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Brazilian Cerrado plant (arnica) Lychnophora ericoides Mart. (Asteraceae) toxicity characterization in mice

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ABSTRACT

Background: Lychnophora ericoides Mart. (Asteraceae) is a Brazilian plant commonly used in folk medicine to treat pain and inflammation by topical administration. In recent years, this medicine has begun to be used orally. However, no study concerning its toxicity profile has been reported.
Objective: The study evaluates the potential toxicity of the ethanolic extract from leaves of the *Lychnophora ericoides*, through the methods of acute and sub-acute oral administration in mice.
Method: An acute toxicity study was performed according to the Organization for Economic Cooperation and Development protocol (OECD 423). A single extract dose of 50, 300, and 2000 mg/kg (n = 3/group) was administered orally to female Swiss mice. For subacute toxicity, the protocol OECD 407 was followed. Doses of 50, 300, and 500 mg/kg (n = 10/group) of the extract were administered daily to Swiss mice of both sexes for 28 days. Abnormal behavior, muscle strength, toxic symptoms, weight, and death were observed when assessing toxicity. Biochemical analysis, hematological analysis, macroscopic examination, and histopathological examination of several organs were conducted at the end of the treatment period.
Results: In acute and subacute toxicity, the extract did not produce mortality. The acute toxicity study revealed alterations in the behavioral test and histopathological changes in the liver, kidney, lung, and spleen. The subacute oral toxicity test showed changes in hematologic and biochemical parameters. Histopathological examination of liver, kidney, spleen, lung, and heart indicated degenerative characteristics with inflammatory infiltrates focus.
Conclusion: The study results suggest that oral use of *Lychnophora ericoides* leaves extract should be performed with caution in low doses in both single and repeated administrations.

Introduction

The use of medicinal plants is an ancient human practice that aims to treat and/or prevent several diseases. Plants are considered the primary diversity chemical source in traditional medicine (Sousa et al., 2020).

The public's acceptance and interest in natural therapies is growing in both developing and developed countries. Approximately 80% of the world's population use medicinal plants and herbal products as a health

Abbreviation: ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BW, body weight; CEEBEA, ethics committee on animal experimentation and welfare; AP, alkaline phosphatase; HDL, high-density lipoprotein; H&E, haematoxylin and eosin; LDL, low-density lipoprotein; SISBIO, Sistema de Autorização e Informação em Biodiversidade; SisGen, Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado; SE, standard error; EELE, Ethanolic extract of Lychnophora ericoides; TG, triglycerides; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; WBC, White blood cells.

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Table 1
Effect of the ethanolic extract of L. *ericoides* in muscle traction strength of the front limbs female swiss mice in the acute toxicity study.

Parameter	Control	Lychnophora ericoides		
		50 mg/Kg	300 mg/Kg	2000 mg/Kg
4 h	96.67 ± 4.256	98.33 ± 3.383	93.00 ± 3.055	115.3 ± 6.888
24 h	75.00 ± 14.47	104.7 ± 14.68	67.67 ± 23.25	84.00 ± 11.14
Week 1	85.00 ± 3.055	74.33 ± 14.33	61.67 ± 7.311	71.67 ± 4.667
Week 2	76.33 ± 13.48	65.00 ± 6.928	78.33 ± 4.910	74.00 ± 11.27

Values are mean/standard error ($n = 3$). n significantly different from the control, . * $p < 0.05$ using Two way ANOVA, followed by Bonferroni test.

significance, including its antioxidant, anti-inflammatory (Gobbo-Neto et al., 2005b) and analgesic properties (Dos Santos et al., 2010).

Phytochemical studies of *Lychnophora ericoides* have demonstrated the presence of flavonoids, sesquiterpene lactones, triterpenes, lignans, and caffeoylquinic acids. These compounds have several therapeutic benefits, such as analgesic, anti-inflammatory, and anti-tumor properties (Borella et al., 1998; Dos Santos et al., 2005; Gobbo-Neto et al., 2005a), as well as improving metabolic profiles (Paraíso et al., 2019). Although bioactive compounds have therapeutic benefits, some secondary metabolites, such as sesquiterpene lactones (STL), can accumulate in tissues causing toxicity (Schmidt, 2006). The systematic

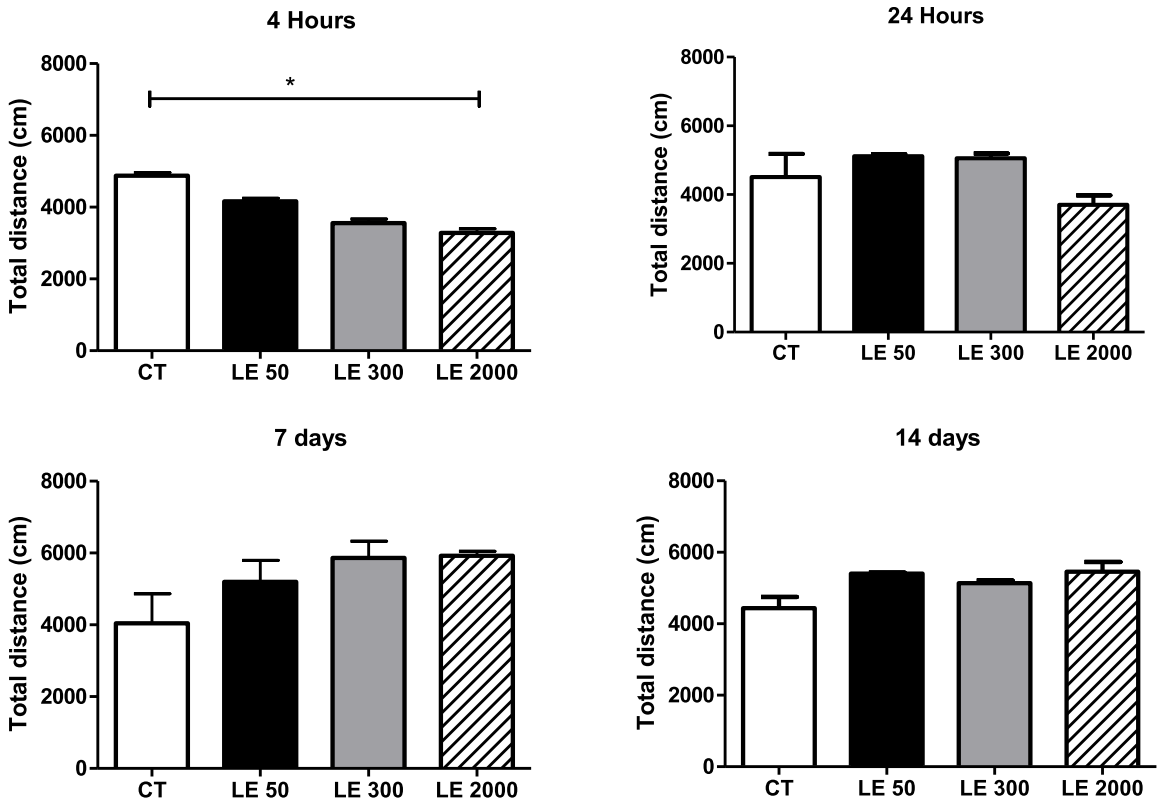

Fig. 1. Total distance passed (cm) in 300 s evaluated in the behavioral test by Open Field after administration of a single dose of EELE in doses of 50, 300 and 2000 mg/Kg ($n = 3$). Columns represent mean ± SEM. *$p < 0.05$ using Kruskal-Wallis test, followed by post-Dunńs test.

source due to easy access and low cost (Ekor, 2014; Hosseinzadeh et al., 2015). Knowledge about medicinal plants often symbolizes the only therapeutic resource for many communities and ethnic groups. Plant species can be considered an invaluable source of biologically active secondary metabolites (Singh et al., 2020).

