Asian Journal of Biotechnology and Genetic Engineering

Volume 6, Issue 1, Page 39-45, 2023; Article no.AJBGE.99527

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

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/99527

Original Research Article

Received: 03/03/2023
Accepted: 04/05/2023
Published: 16/05/2023

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ABSTRACT

This study looks at the chemical makeup and antibacterial activity of a methanol crude leaf extract of *Psidium guajava Linn* against clinical isolates of Staphylococcus aureus from FMC Umuahia, Abia State, that are resistant to multiple drugs. For further antibacterial testing, we chose strains that showed resistance to every antibiotic we tried. Inhibitory concentrations (MICs) of methanol and water extracts were determined to be 625 ug/ml and 7.5 mg/ml, respectively. The MBC for methanol extracts was 1.25 mg/ml, whereas the MBC for aqueous extracts was 12.5 mg/ml. The maximum bactericidal concentration of methanol extract was found to suppress the development of the MDR strain by 85%. Results from a time-kill study showed that mat methanol extract (5 mg/ml) was effective against MDR bacteria within 10 hours. The extract showed potent protein degradative activity when tested on the total polypeptide profiles of bacterial cultures using SDS-PAGE. Last but not least, a human red blood cell (RBC)-based hemolytic test demonstrated no hemolysis at doses greater than MBC, supporting its safety for therapeutic application.

Keywords: Bactericidal; haemolysis; MDR strains; Psidium guajava; Staphylococcus aureus.

1. INTRODUCTION

The term "multidrug-resistant bacteria" (MDRB) refers to germs that have developed resistance to one or more antimicrobial substances. All commercially available antimicrobial drugs, with the exception of one or two, are often ineffective against them. Included in this category are microorganisms that have developed resistance to at least one substance from three or more antimicrobial groups. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* resistant to *vancomycin* (VRSA), *vancomycin-resistant enterococci* (VRE), extended spectrum beta-lactamases (ESBLs) producing gramme-negative *bacilli,* multidrug-resistant *Streptococcus pneumoniae* (MDRSP), and *carbapenem-resistant Enterobacteriaceae* (CRE) are some of the MDRB [1-3].

Globally, infectious illnesses brought on by MDRB represent a significant burden. They have long been among the primary causes of mortality and disability, as well as one of the biggest threats to human development and health security, particularly in developing nations [4]. Although there have been numerous new antibacterial drugs developed, bacteria that are resistant to them have grown and are now a worldwide concern as we are rapidly running out of therapeutic options [5]. Antimicrobial resistance concerns exist in both healthcare and community settings, requiring a comprehensive strategy involving several partners along the continuum of care. For instance, MRSA infections later appeared in 18–33% of individuals who had MRSA colonisation.

Scientists are looking into the antibacterial properties of medicinal plants since microbes are becoming resistant to existing antibiotics [6]. The guava, *Psidium guajava* L., is an evergreen plant that originated in tropical America and has now colonised southeast Asia [7]. According to reports, guava leaf extract offers a broad range of therapeutic effects against a number of human diseases. The leaf is said to contain more than 20 different chemicals [8]. Tannin is present in aqueous leaf extract, but ethanolic extract is more abundant in anthocyanins, alkaloids, flavonoids, tannins, steroids, and terpenes [9,10].

In the current investigation, the ATCC strain was used as a control to assess the antibacterial effects of methanol and the organic extracts of Psidium guajava leaves on clinical isolates of *Staphylococcus aureus* that were obtained at the Federal Medical Centre Umuahia. The MDR strain was chosen for antimicrobial testing because it showed resistance to all of the tested antibiotics.

2. MATERIALS AND METHODOLOGY

2.1 Plant Extract Preparation

Psidium guajava leaves were picked right from the plant at Lodu-Ndume. The Michael Okpara University of Agriculture in Umudike, Abia State, is where the plant's identification and taxonomic authentication took place. To make a powder, fresh leaves were air-dried at room temperature and then pulverised. We used Whatman No. 3 filter paper to enclose 5 grammes of leaf powder before simmering it in 250 millilitres of methanol or acetone at 80 degrees Celsius for 12 hours. The extracts were filtered using Whatman No. 1 filter paper, dried at 37 degrees Celsius, and then kept at 4^{O}C. The methanolic extract yielded

34.37 percent w/w, whereas the acetone extract yielded 19.56 percent w/w. To get the stock solution of 25 mg/ml, it was dissolved in 50% dimethyl sulfoxide (DMSO, 99.7% pure, Sigma-Aldrich, USA). Modifications were made to the methods previously reported (12, 13) for preparing an aqueous extract. We used 25 ml of sterile distilled water to soak 5 g of dry leaf powder for 4 hours. It was then collected by pressing it through a sterile muslin cloth, steamed for 5 minutes, filtered through sterile Whatman No. 1 filter paper, and utilised right away [11-13].

2.2 Bacterial Strains Used

Standard antimicrobial susceptibility test discs were used to determine the level of drug resistance in *Staphylococcus aureus* clinical isolates (NCCLS recommendations were followed to determine resistance and sensitivity; they were given by the manufacturer) [14,15]. We employed antibiotic discs containing ampicillin/sulbactam, cloxacillin, cotrimoxazole, gentamycin, penicillin, and vancomycin. Strain I, a clinical isolate, showed resistance to ampicillin/sulbactam and penicillin; Strain II, all antibiotics tested; Strain III, all except vancomycin; and Strain IV, ampicillin/sulbactam, cloxacillin, and penicillin. The antibacterial activity of P. guajava organic and aqueous leaf extracts was first screened using these four MDR strains; *S. aureus* ATCC strain 25833 was used as a reference.

