Topical application of the hexane fraction of *Lacistema pubescens* reduces skin inflammation and cytokine production in animal model

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**Keywords**
cytokine; dermatoxicity; *Lacistema pubescens*; myeloperoxidase; skin inflammation

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**Received** February 5, 2015  
**Accepted** June 21, 2015

doi: 10.1111/jphp.12463

**Abstract**

The aim of this study was to investigate the acute topical anti-inflammatory effect of the hexane fraction (HLP) of *Lacistema pubescens* in mice.

**Methods** Ear oedema models induced by croton oil, arachidonic acid, phenol, histamine, ethyl phenyl propiolate and capsaicin. Histopathological analyses of ear tissue samples sensitized with croton oil were performed. Myeloperoxidase activity (MPO), the pro-inflammatory cytokine-inhibitory effect and dermatoxicity were also evaluated.

**Key findings** HLP (1, 0.5 and 0.1 mg/ear) resulted in a substantial reduction in skin thickness or tissue weight on all models tested, except for capsaicin-induced ear oedema, similar to dexamethasone (0.1 mg/ear) and/or indomethacin (0.5 mg/ear). Histopathological analyses and neutrophil-mediated MPO activity confirmed the topical anti-inflammatory effect of HLP. In addition, HLP reduced IL-1β, IL-6 and tumour necrosis factor-α cytokine levels. Sitosterol-rich fraction (SRF), obtained from HLP fractionation, reduced ear oedema on croton oil and phenol models at the same dose of dexamethasone (0.1 mg/ear). No dermatoxicity was observed.

**Conclusions** The mechanism of action of HLP was associated with the inhibition of several pro-inflammatory mediators, including cytokines, arachidonic acid metabolites and histamine, which suggested a glucocorticoid-like effect, reinforced by the presence of the steroid sitosterol. This is the first report on anti-inflammatory activity of *L. pubescens* leaves.

**Introduction**

The skin is the body organ responsible for a direct interaction between the environment and the organism, and its main function is to form an effective barrier to protect the organism from several external stimuli. Thus, as a mechanism of defence, the skin is able to recognize, discriminate and integrate specific signals from the environment and generate appropriate responses to maintain the body homeostasis.[1] Normally, this defence mechanism does not cause serious damage; however, an inappropriate or misdirected immune activity can implicate in the pathogenesis of a large variety of inflammatory skin disorders.[2] For example, the most common inflammatory dermatosis are psoriasis and atopic dermatitis, which have a high impact on the life quality, as psychological, self-esteem and body image disturbances.[3]

It is widely recognized that the modulation of the synthesis of inflammatory mediators may be used therapeutically against skin inflammations.[4] For this purpose, skin diseases can be treated either topically or systemically with glucocorticoids, antihistamines, non-steroidal...
anti-inflammatory drugs, and with monoclonal antibodies and recombinant cytokines. However, these therapeutic alternatives are usually aggressive and not effective in all cases, limiting their use. As alternatives, extracts and isolated compounds from herbal medicine have been studied to discover new effective and safe topical anti-inflammatory drugs.

*Lacistema pubescens* Mart. (Lacistemataceae), popularly known as ‘canela-vermelha’ is a native tree from Brazil, also found in other countries of South America. Species of the genus *Lacistema* have been traditionally used for some ethnopharmacological purposes mainly by indigenous peoples of Brazil and Peru to combat rheumatism, vomiting, dysentery, fever and body aches.

Recently, pharmacological studies performed by our group reported some preliminary results indicating a potential in-vivo topical anti-inflammatory activity using croton oil-induced ear oedema model of the hexane fraction (HLP) of *L. pubescens* leaves. Compounds like tocopherol, sitosterol, phytol among others were identified in the hexane extract using gas chromatography and mass spectrometry (GC-MS). This study also reported the antinociceptive effect of this species. Furthermore, a potential antioxidant, antileishmanial and anti-proliferative activities of the crude methanol extract of the leaves and its fractions were reported.

