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A novel strategy for disarming quorum sensing in *Pseudomonas aeruginosa-Chlorella emersonii* KJ725233

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ABSTRACT

Pseudomonas aeruginosa has emerged as a serious nosocomial threat with a high morbidity and mortality rate, especially in immunocompromised patients. Its pathogenicity is attributed to the virulence modulating – quorum sensing system. The coexistence of *Chlorella emersonii* KJ725233, a novel microalga with *P. aeruginosa,* pointed to its subterfuge to alter the pathogenicity of its partners. The study was, thus, aimed at determining the ability of *CEK* to modulate the pathogenesis of the aggressor *P. aeruginosa*. With a MIC of 0.5 mg mL^{-1}, the methanolic extract of *C. emersonii* KJ725233 was able to inhibit the synthesis of pyocyanin (62.48 ± 1.11%), protease (84.72 ± 1.11%), elastase (73.47 ± 0.11%), pyochelin (69.95 ± 7.12%), rhamnolipid (86.76 ± 0.48%), and polysaccharide (44.72 ± 1.58%) which are established virulence factors *of P. aeruginosa.* RT-PCR studies indicated the downregulation of its quorum sensing genes, *lasI* (84.63 ± 5.98%), *lasR* (85.56 ± 3.45%), *rhlI* (88.33 ± 3.56%), *rhlR* (88.73 ± 2.91%), and *pqsA* (72.61 ± 1.91%) which are known to play a crucial role in the pathogenesis of the organism. The presence of phytol in the methanolic extracts was indicated by its GC-HRMS analysis. With an ability to effectively incapacitate the virulence system of *P. aeruginosa*, *C. emersonii* KJ725233 presents itself as an efficient disarming agent with a potential use in pharmaceutical formulations.

1. INTRODUCTION

Pseudomonas aeruginosa, an opportunistic pathogen known to readily develop antibiotic resistance, has been widely implicated in nosocomial infections [1]. The CDC ESKAPE lists this as a priority pathogen not only due its clinical relevance in various disease and conditions such as cystic fibrosis, cancer, immunocompromised individuals, burns, and implanted medical devices [2,3] but also due to its association with high morbidity and mortality rates in such individuals [4]. *P. aeruginosa* is known to regulate its pathogenicity using the cell to cell communication called quorum sensing (QS) [1]. QS permits these bacteria to scrutinize their cell population through the release of signaling molecules called autoinducers [5] and establish infection.

QS systems in *P. aeruginosa* are systematized hierarchically with *las* system consisting of LasI and LasR as the synthase and regulator, respectively, at the top [6,7]. It uses *N*-3-(oxo-dodecanoyl)-L-homoserine lactone (OdDHL) as a signaling molecule and further regulates the *rhl* and the *pqs* systems. The *rhl* system consists of RhlI and RhlR as the synthase and regulator, respectively. It uses *N*-butanoylhomoserine (BHL) lactone as a signaling molecule, whereas the *pqs* system uses 2-heptyl-3-hydroxy-4-quinolone (PQS) as a signaling molecule [7]. During bacterial growth, these autoinducers (OdDHL, BHL, and PQS) accrue in the culture environment till it attains the threshold concentration that instigates the signaling necessary for the regulation of virulence genes expression [5]. QS has, thus, become a primary target in therapeutics [8].

At the source, freshwater microalga is known to exist in symbiotic relationship with the bacterial inhabitants. *C. emersonii* KJ725233 is one such microalga that coexisted with bacterial population in an artificial pond in the Western regions of Maharashtra. It has been isolated, identified by 18s rDNA sequencing, and has exhibited the presence of phenolics such as flavonoids [9,10]. The present study is one of the first to report the anti-quorum sensing potential of a microalga against *P. aeruginosa*. The methanolic extract of *C. emersonii* KJ725233 has displayed anti-pseudomonas activity [9] making it worthwhile to determine its anti-quorum sensing potential. The study for the 1st time reports the downregulation of quorum sensing system of the priority pathogen *P. aeruginosa* by any microalga so far to the best of our knowledge.

The present study was undertaken after identifying coexistence of *C. emersonii* KJ725233 with *P. aeruginosa*. This observation necessitated the investigation of the defensive stratagem of this microalga with pathogenic bacterial neighbors.

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2. MATERIALS AND METHODS

2.1. Culture and Culturing Conditions

C. emersonii KJ725233 was isolated from Western regions of Maharashtra, India [10]. *C. emersonii* KJ725233 was mass cultured in BG-11 media with 12h:12 h light:dark conditions and with 12 h of photoperiod for a period of 30 days.

