Healing effects of *Lavandula officinalis* essential oil associated with *Arctium lappa* extract in a second degree burn model

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ABSTRACT

Burns present major impacts worldwide which stimulate the search for therapeutic alternatives, and the herbal medicines with anti-inflammatory and healing properties are potential targets. In the present study, we evaluated topical treatments with *Lavandula officinalis* essential oil (EO) and *Arctium lappa* extract in a second degree burn model. Wistar rats were anesthetized and submitted to thermal burns. The lesions were treated 2x/day with a cream containing 0.5% of *L. officinalis* EO plus 10% of *A. lappa* extract. Healing process evolution was evaluated on days 3, 7, 14, 21 and 30 after burns. Macroscopic and histopathological analyses showed more discreet edema and better reepithelialization in herbal treated animals. Higher expression of epidermal growth factor receptor in all phases of wound healing and lower expression of matrix metalloproteinases 2 and 9 especially at remodeling stages were observed in treated animals. Phytotherapeutic treatment reduced levels of interleukin-1β and tumor necrosis factor-α in scar tissues after 30 days. Aspartate aminotransferase levels were also decreased in herbal-treated animals. Our data showed better healing in animals treated with *L. officinalis* EO in association with *A. lappa* extract, pointing these phytotherapics as strategies for skin burns treatment.

**Key words:** Inflammation, mast cells, matrix metalloproteinases, medicinal herbs, wound healing.

Abbreviations: ANOVA, Analysis of variance; AST, aspartate serum aminotransferase; DAB, dianinobenidine; EO, essential oil; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ECM, extracellular matrix; HE, hematoxylin and eosin; IL-1β, interleukin-1 beta; MCs, mast cells; MMPs, matrix metalloproteinases; PDGF-A, platelet-derived growth factor A; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor-alpha.

INTRODUCTION

Burns are complex traumas that present high mortality rate and generate strong economic and psychosocial impact due to prolonged treatment (Richardson and Mustard, 2009; Xue et al., 2018). To restore the skin and prevent sequelae, the degree and extent of injury, infection, techniques and drugs available are considered in selecting the treatment of burns (Summer et al., 2007). In this sense, easily reproducible animal models that show clinical and histopathological similarities with second degree burns in human were established to evaluate the use of therapeutic agents in healing evolution (Meyerholz et al., 2009; Souza et al, 2017).

Wound healing is a highly complex process orchestrated by different cell types (Evers et al., 2010). Mast cells (MCs)
have important functions both in the acute and remodeling phases of burn healing (Dong et al., 2013; Douaiher et al., 2014; Iba et al., 2004; Wernersson and Pejler, 2014).

In degranulation process, MCs release chemotactic factors, pro-inflammatory cytokines as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), growth factors and proteases as tryptases and chymases into the extracellular environment, contributing to extracellular matrix (ECM) degradation, angiogenesis and tissue remodeling by selective proteolysis and activation of matrix metalloproteinases (MMPs) (Barrientos et al., 2008; Wernersson and Pejler, 2014).

The proteolytic degradation of ECM, through MMPs, is one of the key factors in repair and remodeling in wound healing and the imbalance between the synthesis and degradation of collagen results in abnormal scars. MMP2, anti-inflammatory, is observed primarily in fibroblasts, related to ECM remodeling. While MMP9, pro-inflammatory, is secreted by keratinocytes and inflammatory cells and is associated with the epithelialization (Gillard et al., 2006). Furthermore, the expression of epidermal growth factor (EGF) is extremely important for rapid wound closure and the action of this factor depends on the interaction with its receptor, EGFR (Barrientos et al., 2008).