The aim of this study was to confirm, using different irritants agents, the preliminary anti-inflammatory activity previously observed for the HLP of the leaves of *L. pubescens* and its possible mechanism of action. A sitosterol-rich fraction (SRF) obtained from chromatographic fractionation of HLP was also evaluated to verify whether this compound contributed for the anti-inflammatory activity observed for HLP.

**Materials and Methods**

**Plant material**

*L. pubescens* leaves were collected in Juiz de Fora, Minas Gerais, Brazil, in December 2011. A voucher specimen (CESJ 49751) has been deposited at the Leopoldo Krieger Herbarium of the Federal University of Juiz de Fora.

**Extraction and fractionation**

Preparation of the HLP was previously described in detail. Fractionation of HLP was performed by the following manner: HLP (6 g) was chromatographed on a 42 × 5 cm column contained silica-gel (70–230 mesh) with a gradient of hexane-ethyl acetate (EtOAc) (90:10 v/v – 100% EtOAc) and EtOAc-MeOH (90:10 v/v – 100% MeOH) to obtain a total of 17 fractions (F1–F17). Fraction F9 (802 mg) was rechromatographed and eluted with hexane-EtOAc (90:10 v/v to 20:80 v/v) and EtOAc-MeOH (90:10 v/v to 50:50 v/v) to obtain 12 fractions (FF1–FF12).

**Chemical analysis**

The chemical constitutions of the fractions were analyzed by GC-MS by computer comparison of the mass spectra with those in the Wiley and NIST libraries, mass fragmentation and retention indices in reference to an n-alkane series in a temperature-programmed run. High pressure liquid chromatography (HPLC) analysis was performed for HLP. Detection was performed at 210 nm. Sitosterol standard (Sigma-Aldrich, Saint Louis, MO, USA) was used in this experiment as marker under the same conditions used for HLP.

**Animals**

Male Swiss mice (*Mus musculus*) weighting 25–35 g and male Wistar rats (*Rattus norvegicus*) weighing 160–200 g, obtained from the Reproduction Biology Center of the Federal University of Juiz de Fora, were housed in a room kept under controlled conditions at 23 ± 2°C and on a 12 h light/12 h dark cycle. They were provided with standard pellets and tap water ad libitum. Throughout the experiments, animals were processed according to the ethical guidelines for the care of laboratory animals. The study was approved by the Brazilian College of Animal Experimentation (COBEA-protocols n° 021/2012 and 013/2013). Animals were divided in groups of six to eight animals each.

**Acute dermal irritation/corrosion test**

The acute dermal irritation/corrosion study was carried out in accordance with the OECD Guideline 404 with minor modifications. Six rats with intact skin were assigned to two treatment groups: control and HLP. On day 0 of the test period, hair was shaved from the back of each rat and HLP (0.5 g) applied to a small area of the skin. After a 4-h exposure period, animals were examined for signs of erythema and oedema at grading intervals of 60 min, and then at 24, 48 and 72 h.

**Croton oil-induced mouse ear oedema**

Oedema was induced on the right ear by topical application of 20 μl of croton oil 5% (v/v) in acetone. After 15 min, HLP (1, 0.5 and 0.1 mg/ear), SRF (0.1 mg/ear), dexamethasone (0.1 mg/ear, used as reference drug) or vehicle acetone was applied topically on the right ear, whereas the left ear of all animals received 20 μl of vehicle. The thickness of the ears was measured before and 6 h after induction of inflammation. After 24 h, animals treated with HLP were euthanized and ear punch biopsies were collected and subjected to histopathological analysis or were snap frozen in...
liquid nitrogen and stored at \(-80^\circ C\) until further processed for myeloperoxidase (MPO) determination.

