

African Journal of Pharmacy and Pharmacology

Volume 7 Number 27 22 July, 2013

ISSN 1996- 0816



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.*

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 University, Turkey.

Dr Nafisa Hassan Ali

Assistant Professor, Dow institute 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.

ARTICLES

Review

- Vancomycin as a risk factor for anaphylactoid reaction (Red Man Syndrome)
: Literature review** 1854

Rosa Eugenia Reyes R, Miguel Ángel Becerril G, Ma. Cristina Fresan O,
Guadalupe A. Camacho V, Adrián de Jesús Guillé P, Ma. Gabriela Pérez G

- An emergency response plan to control emerging infectious disease hazards
in Taiwan's Hospitals** 1860

Hung-Chang Liao, Lien-Hsiung Lee, Shao-Ping Yuan, Ya-huei Wang

Research Articles

- A survey of the antibacterial activity of three plants used in the Congolese
herbal medicine practiced by the healers in the city of Lubumbashi** 1870

Shengo L. M., Mundongo T.H., Kasamba I.E., Malonga K. F., Kapend A. K. L.

- Antimicrobial activity of sequential extracts from leaves of *Cassia nodosa* bunch** 1876

Sharma, R. A., Richa Bhardwaj, Pallavi Sharma

- A preliminary study on the toxicity and novelty-induced behavioral effects
of herbal medicine (*Mama Decoction*[®]) in rats** 1880

M.A. Akanmu, A. O. Akanmu A.O., Adepiti, S.A. Osasan, E.M. Obuotor,
A.A. Elujoba

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 7 Number 27 22 July, 2013

ARTICLES

Research Articles

- Antinociceptive and anti-inflammatory activities of the hexane extract from *Hortia brasiliana* Vand. leaves on experimental animal models** 1886
Carlos Cerqueira Magalhães, Carolina Miranda Gasparetto, Luciana Moreira Chedier, Daniel Sales Pimenta, Maria Silvana Alves, Orlando Vieira de Sousa
- Neuroprotective potential of *Ocimum sanctum* (Linn) leaf extract in monosodium glutamate induced excitotoxicity** 1894
Shanmuga Sundaram Rajagopal, Gowtham Lakshminarayanan , Ramdass Rajesh, Senthil Rajan Dharmalingam, Srinivasan Ramamurthy, Kumarappan Chidambaram, Suresh Shanmugham
- Target propofol concentration required for laryngeal mask airway insertion after pretreatment with dexmedetomidine** 1907
Xiao-Bo Liu, Xi-Ge Yang, Xin-Bai Li, Hai-Chun Ma, Wei Han, Zhuang Zhao, Chun-Ying Han, Long-Xin Luo
- Curcumin inhibits cell survival and migration by suppression of Notch-1 activity in prostate cancer cells** 1911
Tao Kong, Yongxing Wang, Li Xiao, Limin Liao
- Melatonin changes tularemia progression in a BALB/c mouse model** 1917
Miroslav Pohanka, Oto Pavlis

ARTICLES

Research Articles

- Comparison of cytotoxic and genotoxic effects of the synthetic fungicide nimrod and the natural fungicide rhizo-N** 1924
Hala M. Mahfouz, Hoda M. Barakat, Maher abd el fatah
- Academic dishonesty among Nigeria pharmacy students: A comparison with United Kingdom** 1934
Ubaka Chukwuemeka, Fajemirokun Gbenga, Nduka Sunday,
Ezenwanne Ndidiamaka

Review

Vancomycin as a risk factor for anaphylactoid reaction (Red Man Syndrome): Literature review

Rosa Eugenia Reyes R^{1,2}, Miguel Ángel Becerril G³, Ma. Cristina Fresan O², Guadalupe A. Camacho V⁴, Adrián de Jesús Guillé P⁴ and Ma. Gabriela Pérez G^{4*}

¹Farmacia Hospitalaria, Instituto Nacional de Pediatría, CP 04530, Mexico City, México.

²Departamento de Sistemas Biológicos, División CBS. Universidad Autónoma Metropolitana Unidad Xochimilco, Cp. 04960, Mexico City, México.

³Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad de Desarrollo e Investigación en Bioprocesos. Prolongación de Carpio y Plan de Ayala S/N, Col Santo Tomas. Cp 11340, Mexico City, México.

⁴Laboratorio de Farmacología, Instituto Nacional de Pediatría, CP 04530, Mexico City, México.

Accepted 11 June, 2013

Vancomycin is the antibiotic of first choice for the treatment of methicillin resistant *Staphylococcus aureus* (MRSA) infection. Its use is associated with adverse effects in children with a frequency of 5 - 14% and in adults with 1.6 - 35%. Of these adverse effects to vancomycin, two kinds have been described. The first is anaphylactic immunologic or immediate hypersensitivity mediated by immunoglobulin type E (IgE). The second is IgE independent anaphylactoid reaction, also referred to as nonallergic drug hypersensitivities or pseudoallergic reactions, known as red man syndrome (RMS). The signs and symptoms of anaphylactoid reaction principally occur in the first dose of vancomycin and could be accompanied by hypotension and cardiac arrest. The severity of the reaction is proportional to the dose administered, infusion velocity, and liberation of histamine in blood.

Key words: Vancomycin, adverse effects, hypersensitivity, red man syndrome, histamine, infant population.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a public health problem. In U.S.A., the incidence of infection by MRSA in 2005 was estimated to be 31.8 per 100,000 inhabitants with mortality rate of 6.3/100,000 inhabitants (Svetitsky et al., 2009). Vancomycin is the drug of choice for the treatment of MRSA, *Corynebacterium jeikeium* and, resistant strains of *Streptococcus pneumoniae*. (An et al., 2011).

Vancomycin is a glycopeptide obtained from *Amycolatopsis orientalis*. It was developed as an active antimicrobial agent for Grampositives, above all, *staphylococci* producers of β -lactamase. It is an alternative drug for penicillin and/or cephalosporin allergic patients (Núñez et al., 2006) with bactericidal activity. Its

mechanism of action is to inhibit the synthesis of Gram positive bacterial cell wall at the formation level of polymers of *N*-acetylmuramic acid and *N*-acetylglucosamine or the aminoacid chains interwoven inside peptidoglycan structure (Pootoolal et al., 2002). The use of vancomycin is associated with adverse effects. An adverse reaction to medicines (ARM) is unintentional harmful response to drugs occurring at normal dose when administered in humans for prophylaxis, diagnosis, and treatment of sicknesses, or for the modification of physiologic function. ARMs are public health problem due to the fact that these reactions are often unpredictable and put at risk the life of patients (Limsuwan and Demoly, 2010). Their knowledge could help in predicting the risk

of administration and prevention of specific treatments (McAuley, 2012, Edwards and Aronson, 2000). Of these adverse effects to vancomycin, two kinds have been described. The first is anaphylactic immunologic or immediate hypersensitivity mediated by immunoglobulin type E (IgE). The second is IgE independent anaphylactoid reaction, also referred to as non-allergic drug hypersensitivities or pseudoallergic reactions, known as red man syndrome (RMS) (Riedl and Casillas, 2003; Farnam et al., 2012). Nonallergic drug hypersensitivities reaction could occur on first exposition to the drug (Brown, 2009).

Vancomycin could directly activate priming cells or mastocytes, also denominated as granular cells of connective tissue. Mastocytes come from CD34⁺ precursor cells of the bone marrow. The granules of priming cells or mastocytes contain heparin, histamine, and three classes of neutral protease (tryptase, cymase, and carboxypeptidase). The degranulation could be induced by IgE dependent or independent immune mechanisms (Payne and Kam, 2004). The objective of the present revision work is to bring to knowledge the role of vancomycin in the development of hypersensitivity reactions as well as its underlying mechanisms, and which are the risk factors, its prevention, and treatment so as to improve the administration protocol and avoid iatrogenic events concomitant with its use.

MOLECULAR EVENTS AND CLINICAL MANIFESTATIONS OF ANAPHYLAXIS ASSOCIATED TO VANCOMYCIN

ARM require the creation of awareness on its mechanism of action, especially when anaphylaxis is suspected. Independently of infusion velocity of the medicine, vancomycin induces anaphylaxis through immediate hypersensitivity reaction mediated by IgE. As a consequence of the union of Fc portion of IgE to the receptors of high affinity, FcεRI-α, of priming cell or mastocyte membranes, or the membranes of basophilic cells, a massive release of inflammatory mediators such as histamine, tryptase, prostaglandins, leukotrienes, among others is induced (Bischoff, 2007). The FcεRI-α receptor is expressed in priming cell or mastocyte, and basophilic cells as a heterotetramer composed of one α and β, and two γ chains with the last being bonded by disulphuric bridge. The β and γ chains are responsible for propagation of signals to the inner part of cells through phosphorylation of the thyroxin-rich activation sites.

The system of signal transductions of FcεRI-Fc receptor induces the activation of kinase of Src family, the formation of macromolecule complexes in the plasmatic membrane, activation of phosphoinositol-calcium exchange route, the phosphorylation of diverse MAP kinases, and controlled transcription of a great number of genes. This signal transduction system regulates degra-

nulation of priming cell or mastocyte and production of cytokines (González et al., 2005).

Many cases of anaphylaxis are due to activation of histamine receptors, and interaction between H1 and H2 receptors, all of which can lead to acute bronchospasm, and wheezing dyspnea as a result of bronchial smooth muscles constriction and mucosal viscosity increase. The combination of the stimulation of H1 and H2 receptors increases vascular permeability, hypotension, tachycardia, and headache. Histamine is not the only agent that produces symptoms of anaphylaxis. The prostaglandins, leukotrienes, and platelet activating factor also play an important role in bronchospasm by increasing vascular permeability and aiding vasodilatation (Bertolissi et al., 2002).

Early diagnosis of anaphylaxis could be achieved by measuring the blood histamine. Unfortunately, the half-life of histamine is short. The tryptase of priming cells or mastocytes could provide an alternative approach to histamine determination (Laroche et al., 1991).

The tryptases are serine tetrameric proteases with a molecular weight of approximately 134 kDa. The enzyme has four subunits, each unit having its active site. The priming cells or mastocytes principally express two types of tryptases: α and β tryptases. β tryptase serves as a marker of the activation of priming cells or mastocytes. The measurement of the concentration of blood tryptase is used to distinguish the reactions dependent on priming cells or mastocytes activation such as anaphylactic and anaphylactoid reactions of other systemic alterations that could simulate similar clinical manifestations (Payne and Kam, 2004).

MOLECULAR EVENTS AND CLINICAL MANIFESTATIONS OF ANAPHYLACTOID REACTIONS ASSOCIATED TO VANCOMYCIN

The red man syndrome is a common adverse event in children receiving vancomycin (Myers et al., 2012). The RMS is characterized by maculopapular rash on the neck, face, and upper part of the trunk, upper and lower extremities and could affect big areas of the body. It could be accompanied by tachycardia or bradycardia which could sometimes lead to cardiac arrest, increase in temperature, scratches, and hypotension with the last being caused by the liberation of histamine which leads to vasodilation and therefore myocardial hypotension and direct inhibition of its function. In majority of the patients, the presence of tachiphylaxis is seen (Kupstaité et al., 2010).

In lactates, the rash is associated with the reduction of tissue perfusion, cold extremities, increase in the necessity of oxygen and lethargy. This adverse effect could appear during and between 4 - 10 min after the parenteral administration of the first dose of vancomycin. The reaction could disappear after 20 min or persist for

various hours. The severity of the reaction to vancomycin is proportional to the dose administered and the velocity of infusion (Sivagnanam and Deleu, 2003).

Vancomycin induces hypersensitivity reaction (RMS) through IgE independent pathway (Kupstaité et al., 2010). The activation of priming cells or mastocytes, and basophilic leucocytes triggers a cascade of liberation of various pro-inflammatory and vasoactive substances such as histamine, leukotrienes, tumor necrosis factor (TNF α) and platelet activating factor. Histamine is liberated by the granules of priming cells and basophils due to different stimuli such as inflammatory processes, physical agents, drugs like morphine and vancomycin (López, 2009). Great varieties of medicines induce the liberation of histamine from the mastocytes in a direct form and without previous sensitization. All the substances that induce histamine secretion activate the secretory response of the mastocytes or basophils by increasing intracellular ion (Sivagnanam and Deleu, 2003).

Vancomycin is a prototype activator of priming cells or mastocytes, and seems to be partially dependent on calcium for the liberation of histamine and tryptase via mechanisms depending on phospholipase C (PLC) and phospholipase A2 (PLA2). This could be the result of direct action of vancomycin on PLC and PLA2 or by unknown receptor stimulation mechanism (Veien et al., 2000).

The amount of liberated histamine is associated with the velocity of administration and to the concentration of vancomycin in blood (Riedl and Casillas, 2003). Vancomycin prolongs the systemic effect of histamine liberation by inhibiting the action of N-methyltransferase, an enzyme responsible for histamine metabolism (Myers et al., 2012).

Shuto et al. (1999) reported in an *in vivo* and *in vitro* study that muscle relaxing medicines (pancuronium, vecuronium, and succinylcholin) and morphine increase the release of histamine when combined with vancomycin. The intravenous injection of vancomycin in rats (1.25 to 10 mM / 30 min) after morphine administration led to the increase in blood histamine level. These results suggest that vancomycin directly opens calcium Ca⁺⁺ channels and induces the release of histamine. On the other hand, it was believed that morphine activates G proteins. The release of intracellular Ca⁺⁺ induced by these two different mechanisms could contribute synergically to the release of histamine by priming cells or mastocytes. This is observed in the area below histamine curve in correlation to the severity of vancomycin induced erythema. These results show experimental evidences that the combination of muscle relaxing medicines or morphine with vancomycin could increase the risk of anaphylactoid reaction for the increase in the liberation of histamine (Shuto et al., 1999). In 1990, Healy et al. suggested that histamine receptors begin to desensitize due to previous

vancomycin exposition and/or other cofactors such as serotonin, bradykinin, cyclic GMP, and PGD2. Moreover, interindividual differences in the sensitivity of histamine receptors could explain why some people release great amount of histamine without presenting a significant reaction with the administration of vancomycin (Healy et al., 1990).

EPIDEMIOLOGY

One of the causes of morbi-mortality in the world is adverse reaction of the medicines provoked by immune and non-immune mechanisms, where iatrogenic represents 5 -15%. In the United States of America, more than 100,000 deaths are attributed to ARM. ARM, including hypersensitivity reactions to medicines, is among the fourth or sixth largest causes of death (Lazarou et al., 1998). Hypersensitivity reactions to medicines (RAM) are seen in 10 - 20% of hospitalized patients (one-third of all the adverse reactions to medicines) and more than 7% of the general population (Limsuwan and Demoly, 2010).

In a retrospective study carried out in a series of 650 children who received vancomycin (12.9 mg/K/dose), 11 cases of RMS were reported accounting to a prevalence of 1.6%. Two of the 11 cases were children less than 8 years old (Levy et al., 1990). On the other hand, in a study of 224 patients (110 males and 114 females) with age range of 19 - 56 years, who were treated with vancomycin for 7.5 \pm 9.3 days, the global incidence of adverse reactions was 3 - 6% while the incidence on healthy volunteers who received 1 g of vancomycin in infusion was 80 - 90%. Polk (1991) suggested that the patients who received vancomycin could present less risk of adverse reaction than healthy volunteers possibly for the liberation of histamine that accompany the infection process, malignancy or renal insufficiency.

In a study carried out in healthy volunteers (vancomycin 1g/60min), the incidence of ARM was 80 - 95 and 30% in patients treated with vancomycin 1 g / 120 min. One possible reason for this result is that the infection induced a certain amount of histamine as part of natural immune response that increased histamine concentration which could be associated to a downward regulation of the effect of vancomycin in the mastocytes and basophilic leukocytes. Literature reports indicated that a more severe reaction occurs in patients less than 40 years old, specifically in children (McAuley, 2012). RMS seems to occur with the same frequency not only in men but also in women. The incidence of clinically significant cutaneous reaction related with infusion of vancomycin in patients was less than 5%. Age was a risk factor and could explain the high incidence of 6% in youths with prolonged therapy (Kupstaité et al., 2010). In another study of 20 children that received 15 mg/kg of vancomycin for 60 min, the patients were monitored along infusion time with the finding that 7 of the children

(35%) developed anaphylactoid reaction induced by glycopeptides, presented hypotension. RMS had an incidence of 3.7 to 47% in patients treated with vancomycin and higher than 90% in healthy patients. In summary, the estimation of RMS incidence widely varies in the studies but all reflect coincidence in the infusion velocity, vancomycin concentration, definition of reaction, observation diligence, study design, inclusion criteria (healthy volunteers or patients), and concomitant medication (Korman et al., 1997).

PREVENTION AND TREATMENT OF RED MAN SYNDROME

In the recent decades, increase in antimicrobial resistance has been observed. It is believed that 25 - 50% of all prescribed antimicrobials are inappropriate with respect to the selection of the medicine, administration doses, and the duration of treatment (Junior et al., 2007). In a random trial of 33 patients treated with vancomycin (1 g/ 60 min) who were previously administered oral diphenhydramine (50 mg), it was found that none of the patients presented RMS. The reaction was seen in 47% of the patients who did not receive pretreatment with diphenhydramine (Wallace et al., 1991).

Fast infusion of vancomycin provokes histamine mediated effects. In a random study of fast infusion of vancomycin (1 g/ 10 min) in 30 pre-operative patients, oral premedication of antihistamines reduced the incidence and severity of RMS. In this study, oral diphenhydramine (≤ 1 mg / kg) administered for 1 h and oral cimetidine (≤ 4 mg / kg) also for 1 h before the infusion were used. The findings were that 50% of the patients treated with placebo developed hypotension while none in the group treated with antihistamines suffered this, although one patient in the latter group complained of mild scratches (Renz et al., 1998). In majority of the patients receiving first-time treatment with vancomycin at an infusion velocity of ≤ 10 mg/min, premedication with antihistamines for prevention of RMS is not needed. In general, at a dose of ≤ 500 mg/h, or 500 mg to 1 g administrated for more than 2 h, premedication is not necessary. However, an even lower infusion velocity is advised for patients receiving treatment of opioids or other medicines that predispose the activation of priming cells or mastocytes (Kupstaité et al., 2010). Therefore, RMS could be prevented by decreasing the infusion velocity of vancomycin for at least 1 h (maximum velocity of 1 g for 90 min), monitoring of blood pressure during the infusion, and administration of premedication of antihistamines that block H1 and H2 receptors. The combination of H1 and H2 antagonists is more effective than using only H1 (Sivagnanam and Deleu, 2003). Wazny and Daghigh (2001) suggest that a vancomycin desensitization should be considered for severe RMS and anaphylactic reactions, that is not responding to

usual measures, when substitution of another antibiotic is not feasible. A rapid desensitization is preferred as it is effective in the majority of patients, in patients who fail to desensitize rapidly; a slow desensitization protocol may be tried (Wazny and Daghigh, 2001).

VANCOMYCIN AND RISK FACTORS FOR HYPERSENSITIVITY REACTION DEVELOPMENT

The treatment of systemic infections with vancomycin requires the intravenous administration of the medicine, usually by intermittent infusion due to its minimal absorption when orally administered (Revilla, 2009). The interindividual variability of a population in terms of efficacy and safety of therapeutic agents depends on one part on the following factors: age, sex, weight, renal and hepatic functions, co-medication, heterogeneity of the sicknesses, and nutritional state. With respect to the influence of physiologic factors, age is the most important since the expression of maturity state affects pharmacokinetic of medicines suggesting the necessity of establishment of strategies for dose adjustment (Balboa and Rueda, 2004). In newborns, there is a relation between depuration of vancomycin, gestational and postnatal age (Anderson et al., 2007). There are differences in the pharmacokinetic parameters of vancomycin for age effect. In premature (gestational age ≤ 32 weeks), the depuration of vancomycin is less due to a lower glomerular filtration capacity. It is known that filtration and depuration volumes proportionally increase with age due to maturity of the elimination system. The distribution volume is relatively higher in children than in adults for the presence of a higher percentage of water.

Intravenous administration of vancomycin with a unique dose of 500 mg in adults reaches plasma concentrations of 6 to 10 mg/100 ml in 1 or 2 h. The half-life ($T_{1/2}$) of vancomycin varies depending on the age group. In newborns, it is from 6 - 10 h (10 - 15 mg/kg every 6 - 18 h depending on the age). In infants of 3 - 4 years old, it is 4 h. In children older than 4 years, it is 2.2 - 3 h while in adolescents, it has not been defined. However, there is a hypothesis that puts it at the same with infants (doses for infants, children, and adolescents 10 mg/kg every 6 h) and 5 - 8 h in adults (doses of 15 - 20 mg/kg every 6 - 12 h) (Broome and So, 2011). The factors that affect the clinical activity of vancomycin are its tissue distribution, the inoculation site, the bond to protein, and the infusion velocity (Rybak, 2006). The efficacy and toxicity are related to the plasma concentration of the drug. For this, the present clinical guide recommends maintaining the minimal concentration (C_{min}), determined at the end of administration interval, at a therapeutic range of 5 and 10 μ g/ml, and maximum concentration (C_{max}), determined 3 h after intravenous administration, at not more than 40 μ g/ml. Apart from RMS, other principal adverse reactions of vancomycin include ototoxicity, nephrotoxicity, and

phlebitis (Rocha et al., 2002).

The kinetic behavior of vancomycin could be modified by different clinical and physiologic factors. The penetration of vancomycin into cerebrospinal fluid is favored by the presence of meningeal inflammation, but the variability of the concentration and minimal inhibitory concentration (MIC) that should be reached needs intraventricular or intrathecal administration in the case of central nervous system infections. Also, access of vancomycin to lung tissue is variable. For this reason, its use by inhalation has been in promotion (Revilla, 2009).

Epidemiological data report the existence of factors increasing the risk of adverse effects such as concomitant sickness like asthma, erythematous lupus, the use of beta blockers, and drug administration. The most important risk factors related to drug hypersensitivity reactions are associated to the chemical properties and to the molecular weight of the drug (Riedl and Casillas, 2003; Schnyder, 2009). The presence of scratch during the administration of vancomycin in infant population could be a sign of alarm indicating the presence of peripheral vasodilatation. This could be opportunely detected in patients that are at a risk of presenting hypotension. The lack of hemodynamic and respiratory changes in patient treated with β -blockers before any surgery probably show that these agents could confer protection against anaphylactoid reactions mediated by histamine liberation (Bertolissi et al., 2002). Women present values of distribution volume greater than men. The difference is even much greater in case of obese women. These data suggest that vancomycin distribution in fat is higher in female. The influence of obesity in the kinetic behavior of this antibiotic has been established showing an increase in the distribution volume and depuration of vancomycin (Penzak et al., 1998).

Conclusion

The red man syndrome is an idiosyncratic phenomenon and does not depend on concentration of vancomycin. To avoid the possible presentation of RMS induced by vancomycin, it is necessary to administrate the drug at an infusion time of at least 1 h. The benefit of antihistaminic prophylaxis to reduce the incidence and severity of RMS, and elaboration of specific guide for its use should be considered.

ABBREVIATIONS

MRSA, Methicillin resistant *Staphylococcus aureus*; **ARM**, adverse reaction to medicines; **IgE**, immunoglobulin type E; **RMS**, red man syndrome; **TNF**, tumor necrosis factor; **PLC**, phospholipase C; **PLA2**, phospholipase A2; **MIC**, minimal inhibitory concentration.

REFERENCES

- An SY, Hwang EK, Kim JH, Kim JE, Jin HJ, Jin SM, Kyun JO, Lee YH, Park HS, Choi YW, Lim SK, Ye YM (2011). Vancomycin-associated spontaneous cutaneous adverse drug reactions. *Allergy Asthma Immunol. Res.* 3(3):194-198.
- Anderson BJ, Allegaert K, Van den Anker JN, Cossey V, Holford NH (2007). Vancomycin pharmacokinetics in preterm neonates and the prediction of adult clearance. *Br. J. Clin. Pharmacol.* 63(1):75-84.
- Balboa de Paz F, Rueda ES (2004). Características farmacocinéticas de los fármacos en la edad pediátrica. *AFT.* 2(2):89-98.
- Bertolissi M, Bassi F, Cecotti R, Capelli C, Giordano F (2002). Pruritus: a useful sign for predicting the haemodynamic changes that occur following administration of vancomycin. *Crit Care.* 6(3):234-239.
- Bischoff SC (2007). Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* 7(2):93-104.
- Broome L, So TY (2011). An evaluation of initial vancomycin dosing in infants, children, and adolescents. *Int. J. Pediatr.* 2011:470364.
- Brown AFT (2009). Current management of anaphylaxis. *Emergencias.* 21:213-223.
- Edwards IR, Aronson JK (2000). Adverse drug reactions: definitions, diagnosis, and management. *Lancet.* 356(9237):1255-1259.
- Farnam K, Chang CH, Teuber S, Gershwin ME (2012). Nonallergic drug hypersensitivity reactions. *Int Arch Allergy Immunol.* 159(4):327-345.
- González EC, Medina TJ, Benítez GJP (2005). Transduction signals in mast cells. Mechanisms controlling the degranulation and cytokines synthesis differential. *MENSAJE BIOQUÍMICO.* XXIX:225-240 (<http://bq.unam.mx/mensajebioquimico>).
- Healy DP, Sahai JV, Fuller SH, Polk RE (1990). Vancomycin-induced histamine release and "red man syndrome": comparison of 1- and 2-hour infusions. *Antimicrob. Agents Chemother.* 34(4):550-554.
- Junior MS, Correa L, Marra AR, Camargo LF, Pereira CA (2007). Analysis of vancomycin use and associated risk factors in a university teaching hospital: a prospective cohort study. *BMC Infect. Dis.* 7:88.
- Korman TM, Turnidge JD, Grayson ML (1997). Risk factors for adverse cutaneous reactions associated with intravenous vancomycin. *J. Antimicrob. Chemother.* 39(3):371-381.
- Kupstaitė R, Baranauskaitė A, Pileckytė M, Sveikata A, Kadusevicius E, Muckienė G (2010). Severe vancomycin-induced anaphylactic reaction. *Medicina (Kaunas).* 46(1):30-33.
- Laroche D, Vergnaud MC, Sillard B, Soufarapis H, Bricard H (1991). Biochemical markers of anaphylactoid reactions to drugs. Comparison of plasma histamine and tryptase. *Anesthesiology.* 75(6):945-949.
- Lazarou J, Pomeranz BH, Corey PN (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA.* 279(15):1200-1205.
- Levy M, Koren G, Dupuis L, Read SE (1990). Vancomycin-induced red man syndrome. *Pediatrics.* 86(4):572-580.
- Limsuwan T, Demoly P (2010). Acute symptoms of drug hypersensitivity (urticaria, angioedema anaphylaxis, anaphylactic shock). *Med. Clin. North Am.* 94(4):691-710.
- López PN (2009). Study of genetic polymorphism of a single nucleotide not synonymous with the diamine oxidase (REFSNP ID RS1049793) in Crohn's disease. PhD, Complutense University, Spain. pp. 22-24.
- McAuley MA (2012). Allergic reaction or adverse drug effect: correctly classifying vancomycin-induced hypersensitivity reactions. *J. Emerg. Nurs.* 38(1):60-62.
- Myers AL, Gaedigk A, Dai H, James LP, Jones BL, Neville KA (2012). Defining risk factors for red man syndrome in children and adults. *Pediatr. Infect. Dis. J.* 31(5):464-468.
- Núñez BA, Morales RC, Rivera MM, González GA (2006). Vancomycin. *A Winner Expired. Medicrit.* 3(6):136-138.
- Payne V, Kam PC (2004). Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia.* 59(7):695-703.
- Penzak SR, Gubbins PO, Rodvold KA, Hickerson SL (1998). Therapeutic drug monitoring of vancomycin in a morbidly obese patient. *Ther. Drug Monit.* 20(3):261-265.
- Polk RE (1991). Anaphylactoid reactions to glycopeptide antibiotics. *J.*

- Antimicrob. Chemother. 27(Suppl B):17-29.
- Pootoolal J, Neu J, Wright GD (2002). Glycopeptide antibiotic resistance. *Annu. Rev. Pharmacol. Toxicol.* 42:381-408.
- Renz CL, Laroche D, Thurn JD, Finn HA, Lynch JP, Thisted R, Moss J (1998). Tryptase levels are not increased during vancomycin-induced anaphylactoid reactions. *Anesthesiology.* 89(3):620-625.
- Revilla CN (2009). Pharmacokinetic-pharmacodynamic analysis of vancomycin in UIC patients PhD, Salamanca University, Spain. p. 34
- Riedl MA, Casillas AM (2003). Adverse drug reactions: types and treatment options. *Am. Fam. Physician.* 68(9):1781-1790.
- Rocha JL, Kondo W, Baptista MI, Da Cunha CA, Martins LT (2002). Uncommon vancomycin-induced side effects. *Braz. J. Infect. Dis.* 6(4):196-200.
- Rybak MJ (2006). The pharmacokinetic and pharmacodynamic properties of vancomycin. *Clin. Infect. Dis.* 42(Suppl 1):S35-S39.
- Schnyder B, Pichler WJ (2009). Mechanisms of drug-induced allergy. *Mayo Clin. Proc.* 84(3):268-272.
- Shuto H, Sueyasu M, Otsuki S, Hara T, Tsuruta Y, Kataoka Y, Oishi R (1999). Potentiation of vancomycin-induced histamine release by muscle relaxants and morphine in rats. *Antimicrob. Agents Chemother.* 43(12):2881-2884.
- Sivagnanam S, Deleu D (2003). Red man syndrome. *Crit. Care* 7(2):119-120.
- Svetitsky S, Leibovici L, Paul M (2009). Comparative efficacy and safety of vancomycin versus teicoplanin: systematic review and meta-analysis. *Antimicrob. Agents Chemother.* 53(10):4069-4079.
- Veien M, Szlam F, Holden JT, Yamaguchi K, Denson DD, Levy JH (2000). Mechanisms of nonimmunological histamine and tryptase release from human cutaneous mast cells. *Anesthesiology.* 92(4):1074-1081.
- Wallace MR, Mascola JR, Oldfield EC 3rd (1991). Red man syndrome: incidence, etiology, and prophylaxis. *J. Infect. Dis.* 164(6):1180-1185.
- Wazny LD, Daghigh B (2001). Desensitization protocols for vancomycin hypersensitivity. *Ann Pharmacother.* 35(11):1458-1464.

Review

An emergency response plan to control emerging infectious disease hazards in Taiwan's Hospitals

Hung-Chang Liao^{1,2}, Lien-Hsiung Lee³, Shao-Ping Yuan^{2,4} and Ya-huei Wang^{2,5*}

¹Department of Health Services Administration, Chung Shan Medical University, Taiwan.

²Department of Medical Education, Chung Shan Medical University Hospital, Taiwan.

³Institute of Occupational Safety and Health, Council of Labor Affairs, Executive Yuan, Taiwan.

⁴Institute of Medicine, Chung Shan Medical University, Taiwan.

⁵Department of Applied Foreign Languages, Chung Shan Medical University, Taiwan.

Accepted 10 June, 2013

This study is a proposal for a framework for an emergency response plan (ERP) to tackle emerging infectious diseases (EIDs) in order to reduce possible hazards to medical personnel. This ERP was formulated and amended based on the PDRA-Cycle mechanism, which was developed from Deming's PDCA Management Cycle (1986). A total of 11 emergency response management items were drawn up using document analysis and in-depth interviews to facilitate the making of applicable standard operation procedures (SOPs) for each hospital. This study includes key recommendations for the implementation of ERP, specifically for the government and hospital administrations, as follows: 1) The existing Labor Health and Safety Law should be amended to include specific guidelines for biological hazards; 2) labor should be coordinated and divided between infection control departments and occupational health and safety departments to jointly prevent the transmission of EIDs; 3) hospitals should set up SOPs based on the emergency response framework proposed in this study; 4) hospitals should establish a risk-assessment mechanism for EID hazards; 5) hospitals should strengthen the training, education and practice programs required to prevent EID hazards; and 6) hospitals should strengthen communication/coordination, setting up a framework and a center for incident command structure to deal with various emergency activities regarding EIDs.

Key words: Emergency response plan (ERP), emerging infectious disease (EID), occupational health and safety, infection control, hospital management, biological hazard.

INTRODUCTION

The phrase "emerging infectious diseases (EIDs)" refers to infectious diseases that have increased in incidences in the past two decades, or that may affect humans in the future. Each occurrence of an emerging or reemerging infectious disease causes great damage to a nation's and/or the world's economies and the lives of its citizens. Cases such as the outbreaks of the Ebola virus (Hoenen et al., 2006), H5N1, the avian influenza virus in Hong Kong (Li et al., 2004), the Nipah virus in Malaysia (Adam, 1999), the West Nile virus (Mostashari et al., 2001), and

severe acute respiratory syndrome (SARS) in China, Hong Kong, Singapore, Vietnam, Taiwan and Canada in 2002, are all examples of EIDs (Centers for Disease Control and Prevention, 2005). EIDs are highly unpredictable, because it is unknown when, where or what infectious disease may occur, so no specific corresponding preparation may be taken in advance (Su et al., 2003; Wilson, 1999; Alleyne, 1998). Michael et al (2012) studied International Health Regulations in reporting WHO for EIDs and they found that by setting up

*Corresponding author. E-mail: yhuei@csmu.edu.tw.

more prescriptive criteria, the validity could be improved. However, the criteria should be adjusted in order to adapt to future unknown EIDs' threats. Attacks of EIDs in hospitals and among healthcare workers are far-reaching. Hospitals in the post-SARS era must have proper and complete response measures. There must be a well-structured standard operating procedure (SOP) for each department in a hospital: whether it is the infectious response organization, personnel training, logistic planning and mobilization, infectious disease operation standards, staff protection measures, or others, action must be taken to prevent the re-emergence of infectious diseases so that hospitals are not part of the transmission chain. According to studies (Wilson, 1999; Alleyne, 1998), when there is an EID outbreak, about 3 to 5 percent of patients contract nosocomial infections, leading to longer hospital stays which consume limited hospital resources, expand the actual disease incident and mortality rates, and potentially increase the pressure on hospital healthcare cost controls. Furthermore, the surge of EIDs, the re-emergence of existing infectious diseases, and the increase of multi-drug-resistant bacteria are demonstrating the importance of infectious disease control and the development of SOP guidelines to hospitals (Gamage et al., 2005; Lu et al., 2006; Carrico et al., 2008; Rebmann, 2009a).

Effective emergency response strategies are indispensable in reducing EID transmission in hospitals. Although EIDs are not a prevalent threat, their occurrence often causes panic in a community; consequently, hospitals must invest a considerable amount of resources to curb the crisis brought on by the EIDs, and develop effective emergency response plans (ERPs) to minimize the damage. Rebmann (2009c) points out that hospital emergency management plans not only stop the transmission of infectious diseases—more importantly, they also reduce healthcare workers' fears of becoming infected. However, the establishment of an ERP must be supported within the concept of risk management. Based on the Australian Standard (AS/NZS 4360: 1999), risk control is defined as "part of risk management, which involves the implementation of policies, standards, procedures and physical changes to eliminate or minimize adverse risks".

The purpose of risk control is to reduce the damage caused by catastrophic hazards because the occurrence of a disaster may result in human casualties, financial ruin or environmental damage. Formulating a plan that encompasses staff, facilities and management thus has far-reaching impact for hospitals.

In this study the researchers aim to design and develop an emergency response framework to minimize the probability of healthcare workers becoming infected when a hospital is experiencing an EID outbreak. The results of the study may be used as a reference for hospitals to formulate EID hazard management plans, and the emergency response components proposed in this study may be used to compile more detailed SOPs.

RESEARCH METHODS

The research methods used in this study are document analysis and in-depth interviews, described as follows:

Document analysis

A literature review and document research were conducted for this study. Journals and professional reports were the focus of the literature review and were used to understand EID hazard management measures used overseas. Document research focused on infectious control guidelines and ERPs provided by the World Health Organization (WHO), the U.S. CDC, CDC Taiwan, the Institute of Occupational Safety and Health, and by ten different hospitals in Taiwan.

In-depth interviews

This study conducted in-depth interviews with a total of 15 hospital staffers, including infectious disease specialists, infection control personnel, and occupational safety and health management staff. The interviews primarily focused on the tasks of the infection control department, and the occupational health management procedures, protocols, and tasks of each hospital during the SARS outbreak, in order to gain insights from key personnel from each hospital concerning EID hazards and gather their insights on system design, which was then used as the basis for an emergency response framework.

Formulating and revising the ERP-The PDRA

After reviewing and analyzing the emergency response documentation developed by various hospitals, the researchers selected the management cycle theory proposed by Deming (1986) in which he suggested that a planned management action, *PDCA* (Plan, Do, Check, Action), should be implemented. The *PDCA* is illustrated in Figure 1.

In this approach, *P* stands for plan: all management activities begin with planning, and only with a detailed plan can obstructions to implementation be minimized. *D* stands for do: a perfect plan relies on its execution, and Deming (1986, 1993) stresses that execution is necessary to follow a plan through to completion. *C* stands for check, checking the result of doing: administrators should have a checklist to monitor how many elements of an ERP had been implemented, and to identify obstacles or items that require improvement. *A* stands for improvements and corrections made to the checked results and the management activities after implementation. The *PDCA* management cycle is thus repeated, each time bringing it closer to the goal and achieving the best management results. To apply the *PDCA* approach to formulating and

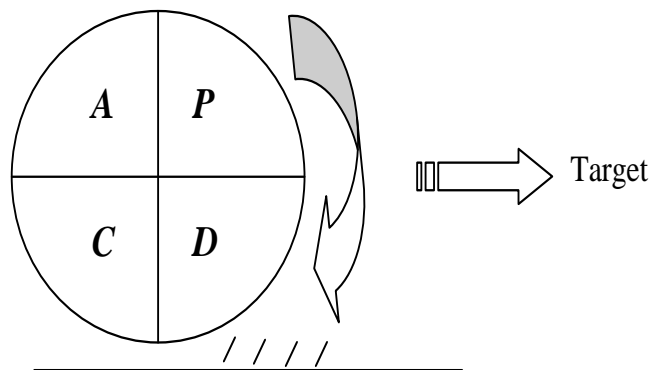


Figure 1. PDCA-management cycle.

improving hospital ERPs, the researchers drew up a PDRA Emergency Response diagram, shown in Figure 2. The PDCA approach was modified to PDRA in this study, where C (Check) was replaced by R (Research) for research improvement. Its significance lies in that infectious control personnel and occupational health management staff must continuously research and absorb new information in the process of handling EID hazards. For example, the information gathered from the websites of organizations such as the WHO, the U.S. CDC, the CDC in Taiwan, and other professional medical associations, is used to educate the public and to plan amendments. Furthermore, infectious control personnel are constantly involved in computing the infection rates of various viruses for each clinic, ward, and department, to help find possible infection sources and prevention measures with the help of statistical analysis. These tasks are beyond the description of Checking, and are more in line with Research.

The design of the PDRA diagram below is based on the idea of loss control: a hospital is represented in the center as the main structure, surrounded by an inner circle and an outer circle, indicating that an emergency response mechanism activated during an EID outbreak will perform as an elaborate protection system to defend healthcare workers from infection. If a case of nosocomial infection occurs, this emergency response mechanism will contain the damage within the hospital, preventing the spread of the illness to other hospitals or to the public and thus avoiding panic. Arrows of the inner circle point to PDRA, while the outer circle contains the emergency response framework and its components.

Prior to field-testing the framework, experts in the relevant fields were convened for a forum discussion, and thereafter the emergency response management framework was divided into 11 components: 1) emergency response measures; 2) nosocomial infection control of the healthcare unit; 3) patient transfer within the hospital, patient transfer in and out, and patient transport; 4) hospital staff protection; 5) fever screening operation; 6) quarantine area and (negative pressure) isolation ward

infection control; 7) protection and safety measures; 8) logistic support operations; 9) training education; 10) audit procedures; and 11) occupational hazard compensation. The P in PDRA means that hospitals must formulate more detailed SOPs, while following the 11 components of the outer circle of this study. D means that ERP drills, or the activation of the emergency response mechanism, must be managed by observing SOPs. R means that if an SOP needs improvement after going through the above-mentioned D process, the infectious control department or occupational health department should study and continuously improve the SOP. A means that in order to verify the ease of implementation of the amended SOP, simulations and drills must be conducted. The 11 components are assessed and improved by using the PDRA periodical cycle mechanism, and include a feedback mechanism. The outer circle indicates that although each of the 11 components operates independently, situations may arise where one can impede another. As such, mutual assistance and support are required to maintain the integrity of the goal.

Formulating the emergency response framework and its components

To further understand the content encompassed in the 11 components, in-depth interviews were conducted. Summaries of these interviews are listed below.

Item 1: What are the top components of the emergency response measures (ERM)?

The ERM in hospitals is determined by top management. In an emergency, the hospital staff would be divided into a logistics group, a medical group, an infection group and other groups, based on their job assignment. Most importantly, there must be clearly defined authority and responsibility.

There must be a clearly defined command center with full-time secretaries who can keep abreast of the immediate situation within the hospital. There must be a complete and thorough reporting process such as the 5W1H (Why, What, Where, When, Who, How) method, and the reporting protocol must be clearly defined.

There must be a drill template setup for the ERM, so that confusion can be avoided when a crisis occurs. Hospitals must set up a quarantine area to avoid transmission between healthcare workers and patients. When hospitals cannot handle infectious patients, a relevant medical care plan may be required. Perhaps patients could be sent to a dedicated hospital, experts could be invited for assistance, or an emergency medical team could be called to help. The ERM should be revised periodically, must comply with the WHO's or the CDC's EID monitoring system, and drills must be conducted so that the staff can cope with the impact of the next wave.

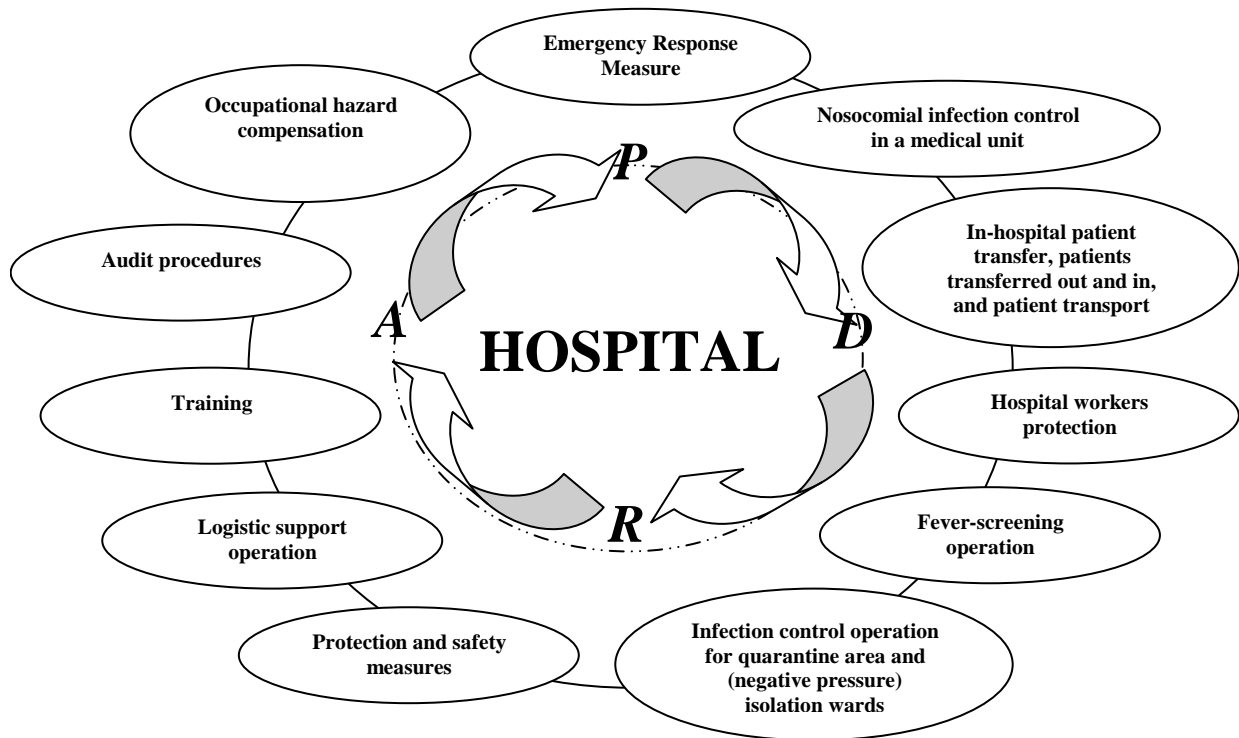


Figure 2. PDRA of the emergency response framework.

Item 2: What should be included in the nosocomial infection control plan in a healthcare unit?

Departments should be separated based on their infection probability to avoid confusion.

The emergency room, being the frontline of the impact, is particularly important. Additionally, the hemodialysis room, burn unit, maternity ward, nursery, intensive care units (ICU), operating rooms, pediatric ICU, respiratory care wards, laboratory unit, pathology unit, and radiology unit are all highly susceptible to infection and must have special attention.

The outpatient clinic, general wards, supply center and nutrition department are less susceptible, but they could also be part of the viral transmission chain and will need special attention.

The morgue is also very important. It has been reported that workers at a mortuary contracted SARS when handling the remains of a SARS patient.

Item 3: What should be included in the guidelines for patient transfer within the hospital, patient transfer in and out of the hospital, and patient transport?

Patients with suspect infections may be transferred to isolation wards. It is possible that patients with suspect infections could be transferred in from areas outside of the hospital.

If the hospital is not a dedicated hospital for EID, and is

visited by patients with suspected infections, these patients must be transferred to a dedicated hospital for care.

Personnel responsible for transporting specimens taken from hospitalized patients with suspect infections must take precaution to avoid contamination. Precaution should also be taken when taking X-rays of these patients. A mobilized X-ray machine is preferable; however, a hospital without such equipment must have an operating protocol in place to avoid contamination.

Item 4: What kind of personal protection equipment (PPE) should be provided for hospital staff?

Proper use of PPE played an important role in protection during the SARS outbreak. During the outbreak, government agencies issued equipment to hospitals, including a variety of facemasks and other protective equipment, which many healthcare workers did not know how to use correctly.

When several employees come down with fevers, it indicates the possibility of a nosocomial infection outbreak. These cases must be handled carefully. Other people with whom the affected employee(s) may have come into contact must be traced, body temperatures checked and reports submitted. Relevant operation standards must be clearly defined.

Outsourced staff and pharmaceutical representatives go

in and out of various units in the hospital. They can easily become part of the viral transmission chain. Their non-hospital employee status makes them blind spots in a disease-prevention plan, so relevant guidelines must be developed to handle non-hospital employees.

Item 5: What processes are to be included in the fever-screening operation?

Fever-screening centers should be established and SOPs developed. During the outbreak of SARS, fever-screening centers were established to prevent the shutdown of emergency rooms due to contamination.

Outpatient nurses must be trained to handle patients with fevers, or lead them to the fever-screening center. If patients are confirmed as suspected infection cases after the screening process, they should be assisted with hospitalization. The ER should be divided into two areas: a general area and a quarantine area. Patients with fevers should be in the quarantine area, in case they are confirmed to be suffering from an infectious disease.

Item 6: What should be included in the infection control operation for the quarantine area and negative-pressure isolation wards?

Hospitals must isolate an area for quarantine, and have an effective control protocol to manage patients and healthcare workers. Thorough disinfection and wearing PPE are absolutely required when entering negative-pressure isolation wards. The protocol for this procedure must be strictly enforced. Isolation wards must be regularly disinfected. A strict infection-control operation standard is required. Moving between isolation wards will require a set of protocols to avoid escalating the suspected cases into confirmed cases.

Item 7: What should be included in protection and safety measures?

There must be a system for patient monitoring, and for keeping abreast of the nosocomial infection issues. Data analysis should also be conducted to detect potential outbreaks or epidemics. Once detected, any incident must be immediately investigated. Environmental facilities and medical equipment that may be contaminated or pose a potential threat to health should also be monitored. Fever-screening stations and negative-pressure isolation wards must be established.

Item 8: What should be included in a logistic support operation?

The handling of clothing, blankets, bed sheets, and

hospital gowns of patients with suspected infections is utterly important. The dirty clothing of the patients in the isolation wards must be disinfected before it is taken out of the ward. Process procedures are required to prevent viral transmission via clothing.

There should be a stricter procedure than the general rules for handling hospital waste and infected objects: for example, waste collectors should also wear PPE, and waste and infected objects should be sprayed with high concentrations of disinfectant before collection.

Cautions should be taken for terminal disinfection of the isolation ward. There must be a strict disinfecting operation procedure in place to eradicate any residual virus.

When ERM is activated, special attention must be given to material supplies and control management. For instance, during the SARS outbreak, some healthcare workers had to supply their own facemasks, while there was a significant shortage of protection gear supplies on the open market. Necessary medicine and medical equipment should be delivered on time once a request is submitted.

