



RESEARCH ARTICLE

Antimicrobial efficacy and activity of ethanolic extract of *Piper betle* L. on *Staphylococcus aureus*-infected wound in mice and clinical isolates of multiple drug-resistant bacterial pathogens

Valle, D.L. Jr.^{1,2,3}, Puzon, J.J.M.¹, Cabrera, E.C.⁴, Cena-Navarro, R.B.³, Rivera, W.L.^{1*}

¹Institute of Biology, College of Science, University of the Philippines Diliman, Quezon City 1101, Philippines

²Makati Medical Center, Makati City 1229, Philippines

³Institute of Molecular Biology and Biotechnology, National Institutes of Health, University of the Philippines Manila, City of Manila 1000, Philippines

⁴Biology Department, De La Salle University, City of Manila 0922, Philippines

*Corresponding author: wlriviera@science.upd.edu.ph

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ABSTRACT

This study aimed to determine the *in vivo* effectiveness of the ethanolic extract of *Piper betle* L. leaves against *Staphylococcus aureus*-infected wounds in mice and its antimicrobial properties on clinical isolates of multiple drug-resistant bacterial pathogens. Twenty mice were divided into four groups. Wounds were created in all mice under anesthesia by excision from the dorsal skin down to the subcutaneous fat and inoculating with *S. aureus*. After 24 h, the wound of each mouse was treated once daily by application of the respective cream. Group I was treated with mupirocin antibacterial cream; Group II received a cream base containing no active ingredient; Groups III and IV were treated with 2.5% and 5.0% concentrations of *P. betle* cream, respectively. Further, an *in vitro* study was performed by adding undiluted, 1:50 and 1:100 dilutions of the four studied creams in normal saline containing 1.5×10^8 CFU/mL of the following bacteria: antimicrobial-susceptible *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *S. aureus*, extended-spectrum β -lactamase-producing *Escherichia coli*, vancomycin-resistant *Enterococcus*, metallo- β -lactamase-producing *P. aeruginosa* and carbapenem-resistant *Klebsiella pneumoniae*. The mice in Groups III and IV had significantly faster wound contraction and significantly shorter re-epithelialization time than Group II ($p < 0.05$), which were not significantly different from Group I ($p > 0.05$). *P. betle* creams inhibited all studied bacterial strains at full concentration and at a dilution of 1:50. The inhibitory effect was more significant than Groups I and II ($p < 0.05$), except on *S. aureus*. Specifically, *S. aureus* inhibition was not significantly different for Groups III and IV ($p > 0.05$) when compared with Group I. Cream formulations derived from *P. betle* ethanolic extract have great potential as antimicrobial agents for the treatment of wound infection. Further clinical tests are recommended to determine the safety and efficacy of these formulations in other mammalian species.

Keywords: Antimicrobial; mice; multiple drug-resistant bacteria; *Piper betle* L.; wound healing.

INTRODUCTION

Despite tremendous advances in the pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound repair is still limited, which poses a challenge in the prevention and management of wound infection (Madden, 2012). Only 1% to 3% of drugs listed in Western pharmacopoeias are used to treat wound infections, most of which are intended to treat chronic illnesses and lifestyle diseases (Pravin *et al.*, 2016). There is an increasing interest in natural remedies, especially in developing countries or in rural areas where health care facilities are inadequate (Atanasov *et al.*, 2015). Antibacterials that are

commonly used to treat wound infections may be expensive and at times ineffective, so the local population prefer to use traditional or herbal medicines. Some herbs have been used to facilitate wound healing with a high degree of success by preventing wound infection (Shedoeva *et al.*, 2019). Some countries have developed herbal plants into formulations to treat wound infections (Romero-Cerecero *et al.*, 2014; Thomas *et al.*, 2014; Farahpour *et al.*, 2015; Gould *et al.*, 2015; Kisseih *et al.*, 2015). The Philippines has a variety of medicinal herbs to treat various diseases.

Piper betle L. is a tropical vine belonging to the family Piperaceae. It is locally called "ikmo", and is cultivated throughout the Philippines (Quisumbing, 1978) and in other

East Asian countries such as India, Thailand, Malaysia, China, Pakistan, Sri Lanka, Nepal, and Indonesia (Guha, 2006). In the Philippines and other Asian countries, a mixture of *P. betle* leaves, lime and betle nut is used as a masticatory to treat oral infections and preserve the teeth, and as prophylaxis against stomach disorders (Quisumbing, 1978; Maramba et al., 1993). *P. betle* has traditionally been used for wound infections and wound healing. The leaves are crushed and directly applied to wounds, boils, and bruises (Valle et al., 2015).

