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Effect of pequi tree Caryocar coriaceum Wittm. leaf extracts on different mouse skin inflammation models: inference with their phenolic compound content

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Caryocar coriaceum Wittm. (pequi) has been popularly used in Northeastern Brazil in the treatment of inflammation, pain and respiratory affections. This study evaluated the topical anti-inflammatory activity of C. coriaceum hydroethanolic extract (CCHE) and methanolic fraction (CCMF) from pequi tree leaves against different skin sensitizer agents (arachidonic acid, croton oil, phenol and histamine), as well their antioxidant activity and phenolic compound profile. High performance liquid chromatography with diode array detection (HPLC-DAD) analysis pointed the presence of chlorogenic acid, rutin, quercetin and lower concentrations of caffeic and gallic acids. Both CCHE and CCMF 1 mg/ear demonstrated significant topical anti-inflammatory effect against arachidonic acid, phenol and histamine single application (antiedematous effect ranging from 48 to 69% for CCHE and 36 to 64% for CCMF; P < 0.05 vs. negative control). In contrast, both extracts did not antagonize the croton oil single application-induced ear edema when compared with the control group (P > 0.05). Additionally, both extracts exhibited strong DPPH free radical scavenging activity in vitro (EC50 = 5.02 ± 4.37 μg/ml for CCMF and 6.06 ± 4.03 μg/ml for CCHE). Together, the results give important support that CCHE and CCMF (and their phenolic compounds, according to previous data from literature) could exert topical anti-inflammatory activity by possibly modulate the local production of inflammatory mediators including histamine, arachidonic acid metabolites and reactive oxygen species.

Key words: Topical anti-inflammatory activity, mouse ear edema, dermatitis, Caryocar coriaceum, polyphenols, skin disorders.
INTRODUCTION

The skin is a large and complex organ that is able to provide a set of defense mechanisms in response to a variety of external stimuli such as physical injury, microbial invasion, environmental pollutants or solar irradiation (Freinkel and Woodley, 2000). These defense mechanisms are normally supposed to repair the damaged tissue or destroy the hazard agent (Lawrence and Gilroy, 2007). However, a misdirected or inappropriate immune activity can lead to a set of different inflammatory skin disorders, including atopic dermatitis, eczema and psoriasis (Novak and Leung, 2011; Stevenson and Lebwohl, 2011).

Skin inflammation is produced and maintained by the interaction of various inflammatory cell populations that migrate to the injury site in response to a variety of released pro-inflammatory mediators such as cytokines, interleukins, neuropeptides, histamine, serotonin, prostaglandins, leukotrienes, reactive oxygen species, among others (Sacca et al., 1997). Hence, the modulation on the production of these pro-inflammatory mediators has been applied in the treatment of cutaneous disorders. For this purpose, drugs including glucocorticoids, antihistamines and non-steroidal anti-inflammatory agents are employed. However, these drugs have demonstrated some limitations since they can exhibit some undesirable side effects and cannot be effective in all cases (Kupper and Fuhlbrigge, 2004; Schoepe et al., 2006).

Additionally, a steadily growing interest has been noticed in skin protection from excessive inflammatory insults by phenolic compounds, since their benefits are largely attributed to their classical chain-breaking antioxidant or free radical scavenging activities (Ismail et al., 2004; Nazaruk, 2008; Potapovich et al., 2011).

Caryocar coriaceum Wittm. (Caryocaraceae) is a tree widely found in Cerrado (savannah) areas from Araripé plateau, Southern Ceará State, Northeastern Brazil (Costa et al., 2004). Its fruit, popularly known as “pequi”, is used as food by local population and it is a source of essential nutritional components including antioxidant vitamins (A and E) and unsaturated fatty acids (De Oliveira et al., 2010; Sena Jr. et al., 2010). Previous pre-clinical studies evidenced that the fixed oil from “pequi” pulp fruit presents gastroprotective, wound healing (Quirino et al., 2009) and topical anti-inflammatory activities in vivo (Saraiva et al., 2011a) and potential synergistic antibacterial effects when combined with antibiotic drugs (aminoglycosides) in vitro (Saraiva et al., 2011b).