**Arachidonic acid (AA), phenol, capsaicin and ethyl phenylpropiolate (EPP)-induced mouse ear oedema**

Inflammation was induced applying on the inner surface of the right ear 20 \(\mu l\) of the following irritants diluted in acetone: AA 2 mg/ml, 10% phenol (v/v), capsaicin 0.5 mg/ear, and EPP 50 mg/ml. After 15 min, 20 \(\mu l\) of HLP (1, 0.5 and 0.1 mg/ear), indomethacin (0.5 mg/ear, used as a positive control for AA) or dexamethasone (0.1 mg/ear, used as a positive control for phenol and EPP) or SRF (0.1 mg/ear – only phenol model) was applied topically on the right ear, whereas the left ear received 20 \(\mu l\) of vehicle. The ear oedema was evaluated 30 min after capsaicin, 1 h after AA and EPP, and 2 h after phenol application.\(^{[18–22]}\)

**Subcutaneous histamine-induced mouse ear oedema**

Right ears were treated topically with HLP (1, 0.5, 0.1 mg/ear), vehicle (20 \(\mu l/ear\)) or dexamethasone (0.1 mg/ear). After 15 min, the oedema was induced in by intradermal application of 10 \(\mu l\) of histamine dihydrochloride (0.1 mg/ml) dissolved in 0.9% saline, whereas the left ear was injected with 10 \(\mu l\) of 0.9% saline. Ear oedema was evaluated 2 h after histamine application.\(^{[23]}\)

**Ear oedema measurement**

Oedema was expressed as increase in ear weight (all models) or ear thickness variation (croton oil), which was measured using a digital micrometre. To evaluate the ear weight, animals were euthanized, 6-mm diameter of ear biopsies were collected using a metal punch, and the biopsies were individually weighed on a precision balance. The extent of the oedema was expressed as the difference between the weight of the section removed from the right ear (which received the irritant agent) and the weight of the section obtained from the left ear (which received vehicle used to dilute the irritant agent). The mean oedema inhibition percentage (\%) was calculated using the following formula: inhibition (\%) = 100 – ((A x 100)/B), where ‘A’ is the mean of oedema weight (mg) of the group treated with HLP, indomethacin or dexamethasone, and ‘B’ is the mean of oedema weight of the untreated group (negative control).

**MPO determination**

Ear neutrophil infiltration was quantified by measuring myeloperoxidase (MPO) activity as described previously.\(^{[24]}\) The results were expressed as specific activity (mUE/mg protein).

**Cytokines determination**

Quantitative cytokine assays were performed by standard capture enzyme-linked immunosorbent assay (ELISA). The ears were collected, minced, homogenized in extraction buffer 0.5% bovine serum albumin (BSA), 0.1 M phenylmethylsulphonyl fluoride, 0.1 M benzethonium chloride, 10 mM ethylenediaminetetraacetic acid and 20 kIU/ml aprotinin and centrifuged at 10 000 g for 15 min at 4°C. The supernatants were stored and used to dosage of IL-1\(\beta\), tumour necrosis factor (TNF)-\(\alpha\), IL-6 using commercial ELISA kits according to the procedures supplied by the manufacturer (Life Technologies do Brasil Ltda Alto de Pinheiros, São Paulo, SP, Brazil). The levels of the cytokine proteins were determined in duplicate by an ELISA reader at 450 nm.

**Histopathology**

Ear biopsies from croton oil-induced ear oedema were collected and fixed in 70% ethanol for 24 h and then preserved in 10% formalin. Subsequently, the ears were dehydrated, blocked in paraffin and then sectioned with a microtome (4 \(\mu m\)). The cross-sections were stained with haematoxylin and eosin for the evaluation of histopathological changes related to acute inflammatory process. A representative area was selected for qualitative light microscope analysis (100× and 400× magnification).

**Statistical analysis**

The results were expressed as mean ± standard error of mean (SEM). The comparison between groups was assessed by ANOVA followed by Student–Newman–Keuls test using the software GraphPad Prism 5.0 (San Diego, CA, USA). Values of \(P < 0.05\) were considered statistically significant compared with negative control.

**Results**

**Chemical analysis**

The HPLC chromatogram profile of the HLP was performed and the presence of sitosterol was confirmed by external standard (Figure 1). GC-MS analysis of the fractions identified FF3 as sitosterol-rich fraction with 70% purity, named in this work as SRF.

