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In vitro antibacterial activity of *Psidium guajava* Linn. leaf extract on clinical isolates of multidrug resistant *Staphylococcus aureus*

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In the present study, antibacterial activity of aqueous and organic extracts of *Psidium guajava* leaves was evaluated against multidrug resistant (MDR) clinical isolates of *Staphylococcus aureus* strains collected from hospitals in northern (Malabar region) Kerala. The strains which exhibited resistance against all the antibiotics tested was selected for antibacterial assays. Minimum inhibitory concentration (MIC) for methanolic and aqueous extracts was found to be 625ug/ml and 7.5mg/ml, respectively. Minimum bactericidal concentration (MBC) recorded for methanolic and aqueous extracts was 1.25 and 12.5mg/ml, respectively. Methanolic extract at minimum bactericidal concentration inhibited the growth of MDR strain by 80%. Time-kill assay revealed that methanolic extract (4mg/ml) killed MDR bacteria within 10 hr. Total polypeptide profiling of bacterial cultures by SDS-PAGE indicated a high degree of protein degradative activity of the extract. Finally, a human RBC based haemolytic assay showed absence of haemolysis even at concentrations higher than that of MBC, advocating thereby its safety in therapeutic use.

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

Development of microbial resistance to the available antibiotics have led scientists to investigate the antibacterial activity of medicinal plants[1,2] *Psidium guajava* L. (Guava) is an evergreen shrub native to tropical America that has naturalized in south east Asia. The Guava leaf extract has been reported to possess a wide spectrum of activities against a variety of human ailments[3,4] Over twenty compounds have been reported to be present in the leaf[5-8]. Aqueous leaf extract contains tannin, while ethanolic extract is enriched with anthocyans, alkaloids, flavonoids, tannins and steroids/terpenoids[9].

Although antibacterial activity of guava leaf extract has been demonstrated against a number of strains[10, 11], reports on the effect of these extracts on multidrug-resistant bacterial strains responsible for nosocomial infections have been found lacking in the literature. The present study was undertaken to evaluate the antibacterial activity of the aqueous and the organic extracts of *Psidium guajava* leaves on four multidrug resistant (MDR) clinical isolates of *Staphylococcus aureus* collected from hospitals in northern (Malabar region) Kerala taking ATCC strain as control. MDR strain which showed resistance to all the antibiotics tested against was selected for antibacterial assays.

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Materials and Methods

Plant extract preparation — *Psidium guajava* leaves used in the study were collected from the University campus. The plant was identified and taxonomically authenticated by the Dept. of Botany, Calicut university (Voucher specimen no.CU 90086 herbarium). Fresh leaves were shade-dried and powdered. Leaf powder (5 g) was packed in Whatman No.3 filter paper and soxhletted in 250 ml of methanol or acetone at 80°C for 12 hr. The extracts were then filtered through a layer of Whatman No.1 filter paper, dried at 37°C and stored at 4°C. The yield of methanolic and acetone extracts were 34.37 and 19.56 % w/w, respectively. It was then dissolved in 50% of dimethyl sulfoxide (DMSO, 99.7% pure, Sigma-Aldrich, USA) to prepare the stock solution at 25mg/ml. Aqueous extract was prepared as described earlier[12,13] with slight modification. Dry leaves powder (5 g) was soaked in 25 ml of sterile distilled water for 4 hr. It was then steam-sterilized for 5 min and collected by squeezing through a sterile muslin cloth followed by filtration through a sterile Whatman No.1 filter paper and used immediately.

Bacterial strains used — Clinical isolates of *Staphylococcus aureus*, were screened for drug resistance using standard antimicrobial susceptibility test discs (HiMedia Lab. Pvt. Ltd. The interpretative chart, based on NCCLS guidelines[14], used for identifying resistance/sensitivity was supplied by the manufacturer). 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 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 antibacterial activity of the organic and aqueous leaf extracts of *P.guajava* *S. aureus* ATCC strain 25923 was taken as a control.

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

Antibacterial assays

Agar-well Diffusion — The four MDR strains I, II, III and IV were exposed to aqueous and organic extracts by an adaptation of the agar well-diffusion method[15]. 75 µl 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 µl of 50% DMSO. The inhibition zones were observed after 24h of growth at 37°C.

MIC and MBC determinations — It was carried out using the aqueous and methanolic extracts by macro-broth dilution method[16, 17].

