academicJournals

Vol. 15(8), pp. 221-235, 24 February, 2016
DOI: 10.5897/AJB2014.14358
Article Number: 2B4DAA857327
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Toxicity effects of ethanol extract of *Simarouba versicolor* on reproductive parameters in female Wistar rats

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Received 9 December, 2014; Accepted 12 November, 2015

***Simarouba versicolor* is popularly known as pau-paraiba. It belongs to the Simaroubaceae family and is found in the Northeast of São Paulo and in some parts of the states of Pará and Mato Grosso do Sul. It is known that this and other species from this genus have several activities: insecticides, anti-inflammatory, antitumor, anthelmintic, among others. The effects of ethanolic extract of the bark of *S. versicolor* (Sv-EtOH) on the reproductive system of rats were investigated; also the influence of estrogenic and/or antiestrogenic activity, estrous cycle, pregnancy, lactation and offspring development was observed. In the experimental protocols with ethanolic extract of *S. versicolor*, the doses were 10, 20 and 40 mg/kg. To evaluate estrogenic and anti-estrogenic activity, uterotrophic assay and histopathology of the uterus were performed. In the estrous cycle tests, animals were treated daily, for 30 days, to count number of cycles and record the biochemical profile. During pre-implantation and post-implantation, the effect of the extract on pregnancy, lactation and development of the progeny was observed. Uterotrophic assay exhibited anti-estrogenic activity at extract doses of 10, 20 and 40 mg/kg. The animals exposed to all doses had no changes in their estrous cycle, pregnancy, lactation and biochemical profile. All results were within normal range; however, creatinine concentration increased significantly at all doses as compared to the control. The ethanol extract of *S. versicolor* presented antiestrogen activity, low systemic toxicity and had no reproductive toxicity at investigated doses.**

Key words: *Simarouba versicolor*, reproduction, rats.

INTRODUCTION

Medicinal plants are important and they supply raw materials to synthetize medicines, besides being used as therapeutics. The use of plants is overvalued traditionally based on their medicinal benefits, and often they are the only therapeutic resource of many communities and ethnic groups (Tresvenzol et al., 2006; Garcia et al., 2010). Herbal remedies are often indiscriminately ingested during pregnancy due to popular belief that

natural products are not harmful to health. Therefore, it is necessary to carry out toxicity studies with respect to this class of users. Thus, reproductive toxicity evaluation tests comprise the exposure of sexually mature animals before conception, during prenatal development and after birth (Lourenço et al., 2009).

Among the commonly used plants, *Simarouba versicolor*, from Simaroubaceae family, stands out in folk medicine. Its bark and fruit are used as anthelmintic and the infusion of its bark has anti poisonous effect (Pires et al., 2006). Furthermore, they may be useful in the treatment of dyspepsia, diarrhea and fever. *S. versicolor* inhibits acetylcholinesterase enzyme (Carvalho, 2008), and causes an outbreak of cattle poisoning in Agua Clara County, Mato Grosso do Sul (Carvalho et al., 2013). Regarding its acute toxicity, Fernandes et al. (2004) determined the median lethal dose (DL_{50}) of the aqueous extract of *S. versicolor* in mice; it was approximately 185.88 and 68.80 mg/kg administered through oral and intraperitoneal routes, respectively. Mesquita (2009), studying an anticancer substance (glaucarubinona, extremely active on cancer cells) obtained from hexane extract of the bark and root of *S. versicolor*, saw that the substance gave significant results.

Mesquita (1997), through chromatographic analysis of chloroform and ethyl acetate fractions removed from the wood of *S. versicolor*, isolated two quassinoids, excelsina and 11-ethyl-amarolídio, where excelsina was first isolated in this genus. Arriaga et al. (2002), through hexane, chloroform, ethyl acetate and methanol extracts, isolated a mixture of quassinoids triterpenoids and steroids, in addition to kaempferol flavonoid and squalenic derivatives from roots, branches and fruits of this plant. According to Almeida et al. (2007), quassinoids present in *S. versicolor* show a wide range of biological activities, such as antitumor, antimalarial, antioxidant, amebicides, antiviral and also a male anti-fertility, a fact that aroused the interest in studying *S. versicolor* effect on female reproduction.

From this perspective, this study aimed to investigate the effects of *S. versicolor* bark ethanolic extract (Sv-EtOH) on the reproductive system of rats, observing the influence of estrogenic and/or antiestrogenic activity, estrous cycle, pregnancy, lactation and progeny development, as well as the effects of this extract on biochemical profile, histologic and morphometric aspects of system reproductive female Wistar rats.

MATERIALS AND METHODS

Animals

Female rats (*Rattus norvegicus*, Wistar variety) were provided by the Central Biotherium of the Federal University of Piauí. Animals were kept in standard cages at controlled temperature (24 ± 1°C) in a 12 h light/dark cycle; there was free access to water and feed. Experimental protocols were approved by the Ethics Committee in Animal Experimentation of the Federal University of Piauí (EAEC-PI 029/11).

Chemicals and drugs

The following constituted the chemicals and drugs used in this study: dimethylsulfoxide (DMSO) - DYNAMIC, BRAZIL), estradiol cyprionate (Pfizer), Tamoxifen (DEG, CHINA), sodium thiopental and corn oil. For protocols application, the extract was diluted in DMSO and distilled water.

Preparation of the ethanol extract

Barks of *S. versicolor* were collected in Angical (6°05'41°28'), Piauí, Brazil. A voucher of the plant was identified and deposited in the Herbarium Graziela Barroso acquis (TEPB) of the Federal University of Piauí, Teresina, Piauí under number TEPB-20.883. It was dried in a forced air circulation drying oven for three days at 45 ± 1°C. After complete drying, the material was crushed in a Willi type mill. The ethanol extract was obtained by placing 1 kg of plant feedstock into ethanol for four days at room temperature in cold maceration followed by filtration. After four successive extractions, it was homogenized and placed in a rotary evaporator at 45 ± 1°C coupled to a thermostatic bath, followed by lyophilization. The ethanol extract lyophilized was diluted in dimethylsulfoxide and distilled water (DMSO = 6%).

Uterotrophic test

To uterotrophic assay, 100 immature Wistar rats (21 ± 1 postnatal day), weighing from 40 - 55 g, were divided into 10 groups of 10 animals treated after 22 to 28 post-natal days (once per day). Treatments were performed according to Table 1, wherein the extract (Sv-EtOH) vehicle was distilled water and dimethyl sulfoxide (6%); for estradiol, it was corn oil and for tamoxifen, distilled water. After treatment, animals were euthanized with an overdose of sodium thiopental (100 mg/kg, ip). They had uterus and ovaries removed, dissected and weighed on an analytical balance (0.001g) and then Formalin fixed (10% buffered formaldehyde). After 24 h, organs were re-sectioned for histology processing: they were dehydrated with alcohol (70 to 100%), diaphanized in xylene, submitted to impregnation and included in paraffin. Tissue fragments were cut with 5.0 µM thickness in a microtome, stained with hematoxylin-eosin and examined by light microscopy. Uterine sections were submitted to morphometric analysis using computerized image analyzer (Qwin Leica D-1000, version 2.1.0: Cambridge, UK). Five to six fields of uterine epithelium of each animal were captured and measured using Image-Pro Plus, version 4.5.0 Windows 98/NT/200.

Test involving estrous cycle

To evaluate the effect of Sv-EtOH treatment on the estrous cycle of rats, 32 mice were randomly assigned into 4 groups of 8 animals of

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Table 1. Experimental design of uterotrophic assay with ethanol extract of *Simarouba versicolor* in rats.

