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ORIGINAL ARTICLE

Quorum sensing-regulated functions of *Serratia marcescens* are reduced by eugenol

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ABSTRACT

Background and Objectives: *Serratia marcescens* has emerged as a nosocomial pathogen responsible for human infections, where antibiotic resistance further complicates the treatments. In *S. marcescens*, biofilm formation and virulence factor production are controlled via quorum sensing (QS) system. QS is a signaling system that enables gene regulation to control diverse physiological functions in bacteria. Essential oils have shown to be potential in diminishing the pathogenicity and virulence of drug-resistant bacteria. This study was performed to determine whether eugenol would affect QS system, biofilm formation and virulence factor production of *S. marcescens.*

Materials and Methods: Biofilm formation, extracellular virulence factor production (hemolysin and protease), swarming motility and pigment formation of *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 were assessed after eugenol exposure at 1.25 and 2.5 µg/ml concentrations. The expression of genes involved in motility *(flhD)*, attachment *(fimC)*, biofilm formation *(bsmB, bsmA)*, and QS regulatory *(swrR)* were also evaluated.

Results: Eugenol treatment at 1.25 and 2.5 µg/ml concentrations caused a significant reduction in biofilm formation. The pigment, hemolysin and protease production of two studied *S. marcescens* strains, also reduced significantly by eugenol treatments ($p<0.05$). The *bsmA, bsmB, flhD* and *fimC* genes were down-regulated after eugenol treatment. The *swrR* gene expression was also reduced significantly by eugenol in both *S. marcescens* strains ($p<0.05$).

Conclusion: Eugenol inhibited quorum sensing-regulated functions of two studied *S. marcescens* strains.

Keywords: *Serratia marcescens*; Virulence factors; Biofilm formation; Quorum sensing; Eugenol

INTRODUCTION

Serratia marcescens is a major nosocomial pathogen which is often isolated from human respiratory and urinary infections. Immunocompromised patients are more susceptible to infection by this opportunistic pathogen than healthy individuals (1). Infections with *S. marcescens* are hard to treat owing to its broad-spectrum resistance to antibiotics including β-lactam, aminoglycosides, quinolones and polypeptide antibiotics (2). Development of antibiotic resistance in *S. marcescens* compels the search of novel and effective treatment approaches.

Recent discoveries in the field of bacterial communication system known as quorum sensing (QS) has attracted a great attention in this alternative treatment to conventional chemotherapy. QS system in bacteria regulates the expression of specific genes and depends on the cell density. The QS system is mediated by auto-inducers (AIs), secreted from each bacteria (3). Two acyl homoserine lactone (AHL)-dependent quorum sensing systems has been identified in *Serratia* spp. as *swrI/swrR* (4) and *smaI/smaR* (5). *Serratia* strains utilize AHLs like C4-homoserine lactone (C4-HSL), and C6-HSL to manage the expression of genes encoding virulence factors such as production of prodigiosin, protease, lipase, nuclease,

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hemolysin and biofilm formation (6, 7). Therefore, the quenching of AHL-mediated QS develops into a promising therapeutic target to attenuate the pathogenicity of *S. marcescens* (8). Recently, substantial efforts have been made for the identification of effective anti-QS and anti-biofilm agents (9). The anti-QS compounds, unlike antimicrobial agents, attenuate virulence of the pathogens rather than their growth, hence the risk of development of antibiotic resistance is almost nullified (10).

Most anti-QS agents have not been applied in clinic because of their toxicity and instability. So, studies have been focused on searching for new anti-QS agents from herbal extracts, which are rich source of bioactive and low toxic compounds (11). Essential oils (oily aromatic liquids) are extracted from natural plant materials and exhibit antimicrobial effects against bacteria, viruses, fungi and yeasts (12-14). Some of the antimicrobial properties of plant biologically active compounds may be attributed to QS inhibition, whereas the growth of the microorganisms may not be inhibited (15). Eugenol (4-allyl-2-methoxyphenol), the major clove extract constituent, exhibited significant bactericidal activity against different organisms like *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* (16). Also, it has been reported that it possesses anti-QS properties (17, 18). Therefore, in the present study the anti-QS activity of eugenol against *S. marcescens* was evaluated.

