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Antimicrobial and wound healing activities of *Piper hayneanum*

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ABSTRACT

In traditional medicine Piper species are widely used for the treatment of various ailments including anti-inflammatory, infectious skin diseases and healing, particularly cuts, wounds, sprain, hematoma, and ecchymosis. This study evaluated for the first time the in vitro antimicrobial and wound healing activities of extracts and fractions from leaves, stems and roots of Piper hayneanum C.DC (Piperaceae). In vitro antimicrobial activity was evaluated by disk diffusion method while wound healing effect in rats was evaluated using the excision wound model infected. Fractions from stems and roots showed in vitro antimicrobial activity against Staphylococcus aureus and Candida albicans (MICs ranging from 1.0 to 500 µg/disc). Of these fractions, two ointments were prepared (5% w/w) and assessed for in vivo wound healing on infected rat model. Wound healing efficacy for 15 days was measured by determining the physiological and histological parameters. Topical application of the ointments fractions significantly improved wound contraction when compared with control group of rats. The results showed that two fractions of P. hayneanum, promising in vitro as antimicrobial, were effective in rats with dorsal injuries infected with S. aureus and C. albicans. Animals treated with fractions showed better wound healing compared with those treated with the gentamicin (S. aureus) and miconazole (C. albicans). From the results obtained, it may be concluded that P. hayneanum fractions has the potential to be developed into new therapeutic agent for use in topical infections.

Keywords: *Piper hayneanum*; Piperaceae; antimicrobial activity; wound healing; excision wound.

INTRODUCTION

The wound healing is a complex process, which is initiated by the stimulation of injury to the tissue [1]. Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue [2]. These events may be artificially categorized into separate steps: the inflammatory, proliferative, and remodeling phases [3]. In the inflammatory phase, bacteria and

debris are phagocytized and removed. Following an injury, a series of events takes place in a predictable fashion to repair the damage. In the subsequent inflammatory response following an injury the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production reaching the last stage of regeneration of epithelial tissue (the outer skin layer) [3]. The proliferative phase is characterized by angiogenesis, collagen deposition, epithelialization, granulation tissue formation, and wound contractions. Though healing process takes place by itself and does not require much help, but various risk factors such as infection and delay in healing has brought attention to promote this process [4]. In spite of many advances in the pharmaceutical sciences, the availability of drugs capable of stimulating the process of wound repair is still limited. Moreover, the management of chronic wounds is another major problem due to the high cost of therapy and the presence of unwanted side effects [2]. Consequently, there is increasing interest in finding plant extracts with wound healing efficacy.

The genus *Piper*, belonging to the Piperaceae family, possess about 700 species, distributed in tropical and subtropical regions [5]. Some of these species are economically important [*P. nigrum* L. (black pepper), *P. methysticum* G. Forst., (kava), and *P. betle* L. (betel leaf)] and others are used as condiments and as medicinal [6, 7]. In Brazil, several *Piper* species have been described in popular medicine for different purposes [8-11]. Among them are *P. marginatum* Jacq. and *P. umbellatum* L. which leaves are used as anti-inflammatory [12], healing (cuts, wounds, sprain, hematoma and ecchymosis) [12, 13] and infectious skin diseases [13, 14]. Whereas in Brazil the use of medicinal plants for treatment of cuts and wounds is a common practice in traditional folk medicine, we chose *P. hayneanum* C.DC. extracts for investigation of the *in vitro* and *in vivo* antimicrobial activities and for the influence of topical application on wound healing in rats.

EXPERIMENTAL SECTION

General experimental procedures

Chloroform, ethyl acetate, hexane, and methanol analytical grade were purchased from Quimex (F. Maia Indústria e Comércio Ltda, Brazil) or Vetec (Vetec Química Fina Ltda, Rio de Janeiro, Brazil). Brain Infusion Heart (BHI), Mueller Hinton Agar (MHA) and Saboraud Dextrose Agar (SDA) medium were purchased from Acumedia Manufacturers Inc. (MI, USA). Sterile dishes of vancomicin, ciprofloxacin and miconazole were purchased from Sigma Co (St Louis, MO, USA). Gentamicin and miconazole ointments were purchased from Schering-Plough S/A (Rio de Janeiro, RJ, Brazil) and Farmácia Homeopática Natural (Maceió, AL, Brazil), respectively. Column chromatography (CC) for filtration was performed on silica gel 60 (70-230 mesh, Merck, Darmstadt, Germany).

