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

<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof. Fen Jicai</td>
<td>School of life science, Xinjiang University</td>
<td>China</td>
</tr>
<tr>
<td>Dr. Ana Laura Nicoletti Carvalho</td>
<td>Av. Dr. Arnaldo, 455, São Paulo, SP</td>
<td>Brazil</td>
</tr>
<tr>
<td>Dr. Ming-hui Zhao</td>
<td>Professor of Medicine, Director of Renal Division, Department of Medicine</td>
<td>China</td>
</tr>
<tr>
<td>Prof. Ji Junjun</td>
<td>Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences</td>
<td>China</td>
</tr>
<tr>
<td>Prof. Yan Zhang</td>
<td>Faculty of Engineering and Applied Science, Memorial University of Newfoundland</td>
<td>Canada</td>
</tr>
<tr>
<td>Dr. Naoufel Madani</td>
<td>Medical Intensive Care Unit, University hospital Ibn Sina, University Mohamed V</td>
<td>Morocco</td>
</tr>
<tr>
<td>Dr. Dong Hui</td>
<td>Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University</td>
<td>China</td>
</tr>
<tr>
<td>Prof. Ma Hui</td>
<td>School of Medicine, Lanzhou University</td>
<td>China</td>
</tr>
<tr>
<td>Prof. Gu Huijun</td>
<td>School of Medicine, Taizhou University</td>
<td>China</td>
</tr>
<tr>
<td>Dr. Chan Kim Wei</td>
<td>Research Officer, Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra</td>
<td>Malaysia</td>
</tr>
<tr>
<td>Dr. Fen Cun</td>
<td>Professor, Department of Pharmacology, Xinjiang University</td>
<td>China</td>
</tr>
<tr>
<td>Dr. Sirajunnisa Razack</td>
<td>Department of Chemical Engineering, Annamalai University, Annamalai Nagar, Tamilnadu</td>
<td>India</td>
</tr>
<tr>
<td>Prof. Ehab S. EL Desoky</td>
<td>Professor of pharmacology, Faculty of Medicine, Assiut University, Assiut</td>
<td>Egypt</td>
</tr>
<tr>
<td>Dr. Yakisich, J. Sebastian</td>
<td>Assistant Professor, Department of Clinical Neuroscience, Karolinska University Hospital</td>
<td>Sweden</td>
</tr>
<tr>
<td>Prof. Dr. Andrei N. Tchernitchin</td>
<td>Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA</td>
<td>Chile</td>
</tr>
<tr>
<td>Dr. Sirajunnisa Razack</td>
<td>Department of Chemical Engineering, Annamalai University, Annamalai Nagar, Tamilnadu</td>
<td>India</td>
</tr>
<tr>
<td>Dr. Yasar Tatar</td>
<td>Marmara University, Turkey</td>
<td></td>
</tr>
<tr>
<td>Dr Nafisa Hassan Ali</td>
<td>Assistant Professor, Dow institute of medical technology, Dow University of Health Sciences, Chand bbi Road</td>
<td>Pakistan</td>
</tr>
<tr>
<td>Dr. Krishnan Namboori P. K.</td>
<td>Computational Chemistry Group, Computational Engineering and Networking, Amrita Vishwa Vidyapeetham, Amritanagar</td>
<td>India</td>
</tr>
<tr>
<td>Prof. Osman Ghani</td>
<td>University of Sargodha, Pakistan</td>
<td></td>
</tr>
<tr>
<td>Dr. Liu Xiaoji</td>
<td>School of Medicine, Shihezi University, China</td>
<td>China</td>
</tr>
</tbody>
</table>
ARTICLES

Research Articles

Khamira Marwarid Khas, a herbo-mineral prescription, as mucosal immunopotentiator in murine model  
Farah Khan, Asif Elahi, Shakir Ali  
598

Maternal exposure to aqueous extract of Mentha pulegium L. inducing Toxicity to embryo development in rats  
Joshua Ikoni Ogaji, Anthony Olaniyi Alawode and Titus Afolabi Iranloye  
609

Antibacterial activity of organic solvent fraction from Euphorbia supina  
Suzy A. El-Sherbeni, Souzan M.I. Moustafa, Abdel-Rahim S. Ibrahim, Kamilia A. El Seoud and Farid A. Badria  
615

Effect of rice bran extract on immunological and physiological parameters of Biomphalaria alexandrina snails infected with Schistosoma mansoni  
Suzy A. El-Sherbeni, Souzan M.I. Moustafa, Abdel-Rahim S. Ibrahim, Kamilia A. El Seoud and Farid A. Badria  
621
Khamira Marwarid Khas, a herbo-mineral prescription, as mucosal immunopotentiator in murine model


Department of Biochemistry, Hamdard University (Jamia Hamdard), Hamdard Nagar, New Delhi-110 062, India.

Received 11 December, 2012; Accepted 30 May, 2014

Khamira Marwarid khas (KMK) is a compound herbo-mineral preparation consisting of pearl extract and 7 plant extracts, widely prescribed by Unani physicians for various ailments related with immune suppression. Though it is a known Unani formulation, no attempts have been made to validate its mechanism of action. In this regard, we attempted to elucidate its role in modulation of the immune response. KMK was administered to mice orally at a dose level of 2 g/kg body weight for 15 days, following which hematology and immune function including the lymphoid organ weight and cellularity of lymphoid organs were analyzed. Humoral and cell mediated immune responses were evaluated by assessing the IgG levels and titres, IgG subtypes, comparative levels of IgG and IgE, delayed type of hypersensitivity, lymphocyte proliferation using 3H-thymidine incorporation assay and cytokine analysis. Innate immune responses were analyzed using production of nitrogen oxide (NO) by macrophages and phagocytosis. KMK treated mice showed a significant increase (p < 0.05) in the cellularity of the bone marrow. Ovalbumin-specific serum IgG level (p<0.05) and levels of IgG2a and IgG2b increased significantly. KMK enhanced significantly (p < 0.05) lymphocyte proliferation and delayed type of hypersensitivity response. An upregulation in the production of Th-1 cytokine (IFN-γ) by concavalin A (Con A) stimulated splenocytes was observed while the level of inflammatory cytokines like TNF-α and IL-1β were non-significantly increased. Oral administration of KMK by itself did not induce the production of NO by macrophages and suppressed the production of NO in response to LPS. Increased phagocytic rate and phagocytic index was observed. Taken together, the results suggest the immunostimulatory effect of KMK through a mechanism, leading to a Th1 dominant immune state.

Key words: Immunopotentiation, herbal prescription, Khamira Marwarid Khas (KMK).

INTRODUCTION

One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses. The basic strategy underlying immunomodulation is to identify aspects of the host response that can be enhanced or suppressed in such a way as to augment or complement a desired immune response. It allows the host to better defend itself against invading microorganisms during the course...
of infection, and this is attractive because it allows for enhanced host-derived mechanisms to take part in the immune response and it does not involve the use of organism-specific therapies such as antibiotics (Tzianabos, 2000). The targets which can be considered for any interaction with immunomodulators range from the prostaglandin metabolism, NO-mediators, and endocrinal systems to complement receptors, adhesion molecules and leukocyte chemotaxis (Wagner et al., 2003).

Immunomodulation is also coming up as a convenient alternative for chemotherapies and biological therapies to treat cancer. Several types of immunomodulators have been identified, including mammalian proteins such as interferon gamma (IFN-γ) (Murray, 1996), granulocyte colony-stimulating factor (Nemunaitis, 1997) and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Hamilton and Anderson, 2004), as well as substances isolated and purified from botanical sources (Tzianabos, 2000). Immunomodulators of natural origin are important in this scenario owing to the least side effects compared with the constitutional, neurological side effects of other treatments for cancer and autoimmune disorders.

In recent decades, an increasing number of patients are resorting to complementary and alternative systems of medicine, in the majority of instances to complement conventional care arena, because of their broad spectrum of therapeutic properties and relatively low toxicity (Tzianabos, 2000; Paulsen, 2001; Wasser, 2002). A number of prescriptions of one of the Indian traditional systems of medicine, the Unani system, are thought to possess immunomodulatory properties. Khamira Marwarid Khas (KMK) is also one such herbo-mineral prescription that is extensively prescribed as a recuperating agent after disease condition, given specially to convalescing patients of typhoid fever and chicken pox. Pearl is the major constituent of this formulation along with the extracts of seven plants namely Salvia haemato dell, Centaurea behen, Santalum album, Lepidium iberis, Crocus sativus, Punica granatum and Pyrus malus. KMK is claimed to boost body’s capacity to fight infection, strengthen the vital organs of the body and as a rejuvenator. Owing to its properties, it was speculated by us, the potential role of this prescription as an immunomodulating agent. The present study was designed to study the effect of KMK on immune responses.

MATERIALS AND METHODS

Animals and chemicals

Swiss albino male mice, 6 to 8 weeks old, weighing 20 to 25 g were used throughout the study. Mice were kept in an environmentally controlled room in polypropylene cages in groups of six each in the University’s Central Animal House Facility. They were fed a commercial pellet diet and water ad libitum. The study was approved by the Animal Ethics Committee and conformed to the national guidelines on the care and use of laboratory animals. Hank’s balanced salt solution (HBSS), ovalbumin, guinea pig complement, o-phenylene diamine dihydrochloride (OPD) and immunochemicals were purchased from Sigma-Aldrich Co., USA. [3H] Thymidine was procured from Amersham Biosciences, UK. Goat anti mouse IgE - HRPO was procured from Serotech. The cytokines kits were procured from e-biosciences, USA. All other chemicals were of analytical grade procured from the standard commercial sources in India.

Preparation of KMK

KMK is a product of Hamdard Wakf Laboratories (New Delhi, India). It is a polypharmaceutical preparation containing crude drugs of plant and mineral origin in the form of fine powder or their infusion/decoctions in sugar syrup. KMK is prepared by adding microfine powder of pearl and crude aqueous extracts of S. haematodes, C. behen, S. album, L. iberis, C. sativus, P. granatum and P. malus to sugar syrup of a particular consistency. The cooled mixture is then stirred continuously till the required consistency is achieved. Finally Warq-e-Nuqrah (Argentum) is added. The composition of the prescription is shown in Table 1.

Dosage and treatment protocol

The medicine was suspended in normal saline and administered orally to mice, once daily for 15 days, with the help of canula at a dose of 2 g/kg body weight in a volume not exceeding 0.2 ml per animal. The dose was based on human equivalent. Mice in the control group received a similar volume of normal saline.

