Evaluation and Comparison of Wound Healing Properties of an Ointment (AlpaWash) Containing Brazilian Micronized Propolis and *Peucedanum ostruthium* Leaf Extract in Skin Ulcer in Rats

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ABSTRACT

Several previous studies have demonstrated improved wound healing associated with natural-based formulations. Therefore, the purpose of this study was to assess the efficacy of a topical formulation containing both a Brazilian micronized propolis extract and a *Peucedanum ostruthium* leaf extract for the treatment of wounds created by surgical punch in rats. The study was conducted for 14 days and animals were treated as follows: gauze group (G), polyethylene glycol base ointment (Control), AlpaWash (an ointment containing a Brazilian micronized propolis extract and *Peucedanum ostruthium* leaf extract [Treatment]), and Polysporin (one of the most commonly used topical antibiotic ointments, based on bacitracin zinc and polymyxin B sulfate [Reference Standard]). In general, the results demonstrated that ointments, due to occlusiveness and the ability to maintain moisture under the damaged area, offered improvements when compared to lesions without any treatment. Additionally, the presence of phenolic and flavonoid compounds, as well as antioxidants and antimicrobials, offered improved stimulation and could accelerate wound healing. The Control, Treatment, and Reference Standard groups were able to close the lesion, as measured by the wound healing rate determination and follow-up photographs. However, AlpaWash and Polysporin presented some additional benefits—anti-inflammatory activity, measured using myeloperoxidase and histological count, as well as fibroplasia and hydroxyproline production, suggesting that skin with a better quality could be formed following these two treatments. Therefore, based on the current concern of antibiotic overuse in wound healing, the emergence of multi-resistant organisms and the decrease in newer antibiotics, AlpaWash is considered a prominent formulation to be employed in wound-healing applications.

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INTRODUCTION

Wound-healing disorders and impaired wound healing affect about six million people around the world, leading to decreased quality of life and serious consequences for these individuals.[1] By definition, a wound is a disruption of the anatomical, normal cellular, and functional continuity of a structure. Consequently, the wound-healing process is the succession of complex biochemical and cellular events aiming to restore its integrity.[2] The healing process presents a multifaceted biological process that can be divided into three overlapping phases:

- Inflammation
- Tissue formation
- Tissue remodeling

During this process, both extracellular matrix synthesis and remodeling take place.[2] In general, wound management is based on the use of topical antibiotic ointments to minimize infection, mainly based on bacitracin zinc (500 U/g) and polymyxin B sulfate (10,000 U/g) ointment. In the U.S., Polysporin (polymyxin B sulfate and bacitracin zinc; Johnson & Johnson, New Brunswick, New Jersey) and Neosporin (neomycin sulfate, polymyxin B sulfate and bacitracin zinc; Johnson & Johnson) are the most commonly used antibiotic-based ointments. On the other hand, current literature has raised the issue that treatment practices pertaining to infected wounds are based on studies from the 1960s and 1970s. Therefore, up-to-date studies deserve consideration, and wound-management practices must be revisited.[3] In this regard, several studies have shown no difference in infection rates and clinical grading assessments (i.e., erythema, edema, epithelial confluence, crusting, scabbing) between wounds treated with topical antibiotics versus those treated with antibiotic-free formulations.[3-5]

Along with the overuse of antibiotics in wound healing, the emergence of multi-resistant organisms and the decrease in the discovery of new antibiotics have shifted the focus to searching for ancient healing methods based on traditional and alternative medicines in wound management.[6] Moreover, affordable and easily accessible treatment options have gained importance; this is mainly due to the associated treatment time and high medical-care costs of extensive and/or long-term wounds.[7] In this context, Brazilian propolis, a resinous balsamic material with a complex chemical composition, and *Peucedanum ostruthium (P. ostruthium)*, a traditional Alpine leaf extract used for its wound healing and anti-inflammatory properties, have appeared as outstanding options in this field.

Brazilian propolis has demonstrated antimicrobial activity and favorable results on the wound-healing process in poloxamer gel treatment.[8] Collagen-based films containing hydroalcoholic Brazilian propolis extracts improved the biological events associated with burn healing, without toxic effects.[9] Additionally, a biocellulose membrane containing Brazilian propolis was efficient in improving the wound-healing process for dermal burn healing and demonstrated antimicrobial effectiveness.[10] Several biological properties have been associated with Brazilian propolis, which, in turn, favor wound healing and promote anti-inflammatory,[11] immunomodulatory,[12] antioxidant,[13] and antimicrobial properties.[8,10,14,15]

