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Targeting Multidrug Resistant A. *Baumannii* to Evaluate Anti-Biofilm and Anti-Quorum Sensing Potential of Some Compounds

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ABSTRACT

Background: *The continuing search for new and novel antimicrobials for their potential as anti-biofilm and anti-quorum sensing agents has turned to many plant products possessing broad-spectrum anti-microbial activities. This potential has gained importance recently because the plant secondary metabolites exhibit anti-quorum sensing activity without interfering with the growth of the organism which thus would minimize the development of multidrug resistance. A. baumannii was the test organism in this study and we used carbapenem-resistant and sensitive clinical isolates. We have evaluated the anti-biofilm and anti-quorum sensing potential of two popular plant sources, namely mango seed kernels and guava leaves. Commonly used antimicrobial food preservative ε-Poly lysine was also evaluated for its anti-quorum sensing potential.* ***Methods:*** *Ethanolic extracts of these plant materials and ε-Poly lysine were tested for their Minimum Inhibitory Concentration (MIC) by disc diffusion method. The antibiofilm activity of the compounds was determined using a Microtitre Plate Test (MTP) and Scanning Electron Microscopy (SEM). The compounds were also evaluated for their anti-quorum sensing activity by the MTP method. Finally, these plant extracts were partially characterized by Gas Chromatography-Mass Spectrometry (GC-MS), and the major components were identified.* ***Results:*** *The MICs of GLE, MSKE and ε-Poly lysine against planktonic cells of the isolates were 60 µg/mL, 40 µg/mL, and 60 µg/mL respectively. The MTP test showed a significant inhibition by the natural extracts on the biofilm formation at sub-MIC concentrations. MSKE and ε-Poly lysine were the strong biofilm inhibitors that could hinder biofilm growth by over 60%-80% in both Carbapenem-Resistant Acinetobacter Baumannii (CRAB) and CSAB isolates. GLE exhibited a moderate effect on 54.5% of CRAB and 25% of CSAB isolates respectively. These results were confirmed by SEM where the biofilm has been reduced to individual cells scattered over the matrix surface. In addition, the relative number of bacteria in the biofilm matrix was significantly less compared to the untreated samples. A. baumannii cells after exposure have lost the biofilm integrity and the cells became flat and elongated. Inhibition of the quorum sensing signal molecule AHL, in terms of blue-green complex production, by MSKE was 47% in CRAB and 82% in CSAB isolates. However, GLE extract showed 42% and 69% inhibition of AHL production in CRAB and CSAB isolates respectively. Streptomycin and ε-Poly lysine could inhibit the production of AHL by about 40% in CRAB and 80% in CSAB isolates.* ***Conclusions:*** *The present study demonstrated that MSKE, GLE ethanolic extracts, and ε-Poly lysine have strong potential as*

antibiofilm and anti-QS compounds which could be developed further as adjunct drugs for treating multi-drug resistant A. baumannii and for co-therapy-with-other-antibiotics-to-eliminate-development of resistance.

Keywords: Biofilms, Anti-biofilm agents, Anti-quorum sensing, Acyl homoserine lactone, Adjunct drug, A. *baumannii*.

