

Article

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Development of hydroethanolic extract of *Ipomoea pes-caprae* using factorial design followed by antinociceptive and anti-inflammatory evaluation

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Abstract: *Ipomoea pes-caprae* (L.) R. Br., Convolvulaceae, is a medicinal plant that grows abundantly as a pan-tropical stand plant. The 3² (two factors and three levels) factorial design, was applied to determine the best time and drug/solvent proportion to maximize the flavonoid content in the hydroethanolic extract by maceration process. The antinociceptive and anti-inflammatory effects were studied at 5-20 mg/kg, *i.p.*, using the writhing test and carrageenan-induced pleurisy models in mice. The optimized extract was able to inhibit more than 50% of abdominal writhing at 20 mg/kg, with 55.88%±2.4 of maximum inhibition. Indomethacin, used as positive control, inhibited 64.86% at 10 mg/kg. In the pleurisy model, the extract produced dose-dependent inhibition of the first phase of inflammation (4 h) in the pleural cavity induced by injection of carrageenan (1%) in mice. It inhibited 50%±0.82 ($p<0.01$) of exudation induced by carrageenan, and 60.88%±0.14 ($p<0.01$) of leukocyte migration to the pleural cavity. In conclusion, the results validate the technological conditions of the maceration process to produce an optimized bioactive herb extract for the development of analgesic and anti-inflammatory phytopharmaceuticals using 70 °GL ethanol, a plant to solvent ratio of 12.5% (w/v), and ten days of maceration.

Introduction

Ipomoea pes-caprae (L.) R. Br., Convolvulaceae, known in English as bayhops, beach morning glory, and goat's-foot-convolvulus, and in Brazil as "salsa-da-praia", "batateira-da-praia" or "pé-de-cabra", has been used as a herb remedy in many countries to treat inflammation, colic, diuretic disorders, gonorrhea, pain etc. (Pongprayoon et al., 1989; Assis et al., 1994; Khan et al., 1994).

Previous studies have confirmed some pharmacological properties of this plant. Extract of petroleum ether caused concentration-dependant inhibition of the contraction of guinea-pig ileum contractions stimulated by different neurotransmitters (Pongprayoon et al., 1989). Bioassay-guided fractionation of this extract resulted in the isolation of β -damascenone and *E*-phytol, which were equipotent to papaverine (Pongprayoon et al., 1992). Another interesting action demonstrated by this plant was its ability to neutralize or inactivate jellyfish venoms (Pongprayoon et al., 1991a). Khan et al. (1994)

reported the insulinogenic and hypoglycemic activities of the alcohol extract of the leaves. Previous studies carried out by our research group have revealed that the methanol extract from the whole plant exhibits potent and significant antinociceptive properties against acetic acid-induced abdominal constrictions and neurogenic and inflammatory phases of formalin-induced pain, probably associated to ethyl acetate fraction, as well as to glochidona, α and β -amyrin acetate, betulinic acid and isoquercitrin, being equipotent to some analgesic drugs (Krogh et al., 1999; Souza et al., 2000).

Considering the popular use of this plant to treat pain and inflammation, as well as the promising previous experimental data and its abundance in Brazil, the first end point of the study was to develop an herb extract using an adequate solvent for industrial scaling up, with lower toxicity, less environmentally hazardous, associated with a simple extraction method. Then, we attempted to develop hydroethanolic extracts by maceration process, using factorial design to find

out the extraction conditions for the highest flavonoid content, and antinociceptive and anti-inflammatory effects analysis of the selected extract.

Material and Methods

Plant material

The botanical material was collected from Esplanada Beach (Jaguaruna-SC, Brazil) in February 2006. A voucher was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC, Brazil) under number V.C. Filho 009. The leaves, stems and other aerial parts (flower buds, seeds and flowers) were manually separated, cleaned and dried in an air oven at 35 °C for eleven days. After drying, each set of each plant material was ground in a hammer mill (outlet sieve =3 mm) and the average size of the particles was determined by sieving.

Previous studies for extraction

The ethanol graduation was first investigated by ethanol extractable matter (EM) from 1.0 g of aerial parts decocted with 100 g of 40, 50, 60, 70 or 80 °GL ethanol, under reflux for 10 min. In another set of experiments, an extract (5%, w/w) of each solvent was prepared by maceration for seven days, in a hermetically sealed amber glass bottle, stirred twice a day.

