ABOUT JMPR

The Journal of Medicinal Plant Research is published weekly (one volume per year) by Academic Journals.

The Journal of Medicinal Plants Research (JMPR) is an open access journal that provides rapid publication (weekly) of articles in all areas of Medicinal Plants research, Ethnopharmacology, Fitoterapia, Phytomedicine 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 JMPR are peerreviewed. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

Submission of Manuscript

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

The Journal of Medicinal Plant Research 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

Prof. Akah Peter Achunike
Editor-in-chief
Department of Pharmacology & Toxicology
University of Nigeria, Nsukka
Nigeria

Prof. Parveen Bansal
Department of Biochemistry
Postgraduate Institute of Medical Education and Research
Chandigarh
India.

Associate Editors

Dr. Ugur Cakilcioglu
Elazig Directorate of National Education
Turkey.

Dr. Jianxin Chen
Information Center,
Beijing University of Chinese Medicine,
Beijing, China
100029,
China.

Dr. Hassan Sher
Department of Botany and Microbiology,
College of Science,
King Saud University, Riyadh
Kingdom of Saudi Arabia.

Dr. Jianxin Chen
Information Center,
Beijing University of Chinese Medicine,
Beijing, China
100029,
China.

Dr. Sayeed Ahmad
Herbal Medicine Laboratory, Department of Pharmacognosy and Phytochemistry,
Faculty of Pharmacy, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi, 110062,
India.

Dr. Cheng Tan
Department of Dermatology, first Affiliated Hospital of Nanjing University of Traditional Chinese Medicine.
155 Hanzhong Road, Nanjing, Jiangsu Province,
China 210029

Dr. Naseem Ahmad
Young Scientist (DST, FAST TRACK Scheme)
Plant Biotechnology Laboratory
Department of Botany
Aligarh Muslim University
Aligarh- 202 002,(UP)
India.

Dr. Isiaka A. Ogunwande
Dept. Of Chemistry,
Lagos State University, Ojo, Lagos,
Nigeria.
Editorial Board

Prof Hatil Hashim EL-Kamali
Omdurman Islamic University, Botany Department, Sudan.

Prof. Dr. Muradiye Nacak
Department of Pharmacology, Faculty of Medicine, Gaziantep University, Turkey.

Dr. Arash Kheradmand
Lorestan University, Iran.

Prof Dr Cemşit Karakurt
Pediatrics and Pediatric Cardiology, Inonu University Faculty of Medicine, Turkey.

Dr. Sadiq Azam
Department of Biotechnology, Abdul Wali Khan University Mardan, Pakistan.

Samuel Adelani Babarinde
Department of Crop and Environmental Protection, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.

Kongyun Wu
Department of Biology and Environment Engineering, Guiyang College, China.

Dr. Wafa Ibrahim Rasheed
Professor of Medical Biochemistry National Research Center, Cairo, Egypt.

Prof Swati Sen Mandi
Division of plant Biology, Bose Institute, India.

Dr. Ujjwal Kumar De
Indian Veterinary Research Institute, Izatnagar, Bareilly, UP-243122 Veterinary Medicine, India.
ARTICLES

Research Articles

Anti-arthritic and cytotoxic effects of methanolic extract of Ixora nigricans leaf
Mohammad Nazmul Alam, Md. Shahrear Biozid, Ahmad Ibtehaz Chowdhury, Muhammad Moin Uddin Mazumdar, Sudipta Chowdhury and Md. Irfan Amin Chowdury

Testicular damage in Wistar rats caused by methanolic extracts of plants from the North of Mexico
Rubén García Garza, Adolfo Soto Domínguez, Miguel Angel Tellez López, Nadia Denys Betancourt Martínez, Ruben Daniel Arellano Perez-Vertti, Carlos Leyva Orasma, Benjamín Serrano Gallardo and Javier Morán Martínez
Full Length Research Paper

Anti-arthritis and cytotoxic effects of methanolic extract of *Ixora nigricans* leaf

Mohammad Nazmul Alam*, Md. Shahrear Biozid, Ahmad Ibtehaz Chowdhury, Muhammad Moin Uddin Mazumdar, Sudipta Chowdhury and Md. Irfan Amin Chowdury

1 Department of Pharmacy, Faculty of Science and Engineering, International Islamic University Chittagong, 154/A, College Road, Chittagong-4203, Bangladesh.

