Chapter I

Screening of south Indian dietary sources for their quorum sensing inhibitory and antibiofilm potential against bacterial pathogens
INTRODUCTION

World Health Organization (2008) reported that 70-80% of the world’s population still relies upon the herbal drugs for their health concern. Ethnopharmacology is the science of exploring the medicinal potential of herbs and appraising them as prospective leads in drug development. About 25% of available drugs in the market comprise at least one or more compounds derived from plant sources. It is noteworthy to mention that herbal medicine is widely acknowledged and accomplished by people worldwide (Meena and Kumar, 2009). Plant based therapy are highly appreciable because of their cost effectiveness, easy availability and the traditional knowledge transfer. Therefore, there is an increasing demand globally for the pharmaceutical products based on herbal products due to their added advantages. On the other hand, medicinal and dietary plants living in an environment amidst of incredibly high microbial density are long assumed to have defensive mechanism against microbial infections (Huerta et al., 2008). Consequently, these plants are recognized to offer a great and attractive phytochemical repertoire for the discovery of novel microbial disease control agents. The potential of these phytochemicals in managing infectious diseases has attracted considerable interest among the scientific community. Since, these phytochemicals are derived from dietary sources, they are generally recognized as safe (GRAS) and are not or rarely associated with any side-effects (Huerta et al., 2008). It is also known that the phytochemicals with well known antibiotic properties could potentially possess antipathogenic activities too (Vattem et al., 2007). Such antipathogenic compounds neither kill nor stop their growth of bacteria and do not leave any chance for the development of resistant strains (Truchado et al., 2009). Instead, these compounds attenuate the expression of genes responsible for the pathogenesis by interfering with bacterial communication system.
In QS system of Gram negative bacteria, the complex of signalling molecules and receptor proteins triggers the expression of specific genes responsible for various phenotypes including violacein pigment production in \(C. violaceum\) (CviI/R), virulence factors production in \(E. coli\) (LsrR and LsrK), \(P. aeruginosa\) (LasI/R), flagellar motility in \(P. mirabilis\) (RsbA) and \(S. marcescens\) (SwrI/R), and development of biofilms in all these organisms (Cha et al., 1998; De Kievit et al., 2001; Miller and Bassler, 2001; Schneider et al., 2002; Waters and Bassler, 2005, Li et al., 2007). Hence, all these bacterial organisms were selected for the present study.

QS mediated biofilm is the major cause of bacterial pathogenesis in human as well as other animals. Biofilms are complex aggregation of microorganisms encased in a self secreted extracellular matrix consisting of EPS (Costerton et al., 1995). Centre for Disease Control and Prevention, USA, states that 65% of all infections are originated from biofilms (Lewis, 2007). It has also been found that bacteria living in the biofilm mode of growth are often up to 1000 times more resistant to antibiotics than their planktonic counterparts (Caraher et al., 2007). Considering the emergence of increasing antibiotic resistance in bacteria, the use of drugs based on disruption of cell–cell communication to attenuate bacterial pathogenicity rather than bacterial growth is very attractive. Since, biofilm formation is generally determined by the QS mediated phenomenon such as production of EPS (Vu et al., 2009) and flagellar-driven swimming and swarming motility (Swift et al., 2001), the interference with such phenomenon by means of QSI compounds could be a suitable alternative strategy to reduce or to prevent biofilm based infections. Hence, such phytochemicals holding QSI potential hopefully will provide new insight in developing next wave of novel and more effective drugs. Considering the multiple therapeutic properties of these dietary sources and their extensive usage against infectious diseases both in
traditional and modern medicines, this investigation has been made with a primary objective to determine the QSI potential of common dietary products including cereals, vegetables, herbs and spices. In this view of fact, the development of this traditional Indian system of medicines with perspectives of safety, efficacy and quality will aid not only to conserve this traditional heritage but also to develop novel antipathogenic drugs to overcome biofilm based infections without any side effects.

MATERIALS AND METHODS

Dietary sources and their extract preparation

The dietary products such as vegetables including Beta vulgaris, Sechium edule, Raphanus Sativus, Momordica charantia, Vigna radiate, cereals like Hoideum vulgare, Zea mays, Hordeum vulgare, Eluesine coracana, herbs and spices such as Brassica juncea, Capsicum annum, Capparis spinosa, Carum coticum, Cuminum cyminum, Curcuma longa, Coriandrum sativum L, Elettaria Cardamomum, Eugenia caryophylata, Ferula asfoetida, Mentha piperita Ocimum sanctum, Papaverum somniferum, Pimpinella anisum L, Pepper nigrum and Piper longum L were obtained from a local market located in Karaikudi, Tamil Nadu, India. The test samples were washed twice with sterile water followed by a final rinse with 70% (v/v) ethanol. The samples were then shade dried until brittleness. The dried samples were ground to a fine powder and extracted twice with methanol for 24 h. All the extracts were filtered through Whatman No.1 paper and evaporated to dryness. The residues were suspended in appropriate volume of sterile milli-Q water and stored at -20 °C until further use.

Bacterial strains and culture conditions

The QS biomarker strains C. violaceum (ATCC 12472) and mutant strain C. violaceum CV026 were used for the screening of QSI activity of extracts.
Human pathogenic bacteria such as *E. coli* (ATCC 10536), *P. aeruginosa* PAO1, *P. mirabilis* (ATCC 7002), clinical isolate *S. marcescens* (FJ584421) were used as target pathogens in studying the antibiofilm and other QSI potential of crude extracts obtained from dietary sources. For experimental analysis, all the aforementioned bacterial pathogens were sub cultured in Luria Bertani (LB) medium at 30°C except PAO1, which was maintained at 37°C.

