Clove bud oil reduces kynurenine and inhibits *pqs A* gene expression in *P. aeruginosa*

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Abstract Quorum sensing (QS), a communication system involved in virulence of pathogenic bacteria like *Pseudomonas aeruginosa* is a promising target to combat multiple drug resistance. In vitro studies using clove bud oil (CBO) in *P. aeruginosa* revealed a concentration dependent attenuation of a variety of virulence factors including motility, extracellular DNA, exopolysaccharides and pigment production. Furthermore, treatment with CBO demonstrated a distinct dose-dependent reduction in biofilm formation as well as promoting dispersion of already formed biofilm, observations that were also supported by porcine skin ex vivo studies. Expression studies of genes involved in signalling systems of *P. aeruginosa* indicated a specific decrease in transcription of *pqsA*, but not in the *lasI* or *rhlI* levels. Additionally, the expression of *vfr* and *gacA* genes, involved in regulation, was also not affected by CBO treatment. CBO also influenced the PQS signalling pathway by decreasing the levels of kynurenine, an effect which was reversed by the addition of exogenous kynurenine. Though the synthesis of the signalling molecules of the Las and Rhl pathways was not affected by CBO, their activity was significantly affected, as observed by decrease in levels of their various effectors. Molecular modelling studies demonstrated that eugenol, the major component of CBO, favourably binds to the QS receptor by hydrophobic interactions as well as by hydrogen bonding with Arg61 and Tyr41 which are key amino acid residues of the LasR receptor. These results thus elucidate the molecular mechanism underlying the action of CBO and provide the basis for the identification of an attractive QS inhibitor.

Keywords Quorum sensing · *P. aeruginosa* · Biofilm · Clove bud oil

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Introduction

Pseudomonas aeruginosa is the foremost cause of secondary infections in immune-compromised patients (Meynard et al. 1999) and accounts for 57 % of total nosocomial infections (Oncul et al. 2009). *P. aeruginosa* can grow as a planktonic, free-living organism or as attached matrix-enclosed communities called biofilm, which are important for microbial endurance in hostile settings, representing a secluded mode of growth. In clinical settings, the major challenge presented by biofilm forming bacteria is the increased protection they enjoy against host immune responses (Cady et al. 2012). Additionally, this makes them significantly more tolerant to various anti-microbial treatments, resulting in multidrug-resistant organisms with major public health implications (Tamma et al. 2012). Furthermore, *P. aeruginosa* possesses an array of cell associated and extracellular virulence factors (Jimenez et al. 2012), which makes it a versatile pathogen responsible for life-threatening infections. Production of most of these virulence factors are regulated by quorum sensing (QS) (Rumbaugh et al. 2000). Since QS regulates factors contributing to the virulence of a pathogen, it is a promising target

for attenuation of bacterial pathogenicity. Hence, inhibitors that regulate biofilm formation and other virulence factors without interfering with bacterial growth have been the focus of recent studies.

The three major QS systems which have been found to control the virulence and biofilm formation in *P. aeruginosa* are LasI-LasR, RhlI-RhlR and PQS-MvfR (Venturi 2006). LasI produces an extracellular diffusible acyl homoserine lactone (AHL) signal molecule, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), which is recognized by the transcriptional regulator LasR, resulting in diverse gene expression in the RhlI-RhlR system. Similarly, RhlI produces the *N*-butyryl-L-homoserine lactone (BHL) signal molecule which binds to its cognate transcriptional regulator RhlR. LasR and RhlR transcriptional regulators are activated when adequate levels of OdDHL and BHL are attained, resulting from a high population density of *P. aeruginosa* (Pesci et al. 1997). In the PQS-MvfR system, 2-heptyl-3-hydroxy-4(1H) quinolone (PQS), and its precursors bind to the transcriptional regulator MvfR and control the transcription of downstream targets (Heeb et al. 2011). The Las system regulates a complex hierarchy of other QS pathways that are regulators of many cellular processes. The PQS pathway is one such pathway that is directly regulated by the Las system (Bacalso et al. 2011). LasR upregulates the expression of *pqs*A/B/C/D/H and R genes. Of these, *pqs*A/B/C and D are involved in the synthesis of the precursor of PQS. In view of the fact that QS regulates factors contributing to the virulence of *P. aeruginosa*, it is a promising target for attenuation of bacterial pathogenicity.

Plant-derived compounds have traditionally been used to treat microbial infections (Mahady, 2005). Essential oils (EOs) are aromatic liquids obtained from plants and are rich sources of biologically active compounds. Extracts of various natural products and EOs of several plants (e.g., lavender, eucalyptus, cinnamon and citrus) (Szabo et al. 2010) exhibit anti-QS effects. Clove bud oil (CBO) demonstrates many useful properties and has been used extensively in dental remedies (Bhowmik et al. 2012). Applications of CBO in aromatherapy to aid with digestive problems are also well established (Sanlin et al. 2011). Additionally, CBO has also been used to alleviate muscular aches and pains (Shaaban et al. 2012) and used in vapour therapy for relief from respiratory problems (Bhowmik et al. 2012). The present study provides convincing evidence that CBO significantly inhibits *P. aeruginosa* biofilm formation, as well as promotes dispersion of formed biofilm. This effect is mediated by attenuation of a variety of virulence factors including motility, extracellular DNA, exopolysaccharide and pigment formation. CBO also causes a specific decrease in *pqsA* gene transcription and reduction in kynurenine levels. The hydrogen bonding and hydrophobic interactions of eugenol, the major component of CBO with Arg61 and Tyr47, which are key amino acid residues of the Las R receptor, are also critical for its inhibitory

effect. The anti-QS effect of CBO towards *P. aeruginosa*, thus provides a better insight into infections caused by this pathogen, and the basis for identification and characterization of an effective means of QS inhibition.

Materials and Methods

Bacterial strains and treatments *P. aeruginosa* wild type strain, PAO1 (ATCC 15692), obtained from American Type Culture Collection, Manassas, VA, USA was grown in Luria Bertani (LB) broth at 37 °C and treated with commercially obtained CBO (*Syzygium aromaticum*) (Plant Lipids Pvt. Ltd., Cochin, Kerala, India) in LB broth for 24 h.

