

Evaluation of healing wound and genotoxicity potentials from extracts hydroalcoholic of *Plantago major* and *Siparuna guianensis*

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Abstract

Despite the large use of the *Plantago major* and *Siparuna guianensis* in traditional medicine, there are no studies demonstrating the effectiveness from extracts of these plants in the healing process by the present methodology. This study reported the effects and toxicity of the *P. major* and *S. guianensis* extracts in the wound healing compared with a commercial product used in Brazil by macroscopic and microscopic analysis. Following injury in cervical dorsal area of the mice, the extract from *P. major* and *S. guianensis* and ointment was applied after an injury in cervical dorsal area of the mice. Wound healing rates were calculated at 4, 9, 15 and 21 d after the wounding, and tissues were obtained on the ninth day for histological analysis. Moreover, mutagenic assay of extracts was performed. Mutagenicity studies carried out with plant extracts showed not mutagenic with or without metabolic activations. Reduction of the wound area occurred earlier in mice treated with *P. major* and control treatment. On the 15th day, the complete wound closure occurred in *P. major*-treated wounds. Throughout ointment and *S. guianensis* treatment it was not observed the wound closed. Microscopic analyses of the wound, on the ninth day, showed the more efficient formation of the neoepithelium and skin appendages in animals treated with *S. guianensis* and *P. major*, while ointment treatment presented no re-epithelialization and absent skin appendages in wound. Thus, *P. major* extract showed good effects on wound healing processes rendering it a promising candidate for the treatment of wounds what also justified its traditional usage in wound treatment.

Keywords: *Plantago major*, *Siparuna guianensis*, neoepithelium, mutagenic, phytochemical assay, mast cells

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Introduction

The wound healing is a dynamic and continuous process, performed and regulated by complex signaling cascades, involving three well-defined phases with each phase overlapping the next: inflammatory phase, proliferative phase and re-modeling phase.¹ Inflammatory cells migrate into the wound attracted by chemotactic factors and promote the inflammatory phase, which is characterized by the sequential infiltration of neutrophils, macrophages and lymphocytes.² In the proliferative phase, re-epithelialization, formation of pilous follicle and angiogenesis are important activities determining the wound lesion.³ In the re-modeling

phase, the imbalance of collagen synthesis and degradation, resulting in excess accumulation of dermal collagen^{4,5} is frequently associated with scar complications. Chemical factors (drug substances), physical and biological (growth factors, cytokines, chemokines and nutritional status of the patient), can accelerate or delay the cicatrization.^{6,7} It is known that the goal for wound treatment is a fast and scarless healing and among the substances used to accelerate the induction of this process is the use of medicinal plants.⁸

A large number of plants are used by tribal and folklore in many countries for the treatment of wounds and it showed auspicious effects.⁹ Over the years, various natural extracts were tested in the healing process such as the extract of

Schinus terebinthifolius Raddi,¹⁰ *Calendula officinalis* L.¹¹ *Centaurea iberica*¹² and the extract of *Ixora coccinea*.¹³ In this sense, Brazil, for having one of the richest biodiversity on the planet, has many natural resources that could be used for curative purposes.¹⁴ However, there are few controlled studies demonstrating the effectiveness of extracts in the healing process of lesions.

The *Plantago major* is a perennial plant that belongs to the Plantaginaceae family. It is a wild plant found worldwide and its leaves have been associated with various biological properties ranging from astringent, anesthetic, antihelminthic, analgesic, analeptic, antiviral, antihistaminic, anti-inflammatory, antirheumatic, antitumor, antiulcer, diuretic, hypotensive and expectorant.^{15,16} The *Siparuna guianensis* is an upright shrub or small tree 3–5 m tall, aromatic, almost all native Brazil, and that belongs to the Siparunaceae. The leaves and flowers of the *S. guianensis* are considered carminative, aromatic, stimulant, antispasmodic and diuretic. It is also used for back pain, rheumatism and arthritis.¹⁷

Despite the large use of the *P. major* and *S. guianensis* in traditional medicine, there are no studies demonstrating the effectiveness from extracts of these plants in the healing process by the present methodology. The aim of the present study was to evaluate the toxicity and effects of the *P. major* and *S. guianensis* extracts in the wound healing compared with a commercial product used in Brazil.

