Topical Anti-inflammatory Potential of Pumpkin (Cucurbita pepo L.) Seed Oil on Acute and Chronic Skin Inflammation in Mice

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ABSTRACT

Background: Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, which involves interactions amongst many cell types and mediators, and underlies many pathological processes. Unsaturated fatty acids (UFAs) can influence inflammation through a variety of mechanisms, and have been indicated as alternative anti-inflammatory agents to treat several inflammatory skin disorders. Pumpkin seed oil (PSO) is rich in UFAs, but its topical anti-inflammatory properties have not been investigated. Therefore, the aim of this paper was to evaluate the effects of PSO on acute and chronic cutaneous inflammation experimental models.

Materials, Methods & Results: PSO was purchased commercially and analyzed phytochemically. The topical anti-inflammatory activity of PSO at different concentrations was evaluated on acute models (xylene- and 12-O-tetradecanoylphorbol acetate (TPA)-induced ear edema) and chronic model (multiple applications of oxazolone-induced dermatitis) in mice. Indomethacin and dexamethasone were used as reference drugs. The ear swelling was measured in both ear thickness (µm) and weight tissue (mg) at 1 and 4 h after xylene and TPA application, respectively. In the chronic model, the effectiveness of treatments was measured each 24 h post-challenge with oxazolone for 4 days. At the end of experiments, ear biopsies were assessed by histological analysis on hematoxylin-eosin- and toluidine blue-stained slides. Data were submitted to ANOVA followed Student Newman Keuls test (P < 0.05). PSO was characterized by a high content of unsaturated fatty acids (UFAs) (79.80%), including linoleic acid (ω-6, 55.83%) and oleic acid (ω-9, 23.47%). PSO caused a dose-dependent inhibition of xylene and TPA-induced ear edema in both skin thickness and weight when compared to respective positive controls (P < 0.05). This anti-inflammatory effect was maximum when PSO was applied in nature (inhibition of 69.9 ± 2.8% and 78.1 ± 7.7% for inflammation induced by xylene and TPA, respectively; P < 0.05), and was similar to, at least, one drug reference (P < 0.05). In addition, the topical treatment with PSO caused the inhibition of inflammation-induced by oxazolone in 60.9 ± 9.8% when compared to control positive (P < 0.05), which was similar to dexamethasone (68.7 ± 8.1%, P < 0.05). In histological analysis, PSO reduced the inflammatory parameters (edema, congestion, epidermal hyperplasia and cellular infiltration) in inflammation models studied. However, the number of mastocytes in cell infiltration was reduced (17.6 ± 4.0) when compared to positive control (39.4 ± 5.8 cells) in chronic model (P < 0.05), but no differences were observed in acute models.

Discussion: Topical anti-inflammatory activity of plant-originated substances can be evaluated in several experimental models. In this study, we used as phlogistic agents: xylene, a promoter of neurogenic inflammation; TPA, a phorbol ester that activate protein kinase C, leading to production of lipid-derived mediators; and oxazolone, an inductor of contact delayed-type hypersensitivity. Our results suggest that PSO alter inflammatory response via modulation of cellular and molecular mediators involved in inflammatory pathways activated by theses phlogistic agents. In addition, this oil was able to resolve a persistent inflammatory lesion similar to dexamethasone, but we did not observe any cutaneous alterations caused by its topical use as related for corticosteroids. This is the first report on topical anti-inflammatory potential of PSO in acute and chronic skin inflammation. This activity may be attributed the proper balance of ω-6 and ω-9 UFAs present in PSO, suggesting this oil as alternative therapy for the treatment of inflammatory skin diseases. Further investigations are needed to support its application in clinical practice.

Keywords: Vegetable oils, unsaturated fatty acids, pumpkin, topical inflammation, skin disorders.
**INTRODUCTION**

Inflammation is a normal defense mechanism that protects the host from infection and other insults. It involves interactions amongst many cell types and chemical mediators for restoring homeostasis [13]. These mediators include lipid-derived mediators, peptide mediators, enzymes, and adhesion molecules, depending upon the cell type involved and the nature of the injurious stimulus [3,10,15]. However, when produced in an unregulated fashion, they can cause damage to host tissues, leading to disease [3].

