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Topical anti-inflammatory activity of semisolid containing standardized *Aleurites moluccana* L. Willd (Euphorbiaceae) leaves extract

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ABSTRACT

Ethnopharmmacological relevance: *Aleurites moluccana* is a medicinal plant popularly used to treat pain, fever, asthma, hepatitis, gastric ulcer and inflammatory process in general. Recently, pre-clinical studies have demonstrated that the dry extract obtained from *A. moluccana* leaves was effective as analgesic, anti-inflammatory and wound healing.
Aim: The present study has aimed to evaluate the mechanisms involved in the topical anti-inflammatory effects of the semisolid containing 10 mg/g of *A. moluccana* dried extract.
Material and Methods: Ear edema induced by croton oil (2.5%) in mice was used throughout the study. The level of cytokines tumour necrosis factor (TNF) and interleukine-1β (IL-1β) and chemokine keratinocyte chemoattractant (CXCL1/KC), and neutrophil migration were quantified. The histological analysis has also been performed.
Results: The topical treatment with the semisolid was able to significantly inhibite the ear edema (35.77 ± 7.35%). This effect was accompanied by the reduction of leukocyte migration, as well as TNF (53.75 ± 12.96%), IL-1β (38.36 ± 5.92%), and CXCL1/KC (62.29 ± 11.65%) decreased levels.
Conclusions: This study demonstrated for the first time the mechanisms involved in the topical anti-inflammatory effect presented by the semisolid containing *A. moluccana* dried extract pointing as the main mechanism is the reduction in the leukocyte migration and consequently resulting in diminished levels of cytokins and chemokines, indicating this herbal product as a promissor anti-inflammatory phytomedicine to treat skin inflammatory diseases.

1. Introduction

The skin inflammation is widely studied due to its complexity and relevance to human health, considering the high number of people that suffer from chronic inflammation (Alam et al., 2011). Skin damage is generally associated with inflammation due to skin burns or inflammatory illness such as psoriasis, contactor atopic dermatitis, vulgaris acne, skin wounds, among others (Gittler et al. 2013; Haertel et al., 2014; Kendall and Nicolaou, 2013; Wagener et al., 2013).

The skin is physiologically considered as an active barrier, consisting of cellsthat regulate the chemical mediators secretion, such as cytokines and bioactive lipids. These mediators are responsible forinitiatingtheimmune response and forcontroling the inflammatory cascade, leading to an efficient inflammatory resolution. However, in some cases the inflammatory process can become uncontrolled, hindering the process resolution, culminating in a pathological condition (Kendall and Nicolaou, 2013). In this context, the search for new therapies that control the skin inflammatory process, including the release of cytokines and others inflammatory mediatorsmight become an important therapeutic strategy for chronic disease (Hilling, 2010).

The medicial plants have a great relevance in the treatment or relief of inflammation, mainly in countries in developmentwhere the folk medicine is largely used to treat many diseases. In Brazil, the use of phytomedicine has received the encouragement of the National Policy on Integrative and Complementary Practices of the

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Unified Health System (SUS-PNPIC), which integrates the National Policy on Medicinal Plants and Herbal Medicines (PNPMF) (Carvalho et al., 2011).

Aleurites moluccana (L.) Willd, belonging to the Euphorbiaceae family and popularly known in Brazilas "nogueira de iguape" and "noz da India", is a tree native from Indonesia that has became acclimatized and well-adaptated to the south and southwest of Brazil. In folk medicine, *A. moluccana* leaves have been used to treat fever, asthma, hepatitis, headache, gastric ulcer and inflammatory process in general (Villalobos and Castellanos, 1992; Foster, 1996; Quintão et al., 2014). Recently, Cesca et al. (2012) demonstrated the topical anti-inflammatory effect of *A. moluccana* leaves dried extract. The semisolid presented reduction of ear edema induced by croton oil and also improved the wound healing.This study had the aim of improve the knowledge about the mechanisms throughout the *A. moluccana* dried extract presented significant topical anti-inflamatory effects providing more scientific evidences to support its use in the folk medicine.

2. Material and methods

2.1. Reagents and samples

The ELISA kits for mouse IL-1β, TNF, and CXCL1/KC were purchased from R&D Systems (Minneapolis, MN). The hydrogen peroxide (H_2O_2), Tween 20, EDTA, aprotinin, and PBS tablets were purchased from Sigma Chemical Co. (St. Louis, MO). The drugs were prepared in saline solution (0.9% NaCl). Ethanol P.A. (Solven), Ethanol Absolute (Meta Química), DMSO (Emplura), Yellowish eosin-C/45380 (fine chemicals Vetec), Entelan (Merck), Ethyl Ether (Diethyl) (Vetec Chemistry), Croton oil (Sigma), Triton X-100 (Sigma), Xylene (Solven).

