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Eugenol exhibits anti-virulence properties by competitively binding to quorum sensing receptors

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ABSTRACT

The primary objective of this study was to ascertain the anti-biofilm and anti-virulence properties of sub-minimum inhibitory concentration (MIC) levels of eugenol against the standard strain PAO1 and two multi-drug resistant P. aeruginosa clinical isolates utilizing quorum sensing inhibition (QSI). Eugenol at 400 μM significantly reduced biofilm formation on urinary catheters and the virulence factors (VF) including extracellular polysaccharides, rhamnolipid, elastase, protease, pyocyanin, and pyoverdine ($p < 0.001$). Further, eugenol exhibited a marked effect on the production of QS signals (AIs) ($p < 0.001$) without affecting their chemical integrity. In silico docking studies demonstrated a stable molecular binding between eugenol and QS receptor(s) in comparison with respective AIs. Investigation on reporter strains confirmed the competitive binding of eugenol to a QS receptor (LasR) as the possible QSI mechanism leading to significant repression of QS associated genes besides the VF genes ($p < 0.001$). This study provides insights, for the first time, into the mechanism of the anti-virulence properties of eugenol.

Introduction

Pseudomonas aeruginosa, a pathogen with superior metabolic versatility, can colonize biotic and abiotic surfaces as a biofilm, where surface-attached microbial cells are enveloped in self-produced extracellular polymeric substances (EPS) (Lyczak et al. 2000; Flemming and Wingender 2010). Urinary tract infections (UTI) due to P. aeruginosa biofilms on urinary catheters account for 35% of nosocomial infections and can lead to cystitis and severe pyelonephritis (Johnson et al. 2006). Antibiotics and host immune mechanisms often fail to combat biofilms and associated bacteria due to the physical permeability barriers that are attributed to biofilms (Abbas et al. 2012; Soto 2014). Due to these permeability hurdles, antibiotics take more time to reach their target than the treatment duration or the antibiotic lifetime, resulting in insufficient antibiotic and facilitating the development of multidrug resistant pathogens and their survival (Bosso 2005). Along with alarming rates of multidrug resistance, new means of anti-infective methods are needed to contain these biofilm-associated nosocomial infections.

Cell density dependent bacterial communication, known as quorum sensing (QS), permits P. aeruginosa to coordinate with various virulence factor determinants, facilitating in-host colonization and antibiotic resistance. Quorum sensing is a signal-response based system, where concentrations of secreted signal molecules (autoinducers, AIs) increase with population density and are accepted by transcriptional regulators managing specific genes (Davies et al. 1998). P. aeruginosa mainly possesses two QS systems: lasI/lasR and rhlI/rhlR in which LasI and RhlI (signal synthase genes) synthesize AIs like N-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and N-butyryl-L-HL (BHL), respectively, which are correspondingly bound to intracellular LasR and RhlR receptors (Pesci et al. 1997). P. aeruginosa also has a third QS system, Pqs, which responds only to the Pseudomonas quinolone signal (PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone), and binds to the transcriptional regulator PqsR (MvfR) (Lee and Zhang 2015). Once activated by their native signals, these receptors will homodimerize and act as transcription factors to regulate specific gene(s). Under standard conditions, the Las system has typically been viewed as the master regulator of the QS systems in P. aeruginosa and induces expression of both the Rhl and Pqs circuits (Pesci et al. 1997; Venturi 2006).

Due to the significant involvement of the QS mechanism in microbial pathogenicity, QS inhibition (QSI) is
an alternative approach to controlling biofilm-associated nosocomial infections. Indeed, anti-QS agents (synthetic and natural) have already shown promise in the battle against *P. aeruginosa* infections with thinner biofilms and reduced virulence (Kumar et al. 2009). Synthetic furanones and autoinducer analogs are attractive man made anti-QS compounds. They have been used only in laboratory studies and have been effective in reducing the virulence of *P. aeruginosa* (Hodgkinson et al. 2012). Similarly, dietary phytochemicals (phenolics, quinones, flavonoids, alkaloids, essential oils, terpenoids, and tannins) and essential oils of several plants (lavender, eucalyptus, citrus, and pepper) have anti-QS effects (Viswanathan et al. 2015). Synthetic furanones and autoinducer analogs are assumed to bind to *P. aeruginosa* LasR and/or RhlR competitively, which inhibits the OdDHL and/or BHL from binding to the respective cognate transcriptional regulator (McInnis and Blackwell 2011).

Eugenol, one of the major constituents of essential oils from clove, nutmeg, cinnamon, basil, and bay leaf, has been reported to possess various pharmacological properties such as anti-cancer, anti-inflammatory, analgesic, anesthetic, antioxidant and antibiotic effects (Kong et al. 2014). Previously, Zhou et al. (2013) reported the anti-QS effects of eugenol against reporter and laboratory reference strains. Recent studies from the authors’ laboratory identified the anti-biofilm and QS inhibitory effects of eugenol rich extract(s)/fraction(s) against catheter isolates from patients with catheter-associated urinary tract infections (CAUTI) (unpublished data). However, the prospect of eugenol as an anti-virulence agent against biofilm-associated nosocomial UTIs and its detailed mechanism has not been studied in detail. Like the AIs-based QS inhibitors, eugenol has an alkyl chain which may provide the binding specificity for QS receptors. Additionally, eugenol's aromatic functionality may mediate either agonistic or antagonistic properties. Hence, it was hypothesized that eugenol might bind to the ligand binding domain of QS receptor(s) in a fashion similar to their endogenous ligands.

The primary objective of this study was to understand the anti-biofilm and anti-virulence properties of sub-MIC levels of eugenol against *P. aeruginosa* PAO1 and catheter isolates from patients with CAUTI mediated through QSI. The effect of eugenol on the expression of virulence factors (VFs) genes was analyzed using reverse transcription quantitative PCR (RT-qPCR). In silico docking studies compared the molecular binding affinity of eugenol and AIs with their respective QS receptor(s). A quorum sensing competition assay was performed using reporter strains to gain insight into the molecular mechanisms of eugenol's QS inhibition.