Materials and methods

Chemicals

Dimethylsulfoxide (DMSO, CAS no. 67-68-5), methanol (CAS no. 67-56-1), dichloromethane (CAS no. 75-09-2), nicotinamide adenine dinucleotide phosphate sodium salt (CAS no. 11-84-16-3), D-glucose-6-phosphate disodium salt (CAS no. 3671-99-6), magnesium chloride (CAS no. 7786-30-3), L-histidine monohydrate (CAS no. 7048-02-4), D-biotin (CAS no. 58-85-5), sodium azide (CAS no. 26628-22-8), 2-anthramine (CAS no. 613-13-8) and 4-nitro-o-phenylenediamine (CAS no. 99-56-9) were purchased from Sigma Chemical Co (St Louis, USA). Nutrient Broth No. 2 (Oxoid, UK), Difco Bacto Agar (Difco Laboratories, Detroit, MI, USA) and Mueller Hinton agar (Difco Laboratories) were used as bacterial media. D-Glucose (CAS no. 154-17-6), magnesium sulfate (CAS no. 7487-88-9), citric acid monohydrate (CAS no. 5949-29-1), ointment Collagenase ([DCB 0341.01-0] + Chloranphenicol [DCB 0296.01-5]), potassium phosphate dibasic anhydrous (CAS no. 7758-11-4), sodium ammonium phosphate (CAS no. 13011-54-6), sodium phosphate monobasic (CAS no. 7558-80-7), sodium phosphate dibasic (CAS no. 7558-79-4), sodium chloride (CAS no. 7647-14-5) were purchased from Merck (Whitehouse Station, NJ, USA).

Plant material and extraction

Samples of *P. major* and *S. guianensis* were collected in Minas Gerais State (Brazil) and vouchers were deposited at Universidade Federal de Minas Gerais (UFMG) Herbarium and Universidade Federal de Uberlândia (UFU) Herbarium (No. 143405, HUFU 49.454, respectively).

Leaves (100 g) were washed with clean control at room temperature, dried, triturated and added to a hydroalcoholic solution (70%). After maceration (48 h) the extract obtained was filtered and lyophilized (Liobras equipment, model K 105). The treatment was prepared as 10% (w/w) concentration, e.g. 5 g of extract was incorporated in 45 g of ointment base (lanolin and liquid paraffin).

Phytochemical assay

Phytochemical tests to detect the presence of secondary metabolites, such as tannins, flavonoids, esters, terpenes, coumarins, saponins and alkaloids were performed according to Matos.¹⁸ These tests are based on the visual observation of color modification or precipitate formation after the addition of specific reagents.

Ames mutagenicity assay

The Ames/*Salmonella* mutagenicity assay was performed using the plate incorporation protocol with the *Salmonella typhimurium* strains TA98 and TA100^{19,20} and five different concentrations of the hydroalcoholic extracts, obtained from leaves of *S. guianensis* (0.68, 1.36, 2.72, 5.45 and 10.90 mg plate⁻¹) and *P. major* (1.78, 3.56, 7.11, 10.67 and 14.22 mg plate⁻¹) were evaluated in this assay, with or without metabolic activation (S9 fraction). The various extract concentrations were added to 500 µL of phosphate-buffered saline (pH 7.4), plus 100 µL of bacterial culture and 2 mL of top agar. After agitation, the mixtures were poured on to plates containing minimum agar. The plates were incubated at 37°C for 48 h and the his⁺ revertant colonies were counted. All experiments were performed in triplicate. To the experiments with metabolic activation, the S9-mix was freshly prepared before each test from an Aroclor-1254-induced rat liver fraction purchased (lyophilized) from Moltox (Molecular Toxicology Inc, Boone, NC, USA). The obtained results were evaluated with the statistical software Salanal (developed by Dr. L. Myers, Research Triangle Institute, NC, USA) (analysis of variance [ANOVA] followed by a linear-regression). The mutagenic index (MI), which is the average number of revertant mutants per plate for treated groups divided by the average number of revertant mutants per plate for the negative control (solvent), was calculated for each concentration. A sample was considered mutagenic when MI ≥ 2 for at least one of the tested doses and if the response was dose dependent.^{21,22}

Animals

A total of 56 male mice of Swiss line (26–30 g) with six weeks were housed individually in plastic cages for one week prior to the experiment. The animals were fed with a standard commercial diet (Purina® Labina, Campinas - SP, Brazil) and control *ad libitum*. All of the experimental procedures were in accordance with the Brazilian College of Animal Experimentation (COBEA) ethical guidelines for the welfare of experimental animals. The experimental protocols were approved by the UFSJ-CEPEA protocol no

Table 1 Phytochemical tests with the hydroalcohol extract of plant *P. major* and *S. guianensis*

