academicJournals

Vol. 12(24), pp. 3864-3871, 12 June, 2013
DOI: 10.5897/AJB12.1742
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Studies of *Copaifera luetzelburgii* Harms in reproductive pharmacology: *In vivo* and *in vitro* approaches

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Accepted 14 June, 2013

The objective of this study was to evaluate the estrogenic and anti-estrogenic actions, as well as the reproductive and foetal toxicity, of the ethanol extract from *Copaifera luetzelburgii* (EEtOH-Cl). In the experiment of (anti) estrogenicity, nulliparous Wistar rats were treated for 3 days with EEtOH-Cl (125, 250 and 500 mg/kg); estradiol (E, 5 µg/kg); E + EEtOH-Cl; tamoxifen (T, 4mg/kg). This extract presented estrogenic activity by increasing the relative weight (%) of the uterus of rats treated at doses of 125, 250 and 500 mg/kg (0.267 ± 0.016*, 0.231 ± 0.014*, 0.242 ± 0.015*), and it showed anti-estrogenic activity when associated with estradiol (0.116 ± 0.006*, 0.103 ± 0.06*, 0.098 ± 0.05*), respectively. For assessment of toxicity in pregnancy, the animals were divided into two groups and treated daily with EEtOH-Cl. In the first group, the effect of the extract on the development of pregnancy from first to seventh day was observed, and in the second group, from 8 to 21 days, there was no change of these parameters or the viability of the progeny when the study assessed reproductive and foetal toxicity; however, there was shortening of pregnancy (125 mg/kg) without affecting the progeny. In the *in vitro* study, uterine strips of pregnant (P) and non-pregnant (NP) females were used. In both groups, half received EEtOH-Cl (vo) for 13 days (treated females - T), and the other half received EEtOH-Cl directly to the isolated organ bath system (untreated - NT). *In vitro* study on the uterus of pregnant animals pretreated with doses of 250 and 500 mg/kg showed that there was inhibition of KCl 80-induced phasic contractions (0.490 ± 0.110, 0.540 ± 0.092), respectively. Also, the contractions induced by oxytocin were inhibited at a dose of 500 mg/kg (0.380 ± 0.109). In non-pregnant, non-treated females, the extract at a concentration of 125 µg/mL (0.180 ± 0.062) also inhibited the contractions induced by oxytocin. Thus, EEtOH-Cl demonstrated estrogenic activity, but when combined with estradiol, it demonstrated anti-estrogenic activity. It did not induce toxicity in the progenitors or in the progeny, and it inhibited isometric contractions induced by oxytocin and KCl 80 mM in pregnant and non-pregnant rats.

Key words: *Copaifera luetzelburgii*, (anti-)estrogenicity, reproductive toxicity, phasic contractions.

INTRODUCTION

The use of medicinal plants for treatment, cure and prevention of diseases has been described by many people since ancient times. Because of this use, scientific and commercial interests have emerged, and therefore it has become necessary to evaluate the efficacy and safety of these plants. For many, the concept of natural means the

absence of chemical products, products that can be dangerous. This concept is misleading, however, since many plants themselves contain substances with toxic action on living organisms (Lourenço et al., 2009).

During pregnancy, the use of any medication should be done carefully because some toxic substances can cross the hematoplacental barrier and interfere with embryo-foetal development, often in a negative way (Oestensen et al., 2006). Thus, according to the study of Lourenço et al. (2009), the same caution should apply to the use of medicinal plants, mainly due to lack of information about them.

A plant that has been recently attracting scientific interest because of the multiple therapeutic properties attributed to it is 'copaibeira'. It is a tree of the *Caesalpiniaceae* family, genus *Copaifera* L-1762 - also popularly known as amaranth, copaiba, 'copiuba', 'pau-de-óleo' and 'jatobá–merim' - which produces an oleo-resin known as 'bálsamo', whose physicochemical and organoleptic characteristics tend to vary depending on the species. There are more than 100 species described for the genus *Copaifera*, with some named *C. officinalis, C. langsdorffii, C. glabra, C. multijuga* and *C. luetzelburgii* (Vieira, 1992; Brito et al., 2005).

The properties attributed to copaiba are anti-inflammatory, analgesic, antibacterial, astringent, healing, emollient, diuretic, expectorant, laxative, anticarcinogenic and abortive, among others, although there is still no scientific proof for most of them (Brito, 1995). Among the various properties of the oil from copaiba that have already been studied are anti-inflammatory (Basilie et al., 1988), analgesic (Fernandes et al., 1992), antitumor (Ohsaki et al., 1994), acute cytotoxicity and toxicity (Moura et al., 2010), and insecticidal activity (Alves et al., 2012).