Wound healing involves an intricate sequence of events that is associated with biochemical and cellular processes, aiming to reconstruct the structural damage and restore the functional status of the skin (Sasidharan et al., 2012; Bakht et al., 2014; Nilugal et al., 2014; Anani et al., 2015; do Nascimento-Neto et al., 2015; Romana-Souza et al., 2015; Valizadeh et al., 2015). It is characterized by hemostasis, re-epithelialization, granulation, remodeling of the extracellular matrix, and scar formation. The wound healing process has been enhanced by various synthetic topical formulations manufactured by the pharmaceutical industry. Similarly, several reports also attest to the increased or improved wound healing activities of various medicinal plant extracts. Herbal ointments containing different medicinal plants are used in folk medicine and have been reported to be beneficial in wound care, promoting wound healing, minimizing pain, discomfort, and scarring (MacKay and Miller, 2003).

In this study, we aimed to evaluate the wound healing ability of Philippine *P. betle* leaf ethanolic extract on *Staphylococcus aureus*-infected wounds in mice, as well as its *in vitro* antibacterial activity against the clinical isolates of multiple drug-resistant (MDR) bacterial pathogens.

MATERIALS AND METHODS

Ethical statement

All animal procedures were conducted in accordance with the Philippine Republic Act No. 8485, otherwise known as "The Animal Welfare Act of 1998" and approved by the Institutional Animal Care and Use Committee, University of the Philippines Manila (RGAO-2015-0169).

Plant and extract preparation

P. betle leaves were collected in the Sierra Madre Mountain Range, Municipality of General Nakar, Quezon Province, Philippines (geographical coordinates: 14°47'14.5"N 121°34'06.2"E). The plant was identified and authenticated at the Jose Vera Santos Memorial Herbarium of the Institute of Biology, College of Science, University of the Philippines Diliman. The ethanolic extract of the powdered dried leaves of *P. betle* was prepared following the methods of Basri and Fan (2005).

P. betle cream preparation

The ethanolic plant extract was used in the preparation of *P. betle* cream according to the methods described by Lai et al. (2011). The ethanol in the extract was completely evaporated using a rotary evaporator to ensure that it would not interfere with the results. The creams were prepared using two different amounts of extract (2.5 g and 5.0 g) along with 100 g aqueous cream base to obtain either 2.5% and 5.0% active ingredient. The aqueous cream base was prepared by mixing emulsifying wax (9%), white soft paraffin (15%), liquid paraffin (6%), chlorocresol (1%), glycerin (5%), and purified water. The mixture was heated at 50-55°C, with constant stirring until a homogeneous cream was obtained. The cream was then transferred to a sterile tube.

In vitro bacterial sensitivity testing

The sensitivity testing was conducted following the modified method of Gilbert et al. (1987). Specifically, the antibacterial activity of the cream preparation was tested in a controlled medium containing known concentrations of *S. aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, methicillin-resistant *S. aureus* (MRSA), extended-spectrum β -lactamase (ES β L)-producing *E. coli*, metallo- β -lactamase (M β L)-producing *P. aeruginosa*, carbapenemase-resistant *Klebsiella pneumoniae* and vancomycin-resistant *Enterococcus* (VRE). A 1:50 and 1:100 dilution of each concentration of the *P. betle* cream (2.5% and 5.0%), mupirocin (positive control), and negative control (only the cream base) were tested against each of the study organisms. The study organisms were prepared as follows: colonies from cultures of each of the bacteria grown on blood agar plates at 37°C for 16-18 h were suspended in sterile normal saline solution. The turbidity was adjusted to 0.5 McFarland standard to give a final inoculum concentration of 1.5×10^8 CFU/mL. One mL each of the prepared bacteria and the homogenized cream were mixed and allowed to stand for 1 h. The bacterial concentration of each mixture was then determined on the blood agar plates. One μ L of the bacterial suspension alone was used as negative control. The plates were incubated at (35 ± 2) °C for 24 h. Each test was performed in triplicate.

In vivo wound healing in mice

Study mice

Male BALB/c mice weighing 20-25 g were obtained from the National Institutes of Health, University of the Philippines Manila. All animals were kept under standard laboratory conditions and acclimatized for one week before the experiment. Each mouse was housed in a cage (30 cm \times 30 cm \times 30 cm) with autoclaved bedding and allowed access to a commercial feed diet and water *ad libitum*.

