Asian Pac J Trop Biomed 2016; 6(3): 202–210

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Floral research http://dx.doi.org/10.1016/j.apjtb.2015.12.003

Sub-chronic effects of a *Phthirusa pyrifolia* aqueous extract on reproductive function and comparative hormone levels in male rats

Romero Marcos Pedrosa Brandão-Costa[1,2*], Vivianne Ferreira Araújo[1], Elizabeth Neves[3], Maria Tereza Santos Correia[1], Ana Lúcia Figueiredo Porto[4], Maria das Graças Carneiro-da-Cunha[1,2]

[1]*Department of Biochemistry, Center of Biological Sciences, Federal University of Pernambuco, Prof. Moraes Rego Avenue, s/n, CEP 50.670-420 Recife, PE, Brazil*

[2]*Keizo Asami Laboratory of Imunopathology, LIKA/UFPE, CEP 50.670-901 Recife, PE, Brazil*

[3]*Department of Anatomy, Federal University of Pernambuco, Prof. Moraes Rego avenue, s/n, CEP 50.670-420 Recife, PE, Brazil*

[4]*Department of Animal Morphology and Physiology, Federal Rural University of Pernambuco, Dom Manoel de Medeiros Street, s/n, Dois Irmãos, CEP 52171-900 Recife, PE, Brazil*

ARTICLE INFO

Article history:
Received 17 Aug 2015
Received in revised form 9 Oct, 2nd revised form 4 Nov 2015
Accepted 15 Nov 2015
Available online 31 Dec 2015

Keywords:
Reproductive function
Phytochemical
Testosterone
Finasteride
Phthirusa pyrifolia

ABSTRACT

Objective: To explore the effects of aqueous extract from *Phthirusa pyrifolia* leaves (67 mg/kg body weight for 12 days) on the reproductive function of male Wistar rats through oral administration.

Methods: Animals (n = 30), aged 13 weeks and weighing (378.5 ± 5.0) g, were housed in a vivarium under controlled environmental conditions [photoperiod of 12 h light/dark, temperature of (23 ± 1) °C] and were fed standard rations *ad libitum*. The experiment ran for 12 days, wherein animals were divided into three groups: negative control (n = 6) received water, positive control (n = 12) with finasteride at a concentration of 1.0 mg/kg; and a test group (n = 12) submitted to aqueous extract. At the end of the experiment, the animals were sacrificed and submitted to analyses.

Results: The morphological results of the testes showed that the aqueous extract induced significant changes in the diameter and cross-sectional area of the seminiferous tubules as well as the thickness of the seminiferous epithelium. Furthermore, the extract was able to abruptly decrease testosterone concentrations by about 81.88% in the treated group when compared with the negative control, (47.0 ± 4.8) ng/dL and (255.0 ± 2.0) ng/dL, respectively, and 76.8%, (211.0 ± 8.7) ng/dL, when compared with finasteride. However, the extract causes neither liver damage nor impairment of renal function.

Conclusions: These results suggest that the high amounts of flavonoids shown to be in the extract may be responsible for its hepato-protective effects and suggest a possible decrease in the libido and reproduction of rats.

1. Introduction

Ethnopharmacological studies have suggested medicinal uses for a large number of plants and provided a source for novel biological assays which may even affect reproduction in rats and various mammals through the presence of secondary metabolites [1,2]. These molecules can be classified based on their chemical structure (*e.g.*, rings with or without sugar moieties), composition (*e.g.*, presence or absence of nitrogen), the pathways by which they are biosynthesized or their solubility in various solvents [3]. According to the literature, plant extracts and secondary metabolites have been shown to have excellent biological activity, including antihypertensive, anti-inflammatory,

*Corresponding author: Romero Marcos Pedrosa Brandão-Costa, Department of Biochemistry, Center of Biological Sciences, Federal University of Pernambuco, Prof. Moraes Rego Avenue, s/n, CEP 50.670-420 Recife, PE, Brazil.
Tel: +55 81 2126 8540
Fax: +55 81 2126 8576
E-mails: romero_brandao@outlook.pt, romero.bcosta@ufpe.br

All experimental procedures involving animals were conducted in accordance to the Brazilian College of Animal Experimentation - (BCAE) and approved by the Committee for Ethics in Animal Experimentation of the Federal University of Pernambuco (No. 23076.012671/2006-09)The Animal Ethics Committee of the Federal University of Pernambuco.

Foundation project: Supported by the National Research Council (CNPq), Foundation for Science and Technology of the State of Pernambuco (FACEPE) and Personnel Improvement Coordination-CAPES/PROCAD/NF/no 1415/2007, Brazil.

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

hepatoprotective, anti-tumor, antioxidant, anti-diarrheic, anti-spermatogenic or may stimulate spermatogenesis, and increase or decrease serum testosterone levels [4]. Several animal studies using rats as models have indicated that some plant extracts, for example from *Andrographis paniculata* and *Tulbaghia violacea*, feature contraceptive or anti-fertility effects following long-term treatment at low or high doses, respectively [5,6]. Generally, these researchers have observed changes in morphological-morphometric testicular parameters and levels of testosterone hormone [7]. Frequently used in these studies, finasteride, a type-2 5a reductase inhibitor that blocks the conversion of testosterone (T) to its more potent form, dihydrotestosterone, is the principal synthetic drug used today. At a dosage of 5 mg/day, finasteride has been approved since 1992 to treat benign prostatic hyperplasia. Since 1997, a dosage of 1 mg/day has been approved to treat androgenic alopecia (male pattern baldness). Studies of the effects of finasteride on male fertility are few, and those that do exist often comprise small samples and case reports [8].

Phthirusa pyrifolia (*P. pyrifolia*) is a hemi-parasitic plant that belongs to the Loranthaceae family (mistletoe). This species is popularly known in Brazil as "erva de passarinho" (bird herb), due to its dependence on bird dispersal. The leaves have been used, in Brazil and Venezuela, as a popular medicine against respiratory diseases, liver damage and for their antimicrobial properties. Recently, our research group isolated and purified a lectin from an aqueous extract (AE) of the leaves of *P. pyrifolia* that presented effective antimicrobial activities and a potentially antioxidant effect with non-toxic action on *Artemia saline* and *Aedes aegypti* biological models [9].

With regard to the reported properties of plant extracts on fertility and libido, the current investigation proposed to evaluate the effects of AE from *P. pyrifolia* leaves on reproductive functions of male Wistar rats submitted to oral ingestion.

2. Materials and methods

2.1. Chemicals

All reagents and solvents were of analytical grade. Finasteride was purchased from the chemical company LIBBS.