Inflammatory skin disorders can be treated with some success by pharmaceutical agents, such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). However, they can frequently cause a set of undesirable side effects [31]. Because of these risks, alternative bioactive molecules are being intensively investigated. In this scenario, fatty acids are highlighted as important effectors and regulators molecules in the immune-inflammatory response [18]. Unsaturation fatty acids (UFAs) are found in significant quantities in vegetable oils. Pumpkin (*Cucurbita pepo* L.; Cucurbitaceae) seed oil (PSO) is extraordinarily rich in UFAs, which represent about 84% of the total fatty acids. It is also rich in minerals and antioxidants as tocopherols and carotenoids [20]. Despite several vegetable oils rich in UFAs have been related as promising alternative anti-inflammatory agents on skin disorders [17,19,22], information is not available about the anti-inflammatory properties of PSO.

The aim of the present study was to investigate the effects of PSO on acute and chronic cutaneous inflammation models in mice, in order to verify its topical anti-inflammatory potential.

**MATERIALS AND METHODS**

**Phytochemical analysis**

The oil from the seeds of pumpkin (PSO) was purchased commercially and used for transesterification reactions [5]. The recovered fatty acid methyl esters were analyzed by gas chromatography coupled to mass spectrometry, using a CGC AGILENT 68950 series GC system under the following conditions-column: Methylpolysiloxane DB-23 AGILENT capillary column (60 m x 0.25 mm); carrier gas: He (1 mL/min); injector temperature: 250°C; detector temperature: 280°C; column temperature: 110°C/5 min, 110-215°C at 5°C/min and then 215°C/24 min; mass spectrum: electronic impact 70 eV. The identification of the constituents was performed by a computer-based library search, retention indices and visual interpretation of the mass spectra [1,2].

**Animals**

Female Swiss mice weighing 25-30 g, obtained from Central Animal House of the Federal University of Ceará were used for this study. Mice were housed in standard polypropylene cages under controlled conditions of temperature (23-25°C), relative humidity (40-45%) and 12 h light/dark cycle, with free access to commercial pellet diet and water.

**Xylene and 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced acute ear edema**

Ear edema was induced in mice (n = 7/group) by topical application on the inner and outer surfaces of the right ear of the following phlogistic agents: xylene (20 µL/ear) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) 2.5 µg/ear in acetone. Immediately after the application of each phlogistic agent, the right ears were topically treated with PSO at 100% (in nature), 50% and 25% (v/v) in acetone, vehicle (acetone, 20 µL/ear, positive control), indomethacin (0.5 mg/ear) and dexamethasone (0.05 mg/ear). The left ear was considered as contralateral control and received only vehicle or saline solution (negative control, 20 µL/ear). The ear edema was evaluated 1 h after xylene and 4 h after TPA application [8,14,19].

**Ear edema measurement**

Ear thickness was measured before and after induction of the inflammatory response using a digital micrometer. The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges and the thickness was recorded in µm [22]. To evaluate the ear weight, animals were euthanized, and then both ears biopsies were removed and individually weighed [19]. Edema was expressed as right ear thickness variation and as the weight difference between the right and left ears. The inhibition edema percentage was calculated as weight reduction in comparison to positive control.

**Oxazolone-induced chronic dermatitis**

Contact dermatitis induction was performed according to Ginner et al. [8]. Briefly, mice (n = 7/group) were sensitized by topical application of 2% solution (w/v) oxazolone in acetone (50 µL) on the shaved abdo-
men skin for two consecutive days (days 1 and 2). On
day 6, the challenge was performed by application of
2% oxazolone (30 µL) on both sides of the mouse right
ear (20 µL/ear). PSO at 100% (in nature), 50% and 25% (v/v)
in acetone (20 µL/ear), vehicle (acetone, 20 µL/ear, positive control) and dexamethasone (0.05 mg/ear)
were topically applied (20 µL) to right ear 6, 24, 48, 72
and 96 h after challenge. The left ear was considered as
contralateral control (negative control). Ear thickness
was measured before treatment application each 24 h for
4 days using a digital micrometer as described above.
The final measurement (102 h after challenge) was per-
formed immediately before the animals were euthanized.
The weight of ears was measured as described for xylene
and TPA tests, and activity was expressed as inhibition
percentage referred to the positive control.

**Histological analysis**

Ear samples were fixed in 10% neutral buff-
ered formalin (pH 7.0) for 48 h and then processed
by standard histological methods. Each sample was
embedded in paraffin, cut into 5 µm sections and
stained with hematoxylin-eosin (HE) and toluidine
blue (TB). On the cross-sections stained with HE, a
representative area was selected for qualitative light
microscopic analysis at 200× magnification [22]. Mas-
tocytes were evaluated with a semiquantitative method
on TB-stained slides in five areas randomly selected at
magnification of 400× [8].