Determination of the growth curve and viable counting — It was evaluated as per standard protocols[18]. Master culture of MDR strain II (0.1 ml) was inoculated into LB medium (10 ml) containing 1.25mg/ml (MBC) of methanolic extract (500 µl from a stock containing 25mg/ml), LB without the extract and LB with 500µl 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 hr 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 hr after inoculation. At each time point, serial dilutions (10^{-2} to 10^{-12}) were prepared using sterile saline and 0.5ml of the diluted samples were plated on LB agar by pour plate method. The plates were then incubated for 48 to 72 hr before the colonies were counted.

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

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

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

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 *Psidium guajava* as evidenced by the inhibition zones around the agar well. No growth inhibition was observed in the control wells containing 50% DMSO (Fig.1, 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 methanolic and aqueous extracts, respectively (Table 2). MBC values were found to be two folds higher than that of MIC. The results of the growth curve analysis are given in Fig. 2. Comparison of the growth patterns of untreated ATCC and clinical

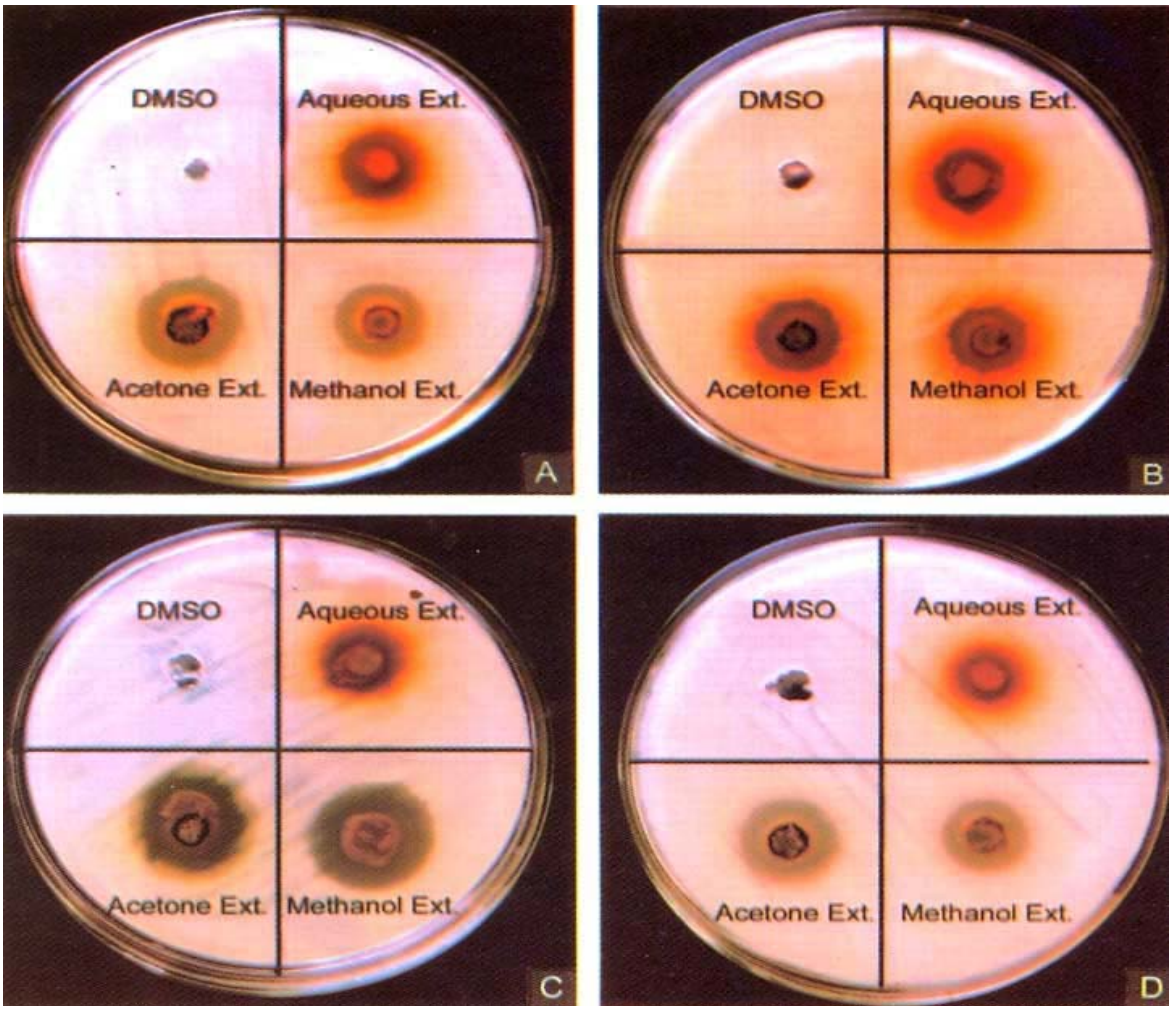

Table.1—. Sensitivity zone (mm) of *S. aureus* (ATCC and MDR) strains against different extracts of *Psidium guajava* leaves.

[Values are mean of 3 replications]

Different Strains of *S. aureus*	Aqueous extract (15 mg)	Methanol extract (1.8 mg)	Acetone extract (1.8mg)	DMSO (50%)
