Chapter III

Evaluation of identified QSI compounds for their \textit{in vitro} and \textit{in vivo} potential
INTRODUCTION

Ethnopharmacological searches evidence that plants have evolved several strategies to counter pathogen attack including the production of antibacterial, antifungal and anti-infective compounds which lead to their wide usage in therapeutic medicines (Lewis and Ausubel, 2006). In particularly, numerous herbs including oregano, rosemary, thyme and spices such as turmeric, clove, black pepper, garlic have been known for their bioactive potential in preventing gastrointestinal, pulmonary and urinary tract infections (Shetty et al., 2005). Such dietary sources offer a great spectrum of secondary metabolites ranging from phenolics, quinones, flavonoids, alkaloids, terpenoids and polyacetylenes. Even though, phytochemicals from these dietary sources are known to have antimicrobial activities against several important bacterial pathogens such as Escherichia, Helicobacter, Streptococcus and Salmonella species, their precise mechanism of action has not yet been completely understood (Stapleton and Taylor, 2002; Vattem and Shetty, 2005). Accumulating evidences confirm the possible actions of phytochemicals with well known antibiotic properties to possess antipathogenic potential (Vattem et al., 2007; Rudrappa and Bais, 2008). Such antipathogenic compounds, in contrast to antibacterial compounds will not allow the resistance to develop as it has no effect on bacterial growth (Otto, 2004; Truchado et al., 2009). Instead, these compounds attenuate the expression of genes responsible for pathogenesis by possibly interfering with their QS mechanisms. Interestingly, several QS inhibitory compounds have already been reported from various dietary resources (Rasmussen and Givskov, 2005; Zeng et al., 2008; Khan et al., 2009).

In our earlier work, we demonstrated that the dietary plants including herb and spices such as O. sanctum, C. longa, C. cuminum and C. spinosa to exhibit QSI
activity. In continuation of such work, it was attempted to identify the active principles present in these dietary sources through various techniques including conventional purification and CAMD analysis. Consequently, it was identified that the QSI activity of the herb *O. sanctum* was contributed by eugenol. In case of spices, the QSI compounds were identified as methyl eugenol and methyl geranate from *C. cyminum* and homovanillic acid from *C. spinosa*. The QSI activity exhibited by homovanillic acid was very meager and thus ruled out. Hence, in the present study, the QSI compounds such as eugenol, methyl eugenol and geranate were selected for further evaluation. In addition to these identified compounds, the well known QSI compound curcumin, a major compound present in *C. longa* was also selected in the present study. All these compounds were evaluated for their QSI potential in inhibiting QS mediated biofilm formation and virulence factors production in human pathogens such as *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* as well as aquatic pathogens including *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*. Further, to confirm the antipathogenic potential of these identified compounds against *V. harveyi* and PAO1 infection, *in vivo* challenging experiments were done using *Artemia* nauplii and *C. elegans*, respectively as live animal models.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

CV026 was used as biomarker strain to evaluate the QSI activity of identified compounds present in the dietary extracts. In addition to this marker strain, human pathogens such as *E. coli* (ATCC 10536), PAO1, *P. mirabilis* (ATCC 7002), clinical isolate *S. marcescens* (FJ584421) and aquatic pathogens including *V. harveyi* (MTCC 3438), *V. parahaemolyticus* (ATCC 17802) and *V. vulnificus* (MTCC 1145) were used as target pathogens in studying the antibiofilm and QSI properties of the
identified compounds. For experimental analysis, all human pathogens were cultivated in LB medium with pH-7.0 at 30°C except PAO1, which was maintained at 37°C, whereas, the aquatic bacterial pathogens were cultivated in marine LB (mLB) medium with pH-7.4 and maintained at 30°C. In every experiment, the cell density of these test bacterial pathogens was adjusted to 0.4 OD at 600 nm (1 × 10^8 CFU/ml).

**Determination of MIC for identified QSI compounds**

The test QSI compounds such as eugenol, methyl eugenol and methyl geranate were purchased from Alfa Aesar, USA, whereas curcumin was purchased from Sigma, St. Louis, MO, USA. Stock solutions of all test QSI compounds were prepared by dissolving 10 mg of test compounds in 1 ml of 95% ethanol and stored at -20°C until further use. Working concentrations were made from the stock solutions by diluting with sterile milli-Q water and used for further assays. MIC for the test compounds was determined by double dilution method in 24-well MTP as described previously by Thenmozhi et al. (2009). Briefly, 1% of test bacterial pathogens at the above mentioned cell density were added to appropriate growth medium supplemented with two fold serially diluted test QSI compounds except eugenol to attain the final concentrations ranging from 600-0.125 µg/ml, whereas, the concentrations of eugenol were ranging from 200-0.195 µg/ml into the wells of MTP and incubated for 24 h at their appropriate temperature. The MIC was recorded as the minimum concentration which inhibits visible growth of the bacterial pathogens. Further, all the experiments in the present study were performed with the concentrations well below of this MIC (sub-MICs).

**Quantification of violacein inhibition assay in C. violaceum CV026**

In violacein inhibition assay, 1% of overnight culture of CV026 was added to the MTP wells containing 1 ml of LB supplemented with QSI compounds in varying
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sub-MIC concentrations (2.5, 5, 7.5 and 10 µg/ml for eugenol, 25, 50, 75 and 100 µg/ml for curcumin, 50, 100, 150 and 200 µg/ml for methyl eugenol and 25, 50, 75 and 100 µg/ml for methyl geranate). C6-HSLs were supplemented exogenously at the final concentration of 50 μM. The culture without QSI compounds was maintained as control. The MTPs were incubated at 30°C for 18 h. After incubation, the inhibition of violacein production was quantified by following the method of Choo et al. (2006).

Effect of QSI compounds on biofilm development of target bacterial pathogens

Assessment of biofilm biomass

The efficiency of test QSI compounds in lessening the biofilm biomass was assessed through crystal violet (CV) assay. Briefly, 1% of target bacterial pathogens were added into 1 ml of respective growth medium and cultivated in the absence and presence of QSI compounds at their respective sub-MICs as mentioned earlier without shaking to allow cell attachment and subsequent biofilm development for 16 h. After incubation, the biofilm biomass inhibition was quantified by following the method of Thenmozhi et al. (2009).

Microscopic analyses of biofilm developed on cover glasses

Light microscopic analysis of the bacterial biofilms was performed as described previously by Bakkiyaraj and Pandian (2010). Briefly, 1% of the test bacterial pathogens were incubated with cover glasses (1cm²) in the wells of MTP containing 1 ml of respective growth medium at 30°C for 24 h in the absence and presence of QSI compounds at the higher concentration of their respective sub-MICs. After incubation, the biofilms were processed as mentioned earlier and visualized under CLSM and light microscope.
Disintegration of mature biofilm

This assay was carried out to ensure the ability of test QSI compounds in disintegrating the mature biofilms of target pathogens. Briefly, 1% overnight cultures of test bacterial pathogens at above said cell density were inoculated into the wells of MTP containing 1 cm² cover glasses and 1 ml of respective growth medium. The MTPs were then incubated at appropriate temperature for 8 h to allow the development of biofilms on cover glasses. To examine the efficiency of test QSI compounds in disintegrating the mature biofilms, the cover glasses with mature biofilm of test bacterial pathogens were incubated with test QSI compounds at their respective highest sub-MIC (10 µg/ml for eugenol, 100 µg/ml for curcumin, 200 µg/ml for methyl eugenol and 100 µg/ml for methyl geranate) for 5 h at appropriate temperature. After incubation, the biofilms on the cover glasses were stained with 0.4% CV solution (w/v) for 10 min, the excess stain was removed by washing with distilled water and air dried. The stained biofilms were observed under a light microscope with 40X objective (You et al., 2007).

