Original Research Article

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

Steve Endeguele Ekom and Jean-De-Dieu Tamokou*

Unit of Microbiology and Antimicrobial Substances, Department of Biochemistry, Faculty of Science, University of Dschang, P.O. Box 67 Dschang, Cameroon

*Corresponding author

**Abstract**

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.

**Keywords**

*Psidium guajava*, Methanol extract, Antibacterial, Wound healing, Synergy, Osmotic stress, Toxicity

**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 °C 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)/WA0] 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 20th 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 mm² 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 (G1, G2, G3, G4 and G5) as compared to the untreated control groups (G6, 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               | +                                |
| Triterpenes           | +                                |
| 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               | MBC >2048                  | 64          |
|                           | MBC/MIC /             | 1                          |             |
| *P. aeruginosa* PA01      | MIC 512               | MBC >2048                  | 2           |
|                           | MBC/MIC /             | 2                          |             |
| *B. subtilis*             | MIC 256               | MBC 2048                   | 32          |
|                           | MBC/MIC 8             | 1                          |             |
| *S. aureus ATCC 25923*    | MIC 256               | MBC >2048                  | 1           |
|                           | MBC/MIC /             | 1                          |             |
| *S. aureus MSSA01*        | MIC 256               | MBC 1024                   | 4           |
|                           | MBC/MIC 1             | 4                          |             |
| *S. aureus MRSA03*        | MIC 512               | MBC 1024                   | 16          |
|                           | MBC/MIC 2             | 1                          |             |
| *S. aureus MRSA04*        | MIC 256               | 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 amoxicillin at 1/8 MIC | MeOH extract of *P. guajava* with amoxicillin at ½ MIC |
|------------------------|--------------------|----------------------------------------------------------|----------------------------------------------------------|
|                        | MIC | FIC | MIC | FIC |
| *E. coli*              | 256 | 1   | 256 | 1   |
| *P. aeruginosa*        | 1024 | 0.25 | 128 | 0.125 |
| *S. flexneri*          | 256 | 1   | 128 | 2   |
| *P. aeruginosa PA01*   | 512 | 0.5 | 256 | 0.5 |
| *B. subtilis*          | 256 | 1   | 256 | 1   |
| *S. aureus ATCC 25923* | 256 | 1   | 256 | 1   |
| *S. aureus MSSA01*     | 256 | 1   | 128 | 0.5 |
| *S. aureus MSSA03*     | 512 | 0.5 | 128 | 0.25 |
| *S. aureus MSSA04*     | 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 | FIC | MIC | FIC |
| *E. coli*              | 64  | 0.25 | 8   | 0.25 |
| *P. aeruginosa*        | 128 | 0.5  | 32  | 0.25 |
| *S. flexneri*          | 1   | 0.25 | 0.125 | 0.125 |
| *P. aeruginosa PA01*   | 2   | 0.25 | 0.125 | 0.0625 |
| *B. subtilis*          | 32  | 0.5  | 8   | 0.25 |
| *S. aureus ATCC 25923* | 1   | 0.25 | 0.125 | 0.125 |
| *S. aureus MSSA01*     | 4   | 0.25 | 0.125 | 0.0312 |
| *S. aureus MRSA03*     | 16  | 0.125 | 0.5 | 0.0312 |
| *S. aureus MRSA04*     | 16  | 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* PAO1      | 512     | 16      | 8       | 2       | 2       | 256     |
| *B. subtilis*             | 256     | 16      | 16      | 8       | 32      | 2       |
| *S. aureus ATCC 25923*    | 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-treatment |
|-----------|------------------------------------------------------|
|           | Day 4 | Day 8 | Day 12 | Day 16 | Day 20 |
| *G*1      |       |       |        |        |        |
| *G*2      |       |       |        |        |        |
| *G*3      |       |       |        |        |        |
| *G*4      |       |       |        |        |        |
| *G*5      |       |       |        |        |        |
| *G*6      |       |       |        |        |        |
| *G*7      |       |       |        |        |        |

