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Quorum quenching as a strategy for treating Methicillin Resistant *S. aureus* (MRSA) - Effect of ε-Polylysine, ethanolic extracts of guava leaves and mango seed kernel

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Staphylococcus aureus accessory gene regulatory Quorum Sensing (QS) system facilitates biofilm formation and biofilms are major contributors for antimicrobial resistance. Quorum quenchers could be used as an adjunct to antibiotics to reduce the development of drug resistance. The objective of this study was to evaluate the sub-inhibitory concentration of ethanolic extracts of Mango Seed Kernel Extract (MSKE), Guava Leaf Extract (GLE) and ε-Polylysine (ε-PL) against quorum sensing mediated virulence factors of *S. aureus*.

We evaluated the antibacterial and Quorum Quenching (QQ) activity of these compounds on 10 MRSA and 10 MSSA isolates. The antibacterial activity was tested by disc diffusion assay. The Minimum Inhibitory Concentrations (MIC) for MRSA isolates were: 20 µg/mL for ε-Polylysine, 20 µg/mL for MSKE extract and GLE and for MSSA isolates were: 12.5 µg/mL for ε-Polylysine, 20 µg/mL for MSKE extract and GLE. Quorum Sensing Inhibition (QSI) activity was determined at sub-MIC of 10 µg/mL of all the compounds by studying inhibition of the delta hemolysin on sheep blood agar plate and motility on soft agar media. All these compounds affected the motility and expression of δ-hemolysin activity which confirms that these compounds interfere with the QS activity. The QQ effect was confirmed by quantitation of *hld* (delta hemolysin) transcript and *rnaIII* transcript by qPCR. The results of this study will be presented as proof of QQ activity by ε-PL, MSKE, and GLE on *S. aureus* in general and MRSA in particular, as an attractive proposition for adjunct therapy and anti-virulence therapy of MRSA infections.

Keywords: Delta hemolysin, Motility, MRSA, MSSA, Quorum quenching

Staphylococcus aureus is a gram-positive bacteria causing several diseases like simple skin infections to life-threatening illness like endocarditis, osteomyelitis, and some infections associated with medical devices[1].The development of multidrug resistance by these bacteria has become a challenge for infection control[2,3]. The bacterial communication system, known as QS, appears to be a useful alternative target for new drug development. Natural products are being investigated by several groups in the world for their effect on drug- resistant bacteria[4-6]. Biofilms are major contributors to multi-drug resistance in bacteria[7]. Since QS initiates biofilm formation, inhibiting the QS pathways seems to be an important strategy for the development of new antimicrobials. Various parts of *Mangifera indica* have been scientifically proven to have therapeutic properties[8]. The extracts of guava (*Psidium guajava*) leaves are known for their antimicrobial properties[9]. ε-Polylysine is widely used to preserve food for its broad antimicrobial activity against bacteria that are both gram-positive and gram-negative[10] though its effect on QS and biofilms has not yet been investigated.

Bacteria generate and detect signal molecules through QS and thereby coordinate their behavior depending on cell number. *S. aureus* uses *agr* QS system comprising of *agr* locus. The latter consists of a cluster of five genes (*hld*, *agr A*, *agr B*, *agr C*, *agr D*) including the gene for δ-hemolysin[11]. The *agr* genotype relates to specific virulence factors and some *Staphylococcal* diseases[12].

Quantification of δ-hemolysin can be used as a marker to assess the *agr* activity at the translational level, in addition to the transcriptional level. Lack of hemolytic activity indicates *agr* dysfunction or mutations resulting in delayed *agr* function[13]. Moreover, an intact *agr* locus is essential for *S. aureus* motility called colony spreading[14] on soft-agar media.

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We used delta hemolysin gene (*hld*) and *rna*III as markers for *agr* QS system. We not only genotyped *hld* in all these isolates but also present data to show that the delta hemolysin transcription is down-regulated, its activity severely inhibited and the colony spreading also significantly inhibited when exposed to these compounds.

