Preclinical studies of hydroalcoholic extract of Calea uniflora Less
Estudios preclínicos de extracto hidroalcohólico de Calea uniflora Less

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Abstract. Calea uniflora Less known popularly as Arnica in Brazil, is a native plant from Brazil, popularly used by coastal populations from south of Santa Catarina. The purpose of this study was to verify the safety profile in of hydroalcoholic extract of C. uniflora in florescences. The hydroalcoholic extract of C. uniflora in florescences was evaluated for its acute and sub-acute toxicity. Acute topical toxicity was performed using the methodology of guideline 402 from OECD. Acute oral toxicity was performed using the methodology of guideline 423 from OECD and sub-acute toxicity was performed using the methodology adapted of guideline 407 from OECD. The single dose for oral or topical administration of C. uniflora showed DL50> 5000 mg/kg b.w. The sub-acute treatment induced animal death in groups, which was administered extract in the doses 100, 250, 500 and 1000 mg/kg. The main signs of toxicity observed were respiratory difficulty, increase in lung weigh, lung damage and muscular relation. The topical or oral administration of C. uniflora extract in short period did not caused toxicological effects in animals, however, when administered for a longer period and in concentrations of 250, 500 and 1000 mg/kg (oral.) caused lung damage and even the death of the animal.

Keywords: Arnica; Inflorescences; Medicinal plant; Calea uniflora

Introduction
The Astereceae family presents some plants that have a toxic effect such as Solidago microglossa (Neto et al. 2004) and Lychnophoratricho carpha (Ferrari et al. 2012). In this family we found the genus Calea that occurs in both, tropical and subtropical regions of the world, and contains about 110 species (Pruski and Urbatsch 1988, Amaral et al. 2017). Plants of Calea genus are rich in components such as lactones, sesquiterpenes (Ober et al. 1987) and derivatives of p-hydroxyacetophenone (Bohlmann et al. 1981). Besides, some species of Calea have scientific studies such as C. Hymenolepis (Bohlmann et al. 1982), C. pruivifolia (Castro et al. 1989) and C. leptocephala (Ober et al. 1986) about terpene identification; as also biological actitivity of C. urticifolia (Torres-Rodríguez et al. 2016) and C. clematidea (Ferraz et al. 2009). Extract of aerial parts C. clematidea did not induce DNA damage in brain tissue from treated animals in doses 100 and 150 mg/kg/ip (in vivo), assessed by comet assay (Ferraz et al. 2009). Extract from the leaves of C. urticifolia has anti-inflammatory and antioxidant activity in vitro test, RAW 264.7 macrophages stimulated with lipopolysaccharide (Torres-Rodríguez et al. 2016).
In the southern Brazil, *Calea uniflora* L. is known as a medicinal plant, popularly called Arnica (Hanazakiet et al. 2012). *C. uniflora* is heliophyta and develops in a subtropical climate. This species is native to Argentina, Brazil, Paraguay and Uruguay. In Brazil, is found in the center-south in Mato Grosso do Sul, Minas Gerais, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul (Flora do Brasil 2014). In particular, the plant is well distributed in the south of Santa Catarina in the coastal area in well-drained soil regions, where usually is collected by the local population and neighboring municipalities. *C. uniflora* is morphologically similar to *Arnica montana*, European plant, used popularly as anti-inflammatory. In the years 1820-1830 this plant appeared in various European pharmacopoeias. There are records of therapeutic use of *A. montana* (Asteraceae) in Portugal, as in the Island of the Azores and Madeira (Rivera et al. 2010; Obónet et al. 2012). The colonization of the Brazilian South by Europeans may have provided this comparison of species and thus the popular name of *C. uniflora* have emerged, as well as its mode of preparation and therapeutic use. According whit Ramos et al. (2016), 65.4% of the people who use *C. uniflora* have known the plant since childhood and 84.6% have obtained knowledge about the plant through their relatives. Some studies with *C. uniflora* have presented significant results as leishmanicidal (Do Nascimento et al. 2007), antifungal, trypansomocidal activity (Do Nascimento and De Oliveira 2004), antinociceptive activity (Rodrigues-Torres et al. 2016) and anti-inflammatory activity (Da Rosa et al. 2017). Phytochemical studies with *C. uniflora* identified many compounds for example, 2,2-dimethyl-6-(1-hydroxyethyl)-chroman-4-one, uniflorol-A, uniflorol-B (Do Nascimento et al. 2007), 2-senecioyl-4-(hydroxyethyl)-phenol, 2-senecioyl-4-(angeloyloxyethyl)-phenol, 2-senecioyl-4-(methoxyethyl)-phenol and 2-senecioyl-4-(pentadecanoyloxyethyl)-phenol (Do Nascimento et al. 2002).