**Screening of dietary sources for QSI Potential**

The methanolic extract of dietary sources was subjected to the qualitative analysis to find out their QSI potential against violacein production in *C. violaceum* (ATCC 12472). It synthesizes a violet color pigment called violacein by responding to its QS signal molecule C6-HSL produced by the AI synthase CviI. This C6-HSL binds to its receptor CviR and this complex triggers the expression of genes responsible for violacein production (McLean et al., 2004). For experimental analysis, 1% overnight culture of *C. violaceum* (ATCC 12472) at the cell density of 0.4 OD at 600 nm was added into wells of sterile micro titre plates (MTP) containing 1 ml of LB broth and incubated in the presence and absence of various concentrations of test extracts. The MTPs were incubated at 30°C for 16 h and observed for the reduction of violacein pigment production in the presence of test extracts.

Dietary extracts those exhibited consistent QSI activity by inhibiting the violacein production in qualitative assay with biomarker strain *C. violaceum* (ATCC 12472) without exerting any inhibitory effect on bacterial growth alone were selected for further experiments related to QS mediated biofilm development and its associated behaviours.
Determination of minimum inhibitory concentration (MIC)

The MIC for the selected test extracts was determined as per the guidelines of Clinical and Laboratory Standards Institute (2006). Briefly, 1% of test pathogens (0.4 OD at 600 nm) were added to LB supplemented with the serially twofold diluted dietary extracts to attain the final concentrations ranging from 50 to 0.012 mg/ml and incubated at their optimum temperature for 24 h. The MIC was recorded as the lowest concentration at which it showed complete inhibition of visible growth of the bacterial pathogens. For further QSI experimental analyses, the concentrations well below the MIC of the test extracts, i.e., sub-MICs were used. Based on the MIC values the sub-MIC concentrations were fixed and used in subsequent assays including violacein quantification, biofilm biomass and EPS inhibition. The selected sub-MIC concentrations were ranging from 0.5, 1, 1.5 and 2 mg/ml for both *C. cyminum* and *C. spinosa*, whereas the concentrations were 3, 6, 9 and 12 mg/ml for *O. sanctum*. In case of *C. longa* the concentrations were 25, 30, 35 and 40 µg/ml.

Quantitative analysis of violacein inhibition

The selected test extracts were subjected to quantitative analysis against violacein inhibition in *C. violaceum* CV026. It is a violacein-negative, double mini-Tn5 mutant of *C. violaceum* (ATCC 31532), deficient in the AI synthase CviI and, therefore, it requires exogenous addition of C6-HSL to induce violacein production (McLean et al., 2004). In this assay, 1% of CV026 was supplemented with 5 µM of C6-HSL (Sigma-Aldrich, Switzerland) and cultivated in the presence and absence of selected test extracts at their respective sub-MIC concentrations in MTPs and incubated at 30ºC for 18 h. At the end of incubation, the treated and untreated cultures were centrifuged at 10,000 rpm for 10 min to precipitate the insoluble violacein. The culture supernatant was discarded and 1 ml of dimethyl sulfoxide (DMSO) was added
to the pellets to extract violacein pigment. The solution was vortexed vigorously for 30 s to completely solubilize violacein and centrifuged at 10,000 rpm for 10 min to remove the cells. The concentration of violacein in the cell free supernatant of CV026 was quantified spectrophotometrically (Hitachi U-2800, Japan) at 585 nm (Choo et al., 2006). The percentage of violacein inhibition was calculated by following the formula: percentage of violacein inhibition = (control OD$_{585 \ nm}$ - test OD$_{585 \ nm}$/control OD$_{585 \ nm}$) × 100.

**Microscopic analyses of bacterial biofilms**

**Light microscopic analysis**

Briefly, 1% overnight cultures of test pathogens (0.4 OD at 600 nm) were added into individual wells of 24 well MTP plates containing 1 ml of fresh LB medium and cover glass of 1 cm$^2$ along with and without test extracts at their respective sub-MICs. After 16 h of incubation, the cover glasses were rinsed thrice with distilled water to remove the planktonic cells and the biofilms adhered in the cover glasses were stained with 0.4% crystal violet (CV) solution (Hi Media, Mumbai, India) for 10 min. Stained cover glasses were placed on slides with the biofilm pointing up and the biofilms were visualized under light microscope (Nikon Eclipse Ti 100, Japan) at 40x magnification (Bakkiyaraj and Pandian, 2010).

**Confocal laser scanning microscopic (CLSM) analysis**

For CLSM analysis, bacterial strains were allowed to form biofilms in the absence and presence of test extracts at their respective sub-MICs on the cover glass slides as described in light microscopic analysis. After 16 h of incubation, biofilm formed in the cover glasses were stained with 20 µl of 1% acridine orange (Sigma Aldrich, Switzerland) for few seconds. The excess stain was washed out and the
stained biofilms in cover glasses were visualized with CLSM (LSM 710, Carl Zeiss, Germany) equipped with an excitation filter 515–560 and magnification at 20× (Nithya and Pandian, 2010).

Biofilm biomass inhibition assay

The methanolic extracts of C. cyminum, C. spinosa, C. longa and O. sanctum with QSI activity were further examined for their efficacy to inhibit the biofilm biomass of the test bacterial pathogens (Thenmozhi et al., 2009). Briefly, 1% overnight cultures (OD adjusted to 0.4 at 600 nm) of test pathogens were added into 1 ml of fresh LB medium in the wells of MTP and cultivated in the absence and presence of test extracts at their respective sub-MICs without agitation for 16 h at appropriate temperature. After incubation, the spent media along with planktonic cells were discarded and all the wells were gently rinsed twice with sterile distilled water. The surface-adhered cells in the MTP wells were stained with 200 μl of 0.4% CV solution for 10 min, unbound CV was discarded and stained biofilms were washed three times with sterile distilled water to remove excess dye. CV in the stained biofilm was then solubilized with 1 ml of 95% ethanol. The biofilm biomass was quantified by measuring the intensity of solubilized CV solution at OD 650 nm using UV–visible spectrophotometer.