MATERIALS AND METHODS

Bacterial strains. *S. marcescens* ATCC 13880 and a clinical isolate (blood sample, *Serratia* sp. named Sm2) were studied in this research. *S. marcescens* ATCC 13880 was obtained from Iranian Biological Resource Center. Clinical isolate was identified by *16S rRNA* gene sequencing (GenBank accession number MK371794). Luria-Bertani (LB) agar was used to culture the bacterial strains at 37°C. For all assays, bacteria from pure bacterial colonies (overnight culture) were inoculated in phosphate-buffered saline (PBS, pH 7.2). The density of inoculum was adjusted to 0.5 McFarland standard (10^8 CFU/ml). For Biofilm biomass quantification assay, the bacteria were cultured in tryptic soya broth (TSB). All assays were carried out two times in triplicate.

Minimum inhibitory concentration (MIC) determination and growth analysis. Eugenol was purchased from Merck (Germany). Stock solutions were prepared by adding 2 µl dimethyl sulfoxide to 1 ml of eugenol. The MIC and minimum bactericidal concentration (MBC) of eugenol was determined against *S. marcescens* strains using the Mueller–Hinton broth microdilution method (19). The effect of ½ MIC (2.5 µg/ml) concentration of eugenol on bacterial proliferation was determined by monitoring the growth curve of *S. marcescens* strains. Briefly, 1% of overnight cultures of microorganisms (OD_{600} nm ~ 0.4) was inoculated into LB broth (25 ml) supplemented with ½ MIC of eugenol. The flasks were incubated at 37°C with shaking at 150 rpm. The cell density of samples were assessed by spectrophotometer at 600 nm every 1-h interval (20).

Biofilm biomass quantification assay. Strains were cultured in TSB at 37°C for 24 h. Bacterial suspensions were diluted (1:50) in TSB plus 0.2% glucose and incubated for 24 h at 37°C with different eugenol concentrations (final concentrations: 1.25 and 2.5 µg/ml). The wells were rinsed with PBS until the planktonic cells were removed. Biofilm formation was quantified by staining with Crystal Violet (0.4%) and a spectrophotometric assay (21).

Scanning electron microscopy (SEM). Biofilms formed by eugenol-treated (1.25 µg/ml) and untreated cells on glass slides were treated and fixed with glutaraldehyde (2.5%, 2 h). After washing with distilled water, dehydration carried out by adding ethanol (35, 50, 70, 90 and 100%). After critical point drying and gold sputtering, the biofilm samples were tested under a scanning electron microscope (Philips, Japan) (22).

QS inhibition assays in *S. marcescens*. QS inhibition assays (including prodigiosin, protease and hemolysin production) were performed by inoculating *S. marcescens* cells in LB medium and treatment with eugenol (1.25 and 2.5 µg/ml) at 37°C for 24 h. The pigment production was examined at 30°C.

Prodigiosin production assay. Prodigiosin was extracted from treated and untreated cells. Two ml of cells were centrifuged (10,000 rpm, 10 min). To the pellet, 1 ml of acidified ethanol (4% of 1 M HCl in absolute ethanol) was added and vortexed. The mixture was centrifuged (10,000 rpm, 10 min) and the extracted pigment absorbance was measured at 534 nm (23).

Proteolytic assay. Treated and untreated cells were centrifuged (11,000 rpm, 20 min, 4°C) and the proteolytic activity of supernatants were examined using azocasein as the substrate. 500 µl of substrate [0.3% azocasein (Sigma, USA) in 1 M PBS] was added to 500 µl of each supernatant. The mixture was incubated at 37°C for 1 h. After incubation, 500 µl of trichloro acetic acid (10%) was added and the reaction was terminated by incubation at -20°C (20 min) and then centrifuged (12,000 rpm -15 min). 100 µl of 1 M NaOH was added to supernatant (200 µl), and the absorbance was read at OD 440 nm (24).

Hemolysin assay. Each bacterial group (treated and untreated cells) were centrifuged (11,000 rpm, 20 min, 4°C). 900 µl of sheep blood suspension (2%) in PBS was added to the supernatant (100 µl) and incubated at 37°C for 1 h. After centrifugation (3,000 rpm, 10 min), the supernatant absorbance was measured at 530 nm (25).