Number	Groups	Dosage	Routes of administration
10	Negative control (H_2Od + DMSO)	10 mL/kg	o.a.
10	Sv-EtOH 10	10 mg/kg	o.a.
10	Sv-EtOH 20	20 mg/kg	o.a.
10	Sv-EtOH 40	40 mg/kg	o.a.
10	Estradiol	5 µg/kg	i.m.
10	Tamoxifen	4 mg/kg	i.m
10	Estradiol + Tamoxifen	5 µg/kg + 4 mg/kg	o.a + i.m
10	Sv-EtOH 10 + Estradiol	10 mg/kg + 5µg/kg	o.a. + i.m
10	Sv-EtOH 20 + Estradiol	20 mg/kg + 5µ/kg	o.a. + i.m
10	Sv-EtOH 40 + Estradiol	40 mg/kg + 5µ/kg	o.a. + i.m

Distilled water (H_2Od); dimethylsulfoxide (DMSO) = dilution vehicle, n = number of animals; o.a = oral admnistration; im = intramuscular.

Table 2. Experimental design of treatment with ethanol extract of *S. versicolor* to evaluate its effect on estrous cycle of female rats.

Number	Groups	Dosage	Routes of administration
8	G1 - Control (H_2Od + DMSO)	10 mL/kg	o.a.
8	G2 - Sv-EtOH 10	10 mg/kg	o.a.
8	G3 - Sv-EtOH 20	20 mg/kg	o.a.
8	G4 - Sv-EtOH 40	40 mg/kg	o.a.

Distilled water (H_2Od); dimethylsulfoxide (DMSO) = dilution vehicle; number of animals (n); oral administration (o.a.).

90 days and weighing from 180 – 250 g, having regular estrous cycle. From the beginning of estrus, animals were treated for 30 consecutive days according to the protocol outlined on Table 2.

Daily, rats were submitted for vaginal lavage with saline solution of 0.9% (50 µL release and immediate recovery). Then, fresh confectioned smears were used to verify cycle phase. This was observed in light microscopy at 40x magnification. Phases were determined according to Hankness and Wagner (1993) (diestrus, proestrus; onset of estrus; end of estrus and meta-oestrus). Estrus visualization was considered as the starting point of each estrous cycle, which was recorded during each individual cycle phase.

After treatment, animals were euthanized with sodium pentobarbital overdose (100 mg/kg, i.p.). Then, there was blood and serum collection, which was stored for later evaluation of the biochemical profile: glutamic oxalacetic transaminase (TGO), transaminase glutamic pyruvate (TGP), triglycerides, urea, creatinine, total cholesterol and total protein. Colorimetric method was performed to determine contents in a Quick Lab (Drake) equipment.

Assays involving pregnancy and lactation

Mating

Nulliparous females were placed with adult males in the dark phase of the cycle consisting of 1 male for 2 females. In the morning after mating, vaginal lavage was performed looking for evidence of sperms. Smears were evaluated by optical microscopy (10 - 40x). The presence of vaginal plug or sperm in vaginal washes confirmed copulation, and it is considered the first day of pregnancy.

Preimplantation period (1st to 7th day of pregnancy)

In this protocol, 32 pregnant rats with 160-250 weights were divided into 4 groups of 8 animals. Rats were treated with extract (10, 20 and 40 mg/kg) from the first to seventh day of pregnancy, as well the control group (distilled water and 6% DMSO/10 g body weight). During the treatment, animals were weighed daily. After eight days, progenitors were euthanized (sodium thiopental 100 mg/kg, ip), observing organ weights, pre-implant and visible signs of toxicity losses: piloerection, fur loss, shivers, salivation, convulsions, hypo activity, reduction of normal feed intake, weight loss, and presence of diarrhea. Liver, kidneys, adrenals, uterus and ovaries had their absolute masses weighed in analytical balance.

Pre-implant losses were determined according to the following formula: pre-implant losses = (number of corpus luteum - number of implants/number of corpus luteum) × 100. Corpus luteum was counted with Lupa Olympus SZ40 and implants with naked eye.

Gestational period (1st to 20th day of pregnancy)

In this experiment, 32 pregnant rats, weighing 160-250 g, divided into 4 groups (n = 8) were housed in individual cages and treated daily from the first to the twentieth day of gestation with control (vehicle), SvEtOH 10, 20 and 40 mg/kg.

Females' weight gain was evaluated daily and extract doses

were adjusted. On the 20th day of gestation, females were anesthetized (ketamine 50 mg + xylazine 5 mg) and submitted to cesarean for removal and evaluation of the pregnant uterus and its contents. Fetuses and implantation sites were counted and weighed individually, so were gravid uterus, fetus and placenta. Fetuses were examined and analyzed macroscopically looking for anomalies and/or congenital malformations. After caesarian, animals were euthanized by overdose of anesthetic association (1 mL/100 g/bw/ip) to remove and weigh the following organs: ovaries, liver, kidneys, spleen, heart, lung and adrenal glands, which had their relative masses calculated. Corpus luteum and implantations sites were counted. Reproductive parameters were evaluated using the following formulas:

Implementation contents = (number of deployments/number of corpora luteum) x 100

Resorption index = (number of resorption*/number of implants) x 100

*Number of resorption = (number implants) - (number of fetuses)

Pre-implant losses = (number of corpus luteum - number of implants/number of corpus luteum) x 100

Post-implant losses = (number of implants - number of births/number of implants) x 100

Piloerection, fur loss, shivers, salivation, convulsions, hypo activity, reduction of normal feed intake, weight loss, abortion and presence of diarrhea were used as systemic toxicity indicators (Muller et al., 2013).

Post-implantation, lactation and development of progeny period

Pregnant rats divided into 4 groups of 5 animals, weighing 160-250, were treated once daily with 10, 20 and 40 mg/kg of Sv-EtOH and with the vehicle; administrations started on the 8th day of pregnancy, beginning of fetus post-implantation period. Treatment was stopped only on progeny birth (21 ± 1 day of pregnancy), and continued on the day after birth until the 21th day of lactation (weaning).

On the 18th day of pregnancy, progenitors were separated into individual cages until delivery and inspected twice daily until offspring birth (1st postnatal day). On 21st postnatal day, pups were separated and placed in new cages (Muller et al., 2009) and progenitors were euthanized (Thiopental, 100 mg/kg/i.p.) for organ gathering. During pregnancy and breastfeeding were observed: weight gain, duration of pregnancy, birth rate, number of births, viability thereof, besides indicators of systemic toxicity (Muller et al., 2013). Birth delivery, birth rates and viability were determined according to US-EPA (1996) formulas:

Childbirth index = (number of females who delivered/number of females with evidence of pregnancy) x 100

Index birth = (number of births/number of litters) x 100

Index viability = (number of live young on the fourth day post-natal/number of live births) x 100

Post-implant losses = (number of implants – number of births/number of implants) × 100

Progeny was observed twice daily as the evolution of characteristics: emergence of fur (5 to 7 days), bilateral eyelid opening (12 to 16 days), displacement of pinna (6 to 8) and opening of vaginal canal in female puppies (about 35 days), based on Mello (2007).

Statistical analysis

For tests conducted during pre and post-implantation periods, pregnancy and uterotrophic data were expressed as mean standard error of the mean (SEM). Statistical analyses were performed with Graphpad PRISM ® software, version 5.0, by ANOVA (One Way), followed by Tukey post-test; significance was set at $p<0.05$. Comparison of data in the same group was made by paired student's test with significance of $p<0.05$.

Estrous cycle length data were analyzed and processed using SPSS (Statistical Package for Social Sciences) software for Windows, version 15. Initially, a descriptive analysis of the sample was done by calculating the average. Thereafter, Kolmogorov-Smirnov test was applied to test the normality of the results. When the data set to compare the results met the requirements for applying parametric statistics, we used the parametric analysis of variance (ANOVA). When application of non-parametric statistics was necessary, comparison between groups was performed using Kruskal-Wallis test. A significance level of 0.05 was adopted.

RESULTS

Uterotrophic assay

Rats treated with SV-EtOH at all doses tested did not show any increase in relative masses of uterus, which is similar to negative control, that shows no estrogenic activity. On the other hand, the group treated with estradiol (positive control) showed a significant increase in the relative mass of uterus ($p <0.05$) as compared to negative control and treated group.