Body weight, lymphoid-organ weight and cellularity and hematological parameters

Body weight gain and relative organ weight (organ weight/100 g of body weight) of kidney, liver, spleen and thymus were determined for each mouse. Single cell suspension was prepared in HBSS from bone marrow, spleen and thymus for cell count. Body weights of all mice were recorded prior to sacrifice, that is, 24 h after administering the last dose of KMK. Blood was collected from the treated and the control group of mice 24 h after the last dose. Total white blood cell (WBC) count, differential leukocyte count, red blood cell (RBC) count and hemoglobin content were measured using automated hematology cell counter (Sysnex K-1000).

Humoral immunity

The haemagglutination (HA) and plaque forming cell (PFC) assay assay was performed using the reported protocol by Bin Hafeez et al. (2003).

Estimation of ovalbumin-specific IgG, IgG end point titer, IgG subclass and IgE in serum using enzyme linked immunosorbent assay (ELISA)

For this analysis, mice were injected intramuscularly with 50 μg ovalbumin emulsified with complete Freund’s adjuvant (CFA) after
the completion of drug administration. A booster dose (25 mg of ovalbumin in CFA) was given on days 21 and 35 with incomplete Freund’s adjuvant (IFA). Mice were bled from the retro-orbital vein on days 27, 42 and 60 and the serum was separated. Ovalbumin-specific IgG, IgG end point titer, IgG subclass and IgE antibodies in the serum were detected by the indirect ELISA method given by Gupta et al. (2011).

Delayed type of hypersensitivity (DTH) response

The DTH response was determined as described earlier (Jazani et al., 2010). Briefly, after completing the treatment, animals were immunized with 1 × 10⁶ gRBC subcutaneously. After 5 days, all animals were again challenged with 1 × 10⁶ gRBC in the left hind footpad. The right footpad, injected with the same volume of normal saline served as control. Increase in the footpad thickness was measured 24 h after the last challenge with gRBC using dial calipers.

Lymphocyte proliferation assay

Mice, treated and control were injected intramuscularly and in the footpad with the antigen (ovalbumin) on day 0 (primary dose). Booster dose was given on day 8th and the animals were sacrificed on day 12th. Spleen was aseptically taken out and cleared of all extraneous tissues. Single cell suspension of it was made in RPMI-1640. RBC was lyzed by addition of 0.9% NH₄Cl and resuspended in complete RPMI-1640 media with 10% fetal calf serum (Sigma, USA). Viability of cells was checked through trypan blue dye exclusion test. Cells were then cultured at a final concentration of 2 × 10⁵ cells/100 µl/well in triplicates in flat bottom microtitre tissue culture plates (Tarson, India). The optimal concentration of antigen ovalbumin (5 µg/ml), positive control (Concavalin A 2 µg/ml) and negative control (plain media) was added to the wells to a final volume of 200 µl. After 3 days of incubation at 37°C under humidified air supplemented with 5% CO₂, 50 µl of supernatant was aspirated from each well and stored at −20°C for cytokine analysis. 0.5 µCi ³H thymidine (Amer sham, Germany) was then added to each well. 18 h later cells were harvested and aspirated onto glass-fibre filter papers using automatic cell harvester (NUNC, The Netherlands) and the radiolabel incorporated into DNA was counted using β-Scintillation counter (Beckman, U.S.A.). The stimulation index was calculated as mean counts per minute of cells stimulated with antigen divided by mean counts per minute of cells without antigen.

Cytokine analysis

The cytokines IL-4, IFN-γ, IL-1β and TNF-α were measured in the culture supernatants collected during T cell proliferation assay. The culture supernatants were centrifuged at 5000 rpm for 15 min, filtered through 0.22 µM membranes and assayed for cytokine levels using DuoSet™ ELISA development system kits (R & D Systems, Minneapolis, USA) as per manufacturer’s instructions.

Measurement of induction of NO in macrophages

Murine peritoneal macrophage cells were collected by the given method. Cells were centrifuged and washed thrice with plain media and suspended in complete RPMI-1640 supplemented with 10% fetal calf serum (Sigma-Aldrich, USA). Cells were counted and plated at a final concentration of 1 × 10⁶ cells/ well. Plates were incubated for 4 h at 37°C in humidified CO₂ incubator. Floating cells were removed and plates were washed twice with warm media to avoid the leaching of adhered macrophages. The final volume of all the wells was made up to 200 µl and incubated at 37°C for 24 h in presence or absence of positive stimulator of nitric oxide production (LPS 5 µg/ml). Culture soup was collected for estimation of NO production by Griess reagent method. Briefly, 59 µl of cell supernatant mixed with 1% sulphanilamide, 0.1% naphthylethylene diamine, 2.5% H₃PO₄ were incubated at room temperature for 10 min to form a chromophore. The absorbance was read at 550 nm and NO measured using NaNO₂ as standard.

Phagocytosis by peritoneal macrophages

Phagocytosis by peritoneal macrophages in mice was detected using method described by Elahi et al. (2014) with slight modification. Briefly, 0.5 ml of 5 × 10⁶ goat red blood cells (gRBC) were intraperitoneally injected into each mouse and mice were euthanized 1 h later. The fluid of abdominal cavity was collected to make a smear for each mouse. The smear were incubated at 37°C for 30 min in a wet box, fixed with 95% ethanol, and then stained by Wright-Giemsa dye. The number of macrophages ingesting gRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The phagocytic rate (PR) and phagocytic index (PI) were calculated using the following formula:

$$PR\% = \frac{\text{Number of macrophages ingesting gRBC}}{\text{Total number of macrophage}} \times 100$$

### Table 1. Constituents of Khamira Marward Khas (KMK).

<table>
<thead>
<tr>
<th>S/No.</th>
<th>Name</th>
<th>Major chemical composition</th>
<th>Content g/10 gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pearl</td>
<td>Calcite or calcium carbonate</td>
<td>0.35</td>
</tr>
<tr>
<td>2</td>
<td>Salvia haematodei</td>
<td>Fat, tannic acid and Bahmanine (crystalline alkaloid)</td>
<td>0.35</td>
</tr>
<tr>
<td>3</td>
<td>Centaurea behen Linn.</td>
<td>Behemnin, taraxasterol, γ- taraxasterol, inulin</td>
<td>0.35</td>
</tr>
<tr>
<td>4</td>
<td>Santalum album Linn.</td>
<td>A- Santalol and B- Santalol</td>
<td>0.12</td>
</tr>
<tr>
<td>5</td>
<td>Lepidium ibiris</td>
<td>Lepidin, sulphur containing volatile oil</td>
<td>0.35</td>
</tr>
<tr>
<td>6</td>
<td>Crocus sativus</td>
<td>α- crocin, β- crocin, γ- crocin</td>
<td>0.12</td>
</tr>
<tr>
<td>7</td>
<td>Punica granatum Linn.</td>
<td>Punicalagin</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>Pyrus malus Linn.</td>
<td>Quercetin</td>
<td>0.01</td>
</tr>
<tr>
<td>9</td>
<td>Argentum</td>
<td>Silver</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Table 2. Effect of KMK on relative organ weights, cellularity of lymphoid organs, hematology and plaque forming cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=6)</th>
<th>Treated group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relative organ weight (g/100 g body weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.84±0.05</td>
<td>1.06±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.24±0.01</td>
<td>0.28±0.009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>6.78±0.84</td>
<td>6.76±0.26</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.02±0.10</td>
<td>0.96±0.13</td>
</tr>
<tr>
<td><strong>Cellularity (×10&lt;sup&gt;6&lt;/sup&gt;)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>16.03± 0.76</td>
<td>30.20±1.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thymus</td>
<td>56.14±7.39</td>
<td>63.13±4.20</td>
</tr>
<tr>
<td>Spleen</td>
<td>316.26±20.78</td>
<td>442.94±16.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Hematology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g%)</td>
<td>12.11± 0.26</td>
<td>15.70±0.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RBC count (Million/cmm)</td>
<td>07.4±0.15</td>
<td>09.03±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WBC count (thousand/cmm)</td>
<td>11.88±0.92</td>
<td>17.10±1.36&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PFCs/10&lt;sup&gt;6&lt;/sup&gt; cells</td>
<td>122.22± 6.7</td>
<td>237.77±6.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Animals were sacrificed 24 hrs after the last dose of the medicine. Blood was collected from retro-orbital plexus for hematology. Organ weights were recorded and calculated as g/100 g body weight. Bone marrow cells were collected from femur bone. Single cell suspension of bone marrow, thymus and spleen were prepared in HBSS and counted using Neubauer chamber. PFC assay was performed in goat RBC immunized mice, administered with KMK for 15 days. Splenocytes were prepared and used for PFC (IgM) antibody production assay as described in text. Data represent mean ± S.E. (n = 6). <sup>a</sup>p<0.05 when compared with respective control.

Statistical analysis

The statistical significance of difference between treated and the control group was determined using the student’s t-test; p < 0.05 was chosen as the level of significance.

RESULTS

Effect of KMK on the body weight, lymphoid organ weight, cellularity, haematology and toxicity

Mice administered with the KMK did not show any significant gain in body weight when the animals were compared with the untreated control group. However, a significant increase (p < 0.05) in the weight of spleen was found in mice receiving the drug for 15 days (Table 2). The size and weight of thymus also increased significantly (p < 0.05). Administration of KMK resulted in a significant increase (p < 0.05) in the hemoglobin content and RBC count of normal Swiss albino mice. A significant increase (p < 0.05) in the bone marrow cellularity was a prominent feature (Table 2). The cellularity of spleen also enhanced significantly (p < 0.05). A non significant increase in the cellularity of thymus was observed. Oral administration of KMK did not produce mortality or show any sign of toxicity. No significant difference in the weight of liver and kidney of treated and untreated mice was observed (results not shown).

Effect on IgM titer measured by plaque forming cells (PFC) assay

The primary antibody IgM response to SRBC was also evaluated (Table 2). The numbers of plaque forming cells per million splenocytes were significantly increased from 122.22 in control group to 237.77 in animals treated with KMK for 15 days.

Effect on ovalbumin specific serum IgG levels and IgG titre

Oral administration of KMK enhanced significantly the
induction of ovalbumin specific IgG antibody responses in mice as determined by ELISA (Figure 1). The secondary immune response seen in the second bleed was statistically higher (p < 0.05) compared to that of the first bleed in all treated groups. Interestingly, the high ovalbumin specific IgG levels were maintained in the third bleed, that is 60 days post primary immunization (Figure 2). The antibody titre as determined by ELISA in the pooled serum also increased in mice administered with the medicine (Figure 2). The maximum end-point titre value peaked to 128,000 in the bleed II of mice treated for 15 days.