### Materials and methods

#### Bacterial strains and culture conditions

*Pseudomonas aeruginosa* standard strain PAO1 and two clinical isolates of *P. aeruginosa* (RRLP1, GenBank ID: KR149278 and RRLP2, GenBank ID: KT309033) were used in this study. The clinical isolates were isolated from urinary catheters of patients having CAUTI who were admitted to the Government Vellore Medical College and Hospital, Adukkamparai, Tamil Nadu, India. These clinical isolates are maintained in the authors’ laboratory, and have been shown to have intact lasI/R and rhlI/R QS systems and could produce various quorum-regulated VFs (Rathinam and Viswanathan 2014). *P. aeruginosa* reporter strains PAO-JP2/plasLVAgfp and PAO-JP2/phaI-LVAgfp, for LasR and RhlR for the competitive assay, were originally from Dr Helen E. Blackwell’s Laboratory, Department of Chemistry, University of Wisconsin–Madison (USA). Culturing of all the test organisms was done in Luria-Bertani (LB) broth, pH 7.0 (Himedia Laboratories Pvt Ltd, Bangalore, India), whereas the reporter strains were grown under antibiotic pressure (LB broth with 300 μg ml⁻¹ carbenicillin, Himedia Laboratories Pvt Ltd) and cultured at 37°C with 120 rpm (Moore et al. 2014). All the bacterial cultures were sub-cultured to achieve an optical density (OD) of 0.4 at a wavelength of 600 nm. Fresh subcultures were prepared for each new experiment from glycerol stocks maintained at −80°C.

#### MIC determination and sub-MIC selection

The MICs of eugenol (Sigma-Aldrich, Bangalore, India) and gentamicin (Himedia Laboratories Pvt Ltd) were determined with the test organisms *P. aeruginosa* PAO1, RRLP1, and RRLP2, following the micro-dilution method as per standard guidelines (CLSI 2006). Sterile medium and medium with eugenol and gentamicin served as controls. Further experiments used sub-MIC levels of eugenol whereas gentamicin (at the MIC and ½ MIC) was used in the biofilm eradication assay.

#### Anti-biofilm effect of sub-MIC levels of eugenol

##### Static biofilm inhibition assay

The biofilm inhibition assay was performed as described previously (Rathinam and Viswanathan 2014). Biofilms induced by PAO1, RRLP1, and RRLP2 were grown in 96-well standard microtiter plates (MTP) having U-shaped wells (Code: 941296, Tarsons, Chennai, India) without (Control) and with (Test) sub-MIC levels of eugenol (200, 400, and 600 μM). Approximately 1 × 10⁶ CFU ml⁻¹ of test bacterial cultures were inoculated into LB broth and
incubated for 24 h at 37°C. After incubation, the planktonic cells were removed, and the wells were washed three times with distilled water and air dried. The adherent biofilms in the MTP wells were stained with 0.4% (w v⁻¹) crystal violet (CV, Himedia Laboratories Pvt Ltd) solution. Excess CV from the wells was removed using distilled water. Further, CV from the cells was solubilized using 95% (v v⁻¹) ethanol. Absorbance was measured at 570 nm (ELx-800 absorbance reader, BioTek’, Mumbai, India) and the percentage biofilm inhibition was calculated using the formula:

\[
\% \text{inhibition} = \left( \frac{\text{Control OD}_{570} - \text{Test OD}_{570}}{\text{Control OD}_{570}} \right) \times 100
\]

**Biofilm eradication assay**

The biofilm eradication assay was performed as described previously (Kumar et al. 2013). Biofilms induced by PAO1, RRLP1, and RRLP2 were first grown on coverslips. The next day the established biofilms were treated with eugenol (400 μM) and gentamicin (10 μg ml⁻¹, at MIC) alone as well as in combination (eugenol, 400 μM and 7 μg ml⁻¹ gentamicin, ½ of the MIC). The coverslips were transferred daily into fresh medium with and without the respective agents for three days. On the third day, the coverslips were aseptically removed from the flasks and washed with sterile saline. The coverslips were then stained with acridine orange/ethidium bromide (AO/EB) and washed to remove the excess stain (Das et al. 2016). These were then visualized using a fluorescent microscope (WESWOX OPTIK-FM3000 equipped with WESWOX DG-140 digital high-resolution camera, India) under 10× magnification to visualize viable and non-viable cells.

**Biofilm development on urinary catheter surfaces in the presence of eugenol**

Urinary Foley catheters (Bard International Inc., New Providence, NJ, USA) were cut into 1.0 cm pieces and placed in LB medium with (400 μM) and without eugenol supplementation. These were inoculated with 100 μl of overnight cultures of the test organisms (PAO1, RRLP1, and RRLP2) and incubated at 37°C. After every 24 h, catheter pieces were removed from the flask, rinsed three times with sterile saline (pH 7.2) and transferred into new flasks containing fresh medium for three days. Biofilm formation on the catheter pieces was visualized using scanning electron microscopy (SEM) (ZEISS-EVO18, Carl Zeiss, Jena, Germany) as per the method described previously (Kumar et al. 2013).

**Effect of eugenol on the virulence of tested strains in vitro**

**Extra cellular polysaccharide (EPS) estimation**

Biofilms from all test organisms were allowed to form on (1 × 1× cm) glass cover slips in the presence and absence of sub-MIC levels of eugenol and incubated at 37°C. EPS quantification was carried out using a total carbohydrate assay (Nielsen 2003). In brief, cover slips were washed in 0.9% NaCl (0.5 ml) and then incubated in an equal volume of concentrated H₂SO₄. The mixture was incubated for 1 h in the dark and absorbance was measured at OD₄₀₀ (ELx-800 absorbance reader, BioTek’).