Wound preparation

The mice were weighed and randomly divided into four groups, each consisting of five mice. The sample size was computed using the formula: $n = \log 0.05 / \log 0.5 = 4.32$ which is based on the assumption that 50% of the animals will be infected and that there will be a 95% chance of detecting the infection (Dell et al., 2002).

The procedure for the wound excision was conducted according to the modified method by Lai et al. (2011) and Li et al. (2011). Specifically, the mice were anesthetized via intramuscular injection of 0.1 mL Zoletil 50 (tiletamine HCl/Zolazepam). The dorsal fur of each mouse was shaved off and the underlying skin sterilized with povidone-iodine. An area of 64 mm² was excised from the dorsal skin down to the subcutaneous fat. The wound of each mouse was carefully inoculated with 100 μ L of *S. aureus* ATCC 25923 at a concentration of 1.5×10^8 CFU/mL.

After wound creation and inoculation, we waited for 24 h to ensure wound colonization and infection. The four groups were then treated as follows: Group I was treated with mupirocin cream (positive control). Group II was treated with base cream only (negative control). Groups III and IV were treated with 2.5% *P. betle* cream and 5.0% *P. betle* cream, respectively. Cream (500 mg) was applied to the wound of each mouse daily for 14 d.

Wound contraction and re-epithelialization time

The area of each wound was measured on days 0, 3, 6, 10, and 14 after treatment using an autoclaved sterile transparent paper and then placed on a graph paper to

determine the area. The percent wound contraction was calculated according to the method of Shenoy *et al.* (2011) using the following equation: % wound contraction = (initial wound size - specific day wound size)/initial wound size × 100.

Complete re-epithelialization time was calculated as the number of days required for the scar to fall off leaving no raw wound behind.

Wound bacterial load determination

The wound swab was cultured using the standard plate count method on days 3, 6, 10, and 14 after treatment to determine the bacterial load. The culture swabs were inoculated into sterile normal saline solution and diluted using tenfold serial dilutions. Subsequently, 0.1 mL of each of the serial dilutions was inoculated evenly over the entire surface of the nutrient agar plate using a sterile glass spreader. Each plate was then incubated at (35 ± 2) °C for 24 h and colonies were counted using a colony counter.

Histopathological evaluation of the wound

At the end of treatment, each animal was euthanized by cervical dislocation. The gross appearance of each wound was recorded and then a cross-section of each wound was obtained after excising the wound and the tissue was fixed in 10% neutral buffered formalin. Tissue samples were then dehydrated with graded ethanol, cleared in xylene, and embedded in paraffin. The blocks were sectioned at 5 µm-thickness, stained with haematoxylin-eosin and Masson's trichrome stain, and observed under a light microscope at 100× magnification. The samples were examined for epithelial remodeling (re-epithelialization), granulation tissue formation, and collagen organization. Two Philippine Board-certified anatomic pathologists assessed and scored each sample (Simonetti *et al.*, 2012). The pathologists were blinded to the treatment groups.

Statistical analysis

The data were analyzed using SPSS for Microsoft Windows version 17. All data were expressed as mean ± SEM (standard error of the mean). Normal distribution (Shapiro-Wilk) of the data and homogeneity of variances (Levene's) were tested. The results were then analyzed using the one-way ANOVA (analysis of variance). A *p*-value < 0.05 was considered statistically significant.

RESULTS

In vitro antibacterial activity of *P. betle* cream formulations

The 2.5% and 5.0% *P. betle* creams completely inhibited all bacterial growth at a 1:50 dilution and exhibited varying inhibition against the bacterial strains at a 1:100 dilution (Table 1). The 5.0% *P. betle* cream showed complete inhibition of MRSA and VRE at a 1:100 dilution. Likewise, the 2.5% *P. betle* cream showed complete inhibition of VRE up to a 1:100 dilution. Both 2.5% and 5.0% *P. betle* creams had significantly (*p*<0.05) greater inhibition on all tested bacterial isolates than mupirocin, except on *S. aureus* ATCC 25923, where they were less effective with no significant difference (*p*>0.05).

Wound bacterial counts

Both 2.5% and 5.0% *P. betle* creams significantly reduced wound bacterial counts compared to the negative control and this reduction was not significantly different from the positive control (*p*>0.05) (Table 2).

Wound contraction and re-epithelialization

The percent wound contraction of 2.5% and 5.0% *P. betle* cream-treated wounds was significantly increased compared to the wounds treated with the negative control (*p*<0.05), which was comparable to those of the positive control (Table 3). The negative control-treated wounds showed no re-epithelialization during the 14-day study period. The wound re-epithelialization time was not significantly different among the 2.5% *P. betle* cream-treated, 5.0% *P. betle* cream-treated, and positive control groups (*p*>0.05) (Table 3).