**Effect of KMK on the levels of ovalbumin-specific IgG subtypes**

The IgG subclasses were estimated to get an idea about the quality of immune response generated as well as to delineate the Th1-Th2 dichotomy, which is known to play a crucial role in development or containment of the disease. Oral administration of KMK resulted in generation of all four IgG isotypes (Figure 3) albeit at very low levels. Pre-dosing of mice with KMK resulted in marked upregulation of ovalbumin-specific IgG2a and IgG2b for all the treated groups as compared to control. A strong reduction in the level of IgG3 was observed in mice pretreated with KMK.

**Effect of KMK on comparative levels of IgE and IgG**

Immunization of mice with ovalbumin is known to exhibit up-regulation of Th-2 cytokine and serum anti-ovalbumin IgE levels. The effect of KMK administration on comparative IgE and IgG production in vivo was examined to analyze any shift, if present, in the levels of IgE to IgG or vice-versa. However, levels of anti-ovalbumin IgE decreased in mice pre-dosed with KMK for 15 days as compared to control mice receiving normal saline. This decrease in the level of IgE was accompanied by an upregulation in the level of IgG, which was maintained till the last bleed of the treated group (Figure 4).

**Effect of KMK on cell mediated immunity**

To assess the effect of oral administration of KMK to mice on cell mediated immune response, DTH response
**Figure 2.** Effect of KMK on ovalbumin-specific IgG titre. Serial dilutions of the pooled sera were done and titers of ovalbumin-specific IgG isotypes were measured using ELISA. Data represented as means ± S.E., (n = 5). A, treated with KMK; C, control. *p<0.01 when compared with its respective control.

**Figure 3.** Effect of KMK on ovalbumin specific IgG subtypes. After the final dose of the medicine, animals were immunized with ovalbumin (50µg/mice) and booster doses were given on 21st, 35th and 60th day of immunization and ovalbumin specific IgG subtypes were measured using IgG isotyping kit. Data represented as means ± S.E., (n = 5). A, treated with KMK; C, control. *p<0.01 when compared with its respective control.
Figure 4. Effect of KMK on comparative ovalbumin specific IgE and IgG levels. After the final dose of the medicine, animals were immunized with ovalbumin (50µg/mice) and booster doses were given on 21st, 35th and 60th day of immunization. Ovalbumin specific IgE and IgG levels were measured simultaneously using ELISA. Data represented as means ± S.E., (n = 5). A, treated with KMK; C, control. *p<0.01 when compared with its respective control.

to immunization with antigen was studied. Significant increase in the footpad thickness was observed in the gRBC immunized mice. The DTH response was further enhanced significantly (p < 0.05) in gRBC immunized mice treated with KMK (Table 3). The increase in footpad thickness of mice treated with KMK for 15 days was 61.65% as compared to 32.56% increase in the control group (Table 3).

Effect on proliferation of splenocytes using lymphocyte proliferation assay

The effect of oral administration of KMK on proliferation of splenic lymphocytes was examined in 3H thymidine incorporation assay. The results, presented in Table 3, demonstrate the effect of KMK on antigenic (ovalbumin) and mitogen (concanavalin A) induced proliferation of splenic lymphocytes. Prior dosing of mice with KMK for a period of 15 days showed an enhanced antigenic and mitogenic in vitro T cell proliferation as compared to control. Maximum antigenic and mitogenic stimulation index of 4.7 and 13.23, respectively was recorded for mice pretreated with KMK for 15 days.

Effect on production of Th1 and Th2 cytokine by splenocytes

Results are presented in Figure 5. Splenocytes from untreated control mice and KMK treated mice did not produce detectable amounts of Th1 cytokine (IFN-γ) or Th2 cytokine (IL-4) without antigenic or mitogenic stimulation. When splenocytes were incubated with antigen (ovalbumin) or mitogen (Con A) detectable amounts of these cytokines were found in cell free supernatant. The oral administration of KMK resulted in a significant enhancement of IFN-γ. A decrease in the level of IL-4 production as compared to untreated control was observed. Thus oral administration of KMK may lead predominantly to the production of Th1 type cytokines.

Effect on inflammatory cytokines (TNF-α and IL-1β) by splenocytes

The effect of KMK on inflammatory cytokines (IL-1β and TNF-α) level is shown in Figure 5a and b. No significant change in the level of IL-1β and TNF-α in the spleen cell culture isolated from KMK treated mice was observed.
Figure 5. Effect of KMK on levels of Th1 and Th2 cytokines. After the final dose of the medicine, mice were immunized with ovalbumin on day 0 and 8. Animals were sacrificed on day 12th and splenocytes were cultured for 3 days, in RPMI supplemented with 10% FCS, in presence of concavalin A (2 µg/ml). The cell free supernatant was collected from each well and the amounts of (a) IFN-γ and (b) IL-4 were measured using ELISA. Data represents the mean cytokine level of triplicate experiments and the S.E. for all the values were less than 0.04.
Table 3. Effect of KMK on cell mediated immune response.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=6)</th>
<th>Treated group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delayed type of hypersensitivity (% increase in footpad thickness)</td>
<td>32.56±3.2</td>
<td>61.65±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proliferation of lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulation index in presence of ovalbumin</td>
<td>1.23±0.12</td>
<td>4.70±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stimulation index in presence of Conavalin A</td>
<td>6.89±0.32</td>
<td>13.23±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

After the final dose of the medicine, mice were immunized with gRBC subcutaneously. On the 5th day, mice were again challenged with gRBC in the left hind footpad; normal saline injected in the right footpad served as trauma control. Increase in footpad thickness was measured 24 h after the last challenge as a measure of delayed type of hypersensitivity response. Influence of KMK on T cell proliferation. After the final dose of the medicine, mice were immunized with ovalbumin on day 0 and 8. Animals were sacrificed on day 12th and splenocytes were cultured for 3 days, in RPMI supplemented with 10% FCS, in presence of concavalin A (2 µg/ml) or ovalbumin (5 µg/ml). Cultures were pulsed with <sup>3</sup>H-thymidine incorporation was detected by liquid scintillation spectroscopy. Data are represented as means + S.E. (n = 6). *p<0.05 when compared with its respective control.

Table 4. Effect of KMK on innate immune response

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=6)</th>
<th>Treated group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO from peritoneal macrophages (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>8 ± 0.01</td>
<td>6 ± 0.01</td>
</tr>
<tr>
<td>Stimulated in-vitro with LPS (5 µg/ml)</td>
<td>22 ± 1.06</td>
<td>20 ± 0.09</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phagocytic rate</td>
<td>19.58 ± 2.16</td>
<td>43.09 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>1.00 ± 0.00</td>
<td>1.13 ± 0.048&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Nitrite production by peritoneal macrophages from mice treated with KM and stimulated in-vitro with LPS (5µg/ml) was measured using Griess reagent. Peritoneal macrophages were collected from mice treated with KM and immunized i.p. with gRBC (5 × 10<sup>6</sup>). Smear of cells were stained with Wright-Giemsa dye and enumerated using light microscope. Data represented as mean ± S.E., where n=6.

(Figure 6a, b) when compared with the respective control. The level of both TNF-α and IL-1β were non significantly elevated in un-stimulated and OVA stimulated splenocytes culture while in Con-A stimulated cell culture the level of TNF-α was non-significantly reduced and the level of IL-1β was slightly increased. These results suggest that KMK treatment does not affect the inflammatory cytokines profile.

Effect on phagocytosis

Table 4 shows the phagocytic activity of peritoneal macrophages isolated from KMK treated mice. The uptake capacity of peripheral phagocytes was increased by KMK. The phagocytic rate (PR) and phagocytic index was significantly elevated after 15 days of oral administration of KMK.

DISCUSSION

The main focus of this study was to identify the effect of KMK, identified as rejuvenating formulation by Unani physicians. The results indicate that administration of KMK viability was not affected by exposure to LPS (data not shown).
KMK for 15 days at a dose of 2 g/kg body weight improved the overall health of mice as indicated by increase in hemoglobin content and RBC count, and strengthened the specific immune system. The common hemopoietic stem cells of bone marrow are the source of major cell types involved in the immune system (Singh et al., 2006). Bone marrow also provides microenvironment for the antigen independent differentiation of B-cells. The increase in bone marrow cellularity and leukocyte counts in the peripheral blood by KMK point towards its stimulating effect on the leukopoietic activity. Also, the enhanced weight of thymus and spleen, which represent primary and secondary organs of specific immune system, suggested an immunopotentiating effect of KMK. Plaque forming cell (PFC) response to gRBC is often used as a monitor of the primary effectors function of the B lymphocyte that is, the synthesis and secretion of antigen-specific antibody, IgM (Holsapple, 1995). KMK produced a significant increase in primary antibody IgM titer measured by PFC.

KMK enhanced the humoral immunity as determined by the level of anti-ovalbumin antibody of IgG type, which increased significantly in treated mice. High levels of IgG antibody against ovalbumin were maintained till 60th day after immunization, suggesting that the drug had a prolonged effect on the potentiation of humoral immune response. The augmentation of the humoral immune response to ovalbumin and gRBC by KMK may be due to enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in the antibody synthesis (Coutelier et al., 1987). Oral administration of KMK was found to influence the Ig specific isotype profile. A comparison of isotype produced by mice to immunization with ovalbumin between treated and control showed induction of two major IgG subclasses IgG2a and IgG2b.

The regulation of IgE production is of clinical importance since this antibody plays a major role in type I allergic response. In this study we demonstrate, using murine in vivo model system, that KMK causes a reduction in ovalbumin specific serum IgE. Moreover a strong up regulation of OVA-specific IgG response was observed. Interestingly these results, in terms of augmentation of IgG and other subclasses and suppression of OVA-specific IgE antibody response may be comparable with that of the immunomodulatory activity of Quillaja saponaria (Molinaceae) (Kensil et al., 1991). This reduction of OVA-specific IgE and IgG1, in addition to the enhancement of IgG2a, suggests that KMK modulates the Th1/Th2 balance in favour of Th1 type response.

Several lines of evidence suggest that DTH is an important component of the host defense system. DTH immune reaction is usually represented by T-cell response (Mu and Sewell, 1994) and in the present study significant enhancement in the paw thickness in KMK treated mice was observed when compared to the mice in control group (P < 0.05). The formulation appeared to activate and differentiate T cells into Th1 sub-population. This polarization of T cells into the Th1 cells is accompanied by secretion of Th1 like cytokines such as IFN-γ and IL-2 by these cells. The overall effect of these cytokines is to recruit macrophages into the inflammatory site and promote phagocytic activity. The increased rate of proliferation of lymphocytes on stimulation by mitogen Con A signifies that in addition to showing an increase in the number of effector cells, KMK also functionally stimulates the lymphocytes. This indicates that KMK administration induces a sizeable peripheral pool of Con-A sensitive, naive T lymphocytes and cytotoxic cells which ensures an improved immune response.

