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TOXICOLOGICAL, GENOTOXIC AND ANTIOXIDANT POTENTIAL OF *PYROSTEGIA VENUSTA*

POTENCIAL TOXICOLÓGICO, GENOTÓXICO E ANTIOXIDANTE DE PYROSTEGIA VENUSTA

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ABSTRACT: *Pyrostegia venusta* is usually found in the secondary growth of the Atlantic forests, and in the Brazilian Savanna. Flowers and leaves of this plant are used in folk remedies for treating a wide variety of healthy conditions, this way is important evaluate its safety and antioxidant potential for this applications. For this, was made a ethanolic extract from its flowers and analyzed with toxicological, genotoxicity and antioxidant tests, the toxicological analysis was made by reproductive toxicity in rats and clatogenicity/aneugenicity in human lymphocytes. The genotoxicity was studied by micronucleus test mice bone marrow. The antimutagenic test in root cells of *Allium cepa,* the antioxidant assays used was DPPH, FRAP, Lipid Perxidation and REM, beyond of that the extract was analyzed in HPLC showing the profile of its compounds. The toxicological analysis showed that *P. venusta* has no negative significant effect on reproductive and cellular level. The micronucleus test in mouse bone marrow, the extract protected cells from cyclophosphamide, mutagenic compound, in a similar way. The *A. cepa* test showed that the extract reduced chromosomal disorders formations. The antioxidant activity of extract was significant, except in REM test. The phytochemical analysis showed the presence of flavonoids compounds. *P. venusta* extract does not present reproductive toxicity and genotoxic effects. However, the extract of this species showed antigenotoxic and antioxidant potential, possibly due to the different flavonoid compounds present in its extract.

KEYWORDS: Reproductive toxicology. Cytotoxicity. Oxidation. Mutagenesis. Flavonoids. Phenol compounds.

INTRODUCTION

Pyrostegia venusta (Ker-Gawl) Miers, is popularly known as "flor-de-São-João" or "cipó-de-São-João" and in folk medicine (SOUSA, 2016), a neotropical heliophyte bignoniaceous vine is a typical colonizing species. It is usually found in the secondary growth of the Atlantic forests, and in the Brazilian Savanna with characteristic savanna-like vegetation. Its geographical distribution reaches from southern Brazil to Paraguay, Bolivia, and northeastern Argentina (POOL, 2008). Ethnomedically, flowers and leaves of this plant are used in folk remedies for treating white patches on the skin (leukoderma, vitiligo) (FERREIRA et al., 2000). An infusion prepared from the bark and roots is widely used in the treatment of erysipelas, jaundice (hyperbilirubinemia and deposition of bile pigments) and infections of female genital tract in newborn babies (ROY et al., 2012). In addition, *P. venusta* is also used for cough and common diseases of the respiratory system that are related to infections, such as bronchitis and influenza (FIGUEIREDO et al., 2014). Roy et al. (2011) demonstrated that the extracts of flowers and roots of *P. venusta* contain significant amounts of phytochemicals with antioxidant properties that could act as inhibitors or scavengers of free radicals.

Moreover, no genotoxic effect was observed for extracts of *P. venusta* on bone marrow of mice using the micronucleus and

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chromosome aberration assay (MAGALHÃES et al., 2010).

Major chemical components reported in the methanolic extract *P. venusta* flowers are sitosterol, n-hentriacontane (n-$C_{31}H_{64}$), 7-O-d-glicopiranosilacacetina and mesoinositol (myoinositol) hexadecanoic acid, linoleic acid, oleic acid, stigmasteryl tosylate, diazoprogesterone, arabipyranose, propanoic acid, pentamethyldisilanyl ester, acetophenone and trans-3-hexenedioic acid, and 9-octadecenoic acid (Z)-methyl ester (ROY et al., 2011). Analysis of the ethanolic extract of the roots performed by Ferreira et al. (2000) identified allantoin, steroids, sitosterol and 3-O-d-glicopiranosilsitosterol and flavanone hesperidin and others studies performed by Santos and Blatt (1998) have identified the presence of different classes of flavonoids and phenols in the leaves evaluated, in forty species of Bignoniaceae, including *P. venusta*, revealing the presence of phenolic components and syringyl groups (phytochemicals).

Given the medicinal effects attributed to *P. venusta,* and the diverse secondary compounds of *P. venusta*, studies on the toxic, cytotoxic and mutagenic potential of this plant species should be carried out. Based on their traditional use for long periods of time vegetable preparations are often assumed to be safe. However, research has shown that many plants that are used for food or used in traditional medicine have *in vitro* mutagenic properties (MOHD-FUAT et al., 2007) or toxic and carcinogenic properties (FERREIRA; VARGAS, 1999).

Within this context, it is also important to screen medicinal plants for their mutagenic properties. Plants exhibiting clear mutagenic properties should be considered as potentially unsafe and certainly require further testing before their continued use can be recommended. On the other hand, plants with obvious antimutagenic potential may be of interest for therapeutic use and are worthy of further in-depth investigations of their pharmacological properties.

The purpose of the present study was to evaluate the *in vivo* and *in vitro* toxicity, cytotoxicity and genotoxicity of extracts of the flowers of *P. venusta* on tissues of female rat reproductive organs, cultured human lymphocytes and bone marrow of mice. In addition, the antioxidant potential and phytochemical profile of the extract was investigated.

MATERIAL AND METHODS

Collection of plant material

The flowers of *P. venusta* were collected from specimen of remaining Savanna vegetation near the campus of UNESP/Assis, SP, Brazil (22°32'26"S and 50º22'31"N and 22°32'18"S; 50°22'47"N) and a copy was taxonomically identified at the Herbarium of the Forestry Institute of Assis, São Paulo, Brazil (voucher specimen: SPSF-40207). The collection was made in a sustainable manner and without causing environmental impact to the specie collected.

Preparation of plant material in the laboratory, production and storage of ethanolic extract

Flowers of *P. venusta* were selected and dried with airflow at a temperature of 40 °C, and then crushed and pulverized to prepare the ethanolic crude extract. The mechanical extraction of the powdered plant under stirring with ethanol PA at 1:10 (w:v) was carried out over a 24 h period and repeated twice. After obtaining the extract, the sample was taken to a rotary evaporator to remove all of the alcohol, and the resulting concentrated extract was taken to the drying chamber to obtain a dry extract.

