Influence of clove oil on certain quorum-sensing-regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*

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Quorum sensing (QS) plays an important role in virulence, biofilm formation and survival of many pathogenic bacteria including *Pseudomonas aeruginosa*. This signalling pathway is considered as novel and promising target for anti-infective agents. In the present investigation, effect of the Sub-MICs of clove oil on QS regulated virulence factors and biofilm formation was evaluated against *P. aeruginosa* PAO1 and *Aeromonas hydrophila* WAF-38 strain. Sub-inhibitory concentrations of the clove oil demonstrated statistically significant reduction of *las*- and *rhl*-regulated virulence factors such as LasB, total protease, chitinase and pyocyanin production, swimming motility and exopolysaccharide production. The biofilm forming capability of PAO1 and *A. hydrophila* WAF-38 was also reduced in a concentration-dependent manner at all tested sub-MIC values. Further, the PAO1-preinfected *Caenorhabditis elegans* displayed an enhanced survival when treated with 1.6% v/v of clove oil. The above findings highlight the promising anti-QS-dependent therapeutic function of clove oil against *P. aeruginosa*.

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1. Introduction

Multi-drug resistant (MDR) bacteria have become a global health problem resulting in mortality of millions of people annually due to infections. Since the progress in the discovery of new antibacterial drugs with novel mode of actions is poor globally, alternative approaches to combat these resistant strains are the need of the hour (Rasmussen *et al.* 2005; Ahmad *et al.* 2008). It is important to emphasize that of all the infectious diseases, at least 65% are associated with the bacterial communities which proliferate by forming biofilms (Lewis 2007). Biofilm formation by many pathogens is closely linked to a density-dependent cell–cell communication known as quorum sensing (QS), in which small diffusible signalling molecules globally regulate expression of various genes including virulence genes (Fuqua *et al.* 2001; Rumbaugh *et al.* 2009). *Pseudomonas aeruginosa* is an opportunistic pathogen in which the role of QS-regulated virulence factors and biofilm is well studied in disease development (Rutherford and Bassler 2012). It is well known that *P. aeruginosa* employs the *las* and *rhl* AHL-based QS systems. LasR is a transcriptional regulator protein that recognizes its ligand (3-oxo-C12-HSL) and triggers the expression of LasB elastase, toxin production and biofilm formation (Pesci *et al.* 1997; Rumbaugh 2004). RhlR is the other transcriptional regulator protein which responds to *N*-butanoyl-L-homoserine lactone (C4-HSL) and regulates pyocyanin production (Williams 2007). Recently, a new QS signal, IQS, has been identified which is tightly controlled by *las* under normal culture conditions but is also activated by phosphate limitation, a common stressor that bacteria encounter during infections, indicating a more complex QS system in bacteria (Lee *et al.* 2013). *Aeromonas hydrophila* has also been described as an opportunistic pathogen that produces *N*-butanoyl-L-homoserine lactone (C4-HSL) as the principal AHL (Swift *et al.* 1997). The AHL-dependent QS plays a crucial role in the virulence and biofilm development of this pathogen (Williams, 2007). Since QS plays an important role in virulence and survival of *P. aeruginosa* and other pathogenic bacteria, this signalling pathway is a novel and potential target for anti-infective agents (Hentzer and Givskov 2003;

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Rasmussen and Givskov 2006). Interference of QS occurs by different ways, such as by acting as enzymes (e.g. AHL-lactonase or AHL acylase) which destroy signal molecules, or as enzymes that degrade LuxR protein, or as AHL mimics that block signal molecules (Zhang and Dong 2004, Kalia 2013). However, safe broad-spectrum stable anti-QS compound with proved therapeutic uses is yet to be discovered and exploited.