2.2. Preparation of extract and protein assay

P. pyrifolia leaves were collected at the Federal University of Pernambuco campus, Brazil, from the top of a *Bauhinia monandra* host plant, and the botanical specimen was identified at the Dárdano de Andrade Lima herbarium of the Agronomic Institute of Pernambuco (AIP), where a voucher was deposited [botanical identification No 18/2006; identification code IPA-80.066; *P. pyrifolia* (Kunth) Eichler]. Briefly, the leaves were washed with distilled water and left to dry at 25 °C for 3 days. Dried leaves were then powdered and submitted to extraction with water [10% (w/v)], and maintained under agitation at 4 °C for 2 h in a dark room (in order to prevent photo-oxidative processes). Afterward, the mixture was filtered through gauze and centrifuged at 12 000 r/min at 25 °C for 15 min and the supernatant, identified as AE, was used. For animal assays, standardization of the plant extract was realized based on protein content. The protein content was spectrophotometrically determined using a protein calibration curve with bovine serum albumin as a standard (100–1 000 μg/mL).

2.3. Phytochemical analysis of aqueous extract

Phytochemical evaluation of AE was carried out using thin layer chromatography (TLC) on silica and cellulose sheets (Merck, Germany) with several systems of development as mobile phase, reagents for adequate revelation and *Cynara scolymus* AE as a chromatographic standard. Chromatograms were evaluated under UV light at 254 and 365 nm to detect the presence of chemical compounds according to Harborne [10]. Subsequently, quantitative assays for phenolic contents and tannin compounds were determined according to Julkunen-Titto [11] using gallic acid (0.025–0.6 mg/mL) as a standard. Total flavonoids were determined according to Jia *et al*. [12], using catechin (0.05–1.0 mg/mL) as a standard. The results were expressed as mg of catechin equivalent (CE) or gallic acid equivalent (GAE)/g AE.

2.4. High performance liquid chromatography (HPLC) apparatus and chromatographic conditions

Analysis of AE was performed on an HPLC (Shimadzu Cooperation, Tokyo, Japan) system equipped with a C-8 reversed phase column. The binary solvent system used was including solvent A [trifluoroacetic acid/water (0.1:99.9)] and solvent B [trifluoroacetic acid/water/acetonitrile (0.1:9.99:90)]. A total of 100 μL of AE was dissolved in 1 900 μL of solvent A and injected into an HPLC C-8 reversed phase column. A linear gradient of 0–100% solvent B from 0 to 68 min was carried out at a flow rate of 0.7 mL/min. Multi-wavelength detection was monitored at 215–254 nm. The flavonoid quercetin was used as a standard.

2.5. Animals and experimental treatment

Male Wistar rats (n = 30), aged 13 weeks and weighing (378.5 ± 5.0) g, were used. They were raised and housed (two rats in each cage) at a vivarium of the Anatomy Department of Federal University of Pernambuco, under controlled environmental conditions [photoperiod of 12 h light/dark, temperature of (23 ± 1) °C]. The animals were divided into three groups, including negative control (n = 6), positive control (n = 12) using finasteride at a concentration of 1.0 mg/kg, and test group (n = 12) using AE, and the groups were fed standard rations *ad libitum*. The control group had free access to water while the test group had free access to the AE (67 mg of protein/kg body weight) by natural ingestion. Animals consumed, on average, 30 mL/rat/day over 12 days. The bottles containing the AE were washed daily, the liquid contents were replaced, and the initial and final volumes were measured and recorded. The positive control group treated with finasteride was submitted to the gavage technique for drug ingestion. The drug was diluted in purified water to a final concentration of 1 mg/mL, and 1.0 mL was administered in each animal. After the treatment period, the rats were subcutaneously anesthetized using two anesthetic-sedative drugs: 0.02 mL (23 mg/mL) xylazine chloride and 0.07 mL (50 mg/mL) ketamine chloride, for each 40 g of body weight. Blood samples were extracted through intracardiac

puncture and then the animals were euthanized through left ventricular perfusion with a fixing solution of 4% glutaraldehyde in 0.05 mol/L phosphate buffer, pH 7.4. Subsequently, animals were orchidectomized and their kidneys were removed.

All experimental procedures involving the animals were approved by the Committee for Ethics in Animal Experimentation of the Federal University of Pernambuco (No. 23076.012671/2006-09). All animal procedures were in accordance with the Brazilian College of Animal Experimentation and approved by the Animal Ethics Committee of the Federal University of Pernambuco.

2.6. Hematological analysis

Blood samples were obtained by intracardiac puncture and placed into micro-blood tubes of 500 μL capacity containing 0.1% ethylene diamine tetraacetic acid. The samples were mixed gently to protect the blood from coagulation and hemolysis. Assays were carried out by optical microscopy, in which the morphology of red blood cells, leukocytes and platelets stained by the Giemsa method were observed. Hematological indices were determined by a Coulter STK-S random-access clinical hematological analyzer (Beckman & Coulter), according to techniques commonly used in clinical laboratories. The analysis comprised red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT) and mean platelet volume (MPV), white blood corpuscles (WBC), basophils (BA), eosinophils (EO), monocytes (MO), lymphocytes (LY) and neutrophils (NE).

2.7. Measurement of biochemical and hormonal constituent levels

Blood samples for biochemical analyses were obtained by intracardiac puncture at the end of the experiment and placed into coagulant micro-blood tubes of 500 μL capacity. After 15 min, the clotted blood was centrifuged (Kubota KR20000T) at 3480 r/min, for 15 min, at 25 °C. The serum was separated and used to evaluate urea (Urease GLDH Diasys), creatinine (Architect Abbot), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (UV Diasys-Architect c8000), alkaline phosphatase (ALP) (*p*-NPP Diasys), total and direct bilirubin (Dichlorophenyldiazonio Diasys) by an automated random-access clinical chemistry analyzer (Abbott Aerosets).

Blood samples for hormonal analyses were obtained daily by retro-orbital sinus puncture using a topical ophthalmic anesthetic tetracaine (drops). The hormonal assay kits for testosterone and cortisol were obtained from Beckman Coulter™, Inc. Samples were analyzed by the chemiluminescent method (Immulite 2000) using the access immunoassay system testosterone calibrator set for animal blood. Furthermore, to evaluate the effect of the extract on testosterone hormone, the animals were submitted every 3 days to blood collection for analysis.

2.8. Histopathological studies

2.8.1. Estimation of testicular parameters, histomorphological study and light microscopy

A laparotomy was done and the testes were clearly dissected out, blotted dry to remove any blood, weighed, processed histologically, fixed in buffered formalin and then embedded in paraffin. Fixed tissue was dehydrated, cleared, infiltrated, and paraffin embedded by an automatic tissue processor and subsequently cut into thin slices (4–6 μm in thickness) with a microtome (Leica, model RM 2245). For the histomorphological study, the sections were stained with hematoxylin-eosin and examined under a light microscope. The specimen was then subjected to morphological and morphometric testicular analysis. Afterward, gonadosomatic indices (GSI) were calculated to show the testes body weight ratios expressed as a percentage according to the expression below:

$$GSI = \text{Weight of testes} \times 100/\text{Weight of the animal}$$

Morphological analysis included observation of testicular compartment organization, interstice and seminiferous tubules in rats. Morphometric analysis involved the measurement of diameters of seminiferous tubules (DST) and height of the seminiferous epithelium (HSE). These data were obtained from the measurement of the coincidence of 30 cross-sections of seminiferous tubules for each animal, which showed the most circular contour possible.