Evaluation of reproductive toxicity in rats

Wistar adult female rats that were 12 weeks of age and weighed 250 g were kept at the Faculty of Sciences and Letters (UNESP- Assis, SP, Brazil), and maintained under controlled conditions of temperature (23 ± 1 ºC) and lighting. (12L, 12D photoperiod, lights switched on at 7 a.m.). Tap water and commercial chow were supplied *ad libitum.* The experimental protocol followed the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Ethical Committee for Animals Use (Permit number: 002/2010).

Females were weighed and randomly distributed in two groups (n = 14/group), either a control group (given 0.5 mL distilled water) or the experimental group (given *P. venusta*, 100 mg/Kg body weight (bw) of ethanolic extract that was diluted in 0.5 mL distilled water). The rats of each group received the treatment by via an oral (gavage), single dose daily, during 20 consecutive days.

During the experiment, animals were given a cytological examination (vaginal swabs), according (MARCONDES et al., 2002). The time collection was fixed at 7 a.m. Each slide was analyzed under a light microscope (Olympus CX31 RBSFA, Japan).

The consumption of water and food were registered daily. After the treatment period, the females in estrus phase of the estrous cycle

(n=7/group) were sacrificed through a lethal intraperitoneal (i.p.) dose of sodium thiopental (Thiopentax®, Cristália, São Paulo, Brasil). The ovaries and uterine horns were collected and weighed, and then fixed in Bouin's solution, and processed in the usual histological routine for paraffin embedding (Paraplast Labware-Oxford, St. Louis, MO, USA). The blocks were sliced into 5 µm-thick sections and stained with Hematoxilin-Eosin (H&E) or Mallory's Tricromic. The types of follicles were identified according (PEDERSEN; PETERS, 1968) as presented (PLOWCHALCK et al., 1993; VIEL et al., 2017). Ovarian and uterine sections were analyzed using a Zeiss microscope (Scope A1-Axio coupled with video-camera AxioCam ICc3 and digitalized by the software Axio Vision, version 4.7.2).

At the end of the treatment period, other 7 females of each group were mated with untreated males. After mating, the presence of sperm in the morning vaginal smear was indicative of fertilization and was considered as day one of gestation (GD1). Pregnant females were kept in individual cages. On the 19th gestational day (GD19) a laparotomy was performed after an intramuscular administration of ketamine (40 mg/kg bw) and xylazine (20 mg/kg bw), and records were obtained for the number of implantations, the number of corpora lutea, the litter size, the litter weight and the number of resorptions. Copulation rate (number of females with sperm in the smear/number of mated females in the group) x 100). Fertility rate (number of pregnant females/number of copulated females) x 100). Pre-implantation loss rate (number of corpora lutea - number of implants/number of corpora lutea) x 100) and post-implantation loss rate (number of implants – number of fetuses/number of implants) x 100) were calculated as described (DAMASCENO, 2008; VIEL et al., 2017). The evaluation of fitotoxicity was performed based on a analysis of fetal external morphology with a stereomicroscope (Carl Zeiss, Citoval 2, Germany).

The results were analyzed using a Students t-test or a Mann-Whitney non-parametric test. The results were considered significant when $p< 0.05$.

Clatogenicity/Aneugenicity in human lymphocytes

Collection of lymphocytes

Lymphocytes were obtained from human blood as approved by the Ethics Committee of the Assis Faculty of Sciences and Letters of Assis (UNESP – SP, Brazil), No. 833386. The blood was collected from healthy volunteers aged between 18 and 25 years that were not using drugs, tobacco or alcohol. Blood was collected from the antecubital vein between 7:00 and 10:00 am in heparinized tubes and under sterile conditions. The material was processed within 4 h of collection.

Lymphocyte isolation of whole blood

Whole blood collected was homogenized by inversion and diluted with saline RPMI with unsupplemented culture for experiments performed in cell culture in the proportion of 1:1 (v/v). The diluted blood (7 mL) was added slowly into a centrifuge tube containing 3 mL of Ficoll-Paque TM Plus and centrifuged for 30 min at 1,000 rpm and refrigerated at 4 °C. Then, the lymphocyte cloud was removed with a Pasteur pipette and washed 2 times with RPMI 1640 medium.

Culture of human lymphocytes

The culture was performed according (TITENKO-HOLLAND et al., 1997; FAREED; AFZAL; SIDDIQUE, 2011) with an initial density of 10^6 cells in 2 mL of culture medium. Then, the lymphocytes were cultured. Culture medium consisted of RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM of L-glutamine, 100 units mL^{-1} of penicillin, 100-µgmL^{-1} of streptomycin and 1.5% phytohemagglutinin.

Treatment conditions

For each individual 5 culture vials were mounted followed by 8 h of incubation at 37 °C with the addition ethanolic extract of *P. venusta* at a concentration of 50, 100 or 200 mgL^{-1}. Two culture flasks were reserved for the positive control (50 mgL^{-1} of cyclophosphamide) and the negative control (without a test substance).

Micronucleus assay

After 48 h of incubation, 0.2 mL of cytochalasin B was added to 5mL of medium to prevent cytokinesis of the dividing cells. After 72 h the culture was stopped with 0.5 mL of methanol-acetic acid fixative (3:1) for 5 min at room temperature. The material was centrifuged at 800 rpm, discarding the supernatant. Next, 5 mL of fixative was added with stirring, and then the material was centrifuged. This procedure was repeated 3 to 4 times until the precipitate remained clean. The material was dripped onto slides, allowed to dry at room temperature, and then stained with Giemsa at a ratio of 1:3 in phosphate buffer (pH = 6.8) for 5 to 8 min.

The slides were examined in a blind condition. For each individual in the sample two blades were selected and MN (micronucleus) was recorded for 1,000 bi-nucleated cells (500 per replicate/blade). The evaluation/cell count of MN

followed the criteria according (FENECH et al., 2003), such that the chromatin structure and similar refractions between nucleus were evaluated, the cytoplasm was well preserved, the staining intensity was similar between nucleus, the definition of the nuclear contours were oval or round in shape, there were no links to the main nucleus, and the diameter was between 1/3 and 1/16 of the average diameter of the main core corresponding to 1/256 and 1/9 of an area of a nucleus of a main bi-nuclear cell.

Significant differences between the controls and treated samples were determined with the Fisher exact test for MN frequencies and with the Chi-square test.