The first QS inhibitory activity was characterized in a seaweed *Delisea pulchra* (Rasmussen *et al.* 2000), and since then, QS inhbitors have also been reported in various natural products including medicinal plant species from South Florida (Adonizio *et al.* 2006; 2008a, b) and India (Sameena 2006; Zahin *et al.* 2010) in fruits and spices (Vattem *et al.* 2007; Packiavathy *et al*. 2012) and phytocompounds (Vandeputte *et al.* 2011). Essential oils possessing anti-QS property have also been reported by many workers (Szabo´ *et al.* 2010; Jaramillo-Colorado *et al.* 2012). In a previous study we also screened 21 essential oils and reported varying levels of anti-QS activity of four essential oils using biosensor strains of *Chromobacterium violaceum* (Khan *et al.* 2009). As QS inhibitors do not kill or inhibit bacterial growth, these agents have an advantage because they do not impose strong selective pressure for development of resistance, as compared to antibiotics (Rasmussen and Givskov 2006). In this study we have examined the effect of *Syzygium aromaticum* (clove) oil on the QS-regulated virulence and biofilm formation in *P.aeruginosa* PAO1 and *A. hydrophila* WAF-38 strain. Further, *in vivo* assessment of the efficacy of clove oil at sub-MICs to attenuate *P. aeruginosa*–mediated killing of the *Caenorhabditis elegans* (nematode model) is also reported.

2. Materials and methods

2.1 *Bacterial strain and growth conditions*

P. aeruginosa PAO1 is pathogenic and many of its virulence factors and traits are QS controlled. *Aeromonas hydrophila* WAF-79 used in the study is isolated from hospital wastewater (GenBank accession number JX416386). The strains were maintained on Luria Bertani or LB broth (15.0 g tryptone, 0.5% yeast extract, 0.5% NaCl) solidified with 1.5% agar (Hi-media) and was cultivated at 37°C. *Escherichia coli* MG4 pKDT17 (Pearson *et al*. 1994) reporter strain was grown on nutrient agar containing 100 µg/mL ampicillin. The plasmid pKDT17 contains a *lasB´-lacZ* detection system by which the lasB promoter driven lacZ expression by LasR is monitored and the *lasR* gene is under the control of the *lac* promoter.

2.2 *Determination of minimum inhibitory concentration*

Minimum inhibitory concentration (MIC) of essential oil was determined against PAO1 by broth macrodilution method (CLSI 2007). Sub-MICs were selected for the assessment of anti-virulence and anti-biofilm activity in the above-mentioned strain.

2.3 *Growth curve analysis*

The growth curve analysis was performed to test the antibacterial activity of sub-MICs of clove oil against PAO1. Briefly, cells were inoculated into 100 mL LB broth and cultivated in the presence or absence of test concentrations of oil. The culture setup was incubated at 37°C and the OD_{600} was monitored at 2 h intervals for up to 24 h.

2.4 *LasB elastolytic activity assay*

The elastolytic activity was determined using the method described by Adonizio *et al*. (2008a). Briefly, 100 µL of treated and untreated bacterial culture supernatant was added to 900 µl of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of elastin congo red (ECR, Sigma, USA) and then incubated with shaking at 37°C for 3 h. Insoluble ECR was removed by centrifugation, and the absorption of the Congo red in supernatant was measured at 495 nm. Cell-free LB medium with or without clove oil was used as negative control.

2.5 *Azocasein-degrading proteolytic activity*

Proteolytic activity of cell-free supernatant of PAO1 culture cultivated in the presence and absence of sub-MICs of clove oil was determined by azocasein assay as described by Kessler *et al*. (1993). Briefly, 150 µL of both treated and untreated PAO1 culture supernatants was added to 1 mL of 0.3% azocasein (Sigma, USA) in 0.05 M TrisHC1 and 0.5 mM $CaCl_2$ (pH 7.5), and incubated at 37°C for 15 min. The reaction was stopped by the addition of trichloroacetic acid (10%, 0.5 mL) followed by centrifugation, and the absorbance was measured at 400 nm.

2.6 *Pyocyanin assay*

Levels of pyocyanin in treated and untreated cultures of *P. aeruginosa* PAO1 was assayed using the method of Essar *et al*. (1990). Briefly, 5 mL supernatant from the liquid broth cultures (with or without clove oil) of bacterial cells was extracted with 3 mL of chloroform and then re-extracted in 1 mL of 0.2 M HCl to get a pink to deep-red colored solution. The absorbance was measured at 520 nm.

