Original Research Article

In vitro Antimicrobial Activities of Methanol Crude Leaf Extract of *Psidium guajava L* on Clinical Isolates of Multidrug Resistant *Staphylococcus aureus*

ABSTRACT

This study examines the chemical composition and in vitro antimicrobial potential of methanol crude leaf extract of Psidium guajava Linn against multidrug-resistant (MDR) clinical isolates of *Staphylococcus aureus* strains collected from FMC Umuahia, Abia State. The strains that exhibited resistance against all the antibiotics tested were selected for antibacterial assays. The minimum inhibitory concentration (MIC) for methanol and aqueous extracts was found to be 625 ug/ml and 7.5 mg/ml, respectively. The minimum bactericidal concentration (MBC) recorded for methanol and aqueous extracts was 1.25 and 12.5 mg/ml, respectively. Methanol extract at the minimum bactericidal concentration inhibited the growth of the MDR strain by 85%. A time-kill assay revealed mat methanol extract (5 mg/ml) killed MDR bacteria within 10 hours. Total polypeptide profiling of bacterial cultures by SDS-PAGE indicated a high degree of protein degradative activity in the extract. Finally, a human RBC-based hemolytic assay showed the absence of hemolysis even at concentrations higher than those of MBC, advocating thereby its safety in therapeutic use.

Keywords: Bactericidal, Haemolysis, MDR Strains, *Psidium guajava*, *Staphylococcus aureus*

1.0 Introduction

Multidrug-resistant bacteria (MDRB) are microorganisms that are resistant to one or more antimicrobial agents. They are usually resistant to all but one or two commercially available antimicrobial agents. This definition includes microbes that have acquired resistance to at least one agent in three or more antimicrobial categories. The MDRB of clinical interest include: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* with resistance to vancomycin [these are Vancomycin-intermediate *Staphylococcus aureus* (VISA) and Vancomycin-resistant *Staphylococcus aureus* (VRSA)], Vancomycin-resistant enterococci (VRE), Extended spectrum beta-lactamases (ESBLs) producing gramme-negative bacilli, Multidrug-resistant *Streptococcus pneumoniae* (MDRSP), Carbapenem-resistant Enterobacteriaceae (CRE), and Multidrug-resistant *Acinetobacter baumannii* [1]–[3]

Infectious diseases caused by MDRB are an important burden globally. They have for centuries been among the leading causes of death, disability, and growing challenges to health security and

human progress, especially in developing countries [4]. Although many new antibacterial drugs have been produced, bacteria exhibiting resistance to them have increased and are becoming a global concern as we are fast running out of therapeutic options [5]. The challenges of antimicrobial resistance are faced in both health care and community settings, necessitating a broad approach with multiple partners across the continuum of care. For example, 18–33% of MRSA-colonised patients subsequently developed MRSA infections.

Microbial resistance to the available antibiotics has led scientists to investigate the antibacterial activity of medicinal plants [6]. *Psidium guajava L.* (guava) is an evergreen shrub native to tropical America that has naturalised in southeast Asia [7]. Guava leaf extract has been reported to possess a wide spectrum of activities against a variety of human ailments. Over twenty compounds have been reported to be present in the leaf[8]. Aqueous leaf extract contains tannin, while ethanolic extract is enriched with anthocyans, alkaloids, flavonoids, tannins, and steroids or terpenes [9, 10].

The present study was undertaken to evaluate the antimicrobial activity of methanol and the organic extracts of *Psidium guajava* leaves on multidrug-resistant (MDR) clinical isolates of *Staphylococcus aureus* collected at the Federal Medical Centre Umuahia, taking the ATCC strain as a control. The MDR strain, which showed resistance to all the antibiotics tested, was selected for antimicrobial assays.

2.0 Materials and Methodology

2.1 Plant Extract Preparation

Fresh leaves of *Psidium guajava* were collected from Lodu-Ndume. The plant was identified and taxonomically authenticated in the Dept. of Plant Science and Biotechnology, College of Natural Sciences, Michael Okpara University of Agriculture, Umudike, Abia State. Fresh leaves were dried at room temperature and ground into powdered form. Leaf powder (5 g) was packed in Whatman No. 3 filter paper and simmered in 250 ml of methanol or acetone at 80°C for 12 hours. The extracts were then filtered through a layer of Whatman No. 1 filter paper, dried at 37 °C, and stored at 4°C. The yields of methanolic and acetone extracts were 34.37 and 19.56% w/w, respectively. It was then dissolved in 50% dimethyl sulfoxide (DMSO, 99.7% pure, Sigma-Aldrich, USA) to prepare the stock solution at 25 mg/ml. Aqueous extract was prepared as described earlier (12, 13), with slight modifications. Dry leaf powder (5 g) was soaked in 25 ml of sterile distilled water for 4 hours. It was then steam-sterilised for 5 minutes and collected by squeezing through a sterile muslin cloth, followed by filtration through a sterile Whatman No. 1 filter paper, and used immediately.

