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EVALUATION OF *IN VIVO* HEALING AND ANGIOGENIC EFFECTS OF GELS CONTAINING SCHINUS TEREBINTHIFOLIUS RADDI EXTRACT ON OPEN WOUNDS

[1]Jaleska Santos Olinto Trindade, [2]Kalline Alves de Araújo, [3]João Carlos da Silva, [4]Ciro Dantas Soares, [5]Cypriano Galvão da Trindade Neto, [6]Eduardo Pereira de Azevedo, [6]Amália Cínthia Meneses do Rêgo, [6]Irami Araújo-Filho, [6]Heryka Myrna Maia Ramalho

[1]Department of Biotechnology, Potiguar University, Laureate International Universities.
[2]Department of Biology, Potiguar University, Laureate International Universities.
[3]Department of Biomedicine, Potiguar University, Laureate International Universities.
[4]Department of Oral Pathology, State University of Campinas.
[5]Department of Pharmacy, Potiguar University, Laureate International Universities.
[6]Department of Biotechnology, Potiguar University, Laureate International Universities.

Abstract*–*Objective: To assess the healing and angiogenic effects of* SchinusterebinthifoliusRaddi *(STR) extract embedded in chitosan and Carbopol® gels in an experimental model using Wistar rats.
Methods: Fifteen Wistar rats (mean age, 90 ± 15 days; weight, 270 ± 70 g) were subjected to a skin wound model and were then divided into three groups of 5 rats each based on the topical treatment received: CG (control group), sodium chloride (NaCl) 0.9%; CTE, treated with chitosan gel containing STR extract once per day; and CPE, treated with Carbopol® gel containing STR extract once per day. A morphometric analysis was performed on the first and seventh days of treatment. After euthanasia, an excisional biopsy was performed for histological and histomorphometric analysis.
Results: The qualitative microscopic analysis revealed that there was increased angiogenesis and elevation in the number of fibroblasts and collagen in the CTE group compared with the other groups.
Conclusion: In the present* in vivo *study, chitosan gel with STR extract repaired wounds more effectively than NaCl solution or Carbopol® formulation, with greater amounts of collagen and more angiogenesis stimulation.

Keywords -Wound healing; *Schinus;Anacardiaceae*; Angiogenesis; Chitosan

I. INTRODUCTION

For thousands of years, humans have been using plants for the treatment of several diseases[1-2]. The interest in the use of medicinal plants have increased in recent decades; they are often used as the sole therapeutic resource in many countries [3]. Numerous plant species are known for being important sources of bioactive compounds, and therefore several studies have been conducted to investigate the effectiveness of these plants in the treatment of chronic diseases [4].

Brazil has at least 20% (around 40,000) of the world's plant species [4]. Among them is the Brazilian pepper (*Schinusterebinthifolius*Raddi [STR]), popularly known as aroeira, rose pepper, and Christmas berry [5]. The plant belongs to the *Anacardiaceae* family, and is considered a native flora species [6]. Parts of this plant, such as the bark, leaves, and fruits [7], are being researched for their potential activities as antibacterial [7-14], antitumor [15-17], antioxidant [7,13,18-21], anti-inflammatory, and wound healing [18,22,23].

Skin wounds are lesions with high prevalence worldwide. In addition, they are a frequent cause of morbidity and mortality, especially in the case of chronic wounds where the normal healing process is delayed. After an injury, a sequence of complex and dynamic biological events is initiated, aiming to promote lesion repair, which characterise healing. Although tissue repair (both by regeneration and tissue replacement) is a systemic process, it is essential to stimulate favourable local physiological conditions through appropriate topical therapy[22, 24]. A chronic wound is characterised by a granulation tissue composed mainly of inflammatory cells and few fibroblasts. Besides, there is a significant reduction in angiogenesis and collagen formation. Thus, there is a continuous research for new formulations that can positively modulate these processes.

Research for natural products that have the ability to improve the wound healing process has been intensified. The anti-inflammatory and immunoregulatory properties of natural products have been tested in various tissues, in order to aid the process of tissue repair [25-26]. Chitosan (a polysaccharide obtained from the exoskeleton of crustaceans) has been extensively used as a vehicle for pharmaceutical preparations due to its biocompatibility, biodegradability and non-toxicity [27].