Plant material

Leaves, roots and stems of *P. hayneanum* were collected in March 2005, at the farm Lamarão, municipality of Pilar, Alagoas State, Brazil, and identified by Rosangela P.L. Lemos of the Instituto do Meio Ambiente do Estado de Alagoas, where a voucher specimen was deposited (MAC-22138).

Extraction procedure

The air-dried and powdered leaves (119.4 g), stems (233.1 g) and roots (129.7 g) were extracted at room temperature with ethanol (EtOH) 90%. The solution was filtered using Whatman Nº 1 filter paper under suction and concentrated to dryness at 50ºC under reduced pressure. The EtOH obtained extracts (leaves: 10.3 g; roots: 10.2 g and stems: 12.9 g) were evaluated *in vitro* as antimicrobial by disk diffusion method and the most promising as antimicrobial (Table 1) were partitioned between hexane, $CHCl_3$ and MeOH-H_2O (7:3) solution. After that, the methanol was

removed under vacuum and the aqueous portion was further extracted with EtOAc [15]. All extracts from these procedures [stems: hexane (1.3 g), $CHCl_3$ (9.4 g), EtOAc (0.2 g), and MeOH-H_2O (1.5 g); roots: hexane (0.4 g), $CHCl_3$ (7.6 g), EtOAc (0.7 g), and MeOH-H_2O (0.7 g)] were evaluated *in vitro* as antimicrobial by disk diffusion method [16]. The $CHCl_3$ extracts from roots and stems, promising in testing antimicrobial, were further fractionated on silica gel column (70-230 mesh) using solvents and binary mixtures of solvents of different polarities (Table 1). These fractions were also evaluated and two of them [$CHCl_3$-EtOAc 1:1 (stems, A) and $CHCl_3$-MeOH 1:1 (roots, B), with promising results against *S. aureus* and/or *C. albicans* were topically evaluated on wound healing infected in rats.

***In vitro* antimicrobial activity**

Staphylococcus aureus (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Candida albicans* (ATCC 10231) strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The microorganisms cultures were grown in BHI liquid medium at 35°C and 28°C for bacteria and yeast, respectively, and the microorganisms were kept under refrigeration (4°C) until use. Bacteria and yeast were grown in the MHA and SDA medium under aerobic conditions for 24 and 48 h, respectively. Sterile dishes (6 mm diameter) of vancomicin (30 μg, *S. aureus*), ciprofloxacin (5 μg, *P. aeruginosa*) and miconazole (50 μg, *C. albicans*) were used as positive controls. In order to avoid any effect of the solvent, methanol and chloroform were used as negative controls.

In this study, disk diffusion method [16] was used to investigate the *in vitro* potential of the extracts and fractions. Stock solutions of the samples [crude extract (50 mg/mL) and fractions (25 mg/mL)] were prepared in chloroform or methanol (HPLC degree, Merck). Further, these solutions were then diluted to give final concentrations ranging from 1.0 to 1000 μg/disc and kept at 4°C prior to use. After 24h (bacteria) and 48h (yeast) of growth, each microorganism, at a concentration of 1.5×10^{-6} cells/mL [17], was inoculated on the surface of MHA and ASD plates. Sterile filter paper dishes (6 mm in diameter) saturated either with extract or fractions were impregnated with 20 μL of each solution on surface of each inoculated plate. After holding the plates at room temperature for 1h to allow diffusion of test samples into the agar, they were incubated [18]. All tests were performed in triplicate and zones of inhibition were measured from the edge of each disc after the incubation period and presented as the arithmetic average (mean value ± standard deviation). Overall, cultured microorganisms with halos equal to or greater than 7 mm were considered susceptible to samples tested. The data were analyzed using Statistical Package for Social Sciences (SPSS), version 11.5.