In CLSM analysis, initially the biofilms were allowed to develop on cover glasses for 12 h. The test QSI compounds at their higher sub-MIC (10 µg/ml for eugenol, 100 µg/ml for curcumin, 200 µg/ml for methyl eugenol and 100 µg/ml for methyl geranate) were added to the mature biofilms and incubated at 30°C for 5 h as described in light microscopy analysis. At the end of incubation, the biofilms on the cover glasses were stained with 0.1% acridine orange (w/v) for 3 min and washed with deionized water and air dried. The stained biofilms were observed under CLSM with an excitation filter at 515-560 nm and the magnification at 20X (Nithya et al., 2011).
EPS inhibition assay

The estimation of EPS inhibition was carried out by quantifying the total carbohydrates. Briefly, cover glasses immersed in the test cultures in the absence and presence of test QSI compounds at their respective sub-MICs in 24-wells MTP were incubated for 24 h. After incubation, the total EPS was quantified by following the method of Nithya et al. (2011) as described previously in chapter 1.

Swimming and swarming inhibition assays

The swimming and swarming motility assays were performed by following the method of Liaw et al. (2000) as described previously. For swimming and swarming assays, two different sub-MICs were selected for each test compound to assess the dose dependent inhibitory activity. The concentrations were 50 and 100 µg/ml for curcumin and methyl eugenol, whereas the concentrations were 100 and 200 µg/ml for methyl eugenol. In case of eugenol, the test concentrations were 5 and 10 µg/ml. The reduction in swimming and swarming migration was recorded by measuring the swim and swarm zones of the bacterial cells after 16 h.

QS inhibition assays in target pathogens

Bioluminescence inhibition in *V. harveyi*

One percentage of *V. harveyi* cells with aforementioned cell density were inoculated into 5 ml of alkaline peptone water (APW) and cultivated in the absence and presence of test QSI compounds at their sub-MICs and incubated at 30°C for 16 h. After incubation, the intensity of bioluminescence was measured in terms of relative light units (RLUs) using luminometer (Turner Biosystem Inc, USA) (Teasdale et al., 2009). The percentage inhibition of bioluminescence was calculated based on the obtained RLU.
Prodigiosin inhibition in *S. marcescens*

Prodigiosin assay in *S. marcescens* was performed by following the method of Morohoshi et al. (2007). Briefly, 1% of *S. marcescens* cells was inoculated into 2 ml of fresh growth medium and cultivated without and with test QSI compounds at their respective sub-MICs. The experimental set up was incubated for 18 h at 30ºC. During late stationary phase, the cultures from each tube was collected in 2 ml sterile tube and centrifuged at 10,000 rpm for 10 min to pelletize bacterial cells along with prodigiosin. Prodigiosin from the cell pellet was extracted with acidified ethanol solution (4% 1 M HCl in ethanol) and the absorbance of the extracted prodigiosin in ethanol was measured at 534 nm using UV–visible spectrophotometer.

Alginate inhibition in PAO1 and *Vibrio* spp.

Alginate was extracted from QSI compounds treated and untreated cultures of PAO1 and *Vibrio* spp. and subsequently quantified by following the method of Owlia et al. (2007). Briefly, 70 µl of test bacterial cultures were gradually added to 600 µl of boric acid-H$_2$SO$_4$ solution in a test tube placed in an ice bath and vortexed for 10 sec. Further, 20 µl of 0.2% carbazole solution in ethanol was added to each test tube and mixed thoroughly. The mixtures were placed in a water bath at 55ºC for 30 min. After incubation, the absorbance was measured spectrophotometrically at 530 nm. The percentage inhibition of alginate was calculated based on the obtained OD values.

Rhamnolipid inhibition in PAO1

Qualitative analysis of rhamnolipid production was assessed by following the method of Caizza et al. (2005). Briefly, 50 µl of overnight cultures from QSI compounds treated and untreated PAO1 cells were inoculated into M8 medium supplemented with MgSO$_4$ (1 mM), glucose (0.2%), tryptone (0.5%),
cetyltrimethylammonium bromide (CTAB) (0.02%), methylene blue (0.0005%) and agar (1.5%). Plates were incubated at 37°C for 48 h and observed for the production of rhamnolipid.

**Synergistic effect of QSI compounds with antibiotics**

In order to assess the synergistic activity of the identified QSI compounds with existing antibiotics, 1% cultures of *C. violaceum* and *S. marcescens* were added at above said cell density to 1ml of LB broth in 24-well MTP containing antibiotic discs such as bacitracin (10 µg/disc) and erythromycin (10 µg/disc) (Hi-Media, India) along with QSI compounds at their respective sub-MICs. The controls were maintained with respective antibiotic discs and without QSI compounds. The plates were incubated overnight at appropriate temperature and growth was measured after 24 h at 600 nm using a UV-visible Spectrophotometer (Rogers et al., 2010).

**Growth curve analysis**

To confirm the non-antibacterial activity of the QSI compounds at their sub-MICs the growth curve analysis was performed. Briefly, 1% overnight cultures of bacterial pathogens (0.4 OD at 600 nm) such as *E. coli*, PAO1, *P. mirabilis*, *S. marcescens*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* were inoculated individually in 250 ml Erlenmeyer flask containing 50 ml of respective growth medium supplemented with QSI compounds at their respective highest sub-MIC (10 µg/ml for eugenol, 100 µg/ml for curcumin, 200 µg/ml for methyl eugenol and 100 µg/ml for methyl geranate). The flasks were incubated at the optimum temperature of respective pathogens under 180 rpm in a rotatory shaker. The cell density was measured in UV–visible spectrophotometer at one hour interval up to 16 h.
Antipathogenic potential of QSI compounds

In vivo challenging experiments with Artemia nauplii

In-vivo experiments were executed with freshly hatched nauplii of Artemia franciscana (San Frassico, Bay Brand, USA). 100 mg of dehydrated cysts were hydrated in 100 ml of sterile seawater for 2 h and aerated for 24 h to hatch out Artemia nauplii. The freshly hatched healthy Artemia nauplii were collected and used for challenging test (Ravi et al., 2007; Brackman et al., 2008). Briefly, groups of 10 Artemia nauplii were introduced into four sets of bowls filled with 50 ml of filtered seawater with the salinity of 35 ppt at room temperature. Set 1 was maintained with QSI compounds at a concentration of 25 µg/ml of curcumin, 5 µg/ml of eugenol, 50 µg/ml of methyl eugenol and 50 µg/ml methyl geranate in rearing medium to facilitate the intake of the QSI compounds by Artemia nauplii. The compound treated Artemia nauplii were exposed to V. harveyi infection at aforementioned cell density. Set 2 was maintained with V. harveyi in the absence of QSI compounds in rearing medium to serve as positive control. Set 3 was maintained only with QSI compounds in rearing medium in order to check for toxicity to Artemia nauplii, if any. Set 4 was maintained only with Artemia nauplii as control. Three independent experiments were performed and mortality in each set was recorded for 144 h.

