Rev Bras Farmacogn 23(2013): 920-926

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Systemic and reproductive toxicity induced by *Parkia platycephala* ethanolic extract in female Wistar rats

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ARTICLE INFO

Article history:
Received 3 September 2013
Accepted 28 November 2013

Keywords:
Parkia platycephala
Systemic toxicity
Reproductive toxicity
Estrous cycle
Biochemistry analysis
Histopathology

ABSTRACT

The present study was conducted to evaluate the toxicity of the ethanolic extract of leaves of *Parkia platycephala* Benth., Fabaceae, on systemic and reproductive parameters. In toxicity on the estrous cycle, four groups of not-pregnant Wistar rats received distilled water and the doses 250, 500 and 1000 $mg.kg^{-1}$ of plant extract for thirty days, at the end of which they were examined as to the frequency of their phases. The systemic toxicity was assessed through the consumption of water and food and by measuring body mass. After the extract was administered, serum AST, ALT, ALP, bilirubin (total, direct and indirect), urea and creatinine were dosed. The evaluation of the organs (brain, heart, hypophysis, adrenal glands, liver, spleen, uterus and ovaries) in their macroscopic aspects, relative and absolute masses and histological structure showed that the plant extract induced a decrease of water and food consumption and of body mass. It caused an increase in the luteal phase and a decrease in the follicular phase of the estrous cycle and rose serum alkaline phosphatase levels. The data exhibit systemic and reproductive toxicity induced by plant extract in female Wistar rats.

Introduction

According to the World Health Organization (WHO), more than 4 billion people worldwide (or 80% of the global population) use medicinal plants in the primary health care (Quirish et al., 2010). However, evidences suggest that although the adverse effects of using medicinal plants are less frequent than those from conventional drugs, scientific studies confirm that they exist (Haq, 2004; George, 2011). This fact was corroborated with the 18579 cases record of plant poisoning in Brazil, between 1999 and 2009 (National System of Toxico-Pharmacological Information - Sinitox, 2011).

Parkia platycephala Benth, member of the family Fabaceae and popularly known as "faveira", "faveira-preta", "visgueira" and "fava-de-bolota", is found in the mid-west, north and northeast, mainly in the states of Maranhão, Piaui and Ceará. Its pods are widely used in supplementary feeding of ruminants, especially sheep and cattle in the Brazilian savannah areas. Phytochemical analysis showed the presence of total phenols, flavonoids and terpenes in ethanolic extracts and fractions from the leaves of *P. platycephala* (Bezerra et al., 2009). These

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phytochemicals may have therapeutic or toxic characteristics (Pandey et al., 2011). Pharmacological study from Fernandes et al. (2010), with *P. platycephala* extract, showed gastroprotective activity.

Given the observed therapeutic property, scientific research is needed to validate the effective and safe use of this species, enabling it in the future to become an herbal medicine, according to the Brazilian National Agency of Sanitary Vigilance's standards. Thus, the aim of this research is to evaluate the possible toxicity of the ethanolic extract of the leaves of *P. platycephala* on systemic and reproductive parameters in Wistar female rats.

Materials and methods

Plant material and extract preparation

The collection took place in the city of Caxias-MA, in October 2010. Representative material was sent to Graziela Barroso Herbarium in the Federal University of Piaui, where it was identified under the number 27869. The plant material was prepared with the leaves, which were dried in oven at 45ºC and pulverized in an electric mill (2400 g of powder). The extraction was performed with ethanol by maceration for 72 h at room temperature in shade. The extract (EtOH-Pp) was then concentrated by a rotary evaporator apparatus *in vacuo* (50°C). This procedure was repeated seven times with fresh solvent. The resulting extract was lyophilized and stored in an amber bottle under refrigeration (4-8°C).

The doses of EtOH-Pp used in all protocols were 250, 500 and 1000 $mg.kg^{-1}$. These doses increase in arithmetic progression ratio at two, trying to establish themselves the lowest dose that manifests toxic effects without severe damages (LOAEL) and obeying to limit of 1000 $mg.kg^{-1}$ for toxicological tests in accordance with OECD 407 (OECD, 1995).