However, according to the State Program for Medicinal Plants of the State Health Secretariat of Rio de Janeiro, some genera like *Copaifera* L. enter the list of medicinal plants contraindicated in pregnancy or lactation, with no certainty that they induce abortion or teratogenicity (Soares et al., 2003). The ability to induce abortion for most plants is attributed to possible estrogenic, cytotoxic and/or stimulant activities of uterine contractility (Montanari, 2008). In several articles about medicinal plants, copaiba is cited as being oxytocic and cathartic, but scientific studies have demonstrated its anti-spasmodic and relaxing effect on smooth muscle of the stomach (Cunha et al., 2003). Another important factor is that most of the studies so far have used only the oil, which has had the most popular use, leaving open the possibility of studies with other plant parts such as the bark, leaves and fruit. Within this perspective, the objective of this study was to evaluate the estrogenic and anti-estrogenic actions as well as the reproductive and foetal toxicities of the ethanol extract from *Copaifera luetzelburgii*.

MATERIALS AND METHODS

Animals

The Wistar rats used in this study were kept in cages at a controlled temperature (24 ± 1°C) and 12 h light/dark cycle, with free access to food and water. Following the experimental procedures, the animals were euthanized by cervical dislocation. Experimental protocols were approved by the Ethics Committee for Animal Experimentation at the Federal University of Piauí (ECAE-PI 026/09).

Chemicals and drugs

All reagents were of high quality and obtained from accredited suppliers: tamoxifen (DEG, China); estradiol cypionate (ECP), KCl and oxytocin (Sigma, USA).

Preparation of the ethanol extract of *Copaifera luetzelburgii*

The collection of the stem bark of *Copaifera luetzelburgii* was procurec in February of 2008 and 2009 from the community of 'Quilombola dos Macacos', near the municipality of 'São Miguel do Tapuio' (5°30'14" S, 41°19'22" W), Piauí State, Brazil, and identified by Professor Dr. Ana Luísa Du Bocage Neta. A voucher specimen was deposited under No. 26.235 in the Graziela Barroso Herbarium, Federal University of Piauí, Teresina, Piauí.

The bark of the plant was cut and dried in a forced-air circulation oven for three days at a maximum temperature of 45°± 1°C. Then, this material was triturated in a laboratory knife mill, type Willy. The extract was prepared by placing 1000 g of the plant material in ethanol 99.5% for four days at ambient temperature. This was subjected to a process of cold maceration, followed by filtration. After four successive extractions, it was placed in a rotary evaporator coupled with a thermostat set to 45°C for removal of the ethanol, and subsequently freeze-dried to remove water.

Uterotrophic test

In this test, 100 nulliparous Wistar rats used (21 ± 1 day, postnatal), were divided into groups of 10 animals and treated for three consecutive days (once daily). For the tests of estrogenicity, the animals received the ethanol extract of *Copaifera luetzelburgii* (EEtOH-Cl) at doses of 125, 250 and 500 mg/kg (per orally (p.o.); estradiol (E) 5 µg/kg (i.p.) was positive control. For the estimation of anti-estrogenic activity, the following treatments were performed: E+EEtOH-Cl 125 mg/kg; E+EEtOH-Cl 250 mg/kg; E+EEtOH-Cl 500 mg/kg; tamoxifen (T) 4 mg/kg (p.o.); and Tamoxifen + Estradiol (positive controls). The negative control was performed with distilled water at 1 ml 100 g (p.o.) + corn oil 0.05 ml/animal (i.p). In the treatments with the combination of substances, the second substance was administered immediately after the first. After three days of treatments, the animals had their body weights measured and then were euthanized by cervical dislocation. The uterus and ovaries were removed by cutting just below the connection with the cervix and above the ovaries. After discarding the liquid present

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Abbreviations: EEtOH-Cl, Ethanol extract of *C. luetzelburgii;* **E,** estradiol; **T,** tamoxifen; **P,** pregnant; **NP,** non-pregnant; **TT,** treated; **NT,** non-treated.