In vivo studies with Caenorhabditis elegans animal model

Young adult worms were maintained by the method of Sivamaruthi et al. (2011). The young adult animals were infected with PAO1 for 12 h at 25ºC in the wells of 24-well MTP. The infected worms in the wells were washed thrice with M9 buffer (KH₂PO₄-3 g, Na₂HPO₄- 6 g, NaCl- 5 g, 1 M MgSO₄ -1 ml and distilled water-1000 ml) to remove surface-bound bacterial cells. Approximately, 10 preinfected worms were transferred to the wells of MTP containing 10% of LB broth in M9
buffer along with *E. coli* OP50 and incubated without or with QSI compounds at a concentration of 20 µg/ml of curcumin, 5 µg/ml of eugenol, 40 µg/ml of methyl eugenol and 40 µg/ml methyl geranate, separately. Each assay was performed in triplicate; the plates were incubated at 25°C and scored for live and dead worms. A control set consisting of uninfected *C. elegans* with QSI compounds alone was maintained to assess the toxicity of test compounds on *C. elegans*, if any. The survival rate of *C. elegans* was scored by following the previous method of Musthafa et al. (2012b).

**Statistical Analysis**

All experiments were performed independently in triplicates and the data obtained were analyzed by one-way analysis of variance, with a *P* value of 0.05 being significant, using student's *t* test.

**RESULTS**

**Determination of MIC for identified compounds**

MIC for identified QSI compounds against all test pathogens were assessed by performing doubling dilution method with the concentrations varying from 600-0.125 µg/ml for curcumin, methyl eugenol and methyl geranate, whereas the concentrations for eugenol were in the range of 200-0.195 µg/ml. The MIC for all the identified QSI compounds against human pathogens such as *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* and aquatic pathogens such as *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* were given in Table 3.1.
Table 3.1: MICs for identified QSI compounds against bacterial pathogens

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>MIC of QSI compounds (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Curcumin</td>
</tr>
<tr>
<td><strong>C. violaceum</strong></td>
<td>300</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>150</td>
</tr>
<tr>
<td><strong>PAO1</strong></td>
<td>150</td>
</tr>
<tr>
<td><strong>P. mirabilis</strong></td>
<td>150</td>
</tr>
<tr>
<td><strong>S. marcescens</strong></td>
<td>300</td>
</tr>
<tr>
<td><strong>V. harveyi</strong></td>
<td>150</td>
</tr>
<tr>
<td><strong>V. parahaemolyticus</strong></td>
<td>150</td>
</tr>
<tr>
<td><strong>V. vulnificus</strong></td>
<td>300</td>
</tr>
</tbody>
</table>

**Violacein inhibition assay in C. violaceum CV026**

The quantitative assay was performed with the respective sub-MICs of all test compounds. The concentrations well below the MIC were considered as sub-MICs at which the compounds exhibit QSI activity without affecting bacterial growth. The sub-MICs for curcumin and methyl geranate were ranging from 25-100 µg/ml, whereas it was 100-200 µg/ml for methyl eugenol. In case of eugenol, the sub-MICs were ranging from 2.5-10 µg/ml. In quantitative analysis, all the identified compounds at their sub-MICs exhibited a concentration dependent inhibitory effect on
AHL mediated violacein production in CV026. Further the violacein inhibition in CV026 was observed to a maximum of 89%, 97%, 98% and 91% upon treatment with curcumin at 100 µg/ml, eugenol at 10 µg/ml, methyl eugenol at 200 µg/ml and methyl geranate at 100 µg/ml, respectively (Fig. 3.1). The respective sub-MICs of the identified compounds were used for further experiments in the present study.

**Fig. 3.1.** Quantitative analysis of violacein inhibition in CV026 by QSI compounds. Cultures were grown in the absence and presence of (A) curcumin, (B) eugenol, (C) methyl eugenol and (D) methyl geranate. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$. 
Microscopic analyses of bacterial biofilm

The direct microscopic observations of biofilms treated with QSI compounds are expected to provide valuable information about the efficiency of QSIs on biofilm development; therefore light microscopic and CLSM analyses were performed. A thick coating of dense biofilms which aggregated into distinct clusters was observed in untreated controls; in contrast a progressive reduction in number of microcolonies was observed in the biofilms treated with increasing concentrations of QSI compounds in human as well as aquatic bacterial pathogens. Upon treatment with higher sub-MIC of QSI compounds, the biofilm appeared to more diffuse and the number of bacterial microcolonies was diminished in all strains examined (Fig. 3.2).

To investigate further the effect of QSI compounds on biofilm architecture, the biofilms were imaged under CLSM. The obtained results clearly evident that besides reducing the number of microcolonies the identified QSI compounds effectively deteriorated the architecture of the biofilm as it was more evident from Fig. 3.3. This analysis demonstrated that in all bacterial pathogens the test QSI compounds disintegrate the matrix of bacterial biofilms and their architecture. The maximum deterioration in biofilm architecture was contributed by eugenol followed by methyl eugenol (Fig. 3.3).
Fig. 3.2. Light microscopic images of (A) *E. coli*, (B) PAO1 (C) *P. mirabilis* (D) *S. marcescens*, (E) *V. harveyi*, (F) *V. parahaemolyticus* and (G) *V. vulnificus* biofilms in the absence and presence of QSI compounds. (a) untreated control; (b) curcumin treated (100 µg/ml); (c) eugenol treated (10 µg/ml); (d) methyl eugenol treated (200 µg/ml) and (e) methyl geranate treated (100 µg/ml).
Fig. 3.3 Confocal laser scanning micrographs of (A) *E. coli*, (B) PAO1, (C) *P. mirabilis*, (D) *S. marcescens*, (E) *V. harveyi*, (F) *V. parahaemolyticus* and (G) *V. vulnificus* biofilms in the absence and presence of QSI compounds. (a) untreated control; (b) curcumin treated (100 µg/ml); (c) eugenol treated (10 µg/ml); (d) methyl eugenol treated (200 µg/ml) and (e) methyl geranate treated (100 µg/ml). Scale bar represents 50 µm.
Inhibition of biofilm biomass

