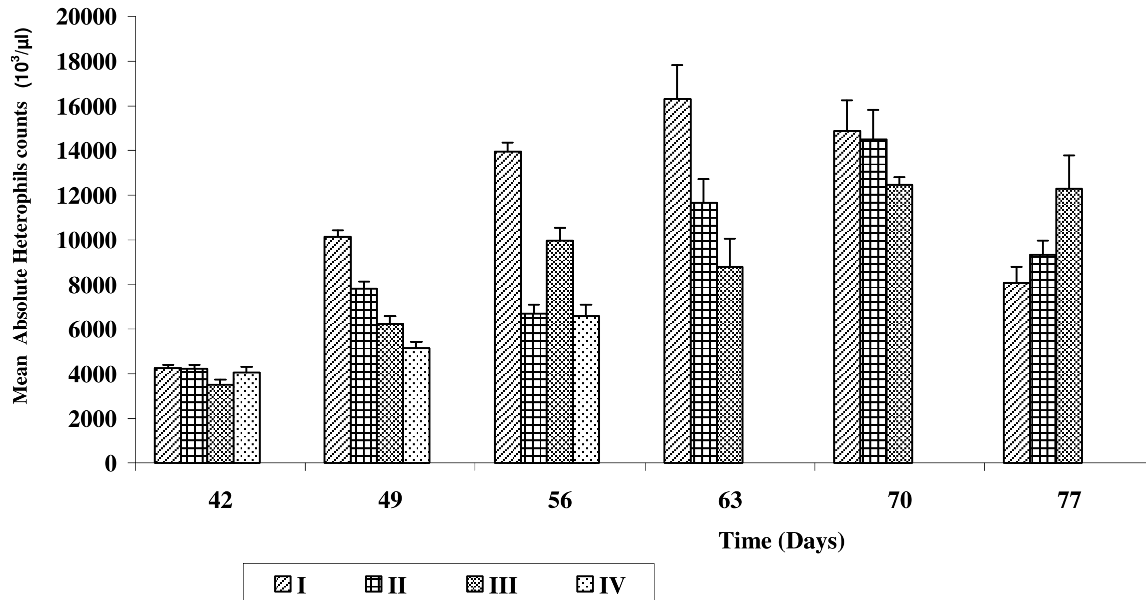


**Figure 4.** Absolute Heterophils counts of the different groups of the birds treated with *M. oleifera* and or NDV vaccination. Group I = treated, unvaccinated, challenged; Group II = treated, vaccinated, challenged; Group III = untreated, vaccinated, challenged; Group IV = untreated, unvaccinated, challenged.

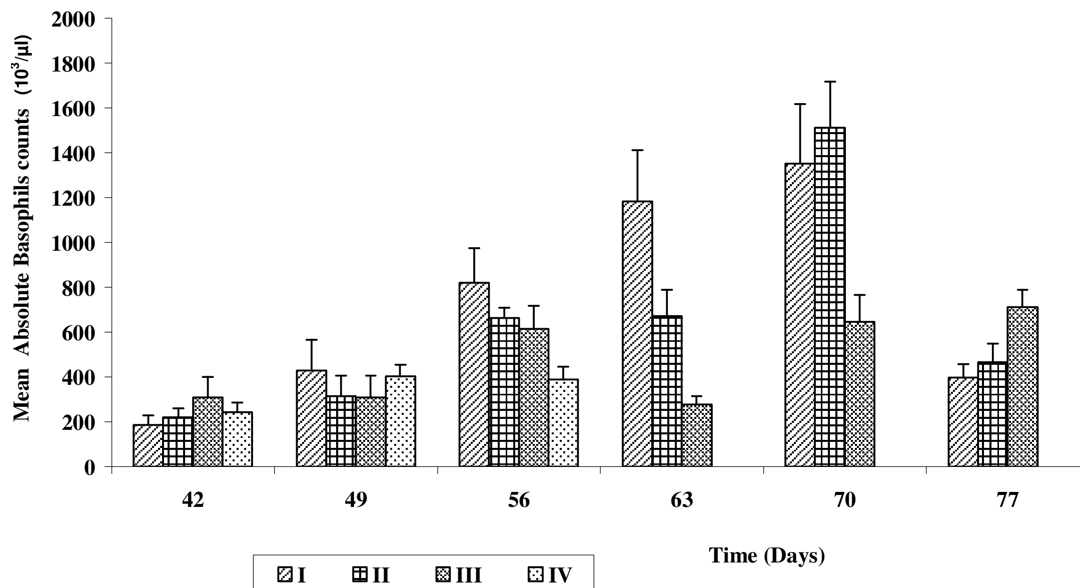


**Figure 5.** Graph showing the absolute basophils counts of the different groups of the birds treated with *M. oleifera* and or NDV vaccination. Group I = treated, unvaccinated, challenged; Group II = treated, vaccinated, challenged; Group III = untreated, vaccinated, challenged; Group IV = untreated, unvaccinated, challenged.

maternally derived antibodies to NDV acquired from their parents had waned at 42 days of age. This progressive decrease in maternally derived antibody titers was in agreement with earlier observations of (Alexander, 2003; Facon et al., 2005), who reported that chicks from vaccinated parent stocks have high levels of maternally derived antibodies at day old which continued to decline in its protection level within 15 to 20 days after hatching. Following vaccination with La Sota vaccine and challenge with VNDV strain Kudu 113, the HI titre was significantly

high in all the vaccinated and challenged Groups II and III than in the unvaccinated and challenged Groups I and IV. This is similar to what was reported that following challenge with VNDV, the HI titre is usually high (Illango and Olaho-Mukani, 2005; Kakengi et al., 2007). The presence of this high NDV HI antibody is necessary to provide long-term protection against ND (Ritchie et al., 1994; Sa' idu, 2006). Throughout the period of experimentation, there was progressive increase in the HI values of groups II and III, while there was equally an appreciable increase in the HI value of the *M. oleifera* treated group I chicks. It is important to note that on 56 weeks post challenge, the antibody titre of Group III fell below the HI value obtained on day 63 of age. This may be due to the continuous challenge by the NDV that was being secreted by the birds within their confined environment (Alexander, 2003). All the chicks in Group II in this study did not responded to the vaccination in the same way. Individual variation in the production of HI antibody could have occurred following vaccination. This variation most often is due to the presence of variable passive immunity in chicks or to the varying degree of susceptibility of immune mechanism to antigen as was also suggested by Hunduma et al. (2010). This might be due to genetical incapability of some birds to produce any reaction to NDV antigen (Alexander, 2003; Herholz et al., 2006). Other possible reasons included impaired immune-competence due to immunosuppressive agents in the feed or due to immunosuppressive diseases such as Infectious bursal disease, etc (Herholz et al., 2006).

Throughout the study, the mean TLC in Group I was significantly ($P < 0.05$) higher than the mean TLC in Group IV. The leucocytosis may be attributed to increased production of leukocytes in the haematopoietic tissues (Yongola et al., 2006; Ravindraa et al., 2009). The primary consequence of low leukocyte count in stressed chickens is suppression of the immune system and increased susceptibility to disease (Wambura, 2009). The AHC started increasing on day 49 and reached the peak level on day 63 in Group I and day 70 in Groups II and III. The value in Group I was significantly ($P < 0.05$) higher than that of Group IV and this might have resulted from the fact that heterophils exhibit high level of apoptosis when infected by NDV (Ravindraaa et al., 2009). The level of the heterophils usually indicates the severity of the initial immune response; therefore their high values in Group I showed that *M. oleifera* possibly protected them from apoptosis (Fahey, 2005).

The mean ALC increased from day 49 to day 63 in Groups I and II, but subsequently decreased. This is in agreement with the report that the increase in lymphocytes might be physiologic, reactive, or proliferative in disease conditions (Wambura, 2009). Birds that normally have high numbers of circulating lymphocytes in the initial response to infective pathogens might develop leucopenia due to lymphopenia (Ritchie et al., 1994). The low values of mean ALC in Groups III and IV is in agreement with the report that NDV has the ability to cause agglutination and lyses of the lymphocytes of affected birds thereby reducing the number of circulating lymphocytes (Bennett et al., 2003; Khesorn, 2009).

Conclusively, *M. oleifera* extract increased ND HI titre and the total and differential leukocyte counts in the treated and unvaccinated Group I birds much more than those of treated and vaccinated Group II, hence, it could be recommended as an immune-booster treatment against ND in non vaccinated birds.

**REFERENCES**

Aldous EW, Alexander DJ (2001). Detection and differentiation of Newcastle disease virus (*Avian paramyxovirus* Type 1). Avian Pathol. 30:117-128.

Alexander JD (2003). Newcastle disease, other avian paramyxoviruses, and pneumovirus infections. In Diseases of Poultry. Y.M. Saif eds, Iowa State Press, Ames Iowa, pp 63-99.

Anwar F, Latir S Ashraf M Gilan A (2007). *Moringa oleifera* a food plant with multiple medicinal uses. Phytother. Res. 21:17-25.

Beard CW (1989). Serologic procedures. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 3rd. H. G. Purchase, L H. Arp, C. H. Domermuth, and J. E. Pearson (eds.), Kennett Square, PA: Amer. Assoc. Avian Pathol. pp. 192-200.

Bennett RN, Mellon FA, Foidl N, Pratt JH, DuPont MS, Perkins L, Kroon PA (2003). Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish tree) and *Moringa stenopetala.* J. Agric. food chem. 51:3546-3553.

Coles EH (1986). Veterinary Clinical Pathology, 4th ed. W. B. Saunders Co., Philadelphia pp 15-40.

Duncan DB (1955). New Multiple Range and Multiple F. Tests. Biometrics 11:1-42.

Evans WC (2002). In Trease G, Evans W. Pharmacognosy. 5th ed. Haarcourt Brace, Company p 336.

Facon C, Jean-LucG, Lacroix F (2005). Assessment of Newcastle Disease Vaccination of Houbara Bustard Breeders (*Chlamydotis undulata undulata*). J. Wildl. Dis. 41(4):768-774.

Fahey J (2005). *Moringa oleifera*. A review of the medical evidence for its nutritional, therapeutic and prophylactic properties. Trees life J. 1: 5.

Herholz C, Jemmmi T, Stark K, Griot C (2006). Pattern of Animal Disease and their control. Vet. Ital. 42(4):295-303.

Hunduma D, Regassa C, Fufa D, Endale B, Samson L (2010). Major Constraints and Health Management of Village Poultry Production in Rift Valley of Oromia, Ethiopia. Am. Eurasian J. Agric. Environ. Sci. 9 (5): 529-533.

Illango J, Olaho-Mukani W (2005). Immunogenicity of a locally produced Newcastle disease I-2 thermostable vaccine in chickens in Uganda. Trop. Anim. Health. Prod. 37(1):25-31.

Kakengi A, Kaijage J, Sarwatt S, Mutayoba S, Shem M, Fujihara T (2007). Effect of *Moringa oleifera* leaf meal as a substitute for sunflower seed meal on performance of laying hens in Tanzania. Livestock Res. Rural Dev. 19: 120.

Khesorn N (2009). Antibacterial activity of the capsules of *Moringa oleifera* Lam. (Moringaceae). J. Ethnopharmacol. 36:233-7.

Office International des Epizooties/World Organization for Animal Health (OIE) (2005). Newcastle disease. In: Manual of standards for diagnostic tests and vaccines 2.1.15.

Ravindraa PV, Ashok, Tiwaria K, Barkha R, Manish V, Baisa B, Uttara C, Sudesh KP, Bhaskar S, Chauhana RS (2009). Time course of Newcastle disease virus-induced apoptotic pathways. Virus Res. 144 (1-2):350-354.

Ritchie BW, Harrison JG, Harrison RL (1994). Avian Medicine. Winger's Publishing, Inc, Florida pp 176-198.

Sa' idu L, Bisalla M, Moumini B (2006). Response of Local Breeds of Chickens to Challenge with Newcastle Disease Virus (Kudu 113). J.

Anim. Vet. Adv. 5(11):975-979.
Sarwatt SV, Kapange SS, Kakengi AMV (2002). Substituting sunflower seed-cake with *Moringa oleifera* leaves as supplemental goat feed in Tanzania. Agroforest. Syst. 56:241-247.
Talebi A (2006). Biochemical parameters in broiler chickens vaccinated against ND, IB and IBD. Inter. J. Poult Sci. 5:1151-55.
Umar MD (1998). Antimicrobial Activity of Small Protein of *Moringa oleifera* leaves. J. Islamic Acad. Sci. 11:27-32.
Villegas P, Purchase HG (1989). Titration of biological suspension. In: A laboratory manual for the isolation and identification of avian pathogens. Iowa. USA. Kendal Hunt. Am. Assoc. Avian Pathol.186-190.
Wambura PN (2009). Protective antibody response produced by the chickens vaccinated with green coloured thermostable Newcastle disease virus. Trop. Anim. Health. Prod. 41:149-152.
Yongola MGS, AP Muhairwa, MMA Mtambo, MU Minga, M Minja, RH Mdegela (2006). Immunogenecity and protection ability of candidate Newcastle disease virus isolated for vaccine production. Livestock Res. Rural Dev. 18:10.

academicJournals

Vol. 7(31), pp. 2238-2245, 22 August, 2013
DOI 10.5897/AJPP2013.3765
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Cytotoxic and anticoagulant peptide from *Scolopendra subspinipes mutilans* venom

**Yi Kong[1,2]*, Jing Hui[1,2], Yu Shao[1], Shilong Huang[1], Hao Chen[1,3] and Ji-Fu Wei[3]**

[1]School of Life Science and Technology, China Pharmaceutical University, 24 Tong Jia Xiang,Nanjing 210009, PR China.
[2]State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, PR China.
[3]Research Division of Clinical Pharmacology, the First Affiliated Hospital with Nanjing Medical University, 300 Guangzhou Road, 210029 Nanjing, PR China.

Accepted 1 August, 2013

***Scolopendra subspinipes mutilans*** **venom is a complex mixture of peptides and proteins with many biochemical and pharmacological activities. In this study, a novel peptide was identified by ultra filtration and reverse-phase high performance liquid chromatography (RP-HPLC). The amino acid sequence was FTGGDESRIQEG determined by Edman degradation. The molecular mass was 1296.05 Da determined by electrospray ionisation mass spectrometry (ESI-MS). The novel peptide displayed specific inhibitory effects on the proliferation of human liver cancer (HepG2) and human gastric cancer cells (MGC). It also showed antibacterial activity against the tested bacteria *Clostridium perfringens, Staphylococcus epidermidis,* and *Escherichia coli*. Moreover, this peptide prolonged the whole blood clotting time *in vivo*. This peptide is the first peptide showing cytotoxic and anticoagulant activities identified from *S. subspinipes mutilans* venom.**

**Key words:** *Scolopendra subspinipes mutilans* venom, cytotoxicity, antibacterial activity, clotting time.

## INTRODUCTION

Centipedes are terrestrial arthropods belonging to the class Chilopoda, being characterized by the presence of a head and an externally segmented body containing a pair of articulate legs in each segment (Malta et al., 2008). About 2,800 species are known in the world, and several species are medically important (Malta et al., 2008). Centipedes are predatory, elongated and dorso-ventrally flattened arthropods. Venoms are extracted directly from the venom gland connected to the first pair forceps of centipedes to kill or immobilize prey as a defense mechanism against predators (Rates et al., 2007). Centipede envenomations are capable of inflicting severe symptoms in humans, including myocardial ischemia and infarction, hemoglobinuria and hematuria, hemorrhage and rhabdomyolysis, itching, fever and chills, general rash, eosinophilic cellulitis, and anaphylaxis (Mohri et al., 1991; Knysak et al., 1998; Rodriguez-Acosta et al., 2000). The long list of symptoms and complications induced by centipede envenomations suggests that centipede venoms contain a variety of different components with diverse functions (Mohamed et al., 1983; Fang et al., 1999).

You et al. (2004) isolated a 25 kDa serine protease from the venom of *S. subspinipes mutilans*, which demonstrated fibrinolytic activity by converting human Glu-plasminogen to activated plasmin. Wu et al. (2006) found that centipede acidic protein (CAP) significantly suppress the development of atherosclerosis, improves the hemorrheological disturbances and histopathological changes in the atherogenic diet fed rat model. Wenhua et al. (2009) isolated an antibacterial peptide named scolopendrin I from the venom of *S. subspinipes mutilans*. González-Morales et al. (2009) isolated a phosholipase $A_2$ from the venom of *S. viridis Say*. Peng et

*Corresponding author. E-mail: yikong668@163.com. Tel: 0086-25-83271030. Fax: 0086-25-83271030.

al. (2010) reported the structural and functional characterization of two antimicrobial peptides (scolopin 1 and 2) identified from the venoms of *S. subspinipes mutilans*. Rates et al. (2007) identified more than 60 proteins/peptides in *S. viridicornis nigra* and *S. angulata* venoms by a proteomic approach. Liu et al. (2012) further purified and characterized 40 proteins/peptides from crude venom of *S. subspinipes dehaani*. The purified proteins/peptides showed different pharmacological properties, including platelet aggregating, anticoagulant, phospholipase $A_2$, trypsin inhibiting, voltage-gated potassium channel, voltage-gated sodium channel, and voltage-gated calcium channel activities. Yang et al. (2012) identified 26 neurotoxin-like peptides from the venom of *S. subspinipes mutilans*.

The dried whole body of *S. subspinipes mutilans* L. Koch has been used for cancer treatment in traditional Chinese medicine for hundreds of years. The water extracts of the organism were reported to possess antitumor and immunopotentiating activities (Cohen and Quistad, 1998; Xu et al., 2010; Zhou et al., 2011; Zhao et al., 2012). Zhao et al.(2012) found that polysaccharide-protein complex from *S. subspinipes mutilans* could inhibit tumor growth *in vivo* by improving antitumor immune responses at least partly via down-regulating arachidonic acid metabolic pathways in tumor-associated macrophages. We recently identified an anticoagulant peptide from the *S. subspinipes mutilans* body (Kong et al., 2013). Cohen and Quistad (1998) found that centipede venom showed higher cytotoxic activities compared with spider venom. However, to our knowledge, the cytotoxic components in centipede venom remain largely unexplored.

In this study, a novel peptide was discovered and the amino acid sequence was determined by Edman degradation. This peptide displayed specific inhibitory effects on the proliferation of human liver cancer (HepG2) and human gastric cancer cells (MGC). It also showed antibacterial activity against the tested bacteria. Moreover, this peptide prolonged the whole blood clotting time *in vivo*.

## MATERIALS AND METHODS

### Venom collection

Adult *S. subspinipes mutilans* L. Koch (both sexes, *n* = 1,000) were from Chuzhou centipede farm (Anhui province, China). Venom was collected manually by stimulating the venom glands in the first pair forceps of centipedes using the multi-purpose electrical instrument with the conditions that the frequency was 7.8 ms (that is, 128 Hz). The voltage was 10 to 20 V, and the pulse width was 2 to 4 ms. Each milking occurred 1 week after the previous milking. Venoms were stored at –20°C until further use.

### Peptide purification

The collected venom (400 mg) was diluted in 20 ml phosphate buffer (PBS), pH = 7.3 and then applied to an ultra filtration tube of 10 kDa, centrifuged at 10,000 rpm for 5 min. The low molecular weight fraction after ultra filtration was further purified by preparative-scale reverse-phase HPLC (BioLogic Duoflow System) on a Lichrospher $C_{18}$ column (25 × 0.46 cm). Elution was performed using a gradient (0% B for 20 min, 0 to 60% B for 30 min) of 0.05% trifluoroacetic acid (TFA) in 5% acetonitrile (A) and 0.05% TFA in 95% acetonitrile (B) at a flow rate of 1 ml/min. The absorbance of elute was monitored at 214 nm.

### Determination of molecular mass and peptide sequence

The molecular mass of the purified peptide was determined using an electrospray ionisation mass spectrometry (ESI-MS). Complete peptide sequencing was undertaken by Edman degradation on an applied biosystems pulsed liquid-phase sequencer, model 491.

### Peptides synthesis

All peptides used for the following bioactivity assays were synthesized by the Fmoc (N-[9-fluorenyl]-methoxycarbonyl) chemistry in solid-phase synthesis. Usually, peptides are synthesized from the carbonyl group side (C-terminus) to amino group side (N-terminus) of the amino acid chain. The solid supports were preloaded with Wang resin for c-terminal acid in this synthesis. Protected amino acids were coupled by in situ activation with diisopropylethylamine (DIEA) and N-hydroxybenzotriazole (HOBt). Then dimethylformamide (DMF) with 20% piperidine was performed in deprotection for 20 min. Cleavage of the peptide from the Wang resin was performed by reagent (95% TFA/2.5% triisopropylsilane (TIS)/2.5% water) for 1 h. The synthesized peptides were purified by preparative-scale reverse-phase HPLC on a Kromasil $C_{18}$ column (250 × 10 mm). Elution was performed using a linear gradient (30 to 100% B for 20 min) of 0.05% TFA in 5% methanol (A) and 0.05% TFA in 95% methanol (B) at a flow rate of 2 ml/min. The absorbance of elute was monitored at 214 nm. The main peak was pooled, dried in vacuum, lyophilized and stored at -20°C. The purity was analyzed by HPLC and ESI-MS.

### Cytotoxicity of purified peptide on cancer cells

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used to measure the cytotoxicity of purified peptide on cancer cells (Wei et al., 2009). Briefly, murine prostate cancer cells (RM-1), human liver cancer (HepG2), human gastric cancer cells (MGC) and human umbilical vein endothelial cells (HUVEC) were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (FBS, 10%) (Gibco, Grand Island, NY, USA), gentamicin (25 mg/ml, Sigma) and L-glutamine (200 mM, Sigma), cultured in a humidified atmosphere of 5% $CO_2$ in air. Cells (4000 cells/100 μl/well) were seeded into a 96-well plate in triplicate. Peptides with concentrations of 6 to 256 μg/ml were added into the wells after 6 h. Non-treated culture cells were used as a negative control. After incubation with peptides for 72 h, the medium in each well was replaced with 20 μl of MTT with final concentration of 5 mg/ml. 150 μl diethylsulfoxide (DMSO)/well was added to dissolve the formed violet formazan crystals within metabolically viable cells after 4 h. The plates were incubated at 37°C for 15 min and then read at 570 nm with a microplate reader. Percent of growth inhibition was calculated as:

(OD of the control − OD of the experiment samples) / OD of the control × 100

$IC_{50}$ was calculated as the concentration (μg/ml) of peptides causing

a 50% inhibition of cell viability.

### *In vitro* antibacterial and antifungal activity test

Antibacterial activities of the purified peptide was determined against bacteria *(Staphylococcus aureus*, *Clostridium perfringens*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and fungi (*Monilia albican, Aspergillus niger*) by an inhibition zone assay with some modifications as described previously (Lu and Chen, 2010). Briefly, agarose plates were seeded with microorganisms (about $10^6$ cells in 10 ml of 1% agarose medium). Wells (3 mm in diameter) were punched out and a 5 µl peptide sample dissolved in water was loaded. Negative controls were loaded with water. After incubation overnight at 37°C, the diameter of the inhibition zone was determined. Liquid growth inhibition assay was further used for determination of the minimal inhibitory concentration (MIC). After microorganisms were grown to log phase, approximately 2000 bacteria or fungi were incubated with a series of peptide concentrations (512, 256,128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/ml) in a 96-well microtiter plate for 24 h. The lowest concentration inhibiting bacterial growth was taken as the MIC value. Ampicillin and streptomycin sulfate were used as positive controls.

### Hemolysis activity

Hemolysis assays were performed using human red blood cells (RBCs) in liquid medium as previous reported (Wei et al., 2006a, b). The RBCs were prepared from freshly collected blood by centrifugation at 4,000 rpm for 10 min. The cells were washed three times with 0.01 M PBS solution (pH = 7.4) and suspended as 1% suspension in PBS to prepare human erythrocyte solution. 100 µl of serial dilutions of the peptide (10, 40, 160, and 640 µg/ml) were added to 100 µl of human erythrocyte solution and incubated at 37°C for 60 min. The human erythrocyte solution were then centrifuged at 4,000 rpm for 10 min and measured at 540 nm with an enzyme linked immunosorbent assay (ELISA) plate reader. A parallel incubation in the presence of 0.1% (v/v) Triton X-100 was carried out to determine the absorbance associated with 100% hemolysis.