Extraction and quantification of EPS

EPS inhibition assay was carried out by following the method of Nithya et al. (2011). Briefly, 1 cm² cover glasses were immersed in the cultures of both untreated and treated with test extracts at their respective sub-MICs in the wells of 24-well MTPs and incubated for 24 h. After the incubation, cover glasses were removed and suspended with 0.5 ml of 0.9% NaCl. The cell suspensions (0.5 ml) were incubated in
test tubes with an equal volume of 5% phenol to which 5 volumes of concentrated 
H₂SO₄ was added. The mixture was then incubated for 1 h in the dark and centrifuged 
at 10,000 rpm for 10 min and the absorbance was measured at 490 nm.

Swimming and swarming assay

Since, QS mediated swimming and swarming motility play a major role in the 
biofilm development, the test extracts with QSI activity was assessed for their ability 
in inhibiting the motility behaviour of bacterial pathogens by following the method of 
Liaw et al. (2000). In swimming assay, overnight cultures of the test bacterial 
pathogens were point inoculated at the center of the medium consisting of 1% 
tryptone, 0.5% NaCl and 0.3% agar supplemented with test extracts at the following 
concentrations; 60 µg/ml for *C. cyminum*, 2 mg/ml for *C. spinosa*, 45 µg/ml for *C. 
longa* and 12 mg/ml for *O. sanctum* extract. After incubation at appropriate 
temperature for 16 h the plates were observed for swimming inhibitory zone.

For swarming assays, test bacteria were point inoculated at the center of the 
medium consisting of 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% of filter sterilized 
D-glucose with test extracts at the concentrations as mentioned earlier for swimming 
motility. The plates were incubated in an undisturbed upright position at appropriate 
temperature for 16 h and observed for swarming migration zone.

Nature of quorum sensing compounds

In order to assess the nature of QSI compounds present in the methanolic 
extracts of test dietary extracts, proteolytic digestion and temperature-sensitivity assay 
were performed by following the method of Musthafa et al. (2011). In proteolytic 
digestion, 10 µl of proteinase K (Sigma Aldrich, St. Louis, MO) at the concentration 
of 1 mg/ml was added to 1 ml of test extracts and incubated for 18 h at 55 °C. In
temperature sensitivity assay, the test methanolic extracts were incubated at 100 °C for 30 min. Controls were maintained with 1mg/ml of test extracts without the addition of proteinase K and without heat treatment. After incubation, samples were observed for the inhibition of violacein production by CV026 as described previously.

**Antibacterial assay**

The effect of test extracts on bacterial growth was assessed through well diffusion agar assay (WDAA) in Muller-Hinton agar (MHA) (Hi-media, Mumbai, India) by following the method specified by the Clinical and Laboratory Standards Institute (USA, 2006). Briefly, 100 µl of test bacterial suspensions such as *C. violaceum* (ATCC 12472), *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* with the cell density equivalent to 0.5 McFarland standard units (1×10⁸ CFU/ml) were uniformly spread over the surface of MHA plates. The plates were kept undisturbed for 10 min for the absorption of excess moisture. The sterile paper disks (Hi-media, Mumbai, India) with a diameter of 10 mm loaded with various concentrations (as described above) of test extracts were placed over the plates. The plates were incubated at 30°C and the zone of growth inhibition was observed after 24 h.

**Growth curve analysis**

This assay was performed to confirm the non-antibacterial activity of the QSI extracts at their respective highest sub-MIC. Briefly, 1% overnight culture of test pathogens (0.4 OD at 600 nm) were inoculated in 250 ml Erlenmeyer flask containing 50 ml of LB broth supplemented with highest sub-MIC of test extracts. 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 (Hitachi U-2800) at an interval of one hour up to 16 h.
Statistical Analysis

All experiments were performed in triplicate and the data obtained from the experiments were presented as mean values and the differences between control and test were analyzed using Student’s $t$ test.

RESULTS

Qualitative screening for QSI activity-Violacein inhibition assay

In qualitative screening, among 25 samples tested which includes five vegetables, four cereals, 16 samples of herbs and spices, the methanolic extracts of *C. cyminum*, *C. spinosa*, *C. longa* and *O. sanctum* showed consistent as well as maximum inhibition of violacein production in *C. violaceum* (ATCC 12472) (Fig. 1.1), whereas, *Eugenia caryophylata* and *Elettaria cadamomum* showed very minimal and inconsistent reductions (Table 1.1). Hence, the extracts of *C. cyminum*, *C. spinosa*, *C. longa* and *O. sanctum* alone were used in quantitative assessment of violacein inhibition with CV026 and subsequent assays in the present study.

![Fig. 1.1. Qualitative analysis of violacein inhibition in *C. violaceum* (ATCC 12472) by herbs and spices. Cultures were grown in the absence (A) a and b: untreated controls and presence of (B) *C. cyminum*; (C) *C. spinosa*; a and b: 1 and 2 mg/ml (D) *C. longa*; a and b: 30 and 40 µg/ml and (E) *O. sanctum*; a and b: 6 and 12 mg/ml.](image-url)
Table 1.1. Quorum sensing inhibitory property of common south Indian cereals spices and vegetables against inhibition of violacein pigment production in *C. violaceum* (ATCC 12472).

