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Methanol Leaves Extract of *Psidium guajava* Linn. Exhibited Antibacterial and Wound Healing Activities

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A B S T R A C T

The objective of this study was to investigate the antibacterial activity and healing efficacy of *Psidium guajava* leaf on an excision-wound infected with *Staphylococcus aureus* using a rat model. The antibacterial activities of the methanol leaf extract alone and combinations of the plant extract with amoxicillin as well as the effect of osmotic stress were determined by using broth microdilution method. The therapeutic effect of the methanol extract was evaluated on an excision-wound infected with *Staphylococcus aureus*. The plant extract displayed antibacterial activity (MIC = 256 – 1024 µg/ml) that varied according to the tested bacterial species. Synergistic effect between amoxicillin and *P. guajava* extract was observed. The antibacterial activity of the plant extract and chloramphenicol increased under osmotic stress condition whereas that of amoxicillin decreased under this condition. *P. guajava* extract and Baneocin ointments gave the shortest epithelization times and highest wound contraction rates as well as the greatest weights and total protein contents of granulation tissues as compared to the negative control. The *P. guajava* methanol extract ointment is non-irritating to the skin and slightly irritating to the eyes. The results of the present study demonstrate the wound healing and antibacterial properties of *P. guajava* and confirm its traditional use in the treatment of wounds and infectious diseases.

Introduction

Wound healing is the process of repair that follows injury of the skin and other soft tissues. Many factors can influence wound healing such as bacterial infection, nutritional deficiency, drugs, sterility, obesity and site of wound (Karl *et al.,* 1995). The treatment of wound can be done by the use of antibiotics which is widely employed in combating post-operative infections in man and animals (Gyang, 1986). The antibiotics are chosen based on their ability to destroy or inhibit the growth of pathogenic organisms, while the tissue is left unharmed (Brander and Pugh 1991). Plant remedies are increasingly being recognized by scientists as a very important low cost alternative to industrially-produced antibiotics which are not available to all who need them because their high price (Huebner *et al.,* 1998). Publishing findings on the antimicrobial activity of plant remedies is important because it raises awareness of alternative medicines which in turn drives biotechnology development (Vieira *et al.*, 2001). Several mineral products and herbal medicines are described in ayurveda for their healing properties against wounds (Sharma *et al.*, 2003). This observation motivated us to evaluate the healing properties of medicinal plants in order to scientifically justify their traditional use.

The guava plant, *Psidium guajava* Linn. (Myrtaceae), is an evergreen small tree. The guava leaves are 2 to 6 inches long and 1 to 2 inches wide, aromatic when crushed, and appear dull-green with stiff but coriaceous with pronounced veins (Morton, 1987). Many bioactive constituents have been found in the guava leaf that can fight against pathogens, regulate blood glucose levels, and can even aid in weight loss. The leaves of guava contain eugenol, fat, cineol, malic acid, triterpenes, flavonoids, tannins, resin, cellulose, chlorophyll, mineral salts, and a number of other fixed substances (Burkill, 1997; Nadkarni *et al.*, 1999; Ncube *et al.*, 2008). The methanolic extract of *P. guajava* is reported for various activities including antipyretic, antispasmodic (Morales *et al.*, 1999), antidiarrheal (Fortin *et al.*, 1990), antidiabetic (Rai *et al.*, 2007) and antimicrobial (Hidetoshi and Gen-ichi, 2002). Traditionally, *Psidium guajava* is used for the healing of wounds. So far, no scientific evidence was found during literature survey for that activity. So, the present study was focused on the antibacterial and wound healing activities of *P. guajava* leaves methanolic extract on excision wound models using *Wistar* rats, to justify its traditional use.

Materials and Methods

Plant material

The leaves of *Psidium guajava* Linn. were collected from local area of Dschang during February 2017. This plant was identified and authenticated at the Cameroon National Herbarium, where the voucher specimen was kept under the reference number 2884/SRF/Cam.

Preparation of the crude extract

The leaves of *P. guajava* were cleaned under running water, air dried under room temperature. They were powdered in an electric blender. Then, 180 g of the powder was macerated in 2 l of methanol for 48 h at room temperature with occasional shaking. After 48 h, the mixture was filtered using a filter paper (Whatman No 1). The filtrate was concentrated using a rotavapor at 65 °C and placed in an oven and dry at 40 °C to give a residue which constituted the methanol extract. The extraction yield (13.46%) was calculated by dividing the amount of extract obtained by the amount of plant material used multiplied by 100. The crude extract was kept at +4 °C until further use.