**Pyocyanin estimation**

Pyocyanin concentrations were estimated using a previously described method (Huerta et al. 2008). All the test organisms were grown without and with eugenol (200, 400, and 600 μM). After 24 h incubation, the culture supernatants were collected from cells after adjusting the absorbance (A₆₀₀) to 1. In addition, chloroform was added to the cell-free culture supernatants and acidified. The absorbance of this acidified chloroform layer was measured at a wavelength of 690 nm and compared with the control (eugenol untreated) cell-free supernatants.

**Pyoverdine estimation**

The pyoverdine assay was adopted from the method of Cox and Adams (1985). Cell-free culture supernatant of the tested organisms (grown with and without eugenol) was diluted 10-fold in Tris-HCl buffer (pH 7.4). The relative concentration of pyoverdine was estimated based on the fluorescence at an excitation wavelength of 405 nm and an emission wavelength of 465 nm (FP-8000 fluorescence Spectrometer, Jasco International Co. Ltd, Tokyo, Japan) and compared with that of the control (eugenol untreated) cell-free supernatants. The pH was maintained at 7.4 throughout the experiment, to avoid false positives.

**LasA protease estimation**

LasA protease activity was determined by measuring the staphylolytic activity (Kong et al. 2005) of the cell-free culture supernatants of the test organisms (treated with and without eugenol). One hundred microliter aliquots of the culture supernatants were added to 900 μl of boiled *Staphylococcus aureus* cells. The OD at the wavelength of 600 nm was measured after incubation for 60 min and compared that of the eugenol untreated culture supernatant. Activity was measured as the change in the OD₆₀₀ h⁻¹ μg⁻¹ protein.

**LasB elastase estimation**

The elastolytic activity of the culture supernatants (treated with and without eugenol) was determined using the Elastin Congo Red (ECR) method (Ohman et al. 1980). A 100 μl aliquot of the cell-free supernatant was added to 900 μl of ECR buffer containing 20 mg of ECR (Himedia Laboratories Pvt Ltd). The mixture was incubated under continuous shaking condition for 3 h at 37°C. Insoluble
ECR was removed and the absorption was measured at a wavelength of 495 nm. The elastolytic activity was measured as a function of the change in OD_{495 μg⁻¹} of protein in comparison with the control (eugenol untreated).

**Rhamnolipid estimation**
Rhamnolipid produced by the test organisms in the presence and absence of eugenol was quantified using the orcinol method as described by Zhu et al. (2002). The cell-free culture supernatants treated and untreated with eugenol were diluted in orcinol reagent and heated at 80°C for 30 min, and the OD was measured at 421 nm and compared with the control.

All the results were presented as % reduction and calculated using the formula:
\[
\%\text{Inhibition} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100
\]

where the test organisms (PAO1, RRLP1 and RRLP2) were treated with sub-MIC levels of eugenol; control organisms were not treated with eugenol.

**RNA extraction and quantitative real-time PCR (RT-qPCR)**
The test organisms (PAO1, RRLP1, and RRLP2) were grown in sterile LB medium supplemented with (400 μM) or without eugenol at 37°C with agitation for 24 h. Total RNA was extracted using TRIzol reagent (Sigma-Aldrich) as per the manufacturer’s protocol. The concentration and purity of extracted total RNA was determined by ultraviolet absorption (260/280 nm). The first strand cDNA was generated from a purified mRNA sample using the Verso cDNA synthesis kit (Thermo Scientific, Bangalore, India) as per the manufacturer’s instructions. Quantitative real-time PCR was carried out using the PrimeScript™ RT master mix (TaKaRa Bio Inc., Mountain View, CA, USA) with a real-time PCR instrument (StepOne™, Thermo Fisher Scientific, Tewksbury, MA, USA). The reaction procedure was performed as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 5 s and a final melting curve analysis from 65 to 95°C (with increments of 0.5°C every 5 s) (Kim et al. 2015). All the RT-qPCR analyses were performed in triplicate.

Primer sequences for the VFs genes; lasA (encoding protease), lasB (encoding elastase), phzA1 (encoding pyocyanin), pvdA (encoding pyoverdine), rhlA (encoding rhamnosyl transferase) and katA (encoding catalase) for the test organisms are described in Supplemental Material, Table 1. The ribosomal gene 16s rRNA was chosen as a housekeeping gene to normalize the RT-qPCR data. Amplification profiles were analyzed using StepOneDataAssist® software (Thermo Fisher Scientific), and cycle threshold (Ct) values for each target gene were normalized to the geometric mean of the Ct of 16s rRNA amplified from the corresponding sample. The fold change (RQ) of the target genes for each group to the control group was calculated using the 2^−ΔΔct method (Sarabhai et al. 2013) and compared to the control (without eugenol).

**Mechanism of the anti-virulence activity of eugenol**

**Screening of QS signal molecules (AIs) from eugenol treated test organisms**
Extraction of QS signal molecules. Extraction of AIs from eugenol treated (400 μM) and untreated stationary phase liquid cultures of PAO1 was performed as described previously (Brelles-Mariño and Bedmar 2001). The cell suspensions were centrifuged at 10,640 x g for 5 min and the supernatants were then filtered through 0.22 μm filters (Himedia Laboratories Pvt Ltd). The filtrate was extracted twice with equal volumes of acidified ethyl acetate and the pooled extracts were evaporated to dryness and suspended in 50–100 μl of HPLC-grade ethyl acetate. Before the quantification of AIs, their presence in the extracts was evaluated by gas chromatography–mass spectrometry (GC-MS) analysis.

**Detection of AHLs by GC-MS**
Quorum sensing signal extracts from eugenol treated and untreated bacterial cells (P. aeruginosa PAO1) were analyzed by GC-MS (Clarus 680, PerkinElmer, Branford, CT, USA) using the Elite 5MS column (PerkinElmer) (30 m × 0.25 mm ID and 250-μm film thickness) operating in an electron impact mode at 70 eV with helium as the carrier gas at a flow rate of 1 ml min⁻¹ (Seghal Kiran et al. 2016). The compounds were identified based on the molecular structure, molecular mass and by comparing the calculated fragment ratio of resolved spectra with that from the library. Spectral data were interpreted using the National Institute Standard and Technology (NIST) database. The obtained mass spectra of the eugenol treated QS signal extracts were compared with the mass spectra of eugenol untreated signal extracts.