**Dermatotoxicity test**

It was assessed a possible acute dermal irritation of HLP when administered on skin. No dermal responses, including erythema/eschar or oedema, were found in rats treated with HLP during the evaluation period.
Effect of HLP on croton oil, AA, phenol, histamine, EPP and capsaicin-induced mice ear oedema

As demonstrated in Figure 2a, HLP in all doses tested (1, 0.5 and 0.1 mg/ear) significantly reduced the ear oedema 6 h after topical application of croton oil when compared with the group treated with acetone (negative control). As a positive control, dexamethasone (0.1 mg/ear) significantly reduced the oedema (inhibition of 72%) compared with negative control group. The calculated inhibition to HLP was 74%, 67% and 65% at a dose of 1, 0.5 and 0.1 mg/ear respectively (P < 0.001). HLP also caused significant oedema reduction against AA (inhibition of 74% and 70% at 1.0 and 0.5 mg/ear, respectively, P < 0.001) (Figure 2b), phenol (inhibition of 95%, 50% and 81% at 1, 0.5 and 0.1 mg/ear, respectively, P < 0.001) (Figure 2c), histamine (inhibition of 64% at 1.0 mg/ear, P < 0.001) (Figure 2d) and EPP (inhibition of 67% and 66% at 1 and 0.5 mg/ear, respectively, P < 0.05) (Figure 2e). In contrast, the topical application of HLP did not present significant reduction on capsaicin-induced ear inflammation (P > 0.05) (Figure 2f).

Effect of HLP on myeloperoxidase activity

Myeloperoxidase activity was measured in the ear punch biopsies taken 24 h after oil croton administration. As Figure 3 shown, the MPO activity of the negative control group was significantly increased. However, treatment with both dexamethasone and HLP produced a remarkable inhibition of MPO activity. Dexamethasone at 0.1 mg/ear and HLP at 1, 0.5 and 0.1 mg/ear doses inhibited cell infiltration by 49%, 58%, 51% and 54% (P < 0.01, Figure 3). This inhibition was confirmed by histopathological analysis.

Effect of HLP on pro-inflammatory cytokines production

The pro-inflammatory cytokine-inhibitory effect of HLP was investigated of the ear punch biopsies taken 6 h after oil croton administration to assess their effectiveness at the molecular level. As shown in Figure 4, topical application of croton oil caused an increase in the production of IL-1β (A), IL-6 (B) and TNF-α (C) 6 h after challenge. In contrast, treatment with HLP or dexamethasone reduced IL-1β (P < 0.05), IL-6 (P < 0.001) and TNF-α (P < 0.05) cytokines levels significantly.

Effect of SRF on croton oil and phenol-induced mice ear oedema

To investigate whether SRF contributed for the anti-inflammatory activity observed for HLP, we employed croton oil and phenol-induced mice ear oedema considering the most significative anti-inflammatory effect of HLP on these models. As demonstrated in Figure 5a, SRF in the same dose of dexamethasone (0.1 mg/ear), significantly reduced the ear oedema (inhibition of 65%), 6 h after topical application of croton oil when compared with the negative control group (P < 0.001). As demonstrated in Figure 5b, SRF caused significant oedema reduction on sensitized ears with phenol, in the same dose of the reference drug (0.1 mg/ear), showing an inhibition effect of 70% (P < 0.05).
Histopathological analysis

Histopathological analysis of ear tissue 24 h after croton oil treatment revealed a significant increase in the dermis thickness associated a vasodilatation, oedema and marked infiltration of inflammatory cells associated (Figure 6b) when compared with non-inflamed ear (Figure 6a, vehicle acetone). The ears treated with dexamethasone (Figure 6c) and HLP at all doses (Figure 6d–f) demonstrated a decrease in these inflammatory parameters when compared with the ears that received croton oil and vehicle.

Discussion

Previous studies from our group have showed that HLP of L. pubescens leaves possesses in-vivo topical anti-inflammatory activity using croton oil-induced ear oedema model; however, ear oedema models induced by different irritant agents allow the proposition of the possible mechanism of action. Besides, they promote conditions that resemble some types of dermatitis observed in humans. The present work provides evidence that HLP clearly exerted anti-inflammatory effect on all models tested.
except for capsaicin-induced ear oedema, in a manner similar to dexamethasone and indomethacin. Furthermore, the acute dermal irritation study of the HLP showed no dermal responses, such as erythema or oedema.