Determination of Minimum Inhibitory Concentration (MIC)

The MICs values, defined as the lowest concentration of sample which inhibits the visible growth, were also determined using the disk diffusion method as previously described.

Wound healing experiments

The most promising fractions [$CHCl_3$-EtOAc 1:1 (stems, A) and $CHCl_3$-MeOH 1:1 (roots, B)] as *in vitro* antimicrobial were topically evaluated for wound healing properties in rats infected with *S. aureus* and *C. albicans*. The samples A and B (20 g) were prepared at 5% under ointment form. Each sample was homogenized with propyleneglycol at 10% and incorporates in non-ionic cream. Gentamicin (bacteria, 1.0 mg/g), miconazole (yeast, 20.0 mg/g) and non-ionic cream were used as positive and negative controls, respectively.

All protocol for conducting the wound healing experiments in rats was performed with prior approval of the Institutional Animal Ethics Committee of the Universidade Federal de Alagoas, Maceió, Alagoas, Brazil (Number 004150/2007-18). For each microorganism, twelve male albino rats (Wistar strain, *Ratus novergicus albinus*) of four months age, weighing 300-320 g each, obtained from the Biotério Central of the Universidade Federal de Alagoas, were used in the experiments. In order to identify possible variables that could influence the analysis results, each rat was housed individually in a standard polypropylene rat cage with stainless steel top grill and maintained in standard laboratory conditions of temperature (22 ± 2°C), 65 ± 5% humidity, and a 12-hour light/12-hour dark cycle for 15 days before and during the experiments. The animals were fed with a standard laboratory diet (Ralston Purina Co., St. Louis, MO, USA) and water *ad libitum*.

Excision wound model

For excision and wound creation, the animals were weighed individually and anesthetized by inhalation with diethyl ether at 70% [19]. The animals were shaved in the thoraco-lumbar region and wiped with 70% alcohol and four circular wounds were punched on each side by excising the skin and panniculus carnosus with the help of biopsy punch of 6 mm diameter. Excision wounds were created on the dorsal thoracic region 1.5 cm from the vertebral column on either side [20]. Homeostasis was achieved by blotting the wound with a cotton swab soaked in normal saline. After 24 h of the contamination with 1 mL of *S. aureus* and *C. albicans* suspensions (1.5 x 10^6 cells/mL), the experimental animals were randomized into four groups (1-4) [G*roups 1 and 2*: the animals of these groups were treated with 20.0 mg of the samples (ointments A and B, respectively); g*roup 3*: gentamicin (1.0 mg/g, *S. aureus*) and miconazole (20.0 mg/g, *C. albicans*) as positive control drugs; and g*roup 4*: only with non-ionic cream (negative control)], containing three each and treated topically one time per day for 15 days [21]. The decrease in wounds diameters during the healing process was measured with an analytical pakimeter. After 15 days, the animals were anesthetized and sacrificed.

Throughout the experiments, the animals were monitored every 48 h based on the three parameters: a) clinical observation; b) microbiological analysis - the materials collected in the 3rd, 9, 12, and 15th days of post surgery (DPS) and were seeded in Petri dishes containing culture media and placed in suitable glass for observation of microbial growth (*S. aureus* and *C. albicans*) and compared with gentamicin (1.0 mg/g) and miconazole (20.0 mg/g) ointments; and c) Histopathological analyses - the tissue samples were taken from the wound surface of one animal per group at 3rd, 9, 12, and 15th day's after the beginning of treatment [21]. The material was fixed in a 10% formalin solution and studied by conventional histochemical methods. The preparations were subjected to hematoxylin–eosin staining and examined under microscope. The results of objective and histopathological investigations were compared between the test and positive and negative control groups. In this analysis the data were transformed into scores and quantified according to the model previously proposed [22] and further modified [23].

Statistical analysis

All treated groups (ointments A and B and positive controls) were compared with untreated groups. Analysis of variance (ANOVA) and Tukey test were used to identify differences between groups ($p < 0.05$), using the program Microcal OriginPro version 7.0.