**Statistical analysis**

Data were initially submitted to Kolmogorov-
Smirnov and Bartlett tests to confirm normal distribu-
tion and homogeneity of variance, respectively. There-
after, multiple comparisons were performed using
one-way analysis of variance (ANOVA) followed by
Student Newman Keuls test. All analyses were made
using GraphPad Prism 5.0 software. Differences were
considered significant at $P < 0.05$ and the results were
expressed as means ± SD.

**RESULTS**

Chemical analysis of PSO is displayed in Table 1. This oil was characterized by a high content of UFAs
(79.80%). The main fatty acids found were linoleic,
oleic and palmitic acids.

In the acute models of inflammation, exposure
to xylene and TPA on the ear of the mouse resulted in
marked increases in both ear thickness (Figures 1A and
2A) and tissue weight (Figures 1B and 2B). Topical
application of the vehicles (acetone or saline solution)
or PSO alone on the ear did not alter the skin thickness
significantly (data not shown). However, PSO inhibited
the xylene and TPA-induced ear edema in both skin thick-
ness and weight when compared to respective positive
controls ($P < 0.05$) at times 1 and 4 h, respectively. This
inhibition occurred in a dose-dependent fashion within
the range of evaluated concentrations and was similar to
indomethacin and/or dexamethasone results (Figures 1
and 2). The maximal anti-inflammatory effects of PSO
were verified at concentration 100% (in nature) on the
TPA-induced ear edema model. In this model, in nature
PSO reduced the ear edema in 78.71%, which was sig-
ificantly higher as compared to inflammation inhibition
promoted by others phlogistic agents (Table 2).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Yield (% m/m)</th>
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<tbody>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>12.87</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>6.24</td>
</tr>
<tr>
<td>Others</td>
<td>1.09</td>
</tr>
<tr>
<td><strong>Unsaturated fatty acids</strong></td>
<td></td>
</tr>
<tr>
<td>Oleic acid (C18:1; ω-9)</td>
<td>23.47</td>
</tr>
<tr>
<td>Linoleic acid (C18:2; ω-6)</td>
<td>55.83</td>
</tr>
<tr>
<td>Linolenic acid C18:3; ω-3)</td>
<td>0.21</td>
</tr>
<tr>
<td>Others</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Table 2. Topical anti-inflammatory effect of pumpkin seed oil (PSO) and reference drugs in acute and chronic models of inflammation at the end of the experimental period.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Inflammation inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Xylene</td>
</tr>
<tr>
<td>PSO 100% (<em>in nature</em>)</td>
<td>69.61 ± 2.79a</td>
</tr>
<tr>
<td>PSO 50%</td>
<td>56.54 ± 4.81a</td>
</tr>
<tr>
<td>PSO 25%</td>
<td>49.57 ± 7.52a</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>51.00 ± 12.08a</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>65.07 ± 8.98a</td>
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Results are expressed as means ± SD (n = 7) of ears weight reduction in relation to the positive control of each model. Different superscripts letters within the same line indicate statistically significant difference among different inflammation models (P < 0.05).

Figure 1. Topical activity of pumpkin seed oil (PSO), indomethacin (Indo) and dexamethasone (Dexa) on xylene (Xyl)-induced ear edema in mice. Ear edema was measured at 1 h after induction of inflammation in both ear thickness (A) and tissue weight (B). The positive control only received acetone topically after the challenge with xylene. The bars represent the mean ± SD for seven animals. Different small letters indicate statistically significant differences among groups (P < 0.05).

Figure 2. Topical activity of pumpkin seed oil (PSO), indomethacin (Indo) and dexamethasone (Dexa) on TPA-induced ear edema in mice. Ear edema was measured at 4 h after induction of inflammation in both ear thickness (A) and tissue weight (B). The positive control only received acetone topically after the challenge with TPA. The bars represent the mean ± SD for seven animals. Different small letters indicate statistically significant differences among groups (P < 0.05).
In the chronic model of inflammation, the topical application of oxazolone to ear of sensitized mice caused a marked inflammatory response as verified by presence of erythema, edema and sometimes induration. Ear thickness significantly increased from 24 h and persisted until 102 h after the challenge (Figure 3A). At the end of experimental period, it was also observed a significant increase of ear weight (Figure 3B). The topical treatment with PSO significantly reduced the oxazolone-induced increase in both ear thickness (Figure 3A) and tissue weight (Figure 3B) during the period of study. In addition, this inhibition was dose-dependent and reached its maximum 102 h after the challenge at concentration 100% ($P < 0.05$), showing a reduction of inflammatory response of 60.86% (Table 2).