Lychnophora ericoides Mart. (Asteraceae, Vernonieae) is a Brazilian native species distributed in the Cerrado (Brazilian-savanna) biome, popularly known as "arnica da serra" or "falsa arnica". Traditional medicine employs alcoholic and hydroalcoholic preparations of leaves for the treatment of wounds, inflammations and pain (Ferreira et al., 2014; Gobbo-Neto et al., 2005b). L. *ericoides* leaves are also used as flavorings in "cachaças[1]", officially called "aguardente de cana". The cachaça macerate of L. *ericoides* leaves is administered orally for therapeutic purposes (Gobbo-Neto and Lopes, 2008).

Report on plant bioactivity indicates its pharmacological evaluation of medicinal plants regarding the potential for toxicity is a fundamental step for the validation of their therapeutic use (El Kabbaoui et al., 2017)

Despite the available knowledge and the widespread use of L. *ericoides* in Brazilian folk medicine, no reported studies approach the toxicological profile of the leaves extract. Thus, the present study was performed to assess the possible toxic effects of the L.*ericoides* leaves alcoholic extract after acute and sub-acute oral administration in mice.

Materials and method

Plant material

Fresh leaves of L. *ericoides* were obtained from Catuni, municipality of Francisco Sá, MG. Brazil (16′21′51′S 43′20′6′W) in September 2019. The harvest was previously authorized by the license n° 66,693–1 (SISBIO/IBAMA) and registered under registration AF73E03 on SisGen (National System for the Management of Genetic Heritage and Associated Traditional Knowledge). The species was identified by Dr. Santos

[1] typical Brazilian alcoholic beverage produced from the fermentation of cana-de-açúcar.

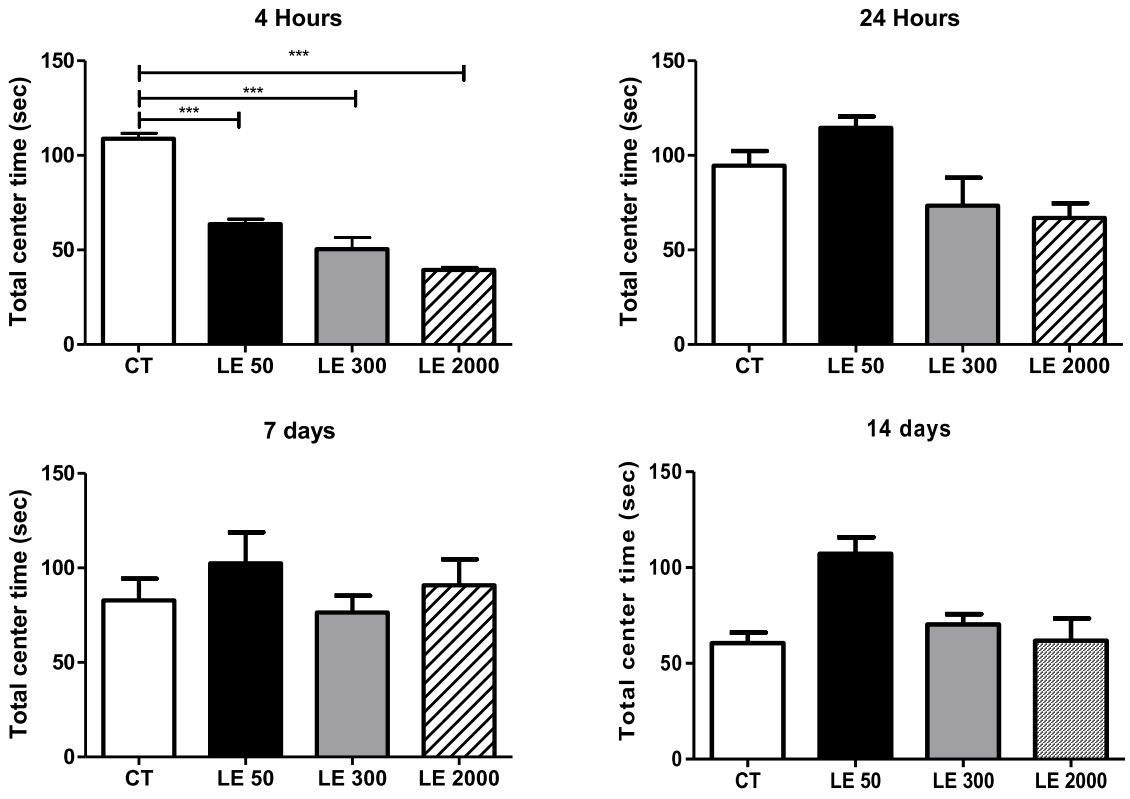

Fig. 2. Total center time (*sec*) in 300 s assessed in the behavioral test by Open Field after administration of a single dose of EELE in doses of 50, 300 and 2000 mg/Kg ($n = 3$). Columns represent mean ± SEM.*** $p < 0.01$ using Kruskal-Wallis test, followed by post-Dunńs test.

D'Angelo Neto, and the voucher specimen was deposited and registered in the herbarium Norte Mineiro - MCCA (http://mcca.jbrj.gov.br) with the identification MCCA 03,405. Plant names were checked using the following website: www.theplantlist.org.

Preparation of the extract

L. *ericoides* leaves were dried using an incubator (model 400/ND/Nova ética) with strengthened air circulation at 40 °C. Following drying, the material was ground in a knife mill. The plant (100 gs) powder was macerated at a ratio of 1:10 (m/v),[16] in a static form in 1-liter absolute ethyl alcohol P.A for 14 days. After extraction, the macerate was filtered and the solvent eliminated in an oven at 35 °C. The extract was stored under refrigeration. Finally, the leaves extract (15,76% yield) was reconstituted on a daily basis in 5% Tween 80 solution to the final required concentrations prior to the experiment.

Animals

Adult Swiss mice (*Mus musculus*) obtained from the bioterium of the State University of Montes Claros (UNIMONTES) were used for the acute and subacute toxicity tests. Animals were kept in standard conditions (22 ± 3 °C; 30 to 70% air humidity; 12 h light /12 h dark cycles), and given food and water ad libitum. All procedures were completed in accordance with the Ethical Principles in Animal Research and approved by the Ethics Committee on Animal Experimentation and Welfare at the State University of Montes Claros, under protocol number 210/2020.

Acute oral toxicity study

The assay of acute oral toxicity of ethanolic extract L. *ericoides* (EELE) leaves was carried out according to Organization for Economic Cooperation and Development protocol (OECD) Directive No. 423 (OECD, 2001). The EELE was administered as a single dose, by oral gavage, in Swiss 8–12 weeks old mice, nulliparous and non-pregnant females were randomly allocated to 4 groups ($n = 3$). The groups were divided as follows: Group I or control group ($n = 3$): 5% Tween 80 solution, diluted in fresh water. Group II ($n = 3$): treated with EELE at the dose of 2000 mg/kg of body weight (BW).

Behavioural manifestations of acute oral toxicity were also noted. Clinical observations were made using the open field test and strength assessment using the traction test. The animals were fasted overnight day 14 after treatment and were subsequently euthanized. Blood samples were collected for biochemical and hematological investigations. The organs (lung, liver, spleen and kidneys) were removed for necropsy and histopathological analysis.

Subacute oral toxicity study

The subacute toxicity study was carried out by oral administration of the extract for 28 days according to OECD Test Guidelines 407 for testing chemicals(OECD/OECDE, 2008). Animals (6–8 weeks) of both sexes, being nulliparous and non-pregnant females, were divided randomly into 4 groups of 10 animals each (5 males and 5 females). The ethanolic extract of L. *ericoides* was administered daily by gavage at concentrations of 50, 300, and 500 mg/kg.BW. Doses of the treatment and 5% Tween 80 solution (vehicle) were administered daily for 28 consecutive days by oral gavage in a single dose. Doses were adjusted weekly according to the animals' weight.