**Quantification of QS signal extracts after eugenol treatment**
The quantification of AIs extracted from eugenol treated (Test), and untreated (Control) bacterial cultures (P. aeruginosa PAO1) were previously (Yang et al. 2005). Briefly, 40 μl of the signal extracts were inoculated into wells of 96-well polystyrene microtiter plates supplemented with 50 μl of a 1:1 mixture of hydroxylamine (2 M): NaOH (3.5 M). Subsequently, the same amount of a 1:1 mixture of ferric chloride (10% in 4 M HCl):95% ethanol was added, and the OD was measured at 520 nm. A dark brown color was observed in all
the samples containing lactone compounds which then turned yellow after repetitive pipetting, depending on the lactone concentrations. Further, the concentration of the AHLs was calculated from a linear graph of homoserine lactones with known concentrations ($r^2 = 0.989$). A neutral pH was maintained to overcome pH dependent lactonolysis. The results were compared with the control and presented as % reduction and calculated using the formula:

$$\text{%\ reduction} = \left(\frac{\text{Control OD}_{520} - \text{Test OD}_{520}}{\text{Control OD}_{520}}\right) \times 100$$

**RT-qPCR analysis of QS genes**
The effects of eugenol (400 μM) on the expression of QS genes lasR and rhlR (codes for QS receptors), lasI and rhlI (QS signal synthase genes) for *P. aeruginosa* PAO1 was measured using RT-qPCR as described in the above section (Sarabhai et al. 2013). Primer sequences for the QS associated genes are described in Supplemental material (Table 1).

**P. aeruginosa PAO-JP2 reporter gene antagonism assay**
The reporter *P. aeruginosa* strains, PAO-JP2/plasILVAgfp and PAO-JP2/rhlI-LVAgfp, were grown overnight in LB broth containing 300 μg ml$^{-1}$ of carbenicillin at 37°C for 20 h and were diluted to an OD$_{600}$ of 0.1. After incubation for 1 h, 200 μl cell cultures were added to individual wells of a 96-well black MTP (96-well plate MicroFluor, Thermo Fisher Scientific) containing 400 μM of eugenol in DMSO, with a final DMSO concentration not exceeding 1%. For the LasR antagonism assay, OdDHL (200 nM) was added along with eugenol. For the RhlR antagonism assay, 100 nM of OdDHL was added along with 30 μM BHL and eugenol to the MTP wells. Cells were then incubated for 6 h at 37°C with vigorous shaking at 200 rpm. Green fluorescent protein (GFP) production was detected at an excitation of 500 nm and emission of 540 nm with a fluorescence spectrometer (FP-8000 fluorescence Spectrometer, Jasco International Co. Ltd). The OD of cultures at 600 nm was determined to normalize the GFP expression to cell density. A subculture containing 1% DMSO and without OdDHL and/or BHL was used as a control to mimic fully inhibited LasR and/or RhlR. The fluorescence/OD$_{600}$ of eugenol treated cells competing against AIs was reported relative to the fluorescence/OD$_{600}$ of the respective AIs alone, considered as the positive control and the GFP expression was deemed to be 100%. The entire experiment was performed in triplicate, and ≥ 3 separate trials were conducted using unique cultures (Moore et al. 2014). The data were represented as % antagonism and calculated using the formula:

$$\text{%\ Antagonism} = \left(\frac{\text{Positive control fluorescence/OD}_{600} - \text{Test fluorescence/OD}_{600}}{\text{Positive Control}}\right) \times 100$$

**Molecular docking analysis**
Molecular docking studies have been extensively employed to identify the best candidate(s) (ligands) which bind to the target protein(s) and to discover their most favorable conformation against the target binding site(s). The Autodock version 4.2 (AD4.2) (Morris et al. 2009) was employed to find the binding affinities between the ligands and the target proteins (Morris et al. 1998; Chang et al. 2010). The crystal structure of the target protein, LasR (PDB ID: 2UV0) was retrieved from Protein Databank. Due to non-availability of the crystal structure of the target protein, RhlR (UniProt ID: P54292.1), a modeled protein was derived from protein model portal. During the preparation of protein, hetero atoms such as bound ligands and water molecules were removed. Then, Kollman charges, polar hydrogen atoms were incorporated into the target proteins by extending the PDB coordinate file, assigning AD4.2 atom type, to the pdbqt file format.

The 3-D structures of eugenol (CHEBI: 4917), and two natural ligands of LasR and RhlR, respectively, OdDHL (CHEBI: 44534) and BHL (CHEBI: 29643) were derived from PubChem and Chemical Entities of Biological Interest (ChEBI). These structures were converted to the pdb file format using OpenBabel. The structures were optimized by Autodock Tool (ADT), where Gasteiger charges and all hydrogen atoms were added. Finally, every atom in each ligand was assigned an ADT atom type and saved in a pdbqt file format for docking analysis.

Ahead of the docking analysis, a grid map was created, by placing the grid center with dimensions 80, 80, 80 Å around the active site of LasR (PDB ID-2UV0) and RhlR (UniProt ID-P54292.1) proteins, with a spacing of 0.375 Å. Once grid maps were generated by the Autogrid 4 subroutine of ADT, a docking parameter file was created by setting the parameters, viz the number of runs (60) and the searching algorithm (Lamarckian genetic algorithm). Finally, docking was performed with the help of the docking parameter file and AD4.2 subroutine of ADT to derive the docking scores. The post-docking analysis was carried out with the aid of visualization software (Discovery Studio Visualizer, Accelrys Software Inc., San Diego, CA, USA) and Pymol (DeLano 2015).

