

Analyses of chemical composition and gastroprotective and antinociceptive properties of *Eugenia involucrate* DC. leaves

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ABSTRACT

Eugenia genus has shown relevant therapeutic results as a medicinal plant, and that is why this study aimed to know the chemical composition of *E. involucrata* leaves and its gastroprotective and antinociceptive potential. The leaves were collected at two moments, January and July of 2014. Leaves were macerated in methanol, resulting in Crude Methanol Extracts (CME), denominated CME-jan and CME-jul. The extracts were partitioned with chloroform and ethyl acetate. Phytochemical analysis was performed by conventional chromatographic techniques and the isolated substances were analyzed by usual spectrometric techniques. The major substance isolated from the ethyl acetate fraction was (-)-catechin and phytol from the chloroform fraction. The gastroprotective evaluation was performed by the ulcer model induced by ethanol, and the extract reduced significantly the total damaged area and the area of injury relative to the treatments with doses of 125 and 250 mg/kg, but the 125 mg/kg dose was more effective. The antinociceptive activity was evaluated by the writhing test induced by acetic acid. Extract and fractions reduced the nociception (26.5 to 64.4% at 10 mg/kg). Chloroform fraction collected in July exhibited more pronounced effect (DI_{50} of 2.33 mg/kg).

INTRODUCTION

Since many years ago, plants have been employed with success to cure the more distinct ailments. Currently, modern medicinal chemistry is using new approaches and technologies that confirm the scientific evidence on the therapeutic efficacy of various medicinal plants, their isolated substances and derivatives (Filho, 2015; Alves, 2013; Cragg and Newman, 2013).

The genus *Eugenia* comprises several species that possesses relevant therapeutic properties. *Eugenia involucrata*, popularly known as cerejeira, cereja-do-Rio Grande or araçazeiro, is commonly found in the south of Brazil, is used in traditional medicine as infusions to treat infections, diarrhea, indigestion, and bleeding. However, it was not

found previous studies regarding the chemical and biological aspects of this plant (Carvalho, 2010; Lorenzi, 2002; Sausen *et al.*, 2009).

The present study aimed to investigate the chemical composition of *E. involucrata* leaves and the gastroprotective and antinociceptive potential of its methanolic extract and ethyl acetate and chloroform fractions, as well to compare two different collects (summer and winter).

MATERIAL AND METHODS

Plant material

The leaves of *E. involucrata* were collected in two different moments, January and July of 2014, in the city of Itajaí-SC. The plant material was identified by Prof. Oscar Iza (UNIVALI) and a voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí) under n°55283.

Phytochemical analysis

Fresh leaves of *E. involucrata* were macerated at room temperature in methanol for 7 days. After, the extracts were

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concentrated by using a rotatory evaporator to obtain the crude methanolic extract of January (CME-Jan, yield = 5.63%) and July (CME-Jul, yield = 3.75%).

The extracts were partitioned with chloroform and ethyl acetate furnishing the respective fractions (CLF-Jan and CLF-Jul; EAF-Jan, EAF-Jul,) and submitted to conventional chromatography methods such as chromatography column (CC) and thin-layer chromatography (TLC). TLC was used to evaluate the chemical composition of studied plants. The following solvent systems were used: hexane: ethyl acetate (7:3) and chloroform: methanol (8:2) and revealed with the following specific reagents: sulfuric anisaldehyde for steroids, terpenoids and glycosides compounds and ferric chloride for phenolic compounds.

Part of the CLF-Jan (2.5 g) was subjected to CC over silica-gel (40 g) eluted with hexane: acetone gradient. Fractions of 10 mL were collected and evaluated by TLC, visualizing the spots by UV (254 nm) and reaction with sulfuric anisaldehyde heated at 100°C. Subfraction 29-40 (280 mg) was re-chromatographed as before, yielding new 36 subfractions, which were combined according to their TLC profiles furnishing 82 mg of a nonpure compound, which was re-chromatographed again with CC flash over silica-gel (12 g) eluted with hexane: acetone (8:2). It was obtained 4 mg of a pure oil, identified as phytol, by using mass spectrometry and comparison with literature data (Souza *et al.*, 2012).