Cytokines play a central role in the immune response by promoting the activation of antigen specific and non-specific effector mechanism and tissue repair. Selective cytokine production, in particular, can determine the outcome of a response by stimulating protective or exacerbative immune mechanism (D’EliOS and Del Prete, 1998; Kidd, 2003). In the current study, oral administration of KMK caused the increased production of IFN-γ stimulated with Con A compared with untreated control while the levels of IL-4 declined. These results indicate that oral administration of KMK can lead to Th-1 type dominant immune responses possibly through induction of IL-12 and IFN-γ production in mice. The enhanced secretion of selective Th1 like cytokines by KMK may be because of various chemical constituents present in the extract. This perhaps could be either because of inactivation of IL-4 receptors on CD4+ cells or selective regulation of costimulatory molecules and downstream up-regulation of transcriptional factors favoring Th1 polarization. The level of inflammatory cytokines (TNF-α/IL-1β) was observed as non-significantly increased in KMK treated mice which indicate that KMK may not stimulate the chronic inflammatory response and may be able to stimulate Th1 type of immune response.

The activation of the ingesting capacity of the macrophages observed here indicates that the KMK is able to activate both the non-specific and specific arms bringing in a total activation of the immune response. Among the earliest cell types to respond to invasion by pathogenic organisms are the phagocytes, which are key participants in the innate immune response (Janeway and Medzhitov, 2002). Macrophages together with neutrophils represent the first line of host defense after the epithelial barrier. In addition, macrophages can function as antigen presenting cells and interact with T lymphocytes to modulate the adaptive immune response (Schepetkin and Quinn, 2006). In addition, activated macrophages secrete various antimicrobial and cytotoxic substances that can destroy phagocytosed microorganisms. In the present study, macrophage function was analyzed through NO production and phagocytosis. Low concentrations of NO from activated macrophages are beneficial, along with other reactive nitrogen intermediates, they are responsible...
for cytostatic and cytotoxic activity against infectious organisms and tumor cells. In addition, NO plays a regulatory role in function of natural killer cells and the expression of cytokines such as IFN-γ and transforming growth factor-β (Bogdan et al., 2000). However, over production of NO has been found to be associated with various diseases such as septic shock, autoimmune diseases, and chronic inflammation by increasing vascular permeability and the extravasations of fluid and proteins at the inflammatory site (Moncada et al., 1991; Kolb and Kolb-Bachofen, 1992; Ileni et al., 1992). Therefore, inhibition of high-out-put NO production could be a useful strategy for treatment of various inflammatory diseases. The in vivo studies presented here demonstrated the suppressive effect of KMK on NO production using freshly isolated peritoneal macrophages. The result is similar to polysaccharide isolated and purified from the seeds of T. Indica that exhibited in vitro immunomodulatory activity such as phagocytic enhancement (Sreelekha et al., 1993) and suppressive effect on NO production in macrophage cell line and freshly isolated peritoneal macrophages.

The overall results of this study suggest the Unani formulation, KMK, to be a multipotent inducer of immune responses acting by way of stimulating leukopoiesis and activating the non-specific and specific immune mechanisms of the host. As believed by the Unani physicians, the drug may show this effect only when all the plants are used in combination, suggested by its effects on the different arms of the immune response.

The results presented here are a scientific evaluation of its biological effects, substantiating its use by humans for centuries, suggesting that it has the potential to be safely used as an immunopotentiating drug. It is not possible at this junction to single out the most effective immunostimulatory constituent of KMK. Further investigations on major constituents of KMK are in progress.

ACKNOWLEDGEMENTS

FK acknowledges the Council of Scientific and Industrial Research (CSIR) for fellowship.

Conflict of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


Mu HH, Sewell WA (1994). Regulation of DTH and IgE responses by IL-4 and IFN-γ in immunized mice given pertussis toxin. Immunology 83:639-645.


Maternal exposure to aqueous extract of *Mentha pulegium* L. inducing toxicity to embryo development in rats

Marli Gerenutti*, Lívia Modesto, Vanessa Alessandra Carrara, Stefani Alves Magalhães, Nobel Penteado de Freitas and Magali Glauzer Silva

Laboratory for the Development and Evaluation of Bioactive Substances, Laboratory for the Toxicological Research (Lapetox), University of Sorocaba (UNISO), Cidade Universitária, Rod. Raposo Tavares km 91, CEP 18023-000 Sorocaba, São Paulo, Brasil.

Received 15 January, 2014; Accepted 16 May, 2014

The aim of this study was to observe the effect of orally administrated aqueous extract of *Mentha pulegium* L. on female rats pregnancy and on the physical development of fetuses. It was administered daily doses of 1.0, 2.5 and 5.0 g per kg of body weight or alternates doses of 2.0 and 4.0 g/kg of the *M. pulegium* L. aqueous extract, to sixty pregnant rats from the 4th day to the 20th day of pregnancy. On the 21th day of pregnancy, the intact rat fetuses were isolated. The aqueous extract of *M. pulegium* L. induced several changes in the reproductive performance of female rats and significant alterations in the skeletal development of fetuses.

**Key words:** *Mentha pulegium*, medicinal plants, abortifacient agents, reproduction, teratology.

INTRODUCTION

In Brazil, the National Program of Medicinal Plants and Phytoterpatics (PNPMF) aims to ensure the population safe access and rational use of medicinal plants and phytoterpatics and promote the sustainable biodiversity use, the productive chain and the national industry development. Some of the PNPMF guiding principles are the sustainable use of Brazilian biodiversity; the valuation and the preservation of the knowledge of the traditional and indigenous communities and the strengthening family agriculture (Brazil, 2007).

The Secretariat of Science, Technology and Strategic Inputs, through the Department of Pharmaceutical Care and Strategic Inputs (DAF/SCTIE/MS), in 2005, drew up a list of plant species that supported in 2008, the definition of RENISUS (National List of Medicinal Plants of Interest to Brazilian Health System). *Mentha pulegium* L. (Lamiaceae) commonly known as “pennyroyal” or “poejo” is a plant native to Europe and Western Asia that was one of the selected to compose the RENISUS.

The *M. pulegium* L. leaf infusion is commonly used as aromatic stimulant, expectorant, antitussive, carminative, emmenagogue and for treating sinusitis and bronchitis (Mahboubi and Haghi, 2008). The flowering aerial parts of *M. pulegium* L. has been traditionally used for its antiseptic properties for treatment of infectious diseases (Mahboubi and Haghi, 2008). The effects of the essential
Mentha pulegium L. are well known in folk medicine as an abortifacient, due to its characteristic as a uterine musculature stimulant (Chitturi and Farrel, 2000; Conover, 2003). The monoterpene pulegone is the major component of the M. pulegium L. essential oil (Aghel et al., 2004; Petrakis et al., 2009; Koutroumanidou et al., 2013).

Despite the fact of M. pulegium L widely used in folk medicine, there are no records of embryo/fetotoxicity studies with its extract. This study seeks to correlate through biological assay in vivo, pharmacological and toxicological data of the extract of M. pulegium L. (Mp), taking into account its medicinal potential, focused in the safety of its aqueous extract on the reproductive performance of female rats and over some morphological parameters of fetuses.

MATERIALS AND METHODS

The plant samples

M. pulegium L. (Figure 1) was grown in the medicinal plants flower bed of University of Sorocaba – UNISO (São Paulo, Brasil). A voucher specimen has been deposited on the herbarium at University of Sorocaba and subsequent identification was carried out by the São Paulo Botanical Institute (IBI), authenticated by Dr. Sérgio Romaniuc Neto (PqC VI).

Preparation of the extract and pharmacognosy assays

Whole aerial flowering parts of the plant (3.60 kg) were dried for 48 h in a forced air incubator at 40°C. The material was then ground in a mill (MA 340®, Marconi, Brazil), macerated for three days (1120.00 g) in 70% ethanol (11.2 L). The suspension, protected from light, was percolated at 20 drops/min resulting in a 38% (w/v) hydro alcoholic extract as described by Gerenucci et al. (2008). The obtained extract was concentrated in a rotary evaporator (TE-210®, Tecnal, Brazil) and lyophilized (Multi-Tasking Freeze Drying S, SNL216V-115, Thermo Fisher Scientific, USA), resulting in 352 g. Dry extract polyphenols (Y = 0.1318, X + 0.0473, r = 0.9984, 720 nm, calculated as pyrogallol) and total flavonoids (Y = 0.0279, X + 0.0104, r = 0.99979, 420 nm, calculated as rutin) were measured (Woisky and Salatino, 1998). The volatile oily fraction of the lyophilized extract was reassembled with 5% v/w oil obtained by hydro distillation (Wasicky 1989) for each dose of lyophilized extract employed in biological assays. The oil was characterized by thin layer chromatography (TLC) (stationary phase: silica gel - mobile phase: mixed solvent of toluene with ethyl acetate - 93:7 - visualization reagent: p-anisaldehyde–sulfuric acid).

Animals

This study was approved by the UNISO Institutional Committee for Ethics in Research and the experiments were carried out according to the guidelines of Brazilian College for Animal Experimentation – COBEA, “The Guide for the Care and Use of Laboratory Animal” (National Research Council 1996) and “European Community guidelines” (EEC Directive of 1986; 86/609/EEC). All animals, supplied by UNICAMP’s vivarium and kept in UNISO’s vivarium, were maintained in groups of 5 rats per cage, housed in the laboratory conditions a week before the experiments starts, at 25 ± 3°C on a 12 h light/dark cycle with access to food and water ad libitum during all the period.

Acute toxicity assay

Fifty Swiss mice (50% of each gender) were distributed into 5 groups (1 control and 4 experimental) composed of five animals of each gender. Experimental groups received 0.5, 1.0, 2.5 and 5.0 g/kg of body weight of Mp aqueous extract (w/v). Control group received the vehicle (deionized water). After dosing, each group was observed for 30, 60, 120, 240 and 360 min every day for fourteen days. The parameters used for acute toxicity assay studies were: general activity, contortion, tremors, convulsions, straub tail, hypnosis, ptosis, urination, defecation, piloerection and hypothermia.
Figure 2. Embryo-lethal lesions resulting in resorption (A), fetus fixed in Bouin’s solution (B) and diaphanized fetus for skeletal analysis (C).

(Amos et al., 2001).