2.2. Plant extract and semisolid preparation

The standardized hydroalcoholic spray dried extract of *A. moluccana* leaves, with 20% of dioxide colloidal silicon, was produced by Eurofarma industry, and donated by the Pharmaceutical Technology Laboratory of UNIVALI. The semisolid, used as vehicle, was prepared by mixing Sepigel 305, vaseline, crodalan, isopropyl myristate, BHT, cetostearyl alcohol, Phenonip®, EDTA and water with slight warming. The extract was added at 1% with the aid of propylene glycol. The formulations were packed in aluminium tubes, with a seal and polypropylene cover.

Semisolid containing dexamethasone acetate 1 mg/g, manufactured by EMS laboratory S/A (batch 603 479), was commercially purchased and used as positive control drug.

2.3. Pharmacological assays

2.3.1. Animals and ethics statement

Male Swiss mice (25–30 g) were provided by Central Animal House of the Universidade do Vale do Itajaí (UNIVALI, Itajaí, Brazil). The animals were housed in standard cages, at room temperature (25 °C ± 2 °C) with 12 h dark/12 h light cycles, and supplemented with food and water *ad libitum*.

The project was approved by the Institutional Animal Care and Use Committee at the Universidade do Vale do Itajaí (Protocol number: 019/2014). All procedures were performed according to the Brazilian Socienty of Science of Laboratory Animals guidelines for the proper care and use of experimental animals.

2.3.2. Croton oil-induced ear edema

To evaluate the topical anti-inflammatory effect, the semisolid containing standardized spray dried extract of *A. moluccana* (10 mg/g), dexamethasone (1 mg/g) or vehicle was topically applied (50 μL) to the inner surface of the mouse right ear. The croton oil (2.5% diluted in acetone; 20 μL/ear) was applied to the outside surface of the right ear 30 min after the treatment with the semisolid. The ears thickness was measured before and 6 h after inflammation induction, using a micrometer (Mitutoyo Series 293). The micrometer was applied near the tip of the ear, slightly distal to the cartilaginous ridges, and the thickness was recorded in μm (Calixto et al., 1991).

2.3.3. Determination of cytokine levels

The IL-1β, TNF and CXCL1/KC levels were determined in the ear tissue collected 6 h after croton oil application. The samples (6 mm punch) were homogenized in PBS containing 0.05% Tween 20, 0.1 mM phenylmethylsulphonyl fluoride (PMCF), 0.1 mM benzamethonium chloride, 10 mM ethylenediaminetetraacetic acid (EDTA) and 20 UI aprotinin A.The homogeinate was centrifuged at 3000 g for 10 min before being stored at -70 °C until further analysis. IL-1β, TNF, and CXCL1/KC levels were quantified using enzyme-linked immunosorbent assay (EIA) kits accoding to the manufacturer's specifications (R&D Systems). The resuls were expressed as pg/mL of IL-1β, TNF or CXCL1/KC.

2.3.4. Myeloperoxidase (MPO) assay

The MPO, a hemoprotein located in the azurophilic granules of neutrophils, was used as a biochemical marker for neutrophil infiltration in the studied tissues. MPO activity was mensured according to the method originally described by Bradley et al. (1982). Animals were treated with 50 μL of semisolids. One group received the semisolid containging *A. molucana* extract (1%), other received vehicle, and another dexamethasone commercial semisolid (1 mg/g) 30 min before the croton oil (2.5%) application. The ear tissue samples (6 mm punch) were collected 6 h after the croton oil application and then homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was re-suspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (HTAB) and the samples were frozen and thawed three times in liquid nitrogen. The samples were centrifuged (10.000 rpm, 15 min, 4 °C) and 25 μL of the supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine (TMB), 80 mM sodium phosphate buffer pH 7.2, and 0.3 mM hydrogen peroxide. The absorbance was measured at 650 nm. The results are expressed as the optical density (OD) per milligram of tissue.

2.3.5. Histological analysis

Ear tissue samples (6 mm punch) were collected 6 h after croton oil treatment and fixed in 4% paraformaldehyde solution in PBS for 24 h and preserved in 70% EtOH until the beginning of the dehydration process (Rulih et al., 2011). Each sample was embedded in paraffin, sectioned in 3 μm slices andstained with haematoxylin–eosin (HE). The integrity of the epidermis, leukocyte infiltration, dermis thickness and edema were assessed in representative areas using 100x and 400x of increase.

