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Quorum quenching activity of *Syzygium cumini* (L.) Skeels and its anthocyanin malvidin against *Klebsiella pneumoniae*

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ABSTRACT

Many bacterial species use their intercellular signaling mechanism called quorum sensing (QS), which is found to be implicated in various factors including bacterial pathogenicity and food spoilage. Interrupting the bacterial communication is an attractive strategy to develop novel QS-based antibacterial drugs. Present study is aimed to investigate the quorum sensing inhibitory activity of *Syzygium cumini* and its anti-biofilm property against opportunistic pathogen using a biosensor strain *Chromobacterium violaceum* CV026. Ethanol extract of *S. cumini* was investigated for its anti-QS activity, and the possible active component was identified by docking with LasR receptor protein. Based on docking analysis, methanol extract was enriched for its total anthocyanin (STA) and its effect on QS regulated phenotypes was assessed. STA specifically inhibited the violacein production in *C. violaceum*; biofilm formation and EPS production in *Klebsiella pneumoniae* up to 82, 79.94 and 64.29% respectively. Synergistic activity of conventional antibiotics with STA enhanced the susceptibility of *K. pneumoniae* up to 58.45%. Molecular docking analysis of active components attributes the QSI activity of *S. cumini* to malvidin. Malvidin exhibited highest ligand binding with LasR receptor protein with docking score more than −7. Effect of malvidin to interrupt the QS regulated phenotypes was also assessed, and it was found to reduce the violacein production, biofilm formation and EPS production of *K. pneumoniae* in a concentration-dependent manner. These findings suggest that *S. cumini* can be used as novel QS-based antibacterial/anti-biofilm agent to manage food-borne pathogens and to increase food safety.

1. Introduction

Many bacterial species are commonly known to control their expression of gene circuits in a population dependent manner through the release of extracellular signaling molecules called auto-inducers, usually oligopeptides in Gram-positive and N-acyl-homoserine lactones (AHL) in Gram-negative bacteria [1]. At a threshold level of population, AHL interacts with the receptors and triggers the target gene expression, including virulence, antibiotic production, biofilm formation, swarming and bioluminescence. Various bacterial models such as *Chromobacterium violaceum* CV026, *Agrobacterium tumefaciens*, *Vibrio fischeri* has been used to study bacterial quorum sensing [2,3]. *Klebsiella pneumoniae* is recognized as an important pathogen in most of the reported outbreaks of food-borne diseases, causing serious illness like destructive lung inflammation, gastroenteritis, and multi-organ failure. Genes associated with biofilm formation through the release of type-2 QS regulatory molecules and AI-2 transport genes in *K. pneumoniae* were also identified [4]. Role of LuxS dependent signal molecules in the earlier stages of biofilm formation in *K. pneumoniae* was also elucidated [5].

Many synthetic compounds like macrolides [6,7], furanyl hydrazide [8], cyclohexanone [9], furanones [10], fimbrolide [11] have been shown to effectively inhibit microbial interaction. But, many of these compounds may be too reactive or highly toxic for treatment of bacterial infections in humans. Thus, there is an increasing demand for the identification of natural compounds to inhibit the QS regulation which could result in the development of novel QS based antibacterial drugs for the management of microbial diseases in humans, food industries, agriculture and aquaculture [12].

Syzygium cumini commonly called as *Jamun*, black plum or Indian blackberry belonging to the family Myrtaceae, is an important underutilized tropical fruit found over the greater part of India and Sub-Himalayan tract. It has been widely used to treat diabetes,

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asthma, sore throat, bronchitis and dysentery by the traditional practitioners over many centuries [13]. Though extensive work has been done on anti-oxidant, free radical scavenging activities, anti-fungal, anti-diabetic and anti-inflammatory activities [14–16], *S. cumini* as a source of anti-QS and anti-biofilm has not been well studied. Considering its multiple therapeutic properties, this investigation has been made with the primary objective to identify the QS inhibitory activity of *S. cumini*. Further to identify the compound(s) responsible for the QSI activity, molecular docking analysis was performed with the ligand binding domain the LasR receptor protein. Furthermore, top ranking compound on docking analysis was selected for screening its potential to reduce the QS dependent factors production in *K. pneumoniae* [17,18].

2. Materials and methods

2.1. Plant materials and extraction

S. cumini fruits were collected in and around the Pondicherry, India. Fruit pulp was separated manually, and tray dried at 55 °C for overnight. Dried materials were milled to yield a finely ground substance; crude methanolic extracts were prepared by dissolving 100 g of powder in 70% methanol (v/v) and kept in the orbital shaker for 24 h at room temperature and filtered through Whatman No. 1 filter paper. Extracts were concentrated by rotary evaporator and redissolved in appropriate concentrations of de-ionized water to obtain desired dilutions. Extracts were filtered through 0.22 μm syringe-filter and stored at −40 °C for further use.

2.2. Bacterial strains and culture conditions

Bacterial cultures used in this study include *C. violaceum* strain CV026 (CECT 5999), *C. violaceum* MTCC2656 and *K. pneumoniae* strain PUFST23 (GenBank: KF817575) dry fish isolate from the departmental culture collection. All the cultures were selected based on their QS dependent phenotypes. CECT 5999 and MTCC2656 were grown in Luria–Bertani (LB) medium and *K. pneumoniae* was grown at nutrient broth. CECT 5999 and MTCC2656 were routinely cultured aerobically in LB broth supplemented with kanamycin (20 μg/ml) in shaking incubator at 30 °C prior to experiments. N-hexanoyl-DL-homoserine lactone (HHL) was added to the medium to induce the violacein production in CV026, when required.