Antimutagenic assay

Micronucleus test in mice bone marrow

Antigenotoxic effects of the extract of *P. venusta* on cyclophosphamide-induced micronucleus in mice was tested using 7-week to 12-week old male Swiss albino mice (Mus musculus Rodentia, Muridae) weighing 25-35 g. The mice were provided by the central bioterium (Instituto de Biociências/UNESP/Botucatu) and acclimatized in cages at 24 + 1 °C under a 12 h light period for one week. During acclimatization and throughout the experiments the mice had free access to standard granulated chow and drinking water. Each cage contained 5 mice that were randomly assigned to 1 of the 4 following groups: 1) a negative control group that was given distilled water by oral gavage; 2) a positive control group that was given a single intraperitoneal injection of the equivalent of 0.2 mg per 100 g of body weight (bw) of cyclophosphamide (CAS n.50-18-0; Endoxan, Baxter Oncology Gmb, Germany) dissolved in distilled water; a extract group that was given the equivalent of 50 mg, 100 mg or 200 mg per kg bw during 7 days by oral gavage. However, on the seventh day, the mice also received the same treatment as the positive control group.

All mice were killed by cervical dislocation on day 8. This study conforms to the relevant Brazilian guidelines regarding the ethical use of live animals. Genotoxic effects were evaluated in the mouse bone marrow by the micronucleus test (BAESSE et al., 2015). Immediately after sacrifice the mice both femurs were removed from each mouse and the bone marrow flushed out into centrifuge tubes containing 2 mL of fetal calf serum at 1,000 revolutions per min for 10 min, after which the supernatant was discarded and the pellet resuspended in a drop of serum, and a smear was made on a clean slide. The smear was air-dried, fixed with absolute methanol for 5 min, then air-dried and either stored at room temperature or directly stained for 5 min with a freshly prepared working solution of Giemsa stain diluted 1:1 v/v in 0.06 M of sodium phosphate buffer and 0.06 M of potassium phosphate buffer (both at pH 6.8). After staining, the slides were rinsed in distilled water, dried at room temperature and scored for micronucleus according to the criteria of Krishna and Hayashi (2000) using 100X magnification and a Carl Zeiss optical microscope. We scanned 2000 polychromatic erythrocytes (PCE) per mouse and recorded the number of micronucleated PCE (MNPCE).

To compare the frequencies of MNPCE and normal PCE between treated and control groups the results were expressed as the mean ± standard deviation and analyzed statistically using the nonparametric Mann-Whitney U-test with the significance level set at $\alpha = 0.05$. The statistical analyses were carried out using the SPSS 12.0 statistical package for PCs (SPSS, Chicago, IL).

Antimutagenic test in root cells of *Allium cepa*

Onion bulbs (*A. cepa* L., 2n = 16) were obtained commercially in Assis, São Paulo, Brazil. They were cleaned and the outer scales were removed, leaving the ring intact with primordial roots. The bulbs were used for the bioassay according to standard procedures (BABATUNDE; BAKARE, 2006). The growth of the roots was used a culture solution (Hoagland's solution). The bulbs were kept suspended in a 100-mL beaker leaving the ring of roots in contact with the solution, changed every 24 h for a period of 72 h, and maintained at a photoperiod (18 h/6h light/dark) and temperature (22 ± 2 °C) in controlled chamber B.O.D. Bulbs with roots approximately 2 cm were used in the experiment.

To evaluate the mitotic index-MI, micronucleus-MN and induction of chromosomal aberrations-CA (c-metaphase; chromosome delay; chromosomal bridge; aberrant telophase; nucleolus outside the nucleus; amorphous nucleus), 6 onion bulbs were exposed to each concentration of ethanolic extract (0.1, 5 and 10 mg/mL) of *P. venusta*. Mineral water and positive control solution of MMS (methyl methanesulfonate) at 10 mg/L was used for the negative control, as described (CARITÁ; MARIN-MORALES, 2008). At the end of 48 h of exposure and 24 h recovery in culture solution, the roots of treated and control bulbs were cut and fixed in ethanol: glacial acetic acid (3:1, v/v). These were hydrolyzed in 1 N of HCl at 60 °C for 8 min, after which they were rinsed in distilled water. The roots were stained with acetic carmine for 10 min, the tips were

removed and the roots carefully crushed between slide and coverslip were sealed, as suggested (AKINBORO; BAKARE, 2007). Five slides were prepared for each treatment and controls were analyzed at 1000× magnification. The mitotic index was calculated on the number of dividing cells per 1000 cells observed (FISKESJÖ, 1985; DE CAMPOS VENTURA-CAMARGO; MARIN-MORALES, 2016). The frequency was calculated based on the number of aberrant cells and micronucleus per total cells analyzed for each treatment and controls (BAKARE et al., 2000). The MI, CA e MN obtained were compared with the controls and statistically analyzed using the Kruskal-Wallis test ($p < 0.05$), as described (BAKARE et al., 2000).

Antioxidant potential

DPPH radical scavenging activity

The stable 1.1-diphenyl-2-picrylhydrazyl (DPPH, Sigma, USA) radical scavenging activity was determined according to the methodology proposed (HELM et al., 2008). The dry ethanolic extract of each sample was dissolved in ethanol (75%) at different concentrations (25, 50, 75, 100, 250, 500 and 1,000 $\mu g/mL^{-1}$) and then mixed with 5 mL of DPPH solution (1.5 x 10-4 M). The extract reacted with the DPPH radical for a period of 30 min in a low luminosity, and then they were submitted to the UV-Vis spectrophotometer (model: SP220, BIOSPECTRO, Brazil) at 517 nm wavelength. The calculation of the antioxidant activity was performed according to the following formula: Scavenging effect

$$(I\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \times 100$$

Where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of sample, with extract. Triplicates were made to the analyses

Ferric reducing antioxidant power (FRAP test)

The FRAP test was performed as previously described (MANIAN et al., 2008) with some modifications. Up to 2.7 mL of FRAP reagent, freshly prepared was mixed with 270 µl of distilled water and 90 µl of each sample. Then, this mixture was maintained in water bath at 37 °C for 30 min. The FRAP reagent contained 2.5 mL of 10 mM of TPZ solution in 40 mM of HCl, plus 2.5 of 20 mM of $FeCl_26H_2O$, plus 25 mL of 0.3-M acetate buffer (pH 3.6). Readings was performed at the absorption maximum (595 nm). Solutions of known Trolox concentration were used for calibration. The final results were expressed as micromole Trolox equivalents (TE) per grams of extract.