Cross-sectional area (AC) of the seminiferous tubules was determined from the formula:

$$AC = \pi D2/4$$

where π is equivalent to 3.14 and D is the mean diameter of the seminiferous tubules. Additionally, the cross-sections of seminiferous tubules were captured by an image capture system and examined by the image processing program Scion Image at a magnification of 360. The values obtained for tubular diameter and height of the epithelium were increased by 15%, which represents the factor of linear shrinkage of seminiferous tubules due to histological processing.

2.8.2. Kidney histological analyses

The kidneys from the normal and experimental mice were kept in cold isotonic buffer [250 mmol/L sucrose, 10 mmol/L Hepes-Tris (pH 7.4), 2 mmol/L ethylene diamine tetraacetic acid and 0.15 mg/mL trypsin inhibitor (type II-S) supplemented with 1 mmol/L phenylmethanesulfonyl fluoride]. Afterward, kidneys were fixed in 10% buffered formalin and processed for paraffin sectioning. Thin (0.5 mm) transverse slices of the cortex corticis were removed with a Stadie–Riggs microtome and carefully dissected with small scissors to eliminate contamination from the rest of the tissue. Subsequently, sections were stained with hematoxylin and eosin to evaluate under a light microscope.

2.9. Statistical analysis

The rats' body weights were analyzed using the Mann–Whitney test with the SigmaStat program for repeated measures. Testicular/body weight ratio, hematological and biochemical blood parameters were analyzed using the parametric student's *t*-test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Phytochemical contents and HPLC analyses of AE

From a qualitative point of view, the data reported in Table 1 show a high phenolic content and low saponin content for the

Table 1
Phytochemical screening by presence of compounds in AE of *P. pyrifolia* leaves.

Chemical compounds	AE[a]
Anthocyanidins	ND
Anthocyanin	ND
Aurones	ND
Chalcones	ND
Flavones	ND
Flavanonas	ND
Flavonoids	+
Leucoanthocyanidins	ND
Saponins	+
Total phenols	++++
Xanthones	ND

++++: Strong presence; +: Trace; ND: Not detected.
[a] : AE obtained using 10% (w/v) dried leaves in distilled water.

AE. Quantitative assays showed total phenols of (98.12 ± 0.04) mg GAE/g AE, total flavonoids of (39.59 ± 0.12) mg CE/g AE, condensed tannins of (9.08 ± 0.02) mg CE/g AE and total protein of (11.4 ± 1.2) mg/mL. TLC results showed a chemical compound easily identified as a flavonoid (Figure 1A). This flavonoid was identified as quercetin, a plant-derived flavonoid, as demonstrated by the HPLC chromatogram (Figure 1B). Five, strongly concentrated peaks were evidenced in this assay. Peaks 1 and 2 were characterized as the flavonoid quercetin according to a standard (Figure 1C).

3.2. Experimental treatment and animal performances

Throughout the experimental period, the daily intake of AE dose in the test group was 67 mg of protein/kg body weight through natural ingestion (30 mL/rat/day), representing about 603 mg of protein/rat/day. During the treatment period with the AE from *P. pyrifolia* leaves, the ponderal curve of the animals presented a profile that depicts the significant loss of body weight in comparison with the control group. After the 12 days of this course of feeding, the average body weight of the test group fed with AE [(369.0 ± 4.0) g] was significantly less than the control group [(409.0 ± 6.0) g]. The administration of the AE did not induce behavioral modification at any point in time in treated rats compared to their respective control group. No death was recorded during the experimental period. Results for the finasteride group showed neither a significant loss of weight nor behavioral modification compared with negative control (data not shown).

3.3. Hematological evaluation

AE affected neither the erythrocyte indices, the platelet count, nor the cell morphology in the test group (Table 2). The