2.7 *Chitinase assay*

Chitinase activity was measured by a modified chitin azure assay (Skindersoe *et al*. 2008). The filter-sterilized supernatants were mixed 2:1 with sodium citrate buffer (0.1 M, pH 4.8), 0.5 mg mL^{-1} chitin azure (Sigma) and were incubated at 37°C with shaking for 1 week. The samples were then centrifuged and the absorbance at 570 nm was determined.

2.8 *Swimming motility assay*

Method of Bala *et al.* (2011) was adopted for swimming assay. Briefly, overnight culture of PAO1 was point inoculated at the center of the medium consisting of 1% tryptone, 0.5% NaCl and 0.3% agar with or without various concentrations of clove oil (0.2–1.6% v/v).

2.9 *Extraction and quantification of exopolysaccharide*

PAO1 and WAF-38 grown in the presence and absence of clove oil were centrifuged and the resulting supernatant was filtered. Three volumes of chilled 100% ethanol were added to the filtered supernatant and incubated overnight at 4°C to precipitate extraction and quantification of exopolysaccharide (EPS) (Huston *et al.* 2004). EPS was then quantified by measuring sugars following the method of Dubois *et al*. (1956).

2.10 *Assay for biofilm inhibition*

The effect of sub-MICs of clove oil on biofilm formation was measured using the polyvinyl chloride biofilm formation assay (O'Toole and Kolter 1998). Briefly, overnight (treated and untreated) cultures of PAO1 and WAF-38 were visualized for biofilm formation by staining with 0.1% crystal violet solution. The microtitre plates were rinsed to remove planktonic cells, and the surface-attached cells were then quantified by solubilizing the dye in ethanol and measuring the absorbance at OD_{470}.

2.11 *In situ visualization of biofilms*

2.11.1 *Light microscopic analysis:* Briefly, 1% of overnight cultures of the PAO1 and WAF-38 (0.4 OD at 600 nm) were added to 1 mL of fresh LB medium containing cover glass of 1 cm^2 along with and without clove oil. After 24 h of incubation, the cover glasses were rinsed with distilled water to remove the planktonic cells and biofilms on the cover glasses were stained with 0.1% crystal violet solution. Stained cover glasses were visualized by light microscope (Nikon Eclipse Ti 100, Japan) (Packiavathy *et al.* 2012).

2.11.2 *Scanning electron microscopy:* Biofilms were grown on glass coverslips, in the treated and untreated cultures of PAO1. After 24 h of incubation, the cover slips were rinsed with distilled water to remove planktonic cells and processed for scanning electron microscopy (SEM) examination as described by Nakamiya *et al*. (2005) with some modifications. Samples were analysed by SEM (Hitachi S-3000 N; High Technology Operation, Japan).

2.12 *β-Galactosidase activity assay for quorum-sensing signal*

β-Galactosidase reporter activity was assayed as described by Harjai *et al*. (2010). Briefly, culture supernatant was extracted by ethylacetate for quorum-sensing signal molecules (AHLs) from overnight cultures of PAO1 grown in the presence and absence of sub-MICs of oil. Then, 2 mL of reporter *E. coli* MG4 (pKDT17) strain (OD_{600} 0.7) and 0.5 mL of the ethylacetate extracted supernatant was incubated at 30°C in a water bath for 5 h with rotation at 100 r.p.m. After centrifugation (3200g for 15 min) of the reporter cell cultures, cell pellet was suspended in an equal volume of Z buffer (Na_2HPO_4 .$7H_2O$, 0.06 M; $NaH_2PO_4.H_2O$, 0.04 M; KCl, 0.01 M; $MgSO_4.7H_2O$, 0.001 M; β-mercaptoethanol, 0.05 M; pH 7.0). To 1 mL of cell suspension 1 mL of Z buffer, 200 µL chloroform and 100 µL of 0.1% sodium dodecyl sulphate was added to lyse cells, and 0.4 mL of *O*-nitrophenol-β-D-galactopyranoside [4 mg/mL in phosphate buffered saline (PBS)] was also added. Reaction was stopped after the development of yellow colour by the addition of 1mL of 1M Na_2CO_3. OD of the reaction samples was measured at 420 and 550 nm. Units of β-galactosidase were calculated as $1000 \times OD_{420}\ nm - (1.75 \times OD_{550nm})/time \times volume \times OD_{600nm}$.