The ideal properties of a natural healing agent comprise immunomodulatory and neocollagenesis activities. In addition, it should also be able to accelerate the wound repair process, as well as to stimulate angiogenesis.

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However, obtaining such a compound is difficult, and research continues in this regard.

Therefore, the present study aimed to assess the healing and angiogenic effects of STR extract embedded in chitosan and Carbopol® gels in a skin wound model.

II. MATERIAL AND METHODS

Botanical Materials

Botanical STR materials were collected at the Agreste region of the state of Rio Grande do Norte, Brazil, in March 2014. The staff of Sebastião Urias de Paula Herbarium of Potiguar University performed the botanical identification of the species for sample preparation and material categorization.

Preparation of the extract

STR leaves were dried in an oven at 60°C for 48 h and then crushed into a powder, where it was weighed and placed in a glass container. Further, ethanol 70% was added at a proportion of 1:3 v:w relative to the powder, at room temperature (~25°C) [28]. The plant material was macerated for 7 days using the percolation process. Then, it was filtered and the extract was concentrated to 10% in a rotary evaporator.

Preparation of chitosan gels containing STR

Water, acetic acid, ethylenediaminetetraacetic acid (EDTA), chitosan, and the hydroalcoholic STR extract were used for the preparation of chitosan gels, using the phase overlay process with controlled speed (Table 1). Briefly, acetic acid and EDTA were dissolved in sufficient water, homogenised, and the STR extract was then added, followed by the addition of chitosan until the preparation was completely homogeneous and gelation could be observed.

Preparation of the Carbopol® gels

The formulation containing Carbopol®, a hydrophilic Galenic gelling base, was prepared using water, STR extract, EDTA, Nipagin®, Carbopol®, propylene glycol, and AmP 95 (2-amino-2-methyl-1-propanol, 95%; Table 1).

The formulation was prepared by dissolving STR extract in water, with further addition of EDTA and Nipagin® previously dissolved in water and alcohol, respectively. Carbopol® was added until the solution was completely homogenised. After 24 hours, propylene glycol and AmP 95 were slowly added via mechanical stirring until the characteristics of a gel were observed.

TABLE 1. FORMULATIONS CONTAINING STR EXTRACT.

Components	Chitosan formulation	Carbopol® formulation
Acetic acid	2%	-
Chitosan	2.5%	-
Water	qs	qs
STR extract	10%	10%
Ethylenediaminetetraacetic acid (EDTA)	0.05%	0.05%
Nipagin®	-	0.1%
Carbopol®	-	0.5%
Propylene glycol	-	2%
AmP 95	-	1%

In vivo experiment

The research was conducted in accordance with the Animal Experimentation Code of Ethics and the Brazilian College of Animal Experimentation. It was approved by the Ethics Committee for Animal Use in Research of Potiguar University (No. 005/2014).

Fifteen male Wistar rats (mean age, 90 ± 15 day; mean weight 270 ± 70 g) from Potiguar University's vivarium, were kept in individual polypropylene boxes, with balanced feed (Labina® Purina) and water *ad libitum*, following light and dark cycles (12 h) with ventilation and controlled temperature (21°C).

For the surgical procedure, the animals were anaesthetised using an intramuscular Zoletil® 50 solution, at a dose of 0.1 mL/100 g. After anaesthesia, trichotomy and antisepsis of the animals' back were performed with 2% chlorhexidine. Then, an incision was performed, producing a clean wound of approximately 1 cm².

After surgery, the rats were divided into three groups: control group (CG, n = 5), treated with topical application of sodium chloride (NaCl) 0.9%; chitosan + extract group (CTE, n = 5), treated with chitosan gel containing STR extract; and Carbopol® + extract group (CPE, n = 5), treated with a Carbopol® gel containing STR extract.

Treatment was initiated 24 hours after the surgical procedure.

Microscopic evaluation

After 7 days of treatment, the animals were anaesthetised and an excision biopsy was performed, containing normal and scar tissue. Then, the skin samples were randomly numbered and placed into vials containing 20 mL of 10% formaldehyde solution. The animals were euthanised with a lethal intracardiac dose of sodium thiopental. After 48 hours of fixation, the samples were sent for histopathologic assessment. The samples were processed in accordance with the histological routine (dehydration, clarification, and rehydration), embedded in paraffin, and cut into 4-µm slices by a rotary microtome. Further, they were stained using haematoxylin and eosin stain (H&E) and Masson's trichrome stain—the most commonly used staining method for identifying collagen fibres. Afterwards, the slides were examined under a Kodak® optical microscope (EasyShare C183; 2010) by two pathologists, in a blinded manner. Data was analysed and tabulated. In addition, photomicrographs were taken with a Kodak® digital camera (EasyShare C183; 2010) attached to the microscope.

