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Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against Gram negative bacterial pathogens

Issac Abraham Sybiya Vasantha Packiavathy, Palani Agilandeswari, Khadar Syed Musthafa, Shunmugiah Karutha Pandian, Arumugam Veera Ravi *

Department of Biotechnology, Alagappa University, Karaikudi 630 003, Tamil Nadu, India

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ABSTRACT

Quorum sensing inhibitory (QSI) activity of common South Indian spices and vegetables were evaluated using the bacterial model *Chromobacterium violaceum*. Among the 22 samples tested the QSI compound present in the methanolic extract of *Cuminum cyminum* at 2 mg/ml inhibited violacein production in *C. violaceum*. Further, the outcome of the present investigation reveals that *C. cyminum* extract strongly interferes with acyl homoserine lactone (AHL) regulated physiological functions coupled with biofilm formation such as flagellar motility and exopolysaccharides (EPS) production. It promotes the loosening of biofilm architecture and powerfully inhibits in vitro biofilm formation in *Pseudomonas aeruginosa* PAO1, *Proteus mirabilis* and *Serratia marcescens* at sub-MIC levels. The result of molecular docking analysis attributes the QSI activity exhibited by *C. cyminum* to methyl eugenol (ME). The ability of ME to interfere with quorum sensing (QS) systems of various Gram-negative bacterial pathogens comprising diverse AHL molecules was also assessed and ME was found to reduce the AHL dependent production of violacein, bioluminescence and biofilm formation.

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1. Introduction

Medicinal and dietary plants living in an environment with a very high bacterial cell density were long suspected to have protective mechanisms against microbial infections (Vattem, Mihalik, Crixell, & McLean, 2007). These plants are known to offer a large and attractive phytochemical repertoire for the discovery of novel microbial disease control agents. The potential of these dietary phytochemicals in managing infectious diseases has attracted considerable interest among the scientific community. Several spices such as turmeric, clove, black pepper, garlic, etc., have been used since ancient times to prevent gastrointestinal, pulmonary and urinary tract infections. They also produce 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 including *Escherichia*, *Helicobacter*, *Streptococcus*, and *Salmonella* species, their precise mechanism of antimicrobial functionality has not been completely understood (Stapleton & Taylor, 2002; Vattem & Shetty, 2005). It is possible that phytochemicals with well known antibiotic properties could also potentially possess antipathogenic activities too. Such antipathogenic compounds, in contrast to antibacterial compounds neither kill bacteria nor stop their growth and are assumed to not result in the development of resistant strains (Otto, 2004; Truchado, Galvez, Gil, Barberan & Allende,2009). Instead, these compounds attenuate the expression of genes responsible for pathogenesis by interfering with bacterial communication system. The ability of bacteria to sense and respond to their population density is termed as "cell–to cell communication" or "quorum sensing" (QS), mediated by autoinducer molecules (Fuqua, Winans, & Greenberg, 1994). In Gram negative bacteria the most intensively studied autoinducer molecules are AHLs (Whitehead, Barnard, Slater, Simpson, & Salmond, 2001). These signal molecules are being synthesized by the members of autoinducer synthases (LuxI homologues). The synthesized signaling molecules secreted out of the cell and binds with specific receptor proteins (LuxR homologues) of neighboring bacterial cell wall. The complex of signaling molecules and receptor proteins trigger the expression of specific genes responsible for various phenotypes including violacein pigment in *Chromobacterium violaceum* (CviI/R), virulence factors production in *Pseudomonas aeruginosa* (LasI/R), flagellar motility in *Proteus mirabilis* (RsbA) and *Serratia marcescens* (SwrI/R), bioluminescence in *Vibrio harveyi* (LuxM/N) and development of biofilms in all these organisms (Cha, Gao, Chen, Shaw, & Farrand, 1998; De Kievit, Gillis, Marx, Brown, & Iglewski, 2001; Miller & Bassler, 2001; Schneider, Lockatell, Johnson, & Belas, 2002; Waters & Bassler, 2005). Hence, these bacterial organisms were selected for the present study.

QS mediated biofilm formation is the major cause of bacterial pathogenesis. Biofilms are complex aggregation of microorganisms

* Corresponding author at: Department of Biotechnology, Alagappa University, Karaikudi- 630 003, Tamil Nadu, India. Tel.: +91 4565 225215; fax: +91 4565 225202.
E-mail address: aveeraravi@rediffmail.com (A. Veera Ravi).

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encased in a self secreted exopolymeric matrix consisting of EPS (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995). Centers for Disease Control and Prevention, USA, states that 65% of all infections are caused by 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 antibiotic than their planktonic counterparts (Caraher, Reynolds, Murphy, McClean, & Callaghan, 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, flagellar-driven swimming and swarming motility (Swift et al., 2001; Vu, Miao, Russell, Elena, & Ivanova, 2009), the interference with this phenomenon by means of QSI compounds could be a suitable alternative strategy to reduce or to prevent biofilm based infections.