Histopathological results

Results of histopathological evaluation on the treated and the untreated groups stained with haematoxylin-eosin and Masson's trichrome are presented in Figures 1-4. A complete, normal epidermis (Figure 1A and Figure 1B), collagen (Figure 1C), and abundant fibers and fibroblasts in the dermal layer (Figure 1D) were observed in the mupirocin cream-treated group on day 14. On the other hand, the excised wound treated with cream base had no epithelial formation on day 14 (Figure 2A). The superficial layer of the dermis was composed of suppurative inflammatory cells and focal granulation tissue (Figures 2A-2C). In addition, there were scanty collagen fibers in the dermis (Figure 2D). Histological specimens of the excised wound treated with 2.5% (Figures 3A-3D) and with 5.0% (Figures 4A-4D) *P. betle* creams exhibited the presence of normal epidermis, large amount of collagen, and large numbers of fibers and fibroblasts in the dermal layer.

The effects of the two concentrations of *P. betle* cream formulation on wound healing parameters, which included re-epithelialization, granulation tissue formation, and collagen organization are summarized in Table 4. The cream base-treated group exhibited the lowest scores on wound re-epithelialization, granulation tissue formation, and collagen organization during the 14-day study period, when compared to those of the mupirocin cream and *P. betle* cream treatment groups. Notably, there were no significant differences in the scores on wound re-epithelialization, granulation tissue formation, and collagen organization of the mupirocin cream treatment group, when compared with those of 2.5% and 5.0% *P. betle* creams (*p*>0.05).

DISCUSSION

P. betle leaves have been traditionally used in the Philippines to treat wound infections and to improve wound healing. We explored the *in vivo* efficacy of the ethanolic extract of *P. betle* leaves against *S. aureus*-infected wounds in mice and its antimicrobial activities on clinical isolates of MDR bacterial pathogens.

The wound healing efficacy of the *P. betle* ethanolic extract cream formulations on *S. aureus*-infected wounds in mice is very promising since it is comparable to the standard drug mupirocin cream. The 2.5% and 5.0% formulations demonstrated similar potency to mupirocin cream as evident from the different wound healing parameters measured, including re-epithelialization, granulation tissue formation, and collagen organization.

The re-epithelialization time was found to be significantly shorter in mice treated with *P. betle* cream, as compared with the negative control, where no re-epithelialization was observed at the end of the 14-day observation period. Re-epithelialization involves the proliferation and migration of epithelial cells across the wound bed. Therefore, the

Table 1. Culture results of bacteria treated with creams at 24 h

Bacterium	Cream base			Mupirocin cream			5.0% <i>P. betle</i> cream			2.5% <i>P. betle</i> cream		
	1:1	1:50	1:100	1:1	1:50	1:100	1:1	1:50	1:100	1:1	1:50	1:100
<i>S. aureus</i> ATCC 25923	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	5 666.7 ± 931.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	7 000.0 ± 1 183.2 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	9 000.0 ± 1 183.2 ^a
<i>E. coli</i> ATCC 25922	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	21 666.7 ± 3 415.7 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	20 000.0 ± 2 236.1 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	28 333.3 ± 1 291.0 ^a
<i>P. aeruginosa</i> ATCC 27853	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	21 666.7 ± 1 291.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	26 666.7 ± 1 291.0 ^a
MRSA	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	6 000.0 ± 447.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	3 666.7 ± 683.1 ^a
ESβL <i>E. coli</i>	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	7 666.7 ± 1 125.5 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	12 333.3 ± 1 125.5 ^a
MβL <i>P. aeruginosa</i>	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	5 333.3 ± 683.1 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	7 666.7 ± 1 125.5 ^a
CRE <i>K. pneumoniae</i>	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	7 666.7 ± 775.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	13 333.3 ± 1 291.0 ^a
VRE	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	100 000.0 ± 0.0 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	14 000.0 ± 774.6 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a

Data are expressed as mean ± SEM in CFU/mL. ^{a,b}Different superscripts across a row indicate significant differences at $p < 0.05$. Cream base was used as negative control while mupirocin as positive control.