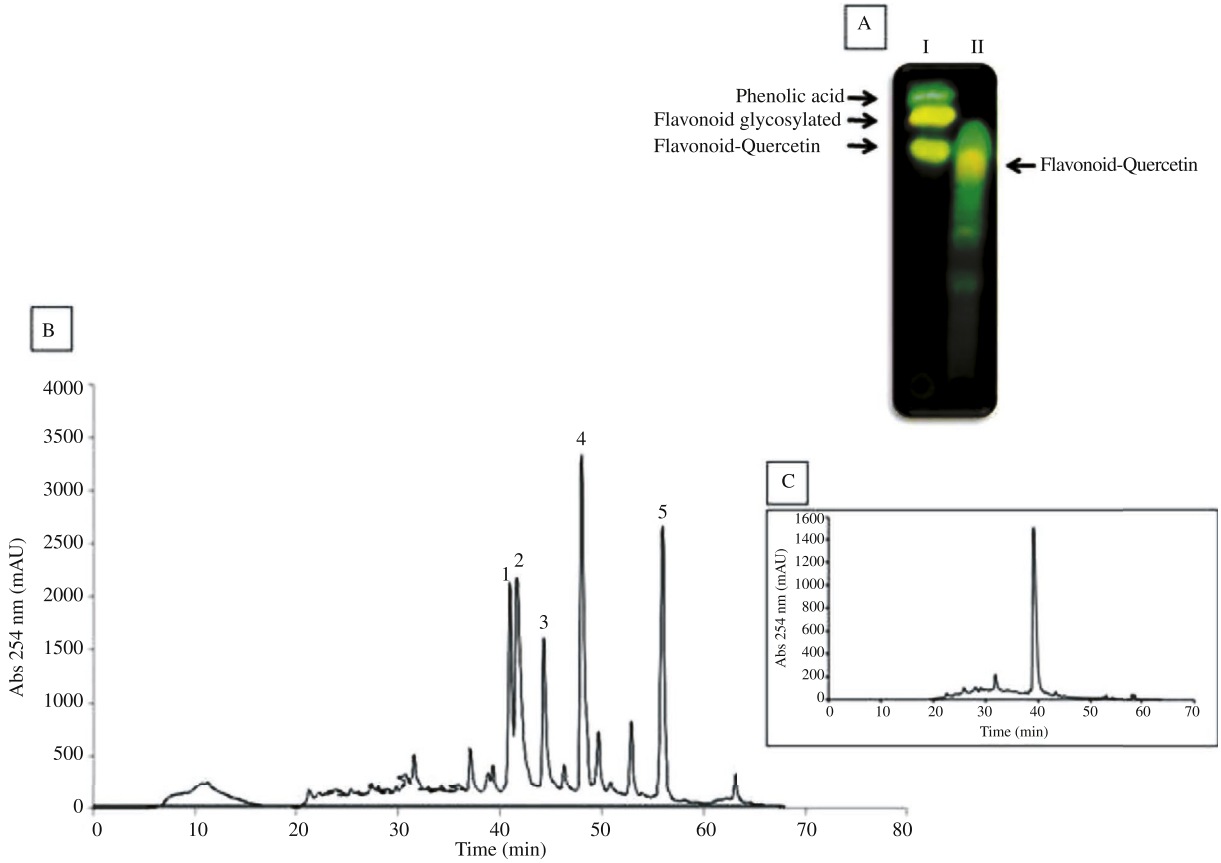


Figure 1. Assays for identification of biomolecules present in aqueous extract from *P. pyrifolia* leaves.
A: I-TLC analysis using a *Cynara scolymus* aqueous extract as a standard and II-TLC analysis of *P. pyrifolia* aqueous extract; B: HPLC chromatogram of the crude extract from the leaves of *P. pyrifolia* after the previously described extraction. HPLC was performed on a system equipped with a C-8 reverse phase column. The binary solvent system used was solvent A [trifluoroacetic acid/water (0.1:99.9)] and solvent B [trifluoroacetic acid/water/acetonitrile (0.1:9.99:90)]. A linear gradient of 0–100% B from 0 to 68 min was carried out at a flow rate of 0.7 mL/min. Multiwavelength detection was monitored at 215–254 nm; C: HPLC chromatogram of quercetin was used as a standard.

administration of AE significantly decreased ($P < 0.05$) the mean values of WBC [(4.44 ± 0.60) × 10^3/μL], MO (5.00% ± 1.00%) and BA (6.00% ± 1.00%) in the test group in comparison with the control group [(5.22 ± 0.50) × 10^3/μL, (7.00% ± 1.00%), (10.00% ± 2.00%), respectively]. However, a significant increase ($P < 0.05$) in the relative count of NE in the test group (19.00% ± 3.00%) in relation to the control group (11.00% ± 2.00%) was observed. Results for the finasteride group showed no significant changes in hematological parameters compared with the negative control (data not shown).

Table 2

Effect of the aqueous extract of *P. pyrifolia* leaves on rat hematological constituents.

Parameters	Control	AE
RBC (10^6/μL)	7.37 ± 0.10	7.64 ± 0.30
HGB (g/dL)	14.20 ± 0.20	14.40 ± 0.50
HCT (%)	42.60 ± 0.50	43.36 ± 2.80
MCV (fL)	57.17 ± 0.80	56.63 ± 1.60
MCH (pg)	19.04 ± 0.10	18.86 ± 0.30
MCHC (g/dL)	33.33 ± 0.30	33.25 ± 1.10
RDW (%)	13.92 ± 0.10	14.20 ± 0.10
PLT (10^3/μL)	906.50 ± 1.20	910.00 ± 0.10
MPV (fL)	5.50 ± 0.10	5.50 ± 0.10
WBC (10^3/μL)	5.22 ± 0.50	4.44 ± 0.60[a]
NE (%)	11.00 ± 2.00	19.00 ± 3.00
LY (%)	71.00 ± 2.00	70.00 ± 2.00
MO (%)	7.00 ± 1.00	5.00 ± 1.00[a]
EO (%)	1.00 ± 0.10	1.00 ± 0.10
BA (%)	10.00 ± 2.00	6.00 ± 1.00[a]

Values are represented as mean ± SD, $n = 6$. Aqueous extract obtained using 10% (w/v) dried leaves in distilled water.
[a] : $P < 0.05$ compared with control group; Student's *t*-test was used to assess the statistical significance.

3.4. Biochemical and hormonal serum analysis

The biochemical analysis shown in Table 3 revealed that the AE did not affect either renal parameter in rats represented by urea and creatinine concentrations. Also, it was observed that AST, ALT and ALP levels were reduced by about 36.95%, 27.06% and 52.63%, respectively.

Table 3

Effect of the aqueous extract of *P. pyrifolia* leaves on rat biochemical parameters.

Parameters	Control	AE
AST (IU/L)	122.60 ± 7.70	77.30 ± 10.10[a]
ALT (IU/L)	38.80 ± 6.10	28.30 ± 2.10[a]
ALP (IU/L)	228.00 ± 4.28	108.00 ± 9.20[a]
Total bilirubin (mg/dL)	0.20 ± 0.01	0.20 ± 0.01
Direct bilirubin (mg/dL)	0.10 ± 0.01	0.10 ± 0.01
Urea (mg/mL)	36.90 ± 2.00	34.30 ± 1.80
Creatinine (mg/mL)	0.60 ± 0.08	0.60 ± 0.03
Sodium (mEq/L)	144.10 ± 2.60	145.10 ± 1.90
Potassium (mEq/L)	6.75 ± 0.22	5.86 ± 0.90
Chloride (mEq/L)	103.70 ± 1.10	104.50 ± 1.10

Values are represented as mean ± SD, $n = 6$.
[a] : $P < 0.05$ compared with control group; Student's *t*-test was used to assess the statistical significance.

The analysis of testosterone hormone levels present in serum of rats treated showed a significant decrease ($P < 0.05$) of about 81.88% when compared with control rats, (47.0 ± 4.8) ng/dL and (255.0 ± 2.0) ng/dL, respectively (Figure 2). However, as seen in Figure 2, there was a significant decrease of 76.8%, (211.0 ± 8.7) ng/dL, when compared with finasteride, the positive control. On the 3rd day of exposure, AE was found to reduce testosterone levels by 10.54% while no changes were observed in the negative and positive controls. Furthermore, serum levels of cortisol did not significantly change with the test group compared to the control group, (1.16 ± 1.00) μg/dL and (1.13 ± 1.00) μg/dL, respectively.

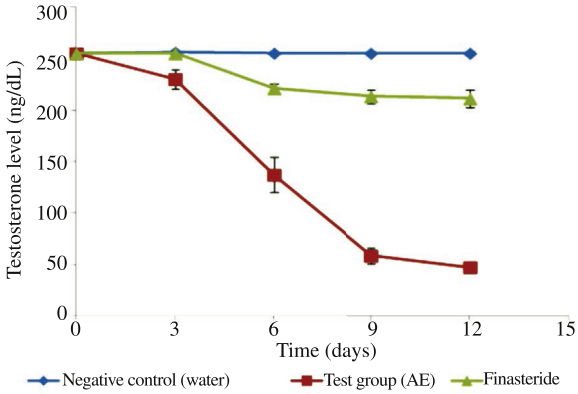


Figure 2. Hormonal curve of testosterone in male rats treated with aqueous extract of *P. pyrifolia*.
Serum levels were presented as ng/dL and time as days. Analyses were realized every 3 days. Each data point is the average of three determinations and the error bars show the SD.