During the 28-day study period, the animals were observed daily for abnormal clinical signs and death. The animals were evaluated for behavioral changes by the open field test and for changes in neuromuscular activity by the traction test. Body weights were measured and recorded at the beginning and then after every week of the experiment, and food and water consumption determined daily.

At the end of the study, all animals were fasted overnight (water was

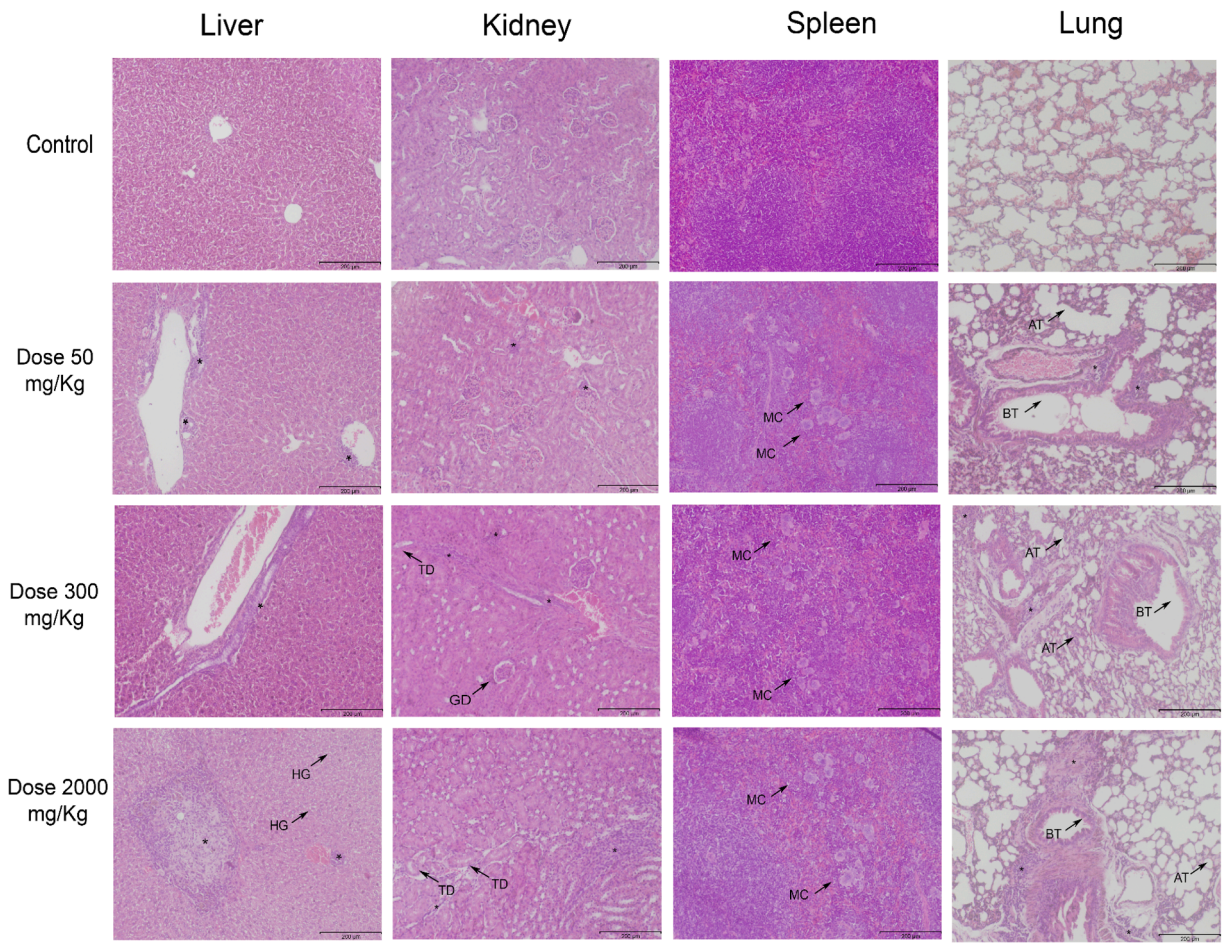

Fig. 3. Photomicrograph of the liver, kidney, spleen and lung of female mice control and treated with 50, 300 and 2000 mg/kg of ethanolic extract of Lychnophora ericoides in the acute oral toxicity test. Size bar for comparison 200 μm. H& E (40 x). * Inflammatory infiltrate; HG: hydropic degeneration; GD: Glomerular Degeneration; TD: Tubular Dilation; MC: Multinuclear cells; BT: thickening of the bronchi; AT: Alveolar thickness.

not restricted) and then weighed. After euthanasia, blood samples were collected for biochemical and hematological analyses. The organs (liver, heart, spleen, kidneys, lung, pancreas, testicles, prostate, thymus, epididymal adipose tissue, brain, stomach, small intestine, large intestine, sciatic nerve, uterus and ovaries) were removed for necropsy and histopathological analysis.

Histopathology analysis

Histopathological examination was performed in the organs of all animals in the acute toxicity test. For the subacute toxicity test six animals from each group (3 females and 3 males) were randomly selected for histopathological studies, according to the methodology described by (Bakrania et al., 2017; Variya et al., 2019). The organs samples were fixed in 10% neutral formalin buffer and underwent a routine histological process for paraffin embedding. Sections (5 μm thick) were stained by hematoxylin and eosin (H.E) method. The sections were examined under a light microscope under different (x100 and x400) magnifications. Photomicrographs of lesions were taken (x 200) with an Olympus photo microscope for observations and documentation of histopathological lesion (Ezeja et al., 2014; Ferreira et al., 2014; Jeena et al., 2011; Mishra et al., 2018).

Open field test

The test was carried out in a multi-unit open field box composed of four activity chambers with dimensions of 50 cm (length) x 50 cm (width) x 38 cm (height) made of MDF and EVA-coated floors. Mice were placed individually in the central area of the box and monitored for five minutes. Animal's trajectory was quantified in centimeters covered using the Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD) (Gomes et al., 2019).

Traction strength test

Muscular traction strength of the front limbs was assessed by means of the grip test, using the Grip-Strength Meter Bonther® according to the methodology described by Takeshita and contributors (Takeshita et al., 2017).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 5.0®, San Diego, California, USA), with 95% ($p < 0.05$) confidence. Data was given as mean ± Standard error of the mean (SEM). The normality was verified by Shapiro Wilk test. The statistical significance of the values for the different groups were estimated by one-way ANOVA followed by the post-D'unnets. Two-way ANOVA was used to evaluate Traction test and open field followed by the post-Bonferroni control-comparison test.

Table 2
Effect of the ethanolic extract of L. *ericoides* in muscle traction strength of the front limbs males e females swiss mice in the subacute oral toxicity study.

Parameter	Control	Lychnophora ericoides		
		50 mg/Kg	300 mg/Kg	500 mg/Kg
4 h	146.200 ± 11.364	135.800 ± 16.850	98.000 ± 8.735	98.000 ± 8.735
24 h	91.25 ± 9.286	124.30 ± 5.935	106.200 ± 10.071	84.75 ± 10.260
7 days	92.600 ± 8.298	102.600 ± 9.558	78.000 ± 8.532	73.400 ± 12.123
14 days	89.200 ± 6.865	86.200 ± 9.876	95.00 ± 5.148	85.200 ± 7.276
21 days	89.600 ± 10.127	97.000 ± 8.619	66.50 ± 11.380	65.600 ± 13.750
28 days	76.200 ± 12.607	75.400 ± 15.827	79.400 ± 17.873	73.000 ± 8.307
Female animales				
4 h	99.800 ± 5.757	98.200 ± 6.591	86.000 ± 4.604	125.000 ± 7.0356
24 h	85.600 ± 8.920	70.000 ± 2.280	81.400 ± 8.059	97.200 ± 8.493
7 days	95.750 ± 8.985	87.600 ± 3.356	71.000 ± 10.75	88.400 ± 5.372
14 days	95.600 ± 4.261	83.000 ± 8.879	51.800 ± 2.956***	58.750 ± 6.088**
21 days	100.800 ± 5.410	56.600 ± 3.279*	56.250 ± 12.370*	60.00 ± 11.710*
28 days	93.500 ± 3.926	41.000 ± 6.181***	47.600 ± 5.134***	49.200 ± 3.169***

Values are mean/standard error($n = 5$). Two way ANOVA, followed by Bonferroni test. * $p < 0.05$, ** $p < 0.1$ and *** $p < 0.01$ when compared to the control group.