Nevertheless, in anti-estrogenic activity test of females treated with Sv-EtOH and estradiol in all the doses, there was an inhibition of uterine growth and statistically significant differences ($p <0.05$) compared to animals treated only with estradiol (Figure 1). Their behavior was equal to groups treated with tamoxifen and tamoxifen + estradiol, which showed a significant inhibition ($p <0.05$) in uterine relative masses compared to those treated with estradiol.

Histological evaluation of uterine epithelium in estrogenic activity tests showed that rats treated with estradiol had 90% eosinophilic inflammatory infiltration of mean intensity reaching muscular layer (Table 3 and Figure 1a). In contrast, animals treated with 10 and 20 mg/kg of Sv-EtOH had a percentage less of eosinophilic infiltration (60 and 50%, respectively). Animals treated with 40 mg/kg presented 100% of eosinophilic infiltration in lamina propria with moderate intensity and swollen mucosal epithelial cells (Table 3).

Antiestrogenic activity in the groups treated with Sv-EtOH + estradiol at doses of 10 and 20 mg/kg (Figure 1d, e and 1f) revealed eosinophilic infiltration of moderate intensity reaching muscle layer with 90 and 100%, respectively. Different behavior was observed at 40 mg/kg, which inhibited infiltration by 50% (Table 3).

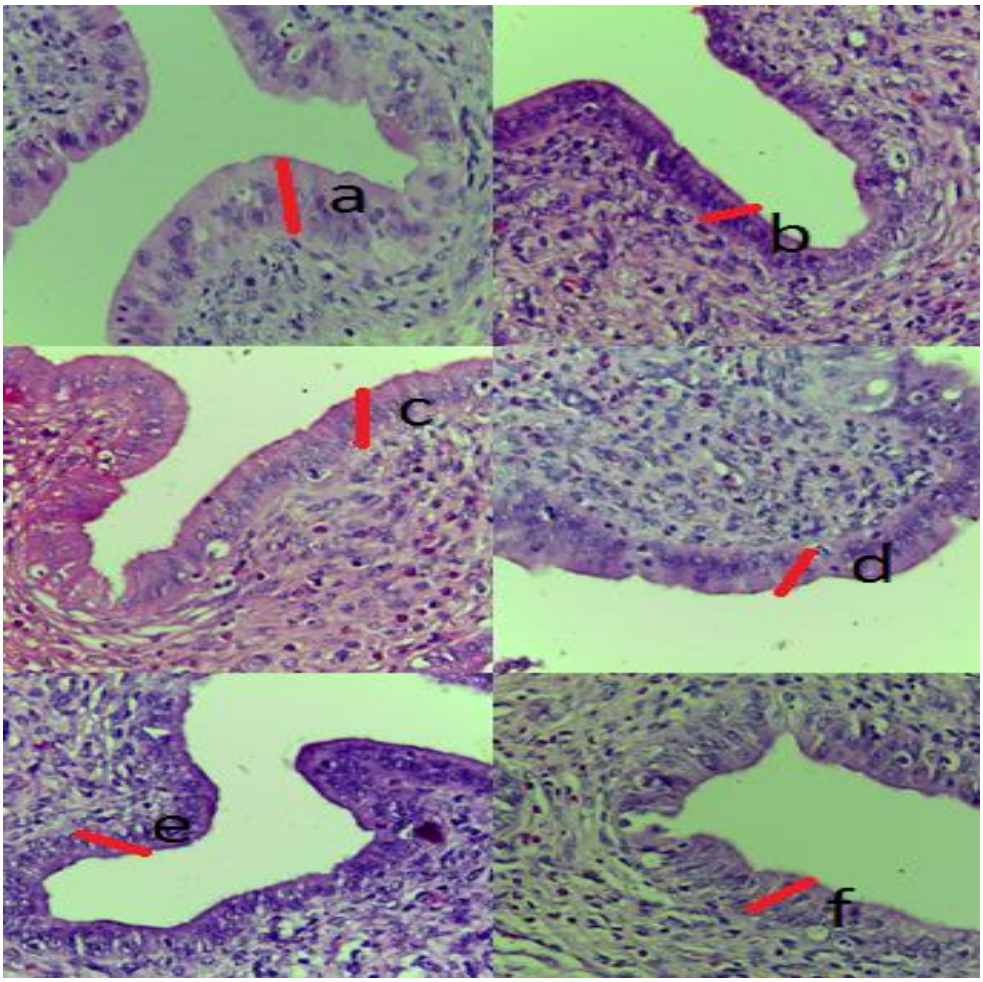

Figure 1. Photomicrographs showing longitudinal sections of the endometrium of rats: **a**, treated with estradiol (5µg); **b**, treated with tamoxifen (4 mg/kg); **c**, treated with estradiol + tamoxifen **d**, **e**, **f**, treated with estradiol + Sv-EtOH (10, 20, 40 mg / kg), respectively.

Table 3. Histopathological uterus of female rats treated with ethanolic extract of *S. versicolor* when compared with negative and positive controls in uterotrophic assay.

Number	Groups	Uterus histopathology	Percentage
10	Negative control (ad + DMSO)	Normal	100
10	Sv-EtOH 10	Eosinophilic inflammatory infiltration of the lamina propria and muscle layer	40
10	Sv-EtOH 20	Eosinophilic infiltrate of moderate intensity reaching the muscle layer	50
10	Sv-EtOH 40	Eosinophilic infiltration of lamina propria of moderately severe intensity and swelling of epithelium cells mucosa	100
10	Estradiol	Eosinophilic infiltration of moderate intensity reaching the muscle layer.	90
10	Tamoxifen	Normal	100

Table 3. Contd.

10	Tamoxifen + Estradiol	Eosinophilic inflammatory infiltrate of lamina propria and muscle layer.	70
10	Sv-EtOH 10 + Estradiol	Eosinophilic inflammatory infiltrate of moderate intensity reaching the muscle layer	90
10	Sv-EtOH 20 + Estradiol	Eosinophilic infiltration of the lamina propria of moderate moderately severe intensity reaching the muscle layer and swelling of the epithelial cells of the mucosa	100
10	Sv-EtOH 40 + Estradiol	Eosinophilic infiltrate of moderate intensity reaching the muscle layer	50

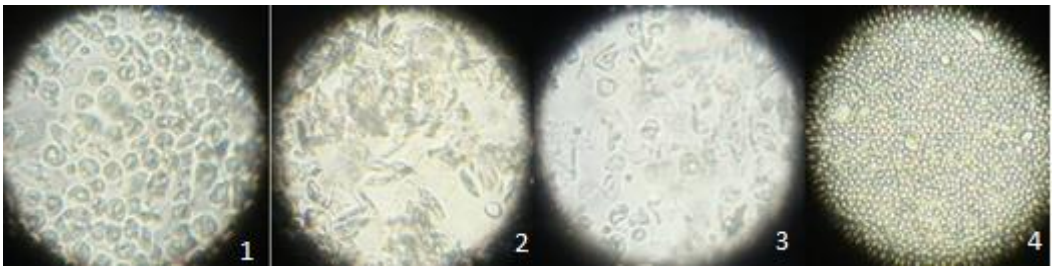

Figure 2. Vaginal smears of rats treated with Sv-EtOH showing the four phases of estrous cycle (400x); proestrus (1), estrus (2), metaestrus (3), diestrus (4). Source: Author.

Rats treated only with tamoxifen showed normal and undeveloped epithelium similar to negative control (Figure 1b). However, 70% of animals treated with tamoxifen + estradiol presented eosinophilic infiltration in the simple columnar epithelium of the lamina propria with moderate intensity and swollen mucosa epithelial cells (Figure 1c).