The total flavonoid content (TFC), expressed as quercetin (g%, w/w), was determined spectrophotometrically (420 nm) for 15.0 g of each sample solution, after selective extraction, acid hydrolysis and complexation with $AlCl_3$, following the method based on the Brazilian Pharmacopeia (Farmacopeia Brasileira, 2001).

Factorial design for extraction by maceration

This study was carried out following a 3^2 factorial model, taking into account the extraction time (5, 10 and 15 days) and the drug/solvent ratio (2.5, 7.5 and 12.5%, w/v) as independent variables, and the total flavonoid content and dry residue as dependent variables.

Chromatographic profile of selected extractive solution

A new batch of extractive solution was produced, according to selected extraction conditions, in order to characterize its chromatographic profile by HPLC, using a Shimadzu LC-10AD HPLC system (Shimadzu, Tokyo, Japan) equipped with a binary pump and a SPD-M10A photo diode array detector, a CTO-10A column oven, and an automatic injection system.

The mobile phase consisted of water pH 3.2 (acidified with formic acid) as solvent A and acetonitrile: methanol (50:50, v/v) as solvent B, gradient system of 75:25 for 30 min, to 60:40 on 20 min, 35 °C and flow rate of 0.8 mL, through a C_{18} 5 μ m 100Å (250 x 4.6 mm) column (Phenomenex®, Torrance, USA), with detection at 254 nm and injection of 20 μ L.

All the solvents were HPLC grade and were previously degassed. The water was purified using an Easy Pure device (Waltham, Massachusetts, USA). For the LC injections, hydroethanolic extract was diluted in methanol and purified water (50:50) (5 mg/mL, according to its dry residue weight). Isoquercitrin (Chromadex®, Irvine, CA), diluted in methanol and purified water (50:50), at six concentrations from 10 to 80 μ g/mL, was used as a reference substance to elaborate an analytical curve. All the samples were filtered through a 0.45 μ m regenerated cellulose membrane (Millipore Maidstone, Kent, UK) before injection. Each analysis was repeated three times and the calibration curves were fitted by linear regression.

Animals

Male wistar mice, 25-30 g, were used. The animals were kept in a controlled temperature room (23±2 °C), with 12 h light-dark cycles, and were allowed free access to food and water. The experiments were performed with the approval of the Committee for the Use of Animals in Experiments of the University of Itajaí Valley (Brazil) (CEUA 407/06), and were carried out in accordance with current guidelines for the care of laboratory animals, and the ethical guidelines for investigations of experimental pain in conscious animals, as specified by Zimmermann (1983). The number of animals (8-10 per group) and intensity of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Antinociceptive activity

The writhing test-abdominal constrictions resulting from intraperitoneal injection of acetic acid (0.6%), consisting of a contraction of the abdominal muscle together with a stretching of hind limbs-was carried out according to the procedures previously described (Collier et al., 1968; Santos et al., 2005). The animals were pretreated with the hydroethanolic extract through intraperitoneal (5-20 mg/kg) administration, 30 min prior to acetic acid injection. The control animals received a similar volume of 0.9% NaCl (10 mL/kg). Pairs of mice were then placed in separate boxes, and the number of abdominal constrictions was cumulatively counted over a period of 20 min.

Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions between the control animals (saline pre-treated) and the mice pre-treated with extract. The nociceptive response caused by acetic acid injection was analyzed 30 min after the administration of hydroethanolic extract or saline injection. All experiments were carried out at 20-22 °C.

Anti-inflammatory activity

The carrageenan (Cg) induced pleurisy model was used. Pleurisy experiments were carried out as previously described (Saleh et al., 1996) and carrageenan was used as the phlogistic agent, by a single intrapleural injection of 0.1 mL of Cg (1%). The animals were intraperitoneally treated with different doses of *Ipomoea pes-caprae* (L.) R. Br., Convolvulaceae, extract (5-20 mg/kg) or vehicle, used to dilute the extracts, or indomethacin (10 mg/kg) as a reference drug, 30 min before the pleurisy induction. The inflammatory parameters (leukocytes and exudation) were analyzed 4 h after Cg injection.

After 4 h, the animals were killed with an overdose of pentobarbital, the thorax was opened, and the pleural cavity was washed with 1.0 mL of sterile PBS (pH 7.6, NaCl 137 mM:KCl 2.7 mmol and phosphate buffer salts 10 mM) containing heparin (20 IU/mL). Samples of the pleural lavage were collected to determine the exudation and total and differential leukocyte counts (Saleh et al., 1996; Dalmarco, 2002). Total leukocyte counts were performed in a Neubauer chamber after diluting the pleural fluid with Turk solution (1:20). The cytospin preparations of pleural wash were stained with May-Grunwald Giemsa for the differential count, which was performed under an oil immersion objective.