Results

Acute toxicity study

The administration of a single dose 50, 300 and 2000 mg/Kg of EELE in Swiss mice did not produce any mortality or signs of toxicity over the observation period.

Neither of the doses evaluated altered neuromuscular activity assessed by the tensile strength test (Table 1). However, dose of 2000 mg/kg resulted in significant decrease ($p < 0.05$) in the total distance covered (cm) after four hours of extract administration (Fig. 1). A reduction in total center time was also observed when animals were treated, after four hours of extract administration (Fig. 2).

No significant changes were found in water consumption and food intake (Table S1-Supplementary material), relative weights of internal organs (Table S2- Supplementary material), and biochemical and hematological parameters (Table S3 and S4- Supplementary material) during the 14 days of acute oral administration of L.*ericoides* compared to control.

The results obtained in the acute toxicity study suggest that acute administration of EELE did not produce death in 50% of mice at the maximum dose of 2000 mg/kg. Therefore, the LD50 of extract could not be estimated, and was considered to be greater than 2000 mg/kg.

Histological analysis

Acute administration of EELE produced dose-dependent histological changes in the liver, kidney, lung and spleen. Liver presented inflammatory infiltrate at doses 50, 300 and 2000 mg/Kg, and hydropic degeneration at the dose 2000 mg/Kg. The kidneys showed mild inflammation at dose 50 mg/kg and, moderate at dose 300, 2000 kg/mg, as well as glomerular degeneration and, tubular dilation. Hydropic degeneration was observed at the dose 2000 mg/kg. In the spleen, at all doses evaluated, it found the presence of multinucleated giant cells. Lung showed inflammatory foci, thickening of the bronchi and increased thickness of alveolar septa (Fig. 3).

28-Days sub-acute toxicity study

General observations

The ethanolic extract of L. *ericoides* administered orally at daily doses of 50, 300 and 500 mg/kg for 28 days, did not produce mortality. Male mice did not show any clinical signs of toxicity. However, female mice treated with EELE 300 mg/Kg, showed excitability from the 5th day until the end of the experiment.

Muscle traction strength

After 28 days of treatment with L. *ericoides* extract, there were no significant changes in the traction strength of the forelimbs in male mice in any of the doses evaluated in this study. However, female mice from the 14th day of oral administration EELE, decrease in the traction strength of the forelimbs was observed until the end of the experiment, when compared to the control group (Table 2).

Open field test

In the open field test, oral administration for 28 days of ethanolic extract of L. *ericoides* doses 50, 300 and 500 mg/kg in males did not induce changes in the total distance covered and in the time that the animal remained in center field during the study (Fig. 4).

In females, oral administration of EELE at doses 50, 300 and 500 mg/Kg significantly decreased the total center time after 4 h (Control: 106.52 ± 3.06; EELE 50 mg/Kg: 68.48 ± 9.39; EELE 300 mg/Kg: 63.14 ± 1.71; EELE 500 mg/Kg: 62.20 ± 15.82). In the other times, there was no significant change (Fig. 5).

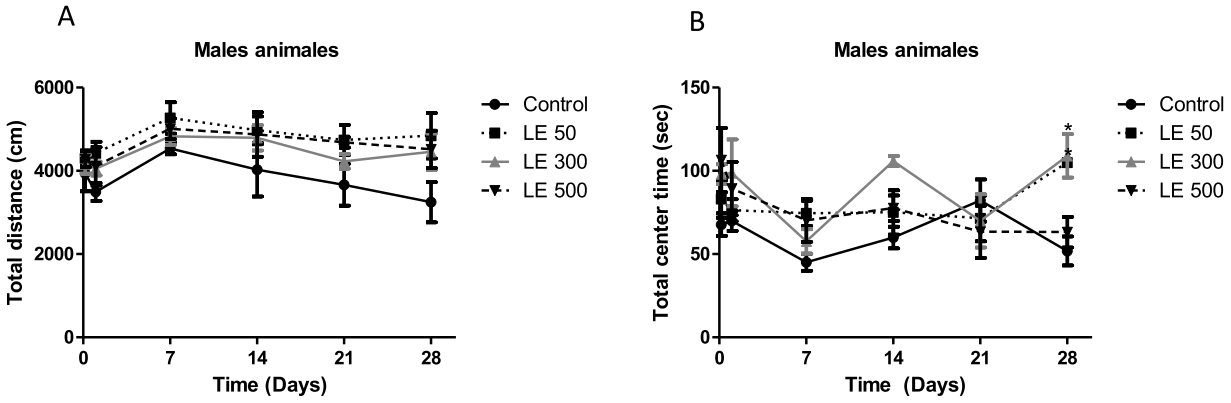

Fig. 4. Spontaneous general activity of male mice ($n = 5$), as recorded in the open field test, after oral administration for 28 days at doses 50, 300 and 500 mg / kg. (A) Total distance; (B) Total center time was recorded for 300 s. Values expressed as mean ± S.E.M Bidirectional ANOVA followed by the Bonferroni test. * $p < 0.05$, ** $p < 0.1$ and *** $p < 0.01$ when compared to the control group.

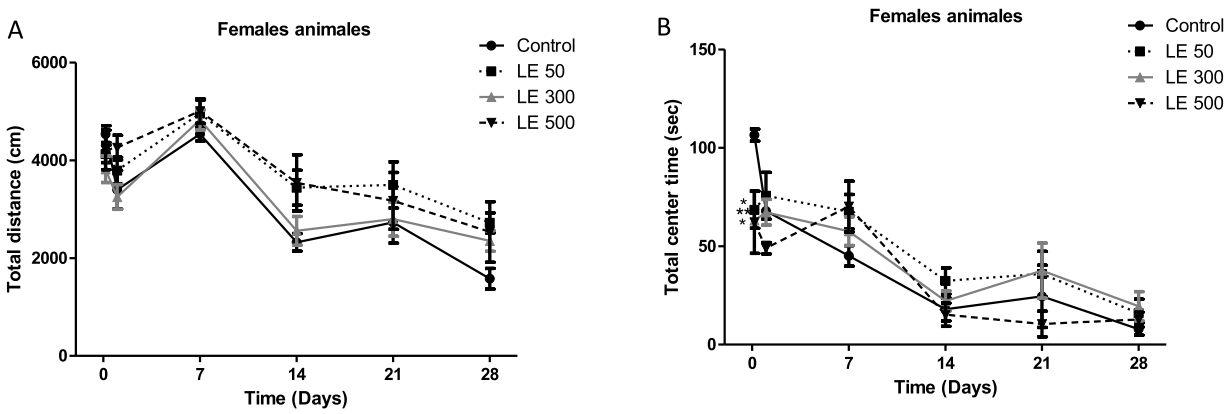

Fig. 5. Spontaneous general activity of female mice ($n = 5$), as recorded in the open field test, after oral administration for 28 days at doses 50, 300 and 500 mg / kg. (A) Total distance; (B) Total center time was recorded for 300 s. Values expressed as mean ± S.E.M Bidirectional ANOVA followed by the Bonferroni test. * $p < 0.05$, ** $p < 0.1$ and *** $p < 0.01$ when compared to the control group.

