Effects of aroeira (*Schinus terebinthifolius* Raddi) oil on cutaneous wound healing in rats

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ABSTRACT

PURPOSE: To evaluate the effects of aroeira (*Schinus terebinthifolius*) ointment on skin wound healing in rats.

METHODS: Adult male rats (n=20) were divided into four groups of five animals each, as follows: G4, G7, G14 and G21, which corresponds to 4th, 7th, 14th and 21st days postoperatively. Each animal were made two incisions on the skin, including the subcutaneous tissue, in the right and left sides of thoracic region, separated by a distance of two inches. The right lesion was treated with base ointment (vaseline, lanolin); the left one was treated with base ointment containing 5% of aroeira oil. At the end of each experimental period the lesions were evaluated for the contraction degree. Then held the collection of fragments that were fixed in 10% formalin and processed for paraffin embedding. In the histological sections (5μm) was evaluated the morphology and quantified the collagen and blood vessels. The data obtained were submitted to ANOVA test complemented by Tukey-Kramer test (*p*<0.05).

RESULTS: The contraction of the lesions was higher in wounds treated with aroeira oil than in controls at 7th and 14th days (*p*<0.01), whereas in the 21st day all lesions were already completely healed. The morphology showed granulation tissue more developed, with fibroblasts more bulky and collagen fibers more arranged in the experimental group at 4th, 7th and 14th days. The morphometry showed a significant increase in the quantification of collagen fibers in the experimental group at 7th and 14th days (*p*<0.05).

CONCLUSION: The aroeira oil accelerates the healing process of wounds as a macroscopic, morphological and morphometrical analysis.

Key words: Wound Healing, Angiogenesis Inducing Agents, Skin, Phytotherapy, Anacardiaceae, Rats.
Introduction

Plants are an unlimited source of potentially active substances and many of them are used to help promote healing and angiogenesis\(^1\). They must be considered as raw material, the first step in discovering new molecules and developing new phytotherapeutics\(^1\). Although popular knowledge has contributed significantly on understanding the effects of medicinal plants, their active principles, mechanism of action and toxicity are still poorly understood\(^1,2\), justifying scientific research to prove their effectiveness.

The aroeira tree (\textit{Schinus terebinthifolius} Raddi) belongs to the Anacardiaceae family and is widely used in popular medicine possessing high capacity in the production of allelopathic compounds\(^3,4\). In Brazil it is found in various vegetations, from Pernambuco to Rio Grande do Sul\(^4\) it is popularly known as Brazilian Pepper, Aroeira, Florida Holly, Rose Pepper, and Christmasberry. It is used to treat wounds and ulcers of skin and mucous membranes, against infections of the respiratory system, digestive system, genito-urinary tract, hemoptysis and metrorrhagia\(^1\). Aroeira has been the subject of several studies involving the use of extracts of bark, leaves and fruit as a healing promoter\(^5\) and as an antibacterial and antifungal agent\(^6,9\).

Healing is a complex, systemic, physiological process where the body is required to activate, inhibit, and produce a number of cellular and molecular components that are organized in order to contribute to the restoration process and the function of damaged tissues. It is divided into stages; at first the cells are destroyed, characterizing the catabolic phase followed by the formation of new cellular elements, constituting the anabolic phase\(^10\).

Under normal conditions, the healing process follows a pattern that can be divided into three specific phases: inflammatory, fibroplastic phase with extracellular matrix deposition and remodeling phase\(^11\). The characteristics assumed by the injury during its evolution results of succession or overlapping cellular and tissue events resulting from cell activation by chemical mediators\(^11,12\).

In the inflammatory phase, the predominant events are related to blood clotting with thrombosis formation and inflammatory process. This phase is characterized by vasoconstriction, platelet aggregation and activation of clotting systems\(^13\). The complete repair of tissue depends on the action of leukocytes, which in addition to their immune activities are closely involved in the anabolic and catabolic reactions, in tissue degradation by proteases and production of reactive oxygen and nitrogen in tissue formation and growth factors production\(^11\).