**Data analysis**
All experiments were performed independently, and the data are expressed as mean ± standard deviation (SD) with the p value of 0.05 being significant, using GraphPad Prism 5 (Software, Inc. La Jolla, CA, USA). All the results of the virulence factor estimation assays,
biofilm inhibition, and competition assays were analyzed using two-way ANOVA with Tukey’s multiple comparison post-tests. Comparisons were performed within the virulence factor production in different eugenol concentrations as ‘a’ – 200 μM vs 400 μM, ‘b’ – 200 μM vs 600 μM and ‘c’ – 400 μM vs 600 μM. The gene expression results were analyzed by two-way ANOVA with Sidak’s multiple comparison post-tests in which comparisons were performed as eugenol untreated vs eugenol treated (400 μM).

Results

**Determination of minimum inhibitory concentration (MIC)**

The MIC of eugenol was assessed for all the test pathogens using the doubling dilution method with the concentrations varying from 1.0 μg ml⁻¹ to 1,000 μg ml⁻¹. The MIC of eugenol was found to be 275 μg ml⁻¹ (1.67 mM), 300 μg ml⁻¹ (1.83 mM) and 150 μg ml⁻¹ (0.91 mM) for *P. aeruginosa* PAO1, RRLP1, and RRLP2 respectively. For all further experiments, sub-MIC levels of eugenol (200, 400, and 600 μM) were used. The growth profile of the standard strain PAO1 and the clinical strains (RRLP1 and RRLP2) at the sub-MIC of eugenol (600 μM), did not show any significant growth inhibition in comparison with the corresponding growth controls (1% DMSO) (Supplemental material Figure 1). The MIC of gentamicin, assayed with concentrations varying from 0.1 μg ml⁻¹ to 32 μg ml⁻¹, was found to be 10 μg ml⁻¹ (PAO1), 13.5 μg ml⁻¹ (RRLP1) and 12 μg ml⁻¹ (RRLP2) respectively. Similarly, gentamicin at 7 μg ml⁻¹ (approximately ½ the MIC) was selected for the biofilm eradication assay in combination with eugenol.

**Eugenol affects biofilm formation at sub-MIC levels**

The CV staining results indicated that microbes not treated with eugenol exhibited increased absorbances corresponding to greater biofilm formation. However, eugenol treatment (200, 400, and 600 μM) markedly inhibited biofilm formation in all the tested strains compared to the respective control(s). A maximum reduction in biofilm formation was found at 400 μM (p < 0.001) (Figure 1). The % inhibition by eugenol (400 μM) on PAO1, RRLP1, and RRLP2 was found to be 65.6, 67.8, and 64.4% respectively.

**Eugenol increases gentamicin sensitivity and causes biofilm eradication (AO/EB dual staining)**

In view of their promising anti-biofilm properties, the biofilm eradication activity of eugenol (400 μM) and gentamicin (10 μg ml⁻¹, at MIC) alone and in combination was examined in three-day-old biofilms and documented with acridine orange and ethidium bromide staining (AO/EB). Eugenol treatment modulated biofilm formation, demonstrating smaller biofilms with less extracellular matrix, micro and macro colonies and adherent cells (indicated by cells stained green). In contrast, gentamicin treatment (at MIC level) exhibited mild bactericidal activity (indicated by the green and red stained cells) without affecting the complexity of the biofilm. Interestingly, the combined therapy (eugenol, 400 μM and gentamicin, 7 μg ml⁻¹, ½ of MIC) enhanced the bactericidal action of gentamicin on biofilm bacteria at a reduced dosage, acting synergistically with eugenol, resulting in complete bacterial clearance (indicated by cells stained red) (Figure 2A).

**Biofilm development on urinary catheters was inhibited by eugenol**

In the light of notable biofilm inhibition and the eradication properties of eugenol (400 μM), it was hypothesized that eugenol might affect the biofilm forming capability of PAO1, RRLP1, and RRLP2 on urinary catheter surfaces. The test microbe biofilms were grown on urinary catheter surfaces in the presence and absence of eugenol. Scanning electron microscopy of these catheter surfaces after day 3 was performed to observe biofilm formation (Figure 2B). Biofilms formed by the test organisms without eugenol treatment (control) demonstrated a complex architecture. Notable extracellular matrix formation and thread-like
structures were present throughout the biofilm formed by PAO1. Biofilms of both the clinical strains also had solid extracellular matrix composition with well-developed nutrient channels. Interestingly, after eugenol treatment, test organisms exhibited architecturally simpler biofilms with a reduced cell density and complete inhibition of biofilm components such as an extracellular matrix and nutrient channels.

**Sub-MIC levels of eugenol exhibits anti-virulence property in vitro**

To understand the anti-virulence properties of eugenol through QSI, the effects of sub-MIC levels of eugenol on the production of quorum-controlled VFs such as EPS, pyocyanin, pyoverdine, protease (LasA), elastase (LasB) and rhamnolipid by the test organisms (PAO1, RRLP1 and RRLP2) (Figure 3) were examined. Extracellular polysaccharide, an important factor for biofilm formation, was produced by PAO1, RRLP1, and RRLP2 in the absence of eugenol. However, eugenol treatment at all doses significantly inhibited biofilm formation in all the tested microbes (Figure 3A). Indeed, eugenol at a dose of 400 μM exhibited the greatest reduction in EPS production in PAO1 (66%, \( p < 0.001 \)), RRLP1 (64.4%, \( p < 0.001 \)) and RRLP2 (65.5%, \( p < 0.001 \)).

Pyoverdine and pyocyanin are VFs produced by *P. aeruginosa*. In the present study, all the tested organisms (PAO1, RRLP1, and RRLP2) showed very high pyoverdine and pyocyanin levels while eugenol treatment significantly reduced the production of both VFs. Eugenol at 400 μM exhibited the maximum percentage reduction (\( p < 0.001 \)) in pyoverdine production following the order of RRLP2 (69.58%) > RRLP1 (68.73%) > PAO1 (62.86%) as shown in Figure 3B. The percentage reduction in pyocyanin production was 67.86 ± 3, 61.54 ± 4.8 and 37.52 ± 1.6 by PAO1, RRLP1 and RRLP2, respectively (Figure 3C).