INTRODUCTION

Acinetobacter baumannii an obligate Gram-negative and aerobic *coccobacillus*, has emerged as the most important organism causing deadly bacterial infections [1]. This organism's resistance to almost all the current generation of antibiotics further decreases its treatment options for hospital-acquired infections. Antibiotic resistance can be transferred among these bacteria, leading to the rapid spread of resistance by the day [2]. Many studies have been done to investigate the virulence factors of this bacterium; however, the most important virulence factors which contribute to the pathogenesis of A. *baumannii* disease are the ability to form biofilms, the presence of lipopolysaccharide in the cell wall, phospholipases, presence of siderophore mediated iron acquisition system, chaperone-usher pili assembly system, OmpA outer membrane proteins and penicillin-binding protein [3]. It is a fact that A. *baumannii* due to its biofilm-forming ability, survives on artificial surfaces for a long period thereby, allowing it to persist in the hospital environment [4]. Biofilms provide bacteria with three main advantages, including trapping the essential elements and nutrients for bacterial usage, tolerating harsh conditions as well as protecting against the host's immune system and the opportunity to transfer antibiotic resistance genes [5]. Furthermore, the availability of fewer nutrients in deeper space within the biofilm and consequently a slower metabolism can prevent the bacteria from succumbing to antibiotic and antimicrobial effects [6]. The process of biofilm formation in many bacteria is facilitated by flagella; however, for A. *baumannii*, pili seem to be involved in this process under the regulation of the CsuA/BABCDE chaperone-usher pilus system which is necessary for the initiation of biofilm formation on abiotic surfaces. It has been shown that inactivation of the csuE gene eliminates pilus production and biofilm formation [7]. Among the several factors, the biofilm-associated protein encoded by the bap gene plays an important role in intercellular adhesion, accumulation of bacterial cells, and establishment of biofilm. This is one of the cell surface-associated proteins involved in biofilm maintenance and maturation. Mutation in the bap gene showed the inhibition in the biofilm formation by A. *baumannii* [8]. Currently, the need for a novel therapeutic strategy to combat the biofilm-associated infection of A. *baumannii* has gained more attention. Anti-biofilm agents attenuate adherence and virulence factors of the pathogen, instead of affecting its growth, and hence, the possibility of resistance development is much lower [9]. In addition, anti-biofilm therapy enhances the sensitivity of bacteria to antibiotics and the host immune system. This indicates the importance of anti-biofilm therapy and the urgent need for the discovery of novel anti-biofilm agents. Previously, numerous anti-biofilm agents from various natural sources have been explored against A. *baumannii* biofilms. However, the present study is the first report on the anti-biofilm and anti-quorum sensing potential of Mango Seed Kernel Extract (MSKE), Guava Leaf Extract (GLE) and a synthetic compound, ε-Poly lysine on A. *baumannii* clinical isolates.

METHOD

Bacterial Strains and Culture Conditions

Thirty clinical isolates of *Acinetobacter calco* Aceticus-Acinetobacter Baumanni (ACB) complex were obtained from the Department of Clinical Microbiology Department of Gleneagles Global Hospitals, Hyderabad. The source of these isolates were: respiratory secretions, blood, wound swabs, sputum, and body fluids including urine and aspirated fluids. *Acinetobacter baumannii* ATCC 19606 reference strain was obtained from Culture collections of Himedia Pvt. Ltd. These specimens were inoculated into Mueller Hinton Agar (MHA) and incubated overnight at 37°C. Pure and isolated colonies of the cultures were diluted to 1.0 McFarland (A600 nm=0.2-0.4) and used for all

the experiments. Agrobacterium tumefaciens NTL4 (pZLR4), a monitor strain was kindly given by Dr. Clay Fuqua, Indiana University, Indiana, USA was utilized to detect AHLs with long acyl chains.

Antimicrobial Drug Sensitivity Testing by Disc Diffusion Method

Antimicrobial Sensitivity Testing (AST) was performed on the A. *baumannii* isolates against two antibiotics- Imipenem (10 μg) and Meropenem (10 μg) (Himedia, Mumbai, India) as per BSAC guidelines (2015) by Kirby=Bauer disk diffusion method to confirm sensitivity to carbapenems.

Preparation of Mango Seed Kernel Extract and Guava Leaf Extract

Mango seed kernel from a popular variety of mango namely Mallika (Mangifera indica sp. Mallika') and guava (Psidium guajava) leaves was obtained from Fruit Research Station, Sangareddy, Telangana. Ethanolic extracts of Mango seed kernels and Guava leaves were prepared as per the protocol published earlier from our laboratory [10]. Briefly, the air-dried kernels and leaves were coarsely powdered and subjected to serial extraction with absolute alcohol in a Soxhlet apparatus and the distillate was collected at 80°C [11]. The extracts were filtered and dried in a rotary evaporator. A stock solution of 100 mg/mL was prepared in dimethyl sulfoxide (DMSO, Cat. No. 41639, Sigma-Aldrich) and stored at -20ºC for further use.

Preparation of ε-Poly Lysine Solution

ε-Poly lysine (food grade, Cat. No. FP-155) was purchased from Bimal Pharma Pvt. Ltd., Mumbai. Sterile stock solutions of 100 mg/mL were made in molecular biology grade water and stored at -20ºC for further use.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of MSKE, GLE and ε-Poly lysine on Reference strain and clinical *A. baumannii* isolates were determined using the Kirby-Bauer Disc Diffusion method [12]. Filter paper discs impregnated with different concentrations within a range of 10 μg mL^{-1}-100 μg mL^{-1} of the compounds, were positioned on the *A. baumannii* bacterial lawn and incubated at 37°C for 24h. The lowest concentration which showed the zone of growth inhibition around the discs was reported as the MIC of the compounds. Willing to participate after giving written informed consent.