Taking into account the ethnopharmacological evidences in relation to the use of pequi tree leaves as an herbal drug, we were encouraged to investigate the potential therapeutic use of C. coriaceum leaves hydroethanolic extract (CCHE) and methanolic fraction (CCMF) by evaluating their topical anti-inflammatory effect on different acute and chronic cutaneous inflammation models in mice; besides, to unveil the possible mechanisms of action involved in their activity.

MATERIALS AND METHODS

Plant

Leaves from C. coriaceum Wittm. trees were previously collected in a Cerrado area from Araripé plateau, municipality of Crato, Ceará State, Brazil (S 7°21'53,1"; W 39°28'42,6") in March, 2010. A voucher specimen was prepared, identified by Prof. Dr. Lígia Q. Matias (Federal University of Ceará) and deposited in the Prisco Bezerra Herbarium (Federal University of Ceará, Brazil), under the number 44523.

Animals

Swiss mice (Mus musculus) from both sexes (25 to 35 g), with free access to water and food (Rodent Chow Labina, Brazil) and housed in standard polypropylene cages under controlled conditions of temperature (22 ± 2°C) and 12 h light/dark cycle, were used in this study. All the protocols concerned in this research were previously evaluated and approved by Research Ethics Committee from Fortaleza University (UNIFOR, Brazil), under number 10 to 020.

Chemicals and instruments

All chemicals were of analytical grade. Acetone and ethanol were purchased from Dinâmica (Brazil). Methanol, acetic acid, gallic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Croton oil, arachidonic acid, phenol, histamine, indomethacin, quercetin, rutin, gallic acid, chlorogenic acid and L-ascorbic acid were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (Decadron®) was purchased from Aché (Brazil). Ketamine chloride and xylazine chloride were purchased from Syntec (Brazil). High performance liquid chromatography diode array detection (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1.

Extraction and fractionation procedures

The extract from C. coriaceum leaves was first obtained by CCHE, in contact with a solution of ethanol and water at ratio 1:1, for a period of 72 h (room temperature). After that, CCHE was filtered, and the solvent was evaporated in vacuum at 50°C. CCMF was obtained from CCHE, using 90% methanol extraction and then evaporated in vacuum. After that, the residues was freeze-dried by

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DPPH free radical scavenging assay

The antioxidant activity of the extracts was evaluated by monitoring their ability in quenching the stable free radical 2,2-diphenyl-1-pikrylhydrazyl (DPPH), according to Choi et al. (2002), with minor modifications. CCHE or CCMF at concentrations ranging from 0.78 to 100 μg/ml diluted in ethanol vehicle were incubated in absence (blank) or presence of 0.3 mmol/L DPPH' ethanol solution in the dark and at room temperature. Vitamin C (L-ascorbic acid) and the flavonoid rutin at concentrations ranging from 0.78 to 100 μg/ml were used as positive controls. After 30 min, the absorbance was measured at 518 nm.

The concentration of DPPH' free radicals (DPPH'), in the percent of control) in the absence or presence of compounds was calculated using the following equation: [DPPH'] = [(AbsSample - AbsBlank) × 100/AbsControl], where AbsSample is the absorbance obtained in the presence of different CCHE or CCMF concentrations in the presence of DPPH' ethanol solution, AbsBlank is the absorbance obtained in the presence of different CCHE or CCMF concentrations in the absence of DPPH' ethanol solution (blank) and AbsControl is that obtained in the absence of extracts and in the presence of DPPH' ethanol solution without any extract or positive control. The free radical scavenging capacity of CCHE and CCMF was calculated as their EC_{50} values (the concentration necessary to inhibit 50% radical formation), using non-linear regression fit of plots where the abscissa axis represented the logarithm of concentration of tested plant extracts and the ordinate axis of the mean percentage of concentration of free radicals DPPH' in the well after 30 min. Tests were carried out in quadruplicate.

