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Is Quorum sensing inhibition a potential target to combat multidrug resistant *Acinetobacter baumannii*? -A Proof of concept study

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Abstract:
Background*:Acinetobacter baumannii has emerged as a major nosocomial pathogen as it exhibits multidrug resistance. One of the major contributors for this multidrug resistance is the remarkable ability of A. baumannii to attract members of same species through quorum sensing to form biofilms. It is therefore possible that inhibiting Quorum Sensing (QS) or biofilm formation would reduce or eliminate development of drug resistance. Swarming motility is a response initiated by quorum sensing signal molecules and this phenomenon may therefore be used as an indicator for any interference in the quorum sensing system. Detection or measurement of signal molecules like violacein or acyl homoserine lactone also serves to monitor QS. Many natural products exhibit extraordinary properties which deserve to be investigated for their effect on QS. This also applies to vastarrays of antimicrobial compounds already in useif they could be repurposed for their effect on QS.*
Materials and Methods*: In the present study, we evaluated three compounds namely 1) Ethyl alcohol extract of kernel of seeds of mango (Mangiferaindica, MIC=40µg/ml), 2) Streptomycin sulfate (MIC=40µg/ml), a well-known antibiotic and 3) ε-Polylysine (MIC=60µg/ml), acommon food preservative, for their effect on QS.ChromobacteriumviolaceumATCC12472 was used as a reference bacterium to study QS signal AHL (as indicated by violacein)) production in the presence of these compounds. Swarming motility of the colonies was also used as a visible physiological marker for QS.Thirty clinical isolates, 25 Carbapenem resistant and 5 Carbapenem sensitive, were used in this study. They were, treated with sub-MIC concentration of each compound: Streptomycin at 20µg/mL, Mango seed kernel ethanolic extract at 20µg/mL, and ε-Polylysine at 20µg/mL.*
Results*:All the three compounds showed inhibition of QS irrespective of whether the cells were carbapenem resistant or sensitive. The potency for inhibitionof violacein production was as follows: Streptomycin>Mango>Polylysine and for swarming motility inhibitory potency the order was Polylysine>Streptomycin>Mango. Inhibition of swarming was correlated and confirmed by qRTPCR of abaI gene which controls this phenomenon.*
Conclusion:*It is remarkable that all these compounds were not only effective in inhibiting growth but also quorum sensing in clinical isolates of multidrug resistant (MDR) A. baumannii. Therefore, this opens up an opportunity to evaluate these compounds systematically as drugs adjunct to standard antibiotics to which A. baumannii otherwise quickly develops resistance. This opens up an opportunity to repurpose the existing antimicrobials and also evaluate mango seed kernel to treat A. baumannii infections, to enhance the efficacy of the antibiotic therapy above all,*
toreduce development of antibiotic resistance.
Key Word: *Acinetobacter baumannii, Quorum sensing inhibitors, ε-Polylysine, Streptomycin sulfateMangiferaindica, Swarming motility*

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I. Introduction

Emergence of resistance to most of the current drugs in bacteria has created humungous problem for antibiotic therapy[1-2]. Therefore, there is a burning need for identification of new targets and to discover new drugs for effective treatment of such drug resistant bacterial infections. Quorum Sensing (QS) seems to be a promising antimicrobial drug target as it controls the expression of many virulence traits in pathogenic bacteria[4-5]. Quorum Sensing is regulated by diffusible signalling Auto-Inducer (AI) molecules which induce the bacteria

to behave in a population density-dependent manner[6-8]. N-Acyl homoserine lactones (AHLs) of varying chain lengths are the AIs produced by most of the gram-negative bacteria which has a key role in virulence of pathogenic bacteria such as *A. baumannii*.Quorum Sensing system also influences the virulence of the organism. Therefore, quorum quenching would potentially reduce the severity and invasiveness of the infection. Control of virulence through quorum quenching is a desirable weapon[3-4]as chances of development of resistance to a non-lethal compound is rare.Quorum sensing results in communities of the bacteria and eventually biofilms. Biofilm is a characteristic trait responsible for enhancing the virulence and pathogenicity of *A. baumannii*. This property along with its resistance to desiccation and disinfection enables *A. baumannii* survival on harsh surfaces for several months[9].