Animals

Thirty-two adult non-pregnant female Wistar rats (180-220 g) were used in this experiment. They were raised and maintained in the experimental vivarium of the Department of Veterinary Morphophysiology (Center of Agricultural Sciences) at the Federal University of Piaui, Brazil. The animals were housed in individual standard cages, in a controlled temperature room (22 ± 2°C) with 12 h light/dark and free access to water and food (FRI-LAB Rats - Fri-Ribe). All protocols were carried out in accordance with the requirements of the Committee on Ethics Animal Experimentation in the adopted by the Federal University of Piaui (Protocol number: 33/2009). The experimental protocols were elaborated and developed based on the principle of the three R's (Refine, Reduce and Redesign) and according to CFMV Resolution Number 714 of June 20th, 2002.

Estrous cycle toxicity evaluation

The experiment consisted of four groups of eight animals each (n = 8) randomly divided. They were: control group, to which distilled water was administered, and three groups received EtOH-Pp 250, 500 and 1000 $mg.kg^{-1}$ via the oral route (10 $ml.kg^{-1}$ of body weight) for thirty days.

The protocol used in the estrous cycle toxicity evaluation followed the methodology developed by Goldman et al. (2007). The rats were examined daily, between 8 and 9 am for seven days initially verify whether they were cycling normally. Only those with normal cycle were included in the experiment. To evaluate the estrous cycle, the vaginal smear was collected with a plastic pipette containing nearly 10 µl of saline (NaCl 0.9 %) and deposited on a glass slide, which was observed through light microscopy using the objectives of 10 and 40×.

The estrous cycle phases (proestrus, estrus, metestrus and diestrus) used in the investigation of the influence of EtOH-Pp was classified according to the cellular profile of the vaginal smear observed in microscopy.

The determination of the estrous cycle phase frequency was established by the sum of the occurrences of the registered phases during the treatment period. The interestrus interval was calculated through the sum of the number of days between the estruses, divided by the total of days of treatment. The diestrus index was calculated by the sum of the numbers of diestrus occurrences, multiplied by 100 and divided by the days of treatment.

Systemic toxicity evaluation

Concomitant with the evaluation of the estrous cycle, the systemic toxicity of EtOH-Pp was evaluated in the same animals, using the method proposed by Park et al. (2010). Water and food consumption, body mass and biochemical parameters also were analysed.

Water (ml) and food (g) consumption of the animals under treatment was measured in each cage on the days 1, 4, 7, 10, 13, 16, 19, 22, 25, 28 after the beginning of the treatment. These parameters were quantified by the difference in the amount available for consumption (250 ml of water and 50 g of food) on the day before and on the days mentioned above.

The body mass of the animals (g) was measured on the days 0, 1, 4, 7, 10, 13, 16, 19, 22, 25, 28 and 29 after the beginning of the treatment, in which day 0 represents the day before the first day of treatment. The animals' body mass was registered also on the day after the last day of treatment (day 31).

On the day subsequent to the end of the 30 day-treatment with EtOH-Pp, the rats were anesthetized with an association of ketamine (50 $mg.kg^{-1}$) and xylazine (11.5 $mg.kg^{-1}$) and then the blood collection was proceeded through cardiac puncture, into the flask containing no anti-coagulant, with clot activator (BD- Vacuette Z serum clot activator), for the evaluation of biochemical parameters.

The blood samples were centrifuged at 1917 × *g* for 5 min and the serum was separated. The biochemical assays of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and urea were performed through dry chemistry (Reflotron, Roche Diagnostics, Brazil). The alkaline phosphatase (ALP), creatinine (CREA), direct bilirubin (BIL D), indirect bilirubin (BIL IND) and total bilirubin (BIL T) assays were performed in Semi-automatic Biochemistry Analyzer RA-50 (Bayer), using Labtest kits (Labtest Diagnóstica, Lagoa Santa, MG, Brazil).

Macroscopic aspect and organ mass measurement

After the blood collection, the rats were euthanized, using the same anesthetics in excess and were necropsied. Brain, hypophysis, adrenal glands, heart, liver, kidneys, spleen, ovaries and uterus were dissected and macroscopically evaluated for color and morphology. Also there was a measurement of the organs' absolute masses and relative masses according to brain weight. The material was fixed in a buffered 10% formalin solution until the histopathological processing procedures.

Histopathological analysis

After the fixation of the organs, their fragments were dehydrated in alcohol solutions of increasing concentrations; diafanized in xylol; included in paraffin, CUT at µm, colored by hematoxylin-eosin and examined under a light microscope.

Statistical analysis

The numerical results were evaluated through variance analysis, followed by the Tukey test at 5% to compare the means. The data were presented mean ± SEM. Statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA, USA).