Particular attention must be taken in the handling of suspect patients' remains. After the remains are placed in body bags, they should be sprayed with disinfectant. It is preferable to double-bag the remains to avoid contaminating mortuary personnel.

Item 9: What should be included in the training education of the ERM?

The ERM drill should be held at least once every six months, or upon a plan being amended. However, to work around busy schedules, each unit may submit its own drill schedule and supervision may be arranged accordingly. The ERM drill should be designed with various conditions in mind, specifically the use of protection gear. The PPE worn by healthcare workers in quarantine areas, negative-pressure isolation wards, and general areas varies. Proper usage of PPE is an indispensable element in epidemic prevention and should be taught in advance.

There should be a standardized process for checking body temperature. Staff responsible for checking temperatures should be able to operate and read the equipment correctly. It is also very important for the healthcare workers to monitor their own temperatures. Workers should be trained to correctly mix and use disinfectants.

Item 10: What should be included in auditing procedures?

There should be a performance-assessment system to check the effectiveness of the implementation.

The assessment system should include internal and external audits, as well as corrective measures and post-correction assessment methods.

Item 11: What should be included in occupational hazard compensation?

Occupational hazard compensation should comply with relevant occupational hazard regulations, which include the Labor Standards Act (2011), Enforcement Rules of the Labor Standards Act (2009), and the Act for Protecting Workers of Occupational Accidents (2001). Summarizing the above, the emergency response framework and its components are presented in Table 1.

DISCUSSION

To prevent EID hazards, discussion on how government and hospitals could implement the ERP are proposed in this study:

Regulations on the handling of biological hazards should be added to the existing Labor Safety and Health Act (2002)

Article 1 paragraph 7, and article 2 of the current Labor Safety and Health Act require that employers provide the necessary safety and health facilities and measures for handling biological pathogen hazards (Council of Labor Affairs, 2002). However, the Act lacks relevant emergency response guidelines, which means that hospitals can only apply nosocomial infection control measures for the prevention of EID hazards. The existing infection control measures have blind spots, that is, when sources of EID hazard are unclear, the means of controlling infection may be inadequate (Rise and Fall of Diseases, 1993; Chang, 2003). As mentioned in the editorial "Rise and Fall of Diseases" (Rise and Fall of Diseases, 1993), EID hazards and relevant information (that is, alerting or not alerting the public to the threat) must be controlled with an epidemiological approach. There is no "gold standard" of quickly acquiring relevant information. Chang (2003) points out that the speed and accuracy of diagnosing SARS is an important factor in controlling an epidemic; thus a rapid screening test method is necessary. Obviously, EID hazard management refers only to the application of infection-control methods. Before EID hazards can be identified, the discrepancies between the various infection control methods must be bridged by an effective ERP.

The division of labor between infection control departments and occupational health departments must be coordinated to prevent the outbreak of EIDs

Bryce et al. (2008) suggest that good resource allocation among a hospital's infection control department and occupational health department will promote healthcare workers' understanding of infection prevention practices.

Bryce et al. (2008) research indicates that infection control departments and occupational health departments perform core tasks of EIDs prevention. Infection control departments' tasks to prevent EIDs currently include: establishing an infection-control monitoring system and infection-control measures; using an epidemiological approach to investigate and process nosocomial infection incidents; providing information for internal hospital inquiries concerning nosocomial infections; implementing nosocomial infection monitoring and data analysis; and reporting to the relevant authorities concerning the status of nosocomial infection (Rebmann, 2009). The responsibilities of occupational health departments with respect to EID prevention focus on effectively blocking the transmission of pathogens and performing successful risk management, such as: 1) formulating a respiratory protection plan; 2) assisting employees in selecting protective gear to avoid getting infected, such as goggles, gloves, isolation gowns, face masks, and face shields; 3) assisting in the design of negative-pressure isolation wards or negative-pressure workplaces to avoid spreading biological pathogens within the hospital; 4) formulating an ERP so that each hospital department knows how to respond to pathogens in a "rapid" transmission mode; and 5) formulating training education plans so that employees understand the hazards of biological pathogens and their response measures (Chang, 2003). There seems to have been little literature concerning how an occupational health department can contribute to the prevention of EID hazards before the outbreak of SARS. However, since the outbreak of SARS, prevention measures originally designed for occupational hazards (such as the selection and use of PPE, and the industrial ventilation technique, which was adopted in the design of negative-pressure wards) have become effective methods of blocking the spread of EIDs. Subhash and Radonovich (2011) point out that future EID prevention programs may adopt the methods used in the 2003 SARS outbreak, and that prudent planning used in the design of negative-pressure wards successfully blocked the transmission of SARS.

Loss control is an important concept in occupational health management (Badri et al., 2012). A hospital's management measures in responding to EID hazards may include occupational prevention measures such as those used in a factory against the hazards of unknown chemical agents. By introducing effective ventilation techniques and selecting and using proper PPE, a disease may be controlled. In the selection and the usage of PPE, the factory know-how may: 1) introduce healthcare workers to the correct method of using face masks, directing their attention to the different grades of the product and the snug fit of the masks; 2) provide healthcare workers with information on identifying various masks and selecting the appropriate mask to avoid inadequate protection; and 3) advise healthcare workers on protection gear issues and recommend tighter protection measures when providing high-risk medical

Table 1. Emergency response management framework and its components.

Emergency response management framework	Components
Emergency Response Measure	Emergency response organization personnel grouping and responsibility
	Emergency response command center
	Emergency response reporting procedure
	Medical care and recovery plan
	Closure or partial closure plan
	In-hospital traffic planning and quarantine area set-up
	Emergency response drill script
Nosocomial infection control in a medical unit	Emergency response plan revisions
	Infection control of entire hospital
	Infection control of general wards
	Infection control of ICU
	Infection control of surgical rooms
	Infection control of ER
	Infection control of clinic department
	Infection control of hemodialysis room
	Infection control of burn center
	Infection control of supply center
	Infection control of nutrition department
	Infection control of maternity wards
	Infection control of nursery
	Infection control of pediatric ICU
	Infection control of respiratory care wards
	Infection control of morgue
Infection control of laboratory	
In-hospital patient transfer, patients transferred out and in, and patient transport	Infection control of pathology department
	Infection control of radiology department
	In-hospital bed transfer process for patients with suspected infections
	Transfer-out process for patients with suspected infections
	Transfer-in process for patients with suspected infections
Hospital workers protection	X-ray process for hospitalized patients with suspected infections
	Specimen transporting process for patients with suspected infections
	The selection, usage, and maintenance of PPE
	Temperature checking and reporting
	Employee quarantine measures
	Medical treatment for employees with fevers
	Entering and leaving procedures for business related personnel
Fever-screening operation	Protection procedures for outsourced staff (i.e., caretakers, cleaners, laundry workers)
	Entering and leaving a fever screening center
	Fever-screening operation in outpatient clinic areas
	Fever-screening operation in ER areas
Infection control operation for quarantine area and (negative pressure) isolation wards	Hospitalization after fever- screening operation
	Infection control operation for quarantine area
	Infection control operation for isolation wards

Table 1. Contd.

	Entering and leaving various isolation wards
	Entering and leaving (negative-pressure) isolation wards
Protection and safety measures	Setting up a fever-screening station
	Setting up (negative-pressure) isolation wards
	Constructing monitoring systems
	Constructing an information exchange platform
Logistic support operation	Handling the remains of patients with suspected infections
	Handling the clothing of hospitalized patients with suspected infections
	Cleaning and handling infected sheets and gowns
	Handling waste and infected objects
	Final cleaning of isolation wards
Training	Logistic supply operation
	Emergency response plan training education
	Temperature checking training education
	Protection gear usage training education
	Disinfection process training education
Audit procedures	Performance assessment and monitoring
	Corrective measures and records
	Records and record keeping
Occupational hazard compensation	Audits
	The Labor Standards Act
	Enforcement Rules of the Labor Standards Act
	Act for Protecting Worker of Occupational Accidents

care (Chang, 2003). Maurer et al. (2003) suggest that many healthcare workers were infected despite wearing masks during the SARS outbreak, and therefore many suffered a great psychological impact. Therefore, the guidance on selecting and using masks is necessary.

For ventilation techniques, factory know-how can assist the hospitals in building isolation wards or negative-pressure wards. In terms of isolation ward function tests and the ventilation system assessment of fever clinics and screening stations, factory know-how can provide models for the use of assessment checklists and assist hospitals in performing on-site ventilation system assessments and isolation ward personnel management (Chang, 2003).

Hospitals can refer to the emergency response framework developed in this study to formulate their own SOPs

In this study the researchers developed an ERP based on the concept of loss control, which correspond to logical management processes. With this emergency response framework, each hospital may be in charge of its own detailed operation standards, processes and procedures based on their organization structures and respective

tasks, to implement infection control and occupational health management. Corner and Shaw (1989) point out that designing a set of procedures and an advanced warning system will reduce the risk of infection.

Hospitals should set up an EID hazard risk assessment mechanism

To quantify loss-control performance, hospitals can set up a quantified risk assessment mechanism for possible EID damage and its consequences, such as Fault Tree Analysis (FTA) and Preliminary Hazard Analysis (PHA) (among others), which may also further promote the functionality of ERP (Cameron et al., 2008; Fung et al., 2010).

Hospitals should strengthen training, education, and drills to implement EID hazard prevention

The key to implementing ERM is training education and practice, the purpose of which is to let healthcare workers familiarize themselves with the content and processes of ERM, and to have sufficient knowledge on the use of PPE, so as to avoid panic when an ERM is activated.

Carrico et al. (2008) proposed that infection control training education must be used with a well-defined training method, and the comprehension levels of medical and non-medical personnel concerning the training content must be closely monitored. John and Zambrano (2004) propose that an ERM must include assessment and continuous updating of the training, education methods and content, and must provide for continuous education.

Hospitals should strengthen communication/coordination, setting up a framework and a center for incident command structure to deal with various emergency activities regarding EIDs

The communication and coordination network in the emergency response framework is crucial to successfully control EIDs hazards. Rebmann (2009a) emphasized that infection preventionists should play an important role to facilitate emergency response system, including internal/external communication/coordination. Also, each hospital should have their own organizational structures establish an incident command structure and a center to deal with various emergency activities regarding EIDs.

Conclusion

For global EID monitoring, hospitals must pay close attention to information from the World Health Organization (WHO), and reports from newspapers and magazines, in addition to officially provided information, in order to facilitate the activation of ERM. In this study, the researchers adopted Deming (1986, 1993) PDCA management cycle theory to formulate an emergency response framework, which contained various components for an ERM that correspond to logical management processes. Each hospital may take charge of its own detailed operation standards, processes, and procedures based on its organizational structures and their respective tasks to implement infection control and occupational health management. Bryce et al. (2008) studied infection control and occupational health human resource allocations of hospitals in British Columbia and Ontario in Canada in the post-SARS period. Their results show that human resource allocation was sufficient to control the spread of the infection, but it was inadequate for occupational health, and that this could pose a leak in an EID prevention plan for occupational health management. Information on teaching materials for training education and the usage and maintenance of respiratory protection gear may be placed on an information exchange platform, built by a government or by hospitals, to facilitate healthcare workers easy access to new information and to implement training and education. Rebmann (2009b) also proposed that there must be an internet communication platform for delivering

information on infection incident reports, so that healthcare workers may have an immediate grasp of the updated information, and that relevant information on PPE and the risk of EID infection transmission may be provided. Furthermore, Item 2 of the emergency response framework, "medical unit nosocomial infection control guidelines", is included particularly for departments with highly infectious or invasive treatment. If there are specific departments in a hospital not covered by this item, the hospital can still refer to the critical concept or logic proposed here in setting up relevant measures or SOPs. Lastly, hospitals must evaluate their existing nosocomial infection control measures and SOPs, analyze a potential crisis and its impact, strengthen the case monitoring function and infection information judgment, restructure and strengthen their EID prevention system, and establish a brand new EID emergency response system.

ABBREVIATIONS

EID, Emerging infectious diseases; **SOP**, standard operating procedure; **ERPs**, emergency response plans; **SARS**, severe acute respiratory syndrome; **ICU**, intensive care units; **PPE**, personal protection equipment

REFERENCES

- Adam E (1999). New virus is identified in Malaysia epidemic. *British Med. J.* 318(7193): 1232.
- Alleyne GA (1998). Emerging diseases-what now? *Emerg. Infect Dis.* 4: 498-500.
- Badri A, Nadeau S, Gbodossou A (2012). Proposal of a risk-factor-based analytical approach for integrating occupational health and safety into project risk evaluation. *Accident Anal. Prevent.* 48: 223-234.
- Bryce E, Copes R, Gamage B, Lockhart K, Yassi A (2008). Staff perception and institutional reporting: tow views of infection control compliance in British Columbia and Ontario three years after an outbreak of severe acute respiratory syndrome. *J. Hospital Infect.* 69:169-176.
- Cambridge, MA: Massachusetts Institute of Technology. Center for Advanced Engineering Study.
- Gamage B, Moore D, Copes R. (2005). Protecting health care workers from SARS and other respiratory pathogens: A review of the infection control literature. *Am. J. Infect. Control* 33(2): 114-121.
- Cameron I, Hare B (2008). Planning tools for integrating Health and safety in construction. *Construct. Manage. Econ.* 26: 899-909.
- Carrico RM, Rebmann T, English JF, Mackey J, Cronin SN (2008). Infection prevention competencies for hospital-based healthcare personnel. *Am. J. Infect. Control* 36: 691-701.
- Centers for Disease Control and Prevention. *Severe acute respiratory syndrome. Supplement C: preparedness and response in healthcare facilities.* Appendix C2: checklist for SARS preparedness in healthcare facilities 2005. Available from: <http://www.cdc.gov/ncidod/sars/guidance/Cpdf/app2.pdf>. Accessed November 12, 2005.
- Chang CW (2003). From a SARS Incident: A Look at Medical Institutions' Occupational Health Response Strategies. The Institute of Occupational Safety and Health.
- Corner GA, Shaw A (1989). Infection control when servicing medical and laboratory equipment. *J. Med. Eng. Technol.* 13(3): 177-180.
- Council of Labor Affairs, Feb. 27, 2009. Enforcement Rules of the Labor Standards Act (Revised Ed.) Executive Yuan, Taiwan. <http://laws.cla.gov.tw/Eng/FLAW/FLAWDAT01.asp?Isid=FL014931>.
- Council of Labor Affairs, Jan. 12, 2002. Labor Safety and Health Act

- (Revised Ed.) Executive Yuan, Taiwan. <http://www.iosh.gov.tw/Law/LawPublish.aspx?LID=1>
- Council of Labor Affairs, Jun. 29, 2011. Labor Standards Act (Revised Ed.) Executive Yuan, Taiwan. <http://laws.cla.gov.tw/Eng/FLAW/FLAWDAT01.asp?Isid=FL014930>
- Council of Labor Affairs, Oct. 31, 2001. Act for Protecting Worker of Occupational Accidents. Executive Yuan, Taiwan. <http://laws.cla.gov.tw/Eng/FLAW/FLAWDAT01.asp?Isid=FL015056>
- Deming WE (1986). *Out of the crisis*. Cambridge, MA: Massachusetts Institute of Technology. Center for Advanced Engineering Study.
- Deming WE (1993). *The new economics for industry, government, education*.
- Fung I-W, Tam V-W-Y, Lo T-Y, Lu L-L-H (2010). Developing a risk assessment model for construct safety. *International Journal of Project Management* 1-8.
- Hoenen T, Groseth A, Falzarano D, Feldmann H (2006). Ebola virus: unravelling pathogenesis to combat a deadly disease. *Trends Mol. Med.* 12(5): 206-215.
- John C, Zambrano J (2004). Continuing medical education programs for oilfield health personnel: Strategy to improve solving capacities on-site and strengthen the emergency response plan. *International Conference on Health, Safety and Environment in Oil and Gas Explorat. Product.* 1531-1544.
- Li KS, Guan Y, Wang J, et al. (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 430(6996): 209-13.
- Lu Y-T, Chen P-J, Sheu C-Y, Liu C-L (2006). Viral load and outcome in SARS infection: The role of personal protective equipment in the emergency department. *J. Emerg. Med.* 30(1): 7-15.
- Maunder R, Hunter J, Vincent L (2003). The immediate psychological and occupational impact of the 2003 SARS outbreak I a teaching hospital. *CMAJ* 168: 1245-1251.
- Michael E, David LH, Johan G. (2012). Validity of International Health Regulations in reporting emerging infectious diseases. *Emerg. Infect. Dis.* 18(7): 1115-1120.
- Mostashari F, Bunning ML, Kitsutani PT (2001) Epidemic West Nile encephalitis, New York, 1999: results of a household-based sero-epidemiological survey. *Lancet* 358: 261-264.
- Rebmann T (2009a). APIC State-of-the-Art report: The role of the infection preventionist in emergency management. *Am. J. Infect. Control* 37: 271-281.
- Rebmann T (2009b). Assessing hospital emergency management plants: A guide for infection preventionists. *Am. J. Infect. Control* 37(9): 708-714.
- Rebmann T (2009c). Emergency management. In: Carrico R. editor. *APIC text of infection control and epidemiology*. 3rd ed., Chapter 119. Washington, DC: Association for Professionals in Infection Control and Epidemiology. Rise and fall of diseases . *Lancet.* 341: 151-152.
- Sbuhash SS, Radonovich LJ (2011). Hospital surge capacity. *ASHRAE* 53(9): 76-78.
- Standards Australia (1999). *Risk Management AS/NZS 4360:1999*. Strathfield, NSW: Standards Association of Australia.
- Su XB, Deng ZH, Su ZY (2003). The discovery and identification of the SARS virus. *Infect. Contr. J.* 13(6):354-358.
- Wilson ME (1999). Emerging infections and disease emergence. *Emerg. Infect. Dis.* 5: 308-309.

Full Length Research Paper

A survey of the antibacterial activity of three plants used in the Congolese herbal medicine practiced by the healers in the city of Lubumbashi

Shengo L. M.^{1*}, Mundongo T.H.³, Kasamba I.E.², Malonga K. F.³, and Kapend A. K. L.²

¹Faculty of the Sciences, Department of Chemistry, University of Lubumbashi, P.O. Box 1825, Likasi Avenue, Katanga province, The Democratic Republic of Congo.

²Faculty of Medicine, Internal Medicine Department and Biological Laboratory of University Of Lubumbashi Clinics, University Of Lubumbashi, P.O. Box 1825, Kasapa Road, Katanga province, The Democratic Republic of Congo.

³Faculty of Medicine, Department of Public Health, University of Lubumbashi, P.O. Box 1825, Kasapa Road, The Katanga province, Democratic Republic of Congo.

Accepted 10 June, 2013

In order to promote Congolese folk medicine, which has been proven to be a viable alternative for our population confronted with health problems due especially to the lack of easy access to primary care and considering the multiplicity of diseases which daily torment them, a survey of the antibacterial activity of three medicinal plants used in herbal medicine by the healers in the city of Lubumbashi has been carried out. Tests of sensitivity of the microbes towards the plant extracts have been achieved in order to search for their therapeutic efficiency since according to the ethnobotanical information, they are used against many diseases. After analysis, the results showed that, as far as their inhibitory capacity on the microbes is concerned, the three plants (*Terminalia mollis*, *Diospyros batocana* and *Antidesma venosum*) were bioactive. *Proteus mirabilis* is the microbe more sensitive to the extracts of *T. mollis* whereas *Salmonella typhi* showed greater sensitivity to extracts of *D. batocana* and *A. venosum*. Concerning *Klebsiella pneumoniae pneumoniae*, no sensitivity was observed towards extracts of *A. venosum*.

Key words: Antibacterial activity, medicinal plants, microbe sensitivity.

INTRODUCTION

No one can minimize the important role played by the healers in the improvement of the health's system in many African countries (Barakat et al., 2013; Samy et al., 2013; Steenkamp et al., 2013). Recently, a lot of countries throughout the world have succeeded in integrating their traditional medicine into their health system in order to satisfy with efficiency the health needs expressed by their population. Indeed, modern medicine has showed its limits in some cases. This is the reason why, in a research on the healers and the medicinal plants in Lubumbashi published in 2004, Petit and

Vakyanakazi (2004), indicated the importance of folk medicine and the fact that WHO have encouraged the Democratic Republic of Congo (DRC) to promote its herbal medicine as is the case in China, India, Argentina, Nigeria and Senegal which are now considered as examples of a succeeded cohabitation between the modern and the traditional medicines. In the DRC, the effort of integration of the traditional medicine into our health policy has been concretized in 2002 by the recognition of the Congolese Healers Association by the Ministry of Health.

*Corresponding author. E-mail: shengolutandulamichel@yahoo.fr.

It is true that folk medicine remains as an alternative for our population greatly impoverished and preoccupied by the problem of access to primary health care. To support this statement especially in the case of the city of Lubumbashi, one can note with bitterness, as Petit and Vakyanakazi (2004) did after their investigation, that the healers practice their professions successfully in the poor media of our city, that means the areas without hospitals and where the greatest part of our fellow citizens lives. Indeed, in our city the majority of inhabitants are confronted with serious health problems. This is indicated when one considers the analysis of a small sample of sick persons which led to the identification of enterobacteria pathogens and their cure in the University Of Lubumbashi clinics (Cliniques Universitaires de Lubumbashi 2006). These statistics had revealed that, among the patients sampled, about 25% cases of gastro-enteritis, 25% of sharp enteritis, 15% of septicemia and 5% of cystitis, have been observed, respectively. Besides, these pathologies were generally observed during the rainy seasons.

Based on inquiries by Petit and Vakyanakazi (2004), it is established that these pathogens found among the population in the city of Lubumbashi are cured by modern physicians but also by the herbal medicine of the healers. However, it is true that an expert, involved in the health sector or its related field, will wonder about the rationality of the curative scheme and the efficiency of the therapeutic choice that the healer uses in their medicine. Here is the fundamental question to which this study would like to give answer. The present survey aims at evaluating the antibacterial activity of three medicinal plants used in the Congolese herbal medicine in view to check the therapeutic validity of the medicinal practices that the healers use in the city of Lubumbashi. It is based on a preliminary research consisting in tests of the sensitivity of three reference pathogenic microbes (*Klebsiella pneumoniae pneumoniae*, *Salmonella typhi* and *Proteus mirabilis*) towards extracts of *Terminalia mollis*, *Diospyros batocana* and *Antidesma venosum* since they are extensively used by the healers in the herbal medicine (Petit and Vakyanakazi, 2004). Indeed, based on the literature and the results from ethnobotanical investigations (Kitwa, 2002; Kruger, 2004; Petit and Vakyanakazi, 2004; Tshibangu, 1997), *T. mollis* is a plant of interest from a medical point of view because it acts against intestinal worms, diarrhea, tuberculosis, malaria, cholera, cancer and dysentery (Mustopha et al., 2000; Sanin et al., 2003; Sumesh et al., 1999). Literature also indicates the use of another plant species in the family, *Terminalia sericea*, against human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), diarrhea, coughing and cutaneous infections (Bessong et al., 2004). Concerning *D. batocana*, it is in a family whose species have innumerable usages in phytomedicine (Borsub et al., 1976; Hostettmann et al., 2000; Khang and Timi, 1999(a-c); Khang et al., 1987; Mallavadhani et al., 1998; Moody and Roberts, 2002; Xing-Cong et al., 1998).

It is used to cure gonorrhea and infertility, particularly the azospermia. Talking about the medicinal use of *A. venosum*, as with other species of its family, it is a plant of great pharmacological interest (Buske et al., 2001, Bringmann et al., 2000, 2001) owing to its efficient action against gonorrhea, tooth decay, abdominal pains, gastritis, infertility, precocious abortion, threats of abortion and sexual impotence.

MATERIALS AND METHODS

Based on the ethnobotanical investigations, three plants used in Congolese phytomedicine were harvested and studied: *T. mollis* (Chrysobalanceae), *D. batocana* (Ebenaceae) and *A. venosum* (Euphorbiaceae). They have been selected in order to determine their antibacterial activity towards a group of well identified pathogenic microbes. The bioactivity tests of these plants have been achieved as described below:

Plant identification

Botanical identification and harvest of the studied plants was carried out by the Geomorphology and Botany laboratory, the Unit of Ecology and Environment (Department of Geography, Faculty of the Sciences) at the University of Lubumbashi (DRC). They come from the forest of the FIKUPA locality found in the mining hinterland of Katanga (DRC) and were identified under the following herbarium numbers: N° 4367 – *T. mollis* Lacus (Combretaceae), N° 4368 – *D. batocana* Hiern. (Ebenaceae), N° 4369 – *A. venosum* E.mey.ex Hoffn. (Euphorbiaceae).

Choice of the microbes of interest

The microbes that we used for the antibacterial tests were identified by their ATCC codes as described below and are usually used as reference for microbial testing in the Clinical Biology laboratory at the University of Lubumbashi Clinic. *P. mirabilis*: ATCC 35659; *K. pneumoniae pneumoniae*: ATCC 35657; *S. typhimurium*: ATCC 14028.

The preparation of the excerpts

For the preparation of the plant extracts, 50 g of plant material constituted of stem peels were dried, ground using an agate mortar and submitted to steeping for 72 h with 750 ml of methanol as solvent in view of extracting the bioactive molecules. The obtained alcoholic solution undergone filtration prior to the solvent removal by vacuum evaporation (40°C) using a BIBBY RE200B rotavapor connected to a KNF Neuberger LABOPORT vacuum pump. The preparation of the plant's extracts was done using 50 g of plant material and the following outputs were obtained: *T. mollis* (9.0%), *D. batocana* (15.6%) and *A. venosum* (8.1%). These plants' extracts were sampled and resuspended in distilled water to prepare solutions to be tested with bacterial strains of our choice.

Isolation of bacteria and inoculation of the plants extracts

The bacteria were cultured in liquid Mueller Hinton medium with a concentration equal to 2 units of Mac Farland (BioMérieux, 1996). This bacterial concentration corresponds to an optical density of 0.250 which was measured spectrophotometrically using the 550 nm wavelength (Spectrophotometer Genesis 20); 2 ml of this

Table 1. Result of microbe sensitivity tests towards *T. mollis* extracts.

Plant's extracts dilution (mg/ml)	<i>K. pneumoniae pneumoniae</i>	<i>S. typhii</i>	<i>P. mirabilis</i>	Number of positive tests	Proportion (%)
50	+	+	+	3/3	100
25	+	+	+	3/3	100
12.5	+	+	+	3/3	100
6.25	-	-	+	1/3	33.3
Number of positive tests	3/4	3/4	4/4	10/12	83.3
Proportion (%)	75	75	100		

+, Inhibition of the bacterial activity; -, failure or resistance to the extract action.

preparation were placed in tests tubes containing 2 ml distilled water containing the medicinal plant's extracts. These extracts were diluted with distilled water progressively in half of a mother solution with a concentration equal to 50 mg/ml to give extracts successively to concentrations equal to 50, 25, 12.5 and 6.25 mg/ml. For each test, three replicates were done per plant extract and concentration per bacterial strain. Incubation of the tests tubes was carried out for 24 h between 35 and 37°C in a stream room.

The reading of the sensitivity of the microbes towards the plant extracts was achieved by visual observation of the turbidity development in the test tubes (Figure 1) and the obtained result validated using the Petri dishes technique for the numbering of the bacteria colonies (Ayres and Mara, 1996). The observation of the turbidity developed in the tests tubes has enabled us determine the minimum inhibitory concentration (MIC). As for the minimum bactericidal concentrations (MBC) in view establishing whether the studied extracts are bacteriostatics or bactericidal, it was determined through the seeding of bacteria, contained in the precipitate recovered from the test tubes, in the Petri dishes using the Mueller Hinton Agar.

RESULTS AND DISCUSSION

The concerned results are related to the determination of sensitivity of *K. pneumoniae pneumoniae*, *S. typhii* and *P. mirabilis* towards the extracts of *T. mollis*, *D. batocana* and *A. venosum*. The active dilutions of the extracts which ranged from 50 to 6.25 mg/ml were incubated for 24 h at between 35 and 37°C.

Bioactivity of *T. mollis*

The sensitivity of the studied bacterial strains towards the extracts of *T. mollis* is shown in Table 1. It is obvious that *T. mollis* extracts have a substantial inhibitory action on all three microbes because about 83.3% of tests were successful. As a matter of fact, the bacterial activity was inhibited for all dilutions in 75 to 100% of the achieved bioassays. The sensitivity of *P. mirabilis* towards these extracts was the largest of all because the MIC of 6.25 mg/ml which corresponds to the greatest dilution of the extract. Concerning the sensitivities of *K. pneumoniae pneumoniae* and *S. typhii* towards the extracts, they seemed to be similar. The total inhibition of microbes was gotten at a dilution of 12.5 mg/ml. It is to this concen-

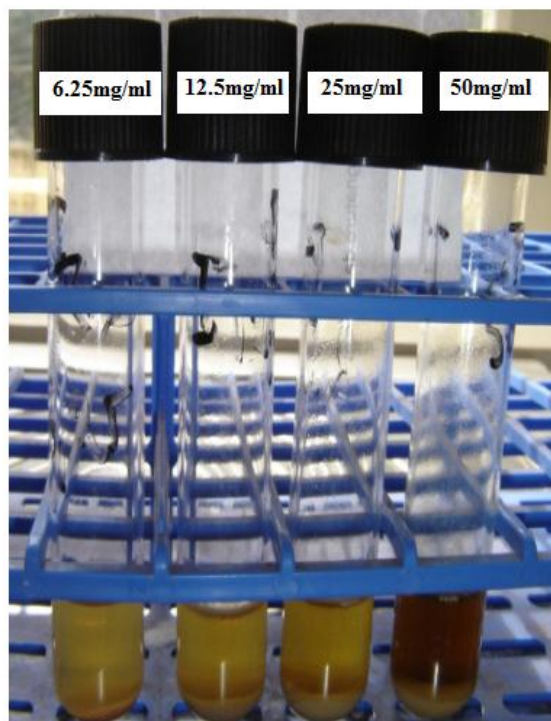


Figure 1. Tests of the sensitivity of *P. mirabilis* towards the extracts of *A. venosum*.

tration that the microbes begin to resist the extracts inhibitory action. Additionally, the bioassays reveal the broadening of the antibacterial spectrum of the extracts of *T. mollis* when the concentration increases or surpasses the MIC which is below 6.25 mg/ml for *P. mirabilis*. Concerning *K. pneumoniae pneumoniae* and *S. typhii*, the MIC was 6.25 mg/ml of the extracts. As for the MBC, it is nil because the bacterial developed on the Mueller Hinton agar.

Bioactivity of *D. batocana*

The tests of sensitivity of the microbes towards the extracts of *D. batocana* led to the results in Table 2.

Table 2. Result of microbe sensitivity tests towards *D. batocana* extracts.

Plant's extracts dilution (mg/ml)	<i>K. pneumoniae pneumoniae</i>	<i>S. typhii</i>	<i>P. mirabilis</i>	Number of positive tests	Proportion (%)
50	+	+	+	3/3	100
25	+	+	+	3/3	100
12.5	-	+	-	1/3	33.3
6.25	-	+	-	1/3	33.3
Number of positive tests	2/4	4/4	2/4	8/12	66.7
Proportion (%)	50	100	50		

+, Inhibition of the bacterial activity; -, failure or resistance to the extract action.

Table 3. Results of microbe sensitivity tests towards *A. venosum* extracts.

Plant's extracts dilution (mg/ml)	<i>K. pneumoniae pneumoniae</i>	<i>S. typhii</i>	<i>P. mirabilis</i>	Number of positive tests	Proportion (%)
50	-	+	+	2/3	66.7
25	-	+	+	2/3	66.7
12.5	-	+	+	2/3	66.7
6.25	-	+	-	1/3	33.3
Number of positive tests	0/4	4/4	3/4	7/12	58.3
Proportion (%)	0	100	75		

+, Inhibition of the bacterial activity; -, failure or resistance to the extract action.

These results indicate that *S. typhii* showed a very great sensitivity (100%) towards the excerpt of *D. batocana* because the minimal inhibitory concentration is the lowest possible for this microbe since it resists even at the greatest used dilution. For the other microbes, *K. pneumoniae pneumoniae* and *P. mirabilis*, displayed a similar behavior (50%) towards these extracts to which they resisted for dilutions below 25 mg/ml. Their MIC is equal to 12.5 mg/ml of the extracts of *D. batocana*. Globally, the inhibitory action of the extracts on the microbes is 50%. However, it is worth mentioning that the MBC is also nil in this case.

Bioactivity of *A. venosum*

The test of sensitivity of the microbes on *A. venosum* extracts is shown in Table 3. These results show that *S. typhii* is more sensitive to the extracts of *A. venosum* because for this microbe, the proportion of positive tests is equal to 100% whatever may be the dilution. As far as the sensitivity of the other microbes towards the extract is concerned, one can notice that *P. mirabilis* (Figure 1), with 75% of positive tests, came second in position. On the other hand, the resistance shown by *K. pneumoniae pneumoniae* to the plant extract was the largest noted while analyzing the bioassay results. Nevertheless, the inhibitory action on the microbes shown by the extracts of *A. venosum* is smaller than for *T. mollis* and *D. batocana*

because only 58.3% positive tests were recorded. The MIC for *S. typhii* is below 6.25 mg/ml of the extracts of *A. venosum* contrarily to *P. mirabilis*. However, the MBC is nil due to the growth of bacteria on the Mueller Hinton Agar.

Globally, it was deduced from the results that the three plants, according to their inhibitory action on the microbes, may be classified as follows: *T. mollis*, *D. batocana* and *A. venosum*. On the other hand, one can see that *P. mirabilis* is more sensitive to the extracts of *T. mollis* whereas *S. typhii* showed a bigger sensitivity towards *D. batocana* and *A. venosum*. With regard to *K. pneumoniae pneumoniae*, it did not have any sensitivity to the extracts of *A. venosum*. Its sensitivity to those of *T. mollis* was more pronounced than it demonstrated towards the extracts of *D. batocana*. To all dilutions and whatever may be the excerpt, the sensitivities of the microbes were classified in the following order: *S. typhii* with 91.7% of the positive tests, *P. mirabilis* with 75% and *K. pneumoniae pneumoniae* with 41.7%, respectively. Considering that the MBC is nil in all the cases, it can be concluded that the plants' extracts tested against the bacterial strains of our interest are bacteriostatics. Based on the sensitivity shown, at different dilutions, by the studied bacterial strains towards the plants' extracts, only the result related to the action of the extracts of *A. venosum* against *K. pneumoniae pneumoniae* shows a difference statistically significant ($p = 0.021$). No significant difference is observed with regard to the results

given by the sensitivity of bacterial strains towards the remaining plants' extracts ($p > 0.05$).

Conclusion

On the antibacterial efficacy standpoint, the three plants used in the Congolese folk medicine can be classified as follows: *T. mollis*, *D. batocana* and *A. venosum*. Their use by the healers against illnesses such as diarrhea, gastroenteritis, urinary infections, typhoid fever and dysentery is well justified. *P. mirabilis* is the microbe most sensitive to the extracts of *T. mollis* whereas *S. typhii* showed more sensitivity to *D. batocana* and *A. venosum*. However, *K. pneumoniae pneumoniae* showed a strong resistance to the extracts of *A. venosum*. The sensitivity of the germs to the extracts of *T. mollis* was bigger than the one shown towards those of *D. batocana*. To all dilutions of the extracts, sensitivity of the microbes was classified as follows: *S. typhii*, *P. mirabilis* and *Klebsiella pneumoniae pneumoniae*. Only the extracts from *T. mollis* and *D. batocana* possess antibacterial activities enabling them to be used as sources of new drugs. The plants extracts tested against the reference bacteria of our interest are bacteriostatic.

ACKNOWLEDGMENTS

We are grateful to the technicians *Emile KISIMBA KIBUYE* (the Geomorphology and Botany Laboratory - Department of Geography) and *Jean-Pierre KAPONGO MAYEBA* (The bio-organic chemistry laboratory – Department of Chemistry) for their technical support and collaboration during the identification and harvesting of the studied medicinal plants and the preparation of the extracts used for bioassays.

ABBREVIATIONS

DRC, Democratic Republic of Congo; **HIV/AIDS**, human immunodeficiency virus/ acquired immunodeficiency syndrome; **MIC**, minimum inhibitory concentration; **MBC**, minimum bactericidal concentrations.

REFERENCES

- Ayres RM, Duncan MD (1996). *Analysis of Wastewater for Use in Agriculture - A Laboratory Manual of Parasitological and Bacteriological Techniques*, World Health Organization, Geneva, pp.24-26.
- Barakat BB, Muahmmad I, Niaz A, Naveed M, Rehmanullah (2013). *Antispasmodic potential of leaves, barks and fruits of Zanthoxylum armatum DC*. Afr. J. Pharm. Pharmacol. 7(13):685-693.
- Bessong PO, Obi CL, Igumbor E, Andreola M-L, Litvak S (2004). *In vitro activity of three selected South African medicinal plants against human immunodeficiency virus type 1 reverse transcriptase*. Afr. J. Biotechnol. 3(10):555-559.
- BioMérieux (1996). *Livret technique, Identification et antibiogrammes, Méthodes manuelles*, Marcy-Etoile-France, pp. 193.
- Borsub L, Thebtaranoth Y, Ruchirawat S, Sadavongvivad C (1976). *A new diglycoside from the anthelmintic berries of Diospyros mollis*, Tetrahedron letters, (2):105-108.
- Bringmann G, Rischer H, Wohlfarth M, Schlauer J (2000). *Biosynthesis of Antidesmone in Cell Cultures of Antidesma membranaceum (Euphorbiaceae): An Unprecedented Class of Glycine-Derived Alkaloids* Institute of Organic Chemistry, University of Wuerzburg, Wuerzburg, Germany. J. Am. Chem. Soc. 122(41):9905- 9910.
- Bringmann G, Schlauer S, Rischer H, Wohlfahrt M, Haller R, Bar S, Brun R (2001). *Antidesmone, a novel antitrypanosomal Alkaloid*, Institut für Organische Chemie, Universität Würzburg, Würzburg, Germany. Pharmaceut. Pharmacol. Lett. 11(2):47-48.
- Buske A, Schmidt J, Porzel A, Adam G (2001). *Alkaloidal, megastigmane and lignan glucosides from Antidesma membranaceum (Euphorbiaceae)*, Department of Natural Products Chemistry, Leibniz-Institute of Plant Biochemistry, Halle/Saale, Germany. Eur. J. Organ. Chem. 18:3537-3543.
- Cliniques Universitaires de Lubumbashi (CUL), Archives and reports from the Internal Medicine Department (2006).
- Hostettmann K, Marston A, Ndjoko K, Wolfender J-L (2000). *Potential of African plants as source of drugs*, Cur. Organ. Chem. 4:973-1010.
- Khang MR, Timi D (1999a). *Constituents of Diospyros lolin, D. maritima and D. novoguineensis*, Fitoterapia, 70(2):194-196.
- Khang MR, Timi D (1999b). *Constituents of Root and Stem Barks of Diospyros hallieri*, Fitoterapia, 70(3):320-321.
- Khang MR, Timi D (1999c). *Constituents of Root and Stem Barks of Diospyros villosiuscula*, Fitoterapia, 70(2):209-211.
- Khang MR, Kishimba MA, Locksley LD (1987). *Naphthoquinones and triterpenoids from Diospyros zombensis, D. Consolatae, D. Squarria*, Fitoterapia, 58(6):424-425.
- Kitwa K (2002). *A survey on dyes from vegetable species, The case of the violet dye from Diospyros batocana*, Research report, Department of Chemistry, Faculty of the Sciences, University OF Lubumbashi, The Democratic Republic of Congo, p.13.
- Kruger J (2004). *Isolation and characterization of antibacterial compounds from three Terminalia spp*, Ph. D., Department of Paraclinical Sciences – Phytomedicine, Faculty of Veterinary Science, University of Pretoria, Pretoria.
- Mallavadhani UV, Panda AK, Rao YR (1998). *Pharmacology and chemotaxonomy of Diospyros*, Phytochemistry 49(4): 901-951.
- Moody JO, Roberts VA (2002). *Antiviral effect of selected medicinal plants 1: effect of Diospyros bateri, Diospyros monbutensis and Sphenocentrum jollyanum on polio viruses*, Niger. J. Natl. Prod. Med. 6:4-6.
- Mustopha VA, Benoit-Vical F, Pelessier Y, Kone-Bamba D, Mallu M (2000). *Antiplasmodial activity of plant extracts in West African traditional medicine*, J. Ethnopharmacol. 73(1-2):145-151.
- Petit P, Vakyanakazi M (2004). «*Bunganga bwa miki* », *The Healers and medicinal plants in Lubumbashi*, Report of researches carried out during the 12th work session (October, 2003-March, 2004) of the Urban Change Observatory financed by the Belgian Unversitary Development Cooperation (UDC), Lubumbashi, The Democratic Republic of Congo, p.7, 12, 17-19, 98, 108.
- Samy AS, Mohamed HAA, Mona SM, Mona FW (2013). *Antibacterial activities, chemical constitutes and acute toxicity of Egyptian Origanum majorana L., Peganum harmala L. and Salvia officinalis L. essential oils*, Afr. J. Pharm. Pharmacol. 7(13):725-735.
- Sanin S, Ollivier E, Azas N, Mathieu V, Gasquet M, Outtara CT, Nebie I, Traore AS, Esposito F, Bolanserd G, Timin-David P, Fumaux F (2003). *Ethnobotanical survey and in vitro antiplasmodial activity of plants in traditional medicine in BURKINA FASO*, J. Ethnopharmacol. 86(2-3): 143-147.
- Steenkamp V, Nkwane O, van Tonder J, Dinsmore A, Gulumian M (2013). *Evaluation of the phenolic and flavonoid contents and radical scavenging activity of three southern African medicinal plants*, Afr. J. Pharm. Pharmacol. 7(13):703-709.
- Sumesh CC, Caroline C (1999). *Kenyan and Tanzanian traditional medicines for treatment of malaria*, First international meeting of the Research Initiative on traditional Anti- Malarials (RITAM), Moshi, Tanzania, 8-11 December 1999, p.41.
- Tshibangu K (1997). *Contribution to ethnobotanical knowledges and determination of the antibacterial activities of some vegetable species*

used in the folk medicine in the city of Lubumbashi, A research report, The Department of Chemistry and biology, the Pedagogic High School of Lubumbashi, The Democratic republic of Congo, pp. 1-48.

Xing-Cong P, Van Der B, Wu DC (1998); *Binaphthalenone glycoside from African Chewing Sticks*, *Diospyros lycioides*, J. Nat. Prod. 61(6):817-820.

Full Length Research Paper

Antimicrobial activity of sequential extracts from leaves of *Cassia nodosa* bunch

Sharma, R. A.*, Richa Bhardwaj and Pallavi Sharma

Plant Physiology and Biochemistry Laboratory, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India.

Accepted 27 May, 2013

The present study deals with the antimicrobial activity of the sequential extracts (petroleum ether, benzene, acetone, chloroform, ethanol, water, etc.) of the leaves of *Cassia nodosa* carried out against certain bacteria which are: *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa* and fungi including, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium moniliformae* and *Rhizoctonia bataticola* using disc diffusion technique. Results showed that most of the extracts were effective against all the test microorganisms. The minimum inhibitory concentrations of the extracts of chloroform, benzene, acetone and ethanol were found to be 2×10^4 µg/ml, while the petroleum ether and water showed no inhibition. The results of the study provide scientific basis for the use of the plant extracts in the treatment of fungal and bacterial diseases. Therefore, the various active principles possessing antimicrobial activity may be extracted from the leaves of *C. nodosa* by various organic solvents.

Key words: *Cassia nodosa*, antimicrobial activity, petroleum ether, benzene, chloroform, acetone, ethanol, water, bacteria, fungi.

INTRODUCTION

One big concern the world is facing is the development of resistance to the antibiotics in current clinical use. Traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries, and moreover, the use of herbal remedies has risen in the developed countries in the last decade. Natural products are still major sources of innovative therapeutic agents for various conditions, including infectious diseases (Selim et al., 2013). In this regard, *Cassia* species have been of keen interest in phytochemical and pharmacological research due to their excellent medicinal values. Different classes of natural products, possessing potent physiological and pharmacological activities have been isolated from *Cassia* species, and they include anthracene

derivatives, flavonoids and polysaccharides (Jain et al., 1997). Some of these compounds have been shown to possess considerable antimicrobial activity (Ayo et al., 2007).

The world health organization (WHO) highly appreciated the conventional medical practices for treatment and precautionary measure of various diseases (Barkatullah et al., 2013). *Cassia* species are well known in folk medicine for their laxative and purgative activities (Elujoba et al., 1999). *Cassia nodosa* is one of the beautiful fast growing ornamental exotic plants with pink flowers found along the roadsides and gardens in Jaipur. It is widespread in world's tropical and sub-tropical regions. Phytochemically, *C. nodosa* have been studied for fixed oils of the seeds (Rizvi et al., 1968),

*Corresponding author. E-mail: sharma_ra2007@yahoo.com. Tel: 09461164336.

glactomann and nodoside, a new anthraquinone glycoside from the flowers (Rizvi et al., 1971a, b.) and bark (Yadav et al., 1998).

The phytochemical and cytotoxic screening of the plant has been carried out earlier, and the four crude extracts showed strong cytotoxic activity. The extracts were found to be positive for carbohydrate, anthracene derivatives, cardiac and saponin glycosides as well as alkaloid. The aim of the present study is to investigate the antimicrobial activity of the various sequential extracts of the leaves of *C. nodosa* in different solvents.

MATERIALS AND METHODS

Plant

The leaves of *C. nodosa* were collected from Central Park, Jaipur. Leaves were washed with tap water, dried at room temperature and ground to fine powder. The species specimen was authenticated and submitted in herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India and got the voucher specimen No. RUBL15487.

Extraction procedure

Powdered 50 g of *C. nodosa* leaves were soxhlet extracted with petroleum ether, benzene, chloroform, acetone, alcohol, and water successively for 36 to 48 h. Each of the resultant extract was filtered, dried *in vacuo* and stored at 4°C, until screened for antimicrobial activity and weighed to calculate the extractive value (%) on dry weight basis. Later, following the established protocols (Paech and Tracey, 1955), each of the test samples was used against test microorganisms.

Test microorganisms

Standard strains of *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from microbiology Lab, SMS medical college College, Jaipur and *Aspergillus flavus*, *Aspergillus niger*, *Fusarium moniliformae* and *Rhizoctonia bataticola* were obtained from seed pathology lab, Department of Botany, University of Rajasthan, Jaipur (Figure 1).