Swarming assay. For swarming assay, each bacterial suspension (5 µl) was inoculated at the center of the swarming agar medium. The medium contains 1% peptone, 0.5% NaCl and 0.3% agar and eugenol at different concentrations. The plates were incubated in upright position at 30°C. The bacterial swarm zones were measured after 24 h (26).

Extracellular polysaccharide (EPS) assay. Bacterial cells were inoculated with eugenol in 96-well sterile polystyrene microtitre plate and incubated for 24 h to form biofilm as described previously. To remove planktonic cells, wells were washed with PBS. A mixture composed of 0.9% NaCl (40 µl), 5% phenol (40 µl) and concentrated sulfuric acid (200 µl) were added to each well. After incubation of the mixture at dark for 1 h, absorbance was measured at 490 nm (27).

Quantitative real time PCR (qPCR). To evaluate the effect of eugenol (2.5 µg/ml) on the expression level of QS-regulated genes, the total RNA of cells was extracted by RNX-PLUS (Sinaclone). Total RNA was dissolved in 0.1% diethylpyrocarbonate (DEPC)-treated water and treated by DNase I (1 U/µl, Thermo Scientific). 1 µl of 50 mM EDTA was added and the DNase was denatured. Treated RNA was reverse transcribed into cDNA by using cDNA reverse transcription kit (BIOFACT). The qPCR reactions were carried out by PCR Master Mix (Power SYBR Green, BIOFACT) and the real-time PCR system (MIC, Bio Molecular Systems). Targeted genes expression (*fimC, SwrR, flhD, bsmB* and *bsmA*) was normalized by an internal control (*rplU* gene, 50S Ribosomal gene) (28). The sequences of the gene-specific primers are given in Table 1.

Statistical analysis. All of the obtained values were displayed as mean ± standard error and were statistically analyzed by post hoc Tukey tests. $P < 0.05$ using GraphPad Prism 6. The statistical significance of real time PCR data was evaluated by the Relative Expression Software Tool (REST 2009).

RESULTS

Determination of MIC and growth curve analysis. The MIC against two *S. marcescens* strains was 5 µg/ml (0.05% v/v). Since complete killing was observed at 10 µg/ml (0.1% v/v), this concentration was determined as MBC.

As shown in Fig. 1, there was no difference in cell density of *S. marcescens* strains in the presence of ½ MIC (2.5 µg/ml) of eugenol as compared to the untreated controls, indicating that eugenol at this concentration had no bactericidal effect on both studied *S. marcescens* strains. Hence, in the present study, 1.25 and 2.5 µg/ml of eugenol were used for further experiments.

Biofilm formation inhibition. As shown in Fig. 2, after eugenol treatments, the biofilm biomass of both strains decreased significantly ($p<0.05$). Eugenol concentrations of 1.25 and 2.5 µg/ml caused a dose-dependent decrease in biofilm formation (60 to 75%), when compared with the untreated controls.

Scanning electron micrographs. The SEM images of untreated controls clearly showed the coherent and integrated biofilm architecture, whereas the treated samples with 1.25 µg/ml eugenol exhibited a complete destruction of biofilm structure (Fig. 3). Furthermore, the eugenol-treated samples demonstrated considerable disruption of bacterial aggregation, in contrast to the elaborate aggregation of bacterial colonies in the untreated controls.

Prodigiosin inhibition. The production of pro-

Table 1. Primer sequences used in this study

Gene	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)	Annealing temperature (°C)
flhD	TTGCCACTTCCGCTTTAACG	CTCTTTTCTTCGTCGGGCTAG	60
bsmA	TAGTCCGCACACTCATCGC	GATCTCCTGCGCCTGTGC	60
bsmB	GCGGATGTGTATGCCTTCG	GCCACGCATTTCTTCACTCA	60
fimC	ACCAGCCGTTTCAACAACAA	GGTTTGTACGGTGCGATCTT	60
swrR	GCCGAAATTCAAATGCTGCT	CTTACCTAAGCTCGCCCAGT	48
rplU	AAATCGGCGTTCCTTTCGTC	TGCTTACGGTGGTGTTTACG	60