Arachidonic acid, croton oil and phenol single application-induced mouse ear edema

In order to evaluate the topical anti-inflammatory effect of CCHE and CCMF with 1 and 2 mg/ear or 50 and 100 mg/ml, different acute models of cutaneous inflammation in vivo (Gábor, 2003; Ferreira et al., 2010) were performed. Swiss mice (n = 7/group) received 20 μl of arachidonic acid 0.1 mg/μl, 5% croton oil (v/v) and 10% phenol (v/v) diluted in acetone onto the inner and outer surfaces of the right ears previously pre-treated with a topical application of CCHE or CCMF diluted in ethanol (20 μl/ear), ethanol (negative control, 20 μl/ear), indomethacin (positive control for arachidonic acid), 2 mg/ear) or dexamethasone (positive control for phenol and croton oil, 0.08 mg/ear). The left ear received 20 μl of acetone (vehicle for irritant agents). The ear edema was evaluated 1 h after arachidonic acid, 1 h after phenol and 6 h after croton oil single application.

At the end of the stipulated period of exposure to each of the irritant substances, the mice were killed by cervical dislocation. Six-millimeter diameter sections of the right and left ears were removed using a circular punch and weighed on a precision balance. The extent of the edema was expressed as the difference between the weight (in mg) of the section removed from the right ear (which received the irritant agent) and the weight (in mg) of the section removed from the left ear (which received vehicle acetone). The percentage of antiedematous effect (% was calculated using the following formula:

\[
\text{Inhibition} \% = 100 - \left[ \frac{A \times 100}{B} \right]
\]

where "A" is the mean of edema weight (mg) of the group treated with the drug (CCHE, CCMF, indomethacin or dexamethasone) and "B" is the mean of edema weight in the negative control.

Histamine subcutaneous application-induced mouse ear edema

In this model, mice (n = 7/group) were previously anesthetized with ketamine 20 mg/kg intraperitoneally (i.p.) and xylazine 10 mg/kg i.p. After that, their right ears were treated topically with ethanol (20 μl/ear, negative control), dexamethasone (0.08 mg/ear, positive control), CCHE or CCMF diluted in ethanol (1 and 2 mg/ear). Fifteen minutes later, the edema was induced on the right ear by intradermal application of 5 μl of histamine dihydrochloride 0.1 mg/μl using a syringe with a 29G-hypodermical needle, while the left ear received 5 μl of saline solution by the same procedure described (Sham). The ear edema was evaluated and measured 2 h after the histamine solution application by the same procedures described in section 2.7 (Brand et al., 2002).

Croton oil multiple application-induced mouse ear edema

The following model is representative of a chronic cutaneous inflammation (Stanley et al., 1991), with duration of 9 days (days 0 to 8). Croton oil 5% (v/v) in acetone (20 μl/ear) was applied on the right ears and acetone on the left ears of Swiss mice (n = 6/group) with a micropipette on alternate days (days 0, 2, 4, 6 and 8). On day 4 to 7, the mice were treated on the inner and outer surfaces of the right ear with CCHE or CCMF 1 mg/ear, saline solution (NaCl 0.9%, 20 μl/ear, negative control) or dexamethasone (0.08 mg/ear, positive control) twice a day. The ear edema was evaluated 4 h after the first application of croton oil solution and daily by measuring the ear thickness with a digital caliper. The digital caliper was applied near the tip of the ear just distal to the cartilaginous ridges and the thickness was recorded in micrometer.
Figure 1. Profile of phenolic compounds by high-performance liquid chromatography (HPLC) of CCHE (A) and CCMF (B). Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4) and quercetin (peak 5).