Medicinal herbs, as lavender (Lavandula officinalis) essential oil (EO) (Alnamer et al., 2012; Koca Kutlu et al., 2013; Mori et al., 2016) or burdock (Arctium lappa) extracts (Knott et al., 2008; Sohn et al., 2011; Gilca et al., 2018) are considered alternative treatments in wound healing and skin affections because of their anti-inflammatory and cicatrising properties. The wound healing potential of lavender oil was demonstrated by acceleration of granulation and wound contraction through induction of transforming growth factor (TGF)-β in a rat model (Mori et al., 2016). Another research showed that wound closure progressed more rapidly with topical application of lavender oil associated with acceleration of platelet-derived growth factor (PDGF)-A and EGF (Koca Kutlu et al., 2013). Moreover, natural A. lappa fruit extract significantly improved the metabolism of dermal ECM and led to visible wrinkle reduction in vivo (Knott et al., 2008). The anti-allergic and anti-inflammatory effects of butanol extract from A. lappa were demonstrated by checking the release of b-hexosaminidase in activated RBL-2H3 MCs and the levels of IL-4 and IL-5 in primary splenocytes after treatment with concanavalin A (Sohn et al., 2011; Yang et al., 2016).

Although previous studies have suggested the curative potential of medicinal herbs on wound healing (Das et al., 2017; Dattner, 2003), to the best of our knowledge, there is no report on the use of L. officinalis and A. lappa in burns. Due to this and in view of the great impact of burns on public health, we evaluated the efficacy and safety of topical use of L. officinalis EO pure or associated with A. lappa extract in a model of second degree thermal injury in rats.

**MATERIALS AND METHODS**

**Animals**

Wistar rats (350 g) were divided into 2 groups (n=7/group) and kept in individual cages in a controlled environment (24 to 25°C, 12 h light/dark cycle) with water and food *ad libitum*. All experimental procedures were conducted according to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and the European legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and approved by the Ethic Committee on Animal Use at University Center Padre Albino (Certificate nº 12/14). The experiments were designed to minimize the number of animals used and their suffering during the execution of the protocols. All animals were daily evaluated by the institution’s veterinarian.

**Herbal medicines**

An ointment was prepared containing L. officinalis EO (By Samia®, São Paulo, Brazil) in the concentration of 0.5% and A. lappa extract of 10% incorporated into a cream (Biocap®, Trindade, Brazil). The L. officinalis EO was evaluated for its color, density and evaporation residue, being within the expected. Fresh leaves of A. lappa were collected at the University Center Padre Albino medicinal herbs garden and the vouchers specimens were deposited in the Institution herbarium. For the alcoholic extract preparation, 40 g of chopped dried herbs were placed in a Soxhlet extractor (Prolab, São Paulo, Brazil) with 160 ml of ethanol.

The A. lappa extract was standardize by phytochemical analysis performed for the identification of tannins (reaction of ferric chloride, neutral lead acetate and copper acetate), flavonoids (Shinoda reaction, reaction of aluminum chloride, sodium hydroxide reaction and ferric chloride reaction) and saponins (foaming by shaking) (Abdulla and Lutfi, 2016). The presence of all these actives were confirmed.

The concentration was chosen after *in vitro* cytotoxicity evaluation (Campos et al., 2016) by the analysis of blood cells exposed to different concentrations (5, 10, 25 and 50%), followed by the reading of absorbance at 540 nm in spectrophotometer. Cytotoxicity of 50% was observed from the 25% concentration, as a result, and knowing the possible synergistic action of medicinal herbs when administered together (Bahri et al., 2017), the 10% concentration was selected.
Burn injury model and treatments

Animals were anaesthetized intraperitoneally with 0.2 ml/100 g of ketamine (BioChimico, Itatiaia, Brazil) and 0.05 ml/100 g of xylazine (Ceva Santé Animale, Paulínea, Brazil) and subjected to shaving the dorsal area for application of a metal block 2 cm² for 10 s, preheated to 100°C with boiling water, setting the second degree burn (Meyerholz et al., 2009; Souza et al., 2017). Immediately, trauma lesions were covered with gauze moistened in cold saline solution. Rats were given analgesics codeine (1 ml/kg) (Cristália, Itapira, Brazil) by gavage, after injury induction and, in the following days, codeine was provided in the water (30 mg/L). The control of water intake and weight was daily performed.