Two analyses were proposed for this study: qualitative and quantitative histomorphometry. In the qualitative analysis, data were described according to the morphological events observed in each specimen. The following parameters were assessed in the quantitative analysis: inflammatory cells and fibroblast counts, which were performed in order to identify the prevailing repair stage (whether inflammatory or proliferative); blood vessel count, which was conducted with the purpose of assessing angiogenesis activity in the animals treated with the gels;

measurement of the amount of collagen in the specimens in order to assess neocollagenesis.

The inflammatory cells, fibroblasts, and blood vessels counting was performed in eight 400× magnification fields for each specimen and each slide (in sections stained with H&E), while the collagen quantification was performed in two 100× magnification fields for each specimen (slides stained with Masson's trichrome stain).

Statistical analysis

The fibroblast and inflammatory cell counts, vessel density, percentage of collagen and wound size were normally distributed, as assessed by Kolmogorov–Smirnov tests. Thus, the data were subjected to analysis of variance (ANOVA) and Tukey's post hoc test to compare the means. All statistical tests were performed using GraphPad® (Prism), version 5.0 for Windows (Graph-Pad Software, San Diego, California, United States). $p < 0.05$ was considered significant.

III. RESULTS

Macroscopic analysis

The surgically induced open-skin lesions were analysed daily for 7 consecutive days, during which the secondary-intention tissue repair process was assessed. The following parameters were assessed: presence or absence of solution of continuity, signs of bleeding, necrotic tissue formation, signs of inflammation in the wound bed, and re-epithelialisation.

According to the results, wound size on the 7th day of treatment was larger in the CG and CPE groups, while the CTE group presented the smallest wound area, significantly different than that of the CG (Figure 1).

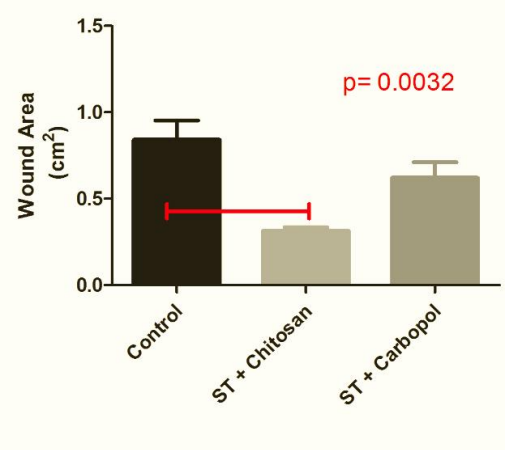

Fig. 1: Wound area (cm²) 7 days postoperatively.

Histomorphometric analysis

Cell count

The results demonstrated a lower count of inflammatory cells in the CTE group. It is important to note that this reduction was significant when compared with the CG. A reduction in the number of inflammatory cells in relation to the CG was also observed in the CTE group (Table 2).

TABLE 2. MEAN NUMBER OF INFLAMMATORY CELLS AND FIBROBLASTS.

	Groups		
Cells	Control	CPE	CTE
Inflammatory cells*	59.53^{A}	54.43^{AB}	47.45^{B}
Fibroblasts*	40.47^{A}	45.57^{AB}	52.55^{B}
Total	100.00	100.00	100.00

Means followed by two different letters are significantly different ($p < 0.05$). Analysis of variance (ANOVA), 95% confidence interval, Tukey's post-test. Asterisks indicate the significance levels compared to the control values: *$p < 0.0001$.

In addition, a significant increase in the number of fibroblasts was observed in the CTE group when compared with the CG (Table 2).

According to the photomicrographs depicted in Figure 2, it can be assumed that in the CG (Figure 2A), events in the inflammatory phase were predominant, while in the CPE group (Figure 2B), those in a transition stage –between the inflammatory and proliferative phase– prevailed. On the other hand, the wounds were in the proliferative phase in the CTE group (Figure 2C).