Table 2. Culture results of total wound bacterial counts over time

Group	Day 3	Day 6	Day 10	Day 14
Cream base	38 600.0 ± 2 111.9 ^b	21 000.0 ± 948.7 ^b	11 000.0 ± 1 000.0 ^b	5 600.0 ± 894.4 ^b
Mupirocin cream	10 000.0 ± 316.2 ^a	4 600.0 ± 245.0 ^a	1 000.0 ± 0.0 ^a	0.0 ± 0.0 ^a
5% <i>P. betle</i> cream	10 600.0 ± 600.0 ^a	4 800.0 ± 374.2 ^a	1 400.0 ± 245.0 ^a	0.0 ± 0.0 ^a
2.5% <i>P. betle</i> cream	10 800.0 ± 583.1 ^a	5 400.0 ± 245.0 ^a	1 600.0 ± 245.0 ^a	200.0 ± 200.0 ^a

Data are expressed as mean ± SEM. ^{a,b}Different superscripts across a row indicate significant differences at $p < 0.05$.

Table 3. Wound area, % wound contraction and re-epithelization time for wounds of mice from different treatment groups

Group	Day 3		Day 6		Day 10		Day 14		Re-epithelization time (days)
	Wound area (mm ²) ± SEM	% wound contraction	Wound area (mm ²) ± SEM	% wound contraction	Wound area (mm ²) ± SEM	% wound contraction	Wound area (mm ²) ± SEM	% wound contraction	
Cream base	59.2 ± 2.0 ^b	7.5% ^b	57.6 ± 3.6 ^b	10.0% ^b	51.8 ± 1.7 ^b	19.1% ^b	36.4 ± 1.7 ^b	43.1% ^b	–
Mupirocin cream	46.2 ± 1.7 ^a	27.8% ^a	34.8 ± 2.9 ^a	45.6% ^a	16.8 ± 0.8 ^a	73.8% ^a	4.8 ± 0.8 ^a	92.5% ^a	11.0 ± 0.32 ^a
5.0% <i>P. betle</i> cream	46.2 ± 1.7 ^a	27.8% ^a	37.2 ± 3.0 ^a	41.9% ^a	17.6 ± 1.0 ^a	72.5% ^a	5.6 ± 1.0 ^a	91.3% ^a	11.4 ± 0.24 ^a
2.5% <i>P. betle</i> cream	47.6 ± 1.4 ^a	25.6% ^a	37.2 ± 2.9 ^a	41.9% ^a	18.4 ± 1.0 ^a	71.3% ^a	6.4 ± 1.0 ^a	90.0% ^a	11.8 ± 0.45 ^a

Data are expressed as mean ± SEM. ^{a,b}Different superscripts across a row indicate significant differences at $p < 0.05$. –: no re-epithelization was observed during the 14-day study period.

Table 4. Histological results on day 14 after treatment with creams

Treatment group	Re-epithelialization	Granulation tissue formation	Collagen organization
Cream base	0.50 ± 0.16 ^b	0.90 ± 0.24 ^b	1.00 ± 0.00 ^b
Mupirocin cream	4.00 ± 0.00 ^a	3.60 ± 0.19 ^a	3.60 ± 0.19 ^a
5.0% <i>P. betle</i> cream	3.80 ± 0.12 ^a	3.70 ± 0.20 ^a	3.90 ± 0.10 ^a
2.5% <i>P. betle</i> cream	3.60 ± 0.24 ^a	3.40 ± 0.19 ^a	3.40 ± 0.19 ^a

Data are expressed as mean ± SEM. ^{a,b}Different superscripts within a column indicate significant difference at $p < 0.05$.

shorter re-epithelialization time in the *S. aureus*-infected wounds treated with the *P. betle* cream formulations suggests increased proliferation of epithelial cells and viability of epithelial cells (Esimone et al., 2008).

An increased rate of wound contraction and a reduction in healing time on mice treated with the *P. betle* cream formulations are remarkable, which are consistent with the report on wound healing activity using the ethanol extracts from the leaves and stems of *P. betle* (Nilugal et al., 2014); thus, the wound healing potential of *P. betle* was further confirmed. Wound contraction is important in the evaluation of wound healing because it improves healing time by decreasing the size of the wound and reducing the amount of extracellular matrix needed to repair the defect (Chandika et al., 2015). Contraction of the wound edges can lead to a significant reduction in the area requiring re-epithelialization and in the quantity of granulation tissue required to fill the wound defect; with a consequent reduction in scar volume. Contraction facilitates re-epithelialization by shortening the distance that the migrating keratinocytes must travel (Mackay & Miller, 2003).

As wound contraction is a fibroblast-dependent process involving the deposition and maturation of collagen, other parameters that are evaluated and analyzed histologically in wound healing studies include granulation tissue formation and collagen organization, aside from re-epithelialization. Granulation tissue consists of collagen, fibroblasts, inflammatory cells, and small blood vessels; and its tensile strength increases proportionally with collagen deposition. The increase in granulation tissue in the *P. betle* cream-treated mice, as compared with the negative control, probably indicates an enhanced collagen maturation which is secondary to the increase in the cross-linking of collagen fibers.

