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Original Research

The administration of hydroethanolic extract of *Cordia ecalyculata* Vell. at different doses promotes reproductive toxicity in adult male rats

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ABSTRACT

Popular use of *Cordia ecalyculata* extract is increasing due to its potential in inhibiting appetite. Its side effects on reproduction have not yet been reported. Thus, for the first time, this study investigated the potential reproductive toxicity of different doses of *C. ecalyculata* extract in adult male rats ($n = 12$/group), which received hydroethanolic extract of *C. ecalyculata* at 20, 100, or 400 mg/kg of body weight (b.w.) or distilled water (control group), orally (gavage) for 60 consecutive days. The testicular and epididymal tissue, sperm morphology, fertility, and pregnancy outcome were evaluated. The results showed that food consumption decreased significantly in the groups that received 20 or 400 mg/kg of extract, but b.w. was similar ($P > 0.05$) in all experimental groups. No significant alteration was observed in testicular weight. At the 400 mg/kg dose, the epididymal relative weight increased ($P < 0.05$), compared to the control group. There were several histopathological changes in testes and epididymis at all extract doses. In addition, the percentage of abnormal gametes increased ($P < 0.05$) and the gestation rate decreased ($P < 0.05$). In conclusion, the *C. ecalyculata* extract promotes reproductive toxicity in rats, but with no dose-dependent effect.

KEY WORDS: *Cordia ecalyculata*, epididymis, fertility, gametes, testis

INTRODUCTION

Since antiquity, natural substances have been used to treat and prevent various clinical diseases, whereas in recent years, the use of herbal medicines has increased considerably due to their wide availability and low cost, compared with synthetic substances. The number of men and women looking for natural or synthetic products claiming to reduce body weight (b.w.) has increased every year worldwide. *Cordia ecalyculata*, known popularly as "Porangaba," constitutes a natural means of decreasing b.w. [1,2]. Due to the large repercussion in the media, especially in magazines and websites that address the topic of weight loss, this herbal medicine has been widely used in recent years.

C. ecalyculata Vell., *Boraginaceae* family, is found from the Northeast to the South of Brazil, and it is also encountered in Argentina and Paraguay [2]. Its fruit is similar to that of coffee, which explains its many popular names such as "café-de-bugre" and "café-do-mato" [3]. The therapeutic properties of this herbal medicine are attributed to the presence of various active compounds including caffeine, allantoin, allantoic acid, consolidine glycoside, tannins, saponins, steroids, and potassium mineral [1,2]. *C. ecalyculata* has been utilized since ancient times as a herbal medicine, especially as a diuretic, circulation stimulant, cardiotonic, anti-edema agent, appetite suppressant, and arterial fat deposition inhibitor [4,5], although such activities have not yet been fully proven in the scientific literature.

Despite widespread pharmacological and toxicological studies of a great diversity of medicinal plants, there are still few reports addressing their side effects on reproduction, especially in relation to the histopathology of organs. The effectiveness of *C. ecalyculata* in reducing the b.w., as well as the lethal dose (LD) in rodents and its genotoxic and cytotoxic activities has been reported previously. However, its adverse effects on reproduction are unknown. To the best of our current knowledge, there are no published data showing the effects of *C. ecalyculata* on male reproduction. Therefore, this study evaluated the possible reproductive toxicity of different doses of hydroethanolic extract of *C. ecalyculata* in adult male rats.

MATERIALS AND METHODS

Preparation of Plant Extract

The Pholia Magra™ (Brazil), a product acquired commercially without excipient but with the presence of a certificate of quality control analysis, was prepared from the leaves and stalks of *C. ecalyculata* by hydroethanolic solvent extraction. The raw material certificate attests to the presence of tannin and caffeine in its composition. The solutions were prepared immediately prior to administration to the animals, with the dilution of Pholia Magra™ in 1.5 ml of distilled water.