RESULTS AND DISCUSSION

In vitro antimicrobial activities

Table 1 shows the zones of inhibition as well as the average values of MIC of the crude extracts, fractions and positive controls. In this assay, no negative controls exhibited antimicrobial activity and when compared with positive controls [vancomicin (15.0 ± 0.0 mm) and miconazole (26.0 ± 0.0 mm)] some extracts had significant activities against *S. aureus* (7.0 ± 0.27 to 12.0 ± 0.0 mm and MIC from 1.0 to 500 µg/disc) and *C. albicans* (7.0 ± 0.0 to 13.6 ± 0.04 mm and 1.0 to 350 µg/disc). Among them, two fractions from chromatographic fractionation from stem [A, $CHCl_3$-EtOAc 1:1 (MIC 1.0 µg/disc, *C. albicans*)] and roots [B, $CHCl_3$-MeOH 1:1 (MICs 1.0 µg/disc, *S. aureus* and *C. albicans*)] effectively inhibited the growth of the microorganisms. Although there are some reports showing that polar extracts inhibited the growth of both Gram-positive and Gram-negative bacteria [24-27], in this study no extract inhibited *P. aeruginosa* growth.

Table 1. Zones of growth inhibition and Minimum Inhibitory Concentrations (MIC) values of the extracts and fractions from *P. hayneanum*.

Extracts and fractions	Mean zone of growth inhibition (mm ± SD)*		MIC (µg/disc)	
	S. aureus	*C. albicans*	*S. aureus*	*C. albicans*
EtOH (Leaves)	8.0 ± 0.53	7.0 ± 0.18	1000	350
EtOH (Stems) and its fractions	8.0 ± 0.41	9.0 ± 0.08	250	250
Hexane	0.0 ± 0.0	7.0 ± 0.25	0.0	350
$CHCl_3$	12.0 ± 0.0	8.0 ± 0.01	350	31.2
EtOAc	7.0 ± 0.53	7.0 ± 0.08	450	350
MeOH-H_2O	7.0 ± 0.16	0.0 ± 0.0	500	0.0
Fractions from $CHCl_3$ extract:				
$CHCl_3$	0.0 ± 0.0	8.0 ± 0.20	0.0	7.8
$CHCl_3$-EtOAc 1:1	0.0 ± 0.0	8.0 ± 0.40	0.0	1.0
EtOAc	0.0 ± 0.0	7.0 ± 0.0	0.0	1.0
MeOH	0.0 ± 0.0	7.0 ± 0.36	0.0	1.0
EtOH (Roots) and its fractions	7.0 ± 0.36	9.0 ± 0.03	450	250
Hexane	0.0 ± 0.0	8.0 ± 0.06	0.0	350
$CHCl_3$	8.0 ± 0.0	10.0 ± 0.03	62.5	125
EtOAc	7.0 ± 0.27	8.0 ± 0.20	125	125
MeOH-H_2O	9.7 ± 0.41	0.0 ± 0.0	0.0	0.0
Fractions from $CHCl_3$ extract:				
Hexane-$CHCl_3$ 1:1	0.0 ± 0.0	0.0 ± 0.0	0.0	0.0
$CHCl_3$	8.0 ± 0.0	8.0 ± 0.0	3.9	3.9
$CHCl_3$-MeOH 1:1	7.0 ± 0.61	8.0 ± 0.18	1.0	1.0
MeOH	0.0 ± 0.0	7.0 ± 0.25	0.0	1.0
Vancomicin (30 µg)	15.0 ± 0.0			
Miconazole (50 µg)		26.0 ± 0.0		

**Standard Deviation (SD, mean of three assays); Solvents (negative controls) did not show inhibitory activity.*

Wound healing activity

In this experiments, the animals were evaluated every 48h based on the following parameters: a) clinical observations (basal temperature, size of the injury and inflammatory reaction); b) microbiological analysis - through antimicrobial analysis of exudates in the 3rd, 9, 12, and 15th days of post surgery (DPS); and c) Histopathological analyses - considering epitheliazation degree and intensity, the predominant cell type of inflammatory reaction, granulation tissue formation, neovascularization, presence of network of fibrin, and presence of collagen fibers.