Determination of Biofilm Inhibition

The bacterial cells were allowed to adhere to the base of a 96-well microtiter polystyrene plate and the biofilm formed was estimated by the method of Stepanovic et al.13 with some modifications. Overnight culture of A. *baumannii* was used to inoculate 200 μL of MHB in the 96-well plate without and with candidate drug compounds and incubated at 37°C for 24 h. *A. baumannii* type strain ATCC 19606 was used as a positive control, while un-inoculated MHB media was used as a negative control. Streptomycin was used as a reference antibiotic for demonstrating the anti-biofilm effect. After incubation, the cell density was measured at 610 nm. The non-adherent cells were discarded and the biofilms were washed using sterile Phosphate-Buffered Saline (PBS) to remove unbound cells followed by cold methanol fixation and air drying. The plate was then stained with 0.1% (w/v) crystal violet for 10 minutes and washed under running tap water to remove excess stain. The biofilm-bound dye was extracted in 30% glacial acetic acid and its absorbance was measured at 610 nm. Finally, the percentage of biofilm inhibition was calculated for both CRAB and CSAB isolates using the following formula: % inhibition=Treated biofilm A610-Untreated biofilm A610/Untreated biofilm A610 × 100.

Direct Demonstration of Quorum Quenching on Clinical Isolates of A. *baumannii* by Candidate Drugs

The blue-green complex formed by AHL was estimated in a 96-well plate format as described previously with some modifications [13-16]. A single isolated colony of Agrobacterium tumefaciens monitor strain, NTL4pZLR4 was cultured overnight in 3mL of LB broth substituted with antibiotics streptomycin (50 µg/mL) and tetracycline (5 µg/mL). 180 µL of 1.0 McFarland NTL4pZLR4 culture was transferred to a 96-well flat bottom polystyrene plate containing X-Gal of 40 µg/mL final concentration. 20 µL A. *baumannii* culture supernatant and 2 µL each of the candidate drugs (sub-MIC concentrations of 10 µg/µL) was added to each well in duplicates. Cultures unexposed to the drug candidates were used as control. The plate was incubated at 28ºC for 24 h. After 24 h, absorbance was measured at 610 nm and inhibition of AHL (Blue-green complex) was calculated for both CRAB and CSAB isolates with respect to the absorbance of the untreated control. The AHL inhibition was measured using the formula: % inhibition=Treated culture OD-Untreated culture OD/Untreated culture OD × 100.

Scanning Electron Microscopy (SEM)

Biofilms from CRAB and CSAB isolates of A. *baumannii* were grown on glass coverslips by adding 4 mL of A. *baumannii* culture (1.0 McFarland) supplemented with a sub-MIC concentration of the extracts (10 µg/mL) in a 6-well microtiter plate while the control cultures had an equal amount of DMSO. After 24 h of incubation at 37ºC, the coverslips were rinsed with distilled water to remove non-adherent cells and processed for Scanning Electron Microscopy (SEM) examination. Samples were analyzed by SEM as described previously [17]. The samples were fixed overnight at 4°C in cold 2.5% (v/v) glutaraldehyde containing 0.2 M Sodium Cacodylate Buffer (SCB pH=7.2). The coverslips were washed thrice with 0.1 M SCB buffer every 30 minutes to get rid of the excessive fixative and dehydrated with increasing concentrations (30%, 50%, 70%, 80%, 90%, and 100%) of ethanol. All the coverslips were removed from the ethanol and air dried under high vacuum (10 Torr-7 Torr) at room temperature (25°C) for a day. All dried samples were mounted on aluminum stubs (SPI supplies division of Structure Probe INC, USA no. 05072-AB) with double-sided adhesive tape and coated with ionic gold (300Aº) in sputter coating unit Model: E-1010 Hitachi Japan) at high vacuum. The processed samples were examined under a scanning electron microscope (SEM) (S3400N Hitachi Japan) at 15 Kv and high vacuum (10 Torr-7 Torr) and the pictures were taken at different magnifications.