Components	<i>P. major</i>	<i>S. guianensis</i>
Steroids and triterpens	++ +	–
Flavonoids	+	+++
Condensed tannins	–	+++
Alkaloid	++	+++
Coumarins	+	+++
Saponins	–	–

– absent, + low presence, ++ medium presence, +++ high presence

08/2009. The mice were anesthetized with Ketamina 50 mg kg⁻¹ and Xilazina 20 mg kg⁻¹. After trichotomy, a cutaneous incision with 2 cm of diameter was made in the cervical dorsal area. Following the injury, the animals were divided in four groups with 14 individuals each. They received local treatment with 100 µL of: group 1 control (water); group 2 *P. major* extract; group 3 *S. guianensis* extract; and group 4 ointment. Except for the drugs under study, no local/systemic therapy was provided to animals bearing any of the wounds.

Macroscopic analysis

The wound closure was measured every day using a pachymeter from first after injury until 21 days. The data were converted in percentage. The period of cicatrisation was calculated as the number of days required for the wounds to heal completely without any raw wound left behind.

Microscopic analysis

Skin samples were collected on the ninth day,²³ fixed in 10% formalin, embedded in paraffin, sectioned with 5 µm thickness and stained with hematoxylin–eosin (HE). For analysis of wound healing, it was considered the presence or absence of epithelium and skin appendages throughout the wound, amount of edema and inflammatory infiltrate. To quantify the edema, it was used a score: 0 (physiological), 1 (tenuous), 2 (moderate) and 3 (severe). The score for inflammatory infiltrate intensity ranged from 0 (physiological), 1 (tenuous), 2 (moderate), 3 (severe) and 4 (very severe).

For mast cells quantification, 10 consecutive fields of histological sections stained with toluidine blue–sodium borate were acquired using an optical microscope and the AxioVision Rel 4.8 software (Carl Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad InStat software (Graphpad Software Inc., San Diego, CA). The data was statistically analyzed by ANOVA followed by Tukey–Kramer for multiple comparison and *t* Student's *t*-test was used followed by Kolmogorov and Smirnov correction for two groups comparison. The difference was considered significant when *P* < 0.05. All the values were expressed as mean ± standard deviation (SD).

Results

Phytochemical assay

The extracts of *P. major* and *S. guianensis* were subjected to phytochemical analysis for the identification of its major components. The presence of steroids and triterpens, and condensed tannins were different in extracts (Table 1).

Ames mutagenicity assay

The mutagenicity studies carried out with plant extracts obtained from *P. major* (Table 2) and *S. guianensis* (Table 3) showed that these compounds were not mutagenic with or without metabolic activations and there is no statistical difference between assays. The negative effects observed in all concentrations evaluated (MI < 2.0) indicated that these extracts did not present in its constitution substances able to induce substitutions, additions or deletions of bases in DNA structure.

Macroscopic analysis

Wound healing of the skin incision was determined by the percentage of wound surface covered by regenerating epidermis at different treatments and the appearance of the

Table 2 Revertant/plate, standard deviation and mutagenicity index (MI) in strains TA98 and TA100 of *S. typhimurium* after treatment with various doses of *P. major* extract with (+ S9) and without (– S9) metabolic activation

Treatment	TA98				TA100			
	– S9		+ S9		– S9		+ S9	
	Mean ± SD	MI	Mean ± SD	MI	Mean ± SD	MI	Mean ± SD	MI
NC	25.33 ± 4.04	–	33.67 ± 3.1	–	172.0 ± 14.8	–	33.7 ± 3.1	–
Extract (mg/plate)								
1.78	29.67 ± 2.1	1.2	30.33 ± 6.0	0.9	178.7 ± 21.1	1.0	30.3 ± 6.0	0.9
3.56	27.00 ± 4.0	1.1	36.67 ± 5.5	1.1	183.0 ± 8.7	1.1	36.7 ± 5.5	1.1
7.12	26.33 ± 3.8	1.0	45.67 ± 5.5	1.4	183.3 ± 18.5	1.1	45.7 ± 5.5	1.4
10.95	24.67 ± 2.9	0.97	36.00 ± 5.0	1.1	135.3 ± 22.0	0.8	36.0 ± 5.0	1.1
14.25	23.33 ± 2.3	0.92	49.67 ± 11.8	1.5	220.3 ± 53.5	1.3	49.7 ± 11.9	1.5
PC	2924.3 ± 383.6		2693 ± 360.5		751.3 ± 140.0		2693.0 ± 360.5	