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) |
|--------|-----------------------------|
| G₁     | 18.66 ± 3.05<sup>ac</sup>   |
| G₂     | 15.00 ± 3.00<sup>ab</sup>   |
| G₃     | 13.00 ± 1.73<sup>b</sup>    |
| G₄     | 15.33 ± 2.30<sup>ab</sup>   |
| G₅     | 16.66 ± 3.05<sup>abc</sup>  |
| G₆     | 18.00 ± 2.00<sup>ac</sup>   |
| G₇     | 21.33 ± 2.30<sup>c</sup>    |
| G₈     | 19.33 ± 4.16<sup>ac</sup>   |

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) |
|--------|------------------------------------|----------------------------------|-----------------------------------------|
| G₁     | 0.015                              | 0.0065                           | 735.28 ± 8.27<sup>a</sup>               |
| G₂     | 0.017                              | 0.0076                           | 759.37 ± 17.21<sup>a</sup>              |
| G₃     | 0.027                              | 0.0095                           | 988.96 ± 8.04<sup>b</sup>               |
| G₄     | 0.023                              | 0.0087                           | 855.52 ± 10.44<sup>c</sup>              |
| G₅     | 0.018                              | 0.0079                           | 791.82 ± 15.24<sup>d</sup>              |
| G₆     | 0.013                              | 0.0066                           | 673.31 ± 9.49<sup>e</sup>               |
| G₇     | 0.0059                             | 0.0042                           | 374.02 ± 11.32<sup>f</sup>              |
| G₈     | 0.0016                             | 0.0016                           | 421.39 ± 13.43<sup>g</sup>              |

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 $100 \leq \text{MIC} \leq 512$ μg/ml; moderately active when $512 < \text{MIC} \leq 2048$ 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 (Verras 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.

**Acknowledgements**

The study was supported in part by the University of Dschang and the Cameroonian Ministry of Higher Education.

**References**

Aiyegoro, O. A. and Okoh, A. I., 2009, Use of bioactive plant products in combination with standard antibiotics: implications in antimicrobial chemotherapy. _Journal of Medicinal Plants Research_, 3: 1147-1152.