### Clotting time *in vivo*

Whole blood clotting time (CT) in mice was measured by capillary glass tube method with some modification (An et al., 2011). All the experimental protocols to use animals were approved by the Animal Care and Use Committee at China Pharmaceutical University. Mice (18 to 22 g body weight) were divided into four groups (both sexes, six per group). Two groups were intraperitoneally injected with 16 and 8 mg/kg body weight of the purified peptide for 4 consecutive days. Other groups received normal saline and 12 mg/kg body weight of clopidogrel, respectively. One hour after the last administration, blood samples were collected through the retro-orbital plexus with a glass capillary and kept on a slide to allow for clotting. Stirring the blood with a dry needle every 30 s until needle wire can provoke a fibrous protein. So far, that is clotting time.

### Statistical analysis

Data are shown as mean ± SE for the number of experiments indicated, and analysis of variance (ANOVA) followed by Tukey's tests were used for statistical comparison of the data. In all analyses, $P < 0.05$ was taken as statistically significant.

## RESULTS

### Peptide purification

The collected *S. subspinipes mutilans* venom (400 mg) was fractionated into the low molecular weight fraction (mainly peptides and small compounds) and high molecular weight fraction (mainly protein) by an ultra filtration tube of 10 kDa. The low molecular weight fraction was further purified by RP-HPLC and fractionated into ten peaks as illustrated in Figure 1. Fraction A showed cytotoxic activity.

### Determination of molecular mass and peptide sequence

The purified peptide was collected, dried in vacuum and subjected to automated Edman degradation. This peptide was composed of 12 residues with an amino acid sequence as Phe-Thr-Gly-Gly-Asp-Glu-Ser-Arg-Ile-Gln-Glu-Gly (FTGGDESRIQEG). The molecular mass of the purified peptide was determined by ESI-MS, giving an observed molecular mass of 1296.05 Da (Figure 2). It matched the theoretical molecular mass of 1295.33 Da deduced by amino acid sequence of the purified peptide.

### Peptides synthesis

All peptides used for the following bioactivity assays were synthesized by the Fmoc (N-[9-fluorenyl]-methoxycarbonyl) chemistry in solid-phase synthesis. The synthesized peptides were purified by RP-HPLC. The main peak was pooled (Figure 3). The purity was higher than 95% analyzed by HPLC. It is homologous to the purified peptide confirmed by ESI-MS.

### Cytotoxicity of purified peptide on cancer cells

After incubation with 16 to 256 µg/ml peptides for 72 h, the proliferation of HepG2 and MGC cells were inhibited in a dose-dependent manner (Figure 4). The calculated $IC_{50}$ value of peptide against HepG2 and MGC cells were 80 and 65 µg/ml, respectively. The survival ratio of cancer cells was decreased to about 10 to 40% after incubation with 256 µg/ml peptide for 72 h. However, RM-1 and HUVEC cells were not obviously affected (Figure 4). These results indicate that this peptide shows specific activities against cancer cells.

### *In vitro* antibacterial and antifungal activity test

The MICs of purified peptide against bacteria and fungi were determined and summarized in Table 1. Among the

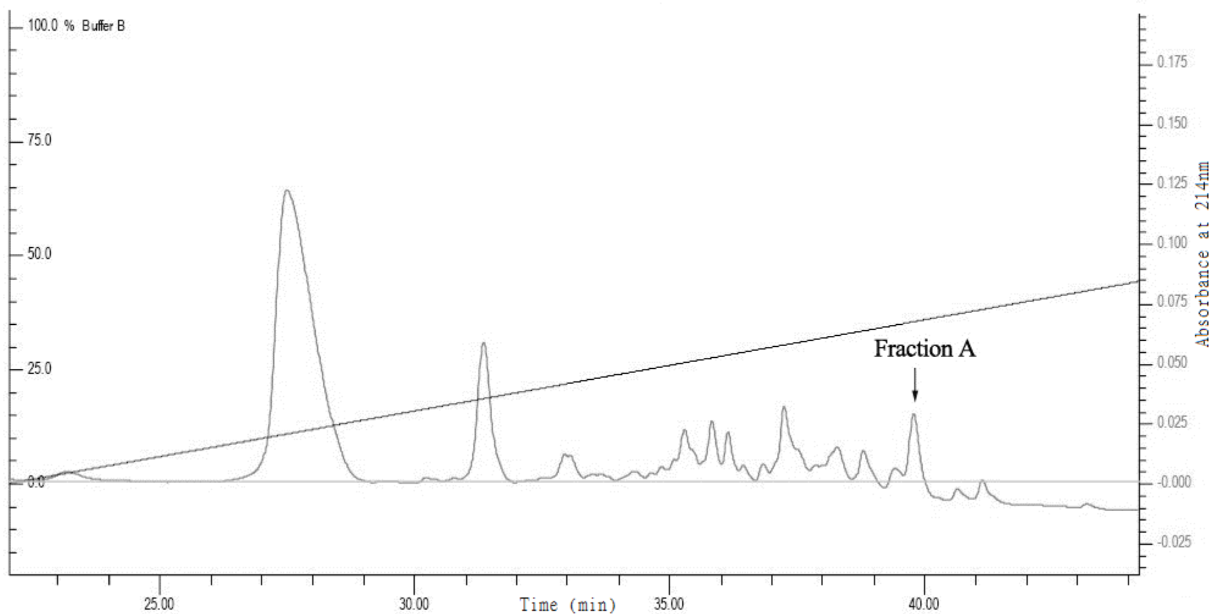


**Figure 1.** Purification of the cytotoxic and anticoaglant peptide from centipede venoms. The crude venoms were fractionated on a Ultra filtration tube. The low molecular weight fraction after ultra filtration was further purified by preparative-scale reverse-phase HPLC on a Lichrospher $C_{18}$ column. The low molecular weight fraction of crude centipede venom was fractionated into ten peaks by RP-HPLC. Fraction A showed cytotoxic activity.

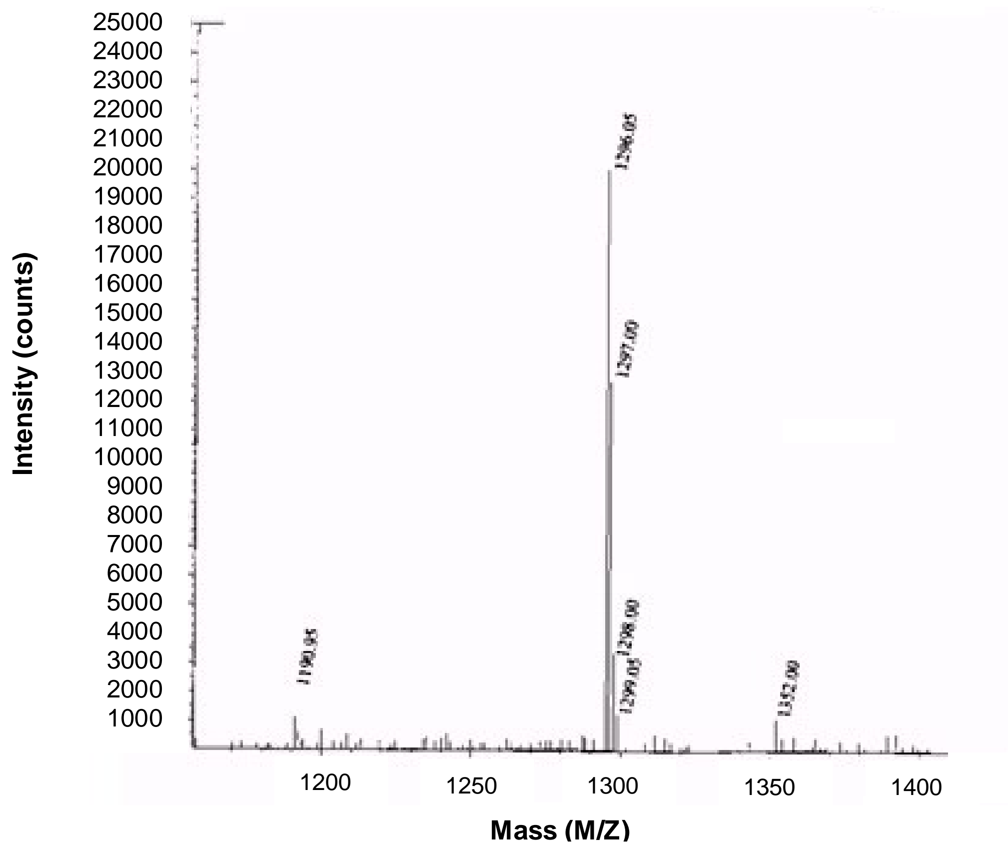


**Figure 2.** The molecular mass of the purified peptide determined by ESI-MS. The molecular mass of purified peptide is 1 296.05Da determined by ESI-MS. The 1297.0 m/z is peptide' isotope peaks

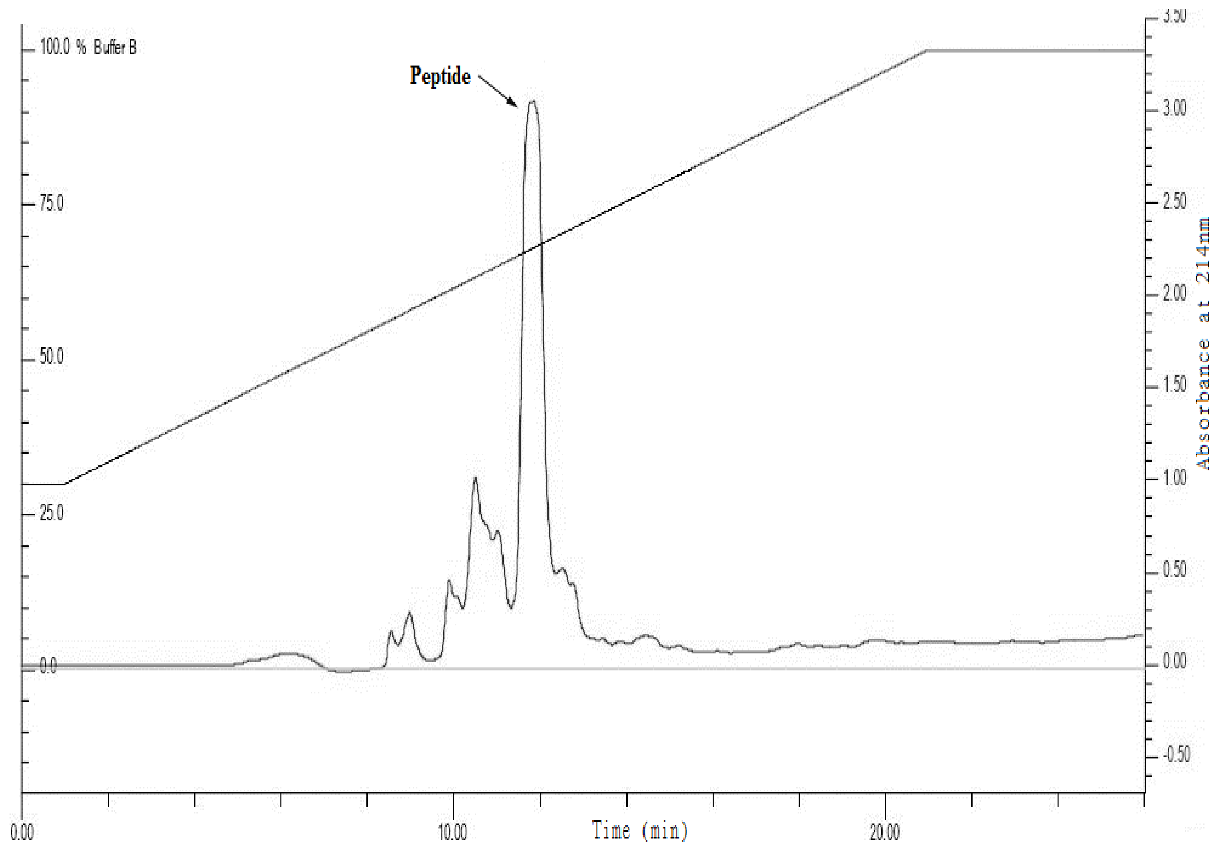


**Figure 3.** Purification of the synthetic peptide. The synthetic peptide solution was further purified by RP-HPLC on a Lichrospher $C_{18}$ column.

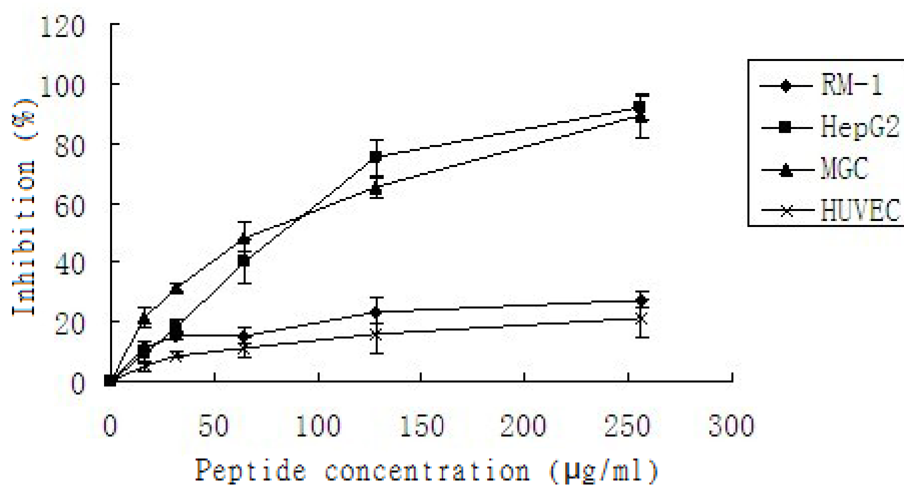


**Figure 4.** Cell inhibition curve as measured by MTT assays for purified peptide against cancer cell lines RM-1, HepG2, MGC and HUVEC cells, respectively. Error bars represent the standard deviation from the mean cell inhibition as determined by three independent experiments.

tested microorganisms, the purified peptide showed weak antimicrobial activities against *C. perfringens* and *E. coli,*

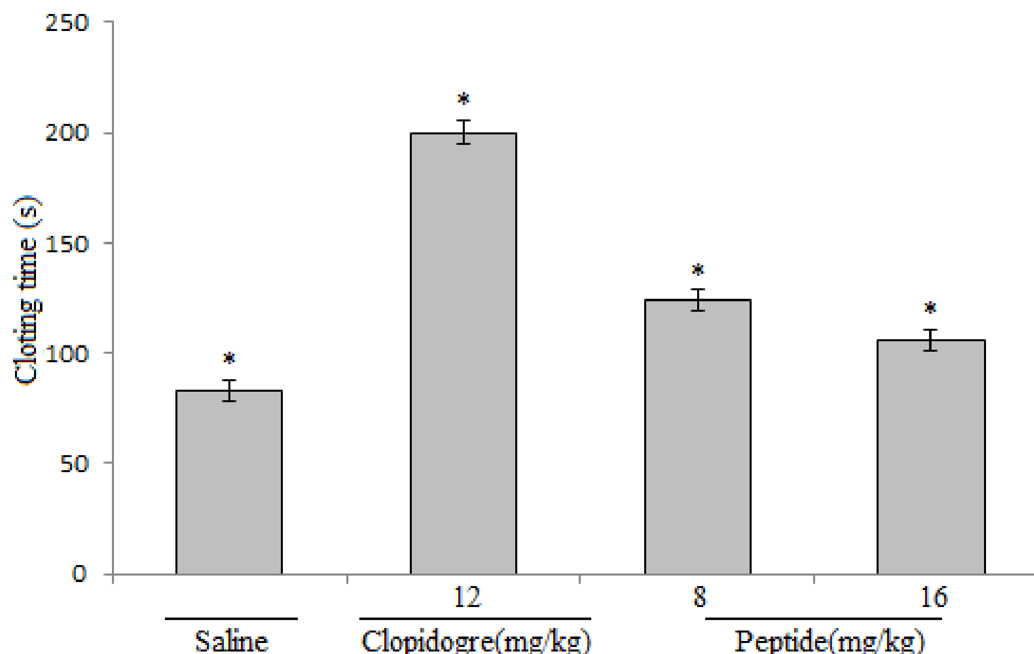


**Figure 5.** Clotting time prolonging activity of purified peptide. The peptide prolonged clotting time. Clopidogre as positive control. *P < 0.05 in comparison with those of control group (saline group).

**Table 1.** Antibacterial activities of purified peptide.

| MIC (µg/ml) | *S. aureus* | *C. perfringens* | *S. epidermidis* | *E. coli* | *K. pneumoniae* | *P. aeruginosa* |
|---|---|---|---|---|---|---|
| Peptide | NA | 256 | 256 | 256 | NA | NA |
| Ampicillin | 0.5 | 0.5 | 2 | 16 | >64 | >64 |
| Streptomycin Sulfate | 8 | 16 | 0.5 | >64 | 4 | 8 |

Ampicillin and Streptomycin sulfate are positive control.

and no activities against other microorganisms.

## Hemolysis activity

When the purified peptide in gradient concentrations from 10 to 640 µg/ml was incubated with human RBCs, only about 0.01% hemolytic activity was observed after 1 h incubation at 640 µg/ml. Our results indicated the purified peptide was not toxic to human RBCs.

## Clotting time *in vivo*

In comparison with the control group, 16 and 8 mg/kg group had significantly prolonged whole blood clotting time ($P < 0.05$) (Figure 5). It indicated that the purified peptide had anticoagulant effects.

## DISCUSSION

Natural plants and animals are rich source for drug development (Atef et al., 2013; Tao et al., 2013; Malik et al., 2013). The dried whole body of *S. subspinipes mutilans* L. Koch has been used for cancer treatment in traditional Chinese medicine for hundreds of years. In this study, a novel peptide was identified by ultra-filtration and reverse-phase high performance liquid chromatography (RP-HPLC). The amino acid sequence was FTGGDESRIQEG determined by Edman degradation. The molecular mass was 1296.05 Da determined by electrospray ionisation mass spectrometry (ESI-MS). The novel peptide displayed specific inhibitory effects on the proliferation of human liver cancer (HepG2) and human gastric cancer cells (MGC). It also showed antibacterial activity against the tested bacteria *C. perfringens*, *S. epidermidis*, and *E. coli*. Moreover, this peptide prolonged

the whole blood clotting time *in vivo*.

Centipedes' venom are rich resource of peptides. Yang et al. (2012) identified twenty-six neurotoxin-like peptides from the venoms of *S. subspinipes mutilans*. Liu et al. (2012) also identified many peptides from another centipede, *S. subspinipes dehaani* venom. These peptides were found to act on voltage-gated sodium, potassium, and calcium channels, respectively. In the present study, we firstly identified a novel cytotoxic and anticoagulant peptide from *S. subspinipes mutilans* venom. The recent study of the closely related species *Scolopendra subspinipes dehaani* performed by Liu et al. (2012) uncovered multiple transcripts that contain the sequence of the described peptide, e.g: KC145039.1 .Given that centipede venoms are known to contain numerous proteases, the purified peptide may be a proteolytic fragment of centipede venom. Centipedes have been used for cancer treatment in traditional Chinese medicine for hundreds of years. Many components were identified from the centipede's body to be antitumor activity in several studies (Cohen and Quistad, 1998; Xu et al., 2010; Zhou et al., 2011; Zhao et al., 2012). We recently identified an anticoagulant peptide from the *S. subspinipes mutilans* body (Kong et al., 2013).

In the present study, we further confirmed that the centipede's venom also contains antitumor and anticoagulant components. However, the cytotoxic activity of the purified peptide is limited. The traditional usefulness of centipedes venom as anticancer agents may be due to indirect cytotoxity of this venom.

## ACKNOWLEDGEMENTS

This study was sponsored by National Science and Technology Major Projects for “Major New Drugs Innovation and Development” (2009ZX09103-091), Chinese National Natural Science Foundation (30972822, 81273274,81273375), Jiangsu Province's Key Provincial Talents Program (RC201170) , The innovative plan of Jiangsu provincial graduate(No. CXZZ11-0822), National Major Scientific and Technological Special Project for "Significant New Drugs Development" (2011ZX09302-003-02), Jiangsu Province Major Scientific and Technological Special Project (BM2011017), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. We also thank Mr.Yu for assistance with ESI mass spectrometry analysis of the purified peptide.

## REFERENCES

Atef M, Attia M, Fatma A, Ibrahim A, Ghada M, Nabil , Samir W Aziz (2013). Antioxidant effects of ginger (*Zingiber officinale Roscoe*) against lead acetate-induced hepatotoxicity in rats. Afr. J. Pharm. Pharmacol. 7:1213-1219.

An S, Ma D, Wei JF, Yang X, Yang HW, Yang H, Xu X, He S, Lai R (2011). A novel allergen Tab y 1 with inhibitory activity of platelet aggregation from salivary glands of horseflies. Allergy 66:1420-1427.

Cohen E, Quistad GB (1998). Cytotoxic effects of arthropod venoms on various cultured cells. Toxicon. 36: 353-358.

Fang H, Deng F, Yan Y, Wang K (1999). Chemical constituents of *Scolopendra negrocapitis*. Zhong Yao Cai 22:226-228.

González-Morales L, Diego-García E, Segovia L, Gutiérrez Mdel C, Possani LD (2009). Venom from the centipede *Scolopendra viridis Say*: purification, gene cloning and phylogenetic analysis of a phospholipase $A_2$. Toxicon. 54:8-15.

Knysak I, Martins R, Bertim CR (1998). Epidemiological aspects of centipede (Scolopendromorphae: Chilopoda) bites registered in greater S. Paulo, SP, Brazil. Rev. Saude Publica. 32:514-518.

Kong Y, Huang SL, Shao Y, Li S, Wei JF (2013). Purification and characterization of a novel antithrombotic peptide from *Scolopendra subspinipes mutilans*. J. Ethnopharmacol. 145:182-186.

Liu ZC, Zhang R, Zhao F, Chen ZM, Liu HW, Wang YJ, Jiang P, Zhang Y, Wu Y, Ding JP, Lee WH, Zhang Y (2012). Venomic and Transcriptomic Analysis of Centipede *Scolopendra subspinipes dehaani*. J. Proteome. Res.11:6197-6212.

Lu J, Chen ZW (2010). Isolation, characterization and anti-cancer activity of SK84, a novel glycine-rich antimicrobial peptide from Drosophila virilis. Peptides 31:44-50.

Malik KJ, Gadekar DH, Jain S (2013). Evaluation of hydro-alcoholic extract of leaves of *Boerhaavia diffusa* for anxiolytic activity in rats. Afr. J. Pharm. Pharmacol. 7:1071-1074.

Malta MB, Lira MS, Soares SL, Rocha GC, Knysak I, Martins R, Guizze SP, Santoro ML, Barbaro KC (2008). Toxic activities of Brazilian centipede venoms. Toxicon. 52:255-263.

Mohamed AH, Abu-Sinna G, El-Shabaka HA, El-Aal AA (1983). Proteins, lipids, lipoproteins and some enzyme characterizations of the venom extract from the centipede *Scolopendra morsitans*. Toxicon.21:371-377.

Mohri S, Sugiyama A, Saito K, Nakajima H (1991). Centipede bites in Japan. Cutis. 47:189-190.

Peng K, Kong Y, Zhai L, Wu X, Jia P, Liu J, Yu H (2010). Two novel antimicrobial peptides from centipede venoms. Toxicon. 55:274-279.

Rates B, Bemquerer MP, Richardson M, Borges MH, Morales RA, De Lima ME, Pimenta AM (2007). Venomic analyses of *Scolopendra viridicornis nigra* and *Scolopendra angulata* (Centipede, Scolopendromorpha): shedding light on venoms from a neglected group. Toxicon. 49:810-826.

Rodriguez-Acosta A, Gassette J, Gonzalez A,Ghisoli M (2000). Centipede (*Scolopendra gigantea Linneaus* 1758) envenomation in a newborn. Rev. Inst. Med. Trop. Sao Paulo. 42:341-342.

Tao C, Wei Q, Yin ZQ, Zhou LJ, Jia RY, Xu J, Shi DX, Zhou Y, Du YH , Deng YX (2013). Antifungal activity of essential oil from *Cinnamomum longepaniculatum* leaves against three dermatophytes *in vitro*. Afr. J. Pharm. Pharmacol. 7:1148-1152.

Wei JF, Li T, Wei XL, Sun QY, Yang FM, Chen QY, Wang WY, Xiong YL, He SH (2006a). Purification, characterization and cytokine release function of a novel Arg-49 phospholipase $A_2$ from the venom of *Protobothrops mucrosquamatus*. Biochimie. 88:1331-1342.