The antibiofilm activity of identified QSI compounds against pathogenic bacteria was further assessed by performing a quantitative biofilm biomass assay. The obtained results revealed a concentration dependent reduction in biofilm biomass of bacterial pathogens when treated with QSI compounds. Curcumin at 100 µg/ml efficiently dislodged the biofilm biomass by 52%, 89%, 52% and 76% in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively. In aquatic pathogens it exhibited a maximum reduction to the level of 69%, 56% and 79% in *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* (Fig. 3.4a). Eugenol at 10 µg/ml inhibited the biofilm biomass by 78%, 72%, 75%, 84%, 76%, 69% and 71% in *E. coli*, PAO1, *P. mirabilis*, *S. marcescens*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*, respectively (Fig. 3.4b). Similarly, the other identified compounds including methyl eugenol and methyl geranate displayed a significant inhibitory activity against the biomass of test bacterial biofilms. A maximum inhibition was recorded at higher concentrations such as 200 µg/ml for methyl eugenol and 100 µg/ml for methyl geranate. The inhibitory activity range of methyl eugenol was found as 65%, 67%, 80%, 67%, 77%, 83% and 65%, whereas 49%, 67%, 71%, 76%, 73%, 81% and 78% inhibition was observed with methyl geranate in respective pathogens such as *E. coli*, PAO1, *P. mirabilis*, *S. marcescens*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* (Fig. 3.4c and d).
Fig. 3.4. Effect of QSI compounds on biofilm biomass of bacterial pathogens. Bacterial cultures of (A) *E. coli*, (B) PAO1, (C) *P. mirabilis*, (D) *S. marcescens*, (E) *V. harveyi*, (F) *V. parahaemolyticus* and (G) *V. vulnificus* were grown in the absence and in the presence of QSI compounds such as (a) curcumin, (b) eugenol, (c) methyl eugenol and (d) methyl geranate at their respective sub-MICs. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

**Inhibition of EPS**

Since, the development of biofilms in bacterial pathogens has been positively correlated with EPS production, an attempt was made to determine the effect of identified QSI compounds on EPS production of test pathogens. The identified QSI compounds showed an increased inhibitory activity on EPS production with increasing concentration of the test compounds such as curcumin (25-100 µg/ml), eugenol (2.5-10 µg/ml), methyl eugenol (50-200 µg/ml) and methyl geranate (25-100 µg/ml). Curcumin at 100 µg/ml inhibited EPS production in *E. coli*, PAO1, *P. mirabilis*, *S. marcescens*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* by
50%, 97%, 96%, 65%, 60%, 75% and 62%, respectively (Fig. 3.5a). Similarly, eugenol (10 µg/ml) identified from *O. sanctum* showed the inhibition of 30%, 33%, 60%, 32%, 91%, 75% and 78% in respective pathogens (Fig. 3.5b). The remaining two compounds methyl eugenol (200 µg/ml) and methyl geranate (100 µg/ml) showed pronounced inhibitory effect on the EPS production of human pathogens such as *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* by 59%, 56%, 49% and 66%; 76%, 64%, 42% and 49%, respectively. In case of aquatic pathogens methyl eugenol and methyl geranate inhibited the EPS production by 71%, 56% and 48%; 57%, 81% and 57%, respectively, in *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* (Fig. 3.5c and d).

![Fig. 3.5](image_url)  
*Fig. 3.5. Effect of QSI compounds on EPS production of bacterial pathogens. Bacterial cultures of (A) *E. coli*, (B) PAO1, (C) *P. mirabilis*, (D) *S. marcescens*, (E) *V. harveyi*, (F) *V. parahaemolyticus* and (G) *V. vulnificus* were grown in the absence and in the presence of QSI compounds such as (a) curcumin, (b) eugenol, (c) methyl eugenol and (d) methyl geranate at their respective sub-MICs. Mean values of triplicate independent experiments and SD are shown. * indicates significance at *p*≤0.025, ** indicates significance at *p*≤0.01 and *** indicates significance at *p*≤0.005.*
Inhibition of mature biofilm

Since, mature biofilm plays a key role in the virulence of bacterial pathogens (Faruque et al., 2006), the effect of QSI compounds in disrupting mature biofilms was examined. In the untreated cover glasses, a thick cellular aggregates were observed on the surface, whereas in QSI compounds treated cover glasses a thin and less surface coverage was observed in light microscopic images (Fig. 3.6). Moreover, the addition of QSI compounds disintegrated the mature biofilm with 5 h of incubation, while the untreated pathogenic bacteria continued to make dense biofilms.

Identical to the results obtained in light microscopic analysis as described above, the addition of QSI compounds caused a pronounced disintegration of mature biofilms as apparent from CLSM images. It was observed that untreated bacterial pathogens developed thick biofilms, whereas QSI compounds treated pathogens displayed sparse distribution of biofilms in cover glasses (Fig. 3.7 and 3.8). Adding up, the treatment with QSI compounds displayed biofilm architectures with reduced thickness when compare to untreated control.
Fig. 3.6. Disintegration of mature biofilms by QSI compounds. Light microscopic images displaying the mature biofilms of (A) *E. coli*, (B) PAO1, (C) *P. mirabilis* (D) *S. marcescens*, (E) *V. harveyi*, (F) *V. parahaemolyticus* and (G) *V. vulnificus* biofilms in the absence (a) untreated control and presence of QSI compounds such as (b) curcumin (100 µg/ml); (c) eugenol (10 µg/ml); (d) methyl eugenol (200 µg/ml) and (e) methyl geranate (100 µg/ml).
Fig. 3.7. Disintegration of mature biofilms of human pathogens by QSI compounds. CLSM images displaying the mature biofilms of (A) *E. coli*, (B) PAO1, (C) *P. mirabilis* and (D) *S. marcescens* in the absence (a) untreated control and presence of QSI compounds such as (b) curcumin (100 µg/ml); (c) eugenol (10 µg/ml); (d) methyl eugenol (200 µg/ml) and (e) methyl geranate (100 µg/ml).

Fig. 3.8. Disintegration of mature biofilms of aquatic pathogens by QSI compounds. CLSM images displaying the mature biofilms of (A) *V. harveyi*, (B) *V. parahaemolyticus* and (C) *V. vulnificus* in the absence (a) untreated control and presence of QSI compounds such as (b) curcumin (100 µg/ml); (c) eugenol (10 µg/ml); (d) methyl eugenol (200 µg/ml) and (e) methyl geranate (100 µg/ml).
Swimming inhibition assay

It is known fact that QS plays a vital role in the formation of bacterial biofilms, especially it has control over the swimming migration of bacterial cells, which facilitates the initial attachment of bacterial cells to the substrate (Niu and Gilbert, 2004; Atkinson et al., 2006). Hence, an effort was made to examine the QSI potential of the identified compounds against QS dependent swimming motility in human pathogens such as *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* as well as aquatic pathogens including *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*. Interestingly, the obtained results indicated an effective inhibition in the swimming behaviour of all test bacterial pathogens when treated with QSI compounds. The QSI compounds at higher concentration significantly inhibited the swimming motility to a greater level compare to that of control (Table 3.2).