2.3. AHL bioassay

K. pneumoniae inducing the violacein production in *C. violaceum* CV026 was tested by cross and parallel streaking against the reporter strain on LB agar plates. Plates were incubated at 30 °C for 24 h. Induction of violacein pigment in the reporter strain inferred the positive result; wild strain of *C. violaceum* (MTCC 2656) served as a positive control. Experiment was repeated twice to ensure the continuous production of violacein pigment by the reporter strain.

2.4. Bioassay for QS inhibitory activity

Disc diffusion assay was carried out to detect anti-QS activity of pulp extracts. One hundred microliter of exogenous HHL (1 mg/ml) was added to 200 ml of sterilized LB media at appropriate temperature; this was gently mixed and poured into the petri plates. Overnight culture of *C. violaceum* CV026 was swabbed evenly onto the solidified agar surface. Sterile discs were loaded with 20 μl of extract at different concentrations and placed onto the agar plates which were then incubated at 27 °C for 24 h. QSI activity was scored as an obscure, colorless, but doable halo around the discs. Sterile deionized water was used as a control.

2.5. Docking analysis

Phytochemical constituents of *S. cumini* were taken from the published literature (Sah & Verma, 2011 [19]; Sikder et al., 2012 [20]). Compound structure of LasR receptor protein (PDB ID 2UV0) was obtained from protein data bank, which was docked with the three-dimensional structures of 43 active components of *S. cumini*, which was obtained from Pubchem database (http://pubchem.ncbi.nlm.nih.gov). PDB 2UV0 structure contains four chains (E, F, G and H) whose confirmation was similar which was analyzed by superimposing with chimera. Since, the H chain is longest and contained the preferred binding site for the natural ligand N-Hexanoyl DL-homoserine lactone, all the water molecules and other chains were removed from the LasR receptor protein for docking analysis to select the potential QSI compound from *S. cumini*. Docking studies were performed with the Schrodinger (ver. 9.2).

2.6. Enrichment and quantification of total anthocyanins

Jamun pulp powder was extracted with three volumes of 75% aqueous ethanol containing 10 mM HCl. The mixture was sonicated for 15 min to increase the extraction efficiency which was then centrifuged at 1000× *g* for 10 min. Above procedure was repeated for three times, and the pooled supernatants were concentrated in rotary vacuum evaporator. Anthocyanins were enriched by loading the concentrated extract on amberlite XAD-7 column. Free sugars were eluted with 15 ml of 10 mM HCl to remove sugars. Anthocyanins were eluted with 34 ml of methanol containing 0.1% HCl and dried under vacuum, which was then stored at −20 °C.

Total anthocyanin content was determined using the pH differential method and expressed as cyanidin-3-glucoside equivalent using molar extinction coefficient of 26,900 L cm^{-1} mol^{-1} and molecular mass of 449.2 g mol^{-1} [21]. Briefly, 0.2 ml samples containing different concentrations of extracts were mixed separately with 0.8 ml each of 0.025 M potassium chloride, pH 1.0 (adjusted with HCl) and 0.4 M sodium acetate, pH 4.5. The reaction mixtures were allowed to equilibrate at room temperature for 15 min and absorbance was measured at 510 and 700 nm. The difference in absorbance between the two samples was then calculated using the formula:

$$\text{Absorbance (A)} = (\text{A 510} - \text{A700})\text{pH1.0} - (\text{A 510} - \text{A700})\text{pH4.5}$$

Finally, total anthocyanins/anthocyanidins in the samples were calculated by the following formula:

$$\text{Total anthocyanins} = \frac{\text{Absorbance (A)} \times 449\ \text{g/mol} \times \text{dilution factor}}{26900\ \text{L/cm/mol}}$$

2.7. Minimum inhibitory concentration of S. cumini total anthocyanin

MIC of STA was determined against *C. violaceum* CV026 and *K. pneumoniae* as recommended by the Clinical and Laboratory Standards Institute, USA (2006). Bacterial cultures inoculated into 20 ml of LB medium added with extracts to attain the final concentrations ranging from 0.1 to 1 mg/ml and incubated for 24 h. Before and after incubation, the absorbance of the medium was measured at wavelength of 600 nm. Lowest concentration of STA,

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which showed inhibition of visible growth, represented the MIC. All further experiments were carried out only at Sub-MIC level and were repeated thrice.

2.8. Bioassay for QS inhibitory activity

Disc diffusion assay was carried out to detect anti-QS activity of STA. One hundred microliter of exogenous HHL (1 mg/ml) added with 200 ml of sterilized LB at appropriate temperature; this was gently mixed and poured into the petri plates. Overnight culture of *C. violaceum* CV026 was swabbed evenly onto the solidified agar surface. Sterile discs were loaded with 20 μl of extract at the concentration below 1.4 mg/ml, which was MIC level for STA. Loaded discs were placed onto the agar plates which were then incubated at 27 °C for 24 h. QSI activity was scored as an obscure, colorless, but doable halo around the discs.