Another feature under study that might determine the virulence of *Acinetobacter* species, more specifically, *A. baumannii* is its ability to acquire motility. Characteristically, *A. baumannii* are non-motile bacteria as they are morphologically non-flagellated. But recent studies indicate that it exhibits twitching, and swarming motilities, when inoculated onto the surface of solid or semi-solid media. It was noted by Eijkelkamp et al,[10] that when an overnight grown culture of *Acinetobacter* was inoculated onto the bottom of a 1% Mueller-Hinton Agar medium by piercing the agar with a sterile toothpick, the strain demonstrated characteristic twitching motility after overnight incubation at 37 °C. Swarming motility was also observed by these strains when single colonies were inoculated onto 0.25% Luria-Bertani (LB) Agar and left overnight at 37°C. They exhibited a characteristic halo zone of >20 mm in their growth. A study[11] reported that the M2 strain of *A. baumannii* exhibited twitching motility as a result of production of a Type IV Pili (TFP).Another study concluded that *A. baumannii* isolated from blood exhibited greater motility than the ones which were isolated from respiratory sources though the later formed robust biofilms[12].

All thesephenotypes, viz. biofilm formation, resistance to various antibiotics and motility are influenced and regulated by QS system of *A. baumannii*. Quorum Sensing is a phenomenon of inter-cellular communication among bacteria present in a colony, enabling them to function like a multicellular organism. This system is characteristically different for gram-negative and gram-positive bacteria. Typically, in gram-negative bacteria, the QS system works by producing Auto Inducer (AI) signal molecules to help in cell-cell communication[13]. The production of AI is directly related to the cell density of bacteria in a given environment. Increase in number of bacteria leads to accumulation of AI in the environment. The bacteria thus detect the AIs in the environment and collectively regulate their gene expression accordingly. Biofilm formation and motility are thus under the control of the QS. In gram-negative bacteria such as *A. baumannii*, the auto inducers are N-acyl-homoserine lactones (AHLs). These AIs are formed within the cell and can easily diffuse out of the cell across the cell membrane. The AHL, whose concentration increases with the biomass, eventually binds to transcription factors present in the cytoplasm of the cellto regulate the expression of other genes under the QS system.

Since bacteria are rapidly and continuouslyevolving to become antibiotic resistant, targeting the QS system may be a unique therapeutic strategy to control the spread of infection.This would inhibit cell-to-cell communication potentially attenuating the QS associated virulence mechanisms while simultaneously enhancing the efficacy of any antimicrobial therapy This may prove to be an effective method of controlling the emergence and spread of MDR and XDR in nosocomial pathogens such *A. baumannii* and other pathogens that utilize the QS system. World over repurposing of existing antimicrobials is being investigated as no new antibiotics have been approved in the last couple of decades.A study carried out in Atlanta by Saroj and Rather[14]postulated that Streptomycin, at sub-inhibitory concentrations, inhibits the QS in *A. baumannii*. Streptomycin reduces the expression of *abaI*thereby reducing thesynthesisof a specificauto inducer, 3-OH-C12-HSL. The dependence of motility on the auto inducer, 3-OH-C12-HSL and in turn the QS system was previously proved by Clemmer et al[15].

The effect of the QS system on motility had been established by the usage of an abaI::Km mutant (lacking the *abaI* gene) and an M2 wild type strain of the bacteria (possessing the *abaI* gene). It was observed that the mutant showed a significant reduction in motility (75% reduction) compared to wild type in Eiken or Difco Agar. However, this defect in the mutant could be reversed when 3-OH C12-HSL was added to the agar media[15]. This proved that the regulation of the *abaI* gene, responsible for the QS system directly controlled the motility of the organism, and that the gene is a novel target for controlling the spread of *A. baumannii*.

ε-Polylysine (ε-poly-L-lysine, EPL) has been used as a natural preservative in food industry because of its anti-bacterial activity. It is essentially produced as a homo-polymer of 25-35 units of L-lysine. Electrostatic adsorption of ε-Polylysine into the bacterial cell causes shedding of the outer membrane and cytoplasmic damage. This eventually leads to cell death[16]. Its influence on QS and biofilm formation in bacteria has not yet been demonstrated.

Hyper-branched poly (ε-lysine) dendron, RG3K was reported to inhibit biofilm formation and pyocyanin production in *P. aeruginosa*. Further, these dendrons were found to synergistically enhance the antibacterial potency of ciprofloxacin by almost 50% in ciprofloxacin-resistant 48h old biofilm containing *P.*

aeruginosa. The 3rd generation dendrons also reduced the production of pyocyanin by approximately 86% in bacteria[17].