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Common name</th>
<th>Violacein inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vegetables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Beta vulgaris</em></td>
<td>Beetroot</td>
<td>-</td>
</tr>
<tr>
<td><em>Sechium edule</em></td>
<td>Chayote</td>
<td>-</td>
</tr>
<tr>
<td><em>Raphanus sativus</em></td>
<td>Radish</td>
<td>-</td>
</tr>
<tr>
<td><em>Momordica charantia</em></td>
<td>Bitterguard</td>
<td>-</td>
</tr>
<tr>
<td><em>Vigna radiata</em></td>
<td>Mung bean</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cereals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>Wheat</td>
<td>-</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>Corn</td>
<td>-</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>Barley</td>
<td>-</td>
</tr>
<tr>
<td><em>Eluesine coracana</em></td>
<td>Ragi</td>
<td>-</td>
</tr>
<tr>
<td><strong>Herbs and Spices</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brassica juncea</em></td>
<td>Mustard</td>
<td>-</td>
</tr>
<tr>
<td><em>Capsicum annum</em></td>
<td>Chilli</td>
<td>-</td>
</tr>
<tr>
<td><em>Carum copticum</em></td>
<td>Carum</td>
<td>-</td>
</tr>
<tr>
<td><em>Capparis spinosa</em></td>
<td>Capper</td>
<td>+++</td>
</tr>
<tr>
<td><em>Cuminum cyminum</em></td>
<td>Cumin seeds</td>
<td>+++</td>
</tr>
<tr>
<td><em>Curcuma longa</em></td>
<td>Turmeric</td>
<td>+++</td>
</tr>
<tr>
<td><em>Coriandrum sativum L</em></td>
<td>Coriander</td>
<td>-</td>
</tr>
<tr>
<td><em>Elettaria cardamomum</em></td>
<td>Elachi</td>
<td>+</td>
</tr>
<tr>
<td><em>Eugenia caryophylata</em></td>
<td>Clove</td>
<td>+</td>
</tr>
<tr>
<td><em>Ferula asfoetida</em></td>
<td>Asfoetida</td>
<td>-</td>
</tr>
<tr>
<td><em>Mentha piperita</em></td>
<td>Mint</td>
<td>-</td>
</tr>
<tr>
<td><em>Ocinum sanctum</em></td>
<td>Holy Basil</td>
<td>+++</td>
</tr>
<tr>
<td><em>Papaverum somniferum</em></td>
<td>Poppy seeds</td>
<td>-</td>
</tr>
<tr>
<td><em>Pimpinella anisum L</em></td>
<td>Fennel seeds</td>
<td>-</td>
</tr>
<tr>
<td><em>Pepper nigrum</em></td>
<td>Pepper</td>
<td>-</td>
</tr>
<tr>
<td><em>Piper longum L</em></td>
<td>Long pepper</td>
<td>-</td>
</tr>
</tbody>
</table>

Moderate activity is represented as (+), very good activity is represented as (+++).
Determination of MIC

Based on the fact that the QSI compounds should not have any adverse effect on bacterial growth, the MIC assay was performed to assess QS inhibitory concentration of the selected test extracts. The lowest concentration of each extract that showed complete inhibition of visible growth was determined as MIC. The MIC of test extracts for each pathogen was determined by doubling dilution method and the obtained results were tabulated in Table 1.2. The concentrations well below the MIC were considered as sub-MICs which were not expected to inhibit the bacterial growth. Therefore, for QS inhibition studies, the sub-MICs were selected and used in subsequent assays including violacein quantification, biofilm biomass and EPS inhibition. The selected sub-MICs were ranging from 0.5, 1, 1.5 and 2 mg/ml for C. cyminum and C. spinosa, whereas the concentrations were 3, 6, 9 and 12 mg/ml for O. sanctum. In case of C. longa the concentrations were 25, 30, 35 and 40 µg/ml.

Table 1.2. MIC of the selected herb and spices extracts against test bacterial pathogens

<table>
<thead>
<tr>
<th>Bacterial pathogen</th>
<th>C. spinosa (mg/ml)</th>
<th>C. cyminum (mg/ml)</th>
<th>C. longa (µg/ml)</th>
<th>O. sanctum (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. violaceum</td>
<td>6.25</td>
<td>3.12</td>
<td>48.5</td>
<td>25.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>6.25</td>
<td>3.12</td>
<td>48.5</td>
<td>25.0</td>
</tr>
<tr>
<td>PAO1</td>
<td>3.12</td>
<td>6.25</td>
<td>48.5</td>
<td>12.5</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td>6.25</td>
<td>3.12</td>
<td>48.5</td>
<td>12.5</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>12.5</td>
<td>6.25</td>
<td>97.0</td>
<td>12.5</td>
</tr>
</tbody>
</table>
Quantitative assessment of violacein inhibition

The samples including the spices extracts such as *C. spinosa*, *C. cyminum*, *C. longa* and herb extract *O. sanctum* which showed effective inhibitory activity on violacein production in *C. violaceum* (ATCC 12472) without any impact on its growth were selected for the quantitative assessment of violacein inhibition. The quantitative assay was performed in CV026 with the exogenous supplement of C6-HSL in the presence and absence of test extracts. The obtained results revealed that all the test extracts exerted a dose dependent inhibitory activity on violacein pigment production. Compared to the control, all the test extracts except *C. longa* showed a significant drop in violacein production, even at their lowest test concentrations such as 0.5 mg/ml of *C. spinosa* and *C. cyminum* and 3 mg/ml of *O. sanctum*. However, the maximum inhibition in violacein production was observed with *C. spinosa* and *C. cyminum*, at the concentration of 2 mg/ml by 88% and 90% respectively. The methanolic extract of *O. sanctum* at the concentration of 12 mg/ml inhibited the violacein production by 95%. At higher concentration of 40 µg/ml, *C. longa* exhibited a maximum of 86% inhibition. However, the highest level of inhibitory activity towards violacein production was contributed by the methanolic extract of *O. sanctum* while all other spice extracts showed the little lower inhibitory activity as depicted in Fig. 1.2.
Fig. 1.2. Quantitative assessment of violacein inhibition in CV026 by herb and spices extracts. The cultures were grown in the absence and in the presence of (A) *C. spinosa*; (B) *C. cyminum*; (C) *C. longa* and (D) *O. sanctum* at their respective sub-MIC concentrations. 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 biofilm inhibition