2.9. Broth assay

Quorum sensing inhibitory activity of STA was quantitatively determined by broth assay. LB broth supplemented with HHL and STA at different concentration (0.1, 0.5 and 1 mg/ml) were inoculated with reporter strain, which was then incubated at 30 °C for 24 h.

Violacein extraction was carried out as described by Choo et al. [22]. Briefly, 1 ml of culture from each flask was centrifuged at 8000 *g* for 5 min to precipitate the insoluble violacein. Obtained pellet was dissolved in 1 ml of DMSO and vortexed robustly to solubilize the violacein completely. Above mixture was centrifuged again to remove the cells and quantified at 585 nm using microplate reader (Biotek, USA). The experiment was repeated for triplicate values and the percentage of inhibition was calculated by the formula:

$$\frac{\text{Control OD585 nm} - \text{Test OD585 nm}}{\text{Control OD585 nm}} \times 100$$

2.10. Reduction in exopolysaccharide

K. pneumoniae was grown in LB broth at 30 °C in static condition; biofilms adhered to the walls of the test tubes were harvested at late-log phase by centrifugation at 8, 500 rpm for 30 min at 2 °C to obtain the crude EPS. Supernatant was filtered through 0.22 μm syringe filter and added with three volumes of chilled ethanol and incubated overnight at 2 °C to precipitate the dislodged EPS. Precipitated EPS was collected by centrifugation at 8500 rpm for 30 min which was then dissolved in 1 ml of deionized water, and stored at −40 °C until further use. Total carbohydrate content in the EPS was measured by phenol-sulfuric acid method using glucose as standard [23].

2.11. Inhibition of biofilm formation by microtitre plate assay

Microtitre plate assay was performed to quantify the effect of STA on the biofilm formation by *K. pneumoniae*. Briefly, 1% of overnight test bacterial culture was inoculated to LB broth with and/or without the extract and incubated at 30 °C in a 12-well microtitre plates. After incubation, planktonic cells were removed by carefully rinsing with double-distilled water and the surface adhered cells were stained with 100 μl of 0.2% crystal violet solution (HiMedia, India) for 10 min. Excess stain was removed and CV in the stained cells were solubilized with 100 μl of 95% ethanol [24]. Biofilm biomass was quantified by measuring the intensity of CV at OD_{650nm} by using a microplate reader (Biotek, USA).

2.12. In-situ visualization of biofilm

One milliliter of sterilized LB broth containing glass slide 1 × 1 cm in 12 well microtitre plate with and/or without the extracts were inoculated with 1% of overnight culture. After 24 h of incubation, slides were carefully washed with distilled water to remove loosely attached cells. Biofilm in the glass slides were tainted by crystal violet and visualized under light microscope at 100× magnification (Nikon, Japan).

For scanning electron microscopy (SEM) sample preparation was done as described by Lembke et al. [25]. Briefly, biofilms formed on the glass slides were fixed with 2.5% glutaraldehyde for 1 h. Fixed glass slides were washed using 0.1 M sodium acetate buffer (pH 7.3). Slides were then dehydrated with ethanol; air dried, carbon sputtered and analyzed using a scanning electron microscope (Hitachi S-3000H, Japan).

Bacterial biofilms were allowed to develop on the glass slides 1 × 1 cm with and without the plant extract for Confocal Laser Scanning Microscopy (CLSM). After 24 h of incubation, glass slides were stained with acridine orange (1%) for 1 min. Stained glass slides were rinsed with distilled water to remove the excess stain. Glass slides were then dried and visualized under advanced confocal microscope at 10× (LSM 710, Zeiss, Germany).

2.13. Synergistic effects of STA and antibiotics against K. pneumoniae

Twelve well microtitre plates containing 1 ml of LB broth along with STA and antibiotics was inoculated with 1% positive culture. Inoculated plates were incubated at 32 °C for overnight. Wells without STA were maintained as control [26]. Antibiotics that were tested include ofloxacine (10 μg), tetracycline (30 μg) and chloramphenicol (10 μg). After incubation, plates were measured at OD_{600} using a microplate reader (Biotek, USA).

2.14. Extraction and quantification of AHLs from K. pneumoniae

Overnight bacterial culture of *K. pneumoniae* was inoculated into 100 ml appropriate growth medium containing, STA (0.1, 0.5 and 1 mg/ml), the culture broth without the above extract was served as control. Spent culture supernatants were extracted with ethyl acetate and pooled extracts were dried over anhydrous magnesium sulphate and evaporated to dryness. Residues were resuspended in 100 μl of HPLC grade acetonitrile and stored at −40 °C until further use [27].

High-performance thin layer chromatographic method was optimized for the quantitative evaluation of AHL profile in treated and untreated culture broth of *K. pneumoniae*. Two microliters ethyl acetate extract of both treated and control broth were applied onto C18 RP − Silica gel 60 F254 (10 × 10 Merck, Germany) using automatic TLC sampler 4 (ATS4) and the chromatograms were developed using methanol and water (60:40 v/v). Developed plate was air dried and overlaid with a thin film of soft agar seeded with reporter strain *C. violaceum* CV02, incubated for 24 h at 32 °C. AHLs detected by blue pigmentation were scanned at 585 nm by TLC scanner (CAMAG, Switzerland), and results were recorded.