Animals

Male albino rats, Wistar lineage (n = 48), 90-day-old and approximately 350 g b.w., were supplied by the Central Biotherium of Univ Estadual Paulista - UNESP (Botucatu, São Paulo, Brazil) and kept at the Faculty of Sciences and Letters (UNESP, Assis, São Paulo, Brazil). The animals were maintained under controlled temperature and luminosity (22 ± 2°C; 12 h-light/12 h-dark, respectively). Rat chow and filtered tap water were provided *ad libitum*. The experimental protocol followed the Ethical Principles in Animal Research of the Brazilian College of Animal Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and was approved by the Ethics Committee for Animals Use (Register Number 010/2011, CEUA).

Experimental Groups

The rats were weighed and randomly distributed into four experimental groups (n = 12/group): C20, C100, and C400, treated, respectively, with 20, 100, and 400 mg/kg b.w. of *C. ecalyculata* extract and treated with 1.5 mL of distilled water (control - Co). The doses were administered orally (gavage), once daily, for 60 consecutive days, at the same time (8:00-9:00 a.m.). The choice of doses was based on a previous study [6].

B.w. and Obtaining Reproductive Organs

All rats were weighed weekly, and at the end of experimental period, six animals from each group were euthanized intraperitoneally via an overdose of anesthesia (Thiopentax™, Cristalia, São Paulo, Brazil). The testes and epididymis were removed and weighed, and the absolute (g) and relative (g%; organ weight divided by b.w., ×100) weights were obtained.

Histological Routine and Analysis

The testes and left epididymis were fixed in Bouin's solution, dehydrated in ethyl alcohol, clarified in xylol, and embedded in paraffin (Paraplast, Labware-Oxford, St. Louis, MO, USA). The 5 μm thick sections were stained with hematoxylin and eosin, Mallory's Trichrome, and Periodic acid-Schiff-hematoxylin for the analysis of cellular components and extracellular matrix. In the epididymis, each anatomical region was examined (caput, corpus, and cauda). The slides were analyzed qualitatively, using a Zeiss Scope A1-Axio microscope connected to an AxioCam ICc3 camera, and the digitalized images were obtained by the image analyzer AxioVision, Version 4.7.2.

Morphometric Analysis

In the testes of each rat, 20 cross-sections of seminiferous tubules were randomly chosen to measure the tubular diameter, height of the germinal epithelium, and luminal and tubular area, at ×20 magnification. In epididymis, 10 cross-sections of each anatomical region were randomly chosen to measure the epithelial height and luminal and ductal area at ×10 magnification. The morphometric analyses were conducted using a Zeiss Scope A1-Axio microscope connected to an AxioCam ICc3 camera, with image analyzer AxioVision, Version 4.7.2.

Sperm Morphology

In the right cauda epididymis of each animal, spermatozoa were obtained according to the technique described previously [7]. The smears were stained with hematoxylin and Shorr, for morphological analysis of gametes, in a light microscope, at ×100 magnification. 100 spermatozoa were examined randomly from each animal, and classified into two groups: Normal (normotype) and abnormal (teratospermy), according to the criteria for evaluating the morphology of the gamete head and flagellum [8].

Fertility Test and Fetal Parameters

Six rats from each group were subjected to a fertility test at the end of the treatment period. The animals were mated with non-treated females, at a proportion of 1:1. The presence of sperm in vaginal smears was designated as gestation day 1 (GD). In GD 19, the females were previously anesthetized with ketamine (40 mg/kg; Dopalen™, Ceva Lab., São Paulo, Brazil) and xylazine (20 mg/kg; Dorcipec™, Vallée Lab., São Paulo, Brazil), intramuscularly. The laparotomy was carried out, and the following records were obtained: Size and weight of litter, fetal weight, number of implants, number of corpora lutea, number of resorptions, and placental weight. Pre-implantation loss rate (number of corpora lutea-number of implantations/number of corpora lutea ×100), post-implantation loss rate (number of implantations-number of fetuses/number of implantations

×100), fertility rate (number of females with sperm in smears/number of females mated ×100), and gestation rate (number of females with viable fetuses/number of females having copulated ×100) were calculated. The fetuses were examined under stereomicroscopy to evaluate the external morphology.