Like as male animals, the ethanolic extract of L. *ericoides* did not significantly change the total distance covered by females during the study.

Body weight, relative organ weight, food and water intake

The treatment for 28 days did not result in significant changes in the weight gain (%) of the male animals (Fig.S1-Supplementary material).

Females treated with EELE doses 50 and 500 mg/Kg did not show significant difference in the variation of body weight in this study. In contrast, the group treated with 300 mg/kg of EELE showed a significantly greater weight gain than the control group from the 7th day of treatment. This group showed 11.50% of body weight gain when compared to the control group (Fig. S1-Supplementary material).

At the end of subacute treatment, the relative weight of prostate was statistically lower from those of the control group. In females, a significant increase ($p<0.05$) in the relative organ weight of spleen and thymus were observed at dose of 500 mg/Kg compared with control group (Table 3).

Consumption of food and water, in both sexes, did not change throughout the study period, when compared to the control group.

Hematological parameters

The hematological parameters of control and treatment groups with ethanolic extracts of L. *ericoides* (50, 300 and 500 mg/kg) were presented in table 4. Results of hematological analysis revealed a significant increase in WBC count at doses 300 and 500 mg/kg in male and at dose 500 mg/Kg in female. Other alteration such as significant increase in the Mean Corpuscular Hemoglobin Concentration (MCHC) was observed in female mice.

Biochemical parameters

The biochemical parameters evaluated after 28 days of oral administration of the extract of L. *ericoides*. The administration of EELE dose 500 mg/Kg in male animals resulted in a significant increase in alkaline phosphatase. However, in females there were no significant changes in the biochemical parameters evaluated when compared to the control group (Table 5).

Histopathological observations

Microscopic examination of the organs from mice exposed to subacute treatment with ethanolic extract of L. *ericoides* in doses 50, 300 and 500 mg/Kg showed changes in the liver, kidneys, lung, spleen, thymus and heart in all treated animals (Fig. 6). Consistent histopathological changes related to treatment in these organs were found in both sexes. Alterations evidenced in the other organs were not observed in all animals in the group.

Liver histological analysis in the control group showed hepatocyte strings, centrilobular vein and sinusoid capillaries with normal features. However, the animals treated with EELE presented infiltration of inflammatory cells and cytoplasmic vacuolization.

Renal sections showed changes in the kidneys of the animals treated with the three doses, glomerular degeneration, inflammatory infiltrate foci, hemorrhagic foci and tubular dilation, unlike the control group that presented normal glomerular architecture with normal distal and proximal tubules organization.

The lung showed thickening of the alveolar walls, infiltration of inflammatory cells, exfoliated bronchiolar cells were observed in some animals that received continuous administration of EELE.

In the spleen, it was observed hemosiderin deposition, presence of multinucleated giant cells in all evaluated doses. Histological manifestations of the thymus in males were dose dependent, showed an increase in the number of lymphocytes in the medullary region when compared to the control group. All groups had a defined limit between the cortex and the medulla.

Small inflammatory foci were observed in the heart at 500 mg/Kg dose evaluated in both males and females.

Histopathological analyzes of the prostate showed some foci of inflammatory cell infiltration at all doses evaluated (Fig. 7).

Discussion

Lychnophora ericoides (Mart). leaves and roots are traditionally used medicinally and have analgesic and anti-inflammatory effects (Borsato et al., 2000). Although this plant is widely used in traditional medicine in “cachaça” macerates (oral and topic use), there is a gap in the scientific literature on its oral toxicity. Therefore, the present study is the first to assess the ethanolic toxicological profile of *Lychnophora ericoides* leaves by performing acute oral toxicity in mice for 14 days and subacute oral toxicity for 28 days.

Mortality is the main criterion to evaluate the acute drug toxicity (LD50) (Asare et al., 2012). No deaths were observed after extract administration in single-dose or in continuous-dose tests for 28 days. According to OECD 423 (OECD, 2001), EELE has low acute toxicity and should be included in category 5 with an estimated LD50 greater than 2000 mg/kg.

The evaluation of single-dose toxicity is important, but does not ensure the safety of medicinal plants used for prolonged periods. Since the use of medicinal plants normally occurs for continuous or prolonged periods, a long-term repeated study would provide useful safety information (da Silva Moreira et al., 2019; Moreira et al., 2014). Therefore, the subacute oral toxicity and animal behavior were evaluated for 28 days.

Table 3
Effect of the ethanolic extract of L. *ericoides* on relative organ weights in male and female swiss mice treated for 28 days with diferents doses.

Parameter	Control	Lychnophora ericoides		
		50 mg/Kg	300 mg/Kg	500 mg/Kg
Males animals				
Adipose (g/BW)	0.0160 ± 0.0023	0.0260 ± 0.0013	0.0195 ± 0.0025	0.0148 ± 0.0019
Spleen (g/BW)	0.0028 ± 0.0003	0.0031 ± 0.0002	0.0062 ± 0.0015	0.0029 ± 0.0003
Brain (g/BW)	0.0102 ± 0.0004	0.0092 ± 0.0011	0.0119 ± 0.0009	0.0109 ± 0.0004
Pancreas (g/BW)	0.0049 ± 0.0007	0.0055 ± 0.0006	0.0063 ± 0.0014	0.0059 ± 0.0005
Thymus (g/BW)	0.0017 ± 0.0004	0.0018 ± 0.0002	0.0015 ± 0.0001	0.0019 ± 0.0003
Kidney (g/BW)	0.0147 ± 0.0006	0.0138 ± 0.0007	0.0145 ± 0.0006	0.0139 ± 0.0004
Heart (g/BW)	0.0047 ± 0.0003	0.0042 ± 0.0002	0.0053 ± 0.0004	0.0042 ± 0.0002
Stomach (g/BW)	0.0217 ± 0.0064	0.0131 ± 0.0023	0.0122 ± 0.0015	0.0138 ± 0.0015
Lung (g/BW)	0.0069 ± 0.0009	0.0092 ± 0.0012	0..0101 ± 0.0017	0.0072 ± 0.0007
Bone (g/BW)	0.0035 ± 0.0006	0.0055 ± 0.0013	0.0058 ± 0.0012	0.0047 ± 0.0011
Liver (g/BW)	0.0647 ± 0.0104	0.0457 ± 0.0019	0.0537 ± 0.0053	0.0463 ± 0.0491
Prostata (g/BW)	0.0021 ± 0.0002	0.0009 ± 0.0001**	0.0007 ± 0.0000**	0.0012 ± 0.0002*
Epididymo (g/BW)	0.0134 ± 0.0032	0.0307 ± 0.0076	0.0195 ± 0.0025	0.0148 ± 0.0018
Female animales				
Adipose (g/BW)	0.1383 ± 0.0277	0.1093 ± 0.0189	0.1008 ± 0.0175	0.0889 ± 0.0173
Spleen (g/BW)	0.0099 ± 0.0007	0.0098 ± 0.0005	0.0086 ± 0.0027	0.0114 ± 0.0022*
Brain (g/BW)	0.03408 ± 0.0014	0.04192 ± 0.0028	0.03986 ± 0.0028	0.04038 ± 0.0016
Pancreas (g/BW)	0.0156 ± 0.0015	0.0161 ± 0.0015	0.0104 ± 0.0004	0.0147 ± 0.0019
Thymus (g/BW)	0.0069 ± 0.0007	0.0090 ± 0.0009	0.0106 ± 0.0010	0.0127 ± 0.0013 **
Kidney (g/BW)	0.0316 ± 0.0018	0.0288± 0.0009	0.0296 ± 0.0016	0.0268 ± 0.0022
Heart (g/BW)	0.0118 ± 0.0008	0.0015 ± 0.0012	0.0124 ± 0.0018	0.0118 ± 0.0007
Stomach (g/BW)	0.0452 ± 0.0055	0.0572 ± 0.0087	0.0458 ± 0.0041	0.0521 ± 0.0015
Lung (g/BW)	0.0187 ± 0.0023	0.0201 ± 0.0008	0.0201 ± 0.0045	0.0199 ± 0.0023
Bone (g/BW)	0.0084 ± 0.0008	0.0125 ± 0.0020	0.0101 ± 0.0025	0.0087 ± 0.0021
Liver (g/BW)	0.1340 ± 0.0053	0.1273 ± 0.0025	0.1309 ± 0.0040	0.1130 ± 0.0052
Ovarios (g/BW)	0.0021 ± 0.0002	0.0034 ± 0.0005	0.0032 ± 0.0002	0.0026 ± 0.0001
Uterus (g/BW)	0.0118 ± 0.0006	0.0079 ± 0.0022	0.0071 ± 0.0012	0.0119 ± 0.0015