Antimicrobial screening

The disc diffusion method was used to determine the antimicrobial activities of the crude extracts of petroleum ether, benzene, acetone, chloroform, ethanol and water using standard procedure (Ali et al., 1999; Crockett et al., 1992). The discs of 6 mm size were prepared using Whatmann's filter paper no. 1. Solutions of varying concentrations ranging from 1.0×10^4 to 5.0×10^4 mg/ml/disc were prepared. The treated discs were air dried at room temperature to remove any residual solvent which might interfere with the determination, sterilized and inoculated. For the cultivation of bacteria, nutrient broth medium (NBM) was prepared using 8% nutrient broth (Difco) in distilled water and agar-agar and sterilized at 15 lbs psi for 25 to 30 min. However, for the cultivation of fungi, potato dextrose agar medium was prepared and the test fungi were incubated at 27°C for 48 h, and the cultures were maintained on same medium by regular sub-culturings. To prepare the test plates,

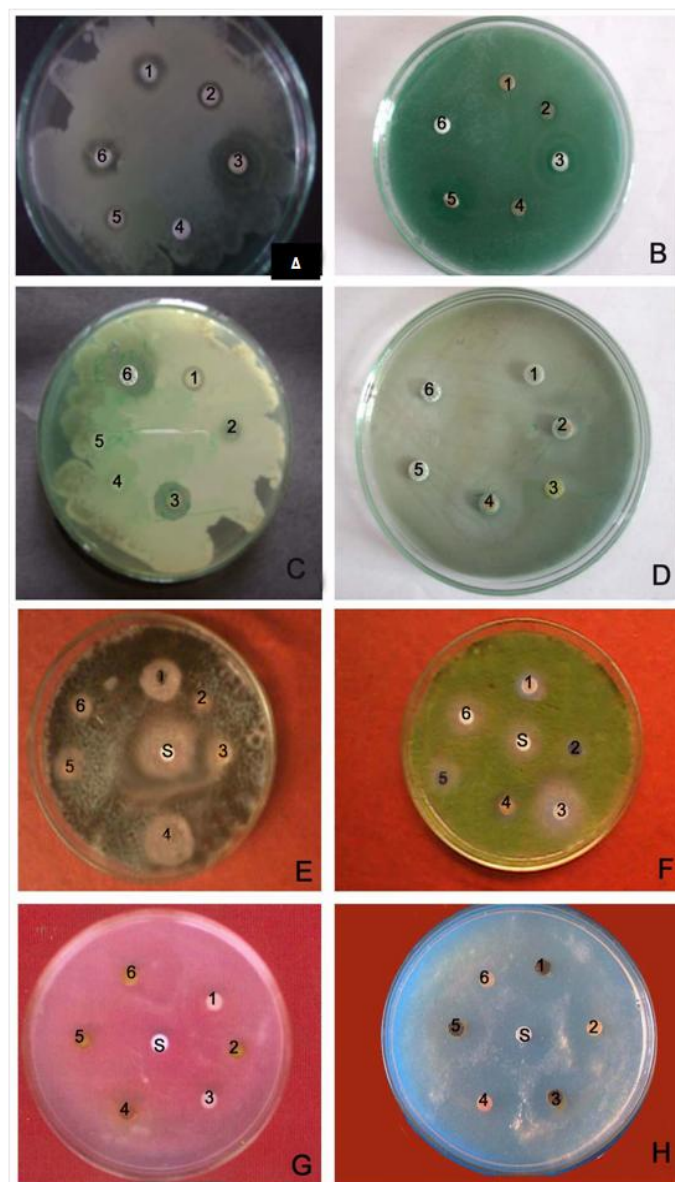


Figure 1. Photographs of antimicrobial activity of sequential extracts of leaves of selected *Cassia nodosa*. Bacteria (A = *E. coli*, B = *S. aureus*, C = *P. aeruginosa*, D = *S. typhi*); Fungi (E = *A. flavus*, F = *A. niger*, G = *R. bataticola*, H = *F. moniliforme*, S = Standard). 1 = Petroleum ether extract of leaves, 2 = benzene extract of leaves, 3 = chloroform extract of leaves, 4 = acetone extract of leaves, 5 = alcohol extract of leaves, 6 = aqueous extract of leaves.

in both bacteria and fungi, 15 to 20 ml of the respective medium was poured into the petri dishes under aseptic conditions and used for screening. The different test organisms were proceeded separately using a sterile swab over sterilized culture medium plates and the zone of inhibition were measured around sterilized dried discs of whatman no. 1 paper. These plates were initially placed at low temperature for 1 h so as to allow the maximum diffusion of compounds from the test disc into the agar plate and later incubated at 37°C for 24 h in case of bacteria and 48 h for

Table 1. Results zone of inhibition of *Cassia nodosa* leaves against microorganism.

Text microorganism	Diameter of zone of inhibition (mm)					
	Petroleum ether	Benzene	Acetone	Chloroform	Ethanol	Water
Bacteria						
<i>Staphylococcus aureus</i>	07	13	14	17	16	06
<i>Salmonella typhi</i>	04	10	12	12	13	00
<i>Pseudomonas aeruginosa</i>	07	13	24	21	10	07
<i>Escherichia/ coli</i>	04	11	20	12	16	06
Fungi						
<i>Aspergillus niger</i>	00	18	20	16	18	00
<i>Aspergillus flavus</i>	00	15	18	23	21	00
<i>Fusarium monilliformae</i>	03	11	12	15	17	00
<i>Rhizoctonia bataticola</i>	00	10	09	10	11	00

fungi, after which the zone of inhibition could be easily observed. Five replicates of each text extract were examined and the mean values were then referred.

Microdilution test

The minimum inhibitory concentrations (MICs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^7 cfu/ml. Compounds to be investigated were dissolved in broth LB medium (100 μ l) with bacterial inoculums (1.0×10^5 cfu) to achieve the wanted concentrations (1 mg/ml). The microplates were incubated for 24 h at 48°C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The optical density of each well was measured at a wavelength of 655 nm by Microplate reader (Perlong, ENM8602) and compared with a blank and the positive control.

RESULTS AND DISCUSSION

Table 1 shows the results of the antimicrobial activities against the test microorganisms. The zones of inhibition for each case were measured in mm of the diametrical sections of the respective sequential extracts. Table 2 shows the results of MIC. From the results, it was observed that the chloroform, benzene, acetone and ethanol had very high growth inhibitory effects on all microorganisms. The MIC values for the chloroform extract on *S. aureus*, *E. coli*, *P. aeruginosa*, *A. flavus* and *A. niger* was found to be 2×10^4 μ g/ml, while for *S. typhi*, *F. monilliformae* and *R. betaticola* the MIC value was 3×10^4 μ g/ml. The MIC value of acetone was found that 2×10^4 μ g/ml against *S. aureus*, *P. aeruginosa*, *E. coli*, *A. flavus*, *A. niger* and for *S. typhi*, *F. monilliformae*

and *R. betaticola* the MIC value was 3×10^4 μ g/ml. The MIC for the benzene and ethanol was found to be 2×10^4 μ g/ml for all the test microorganisms, except for *S. typhi* and *R. betaticola* (3×10^4 μ g/ml).

The petroleum ether and water extracts did not show any inhibition against the test microorganisms. Low MIC is an indication of high efficacy of the plant extract while high MIC may indicate low efficacy or possible development of resistance by the microorganisms to the antimicrobial. Reports on the antibacterial and antifungal activity have been evaluated by number of workers on the *C. nodosa* species (Mohtar and Sharri, 2000; Sayed et al., 2011). The test organisms used in this study are associated with various forms of human infections. Infection caused by *S. typhi* is a serious public health problem in developing countries and represents a constant concern for the food industry (Mastroeni, 2002). The results of the antimicrobial activity of the various sequential extracts were in agreement with the uses of the extract of the leaves of *C. nodosa* in traditional medicine for the treatment of bacterial and fungal diseases. Secondly, different solvents have different solubility capacities for different phytoconstituents, hence the differences in the activities of the various extracts (Majorie, 1999). The leaves of the plant were found to be a potential source of broad spectrum antibiotics. Studies are in progress to purify and characterize the active principles in the leaves.

Conclusion

Active principles possessing antimicrobial activity may be extracted from the leaves of *C. nodosa* by benzene, acetone, chloroform and ethanol.

Table 2. Zones of inhibition of different concentration of *Cassia nodosa* (µg/ml).

Text organisms	Petroleum ether					Benzene					Chloroform					Acetone					Ethanol					Water									
	1×10 ⁴	2×10 ⁴	3×10 ⁴	4×10 ⁴	5×10 ⁴	1×10 ⁴	2×10 ⁴	3×10 ⁴	4×10 ⁴	5×10 ⁴	1×10 ⁴	2×10 ⁴	3×10 ⁴	4×10 ⁴	5×10 ⁴	1×10 ⁴	2×10 ⁴	3×10 ⁴	4×10 ⁴	5×10 ⁴	1×10 ⁴	2×10 ⁴	3×10 ⁴	4×10 ⁴	5×10 ⁴	1×10 ⁴	2×10 ⁴	3×10 ⁴	4×10 ⁴	5×10 ⁴					
Bacteria																																			
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
<i>Salmonella typhi</i>	-	-	-	-	-	-	±	+	+	-	±	+	+	+	-	±	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	
<i>Escherichia coli</i>	-	-	-	-	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	
Fungi																																			
<i>Aspergillus niger</i>	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	
<i>Aspergillus flavus</i>	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	
<i>Fusarium moniliformae</i>	-	-	-	-	-	-	±	+	+	-	-	+	+	+	-	+	+	+	+	-	-	±	+	+	-	-	-	-	-	-	-	-	-	-	
<i>Rhizoctonia bataticola</i>	-	-	-	-	-	-	±	+	+	-	-	+	+	+	-	+	+	+	+	-	-	±	+	+	-	-	-	-	-	-	-	-	-	-	

REFERENCES

Ali MS, Azhar I, Amtul Z, Ahmad VU, Usmanghani K (1999). Antimicrobial screening of some Caesalpiniaceae. *Fitoterapia* 70:299-304.

Ayo RG, Amupitan JO, Zhao Y (2007). Cytotoxicity and antimicrobial studies of 1, 6, 8- trihydroxy-3-methyl-anthraquinone (emodin) isolated from the leaves of *Cassia nigricans* Vahl. *Afr. J. Biotechnol.* 6(11):1276-1279.

Barkatullah BB, Muahmmad I, Niaz A, Naveed M, Rehmanullah (2013). Antispasmodic potential of leaves, barks and fruits of *Zanthoxylum armatum* DC. *Afr. J. Pharm. Pharmacol.* 7(13):685-693.

Crockett CO, Guede-Guina F, Pugh D, Vangah- Manda M, Robinson J, Qlubadewo JO, Ochillo RF (1992). *Cassia alata* and the preclinical search for therapeutic agents for the treatment of opportunistic infections in AIDS patients, *Cell Mol. Biol.* 35:505-511.

Elujoba AA, Abere AT, Adelusi SA (1999). Laxatives activities of Cassia pods sourced from Nigeria. *Nig. J. Nat. Prod. Med.* 3:51-53.

Jain SC, Jain R, Sharma RA, Cappasso F, Mecudo N (1997). Ethnopharmacological investigation of *Cassia italica*. *J. Ethnopharmacol.* 58(2):135-142.

Majorie MC (1999). Plant Products as Antimicrobial Agents. *Clinical. Microbiol. Rev.* 12:564-582.

Mastroeni P (2002). Immunity to systemic Salmonella infections. *Curr. Mol. Med.* 2:393-406.

Mohtar M, Shaari K (1999). Antimicrobial activity of *Senna alata* and *Cassia javanica* subsp. *nodosa* extracts against micro-organisms related to skin infections. *J. Trop. Forest Products* 6(2):218-221.

Paech K, Tracey MV (1955). *Modern Methods of Plant Analysis*. Vol. III. Springer-Verlag, Berlin.

Rizvi SA, Gupta PC, Kaul RK (1968). Chemical and spectral studied of nodoside. A new anthraquinone glycoside from the flowers of *Cassia nodosa*. *Plant Med.* 19(3):221-233.

Rizvi, SA, Gupta PC, Kaul RK (1971a). Chemical examination of the fixed oils from the seeds of *Cassia nodosa*. *Plant Med.* 16(3):317-322.

Rizvi, SA, Gupta PC, Kaul RK (1971b). The structure of glectomannan from the seeds of *Cassia nodosa*. *Plant. Med.* 20(10):24-32.

Sayed MME, Salah AAS, Nahas HAE, Gawad MME, Aziz MMA, Hameed ESA, Ahmed WS, Abdel-Lateef EE (2011). *Austr. J. Basic Appl. Sci.* 5(9):344-352

Selim SA, Abdel Aziz MH, Mashait MS, Warrad MF (2013). Antibacterial activities, chemical constituents and acute

toxicity of Egyptian *Origanum majorana* L., *Peganum harmala* L. and *Salvia officinalis* L. essential oils. *Afr. J. Pharm. Pharmacol.* 7(13):725-735.

Yadav NK, Singh BK, Kumar A (1998). A new anthraquinone from the root bark of *Cassia nodosa*. *Asian J. Chem.* 10(1):204-206.

Full Length Research Paper

A preliminary study on the toxicity and novelty-induced behavioral effects of herbal medicine (*Mama Decoction*[®]) in rats

M.A. Akanmu^{1*}, A. O. Akanmu¹, A.O. Adepiti², S.A. Osasan³, E.M. Obuotor⁴, A.A. Elujoba²

¹Department of Pharmacology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.

²Department of Pharmacognosy, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.

³Department of Morbid Anatomy, Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria.

⁴Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria.

Accepted 24 June, 2013

Mama decoction (MD) is a commonly used formulated herbal product in Nigeria for the management of malaria; no study on its central and possible toxicological effects have been investigated, hence this study. MD was administered orally to rats at 143, 286, and 572 mg/kg daily for 30 days. Novelty-induced behavior was observed and recorded on both day 1 and day 30 of administration. Furthermore, mortality, biochemical and histopathological tests were evaluated appropriately. The animals were sacrificed on day 30 after the behavioral scoring and blood samples obtained for biochemical assays. Histopathological examinations of the liver, kidney, brain, spleen, testes and lungs were carried out. The results showed that acute oral administrations of MD had no significant effect on locomotion at all dose levels used on Day 1 while during the subchronic administration of MD, only the dose of 286 mg/kg had significant effect on locomotion. Furthermore, the grooming behavior was significantly ($p < 0.01$) decreased dose-dependently. Biochemical analysis showed that sub-chronic administration of MD caused significant decrease in both triglyceride and cholesterol levels but caused a significant increase in alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in the plasma. In the liver, triglyceride, cholesterol and ALT were significantly decreased while AST was significantly increased but it had no effect on the ALP. The histopathological analysis revealed that most of the organs were essentially normal. In conclusion, the study showed that oral administration of MD is relatively safe when used within the recommended maximum dose of 286 mg/kg, however, there is need for caution in using it for a long period.

Key words: *Azadirachta indica*, *Alstonia boonei*, *Morinda lucida*, *Mangifera indica*, biochemical, sub-chronic toxicity.

INTRODUCTION

Mama decoction (MD) (prepared from the mixture of leaves of *Mangifera indica*, *Azadirachta indica*, *Morinda lucida* and *Alstonia boonei*) is a herbal medicine that has been used to treat malaria traditionally in Nigeria. Since there has been no study on its central behavioral and possible toxicological effects, this study was necessary.

M. indica Linn. (Anacardiaceae, Mango tree) is a large evergreen tree, with a heavy, dome-shaped crown. Its leaf, which is used as antimicrobial in the treatment of burns, scalds, sores, wounds, abscesses and other infections in humans and animals, had been reported to contain saponins, glycosides, unsaturated sterols,

polyphenols, euxanthin acid, mangiferine, mangin and gallo-tannins (Ngo, 2001). Mangiferin possessed antiviral (Zheng and Lu, 1990), antitumor, immunomodulatory and antiHIV (Guha et al., 1996), antidiabetic (Ichiki et al., 1998), anti-inflammatory (Garrido et al., 2004) and antioxidative activities (Martnez et al., 2000). *M. lucida* Benth (Rubiaceae) leaves are used as an ingredient of "fever teas", as antimalarial as well as febrifuge, analgesic and laxative remedies. The antiplasmodial and antibacterial activities of the plant had been associated with the anthraquinones (Sittie et al., 1999; Omar et al., 2003). *A. indica* A. Juss. (Meliaceae; Neem) is widely distributed in the tropics especially in Africa and Asia. Extracts are used traditionally to treat malaria and other illnesses in several malaria endemic countries (Soh and Benoit-Vical, 2007). The efficacy of its extracts against *Plasmodium falciparum* and *Plasmodium berghei* microgamete exflagellation *in vitro* is attributed to limonoids, a class of highly oxygenated terpenoids which possess insecticidal, anti-microbial, anti-inflammatory and immuno-modulatory activities (Biswas et al., 2002; Roy and Saraf, 2006; Jones et al., 1994). Several studies demonstrated that *A. indica* leaf, seed and stem bark extracts possessed *in vitro* inhibitory activity on *P. falciparum* asexual stages (Udeinya et al., 2008). Its leaves combined with those of *M. indica* is used for the treatment of malaria in Uganda (Tabuti, 2007) while in Togo, they are added to those of *Picralima nitida* and *A. boonei* for malaria therapy (Gbeassor et al., 1996). *A. boonei* De Wild (Apocynaceae) is widely distributed in Africa and used in folklore medicine as antimalaria, anti-inflammatory, analgesic and antipyretic among other uses (Olajide et al., 2000; Betti, 2004). The extract of the stem bark have also been known to possess potent neuroleptic and anxiolytic properties in behavioral studies using mice, probably due to alstonine content, its major chemical constituent (Elisabetsky and Costa-Campos, 2006). Therefore, the present study investigated the toxicity potential and behavioural effect of MD in rats.

MATERIALS AND METHODS

Reagents and materials

Aspartate, alanine transferase, triglyceride and cholesterol assay kits (RANDOX®) were purchased from Randox Laboratories Limited, Atrium, U.K. All other chemicals were of analytical grade, formalin (BDH), diethylether (BDH) and 5% alcohol (BDH).

Animals

Twelve female and twelve male albino rats, weighing between 111-121 g (3 months old) were used. The animals were procured from the animal house of Pharmacology Department, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The animals, divided into four groups (6 rats per group) of both sexes, were kept in plastic cages in the animal house with free access to both food and water. All animals were cared for in accordance with the Guide for the Care and Use of Laboratory

Animals, published by the US National Institute of Health (NIH Publication 85-23, revised 1996).

Plant collection

The leaves of *M. lucida* Benth (Rubiaceae), *A. boonei* De Wild (Apocynaceae), *M. indica* Linn (Anacardiaceae) and *A. indica* A. Juss (Meliaceae) were collected from the Obafemi Awolowo University campus and authenticated at the Botany Department herbarium, Obafemi Awolowo University, Ile-Ife where their specimens were deposited. The leaves were oven-dried at 40°C and powdered.

Preparation of Mama decoction (MD)

Each powdered leaf (25 g) was weighed respectively into a round bottom flask and the mixture boiled together with 1,750 ml of distilled water on a heating mantle for 1 h. After cooling, the decoction was filtered in order to remove the plant residues, followed by the addition of 0.2% sodium benzoate as preservative and 5% Simple Syrup B.P. as a sweetner (following the preparation method used for the commercial production of MD by the Village Chemist, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria).

Administration of Mama decoction

The doses used for this work were calculated based on the adult human dose of the decoction (300 ml of the decoction containing 17.14 g of the leaf mixture of the four plants equivalent to 286 mg/kg, taken twice daily where the average adult weight was taken as 60 kg). Thus, three dose levels (143 mg/kg (low dose), 286 mg/kg (medium dose) and 572 mg/kg (high dose) were chosen for oral administration in rats. Equivalent volume for the dose selected in each case was administered to the animals based on the body weight. The LD₅₀ value of MD was earlier obtained as 3,807 mg/kg per oral in a preliminary study by using Lorke (1983) method. The first three animal groups were assigned to 143 mg/kg (low), 286 mg/kg (medium) and 572 mg/kg (high) doses, respectively while the fourth group served as the control. Freshly prepared MD was administered orally twice daily at those three different doses (143, 286 and 572 mg/kg) for 30 days, using an oral syringe, based on the animal body weights. The dose volumes were adjusted every week to compensate for changes in body weights of the animals. The control group was similarly dosed orally with distilled water twice daily. The dosing procedures continued on the four animal groups for thirty days while each animal was daily assessed for any sign of toxicity or death.

Novelty-induced behavior

Each of the 24 animals was separately put in the open field box and was scored for locomotion, rearing and grooming. The locomotion (number of floor units entered; crossed with all the paws), rearing (number of times the animal stood on its hind limbs or with the forearm against the wall of the observation box or in free air or frequency of standing on hind limbs) and grooming (the number of body cleaning with paws, picking of the body and pubis with mouth and the face washing actions). The behavioral activities of each rat were thus scored and recorded (Suarez et al., 1996; Ajayi and Ukponmwan, 1994). After an animal has been studied for its novelty-induced behavior, the box was cleaned with cotton wool, soaked with ethanol to prevent interference of any odor with the subsequent animal to be studied. The behavioral tests were

commenced on each animal after 1 h of administration of the MD on the day 1 of the experiment while the same behavioral tests were carried out on the control animals which were administered with distilled water. The behavioral testing was repeated for the entire groups on day-30 of the experiment.

Sacrificing the animals

After the novelty-induced behavior (NIB) testing on day 30, the animals were each anaesthetized with diethylether. When the animals were confirmed completely anaesthetized, they were carefully dissected. The blood for biochemical analysis was obtained using cardiac puncture technique and transferred into the pre-labeled ethylenediaminetetraacetic acid (EDTA) sample bottles which were gently rolled between the palms to allow the blood to mix thoroughly with EDTA anticoagulant. The brain, kidney, lungs, liver, spleen and testes were removed, stored in sample bottles containing formalin solution and were all preserved for histopathological analysis.

Biochemical analysis of blood samples

Collection of plasma

Heparinised blood samples were centrifuged at 5000 revolution per min for 5 mins. The supernatant layer (containing the plasma) was aspirated using a 1000 μ L micropipette and transferred into a well labeled plastic container with a stopper. The entire plasma samples were kept in a freezer until ready for use. The following biochemical parameters were determined: triglyceride (TAG), total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) using a commercially available assay kits made by RANDOX Laboratory Ltd, Antrim, UK in accordance with the standard procedures (Kuetze et al., 2010).

Preparation of the liver homogenates

Each liver sample was taken out of the freezer, blotted out of any blood in it and weighed using electronic balance. It was homogenized using an electronic homogenizer (Stir-R) at 1600 rpm and a 10% homogenate was prepared in 0.25 M sucrose.

Histopathology

The histopathological examination was carried following the standard procedures (Abdel Salam and Sleem, 2010; Soujanya et al., 2013). Tissue biopsies from the brain, kidney, liver, lungs and the testes were fixed in formalin solution and were processed with automated tissue processor (Handon citadel 2000). Sections were cut at 4 microns thick with the rotary microtome and stained with haematoxylin and eosin while additional thin sections of the kidney were cut at 3 microns and stained with periodic acid (Schiff). Histological sections were examined using Leica light microscope.

Statistical analysis

All behavioral data were analyzed by ANOVA (SAA Institute Cary, NC), post hoc tests (Student-Newman-Keuls test) were carried out to determine the source of a significant mean effect. Results are expressed as mean \pm S.E.M. while p values $<$ 0.05 were taken to indicate statistically significant differences.

RESULTS

The body weight

Sub-chronic administration of MD did not cause any significant [F(3,23)=1.525, p =0.2387; n =6 per group] change in the body weight of rats used in this study. (Control: 26.85 ± 5.59 ; 143 mg/kg: 22.56 ± 4.33 ; 286 mg/kg: 26.65 ± 3.70 ; 572 mg/kg: 12.41 ± 7.5).

Novelty-induced behavior (open field)

The results obtained showed that there was no significant effects of MD administration on locomotion [F(3,20)=2.46, p =0.098], rearing [F(3,20)=0.70, p =0.563] and grooming [F(3,20)=0.57, p =0.640] on day 1 when compared with the control group (Table 1). However, at Day 30, after the sub-chronic administration of MD, there was a significant [F(3,20)=4.60, p =0.015] increase in locomotor activity at the medium dose level of 286 mg/kg when compared with the control whereas the other two doses did not exhibit any significant effects on locomotion. In rearing, the results showed that there was no significant difference [F(3,20)=1.24, p =0.326] due to treatment. In grooming, it was observed that there was a significant [F(3,20)=14.98, p =0.0001] dose-dependent decrease in this behavior when compared with the control (Table 2).

Biochemical analysis

Plasma

Biochemical analysis of the plasma revealed that there was significant difference in both triglyceride [F(3, 23) = 6.09, p =0.004; n =6] and cholesterol [F(3, 23) = 12.94, p =0.0001; n =6] levels of treated animals only at the low dose of 143 mg/kg only when compared with the vehicle-treated rats (control). Furthermore, the results showed that other biochemical parameters such as ALT [F(3, 23) = 25.38, p =0.0001; n =6], AST [F(3, 23) = 19.58, p =0.0001; n =6] and ALT [F(3, 23) = 42.83, p =0.0001; n =6] were significantly increased in all treatment groups when compared with the control group (Table 3).

Liver

In the assay of triglyceride, one-way ANOVA indicated that a significant [F(3,23)=11.19, p =0.0002; n =6] decrease was produced by both 143 and 572 mg/kg dose levels with a dose-dependent significant decrease in cholesterol level that was significant [F(3,23)=140.66, p =0.0001; n =6] when compared with the control. Similarly, ALT was significantly [F(3,23)=297.69, p =0.0001; n =6] decreased in a dose-dependent manner in the treatment groups that

Table 1. Novelty-induced behavior (Day 1).

Dose (mg/kg, p.o.)	Locomotion(30 min)	Rearing(30 min)	Grooming(30 min)
Control	44.2 ± 14.0	24.5 ± 6.4	16.3 ± 5.5
143	42.5 ± 9.2	30.3 ± 13.3	15.3 ± 2.5
286	88.8 ± 17.1	36.7 ± 7.5	15.2 ± 1.7
572	60.8 ± 10.2	22.2 ± 5.9	10.4 ± 0.5

Data presented as mean ± SEM, n=4-6.

Table 2. Novelty-induced behavior (Day 30).

Dose (mg/kg, p.o.)	Locomotion(30 min)	Rearing(30 min)	Grooming(30 min)
Control	36.2 ± 7.0	25.5 ± 2.3	33.3 ± 3.1
143 mg/kg	36.8 ± 4.8	18.3 ± 4.7	17.3 ± 3.2*
286 mg/kg	97.5 ± 16.6*	40.2 ± 12.8	12.0 ± 2.4*
572 mg/kg	59.2 ± 19.0	24.0 ± 7.1	10.4 ± 2.6*

Data arranged as mean ± SEM, n=4-6; * p<0.05 statistically significant compared to the control animals (ANOVA followed by Newman-Keuls' test).

Table 3. Effects of MD (143, 286, 572 mg/kg, p.o.) after the 30 days subchronic administration on biochemical parameters in plasma.

Group	TRG (mg/dl)	CHOL (mg/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	153.37 ± 2.83	165.95 ± 6.23	38.37 ± 1.05	120.09 ± 1.00	122.87 ± 0.91
143 mg/kg	125.17 ± 1.92*	128.83 ± 5.73*	58.83 ± 1.85*	141.17 ± 2.20*	147.17 ± 3.42*
286 mg/kg	149.67 ± 9.41	165.83 ± 3.36	54.00 ± 1.69*	136.33 ± 3.12*	148.83 ± 1.92*
572 mg/kg	137.17 ± 2.76	156.00 ± 3.45	57.00 ± 2.52*	148.50 ± 3.75*	165.00 ± 3.46*

TRG, Triglyceride; CHOL, cholesterol, ALT, alanine aminotransferase; AST, aspartate transaminase and ALP, alkaline phosphatase. Values are mean ± SEM (n=6 per group), *p<0.05 compared to control animals (ANOVA followed by Newman-Keuls' test).

were administered with MD sub-chronically when compared with the vehicle-treated rats. However, the results showed that AST was significantly [F(3,23)=28.04, p=0.0001; n=6] increased in all the MD treated rats. In the assay of ALP, the significant [F(3,23)=3.47, p=0.035; n=6] difference was only noted among the treated groups but not with vehicle-treated rats (Table 4).

Histopathology

The result of the histopathology of the organs studied revealed that there was a moderately interstitial expansion of the lungs with infiltration by lymphocytes in one out of six rats [control and MD: 143, 286 and 582 mg/kg]. Similarly, there was significant expansion of the white pulp with reactive germinal center in the spleen of one out of six rats [control and MD: 143, 286 and 582 mg/kg]; while the other organs (liver, kidney, heart, brain and testes) were essentially normal in all the treated animals with MD and control.

DISCUSSION

In the present study, investigations on the potential toxicity and central nervous system effects of both acute and sub-chronic administration of MD were carried out. In our study, biomarkers were selected to include a wide range of behavioral, biochemical and histological parameters. Both acute and sub-chronic oral administration of MD in rats revealed neither mortality nor any sign of physical toxicity. The novelty-induced behavioral studies on days 1 and 30 showed that locomotion was significantly increased at the dose of 286 mg/kg, p.o. Generally, the rearing behavior was unaffected, although it was non-significantly increased at 286 mg/kg. The grooming behavior was not significantly affected during the acute administration (Day 1) but was significantly decreased dose-dependently during the sub-chronic administration. Thus, the overall results showed that MD significantly possessed central effects suggesting the need for further investigation to unravel its possible central mechanism (s) of action.

Table 4. Effects of MD (143, 286, 572 mg/kg, p.o.) after the 30 days subchronic administration on biochemical parameters in liver of rats.

Group	TRG (mg/dl)	CHOL (mg/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	4.82 ± 0.32	11.85 ± 0.47	25.54 ± 1.07	15.65 ± 0.83	46.13 ± 2.03
143 mg/kg	3.56 ± 0.15*	4.11 ± 0.41*	4.79 ± 0.26*	28.53 ± 0.73*	41.06 ± 2.78
286 mg/kg	4.23 ± 0.30	4.41 ± 0.26*	5.15 ± 0.38*	29.80 ± 2.35*	47.65 ± 0.96
572 mg/kg	2.94 ± 0.15*	3.00 ± 0.10*	5.88 ± 0.16*	30.24 ± 0.44*	49.33 ± 1.39

TRG, Triglyceride; CHOL, cholesterol; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase. Values are mean ± SEM (n=6 per group), *p<0.05 compared to control animals (ANOVA followed by Newman-Keuls' test).

The biochemical analysis showed that all the biochemical parameters were significantly affected by the sub-chronic administration of MD as revealed in the results obtained. Both triglyceride and cholesterol levels were significantly decreased in both the plasma and liver only at 143 mg/kg (low dose level). At 572 mg/kg dose level, the two biochemical parameters were decreased in the liver, whereas at 286 mg/kg (medium dose level) no significant effect on these biochemical parameters were observed. This clearly suggests that sub-chronic administration of MD at the medium (human therapeutic) dose has no significant effect on these biochemical parameters. High cholesterol production is known to occur in the liver; other sites of high as well as in other body organs such as intestines, adrenal glands and reproductive organs (Kumar and Clark, 2005). It occurs free or as an ester which is formed with the help of an enzyme called lecithin cholesterol acyltransferase (LCAT). In a severe liver damage, the level of this enzyme reduction can lead to an increase in the concentration of cholesterol in the plasma. The implication of this is the accumulation of these lipids in the arterial walls leading to atherosclerosis due to obstruction and distortion of the arteries with serious implications related to cardiac disorders such as angina, myocardial infarction, strokes, peripheral vascular disease and hypertension (Brunzell et al., 2008). Conversely, abnormally low levels of cholesterol, known as hypocholesterolemia, have been reported to be associated with depression, cancer and cerebral hemorrhage (Lewington et al., 2007). From the results obtained in this study, the low levels of both triglyceride and cholesterol obtained are still within the normal range. Thus, MD can not cause any cardiovascular disease that may be attributed to lipid concentrations. The ALT, AST and ALP results showed that all these biochemical parameters were significantly increased dose-dependently in serum while in the liver only ALT was significantly decreased dose-dependently. There was no significant effect on ALP but AST was significantly increased in the liver. The significant increase in ALT, AST and ALP levels in plasma, following the administration of MD clearly suggested the possibility of liver or bone damage since these are diagnostic markers for the detection of possible liver or bone damage as a diagnostic tool (Attiah et al., 2013).

However, further biochemical analysis of the liver did not show this pattern completely except for the increase in AST similarly observed in the liver. Slight-to-moderate elevations of ALT (usually with higher increases in AST levels) may appear in any condition that produces acute hepatocellular injury. Confirmatory procedures to ascertain the liver status were performed and it was observed that there were no significant histopathological effects on the liver. In fact, it was very clear that most of the organs were essentially normal. However, one of the major differences observed was that there was a moderately interstitial expansion with infiltration by lymphocytes in the lungs of one out of six rats [(control and MD: 143, 286 and 572 mg/kg) during the subchronic administration of MD. Similarly, there was significant expansion of the white pulp with reactive germinal center in the spleen of one out of six rats (control and MD: 143, 286 and 572 mg/kg). The remaining organs namely liver, kidney, heart, brain and testes were essentially normal in all the animals treated with MD and the control. Thus, the observed effects on the lungs and spleen could not have been due to the treatment with MD since one of the control animals also manifested those effects in its lungs and spleen. Indeed, it had earlier been reported that infiltration of lymphocytic cells into any organ usually occurred as a result of some hypersensitivity reaction, possibly due to an antigen-immune response (Cotran et al., 1999). In conclusion, therefore, MD possessed a central effect as well as with relative safety at the recommended doses but there is need for caution when used at high doses or on prolonged periods. In humans, it is normally taken twice daily at a dose of 286 mg/kg for only 2-4 days to treat malaria.

ABBREVIATIONS

NIB, Novelty-induced behavior; **TAG**, triglyceride; **AST**, aspartate aminotransferase; **ALT**, alanine aminotransferase; **ALP**, alkaline phosphatase.

REFERENCES

Abdel Salam OM, Sleem AA, Shafee N (2010). Effect of trazodone and nefazodone on hepatic injury induced by carbon tetrachloride.

- Drug Discov. Ther. 4(4):285-297.
- Attiah AMM, Ibrahim FAA, Nabil GM, Aziz SW (2013). Antioxidant effects of ginger (*Zingiber officinale* Roscoe) against lead acetate-induced hepatotoxicity in rats. *Afr. J. Pharm. Pharmacol.* 7(20):1213-1219.
- Ajayi AA, Ukponmwan OE (1994). Possible evidence of angiotensin II and endogenous opioid modulation of novelty-induced rearing in the rat. *Afr. J. Med. Sci.* 22:287-290.
- Betti JL (2004). An ethnobotanical study of medicinal plants among the Baka Pygmies in the dja biosphere reserve, Cameroon. *Afr. Stud. Monographs* 25(1):1-27.
- Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U (2002). Biological activities and medicinal properties of neem (*Azadirachta indica*). *Curr. Sci.* 82:1336-1345.
- Brunzell JD, Davidson M, Furberg CD, Goldberg RB, Howard BV, Stein JH, Witztum JL (2008). Lipoprotein management in patients with cardiometabolic risk: consensus conference report from the American Diabetes Association and the American College of Cardiology Foundation. *J. Am. Coll. Cardiol.* 51(15):1512-1524.
- Cotran RS, Kumar V, Collins T (1999). Diseases of Immunity. In: Cotran RS, Kumar V, Collins T, editors. Robbins pathologic basis of disease. Philadelphia: Saunders, p.188-259.
- Elisabetsky E, Costa-Campos L (2006). The Alkaloid Alstonine: A Review of its Pharmacological properties. *CAM.* 3(1):39-48.
- Garrido G, Gonzalez D, Lemus Y, Garcia D, Lodeiro L, Quintero G, (2004). *In vivo* and *in vitro* anti-inflammatory activity of *Mangifera indica* L. extract (VIMANG). *Pharmacol. Res.* 50:143-149.
- Gbeassor M, Koumaglo HK, Awang DVC, Durst J, Mackinnon S, Arnason JT (1996). Development of ethical phytomedicines for Togo, West Africa. In: Chemistry, Biological and Pharmacological Properties of African Medicinal Plants. University of Zimbabwe Publications, p. 336.
- Guha S, Ghosal S, Chattopadhyay U (1996). Antitumor, immunomodulatory, and antiHIV effect of mangiferin: A naturally occurring glucosylxanthone. *Chemotherapy* 42:443-451.
- Jones IW, Denholm AA, Ley SV, Lovell H, Wood A, Sinden RE (1994). Sexual development of malaria parasites is inhibited in vitro by the neem extract azadirachtin, and its semi-synthetic analogues. *FEMS Microbiol. Lett.* 120:267-273.
- Kuete V, Manfouo RN, Beng VP (2010). Toxicological evaluation of the hydroethanol extract of *Tabernaemontana crassa* (Apocynaceae) stem bark. *J. Ethnopharmacol.* 130(3):470-476.
- Kumar P, Clark M (eds.) (2005). Kumar & Clark Clinical medicine. 6th ed. Edinburgh: W. B. Saunders, 2005.
- Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, Halsey J, Qizilbash N, Peto R, Collins R (2007). Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. *Lancet* 370(9602):1829-39.
- Lorke D (1983). A new approach to practical acute toxicity testing. *Arch Toxicol.* 54(4):275-287.
- Martnez G, Delgado R, Perez G, Garrido G, Nenez-Sells AJ, Leon OS (2000). Evaluation of the *in vitro* antioxidant activity of *Mangifera indica* L. extract (VIMANG). *Phytother. Res.* 14:424-427.
- Ngo T (2001). Contribution to researches on mango leaves raw materials in northern Vietnam. *Sci. Technol.* 563-565.
- Olajide OO, Awe SO, Makinde M, Ekhehar AI, Olusola A, Morebise O, Okpako DT (2000). Studies on the anti-inflammatory, antipyretic and analgesic properties of *Alstonia boonei* stem bark. *J. Ethnopharmacol.* 71:179-186.
- Omar S, Zhang J, MacKinnon S, Leaman D, Durst T, Philogene BJ, Arnason JT, Sanchez-Vindas PE, Poveda L, Tamez, P.A., Pezzuto, J.M. 2003. Traditionally used antimalarials from the Meliaceae. *Curr. Top. Med. Chem.* 3:133-139.
- Roy A, Saraf S (2006). Limonoids: Overview of significant bioactive triterpenes distributed in plants kingdom. *Biol. Pharm. Bull.* 29:191-201.
- Sittie AA, Lemmich E, Olsen CE, Hviid L, Kharazmi A, Nkrumah FK, Christensen SB (1999). Structure-activity studies: *in vitro* antileishmanial and antimalarial activities of anthraquinones from *Morinda lucida*. *Planta Med.* 65:259-261.
- Soh PN, Benoit-Vical F (2007). Are West African plants a source of future antimalarial drugs? *J. Ethnopharmacol.* 114:130-140.
- Soujanya S, Lakshman M, AnaadKumar A, Gopala Reddy A (2013). Evaluation of the protective role of vitamin C in imidacloprid-induced hepatotoxicity in male Albino rats. *J. Nat. Sci. Biol. Med.* 4(1):63-67.
- Suarez M, Perassi N, Dal Zotto S (1996). Adrenocortical response to open-field test in rats with anterodorsal thalamic nuclei lesion. *Arch. Physiol. Biochem.* 104(1):81-86.
- Tabuti JRS (2007). Herbal medicines used in the treatment of malaria in Budiope county, Uganda. *J. Ethnopharmacol.* 116:33-42.
- Udeinya JI, Shu EN, Quakyi I, Ajayi FO (2008). An antimalarial neem leaf extract has both schizonticidal and gametocytocidal activities. *Am. J. Ther.* 15:108-110.
- Zheng MS, Lu ZY (1990). Antiviral effect of mangiferin and isomangiferin on Herpes simplex virus. *Chin. Med. J.* 103:160-165.

Full Length Research Paper

Antinociceptive and anti-inflammatory activities of the hexane extract from *Hortia brasiliiana* Vand. leaves on experimental animal models

Carlos Cerqueira Magalhães¹, Carolina Miranda Gasparetto¹, Luciana Moreira Chedier³, Daniel Sales Pimenta³, Maria Silvana Alves² and Orlando Vieira de Sousa^{2*}

¹Graduate Program in Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Juiz de Fora, Campus Universitário, Juiz de Fora, Minas Gerais, 36036-330, Brazil.

²Department of Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Juiz de Fora, Campus Universitário, Juiz de Fora, Minas Gerais, 36036-330, Brazil.

³Department of Botany, Institute of Biological Sciences, Federal University of Juiz de Fora, Campus Universitário, Juiz de Fora, 36036-330, Brazil.

Accepted 11 July, 2013

Antinociceptive and anti-inflammatory activities of the hexane extract from *Hortia brasiliiana* Vand. leaves were investigated by experimental animal models. Guaiol (9.72%), nonacosane (25.57%) and eicosane (10.80%) were the most abundant components identified by GC/MS. The extract, with LD₅₀ of 2.40 g/kg, reduced the number of abdominal contortions by 13.86 (100 mg/kg) and 18.51% (200 mg/kg). Doses of 100 and 200 mg/kg inhibited both phases of the time paw licking: First phase (9.44% and 16.32%) and the second phase (11.97 and 23.49%), respectively. The extract increased the reaction time on a hot plate at doses of 100 (24.92%) and 200 mg/kg (55.69%) after 90 min of treatment. The paw edema was reduced by the hexane extract at doses of 100 (10.42 and 8.23%) and 200 mg/kg (15.62 and 17.65%) after 3 to 4 h of application of carrageenan, respectively. At the dose of 200 mg/kg, the extract reduced the exudate volume by 19.44%, while the leucocyte migration was inhibited at the doses of 100 and 200 mg/kg (6.13 and 13.84%, respectively). These results suggest that *H. brasiliiana* can be an active source of substances with antinociceptive and anti-inflammatory activities supporting the use in the Brazilian folk medicine.

Key words: *Hortia brasiliiana*, Rutaceae, antinociceptive activity, anti-inflammatory activity.

INTRODUCTION

One of the cardinal features of the inflammatory states is that normally innocuous stimuli produce pain (Stankov, 2012). The physiological mechanisms of pain involve peripheral sensitization and neuroplasticity in the perpetuation of this event, with action through chemical mediators on nociceptive pathways (Stankov, 2012). Based on the origin of these mediators, these substances

can contribute to the cycle of pain causing hypersensitivity by synthesis of cytokines (White et al., 2005). Usually, the pain is treated with opioids and non steroidal anti-inflammatory drugs (NSAIDs). However, the adverse effects as constipation and respiratory depression and irritation of gastric mucosa and ulcer, water retention and nephrotoxicity for opioids and NSAIDs, respectively, have

*Corresponding author. E-mail: orlando.sousa@ufff.edu.br.

prevented the application of these therapeutic agents (Kawada et al., 2012; Benyamin et al., 2008). Another important option in the treatment of pain and inflammation is the use of medicinal plants that is a common practice worldwide (Shah et al., 2011). Therefore, the evaluation of the pharmacological effects of these plants can be used as a strategy and support to find new drugs with scientific sustainability in the treatment of many disorders.

Hortia brasiliiana Vand. (Rutaceae), proposed as synonyms *Hortia arborea* Engl., *Hortia badinii* M. Lisboa and *Hortia colombiana* Groppo, is distributed from Panama to the state of São Paulo, Brazil, most of them occurring in the Amazonian region (Groppo, 2010). In Brazilian folk medicine reports, plants of the genus *Hortia*, including *H. brasiliiana*, possess excellent stimulant, stomachic, sedative, hypotensive, antiulcerogenic, analgesic and anti-inflammatory properties (Sobrinho et al., 2011).

In addition, ruteacarpine, an alkaloid found in plants of Rutaceae family as *H. brasiliiana*, has demonstrated cardiovascular, antithrombotic, anticancer, anti-inflammatory, analgesic, antiobesity and thermoregulatory activities and effects on endocrine and smooth muscle (Lee et al., 2008), as well as cyclooxygenase-2 inhibition (Liao et al., 2011).

Considering the phytochemical and pharmacological aspects, *H. brasiliiana* has been described as a large source of alkaloids, coumarins, flavonoids, and limonoids (Suárez et al., 1998; Severino et al., 2009a, 2009b, 2012). Five alkaloids were isolated from wood and identified as flindersine, N-methylflindersine, γ -fagarine, skimmianine and 2,4-dimethoxyquinoline (Suárez et al., 1998). A tetranortriterpenoid and two dihydrocinnamic acid derivatives, as well as alloxanthoxyletin, nerolidol, epoxynerolidol, three known dihydrocinnamic acid derivatives and two amides were obtained from the wood (Suárez et al., 2002). Four dihydrocinnamic acid derivatives, limonoid guyanin and furoquinoline alkaloid dictamnine, obtained from roots, showed antibacterial activity (Severino et al., 2009a). Ruteacarpine, found in leaves and wood of *H. brasiliiana*, has demonstrated different pharmacological properties, including analgesic and anti-inflammatory (Shin et al., 2007; Lee et al., 2008; Liao et al., 2011).

From leaves were identified guyanin, ruteacarpin and dictamnine and dihydrocinnamic acid derivatives, together with the new cinnamic acid derivative, been observed plasmocidal and trypanosomidal activities (Severino et al., 2009b). Moreover, several limonoids were identified in the dichloromethane extracts obtained from taproots and stems (Severino et al., 2012).

In the present study, *H. brasiliiana* was selected because it is one plant among Brazilian biodiversity commonly used as traditional medicine to treat abdominal pain, headache, inflammation, swelling and rheumatism.

However, extracts from leaves have not been evaluated systematically for pharmacological properties to corroborate the traditional uses of this species in folk medicine. In this investigation, we evaluated the antinociceptive and anti-inflammatory activities of *H. brasiliiana* in experimental animal models using hexane extract.

MATERIALS AND METHODS

Plant material and extraction

Leaves of *Hortia brasiliiana* Vand. were collected in the city of Muriaé, Minas Gerais State, Southeast region of Brazil, in September 2010. The species was identified by Dr Milton Groppo Júnior and a voucher specimen (number 59192) was deposited in the Herbarium CESJ, Federal University of Juiz de Fora, Brazil. Dried and cut leaves (20 g/100 ml) were exhaustively extracted in hexane by static maceration at room temperature with renewal of solvent every day. The hexane was filtered and evaporated under a rotary evaporator at controlled temperature (40 to 45°C).

Gas chromatography/mass spectrometry analysis (GC/MS)

This analysis was carried out using a Hewlett-Packard 6890 gas chromatograph equipped with a fused silica capillary column (HP-5, 30m \times 0.25 mm, 0.25 μ m film thickness), helium as carrier gas with a flow rate 1.0 ml/min; temperature programming from 70°C to 290°C (2°C/min), coupled to a Hewlett-Packard 5972 mass spectrometer. The MS operating parameters were: 70 eV, ion source 250°C equipped with EI. The compound identifications were carried out by comparison of their retention indices (RI) with literature values; and the MS data with those from Wiley 275.1 mass spectral data base besides literature records (Adams, 1995). The retention indices were calculated using a GC data of a homologous series of saturated aliphatic hydrocarbons within C8 to C22.

Chemicals

Drugs and reagents used in this study (and their sources) were as follows: Acetic acid and hexane (Vetec Química Farm Ltda, Rio de Janeiro, RJ, Brazil), formaldehyde and acetylsalicylic acid (Reagen Quimibrás Ind. Química S.A., Rio de Janeiro, RJ, Brazil), morphine hydrochloride (Merck Inc., Whitehouse Station, NJ, USA), naloxone and indomethacin (Sigma Chemical Co, St Louis, MI, USA).

Animals

Male Wistar rats (90-110 days) weighing 200 to 240 g and male Swiss albino mice (50-70 days) weighing 25 to 30 g were used in the experiments. The animals were provided by the Central Biotery of the Federal University of Juiz de Fora. The animals were divided into groups and kept in plastic cages (47 \times 34 \times 18 cm) under a 12 h light/12 h dark cycle at room temperature (22 \pm 2 °C), with free access to Purina® rations and water. Animal care and the experimental protocol followed the principles and international guidelines suggested by the Brazilian College of Animal Experimentation (COBEA) and were approved by the local ethical committee (protocol number 036/2010).

Acute toxicity

Groups of ten mice received oral doses of 0.5, 1, 1.5, 2 and 3 g/kg of hexane extract from *H. brasiliiana*, while the control group received the vehicle (saline). Due to solubility of the extract, the volume changed from 0.3 to 0.9 ml. After administration, the groups were observed for 48 h and 50% lethal dose (LD₅₀) was the mortality at the end of this period was recorded for each group (Lorke, 1983). The LD₅₀ determined by probit test using a log plot of percentage death *versus* dose (Litchfield and Wilcoxon, 1949).

Acetic acid-induced writhing test

The acetic-acid writhing test is used for the evaluation of the analgesic activity (Schmidt et al., 2010). Mice (n = 8 per group) were injected (i.p.) with 0.6% acetic acid (10 ml/kg body weight), and the intensity of nociception was quantified by counting of the total writhes number that occurred between 10 and 30 min after injection. Animals received hexane extract (50, 100 or 200 mg/kg, p.o.) or sterile saline (control group, 0.9%, w/v) 60 min before acetic acid injection. Acetylsalicylic acid (200 mg/kg, p.o.) and Indomethacin (10 mg/kg, p.o.) were administered 60 min before acetic acid as reference compounds.

Formalin test

Groups of mice (n = 8) were treated (p.o.) with hexane extract (50, 100 or 200 mg/kg) or sterile saline (0.9%) 60 min before formalin injection. Twenty microliters of 1% formalin was administered (i.pl.) in the mouse's right paw and the licking time was recorded from zero to 5 min (phase 1, neurogenic) and from 20 to 25 min (phase 2, inflammatory) (Hunskar and Hole, 1987). Morphine (1 mg/kg, s.c.) and indomethacin (10 mg/kg, p.o.) were also administered 60 min before the formalin injection and used as reference compounds.

Hot plate test

Animals were placed on a hot-plate (Model LE 7406, Letica Scientific Instruments, Barcelona, Spain) heated at 55 ± 1°C (Eddy and Leimbach, 1953). Three groups of mice (n = 8) were treated (p.o.) with hexane extract (50, 100 or 200 mg/kg; 0.1 ml per 10 g body weight); the control group received sterile saline (10 ml/kg). Measurements were performed at zero, 30, 60 and 90 min after drug administration, with a cut-off time of 40 s to avoid lesions in the animals' paws. The effect of pretreatment with naloxone (1 mg/kg, subcutaneously) on the analgesia produced by the hexane extract (200 mg/kg) was determined in a separate group of animals. Morphine (1 mg/kg, s.c.), in the absence and presence of naloxone (1 mg/kg, s.c.) treatment, was used as a reference.