Part of the EAF-Jan (4.8 g), was subjected to CC over silica-gel (80 g) and eluted with chloroform: methanol gradient. Fractions of 10 mL were collected and evaluated by TLC, visualizing the spots by UV (254 nm) and, ferric chloride 3%. Subfraction 25-34 (150 mg) was re-chromatographed as before, given 72 mg of a pure solid, identified as (-)-catechin by direct comparison with an authentic sample and spectral data (NMR) reported previously (Ayes *et al.*, 2009).

High-performance liquid chromatography (HPLC)

HPLC analysis was realized to compare the seasonal variation, CME-Jan (summer) and CME-Jul (winter), in relation to the (-)-catechin and to define (fingerprint) the compounds present in the extracts.

A Shimadzu LC-20AT LC system (Shimadzu, Tokyo, Japan) was used, consisting of an SPD-M20A photodiode array detector, an SIL-20AHT autosampler and a software LC-Solution (Shimadzu, Tokyo, Japan). The samples were diluted in methanol at 1 mg/ml. The injections of samples (20 µL) were carried out on a C18 column (Luna Phenomenex, 250 × 4.5 mm²; 0.5 µm film thickness and 100 Å), conditioned at 35°C. The mobile phase consisted of acetonitrile and ultra-pure water (pH 2.5, phosphoric acid) eluted in a gradient system (Table 1), monitored at 280 nm.

Pharmacological assays

Acute gastric lesions induced by ethanol/HCl in mice

In this study, were used 30 female Swiss mice (25–50 g), provided by the Central Animal House of Universidade do Vale do Itajaí (UNIVALI - Itajaí, SC, Brazil), housed at room temperature of 22° ± 2°C with 12 h dark/ light cycle and received water and food *ad libitum*.

Table 1: Gradient system used in HPLC analyses at 280 nm.

Time	Acetonitrile (%)	Water* (%)
0	15	85
25	35	65
30	80	20
32	15	85
35	15	85

*pH 2.5, phosphoric acid.

The animals were housed and cared for in accordance with the Federal Government legislation on animal care. The experiment was authorized by the Ethical Committee for Animal Care of the Universidade do Vale do Itajaí (process number 019/13).

The experiment was performed according to the previously described method (Mizui and Doteuchi, 1983), with some modifications. After 12 h of fasting, the animals were randomly divided into different groups of six animals each and pre-treated orally with cimetidine (positive control – 100 mg/kg), vehicle (negative control – distilled water) and the CME-Jul at doses of 50, 125 e 250 mg/kg. All the treatments were administered by gavage.

One hour after treatment, all the animals received 0.1 mL/10 g (body weight) of a 0.3 mol/L HCl/60% ethanol solution (ethanol/HCl) to induce a gastric ulcer. Another hour later, the animals were sacrificed by cervical dislocation, and the stomachs removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots, for subsequent scanning. The images obtained were analyzed using specific “EARP” software to measure each lesion point. The results were expressed as total lesion area (mm²) and relative lesion area (%).

Acetic acid-induced writhing

Abdominal constriction was induced by intraperitoneal injection of acetic acid (0.6%), according to the procedure described previously (Collier *et al.*, 1968) with minor modifications. Male Swiss mice (25-35 g) were pre-treated with CME-Jul and EAF-Jul (10 mg/kg) and CLF-Jul (10, 3 and 1 mg/kg), intraperitoneally (i.p.), 30 min before acetic acid injection (six to eight animals in each group). The control animals received a similar volume of 0.9% NaCl (10 ml/kg, i.p.).

All the experiments were carried out at 23 ± 2°C. After the challenge, pairs of mice were placed in separate glass funnels and the number of contractions of the abdominal muscles, together with stretching, were counted cumulatively over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal contractions between the control animals and the mice pretreated with the test materials.

Statistics

The data are reported as mean ± standard error of the mean (SEM) and compared using one-way analysis of variance (ANOVA) followed by Dunnett’s pairwise test. Values of p < 0,05 or less were considered significant.

RESULT AND DISCUSSION

Preliminary studies by using TLC revealed with specific reactive including sulfuric anisaldehyde and ferric chloride, indicated the presence of steroids, terpenes and phenolic compounds as main constituents of this plant.

Fractionation chloroform and ethyl acetate fractions (plant collected in January) by column chromatographic permitted the isolation of phytol, an acyclic diterpene, and a phenolic compound, (-)-catechin (Figure 1). Although both compounds are common in plants, this is the first report from *E. involucrata*.