**Embryo- and feto-toxicity assay**

The method of reproductive evaluation previously applied elsewhere (Esteves-Pedro et al., 2012), consists in 60 sexually naive female rats mated with males (Rattus norvegicus albinus, Wistar), 5 females per cage with 2 males, weighing between 160 and 200 g. Pregnancy was confirmed by the presence of sperm in vaginal-washing rubbing observed in microscopic analysis. Presence of spermatozoids was considered the first day of pregnancy (Vickery and Bennett, 1970). Pregnant females were kept in separate cages. Water and food were supplied *ad libitum* during the experiment and the consumption was daily monitored. For reproductive evaluation, each group of 10 females received from the 4th day till the 20th day of pregnancy: 1.0 g/kg/day; 2.5 g/kg/day and 5.0 g/kg/day of Mentha pulegium L. aqueous extract or deionized water (Control A), by gavage. After the results analysis, we started a second test with doses of 2.0 and 4.0 g/kg aqueous extract of *M. pulegium* or deionized water (Control B) on alternate days, from the 4th day till the 20th day of pregnancy. In both studies, to evaluate teratogenicity, mothers were anesthetized with halothane (Halotano®, Cristalia, Brazil), killed and submitted to a rapid excision of their uterus. The following macroscopic parameters were evaluated in order to observe the rats' reproductive performance (Randazzo-Moura et al., 2011): (1) mothers' weight gain (g); (2) post-implantation loss (%) = implantation no. - alive fetus no. / implantation no. × 100; (3) offspring vitality (%) (Figure 2A). To study the fetuses development they and the placentas were weighed (g); after that, the offspring was anesthetized, killed and fixed in Bouin’s solution (Figure 2B) for 24 to 48 h, replacing it by a 70% hydro alcoholic solution to measure the following parameters (cm): anteroposterior and lateral-lateral of the skull; anteroposterior and lateral-lateral of the thorax; cranio-caudal and tail. Other offspring group was anesthetized, killed, eviscerated and diaphanized for posterior skeletal examination (Figure 2C). Fetuses selected were fixed in ethanol, then “cleared” and stained by koh-alizarin red method (Damasceno and Kempinas, 2008). The examination included: enumeration of the vertebra, ribs and other bone structures; degree of ossification and any fusions or abnormalities in bone's shape or position (Keller, 2001).

**Statistical analysis**

The results were submitted for statistical analyses, considering a significance level of 5%. Tukey-Kramer test was used to compare experimental and control groups, considering body-weight gain, placenta’s weight, fetuses’ weight and offspring’s morphological parameters. Chi-square test was used to evaluate changes (%) in osseous development parameters, post-implantation losses and fetuses’ vitality.

**RESULTS AND DISCUSSION**

**Qualitative phytochemical constituents of the hydro alcoholic extract of the Mentha pulegium L.**

Briefly, the obtained lyophilized extract containing: 13.04% of polyphenols, 5.22% of flavonoids totals, 10.85 ± 0.0064 of total ashes, 0.96 ± 0.0081 of insoluble ashes, 0.4% by hydrodestilation of volatile oil content. The thin layer chromatography (TLC) indicated 4 spots: Rf 0.98 (brown-orange); Rf 0.72 (orange); Rf 0.49 (nut-brown, majority, probably pulegone) and Rf 0.28
The acute toxicity of hydro alcoholic extract of the Mentha pulegium L.

Sztajnkrycer et al. (2003) suggested that pullegone intake is associated with severe hepatotoxicity and death. However our results of the acute toxicity assay showed that, despite the fact that with the Mp 5.0 g/kg dose the general activity of rats was slightly reduced but no death observed with any of the tested doses.

Effects of varying doses of hydro alcoholic extract of the Mentha pulegium L. in the embryo development in rats

Toxicology related to reproduction and development is an area that has achieved great scientific advances in recent decades. Studying the actions of toxic agents on the different stages of the reproductive process and development allows us to evaluate its effects on: fertility, transport and implantation of the egg, embryogenesis and fetal stage, birth, the newborn, lactation, weaning and care for the brood, delayed postnatal development, sexual behavior, estrus cycles or rhythms of design and placental and uterine functions (Oliveira et al., 2010; Tanaka et al., 2012; Behl et al., 2013; Repo et al., 2014).

Ensuring security in using bioactive compounds sometimes becomes more necessary than the pharmacological use itself, mainly considering the cultural application of certain compounds. In this sense, study models that ensure security in using vegetal drugs during pregnancy are fundamental in developing new drugs. Reproductive toxicity studies reveal possible effects of one or more active substances on reproduction of mammals and therefore investigations and interpretations of the results should always be related to other pharmacological and toxicological data available. Many of the changes observed during birth, growth and development are especially due to the mother’s exposure to chemical agents. Table 1 shows that, despite the statistically significant differences between the groups in some periods, the Mp extract did affect the reproductive capacity of female rats, reducing the weight gain when exposed to daily doses (F: 669.583, p < 0.0001) and to alternate doses (F: 404.96, p < 0.0001); increased post-implantation loss for all daily doses (Chi-square df: 40.943, p < 0.0001) and for the alternate dose of 4 g/kg (Chi-square df: 12.252, p: 0.0002); beyond reducing offspring’s vigor (df Chi-square: 54.553, p < 0.0001, and df Chi-square: p 10.552; 0.0051). There are no statistically significant differences between the number of liveborn pups in the groups (F: 0.58; p: 0.9433).

Developmental abnormalities can be assessed as: embryo-lethal lesions that may culminate in resorptions, miscarriages or stillbirths; teratogenic lesions that can result to structural or functional anomalies; and embryo- and fetotoxic lesions that cause growth retardation or alterations in physiology (Schwarz et al., 2007). Table 2 shows significant changes in external measurements of morphological parameters in fetuses that all Mp doses caused, in addition to promoting a delay period of sternum’s ossification, and skull flatting. Pigmentation in liver and kidney was also observed. Nevertheless, other common alterations such as syndactyly, cleft palate and abnormal eyes/ears implantation have not been noted.

Table 1. Effects of Mentha pulegium L. aqueous extract on the reproductive performance of female rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Weight gain of pregnant rats (g)</th>
<th>Offspring vitality (%)</th>
<th>Weight of placenta (g)</th>
<th>Weight of fetus (g)</th>
<th>Fetus/pregnancy rats</th>
<th>Implantation losses Post (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>115.00 ± 2.17</td>
<td>100</td>
<td>0.51 ± 0.04</td>
<td>2.91 ± 0.27</td>
<td>10.0 ± 1.41</td>
<td>0.99</td>
</tr>
<tr>
<td>II</td>
<td>102.1 ± 2.14*</td>
<td>91.81*</td>
<td>0.53 ± 0.09</td>
<td>2.44 ± 0.22</td>
<td>10.1 ± 1.10</td>
<td>7.27*</td>
</tr>
<tr>
<td>III</td>
<td>97.1 ± 1.69*</td>
<td>87.25*</td>
<td>0.52 ± 0.08</td>
<td>2.53 ± 0.19</td>
<td>9.90 ± 1.4</td>
<td>11.76*</td>
</tr>
<tr>
<td>IV</td>
<td>85.4 ± 3.46*</td>
<td>100</td>
<td>0.46 ± 0.05</td>
<td>1.58 ± 0.29</td>
<td>9.0 ± 0.31</td>
<td>5.26*</td>
</tr>
<tr>
<td>V</td>
<td>74.2 ± 4.78*</td>
<td>90.14*</td>
<td>0.46 ± 0.06</td>
<td>1.56 ± 0.20</td>
<td>10.67 ± 2.06</td>
<td>10.29*</td>
</tr>
<tr>
<td>VI</td>
<td>47.9 ± 3.87*</td>
<td>73.13*</td>
<td>0.41 ± 0.06</td>
<td>1.47 ± 0.29</td>
<td>9.8 ± 2.58</td>
<td>26.86*</td>
</tr>
</tbody>
</table>

Groups: I:Control; II: Mp 2.0 g/kg/alternate day; III: Mp 4.0 g/kg/alternate day; IV: Mp L 1.0 g/kg/day; V: Mp L 2.5 g/kg/day; VI: Mp 5.0 g/kg/day. N: 10 animals per group, * p<0.05 (Chi-square test or Tukey-Kramer test).
Table 2. Offspring external and internal morphological parameters whose mothers were exposed to Mentha pulegium L. aqueous extract.

<table>
<thead>
<tr>
<th>Fetus external morphological parameters (cm)</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Skull antero-posterior</td>
<td>1.42 ± 0.03</td>
</tr>
<tr>
<td>Skull latero-lateral</td>
<td>1.02 ± 0.04</td>
</tr>
<tr>
<td>Thorax antero-posterior</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>Thorax latero-lateral</td>
<td>1.12 ± 0.01</td>
</tr>
<tr>
<td>Cranio-caudal</td>
<td>4.22 ± 0.03</td>
</tr>
<tr>
<td>Tail</td>
<td>1.08 ± 0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fetus osseous structure and soft tissue (%)</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Renal pigmentation</td>
<td>0</td>
</tr>
<tr>
<td>Liver pigmentation</td>
<td>2.27</td>
</tr>
<tr>
<td>Flattening (skull soft/bones)</td>
<td>0</td>
</tr>
<tr>
<td>Parietal ossification</td>
<td>100</td>
</tr>
<tr>
<td>Sternumossification</td>
<td>100</td>
</tr>
</tbody>
</table>

Groups: I:Control; II: Mₚ 2.0 g/kg/alternate day; III: Mₚ 4.0 g/kg/alternate day; IV: Mp L 1.0 g/kg/day; V: Mp L 2.5 g/kg/day; VI: Mₚ 5.0 g/kg/day. N: 60 animals per group, *p<0.05 (Chi-square test or Tukey-Kramer test).

Conclusion

Overall, the results indicate that the oral administration of M. pulegium L. aqueous extract in daily doses of 1.0, 2.5 and 5.0 g per kg of body weight and alternates doses of 2.0 and 4.0 g/kg promotes changes in reproductive performance of female rats and induces fetotoxicity, proving to be unsafe.

REFERENCES


Antibacterial activity of organic solvent fraction from *Euphorbia supina*

Yeong-Ho Choe¹, Youn-Jin Park¹, Xiao-Wan Zhang¹, Sei-Ryang Oh²* and Byeong-Soo Kim¹*

¹Department of Companion and Laboratory Science, Kongju National University, Daehak-ro 54, Yesan-gun, Chungcheongnam-do, Republic of Korea.
²Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, 30- Yeongudanji-ro, Ochang-eup, ChungBuk 363-883, Republic of Korea.

