ACUTE TOXICITY AND ANTIEDEMATOGENIC ACTIVITY FROM STEMS OF *Pisonia aculeata* L. (NYCTAGINACEAE)

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ABSTRACT

Pisonia aculeata L. (Nyctaginaceae) is a shrub widely used in Brazilian folk medicine, where the roots are used as purging and barks and leaves have anti-inflammatory properties. The aim of this study is to evaluate the antiedematogenic activity of crude extract and derived fractions from stems of *P. aculeata* and the acute toxicity of crude methanolic extract. From methanolic extract from stems of *P. aculeata* were obtained steroids, triterpenoids and nitrogenous compounds. All fractions tested show inhibitory activity of mice edema ear induced by croton oil, mainly hexane and chloroform fractions. Crude methanolic extract was also active, inhibiting 27.9% of the swelling occasioned by croton oil. In addition, treatment of mice with the crude extract of the stems of *P. aculeata* in the acute toxicity test did not cause death of animals or change in behavioral parameters, indicating that the extract may be considered non-toxic. *www.relaquim.com*

Keywords: Nyctaginaceae, *Pisonia aculeata,* triterpenes, antiedematogenic activity, acute toxicity.

RESUMEN

Pisonia aculeata L. (Nyctaginaceae) es un arbusto ampliamente utilizado en la medicina popular de Brasil, donde las raíces se utilizan como purgante, mientras que la corteza y hojas por sus propiedades anti-inflamatorias. El objetivo de este estudio es evaluar la actividad antiedematogénica del extracto crudo y las fracciones derivadas de los tallos de *P. aculeata* y la toxicidad aguda del extracto metanólico crudo. Del extracto metanólico obtenido de los tallos de *P. aculeata* se obtuvieron esteroides, triterpenoides y compuestos nitrogenados. Todas las fracciones probadas

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mostraron antiinflamatoria actividad en el edema de oreja inducido por aceite de crotón en ratones, sobre todo las fracciones de hexano y cloroformo. El extracto metanólico crudo también fue activo, inhibiendo el 27,9% de la inflamación ocasionada por el aceite de crotón. Además, el extracto crudo de los tallos de *P. aculeata* en el ensayo de toxicidad aguda no causaron la muerte de los animales o el cambio en su comportamiento, lo que indica que el extracto puede ser considerado como no tóxico. *www.relaquim.com*

Palabras clave: Nyctaginaceae, *Pisonia aculeata*, triterpenos, actividad antiedematogénica, toxicidad aguda.

INTRODUCTION

The use of natural products for the treatment of inflammatory diseases has a long history and medicinal plants and their extracts or isolates compounds are still used all over the world as unique available alternative for this purpose. Various chemical constituents present in plants may have anti-inflammatory activity as triterpenes and phenolic compounds (Ahmadiani *et al.*, 1998). Therefore, chemical study of plants for discovery of new pain killers compounds is still a logical and necessary strategy to the search for new analgesic drugs.

Nyctaginaceae family comprises about 30 genera and 300 species distributed predominantly in tropical and subtropical America. The genus *Pisonia* comprises about 50 species having as representatives trees and small shrubs. *Pisonia aculeata* is a shrub distributed in tropical and subtropical America known in Brazil as "espora-de-galo", "cipó-mole" or "tapaciriba". This specie has a geographic distribution restricted to the states of Rio de Janeiro, São Paulo and Minas Gerais, not reaching scale or size of leaves from those found in other regions of the World (Barroso, 1986). Its roots are used as purging and decoction or infusion of the barks and leaves is used externally or internally for the treatment of rheumatism, inflammation of joints and venereal diseases (Correa, 1984), treating swelling, jaundice and tumors (Nadkarni, 2005; Ghode *et al.*, 2011; Anbarasu *et al.*, 2012).

Preliminary phytochemical screening of the extracts of *P. aculeata* revealed the presence of alkaloids, phenolic compounds, tannins, saponnins and flavonoids (Wagner, 1984). Recently was reported the isolation of flavonoids, isoflavonoids, chromones and alkaloids as well as antitubercular activity against *Mycobacterium tuberculosis* for isolated compounds for this specie (Wu *et al.*, 2011). Furthermore, the leaves of *P. aculeata* have been reported to have antioxidant, hepatoprotective properties (Palinavel *et al.*, 2008) and antitumor activity (Senthilkumar *et al.*, 2008).