Assessment of biofilm formation and biofilm dispersal Biofilm formation was determined as previously described (Adonizio et al. 2008). Briefly, the stationary phase culture of PAO1 was diluted in fresh medium, followed by overnight incubation with CBO in polystyrene microtitre plates (BD, USA) at 37 °C for 24 h. Surface-attached cells were quantified using 0.1 % (*w/v*) aqueous solution of crystal violet, and the absorbance was measured at 600 nm. For biofilm dispersal assays, the biofilm was first allowed to develop in polystyrene microtitre plates for 24 h in the absence of CBO treatment. Following incubation, the plates were washed, and fresh media containing CBO was added and incubated for an additional 24 h (Quave et al. 2008). The staining techniques were the same as those described above.

Microscopic observation of biofilm To observe the dispersal of mature biofilm, glass slides were used to develop the biofilm from cultures grown at 37 °C at 100 rpm for 24 h. The mature biofilm was incubated with CBO for an additional 24 h, and the unbound bacteria were removed by rinsing the slides with sterile saline. The samples were air dried and processed for atomic force microscopy (AFM) (Oh et al. 2009).

Growth curve The effect of CBO on cell proliferation of *P. aeruginosa* PAO1 was determined as per Thenmozhi et al. (2009). CBO was added to 50 ml LB, to which a 1 % inoculum from the overnight culture was added. This was incubated at 37 °C. Growth medium, without the addition of *P. aeruginosa* PAO1, served as a negative control, as compared to medium with the addition of bacteria, which served as a positive control. The growth rate was monitored by taking the OD at 600 nm (OD_{600}) for 24 h at hourly intervals.

Swarming motility The bacteria was cultured in media containing 0.5 % agar, 0.5 % peptone, 0.2 % yeast extract and 1 % glucose in distilled water with or without CBO. Two microlitres of late log phase cultures of PAO1 diluted to 0.1

OD$_{600}$ was inoculated at the centre of the agar, following which the plate was incubated for 16 h at 37 °C. The extent of swarming motility was determined by measuring the diameter of the swarming migration from the point of inoculation (Rashid and Kornberg 2000).

Swimming and twitching motility Determination of swimming and twitching motility was carried out, according to the method described by Rashid and Kornberg 2000. The bacterial culture was inoculated in media containing 1 % tryptone, 0.5 % NaCl and 0.3 % agar with or without 1 % CBO and incubated for 16 h at 37 °C. Motility was assessed by the circular turbid zone formed by bacteria migrating away from the point of inoculation. To study twitching motility, cells were stab inoculated through 1 % LB agar layer, to the bottom of the petri dish. After incubation for 24 h at 37 °C, the extent of motility was determined by measuring the hazy zone of growth at the interface between the agar and the polystyrene surface.

Isolation and quantification of exopolysaccharides Isolation and quantification of exopolysaccharides (EPS) was done as described previously (Myska and Czaczk, 2009). Bacteria treated with or without 1 % CBO was harvested by centrifugation at 8000g for 20 min at room temperature. The cells were resuspended in 1.5 ml of 30 % (w/v) NaOH, boiled for 15 min and centrifuged at 15,000g for 15 min, and the supernatant was added drop wise to 60 % (v/v) ethanol. The total EPS was determined using the acid hydrolysis method (Myska and Czaczk, 2009). The precipitated EPS was collected by centrifugation at 15,000g for 20 min. The pellet obtained was resuspended in 1 ml sterile water, mixed with 7 ml of 77 % (v/v) H$_2$SO$_4$ and cooled for 10 min in an ice bath. Subsequently, 1 ml of 1 % (w/v) of cold tryptophan was added, followed by heating in a boiling water bath for 20 min. The acid hydrolysis of EPS produced a furan, which condenses the tryptophan to form a coloured product that was read at OD$_{500}$ after cooling. The calibration curve was prepared using standard dextran solution.

Measurements of extracellular DNA Overnight cultures of PAO1 were grown in LB broth and were subsequently diluted to an OD$_{600}$ of 0.1 in LB medium containing 0.05 mM propidium iodide. The diluted cultures were transferred to wells of polystyrene microtitre plates (100 μl/well) and incubated for 24 h at 37 °C. After incubation, the absorbance of propidium iodide was measured at OD$_{480}$, and the cell density was measured at OD$_{600}$(Yang et al. 2007).

Quantification of pigment production Pyocyanin was extracted from culture supernatants and measured using previously reported methods (Liang et al. 2011). Briefly, 3 ml of chloroform was added to 5 ml culture supernatant. Following

extraction, the chloroform layer was transferred to a fresh tube and mixed with 1 ml of 0.2 M HCl. After centrifugation, the top layer (0.2 M HCl) was removed, and the absorbance at 520 nm was measured. The pyocyanin concentration was determined by multiplying the A$_{520}$ by 17.072. To estimate Pyoverdin, 1 ml of culture supernatant was diluted in 50 mM Tris-HCl (pH 7.4), and the resulting fluorescence was measured at 465 nm by exciting the sample at 405 nm (Adonizio et al. 2008).

Kynurenine production assay *P. aeruginosa* PAO1 was cultured in LB broth at 37 °C with a starting OD$_{600}$ of 0.2. At the indicated incubation time, the supernatant recovered after centrifugation at 10,000g for 10 min was mixed with an equal volume of Kovac's reagent. The amount of kynurenine produced was measured at 490 nm and computed from the standard curves (Genestet et al. 2014).

RNA isolation and RT PCR To examine the effect of CBO on QS genes, the bacterial cultures were treated with different concentrations of CBO. Aliquots (1.5 ml) of bacterial cultures to be used for RNA extraction were transferred to a pre-chilled vial and the cells were harvested by centrifugation at 6000 g for 10 min to remove extracellular membranes, polysaccharides, and DNA. The RNA was extracted by TRIZOL (Life technologies) reagent, which was added to the pellet and incubated for 3 min at room temperature. Ice-cold chloroform (0.1ml) was added, mixed for 15 s and incubated for 15 min followed by centrifugation (8000g) at 4 °C for 15 min. The upper chloroform layer was transferred to a fresh tube to which 0.25 ml of 100 % isopropyl alcohol was added and incubated at −20 °C for 30 min. The mixture was then centrifuged at 14,000 rpm for 10 min at 4 °C to form pellets. The pellets were washed with 1 ml of 70 % ethanol and centrifuged at 14,000 rpm for 5 min at 4 °C. Washed pellets were dissolved in nuclease free water, and the quantity of total RNA was measured using Nanodrop spectrophotometer (Thermo Scientific). One microgram of template RNA was mixed with 1 pM/μl of specific primer (Table S1) and the PCR master mix, as described by the manufacturer (Origin, India). Amplification and detection of the product was performed by employing the one step reverse transcription PCR using Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Primers were designed as reported by Kruczek et al. 2014.