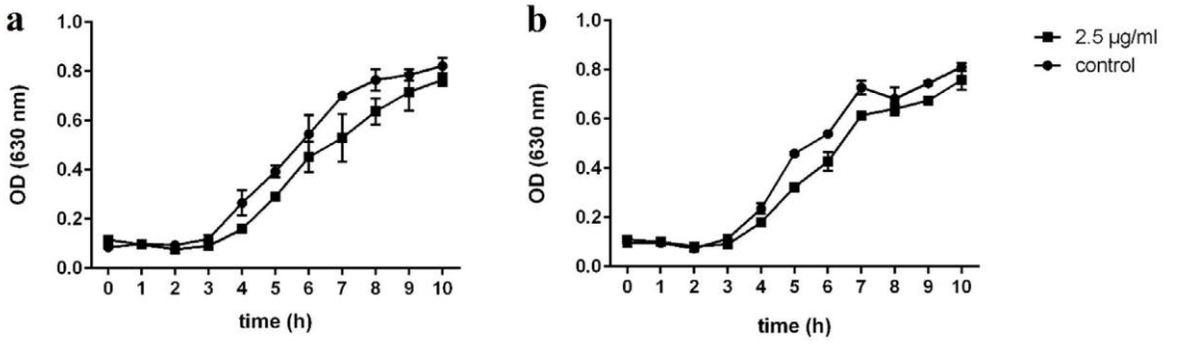

Fig. 1. Effect of eugenol (1/2 MIC:2.5 µg/ml) on growth of (a) *S. marcescens* ATCC 13880 and (b) *S. marcescens* Sm2. (Control: untreated group)

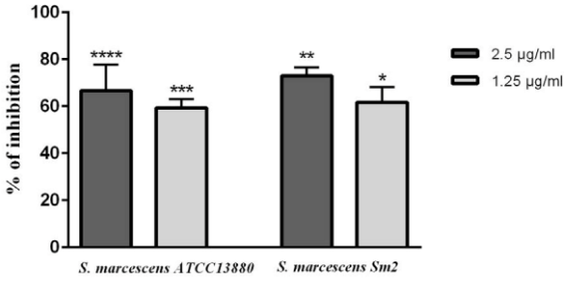

Fig. 2. Biofilm formation inhibition of *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 by eugenol treatments. The biofilm formation was estimated by crystal violet assay. Absorbance was measured at 492 nm. *significant at $p < 0.05$, **significant at $p < 0.005$, ***significant at $p < 0.0005$ and ****significant at $p < 0.0001$.

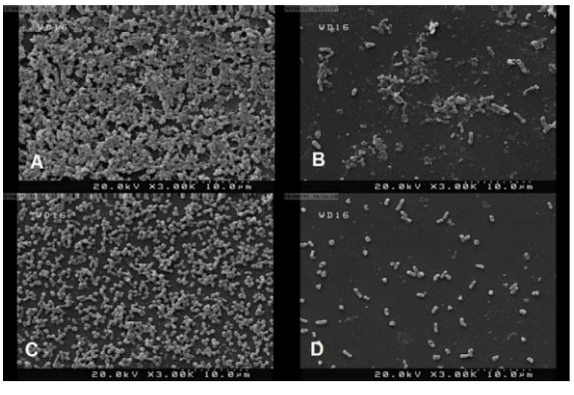

Fig. 3. Scanning electron images of *S. marcescens* biofilms in the presence and absence of eugenol (1.25 µg/ml). A) *S. marcescens* ATCC 13880 untreated cells, B) *S. marcescens* ATCC 13880 treated control cells, C) *S. marcescens* Sm2 untreated control cells, D) *S. marcescens* Sm2 treated cells.

digiosin pigment decreased 30-50% by using 1.25 and 2.5 µg/ml concentrations of eugenol in *S. marcescens* ATCC 13880. Pigment production in *S. marcescens* Sm2 also decreased 45-70% after using the same concentrations of eugenol (Fig. 4).

Protease inhibition. As shown in Fig. 5, eugenol causeda significant decrease in protease production to about 20-35% and 25-35% by using 1.25 and 2.5 µg/ml eugenol in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2, respectively.