In another set of experiments, the animals were treated 30 min before Cg, with a solution of Evans blue dye (2.5 mg/kg, 0.2 mL, i.v.) in order to evaluate the degree of exudation in the pleural space (Calixto & Campos, 1995). A sample (500 µL) of the fluid leakage collected from the pleural cavity was stored in a freezer (-20 °C) to further determine the concentration of Evans blue dye. To this end, on the day of the experiments, a batch of samples was thawed at room temperature and the amount of dye was estimated by optical density using a spectrophotometer (Pharmacia Biotech-Ultrospec 2000) at 600 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50 µg/mL (Cordeiro et al., 1999).

Statistical analysis

The factorial design 3² data were analyzed

through response surface plots from adjusted equations by multiple regressions (Cochran & Cox, 1978; Montgomery, 1991), with the aid of Statgraphics Plus® (software version 5.1).

The pharmacological results are presented as mean±standard error deviation (SEM). The statistical significance between groups was analyzed by means of t test or analysis of variance, followed by the Dunnett's with the aid of GraphPad Instat® software. *p* values<0.05 were considered as indicative of significance.

Results and discussion

The batch of herb raw material used for this study was consisted by 52.24% of stems, 32.24% of leaves and 15.46% of buds, seeds and flowers, with a moisture content of 8.99±0.06, 11.76±0.81 and 8.20±0.46, respectively, and was in accordance with acceptable values for preservation of the raw plant material in storage (Zhi-Chen, 1980). The mean particle size was 0.642 mm for the leaves, 0.657 mm for the stems and 0.698 mm for the buds, seeds and flowers together.

There were no statically differences among all the results of ethanol extractable matter, making this assay poorly discriminative (Table 1). However, the extraction of flavonoids was higher at 70 or 80 °GL ethanol. As no statistically differences were observed between the results, the 70 °GL was chosen due to its superior microbiological preservation properties.

Table 1. Ethanol extractable matter (EM) and total flavonoid content (TFC) of extractive solutions (1%, w/w and 5%, w/w, for EM and TFC assay, respectively). The results are expressed as mean±standard deviation (variation coefficient).

Solvent	EM (% w/w)	TFC (g%)
40° GL	32.15±0.85 (2.65)	0.0739±0.0037 (4.98)
50° GL	35.13±2.56 (7.27)	0.0574±0.0020 (3.45)
60° GL	32.34±4.43 (13.68)	0.0530±0.0115 (21.77)
70° GL	36.88±3.68 (9.97)	0.0921±0.0100 (10.90)
80° GL	30.85±4.68 (15.16)	0.0877±0.0011 (1.29)

The factorial design was then applied, in order to find out the best drug/solvent proportion and extraction time for the maceration process. Dry residue was investigated for its importance in yield calculations in further studies with technological purposes, such as the drying process. Flavonoids were chosen as chemical markers in this study because of their well-known antinociceptive and anti-inflammatory properties (Muschiatti & Martino, 2007). The main flavonoid from this plant, isoquercitrin, proved to be one of the active principles of this plant (Krogh et al., 1999).

The studied responses (Dry residue

or TFC) (Table 2) were adjusted by a second order mathematic model, according to the following equation: $Y = \beta_0 + \beta_1 * Conc + \beta_2 * T + \beta_{12} * Conc * T + \beta_{11} * Conc^2 + \beta_{22} * T^2$, where y = studied response, $\beta_0 \dots \beta_{22}$ = coefficients, $Conc$ = concentration (% w/v) of *I. pes-caprae* aerial parts, T = extraction time. The experimental data were adjusted by the quadratic model, resulting in the following equations: $y = -0.372 + 0.637 * Conc + 0.078 * T - 0.014 * Conc^2 - 0.014 * Conc * T - 0.0015 * T^2$ for dry residue ($r^2 = 0.9908$) and $y = -0.058 + 0.010 * Conc + 0.017 * T - 4 * 10^{-5} * Conc^2 - 4.04 * 10^{-4} * Conc * T - 8.64 * 10^{-4} * T^2$ for TFC ($r^2 = 0.9143$), where $Conc$ = herb drug concentration (% w/v) and T = extraction time (days).