Phytochemical Screening of the MeOH extract

The phytochemical screening of the methanol extract from *P. guajava* was carried out according to the methods described by Trease and Evans (1989). The plant extract was screened for the presence of different classes of compounds including triterpenes, flavonoids, anthraquinones, alkaloids, tannins, polyphenols, steroids, anthocyanins and saponins.

Test microorganisms and growth conditions

The microorganisms used in this study included: Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus* ATCC25923, methicillin sensitive *S. aureus* MSSA01, methicillin resistant *S. aureus* MRSA03, methicillin resistant *S. aureus* MRSA04) and Gram-negative (*Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* PA01, *Escherichia coli* S2 (1), *Shigella flexneri* SDINT) bacteria. These microorganisms were taken from our laboratory collection. The bacteria were stored and activated on nutrient agar.

Antibacterial assay under normal condition

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extract were determined using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 1997; 1999) with slight modifications. The plant extract was dissolved in dimethylsulfoxide (DMSO) and serially diluted twofold with Mueller Hinton Broth (MHB) in a microculture plate (Nunclon, Roskilde, Denmark, 96 wells) to obtain a concentration range of 4 - 2048 µg/ml. The inoculum was standardized at 10^6 CFU/ml by adjusting the optical density to 0.1 at 600 nm using a JENWAY 6105 UV/Vis spectrophotometer. The final concentration of DMSO in each well was less than 1%. Preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of test organisms. The negative control well consisted of 195 µl of MHB and 5 µl of standard inoculums whereas dilutions of amoxicillin (Sigma-Aldrich, Steinheim, Germany) served as positive control. The MIC values of the plant extract were determined by adding 50 µl of a 0.2 mg/ml *p*-iodonitrotetrazolium (INT) violet solution whose principle is based on the capture of protons emitted by dehydrogenases of living bacteria after metabolizing glucose; the INT is reduced to pink after 30 minutes of re-incubation.

MIC values were defined as the lowest plant extract concentrations that prevented this change in color indicating a complete inhibition of bacterial growth. For the determination of MBC values, each well that showed no growth of bacteria was mixed with the pipette tips, then 10 µl was loaded and spread on Mueller Hinton Agar (MHA) followed by incubation at 37 °C for 24 h. The lowest concentrations that lead to failure in bacterial growth after this subculture process were considered as the MBC values. All the experiments were performed in triplicate.

The antibacterial assay under osmotic stress (5% NaCl) condition

Osmotic stress condition was induced by adding 5% NaCl (w/v) to MHB. The MHB supplemented with 5% NaCl was then sterilized and used for the determination of the new MIC and MBC values of the samples as described above. The incubation time was increased from 24 hours to 48 hours at 37 °C.

Combined effect of the MeOH extract of *P. guajava* leaf and amoxicillin

The interaction between the MeOH extract of *P. guajava* leaf and amoxicillin was examined by using the broth microdilution method as described above. The antibacterial activities of the MeOH extract of *P. guajava* leaf in the presence of amoxicillin (1/8xMIC and ½xMIC) and that of amoxicillin in the presence of the MeOH extract of *P. guajava* leaf (1/8xMIC and ½xMIC) were performed as described above. The preliminary tests allow the selection of MIC/8 and MIC/2 as the sub-inhibitory concentrations of the samples.

The fractional inhibitory concentration (FIC) index for combinations of two antibacterial agents was calculated according to the following equation: FIC index = FIC A + FIC E; where FIC A = MIC of antibiotic in combination / MIC of antibiotic alone; FIC E = MIC of the extract in combination / MIC of the extract alone. The FIC indices were interpreted as follows: ≤ 0.5, synergy; > 0.5 to 1, addition; > 1 and ≤ 4, indifference and > 4, antagonism (Bone, 1994). All the experiments were performed in triplicate.

Wound healing assay

Experimental animals

Twenty four males Wistar albino rats aged 6 – 8 weeks and weighing 180-200 g were used. They were bred in the animal house of the Department of Biochemistry, University of Dschang, Cameroon. The rats were housed individually in polypropylene cages at 23 ± 1 ºC in 12 h: 12 h, dark: light cycle. The animals were provided with standard diet and water *ad libitum* and the food was withdrawn 12 h before the start of the experiment. The study was conducted according to the ethical guidelines of the Committee for Control and Supervision of Experiments on Animals (Registration no. 173/CPCSEA, dated 28 January, 2000), Government of India, on the use of animals for scientific research.