ATCC 25923	15	14	15	No zone
MDR strain I	12	14	14	No zone
MDR strain II	13	21	20	No zone
MDR strain III	13	14	15	No zone
MDR strain IV	No zone	Zone with turbidity	Zone with turbidity	No zone

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/lethal 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 CFUs, 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 methanolic extract indicating the bactericidal activity of the extract. The results of the time kill assay showed that 4 mg/ml of the methanolic extract of *Psidium guajava* 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 identify variations. Interestingly, the molecular weight of some of the polypeptides match with those reported to be involved in drug resistance[22,23].

Table 2 — MIC and MBC values of methanolic and aqueous extracts of *P. guajava* leaves using the MDR strain II

Concentration of extract (mg/ml)		MIC		MBC	
Methanol	Aqueous	Methalolic extract	Aqueous extract	Methanolic extract	Aqueous extract
Stock – 25	Stock – 100				
25µl (312 µg/ml)	25µl (2.5)	+	+	+	+
50µl (625 µg/ml)	50µl (5)	-	+	+	+
75µl (937.5µg/ml)	75µl (7.5)	-	-	+	+
100µl (1.25)	100µl (10)	-	-	-	+
125µl(1.5)	125µl(12.5)	-	-	-	-
150µl (1.8)	150µl (15)	-	-	-	-
175µl (2)	175µl (17.5)	-	-	-	-
200µl (2.5)	200µl (20)	-	-	-	-

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

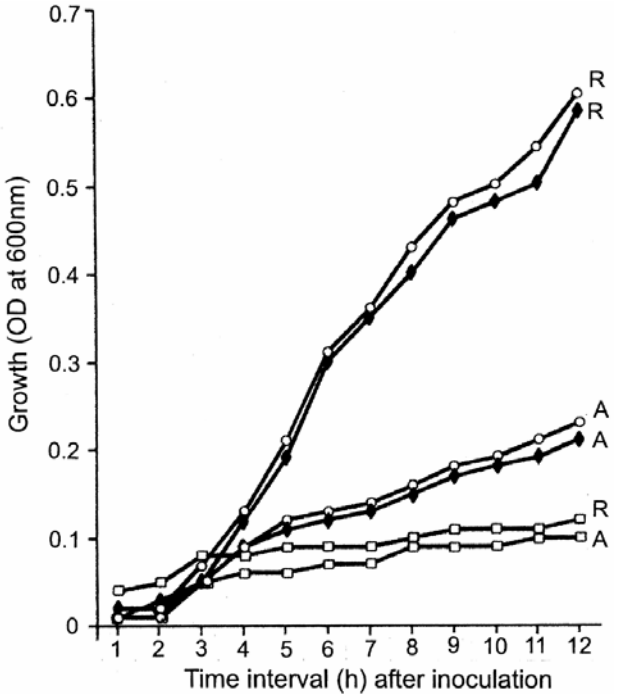

Fig.2 — Effect of the methanolic extract of the *P. guajava* leaves on the growth pattern of ATCC (A) and MDR strain II (R) of *S. aureus*. [(-○-) untreated control; (-♦-) culture exposed to 50% DMSO; (-□-) culture exposed to the extract].