Materials and Methods

Bacteria and compounds

MRSA (ATCC 43300) and MSSA (ATCC 29213, ATCC 25923) strains were provided by the Infection Biology Lab, Department of Animal Biology, School of Life Sciences, University of Hyderabad, and Hyderabad, India. Clinical isolates were obtained from the Department of Microbiology, Gleneagles Global Hospital, Hyderabad. Mango (*Mangifera indica*) seed kernel and Guava (*Psidium guajava*) leaves were obtained from the Fruit Research Station Sangareddy, Telangana, washed in water, and dried in shade. Epsilon Polylysine (Bimal Pharma Private Limited), DMSO (Sigma Aldrich), Mannitol Salt Agar (MSA), Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), Tryptic Soy broth (TSB) (HiMedia) and Columbia Blood Agar (Difco Laboratories) were used in this study.

Preparation of mango seed kernel and guava leaves extract

Mango seed kernels and guava leaves were washed in running tap water and then with distilled water thoroughly. They were then chopped into small pieces, allowed to dry completely in shade and ground with a homogenizer to a fine powder. The dried powder (100 g) was used for serial extraction with absolute alcohol in a soxhlet apparatus and the distillate was collected. The extracts were filtered and evaporated to dryness in a rotary vacuum evaporator[15]. A stock solution containing 100 mg/mL of MSKE and GLE was prepared separately in dimethyl sulphoxide (DMSO, Cat. No. 41639, Sigma- Aldrich) and stored at −20°C till further use.

Preparation of ε-Polylysine

50 mg of ε- PL was dissolved in 1 mL of Molecular Biology Grade Water (MBGW, 50 mg/mL) and kept at −20°C.

Kirby Bauer disk diffusion

Susceptibility of clinical isolates to antibiotics including Oxacillin (1 µg) and Cefoxitin (30 µg), was evaluated by the agar disk diffusion method on Muller Hinton Agar (MHA) plates as recommended by CLSI[16].

Bacterial DNA isolation

Single colonies were inoculated into Muller Hinton Broth (MHB) and incubated at 37°C. Overnight cultures were grown to 0.5 McFarland, corresponding (10^8 CFU/mL), and used for DNA isolation by standard methods[17]. Briefly, the cell pellets were subjected to initial lysis with 50 µL of lysozyme (10 mg/mL) and incubated at 37°C for 1 h. 70 µL of 10% Sodium Dodecyl Sulfate (SDS), 6 µL of Proteinase K (20 mg/mL) was added and incubated at 50°C for 1 h. 100 µL of 5M NaCl and 600 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) were added to the lysate and vortexed gently for 10 seconds. The lysate was centrifuged at 10000 g for 10 min. The DNA was precipitated from the supernatant by adding 450 µL of chilled isopropanol and centrifuged at 12000 *g* for 10 min. The pellet was carefully washed with 70% ethanol and dissolved in Tris-EDTA buffer. DNA concentrations were checked in Nanodrop (Model No. ND2000, Thermo Scientific) as well as visually in 0.8% agarose gel electrophoresis.

mecA gene amplification by Polymerase Chain Reaction (PCR)

mecA PCR for identification and confirmation of MRSA was first optimized with the reference strain of *S. aureus* ATCC 43300 (MRSA) using the following primers[18].

mecA P4 TCCAGATTACAACTTCACCAGG
mecA P7 CCACTTCATATCTTGTAACG

Amplification of the *mecA* gene was performed in 25 µL reaction volume. The reaction mixture comprised of 2.5 µL 10X PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 2 µL of 10 mM dNTPs, 2 µL of 10 pmol *mecA* forward and reverse primers, 0.2 µL of Kappa Taq DNA polymerase (5U/µL), 1 µL of template DNA in a final volume of 25 L with molecular biology grade water. Initial denaturation of template DNA was done for 5 min at 94°C, followed by 35 amplification cycles, each consisting of: 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C. The final extension was 7 min at 72°C (G-Storm GT-1197). The amplicons were resolved in agarose (2%) gel by electrophoresis and analyzed.

Detection of *hld* gene

Oligonucleotide Primers for identification of δ-hemolysin gene were selected from the published sequences of *S. aureus*[19] and the reaction was first optimized with the reference strain of *S. aureus* ATCC 43300 (MRSA).

hld F.P 5′-AAGAATTTTTATCTTAATTAAGGAAG GAGTG-3′
hld R.P 5′-TTAGTGAATTTGTTCACTGTGTCGA-3′

Amplification of *hld* gene was performed in a total of 25 µL reaction volume consisting of: 1 µL template DNA, 2.5 µL 10X PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 2 µL of 10 mM dNTPs, 2 µL of 10 pmol of each primer, 0.2 µL of Kappa Taq DNA polymerase (5 U/µL) made up to 25 µL with DNase free water. Template DNA was initially denatured in a thermo-cycler at 94°C for 7 min followed by a total of 35 amplification cycles. Each cycle consists of 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C. The final extension was 7 min at 72°C. The amplicons were analyzed in 2% agarose gel.

