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Pereskia aculeata Miller leaves present *in vivo* topical anti-inflammatory activity in models of acute and chronic dermatitis

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115250
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Stigmasterol
PubChem CID
5280794
Phytol
PubChem CID
9018

ABSTRACT

Ethnopharmacological relevance: The leaves of *Pereskia aculeata* Miller (Cactaceae), known as Barbados gooseberry, are used in Brazilian traditional medicine as emollients and to treat skin wounds and inflammation. This study investigated the topical anti-inflammatory activity of the hexane fraction (HF) obtained from the methanol extract of the leaves of this species in models of acute and chronic ear dermatitis in mice.

Material and methods: Mice ear edema was induced by topical application of croton oil, arachidonic acid, capsaicin, ethyl-phenylpropiolate and phenol; and by subcutaneous injection of histamine. Ear biopsies were obtained to determine the levels of IL-1β, IL-6 and TNF-α cytokines by ELISA assay. Histopathological analysis was also performed to evaluate the HF activity in croton oil multiple application test. In addition, acute dermal irritation/corrosion test in rats was accomplished. HF chemical characterization was performed by GC–MS analysis.

Results: HF intensively reduced the inflammatory process induced by all irritant agents used, except for arachidonic acid. This activity is related, at least in part, to the reduction of IL-6 and TNF-α cytokines levels. Moreover, when the glucocorticoid receptor antagonist mifepristone was used, HF failed to respond to the croton oil application.The results strongly suggested a glucocorticoid-like effect, which was reinforced by the presence of considerable amounts of sterol compounds identified in HF. The acute dermal irritaton/corrosion test showed no signs of toxicity.

Conclusions: This study showed that the acute and chronic anti-inflammatory activity of *P. aculeata* leaves is very promising, and corroborates to better understand their ethnopharmacological applications.

1. Introduction

The skin, the largest organ of the human body, plays an essential role as a protective barrier against microorganisms, and chemical and physical injuries from the external environment. This physiological barrier consists of a complex network of structural, cellular and molecular components which provide an effective defensive immunological reaction against harmful stimuli (Nestle et al., 2009; Bangert et al., 2011). Nevertheless, in some circumstances, alterations in the skin immunological balance may elicit inappropriate defensive responses which lead to an

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inflammatory process or chronic skin disorders, including psoriasis, eczema, atopic dermatitis and allergic contact dermatitis (Lee and Hwang, 2012). The current therapeutic agents available to treat skin inflammation are mainly glucocorticoids, which often exhibit adverse reactions that affect the hypothalamic–pituitary axis, the lipids, carbohydrates, proteins and bone metabolisms, and the organs related to the immune system (Poetker and Reh, 2010; Sarnes et al., 2011; Rang et al., 2012).

In this context, plant extracts and natural substances remain as an alternative in the search for new safer and more effective topical anti-inflammatory drugs (Newman and Cragg, 2012). There are reports that leaves of *Pereskia aculeata* Miller (Cactaceae), known as Barbados gooseberry, are used in folk medicine as emollients and to treat skin wounds and inflammatory process (Duarte and Hayashi, 2005; Sartor et al., 2010; Pinto and Scio, 2014). *P. aculeata* is a climbing cactus shrub distributed in Central and South America, covering mainly from south to northeast of Brazil, where its leaves are also used by natives as a vegetable in traditional cuisine (Takeiti et al., 2009; Rosa and Souza, 2003; Paterson et al., 2009). However, there are only few studies about *P. aculeata* leaves phytochemicals and their therapeutic potential (Pinto et al., 2012; Pinto and Scio, 2014). Recently, Carvalho et al. (2014) reported the *in vitro* wound healing activity of the mucilage extracted from the leaves of this species, and Pinto et al. (2015) showed that the hydromethanolic fraction from the crude extract of the leaves presented central and peripheral antinociceptive activity in mice.

Preliminary studies performed by our group showed that, among the different fractions obtained from the methanol extract of *P. aculeata* leaves by solvent partition, the hexane fraction showed the most remarkable anti-inflammatory potential (data not shown).

For that reason, in order to contribute to the search for new plant extracts or natural substances with anti-inflammatory potential, and for better understanding the ethnopharmacological relevance of *P. aculeata*, this study was conducted to further investigate the topical anti-inflammatory activity of the hexane fraction (HF) obtained from the methanol crude extract of the leaves of this species in models of acute and chronic ear dermatitis in mice, using different phlogistic agents. In addition, an established acute dermal irritation and corrosion test in rats was accomplished to predict a possible dermal toxicity of HF. Chemical characterization of HF was also performed.

2. Materials and methods

2.1. Plant material

The plant material was collected in Juiz de Fora (MG, Brazil) in August 2010, in the morning. A voucher specimen (No. 57539) was deposited in the Herbarium Leopoldo Krieger of the Federal University of Juiz de Fora for future evidence.

The leaves were air-dried in a well-ventilated place at room temperature (25 °C) for 15 days. Once dried, the material (approximately 1 kg) was powdered using a knife mill and then extracted by maceration with methanol until exhaustion. The extract was concentrated on a rotary evaporator to obtain the crude methanol extract (140 g), which was dissolved in methanol/water (8:2 v/v) and then fractionated with hexane by solvent partition. The hexane fraction (38 g) was stored in a refrigerator at 4 °C.