The fibroplastic phase and deposition of extracellular matrix is characterized by granulation, contraction and epithelialization of the wound. Beginning approximately four days after injury and according to Rodrigues \textit{et al.}\(^13\), can continue for two weeks. Granulation is the formation of a tissue composed of capillaries, collagen and proteoglycans\(^13\). The activation of fibroblasts in this phase is intensified and activated to produce collagen. Thus, fibroplasia is necessary to neovascularization in the region. Capillary formation is a result of angiogenic factors secreted by macrophages and mast cells that stimulate the proliferation of endothelial cells from blood vessels. It is essential at this stage because it allows gas exchange and nutrition of metabolically active cells\(^11\). In addition to the direct action of growth factors, particularly VEGF (vascular endothelial growth factor) on the endothelial cells vessels, induction of angiogenesis is also influenced by low oxygen tension that occurs in the center of a wound\(^12,14\). Reepithelialization, migration and mitotic division of basal cells at the edges of the wound occurs along with the granulation process\(^15\). Finalizing with the process of contraction, this occurs by spontaneous closure of skin wounds and by the action of specialized myofibroblasts\(^13\).

The maturation or remodeling stage remains for months or years, in which acute and chronic inflammatory cells gradually decrease and stop angiogenesis and fibroplasia. It is also during this period that we see the balance between synthesis and degradation of collagen, and this remodeling is responsible for increasing the tensile strength of scar tissue\(^11,13\).

Based on popular medicine and on the previously known healing action of hydroalcoholic extracts of \textit{Schinus terebinthifolius} Raddi and the action of its essential oils against bacteria and fungi, also, in order to study the manipulation of its ointment with a percentage of concentration that promotes the tissue repair process, this study was conducted to evaluate the effect of an ointment manufactured with essential oil of \textit{Schinus terebinthifolius} Raddi leaf 5% in the treatment of cutaneous wound healing in rats.

Methods

The study protocol was approved by the Animal Ethics Committee of the Federal Rural University of Pernambuco (UFRPE) (process n° 23082.014123/2011). At the end of the experiment the animals were euthanized, prepared and discarded according to the requirements of ethical principles for experimental work of the Brazilian College of Animal Experimentation.
Twenty adult male rats (Rattus norvegicus albinus), weighing 250g, with three months of age were obtained from the Morphology and Physiology Department of Federal Rural University of Pernambuco (UFRPE). The animals were housed in individual boxes with commercial chow (Presence®, Purina) and water ad libitum, maintained at 23-25°C, under 12 hour light/dark cycle, in the animal colony at the Pharmacy Department of Rural Federal University of Pernambuco (UFRPE).

After a week of adaptation to the new environment, the animals were anesthetized with a combination of xylazine (20 mg/Kg) and ketamine (100 mg/Kg), administered intramuscularly12. The animals furry thoracic region was shaved and antisepsis was performed with topical alcoholic chlorhexidine 0.5%. The area was initially marked with the aid of 1.3 cm diameter cylinder. With a surgical blade and blunt scissors, incisions were made in skin and subcutaneous tissue on the right and left sides of the thoracic region, separated by a 2 cm distance. The tissue was dissected and removed leaving adjacent fascia exposed.

Immediately after surgical excision the wounds located on the right antimere, received a daily topical applications of the ointment lanolin and Vaseline base (vehicle) - control group (GCtrl) and the located on the left antimere, received a daily topical application of the ointment containing aroeira Leaf oil at 5% treated group (GTreat). The rats were then divided into four groups of five animals each according to the time of application; G14 - 14 days of application and G21 - 21 days of the ointment. G4 - four days application; G7 - seven days application.

Preparation of the extract of Schinus terebinthifolius

Leaves of the pepper tree (Schinus terebinthifolius Raddi, Anacardiaceae) were collected in the morning of September 2010 on the campus of Federal Rural University of Pernambuco (UFRPE). The plant was identified by comparison with a previously identified specimen and deposited in the Herbarium Vasconcelos Sobrinho UFRPE under the number 49259.