2.13 *C. elegans survival assay*

The method described by Musthafa *et al*. (2012) was adopted to study the *in vivo* efficiency of clove oil in *C. elegans* nematode infection model. Briefly, the young adult nematodes were infected with PAO1 in the 24-well microtitre plate and incubated at 25°C for 12 h. After incubation, *C. elegans* from the wells were washed thrice with M9 (KH_2PO_4 3 g, Na_2HPO_4 6 g, NaCl 5 g, 1 M $MgSO_4$ 1 mL, and distilled water 1000 mL) buffer to remove surface-bound bacteria. Around 10 infected worms were transferred to the wells of micro titre plate containing 10% LB broth in M9 buffer and incubated at 25°C with or without 1.6% v/v clove oil treatment. The plate was scored for live

and dead worms every 12 h for 4 days. To assess the toxicity if any of the oil, *C. elegans* with clove oil was maintained. Worms were scored for survival by following the method of Moy *et al.* (2006), in which the plate was shaken by hand and nematode was considered dead if it did not show any muscle tone or movement.

2.14 *Statistical analysis*

All experiments were performed in triplicates and the data obtained from experiments were presented as mean values and the difference between control and test were analysed using Student's *t*-test.

3. Results

Minimum inhibitory concentration (MIC) of clove oil was determined to select the sub-MICs to study the effect on growth and inhibition of QS-regulated functions. MIC of the oil was found to be 3.2% and 0.8% v/v against PAO1 and *A. hydrophila* WAF-38 strain, respectively. The growth curve study showed no significant change in cell densities between treated and untreated PAO1 and WAF-38 cultures, suggesting that clove oil at selected sub-MICs does not inhibit significant growth of the test strain.

Effects of clove oil were studied at different sub-MICs on the QS-regulated virulence factors and biofilm formation in *P. aeruginosa* PAO1 strain. The investigation revealed a concentration-dependent decrease in all the tested QS linked phenotypes as depicted in table 1. Elastase activity was reduced significantly ($p \leq 0.05$) in the test strain at 1.6% (v/v) concentration of the oil and a reduction of 70% over control was recorded. Total protease activity was also reduced considerably at tested concentrations. A decrease of 48%, 61%, 77% and 85% was recorded at 0.2, 0.4, 0.8 and 1.6% (v/v) oil concentration respectively over untreated control (OD_{495} 0.132). The chitinase activity was inhibited to a maximum of 80% ($p \leq 0.005$) when PAO1 was grown in the presence of 1.6% (v/v) oil concentration. However, the other tested concentrations showed low to moderate reduction (16–58%) in the activity of the enzyme (table 1).

The oil was further tested for its ability to reduce QS-dependent pyocyanin production in PAO1. To analyse the efficiency, PAO1 cells were cultivated in the presence or absence of sub-MICs of the clove oil. All tested sub-MICs produced statistically significant ($p \leq 0.005$) reduction in the levels of pyocyanin production in comparison with the untreated control. The decrease in the production of pyocyanin ranged from 37% to 75% at varying sub-MICs (0.2–1.6% v/v) of oil concentration. Spectrometric analysis of the extracted exopolysaccharide (EPS) revealed that the concentration of EPS decreased with increasing

Table 1. Effect of sub-MICs of clove oil on inhibition of virulence factors in *P. aeruginosa*

Clove oil concentration(% v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Biofilm formation[f]	Swimming motility[g]
Control	0.132±0.018	1.169±0.034	5.91±0.47	0.158±0.013	1.278±0.02	0.406±0.017	50±2.0
0.2	0.113±0.008 (14)	0.606±0.017 (48)**	3.74±0.41 (37)	0.132±0.014 (16)	0.875±0.017 (31)***	0.326±0.018 (20)*	24±1.2 (52)**
0.4	0.088±0.012 (33)	0.457±0.016 (61)**	3.15±0.27 (47)**	0.114±0.008 (28)*	0.514±0.021 (60)***	0.279±0.021 (31)*	20±0.5 (60)**
0.8	0.067±0.014 (49)*	0.271±0.013 (76)**	2.35±0.29 (60)**	0.066±0.010 (58)*	0.448±0.012 (65)***	0.203±0.019 (50)*	17±1.0 (66)***
1.6	0.040±0.008 (69)*	0.177±0.014 (85)***	1.47±0.25 (75)**	0.031±0.003 (80)**	0.290±0.013 (77)***	0.140±0.004 (65)**	10±0.5 (80)***