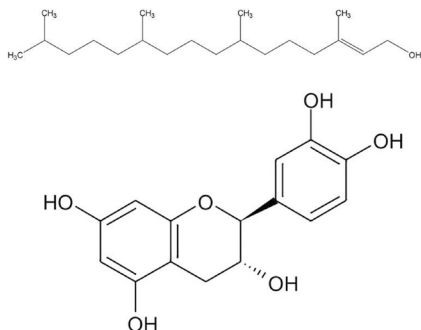


Fig. 1: The molecular structure of Phytol (a) and (-)-Catechin (b).

Considering the importance of the seasonality in the influence of secondary metabolism (Gobbo-Neto and Lopes, 2007), we have examined the chemical constitution of the plant collected in two seasons, summer and winter.

HPLC analysis showed the similarity of substances in the summer and winter extracts, but also important differences, such as the substances that have retention time in ~7, ~10, ~14.5 and ~35 minutes, that are visible in winter extract but doesn't appear in the summer or appear in fewer amounts. The isolated compound (-)-catechin is the major compound in both extracts, but apparently, the winter extract shows higher amounts (Figure 2).

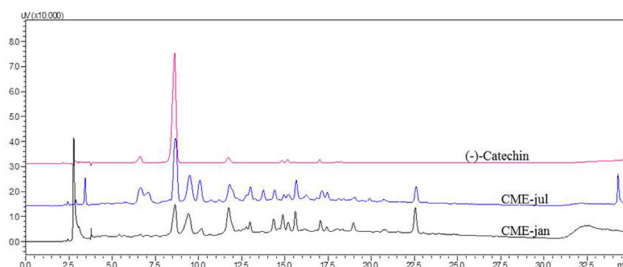


Fig. 2: HPLC analysis of CME-Jan, CME-Jul and (-)-Catechin from the leaves of *E. involucrata*.

The oral administration of CME-Jul extract to the mice significantly reduced the gastric injury caused by administration of the ethanol/HCl solution compared to the control group (vehicle). The doses of 125 and 250 mg/kg reduced significantly the total lesion area (mm²) and relative lesion area (%) when compared to the negative control, while the dose of 50 mg/kg did not show significance. As expected, in the positive control group, treated with cimetidine, it was observed a significant gastroprotective effect (Table 2).

Table 2: Effect of oral administration of cimetidine (100 mg/kg) and different doses of CME-Jul of *E. involucratal* leaves (50, 125 e 250 mg/kg) in ethanol-HCl induced ulcer in mice (n = 6).

Treatment	Dose (mg/kg)	Total lesion area (mm ²)	Relative lesion area (%)
Control	Vehicle	42.86 ± 10.60	10.57 ± 3.61
CME-jul	50	23.76 ± 5.63	6.31 ± 1.96
of	125	4.61 ± 2.03**	1.21 ± 0.56*
<i>E. involucrata</i>	250	6.67 ± 1.81**	2.13 ± 0.54*
Cimetidine	100	1.98 ± 0.44**	0.75 ± 0.17**

Significant difference in relation to the control group (**p < 0,01 and *p < 0,05), ANOVA, expressed as mean ± SEM, a *posteriori* test of Dunnett.

The model of gastric lesion induced by ethanol mimics one of the main causes of the gastric ulcer and gastritis in humans, the excessive use of alcoholic drinks. The administration of ethanol acidified provokes extensive areas of necrosis and hemorrhage in the gastric mucosa, which proves the toxicity of this agent. The ethanol toxicity changes the gastric cell homeostasis and damages the tissue, beyond increasing formation of free radicals and reactive oxygen species (Basting *et al.*, 2014).

Antioxidants have the power to control free radicals, and many secondary metabolites of plants have this property, such as phenolic compounds and terpenoids (Klein-Júnior *et al.*, 2012).

Other *Eugenia* species studied previously showed gastroprotective properties, such as *E. umbelliflora* (Meyre-Silva *et al.*, 2009), *E. punicifolia* (Basting *et al.*, 2014) and *E. jambolana* (Donatini *et al.*, 2009). In general, the activity can be related to the

presence of tannins, flavonoids, and terpenes, which were the main class of substances evidenced in the leaves of *E. involucrata*. In this mode, the present study pointed that, as well as other *Eugenia* species, the *E. involucrata* presents gastroprotective potential.