To obtain the essential oil, fresh leaves (200g) were crushed and subjected to hydrodistillation technique in a modified Clevenger apparatus. After two hours of hydrodistillation the oil obtained was separated from water by density difference and excess moisture was removed with anhydrous sodium sulfate (Na₂SO₄)16. The total oil amount was calculated based on the weight of fresh leaves. The oil was stored in amber glass container, tightly closed kept in freezer, until the experiment, at a temperature of -20°C. The study of the leaves oil had its chemical profile reported by Silva et al.17, which identified thirty-three components, representing 95.5% of the oil. Among the majority of this oil compounds are p-Cymen-7-ol (22.5%), 9-epi-(E)-cariophyllene (10.1%), carvone (7.5%) and Verbenone (7.4%).

With the oil, an ointment was manipulated with a lanolin-vaseline formulation: lanolin anhydrous - 30%; essential oil of aroeira leaf - 5% VIT; e-oily acetate - 0.5%; solid vaseline qsp 100g.

The formulation was based on a mixture of anhydrous lanolin and vaseline solid and the antioxidant of the essential oil itself (anhydrous lanolin purchased from Pharma Special, solid vaseline and BHT acquired from DEG), as recommended by Gulcan et al.18.

Morphology and morphometry

On 4th, 7th, 14th and 21st days after surgery, the wounds of each group were measured with the aid of a caliper graph (King Tools). To calculate the injured areas the major and minor diameters were observed. From these data the wound area (A) was calculated as $A = \pi R_r r$, where $R$ the larger radius and $r$ the smaller radius of the wound.

The calculation of the average degree of contraction (C) was expressed as percentuals, being $C = \frac{(A_0 - A_i)}{A_0} \times 100$, where $A_0$ is the initial area of mm the wound (day 0) and $A_i$ is the area of the wound in the 4th, 7th, 14th and 21st days postoperatively.

After macroscopic analysis of the respective groups the animals were anesthetized with isoflurane and a fragment was collected for histological analysis was administered to animals from each group (G4, G7, G14 and G21), the wound was dissected with a 0.5 cm margin of healthy skin around the lesion.

Histological analysis

After 24h in 10% formaldehyde, tissue samples were dehydrated in increasing concentrations of ethyl alcohol and diaphanized in xylene. Samples were then processed for paraffin inclusion. For each animal cuts were made in the middle region of the flap Sections longitudinal samples (5µm) were obtained parallel to the greater axis of fragments and stained with hematoxylin-eosin (H.E) and Gomori trichrome for morphological and histometric analysis.
**Immunohistochemistry analyses**

Two sections (5µm) were dried overnight at 37°C and then during 30 minutes at 60°C. Slides were immersed and dehydrated with xylene and alcohol. Afterwards, they were left in 3% H₂O₂ for five minutes to prevent endogenous peroxidase activity. Slides were transferred to citrate-buffered solution (pH 6.0) and processed in a microwave (750w) for five minutes twice. Then, slides were submitted to immunohistochemical staining with classic avidin-biotin peroxidase method for VEGF (VEGF -LabVision; 1/100). Immunohistochemical staining was performed with streptavidin-biotin-peroxidase method (UltraVision Polyvalent [rabbit-mouse], LabVision Products) with horseradish peroxidase (HRP) kit. Diaminobenzidin was used as the chromogen.

Six images of each slide was obtained, always immediately below the crust, with the aid of a trinocular biological microscope (NIKON 50i) under 400X magnification and adjusted to a system that captures images. Quantification of newly formed vessels and the collagen content was performed in the center of the lesion in an area of 0.66 mm² (imaging), with the aid of an image analyzer (Imagelab 2000) in a Windows operational system.

**Statistical analysis**

The data was evaluated by ANOVA complemented by Tukey-Kramer test (p<0.05). Statistical analysis was performed using Assistat software, version 7.6 beta2.0.

