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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.

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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 [19,20]. 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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