2.2. Chemicals

Croton oil, arachidonic acid (AA), capsaicin, ethyl-phenylpropiolate (EPP), phenol, histamine, indomethacin, dexamethasone, mifepristone, bovine serum albumin (BSA), phenylmethylsulphonyl fluoride (PMSF), benzethonium chloride, ethylenediaminetetracetic acid (EDTA) and aprotinin were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Ketamine chloride and xylazine chloride were obtained from Syntec® (Hortolândia, SP, Brazil). Animal commercial chow was from Nuvitalis® (Colombo, PR, Brazil). All other reagents were of the highest quality available.

2.3. Pharmacological assays

2.3.1. Animals

Male Swiss mice (20–30 g) and Male Wistar rats (160–200 g) bred in the Center of Reproductive Biology (Federal University of Juiz de Fora, Brazil) were used. The animals were kept under standard temperature (22 °C), 12/12 h light/dark cycle, and had food and water *ad libitum*. The groups consisted of 6–8 animals. All experimental procedures are in accordance with the Ethical Principles of Animal Research adopted by Brazilian College of Animal Experimentation (COBEA – Protocols no 013/2013, 021/2013, 016/2013 and 028/2014).

2.3.2. Topical anti-inflammatory activity on acute inflammation

2.3.2.1. Croton oil single application-induced ear edema test. This test was carried out in accordance to the method described by Schiantarelli et al. (1982). Each mouse was immobilized by hands, and 20 μL of a fresh solution of croton oil 2.5% (v/v) were topically applied on the inner surface of the right ear and the same volume of acetone (vehicle) on the inner surface of the left ear. Immediately after the application of the phlogistic agent, the animals received topical treatment with 20 μL of HF (0.1, 0.5, and 1.0 mg/ear), dexamethasone (reference drug) 0.1 mg/ear, or acetone. Four hours after the topical applications, the animals were euthanized and 6 mm diameter ear punch biopsies were collected and subjected to ear edema measurement. Subsequently, the fragments obtained from the right ears of dexamethasone, vehicle and HF 1.0 mg/ear groups were stored at −80 °C in order to measure interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) cytokines levels.

2.3.2.2. AA, EPP, capsaicin and phenol-induced ear edema tests. Each mouse was immobilized by hands. Then, 20 μL of fresh solutions of AA 2.0 mg/ear (Young et al., 1984), EPP 1.0 mg/ear (Giner et al., 2000), capsaicin 1.0 mg/mL (Gábor and Razga, 1992), or phenol 10% (v/v) (Gábor, 2000) were topically applied on the inner surface of the right ear, and 20 μL of acetone (vehicle) on the inner surface of the left ear. Immediately after the application of the phlogistic agents, the animals received topical treatment with 20 μL of HF 1.0 mg/ear, indomethacin 0.5 mg/ear (reference drug for AA test), dexamethasone 0.1 mg/ear (reference drug for EPP, capsaicin and phenol tests) or acetone. One hour after topical applications of AA, EPP and phenol, and 30 min after topical application of capsaicin, the animals were euthanized and 6 mm diameter ear punch biopsies were subjected to ear edema measurement.

2.3.2.3. Histamine-induced ear edema test. The animals were treated topically with 20 μL of HF 1.0 mg/ear, dexamethasone 0.1 mg/ear (reference drug) or acetone on the inner surface of the right ear and the same volume of acetone on the inner surface of the left ear. After 20 min, mice were anesthetized by intraperitoneal application of ketamine 80 mg/kg and xylazine 15 mg/kg. Thirty minutes after the topical treatments, 10 μL of histamine dihydrochloride 0.1 mg/μL diluted in NaCl 0.9% were applied subcutaneously with a 29-G needle syringe in the right ear of each mouse, whereas only NaCl 0.9% was injected in the left ear. Two hours after the treatments applications, the animals were euthanized and 6 mm diameter ear punch biopsies were collected for

edema measurement (Brand et al., 2002).

2.3.2.4. IL-1β, TNF-α and IL-6 levels determination. The ear biopsies obtained from the croton oil test were used to evaluate the HF ability to influence the IL-1β, TNF-α and IL-6 cytokines levels. The ears were homogenized in 800 μL of extraction buffer (BSA 0.5%, PMSF 0.1 M, benzethonium chloride 0.1 M, EDTA 10 mM, and aprotinin 20 kIU/ml) and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatants were stored and used to measure the cytokine levels using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the procedures supplied by the manufacturer. The levels of the cytokines were determined in duplicate by an ELISA reader at 450 nm.

2.3.2.5. Croton oil single application-induced ear edema test after glucocorticoid antagonism. To verify whether HF mechanism of action is glucocorticod-like, the croton oil single application-induced ear edema test was accomplished as previously described (Section 2.3.2.1) 15 min after subcutaneous injection of mifepristone, an glucocorticoid antagonist, diluted in polyethylene glycol 400 (PEG 400), at 50 mg/kg, 100 μL (Horinouchi et al., 2013). Animals were treated with vehicle (acetone), HF 1.0 mg/ear, dexamethasone 0.1 mg/ear or indomethacin 0.5 mg/ear.