Ointment formulation

A mass of the MeOH extract of *P. guajava* leaf was weighed using an electronic balance and introduced into the porcelain mortar. A volume of palm kernel oil (excipient) previously heated at 60 ° C was taken with a test piece and added. The whole was mixed with the pestle until complete curing in order to obtain homogeneous extract ointments at the concentrations of 1.25%, 2.5% and 5%. The 5% extract + 5% NaCl ointment was prepared by incorporating 5% NaCl in the preparation of the 5% extract ointment. The test doses were prepared freshly on the day of the experiment.

Bacteria and preparation of bacterial inoculum

Staphylococcus aureus was used as infecting bacterium during the infected excision wound assay. The bacterial inoculum was prepared from an overnight culture by picking numerous colonies and suspending them in sterile saline (NaCl) solution (0.90%). Absorbance was read at 600 nm and adjusted with the saline solution to match that of a 0.50 McFarland standard solution. From the prepared microbial solution, other dilutions with saline solution were prepared to give a final concentration of 10^8 CFU/ml.

Creation and contamination of excision wound

The animals were starved for 12 h prior to wounding. The wound site was prepared following the excision wound model. Dose of ketamine anaesthesia (100 mg/kg body wt, ip) for wounding procedure was selected. The rats were anesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions. The dorsal fur of the animals was shaved with an electric clipper and the area where the wound will be created was outlined on the back of the animals using a marker, then, disinfected with alcohol 95°. On the shaved region of the animal, the excision wound was made by cutting away a circular area of 350 mm^2 and 1- 2 mm depth full thickness of skin from the depilated area along the marking using toothed forceps, scalpel and sharp scissors. Post wounding, the rats were inoculated with 1 ml of 10^8 UFC/ml of *S. aureus* suspension at the site of excision wounds. The wound was left undressed to open environment. To minimize further microbial contamination of wound, each animal was carefully placed individually in disinfected cages kept in a disinfected, clean and dust-free animal house in the Department of Biochemistry, Faculty of Science, University of Dschang. The wounds were not treated for 48 hr post contamination to ensure colonization and establishment of infection. Animals were randomly assigned into eight groups of three animals per group. Group 1: infected and treated topically with 1,25% extract; group 2: infected and treated topically with 2,5% extract; group 3: infected and treated topically with 5% extract; group 4: infected and treated topically with 5% NaCl + 5% extract; group 5: infected and treated topically with Baneocin® 250 UI/5000 UI; group 6: infected and treated topically with palm kernel oil; group 7: infected and untreated control group and group 8: uninfected and untreated control group.

Wound healing assay with the MeOH extract of *P. guajava* leaf

The ointment was topically applied once a day starting from 48 hr post contamination till complete epithelization. This model was used to monitor wound contraction and wound closure time. The progressive changes in wound area were monitored planimetrically by measuring the diameter every alternate day.

The epithelization period was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound (Bhaskar and Nithya, 2012). The epithelization period was recorded at the end of the study. Wound contraction (%) was calculated as percentage reduction in wound area using the following formula (Okoli *et al.,* 2009): Wound contraction (%) = [(WA0-WAt)/WAo] x 100, where: WA0 is the wound area on day zero and WAt, the wound area on day t. The granulation tissue formed on the wound, was excised on the 20^{th} post-operative day and its fresh weight was measured using a precision balance. The granulation tissue was then dried in an oven at 60 °C and its dry weight was weighted as described above.

Estimation of total proteins

0.008 mg of dry granulation tissue was weighed and ground in a porcelain mortar in the presence of 1 ml of the physiological saline solution (0.9% NaCl). The homogenate obtained was centrifuged at 3000 rpm for 15 min and then the supernatant was decanted and used for the determination of total proteins using the Bradford method (Bradford, 1976).

Skin irritation test

The skin irritation test with *P. guajava* extract ointments was conducted on rats using the protocol described by Luepke (1986). Five rats were employed for each ointment and their skin was shaved on the dorsal side, each about 600 mm^2 areas 24 h before application of the sample. The test ointment was applied in a single dose to the skin of each experimental animal. An area of untreated skin served as a control. 500 mg of *P. guajava* extract ointment were applied uniformly to a shaved area of skin. After application of the ointment, the shaved dorsal areas of the animal were covered with an adhesive tape. Reactions related to the application of the tested cream were observed after 1 h of application and then 24 h, 48 h and 72 h after removing the adhesive tape (OECD, 1987). The formation of edema, erythema and pressure sores in the treated skin were observed and the skin reactions evaluated by grades of skin irritation.