Statistical Analysis

The data were analyzed by the ANOVA, complemented by Tukey's test or by non-parametric Kruskal-Wallis test complemented by Dunn test. Fertility and gestation rates were analyzed by Chi-square test. Significance was set at $P < 0.05$.

RESULTS

Effects of *C. ecalyculata* on Water and Food Intake

The food and water consumption was similar ($P > 0.05$) between the Co and C100 groups [Table 1]. There was a higher food consumption in the C20 group, while the C400 group presented reduced intake. The water consumption increased significantly ($P < 0.05$) in the C20 and C400 groups, compared to Co and C100 groups [Table 1].

Extract of *C. ecalyculata* Alters Epididymis but not Testis Weight

The administration of extract at doses of 20, 100, and 400 mg/kg did not cause a significant change in final b.w. of rats, compared to the Co group [Table 1]. Furthermore, the b.w. of animals receiving the highest dose (C400) was significantly lower ($P < 0.05$) than the group treated with the lowest dose (C20).

Table 1: Food (g) and water (mL) consumption, initial and final b.w. (g), and absolute (g) and relative (g%) weights of reproductive organs in the different experimental groups

Parameters	Co	C20	C100	C400
Food consumption[1]	105±11[a]	115±11.5[b]	107±16[a]	93±18[c]
Water consumption[1]	150±22.7[a]	185±41.2[b]	150±25[a]	200±110[b]
b.w.[1] (g)				
Initial	368.0±45.7[a]	343.6±24.8[a]	333.8±24.8[a]	332.5±38.4[a]
Final	457.1±69.3[ab]	479.3±42.8[a]	441.5±37.8[ab]	410.1±51.2[b]
Testicular weight				
g	3.15±1.17[a]	3.71±0.32[a]	3.29±0.03[a]	3.53±0.09[a]
g%*	0.69±0.11[a]	0.72±0.07[a]	0.76±0.11[a]	0.84±0.11[a]
Epididymal weight				
g	1.37±0.38[a]	1.41±0.11[a]	1.48±0.05[a]	1.66±0.10[a]
g%*	0.29±0.03[a]	0.28±0.02[a]	0.32±0.03[ab]	0.38±0.05[b]

Within each line, values followed by different letters indicate statistical differences between the groups ($P<0.05$). Data are expressed as the median±interquartile deviation (Kruskal-Wallis, Dunn test). *Data are expressed as the mean±standard deviation (ANOVA, Tukey's test). Co: Control group; C20: Treated with 20 mg/kg b.w. of extract; C100: Treated with 100 mg/kg b.w. of extract; C400: Treated with 400 mg/kg b.w. of extract. [1](n=12/group); other parameters (n=6/group)

The absolute and relative weights of testes were significantly similar ($P > 0.05$) in all experimental groups, but there was an increase ($P < 0.05$) in the relative weight of epididymis at the 400 mg/kg dose, in contrast to the Co and C20 groups [Table 1].

C. ecalyculata Promotes Morphological Changes in Testes and Epididymis

The gonad tissue of groups treated with *C. ecalyculata* [Figure 1C-H] showed alterations in the seminiferous tubules, compared to the control group [Figure 1A and B]. There was a loss of seminiferous cytoarchitecture integrity, desquamation of germ cells, infiltration of immature cells into the lumen [Figure 1C, E and F], and epithelial area devoid of cells [Figure 1D]. In addition, the amount of spermatozoa inside the tubular lumen was apparently reduced, as shown in the C400 group [Figure 1G]. Degenerative cells were observed in periluminal region [Figure 1H], characterized by intense nuclear basophilia and cytoplasmic acidophilia. The tubular area of C400 group increased ($P < 0.05$) comparatively to the Co group [Table 2]. However, the luminal area, tubular diameter, and epithelial height were similar ($P > 0.05$) in all experimental groups.