Hemolysin inhibition. Hemolysin production of two studied strains were decreased in the presence of eugenol ($p<0.05$). At 1.25 and 2.5 µg/ml eugenol, about 25% and 35% of hemolysin inhibition was observed in *S. marcescens* Sm2 and *S. marcescens* ATCC 13880, respectively (Fig. 6).

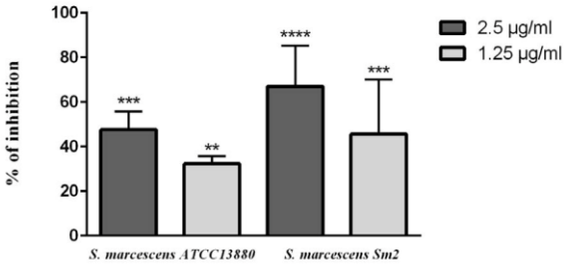

Fig. 4. Prodigiosin inhibition in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 by eugenol. **significant at $p < 0.005$ and ***significant at $p < 0.0005$ and ****significant at $p < 0.0001$.

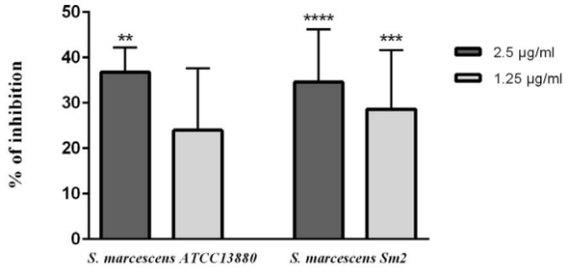

Fig. 5. Inhibition of protease in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 by eugenol. **significant at $p < 0.005$ and ***significant at $p < 0.0005$ and ****significant at $p < 0.0001$.

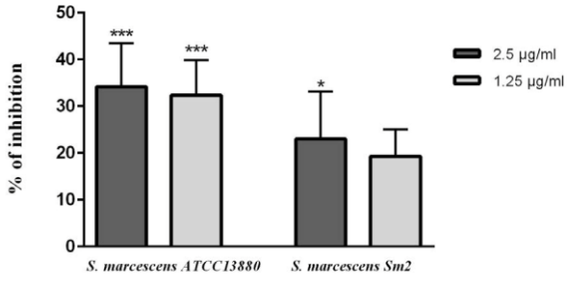

Fig. 6. Hemolysin inhibition in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 by eugenol. *significant at $p < 0.05$ and ***significant at $p < 0.0005$.

Swarming inhibition. As shown in Fig. 7, untreated control groups of *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 exhibited swarming on the soft agar plates. The addition of eugenol (1.25 and 2.5 µg/ml) decreased swarming motility of the two studied strains.

EPS inhibition. At 1.25 and 2.5 µg/ml concentrations, eugenol showed significant inhibition in EPS production of about 50-60% in both studied strains (Fig. 8).

qPCR analysis. The qPCR data was presented as fold changes in transcriptional level of targeted genes of treated samples compared to their untreated controls (Fig. 9). Eugenol treatment resulted in down-regulation of the expression of flagellar gene *(flhD)*, biofilm formation genes (*bsmA* and *bsmB*), fimbrial gene *(fimC)*, and QS regulatory gene *(swrR)* in both *S. marcescens* strains ($p<0.05$).

DISCUSSION

Traditional medicines such as essential oils have been found to have anti-QS function (29, 30). In the

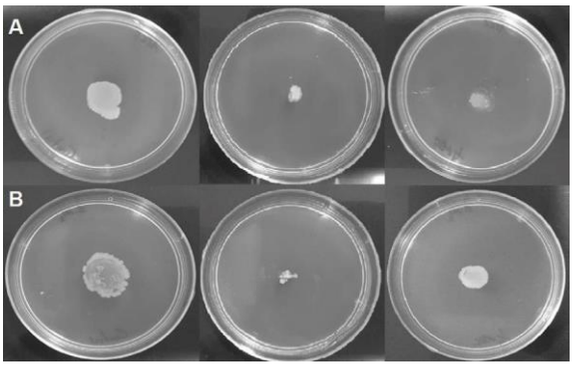

Fig. 7. Swarming inhibition in (A) *S. marcescens* ATCC 13880 and (B) *S. marcescens* Sm2 by eugenol. Left to right: untreated cells, 2.5 µg/ml-treated cells, and 1.25 µg/ml-treated cells.