Eye irritation test

For this test, 5 rats were used per group. The animals were immobilized and placed individually in a compression box. 100 mg of the ointment to be tested was instilled into the conjunctival sac of one of the animal's eyes after removing the hairs from the eyelids. The untreated eye served as a control. Observations of the ocular irritation were made at 1, 24 and 48 h after the instillation of the ointment (OECD, 2012). Eye lesions were evaluated according to the nature and severity of the lesions and their reversibility or not, and numerically by scores.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Tukey's multiple range tests with SPSS 16.0 for windows. $P < 0.05$ was considered significant and all data was expressed as mean ± standard deviation.

Results and Discussion

Phytochemical analysis of the MeOH extract of *P. guajava* leaf

The phytochemical investigation of the MeOH extract of *P. guajava* leaf showed the presence of alkaloids, polyphenols, flavonoids, anthraquinones, tannins, triterpenes, and saponins while anthocyanins and steroids are absent (Table 1).

Antibacterial activity of the MeOH extract of *P. guajava* leaf under normal conditions

The antibacterial activity of the MeOH extract of *P. guajava* leaf was evaluated through the determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against the bacterial species tested (Table 2).

The results of MIC and MBC determinations showed that the antibacterial activity of the MeOH extract of *P. guajava* leaf varies according to the tested bacteria (MIC = 256-1024 µg/ml; MBC = 1024 - 2048 µg/ml).

The lowest MIC value of 256 µg/ml; indicating the best antibacterial activity, was recorded on *B. subtilis*, *E. coli*, *S. flexneri*, *S. aureus* ATCC 25923, *S. aureus* MSSA01 and *S. aureus* MRSA04 whereas the highest MIC value of 1024 µg/ml; indicating the lowest antibacterial activity, was obtained against *P. aeruginosa*. The MIC and MBC values of the tested plant extract were higher when compared to those of amoxicilline, used as reference antibacterial drug.

Combined effect of the *P. guajava* MeOH extract and amoxicillin

The effect of the association between *P. guajava* MeOH extract and amoxicillin has been studied and the results are presented in Tables 3-5. The MIC values of the *P. guajava* MeOH extract in combination with amoxicillin at ½ and 1/8 MICs are smaller than that of the plant extract used alone against *P. aeruginosa*, *S. flexneri*, *P. aeruginosa* PA01, *S. aureus* MSSA01 and *S. aureus* MRSA03; suggesting an increase in the activity of this extract in combination with amoxicillin (Table 3). The other MIC values of *P. guajava* MeOH extract in combination with amoxicillin are equal to those of the extract used alone (Table 3).

The MIC values of amoxicillin in combination with *P. guajava* MeOH extract at ½ and 1/8 MICs are smaller than those of amoxicillin alone (Table 4). This result indicates an increase in the activity of amoxicillin in combination with the *P. guajava* MeOH extract at 1/8 and ½ of its MICs.

The *P. guajava* MeOH extract and amoxicillin exhibited in association indifference effects against *B. subtilis*, *E. coli*, *S. aureus* ATCC 25923 and *S. aureus* MRSA04; antagonism effects against *S. flexneri* SDINT; additive effects against *P. aeruginosa* PAO1 and *S. aureus* MSSA01 as well as synergistic effects on *P. aeruginosa* and *S. aureus* MRSA03 (Table 5).

Antibacterial activity of the *P. guajava* MeOH extract under osmotic stress condition

The MIC values of the extract obtained under osmotic stress condition (in the presence of

5% NaCl) are generally smaller than those obtained under normal conditions (0% NaCl); suggesting an increase in the activities of the extract under osmotic stress condition (Table 6). With the exception of *P. aeruginosa*, the MIC values of chloramphenicol determined under osmotic stress conditions are smaller than those determined under normal conditions. However, under osmotic stress condition, the MIC values of amoxicillin against *P. aeruginosa* PA01, *S. aureus* MSSA01, *S. aureus* MRSA04, *S. aureus* and *S. flexneri* are higher than those determined under normal conditions. Interestingly, the antibacterial activity of *P. guajava* extract against *S. aureus* ATCC25923 (MIC = 16 µg/ml) and *P. aeruginosa* PA01 (MIC = 16 µg/ml) under osmotic stress conditions, was higher than that of amoxicillin (MIC = 32 and 256 µg/ml) on the corresponding microorganisms (Table 6).

Wound healing effect of the *P. guajava* MeOH extract in excision wound model

The therapeutic effect of the *P. guajava* extract was evaluated on a *S. aureus*-infected wound in rats. The topical application of *P. guajava* extract ointment on infected excision wounds resulted in a concentration-dependent increase in the percentages of wound contraction (Table 7). Moreover, the percentages of wound contraction increased with the duration of treatment whatever the tested ointment. The highest percentages of wound contraction were obtained with extract ointments (G2, G3 and G4), Baneocin ointment (G5) and palm kernel oil (G6) when compared to group treated with extract ointment at 1.25% as well as infected and untreated group (G7) and uninfected and untreated group (G8). After 20 days of treatment, only the groups treated with extract ointments (G2, G3 and G4), palm kernel oil (G6) and Baneocin ointment (G5) recorded 100% of wound contraction rate.