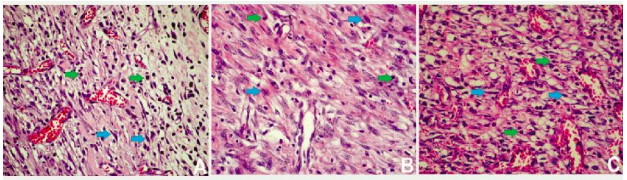

Fig. 2: Photomicrography of the skin of the rats 7 days postoperatively. A: CG, B: CTE, C: CPE. Green arrows represent inflammatory cells and blue arrows represent fibroblasts. H&E (Magnification: 400× for all images).

Density of blood vessels

Significant angiogenesis was observed in the CTE and CPE groups, when compared with the CG (Figure 3). Figure 4 shows the blood vessels densities in the assessed areas, showing a higher density in the sample taken from the CTE group.

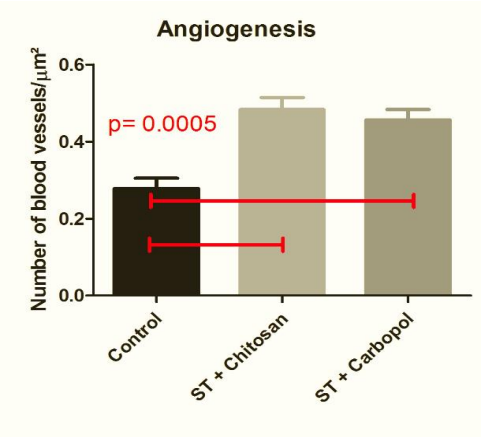

Fig. 3: Angiogenesis 7 days postoperatively.

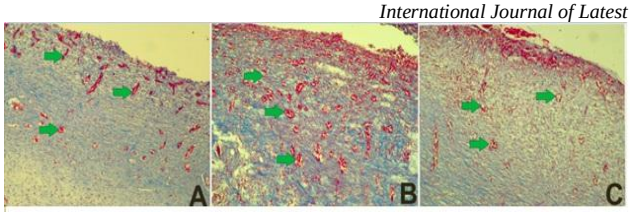

Fig. 4: Photomicrography of the skin of the rats 7 days postoperatively, emphasising the density of blood vessels in the groups. A: CG, B: CTE, C: CPE. Green arrows represent blood vessels. Masson's trichrome stain (Magnification: 100× for all images).

Collagen in the tissue

Figure 5 presents the percentage of collagen in the scar area of the samples, where the CTE group presented significantly higher collagen formation when compared with the CG (Figure 6).

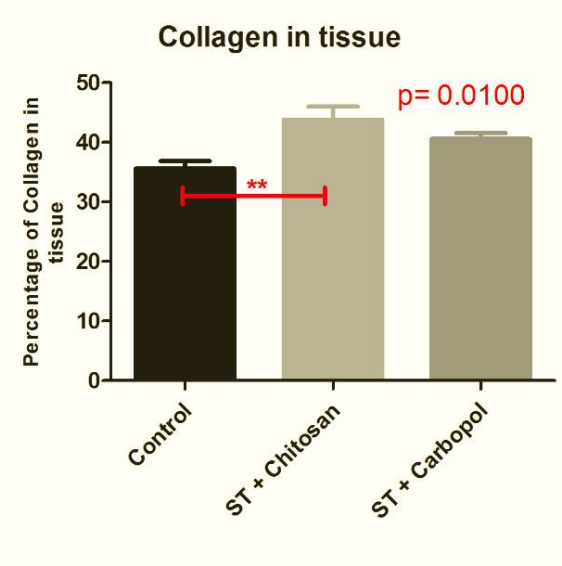

Fig. 5: Collagen in tissue 7 days postoperatively.

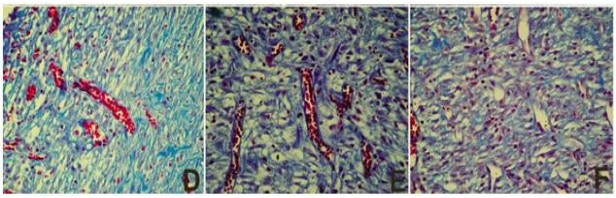

Fig. 6: Photomicrography of the skin of the rats 7 days postoperatively, emphasising the collagen fibres. D: CG, E: CTE, F: CPE. Masson's trichrome stain (Magnification: 400× for all images).