Mean ± SD: Revertants mean frequency per plate ± standard deviation; MI, mutagenicity index; NC, negative control (DMSO – 80 µL/plate); PC, positive control (4-nitro-*o*-phenylenediamine (10 µg/plate – TA98 (– S9)), methylmethane sulfonate (260 µg/plate – TA100 (– S9) or 2-aminoanthracene (5 µg/plate – TA98 (+ S9) and TA100 (+ S9))

**P* < 0,05

Table 3 Revertant/plate, standard deviation and mutagenicity index (MI) in strains TA98 and TA100 of *S. typhimurium* after treatment with various doses of *S. guianensis* extract with (+ S9) and without (– S9) metabolic activation

Treatment	TA98				TA100			
	– S9		+ S9		– S9		+ S9	
	Mean $\pm$ SD	MI	Mean $\pm$ SD	MI	Mean $\pm$ SD	MI	Mean $\pm$ SD	MI
NC	24.0 $\pm$ 4.4	–	32.3 $\pm$ 2.1	–	146.3 $\pm$ 29.7	–	97.0 $\pm$ 40.1	–
Extract (mg/plate)								
0.68	25.7 $\pm$ 4.7	1.1	30.0 $\pm$ 11.5	0.9	117.7 $\pm$ 27.7	0.8	105.0 $\pm$ 35.2	1.1
1.36	29.7 $\pm$ 3.8	1.2	26.3 $\pm$ 3.1	0.8	108.7 $\pm$ 5.7	0.7	95.0 $\pm$ 11.0	1.0
2.73	30.7 $\pm$ 3.2	1.3	32.7 $\pm$ 4.5	1.0	118.0 $\pm$ 25.9	0.8	153.3 $\pm$ 8.4	1.6
5.45	21.0 $\pm$ 6.1	0.9	32.0 $\pm$ 13.2	1.0	121.7 $\pm$ 9.3	0.8	122.3 $\pm$ 31.6	1.3
10.9	25.7 $\pm$ 8.5	1.1	40.0 $\pm$ 5.7	1.2	118.3 $\pm$ 15.7	0.8	110.3 $\pm$ 10.9	1.1
PC	1160.0 $\pm$ 162.6				900.3 $\pm$ 211.3		1257.3 $\pm$ 159.3	

Mean $\pm$ SD, Revertants mean frequency per plate $\pm$ standard deviation; MI, mutagenicity index; NC, negative control (DMSO – 80 μ L/plate); PC, positive control (4-nitro-o-phenylenediamine (10 μ g/plate – TA98 (– S9)), methylmethane sulfonate (260 μ g/plate – TA100 (– S9) or 2-aminoanthracene (5 μ g/plate – TA98 (+ S9) and TA100 (+ S9))

* $P < 0.05$

wound was observed during the period studied (Figure 1a). The reduction of the wound area occurred earlier in mice treated with *P. major* and control treatment (no statistically significant differences). In the mice submitted to ointment treatment it was observed an increase in the wound healing area on the fourth day. On the 15th day, the complete wound closure occurred in *P. major* and in the control group. Throughout ointment and *S. guianensis* treatment it was not observed the wound closed (Figure 1b).

Microscopic analysis

On the ninth day, regarding edema, *P. major* and control were classified as moderate, the *S. guianensis* was between tenuous and moderate, ointment was between moderate and severe with accumulation of fluid in the extravascular compartment and in the connective tissue (Figure 2a). The inflammatory infiltrate intensity of the *S. guianensis* was tenuous, *P. major* and control presented moderate, and ointment were between moderate and severe with remarkable presence of neutrophils and lymphocytes which were identified by nuclear morphology (Figure 2b). The mast cells with normal and degranulated morphology were recorded surrounding the wounds in all treatments. The quantitative analyses of the mast cells population showed the following values: control (range 0.0–46.0; mean 17.7 ± 1.3 cells/field), *P. major* (range 0.0–45.0; mean 18.5 ± 1.3 cells/field), *S. guianensis* (range 3.0–42.0; mean 22.0 ± 1.5 cells/field) and ointment (range 0.0–43.0; mean 15.8 ± 1.4 cells/field). There was a statistically significant difference only between the group treated with *S. guianensis* extract (Figure 2c) and the ointment treatment (Figure 2d).