Although some investigations of the activities of *P. aculeata* have been performed, no references were detected concerning pharmacological studies of the antiedematogenic effects of the extract and fractions of this specie. In view of the extensively use of *P. aculeata* for these purposes in the Brazilian folk medicine, the objective of the present study was to evaluate antiedematogenic effects of the crude extract and fractions derived of *P. aculeata* stems. The acute toxicity of crude extract of stems was also investigated.

MATERIAL AND METHODS

Plant material

The stems of *P. aculeata* L. were collected in Maringá (Paraná state, Brazil) and identified by Prof. Maria Conceição de Souza. A specimen was deposited in the Herbarium

of State University of Maringá under the code # 6578.

Instrumentation

The NMR spectra (single-and two-dimensional experiments) were obtained with a Varian spectrometer, model Mercury plus BB, operating at 300.06 MHz for ^{1}H and 75.5 MHz for ^{13}C. Chemical shifts were recorded in ppm with reference to internal tetramethylsilane (TMS δ = 0.0 ppm) or to the solvent signal itself. The solvent used was $CDCl_3$ or methanol, CD_3OD (Aldrich). The IR spectrum were recorded in Bomem-MB spectrophotometer. Mass spectrum were recorded in Shimadzu-GC/MS spectrometer, QP 2000A model with electron impact ionization (70eV). Column chromatography (CC) was performed using silica gel 60 (0.063 to 0.020 mm, Merck), neutral alumina (Merck) or Sephadex LH-20 (Sigma) as the stationary phase. For thin layer chromatography (TLC) silica gel 60 G or 60 GF254 (Merck) were employed. Visualization of spots was carried out on CCD by irradiation with ultraviolet light at 254 and 366 nm and/or by acetic acid/H_2SO_4/anysaldehyde solution (1:0.5:48.5 mL) spraying followed by plate heating or Dragendorff reagent.

Preparation of extracts

The steams (605 g) of *P. aculeata* were dried, pulverized and exhaustively extracted with methanol at room temperature. Extracts were filtered and concentrated by evaporation under reduced pressure at a temperature of about 40°C, yielding crude extracts of stems (31.4 g).

Antiedematogenic activity assays

The antiedematogenic activity assays were performed using the experimental model of ear edema induced by croton oil, according to the methodology described in Van Harman (1974). For testing, Swiss mice (n = 6), weighing between 30-35 g were employed. Edema was induced by application of croton oil (20 μL, 200 μg) diluted in 70 % acetone or chloroform (vehicle) on the inner surface of the left ear of each mice. The right ear (negative control) received only vehicle (20 μL). Immediately after applying the phlogistic agent, the crude extracts of stems and fractions from *P. aculeata* at doses of 1.25, 2.5 and 5.0 mg, were applied topically to the left ear. Indomethacin was employed as positive control, at a dose of 1 mg applied topically. After six hours the animals were sacrificed, the ears cut in 7 mm diameter disks and weighted on an analytical balance. Butanol fraction was not tested due to difficulties in solubilization.

Acute toxicity assays

For the acute toxicity tests it were used Swiss mice (n = 5), weighing 20-25 g, fasted for 12 hours. Each animal was treated orally with the crude extract of *P. aculeata* dissolved in 15% DMSO/water at a dose of 4.0 g per kg based on body weight. In this experiment, it was observed the following behavioral parameters: motor activity, convulsions, piloerection, salivation and sedation, according to the methodology described by Malone and Robichaud (1962). The evaluation was performed during the first four hours daily during seven days after administration of the crude extract. Both acute toxicity and antiedematogenic activity assays were approved by the Ethics Committee for Animal Experimentation of the Universidade Estadual de Maringá (PRO 021/2009 CEAE). The results were statistically analyzed using the gamma Tukey range, and were considered significant if $p < 0.05$.