Gc-Ms Analysis and Identification of Components

GC-MS analysis was carried out in Shimadzu, GasChromatography-Mass Spectroscopy GC-MS 2010QP plus. ZB-5 column capillary column (30 m × 0.25 mm × 0.25 µm) of 5% -Phenyl-95% Dimethyl poly-siloxane was used for the identification of metabolites in the candidate compound extracts. Helium was used as carrier gas with a consistent flow rate of 1.10 ml min^{-1} with a sample injection volume was 1µl and ion source temperature was 200°C. The oven temperature was programmed from 100°C (isothermal of 4 min), with an increase of 4°C min^{-1}, to 280°C, then hold it for 12.95 minutes. Mass spectra were taken at 70 eV: a scan interval of 10 spectra s^{-1} and fragments from 50 Da to 500 Da. The relative percentage of each component was calculated by comparing its average peak area to the total areas. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST and Wiley library to ascertain the name, molecular weight, and structure of the components of the test materials [18].

RESULTS

Antimicrobial Sensitivity Testing (AST) Profile

The 30 clinical isolates were characterised for carbapenem sensitivity and grouped as Carbapenem Resistant (CRAB) (n=22) and Carbapenem Sensitive (CSAB) (n=08). The isolates were considered CRAB if the inhibition zone diameter was ≤ 13mm (Imipenem) and ≤ 12mm (Meropenem). CSAB isolates showed an inhibition zone of ≥ 25 mm (Imipenem) and ≥ 20mm (Meropenem).

Impact of Candidate Drug Compounds on Biofilm Formation by A. *baumannii*

The effect of the 3 candidate drug compounds was initially investigated on 30 clinical isolates of A. *baumannii* biofilm formation in 96-well polystyrene plates using a crystal violet assay. All compounds were tested at Sub-MIC (Figure 1). The percentage inhibition of biofilm by the compounds was categorized as weak (<20%), moderate (20%-60%) and strong inhibition (>60%). These compounds were equally effective in inhibiting the biofilms of both Carbapenem resistant and sensitive isolates. Of these three compounds, MSKE and *ε-Poly lysine* were the most effective biofilm inhibitors as they showed strong biofilm inhibition in more than 60% (both CRAB and CSAB) of A. *baumannii* isolates (Table 1).

Table1 Categorisation of the test compounds based on the extent of Biofilm inhibition in clinical isolates of CRAB and CSAB A. *baumannii*

Compound	Biofilm Inhibition, the number of positive isolates is presented as % total number of CRAB isolates (n=22)/CSAB isolates (n=8)		
	Weak inhibition	Moderate inhibition	Strong inhibition
MSKE	4.5/0	31.8/37.5	63.6/62.5
GLE	45.4/62.5	54.5/25	0/12.5
ε-Poly lysine	0/0	36.3/25	63.6/75
Streptomycin	18.1/12.5	63.6/50	18.1/37.5
Weak Inhibition ≤ 20% biofilm inhibition, Moderate inhibition=20%-60% biofilm inhibition, Strong inhibition ≥ 60% biofilm inhibition.			

Figure 1 Inhibitory effect of compounds on biofilm formation by clinical isolates A. *baumannii* from crystal violet micro titre plate assay. Biofilm formation by A. *baumannii* clinical isolates were quantified in the presence of MSKE, GLE, ε-Poly lysine at 10 µg mL^{-1} for 24 h in 96-well plates vs untreated controls in triplicates. Streptomycin was used as a reference antibiotic

Morphological and Structural Impact on the Biofilm by the Drug Candidates

SEM images revealed a reduced number of A. *baumannii* cells in all four test groups compared with the control, specifically in terms of biofilm biomass, thickness, integrity of the film and substrate coverage were approximately dramatically reduced compared to untreated controls (Figure 2). The untreated cells appeared to be regular spherical in shape with smooth surfaces and intact cell walls. The SEM images of the biofilm specimens after they were

exposed to compounds revealed that A. *baumannii* cells exhibited membrane disruption and irregular cell morphology (Figure 3).