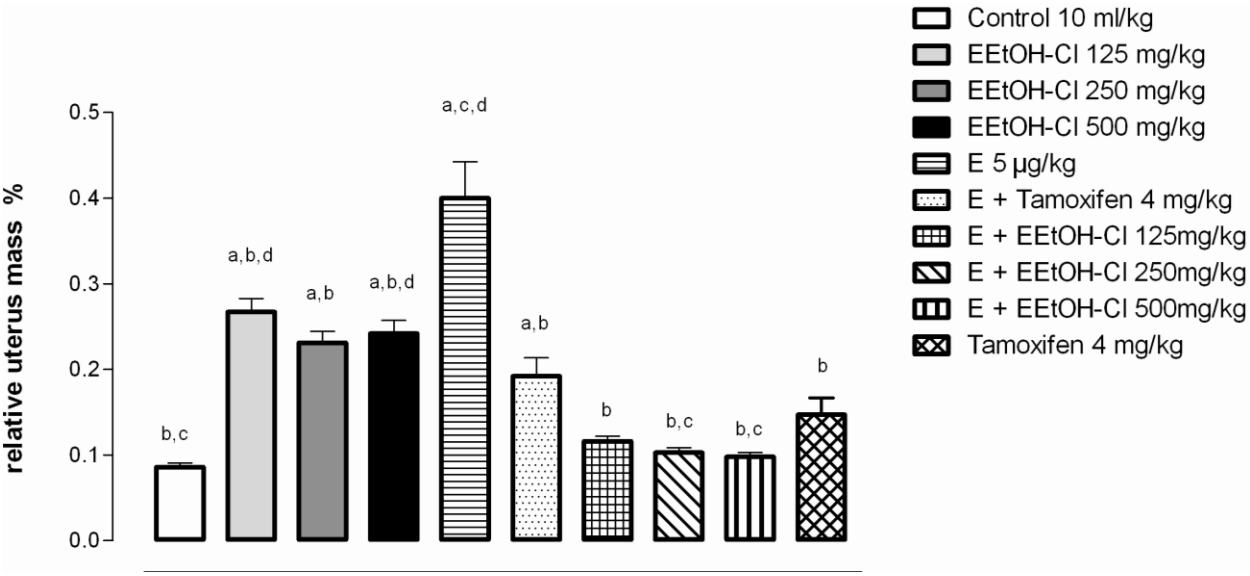

Figure 1. EEtOH-Cl effect on uterine weight, relative to body weight, in percentages, for prepubertal female rats treated with control (H_2Od, P.O.) or EEtOH-Cl (125, 250 and 500 mg/kg, P.O.) or estradiol (5 µg/kg, I.P.) or estradiol (5 µg/kg, I.P.) + tamoxifen (4 mg/kg, P.O.) or estradiol (5 µg/kg, I.P.) + EEtOH-Cl (125, 250 and 500 mg/kg, P.O.) or tamoxifen (4 mg/kg). Data expressed as mean ± SEM, n = 10.

inside the uterus, both the uterus and ovaries were weighed, on an analytical balance.

Pregnancy test

The pre-implantation and post –implantation period and progeny were evaluated to study the toxicity during pregnancy. In these tests, we used 80 females divided into two equal groups. In the first group, we observed the evolution of pregnancy from day 1 to day 7 (post –implantation); in the 2nd group, pregnancy from day 8 to day 21(pregnancy), and the weaning period (21 days postnatal). The groups (n = 10) were treated daily per orally with EEtOH-Cl at doses of 125, 250 and 500 mg/kg and distilled water 1.0 mL/100 g (negative control = solvent extract). They received the extract daily at fixed times; however, in the second group, there was a pause in the administrations on the day of parturition, when the mother was placed in an isolated cage together with its offspring, and the administration continued until the date of weaning (day 21 of life).

The females from the first group were euthanized on day 7 of the pregnancy, and those from the second group on the day of weaning their progeny. During pregnancy and lactation, we observed the following parameters: Weight gain, duration of pregnancy, birth rate, number of live births, and their viability. The progeny were observed twice a day for the following characteristics of evolution: the emergence of fur (5 to 7 days), bilateral eyelid opening (6 to 8 days), displacement of the pinna (12 to 16 days), and opening of the vaginal canal in the female offspring (why about 35 days), in accordance to the study of Mello (2007).

In vitro study

In the *in vitro* study, we used uterine strips from 30 pregnant (P) and 30 non-pregnant females (NP) for isolated organ testing. The first group, comprising pregnant (n = 15) and non-pregnant animals (n = 15), received EEtOH-Cl (p.o.) at doses of 125, 250 and 500 mg/kg for 13 days (treated - TT); and for the second group, the EEtOH-Cl was added directly to the vat containing uterine strips from pregnant (n = 15) and non-pregnant females (n = 15) (non-treated - NT) at concentrations adjusted to 125, 250 and 500 µg/ml.