The epididymal tissue exhibited under the three extract doses [Figure 2D-L] characteristics different from those observed in the control group [Figure 2A-C]. In corpus, the ductal epithelium presented degenerative cells, with pyknotic nucleus and vacuolated cytoplasm [Figure 2K]. Spermatozoa with short or absent flagellum, immature germ cells, and multinucleated cells were observed in the lumen [Figure 2E and G]. In all groups treated with *C. ecalyculata*, the three anatomical regions of the organ showed interstitium with edematous and hemorrhagic aspect, having congested blood vessels and immune cells dispersed in the tissue [Figure 2D and F-J]. In the C400 group, there was thickening of the muscle layer surrounding the duct in the cauda [Figure 2L]. The ductal and luminal area and epithelial height of the caput were equal ($P > 0.05$) in all experimental groups [Table 2]. There was a significant increase in the luminal area of corpus in the C20 group, compared to the Co and C400 groups. The caudal epithelial height was decreased ($P < 0.05$) in the C100 and C400 groups, compared to the Co group.

C. ecalyculata Affects the Sperm Morphology

The percentage of morphologically abnormal gametes was significantly higher ($P < 0.05$) in the groups treated with each dose of extract, compared to the Co group [Table 2]. The spermatozoa presented mainly anomalies of the flagellum, characterized by absence or shortening of this morphological portion of the cell.

C. ecalyculata Promotes a Decrease in Gestation Rate

Fertility rates were similar ($P > 0.05$) among all experimental groups [Table 3]. However, the gestation rate was significantly decreased ($P < 0.05$) in the C20, C100, and C400 groups

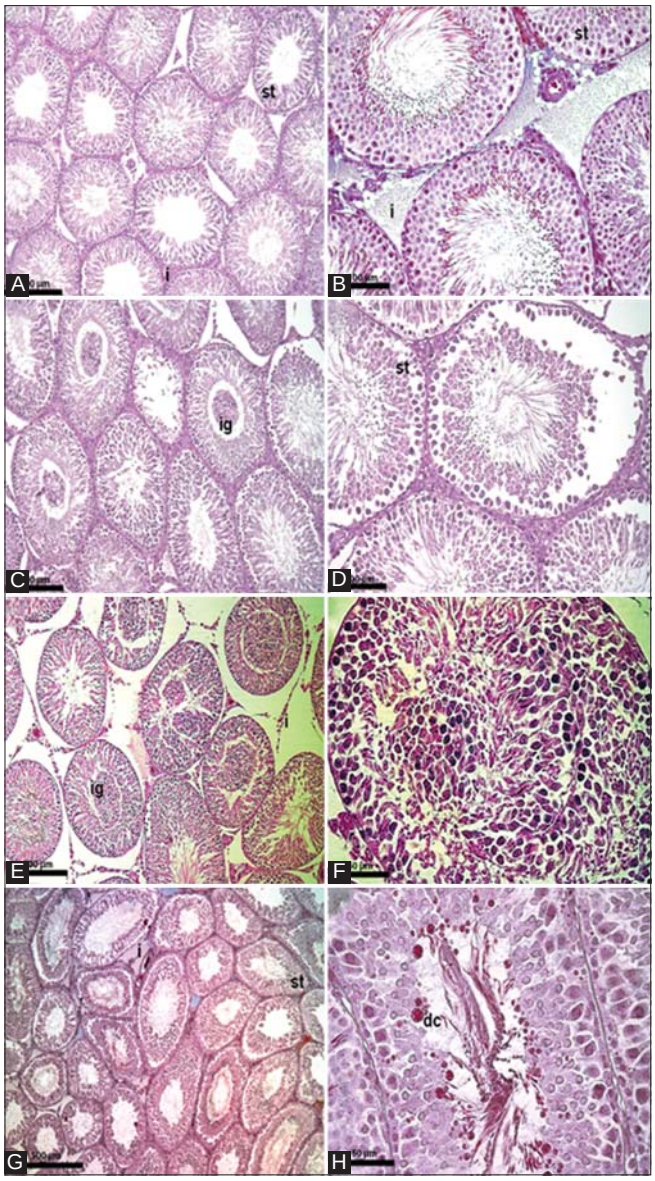

Figure 1: Photomicrographs of testes in the rats of groups: Co (A and B), C20 (C and D), C100 (E and F), and C400 (G and H). Observe the morphological integrity of the seminiferous tubules (st) in the control group. Animals treated with *Cordia ecalyculata* extract show seminiferous epithelium disorganization, immature germ cells (ig) in lumen, epithelial area devoid of cells (in D), and scarcity of spermatozoa in lumen and degenerative cells (dc) near the lumen. st; interstitium (i). Periodic acid-Schiff-Hematoxylin (A, C and D); Mallory's trichrome (B, G and H); hematoxylin and eosin (HE) (E and F). Bars=50 μm (F and H), 100 μm (B and D), 200 μm (A, C and E); 500 μm (G)