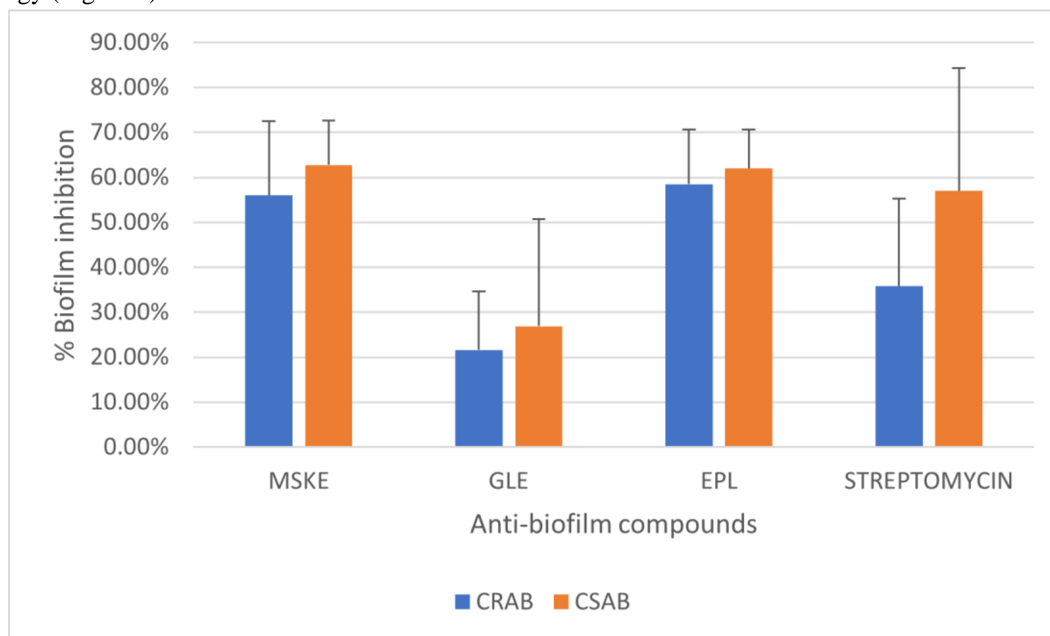

Figure 2 Graph representing inhibitory effect of compounds on biofilm formation by A. baumannii clinical isolates. Biofilm formation by A. *baumannii* clinical isolates was quantified in the presence of MSKE, GLE, ε-Poly lysine at 10 µg mL^{-1} after 24 h in 96-well plates vs untreated controls. Streptomycin was used as reference antibiotic control to show the anti-biofilm effect. Error bars indicate standard deviation of the mean from three experiments of biofilm inhibition, values are in %