Table 2. Total flavonoid content (TFC, g%) and dry residue for extractive solutions (ES) obtained according 3² factorial design.

ES (Conc+T)	TFC (g%)	Dry Residue (%)
1 (2.5+5)	0.0273±0.0025	1.399±0.130
2 (7.5+5)	0.0781±0.0005	3.311±0.062
3 (12.5+5)	0.0926±0.0006	4.812±0.180
4 (2.5+10)	0.0343±0.0006	1.370±0.045
5 (7.5+10)	0.0687±0.0040	3.047±0.045
6 (12.5+10)	0.1125±0.0007	4.321±0.022
7 (2.5+15)	0.0266±0.0004	1.358±0.058
8 (7.5+15)	0.0277±0.0004	3.023±0.085
9 (12.5+15)	0.0519±0.0003	3.341±0.209

Conc: herb drug concentration (% w/v); T: extraction time (day). The results are expressed as mean±standard deviation.

According to the statistical analysis for the dry residue, concentration ($p = 0.0004$) had the highest significance compared to the other variables, like time ($p = 0.0347$) and the interaction of these factors (0.0372) (Figure 1A). For the TFC, only the linear coefficient of concentration demonstrated significance ($p = 0.0204$) as reflected by the surface plot (Figure 1B).

Both response surface plots indicated the best conditions for extraction in order to obtain higher levels of dry residue (Figure 1A) and flavonoid content (Figure 1B): 12.5% (w/v), due to their drug:solvent ratio and extraction time of about seven days.

Through the factorial experiment, it was possible to choose the most favorable extraction conditions, with a view to achieving greater efficiency in terms of flavonoids and dry residue. Both the total flavonoid content and the dry residue were strongly influenced by extraction time and drug:solvent ratio, particularly the latter. According to the response surface plot, the dried, powdered plant matter of 12.5% (w/v), placed in contact with 70 °GL ethanol over a period of five to seven days, obtained higher yields in terms of dry residue and total flavonoid content.

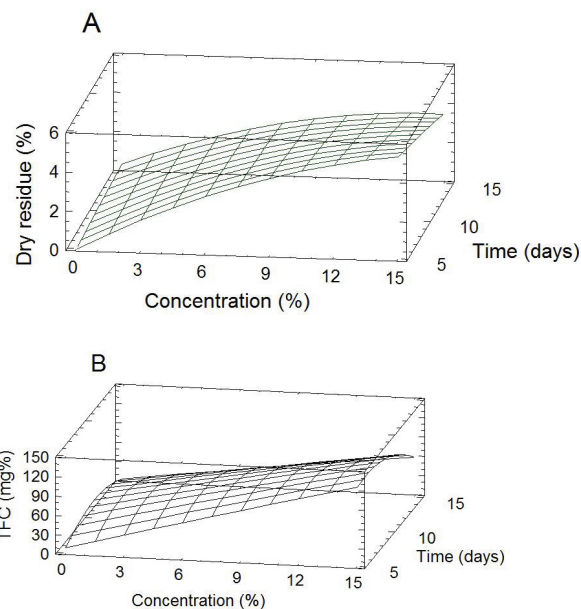


Figure 1. Response Surface plot for evaluating the influence of herb drug concentration and extraction time on the dry residue (A); and on the total flavonoid content (TFC) (B).

Chromatographic profile of optimized extractive solution

Another batch was also produced, for the sole purpose of identifying a possible flavonoid marker. The new batch was produced using a drug/solvent ratio of 12.5% (g/mL) and extraction time of seven days, taking into account the conditions set by the response surface plot (Figure 1B).

Figure 2 shows the chromatographic profile of ES with six peaks. Only two of them (1 and 2) have a characteristic UV absorbance profile of flavonoids. The second peak was identified as isoquercitrin, since it was eluted at the same retention time (about 17.66 min) and had the same UV absorbance profile (data not show). The isoquercitrin concentration was calculated from the linear regression equation $y = 48487x - 3145.3$ ($r^2 = 0.9999$). In this batch, there was $71.3 \pm 7.7 \mu\text{g/mL}$ of isoquercitrin, corresponding to 3.1 mg/g, considering its extractive solution dry residue weight of 2.3 g% (w/w).

Pharmacological evaluation

The extractive solution that showed the highest TFC (ES6, Table 2) was chosen for the evaluation of antinociceptive activity by the writhing test, and anti-inflammatory activity by the pleurisy model.