Croton oil contains 12-o-tetradecanoylphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents, and once topically applied, results in an increased vascular permeability, migration of polymorphonuclear leukocytes (mainly neutrophils), liberation of histamine and serotonin, and a moderate synthesis of eicosanoids.[25] Croton oil is able to activate protein kinase C (PKC), which activates other enzymatic cascades in turn, such as mitogen-activated protein kinases and phospholipase A2 (PLA2), leading to the release of platelet activation factor and AA. These metabolites, as well as cytokines, are mediators of inflammatory pathways and are responsible for triggering and maintaining inflammation.[26] Thus, as croton oil induces an

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**Figure 3**  Effect of hexane fraction (HLP) on production of myeloperoxidase (MPO). Dexamethasone (DEX) was used as reference drug. Statistical analysis: one-way ANOVA followed by Newman–Keuls test ($n = 6–8$). **$P < 0.01$ compared with negative control group (C). ##Statistically equal to reference drug.

**Figure 4**  Effect of hexane fraction (HLP – 1.0 mg/ear) on production of IL-1β (a), IL-6 (b) and tumour necrosis factor-α (c). Dexamethasone (DEX – 0.1 mg/ear) was used as reference drug. Statistical analysis: one-way ANOVA followed by Newman–Keuls test ($n = 6–8$). *$P < 0.05$, ***$P < 0.001$ compared with negative control group (C). ##Statistically equal to reference drug.

**Figure 5**  Effect of sitosterol-rich fraction (SRF – 0.1 mg/ear) on croton oil (a) and phenol (b) induced mice ear oedema. Dexamethasone (DEX – 0.1 mg/ear) was used as reference drug. Statistical analysis: one-way ANOVA followed by Newman–Keuls test ($n = 6–8$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with negative control group (C). ##Statistically equal to reference drug.
Figure 6 Photomicrograph of transverse sections of mice ears sensitized with topical application of croton oil 5% (v/v) in acetone (b–f) or vehicle acetone (a, not inflamed), (haematoxylin and eosin 100× e 400×). Treatments: acetone (b), dexamethasone 0.1 mg/ear (c), HLP 1.0 mg/ear (d) 0.5 mg/ear (e) and 0.1 mg/ear (f). Keys indicate epidermis, and arrows indicate leukocyte infiltration in the dermis. The shown sections are representative of five animals per group.
**L. pubescens** reduces skin inflammation

Anti-inflammatory action. [36] HLP was able to reduce the inflammatory response by EPP (Figure 2a). As described, HLP evidenced a significant decrease on ear oedema at all doses tested (Figure 2a).

AA is a precursor of inflammatory eicosanoids such as prostaglandin E2 and leukotrienes (produced via COX-1, COX-2 and 5-LOX enzymes). Indomethacin is related to the non-selective inhibition of the isoforms of COX and clearly reversed the oedema induced by AA.[28] Dexamethasone, likewise, modulates the production of AA metabolites, probably via inhibition of COX and LOX enzymes synthesis.[30] HLP 1 and 0.5 mg/ear decreased the ear oedema in this model (Figure 2b).

Phenol-induced ear oedema is an appropriate model for simulating contact dermatitis. When phenol is topical applied, keratinocytes produce chemical mediators that are important in primary contact irritation responses, including cytokines, such as IL-1α, TNF-α and IL-8.[29] This mechanism is independent of PKC activation pathway, which in turn results in the release of inflammatory mediators such as AA metabolites and reactive oxygen species.[30] HLP significantly reduced the phenol-induced oedema at all doses, suggesting a probable activity against contact dermatitis (Figure 2c). This activity may be related to the influence on the production of AA metabolites and cytokines.