Crude methanolic extract of mango seed kernel (100mg/mL), showed antimicrobial activity on MRSA and *E. coli*[18]. Ethanolic extract of mango seed kernel inhibited the bacterial adhesion on the glass tube indicating its anti-biofilm activity in certain gram-negative organisms such as *P. aeruginosa* and *E. coli*[19] though its effect on drug resistant bacteria, especially on the QS system of multidrug resistant *A. baumannii* has not yet been reported. Therefore we proposed in this study to investigate and demonstrate for the first time the effect of a) to re-purpose a well-known antibiotic Streptomycin which is losing its importance due to emergence of multidrug resistance among several species of bacteria b) to re-purpose a commonly used food preservative, ε-poly-L-lysine and c) to investigate the seed kernels of a local variety of Mango (*Mangiferaindica*) for their effect on the QS system of an important nosocomial gram-negative pathogenic bacteria, *A. baumannii*.

II. Material and Methods

Preparation of bacterial cultures

Clinical isolates of *Acinetobacter calcoaceticus-Acinetobacter baumannii* (ACB) complex were kindly provided by the Department of Clinical Microbiology of Gleneagles Global hospital, Hyderabad (n=30). These isolates were initially isolated from different clinical specimens, which included respiratory secretions, blood, wound swabs, sputum, body fluids including urine and aspirated fluids. *Acinetobacter baumannii*ATCC 19606 was used as the reference strain. These specimens were inoculated onto Mueller Hinton Agar (MHA) and incubated overnight at 37 °C.

A single, isolated pure colony was picked off the plate using a sterile inoculating loop and inoculated into a 5mL of sterile Mueller-Hinton Broth (MHB)Falcon tube. The tubes were incubated at 37 °C for 16 h in a shaker incubator at 150 rpm**.** The cultures were diluted to 1.0 McFarland (A_{600nm} = 0.2 - 0.4) for all the assays.

Bio-monitor strain used for anti-QS activity

Chromobacteriumviolaceum, ATCC 12472 is a wild type strain which produces a characteristic purple pigment called violacein. It was procured from National Centre for Microbial Resource (NCMR, Pune). It was maintained on Nutrient Broth Agar plates at 28 ºC.

Preparation of Mango seed kernel extract

Mango variety, Mallika (*Mangifera indica sp.Mallika*') was obtained from Fruit Research Station, Sangareddy, Telangana. The seed kernels were taken and washed thoroughly under running water tap and then with sterile water. They were then chopped into small pieces and allowed to dry completely in shade. The dried kernels were finely powdered and stored in air-tight containers. The dried powder was then subjected to serial extraction with absolute alcohol in a soxhlet apparatus and the distillate was collected. The extracts were ultimately filtered and dried in a rotary evaporator[20-23]. A stock solution of 100mg/mL was prepared in dimethyl sulphoxide (DMSO, Cat. No. 41639, Sigma- Aldrich) and stored at -20ºC for further use.

Preparation of ε-Polylysine solution

ε-Polylysine (food grade) was bought from BimalPharmaPvt. Ltd., Mumbai. Sterile stock solutions of 100mg/mL were made in molecular biology grade water and stored at -20ºC for further use.

Preparation of Streptomycin solution

Streptomycin sulphate was obtained from HiMedia Laboratories Pvt. Ltd. (Cat. No. TC035-25G). Sterile stock solutions of 100mg/mL were made in water and stored at -20ºC for further use.

Antimicrobial (carbapenem) Susceptibility Testing (AST)

AST was performed on the *A. baumannii* isolates against two antibiotics – Imipenem (10µg) and Meropenem (10µg) as per EUCAST guidelines[24] (2016) by Kirby-Bauer disk diffusion method.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) determination of Streptomycin, Mango extract and ε-Polylysine in clinical *A. baumannii* isolates was done using Kirby-Bauer Disc Diffusion method. Bacterial lawns were prepared on MHA plates with the help of sterile cotton swabs from overnight grown broth cultures of *A. baumannii*. Filter paper discs impregnated with different concentrations (10µg/mL to 100µg/mL) of the compounds were positioned on the bacterial lawn. Minimal Inhibitory Concentration of the compounds against *A. baumannii* clinical isolateswas determined after incubation of the agar plates for 24 h at 37 °C. The lowest concentration which showed the zone of growth inhibition around the discs was reported as the MIC of the compounds[21-25].