Results

The Pp-EtOH extract toxicity in the estrous cycle demonstrated that proestrus frequency was significantly reduced at doses of 500 and 1000 mg.kg^{-1} compared to vehicle. The frequency of the other phases, as well as the interestrus interval, was not significantly altered (Table 1).

The water and food consumption were significantly reduced between the doses of 500 and 1000 mg.kg^{-1} vs. vehicle and in the dose of 250 mg.kg^{-1}, there was a reduction in the water consumption. Although, the difference among the doses was not significant, there was a significant reduction in body mass in the doses of 500 and 1000 mg.kg^{-1} of EtOH-Pp when compared to vehicle (Table 2).

Increased ALP enzyme dosages in the doses of 500 and 1000 mg.kg^{-1} compared to vehicle, while the other biochemical parameters did not show significant alteration, those presented in Table 3. The macroscopic analysis of the heart, brain, hypophysis, adrenal glands, liver, kidneys, ovaries and uterus did not demonstrate morphologic alterations among the groups (results not shown).

Among the organs analyzed, the uterus had significant reduction in its relative mass at the doses of 250, 500 and 1000 mg.kg^{-1} (Table 4). There were no significant alterations in the relative weight of the other organs evaluated.

Regarding histopathological analysis, heart, brain, liver, kidneys, uterus and ovaries did not demonstrate significant alteration in the groups treated with extract, compared to vehicle (data not shown).

Discussion

Several potentially active substances were described in Pp-EtOH extract, such as phenolics compounds, flavonoids and triterpenes (Bezerra et al., 2009). Besides these, other substances such as steroids, tannins, saponins, cardiac glycosides have been found in the genus *Parkia* according

Table 1
Frequency of estrus cycle phases, interval between estrus and diestrus index after 30 days of treatment with different doses of ethanolic extract of *Parkia platycephala* (EtOH-Pp).

Phase	Frequency			
	Treatments			
	Vehicle (H20d)	EtOH-Pp 250 mg.kg^{-1}	EtOH-Pp 500 mg.kg^{-1}	EtOH-Pp 1000 mg.kg^{-1}
Proestrus	7.375 ± 0.263	6.750 ± 0.250	5.500 ± 0.423[a]	5.750 ± 0.313[a]
Estrus	7.375 ± 0.324	7.125 ± 0.350	6.750 ± 0.453	6.500 ± 0.500
Interestrus Interval	4.093 ± 0.194	4.423 ± 0.316	4.775 ± 0.293	5.068 ± 0.525
Metaestrus	8.125 ± 0.549	8.000 ± 0.423	7.750 ± 0.366	7.375 ± 0.375
Diestrus	8.125 ± 0.295	9.125 ± 0.895	11.000 ± 0.707[a]	11.375 ± 0.90[a]
Diestrus index (%)	26.200	29.438	30.587	38.338[a]

Diestrus index, Number of days with clear diestrus smear × 100/total duration of treatment (days).The data represent the mean ± SEM animals, n = 8.
[a] $p < 0.05$ vs. vehicle.

Table 2
Water and food consumption by female rats during 30 days of treatment with ethanol extract from leaves of *Parkia platycephala* Benth (Pp-EtOH) at different doses.

Parameters	Consumption			
	Treatments			
	Vehicle (H_2O)	EtOH-Pp 250 mg.kg^{-1}	EtOH-Pp 500 mg.kg^{-1}	EtOH-Pp 1000 mg.kg^{-1}
Water (ml)	24.930 ± 1.215	22.130 ± 0.886 [a]	20.830 ± 0.493[a]	23.800 ± 1.201[a]
Food (g)	14.750 ± 0.307	13.200 ± 0.401	11.92 0 ± 0.545[a]	12.310 ± 0.438[a]

The data represent the mean ± standard error of the mean of animals, n = 8.
[a] $p < 0.05$ vs. vehicle.

Table 3
Serum concentrations of aspartate transferase (AST), alanine transferase (ALT) and alkaline phosphatase (ALP) in U/l, direct bilirubin (BIL D), bilirubin (BIL IND), total bilirubin (T-BIL), urea, creatinine (CREA) in mg.dl^{-1} in female rats after 30 days´ administration of ethanol extract from leaves of *Parkia platycephala* (Pp-EtOH) at different doses.