Received 18 December, 2013; Accepted 29 April, 2014

In this study, the value of *Euphorbia supina* antibiotics and disinfectants was investigated. *E. supina* was cultivated in Wonju, Gangwon-do, Republic of Korea, until August to November, 2013. After the harvest, *E. supina* was extracted with methanol. Extracts of MeOH were partitioned with different polaric organic solvents (n-hexane, CHCl₃, EtOAc, n-BUOH and distilled water fractions). The antibacterial activity of each fraction used disk diffusion. First, all fractions of *E. supina* extracts showed antibacterial activity against Gram positive bacteria. Especially, EtOAc fractions were 14±8 mm against *Staphylococcus aureus* and 10±0 mm against *Bacillus subtilis*. EtOAc fractions were partitioned into 6 sub-fractions by high performance liquid chromatography (HPLC). Each subfraction investigated the antibacterial activity by minimum inhibitory concentration (MIC). As the second result of subfraction, number 2 subfraction showed 2.5 mg/ml against *S. aureus*, 0.62 mg/ml against *B. subtilis* and number 5 subfraction showed 2.5 mg/ml against *S. aureus*, 0.62 mg/ml against *B. subtilis*. Through this study, *E. supina* has utility as a natural pharmacological raw material for natural antibiotics and disinfectants.

**Key words:** *Euphorbia supina*, antibacterial activity, antibiotics, disinfectant.

**INTRODUCTION**

*Euphorbia supina* is an annual prostrate herb that grows in fields. Its main stream spreads on the ground and its length is about 10 to 25 cm, stem soft, curved, branched, white pilose, containing leaves and red-colored hair. On the center of it, there is red-colored macule with white latex. Fern fruit prismatic spherical, diameter 2 mm was red, white pilose; seed ovoid and angled, florescence 3 to 5 months, fruit period is from 6 to 9 month (The State Administration of Traditional Chinese Medicine “Chinese Herbal Medicine” Editorial Board, 1999; Lee, 1989).

The Dictionary of Common Chinese Herbal Medicines reported that *E. supina* can treat hemostasis, diuretic, stomachic, invigorate the circulation of blood, detoxification, jaundice, and dysentery (Tanaka et al., 1990). Also, the plant affects diarrhea, urinary tract infection, hematochezia, hematuria, uterine bleeding, bleeding...
hemorrhoids, plot rickets in children, traumatic swelling, snake bites, head sore, skin Chuangdu, trauma hemorrhage. In addition, in vitro has been reported to have an anti-cancer effect (Tanaka et al. 1990). So far, however, there have been no reports on antibacterial activity of subfraction about antibacterial activity fraction of E. supina. Chemical composition of E. supina has been reported as non-polar, triterpenoid derivatives (Tanaka and Matsunaga, 1991, 1989, 1988; Tanaka et al., 1990, 1989, 1987; Matsunaga and Morita 1983), monoterpenic lactone (Tanaka and Matsunaga, 1989), tannins, flavonoids (Lee et al. 1991), and phenol. In addition, polar compound supinaonodies A and B have been recently revealed (Cai et al., 2009).

Existing antibacterial agents were consistently raised regarding safety and anti-methanogenic properties (Olajuyigbe and Afolayan, 2012), antibacterial activity of honey has been attributed to hydrogen peroxide, osmolarity acidity, aromatic acids and phenolic compounds, a lot of researches were conducted using natural chemical compound about antibacterial agent. Among the pathogenic bacteria, millions of dollars annually for food processing and healthcare care were caused by Escherichia coli, a food-borne pathogen in North America (Liu et al., 2013), Salmonella species can be classified as a potential microorganism for bioterrorism and there are more than 2500 serovars of Salmonella spp. and all are potential pathogens (Chattopadhyay et al., 2013). Staphylococcus aureus is one of the important causes of nosocomial and cause life-threatening diseases, such as pneumonia, osteomyelitis, septicemia and endocarditis (Soromou et al., 2013). Bacillus subtilis have been used for the fermentation of soybean as a non-pathogenic microorganism that produced psbutilin, surfactin, fengycin, gramicidins, tyrocidine, iturine, and bacitracin. Taste of food can improve the natural antibacterial agent about B. subtilis for decision of fermentation period.

Here we report that E. supina extract, fraction, and subfraction have antibacterial activity against Gram positive and negative bacteria. This study investigated E. supina utility value of antibiotics and disinfectants about pathogenic bacteria and B. subtilis.

MATERIALS AND METHODS

Plant collection

E. supina was cultivated in Dongsu Farm, Wonju, Gangwon-do, Republic of Korea on autumn season August to November in 2012 and were harvested.

Test organism

Four bacterial strains were used to assess the antibacterial properties of the test samples, two Gram-positive and two Gram-negative bacteria.

The Gram-positive bacterial strains (2) used were S. aureus (KCCM 11335), B. subtilis (ATCC 11774) and Gram-negative bacterial strains (2) used were E. coli (ATCC 8739), Salmonella typhimurium (KCCM 11862).

Before use, all bacteria were cultivated in Mueller-Hinton Broth (MHB) (Difco Laboratories, Sparks, MD, USA) and then stored in 15% glycerol and frozen at -70°C.

Preparation of E. supina extract and fraction

The plants was dried at room temperature until extraction. Dried plant was ground to a powder. The plants of 3,000 g were extracted with 60,000 ml methanol. The plant was macerated three times at room temperature using fresh methanol every 24 h. The plant extracts were filtered through filter paper (Whatman, 47 mm, USA) and then evaporated to dryness using a rotary vacuum evaporator at 45°C on water bath. The methanol extract of plant was partitioned with organic solvents of different polarities to yield n-hexane, CHCl3, EtOAc, n-BUOH and water fractions, in sequence. E. supina fractions were concentrated by rotary evaporation. The concentrate was recovered with a small volume of solvent and kept open at room temperature until all the residual solvent had evaporated. The dried crude extracts was dissolved in dimethyl sulfoxide (DMSO, Biosesang, Korea, >99%) at final concentration 20 mg/ml. The samples were stored at 4°C (Figure 1).

Paper disk diffusion assay

The stored pathogenic microorganisms were cultured in Mueller-Hinton agar (MHA) at 37°C at 24 h. After 24 h of culture, selected colony from cultured MHA plate inoculated in Mueller-Hinton broth (MHB) and then cultured at 37°C with shaking. The bacterial cultures were adjusted to 10⁶ CFU/ml and inoculated onto MHA plates by streaking the swab (Wikler, 2006).

For the determination of antibacterial activity from E. supina, 10 µl of each extract and fraction solution absorbed sterilized paper disk (Whatman, 6 min diameter). The disks were placed on the surface of each inoculated plate. Amoxicillin (10 µg/ml) was used as positive control (P.C) of Gram positive, and colistin sulfate (10 µg/ml) were used as positive control of Gram negative bacteria. Negative control (N.C) prepared DMSO. The plates were incubated at 37°C for 18 h. The inhibition zone diameter around each of the disks was measured and recorded at the end of the incubation period.

Preparation of E. supina subfraction

The ethyl acetate fraction showed the highest antibacterial activity against S. aureus and B. subtilis. The fraction was partitioned by High Performance Liquid Chromatography (Dongilshimaz, Japan), Zeoprep C18, 45 to 60 mm, 7 x 50 cm, step-gradient elution of water/methanol (10 to 100%, 200 min), UV detector: 245 mm. Solvent in each subfraction was evaporated to dryness under vacuum and freeze drying.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined by microdilution in a 96-well microculture plate (Coylue, 2005). Mueller Hinton broth (100 µl) was pipetted into each required well
and plant extracts was added serial two fold dilutions (10 mg to 0.075 mg/ml).

Bacteria were inoculated into each well (10^6 cells/ml) except for negative control. Positive control inoculated only bacteria and negative control pipetted only Mueller Hinton broth. The plates were incubated at 37°C for 24 h. During incubation, absorbance was measured at 600 nm with an Eon Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA) after 4, 8, 12, 16, 20, and 24 h.

RESULTS

Plant extract

E. supina was isolated with different polaritic organic solvents (n-hexane, CHCl_3, EtOAc, n-BUOH and Distilled water fractions). Mass of each fraction was MeOH extract 285.5 g, n-Hexane fraction 63.1 g, CHCl_3 fraction 3.8 g, EtOAc fraction 50 g, n-Buthyl alcohol fraction 65.6 g, dry weight fraction 34.9 g (Table 1).

Antibacterial effect of E. supina organic solvent fraction

Antibacterial activity of E. supina used disk diffusion assay. First, E. supina had no activity against S. typhimurium and E. coli of Gram negative. But all fraction of E. supina was against Gram positive bacteria. Clean zone of each fraction was MeOH 8.8±1.6 mm, n-Hexane 8±0.9 mm, chloroform 8.8±2.8 mm, ethyl acetate 14±8 mm, n-Buthylalcohol 12±4.4 mm for S. aureus and MeOH 8.5±0.7 mm, n-Hexane 8.5±0.21 mm, chloroform 8.0±0 mm, ethyl acetate 10±0 mm, n-Buthylalcohol 9±1.4 mm for B. subtilis. Especially, EtOAc fraction showed the best antibacterial activity about S. aureus and B. subtilis (Table 2).

Isolation of ethyl acetate layer

EtOAc fraction of E. supina was isolated by High Performance Liquid Chromatography (Dongilshimaz, Japan), Zeoprep C_18, 45 to 60 mm, 7×50 cm, step-gradient elution of water/methanol (10 to 100%, 200 min), UV detector: 245 mm. The EtOAc fraction result was partitioned into six subfractions (Fraction 1 to 6). Each subfraction configured chemical compound (Figure 2).

| Table 1. Euphorbia supina Rafinesque extract mass of each solvent. |
|------------------|------------------|
| Solvent          | Mass of extracts (g) |
| Methyl alcohol   | 285.5             |
| n-Hexane         | 63.1              |
| Chloroform       | 3.8               |
| Ethyl acetate    | 50                |
| n-Buthylalcohol  | 65.6              |
| Distilled water  | 34.9              |

Antibacterial activity of EtOAc subfraction fraction (1 to 6)

Antibacterial activity of EtOAc subfraction 1 to 6 conducted MIC. The result of subfraction 2 was 2.5 mg/ml against S. aureus, 2.5 mg/ml against B. subtilis and subfraction number 5 was 0.62 mg/ml against S. aureus, 0.62 mg/ml against B. subtilis (Table 3).