2.15. Evaluation of QSI potential compound

It has been already reported that malvidin comprises 15% of total anthocyanin in *S. cumini* fruit [28]. In the current study molecular docking analysis was performed to identify the QSI potential of malvidin and subsequently through experimental analysis like

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violacein inhibition, EPS production and biofilm formation in *K. pneumoniae* as mentioned previously with active components and STA extract respectively.

2.16. Statistical analysis

All the experiments were repeated for triplicate values, and the figures obtained were represented as mean value. Differences among control and test were analyzed by means of one way ANOVA.

3. Results

3.1. QS inhibitory bioassay

K. pneumoniae (KF817575) induced AHL mediated violacein production both in cross and parallel streaking method. Methanol extract of *S. cumini* pulp showed anti-QS activity against the reporter strain *C. violaceum* CV026 indicated by the loss of purple pigmentation. Methanol extract at the concentration of 4 mg/ml exhibited anti-QS activity through violacein inhibition around the discs. Pulp extract did not show any antibacterial activity even at the concentration of 5 mg/ml. Upon enrichment strong anti-QS activity was observed at the concentration of 0.1 mg/ml, indicated by the zone of pigmentation loss. No significant changes were observed in the bacterial growth pattern up to the concentration of 1 mg/ml (Fig. 1).

3.2. Molecular docking and total anthocyanin content

Molecular docking of 43 active components of *S. cumini* with the receptor protein showed that anthocyanins like petunidin, malvidin and cyanidin displayed docking score more than −7.7 most comparable with that of the natural ligand (Table 1). Upon estimation by pH-differential method enriched extract of *S. cumini* pulp powder contained 0.91% anthocyanins.

3.3. Quantitative inhibition of violacein production

In flask incubation assay all tested concentration (0.1, 0.5 and 1 mg/ml) showed a significant drop in violacein content without inhibition of bacterial growth. At the concentration of 0.1 mg/ml, 31.95% inhibition was observed. Gradual increase in the inhibitory activity was observed with increasing concentration of enriched extract and maximum of 82% inhibition was observed at the concentration of 1 mg/ml (Fig. 2).

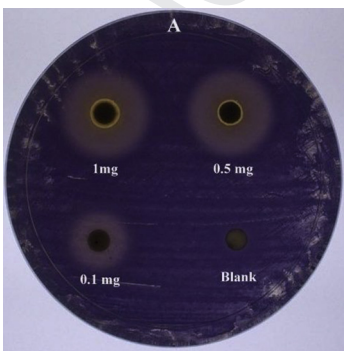

Fig. 1. Quorum sensing bioassay of *S. cumini* methanolic extracts at different concentration (0.1, 0.5 and 1 mg/ml) showing inhibition of HHL mediated violacein inhibition.

Table 1
Docking scores of active components in *S. cumini*.

Pubchem ID	Compound	Docking score	Glide Emodel score
3474204	N-Hexanoyl-DL-homoserine lactone	−4.44	−4.44
73386	Malvidin	−8.84	−8.87
159287	Petunidin	−8.38	−8.41
128861	Cyanidin	−7.76	−7.78

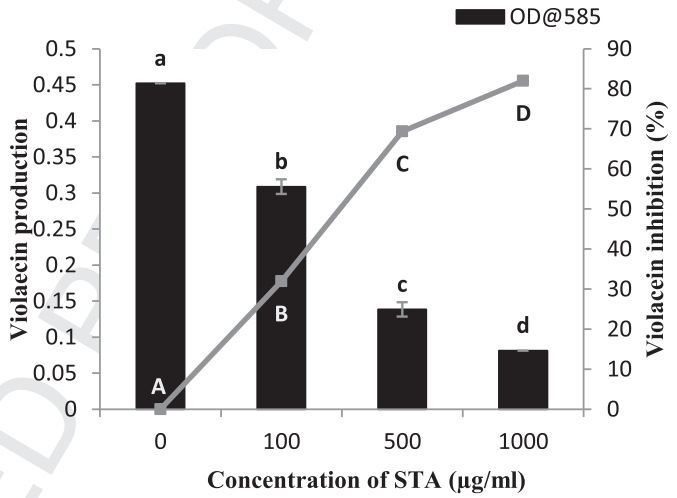

Fig. 2. Inhibition of violacein production in *C. violaceum* by enriched extract at different concentration (0.1, 0.5 and 1 mg/ml). Line graph represents the percentage inhibition. Vertical bars represent the mean values of triplicates with a standard deviation. Same letters in the column are not significantly different ($p < 0.05$).

3.4. Inhibition of biofilm formation and EPS production

Fig. 3 represents the effect of STA on biofilm formation and EPS production. Concentration dependent decrease in the formation of biofilm biomass by *K. pneumoniae* was observed. Enriched extract at the concentration of 0.1, 0.5 and 1 mg/ml extricated the biofilm biomass by 35.85, 64.03, and 79.94% respectively. It was observed