n = 5 males and 5 females. Unidirectional ANOVA followed by Bonferroni multiple comparison test. * p <0.05, ** p <0.1 and *** p <0.01 when compared to the control group.

Open field testing (OFT) is a classic experimental model used to investigate exploratory activity and emotional behavior in rodents (Gamberini et al., 2015). In the present study, a reduction in the OFT central time in the acute test (50, 300 and 2000 mg/kg), as well as a reduction in the total distance after the administration of 2000 mg/kg were observed without change in muscle tone. The altered behavior occurred 4 h after oral administration of the plant extract and disappeared 24 h after treatment, presenting a transitory characteristic. A study conducted with mice treated with ethanol extract of *Lychnophora tricocarpha* (0.750 g/kg) reduced the animals' spontaneous locomotor activity and exploratory behavior 1 and 4 h after administration (Ferrari et al., 2012). Similar results were obtained in the open field test with Swiss mice treated with a single dose of *Lychnophora pinaster* (Ferreira et al., 2014).

Table 4
Hematological parameters of male and female mice treated with L. *ericoides* extract in the subacute toxicity study.

Parameter	Control	Lychnophora ericoides		
		50 mg/Kg	300 mg/Kg	500 mg/Kg
Male animales				
Hemoglobin (gm/dl)	11.55 ± 1.237	14.60 ± 0.56	14.26 ± 0.48	14.38 ± 0.51
Hematocrit (%)	37.33 ± 3.82	47.60 ± 1.92	46.52 ± 1.20	46.35 ± 1.73
MCH (pg)	16.46 ± 0.63	16.33 ± 0.35	16.25 ± 0.18	16.30 ± 0.12
MCHC (g/dL)	30.89 ± 0.21	30.68 ± 0.07	30.62 ± 0.33	31.02 ± 0.09
MCV (fL)	53.27 ± 2.01	53.25 ± 1.18	53.10 ± 0.89	52.56 ± 0.50
Total Plateletes count	1,679,000 ± 100,500	1,527,000 ± 209,200	1,570,000 ± 133,600	1,625,000± 129,100
Total WBC count $10^3/\mu L$	4100 ± 400	5667 ± 405.5	8467 ± 569.6**	7200 ± 611*
Neutrophils (%)	18.75 ± 2.95	22.33 ± 0.8819	25.40 ± 2.015	21.00 ± 0.7071
Lymphocytes (%)	81.25 ± 2.95	76.33 ± 1.20	73.80 ± 3.184	78.50 ± 1.555
Eosinophils (%)	0.00± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Monocytes (%)	0.00 ± 0.00	1.33 ± 0.67	0.80 ± 0.58	0.50 ± 0.29
Female animales				
Hemoglobin (gm/dl)	9.60 ± 1.4	13.00 ± 0.38	13.68 ± 0.47	13.07 ± 0.97
Hematocrit (%)	46.90 ± 3.52	44.50 ± 1.53	47.03 ± 2.56	42.83 ± 2.68
MCH (pg)	12.14 ± 1.63	15.01 ± 0.90	15.65 ± 0.85	16.13 ± 0.34
MCHC (g/dL)	20.02 ± 1.94	29.31 ± 1.64*	29.26 ± 1.38**	30.46 ± 0.38**
MCV (fL)	51.21 ± 4.43	51.19 ± 0.29	53.43 ± 0.60	52.96 ± 0.47
Total Plateletes count	836,500 ± 7794	754,300 ± 73,170	849,500 ± 67,650	883,000 ± 56,670
Total WBC count $10^3/\mu L$	4550 ± 317.5	5367 ± 1189.00	6100 ± 985.70	9033 ± 318.0*
Neutrophils (%)	33.00 ± 2.89	30.00 ± 0.58	32.50 ± 3.57	38.67 ± 1.45
Lymphocytes (%)	66.00 ± 3.464	69.67 ± 5.78	66.25 ± 4.05	61.00 ± 1.53
Eosinophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.33
Monocytes (%)	1.00 ± 0.58	0.33 ± 0.33	1.25 ± 0.48	0.00 ± 0.00

MCH = Mean corpuscular hemoglobin; MCHC = Mean corpuscular hemoglobin concentration; MCV = Mean corpuscular volume. WBC= White blood cells; Values expressed as mean ± SEM. Unidirectional ANOVA followed by Bonferroni multiple comparison test. * p <0.05, ** p <0.1 and *** p <0.01 when compared to the control group., n = 5 males/group and 5 females/group.

Changes in body weight without metabolic regulation may be a toxicity indicator (Yi-Chen et al., 2018). In the sub-acute toxicity study, daily oral administration of EELE caused no significant body weight changes in males; however, a non-dose-dependent response was observed in females. The increase in female weight gain treated with a 300 mg/kg dose might not be attributed to the toxic effect of EELE. Regarding internal organ weight, the subacute toxicity study revealed a significant increase in the relative weight of the spleen and thymus in female mice treated daily with a dose of 500 mg/kg. Organ enlargement may be associated with the stimulatory/proliferative effect of the plant extract on the thymus and spleen. The increase in relative weight was accompanied by the increase in WBC count, as noted in the previous toxicity study of *Ludwigia octovalvis* extract (Kadum Yakob et al., 2012).

The thymus and spleen are important lymphatic organs closely related to the immune system. The thymus is the main site where stem cells produced by the bone marrow are transformed into T cells (Carrio and Lopez, 2013). In both organs, an increase in defense cells was observed in the histopathological analysis. The results corroborate the hypothesis that EELE stimulates the lymphoid organs action.

The male and female mice's reproductive organs were evaluated to assess possible morphological changes that would influence the animals' reproduction after treatment with L. *ericoides*. We observed a significant decrease in the prostate relative weight and histological morphology

Table 5
Biochemical parameters of male and female mice treated with L. *ericoides* extract in the subacute toxicity study.