2.2 Bacterial Strains Used

Clinical isolates of *Staphylococcus aureus* were screened for drug resistance using standard antimicrobial susceptibility test discs (NCCLS guidelines were used for identifying resistance and sensitivity; these were supplied by the manufacturer) [14]. Ampicillin/sulbactam, cloxacillin, cotrimoxazole, gentamycin, penicillin, and vancomycin discs were used. The clinical isolate designated as Strain I was resistant to ampicillin/sulbactam and penicillin; Strain II was resistant

UNDER PEER REVIEW

to all the antibiotics tested; Strain III was resistant to all except vancomycin; and Strain IV was resistant to ampicillin/sulbactam, cloxacillin, and penicillin. These four MDR strains were used for a preliminary screening of the antibacterial activity of the organic and aqueous leaf extracts of *P. guajava; S. aureus* ATCC strain 25833 was taken as a control.

2.3 Growth and Maintenance of the Bacterial Strains

The isolated MDR bacterial strains were maintained on Luria Bertani (LB) agar slants and sub-cultured on LB-agar plates by the streak method to obtain a single colony. A single colony was inoculated into LB medium and incubated overnight at 37°C. The overnight culture was diluted with sterilised medium to obtain an OD value of 0.1 at 600 nm, and this was used as the master culture. Fresh master cultures were prepared for each set of experiments.

2.4 Antimicrobial Assays

2.4.1 Agar-Well Diffusion

The four MDR strains I, II, El, and IV were exposed to aqueous and organic extracts by an adaptation of the agar well-diffusion method". 75 of the three types of extracts were placed in 6 mm wells cut in the Mueller-Hinton agar (MHA) plates; the control well received only 75 gels of 50% DMSO. The inhibition zones were observed after 24 hours of growth at 37°C.

2.4.2 MIC and MBC Determinations

It was carried out using aqueous and methanol extracts by the macro-broth dilution method [16].

2.4.3 Determination of the Growth Curve and Viable Counting

It was evaluated as per standard protocols [18]. A master culture of MDR strain n (0.1 ml) was inoculated into LB medium (10 ml) containing 1.25 mg/ml (MBC) of methanol extract (500 l from a stock containing 25 mg/ml); LB without the extract and LB with 500 l of 50% DMSO served as the vehicle. The cultures were incubated at 37°C. OD measurements at 600 nm of the culture against the respective blanks were taken every hour for a period of 12 hours after inoculation. A similar analysis was also carried out in parallel using ATCC strain 25923 for comparison. Samples were removed for determination of viable counts at 3, 6, 12, and 24 hours after inoculation. At each time point, serial dilutions (10" to 1012) were prepared using sterile saline, and 0.5 ml of the diluted samples were placed on LB agar by the pour-plate method. The plates were then incubated for 48 to 72 hours before the colonies were counted.

2.4.4 Time-kill Assay of Bacterial Cells

Death time was used to determine the rate of bactericidal activity [19]. The extract was used at 4 mg/ml, about 3 times the MBC value. Culture (0.1 ml) was withdrawn every *2* hours for 24 hours after inoculation and diluted up to 10 times. At each time point, 0.5 ml of both the diluted and undiluted cultures was plated. Emergent bacterial colonies were counted after 48 hours of incubation at 37°C.

UNDER PEER REVIEW

2.4.5 SDS-PAGE Analysis

Polypeptide profiles of the control and extract-treated cultures were obtained by SDS-PAGE followed by silver staining [20]. Cultures were harvested at 6 h after exposure to methanol (1.25 mg/ml) and aqueous extract (12.5 mg/ml). Following two saline washes, the bacterial pellets were lysed in sample buffer and heated at 95 °C for 5 minutes prior to loading onto gels.

2.4.6 Determination of Cellular Toxicity to Human Erythrocytes

Human erythrocytes suspended in PBS (10 mm phosphate, 150 mm NaCl, and pH 7.4) at 10^8 cells/ml were used to determine the cellular toxicity of aqueous and methanol extracts on human erythrocytes [21]. Serial dilutions of the plant extract (for 3 times the MBC value) were used for the assay.

3. Results and Discussion

Three of the four clinical isolates *of S. aureus*and the control ATCC strain were found to be sensitive to the different leaf extracts of *Psidiumguajava*asevidenced by the inhibition zones around the agar well. No growth inhibition was observed in the control wells containing 50% DMSO (Table 1).

MIC value of 625 µg/ml and 7.5 mg/ml and MBC value of 1.25 and 12.5 mg/ml were obtained for methanol and aqueous extracts, respectively (Table 2). MBC values were found to be two folds higher than that of MIC.

Table 1: Sensitivity zone (mm) of S. *aureus* (ATCC and MDR) strains against different extracts *of guava* leaves

[Values are mean of 3 replications]

Different Strains of *S. aureus*	Aqueous Extract (15 mg)	Methanol Extract (l.Smg)	Acetone Extract (1.8mg)	DMSO (50%)
ATCC 25923	15	15	16	No zone
MDR strain I	11	13	13	No zone
MDR strain II	11	19	18	No zone
MDR strain III	12	16	15	No zone
MDR strain IV	No zone	Zone with turbidity	Zone with turbidity	No zone
Conc. extract (mg/ml)		MIC	MBC	

Results is expressed as mean ± Standard Deviation (n=5).