Lipid peroxidation test

For the lipid peroxidation test a source of homogenized egg yolk lipid was used according to the method proposed (PULIDO et al., 2000). In Pirex® screw capped tubes 1,000µL of homogenized egg yolk was added in 1% (v/v) to saline solution. To this medium 100 µL of different extracts in different concentrations were included, which constituted the different treatment groups. Next, 100 µL of AAPH (2,2-azobis-amidinopropane dihydrochloride, 120 mM) in buffer TBA (Thiobarbituric acid) was added to the samples. Finally TBA buffer at volume of 1.2 mL was added. This mixture was incubated at 37 °C for 30 min. After this period, 300 µL was removed from each tube and transferred to Eppendorf tubes. Then, 600 µL of TCA (trichloroacetic acid) at 15% was added to the Eppendorf tubes, and the tubes were centrifuged at 10,000 g at 4 °C. Then, 500 µL of supernatant from each reaction was transferred to Eppendorf tubes followed by the addition of 500 µL of 0.67% TBA in Milli-Q water. The final mixture was heated in a water bath for 30 min. Afterwards the absorbance was measured at 532 nm.

The antioxidant activity was calculated as the percentage of inhibition of lipid peroxidation, based on the following equation: Inhibition of lipid

$$\text{peroxidation } (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \times 100$$

Where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of sample. The concentration of the extract ($mg.mL^{-1}$) required to inhibit 50% of the lipid peroxidation (IC50) was calculated. The TMP standard curve was prepared from an aqueous solution at a concentration of 2.0 $nmol.mL^{-1}$. Aliquots of 30, 60, 120 and 180 µL of TMP solution are transferred to glass tubes and the volume was adjusted to 500 µL with distilled water. The solutions were prepared in triplicate.

Relative electrophoresis mobility (REM) test

REM was adapted (HSIEH et al., 2005), bovine serum albumin (BSA, 2 mg/mL) was diluted in PBS (10 mM, pH 7.4) and incubated with Cu^{2+} (2 mM) at 37 ◦C for 24 h in the presence or absence of the herbal ethanolic extract (1000 µg/mL), H_2O_2 (0.25 mM) was used as a positive control for oxidation. Electrophoresis of BSA was performed using polyacrylamide gels (SDS-PAGE). Running gel solution was comprised of 12% acrylamide, and the stacking gel was 5% acrylamide. Proteins were stained with 0.25% Coomassie Blue R-250. The results were

expressed in the REM in mm using that of native BSA as the base.

Phytochemical compounds

Total phenols and flavonoids quantification

The quantification of total phenols and flavonoids was performed for extract diluted in ethanol concentrations of 25, 50, 75, 100, 250, 500, 1,000, 3,000, 5,000 and 10,000 µg/mL-1. For the determination of total phenols, the Folin-Ciocalteu method was performed. For each 0.5 mL of extract at the different concentrations 5 mL of distilled water was added in addition to 0.25 mL of Folin-Ciocalteu Reagent. After 3 min, 1 mL of saturated Na2CO3 solution was added at 10% and the mixture was stored for 1 h. The absorbance was measured at 725 nm using a UV-Vis spectrophotometer (model: SP220, BIOSPECTRO, Brazil). All tests were performed in triplicate and the results were expressed in mg of gallic acid equivalent (AGE) per gram of extract.

Total flavonoid quantification of the extract was determined based on the UV-Vis spectrophotometer and the samples were prepared as described (TODA, 2005), based on the flavonoids complexation with AlCl3. An aliquot of 250 µL of extract at the different concentrations was mixed with 1.25 mL distilled water and 75 µL NaNO2 solution at 5%. After 6 min, a 150 µL AlCl3/H2O solution at 10% was added. After 5 min, 0.5 mL of a 1 M NaOH solution was added and then the total volume was adjusted by adding 2.5 mL of distilled water. The samples were shaken in a vortex mixer and the absorbance was measured at 510 nm. All tests were performed in triplicate and the results were expressed in mg of rutin equivalent (RE) per gram of extract.

Analysis in HPLC

The fingerprint of the crude ethanolic extract of the flowers of *P. venusta* was analyzed using HPLC-ESI-IT-MS (Accela High Speed LC from Thermo Scientific®, coupled to an Accela Thermo Scientific® LCQ Fleet with Ion Trap 3D and ionization by electrospray (Column Phenomenex® Luna C18 (2)). The conditions for HPLC were a 250 mm x 4.6 mm x 5 µm column; a mobile phase with water ultra-pure + formic acid 0.1% (A) and methanol + formic acid 0.1% (B); a gradient of 25% of A to 100% of B in 80 min; an injection volume of 20.0 µL; a column temperature of 25 ºC; a flow ratio of 0.8 $mL.min^{-1}$, λ= 254 nm. FIA-ESI-IT-MSn was in the negative mode.

RESULTS

Evaluation of reproductive toxicity

There was no significant difference ($p>0.05$) in the water and food consumption and ovarian weight between the two groups (Table 1). The females treated with *P. venusta* had a significant increase ($p<0.05$) in uterine weight compared to the control females. The estrous cycle was regular during the treatment period, being that the number of estrus phase was similar ($p>0.05$) in the control and experimental groups (5.0 ± 0.0 and 5.0 ± 0.50, respectively).

Table 1. Water and food consumption and reproductive organs relative weight in the control group (0.5 mL distilled water) and experimental group (*P. venusta*, 100 mg/Kg bw).

Parameters	Control group (n=7)	Experimental group (n=7)
Water consumption[1] (mL)	241.05 ± 30.94	244.21 ± 25.18
Food consumption[2] (g)	128.00 ± 29.50	125.00 ± 29.50
Ovaries weight[1] (g%)	0.017 ± 0.007	0.021 ± 0.004
Uterus weight[1] (g%)	0.193 ± 0.048	0.245 ± 0.037*

[1]Values express as the mean ± standard deviation. t-Student test .[2]Values express as the median ± interquartile deviation. Mann-Whitney test. *$p<0.05$ in comparison between the groups.