About the neoepithelium in skin repair, 42.9% of *S. guianensis*, 14.3% in control, 33.3% for *P. major* animals presented closed wound (Figure 3a) while ointment treatment showed no re-epithelialization (Figure 3d). Considering the skin appendages in wound, 42.9% in control, 28.6% for *S. guianensis*, 16.7% of *P. major* of the mice showed cutaneous structures in formation (Figure 3b) and completely established (Figure 3c) while in ointment this appendages were not observed.

Discussion

This study reported the effect of *P. major* and *S. guianensis* extracts on the wound healing by macroscopic, microscopic analysis and mutagenic activity. The reduction of the wound area occurred earlier in mice treated with *P. major*. Histological analyses of the wound, on the ninth day, showed neoepithelium and skin appendages formation a more efficient in animals treated with *P. major* and *S. guianensis*, while ointment treatment presented no re-epithelialization and absent skin appendages in wound. Mutagenicity studies carried out with plant extracts showed not mutagenic with or without metabolic activations.

The *P. major* extracts showed a high presence of steroids and triptenes, while the *S. guianensis* extracts presented condensed tannins. The steroids and triptenes were related with anti-inflammatory activity.²⁴ The triterpenoids inhibits cyclooxygenase-1 and -2 catalyzed of prostaglandin biosynthesis *in vitro* while the structural isomer oleanolic acid is less active.²⁵ Tannins promote wound healing through several cellular mechanisms: scavenging of free radicals and reactive oxygen species, promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts.²⁶ However, the wound healing potential of these extracts is a result of the mixture of the phyto-constituents and the real contribution of each one needs to be better elucidated. Coumarins have multiple biological activities including disease prevention, growth modulation and antioxidant properties. These compounds are known to exert antitumor effects and can cause significant changes in the regulation of immune responses, cell growth and differentiation.²⁷ The alkaloids have been described as being responsible for the improvement in the healing of incisions by increasing the tensile strength of the scar tissue.²⁸ Moreover, it enhanced the wound healing by hastening the period of epithelialization and wound contraction on fourth and 16th days.²⁸ Furthermore, it accelerates soft tissue repair in a dose-dependent manner at initial phases of the process, probably by chemotactic properties toward fibroblasts.²⁹

The mutagenicity studies carried out with plant extracts obtained from *S. guianensis* and *P. major* showed that these