Wei JF, Wei XL, Chen QY, Huang T, Qiao LY, Wang WY, Xiong YL, He SH (2006b). N49 phospholipase A2, a unique subgroup of snake venom group II phospholipase $A_2$. Biochim. Biophys. Acta. 1760:462-471.

Wei JF, Yang HW, Wei XL, Qiao LY, Wang WY, He SH (2009). Purification, characterization and biological activities of the L-amino acid oxidase from *Bungarus fasciatus* snake venom. Toxicon. 54:62-271.

Wenhua R, Shuangquan Z, Daxiang S, Kaiya Z, Guang Y (2006). Induction, purification and characterization of an antibacterial peptide scolopendrin I from the venom of centipede *Scolopendra subspinipes mutilans*. Indian J. Biochem. Biophys. 43:88-93.

Wu Y, Li J, Wang J, Si Q, Zhang J, Jiang Y, Chu L (2009). Anti-atherogenic effects of centipede acidic protein in rats fed an atherogenic diet. J. Ethnopharmacol. 122: 509-516.

Xu XL, Wang CM, Geng D, Zhang L, Zhang L, Hu B (2010). Effects of centipede extracts on normal mouse and S180, H22 bearing mouse. Zhong Yao Cai 33:499-503.

Yang S, Liu Z, Xiao Y, Li Y, Rong M, Liang S, Zhang Z,Yu H, King GF, Lai R (2012). Chemical punch packed in venoms makes centipedes excellent predators. Mol. Cell. Proteomics. 11:640-650.

You WK, Sohn YD, Kim KY, Park DH, Jang Y, Chung KH (2004). Purification and molecular cloning of a novel serine protease from the centipede, *Scolopendra subspinipes mutilans*. Insect Biochem. Mol. Biol. 34:239-250.

Zhao H, Li Y, Wang Y, Zhang J, Ouyang X, Peng R, Yang J (2012). Antitumor and immunostimulatory activity of a polysaccharide-protein complex from *Scolopendra subspinipes mutilans* L. Koch in tumor-bearing mice. Food Chem. Toxicol. 50:2648-2655.

Zhou YQ, Han L, Liu ZQ, Du KC, Li KY (2011). Effect of centipede extract on cervical tumor of mice and its mechanism. Zhong Yao Cai 34:859-864.

academicJournals

Vol. 7(31), pp. 2246-2251, 22 August, 2013
DOI 10.5897/AJPP12.435
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Preparation of curcumin ethosomes

**CHEN Jin-guang[1]*, LAI Wei[2] and JIANG Yu[1]**

[1]Department of Dermatology, People's Hospital of Zhongshan City, Zhongshan, 528403, China.
[2]Department of Dermatology, Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China.

Accepted 3 September, 2012

**This study aims to prepare a novel curcumin ethosomes and investigate its properties as a drug delivery system of curcumin. A new modified method combining thin-film hydration and ultrasound was used to prepare ethosomes. Particle size was determined using Mastersizer 3000 laser diffraction analyzer. Average particle size was calculated based on the measurements of five different batches of ethosomes. Free curcumin was isolated by ultracentrifugation to determine the entrapment efficiency. High performance liquid chromatography (HPLC) was performed to determine the concentration of curcumin. The average particle size of curcumin ethosomes decreased with the increase of ethanol concentrations, whereas it increased with the increase of phospholipid concentration. The entrapment efficacy increased with the increase of ethanol and phospholipids concentration. The particle size of the curcumin ethosomes decreased with the increase of ethanol concentration, and increased with the increase of phospholipids contents. The concentration of ethanol and phospholipids showed a positive impact on entrapment efficiency. Finally, curcumin ethosomes showed high entrapment efficiency and good percutaneous permeability.**

**Key words:** Ethosomes, curcumin, liposome, high performance liquid chromatography (HPLC).

## INTRODUCTION

Curcumin (diferuloylmethane), a phenolic compound extracted from the root of *Curcuma longa*, has been widely used as a spice and coloring agent in food industry. According to our knowledge, curcumin showed anti-tumor, anti-oxidant, immunomodulatory, enhancing of apoptosis process, and antiangiogenic properties (Schaffer et al., 2011). Also, it has been reported as a mediator of chemo-resistance and radio-resistance (Bar-Seal et al., 2010). However, its limitations include easy susceptibility to oxidization and low bioavailability have prevented its further application in clinical practices.

Ethosome, a new type of liposome carrier in drug delivery system to emerge in recent years, showed characteristics of high deformability, high entrapment efficiency and percutaneous permeability through the keratoderma barrier. Compared with conventional liposome, ethosome showed more stable structure and quality which can promote the percutaneous drug absorption, increase drug storage in the skin cells and drug mobility to the cells. Additionally, it has the characteristics of prolonged action and avirulent. Therefore, it has been considered as an important drug carrier (Fang et al., 2009; de la Presa et al., 2009).

Ethosome could increase its percutaneous capability and promote the curative efficacy through gradual release of the drugs. Local administration of the drugs delivered by ethosome can avoid the first-pass effect on liver and the degradation within gastrointestinal tract, maintain the stability of the medicine in focal zone, reduce the toxicity and adverse reactions, attenuate the administration frequency, and increase the clinical efficiency and patient compliance.

In this study, ethosomes with a small particle size, even distribution and a high entrapment efficiency (EE) were

*Corresponding author. E-mail: tongly0422@126.com.

prepared in combination of filming-rehydration and ultrasonic methods. Based on that, curcumin ethosome was produced using ethosome as a drug delivery system of curcumin.

## MATERIALS AND METHODS

### Preparation of ethosomes

Curcumin ethosomes was prepared by filming-rehydration and ultrasonic method (Chen Jin et al., 2010). The prepared ethosome consist of 1~3% (w/v) lecithin, 30 to 45% ethanol (v/v), curcumin (0.1%) and water. For preparation of ethosome, an amount of lecithin and that of curcumin were dissolved in a glass bottle and mixed well with a magnetic stirrer. The glass bottle was connected to an injector and sealed; thereafter ethanol was added without vaporization. The mixture was poured into a round bottom flask and a thin film was prepared using roto-evaporator. The above mentioned procedures was repeated. Double distilled water (100 ml) was added to rehydrate the film to obtain the methyl nicotinate ethosomes. Then the ethosomes were homogenized for 5 min using a sonde-type ultrasonic instrument. Subsequently, the ethosomes were filtered using a 0.22 μm disposable filter. All the procedures in this test were carried out under gaseous nitrogen at room temperature. The quality fractions of curcumin and methyl nicotinate were 0.1 and 0.2%, respectively. Curcumin was not added in the aforementioned preparation process to produce empty ethosome suspension.

### Particle size of ethosomes

Particle size was measured using the Mastersizer 3000 laser diffraction particle size analyzer (Malvern, Worcestershire, UK) immediately after diluting and filtering the ethosomes with a 0.22 μm filter. The average particle size of ethosomes was calculated based on the measurements of 5 batches of ethosomes.

### Entrapment efficiency of ethosomes

To obtain the curcumin ethosomes, the unentrapped part was removed using ultracentrifuge (56,000 rpm at 4℃ for 40 min) after overnight storage at 4℃. The sediments of curcumin ethosomes were kept after removal of the supernatant. High-performance liquid chromatography (HPLC) was performed to evaluate the drug absorption from the ethosomes. The entrapment efficiency (EE) was calculated according to the following formula:

$$EE = DE / (DE + DS) \times 100$$

Where DE stands for the drug content measured from the parvules; Ds stands for the drug content measured from supernatant. All the results obtained were presented as the mean±standard deviation.

### High performance liquid chromatography (HPLC) analysis

HPLC analysis was performed using Dionex U3000 system. The curcumin ethosomes were separated on a μ-Bondapak C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was 43 : 57 (v/v) methanol-water at a flow rate of 1 ml/min. The wavelength for UV detection was 219 nm. The column temperature was set at 30℃. A 20-μl sample was injected to the column. The measuring range was 0.04 AUFS. The paper speed was set at 3 mm/min. Finally, the number of theoretical plates was counted based on the peak value of curcumin.

### Optimized design of ethosome prescription

The basic prescription and conditions for the preparation of curcumin ethosome were selected by single factor analysis followed by optimization by orthogonal experiment. The optimized prescription design included the concentration of phospholipid, the content of ethanol, and ultrasonic processing time, which were counted as the major investigating factors (3 levels from each factor were taken). The experiment was carried out according to the orthogonal array. The prediction schemes were presented in Tables 1 and 2.

## RESULTS

### Effects of ultrasonic processing time on entrapment efficiency

Table 3 summarizes the effects of ultrasonic processing time on the entrapment efficiency and particle size. The results indicated that the enhanced EE was noted with increase of the ultrasonic processing time, and reached the peak value at 8 min. No significant difference was noted in the particle size with increase of the ultrasonic processing time.

### Effects of ethanol content on entrapment efficiency

Table 4 summarizes the effects of ethanol content on the entrapment efficiency, which indicated that enhanced EE was detected with the increase of ethanol content. In addition, the average particle size and the gathering probability among ethosomes decreased with increase of the ethanol content. A particle size of less than 100 nm would promote the percutaneous effect of the drug. Thus, on the premise of stable ethosome structure, ethanol content should be maximized to increase the percutaneous capability.

### Optimization of preparation process of ethosomes

For the single factor analysis, phospholipids concentration (A), ethanol content (B) and ultrasonic processing time (C) was used as the major investigating factors, respectively. Three levels (A1~A3, B1~ B3, C1~C3) were set for each investigating factor. The experiment was carried out according to the orthogonal array. The entrapment efficiency (EE) obtained from various factors has been shown in Table 5. The effects of the three factors on EE ranked in an order of phospholipids concentration > ethanol content > ultrasonic time. According to the medium values, the most optimized prescription was A2B3C1, based on which the entrapment efficiency was (94.5±3.1)%.

**Table 1.** Optimization of curcumin ethosome preparation by orthogonal array.

| Number | Phospholipids concentration (w/v, %) | Ethanol content (v/v, %) | Ultrasonic time (min) |
|---|---|---|---|
| 1 | 1 | 1 | 1 |
| 2 | 1 | 2 | 2 |
| 3 | 1 | 3 | 3 |
| 4 | 2 | 1 | 2 |
| 5 | 2 | 2 | 3 |
| 6 | 2 | 3 | 1 |
| 7 | 3 | 1 | 3 |
| 8 | 3 | 2 | 1 |
| 9 | 3 | 3 | 2 |

**Table 2.** Optimization of curcumin ethosome preparation by orthogonal array factor-level.

| Level | Phospholipids concentration (w/v, %) | Ethanol content (v/v, %) | Ultrasonic time (min) |
|---|---|---|---|
| 1 | 1.5 | 35 | 5 |
| 2 | 2.0 | 40 | 8 |
| 3 | 2.5 | 45 | 11 |

**Table 3.** Effects of ultrasonic processing time on entrapment efficiency.

| Ultrasonic processing time (min) | EE (%) | Particle size (nm) |
|---|---|---|
| 2 | 52.41±2.80 | 77.41±7.33 |
| 5 | 62.55±2.63 | 74.32±7.02 |
| 8 | 66.11±1.19 | 80.53±8.01 |
| 11 | 60.10±3.61 | 81.87±8.13 |
| 15 | 56.40±1.41 | 70.02±5.62 |

**Table 4.** Effects of ethanol content on entrapment efficiency (n=5, mean ± sd).

| Ethanol content (v/v, %) | EE (%) | Particle size (nm) |
|---|---|---|
| 30 | 37.26±1.21 | 84.41±7.83 |
| 35 | 44.32±1.25 | 74.32±7.02 |
| 40 | 57.72±2.32 | 69.53±5.21 |
| 45 | 70.05±3.17 | 50..32±5.13 |
| 50 | 75.41±2.35 | 46.02±4.22 |

## Evaluation of the quality of curcumin ethosomes

Ten grams of curcumin ethosomes was centrifuged at 3000 r/min for 30 min, and then incubated at 60℃ or -10℃ for 24 h. After that, the curcumin ethosomes were brought to room temperature. The ethosomes were comparatively stable and not easily delaminated or precipitated at normal temperatures as no delamination was observed. For the physical properties, the ethosomes was ivory white suspension with proper viscosity and good ductility. In addition, the colloid was smooth and evenly-distributed. After probe-type ultrasound, it turned into transparent colloid solution.

Percutaneous capability through microporous membrane (orifice diameter of 0.15 μm) after deformation under external pressure (0.1 ~ 0.3 MPa) was analyzed. The passing rate (P) was calculated according to the following formula:

$$P = V_{ethosomes} / V_{water} \times 100\%$$

where $V_{ethosomes}$ stands for the time for flexible liposome colloid solution to penetrate the filter membrane; $V_{water}$ stands for the time for water to penetrate the filter mem-

**Table 5.** Optimization of curcumin ethosome preparation process.

| Number | Phospholipids concentration (w/v, %) | Ethanol content (v/v, %) | Ultrasonic time(min) | EE(%) |
|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 86.22 |
| 2 | 1 | 2 | 2 | 89.53 |
| 3 | 1 | 3 | 3 | 90.71 |
| 4 | 2 | 1 | 2 | 90.91 |
| 5 | 2 | 2 | 3 | 92.02 |
| 6 | 2 | 3 | 1 | 94.15 |
| 7 | 3 | 1 | 3 | 91.64 |
| 8 | 3 | 2 | 1 | 89.79 |
| 9 | 3 | 3 | 2 | 87.01 |
| Ij | 272.96 | 277.95 | 282.64 | - |
| IIj | 289.35 | 283.83 | 280.57 | - |
| IIIj | 283.44 | 283.97 | 282.54 | - |
| Average Ij | 90.99 | 92.65 | 94.21 | - |
| Average IIj | 96.45 | 94.61 | 93.52 | - |
| Average IIIj | 94.48 | 94.66 | 94.18 | - |
| Rj | 5.46 | 2.01 | 0.69 | - |

brane. The passing rate increased with the increase of external pressure. The peak value of passing rate reached 91% under an external pressure of 0.3 MPa, which indicated that curcumin ethosomes showed satisfactory deformability.

Average particle size played a pivotal role in the curative effect of the medicine. In our study, five batches of curcumin ethosome were separately prepared according to the optimized prescription. The average particle size of curcumin ethosomes after dilution was 54.3±5.2 nm using laser particle analyzer (Malvern Instruments Corporate, Worcestershire, UK). Additionally, the distribution of ethosomes was found to be tight with an averaged particle size of 51.8±4.7 nm.

## Measurement of curcumin content

To prepare the curcumin stock, 4 mg of standard curcumin was dissolved in methanol in a 100-ml volumetric flask. To dilute the curcumin stock, different volumes of stock were added in 10-ml volumetric flasks, and diluted with methanol. After filtering with Millipore, 10 µl from each dilution was separately injected into liquid chromatography. The peak areas were recorded respectively.

Standard curves were drawn with sample weight (µg) as X-axis and average value peak area as Y-axis. The equation of linear regression was:

$$Y = 75.3261 + 211.8860X; \ r = 0.9999.$$

Curcumin ethosome sample (1 ml) from 3 batches prepared according to the optimized prescription was separately added into a 100-ml measuring flask, diluted with methanol and mixed well. 20 µl of sample was injected into high-performance liquid chromatography (HPLC) column. Then the peak area was recorded and subjected to regression analysis. The contents of the 3 batches of samples were calculated as 99.01, 96.90 and 97.91%, respectively.

## Measurement of entrapment efficiency

According to the optimized prescription, three batches of samples of curcumin ethosomes were prepared. The unentrapped part was removed using ultracentrifuge at 6000 r/min for 45 min after storing overnight at 4℃. The parvules of curcumin ethosome were kept after removal of the supernatant. The drug content was determined using HPLC. The entrapment efficiency was calculated with the following formula:

$$EE = DE / (DE + DS) \times 100$$

where DE stands for the drug content measured from the parvules; Ds stands for the drug content measured from supernatant. The average entrapment efficiency in this study was 94±3% (n = 3).

## DISCUSSION

As a new type of vesicle-structured drug carrier, ethosome showed the characteristics of good deformability, high entrapment efficiency, satisfactory permeability and reliable stability, which enabled the delivery of drugs into deeper skin layers and/or the stystemic circulation. Meanwhile, it could promote the

internal transmission of lipophilic drugs *in vivo*. Furthermore, the ethosomes could deliver the drugs through the eyelets which was 1/10 to 1/5 less than its size without changing its shape under the hydration pressure (Godin and Touitou, 2003). The high concentration of ethanol contained in the ethosomes could increase the flexibility and fluidity of lipid bilayer, based on which the liposomes with ethanol could penetrate the cuticle. In addition, the interaction between ethanol and cuticle could increase the solubility of the drug, decrease the phase-transmission temperature, alternate the arrangement of lipid molecule, and increase the fluidity and flexibility of ethosome membrane. As phospholipid was apt to be dissolved by ethanol, the concentration of ethanol in ethosome should be less than 45% (Touitou et al., 2000; Barry, 2001).

The study presented the preparation of curcumin ethosomes based on optimal prescription. Single factor method was used to select the basic prescription and conditions for the preparation of curcumin ethosome. The phospholipid concentration, ethanol content and ultrasonic processing time were considered as the major investigating factors. Orthogonal experiment was carried out to select the optimized condition for the preparation of ethosomes with entrapment efficiency. After that the quality of curcumin ethosomes was analyzed. The results showed that the effects of three factors on the entrapment efficiency ranked in an order of concentration > ethanol content > ultrasonic processing time. The analysis of the average particle size using laser dynamic scattering equipment indicated that the particle size of ethosome decreased with the increase of ethanol concentration. However, its size increased with the increase of phospholipids concentration. No restrict correlation was identified between the ultrasonic processing time and the average particle size. Previous study indicated that entrapment efficiency and stability were correlated with particle size and distribution, and they could directly influence the activities of ethosomes in the organism (Esposito et al., 2004). Our study indicated that the entrapment efficiency of the drug increased with the increase of phospholipid and ethanol concentration as well as the ultrasonic processing time. In addition, the aggregation probability among ethosomes increased with increase of phospholipid concentration and decrease of ethanol concentration. As the particle size of ~100 nm showed negative effects on the percutaneous capability of the drug, phospholipid concentration must be controlled to modulate the percutaneous capability of the ethosomes. Additionally, the ethanol content should be increased as high as possible on the premise of the stability of ethosomes, in order to increase the percutaneous capability of the agent (Esposito et al., 2004).

In our study, significant decrease was noted in the particle sizes of ethosome compared with those of general liposomes. Presumably, the addition of ethanol in the prescription may give rise to a change in the nature of electrical charge, which may strengthen the space stability of vesicle and shrink its size. Curcumin is a fat soluble drug with relatively high entrapment efficiency. According to our knowledge, the entrapment efficiency is usually measured with dialysis or ultracentrifugation. Once the entrapment efficiency measured by ultracentrifugation is lower than that obtained from dialysis, it indicated that the drug had been partially lost due to the deformation and disruption of the lipid layers in the process of ultracentrifugation (López-Pinto et al., 2005). In the present study, the entrapment efficiency of curcumin ethosomes prepared with the mixture ratio of 45% (v/v) of ethanol to 2% (w/v) of phospholipids was over 85%. The ethosomes were small in size with good distribution and a high entrapment efficiency of ~95%. Results obtained from the minimal inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and antibiosis-seasoning experiments proved that the speed rate and the percutaneous capability of ethosomes was higher and the time lag was shorter, compared with those of liposomes, demonstrating the drug could exert its effects effectively in antibiosis and the treatment of deep mycotic infections (Godin and Touitou, 2005).

## Conclusion

In this study, a novel ethosome with lecithin, ethanol and water was produced, which showed characteristics of well distribution, small particle size and high entrapment efficiency. The combination of the thin-film hydration and ultrasound technology made these ethosomes suitable to serve as liposome drug carriers. This study may provide a new orientation for the research and development of external curcumin agents.

## ACKNOWLEDGEMENTS

This work was supported by the Fund of Zhongshan Administration of Science and Technology (NO. 20122A012).

### REFERENCES

Barry BW (2001). Novel mechanisms and devices to enable successful transdermal drug delivery. Eur. J. Pharm. Sci. 14:101-114.

Bar-Seal G, Epelbaum R, Schaffer M (2010). Curcumin as an anti-cancer agent: review of the gap between basic and clinical applications. Curr. Med. Chem. 3:190-197.

Chen JG, Liu YF, Gao TW (2010). Preparation and anti-inflammatory activity of triptolide ethosomes in an erythema model. J. Liposome Res. 20:297-303.

de la Presa P, Rueda T, del Puerto Morales M, Javier Chichón F, Arranz R, Valpuesta JM, Hernando A (2009). Gold nanoparticles generated in ethosome bilayers, as revealed by cryo-electron-tomography. J. Phys. Chem. B. 113:3051-3057.

Esposito E, Menegatti F, Cortesi R (2004). Ethosomes and liposomes as topical vehicles for azelaic acid. Int. J. Cosmet. Sci. 26:270-271.
Fang YP, Huang YB, Wu PC, Tsai YH (2009). Topical delivery of 5-aminolevulinic acid-encapsulated ethosomes in a hyperproliferative skin animal model using the CLSM technique to evaluate the penetration behavior. Eur. J. Pharm. Biopharm. 73:391-398.
Godin B, Touitou E (2003). Ethosomes：New prospects in transdermal delivery. Crit. Rev. Ther. Drug Carrier. Syst. 20:63-102.
Godin B, Touitou E (2005). Erythromycin ethosomal systems:physicochemical characterization and enhanced antibacterial activity. Curr. Drug Deliy. 2:269-275.
López-Pinto JM, González-Rodríguez ML, Rabasco AM (2005). Effect of cholesterol and ethanol on dermal delivery from DPPC liposome. Int. J. Pharm. 298:1-12.
Schaffer M, Schaffer PM, Zidan J, Bar Sela G (2011). Curcuma as a functional food in the control of cancer and inflammation. Curr. Opin. Clin. Nutr. Metab. Care 14:588-597.
Touitou E, Dayan N, Bergelson L, Godin B, Eliaz M (2000). Ethosomes ovel vesicular carriers for enhanced delivery: characterization and skin penetration properties. J. Control Release 65:403-418.

academicJournals

Vol. 7(31), pp. 2252-2257, 22 August, 2013
DOI 10.5897/AJPP12.438
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Antioxidant markers in guinea pig exposed to obidoxime and HI-6 acetylcholinesterase oxime reactivators containing oxime moiety

**Lucie Drtinova and Miroslav Pohanka***

Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 50001, Hradec Kralove, Czech Republic.

Accepted 10 August, 2012

**Obidoxime and asoxime (HI-6) are considered to be the most important acetylcholinesterase (AChE) reactivators applicable for treatment of poisoning by nerve agents. Unfortunately, toxicology of the oximes is not well known. For this reason, we decided to investigate the pertinent adverse effects on guinea pigs which are close to humans in toxicological point of view. HI-6 and obidoxime were administered intramuscularly in 5% of the median lethal dose. The animals were sacrificed 15, 30, 60, 120, and 240 min after exposure, and the brain, liver, spleen and kidneys were collected. Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST) and glutathione reductase (GR) were measured. Results indicated that obidoxime acted on oxidative stress than HI-6. We found evidence of low molecular weight antioxidants depletion after obidoxime administration. On the other hand, TBARS assay showed significant decrease in brain and little increase in spleen and liver. The effect of HI-6 was more striking than the effect of obidoxime. There was a sign of higher metabolism and production of antioxidants in liver because GR was significantly increased after HI-6 exposure, and it is another sign of ongoing oxidative stress. Owing to the achieved results, obidoxime can be considered as a less toxic drug in counteracting oxidative stress despite its higher toxicity.**

**Key words:** Oxidative stress, obidoxime, HI-6, acetylcholinesterase, butyrylcholinesterase.

## INTRODUCTION

Nerve agents and organophosphorus pesticides are organic compounds obtained from phosphorus and characterized by a high toxicity to mammals. Acute organophosphorus pesticide poisoning causes tens of thousands of deaths each year across the developing world (Buckley et al., 2011). Their main toxic effect is inhibition of enzyme acetylcholinesterase (AChE; EC 3.1.1.7); the phosphorous substances covalently bind to hydroxyl group of serine molecule in catalytic site of AChE. When AChE becomes inhibited, it cannot proceed in physiological function - splitting of neurotransmitter acetylcholine (ACh) (Marrs, 1993). ACh as a substrate of AChE become accumulated in the neurosynaptic cleft and causes cholinergic crisis due to the overstimulation of both ACh receptors: muscarinic and nicotinic. The therapy after organophosphorus poisoning is symptom-matic (atropine and/or anticonvulsants) and causative (oxime reactivators). Oxime reactivators are a group of antidotes suitable for causal treatment after exposure to these phosphorous compounds (Bajgar, 2010). Oximes react with nerve agents and pesticides bind the AChE active site (Pohanka, 2011). The covalent bound is broken due to nucleophilic substitution (Petroianu et al., 2012). On the other hand, oximes are inhibitors of AChE

*Corresponding author. E-mail: miroslav.pohanka@gmail.com.