Clinical observations

From 3rd to 9th days, animals of the treated groups (ointments A and B and positive controls) remained within the parameters of normality (36.5 to 36.7ºC) and only animals of the untreated groups suggested an inflammatory process (> 37.5°C). So, as shows Figure 1, on the first DPS, the basal temperatures of the infected animals with both microorganisms ranged from 36.1 to 36.3°C. According to Simões and Martino [28], basal temperatures between 37.2 to 37.8°C can be considered as a beginning of a protective response of the organism before infection and injury.

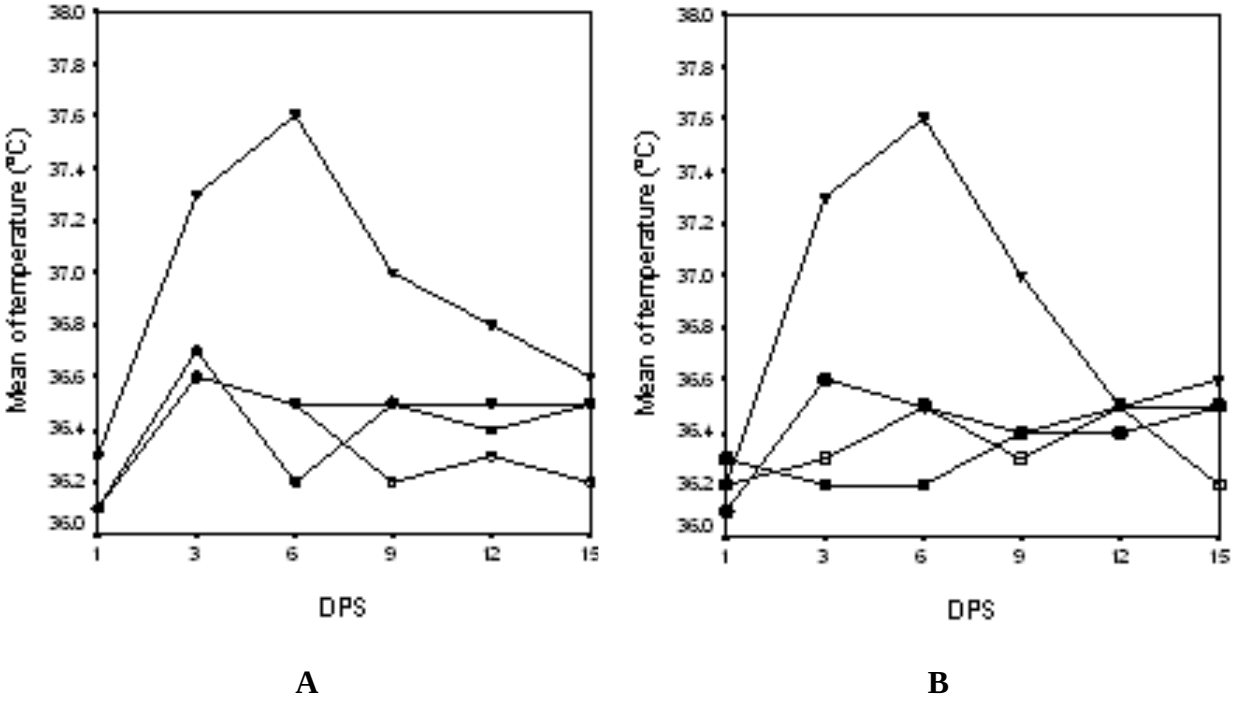
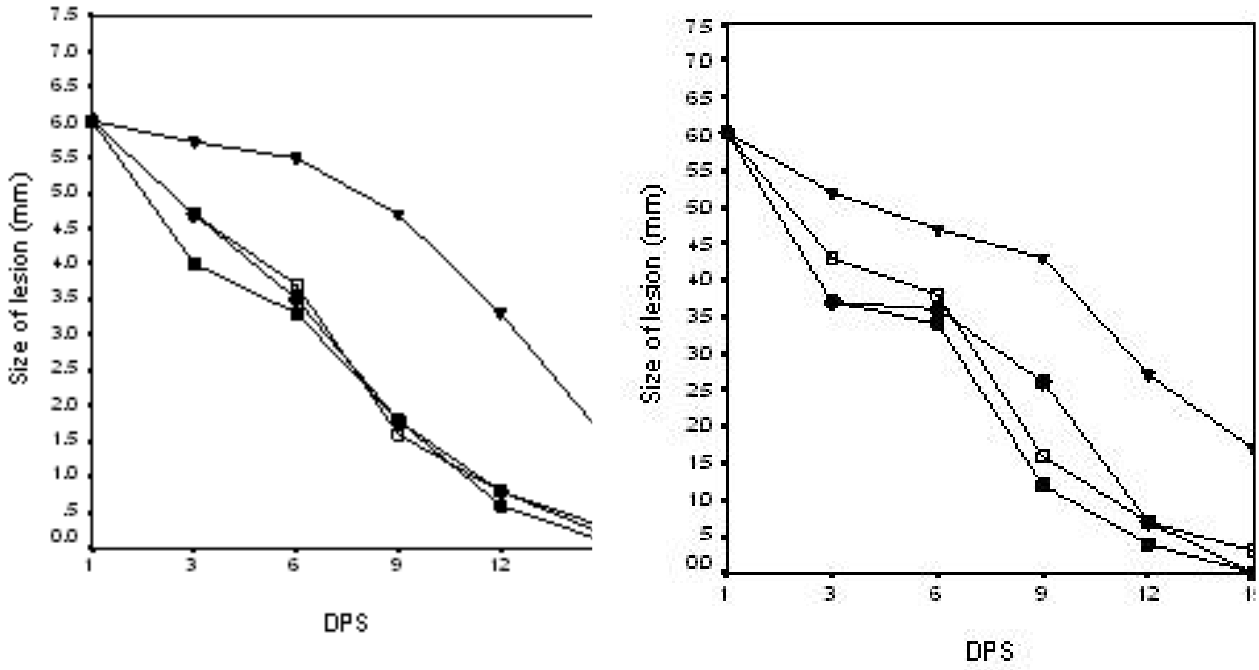