Received 13 June, 2015; Accepted 26 June, 2015

This study aimed to evaluate anti-arthritis and cytotoxic effects of methanolic extract of *Ixora nigricans* leaf. Anti-denaturation method was performed by using bovine serum albumin (BSA) to evaluate the anti-arthritis potential. The screening of cytotoxic activity was done using brine shrimp lethality bioassay. The *in vitro* study on *Ixora nigricans* leaf showed the presence of significant anti-arthritis activity. Here, the *Ixora nigricans* leaf showed 79.35% at 1000 µg/ml and 46.77% at 31.25 µg/ml concentration, whereas the standard drug showed 85.49% at 1000 µg/ml and 51.61% at 31.25 µg/ml concentration. Moderate cytotoxicity was found for methanolic extract and it was compared with the standard drug vincristine sulfate in the brine shrimp bioassay. In the present study, the LC$_{50}$ values of methanolic crude extract of *Ixora nigricans* leaf and vincristine sulfate were 179.18 and 12.59 µg/ml, respectively. The results of this study demonstrated that methanolic extract of *I. nigricans* leaf contains significant anti-arthritis activity and moderate cytotoxic activity.

Keywords: *Ixora nigricans*, cytotoxicity, brine shrimp, anti-arthritis, inhibition, protein denaturation.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune chronic, systemic inflammatory disease predominantly affecting joints and peri-articular tissues. RA still remains a formidable disease, being capable of producing severe crippling deformities and functional disabilities. The association of blood clotting mechanisms with inflammation is more firmly established in some types of injury. Prostaglandin E1 (PGE1) was found to act on erythrocytes in such a way that it causes phospholipids disruption. Changes in protein or lipoprotein structure might account for the development of erythrocyte membrane destabilization in polyarthritis and rheumatoid arthritis (Sadique et al., 1989). Brine shrimp lethality evaluation is a bench top bioassay method for evaluating anticancer, antimicrobial and other pharmacological activity of natural products. Natural products extracts...
fractions or pure compounds can be tested for their bioactivity by this method (Dockery et al., 2000). *Ixora nigricans* R. Br. (Rubiaceae) is a large shrub which is found throughout Bangladesh, the forests of India and Indo-malaysia (Barbhuiya et al., 2014). In local tribes of Bangladesh, it is known as Dikranga Chuillya (Chakma, Tripura), Rongma, Frareko (Marma). Extract of root is used to treat diarrhoea and ear infections by the Chakma. A paste of the leaves is applied to affected areas for the treatment of boils, pills prepared from the paste of the leaves are taken thrice daily for dysentery by the Tanchangya. Extract prepared from leaf taken and paste prepared from root is applied in the whole body as a remedy for unconsciousness of little child and extract prepared from root, taken one cupful four times daily for two days against vomiting over bleeding by the Marma (Yusuf et al., 2009). EtOH (50%) extract of aerial parts is antiviral, spasmylytic and CNS depressant (Asolkar et al., 1992).

**MATERIALS AND METHODS**

**Collection and proper identification of plant**

*Ixora nigricans* R. Br. was collected from Chittagong Hill near Mimi super market area, Chittagong. The sample was identified by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong. (12th October, 2013).

**Chemicals and drugs**

The chemicals used were bovine serum albumin (BSA), diclofenac sodium, absolute methanol (99.50%) and vincristine sulfate (VS) were purchased from Sigma-Aldrich, Munich, Germany. All chemicals in this investigation were of analytical reagent grade.

**Preparation of extract**

Plant materials were dried and ground (Moulinex Blender AK-241, Moulinex, France) into powder (40 to 80 mesh, 500 g) and soaked for 7 days with 2 to 3 days interval in 2.0 L of methanol at room temperature (23 ± 0.5°C). Filtrate obtained through cheese cloth and Whatman filter paper No. 1 was concentrated under reduced pressure at a temperature below 50°C using a rotary evaporator (RE 200, Sterling, UK). The extracts (yield 4.4 to 5.6% W/W) were all placed in glass petri dishes (90 × 15 mm, Pyrex, Germany). A 100 mg each of the extracts was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer. In this way, the concentration (10 mg/ml) of extracts was prepared for screening the cytotoxic properties.