The ovarian histological structure showed that in the control (Figure 1A) and experimental (Figure 1B) groups, there were several corpora lutea and health follicles inserted into a fibrocellular stroma. Comparing the groups, there was no significant difference ($p>0.05$) in the number of corpora lutea (control, 3.0 ± 3.0 and experimental, 2.5 ± 4.0), growth follicles (control, 2.0 ± 2.0 and experimental, 2.0 ± 1.2), antral follicles (control: 2.0 ± 2.2 and experimental: 2.0 ± 2.0) and atretic follicles (control: 5.0 ± 4.0 and experimental, 5.0 ± 3.0). Data are expressed as the median and interquartile deviation. The uterus of untreated females (Figure 1C) presented with endometrium composed of loose connective tissue, with many leukocytes scattered throughout the interstitium. Nevertheless, in the females treated with *P. venusta* (Figure 1D), the endometrial stroma exhibited a predominantly fibrous appearance, with leukocytes located mainly in the subepithelial region. In this group, there was an increase in the tissular vascularization and

vasodilatation. Moreover, a significant decrease ($p<0.05$) in luminal epithelium height and thickness of the endometrial stroma and perimetrium was observed in the group that received the extract (experimental *vs.* control: 29.3 ± 8.7 *vs*. 32.3 ± 9.0 µm; 256.2 ± 269.0 *vs*. 424.6 ± 331.4 µm; 17.3 ± 6.9 *vs.* 26.4 ± 11.0 µm, respectively). The myometrium thickness was similar ($p>0.05$) in the control (398.8 ± 170.5 µm) and experimental (360.2 ± 181.7 µm) groups. Data are express as the median and interquartile deviation.

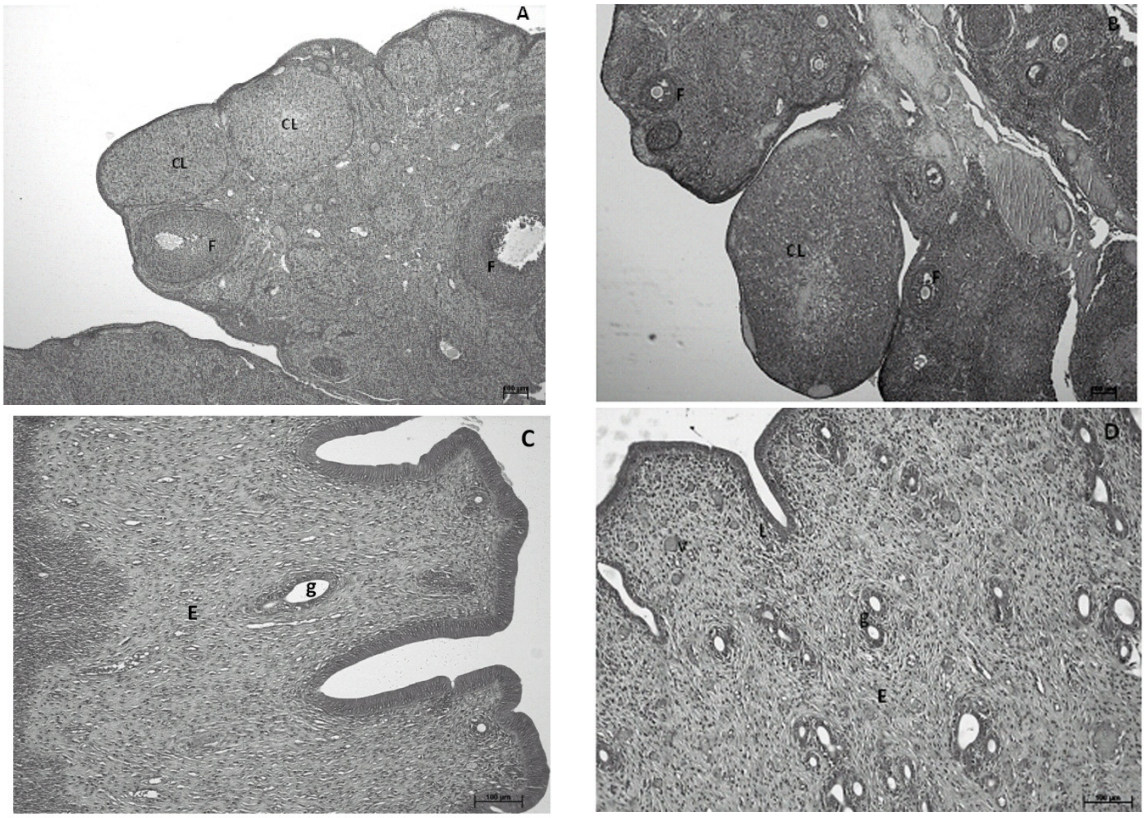

Figure 1. Photomicrographs of the ovarian tissue (A and B) and uterine tissue (C and D). In A and B, observe the similarity of ovaries in the two groups, with corpora lutea (CL) and health follicles (F) in stroma. In C, observe the glands (g) in the endometrium (E). In D, are shows glands (g) and blood vessels (v) inserted in endometrium (E) of fibrous aspect and leukocytes (L) in subepithelial region. Hematoxylin-eosin. Bars = 100 µm.

Table 2 shows that there was no effect of treatment with *P. venusta* extract in maternal and fetal parameters. No external morphologic anomaly was observed in the fetuses of the experimental group.

Table 2. Evaluation of maternal and fetal parameters.

Parameters	**Control group (n=7)**	**Experimental group (n=7)**
Copulation rate (%)	100	100
Fertility rate (%)	71.4	71.4
Body weight of dams (g)	344.0 ± 36.0	350.0 ± 15.0
Gravid uterus weight (g)	44.9 ± 10.0	43.0 ± 7.4
Placentas weight (g)	5.7 ± 0.9	5.9 ± 0.8
Litter weight (g)	21.9 ± 5.4	22.2 ± 3.6
Size litter	13 ± 4	12 ± 2
Pre-implantation loss (%)	0	0
Post-implantation loss (%)	0	0

Values are expressed as median ± interquartile deviation. Mann-Whitney test. None statistical difference between the groups ($p>0.05$).

Clatogenicity/aneugenicity in human lymphocytes

Table 3 shows the micronucleus frequency in bi-nucleated lymphocytes. There were no significant differences between extract treatments, the different concentrations, and the negative control group. Compared to the positive control, the extract-treated groups showed a significant difference; the PC presented the highest frequency of micronucleated bi-nucleated lymphocytes (26.17 ± 2.14), as the group treated with 200 $mg.Kg^{-1}$ bw (highest concentration) showed a frequency of 7.83 ± 2.64.