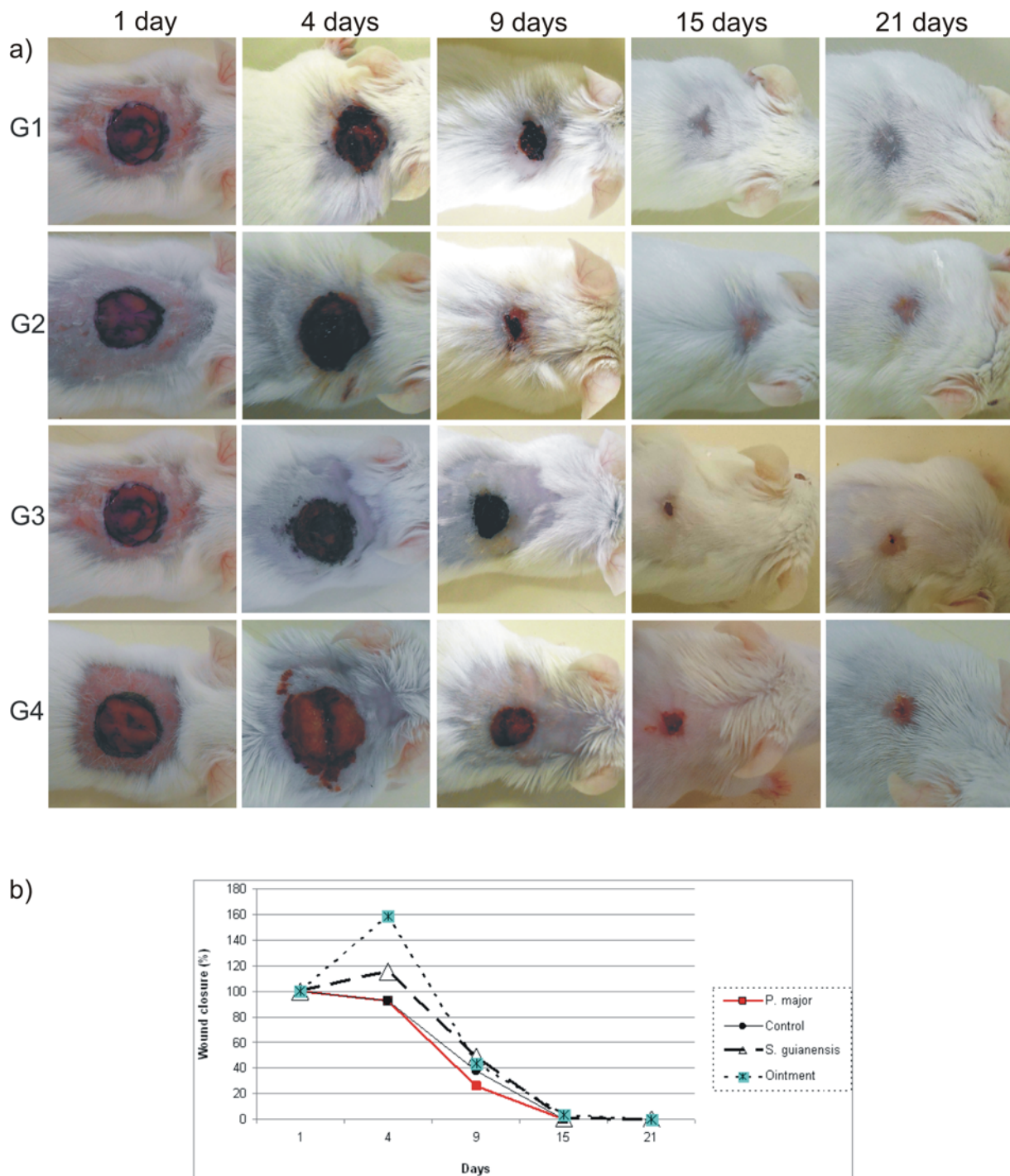


Figure 1 Wound healing effect after different treatments. (a) photographic representation of wound contraction and appearance observed throughout of the period studied G1 (control); G2 (*P. major*); G3 (*S. guianensis*); G4 (ointment); (b) curve of wound closure percentage, with *P. major* contributed to the wound healing compared with control, *S. guianensis* and ointment treatment. (A color version of this figure is available in the online journal)

compounds were not mutagenic with or without metabolic activations. Mutagenic activity is an important parameter to evaluate the viability of topical administration,³⁰ as the epithelial tissue has a high-proliferation rate during the regeneration process.³¹ The absence of a mutagenic response by plant extract is a positive step forward in determining the safe use of plants in traditional medicine.³² The Ames test is a widely accepted method to identify various chemicals

and drugs that can cause gene mutations and has a high-predictive value for *in vivo* carcinogenicity.²⁰

In the present study, the animals treated with *S. guianensis* presented the inflammation stage tenuous, while *P. major* presented moderate inflammation, and with the ointment it showed inflammatory response between moderate and severe. The time of inflammation can range from 24–48 h after injury, but in chronic inflammation the inflammatory