P. ostruthium rhizomes have long been used in Austrian traditional medicine for the treatment of inflammatory diseases. Several synonyms have been reported for this species, including *Imperatoria ostruthium, Selinum ostruthium, Angelica officinalis,* and Masterwort. Several biological activities have also been reported, such as a diuretic for chronic indigestion, a stimulant, a stomachic, as well as for typhoid, intermittent fever, paralytic conditions, and as a component of delirium treatments. *P. ostruthium* was also applied topically as a powder for ulcers and cancer.[16] Aside from the biological properties presented earlier, the plant demonstrated antimycobacterial activity against *Mycobacterium fortuitum* and *Bacillus cereus,* results that were attributed to a coumarin-related compound identified as ostruthin and another compound, oxypeucedanin hydrate.[16] Hiermann and Schante have also demonstrated antiphlogistic and antipyretic activity of *P. ostruthium*.[17]

Based on the aforementioned context, a formulation containing Brazilian micronized propolis and *P. ostruthium* leaf extract (AlpaWash) was developed as an alternative solution for wound treatment. This *in vivo* study compared the wound-healing properties using AlpaWash, an antibiotic-free ointment base, versus Polysporin, a widely prescribed first-aid antibiotic ointment, for the treatment of dorsal wounds in rats. Wounds were created by surgical punch, and wound-healing assessments included wound healing measurements by digital photography, histological analysis (i.e., inflammatory infiltrate evaluation, blood vessel analysis, fibroblast response), myeloperoxidase (MPO) assay, and hydroxyproline determination.

MATERIALS AND METHODS

SAMPLES

The study involved the following groups:

- Polyethylene glycol (PEG) ointment base (Control [C] group) (Batch 0055; MEDISCA Pharmaceutique Inc., Canada)
- AlpaWash ointment with Brazilian micronized propolis and (P. ostruthium) leaf extract in PEG ointment (Treatment [T] group) (Batch QF98, MEDISCA Pharmaceutique Inc.)
- Polysporin (Reference Standard, RS) (Batch 1946LZ, Johnson & Johnson)
- Group without treatment (Gauze [G] group)

PEG base and AlpaWash were kindly donated by MEDISCA Pharmaceutique Inc. PEG base and AlpaWash ointments consisted of a mixture of two PEGs of different molecular weights.

Polysporin is an ointment composed of the following:

- 10,000 units polymyxin B (as sulfate)
- 500 units bacitracin zinc
- Butylated hydroxytoluene
- Cocoa butter
- Cotton seed oil
- Olive oil
- Petrolatum
- Sodium pyruvate
- Vitamin E

The G group did not receive any treatment; gauze was employed as a dry-coating agent. All groups were coated with gauze after respective treatments.

ANIMALS

This protocol was conducted in accordance with the Brazilian Committee for animal care and use (COBEA) guidelines and approved by the Ethics Committee on Animal Experimentation (CETEA), Ribeirão Preto School of Medicine, University of São Paulo (FMRP-USP), registry number 028/2015-1. A total of 90 adult, male Wistar rats (200 g to 250 g), aged 6 weeks to 7 weeks, were obtained from the central Bioterium of the Medical School, Ribeirão Preto, University of São Paulo (FMRP-USP). Animals were fed with a standard diet and water *ad libitum* and properly housed in individual cages at a temperature of 25°C ± 5°C, and with a 12-hour light-dark cycle; this was followed throughout the experimental period with 1 week before the protocol for adaptation of the animals to the laboratory facility.

SURGICAL PROCEDURE AND TREATMENTS

The animals were weighed and anesthetized by intraperitoneal administration of ketamine (80 mg/kg) and xylazine (15 mg/kg). After shaving and cleaning the skin with 70% ethanol, two full-thickness excision wounds were made on the dorsal cervical region of each rat with sterile histological punch (1.5 cm diameter) with thickness comprising all the skin layers.[7] Post-surgery, dipyrone (50 mg/kg, diluted in saline) was administered in the intraperitoneal space 2 times per day during the first 24 to 48 hours, depending on behavioral changes and perception of pain in the animals. Immediately after the surgery and daily afterwards at the same hour, the groups received their respective treatments.

The excised skin of each animal was properly stocked for subsequent histological and biochemical assays, representing healthy-skin samples of the tissue before the surgery.

Groups, with six animals each, were separated according to the wound treatment:

- C group, wounds that were treated with PEG ointment base
- T group, wounds that were treated with AlpaWash
- Reference standard (RS) group, wounds that were treated with Polysporin
- G group, wounds that were untreated

All animals' wounds were covered with gauze, and the tape was changed daily after each administration. The animals were euthanized at the 2nd, 7th, and 14th post-wounding days in a CO_2 chamber; the wounds and their surrounding areas were cut with sterile scissors. One wound sample of each animal was stored at -70°C for biochemical analysis (hydroxyproline and MPO assays), and the other wound sample was used to perform histological assays.