Minimum Inhibitory Concentration (MIC) determination

The MIC of MSKE, GLE, and ε-PL were assessed on Muller Hinton Agar plates. 100 mg/mL (DMSO) of MSKE and GLE, 50 mg/mL of ε-PL (MBGW) as stock solutions were used for MIC determination. The cultures were grown overnight at 37°C in MH broth to a McFarland 0.5 and spread evenly using a sterile cotton swab over the entire surface of the agar plates[20]. Whatman Filter paper discs containing 5 µg to 100 µg of each compound were positioned on the agar surface. Each extract was checked twice. Then, the plates were incubated for 24 h at 37°C. The MIC was described as the minimum concentration of drugs which inhibited the growth of *S. aureus*[21].

Bacterial cultures

All the experiments in this study, the cultures were retrieved from the glycerol stocks and streaked on Mannitol Salt Agar (MSA) plates for isolating the *S. aureus*. Single colonies were inoculated into Muller Hinton Broth (MHB) and incubated overnight at 37°C and adjusted to a 0.5 McFarland standard (10^8 CFU/mL), sub-MIC (10 µg/mL) concentrations of extracts were added to the test cultures and equivalent volume of DMSO has been added to the control cultures. All the cultures were incubated with shaking, at 37°C. Aliquots were taken for assessing the motility, delta hemolysin activity, and for RNA extraction.

Colony Spreading Activity of *S.aureus*

Tryptic Soy broth supplemented with 0.24% (*w/v*) agar was autoclaved and poured into petriplates. Plates were allowed to dry in laminar air flow for 2-3 h before inoculating the bacteria. Aliquots (2 µL) of both treated (MSKE, GLE, and ε-PL) and control (untreated) cultures were spotted onto the center of the plate using a pipette. The plates were incubated at 37°C for overnight[15].

Assessment of delta-hemolysin activity on SBAP

Columbia blood agar supplemented with 5% (*v/v*) sheep blood was poured intopetri plates. The ability to produce δ-hemolysin in the test isolate was measured by cross-streaking the test isolate perpendicular to a δ-hemolysin reference culture of *S. aureus*, ATCC 25923. Aliquots of both treated (MSKE, GLE and ε-PL), and untreated cultures were streaked on sheep blood agar plates at right angles to *S. aureus* ATCC 25923. δ-hemolysin positive isolates produce a clear zone at the confluence of the two streaks (the area around the intersection) due to enhanced hemolysis[22] and any inhibition of this will result in the disappearance of the confluent clear hemolytic zone.

RNA Extraction and cDNA synthesis

Total RNA was isolated from both treated and untreated cultures using the RN easy mini kit (Qiagen Cat No. 74104) based on the manufacture's protocol. RNA concentrations and purity were determined by measuring the absorbance at 260 nm and 280 nm (Nanodrop, Model No. ND2000, Thermo Scientific). Extracted RNA was preserved at −70°C. Conversion of RNA to cDNA was synthesized using Reverse Transcriptase Core Kit (Eurogentec) following the manufacturer's instructions in q-Tower 2.2 (Analytika Jena).

Quantitative Real-Time PCR

Takyon™ Rox SYBR® Mastermix dTTP Blue (Eurogentec) PCR master mix was used for the quantification and the real- time PCR analysis, according to the manufacturer's instructions. The primers (Eurofins Bangalore) used in this study were *hld*[19], *rnaIII*[23], and 16S *rRNA*[24] (Housekeeping gene as internal control).

rna III F.P 5′- GAAGGAGTGATTTCAATGGCACA AG-3′
rna III R.P 5′-GAAAGTAATTAATTATTCATCTTA TTTTTAGTGAATTTG-3′
16S rRNA FP5′-TGT CGT GAG ATG TTG GG-3′
16S rRNA RP5′-CGA TTC CAG CTT CAT GT-3′

Amplification was done under following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 15s and 56°C for the 30s and at 60°C for 1 min. All the samples were analysed in triplicate, and the housekeeping gene 16S *rRNA* used as an internal control to normalize the expression levels of the samples. Relative expression levels were analyzed by the **ΔΔ**CT method.