DISCUSSION

Nowadays, the spread of multidrug resistance (MDR) pathogenic bacteria is a threat to our future, leading to an urgent requirement for new antibacterial compounds (Bassetti et al., 2013). Therefore, in this study we studied the antibacterial activity of multidrug-resistant, β-lactamase-producing methicillin-resistant S. aureus (MRSA) strains and a βlactamase producing methicillin sensitive S. aureus (MSSA) strain (Aqil et al., 2006), antibacterial activity against MDR pathogenic bacteria of local herbs collected from Murre, Pakistan (Mansoor et al., 2013) and volatile oils of plant was used as the antibacterial agents (Dorman and Deans, 2000). However, despite these studies, scarcely natural product was used as the natural material of antibiotics and disinfectants (Olajuyigbe and Afolayan, 2012; Khan et al., 2011). Thus, development of antibiotics and disinfectants by natural material is an urgent situation. Antibacterial activity of E. supina was reported as an antibacterial activity conforming to light intensity (Joung et al., 2011), but was not reported to be an antibacterial activity of the subfraction of antibacterial activity fraction.

Thus, this study investigated the antibacterial activity of E. supina fraction and subfraction of pathogenic bacteria. MeOH extract of E. supina was partitioned on n-hexane, CHCl_3, EtOAc, n-BUOH and Distilled water fractions. Antibacterial activity of each fraction used disk diffusion assay. First, E.supina did not have antibacterial activity against Gram negative bacteria, but all fraction of E. supina was against S. aureus, B. subtilis of Gram positive bacteria. Among the fraction, inhibition zone of EtOAc fraction was 14±8 and 10±0 mm most excellent and at least, the fraction was partitioned into 6 chemical compounds. Antibacterial activity against Gram positive bacteria of subfraction were used as MIC. The result of subfraction number 2 was 2.5 mg/ml against S. aureus and B. subtilis, and for subfraction number 5 was 0.62 mg/ml against S. aureus and B. subtilis.
Table 2. The paper disk diffusion assay of organic solvent fraction (MeOH, n-hexane, CHCl₃, EtOAc, n-BuOH and H₂O) from E. supina.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Strain</th>
<th>Methyl alcohol</th>
<th>n-Hexane</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>n-BuOH</th>
<th>PC*</th>
<th>NC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (-)</td>
<td>S. Typhimurium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30±2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>24±4</td>
<td>-</td>
</tr>
<tr>
<td>Gram (+)</td>
<td>S. aureus</td>
<td>8.8±1.6</td>
<td>8±0.9</td>
<td>8.8±2.8</td>
<td>14±8</td>
<td>12±4.4</td>
<td>42±2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td>8.5±0.7</td>
<td>8.5±2.1</td>
<td>8±0</td>
<td>10±0</td>
<td>9±1.4</td>
<td>35±0</td>
<td>-</td>
</tr>
</tbody>
</table>

*P.C: Positive control (gram positive: amoxicillin, gram negative: colistin sulfate 10μg/ml), N.C: Negative control (99%, DMSO). Na: None antibacterial activity at the highest concentration (20 mg/ml) test in this study.

Table 3. The Minimum Inhibitory Concentration (MIC) of the subfraction at EtOAc fractions from MeOH extracts of E. supina.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Microorganism</th>
<th>*Fraction 1</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
<th>Fraction 5</th>
<th>Fraction 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram (+)</td>
<td>S. aureus</td>
<td>10</td>
<td>2.5</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>&gt;10</td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td>10</td>
<td>0.62</td>
<td>5</td>
<td>&gt;10</td>
<td>0.62</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

**Euphorbia supina** Rafinesque

extract with Methyl alcohol
Methyl alcohol extract
extract with n-Hexane
Evaporation
Hexane extract
H₂O layer
extract with Chloroform
Evaporation
Chloroform extract
H₂O layer
extract with Ethyl acetate
Evaporation
Ethyl acetate extract
H₂O layer
extract with n-BuOH acetate
Evaporation
n-BuOH acetate extract
H₂O layer

Figure 1. Fraction of extraction of *Euphorbia supina* Rafinesque extract.
Figure 2. Qualitative analysis of subfraction number 1 to 6 about EtOAc fraction of *E. supina*. 
Many studies of medicinal herbal plants have reported the antibacterial activity of pathogenic bacteria. Stem bark extracts from *Jatropha curcas* was 5 mg/ml against *S. aureus* and *B. subtilis* (Igbinosa et al., 2009), ethanol extracts of *Tamarindus indica* was 20 mg/ml against *S. aureus* and 18 mg/ml against *B. subtilis* (Doughari, 2007). Depending on the result of this study, the antibacterial activity of subfraction from EtOAc fraction of *E. supina* showed relatively excellent effect. For future study, effective single compound of *E. supina* was isolated and then each single compound was examined for every kind of *in vitro* and *in vivo* test. Depending on the result of this study, *E. supina* was expected to be natural pharmacological raw material antibiotics and disinfectants.

**ACKNOWLEDGMENT**

This research was financially supported by the Ministry of Knowledge Economy (MKE), Korea Institute for Advancement of Technology (KIAT) through the Inter-ER Cooperation Projects.

**REFERENCES**


Full Length Research Paper

Effect of rice bran extract on immunological and physiological parameters of Biomphalaria alexandrina snails infected with Schistosoma mansoni

Hanan S. Mossalem1 and Abdel-Tawab H. Mossa2

1Medical Malacology, Theodor Bilharz Research Institute, Warak El Hadar, Imbaba, Giza, Egypt.
2Environmental Toxicology Research Unit (ETRU), Pesticide Chemistry Department, National Research Centre (NRC), Tahirr Street, Dokki, Cairo, Egypt.

Received 30 November, 2013; Accepted 15 April, 2014

The present study was undertaken to evaluate the effect of rice bran extract on immunological and physiological parameters of Biomphalaria alexandrina snails and Schistosoma mansoni infection. Rice bran methanolic extract showed the strongest antioxidant activity. IC50 values for scavenging DPPH was 344.22 µg/ml and reducing power was increased with increasing amounts of the extract. Total phenolic and flavonoid contents were 4.23 mg gallic acid-equivalent (GAE)/g bran and 2.11 mg quercetin-equivalent (QE)/g bran, respectively. The presence of rice bran during infection causes reduction in infection rate by 33.3%. The exposure of snails to infection for 24 h followed by treatment with 500 ppm of rice bran for 24 h caused a reduction in infection rate by 56.5% as compared to the control (80%). The reduction in infection rate of Schistosoma haematobium may be due to the increase in immune activity response, activation and vacuolation of haemocytes distributed in hemolymph of snails which represent the important system in immunity defense against any invaders. These results could provide useful information for control of Schistosomiasis in integrated pest management program and environmental protection.

Key words: Schistosomiasis, antioxidant, rice bran, control, immunological and physiological parameters, snail.

INTRODUCTION

Schistosomiasis is still a prime health problem in many tropical and subtropical countries (WHO, 1992). It is affecting about 200 million people infected worldwide with more than 95% of the infections concentrated in Africa (Gryseels et al., 2006; Steinmann et al., 2006). Currently, almost 600 million people at schistosomiasis risk in 76 endemic countries and an estimated 280,000 deaths deaths are directly or indirectly attributable to the disease annually (van der Werf et al., 2003; Gryseels et al., 2006).

Schistosomiasis is a group of diseases caused by parasitic worms of the genus Schistosoma. These blood-dwelling flukes have a complicated life cycle involving freshwater snail intermediate hosts and transmission of
transmission of the parasite is governed by social-ecological systems and intimately linked with conditions of poverty (King, 2010; Utzinger et al., 2011). In fact, fresh water pulmonate snails of the genus *Biomphalaria* are best known for their role as intermediate hosts of the widely distributed parasite, *Schistosoma mansoni* (Ross et al., 2000). One of the keys to understand the present and future of *S. mansoni* infection in Egypt is to understand more about the snails that play an indispensable role in its transmission (Lotfy et al., 2005).

The defense mechanisms and immunological responses which consist of the immune system have been considered as biomarkers of pollution in aquatic invertebrates (Galloway and Depledge, 2001). Despite the lack of an adaptive immune system, invertebrates are able to survive among potential pathogens and respond to infection by activation of various defense mechanisms (Little et al., 2005). Phagocytosis is one of the various functions of hemocytes, which is a nonspecific immune mechanism against non-self materials (Galloway and Depledge, 2001; Negrao-Correa et al., 2007). The response patterns, density and functions, of hemocytes can be affected by xenobiotics and parasites (Livingstone et al., 2000). The *Biomphalaria* internal defense system is composed of soluble components of hemolymph and circulating cells, termed hemocytes, which work in association during the snail responses against infectious agents (Van der Knaap and Loker, 1990). In snails, circulating hemocytes, especially the phagocytic cell population, are the principal line of cellular defense involved in destruction of *S. mansoni* larvae inside the intermediate host (Bayne et al., 1980; Noda and Loker, 1989; Negrao-Corrêa et al., 2007).

Histopathological analysis of *S. mansoni* infected *Biomphalaria* showed that hemocyte infiltration around parasite larvae was faster and stronger in snail strains that are more resistant to parasite infection (Negrao-Correa et al., 2007). The effector mechanisms by which hemocytes are able to kill trematode larvae are partially dependent on the capability of these cells to recognize sporocyst tegument molecules, leading to parasite encapsulation and cellular activation, that result in production of highly toxic metabolites of oxygen and nitrogen associated with parasite killing (Hahn et al., 2000).

Particular attention has been directed to immunological changes induced by environmental pollution in aquatic invertebrates. The response patterns, density and functions, of hemocytes can be affected by xenobiotics and parasites (Livingstone et al., 2000). Furthermore, the development of an infectious disease results from an imbalance between the host and the pathogen due to external factors, like pollutants, and/or internal factors, like susceptibility of the host (Snieszko, 1974).

Rice bran, a byproduct of rice milling, is a constituent (approximately 10%) of the whole rice grain and consists of the bran layers (pericarp, seed coat, nucellus, and aleurone) and the germ (Rohrer and Siebenmorgen, 2004). It is a rich source of natural antioxidants which can be used as free radical scavengers. It is widely recognized that many of the today's diseases are due to the oxidative stress that results from an imbalance between formation and neutralization of free radicals (Arab et al., 2011).

It is hypothesized that antioxidant improved immunity system in snails e.g. *Biomphalaria alexandrina* and reduced infection by pathogen e.g. *S. mansoni*. Therefore, this study was undertaken to evaluate the effect of rice bran extract on immunological and physiological parameters of *B. alexandrina* snails and *S. mansoni* infection.

**MATERIALS AND METHODS**

**Preparation of rice bran extract**

The rice bran was extracted with 95% methanol overnight with shaking at a bran-to-solvent ratio of 1:10 at room temperature. The next day, the mixture was centrifuged at 3000 rpm for 10 min and then the supernatant was collected. The pellet was extracted with 95% methanol one more time. Both supernatants were pooled and filtered, and then methanol was evaporated under reduced pressure using rotary evaporator. The crude extract residues were stored in clean dry dark vessel till use.