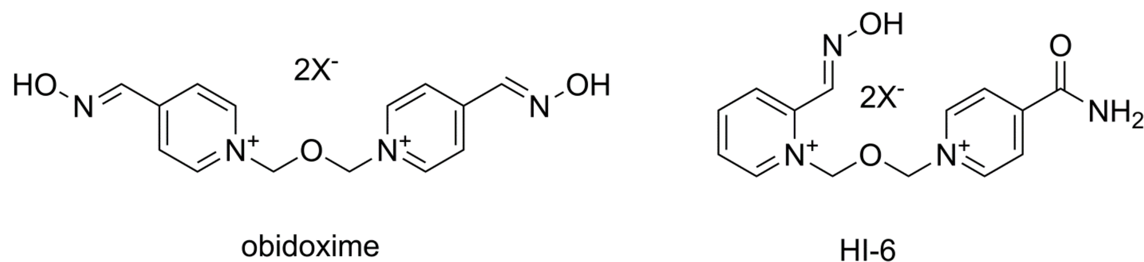


**Figure 1.** Structures of the oxime reactivators: obidoxime and asoxime (HI-6).

as well and also protect the active site of AChE (Mercey et al., 2012). Oxidative stress is an imbalance between formation and removal of reactive oxygen species. The reactive oxygen species products are free radicals and peroxides. The reactive forms are covered by antioxidants as the reactive forms are scavenged. Redox balance is considered as another parameter of homeostasis. Oxidative stress in humans results in many diseases - including the neuro-degenerative ones such as Parkinson´s disease, Alzheimer´s disease, and Huntington's disease (Guglielmotto et al., 2010); cardiovascular disease – myocardial infarction, atherosclerosis, heart failure (Ramond et al., 2011); and others – fragile X syndrome or chronic fatigue syndrome (Kennedy et al., 2005).

The prevention of oxidative stress is based on up-regulation of antioxidant enzymes. We may achieve up-regulation with some trace elements in food supplements, such as selenium, zinc, copper or combination of the elements (Micke et al., 2009). Alteration in the antioxidant enzymes provide information about relationship with some diseases such as Alzheimer´s disease, atherosclerosis and in treatment of diseases linked with chronic inflammation and persisting generation of reactive species (Valko et al., 2005).

Oxidative stress plays a significant role in poisoning with inhibitors of the AChE enzyme. Although the mechanism of inhibition of enzymes is known, but the overall effect on the organism is not completely understood. In previous experiments, we examined some side effects of oxime reactivators. Especially, oxidative stress was plausibly proven to follow administration of oxime reactivators. Unfortunately, complete evidence of the link between oxidative stress and the time pathogenesis starts has not been extensively investigated yet. Hence, in order to recognize scale and bulk of oxidative stress, we decided to investigate it in guinea pigs as a model organism with good extrapolation to humans. Obidoxime (1,3-bis(4-hydroxy-iminomethylpyridinium)-2-oxapropane dichloride) and HI-6 (4-carbamoyl-1-[({2-[(E)-(hydroxyimino)-methyl]pyridinium-1-yl}methoxy)methyl] pyridinium dichloride), as shown in Figure 1, were chosen as the representative oxime reactivators for the experiment purposes.

## MATERIALS AND METHODS

### Animal exposure

The experiment was done using three months old guinea pig (*Cavia porcellus*) weighing 260 ± 20 g at the start of the experiment. The animals were received from Velaz Company (Prague, Czech Republic) and kept under standard room temperature 22 ± 2°C, humidity 50 ± 10% and light period 12 h per a day. Food and water were provided *ad libitum*. The experiment was permitted and supervised by the Ethical Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic. Obidoxime and HI-6 both received from Sigma-Aldrich (Saint Louis, Missouri, USA), were injected intramuscularly (i.m.) in an amount of 100 µL into the rear limb of the animals. The animals were divided into 5 groups each of 5 specimens, while 8 animals were disposed for control purposes. The control group was used for both reactivators and it was exposed to saline solution in the same volume: 100 µl. Animals in the 5 groups were i.m. exposed to either obidoxime or HI-6 in doses 5% of $LD_{50}$; it was 4.15 mg/kg for obidoxime and 45.1 mg/kg for HI-6. The animals were sacrificed by light $CO_2$ narcosis 15, 30, 60, 120, and 240 min after the start of the experiment for both reactivators.

### Preparation of samples for antioxidant markers assessment

The frontal lobe, cerebellum, spleen, liver and kidney were collected after euthanasia from the animals. In total, 100 mg of freshly collected tissue was used for sample preparation. The tissue sample was placed in 1 ml of saline solution (0.9 w/v of sodium chloride in deionized water) and processed immediately. The individual samples were homogenized using a T10 basic ULTRA-TURRAX® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) mechanical homogenizer for 1 min.

### Methods of antioxidants markers assessment

Ferric reducing antioxidant power assay (FRAP), thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST) and glutathione reductase (GR) were assessed using standard protocols previously reported (Pohanka et al., 2011).

### Statistical analysis

The experimental data were processed by the statistical software Origin 8 SR2 (OriginLab Corporation, Northampton, MA, USA). The results were tested for their significance. Significant differences between the experimental groups were estimated by one-way analysis of variance (ANOVA) with Bonferroni's test, considering

**Table 1.** Results on the ferric reducing antioxidant power (FRAP) for obidoxime.

| Parameter (µmol/g) | Control | 15 min | 30 min | 60 min | 120 min | 240 min |
|---|---|---|---|---|---|---|
| Liver | 1.46±0.28 | 1.09±0.14 | 1.21±0.27 | 1.09±0.16 | 1.11±0.18 | 1.29±0.18 |
| Spleen | 1.78±0.17 | 1.38±0.25 | 1.44±0.34 | 1.30±0.18* | 1.27±0.08* | 1.48±0.34 |
| Kidney | 0.885±0.215 | 0.863±0.253 | 0.885±0.311 | 0.829±0.223 | 0.843±0.202 | 0.862±0.098 |
| Cerebellum | 0.800±0.280 | 0.614±0.222 | 1.33±0.05 (*) | 0.476±0.045 | 0.548±0.197 | 0.493±0.051 |
| Frontal lobe | 0.655±0.188 | 0.411±0.031** | 0.384±0.025** | 0.411±0.024** | 0.374±0.022** | 0.408±0.020** |

*=p<0.05. **=<0.01.

**Table 2.** Results on the thiobarbituric acid reactive substances (TBARS) for obidoxime.

| Parameter (µmol/g) | Control | 15 min | 30 min | 60 min | 120 min | 240 min |
|---|---|---|---|---|---|---|
| Liver | 0.567±0.101 | 0.600±0.039 | 0.717±0.147 | 0.722±0.088 | 0.569±0.043 | 0.509±0.030 |
| Spleen | 0.491±0.066 | 0.409±0.051 | 0.389±0.059 | 0.559±0.079 | 0.510±0.076 | 0.499±0.045 |
| Kidney | 0.800±0.113 | 0.679±0.032 | 0.654±0.043* | 0.586±0.040** | 0.597±0.111** | 0.495±0.041** |
| Cerebellum | 0.873±0.135 | 0.575±0.066** | 0.577±0.023** | 0.639±0.030** | 0.571±0.026** | 0.630±0.032** |
| Frontal lobe | 0.816±0.084 | 0.643±0.052** | 0.611±0.049** | 0.660±0.070** | 0.642±0.060** | 0.667±0.066** |

*=p<0.05. **=<0.01.

**Table 3.** Results on glutathione reductase (GR) for obidoxime.

| Parameter (µmol/g) | Control | 15 min | 30 min | 60 min | 120 min | 240 min |
|---|---|---|---|---|---|---|
| Liver | 0.128±0.052 | 0.253±0.021** | 0.265±0.025** | 0.221±0.017** | 0.225±0.041** | 0.219±0.059** |
| Spleen | 0.146±0.059 | 0.196±0.017 | 0.177±0.090 | 0.218±0.075 | 0.186±0.100 | 0.259±0.017 |
| Kidney | 0.129±0.031 | 0.217±0.105 | 0.250±0.029 | 0.227±0.092 | 0.312±0.134* | 0.247±0.017 |
| Cerebellum | 0.164±0.046 | 0.114±0.018 | 0.140±0.058 | 0.145±0.024 | 0.168±0.047 | 0.184±0.029 |
| Frontal lobe | 0.114±0.114 | 0.239±0.059 | 0.166±0.059 | 0.238±0.086 | 0.215±0.028 | 0.282±0.068* |

*=p<0.05. **=<0.01.

**Table 4.** Ferric reducing antioxidant power (FRAP) for HI-6.

| Parameter (µmol/g) | Control | 15 min | 30 min | 60 min | 120 min | 240 min |
|---|---|---|---|---|---|---|
| Liver | 1.46±0.28 | 1.02±0.206** | 0.790±0.050** | 1.03±0.060** | 0.925±0.123** | 0.999±0.203** |
| Spleen | 1.78±0.17 | 0.889±0.111** | 0.934±0.344** | 1.05±0.18** | 0.968±0.170** | 0.932±0.272** |
| Kidney | 0.885±0.211 | 0.562±0.133 | 0.436±0.120* | 0.456±0.050* | 0.339±0.020* | 0.591±0.333 |
| Cerebellum | 0.800±0.282 | 0.432±0.181 | 0.504±0.165 | 0.342±0.323 | 0.418±0.275 | 0.672±0.243* |
| Frontal lobe | 0.655±0.180 | 0.499±0.221 | 0.481±0.244 | 0.346±0.162 | 0.364±0.160 | 0.362±0.201 |

*=p<0.05. **=<0.01.

probability level p=0.05 ($0.01<p\leq0.05$) and p=0.01 ($p\leq0.01$) probability levels for each group.

## RESULTS

The animals were in a good condition and they exerted no symptoms of poisoning. Moreover, no convulsions occurred during the whole experiment. FRAP was used for the assessment of antioxidants content (Tables 1 and 2), lipid peroxidation was represented by TBARS (Tables 3 and 4) and enzymatic marker by GR (Tables 5 and 6). All the experiments were assessed on liver, spleen, kidney, cerebellum and frontal lobe for the both reactivators. FRAP value was extensively altered for the both reactivators. Beside the difference in the reactivators exposed animals, the tested organs had variation in the

**Table 5.** Thiobarbituric acid reactive substances (TBARS) for HI-6.

| Parameter (µmol/g) | Control | 15 min | 30 min | 60 min | 120 min | 240 min |
|---|---|---|---|---|---|---|
| Liver | 0.567±0.101 | 0.687±0.062 | 0.651±0.107* | 0.647±0.084 | 0.680±0.061 | 0.682±0.035 |
| Spleen | 0.491±0.066 | 0.471±0.015 | 0.561±0.054 | 0.584±0.057 | 0.584±0.049 | 0.548±0.042 |
| Kidney | 0.800±0.114 | 0.888±0.033 | 0.911±0.059 | 0.979±0.091 | 0.937±0.039 | 0.916±0.118 |
| Cerebellum | 0.873±0.135 | 1.13±0.05** | 1.29±0.13** | 1.36±0.06** | 1.22±0.15** | 1.25±0.08 |
| Frontal lobe | 0.816±0.084 | 1.09±0.07** | 1.04±0.06** | 1.16±0.10** | 1.00±0.10** | 1.12±0.03** |

*=p<0.05. **=<0.01

level of low molecular weight antioxidants. However, the effect was unequal in the different organs. Obidoxime caused a decrease of FRAP (significant on p=0.01) in frontal lobe from 15 min and in spleen after 60 min (significance at p=0.05). A significant decrease (p=0.01) was also observe in the liver and in the spleen from 15 min after HI-6 exposure as well. The second decrease were evident about half of the value beside control group (p=0.05) in kidney after 30 min.

TBARS levels were also detected by the same organs for the both reactivators. The significance was the same (p=0.01) for the reactivators in cerebellum and in frontal lobe from 15 min. It is interesting to note that the level of lipid peroxidation was reduced for obidoxime, but increased for HI-6 compared to the control. In obidoxime exposed animal, one more decrease was found in kidney after 30 min (significant on p=0.05) respective from 60 min (significant on p=0.01). Furthermore, GR significantly (p=0.01) increased when compared to controls in obidoxime group in liver after 15 min. In the HI-6 exposed animals, GR activity was significantly altered in kidney on probability level p=0.05 after 30 min and on p=0.01 after 60 min. Beside the kidney, HI-6 caused significant alteration of glutathione reductase activity in spleen after 30 min (p=0.05). Compared to the other markers, GST activity in the examined organs has no tendency and shift in GST activity appears to be random. The marker fluctuated in a range 283 to 325 µmol/g in kidney, 480 to 553 µmol/g in liver, and 11.5 to 28.4 µmol/g in the other organs. No statistically significant result was provided for the marker.

## DISCUSSION

Obidoxime and HI-6 are bispyridinium oximes. They are used in the treatment of acute nerve agents poisoning. The oxime substances are called reactivators and they are known for their ability to reverse the binding of organophosphorus compounds to the enzyme AChE. At present, we know that the effectiveness of the oximes is too low to resolve the acute toxic effects of some organophosphorus nerve agents. This finding is supported by a long term research resumed by Kassa (2002). Our main interest was to find out adverse effect after intramuscular administration in guinea pig model.

Our results indicated that the level of low molecular weight antioxidants decreased in all the organs after administration of obidoxime with the exception of kidneys. Based on the FRAP measurement results, we can infer that variation in metabolism took place in the guinea pigs after administration of obidoxime. Liver is obviously more sensitive to the impact of oxime reactivators in terms of the FRAP value compared to brain (Pohanka et al., 2011). However, it was observed that the decrease of low molecular weight antioxidants was significant in frontal lobe tissue in the obidoxime case. This is sign of non-normal homeostasis or antioxidant imbalance in the brain. In clinical studies, evidence of initiated pathological processes is linked to alteration of antioxidant balance and antioxidants are typically depleted in the brain of patients that suffers from neurodegenerative disease (Fujita et al., 2012).

The TBARS value indicates damage to membranes by reactive oxygen species and in response to the molar fragment of oxidized lipids (malondialdehyde) (Pohanka et al., 2011). The increase of malondialdehyde is common once oxidative stress is uncovered by antioxidants (Jomova et al., 2010). From the results of TBARS, it was clearly evident that the lipid peroxidation significantly decreased in kidney, cerebellum and frontal lobe. On the other hand, we observed a little increase in the liver and spleen in 30, 60 and 120 min after the administration of the oximes. Though the TBARS level was not extensively altered after application of the oxime reactivators, depletion of antioxidants evident from the FRAP level point to increased sensitivity to a pro-oxidant effect. Moreover, liver is a source of low molecular weight antioxidants such as reduced glutathione (GSH) distributed by blood system to the other organs where it helps to maintain oxidative homeostasis (Hermes-Lima et al., 2012; El-Demerdash et al., 2012). Depletion of antioxidants in liver causes aggravation of antioxidant balance in the other organs. GR is a ubiquitous enzyme that catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. GSH is an important molecular antioxidant; it is dominantly involved in the destruction of free reactive oxygen species and in the metabolism of exogenous and endogenous compounds (Rana et al., 2002). As seen in the experi-

**Table 6.** Glutathione reductase (GR) for HI-6.

| Parameter (μmol/g) | Control | 15 min | 30 min | 60 min | 120 min | 240 min |
|---|---|---|---|---|---|---|
| Liver | 0.128±0.052 | 0.169±0.036 | 0.120±0.023 | 0.156±0.038 | 0.155±0.028 | 0.162±0.031 |
| Spleen | 0.146±0.059 | 0.247±0.053 | 0.266±0.060* | 0.284±0.036* | 0.203±0.033 | 0.284±0.080* |
| Kidney | 0.129±0.031 | 0.156±0.026 | 0.199±0.034* | 0.254±0.036** | 0.258±0.034** | 0.324±0.030** |
| Cerebellum | 0.164±0.046 | 0.142±0.035 | 0.108±0.054 | 0.125±0.058 | 0.152±0.055 | 0.145±0.022 |
| Frontal lobe | 0.114±0.114 | 0.153±0.026 | 0.183±0.060 | 0.142±0.047 | 0.134±0.051 | 0.164±0.096 |

*=p<0.05. **=<0.01.

mental data, there was significantly increased GR activity only in liver, but the non-significant increase was found in all the organs of the exposed animals. Hence, it can be concluded that oxidative stress took place after the administration of oxime reactivators.

The examination of low molecular weight antioxidants were conducted in all selected organs using the FRAP test. The significant variations of the marker in animals treated by HI-6 are interesting when compared with each other. For both reactivators, we detected a decrease of low molecular weight antioxidants in organ's tissues. In HI-6 exposed animals, we found significantly decreased low molecular weight antioxidants in the liver, spleen and in kidney. The decrease was almost double compared to the controls. And although the depletion of low molecular weight antioxidants is not necessary a pathological process, however, the depletion increases vulnerability of organism to the other pathological processes such as degradation of macromolecules (Valachova et al., 2010).

In TBARS assay, we found increased value of malondialdehyde in all the organs. The sensitivity of the brain to impact of oxime reactivators was a surprising fact. Owing to the obidoxime, the increase of lipid peroxidation uncovered oxidative stress in organism. On the other hand, HI-6 initiated a depletion of antioxidants in the examined organs. The depletion was more extensive for HI-6 than was evident in obidoxime-exposed animals. We must emphasize that the more extensive effect of HI-6 could be caused by application of the equitoxic doses into the animals. The animals treated with HI-6 received larger mass than the animals treated with obidoxime. Application of equitoxic doses have drawback in the more complicated investigation of some side effects of the tested compounds. However, the animals had similar adverse effects as toxic doses were the same. Therefore, further researches should be aimed at providing a link between the equitoxic and equimolar doses for oxime reactivators. Considering chemical structure of the oxime reactivators, obidoxime has two oxime moieties, while HI-6 has only one oxime and one carbamoyl moieties. The presence of the carbamoyl group would be considered as a risk factor for the reported depletion of antioxidants.

In the GR assessment, we obtained an increase of enzyme activity in all organs. Though GR activity was increased in all of the examined organs, the alteration was significant in kidneys only. It confirms that the organs met reactive oxygen species evoking GR expression as GR is considered as a plausible marker of appeared oxidative stress (Anand et al., 2012). This finding can be interpreted that the two tested reactivators acted as pro-stressogenic factors in the kidney and the detrimental processes would be initiated once the reactivators administered as a prophylactic. The link between oxidative stress in kidneys and nephropathy has been reported by several studies (Yiu et al., 2010). The fact that kidneys are influenced by the oxime reactivators is therefore not surprising, considering the renal elimination and temporal retention of the compounds (Ligtenstein and Kossen, 1983).

## Conclusion

It can be approved that both reactivators have significant effect on oxidative homeostasis in the body. Thus far, we have proven that obidoxime is less implicated in oxidative stress than HI-6. This is an interesting fact because HI-6 is commonly considered as a low toxic oxime reactivator when taking acute toxicity into account. In contrast, HI-6 has potential adverse effects when administered repeatedly.

## ACKNOWLEDGEMENT

The contribution of the research project: Assessment of biochemical and pharmacokinetics parameters of selected acetylcholinesterase inhibitors is gratefully acknowledged.

### REFERENCES

Anand KV, Mohamed JMS, Thomas PA, Geraldine P (2012). Protective role of chrysin against oxidative stress in D-galactose-induced aging in an experimental rat model. Geriatr. Gerontol. Int. 12(4):741-50.

Bajgar J (2010). Optimal choice of acetylcholinesterase reactivators for antidotal treatment of nerve agent intoxication. Acta Medica 53:207-211.

Buckley NA, Eddleston M, Li Y, Bevan M, Robertson J (2011). Oximes for acute organophosphate pesticide poisoning. Cochrane database

Syst. Rev. 16(2).
El-Demerdash F, Attia AA, Elmazoudy RH (2012). Biochemical and histopathological changes induced by different time intervals of methomyl treatment in mice liver. J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng. 47:1948-1954.
Fujita K, Yamafuji M, Nakabeppu Y, Noda M (2012). Therapeutic approach to neurodegenerative diseases by medical gases: focusing on redox signaling and related antioxidant enzymes. Oxid. Med. Cell. Longev. 2012:324256.
Guglielmotto M, Giliberto L, Tamagno E, Tabaton M (2010). Oxidative stress mediates the pathogenic effect of different Alzheimer´s disease risk factors. Frontiers Aging Neurosci. 2:1-8.
Hermes-Lima M, Carreiro C, Moreira DC, Polcheira C, Machado DP, Campos EG (2012). Glutathione status and antioxidant enzymes in a crocodilian species from the swamps of the Brazilian Pantanal. Comp. Biohcem. Physiol. A Mol. Integr. Physiol. 163:189-198.
Jomova K, Vondrakova D, Lawson M, Valko M (2010). Metals, oxidative stress and neurodegenerative disorders. Mol. Cell. Biochem. 345:91-104.
Kassa J (2002). Review of oximes in the antidotal treatment of poisoning by organophoshorus nerve agents. J. Toxicol. Clin. Toxicol. 40:803-816.
Kennedy G, Spence VA, McLarren M, Hill A, Underwood C, Belch JJF (2005). Oxidative stress levels are raised in chronic fatique syndrome and are associated with clinical symptoms. Free Radic. Biol. Med. 39:584-589.
Ligtenstein DA, Kossen SP (1983). Kinetic profile in blood and brain of the cholinesterase reactivating oxime HI-6 after intravenous administration to the rat. Toxicol. Appl. Pharmacol. 71:177-183.
Marrs TC (1993). Organophosphate poisoning. Pharmacol. Ther. 58:51-66.
Mercey G, Verdelet T, Renou J, Kliachyna M, Baati R, Nachon F, Jean L, Rehard PY (2012). Reactivators of acetylcholinesterase inhibited by organophoshorus nerve agents. Acc. Chem. Res. 45:756-766.
Micke O, Schomburg L, Buentzel J, Kisters K, Muecke R (2009). Selenium in oncology: from chemistry to clinics. Molecules 14:3975-3988.
Petroianu GA, Lorke DE, Kalasz H (2012). Comparison of the ability of pyridinium aldoximes to reactivate human red blood cell acetylcholinesterase inhibited by ethyl- and methyl-paraoxon. Curr Organ. Chem. 16:1359-1369.
Pohanka M (2011). Cholinesterases, a target of pharmacology and toxicology. Biomed. Pap. Fac. Univ. Palacky Olomouc Czech Repub. 155:219-230.
Pohanka M, Novotny L, Fusek J, Pikula J (2011). HI-6 and obidoxime implication in oxidative stress, antioxidants level and apoptosis. Afr. J. Pharm. Pharmacol. 5:1145-1149.
Ramond A, Godin-Ribuot D, Totoson P, Koritschneva I, Cachot S, Levy P, Joyeux-Faure M (2011). Oxidative stress mediates cardiac infarction aggravation induced by intermittent hypoxia. Fundam. Clin. Pharmaco. 27(3):252-61.
Rana SV, Allen T, Singh R (2002). Inevitable glutathione, then and now. Indian J. Exp. Biol. 40:706-716.
Valachova K, Hrabarova E, Drafi F, Juranek I, Bauerova K, Priesolova E, Nagy M, Soltes L (2010). Ascorbate and Cu(II)-induces oxidative degradation of high-molar-mass hyaluronan. Pro and antioxidative effects of some thiols. Neuro. Endocrinol. Lett. 31:101-104.
Valko M, Morris H, Cronin MTD (2005). Metals, toxicity and oxidative stress. Curr. Med. Chem. 12:1161-1208.
Yiu W, Mead PA, Mansfeld BC, Chou JY (2010). Oxidative stress mediates nephropathy in type Ia glycogen storage disease. Lab. Invest. 90:620-629.

academicJournals

Vol. 7(31), pp. 2258-2263, 22 August, 2013
DOI 10.5897/AJPP12.479
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Anti-*Plasmodium falciparum* activity of *Aloe dawei* and *Justicia betonica*

**Godfrey S. Bbosa[1]*, David B. Kyegombe[3], Aloysius Lubega[1], Nathan Musisi[2], Jasper Ogwal-Okeng[1] and Olwa Odyek[4]**

[1]Department of Pharmacology and Therapeutics, Makerere University College of Health Sciences, Kampala, Uganda.
[2]Department of Veterinary Microbiology and Parasitology, Makerere University, College of Veterinary Medicine, Animal Resources and Biosecurity, Kampala, Uganda.
[3]Department of Pharmacology and Toxicology, Faculty of Medicine, Ishaka Campus, Kampala International University, Uganda.
[4]Department of Chemistry, Faculty of Science, Gulu University, Uganda.