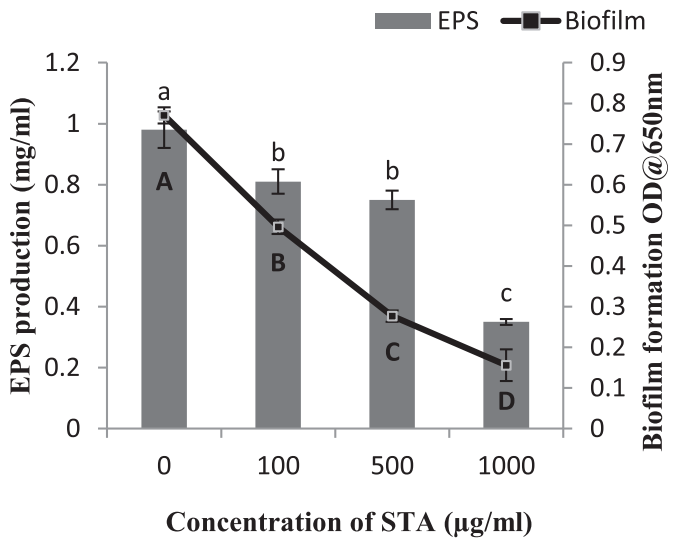

Fig. 3. Effect of STA on biofilm formation and EPS production in *K. pneumoniae*. Vertical bars represent the mean values of triplicates with a standard deviation. Same letters in the column are not significantly different ($p < 0.05$).

that STA at the tested concentrations (0.1, 0.5 and 1 mg/ml) inhibited EPS production by 17.34, 23.47 and 64.29% respectively (Fig. 3).

3.5. In-situ visualization of biofilm

In-situ visualization of biofilm developed with and without STA was analyzed using light microscopy, scanning electron microscopy and confocal laser microscopy. Light microscopic images showed a thick biofilm biomass on the control slide, whereas slide treated with enriched extract at 1 mg/ml concentration exhibited the dislodged biofilm. SEM analysis also revealed that; there was deterioration in the biofilm architecture and cells were loosely attached to the surface in the slides treated with an extract. Three-

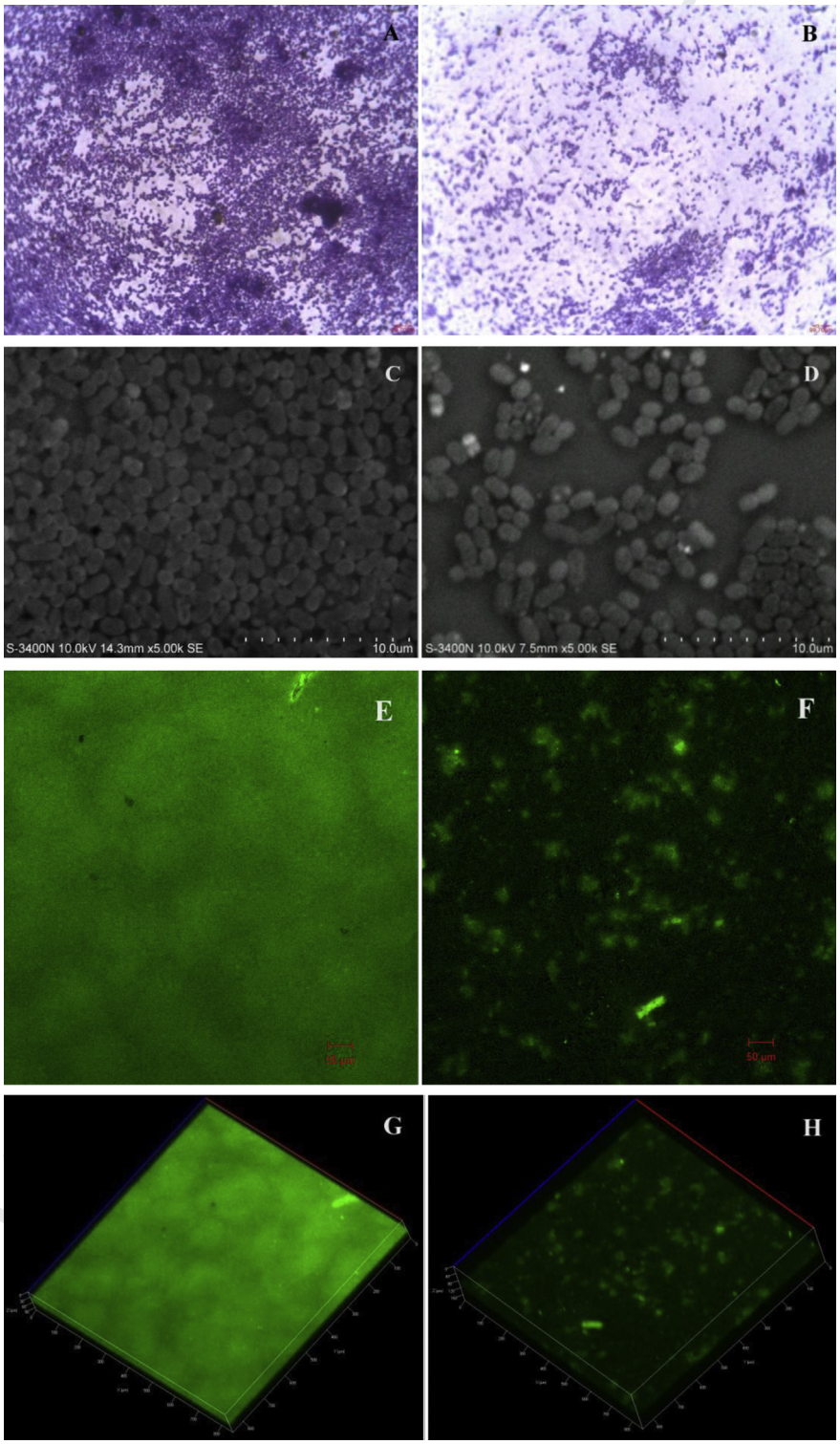

Fig. 4. Microscopic images of test bacterial biofilm grown in the presence and absence of enriched extracts (1 mg/ml). Light microscopic images, scanning electron microscopic images, Confocal laser scanning microscopic images, two-dimensional and three-dimensional. Images A, C, E and G – Untreated slide, Images B, D, F and H – treated slide.