**Results**

Our results demonstrated an earlier degree of wound contraction in the treated group at day 14 postoperatively (PO). In this period three GTreat wounds had reepithelialized and two had epithelia almost complete. In the GCtrl only two wounds were completely healed and three were approaching reepithelization. At day 21 PO all wounds were completely healed with integrate epithelium. Analyzing the averaged percentages of the results, the GTreat wounds contracted more than GCtrl on days 7 and 14 PO, with statistical difference in the latter (Table 1).

**TABLE 1 - Skin wound contraction (expressed as mean ± SD relative to the initial area of the lesion) upon different periods of treatment of the lesions with drug vehicle or with an Aroeira oil ointment.**

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<thead>
<tr>
<th>Treatment</th>
<th>Postoperative days</th>
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<tr>
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<td>4</td>
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<tr>
<td>Drug vehicle</td>
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<td></td>
<td>7.5</td>
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<tr>
<td>Aroeira oil ointment</td>
<td>±</td>
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<td></td>
<td>8.1</td>
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<td>13.6%</td>
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*p<0.05 in comparison with the respective control (drug vehicle) value.

Morphological analysis demonstrated a high concentration of polymorphonuclear cells observed in both groups at day four postoperative. Mast cells were visualized both on the side that received the ointment base (GCtrl) and the side that received ointment containing aroeira leaf oil at 5% (GTreat) in the 4th, 7th, 14th and 21st days after the formation of skin wound. However, the control group showed some mast cells scattered through the granulation tissue or in between the newly formed capillaries while the treated group showed multiple aggregates of mast cells in the fibrovascular tissue in all periods.

The concentration of collagen was higher in the GTreat on days 7 and 14 PO (p<0.007). On the fourth postoperative day the wound showed granulation tissue approaching themselves, with low concentration of collagen fibers in both groups. Morphologically, the collagen fibers of the GTreat were more exuberant and organized when compared to GCtrl (Figures 1 and 2) at all times.
Histometric assessment of neoangiogenesis demonstrated no differences between the mean number of blood vessels in the treated and control groups in different periods. Morphologically, at 4th PO day, the GCtrl had newly formed, dilated and congested capillaries, demonstrating a greater degree of immaturity in relation to GTreat where the tissue had newly formed capillaries of smaller diameter (Figures 3 and 4). In the other periods, the GTrat, with evident neoangiogenesis, had a higher concentration of collagen and the epithelization process occurred earlier.

Discussion

Initially we would mention that extracts of pepper tree (aroeira) are already used in popular medium as cicatrizing agent, however, few works are of a scientific showing effective results. Accordingly our study showed smaller scar area on the 7th and 14th days in wounds treated with ointment containing areoeira oil than in the control group, indicating an early shrinkage. Concerning high concentration of polymorphonuclear cells observed in the fourth postoperative day coincide with the findings of Branco-Neto et al.6 and their work with hydroalcoholic extract of pepper tree. According to Mandelbaum et al.19, polymorphonuclear leukocytes arrive at the time of tissue injury and remain for a period of three to five days, primarily with the function to elimination possible microorganisms by phagocytosis.

According to Ng12, mast cells release inflammatory mediators (TNF-α, IL-1), growth factors (TGF-β1, PDGF) and proteases (chymase and tryptase) in early stages (inflammatory phase) by altering the regulation of vascular permeability and leukocyte infiltration into the wound. They are also able to induce and exacerbate the process of angiogenesis by releasing tumor necrosis factor, tryptase and heparin20. Moreover, mast cells
accumulate in the wound edge and participate in the remodeling of collagen.

Fibroplasia is extremely important in the formation of granulation tissue. With an increase of activated fibroblasts to produce collagen, the extracellular matrix begins to be replaced by a connective tissue stronger and more elastic. A similar result was observed by Ribas et al. where the pepper trees extract increased fibroblast proliferation in the oral mucosa of rats.

The angiogenesis phenomenon is essential to carry oxygen to ischemic or newly formed healing tissues. Stimulation of growth factors and other mediators, endothelial cells of intact vessels inside the margins of the wound migrate toward the injured area, differentiate to form new capillaries that sprouts form on the outer side of the vessels. These join the original capillaries and restore blood flow. In this experiment, the number of newly formed vessels was enough to keep the healing process, aiding the production of collagen by fibroblasts and epithelialization with a superior time on treated lesions.