Fig. 1. Basal temperature of infected animals with *S. aureus* (stems, A) and *C. albicans* (roots, B). Data represent animals treated with ointment A (■), ointment B (□), positive controls (●), and negative controls (▼).

Figure 2 shows cicatrizing activities in skin lesions of animals infected with *S. aureus* (A) and *C. albicans* (B). In both experiments, before treatment the model wounds in all animals were about 6.2-6.8 mm in size, exhibited phlogistic characteristics (infiltration, hyperemia, edema and exudates). In the 15th day of observation, in contrast to the untreated animals (1.5-1.7 mm), the edges of the wounds treated animals (ointments A and B) were contracted (0.1-0.4 mm) as the animals of the positive controls (0.1-0.2 mm).

According to Vasconcelos et al. [29], the inflammation comprises the first phase of the process of tissue regeneration, with the participation of cells of the immune system. However, this phase may be prolonged due to various factors including the wound contamination by pathogens. Treatments that promote the expulsion or death of the foreign body may shorten the process, accelerating the healing of the lesion. In relation to inflammatory reaction, until the 3rd DPS, the phlogistic characteristics were observed in all animals. From the 6th day, only the untreated animals showed these characteristics. Although one of the fractions tested was inactive *in vitro* against *S. aureus* (Table 1), based on the results, both the ointments A and B from *P. hayneanum*, in relation to untreated controls, were able to significantly reduce the time for healing of infected wounds and also were as effective as the antibiotic (gentamicin) and

antifungal (miconazole) in the containment of the inflammatory process, probably due its antimicrobial action on the wounds.



A **B**

Fig. 2. Cicatrizing activity in skin lesions of animals infected with 1.5 ×10^6 CFU/mL of *S. aureus* (stems, A) and *C. albicans* (roots, B). Data represent animals treated with ointment A (■), ointment B (□), positive controls (●), and negative controls (▼).

In vivo microbiological analysis

Microbial infection of the wound can significantly compromise the healing process and in many cases antibiotics are used for topical treatment of infected wounds. The control of infection by microbiological evaluation is a parameter that should be used routinely to treat patients with wounds [30]. In this study, on 3rd DPS all cultures were positive for *S. aureus* and *C. albicans*, confirming the contamination of all injuries. From the 9th DPS only the untreated animals showed growth of microorganisms.