Parameter	Reference Value	Serum concentrations			
		Treatments			
		Vehicle (H_20d)	EtOH-Pp 250 mg.kg^{-1}	EtOH-Pp 500 mg.kg^{-1}	EtOH-Pp 1000 mg.kg^{-1}
AST	76 - 100	60.633 ± 2.082	74.417 ± 5.901	65.167 ± 3.832	69.400 ± 2.440
ALT	45 - 61	34.967 ± 1.684	37.883 ± 3.694	34.850 ± 3.296	28.933 ± 3.017
ALP	57 - 61	39.250 ± 5.799	68.375 ± 10.765	90.500 ± 13.639 [a]	89.500 ± 4.668[a]
BIL D	-	0.225 ± 0.056	0.163 ± 0.026	0.188 ± 0.040	0.200 ± 0.038
BIL IND	-	0.575 ± 0.121	0.638 ± 0.096	0.712 ± 0.074	0.675 ± 0.080
BIL T	-	0.800 ± 0.093	0.925 ± 0.136	1.000 ± 0.127	0.875 ± 0.077
UREA	52 - 62	47.750 ± 1.968	48.225 ± 1.009	44.187 ± 2.137	43.513 ± 1.160
CREA	0.4 - 0.6	0.375 ± 0.065	0.325 ± 0.031	0.400 ± 0.033	0.388 ± 0.030

The data represent the mean ± standard error of mean, n = 8.
[a] $p < 0.05$ vs. vehicle.

Ajaiyeoba (2002) and could be responsible for therapeutic or toxic activities on reproductive and systemic parameters. There are no records of studies on systemic and reproductive toxicity of *Parkia platycephala* Benth. Thus, this study about the EtOH-Pp aimed to establishing doses note possible toxic effects over systemic and reproductive parameters in non-pregnant rats.

The short length of the estrous cycle of rats makes them ideal for investigation of changes occurring during the reproductive cycle (Marcondes et al., 2002). The results of the estrous cycle evaluation demonstrate the probable reproductive toxicity of EtOH-Pp in the doses of 500 and 1000 mg.kg^{-1}, for it has altered the frequency of the phases of estrous cycle diestrus prolongation and reduction of proestrus, as suggested in Goldman et al. (2007). This is similar to reports from Soni et al. (2013) wherein the treatment of rats with ethanol extract of stem of *Musa paradisiaca* L., Musaceae, at doses of 250 and 500 mg.kg^{-1}

Table 4
Relative mass of the organs of rats relative body mass (g/100 g body weight) after 30 days of exposure to different doses of the extract of *Parkia platycephala* (EtOH-Pp).

Organ	Relative mass of the organs (g/100 g of body mass)			
	Treatments			
	Vehicle (H_20)	EtOH-Pp 250 $mg.kg^{-1}$	EtOH-Pp 500 $mg.kg^{-1}$	EtOH-Pp 1000 $mg.kg^{-1}$
Brain	0.872 ± 0.031	0,884 ± 0.034	0,907 ± 0.027	0,953 ± 0.025
Hypophysis	0.06 ± 0.0007	0.006 ± 0.001	0.013 ± 0.008	0.006 ± 0.0006
Adrenal	0.019 ± 0.002	0.016 ± 0.002	0.016 ± 0.002	0.018 ± 0.0009
Liver	2.846 ± 0.107	2.698 ± 0.011	2.741 ± 0.096	2.750 ± 0.076
Kidneys	0.346 ± 0.008	0.353 ± 0.007	0.347 ± 0.010	0.336 ± 0.009
Spleen	0.325 ± 0.021	0.332 ± 0.016	0.331 ± 0.010	0.323 ± 0.015
Heart	0.380 ± 0.008	0.403 ± 0.021	0.365 ± 0.007	0.357 ± 0.011
Uterus	0.492 ± 0.057	0.229 ± 0.009[a]	0.253 ± 0.024[a]	0.287 ± 0.025[a]
Ovary	0.028 ± 0.020	0.026 ± 0.002	0.055 ± 0.029	0.034 ± 0.003

The data represent the mean ± standard error of the mean of animals, n = 8.
[a] $p < 0.05$ vs. vehicle.

body weight for five days caused a prolonged diestrous stage of the estrous cycle.

These alterations can be explained possibly by the presence of progestogen in EtOH-Pp, capable of inhibiting the hypothalamic-pituitary axis and the follicular development, similar to what was observed by Bakry et al. (2010) in a treatment of mice with medroxyprogesterone acetate. Those substances can be among the steroid compounds described in the extract of plant of the same genus in the study of Ajaiyeoba (2002). This effect similar to the progestogen's must be investigated for the possibility of provoking anestrus in farm animals that ordinarily ingest the pods of the plant, and also for the potential use in phytotherapy.