**Inhibition of protein denaturation in vitro**

For the inhibition of protein denaturation, in vitro diclofenac sodium was used as standard. The test solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of methanol extract of *Ixora nigricans*. The control solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of methanolic extract of *Ixora nigricans*. Standard solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of diclofenac sodium. Various concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/ml) of methanol extract of *Ixora nigricans* (IN) and diclofenac sodium (standard) were taken, respectively.

All the solutions were adjusted to pH 6.3 using 1 N HCL. Samples were incubated at 37°C for 20 min and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5 ml of phosphate buffer was added to the previous solutions. The absorbance was measured using UV-Visible spectrophotometer at 416 nm. The control represents 100% protein denaturation. The results were compared with diclofenac sodium. The percentage inhibition of protein denaturation of different concentrations is tabulated in Table 1. The percentage inhibition of protein denaturation can be calculated as:

\[
\% \text{ inhibition} = \left[100 - \left( \frac{\text{OD of test solution} - \text{OD of product control}}{\text{OD of control}} \right) \right] \times 100
\]

Where OD = optical density.

The control represents 100% protein denaturation. The results were compared with diclofenac sodium.

**Cytotoxicity screening**

Cytotoxicity of the methanol extracts of *Ixora nigricans* was evaluated by the brine shrimp lethality bioassay (Figure 2), which is widely used for screening bioactive compounds (Meyer et al., 1982; Zhao et al., 1992). In this study, a simple zoological organism (*Artemiasalina*) was used as a convenient monitor for the experiment. The eggs of the brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 h to develop into larval shrimp called nauplii. The cytotoxicity assay was performed on the brine shrimp nauplii using the Meyer method.

The test samples (extract) were prepared by dissolving them in DMSO (not more than 50 µl in 5 ml solution) plus seawater (3.8% NaCl in water) to attain concentrations of 10, 50, 100, 150, 200 and 300 µg/ml. A vial containing 50 µl DMSO diluted to 5 ml was used as a control. Standard vincristine sulfate was used as a positive control. Mature shrimps were placed into each of the experimental vials. After 24 h, the vials were inspected using a magnifying glass, and the number of surviving nauplii in each vial was counted. From these data, the percentage lethality of the brine shrimp nauplii was calculated for each concentration using the following formula:

\[
\% \text{ Mortality} = \left( \frac{N_1}{N_0} \right) \times 100
\]

Where \(N_1\) = Number of dead nauplii after a 24 h incubation; \(N_0\) = Number of total nauplii transferred (10).

The LC50 (median lethal concentration) was determined from the log concentration versus % mortality curve.
Table 1. Percentage inhibition of protein denaturation of *Ixora nigricans*.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Percentage inhibition in protein denaturation</th>
<th>Diclofenac sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.25</td>
<td>46.77 ± 1.38*</td>
<td>51.61 ± 0.86</td>
</tr>
<tr>
<td>62.5</td>
<td>52.65 ± 1.66*</td>
<td>61.29 ± 0.67</td>
</tr>
<tr>
<td>125</td>
<td>59.43 ± 1.48</td>
<td>64.52 ± 0.71</td>
</tr>
<tr>
<td>250</td>
<td>68.52 ± 1.77</td>
<td>74.19 ± 1.19</td>
</tr>
<tr>
<td>500</td>
<td>74.84 ± 1.30*</td>
<td>80.65 ± 0.91</td>
</tr>
<tr>
<td>1000</td>
<td>79.35 ± 0.81**</td>
<td>85.49 ± 0.86</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M. of three replicate (n = 3); Here, **P < 0.01, *P < 0.05.

Table 2. Percentage mortality of brine shrimp extract at six concentrations.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Log C</th>
<th>Percentage mortality</th>
<th>Vincristine sulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>50</td>
<td>1.699</td>
<td>30</td>
<td>80</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>200</td>
<td>2.301</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>300</td>
<td>2.477</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>2.699</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>LC50</td>
<td>-</td>
<td>179.18</td>
<td>12.59</td>
</tr>
</tbody>
</table>

*IN = Ixora nigricans.*

RESULTS AND DISCUSSION

Anti-arthritic study

Different concentrations of methanolic extract of *Ixora nigricans* and diclofenac sodium were tested for anti-arthritic activity and found significant percentage inhibition in protein denaturation (Table 1). In this study, methanolic extract of *Ixora nigricans* showed 46.77%, where the standard drug diclofenac sodium showed 51.61% of inhibition at 31.25 µg/ml. The extract of *Ixora nigricans* exhibited 79.35% inhibition, while the diclofenac sodium exhibited 85.49% inhibition of protein denaturation at 1000 µg/ml.