Swarming inhibition assay

Like swimming motility, the flagellar mediated swarming motility has also been reported to be involved in bacterial surface translocation and subsequent colonization (Kohler et al., 2000). The swarming motility of bacterial pathogens also relies on the expression of QS dependent genes. Therefore, the QSI potential of the identified compounds were evaluated for their efficacy in interfering with the QS mediated swarming motility. The exposure of QSI compounds decreased the swarming velocity of the bacterial pathogens in a dose dependent manner. At the highest sub-MIC such as 100 µg/ml of curcumin, 10 µg/ml of eugenol, 200 µg/ml of methyl eugenol and 100 µg/ml of methyl geranate significantly inhibited the swarming migration (Table 3.3) without affecting the bacterial growth.
### Table 3.2: Effect of identified compounds on swimming motility of bacterial pathogens

<table>
<thead>
<tr>
<th>Bacterial pathogens</th>
<th>Control</th>
<th>Curcumin (µg/ml)</th>
<th>Eugenol (µg/ml)</th>
<th>Methyl eugenol (µg/ml)</th>
<th>Methyl geranate (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>5</td>
<td>10</td>
<td>100</td>
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<tr>
<td><strong>Human pathogens</strong></td>
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<tr>
<td><em>E. coli</em></td>
<td>45.8±2.8</td>
<td>23.3±1.8</td>
<td>8.4±1.5</td>
<td>12.4±1.5</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>PAO1</td>
<td>46.1±0.8</td>
<td>20.0±1.9</td>
<td>6.6±0.7</td>
<td>18.9±2.6</td>
<td>8.2±2.4</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>43.4±0.4</td>
<td>36.2±1.2</td>
<td>20.8±1.5</td>
<td>28.6±1.9</td>
<td>20.3±0.9</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>44.6±1.1</td>
<td>26.7±1.3</td>
<td>15.5±1.9</td>
<td>24.3±0.7</td>
<td>12.3±0.6</td>
</tr>
<tr>
<td><strong>Aquatic pathogens</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. harveyi</em></td>
<td>19.6±3.1</td>
<td>10.3±1.0</td>
<td>3.7±0.8</td>
<td>12.8±0.4</td>
<td>8.9±0.8</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>28.4±1.4</td>
<td>9.6±1.4</td>
<td>7.8±0.4</td>
<td>18.6±1.2</td>
<td>12.6±1.9</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>45.2±3.2</td>
<td>12.3±2.8</td>
<td>10.8±1.6</td>
<td>38.8±1.7</td>
<td>15.3±1.3</td>
</tr>
</tbody>
</table>
Table 3.3: Effect of identified QSI compounds on swarming motility of aquatic bacterial pathogens

<table>
<thead>
<tr>
<th>Bacterial Pathogens</th>
<th>Control</th>
<th>Curcumin (µg/ml)</th>
<th>Eugenol (µg/ml)</th>
<th>Methyl eugenol (µg/ml)</th>
<th>Methyl geranate (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>5</td>
<td>10</td>
<td>100 200</td>
</tr>
<tr>
<td>Human pathogens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>24.0±2.6</td>
<td>9.2±1.5</td>
<td>5.9±1.7</td>
<td>18.8±1.1</td>
<td>7.6±0.9</td>
</tr>
<tr>
<td>PAO1</td>
<td>22.3±1.0</td>
<td>12.8±1.3</td>
<td>4.4±0.7</td>
<td>12.7±1.2</td>
<td>5.5±0.7</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>43.7±0.5</td>
<td>16.0±1.8</td>
<td>7.8±2.3</td>
<td>23.5±1.9</td>
<td>13.7±1.1</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>45.4±0.8</td>
<td>18.8±1.9</td>
<td>9.0±0.8</td>
<td>10.9±1.5</td>
<td>6.3±1.2</td>
</tr>
<tr>
<td>Aquatic pathogens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. harveyi</td>
<td>15.2±1.6</td>
<td>8.3±0.4</td>
<td>2.4±0.5</td>
<td>7.8±0.4</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>18.5±3.4</td>
<td>12.2±1.6</td>
<td>8.1±1.1</td>
<td>28.5±1.6</td>
<td>14.4±0.8</td>
</tr>
<tr>
<td>V. vulnificus</td>
<td>42.5±2.8</td>
<td>10.7±0.2</td>
<td>6.9±0.9</td>
<td>16.6±1.4</td>
<td>8.2±0.6</td>
</tr>
</tbody>
</table>
QS inhibition assays in bacterial pathogens

Inhibition of alginate production in PAO1 and *Vibrio* spp.

As alginate constitutes the major portion in the EPS of PAO1 and *Vibrio* spp. biofilm (Owlia et al., 2007; Shi et al., 2008), the efficacy of QSI compounds in reducing the production of alginate in bacterial pathogens was examined. The attained results exhibited a dose dependent inhibition in the production of alginate in the presence of QSI compounds. However, a maximum level of inhibition was obtained with higher concentrations of QSI compounds. Curcumin at 100 µg/ml inhibited alginate production by 63% in PAO1, 45% in *V. harveyi*, 78% in *V. parahaemolyticus* and 65% in *V. vulnificus* (Fig. 3.9a). Similarly, eugenol (10 µg/ml) displayed an inhibitory effect of 56%, 42%, 62% and 53% on alginate production by PAO1, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*, respectively (Fig. 3.9b). The remaining two compounds derived from *C. cyminum* such as methyl eugenol and methyl geranate also showed a significant reduction in alginate production as depicted in Fig. 3.9c and d. At 200 µg/ml, methyl eugenol exhibited 66%, 45%, 81% and 53% reduction, whereas methyl geranate at 100 µg/ml exerted 61%, 58%, 53% and 85% inhibition in the production of alginate respectively by PAO1, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus*. 
Fig. 3.9. Effect of QSI compounds on alginate production of bacterial pathogens. Bacterial cultures of (A) PAO1, (B) *V. harveyi*; (C) *V. parahaemolyticus* and (D) *V. vulnificus* were grown in the absence and presence of (a) curcumin (b) eugenol (c) methyl eugenol and (d) methyl geranate. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

**Rhamnolipid inhibition in PAO1**

In CTAB plate assay, the interaction between CTAB with surfactants resulted in the formation of dark blue colonies around the culture containing wells indicating the presence of rhamnolipid. In the obtained results, when compared to the control, QSI compounds treated wells showed reduced level of halo zone around dark blue colonies, which indicated the reduction in the production of rhamnolipid (Fig. 3.10).
Fig. 3.10. Effect of QSI compounds on rhamnolipid production of PAO1. Cultures of PAO1 were grown in the presence of (A) curcumin; (B) eugenol; (C) methyl eugenol and (D) methyl geranate. In all CTAB plates (a) untreated control (b) and (c) treated with respective QSI compounds at their two different sub-MICs (5 and 10 µg/ml for eugenol, 100 and 200 µg/ml for methyl eugenol, 50 and 100 µg/ml for curcumin and methyl geranate).