Parameter	Control	Lychnophora ericoides		
		50 mg/Kg	300 mg/Kg	500 mg/Kg
Male animales				
Random blood glucose (mg/dl)	178.00 ± 20.83	185.60 ± 9.60	181.50 ± 16.58	200.40 ± 9.54
Total cholesterol (mg/dl)	144.00 ± 8.10	134.80 ± 7.17	174.50 ± 15.47	120.80 ± 10.61
Tryglicerides (mg/dl)	201.20 ± 12.92	165.60 ± 10.91	228.50 ± 51.38	223.60 ± 49.45
HDL (mg/dl)	123.80 ± 7.81	109.1 ± 5.89	110.70 ± 10.83	117.2 ± 11.20
GOT (U/L)	438.00 ± 120.40	328.00 ± 58.54	409.50 ± 65.70	404.00 ± 35.67
GTP (U/L)	88.80 ± 15.56	55.20 ± 14.37	81.50 ± 13.67	64.80 ± 3.50
Total Protein (mg/dl)	6.88 ± 0.20	6.44 ± 0.24	6.45 ± 0.51	8,46 ± 1,73
alkaline phosphatase (U/L)	293.00 ± 18.93	446.80 ± 50.71	343.30 ± 37.58	555.5 ± 90.25*
Female animales				
Random blood glucose (mg/dl)	144.90 ± 16.47	143.90 ± 8.06	142.4 ± 19.52	136.9 ± 6.52
Total cholesterol (mg/dl)	95.45 ± 2.88	117.4 ± 7.35	127.3 ± 16.79	99.65 ± 6.46
Tryglicerides (mg/dl)	185.40 ± 28.84	192.1 ± 13.67	165.1 ± 7,88	137.6 ± 15.73
HDL (mg/dl)	76.85 ± 6.24	98..5 ± 5.14	104.7 ± 8.32	86.86 ± 5.14
GOT (U/L)	373.3 ± 83.60	453.3 ± 44.15	430.6 ± 38.91	449.6 ± 100.2
GTP (U/L)	71.98 ± 15.10	82.96 ± 6.88	78.50 ± 11.59	90.53 ± 12.04
Total Protein (mg/dl)	7.26 ± 0.63	6.58 ± 0.23	6.47 ± 0.38	5.77 ± 0.60
Alkaline phosphatase (U/L)	521.80 ± 91.30	488.00 ± 59.45	362.2 ± 24.02	635.1,00 ± 71.65

HDL= high-density lipoprotein; LDL = low-density lipoprotein; GOT= glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase. Values are mean ± SEM. (n = 3). n significantly different from the control, $p<0.05$ using Kruskal-Wallis test, followed by post-Dunnś test. Values expressed as mean ± SEM. Unidirectional ANOVA followed by Bonferroni multiple comparison test. * $p<0.05$, ** $p<0.1$ and *** $p<0.01$ when compared to the control group., $n = 5$ males/group and 5 females/group.

modifications were observed in male mice treated daily with 500 mg/kg. The atrophy of reproductive organs is one of the main causes of sex hormone metabolic disorders (Wang et al., 2019).

The hematological parameters generally indicate the pathological status of animals and are highly sensitive indicators of drug-induced toxicity. In the present study, the administration of single doses of EELE did not result in hematological and biochemical changes as observed in the *Lychnophora pinaster* acute oral toxicity study results (Ferreira et al., 2014). However, hematological and biochemical changes occur relatively slowly and the experimental time and administration profile may not be sufficient to identify all possible changes (Ferreira et al., 2014). This fact is evidenced by the results obtained in the subacute toxicity study. WBC changes were observed in both sexes. The total increase in leukocytes is a stress marker and an indicator of immune response. Generally, an increase in white blood cell activity occurs in response to the toxic environment (Chanda et al., 2015). MCHC female changes are within the normal range for the species (Santos et al., 2016). Therefore, the observed changes may not have toxicological significance.

A clinical blood chemistry examination was performed to evaluate toxic effects on the pancreas, kidney and liver function (Sireeratawong et al., 2012). In male animals, the increase in serum level of alkaline phosphatase, an important blood biochemical marker, may be an indicative of liver disfunction (Zárybnický et al., 2018).

Histopathological changes in liver and kidneys were observed in the acute and subacute toxicity test. The liver is responsible for protecting the body from potentially toxic chemical aggressions (da Silva Moreira et al., 2019). Hepatocytes are exposed to xenobiotics, without systemic modification or dilution when administered orally, which makes them highly susceptible to toxic effects (Zárybnický et al., 2018). Like the kidneys, they are also frequent targets of toxic compounds, receiving about 25% of the cardiac blood flow (Araújo et al., 2017). Several metabolites present in medicinal plants, such as flavonoids, can be toxic to the kidneys, since renal tubular alterations have been described after exposure to these compounds (Yang et al., 2018). The presence of flavonoids in the extract (Gobbo-Neto et al., 2008) may also be responsible for the hyperemia and vascular congestion evidenced in the subacute test of the histopathological analysis, due to vasodilator effect (Pérez-Vizcaíno et al., 2002).

We observed changes in pulmonary histology at all doses assessed in both the acute and subacute toxicity studies. Although, this fact was not observed in toxicity studies with species of the same genus. Though, Li et al. and Peibo Li et al. observed similar histopathological changes in the lung, using the glycosylated flavonoids hesperidin and naringin, both isolated from Citrus grandis (Li et al., 2013, 2019). We hypothesize that different plant metabolites when administered orally might cause lung damage. The results presented in this study should be considered as evidence to avoid the oral use of L. *ericoides* leaves added in spirits (cachaça).

To our knowledge, this is the first study to investigate the oral toxicity effects of *Lychnophora ericoides* Nevertheless, our study has limitations. The analysis of the chromatographic profile of the species under study were not performed by HPLC. However, a previous study described the chromatographic profile of the *Lychnophora ericoides* species (Borella et al., 1998; Fernandes et al., 2011; Gobbo-Neto and Lopes, 2008). We understand that there may be some variation in the number of compounds due to region and seasonality, however the presence of the main compound classes (such as Chlorogenic Acids, Sesquiterpene Lactones, and Di-C-glucosylflavones) was maintained.

The literature describes the toxic characteristics of chemical active principles (substances) evaluation through determination of oral toxicity using repeated doses (subacute test) after obtaining information about the toxicity by acute toxicity test (Atchou et al., 2021; Auti and Kulkarni, 2019; Bakrania et al., 2017; Betti et al., 2012; Figueredo et al., 2018). The present study provides information about the possible health hazards that can arise from repeated exposure for a relatively limited period of time. The study can also provide data on substances which may affect the male and/or female reproductive organs of young adult animals and may give an indication of immunological effects. The present data from the repeated dose provides important first information regarding hematological (total WBC increase), biochemical (increase in serum level of alkaline phosphatase) and behavioral changes (decrease of muscle traction force from day 14 onwards in females) which were not evidenced in the single dose test (acute toxicity test). In addition, the data can support dose selection in future chronic toxicity tests. A chronic toxicity test is the next step using the lower and safe doses obtained in the present study; however, it would be another goal for future studies.

Conclusion

The present toxicity study revealed that oral acute lethal dose of the L. *ericoides* ethanolic extract is greater than 2000 mg/kg. However, single doses should be used with caution, since single doses of 50, 300 and 2000 mg/kg resulted in histological changes in the spleen, kidney, lung and liver of mice.

The subacute toxicity study revealed that administration of 50, 300 and 500 mg/kg doses for 28 days of EELE results in biochemical, hematological, and histological changes in the liver, kidney, spleen, thymus, lung, and heart of mice. The results indicate that the extract is not highly safe when used orally on successive days, at the doses