The *P. guajava* extract and Baneocin ointments (G1, G2, G3, G4 and G5) exhibited the shortest epithelization times compared to the controls (G6, G7 and G8) (Table 8). The ointment containing 5% extract displayed the shortest epithelization time compared to the other extract ointments. However, no apparent difference in the period of epithelization was found between extract ointments and Baneocin as well as between different concentrations of the extract. The highest fresh and dry granulation tissue weights as well as the greatest total protein contents of granulation tissues were obtained with extract ointments, Baneocin and palm kernel oil (G1, G2, G3, G4 and G5) as compared to the untreated control groups (G7 and G8) (Table 9). In addition, the extract ointments resulted in a concentration-dependent increase in the weights and total protein contents of granulation tissues.

Toxicological effect of *P. guajava* extract on the skin and eye

The effect of *P. guajava* extract on the skin and eye was assessed through skin and eye irritation tests in rats. Topical application of extract ointments revealed no irritation (no edema, erythema and eschar) on healthy skin after 72 h post-application. Similarly, the application of extract ointments to the eyeball followed by clinical examinations of the conjunctiva (for the presence of chemosis, lacrimation and enanthema), iris (by evaluation of the direct photomotor reflex of the pupil and the degree of congestion) and cornea (by evaluation of the degree of opacity, the area of attack, ulceration and granulation) revealed no ocular irritation effect of the extract ointments after 48 h post application. The phytochemical analysis of the MeOH extract of *P. guajava* leaf was carried out with the aim of highlighting the different classes of secondary metabolites that can explain its wound healing and antibacterial properties.

Table.1 Distribution of the main classes of secondary metabolites in the MeOH extract of *P. guajava* leaf

Secondary metabolites	MeOH extract of *P. guajava* leaf
Alkaloids	+
Polyphenols	+
Flavonoids	+
Anthraquinones	+
Anthocyanines	-
Tannins	+
Triterpens	+
Steroids	-
Saponins	+

(+): Présent; (-): Absent

Table.2 Antibacterial activity (MIC and MBC in µg/mL) of the *P. guajava* MeOH extract and amoxicillin

Bacteria	Inhibition parameters	*P. guajava* MeOH extract	Amoxicillin
E. coli	MIC	256	64
	MBC	>2048	64
	MBC/MIC	/	1
P. aeruginosa	MIC	1024	128
	MBC	>2048	256
	MBC/MIC	/	2
S. flexneri	MIC	256	1
	MBC	>2048	4
	MBC/MIC	/	4
***P. aeruginosa* PA01**	MIC	512	2
	MBC	>2048	2
	MBC/MIC	/	1
B. subtilis	MIC	256	32
	MBC	2048	32
	MBC/MIC	8	1
***S. aureus* ATCC 25923**	MIC	256	1
	MBC	>2048	1
	MBC/MIC	/	1
***S. aureus* MSSA01**	MIC	256	4
	MBC	1024	4
	MBC/MIC	4	1
***S. aureus* MRSA03**	MIC	512	16
	MBC	1024	16
	MBC/MIC	2	1
***S. aureus* MRSA04**	MIC	256	16
	MBC	2048	16
	MBC/MIC	8	1

/: not determined; MIC: Minimum Inhibitory Concentration; MBC Minimum Bactericidal Concentration.

Table.3 Antibacterial activity of the combination between *P. guajava* MeOH extract and amoxicillin at ½ and 1/8 MIC as a function of bacteria

Bacteria	MeOH extract alone	MeOH extract of *P. guajava* with of amoxicillin at 1/8 MIC		MeOH extract of *P. guajava* with amoxicillin at ½ MIC	
	MIC	MIC	FIC	MIC	FIC
E. coli	256	256	1	256	1
P. aeruginosa	1024	256	0.25	128	0.125
S. flexneri	256	256	1	128	2
P. aeruginosa PA01	512	256	0.5	256	0.5
B. subtilis	256	256	1	256	1
S. aureus ATCC 25923	256	256	1	256	1
S. aureus MSSA01	256	256	1	128	0.5
S. aureus MSSA03	512	256	0.5	128	0.25
S. aureus MSSA04	256	256	1	256	1

MIC: minimum inhibitory concentration in µg / ml; FIC: Fractional Inhibitory Concentration index.