Results and Discussion

Screening of clinical isolates for antibiotic sensitivity by Kirby Bauer disk diffusion method

Isolates displaying inhibition zone <22 mm with Cefoxitin and <10 mm with Oxacillin were considered resistant and >23 with Cefoxitin and >13 with Oxacillin were sensitive as per CLSI guidelines. *S. aureus* isolates (n=152) were categorized as MRSA 63.15% (n=96) and MSSA 36.84% (n=56).

Confirmation of MRSA by *mecA* PCR

MRSA isolates were confirmed by the presence of the *mecA* gene (Fig. 1).

Genotyping of delta hemolysin gene in the clinical isolates of *S. aureus*

All the 20 isolates included in this study contained the δ-hemolysin gene (Fig. 2).

Minimal Inhibitory Concentrations (MIC) determination

The antibacterial activity and MIC were determined by the disc diffusion assay. The MICs on MSSA isolates were: 12.5 µg/mL for ε-Polylysine, 20 µg/mL for mango seed kernel extract and guava leaves extract. The MICs on MRSA isolates were: 20 µg/mL for ε-Polylysine, 20 µg/mL for mango seed kernel extract, and guava leaves extract (Fig. 3).

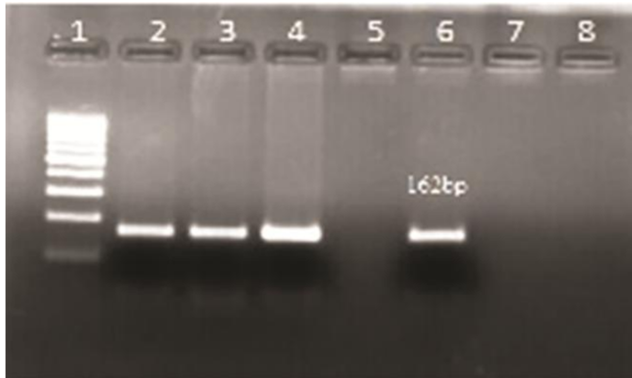

Fig. 1 — *mecA* gene amplification in selected isolates of *S. aureus* by PCR. Agarose (2% *w/v*) gel electrophoresis showing the *mecA* amplicon from MRSA isolates. Lane 1: 100 bp ladder; Lane 2, 3, 4: *mecA* gene PCR product (162 bp) in clinical isolates of *S. aureus*; Lane 5: *mecA* gene PCR product (162 bp) of *S. aureus* ATCC 43300

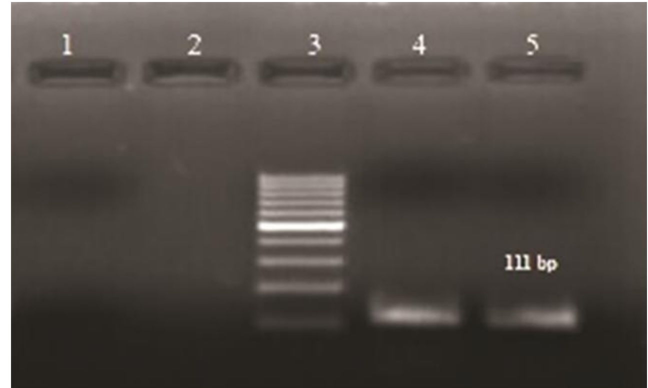

Fig. 2 — PCR amplification of *hld* in clinical isolates of *S. aureus.* Agarose (2%) Gel Electrophoresis of *hld* amplicons (111bp) from a couple of representative isolates. Lane 1: Negative control; Lane 3: 100 bp Ladder; Lane 4: Positive control (ATCC 43300); Lane 5: Clinical isolate of *S. aureus*