Annan, K. and Houghton, P. J., 2008, Antimicrobial, antioxidant and fibroblast growth stimulation of aqueous extracts of _Ficus asperifolia_ Miq. and _Gossypium_
arboretum L., wound healing plants of Ghana. Journal of Ethnopharmacology, 119:141–144.
Beales, N., 2004, Adaptation of microorganisms to cold temperatures, weak acid reservaties, low pH, and osmotic stress: A review. Comprehensive Reviews in Food Science and Food Safety, 3(1): 1-20.
Besten, H. M. W., Mols, M., Moezelaar, R., Zwietering, M. H. and Abee, T., 2009, Phenotypic and transcriptomic analyses of mildly and severely salt-stressed Bacillus cereus ATCC 14579 cells. Applied Environment Microbiology, 75: 111-119.
Bhaskar, A. and Nithya, V., 2012, Evaluation of the wound healing activity of Hibiscus rosasinensis L (Malvaceae) in Wistar albino rats. Indian Journal of Pharmacology, 44(6): 694–698.
Biswas, B., Rogers, K., McLaughlin, F., Daniels D. and Yadav, A. 2013, Antimicrobial Activities of leaf extracts of guava (Psidium guajava L.) on two Gram-negative and Gram-positive bacteria. International Journal of Microbiology, Article ID 746165, pp. 1-7, 2013.
Bone, R. C., 1994, Gram-positive organisms and sepsis. Archives of Internal Medicine, 154: 26-34.
Bradford, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248 – 256.
Blander, G. C. and Pugh, D. M., 1991, Veterinary Applied Pharmacology and Therapeutics, Bailliere Tindall, London, pp. 424-427.
Burkill, H. M., 1997, The Useful Plants of West Tropical Africa, 2nd edition.
Chah, K. F., Eze, C. A., Emuelosi, C. E. and Esimone, C. O., 2006, Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. Journal of Ethnopharmacology, 104(1–2): 164–167.
CLSI, 1997, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standards, M7-A4, Clinical and Laboratory Standards Institute, Wayne, PA.
CLSI, 1999, Methods for determining bactericidal activity of antimicrobial agents. Approved guideline, M26-A, Clinical and Laboratory Standards Institute, Wayne, Pa.
Cushnie, T. P. and Lamb, J. A., 2005, Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents, 26: 343–356.
Ejaz, S., Chekarova, I., Cho, J. W., Lee, S. Y., Ashraf, S. and Lim, C. W., 2009, Effect of aged garlic extract on wound healing: A new frontier in wound management. Drug and Chemical Toxicology, 32(3): 191–203.
Esimone, C., Nworu, C. and Jackson, C., 2008, Cutaneous wound healing activity of a herbal ointment containing the leaf extract of Jatropha curcas L. (Euphorbiaceae). International Journal of Applied Research in Natural Products, 1: 1–4.
Fortin, D., Lo, M. and Maynart, G., 1990, Plantes médicinales du Sahel CECI / ENDA.
Gyang, E. O., 1986, Introduction to Animal Surgery, Agitab Pub. Nigeria.
Hidetoshi, A. and Gen-ichi, D., 2002, Isolation of Antimicrobial Compounds from Guava (Psidium guajava L.) and their Structural Elucidation. Bioscience, Biotechnology, and Biochemistry, 66(8): 1727-1730.
Huebner, R. R. E., Wasas, A., Mushli, A., Mazhani, L. and Klugman, K., 1998, Nasopharyngeal carriage and antimicrobial resistance in isolates of Streptococcus pneumoniae and Hemophilus influenzae Type b in children under 5 years of age in Bostswana. International Journal of Infectious Diseases, 3(1): 18-25.
Karl, M., Lacrix, J. V. and Preston, H. H., 1995, Canine surgery, 4th edition, American
Veterinary Publications, California. Pp 42-45.
Lopez-Malo, V. A., Palou, E. and Alzamora, S. M., 2005, Naturally occurring compounds-plant sources. In: Antimicrobials in Food. 3rd edition. Davidson, P.M.; Sofos, J.N.; and Branen, A.L. (eds.) CRC Press, Taylor & Francis Group, Boca Raton, FL, USA, Pp. 429-451.
Luepke, N. P., 1986, The Hen’s egg test (HET): An alternative toxicity test. British Journal of Dermatology, 115(3): 133-135.
McMahon, M. A. S., Xu, J., Moore, J. E., Blair, I. S. and McDowell, D. A., 2007, Environmental stress and antibiotic resistance in food-related pathogens. Applied and Environmental Microbiology, 73: 211–217.
Mims, C. A., Playfair, J. H. L., Roitt, I. M., Wakelin, D. and Williams, R., 1993, Antimicrobials and chemotherapy. In: Mims, C.A., et al., (Eds.). Medical Microbiology Review, 35: 1–34.