According to local knowledge, this plant is widely used for wound healing, muscle pain, bruises/hematomas, flu/cold and insect bites. The part of the plant most used is the inflorescences prepared mainly by maceration in hydroalcoholic solution, used topically and orally (Ramos et al. 2016). Some biological activities of *C. uniflora* are already known, but little is known about their toxicological effects. Knowing the toxicity of several plants of the family Asteraceae, the purpose of this study was to verify the safety profile of the hydroalcoholic extract of *C. uniflora* inflorescences through the acute and sub-acute extract administration in rodents.

**Materials and methods**

**Plant material and extraction**

Inflorescences of *C. uniflora* were collected in January/February 2017 in BalneariRincão (Santa Catarina), located in the southern Brazil (28°48'20.0"S and 49°14'45.3"W). The plant was identified and authenticated by Dr. Vanilde Citadini-Zanette and Dr. Mara Rejane Ritter, and a voucher specimen (dry plant species fixed on white paper with its botanical data, stored in a closed cabinet at temperature 18-23 °C with humidity 40-55 %) was deposited in the Herbarium of Dr. Raúlino Reitz (CRI) of the University of the Extremo Sul Catarinense (UNESC-SC), Brazil, CRI 10304. The material plant was dried in a drying oven at approximately 50–60 °C. Briefly, material plant was extracted with ethanol (70%) during fifteen days with occasional stirring, followed by the filtration and evaporated to dryness under reduced pressure. The inflorescences extract of *C. uniflora* (ECU) was kept at 4–8 °C, dissolved in distilled water/corn oil/tween 80 prior to administration oral and dissolved in acetone prior to administration topical.

Total flavonoid content was estimated by a colorimetric method using aluminium chloride. ECU (2 mg/ml) was mixed with 1.5 mL methanol, 0.1 mL of potassium acetate 1M and 2.8 mL of water, mixed and allowed to stand for 3 min, and 0.1 mL 10% aluminium chloride solution was added. The absorbance at 415 nm was measured after 30 min, in triplicate. Quercetin was used as standard to construct a calibration curve (12.5–200 μg/mL). Total flavonoid content was expressed µg/ml quercetin equivalents (Chang et al. 2002).

**Animals**

Young adult male and female *Wistar* rats (a total of 118 animals, 50 male and 68 female, 8 to 10 weeks old, weight 200-300 g) were supplied by vivarium of the University of the Extremo Sul Catarinense (UNESCO). Animals were segregated according to the genus and housed in plastic cages with access to food and water ad libitum, under a 12 h light/dark cycle at a constant temperature of 21 ± 2 °C. Animals were handled and experiments carried out in conformity with the European Union on Animal...
Care (CEE 86/609). The experimental protocol was approved by the local ethics committee (CEUA UNESCO), registered with protocol No. 050/2014. The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions. In the acute topical test, a total of 6 female animals were used, 3 in the control group and 3 in the 2000 mg/kg ECU group. In the acute oral test, 12 female animals were used, 6 in the control group and 6 in the 2000 mg/kg ECU group. The doses for the acute tests were established by the diagram of guides 402 and 423 (OECD) starting at the 2000 mg/kg. In the sub-acute 100 rats were allocated in five groups (1 control group and 4 ECU groups) of 20 animals each (10 males and 10 female). Rats received ECU in doses 100, 250, 500 or 1000 mg/kg (oral) by gavage. Doses for sub-acute tests were established by the recommendations of the guide 407 (OECD) and based on the doses used in the antinociceptive test of the inflorescence extract of C. uniflora (Rodrigues-Torres et al. 2016).