Carrageenan-induced rat paw edema

Anti-inflammatory activity was assessed on the basis of inhibition of paw edema induced by the injection of 0.1 ml of 2% carrageenan (an edematogenic agent) into the subplantar region of the right hind paw of the rat (Winter et al., 1962). Male Wistar rats were divided into groups of six animals which received (p.o.) doses of hexane extract (50, 100 and 200 mg/kg; 0.1 ml per 10 g body weight), saline or indomethacin (10 mg/kg) 1 h before the injection of carrageenan. In the left paw, used as a control, 0.1 ml of sterile

saline was injected. 1, 2, 3 and 4 h after injection of carrageenan, the measure of edema was made by the difference between the volume displaced by the right paw and the left paw using a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain).

Carrageenan-induced pleurisy in rats

Pleurisy was induced in male Wistar rats by intrapleural administration of 0.5 ml 2% carrageenan suspension in saline solution between the third and fifth ribs on the right side of the mediastinum (Vinegar et al., 1973). Hexane extract (50, 100 or 200 mg/kg), saline or indomethacin (10 mg/kg) (p.o.) were given 60 min before injection of the irritant agent. Animals were killed 4 h after carrageenan injection, and the skin and the pectoral muscles were retracted. A longitudinal incision was made between the third and fifth ribs on each side of the mediastinum. The exudate was collected and transferred to a 15 ml conical centrifuge tube and the total volume determined. A 20 µl aliquot was used to determine the total leucocyte count by Neubauer chambers.

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical significance was analysed by the one-way analysis of variance followed by the Student Newman-Keuls test. *P* values below 0.05 were considered significant.

RESULTS

Chemical constituents

According Table 1, based on the fragmentation pattern obtained in the mass spectrometry, constituents belonging to the classes of sesquiterpenes (20.28%), diterpenes (3.94%), hydrocarbons (36.37%) and pentacyclic triterpenes (35.12%) were detected in the hexane extract from *H. brasiliiana* leaves. Among the identified compounds, eight were sesquiterpenes and two hydrocarbons. Guaiol (9.72%), nonacosane (25.57%), eicosane (10.80%) and one pentacyclic triterpene (18.49%) were the most abundant components found in the extract.

Acute toxicity

At the doses administered per oral route (p.o.), the hexane extract from *H. brasiliiana* leaves was toxic to animals with LD₅₀ of 2.40 g/kg (95% confidence intervals 1.51-3.83 g/kg). However, in the evaluated period, the animals did not show cyanosis, piloerection, writhing, ptosis, tremors, convulsions, ataxia, hypnosis, red urine or diarrhea. The parameters like motor activity, respiration, corneal reflex, righting and withdrawal, and body tone were not affected.

Table 1. Constituents of the hexane extract from *Hortia brasiliiana* leaves.

Compound	Chemical class	Retention time	Concentration (%)
Alpha-curcumene	Sesquiterpene	18.67	2.70
Beta-bisabolene	Sesquiterpene	19.32	0.86
Beta-sesquiphellandrene	Sesquiterpene	19.72	1.16
Caryophyllene oxide	Sesquiterpene	21.27	0.94
Guaiol	Sesquiterpene	21.50	9.72
Dihydrolinalool	Sesquiterpene	23.47	1.61
Trans-alpha-bergamotene	Sesquiterpene	23.63	1.30
Trans-bergamotol	Sesquiterpene	23.99	2.53
Unidentified	Diterpene	24.67	1.62
Unidentified	Diterpene	26.10	2.32
Nonacosane	Hydrocarbon	44.33	25.57
Eicosane	Hydrocarbon	46.95	10.80
Unidentified	Pentacyclic triterpene	50.33	5.12
Unidentified	Pentacyclic triterpene	50.56	2.73
Unidentified	Pentacyclic triterpene	51.05	18.49
Unidentified	Pentacyclic triterpene	54.89	8.78
Total			96.25

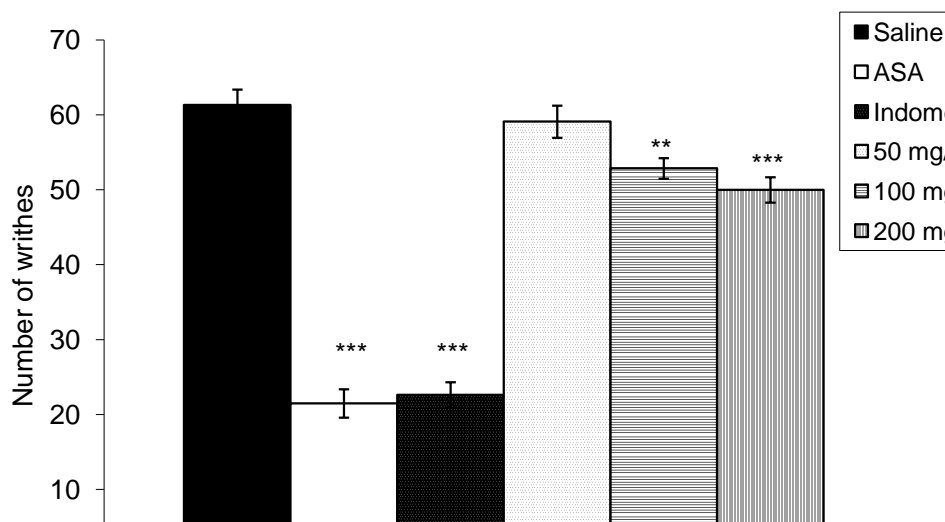


Figure 1. Effects of the hexane extract from *H. brasiliiana* leaves on acetic acid-induced writhing in mice. ASA, Acetylsalicylic acid. Data are mean \pm S.E.M. of eight mice. ** $p < 0.01$; *** $p < 0.001$ vs control group (saline).

Acetic acid-induced writhing response in mice

The treatment of animals with hexane extract (100 and 200 mg/kg, p.o.) produced a significant ($p < 0.01$ and $p < 0.001$, respectively) and dose-dependent inhibition in abdominal writhes induced by acetic acid (Figure 1). The control group caused 61.36 ± 2.05 abdominal contortions. Acetylsalicylic acid (200 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) decreased the abdominal contortions by

64.96 and 63.13%, respectively, when compared with the control group.

Formalin-induced paw licking in mice

The Figure 2 shows that pretreatment with morphine (1 mg/kg) or with the hexane extract of *H. brasiliiana* (100 and 200 mg/kg, p.o.) produced significant changes of

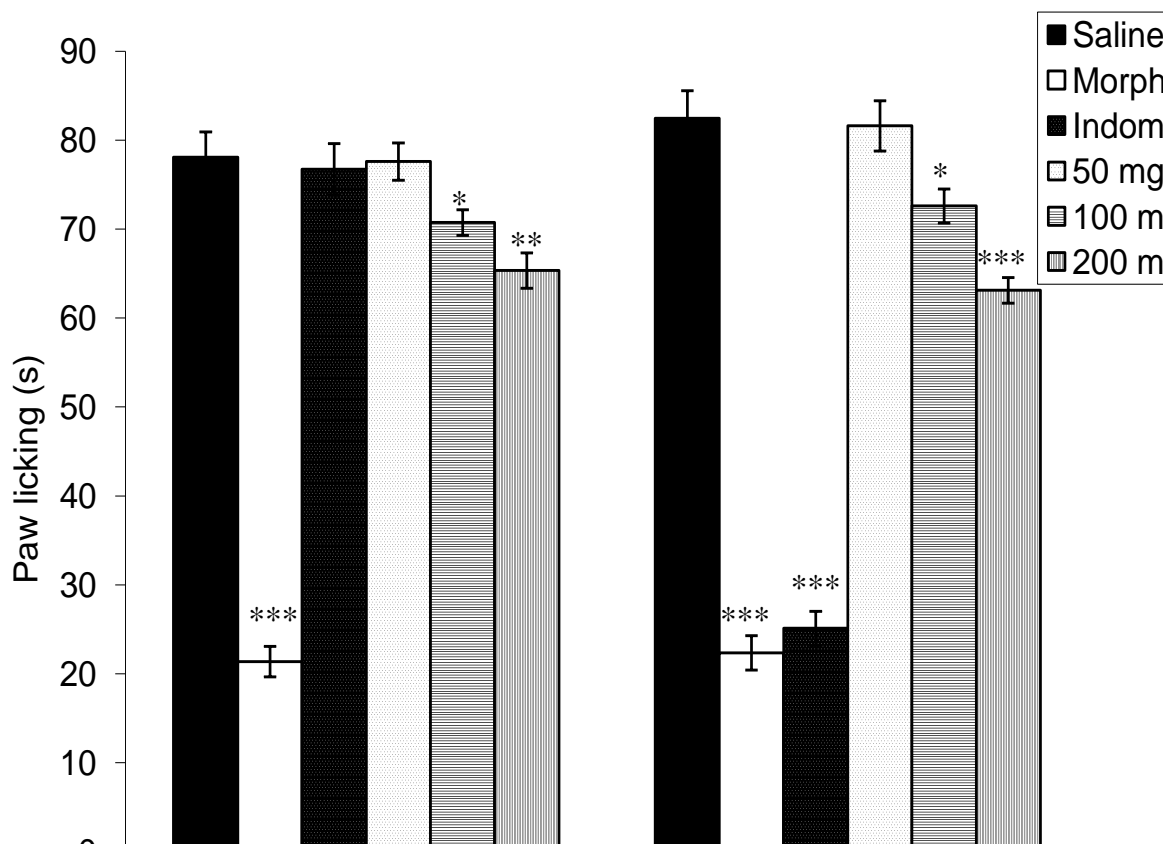


Figure 2. Effects of the hexane extract from *H. brasiliensis* leaves on formalin-induced nociception in mice. Data are mean \pm S.E.M. of eight mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs control group (saline).

paw licking time in the first phase of pain response. In the second phase, a dose-dependent and significant ($p < 0.05$ or $p < 0.001$) reduction was observed in mice treated with extract (100 and 200 mg/kg, p.o.) as well as with indomethacin (10 mg/kg, p.o.) and morphine (1 mg/kg, s.c.). For the control group, the time spent was 78.12 ± 2.82 s and 82.50 ± 3.10 s in the first and second phases, respectively.

Effects on hot-plate latency assay in mice

In consequence of the analgesic effect observed in the first phase of formalin test, we decided to evaluate the hexane extract using hot plate test, a model of central antinociceptive activity.

After 60 min of treatment, doses of 200 mg/kg ($p < 0.01$) increased significantly the latency time in the respective control group (Table 2). Morphine (1 mg/kg) proved to be a potent analgesic increasing the latency time within the evaluated periods. Naloxone (1 mg/kg, s.c.), an opioid antagonist, blocked the effect of morphine (1 mg/kg, s.c.) and extract (200 mg/kg, p.o.).

Effects on carrageenan-induced edema in rats

The anti-inflammatory effect of the hexane extract from *H. brasiliensis* evaluated by the paw edema method induced by carrageenan is shown in Table 3. Edema inhibition was observed 3 h after carrageenan application in the doses of 100 (0.86 ± 0.02 ; 10.42%; $p < 0.05$) and 200 mg/kg (0.81 ± 0.02 ; 15.62%; $p < 0.01$). Four hours after carrageenan injections, the doses of 100 (0.78 ± 0.02 ; $p < 0.05$) and 200 (0.70 ± 0.02 ; $p < 0.001$) reduced the paw edema in 8.23 and 17.65%, respectively. Indomethacin (10 mg/kg), reference drug, was active from 2 h of treatment.

Effects on carrageenan-induced pleurisy in rats

The anti-inflammatory effect of the hexane extract from *H. brasiliensis* was confirmed by a decrease in exudate volume and leucocyte migration to the pleural cavity of rats. The pleurisy effects demonstrated that dose of 200 mg/kg (0.87 ± 0.05 ; $p < 0.05$) of the extract significantly reduced the exudate volume by 19.44% when compared

Table 2. Effects of the hexane extract from *H. brasiliiana* leaves on the latency time of mice exposed to the hot plate test.

Group	Dose (mg/kg)	Time after drug administration (s)			
		0 min	30 min	60 min	90 min
Control	Saline	6.12±0.64	6.37±0.60	6.25±0.65	6.50±0.60
	50	6.50±0.50	6.50±0.57	7.12±0.58	7.50±0.42
Extract	100	6.75±0.65	6.87±0.51	7.50±0.42	8.12±0.40*
	200	6.62±0.65	7.37±0.60	8.87±0.48**	10.12±0.51***
Morphine	1	6.25±0.65	10.25±0.67***	12.37±0.86***	15.25±0.80***
Naloxone+morphine	1 + 1	6.00±0.71	9.15±0.64**	8.75±0.67*	7.87±0.80
Naloxone+extract	1 + 200	6.62±0.50	7.12±0.72	8.12±0.44*	7.75±0.53

Data are mean ± S.E.M. of eight mice. *p < 0.05; **p < 0.01; ***p < 0.001 vs control group (saline).

Table 3. Effects of the hexane extract from *H. brasiliiana* leaves on carrageenan-induced paw edema in rats.

Group	Dose (mg/kg)	Volume of hind paw (ml)			
		1 h	2 h	3 h	4 h
Control	Saline	0.55±0.02	0.76±0.02	0.96±0.03	0.85±0.02
Indomethacin	10	0.53±0.02	0.56±0.02***	0.62±0.02***	0.47±0.01***
	50	0.56±0.02	0.77±0.02	0.94±0.03	0.84±0.02
Extract	100	0.56±0.01	0.76±0.02	0.86±0.02*	0.78±0.02*
	200	0.54±0.02	0.75±0.02	0.81±0.02**	0.70±0.02***

Data are mean ± S.E.M. of six rats. *p < 0.05, **p < 0.01, ***p < 0.001 vs control group (saline).

to the control group (Table 4). The number of total leucocytes was inhibited significantly at the doses of 100 ($12.55 \pm 0.22 \times 10^3$ cells/mm³; p < 0.05) and 200 mg/kg ($11.52 \pm 0.25 \times 10^3$ cells/mm³; p < 0.001) (Table 4). Indomethacin (10 mg/kg) reduced the exudate volume and the leucocyte migration.

DISCUSSION

Considering the phytochemicals, the major components identified in *H. brasiliiana* were the alkaloids and these are described as chemical markers of the species (Suárez et al., 1998; Shin et al., 2007; Lee et al., 2008; Severino et al., 2009a; Liao et al., 2011). However, based on the polarity of the compounds extracted in the hexane extract, sesquiterpenes, diterpenes, hydrocarbons and pentacyclic triterpenes were detected in the hexane extract from *H. brasiliiana* leaves and these chemical classes may be responsible for the pharmacological activities reported in the present study.

The acute toxicity assay showed that the tested doses of the hexane extract were toxic to mice. However, the largest dose administered (200 mg/kg) is less than the lowest dose applied for determination of the LD₅₀ (0.5

g/kg or 500 mg/kg). Probably, the toxic effect was due to the presence of compounds, such as terpenes, detected in the hexane extract, as well as identified substances from this species (Suárez et al., 2002). In the present study, the LD₅₀ was used to define the doses that were administered to the animals.

Intraperitoneal injection of acetic acid has been reported to significantly increase level of prostanoids, particularly PGE₂ and PGF_{2α} as well as lipoxigenase products in the peritoneal fluid (Ahmed et al., 2011; Ricciotti and FitzGerald, 2011). Regarding this test, our results clarified that the hexane extract from *H. brasiliiana* (Figure 1) presented antinociceptive property by reducing abdominal writhing based on this explanation.

The hexane extract also produced significant inhibition in the both phases of formalin-induced pain. The formalin test is a valid and reliable model for nociception investigation and it is sensitive for several classes of analgesic drugs. This important experiment produces a distinct biphasic response that is characterized by two phases (first and second phases) (Zouikr et al., 2013). Centrally acting drugs such as opioids inhibit both phases equally, while peripherally acting drugs such as nefopam and ketoprofen only inhibit the second phase (Girard et al., 2008). According to the Figure 2, the effect observed

Table 4. Effects of the hexane extract from *H. brasiliiana* leaves on pleural exudation and number of leucocytes in carrageenan-induced pleurisy in rats.

Group	Dose (mg/kg)	Exudate volume (ml)	N ^o Leucocytes ($\times 10^3$ cells/mm ³)
Control	Saline	1.08 \pm 0.06	13.37 \pm 0.24
Indomethacin	10	0.62 \pm 0.07***	9.27 \pm 0.53***
	50	1.07 \pm 0.07	13.42 \pm 0.27
Extract	100	1.03 \pm 0.08	12.55 \pm 0.22*
	200	0.87 \pm 0.05*	11.52 \pm 0.25***

Data are mean \pm S.E.M. of six rats. *p < 0.05; ***p < 0.001 vs control group (saline).

with the hexane extract suggests that antinociceptive activity may be resulting from central and peripheral actions confirming the writhing test.

In the hot plate procedure, a central model that has a selectivity for opioid-derived analgesics, the oral treatment with hexane extract exerts an antinociceptive action confirming the central activity observed in the first phase of formalin test (100 and 200 mg/kg). This experiment is also considered to be sensitive to drugs acting at the supraspinal modulation level of the pain response (Little et al., 2012) suggesting at least a modulatory effect of the investigated extract. Our results indicated that the analgesia induced by the extract could be dependent on the opioid system, since previous treatment with naloxone reversed the effect (Table 2). In addition, it is possible that this effect was due to a synergistic action of the constituents presented in the hexane extract.

The anti-inflammatory activity of the hexane extract from *H. brasiliiana* suggested in the formalin test was confirmed by the carrageenan-induced paw edema model through the reduction on the displaced volume (Table 3). This experiment is a suitable model for evaluating anti-inflammatory drugs, which has frequently been used to assess the anti-edematous effect of natural products (Omar et al., 2012). Moreover, carrageenan-induced rat paw edema is associated with three distinct phases (Patel et al., 2012). The first phase is early mediated by mast cell degranulation and histamine and serotonin release (1 h), the second phase (60 to 150 min) is characterized by bradykinin release and pain, and further eicosanoid (prostaglandins) production in the late phase (3-4 h) (Moore et al., 2010; Patel et al., 2012). The treatment with the hexane extract from *H. brasiliiana* reduced the paw edema demonstrating a possible inhibition of the inflammatory mediators in the late phase (Table 3).

Carrageenan-induced pleurisy in rats is considered to be an excellent acute inflammatory model in which fluid extravasation, leukocyte migration and the various biochemical parameters involved in the inflammatory response can be measured easily in the exudate (Patel et

al., 2012). Therefore, this method assesses the inflammatory infiltrate and confirms the paw edema results. Non-steroidal anti-inflammatory drugs inhibit the accumulation of exudate and mobilization of leucocytes between 3 and 6 h after the application of carrageenan (Vinegar et al., 1973). By reducing the both volume of exudate and leucocyte migration (Table 4), the hexane extract from *H. brasiliiana* reinforced the anti-inflammatory effect observed in the formalin (Figure 2) and paw edema tests (Table 3).

Antinociceptive and anti-inflammatory activities verified in the present study were also reported with plants belonging Rutaceae family (Lee et al., 2008; Liao et al., 2011). Probably, similar components detected in our experiments could be responsible for these properties, because the chemical analysis of the hexane extract from *H. brasiliiana* demonstrated the presence of sesquiterpenes, diterpenes, hydrocarbons and pentacyclic triterpenes, suggesting a synergistic biological action (Heras and Hortelano, 2009). Interestingly, compounds like triterpenes, common in hexanic extracts, have been shown to possess antinociceptive and anti-inflammatory activities (Heras and Hortelano, 2009; Gomes et al., 2010). Based on the classes of compounds detected in *H. brasiliiana*, mechanisms of action could be applied to explain the activities observed with hexane extract. For example, the anti-inflammatory activity of extracts from *H. brasiliiana* could be associated with the inhibitory effect of triterpenes on the nuclear factor- κ B (Harikumar et al., 2010). The anti-inflammatory mechanisms of asiatic acid, a pentacyclic triterpene, have been related to the decrease in the level of MDA, iNOS, COX-2, and NF- κ B in the paw edema via increasing the activities of CAT, SOD, and GPx in the liver (Huang et al., 2011).

Therefore, the hexane extract from *H. brasiliiana* leaves showed antinociceptive and anti-inflammatory effects as demonstrated by well established methods suggesting a potential alternative for therapeutic purposes and supporting the use of this plant in the Brazilian folk medicine. However, further studies need to be conducted to ensure the safe use.

ACKNOWLEDGEMENTS

This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil. We are grateful to the Plataforma Analítica de Farmanguinhos/FIOCRUZ for performing the analysis by GC/MS. Thanks very much to Dr. Milton Groppo Júnior for plant identification.

REFERENCES

- Adams RP (1995). *Identification of essential oil components by gas chromatography/mass spectrometry*. 4th ed.; Allured Publishing Co., Carol Stream, IL, USA, 2007.
- Ahmed TS, Magaji MG, Yaro AH, Musa AM, Adamu AK (2011). Aqueous methanol extracts of *Cochlospermum tinctorium* (A. Rich) possess analgesic and anti-inflammatory activities. *J. Young Pharm.* 3: 237–242.
- Kawada N, Moriyama T, Kitamura H, Yamamoto R, Furumatsu Y, Matsui I, Takabatake Y, Nagasawa Y, Imai E, Wilcox CS, Rakugi H, Isaka Y (2012). Towards developing new strategies to reduce the adverse side-effects of nonsteroidal anti-inflammatory drugs. *Clin. Exp. Nephrol.* 16: 25–29.
- Eddy NB, Leimbach D (1953). Synthetic analgesics. II. Dithienylbutenyl and dithienylbutylamines. *J. Pharmacol. Exp. Ther.* 107: 385–393.
- Girard P, Verniers D, Coppé MC, Pansart Y, Gillardin JM (2008). Nefopam and ketoprofen synergy in rodent models of antinociception. *Eur. J. Pharmacol.* 584: 263–271.
- Gomes N. M, Rezende CM, Fontes SP, Matheus ME, Pinto Ada C, Fernandes PD (2010). Characterization of the antinociceptive and anti-inflammatory activities of fractions obtained from *Copaifera multijuga* Hayne. *J. Ethnopharmacol.* 128: 177–183.
- Groppo M (2010). New synonyms in *Hortia* and *Dictyoloma* (Rutaceae), with validation of the name *Hortia badinii*. *Novon.* 20: 63–165.
- Harikumar KB, Sung B, Pandey MK, Guha S, Krishnan S, Aggarwal BB (2010). Escin, a pentacyclic triterpene, chemosensitizes human tumor cells through inhibition of nuclear factor- κ B signaling pathway. *Mol. Pharmacol.* 77: 818–827.
- Heras B, Hortelano S (2009). Molecular basis of the anti-inflammatory effects of terpenoids. *Inflamm. Allergy Drug Targets.* 8: 28–39.
- Huang S-S, Chiu C-S, Chen H-J, Hou W-C, Sheu M-J, Lin Y-C, Shie P-H, Huang G-J. (2011). Antinociceptive activities and the mechanisms of anti-inflammation of asiatic acid in mice. *Evid-Based Complement. Alternat. Med.* 2011: 1–10.
- Hunnskaar S, Hole K (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 30: 103–114.
- Lee SH, Son J-K, Jeong BS, Jeong T-C, Chang HW, Lee E-S, Jahng Y (2008). Progress in the studies on rutaecarpine. *Molecules* 3: 272–300.
- Liao J-F, Chiou W-F, Shen Y-C, Wang G-J, Chen C-F (2011). Anti-inflammatory and anti-infectious effects of *Evodia rutaecarpa* (Wuzhuyu) and its major bioactive components. *Chin. Med.* 6: 1–8.
- Litchfield JT, Wilcoxon F (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Therap.* 96: 99–113.
- Little JW, Chen Z, Doyle T, Porreca F, Ghaffari M, Neumann WL, Salvemini D (2012). Spinal peroxynitrite modulates pain signaling by suppressing the endogenous opioid pathway. *J. Neurosci.* 32: 10797–10808.
- Lorke D (1983). A new approach to practical acute toxicity testing. *Arch. Toxicol.* 54: 275–287.
- Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE, Vallejo R (2008). Opioid complications and side effects. *Pain Physician.* 11: S105–120.
- Moore AR, Ayoub SS, Seed MP (2010). Cyclooxygenase enzymes and their products in the carrageenan-induced pleurisy in rats. *Methods Mol. Biol.* 644: 201–205.
- Omar HM, Ibraheim ZZ, El-Shimy NA, Ali RS (2012). Anti-inflammatory, antipyretic and antioxidant activities of the earthworms extract. *J. Biol. Earth Sci.* 2: B10-B17.
- Patel M, Murugananthan G, Gowda KPS (2012). *In vivo* animal models in preclinical evaluation of anti-inflammatory activity - a review. *Int. J. Pharm. Res. Allied Sci.* 1: 1–5.
- Ricciotti E, FitzGerald GA (2011). Prostaglandins and Inflammation. *Arterioscler. Thromb. Vasc. Biol.* 31: 986–1000.
- Severino VGP, Silva MFGF, Lucarini R, Montanari LB, Cunha WR, Vinholis AHC, Martins CHG (2009a). Determination of the antibacterial activity of crude extracts and compounds isolated from *Hortia oreadica* (Rutaceae) against oral pathogens. *Braz. J. Microbiol.* 40: 535–540.
- Severino VGP, Cazal MC, Forim, MR, Graças MF, Silva F, Rodrigues-Filho E, Fernandes JB, Vieira PC (2009b). Isolation of secondary metabolites from *Hortia oreadica* (Rutaceae) leaves through high-speed counter-current chromatography. *J. Chromatogr. A.* 1216: 4275–4281.
- Severino VGP, Braga PAC, Silva MFGF, Fernandes JB, Vieira PC, Theodoro JE, Ellena JÁ (2012). Cyclopropane- and spirolimonoids and related compounds from *Hortia oreadica*. *Phytochemistry* 76: 52–59.
- Shah BN, Seth AK, Maheshwari KM (2011). A review on medicinal plants as a source of anti-inflammatory agents. *Res. J. Med. Plant* 5: 101–115.
- Schmidt AP, Böhmer AE, Schallenger C, Antunes C, Tavares RG, Wofchuk ST, Elisabetsky E, Souza DO (2010). Mechanisms involved in the antinociception induced by systemic administration of guanosine in mice. *Brit. J. Pharmacol.* 159: 1247–1263.
- Shin YW, Bae EA, Cai XF, Lee JJ, Kim DH (2007). *In vitro* and *in vivo* antiallergic effect of the fructus of *Evodia rutaecarpa* and its constituents. *Biol. Pharm. Bull.* 30: 197–199.
- Sobrinho FAP, Guedes-Bruni RR, Christo AG (2011). Uso de plantas medicinais no entorno da Reserva Biológica de Tinguá, Nova Iguaçu, RJ. *Rev. Acad., Ciênc. Agrár. Ambient.* 9: 195–206.
- Stankov SV (2012). Definition of inflammation, causes of inflammation and possible anti-inflammatory strategies. *The Open Inflamm. J.* 5: 1–9.
- Suárez LEC, Martínez JC, DelleMonache FM (1998). Alcalóides presentes en *Hortia colombiana*. *Rev. Colomb. Quím.* 27: 23–29.
- Suárez LEC, Menichini F, Dellemonache F (2002). Tetranortriterpenoids and dihydrocinnamic acid derivatives from *Hortia colombiana*. *J. Braz. Chem. Soc.* 113: 339–344.
- Vinegar R, Truax JF, Selph JL (1973). Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. *Proc. Soc. Exp. Biol. Med.* 143: 711–714.
- Winter CA, Risley EA, Nuss GW (1962). Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. *Proc. Soc. Exp. Biol. Med.* 111: 544–547.
- Zoukr I, Tadros MA, Clifton VL, Beagley KW, Hodgson DM (2013). Low formalin concentrations induce fine-tuned responses that are sex and age-dependent: a developmental study. *Plos One* 8: e53384–e53384.

Full Length Research Paper

Neuroprotective potential of *Ocimum sanctum* (Linn) leaf extract in monosodium glutamate induced excitotoxicity

Shanmuga Sundaram Rajagopal¹, Gowtham Lakshminarayanan², Ramdass Rajesh³, Senthil Rajan Dharmalingam^{4*}, Srinivasan Ramamurthy⁴, Kumarappan Chidambaram⁴, Suresh Shanmugham⁴

¹Department of Pharmacology, J.S.S.College of Pharmacy, Ootacamund, Tamilnadu, India.

²Department of Pharmacology, P.S.G. College of Pharmacy, Coimbatore, Tamilnadu, India.

³Bioequivalence Centre, Apotex Research Pvt. Ltd., Bangalore-560 099, Karnataka, India.

⁴School of Pharmacy, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia.

Accepted 24 June, 2013

The aim of this study was to investigate the potent neuroprotective property of ethanol extract of *Ocimum sanctum* (EEOS) leaf (Holy basil, Family: Labiataea) against excitotoxicity induced neurodegeneration by using monosodium-L-glutamate (MSG) in Sprague-Dawley rats. The animals received EEOS (50, 100 and 200 mg/kg) and memantine (MMT, 20 mg/kg) daily for 7 days. On all the 7 days, MSG (2g/kg, i.p.) was administered one hour before drug treatment. The animals were observed for neurobehavioral performance on 1st, 3rd, 5th and 7th day. Oxidative damage and histopathological analysis were also assessed. EEOS (100 and 200 mg/kg, p.o.) and MMT (20 mg/kg, i.p.) administration significantly improved body weight and attenuated locomotor activity, rotarod performance and foot-fault test as compared with MSG treated group. In addition, EEOS was found to restore reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), super oxide dismutase (SOD) and Na⁺-K⁺ ATPase. Conversely, the elevated level of lipid peroxidation and nitrite concentration in MSG treated group was attenuated significantly in EEOS group in comparison to MSG treated group. Histopathological evaluation showed that treatment with EEOS and MMT significantly attenuated neuronal death and increased the density of neurons after MSG treatment. Thus, these findings suggest that EEOS contains rosmarinic acid and ursolic acid in addition to other bioactive principles may have utility in the preventing and/or treating the neurodegenerative diseases and its protective effects may be due to the amelioration of excitotoxicity, oxidative stress, neurological and behavioral alterations. However, further studies are necessary to clearly define mechanism responsible.

Key words: *Ocimum sanctum*, F Holy basil, sodium glutamate, neurological, neurodegeneration. rosmarinic acid

INTRODUCTION

Glutamate is present in very high concentrations in the brain and is believed to be a major excitatory neurotransmitter in the mammalian central nervous system

(CNS) (Fonnum, 1984). L-monosodium glutamate (MSG), however, has been shown to be toxic to neurons *in vivo* (Mcbean and Roberts, 1984; Kubo et al., 1993) and *in*

*Corresponding author. E-mail: senthil_rajan@imu.edu.my; dsenthilrajan@yahoo.co.in

vitro (Zhou et al., 2009; Michaels and Rothman, 1990). This neurotoxicity of L-glutamate has been implicated both in the acute degenerative changes that occur after status epilepticus, hypoglycemia, ischemia, and trauma, and in such chronic neurodegenerative disorders as Huntington's disease, olivopontocerebellar atrophy, Alzheimer's dementia, Parkinsonism, and amyotrophic lateral sclerosis (ALS) (Hynd et al., 2004; Coyle and Puttfarcken, 1993). Although the detailed mechanisms are still not fully clarified, growing evidence points to a key role of receptor-mediated intracellular Ca^{2+} overload and increased reactive oxygen species (ROS) production in governing glutamate-mediated neurotoxicity. This type of over excitation-induced oxidative stress and intracellular Ca^{2+} metabolism disorders have been identified to execute cell death via distinct downstream signaling cascades, including activation of potentially lethal second messengers and enzymes, disturbance of mitochondrial function, and inhibition of anti-apoptotic pathways (Greenwood and Connolly, 2007; Montal, 1998). Thus, neuroprotection against glutamate-induced neurotoxicity has been therapeutic strategy for preventing and/or treating both acute and chronic forms of neurodegeneration (Meldrum, 2002).

In spite of its ubiquitous role as a neurotransmitter, glutamate is highly toxic to neurons, a phenomenon dubbed 'excitotoxicity' (Choi, 1988). Studies in tissue culture indicate that glutamate receptor mediated neuronal degeneration can be separated into two distinct forms: acute and delayed form of neuronal degeneration. Studies have demonstrated that synaptic glutamate release and uptake are energy (ATP)-dependent, and any impairment or breakdown may lead to generation of ROS and inactivation of glutamate reuptake mechanism leading to excessive glutamate accumulation. If the circumstance continues unabated, there is excessive influx of Na^+ , Cl^- and Ca^{2+} , via post-synaptic ion channels producing swelling and destruction of post synaptic elements not only in the immediate vicinity but also the entire neuron as well. Upon destruction of neurons by this mechanism, additional glutamate may be released further increasing the level of extracellular glutamate and thereby propagating the excitotoxicity and death of additional glutamate-sensitive neurons in the region of involvement (Nicholis and Attwell, 1990; Novelli et al., 1988).

Published reports indicates that several medicinal plants have been found to possess *in vitro* neuroprotection against glutamate excitotoxicity (Li et al., 2007; Nobre et al., 2008), may open a promising approach for treating glutamate-associated neurodegenerative disease. *Ocimum sanctum* (*O. sanctum* Linn. Family: Labiatae) is a well-known, widely distributed, and highly esteemed sacred medicinal herb especially for Hindus in the Indian subcontinent. Traditionally, it has been used as nerve tonic to alleviate the problems related to the nervous system. In Ayurveda, *O. sanctum* is described

as rasayana (plants having adaptogen like properties). Ayurvedic rasayanas are those drugs, which prevent ageing, increase longevity, impart immunity, improve mental functions and add vigor and vitality to the body (Bhargava and Singh, 1981). Recently, it has been demonstrated that *O. sanctum* exhibited significant neuroprotective effect in models of cerebral reperfusion injury and long-term hypoperfusion (Yanpallewar et al., 2004), anticonvulsant (Jaggi et al., 2003), antistress (Samson et al., 2006), anti-inflammatory (Godhwani et al., 1987), antioxidant (Kelm, 2000), and antidepressant (Sudhakar et al., 2010) properties.

Major phytochemical compounds in *O. sanctum* leaf include eugenol (volatile oil), palmitic, stearic, oleic, linoleic, linolenic acids (fixed oil), luteolin, orientin, vicenin (flavonoids), rosmarinic acid (RA, phenylpropnoid) and ursolic acid (UA, triterepene) (Kelm, 2000). Therefore, the present study was planned to standardize the ethanol extract of *O. sanctum* (EEOS) leaf for RA and UA and evaluate its possible effects on glutamate-induced general behavioral and biochemical alterations for the first time, in an attempt to assess and validate the central neuroprotective effects of *O. sanctum* leaf.

MATERIALS AND METHODS

Plant material

The aerial parts of the plant *O. sanctum* was collected from Bhavani, Erode district, Tamil Nadu, India. It was taxonomically identified by Survey of Medicinal Plants and Collection Unit, Ooty, Tamilnadu, India, and an herbarium of the plant is preserved (OS/234) in the Department of Pharmacognosy, J.S.S. College of Pharmacy, Ooty, Tamil Nadu, India. The plant was identified and authenticated by Dr. S. Rajan and Dr. D. Suresh Baburaj, Survey of Medicinal Plants and Collection Unit, Ootacamund, Tamilnadu, India.

Dried leaves of *O. sanctum* were coarsely powdered (1.9 kg \pm 0.5 dry basis) and subjected to extraction by cold maceration with 90% ethanol at room temperature with continuous stirring (300 rpm) for 7 days, after defatting with petroleum ether (60-80°C). The solvents were evaporated with rotary vacuum evaporator until a solid residue was formed and was stored in desiccator. The solid residue was then made into a fine suspension using 0.5% Tween 80. The extract yield of the *O. sanctum* was found to be 17.38 % w/w. (Joshi and Parle, 2006).

Estimation of rosmarinic acid (RA) and ursolic acid (UA) in EEOS by LC-MS

The liquid chromatography (LC) separation was carried out on a Phenomenex C_{18} (150 x 4 mm i.d., 5 μ) with single quadrupole MS analyzer. The mobile phase consisting of 0.5% formic acid-acetonitrile (75:25%) and ammonium acetate-acetonitrile (70:30%) were used for RA and UA respectively, and delivered at a flow rate of 0.5 ml/min. The column temperature was maintained at 30°C. The optimum operating parameters of the APCI interface in negative mode were: Heat block temperature 400°C, CDL temperature 450°C, nebulizing gas (N_2) 1.5 L/min, drying gas (N_2) 10 L/min, and detector voltage 1.3 kV. Quantification was achieved using selected ion-monitoring (SIM) mode of ion at m/z 359 for RA and 455 for UA.

Animals

Healthy Sprague-Dawley rats (age in the range of 4-6 weeks) with weight in range between 120-160 g, were used. Animals were obtained from the Central Animal House of the J.S.S. College of Pharmacy, housed in colony cages at an ambient temperature of $25 \pm 2^\circ\text{C}$ and 45-55% relative humidity with 12 h light / dark cycles with lights on at 20:00. Pelleted chow (Brook Bond, Lipton, India) and tap water were freely available in the home cages. A protocol for the use of animals study was approved by the Institutional Animal Ethical Committee, under the regulation of CPCSEA, New Delhi (JSSCP/IAEC/PhD/Ph.Cology/01/2005-06).

MSG administration

MSG (2 g/kg body weight) dissolved in physiological saline solution or sodium chloride solution at an equimolar concentration (control group) was injected intraperitoneally (i.p.) for 7 consecutive days, according to a previous protocol used to determine the MSG effects on neurodegeneration (Ramanathan et al., 2007).

Drug and treatment schedule

Memantine (MMT, Sigma Chemicals, St. Louis, USA) and EEOS was prepared as fine suspension in 0.5% Tween 80 and administered depending upon the body weight (5 ml/kg) of the rats. Experimental study included various treatment groups, consisting of 6 animals in each group. Group-1: Control (vehicle administered); Group-2: MSG (2 g/kg, i.p.); Group-3: MSG (2g/kg, i.p.) + EEOS (50 mg/kg, p.o.); Group-4: MSG (2g/kg) + EEOS (100 mg/kg, p.o.); Group-5: MSG (2 g/kg) + EEOS (200 mg/kg, p.o.); Group-6: MSG (2 g/kg, i.p.) + MMT (20 mg/kg, i.p.); Group-7: EEOS (200 mg/kg, p.o.) per sec (only) treatment. All the groups were subjected daily to drug treatment at 0900 h, for 7 days, starting from day 1 after one hour of MSG treatment (0800 h).

Examination of general behavior

Body weight

The body weight of the animals was recorded immediately before administration of MSG or drug treatment, from the day of first injection (day 1) and continued for 7 days thereafter, by weighing on a top loading balance with accuracy to ± 0.1 g. Changes in body weight were calculated by subtracting the weight of the animal obtained on every day from that of the animal weight immediately before the first MSG injection and expressed as % change (changes in body weight per 100 g).

Locomotor activity

The locomotor activity of each animal was assessed using a photoelectric actimeter, 1 h after administration of MSG or drug treatment, on 1st, 3rd, 5th and 7th day. The apparatus consists of a stainless steel box containing transparent cages (270 × 220 × 110 mm) in which the animals' horizontal activity is measured by two light beams connected to a photoelectric cell. The total number of beam crossings is recorded over a period of 5 min (Ramanathan et al., 2007).

Rotarod activity

Animals were tested for muscular coordination and balance using the rotarod on 1st, 3rd, 5th and 7th day, 1 h after MSG or drug treat-

ment. The animals were given a prior training session before actual recording on rotarod apparatus (Inco, India). The animals were placed individually on the horizontal rotating bar (diameter 2.5 cm, 20 r.p.m.) of the rotarod apparatus and the time the animal could stay on the rotarod was measured (max. 2 min). Total time spent on the rotating bar was registered using a stopwatch, and the number of falls during the session was also recorded (Dunham and Miya, 1957).

Foot-fault test

The animals were acclimatized for 2 min on an elevated stainless steel grid floor before MSG administration for one week. The foot-fault test was performed 1 h after administration of MSG or drug treatment on 1st, 3rd, 5th and 7th day, according to a published method of Barth and Stanfield (1990). Rats were placed on an elevated stainless steel grid floor 50×40 cm, 1 m above the floor with 3 cm² holes and a wire diameter of 0.4 cm. Each animal was placed on the grid and observed for 2 min. An animal is said to have satisfactorily performed a foot-fault when it misplaced a fore or hind limb and the paw falls through between the grid bars. The excess of left (ipsilateral foot-faults) to right (contralateral foot-faults) was recorded. Only the side difference of foot-faults was used for the statistical evaluation to eliminate the influence of the extent of activity in different rats.

Biochemical estimations

Dissection and homogenization

On day 8, following behavioral testing, animals were sacrificed by decapitation under ether anesthesia and the brains were removed immediately and dissected. The hippocampus and striatum, of all the animals were isolated, rinsed in ice-cold isotonic saline and packed with ice and stored at -70°C . A 25% homogenate was prepared in potassium phosphate buffer (100 mM, pH 7.5) containing 0.15 M KCl. The homogenate was then centrifuged at 10000 × g at 4°C for 20 min and the resultant supernatant was separated and used for biochemical estimations.

Total glutathione estimation (GSht)

GSht was measured by the enzymatic recycling procedure in which reduced glutathione (GSH) is sequentially oxidized by 5, 5-dithiobis-(2- nitrobenzoic acid) (DTNB) to oxidized glutathione (GSSG) which is then reduced by NADPH in the presence of glutathione reductase (GR) back to GSH (Griffith, 1980). GSH was used as an external standard, and the level of GSht in the samples was expressed as nanomoles of GSH/mg protein.

Glutathione peroxidase estimation (GPx)

The reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA), sodium azide (1 mM), GR (1 EU/ml), GSH (1 mM), NADPH (0.2 mM), hydrogen peroxide (0.25 mM), and 0.1 ml of supernatant in a final volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram of protein using a molar extinction coefficient $6.22 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ (Mohandas et al., 1984).

Glutathione reductase estimation (GR)

The assay mixture consisted of phosphate buffer (0.1 M, pH 7.6),

NADPH (0.1 mM), EDTA (0.5 mM), GSSG (1 mM), and 0.05 ml of supernatant in a total volume of 1 ml. The enzyme activity was quantified at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nanomoles of NADPH oxidized per minute per milligram of protein using molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Mohandas et al., 1984).

Superoxide dismutase estimation (SOD)

Brain SOD activity was determined colorimetrically according to the method of Kakkar et al. (1984). This assay relies on the ability of the enzyme to inhibit the phenazine methosulfate-mediated reduction of nitroblue tetrazolium dye which can be measured at 560 nm.

Catalase estimation (CAT)

In brief, potassium phosphate buffer (65 mM, pH 7.8, 2.25 ml) and 100 μl supernatant or sucrose (0.32 M) were incubated at 25°C for 30 min. Hydrogen peroxide (7.5 mM; 650 μl) was added to initiate reaction. Decomposition of hydrogen peroxide in the presence of CAT was followed at 240 nm. The results are expressed as units (U) of CAT activity/mg of protein (Beers and Sizer, 1952).

Lipid peroxidase estimation (LPO)

LPO in terms of thiobarbituric acid reactive substances (TBARS) was measured using the method of Okawara et al. (1979). Lipid peroxide content was expressed as nanomoles of malondialdehyde (MDA) / mg of protein. The calibration curve was prepared by using 1,1,3,3-tetra ethoxypropane as standard.

Nitrite estimation

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as described by Green et al (1982). Equal volumes of supernatant and Greiss reagent were mixed, and then the mixture was incubated at 25°C for 10 min in the dark. The concentration of nitrite was assayed at 540 nm and calculated with reference to the absorbance of the sodium nitrite standard curve.

Na⁺-K⁺ ATPase estimation

Na⁺-K⁺ ATPase was assayed by taking 250 μl of Tris HCl (184 mM; pH 7.5) buffer followed by the addition of 50 μl of 600 mM NaCl, 50 μl of 50 mM KCl, along with 50 μl of 1 mM Na-EDTA and 50 μl of 80 mM ATP. The reaction mixture was pre-incubated at 37°C for 10 min. Then 25 μl of 10% homogenate was added to the test alone and further incubated at 37°C for 1 h. The reaction was immediately arrested by the addition of 10% trichloroacetic acid (TCA). Control samples were carried out under the same conditions with the addition of 0.1 mM ouabain. The precipitate was removed by centrifugation at 3500 rpm for 10 min. The supernatant (0.5 ml) was used for the estimation of inorganic phosphorous according to method of Sovoboda and Mossinger (1981). The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain (ouabain-resistant activity).

Protein estimation

Protein was assayed according to Lowry et al. (1951) using the

Folin-Ciocalteus phenol reagent with bovine serum albumin as standard.

Histological examination

On the 8th day, the animals were anesthetized with diethyl ether and underwent transcardial perfusion with 0.9% saline followed by 4% para-formaldehyde in 0.1 M phosphate buffer (pH7.3). The hippocampus, harvested from the rat brain, was post-fixed overnight in paraformaldehyde, processed and embedded in paraffin. The fixed brains were cut into 5 μm sections on a microtome. Levels of sections and CA1 area in the hippocampus were found with the help of the stereotaxic atlas (Paxinos and Watson, 2007), and were stained with cresyl-violet. The hippocampal damage was determined by counting the number of intact neurons in the stratum pyramidale within the CA1 subfield at a magnification of 400 and the counts of neurons were determined per square millimeter by using a standardized ocular grid. The neurons counted in random high-power fields using a light microscope (Olympus BH-2, Japan) incorporating a square graticule in the eyepiece (eyepiece x10, objective x40, a total side length of 0.225mm). Neurons density was assessed by counting the number of cells in 200 high power field in hippocampal tissue preparations of each group. The neurons density in each site was calculated and recorded as number of NCs/mm². The tissue compartments were used to record the neurons distribution in hippocampal tissue. Six sections from each animal were used for counting.

Histological examination

The brain tissue was fixed in 10% formalin for one week, washed in running tap water for 24 h and dehydrated in ascending series of ethanol (50–90 %), followed by absolute alcohol. The samples were cleared in xylene and immersed in a mixture of xylene and paraffin at 60°C. The tissue was then transferred to pure paraffin wax of the melting point 58°C and then mounted in blocks and left at 4°C. The paraffin blocks were sectioned on a microtome at thickness of 5 μm and mounted on clean glass slides and left in the oven at 40°C to dryness. The slides were deparaffinized in xylene and then immersed in descending series of ethanol (90–50%). The ordinary haematoxylin and eosin stain was used to stain the slides (Paxinos and Watson, 2007).

Liquid chromatography-mass spectrophotometry (LC-MS) method validation

The assay was shown to be linear over the range of 100-1000 ng/ml ($r^2 = 0.9997$ and 0.9999 for RA and UA respectively). The limit of detection (LOD) and limit of quantification (LOQ) for RA and UA were found to be 1 and 3 ng/ml, and 2 and 6 ng/ml, respectively. The method was shown to be reproducible and reliable with intraday precision below 0.14 and 0.11% and interday precision below 0.71 and 0.46% and mean recovery excess of 94.38 and 92.92% for RA and UA, respectively.

Statistical analysis

All data are presented as mean \pm SEM. The behavioral and biochemical data was analyzed using two ways ANOVA and one way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc analysis was used to analyze differences between the groups. A probability level (p) of value of less than 0.05 was considered to be statistically significant. The statistical analysis was performed using GraphPad Prism for Windows (Graph Pad Prism Software (version 4.03), San Diego, California, USA).