Topical treatments were initiated 24 h after burns and maintained for 30 days. Control group animals were treated with two daily applications of 0.9% saline, while herbal medicine treated group received topical administrations of the cream containing L. officinalis EO plus A. lappa extract. To follow healing process evolution, there were performed excisions of the lesions in the upper and lower quadrants, right and left, respectively after 3, 7, 14 and 21 days of injury. After 30 days, the remaining lesions, Normal skin fragments, were also taken (n=7).

Histopathological and immunohistochemical studies

Fragments of normal skin and lesions were fixed in 4% formalin, processed for inclusion in paraffin and sectioned at 5 μm for histopathologic, quantitative and immunohistochemical analysis. The repair process was evaluated histologically by Hematoxylin-Eosin (HE) and the organization of the collagen fibers was evidenced by Picrosirius Polarization method.

MCs were stained with 0.1% toluidine blue and evaluated according to their morphological characteristics in intact or degranulated. MCs quantification was performed in 10 images per slide obtained by 40 X objective in a Leica microscope (DM500). Tissue areas were obtained using the Leica Image Analysis Software. Data were expressed as mean ± standard error mean (S.E.M.) of the number of MCs per mm².

Expressions of EGFR, MMP2 and MMP9 were evaluated by immunohistochemistry and subsequent optical densitometry of the immunostaining (Souza et al., 2017). For this, sections of the different samples were prepared on gelatinized slides, deparaffinized and rehydrated. After antigen retrieval (citrate buffer pH 6.0, to 96°C, for 20 min), endogenous peroxidase blockade and washing in PBS, the slides were incubated overnight in a humid chamber at 4°C with the polyclonal primaries antibodies: rabbit anti-EGFR (1:100; Abcam, Cambridge, MA, US), rabbit anti-MMP2 (1:100; Abcam) and rabbit anti-MMP9 (1:100; Abcam) diluted in 1% BSA. In the next day, slides were incubated with biotinylated secondary antibody (kit Zymed Invitrogen, Carlsbad, CA, US) and then in dianaminobenzidine (DAB) substrate (Zymed Invitrogen kit) for revelation. Subsequently, the sections were counterstained with hematoxylin.

For densitometric analysis, 10 distinct points of epidermis and dermis were evaluated to obtain an average related to immunostaining intensity. Values were obtained as arbitrary units (0 to 255) using the Leica Image Analysis software.

Quantitative analysis of cytokines and AST levels

Normal and wound skin fragments collected 30 days after burn were macerated in liquid nitrogen and added 500 μl of a solution containing protease and phosphatase inhibitors (Merck Millipore Corporation, USA). After, the material was incubated for 20 min at 4°C under agitation and then centrifuged at 14.000 rpm for 10 min at 4°C. TNF-α and IL-1β were quantified in the supernatant using the milliplex map Kit (recymag-65K; Merck Millipore) and analyzed on the Luminex xMAP Magpix device (Merck Millipore). The analytes concentration was determined by the Magpix x ponent software and expressed in pg/ml.

For biochemical analysis of aspartate serum aminotransferase (AST), blood was collected by cardiac puncture, centrifuged for 15 min at 3.000 rpm and the plasma was used for AST measurements using a comercial Kit (Labtest, Lagoa Santa, Brazil) according to the manufacturer’s instructions. The dosages were compared with animals not subjected to burns.

Statistical analysis

The results were previously submitted to descriptive analysis and determination of normality, and the means were compared by Analysis of Variance (ANOVA, two-way for MC quantification and densitometry and one-way for biochemical analyses), followed by the Bonferroni post-test. P values less than 0.05 were considered statistically significant (Zhang et al, 2016; Zike et al., 2017).

RESULTS

Macroscopic and histopathological analyses

Macroscopic analysis indicated better healing in herbal medicines treated group, which showed mild edema and faster falling of burned skin crusts, wounds closing and reappearance of attachments (Figure 1A and F).