Comparative infrared analysis of CBO and eugenol Infrared spectra were recorded (cm^{-1}) on FT-IR spectrophotometer (Shimadzu model no. 01080). Sample spectrums were recorded on film. CBO was compared with the standard compound, eugenol.

In silico docking study of Las R and eugenol In order to identify the binding mode of eugenol, Auto Dock Tools (http://mgltools.scripps.edu) was used (Nanjan et al. 2015). A PDB coordinate file of LasR (PDB ID: 2UVO) was downloaded from the RCSB PDB data bank and then utilized for the docking studies. 2UVO is the X-ray structure of the N-terminal OdDHL binding domains of LasR complexed with OdDHL. The best docking pose of eugenol was selected and analysed.

Ex vivo porcine skin model Hides from carcasses of freshly killed adult pigs were obtained from a local slaughterhouse. Prior to tests, the hides were thoroughly washed with sterile water and de-haired. The excess fat deposits were removed, and the skin was cut into sections of 1.5 cm × 1.5 cm. The cut sections were soaked in 70 % ethanol for 5 min and washed thoroughly with sterile water. The disinfected sections were then placed on sterile petri plates and allowed to dry. The log phase culture (OD_{600} between 0.2 and 0.4, 10 µl) was added to the skin explants. The explants were then placed in culture plates and incubated (37 °C for 24 h, 5 % CO_2) for the development of the biofilm. Following incubation with CBO for 24 h, the skin explants were processed for SEM analysis. Subsequently, the explants were processed by sonication in phosphate-buffered saline (PBS) with 5 µl/L Tween-80 to ensure the complete dispersal of biofilm. The accurate colony-forming unit (CFU) counts in the resulting bacterial

suspensions were determined by plating serial dilutions on tryptic soy agar (TSA) in triplicates, and the data was reported as CFU/millilitre.

The skin explants were washed with PBS, fixed in Trump's solution for 1 h and washed three times with PBS at 10 min intervals followed by a single 10-min wash with distilled water. Each fixed and washed explant was dehydrated in a graded ethanol series of 25, 50, 75, 95 and 100 % at 10-min intervals. The explants were then air dried overnight and processed for SEM analysis (Nataraj et al. 2013, Yang et al. 2013).

Statistical analysis

Statistical analyses were performed with the GraphPad Prism 5 software. Values were expressed as mean ± SD. Dunnett-ANOVA test was employed to compare the tests and control. Significance levels were at *$p < 0.05$ vs control, **$p < 0.01$ vs control and ***$p < 0.001$ vs control.

Results

Effect of CBO on biofilm formation and dispersion of pre-established biofilm of *P. aeruginosa* A static biofilm assay was employed to evaluate the biofilm formation of *P. aeruginosa* in the presence of CBO. As illustrated in Fig. 1a, cultures treated with CBO concentrations ranging

Fig. 1 **a** Effect of CBO on biofilm formation and biofilm dispersion. *Pseudomonas aeruginosa* (PAO1) was grown in medium containing 0.01–1.0 % CBO to study the effect on biofilm formation and biofilm dispersion. **b** AFM images of *P. aeruginosa* PAO1 mature biofilm grown on glass slide treated with or without CBO (**c**). To study the effect of CBO on bacterial growth, PAO1 was treated with 1.0 % CBO and growth was monitored by taking the OD_{600}. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at *$p < 0.05$ vs control, **$p < 0.01$ vs control, ***$p < 0.001$ vs control
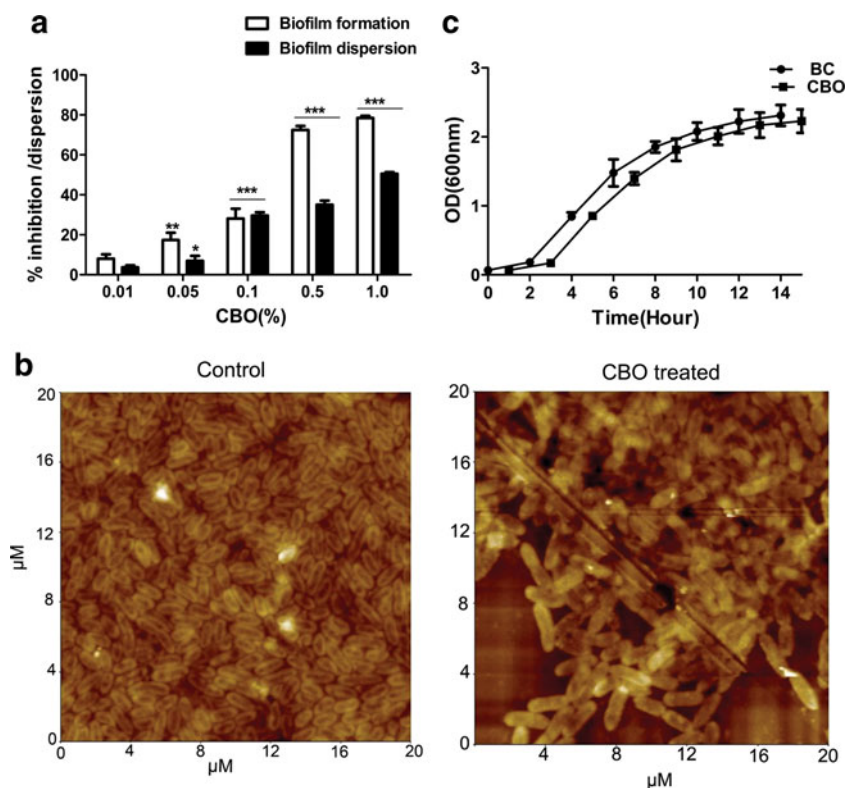
from 0.01 to 1.0 % demonstrated a dose-dependent reduction of biofilm formation by 5 to 85.3 %, respectively, when compared with the untreated control, indicating that this effect was mediated by CBO. In order to determine if CBO could also affect previously formed biofilm, cultures with pre-established PAO1 biofilm was treated with CBO, at concentrations ranging from 0.01 to 1.0 %. Figure 1a shows a dose-dependent increase in biofilm dispersal ranging from 2.7 to 50.4 %, as compared to the untreated control, demonstrating that CBO also abolished pre-established biofilm, in addition to inhibiting biofilm formation. Atomic force microscopy (AFM) was also utilized to visualize the effect of CBO on dispersion of pre-established biofilm on glass slides. The AFM images revealed distinct differences in surface morphology and topography. The topography of the biofilm treated with CBO showed fewer adherent cells on the surface as compared with the untreated control. Additionally, the thickness and architecture of biofilm in the presence of CBO indicated that the bacterial cells were scattered on the substratum in a thin layer, while in the untreated control, the biofilm appeared mature and stacked densely in multiple layers (Fig. 1b and Fig. S1).