Rhamnolipids are biosurfactants that aid in epithelial cell destruction and help to mask pathogens to avoid phagocytosis. Supernatants from eugenol treated (200, 400, and 600 μM) cultures showed significantly reduced levels of rhamnolipids compared with the control (eugenol, 0.0 μM) levels. Among the measured doses, 400 μM of eugenol exhibited a maximum reduction in rhamnolipids levels from PAO1, RRLP1, and RRLP2 (55.96, 57.14 and 50.95% respectively) (\( p < 0.001 \)).

Elevated levels of the extracellular enzymes protease and elastase directly relate to microbial pathogenicity by inducing host tissue degradation, thereby facilitating bacterial invasion and proliferation. In the present study, *P. aeruginosa* PAO1 and the clinical isolates produced increased protease and elastase levels in the absence of eugenol (control), whereas eugenol treatment significantly reduced production of these exoenzymes. Furthermore, the maximum percentage reduction was observed with 400 μM eugenol (\( p < 0.001 \)). The decrease in protease production followed the order of RRLP2 (65.18%) > RRLP1 (48.32%) > PAO1 (42.18%) (Figure 3E). Similarly, the % reduction in the elastase production was found to be 70.72 (PAO1), 62.49 (RRLP1) and 53.0 (RRLP2) (Figure 3F).

**Eugenol exhibited a decline in mRNA levels of virulence determinants**

The effect of eugenol on the expression of QS regulated VF genes was monitored with quantitative real time PCR. The
mRNA levels of virulence genes including lasA (encoding protease), lasB (encoding elastase), phzA (encoding pyocyanin), pvdA1 (encoding pyoverdine), rhlA (encoding rhamnosyl transferase) and katA (encoding catalase), decreased after eugenol treatment (400 μM) in comparison with control (eugenol, 0.0 μM), as represented in Figure 4). In PAO1, the relative fold expression levels of pvdA, phzA1, lasA, rhlA, katA and lasB were found to be 0.076, 0.096, 0.081, 0.079, 0.077 and 0.082, respectively. Similarly, eugenol at 400 μM exhibited relative fold levels of 0.09 and 0.035 (pvdA), 0.084 and 0.072 (phzA1), 0.1 and 0.029 (lasA), 0.08 and 0.028 (rhlA), 0.095 and 0.017 (katA), 0.058 and 0.021 (lasB) in the clinical isolates RRLP1 and RRLP2 respectively. These results were statistically significant in comparison with the respective growth control (p < 0.001).

**Mechanism of anti-QS activity by eugenol**

*Eugenol affects the QS signal levels in PAO1 without affecting the signal integrity*

Signal molecules were extracted from PAO1 culture supernatants grown with and without eugenol (400 and 0.0 μM). These extracts were quantified and compared with corresponding growth control AIs extracts. Culture supernatants of PAO1, untreated with eugenol, exhibited signal levels of 1.223 μM unlike the eugenol treated extracts which showed significant (p < 0.001) reduction on signal levels as 0.482 μM (% reduction=60.6).

Using optimized experimental conditions, analysis of QS signal extracts of *P. aeruginosa* was performed to examine the integrity/degradation of QS signal molecules. From the retention time and the characteristic fragment ions, the presence of BHL and OdDHL was confirmed. The electrical ionization mass spectra of the extracts are shown in Figure 5. GC-MS analyses of eugenol untreated and treated culture extracts yielded peaks of QS signals at retention times of 8.63 min (m/z 171) and 16.7 min (m/z 283) corresponding to N-butryl-homoserine lactone (Figure 5A and C) and N-dodecanoyl homoserine lactone (Figure 5B and D), respectively. A typical abundant fragmentation ion was observed from all the AIs extracts at m/z 143 and other minor peaks at 73, 57 and 43, as seen in the control (eugenol untreated) AIs.
the growth control (eugenol, 0.0 μM) (Figure 6). In PAO1, eugenol treatment has significantly reduced the mRNA levels and the relative fold expression of lasI, lasR, rhlI and rhlR were 0.22, 0.19, 0.17 and 0.15, respectively exhibiting a transcriptional level inhibition of QS associated genes.

**Expression of QS associated genes after eugenol treatment**

A reduction in the QS signal levels after eugenol treatment corresponded well with the decreased mRNA expression levels of QS-associated genes in PAO1 in comparison with the growth control (eugenol, 0.0 μM) (Figure 6). In PAO1, eugenol treatment has significantly reduced the mRNA levels and the relative fold expression of lasI, lasR, rhlI and rhlR were 0.22, 0.19, 0.17 and 0.15, respectively exhibiting a transcriptional level inhibition of QS associated genes.
Eugenol competitively binds with the QS receptors

The promising docking results of eugenol with QS receptors (LasR and RhlR) were further evaluated for their ability to affect LasR and/or RhlR activities by competing with the individual native signal molecules. In order to facilitate this, two mutant strains of *P. aeruginosa* PAO-JP2 that lack functional AHL synthases (*lasI* and *rhlI*), harboring the GFP receptor plasmids *plas*-LVA<sub>gfp</sub> or *prhl*-LVA<sub>gfp</sub> were utilized. Screening of ligand (eugenol) at sub-MIC levels (200, 400, and 600 μM) in these two reporter strains for antagonistic activity was performed as detailed in the experimental section. Eugenol, (400 μM) demonstrated reductions in GFP levels in the presence of their respective AIs by both the reporter strains, indicating notable competition with both AIs to both (Table 1). The percentage reduction in GFP production after treatment with sub-MIC doses of eugenol in *P. aeruginosa* *plas*-LVA<sub>gfp</sub> followed the order of 83.2 ± 9.3 (400 μM) > 71.4 ± 1.8 (600 μM) > 19.3 ± 2.6 (200 μM), respectively. The percentage reduction in GFP production by *P. aeruginosa* *prhl*-LVA<sub>gfp</sub> after eugenol treatment (200, 400, and 600 μM) was less in comparison with that of *plas*-LVA<sub>gfp</sub>, 27.6 ± 5.2, 29.8 ± 4.6 and 25.3 ± 8.2 respectively.