2.3.2.6. Ear edema measurement and statistical analysis. The ear punch biopsies were individually weighed using an analytical balance. The weight difference between the inflamed right ear and the non-inflamed left ear of each mouse indicated the intensity of the edema. All results were expressed as mean ± S.E.M. One-way ANOVA followed by Newman–Keuls test was applied for statistical analysis using the software GraphPad Prism® 5.0. $p < 0.05$ was considered significant.

2.3.3. Topical anti-inflammatory activity on chronic inflammation

2.3.3.1. Croton oil multiple application-induced ear edema test. This test was carried out for 10 consecutive days (days 0–9) as described by Stanley et al. (1991) with minor modifications. On the day 0, each mouse was anesthetized by intraperitoneal application of ketamine 80 mg/kg and xylazine 15 mg/kg for the measurement of the natural ear thickness of the right ear using a digital micrometer. On the day 1, 20 μL of a fresh croton oil 2.5% (v/v) solution were topically applied on the inner surface of the right ear. This same procedure was conducted on alternate days. The animals were treated with 20 μL HF 1.0 mg/ear, dexamethasone 0.1 mg/ear and acetone (vehicle) twice a day, between days 5 and 9. The ear thickness was measured on alternate days (days 1, 3, 5, 7 and 9). On the day 9, animals were euthanized and 6 mm diameter ear punch biopsies were collected for edema measurement as previously described (Section 2.3.2.6) and for histopathological analysis. The results were expressed as mean ± S.E.M, and two-way ANOVA followed by Bonferroni test was applied using the software GraphPad Prism® 5.0 for statistical analyses. $p < 0.05$ was considered significant.

2.3.3.2. Histopathological analysis. Ear biopsies were fixed in 70% ethanol for 24 h and then preserved in 10% formalin. Subsequently, the ears were dehydrated, blocked in paraffin, and transversely sectioned with a microtome (4 μm). The cross-sections were stained with hematoxylin and eosin for the evaluation of edema intensity, vasodilation and leukocyte infiltration. Representative areas were selected for qualitative light microscopic analysis. Images were captured using the Image-Pro Plus® 6.0 software (40, 100 and 400 × magnification).

2.3.4. Acute dermal irritation/corrosion test

The acute dermal irritation/corrosion test for HF was conducted as recommended by the OECD Guideline 404 (2002) in rats. The animals were anesthetized with ketamine 90 mg/kg and xylazine 10 mg/kg intraperitoneally and the dorsocostal area was shaved. The next day, animals with intact skin ($n=3$) received 0.5 g of HF without any dilution, which was applied uniformly on a small shaved region (3 cm^2). The area was covered with gauze and a hypoallergenic tape. HF was maintained in contact with the skin for 4 h, and then removed. After 1, 24, 48 and 72 h all animals were examined for signs of erythema, edema, dry skin and any local or systemic clinical adverse effects, including body weight and water and food consumption, which were evaluated daily for 14 days. Distilled water was used as negative control.

2.4. Phytochemical characterization by GC–MS analysis

The phytochemical characterization of HF was performed by gas chromatography–mass spectrometry (GC–MS) analysis using Agilent Technologies GC Hewlett Packard 6890 and MS Hewlett Packard P 5972 devices. An analytical DB-5MS capillary column (30 m length × 0.25 mm inner diameter; 0.25 μm film thickness) was connected to the system. Helium was used as the carrier gas with a flow rate adjusted to 0.5 ml/min. The oven temperature was programmed as a gradient from 50 °C to 300 °C for 50 min, and an isotherm at 300 °C was maintained for more 15 min, so that the total run time used was 65 min. The injector temperature was set to 270 °C. The mass spectrometer operated by electron impact (70 eV) with the ion source at 230 °C. The individual compounds were identified by mass spectra comparison using the mass spectrometer library database (WILEY 275.1).

3. Results

3.1. Topical anti-inflammatory activity on acute inflammation

In croton oil-induced ear edema test HF, at all the doses tested, showed expressive activity, however the dose of 1.0 mg/ear was the most effective, inhibiting the edema formation by 75% (Fig. 1A). Nevertheless, HF did not show any action in AA challenge test (Fig. 1B). As shown in Fig. 1C–F, HF efficiently inhibited the EPP, capsaicin, phenol and histamine-induced ear edema by 45%, 46%, 82% and 55%, respectively.

As shown in Fig. 2, HF reduced significantly the IL-6 and TNF-α levels in ear biopsies obtained from the croton oil single application test, in a manner similar to dexamethasone. On the other hand, the levels of IL-1β were similar to the vehicle group.

HF and dexamethasone did not respond to the croton oil topical application after the pretreatment with mifepristone, a glucocorticoid receptors inhibitor, as shown in Fig. 3.

3.2. Topical anti-inflammatory activity on chronic inflammation

As shown in Fig. 4, HF and dexamethasone showed similar activity in the croton oil-induced ear edema multiple application test. The histopathological analysis emphasized that HF inhibited the edema considerably (Fig. 5A–C). Leukocytes infiltration (Fig. 5G–I) and vasodilation (Fig. 5D) were also less intense in HF group.