Growth curve analysis The growth of PAO1 was monitored for 24 h at 37 °C to determine the effect of CBO on bacterial growth. As observed in Fig. 1c, there was no significant difference in the end point cell density of PAO1 in the presence of 1 % CBO as compared to the untreated control, indicating that CBO did not affect the growth of *P. aeruginosa*. Based on these observations, we concluded that the inhibitory effect exhibited by CBO, on biofilm formation and increased dispersal of already formed biofilm, is not due to alteration in growth patterns.

Effect of CBO on factors that influence biofilm formation

Effect on swarming, swimming and twitching motilities of PAO1 The effect of CBO on swarming, swimming, and twitching motilities of PAO1, all of which play a crucial role in biofilm formation, was tested (Fig. 2a). The swarming motility was substantially decreased by 59.5 % in the presence of 1 % CBO (Fig. 2b). When compared with the swarming motility, the diameter of the swimming zone was decreased by 35 % in the presence of 1 % CBO, while the twitching motility was reduced by 24 % as compared to the untreated control. These results indicated that CBO differentially regulated all three types of motility exhibited by *P. aeruginosa*.

Effect on exopolysaccharide level Quantitative determination of EPS was carried out to determine the potential of CBO on EPS production. As indicated in Fig. 3, the amount of EPS produced by PAO1 was significantly reduced in a dose-dependent manner, i.e. 48.6, 85.9 and 91.9 % reduction in the presence of 0.1, 0.5 and 1 % of CBO, respectively, as compared to the untreated control.

Effect on extracellular DNA In order to quantify the extracellular DNA (e DNA), the level of fluorescence in the cultures,
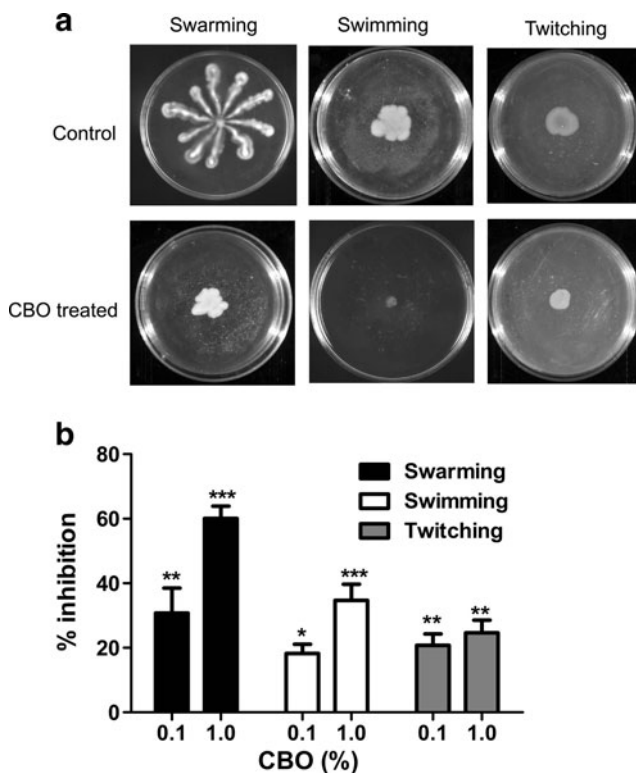
Fig. 2 **a** Impact of CBO on swarming, swimming and twitching motility exhibited by PAO1. **b** Motility was measured by taking the diameter of movement of bacteria. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at *$p < 0.05$ vs control, **$p < 0.01$ vs control, ***$p < 0.001$ vs control
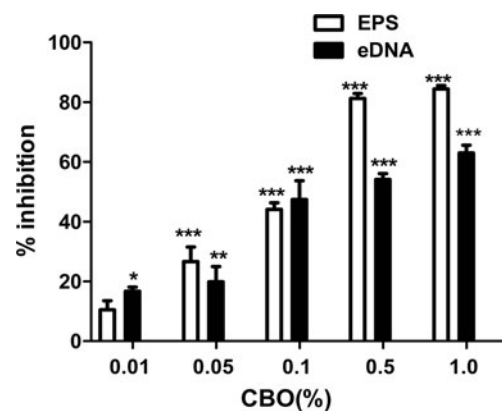
Fig. 3 Effect of CBO on exopolysaccharide and extracellular DNA content. *P. aeruginosa* (PAO1) was treated with 0.01–1.0 % CBO for 24 h and exopolysaccharide (EPS) and extra cellular DNA (e DNA) content was determined. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at *$p < 0.05$ vs control, **$p < 0.01$ vs control, ***$p < 0.001$ vs control

which was indicative of e DNA, was measured. The amount of e DNA of PAO1 was found to be decreased in a dose-dependent manner with increasing concentration of CBO (Fig. 3). Treatment with 0.5 and 1 % CBO resulted in 56.2 and 65.5 % reduction in the amount of e DNA respectively.

Effect on pigments of PAO1 The levels of the pyocyanin and pyoverdine in PAO1 cultures treated with CBO were measured. As indicated in Fig. 4, both pyocyanin and pyoverdine concentrations were decreased by 49.9 and 54.3 %, respectively, in the presence of 1 % CBO as compared to the untreated control.

Effect of CBO on expression of QS genes To determine whether the inhibition of biofilm formation was due to the decreased expression of QS genes, reverse transcription polymerase chain reaction of RNA from cultures grown in the presence of different concentrations of CBO was performed. *P. aeruginosa* has three QS systems—namely Las, Rhl and PQS. As shown in Fig. 5a and b our studies demonstrated that CBO did not affect the transcriptional activation of *lasI* or *rhlI* genes, indicating that the synthesis of the signalling molecules, OdDHL and BHL, was not affected by CBO. Additionally, the expression levels of *gacA* and *vfr*, the positive regulators of QS, were also not significantly affected by CBO. However, the transcriptional activation of *pqsA*, which is a part of *pqsA-E* operon, was specifically down regulated by CBO when compared to the untreated control (0.3 fold decrease in the expression compared to the control).