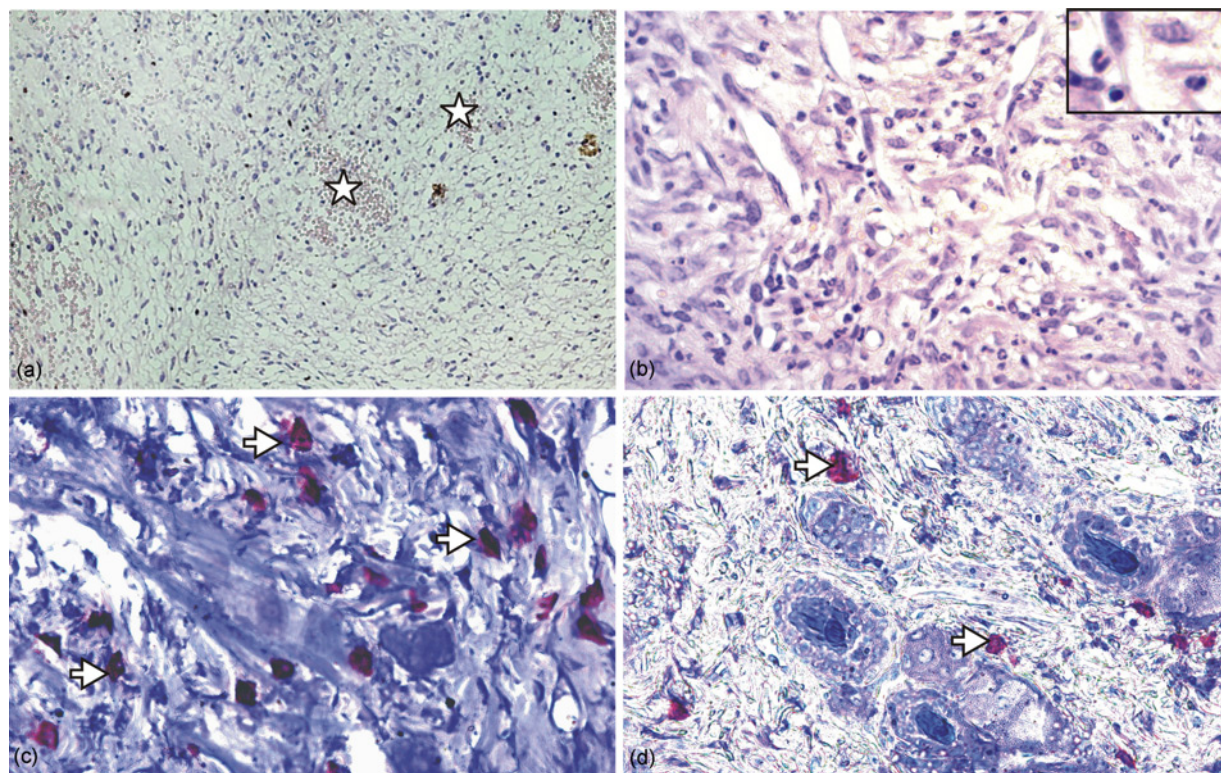


Figure 2 Histological sections of wound area at ninth day evidencing the remarkable findings, stained with hematoxylin–eosin (a, b) and toluidine blue (c, d). (a) edema and hyperemia (star) in individuals of the G4; (b) presence of inflammatory infiltrate in G4, detail with neutrophils and lymphocytes; (c) mast cells (white arrow) in G3; (d) mast cells (white arrow) in G4. (A color version of this figure is available in the online journal)