3.3. Acute dermal irritation/corrosion test

The rats subjected to topical application of HF in the acute dermal irritation/corrosion test showed no clinical signs of local or systemic toxicity.

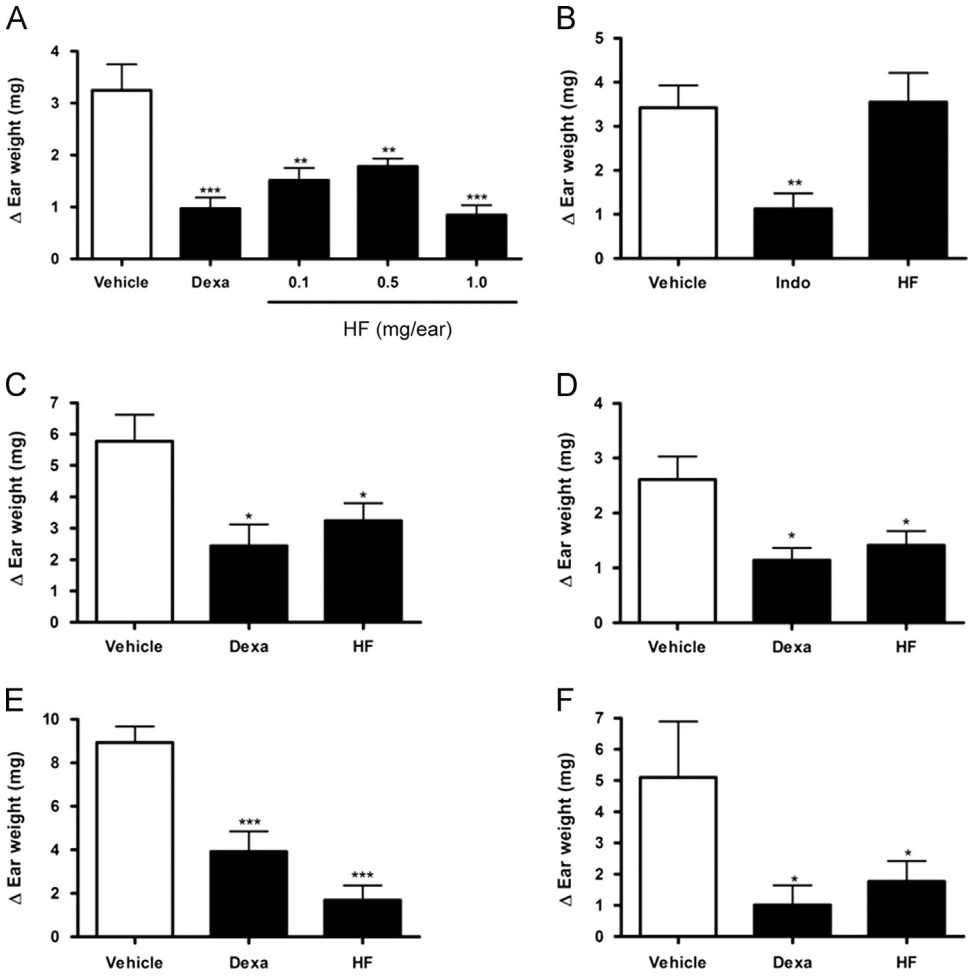

Fig. 1. Effect of HF in mice ear edema induced by different phlogistic agents. (A) croton oil single application; (B) AA; (C) EPP; (D) capsaicin; (E) phenol; (F) histamine. HF at 0.1, 0.5 and 1.0 mg/ear was used in croton oil single application test, and HF at 1.0 mg/ear was used in further studies. Dexamethasone (Dexa) or indomethacin (Indo) were used as reference drugs at 0.1 and 0.5 mg/ear, respectively. Values in each column represent the mean ± S.E.M. of the weight difference between right and lefts ears. One-way ANOVA followed by Newman–Keuls test was used for statistical analysis. Significant values: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs* vehicle.