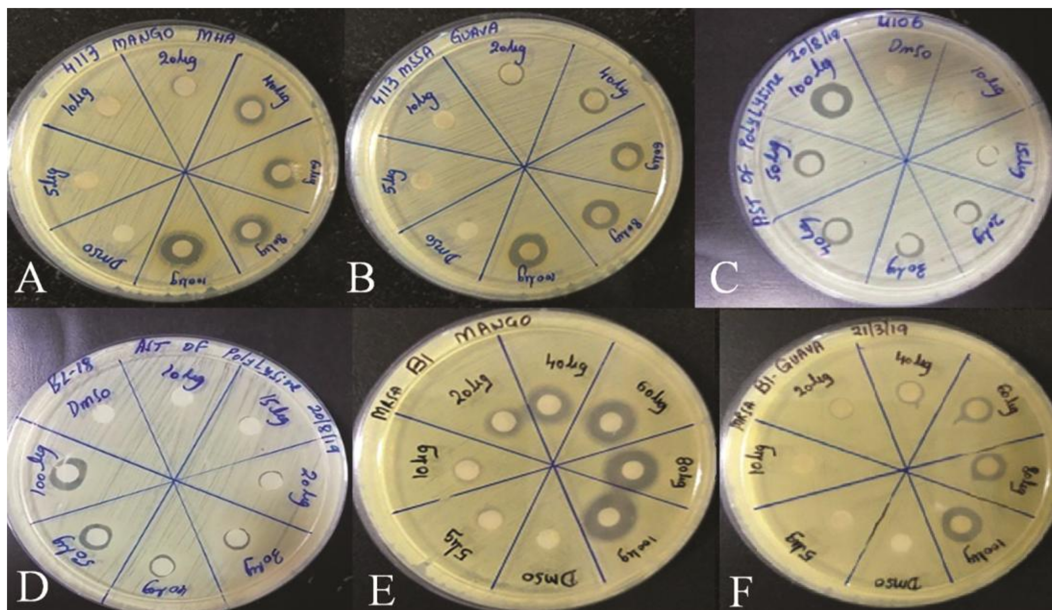

Fig. 3 — MIC of MSKE, GLE and ε-PL on MSSA and MRSA. Zone of inhibitions produced on MSSA by (A) MSKE; (B) GLE; (C) ε-PL & on MRSA by (D) ε-PL; (E) MSKE; and (F) GLE

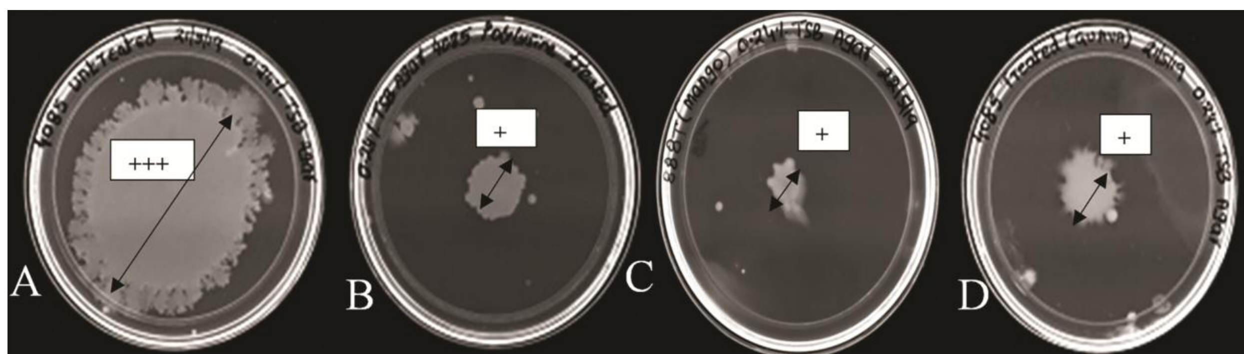

Fig. 4 — Motility profile of clinical isolates of *S. aureus*. The diameter of the motility zone decreased when cultures were exposed to (A) Untreated (> 60 mm zone diameter +++); (B) Treated with ε-PL (10-20 mm zone diameter +); (C) Treated with MSKE (10-20 mm zone diameter +); and (D) Treated with GLE (10-20 mm zone diameter +)

Effect of candidate drugs on colony spreading of *S. aureus* isolates

S. aureus spreads rapidly on the soft agar surface. The rate of spreading expansion was distinct for different clinical isolates. All the tested *Staphylococcal* isolates demonstrated a strong ability to spread on the 0.24% agar. All the compounds MSKE, GLE, and ε-PL were able to inhibit colony spreading activity at a sub-MIC concentrations of 10 µg/mL when compared to untreated controls (Fig. 4).