2.2. Preparation of Extract

After 30 days of incubation, the biomass was harvested by centrifugation at 5000 rpm for 20 min and dried at 60°C for a period of 24 h. The extract was prepared by sonicating (LABMAN sonicator – Digital Ultrasonic Cleaner LMUC Series, India) dried biomass in methanol at a concentration of 0.1 g mL^{-1} for 40 min. These suspensions were centrifuged at 5000 rpm for 20 min and the supernatant was transferred to a crucible. The extraction was repeated thrice and all the supernatants were pooled together. These were dried at 28 ± 1°C and reconstituted in dimethyl sulfoxide at a concentration of 5 mg mL^{-1}.

2.3. Minimum Inhibitory Concentration

The antimicrobial potential of *C. emersonii* KJ725233 against *P. aeruginosa* MTCC1688 was determined by agar well diffusion method [9]. The MIC of the methanolic extract was determined by broth microdilution method [11] using an inoculum density of $1*10^4$ cells mL^{-1}. The methanolic extract was used at concentrations from 0.1 to 1 mg mL^{-1}, whereas quercetin was used as a positive control at concentrations of 2–20 µg mL^{-1}. To determine whether the MIC was bactericidal or bacteriostatic, the culture from both the MIC tube of the extract and quercetin was plated on sterile LB agar plates.

2.4. Spectroscopic Determination of Anti-Quorum Sensing Potential of *C. emersonii* KJ725233 against *P. aeruginosa* MTCC1688 in terms of Quantitative inhibition of its Virulence Factors

A 3 mL of $1*10^6$ cells mL^{-1} were inoculated with varying concentrations, namely, 0.042, 0.083, 0.125, 0.166, and 0.333 mg mL^{-1} of the methanolic extract and 3.33 µg mL^{-1} of quercetin in glycerol supplemented nutrient broth medium [glycerol (3%), meat extract (0.1%), yeast extract (0.2%), peptone (0.5%), and sodium chloride (0.5%)] for pyocyanin inhibition assay, CYKN [casein (1%), dipotassium dihydrogen phosphate (0.2%), yeast extract (0.5%), and sodium chloride (0.5%)] for protease and elastase inhibition assay; LB broth for pyochelin inhibition and in tryptone soy broth [tryptone (1.7%), peptone (0.15%), sodium chloride (0.5%), dipotassium hydrogen phosphate (0.25%), dextrose (0.25%), and malt extract (0.15%)] for polysaccharide and biofilm inhibition. These were incubated at 37°C for 24 h. At the end of incubation, the supernatant was obtained by centrifugation at 5000 rpm for 20 min and processed as earlier described for pyocyanin [12-14], protease [14-17], elastase [18], rhamnolipid [19], pyochelin [20,21], polysaccharide [22], and biofilm formation [23,24]. Alterations in quorum sensing gene expression of *P. aeruginosa* KJ725233 by quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the alterations in the quorum sensing genes of *P. aeruginosa* MTCC1688, $1*10^6$ cell mL^{-1} were incubated with 1 mg mL^{-1} of CEME at 37°C for 24 h. The total RNA was extracted using TRI reagent (Sigma, USA) by following the manufacturer's protocol. cDNA was synthesized from RNA using Thermo Fisher Scientific – RevertAid First Strand cDNA synthesis kit. The PCR reaction was performed on LightCycler® 480 Instrument II – Roche (Switzerland) using LightCycler® 480 SYBR Green I master mix. PCR conditions employed were –94°C for 5 min for 1 cycle, 40 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s using primers mentioned in Table 1. The primers were designed using *Pseudomonas* genome database (https://www.pseudomonas.com/) and Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). RpoD was used as a housekeeping gene. Melting curves analysis was performed at the end of amplification to confirm data quality. Fold change in target gene expression was translated into critical threshold cycle (C_T) calculated by delta-delta C_T algorithms [25].

2.5. Identification of Bioactives in CEME by GC-HRMS

GC-HRMS analysis was carried out using GC (Agilent Technologies, USA) equipped with Accutoff MS with HP-5 MS capillary column 30 m in length with an internal diameter of 0.32 mm and film thickness of 0.25 µm containing 5% phenyl polysiloxane [9].

2.6. Statistical Analysis

All the experiments were performed in triplicates. Data were analyzed by one-way ANOVA and are presented as mean±SD. $P < 0.05$ was considered less significant.