WOUND HEALING MEASUREMENTS

Using digital photography, the two wound samples for each animal were evaluated on days 0, 2, 7, and 14 post-wounding. Image capture was standardized using a 30-cm-high aluminum support to which the camera was fixed perpendicularly to the wound. The wound areas were calculated using the ImageJ software to analyze the re-epithelialization with the wound healing rate (WHR) according to the formula:

$$WHR = (A_0 - A_n)/A_0$$

where A_0 corresponds to the wound area obtained on the day of the surgery (day 0) and A_n corresponds to the area obtained on the day of euthanasia (day 2, 7, or 14).[7,18]

HISTOLOGICAL ANALYSIS

Wound samples were stored in histological cassettes and fixed with buffered formaldehyde solution at 3.7% (pH 7.4) for 24 hours. Subsequently, they were processed according to the standard light microscope tissue protocols. The processed tissues were embedded in paraffin; 5-µm-thick paraffin sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for the evaluation of inflammatory infiltrate, blood vessel, and fibroblast response to treatment. Then, sections were photographed at 100× and 400× with an Olympus BX41 microscope equipped with an Olympus DP70 camera (Olympus America Inc., Melville, New York).[7,19]

MYELOPEROXIDASE DETERMINATION

The presence of polymorphonuclear cells (PMNs), mainly neutrophil infiltration in tissues, was measured indirectly by the MPO determination assay according to Souza et al.[20] Wound samples were weighed and homogenized using a Polytron PT 3100 homogenizer (Kinematica, Inc., Bohemia, New York) with ice-cold buffer (0.1 M NaCl, 15 mM EDTA, 20 mM NaPO4, pH 4.7). The pellet was lysed with 1 mL 2% NaCl solution for 30 seconds, followed by centrifugation, the pellet was then re-suspended in 0.05 M $NaPO_4$ buffer, pH 5.4, containing 0.5% hexadecyl trimethylammonium bromide, and re-homogenized. Then, the samples were centrifuged for 15 minutes at 5000 x *g*. MPO activity in the supernatant was detected by diluting the samples in buffer (0.08 M $NaPO_4$), then adding 25 µL of 1.6 mM tetramethylbenzidine (Lot 098K1434; Millipore Sigma, Milwaukee, Wisconsin) followed by 100 µL of 0.5 mM H_2O_2. Samples were aliquoted into a 96-well plate and absorbance was measured at 450 nm. The results were reported as the total number of neutrophils/mg tissue by comparing the absorbance of the tissue supernatant to a standard curve.[21]

HYDROXYPROLINE DETERMINATION

The hydroxyproline determination was performed by following an established protocol.[7,19] The samples were dried overnight at 60°C until a constant weight. The dried samples were weighed and

homogenized using a Polytron tissue homogenizer with 6 N hydrochloric acid (100 µL per milligram of dry tissue) and incubated for 4 hours at 130°C to promote acid hydrolysis. Following adjustment to neutral pH, 10 µL of the hydrolysate was added to a 96-well microplate. Hydroxyproline standard solutions were prepared at concentrations from 1.0 µg/mL to 100 µg/mL, and 10 µL of each standard solution was transferred to the wells of a 96-well microplate. Then, 90 µL of 0.056 M chloramine-T solution was added to each well. After 25 minutes, 100 µL of Ehrlich's reagent was added to the microplates. Subsequently, samples were incubated at 60°C for 20 minutes to allow for chromophore development; the absorbance values of the reddish-purple complex were then measured at 550 nm at room temperature and compared to those of the standard curve to determine the concentration of hydroxyproline in the samples.[7,19]

STATISTICAL ANALYSIS

Statistical analyses were done using one-way ANOVA with α=5%. The Tukey test was used to identify statistically significant differences between the mean values determined for each group using the GraphPad Prism 5 software (GraphPad Software, San Diego, California).

RESULTS AND DISCUSSION

The use of natural and traditional medicines has been increasing in recent times, especially considering the scientific evaluation and demonstrated effectiveness of these compounds. In addition, nowadays the population is more concerned with environmental issues. Such concerns have piqued interest in the consumption of eco-friendly products. Moreover, the immense biodiversity that remains to be studied and explored contains as-yet undiscovered herbs and natural products that could potentially yield new medicines and treatment options. In this context, natural extracts like propolis,[9] *(P. ostruthium)*,[16-17] *Calendula officinalis, Centella asiatica, Aloe vera,* and others are emerging as interesting alternatives to stimulate wound healing in surgical and burn lesions.[22-25]

Considering the opportunities available, Brazilian micronized propolis was developed[26] with the intention of creating a propolis formulation that could be used in cosmetic and pharmaceutical preparations. This formulation takes into account the advantages of the micronized form, including the minimization of the odor and color properties of a propolis extract, increased stability, the absence of alcohol in the formulation and producing an active effect against *Staphylococcus aureus*.[14,26]

Although the main focus of the present study was not the antimicrobial effect on the wounds, it is well established that when a lesion is infected, the wound-healing process is compromised. Therefore, it is beneficial to include ingredients capable of controlling infection in the formulation in an effort to promote wound healing. It is probable that the antimicrobial activity of propolis would be maintained against other microbes, providing certain benefits in treating infected wounds, which could also be corroborated by *P. ostruthium* antimicrobial properties, considering its effectiveness against *Bacillus* and some species of mycobacterium.[16,17]