Antinociceptive activity by the Writhing test

The results (Figure 3) demonstrate that *I. pes-caprae* hydroethanolic extract, given by *i.p.* 30 min prior to testing, produced dose-related inhibition of acetic acid-induced abdominal constrictions in mice, with MI (Maximum inhibition) and calculated DI50 of $55.88 \pm 2.4\%$ and 17.34 (12.23-24.30) mg/kg, respectively. Indomethacin, used in this experiment as positive control, also reduced the number of writhings induced by acetic acid, with inhibition of 64.86% at 10 mg/kg.

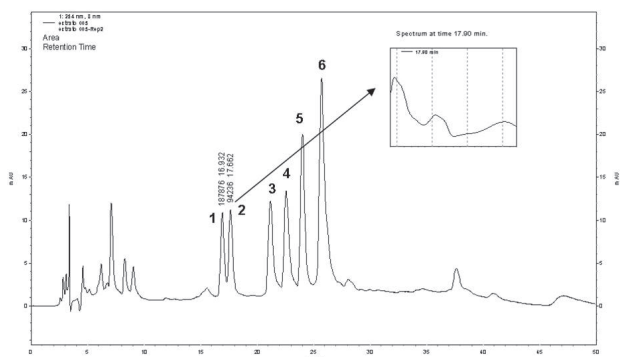


Figure 2. Chromatographic profile of *Ipomoea pes-caprae* hydroethanolic extract solution (new batch obtained as established by the response surface plot) at $5 \text{ mg} \cdot \text{mL}^{-1}$ of dried residue, monitored at 254 nm (2=isoquercitrin).

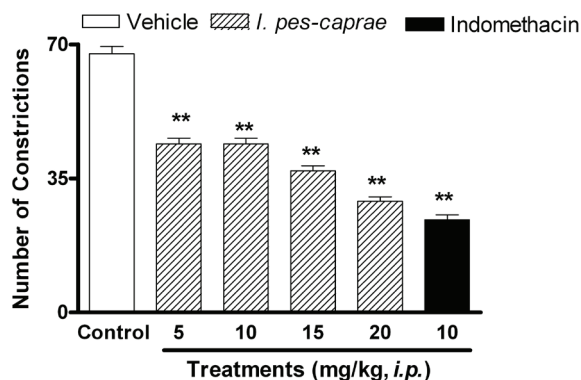


Figure 3. Effects of hydroethanolic extract (ES6, Table 2) of *I. pes-caprae* on the number of acetic acid induced writhings in mice. Each column represents the average of 8 animals per group and the vertical bars represent the EPMs. The asterisks denote statistical significance (** $p < 0.01$) when compared with the control group (vehicle). ANOVA, complemented by the Dunnett's test.

Our results show that the optimized hydroethanolic extractive solution was able to inhibit more than 50% of abdominal writhings at 20 mg/kg, being more effective than the methanol extract evaluated earlier by Krogh et al. (1999) and Souza et al. (2000), which showed a DI50 of 33.8 mg/kg under the