Interestingly, *P. betle* cream formulations at both concentrations showed greater antibacterial effects against MDR bacteria tested in comparison to mupirocin cream, indicating the relatively weaker activity of mupirocin against MDR bacteria, particularly the Gram-negative ones. Mupirocin has been documented to be highly antagonistic against streptococci and staphylococci, including MRSA, but has no activity against enterococci, and weak effects against Gram-

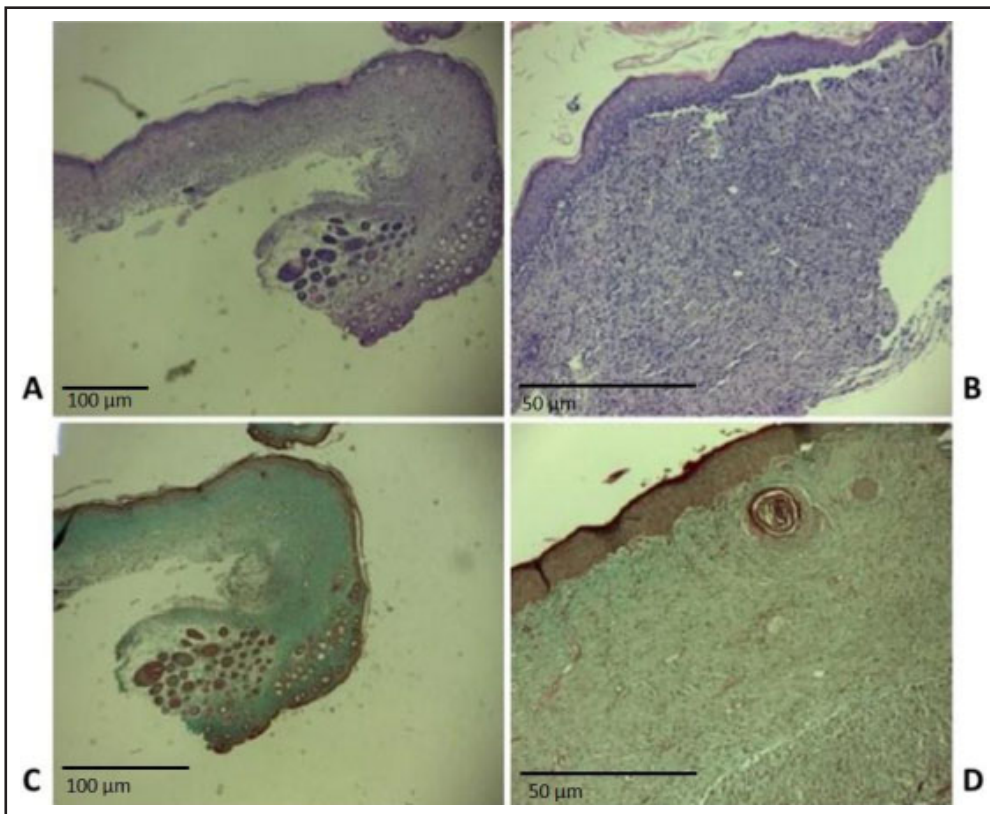


Figure 1. Histological specimen of wound on day 14 after treatment with mupirocin cream stained with haematoxylin-eosin and Masson's trichrome (100× magnification). The mupirocin cream-treated group shows complete and normal epidermis (A, B) and the presence of collagen (C), abundant fibers and fibroblasts in the dermal layer (D).