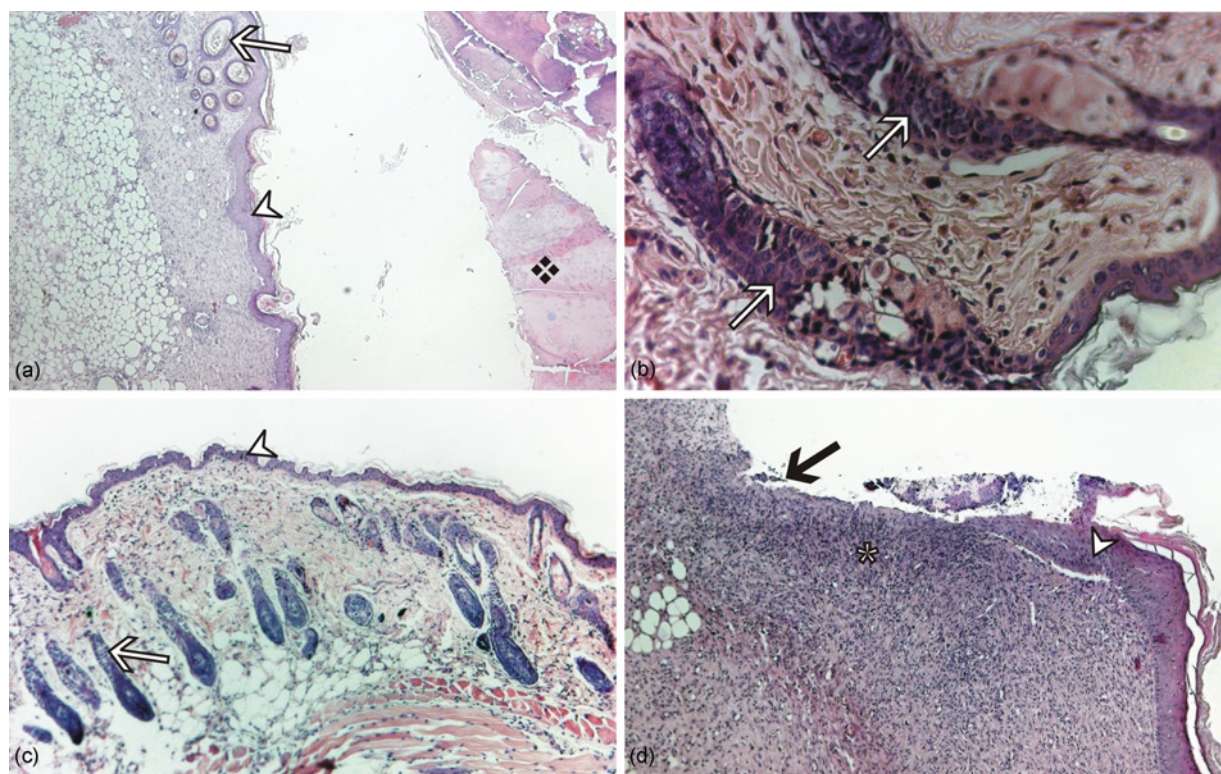


Figure 3 Histological sections of wound area at ninth day, stained with hematoxylin–eosin (a–d) and picosirius red (e, f). (a) re-epithelialization: epithelium (white arrowhead) stratified with many layers and presence of crust (◆) in G1, G2 and G3 treatment; (b) pilous follicles and sebaceous glands (white arrow), formation early in G2; (c) neoepithelium with skin appendages; (d) ointment treatment showed incomplete epithelialization (black arrow) and inflammatory infiltrate (*). (A color version of this figure is available in the online journal)

response can be prolonged for weeks, months, or even indefinitely. This extended time course is caused by persistence of the causative stimulus to inflammation in the tissue, repetitive use of anti-inflammatory drugs, a weakened immune system, or an improper nerve supply. Thus, wound healing delayed by healing of chronic wounds often results from an imbalance in the wound preventing progression from one phase to another.³³ The stage of new tissue formation (proliferative phase), that happens from two days up to three weeks after the inflammatory phase, is characterized by a re-epithelialization process, activation and migration of fibroblast, production of collagen, glycosaminoglycan and proteoglycans by fibroblasts, angiogenesis and later, some of the fibroblasts differentiate into myofibroblasts.³⁴ In this work, on the ninth day, the neoepithelium and skin appendages formation was more efficient in animals treated with *S. guianensis* and *P. major* while ointment treatment showed no re-epithelialization and absent skin appendages in the wound. This step becomes the center point of wound healing, since the closure of the wound prevents the entry of pathogenic organisms that can cause various disorders including contamination and delay in the healing process.³⁵ The findings for *S. guianensis* and *P. major*, indicate that the compounds presents in the extracts of this species probably promote a proliferative phase more efficient, preventing a delay in phase and consequently the healing.

In the present work it was analyzed mast cells population during a wound healing where the animals treated with ointment had a lower density (15.8 ± 1.4 cells/field) since that the treatment with *S. guianensis* showed higher density for mast cell (22.0 ± 1.5 cells/field). There was a statistically significant difference only between the group treated with *S. guianensis* extract and the ointment treatment. These findings were expected since the ointment is a pharmaceuticals industrial product, which decreases the allergen effects. The mast cell is involved in the various phases of skin repair (mainly proliferative and re-modeling phases), secreting pro-inflammatory molecules and also regulating the process of the healing.^{36,37} In fact, in mast cell deficient-mice, the healing process was partially impaired after skin scald injury.³⁷ The significance of this cellular type in the process of wound healing is in fact that an increased or a decreased of degranulated biological mediators causes impaired skin repair, with the formation of exaggerate granulation tissue, delayed closure and chronicity of the inflammatory stage.³⁶ The re-modeling phase is featured by the regression of most of the newly formed capillaries, thus the vascular density of the wound returns to normal. Another critical point of the re-modeling step is extracellular matrix re-modeling to an architecture that is similar to that of the normal tissue. Moreover, the wound undergoes physical contraction throughout the entire wound healing process, which probably can be performed by contractile fibroblasts (myofibroblasts) that appear in the wound.³⁵

The wound area was measured up to 21 days to determine whether the treatment with *P. major* and *S. guianensis* extracts affects the progress of wound healing. The control and group treated with *P. major*

showed the wound closure on the 15th day. However, from the fourth day until the 15th day it was observed that the wound closure evolution in *P. major* treatment was better than others fact evidenced on ninth day. In the ointment and *S. guianensis* treatment the wound closure was not observed. If the goal for wound treatment is the fast healing, the *P. major* extract was more effective than ointment treatment. In fact, the *P. major* was suggested related with wound healing and some of these effects may attribute to the use of this plant in folk medicine.¹⁵

Conclusions

In summary, the study has showed that the *P. major* extract effectively stimulated wound-healing processes as compared with the others treatments. These finding could justify its traditional usage in wound treatment.

Author contributions: RIMAR and HBS were involved in conception and design; RGT, HBS and FVS conducted analysis and interpretation; RJO, LFC, MNP, TRL, CMS, CSF, ROAS were involved in data collection; RGT, HBS and RIMAR wrote the article, carried out statistical analysis; RIMAR obtained funding and took overall responsibility.

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