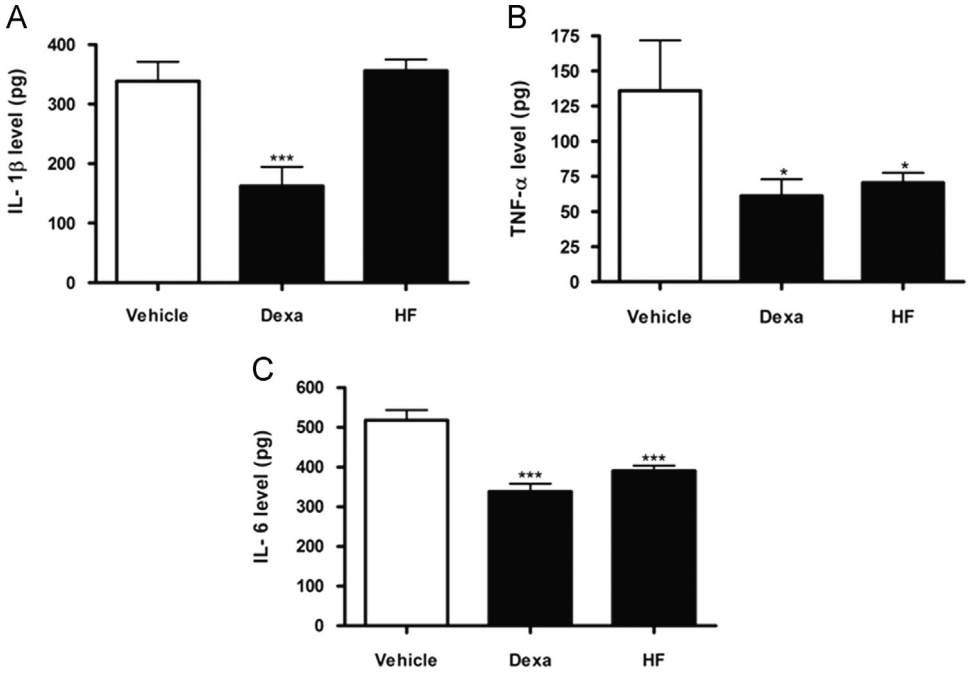

Fig. 2. Effect of HF 1.0 mg/ear on cytokines levels in ear biopsies obtained from croton oil single application test. (A) IL-1β; (B) TNF-α; (C) IL-6. Values in each column represent the mean ± S.E.M. One-way ANOVA followed by Newman–Keuls test was used for statistical analysis. Significant values: $^{*}p < 0.05$, and $^{***}p < 0.001$ *vs* vehicle.

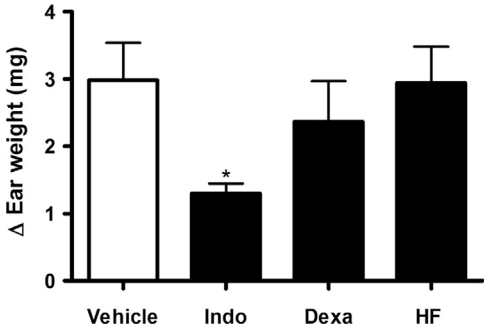

Fig. 3. Effect of HF in mice ear edema induced by croton oil after pretreatment with mifepristone. HF, dexamethasone (Dexa) and indomethacin (Indo) at 1.0, 0.1 and 0.5 mg/ear, respectively, was topically applied. Mifepristone 50 mg/kg was subcutaneously injected 15 min before croton oil single application. Values in each column represent the mean ± S.E.M. of the weight difference between right and lefts ears. One-way ANOVA followed by Newman–Keuls test was used for statistical analysis. Significant values: $^{*}p < 0.05$ *vs* vehicle.

3.4. Chemical characterization by GC–MS analysis

The GC–MS chemical analysis of HF showed considerable amounts of phytosterols, including campesterol, stigmasterol and sitosterol (Table 1).

4. Discussion

The mice ear edema is an appropriate animal model for the evaluation of topical anti-inflammatory activity of plant-related compounds as it is rapidly accomplished, provides reproducible results and requires a small amount of the natural substances, which obtainment is, in some cases, time-consuming and expensive (Gábor, 2003).

The main irritant constituent in croton oil, 12-o-tetradecanoilphorbol-13-acetate (TPA), is an activator of the protein kinase C (PKC) and the mitogen-activated protein kinases (MAPK), which stimulate the release of transcription factors, such as the nuclear factor κB (NF-κB) and the activating protein-1 (AP-1), which are essential for the activation of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6, and other mediators, such as phospholipase-A_2, (PLA_2), platelet activating factor (PAF) and arachidonic acid (AA). These mechanisms increase the vascular permeability, vasodilation, leukocyte migration, release of histamine and serotonin, and the levels of eicosanoids synthesized by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes (Pascual and Glass, 2006; Saraiva et al., 2011). As several different inflammatory pathways are stimulated by croton oil, anti-inflammatory drugs with distinct mechanisms of action may respond effectively to this model (Murakawa et al., 2006; Garg et al., 2008). For this reason, this phlogistic agent was used in this study to identify HF anti-inflammatory activity and to evaluate its dose–response using three different doses. The dose of 1.0 mg/ear was the most effective (Fig. 1A) and, for that reason, it was used in the further tests to better understand HF anti-inflammatory action.

AA, as an inductor of skin inflammation, stimulates the eicosanoids production, including PGE_2 and leukotrienes, which are synthesized by COX and 5- LOX enzymes respectively, and induces a rapid and intense inflammatory response. Therefore, AA is often used to identify compounds capable to suppress the eicosanoids synthesis, as indomethacin, a COX inhibitor (Carlson et al., 1985; Crummey et al., 1987; Gábor, 2000). As HF clearly did not respond to this test (Fig. 1B), it can be concluded that its anti-inflammatory mechanism is not related to the AA metabolism, which encouraged further studies to better understand its action. EPP topical application induces an inflammatory process by AA pathway; however this phlogistic agent also elicits the release of histamine, serotonin and bradykinin relevantly, activating mainly a vascular response (Brattsand et al., 1982; Just et al., 1998). As shown in Fig. 1C, HF inhibited the EPP-induced edema formation, which suggested that HF may have influence in EPP pro-inflammatory mediators, including histamine. For this reason, histamine was also used as an irritant agent in this study.