Table.4 Antibacterial activity of amoxicillin in combination with the *P. guajava* MeOH extract at 1/8 and ½ MICs

Bacteria	Amoxicillin alone	Amoxicillin with *P. guajava* MeOH extract at 1/8 MICs		Amoxicillin with *P. guajava* MeOH extract at ½ MICs	
	MIC	MIC	FIC	MIC	FIC
E. coli	64	16	0.25	8	0.25
P. aeruginosa	128	64	0.5	32	0.25
S. flexneri	1	0.25	0.25	0.125	0.125
P. aeruginosa PA01	2	0.25	0.125	0.125	0.0625
B. subtilis	32	16	0.5	8	0.25
S. aureus ATCC 25923	1	0.25	0.25	0.125	0.125
S. aureus MSSA01	4	0.25	0.125	0.125	0.0312
S. aureus MRSA03	16	2	0.125	0.5	0.0312
S. aureus MRSA04	16	2	0.125	0.5	0.0312

MIC: minimum inhibitory concentration in µg / ml; FIC: Fractional Inhibitory Concentration index.

Table.5 Fractional inhibitory concentration (FIC) indices calculated for the combination of amoxicillin and *P. guajava* MeOH extract as a function of studied bacteria

Bacteria	∑ FIC	Interpretation
E. coli	1.25	Indifference
P. aeruginosa	0.375	Synergy
S. flexneri	2.125	Antagonism
***P. aeruginosa* PAO1**	0.5625	Additive
B. subtilis	1.25	Indifference
***S. aureus* ATCC 25923**	1.125	Indifference
***S. aureus* MSSA01**	0.5312	Additive
***S. aureus* MRSA03**	0.2812	Synergy
***S. aureus* MRSA04**	1.312	Indifference

FIC: Fractional Inhibitory Concentration index.

Table.6 Effect of the osmotic stress on the antibacterial activity of the *P. guajava* MeOH extract and reference antibacterial drugs (MIC in µg/ml)

Bacteria	MeOH extract of *P. guajava*		Chloramphenicol		Amoxicillin	
	0% NaCl	5% NaCl	0% NaCl	5% NaCl	0%NaCl	5% NaCl
E. coli	256	512	4	4	64	16
P. aeruginosa	1024	1024	64	64	128	64
S. flexneri	256	256	64	1	1	256
***P. aeruginosa* PA01**	512	16	8	2	2	256
B. subtilis	256	16	16	8	32	2
***S. aureus* ATCC25923**	256	16	32	1	1	32
***S. aureus* MSSA01**	256	1024	32	8	4	256
***S. aureus* MRSA03**	512	1024	64	16	16	4
***S. aureus* MRSA04**	256	512	64	16	16	128

Table.7 Effect of *P. guajava* extract ointments on the percentages of wound contraction on rat excision wound infected with *S. aureus* as a function of the duration of treatment

Treatment	Percentage of wound contraction at days post-treatement				
	Day 4	Day 8	Day 12	Day 16	Day 20
G_1	7.06 ± 0.14^a	55.44 ± 0.26^a	74.17 ± 0.31^a	85.13 ± 0.22^a	93.80 ± 0.18^a
G_2	6.12 ± 0.26^b	50.06 ± 0.34^b	66.37 ± 0.20^b	89.69 ± 0.33^b	100 ± 0.00^b
G_3	7.28 ± 0.06^c	54.85 ± 0.22^a	68.93 ± 0.19^c	100 ± 0.00^c	100 ± 0.00^b
G_4	12.34 ± 0.28^d	55.31 ± 0.42^a	66.81 ± 0.30^b	90.07 ± 0.33^b	100 ± 0.00^b
G_5	6.94 ± 0.15^a	45.37 ± 0.16^c	58.33 ± 0.29^d	86.88 ± 0.40^d	100 ± 0.00^b
G_6	8.45 ± 0.17^e	50.23 ± 0.17^b	74.06 ± 0.18^a	85.91 ± 0.26^e	100 ± 0.00^b
G_7	11.24 ± 0.16^f	42.05 ± 0.26^d	62.16 ± 0.14^e	75.05 ± 0.10^f	92.06 ± 0.24^c
G_8	6.51 ± 0.023^g	40.46 ± 0.40^e	59.12 ± 0.38^f	88.37 ± 0.35^g	92.55 ± 0.24^c

The data represent the mean ± Standard deviation; on the same column, the values affected different superscript letters (a-g) are significantly different at $P < 0.05$; Group 1: infected and treated topically with 1,25% extract; group 2: infected and treated topically with 2,5% extract; group 3: infected and treated topically with 5% extract; group 4: infected and treated topically with 5% NaCl + 5% extract; group 5: infected and treated topically with Baneocin® 250 UI/5000 UI; group 6: infected and treated topically with palm kernel oil; group 7: infected and untreated control group and group 8: uninfected and untreated control group.