The strips of uterus - removed, cleaned and devoid of any surrounding tissue - were suspended on cotton thread attached to a force transducer coupled to an acquisition system (AECAD 1604, AQCAD 2.0.5; AVS Projects, SP, Brazil) to record the isometric tensions. The strips were maintained in vats containing 10 ml Jalon solution at 32°C, under aeration with carbogenic mixture (95% O_2 and 5% CO_2), and subjected to a tension of 1.0 g of load. Stabilization was carried out for 60 min, and the nutrient solution was replaced every 15 min to prevent interference of metabolites. The contractions were induced by KCl 80 mM and oxytocin for comparison between groups.

Statistical analysis

The results were presented as mean ± SEM and, in the *in vivo* stage, the data were subjected to analysis of variance by the program ANOVA (one-way), followed by the Bonferroni test. In the *in vitro* stage, the data were statistically analyzed by ANOVA (one-way), followed by Tukey's test. For the analysis of weights in the preimplantation period, we used Student's t-test. For all stages, the level of statistical significance was 5% ($p < 0.05$). The construction of graphics was done by *GraphPad Prism*® software, version 5.03.

RESULTS

Uterotrophic test

The uterotrophic test results are expressed in Figure 1, It was observed that in the tests of estrogenicity, the rats treated with EEtOH-Cl at doses of 125, 250 and 500 mg/kg and with estradiol alone developed a significant increase ($p < 0.005$) in relative weights of the uterus

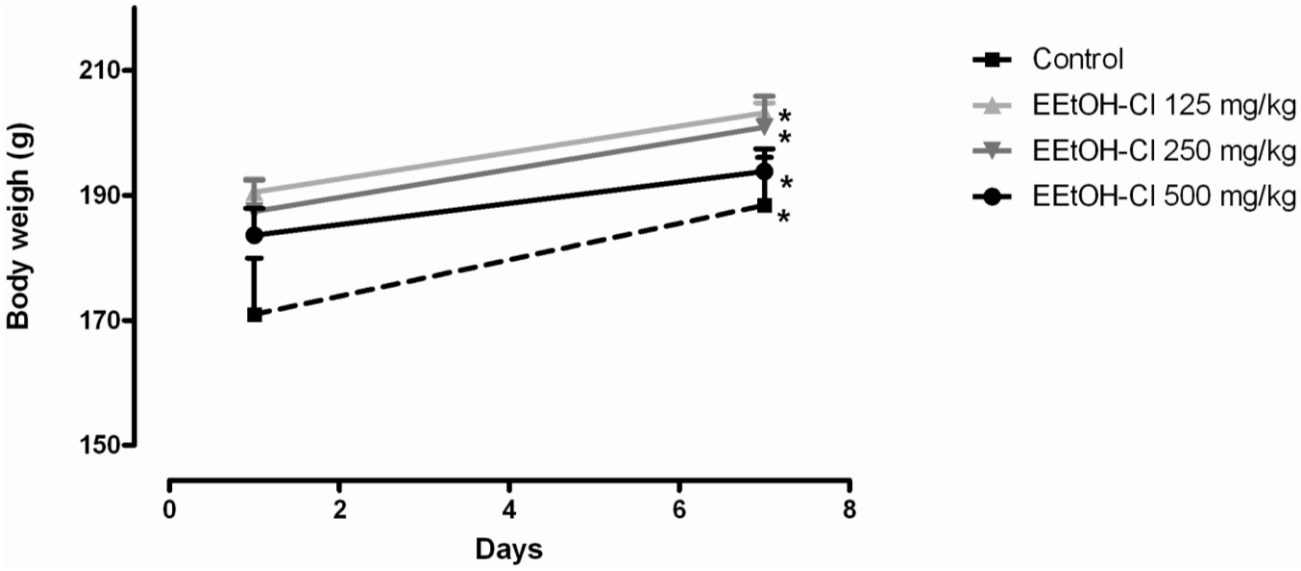

Figure 2. Weight gain of progenitors exposed to EEtOH-Cl in the preimplantation period (1st to 7th day of pregnancy). The animals were treated with control (H_2Od, P.O.) and EEtOH-Cl (125, 250 and 500 mg/kg, P.O.). Data expressed as mean ± SEM, n = 10, one-way ANOVA / Tukey. *p < 0.05 vs. initial weight (paired Student's t-test).