Histopathological analysis confirmed the characteristics
of second-degree burns. In all groups, after 3 days of burn induction (acute phase of inflammation) (Figure 1B and G), an inflammatory influx was observed, especially of polymorphonucleates. On day 7 (Figure 1C and H), the reepithelialization and fibroblasts proliferation processes were observed. Whereas on days 14 (Figure 1D and I) and 21 (Figure 1E and J) post injury, the remodeling phase was characterized by restructuring of the epidermis, epithelial appendages and dermis with proliferation of the ECM, that was better in animals treated with the ointment containing L. officinalis EO plus A. lappa extract.

The changes in the patterns of birefringence of collagen fibers, observed after polarization, indicating reorganization, were more evident in the dermis of the herbal-treated animals (Figure 2).

After showing, macroscopically and histopathologically, the better healing of wounds treated with phytotherapeutic ointment, we quantified the MCs in the region of the lesion as evidenced by the Toluidine Blue as intact mast cells, with well-defined contour and this was not in the clear process of releasing the contents of their cytoplasmic granules (Figure 3B) and degranulated MCs with irregular contours and dispersed granules (Figure 3A).

On day 7 after burn, the largest amounts of mast cells were observed in untreated animals (Figure 3). Few total, intact and degranulated MCs were found in the herbal-treated group on days 3, 7, 14 and 21 after burn (Figure 3C and D).

**MMPs and EGFR expressions**

MMP2 (Figure 4) and MMP9 (Figure 5) immunostainings were analyzed in the epidermis and dermis of both groups during the tissue repair process. After 3 days of injury, the MMPs expressions were reduced and observed only in a few animals that had already initiated the reepithelialization process. From day 7, the immunostaining was verified in all groups.

MMP2 expression in epidermis was increased in the control group (Figure 4A, B and C) as compared with herbal-treated group on days 7 (< 0.01; Figure 4A, D, G) and 14 (< 0.001; Figure 4B, E and G) post injury. The dermis of treated animals showed variation in MMP2 expression which was reduced on day 7 (< 0.05; Figure 4A, D, H) as compared with the control group and on day 14 (< 0.01; Figure 4B, E, G).

Higher MMP9 expression was found in the control animals in epidermis (< 0.001; Figure 5B, E and G) and dermis (< 0.01 Figure 5B, E and H) after 14 days of repair process as compared with the herbal-treated group. On day 21, the increase was only observed in the epidermis of the control group (< 0.001; Figure 5G) as compared with the L. officinalis EO plus A. lappa extract cream treated animals.

Similar to MMPs, the expression of EGFR on day 3 was reduced and only observed in a few animals of the treated group, but from day 7 of injury, with the evolution of wounds closure, significant increase of EGFR expression...
Figure 2: Collagen fibers distribution during burn healing process. More organized dermis and more stained collagen fibers in the group treated with L. officinalis OE + A. lappa extract (I, J, K, L). In the remodeling phase, higher birefringence in the treated group (O, P). Picrossiúrus Staining (A, B, C, D, I, J, K, L) and Polarization (E, F, G, H, M, N, O, P). Bars: 500 μm.

was observed in animals treated with EO in cream associated with A. lappa extract ($p < 0.001$) as compared with the control (Figure 6A, D and H). The receptor immunostaining remained increased in herbal-treated group as compared with the control on days 14 ($p < 0.01$; Figure 6B, E and H) and 21 after injury induction ($p < 0.01$; Figure 6C, F and H). The specificity of the immunostainings was confirmed by the reaction controls (Figure 6G).

TNF-α, IL-1β and AST levels

TNF-α quantification showed increase in the control group ($p < 0.001$) as compared with measurements from normal skin fragments. Lower expressions of this cytokine were observed in EO plus A. lappa treated group ($p < 0.001$) (Normal: 0.38 ± 0.04; Control: 19.4 ± 2.65; L. officinalis EO and A. lappa: 3.21 ± 1.13; Figure 7A). Similar results ($p <$
Figure 3: MCs quantification in thermal second degree burns treated with phytotherapics. Few total MCs (C) on days 3 and 7 of the injury repair in herbal treated group (B) compared to control group (A). On days 07 and 21, the quantification of degranulated MCs (D) shows more cells in control group. The values are presented as mean ± S.E.M.