Demonstration of δ-hemolysin activity on Sheep Blood Agar Plates

An enhanced area of hemolysis at the intersection of ATCC 25923 (Reference strain for β-hemolysin) and test isolate streak indicates the isolate is positive for δ-hemolysin. All the isolates tested in this study were positive for delta-hemolysin production (Fig. 5).

Effect of candidate drugs on the expression of *hld* and *rnaIII* by q-RT PCR

The effect of sub-inhibitory concentrations of the compounds (10 µg/mL) was assessed by measuring the expression of *hld* and *rnaIII* gene transcription by q-RTPCR. q-RTPCR was performed to evaluate the relative expression levels of the *hld* and *rnaIII* in cultures that were exposed to the candidate drugs in comparison to those not exposed to them. *16s rRNA* was used as a housekeeping gene for normalization and as a reference gene to determine the relative impact on the transcription of *hld* and *rnaIII* (Fig. 6).

Vertical bars represent mean transcript levels and the error bars represent the standard deviation from 3 separate experiments. The *hld* transcript levels were varied in the treated cultures. GLE treated cultures showed a remarkable down-regulation in the *hld* transcript (A3-0.67) whereas MSKE & ε-PL treated cultures showed marginally decreased *hld* transcript levels A4-0.71 & A8-0.77 (MSKE), A5-0.76 & A9-0.82 (ε-PL). In the case of *rnaIII* transcript, MSKE treated cultures showed a remarkable down-regulation in the *rnaIII* transcript (B4-0.67) whereas GLE & ε-PL treated cultures showed marginally decreased *rnaIII* transcript levels B3-0.87 & B7-0.89 (GLE), B5-0.76 & B9-0.82 (ε-PL).

Fig. 5 — Delta hemolysin activity, see the enhancement and extension of beta hemolytic zone at the intersection in clinical isolates of *S. aureus*. The enhanced zone of activity which is due to delta hemolysin is scored as 1+, 2+ and 3+ depending on the area of enhancement. 1+ = Low activity of δ-hemolysin # 1021, B1; 2+ = Moderate activity of δ-hemolysin # BL-53; 3+ = High activity of δ-hemolysin # 1720, 4171

Effect of candidate drugs on the *agr* function

δ-Hemolysin activity from the *Staphylococcus aureus* can be used as markers to assess *agr* function or QS system. MSKE, GLE, and ε-PL influenced the