2.4. Statistical analysis

Data were expressed as means ± S.E.M. Differences between means were determined by analysis of variance (ANOVA) followed by Dunnett's post hoc using the software GraphPad Prism 5. The data was considered significant when $p < 0.05$.

3. Results and discussion

The analiysis of the mechanism involved in the topical anti-inflammatory effect obtained with the semisolid containing *A.*

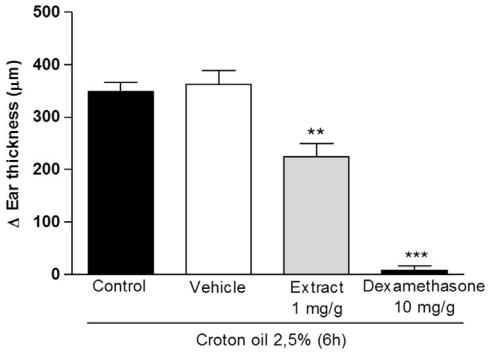

Fig. 1. Influence of pretreatment with topical cream containing dry extract of A. moluccana 1% on the ear edema induced by topical administration of croton oil. Mice received topic pre-treatment (50 μL/right ear, inner surface) for treated groups except the control, 30 min before application of croton oil (20 μL/right ear, l outer surface). Values represent the mean ± SEM of the difference between the measurements of time zero and after installation of the inflammatory response in animal groups (n = 6). Statistical analysis was performed using ANOVA followed by Newman-Keuls test. Asterisks denote **p < 0.01, *** p < 0.001) for treatments that differ from the control group.

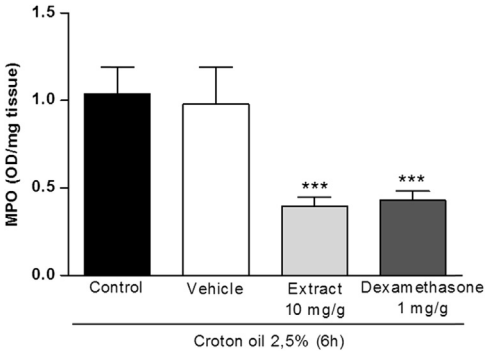

Fig. 3. Effect of topical cream containing dry extract of A. moluccana 1% on the activity of myeloperoxidase after 6 h of application of croton oil. Mice received topic pre-treatment (50 μL/right ear, inner surface) for the treated groups, except for control 30 min before the application of the cróton oil (20 μL/ right ear, outer surface). Values represent the mean of 4-5 animals and the vertical lines indicate SEM Statistical analysis was performed with ANOVA followed by Newman-Keuls test. Asterisks denote p < 0.001 for treatments that differ from the control group.

moluccana dried extract (1%) was performed using the ear-edema model induced by croton oil (2.5%) in mice (Fig. 1). The topical croton oil (2.5%) application on the ear skin resulted in the increase of the ear thickness, as presented in Fig. 1. The animals pretreated with the semisolid containing the plant extract or dexamethasone, the drug used as positive control, was able to significantly reduce the edema with inhibition values of 35.77 ± 7.35% and 97.69 ± 2.30%, respectively. Cesca et al. (2012) have demonstrated that a semisolid of *A. moluccana* (1%) significantly prevented the ear thickness increase (64.80 ± 6.20%) when compared with the vehicle. It is important to mention that the semisolid composition previously used by the author was different, what could justify the difference between the achived inhibitions.

It is important to analyse the pharmacological events iniciated by the croton oil administration, since the mechanism involved in the topical anti-inflammatory effect of *A. moluccana* dried extract is the main goal of this study. The ear-edema induced by croton oil is largely used in pre-clinical studies (Carlson et al., 1985). The croton oil is an irritant agent that promotes tissue acute reaction by the release of several inflammatory mediators (Cabrini et al., 2011; Baumgartner et al., 2011; Domiciano et al., 2013; Chen et al., 2012; Fabri et al., 2013; Borges et al., 2013; Chibli et al., 2014). The croton oil-induced response after topical application is characterized by edema caused by vasodilation and leukocyte infiltration. These events are evoked by protein kinase C (PKC) phosphorylation and subsequent phospholipase A2 (PLA2) activation. In the sequence, the arachidonic acid (AA) cascade is initiated and leukotrienes, prostaglandins and cytokine are produced, leading to the inflammatory processinstallation (Carlson et al., 1985; Cabrini et al., 2011; Domiciano et al., 2013; Chibli et al., 2014).