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Table 2
Synergistic effect of STA with antibiotics against *K. pneumoniae*.

Bacterial strain	Antibiotics	Growth inhibition (%)	Increase in sensitivity with STA(%)		
			100 μg	500 μg	1000 μg
K. pneumoniae	Ofloxacine (10 mcg)	25.10	2.29	18.35	55.59
	Tetracycline (30 mcg)	17.38	3.08	28.51	41.69
	Chloramphenicol (10 mcg)	44.42	1.29	20.78	41.29

dimensional images of CLSM clearly showed a reduction in the thickness of the biofilm in the treated slides when compared with untreated one (Fig. 4).

3.6. Synergistic effects of STA with antibiotics

As a new strategy to combat bacterial infections, this assay was performed to examine the synergistic activity of *S. cumini* extracts with selected antibiotics. On testing the bacterial growth inhibition with antibiotics 25.10, 17.38 and 44.42% inhibition was observed with ofloxacine, tetracycline and chloramphenicol respectively. Enhanced susceptibility was observed towards all antibiotics when treated with *S. cumini* at different concentration (0.1 0.5 and 1 mg/ml). It was also revealed that increasing concentration of enriched extract with antibiotics enhanced the sensitivity of *K. pneumoniae* towards relevant antibiotics in dose-dependent manner (Table 2). STA at the concentration of 1 mg/ml increased the sensitivity of *K. pneumoniae* up to 55.59% towards ofloxacine. Percentage increase in the sensitivity was calculated by considering the OD values of antibiotic treated ones as control.

3.7. Confirmation of AHL inhibition by HPTLC

HPTLC was optimized to detect and quantitate the levels of AHLs in treated and untreated culture broths of *K. pneumoniae*. It was found that *K. pneumoniae* produced AHL compounds that caused activation of the reporter strain CV026 to induce violacein pigmentation. Sharp, well resolved peaks were obtained at R_f value of 0.61with different peak area corresponding to their concentration (Fig. 5).

Addition of STA to culture media at tested concentrations (0.1, 0.5 and 1 mg/ml) significantly reduced the AHL production up to 24.19, 29.77 and 33.59% respectively. This corroborates with the QS inhibitory activity detected in the violacein inhibition assay. When the percent reduction in the peak area was calculated, it was observed that the concentration of AHL produced by *K. pneumoniae* was significantly reduced with the addition of STA.

3.8. Evaluation of QSI potential compound

Among the four components which showed significant docking score, malvidin exhibited the highest docking and Glide Emodel score of −8.84 and −8.87 respectively. In docking analysis interaction between the receptor protein and malvidin is of hydrophobic nature, no hydrogen interactions were found. All the residues of protein present in the complex were displayed in stick, and ball representations are indirect interaction to the ligand (Fig. 6).

In quantification assay, malvidin inhibited the violacein production in *C. violaceum* CECT5999 up to 85.40% at the maximum concentration of 20 μg/ml. Furthermore at the Sub-MIC level tested compound reduced the EPS production (81.16%) and biofilm formation (72.70%) (Fig. 7a and b).

4. Discussion

To the best of our knowledge this is the first report demonstrating the anti-quorum sensing potential of tropical plant *S. cumini* against *K. pneumoniae* (KF817575). Upon screening *K. pneumoniae*, (KF817575) showed consistent induction of violacein production in reporter strain indicating its ability to produce AHL. Results obtained in this study is comparable with those of Yin et al. [29] who reported the production of both short and long chain AHLs in *K. pneumoniae*. Limitations were there in screening the spoilage microflora for their signal molecule production as *C. violaceum* CECT5999 responds only to short range of AHLs [30]. These limitations may be overcome by using monitor systems that respond to a wide range of AHLs.

On docking with LasR receptor protein, active components like petunidin, malvidin and cyanidin belongs to anthocyanins of *S. cumini* showed highest docking score than that of the natural signal