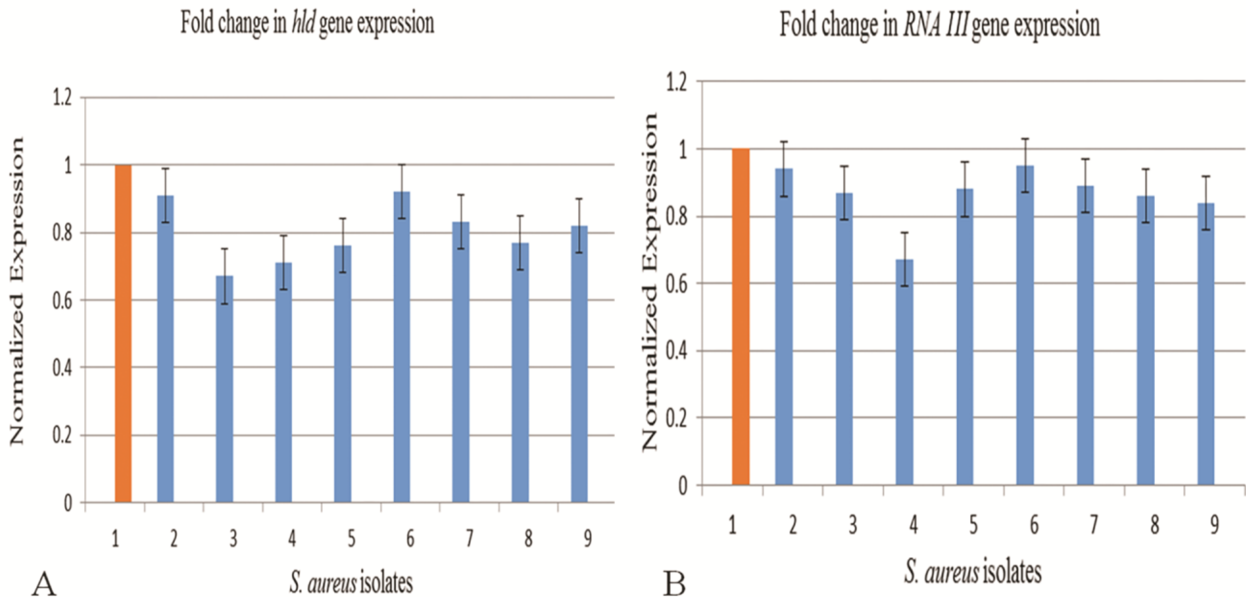

Fig. 6 — Transcription levels of (A) *hld* and (B) *rnaIII* in clinical isolates of *S. aureus* and the effect of exposure to candidate drugs. 1= calibrator (Housekeeping gene) normalized to unity; on MRSA: 2= untreated; 3= GLE treated; 4= MSKE treated; 5= ε-PL treated; on MSSA: 6= untreated; 7= GLE treated; 8= MSKE treated; 9= ε-PL treated

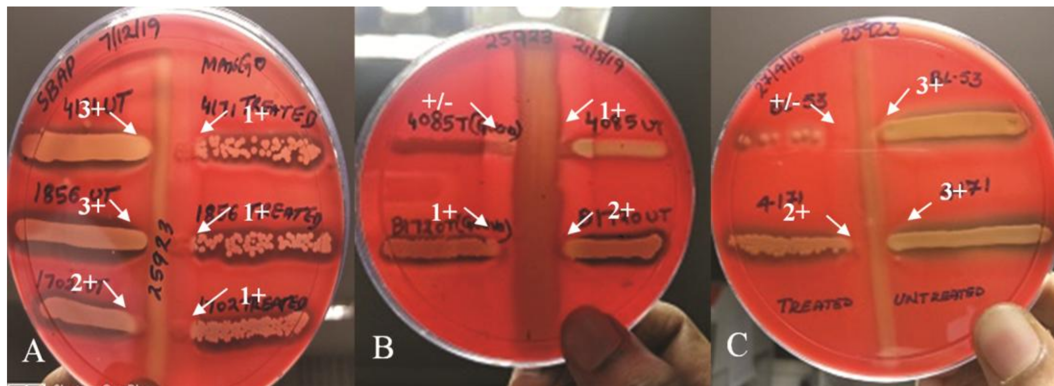

Fig. 7 — Effect of MSKE, GLE and ε-PL on *agr* function. Inhibition of δ-hemolysin production by (A) MSKE compared with controls; (B) GLE compared with controls; and (C) ε-PL compared with controls in clinical isolates of *Staphylococcus aureus*. (1+ = Low activity of δ-hemolysin, 2+ = Moderate activity of δ-hemolysin, 3+ = High activity of δ-hemolysin, +/− = Marginal activity of δ-hemolysin)

δ-hemolysin production in all isolates. δ-hemolysin production was partially inhibited in the treated samples compared to controls (Fig. 7).

Conclusion

In conclusion, our results showed that mango seed kernel, guava leaves extract, and ε-PL at sub-MIC concentrations quench the *agr* QS system. QQ happens by attenuation of signal transduction. Such attenuation would cause the observed reduction in δ-hemolysin production at both transcription and translation levels and inhibit the motility of *S. aureus* by the compounds when exposed. Interestingly MRSA isolates were more sensitive to QQ than MSSA isolates which probably needs to be investigated further. Guava Leaf Extract and Mango Seed Kernel Extract seem to be more potent in inhibiting the transcription of *hld* compared to ε-PL of MRSA isolates while Mango seed kernel extract strongly down-regulated the *rnaIII* transcription compared to GLE or ε-PL. Delta hemolysin activity seems to more susceptible to inhibition by mango seed kernel and ε-PL than GLE. It is possible that all three compounds have some unique mechanism of action in addition to their effect on the *agr* QS system. QQ and anti-virulence therapy would play a significant role in controlling and managing infectious diseases in the

future. This strategy would help identify compounds which not only help to reduce Multi-Drug Resistance (MDR) development but also control the severity of infections. Anti-virulence targets are unlikely to become resistant as they do not pose unusual metabolic pressure on the host bacterium. Such compounds are good candidates for adjunct therapy. It is promising that extracts of MSKE and GLE and a widely used food preservative, the ε-PL show strong quorum quenching properties. Differential fractionation, isolation of active components from these extracts, and studies to understand the mechanism of action of active components are in progress.

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Conflicts of Interest

All authors declare no conflict of interest.

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