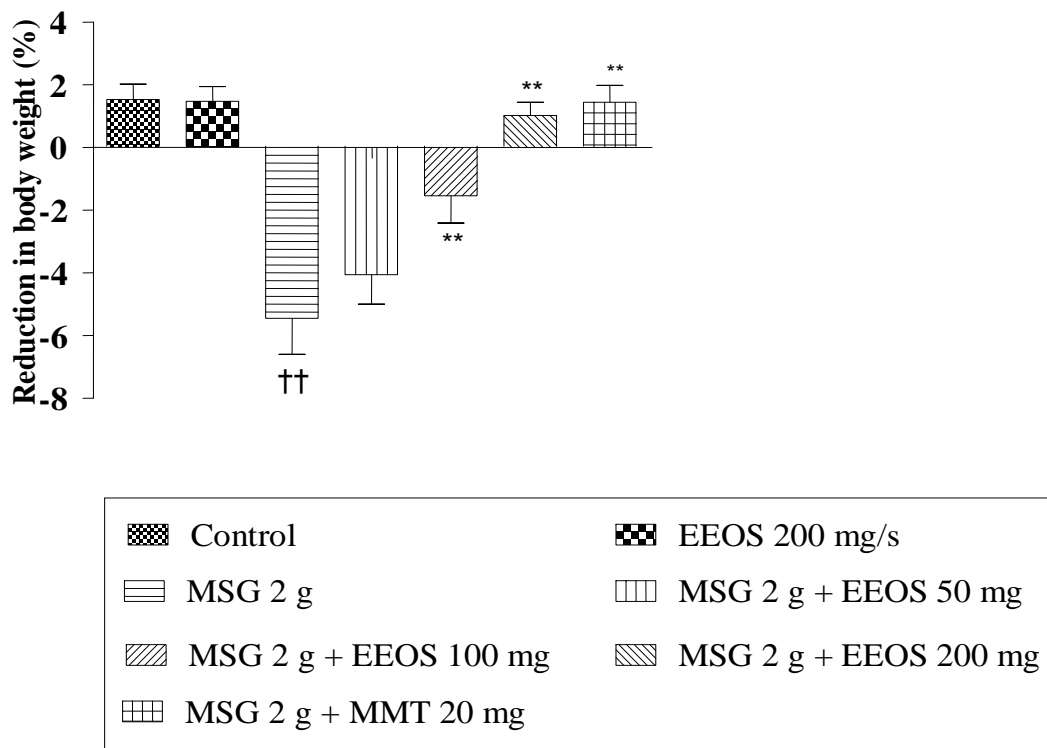


Figure 1. Effect of EEOS and memantine on MSG induced change in rat body weight. Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA is indicated as †p<0.05 and ††p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

RESULTS

LC-MS estimation of RA and UA

LC-MS assay achieved higher sensitivity and better specificity for analysis of RA and UA. The method offers sensitivity, with a LOQ of 3 and 6 ng/ml for RA and UA, respectively, without interference from other phytoconstituents present in the herb. Using the optimized conditions, the quantity of RA and UA in EEOS was found to be 0.27 and 0.40% w/w, respectively. The method is suggested to be ideally suited for rapid routine analysis of RA and UA.

Effect of EEOS and MMT on MSG induced change in body weight

The body weights of the experimental animals are shown in Figure 1. Administration of MSG displayed significantly greater decrease in body weights (-11.45) of the rats as compared to control group. EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) co-treatment significantly improved the body weight (P<0.001) as compared to MSG treated group. EEOS (200 mg/kg) per se treatment demonstrated had no statistically significant effect on the body weight as compared to control group.

Effect of EEOS and MMT on MSG induced alterations in locomotor activity and rotarod performance

Co-administration of MSG for 7 days significantly reduced locomotor activity and rotarod performance as compared to the control group. EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) treatment significantly improved the locomotor activity (Figure 2) (P<0.001) and fall off time (rotarod performance) (Figure 3) as compared to MSG treated animals (P<0.001). EEOS (200 mg/kg) per se treatment exhibited no significant effect on locomotor activity and fall off time (rotarod performance) as compared to control group.

Effect of EEOS and MMT on MSG induced alterations in foot-fault test

Figure 4 shows the number of foot-faults in each group. The number of foot-fault was significantly greater in the MSG treated group than that in the control group. The number of foot-fault was significantly (P<0.001) attenuated in EEOS (100 and 200 mg/kg) and MMT (20mg/kg) co-treated group than that in the MSG treated group. EEOS (200 mg/kg) per se treatment demonstrated no statistically significant effect on foot-fault test as compared to control group.

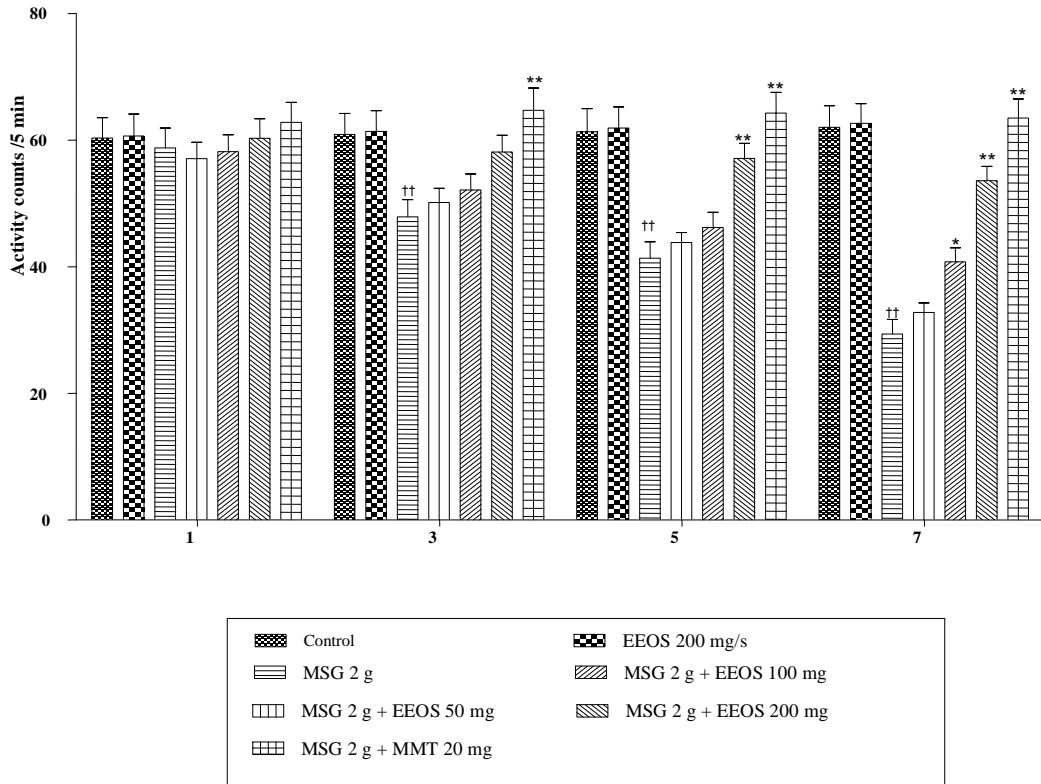


Figure 2. Effect of EEOS and memantine on locomotor activity in MSG treated rats. Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA is indicated as †p<0.05 and ††p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

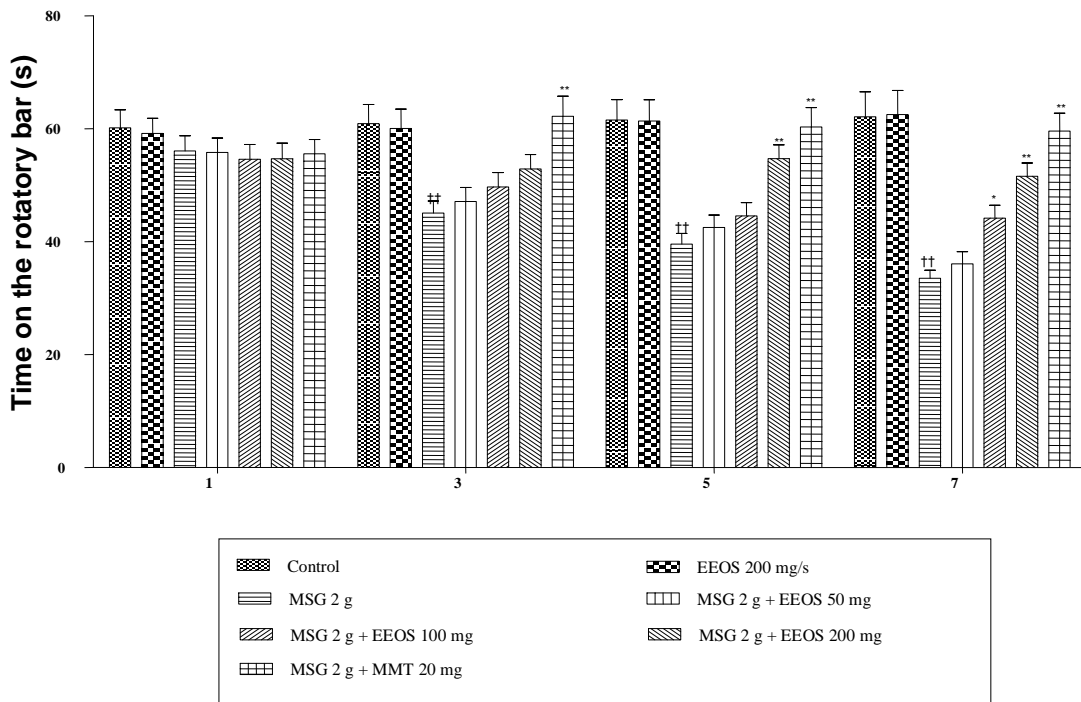


Figure 3. Effect of EEOS and memantine on performance of rota-rod test in MSG treated rats. Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA is indicated as †p<0.05 and ††p<0.01 compared to control or *p<0.05 or **p <0.01 compared to MSG.

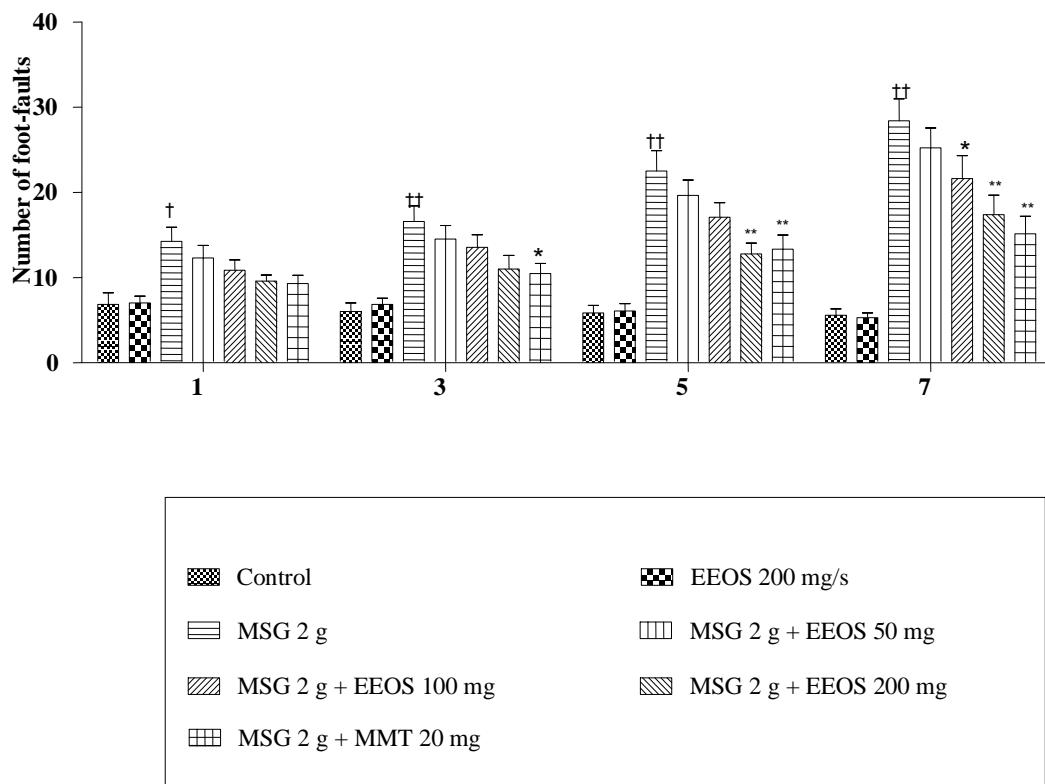


Figure 4. Effect of EEOS and memantine on the performance of foot-fault test in MSG treated rats. Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA is indicated as [†]p<0.05 and ^{††}p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

Effect of EEOS and MMT on MSG induced GST, GPx, and GR enzymes level

A significant decrease in the GSht, GPx and GR level was observed in hippocampus and striatum of the MSG treated groups as compared to the control group (Table 1). On the other hand, EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) treatment significantly stimulated GSht, GPx and GR levels both in hippocampus (P<0.001) and striatum (P<0.001) in comparison to MSG treated groups, whereas EEOS (200 mg/kg) per se treatment demonstrated no significant change in GSht, GPx and GR levels, as compared to control group.

Effect of EEOS and MMT on MSG induced SOD and CAT enzyme level

The data in Table 2 indicates that SOD and CAT enzyme levels in hippocampus and striatum were significantly decreased (P<0.001) in MSG treated animals with respect to control group and significantly increased in EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) treated groups (P<0.001) with respect to MSG treated group both in the hippocampus (P<0.001) and striatum (P<0.001), whereas EEOS (200 mg/kg) per se treated animals

demonstrated no significant change in SOD and CAT enzymes level as compared to control group.

Effect of EEOS and MMT on MSG induced lipid peroxidation (MDA) and nitrite content

A significant increase in MDA and nitrite content in hippocampus and striatum was observed in MSG treated group when compared to control group. EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) demonstrated significant attenuation in lipid peroxidation and nitrite concentration both in hippocampus (P<0.001) and striatum (P<0.001) comparison to MSG treated group (Table 3). EEOS (200 mg/kg) per se treatment demonstrated no significant effect on MDA and nitrite content as compared to control group.

Effect of EEOS and MMT on Na⁺-K⁺ ATPase activity

Figure 6 shows that MSG administration significantly decreased Na⁺-K⁺ ATPase activity in hippocampus and striatum as compared to the control group. However, EEOS (100 and 200 mg/kg) and MMT (20mg/kg) treatment enhanced Na⁺-K⁺ ATPase activity significantly,

Table 1. Effect of EEOS and memantine on MSG induced change in GSH, GPx and GR levels of brain hippocampus and striatum.

Treatment	nmol GSH/mg protein		GPx (nmol NADPH oxidized/min/mg protein)		GR (nmol NADPH oxidized/min/mg protein)	
	Hippocampus	Striatum	Hippocampus	Striatum	Hippocampus	Striatum
Control	3.54±0.52	2.86±0.47	42.56±2.17	37.89±2.25	50.14±2.65	54.14±3.13
EEOS 200 mg per sec	3.82±0.60	3.25±0.62	44.24±2.08	40.24±2.40	54.62±3.30	58.55±3.50
MSG 2g	1.04±0.12 ^{††}	0.92±0.07 ^{††}	18.70±1.22 ^{††}	16.60±1.06 ^{††}	28.73±1.72 ^{††}	28.58±1.59 ^{††}
MSG 2 g + EEOS 50 mg	1.14±0.15	1.03±0.09	21.42±1.28	18.40±1.30	31.13±2.14	30.84±2.35
MSG 2 g + EEOS 100 mg	2.69±0.20*	2.57±0.16*	30.05±1.45*	26.19±1.53*	43.65±2.54*	43.51±2.28*
MSG 2 g + EEOS 200 mg	3.26±0.24*	2.86±0.28*	38.30±1.72**	33.58±1.84**	55.82±2.70**	50.77±2.94**
MSG 2 g + MMT 20 mg	3.70±0.35**	3.24±0.35**	42.53±2.29**	40.13±2.42**	58.45±3.14**	55.45±2.66**

Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA and indicated as [†]p<0.05 and ^{††}p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

Table 2. Effect of EEOS and memantine on MSG induced change in SOD and catalase levels of brain hippocampus and striatum.

Treatment	SOD levels (U of SOD/mg protein)		Catalase levels (µmol of catalase/mg protein)	
	Hippocampus	Striatum	Hippocampus	Striatum
Control	1.71±0.17	1.25±0.12	36.8±2.85	29.2±2.50
EEOS 200 mg/s	1.83±0.27	1.30±0.15	37.1±2.60	32.5±2.72
MSG 2 g	0.84±0.06 ^{††}	0.64±0.05 ^{††}	15.3±1.32 ^{††}	13.5±1.16 ^{††}
MSG 2 g + EEOS 50 mg	1.07±0.08	0.86±0.07	17.4±1.63	15.1±1.24
MSG 2 g + EEOS 100 mg	1.63±0.13*	1.34±0.12*	27.2±2.40*	26.2±2.49*
MSG 2 g + EEOS 200 mg	1.86±0.20*	1.52±0.18*	33.8±2.84**	32.5±3.18**
MSG 2 g + MMT 20 mg	2.25±0.21**	1.80±0.21**	39.4±3.25**	38.6±3.51**

Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA is indicated as [†]p<0.05 and ^{††}p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

Table 3. Effect of EEOS and memantine on MSG induced change in MDA and nitrite levels of brain hippocampus and striatum.

Treatment	MDA levels (µmol of MDA/mg protein)		Nitrite levels (µmol of nitrite/mg protein)	
	Hippocampus	Striatum	Hippocampus	Striatum
Control	0.97±0.05	0.76 ± 0.04	098.20 ± 4.12	094.53±4.12
EEOS 200 mg/s	0.87±0.04	0.68 ± 0.06	092.45 ± 4.65	089.08±3.95
MSG 2 g	1.86±0.14 ^{††}	1.47 ± 0.15 ^{††}	224.93 ± 9.71 ^{††}	195.73±8.94 ^{††}
MSG 2 g + EEOS 50 mg	1.73±0.10	1.26±0.12	208.52±9.39	173.26±8.44
MSG 2 g + EEOS 100 mg	1.31±0.08*	1.05±0.09*	171.58± 6.73*	146.44±7.09*
MSG 2 g + EEOS 200 mg	1.18±0.06**	0.95 ± 0.07**	129.96 ± 5.94**	118.16±5.60**
MSG 2 g + MMT 20 mg	1.05±0.04**	0.83 ± 0.05**	112.23±5.23**	105.35±4.38**

Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA is indicated as [†]p<0.05 and ^{††}p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

both in hippocampus (P<0.001) and in striatum (P<0.001) when compared with MSG treated groups (Table 4).

Further, there was no significant change found in EEOS (200 mg/kg) per se treated group of rats as compared to

Table 4. Effect of EEOS and memantine on MSG induced change in Na⁺/K⁺-ATPase activity levels of brain hippocampus and striatum.

Treatment	Na ⁺ /K ⁺ -ATPase activity (nmol of inorganic phosphorus liberated/min/mg protein)	
	Hippocampus	Striatum
Control	360.53±22.25	301.53±17.20
EEOS 200 mg/s	388.49±23.54	321.15±19.13
MSG 2 g	138.71±08.78 ^{††}	127.16±07.44 ^{††}
MSG 2 g + EEOS 50 mg	186.20±10.30	161.24±09.60
MSG 2 g + EEOS 100 mg	278.67±13.25 ^{**}	240.22±12.48 ^{**}
MSG 2 g + EEOS 200 mg	347.02±18.18 ^{**}	293.43±17.30 ^{**}
MSG 2 g + MMT 20 mg	356.82±21.40 ^{**}	298.17±18.58 ^{**}

Values are expressed in mean±SEM; significance with Tukey's test following one way ANOVA and indicated as [†]p<0.05 and ^{††}p<0.01 compared to control or *p<0.05 or **p<0.01 compared to MSG.

control group.

Histological studies

The histological results are shown in Figure 5a-g. In the control group, the morphology of neurons CA1 region of the hippocampal tissues were normal, borders distinct and cell membrane integrity was preserved. Nucleus borders were regular and nucleolus was distinctively observed EEOS (200 mg/kg, Figure 5a) per se treatment demonstrated no significant effect on the morphology of neurons in CA1 region of the hippocampal tissues as compared to control group (Figure 5b). The observations of cresyl-violet stained Figure 5c showed unequivocal signs of neuron death with extensively dark pyknotic and shrunken nuclei located in the CA1 pyramidal cell layer of rats following 7 days of MSG treatment, in comparison to control group animals. In contrast to this, the cellular structures of neurons in EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) treated group were found to be normal and the nuclei and the nucleoli were observed to be more distinct in hippocampal CA1 region in comparison with the control group (Figure 5e,f and g). On the other hand, animals treated with EEOS (50 mg/kg,) exhibited very little neuroprotection as evidenced from the extensive loss of hippocampal CA1 cell bodies and decreased neuronal density in MSG treated animals in comparison with control group animals (Figure 5d). The effect of EEOS and MMT treatment on neuron density was examined by quantitative histopathological analysis. In MSG treated group, hippocampal neuron density was significantly decreased (Figure 6) in comparison with the control group (p <0.001). However, treatment with EEOS (100 and 200 mg/kg) and MMT (20 mg/kg) significantly preserved the neurons of hippocampal regions (P<0.001) as compared to MSG treated group. Further, treatment with EEOS (50 mg/kg) did not show any significant inhibition against the decreased neuronal cell density due to MSG treatment. EEOS (200 mg/kg) per se treatment

exhibited no significant effect on hippocampal neuron density as compared to control group.

DISCUSSION

The present study highlights the neuroprotective effect of EEOS against MSG-induced neurotoxicity. The results of present study indicate that the treatment with EEOS significantly improved body weight and motor deficits, marked reduction in oxidative stress, restored antioxidant defense mechanisms and reduction in histological changes characterized by MSG-induced neurodegeneration study. Administration of MSG for seven days exhibited significantly reduced body weight, locomotor activity, muscle grip strength test and foot fault test in rats. These findings are consistent with earlier reports including those from our laboratory, which showed a variety of neurobehavioral abnormalities and motor deficits in rats following MSG administration (Ramanathan et al., 2007). In the present study, EEOS treatment significantly attenuated changes in body weight and motor function.

Glutamate toxicity is a major contributor to pathological cell death within the nervous system and appears to be mediated by ROS (Coyle et al., 1981). There are two forms of glutamate toxicity: excitotoxicity pathway relies on the hyper-activation of glutamate receptors and non-receptor mediated oxidative glutamate toxicity (Murphy et al., 1989). Reports indicated that there may be a close relation between activation of glutamate receptor and oxidative stress. Hyperactivation of glutamate receptor has been implicated in inhibition of cystine transport, GSH depletion, and lipid peroxidation (Bondy and Lee, 1993). Oxidative glutamate toxicity is initiated by high concentrations of extracellular glutamate that prevent cystine uptake into the cells via the cystine/glutamate antiporter system, resulting in depletion of intracellular cysteine and glutathione. Glutathione (GSH) depletion induces cellular accumulation of ROS, leading to cell

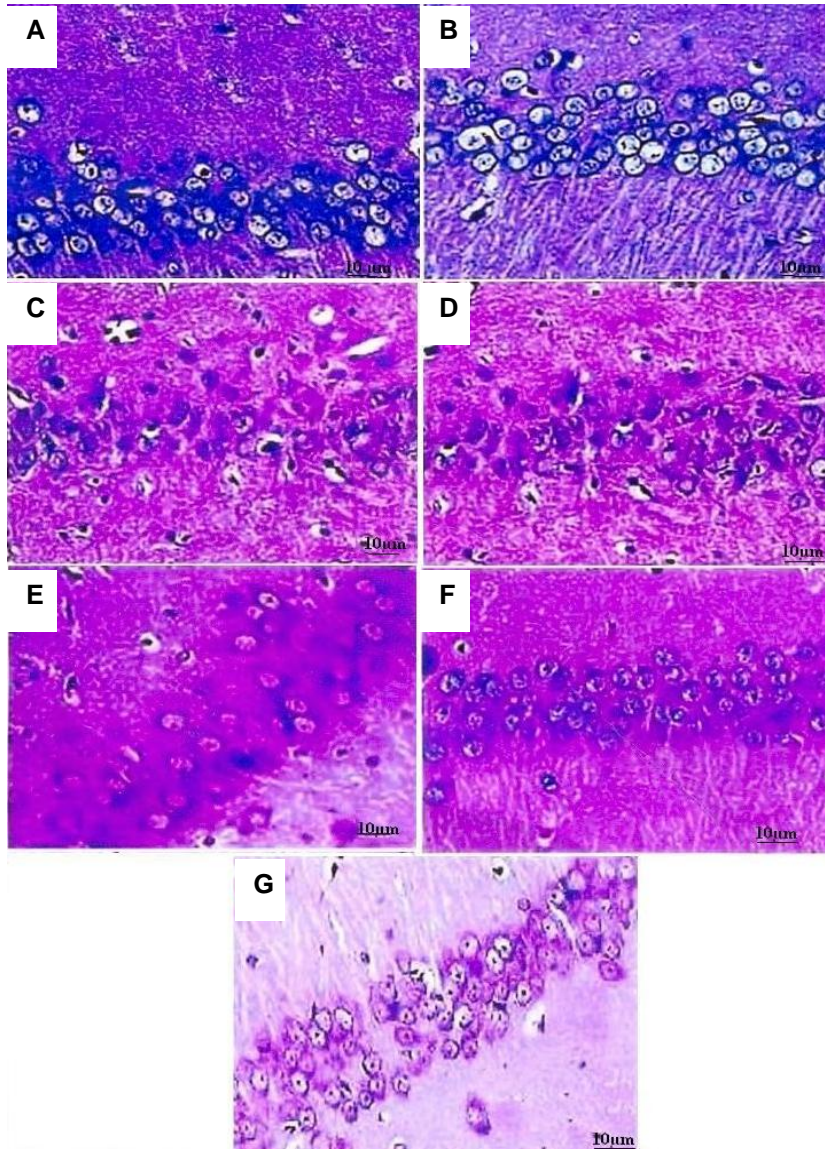


Figure 5. (Panel a–g) The effect of EEOS and memantine on the density of neurons in hippocampal CA1 region of MSG and drug treated rat brains. Photomicrographs of cresyl-violet stained brain hippocampus CA1 sections. (Panel a) Vehicle-treated control group showing healthy neurons; (Panel b) EEOS (200 mg/kg) *per se* treatment showing intact neurons; (Panel c) MSG treated group showing signs of neuron death with extensively dark pyknotic and shrunken nuclei located in the CA1 pyramidal cell; (Panel d) EEOS (50 mg/kg) little/no protective effect on MSG induced neurodegeneration; (Panel e) EEOS (100 mg/kg.) showing neuroprotection against MSG induced neurotoxicity; (Panel f) EEOS (200 mg/kg) showing more neuroprotection against MSG induced neurotoxicity; (Panel g) memantine (20 mg/kg) showing more protection against MSG induced neurodegeneration (Scale bars represent 10µm, magnification:40×).

injury. GSH is one of the most important tripeptide and plays an important role in maintaining cellular oxidant homeostasis by detoxifying ROS with antioxidative enzymes such as SOD, GPx, CAT and GR (Dringen, 2000). The Na⁺-K⁺ ATPase activity was reduced or insufficient to maintain ionic balances during and immediately

after episodes of ischemia, hypoglycemia, epilepsy, and after administration of glutamate agonists (LEES) (Lees, 1991). In the present study, intra-peritoneal administration MSG caused oxidative damage as evidenced by decreased levels of GSH, GPx, GR, SOD, CAT, Na⁺-K⁺ ATPase activities and elevated melondialdehyde and

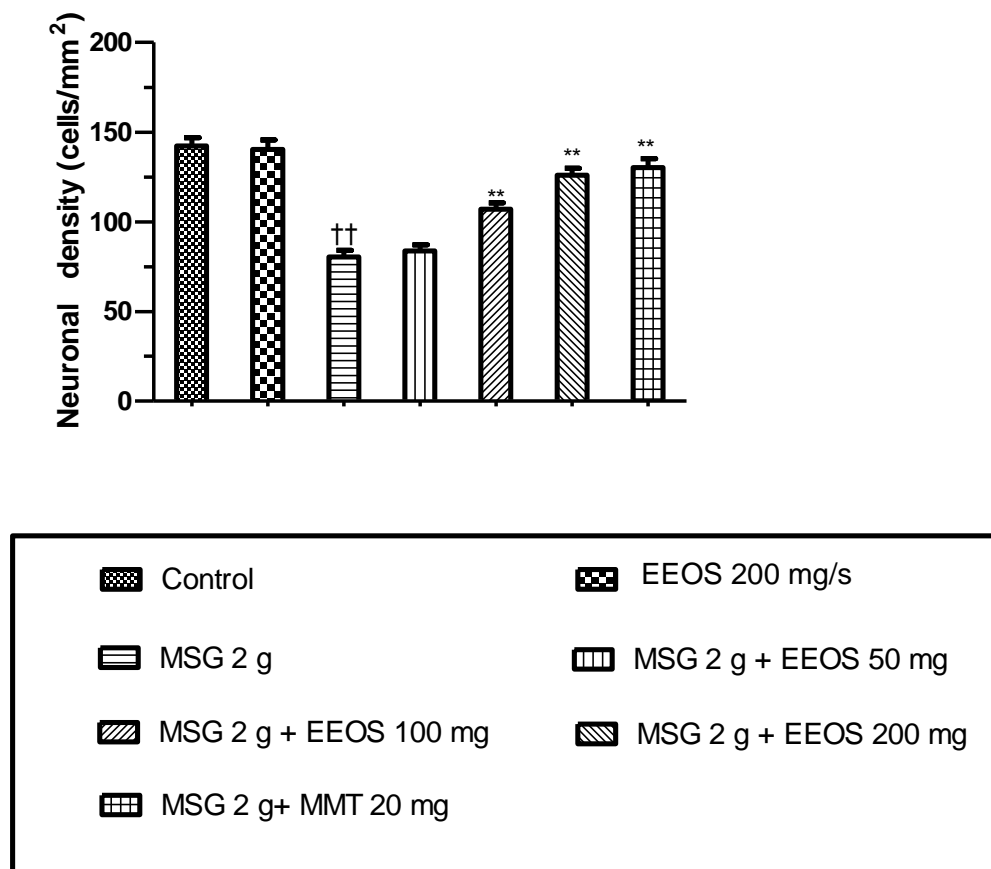


Figure 6. Effect of EEOS and memantine on neuronal density in the cresyl-violet stained hippocampal CA1 section of MSG treated rats. Values are expressed in mean \pm SEM; significance with Tukey's test following one way ANOVA is indicated as $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ compared to control or $^*p<0.05$ or $^{**}p<0.01$ compared to MSG.

nitrite content in the brain, as compared with the vehicle control rats, these results are consistent with earlier report (Ramanathan et al., 2007; Lees, 1991). Previous studies demonstrated that *O. sanctum* has a potent antioxidant activity by scavenging free radicals (Kelm, 2000). *O. sanctum* extracts and their fractions showed strong inhibitors of *in vitro* lipid peroxidation of erythrocytes membranes and also exhibited anti-lipidperoxidative effects *in vivo* both in normal and in hypercholesterolemia-induced stress conditions (Geetha et al., 2004). Yanpallewar et al. (2004) reported that pretreatment of *O. sanctum* prevented the oxidative stress caused by cerebral reperfusion injury as well as attenuated the behavior deficits and histopathological alterations secondary to long-term hypoperfusion. Strikingly, EEOS treatment significantly increased the activities of all these enzymes and decreased the level of LPO and nitrite in the brain of MSG treated rats. Our results strongly suggest that EEOS can strengthen antioxidant defense against free radicals induced by MSG *in vivo*.

It is well reported that MSG administration produces

morphological and histological changes in rat brain that further results in learning and memory impairment (Coyle et al., 1981; Ali et al., 2000). Furthermore, neonatal administration of MSG has been reported to destroy the hippocampal CA1 structure while other neurons such as CA3, the dentate gyrus and the frontal cortex are much less vulnerable in rodents and impair the acquisition of discrimination learning (Kubo et al., 1993). In the studies carried out with MSG treated rat, unequivocal signs of neuron death with extensively dark pyknotic and shrunken nuclei located in the CA1 pyramidal cell layer have been determined. Oral administration of EEOS remarkably attenuated MSG induced neuronal loss and also decreased pyknotic cell density in a dose dependent manner. The cell counts showed that the number of cells in the hippocampus sections were significantly lower in the MSG treated groups than in the control group. On the other hand, the number of cells in the treatment groups was closer to the values of the control group compared to the MSG treated group. This result suggests that EEOS has neuroprotective effects against MSG induced neuronal

death.

Previous phytochemical studies of *O. sanctum* have led to the isolation of terpenoids, fatty acids, triglycerides esters and phenolic derivatives (Norr and Wagner, 1992; Sukari et al., 1995). The content of RA and UA in the leaves of EEOS was quantified by LC-MS and was found to be 0.27 and 0.40% w/w, respectively. Therefore it can be assumed that EEOS attenuates behavioral and biochemical alterations against MSG induced excitotoxicity depending on rosmarinic acid, ursolic acid and other active principles present in EEOS leaves.

The effect of EEOS was comparable and equipotent to that of MMT, a known N-methyl-D-aspartate receptor (NMDAR) antagonists. MMT (20 mg/kg) administration significantly improved body weight and attenuated the altered neurofunctional paradigms (locomotor activity, rotarod performance and foot-fault test) significantly and prevented the neurodegeneration on MSG treatment. In addition, MMT significantly increased the activities of antioxidant enzymes and decreased the level of lipid peroxidation and nitrite in the brain of MSG treated rats. These effects could be attributed to the antagonizing activity at NMDAR, leading to controlling the glutamate excitotoxicity resulting in the improved body weight, motor functions and oxidative defense. These findings are in line with previous reports regarding modulatory effect of MMT on neurological deficits, anxiogenic behavior, histological changes, lipid peroxidation, Na⁺-K⁺ ATPase activity and antioxidant enzymes following injuries such as hypoxia/ischemia and CNS injuries (Ozsuer et al., 2005; Liu et al., 2009).

The effect of EEOS was comparable and equipotent, to that of known NMDAR-antagonists memantine. The most acceptable explanation for the neuroprotective action of memantine is to block preferentially the opening of the NMDA channel due to prolonged exposure to extracellular glutamate while still allowing for physiological activation of the NMDAR. It was also found that memantine decreased the levels of SOD, CAT and GSH with a corresponding increase in TBAR levels in all the regions studied. These effects could be attributed to the antagonizing activity at NMDAR, leading to controlling the glutamate excitotoxicity resulting in the preservation of brain antioxidant system.

The present study has provided experimental evidence for neuroprotective effects of EEOS against MSG induced excitotoxicity might caused at least in part by the increase in the activity of antioxidant enzymes with reduction in lipid peroxidation and nitrite concentration and improvement in behavioral activities.

Thus, these findings suggest that EEOS may have utility in the preventing and/or treating the neurodegenerative diseases and its protective effects may be due to the amelioration of excitotoxicity, oxidative stress, neurological and behavioral alterations. Further study is required to understand, more fully, the mechanisms of neuropharmacological effects of *O. sanctum*.

ABBREVIATIONS

CNS, Central nervous system; **MSG**, monosodium glutamate; **ALS**, amyotrophic lateral sclerosis; **ROS**, reactive oxygen species; **EEOS**, ethanol extract of *Ocimum sanctum*; **RA**, rosmarinic acid; **UA**, ursolic acid; **SIM**, selected ion-monitoring; **MMT**, memantine; **GSH**, reduced glutathione; **DTNB**, 5, 5-dithiobis-(2-nitrobenzoic acid); **GSSG**, oxidized glutathione; **GR**, glutathione reductase; **GPx**, glutathione peroxidase; **SOD**, superoxide dismutase; **CAT**, catalase; **LPO**, lipid peroxidase; **LOD**, limit of detection; **LOQ**, limit of quantification; **LC-MS**, liquid chromatography-mass spectrophotometry; **NMDAR**, N-methyl-D-aspartate receptor.

REFERENCES

- Ali MM, Bawari UK, Babu MGN (2000). Locomotor and learning deficits in adult rats exposed to monosodium-l-glutamate during early life. *Neurosci. Lett.* 284:57-60.
- Barth TM, Stanfield BB (1990). The recovery of forelimb-placing behavior in rats with neonatal unilateral cortical damage involves the remaining hemisphere. *J. Neurosci.* 10:3449-3459.
- Beers RF, Sizer IW (1952). A Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 195:133-140.
- Bhargava KP, Singh N (1981). Anti-stress activity of *Ocimum sanctum* Linn. *Indian J. Med. Res.* 73:443-451.
- Bondy SC, Lee CP (1993). The relationship between excitotoxicity and oxidative stress in the central nervous system. *Free. Rad. Biol. Med.* 14:633-642
- Choi DW (1988). Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci.* 11:465-467.
- Coyle JT, Bird SJ, Evans RH, Gulley RL, Nadler JV, Nicklas WJ, Olney JW (1981). Excitatory amino acid neurotoxins: selectivity, specificity, and mechanisms of action. *Neurosci. Res. Prog. Bull.* 19:421-427.
- Coyle JT, Puttfarcken P (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689-695.
- Dringen R (2000). Metabolism and functions of glutathione in brain. *Prog. Neurobiol.* 62:649-671.
- Dunham NW, Miya TS (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *J. Am. Pharm. Assoc.* 46:208-209.
- Fonnum F (1984). Glutamate: a neurotransmitter in mammalian brain. *J. Neurochem.* 42:1-11.
- Geetha M, Kedlaya R, Vasudevan DM (2004). Inhibition of lipid peroxidation by botanical extracts of *Ocimum sanctum*: *In vivo* and *in vitro* studies. *Life Sci.* 76:21-28.
- Godhwani S, Godhwani JL, Vyas DS (1987). *Ocimum sanctum*: An experimental study evaluating its anti-inflammatory, analgesic and antipyretic activity in animals. *J. Ethnopharmacol.* 21:153-163.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannebaum SR (1982). Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal. Biochem.* 126:131-138.
- Greenwood SM, Connolly CN (2007). Dendritic and mitochondrial changes during glutamate excitotoxicity. *Neuropharmacology* 53:891-898.
- Griffith OW (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106:207-212.
- Hynd MR, Scott HL, Dodd PR (2004). Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. *Neurochem. Int.* 45:583-595.
- Jaggi RK, Madaan R, Singh B (2003). Anticonvulsant potential of holy basil, *Ocimum sanctum* Linn., and its cultures. *Indian. J. Exp. Biol.*

- 41:1329-33.
- Joshi H, Parle M (2006). Evaluation of nootropic potential of *Ocimum sanctum* Linn. in mice. *Indian J. Exp. Biol.* 44:133-13.
- Kakkar P, Das B, Viswanathan PNA (1984). Modified spectrophotometric assay of superoxide dismutase. *Indian J. Biochem. Biophys.* 21:130-132.
- Kelm MA, Nair MG, Strasburg GM, De WDL (2000). Antioxidant and COX inhibitory phenolic compounds from *Ocimum sanctum* Linn. *Phytomedicine* 7:7-13.
- Kubo T, Kohira R, Okano T, Ishikawa K (1993). Neonatal glutamate can destroy the hippocampal CA1 structure and impair discrimination learning in rats. *Brain Res.* 616:311-314.
- Lees GJ (1991). Inhibition of sodium-potassium-ATPase: a potentially ubiquitous mechanism contributing to central nervous system neuropathology. *Brain Res. Rev.* 16:283-300.
- Li N, Liu B, Dluzen DE, Jin Y (2007). Protective effects of ginsenoside Rg2 against glutamate-induced neurotoxicity in PC12 cells. *J. Ethnopharmacol.* 111:458-463.
- Liu C, Lin N, Wu B, Qiu Y (2009). Neuroprotective effect of memantine combined with topiramate in hypoxic-ischemic brain injury. *Brain Res.* 1282:173-182.
- Lowry OH, Rosenborough NJ, Farr AL, Randal RJ (1951). Protein measurement with Folin- phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mcbean GJ, Roberts PJ (1984). Chronic infusion of L- glutamate causes neurotoxicity in rat striatum. *Brain. Res.* (1984) 290:372-375.
- Meldrum BS (2002). Implications for neuroprotective treatments. *Prog. Brain. Res.* 135:487-495.
- Michaels R, Rothman SM (1990). Glutamate neurotoxicity in vitro: antagonist pharmacology and intracellular calcium concentrations. *J. Neurosci.* 10:283-292.
- Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller D (1984). Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney possible implications in analgesic nephropathy. *Biochem. Pharmacol.* 33:1801-1807.
- Montal M (1998). Mitochondria, glutamate neurotoxicity and the death cascade. *Biochem. Biophys. Acta.* 1366:113-126.
- Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT (1989). Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neurosci.* 2:1547-1558.
- Nicholis D, Attwell D (1990). The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* 11:462-468.
- Nobre Jr HV, Maia FD, De Oliveira RA (2008). Neuroprotective actions of tannins from *Myracrodruon urundeuva* on 6-hydroxydopamine-induced neuronal cell death. *J. Herbs Spices Med. Plants* 13:41-57.
- Norr H, Wagner H (1992). New constituents from *Ocimum sanctum*. *Planta Med.* 58:574.
- Novelli A, Reilly JA, Lysko PG, Henneberry RC (1988). Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Res.* 451:205-212.
- Okawara M, Katsuki H, Kurimoto E, Shibata H, Kume T, Akaike A (1979). Resveratrol protects dopaminergic neurons in midbrain slice culture from multiple insults. *Biochem. Pharmacol.* 73:550-560.
- Ozsuer H, Gorgulu A, Kiris T, Cobanoglu S (2005). The effects of memantine on lipid peroxidation following closed-head trauma in rats. *Neurosurg. Rev.* 28:143-147.
- Paxinos G, Watson C (2007). *The Rat Brain: In Stereotaxic Coordinates*. Elsevier Science, London.
- Ramanathan M, Sivakumar S, Anandvijaykumar PR, Saravanababu C, Pandian R (2007). Neuroprotective evaluation of standardized extract of *Centella asiatica* in monosodium glutamate treated rats. *Indian J. Exp. Biol.* 45:425-431.
- Samson J, Sheela Devi R, Ravindran R, Senthilvelan M (2006). Biogenic amine changes in brain regions and attenuating action of *Ocimum sanctum* in noise exposure. *Pharmacol. Biochem. Behav.* 83:67-75.
- Sovoboda P, Mossinger B (1981). Catecholamines and brain microsomal Na⁺-K⁺ ATPase protection against lipoperoxidative damage. *Biochem. Pharmacol.* 30:427-432.
- Sudhakar P, Gopalakrishna HN, Akshaya A (2010). Antidepressant activity of ethanolic extract of leaves of *Ocimum sanctum* in mice. *J. Pharm. Res.* 3:624-626.
- Sukari MA, Rahmani M, Lee GB, Takahashi S (1995). Constituents of stem barks of *Ocimum sanctum*. *Fitoterapia* 66:552-553.
- Yanpallewar SU, Rai S, Kumar M, Acharya SB (2004). Evaluation of antioxidant and neuroprotective effect of *Ocimum sanctum* on transient cerebral ischemia and long-term cerebral hypoperfusion. *Pharmacol. Biochem. Behav.* 79:155-164.
- Zhou S, Chena X, Gu X, Dinga F (2009). *Achyranthes bidentata* Blume extract protects cultured hippocampal neurons against glutamate-induced neurotoxicity. *J. Ethnopharmacol.* 122:547-554.

Full Length Research Paper

Target propofol concentration required for laryngeal mask airway insertion after pretreatment with dexmedetomidine

Xiao-Bo Liu, Xi-Ge Yang*, Xin-Bai Li, Hai-Chun Ma, Wei Han, Zhuang Zhao, Chun-Ying Han, and Long-Xin Luo

Department of Anesthesiology, First Hospital of Jilin University, Changchun 130021, China

Accepted 17 June, 2013

The aim of this study was to determine the target-controlled concentration of propofol required for successful laryngeal mask airway (LMA) placement after dexmedetomidine pre-injection. Twenty ASA physical status I-II patients aged 20–60 years old, who were scheduled for general anesthesia, were studied. After receiving a loading dose of 1.0 µg/kg dexmedetomidine over 10 min, propofol was infused using a target-controlled infusion as determined by a modified Dixon's up-and-down method. The first patient received a target-controlled infusion of 3.0 µg/ml propofol. The response of each patient determined the propofol concentration given to the next patient. Cough, body movement, laryngospasm, intentional movement, mouth opening, and difficulty of LMA insertion indicated failure, and the propofol concentration was increased by a step of 0.2 µg/ml. If the insertion of the LMA was successful, then the target concentration was decreased by the same dose. The effect-site propofol concentration for successful LMA insertion was determined to be 2.351 µg/ml in 50% of the patients (EC50) with pre-injection of dexmedetomidine without muscle relaxant. Subsequent probit analysis showed an EC95 (95% CI) of 2.854 µg/ml (2.588–2.944 µg/ml). Thus, dexmedetomidine combined with target-controlled infusion of propofol can be used for LMA placement, with few adverse reactions. In addition, dexmedetomidine sedation can effectively reduce the target-controlled plasma concentration of propofol.

Key words: Dexmedetomidine, propofol, target-controlled infusion, laryngeal airway mask.

INTRODUCTION

A laryngeal mask is widely used in clinical anesthesia to establish an effective airway. Successful insertion of a laryngeal mask airway (LMA) requires an adequate depth of anesthesia to reduce the laryngeal response and prevent coughing, laryngospasm, and other adverse events. Propofol is a short-acting intravenous anesthetic that can effectively reduce laryngeal responses and is widely used to induce anesthesia for laryngeal mask placement (Wang et al., 2010). However, anesthetic induction using propofol alone often requires large doses to achieve enough depth of anesthesia for LMA insertion,

resulting in hemodynamic fluctuations and transient respiratory depression. Clinical trials have shown that separate applications (2.5–3 mg/kg) or plasma concentrations (7–9 µg/ml) of propofol cannot meet the LMA insertion anesthetic requirements (Hickey et al., 1990; Higuchi et al., 2002; Richebe et al., 2005; Taylor and Kenny, 1998). To avoid this problem, propofol is usually combined with other drugs like fentanyl or remifentanyl.

Dexmedetomidine is a highly selective, α₂ receptor agonist with sedative and analgesic properties. It reduces the amount of anesthetic required and provides

*Corresponding author. E-mail: xige_yang@163.com; Tel: +86-13756661628.

hemodynamic stability without respiratory depression. The purpose of this study was to determine, following premedication with dexmedetomidine, the optimal plasma concentration of propofol required for successful laryngeal mask placement.

MATERIALS AND METHODS

After approval from the ethics committee of Jilin University and patients' written informed consent, 22 patients, ASA status I–II, aged 20 to 60 years old, were included in the study. Patients were excluded if they were suspected of having difficulty opening their airways (Mallampati score of III–IV, or a mouth opening of <2.5 cm). Patients were also excluded if they had a history of upper respiratory tract infection in the past two weeks, serious cardiovascular disease, gastroesophageal reflux disease (GERD), or a body mass index ≥ 30 kg/m².

The patients were not given premedication. On arrival to the operating room, each patient was attached to routine monitors and Ringer's lactate solution (10 ml/kg) was infused over 20 min and then maintained at a rate of 100 ml/h. Following the initial fluid bolus, patients were infused with dexmedetomidine (Jiangsu Hengrui Medicine Co., Ltd., China) at 1.0 μ g/kg over 10 min. The target-controlled infusion of propofol (AstraZeneca, Italy) was then started. The LMA "Supreme" (The Laryngeal Mask Company, Ltd., Singapore) was inserted when the infusion and target-controlled infusion concentrations reached equilibrium at the adjusted concentration, and the BIS value was 40–50. A size 3 LMA was selected for patients weighing 30–50 kg, a size 4 LMA was used for patients weighing 50–70 kg, and a size 5 LMA was used for patients weighing >70 kg. Target-controlled infusion (TCI) anesthesia with propofol was administered using a Graseby 3500 target-controlled infusion pump (Smiths Medical, USA). The target concentration of propofol was adjusted according to Dixon's up-and-down sequential method (Kim et al., 2008; Lu et al., 2003). The first patient's initial target-controlled infusion concentration of propofol was 3.0 μ g/ml. The target-controlled infusion effect-site concentration of propofol for subsequent patients was based on the previous patient's response to insertion of the laryngeal mask. If the insertion was successful, for the next patient, the target-controlled infusion concentration of propofol was decreased by 0.2 μ g/ml. If the placement failed, the target-controlled infusion concentration of propofol was increased by 0.2 μ g/ml.

The following variables were observed and recorded:

1. Response to LMA insertion: cough, holding of breath, laryngospasm, or conscious movement of the whole body were considered as a positive response (Yu et al., 2006).
2. Ease of LMA insertion was graded as follows: 1. Insertion without resistance, 2. mild resistance, 3. more resistance but mouth opened, and 4. resistance required additional doses of propofol for LMA insertion. Grades 1 and 2 were considered successful, while grades 3 and 4 were defined as failure of LMA insertion.
3. MAP, heart rate (HR), SpO₂, P_{ET} CO₂, and BIS values were recorded before anesthesia (T0), after dexmedetomidine infusion (T1), when the plasma concentration and effect-site concentration of propofol reached a balance at the set level (T2), and 1 min after LMA insertion (T3).
4. Induction time from the start of anesthesia until LMA insertion.
5. Adverse effects: hypotension, bradycardia, and apnea. Hypotension was defined as mean arterial pressure <60 mmHg or a decrease of more than 30% from baseline values for 1 min. Bradycardia was defined as having a HR below 50 beats/min or the HR decreased more than 30% from the baseline value for 1 min. Apnea was defined as P_{ET} CO₂ = 0 mmHg and RR = 0 breaths/min for more than 1 min. In cases of apnea, assisted

ventilation was performed. Bradycardia was defined as a HR below 50 beats/min or the HR decreased by more than 30% from the baseline value for 1 min. In cases of bradycardia, 0.5 mg of atropine was administered. Hypotension was defined as a mean arterial pressure <60 mmHg. In cases of hypotension, 1–2 mg of dopamine was administered.