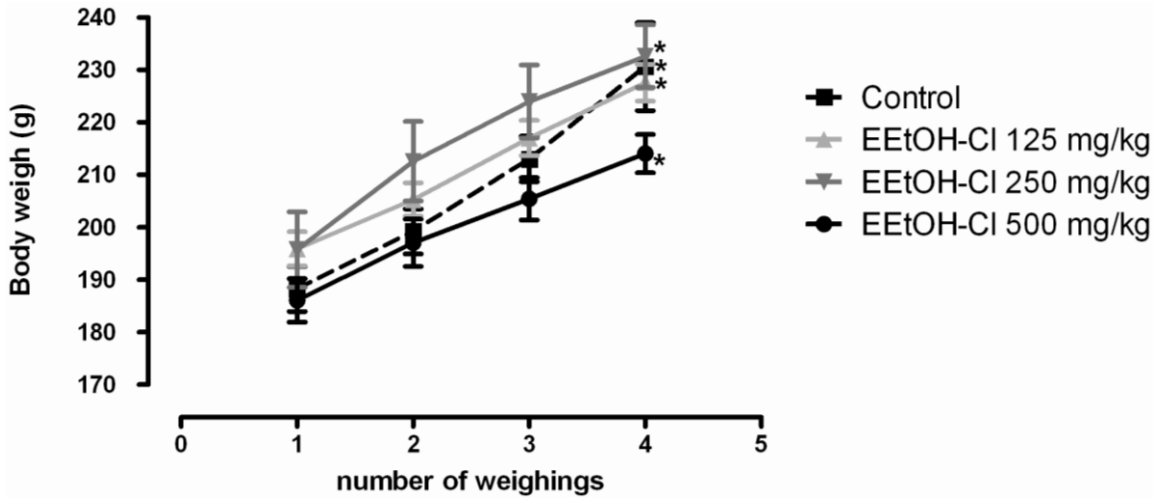

Figure 3. Weight gain of progenitors exposed to EEtOH-Cl in the post-implantation period. The animals were treated with control (H_2Od, P.O.) and EEtOH-Cl (125, 250 and 500 mg/kg, P.O.). Data expressed as mean ± SEM, n = 10, one-way ANOVA / Tukey. *p < 0.05 vs. initial weight (paired Student's t-test).

(0.267 ± 0.016, 0.231 ± 0.014, 0.242 ± 0.015, 0.400 ± 0.042, respectively), compared to the negative control group (0.086 ± 0.05), and were statistically similar (Figure 1).

In the anti-estrogenic treatments, the animals that received E + T (0.192 ± 0.021), E + EEtOH-Cl at doses of 125, 250 and 500 mg/kg (0.116 ± 0.006, 0.103 ± 0.06, 0.098 ± 0.05, respectively) and tamoxifen alone (0.147 ± 0.020) showed a statistically significant reduction ($p < 0.005$) in uterine relative weights when compared with animals that received only estradiol (0.400 ± 0.042).

Pre- and post-implantation tests and evaluation of the progeny

We found that EEtOH-Cl did not alter the organ weights (liver, kidney, uterus and ovaries) in the control group. The weight gain of pregnant animals was not influenced by the extract, as shown in Figures 2 and 3 for the first group (1st to 7th day - preimplantation) and the 2nd group (8th to 21st day - post-implantation).

The number of rat pups born and their viability showed no significant difference ($p > 0.005$) compared to the

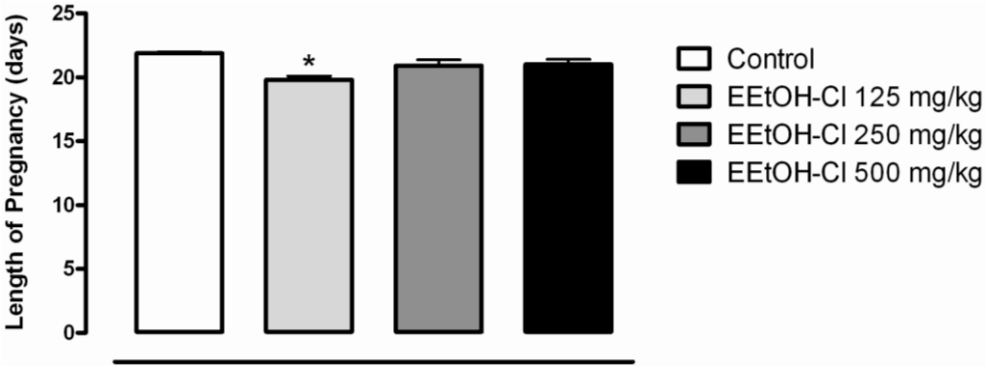

Figure 4. Duration of pregnancy (in days) of progenitors exposed to EEtOH-Cl in the post-implantation period. The animals were treated with control (H_2Od, P.O.) and EEtOH-Cl (125, 250 and 500 mg/kg, P.O.). Data expressed as mean ± SEM, n = 10. *p < 0.05 vs. control, one-way ANOVA / Tukey.