Brine shrimp lethality bioassay

Following the procedure of Meyer, the lethality of methanol *Ixora nigricans* leaf extract was determined on *Artemia salina* after sample exposure for 24 h. The negative control (vehicle only) and vincristine sulfate (positive control) were also used to compare the toxic activities of the extracts. Percentage mortality of brine shrimp at six different concentrations (10 to 500 µg/ml) of the extracts has been presented in Table 2. From Figure 1, it is clear that the percentage mortality is directly proportional to the extract concentrations. LC50 values of methanol extract of *Ixora nigricans* obtained in the present experiment was 179.18 µg/ml. The LC50 value for the standard drug vincristine sulfate was 12.59 µg/ml. However, no mortality was obtained for the negative control group.

Conclusion

Arthritis is a type of joint disorder that involves inflammation of one or more joints, responsible for pain, swelling, stiffness and loss of function in joint. Denaturation of protein which is one of the causes of arthritis was documented. Production of auto antigen in certain arthritic disease may occur due to the denaturation of protein. The mechanism of denaturation
Figure 1. Percentage inhibition of methanolic extract of *Ixora nigricans* and the standard diclofenac on protein denaturation. IN = *Ixora nigricans*.

Figure 2. Brine shrimp lethality bioassay. Determination of LC50 values for methanolic extract of *Ixora nigricans* from a linear correlation between log concentrations versus % of mortality; *IN = Ixora nigricans.*
probably involve alteration in electrostatic hydrogen, hydrophobic and disulphide bonding (Shravan et al., 2011). Here, the methanol extract have shown promising activity at various concentrations and the effects were compared with the standard drug diclofenac sodium.

The maximum percentage inhibition of protein denaturation of *Ixora nigricans* leaf was observed as 79.35% at 1000 μg/ml which were close to the percentage inhibition of diclofenac sodium (85.49%). From the result, it can be stated that this extract is capable of controlling the production of auto antigen to inhibit the denaturation of protein. Toxicity profile of plant materials is mainly an important criteria to experts and medical practitioners (Singh et al., 2005; Fowles et al., 2012; Okwuosa et al., 1993) and cytotoxic assay was conducted in this study to learn about the toxicity study of plant extract through the brine shrimp lethality (LC50, 24 h) test. Logarto et al., (2001) showed a great correlation (r = 0.85; P < 0.05) between the LC50 of brine shrimp lethality test and the severe oral toxicity assay in mice. Based on that correlation, brine shrimp lethality LC50 < 10 μg/ml (LD50 between 100 and also 1000 mg/kg) is measured as cutoff value on cytotoxicity (Logarto et al., 2001; Chew et al., 2012).

In this present experiment, modest brine shrimp cytotoxicity was found for methanolic extract of *Ixora nigricans* compared with the standard drug vincristine sulfate. In conclusion, the present study, using in vitro experiments established that methanolic extract of *Ixora nigricans* (leaf) has moderate anti-arthritic and low cytotoxic effect. This is only a preliminary study but the plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of biologically important drug candidates.

**ACKNOWLEDGEMENT**

Authors wish to thank botanist Dr. Shaikh Bokhtear Uddin, Assistant professor, Department of Botany, University of Chittagong, Bangladesh, who helped to identify these plants. The authors are grateful to the Department of Pharmacy, International Islamic University, Chittagong, Bangladesh, for providing support and research facilities.

**Conflicts of interest**

Authors have none to declare.