Effect of QSI compounds on prodigiosin production in *S. marcescens*

A dose dependent inhibitory activity of the QSI compounds was observed on prodigiosin pigment production in *S. marcescens*. A maximum inhibition level of 63% was attributed by eugenol (10 µg/ml) followed by curcumin (100 µg/ml) to the level of 58%, whereas methyl eugenol (200 µg/ml) and methyl geranate (100 µg/ml) inhibited the prodigiosin production to the level of 42% and 50%, respectively (Fig. 3.11).
Fig. 3.11. Effect of QSI compounds on prodigiosin production of *S. marcescens*. Cultures were grown in the absence and presence of (A) curcumin, (B) eugenol, (C) methyl eugenol and (D) methyl geranate. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

**Bioluminescence inhibition in *V. harveyi***

The QSI potential of the identified compounds was assessed against the QS mediated bioluminescence production in *V. harveyi*. All the four QSI compounds such as curcumin, eugenol, methyl eugenol and methyl geranate exhibited a greater degree of bioluminescence inhibition in a dose dependent manner. However, a maximum level of inhibition in bioluminescence was exhibited by curcumin, eugenol, methyl eugenol and methyl geranate up to 88%, 99%, 94% and 99%, respectively at their highest sub-MIC (Fig. 3.12).
Fig. 3.12. Effect of QSI compounds on bioluminescence of *V. harveyi*. Cultures were grown in the absence and presence of (A) curcumin, (B) eugenol, (C) methyl eugenol and (D) methyl geranate. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

**Effect of QSI compounds on growth of pathogenic bacteria**

In order to confirm the non-antibacterial activity of QSI compounds at tested concentrations, the bacterial growth curve assay was performed for all test bacterial pathogens. The obtained results revealed no considerable changes in the cell densities of both untreated control and cultures treated with curcumin (100 µg/ml), eugenol (10 µg/ml), methyl eugenol (200 µg/ml) and methyl geranate (100 µg/ml) (Fig. 3.13).
Fig. 3.13. Effect of QSI compounds on growth of pathogenic bacteria. Bacterial cultures of (A) *E. coli*, (B) PAO1, (C) *P. mirabilis*, (D) *S. marcescens*, (E) *V. harveyi*, (F) *V. parahaemolyticus* and (G) *V. vulnificus* were grown in the absence and presence of QSI compounds at their respective higher sub-MIC and their growth OD was measured at 600 nm for every one hour.
In vivo challenging experiments with Artemia nauplii

The antipathogenic potential of QSI compounds in protecting Artemia nauplii against *V. harveyi* infection was investigated. The pathogenic *V. harveyi* in the absence of QSI compounds caused complete mortality of the Artemia nauplii, whereas the test QSI compounds alone had no considerable lethal effects on Artemia nauplii. However, the QSI compounds such as curcumin (25 µg/ml), eugenol (5 µg/ml), methyl eugenol (50 µg/ml) and methyl geranate (50 µg/ml) enhanced the survival up to 67%, 60%, 74% and 87%, respectively against *V. harveyi* infections (Fig. 3.14).

![Figure 3.14](image)

Fig. 3.14. Antipathogenic potential of (A) curcumin, (B) eugenol, (C) methyl eugenol and (D) methyl geranate in enhancing the survival of Artemia nauplii against *V. harveyi* infection. Mean values of triplicate independent experiments and SD are shown. *** indicates significance at $p \leq 0.005$. 
Effect of QSI compounds on virulence of PAO1 in *C. elegans*

To investigate the effect of the QSI compounds on the survival of *C. elegans* preinfected with PAO1 an *in vivo* analysis was made. In which, methyl eugenol and methyl geranate were found to be nontoxic towards *C. elegans* when used at 40 µg/ml. However, eugenol and curcumin were found to be toxic at concentrations above 10 µg/ml and 25 µg/ml, respectively and hence these two compounds were tested in the concentrations well below to their threshold limit. A complete mortality of PAO1 infected *C. elegans* was observed at 144th hour in the absence of QSI compounds, whereas, QSI compounds such as curcumin (20 µg/ml), eugenol (5 µg/ml), methyl eugenol (40 µg/ml) and methyl geranate (40 µg/ml) significantly increased survival of nematodes to 67%, 95%, 90% and 75%, respectively after infection. The most significant effect was observed with 5 µg/ml of eugenol and 40 µg/ml of methyl eugenol (Fig. 3.15).

![Graph showing survival rate](image)

**Fig. 3.15.** Antipathogenic potential of identified QSI compounds in enhancing the survival rate of *C. elegans* infected with PAO1. Mean values of triplicate independent experiments and SD are shown. *** indicates significance at \( p \leq 0.005 \).
Synergistic activity of QSI compounds with antibiotics

In synergistic assay, biomarker strain *C. violaceum* was assessed for its sensitivity to the antibiotic bacitracin (10 µg/ml) in the presence and absence of QSI compounds. It was observed that *C. violaceum* was highly resistant to bacitracin in the absence of QSI compounds. Similarly, the QSI compounds had also not shown to inhibit the growth of *C. violaceum* at tested concentrations. Interestingly, the bacteria exposed to combine action of QSI compounds with bacitracin showed an enhanced susceptibility to bacitracin in a dose dependent manner (Fig. 3.16).

**Fig. 3.16.** Synergistic activity of QSI compounds with conventional antibiotics. Increased susceptibility of *C. violaceum* to bacitracin in the presence of (a) curcumin, (b) eugenol, (c) methyl eugenol and (d) methyl geranate. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

Similarly, *S. marcescens* in the presence of QSI compounds showed an increased susceptibility to erythromycin. In contrast, *S. marcescens* in the absence of
QSI compounds showed moderate susceptibility towards erythromycin. Moreover, the synergistic activity of QSI compounds with erythromycin was found to be dose dependent. Thus, the results clearly revealed the increased susceptibility of pathogenic bacteria towards conventional antibiotics in the presence of QSI compounds (Fig. 3.17).

**Fig. 3.17. Synergistic activity of QSI compounds with conventional antibiotics.** Increased susceptibility of *S. marcescens* to erythromycin in the presence of (a) curcumin, (b) eugenol, (c) methyl eugenol and (d) methyl geranate. Mean values of triplicate independent experiments and SD are shown. * indicates significance at $p \leq 0.025$, ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

**DISCUSSION**

In the present investigation, the QSI potential of four identified compounds was examined using the biomarker strain CV026, subsequently these compounds were studied against QS dependent biofilm and production of other virulence factors in human as well as aquatic bacterial pathogens. The quantitative assessment of
violacein pigment production in CV026 clearly indicated that the QSI activity of these identified compounds was concentration dependent one. At highest concentration, the test compounds such as curcumin, eugenol, methyl eugenol and methyl geranate showed higher degree of reduction in violacein pigment production. Thus, the obtained results in the present study are comparable with that of Khan et al. (2009), who have reported about 92% inhibition of QS mediated violacein production in CV026 by clove oil. Brackman et al. (2009) have also reported the inhibitory effect of curcumin on \textit{C. violaceum} CV026 violacein production.