Morales, M. A., Tortoriello, J., Meckes, M., Paz, D. and Lozoya, X., 1999, Calcium antagonist effect of quercetin and its relation with the spasmolytic properties of Psidium guajava L. Pharmacological Research, 39(3): 239-245.
Morton, J. F., 1987, Guava (Psidium guajava L.). In: Fruits of warm climates, Miami, FL, 356-363.
Mulisa, E., Asres, K. and Engidawork, E., 2015. Evaluation of wound healing and anti-inflammatory activity of the rhizomes of Rumex abyssinicus J. (Polygonaceae) in mice. BMC Complementary and Alternative Medicine, 15:341
Nadkarni, K. M. and Nadkarni, A. K., 1999, Indian Materia Medicawith Ayurvedic, Unani-Tibbi, Siddha, Allopathic, Homeopathic, Naturopathic and Home Remedies, Popular Prakashan Private Limited.
Nascimento, G. G. F., Locatelli, J., Freitas, P. C. and Silva, G. L., 2000, Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Brazilian Journal of Microbiology, 31: 247-256.
Ncube, N. S., Afolayan, A. J. and Okoh, A. I., 2008, Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology, 7(12): 1797–1806.
OECD, 1987, Acute dermal toxicity. Guidelines for testing of chemicals, February, 402, pp. 1–7.
OECD, 2012, Test No 405. Acute Eye Irritation/Corrosion, OECD guideline for the testing of chemicals: OECD Publishing. Adopted 2nd October 2012.
Okoli, C. O., Ezike, A. C., Akah, P. A., Udegbunam, S. O., Okoye, T. C., Mbanu, T. P. and Ugwu, E., 2009, Studies on wound healing and antiulcer activities of extract of aerial parts of Phyllanthus niruri L. (Euphorbiaceae). American Journal of Pharmacology and Toxicology, 4(4): 118–126.
Paschapur, M. S., Patil, M. B., Kumar, R. and Patil, S. R., 2009, Evaluation of aqueous extract of leaves of Ocimum kilimandscharicum on wound healing activity in albino Wistar rats. International Journal of Pharm Tech Research, 1(3): 544-550.
Rai, P. K., Singh, S. K., Kesari, A. N. and Watral, G. 2007, Glycaemic evaluation of Psidium guajava in rats. Indian Journal of Medical Research, vol. 126, pp. 224-227, 2007.
Reuben, K. D., Abdulrahman, F. I., Akan, J. C., Usman, H., Sodipo, O. A. and Egwu, G. O., 2008, Phytochemical screening and in vitro antimicrobial investigation of the methanolic extract of Croton Zambesicus Muell ARG. stem bark. European Journal of Scientific Research, 23(1): 134-140.
Sanwal, R. and Chaudhary, A. K., 2011, Wound healing and antimicrobial potential of Carissa spinarum Linn. in albino mice.
Journal of Ethnopharmacology, 135: 792–796.
Sharma, Y., Jeyabalan, G. and Ramandeep, S., 2013, Potential Wound Healing Agent from Medical Plants: A Review. Pharmacologia, 4(5): 349-358.
Tamokou, J. D., Mbaveng, T. A. and Kuete, V., 2017, Antimicrobial activities of African medicinal spices and vegetables. In: "Medicinal Spices and Vegetables from Africa: Therapeutic Potential against Metabolic, Inflammatory, Infectious and Systemic Diseases", 1st Edition, Elsevier, ISBN: 9780128092866, Chapter 8, pp. 207-237.
Tang, T., Yin, L., Yang, J. and Shan, G., 2007, Emodin, an anthraquinone derivative from Rheum officinale Baill, enhances cutaneous wound healing in rats. European Journal of Pharmacology, 567: 177–185.
Trease, G. E. and Evans, W. C., 1989, A textbook of pharmacognosy. 13ème édition, Baillière Tindall Ltd., Londres.
Veras, H. N. H., Rodrigues, F. F. G., Colares, A. V., Menezes, I. R. A., Coutinho, H. D. M., Botelho, M. A. and Costa, J. G. M., 2012, Synergistic antibiotic activity of volatile compounds from the essential oil of Lippia sidoides and thymol. Fitoterapia, 83: 508-512.
Vieira, R. H. S. F., Rodrigues, D. P., Gonçalves, F. A., Menezes, F. G., Aragão, J. S. and Sousa, O. V., 2001, Microbicidal effect of medicinal plant extracts (Psidium guajava Linn. and Carica papaya Linn.) upon bacteria isolated from fish muscle and known to induce diarrhea in children. The Revista do Instituto de Medicina Tropical de São Paulo, 43(3): 145-148.

How to cite this article:
Steve Endeguele Ekom and Jean-De-Dieu Tamokou. 2018. Methanol Leaves Extract of Psidium guajava Linn. Exhibited Antibacterial and Wound Healing Activities. Int.J.Curr.Microbiol.App.Sci. 7(07): 4008-4023. doi: https://doi.org/10.20546/ijcemas.2018.707.467