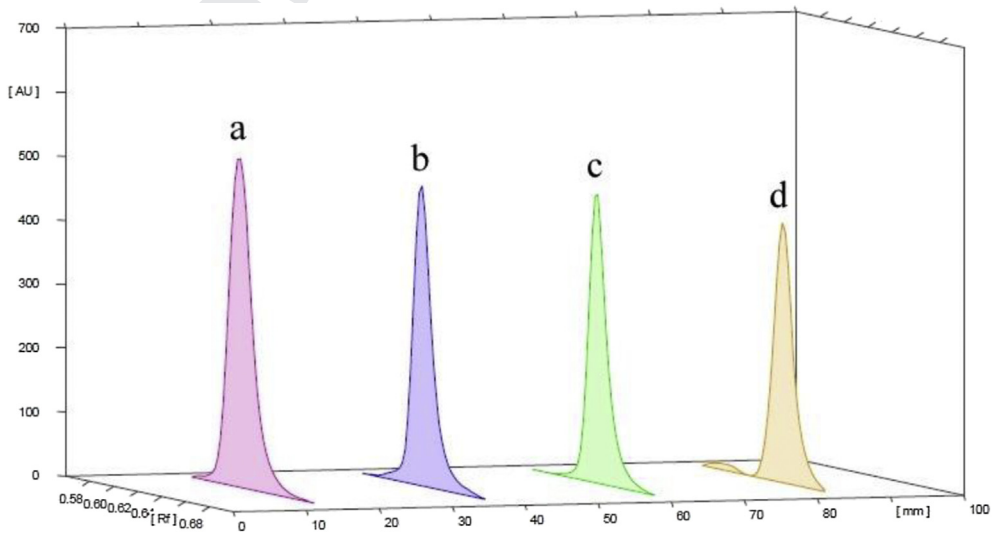

Fig. 5. HPTLC graphs showing the presence of signaling molecule in the culture supernatant of *K. pneumoniae* (a) and supernatant of *K. pneumoniae* treated with 0.1, 0.5 and 1 mg/ml of STA (b–d).

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molecule. It was already reported that competitive molecules showing the nearest or higher affinity than natural ligand may compete to inhibit the QS dependent physiological functions [31]. In quantitative analysis enriched extracts significantly reduced the violacein production up to 82%. Musthafa et al. [32] reported the inhibition of violacein production in *C. violaceum* by certain plants such as *Ananas comosus*, *Manilkara zapota*, and *Musa paradiciaca*. Our work corroborates well with above findings. Even though *S. cumini* is well known for its antimicrobial activity against large number of food-borne pathogens [33], anti-quorum sensing property has not been reported hitherto. There was no bacteriostatic or bactericidal effect observed with the extracts as the entire study was carried out below their minimum inhibitory concentration, which was evidenced by antibacterial and bacterial growth curve study and hence it would not impose a selective pressure to develop resistance among the bacteria tested.

QS mechanism in *K. pneumoniae* plays a positive role in influencing the biofilm formation, initial coverage of substratum and the development of mature biofilm architecture [34]. Biofilm formation through the release of non-specific AI-2 autoinducers molecules were also found to regulate the genes involved in the production of lipopolysaccharide and capsule synthesis in *K. pneumoniae* [5]. These lipopolysaccharides play a major role as the substratum for the initial attachment of cells to the surface. Bacterial communication mediated by LuxS was also found to involve in biofilm formation by *Salmonella enterica* serovar Typhimurium [35] and *Streptococcus mutans* [36]. Treatment with enriched extract effectively reduced the biofilm formation without inhibiting planktonic growth and loosens the attachment of bacterial cells, which might be due to reduced AHL production in *K. pneumoniae* and binding of active components on the LasR receptor protein. Olofsson, Hermansson, & Elwing [37] reported that quorum signals also regulate the production of EPS, which acts as a protective barrier for cells [38] and enhances biofilm formation. Hence, interference with quorum signals may results in reduced EPS production. In this study enriched *S. cumini* extract abruptly reduced the EPS production in *K. pneumoniae* that were more apparent from SEM and CLSM images. Our results are in accordance with Watnick and Kolter [39] who reported the significance of EPS production in

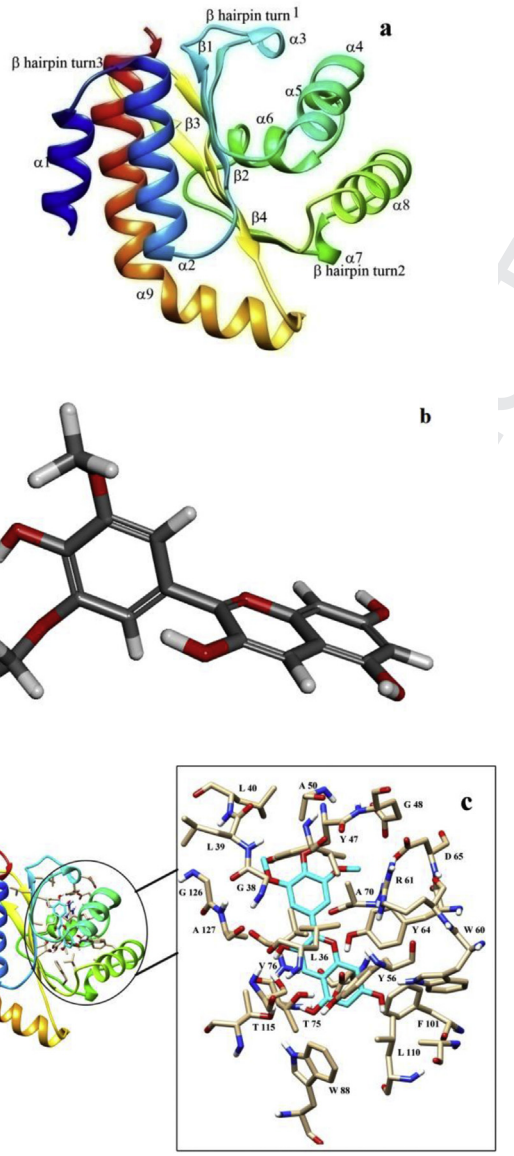

Fig. 6. The docking of active component malvidin on LasR receptor protein. LasR receptor protein labeled with secondary structural components (a), image of active component malvidin (b) and docked image of malvidin on the receptor protein (c).