3. RESULTS

3.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antibacterial activity of the methanolic extract of *C. emersonii* KJ725233 against *P. aeruginosa* MTCC1688 is reported earlier [9]. To determine the MIC of the methanolic extract, broth dilution method was used and the MIC for the methanolic extract of *C. emersonii*

Table 1: Primer sequences for RT-PCR

Primer	Sequences (5'–3')	Amplicon size (bp)
RpoD forward primer	CGATCGGTGACGACGAAGAT	176
RpoD reverse primer	GTTCATGTCGATGCCGAAGC	
LasI forward primer	GCGCGAAGAGTTCGATAAAA	68
LasI reverse primer	GCTCCTTGAACACTTGAGCA	
LasR forward primer	CTGTGGATGCTCAAGGACTAC	210
LasR reverse primer	ACCGAACTTCCGCCGAAT	
RhlI forward primer	GCTCTCTGAATCGCTGGAAG	153
RhlI reverse primer	GTTTGCGGATGGTCGAACTG	
RhlR forward primer	TCGGAAATGGTGGTCTGGAG	219
RhlR reverse primer	GGTCAGCAACTCGATCATGC	
PqsA forward primer	CCGGACCTACATTCTCTCCC	156
PqsA reverse primer	GACTTGGGATTGATCACGGC	

KJ725233 was found to be 0.5 mg mL^{-1} whereas that of quercetin was 6 µg mL^{-1} [Figure 1]. However, the MIC of the methanolic extract (0.5 mg mL^{-1}) was found to be bacteriostatic whereas that of quercetin (6 µg mL^{-1}) was found to be bactericidal in nature.

3.2. Quantitative Inhibition of Virulence factors of *P. aeruginosa* MTCC 1688 by CEME

The present study dealt with the spectrophotometric determination of inhibition of *P. aeruginosa* virulence factors, namely, pyocyanin, protease, elastase, pyochelin, rhamnolipid, polysaccharides, and biofilm production. CEME at 0.33 mg mL^{-1} exhibited 62.48 ± 1.11% pyocyanin inhibition, 84.72 ± 1.11% protease inhibition, 73.47 ± 0.11% elastase inhibition, 69.95 ± 7.12% pyochelin inhibition, 86.76 ± 0.48% rhamnolipid inhibition, 44.72 ± 1.58% polysaccharide inhibition, and 83.77 ± 6.48% biofilm inhibition. On the other hand, quercetin at 3.33 µg mL^{-1} exhibited 42.72 ± 2.04% pyocyanin inhibition, 59.05 ± 3.33% protease inhibition, 30.12 ± 1.81% elastase inhibition, 67.88 ± 7.83% pyochelin inhibition, 50.62 ± 2.20% rhamnolipid inhibition, 28.57 ± 2.58% polysaccharide inhibition, and 26.86 ± 0.52% biofilm inhibition [Figure 2].

3.3. Alteration in the Expression of Quorum Sensing Genes of *P. aeruginosa* MTCC 1688 by CEME

The relative expression of the target genes of *P. aeruginosa* was determined by comparing them with the reference *rpoD* gene. Relative expression levels of these genes in *P. aeruginosa* treated with CEME were compared with that of the untreated *P. aeruginosa* and expressed in terms of fold change by $2^{-\blacktriangle\blacktriangle Ct}$ method. Percent reduction in gene expression was determined by 1-FC*100 [26] [Figure 3]. Although CEME was used at a concentration 100 folds higher than that of quercetin; quercetin was found to affect *las*, *rhl,* and *pqs* system 13.93–27.03 times more as compared to CEME [Figure 3].

3.4. Bioactives in CEME

GC-HRMS analysis enabled the identification of the bioactives present in the methanolic extract of *C. emersonii* KJ725233. Phytol was one of the major compounds identified at 21.51 min with a peak area of 64.77%. In addition to phytol, 1-docosene, hexadecane, bis-cyclohexylidene-2-oxocyclohexyl methane, pentadecanoic acid, 14-methyl, methyl ester, and 9,12-octadecadienoic acid methyl ester were also identified in CEME [9].

4. DISCUSSION

A serious concern about *P. aeruginosa* infections is the higher mortality rate in immunocompromised patients (70–80%) compared to other bacterial infections such as *Staphylococcus* or other Gram-negative bacteria [27]. The ability of pathogenic bacteria such as *P. aeruginosa* to induce a diseased condition depends on its potential to synthesize agents such as toxins and adhesion molecules collectively termed as "virulence factors" that violently destroy the host immune system [28]. *P. aeruginosa* possesses a multitude of virulence factors such as pyocyanin, proteases, siderophores, rhamnolipids, polysaccharides, and biofilm [29]. Pyocyanin acts as a virulence factor primarily because of its ability to generate ROS by oxidizing glutathione with simultaneous reduction of oxygen as well as by interfering in the electron transport by accepting an electron from NAD or NADP and transferring it to oxygen [12,30,31]. It is produced by different strains of *P. aeruginosa*