The results of light microscopic analysis revealed that the untreated cover glasses displayed a well developed biofilm growth of test bacterial pathogens such as *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, while the extracts treated cover glasses exhibited poorly developed thin biofilm. Most notably, a reduction in number of microcolonies was observed when treated with higher concentration of test extracts against all test bacterial pathogens (Fig. 1.3). Among the extracts tested, a higher degree of inhibition in biofilm matrix as well as number of microcolony formation was observed with the methanolic extract of *C. cyminum*. All other extracts exhibited antibiofilm activity in varying degree in a strain dependent manner.
Further, the antibiofilm potential of all the test extracts was confirmed through CLSM analysis. Consistent with the results obtained in light microscopic analysis, images of CLSM also revealed clear differences in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* biofilm structure between treated with methanolic extracts such as *C. cyminum* (2 mg/ml), *C. spinosa* (2 mg/ml), *C. longa* (40 μg/ml) and *O. sanctum* (12 mg/ml) and the respective untreated controls (Fig.1. 4), the former biofilm was less structured and contained only a fewer cells.
Fig. 1.4. CLSM images depict the antibiofilm activity of herb and spice extracts. Bacterial biofilms of (A) *E. coli*; (B) PAO1; (C) *P. mirabilis*; (D) *S. marcescens* grown in the absence (a) and presence of methanolic extracts of (b) *C. spinosa* (2 mg/ml) (c) *C. cyminum* (2 mg/ml) (d) *C. longa* (40 µg/ml) and (e) *O. sanctum* (12 mg/ml).

Biofilm biomass quantification assay

It is well known that QS positively regulates the biofilm formation of bacterial pathogens (Annous et al., 2009). Therefore, the dietary extracts that exhibited QSI activity were further investigated for their influence on biofilm biomass production of selected Gram negative bacterial pathogens. The obtained results clearly evident that the bacterial pathogens including *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* displayed a dose dependent reduction in biofilm mass in MTPs, when grown in the presence of the test extracts such as *C. spinosa* (0.5-2 mg/ml), *C. cyminum* (0.5-2 mg/ml), *C. longa* (25-40 µg/ml), and *O. sanctum* (3-12 mg/ml) as compared to that of respective controls. At 2 mg/ml concentration, *C. spinosa* extract showed a maximum of 79%, 75%, 73% and 70% reduction in biofilm biomass of *S. marcescens*, PAO1,
*E. coli* and *P. mirabilis*, respectively (Fig. 1.5A). Methanolic extract of *C. cyminum* exhibited a maximum reduction of 82%, 94%, 86% and 71% in biofilm biomass production of *E. coli*, PAO1, *P. mirabilis*, and *S. marcescens*, respectively, at the concentration of 2 mg/ml (Fig. 1.5B). As depicted in Fig. 1.5C, the inhibitory effect of *C. longa* was ranging from 7-46%, 24-53%, 14-40% and 36-80% in *E. coli*, PAO1, *P. mirabilis*, and *S. marcescens*, respectively at the test concentrations (25-40 µg/ml). At 12 mg/ml concentration, *O. sanctum* inhibited the biofilm biomass by 46%, 29%, 47% and 92% in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively (Fig. 1.5D). However a marked inhibitory activity in reducing the biofilm biomass of all test pathogens was exhibited by *C. cyminum* extract. Adding up, exposure of these extracts to test pathogens had no inhibitory effect on planktonic growth at the concentrations tested.

**Fig. 1.5.** Inhibition of biofilm biomass by herb and spices extracts against Gram negative human pathogenic bacteria. Cultures were grown in the absence and presence of (A) *C. spinosa* (B) *C. cyminum* (C) *C. longa* and (D) *O. sanctum* extracts at their 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 $p \leq 0.005$. 
EPS inhibition assay

Since, EPS plays a key role in the development of bacterial biofilm the antibiotic potential of these test extracts were further evaluated for their efficiency in reducing the EPS production of test bacterial pathogens. EPS was extracted from extract treated and untreated cultures of test pathogens. Spectrometric analysis of the extracted EPS revealed that the concentration of EPS was decreased with increasing concentration of test extracts such as *C. spinosa* (0.5-2 mg/ml), *C. cyminum* (0.5-2 mg/ml), *C. longa* (25-40 µg/ml), and *O. sanctum* (3-12 mg/ml) as compared to untreated control. *C. spinosa* at 2 mg/ml exhibited a maximum of 66%, 46%, 67% and 58% decrease in EPS production of *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively (Fig. 1.6A). Similarly, at 2 mg/ml of *C. cyminum* extract, the production of EPS in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens* was reduced to the maximum level of 75% 70%, 71% and 70% respectively (Fig. 1.6B). *C. longa* at 40 µg/ml showed 48%, 58%, 66% and 57% reduction in EPS production in respective pathogens (Fig. 1.6C). *O. sanctum*, at 12 mg/ml, reduced the secretion of EPS by 75%, 40%, 78% and 36% in *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*, respectively (Fig. 1.6D).
Fig. 1.6. Inhibition of EPS production by herb and spices extracts against Gram negative human pathogenic bacteria. Cultures were grown in the absence and presence of (A) *C. spinosa* (B) *C. cyminum* (C) *C. longa* and (D) *O. sanctum* extracts 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 $p \leq 0.005$.