Purification of chemical constituents

The crude extract of stems of *P. aculeata* was dissolved in methanol:water (90:10) and partitioned with different solvents, resulting in four fractions: hexane (6.0 g), chloroform (6.6 g), hydromethanol (13.8 g) and butanol (4.1 g). The hexane fraction was chromatographed over silica gel eluting with hexane, hexane:chloroform in

gradient of increasing polarity, resulting in 78 fractions. The fraction eluted with hexane:chloroform (70:30) was recrystallized with acetone resulting in 58 mg of mixture of stigmasterol, β-sitosterol and campesterol. The fraction eluted with hexane: chloroform (50:50) resulting in 20 mg of mixture of triterpenes oleanolic acid (**1**) and ursolic acid (**2**), and the fraction eluted with hexane:chloroform (30:70) resulting in 3 mg of katonic acid (**3**). The chloroform and hydromethanol fractions showed a positive test for Dragendorff reagent. The chloroform fraction was chromatographed over neutral alumina and eluted with hexane gradually increasing the polarity whit chloroform and methanol, resulting in 96 fractions which was monitored by TLC and grouped in 8 new fractions (A-H) according to their chromatographic profile. The fraction D was submeted to preparative TLC eluted with hexane:chloroform (40:60), resulting in 7 mg of *N-trans*-caffeoyl tyramine (**4**). The hydromethanol fraction was submitted to chromatographic column over neutral alumina and eluted with chloroform, chloroform:methanol in gradient of increasing polarity and methanol. The collected fractions were monitored by TLC, furnishing 5 mg of compound 3-(methoxycarbonyl)-pyridine (**5**) by fraction eluted with chloroform:methanol (50:50). Butanol fraction has not resulted in isolation of compounds.

The isolated compounds (Fig. 1) have been identified by NMR spectral data of ^{1}H and ^{13}C/DEPT, IR and MS data as well as data comparison with the literature.

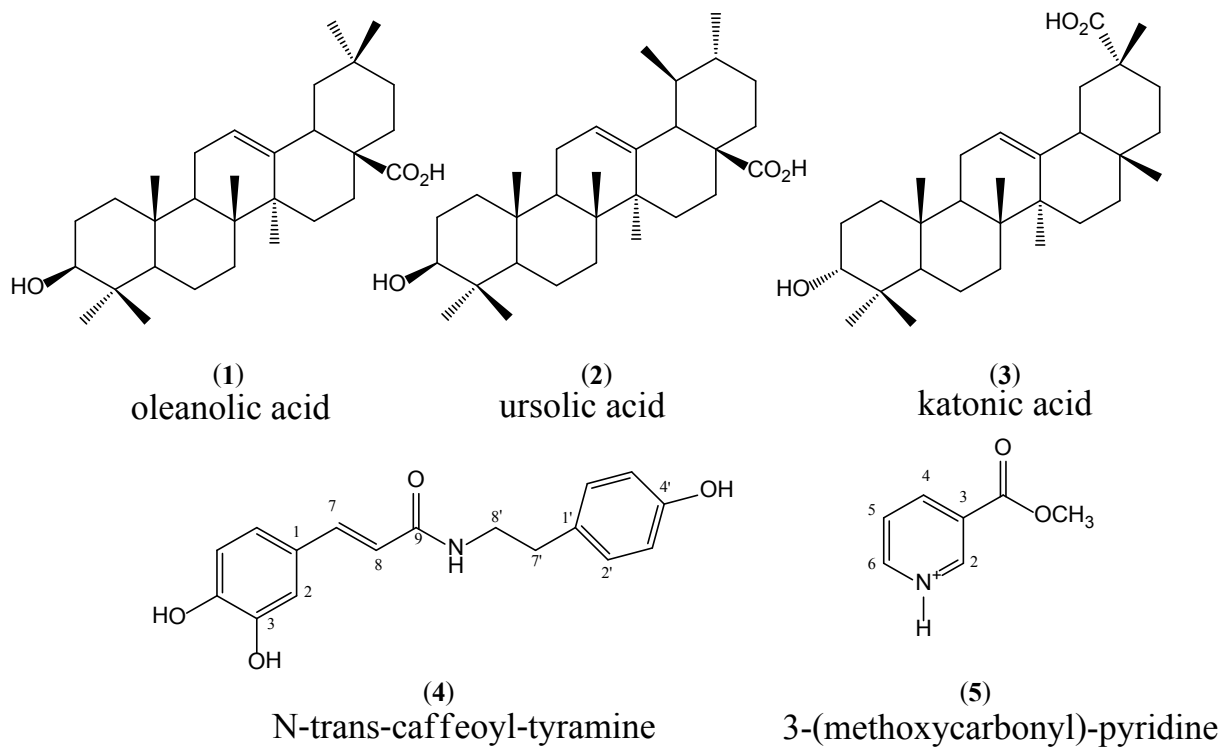

Figure 1. Substances isolated from crude extracts from stems of *P. aculeata*.