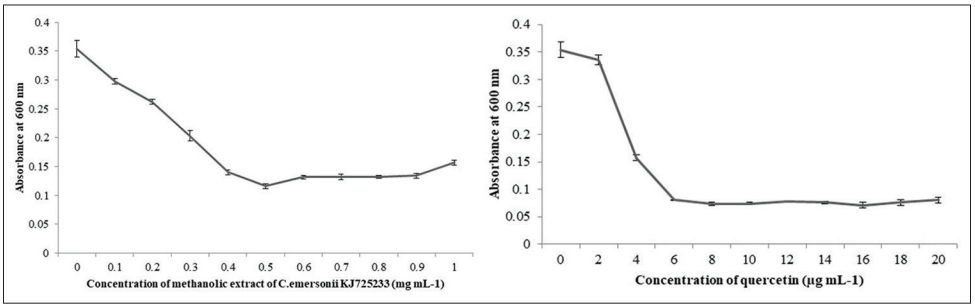

Figure 1: Determination of minimum inhibitory concentration of A. Methanolic extract of *Chlorella emersonii* KJ725233 (0–1 mg mL^{-1}); B. Quercetin (0–20 µg mL^{-1}) against *Pseudomonas aeruginosa* MTCC 1688

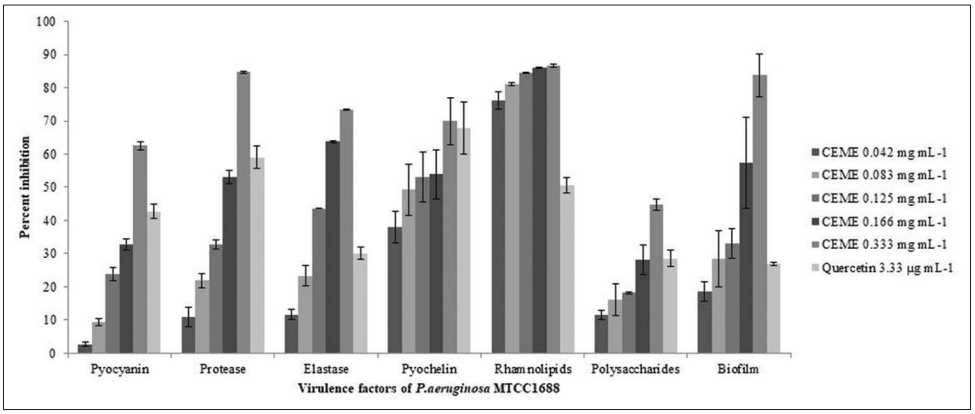

Figure 2: Percent inhibition of the virulence factors of *Pseudomonas aeruginosa* MTCC1688 by varying concentrations of the 0.042–0.33 mg mL^{-1} methanolic extract and quercetin (3.33 µg mL^{-1})

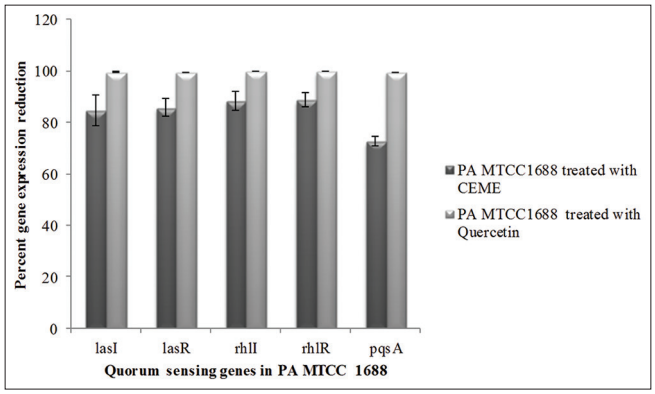

Figure 3: Percent reduction in expression of quorum sensing genes of *Pseudomonas aeruginosa* MTCC1688 on treatment with methanolic extract of *CEK* (0.33 mg mL^{-1}) and with quercetin (3.33 µg mL^{-1})