**REFERENCES**

Asolkar LV, Kakkar KK, Chakre OJ (1992). Second Supplement to...
Full Length Research Paper

Testicular damage in Wistar rats caused by methanolic extracts of plants from the North of Mexico

Rubén García Garza¹, Adolfo Soto Domínguez², Miguel Angel Tellez López³, Nadia Denys Betancourt Martínez¹, Ruben Daniel Arellano Perez-Vertti¹, Carlos Leyva Orasma⁴, Benjamín Serrano Gallardo⁵ and Javier Morán Martínez¹

¹Departamento de Biología Celular y Ultraestructura, Centro de Investigación Biomédica, Facultad de Medicina, Universidad Autónoma de Coahuila, Unidad Torreón. Av. Gregorio A. García 198 Sur, Torreón, Coahuila, México. C.P. 27000, Mexico.
²Departamento de Histología, Facultad de Medicina, Universidad Autónoma de Nuevo León. Av. Madero y E. Aguirre-Pequeño, Monterrey, Nuevo León, México. A. P.1563, Mexico.
⁴Universidad Autónoma Agraria Antonio Narro, Unidad laguna, Periférico Raúl López Sánchez y Carretera a Santa Fé, C.P 27054, Torreón, Coahuila, México.
⁵Departamento de Bioquímica y Farmacología, Centro de Investigación Biomédica, Facultad de Medicina, Universidad Autónoma de Coahuila, Unidad Torreón. Av. Gregorio A. García 198 Sur, Torreón, Coahuila, C.P. 27000, México.

Received 24 May, 2015; Accepted 25 June, 2015

The aim of this study was to evaluate the effect of methanolic extract of plants from the north of Mexico, on testicular morphology and spermatic quality in Wistar rats. Methanolic extracts were orally administered to 4 experimental groups (n=6) of rat for 30 days; a control group not treated with plant extract was included. All animals were sacrificed 24 h after the last dose. Samples of testicles were collected, fixed, processed by histological technique and embedded in paraffin blocks. Histological sections were stained with H&E, Masson's trichrome and histochemical PAS reaction with diastase. Results of the groups treated with methanolic extract of Tagetes lucida, Cynodon dactylon, Lippia graveolens HBK, and Opuntia ficus-indica testicle samples demonstrated epithelial detachment and cell fragmentation, an apparent decrease in the diameter of seminiferous tubules, with large empty spaces between them and areas with fragmentation of basal membrane. Sperm quality analysis (concentration, motility and viability) showed a significant decrease of these spermatic parameters in all treated groups. In conclusion, this paper demonstrated for the first time that methanolic extracts from plants of the north of Mexico, have toxic effect on testicle and affects spermatic parameters of Wistar rats.

Key words: Plants, testicle, spermatic quality, methanolic extract, Wistar rat, toxicity.

INTRODUCTION

In traditional medicine, plants have been used for hundreds of years to treat a wide variety of health-related
disorders. The scientific research of plants used in traditional medicine with a multidisciplinary approach has increased worldwide, with more than 13,000 superior plants having been studied in the past five years, leading to sufficient scientific evidence on the pharmacological properties of these plants (Dahanukar et al., 2000). With this knowledge, the pharmaceutical industry has synthesized from active compounds isolated from plants. About 25% of the most successful drugs in the market are aspirin and tamoxifen (Tripath, 2005). Numerous plants used frequently in traditional medicine have shown effects like antioxidant and antimutagenic activities (Lee et al., 2002; Martinez-Rocha et al., 2008), antidiabetic activity (Frati et al., 1990; Singh et al., 2007, 2008; Jarald et al., 2008), neuroprotective and antidepressant effect (Dok-Go et al., 2003; Guadarrama-Cruz et al., 2008), antiulcer activity (Galati et al., 2001), antimicrobial (Salgueiro et al., 2003; Hernández et al., 2006), antifungal and antibacterial (Céspedes et al., 2006) as well as acarcidal effects (Martinez-Velázquez et al., 2011). Also, it has been described that medicinal plants have reproductive regulatory effects like infertility stress-induced (Chidrawar et al., 2011) and sperm DNA fragmentation induction (Meamar et al., 2012); for this reason, it calls for the opportunity to search new alternatives for fertility regulation in human reproduction (Talwar et al., 1997).