Acute topical toxicity
The analysis was performed using the methodology of guideline 402 from Organization for Economic Co-operation and Development (OECD 2017), starting dose of 2000 mg/kg of C. uniflora extract applied topically in female rats, exposed area of dorsal/flank skin (10% of the total body surface area). For this, it was necessary to removed all fur, 24 hours before the application of the ECU or vehicle. The test started with 2 animals (1 animal from control group and 1 animal from ECU group), after the test according to the diagram of guide 402 with administration in 4 other animals (2 animals from control group and 2 animals from ECU group), the dose depended on the signs of toxicity. After application, the treatment was in contact with the skin for 24 hours, the animals were observed for 24 hours after application and for 14 days were observed daily for 1 hour. The same test was performed using only extract diluents (acetone). Acetone was chosen as the diluent because it is a very volatile solvent, with only the extract remaining in the application area. The skin was removed and fixed in 10% formalin, embedded in paraffin. Then the skin was sectioned and stained with hematoxylin and eosin for microscopic examination (Lameb 2017).

Acute oral toxicity
The analysis was performed using the methodology of guideline 423 from Organization for Economic Co-operation and Development (OECD 2001), starting dose of 2000 mg/kg (oral) of C. uniflora extract in female rats, administered in single dose in bolus by gavage. The same test was performed using only extract diluents (water/corn oil/tween 80). The test started with 6 animals (3 animals from control group and 3 animals from ECU group), after the test according to the diagram of guide 423 with administration in 6 other animals (3 animals from control group and 3 animals from ECU group), the dose depended on the signs of toxicity. After administration of treatment, the animals were observed for 24 hours, and for 14 days were observed daily for 1 hour.

Sub-acute oral toxicity
The analysis was performed using the methodology adapted of guideline 407 from Organization for Economic Co-operation and Development (OECD 2008). C. uniflora extract (100, 250, 500 or 1000 mg/kg/oral) was administered daily for 30 days. Control group received the same amounts of the vehicle (water/corn oil/tween 80). Food and water consumption were measured daily, and the body weight was measured every five days. During the experimental period, all animals were evaluated in order to observe any signs of toxicity daily. Behavioral analysis was performed on the 15th day and 30th day of treatment. The behavioral analyses were performed according to the tests: open-field test (Vianna et al. 2000); elevated plus-maze (Pellow et al. 1985); forced swimming (Detke et al. 1995) and rota-rod (Paul et al. 1994). At the end of treatment period, blood samples were collected for hematological and biochemical parameters analysis. The heart, liver, kidney and lung were dissected and weighed. The results of organs weight were expressed as relative corporal weight (organ weight g/100g body weight). One part of the blood sample was collected by cardiac puncture in heparin tubes for hematological analysis and the other in dry tubes for separation of serum to biochemical analysis. Blood samples in dry tubes were centrifuged at 3500 rpm (15 min) and the serum was collected and introduced into new tubes. Triglycerides, total cholesterol (TC), alanine transaminase (ALT), aspartate transaminase (AST), glucose, uric acid, alkaline phosphatase, urea, creatinine and total proteins were measured according to
commercial kits obtained from Analisa® (Minas Gerais, Brazil) by spectrophotometry (Femto 700). Red blood cells (RBC), white blood cells (WBC) and platelet were analyzed in the Neubauer chamber.

After animals sacrificed by deep anesthesia (Ketamine 80 mg/kg and xylazine 20 mg/kg), and then the heart, liver, lung and kidney were removed and fixed in 10% formalin, embedded in paraffin. The organs and tissues were then sectioned and stained with hematoxylin and eosin for microscopic examination.