To determine a possible antinociceptive effect for this plant, the writhing test was selected, which is common in trials, because of its simplicity, reproducibility e low cost, showing good relation to the pre-clinic and clinic studies (Calixto *et al.*, 2000; Campos-Buzzi *et al.*, 2006).

The results of the evaluation of CME-Jul, CLF-Jul, and EAF-Jul at a dose of 10 mg/kg indicated that all the samples tested significantly reduced the nociception, but a greater result was observed for the non-polar fraction, CLF-Jul (64.4%) (Figure 3).

Common drugs used in clinical treatments, such as aspirin and paracetamol, show inhibition around 35% in same conditions

(Costa *et al.*, 2007). After this trial result, it was determined the DI_{50} (minimal dose able to inhibit 50% of writhings compared to the control group) of CLF-Jul (Figure 4).

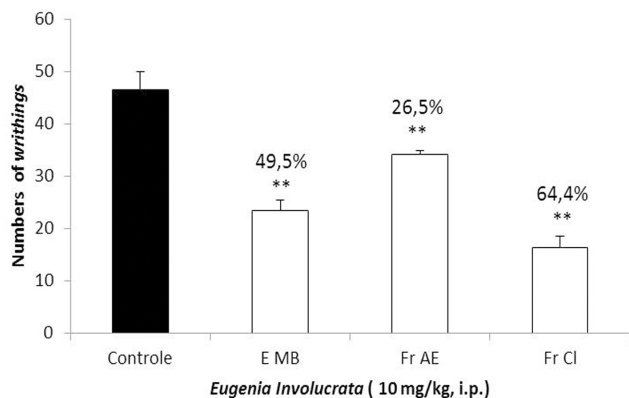


Fig. 3: Inhibition of CME-Jul, CLF-Jul, and EAF-Jul of the leaves of *E. involucreta*, at a dose of 10 mg/kg (via i.p.) in the model of acetic acid-induced writhing in mice (n = 6).

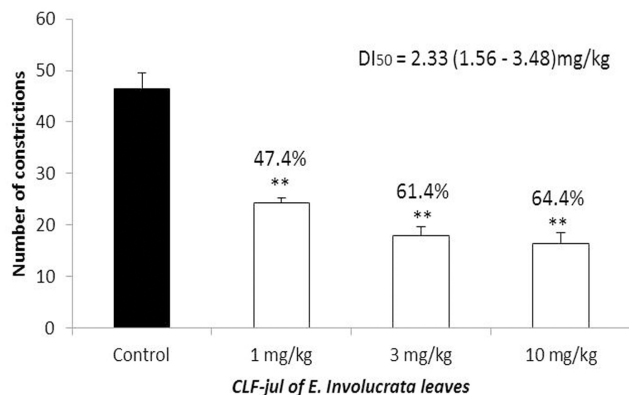


Fig. 4: Inhibition of the chloroform fraction (CLF-Jul) leaves of *E. involucreta*, at doses of 1, 3 and 10 mg/kg (via i.p.) in the model of acetic acid-induced writhing in mice (n = 6).

All the tested doses showed significant reduction of writhings, and the DI_{50} was about 2 mg/kg. Comparing to the same drugs mentioned before, that exhibited a DI_{50} around 25 mg/kg (Serafin *et al.*, 2007), CLF-Jul was about 25-fold more potent than these drugs.

The antinociceptive potential was evidenced previously in leaves of other *Eugenia* species, such as *E. fruticosa* (Shams-Ud-Doha *et al.*, 2012) and *E. punicifolia* (Basting *et al.*, 2014).

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Terpenes present pronounced analgesic potential and they can act in different pathways (Calixto *et al.*, 2000; Guimarães *et al.*, 2013; Niero and Malheiros, 2014). The presence of terpenes in the chloroform fraction of *E. involucreta* leaves can, therefore, to explain, at least in part, its potent antinociceptive action.

CONCLUSION

In conclusion, *E. involucreta* leaves showed gastroprotective and antinociceptive potential, related to the

terpenes and phenolic compounds. However, the studies are in progress to confirm these results in other specific models as well as to isolate the minor constituents of this plant.

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