Effect of CBO on kynurenine production To evaluate the impact of CBO on the kynurenine pathway of *P. aeruginosa*, with a specific focus on kynurenine production, PAO1 (OD600=0.2) was treated with different concentrations of CBO. As shown in Fig. 6, there was a concomitant decrease

in kynurenine levels observed with increasing concentrations of CBO. Kynurenine production was decreased by 61 % in the presence of 1 % CBO as compared to the untreated control. This data suggests that CBO affects the kynurenine pathway, which is essential for the production of PQS signalling molecules.

***P. aeruginosa* pyocyanin production in the presence of externally added kynurenine** Since pyocyanin production was regulated by PQS signalling mediated through the kynurenine pathway, the effect of externally added kynurenine on the levels of pyocyanin was determined in the presence of CBO. The addition of 12.5, 25 and 50 μM kynurenine to *P. aeruginosa* PAO1 cultures containing 1 % CBO led to a corresponding 1.39, 1.46 and 2.16 fold increase in pyocyanin production,
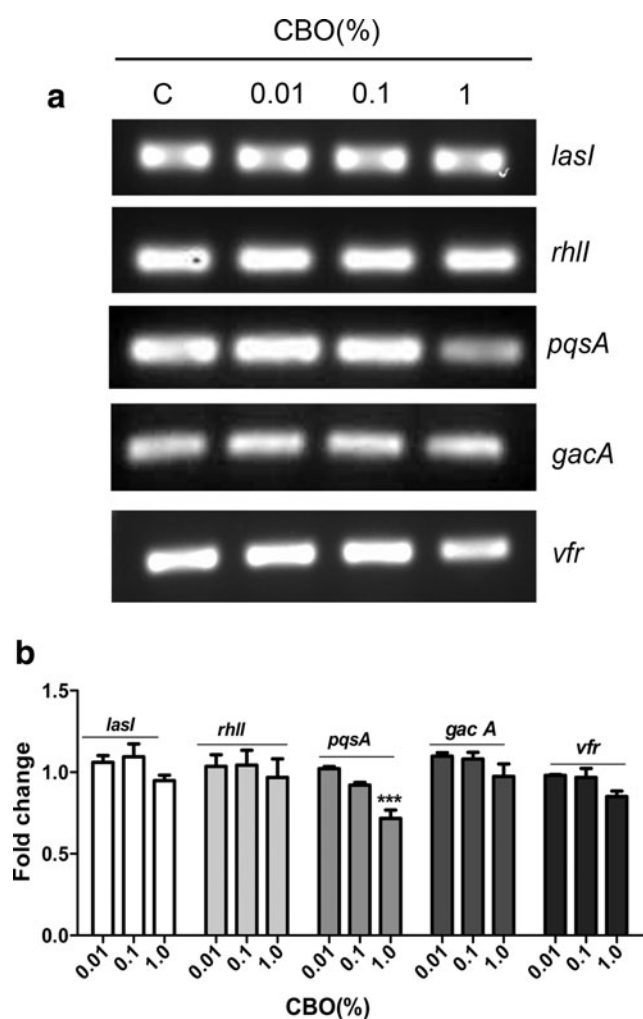
Fig. 5 RT PCR analyses of genes involved in QS system of *P. aeruginosa* PAO1. **a** Gene expression was evaluated in control and cultures treated with 0.01, 0.1 and 1.0 % CBO. **b** Fold change in the expression of QS genes of *P. aeruginosa* treated with CBO. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at ***$p < 0.001$ vs control
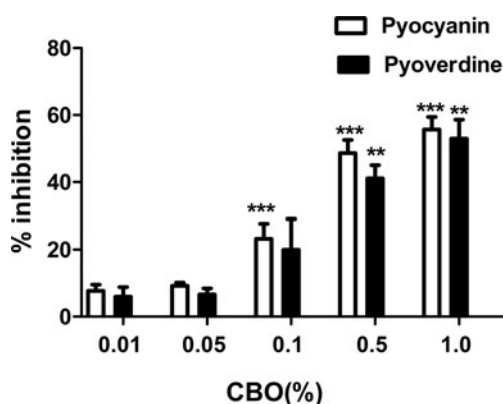

Fig. 4 Effect of CBO on pyocyanin and pyoverdin. *P. aeruginosa* (PAO1) was treated with 0.01–1.0 % CBO and the amount of pyocyanin and pyoverdin were determined. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at **$p < 0.01$ vs control, ***$p < 0.001$ vs control
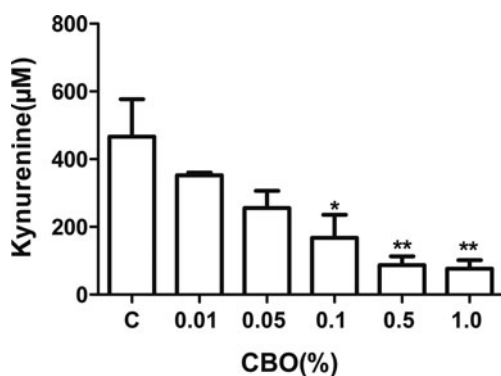
Fig. 6 Effect of CBO on the production of kynurenine. *P. aeruginosa* PAO1 was grown in LB at 37 °C with different concentrations of CBO. The kynurenine production in the supernatant was measured after 8h. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at *$p < 0.05$ vs control, **$p < 0.01$ vs control
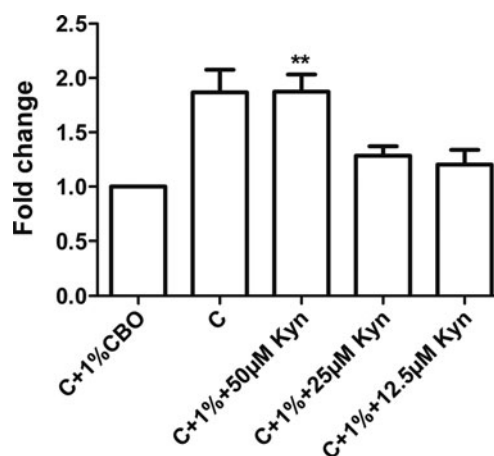

Fig. 7 Change in pyocyanin production by *P. aeruginosa* PAO1 grown in the presence of different concentrations of kynurenine (*Kyn*) with 1 % CBO. The values are presented as fold change relative to *P. aeruginosa* treated with 1 % CBO. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control. Significance at **$p < 0.01$ vs control

relative to cultures treated with 1 % CBO alone (Fig. 7). These results clearly demonstrate that CBO interferes with the kynurenine pathway of *P. aeruginosa*, by decreasing the production of kynurenine, which in turn leads to a diminished level of PQS signalling, resulting in lowered levels of pyocyanin.