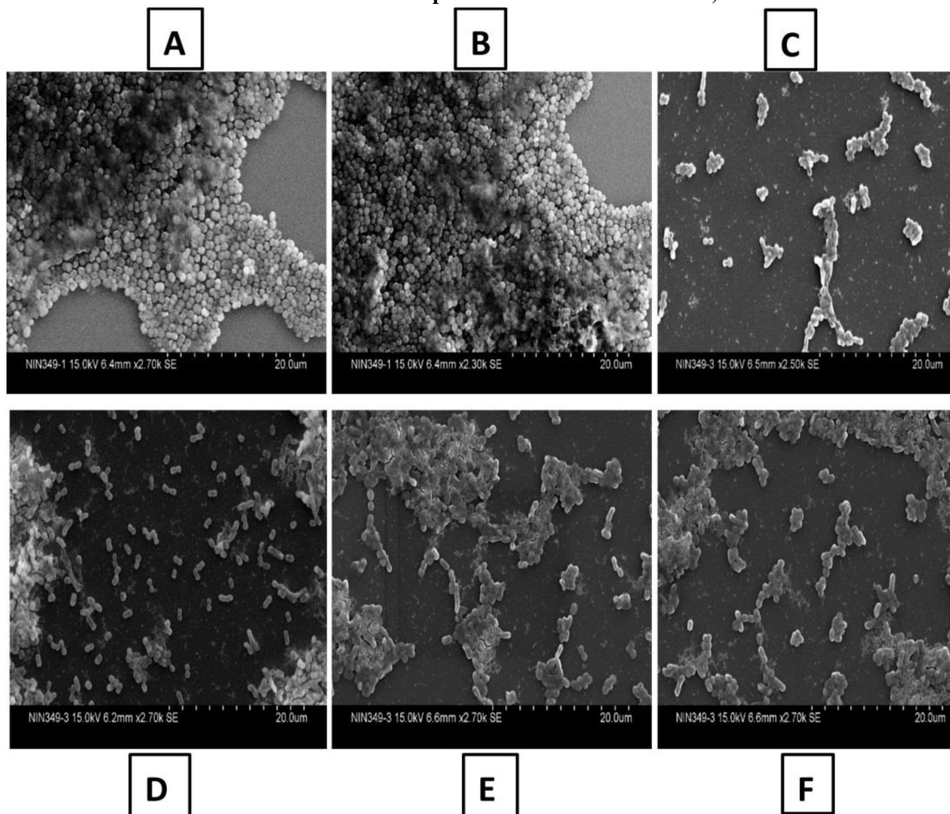

Figure 3 SEM images of 24-hour biofilm of A. *baumannii* isolates. A) Control (no drug), B) Control with DMSO, C) Streptomycin at sub-MIC levels of 10 µg mL^{-1}, D) MSKE (Mango Seed Kernel Extract) at sub-MIC of 10 µg mL^{-1}, E) GLE (Guava Leaf Extract) at sub-MIC of 10 µg mL^{-1}, F) ε-Poly lysine at sub-MIC of 10 µg mL^{-1}

Phytochemical Analysis of Bioactive Compounds

Ten major compounds were identified in the kernel extract of Mangifera indica L. The GC-MS chromatogram is shown in Figure 4 and the corresponding compounds with their retention times, molecular formulae, Molecular Weights (MW) and concentrations (%) are shown in Table 2.

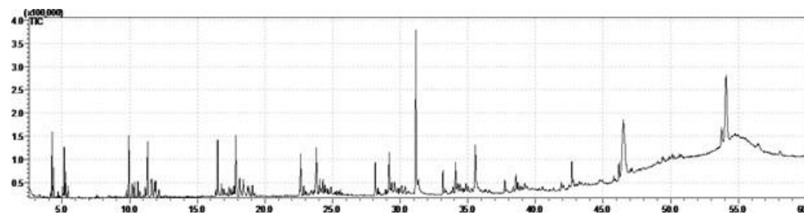

Figure 4 GC/MS Chromatogram of MSKE. Ten major components were identified in the kernel extract of Mangifera indica L. GC-MS analysis was carried out in Shimadzu, Gas Chromatography-Mass Spectroscopy GC-MS 2010QP plus

Table 2 GC-MS analysis of MSKE 4-Methyl-1- decene followed by 2-ethylhexyl ester were the major compounds with highest % found in the MSKE extract

RT	AREA	Compound Name	Molecular Weight	Molecular Formulae	Area%
RT:4.261	95276	4-Methyl-1- decene	154	$C_{11}H_{22}$	9.147266
RT:4.369	38295	4-Methyl decane	156	$C_{11}H_{24}$	3.676629
RT:5.153	76093	3,7-Dimethyl decane	170	$C_{12}H_{26}$	7.305543
RT:9.936	91457	2-ethylhexyl ester	202	$C_{11}H_{22}O_3$	8.780611
RT:10.594	24876	2,6,11-Trimethyl do-decane	212	$C_{15}H_{32}$	2.388297
RT:16.511	82861	n-Penta decane	212	$C_{15}H_{32}$	7.955326
RT:18.394	32103	1-Iodo-2-methyl un- decane	296	$C_{12}H_{25}I$	3.082147
RT:22.636	63727	n-Hepta decane	240	$C_{17}H_{36}$	6.118307
RT:28.161	45135	2,6,10,14-Tetramethyl octa decane	310	$C_{22}H_{46}$	4.333325
RT:29.186	56999	2,3- Dimethyl nona decane	296	$C_{21}H_{44}$	5.472365

Ten major compounds were identified in the leaf extract of guava. The GC-MS chromatogram is shown in Figure 5 and the corresponding components with individual retention times, molecular formulae, Molecular Weights (MW) and relative proportion (%) of the total compound are shown in Table 3.

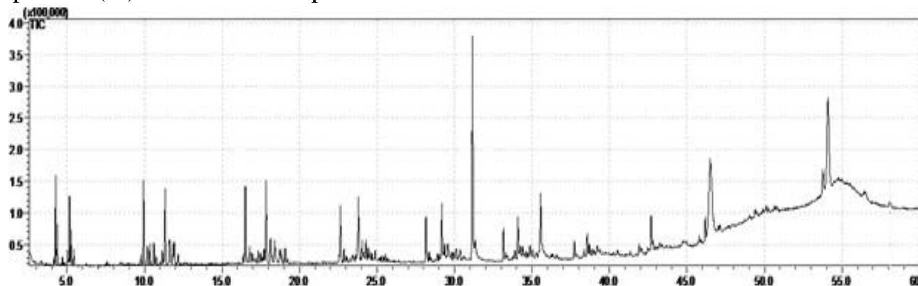

Figure 5 GC/MS Chromatogram of Guava Leaf Extract. Ten major components were identified in the leaves extract of Psidium guajava. GC-MS analysis was carried out in Shimadzu, Gas Chromatography-Mass Spectroscopy GC-MS 2010QP plus