Spectral data for isolated compounds

Compound 4: *N-trans-caffeoyl-tyramine:* Amorphous powder. EI/MS: m/z: 299 [$M^{\cdot+}$]. ^{1}H-NMR (CD_3OD, 300.06 MHz): 7.42 (d, ^{1}H, *J* = 15.6 Hz, H-7), 7.08 (d, 1H, *J* = 1.5 Hz, H-2), 7.02 (d, 2H, *J* = 8.5 Hz, H-2', H-6'), 7.00 (dd, 1H, *J* = 8.4, 1.5 Hz, H-6), 6.81 (d, 1H, *J* = 8.4 Hz, H-5), 6.79 (d, 2H, *J* = 8.5 Hz, H-3', H-5'), 6.36 (d, 1H, *J* = 15.6 Hz, H-8'), 3.53 (t, 2H, *J* = 7.5 Hz, H-8), 2.68 (t, 2H, *J* = 7.5 Hz, H-7), ^{13}C-NMR (CD_3OD): 169.6 (C-9), 156.4 (C-4'), 149.0 (C-4), 147.6 (C-3), 141.6 (C-7), 131.0 (C-1′), 130.8 (C-2', C-6'), 126.9 (C-1), 122.8 (C-6), 118.8 (C-8), 116.3 (C-5), 116.7 (C-3', C-5'), 114.8 (C-2), 42.0 (C-8'), 36.1(C-7').

Compound 5: *3-(methoxycarbonyl)-pyridine:* Colorless crystals. ^{1}H-NMR (CD_3OD, 300.06 MHz): 9.20 (s, 1H, H-2), 8.90 (d, 1H, *J* = 8.5 Hz, H-6); 8.85 (dd, 1H, *J* = 8.5, 1.5 Hz, H-4), 8.05 (dd, 1H, *J* = 8.5, 1.5 Hz, H-5), 4.43 (s, OCH_3) ,^{13}C-NMR (CD_3OD): 167.0 (C=O), 147.7 (C-2), 147.0 (C-4), 146.1 (C-6), 128.6 (C-5), 56.1 (OCH_3).

RESULTS AND DISCUSSION

Structural identification of isolated compounds

Hexanic fraction derived of crude extract of stems of *P.aculeata* were subjected to classical chromatographic purification methods leading to the identification of three known steroids (stigmasterol, β-sitosterol and campesterol) and three triterpenes, oleanolic acid (**1**), ursolic acid (**2**), and katonic acid (**3**), (**1**) and (**3**) belonging to the oleanes class and (**2**) belongs to the ursanes class. The phenolic α,β–unsatured amide N-*trans*-caffeoyl-tyramine (**4**) was isolated from chloroform fraction of stems of *P. aculeata.* Finally, 3-(methoxycarbonyl)-pyridine (**5**) was obtained from methanolic fraction. The triterpenes (**1**-**3**), phenolic amide (**4**) and compound (**5**) were identified according to the NMR spectral data of ^{1}H, ^{13}C, DEPT and COSY and IR and MS data, and by comparison with the literature data (Mahato and Kundu, 1994; Wu *et al.*, 1995; Breitmair and Voelter, 1990).

Acute toxicity and Antiedematogenic activity assay

Topical application of crude methanol extract of stems of *P. aculeata* at a dose of 5.0 mg/ear, caused a significant inhibition of ear edema. The same result was observed when the fractions obtained from the stems of *P. aculeata,* hexane, chloroform and methanolic were evaluated. It was noted that the topical application of these extracts caused a reduction in ear swelling mainly for hexane and chloroform fractions. In acute toxicity study, oral treatment of animals (mice) with the crude extract from the stems of *P.aculeata* produced no mortality (LD_{50} > 4g/kg), demonstrating their safety in use. More importantly, no changes were observed in the behavioral parameters of the animals in comparison with blank and no death occurred within seven days after treatment.

Mice ear edema reached a maximum 6 h after croton oil application. Crude extract at a dose of 5.0 mg significantly inhibited swelling, considerably reducing vascular permeability in response to croton oil (Fig. 2). Inflammation was inhibited by 27.9%. Application of chloroform and hexane fractions at a dose of 5.0 mg each also reduced ear edema, showed inhibitory activity of 26.6% and 25.0%, respectively (Fig. 2). Methanolic fraction showed the lowest inhibition of the mouse ear edema (19.1%). The positive control indomethacin at a dose of 1 mg/ear inhibited inflammation by 41.5% (Fig. 2).