Anti-QS activity assay

Disc-diffusion assays were performed as described previously with some modifications[26]. An overnight culture of *C. violaceum* ATCC12472 was swabbed on Nutrient Broth (NB) Agar to make a lawn. Sterile filter-paper discs (6 mm diameter) were loaded with test compounds of various concentrations and placed on the NB agar. After incubation at 28 °C for 24 h, anti-QS activity was measured as the diameter of violacein clear zone. As a control, a disc with equal volume of DMSO was used.

Determination of Motility

All motility assays were performed on fresh LB broth with 0.28% agar. 1µl of fresh cultures (1.0 McFarland) exposed to sub-MIC concentration of the compounds were stabbed onto the surface of the soft-agar medium as described previously[13]. Corresponding bacterial cultures not exposed to the test compounds were used as reference controls. The petri plates were sealed with para-film and incubated at 37°C for 24 h. The isolates which showed a spread zone of at least >10 mmwas considered as swarming positive.

RNA isolation and reverse transcriptase-quantitative PCR (qRTPCR)

Transcription of *abaI* was determined in bacteria exposed to sub-MIC concentration of the compounds and control bacteria as described[27] with some modifications. An over-night grown bacterial culture was diluted to A_{600} of 0.2-0.4. An aliquot of 10µL was inoculated into a 5mL polystyrene tube with and without test drugs and incubated at 37 ºC overnight in shaker incubator. The culture was adjusted to absorbance (A_{600}) of 0.2-0.4 by dilution with fresh broth medium. Bacterial pellets (10,000rpm x 5min) were collected for RNA extraction. RNeasy Mini Kit (Cat No. 74104, Qiagen, Valencia CA) was used to extract total RNA as per manufacturer's instructions. 200 ng of total RNA was converted into cDNA in 10µL total reaction volume (Reverse Transcriptase Core kit, Cat.No.RT-RTCK-03, Eurogentec). qRTPCR was performed as described previously[29] with the primer sets as listed in Table 1 using the Takyon SYBR Green PCR Master Mix (Cat. No. UF-NSMT-B0701, Eurogentec) in qTower 2.0 Real-Time PCR System (Analytica Jena). Transcripts were measured as fold changes using the comparative C*t* method ($2^{-\Delta\Delta C_T}$) in comparison to *rpoB* as the internal reference control for transcription.

Table 1: Oligonucleotide Primers used in this study

Gene	Primer Sequences	Reference
abaI	F: AATGCCTATTCCCTGCTCAC R: ATTGCTTCTTGCAGAATTGC	8
rpoB	F: ATGCCGCCTGAAAAAGTAAC R: CGAGCGCCTACTGGAATTA	23

F: Forward primer; R: Reverse primer

III. Result

The 30 clinical isolates were confirmed as Carbapenem Resistant (CRAB, n=25) and Carbapenem Sensitive (CSAB, n=5) based on the inhibition zones around the antibiotic discs. The isolates were considered CRAB if the zones were <21mm (Imipenem) and <15mm (Meropenem). CSAB isolates showed an inhibition zone of >/= 24mm (Imipenem) and >/= 21mm (Meropenem).

Determination of MICs of compounds

Drug impregnated filter paper discs of different concentrations (10µg/mL to 100µg/mL) were tested. Minimal Inhibitory Concentration (MIC) values were defined as the lowest concentration of each compound, which inhibited the growth of *A. baumannii*. The results were expressed in µg/mL (Table2). Mango seed kernel extract exhibited the lowest MIC (40µg/mL), followed by Streptomycin and Polylysine which showed an MIC of 60µg/mL (Fig. 1).

Determination of Anti QS activity

The anti-QS activity of the extracts was assessed by disc-diffusion assay with *C. violaceum* ATCC12472. Inhibition of violacein pigment by the test compound resulted in a halo zone of clearance around the disc. Such inhibition zones were clearly visible as the background was a lawn of purple bacteria. The anti-QS activity was determined as the inhibition of violacein pigment zone in the monitor strain *C. violaceum*, ATCC 12472 by the test compounds. All three compounds, Mango seed kernel extract, Polylysine and Streptomycin inhibited the violacein pigment as evident by the colourless hallow around the discs containing various concentrations of the compounds on the lawn of *C. violaceum* (Fig. 2). Of all three compounds, at

10µg/mL concentration, Streptomycin showed the maximum inhibition (12mm diameter) followed by Mango (8mm diameter) and Polylysine (6mm diameter).