Wound healing is a complex sequence of independent and overlapping steps that involve the exudative/inflammatory phase, proliferative phase, and remodeling events. Lesions, either consisting of large dimensions or involving the loss of considerable cutaneous tissue, are typically considered wounds of second intention; consequently, they are slower to close, since it requires the formation of granulation tissues before the formation of collagen.[27]

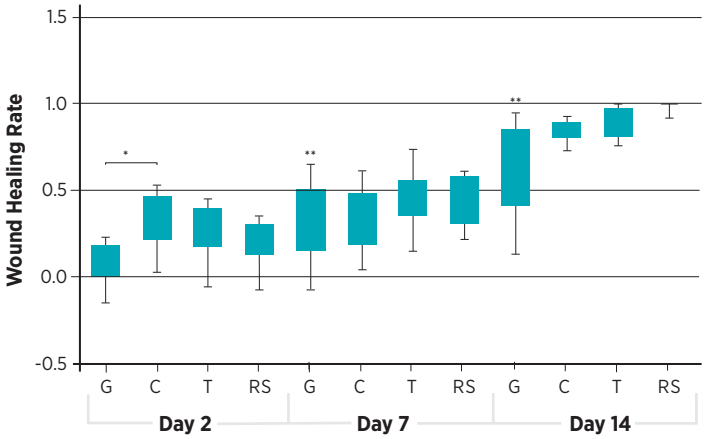
In this study, four independent groups of animals were monitored during 2, 7, and 14 days of treatment. For each time point, a follow-up of the wound was performed by taking digital photographs and measuring the size of wound, using ImageJ software. The WHR showed that for 2 days of treatment, the C group presented a more closed wound than the G group (Figure 1a; Table 1). With 7 days of treatment, all groups showed similar statistical behavior, although the tendency for better closure of the wound could be seen for T and RS groups. Finally, within 14 days, the C, T, and RS groups showed better statistical results when compared to the G group. Observation of the photographs (Figure 1b) showed that the wound of the C group is still not completely closed after 14 days; this observation is corroborated by the statistical difference in the WHR (Table 1) of C and G groups, where the wound is seen to be completely closed in the latter (Figure 1b). As such, it is possible to conclude that with 14 days of treatment, AlpaWash and Polysporin resulted in a better closure of the lesions, indicating that these formulations can accelerate the wound-healing process.

Considering these results, as well as the benefits of the PEG base, it is possible to suggest that medicines, capable of maintaining a physiologically moist microenvironment on the lesion, could stimulate the formation of granulation tissue and could contribute to wound healing.[7] An additional advantage is that some of the substances present in the treatments tested here (such as those found in propolis and *P. Ostruthium* extracts, including phenolic compounds, flavonoids, caffeic and cinnamic acid, terpenoids, and artepillin C,[16,28] all of which are components of AlpaWash) and some antioxidants, like vitamin E, present in Polysporin, could not only stimulate the closure of the lesion but also stimulate newly formed tissue of higher quality, as observed in histology images and through biochemical parameters analyzed.[29] This notion will be further discussed in the present work, since the aforementioned substances are well documented for stimulating re-epithelialization,[30-33] or, in the case of alpha-tocopherol (Vitamin E), reducing the damage caused by oxidant substances generated during the inflammatory phase.[34] Another point to mention is that several phenolic compounds, terpenoids, and flavonoids, naturally present in propolis and *P. ostruthium* extracts, could offer anti-inflammatory[16,35] and antioxidant actions,[36,37] reinforcing the induction of wound healing and closure of the lesions.[9,27,38]

With the information presented in Table 1, it is possible to demonstrate the tendency for improved wound closure with 7 days

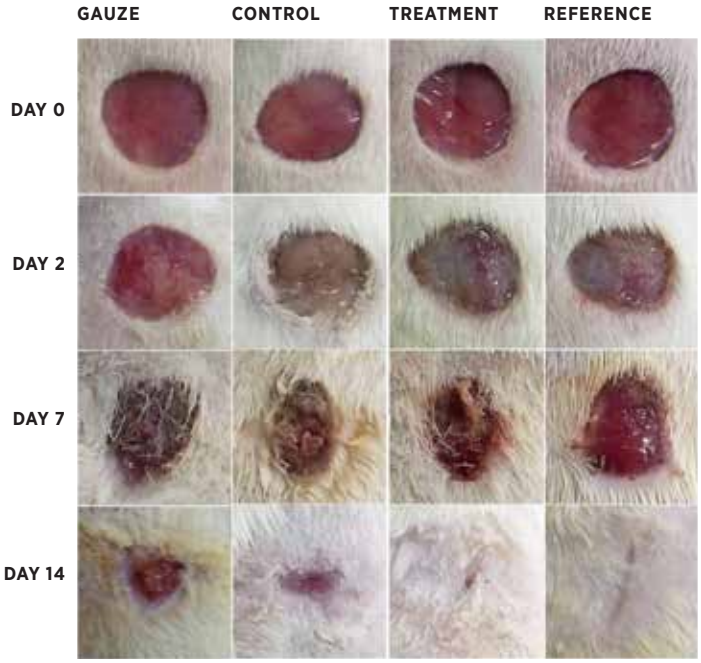
FIGURE 1. EVALUATION OF TREATED WOUNDS.