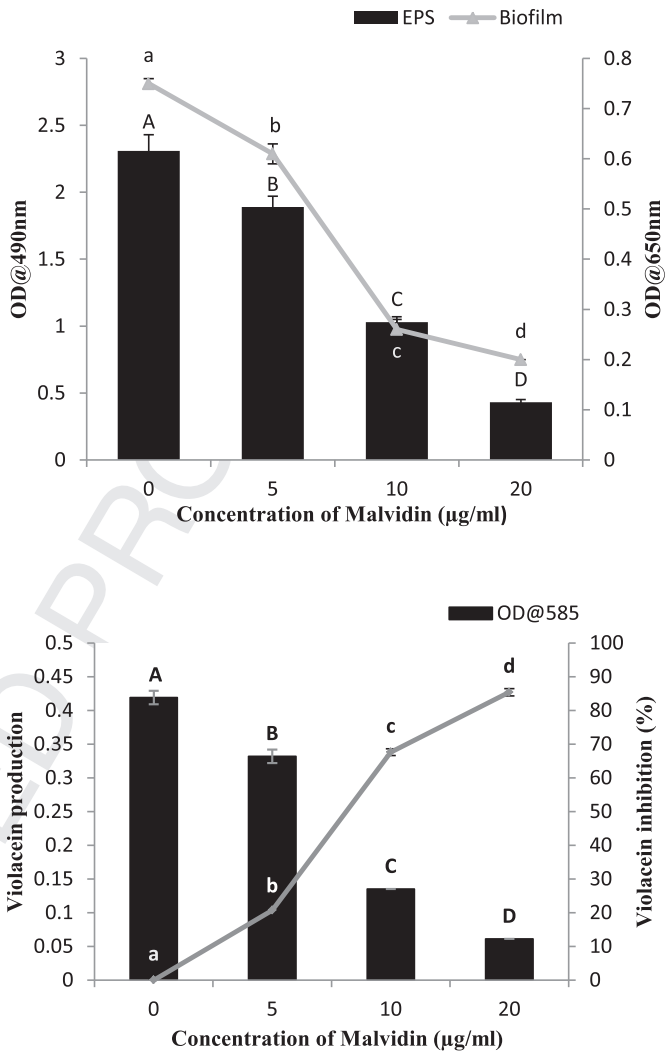

Fig. 7. Quantitative analysis of anti-biofilm and EPS reducing activity in *K. pneumoniae* (a) violacein inhibition in *C. violaceum* CV026 (b) by malvidin at different concentration (0–20 µg/ml). Data represented were mean of triplicate values with a standard deviation. Same letters in the column are not significantly different ($p < 0.05$).

biofilm formation and maturation.

Enhanced susceptibility of microbes towards antibiotics relies on quorum sensing mechanism which was acknowledged by Bjarnsholt et al. [40] in an earlier study. *K. pneumoniae* cells are less sensitive to antibiotics, such as ofloxacine, tetracycline and chloramphenicol. *K. pneumoniae* showed enhanced sensitivity towards all the above-tested antibiotics with added STA. Minimum of 1.29% increase in the sensitivity was observed towards chloramphenicol and a maximum of 55.59% was observed towards ofloxacine against growth inhibition of respective antibiotics as control. This evidenced that the anti-biofilm compound proved to be non-antibacterial may overcome the resistance by acting synergistically with conventional antibiotics, as reported by Rogers et al. [26]. Brackman et al. [41] reported that the cinnamaldehyde enhanced the sensitivity of *Vibrio vulnificus* against doxycycline.

Signaling molecule produced by *K. pneumoniae* was quantified through HP-TLC, and found that STA inhibited around 33.59% of AHL production. Studies reported that plant extracts like *A. comosus*, *M. zapota*, *M. paradiciaca*, *Phaseolus vulgaris*, *Rosa rugosa* and *Centella asiatica* efficiently interfered with AHL mediated QS [32,42,43]. To best of our knowledge, efficacy of plant extracts to inhibit AHL production has not been quantified using HP-TLC so far. Hence, we could not compare the result obtained in the present study with others for its efficacy. Though studies were there for the detection of signaling molecules in *Pseudomonas aeruginosa* using HP-TLC, results suggest that this is an extremely perceptive method for precise detection and quantification as compared to other conventional methods.

In the present study malvidin, anthocyanin of *S. cumini* showed a pronounced inhibitory effect against QS regulated violacein production (85.40%), biofilm formation (72.70%) and EPS production (81.16%) in *K. pneumoniae*. In molecular dynamics simulation, interesting conformational changes were observed, which interested us deeply will be exploited in our further research.

5. Conclusion

In summary, the present study evidenced that anthocyanin-rich *S. cumini* efficiently inhibited the AHL production, and QS regulated phenotypes like EPS production and biofilm formation in *K. pneumoniae*. As extracts used in this study was at the sub-MIC level, it is not expected to impose pressure on the organism to develop resistance, which offers a new hope in combating with multi-antibiotic resistant bacteria. In molecular docking analysis, it is revealed that the QSI activity of *S. cumini* attributes to its malvidin. *S. cumini* known for its medicinal functions like antibacterial and anti-inflammatory properties, the current study appends additional message on its QS inhibitory and antibiofilm properties. This could serve as a cost-effective source for developing novel QS-based antibacterial strategies, for the management of food-borne pathogens and to ensure the food safety.

Conflict of interest

No conflict of interest.

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