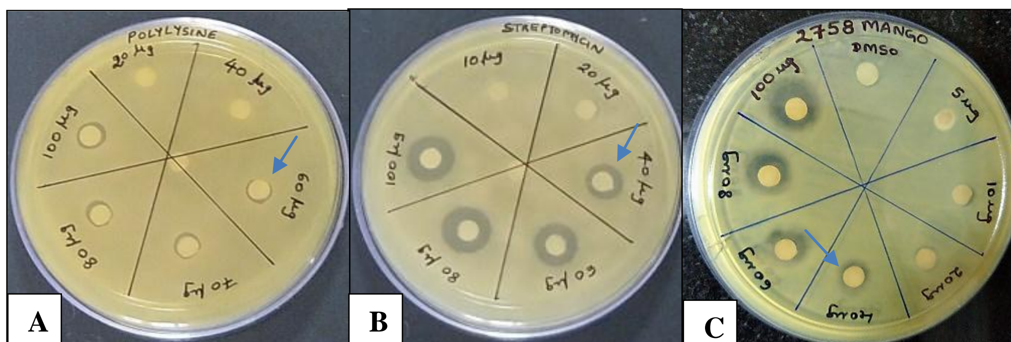

Fig. 1 – MIC determination by disc diffusion method (A)Polylysine (MIC=60µg/mL); (B) Streptomycin (MIC=40µg/mL) and (C) Mango seed kernel extract (MIC=40µg/mL). Blue arrows indicate MIC discs in each image

Table 2: MIC of Compounds

Compound	MIC (µg/mL)
Polylysine	60
Streptomycin	40
Mango	40

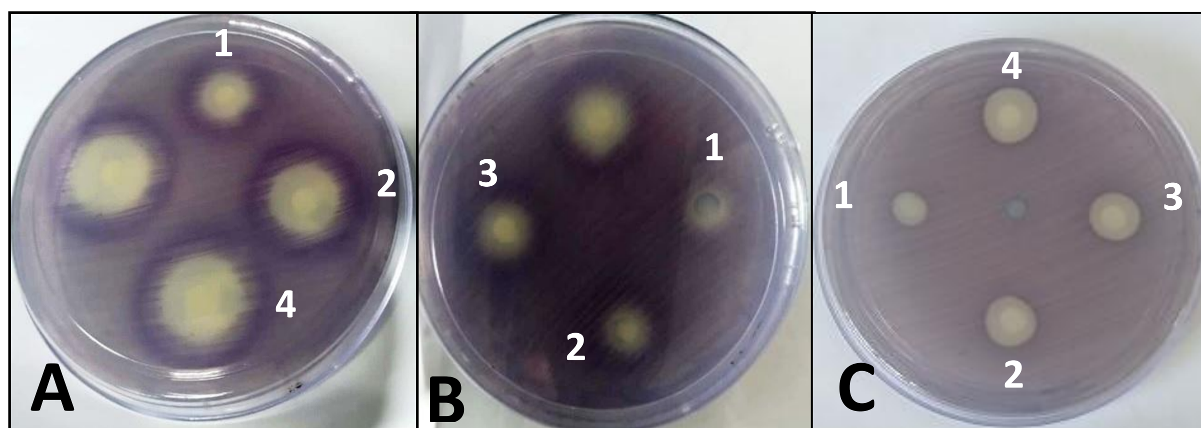

Fig. 2 – Inhibition of violacein pigment production by the test compounds (A) Streptomycin; (B) Mango seed kernel extract and (C) Polylysine; (1=10µg/mL, 2=20µg/mL, 3=30µg/mL, 4=40µg/mL)

Motility assay

Motility positive isolates were those which showed more than 10mm spread of lawn around the point of inoculation. All the isolates were exposed to 20µg/mL (sub MIC) concentration of the compounds whose effect is shown in (Table3). First all the isolates were screened for motility and all the 30 isolates of *A. baumannii*were positive for motility. Mango seed kernel extract completely inhibited the motility of (08/30) 27% isolates and significantly decreased the motility of another (08/30) 27% isolates. It did not show any effect on (14/30) 46% isolates. Polylysine showed strong inhibition of motility in (18/30) 60% isolates and moderate inhibition of motility in another (08/30) 27% isolates. Four (13%) isolates did not show any effect with polylysine. In most of the isolates, Streptomycin could partially inhibit the motility of (20/30) 67% isolates and showed complete inhibition only in 04 (13%) isolates. Motility of six (20%) isolates were unaffected by streptomycin (Fig. 3).