Figure 1a: Wound Healing Rate obtained with different treatments.



Gauze Group (G), Polyethylene glycol ointment control (C), AlpaWash (T) and finally, RS (Reference Standard, Polysporin) with 2, 7 and 14 days of treatment after surgical excision (n=6).
*Statistically different
** Statistically different from the other groups on the same day of treatment, one-way ANOVA statistical analysis, with Tukey post-test (α=0.05).

Figure 1b: Clinical follow up of all groups evaluated during 2, 7, and 14 days.



of treatment for AlpaWash (46.7% closure) and Polysporin (43.7%), although no statistical difference was observed when compared to the G group (30.7%) and PEG base control (33.2%).

Considering the disadvantages of antibiotic use, including adverse reactions and the risk of stimulating microbial resistance (due to the indiscriminate use of antibiotics),[3] the results observed here could suggest that for acute and non-infected wounds, it is not mandatory to use antibiotics for stimulating wound healing. The use of natural extracts with antimicrobial effects, as it is the case with micronized propolis,[14,26,39] could be an outstanding alternative, especially considering that propolis possesses other biological characteristics that could improve wound healing, as previously explained and well documented in the literature.[10-12,32,40] The present study does not involve the use of infected lesions, and, as such, it was not possible to observe any potential benefits of antibiotics on the healing of lesions. It is possible that when treating infected lesions, T and RS could offer an improved WHR, when compared to PEG base and G groups. It is well documented that microorganisms can impede the progression of re-epithelialization in wound healing[41]; in cases where microorganisms are capable of forming a biofilm, skin lesions could become very difficult to heal and close.[42-44] Based on this, several studies are currently underway in order to address the issue of biofilm formation and ultimately improve wound healing.[45,46]

Histological analysis of the groups was performed in all animals, and for such assessment, at least 4 (four) random optic fields per animal were counted using ImageJ software. Fibroblasts and inflammatory infiltrate were counted under 400x magnification and blood vessels with 100x magnification (Figures 2b-c and 3b).

The results obtained (Figures 2a and b) showed that with 2 and 7 days of treatment, groups G, T, and RS showed a more intense presence of fibroblasts than group C. After 14 days, treatments with AlpaWash and Polysporin demonstrated a significantly higher quantity of fibroblasts (Figures 2a and 2b), corroborating the WHR results and the follow-up images (Figure 1b and Table 1). An increased number of fibroblasts were also observed in other studies that have tested Brazilian propolis treatments in lesions and burn areas.[8,9]

To analyze the inflammatory process in the lesions, the histological evaluation of inflammatory infiltrate (Figure 2c) with neutrophil indirect measure using MPO analysis was performed (Figure 3a). The results obtained demonstrated that with 2 days of treatment, all groups possessed similar characteristics for histological analysis (i.e., all groups showed intense inflammatory infiltrate [Figures 2a and 2c]). These data can be corroborated with MPO measure (Figure 3a), where it is possible to consider a high quantity of neutrophil cells. For the G group, it was not possible to demonstrate the same observation considering MPO analysis. We believe that the results could have been influenced by the adherence of some part of the wound tissue to the gauze, since this lesion was very dry in comparison to the others; although the manipulation to remove the gauze was performed very carefully, it is possible that some damage to the

wound tissue occurred, compromising the MPO analysis.[7]

For 7 days of follow-up, only the G group demonstrated higher quantities of inflammatory cells (Figure 2c), which is in accordance with MPO results (Figure 3a) and represented the poorest healing outcome. Interestingly, the results obtained with 7 days of treatment for groups T and RS (Figure 3a) showed a significant reduction in neutrophil cells, since they both showed similar results when compared to closed tissue, therefore demonstrating a better anti-inflammatory action for both of these treatment options. Curiously, the analysis of fibroblasts with 7 days demonstrated an increase of neutrophils but only for T and RS (85.01 ± 22.04 and 89.75 ± 13.65). Careful observation of Figure 1a and Table 1 show a tendency for a higher WHR with these groups, although no significant statistical result was obtained. It is common to observe an intimate relation between epithelialization and fibroplasia (fibroblastic proliferation and collagen deposition)[9] and, since the WHR for AlpaWash and Polysporin were similar, it remains plausible to suggest that better quality tissue can be formed when using one of the products (Figures 2b, 3a, and 4). Further studies could be performed in order to better understand this process.