Accepted 5 August, 2013

**Malaria is a fatal disease caused by different *Plasmodium* species of parasites and has remained the major killer of humans worldwide especially the children under five years of age and pregnant women. In this study, the anti-Plasmodia activities of the crude leaf ether extracts of *Aloe dawei* (AD) and *Justicia betonica* (JB) on *Plasmodium falciparum* were investigated, with chloroquine diphosphate as a positive control. The results showed that ether extracts of JB had $EC_{50}$ of 13.36 (95% CI: 8.032 to 22.23) µg/ml and AD had 7.965 (95% CI: 3.557 to 17.84) µg/ml. The chloroquine diphosphate had $EC_{50}$ of 24.86 (95% CI: 9.239 to 66.89) µg/ml. The qualitative phytochemical analysis of the ether extract showed that JB contains steroids and triterpenoids, alkaloids and saponins while AD contained steroids and triterpenoids, anthraquinolones, alkaloids and saponins. The results provides evidence that JB and AD contain compounds with anti-*P. falciparum* activity and hence their use by the traditional herbalist and local communities in treatment of malaria.**

**Key words:** Anti-*Plasmodium falciparum,* activity, $EC_{50}$, *Aloe dawei, Justicia betonica.*

## INTRODUCTION

Malaria is a life-threatening parasitic disease caused by various protozoan species of *Plasmodia* organisms including *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum*; with *P. falciparum* being the most deadly parasite accounting for most malaria cases in humans worldwide (World Health Organization (WHO), 2012; Kaiser Family Foundation (KFF), 2013). Globally, according to the latest WHO estimates, it is reported that malaria accounted for about 219 million cases with more than 660,000 death in 2010, and in sub-Saharan Africa, it accounted for 174 million (81%) of the total malaria cases with 596,000 (90%) death and mostly the vulnerable groups like children under the age of five years contributing to 85% and pregnant mothers (WHO, 2012; KFF, 2013). Malaria is reported to cost US$ 12 billion annually in Africa and in some African countries, it may cause up to 1.3% growth reduction per year as compared to countries without malaria (WHO, 2012; KFF, 2013).

In Uganda, malaria ranks among the third top killer diseases in the country (MCP, 2010; UMoH, 2010; UMoH and UNICEF, 2010). It is endemic in 95% of Uganda and it accounts for 40% of Ugandan public health expenditure (MCP, 2010; WHO, 2012). Malaria remains the major public health problem in the country with annual estimates of 10 million cases and 70,000 to 110,000

*Corresponding author. E-mail: godfossa@yahoo.com. Tel: +256 702 977587.

annual deaths of which 91% are children below 5 years of age, and annually it accounts for approximately 30 to 50% of outpatient care, 15 to 50% of hospital admissions and 9 to 14% of inpatient deaths (Nankabirwa et al., 2009; UMoH, 2010; UMoH and UNICEF, 2010; WHO, 2012). However, due to the poor and fake antimalarials drugs (Newton et al., 2010; Nayyar et al., 2012; Björkman-Nyqvist et al., 2013), on the market together with the high cost of the available effective antimalarial drugs, many people especially the local communities and traditional herbalist in rural areas use medicinal plants to treat malaria cases (Elujoba, 2005; Pierre et al., 2011; Stangeland et al., 2011).

Traditional herbal medicines have been used for thousands of years to treat malaria worldwide, and about 1,277 medicinal plants from 160 families with antimalarial activities have been reported (Willcox and Bodeker, 2004; Karou et al., 2011; Stangeland et al., 2011; WHO and MSH, 2012). The two modern antimalarial drugs (Artemisins and Quinine) in use currently are derived from herbal medicines (Brunton et al., 2013). It is also reported that the proportion of people who use herbal medicines to treat malaria worldwide rages from 0 to 75%, with an overall average of 20% and this is because they are cheap and easily accessible to most rural people in various countries (Willcox and Bodeker, 2004; WHO and MSH, 2012). Many medicinal plants such as *Justicia betonica* and *Aloes* species have been reported to have anti-malarial activities and are used locally by the traditional herbalist and local communities in various part of Uganda to treat malaria (CITES, 2003; Lamorde et al., 2008; Lubia et al., 2008; Rutebemberwa et al., 2009; Stangeland et al., 2011; Guianas, 2013). The *J. betonica* (Kanchanapoom et al., 2004; Subbaraju et al., 2004) and *Aloes* species (CITES, 2003; Lubia et al., 2008; Karou et al., 2011; Guianas, 2013) have been reported to have various compounds with medicinal properties. However, the efficacy and safety of these medicinal plants is not known and this is coupled with the lack of standard dosages to be administered to malarial patients. This study therefore investigated the anti-*P. falciparum* activity of ether extract of *A. dawei* (AD) and *J. betonica* (JB) medicinal herbs that are locally used by various communities in Uganda to treat various conditions like malaria.

## MATERIALS AND METHODS

An experimental study design was used to determine the activities of the ether leaf extracts of *A. dawei* and *J. betonica* on the *P. falciparum* parasites. These medicinal herbs have been reported to be used in various communities of Uganda to treat malaria (Lamorde et al., 2008; Lubia et al., 2008; Rutebemberwa et al. 2009; Stangeland et al., 2011).

### Processing and extraction

The *A. dawei* (AD) and *J. betonica* (JB) were collected from Wakiso district in Central Uganda. Botanical identification was carried out at the Makerere University herbarium. The leaves of both medicinal herbs were cleaned using water and then were dried in a shade until constant weight was obtained, in the Department of Pharmacology and Therapeutics laboratory, Makerere University College of Health Sciences. The dry leaves were then crushed by pounding in a wooden mortar into a fine powder. A total of 200 g of each medicinal herb powder was soaked in 500 ml of ether in Erlenmeyer flasks for 48 h. The mixture was filtered using a Whatman No.1 filter paper in a Buchner funnel. The filtrate was collected in a conical flask. The dry ether extract for both plants were obtained from the filtrate using a Heidolph model rotary evaporator (BUCHI Rotavapor R-205 model) and to obtain a complete dry ether extract, it was exposed at room temperature in dark sample bottles to prevent direct exposure to light that would cause oxidation of the compounds in the extracts for 24 h in order to allow complete evaporation of the ether solvent.

### Qualitative phytochemical analysis of the ether extracts of *J. betonica* and *A. daweii*

The phytochemical compound in the ether extracts were determined using the following methods (Sofowora, 1993; Trease and Evans, 2002; Usman et al., 2009; Damodaran and Manohar, 2012).

#### *Test for tannins*

About 0.5 g each portion was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate. Occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins (Trease and Evans, 2002; Usman et al., 2009; Damodaran and Manohar, 2012).

#### *Test for anthraquinones*

A few drops of 2% HCl were added to 1 ml of the extract. Appearance of the red color precipitate indicated the presence of anthraquinones (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

#### *Liebermann-Burchard test for steroids*

To 0.2 g of each portion, 2 ml of acetic acid was added; the solution was cooled well in ice followed by the addition of conc. $H_2SO_4$ carefully. Colour development from violet to blue or bluish-green indicated the presence of a steroidal ring that is, aglycone portion of cardiac glycoside (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

#### *Test for triterpenoids*

A little of each portion was dissolved in ethanol. To it, 1 ml of acetic anhydride was added followed by the addition of conc. $H_2SO_4$. A change in color from pink to violet showed the presence of triterpenoids (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

#### *Test for terpenoids*

A little of each portion was dissolved in ethanol. To it 1 ml of acetic

anhydride was added followed by the addition of conc. $H_2SO_4$. A change in colour from pink to violet showed the presence of terpenoids (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

### *Test for saponins*

One gram of each portion was boiled with 5 ml of distilled water, filtered. To the filtrate, about 3 ml of distilled water was further added and shaken vigorously for about 5 min. Frothing which persisted on warming was taken as an evidence for the presence of saponins (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

### *Sodium hydroxide test for flavonoids*

Few quantity of the each portion was dissolved in water and filtered; to this 2 ml of the 10% aqueous sodium hydroxide was later added to produce a yellow coloration. A change in color from yellow to colorless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids (Trease and Evans, 2002; Usman et al., 2009; Damodaran and Manohar, 2012).

### *Test for alkaloids*

Few quantity of the each portion was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 1 ml was taken individually into 2 test tubes. To the first portion, few drops of Dragendorff's reagent were added; occurrence of orange-red precipitate was taken as positive. To the second, 1 ml Mayer's reagent was added and appearance of buff-colored precipitate will be an indication for the presence of alkaloids (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

### *Fehling's test for free reducing sugar*

About 0.5 g each portion was dissolved in distilled water and filtered. The filtrate was heated with 5 ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars (Sofowora, 1993; Usman et al., 2009; Damodaran and Manohar, 2012).

## Anti-*P. falciparum* activity study

The anti-*P. falciparum* activity of the JB and AD ether extracts was evaluated at the department of Microbiology, Makerere University, College of Veterinary Medicine, Animal Resources and Biosecurity, using standard methods (Moll et al., 2008; Kalra et al., 2006; WHO, 2001; Trager and Jensen, 1976).

### *Preparation of culture media and the P. falciparum organisms*

The culture medium was prepared by dissolving 10.4 g of powdered Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich Chemical Company, Munich, Germany) and 5.94 g of HEPES (N-2 hydroxyethyl piperazine-N-2-ethane sulphonic acid) (Sigma-Aldrich Chemical Company, Munich, Germany) in 1 L of sterile distilled water. The medium was filtered using 0.22 mm millipore filter, stored at 4°C ready to be used. The wash medium was prepared by adding 1.6 ml of 7.5 % (w/v) of $NaHCO_3$ (Sigma-Aldrich Chemical Company, Munich, Germany) to 43.4 ml of the medium above (RPMI 1640 with HEPES) in 50 ml centrifuge tubes for use (Moll et al., 2008; Omoregie and Sisodia, 2012; Shujatullah et al., 2012). The wild *P. falciparum* organisms were obtained from the isolated parasites in blood samples with mono-infection in the Microbiology Laboratory which were obtained during the screening of patients with uncomplicated malaria after consent in Mulago hospital prior to malaria treatment. The *P. falciparum* organisms were cultured with the freshly prepared culture medium (Moll et al., 2008; Omoregie and Sisodia, 2012; Shujatullah et al., 2012).

The *in vitro* cultivation of *P. falciparum* isolates followed a modification of the standard culture techniques (Trager and Jensen, 1976). The culture medium consisted of RPMI 1640 (Sigma Aldrich), 2 g glucose, and 40 µg/ml gentamycin sulphate with supplemented 10% $AB^+$ serum. Culture medium was sterilised by filtration through a Millipore filter of 0.22 µm porosity and pH was adjusted to 7.4 by the addition of 4.2 ml of sterile 5% sodium bicarbonate. The plate was put in a candle jar and placed in the incubator set at 37.5°C for 24 to 30 h, depending upon development stage of the parasite. After 24 h incubation, a thin smear was prepared from the control well to see the mature schizonts, and if more than 10% schizonts were seen, it was considered to be valid; thick smears were prepared from each well by discarding the excess media with a micropipette (Moll et al., 2008; Omoregie and Sisodia, 2012; Shujatullah et al., 2012).

### *In vitro bioassay*

The *in vitro* Micro-Test (MARK III) kit was used to determine the anti-*P. falciparum* activity of JB and AD ether leaf extracts (Moll et al., 2008; Omoregie and Sisodia, 2012; Shujatullah et al., 2012). The AD and JB ether extracts were each dissolved in a 0.2 ml of dimethylsulfoxide (DMSO) to facilitate the dissolution and topped up with distilled water to give a stock solution of concentration of 100 mg/ml. The chloroquine diphosphate was obtained from Sigma-Aldrich Chemical Company, Munich, Germany and was dissolved in 10% ethanol to a concentration of 1 mg/ml and diluted subsequently with culture medium to achieve the required concentrations required for the growth of the parasites. The preparation of ether and chloroquine diphosphate concentrations and the design of the test plates were based on the standard methods (Moll et al., 2008; Omoregie and Sisodia, 2012; Shujatullah et al., 2012). Standard drug (chloroquine) and extracts (at different concentrations of 1, 5, 10, 50, 100, 500, and 1000 µg/ml) were prepared in distilled water (chloroquine; Sigma) and DMSO (test extracts) and then serially diluted to achieve the required concentrations. All the culture plates with parasites and the ether extracts and chloroquine diphosphate were incubated at 37°C in a candle jar (5% $CO_2$, 17% $O_2$, 78% $N_2$) according to the method of Trager and Jensen (1976), for 18 to 48 h depending on the time taken by the parasite to develop to schizonts (Moll et al., 2008; Omoregie and Sisodia, 2012; Shujatullah et al., 2012). The synchronized culture with parasitaemia of 1.5 and 3% haematocrit were incubated in 96-well microtitre plate containing multiple concentrations of compounds/extracts for 48 h at 37°C in candle jar. Blood smears from each well were fixed in methanol, stained with Giemsa's stain and the numbers of infected red blood cells (RBCs) per 200 cells were counted. The parasite density was estimated as the number of mature schizonts per 200 white blood cells (WBC) from which the inhibitory concentrations of 50% of *P. falciparum* schizonts ($EC_{50}$) were determined using a log dose-response curve.

### Determination of in *vitro* effective concentration ($EC_{50}$) values of the ether extract of JB and AD

The effective concentration ($EC_{50}$) defined as the drug concentration

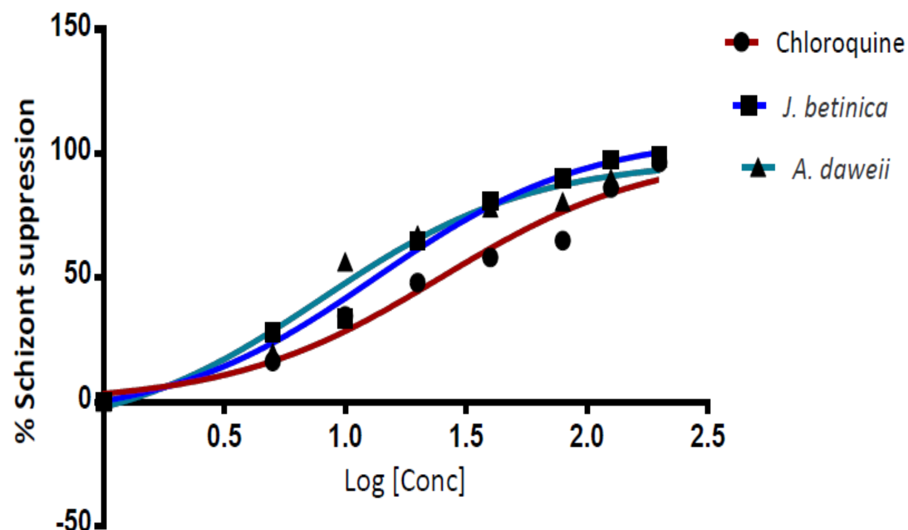


**Figure 1.** Mean percentage schizonts suppression per 200 WBC against log concentration of the ether extracts of *Justicia betonica* and *Aloe dawei*

concentration required to suppress 50% of schizonts development were determined. The anti-malarial activities of the test extracts were expressed as 50% effective concentration ($EC_{50}$) determined from dose-response curve by non-linear regression analysis (curve-fit) using Graph Pad Prism (version 6) software at 95% confidence intervals (CI). All experiments were performed in triplicates and the results were expressed as percentage of *anti-Plasmodial* schizonts suppression. Crude extracts with $EC_{50}$ values > 50 µg/ml were considered to be inactive (Kraft et al., 2003).

## RESULTS

### Anti-*P. falciparum* activity

The results of the anti-*P. falciparum* activity of JB and AD ether leaf extracts using the chloroquine diphosphate as control, showed that these extracts had anti-Plasmodia activity with the 50% schizonts suppression per 200 white blood cell (WBC) ($EC_{50}$) values of 13.36 (95% CI: 9.03 to 22.24) µg/ml, 7.97 (95% CI: 3.56 to 17.85) µg/ml and 24.86 (95% CI: 9.24 to 66.9) µg/ml, respectively (Table 1 and Figure 1). The qualitative phytochemical analysis showed that JB ether extract contained various compounds including the steroids and triterpenoids, alkaloids and saponins while the AD ether extract had steroids and triterpenoids, anthraquinones, alkaloids and saponins (Table 2).

## DISCUSSION

The observed anti-*P. falciparum* activity of the ether extracts of JB and AD leaves with $EC_{50}$ values of 13.36 (95% CI: 9.03 to 22.24) µg/ml and 7.97 (95% CI: 3.56 to 17.85) µg/ml, respectively could be attributed to the presence of the alkaloids, anthraquinolones, steroids and triterpenoids and saponins compounds in both the medicinal herbs. Previous studies have shown that JB and AD contain similar compounds that could be responsible for the anti-*P. falciparum* activity (Kanchanapoom et al., 2004; Subbaraju et al., 2004). Also some of the current antimalarial drugs used in the management and treatment of malaria globally are plant alkaloids derived from medicinal plants such as quinine from *Cinchona* tree of plants and the sesquiterpenes lactone endoperoxides from artemisinins from *Artemisia annua* or sweet wormwood (Willcox and Bodeker, 2004; Brunton et al., 2013). Many medicinal herbs have been reported globally to have anti-malarial activities and most of them have been reported to contain various compounds especially the alkaloids (Stangeland et al., 2011; WHO and MSH, 2012). These herbs have been used traditionally to treat malaria for many years worldwide and currently about 1,277 medicinal herbs have been reported to have *anti-Plasmodia* activities (Willcox and Bodeker, 2004; Karou et al., 2011; Stangeland et al., 2011; WHO and MSH, 2012). The observed low $EC_{50}$ value of AD as compared to the JB could possibly be due to the presence of the high concentrations of alkaloids, steroids and triterpenoids, saponins and anthraquinolones in the herb.

However, since the compounds in the extracts were used in the crude form, their $EC_{50}$ values were not comparable to the $EC_{50}$ values of the chloroquine diphosphate which was in pure form. But also, the $EC_{50}$ value of the chloroquine diphosphate in this study was slightly higher than those from previous studies and this could be due to the fact the *P. falciparum* organisms used in the study, were wild type from clinical isolates and

**Table 1.** *Anti-Plasmodia falciparum* activity values with 50% schizonts suppression per 200 WBC ($EC_{50}$) of ether extracts of *Justicia betonica* and *Aloe dawei.*

| Medicinal herb | $EC_{50}$ (95% CI) µg/ml |
|---|---|
| *Justicia betonica (JB)* | 13.36 (8.032 - 22.23) |
| *Aloe dawei (AD)* | 7.965 (3.557 - 17.84) |
| *Chloroquine disphosphate* | 24.86 (9.239 - 66.89) |

**Table 2.** Different classes of compounds in ether extracts of *J. betonica* and *A. Dawei.*

| Medicinal herb | Various compounds in the ether extracts | | | | | | |
|---|---|---|---|---|---|---|---|
| | Steroids and triterpenoids | Anthraquinolone | Alkaloids | Reducing sugars | Tannins | Saponins | Flavonoids |
| JB | ++ | - | +++ | - | - | +++ | - |
| AD | ++ | +++ | +++ | - | - | +++ | - |

Key: +++ strongly present, + present, - absent, JB - *Justicia betonica*, AD - *Aloe daweii*

possibly these were resistant to chloroquine disphosphate thus requiring slightly a higher concentration to suppress the schizonts development in culture. The study therefore concludes that JB and AD medicinal herbs have compounds with anti-*Plasmodia* activity though their pharmacological mechanisms of action is not known and hence their use by the traditional herbalists and local communities in the management and treatment of malaria in Uganda is justified.

## Conclusion

The medicinal herbs *J. betonica* and *A.daweii* contain compounds with anti-*P. falciparum* activity and this could be the reason why they are used by traditional herbalists and local communities in Uganda to manage and treat malaria.

## ACKNOWLEDGEMENTS

Great thanks go to technical staff in the Department of Pharmacology and Therapeutics, Makerere University College of Health Sciences and Department of Microbiology and Parasitology, Makerere University College of Veterinary Medicines, Animal Resources and Biosecurity for their support in the study.

### REFERENCES

Björkman-Nyqvist M, Svensson J, Yanagizawa-Drott D (2013). "Can Good Products Drive Out Bad? Evidence from Local Markets for (Fake?) Antimalarial Medicine in Uganda." Havard University and Stockholm University School of Economics. http://www.hks.harvard.edu/fs/dyanagi/Research/FakeDrugs.pdf.

Brunton LL, Chabner BA, Knollmann BC (2013). "Chemotherapy of Malaria: Artemisinin and Derivatives; Quinolines and Related Compounds. Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th ed." Chapter 49. http://accessmedicine.com/content.aspx?aID=16676153 (Cited on 16th June 2013).

CITES (2003). "Review of Significant Trade East African Aloes (November 2003). PC14 Doc. 9.2.2 -Annex 4 " cites.org. PC14 Doc. 9.2.2: 93 - 108. http://www.cites.org/eng/com/pc/14/E-PC14-09-02-02-A4.pdf.

Damodaran A, Manohar S (2012). "Qualitative screening for phytochemicals of various solvent extracts of Cassia alata Linn. leaves." Herbal Technological Industry. 11-13. http://www.herbaltechindustry.com/QUALITATIVE%20jan_12.pdf.

Elujoba T (2005). "Traditional medicinal plants and Malaria: Book Reviews. " Afr. J. Trad. Compl. Altern. Med. 2(2):206-207.

Guianas (2013). "Medicinal Plants of the Guianas (Guyana, Surinam, French Guiana)." botany.si.edu. http://botany.si.edu/bdg/medicinal/MedPlantsGui1.pdf.

Kalra BS, Chawla S, Gupta P, Valecha N (2006). Screening of antimalarial drugs: An overview. Indian J. Pharmacol. 38:5-12.

Kanchanapoom T, Noiarsa P, Ruchirawat S, Kasai R, Otsuka H (2004). "Triterpenoidal glycosides from *Justicia betonica*. Phytochemistry 65(18):2613-2618.

Karou DS, Tchacondo T, Ilboudo PD, Simpore J (2011). "Sub-Saharan Rubiaceae: A review of their Traditional uses, Phytochemistry and Biological activities." Parkistan J. Biol. Sci. 14(3):149-169.

KFF (2013). "The Global Malaria Epidemic- Fact Sheet: US Global Health Policy." The Henry J. Kaiser Family Foundation. http://kaiserfamilyfoundation.files.wordpress.com/2012/10/7882-04-2.pdf.

Lamorde M, Tabuti JRS, Obua C, Kukunda-Byobona C, Lanyero H, Byakika-Kibwika P, Bbosa GS, Lubega A, Ogwal-Okeng J, Ryan M, Waako PJ, Merry C (2008). "Medicinal plants used by traditional medicine practitioners for the treatment of HIV/AIDS and related conditions in Uganda." J. Ethnopharmacol. 130(1):43-53.

Lubia IK, Kyalo SN, Mukonyi KW, Lusweti AM, Situma CA (2008). "Strategy for Conservation and Management of Commercial Aloes species in Kenya." Kenya Wildlife Service. http://www.hks.harvard.edu/fs/dyanagi/Research/FakeDrugs.pdf. http://www.kws.org/export/sites/kws/info/publications/strategies/Strategy_of_Conversation_Management_of_ALoe_BOOK_for_Web.pdf.

MCP (2010). "Malaria Control Programme (MCP) (2010). Uganda Malaria Control Strategy 2005/06 –2009/10." Malaria Control Programme (MCP), Uganda Ministry of Health. http://www.rollbackmalaria.org/countryaction/nsp/uganda.pdf.

Moll K, Ljungström I, Perlmann H, Scherf A, Wahlgren M (2008). "Methods in Malaria Research. MR4/ ATCC." Manassas, Virginia,

USA.http://www.biomalpar.org/updatedMethods_In_Malaria_Research_5thedition.pdf.

Nankabirwa J, Zurovac D, Njogu JN, Rwakimari JB, Counihan H, Snow RW, Tibenderana JK (2009). "Malaria misdiagnosis in Uganda – implications for policy change." Malar J. 8(66).