HLP markedly inhibited the histamine (Figure 2d) and EPP (Figure 2e) ear induced oedema. Histamine causes vasodilation and an increase in microvascular permeability[31] as well as stimulates nerve fibres. Antihistamines and corticosteroids reduce the oedema in this model, which is involved in immediate type allergic reactions.[32] The local inflammatory response by EPP is mainly vascular in nature,[33] involving the release of several inflammatory mediators, such as histamine, serotonin, bradykinin and prostaglandins.[34] On the other hand, capsaicin when in contact with epidermis exerts an immediate effect on a TRPV1 receptor target and stimulates a neurogenic inflammatory response.[20] HLP did not present significant oedema reduction in this model, suggesting that it does not interfere in substances or receptors involved in capsaicin-activated inflammatory pathways.

Neutrophils are key players in the recognition and elimination of pathogens, but their improper activation is thought to induce tissue lesions and contribute to the pathophysiology of various inflammatory diseases.[35] MPO is known as a direct marker of neutrophil infiltration; therefore, its activity inhibition can be used as an indicator of anti-inflammatory action.[36] HLP was able to reduce the number of leukocytes in the inflamed tissue evidenced by MPO activity and by the histopathological analysis, which suggests a possible interference in cell migration during the inflammatory process. The histopathological analysis also confirmed that HLP markedly inhibited the oedema intensity (Figure 6).

This study also showed that topical exposure to croton oil resulted in an increased secretion of TNF-α, IL-6 and IL-1β in mouse ear biopsy homogenates. IL-1β, a pro-inflammatory cytokine, in which main sources are the keratinocytes in the skin, activates neutrophils, monocytes, eosinophils and basophils.[37] IL-1β is important in a number of severe inflammatory diseases and most of these diseases can be completely controlled by anti-IL-1β treatment.[38] IL-6 is involved in the growth and differentiation of dermal and epidermal cells, and acts as a chemotactic factor for T cells.[39] TNF-α is also an important cytokine involved in the maintenance of inflammatory processes in the skin, and TNF-α is stressed by its capacity to induce IL-1β and IL-6.[40] HLP (1 mg/ear) significantly decreased the levels of these cytokines (Figure 4), suggesting that this mechanism, at least partially, contributed to its anti-inflammatory response in acute skin inflammation, considering the results obtained from phenol topical application, which induces the production of these inflammatory mediators (Figure 2c).

Considering the most significative anti-inflammatory effect of HLP in croton oil- and phenol-induced ear oedema, these models were selected to verify if SRF contributed, in any manner, to the anti-inflammatory activity observed for HLP. In fact, SRF evidenced a significant decrease on ear oedema at the same dose of dexamethasone (0.1 mg/ear) in both models (Figure 5). Glucocorticoids, including sitosterol and dexamethasone, bind to specific intracellular receptors that activate or repress gene transcription to inhibit the actions of the immune system as a whole. Glucocorticoids inhibit the expression of COX-2, iNOS, adhesion factors, complement factors and cytokines, and induce the expression of other proteins, such as annexin-1, which inhibits the prostanooids synthesis. As dexamethasone, HLP inhibited the action of different phlogistic agents, which induced inflammation by different pathways, suggesting a glucocorticoid-like effect of HLP, which is reinforced by the presence of sitosterol.[41] These data are also supported by the literature, which reported that sitosterol (dose of 0.5 mg/ear), isolated of *Achillea ageratum*, is effective as a topical anti-inflammatory agent in acute inflammatory process.[42]

**Conclusions**

In summary, the results of this study showed the effectiveness and safeness of HLP as a topical anti-inflammatory agent that may serve as a source for developing effective drugs for skin inflammatory disorders. Furthermore, the mechanism of action of HLP was associated with the
inhibition of several pro-inflammatory mediators, including cytokines, AA metabolites and histamine, which suggested a glucocorticoid-like effect, reinforced by the presence of the steroid sitosterol. Further studies are necessary to identify other chemical constituents that may be contributing to this activity. According to our knowledge, this is the first report on anti-inflammatory activity of _L. pubescens_ leaves.

**Declarations**

**Conflict of interest**

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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**Funding**

The authors are grateful to Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) and Universidade Federal de Juiz de Fora (UFJJ), Brazil, for financial support.

**Acknowledgements**

The authors thank Dr Fatima Regina Salimena for the botanical identification of species and Delfino Antonio Campos for technical assistance.
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