**Discussion**

During catheterization, bacterial colonization and biofilm formation is inevitable; 80% of nosocomial UTIs are related to urethral catheterization and associated biofilm formation (Johnson et al. 2006). Hence, biofilm inhibition is one of the primary objectives to prevent these device-associated nosocomial infections. Earlier studies have detailed various phytochemicals with notable anti-biofilm properties (Viswanathan et al. 2015). This study clearly indicated the anti-biofilm activity of sub-MIC levels of eugenol (Figure 1). This effect may be due to the ineffective attachment of organisms leading to failure in biofilm establishment (Figure 2B). Antibiotics show reduced efficacy in preformed biofilms due to the impermeable polysaccharide matrix and active efflux pumps in the cells. These physical and/or cellular barriers do not allow antibiotics to reach their desired target(s) (Saini et al. 2016). Studies have established that combination drug therapies with both anti-virulence agent(s) and antibiotics may result in reduced biofilm formation that make microbes susceptible to a reduced dose of antibiotics.
mechanism is via quorum sensing (QSI) (Saini et al. 2016). Quorum sensing regulated virulence factor production was performed to evaluate the anti-virulence property of sub-MIC levels of eugenol. Quorum sensing-controlled expression of an arsenal of virulence determinants (EPS, pyocyanin, pyoverdine, LasA protease, LasB elastase and rhamnolipid) in \textit{P. aeruginosa} and marked suppression of these VFs by eugenol indicated that it is an active anti-virulence candidate through QSI. In vitro attenuation (Figure 3) of virulence phenotypes correlated well with the individual gene expression analyses (\textit{lasA}, \textit{lasB}, \textit{phzA1}, \textit{pvdA}, \textit{katA}, and \textit{rhlA}) (Figure 4) confirming the transcription level suppression of virulence after eugenol treatment.

Quorum sensing inhibition occurs mainly by two mechanisms; signal degradation and signal mimicry, resulting in inhibition of genetic regulation systems or the interruption of downstream virulence and biofilm genes (Ni et al. 2009). Extracts with significantly reduced QS signal molecules after eugenol treatment indicated the possibility of QS inhibition through the autoinducer(s) degradation mechanism. Lactonases and acylases were identified to cause pH dependent enzymatic degradation

\begin{table}[h]
\centering
\caption{\textit{P. aeruginosa} PAO-JP2 reporter gene antagonism assay.}
\begin{tabular}{lll}
\hline
\textbf{Compound} & \textbf{Antagonism} \textit{(Eugenol) inhibition (mean%±SD)} & \textbf{Antagonism} \textit{(Eugenol) inhibition (mean%±SD)} \\
\hline
200 µM & 19.3±2.6 & Inhibition (mean%±SD) \\
400 µM & 83.4±9.3*** & 27.6±5.2 \\
600 µM & 71.4±1.8*** & 29.8±4.6 \\
\hline
\end{tabular
\end{table}

(Ashraf et al. 2010; Raad et al. 2012). Similarly, in this study, a combination therapy of eugenol and gentamicin showed significantly greater eradication efficiency against adherent \textit{Pseudomonas} cells (Figure 2A). Biofilms formed by the test organisms with eugenol were less dense than those without eugenol treatment and were more susceptible to gentamicin, even at low doses.

Quantification of quorum-regulated virulence phenotypes not only confirms the anti-virulence properties of this agent (natural/synthetic) but also suggests that the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Representation of ligand–receptor interactions. (A) LasR and (B) RhlR. (a) natural signal molecule(s) and (b) eugenol.}
\end{figure}
of AIs, resulting in QS inhibition. The possibility of a pH dependent AI degradation was ruled out since the pH of both the test material (eugenol) and culture supernatants were maintained at neutral (Yates et al. 2002). Interestingly, gas chromatography of AHL extracts (Figure 5) showed characteristic peaks for BHL and N-dodecanoyl homoserine lactone (OdDHL) at retention times that are similar to that of control (eugenol untreated) extract AIs. A typical abundant fragmentation ion was observed from all the AI extracts at m/z 143 and other minor peaks at 73, 57 and 43. The fragment ion at 143 m/z is most likely due to McLafferty rearrangement which is typical of carbonyl groups having a hydrogen atom in the γ-position. This arrangement gives rise to an anolic fragment and an olefin. Two major fragmentation processes involving an inductive effect and α-cleavage produce the other fragment ions at 73, 57 and 43 (Pearson et al. 1995). These observations rule out the likelihood of AI degradation as the mechanism for eugenol’s anti-QS properties.

Decreased AHL levels may also be attributed to the deactivation of the signaling cascade by the binding of QS signal mimics to the active site of respective receptors. Supporting this notion is the finding that synthetic AI mimics (halogenated furanone, meta-bromo-thiolactones, and C-30), natural phyto compounds (vanillic acid, zingerone and gingerol) also have been found to inhibit the QS signal cascade by binding to the respective ligand binding site (Kim et al. 2015; Kumar et al. 2015; Viswanathan et al. 2015). In silico docking studies of eugenol with LasR and RhlR receptors (Figure 7) showed its potential binding mode to the respective ligand-binding domains. From the data obtained in this study, eugenol was found to have more stable binding with LasR in comparison to the original AI through an arsenal of interactions. According to Shi et al. (2013), there is a considerable increase in the ligand–receptor binding stability due to the π–π interactions and π–alkyl interactions, resulting in significant electrostatic potentials that occur between two aromatic rings. Accordingly, more π–π and π–alkyl interactions were found in the case of eugenol–LasR, which was not present in the case of AI–LasR complex. As a consequence, the eugenol–LasR complex has an increased electrostatic binding potential.