**Swimming inhibition assay**

The addition of test extract showed a dose dependent decrease in the swimming velocity of all the test pathogens. However, the maximum level of inhibition over swimming migration was recorded at 60 μg/ml for *C. cyminum*, 2 mg/ml for *C. spinosa*, 40 μg/ml for *C. longa* and 12 mg/ml for *O. sanctum* against all the bacterial pathogens tested (Fig. 1.7). The extract of *C. longa* showed a pronounced inhibition in swimming pattern of all test pathogens, whereas the other extracts exhibited a good inhibitory activity against some pathogens and moderate activity against rest of the pathogens.
Swarming inhibition assay

The efficiency of test extracts over swarming motility of target pathogens was further examined. The attained results lucidly revealed that the extracts of *C. spinosa*, *C. cyminum*, *C. longa* and *O. sanctum* effectively inhibited QS dependent swarming migration in *E. coli*, PAO1 and *S. marcescens* at higher concentrations. However, the extracts of *C. cyminum* and *C. longa* were found to be less effective in reducing the swarming motility of *P. mirabilis* even at higher concentration, whereas the swarming inhibitory effect of *C. spinosa* and *O. sanctum* on *P. mirabilis* was found to be moderate. Compare to all test pathogens, the swarming behavior of *E. coli* was significantly inhibited by all test extracts (Fig. 1.8).
Fig. 1.8. Effect of herb and spices extracts compounds on swarming behaviour of bacterial pathogens. A: *E. coli*, B: PAO1, C: *P. mirabilis* and D: *S. marcescens*. (a) Untreated control, (b-e) treated with *C. spinosa* (2 mg/ml), *C. cyminum* (60 µg/ml), *C. longa* (40 µg/ml) and *O. sanctum* (12 mg/ml) extracts, respectively.

Nature of the QSI compound

In order to sort out the nature of the QSI compound in the dietary extracts, the methanolic extracts of all test samples were subjected to proteinase K and heat treatments. In the obtained results the test extracts such as *C. spinosa*, *C. cyminum*, *C. longa* and *O. sanctum* retained their original level of violacein inhibition in CV026 even after heat treatment at 90°C for 15 min to 85%, 88%, 82% and 92%, respectively. Similarly, the test extracts showed an inhibitory level of 82%, 86%, 83% and 93%, respectively after the treatment with proteinase K for overnight. Thus, the results of the present study confirmed the non-enzymatic nature of the QSI compound present in the test extracts (Fig. 1.9).
Fig. 1.9. Effect of temperature and proteinase K on violacein inhibitory property of the QSI compounds present in herb and spices extracts against CV026. Mean values of triplicate independent experiments and SD are shown. ** indicates significance at $p \leq 0.01$ and *** indicates significance at $p \leq 0.005$.

Antibacterial assay

The WDAA was performed to check the presence of growth inhibitory in the test extracts if any. The obtained results showed even after 16 h of incubation, there was no growth inhibitory zone found around the wells added with the test extracts (Fig. 1.10), which validate the non antibacterial activity of test extracts at tested concentrations.
Fig. 1.10. Antibacterial activity of herb and spices extracts against Gram negative bacterial pathogens. Bacterial cultures of (A) *E. coli*; (B) PAO1; (C) *P. mirabilis* and (D) *S. marcescens* in the absence (a) Milli-Q control and presence of (b) *C. spinosa* (2 mg/ml), (c) *C. cyminum* (2 mg/ml), (d) *C. longa* (40 µg/ml) and (e) *O. sanctum* (12 mg/ml)

Growth curve assay

The test extracts with QSI potential did not exhibit any growth inhibitory activity against all test pathogens at their respective sub-MICs. In order to further confirm the non-antibacterial activity of QSI extracts, the bacterial growth curve assay was performed at sub-MIC concentrations of all test extracts against bacterial pathogens. The obtained result revealed that the growth rates as well as maximum cell densities did not differ in broths without or with test extracts at tested concentrations (Fig. 1.11).
DISCUSSION

The present study demonstrates the QSI potential of the south Indian dietary sources against the QS dependent phenotypic expressions in *C. violaceum*, *E. coli*, PAO1, *P. mirabilis* and *S. marcescens*. As virulence factors such as EPS production, motility and biofilm formation in many pathogenic bacteria are regulated by signal mediated QS system, the inhibition of such QS mechanisms by means of QSI compounds may in turn lead to the attenuation of above said virulence gene expression and subsequent bacterial infections. Therefore, any compound which is able to inhibit the violacein production without growth inhibition of *C. violaceum* will be considered as a promising source of QS inhibitor (Choo et al., 2006). Hence, screening for such compounds with QSI activity was carried out using *C. violaceum*, a biomarker strain that produces violacein pigment by CviIR-dependent QS system.
Among the different vegetables, cereals, herbs and spices screened, the extracts of *C. spinosa*, *C. cyminum*, *C. longa* and *O. sanctum* revealed a strong QSI potential by inhibiting violacein pigment production in *C. violaceum* (ATCC 12472) (Table 1.1). In agreement to this finding, several plant extracts such as *Chlamydomonas reinhardtii* (Teplitski et al., 2003), *Conocarpus erectus* (Adonizio et al., 2006), *Tremella fuciformis* (Zhu and Sun, 2008), *M. lunu-ankenda* (Tan et al., 2012) and aqueous extracts of edible fruits such as *Manilkara sappota*, *Musa paradisiaca* and *Ananus cosmosus* (Musthafa et al., 2010) have found to inhibit the violacein production.