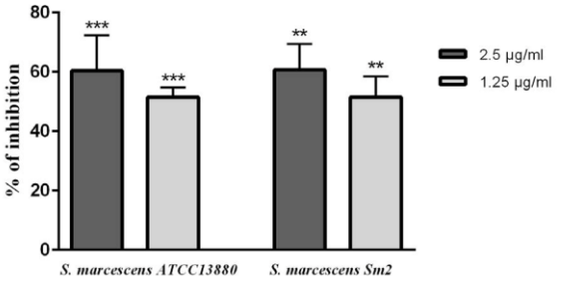

Fig. 8. EPS inhibition in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 by eugenol. **significant at $p < 0.005$, ***significant at $p < 0.0005$.

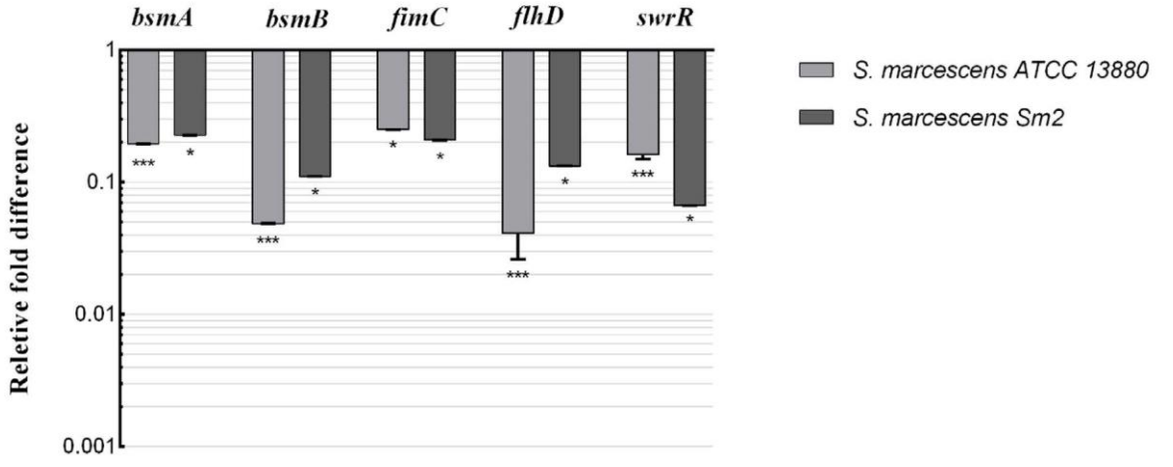

Fig. 9. Fold changes in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 gene expression after eugenol treatment (2.5 µg/ml). *significant at $p < 0.05$ and ***significant at $p < 0.0005$.

present study, the anti-QS potential of eugenol to inhibit QS-mediated virulence factor production and biofilm formation in *S. marcescens* was assessed. The results clearly demonstrate that eugenol hinders the biofilm formation and virulence factors production in *S. marcescens*, in a concentration-dependent manner. Since concentrations greater than 5 µg/ml resulted in bactericidal effects, further studies were performed at a concentration of 1.25 and 2.5 µg/ml eugenol.

Bacterial biofilms have the ability to resist the action of conventional antibiotics as well as the invading host immune responses (31). Therefore, new approaches are required to prevent the infections which are based on the biofilm formation. Our results showed that biofilm formation ability of both studied strains was decreased significantly by using eugenol. The SEM images of biofilms treated with eugenol (1.25 µg/ml) also exhibited very little adherence of cells on the surface. However, untreated cells showed a dense biofilm. Since the QS mechanism controls two stages of bacterial biofilm formation including the initiation and the maturation (32), eugenol acts as an anti-QS agent, which affects the bacterial adherence on the surface and also causes the subsequent dispersion of the biofilm. Thus, eugenol can make sessile cells susceptible to antibiotics. At the same time, eugenol helps the host immune system to clear the bacteria successfully.

Overproduction of EPS leads to changes in biofilm structure, which will eventually be correlated with increased cell resistance to osmotic and oxidative stresses and killing by biocides (33). Herein, we observed that eugenol significantly reduced the production of EPS more than 50%.