TABLE 1. WOUND-HEALING RATE VALUES OF EACH GROUP DURING FOLLOW-UP EVALUATION OF THE ANIMALS UNDER TREATMENT (*n*=6).

GROUPS	GAUZE		CONTROL		TREATMENT		REFERENCE	
	AVERAGE	SD	AVERAGE	SD	AVERAGE	SD	AVERAGE	SD
Day 2	0.099	0.121	0.362*	0.153	0.255*	0.152	0.213	0.120
Day 7	0.307	0.212	0.332	0.186	0.467	0.167	0.437	0.144
Day 14	0.620**#	0.272	0.839#	0.057	0.890	0.084	0.993	0.025

*Statistically different from gauze group on day 2; **Statistically different from control, treatment, and reference groups on day 14; Finally, all groups of day 2 and 7 were different from the closed group, and (#) only on day 14 they were different from the closed group. One-way ANOVA, with Tukey post-test, 95% confidence (α=0.05).

Finally, within 14 days, the T and RS treated groups showed reduced inflammatory cells (43.17 ± 20.12 and 39.80 ± 12.97, respectively) when compared with both the C and G groups, (116.96 ± 19.52 and 121.00 ± 25.13, respectively), in accordance with the results of wound healing (Figure 1 and Table 1). Here, it is possible to demonstrate the advantage of the active substances used in the treatments, since T and RS showed a reduction in neutrophil cells. Again, considering the results obtained for fibroblasts at this time point (14 days), the T and RS groups (90.26 ± 7.95 and 104.15 ± 14.93, respectively) had better fibroblast counts than the G and C groups (59.8 ± 13.99 and 37.85 ± 9.49, respectively).

Although one could think that inflammatory infiltrate cell counts need to correlate with MPO analysis, it is not always the case. For example, during the first steps of the inflammatory process, several different types of cells (including neutrophils) are recruited; the presence of other cell types can certainly account for the observed differences between these two analyses. The absence of correlation was also observed in the results presented by Caetano et al[7] and Fronza et al.[47]

The anti-inflammatory action of propolis treatment has been previously demonstrated,[11,12,48] as well as for *P. ostruthium*.[16,17] Some experiments demonstrated that Brazilian propolis inhibited IL-1β production, suggesting that the inflammasome can be involved in this mechanism.[11] Elegant *in vitro* protocols provided evidence that propolis, including some of its constituents (e.g., quercetin), had a regulatory effect on basic immune cell functions, especially on lymphocytes. The lymphocyte growth regulation was demonstrated to be via the Erk-2 signal pathway, suppressing pro-inflammatory Th1- and Th2-derived cytokines, and inducing the regulatory T lymphocyte-derived TGF-β1 cells.[48] In addition, Hiermann and Schante[17] demonstrated that an ethanol extract of *P. ostruthium* and its main isolated compound (hydroxycoumarin) had significant inhibitory activity on edema, using carrageen-induced edema in rats. The ethanol extract of *P. ostruthium* and coumarin have been characterized as dual inhibitors of cyclooxygenase and 5-lipoxygenase activity.

Considering the blood vessel analysis, the importance of angiogenesis in wound healing is related to the supply of nutrients and oxygen to the newly formed tissue.[21] Here, no specific result was observed in any of the groups, suggesting that the mechanism by which the treatments evaluated in this study were acting was not related to angiogenesis.

Caetano et al[7] demonstrated a wound-healing effect for chitosan-alginate membranes; results for angiogenesis do not differ when compared to the C group, similar to what was observed here.

Collagen is the major component of the extracellular matrix and the main protein of granulation tissue. The role that collagen plays in the closure of a lesion starts immediately after the injury is inflicted and continues for many weeks, even after the wound is closed. The level of hydroxyproline is a value that can provide indirect information regarding collagen production. Results obtained demonstrated that with 2 days of treatment, all groups presented statistically lower values compared to closed tissue. With 7 days, all groups presented values similar to the closure area, demonstrating that the production of collagen is occurring (Figure 4).

Finally, RS showed a higher quantity of hydroxyproline after 14 days of treatment compared to the other groups (Figure 4). This observation is in accordance with the histology results shown in Figure 2a, where it is possible to suggest that this higher quantity of hydroxyproline may involve a better organization of collagen fibers, which would be associated with higher quality of the newly formed tissue.