Comprehensive structure–activity relationship studies of QS receptors vs autoinducer mimics concluded that the homoserine lactone moiety (present in AIs) substituted with an aromatic system was found to have augmented the ligand’s antagonistic properties (McInnis and Blackwell 2011; Hodgkinson et al. 2012). Similarly, the substituted phenyl ring of eugenol might have contributed to the intended antagonistic properties, while the para-positioned allyl group provided the specificity for the QS receptors. Interestingly, the current test compound (eugenol) shared significant structural similarity with the previously reported anti-QS agent, zingerone (Kumar et al. 2015). The possibility of eugenol competing with OdDHL and BHL for binding to their respective receptors (LasR and RhlR) was studied using a PAO-JP2 (pLas/prhl II-LVA/gfp) based antagonistic assay. From the results in Table 1, it was evident that the effect of exogenous OdDHL and/or BHL (indicated by the production of GFP) was significantly decreased by sub-MIC levels of eugenol, especially at 400 μM. These competitive assay results are highly suggestive of the mechanism of the observed QS inhibition in P. aeruginosa being mediated through the competitive binding of eugenol to LasR, the master regulator of the QS systems in P. aeruginosa. These results are concordant with previously reported results (Kim et al. 2015) and the possible molecular mechanism is illustrated in Figure 8.

In P. aeruginosa the QS systems are well regulated in a hierarchical cascade in which the las system activates the secondary QS circuit, rhl and pqs (a third QS circuit in P. aeruginosa) and induces a positive feedback loop to activate lasI to produce more signal molecules. Similarly, the Rhl system induced the expression of rhlI (respective signal synthesis) and has been shown to control PQS(2-heptyl-3-hydroxy-4(1H)-quinolone) which is considered to be a regulatory link between the las and rhl QS system. In a high cell density environment, the conventional QS circuitry of P. aeruginosa initiated the OdDHL and LasR complex which acts as the transcriptional activator and enhances the expression of several genes within the QS regulon (Aswathanarayan and Rai 2015). Earlier studies of anti-QS agents showed reduced AIs levels without affecting their chemical integrity. These low levels of AIs were later associated with the transcriptional repression of QS associated genes (lasI, lasR, rhlI, and rhlR), especially the signal synthase gene(s) (Sarabhai et al. 2013; Kim et al. 2015). These results substantiate previous data showing eugenol treatment compromised AIs levels alongside the suppression of respective signal synthase genes (Figure 6).

The DNA-binding transcription regulator LasR induces expression of both the rhl and pqs QS circuits along with virulence factors such as lasB (elastase), lasA (staphylolytic), aprA (alkaline protease), toxA (exotoxin A) and hcnABC (hydrogen cyanide synthase). Hence, under standard conditions, the las system has typically been viewed as the master regulator of the QS systems in P. aeruginosa (Winzer et al. 2000). Based on the proposed binding stability of eugenol for LasR through π–π and π–alkyl interactions, as evidenced by the docking analysis and competitive assay, it is speculated that the observed anti-QS effects begin with eugenol binding to the LasR receptor. Binding of eugenol to the LasR signal receptor causes it to lose its function as a transcriptional activator.
Thus, the observed reductions in various virulence phenotypes and down-expression of their respective genes as indicated by RT-qPCR results support this speculation (Kim et al. 2015).

In P. aeruginosa, the second QS circuit, the Rhl system induces the expression of rhlI (respective signal synthesis) C4-HSL (BHL), for which the receptor is RhlR. The BHL–RhlR complex is the transcriptional activator for the pqs gene, rhlAB (rhamnolipid synthesis genes), lasB, rpoS (stationary phase sigma factor), lecA (type I lectin), lecB (type II lectin) and hcnABC (Kim et al. 2015). Although eugenol could inhibit GFP production by PAO-JP2 (pRh-lII-LVAgfp) and PAO-JP2 (pLasII-LVAgfp), the competitive binding seems to be less effective with RhlR in comparison with LasR. This observation is well supported by the molecular docking studies which revealed a lesser binding affinity of eugenol for RhlR, compared with the signal molecule, BHL. However, the reduction in rhl regulated VF production observed in the study might be due to the eugenol–LasR binding, which caused a repression in the rhl QS system (Figure 8) (Kim et al. 2015).

In P. aeruginosa the precursor for PQS, 2-alkyl-4-quinolone (AHQ) is controlled by the regulator, PqsR (MvfR), which binds to the pqsA promoter of the pqsAB-CDE operon producing AHQ. The expression of mvfR turns on the cascade of genes involved in the PQS system (phnAB and pqsA-E), pyocyanin (phzA1-GI operon) and biofilm production. The pqsR gene itself is positively regulated by lasR and negatively regulated by rhlR, establishing a further link between AI dependent QS and PQS signaling (Gallagher et al. 2002). However, in the present study, the MvfR regulated genes were repressed, contrary
to the expected overexpression due to the reduced RhlR production. Indeed, the observed repression of mvfR regulated genes could be attributed to less OdDHL–LasR complex formation activating the mvfR expression as detailed in Figure 8. This hypothesis is supported by Choi et al. (2011) who studied the expression of mvfR system regulated genes (pyocyanin and the pqsA-E operon) in P. aeruginosa rhIR and lasRrhIR mutants. They observed inactivation of these genes in lasRrhIR mutants, demonstrating the requirement of OdDHL–LasR in activating the PqsR system.

The significance of this study is confirmation of the anti-virulence properties of eugenol through QS inhibition mechanisms in addition to its anti-biofilm and biofilm eradication properties. Eugenol could enhance biofilm-associated bacterial clearance of gentamicin even at lower therapeutic doses (less than the MIC). This study further evaluated the QSI properties of eugenol not only against P. aeruginosa (reference strain) but also in the catheter isolates P. aeruginosa RRLP1 and RRLP2 from patients with CAUTIs. Furthermore, this study provided insights into the molecular mechanism of the anti-QS property of eugenol via competitive binding to cognate receptors. Eugenol, a major active principle in many essential oils, is considered safe as a medication for treating biofilm-associated health care infections and/or as a cleaning agent for hospital apparatus and surfaces in health care environments, which can contribute to the prevention of biofilm-associated nosocomial infections.

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