Regarding morphometry of uterine epithelium in antiestrogen activity (Figure 1d, e and f), Sv-EtOH at all doses was able to inhibit the increased thickness of estradiol-induced uterine epithelium, behaving like tamoxifen alone and associated with estradiol. The group treated with estradiol had values significantly higher than the other groups (Figure 4 and Figure 1a).

Evaluation of estrous cycle

The four phases of the estrous cycle (Figure 2) were observed through smear vaginal realized during 30 days of treatment. The estrus, being the most characteristic phase, was regarded as the starting point of each cycle; it duration ranged from 1 to 7 days in this experiment. The number of estrous cycles recorded during 30 days for treated groups did not differ significantly ($p>0.05$) compared with the control group. Control average was 6.00 ± 0.26 estrous cycles, and groups treated with Sv-EtOH (10, 20 and 40 mg/kg) presented 5.13 ± 0.39; 4.62 ± 0.39; 4.37 ± 0.65 estrous cycles, respectively (Figure 5).

Cycle duration for group treated with Sv-EtOH (10, 20, 40 mg/kg) was not significant ($p>0.05$), but it was observed that the estrous cycle length is dose dependent. Control group average was 4.83 ± 1.18 and for those treated with Sv-EtOH (10, 20, 40 mg / kg) it ranged from 5.57 ± 1.88, 6.16 ± 2.35, 8.16 ± 1.17, respectively.

Concerning the regularity of cycles, those that exceeded the duration of 5 days were considered irregulars. The total number of estrous cycles was 159, with 77.36% regular and 22.64% irregular. Control group exhibited 87.5% of regular cycles and 12.5% of irregular cycles. Animals treated with Sv-EtOH (10, 20, 40 mg / kg) had frequencies of 73.17, 67.56 and 78.78% for regular estrous cycles and 26.82, 32.43 and 21.21% for irregulars, respectively.

Biochemical analysis

During treatment with Sv-EtOH at 10, 20, 40 mg/kg for 30 days, no clinical signs of toxicity nor mortality were observed. The treatment did not induce changes in the biochemical profile of TGP, TGO, urea, total cholesterol,

Table 4. Effect of ethanolic extract of *S. versicolor* on biochemical parameters of Wistar rats treated for 30 days.

Parameters	Control	Treatments (Sv-EtOH)		
	H_2Od + DMSO	10 mg/kg	20 mg/kg	40 mg/kg
TGP (U/L)	67.37 ± 4.55	55.75 ± 7.34	71.87 ± 15.4	87.00 ±14.0
TGO (U/L)	164.8 ± 10.8	130.3 ± 64.7	98.62 ± 5.19	81.50 ± 7.9
Urea (mg/dL)	48.25 ± 0.79	47.00 ± 1.09	46.00 ± 2.36	60.12 ± 5.9
Creatinine (mg/dL)	0.625 ± 0.037	1.00 ± 0.05***	0.92 ±0.05***	0.87±0.025**
Total cholesterol (mg/dL)	77.50 ± 33,0	56.00 ± 21.33	50.62 ± 8.12	44.37± 11.1
Triglycerides (mg/dL)	25.37± 4.14	30.75± 3.61	31.62 ± 5.72	30.37± 5.70
Total protein (mg/dL)	6.72 ± 0.17	6.088 ± 0.34	6.11± 0.23	5.86 ±0.075

The data express mean (± SEM). **p <0.01, ***p <0.001 (One way ANOVA/Tukey).

triglycerides and total protein, since the parameters were within the reference range, as to the values observed in control animals. The only exception is observed for the dosage of creatinine in animals treated with Sv-EtOH at doses of 10, 20, 40 mg/kg, which increased significantly at 1.00, 0.92, 0.87 (mg/dL), respectively, as compared to those of control animals (Table 4).

Trial involving pregnancy and lactation

Pre-implantation period

The administration of Sv-EtOH (10, 20 and 40 mg/kg) during pre-implantation period (1st to 7th day of pregnancy), as compared to control, had no effect on body weight gain of the mother (Figure 6).

Absolute (g) and relative masses (%) of organs (uterus, ovaries, kidney, adrenal, lung, heart, liver and spleen) from progenitor treated with extract during pre-implantation did not differ significantly (p>0, 01) when compared to control (Table 5).

Pre-implant losses, numbers of corpus luteum and number of deployments found in dams treated with Sv-EtOH (10, 20 and 40 mg/kg) during pre-implantation did not differ from control group (Table 6).

Pregnancy

Administration of Sv-EtOH during gestation did not affect body weight gain of progenitor, and did not differ significantly from control (Figure 7). During this period, absolute (g) and relative masses of uterus, ovaries, adrenals, kidneys, lungs, heart, liver and spleen were not significantly different (P>0.05) (Table 7). In the reproductive parameters evaluated, deployment rate was 100% for animals treated with 10 and 40 mg/kg and 99.03% at 20 mg/kg. Females treated with 10, 20 and 40 mg/kg had resorption rate, pre-implant loss, and post implant loss of 2.50 ± 1.63 to 2.60 ± 1.75; 1.56 ± 1.56 to 2.50 ± 1.63; 1.56 ± 1.56 to 2.50 ± 1.63, respectively, not significantly different from control group.

Post-implantation period (8th to 21st days of lactation) and evaluation of progeny

Administration of Sv-EtOH, at all doses, during gestation did not affect body weight gain of progenitors, and did not differ significantly from control (Figure 8). Absolute (g) and relative masses (%) of organs (uterus, ovaries, kidneys, adrenals, lungs, heart, liver, and spleen) from progenitors treated with Sv-EtOH (10, 20 and 40 mg/kg) on post-implantation period (8th day of pregnancy to weaning) did not differ significantly when compared to control (Table 8).

Childbirth and birth rates were 100 and 98.05%, respectively. Animals treated with Sv-EtOH at different doses in post-implementation period did not differ significantly in relation to pregnancy duration, number of born alive offspring and offspring viability compared to control (Figure 3). The Sv-EtOH administration during lactation, at all doses, interfered significantly reducing weight gain of progenitors as compared to the control (Figure 9).

Evaluation of the progeny: Analysis of daily observations on indicative characteristics of physical development expected in normal offspring from mothers treated with Sv-EtOH (10, 20 and 40 mg/kg) for 45 days did not show significant differences, compared to the control (Table 9).

DISCUSSION

Estradiol, predominant estrogen, is synthesized on ovaries and secreted by granulosa cells of mature follicles, as well by placenta during pregnancy. Secretion in the systemic circulation acts on alpha and beta estrogen receptors. It is important to note that epithelial cells and uterine stroma express ER in early development and adulthood, and therefore both tissues are susceptible to estrogens in these phases (Brown et al., 1999; Brolio et al., 2010). Whereas, estrogens are

Table 5. Evaluation of the absolute (g) and relative masses (%) of organs from progenitors treated with the ethanolic extract of *S. versicolor* during pre-deployment period (1st to 7th day) as compared to the control.