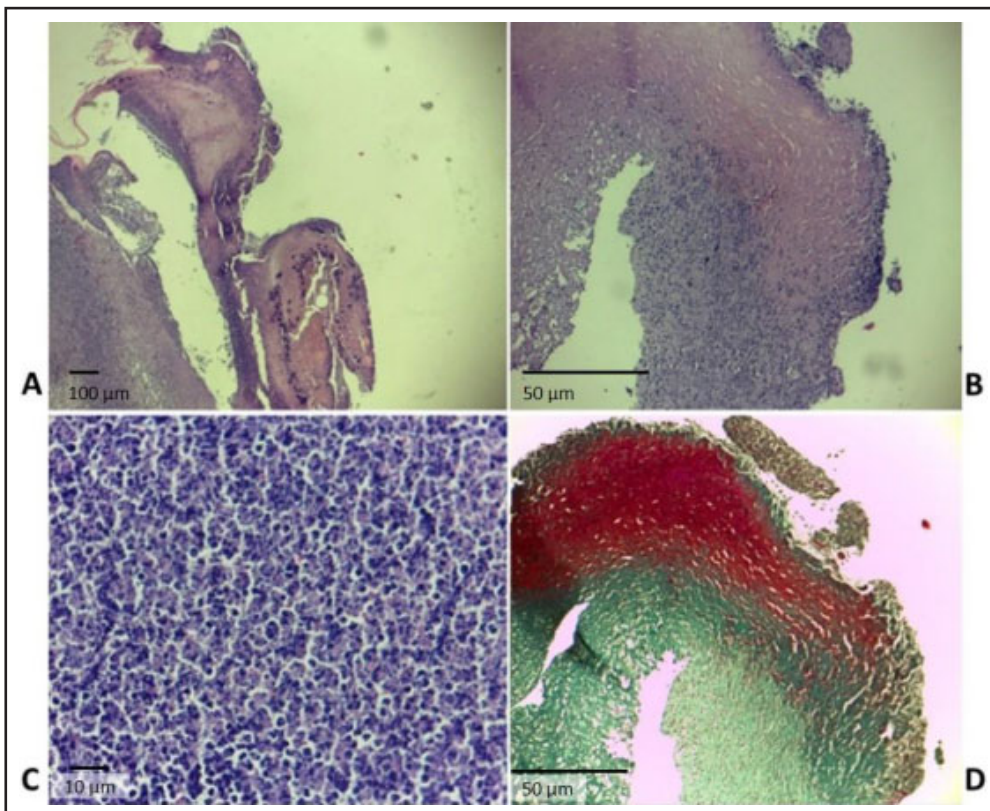


Figure 2. Histological specimen of wound on day 14 after treatment with only cream base stained with haematoxylin-eosin and Masson's trichrome (100× magnification). The cream base-treated group shows no epithelial formation. The superficial layer of the dermis is composed of suppurative inflammatory cells and focal granulation tissue (A-C). The dermis has scanty collagen fibers (D).

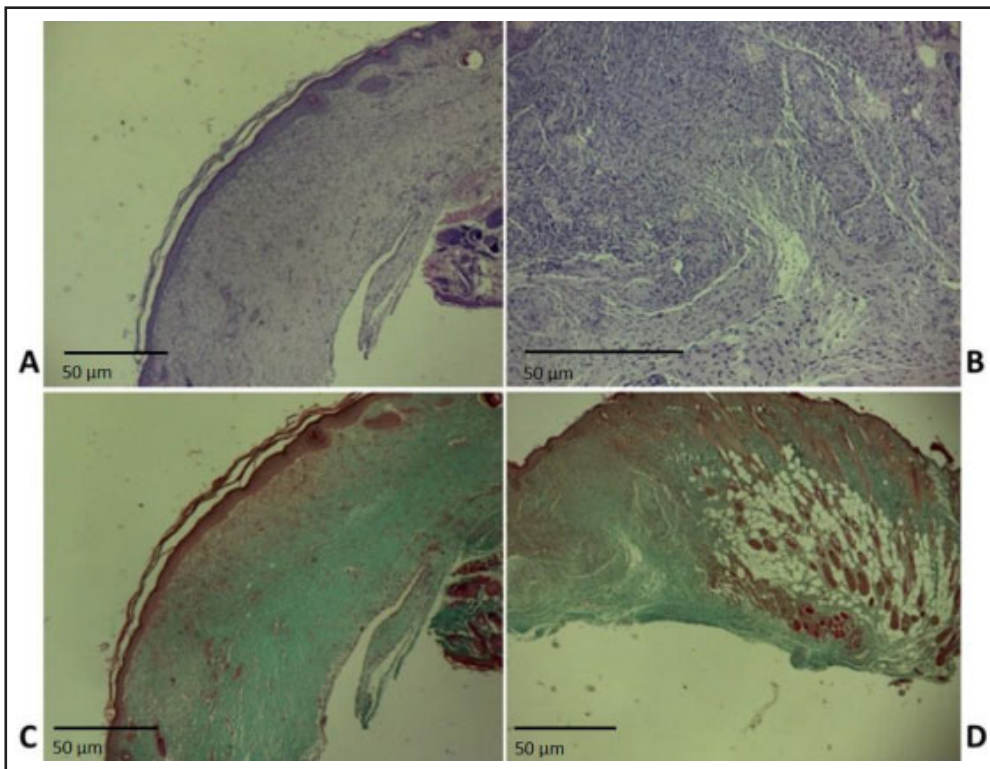


Figure 3. Histological specimen of wound on day 14 after treatment with 2.5% *P. betle* cream stained with haematoxylin-eosin and Masson's trichrome (100× magnification). The 2.5% *P. betle* cream-treated group shows normal epidermis (A, B), a large amount of collagen (C), and large numbers of fibers and fibroblasts in the dermal layer (D).