Polypeptide profiles of the clinical strain exposed to the extracts at the respective MBCs showed that both the extracts (aqueous and methanolic) of the leaves of *Psidium guajava* had a strong proteolytic activity (Fig. 3). A band of approximately 29 KDa was the only one seen intact in the treated category. Whether this was an accumulation of the degradation products of a particular size or was a protease resistant band, remained to be elucidated. ATCC strain was also observed to have the same response towards both the extracts with identical profiles.

Table 3 — Viable counts of MDR strain II taken at different time intervals after incubation in medium containing methanolic extract (1.25 mg/ml)

Time of collection of samples (hr)	Treatment	Dilution	No.of CFU	Reduction in CFU (%)
3	A	10^{-3} (10^{-4})	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^{-6})	70000 (70)	99.9
	B	10^{-3}	58	
24	A	10^{-3}(10^{-9})	43000000 (43)	100
	B	10^{-3}	5	

A–Control; B–Treated with methanolic extract (1.25 mg/ml)
Values given in brackets denote the actual dilution and the corresponding number of CFU taken, which has been normalized to number of CFU 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 ethanolic extracts[9], reportedly have protein-binding activities and can interfere with many substances[24]. Quite interestingly, a new class of antibacterial agents, acyldepsipeptides (ADEPS), reported recently, kill gram-positive bacteria via uncontrolled proteolysis[25]. ADEPS has been shown to activate a major bacterial protease, Clp P (Caseinolytic protease), bypassing the requirement of its ATPase mediated activation. Clp protease

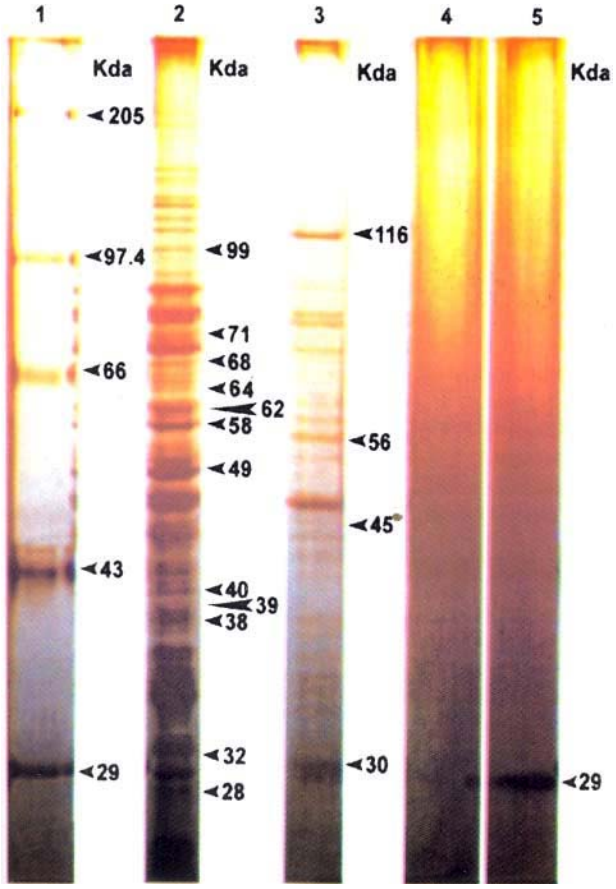

Fig.3 — SDS-PAGE (10%) showing the action of *Psidium guajava* leaf extracts on the total poloypeptide profiles of the MDR strain II [Lane – 1- Protein molecular weight marker; Lane – 2 -Untreated ATCC; Lane – 3 - Untreated MDR strain II; Lane – 4 - MDR strain II treated with aqueous extract; Lane – 5 - MDR strain II treated with methanolic extract]. Arrowheads in lanes 2 & 3 indicate variations in the profiles of ATCC and MDR strain II.

family has a crucial role in the survival and virulence of pathogens during host infection[26,27]. Whether the potent protein-degradation observed by us in the extract treated cultures was a cause or consequence of such activities remained to be elucidated by further studies.

Cellular toxicity of the methanolic extract was studied using human erythrocytes as a test system. Even at 4 mg/ml of the extract used (~three times of the MBC value), no hemolysis was observed, indicating non toxicity to living cells. In the positive control, the erythrocytes treated with 1 % TritonX 100 showed complete lysis. Unlike synthetic antibiotics such as chloramphenicol known to induce RBC lysis[28], the absence of such detrimental effects in these extracts vouch for its safe therapeutic use.

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