responsible for pulmonary and extrapulmonary infections as well as in environmental strains. It promotes *P. aeruginosa* colonization in the respiratory tract by significantly affecting tracheobronchial mucous velocity and hence can be found at concentrations as high as 100 µM in the lungs of cystic fibrosis patients [32,33]. *P. aeruginosa* subspecies secrete an array of proteases such as protease IV, alkaline protease, LasA protease, and LasB elastase that target the host proteins such as plasminogen, fibrinogen, α1-proteinase inhibitor, cytokine, and complement factors. Pyocyanin is known to disable protease inhibitors, thereby affecting the protease-antiprotease balance subsequently resulting in lung epithelial injury. Siderophores, further, aid in iron acquisition that ultimately helps the bacteria in colonization of the host. They are known to induce oxidative damage with a continual inflammatory response [34-36]. Rhamnolipids, on the other hand, are known to play a role in host immune cell and erythrocyte destruction while imparting twitching, swarming motility, and inducing biofilm formation [37]. They are known to inactivate tracheal cilia in the mammalian cell and are found in the sputa of the cystic fibrosis patients indicating their role in virulence [38]. Polysaccharides aid in attachment of the bacterial cells to the surface enabling it to develop thick antibiotic-resistant biofilms and provide protection against dehydration, antibiotics, and predators [39]. Biofilms known to cause chronic infections are structured consortium of bacterium embedded in matrix consisting of polysaccharide, protein, and extracellular DNA. Alginate forms a major part of the biofilms in cystic fibrosis patients. These biofilms, therefore, need to be prevented to avoid the chronic inflammation caused due to a lung tissue damage [40]. The expression of genes responsible for the synthesis of such virulence factors is controlled by the quorum sensing signal molecules regulated by the quorum sensing systems [41]. Antibacterial strategies are thus directed against these virulence factors with an aim to disarm the pathogenicity of the bacteria, thereby allowing the immune system with an improved chance of lessening the severity of infection [28].

Algae are reported to synthesize an array of compounds that are non-antibiotic quorum quenchers that interfere and disrupt the bacterial quorum sensing [31,42-44]. Phenolics such as flavonoids and tannins are widely reported for their quorum quenching potential [45]. Teplitski *et al*., 2004, have earlier reported the synthesis of quorum sensing mimic compounds in *Chlamydomonas mutabilis, Chlorella vulgaris,* and *Chlorella fusca* that regulate luminescence in *Vibrio harveyi* [44]. In addition, Natrah *et al*., 2011, have reported that the extracts of freshwater *Chlorella saccharophila* CCAP211/48 inhibit violacein production in *Chromobacterium violaceum* as well as interfere with the bioluminescence in *Vibrio harveyi* [43]. The halogenated furanones from red alga *Delisea pulchra* have been reported to inhibit biofilm formation by targeting the *las* and *rhl* systems [46].

The presence of phenolics and flavonoids in *C. emersonii* KJ725233 as described earlier [9] may have contributed to its anti-quorum sensing potential in terms of pyocyanin, elastase, protease, pyochelin, rhamnolipid, polysaccharide, and biofilm formation inhibition as seen in the present study. Phytol a quorum sensing inhibitor reported to inhibit biofilm formation, pyocyanin production, and motility of *P. aeruginosa* was identified in the methanolic extract of *C. emersonii* KJ725233 [9,47]. The quorum sensing inhibitors present in the methanolic extract of *C. emersonii* KJ725233 were found to downregulate all the three QS systems [Figure 3]. In addition, the oxidative stress reportedly induced by pyocyanin can also be counteracted by the strong antioxidant potential exhibited by the methanolic extract of *C. emersonii* KJ725233 [9]. However, though methanolic extract of *C. emersonii* KJ725233 was used at a concentration 100 times higher than that of quercetin; quercetin was found to affect *las, rhl,* and *pqs* system 13.93–27.03 folds more. The downregulation of the *lasI* and *rhl* genes of *P. aeruginosa* by natural compounds such as quercetin, halogenated furanones, baicalin, and terpinen-4-ol either alone or in combination with an antibiotic has only been reported so far [48-51].

5. CONCLUSION

C. emersonii KJ725233 interferes with both the AHL and the PQS quorum sensing systems in *P. aeruginosa*. Since these QS systems regulate virulence in *P. aeruginosa*, *C. emersonii* KJ725233 can prove to be a potential biocontrol agent that aids in disarming the pathogenicity of such a virulent microbe. It exhibits itself as a probable candidate for use as an additive in an anti-virulence drug formulation which, however, requires *in vivo* evaluation. Since there are more than 30,000 identified microalgal species with over 15,000 bioactives compounds, the study, thus, sets the podium to unravel another reservoir of pharmaceutically significant quorum sensing disarmer from the sea of untapped microalgal species.

6. CONFLICT OF INTEREST

Authors declared that there are no conflicts of interests.

7. FINANCIAL SUPPORT AND SPONSORSHIP

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