Histamine is an endogenous vasoactive amine which is released by mast cell degranulation and increases vasodilation and vascular permeability in the course of the inflammatory process, leading to plasma extravasation, erythema and nerve fibers sensitization. In addition, histamine regulates the release of several cytokines, including IL-1β, TNF-α and IL-6 (Brand et al., 2002; O'Mahoni et al., 2011). Antihistaminergic and glucocorticoids drugs are frequently effective to reduce histamine-induced edema (Saraiva et al., 2011). As shown in Fig. 1F, HF was efficient in the histamine challenge test.

Capsaicin is the chemical constituent responsible for the pungency of peppers of the genus *Capsicum*. This substance has the ability to activate the transient receptor potential vanilloid type 1 (TRPV1), an ion channel expressed in sensory neurons. TRPV1 activation induces the release of neuropeptides, including substance P and tachykinin, and monoamines, such as serotonin and histamine. Those mechanisms lead to an increase in edema formation, blood flow and leukocytes recruitment (Szolcsányi, 2004; Zegarska et al., 2006; Alawi and Keeble, 2010). Thus, capsaicin-induced ear edema model is adequate to identify compounds capable to inhibit a neurogenic inflammatory process, which is caused by a nociceptive stimulus (Alawi and Keeble, 2010). As shown in Fig. 1D, HF reduced efficiently the capsaicin edema

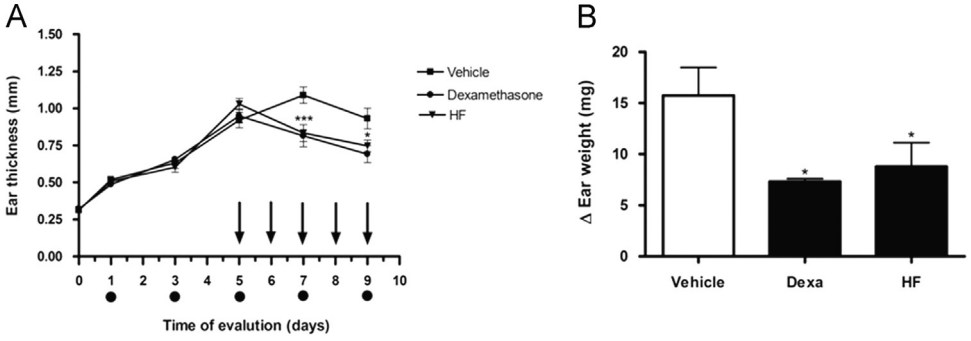

Fig. 4. Effect of HF 1.0 mg/ear in mice ear edema induced by croton oil multiple application test. Dexamethasone (Dexa) at 0.1 mg/ear was used as reference drug. (A) time–response curve on ear thickness during 10 consecutive days; the arrows indicate the days in which the animals were treated, and the circles indicate the days in which the ears thickness were measured; the values represent the mean ± S.E.M of ear thickness; two-way ANOVA followed by Newman–Keuls test was used; (B) effect on ear weight on the last day; values in each column represent the mean ± S.E.M of the weight difference between right and lefts ears; one-way ANOVA followed by Newman–Keuls test was used for statistical analysis. Significant values: $^{*}p < 0.05$, and $^{***}p < 0.001$ *vs* vehicle.