Figure 2. Structures of phenolic compounds identified in CCHE and CCMF by HPLC-DAD. Gallic acid (1), chlorogenic acid (2), caffeic acid (3), quercetin (4) and rutin (5).

Statistical analysis

The results are expressed as mean ± standard error of mean (SEM). The comparison between groups was assessed by one-way analysis of variance (ANOVA) followed by Student–Newmann–Keuls test or by two-way ANOVA followed by Bonferroni test (repeated measures) when appropriated. Values of p < 0.05 were accepted as statistically significant.

RESULTS

Phytochemical studies: Phenolic compound content

According to HPLC analysis (Figures 1 to 2 and Table 1), the presence of gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), the flavonoids rutin (peak 4) and quercetin (peak 5) and other minority compounds (peaks not identified) were detected. Chlorogenic acid (peak 2) and rutin (peak 4) represent the major compounds present in CCMF and CCHE, respectively.

Antioxidant effect of CCHE and CCMF on DPPH free radicals scavenging activity in vitro

CCHE and CCMF exhibited strong DPPH• scavenging activity, which was statistically significant from the concentration of 3.13 (p < 0.01 vs. negative control for CCHE and p < 0.001 vs. negative control for CCMF) to 100μg/ml (p < 0.001 vs. negative control). The positive controls rutin demonstrated a significant DPPH• scavenging activity from the concentration of 1.56 (p < 0.05 vs. negative control) to 100μg/ml (p < 0.001) and L-ascorbic acid from 12.5 to 100μg/ml (p < 0.001 vs. negative control). The EC50 values estimated for CCMF, CCHE, rutin (RUT) and L-ascorbic acid (VITC) were, respectively, 5.02 ± 4.37, 6.06 ± 4.03, 9.225 ± 5.52 and 77.76 ± 20.22μg/ml (Figure 3).

Effect of CCHE and CCMF on arachidonic acid, croton oil, phenol and histamine single application-induced ear edema

The topical single application of arachidonic acid, croton oil, phenol and histamine caused a significant inflammatory response in mouse ears in comparison to ears that received the correspondent vehicle of their respective irritant agent, as determined by the increase of ear weight or thickness. When used as positive control, both the corticosteroid dexamethasone (DEX) and the
non-steroidal anti-inflammatory drug indomethacin (IND) significantly reduced the mouse ear edema when compared with vehicle-treated group (Figures 4 and 5). As shown in Figure 4 and Table 2, both CCHE and CCME at concentrations 1 and 2 mg/ear exhibited significant topical anti-inflammatory activity that reduced inflammation edema in mouse ears caused by arachidonic acid (Figure 4A), phenol (Figure 4C) and histamine (Figure 4D). Although the comparison between the anti-inflammatory effect of both CCHE and CCMF extracts at 1 and 2 mg/ear did not significantly differ on arachidonic acid and phenol-induced edema models (Figure 4A and C), CCHE had better antiedematous effect when compared with CCMF in histamine-induced ear edema model (Figure 4D). In contrast, CCHE and CCME did not reduce the croton oil-induced edema as compared to negative control group, however, this is almost certainly experimental variation between protocols, mice or specific croton oil sample (or all three factors) (Figure 4B). The anti-inflammatory effect of both CCHE and CCME has demonstrated a profile of action similar to that observed using the control IND what can suggest that the participation of anti-inflammatory activity involves the path of prostaglandins and cytokines.