Swarming motility has a crucial role in surface colonization and biofilm formation of *S. marcescens* (34). Eugenol showed a significant reduction in swarming motility in both *S. marcescens* strains.

In the present study, reduction of EPS production and swarming motility along with the inhibition of biofilm formation indicates that eugenol is effective in the early stages of biofilm formation as well as structural design of the biofilm.

QS system regulates the pigment production of *S. marcescens* through two signal molecules, N–butanoyl homoserine lactone and HHL and any interference with these QS systems will be followed by decrease in prodigiosin production (35). This pigment also linked to the hydrophobic surface attachment of bacteria and could be important in the colonization and dissemination of bacteria (36). In our study, the pigment production of both strains diminished significantly after eugenol treatment compared to their untreated controls.

Hemolytic activity and production of extracellular enzymes such as protease in *S. marcescens*, are also under QS control (7). It has been reported that the proteases of *S. marcescens* can induce inflammatory and immune responses and play a significant role as virulence factors (1). Almost all strains of *S. marcescens* secrete a hemolysin that causes hemolysis of

animal and human erythrocytes (37) and the release of inflammatory mediators from leukocytes (38). It also acts as a cytotoxin on human epithelial cells and fibroblasts (39). According to our results, hemolysin and protease production decreased considerably after eugenol treatment in both strains. These results are consistent with the results of Srinivasan et al. who demonstrated that phytol, a diterpene alcohol compound majorly found in essential oils, decreased the level of extracellular enzymes and hemolysin production in *S. marcescens* (40).

According to Labbate et al. the major fimbrial proteins in *S. marcescens* are encoded by *fimA* and *fimC* genes (41). These QS-regulated genes have significant role in the attachment to biotic and abiotic surfaces by *S. marcescens* cells. In this study, the real-time PCR analysis showed that upon treatment with eugenol the expression of *fimC* was down-regulated almost 0.9-fold in both *S. marcescens* strains (Fig. 9). Recently, Salini and Pandian have also observed that methanolic extract of *Anethum graveolens* treatment decreased the expression of *fimC* gene (26).

The flagellar master operon *flhD* regulates swarming motility in *S. marcescens* (42). Eugenol treatment decreased the transcript level of *flhD* more than 0.9-fold in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2 (Fig. 9). Reduction in expression of this gene is in accordance with the report of Srinivasan et al. which showed that the *Piper betle* ethyl acetate extract down-regulated the *flhD* gene expression (43). The C4-HSL mediated QS genes, *bsmA* and *bsmB*, modulate the biofilm formation in *S. marcescens.* Labbate et al. showed that *bsmA* and *bsmB* are involved in adhesion to the abiotic surfaces (41). In this study, the expressions of *bsmA* and *bsmB* in both *S. marcescens* strains were down-regulated by eugenol (Fig. 9). Salini and Pandian have also observed that the *bsmA* gene expression was down-regulated in samples treated with methanolic extract of *A. graveolens* (26). The *SwrI/SwrR* QS system regulates the biofilm formation, swarming motility, as well as production of protease, S-layer protein and serrawettin in *S. marcescens* (44). The expression of *swrI* gene leads to the production of an AHL synthase which synthesis the AHL auto-inducers. An open reading frame, downstream of *swrI*, codes *SwrR* polypeptide. The *SwrR* has a major similarity to other AHL-dependent regulators such as members of the *LuxR* family (45). In the present study, after eugenol treatment, the *swrR* gene was down- regulated in *S. marcescens* ATCC 13880 and *S. marcescens* Sm2.

CONCLUSION

This study demonstrated that eugenol could prevent the production of QS-controlled virulence factors like production of prodigiosin pigment, protease and hemolysin. Moreover, the QS-mediated biofilm formation stages such as swarming motility, formation of microcolony, and extracellular polysaccharide production were inhibited by the eugenol treatment. Further, eugenol down-regulated the expression of genes responsible for QS system, adhesion, motility, and biofilm formation in *S. marcescens*. Thus, these results suggest that eugenol may have potential medicinal value including the anti-QS and anti-biofilm effects against *S. marcescens* strains.

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