In continuation of the results attained in the violacein inhibition assay with \textit{C. violaceum}, the identified QSI compounds were further assessed for their ability to inhibit biofilm formation, EPS production and flagellar motility in human bacterial pathogens such as \textit{E. coli}, PAO1, \textit{P. mirabilis}, \textit{S. marcescens} as well as aquatic bacterial pathogens including \textit{V. harveyi}, \textit{V. parahaemolyticus} and \textit{V. vulnificus}. It has been well proven that the formation of bacterial biofilm plays an important role in the pathogenesis of human (Jones, 2004) as well as aquatic pathogens (Manefield et al., 1999). The development of such biofilms is being controlled through signal mediated QS system (Anetzberger et al., 2009) and such mechanism can possibly interfere with QS inhibitors (Bjarnsholt and Givskov, 2008). The test QSI compounds such as curcumin from \textit{C. longa}, eugenol from \textit{O. sanctum}, methyl eugenol and methyl geranate from \textit{C. cyminum} effectively inhibited the biofilm biomass of all test bacterial pathogens in a dose dependent manner (Fig. 3.4) without affecting their planktonic growth (Fig. 3.13). These findings are in consistent with previous reports made in \textit{Vibrio} spp., wherein the dietary phytochemicals like cinnamaldehyde and its derivatives considerably reduced the total biofilm biomass (Brackman et al., 2008). Our results are also falls in line with the findings of Vikram et al. (2010), in which
limonoids such as obacunone and nomilin from grapefruit have been documented to suppress biofilm formation of *V. harveyi* in non-growth inhibitory fashion and plausibly by inhibition of QS. In another study, Nashikkar et al. (2011) have found that the biofilm formation by the human pathogens such as *P. mirabilis* and PAO1 in the presence of latex extract of *E. trigona* is meager when compared to that of untreated biofilms. Besides reducing the biomass, the QSI compounds used in the present work eventually reduced the microcolony formation, which was more apparent in the light microscopic images (Fig. 3.2). Similar observations have also been documented with the extracts of marine actinomycetes A66 (You et al., 2007) and *Bacillus* spp. from marine sediment (Nithya and Pandian, 2010) to reduce the number of microcolonies and the biofilm architecture of *Vibrio* spp. Yet another interesting mechanism in the biofilm development is the characteristic biofilm architecture (You et al., 2007). In the present study, the results of CLSM analysis clearly revealed that the architecture of the QSI compounds treated biofilms was looser and less in thickness when compared with that of untreated control biofilms (Fig. 3.3). Our results are in accordance with previous reports of Hentzer and Givskov (2003) with furanone of *D. pulchra* and Bjarnsholt et al. (2005) with garlic, wherein a considerable reduction in morphology and thickness of *P. aeruginosa* biofilm has been observed. The obtained results lucidly indicated that the QSI potential of identified compounds was associated with the development of biofilm matrix as well as biofilm architecture.

Accumulating evidences suggest that QS plays a crucial role in the maturation of bacterial biofilms (De Kievit et al., 2001). Mature biofilms are complex communities and the bacterial cells residing inside the biofilms grow slowly than their free-swimming planktonic counterparts. In aquaculture industries, it is well proven
that the biofilm forming ability of bacterial pathogens are highly accountable for enhanced survival and infectivity of *Vibrio* spp. (Milton, 2006). Thus vibrios in turn, will pollute the entire aquaculture environment and enhance the frequency of vibriosis. Most notably, in case of infectious diseases, such biofilms use several different mechanisms to evade killing by host defenses and antibiotics. Consequently, these would result in transient phenotypic changes that allow the bacteria to increase their antibiotic resistance (Brown et al., 1990). Furthermore, bacterial cells in mature biofilm can be up to 1,000-fold more resistant to antibiotic treatment than their counterpart growing planktonically (Brooun et al., 2000). Hence, the bacterial cells reside inside the mature biofilms are harder to eradicate than their planktonic counterparts. Therefore, disintegration of mature biofilm is very important step to overcome the infections out of antibiotic resistant bacterial pathogens. It is worthwhile noting that in the present study, the test QSI compounds were able to disintegrate the mature biofilms of human and aquatic bacterial pathogens (Fig. 3.6). The antibiofilm activity of identified QSI compounds in disturbing the preformed biofilm was evidenced through light and CLSM analyses (Fig. 3.7 and 3.8). Based on the obtained results, it is envisaged that the loosening of architecture and morphology of the preformed biofilms by these compounds ultimately led an increased sensitivity of bacterial pathogens to antibiotics. Similarly, marine actinomycetes A66 was reportedly disintegrated the mature biofilms of *V. harveyi* (You et al., 2007). Rogers et al. (2010) have also revealed the possible role of 2-aminoimidazoles to disrupt the preformed PAO1 biofilms. The obtained results of the present study also evidenced the dual role of identified compounds in inhibiting the initiation as well as maturation of bacterial biofilm development.
The importance of QS dependent EPS production in the biofilm development and its maturation is well reported (Yildiz and Visick, 2009; Vu et al., 2009). EPS affords a protection against antibiotics, disinfectants and host immune system to bacterial cells living within the matrix (Lapidot et al., 2006) by preventing the permeability of such compounds to the matured bacterial biofilm. Moreover, increased production of EPS often correlates with increased resistance among bacterial pathogens (Nakhamchik et al., 2008). Therefore, any interruption with the production of EPS would result in significant implications on the development of biofilm and subsequent emergence of resistance in bacterial pathogens. With this fact, the efficiency of QSI compounds in inhibiting the QS mediated EPS production was assessed and the obtained results revealed a significant inhibition in EPS production of target pathogens in a dose dependent manner as depicted in Fig. 3.5. Hence, it is believed that the reduction in EPS production by QSI compounds will lead to the decreased resistance by allowing the penetration of therapeutic agents into the bacterial cells residing inside the biofilm. Findings of Brackman et al. (2008) have clearly revealed the reduced production of EPS by *Vibrio* spp. upon treatment with cinnamaldehyde and its derivatives. Our results are in agreement with the reports of Nithya and Pandian (2010), wherein the cell free supernatants of marine bacteria effectively inhibited the EPS production of *Vibrio* spp. Similar observations have been made by Musthafa et al. (2012c), in which the QSI compound phenylacetic acid effectively inhibits the EPS production of PAO1. It has also been reported that alginate constitutes the major proportion of EPS and plays vital role in the biofilm formation (Owlia et al., 2007). As described in Fig. 3.9, alginate production of PAO1, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* was also decreased with increasing concentration of QSI compounds. Since, alginate confers increased resistance to
antimicrobial agents an inhibition in alginate production will decrease the rate of bacterial resistance. Previously, essential oil of *M. chamonilla* was shown to inhibit alginate production of *P. aeruginosa* (Owlia et al., 2007).

Flagellar mediated swimming motility is associated with biofilm formation by instigating the cell to surface attachment (Pratt and Kolter, 1998) and plays a key role in the virulence of bacterial pathogens. Therefore, any interruption in swimming motility will possibly affect the formation of biofilm. The results of the swimming assay (Table 3.2) lucidly evident that the swimming motility of tested pathogens was comparatively poor when treated with QSI compounds with that of untreated control. Hence, it is envisaged that test compounds appeared to reduce the biofilm formation in bacterial pathogens by interfering with its ability to reach the substratum. These results are in accordance with the findings of Niu and Gilbert (2004) wherein cinnamaldehyde reduced the biofilm formation of *E. coli* by interfering with its swimming motility. The obtained results are also comparable with the inhibitory effect of furanone C-30 on swimming motility of *V. anguillarum* (Rasch et al., 2004). Similar observations have also been made by Musthafa et al. (2012b), wherein the swimming motility of PAO1 is effectively reduced in the presence of the chemical compound 2,5-piperazinedione.