Table 3 GC-MS analysis of GLE. 4-Methyl-1- decene and 2,4-Dimethyldodecane were the highest found compounds in the extracts of GLE

RT	Area	Compound Name	Molecular Weight	Molecular Formulae	Area%
RT:4.269	79433	4-Methyl-1-decene	154	$C_{11}H_{22}$	7.636944
RT:5.169	63648	2,2,3,3,5,6,6-Heptamethyl heptane	198	$C_{14}H_{30}$	6.119323

RT:5.286	30769	Hendecane	156	$C_{11}H_{24}$	2.958231
RT:9.944	77851	2,4-Dimethyl do-decane	196	$C_{14}H_{30}$	7.484845
RT:11.336	71825	2-Methyl-n-tri decane	198	$C_{14}H_{30}$	6.905486
RT:16.511	67326	Oxalic acid	244	$C_{13}H_{24}O_4$	6.472938
RT:22.636	53815	n-Hepta decane	240	$C_{17}H_{36}$	5.173947
RT:23.803	69933	6-ethyloct-3-ylisobutyl ester	286	$C_{16}H_{30}O_4$	6.723583
RT:28.161	41361	10-Methylnona decane	282	$C_{20}H_{42}$	3.97658
RT:34.078	35559	n-Docosane	310	$C_{22}H_{46}$	3.418757

Quorum Quenching Effect of Compounds on Clinical Isolates of A. *baumannii*

The anti-QS effect of all the four compounds was investigated on just 10 CRAB and 10 CSAB clinical isolates in 96-well polystyrene plates. A. *tumefaciens* monitor strain NTL4pZLR4, with 40 µg mL-1 of X-gal and 20 µL A. *baumannii* culture supernatants was incubated with sub-MIC amounts (10 µg mL^{-1}) of the compounds and allowed to grow at 28°C for 24 hours. Agrobacterium monitor strain utilizes the AHL diffused from the A. *baumannii* culture supernatants and forms a blue-green complex. Untreated (DMSO without candidate drugs) cultures were used as control. After 24 hours, absorbance was measured at 610 nm and inhibition of AHL production (Blue-green complex) was calculated with respect to the absorbance of the untreated control. The formation of Blue-green complex was inhibited by the test compounds and the decrease in absorbance was compared to that of the control untreated well. The inhibition was expressed as % (mean ± SD) for each drug on both CRAB and CSAB isolates (Table 4). The candidate drugs showed good anti-QS effect in carbapenem sensitive isolates than carbapenem resistant isolates. MSKE showed remarkable effect in decreasing the blue-green complex formation. The inhibition of blue-green complex production by MSKE was 47% in CRAB and 82% in CSAB isolates, while GLE showed 42% and 69% inhibition in CRAB and CSAB isolates respectively. *Streptomycin* and *ε-Poly lysine* both could inhibit the production of the blue-green complex by around 40% in CRAB and 80% in CSAB isolates without affecting the growth of A. tumefaciens monitor strain. This data shows the promising potential of these candidate drugs as anti-QS compounds.

Table 4 Quorum quenching effect of compounds on clinical isolates of A. *baumannii*. Data showing the percentage inhibition in production of blue-green complex in the presence of candidate compounds. Mean and SD are calculated for each data sets

Drug Sensitivity	MSKE	GLE	*Streptomycin*	*ε-Poly lysine*
CRAB	47% ± 7%	42% ± 0.08%	43.8% ± 7%	45% ± 9%
CSAB	84 % ± 0.06%	69.7% ± 4%	80.8% ± 4%	81.4% ± 3%