(67, 67, and 50%, respectively), compared to the Co group (100%). The b.w. of the dams mated with treated males was decreased ($P < 0.05$) in C400 in relation to the C20 group. Fetal weight was diminished ($P < 0.05$) in C400 in comparison to the Co group. The treatment with *C. ecalyculata* at doses of 20, 100, and 400 mg/kg did not affect ($P > 0.05$) the gravid uterus weight, placental weight, size and weight of litter, and pre- and post-implantation loss rates. No external anomaly was observed in the fetuses.

Table 2: Morphometric analysis in testes and epididymis (area in mm^2; diameter and height in μm), and percentage of abnormal spermatozoa in the different experimental groups (n=6/group)

Parameters	Co	C20	C100	C400
Testes				
Tubular area	62.4±6.4^{a}	69.2±8.6^{ac}	64.5±4.5^{ab}	68.3±5.1^{bc}
Luminal area	12.1±2.7^{a}	15.6±3.9^{a}	14.6±2.6^{a}	18.1±5.1^{a}
Tubular diameter	277.2±15.7^{a}	293.0±16.2^{a}	286.2±9.4^{a}	294.7±8.3^{a}
Epithelial height	83.1±8.2^{a}	81.6±5.7^{a}	77.6±5.6^{a}	79.3±3.5^{a}
Epididymis				
caput				
Ductal area	29.9±2.7^{a}	30.4±2.8^{a}	35.4±8.5^{a}	29.3±8.6^{a}
Luminal area	12.8±0.1^{a}	14.5±6.2^{a}	17.8±0.6^{a}	12.7±2.3^{a}
Epithelial height	33.0±7.0^{a}	31.0±8.7^{a}	32.9±9.5^{a}	28.8±8.0^{a}
Corpus				
Ductal area	91.9±33.6^{a}	68.5±10.0^{a}	98.6±21.6^{a}	86.3±14.9^{a}
Luminal area	68.6±30.0^{a}	102.2±24.2^{b}	76.3±20.2^{abc}	68.5±19.3^{ac}
Epithelial height	24.3±1.5^{a}	22.8±3.3^{a}	19.8±0.04^{a}	19.4±8.53^{a}
Cauda				
Ductal area	176.4±70.3^{a}	168.1±34.1^{a}	198.7±65.4^{a}	208.4±81.0^{a}
Luminal area	152.2±76.3^{a}	135.7±28.0^{a}	200.8±43.6^{a}	214.2±94.5^{a}
Epithelial height	21.6±3.3^{a}	18.6±1.1^{ab}	14.3±1.3^{b}	14.0±3.5^{b}
Abnormal gametes (%)	1.16±1.60^{a}	20.17±1.72^{b}	18.40±4.39^{b}	11.20±11.38^{b}

Within each line, values followed by different letters indicate statistical differences between the groups ($P<0.05$). Data are expressed as the mean±standard deviation (ANOVA, Tukey's test). Co: Control group; C20: Treated with 20 mg/kg b.w. of extract; C100: Treated with 100 mg/kg b.w. of extract; C400: Treated with 400 mg/kg b.w. of extract

Table 3: Reproductive and fetal parameters in the different experimental groups (n=6/group)