[a] Elastase activity is expressed as the decrease in absorbance at OD_{495} per microgram of protein.
[b] Total protease activity is expressed as the absorbance at OD_{400} per microgram of protein.
[c] Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d] Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}.
[f] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
[g] Swimming motility is expressed as diameter of swim in mm.
The data represents mean values of three independent experiments. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.001$.
Values in the parentheses indicate percent reduction over control.

concentration of clove oil. The test oil exhibited 31%, 59%, 65% and 77% decrease in EPS production of PAO1 at 0.2, 0.4, 0.8 and 1.6% (v/v) concentration respectively (table 1). The motility of PAO1 in the presence and absence of test concentrations of oil was assessed through swimming motility assay. Oil-treated bacterial cells demonstrated poor flagellar motility on the agar plates. All tested concentration exhibited significant reduction (52–80%), compared with the untreated control (table 1).

Similarly, significant ($p \leq 0.05$) decrease in biofilm formation was observed in test bacterial strain when grown in the presence of (0.2–0.8% v/v) clove oil. At 1.6% concentration, clove oil showed a maximum of 65% ($p \leq 0.005$) reduction in biofilm forming capability of PAO1 (table 1). The results of light microscopy and SEM analysis revealed that the untreated control showed a well-developed biofilm growth, whereas,PAO1 treated with the oil (1.6% v/v) developed poor biofilm in comparison with the control (figure 1).

Inhibition of QS-regulated virulence functions by clove oil treatment of *Aeromonas* sp was also addressed in this study. A number of secreted virulence factors are responsible for host tissue destruction during initiation of the infectious process by *Aeromonas* sp. Virulence factors such as production of exoproteases, swarming motility and the formation of biofilm is known to be regulated by *ahyRI* QS system (Williams 2007). Effect of clove oil on virulence factors was studied; a dose-dependent decrease in the total protease production of *A. hydrphila* was recorded. There was statistically significant reduction (19–57% compared with the control) when *A. hydrophila* was grown in the presence of 0.05–0.4% v/v oil concentration (table 2). Extracted EPS showed a decrease of 28–71% over untreated control in the presence of sub-MICs of PMO. In biofilm quantification assay, a concentration-dependent decrease in biofilm formation was observed in the test bacteria when treated with oil at varying concentrations. The oil showed 35–66% reduction in biofilm of *A. hydrophila* at concentrations of 0.05–0.4% v/v (table 2; figure 2).

To examine whether anti-QS activity of clove oil involves the well-known LasR/RhlR regulators and signalling molecules AHL of QS system, we employed the LasR-dependent lasB promoter and reporter gene fusion system (lasB::lacZ) for measuring β-galactosidase activity in cells. The treatment of PAO1 with 1.6% v/v clove oil significantly reduced the AHL levels (337 miller units) as compared to untreated control (770 miller units). Thus, 56% reduction of β-galactosidase activity in *E. coli* MG4/pKDT17 was achieved (figure 3), indicating that clove-oil-mediated inhibition of *lasB* promoter activity involves LasR-controlled transcription.

The anti-infection potential of the sub-MIC of clove oil was assessed using a liquid killing assay of *C. elegans* by