**Effect of CCHE and CCMF on croton oil multiple application-induced ear edema**

The alternate application of 5% croton oil on right ears of mice during the days 0 to 4 significantly increased the ear thickness in comparison to left ears of mice (treated with acetone), maintaining the inflammatory process during all the procedure. It was observed that the application of CCMF on days 4 to 8 (in an established inflammatory process by previous application of croton oil solution) caused a significant reduction (but slight) in ear thickness when compared with negative control group 48 h after the first treatment with CCMF and subsequent days (days 6 to 8) (Figure 5). Dexamethasone was effective in significantly reducing the established edema 24 h after its first application (on days 5 to 8) (Figure 5). The CCHE did not decrease the ear edema as compared to the negative control (Figure 5). The effect following topical administration did not exhibit anti-inflammatory activity, because tannins are capable of coagulate with lipid-protein present in the skin leading to complexes formations. Tannins also aid vasoconstriction, reducing vascular permeability; these facts can be responsible for decreased dermal absorption that leads to the low anti-inflammatory action. On the other hand, this result corroborated with tannin levels present in the CCHE and CCMF (Table 1) since the levels of tannins present CCHE 30% higher than as shown by CCMF.

**DISCUSSION**

The screening of possible natural therapeutic agents from plants in animal models has been used as an efficient strategy in the discovery of novel and safe anti-inflammatory drugs. The croton oil-induced ear edema has been widely used as acute or chronic skin inflammation models in vivo (Stanley et al., 1991; Gábor, 2003). A single application is able to activate protein kinase C (PKC), which in turn activates other enzymatic cascades, such as mitogen activated protein kinases (MAPK) and phospholipase A 2 (PLA2), leading to release of platelet activation factor (PAF) and arachidonic acid (Gábor, 2003). Concomitantly, also induces the activation of several inducible nitric oxide synthase (iNOS)-dependent intracellular signaling pathways that present a key role in the control of inflammatory response in the skin (Medeiros et al., 2009). As consequence, this set of events leads to vascular permeability, vasodilation, polymorphonuclear leukocytes migration, release of histamine and serotonin, and moderate synthesis of eicosanoids (Murakawa et al., 2006).

The use of repeated croton oil application (chronic model) is also associated with intense neutrophil and macrophage infiltration, T cells (CD4+ and CD8+) migration and hyperproliferative epidermis (acanthosis) (Stanley et al., 1991). Corticosteroids and 5-LOX inhibitors decrease the ear edema in these models while anti-histamines and cyclooxygenase (COX) inhibitors show little or no effect (Green and Shuster, 1987). CCMF and CCHE did not have anti-edematous effect against croton oil single application-induced ear edema at concentrations tested. However, CCMF exhibited a slight but significant
Figure 4. Effect of CCHE, CCMF, indomethacin (IND) or dexamethasone (DEX) on mice ear edema induced by the arachidonic acid (A), croton oil (B), phenol (C) and histamine (D) single application. Ears from control group received ethanol vehicle as treatment. Each point represents the mean ± SEM of 7 mice. Means with different lowercase letters differ significantly at least at p < 0.05 (One-Way ANOVA followed by Student-Newman-Keuls test).

anti-edematous effect on croton oil multiple application-induced ear edema (Figure 5).

It is accepted that the inflammatory process also leads to a situation of oxidative stress, where reactive oxygen species (ROS) such as superoxide anion (O$_2^•^−$), hydroxyl (OH$^•$) and peroxyl (ROO$^•$) radicals are generated by neutrophil and macrophage cellular infiltration (Khodra and Khalila, 2001). The mechanism of inflammation injury was attributed by damage of macromolecules and lipid peroxidation of cell membranes (Parejo et al., 2003). In addition, ROS can also stimulate the release of cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and interferon (IF)-γ, which stimulate the recruitment of additional neutrophils and macrophages.

HPLC analysis identified in both extracts the presence of chlorogenic acid, rutin, quercetin and lower concentrations of gallic acid and caffeic acid (Figure 1 and Table 1). The beneficial effect of phenolic compounds on skin has been largely attributed to their classical antioxidant properties, which could decrease the levels of ROS during an inflammatory process (Geronikaki and Gavalas, 2006). Furthermore, several anti-inflammatory drugs have been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity (Sosa et al., 2002; Fu et al., 2010). According to DPPH free radical scavenging activity in vitro, both CCHE and CCMF exhibited strong antioxidant activity as compared to known antioxidants rutin (found in both extracts) and vitamin C (Figure 3).