3.5. Histopathological studies

To evaluate and confirm the results observed in Table 3 on renal function, the histology of kidneys was analyzed. The kidneys of male rats treated with plant extract did not show any significant changes, even days after stopping the treatment (Figure 3). The effects of AE on body weight, testicular weight and GSI of rats are shown in Figure 4. Body weight, testicular weight and GSI were significantly higher in the control group treated with water [(409.0 ± 6.0) g; (1.75 ± 0.30) g; (0.48% ± 0.02%)] than in the test group treated with AE [(369.0 ± 4.0) g; (1.48 ± 0.09) g; (0.41% ± 0.02%)]. The group of animals treated with finasteride did not show significative changes compared with control group. However, there were no significant data related to changes in testicular weight between the two groups. Figure 5 shows these results, and suggests that it could be observed through morphological analysis of testes where AE did not alter organization of testicular compartments (Figure 5A) when compared with the control group (Figure 5B). Additionally, small groups of interstitial Leydig cells were present and the seminiferous tubules showed signs of spermatogenesis in both groups. Seminiferous tubules of animals treated with *P. pyrifolia* were more closely arranged and were smaller than those of control animals (Figure 6). A distinct lumen was often present. The HSE was thick, usually comprised five to six layers of closely packed cells in the test group [HSE = (45.0 ± 1.0) μm] and showed clear signs of spermatogenesis when compared with the control group [HSE = (66.0 ± 1.0) μm]. The DST and AC of seminiferous tubules were significantly lower ($P < 0.05$) in the test group [DST = (247.0 ± 2.0) μm; (54.0 ± 0.6) × 10^3 μm^2)] than in the control group [DST = (262.0 ± 2.0) μm; (72.0 ± 0.7) × 10^3 μm^2).

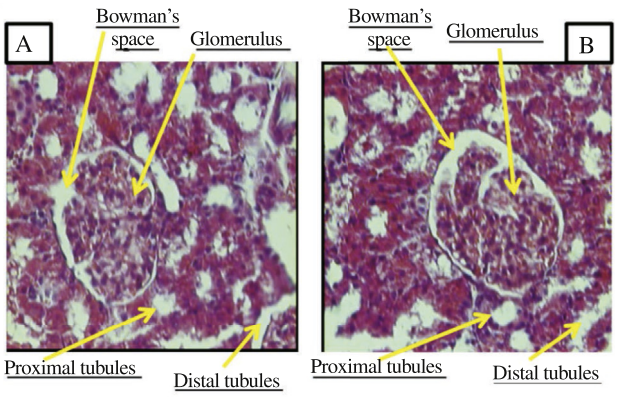
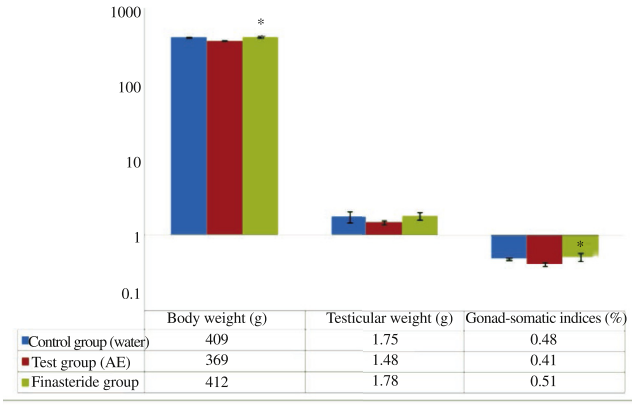


Figure 3. Hematoxylin and eosin stained kidney section of experimental mice.
A: Aqueous extract: kidney section of the mice treated with AE only (light microscope 40×); B: Control: kidney section from normal mice (light microscope 40×). Experimental procedure: Glomerulus apparatus; Proximal tubules; Distal tubules; Bowman's capsule.



	Body weight (g)	Testicular weight (g)	Gonad-somatic indices (%)
Control group (water)	409	1.75	0.48
Test group (AE)	369	1.48	0.41
Finasteride group	412	1.78	0.51

Figure 4. Effect of aqueous extract on body and testis weights of rats, and on GSI.
*: Statistical significance (student's *t*-test) $P < 0.05$. Each data point is the average of three determinations and the error bars show SD.

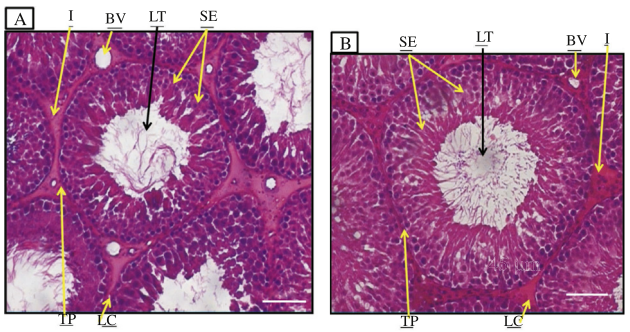


Figure 5. Histochemical analyses of rat testicular compartments.
High magnification of the seminiferous tubules of rats treated with *P. pyrifolia* showed them to comprise about eight layers of cells with many elongated sperm heads (single arrows). A: Test group; B: Control group; I: Interstice; LC: Lymph capsule; BV: Blood vessel; TP: Tunica; SE: Seminiferous epithelium; LT: Lumen. The LT contains sperm tails. Large arrows: Primary spermatocytes; Double small arrows: Spermatids. The SE comprises many layers of sex cells. The lumen is distinct. Numerous spermatogonia with small dark nuclei (squares) are clearly visible at the periphery of each tubule.

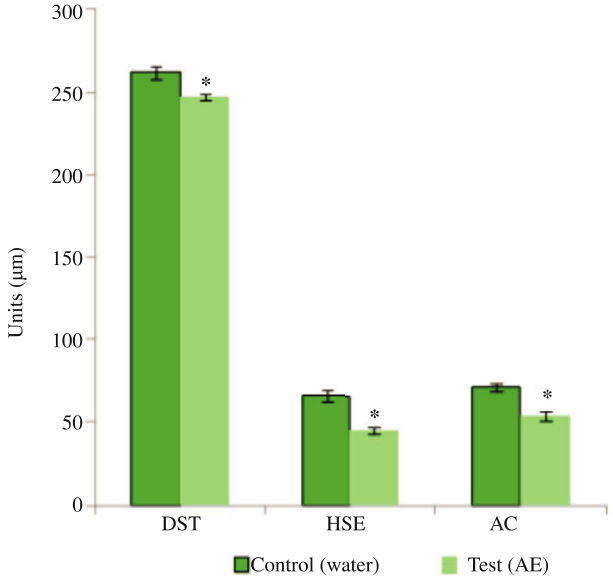


Figure 6. Effects of *P. pyrifolia* leaf extract on the DST, HES and AC of the seminiferous tubules.
*: Statistical significance analyzed by Mann–Whitney test, $P < 0.05$. Each data point is the average of three determinations and the error bars show SD.

4. Discussion

4.1. Phytochemical analyses

Phytochemical screening of AE showed an amount of total phenols identified by HPLC. The use of this system as a tool for characterization of biomolecules in plant extracts is widespread [13]. Our research using the HPLC system presented a small difference in retention times of AE analysis. Such results may have been affected by the high quantities of total phenols observed in the sample, which might have disturbed the reversed phase column interactions. This group of biomolecules has been shown to have numerous biological and pharmacological effects, including anti-oxidant, anti-inflammatory, anti-carcinogenic, cardio-protective, bacteriostatic and secretory properties and is being investigated for a wide range of potential health benefits [14]. According to Wong-Paz *et al.* [15], aqueous extracts of *Jatropha dioica* (2.1 mg GAE/g), *Flourensia cemua* (7.9 mg GAE/g), *Eucalyptus camaldulensis* (12.8 mg GAE/g) and *Turnera diffusa* (2.5 mg GAE/g) exhibited lower content of total phenolics than AE obtained from *P. pyrifolia*.