Table 3. Micronucleus frequency in peripheral blood lymphocytes treated with diferents concentrations of *P. venusta* (50mg/Kg - E50, 100mg/Kg - E100, 200mg/Kg - E200).

Treatments	Number of individuals	MN per 1000 binucleated lynphocytes Mean±SE(%)
NC	6	5.83±2.40a
E50	6	9.33±1.75a
E100	6	8.33±2.06a
E200	6	7.83±2.64a
PC	6	26.17±2.14b

Means followed by same letter in a column not differ significantly by Mann-Whitney and U-test (α=0.05). NC= negative control; PC= positive control (cyclophosphamide).

Antimutagenic assay

Micronucleus test in mice bone marrow

Table 4 shows the results of a micronucleus test in mice bone marrow. The groups treated with different concentrations of extract and cyclophosphamide presented a superior rate of MNPCEs in relation to negative control (NC = 50.0); however compared to positive control group (PC) treated with only with cyclophosphamide, the extract-treated groups showed a significant decrease in the frequency of MNPCEs. The different treatments with the extracts showed no significant difference between them.

Table 4. Frequency of erythrocytes polychromatics micronucleuds (MNPCEs) of bone marrow cells of Swiss mice in experimental groups treated with hydroethanolic extract of *P. venusta* (NC=negative control was distilled water; Animals were treated with the following doses of extract: 50mg/Kg bw (E50), 100mg/Kg bw (E100) and 200mg/Kg bw (E200) or 2mg/Kg bw of cyclophosphamide (PC)).

Treatments	Number of animals	MNPCE per 2000 PCE	
		Nº	Mean ± SD(%)
NC	5	50,0	0,50±2,34a
E50 + PC	5	89,0	0,89±6,46b
E100 + PC	5	76,0	0,76±6,72b
E200 + PC	5	84,0	0,84±4,32b
PC	5	286	2,86 ± 9,01c

Means followed by same letter in a column not differ significantly by Mann-Whitney and U-test (α=0.05).

Antimutagenic test in root cells of *Allium cepa*

The *Allium cepa* test results are shown in Table 5. There was a significant decrease in the micronucleus frequency for roots treated with 5.0 $mg.mL^{-1}$ + MMS (04.4 ± 2.41) compared to other treatments (0.5 $mg.mL^{-1}$ + MMS = 13.8 ± 5.89 and 1.0 $mg.mL^{-1}$ + MMS = 10.2 ± 3.63) and controls (01.0 ± 1.22 = NC and PC = 16.4 ± 2.30), revealing a dose-dependent profile. Increasing extract concentrations resulted in a significant decrease in total chromosomal disorders between the two higher concentrations (0.5 $mg.mL^{-1}$ MMS + = 2.92 ± 0.90, 1 $mg.mL^{-1}$ MMS + = 3.52 ± 0, 66 and 5 $mg.mL^{-1}$ MMS + = 2.60 ± 0.51). Compared to controls sample the results were higher than the NC (1.72 ± 0.59) and lower than the PC (5.10 ± 1.21).

Table 5. Total cells with micronuclei and chromosomal disorders in meristem tissue of *Allium cepa* root under different *P. venusta* extract concentrations (Pv) with Metilmetanosulfonato (MMS), negative control (NC) and positive control (PC).

Treatament (mg/mL)	Micronuclei	c-Metaphase	Chromossomic delay	Cromossomic bridge	Telophase Aberrant	Nucleoli out the Nucleus	Amorphous nucleus	Total chromosomal aberrations
NC	01.0 ± 1.22a	5.0 ± 3.16	3.8 ± 0.45	1.8 ± 0.45	0.8 ± 0.8	03.8 ± 3.27	02.0 ± 2.55	1.72 ± 0.59a
Pv(0.5)+MMS(10)	13.8 ± 5.89b	3.6 ± 2.97	0.8 ± 0.84	5.2 ± 0.45	2.0 ± 1.41	12.2 ± 6.87	05.4 ± 1.82	2.92 ± 0.90b
Pv(1.0)+MMS(10)	10.2 ± 3.63b	3.6 ± 1.52	3.0 ± 1.87	3.4 ± 3.00	4.0 ± 0.70	11.6 ± 3.51	09.6 ± 4.83	3.52 ± 0.66b
Pv(5.0)+MMS(10)	04.4 ± 2.41c	3.0 ± 1.22	2.4 ± 1.52	3.6 ± 1.67	2.4 ± 1.82	07.6 ± 1.82	07.0 ± 3.54	4.60 ± 0.51b
PC	16.4 ± 2.30d	5.6 ± 1.34	2.8 ± 1.92	3.2 ± 2.68	2.6 ± 1.52	15.0 ± 3.54	21.8 ± 6.38	5.10 ± 1.21c

* Equal letters to no significant differences ($p \geq 0.05$).

Antioxidant potential and total phenols and flavonoids DPPH, FRAP and TBARS, total phenols and flavonoids compounds

Table 6 presents the results of the antioxidant tests (DPPH, FRAP and TBARS) and quantification of total phenols and flavonoids in the *P. venusta* extract. For these tests revealed a dose-dependency for the antioxidant activity and phenolic content and total flavonoid. The EC50 was calculated for the DPPH and TBARS tests, resulting in concentrations of, respectively, 131.63 µg and 1.038 mg. The FRAP test found 154.00 µM of TE per extract gram in the extract concentration of 1000 $\mu g/mL^{-1}$.

Table 6. Antioxidant activity, total phenols and flavonoids of *P. venusta* extract.

Concentration ($\mu g.mL^{-1}$)	DPPH (%)[a]	FRAP[b]	TBARS (%)[c]	Total Phenols	Total Flavonoids
100	48.90	54.92	6.78	8.42	40.50
250	61.43	98.96	14.56	21.40	65.34
500	63.23	138.00	23.90	37.64	113.25
1000	65.62	154.00	38.34	51.92	190.52

[a]Average values standard deviation of triplicates for test cleaning of DPPH radical scavenging activity; [b]FRAP: ferric reducing power; [c]lipoperoxidation inhibition percentage (TBARS (%)).