Nayyar GML, Breman JG, Newton PN, Herrington J (2012). "Poor-quality antimalarial drugs in southeast Asia and sub-Saharan Africa." The Lancet Infect. Dis. 12(6):488-496.

Newton PN, Green MD, Fernandez FM (2010). "Impact of poor-quality medicines in the 'developing' World." Trends Pharmacol. Sci. 31(3): 99-101.

Omoregie ES, Sisodia BS (2012). "*In vitro* antiplasmodial activity and cytotoxicity of leaf extracts of *Jatropha tanjorensis J. L. Ellis* and *Soroja."* Bayero J. Pure Appl. Sci. 5(1):90-97.

Pierre S, Toua V, Tchobsala, Tchuenguem FF, Njan NA, Messi J (2011). Medicinal plants used in traditional treatment of malaria in Cameroon. J. Ecol. Nat. Environ. 3(3):104-117.

Rutebemberwa E, Nsabagasani X, Pariyo G, Tomson G, Peterson S, Kallander K (2009). Use of drugs, perceived drug efficacy and preferred providers for febrile children: implications for home management of fever. Malar. J. 8(131).

Shujatullah F, Khan HM, Khatoon A, Khan PA, Ashfaq M (2012). *In vitro* Chloroquine Resistance in *Plasmodium falciparum* isolates from Tertiary Care Hospital.Malar. Res. Treat. 12(12):4.

Sofowora A (1993). Screening Plants for Bioactive Agents; Medicinal Plants and Traditional Medicinal in Africa, 2nd Ed. Spectrum Books Ltd. Sunshine House, Ibadan, Nigeria pp. 134-156.

Stangeland T, Alele PE, Khatoon A, Lye KA (2011). Plants used to treat malaria in Nyakayojo sub-county, western Uganda. J. Ethnopharmacol. 137(1):154-166.

Subbaraju GV, Kavitha J, Rajasekhar D, Jimenez JI (2004). Jusbetonin, the first indolo[3,2-b]quinoline alkaloid glycoside, from *Justicia betonica*. J. Nat. Prod. 67(3):461-462.

Trager W, Jensen JB (1976). Human malaria parasites in continuous culture. Science 193(4254):673-675.

Trease GE, Evans WC (2002). "Pharmacognosy." 15th Ed. Saunders Publishers, London pp. 42-44, 221-229, 246-249, 304-306, 331-332, 391-393.

UMoH (2010). "Health Sector Strategic Plan III 2010/11-2014/15." Uganda Ministry of Health (UMoH). http://www.health.go.ug/docs/HSSP_III_2010.pdf.

UMoH, UNICEF (2010). "Health Sector Strategic and Investment Plan: Promoting People's Health to Enhance Socio-economic Development 2010/11 – 2014/15." Uganda Ministry of Health (UMoH) and UNICEF. http://www.unicef.org/uganda/HSSIP_Final.pdf.

Usman H, Abdulrahman FI, Usman A (2009). Qualitative Phytochemical screening and *In vitro* Antimicrobial effects of methanol stem bark extract of *Ficus thonningii* (Moraceae). Afr. J. Tradit. Complement. Altern. Med. 6(3):289-295.

WHO (2001). *In vitro* Micro-Test (MARK III) for the Assessment of the response of Plasmodium falciparum to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and artemisinin. World Health Organization (WHO), Geneva, Switzerland. http://www.who.int/malaria/publications/atoz/markiii.pdf

WHO (2012). "World Malaria Report 2012: Fact Sheet." World Health Organization (WHO). http://www.who.int/malaria/publications/world_malaria_report_2012/wmr2012_factsheet.pdf.

WHO, MSH (2012). "Traditional and complementary medicine policy." World Health Organization (WHO) and Management Science for Health (MSH). Chapter 5:5.0-5.16.

Willcox ML, Bodeker G (2004). Traditional herbal medicines for malaria. BMJ 329(7475):1156-1159.

academicJournals

Vol. 7(31), pp. 2264-2272, 22 August, 2013
DOI 10.5897/AJPP12.1311
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Monotherapy versus combinations of nucleos(t)ide in treatment-naive hepatitis B decompensated cirrhotic patients: A nested case-control study

**Lei Li[1#], Wei Liu[2#], Yuhan Chen[1], Peng Li[1],Feili Wei[3], Chunlei Fan[1], Peiling Dong[1], Bing Li[1], Dexi Chen[3] and Huiguo Ding[1]***

[1]Department of Gastroenterology and Hepatology, Beijing You'an Hospital Affiliated with Capital Medical University, Beijing 100069, China.

[2]Department of Internal Medicine, Beijing Ji Shui Tan Hospital Affiliated with Peking University, Beijing, Beijing 100035, China.

[3]Viral Laboratory of Liver Diseases Research Institute, Capital Medical University, Beijing 100069, China.

Accepted 3 January, 2013

**The data are limited on the long-term clincial outcome of monotherapy versus combinations of nucleos(t)ide analog (NUCs) for hepatitis B related decompensated cirrhotic patients. This study was to evaluate the efficacy in treatment-naive patients using NUCs monotherapy or combinations. Three hundred and six patients with decompensated hepatitis B cirrhosis were selected from cirrhosis cohort and divided into treatment-naive (n = 260) and control groups (n = 46). Antiviral therapies included monotherapy of lamivudine (LAM, n = 39), adefovir (ADV, n = 73), telbivudine (LDT, n = 36), entecavir (ETV, n = 48), and combinations of LAM+ADV (n = 39) or LDT+ADV (n = 25). Of these patients, 193 in antiviral therapy and 39 in control group were included for analysis over two years. The cumulative drug-resistance rate at two year was higher in the LAM (37.9%), ADV (21.2%), LDT (23.3%) than in the ETV monotherapy (2.6%), and with combinations of LAM+ADV (8.7%) or LDT+ADV (6.3%), respectively, $P < 0.001$. Serum hepatitis B virus (HBV) DNA undetectability in the ETV and the LDT+ADV group was higher than in the LAM, ADV and LAM+ADV group at over two years ($P < 0.05$). The child-pugh score (CPs) in the antiviral therapy group was decreased at two years ($P < 0.05$). In the control group and drug-resistant patients, however, CPs was increased. The two years cumulative incidence of liver failure in the antiviral therapy group was significantly less than the control group (OR 24.9, 95%; CI 6.5 to 94.7, $P = 0.001$). The total cumulative survival rate in the antiviral therapy group was higher than in control group (OR 4.2, 95%; CI 1.4 to 12.9, $P = 0.017$). The combinations of NUCs therapy and ETV momotherapy are optimum management for hepatitis B related decompensated cirrhotic patients.**

**Key words:** Hepatitis B, decompensated cirrhosis, nucleos(t)ide analogs, hepatocellular carcinoma, drug resistance.

## INTRODUCTION

Chronic hepatitis B virus (HBV) infections, the main etiology of liver cirrhosis and hepatocellular carcinoma

*Corresponding author. E-mail: dinghuiguo@medmail.com.cn. Tel: 8610-83997155. Fax: 8610-63295525.
#These authors contributed equally to this work.

(HCC) remain a major public health problem worldwide, especially in China (Lok and McMahon, 2009; Liaw et al., 2008; Tanaka et al., 2011). After infection with HBV, the cumulative 5-year incidence of liver cirrhosis is 8 to 20%; among these cases, the annual incidence of HCC is 2 to 5% (Tanaka et al., 2011; Tan, 2011; Asia-Pacific Working Party on Prevention of Hepatocellular Carcinoma, 2010). The most effective methods to preventing HCC is to control HBV infection through vaccination (Asia-Pacific Working Party on Prevention of Hepatocellular Carcinoma, 2010; Lim et al., 2009). In patients already infected with HBV, antiviral therapy remains the best strategy to prevent liver cirrhosis and HCC (Lim et al., 2009; Chan et al., 2012; Kwon and Lok, 2011; Kim et al., 2011). Major progress in the treatment of chronic hepatitis B has recently been made during the last decade with the development of antiviral drugs, especially nucleos(t)ide analogs (NUCs) (Fung et al., 2011; Liaw et al., 2011; Zhang et al., 2011). Some data supporting the benefit of antiviral therapy on the prevention of HCC in chronic hepatitis B patients has been shown in a few randomized controlled trials (Zhang et al., 2011; Jin et al., 2011; Lim et al., 2011; Tujios and Lee, 2012). Nonetheless, antiviral drug resistance is important in determining the success of long-term therapy for chronic hepatitis B patients (Yeh et al., 2011; Papatheodoridis et al., 2010).

In recent clinical study data, the development of resistance to NUCs is associated with exacerbation of liver disease, including development of cirrhosis and HCC (Yeh et al., 2011), in addition, the risk of HCC remains high in HBV-related cirrhosis in patients who are treatment-naïve using NUCs (Papatheodoridis et al., 2010). Decompensated cirrhosis is an end-stage characterized by high mortility and extremely high risk of HCC. In HBV-related decompensated cirrhosis patients, antiviral therapy using NUCs is recommended by global guidelines from 2005 (Liaw et al., 2008; Chinese Medical Association, 2005; Jhaveri and Murray, 2007; European Association For The Study of The Liver (EASL), 2009). However, antiviral therapy for decompensated cirrhosis patients is still a problem for clinicians because of the lack of clinical evidence-based data. In recent clinical studies (Liaw et al., 2011), ETV and LAM had similar effects on one-year mortality. However, more patients taking ETV tended to attain ALT normalization, HBV-DNA undetectability, and reduction of the model for end-stage liver disease scores, with no drug resistance. Therefore, ETV was recommended as the first-line monotherapy for patients with HBV-related decompensated cirrhosis because of low drug-resistance (Tujios and Lee, 2012; Jhaveri and Murray, 2007; Singal and Fontana, 2001). However, data are limited on the long-term safety, effectiveness and HCC incidence of ETV monotherapy versus combinations of NUCs in HBV-related decompensated cirrhotic patients. Therefore, the aim of this study was to evaluate the long-term outcome in patients with HBV-related decompensated cirrhosis treatment-naive using NUCs in real-life clinical practice.

## MATERIALS AND METHODS

### Patients

The clinical records of 306 patients from hepatitis B-related decompensated cirrhosis cohort from January, 2008 to December, 2011 were enrolled. The clinical parameters of gender, age, presence of liver cirrhosis with portal hypertnsion, alcohol abuse, and HCC family history were recorded. All patients were positive for hepatitis B surface antigen (HBsAg). No patient had detectable liver tumors (except hemangiomas and cirrhotic regenerative nodules) prior to entry into this cohort study. A diagnostic workup of decompensated liver cirrhosis was performed, including a clinical manifestation, physical examination, and laboratory tests according to the criteria suggested by the Chinese Medical Association in 2005 for liver diseases (Chinese Medical Association, 2005). These included (1) chronic hepatitis B history and/or signs; (2) abnormal liver function accompanied by portal hypertension, such as hepatic encephalopathy, ascites or variceal bleeding with child-pugh scores (CPs) score ≥ 7; (3) B-ultrasound scanning (LOGIQ9, GE Company, USA) and CT (GE HISPEED DXI, GE company, USA) consistent with the signs of liver cirrhosis without images of liver cancer; and (4) no NUCs medicine taken prior to study entry. No patients met the exclusion criteria of (1) any co-infection, such as hepatitis A virus, hepatitis C virus, hepatitis E virus, hepatitis D virus, Epstein-Barr virus, cytomegalovirus and human immunodeficiency virus (HIV) or bacterial infections; (2) long-term use of liver toxicity drug; (3) HCC or metastatic liver cancer; (4) poor compliance and uncontrolled serious cardio-vascular, respiratory, digestive and nervous system diseases; (5) pregnant or lactating. (6) CPs ≥ 12. Alcohol abuse was defined as in this study (1) alcohol abuse more than 5 years; or (2) drinking equivalent to ethanol ≥ 40 g/d for men or ≥ 20 g/d for women, or heavy drinking in recent 2 weeks equivalent to ethanol≥80 g/d.

The study protocol was approved by the Ethical Committee of Beijing YouAn Hospital, Capital Medical University. Investigators explained the study in detail to all patients and/or their relatives. Consent forms were obtained from all participants when they were recruited.

### Study endpoints

Endpoints of these patients were death, liver transplantion, loss follow-up, and or HCC diagnosed according to the criteria suggested by the Chinese Anticancer Association (2001). The diagnostic criteria of HCC were (1) serum AFP > 400 ng/ml and B-ultrasound and CT positive findings; or (2) serum AFP < 400 ng/ml and B-ultrasound and CT scanning positive findings or echoguided liver biopsy pathology positive findings. Serum AFP was tested by electrochemiluminescence (Abbott Ltd, USA).

### Antiviral therapies

In HBV-related decompensated cirrhotic patients of this cohort, antiviral therapies were included monotherapy of lamivudine (LAM) 100 mg/d, adefovir (ADV)10 mg/d, telbivudine (LDT) 600 mg/d, or entecavir (ETV) 0.5 mg/d; or combinations of LAM 100 mg/d and ADV 10 mg/d (LAM+ADV), or LDT 600 mg/d and ADV 10 mg/d (LDT+ADV) according to the clinican real-life practices. We divided 306 patients into an antiviral therapy group (n = 260) and a control group (n = 46). The antiviral therapies were LAM (n = 39), ADV (n = 73), LDT (n = 36), ETV (n = 48), LAM+ADV (n = 39), and LDT+ADV (n = 25). Patients were followed up every 3 months and virology, biochemical and clinical parameters were obtained. Of these patients, 74 were excluded because of less than 24 months of follow-up; thus, 193 patients in the antiviral therapy group and 39 in

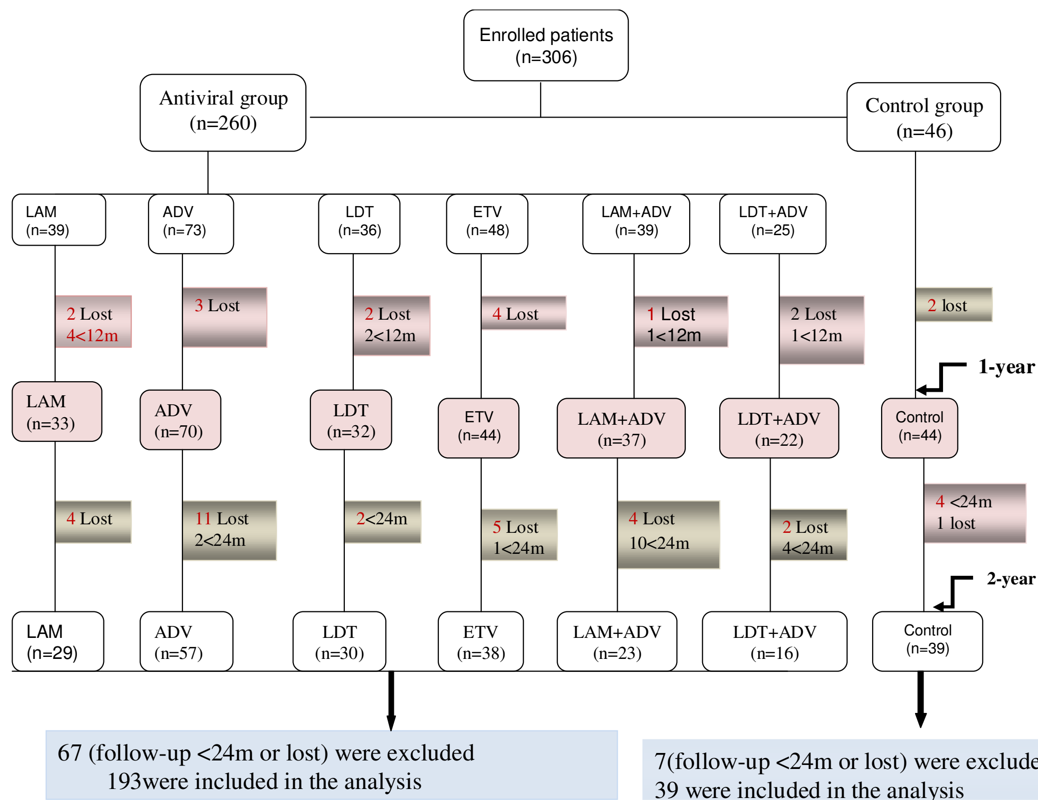


**Figure 1**. A flow chart of enrolled patients.

the control group were included for analysis (Figure 1). Antiviral therapies patients were subdivided into a drug-resistant group (n = 34) and complete virological response (CVR) group (n = 159) according to whether drug-resistance before they reached endpoints. All patients also received individual supporting treatment to prevent complications, for example, correcting water and electrolyte balance; infusion of albumin, plasma, or antibiotics for bacterial infections.

### Assessment of liver and renal function

Parameters of liver and renal biochemical profiles, such as alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), albumin, blood urea nitrogen (BUN), creatinine level (Cr), and creatine kinase (CK) were tested with an Olympus automatic biochemical analyzer (Olympus AU640, Japan). Prothrombin time (PT) and prothrombin activity (PTA) were measured by blood coagulation analyzer (AcL Top, Beckman Coulter, USA). Child-Pugh score (CPs) was calculated according to the parameters (Committee of Liver Cancer, Chinese Anti-cancer Association, 2001). Liver failure was diagnosed including a clinical manifestation, physical examination, and laboratory tests according to the criteria suggested by the Chinese Medical Association for Liver Diseases (Chinese Medical Association, 2005).

### Virology assay

Serum hepatitis B markers were detected by electro-chemiluminescence immunoassay using a Roche E170 modular immunoassay analyzer following the manufacturer's protocols (Roche Diagnostics, Germany). The serum HBV-DNA was quantified by real-time polymerase chain reaction (PCR) (FQ-PCR Kit, DaAn Gene Co., China) using a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, USA). The lower limit of HBV DNA detection was 500 copies/ml. The CVR was defined as the HBV-DNA undetectability and ALT normalization in 24 weeks after antiviral therapies. The HBV DNA-negative (undetectability) was defined as serum HBV DNA < 500 copies/ml twice for two consecutive months.

### Antiviral resistance

Antiviral resistance was defined as the conversion of HBV DNA to a positive, namely virologic rebound or the detection by sequence analysis of mutations know to be related to drug resistance during NUCs treatment. Rescue treatment was performed if antiviral resistance or serum HBV DNA was sustained as positive over the course of 24 weeks of therapy according to guidelines (Chinese Medical Association, 2005; European Association For The Study of The Liver (EASL), 2009).

### Detection of HBV polymerase sequence

Serum HBV DNA was extracted according to the instructions of a commercial kit (Qiagen Blood Kit, USA), using Platinum Taq DNA polymerase high fidelity (Invitrogen, USA) for nested PCR amplification. The first round of the primer sequences were: P54: 5´-TYCCTGCTGGTGGCT CCAGTTC-3´ (nt54-75), P1287: 5-´ CATACTGCGGAACTCCTAGCG-3´ (nt1267-1287). The second round of primer sequences were: P253: 5

**Table 1**. Clinical characterizations of enrolled patients at baseline.

| Group | Gender (M/F) | Age (year) | FHH (%) | AA (%) | CPs | qHBVDNA (log) | HBeAg(+) (%) |
|---|---|---|---|---|---|---|---|
| LAM (n=29) | 20/9 | 50.5±10.1 | 20.6 | 13.7 | 8.3±1.7 | 4.9±1.8 | 65.5 |
| ADV (n=57) | 41/16 | 48.7±10.2 | 19.3 | 12.3 | 8.1±2.4 | 4.7±1.8 | 66.7 |
| LDT (n=30) | 19/11 | 52.5±13.7 | 16.7 | 10.0 | 8.0±2.5 | 4.6±2.0 | 56.7 |
| ETV (n=38) | 29/9 | 54.7±11.8 | 23.7 | 13.1 | 7.8±1.9 | 5.1±1.9 | 60.5 |
| LAM+ADV (n=23) | 15/8 | 54.9±13.6 | 21.7 | 13.0 | 8.6±2.3 | 5.0±1.8 | 65.2 |
| LDT+ADV (n=16) | 11/5 | 51.7±12.6 | 18.7 | 12.5 | 8.3±2.5 | 4.8±1.6 | 68.7 |
| Control (n=39) | 24/15 | 52.2±10.8 | 17.9 | 10.2 | 7.8±2.0 | 4.9±1.7 | 64.5 |

FHH: family history of hepatitisB, AA:alcohol abuse, qHBVDNA:quantified HBV-DNA, CPs: Child-Pugh score

**Table 2.** Summary of the frequency of actites , hospitalization ,CPs in the course of antiviral therapies over 2 years.

| Group | CPs at baseline | CPs at 2-year | Frequency of ascites per year (time) | Frequency of hospitalization per year (time) |
|---|---|---|---|---|
| LAM (n=29) | 8.5±1.9 | 7.0±2.3* | 1.8±0.9 | 1.8±1.1 |
| ADV (n=57) | 8.0±2.1 | 6.1±1.7* | 1.7±1.2 | 1.8±1.2 |
| LDT (n=30) | 8.3±2.2 | 6.2±1.9* | 1.8±0.8 | 1.8±1.2 |
| ETV (n=38) | 8.5±2.0 | 6.7±1.6* | 1.5±1.3 | 1.6±0.6 |
| LAM+ADV (n=23) | 8.7±2.4 | 6.4±1.5* | 1.6±0.8 | 1.8±1.1 |
| LDT+ADV (n=16) | 8.4±2.2 | 6.2±1.6* | 1.4±0.6 | 1.3±1.0** |
| DR (n=34) | 8.4±2.5 | 9.0±2.7 | 1.98±1.0 | 2.42±0.9 |
| CVR (n=159) | 8.6±1.9 | 6.0±1.6** | 1.16±0.8** | 0.63±0.4** |
| Control (n=39) | 7.9±2.1 | 9.2±2.6 | 2.1±1.1 | 2.6±1.7 |

DR: Drug resistance, CVR: complete virological response ,CPs: Child-Pugh score *compared with baseline, P < 0.05; ** compared with control, P<0.05.

-´CTCGTGGTGGACTTCTCTC-3´ (nt253-271), P1000: 5-´GCAAANCCCM AAA GRCCCAC-3´ (nt1000-1019). Primers were synthesized by the Yingjun Biotechnology Company (Shanghai, China). PCR products were sequenced by an ABI 3730XL sequencer using a bi-directional method. Sequencing results were spliced and corrected by Contig Express and Bioedit software. The spliced and corrected results of the sequencing were submitted to the Stanford University webpage for antiviral resistance and genotypic resistance location analysis (http://www.hiv-grade.de /hbvgrade/deployed/).

### Safety assessment

Abnormal renal functions were defined as serum BUN level > 7 mmol/L and serum Cr > 106 umol/L (upper limit of normal range). Abnormal CK was defined as serum CK level > 170 U/L (upper limit of normal range).

### Statistical analysis

Parametrical data were expressed in means with standard deviations (SD) when normal distribution was assumed. Statistical analysis was conducted using statistical package for social sciences (SPSS) (version 16.0). A student's t-test, analysis of variance and log-rank test were used to compare means between groups. A 2-test was used for analysis when appropriate. Logistic regression analysis was performed to evaluate the association of variables with death or liver failure. The Kaplan-Meier method was used to estimate the accumulative survival. Differences were considered to be significant if P < 0.05.

## RESULTS

### Clinical characteristics

No difference in gender, age, family history of hepatitis B, alcohol abuse, CPs or HBeAg positive and HBV DNA level was seen between groups at baseline (P > 0.05; Table1). HBeAg-positive patients were accounted for 63.7%. According to whether drug-resistance occurred by 2 years, 34 (17.6%) patients had antiviral resistance and were regarded as a drug-resistant group.

### Rescue therapies

The rescue therapy at 2 years was higher in the LAM, ADV and LDT monotherapy than in ETV monotherapy or combinations of NUCs (Figure 2A). This suggested that ETV monotherapy and combinations of NUCs were superior to LAM, ADV, and LDT monotherapy.