The decrease in water consumption in the EtOH-Pp doses used can be explained by the presence of phenolic compounds, as described by Bezerra et al. (2009). These compounds reduce water ingest by altering the palatability and not for their direct toxic effects, as it could be inferred. Besides water consumption, also there was a reduction in food ingest and body mass. A possible explanation for the decrease in food ingest concerns alterations in gastrointestinal hormones like glucagon and pancreatic polypeptide, which act like neurotransmitters in the central nervous system, peripheral action by specific receptors in glucagon like receptors (GCGR) and Y2, respectively (Suzuki et al., 2010).

The biochemical to the analysis, there were no significant alterations in AST, ALT enzymes dosages. Also, serum values below the reference range show absence of hepatotoxicity of EtOH-Pp, according to Mukinda and Syce´s (2007) acute and chronic toxicity study of *Artemisia afra* in rats and Oluduro and Aderiye (2009), who evaluated the effects of *Moringa oleifera* extract in albino rats. This fact was also corroborated with the total, direct and indirect bilirubin dosages, which also did not demonstrate significant differences.

Nevertheless, the high levels of alkaline phosphatase in the doses of 500 and 1000 $mg.kg^{-1}$ seem not to be due to EtOH-Pp toxicity with hepatic injury, because the enzymes AST and ALT did not suffer significant alterations, but they can be related to the skeletal muscle metabolism disturbance, according to Amida et al. (2007), in a study of acute and chronic toxicity of *Artemisia afra* in rats or bone alteration, as in Oluduro and Aderiye´s (2009) analysis of the effect of *Moringa oleifera* extract on enzymes' activity in albino rats.

In urea and creatinine serum concentrations, there was no significant difference among the treated groups; also, their values were below the reference range, what implies that EtOH-Pp is not toxic to the kidneys. The macroscopic analysis, like the histopathological analysis of the heart, brain, liver, ovaries, and uterus of rats after exposure to different doses of EtOH-Pp, did not demonstrate significant alterations in their color, morphology and macroscopic structures, what evidences the extract incapacity to induce structural alterations in these organs.

In the analysis of the weight of the organs, both the absolute and the one related to the brain weight, there was only significant reduction in the uterus compared to

vehicle, in the doses of 250, 500 and 1000 mg.kg^{-1}, what demonstrates the toxic effect of EtOH-Pp on uterus. According to Wolfsegger et al. (2009), the weight alteration can indicate direct toxic effect of an extract on an organ even in the absence of morphological changes in the organ. Thus, EtOH-Pp presents evidence of direct toxicity on the uterus.

The fact that the reduction of uterine weight repeats both in the absolute measurements as well as in the ones related to the brain weight corroborates also the possibility of toxic effect of EtOH-Pp, especially because as in Bailey et al. (2004), brain weight does not usually suffer from chemical substance influence. Another possible mechanism that could explain the decrease in uterine weight is the presence of substances with antiestrognic activity in EtOH-Pp, which can reduce the effect of endogenous estrogens, like in a study on the effects of the species *Nelumbo nucífera* on reproductive organs developed by Mutreja et al. (2008), neutralizing their proliferative effects on the uterus. These substances can be flavonoids, compounds possibly present in EtOH-Pp in previous work (Bezerra et. al., 2009), that can present antiestrogenic action through a mechanism that is independent and dependent on estrogen receptor (Collins-Burow et al. 2000).

Briefly, the extract induced more toxicity on water and food consumption, body mass, and an increase in the biochemical parameter of bone alteration, in the dose of 1000 mg.kg^{-1}. Regarding the great organs of blood flow and the biochemical blood parameters, the extract did not produce structural and biochemical alterations that indicate functional alteration in the organs evaluated. In reproductive toxicity, the EtOH-Pp extract, in the highest dose, demonstrated toxicity on estrous cycle with increase in the diestrus phase ratio and reduction of the uterus weight.

Authors' contributions

BAC contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the date and drafted the paper. JMGO contributed inanalysis of the date and drafted the paper. PABS contributed in extract preparation, execution of protocols in the laboratory, analysis of the date and drafted the paper. SRSL assisted in the laboratory work and provided some materials and reagents. SMMSS contributed with histopathological analysis. LMC made the manuscript translation. MCSM contributed to critical reading of the manuscript and the APRC designed study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Acknowledgment

The authors thank teacher Abilio Borghi for the grammar review of the manuscript.

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