These alternatives can associate scientific literature and popular knowledge of plants with spermicidal effect, to suggest a regulator method of fertility from plant origin which retains spermicides advantages without cytotoxicity against epithelial cells (Upadhyay et al., 1993; Kumar et al., 2012). Currently, research has focused on evaluating and developing a regulator of male fertility which is both safe and effective, with reversible production of azoospermia, and suppresses sperm production by hormonal and non-hormonal methods (Lopez et al., 2006). Recently, plant products with low toxicity have been used to regulate fertility (Singh and Singh, 2008). According to Álvarez-Gómez et al. (2007), some plants demonstrating pharmacological fertility control are Abrus precatorius (black color pill), Albizia lebbeck (shadow chickadee or sleepy), Aloe vera (sábila), Ananas comosus (pineapple), Anethum graveolens (dill), Apium graveolens (celery), Azadirachta indica (neem tree) (Upadhyay et al., 1993), Bursera fagaroides (cujote), Calendula officinalis (gold button, crown of king, flamenguilla, dead flower, rose of dead or cempasúchiltl), Carica papaya (papaya), Citrus limon (lemon), Curcuma longa (Indian saffron), Cyclamen persicium and Primula vulgaris (violets from the Andes and springs), Eupatorium brevipes, Gypsophila paniculata (paniculata or wedding veil), Momordica charantia (bitter melon or sibicogén), Passiflora edulis (maracuyá or passion fruit), Pithecellobium saman (saman rain tree), etc.

Studies in cattle reported that incorporation of Cynodon dactylon (Bermuda grass) in the diet decreased the reproduction rate (Banta et al., 2008). Furthermore, ethnobotanical studies in Mexico and Mediterranean area of Europe, reported the traditional use of Ceterach officinarum (Doradilla) as a method of contraception, but this has not been fully tested (Márquez et al., 1999; Guerrera et al., 2008). Same information was reported in studies with an extract of Lippia graveolens (oregano), which is known to be toxic in high doses and is traditionally believed to affect reproduction when ingested as a tea (Longe, 2002). There is empirical evidence that communities in the State of Durango in Mexico use Tagetes lucida (yerbanís) and Opuntia ficus-indica (prickly pear) as reproductive control methods; but to date, there is no scientific evidence of anti-reproductive properties of these plants (González et al., 2004). Little studies have been done on the flora from the North of Mexico, and the literature provides few references on the study of their pharmacological activities and possible contribution to the ethnopharmacology. The objective of this study was to evaluate by morphological methods, the effect of methanolic extracts of T. lucida, O. ficus-indica, C. dactylon, and L. graveolens HBK, from the North of Mexico on testicle of male Wistar rats.

MATERIALS AND METHODS

Collection of plants

About 5 kg of leaves or stalks of T. lucida, C. dactylon, L. graveolens HBK and O. ficus-indica were collected in the rural area of Torrón Coahuila in the north of Mexico, on an altitude of 1,140 m above the sea. To collect plants tested, Guidelines for Good Practice Plant Collection proposed by the World Health Organization (WHO, 2003). Identification of the specimens were performed by a botanic specialist from the Universidad Autónoma Agraria Antonio Narro, Campus Torrón.

Preparation of methanolic extracts

Plant material was dried in a solar drier with aeration for 5days. When dried, leaves and stalk were ground and passed through a sieve with 20 mesh (inch 0030, MM 08600) to obtain a fine powder as proposed by Navarro et al. (2006). Then, 30 g of dry ground plant material was weighed and added to 300 ml of methanol (analytical grade) (JT Baker, USA) in an Erlenmeyer flask (Payrex, USA). Flask was capped with parafilm, stirred at low speed and maintained at 37°C for 24 h. Mash was filtered with Whatman filter paper (Analytical grade) (Whatman, USA), and filtered again 3 times with same solvent. The filtrate obtained was distilled in a rotavapor (Rotavapor Buchii-215®, USA) to evaporate the solvent; crude extract was completely dried in a hot air oven at temperature lower than 50°C for 7 days (Benchmark Scientific Mini Incubator, USA). Dried extracts were stored at 4°C, in amber vials until use (Nostro et al., 2000; Navarro et al., 2006).