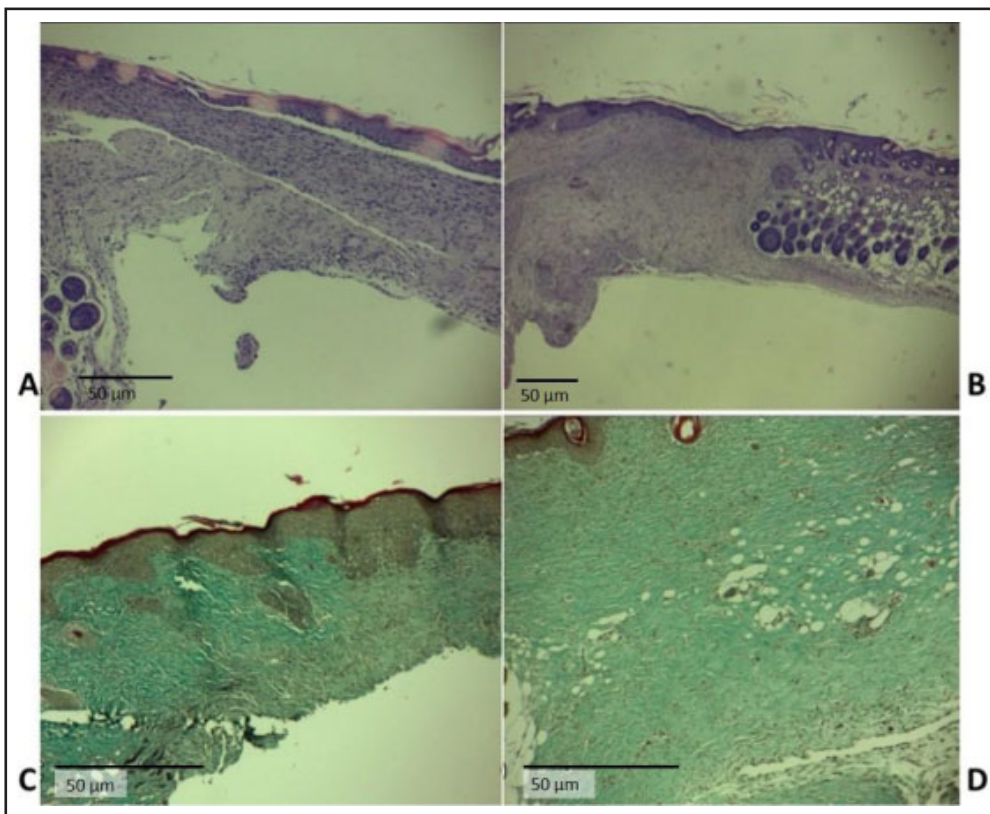


Figure 4. Histological specimen of wound on day 14 after treatment with 5.0% *P. betle* cream stained with haematoxylin-eosin and Masson's trichrome (100×). The 5.0% *P. betle* cream-treated group shows normal epidermis (A, B), a large amount of collagen (C) and large numbers of fibers and fibroblasts in the dermal layer (D).

negative bacilli and anaerobes including *K. pneumoniae* and *P. aeruginosa* (Glasser et al., 2010; Hong et al., 2014; Barakat & Kabil, 2016). In a previous study, mupirocin was tested against isolates of *P. aeruginosa*, MRSA, ES β L *K. pneumoniae*, and *Acinetobacter baumannii* from burn patients. It was reported to have the best activity against MRSA and no or weak activity against the other pathogens, particularly Gram-negative bacteria (Satpathy et al., 2011).

The findings of our study verified the efficacy of the *P. betle* leaf extract in cream formulations, indicating its great potential use as a wound-healing agent, particularly when MDR Gram-negative bacteria are involved. Presently, there are no documented studies on the *in vitro* antibacterial activity of *P. betle* leaf extract in cream formulations. However, a gel formulation containing essential oils extracted from *P. betle* was evaluated *in vitro* by Satpathy et al. (2011), which showed significant antagonistic activities against *S. aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Candida albicans*.

Overall, *P. betle* extract has great potential as an antimicrobial agent to treat infected wounds. The findings provide important information for future studies which will potentially use the extract clinically. Further research on the efficacy and safety of *P. betle* extracts and formulations on human subjects should be encouraged.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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