The QSI positive extracts such as *C. spinosa*, *C. cyminum*, *C. longa* and *O. sanctum* have extensively been known for their medicinal properties and being used as traditional medicines for centuries. *C. spinosa* L. (family Capparidaceae) is one of the most common aromatic plants growing wild in the Mediterranean region. In India, it grows from Punjab and Rajasthan to the Deccan Peninsula. Apart from flavouring agent, *C. spinosa* has been known for centuries in traditional phytomedicine. The aqueous extract from total aerial parts of the plant has been used for its antifungal, anti-inflammatory, antidiabetic and antihyperlipidemic activities (Tesoriere et al., 2007). In addition, caper act as a hepatic stimulant, which has been used for improving the functional efficiency of the liver (Samy et al., 2008). Decotion of the unopened flower buds of caper is laxative and externally used for treating eye infections. As, the buds are a rich source of compounds with aldose-reductase inhibitory activity; these compounds has been used in preventing the formation of cataracts (Sher and Alyemeni, 2010).

*C. cyminum* is an aromatic plant which belongs to Apiaceae family and is used as antispasmodic, carminative and appetite stimulant agent (Morton, 1976). Cumin
oil was proved as an antifungal agent against various pathogenic fungi (Rahman et al., 2000). In addition, cumin is reported to have extensive antibiotic spectrum against both Gram positive and negative bacteria (Sheikh et al., 2010). Besides, cumin seeds are being used as flavoring agent in food industries (Iacobellis et al., 2005). It can also be used as a fumigant or preservative in the storage of foodstuff (Tunc et al., 2000).

*C. longa* commonly known as turmeric belongs to the family Zingiberaceae. Root portion of this plant has been known to possess insect repellent, antimicrobial, antidiabetic and antihelminthetic properties (Negi et al., 1999; Mohamed et al., 2009). Extracts of *C. longa* are traditionally being used in the treatment of rheumatism, body ache, skin diseases, intestinal worms, diarrhea, intermittent fever, hepatic disorders, urinary discharges, dyspepsia, inflammations, constipation, leukoderma and colic inflammatory disorders (Villegas et al., 2008).

*O. sanctum* is being used as medicinal herb since from ancient times without any adverse effects. Several medicinal properties have been attributed to this plant not only in Ayurveda and Siddha but also in Greek, Roman and Unani system of medicines. Traditionally, juice of the leaves of this plant is being used as demulcent, stimulant and expectorant. Besides, this herb is used in the cure of upper respiratory tract infections, bronchitis, skin infections and ear-ache. Infusions of the leaves have been used as anti-spasmodic in gastric disorders of children. A concoction of root of this herb is still being used as a diaphoretic in malarial fevers in remote areas. *O. sanctum* is extensively used for the cure of heart disorders; it stimulates digestion and reduces breathing difficulties. This herb can also offered anti-carcinogenic, anti-inflammatory, hepatoprotective, immunomodulatory, radio-protective, neuroprotective and cardio-protective properties (Mondal et al., 2009).
Despite the rich knowledge on several medicinal properties of these herbs and spices, reports on their QSI activity remain in its infancy. However, the promising results obtained from violacein inhibition assay append the QSI potential of these herb and spices. Our findings of violacein inhibitory potential of these herbs and spices against *C. violaceum* was supported by the report of Vattem et al. (2007), in which it has been mentioned that spices with well known antibiotic properties could also potentially hold antipathogenic activities too, which may not be related to growth inhibition of the microorganism. Consequently, the QSI potential of these extracts was evaluated for their efficiency in inhibiting the biofilm formation and its related behaviour.

Obviously, QS mechanism plays a major role in the formation of biofilm with complex architecture. In the present study, the exposure of pathogens with the test extracts such as *C. spinosa, C. cyminum, C. longa* and *O. sanctum* inhibited the biofilm biomass significantly in dose dependent manner (Fig. 1.5) without affecting the bacterial growth. The earlier reports by Vikram et al. (2010) revealed the inhibitory effect of limonoids from grape fruit extracts on *E. coli* and *V. harveyi* biofilm formation. Similarly, the aqueous extracts of *O. sanctum, M. sappota, M. paradisiaca* and *A. cosmosus* have been found to inhibit biofilm formation of PAO1 (Musthafa et al., 2010). It has also been proven that surface adhesion promotes initial attachment and subsequent microcolony formation (Sandasi et al., 2010). In the present study, the light microscopic images (Fig.1.3) revealed that the test extracts efficiently reduced the number of microcolonies during the biofilm formation of the tested bacterial pathogens. This evidence that biofilm formation was possibly inhibited at the beginning of the attachment stage itself. This is in agreement with previously published report of You et al. (2007), wherein, the extract of *Streptomyces*
*albus* significantly inhibited biofilm formation of *Vibrio* spp. by preventing their initial adherence. Therefore, it is envisaged that the active principle present in test extracts could possibly interfere with the expressions of genes responsible for initial attachment, resulted in the formation of weak biofilms possibly by reducing the surface adhesion and subsequent microcolony formation. Our results are in accordance with the findings of Sandasi et al. (2010), in which the extract of culinary herbs and several plant extracts were found to inhibit bacterial cell adhesion. In addition to reduce the number of microcolonies, the test bacterial biofilms in the presence of test extracts displayed disintegrated architecture compared to control as were more evident from CLSM analysis (Fig. 1.4).