S.No.	Isolate Number	Untreated	Mango Seed Kernel Extract Treated	Polylysine Treated	Streptomycin Treated
1.	19606 (ATCC Reference Strain)	4+	No Inhibition	Inhibited	3+
2.	1674	1+	No Inhibition	+/-	No Inhibition
3.	2575	1+	No Inhibition	+/-	No Inhibition
4.	2469	4+	No Inhibition	Inhibited	+/-
5.	1195	3+	2+	+/-	+/-
6.	945	2+	Inhibited	Inhibited	1+
7.	1066	3+	Inhibited	Inhibited	2+
8.	962	3+	Inhibited	Inhibited	1+
9.	1075	2+	+/-	Inhibited	1+
10.	1103	4+	Inhibited	Inhibited	3+
11.	1229	4+	No Inhibition	No Inhibition	No Inhibition
12.	928	2+	Inhibited	Inhibited	Inhibited
13.	748	4+	No Inhibition	No Inhibition	No Inhibition
14.	930	1+	Inhibited	+/-	+/-
15.	1292	1+	Inhibited	Inhibited	No Inhibition
16.	1571	1+	+/-	Inhibited	1+
17.	1909	3+	2+	Inhibited	2+
18.	1400	3+	1+	Inhibited	1+
19.	1945	1+	Inhibited	Inhibited	1+
20.	1914	1+	No Inhibition	+/-	+/-
21.	2170	1+	No Inhibition	+/-	Inhibited
22.	1992	4+	No Inhibition	+/-	1+
23.	2884	1+	No Inhibition	Inhibited	Inhibited
24.	2699	2+	+/-	+/-	1+
25.	3070	4+	No Inhibition	No Inhibition	Inhibited
26.	2947	3+	No Inhibition	No Inhibition	1+
27.	1678	2+	No Inhibition	Inhibited	1+
28.	1726	3+	1+	Inhibited	1+
29.	1274	2+	+/-	Inhibited	1+
30.	1259	1+	No Inhibition	Inhibited	No Inhibition

Table 3: Effect of compounds on motility in clinical isolates of *A. baumannii*

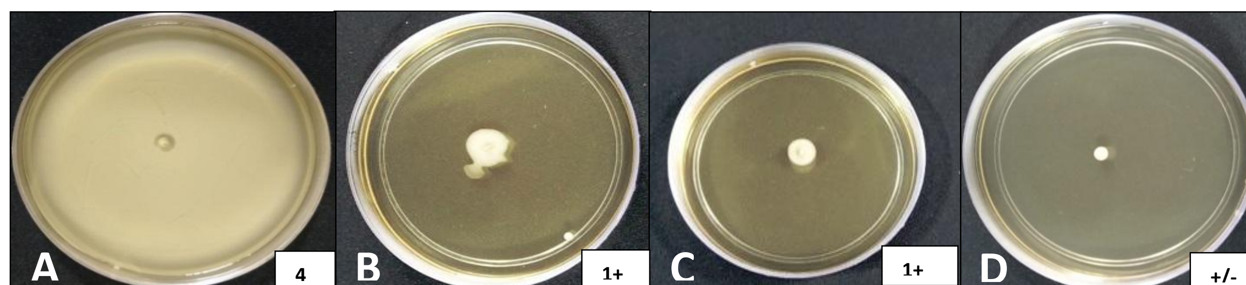

Fig. 3 – Motility pattern of *A. baumannii* (A) Untreated; treated with (B) Streptomycin at 20µg/mL; (C) Mango seed kernel extract at 20µg/mL and (D) Polylysine at 20µg/mL. The motility spread was graded as <10mm=+/-, 10-20mm=+, 21- 40mm=2+, 41- 60mm=3+, >60mm=4+

Although all the three compounds inhibited the motility of the *A. baumannii* isolates, Polylysine was the most effective inhibitorof motility in 87% (26/30) of the isolates. Streptomycin showed its effect on the motility of 80% (24/30) of the isolates. Mango seed kernel extract showed its inhibitory effect on 54% (16/30) isolates (Table4)

Table 4: Summary of Quorum Quenching Screening on Clinical Isolates of *A. baumannii*

Compound	TotalIsolates(n)	No Inhibition	Complete Inhibition	Partial Inhibition	Total Inhibition
Mango	30	14 (46%)	08 (27%)	08 (27%)	54%
Polylysine	30	04 (13%)	18 (60%)	08 (27%)	87%
Streptomycin	30	06 (20%)	04 (13%)	20 (67%)	80%

Reverse transcriptase-quantitative PCR (RT-qPCR)

All relative transcription of *abaI* were normalised to 100% with *rpoB*. Carbapenem sensitive *A. baumannii*showed greater (40%) down regulation of *abaI*compared to the CRAB isolates(20%).This data supports the fact that these compounds decrease the motility of the organisms by targeting the *abaI*of quorum sensing system.