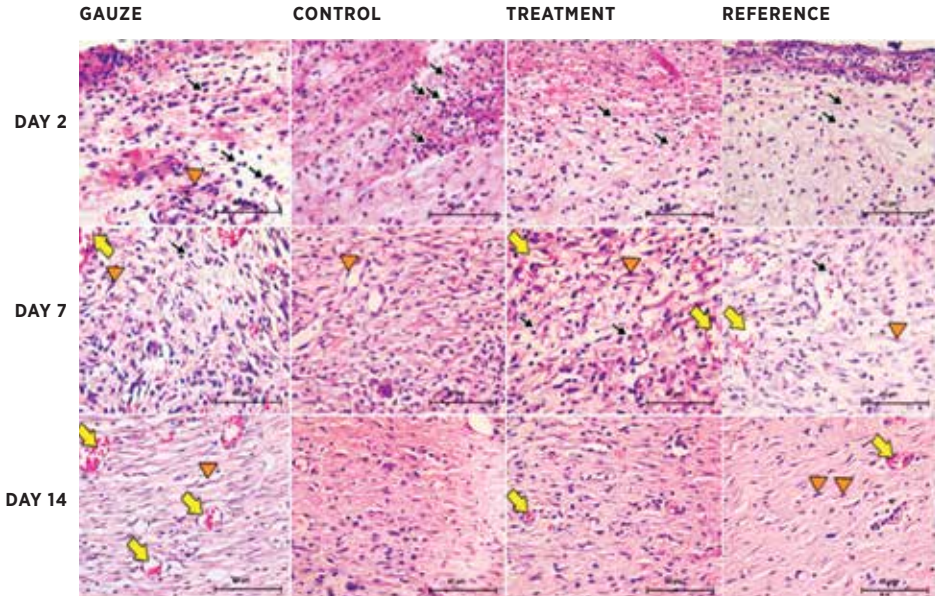
Several studies have demonstrated the therapeutic benefits of natural ingredient formulations in wound healing, external ulcers, burns, shortened healing time, increase of wound contraction, and acceleration of tissue repair.[49-52] The results presented in this study demonstrate the effectiveness of AlpaWash, which contains Brazilian micronized propolis and *P. ostruthium* leaf extract, therefore establishing it as a prominent formulation to be employed in wound healing.

CONCLUSION

The results demonstrated that ointments, due to occlusiveness and the ability to maintain moisture under the damaged area of tissue, offered improvements to the wound healing process when compared to lesions without any treatment. The PEG ointment base, AlpaWash, and Polysporin groups were able to close the lesion, as measured by the WHR determination and follow-up photographs. However, AlpaWash and Polysporin presented some additional benefits, among them the anti-inflammatory activity, measured using MPO, as well as histological count, fibroplasia, and hydroxyproline production, suggesting that the newly formed skin is of better quality with these two treatments. Therefore, based on the results presented, AlpaWash, an antibiotic-free ointment, can be considered a valuable formulation in ameliorating the wound healing process.

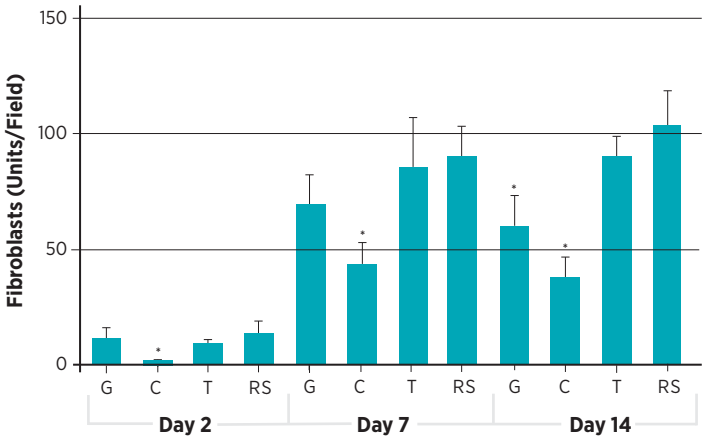
FIGURE 2. HISTOLOGICAL ANALYSIS OF THE WOUND-HEALING AREA (400x).

Figure 2a: Photomicrography of the wound healing of all groups during follow-up stained with hematoxylin-eosin (H&E).



Orange arrow identifies fibroblasts, yellow identifies blood vessels, and black identifies inflammatory infiltrate

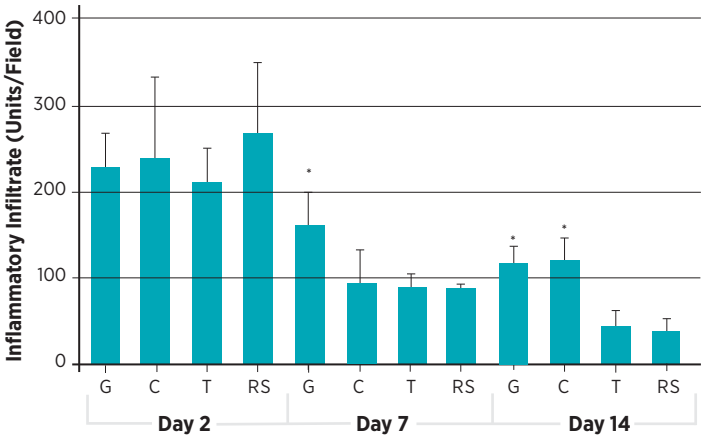
Figure 2b: Quantitative analysis of fibroblast proliferation on wounds treated on 2, 7, and 14 post-operative days.