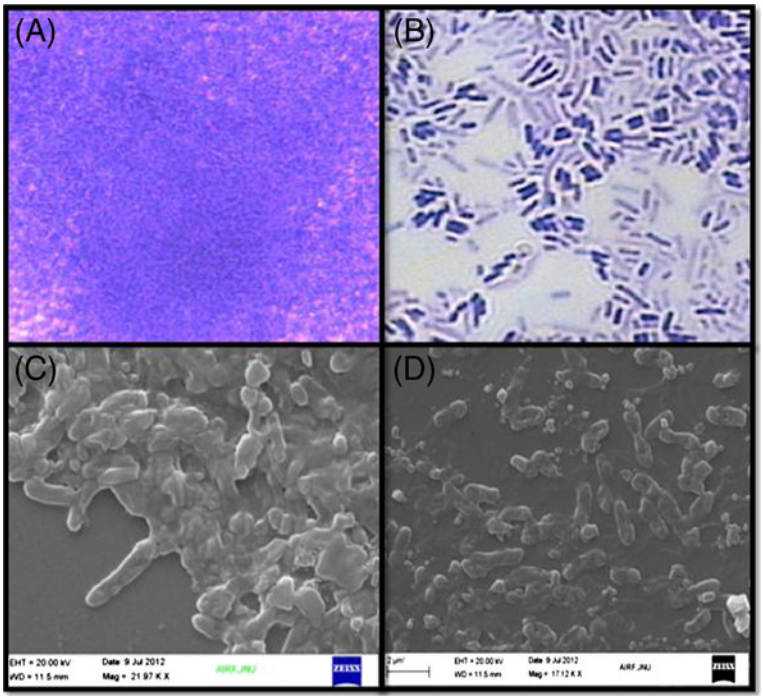

Figure 1. Microscopic images of *P. aeruginosa* PAO1 biofilm in the presence and absence of sub-MICs of clove oil. (**A**) Light microscopic image of untreated control, (**B**) treated with 1.6% (v/v) clove oil, (**C**) scanning electron microscopic image of untreated control and (**D**) treated with 1.6% (v/v) clove oil.

Table 2. Effect of sub-MICs of clove oil on inhibition of virulence factors in *Aeromonas hydrophila*

Clove oil concentration (% v/v)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.747±0.032	0.816±0.038	0.325±0.027
0.05	0.605±0.025 (19)	0.587±0.039 (28)	0.211±0.021 (35)*
0.1	0.515±0.028 (31)*	0.318±0.034 (61)*	0.152±0.014 (53)**
0.2	0.395±0.021 (47)*	0.261±0.022 (68)**	0.130±0.015 (60)**
0.4	0.321±0.018 (57)*	0.236±0.025 (71)**	0.110±0.008 (66)**

[a] Total protease activity is expressed as the decrease in absorbance at OD_{400} per microgram of protein.

[b] EPS production is expressed as absorbance at OD_{480}.

[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.

The data represents mean values of three independent experiments. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$.

Values in the parentheses indicate percent reduction over control.

PAO1 in a 24-well microtitre plate. Complete mortality of the *P. aeruginosa* PAO1 preinfected *C. elegans* was observed within 72 h. However, *C. elegans* preinfected with PAO1 further treated with clove oil (1.6% v/v) displayed enhanced survival rate of 62% (figure 4). However, clove oil alone demonstrated no significant mortality of *C. elegans* at tested concentrations.

4. Discussion

Virulence factors such as elastase, protease, pigment production, motility and biofilm formation in *P. aeruginosa* are regulated by acyl homoserine lactone (AHL)-mediated QS system. It is expected that inhibition of QS-regulated functions will lead to the attenuation of virulence and subsequent eradication of pathogen by host immune response. In the present investigation, attempt has been made to determine the effect of sub-MICs of clove oil on *P. aeruginosa* QS system and its efficacy against bacterial virulence in the *C. elegans* infection model.

The successful establishment of *P. aeruginosa* infection requires an important virulence enzyme, i.e. LasB elastase, whose expression is under the control of QS. This enzyme enhances the invasiveness of *P. aeruginosa* by digesting structural components of host tissue. Hence, a reduction in elastase production will reduce efficiency of the infection. As depicted in table 1, the production of LasB was significantly reduced after treatment with sub-MICs of clove oil. These findings are in agreement with the earlier reports on south Florida plants (Adonizio *et al.* 2008a) and edible fruits (Musthafa *et al.* 2010). Further, the reduction in

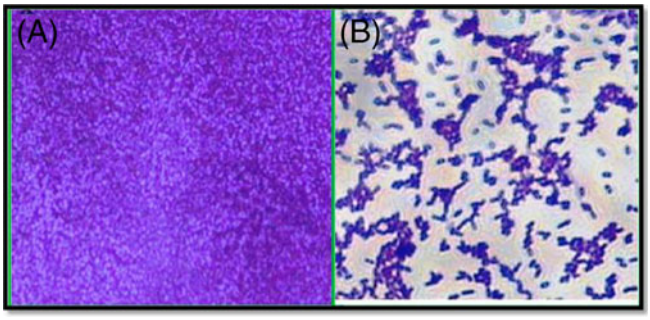

Figure 2. Light microscopy images of *Aeromonas hydrophila* WAF-38 biofilm in the presence and absence of sub-MICs of clove oil. (**A**) Untreated control and (**B**) treated with 0.4% (v/v) clove oil. The cells were stained with crystal violet.