Table.8 Effect of the *P. guajava* extract ointments on wound epithelization time

Groups	Epithelization time (in day)
G_1	18.66 ± 3.05^{ac}
G_2	15.00 ± 3.00^{ab}
G_3	13.00 ± 1.73^{b}
G_4	15.33 ± 2.30ab
G_5	16.66 ± 3.05^{abc}
G_6	18.00 ± 2.00^{ac}
G_7	21.33 ± 2.30^{c}
G_8	19.33 ± 4.16^{ac}

The data represent the mean ± Standard deviation; on the same column, the values affected by different superscript letters (a-c) are significantly different at $p < 0.05$; G1: wound infected and treated with 1.25% ointment; G2: wound infected and treated with 2.5% ointment; G3: infected wound and treated with 5% ointment; G4: infected wound and treated with 5% ointment % + 5% NaCl; G5: wound infected and treated with Baneocin; G6: wound infected and treated with palm kernel oil; G7: infected and untreated wound; G8: uninfected and untreated wound.

Table.9 Effect of *P. guajava* extract ointments on the weights (mg) and total protein contents (µg / ml) of granulation tissues

Groups	Fresh granulation tissue weight (mg)	Dry granulation tissue weight (mg)	Total protein content (µg/mL.g de tissu)
G_1	0.015	0.0065	735.28 ± 8.27^{a}
G_2	0.017	0.0076	759.37 ± 17.21^{a}
G_3	0.027	0.0095	988.96 ± 8.04^{b}
G_4	0.023	0.0087	855.52 ± 10.44^{c}
G_5	0.018	0.0079	791.82 ± 15.24^{d}
G_6	0.013	0.0066	673.31 ± 9.49^{e}
G_7	0.0059	0.0042	374.02 ± 11.32^{f}
G_8	0.0016	0.0016	421.39 ± 13.43^{g}

The data represent the mean ± Standard deviation; on the same column, the values affected by different superscript letters (a-g) are significantly different at $p < 0.05$; G1: wound infected and treated with 1.25% ointment; G2: wound infected and treated with 2.5% ointment; G3: infected wound and treated with 5% ointment; G4: infected wound and treated with 5% ointment % + 5% NaCl; G5: wound infected and treated with Baneocin; G6: wound infected and treated with palm kernel oil; G7: infected and untreated wound; G8: uninfected and untreated wound.

Hence, the results on the phytochemical study of the methanol extract of *P. guajava* revealed the presence of alkaloids, polyphenols, flavonoids, triterpenes, tannins, anthraquinones and saponins. These results partially corroborate those of Biswas *et al.,* (2013) which showed the presence of phenols, tannins, saponins, terpenoids, flavonoids and glycosides in the methanol leaf extract of this plant.

The antibacterial activity of *P. guajava* extract may be due to the different groups of secondary metabolites found present in this extract. Indeed, the antimicrobial activity of medicinal plants is correlated with the presence in their extracts of one or more classes of bioactive secondary metabolites (Reuben *et al.*, 2008). According to Tamokou *et al.,* (2017), a plant extract is considered to be highly active if the MIC < 100 µg/ml;

significantly active when 100 ≤ MIC ≤ 512 µg/ml; moderately active when 512 <MIC ≤ 2048 µg/ml; weakly active if MIC> 2048 µg/ml and not active when MIC> 10 mg/ml. Thus, *P. guajava* extract has moderate activities against *P. aeruginosa* and significant activities against B. *subtilis*, *E. coli* S2 (1), S. *flexneri, S. aureus, P. aeruginosa* PA01, *S. aureus* MSSA01, *S aureus* MRSA03 and *S. aureus* MRSA04. The results on the antibacterial activities of *P. guajava* are comparable to those of the literature (Chah *et al.,* 2006).

We have noted during the MIC and MBC determinations that most of the extracts had MBC values > 2048 µg/ml and four fold greater than their corresponding MICs; indicating that these extracts generally have a bacteriostatic effect (Mims *et al.,* 1993).