Parameters	Co	C20	C100	C400
Fertility rate* (%)	100^{a}	100^{a}	100^{a}	100^{a}
Gestation rate* (%)	100^{a}	67^{b}	67^{b}	50^{b}
B.w. of dams (g)	359±41.3^{ab}	428±28.7^{a}	365±12.2^{ab}	320±42.0^{b}
Gravid uterine weight (g)	41.9±11.8^{a}	45.7±2.8^{a}	37.9±15.3^{a}	33.2±7.1^{a}
Placental weight (g)	5.7±1.1^{a}	6.5±2.0^{a}	5.7±2.0^{a}	4.3±1.4^{a}
Litter weight (g)	21.2±5.4^{a}	21.1±2.7^{a}	18.4±7.3^{a}	15.8±3.3^{a}
Litter size	12.0±2.2^{a}	12.0±1.7^{a}	10.5±4.7^{a}	10.0±1.0^{a}
Fetal weight (g)	1.8±0.24^{a}	1.7±0.20^{ab}	1.7±0.16^{ab}	1.6±0.22^{b}
Pre-implantation loss (%)	0^{a}	4.2^{a}	6.6^{a}	6.8^{a}
Post-implantation loss (%)	0^{a}	0^{a}	0^{a}	0^{a}

Within each line, values followed by different letters indicate statistical differences between the groups ($P<0.05$). Data are expressed as the median±interquartile deviation (Kruskal-Wallis, Dunn test). *Chi-square test. Co: Control group; C20: Treated with 20 mg/kg b.w. of extract; C100: Treated with 100 mg/kg b.w. of extract; C400: Treated with 400 mg/kg b.w. of extract

DISCUSSION

Reports on the effects of *C. ecalyculata* in the organism are scarce. The obtained results in this study showed that different extract doses did not affect the b.w. or testicular weight, but promoted toxic effects in the morphological structure of

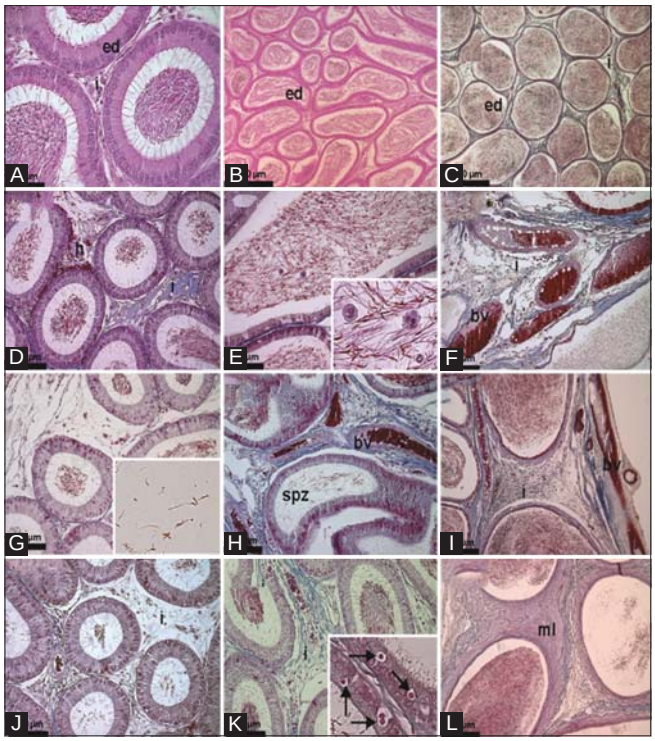

Figure 2: Photomicrographs of epididymis in the rats of groups: Co (A-C), C20 (D-F), C100 (G-I), and C400 (J-L), in caput, corpus, and cauda. In the groups treated with *C. ecalyculata* extract, observe the interstitial hemorrhage (h, in D), congested blood vessels (bv, in F and H), polymorphonucleated cells in lumen (detail in E), gametes with short or absent flagellum (detail in G), degenerative epithelial cells (arrows, detail in K), thick muscle layer (ml) surrounding the duct (in L), and edematous interstitium (in F, G and J). Epididymal duct (ed), interstitium (i), spermatozoa (spz). HE (A and B); Mallory's Trichrome (C-L). Bars=50 µm (A), 100 µm (D-H, J and K), 200 µm (I and L); 500 µm (B and C)

testes and epididymis, increased the frequency of abnormal spermatozoa, and decreased the gestation rate.