Here strains showed that the rate of growth exhibited by the clinical strain is approximately 70% greater than that of ATCC strain. The pattern remained unaffected in the presence of DMSO as well, indicating that the vehicle, at the concentration used, did not have any growth inhibitory

activity on both the strains. Even though, the growth rate differs between the two strains, the extract treatment was found to be inhibitory to both these strains, with increased susceptibility exhibited by the clinical strains. The result of this experiment corroborates with that of agar well diffusion experiment using both these strains. Viable counts, represented as CPUs, are shown in Table 3. The number of CFUs showed a decrease of 70% at 3 hr post-treatment that increased to 98% by 6 hr followed by almost 99 - 100% at 12 hr and 24 hr of incubation with the methanol extract indicating the bactericidal activity of the extract. The results of the tune kill assay showed that 4 mg/ml of the methanol extract of *Psidiumguajava*leaves completely killed the bacteria by 8 to 10 hr of exposure, confirming the potent bactericidal activity of the extract.

Total polypeptide profiling of untreated ATCC and clinical strain was undertaken to identity variations. Interestingly, the molecular weight of some of the polypeptides match with those reported to be involved in drug resistance [22].

Table 2: MIC and MBC Values of Methanol and Aqueous Extracts of *F. guajava* Leaves Using the MDR Strain II.

Methanol stock - 25	Aqueous stock - 100	Methanol Extract	Aqueous Extract	Methanol Extract	Aqueous Extract
25 µl (312 µg/ml)	25 µl (2.5)	+	+	+	+
50 µl (625 µg/ml)	50 µl (5)	-	+	+	+
75 µl (937.5 µg/ml)	75 µl (7.5)	-	-	+	+
100 µl (1.25)	100 µl (10)	-	-	-	+
125 µl (1.5)	125 µl (12.5)	-	-	-	-
150 µl (1.8)	150 µl (15)	-	-	-	-
175 µl (2)	175 µl (17.5)	-	-	-	-
200 µl (2.5)	200 µl (20)	-		-	-

(+) - Presence of growth/colonies; (-) - No growth/colonies

Results is expressed as mean ± Standard Deviation (n=5).

Table 3: Viable Counts of MDR Strain II Taken at Different Time Intervals after Incubation in Medium Containing Methanol Extract (1.25 mg/ml)

Time of collection of samples (hr)	Treatment	Dilution	No. of CFU	Reduction in CFU (%)
3	A	10^{-3} (10^{-3})	450 (45)	70
	B	10^{-3}	55	
6	A	10^{-3} (10^{-5})	6700 (67)	98.8
	B	10^{-3}	74	
12	A	10^{-3} (10^{-5})	70000 (70)	99.9
	B	10^{-3}	58	
24	A	10^{-3} (10^{-9})	43000000 (43)	100
	B	10^{-3}	5	

Results is expressed as mean ± Standard Deviation (n=5).

A-Control: B-Treated with Methanol Extract (1.25 mg/ml).

Values given in brackets denote the actual dilution and the corresponding number of CPU taken, which has been normalized to number of CPU at 10^3 to calculate the percentage reduction in viability.

Polypeptide profiles of cells treated with DMSO (50%) did not show any detectable difference from that of the untreated controls confirming that DMSO did not have any deleterious effect on the cells (data not shown).

The present results indicated that the strong bactericidal activity exhibited by the leaf extracts of *Psidium guajava* was possibly due to protein-degrading activity of the extracts. Tannins, known to be present in the aqueous and ethanol extracts [9], reportedly have protein-binding activities and can interfere with many substances [24]. Quite interestingly, a new class of antibacterial agents, acyldepsipeptide(ADEPS), reported recently, kill gram-positive bacteria via uncontrolled proteolysis[25]. ADEPS has been shown to activate a major bacterial protease, Caseinolytic protease, bypassing the requirement of its ATPase mediated activation.

4. Conclusion

S. aureus is a common microorganism that is widely spread in the human population with many being asymptomatic carriers. It can also cause life-threatening infections and its strains have evolved into MRSA and strains with reduced vancomycin susceptibility (VISA, hVISA and VRSA). These strains cause infections and diseases that are either difficult to treat or resistant to the empiric antibiotics usually prescribed for treatment. The globe is running short of drugs/antibiotics available for therapy as a result of infections associated with this organism.
Many research studies have reported that some medicinal plants in different countries have anti-MRSA activities due to their phytochemical contents. These plants can be employed as alternative candidates for drug development to halt or/and control the infections of multi-drug resistant *S. aureus*. However, there is a need for further studies to adequately determine the safety and clinical efficacy of anti-MRSA plants to man.

Consent

It is not applicable

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