Animals and study groups

In this study, 30 Wistar male rats were used with 280 to 300 g weight, and were 60 days old. Age of animals was selected...
according to events of maturation in reproductive cycle of the rat. Rats per group were placed in plastic boxes. The animals were kept in a controlled environment with room temperature between 25 and 28°C (temperature control: 2H/JT-03, Lennox®, Mexico), relative humidity between 30 and 70% (Minisplit, Lennox®, Mexico), light/darkness cycles of 12 h (Photoperiod was regulated by an electric timer and light intensity was at least 300 lx; positioned laterally to the eyes of the animals; Electric R/F, China), with free access to food (NUTRI-CUBES, Agribrands Purina®, Mexico) and water ad libitum. Rats were subjected to a period of acclimatization for 7 days before the first administration of extracts. Extracts were suspended in distilled water and were orally administered daily with gastric probe for 36 days; a pilot assay was conducted to determine the effective dose that resulted in 50 mg/kg for all extracts; this dose was administered to the following groups.

Animals were organized in 5 groups as follows: Control group (n:6), group without treatment; Group 1 (n:6), treated with 50 mg/kg extract of T. lucida; Group 2 (n:6), treated with 50 mg/kg extract of C. dactylon; Group 3 (n:6), treated with 50 mg/kg extract of L. graveolens HBK; Group 4 (n:6), treated with 50 mg/kg extract of O. ficus-indica.

Experiments were carried out in accordance with the International Guidelines on the Appropriate Use of Experimental Animals, and according to Mexican Norm NOM-062-ZOO-1999 on the Technical Specifications for Production, Care and Use of Laboratory Animals (SAGARPA, 2010) and cared for by expert veterinarian (professional license: 4807528). The protocol was approved by the Bioethical Committee of the Faculty of Medicine of the Autonomous University of Coahuila, Torreon Campus, Coahuila in Mexico (Number of approval by the Secretaría de Salud and Comisión Nacional de Bioética in Mexico: CONBIOETICA07CE100320131015).

Tissue samples collection

24 h after the last dose had been administered, all animals were sacrificed by cervical dislocation. Testicle samples were placed in 10% formalin with phosphate buffered saline (PBS) pH 7.2 to 7.4, processed by conventional histological technique and embedded in paraffin blocks.

Morphological evaluation

5-micron histological sections were obtained, mounted, and stained with hematoxylin and eosin (H&E) and Masson’s trichrome; pretreatment with diastase and periodic acid Shiff (PAS) was used for histochemical analysis. Samples were analyzed by light microscopy to evaluate the morphology of different stages of development of spermatogonia, Sertoli cells and Leydig cells (Gilbert et al., 1986).

Collection of sperm from the epididymis

Sperm cells were collected from the epididymis in test tubes and immediately washed with 3 ml of physiologic solution (NaCl0.9%), then washed again with HamF-10 culture medium (Sigma-Aldrich, San Luis MO, USA) and incubated at 37°C for 30 min. Concentration of cells was carried using bright field microscopy according to Zhou et al. (2006). To determine the percentage of sperm motility, a drop of sperm cell suspension was placed in a Neubauer chamber; sperm were counted in 10 different fields and the number of sperm with lineal mobility was determined with total mobility. A total of 200 sperm per sample were evaluated as described by Zhou et al. (2006).

Data analysis

Analysis of variance, and means comparison Pearson test with P≤0.05 were performed. The statistical software SPSS version 20.0 for Windows was used to analyze the variables.

RESULTS

Morphological alterations

In testicle samples of control group stained with H&E, a normal histology was observed: seminiferous tubules with epithelium without peeling or artifacts (Figure 1A). In group treated with the methanolic extract of T. lucida, destruction of some seminiferous tubules, characterized by epithelial detachment with cell fragmentation was observed (Figure 1B). In group 2 that received extract of C. dactylon, same alterations described earlier were observed alternating with areas without damage in the same seminiferous tubule (Figure 1C and D). In groups 3 and 4 treated with L. graveolens HBK and O. ficus-indica, respectively, same alterations described were observed, with an apparent decrease in the diameter of the seminiferous tubules, accompanied with large empty spaces between them (Figure 1E). With Masson trichrome method, no differences were observed among the control group (Figure 1F) and treated groups (Figure 1G and J). In samples analyzed with histochemical reaction of PAS with diastase from the control group, basal membrane was observed as a continuous line in magenta color (Figure 1K). In treated groups, areas with fragmentation of the basal membrane related to the areas of epithelial disruption were observed (Figure 1L and O).