In addition, several variables including the stage of plant maturation and growing conditions such as temperature and rainfall are known to affect contents of the phenolic compounds which may be responsible for antioxidant and hepato-protective activity of plants. The total flavonoid content reported for green tea (23.5 mg CE/g) by Lee *et al.* was similar to our study [16]. Firdaus *et al.* observed anti-oxidant and hepato-protective effects of an AE of *Murraya koenigii* and attributed these actions to the presence of high concentrations of phenolics and flavonoids in that extract [17]. The presence of high amounts of plant metabolites can contribute to the beneficial effects of plants. This hypothesis is supported by the fact that a wide range of flavonoids, such as myricetin, kaempferol, quercetin, rutin and luteolin, have immuno-modulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors [18].

4.2. Animal performances

P. pyrifolia leaf AE did not cause the death of any animals during the experimental period of the present work, indicating that the extract has no lethal effect. In addition, behavioral changes, rejection of extract ingestion and decreases of thirst and hunger mechanisms (data not shown) were also not detected. The natural ingestion method was chosen because its efficacy inflicts low stress on animal, improving biochemical parameter analysis while not significantly changing their values. In the present study, the average body weight of the rats fed with *P. pyrifolia* extract was observed to be significantly less than the control group. Body weight monitoring is important since its profile can reveal symptoms of changes in the animals' metabolism and in the mechanism of hunger as well. In contrast, the studies with plant extracts from *Jodina rhombifolia* did not show any significant changes in body weight of Wistar rats [19].

4.3. Hematological, biochemical and hormonal studies

Hematological evaluation showed a slight decrease in WBC value of the test group, but this occurrence may not indicate variations in the immune system response. Also, we observed a decrease in the MO count. This would be of concern because the activation of the monocyte-macrophagic response occurs after contact of these cells with live or inert particles which migrate into the internal environment and results in activation of basophiles which appear in large amounts with the presence of allergens. During the experimental period, there were neither changes in the physiological state nor signs of disease or death in the test group, although a decrease in WBC was seen. Cell differentiation after administration of the plant extract was shown to be consistent with the literature according to Granados-Echegoyen *et al*. [20], except for the number of MO, which was lower in the test group than control group. Phenolic and quercetin compounds are not listed in literature related to decreases in hematological parameters. Additionally, we do not believe that slight decreases in relative WBC values observed in the test group were biologically meaningful. However, further studies are needed to elucidate data on the observed reduction in WBC induced by AE.

The study of biochemical parameters in plasma on liver function from hepatic metabolism provides valuable parameters for the evaluation of hepatic function as far as it is concerned with diseases, directly or indirectly, related to the liver. When high plasmatic levels of urea and creatinine are found, they provide evidence of renal overload, acute renal failure, or increase in protein catabolism [20]. Disorders of renal function frequently result in electrolyte imbalances. The concentration of sodium and potassium in the animals of the test group is within the range of normality. In our study, we observed that AE did not cause significant differences when compared with the control. The morphological analyses of the glomerulus, distal tubules, proximal tubules and Bowman's capsule of all treated and untreated rats were in accordance with the literature. The AE reduced the levels of all serum enzymes tested. The results are in agreement with the reports by Ngueguim *et al*. [21], who showed that levels of these enzymes were significantly decreased by pretreatment with an AE of *Spilanthes africana*, implying that the extract prevented liver damage. This conclusion was further supported by the reduced amount of histopathological injuries. The determination of ALT and AST in blood samples is useful in the assessment of liver function, being more sensitive in the detection of hepatocellular injury than biliary obstruction [22]. Increased values of enzymes are usually found in cirrhosis, hepatitis, hepatotoxicity and drug-biliary disease. An increase of plasmatic concentration of ALP is related to liver failure, mainly by obstruction, although a significant increase is also found in situations like pancreatic and bone cancer, leukemia, and pregnancy due to the production of the placental isoenzyme [23]. Yet, in the study of Wang *et al*. [23], they did not find changes in parameters of liver function as well as in liver morphology in rats treated with an extract of *Cortex dictamni*. Similar results to those described in our work were reported by Abirami *et al*. [24], where the protective effects of an extract from the roots of *Citrus hystrix* were confirmed in a mice model of CCl_4-induced acute liver injury. The responsible mechanisms for the protective effects of the root extract must be related to inhibitory effects and anti-oxidative actions mediated by increased regulation of OH^- in the liver. The phytochemical analysis of the plant showed the presence of high contents of phenolics and flavonoids that may be responsible for the activity of the plant, beside other phytochemicals. Quercetin flavonol, which was found in the extract, has been reported as a hepato-protective agent against liver damage [25]. Therefore, treating liver diseases with plant-derived compounds, such as those from *P. pyrifolia*, which are easily available and do not require laborious pharmaceutical synthesis, seems to be highly attractive. Recently, much attention has been focused on the protective biochemical functions of naturally occurring antioxidant agents in biological systems that can either scavenge reactive oxygen species or stimulate the detoxification mechanism within the cells, resulting in the removal of reactive oxygen species.

Concerning the hormone testosterone, we observed a steady decline of serum testosterone in the test group compared to controls. The decrease in serum testosterone may have resulted either from the direct effect of the extract on Leydig cells or indirectly as a consequence of decreased luteinizing hormone levels, thereby affecting steroidogenesis. Testosterone is synthesized in the Leydig cells via several important enzymes, carrier proteins or receptors from cholesterol synthesized *de novo* [26]. According to Mishra and Singh [27], treatment with *Curcuma longa* suppresses spermatogenesis in mice testes, although the mechanism of this suppression remains poorly understood. In immature male rats, *Curcuma comosa*, another species of *Curcuma*, has been suggested to act directly on the testes or indirectly to inhibit gonadotropin secretion, which consequently reduces testosterone production, or to act by both means. Finasteride [N-(1,1-dimethylethyl)3-oxo-4-aza-5ot-androst-1-ene-1713-carboxyamide] is a potent and specific inhibitor of 5ot-reductase, the enzyme responsible for the conversion of testosterone to 5ot-dihydrotestosterone.

From these accounts and the observation that the AE did not cause suppression of spermatogenesis in rats, we can deduce that the reduced levels of testosterone observed are probably due to the effects of the extract on gonadotropin secretion or pituitary levels. In contrast to our results, Yakubu and Jimoh revealed that the AE of *Carpolobia lutea* increased testosterone levels and this may be adduced to the induction of hormone synthesis by the Leydig cells, as these cells are the main source of testosterone [4].