Patient data were reported as the mean \pm standard deviation (SD). Statistical analysis was performed using the SPSS package (SPSS 12.0 for windows, SPSS Inc., Chicago, IL, USA). According to Dixon's up-and-down method (Dixon and Massey, 1983), the study continued until six pairs of successful and failed LMA insertions occurred. The 50% target concentration (EC₅₀) of propofol for LMA insertion was defined as the mean of the median cross-over dose. The data were also subjected to probit regression analysis using the 95% effective target concentration (EC₉₅) and the 95% confidence interval (CI). A P-value less than 0.05 was used to define the level of statistical significance.

RESULTS

All the cases were performed in the First Hospital of Jilin University from October 2011 to January 2012. The patients' ages ranged from 20 to 60 years old. They had an average height \pm SD of 159.95 \pm 3.69 cm and an average weight \pm SD of 61.85 \pm 8.54 kg. The average induction time, including the infusion time of dexmedetomidine and propofol, was 13.25 \pm 0.68 min. The study was performed on 20 patients, and all patient data were included in the analysis.

The laryngeal mask was inserted without difficulty in 12 patients (60.0%), whereas insertion was difficult in 8 cases (40%). During laryngeal mask insertion, SpO₂ and PETCO₂ values did not change significantly compared to before insertion. In addition, postoperative follow-up found that patients had no intraoperative awareness.

The effect-site propofol concentration for successful LMA insertion in 50% of the patients with pre-injection of dexmedetomidine (EC₅₀) was 2.351 μ g/ml (1.737–2.6 μ g/ml), while the EC₉₅ was 2.854 μ g/ml (2.588–2.944 μ g/ml). Figure 1 shows the up-down diagram of the effect-site plasma concentration of propofol for all patients. Table 1 lists the changes in hemodynamic variables from the preoperative values after dexmedetomidine infusion, showing that the HR was significantly reduced after dexmedetomidine infusion.

DISCUSSION

The main finding in the present study was that pre-injection of dexmedetomidine can reduce the target-controlled plasma concentration of propofol required for LMA insertion. In addition, experimental application of a modified Dixon's up-and-down method was applied as this procedure is applicable to small clinical samples and has been widely used for calculating the EC₅₀ values of a variety of drugs (Lu et al., 2003; Yu et al., 2006). In order to determine the EC₅₀, the modified Dixon's up-and-down method requires more than six inflection points (Dixon

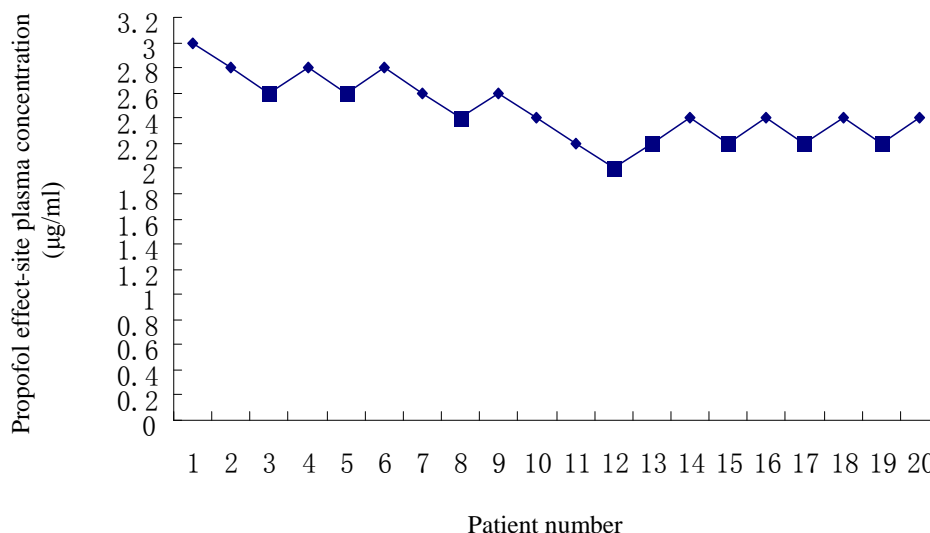


Figure 1. Target-controlled concentration of propofol for sequential patients. ◆, Indicates successful laryngeal mask insertion; ■, indicates failed laryngeal mask placement.

Table 1. Changes in hemodynamic variables at different observation times.

Time	HR (beats/min)	P-value	MAP (mmHg)	P-value
T0	80.15±14.57		94.22±14.09	
T1	65.45±9.37	0.001	93.41±13.46	0.865
T2	66.10±8.43	0.818	85.91±12.53	0.080
T3	64.8±7.38	0.725	86.76±14.75	0.267

MAP and HR values were recorded before anesthesia (T0), after dexmedetomidine infusion (T1), when the plasma concentration and effect-site concentration of propofol reached a balance at the set level (T2), and 1 min after LMA insertion (T3). P values represent the comparison to T0

and Massey, 1983).

Dexmedetomidine is a highly selective α_2 adrenergic receptor agonist. This drug was chosen because it can reduce the doses of opioids and sedatives (Li et al., 2007), and it can inhibit the stress response to intubation. Another significant advantage of dexmedetomidine is that it keeps the wake-up status of sedated patients with almost no inhibitory effect on respiration (Khan et al., 1999). The hemodynamic effects of dexmedetomidine depend on its dosage and injection speed (Li et al., 2007). A rapid intravenous infusion loading dose of 1.0 $\mu\text{g}/\text{kg}$ dexmedetomidine can cause short-term high blood pressure and a reflex decrease in HR. This reaction is more pronounced in a young, healthy population due to direct activation of α_2 receptors in the vascular smooth muscle, leading to vasoconstriction (Pandharipande et al., 2006). Dexmedetomidine at an intravenous infusion loading dose of 1.0 $\mu\text{g}/\text{kg}/10$ min can attenuate a hypertensive reaction. After a subsequent continuous infusion phase, dexmedetomidine has a central anti-sympathetic role and causes increased vagal activity, while blood pressure and HR can be moderately decreased (Triltsch et al., 2002). Dexmedetomidine-

induced hypotension and bradycardia can be corrected by rehydration and by using drugs such as ephedrine and atropine. However, in the presence of hypovolemia or heart block, dexmedetomidine can cause serious consequences (Wang and Cheng, 2010).

Propofol is a short-acting intravenous anesthetic, a perfect sedative with a short half-life, but its analgesic effect is weak. Increasing the dose causes dose-dependent respiratory and circulatory suppression. It reduces the laryngeal responses and is widely used in laryngeal mask placement (Wysowski and Pollock, 2006). It has been reported that the ED₅₀ of propofol was 2.99 $\mu\text{g}/\text{ml}$ (95% CI 2.85–3.12 $\mu\text{g}/\text{ml}$) for smooth laryngeal mask placement when the anesthetic contained 1.5 $\mu\text{g}/\text{kg}$ fentanyl (Yu et al., 2006). Clinical trials have shown that propofol alone (2.5–3 mg/kg) cannot meet the throat mask airway placement conditions; therefore, anesthesia is often combined with opioids (Park et al., 2007). Anesthesia induction with propofol alone requires higher doses with consequent fluctuations in hemodynamics and respiratory depression.

In this study, infusion of dexmedetomidine at 1.0 $\mu\text{g}/\text{kg}/10$ min before propofol induction of anesthesia

could reduce the effect-site concentration of propofol, reduce the amount used, and in turn reduce the cardiovascular responses. Dexmedetomidine also maintains normal breathing; therefore, small doses of dexmedetomidine can be used as an adjuvant in general anesthesia, especially during induction and difficult airway insertion to maintain the awake status and spontaneous breathing while patients are sedated.

In conclusion, dexmedetomidine combined with target-controlled infusion of propofol can be used for LMA placement, with few adverse reactions. In addition, dexmedetomidine sedation can effectively reduce the target-controlled plasma concentration of propofol.

ACKNOWLEDGEMENT

We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

ABBREVIATIONS

LMA, Laryngeal mask airway; **TCI**, target-controlled infusion; **HR**, heart rate.

REFERENCES

- Dixon WJ, Massey FJ (1983). Introduction to statistical analysis. 4th Ed. McGraw-Hill, New York. pp. 426-441.
- Hickey S, Cameron AE, Asbury AJ (1990). Cardiovascular response to insertion of Brain's laryngeal mask. *Anaesthesia* 45:629-633.
- Higuchi H, Adachi Y, Arimura S, Nitahara K, Satoh T (2002). Oral clonidine premedication reduces the EC50 of propofol concentration for laryngeal mask airway insertion in male patients. *Acta Anaesthesiol. Scand.* 46:372-377.
- Khan ZP, Ferguson CN, Jones RM (1999). alpha-2 and imidazoline receptor agonists. Their pharmacology and therapeutic role. *Anaesthesia* 54:146-165.
- Kim JY, Kwak YL, Lee KC, Chang YJ, Kwak HJ (2008). The optimal bolus dose of alfentanil for tracheal intubation during sevoflurane induction without neuromuscular blockade in day-case anaesthesia. *Acta Anaesthesiol. Scand.* 52:106-110.
- Li M, Zhang LP, Wu XM (2007). Research progress of dexmedetomidine in clinical anesthesia. *Chinese J. Clin. Pharmacol.* 23:466-470.
- Lu W, Ramsay JG, Bailey JM (2003). Reliability of pharmacodynamic analysis by logistic regression: mixed-effects modeling. *Anesthesiology* 99:1255-1262.
- Pandharipande P, Ely E, Maze M (2006). Dexmedetomidine for sedation and perioperative management of critically ill patients. *Semin. Anesth. Periop. Med. Pain* 25:43-50.
- Park HJ, Lee JR, Kim CS, Kim SD, Kim HS (2007). Remifentanyl halves the EC50 of propofol for successful insertion of the laryngeal mask airway and laryngeal tube in pediatric patients. *Anesth. Analg.* 105:57-61.
- Richebe P, Rivalan B, Baudouin L, Sesay M, Sztark F, Cros AM, Maurette P (2005). Comparison of the anaesthetic requirement with target-controlled infusion of propofol to insert the laryngeal tube vs. the laryngeal mask. *Eur. J. Anaesthesiol.* 22:858-863.
- Taylor IN, Kenny GN (1998). Requirements for target-controlled infusion of propofol to insert the laryngeal mask airway. *Anaesthesia* 53:222-226.
- Triltsch AE, Welte M, von Homeyer P, Grosse J, Genahr A, Moshirzadeh M, Sidiropoulos A, Konertz W, Kox WJ, Spies CD (2002). Bispectral index-guided sedation with dexmedetomidine in intensive care: a prospective, randomized, double blind, placebo-controlled phase II study. *Crit. Care Med.* 30: 1007-1014.
- Wang D, Cheng Y (2010). Fifty percent of effective concentration of sufentanil for Intubating Laryngeal Mask Airway insertion in obese patients with dexmedetomidine sedation. *China J. Clin. Pharmacol.* 3:614-624.
- Wang Y, Yu WJ, Zhang W, Chen J, Cai RJ (2010). The clinical observation of injection pain of medium and long-chain propofol in intravenous anesthesia. *Shaanxi Med. J.* 39:238-239.
- Wysowski DK, Pollock ML (2006). Reports of death with use of propofol (Diprivan) for nonprocedural (long-term) sedation and literature review. *Anesthesiology* 105:1047-1051.
- Yu AL, Critchley LA, Lee A, Gin T (2006). Alfentanil dosage when inserting the classic laryngeal mask airway. *Anesthesiology* 105:684-688.

Full Length Research Paper

Curcumin inhibits cell survival and migration by suppression of Notch-1 activity in prostate cancer cells

Tao Kong^{1,2}, Yongxing Wang³, Li Xiao² and Limin Liao^{1*}

¹Department of Urology, Beijing Bo'ai Hospital, China Rehabilitation Research Center, School of Rehabilitation Medicine, Capital Medical University, Beijing 100077, China.

²Department of Urological Surgery, Beijing University of Traditional Chinese Medicine Subsidiary, Dongfang Hospital, Beijing 100078, China.

³Department of Urology, Beijing Anzhen Hospital Affiliated with Capital Medical University, Beijing Institute of Heart Lung and Blood Diseases, Beijing 100029, China.

Accepted 5 July, 2013

Previous studies have indicated that Notch-1 activity plays an essential role in prostate tumorigenesis. However, its underlying mechanism is not yet clear. In this study, Curcumin, a drug widely used in Ayurvedic medicine for its antimalarial and anti-inflammatory properties, was used to investigate its impact on cell survival, migration and Notch signaling status in prostate cancer cell lines. Our data show that Curcumin treatment significantly suppressed the proliferation and migration of prostate cancer cell lines DU145 and PC3 in a dose- and time-dependent manner. Two migration-related genes, MT1-MMP and its target-molecule MMP2, were downregulated by Curcumin. There was no significant change of Notch-1 and its cleaved product NICD levels after Curcumin treatment. Furthermore, our chromatin immunoprecipitation assay (ChIP) revealed a remarkable decrease of NICD binding to Hes-1 promoter. Altogether, our results suggest an anti-tumor action of Curcumin in prostate cancer that might be through suppression of the Notch-1 transactivity.

Key words: Prostate cancer, Curcumin, Notch.

INTRODUCTION

Prostate cancer remains the second leading cause of cancer-related death among men in the world (Carlsson et al., 2012). The high mortality of this life-threatening disease results from a bundle of factors, such as late diagnosis, high metastatic potential and lack of effective therapies available, etc. These disappointing facts call for cancer researchers to pay much more attention to prostate cancer. Recent molecular and cellular *in vivo* and *in vitro* studies have indicated that the activation and deactivating of multiple cellular signaling pathways might be involved in the development and progression of prostate cancer (Mellado et al., 2009; Sarker et al., 2009; Yang and Dou, 2010). Among all the possible pathways that might be associated with prostate cancer develop-

ment, Notch pathway is increasingly gaining attention in recent years. Notch signaling pathway is an evolutionarily conserved signaling system essential for embryonic development in metazoan (Dang, 2012). In eukaryotic cells, Notch family consists of four Notch receptors (Notch1-4), three Deltalike ligands (Dll1, Dll3, and Dll4), and two ligands of the Jagged family (Jag1 and Jag2) (Allenspach et al., 2002; Wang et al., 2009). Notch signaling is initiated by the interaction of its ligands and receptors, resulting in subsequent proteolytic digestion of Notch receptor by two enzymes. The first cleavage is conducted by an extracellular matrix metalloprotease, followed by second cleavage mediated by the transmembrane protease complex γ -secretase, releasing

*Corresponding author. E-mail: liminl-kt@hotmail.com. Tel: +8610-87569043. Fax: +8610-87569043.

the Notch intracellular domain (NICD) (Miele et al., 2006). The released NICD can translocate into the nucleus where it, interacting with the CSL family of transcription factors (CBF-1/RBP-Jk, Su (h) and LAG-1), regulates its target genes, such as Hes-1 and Hey-1, which are involved in cell survival regulation. Several lines of evidence showed that suppression of Notch-1 activation contributed to cancer cell growth inhibition and apoptosis onset. More recent data indicated that Notch-1 signal might regulate prostate cell proliferation by targeting Hes-1 (Beatus et al., 2001; Zhang et al., 2009).

Curcumin, a yellow pigment from *Curcuma longa*, is a widely-used spice in Southeast Asian and Middle Eastern cuisine (Ravindran et al., 2009). Several biochemical and functional studies have indicated that Curcumin possesses a potent anti-cancer activity in many types of cancer (Shishodia et al., 2007), especially in prostate cancer (Kurien and Scofield, 2009; Teiten et al., 2010). Since Notch signaling pathway is one of the most essential pathways implicated in prostate cancer development, in this study, our aim is to elucidate if Notch signalling is involved in Curcumin's anti-cancer activity in prostate cancer cell.

MATERIALS AND METHODS

Reagents and kits

All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). BCA protein quantitation kit was purchased from Pierce Company, USA. SYBR® Premix Ex Taq™ II (Perfect Real Time) was purchased from Takara (Dalian, China). Curcumin, purchased from Calbiochem (La Jolla, CA), was dissolved in DMSO and stored at -20°C.

Cell culture

The DU145 and PC3 cells, two prostate cancer cell lines, were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in complete medium (RPMI-1640 medium supplemented with 10% FBS, streptomycin 100 mg/ml and penicillin 100 U/ml) at 37°C in 5% CO₂ humidified incubator.

MTT assay

Cells were seeded with 100 µl of complete medium in 96-well plate (5×10³ cells per well). Different doses of Curcumin were added to each well for indicated times. After 48 h of Curcumin treatment, MTT reagent (5 mg/ml) was added to each well, and incubated for 4 h at 37°C. The formazan crystals were solubilized by the addition of 100 µl of DMSO. The optical density (OD) at 570 nm was measured and cell viability was determined by the following formula. Cell viability (%) = (OD of the treated wells - OD of the blank control wells) / (OD of the negative control wells - OD of the blank control wells) × 100%. All MTT experiments were performed in triplicate and repeated at least three times.

Wound healing assay

Cells were plated into 6-well plates and grown to full confluence.

The cell monolayer was artificially wounded using a 200-µl pipette tip. Cell debris was removed by washing with PBS. After treatment of Curcumin for indicated times, wound closure was photographed with an inverted microscopy equipped with a digital camera. The wound healing extent was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area. All experiments were performed in the presence of 10 µg/ml of mitomycin-C, a cell proliferation inhibitor.

Western blot

The Curcumin-treated and untreated cells were lysed in modified RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 100 mM NaF, and 1 mM Na₃VO₄) containing protease inhibitor cocktail. Cell lysates were spun at 12000 rpm for 30 min to collect supernatant. Protein concentration was determined by the BCA kit. Total proteins were separated by SDS-PAGE and transferred to PVDF membrane. After blockage in 5% nonfat milk, blots were incubated overnight at 4°C with primary antibodies. After washing by PBST (PBS-Tween), the blots were incubated for 1 h with horseradish peroxidase (HRP) conjugated secondary antibody. After washing in PBST, blots were visualized using enhanced chemiluminescence (ECL, Pierce, USA), followed by exposure to Fujifilm LAS3000 Imager (Fuji, Japan). The band densities on the blots were normalized relative to the relevant β-actin band density with Quantity One software (Bio-Rad, USA).

ChIP assay

After treatment of Curcumin for the indicated times, cells were fixed with 1% formaldehyde and nuclei were isolated. After sonication, soluble chromatin DNA was precleared with protein A beads slurry and salmon sperm DNA. Precleared chromatin was immunoprecipitated overnight with 2 µg of antibody for Notch-1 and normal rabbit IgG which serves as negative control. After extensive washing, de-crosslinking and purification, routine PCR was performed to detect the target Hes-1 promoter by using the following primer pair: Forward, 5'-CTGAAAGTTACTGTGGG-3'; reverse, 5'-TGAGCAAGTGCTGAGGG-3'. β-actin (forward 5'-CTGGAACGGTGAAGGTGACA-3'; reverse, 5'-AAGGACTTCCTGTAACAATGCA-3') was used as a loading control. qPCR was also performed to make quantitative analysis. The ChIP qPCR signals of Curcumin treatment are subtracted by the IgG signals. The IgG-normalized ChIP data was presented as percentage of control (0h), which was arbitrarily set as 100%.

Statistical analysis

Results in this study were expressed as mean ± standard deviation (SD). Student's t-test was used for statistical analysis with SPSS 16.0. Differences with P < 0.05 were considered statistically significant.

RESULTS

Curcumin decreased cell viability in prostate cancer cell lines

First, we determined the influence of Curcumin treatment on cell survival. DU145 and PC3 cells were treated with increasing concentration of Curcumin for the indicated time, and MTT assay was performed to examine cell

viability. As shown in Figure 1A, the viability of both DU145 and PC3 cells was significantly inhibited in a dose-dependent manner. Significantly inhibitory effect was noted between the doses of 10 μ M and 50 μ M ($p < 0.01$). Also, we observed cell viability after treatment of 25 μ M of Curcumin for increasing hours. The result was shown in Figure 1B. Obviously, Curcumin decreased cell viability in a time-dependent manner. After 48 h Curcumin treatment, both PC3 and DU145 cells experienced a maximal cell viability loss (~50%) ($P < 0.05$).

The migration of prostate cancer cell lines was inhibited by Curcumin treatment

Next, we assessed the impact of Curcumin treatment on cell migration. We performed a classical cell wound healing assay to determine the cell migration ability. To exclude the impact of cell proliferation, mitomycin-C was included in this assay as described in materials and methods. As shown in Figure 2, Curcumin dramatically suppressed DU145 cell mobility compared to control treatment, with a statistically significant difference ($P < 0.05$).

Curcumin downregulated the expression of MT1-MMP and MMP2, but not of NICD

To test whether Curcumin could affect the expression of Notch-1 and its cleaved form, Notch-1 intercellular domain (NICD), we used western blotting to detect their levels in DU145 cells upon Curcumin treatment. Our study revealed that different concentrations of Curcumin (10 or 50 μ M) resulted in no obvious changes of Notch-1 and NICD levels (Figure 3). Since the above results (Figure 2) showed a migration-inhibitory effect of Curcumin, we also determined two migration-related genes expression levels. As shown in Figure 3, MT1-MMP and MMP2 levels were both decreased after Curcumin treatment (Figure 3). These results suggested that Curcumin could down-regulate the expression of MT1-MMP and MMP2 without affecting Notch-1 and NICD levels.

Curcumin suppressed the binding of NICD to Hes-1 promoter

The above results indicated that the downregulation of Notch pathway target genes induced by Curcumin might be due to the changes of the quality, but not of the quantity of Notch-1. So, we further examined the transactivating ability of NICD upon Curcumin treatment. To this end, chromatin immunoprecipitation assay was conducted to detect the NICD binding to the promoter of Hes-1, one of its target genes. As shown in Figure 4A, in

control treated cells, we using PCR obtained a strong amplicon. In sharp contrast, the amplicon from Curcumin-treated cells showed a fainter amplifying signal, indicating a decreased binding activity of NICD. To better show the changes in NICD binding to Hes-1 promoter, we also performed qPCR. After 10 or 50 μ M Curcumin treatment, NICD binding to Hes-1 promoter was decreased to 53.8 and 14.5% respectively (Figure 4B).

DISCUSSION

Curcumin has gained much attention in cancer research field during the last decade. Curcumin has been demonstrated to inhibit almost many types of cancer cell, such as head and neck carcinoma (Wilken et al., 2011), colon cancer (Patel et al., 2010) and leukemia (Kelkel et al., 2010). Recently, some researchers reported an anti-neoplastic activity of Curcumin in prostate cancer (Hilchie et al., 2010; Piantino et al., 2009; Teiten et al., 2010). Hilchie et al. (2010) found that Curcumin treatment caused a significant PC3 cell death by inducing apoptosis in a dose and time dependent manner. In the present study, we also found that Curcumin can decrease cell survival of PC3 and another prostate cancer cell line DU145. This effect also depended upon dose and duration of treatment. Another study using DU145 as experiment model also obtained a similar conclusion that Curcumin can strongly suppress cell survival (Mukhopadhyay et al., 2001). Therefore, in combination with all these previous findings, our results support an anti-cancer bioactivity of Curcumin in prostate cancer.

In this study, Curcumin suppressed not only cell survival but also cell migration. As we know, increased cell migration is another characteristic of tumor cells. Therefore, curbing tumor cell migration is regarded as a tumor therapeutic target. A recent biochemical study showed that Curcumin bears a strong migration-suppressing activity in lung cancer cells (Yang et al., 2012). Another study using microglial cells as model also gained a parallel conclusion that Curcumin is a negative cell migration regulator in tumor cells (Karlstetter et al., 2011). These findings agree well with our results in prostate cancer cells. Furthermore, we also attempted to interpret its underlying mechanism and found MT1-MMP and MMP2 might be the molecular target of Curcumin, because these two important migration-relating genes can be downregulated by Curcumin.

To date, multiple signaling pathways have been proposed to be implicated in the negative regulation of cancer cell by Curcumin. Indeed, a diverse range of factors have been verified to a molecular target of Curcumin. Curcumin can activate caspases to induce cell apoptosis (Park and Lee, 2007; Tan et al., 2006). Curcumin also can induced cell apoptosis by suppressing the expression of negative apoptosis regulators, such as Bcl-2, Bcl-xL, surviving, or by upregulating protein levels

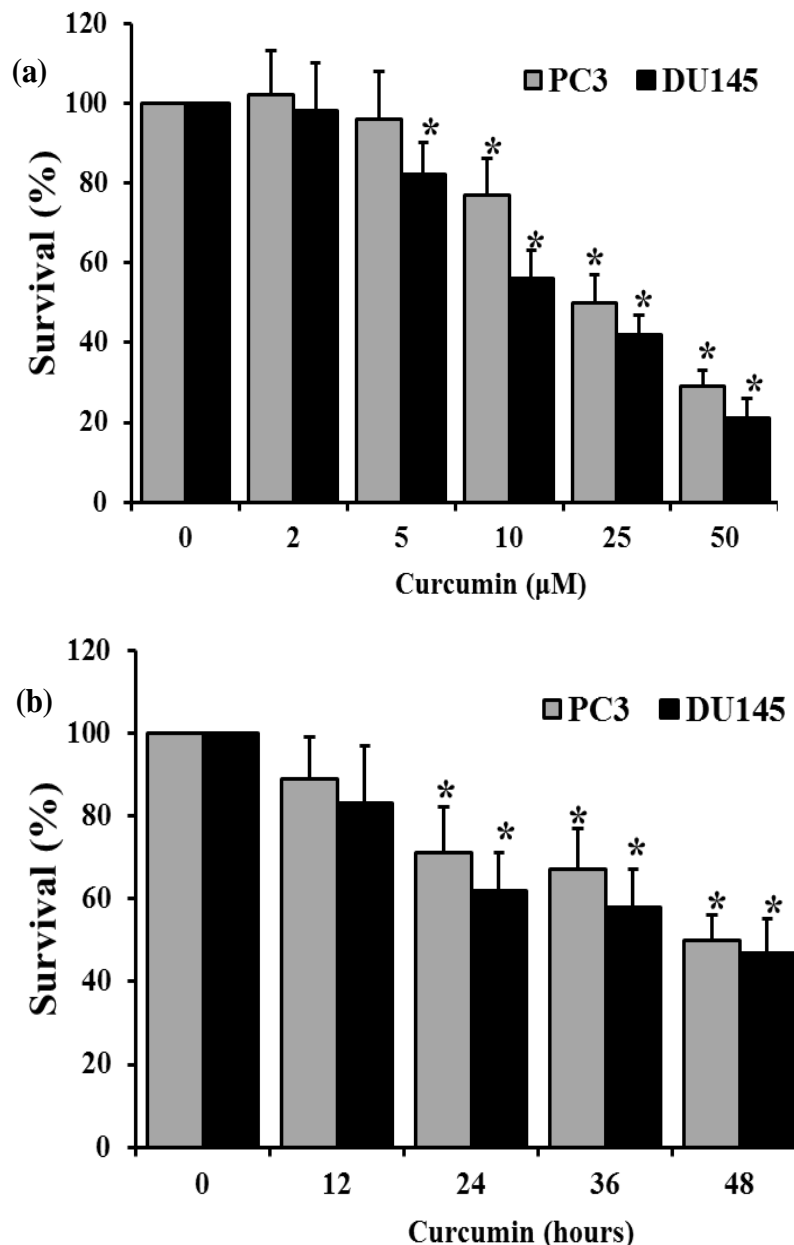


Figure 1. The impact of curcumin treatment on cell survival. **(A)** PC3 and DU145 cells were treated with increasing doses of curcumin for 48 h and MTT assay was performed to examine cell viability. Data from three independent experiments were statistically analyzed and plotted as Mean \pm SD. * denotes $P < 0.05$. **(B)** PC3 and DU145 cells were treated with 25 μM of curcumin for increasing hours and MTT assay was performed to examine cell viability. Data from three independent experiments were statistically analyzed and plotted as mean \pm SD. * denotes $P < 0.05$.

of positive apoptosis regulators, such as Bax, Bim, PUMA (Ravindran et al., 2009). Curcumin also can induce tumor cell apoptosis in a p53/p21-dependent manner (Liu et al., 2007; Srivastava et al., 2007). Here, our results indicated another important cellular pathway, Notch pathway, might be involved in Curcumin's anti-proliferative process, because NICD's transactivating activity was

dramatically suppressed by Curcumin. Of note, Curcumin cannot disturb NICD level or total Notch-1 level, suggesting Curcumin can only affect Notch pathway activity, but not its constitution. Altogether, the present study demonstrates a suppressing role of Curcumin in prostate cancer cells. It can inhibit cell proliferation and migration. The involving mechanism might be duo to

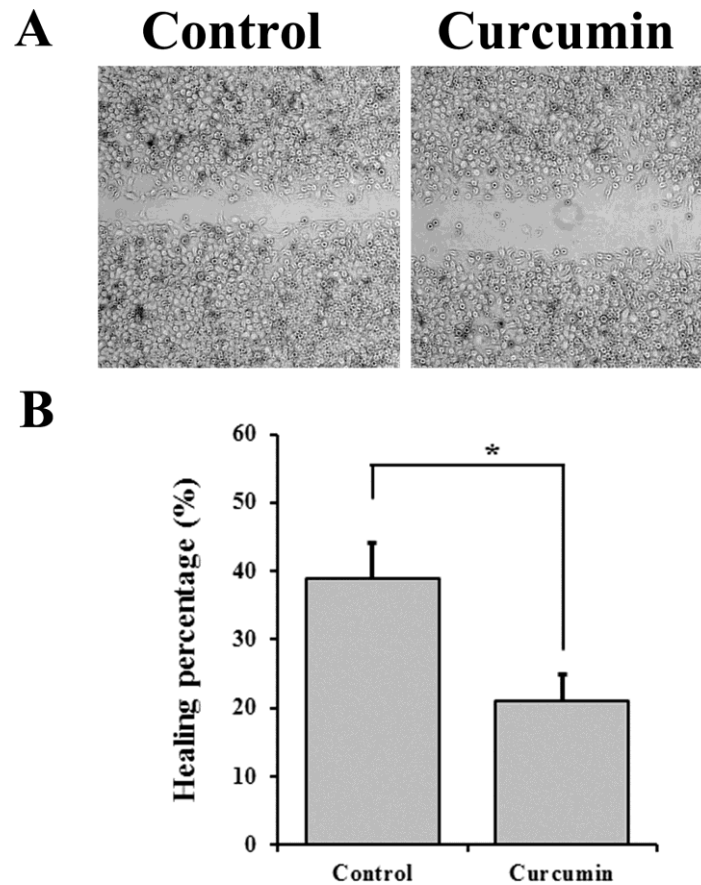


Figure 2. The effect of curcumin treatment on cell migration. Cell wound healing assay was performed to assess the DU145 cell mobility after treatment of 25 μ M of curcumin or DMSO (as control) for 24 h. Data from three independent experiments were statistically analyzed and plotted as Mean \pm SD. * denotes $P < 0.05$.

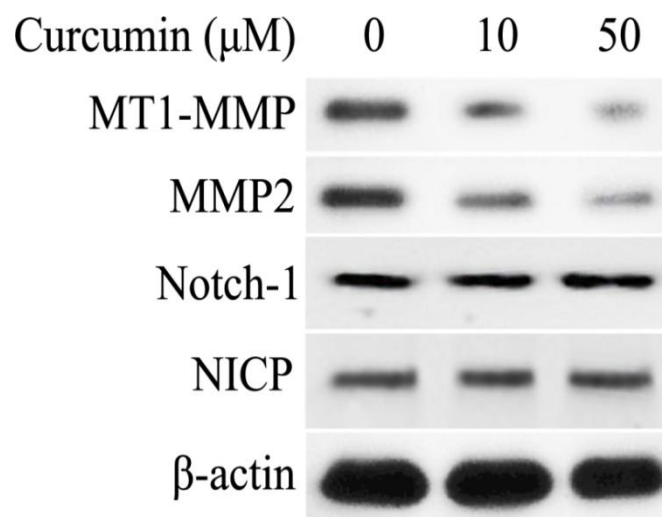


Figure 3. The protein levels of MT1-MMP, MMP2, Notch-1 and NICD after Curcumin treatment. DU145 cells were incubated with 10 μ M or 50 μ M of Curcumin for 24 h and western blot was performed to detect the protein levels of MT1-MMP, MMP2, Notch-1 and NICD. β -actin served as a loading control.

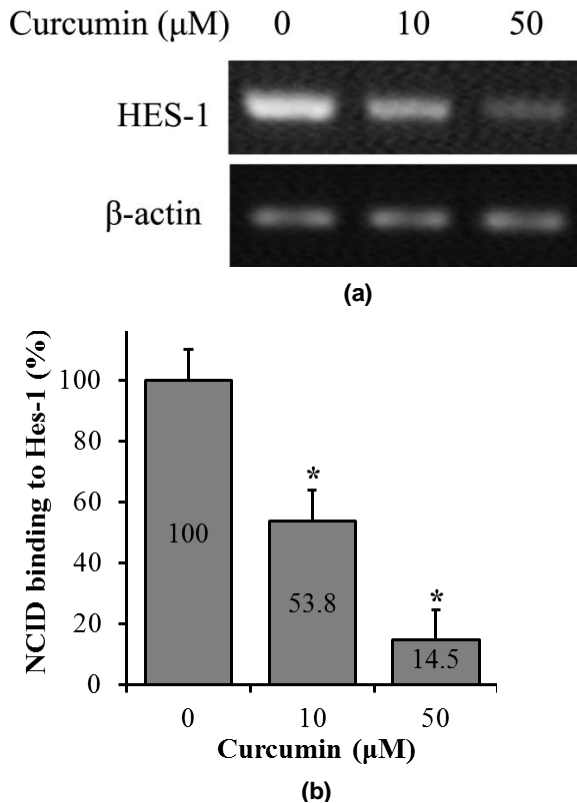


Figure 4. The transactivating ability of NICD after curcumin treatment. DU145 cells were treated with 10 μM or 50 μM of curcumin for 24 h and chromatin immunoprecipitation assay was performed to detect the binding of NICD to Hes-1 gene promoter. (A) PCR was conducted to detect the Hes-1 promoter segments immunoprecipitated by NICD. (B) qPCR was performed to quantitatively determine the abundance of Hes-1 promoter segments immunoprecipitated by NICD, which represents the NCID binding activity to Hes-1. Data from three independent experiments was statistically analyzed and plotted as Mean ± SD. * denotes P < 0.05.

activity loss of Notch signalling. Future study should emphasize the more detailed molecular mechanism underlying this phenomenon.

REFERENCES

- Allenspach EJ, Maillard I, Aster JC, Pear WS (2002). Notch signaling in cancer. *Cancer Biol. Ther.* 1:466-476.
- Beatus P, Lundkvist J, Oberg C, Pedersen K, Lendahl U (2001). The origin of the ankyrin repeat region in Notch intracellular domains is critical for regulation of HES promoter activity. *Mech Dev* 104:3-20.
- Carlsson S, Vickers AJ, Roobol M, Eastham J, Scardino P, Lilja H, Hugosson J (2012). Prostate Cancer Screening: Facts, Statistics, and Interpretation in Response to the US Preventive Services Task Force Review. *J. Clin. Oncol.* 30:2581-2584.
- Dang TP (2012). Notch, apoptosis and cancer. *Adv Exp Med. Biol.* 727:199-209.
- Hilchie AL, Furlong SJ, Sutton K, Richardson A, Robichaud MR, Giacomantonio CA, Ridgway ND, Hoskin DW (2010). Curcumin-induced apoptosis in PC3 prostate carcinoma cells is caspase-independent and involves cellular ceramide accumulation and damage to mitochondria. *Nutr. Cancer.* 62:379-389.
- Karlstetter M, Lippe E, Walczak Y, Moehle C, Aslanidis A, Mirza M, Langmann T (2011). Curcumin is a potent modulator of microglial gene expression and migration. *J. Neuroinflammation.* 8:125.
- Kelkel M, Jacob C, Dicato M, Diederich M (2010). Potential of the dietary antioxidants resveratrol and curcumin in prevention and treatment of hematologic malignancies. *Molecules* 15:7035-7074.
- Kurien BT, Scofield RH (2009). Curry spice curcumin and prostate cancer. *Mol. Nutr. Food Res.* 53:939-940.
- Liu E, Wu J, Cao W, Zhang J, Liu W, Jiang X, Zhang X (2007). Curcumin induces G2/M cell cycle arrest in a p53-dependent manner and upregulates ING4 expression in human glioma. *J. Neurooncol.* 85:263-270.
- Mellado B, Codony J, Ribal MJ, Visa L, Gascon P (2009). Molecular biology of androgen-independent prostate cancer: the role of the androgen receptor pathway. *Clin. Transl. Oncol.* 11:5-10.
- Miele L, Golde T, Osborne B (2006). Notch signaling in cancer. *Curr. Mol. Med.* 6:905-918.
- Mukhopadhyay A, Bueso-Ramos C, Chatterjee D, Pantazis P, Aggarwal BB (2001). Curcumin downregulates cell survival mechanisms in human prostate cancer cell lines. *Oncogene* 20:7597-7609.
- Park K, Lee JH (2007). Photosensitizer effect of curcumin on UVB-irradiated HaCaT cells through activation of caspase pathways. *Oncol. Rep.* 17:537-540.
- Patel VB, Misra S, Patel BB, Majumdar AP (2010). Colorectal cancer: chemopreventive role of curcumin and resveratrol. *Nutr. Cancer* 62:958-967.
- Piantino CB, Salvadori FA, Ayres PP, Kato RB, Srougi V, Leite KR, Srougi M (2009). An evaluation of the anti-neoplastic activity of curcumin in prostate cancer cell lines. *Int. Braz. J. Urol.* 35:354-360; discussion 361.
- Ravindran J, Prasad S, Aggarwal BB (2009). Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *AAPS J.* 11:495-510.
- Sarker D, Reid AH, Yap TA, de Bono JS (2009). Targeting the PI3K/AKT pathway for the treatment of prostate cancer. *Clin. Cancer. Res.* 15:4799-4805.
- Shishodia S, Chaturvedi MM, Aggarwal BB (2007). Role of curcumin in cancer therapy. *Curr. Probl. Cancer* 31:243-305.
- Srivastava RK, Chen Q, Siddiqui I, Sarva K, Shankar S (2007). Linkage of curcumin-induced cell cycle arrest and apoptosis by cyclin-dependent kinase inhibitor p21(WAF1/CIP1). *Cell Cycle* 6:2953-2961.
- Tan TW, Tsai HR, Lu HF, Lin HL, Tsou MF, Lin YT, Tsai HY, Chen YF, Chung JG (2006). Curcumin-induced cell cycle arrest and apoptosis in human acute promyelocytic leukemia HL-60 cells via MMP changes and caspase-3 activation. *Anticancer Res.* 26:4361-4371.
- Teiten MH, Gaascht F, Eifes S, Dicato M, Diederich M (2010). Chemopreventive potential of curcumin in prostate cancer. *Gen. Nutr* 5:61-74.
- Wang Z, Li Y, Banerjee S, Sarkar FH (2009). Emerging role of Notch in stem cells and cancer. *Cancer Lett.* 279:8-12.
- Wilken R, Veena MS, Wang MB, Srivatsan ES (2011). Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. *Mol. Cancer* 10:12.
- Yang CL, Liu YY, Ma YG, Xue YX, Liu DG, Ren Y, Liu XB, Li Y, Li Z (2012). Curcumin blocks small cell lung cancer cells migration, invasion, angiogenesis, cell cycle and neoplasia through Janus kinase-STAT3 signalling pathway. *PLoS One* 7:e37960.
- Yang H, Dou QP (2010). Targeting apoptosis pathway with natural terpenoids: implications for treatment of breast and prostate cancer. *Curr. Drug Targets* 11:733-744.
- Zhang Y, Lian JB, Stein JL, van Wijnen AJ, Stein GS (2009). The Notch-responsive transcription factor Hes-1 attenuates osteocalcin promoter activity in osteoblastic cells. *J. Cell Biochem.* 108:651-659.

Full Length Research Paper

Melatonin changes tularemia progression in a BALB/c mouse model

Miroslav Pohanka^{1*} and Oto Pavlis^{1,2}

¹Faculty of Military Health Sciences, University of Defense, Trebesska 1575, 50001 Hradec Kralove, Czech Republic.
²Centre of Biological Defense, 561 66 Techonin, Czech Republic.

Accepted 10 June, 2013

Melatonin is a hormone with antioxidant properties. In the body, melatonin is involved in regulation of circadian biological rhythm. However, receptors for melatonin are expressed on disparate organs and they can be found on immune cells as well. The present experiment is focused on research whether melatonin would regulate pathogenesis caused by a model intracellular pathogen, *Francisella tularensis*. For this reason, laboratory mice BALB/c were chosen as a suitable model and they were infected with *F. tularensis*. Melatonin was given in two doses: 10 and 100 µg/kg. Animals were sacrificed after either three or five days. Spleen and liver were sampled for bacterial burden. Interferon gamma (IFN-γ), interleukin 2 (IL-2) and total immunoglobulins were assayed from plasma samples. The results showed administration of melatonin reduced bacterial burden in the organs in a dose response manner. Surprisingly, IFN-γ and IL-2 levels were reduced as well, while immunoglobulins remained unchanged. We conclude our experiment that melatonin is potent to reduce tularemia progression.

Key words: Melatonin, *Francisella tularensis*, tularemia, interferon gamma, interleukin 2, oxidative stress, pineal gland, inflammation.

INTRODUCTION

Melatonin (N-acetyl-5methoxytryptamine) is a compound created in the body from amino acid tryptophan (Natarajan et al., 2012). In the body of vertebrates, it comes from pineal gland and it is released to blood system in order to regulate circadian biological rhythms (Hasan et al., 2012). Namely, melatonin is involved in sleep regulation in both humans and animals (Hur et al., 2012). It also regulates seasonal body weight and sexual activities of some animals (Barrett and Bolborea, 2012) and vocal signatures in birds (Deregnacourt et al., 2012). Action of melatonin is mediated through G protein coupled melatonin receptors MT₁, MT₂, and MT₃ expressed in disparate organs including brain, cardiovascular system, liver, intestine, kidney, and immune cells (Ekmekcioglu, 2006). Effect of melatonin is not well understood and it is believed that melatonin can modulate different pathological processes; however, it

needs some more experiments to resolve the issue (Pohanka, 2011).

Melatonin was reported to be able to modulate immune system as stated by many researchers (Carrillo-Vico et al., 2013). Unfortunately, the findings are quite scarce and the results from the reported experiments are quite different in their conclusions. In an animal model, melatonin acting via MT₁ was revealed to be responsible for release of interleukin-2 (IL-2) and MT₂ was excluded from the action (Ahmad et al., 2012). Cardinali et al. (2008) stated that melatonin stimulates production of natural killer cells and CD4+ cells and down regulates CD8+ cells. In another experiment, melatonin was shown to shift the balance between Th-1 and Th-2 lymphocytes to Th-1 (Srinivasan et al., 2008). On the other hand, another study indicated that melatonin can suppress immunity response previously stimulated with

*Corresponding author. E-mail: miroslav.pohanka@gmail.com. Tel.: +420973253091.

lipopolysaccharide (Ban et al., 2011). In the study, suppressive effect of melatonin was proved on macrophages. The cells had reduced expression of genes involved in regulation of immunity when compared to the controls. Wu et al. (2011) investigated effect of melatonin on microglia. They reported anti-inflammatory effect of melatonin and protection of hippocampus from *Klebsiella pneumoniae* induced pathology. Beside regulation of the immune response, melatonin can modulate immune system by inhibition of enzymes involved in killing of pathogens such as myeloperoxidase and NO synthase including the inducible isoform (Galijasevic et al., 2008; Koh, 2008). Beneficial effects of antioxidants including melatonin in infectious diseases are extensively researched and we can found opinions that melatonin is a promising compound suitable for a nonspecific therapy (Fares, 2013; Olegario et al., 2013; Poplawski et al., 2013).

In the present experiment, we focused our effort on research of possible immune response modulation by melatonin in tularemia infected mice. The disease is caused by *Francisella tularensis*, an intracellular pathogen being able to survive in and escape from macrophages (Mahawar et al., 2012). Both innate and adaptive immunity are necessary for resolving of tularemia. Beside innate immunity, interferon gamma (IFN- γ) is necessary for resolving of the disease and activation of the captured bacteria killing (Casbon et al., 2012). We chose a tularemia infected mice model for the test of melatonin effect. The primary intention of the experiment is to answer whether melatonin can improve or make worse the disease.

MATERIALS AND METHODS

Bacterium

Live vaccine strain of *F. tularensis* (American Type Culture Collection 29684) was used throughout. The vaccine strain is fully virulent for rodents (Conlan, 2011). The bacterium was cultivated on McLeod agar supplemented with bovine hemoglobin (Sigma-Aldrich, St. Louis, MO, USA) and IsoVitalaxX (Becton-Dickinson, San Jose, CA, USA). The cultivation was carried out at 37°C for one day. After the cultivation, the cells were harvested using a disposable scraper, re-suspended in saline solution, and re-harvested by centrifugation at 2,000xg for 10 min. Finally, the cells were re-suspended in saline in order to be used in the *in vivo* experiments. Concentration of the cells in the suspension was estimated by calibrated turbidimetric assay and exact concentration 2.77×10^6 CFU/ml of viable cells in the suspension was confirmed by cultivation test one day later.

Laboratory animals

Laboratory female mice BALB/c (Velaz, Unetice, Czech Republic) were chosen for the experiment purposes. The mice are an accepted model for tularemia and results from the experiment can be easily extrapolated to previous findings (Shen et al., 2010). In the experiment beginning, the mice weighed 21 ± 1 g at eight weeks old. The animals were kept in an air conditioned room with tempe-

perature $22 \pm 2^\circ\text{C}$ and humidity $50 \pm 10\%$. In the room, lighting lasted 12 h per day. The animals had free access to food and water. The experiment was approved and supervised by the ethical committee at Centre of Biological Defense in Techonin (Czech Republic).

The animals were divided into eight groups of 12 specimens. For the experimental purposes, melatonin in analytical purity was obtained from Sigma-Aldrich and solved in saline with 10% (v/v) ethanol. Animals in the individual groups received subcutaneous (neck skinfold) application of solutions in compliance with the following overview:

First group received 50 μl of saline with 10% (v/v) of ethanol first and second day of the experiment. The animals in the first group were sacrificed after five days.

Second group received 50 μl of melatonin solution providing dose 100 $\mu\text{g}/\text{kg}$. Melatonin was solved in saline with 10% (v/v) ethanol. The doses were given the first and the same dose the second day after experiment beginning. The animals were sacrificed after five days.

Third group received 100 μl of *F. tularensis* suspension and 50 μl of saline with 10% (v/v) of ethanol one hour later. The application of saline with ethanol was repeated the second day. The animals were sacrificed after three days.

Fourth group received 100 μl of *F. tularensis* suspension and 50 μl of melatonin solution providing dose 10 $\mu\text{g}/\text{kg}$. Melatonin was solved in saline with 10% (v/v). Melatonin was applied one hour after the *F. tularensis* and then again after one day. The animals were sacrificed after three days.

Fifth group received 100 μl of *F. tularensis* suspension and 50 μl of melatonin solution providing dose 100 $\mu\text{g}/\text{kg}$. Melatonin was solved in saline with 10% (v/v). Melatonin was applied one hour after the *F. tularensis* and then again after one day. The animals were sacrificed after three days.

Sixth group received 100 μl of *F. tularensis* suspension and 50 μl of saline with 10% (v/v) of ethanol one hour later. The application of saline with ethanol was repeated the second day. The animals were sacrificed after five days.

Seventh group received 100 μl of *F. tularensis* suspension and 50 μl of melatonin solution providing dose 10 $\mu\text{g}/\text{kg}$. Melatonin was solved in saline with 10% (v/v). Melatonin was applied one hour after the *F. tularensis* and then again after one day. The animals were sacrificed after five days.

Eighth group received 100 μl of *F. tularensis* suspension and 50 μl of melatonin solution providing dose 100 $\mu\text{g}/\text{kg}$. Melatonin was solved in saline with 10% (v/v). Melatonin was applied one hour after the *F. tularensis* and then again after one day. The animals were sacrificed after five days.

The sacrifice was performed by CO₂ anesthesia. Blood was collected from hearth into tubes with lithium heparin (Dialab, Prague, Czech Republic). The freshly collected blood was centrifuged at 1,000xg for 5 min and plasma was separated. The plasma samples were kept at -80°C until *ex vivo* assay. Spleen and liver were sampled from the cadavers as well. The organs were used for bacterial burden assay immediately after the collection.