Arachidonic acid is a precursor of inflammatory eicosanoids such as prostaglandin E$_2$ (PGE$_2$) and leukotrienes via COX-2 and 5-lipoxygenase (5-LOX) enzymes, respectively. Gallic acid alone is considered a contact skin sensitizer (Basketter et al., 1999), recent studies demonstrated that gallic acid can inhibit COX-2 activity in vitro and decrease PGE$_2$ and TxB$_2$ levels at 100 and 200 μmol/L, while increase TNF-α and IL-1β levels only at 200 μmol/L (Del Bufalo et al., 2011). In both extracts used in this study, the concentration of gallic acid is lower in comparison with other phenolic compounds (Figure 1 and Table 1).

Quercetin at 2 mg/ear showed a higher activity against arachidonic acid-induced ear edema than croton oil-induced edema by topical application in vivo (Yasukawa et al., 1989; Kim et al., 1993). COX and LOX inhibitors, leukotriene antagonists, anti-histamines and
immunophilin-ligands are active in reducing the arachidonic acid-induced ear edema, whereas corticosteroids present slow effect in this model (Tramposch, 1999). Quercetin is pointed as a dual COX/LOX inhibitor (Laughton et al., 1991; You et al., 1999) and can also inhibit the histamine release in basophils activated with anti-IgE or with the calcium ionophore A23187 (Trinh et al., 2010). Caffeic acid is considered a classical 5-LOX inhibitor (Boudreau et al., 2012). Both CCHE and CCMF were able to decrease the arachidonic acid-induced ear edema when compared with negative control group (Figure 4A).

Histamine is a vasoactive amine involved in immediate type-hypersensitivity reactions and also plays a key role in human allergic reactions (Lee et al., 2012). It is released by mast cells activated by C3a and C5a protein complements, IgE-activated lymphocytes and arachidonic acid, which increases vascular permeability and vasodilation (Brand et al., 2002). Recent in vitro studies have demonstrated that chlorogenic acid blunted LPS-induced nitric oxide (NO), PGE2, and intracellular ROS production in RAW 264.7 cells (Yu et al., 2009) as well as inhibit compound 48/80-induced systemic anaphylactic shock in mice and anti-dinitrophenyl (DNP) IgE-mediated passive cutaneous anaphylaxis, besides to reduce histamine, cytokines from allergy and TNF-α release from RBL-2H3 cells activated by anti-DNP IgE (Qin et al., 2010; Trinh et al., 2010). Antihistamines and corticosteroids (dexamethasone) can decrease the ear edema in this model (Brand et al., 2002). Both extracts showed a significant antiedematous effect against histamine induced ear edema in mice (Figure 4D).

Phenol is an irritant agent able to mimic pathophysiological conditions similar to contact dermatitis in mice (Lim et al., 2004). A single application of a 10% phenol solution on skin is able to disrupt keratinocyte membranes, leading to release of cytokines such as interleukin (IL)-1α, tumor necrosis factor (TNF)-α and IL-8 via protein kinase C (PKC)-independent mechanism, which in turn release other inflammatory mediators, such as histamine, arachidonic acid metabolites and ROS (Wilmer et al., 1994; Murray et al., 2007). Both CCHE and CCMF decreased the phenol-induced ear edema (Figure 4C).

Taking together the results presented here, our data give important support that CCHE and CCMF could be used as topical anti-inflammatory herb by possibly exerting modulation on production of inflammatory...
mediators such as histamine, reactive oxygen species and arachidonic acid metabolites. The continual efforts on understanding of the potential use of *C. coriaceum* leaves as herb will provide new insight into the anti-inflammatory activity of its extracts, and eventually lead to development of safe and effective treatment on cutaneous inflammation.

**Conflict of Interest**

There is no conflict of interest.

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