IV. DISCUSSION

Skin wound repair is a dynamic process. Various cellular and molecular events dictate the interactions between components that restore the injured area or hinder tissue homoeostasis. The interaction between inflammatory cells, keratinocytes, fibroblasts, and endothelial cells assist in the production, activation, and regulation of the expression of growth factors and enzymes, which are essential for an effective tissue repair [29, 30].

Histologically, the gradual process of wound repair includes sequential and well-determined steps: clot formation, inflammation, re-epithelisation, angiogenesis, formation of granulation tissue, wound contraction, scar formation, and tissue remodelling. An ideal repair can be characterised by three distinct stages: inflammatory, proliferative, and maturation stages [31, 32].

Biomolecular advances have enabled a better understanding on the pathogenesis of injured tissue repair and therapeutic approach for wound healing. However, many deaths are still caused by complications in hospitalised patients as a result of the emergence of wounds with subsequent infection and tissue revascularization failures [33]. These are associated with factors that are both intrinsic and extrinsic to the patient, generating relevant expenses to public health facilities.

Alternative therapies with new drugs and compounds derived from natural plants have emerged. In fact, Branco Neto et al., [22] have reported a promising healing effect after topical administration of STR hydroalcoholic extract on open wounds in the dorsal region of rats. Similar results were observed by other authors who investigated the healing effect of STR extract, obtained from various parts of the plant, in cutaneous lesions in rats [14, 34-37]. To the best of our knowledge, only two studies have used STR extract in different vehicles: Martonelli et al. [23] incorporated the extract of STR in Orabase®, while Moura, Raffin, and Santos [38] incorporated the hydroalcoholic extract in Plurigel®. However, no study used chitosan or Carbopol® gels as vehicles for STR extracts.

Thus, in the present study, Carbopol® and chitosan gels were used to incorporate the STR hydroalcoholic extract, where the influence of these vehicles on the wound repair was investigated.

Macroscopically, wound contraction was observed in all groups after 7 days of treatment, demonstrating that all formulations can be used topically in skin wounds. The CTE group presented a greater contraction of the wound area ($p < 0.05$) when compared with the CG and CPE groups.

Regarding the histomorphometric analysis, all groups showed highly vascularised granulation tissue, which is indicative of inflammatory and/or proliferative stage. These findings are in agreement with most studies on animal models, which feature an evolution process between the phases mentioned at the 7th day [39]. The group that demonstrated the lowest inflammatory cell count and the highest number of fibroblasts was the CTE ($p < 0.05$). Thus, it can be inferred that the use of the chitosan gel can accelerate the repair process and enhance fibroblast recruitment and differentiation.

The proliferation of fibroblasts as well as the modulation of inflammatory response were more efficient in the group treated with STR in chitosan gel then with that treated with STR in Carbopol® gel ($p < 0.05$), within 7 days of treatment. Carbopol® is usually the gelling agent of choice in pharmaceutical gel formulations. However, in this study, chitosan gel demonstrated to be superior for wound healing applications due to its beneficial effects in the tissue repair process.

A relevant event in the wound repair process is the production of collagen fibres, namely neocollagenesis, which are responsible for the physical functions of the skin. The CTE group showed favourable neocollagenic activity, since the percentage of collagen fibres in the tissue was

significantly higher than in the CG ($p < 0.05$). These results corroborate those reported by Stephen et al. [37], who assessed the effect of STR oil on wound healing in rats, where they found a greater concentration of collagen fibres in the group treated with STR.

Another important aspect to be discussed is the ability to stimulate the formation of new blood vessels (angiogenesis) in the newly injured area, which facilitates the recruitment of inflammatory cells and undifferentiated stem cells for proliferation and differentiation at the repair site. This fact is particularly important in diabetic patients, who have difficulty in forming new blood vessels. In the present study, the number of blood vessels was higher in both groups treated with STR (CTE and CPE) in comparison with the CG ($p < 0.05$). Thus, the incorporation of the hydroalcoholic STR extract in Carbopol® gel and especially in chitoan gel may aid in the repair of chronic wounds.

V. CONCLUSIONS

The hydroalcoholic STR extract embedded in chitosan gel accelerated skin wound repair, assisting in the formation of tissue with a higher amount of collagen and faster contraction. It also presented a significant immunomodulatory (anti-inflammatory activity) and angiogenic effect in the experimental model used.

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