Assay of selected markers

IL-6 and IFN- γ were assayed using a commercial enzyme linked immuno sorbent assay (ELISA) kit from Abcam (Cambridge, MA, USA). The kits were processed in compliance with instructions using polystyrene 96-well microplates (Gama, Ceske Budejovice, Czech Republic) and a microplate reader Sunrise (Tecan, Salzburg, Austria). Total immunoglobulins were assayed by a direct ELISA using the aforementioned microplates and device. 20 $\mu\text{l}/\text{well}$ of the serum sample and 180 $\mu\text{l}/\text{well}$ of phosphate buffered saline were mixed and incubated at 37°C overnight. The wells were washed by

phosphate buffered saline and free surface was blocked by 100 μ l of 0.1 % (w/v) gelatin at 37°C for 1 h. 100 μ l of anti-mouse immunoglobulin (specificity to G, A, and M isotypes) polyclonal antibody labeled with horseradish peroxidase (Sigma-Aldrich) diluted 1:1000 were injected per well and incubated at 37°C for 1 h. After that, the microplate was washed with phosphate buffered saline with addition of Tween 20 (Sigma-Aldrich). Fresh solution of 0.5 mg/ml *o*-phenylenediamine and 5 mmol/l H₂O₂ was added for 1 min and reaction was stopped with 100 μ l/well of 2 mol/l H₂SO₄. Optical density was measured at 490 nm. Wells with captured albumin (20 μ l; 5 mg/ μ l) were used for negative control purposes. Standard immunoglobulin from mouse was used for calibration purposes.

The organ samples (spleen and liver) were mechanically homogenized immediately after collection. They were passed through nylon net with holes sized 1 mm². The fresh homogenate was re-suspended into saline and injected over the McLeod Agar with composition and cultivation conditions as mentioned above.

Statistical analysis

The achieved data were processed in Origin 8 Pro (OriginLab Corporation, Northampton, MA, USA) software using one-way ANOVA test with Scheffe test. The both probability levels $P < 0.05$ and $P < 0.01$ were calculated.

RESULTS

The first two (1st and 2nd) groups treated with saline or melatonin only had no clinical manifestation. The last six groups of animals (3rd – 8th) were infected with *F. tularensis*. No difference in clinical manifestation was observed for groups 3rd -8th. The applied dose of *F. tularensis* was sub-lethal so no demission occurred during the experiment. Full manifestation of the symptoms was seen three days after tularemia starting and remained up to the end of the experiment.

Bacterial burden is depicted in Figure 1 for liver and Figure 2 for spleen. The organs were chosen because they are typically invaded by *F. tularensis* (Ellis et al., 2002; Sharma et al., 2011). In the liver, melatonin caused dose dependent decrease of bacterial burden in the both time intervals 3 and 5 days after the experiment beginning. The decrease was significant on probability level 0.01. Effect of melatonin on bacterial presence in the spleen was milder when compared to the liver. The decrease in the spleen was significant (probability level 0.05) three days after the experiment beginning. Five days after the experiment beginning, dose dependent but not significant decrease in the spleen was seen.

Assay of antibodies provided no significant effect of melatonin on immune response (data not shown). Average content of total IgG, IgA and IgM was 8.3±2.5 mg/ml. Melatonin alone did not cause change in the antibodies. Tularemia caused alteration in antibodies level around 20%. However, this alteration was insignificant. Plasmatic level of IL-6 is depicted in Figures 3 and 4. All infected animals had significantly ($P < 0.01$) increased IL-6 when compared to the first two groups (controls and the exposed to melatonin). Melatonin alone had no effect on secretion of IL-6. In the tularemia infec-

ted animals, melatonin caused decline of IL-6 level three days after the infection started. The upper dose of melatonin provided significant ($P < 0.05$) decrease of IL-6 three days after infection started. Effect of melatonin on IL-6 was in a dose dependent manner in the interval three days. However, melatonin had no plausible effect five days after infection started. When compared to the two intervals, it is obvious that inflammatory immune reaction damp out after the longer time.

IFN- γ is the last marker described here. It is a cytokine necessary for resolving of a disease caused by an intracellular pathogen. In the *F. tularensis* invaded macrophages, it can interfere replication of the pathogen and thus suppress progression of the disease (Zhou et al., 2012). In the experiment reported here, we proved significant ($P < 0.01$) increase of IFN- γ in the tularemia infected animals incorporated into the 3rd group, and in the infected animals in the 6th – 8th groups. Melatonin had no effect on IFN- γ five days after experiment beginning. However, tularemia infected animals which received melatonin had IFN- γ level not significant to controls. Three days after experiment beginning, the upper dose of melatonin caused significant ($P < 0.05$) decrease in IFN- γ level when compared to the infected animals that did not received melatonin.

DISCUSSION

The infected animals had typical symptoms of tularemia such as lethargy, and ruffled fur, as has been reported in previous studies (Hepburn and Simpson, 2008; Nigrovic and Wingerter, 2008). Owing to the bacterial burden, the effect cannot be attributed to pertinent melatonin cytotoxic effect on the pathogen as the compound is harmless and there is no evidence that melatonin would kill either host or pathogen cells (Ono et al., 2012; Uguz et al., 2012; Wang et al., 2012).

The level of antibodies corresponded to assumptions from experiments on humoral immunity in tularemia suffered animals (Koskela and Salminen 1985). When effect of melatonin on antibodies production is searched, ambiguous data can be found. Both decrease (Zhou et al., 2010) and increase (Regodon et al., 2009) in course of melatonin can be found in plasma after challenge by an antigen. The findings about antibodies reported here neither confirm nor neglect the mentioned experiments.

Intervention of melatonin in innate immunity is not surprising as attenuation of IL-6 secretion in course of melatonin was described by other researchers as well (Laliena et al., 2012; Lau et al., 2012; Wu et al., 2012). Effect of melatonin on inflammation is not probably privileged to IL-6 only as anti-inflammatory effect of melatonin on other inflammatory cytokines is reported in current literature (Wu et al., 2012). The effect is probably mediated via nuclear factor-kappa B and nuclear erythroid 2-related factor 2 (Negi et al., 2011). When searched literature, desperate knowledge about melatonin

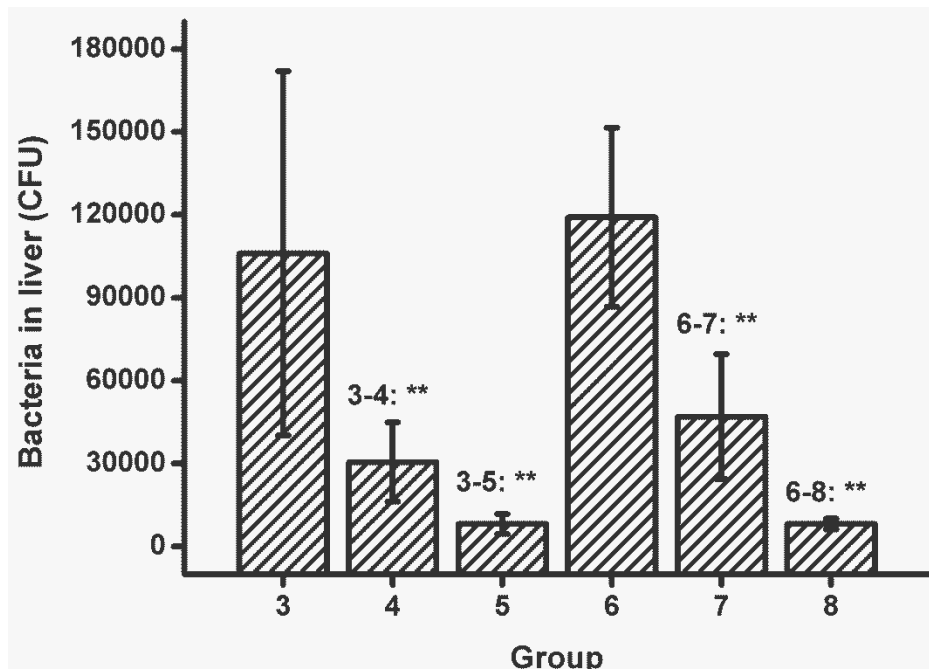


Figure 1. *F. tularensis* LVS viable cells in the livers of mice BALB/c. Significance between the groups was tested for two probability levels $P < 0.05$ (*) and $P < 0.01$ (**).

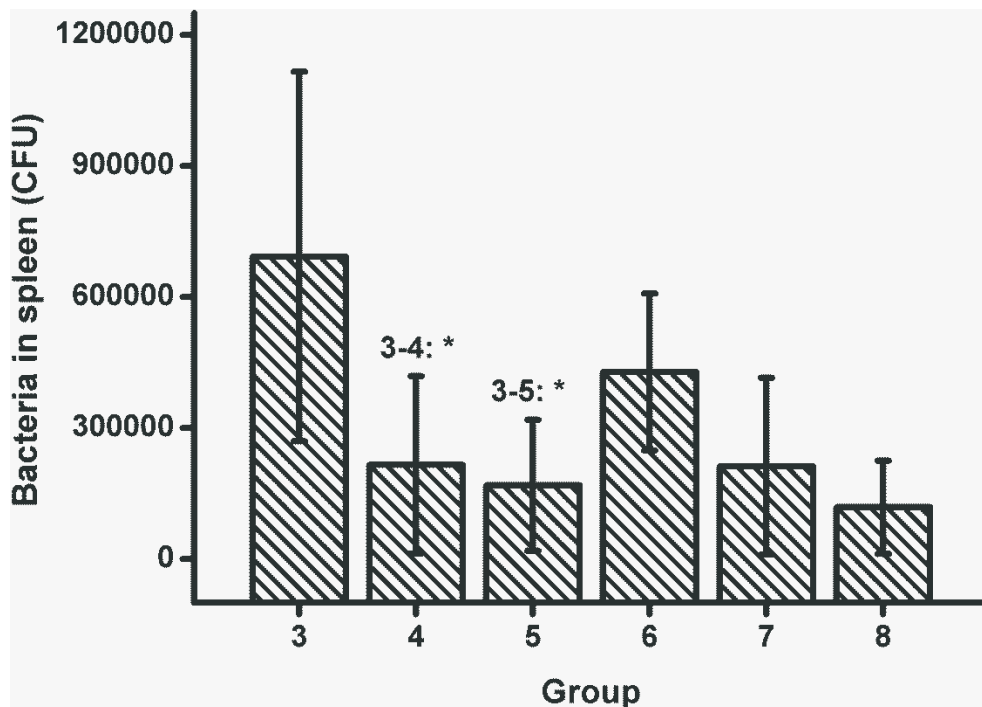


Figure 2. *F. tularensis* LVS viable cells in the spleens of mice BALB/c. Significance between the groups was tested for two probability levels $P < 0.05$ (*) and $P < 0.01$ (**).

effect on IFN- γ level can be learned. While Oliveira et al. (2010) reported release of IFN- γ in course of melatonin in a Wistar rat model, Kim et al. (2012) revealed suppres-

sion of IFN- γ in a mice model three days after melatonin application (Kim et al., 2012).

The findings reported here are quite surprising as the

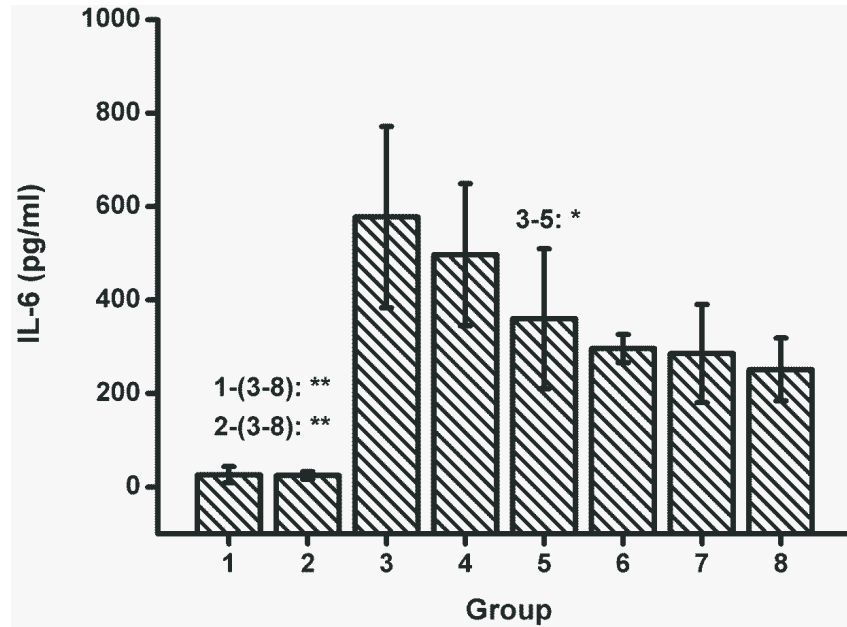


Figure 3. IL-6 level in plasma. Significance between the groups was tested for two probability levels $P < 0.05$ (*) and $P < 0.01$ (**).

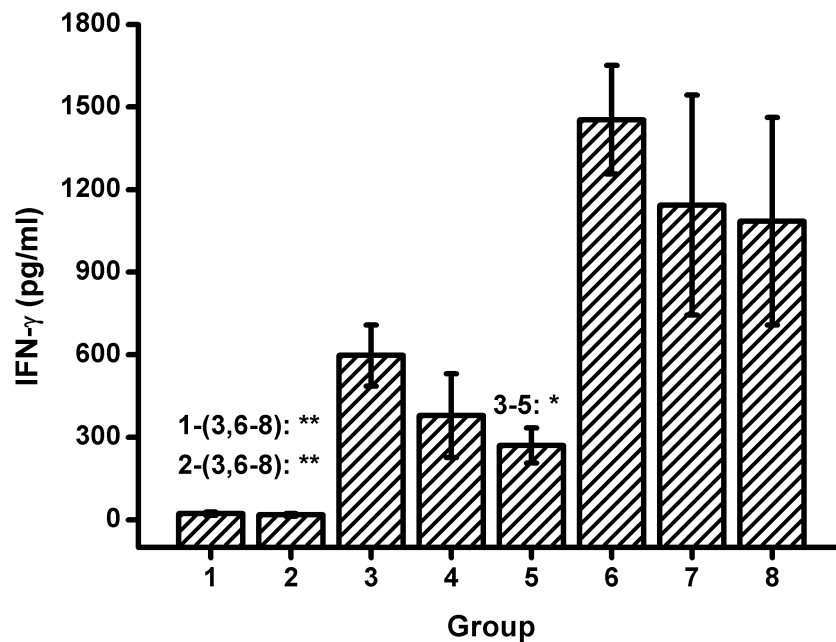


Figure 4. IFN- γ level in plasma. Significance between the groups was tested for two probability levels $P < 0.05$ (*) and $P < 0.01$ (**).

both IFN- γ , IL-6 and bacterial burden were reduced. It means that melatonin can reduce bacterial burden despite suppression of immunity. It is not easy to answer the described phenomenon. The cytokines could be decreased due to reduction of number of invading bacteria. However, we are not able to recognize what cause and consequence is. We can infer that the mela-

tonin effect could lay in another mechanism than immunity control. Some possible mechanisms can be revealed from literature. Melatonin can change level of intracellular calcium and cAMP. It regulates kinase activity and cell cycle in this way (Bagnaresi et al., 2012). Melatonin is also able to initiate expression of extracellular mitogen-activated protein kinase, ERK, and

signal transducer and activator of transcription STAT 3 (Laliena et al., 2012). As a result of the melatonin action, alteration in apoptosis and basal metabolism can be expected (Boga et al., 2012; Pohanka, 2011). It is a question whether melatonin reduces bacterial burden and the here assessed markers IL-6 and IFN- γ are decreased consequently or two independent pathways can be recognized in the body. Aforementioned necessity of IFN- γ for tularemia resolving neglect the idea that melatonin would intermediate its beneficial effect on the disease this way. However, more specific experiment on the melatonin effect on the bacteria should be done prior to give the final answer.

Conclusions

Melatonin is able to modulate tularemia disease. We assume that it acts through cell cycle regulation rather than direct effect on cytokines release. Though evidence of the pathways is not clear enough to provide simple conclusion, melatonin appears to be a perspective compound for pharmacological research on infectious disease treatment. We can conclude our experiment by statement that melatonin would ameliorate progression of tularemia probably by restriction of *F. tularensis* growth. However, research is needed to get more detailed knowledge about melatonin action.

ACKNOWLEDGEMENT

A long-term organization development plan 1011 (Faculty of Military Health Sciences, University of Defense, Czech Republic) is gratefully acknowledged.

REFERENCES

- Ahmad R, Haldar C, Gupta S (2012). Melatonin membrane receptor type MT1 modulates cell-mediated immunity in the seasonally breeding tropical rodent *Funambulus pennanti*. *Neuroimmunomodulation* 19:50-59.
- Bagnaresi P, Nakabashi M, Thomas AP, Reiter RJ, Garcia CRS (2012). The role of melatonin in parasite biology. *Mol. Biochem. Parasitol.* 181:1-6.
- Ban JY, Kim BS, Kim SC, Kim DH, Chung JH (2011). Microarray Analysis of Gene Expression Profiles in Response to Treatment with Melatonin in Lipopolysaccharide Activated RAW 264.7 Cells. *Korean J. Physiol. Pharmacol.* 15:23-29.
- Barrett P, Bolborea M (2012). Molecular pathways involved in seasonal body weight and reproductive responses governed by melatonin. *J. Pineal Res.* 52:376-388.
- Boga JA, Coto-Montes A, Rosales-Corral SA, Tan DX, Reiter RJ (2012). Beneficial actions of melatonin in the management of viral infections: a new use for this "molecular handyman"? *Rev. Med. Virol.* 22:323-338.
- Cardinali DP, Esquifino AI, Srinivasan V, Pandi-Perumal SR (2008). Melatonin and the immune system in aging. *Neuroimmunomodulation* 15:272-278.
- Carrillo-Vico A, Lardone PJ, Alvarez-Sanchez N, Rodriguez-Rodriguez A, Guerrero JM (2013). Melatonin: buffering the immune system. *Int. J. Mol. Sci.* 14:8638-8683.
- Casbon AJ, Long ME, Dunn KW, Allen LAH, Dinauer MC (2012). Effects of IFN-gamma on intracellular trafficking and activity of macrophage NADPH oxidase flavocytochrome b(558). *J. Leukoc. Biol.* 92:869-882.
- Conlan JW (2011). Tularemia vaccines: recent developments and remaining hurdles. *Future Microbiol.* 6:391-405.
- Deregnacourt S, Saar S, Gahr M (2012). Melatonin affects the temporal pattern of vocal signatures in birds. *J. Pineal Res.* 53:245-258.
- Ekmekcioglu C (2006). Melatonin receptors in humans: biological role and clinical relevance. *Biomed. Pharmacother.* 60:97-108.
- Ellis J, Oyston PCF, Green M, Titball RW (2002). Tularemia. *Clin. Microbiol. Rev.* 15:631-646.
- Fares A (2013). Factors influencing the seasonal patterns of infectious diseases. *Int. J. Prev. Med.* 4:128-132.
- Galjasevic S, Abdulhamid I, Abu-Soud HM (2008). Melatonin is a potent inhibitor for myeloperoxidase. *Biochemistry* 47:2668-2677.
- Hasan S, Santhi N, Lazar AS, Slak A, Lo JN, von Schantz M, Archer SN, Johnston JD, Dijk DJ (2012). Assessment of circadian rhythms in humans: comparison of real-time fibroblast reporter imaging with plasma melatonin. *Faseb J.* 26:2414-2423.
- Hepburn MJ, Simpson AJH (2008). Tularemia: current diagnosis and treatment options. *Expert Rev. Anti-Infect. Ther.* 6:231-240.
- Hur SP, Takeuchi Y, Itoh H, Uchimura M, Takahashi K, Kang HC, Lee YD, Kim SJ, Takemura A (2012). Fish sleeping under sandy bottom: Interplay of melatonin and clock genes. *Gen. Comp. Endocrinol.* 177:37-45.
- Kim JY, Lee YD, Kim BJ, Kim SP, Kim DH, Jo KJ, Lee SK, Lee KH, Baik HW (2012). Melatonin improves inflammatory cytokine profiles in lung inflammation associated with sleep deprivation. *Mol. Med. Rep.* 5:1281-1284.
- Koh PO (2008). Melatonin regulates nitric oxide synthase expression in ischemic brain injury. *J. Vet. Med. Sci.* 70: 747-750.
- Koskela P, Salminen A (1985). Humoral immunity against *Francisella tularensis* after natural infection. *J. Clin. Microbiol.* 22:973-979.
- Laliena A, San Miguel B, Crespo I, Alvarez M, Gonzalez-Gallego J, Tunon MJ (2012). Melatonin attenuates inflammation and promotes regeneration in rabbits with fulminant hepatitis of viral origin. *J. Pineal Res.* 53:270-278.
- Lau WWI, Ng JKY, Lee MMK, Chan ASL, Wong YH (2012). Interleukin-6 autocrine signaling mediates melatonin MT1/2 receptor-induced STAT3 Tyr705 phosphorylation. *J. Pineal Res.* 52:477-489.
- Mahawar M, Atianand MK, Dotson RJ, Mora V, Rabadi SM, Metzger DW, Huntley JF, Harton JA, Malik M, Bakshi CS (2012). Identification of a novel *Francisella tularensis* factor required for intramacrophage survival and subversion of innate immune response. *J. Biol. Chem.* 287:25216-25229.
- Natarajan R, Einarsdottir E, Riuitta A, Hagman S, Raunio M, Mononen N, Lehtimäki T, Elovaara I (2012). Melatonin pathway genes are associated with progressive subtypes and disability status in multiple sclerosis among Finnish patients. *J. Neuroimmunol.* 250:106-110.
- Negi G, Kumar A, Sharma SS (2011). Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-kappa B and Nrf2 cascades. *J. Pineal Res.* 50:124-131.
- Nigrovic LE, Wingerter SL (2008). Tularemia. *Infect. Dis. Clin. North Am.* 22:489-504.
- Olegario JG, Silva MV, Machado JR, Rocha LP, Reis MS, Guimaraes CS, Correa RR (2013). Pulmonary innate immune response and melatonin receptors in the perinatal stress. *Clin. Dev. Immunol.* 2013:340959.
- Oliveira LGR, Kuehn CC, Santos CD, Toldo MPA, do Prado JC (2010). Enhanced protection by melatonin and meloxicam combination in experimental infection by *Trypanosoma cruzi*. *Parasite Immunol.* 32:245-251.
- Ono K, Mochizuki H, Ikeda T, Nihira T, Takasaki J, Teplow DB, Yamada M (2012). Effect of melatonin on alpha-synuclein self-assembly and cytotoxicity. *Neurobiol Aging* 33:2172-2185.
- Poplawski T, Chojnacki C, Czubatka A, Klupinska G, Chojnacki J, Blasiak J (2013). *Helicobacter pylori* infection and antioxidants can modulate the genotoxic effects of heterocyclic amines in gastric mucosa cells. *Mol. Biol. Rep. In press.*

- Pohanka M (2011). Alzheimer's disease and related neurodegenerative disorders: implication and counteracting of melatonin. *J. Appl. Biomed.* 9:185-196.
- Regodon S, Ramos A, Morgado S, Tarazona R, Martin-Palomino P, Rosado JA, Miguez Midel P (2009). Melatonin enhances the immune response to vaccination against A1 and C strains of *Dichelobacter nodosus*. *Vaccine* 27:1566-1570.
- Sharma J, Mares CA, Li Q, Morris EG, Teale JM (2011). Features of sepsis caused by pulmonary infection with *Francisella tularensis* Type A strain. *Microb. Pathog.* 51:39-47.
- Shen H, Harris G, Chen WX, Sjostedt A, Ryden P, Conlan W (2010). Molecular Immune Responses to Aerosol Challenge with *Francisella tularensis* in Mice Inoculated with Live Vaccine Candidates of Varying Efficacy. *PLoS One* 5(10): e13349.
- Srinivasan V, Spence DW, Trakht I, Pandi-Perumal SR, Cardinali DP, Maestroni GJ (2008). Immunomodulation by melatonin: Its significance for seasonally occurring diseases. *Neuroimmunomodulation* 15:93-101.
- Uguz AC, Cig B, Espino J, Bejarano I, Naziroglu M, Rodriguez AB, Pariente JA (2012). Melatonin potentiates chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor cells. *J. Pineal Res.* 53:91-98.
- Wang Z, Liu DX, Wang JT, Liu SM, Gao M, Ling EA, Hao AJ (2012). Cytoprotective effects of melatonin on astroglial cells subjected to palmitic acid treatment in vitro. *J. Pineal Res.* 52:253-264.
- Wu UI, Mai FD, Sheu JN, Chen LY, Liu YT, Huang HC, Chang HM (2011). Melatonin inhibits microglial activation, reduces pro-inflammatory cytokine levels, and rescues hippocampal neurons of adult rats with acute *Klebsiella pneumoniae* meningitis. *J. Pineal Res.* 50:159-170.
- Wu WS, Chou MT, Chao CM, Chang CK, Lin MT, Chang CP (2012). Melatonin reduces acute lung inflammation, edema, and hemorrhage in heatstroke rats. *Acta Pharmacol. Sin.* 33:775-782.
- Zhou HW, DeLoid G, Browning E, Gregory DJ, Tan FX, Bedugnis AS, Imrich A, Koziel H, Kramnik I, Lu Q, Kobzik L (2012). Genome-Wide RNAi Screen in IFN-gamma-Treated Human Macrophages Identifies Genes Mediating Resistance to the Intracellular Pathogen *Francisella tularensis*. *PLoS One* 7(2):e31752.
- Zhou LL, Wei W, Si JF, Yuan DP (2010). Regulatory effect of melatonin on cytokine disturbances in the pristane-induced lupus mice. *Mediators Inflamm.* 2010:ID951210.

Full Length Research Paper

Comparison of cytotoxic and genotoxic effects of the synthetic fungicide nimrod and the natural fungicide rhizo-N

Hala M. Mahfouz*, Hoda M. Barakat, Maher abd el fatah

Department of Botany, Faculty of Science, Ain Shams University, Cairo, Egypt.

Accepted 4 June, 2013

The present study was carried out to compare the mutagenic effects of the synthetic fungicide nimrod and the natural fungicide rhizo-N (produced by the bacterium *Bacillus subtilis*) on mitosis of *Allium cepa* cells. This study also concerned with the changes in seed protein profile after treatment with both pesticides using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Different concentrations for each fungicide were applied for 3, 6, 24, and 48 h. The obtained results indicate that the two fungicides caused reduction in mitotic activity and induced different types of mitotic abnormalities such as C-metaphase, laggard chromosomes, bridges, stickiness, and disturbed phases as well as micronuclei and multinucleate interphase cells. These changes appeared in varying degree depending on the duration of treatment and concentration applied. The results showed that the synthetic fungicide nimrod have more mutagenic potentialities than the natural fungicide. At electrophoretic level, these fungicides induced alterations in the protein banding patterns of *A. cepa* seeds as compared with untreated samples.

Key words: *Allium cepa*, mitotic division, chrosomal abnormality, *Bacillus subtilis*, pesticides, sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Chemical pesticides are widely used in Egypt and other countries to minimize the loss of economic crops due to pest invading. Although, pesticides have been useful in pest control and plant disease, there is a considerable risk on human health (Ergonen et al., 2005). The continuous production and use of these pesticides led to many side effects including mutagenic and / or carcinogenic effects in plant, animals, and human (Gopalan, 1999). Evidences accumulated in the past two decades have indicated that a large number of chemical pesticides have mutagenic effects and are capable of inducing chromosomal aberration or DNA damage in cells of different organisms (Singh, 2007; Asya et al., 2012; Mossa and Abbassy, 2012; Sibhghatulla et al., 2012). The danger of these pesticides is not necessary due to

direct contact, since it was found that some of these pesticides may accumulate in the food to a toxic level and therefore could be dangerous on the public health (Ergonen et al., 2005). Santovito et al. (2012) reported that human lymphocytes treated *in vitro* with the fungicide thiran showed a clastogenic effect with respect to residue limits found in some fruits and vegetables in Italy.

Man today is concerned very much with the pollution of his environment. Scientists began trying to use natural compounds to protect plants. Biological control agents are currently being used as alternatives to synthetic pesticides due to their perceived increased level of safety and minimal environmental impacts. Different species of plants, fungi, and bacteria are commercially applied as biological control agents against plant pathogen. The

*Corresponding author. E-mail: hala.mahfouz@yahoo.com; Tel: 01003022394.

extract of bacterium *Bacillus subtilis* is used for the control of a range of seedling fungal pathogens including *Fusarium spp.*, *Pythium spp.* and *Rhizoctonia spp* (Yoshihero et al., 2003). This bacterium is a common component of soil, being particularly abundant in the rhizosphere of germinating plants. This strain is selected for commercialization because of its effectiveness at controlling fungal infections.

A number of bioassay test system have been developed to monitor the action of pesticides and other environmental hazards on living organisms. Higher plants provide valuable genetic assay systems for screening and monitoring of environmental pollutants (Grant and Owens, 2006; Pesnya and Romanovsky, 2013). Plant genotoxicity assays are relatively inexpensive, fast and give reliable results. *Allium cepa* test provides a useful tool to estimate the genetic risk derived from an integrated exposure to different chemicals (Mustafa and Arikan, 2008; Yuzbasioglu et al., 2009; Asita and Matebesi, 2010). Chemicals, which cause chromosomal aberration (CA) in plant cells, also produce CA in cultured animal cells that are frequently identical (Santovito et al., 2012).

The present study was planned to compare the mutagenic effect of the biological pesticide (rhizo-N) which contain the bacterium *Bacillus subtilis* with the synthetic fungicide (nimrod). There is no available published data about the cytotoxic effect of the biological pesticide (rhizo-N). This study aims to investigate the effect of both pesticides on cell division and their capacity to induce chromosomal abnormalities in root tips of *A. cepa*, in addition, their effect on seed protein electrophoretic profiles.

MATERIALS AND METHODS

Bulbs of *A. cepa* (Giza 6) were kindly supplied by the Agricultural Research Center, Ministry of Agriculture, and Giza, Egypt. Two pesticides were used in the present study. Both fungicides have been kindly supplied by the General Administration of Pesticides, Ministry of Agriculture, Egypt. The chemical structure of synthetic fungicide (nimrod) is 5-butyl-2-ethyl amino-6 methyl pyrimidin-4-dimethylsulfamate and its molecular formula is $C_{13}H_{24}N_4O_3S$. The recommended dose is 7 cm³/L water. The biological fungicide rhizo-N contains 30 million living bacterium cells of *B. subtilis* per gram. The recommended dose is 4 gm/L water.

Cytological studies

Young, healthy and uniformed *A. cepa* bulbs were allowed to germinate in tap water. When the roots reached 2-3 cm long, the bulbs were transferred to new bottles which contained the test substances. The roots were treated for 3, 6, 24 and 48 h for each fungicide. Three bulbs were taken for each treatment and 3 meristematic roots were taken from each bulb (9 roots for each treatment). The root tips were then, fixed in Carnoy solution (3:1) absolute ethyl alcohol: acetic acid glacial for 24 h. Each series of experiments included a simultaneous control treated with tap water. After fixation, the roots were stored in 70% ethanol at 20-22°C until cytological analysis. Permanent preparations were made according

to Darlington and La-Cour (1976) using Feulgens quash technique. 90 fields were completely analyzed microscopically for each concentration. The frequencies of the different mitotic phases and the mitotic abnormalities after each treatment were statistically analyzed using (t-test).

$$\pm T = \left[\bar{X}_c - \bar{X}_t \right] \left[\sqrt{\frac{n(n-1)}{\sum (X_c - \bar{X}_c)^2 + \sum (X_t - \bar{X}_t)^2}} \right]$$

Where, c, Control; t, treated.

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{(n - 1)}}$$

Where S, Standard deviation.

$$SE = \frac{s}{\sqrt{n}}$$

Where, SE, Standard error.

Protein banding patterns electrophoresis

Total soluble proteins were extracted from M₂ seeds of *A. cepa* treated with the pesticides under study. Characterization of protein profiles was carried out using one dimensional sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli, 1970. Run was performed in 12% acrylamide slab gel at 15 mA till the tracing bromophenol blue dye reached the gel bottom. Gels were stained with Comassie blue R-250. The banding profile was analyzed.

RESULTS

Cytological studies

Results obtained from the present study showed that, both pesticides examined induced change in the frequency of mitotic phases that is accompanied by a decrease in mitotic index (MI) and appearance of different mitotic abnormalities. In general, there is an inverse correlation between the frequency of prophase and that of metaphase (Table 1). The results indicated that all treatment with the synthetic fungicide nimrod induced marked reduction in mitotic activity. At long treatments for 48 h there was an obvious decrease in MI as compared with those treated for 3, 6 and 24 h. Such reduction was clearly dose and time dependent and showed statistical significant effect in roots treated with most concentrations (Table 1 and Figure 1A). On the other hand, the biological fungicide rhizo-N showed reduction in mitotic index in roots treated with different concentrations for 3, 6, 24 and 48 h as compared with the control. This reduction increased gradually with the increase of the concentrations applied; but there was no clear decrease in mitotic index values with increasing

Table 1. Number and percentage of different mitotic phases and mitotic index after treatment *Allium cepa* roots with rhizo-N and nimrod fungicides.

Time	Conc	Rhizo-N						MI±SE (%)	Nimrod						MI±SE (%)
		Prophase		Metaphase		Anaphas Telophase			Prophase		Metaphase		Anaphase Telophase		
		Number	%	Number	%	Number	%		Number	%	Number	%	Number	%	
3	Cont.	163	49.70	95	28.96	70	21.34	4.67±0.50	310	58.38	129	24.29	92	17.33	3.89±0.69
	0.312	96	41.20	87	37.34	50	21.46	3.30±0.15	261	54.15	115	23.86	106	21.99	2.48±0.69
	0.625	87	38.67	89	39.56	49	21.78	2.61±0.15	103	34.11	128	42.38	71	23.51	1.63±0.47
	1.25	63	28.90	96	44.04	59	27.06	2.46±0.12	24	15.29	79	50.32	54	34.39	1.60±0.20*
	2.50	42	20.00	102	48.57	66	31.43	2.33±0.03	12	9.09	77	58.33	43	32.58	1.30±0.39**
6	Cont.	163	47.66	109	31.87	70	20.46	4.02±0.30	251	53.29	115	24.41	105	22.29	3.15±0.70
	0.312	91	37.45	93	38.27	59	24.28	2.99±0.04	139	53.26	69	26.44	53	20.31	1.57±0.54
	0.625	79	34.50	99	43.23	51	22.27	2.43±0.01	86	35.98	104	43.51	49	20.50	1.29±0.50
	1.25	54	24.66	105	47.94	60	27.40	2.42±0.04	22	11.83	129	69.35	35	18.82	1.20±0.28*
	2.50	38	17.76	112	52.34	64	29.91	2.40±0.03	9	5.14	138	78.86	28	16.00	1.13±0.45**
24	Cont.	149	42.10	103	29.10	102	28.81	3.96±0.30	213	48.97	110	25.29	112	25.75	3.04±0.4
	0.312	82	32.67	113	45.00	56	22.31	2.98±0.08	65	44.83	47	32.41	33	22.76	1.51±0.17*
	0.625	60	25.42	121	51.27	55	23.31	2.42±0.04	29	22.66	72	56.25	27	21.09	1.23±0.22**
	1.25	49	20.16	137	56.38	57	23.46	2.48±0.04	12	9.76	84	68.29	27	21.95	1.06±0.08**
	2.50	32	13.33	149	62.08	59	24.58	2.49±0.06*	3	2.75	88	80.73	18	16.51	0.87±0.07**
48	Cont.	161	43.63	91	24.66	117	31.71	3.76±0.20	193	49.74	100	25.77	95	24.48	3.08±0.06
	0.312	78	30.12	115	44.40	66	25.48	2.89±0.08	36	27.27	65	49.24	31	23.48	1.39±0.12**
	0.625	48	19.75	136	55.97	59	24.28	2.39±0.03	11	9.57	79	68.69	25	21.74	1.10±0.04**
	1.25	41	17.37	139	58.90	56	23.73	2.32±0.04*	6	5.83	81	78.64	16	15.53	0.96±0.03**
	2.50	30	13.16	152	66.67	46	20.18	2.24±0.03**	2	1.98	92	91.09	7	6.93	0.85±0.06**

*Significant from control at 0.05 level (t-test). **Significant from control at 0.01 level (t-test). Conc = concentration, Cont = control.

time of treatment. The statistical analysis of the data reveals that all treatments for 3 and 6 h with the biocide rhizo-N had no significant effect on MI. Only, the highest concentrations applied for 24 and 48 h and the concentration 1.25 gm/L applied for 48 h induced a significant or highly significant decrease in MI value (Table 1 and Figure 2A).

This inhibition of mitotic index may be due to the interference of fungicides with normal process of division by reducing the number of the cell entering mitosis. In the roots treated for 48 h with

the highest concentration of nimrod, the percentage of prophase decreased to the extent that they recorded 1.98% as compared with the control value which was recorded 49.74 (Table 1). It can be concluded that the synthetic fungicide nimrod is more effective in inducing reduction in mitotic activity accompanied with high percentage of chromosomal abnormalities as compared with the natural fungicide rhizo -N (Tables 2 and 3 and Figures 1B and 2B). Treatment of root tips cells of *A. cepa* with the two fungicides produced different

types of chromosomal abnormalities such as C-metaphase, disturbed, laggard chromosomes, chromosome bridge, stickiness, micronuclei and multinucleate (Tables 2 and 3 and Figures 3 and 4). The frequency of total abnormalities produced by nimrod increased as the concentration and the period of treatment was increased (Table 2). On the other hand, the percentages of total abnormalities produced by natural fungicide are slightly increased with the increasing concentration and duration of treatments (Table 3). The

Table 2. Frequency of micronuclei, abnormal mitotic phases and total abnormal mitosis after treatment *Allium cepa* roots with nimrod fungicide.

Time	Conc	Micronuclei	Prophase	Metaphase							Anaphase-telophase				% of total abnormal mitoses \pm SE		
				Stickiness	C-M (2n)	C-M (4n)	Star	Disturbed	Laggards	Total	Stick	Bridge	Disturbed	Multipolar		Laggards	Total
3	Cont.	-	--	--	--	--	--	--	--	--	--	--	1.09	--	--	1.09	0.18 \pm 0.03
	0.312	0.16	0.77	--	29.99	--	--	11.74	--	41.73	--	--	22.50	1.61	7.02	31.13	17.22 \pm 0.25*
	0.625	0.36	8.73	6.25	45.31	--	--	11.72	--	63.28	15.63	14.13	12.82	7.41	2.12	52.11	42.05 \pm 0.21**
	1.25	-	--	37.85	26.7	--	2.53	15.19	--	82.27	23.32	20.37	11.86	5.56	--	61.11	62.42 \pm 0.43**
	2.50	--	--	74.04	5.19	--	6.49	--	--	92.21	45.58	17.21	4.65	2.33	--	69.77	78.78 \pm 0.36**
6	Cont.	--	0.39	--	--	--	--	1.73	--	1.73	--	--	--	--	--	--	0.64 \pm 0.07
	0.312	0.36	2.15	--	11.12	--	2.90	18.90	6.21	39.13	--	2.55	18.18	3.89	18.77	43.39	20.31 \pm 0.39*
	0.625	0.48	2.32	5.77	26.35	--	1.92	28.43	5.80	68.27	16.33	12.24	14.08	10.20	10.41	63.26	43.51 \pm 0.41**
	1.25	0.54	9.09	18.53	30.58	--	5.43	19.38	2.82	76.74	38.57	20.00	14.29	11.45	4.26	88.57	70.96 \pm 0.11**
	2.50	--	22.22	53.62	13.05	--	10.87	15.94	--	93.48	46.43	46.43	--	3.56	--	96.42	90.29 \pm 0.16**
24	Cont.	--	0.46	--	--	--	--	0.91	--	0.91	--	--	--	--	--	--	0.46 \pm 0.02
	0.312	0.49	3.08	2.32	22.94	3.32	8.77	27.80	2.94	68.09	3.11	3.11	23.04	8.34	19.97	57.57	36.55 \pm 0.05**
	0.625	0.25	--	24.89	21.11	--	8.45	12.78	2.21	69.44	28.15	29.63	12.20	--	7.80	77.78	55.47 \pm 0.11**
	1.25	-	--	37.76	21.38	2.23	15.24	3.61	3.11	83.33	38.15	24.81	7.41	11.11	--	81.48	74.80 \pm 0.06**
	2.50	-	--	51.14	19.08	1.14	18.41	3.41	1.14	94.32	57.78	31.10	--	5.56	--	94.44	91.74 \pm 0.07**
48	Cont.	--	--	--	--	--	--	2.00	--	2.00	--	--	2.10	--	--	2.10	1.03 \pm 0.03
	0.312	0.64	--	1.15	22.23	0.54	13.25	20.57	2.26	60.00	--	8.57	22.38	14.28	19.29	64.52	44.70 \pm 0.19**
	0.625	0.36	--	26.11	6.11	--	11.39	18.99	3.22	65.82	42.00	34.00	10.00	4.00	6.00	96.00	66.09 \pm 0.35**
	1.25	0.25	--	34.57	11.11	3.70	19.76	11.11	--	80.25	87.50	12.50	--	--	--	100.0	78.64 \pm 0.14**
	2.50	-	--	66.52	9.57	9.78	13.04	--	--	98.91	90.00	10.00	--	--	--	100.0	97.03 \pm 0.07**

*Significant from control at 0.05 level (t-test). **Significant from control at 0.01 level (t-test).

scored values of abnormalities induced by the natural fungicide were generally low as compared with that of the chemical fungicide (Figures 1B and 2B).

SDS-PAGE studies

The SDS-PAGE protein profiles of M_2 seeds of *A. cepa* plants treated with the nimrod and rizho-N fungicides are illustrated in Figure 5 and Table 4. The total number of the protein bands recorded was 18 bands. The protein patterns showed variations among the investigated samples includ-

ing the appearance of new bands, disappearance of some bands and changes in band intensity (Table 4). The synthetic fungicide nimrod induced 5 new bands and 4 bands disappeared. The treatment with the natural fungicide rizho-N induced only 1 new band with molecular weight of KDa (Table 4 and Figure 5).

DISCUSSION

Many investigators attributed the inhibition of mitotic activity to blocking of mitotic cycle and accumulation of cells at G_1 or G_2 periods due to

the inhibition of nuclear protein synthesis required for the progress of cell cycle (Polit et al., 2003; Cvikrova et al., 2003). Binarova et al. (1998) showed that treatment of *Vicia faba* root tip cells with specific inhibitors to cyclin - dependent kinases leads to abnormal spindle formation; arrest cells at the G_1/S and G_2/M regulatory points; decrease cyclin A and B levels as well as cdc2 kinase indicating the role of this enzyme. This view is in agreement with the result obtained from the present study since the two fungicides led to appearance of c-metaphase and disturbed mitotic phases produced from abnormal spindle formation in addition the disappearance of some

Table 3. Frequency of micronuclei abnormal mitotic phases and total abnormal mitosis after treatment *Allium cepa* roots with rhizo-N fungicide.

Time	Conc	Micronuclei	Prophase%	Metaphase %						Anaphase - telophase%						% of total abnormal mitoses ± SE	
				Stickiness	C-M (2n)	C-M (4n)	Star	Disturbed	Laggards	total	Stickiness	Bridge	Disturbed	Multipolar	Laggards		Total
3	Cont.		--	--	--	--	--	1.05	--	1.05	--	--	1.43	--	--	1.43	0.61±0.60
	0.312	0.04	1.04	--	--	--	--	3.40	1.20	4.60	--	--	3.00	--	1.00	4.00	3.00±1.02
	0.625	0.06	2.30	--	2.24	--	--	11.28	2.21	15.73	--	2.04	3.04	--	2.04	8.16	8.89±1.04
	1.25		1.59	1.04	12.08	--	--	12.92	--	26.04	8.48	5.08	3.39	1.04	--	16.95	16.51±1.03
	2.50		2.38	19.61	4.90	--	--	4.90	--	29.41	15.15	4.55	3.03	--	--	22.73	21.90±0.80
6	Cont.		--	--	--	--	--	0.92	--	0.92	--	--	1.43	--	--	1.43	0.58±0.70
	0.312	0.06	2.20	--	--	--	--	7.52	1.08	8.60	--	--	1.69	--	1.70	3.39	4.94±1.08
	0.625	0.08	3.80	2.02	2.02	--	1.01	13.14	2.01	20.20	1.96	3.92	3.36	1.20	1.32	11.76	12.66±1.07
	1.25	0.07	1.85	9.53	7.70	2.76	--	9.53	--	29.52	11.22	6.67	--	1.67	2.11	21.67	20.55±0.50
	2.50	-	2.63	30.14	3.79	--	--	--	--	33.93	15.63	7.81	--	--	--	23.44	25.23±0.50
24	Cont.		0.67	--	--	--	--	1.94	--	1.94	--	--	0.98	--	--	0.98	1.13±0.60
	0.312	0.07	1.22	--	--	--	1.77	16.34	2.24	20.35	--	--	14.27	--	5.37	19.64	13.94±0.40
	0.625	0.05	1.66	3.31	0.82	--	2.48	28.11	4.12	38.84	5.46	1.82	18.18	1.82	3.63	30.91	27.54±0.90
	1.25	-	4.08	20.50	13.30	--	--	14.38	--	48.18	17.54	15.80	5.26	5.26	--	43.86	38.27±1.04
	2.50	-	6.25	43.70	15.36	--	--	--	--	59.06	25.43	23.73	1.69	3.39	--	54.24	50.38±1.06*
48	Cont.	-	1.24	--	--	--	--	3.29	--	3.29	--	--	0.85	--	--	0.85	1.63±1.30
	0.312	-	2.56	--	6.96	--	3.47	20.00	--	30.43	--	--	25.75	--	1.52	27.27	21.24±0.60
	0.625	0.08	--	7.35	8.09	--	4.22	26.66	--	46.32	10.22	10.17	10.13	1.69	1.69	33.90	34.16±0.60
	1.25	0.06	--	24.46	10.07	3.60	5.04	20.14	--	63.31	20.23	14.28	4.23	5.36	2.32	46.42	48.31±0.80*
	2.50	0.05	6.67	38.04	17.89	7.89	--	--	--	63.82	47.83	28.26	--	--	--	76.09	58.77±0.70**

protein bands in SDS-PAGE profile. These results indicate the potentiality of the investigated fungicides to induce mitotic irregularities, which are in agreement with the results of many investigators studying the genotoxic effect of different pesticides or other agents (Asita and Makhalemele, 2009; Asita and Matebesi, 2010; Asya et al., 2012; Sibghatulla et al, 2012; Sarah et al., 2013). The inhibition of mitotic division may be also attributed to induction of large number of mitotic abnormalities. Mendhulkar (1993) attributed plant inhibition to disturbance in natural growth regulators and mitotic chromosomal irregularities as additional factors.

The most common type of aberration observed after treatments with the two pesticides nimrod

and rhizo-N was C-metaphase. Such type of abnormalities causes inhibition of spindle fiber formation by their action on microtubules, which play the major role in the formation of spindle fibers. On the other hand, the induction of disturbed mitotic configurations by these fungicides may be due to partial inhibition on spindle fiber that affects the orientation of these chromosomes at the equatorial plate. Lagging chromosomes appeared at metaphase, anaphase, and telophase stages. The induction of laggard could be attributed to disturbance in the mechanism of chromosomes movement.

Induction of chromosomal and chromatin bridges at anaphase and telophase stages were also observed. Bridges may be result from chro-

mosome stickiness or from breakage followed by reunion. In the present investigation, occurrence of bridges may be due to stickiness rather than chromosome breakage and reunion. Stickiness appeared in different mitotic phases after treatment roots with higher concentrations of both fungicides. Such stickiness led to the appearance of chromatin masses where the general appearance of chromosomes is lost. Micronuclei and multinucleated cells were observed in the interphase cells. Micronuclei are true mutagenic aspects, which may lead to a loss of genetic material (Fernandes et al., 2007). The formation of multinucleated cells may be result from a preceding multipolar mitosis. Several investigators studying the effect of different pesticides or

Table 4. Effects of the fungicide nimrod and the natural fungicide rhizo-N on the protein banding pattern of *Allium cepa* seeds using SDS – PAGE technique.