**Statistical analysis**
The results are expressed as mean ± standard error of the mean (SEM) or median with range. In parametric analyses, comparison between groups was assessed by one-way analysis of variance (ANOVA) followed by Dunnett. The \( *p < 0.05 \) or \( **p < 0.01 \) was considered statistically significant.

**Results**
The total flavonoid contents of the samples were determined and expressed in terms of quercetin (standard curve equation: \( y = 0.009x + 0.100, R^2 = 0.994 \)), content in the ECU 92.763 µg/ml quercetin equivalents.

The toxicological tests started with application of 2000 mg/kg/topical of ECU in one animal and then the test was repeated with more two animals. The single administration of ECU caused no mortality. The control group and ECU group did not present signs of toxicity. The results show that the ECU has category 5 in GHS (Globally Harmonised Classification System for Chemical Substances and Mixtures) classification or LD\(_{50}\) > 5000 mg/kg/b.w/ topical. Histological analyzes did not show any morphological alteration of skin.

The oral test started with administration acute of 2000 mg/kg/ oral of ECU in three animals and then the test was repeated with more three animals. The single administration caused no mortality. However, one hour after administration of ECU was observed piloerection and agitation signals. The control group did not present signs of toxicity. Toxicity signs were registered in animal throughout the observation period (14 days). The results show that the ECU has LD\(_{50}\) > 5000 mg/kg/b.w or category 5 in GHS classification.

In sub-acute evaluation, the animals present toxicity signs in all groups that received ECU (100, 250, 500 or 1000 mg/kg/oral) as: piloerection, cough signals, respiratory difficulty, nose bleeding, suggestive behavior pain, diarrhea and sedation. These behaviors signs were observed in all groups treated with ECU, however the dose of 100 mg/kg the signs were less frequent. In the control group these effects were not observed. However, 30 deaths have occurred during sub-chronic treatment (Figure 1), therefore male group of 1000 mg/kg was not analyzed in tests: relative weight of organs, biochemical parameters, hematological parameters and behavioral test (open-field, elevated plus-maze, forced swimming and rota-rod).

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**Figure 1.** The effect of ECU on rat’s survival in male (A) and female (B). Animal were treated during 30 days with ECU (100 – 1000 mg/kg/day, oral). Control group receives the same amount of vehicle Results were expressed as per cent survival and are representative of one experiment (n = 10 per group). The 100 mg/kg group line overlaps the control group.
Table 1. Effect of the sub-acute oral administration of different dose ECU on relative weight of organs (g/100g body weight) in male Wistar rats.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>ECU mg/kg</th>
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<tbody>
<tr>
<td></td>
<td>100</td>
<td>250</td>
</tr>
<tr>
<td>Liver</td>
<td>3.512±0.093</td>
<td>3.508±0.135</td>
</tr>
<tr>
<td>Heart</td>
<td>0.428±0.022</td>
<td>0.420±0.017</td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.458±0.013</td>
<td>0.439±0.020</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.461±0.009</td>
<td>0.473±0.021</td>
</tr>
<tr>
<td>Lung</td>
<td>0.664±0.055</td>
<td>0.916±0.049*</td>
</tr>
</tbody>
</table>

Data are expressed as mean weight of organs ±SEM of 6 to 10 animals per group. The animals of 1000 mg/kg group were not present in this table due to high mortality. The statistical analyze was performed by one-way analysis of variance (ANOVA) followed by Dunnett’s test. *Significance against control group: p ≤ 0.05. **Significance against control group: p ≤ 0.01.