Histopathological analysis of wounds

Histopathological analyses were performed by reference to the degree of epitheliazation, the intensity and type of cell predominant inflammatory reaction, presence and degree network of fibrin, granulation tissue formation, neovascularization, and collagen fibers formation. In these analyses the obtained data after 3rd, 9, 12, and 15th DPS were transformed into scores and quantified [ointment A (*S. aureus*: 42.0 ± 2.6; *C. albicans*: 40.7 ± 2.1), ointment B (*S. aureus*: 40.0 ± 3.6; *C. albicans*: 41.7 ± 2.1), positive controls (*S. aureus* and *C. albicans*: 42.7 ± 2.1), and negative controls (*S. aureus*: 31.0 ± 4.6; *C. albicans*: 31.0 ± 3.0)] and the results showed significant difference among treated and untreated groups ($p < 0.05$).

Figure 3 shows the morphological changes of the skin lesions induced in animals on day 15th, after treatment. For treated animals (ointments A and B and positive controls) with both microorganisms (*S. aureus* and *C. albicans*), the analyses showed epitheliazation, presence of granulation tissue and neovascularization, and presence of fibrin. Intense acute inflammatory

reaction was observed only for untreated animals. On the other hand, from the 9th DPS, moderate to strong epitheliazation and granulation tissue were observed for treated animals; while in the untreated animals were absent or very weak. These data were consistent with the macroscopic evaluation, suggesting that fractions A and B from *P. hayneanum* have therapeutic properties against microorganisms studied and contributed to accelerate the healing time.