In silico molecular docking of Las R with eugenol Eugenol is known to be the major component, constituting 88.5 % of CBO (Chaieb et al. 2007). As shown in Fig. S2, IR spectra studies confirmed that the CBO used in the present study also contained eugenol as its major component. Hence, eugenol was utilized for in silico analysis with Las R receptor to understand the binding pattern as compared to its ligand. Molecular modelling of eugenol with Las R receptor indicated that both the methoxy and hydroxyl groups of eugenol hydrogen bond with the amino acids Arg61 and Tyr47, which are key amino acid residues in the active site of Las R. Additionally, eugenol also forms hydrophobic interactions with Ile52, Gly126, Gly127, Lys79, Val76, Ala70, Tyr64 and Leu36. The aromatic hydroxyl and methoxy groups are involved in the inhibition, while the hydrophobic interactions stabilize the overall orientation of eugenol (Fig. 8a and b).

Ex vivo porcine skin explants study SEM analysis of skin explants was prepared as described earlier (Nataraj et al. 2013, Yang et al. 2013). Explants treated with CBO were nearly devoid of the exopolysaccharide (EPS) matrix when compared with the untreated explants. A substantial number of attached bacterial cells were present on the surface of untreated explants, whereas there was a significant decrease in the bacteria attached on the surface after treatment with 1 % CBO (Fig. 9a, Fig S3). These observations were further substantiated by lower levels of CFU in treated explants, as compared with the untreated control (the mean bacterial load decreasing from 17×10^6 log total CFU to 6×10^5 log) (Fig. 9b). Collectively, both the SEM analyses and CFU levels indicated that CBO

significantly reduced the extent of *P. aeruginosa*, in mature biofilm communities.

Discussion

The occurrence of multidrug resistance in pathogens like *P. aeruginosa* is an issue of great clinical concern. Since the antibiotic resistant variants of *Pseudomonas* were established to be more adherent and virulent in most cases (Aliaga et al. 2002; Wei et al. 2011), the identification of substances which attenuate the virulence, without killing the bacteria, is an attractive alternative. Quorum sensing (QS) is a perfect target for this approach, since most of the virulence factors in *P. aeruginosa* are under the control of QS. Traditional medicines, such as essential oils, have over the years, generated a great deal of interest as repositories for the development of new drugs and therapeutics (Kim et al. 2015). The present study clearly demonstrates that clove bud oil (CBO) inhibits biofilm formation as well as promotes dispersion of the formed mature biofilm in *P. aeruginosa*, in a concentration-dependent manner. Since concentrations greater than 1 % resulted in bactericidal effects, further studies were carried out at a concentration of 0.01–1 % clove bud oil.

The AFM images of biofilm treated with CBO revealed distinct differences in surface morphology as well as topography when compared to the untreated control, which showed a dense biofilm with multiple layers of EPS embedded with bacteria. In contrast, the topography of the CBO treated biofilm was thin, less intense and exhibited very little adherence of cells on the surface biofilm. These findings clearly demonstrate that not only does CBO affect the adherence of bacteria from forming biofilms but also causes the eventual dispersion of the biofilm
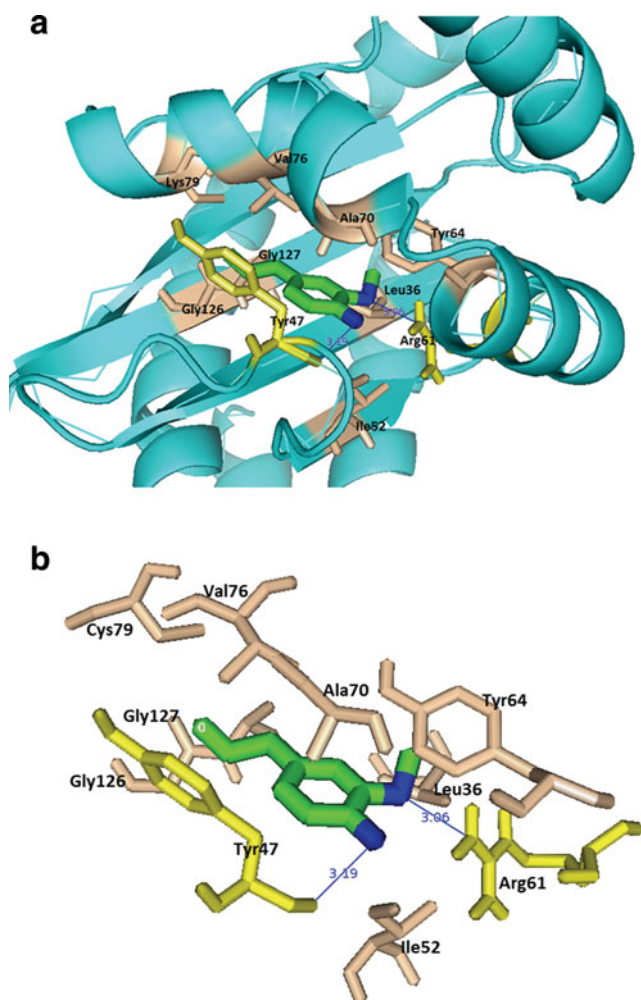
Fig. 8 In silico molecular docking analysis. **a** Docking of eugenol with LasR (2UVO). **b** Interactions of eugenol with the amino acids present in LasR. *Numbers* indicate the distance in A°

matrix, leading to its structural loss. The CBO thereby makes the organism more vulnerable and provides a possible line of attack, by reducing the resistance of sessile cells to antibiotics.