In addition to swimming motility, the swarming behavior of bacteria is also considered to be an important virulence factor, since it is involved in biofilm formation. The test compounds used in this study have shown significant reduction in swarming motility of the bacterial pathogens such as *E. coli*, PAO1, *P. mirabilis*, *S. marcescens*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* with a marked change in the spacing of the radiating tendrils. The dendrites were closely spaced and thin in untreated control but broader and widely spaced in the presence of higher
concentration of QSI compounds, which might in turn, lead to the development of immature or reduced biofilm. The altered pattern of swarming behaviour may have important applications in treating urinary infections in humans as well as vibriosis in aquaculture. It is also proven that the mass translocation of cells during swarming migration relies on the production of biosurfactants molecules, whose expression is controlled by QS (Daniels et al., 2004). This present study also revealed the ability of the QSI compounds to inhibit the production of biosurfactant-rhamnolipid in PAO1 (Fig. 3.10).

Since, the QS dependent production of prodigiosin in *S. marcescens* is considered as a major virulence factor (Liu and Nizet, 2009) for its pathogenicity, the QSI activity of identified compounds was assessed against prodigiosin production. The two signal C4-HSL and C6-HSL are shown to regulate the production of prodigiosin (Morohoshi et al., 2007) in *S. marcescens*. Therefore, any interference with the QS system will result in reduction in prodigiosin production. As shown in Fig. 3.11, the QSI compounds exhibited a concentration dependent reduction in prodigiosin production without any hindrance on its growth. These results are comparable with the findings of Annapoorani et al. (2012a), where in the marine sponges have exhibited inhibitory activity against the production of prodigiosin. Recently, Musthafa et al. (2012a) have also observed a similar effect, in which, the crude extract obtained from marine *Bacillus* spp. inhibits the production of prodigiosin in *S. marcescens* without any reduction in the growth.

It is known fact that QS system triggers bioluminescence in *V. harveyi* (Niu et al., 2006). Therefore, the identified QSI compounds were examined for their efficacy in reducing the production of bioluminescence. In the obtained results, a concentration dependent inhibition in bioluminescence was documented with tested
concentrations of QSI compounds (Fig. 3.12) without inhibiting the growth of pathogenic *V. harveyi*. The results of the present study are in conformity with the findings of Niu et al. (2006) in which the phytochemical cinnamaldehyde exhibited a significant inhibitory effect on bioluminescence of a reporter strain *V. harveyi* BB170. The bioluminescence inhibitory potential of test QSI compounds was also comparable to the reported activity of boronic acid (Ni et al., 2008). Such an observation has also been made by Brackman et al. (2008) wherein, the cinnamaldehyde and its derivatives were effectively interfered with QS mediated bioluminescence production in *V. harveyi*. Based on the obtained results, it is believed that since the expression of bioluminescence is under the control of QS, interference of the QS system by the identified QSI compounds would result in the observed reduction of bioluminescence in *V. harveyi*. As, these QSI compounds have been controlled the bioluminescence, such compounds were expected to control over other virulence factors production in *V. harveyi*. It is worthy to mention that all the identified compounds did not show any antibacterial activity against all test pathogens (Fig. 3.13). Hence, it is envisaged that the identified compounds from dietary sources possibly interfered with the bacterial biofilm development and other virulence factors including pigment production and bioluminescence, but evidently did not inhibit the growth of the bacterial pathogens.

The QSI and antibiofilm activity of the test compounds were further examined *in vivo* for their efficacy in reducing the virulence of *V. harveyi* and PAO1 by challenging experiments with *Artemia nauplii* and *C. elegans*, respectively. In aquatic environment, the biofilm forming potential of *V. harveyi* confers virulence, which is responsible for shrimp mortality (Guzman et al. 2004; Yildiz and Visick, 2009). Hence, an effort was made to evaluate the QSI efficiency of test QSI compounds in protecting the *Artemia* nauplii against the pathogenic *V. harveyi* infections. As shown
in Fig. 3.14, the QSI compounds such as curcumin (25 µg/ml), eugenol (5 µg/ml), methyl eugenol (50 µg/ml) and methyl geranate (25 µg/ml) did not hold any lethal effects on *Artemia* nauplii, whereas a higher rate of mortality was observed when exposing *Artemia* to *V. harveyi*. However, *V. harveyi* in the presence of QSI compounds at determined concentrations showed a marked reduction in the mortalities of *Artemia* nauplii in challenge trials. According to Rasch et al. (2004), there is a marked reduction in mortalities of rainbow trout by *V. anguillarum* cultures in the presence of 0.1 µM of furanone C-30. A similar result has also been recorded with cinnamaldehyde and 2-NO2-cinnamaldehyde to reduce the mortalities of *Artemia* shrimp infected with *V. harveyi* (Brackman et al., 2008). The obtained results of *in vivo* analysis of the present investigation revealed the antipathogenic potential of test QSI compounds in enhancing the survival of *Artemia* by decreasing the virulence of *V. harveyi*.

It has been proven that *C. elegans* can successfully be employed as an alternative host to investigate the virulence of a variety of bacterial pathogens (Moy et al., 2006). It has also been well established that the virulence factors of *P. aeruginosa* responsible for killing *C. elegans* are also relevant to mammalian systems (Tan et al., 1999). Moreover, the strain PAO1 causes nematode death through cyanide poisoning and neuromuscular paralysis (Gallagher and Manoil, 2001). It is well known that the AHL dependent *hcn* operon produces cyanide in PAO1, which leads to paralysis and death of *C. elegans* (Gallagher and Manoil, 2001). Hence, in the present investigation, an attempt was made to study the potential of QSI compounds in reducing the mortality of PAO1 preinfected *C. elegans*. As apparent from Fig. 3.15, an enhanced survival of preinfected *C. elegans* was observed against PAO1 infection when treated with QSI compounds. Thus, from the attained result, it is envisaged that the enhanced
survival of *C. elegans* was probably due to the interference in the AHL system of PAO1 by QSI compounds which led to the reduced death of *C. elegans* caused by cyanide poisoning.

It is well documented by Bjarnsholt et al. (2005) that increased sensitivity towards antibiotics depends on the process of QS. Bacterial cells living inside the biofilm are generally resistant or less sensitive towards antibiotics. In the present study, it has been found that cells of *C. violaceum* and *S. marcescens* in biofilm are less sensitive to antibiotics, such as bacitracin and erythromycin, respectively. Despite such resistance, the exposure of bacteria in the presence of QSI compounds resulted in an increased susceptibility to respective antibiotics (Fig. 3.16 and 3.17). This clearly reveals the synergistic activity of test QSI compounds with that of antibiotics. The obtained results are also supported by the reports of Rogers et al. (2010), stating that compounds which reduce bacterial biofilms without targeting bacterial growth may likely act synergistically with conventional antibiotics to overcome the resistance among bacterial pathogens. Our results are in consistent with previous reports of Hentzer and Givskov (2003), wherein halogenated furanone compounds act synergistically with tobramycin to eradicate *P. aeruginosa* biofilms. Similarly, cinnamaldehyde increases the susceptibility of *V. vulnificus* towards doxycycline (Brackman et al., 2008). Given the widespread occurrence of signal-mediated QS systems, it reveals that interfering with the QS system may pave the way to prevent the development of signal-mediated biofilm formation and subsequent infections in humans as well as aquatic animals.