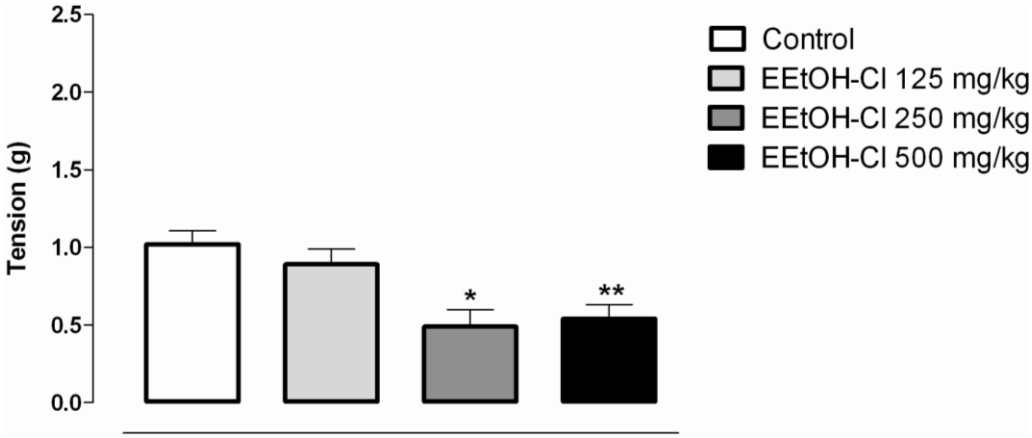

Figure 5. Contraction of the uterine wall (in tension), induced by KCl 80 mM in pregnant rats pretreated with EEtOH-Cl (125, 250 and 500 mg/kg, P.O.). p < 0.05 vs. control, one-way ANOVA / Tukey.

control, although, the duration of pregnancy was reduced at a dose of 125 mg/kg, as shown in Figure 4. The offspring descending from progenitors exposed to EEtOH-Cl, at the three doses tested, did not exhibit changes in their weight gain or development, compared to the control group.

In vitro tests of uterine contractility

In vitro tests showed that for the uteri of pregnant females treated with EEtOH-Cl, at doses of 250 and 500 mg/kg, there was a statistically significant difference ($p < 0.005$) in inhibiting phasic contractions produced by KCl 80 mM, when compared with the control (Figure 5). Likewise, the uteri from non-pregnant females that were not treated with EEtOH-Cl at a concentration of 125 µg/mL had phasic contractions induced by oxytocin inhibited, which was statistically different from the control group (Figure 6). Also, the uteri of pregnant females pretreated with EEtOH-Cl at a dose of 500 mg/kg of extract had inhibited phasic contractions that were induced by oxytocin (Figure 7).

DISCUSSION

Investigations on congenital malformations and on the toxicology tests predictive of foetal development are recent. Reproductive toxicity assessments generally comprise exposure of sexually mature animals before conception, during prenatal development, after birth and continuously until their sexual maturity (Mello, 2007).

Estradiol, which is the predominant estrogen, is released into systemic circulation and will work primarily in the alpha and beta estrogen receptors (ER) Importantly, epithelial and stromal uterine cells express ER both early in development and in adult life, and therefore both tissues are susceptible to the effects of estrogens (Muller et al., 2009; Brolio, 2010). In this study, the *in vivo* uterotrophic