The absence of a treatment effect from the extract in the form of a b.w. reduction in the C20, C100, and C400 groups demonstrated the ineffectiveness of herbal medicine as a natural product for emaciation. Similar results were obtained by other authors, at doses varying from 20 to 2000 mg/kg b.w. [2,5,6], showing the opposite effect from what people generally believe [2]. There were reports that *C. ecalyculata* did not reduce water or food consumption by animals [2,5], but the results are controversial. In the present study, the increase in water consumption by the rats treated with the lowest (20 mg/kg) and highest doses (400 mg/kg) of the extract can be attributed mainly to the active metabolites of caffeine, such as theobromine and theophylline, which act by increasing renal blood flow and glomerular filtration [9]. The food ingestion was decreased significantly in the group receiving the highest dose (400 mg/kg) and increased among the group under the lowest dose (20 mg/kg), a result that demonstrates the ability of this extract to act in a biphasic manner. Caffeine appears to have the same effect when introduced in the diet of Sprague-Dawley male rats [10,11].

The herbal medicine did not change the testicular weight, despite producing histopathological changes in the seminiferous tubules at all doses. There was a significant increase in the relative epididymal weight of rats treated with 400 mg/kg. This result may have been influenced by the increase in the thickness of the muscular layer observed in the histological sections of cauda region. This alteration is probably attributable to the action of caffeine. A previous study [12] reported an increase of the smooth muscle surrounding the ventral prostate of rats treated with caffeine. Similar to the prostate, the epididymis is an androgen-dependent organ, which may have responded likewise to the action of caffeine present in *C. ecalyculata* extract.

The histopathological changes observed in the testes and epididymis of rats treated with different doses of *C. ecalyculata* may be due to caffeine and its metabolites, or to the synergism of these compounds with tannins, which due to their property of binding to proteins and other macromolecules, causes them to act as a toxicity pathway [13]. Rats fed a diet supplemented with caffeine and theobromine presented testicular toxicity, characterized by the degeneration of seminiferous epithelium, necrosis, multinucleated germ cells [14], and edematous interstitium, with apparent vascular proliferation [15]. Caffeine alone promoted epithelial vacuolization [11]. In the present study, several similar changes were observed in the testicular and epididymal tissue of rats treated with any of the three extract doses. The reproductive toxicity verified in these tissues may have resulted from a direct action of one or more plant constituents on the organs, or an indirect action on the neuroendocrine axis. Future studies should be performed to investigate the levels of follicle-stimulating hormone, luteinizing hormone, testosterone, and dihydrotestosterone, in animals treated with *C. ecalyculata*. There is one report [12] that caffeine increases the testosterone levels in rats. In the present study, testicular Leydig cells were not influenced by treatment with *C. ecalyculata*, similarly to the study performed by Ax *et al*. [16] in roosters (*Gallus domesticus*) fed rations supplemented with caffeine.

Sperm abnormalities observed in the groups receiving the extract show that the active metabolites of *C. ecalyculata* interfere with gamete formation, especially in relation to the flagellar apparatus. Ekaluo *et al*. [17] reported that caffeine present in the *Annona muricata* extract decreased the quantity, motility, and viability of gametes, and increased the percentage of spermatozoa with head anomalies in rats.

All females in the present study copulated with males that had received *C. ecalyculata* extract at doses of 20, 100, and 400 mg/kg, but the gestation rate was reduced, indicating that the gamete-fertilizing capacity was impaired by treatment. Only in the progenitor group treated with highest extract dose, there was a significant reduction in the fetal weight. This result may be associated with lower b.w. of dams mated with males of C400 group, observed during the final period of gestation. The mechanisms involved in this result are uncertain.

In each group, 100 and 400 mg/kg, mortality was recorded in only one animal. According to Caparroz-Assef *et al*. [6], the LD50

of *Cordia salicifolia* (a synonym for *C. ecalyculata*) extract in mice treated for 90 days was 2000 mg/kg (oral), a dose exceeding those used in the present study.

In conclusion, the administration of *C. ecalyculata* extract in male rats promotes reproductive toxicity, but with no dose-dependent effect.

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