### Child-pugh score

The CPs of each antiviral therapy group was significantly decreased at one and two years, compared with baseline (P < 0.05) (Table 2). In the control group and drug-resistant

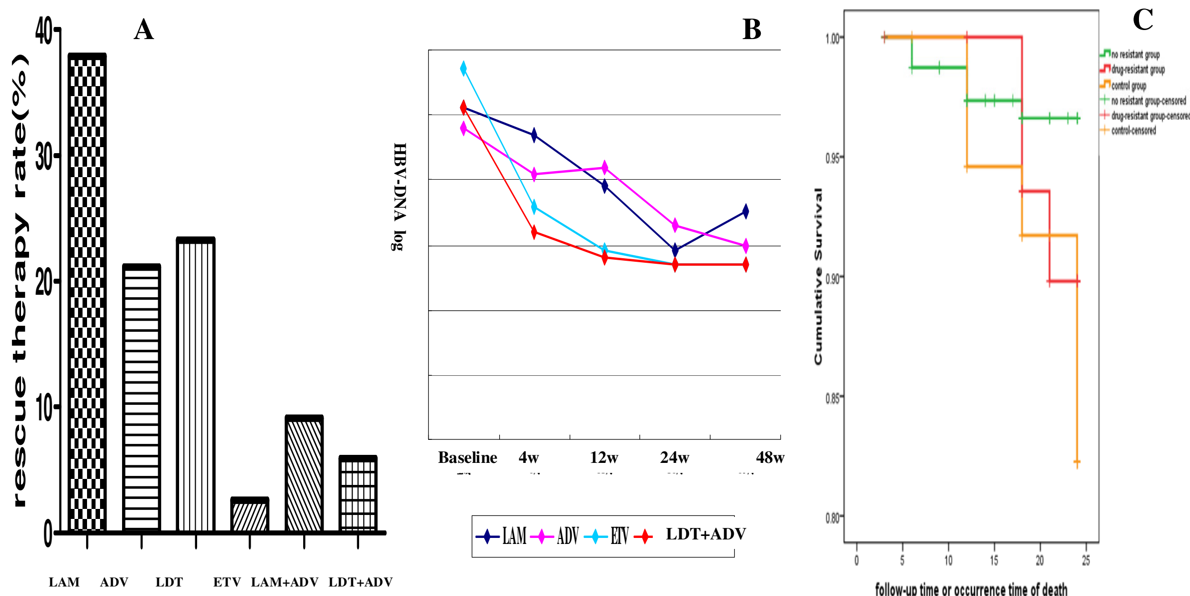


**Figure 2.** (A) Rescued therapy in the course of antiviral therapies over two years were shown; (B) serum HBV DNA dynamics. The ETV monotherapy combination of LDT+ADV shows significant decline in HBV DNA at week 12 compared to the baseline ($P < 0.05$); (C) the cumulative survival was shown in cohort fellow-up over 2 years. DR = drug resistance, CVR = complete virological response.

**Table 3.** Summary of virological parameters and side-effects in the course of antiviral therapy over 2-year follow-up.

| Group | HBeAg seroconversion (%) | HBeAg loss (%) | HBV-DNA undetectability (%) | CDR (%) | abnormal CK (%) |
|---|---|---|---|---|---|
| LAM | 10.5 | 31.5 | 89.5 | 37.9 | 7.0 |
| ADV | 15.8 | 26.3 | 91.2 | 21.2 | 1.8 |
| LDT | 17.6 | 47.1 | 86.7 | 23.3 | 13.3 |
| ETV | 17.4 | 30.4 | 97.3 | 2.6 | 5.3 |
| LAM+ADV | 20.0 | 40.0 | 91.3 | 8.7 | 4.3 |
| LDT+ADV | 27.3 | 72.7 | 93.7 | 6.3 | 6.3 |
| Control | 16.7 | 22.2 | 12.8 | ND | ND |

CDR: cumulative drug-resistance rate. ND: no data.

resistant patients, however, CPs was increased at 2 years ($P > 0.05$).

## Virological response

The patients of monotherapy with ETV and the combinations of LDT+ADV showed a significant decline in HBV DNA level compared to baseline in 12 weeks (Figure 2B) ($P < 0.05$). HBV DNA undetectability in ETV group and LDT+ADV group was higher than in LAM, ADV and LAM+ADV group at one year ($P < 0.05$). HBeAg seroconversion and loss were shown in Table 3. The combinations of LDT+ADV therapy had a higher HBeAg loss at one-year (63.6%), and two-year (72.7%) than LAM, ADV, or ETV monotherapy. HBV DNA undetectability for the combinations of LDT+ADV therapy was similar to ETV monotherapy over the 2 years.

## Drug resistance

The two-year cumulative drug-resistance rate in LAM, ADV, LDT, ETV, LAM+ADV, and LDT+ADV groups were 37.9, 21.1, 23.3, 2.6, 8.7, and 6.3%, respectively. The two-year cumulative drug-resistance rate of ETV monotherapy and combinations of NUCs were significantly lower than for the LAM (OR 22.6, 95%; CI =

2.7 to 188.9, P < 0.001), ADV (OR 9.8, 95%; CI 1.2 to 79.4, P = 0.013) and LDT monotherapy groups (OR 11.3, 95%; CI 1.3 to 97.5, P = 0.018) (Table 3).

## Prognosis

The cumulative survival rate in the drug-resistant, CVR and control group was 91.2, 96.9, and 84.6%. The total cumulative survival rate in the antiviral therapy group was significantly higher than in the control group (Figure 2C) (OR 4.2, 95%; CI 1.4 to 12.9, P = 0.017). No significant difference was seen in two years survival between the drug-resistant group and CVR group (P = 0.132). In the course of the two-year follow-up, eight patients died in the antiviral therapy group. Of those, four died of chronic liver failure, three of HCC, and one of hepatorenal syndrome. Six patients died in the control group, three of chronic liver failure, two of HCC, and one of hepatorenal syndrome. By logistic regression analysis, HCC and liver failure were independent risk factors for predicting poor prognosis in two years (HCC: 95%; CI 2.8 to 37.5, P < 0.001; and liver failure 95%; CI 2.9 to 49.4, P = 0.001). However, no statistically significant difference was seen between mutations related drug-resistance and occurrence of HCC (P > 0.05, Table 4). The cumulative incidence of liver failure in drug-resistant, CVR and control groups were 5.9, 0.6, and 28.2%. The two-year cumulative incidence of liver failure in the antiviral therapy group was significantly less than the control group (OR 24.9, 95%; CI 6.5 to 94.7, P = 0.001).

## Frequency of actites and hospitalization

The frequency of actites per year in the LDT+ADV group was lower (1.4 ± 0.6), but not significantly different than the control group (P = 0.608). The frequency of hospitalization per year in the LDT+ADV group was lower (1.3 ± 1.0) than the control group (Table 2) (P = 0.021, 95%; CI 0.759 to 3.086). The main reasons for hospitalization were liver failure, esophageal variceal bleeding or HCC.

## Safety

Abnormal BUN and Cr in the antiviral therapy group were not significantly changed in the course of treatment over two years compared to baseline (P > 0.05). Eleven patients had elevated CK in the course of antiviral therapy (Table 3).

# DISCUSSION

HBV infection remains a global public health problem. The geographical distribution of the rates of chronic HBV infection and HCC are strikingly parallel. The incidence rate and mortality of HBV-related cirrhosis and HCC, however, have significantly increased (Lok and McMahon, 2009; Liaw et al., 2008; Tanaka et al., 2011). To date, the reasons and mechanisms of the different clinical outcomes after HBV infection are still unknown. Substantial clinical evidence shows that the differences in clinical outcomes after HBV infection might be related to HBV DNA level, antiviral treatment response, and immune activation (Ohishi and Chayama, 2012; Di Marco et al., 2005; Bae et al., 2005). All guidelines for the prevention and treatment of chronic hepatitis B both in China and in other countries clearly state that the main aim of treatment for chronic hepatitis B is to bring down the incidence rate and death rate of liver cirrhosis and liver cancer, to prolong life and to improve living conditions (Tujios and Lee, 2012; European Association for the Study of the Liver, 2012). However, for patients with liver cirrhosis, especially those in the decom-pensation period, clinical outcomes after antiviral therapy with nucleoside analogs are unclear (Kwon and Lok, 2011; Das et al., 2010; Manolakopoulos et al., 2009). NUCs are effective drugs for the suppression of HBV reproduction and a good compliance in most chronic hepatitis B patients, especially in HBV-related cirrhosis patients. Optimizing antiviral drugs for decompensated HBV-related cirrhosis still remains a difficult problem for clinicans.

Di Marcov first reported the clinical effect of LAM treatment with 59 cases of HBV-related cirrhosis (45 child-Pugh with A and 4 with B), showing that a sustained suppression of HBV DNA significantly improved the prognosis of patients with liver cirrhosis (Di Marco et al., 2005). Bae reported that 58.2% of patients show complete antiviral responses using LAM in decompensated HBV-related cirrhosis, which can improve the clinical prognosis (Bae et al., 2005). However, the failure of antiviral treatment or drug resistance are dangerous factors for liver disease progression and increase the incidence of liver cancer (Yeh et al., 2011; Zoulim, 2011). A single randomized double-blind controlled trial of LAM in patients with HBeAg and/or high serum HBV DNA levels showed that antiviral therapy prevented disease progression and reduced the incidence of HCC (Liaw et al., 2011; Nishida et al., 2008). By comparing the clinical efficacy and safety of LDT and LAM in chronic HBV hepatitis, found similar results (Hann, 2010). Therefore, LDT is still suggested to be safe for patients with HBV-related decompensated cirrhosis. For choosing NUCs, however, ETV has a potent antiviral effect and low rates of drug resistance and is the first-line monotherapy for patients with HBV-related decompensated cirrhosis by updated guidelines and publications (Tujios and Lee, 2012; Singal and Fontana, 2001; Keating, 2011).

In this study, we found HBV DNA undetectability in ETV

**Table 4.** The relationship between HCC and mutations related drug-resistance.

| Mutations | HCC (n=10) | non-HCC (n=24) | P value |
|---|---|---|---|
| **rtM204V** | | | |
| + | 2(20.0%) | 0(0.0%) | 0.08 |
| - | 8(80.0%) | 24(100.0%) | |
| **rtM204I** | | | |
| + | 0(0.0%) | 7(29.2%) | 0.078 |
| - | 10(100.0%) | 17(70.8%) | |
| **rtA181T** | | | |
| + | 3(30.0%) | 3(12.5%) | 0.328 |
| - | 7(70.0%) | 21(87.5%) | |
| **rtL180M** | | | |
| + | 1(10.0%) | 3(12.5%) | 1.000 |
| - | 9(90.0%) | 21(87.5%) | |
| **rtL80I** | | | |
| + | 0(0.0%) | 2(8.3%) | 1.000 |
| - | 10(100.0%) | 22(91.7%) | |
| **rtN236T** | | | |
| + | 1(10.0%) | 1(4.2%) | 0.508 |
| - | 9(90.0%) | 23(95.8%) | |
| **rtT184I** | | | |
| + | 0(0.0%) | 1(4.2%) | 1.000 |
| - | 10(100.0%) | 23(95.8%) | |
| **rtS202G** | | | |
| + | 1(10.0%) | 0(0.0%) | 0.294 |
| - | 9(90.0%) | 24(100.0%) | |
| gender (M/F) | 8/2 | 19/5 | 0.956 |
| Age (mean±SD) | 53.9±11.5 | 51.5±13.9 | 0.783 |
| HCC family history | 3(30%) | 2(8.3%) | 0.104 |
| Alcohol abuse | 1(10%) | 3(12.5%) | 0.837 |
| HBVDNA (log) baseline | 5.6±1.7 | 5.1±1.9 | 0.862 |

+: positive, -: negative.

monotherapy and combination LDT+ADV therapy group was higher than in LAM, ADV, and LDT monotherapy over two years. Rescue therapy rates at two years were higher in the LAM, ADV, and LDT monotherapy groups, at 37.9, 21.1, and 23.3%, respectively. However, a recent clinical study found that cirrhotic complications cannot be avoided and reversed with potent antiviral suppression of HBV-DNA by rescue therapy (Yeh et al., 2011). Therefore, optimizing antiviral drugs for treatment-naive HBV-related patients is very important. The combinations LDT+ADV had a higher HBeAg loss rate (63.6% at one year, 72.7% at two years) than the LAM, ADV, and ETV monotherapy in this study. The HBeAg loss with antiviral treatment of chronic hepatitis B are important indications of substantial virological response (Lok and McMahon, 2009; Liaw et al., 2008; Tanaka et al., 2011; Tan, 2011). However, the clinical implications of HBeAg loss in HBV-related cirrhosis, especially for decompensated patients, still remains unclear. No change in renal function related to antiviral drugs was found in the two-year fellow-up. These data strongly suggested that ETV monotherapy and combinations of NUCs are superior to LAM, ADV, or LDT

monotherapy.

A greater effect was observed in patients who achieved sustained virological response, while the benefit in non-responders is unclear (Zhang et al., 2011; Papatheodoridis et al., 2010; Ohishi and Chayama, 2012). In our study, we found that antiviral treatment by both monotherapy and combinations of NUC significantly improved liver function. The CPs in the antiviral therapy group was significantly decreased in two years compared to baseline. If antiviral drug-resistance occurred, however, the CPs increased. Therefore, we can conclude that antiviral therapy might have benefits for decompensated HBV-elated cirrhosis. More effective and more affordable antiviral therapies are needed for patients with HBV-related decompensated cirrhosis. A few studies showed that the failure of antiviral therapy of chronic hepatitis B or drug resistance increases the risk of liver cirrhosis and causes the progression of liver disease (Heo et al., 2010; Greece Cohort Study Group, 2011). Our study found that, although combinations of LDT+ADV and ETV mono-therapy showed a similar clinical efficacy and drug two years. The combinations therapy more quickly controlled the reproduction of HBV-DNA in 12 weeks. Therefore, we hypothesize that the long-term efficacy of combinations of NUCs therapy are more effective than monotherapy in suppressing the reproduction of HBV DNA, reduce the incidence of antiviral drug resistance and prolong survival. The relationship between drug resistance and incidence of HCC or bad prognosis will be further investigated in our next project.

## Conclusion

For HBV-related decompensated cirrhotic patients, an effective antiviral therapy can improve long-term clinical outcomes. However, antiviral resistance can significantly increase incidence of liver failure. The efficacy and safety of combinations of NUCs and ETV momotherapy at two years are similar and superior to the LAM, ADV and LDT monotherapy. The long-term safety and cirrhotic complications warrant further monitoring.

## ACKNOWLEDGEMENTS

This study was funded by the Capital Science and Technology Development Foundation (2007-1021), major Projects on Infectious Disease (2012ZX1002-008-05) and Beijing Science and Technology Commission research projects (No. H010210110129, Z111107058811067), and High-Level Talent Academic Leader Training Program (2011-2-19).

### REFERENCES

Asia-Pacific Working Party on Prevention of Hepatocellular Carcinoma (2010). Prevention of hepatocellular carcinoma in the Asia-Pacific region: consensus statements. J. Gastroenterol. Hepatol. 25:657-63.

Bae SH, Yoon SK, Choi JY, Jang JW, Cho SH, Yang JM, Han NI, Ahn BM, Chung KW, Sun HS (2005). Timing of lamivudine administration according to Child class in patients with decompensated cirrhosis. J. Gastroenterol. Hepatol. 20:1527-1532.

Chan SL, Mo FK, Wong VW, Liem GS, Wong GL, Chan VT, Poon DM, Loong HH, Yeo W, Chan AT, Mok TS, Chan HL (2012). Use of antiviral therapy in surveillance: impact on outcome of hepatitis B-related hepatocellular carcinoma. Liver Int. 32:271-278.

Chinese Medical Association (2005). Guidelines for the Prevention and Treatment of Chronic Hepatitis B. Chin. J. Hepatol. 13: 881-891.

Committee of Liver Cancer, Chinese Anti-cancer Association (2001). The clinical diagnosis and staging criteria of primary liver cancers. J. Chin. Hepatol. 9:324-328.

Das K, Das K, Datta S, Pal S, Hembram JR, Dhali GK, Santra A, Chowdhury A (2010). Course of disease and survival after onset of decompensation in hepatitis B virus-related cirrhosis. Liver Int. 30:1033-1042.

European Association for The Study of The Liver (2009). EASL Clinical Practice Guidelines: management of chronic hepatitis B. J. Hepatol. 50:227-242.

European Association for the Study of the Liver (2012). EASL clinical practice guidelines: Management of chronic hepatitis B virusin fection.J. Hepatol. 57:167-185.

Fung J, Lai CL, Seto WK, Yuen MF (2011). Nucleoside/nucleotide analogues in the treatment of chronic hepatitis B. J. Antimicrob. Chemother. 66:2715-2725.

Greece Cohort Study Group (2011). Virological suppression does not prevent the development of hepatocellular carcinoma in HBeAg-negative chronic hepatitis B patients with cirrhosis receiving oral antiviral(s) starting with lamivudine monotherapy: results of the nationwide HEPNET. Greece cohort study. Gut. 60:1109-16.

Hann HW (2010). Telbivudine: an effective anti-HBV drug for chronic hepatitis B patients with early on -treatment responses. Expert Opin. Pharmacother. 11:2243-2249.

Heo NY, Lim YS, Lee HC, Chung YH, Lee YS, Suh DJ (2010). Lamivudine plus adefovir or entecavir for patients with chronic hepatitis B resistant to lamivudine and adefovir. J. Hepatol. 53:449-54.

Jhaveri R, Murray N (2007). An omission in the AASLD Practice Guidelines on Chronic Hepatitis B. Hepatology 46:280.

Jin YJ, Shim JH, Lee HC, Yoo DJ, Kim KM, Lim YS, Suh DJ (2011). Suppressive effects of entecavir on hepatitis B virus and hepatocellular carcinoma. J. Gastroenterol. Hepatol. 26:1380-1388.

Keating GM (2011). Entecavir: A review of its use in the treatment of chronic hepatitis B in patients with decompensated liver disease. Drugs 71:2511-29.

Kim BK, Han KH, Ahn SH (2011). Prevention of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. Oncology 81:41-49

Kwon H, Lok AS (2011). Does antiviral therapy prevent hepatocellular carcinoma? Antivir. Ther. 16:787-795.

Liaw YF (2011). Impact of hepatitis B therapy on the long-term outcome of liver disease. Liver Int. 31(1)117-121.

Liaw YF, Leung N, Kao JH, Piratvisuth T, Chan HLY, Chien R, Liu C, Gane E, Locarnini S, Lim S, Han K, Amarapurkar D, Cooksley G, Jafri W, Mohamed R, Hou J, Chuang W, Lesmana LA, Sollano JD, Suh D, Omata M (2008). Asian-Pacific consensus statement on the management of chronic hepatitis B. Hepatol. Int. 2:263-283.

Liaw YF, Raptopoulou-Gigi M, Cheinquer H, Sarin SK, Tanwandee T, Leung N, Peng CY, Myers RP, Brown RS Jr, Jeffers L, Tsai N, Bialkowska J, Tang S, Beebe S, Cooney E (2011). Efficacy and safety of entecavir versus adefovir in chronic hepatitis B patients with hepatic decompensation: a randomized, open-label study. Hepatology 54:91-100.

Lim SG, Aung MO, Mak B, Sutedja D, Lee YM, Lee GH, Fernandes M, Low HC, Lai V, Dan YY (2011). Clinical outcomes of lamivudine-adefovir therapy in chronic hepatitis B cirrhosis. J. Clin. Gastroenterol. 45:818-823.

Lim SG, Mohammed R, Yuen MF, Kao JH (2009). Prevention of hepatocellular carcinoma in hepatitis B virus infection. J. Gastroenterol. Hepatol. 24:1352-1357.

Lok AS, McMahon BJ(2009). Chronic hepatitis B. Hepatology 50:661-

662.
Manolakopoulos S, Triantos C, Theodoropoulos J, Vlachogiannakos J, Kougioumtzan A, Papatheodoridis G, Tzourmakliotis D, Karamanolis D, Burroughs AK, Archimandritis A, Raptis S, Avgerinos A (2009). Antiviral therapy reduces portal pressure in patients with cirrhosis due to HBeAg-negative chronic hepatitis B and significant portal hypertension. J. Hepatol. 51:468-74.
Nishida T, Kobashi H, Fujioka SI (2008). A prospective and comparative cohort study on efficacy and drug resistance during long-term lamivudine treatment for various stages of chronic hepatitis B and cirrhosis. J. Gastroenterol. Hepatol. 23:794-803.
Ohishi W, Chayama K (2012). Treatment of chronic hepatitis B with nucleos(t)ide analogues. Hepatol. Res. 42:219-225.
Papatheodoridis GV, Lampertico P, Manolakopoulos S, Lok A (2010). Incidence of hepatocellular carcinoma in chronic hepatitis B patients receiving nucleos(t)ide therapy: a systematic review. J. Hepatol. 53:348-356.
Singal AK, Fontana RJ (2012). Meta-analysis: oral anti-viral agents in adults with decompensated hepatitis B virus cirrhosis. Aliment Pharmacol. Ther. 35:674-89.
Tan YJ (2011). Hepatitis B virus infection and the risk of hepatocellular carcinoma. World J. Gastroenterol. 17:4853-4857.
Tanaka M, Katayama F, Kato H, Tanaka H, Wang J, Qiao YL, Inoue M (2011). Hepatitis B and C virus infection and hepatocellular carcinoma in China: a review of epidemiology and control measures. J. Epidemiol. 21:401-416.
Tujios SR, Lee WM (2012). New advances in chronic hepatitis B. Curr. Opin. Gastroenterol. 28:193-197.
Yeh CT, Chen T, Hsu CW, Chen Y, Lai M, Liang K, Chen T (2011). Emergence of the rtA181T/sW172* mutant increased the risk of hepatoma occurrence in patients with lamivudine-resistant chronic hepatitis B. BMC Cancer 11:398.
Zhang QQ, An X, Liu YH, Li SY, Zhong Q, Wang J, Hu HD, Zhang DZ, Ren H, Hu P (2011). Long-term nucleos(t)ide analogues therapy for adults with chronic hepatitis B reduces the risk of long-term complications: a meta-analysis. Virol. J. 8:72.
Zoulim F (2011). Hepatitis B virus resistance to antiviral drugs: where are we going? Liver Int. 1:111-6.

academicJournals

Vol. 7(31), pp. 2273-2278, 22 August, 2013
DOI 10.5897/AJPP2012.2934
ISSN 1996-0816 ©2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

*Full Length Research Paper*

# Social stratification predicts the risk of ischemic heart disease: A cross sectional study in Pakistani population above 40 years

**Mahtab Ahmad Khan[1,2], Maqsood Ahmad[2], Sohail Safdar[3], Ahmed Subhani[2], Muhammad Usman Minhas[4], Saba Sajjad[5], Muhammad Asadullah Madni[4] and Sajjad Muhammad[6]***

[1]Institute of Experimental and Clinical Pharmacology and Toxicology, University of Lübeck- Germany.
[2]Bahaudin Zakariya University, Multan, Pakistan.
[3]Pakistan Medical Research Council, Research Center, Nishtar Hospital Multan, Pakistan.
[4]The Islamia University of Bahwalpur, Pakistan.
[5]Faculty of Medicine, University Hospital Bonn, 53127-Bonn, Germany.
[6]Department of Neurosurgery, University Hospital Bonn, Sigmund Freud Str.25; 53127-Bonn, Germany.

Accepted 17 June, 2013

**This study was designed to investigate risk of ischemic heart disease in different social classes of healthy Pakistani population above 40 years of age. We stratified healthy Pakistani population into 5 social classes according to their life style and work profile. Different baseline parameters and serum markers were measured to investigate risk factors and to determine the risk of ischemic heart disease (IHD) over next 10 years in different social classes. Baseline measurement of IHD risk factors were obtained from 100 healthy volunteers (age range >40 years). Physical and biochemical parameters namely fasting blood sugar (FBS) levels, total cholesterol (TCH), high density lipoprotein (HDL), triglycerides (TG), low density lipoprotein (LDL), body mass index (BMI), systolic and diastolic blood pressure were investigated. The volunteers were divided into five social classes on the basis of their job profile and life style. IHD risk calculation was done with risk prediction chart for non-diabetic patients as recommended by British heart association. The mean value of serum cholesterol in healthy volunteers was 195.43± 5.22 mg/dl, mean TG, 250.4± 12.4 mg/dl, mean HDL, 83.63± 5.32 mg/dl, mean LDL, 113.88± 4.42 mg/dl, mean FBS, 85.66± 2.98 mg/dl and for the mean BMI was 24.61 ± 0.410 Kg/m². Our data shows that social class IV (labors and hard workers) has significantly lower BMI as compared to class I, II and III and significantly lower FBS as compared to class I. Social class V (farmers) has significantly lower total cholesterol as compared to class I and II and TG in class III were lower as compared to class I. Cumulative IHD risk >30% over next 10 years was significantly lower in class V as compared to class I (p-value <0.05). Cumulative IHD risk >30% over next 10 years was 10 % in Pakistani population. Social class V has significantly lower IHD risk >30% over next 10 years.**

**Key words**: Ischemic heart disease, body mass index, lipid profile, social classes.

## INTRODUCTION

Ischemic heart disease (IHD) is a general term, which refers to narrowing of the major coronary arteries leading to ischemia in the region of supply. Ischemic heart disease is also known as coronary artery disease (CAD) or atherosclerotic coronary artery disease (McPhee et al., 2011). Ischemic heart disease is a form of heart disease

*Corresponding author. E-mail sajjad.muhammad@ukb.uni-bonn.de. Tel: 0049-228-28716509. Fax: 0049-228-28714758.