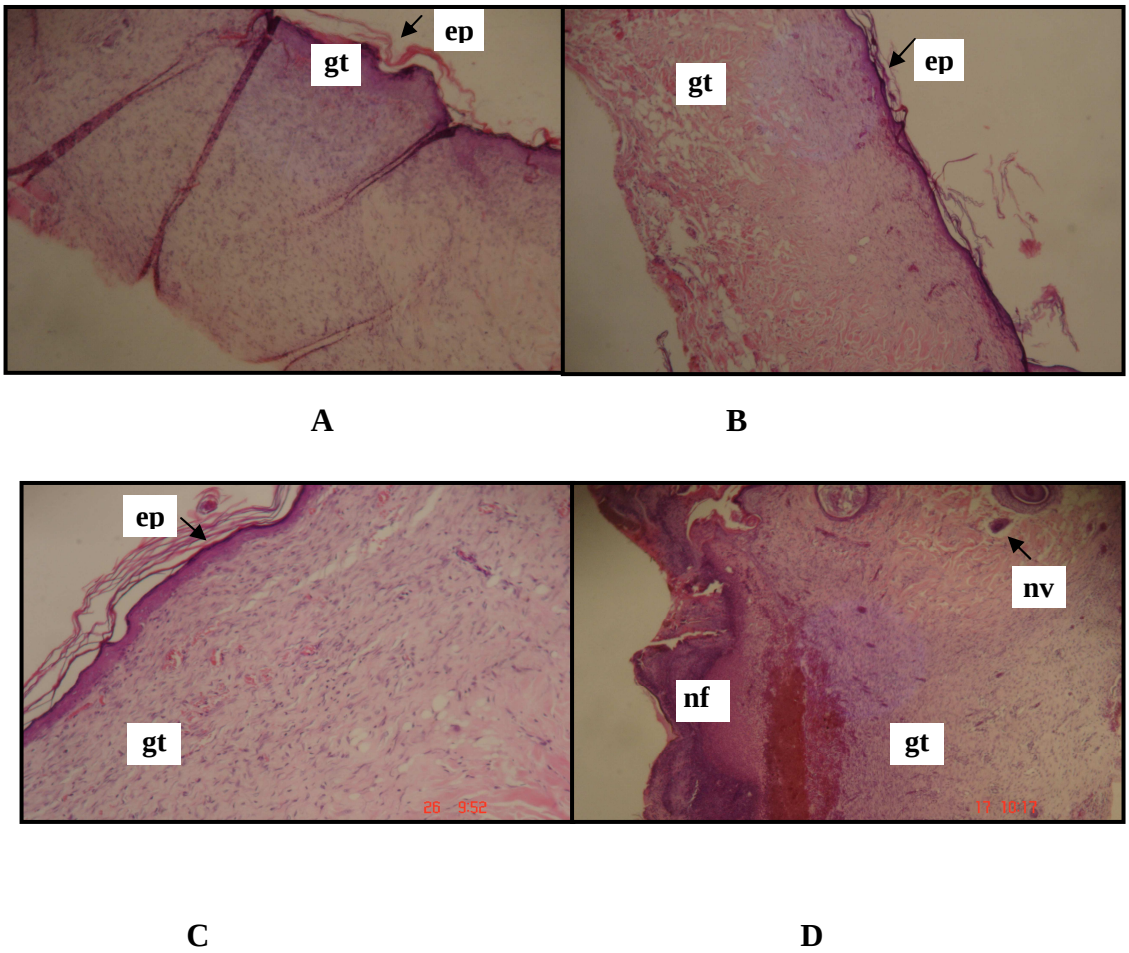


Fig. 3. Morphological changes of the skin lesions induced in animals 15 days after treatment. Epithelialization (ep), granulation tissue (gt), neovascularization (nv), network of fibrin (nf). Ointment A (A), ointment B (B), positive controls (C), and negative controls (D).

Wound healing is a fundamental response to the tissue injury that results in restoration of tissue integrity, which is due to synthesis of the connective tissue matrix [31]. A wound is the result of physical disruption of the skin, one of the major obstacles to the establishment of infections by microorganism pathogens in internal tissues. Its impact on the healing process is not fully understood, but infection is known to interrupt healing and at worst can lead to death. In chronic wounds, it has been suggested that bacteria and fungal delay healing [32]. The therapeutic efficacies of many medicinal plants have been described by traditional herbal medicine practitioners. The presence of various life sustaining constituents in plants has urged scientists to examine these plants with a view to determine potential wound healing properties. Nevertheless, thorough research in wound healing has not yielded, efficient pro-healing agent that could preclude the long hospitalization of patients. The wound healing process begins with the restoration of a damaged tissue as closely as possible to its natural state and wound contraction is the course of shrinkage in wounded area.

Cutaneous healing is an important area of dermatology as it is involved in a large number of common conditions, such as superficial wounds, minor surgery, leg ulcers, scabby lesions or burns. According to Mohtar et al. [33], the antimicrobial activity could also be a key factor in the rapid wound healing process and many attention has been directed toward extracts of the plants with antimicrobial activity in treatment of infected wounds [20] and healing activities have been reported for other topical extracts of plants, but only after longer periods of treatment or higher concentrations after application and protective effects on skin connective tissues also have been reported [34].

In this work, fractions from stems (A) and roots (B) of *P. hayneanum* exhibited *in vitro* significant antimicrobial activity against *S. aureus* and *C. albicans*. The topical application of these fractions on the intentionally excised wounds surface accelerated, after 15 days in the treated rats, the wound healing process by stimulating different biological events such as network of fibrin, epithelialization, granulation tissue, neovascularization, and wound contraction. Thus, it may be presumed that inhibition of infectious wounds by fractions of *P. hayneanum* might accelerate wound healing in rats. Potent antimicrobial activity which is also infectious in skin diseases and wounds also validates the ethnopharmacologic claim of the *Piper* species.

CONCLUSION

Fractions from stems and roots of *P. hayneanum*, promising *in vitro* as antimicrobial, were tested for cicatrizing activity in rats with dorsal injuries infected with *S. aureus* and *C. albicans*. Animals treated with fractions showed better wound healing compared with those treated with the gentamicin (*S. aureus*) and miconazole (*C. albicans*). Subsequently, other studies to clarify the mechanism involved in the process of healing are recommended before proposing its potential application for therapeutic use. This is the first report on the antimicrobial and wound healing activities of *P. hayneanum*. Further phytochemical studies are in progress to isolate, characterize and identify the specific compounds responsible for wound healing activity.

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