Histological analysis of the ear tissue clearly supported the results described above. Ears exposed to xylene and TPA applications revealed an intense dermal edema and congestion, epidermal hyperplasia and a large number of infiltrating inflammatory cells (Figures 4A and 4D). These findings were reduced in ear tissues of animals treated topically with in nature PSO (Figures 4B and 4E) and dexamethasone (Figures 4C and 4F). Repeated applications of oxazolone induced a slight dermal edema, intense congestion, epidermal hyperproliferation and infiltration of leukocytes and mastocytes into the dermis (Figure 4G). The PSO treatment as well as dexamethasone prominently reduced the keratinocyte hyperproliferation as well as other inflammatory parameters, such as edema, congestion and cells infiltration (Figures 4H and 4I).

Additionally, in the oxazolone-induced dermatitis model, the ears treated with in nature PSO showed a significant reduction in number of mastocytes when compared to positive control, but this reduction was inferior ($P < 0.05$) to dexamethasone (Table 3 and Figures 5G, 5H and 5I). On the other hand, no differences were observed in the number of mastocytes among treatment groups in xylene (Figures 5A, 5B and 5C) or TPA-induced ear edema models (Figures 5D, 5E and 5F), except inflamed ears with TPA and treated with dexamethasone (Table 3).
Figure 5. Topical effect of pumpkin seed oil (PSO) and dexamethasone on mastocytes infiltration in acute ear edema induced by xylene (A, B, C) and TPA (D, E, F) and chronic dermatitis induced by oxazolone multiple applications (G, H, I). Photomicrograph of transverse sections from ears collected in the last day of experiments. In chronic model, the treatment with in nature PSO (B, E, H) and dexamethasone (C, F, I) reduced the number of mastocytes in inflammatory cell infiltration when compared to respective positive control (A, D, G). A minimum of two section from five animals for each treatment were analyzed. TB staining. Original magnification: 100x. [Scale bar: 200 µm].

Table 3. Topical effect of pumpkin seed oil (PSO) and reference drug on absolute number of mastocytes in acute and chronic models of inflammation.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Number of mastocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xylene</td>
</tr>
<tr>
<td>Positive control</td>
<td>14.40 ± 3.05a</td>
</tr>
<tr>
<td>PSO (in nature)</td>
<td>12.80 ± 2.68a</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>12.00 ± 3.16a</td>
</tr>
<tr>
<td>Negative control</td>
<td>8.00 ± 1.58b</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SD (n = 5) of mastocytes counting in five fields on toluidine blue-stained slides. Different superscripts letters within the same column indicate statistically significant differences among groups (P < 0.05).
DISCUSSION

Models of topical inflammation induced by different phlogistic agents has been extensively used as pharmacological tools for the investigation of several substances with anti-inflammatory potential in alternative medicine. These compounds include products of plant origin as vegetable oils, which can be useful in the treatment of inflammatory skin disorders [7]. In the present study, we have examined the pharmacological properties of PSO in acute and chronic skin inflammation in order to demonstrate its topical anti-inflammatory potential.

Firstly, we studied the anti-inflammatory activity of PSO in two models of topical acute edema. The xylene is a phlogistic agent that act on target cells in the periphery such as mast cells, immune cells, and vascular smooth muscle via VR1 receptors, and promote neurogenic inflammation, which is characterized by redness and warmth, swelling, and hypersensitivity. This inflammatory response result from the release of neuropeptides from primary sensory nerve terminals [15,21]. Another phlogistic agent, TPA is a phorbol ester able to activate protein kinase C, which activates other enzymatic cascades in turn, such as phospholipase A2, leading to release of arachidonic acid (AA). This signaling pathway stimulates local inflammation with vascular permeability, edema formation and polymorphonuclear leukocytes infiltration as consequence of the production of inflammatory eicosanoids by cyclooxygenase (COX) and lipooxygenase (LOX) enzymes [6,7]. Many drug classes, such as corticosteroids and NSAIDs, can modulate the acute ear edema response in varying degrees, depending on the nature of edematogen and involved mediators [7,21]. In the present work, we observed that the topical application of PSO was able of reducing inflammation in xylene and TPA-induced acute ear edema in a dose-dependent fashion, and this effect was similar to, at least, one drug reference (Figures 1 and 2). Moreover, PSO reduced the inflammatory lesion and cellular infiltration in both acute ear edema models (Figures 4B and 4E). Taken together, these results suggest that this oil alter inflammatory response via modulation of cellular and molecular mediators involved in inflammatory pathways activated by theses phlogistic agents.