The data found in our study about the hormone testosterone may be relevant to the treatment of one of the diseases that most

affect man nowadays, prostate cancer. Hormones, especially androgens, are believed to play a key role in the etiology of prostate cancer [28]. The existence of tumors or metastases in other organs renders useless any attempt to eradicate the original prostate tumor by surgery or radiotherapy. While the growth of malignant cells of the prostate is stimulated by testosterone, any measure that reduces the levels of this substance in the blood also feeds the cancer; this is true for both the initial lesion of the prostate and any locus of metastasis. In practice, we resort to castration (extraction of both testicles) or the administration of anti-hormones called anti-androgenics, which antagonize testosterone. The clinical efficiency of these different alternatives is more or less similar; the differences are their side effects.

Concerning the levels of cortisol, the data showed no significant change, corroborating the data observed on the behavioral and physiological analysis of the treated mice. Cortisol is a glucocorticoid-type hormone produced and secreted by the adrenal cortex. This affects the metabolism of proteins, fats and carbohydrates, and maintenance of muscle integrity and myocardial suppression of allergic and inflammatory activity. Abnormal levels of cortisol occur because of hypothalamic, pituitary or adrenal gland dysfunctions, disturbing the hypothalamo-pituitary gonadal axis. Generally, high levels of cortisol are noted in the presence of stress [29].

4.4. Histopathological parameters

The results of the histopathological testicular studies showed significant differences between the control and test groups. Firstly, we observed variation in GSI parameters (organ-body weight ratio) in the two groups. GSIs were significantly higher in the control group than in the test group. Similar results were reported by Yakubu *et al*. in rats treated with *Bulbine natalensis* [30]. However, our results disagree with another study carried out by Yakabu *et al*. [31], which revealed an increase of GSI in rats treated with *Fadogia agrestis*, a plant used to treat sexual disorders. These parameters can also be used to evaluate the normal functioning capacity of the testes. According to Wankeu-Nya *et al*. [32], alterations in GSI indices may occur due to inflammatory or cell constriction processes. An increase in organ-body weight ratio may either indicate inflammation or an increase in the secretory ability of the organ, while a reduction in this parameter may imply cellular constriction. Secondary metabolites of plants have been studied for their androgenic effects in mammals. Subsequently, the studies carried out by Yakubu and Jimoh showed decreased levels in the weight of reproductive organs in male rats submitted to treatment with extract of seeds from *Vitex negundo*, and suggested that the flavonoids present in high concentrations in the extract may be related to the results [4]. While we did observe differences in testicular parameters, as cited above, once again it is worth noting that a distinct lumen was often present in the test group and clear signs of spermatogenesis were seen in both the test and control groups. According to the DST and AC data, spermatocytogenesis was clearly reflected in the presence of primary spermatocytes undergoing their first meiotic division. It was also observed that the decrease in the DST in the treated animals was proportionate to the proliferation of spermatogenic cells revealed by histology; values of all three parameters were lowest in the test group and highest in the control.

According to Hirai *et al*. [33], male rat infertility and conditions of impaired spermatogenesis may involve different mechanisms of testicular damage. In the seminiferous tubules, it was possible to observe spermatogenic cell lines and Sertoli cells composing the seminiferous epithelium, plus tubular lumen and tunica. Sertoli cells provide mechanical and nutritional support for developing germ cells. In the interstitial compartment around Leydig cells, blood and lymph vessels and conjunctive tissue were observed. As stated by Yang *et al*. [34], changes in basement membrane thickness, degeneration around the tubule, and tubular atrophy were detected in their research when they observed that an ethanol extract of *Salacia chinensis* attenuates testicular and liver toxicity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. The results obtained in our study suggest that the AE of *P. pyrifolia* leaves may have an anti-androgenic effect, delaying sexual maturation and altering normal testicular function.

Associating the results obtained for hormone and histopathological data, we intend in future research to carry out tests in order to observe the effects of the plant on the copulatory behavior and sexual activity in rats of the same lineage, then promoting a comparison with the studies assayed by Toyin and Olaide *et al*. [35], where it was observed that the aqueous crude extract from *Montanoa tomentosa* produced very interesting data, suggesting that the plant may be a sexual stimulant with aphrodisiac properties.

In conclusion, the results of the current study revealed that the oral administration of an AE of *P. pyrifolia* was not lethal, but promoted a severe decrease in testosterone hormone levels. Concerning the phytochemical analyses, the results suggest that flavonoids like quercetins, which were found and identified in the AE, may be the active principle responsible for the pharmacological action of the extract. This finding would be in accordance with the literature. However, further studies are necessary to understand the effect of long-term administration of the extract on spermatogenesis and, consequently, fertility in male rats and the extract's possible use as an adjuvant in the therapy of prostate cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are deeply grateful to the Clinical Hospital of Pernambuco for valuable assistance and Mr. Scott V. Heald for language revision. This research was supported by the National Research Council (CNPq), Foundation for Science and Technology of the State of Pernambuco (FACEPE) and Personnel Improvement Coordination-CAPES/PROCAD/NF/no 1415/2007, Brazil.

References

[1] Tahtamouni LH, Alqurna NM, Al-Hudhud MY, Al-Hajj HA. Dandelion (*Taraxacum officinale*) decreases male rat fertility *in vivo*. *J Ethnopharmacol* 2011; **135**: 102-9.

[2] Asadujjaman M, Mishuk AU, Hossain MA, Karmaka UK. Medicinal potential of *Passiflora foetida* L. plant extracts: biological and pharmacological activities. *J Integr Med* 2014; **12**: 121-6.

[3] Komlaga G, Agyare C, Dickson RA, Mensah ML, Annan K, Loiseau PM, et al. Medicinal plants and finished marketed herbal