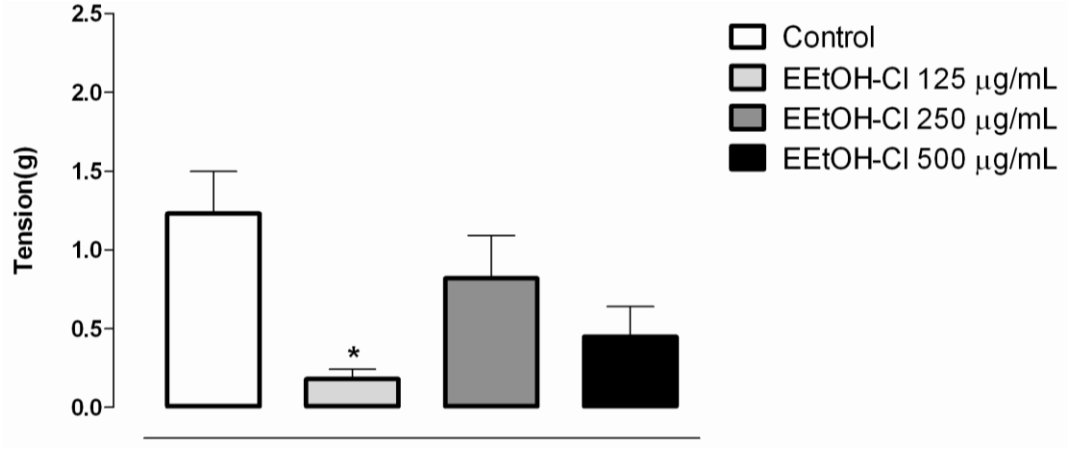

Figure 6. Contraction of the uterine wall (in tension), induced by oxytocin in rats submitted to the following treatments: control (H_2Od, P.O.) and EEtOH-Cl (125, 250 and 500 µg/mL, P.O.). One-way ANOVA / Tukey.

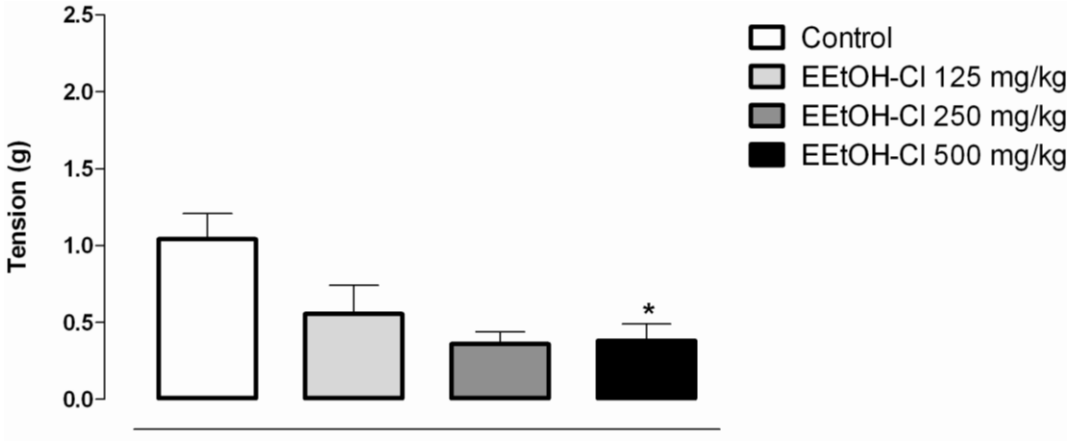

Figure 7. Contraction of the uterine wall (in tension), induced by oxytocin in isolated uterus of pregnant rats pretreated with EEtOH-Cl (125, 250 and 500 mg/kg, P.O.). One-way ANOVA / Tukey.

test was included in the design of the experimental protocol in order to assess the risk of toxicity.

EEtOH-Cl increased the relative weight of the uterus in prepubertal rats, as well as the estradiol alone, inferring that the extract has estrogenic activity at the doses tested. However, the effect of estradiol was greater than that of EEtOH-Cl. Thus, considering that estrogenic actions are responsible for the production of the environment for fertilization, implantation, nutrition of the embryo and parturition, they are necessary for uterine cell development and tissue proliferation (Muller et al., 2009). The combination of the extract with estradiol showed a reduction of the relative weight of the uterus, just as the combination of estradiol with tamoxifen, suggesting anti-estrogenic activity of the extract, and indicating that in this situation, it behaved similarly to tamoxifen, which is considered, primarily, an estrogenic antagonist acting to block the binding site of estrogen at the tissue receptor. It is important to remember that, depending on the orientation of its side chain, tamoxifen can trigger anti-estrogen or estrogen because its activity seems to depend also on the state of the receptor, circulating levels, and target tissue (Ali et al., 2011). Therefore, the likely anti-estrogenic activity of EEtOH-Cl is similar to the ambiguous behavior of tamoxifen, or the competition by the receptors reduced the activity of both estradiol and the extract.