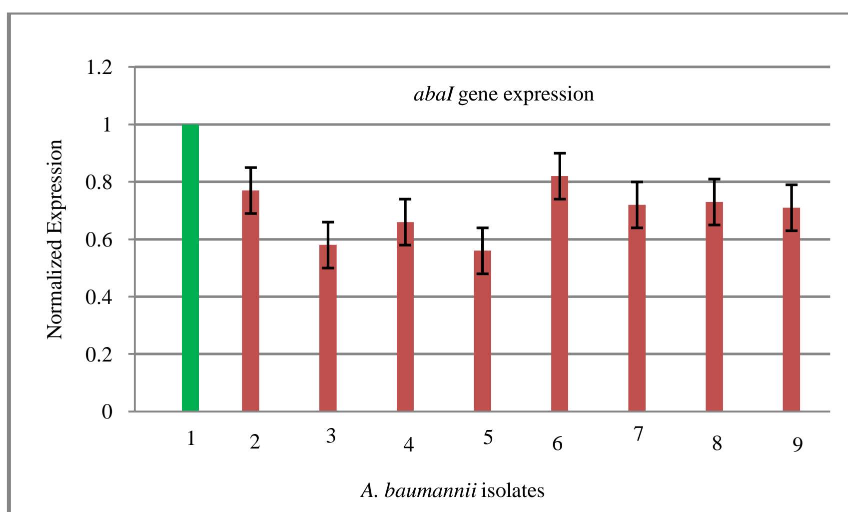

Fig. 4 – Relative expressionof *abaI* by RT-qPCR. Bar 1=calibrator (*rpoB* housekeeping gene) normalized to unity; Bars 2-5 represent mean and SD of 3 determinations with CSAB; 2=Untreated; 3=Streptomycin treated; 4=Mango seed kernel extract treated; 5=Polylysine treated; Bars 6-9 represent mean and SD of 3 independent experiments with CRAB; 6=Untreated; 7=Streptomycin treated; 8=Mango seed kernel extract treated; 9=Polylysine treated

IV. Discussion

Quorum sensing in *A. baumannii* is inhibited by sub-MIC concentrations (20µg/mL each) of Streptomycin, Polylysine and mango seed kernel ethanolic extract. The effect was more pronounced in carbapenem sensitive isolates compared to the resistant isolates. The correlation between down-regulation of the *abaI* gene and motility was remarkable, suggesting possible association between QS and motility in *A. baumannii*. Mango seed kernel extract was tested for the first time for its effect on QS in *A. baumannii* in this study, though Mango Leaf extract has been shown to inhibit pigment production in *C. violaceum* 12472 and the swarming motility in a dose dependent manner in *P. aeruginosa* isolates[29]. ε-Polylysine has so far not been tested for quorum quenching in *A. baumannii*. This study highlights the efficacy of ε-Polylysine on QS and motility for the first time in clinical *A. baumannii* isolates. As Streptomycin was able to inhibit motility and expression of *abaI*, streptomycin itself might act as a 3-OH-C_{12}-HSL antagonist and compete with the native signal molecules binding to the AbaR protein in *A. baumannii* cells thereby disrupting the QS mechanism[5]. These compounds apparently inhibit the production of acyl homoserine lactone synthase directly, which reduces the AHL production and ultimately decreases the QS response. Regardless of the mechanism, our data show that these compounds are effective disrupters of QS in *A. baumannii* and indicate a potential role for these compounds as adjunct drugs in anti-virulence therapy against drug resistant *A. baumannii*and also to reduce the development of drug resistance.

V. Conclusion

Streptomycin at 20µg/mL, Mango seed kernel ethanolic extract at 20µg/mL, and ε-Polylysine at 20µg/mL were effective inhibitors of Quorum Sensing in clinical isolates of *A. baumannii*.

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