Variables	Control (n=8)	Treatment (Sv-EtOH)		
		10 mg/kg (n=8)	20 mg/kg (n=8)	40 mg/kg (n=8)
Body weight (g)	194.08 ± 12.45	170.14 ± 4.93	171.58 ± 4.72	193.42 ± 13.21
Absolute mass				
Uterus	0.412 ± 0.044	0.474 ± 0.012	0.447 ± 0.035	0.528 ± 0.049
Kidneys	1.636 ± 0.083	1.446 ± 0.034	1.619 ± 0.079	1.562 ± 0.093
Ovary D	0.040 ± 0.002	0.036 ± 0.001	0.031 ± 0.004	0.103 ± 0.067
Ovary L	0.041 ± 0.005	0.039 ± 0.002	0.038 ± 0.004	0.073 ± 0.039
Adrenal	0.084 ± 0.037	0.047 ± 0.002	0.053 ± 0.005	0.051 ± 0.005
Lung	1.330 ± 0.086	1.234 ± 0.036	1.163 ± 0.053	1.377 ± 0.096
Heart	0.702 ± 0.035	0.672 ± 0.015	0.650 ± 0.021	0.733 ± 0.046
Liver	8.604 ± 0.534	8.593 ± 0.327	7.119 ± 1.075	8.405 ± 0.484
Spleen	0.815 ± 0.121	0.897 ± 0.073	0.799 ± 0.094	0.798 ± 0.087
Relative mass				
Uterus	0.204 ± 0.022	0.260 ± 0.009	0.247 ± 0.018	0.263 ± 0.015
Kidneys	0.806 ± 0.029	0.796 ± 0.030	0.895 ± 0.030	0.782 ± 0.017
Ovary D	0.020 ± 0.001	0.020 ± 0.001	0.017 ± 0.002	0.047 ± 0.029
Ovary L	0.020 ± 0.002	0.022 ± 0.001	0.021 ± 0.002	0.034 ± 0.017
Adrenal	0.042 ± 0.019	0.026 ± 0.001	0.029 ± 0.002	0.026 ± 0.003
Lung	0.655 ± 0.037	0.676 ± 0.015	0.645 ± 0.025	0.687 ± 0.018
Heart	0.346 ± 0.015	0.368 ± 0.006	0.361 ± 0.013	0.367 ± 0.011
Liver	4.212 ± 0.133	4.712 ± 0.172	3.934 ± 0.578	4.226 ± 0.162
Spleen	0.398 ± 0.055	0.494 ± 0.044	0.448 ± 0.060	0.403 ± 0.041

Data express means ± standard error (ANOVA / Tukey).

Table 6. Effect of ethanolic extract of *S. versicolor* during pre-implantation (1st to 7th days) on pre-implant losses, number of corpus luteum and number of deployments compared to control.

Variables	Control	Treatment (Sv-EtOH)		
		10 mg/kg	20 mg/kg	40 mg/kg
Corps luteum	9.75 ± 0.52	10.75 ± 0.81	10.62 ± 0.68	10.62 ± 0.59
Deployments	9.12 ± 0.66	9.37 ±0.65	10.0. ±0.53	10.12 ± 0.54
Pre-implant loss (%)	6.80 ± 2.89	12.28 ±3.51	6.39 ± 2.57	4.41 ± 2.41

Data express mean (± SEM). $p < 0.05$ (ANOVA / Tukey test), n = 8.

responsible for female secondary characteristics development, they also produce a suitable environment for fertilization, implantation, embryo nutrition and parturition (Lindzey and Korach, 1999). Sv-EtOH effect on endometrium was evaluated through uterotrophic assay. Within this perspective, the extract was not able to increase the uterus mass of rats treated at doses of 10, 20 and 40 mg/kg, when compared to rats treated with estradiol alone, inferring that it has no estrogenic activity. However, Sv-EtOH (10, 20 and 40 mg/kg) associated with estradiol inhibited increase of uterus mass stimulated by estradiol. Similar behavior was observed in animals treated with estradiol association + tamoxifen, suggesting anti- estrogenic activity and indicating that the extract can prevent fertilization processes, implantation, embryo nutrition and childbirth.

Hormonal effects on cervical epithelium are still limited on literature. Valente and Sasso (1992), studying female rats cervix, observed that the proportion of collagen fibers and infiltration of eosinophils in the lamina propria was

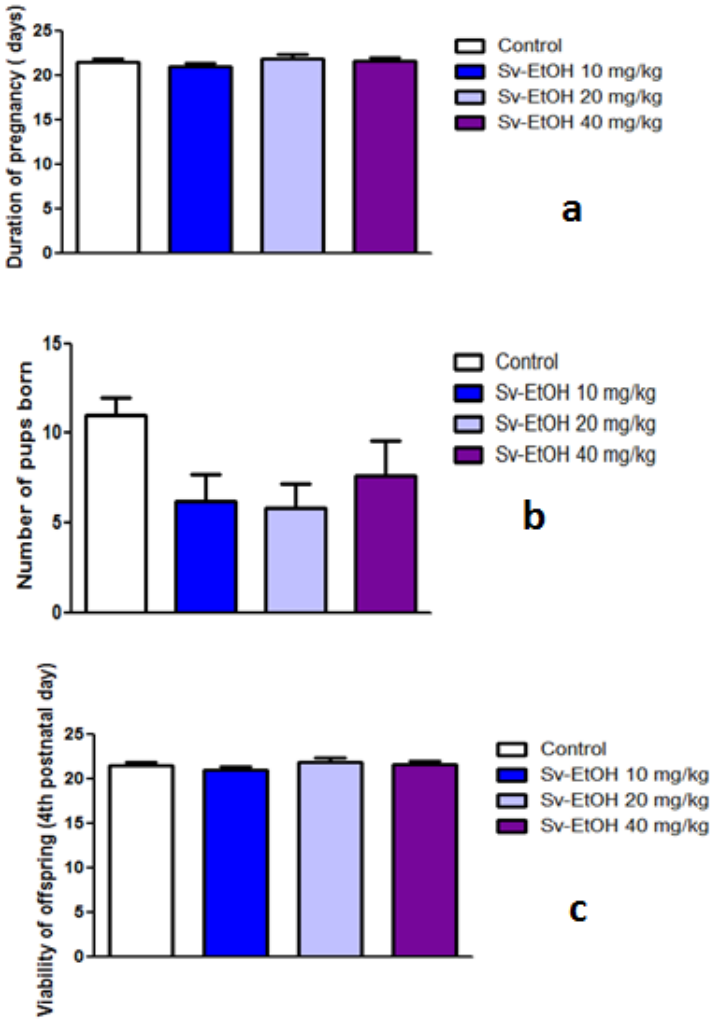

Figure 3. Evaluation of pregnancy duration (a) number of born alive offspring (b) and offspring viability (c) of progenitors treated with ethanolic extract of *S. versicolor* at post-deployment period (8th day pregnant childbirth) compared to control

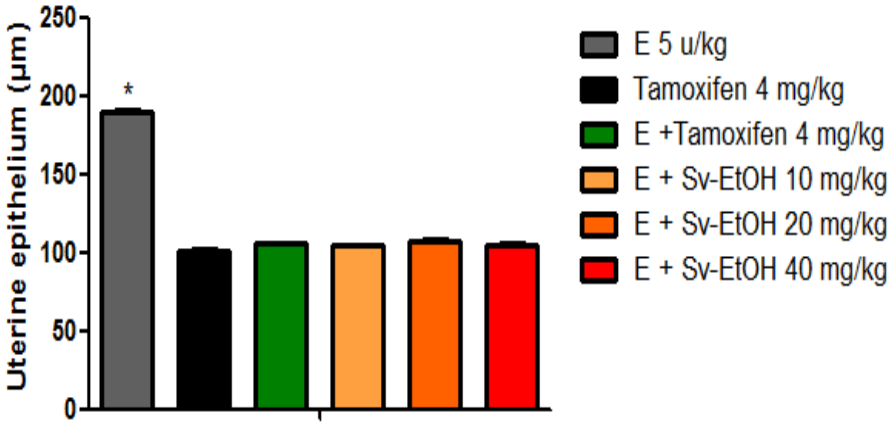

Figure 4. Effect of Estradiol, Tamoxifen, Estradiol+Tamoxifen and Estradiol + Sv-EtOH on the thickness of uterine epithelium of prepubertal rats.Data express mean ± standard error, n = 10, (One Way ANOVA / Tukey).