The appearance of embryofoetotoxic effects induced by various substances may result from their interaction with other factors that affect the mother-placenta-fetus as a whole, interfering in the nutritional state (Mello, 2007).

Monitoring animal body weight is a strong indicator in assessing the toxicity of a substance (Silva et al., 2005). The situation of pregnancy involves weight gain because estrogen and progesterone influence food intake, energy consumption, water retention rates and fat deposition

(Brolio, 2010). In this study, exposure of the progenitor to EEtOH-Cl during pre- and post-implantation did not alter the gain in body weight or the absolute and relative weights of the reproductive organs, that is, uterus and ovaries, or that of the non-reproductive organs such as the liver, kidneys and adrenal glands. There were also no macroscopic signs of toxicity observed in these organs.

The evaluation of the absolute weights of the non-reproductive organs is important, as the liver and kidneys are responsible for metabolism and elimination of xenobiotics and, in cases of intoxication, can have increased weight (Mello, 2007).

Variations in gestation length, regarding its abbreviation, may be indicative of changes in biochemical processes, since at the end of the pregnancy period estrogen levels rise and there is a decrease in progesterone levels. The estrogens initiate increased sensitivity of the myometrium to oxytocin through the induction of its receptors, and they also raise the production of prostaglandins (Wischral et al., 2001; Mello, 2007; Brolio, 2010). Thus, a likely explanation for the reduced gestation time may be these possible estrogenic actions or the increased expression of estrogen receptors, and so, influence the structural characteristics of the cells of the myometrium to promote parturition, especially when exposed in late pregnancy (Muller et al., 2009).

The uterine wall of mammals is comprised of smooth muscle in which calcium dependent mechanisms participate in the process of contraction (Travassos, 2010). This contraction, which is essential for foetal expulsion, involves complex events, which culminate with an increase in intracellular [Ca^{2+}], activation of myosin light chains, phosphorylation and, because of that, changing into chains of actin; however, all the mechanisms involved in uterine smooth muscle contraction are not yet fully understood (Du et al., 2006; Aguilar and Mitchel, 2010).

Observing the effect of EEtOH-Cl on contractions produced by KCl 80, we found that there was no significant inhibition or prolongation of these contractions in non-pregnant, non-treated females (NP, NT) or in non-pregnant, treated females (NPT). Likewise, in pregnant, non-treated females, there was no inhibition of contraction induced by KCl. However, in pregnant females treated with EEtOH-Cl, at doses of 250 and 500 mg/kg, there was significant inhibition of contraction induced by the contracting agent. Thereafter, it is possible to hypothesize that EEtOH-Cl can suppress expression of a receptor or class of proteins involved in the contraction of the uterus. In this case, the gestation period is critical for this likely action, since in pregnancy there is participation of estradiol, prostaglandins, oxytocin, gap junctions and ion channels (Slater et al., 2002). It is known that estrogens stimulate the synthesis and formation of gap junctions between muscle cells, resulting in greater efficiency of the muscle action potential and synchronized contractions of the myometrium (Lindzey and Korach, 1999).

With respect to contractions induced by oxytocin in NP, NT females, we observed an inhibition of contraction at an EEtOH-Cl concentration of 125 µg/mL placed directly into the vat of the isolated organ. This fact suggests that EEtOH-Cl may have, in accordance with its estrogenic activity, sensitized receptors to its action. In this context, it is suggested that EEtOH-Cl can produce interference in labor, decreasing the threshold to stimulate uterine contractility (Ramsey et al., 2011).

In pregnant females treated (PT) with EEtOH-Cl at a dose of 500 mg/kg, we observed inhibition of the phasic contractions induced by oxytocin, probably due to the influence of endogenous prostaglandins and oxytocin, characteristics of the end of pregnancy.

Finally, we can infer that EEtOH-Cl does not seem to impede the achievement of the calcium-dependent events, which is common to both promoting agents of contraction (that is, KCl and oxytocin). While KCl 80 mM, through its depolarizing action, allows the influx of Ca^{2+} into the interior of myometrial cells, oxytocin also does, upon binding to its receptors (Du et al., 2006; Chaves, 2007).

We conclude that EEtOH-Cl has estrogenic activity and, when combined with estradiol, presents anti-estrogenic activity as well. It does not induce toxicity in progenitors or in their offspring, and it inhibits isometric contractions induced by KCl 80 mM and oxytocin in pregnant and non-pregnant rats.

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