Given this result, it can be suggested that the apolar metabolites such as steroids and triterpenes (**1**-**3**) obtained from hexane fraction are responsible in part for the anti-edematogenic activity observed, since the literature reports the anti-inflammatory potential of steroids (Srivastava *et al.*, 1990; Delporte *et al.*,

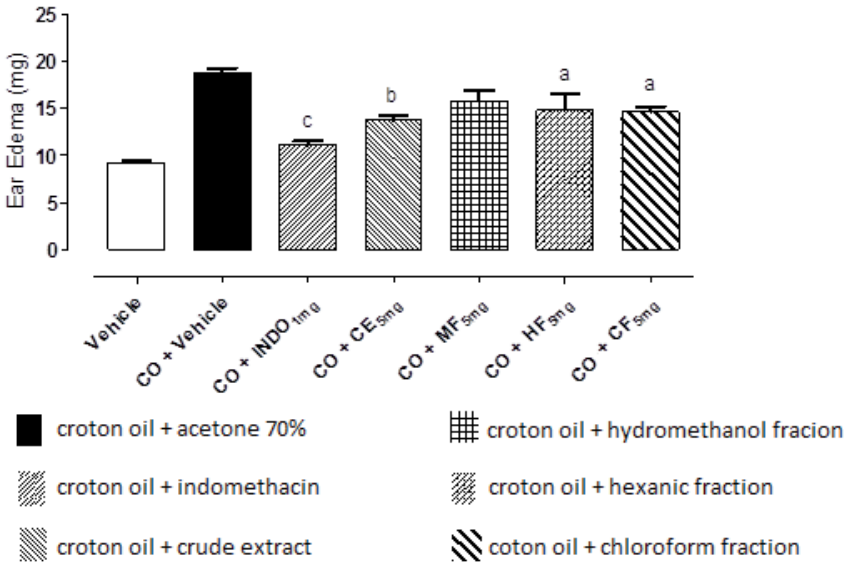

Figure 2. Effect of crude extract (**CE**) and hydromethanol (**MF**), hexane (**HF**) and chloroform (**CF**) fractions from the stems of *P. aculeata* on the ear edema induced by croton oil (**CO**) in mice. Indomethacin (**INDO**) applied topically, 1.0 mg, was used as reference substance (positive control). Acetone 70 % was used as vehicle. Each bar represents the mean weight ± SD for ears, 6 h after application of croton oil. The masses indicated refer to the concentrations used in each sample (5.0 mg / ear). [a] P <0.05, [b] P <0.01compared and [c]P <0.001 to CO + Vehicle group. (ANOVA followed by Tukey test).

2005). The anti-inflammatory effect is also a common property of many triterpenoids found in plant extracts and this activity is attributed to the presence of oleanolic acid (**1**) or ursolic acid (**2**) as main constituents. These triterpenes have confirmed their anti-inflammatory properties through the use of different models and their mechanisms of action have also been elucidated. Moreover, also are reported antitumor, antimicrobial, antileishmania and anti-hyperlipidemic properties for both triterpenes (Liu, 1995). Katonic acid (**3**) was reported with anti-inflammatory properties. Its activity was almost equivalent to that shown by the reference standard, indomethacin (Rasadah *et al.*, 2004). The chloroform fraction of stems of *P. aculeata* showed a chromatographic profile rich in nitrogen compounds. This fraction afforded the compound (**4**) (N-*trans*-caffeoyl-tyramine). This compound has anti-inflammatory activity as well as antioxidant activity and may be one of the compounds responsible for the antiedematogenic activity displayed by extract (Al-Taweel *et al.*, 2012).

Treatment of mice with the crude extract from stems of *P. aculeata* in the acute toxicity test did not cause death of animals or change in behavioral parameters, indicating that the extract may be considered non-toxic in a first instance.

CONCLUSION

In conclusion, the phytochemical study of the crude extract and fractions derived from stems of P. *aculeata* made in this work allowed us to isolate and identify steroids,

triterpenoids and nitrogenous compounds. Studies of the anti-inflammatory activity of these compounds have been reported in the literature and therefore, may be primarily responsible for the antiedematogenic activity verified for the extract and fractions. Evaluation of the antiedematogenic activity showed that the crude methanolic extract, as well as the hexane and chloroform fraction, have a significant inhibitory activity for the ear edema induced by croton oil.

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