**Table 1.** Stratification of social classes.

| Social class | Job profile | Life style |
|---|---|---|
| Social class -I | Office workers | Sedentary life style and working in tense environment |
| Social class -II | House wives | Sedentary life style, work and live at homes |
| Social class-III | Shop keepers | Passing their life without any exercise, work within their shop premises |
| Social class-IV | Labors and hard workers | Work very hard and perform vigorous exercise during their work, in less tense environment |
| Social class-V | Farmers | Work hard in the fields by keeping their body in exertion. |

with primary manifestations that result from myocardial ischemia and may lead to myocardial infarction and abnormal functioning of the heart. This term encompasses a spectrum of conditions, ranging from the asymptomatic preclinical phase to acute myocardial infarction and sudden cardiac death (Liu et al., 2011).

IHD is responsible for 1 out of every 4.8 deaths in the United States. Each year in the USA, more than 1 million patients suffer an acute myocardial infarction. The IHD is among those diseases that are causing the most economic burden. Increasing prevalence of urban cardiovascular disease (CVD) risk factors are reported in many countries with emerging economies (Aziz et al., 2004). South Asian countries are faced with the double burden of infectious diseases, infant mortality, under nutrition and emerging epidemic of CVD, linked in part with obesity (Donin et al., 2010). In United Kingdom, the population and migrants from south Asian subcontinent has higher incidence of cardiovascular problems (Jonnalagadda et al., 1996). The overall mortality risk of IHD in different social classes in Pakistani population has not been investigated yet. We stratified healthy Pakistani population into 5 social classes according to their life style and work profile (Table 1) and analyzed serum IHD risk factors to predict risk of IHD over the next 10 years in different social classes. Our data on social stratification reflects serum biochemical markers and IHD risk prediction and hence may provide valuable information to take preventive measures in specific social class in Pakistani population.

## MATERIALS AND METHODS

### Subjects and Methods

#### Subjects

We enrolled prospectively 100 healthy volunteers above age of 40 years in our study. The volunteers were divided into five social classes on the basis of their job profile and life style (Table 1). The study was approved by local ethical committee and the volunteers have given informed consent accordingly.

#### Methods

In each social class, 20 healthy volunteers with age above 40 years were selected (Table 1). Volunteers with chronic disease including IHD, diabetes, hepatitis and HIV were excluded. Data from each social class was combined. Blood pressure measurements, weight and height were measured twice and recorded in a Performa, anthropometric measurements were taken and fasting blood specimens were taken for plasma sugar and lipid determination. Subjects were considered to have high blood pressure if they had a DBP ≥ 90 mm Hg and SBP ≥ 140 mm Hg. (Carlos Lorenzo et al., 2007). The subjects with a BMI of 25 – 30 kg/m$^2$ were considered overweight (World Health Organization criteria). IHD risk calculation was done by measuring systolic blood pressure (SBP) and serum total cholesterol to high density lipoproteins ratio (TC: HDL) and then comparing these values on risk prediction chart for non-diabetic patients, recommended by British heart association.

### Statistical analysis

Data were analyzed using one –way ANOVA (analysis of variance) followed by Newman - Keuls Multiple Comparison posthoc test. For the prediction of IHD risk over next 10 years, we used IHD risk prediction chart recommended by Joint British Societies Coronary risk prediction charts. A probability value of ≤0.05 was taken as significant.

## RESULTS

Our study shows that in different social classes of Pakistani population above 40 years of age show differences in risk of ischemic heart disease, and hence social stratification of Pakistani population may be helpful to predict the IHD risk and hence to take preventive measure for specific social class. Our data show that about 10% of subjects were at IHD risk >30% over next 10 years, 27% people at IHD risk 15-30% over next 10 years, 26% people at IHD risk of <15% over next 10 years. Out of these 10% subjects having IHD risk >30% over next 10 years (Figure 7), there are 18% smokers and 4% non-smokers reflecting that smoking as a known risk factor of IHD applies similarly to Pakistani population.

### Body mass index of five social classes

The mean values of BMI of five social classes were μ1 = 25.175, μ2 = 25.450, μ3 = 26.830, μ4 = 21.610, μ5 = 24.020. There is a significant difference between the social classes 4 and 1, 4 and 2, 4 and 3 (Figure 1).

Social class-IV has least BMI that reflects the social class of labors and hard workers have comparatively low BMI. However, BMI was still in normal range. Farmers had also lower BMI than social class I, II and III that was not significantly different. Social class I, II and III with similar life style had similar BMI (Figure 1).

**Analysis of fasting blood sugar (FBS) in five social classes:**

The mean values of FBS of five social classes were $\mu1$=100.65, $\mu2$=79.75, $\mu3$=91.90, $\mu4$=77.10, $\mu5$=78.90 (Figure 2). There is a significant difference between the social classes-I and -IV ($\mu4$ and $\mu1$) Figure 2. Social class-IV has significantly lower fasting blood glucose levels. Low normal range glucose reflects the vigorous exercise of social class IV. Social class-V showed lower FBS that was not significantly different from other classes. In short, BMI reflects better metabolism and better range of FBS.

**Analysis of total cholesterol in five social classes**

The mean values of total cholesterol of five social classes were $\mu1$=214.6, $\mu2$=207.7, $\mu3$=198.7, $\mu4$=198, $\mu5$=171.2. There is a significant difference between the social classes I, II and class-V ($\mu1$, $\mu2$ and $\mu5$) (Figure 3). The results show that the social class-V has least cholesterol levels that might be due to vegetarian food style of most of Pakistani farmers. Total cholesterol in class I and II was higher reflecting their life style.

**Analysis of triglycerides in five social classes**

The mean values of TG of five social classes were $\mu1$=316.7, $\mu2$=259.0, $\mu3$=201.0, $\mu4$=245.1, $\mu5$=230.6. Subjects of social class-III had significantly lower triglycerides than class I (Figure 4). However, the BMI and total cholesterol levels of social class-I and -III were similar. Social class-III has lowest level of triglycerides as compared to other social classes.

**Analysis of HDL-cholesterol in five social classes**

The mean values of HDL-cholesterol levels of the five social classes were $\mu1$=80.65, $\mu2$=74.85, $\mu3$=86.6, $\mu4$=84.2, $\mu5$=91.85. There is no significant difference in serum HDL levels between the social classes (Figure 5).

**Analysis of LDL-cholesterol in five social classes**

The mean values of LDL-cholesterol levels of five social classes were $\mu1$ = 110.8, $\mu2$ = 107.8. $\mu3$ = 135.1, $\mu4$ = 109.9, $\mu5$ = 105.95. There is no difference in LDL serum levels between the social classes (Figure 6). Interestingly, serum LDL levels do not reflect BMI.

**Analysis of IHD- risk over the next 10 years in five social classes:**

15% of social class-I is at IHD risk > 30% over next 10 years which is the highest percentage among all classes, while only 5% of social class-V is at IHD risk > 30% over next 10 years (Figure 7). Our data demonstrates that social class-V is at significantly lower risk of IHD in the next 10 years as compared to social class-I. Farmers with regular hard exercise show lower IHD disease risk that confirms the known beneficial effect of exercise in Pakistani population.

## DISCUSSION

Ischemic heart disease is a major public health problem. It contributes to mortality and morbidity in both westernized economics and in countries undergoing economic transition. IHD affects 3.5% of the UK adult population. Worldwide, Asian people are at highest risk for cardiovascular disease as compared to European inhabitants. Jafar et al. (2005) have shown that the prevalence of overweight was 25% and the prevalence of obesity was 10.3% in Pakistani population. Our study showed that the prevalence of overweight was 25% and the prevalence of obesity was 13% in healthy volunteers that is in accordance with the literature. Slight difference in obesity (13 %) might be due to presence of more volunteers living with a sedentary life style with higher BMI. Furthermore, in the former study the subjects were selected between 15 and 65 years of age. But in this study healthy volunteers of age above 40 years were selected.

South Asians have a 40% higher mortality from IHD than Europeans and diabetes is a major risk factor for IHD in south Asians (Jolly et al., 1994). Mortality from IHD increases about 3 to 10 fold and 2 to 4 fold in patients with type-1 and type-2 diabetes, respectively (Gibbons et al., 1999). Chowdhury and Lasker (2002) showed that the mean FBS level of south Asian cohort was 143.24 mg /d l. According to our data on Pakistani population, mean FBS level was 85.67 mg/dl. The difference is because in the present study, all the patients were non-diabetic and healthy volunteers.

Community prevention focusing on primary prevention of CVD risk factors such as hypertension, hyperlipidemia, and obesity is considered the most efficient use of limited recourses in South Asia. Nishtar (2002) showed that 12.6% of people in Pakistani population have highest level of total cholesterol. Our data showed the prevalence

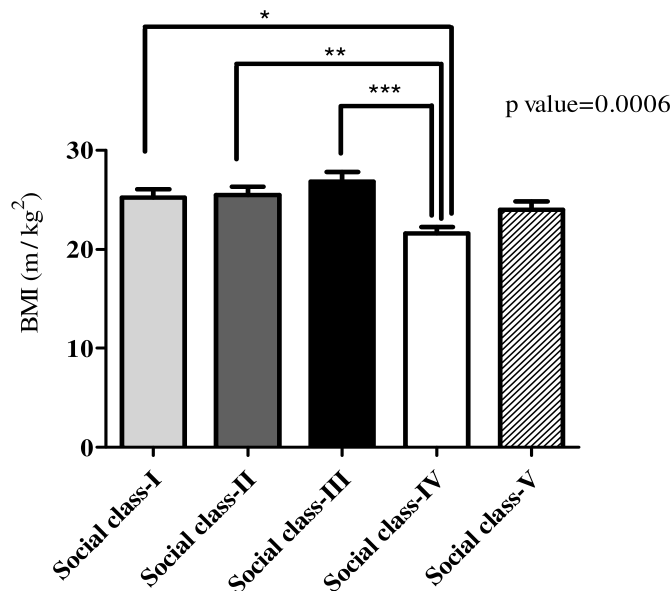


**Figure 1.** Body mass index (BMI) of 100 healthy volunteers of five (I-V) different social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

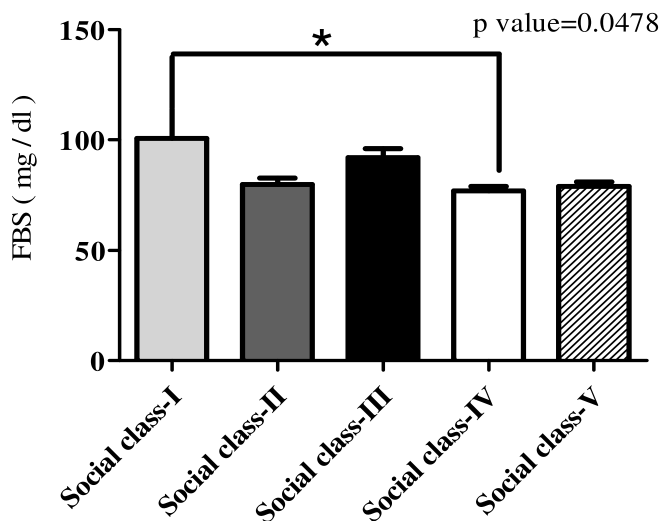


**Figure 2.** Fasting blood sugar (FBS) of 100 healthy volunteers of five (I-V) different social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

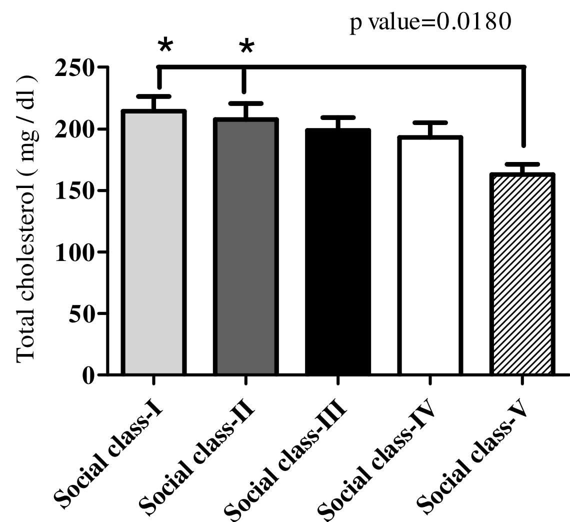


**Figure 3.** Total cholesterol (T.Ch) of 100 healthy volunteers of five (I-V) different social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

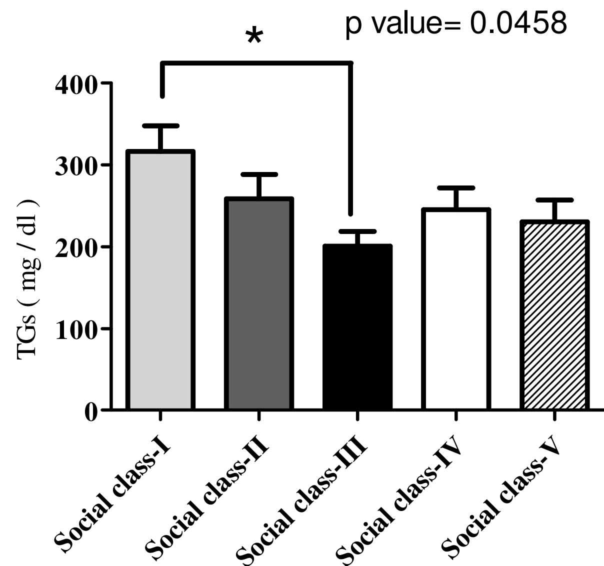


**Figure 4.** Triglyceride (TG) level of 100 healthy volunteers of five (I-V) social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

of hypercholesterolemia of 11% in Pakistani population. Our study focused on social classes and hence included healthy individuals with variety of lifestyle and hence heterogeneity in total cholesterol levels in serum. In our study social class-I with sedentary life style had the highest mean value and the percentage of hyper-cholesterolemia was 30% while the social class-II and social class-IV have 10% hypercholesterolemia while the social class-V has the total cholesterol levels in normal range ( <200 mg/dl). Moreover, in previous studies, the study objects were similar to social class-II or social class-IV of our study. In the Copenhagen male study (CMS) 1997, presence of high fasting plasma triglycerides (TG) concentration and low HDL-cholesterol concentration, the characteristic dyslipidemia in the meta-

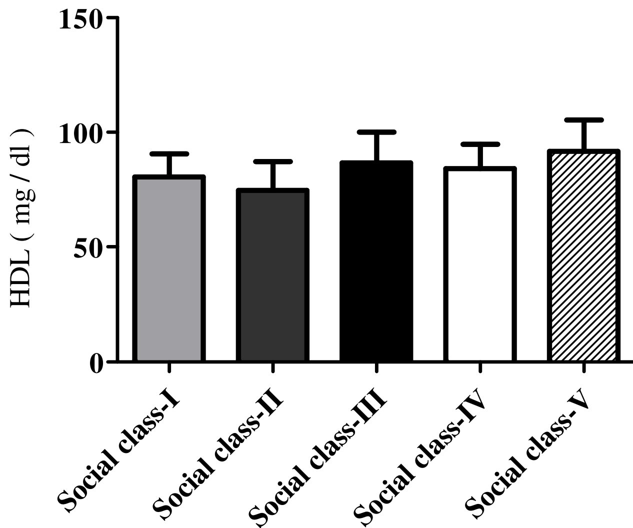


**Figure 5.** High density lipoprotein (HDL) of 100 healthy volunteers of five (I-V) different social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

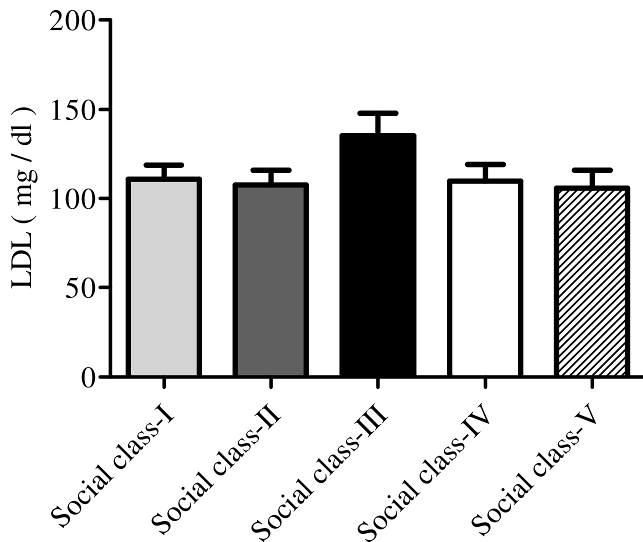


**Figure 6.** Low density lipoprotein (LDL) of 100 healthy volunteers of five (I-V) different social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

bolic syndrome, was associated with a 2-fold higher prevalence of IHD and a 2-fold higher incidence of IHD in men without symptoms of CVD at baseline (Jeppesen et al., 1997). The prevalence of low plasma HDL-cholesterol, hyper-triglyceridemia and diabetes has been reported among South Asians and may be more important than conventional risk factors such as smoking,

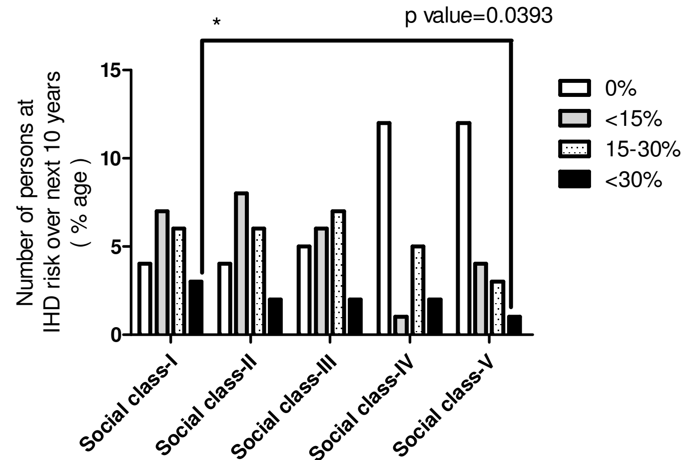


**Figure 7.** Risk of ischemic heart disease over next 10 years in 100 healthy volunteers of five (I-V) different social classes. Class-I, Office workers; Class-II, house wives; Class-III, shop keepers; Class-IV, labors and hard workers; Class-V, farmers. Level of significance was at P<0.05.

high LDL-cholesterol and hypertension. In our study the TG levels of the five social classes were µ1 = 316.7mg/dl, µ2 = 259.0 mg/dl, µ3 = 201.0 mg/dl, µ4 = 245.1mg/dl and µ5 = 230.6 mg/dl, respectively (Figure 4). The social class-I has 80% volunteers with TG level > 200 mg/dl and in the IHD risk prediction analysis, the highest risk of IHD is in this social class.

In India prevalence of IHD has been reported as being 11% in 2001 (Mohan et al., 2001). However, sizeable population such as in Pakistan have no published data for the prevalence or incidence of IHD, and casual and temporal relations between risk factors and this disease have not been established (Geleijnse et al., 2004). Our study showed that in healthy Pakistani population above 40 years of age, 10% are at IHD risk > 30% over next 10 years (Figure 7). Out of these 10%, the social class-I is at 15% IHD risk > 30 % over next 10 years. And this is the highest risk ratio found in these social classes. While the IHD risk in social class-V is 5% which is the least percentage of risk observed in five social classes of Pakistani population. Our data may be valuable to screen Pakistani individuals with higher IHD risk on the basis of social classification that reflects in majority the biochemical analysis.

## ACKNOWLEDGEMENTS

We are thankful to the Faculty of science, Bahaudin Zakariya University and University of Bonn for providing funds for this study.

## ABBREVIATIONS

**IHD,** Ischemic heart disease; **CAD,** coronary artery

disease; **CVD,** cardiovascular disease; **SBP**, systolic blood pressure; **TC,** total cholesterol; **HDL,** high density lipoproteins; **FBS,** fasting blood sugar; **TG,** triglycerides; **LDL,** low density lipoprotein; **BMI,** body mass index.

## REFERENCES

Aziz KS, MA A, Faruqui, Patel N, H Chagani SA, Hafeez SA, Ghuari MF, Memon T, Ashraf, Sultana H (2004). Evaluation and Comparison of Coronary Heart Disease Risk Factor Profiles of Children in a Country with Developing Economy. JPMA 54(7):364-71.

Carlos Lorenzo M, Ken Williams MS, Kelly J, Hunt PHD, Steven M, Haffner MD (2007). The National Cholesterol Education Program–Adult Treatment Panel III, International Diabetes Federation, and World Health Organization Definitions of the Metabolic Syndrome as Predictors of Incident Cardiovascular Disease and Diabetes. Diabetes care 30:8-13.

Chowdhury TA, Lasker SS (2002). Complications and cardiovascular risk factors in South Asians and Europeans with early-onset type 2 diabetes. QJM 95:241-246.

Donin AS, Nightingale CM, Owen CG, Rudnicka AR, McNamara MC, Prynne CJ, Stephen AM, Cook DG, Whincup PH (2010). Nutritional composition of the diets of South Asian, black African-Caribbean and white European children in the United Kingdom: The Child Heart and Health Study in England (CHASE). Br. J. Nutr. 104:276-285.

Geleijnse JM, Kok FJ, Grobbee DE (2004). Impact of dietary and lifestyle factors on the prevalence of hypertension in Western populations. Eur. J.Public Health 14:235-239.

Gibbons RJ, Chatterjee K, Daley J, Douglas JS, Fihn SD, Gardin JM, Grunwald MA, Levy D, Lytle BW, O'Rourke RA, Schafer WP, Williams SV, Ritchie JL, Cheitlin MD, Eagle KA, Gardner TJ, Garson A, Jr., Russell RO, Ryan TJ, Smith SC Jr (1999). ACC/AHA/ACP-ASIM guidelines for the management of patients with chronic stable angina: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on Management of Patients With Chronic Stable Angina). J. Am. Coll.Cardiol. 33:2092-2197.

Jafar TH, Jafary FH, Jessani S, Chaturvedi N (2005). Heart disease epidemic in Pakistan: women and men at equal risk. Am. Heart J. 150:221-226.

Jeppesen J, Schaaf P, Jones C, Zhou MY, Chen YD, Reaven GM (1997). Effects of low-fat, high-carbohydrate diets on risk factors for ischemic heart disease in postmenopausal women. Am. J. Clin. Nutr. 65:1027-1033.

Jolly KS, Pais P, Rihal CS (1996). Coronary artery disease among South Asians: identification of a high risk population. Can. J. Cardiol.12:569-571.

Jonnalagadda SS, Mustard VA, Yu S, Etherton TD, Kris-Etherton PM (1996). Effects of Individual Fatty Acids on Chronic Diseases. Nutr.Today 31(3):90-106.

Mcphee SJ, Michael WR, Maxine AP (2011). Current Medical Diagnosis and Treatment 2011. San Francisco, USA: The McGraw – Hill companies p 1808.

Liu SQ, Tefft BJ, Zhang D, Roberts D, Schuster DJ, Wu A (2011). Cardioprotective mechanisms activated in response to myocardial ischemia. Mol. Cell. Biomech. 8:319-338.

Mohan V, Deepa R, Rani SS, Premalatha G (2001). Prevalence of coronary artery disease and its relationship to lipids in a selected population in South India: The Chennai Urban Population Study (CUPS No. 5). J. Am. Coll. Cardiol. 38:682-687.

Nishtar S (2002). Prevention of coronary heart disease in south Asia. Lancet 360:1015-1018.

## *UPCOMING CONFERENCES*

## International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 24 Dec 2013

## 1st Annual Pharmacology and Pharmaceutical Sciences Conference (PHARMA2013).Conference Dates: 18th – 19th November 2013

## Conferences and Advert

**November 2013**
1st Annual Pharmacology and Pharmaceutical Sciences Conference (PHARMA 2013).

**December 2013**
ICPP 2013 : International Conference on Pharmacy and Pharmacology
Bangkok, Thailand December 24-25, 2013

**December 2013**
46th Annual Conference of Pharmacological Society of India

# 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