QSI compounds have been identified from a wide range of natural resources, particularly medicinal plants, edible vegetables and fruits, marine sponges and seaweeds (Adonizio, Downum, Bennett, & Mathee, 2006; Kim, Kim, Seo, & Park, 2007; Skindersoe et al., 2008; Daglia et al., 2010; Musthafa, Ravi, Annapoorani, Packiavathy, & Pandian, 2010) and even bacteria themselves produce QSI substances (Thenmozhi, Nithyanand, Rathna, & Pandian, 2009; Nithyanand, Thenmozhi, Rathna, & Pandian, 2009; Nithya, Aravindraja, & Pandian, 2010; Bakkiyaraj & Pandian, 2010). Not surprisingly, several plants have been shown to control biofouling in situ by interfering with QS mechanisms (Egan, James, Holmström, & Kjelleberg, 2002). Recently, spices such as garlic, ginger and turmeric have been reported for their QSI potential (Vattem et al., 2007). Similarly, the essential oils of cinnamon (Niu, Alfre, & Gilbert, 2006) and clove (Khan, Zahin, Hasan, Husain, & Ahmad, 2009) are also known to possess QSI potentials.

Natural products are promising sources of QSI compounds that can potentially inhibit QS. The presence of such compounds in natural foods is extremely interesting because, in most cases, vegetables and herbs are non-toxic to humans and readily available (Rasmussen & Givskov, 2006). Though extensive work has been done on the antimicrobial properties of vegetables and dietary plants, reports on dietary phytochemicals as sources of QS modulators are scarce. It is also possible that some of the antimicrobial properties of phytochemicals may be attributed to QS inhibition, which may not be related to growth inhibition of the microorganism (Vattem et al., 2007). Considering the multiple therapeutic properties of these 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 and vegetables. Further, in order to find out the compound(s) with QSI potential, the molecular docking analysis was performed with already reported chemical compounds present in the extract which exhibited strong QSI activity. Based on the literature available and ligand binding domain, the LasR receptor protein from *P. aeruginosa* was used as a target site for molecular docking analysis. Subsequently, on the basis of docking score the top ranking compound was selected and examined for its QSI potential in reducing the QS dependent factors production in selected Gram-negative bacterial pathogens.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this study such as *C. violaceum* ATCC 12472 *C. violaceum* CV026, *P. aeruginosa* PAO1, *P. mirabilis* ATCC 7002, clinical isolate *S. marcescens* FJ584421 and *V. harveyi* MTCC 3438 were selected based on the possession of QS dependent phenotypes. All the aforementioned bacterial strains were cultivated in Luria Bertani (LB) medium at 30 °C except *P. aeruginosa* PAO1, which was maintained at 37 °C.

2.2. Extraction

The vegetables, cereals and spices used in this study were purchased from the local outlets and the extractions were made by following the method of Choo, Rukayadi, and Hwang (2006), with little modifications. Powdered samples (5 g) were soaked in 50 ml of methanol for overnight; then the methanol phase was collected and dried at 55 °C, the residues were redissolved with deionized water and stored at −20 °C until further use.

2.3. Determination of Minimum Inhibitory Concentration (MIC)

The MIC for the 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 extracts to attain the final concentrations ranging from 0.1 to 20 mg/ml and incubated at their optimum temperature for 24 h. The MIC was recorded as the lowest concentration, which showed complete inhibition of visible growth. All the further experiments in the present study were performed at sub-MIC concentrations of each extracts.

2.4. Violacein inhibition assay

The methanol extract of test samples was subjected to the qualitative analysis to find out their QSI potentials against *C. violaceum* ATCC 12472. *C. violaceum* synthesizes the violet colored pigment violacein by responding to its QS signal molecule N-hexanoyl-L-homoserine lactone (HHL) produced by the autoinducer synthase CviI. This HHL binds to its receptor CviR and this complex triggers the expression of violacein production (McClean, Pierson, Leland, & Fuqua, 2004; Choo et al., 2006). Overnight culture (10 μl) of *C. violaceum* (adjusted to 0.4 OD at 600 nm) was added into wells of sterile microtiter plates (MTP) containing 1 ml of LB broth and incubated in the presence and absence of various concentrations of test extracts (0.5–2 mg/ml). These MTPs were incubated at 30 °C for 16 h and observed for the reduction in violacein pigment production. Only the extract which exhibited strong QSI activity against *C. violaceum* ATCC 12472 alone was further considered for subsequent experiments in this study. Further, the quantitative analysis was performed in *C. violaceum* CV026. It is a violacein-negative, double mini-Tn5 mutant of *C. violaceum* ATCC 31532 deficient in the autoinducer synthase CviI and, therefore, requires exogenous addition of HHL to induce violacein production (McClean et al., 2004). In quantitative analysis, CV026 was supplemented with 5 μM of HHL (Sigma-Aldrich, Switzerland) and cultivated in the presence and absence of test extract (0.5–2 mg/ml) by following the method of 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.

2.5. Antibacterial assay

The antibacterial activity of the *Cuminum cyminum* extract with QSI potential was performed in Muller–Hinton agar (MHA) (Hi Media, India) by the method specified by the Clinical and Laboratory Standards Institute (2006). Briefly, 100 μl of test bacterial suspensions with the cell density equivalent to 0.5 McFarland standards (approximately 1×10^8 CFU/ml) were uniformly spread over the surface of the MHA plate. Then, the sterile paper disks (Hi Media, India) with a diameter of 10 mm loaded with various concentrations (0.5–2 mg) of *C. cyminum* extracts were placed over the plates and incubated at 30 °C for 24 h and observed for growth inhibition zone.

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