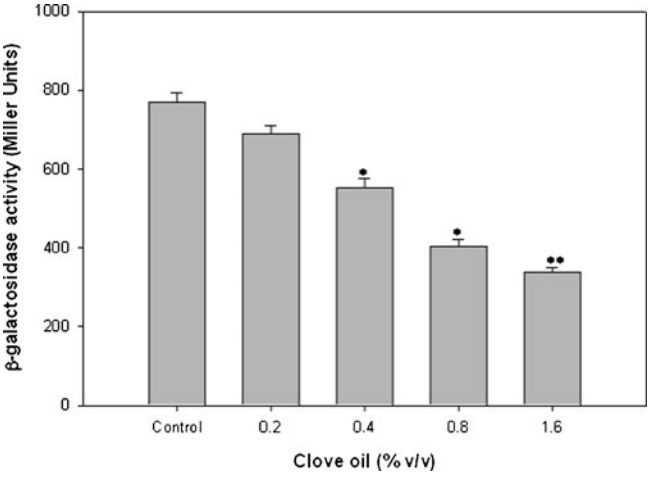

Figure 3. β-Galactosidase activity was measured in the *E. coli* MG4/pKDT17 with and without clove oil. The plasmid pKDT contains the *lacZ* reporter gene fused to *lasB* promoter that is responsive to LasR regulator and AHL signalling molecules, for the production of β-galactosidase. All of the data are presented as mean±SD. *$p \leq 0.05$, **$p \leq 0.005$.

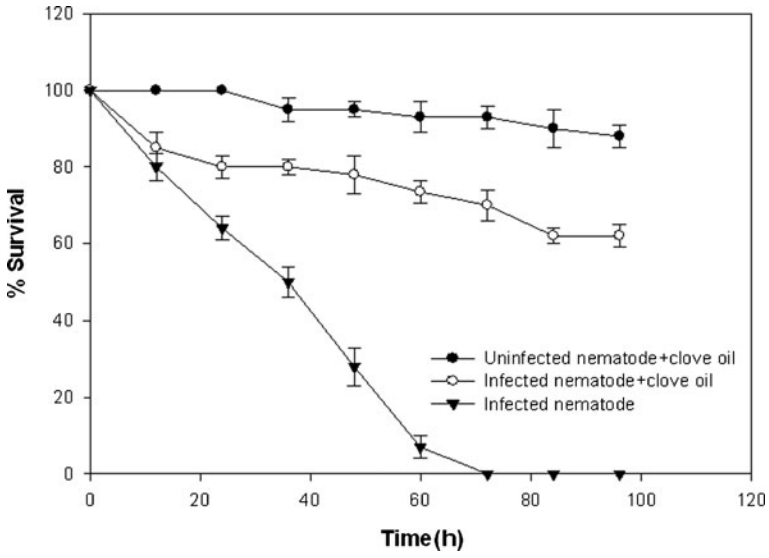

Figure 4. Anti-infection potential of sub-MIC of clove oil (1.6% v/v) in increasing the survival of *C. elegans* preinfected with *P. aeruginosa* PAO1. Means values of triplicate independent experiments and SDs are shown.

β-galactosidase reporter activity shows that the oil at sub-MICs decreases the transcriptional activity of lasB promoter in *E. coli* and that clove oil possibly inhibits the *las* regulatory system. Considering the fact that *las* system regulates the expression of numerous virulence related genes, clove oil at sub-MICs would significantly decrease different virulence factors of *P. aeruginosa*. The findings are in agreement with those reported by Zhou *et al*. (2013). Further, treatment with clove oil (0.2–1.6% v/v) clearly indicated concentration-dependent decrease in the activity of chitinase, total protease, swimming motility pyocyanin and EPS production to varying levels. In the recent years similar reports have appeared indicating potential of various plant extracts and phtyo-compounds inhibiting one or more QS-controlled traits in *P. aeruginosa* (Huerta *et al*. 2008; Musthafa *et al*. 2010, 2012; Vandeputte *et al*. 2011; Tagannaa *et al*. 2011; Packiavathy *et al*. 2012; Husain and Ahmad 2013).