Four random optic fields per animal were counted after the proper coloration with H&E

*Statistically different from the other groups on the same day of treatment, one-way ANOVA statistical analysis, with Tukey post-test (α=0.05)

Figure 2c: Histogram of a quantitative analysis of inflammatory infiltrate counted according to the same procedure in Figure 2b.

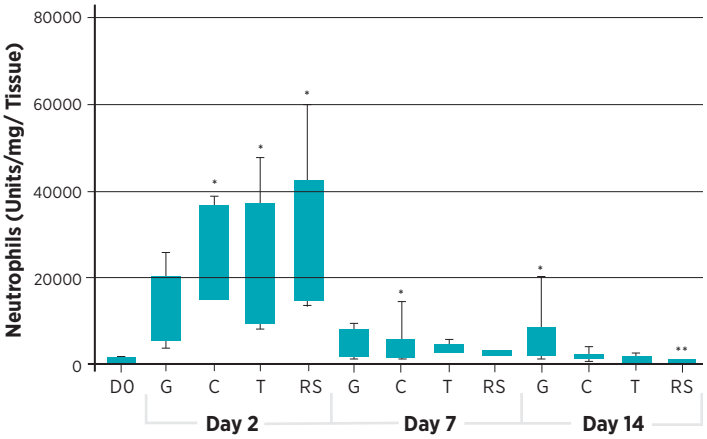


Four random optic fields per animal were counted after the proper coloration with H&E

*Statistically different from the other groups on the same day of treatment, one-way ANOVA statistical analysis, with Tukey post-test (α=0.05).

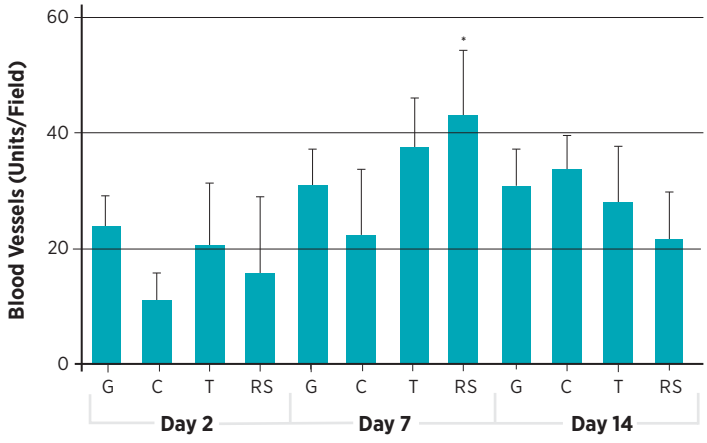
FIGURE 3. QUANTITATIVE ANALYSIS OF THE NEUTROPHIL INFILTRATION AND OF THE BLOOD VESSELS AFTER HISTOLOGICAL PROCEDURE.

Figure 3a. Biochemical analysis of neutrophil infiltration on wounds treated after 2, 7, and 14 post-operative days.



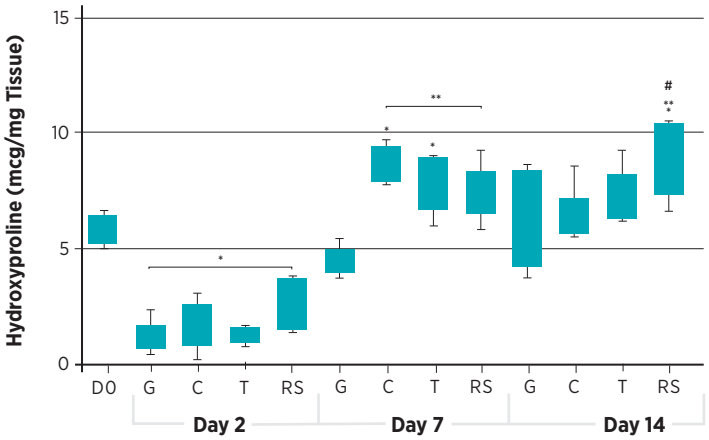
* *P*<0.05 statistically different compared to closed tissue, one-way ANOVA, with Tukey post-test
**Statistically different compared to other groups at the same time point, using the same statistical methodology

Figure 3b. Histogram of a quantitative analysis of blood vessels counted according to the same procedure used in Figure 2b (100x)(*n*=6).



**P*<0.05 statistically different compared with other groups of the same point, one-way ANOVA, with Tukey post-test

FIGURE 4. BIOCHEMICAL QUANTITATIVE ANALYSIS OF WOUND HYDROXYPROLINE CONTENT AS AN INDIRECT MEASURE OF COLLAGEN IN ALL ANIMALS TREATED (*n*=6).



**P*<0.05 statistically different compared to the closed group (D0), one-way ANOVA, with Tukey post-test
**Statistically different compared to G
#Statistically different from C at same time point, one-way ANOVA, with Tukey post-test.

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