A third model used in this study was the chronic skin inflammation induced by repeated exposure to oxazolone. Skin inflammation is persistent in this model, which induces a contact delayed-type hypersensitivity resembling human contact dermatitis. This response is characterized by a sustained ear swelling, marked polymorphonuclear, macrophage, mastocytes and T lymphocytes infiltration, and abundant pro-inflammatory mediators [16]. Increased skin thickening is often the first hallmark of events that occur during cutaneous inflammation, including dermal edema, inflammatory cells infiltration and proliferation of epidermal keratinocytes [12]. Here we verified that the topical application of PSO were active both in the early stage and throughout the inflammatory process and decreased the ear thickness in model of oxazolone-induced chronic dermatitis. This reduction was dose-dependent and similar to dexamethasone results. Corticosteroids are well known to have potent anti-inflammatory effects, however its topical use can cause intense skin atrophy, one of the serious side effects limiting their uses for chronic skin diseases [23]. Thus, our findings support the ability of PSO to resolve a persistent inflammatory lesion, with an efficacy comparable to dexamethasone. In addition, we did not observe any cutaneous alterations when PSO alone was applied to mice skin.

With an extent of activity comparable to reference drug, PSO reduced two important events related to the skin inflammatory response induced by oxazolone: the migration of neutrophils and the number of mastocytes, as determined by HE and TB staining, respectively. Indeed, the accumulation of polymorphonuclear cells and mastocytes plays a critical role in cutaneous inflammatory diseases such as contact dermatitis, and is related to the pathological mechanism of disease [16]. Neutrophils recruitment is an essential factor in the acute inflammatory process, acting as first-line defense cells in the initiation and resolution phases of this process [11]. Another immune cell, the mastocytes act as important sentinels in the skin, helping to limit or even prevent the damage that results from injurious agent due to release of various biochemical mediators [25]. Under condition in which uncontrolled infiltration of these cells occurs, they can become the main aggressor factor and have pathological role [11,25]. Thus, our results directly illustrate the inhibitory effects of PSO on neutrophils and mastocytes within the target tissue, providing further evidence that PSO ameliorates oxazolone-induced contact dermatitis and indicating the therapeutic effect of this oil for chronic skin disorders.
With respect to this study, for the first time, we demonstrate the topical anti-inflammatory potential of PSO in acute and chronic skin inflammation. Altogether, we believed that the results evidenced here may be attributed the promising ratio of ω-6 and ω-9 UFAs (2:1) present in PSO, namely linoleic and oleic acids (Table 1).

Fatty acids are a diverse set of molecules that act as cell membranes constituents, gene expression regulators, signal transduction modifiers and cell proliferation, generating products that modulate a variety of biological mechanisms, including those linked to homeostasis, immune responsiveness, and inflammation [9]. These bioactive molecules have shown to be potential anti-inflammatory agents due, at least in part, to their ability to inhibit enzymes that modulate the production of AA metabolites, or produce anti-inflammatory eicosanoids [4]. In this context, linoleic and oleic acids have been reported to modulate the skin immune-inflammatory responses [4,10,18]. These previous findings are consistent with our results, which we suggest that the proper balance of these UFAs present in PSO can have a great importance in modulation of different inflammatory signaling according to injurious stimulus.

In recent years, plant-derived natural products have become known for its beneficial effects on inflammatory response [12,14,24]. Amongst the natural products as rich source of UFAs, the vegetable oils have been indicated as alternative therapy for treat skin inflammatory process [17,18]. Previously, we reported that Caryocar coriaceum oil (ω-6:ω-9 / 1:24), inhibited the xylene-induced ear edema, but this inhibition was twice less effective compared to the current study [19]. In other study, this same oil demonstrated significant topical antiedematous effect against croton oil, but did not present significant reduction on capsaicin-induced ear neurogenic inflammation [22]. Here we verified that PSO showed topical anti-inflammatory activity similar to reference drugs that inhibit the production of eicosanoids via modulation of phospholipase A2, COX and LOX enzymes [7,23], suggesting that this finding may be focused for future studies aiming to unravel mechanism of action of PSO.

CONCLUSION

In summary, this study represents the first report that PSO acts as a topical anti-inflammatory agent, and it is effective against acute and chronic skin inflammatory processes. The anti-inflammatory activity of PSO may be attributed to promising proportions of ω-6 and ω-9 UFAs present in it, due to either their individual activity or the synergistic effect of these bioactive molecules. Together, these finding suggest that PSO may be an important alternative therapy for the treatment of inflammatory skin diseases, such as psoriasis, contact dermatitis and atopic dermatitis. Nevertheless, further investigations need to be performed to determine the precise mechanism of action and support its application in clinical practice.

REFERENCES