The growth curve of *P. aeruginosa* was not altered in the presence of CBO, demonstrating that this compound did not exhibit any bactericidal or bacteriostatic effect. This indicates that the inhibition of biofilm formation was not due to any lethal effect on bacteria and might, therefore, be expected to reduce the resistance as well as restore sensitivity to antibiotics, in addition to allowing for successful clearance by the host immune system (Johansson et al. 2008). Since QS signalling controls motility, EPS and e DNA production in Gram-negative bacteria like *P. aeruginosa* (Marketon et al. 2003), all of which play a crucial role in biofilm formation, the effect of CBO was also assessed against these properties. Bacterial motility has a pivotal role in both surface colonization and biofilm formation and is proven to be instrumental in spreading to new environments (Rashid and Kornberg 2000). *P. aeruginosa* exhibits different types of motility like swimming, swarming and twitching that help in adherence, formation and expansion of biofilm. The swimming motility, which is mediated by the flagella, is used to approach a chosen surface, while attachment and further spreading of the colony is mediated by swarming and twitching motilities, which are regulated by multiple flagella and type IV pili, respectively (Klausen et al. 2003). Earlier reports have indicated that swarming of *P. aeruginosa* is controlled mainly by the Rhl QS system and partly by the Las system, while the swimming and twitching motilities are under the control of the PQS system (Köhler et al. 2000). Since the adherence and subsequent establishment of biofilm by *P. aeruginosa* is mediated to a great extent by the different types of motility, the effect of CBO in inhibiting the different types of motility provides evidence that CBO impedes biofilm formation, at the beginning of the attachment stage itself.

Another key aspect of biofilm formation by *P. aeruginosa* is the production of the extracellular matrix, which is comprised of EPS, e DNA and protein, and it has typically been credited with structuring the mature biofilm (Wei and Ma 2013). It has been reported that overproduction of EPS leads to variation in biofilm architecture that correlates with an augmented resistance of the cells to osmotic and oxidative stress, as well as susceptibility to killing by biocides (Yildiz and Schoolnik, 1999). Reduction of EPS production and swarming motility in addition to inhibition of biofilm formation suggests that CBO not only affects the initial stages of biofilm formation but also affects structural design of the biofilm. In addition to EPS, e DNA is by far the most abundant polymer and comprises an important component of the *P. aeruginosa* biofilm matrix (Matsukawa and Greenberg, 2004). Production of e DNA, which is regulated by PQS signalling, facilitates expansion of biofilm by mediating twitching motility and facilitates cell aggregation by influencing physio-chemical interactions (Gloag et al. 2013; Das T and Manefield, 2012). The inhibition of e DNA observed in the presence of CBO clearly demonstrates that the expansion of the biofilm is also affected along with the cell aggregation.

Additionally, CBO inhibited the formation of both pyocyanin and pyoverdine, which are also controlled by PQS and Rhl signalling systems, respectively. Synthesis of the iron siderophore, pyoverdine, occurs in the stalk portion of the mushroom-shaped structures of the biofilm and is necessary for development of the cap-forming sub-population (Yang et al. 2009). Pyoverdin can also compete with mammalian transferrin for iron as well as promote pathogenicity by stimulating bacterial growth (Adonizio et al. 2008). Earlier reports have indicated that the production of pyocyanin contributes to virulence in chronic lung infections, enhanced e DNA production, microcolony formation and increased biomass, all of which are necessary for development of biofilm stability in *P. aeruginosa* (Das et al. 2013). Our observation of decreased e DNA and inhibition of biofilm formation is consistent with lowered levels of pyocyanin, which is seen in the presence of

Fig. 9 CBO inhibits biofilm formation in ex vivo porcine skin model. **a** Scanning electron micrographs of PAO1 biofilm attached to porcine skin explants. **b** Difference in colony-forming units in CBO treated and untreated porcine skin explants. Mean values of triplicate independent experiments and SD are shown. *t* test demonstrates significant difference between the test and the control. Significance at **$p < 0.01$ vs control
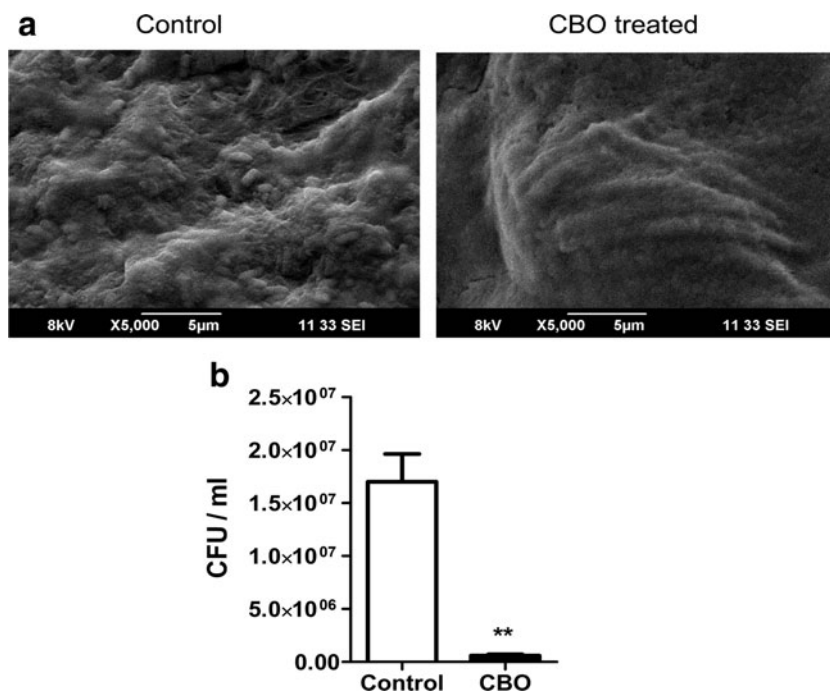
CBO. Taken together, these results clearly indicated that since CBO inhibited biofilm formation in addition to motility, pigment production as well as levels of EPS and e DNA, it could potentially be a very good candidate for attenuating a wide array of virulence factors exhibited by *Pseudomonas*.