Figure 2. Representative microphotograph of the lung at 100x, embedded in paraffin and stained with hematoxylin and eosin. (A) control. (B) 100 mg/kg/day p.o of ECU. (C) 250 mg/kg/day p.o of ECU. (D) 500 mg/kg/day p.o of ECU. (E) 1000 mg/kg/day p.o of ECU.
The relative organ’s weight (g/100g body weight) of male and female rats treated with ECU for 30 days are shown in table 01 and 02. In this analysis was possible to observe an increase of lung organ in groups with ECU (100 and 1000 mg/kg/oral). Histological analyzes showed lung damage characterized by neutrophil infiltration (Figure 2) in dose 100, 250 500, 1000 mg/kg/oral, which may be related to increase lungs weight. The other organs did not show any morphological alteration in the analyzed tissues. Some behavioral changes observed in the oral acute test were noted in oral sub-acute test, however with more frequency. Other possible signs of toxicity also emerged with effect suggestive of pain and respiratory difficulty; this behavior was observed in all groups treated with ECU. Besides, during the sub-acute experiment 30 deaths occurs in groups treated at the doses of 250, 500 and 1000 mg/kg/day (in both, male and female).

The female group shows a decrease in body weight at the dose 1000 mg/kg. However, there was reduction in consumption diet also. Water and food consumption and body weight evolution are summarized in Figure 3. When the animals were treated with ECU, it was observed skeletal muscle relation in rota-rod test at doses 100 and 250 mg/kg/oral in 15 days treatments and 250 mg/kg/oral in 30 days, showed Figure 4. In the elevated plus-maze test were analyzed the parameters: open arm entries, closed arm entries, open arm entries attempts, closed arm entries attempts, frequency of defecation, time open arm and time closed arm; however no significant difference was observed between the control and treatment groups in any of the parameters. In the forced swimming, all groups’ animals swam the time required. In open field test, the parameters: crossing number, rearing number, frequency of defecation, stationary time, center of the field time and edge of field time were analyzed, however no significant difference was observed between the control and treatment groups. Biochemical analysis as well most parameters remained unchanged, as only non-significant variations were observed. In hematological parameters, no significant difference occurs between the control and treated groups with ECU.

**Discussion**

*C. uniflora*, a medicinal plant used in southern Brazil (Ramos et al. 2016), has few studies about the toxicity *in vivo*. However, report the potential therapeutic effect of *C. uniflora* extracts as antifungal, trypanocidal and lechimanicidal (Do Nascimento et al. 2004, Do Nascimento et al. 2007). Test *in vitro* with extract hydroalcoholic from *C. uniflora* did not show a high degree of cytotoxicity compared to controls doxorubicin and vincristine for B16-F1 and HaCaT cell lines, but dichloromethane fraction has significant inhibition in the two cell cells compared to controls (Rodrigues-Torres et al. 2016). Known the biological effect of *C. uniflora*, this study evaluated the acute and sub-acute toxicological profile of the ECU in rats by oral and topical administration *in vivo*.

<table>
<thead>
<tr>
<th>Control</th>
<th>ECU mg/kg</th>
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<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Liver</td>
<td>3.622±0.226</td>
</tr>
<tr>
<td>Heart</td>
<td>0.511±0.021</td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.504±0.034</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.519±0.030</td>
</tr>
<tr>
<td>Lung</td>
<td>0.744±0.043</td>
</tr>
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</table>

Data are expressed as mean weight of organs ±SEM of 4 to 10 animals per group. The statistical analyze was performed by one-way analysis of variance (ANOVA) followed by Dunnett’s test. *Significance against control group: p ≤ 0.05. **Significance against control group: p ≤ 0.01. ***Significance against control group: p ≤ 0.001.
Figure 3. In panel A and B were presented body weight evaluation of males (A) and female (B) rats. In Panel C and D are represented mean food consumption daily of males (C) and female (D). In panel E and F was shown mean daily water consumption of males (E) and female (F). Wistar rats were submitted to 30 days treatments with ECU (100 – 1000 mg/kg/day, oral.). Each point represents the mean ± SEM of 2 to 10 animals per group. The statistical analyze was performed by one-way analysis of variance (ANOVA) followed by Dunnett’s test. **Significance against control group: p ≤ 0.01; ***Significance against control group: p ≤ 0.001.
Figure 4. Effect of ECU in motor coordination evaluated in Hota-test in male (A and C) and female (B and D) rats that received ECU (100 – 1000 mg/kg/day, oral.) about 15 days (A and B) and 30 days treatment (C and D). Data are expressed as mean ±SEM of 4 to 10 animals per group. The statistical analyzes was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test. *Significance against control group: p ≤ 0.05.