In fact, the main goal of this study was to investigate the mechanisms troughtout the extract causes edema inhibition. In this series of experiments we firstly investigated the effect of pretreatment with the semisolid containing *A. moluccana* extract in the production of inflammatory mediators induced by croton oil in mice's ears. The obtained data has shown that both extract and dexamethasone, applied 30 min before the croton oil, signicantly reduced the CXCL1/KC, IL-1β and TNF levels, with inhibition values of 62.29 ± 11.65%, 38.36 ± 5.92% and 53.75 ± 12.96%, respectively for the extract, and 62.58 ± 9.70%, 49.69 ± 6.68% and 86.92 ± 8.47%, respectively for dexamethasone (Fig. 2A, B and C).

The study developed by Lawrence et al. (2002) showed the events related to inflammation after induction by oil croton. The authors reported that in the first 30 min vasoactive amines (histamine and serotonin) are released and are responsible by the edema formation. However, the neutrophil migration is evidenced approximately 1 h after the injury (Calixto et al., 2003; Calder, 2006; Nicolaou et al., 2011).

Neutrophils are the first leukocytes recruited into the injury site. Their recruitment begins by resident macrophages and mast cells that induce several control events that allow this recruitment process, such as changes in endothelium cells, caused by inflammatory mediators, as histamine and cytokines, and chemokinesrelease (Kolaczkowska and Kubes, 2013). At the injury site, these cells phagocyte the causative agentsof injury and release

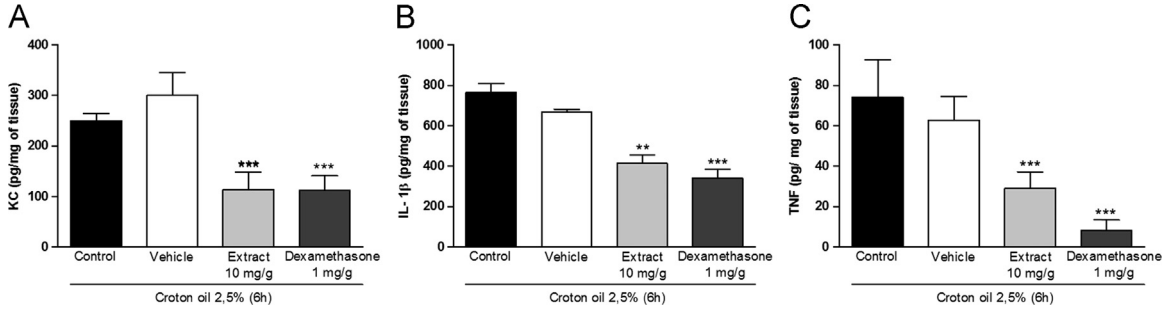

Fig. 2. -Effect of topical pre-treatment cream containing dry extract of A. moluccana 1% on the levels of chemokine KC, cytokines IL-1β and TNF after induction of ear edema induced by topical administration of croton oil. Mice received topic pre-treatment (50 μL/right ear, inner surface) for treated groups except the control, 30 min before application of croton (20 μL/right ear oil outer surface). Values represent the mean of 4-5 animals and the vertical lines indicate S.E.M. All samples were collected 6 h after induction of edema induced by croton oil due to the dosage of processing of KC Chemokine (A) IL-1β (B) and TNF (C). Statistical analysis was performed with ANOVA followed by Newman-Keuls test. Asterisks denote ** p < 0.01, *** p < 0.001) for treatments that differ from the control group.