Sperm quality analysis

Results of sperm quality analysis showed a significant decrease in sperm concentration in the group treated with O. ficus–indica (Figure 2A). Significant decrease in sperm motility was observed in groups treated with T. lucida, L. graveolens HBK and O. ficus–indica (Figure 2B). Groups treated with C. dactylon, L. graveolens HBK and O. ficus–indica showed a marked decrease in sperm viability, this was more evident in group treated with O. ficus–indica (Figure 2C).

DISCUSSION

This study, reports for the first time that methanolic extracts of T. lucida, C. dactylon, L. graveolens HBK and O. ficus–indica from the north of Mexico, have toxic effects on testicle and affect spermatogenic parameters of Wistar rats. These results correlate with other studies that demonstrated these same injuries after administration of extracts of Lagenaria breviflora (Saba et al., 2009), Carica papaya (Lohiya et al., 2002), A. precatorius (Sinha and Mathu, 1990), Martynia annua (Mali et al., 2002),
Figure 1. Methanolic extracts induced histopathological alterations in testicle: (A) Control group shows a normal histology. Groups treated with methanolic extracts: (B) Tagetes lucida, (C) Cynodon dactylon, (D) Lippia graveolens HBK, and (E) Opuntia ficus-indica. Varying degrees of detachment and epithelial cells destruction are observed (white arrows). H&E. Methanolic extract did not cause fibrosis: (F) Control group shows few fibers of collagen, no differences were observed between control and treated groups (G-J). Masson’s trichrome. Fragmentation of basal membrane: (K) Intact basement membrane in control group is observed. In treated groups (L-O), areas of fragmentation of basal membrane (white arrows) are observed in relation to areas of epithelial damage. PAS with diastase. Paraffin embedded, light microscopy. Bar: 50 µm.
among others (Kamal et al., 2003; D’Cruz et al., 2010). The mechanism of damage to the testicular epithelium has not been fully established, but several studies tested other plant extracts and described selective damage to spermatogonia (Sinha and Mathu, 1990), Sertolicells (Lohiya et al., 2002) and Leydigcells (Oluymet et al., 2007), as well as a decrease in serum levels of testosterone (Ashok and Meenakshi, 2004; Takizawa et al., 2004; Yakubu et al., 2007; Shukkani et al., 2007) and spermatic genotoxicity (Tellez-López et al., 2014). In our study, the decrease in sperm quality was more pronounced in groups treated with L. graveolens HBK and O. ficus-indica, this correlates with the degree of damage to the testicular epithelium observed in histopathological sections.

The damage observed in testicular epithelium, as well as the decrease in sperm quality were not in the same intensity in the different study groups; they were more marked in groups treated with L. graveolens HBK and O. ficus-indica, and less evident in group treated with C. dactylon. The degree of damage to the testicular epithelium has been described as dose dependent (Gupta et al., 2004; Tellez-López et al., 2013); our findings suggest that an increased dose of C. dactylon, could cause similar damage to that observed with extracts of L. graveolens HBK and O. ficus-indica. Finally, the accented damage in testicular epithelium, and the adverse effect on sperm quality parameters caused by extracts from L. graveolens HBK and O. ficus-indica, makes them good candidates for male fertility control; however, other physiological studies are required to support their use in humans. Several studies describe that extracts of Azadirachta indica (Shaikh et al., 1999), Dendrophthoe falcate (Gupta and Kachhawa, 2007) and Taraxacum officinale (Tahtamouni et al., 2011), affect the structure and sperm function that results in azoospermia (Purandare et al., 1999). To date, current studies are carried out to elucidate the mechanism of damage on testicular epithelium, and how the methanolic extract evaluated in this study decreased the sperm quality. Also we are investigating if morphological alterations in testicle of treated rats, are reversible or not after discontinuation of treatment.

Conflict of interest

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

The authors acknowledged the work of Biol. René
Garza et al. 729

Reynaga-Piña for technical assistance involved in processing and staining of the samples and Lic. Daniel García for English language review of the manuscript. Miguel Ángel Téllez-López and Nadia Denys Betancourt-Martínez were supported by CONACyT scholarships #305943 and #216334, respectively. Javier Morán Martínez was supported by Fogarty International Center CareerTrac, School of Public Health, University of California at Los Angeles. Grant Number: D43TW0623 Program Name: ITREOH.

REFERENCES