To study the mechanism underlying these observations, we analysed the effect of CBO on the expression of genes in the QS circuitry. Our results indicated that CBO specifically decreased the expression of *pqsA*, implicated in the conversion of anthranilic acid to 2-heptyl-4-quinolone (HHQ), the precursor of PQS (Zhang et al. 2008; Farrow and Pesci 2007). These results support and corroborate our earlier observations (inhibition of e DNA and pyocyanin) that the presence of CBO results in weak biofilm formation. Additionally, CBO also inhibited the levels of kynurenine, the precursor to anthranilate. The downregulation of *pqsA* gene could be due to
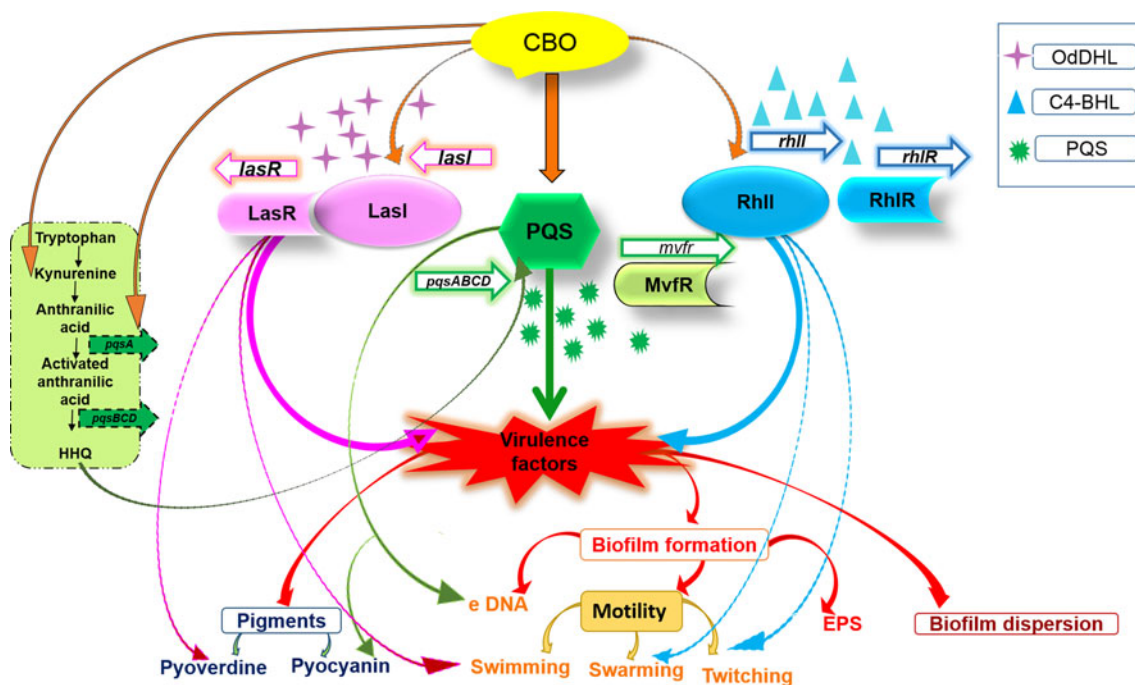

Fig. 10 A schematic representation of effect of the CBO on QS system of *P. aeruginosa* PAO1

the lowered concentration of kynurenine. Anthranilate is also known to induce genes for pyocyanin biosynthesis (Calfee et al. 2001; Mavrodi et al. 2001). The reduced amount of pyocyanin and e DNA levels could possibly be an effect of CBO interfering with the PQS signalling pathway, an observation which was corroborated by the fact that the amount of pyocyanin was increased significantly in response to the external addition of kynurenine. Taken together, these results clearly establish the mechanism by means of which CBO affects tryptophan metabolism, particularly the kynurenine pathway of *P. aeruginosa*, by decreasing the production of kynurenine, which in turn leads to a diminished level of PQS signalling, resulting in lowered levels of pyocyanin.

The other two QS systems in *P. aeruginosa* are Las and Rhl. Our data demonstrated that even though CBO did not affect the transcriptional activation of *lasI* gene which encodes AHL synthase, it reduced QS controlled factors like pyoverdin levels and swimming motility regulated mainly by the Las system (Stintzi et al. 1998). Similarly, the transcriptional activation of the *rhlI* gene was also not affected in response to CBO, whereas swarming and twitching motilities, which are regulated by *rhl* system, were reduced. Furthermore, CBO did not significantly affect the expression of *vfr and gac A* genes. In *P. aeruginosa*, the Gac/Rsm system positively regulates the expression of the quorum-sensing signal *N*-butanoyl-homoserine lactone (C4-HSL) and the extracellular virulence factors, such as hydrogen cyanide (HCN), pyocyanin, and elastase production (Kay et al. 2006). The virulence factor regulator (*vfr*) is a central player in a transcriptional cascade that enhances the pathogenicity of *P. aeruginosa* through its role as a global transcriptional regulator (Serate et al. 2011). *vfr* positively regulates quorum sensing by promoting the transcription of *lasR* and upregulates transcription of the type III secretion system. These results indicated that CBO does not have any effect on the synthesis of the AHL, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL). Since the synthesis of these auto-inducers were not affected, whereas the virulence factors associated with these signalling systems were affected by CBO, we were interested in determining if the binding of these molecules to their receptors was affected by CBO. To address this, we have analysed the binding of eugenol, the major component of CBO, with the Las R receptor using in silico docking analysis. These studies suggest that the binding pattern of eugenol to the Las R receptor was comparable to that of its natural ligand OdDHL. Hence, eugenol could compete with OdDHL for binding to its cognate receptor, Las R, resulting in inhibition of QS.

Presences of appropriate substrates for attachment and adherence, as well as nutritional availability, are factors that are known to contribute to the development of bacterial biofilm. In this study, we used an ex vivo porcine skin explant biofilm model, which more closely replicates conditions in skin wounds as compared to the other models (Yang et al. 2013).

The SEM images clearly demonstrate that CBO was effective in inhibiting the bacterial biofilm attached to the porcine skin with significant differences in the depth of biofilm, as well as the bacterial density.

In conclusion, the present study provides convincing evidence that CBO successfully attenuates a wide array of virulence factors of *P. aeruginosa* and proposes a mechanism by which CBO circumvents QS in PAO1 (Fig. 10). The study further shows that in addition to exhibiting a potent inhibitory effect against the AHL-QS systems, CBO also inhibits the species-specific PQS system in *P. aeruginosa*. The use of CBO to combat *P. aeruginosa* infections should not raise safety concerns since clove traditionally has been used for medicinal and culinary purposes. Furthermore, the present study can also be used as a basis for utilizing eugenol as a suitable template in the design of new drug candidates focused on QS while reducing the possibility of the development of drug resistance in microorganisms.

Compliance with ethical standards This article does not contain any studies with human participants performed by any of the authors. Approval for experiments in this study to be conducted with porcine skin was obtained from the Institutional Ethics Committee.

Conflict of interest The authors declare that they have no competing interests.

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