Relative electrophoresis mobility (REM) test

The electrophoresis gel revealed that there was an extensive BSA protein fragmentation resulting from the combined activity of Cu^{2+} and the extract (Figure 2, lane 3), and fragmentation was much higher compared to the positive control with only Cu^{2+} and hydrogen peroxide in the acting protein (Figure 2, lane 2) and with only Cu^{2+} and BSA (Figure 2, lane 1).

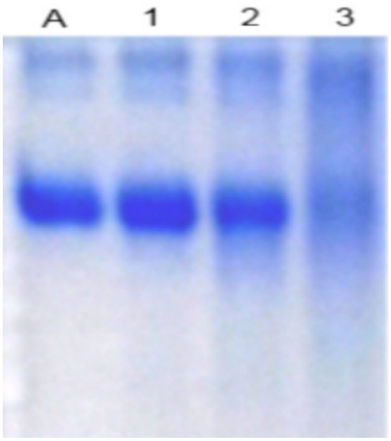

Figure 2. Effect of hydroethanolic extract of *P. venusta* on migration of BSA with PAGE (Incubation period was 24 hours) in oxidative condition. A: Native BSA; 1: BSA with Cu^{2+}; 2: BSA with Cu^{2+} and H_2O_2 3: BSA with Cu2+/H2O2 and hydroethanolic extract (1000 µg/mL).

Phytochemical analysis using HPL

The analysis of the HPLC-PDA chromatogram of *P. venusta* showed two main peaks at 4.11 min and 31.37 min. Based on the *m/z* values, UV spectra and comparison with the literature, we can conclude that peak 2 is quinic acid, and peak 3 is the flavonoid diglycoside. Furthermore, we have propose that peak 1 is a myo-inositol derivative (3).

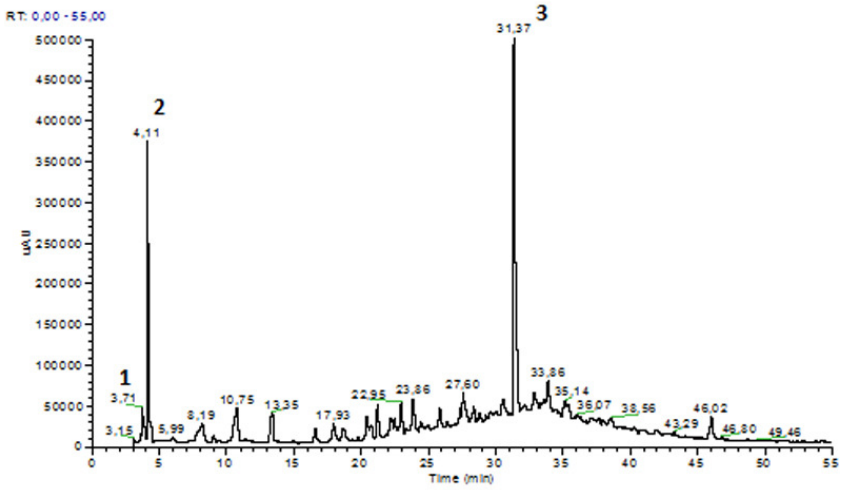

Figure 3. Analytical HPLC-PDA chromatograms at 254 nm of *P. venusta* extract.

Table 7. Tentative identification of compounds in *P. venusta* flowers by LC-PDA, LC-MS and MS/MS data.

Peak	Rt(min)	UV ($\lambda_{máx.}$)	$[M-H]^-$	MS/MS ions	Tentative identification
1	3.71	257	179	225, 195	myo-inositol derivative
2	4.11	269	191	173, 127, 85	quinic acid
3	31.37	331, 249	623	461	flavonoid diglycoside

DISCUSSION

P. venusta is widely used in popular culture for treating various diseases (ALTOÉ et al., 2014). There is promising research that aims treat cancer using *P. venusta* (FIGUEIREDO et al., 2014). Moreover, the flowers and roots extracts of this plant contain significant amounts of phytochemicals with antioxidant properties (VELOSO; SILVA, 2010). Thus, the use of this species could possibly expand to include applications in the pharmaceutical and food industry; but for this expansion, and for its current use, it is necessary to establish its safety.

There are several side effects that may be caused by the chemical compounds found in medicinal plants. Among them include changes in water consumption, diet and body weight, and clinical symptoms of piloerection, changes in behavior, tremors, convulsions and even death (FIGUEIREDO et al., 2014). In studies of the antitumor properties associated with phenolic compounds such as flavonoids, (FERNANDES et al., 2010) there was no change in water and food consumption of Wistar rats treated with a phenolic compound concentration of 50 $mg.kg^{-1}$, as in the present study, treatment of Wistar adult female rats with *P. venusta* did not cause any clinical signs of toxicity.

Female rats treated with isoflavones at concentrations of 3.3 mg/100 mL and 100 mg/100 mL showed a relative weight of the ovaries that was similar to the control group. But the relative uterine weight was higher in the group treated with isoflavones at 100 mg/100 mL compared to groups treated with 3.3 mg/100 mL and controls (IKAKI et al., 2008). In addition, rats treated with both flavonoid concentrations showed no changes in the estrous cycle or in the number of estrus phase in relationship to the control group. In the present study, the same result was obtained with *P. venusta*, which has a high content of flavonoids. The increase in uterine weight observed in this study probably occurred due to discrete changes observed in the endometrium. Currently, no other report is found in the literature on the effects of *P. venusta* extract on the weight and structure of these reproductive organs.

The *P. venusta* chemical constituents did not promote ovarian toxicity, as gonadal tissue showed similar characteristics to that observed in the control group. A study performed (ROMERO et al., 2008) showed that rats treated with *Ginkgo biloba* (120 $mg.Kg^{-1}$), which is rich in flavonoids, showed no histopathological change in ovarian tissue. In a experiment with *Tabebuia avellanedae* (ipe-purple), which is in the same Bignoniaceae family as *P. venusta*, (KUNTZE et al., 2012) examined, in rats, the cicatrizant activity of a topical ointment consisting of plant species extracts. Kuntze et al. (2012) highlighted the importance of flavonoids in tissue healing because the phenolic compound promotes neovascularization and

fibroplasia inhibition in inflammatory process, contributing to increased oxygenation and rapid recovery of the injured tissue. This study indicated that the *P. venusta* flavonoids may have contributed to the fibrous endometrial characteristics and the vasodilation observed in the uterus of female rats. These characteristics did not affect endometrial implantation of the egg or embryo development, indicating that the plant does not present toxicity to the embryo or the fetus.