2.3 Growth and Maintenance of the Bacterial Strains

Slants of the isolated MDR bacterial strains were kept on Luria Bertani (LB) agar, and single colonies were obtained on LB-agar plates using the streak method. The overnight culture was diluted with sterile medium to an OD value of 0.1 at 600 nm, and this was used as the master culture. New 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

The evaluation followed accepted procedures [17,18]. A 0.1 ml master culture of MDR strain n was added to 10 ml of LB medium with 1.25 mg/ml (MBC) of methanol extract (500 l from a stock with 25 mg/ml); LB without the extract and LB with 500 l of 50% DMSO were used as vehicles. All of the culturing action took place at 37 degrees Celsius. The optical density (OD) of the culture was measured at 600 nm against the corresponding blanks hourly for 12 hours following inoculation. The ATCC strain 25923 was likewise subjected to a parallel study with the same goals. At 3-, 6-, 12-, and 24-hours post-inoculation, samples were taken to calculate viable cell counts. At each time point, sterile saline was used to create serial dilutions (10" to 1012), and 0.5 ml of the diluted samples were poured onto LB agar using the pour-plate technique. The plates were then placed in an incubator for another 48–72 hours before the colony count was taken.

2.4.4 Time-kill assay of bacterial cells

The rate of bactericidal activity was calculated based on the time of death [19]. Three times the MBC value (or 4 mg/ml) of the extract was utilised. For the first 24 hours after inoculation, 0.1 ml of culture was taken every 2 hours and diluted up to 10 times. 0.5 cc of both the diluted and undiluted cultures were plated at each time point. After 48 hours of incubation at 37°C, the number of bacterial colonies that had emerged was recorded.

2.4.5 SDS-PAGE analysis

SDS-PAGE with silver staining [20] was used to get polypeptide profiles of untreated and extract-treated cells. At 6 hours, cultures were collected after being treated with 1.25 mg/ml methanol and 12.5 mg/ml aqueous extract. Bacterial pellets were washed twice with saline and then lysed in sample buffer at 95 °C for 5 minutes before being loaded onto gels.

2.4.6 Determination of cellular toxicity to human erythrocytes

The cytotoxicity of aqueous and methanol extracts was tested on human erythrocytes suspended at a concentration of 108 cells/ml in PBS (10 mm phosphate, 150 mm NaCl, pH 7.4) [21]. The plant extract was diluted three times to get the MBC value.

3. RESULTS AND DISCUSSION

Inhibition zones surrounding the agar wells showed that three of the four clinical isolates of S. aureus and the control ATCC strain were susceptible to the various leaf extracts of Psidium guajava. In the wells serving as controls, 50% DMSO had no effect on growth (Table 1).

The minimum inhibitory concentration (MIC) for the methanol extract was determined to be 625 g/ml, while the MBC for the aqueous extract was determined to be 1.25 mg/ml. It was discovered that MBC values were twice as high than MIC values.

Compared to the ATCC strain, the growth rate shown by the clinical strain is almost 70% faster in this study. Since the pattern was unaffected by the presence of DMSO, this suggests that the vehicle does not have any growth-inhibiting activity on both strains at the concentrations tested. Both strains were found to be sensitive to the extract treatment, with the clinical strains showing particularly high susceptibility despite their slower growth rate. The results of the agar well diffusion experiment utilising these same strains complement the findings of this study. Table 3 displays the viable counts as the number of CPUs. Three hours after treatment, the number of colony forming units (CFUs) dropped by 70%; by six hours, that number had risen to 98%; and by twelve and twenty-four hours, it had reached almost 99-100 percent, demonstrating the bactericidal action of the methanol extract. The findings of the tune kill experiment shown that the methanol extract of Psidium guajava leaves exhibited significant bactericidal action, killing bacteria fully after 8-10 hours of exposure. Total polypeptide profiling was used to determine differences between the ATCC and clinical strains. Some of the polypeptides have been linked to drug resistance [22], and their molecular weights are a good match.

In order to compute the % loss in viability, values in brackets indicate the actual dilution and the associated number of CPU used, which has been normalised to the number of CPU at 103.

Polypeptide profiles of cells treated with DMSO at a 50% concentration did not change noticeably from those of untreated controls, indicating that DMSO had no negative effects on the cells (data not shown) [23].

According to the current findings, the *Psidium guajava* leaf extracts' potent antibacterial effects may have resulted from their ability to break down proteins. According to reports, tannins, which are known to be found in the aqueous and ethanol extracts [9], have protein-binding properties and may interfere with a variety of chemicals [24,25]. It's noteworthy to note that acyldepsipeptide (ADEPS), a novel family of antibacterial drugs, recently described, kills gram-positive bacteria by uncontrolled proteolysis25. A significant bacterial protease called caseinolytic protease has been shown to be activated by ADEPS without the need for ATPase-mediated activation.

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 urbidity	No zone
Conc. extract (mg/ml)		MIC	MBC	

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

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)

4. CONCLUSION

S. aureus is a typical bacteria that is extensively distributed in humans, with many of them being asymptomatic carriers. Its strains have developed into MRSA and strains with decreased vancomycin susceptibility (VISA, hVISA, and VRSA), and they may also result in infections that are fatal. Infections and disorders brought on by these strains are either difficult to treat or resistant to the empiric antibiotics that are often provided for therapy. As a consequence of diseases linked to this bacterium, the world is running out of medications and antibiotics that can be used for treatment.

Numerous studies have shown that the phytochemical components of some medicinal plants found in various countries have anti-MRSA properties. To stop or control the infections caused by multi-drug resistant *S. aureus,* these plants may be used as alternative options for drug development. The safety and therapeutic effectiveness of anti-MRSA plants for humans, however, still need additional research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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