0.01) were found for IL-1β (Normal: 4.31 ± 2.29; Control: 352.44 ± 203.48; L. officinalis EO and A. lappa: 231.45 ± 133.62; Figure 7B).

Blood plasma analysis showed elevated AST levels in untreated animals subjected to burn. But treatment with cream containing L. officinalis EO and A. lappa extract significantly reduced AST levels (p < 0.05) (Figure 7C).

**DISCUSSION**

Burns constitute a serious public health problem (Richardson and Mustard, 2009; Summer et al., 2007; Xue et al., 2018) which encourages the search for alternative therapies such as herbal medicine. In this study, we developed experiments with an ointment formulated with essential oil of L. officinalis and extract of A. lappa, herbs not yet explored in burns, and we demonstrated the potential use of them in the treatment of second-degree burns.

First, we performed macroscopic analysis by monitoring the injuries during wound healing process. Our observations showed faster healing in animals treated with herbal medicines when compared with the controls. These results were corroborated by histopathological analysis and are consistent with the data obtained in other investigations, that used different medicinal plants as treatment in burn models (Abe et al., 2003; Chandran and Kuttan, 2008; Das et al., 2017). Several reactions, such as allergies, burning sensation and dermatitis have been documented with the use of other medicinal herbs (Kooshiar et al., 2012). On our phytochemical analyses of the A. lappa extract, it was observed the presence of flavonoids, tannins and saponins, that exhibit anti-inflammatory properties (Abo-dola and Lutfi, 2016), which may have prevented the allergic reaction on our model.

Knowing that in burn injury the interactions between inflammatory cells and ECM coordinate healing and tissue remodeling (Evers et al., 2010), we proceeded to MCs analysis, by quantifying these cells and evaluating their activation state. On days 3, 7, 14 and 21 after injury, we
found fewer MCs in the treatment group. The inflammation in early stages of the wound repair process is important to the overall healing, and this process depends on activation of MCs (Dong et al., 2013; Wernersson and Pejler, 2014). Impaired skin wound closure was observed in a study that used MCs deficient mice, indicating the importance of the mediators released from these cells to wound healing (Souza et al., 2017). In a previous research in the same burn model (Souza et al., 2017), it was found that treatment with silver sulfadiazine reduced the number of MCs, indicating that this as an interesting therapeutic strategy to avoid keloid formation.

Other researchers (Cho et al., 2011) found reduction of the inflammatory response in passive cutaneous anaphylaxis reaction with herbal *Fritillaria ussuriensis* treatment, by reducing the release of histamine and cytokines. Furthermore, another study demonstrated that oleamide, a bioactive present in the alcoholic extract of *A. lappa*, decreased the allergic response by reducing the release of histamine, TNF-α and IL-4 (Yang et al., 2016), which corroborates with our observation on the MCs reduction in the group treated with oil and *A. lappa* extract.

MCs and their proteases are related to matrix remodeling and are present at final stages of repair injuries and burns...
Figure 5: MMP9 expression in healing evolution in second degree burn and treatment. Strong immunostaining in all periods, especially in control animals (A, B, C). Decreased expression after treatment with herbal medicines (D, E, F). Counterstaining: Hematoxylin. Bars 50µm. Densitometric analysis of MMP9 expression in epidermis (G) and dermis (H). Data are presented as ± mean S.E.M.

(Ehrlich, 2013). Although MCs are important for matrix remodeling and cell proliferation (Weller et al., 2006; Wernersson and Pejler, 2014), researches also indicated the MC relation to formation of hypertrophic scars and fibrosis by activating fibroblasts (Douaiher et al., 2014; Dong et al., 2015).