The micronucleus assay with lymphocytes can be efficiently used to measure the cytotoxic and genotoxic effects of different compounds as reactive oxygen species by ionizing radiation (FENECH et al., 2003). Regarding the *P. venusta* clatogenicity/aneugenicity to human lymphocytes, the result was negative, thus it can be considered that the species studied shows low genotoxicity in respect of the formation of the MN of lymphocytes analyzed, which supports the safety of using this species. In cancer patients there is a higher frequency of MN, and there is a correlation between genotoxic agents such as ionizing radiation, cancer on set and MN (GREENROD; FENECH, 2003).

In the micronucleus test in mouse bone marrow, *P. venusta* extract reduced the chances of genetic alterations in the presence of mutagenic compounds. The extract of this species, reduced the formation of micronucleated polychromatic erythrocytes in relation to the controls that showed the mutagen activity of cyclophosphamide. This reduction was similar in all tested concentrations, making this species promising for use in preventing genomic damage because MN in polychromatic erythrocytes (PCE) of mouse bone marrow is a very sensitive measure of the damage caused by chemical mutagens (FENECH, 2002).

Tests of *A. cepa* have a high level of sensitivity in mammals, and thus is suitable to verify chromosome damage and disorders of the mitotic cycle, cytotoxicity, and genotoxicity to a wide variety of compounds, from heavy metals to aromatic compounds (AKINBORO; BAKARE, 2007). In the present study, the results for total chromosomal disorders were similar to those shown for the micronucleus test in mice bone marrow. The number of chromosomal disorders after treatment was lower than that of the positive control. In the same test, regarding the number of micronuclei, unexpectedly, lower concentrations were more effective by inhibiting the formation of micronuclei in the cells analyzed, and the highest contraction (5 mg/mL^{-1}) had a less significant effect. Nonetheless, these results also confirm the results of the previous experiment on the micronucleus of the mouse bone marrow.

In the DPPH assay, the values achieved with *P. venusta* extract were lower than the values reported previously (ALTOÉ et al., 2014) for *P. venusta* extracts, where the results showed a 94.6% antioxidant activity. The reducing capability of the extract is similar to that described (ROY et al., 2011) in a FRAP test for the extract of *P. venusta* flowers. The results of the FRAP test were lower than that for other species. For example, the value found for the carambola residue was 510.3-$\mu Mol/g^{-1}$ of dry weight (PEREIRA et al., 2014). In a study conducted by Guo et al. (2003) the reducing power found for the red grape was equivalent to 670.5-$\mu Mol.g^{-1}$ of dry weight; however the level of the reducing ability of *P. venusta* is still significant, and it can be correlated with the phenolic compounds dosage ($r^2 = 0.9587$). Thus, the test results of FRAP and DPPH confirm that this species has the potential to protect the body from free radicals.

In the work (SHUI; LEONG, 2006) the results obtained with the cyan-carvone, at a concentration of 7.2-$\mu g/mL^{-1}$ caused a 72.1% inhibition of lipid peroxidation. The study, (SHUI; LEONG, 2006) examined different flavonoids at a concentration of 15 μM, and quercetin reached more than 60% inhibition lipid peroxidation and rutin showed 8% inhibition. Because they are single compounds, the results obtained using *P. venusta* extract can be considered high, and it is possible to isolate individual compounds. The value of r^2 (0.988) resulting from the correlation between the dosage of compounds and the ability to inhibit lipid peroxidation show a possible dependence.

In the relative mobility test in an electrophoresis gel in the presence of Cu^{2+} and H_2O_2, the extract induced more intense activity that fragmented the BSA protein. Thus, the most prominent action shown in the gel was that the tested extract can present oxidative action under certain conditions. In the work (COSTA et al., 2013), antioxidants tested differently in a similar oxidation system. The antioxidant with the best results was glutathione, which almost completely inhibited oxidation, but only at higher concentrations.

A phytochemical analysis indicated the likely presence of quinic acid, a flavonoid diglycoside and myo-inositol. Similarly, (ROY et al., 2011) was identified these same classes of compounds in extracts from the flowers and roots of *P. venusta,* and also confirmed the antioxidant potential of these compounds.

CONCLUSION

P. venusta extract does not present reproductive toxicity and genotoxic effects. However, the extract of this species showed antigenotoxic and antioxidant potential, possibly due to the different flavonoid compounds present in its extract.

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RESUMO: *Pyrostegia venusta* é geralmente encontrada no crescimento secundário das florestas atlânticas e na savana brasileira. Flores e folhas desta planta são utilizadas em remédios populares para tratar uma grande variedade de doenças, desta forma é importante avaliar a segurança e o potencial antioxidante para estas aplicações. Para tanto, o extrato etanólico das flores foi avaliado com testes toxicológicos, genotóxicos e antioxidants. A análise toxicológica foi realizada por meio da toxicidade reprodutiva em ratos e a clatogenicidade/aneugenicidade em linfócitos humanos, a genotoxicidade foi estudada por teste de micronúcleo em medula óssea de camundongo. A antimutagenicidade em células da raiz de *Allium cepa*. Os ensaios antioxidantes utilizados foram DPPH, FRAP, TARBS e MRE. O extrato foi analisado em HPLC. A análise toxicológica reprodutiva mostrou que *P. venusta* não tem efeito negativo sobre o nível reprodutivo e cellular. No teste do micronúcleo o extrato protegeu as células da ciclofosfamida, um composto mutagênico. O teste de *A. cepa* mostrou que o extrato reduziu as formações dos distúrbios cromossômicos. A atividade antioxidante do extrato foi significativa, exceto no teste REM. A análise fitoquímica mostrou a presença de compostos flavonoídicos. O extrato de *P. venusta* não apresenta toxicidade reprodutiva e efeitos genotóxicos. No entanto, o extrato desta espécie apresentou potencial antigenotóxico e antioxidante, possivelmente devido aos diferentes compostos flavonoídicos presentes em seu extrato.

PALAVRAS-CHAVE: Toxicologia reprodutiva. Citotoxicidade. Oxidação. Mutagênese. Flavonoides. Compostos fenólicos.

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