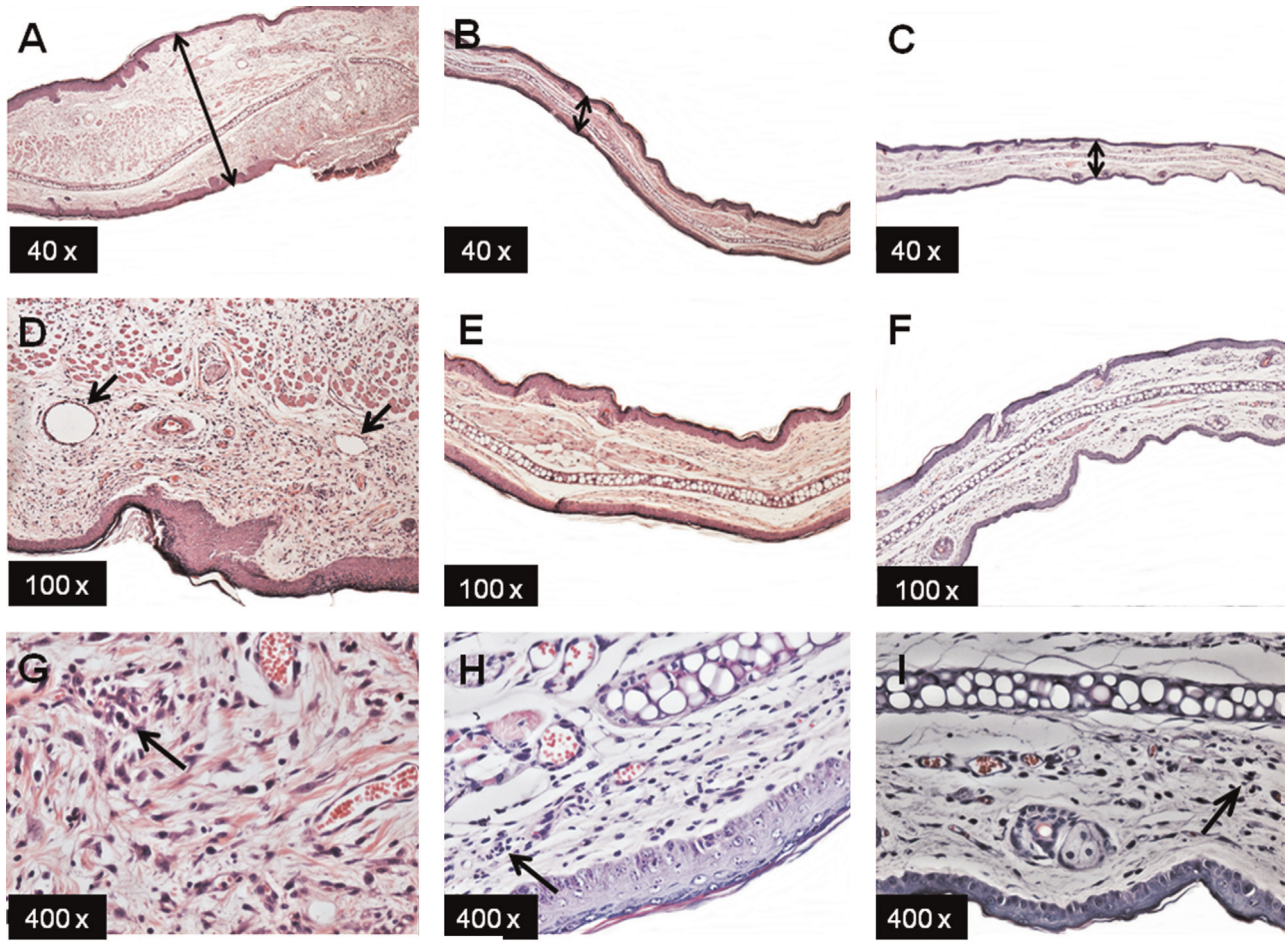

Fig. 5. Representative photomicrographs of transverse sections of mice ear biopsies obtained from the croton oil multiple application ear edema test on the day 9 (40, 100 and 400 × magnification; H&E staining). (A) vehicle 40 × ; (B) dexamethasone 0.1 mg/ear 40 × ; (C) HF 1.0 mg/ear 40 × ; (D) vehicle 100 × ; (E) dexamethasone 0.1 mg/ear 100 × ; (F) HF 1.0 mg/ear 100 × ; (G) vehicle 400 × ; (H) dexamethasone 0.1 mg/ear 400 × ; (I) HF 1.0 mg/ear 400 × . In (A), (B) and (C), the double arrows indicate the edema intensity; in (D) the arrows indicate intense vasodilation; in (G), (H) and (I) the arrows indicate the presence of inflammatory cells. Images were captured using the Image-Pro Plus® 6.0 software.

formation. Glucocorticoids and antihistaminergic drugs may be effective in this test; however COX inhibitors are not capable to respond (Inoue et al., 1993, 1995a, 1995b). Therefore, the results for capsaicin test were important to identify the neurogenic anti-inflammatory potential of HF, and also to corroborate to the other findings presented in this study, more particularly regarding to the lack of HF activity in the AA challenge test.

The inflammatory reaction and tissue damage induced by

Table 1
Chemical constituents identified in HF by GC–MS analysis.

Retention time (min)	Compound	Molecular weight	Area (%)	Main MS fragmentation ions
9.199	Decane	142	0.72	142→113, 85, 71, 57, 43
10.714	4,5-Dimethyl-2,6-octadiene	138	1.11	138→123, 95, 69, 41
12.041	Undecane	156	0.53	156→127, 85, 71, 57, 43
23.516	Nerolidol	222	0.70	222→204, 161, 93, 69, 41
26. 774	1-Methoxy-p-tolyl-propan-2-ol	181	6.81	181→144, 116, 103, 87, 73
30.499	Neophytadiene	278	1.19	278→123, 95, 68, 43
32.308	Methyl palmitate	270	2.34	270→227, 143, 87, 74, 55, 43
32.962	Palmitic acid	256	0.89	256→213, 129, 73, 60, 43
35.648	Linolenic acid methyl ester	292	1,62	292→108, 95, 79, 67, 55, 41
35.821	Phytol	297	4.51	297→123, 95, 81, 71, 57, 43
36.136	Methyl octadecanoate	298	0.44	298→143, 87, 74, 55, 43
39.186	Tricosane	324	0.81	324→113, 99, 85, 71, 57, 43
51.290	Cholesterol	386	1.89	386→301, 275, 213, 145, 44
52.969	Campesterol	400	8.97	400→382, 367, 315, 289, 213, 145, 43
53.415	Stigmasterol	412	6.89	412→351, 271, 255, 159, 133, 83, 69, 55
54.556	Sitosterol	414	24.66	414→396, 329, 303, 213, 145, 107, 81, 57, 43
55.117	Taraxerol	426	7.12	426→302, 287, 204, 135, 95, 69
56.453	Taraxasterol	426	11.59	426→207, 189, 135, 95, 69, 43
Total			84.25	