It is a proven fact that EPS plays a major role in the maintenance of biofilm architecture and the production of EPS in bacterial pathogens is under the control of QS (Vu et al., 2009). Moreover, overproduction of EPS leads to alterations in the architecture of biofilm that correlates with an increased resistance of the cells to osmotic and other oxidative stresses (Yildiz and Schoolnik, 1999). Furthermore, there is increasing evidence suggesting that the interference with the expression of EPS synthesis gene leads to the weakening of biofilm architecture (Bomchil et al., 2003). Herein, it is observed that the test extracts significantly reduced the production of EPS (Fig. 1.6) as well as it caused disintegration of biofilm architecture, as it is more evident from CLSM images (Fig. 1.5). To the best of our knowledge, this is the first report on inhibition of EPS production of bacterial pathogens by dietary extracts. It is important to note that since, the test extract loosens the architecture of the biofilm by inhibiting the synthesis of EPS, it is possible to make resistant bacterial cells become sensitive to antibiotics.
Swimming motility is an important QS-dependent, flagellar mediated behavior which plays a key role in the initial attachment of free living planktonic bacterial cells to the substrate during biofilm development (Pratt and Kolter, 1998). The findings of the present study explored the ability of the test extracts to prevent the swimming migration of test bacterial pathogens in a dose dependent manner (Fig. 1.7). Since, flagellar mediated swimming motility has been controlled by QS, inhibition of QS by the extracts of herbs and spices would result in the reduced swimming migration activity. Thus, the test extracts indirectly demonstrated the inhibition of biofilm formation of all the target pathogens in part by interfering with its ability to reach the substratum and subsequent biofilm formation by disturbing AHL-mediated QS systems. Results obtained in the present study support the findings of Niu and Gilbert (2004), in which cinnamaldehyde inhibited the swimming motility of E. coli by preventing the initial cell-to-surface attachment. Also, extracts from garlic was found to reduce the swimming motility in A. tumefaciens (Al Ghonaiem et al., 2009). Apart from swimming motility, there is another important QS mediated flagellar and pili dependent phenomenon called swarming motility and it is considered as one of the important virulence factors because it involved in biofilm formation. In the present study, test extracts at higher concentrations effectively reduced the QS dependent swarming motility, which accounts for the formation of biofilm in many pathogenic bacteria including E. coli, S. marcescens, P. mirabilis and PAO1 (Fig. 1.8). Perhaps, there are two likely possible mechanisms by which these extracts accomplish the inhibition of swarming motility. The QSI compound present in these extracts might inhibit the motility either by interfering with AHL mediated cell differentiation, where the normal cells got differentiated into swarmer cells rather than inhibiting the bacterial growth as reported previously with resveratrol (Wang et al., 2006) and p-
nitrophenylglycerol (Liaw et al., 2000) or by interfering with the putative AHL receptor and thereby displaces the AHL molecules. Similar observation has also been observed by Tan et al. (2012), wherein the extract of *Melicope lunu-ankenda* arrested the swarming motility by inhibiting the differentiation of vegetative cells into swarmer cells and subsequent swarming migration. In the same way, the possible interference of QSI compounds with the putative AHL receptors has already been reported with the swarming motility of *P. mirabilis* (Gram et al., 1996) and *S. marcescens* (Givskov et al., 1996) by halogenated furanones obtained from *Delisea pulchura*. Few more extracts were also reported to inhibit swarming motility of bacterial pathogens including thyme (Vattem et al., 2007) and clove oil (Khan et al., 2009). Since the swimming and swarming behaviour essentially determines the formation of biofilm (Harshey, 2003), the reduction in these motility facilitates the reduced biofilm formation. Our results also go well with the findings of Swift et al. (2001) wherein mutant strains with altered swimming and swarming motility were shown to be defective in biofilm formation.

It is well proven fact that an effective QS inhibitor does not inhibit the quorum but interfere with “sensing” (Choo et al., 2006). The results of the present study also revealed that all the four dietary extracts with QSI potential are not having any antibacterial activity against test pathogens (Fig. 1.10). Further, it is also confirmed with the bacterial growth curve analysis, wherein no considerable variation in cell densities were observed between the control and extract treated cultures (Fig. 1.11). Therefore, it is confirmed that the QSI compounds present in the herb and spices extracts possibly interfered with the QS mediated biofilm formation but evidently did not inhibit the growth of test pathogenic bacteria. It is envisaged that the biofilm inhibiting activity of these dietary extracts observed in the current study would have a
great implication on medical applications. Furthermore, interruption with bacterial biofilm formation is expected to reduce the development of resistance by allowing the increased sensitivity of pathogenic bacteria towards antibiotics.

Concerning the nature of the QSI compound, the earlier studies have proved that the QSIIs from plant origin may either be enzymatic (Delalande et al., 2005) or non-enzymatic (Choo et al., 2006). However, in our study, even after the heat and proteinase K treatment, the QSI activity of all the dietary extracts was retained against violacein production in CV026 (Fig. 1.9) without any change in their efficiency. Therefore, it is envisaged that the enzymatic degradation was not a contributing factor in the observed inhibition by these test extracts and the inhibitor present in the extracts with QSI potential might be a non enzymatic compound. The presence of such non enzymatic based compound present in the dietary extracts has already been reported in the previous works of Vandeputte et al. (2010), where the QSI activity of Combretum albiflorum extract was attributed to the flavanoid compound catechin.

In summary, the methanolic extracts obtained from the herbs and spices efficiently inhibited QS by interfering with the AHL activity and thus inhibited the production of violacein pigment in C. violaceum. In addition, these extracts inhibited the swimming and swarming motility, EPS production and subsequent biofilm formation in the tested bacterial pathogens. It is worth to mention that these herbs and spices are well known for their medicinal properties and being used regularly as one of the food ingredients in south Indian dishes from time immemorial. Though, these extracts have been explored for their bioactive potentials such as antioxidant, antibacterial, anti-inflammatory, anti-spasmodic, diuretic properties, the present study appends yet another note on their QSI and antibiofilm potentials against a wide range of bacterial pathogens.