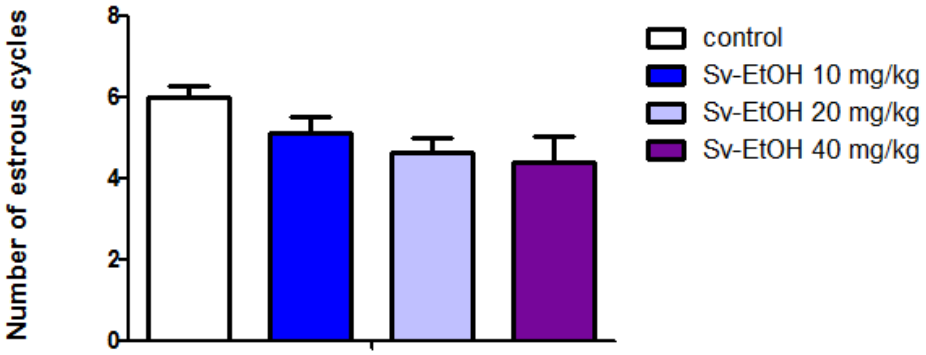

Figure 5. Effect of ethanolic extract of *S. versicolor* on the number of estrous cycles of rats treated for 30 days as compared to the control. Data express mean ± standard error, n = 8, (One Way ANOVA/Tukey).

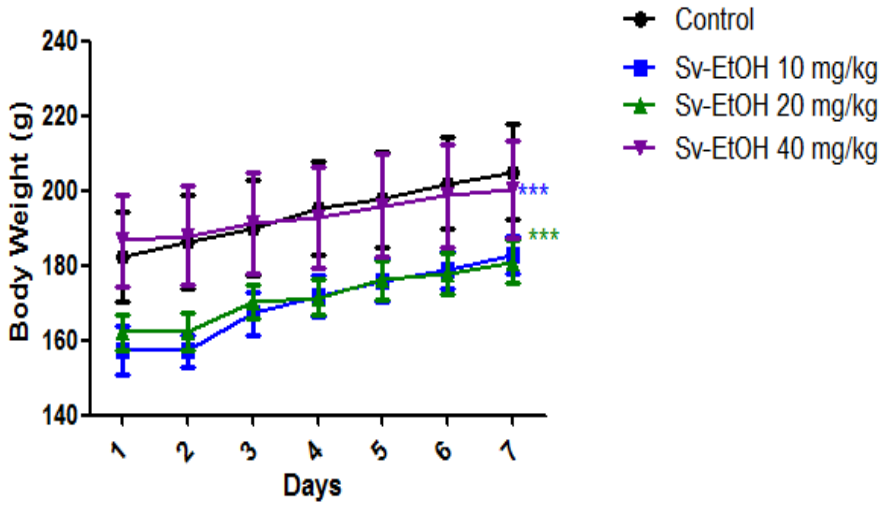

Figure 6. Weigh evolution of progenitors treated with the ethanolic extract of *Simarouba versicolor* during pre-deployment (1º to the 7º day of pregnancy), compared to control .Data express mean ± SEM, n = 8 (One Way ANOVA / Tukey) *** p <0.001 vs baseline weight.

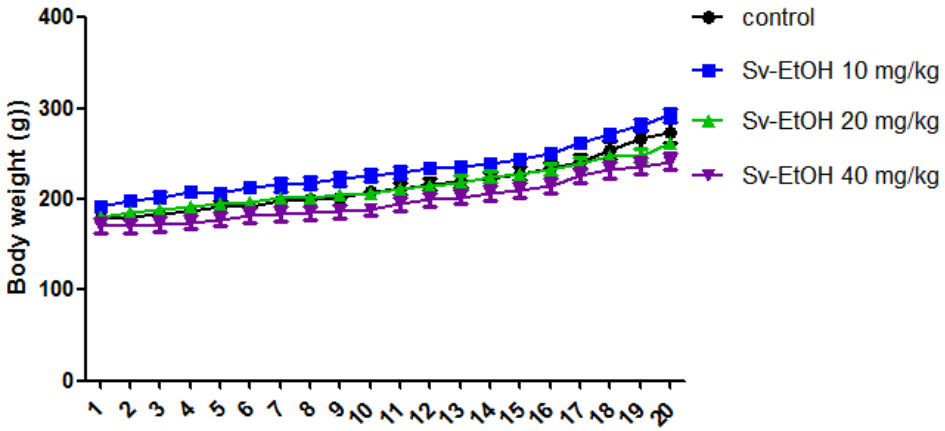

Figure 7. Weight evolution of progenitors treated with the ethanolic extract of *Simarouba versicolor* during pregnancy (1st to 20th day), compared to control. The data is expressed mean ± SEM, n = 8, (One Way ANOVA/Tukey).

Table 7. Evaluation the absolute (g) and relative masses (%) of organs from progenitor treated with ethanolic extract of *Simarouba versicolor* during pregnancy (1st to 20th day) as compared to the control.

Variables	Control (n=8)	Treatment (Sv-EtOH)		
		10 mg/kg (n=8)	20 mg/kg (n=8)	40 mg/kg (n=8)
Body weight (g)	273.25 ± 10.91	292.1 ± 7.27	261.50 ± 5.37	241.28 ± 9.74
Absolute mass				
Uterus	42.18 ± 5.019	44.47 ± 2.529	39.36 ± 2.878	45.58 ± 1.881
Kidneys	1.470 ± 0.060	1.747 ± 0.066	1.640 ± 0.069	1.430 ± 0.044
Ovary D	0.053 ± 0.006	0.064 ± 0.006	0.069 ± 0.003	0.050 ± 0.005
Ovary E	0.066 ± 0.005	0.077 ± 0.006	0.061 ± 0.005	0.059 ± 0.003
Adrenal	0.058 ± 0.005	0.056 ± 0.006	0.068 ± 0.004	0.063 ± 0.003
Lung	1.120 ± 0.067	1.224 ± 0.104	1.393 ± 0.085	1.115 ± 0.049
Heart	0.725 ± 0.040	0.865 ± 0.024	0.818 ± 0.029	0.783 ± 0.028
Liver	11.81 ± 0.437	13.17 ± 0.453	12.18 ± 0.252	11.18 ± 0.495
Spleen	0.858 ± 0.064	0.909 ± 0.032	0.977 ± 0.083	0.731 ± 0.049
Relative mass				
Uterus	15.134 ± 1.50	15.26 ± 0.860	15.10 ± 1.150	18.44 ± 0.627
kidneys	0.541 ± 0.024	0.598 ± 0.016	0.626 ± 0.018	0.595 ± 0.016
Ovary D	0.020 ± 0.003	0.022 ± 0.002	0.027 ± 0.001	0.021 ± 0.003
Ovary E	0.024 ± 0.001	0.026 ± 0.002	0.023 ± 0.002	0.025 ± 0.001
Adrenal	0.022 ± 0.003	0.019 ± 0.002	0.026 ± 0.002	0.026 ± 0.001
Lung	0.417 ± 0.037	0.415 ± 0.031	0.534 ± 0.034	0.463 ± 0.016
Heart	0.266 ± 0.012	0.297 ± 0.009	0.313 ± 0.006	0.326 ± 0.009
Liver	4.345 ± 0.135	4.515 ± 0.122	4.669 ± 0.100	4.646 ± 0.161
Spleen	0.322 ± 0.036	0.312 ± 0.012	0.372 ± 0.027	0.309 ± 0.029

The data express the mean ± standard error. ** = P <0.01 (ANOVA/Tukey).