Biofilm formation in PAO1 is suggested to be positively regulated by AHL-mediated cell to cell signalling (Hentzer *et al*. 2002; Rasmussen *et al*. 2005). Since clove oil demonstrated effective inhibitory activity against the QS-regulated virulence traits, we further hypothesized that the oil may also influence biofilm formation in PAO1. The observations recorded support the hypothesis as the oil inhibited biofilm biomass significantly ($p \leq 0.005$) in a dose-dependent manner (table 1) without affecting the growth of the bacteria. Essential oils of red thyme (Kavanaugh and Ribbeck 2012) showed similar anti-biofilm activity in PAO1. Microscopic images (figure 1) revealed that the oil of clove efficiently reduced the number of microcolonies in the biofilm mode of growth. Therefore, it is expected that treatment of PAO1 with sub-MICs of oil resulted in the formation of weak biofilms possibly by reducing the surface adhesion and subsequent microcolony formation. However, biofilm development and establishment involves several complex processes, which are under the control of various genes and ecological parameters (Kjelleberg and Molin 2002). Therefore, the exact mode of action on specific stages of biofilm formation is to be worked out.

Further, clove oil was tested for activity against *A. hydrophila*. The *A. hydrophila* QS circuit consists of an AhyRI system homologous to the LuxRI system and produces a number of virulence factors which function together to cause diseases in the host (Khajanchi *et al*. 2010). In *A. hydrophila*, AhyR/C4-HSL-dependent QS system regulates both extracellular protease production and biofilm development (Swift *et al*. 1999; Lynch *et al*. 2002). In the present study we have investigated the effect of sub-MICs of clove oil on selected virulence factors (exoprotease, EPS production) and biofilm formation. Substantial reduction in protease and EPS production was observed with bacteria treated with clove oil. Similar results were obtained for biofilm inhibition in *A. hydrophila* which is comparable to the work of Ponnusamy *et al*. (2009) with vanillin. The data obtained for protease production and biofilm inhibition indicates that the oil is possibly acting on the AhyRI system.

The results of the β-galactosidase assay show that the AHL activity might have reduced significantly by the

treatment with clove oil at sub-MICs (figure 3). In a similar study reduction of AHL in PAO1 has been reported with fresh garlic extract (Harjai *et al*. 2010). Reduction in AHL activity indicates interference with bacterial QS system.

Further, to assess directly the anti-infective efficacy of clove oil we used *C. elegans* infection model assay. *C. elegans* has been widely employed as an animal model to investigate the pathogenicity of bacterial pathogens. This nematode has also been used to study the anti-infective potential of antibacterial and antifungal drugs (Moy *et al.* 2006). The death of the nematode by PAO1 is caused by the cyanide asphyxiation and paralysis (Gallagher and Manoil 2001). The *hcn* operon mediates cyanide production in PAO1 and is controlled by the LasR and RhlR QS regulators (Pessi and Haas 2000). As depicted in figure 4, the *in vivo* assay demonstrated an enhanced survival of pre-infected nematode that was treated with clove oil (1.6% v/v). *In vivo* study clearly indicates that the clove oil interferes with the virulence factors of PAO1 leading to reduced mortality of *C. elegans* possibly by cyanide asphyxiation and paralysis. Similar enhanced survival of the nematode (*C. elegans*) after treatment with aqueous extracts of three south Florida medicinal plants has been reported by Adonizio *et al*. (2008b).

In conclusion, the study reveals the anti-quorum-sensing and biofilm inhibitory activity of clove oil against two Gram-negative pathogens. The findings of the *in vivo* effects of clove oil in *C. elegans* model throw light on the anti-infective property of the oil at sub-MICs. The above property could be exploited in developing the oil as an antipathogenic agent alone or in combination with antibiotics against drug resistant pathogenic bacteria.

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