MCs synthesize and release MMP2 and MMP9 (Iba et al., 2004; Wernersson and Pejler, 2014). Furthermore, it is also known that chymase is important to enable the MMP2 and MMP9 synthesis (Weller et al., 2006; Wernersson and Pejler, 2014). For these reasons, we analyzed the expression of these MMPs. Our results showed decreased expression of MMP2 in the epidermis of groups treated with herbal medicines. The expression of MMP9 was stronger in all groups as compared with MMP2, however, in the herbal treated groups, the intensity of labeling was lower in the epidermis and dermis as compared with the control group.

Herbal treated groups showed better healing, which may be related to the control of MMP2 and MMP9 expressions.
by treatment. Investigations indicate that the delay in thermal injury repair processes in the skin of older mice is associated with increased expression of MMP9 (Oriana et al., 2013). Other research (Nessler et al., 2014) showed that MMP9 may be associated with fibrosis and scarring and that degradation of dermal matrix regeneration (Integra®) is related to high levels of MMP2 in the blood plasma.

To better understand the effects of treatment on burn healing, we evaluated the EGFR expression in the epidermis. Our analyses showed reduced receptor expression in inflammatory phase and only observed in herbal treated animals, in which wounds closure occurred earlier. From 7 days after injury, the EGFR immunostaining was observed in all groups. These results indicated again, the efficacy of the used herbal medicines in modulating the tissue repair. The EGF is released mainly by fibroblasts and macrophages and is one of the main growth factors in the tissue regeneration process by promoting the reepithelialization mediated by EGFR (Barrientos et al., 2008).

Figure 6: EGFR expression in healing evolution in second degree burn and treatment with phytotherapics. EGFR expression after 7 (A, D), 14 (B, E) and 21 (C, F) days post injury. The expression of the receptor increases with treatment with herbal medicines. Absence of immunostaining in reaction control (G). Counter-staining: hematoxylin. Bars 50 μm. Densitometric analysis (H) the data are represented as mean ± standard error of the mean (S.E.M) and confirm the increased in immunostaining in groups treated with EO L. officinalis and associated to A. lappa.
Figure 7: Dosage of TNF-α and IL-1β in lesions and AST plasma levels after second degree burn and treatment. Data are presented as mean ± mean S.E.M and show increased levels of (A) TNF-α and IL-1β (B) in burned skin without treatment and reduced levels in herbal-treated animals. Increased levels of AST after burning but reduction with *L. officinalis* OE + *A. lappa* treatment (C). *** p < 0.001 and ** p < 0.01 vs Normal; ### p < 0.001 and # p < 0.05 vs control group 30 days after injury.

In determining the importance of the inflammatory mediators, TNF-α and IL-1β levels were quantified in supernatant of lesions macerated fragments of 30 days after burn. Quantification of both cytokines revealed increased expression only in the control group, showing that treatments with *L. officinalis* EO associated with *A. lappa* extract in cream were able to reduce cytokines levels. The cytokines TNF-α and IL-1β are pro-inflammatory and important for leukocyte recruitment and reepithelialization in early phases of tissue repair, but the extension of
cytokine liberation and the prolonged inflammation can be harmful (Agay et al., 2008; Barrientos et al., 2008). In addition, other studies also observed the herbal anti-inflammatory property by reducing cytokines levels (Abe et al., 2003; Sohn et al., 2011). The Lavandula angustifolia EO using in aromatherapy treatment reduced TNF-α (Abe et al., 2003) and an in vitro study demonstrated that A. lappa extract may reduce IL-4 and IL-5 expression (Sohn et al., 2011).

Another important factor to be considered in the thermal injury is the possibility of deep systemic and metabolic changes (Richardson and Mustard, 2009). Thus, at the end of the study, we evaluated the effects of herbal treatments on AST levels. Our results indicated increased AST levels post burn but significant reduction after treatment with EO associated with A. lappa. This indicates, besides the toxicity absence of the treatment, the protective effect of herbal medicine in reducing injury to internal organs.

Conclusions

All together, our data showed that topical treatments with L. officinalis EO associated with A. lappa extract promoted better tissue repair as compared with untreated animals in a burn model. These results open the possibility for further exploration of these herbal medicines in the treatment of burns.

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