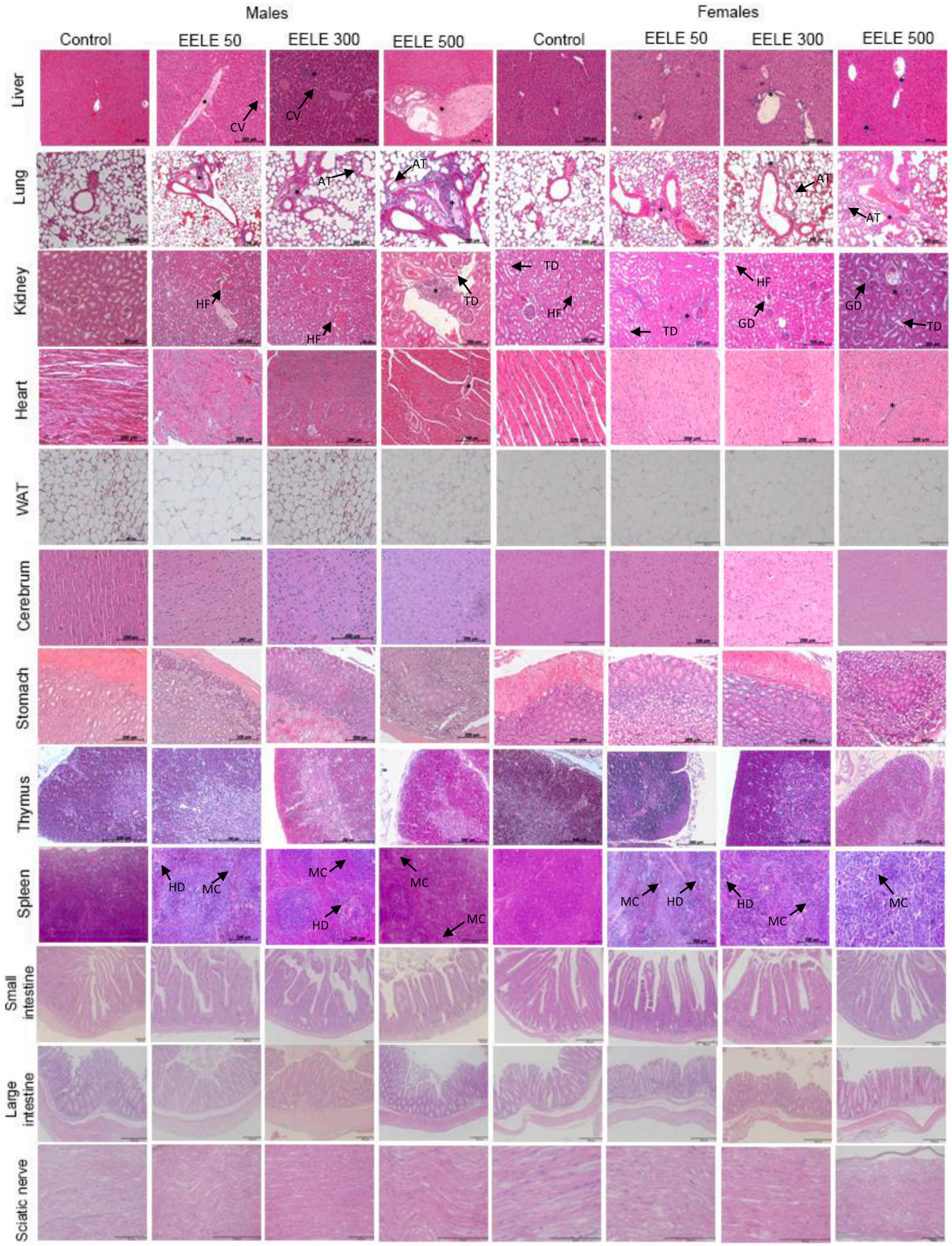

Fig. 6. Photomicrographs of Swiss mice organs sections submitted to the toxicity evaluation of the crude ethanolic extract of Lychnophora ericoides, administered orally at three different doses (50; 300 or 500 mg/kg of body weight), in accordance to gender, after 28 days of treatment. Size bar for comparison 200 μm. H& E (20 x). (EELE) Ethanolic Extract of Lychnophora ericoides, (WAT) White Adipose Tissue. * Inflamatory foci; TD: Tubular dilation; GD: Glomerular degeneration; AT: Alveolar Thickening; CV: Cytoplasmic vacuolization; HD: Hemosiderin deposition; MC: Multinuclear cells.

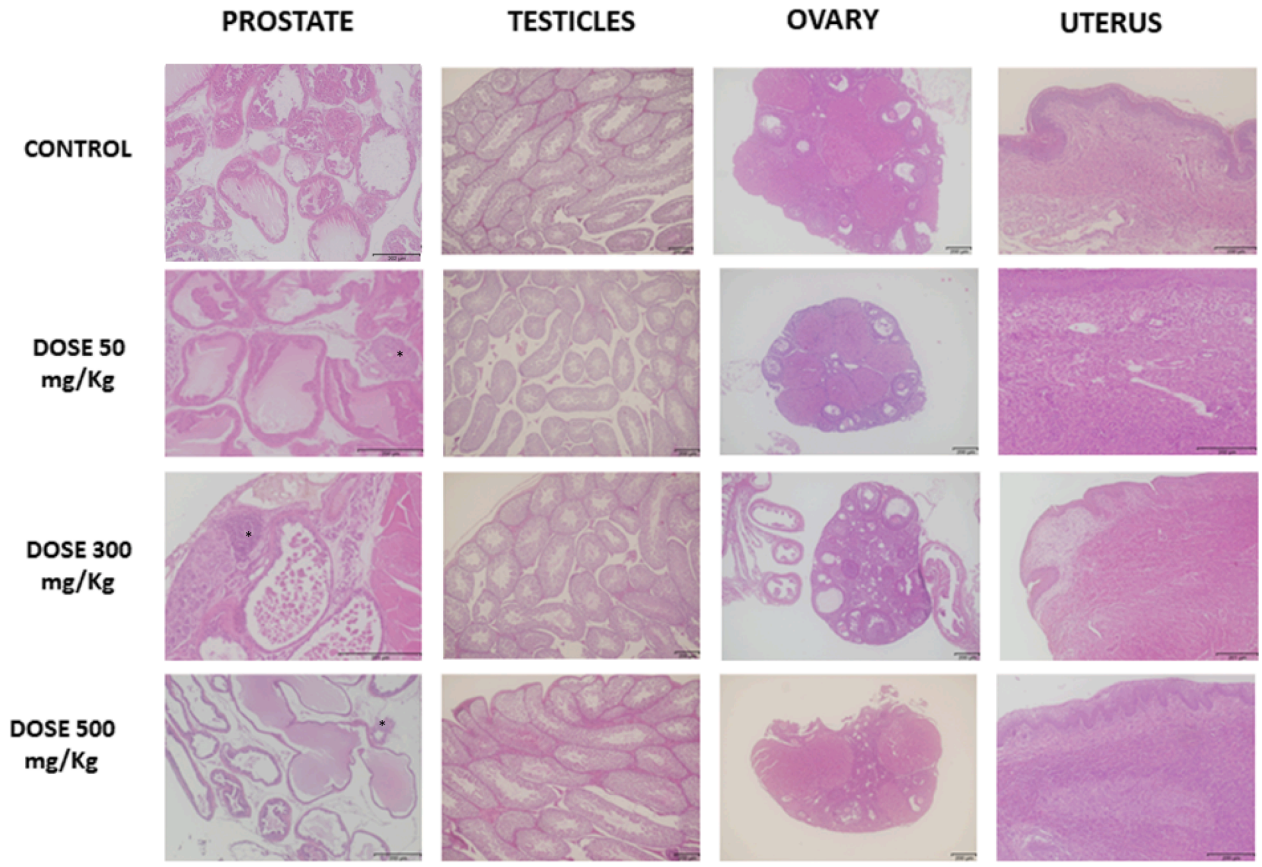

Fig. 7. Histopathological studies of testicles, prostate, ovary and uterus in Swiss mice treated with ethanolic extract Lychniphora ericoides in doses 50, 300 and 500 mg/Kg. Size bar for comparison 200 μm. H& E (20 x). * Inflammatory infiltrate.

evaluated. The results also suggest a possible sedative extract effect in the first four hours after administration.

Considering the fact that L. *ericoides* is already used in Brazilian folk medicine as an analgesic and anti-inflammatory plant, the present results provide valuable primary data on the *Lychnophora ericoides* toxicity profile which may guide future studies. However, further studies are needed to fully determine the plant's toxic profile and should include assessments of genotoxicity, mutagenicity and carcinogenicity. In addition, an evaluation of the toxic effects reversion after discontinuing the extract use should be performed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval

All applicable institutional and/or national guidelines for the care and use of animals were followed.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2021.100154.

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