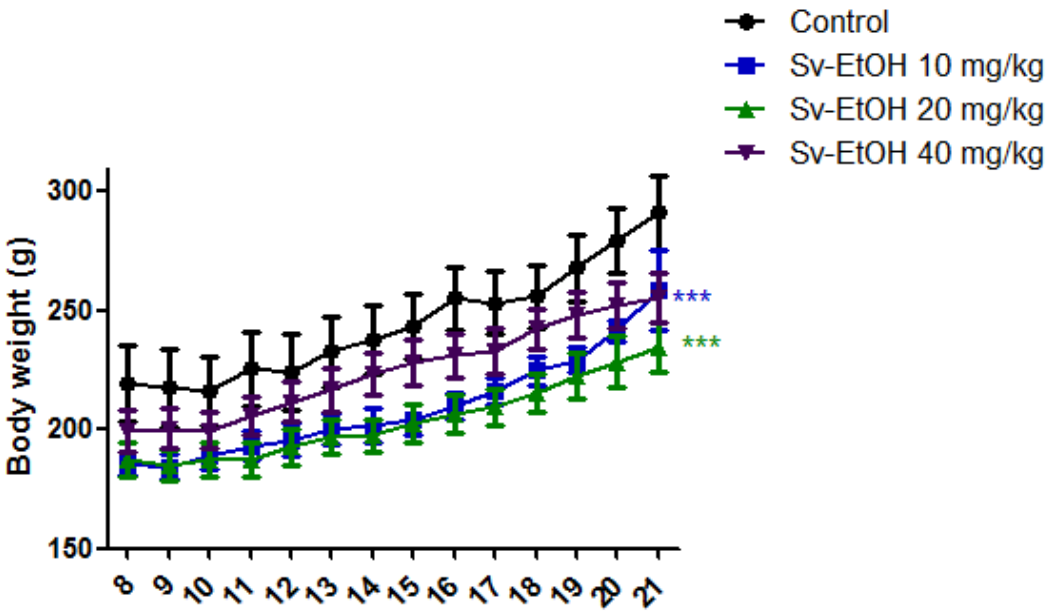

Figure 8. Weight evolution of progenitors treated with ethanolic extract of *Simarouba versicolor* on post-implantation period (8th to 21st days of pregnant) compared to control. The data is expressed mean ± SEM, n = 5, (One Way ANOVA / Tukey); p, *** p <0.001 vs. baseline weight

Table 8. Evaluation absolute (g) and relative masses (%) of organs from progenitors treated with ethanolic extract of *Simarouba versicolor* on post-implantation period (8th to 21st days of pregnancy), compared to control.

Variables	Control (n=8)	Treatment (Sv-EtOH)		
		10 mg/kg (n=8)	20 mg/kg (n=8)	40 mg/kg (n=8)
Body weight (g)	239.41 ± 14.17	209.58 ± 5.54	203.84 ± 7.81	224.71 ± 8.62
Absolute mass				
Ovary D	0.039 ± 0.004	0.035 ± 0.005	0.039 ± 0.005	0.028 ± 0.002
Ovary E	0.041 ± 0.005	1.036 ± 0.004	0.031 ± 0.005	0.046 ± 0.004
Uterus	0.252 ± 0.033	0.237 ± 0.055	0.256 ± 0.053	0.199 ± 0.018
Lung	1.497 ± 0.123	1.273 ± 0.065	1.240 ± 0.065	1.232 ± 0.015
Adrenal	0.070 ± 0.005	0.064 ± 0.006	0.062 ± 0.004	0.075 ± 0.004
kidneys	1.875 ± 0.040	1.706 ± 0.049	1.658 ± 0.064	1.912 ± 0.103
Liver	10.55 ± 0.661	8.698± 0.299	9.942 ± 0.624	10.51 ± 0.543
Spleen	0.565 ± 0.017	0.565 ± 0.036	0.559 ± 0.017	0.507± 0.023
Heart	0.790 ± 0.032	0.630 ± 0.020	0.729 ± 0.034	0.621 ± 0.025
Relative mass				
Ovary D	0.018 ± 0.002	0.018 ± 0.002	0.019 ± 0.002	0.015 ± 0.001
Ovary E	0.018 ± 0.002	0.018 ± 0.002	0.016 ± 0.003	0.024 ± 0.001
Uterus	0.114 ± 0.016	0.119 ± 0.023	0.126 ± 0.023	0.103 ± 0.010
Lung	0.668 ± 0.024	0.651 ± 0.028	0.630 ± 0.053	0.635 ± 0.012
Adrenal	0.032 ± 0.002	0.033 ± 0.003	0.032 ± 0.003	0.039 ± 0.002
Kidneys	0.847 ± 0.036	0.876 ± 0.035	0.841 ± 0.061	0.983 ± 0.036
Liver	4.721 ± 0.078	4.455 ± 0.134	5.049 ± 0.457	5.408 ± 0.206
Spleen	0.256 ± 0.013	0.288 ± 0.013	0.282 ± 0.010	0.262 ± 0.015
Heart	0.355 ± 0.009	0.323 ± 0.012	0.336 ± 0.014	0.320 ± 0.010

The data express the mean ± standard error (ANOVA/Tukey).

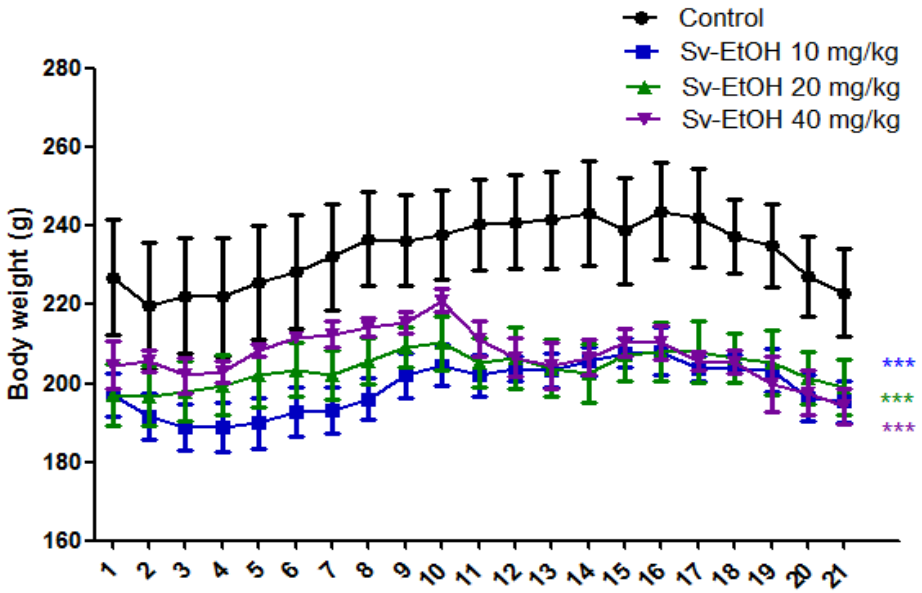

Figure 9. Weight evolution of progenitors treated with ethanolic extract of *Simarouba versicolor* on lactation period (1st to 21st days of lactation) compared to control. The data express the mean ± standard error (ANOVA One Wa /Tukey). The data is expressed mean ± SEM, n = 5, (One Way ANOVA / Tukey).

Table 9. Evaluated parameters of progeny coming from mothers treated with ethanolic extract of *Simarouba versicolor* for 45 days.

Parameters (days)	Control	Treatment (Sv-EtOH)		
		10 mg/kg	20 mg/kg	40 mg/kg
Eye opening	14.40 ± 0.51	11.16 ± 2.24	14.40 ± 0.74	11.00 ± 2.77
Detachment of ears	6.80 ± 0.37	5.83 ± 1.19	7.00 ± 0.316	5.60 ± 1.43
Appearance of hair	6.40 ± 0.24	5.33 ± 1.08	7.40 ± 0.40	5.40 ± 1.36
Opening of vaginal canal (females)	39.40 ± 1.03	33.53 ± 6.70	41.40 ± 2.01	31.80 ± 7.97

The data express mean (± SEM), p> 0.05. (ANOVA, One Way / Tukey).

higher in estrus phase (after hormonal peak of estrogen) compared to the diameter. Ramos et al. (2002) reported that uterine morphological aspects are related directly to receptors' levels of estrogen and progesterone.

However, analyzing histological sections of uterus in uterotrophic assay, animals treated with estradiol in endocervical canal presented simple cylindrical epithelium sorely infiltrated by leukocytes and stratified epithelium nonkeratinized with high concentration of eosinophils. Animals treated with tamoxifen had both epithelium endocervical and ectocervical underdeveloped compared with estradiol groups. In the treatment with estradiol + tamoxifen, simple cylindrical epithelium showed little leukocyte infiltration, low concentration of eosinophils and epithelia undeveloped compared to animals treated with estradiol alone. Likewise, it happened to groups treated with estradiol and Sv-EtOH at all doses.