**Phytochemicals**

**Determination of total flavonoid content**

The total flavonoid content was determined using aluminium chloride (AlCl₃) according to Kähkönen et al. (1999) with some modifications and using quercetin as a standard. Aliquot (250 µl) of the extract (2 mg/ml) was mixed with 250 µl of a 5% NaNO₂ solution. The mixture was allowed to stay at room temperature for 6 min, then 200 µl of a 10% AlCl₃·H₂O solution was added for 6 min followed by the addition of a 2 ml 4% NaOH solution. Distilled water was added to reach a final volume of 5 ml. The solution was mixed and kept at room temperature for 15 min. Absorbance was measured immediately against the prepared blank at 510 nm using a spectrophotometer. Total flavonoid content is expressed as mg of catechin equivalents (CE)/g of extract.

**Determination of the total polyphenolic content**

The total phenolic content of rice bran methanolic extract was determined using the Folin-Ciocalteu reagent, according to the method of Slinkard and Singleton (1977) with some modifications. Aliquots (100 µl) of the extracts (2 mg/ml) were transferred into test tubes and combined with 100 µl of Folin-Ciocalteu reagent; after 3 min, 100 µl of sodium carbonate solution (2% Na₂CO₃) was added and the volume was adjusted to a final volume of 2.5 ml. After 1 h of incubation in the dark at room temperature, the absorbance was read at 760 nm using a Shimadzu UV-VIS Recording 2401 PC (Japan). The amount of total phenolic compounds was calculated as mg of gallic acid equivalents (mEq gallic acid) from the calibration curve of gallic acid standard solution. The data were presented as the average of five replicate analyses.
Antioxidant activity

Free radical scavenging activity by DPPH (1-1-diphenyl 2-picryl hydrazyl)

The hydrogen atom-or-electron donation ability of the extract was measured from the bleaching of the purple colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2,2'-diphenylpicrylhydrazyl (DPPH), as a reagent, according to the method of Amarowicz et al. (2004) with some modifications. One milliliter of different concentrations of the methanolic extract of rice bran (32 to 500 μg/ml) were mixed with 1.0 ml of 0.1 mM DPPH in methanol, and final volume adjusted up to 3.0 ml with MeOH. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min, and then the absorbance was measured at 524 nm using spectrophotometer (Shimadzu UV-VIS Recording 2401 PC, Japan). Methanol was used as blank and ascorbic acid (0.5 to 5 μg/ml) was used as the reference compound. The absorbance of solvent and DPPH radical without extract was measured as control. The radical-scavenging activities of samples, expressed as percentage inhibition of DPPH, were calculated according to the formula:

\[ I (%) = \frac{[A_C - A_S]}{A_C} \times 100 \]

where \( A_C \) and \( A_S \) are the absorbance of the control and sample, respectively.

Reducing power

Total reducing capacity of rice bran extracts was determined according to the method of Oyaizu (1986). One milliliter of extract at different concentrations (300 to 1000 μg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide [K₃ Fe (CN)₆] (1%). The mixture was incubated at 50°C for 20 min, and then a portion (2.5 ml) of trichloroacetic acid (TCA, 10%) was added to mixture, which was centrifuged for 10 min at 1000 x g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml FeCl₃ (0.1%). Then the absorbance was measured at 700 nm. Trolox (10 to 160 μg/ml) was used as the reference compound.

Experimental animals and infection

Miracidia

Schistosoma mansoni miracidia were obtained from The Schistosoma Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Egypt.

Snails

Adult Biomphalaria alexandrina snails (5 to 8 mm in height) used in the present study were obtained from the bred stock in Medical Malacology Laboratory, Theodor Bilharz Research Institute (TBRI), Egypt.

Infection of snails

Snails were infected by miracidia (10 per snail) at 25°C±1 under ceiling illumination. After exposure, snails were washed and placed in clean plastic aquaria of dechlorinated water and supplied with lettuce leaves till shedding cercariae, then calculate the number of dead snails, total livening snails and number of shedding snails after calculating the percentage of rate infection.

Experimental bioassays

Four groups of B. alexandrina snails each group contains 30 adult snails (5 to 8 mm in height).

1 (1) Normal control group.
2 (2) S. mansoni miracidia-infected group (infected for 24 h then recovery in dechlorinated water for 24 h), then divided into two subgroups. (A) First sub-group for withdrawal haemolymph for light and electron microscopy investigation. (B) Second sub-group was kept in container includes dechlorinated water till cercarea shedding, calculate the infection rat and then take samples of haemolymph for light and electron microscopy investigations.
3 (3) Rice bran group (exposed to aqueous solution of rice bran extract 500 mg/L for 3-days), then divided into two sub-groups. (A) First used for haemolymph collections for light and electron microscopy investigations. (B) Second sub-group exposed to S. mansoni miracidia for 24 h (5 to 8 miracidia for each snail) then recovery and after that kept adult B. alexandrina till shedding cercarea and count number of snails producing cercarea, number of living snails at the same time and number of dead snails to calculate rate of infection in both groups the 2nd sub-group and last groups according to the standard method recorded by WHO (1965).

For each group, glass containers each containing one liter dechlorinated water to which 10 snails were introduced. The glass containers were covered by porous plastic sheets and maintained for shedding at normal laboratory conditions (25 ± 1°C). Dead snails were distinguished and counted and feed on lettuce powder.

Hemolymph collection

Haemolymph samples were collected according to the methods of Abdul-Salam and Michelson (1983) by removing a small portion of the shell situated directly above the heart to insert a capillary tube into it. Two milliliters of hemolymph were pooled from each snail group in a vial tube and kept in ice-bath then centrifuged for 10 min at 3000 rpm at 4°C where the fresh supernatant was used for light microscope and sediment cells for electron microscope investigations.

Hemolymph examination

Light microscopy

Haemocytes staining were prepared according to Mossalem (2008). Monolayer of hemocytes were stained with Giemsa stain for 20 min according to the methods of Abdul-Salam and Michelson (1983), examined and counted by light microscopy and photographed using Agfa film RSX 100.

Electron microscopy

Sediment cells were fixed in 4% glutaraldehyde with sodium cacodylate. Two hours later, the cells were post fixed in 2% osmium tetroxide, dehydrated with ascending concentration of alcohol and embedded in epoxy resin according to the technique of Grimaud et al. (1980). Semi-thin and ultra-thin sections were cut with a Leika ultra microtome. Ultra-thin sections were contrasted with uranyl acetate and lead citrate stains then examined by Phillips EM 208 Electron Microscope.
Estimation of IC$_{50}$ values and statistical analysis

The concentrations of the extract induced 50% inhibition (IC$_{50}$) were determined by a linear regression analysis between the inhibition percentages against the extract concentrations by log-probit analysis. Results were analyzed by SPSS (version 14.0) for Windows and expressed as mean ± standard division.

RESULTS AND DISCUSSION

The results of the total phenolic content of rice brain extract using Folin-Ciocalteu method, is presented in Figure 1. TPC was expressed as gallic acid equivalents and 4.23 mg GAE/g rice brain was found. In contrast, total flavonoid content of the methanolic extract was 2.11 mg QE/g rice brain (Figure 1).

The proton radical scavenging action is known to be one of the various mechanisms for measuring antioxidant activity. DPPH radical scavenging is considered a good in vitro model widely used to assess antioxidant efficacy within a very short time. It is one of the compounds that possess a proton free radical.

Antioxidant activity on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, is neutralization its free radical character. However, the scavenging effects were 3.69 and 64.74% for rice bran extract at 31.0 and 500 µg/ml. The lowest IC$_{50}$ indicates the strongest ability of the extracts to act as DPPH scavengers. DPPH radical scavenging abilities of the rice bran extract in our study exhibited appreciable scavenging activity and the IC$_{50}$ accounted for 344.22 µg/ml (Figure 2).

Reducing powers of rice bran extract increased rapidly
at low concentrations from 0.125 to 1.00 mg/ml (Figure 3). Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay et al., 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Chanda and Dave, 2009).

The exposure of snails to rice bran for three days then exposed to infection with S. mansoni causes reduction in infection rate by 40.70% where the rate of infection in control equal 80.00%. The presence of rice bran during infection causes reduction in infection rate by 33.30%. The exposure of snails to infection for 24 h was then treated with 500 ppm of rice bran for 24 h which caused a reduction in infection rate by 56.5%, with no significance at survival rate as shown in Figure 4. So the presence of rice bran in all cases before, du-ring, and after infection with S. mansoni causes reduction in infection rate, and this may be due to the rice bran increasing immune activity response, activation and vacuolation of haemocytes which distributed in hemolymph of snails which represent the important system in immunity defense against any invaders as shown in Figures 5 and 6.
Figure 5. Photomicrograph of snail hemolymph stained by giemsa stain. Control group [A] showing the presence of small distributed cells in the hemolymph of *B. alexandrina* in normal conditions (x100). Haemocytes after exposure to rice bran for 3-days then exposed infected for 24 h [B] showing vacuolation and many pseudopodia for phagosomes (x200). Haemocytes of *B. truncates* infected with *S. mansoni* in the presence of rice bran [C] showing increasing in shape of cells due to activation against infection (x200). Haemocytes after infected for 24 h then exposed to rice bran for 3 days [D] showing haemocytes increased in shape (x200).

**Conclusion**

Rice bran extract indicated the strongest antioxidant activity. It causes reduction in infection rate of *S. mansoni*, this may be due to the increase in immune activity response, activation and vacuolation of haemocytes which distributed in hemolymph of snails which represent the important system in immunity defense.
Figure 6. Electron microscope images of normal *B. alexandrina* haemocytes, [A] showing cells with internal organelles e.g. nucleus, mitochondria, endoplasmic reticulum and cell membrane (x2000). Haemocytes after exposed to rice bran for 3-days and infected for 24 h [B] showing vacuolated cytoplasm (V), dense nuclear chromatin (Ch) and pseudopodia forming phagolysosome vacuole (x2000). Haemocytes of *B. alexandrina* infected with *S. mansoni* in the presence of rice bran [C] showing vacuolation of cells and their nucleus become eccentric (x1000). Haemocytes after infected for 24 h then exposed to rice bran for 3 days [D] showing microgram haemocytes with vacuoles and clear cell membrane forming pseudopodia (x500).
against any invaders. These results could provide useful information for control of Schistosomiasis in integrated pest management program and environmental protection.

REFERENCES