Combinations of antibiotics can lead to synergistic effects especially during the therapy of bacterial infections. These combinations have been recognized as being able to delay the emergence of resistant strains of microorganisms (Aiyegoro and Okoh, 2009). The effect of synergy between plant-derived compounds and antibiotics makes it possible to use antibiotics when their efficacy alone is reduced (Nascimento *et al.*, 2000). These observations could explain the evaluation of the antibacterial activity of the combination of amoxicillin and methanol extract of *P. guajava* leaves. Indeed, in addition to substances having direct antibacterial activity, it has been demonstrated that within plants, other substances can act as adjuvants by modulating the activity of antibacterial agents (Veras *et al.,* 2012). The polyphenols and flavonoids detected in this plant extract would be responsible for the observed potentiating activity with respect to certain tested bacteria. Several studies have shown that polyphenols and flavonoids could improve antibiotic activity against resistant bacterial strains (Cushnie and Lamb, 2005).

The antibacterial activity of MeOH extract of *P. guajava* and chloramphenicol increased under osmotic stress conditions (5% NaCl) while those of amoxicillin decreased under these conditions. This result is an original contribution to the formulation of disinfectants, antiseptics and wound medicine. Previous studies have shown that certain bacteria (*E. coli, S. aureus, P. aeruginosa*) can survive and develop under osmotic stress conditions (Besten *et al.*, 2009). Hence, the presence of the salt in the medium is liable to cause changes in the lipid composition of the membrane (Beales, 2004); making it more permeable to the plant extract and chloramphenicol. This may explain the increased antibacterial activity of these samples. However, the mechanisms that make bacteria more sensitive to certain antibiotics / extracts under osmotic stress conditions are still unknown. The results on amoxicillin activity are similar to those of McMahon *et al.,* (2007) who demonstrated a decrease in the activity of amikacin, ceftriaxone and trimethoprim against *E. coli* and *S. aureus* under osmotic stress conditions. Plant extracts contain a multitude of compounds that can act individually or interact on several targets (Lopez-Malo *et al.*, 2005). This could make it difficult to develop mechanisms of resistance by bacteria to the tested extract.

The results of the present study have revealed that ointment formulated with methanol extract of *P. guajava* leaves showed a significant increase in the percentage of wound closure at the infected wound site and have a significant antibacterial activity against *S. aureus*. Indeed, during the proliferative phase of wound healing, the wound contraction improves the breccia by pulling the edges of the wound towards the center (Paschapur *et al.,* 2009). The effect of *P.*

guajava extract on wound contraction indicates that the extract has a healing action because wound contraction accounts for 88% of the healing process (Ejaz *et al.,* 2009). The findings of the present study also showed that the extract ointments, Baneocin ointment and palm kernel oil recorded the greatest total protein contents of granulation tissues as compared to the untreated control groups. Indeed, tissue proteins such as collagen contribute to the strengthening and support of cellular tissue and are used as biochemical markers, indicative of a better curative quality of drugs in the wounds (Tang *et al.*, 2007; Paschapur *et al.,* 2009).

In this study, we found that epithelization time was significantly shorter in animals treated with extract ointment compared to negative control groups. Indeed, epithelization involves the proliferation and migration of epithelial cells through the wound bed (Sanwal and Chaudhary, 2011). Therefore, a shorter epithelization time could be due to facilitated epithelial cell proliferation and / or increased viability of epithelial cells (Mulisa *et al.,* 2015). Thus, the shorter epithelization periods in the animals treated with the extract reinforce the hypothesis according to which the extract of *P. guajava* has a potential application as a healing agent.

The flavonoids and tannins found in *P. guajava* extract have been shown to be important for wound healing due to their antioxidant, anti-inflammatory and antibacterial activities (Mulisa *et al.,* 2015). Many previous studies have shown that antimicrobial activity correlates with wound healing. Indeed, infection of a wound can seriously delay the healing process by causing the formation of poor quality granulation tissue, causing reduction of the tensile strength of the connective tissue as well as loss of epithelization and the appearance of odor (OECD, 1987; Annan and Houghton, 2008). Therefore, a high rate of wound contraction and a decrease in the epithelization period in the animals treated with the extract in the excisional injury model are also attributed to the antibacterial properties of *P. guajava*.

Toxicological tests have shown that *P. guajava* extract ointment is non-irritating to the skin (primary irritation index of the ointment, PII = 0) and slightly irritating to the eyes (average eye irritation index, AEII = 0) (OECD, 2012).

The overall results of the present study demonstrate the wound healing and antibacterial activities of *P. guajava* and confirm its traditional use in the treatment of wounds and infectious diseases.

Author contribution statement

SEE did the biological assays and helped in manuscript writing and editing. JDT designated the study, supervised the assays and revised the manuscript critically for important intellectual content. All authors read and agreed on the final version of the manuscript.

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