In the topical and oral acute test were observed toxicity of the ECU at 2000 mg/kg, since the extract did not induce mortality in all experimental period. This result contributes with previous studies of *C. uniflora* and *C. clematidea*, which demonstrate that the methanol extract at the doses of 100 and 150 mg/kg/ i.p, did not caused anxiolytic, muscular relaxing and genotoxic effects (Ferraz et al. 2009). Some behavioral alterations were observed in first hour after administration of the ECU in acute test, such as piloerection and agitation signals. These behavioral effects were not observed in the control group. Although the presence of this signs of toxicity, these were not observed with high frequency and extract did not induce mortality of animals. The extract (ECU) content 92.763 µg/ml quercetin equivalents, flavonoids as orobol and quercetin 3-O-glucopyranosyl were identified in the extract from *C. uniflora* leaves, and phenolic compounds as noreugenin, ethyl caffeate, butein, p-hydroxy-butein, caffeic acid, butein 40-O-glucopyranosyl and 3,5-di-O-caffeoylquinic acid (Lima et al. 2016).

*C. uniflora* have identified substances derived of p-hydroxyacetophenone with the chromanones Uniflorol A and Uniflorol B (Do Nascimento et al. 2007). This is important since some chromenes
have the ability to inhibit determined enzymes such as: acetylcholinesterase (Anand and Singh 2013), cyclin-dependent kinase (Lee et al. 2007) e alkaline phosphatase (Al-Rashida et al. 2013). The inhibition of these enzymes may be a toxicity mechanism of some plants of this family. In accordance to that, some studies evince that Asteraceae plants inhibit the enzyme acetylcholinesterase (Gurovic et al. 2010). Study with Calea serrate proved that extract rich in chromenes obtained from leaves and stalk inhibited of acetylcholinesterase enzyme of brain structures (frontal cortex, striatum and hippocampus) in Wistar rats (Ribeiro et al. 2012); two chromenes were isolated from this plant, being precocene and eupaloriochromene (Steinbeck et al. 1997). Other plants of family Asteraceae such as Inula graveolens, Artemisia dracunculus, Eupatorium odoratum and Arnica chamissonis also present inhibitory activity of the acetylcholinesterase enzyme (Bhadra et al. 2015). The skeletal muscle relation showed Figure 4 can reflect an over stimulation of cholinergic system. When acetyl cholinesterase is inhibited at the neuromuscular junction (skeletal muscle), occurs an increased acetylcholine concentration at the synaptic cleft, and the frequency of stimuli on nicotinic receptors occurs. This prolonging stimuli cause a blocking depolarization at neuromuscular transmission (Hibbs and Zambon 2012).

During the sub-acute experiment 30 deaths occurs in groups treated at the doses of 250, 500 and 1000 mg/kg/day, the most relevant behavior observed before death was respiratory difficulty, suggesting a possible extract action in the respiratory systems or in the nervous central system. Another result that collaborates with this hypothesis is the lungs weight that had a significant increase in some groups treated with ECU compared to the control group and lung damage characterized by neutrophil infiltration in dose 100, 250, 500, 1000 mg/kg/oral.

Conclusion
Experimental results show that the ECU administered in topical and oral acute treatment has no toxic effects, with LD50 > 5000 mg/kg (b.w.). However, prolonged use oral of the extract at doses 250, 500 and 1000 mg/kg/oral (b.w) is toxic and cause death of the animal. The signs of toxicity observed were respiratory difficulty, increase in lung weigh, lung damage and muscular relation of ECU group of 100, 250, 500, 1000 mg/kg/oral. This plant is popularly used topically and orally, and the results of this study show important information about the safety of C. uniflora use. Oral or topical (single dose) acute use did not cause toxicity, however, repeated oral use caused low dose toxicity (100 mg / kg).

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