Band No.	M.wt (KDa)	Control	Rhizo-N				Nimrod			
			Lane 1	Lane 2	Lane 3	Lane 4	Lane 1	Lane 2	Lane 3	Lane 4
1	72	-	+	+	+	+	-	-	-	-
2	68	+	+	+	+	+	+	+	+	+
3	64	+	+	+	+	+	+	+	+	+
4	52	-	-	-	-	-	+	+	+	+
5	49	++	++	++	++	++	-	-	-	-
6	48	++	++	++	++	++	++	++	++	++
7	44	+	+	+	+	+	++	++	++	++
8	42	+	+	+	+	+	+	+	+	+
9	40	-	-	-	-	-	+	+	+	+
10	35	++	++	++	++	++	+	+	+	+
11	34	+	+	+	+	+	-	-	-	-
12	33	++	++	++	++	++	++	++	++	++
13	32	+	+	+	+	+	-	-	-	-
14	30	+	+	+	+	+	+	+	+	+
15	29	+	+	+	+	+	+	+	+	-
16	20	-	-	-	-	-	+	+	+	+
17	12	-	-	-	-	-	+	+	+	-
18	10	-	-	-	-	-	+	+	+	-
Total		12	12	12	12	12	14	14	14	11

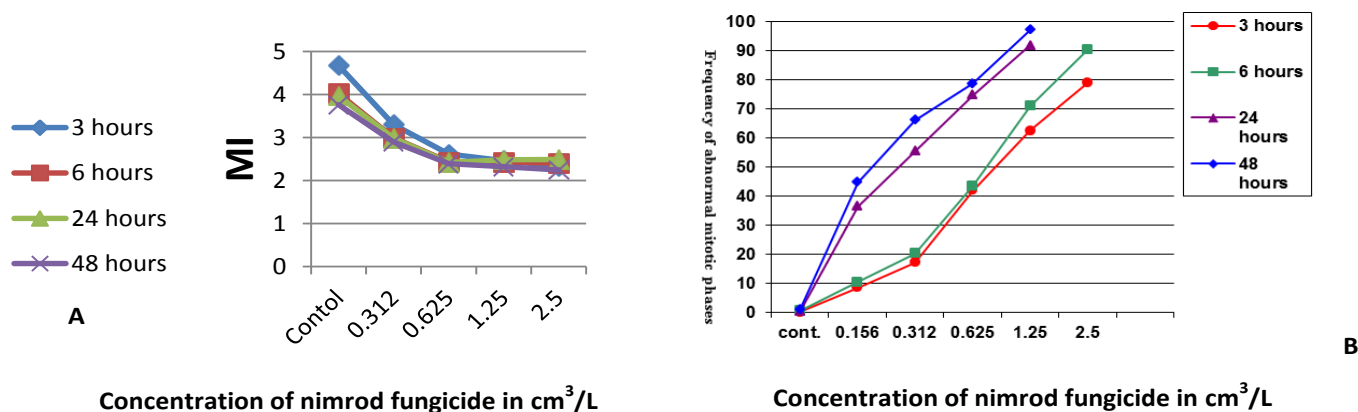


Figure 1. Mitotic index (A) and mean percentage of abnormal mitosis (B) after treating *Allium cepa* root tips with different concentrations of nimrod fungicide for 3, 6, 24, 84 h

chemicals obtained similar types of mitotic abnormalities (Tartar et al., 2006; Lamsal, et al., 2010; Niraikulam et al., 2013; Ozlem et al., 2013; Sarah et al., 2013).

The appearance of new bands may be due to mutational events at the regulatory system of an unexpressed gene(s) that activate it. The disappearance of some bands could be explained on the basis of mutational event at the regulatory genes that prevent transcription. Induction of laggards, micronuclei by these pesticides may lead to the loss of genetic materials. Therefore, some electrophoretic bands were disappeared due to the

loss of their corresponding genes. Similar results were obtained by Prasad and Zha (1992) and George and Ghareeb (2001).

Conclusion

A drastic inhibition in mitotic division was clearly observed in *A. cepa* root tips treated with the chemical fungicides nimrod; such reduction increased as the concentrations and the duration of treatments increased.

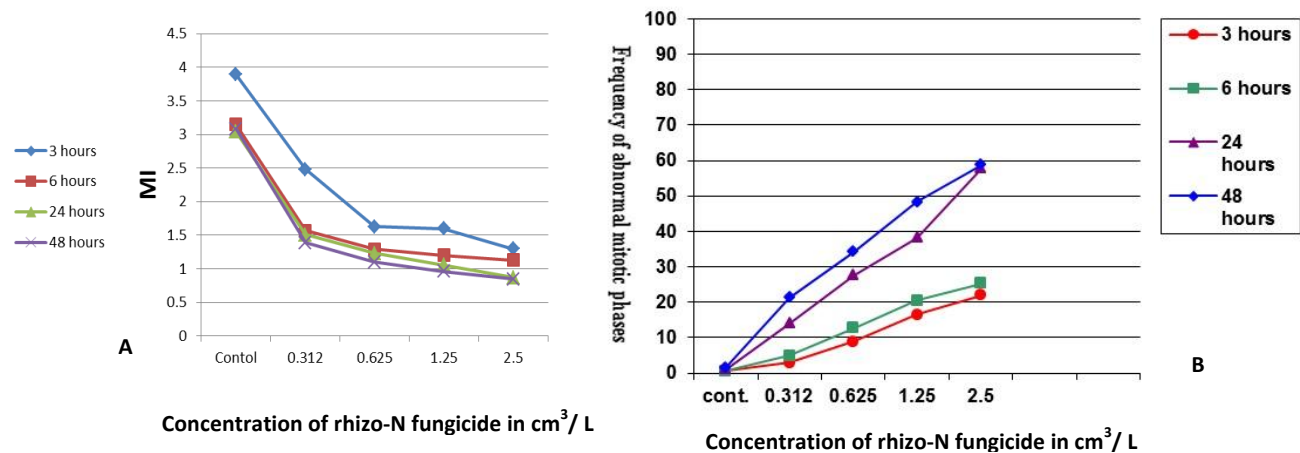


Figure 2. Mitotic index (A) and mean percentage of abnormal mitosis (B) after treating *Allium cepa* root tips with different concentrations of rhizo-N fungicide for 3, 6, 24, 84 h.

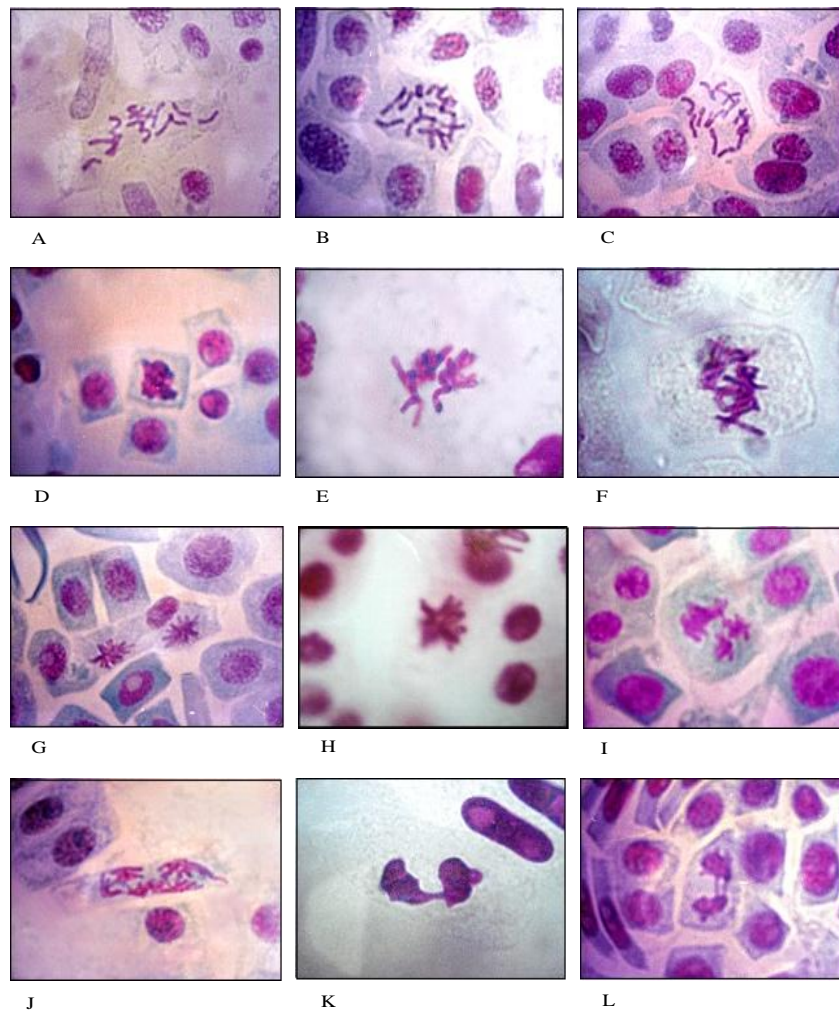


Figure 3. Some types of chromosomal abnormalities after treating *A. cepa* root tips with different concentrations of nimrod. a, b and c, C-Metaphase; d, sticky metaphase; e and f, disturbed metaphase; g and h, star metaphase; i and j, tripolar and multipolar anaphase; k and l, telophase bridge.

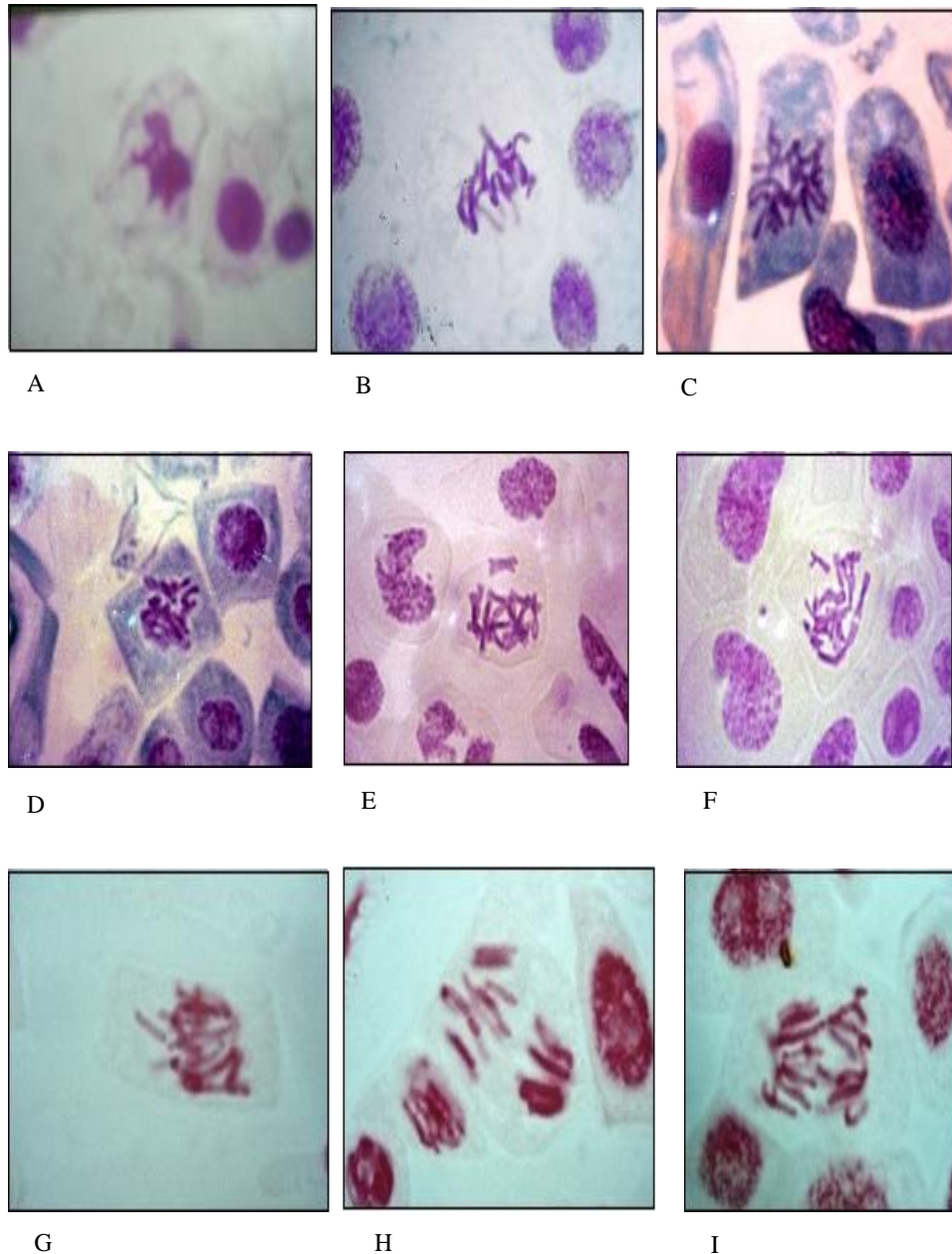


Figure 4. Some types of chromosomal abnormalities after treating *Allium cepa* root tips with different concentrations of natural fungicide rhizo-N. a, Stickiness; b, c and d, disturbed metaphase; e and f, C-metaphase; g, disturbed metaphase with lagging chromosome; h, disturbed anaphase; i, disturbed anaphase with bridge.

The natural fungicide rhizo-N induced reduction in mitotic activity with the increase of concentrations, but there is no clear increase in reduction of mitotic index percentage as duration of treatment increased. On the other hand, the scored values of the induced abnormalities after treatment with the rhizo-N were low as compared with that scored after treatments with the chemical fungicides nimrod. This means that the chemical fungicides are more effective in inducing mitotic abnormalities than that of the biological fungicide. The genotoxicity of the

synthetic fungicide nimrod, as indicated by their capacity to produce chromosomal aberrations, was confirmed by their effect on cell cycle phases as well as protein banding pattern and they are more dangerous than the biological fungicides rhizo-N. The result of the present investigation, point out the importance of taking proper measure in order to avoid contamination with these pollutants. The toxic effect of the synthetic fungicides has created a demand for new environmentally safe fungicides.

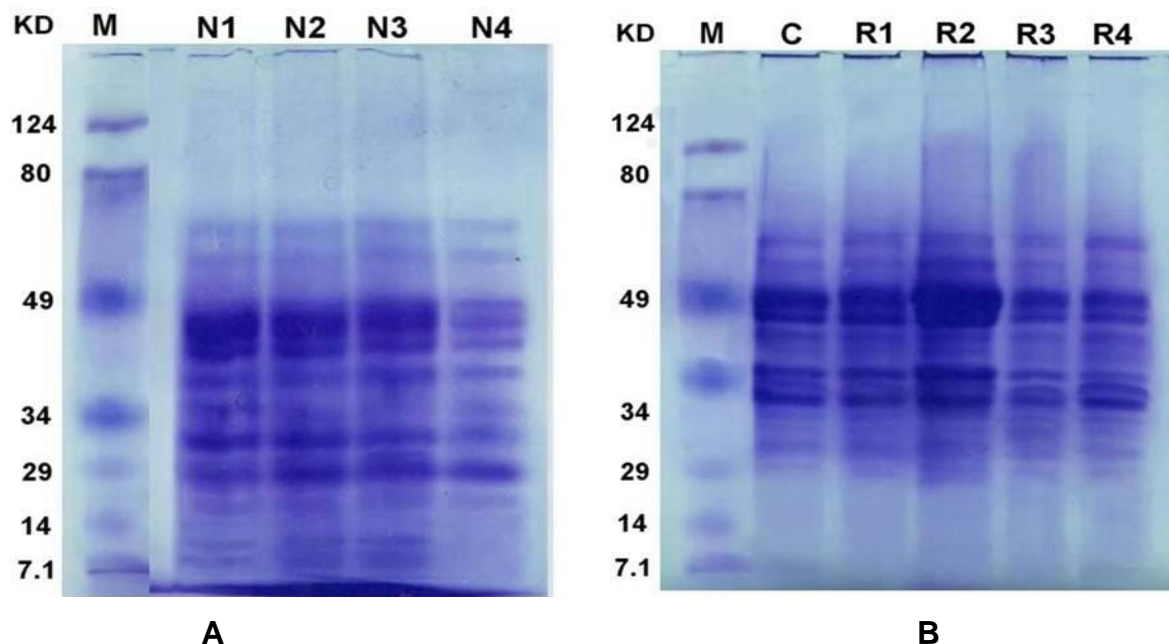


Figure 5. Electrophotographs produced by SDS-PAGE analysis of protein patterns of *Allium cepa* seeds after treatment with different concentrations of nimrod (A) and rizho-N (B) fungicides. M, Marker; C, control; Lane 1, 0.312%; Lane 2, 0.625%; Lane 3, 1.25%; Lane 4, 2.5%.

ACKNOWLEDGEMENTS

We are very grateful to Prof .Dr .Hoda Barakat and Prof .Dr. Soheir El Khodary (Professor of Cytogenetic in Ain Shams University, Faculty of Science, Botany Department) for their valuable suggestions and encouragement.

REFERENCES

- Asita AO, Makhalemele R (2009). Genotoxic effects of dithane, malathion and garden ripcord on onion root tip cells. *Afr. J. Food Agric. Nutr. Dev.* 9(4):1191-1209.
- Asita AO, Matebesi LP (2010). Genotoxicity of hormoban and seven other pesticides to onion root tip meristematic cells. *Afr. J. Biotechnol.* (9):4225-4232.
- Asya D, Vanya K, Nurzhihan H, Stoicho S (2012). Cytotoxic and Genotoxic Effects of Diphenyl-ether Herbicide GOAL (Oxyfluorfen) using the *Allium cepa* test. *Res. J. Mutag.* 2:1-9.
- Binarova P, Dolezel J, Draber p, Heberle-Bors E, Strnad M, Bogre L (1998). Treatment of *Vicia faba* root tip cells with specific inhibitors to cyclin - dependent kinases leads to abnormal spindle formation. *Plant J.* 16(6):697-707.
- Cvikrova M, Binarova P, Cencklova V, Eder J, Dolezel J, Machackova I (2003). Effect of 2-aminoindan-2-phosphonic acid on cell cycle progression in synchronous meristematic cells of *Vicia faba* roots. *Plant Sci.* 164:823-832.
- Darlington CD, La-Cour LF (1976). The handling of chromosomes, sixth ed. George Allene and Unwin, LTD. London.
- Ergonen AT, Salacin S, Ozdemir H (2005). Pesticides use among greenhouse works in Turkey. *J. Clin. Foren. Med.* 12:205-208.
- Fernandes TC, Mazzeo DE, Marin-Morales MA (2007). Mechanism of micronuclei formation in polyploidized cells of *Allium cepa* exposed to trifluralin herbicide. *Pestic. Biochem. Phys.* 88:252-259.
- George NM, Ghareeb A (2001). Genotoxicity of insecticide cyolan on mitosis, meiosis and seed storage proteins of *Vicia faba*. *Cytologia* 66:77-84.
- Gopalan HN (1999). Ecosystem health and human wellbeing: the mission of the international programme on plant bioassays. *Mutat. Res.* (426):99-102.
- Grant WF, Owens ET (2006). *Zea mays* assays of chemical/radiation genotoxicity for the study of environmental mutagens. *Mutat. Res.* 613(1):17-64.
- Laemmli UK (1970). Cleavage of structural proteins during assembly of head bacteriophage T4. *Nature* 227:680-685.
- Lamsal K, Ghimire BK, Sharma P, Ghimirar AK, Kim SW, Yu CY, Chung M, Lee YS, Kim JS, Shakya SR (2010). Genotoxicity evaluation of the insecticide ethion in root of *Allium cepa* L. *Afr. J. Biotechnol.* 9:4204-4210.
- Niraikulam A, Natarajan V, Kannan S, Jansirani D, Abdulrahman A (2013). Evaluation of cytotoxic properties of *Curcuma longa* and *Tagetes erecta* on cancer cell line (Hep2). *Afr. J. Pharm. Pharmacol.* 7(14):736-739.
- Mendhulkar VD (1993). Effect of chemical mutagens in *Desmodium totuosum*. Ph. D. thesis, Kamatak University, Dharvad, India.
- Mossa AH, Abbassy M (2012). Adverse haematological and biochemical effects of certain formulated insecticides in male rats. *Res. J. Environ. Toxicol.* 6:160-168.
- Mustafa Y, Arikan ES (2008). Genotoxicity testing of quizalofop-P-ethyl herbicide using the *Allium cepa* anaphase-telophase chromosome aberration assay. *Caryologia* 61:45-52.
- Ozlem SA, Tülay A (2013). Investigation of antioxidant, cytotoxic and apoptotic activities of the extracts from tubers of *Asphodelus aestivus* Brot. *Afr. J. Pharm. Pharmacol.* 7(11):610-621.
- Pesnya DS, Romanovsky AV (2013). Comparison of cytotoxic and genotoxic effects of plutonium-239 alpha particles and mobile phone GSM 900 radiation in the *Allium cepa* test. *Mutat. Res.* 750:27-33.
- Prasad AB, Zha AM (1992). Mutation effecting seed coat and seed proteins in *Phaseolus vulgaris* L. *J. Cytol. Genet.* 27:147-152.
- Polit JT, Maszewski J, Kazmierczak A (2003). Effect of BAP and IAA on the expression of G1 and G2 control points and G1-S and G2-M transitions in root meristem cells of *Vicia faba*. *Cell Biol. Int.* 27:559-566.

- Santovito A, Cervella P, Delpero M (2012). Chromosomal aberration in cultured human lymphocytes treated with the fungicide Thiram. *Drug Chemotoxicol.* 35(3):347-351.
- Sarah KN, Andy O, Francis BL, Adebola OO, Moganavelli S (2013). Chemical composition, antioxidant activity and cytotoxicity of the essential oils of the leaves and stem of *Tarchoanthus camphorates*. *Afr. J. Pharm. Pharmacol.* 7(7):360-367.
- Sibhghatulla S, Nazia N, Mohammad I, Waseem A (2012). Dichlorophen and Dichlorovos mediated genotoxic and cytotoxic assessment on root meristem cells of *Allium cepa*. *Sci. Diliman* 24(1):13-22.
- Singh P (2007). Comparative sensitivity of barely (*Hordeumvulgare* L.) to insecticide and fungicide on different stages of cell cycle. *Pesticide Biochem. Physiol.* 89(3):216-219.
- Tartar G, Kaymak F, Gokalp FM (2006). Genotoxic effects of avenoxan on *Allium cepa* L. and *Allium sativum* L. *Caryologia* 59:241-247.
- Yoshihero I, Mitsuo H, Hayato H, Futoshi K (2003). Biological control of rice blast disease by *Bacillus subtilis*. *Aannals of Phytopatholsoc Japan.* 69(2):85-93.
- Yuzbasioglu DF, Unal C, Sanca k (2009). Genotoxic effects of herbicide Illoxan (Diclofop-Methyl) on *Allium cepa* L. *Turk. J. Biol.* (33):283-290.

Full Length Research Paper

Academic dishonesty among Nigeria pharmacy students: A comparison with United Kingdom

Ubaka Chukwuemeka^{1*}, Fajemirokun Gbenga², Nduka Sunday³, and Ezenwanne Ndidiamaka¹

¹Department of Clinical Pharmacy and Pharmacy Management, University of Nigeria, Nsukka, Enugu State, Nigeria.

²Department of Clinical Pharmacy, Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria.

³Department of Clinical Pharmacy, Nnamdi Azikiwe University, Agulu, Anambra State, Nigeria.

Accepted 26 June, 2013

Professionalism and integrity are integral attributes of the pharmacy profession. This study was set out to determine the perceptions towards and actual indulgence in academic dishonesty among Nigerian Pharmacy students and to compare these with results obtained in the United Kingdom. Final year and third year pharmacy students of University of Nigeria (UNN) and Olabisi Onabanjo University (OOU), all in Nigeria, were asked to complete a survey instrument consisting of 16 activities considered to be cheating. Data on perceptions and previous indulgence in such activities were collected and analysed with appropriate statistical tools. Three hundred and sixty six students participated (overall participation rate, 72.8%; UNN: n=216, 59.0%; OOU: n=150, 41%) in the study while male and female students were distributed almost equally (male: 48.6% and female: 51.4%). More students cheated in their coursework than in examination (54.2 vs. 45.8%, $p<0.05$), while significantly more final year students in OOU indulged in cheating (74.6 vs. 62.1%, $p<0.05$). Overall, significantly more students in UNN cheated than those in OOU (81.5 vs. 68%, $p=0.002$). In comparison with students of University of Portsmouth, proportionally more Nigerian students participated in all eleven selected scenarios than UK students. Nigerian pharmacy students generally have a poor perception towards academic dishonesty and acts of such dishonesty are prevalent among these students.

Key words: Academic dishonesty, Nigeria, pharmacy practice, professionalism.

INTRODUCTION

Pharmacy as a profession prides itself on high ethical standards and integrity. These qualities endear this profession to patients and other healthcare providers enabling trust to be built upon and subsequently mutual benefits achieved by both parties. Integrity does matter in both learning and practice. If there lacks integrity in the learning (education) process, integrity during practice may suffer adversely and studies have either perceived or demonstrated this association (Taradi et al., 2009; Turrens et al., 2001).

Academic dishonesty (also known as cheating) has been defined by Storch and Storch (2002), to be "the act of giving or receiving unauthorized assistance in an

academic task or receiving credit for plagiarized work". There is evidence that academic dishonesty is on the rise among pharmacy students in developed economies across Europe and North America (Harries and Rutter, 2005; Austin et al., 2005). Findings from such studies have shown that as much as 50-90% of students in some schools of pharmacy have said to have cheated in at least an examination or class work exercise. Some factors have been pointed to promote cheating among students, some of which may be applicable to a setting as ours (Africa). One of such factors is the size of class and number of tutors. Large classes with proportionally fewer tutors, often the case in developing countries, has

*Corresponding author. E-mail: pharmubk@yahoo.com; Tel.: +234 8038246913.

been shown to be favourable to student cheating as students believe they are less likely to be caught in such "disorganised" setting (McCabe and Trevino, 1996). Another study highlighted students' poor awareness of the contents institutional policies on plagiarism and cheating and their effect on continued cheating behaviours (Ryan et al, 2009). The gender of the student has also been reported to play a role in involvement in academic dishonesty, though most of such studies have been largely conflicting. However two interesting studies have significantly identified the male gender as culprit in most incidences of academic dishonesty (Norton et al., 2002; Aggarwal et al., 2002). A study conducted in the US however showed a declining prevalence in cheating as students progressed through the curriculum/professional classes (Hardigan and Ranelli, 2006). Some schools of pharmacy have employed strategies to curb the high prevalence of academic dishonesty among its students, particularly with the use of examination proctors and anti-plagiarism software but these are not within the reach of schools in developing countries (Paiscik and Brazeau, 2010). Even if they eventually became available, their implementation and effective management could be yet another hurdle to climb.

To our knowledge there is no published evidence on the prevalence of cheating among students in any Nigeria school of pharmacy. This study was then conducted to fulfil the following objectives; 1) to evaluate the perceptions of pharmacy students towards academic dishonesty 2) to identify the level of indulgence in cheating among these students 3) to compare these perceptions and indulgence between pharmacy students in Nigeria and those of the United Kingdom.

MATERIALS AND METHODS

Study design

This study is a cross-sectional and descriptive survey designed to assess the perceptions and self-reported prevalence of cheating behaviours among Nigerian pharmacy students and compare them with those of a school of pharmacy in the United Kingdom. The survey in Nigeria included two schools of pharmacy conveniently sampled so as to compare institutional differences. Two professional classes were also used to note if academic progression had any effect on cheating behaviours.

Study site

The study was conducted in two accredited schools of pharmacy in Nigeria, one located at the Southern region and the other at the Eastern region. As at the time of this study, there were eleven council-accredited schools of pharmacy scattered all over Nigeria. The first used in this study; University of Nigeria Nsukka (UNN), is Nigeria's first indigenous university and its school of pharmacy graduates an average of 170 students annually. It is located in the ancient city of Enugu in South eastern Nigeria and it attracts nearly 4,000 applicants for its pharmacy program while accepting only an average of 200 candidates every year. The school of pharmacy in UNN utilizes the "resit-repeat" curriculum where unsuccessful students after a professional examination may have to re-take an

examination or repeat a class. The second university, Olabisi Onabanjo University, Shagamu (OOU), is located in Ogun State. It is a newer school of pharmacy receiving both the Council and Universities' Commission accreditation in 1994. It receives just over 600 applications yearly and it graduates an average of 90 students per year.

Study sample

Third and final year students of both schools of pharmacy formed the population for this study. The third year class represents a class that had successfully completed the first professional examination in the pharmacy curriculum while the final year class had been through three professional examinations. It is possible that differences may lie in the perceptions of these students towards cheating and academic progression may play a role. A non-probabilistic sampling method was used to select student participants such that the inclusion criteria used was being a bona fide member of the class and being present during the lecture at the time of the study.

Study instrument

A questionnaire in English language to be individually filled was used for this study. It consisted of 16 items (scenarios) that were statements considered to be dishonest. Eleven (11) statements were drawn from a previous study (Harries and Rutter, 2005), carried out in the University of Portsmouth, United Kingdom and others derived from graduates and lecturers of both schools of pharmacy under study in Nigeria. The statements covered common activities students participate in during examinations and tests (7 statements) and course and laboratory works (9 statements). The instrument sought to explore students' opinions on cheating behaviours and if they had indulged in any of such behaviours in the past. Demographic data such as age in years, gender and current year of study were also included in the study instrument.

Study procedure

After an approval of the study by the Faculty administration office of both schools, students were approached during a mandatory lecture, mid semester. A briefing was conducted and students were encouraged to provide genuine responses to all statements, as confidentiality and anonymity were promised by the researchers. To further build student trust, ballot boxes and similar marker pens were provided for all participating students. Oral consent for participation was sought from each student and no punishment for non-participation was promised. The questionnaires were distributed under the supervision of a lecturer to pharmacy students present during the third year and final year lectures. The classes were large enough and spacing was encouraged to reduce communication and bias when filling the questionnaire. A maximum allocated time of 20 minutes was given for completion of the questionnaire. The questionnaires were collected after completion. No honorarium was promised or given.

Outcome measures and statistical analysis

Data collected from the questionnaire were entered into the Microsoft Excel 2007 spread sheet independently by two personnel so as to ensure accuracy of entry. Entered data were transferred into the SPSS 16.0 package (SPSS Inc, Version 16, Chicago, USA) and subjected to differential analysis. Demographics of students from the two schools were presented as mean and percentages. Students' perception of dishonest scenarios was assessed using

percentages of students who correctly/incorrectly identified the dishonest behaviours. Prevalence of cheating was assessed by percentages of students who had reported in self-indulgence in cheating in the past year. These were presented by school of pharmacy and year of study. Effect of demographic variables (age, gender and year of study) on perceptions and prevalence were assessed by cross tabulation and differences were analysed using χ^2 -test at P values less than 0.05 being considered significant. Differences in perceptions and prevalence of cheating behaviours between the two Nigerian schools and a school in the UK³ were assessed using percentages in statements similar to both studies' instrument.

RESULTS

Respondents' characteristics and response rate

Three hundred and sixty six students participated in this study (overall participation rate of 72.8%). University of Nigeria accounted for 216 (59.0%) students and Olabisi Onabanjo University, 150 (41.0%) students. These represent an institutional participation rate of 66.4% (216/325) for UNN and 84.3% (150/178) for OOU. A majority of the students were females, 188 (51.4%) and with an overall mean age of 21 years for third years and 24 years for final years in both schools.

Students' opinion of cheating behaviour to cheating in examinations and coursework

Results on students' opinion of cheating behaviours in examinations and coursework are displayed in Tables 1 and 2, respectively. Overall, more than one-tenth of the students (12.8%) from both schools thought that the statements on examinations did not constitute cheating. A majority (>90%) of students of both universities thought that five of the seven statements were cheating behaviours for written examination. While nearly a quarter (23.6%) of students said that "seeing a leaked paper before an exam and solving it for use during the exam" was not cheating, also almost of half of them (42.37%) thought that "writing in an examination after the allotted time was over" was not cheating either. Differences in year of study showed that in OOU, more final year students considered all but one scenario ("writing an exam after the allotted time was over") were cheating than those in the third years. In contrast however to UNN, proportionally more third years students considered nearly all scenarios to be cheating.

Overall, nearly a third (29.3%) of students from both schools thought that the statements on course and laboratory work did not constitute cheating. Specifically, more than 30% of students of both universities thought that five of the nine scenarios were not cheating. Most notably, nearly half (48.2%) of the students said "passing down one's practical workbook for lower years to use" and "one using an old practical workbook to complete a practical work" were not cheating. Differences in year of

study showed that more final year students considered five of the nine scenarios as cheating behaviours than third year students in OOU. In contrast however, fewer students in final years in UNN considered all the coursework scenarios as cheating than third year students.

Student's self admittance to cheating in examinations and coursework

Students in these schools admitted to indulge in all scenarios presented in their last professional examinations. However, the prevalence of cheating indulgence was significantly higher (54.2% vs. 45.8%, $p < 0.05$) with coursework than in examinations. Adjusting for year of study for each school of pharmacy, more final year students in OOU admitted to cheating than the third year students (74.6% vs. 62.1%, $p < 0.05$). In contrast, a non-significantly lesser number of final year students of UNN were involved in these cheating behaviours than their third year counterparts (79.3% vs. 84.0%, $p > 0.05$) (Table 3). From a school of pharmacy point of view, there was a significantly higher number of students in UNN reported to have indulged in at least one of the cheating scenarios than in OOU (81.5% vs. 68.0%, $p = 0.002$). Also there was no significant difference between males and females students who indulged in academic dishonesty irrespective of the school of pharmacy (49.1% vs. 50.9%, $n = 277$; $p = 0.471$).

Differences of perceptions and prevalence of academic dishonesty among pharmacy students in Nigeria and United Kingdom

Results comparing perceptions and self-reported indulgence of academic dishonesty between students of two schools of pharmacy in Nigeria and a school of pharmacy in United Kingdom are displayed in Tables 4 and 5. More students in the Nigerian schools of pharmacy thought that four of the eleven selected scenarios were cheating than their UK counterparts. These included the scenarios "a student continues to write in an exam after the allocated time", "a student gets no results during the practical, then he makes it up (forges it)", "a student uses an old practical workbook to complete his practical report" and "a student passes down his practical workbook to lower groups for them to use to complete their practical reporting". Interestingly, only about half (47.7% to 57.7%) of the students in both countries thought that writing in an exam even after the allotted time and using an old workbook to complete one's assignments should be considered cheating. For admitted indulgence in cheating (Table 5), proportionally more Nigerian students participated in academic dishonesty in all eleven selected case scenarios than UK students.

Passing down practical notes (16.6%) or using practical

Table 1. What Constitutes cheating: Opinion of students in examination scenario (No = believes the scenario does not constitute cheating).

Examination scenario		Olabisi Onabanjo University				University of Nigeria				Total		NP
		Year 3		Year 5		Year 3		Year 5		%	n	
		%	n	%	n	%	n	%	n			
A student uses information written on the arm during a written examination	No	12.6	11	3.2*	2	4.0	4	5.3	6	6.3	23	22
A student takes a revision note ("chips") into a written exam	No	8.0	7	0*	0	2.0	2	5.2	6	6.2	23	12
A student uses electronic storage device (e.g. programmable calculator, phone, blackberry to store data and use it in an exam	No	7.0	6	0*	0	4.0	4	7.8	9	7.0	26	14
Students exchange answers by (signals or orally) during an exam	No	12.8	11	1.7*	1	7.0	7	12.1	14	10.0	33	115
A student sees a "leaked" paper before an exam, solves it and uses the information during the exam	No	33.7	29	26.7	16	20.0	20	17.5	20	23.6	85	38
A student continues to write in an exam after the allotted time was over	No	33.8	33	45.2	28	43.0	43	43	49	42.3	153	116
A student allows another student to copy his paper in an exam	No	16.3	14	3.4*	2	7.0	7	9.5	11	9.4	34	126

*Indicates $p < 0.05$ (3rd versus 5th year); NP = no of students who had indulged in the act.

Table 2. What Constitutes cheating? Opinion of students in course/laboratory work scenario (No= believes the scenario does not constitute cheating).

Course and laboratory work scenario		Olabisi Onabanjo University				University of Nigeria Nsukka				Total		NP
		Year 3		Year 5		Year 3		Year 5		%	n	
		%	n	%	n	%	n	%	n			
Instead of doing his practical work, a student copies from a group member	No	37.6	32	28.3	17	32	32	33	38	33.1	119	155
A student allows another student to copy his/her assignment	No	39.3	33	42.9	22	34	34	37.9	44	37.6	133	199
A student copies a colleague's practical note without their consent	No	20	17	8.1*	5	13	13	18.1	21	15.4	56	57
A student gets no results during the practical, then he makes it up (forges it)	No	26.7	23	20.6	13	24	24	25.2	29	24.5	89	97
A student uses an old practical workbook to complete his practical report	No	42.4	36	42.6	26	44	44	45.7	53	43.9	159	151
A student passes down his practical workbook to lower groups for them to use to complete their practical reporting	No	50.6	41	54.2	32	41.4	41	49.1	56	48.2	170	102
A student does not participate in practical group work, leaving it only for other members	No	41	34	36.1	22	26	26	29.3	34	32.2	116	70
A student gets his colleague to write his assignments for him/her	No	31	26	35.1	20	24	24	26.7	31	28.3	101	86
A student uses electronic devices to get answers from the internet during an exam or class	No	10.5	9	3.2	2	4*	4	12.9	15	8.2	30	15

*Indicates $p < 0.05$ (3rd versus 5th year). NP = no of students who had indulged in the act.

Table 3. Reported rates of dishonesty by students of two Nigerian schools of Pharmacy.

Number of students admitting to undertaking academic dishonesty*	Number of instances students indulged in academic dishonesty		
	Written Examinations	Coursework	Total
OOU 3 rd year (n=54/87, 62.1%)	68	155	223
OOU 5 th year (n=47/63, 74.6%)	57	151	208
UNN 3 RD year (n=84/100, 84%)	185	314	499
UNN 5 th year (n=92/116, 79.3%)	133	312	445
All years of both schools (n=277/366, 75.7%)	443	932	1375

* At least one scenario described as academic dishonesty.

notes to complete one's practical assignment (17.1%) were most prevalent cheating behaviours seen among UK pharmacy students while copying a practical work from a group member (42.3%) and using old practical workbook to complete assignments (41.2%) were most prevalent cheating behaviours among Nigerian students. Overall, the cheating was higher in coursework than in examination for students in both countries.

DISCUSSION

This study explored the perceptions and actual indulgence in academic dishonesty ("cheating") among pharmacy students in two schools of pharmacy in Nigeria. Its results show that the perceptions towards cheating was poor and prevalence of cheating was high, much higher than reported in other schools in the US (Aggarwal et al., 2002). In examination scenarios, poor perceptions of what constitutes cheating in an examination was seen especially in two statements which were "a student continues to write in an exam after the allotted time was over" and "a student sees a leaked paper before an exam, solves it and uses the information during an exam". Interestingly the latter scenario can warrant a student's withdrawal from the pharmacy program in both Nigerian universities, but the students felt it was one's "luck" if such an event were presented to them. More encouragingly, only a few of them accepted to have indulged in such an act. The prevalence of indulgence in cheating was high in three examination scenarios with about half of the students reporting indulgence. Allowing a fellow student copy one's work, writing after allotted time was over and exchanging answers by signals during examinations accounted for the most prevalent acts of cheating amongst these students. Quite worryingly, majority of the students perceived these acts as cheating and still went ahead to indulge in them. Large number of students taking examinations in medium (or small) sized classrooms is common in most schools of the country. Such scenarios encourage cheating among students as they feel (or know) they are not likely to be caught if they cheated. This may cast some doubts on their academic

competencies and professionalism status as they graduate, as some authors have reported that students who cheat in school would most likely be involved in unprofessional acts during practice (Turrens et al., 2001). There were also poor perceptions in course (classroom) work cheating scenarios among students in nearly all the scenarios presented. Copying another's work and assignments, forging laboratory results and using previous workbook to complete assignments were seen to be cheating by more than half the students assessed. These acts of dishonesty were also found to be correspondingly prevalent among these students with majority of them admitting to have indulged in them. There was high indulgence in nearly all the scenarios especially in cases of copying assignments, but there was evidently low use of electronic devices to cheat. The authors believe that this high prevalence in academic dishonesty in class work may be due to very weak or non-existent penalties from the schools' administration, as most schools do not punish students for classroom work cheating.

Prevalence of cheating in our study showed nearly equal proportion of third and fifth year pharmacy students being involved in at least one form of cheating. This contradicts the report of Ng et al. (2006), which reported higher class students being more involved in forms of academic honesty. Hardigan and Ranelli (2006) however had a mixed report, stating that the students from the higher class (third year vs. first year) cheated more before actual examinations with first years more likely to cheat during examinations. The higher prevalence of cheating among third year students of UNN could possibly be attributed to the high attrition rate in that class, and students become desperate to get to the next class. However, for a smaller school like OOU in which attrition rate is similar across all years, more final year students said they were involved in cheating. This may be as a result of spending more years in school or were possibly more aware of opportunities to cheat (Ng et al., 2006).

Students in the larger school (UNN) were significantly more involved in cheating behaviours than the students in the smaller institution (OOU). This trend is supported by

Table 4. Comparison of the perception* toward cheating between students of schools of pharmacy in Nigeria and United Kingdom.

Scenario	Olabisi Onabanjo University/University of Nigeria, Nigeria n=366		University of Portsmouth, United Kingdom n=409	
	N	%	N	%
Examination				
A student uses information written on the arm during a written examination	341	93.7	403	99
A student takes a small piece of revision note (“chips”) into a written exam	351	93.8	402	98.7
A student uses electronic storage device (e.g. programmable calculator, phone, blackberry to store data and use it in an exam	346	93	403	99
Students exchange answers by signals or orally during an exam	328	90	386	95
A student sees a paper before the exam, solves it and uses it in an examination	275	76.4	383	94.3
A student continues to write in an exam after the allocated time	209	57.7	192	47.3
Course work				
Instead of doing his practical work, a student copies from a group member	241	66.9	375	92.4
A student copies a colleague’s practical note without their consent	307	84.5	393	96.8
A student gets no results during the practical, then he makes it up (forges it)	275	75.5	192	48.9
A student uses an old practical workbook to complete his practical report	203	56.1	193	47.7
A student passes down his practical workbook to lower groups for them to use to complete their practical reporting	183	51.8	170	42.2

*Responses that believe the scenario does constitutes cheating.

the works of Thorpe et al. (1999). This difference in cheating prevalence between the schools may also be attributed to a lesser inclination by OOU students to cheat or report they cheated or may have fewer opportunities to cheat than those in UNN. Larger classes and fewer tutors/examiners (typical of some schools in Nigeria) as mentioned above may provide ready atmosphere for students to cheat. Our study found no association of gender of pharmacy students and actual indulgence in academic dishonesty which is similar to results published earlier (Rabi et al., 2006) but contrary to another study assessing a school with predominantly African-Americans (Saulsbury et al., 2011).

Students from schools of pharmacy in UK and Nigeria had poor perceptions of cheating behaviours and both indulged in different examination and coursework malpractice. Quite a large number of students of both schools thought that using previous workbooks to complete one’s assignment or passing down workbooks to lower classes for use and continued writing in an exam after allotted time was not cheating. Using previous workbooks to complete one’s assignment or passing down workbooks to lower classes for use were the most common cheating behaviours both sets of students indulged in. Other cheating behaviours that were common among both students included “forging laboratory results” and

“writing an examination after the allotted time was over”. However, proportionally, more Nigerian students indulged in cheating and their knowledge of what constitutes cheating was poorer than their UK counterparts overall. This may be suggestive of the effect of cultural and regional differences as suggested by Ng et al. (2006). Prevalence of cheating is expected to be lower in UK schools and schools of highly developed economies due to the availability and utilization of cheating technological devices which are evidently non-existent in schools of most developing nations. A major concern we express in this study is the growing use of electronic storage devices to store answers and use during examinations. Though

Table 5. A comparison of the *levels of admitted indulgence in academic dishonesty* between students of the schools of pharmacy in Nigeria and United Kingdom.

Scenario	Olabisi Onabanjo University/University of Nigeria, Nigeria n=366		University of Portsmouth, United Kingdom n=409	
	N	%	N	%
Examination				
A student uses information written on the arm during a written examination	22	6.0	13	3.2
A student takes a revision note into a written exam	12	3.3	3	0.1
A student uses electronic storage device (e.g. programmable calculator, phone, blackberry to store data and use it in an exam	14	3.8	9	2.2
Students exchange answers during an exam	115	31.4	32	7.8
A student sees a leaked paper, solves it and uses it in an examination	38	10.4	3	0.1
A student continues to write in an exam after the allotted time was over	116	31.7	45	11.0
Course work				
Instead of doing his practical work, a student copies from a group member	155	42.3	19	4.6
A student copies a colleague's practical note without their consent	57	15.6	7	1.7
A student gets no results during the practical, then he makes it up (forges it)	97	26.5	67	16.4
A student uses an old practical workbook to complete his practical report	151	41.2	70	17.1
A student passes down his practical workbook to lower groups for them to use to complete their practical reporting	102	27.9	68	16.6

the levels reported in this study was relatively low but they were higher than those reported in schools of pharmacy in more technologically advanced countries like the UK (Harries and Rutter, 2005). Students should be encouraged to embrace new technologies and employ them in purposeful ventures such as literature search, research and academic discussions. An author recounted that some schools have devised strategies such as honour codes and academic integrity committees to enforce such codes, use of examination proctors and anti-plagiarism softwares to reduce academic dishonesty and plagiarism. However these efforts, no matter how stressed, would be useless if students themselves

cannot instil good ethics and honesty in their individual and collective characters. As at the time of this study, schools of pharmacy in Nigeria do not assess its students using performance-based and problem-solving examinations but rather focus hugely on multiple choice and theory-based tests, which shifts focus of learning to a mere jostle for higher grades (Piascik and Brazeau, 2010).

This study had some limitations. Firstly, the sample though representative of a typically small and large pharmacy school in Nigeria, may not typify the perceptions and actions of students in other schools of pharmacy. Secondly, this study assesses perceptions towards a concept our

respondents know is unprofessional, and this may have caused some respondents to report falsely (agreeably) which may have affected validity of these results obtained. Lastly, this study did not set out to understand reasons why students cheat and thus these results should be treated as descriptive and not inferential.

Conclusion

Pharmacy students in the Nigerian schools surveyed have poor perceptions about academic dishonesty and have indulged in it. Educational interventions should be enforced to reduce this

worsening trend so as to preserve the professionalism this profession prides itself on.

ACKNOWLEDGEMENT

The authors want to specially thank all the members of staff and students of both pharmacy schools who participated in this study.

REFERENCES

- Aggarwal R, Bates I, Davies JG, Khan I (2002). A study of academic dishonesty among student at two pharmacy schools. *Pharm J.* 269:529-533.
- Austin Z, Simpson S, Reynen E (2005). The fault lies not in our students but in ourselves: academic dishonesty and moral development in health professions education – results of a pilot study in Canadian pharmacy. *Teach Higher Educ.* 10: 147-60.
- Hardigan PC, Ranelli PL (2006). Perceptions of cheating and self-reported cheating behaviors of first-year and third-year pharmacy students. *Int. J. Pharm Educ.* 1: 1-8.
- Harries R, Rutter PM (2005). Cheating by pharmacy students: perceptions, prevalence and comparisons. *Pharm. Educ.* 5(1): 53-60.
- McCabe DL, Trevino LK (1996). "What we know about cheating in college" *Change* 28(1): 28-33.
- Ng HWW, Davies G, Bates I, Avellone M (2003). Academic dishonesty among pharmacy students. *Pharm Educ* 3: 261–269.
- Norton LS, Tilley AJ, Newstead SE, Franklyn-Stokes A (2002). The pressures of assessment in undergraduate courses and their effect on student behaviors. *Assoc. Eval. High Educ.* 26(3): 269-284.
- Piasek P, Brazeau GA (2010). Promoting a culture of academic integrity. *Am. J. Pharm. Educ.* 74(6): Article 13.
- Rabi SM, Patton LR, Fjortoft N, Zgarrick DP (2006). Characteristics, Prevalence, Attitude and perceptions of Academic dishonesty among pharmacy students. *Am. J. Pharm. Educ.* 70(4): Article 14.
- Ryan G, Bonnno H, Krass I, Scouller K, Smith L (2009). Undergraduate and postgraduate pharmacy student's perceptions of plagiarism and academic dishonesty. *Am. J. Pharm. Educ.* 73(6): Article 105.
- Salsbury MD, Brown UJ, Heyliger SO, Beale RL (2011). Effects of dispositional traits on pharmacy students' attitude towards cheating. *Am. J. Pharm. Educ.* 75(4); Article 69.
- Storch EA, Storch JB (2002). Fraternities, sororities and academic dishonesty. *Coll Student J* 3: 247-51.
- Taradi SK, Taradi M, Dogas Z (2012). Croatian medical students see academic dishonesty as an acceptable behavior: a cross-sectional multicampus study. *J. Med. Ethics* 38: 376-379.
- Thorpe MF, Pittenger DJ, Reed BD (1999). "Cheating the researcher: a study of relation between personality measures and self-reported cheating (statistical data included)". *Coll Student J* 33(1): 49, see also 1-10.
- Turrens JF, Staik IM, Gilbert DK, Small WC, Burling JW (2001). Undergraduate academic cheating as a risk factor for future professional misconduct. In: *Investigating Research Integrity; Proceedings of the First ORI Research Conference on Research Integrity.* Washington, DC.

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**



**1st Annual International Conference on
Pharmacology and Pharmaceutical Sciences
(PHARMA 2013)**

**18th - 19th November 2013
SINGAPORE**

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