The Schinus terebinthifolius Raddi is a public domain plant popularly used for the treatment of lesions and ulcers, inflammation and infections, whose active principles are not fully understood. Morphologically, histologically and chemically the leaves and bark of this species are rich in tannins and essential oils, saponins are only in the bark, and the bark is of a different phenolic composition than the leaves.

A research with extracts conducted by Branco-Neto et al. a delay in reepithelialization of skin wounds was observed in rats after using the hydroalcoholic extract prepared from the aroeira bark (Schinus terebinthifolius Raddi). Ribas et al. demonstrated that Schinus terebinthifolius was effective in the epithelial tissue repair process of ulcers on the oral mucosa of rats. Lucena et al. concluded that the use of hydroalcoholic extract of aroeira was favorable to heal rats that were submitted to cystotomy. Copaiba oil was effective in healing of random skin flaps in rats.

Chemically, essential oils contain mostly terpene substances and eventually phenylpropanoids, together with smaller molecules, such as alcohol, esters, aldehydes and short-chain ketones. Many of the biological activities of terpenoids, among them antimicrobial, are related to its essential oils. Terpenes are the main chemicals responsible for the fragrances, culinary and medicinal uses of plants. According to Lima et al. antibacterial and antifungal activity of aqueous extract of S. terebinthifolius, is possibly associated with the presence of certain chemical compounds present in small amounts, such as alkaloids, steroids, and chalcones urundeuvinas.

The aroeira essential oil promoted antimicrobial activity against Escherichia coli, Shigella dysenteriae, Bacillus subtilis and Staphylococcus aureus and antifungal activity on Aspergillus niger, A. parasiticus, A. flavus, A. oryzae, A. fumigatus, Penicillium digitatum, Trichoderma spp and Helminthosporium oryae analyzing the antimicrobial activity of aqueous and alcoholic extracts obtained from Schinus terebinthifolius fruits learned that the alcoholic extract showed inhibitory effect on Staphylococcus aureus and Bacillus cereus growth, while the aqueous extract promoted no inhibitory effect on microorganisms growth. This result was due to the significant amount of flavone apigenin, and ellagic acid in the alcoholic extract, which the aqueous extract does not contain.

Most of the oil samples analyzed by Barbosa et al. revealed the α-pinene as its major component, especially those collected in India. α-pinene and Cymen were previously reported for its inhibitory effect on microorganisms. Silva et al. observed in vitro a favorable effect of the Schinus terebinthifolius Raddi essential oil on coagulase-positive staphylococci obtained from canine oitis. The antimicrobial activity is usually a result from synergism between various chemical compounds present in the oil, although this may also be due to a particular chemical compound.

Essential oils and extracts may be manufactured as topical dosage forms such as ointments. Semisolid preparations are of soft consistency, for skin and mucous membrane use. Ointments should have plasticity in order to modify its shape with little mechanical effort and easily adapt and adjust to the place where they are being applied. According to Viegas et al. the combination of Vaseline and lanolin anhydrous solid results in a hydrophobic, oily and aqueous excipient mixture, promoting at the same time an occlusive effect (vaseline) and endodermal activity (lanolin) in order to penetrate the skin, improving the absorption of the active principles in deeper tissue layers, but without reaching the bloodstream.

This research contributed to assess the healing process aided by the essential oil of aroeira and to develop an ointment with a concentration that promotes the tissue repair process as an alternative treatment of cutaneous wounds. Other studies and further concentrations of this phytomedicine are being developed as to improve the minimum optimal concentration needed for its use on skin wounds. Additional studies are necessary to establish the direct role of this essential oil in the regulatory mechanisms of collagen synthesis and maturation processes and epithelial regeneration mechanisms.
Conclusion

After analysis of results obtained in this experiment we concluded that the ointment containing aroeira oil at 5% was favorable for the tissue repair process of rat skin wounds.

References

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