products used in the treatment of malaria in the Ashanti region, Ghana. *J Ethnopharmacol* 2015; **172**: 333-46.
[4] Yakubu MT, Jimoh RO. Aqueous extract of *Carpolobia lutea* root ameliorates paroxetine-induced anti-androgenic activity in male rats. *Middle East Fertil Soc J* 2015; **20**: 192-7.
[5] Sattayasai J, Srisuwan S, Arkaravichien T, Aromdee C. Effects of andrographolide on sexual functions, vascular reactivity and serum testosterone level in rodents. *Food Chem Toxicol* 2010; **48**: 1934-8.
[6] Ebrahim M, Pool EJ. The effect of *Tulbaghia violacea* extracts on testosterone secretion by testicular cell cultures. *J Ethnopharmacol* 2010; **132**: 359-61.
[7] Dollah MA, Parhizkar S, Noh NH. Effect of non-polar extract of *Phaleria macrocarpa* (Mahkota dewa) on serum testosterone level and libido behavior in male rats. *Maturitas* 2015; **81**: 208-9.
[8] Samplaski MK, Lo K, Grober E, Jarvi K. Finasteride use in the male infertility population: effects on semen and hormone parameters. *Fertil Steril* 2013; **100**: 1542-6.
[9] Costa RMPB, Vaz AFM, Xavier HS, Correia MTS, Carneiro-da-Cunha MG. Phytochemical screening of *Phthirusa pyrifolia* leaf extracts: free-radical scavenging activities and environmental toxicity. *S Afr J Bot* 2015; **99**: 132-7.
[10] Harborne JB. *Phytochemical methods: a guide to modern techniques of plant analysis*. London: Chapman and Hall; 1988.
[11] Julkunen-Titto R. Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *J Agric Food Chem* 1985; **33**: 213-7.
[12] Jia Z, Tang M, Wu J. The determination of flavonoids contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* 1999; **64**: 555-9.
[13] Nuengchamnong N, Krittasilp K, Ingkaninan K. Characterisation of phenolic antioxidants in aqueous extract of *Orthosiphon grandiflorus* tea by LC-ESI-MS/MS coupled to DPPH assay. *Food Chem* 2011; **127**: 1287-93.
[14] Li C, Li Q, Mei Q, Lu T. Pharmacological effects and pharmacokinetic properties of icariin, the major bioactive component in Herba Epimedii. *Life Sci* 2015; **126**: 57-68.
[15] Wong-Paz JE, Contreras-Esquivel JC, Rodríguez-Herrera R, Carrillo-Inungaray ML, López LI, Nevárez-Moorillón GV, et al. Total phenolic content, *in vitro* antioxidant activity and chemical composition of plant extracts from semiarid Mexican region. *Asian Pac J Trop Med* 2015; **8**: 104-11.
[16] Lee SC, Hwang SY, Kim SW, Kim SK. Ethanol extract of *Allium sativum* attenuates testicular and liver toxicity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats. *J Med Food* 2009; **12**: 93-9.
[17] Firdaus SB, Ghosh D, Chattyopadhyay A, Dutta M, Paul S, Jana J, et al. Protective effect of antioxidant rich aqueous curry leaf (*Murraya koenigii*) extract against gastro-toxic effects of piroxicam in male Wistar rats. *Toxicol Rep* 2014; **1**: 987-1003.
[18] Vauzour D, Martinsen A, Layé S. Neuroinflammatory processes in cognitive disorders: is there a role for flavonoids and *n*-3 polyunsaturated fatty acids in counteracting their detrimental effects? *Neurochem Int* 2015; **89**: 63-74.
[19] Teves MR, Wendel GH, Pelzer LE. Reduction in voluntary ethanol intake following repeated oral administration of *Jodina rhombifolia* lyophilized aqueous extract in male Wistar rats. *J Ethnopharmacol* 2015; **161**: 170-4.
[20] Granados-Echegoyen C, Pérez-Pacheco R, Alexander-Aguilera A, Lagunez-Rivera L, Alonso-Hernández N, Chairez-Martinez EJ. Effects of aqueous and ethanol extract of dried leaves of *Pseudocalymma alliaceum* (Bignonaceae) on haematological and biochemical parameters of Wistar rats. *Asian Pac J Reprod* 2015; **4**: 129-34.
[21] Ngueguim TF, Djouwoug Noussi C, Donfack JH, Gounoue KR, Mbatchou A, Kamtchouing P, et al. Acute and sub-acute toxicity of a lyophilised aqueous extract of the aerial part of *Spilanthes africana* Delile in rats. *J Ethnopharmacol* 2015; **172**: 145-54.
[22] Chikhi I, Allali H, Dib ME, Medjdoub H, Tabti B. Antidiabetic activity of aqueous leaf extract of *Atriplex halimus* L. (Chenopodiaceae) in streptozotocin-induced diabetic rats. *Asian Pac J Trop Dis* 2014; **4**: 181-4.
[23] Wang L, Li Z, Li L, Li Y, Yu M, Zhou Y, et al. Acute and subchronic oral toxicity profiles of the aqueous extract of *Cortex dictamni* in mice and rats. *J Ethnopharmacol* 2014; **158**: 207-15.
[24] Abirami A, Nagarani G, Siddhuraju P. Hepatoprotective effect of leaf extracts from *Citrus hystrix* and *C. maxima* against paracetamol induced liver injury in rats. *Food Sci Hum Wellness* 2015; **4**: 35-41.
[25] Zou W, Liu W, Yang B, Wu L, Yang J, Zou T, et al. Quercetin protects against perfluorooctanoic acid-induced liver injury by attenuating oxidative stress and inflammatory response in mice. *Int Immunopharmacol* 2015; **28**: 129-35.
[26] Nurudeen QO, Ajiboye TO. Aqueous root extract of *Lecaniodiscus cupanioides* restores the alterations in testicular parameters of sexually impaired male rats. *Asian Pac J Reprod* 2012; **1**: 120-4.
[27] Mishra RK, Singh SK. Reversible antifertility effect of aqueous rhizome extract of *Curcuma longa* L. in male laboratory mice. *Contraception* 2009; **79**: 479-87.
[28] Silva RD, Bueno AL, Gallon CW, Gomes LF, Kaiser S, Pavei C, et al. The effect of aqueous extract of gross and commercial yerba mate (*Ilex paraguariensis*) on intra-abdominal and epididymal fat and glucose levels in male Wistar rats. *Fitoterapia* 2011; **82**: 818-26.
[29] Aswar U, Gurav M, More G, Rashed K, Aswar M. Effect of aqueous extract of *Solanum xanthocarpum* Schrad. & Wendl. on postmenopausal syndrome in ovariectomized rats. *J Integr Med* 2014; **12**: 439-46.
[30] Yakubu MT, Afolayan AJ. Reproductive toxicological evaluations of *Bulbine natalensis* Baker stem extract in albino rats. *Theriogenology* 2009; **72**: 322-32.
[31] Yakabu MT, Akanji MA, Oladiji AT. Effects of oral administration of aqueous extract of *Fadogia agrestis* (Schweinf. ex Hiern) stem on some testicular function indices of male rats. *J Ethnopharmacol* 2008; **115**: 288-92.
[32] Wankeu-Nya M, Watcho P, Nguelefack T, Carro-Juarez M, Tapondjou L, Kamanyi A. Effects of *Dracaena arborea* (Dracaenaceae) on sexual dysfunction in 4 weeks hyperglycemic male rats. *Asian Pac J Trop Med* 2014; **7**: 609-19.
[33] Hirai T, Tsujimura A, Ueda T, Fujita K, Matsuoka Y, Takao T, et al. Effect of 1,25-dihydroxyvitamin D on testicular morphology and gene expression in experimental cryptorchid mouse: testis specific cDNA microarray analysis and potential implication in male infertility. *J Urol* 2009; **181**: 1487-92.
[34] Yang J, Luo S, Song J, Kobayashi M, Akaki J, Yamashita K, et al. Effects of *Salacia chinensis* extract on reproductive outcome in rats. *Food Chem Toxicol* 2011; **49**: 57-60.
[35] Toyin YM, Olaide NQ. Effects of aqueous extract of *Cnestis ferruginea* (Vahl ex De Candolle) root on paroxetine-induced sexual dysfunction in male rats. *Asian Pac J Reprod* 2012; **1**: 111-6.