The individual compounds were identified by mass spectra comparison using the mass spectrometer library database (WILEY 275.1).

topical application of phenol are related to the reactive oxygen species (ROS) production and to the rupture of keratinocytes membranes, which elicits the release of pro-inflammatory cytokines, including IL-1α, IL-1β, IL-6, IL-8 and TNF-α (Wilmer et al.,1994; Murray et al., 2007). Skin enzymes, such as COX-2, tyrosinase and LOX, generate a favorable microenvironment for oxidation of the phenol molecule, so that phenol is an appropriate irritant agent to simulate the contact dermatitis inflammation in mice (Lim et al., 2004; Murray et al., 2007). Skin toxic phenolic compounds are often used in resins, paints, varnishes and cosmetic products. Consequently, industrial workers and consumers are commonly exposed to these agents, which may cause several disorders, including erythema, edema, burns, necrosis, skin depigmentation and cancer (Murray et al., 2007). Thus, the use of phenol induced-ear edema model is also relevant in the search for topical anti-inflammatory drugs. HF showed expressive activity in this test (Fig. 1E), which suggested its remarkable potential for the treatment of contact dermatitis. This action may be related to the antioxidant activity of *P. aculeata* leaves reported by Pinto et al. (2012), and to the inhibition of pro-inflammatory cytokines.

Therefore, it was evaluated whether HF is capable to interfere in cytokine levels in an inflamed tissue. As shown in Fig. 2, HF reduced significantly the IL-6 and TNF-α levels in ear biopsies obtained from the croton oil single application test. However, the levels of IL-1β were similar to the vehicle group, which is not surprising, as this cytokine is closely related to the activation of COX enzymes (Akira et al., 1990; Rider et al., 2011), and the results presented in this study strongly suggested that HF did not interfere in the AA metabolism.

The croton oil multiple application test is useful to assess a drug response in chronic inflammatory process. Glucocorticoids and 5-LPOX inhibitors respond efficiently to this test; however COX inhibitors and antihistaminergic drugs are not effective (Green and Shuster, 1987; Stanley et al., 1991). The results of this test (Figs. 4 and 5), along with those obtained from the acute inflammation models, strongly indicated that HF mechanism of action was probably due to a glucocorticoid-like effect. To confirm this proposition, the croton oil single application test was repeated, however, at that time, the animals were pretreated with mifepristone, a glucocorticoid antagonist (Horinouchi et al., 2013). Once the glucocorticoid receptors were inhibited, HF and dexamethasone did not respond to the croton oil test, unlike indomethacin, which mechanism of action is related to COX inhibition (Fig. 3). This result confirmed that, at least in part, HF anti-inflammatory effect was due to glucocorticoid receptors activation.

HF showed no clinical signs of toxicity in the acute dermal irritation/corrosion test. It is noteworthy to mention that *P. aculeata* leaves are commonly used as food in Brazilian traditional cuisine and as a topical remedy in folk medicine, and there are no reports of skin or systemic adverse effects.

The chemical analysis of HF showed considerable amounts of phytosterols (Table 1). The chemical structure of those compounds are very similar to glucocorticoids, which may suggest similar biological actions (Dewick, 2009). In fact, phytosterols anti-inflammatory activities are well described in literature. Their ingestion decreases plasma levels of pro-inflammatory components, including C-reactive protein, phospholipase A_1, IL-6, TNF-α, NF-kB and other cytokines and transcription factors (Othman and Moghadasian 2011). In addition, many studies have reported the efficacy of plant sterols as topical anti-inflammatory agents (Gómez et al., 1999; Navarro et al., 2001; Medeiros et al., 2007; Puglia and Bonina, 2008).

Taraxerol and taraxasterol are anti-inflammatory triterpenes that were also identified in HF. These compounds have been isolated from a variety of plants, and have shown the property of decreasing the production of NO, PGE_2, NF-kB, TNF-α, IL-6 and IL-1β in vitro (Zhang et al., 2012; Yao et al., 2013). Besides, Akihisa et al. (1996) reported the anti-inflammatory activity of taraxerol and taraxasterol in TPA-induced ear edema model in mice.

Phytol may also contribute to HF activity, as Silva et al. (2013) recently reported that this diterpene was able to inhibit rats paw edema, leukocyte migration, oxidative stress and IL-1β and TNF-α cytokines.

5. Conclusion

HF showed intense chronic and acute topical anti-inflammatory activity against different irritant agents with no clinical signs of toxicity. Its mechanism of action is related, at least in part, to the inhibition of IL-6 and TNF-α cytokines. In addition, the pharmacological results strongly suggested a glucocorticoid-like effect, which was reinforced by the presence of significant amounts of phytosterols. Taraxerol, taraxasterol and phytol may also contribute to the HF activity. For this reason, future studies aimed to evaluate typical adverse reaction related to glucocorticoids are important. This study is the first report on the anti-inflammatory activity of *P. aculeata* leaves and corroborates to better understand its ethnopharmacological applications.

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