Morphological and morphometric analysis confirmed that the endometrium was affected by treatments in antiestrogenic activity assay, as animals treated with estradiol + tamoxifen and estradiol + Sv EtOH (10, 20 and 40 mg/kg) presented low uterine epithelium, inhibiting estrogen action and confirming Sv-EtOH antiestrogenic activity. Events pattern in estrous cycle provide a useful normality indicator for neuroendocrine function of non-pregnant female ovaries (Andrews et al.; 2002). Sv-EtOH treatment (10, 20 and 40 mg/kg) for 30 consecutive days did not cause changes in regularity, duration and repeatability of estrous cycles as compared to the control. This indicates that the treatment did not promote disorders of hypothalamic-pituitary-ovarian axis.

Estrous cycle in female rats is regular, consists of several annual cycles and with 4 to 5 days of duration (Cobea, 1996). Due to this short period, rats are considered the ideal animals to study estrous cycle variations (Marcondes et al., 2001). However, control animals may show irregular cycles, usually in the form of cycle stretching or acyclicity (US EPA, 1996). In this study, control rats showed cycle elongation (1 day for treated groups). Although the difference between the groups was not significant ($p > 0.05$), there is a dose-dependent tendency to increase cycles duration. So far, all cycles in the 30 days consecutive period of treatment were in accordance with the literature (Santos et al., 2003; Hollenbach et al., 2008).

Cycle changes can be caused by treatment with certain substances, such as xenobiotics, that can induce acyclicity characterized by persistent estrus, diestrus or irregular standard (Goldman et al., 2007). Some changes in ovarian hormones and extra-ovarian can lead to irregularity in ovarian function causing changes in estrous cycle duration (Shivalingappa et al., 2002).

Alterations in estrous cycle extension can be a response to exposure to an agent with estrogenic property or capable of blocking ovulation. On the other hand, there are substances that can induce changes in estrous cycle at doses that do not impair fertility. Diestrus persistence indicates temporary or permanent interruption of follicular development and ovulation, inducing temporary infertility. Prolonged diestrus or anoestrus can be indicative agents (for example, polycyclic aromatic hydrocarbons) that interfere with follicle development or deplete the pool of primordial follicles or agents such as atrazine, which interrupt support of ovarian gonadotropin (US EPA, 1996; Romero, 2007). In this experiment, it was observed that in irregular cycles, the phase that lasted more was diestrous (72.2%); it is characterized by the lack of cellular elements and high concentrations of leucocytes and mucus. Estrus phase lasted for more than 12 h in 22.2% cases.

Hematological evaluation represents an important area of study on animals' health. Blood analysis helps in the diagnosis and prognosis of various diseases (Jain, 1993). Evaluation of systemic toxicity showed that oral administration of Sv-EtOH at doses of 10, 20 and 40 mg/kg for 30 days produced no behavioral changes in adult Wistar rats nor visible signs of toxicity. Chemistry profile parameters evaluated were within the normal range except for creatinine levels. There was a quantitative increase in creatinine concentration for all groups exposed to Sv-EtOH as compared to the control group. Creatinine is an important parameter for diagnosing various kidney problems. Creatinine is a non-protein nitrogenous organic compound formed from the dehydration of the muscle creatine. This serves to assess glomerular filtration rate, as blood concentration increases

with reduced renal filtration rate. The presence of this substance in blood can be used to identify renal disorders. Creatinine tends to increase slower than urea in renal disease. Thus, this parameter provides us with evidence of renal impairment in animals treated with Sv-EtOH.

Deployment is a process by which the embryo has intimate physical and physiological contact with maternal endometrium to establish pregnancy. Although there are variations of this process between species, certain basic events are similar. The fundamental characteristic of this process is the synchronized embryo development to the blastocyst stage and differentiation from uterus to receptive state. This is followed by interactions between activated blastocysts and the uterine epithelium to start deployment (Muller et al., 2009).

Progenitors exposed to Sv-EtOH (10, 20 and 40 mg/kg) during pre-implantation period did not alter weight gain. Reduction in corporal mass could reflect a variety of responses, including anorexia induced by the treatment or systemic toxicity (US EPA, 1996). In relation to absolute and relative masses of ovaries, uterus, liver, lungs, spleen, kidneys, heart and adrenal did not exhibit significant changes. There were also no physical and macroscopic changes in animals and organs exposed to Sv-EtOH. These results indicate absence of extract toxicity at investigated doses, during this period, which is probably due to short term exposure.

Gestational period is one of the most sensitive stages of reproductive cycle, which results in important responses. Today, it is known that in this period most agents cross through the placenta. Thus, it can be considered that maternal exposure to external agents may result in significant effects on a passive organism, a secondary target of these agents, which is the embryo body (Damasceno et al., 2008). During pregnancy, the exposure of mothers to the ethanol extract of *S. versicolor* at all doses did not affect the body mass gain or the absolute and relative masses of organs (uterus, ovaries, liver, heart, lung, spleen, kidney and adrenal glands). Resorption rates, pre-implant loss and post-implant loss were not significant compared to control results. Groups treated with Sv-EtOH presented implantation rates of 100%, indicating absence of maternal toxicity at doses investigated during pregnancy. Also no macroscopic signs of toxicity were observed in animals or organs.

The evaluation of absolute masses of metabolism and excretion organs is important, since liver and kidney are responsible for metabolism and elimination of xenobiotics intoxication cases, which may lead to an increase in mass (Mello, 2007). In post-implantation period, progenitors exposed to Sv-EtOH during gestation and lactation did not show changes in absolute and relative masses of organs (uterus, ovaries, liver, heart, lung, spleen, kidneys and adrenals), not differing from control animals. Exposure to Sv-EtOH did not affect weight gain of progenitors treated during gestation, compared to control animals, indicating absence of maternal toxicity in the doses investigated.

The same way, during pregnancy and lactation there were no signs of toxicity such as anorexia, shivers, diarrhea, piloerection or seizures. Rats' exposure to Sv-EtOH at different doses did not affect childbirth index (%) and birth index (%), indicating the extract is not toxic to prenatal development. Variations in gestation duration can result in alteration in childbirth labor. A significant prolongation of gestation time can be the cause of failure during childbirth mechanism and can result in death or injury of brood per dystocia.

Exposure in late pregnancy to substances with anti-estrogenic activity can interfere in estrogen action, altering both characteristics structurally and sensitivity of myometrium cells to promote childbirth (Lindzey and Korach, 1999). Administration of Sv-EtOH, at tested doses, did not affect pregnancy duration, number of born alive (cubs alive until the fourth day) and offspring viability. In this way, Sv-EtOH antiestrogenic activity did not impair estradiol action to increase oxytocin expression. It did not inhibit the production of prostaglandins in uterus in late pregnancy and did not also interfere in childbirth labor in rats treated with the extract.

Sv-EtOH at the tested doses, during lactation affected weight gain when compared to control animals. This may be an indication of toxicity but also by caloric burn in parturition and lactation periods. Although results indicate a possible extract systemic toxicity, exposure at doses investigated in pregnancy and lactation periods did not affect the overall development of offspring (detachment period of ears, fur appearance, eye opening, opening of vaginal canal and offspring viability) when compared with control group. Although, exposure route has been altered from fetal-placental membrane to breast milk, indicating a possible reduction in exposure levels to the extract components; it also suggests lack of toxicity in postnatal development because descendants of the progenitors treated with the extract.

Therefore, the ethanol extract of *S. versicolor* presented antiestrogen activity, possible systemic toxicity and showed no reproductive toxicity at the doses investigated.

Conflict of interests

The author(s) did not declare any conflict of interest.

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