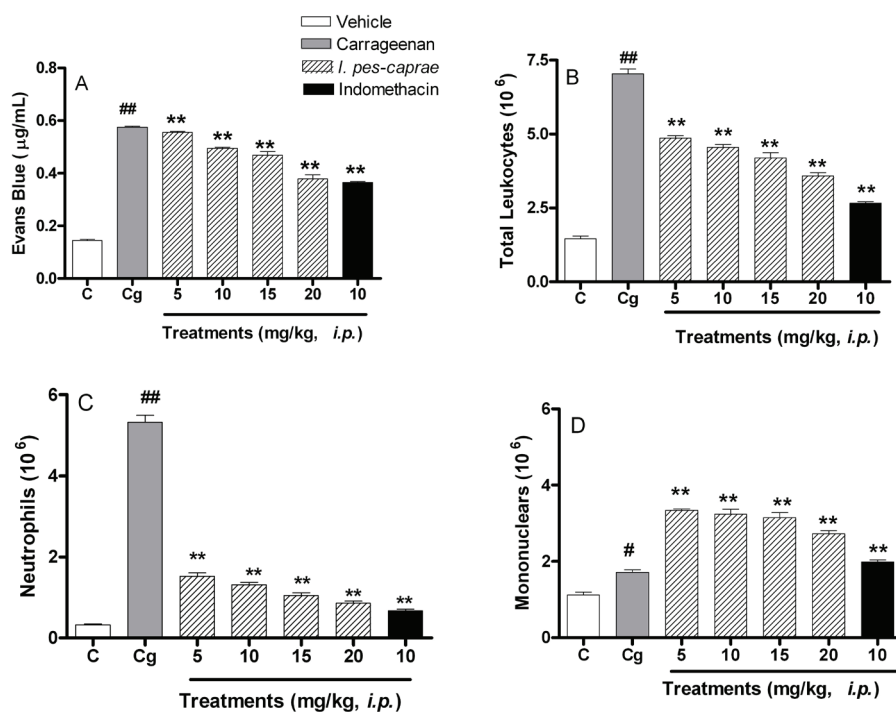


Figure 4. Effects of hydroethanolic extract (ES6, Table 2) of *I. pes-caprae* (5-20 mg.kg⁻¹, *i.p.*) upon inflammatory parameters in the mice model of pleurisy. (A) exudate, (B) total leukocytes, (C) neutrophils and (D) mononuclear cells. Each column represents the average of 6 to 8 animals and the vertical bars represent the EPM. Asterisks denote statistical significance (** $p < 0.01$) when compared with carrageenan and # $p < 0.05$ when compared with the control group (saline). ANOVA, complemented by the Dunnett's test.

same experimental conditions. Moreover, the solvent used in our work presents advantages over other toxic or more polluting organic solvents, being more useful and suitable for the phytopharmaceutical industry.

With respect to the phytochemical constituents isolated from *I. pes-caprae* related to these activities, Krogh et al. (1999) showed that some triterpenes and isoquercitrin significantly inhibited acetic acid-induced abdominal constrictions in mice. The selected hydroethanolic extractive solution also showed significant inhibition, being more active than isoquercitrin itself and some triterpenes, suggesting the occurrence of synergism, and the fact that flavonoids such as isoquercitrin detected in the extract (Figure 2) are not the only substances responsible for the activity.

Carrageenan-induced pleurisy

In the present study, it was found that the extract of *I. pes-caprae* (5, 10, 15 and 20 mg/kg, *i.p.*) was able to induce significant and dose-dependant inhibition of the first phase of inflammation (4 h) of the pleural cavity induced by injection of carrageenan (1%) in mice. It was observed that the extract of *I. pes-caprae* caused, to a lesser extent, a reduction in carrageenan-induced exudation (% inhibition: 50 ± 0.82) ($p < 0.01$) (Figure 4A). Moreover, the extract produced inhibition of cell migration (number of total leukocytes) to the pleural cavity (% inhibition: 60.88 ± 0.14) ($p < 0.01$) (Figure 4B), mainly represented by neutrophils (% inhibition: 88.90 ± 0.12) ($p < 0.01$) (Figure 4C). However, the extract produced an increase in migration of mononuclear cells (Figure 4D) to the inflammatory site.

In carrageenan-induced pleurisy in mice, a model of inflammation, carrageenan promotes an increase in the volume of exudate in the pleural cavity, leukocyte migration of the polymorphonuclear type, and participation of some inflammatory mediators such as histamine, serotonin bradykinin, nitric oxide (NO), TNF α and IL-1 β , substance P (SP) and others (Saleh et al. 1996, 1999; Endo, 2001; Banerjee et al. 2002; Brennan & McInnes, 2008).

Regarding the inflammatory process, there are few reports on *I. pes-caprae* in the literature. It is known that the plant antagonizes the effects of histamine and also inhibits the release of prostaglandins (Pongrayoon et al., 1991b). Rogers et al. (2000) showed that the methanol extract of this plant potently inhibited ADP induced human platelet [^{14}C]5-HT release in vitro, with levels of inhibition ranging from 62 to 95%.

The increase in migration of mononuclear cells to the inflammatory site is probably related to the immunostimulatory activity, and appears to be independent of the type of plant extract used. Philippi

et al. (2010) demonstrated that *I. pes-caprae* methanol extract has already been shown to induce human T lymphocyte proliferation.

The pharmacological results performed with *I. pes-caprae* hydroethanolic extract also suggest analgesic and anti-inflammatory effects similar to those previously demonstrated by methanol extract, ethyl acetate and aqueous fractions of *I. pes-caprae* (Krogh et al., 1999). Indeed, this work was able to validate the technological conditions of the maceration process to produce a bioactive plant extract for the development of phytopharmaceuticals. Further studies are necessary to elucidate the mechanism of action of this extract.

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