DISCUSSION

The medicinal use of P. *guajava* and M. indica has already been reported in native system of medicines and its use continues even today in contemporary home remedies and personal care products. Reports show that P. *guajava* essential oil has capacity to inhibit the growth of both gram-positive and gram-negative bacterial strains [19]. Hence, we chose to look at its anti-biofilm effect on A. *baumannii* isolates. Of these three compounds, MSKE and *ε-Poly lysine* were the most effective biofilm inhibitors. They showed more than 60% inhibition of biofilm formation in maximum number of clinical isolates. A similar effect was observed by Angad and his co-workers at an MIC of 125 µg mL^{-1} on P. *aeruginosa* and E. coli. The antibiofilm test showed the ethanol extract inhibited the bacterial adhesion on glass tube which caused biofilm detachments when compared with control tubes [20]. Our study demonstrated that our candidate drugs were much more effective as biofilm inhibitors at a concentration several times lower (10 µg mL^{-1}) on A. *baumannii* compared to how P. *aeruginosa* and E. coli responded. The results obtained in this study are similar to those reported by several other authors: Adesina *et al*., reported that M. indica, *Psidium guajava* and Ocimum gratissimum leaf extracts prevented the E. coli biofilm formation on catheters [21].

Many studies have showcased the potential of natural extracts as anti-QS compounds where they have shown effect on violacein pigment production in *Chromobacterium violaceum* or blue green colour complex in Agrobacterium tumefaciens [22-24]. This study was the first to demonstrate the direct effect of these compounds on AHL production by A. *baumannii* clinical isolates in a microtiter plate by using A. *tumefaciens* monitor strain NTL4pZLR4.

Our results were further confirmed by a Scanning Electron Microscopy analysis of the biofilms. The SEM images of the biofilms exposed to compounds (sub-MIC of 10 µg mL^{-1}) shows poorly developed biofilms with membrane disruption and irregular cell morphology compared with untreated cells. A. *baumannii* biofilms treated with compounds showed fewer cells of different size scattered than the controls and the matrix was also scarcely visible. Presently, there is no report on the anti-biofilm potential of ε-Poly lysine against A. *baumannii*. However, the anti-biofilm activity of G3 dendrons of *ε-Poly lysine* was demonstrated against P. *aeruginosa* [25].

Ten components were identified in the GLE and MSKE extracts by Gas chromatography Mass spectrometry. The major components reported were: 4-Methyl-1-decene (7.63%), 2,4-Dimethyl dodecane (7.48%), 4-Methyl-1-decene (9.14%) and 2-ethyl hexyl ester (8.78%). All these components were basically essential oils, volatile organic compounds, esters, hydrocarbons, fatty acids like oxalic acid. Many reports confirm the presence of these major components [26-28]. Identifying particularly the most active antimicrobial compounds of the essential oils is burdensome and requires extensive purification of the extracts because essential oils are complex mixtures of up to 30 different components.

Mango Seed Kernel Extract (MSKE), Guava Leaf Extract (GLE) and *ε-Poly lysine* exert strong antimicrobial and antibiofilm activities against carbapenem-resistant A. *baumannii* strains. The active compounds of the crude extract, at sub-MIC, caused damage to the stability and integrity of its biofilm as well as its morphology. The antibiofilm effect of these compounds could be exploited for anti-virulence therapy and as adjunct drugs for treating biofilm associated clinical problems caused by this pathogen.

CONCLUSION

The menstrual cycle was regular in the majority of girls from government, private, and tribal schools girls. Menstrual flow lasted for 2 days-7 days in most girls from government and private schools while it was less than 2 days in the majority of tribal school girls. The menstrual bleeding flow was normal in almost all government and private school girls while it was scanty in most tribal school girls. Nearly all girls from government and private schools knew about menstruation before menarche and the source of information were mothers or sisters for government and private school girls while it was radio and television for tribal school girls.

Almost all girls from government, and private schools used sanitary pads during menstruation but girls from tribal schools used reusable cloth. Most of the girls changed their pads as per need. The majority of government and private school girls disposed of sanitary pads in dust bins while 54% of tribal school girls washed and reused the cloth. Nearly 41% of girls from government schools cleaned their genitalia more than 3 times during menstruation while the majority of girls from private and tribal schools cleaned less than 3 times. Soap and water was the main agent used by government school girls (55.3%) while the majority of the girls from private and tribal schools used only water (44% and 94% respectively).

RECOMMENDATIONS

Menstrual hygiene and the use of sanitary disposable pads during menstruation needs to be emphasized and introducing it through familiar backgrounds such as school and teachers encourages girls to adopt healthy habits. Further detailed investigations need to be carried out to assess the reasons behind scanty menstrual flow and other menstruation-related issues, particularly in tribal girls.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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