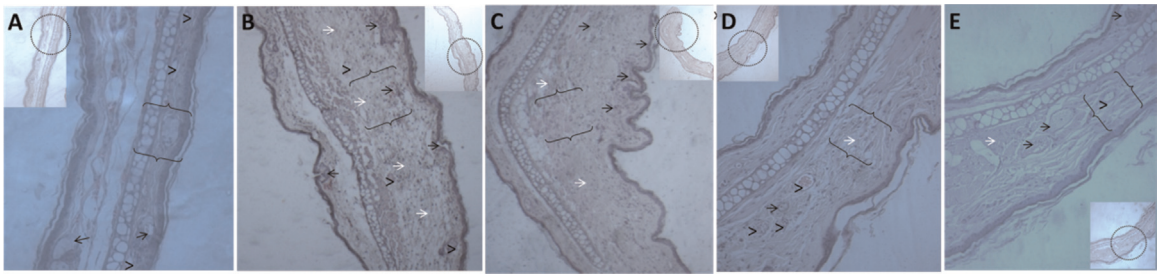

Fig. 4. Microscopic evaluation of pretreatment with topical cream containing A. moluccana 1% on the ear edema induced by topical application of croton oil.Mice received topic pre-treatment (50 μL / right ear, inner surface) for the treated groups, except for control 30 min before the application of the croton oil (20 μL/right ear, outer surface). Samples were collected 6 h after application of croton oil to the control group (B), Vehicle (C), semisolid containing *A. moluccana* extract (1%) (D) and dexamethasone (E). To the naive group (A), samples were collected without there some kind of treatment. The brackets in the images show the thickness of the dermis, the black arrows identify sebaceous glands, arrowhead identify blood vessels and the white arrows identify leukocyte infiltration. All were assessed in representative areas with increased 10x and 40x.

chemical mediators that contribute for the destruction of the damaging agent, such as the reactive oxygen species. However, in the case of noncontrolled inflammation, the interference in the neutrophil mobilization tothe injury site is an important therapeutic strategy (Li and Ng, 2012; Silva et al., 2015). In fact, the resultsobtained shown that after croton oil administration in animals pretreated with *A. moluccana* extract the neutrophil migration was significantly reduced (41.50 ± 9.30%, Fig. 3). Quintão et al. (2012) have also demonstrated that the treatment with *A. moluccana*dried extract, using oral administration route, was capable of drastically reducing the neutrophil migration induced by intraplantar injection of carrageenan. As mentioned before, the neutrophil migration depends on the production and release of chemokines. The release of the chemokine CXCL1/KC occurs rapidly under several experimental conditions and plays an important role in inducing the neutrophils influx, collaborating with their migration to the injured tissue (Vieira et al., 2009; Kolaczkowsha and Kubes, 2013). As mentioned above, our study has demonstrated that the extract administered topically significantly reduced the CXCL1/KC levels induced by croton oil, effect that could be responsible for the leukocyte migration inhibition. Both TNF and IL-1β also present important role in all cardinal signs of inflammation (Sanz and Kubes, 2012; Barioni et al., 2013). This data reinforce the idea that the extract could exerte its anti-inflamatory effects blocking the action of important mediators released prior to the leukocytes migration.

The Fig. 4 shows the microscopy evaluation of the ear-tissue of mice submitted to the croton oil-induced inflammation. Firstly, we have analysed the histological parameters of naive animals, in order to caractherize the skin normal morphology. The histological analysis presented resident cells, without changes in the epidermis and dermis, and cartilage. Dermis also presented a regular connective tissue with abundant extracellular matrix, presence of fibroblasts, sebaceous glands and hair follicles, blood vesselsin regular state (Fig. 4A). As expected, animals from control group and treated with placebo showed significant changes in the tissue morphology when compared with naive mice (Fig. 4B and C). The connective tissue in the dermis presented lower amount of extracellular matrix due to the increase in cell number that migrated to the injured site, which was approximately 4 folds greater than the naive mice, corroborating with the data obtained in MPO assay (Zanusso-Junior et al., 2011). No significant difference was found in the number and morphology of sebaceous glands, hair follicles and blood vessels, when compared to the naive group (Fig. 4A).

The groups treated with the semisolid containing *A. moluccana* extract (1%) or dexamethasone presented higher number of blood vessels (Fig. 4D and E), similar to placebo group. Regarding the epidermis and dermis, dexamethasone-treated mice showed thickness reduction and tissue characteristic quite similar to the naive group. The group of mice treated with the extract presented thickness reduction when compared with placebo-treated group, although a slight way when compared with dexamethasone group. The same thing happened with the cellular infiltrate, characterizing a significant reduction in the inflammatory process.

In fact, the *A. moluccana* extract presents flavonoids as 2″-O-rhamnosylswertisin and α,β-amyrenone, which has demonstrated potent antinociceptive and anti-inflamatory activity in the writhing model induced by acetic acid whemadministered by oral route. The effect promoted by extract was mediated by inhibiting the neutrophil migration and IL-1β release after carrageenan and CFA-induced mechanical nociception (Cechinel-Filho, 2000; Quintão et al. 2012; 2014).

Together, these results presented herein reinforce the popular use of this herbal product presenting significanttopical effect of the semisolid containing *A. moluccana* dried extract against skin inflammatory process. This effect seems to be related with the inhibition of neutrophil migration and inflammatory mediators' release.

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