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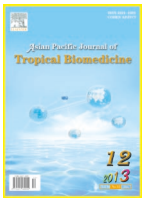

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Inhibition of quorum sensing in *Chromobacterium violaceum* by *Syzygium cumini* L. and *Pimenta dioica* L.

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PEER REVIEW

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Comments

This valuable scientific work has demonstrated the anti-QS activity of two medicinal herbs, *S. cumini* and *P. dioica* using *C. violaceum* in which QS-regulated violacein production was inhibited. The presence of anti-QS sensing compound was confirmed on TLC biosensor overlay.
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ABSTRACT

Objective: To investigated into the anti-quorum sensing (QS) activity of *Syzygium cumini* L. (*S. cumini*) and *Pimenta dioica* L. (*P. dioica*) using *Chromobacterium violaceum* (*C. violaceum*) strains.
Methods: In this study, anti-QS activity of ethanol extract of *Syzygium cumini* L. and *Pimenta dioica* L. were screened using *C. violaceum* CV026 biosensor bioassay. By bioassay guided fractionation of *S. cumini* and *P. dioica*, ethyl acetate fraction (EAF) with strong anti-QS activity was separated. Inhibition of QS regulated violacein production in *C. violaceum* ATCC12472 by EAF was assessed at different concentrations. The effect of EAF on the synthesis of autoinducer like N-acyl homoserine lactone (AHL) was studied in *C. violaceum* ATCC31532 using its mutant *C. violaceum* CV026 by standard methods.
Results: EAF inhibited violacein production in *C. violaceum* ATCC12472 in a concentration dependent manner without significant reduction in bacterial growth. Complete inhibition of violacein production was evidenced in 0.75-1.0 mg/mL concentration of EAF without inhibiting the synthesis of the AHL. TLC biosensor overlay profile of EAF revealed two translucent spots in *S. cumini* and *P. dioica* that inhibited C_6-AHL mediated violacein production in *C. violaceum* CV026.
Conclusions: This study indicates the anti-QS activity of the tested medicinal plants against *C. violaceum*.

KEYWORDS

Anti-quorum sensing, *Chromobacterium*, Medicinal plants, Quorum sensing, Pathogenicity

1. Introduction

Quorum sensing (QS), a population density dependent mechanism present in many bacteria, is mediated through small signal molecules called autoinducers that regulate the target gene expression responsible for the phenotypes essential to pathogenicity/symbiosis[1]. In Gram negative bacteria, QS is mediated through N-acyl homoserine lactones (AHL)[2]. Deeper understanding on QS has led to its role in controlling the production of virulence factors such as exopolysaccharide synthesis, biofilm formation, swarming motility, pigment production and antibiotic production in some pathogenic bacteria[3,4]. Owing to the importance of QS during bacterial pathogenesis, interfering with this mechanism is being considered as a rational strategy to attenuate their virulence[5]. Increasing emergence of antibiotic resistance in Gram negative bacteria also demands alternative strategies to combat bacterial infections and anti-QS approach is being viewed as an attractive alternative. QS inhibitory compounds, unlike conventional antibiotics, do not kill or inhibit microbial growth and are less likely to impose a selective

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pressure for the development of drug resistant bacteria[6].

Plant metabolites having the ability to control the growth of microbes have been traditionally used to treat human diseases including microbial infections[7]. However, the mode of action of many phytocompounds against the target organism is not clearly understood. Recent research has revealed that a few natural products including plant extracts have properties for modulating bacterial QS system, thereby reducing the virulence[8,9]. *Syzygium cumini* (*S. cumini*) and *Pimenta dioica* (*P. dioica*) are widely used medicinal plants having ability to alleviate bacterial infections and are used in traditional healing in different parts of the world. Due to their use in treating microbial infections, it is possible that these plants may possess anti-QS properties.

Chromobacterium violaceum (*C. violaceum*), a Gram negative bacterium having a wide geographic distribution, produces the pigment violacein in response to QS regulated gene expression. Further, the whole genome studies on *C. violaceum* showed that violacein production is regulated by *vioD*, *vioC*, *vioB* and *vioA* genes arranged in an operon through a QS system mediated by AHL[10]. A biosensor strain, *C. violaceum* CV026 (mini-Tn5 mutant of wild type strain), deficient in autoinducer synthase which requires exogenous addition of AHL to produce violacein was developed for studying various QS mechanisms, and it offers a convenient tool for the biological assay of screening QS inhibitors[11]. This study investigated into the anti-QS activity of *S. cumini* and *P. dioica* using *C. violaceum* strains.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

Bacterial strains *C. violaceum* ATCC12472, *C. violaceum* ATCC31532 and *C. violaceum* CV026 were used. All these strains were cultured in Luria-Bertani (LB) medium at 32 °C for 24 h. When required, the medium for *C. violaceum* CV026 was supplemented with autoinducer, C_6-AHL (Sigma). For all the experiments, the inoculum was prepared by growing the bacteria in 10 mL LB broth at 32 °C for 24 h in a shaking incubator (130 r/min).

2.2. Collection of plant materials and extract preparation

Leaves of *S. cumini* and *P. dioica* were collected from Mangalore region, Karnataka (India). The leaves were washed in sterile water, shade dried and pulverized to a fine powder in an analytical mill (IKA, Germany). Plant extracts were prepared by mixing 100 g of powdered leaves in 90% ethanol and kept under agitation at room temperature for 48 h. The extract was filtered through sterile filter paper (Whatman No. 1) and concentrated to complete dryness under a vacuum evaporator.

2.3. Biosensor bioassay for detecting anti-QS activity of the plant extracts

Biosensor bioassay for anti-QS activity was carried out according to the methods described previously[11]. Briefly, plant extracts re-suspended in ethanol were loaded onto 6 mm sterile discs (Himedia, India) at different concentrations (100 μg-3.0 mg/disc). LB agar plates were prepared by supplementing 10 μL of 5 μg/mL of C_6-AHL and inoculated with *C. violaceum* CV026. Discs containing plant extracts were placed on the agar plates and incubated for 24 h at 32 °C in upright position. Discs loaded with solvent (ethanol) were included as vehicle only controls. Inhibition of QS was detected by the presence of a zone of colorless, but viable cells around the discs could clearly be differentiated from the zone of growth inhibition (antibacterial activity).

2.4. Bioassay guided purification of anti-QS compounds

The ethanol extract was subjected to bioassay guided purification of active compounds. In the first step, the ethanol extracts of *S. cumini* and *P. dioica* were defatted with *n*-hexane and treated with water, shaken well to resolve into water soluble and water insoluble parts. The water soluble fraction was repeatedly extracted with ethyl acetate and the final ethyl acetate fraction (EAF) was separated. All the fractions were concentrated and subjected to *C. violaceum* CV026 biosensor bioassay. EAF of *S. cumini* and *P. dioica* showed strong anti-QS activity in bioassay.

2.5. Inhibition of violacein production in *C. violaceum* ATCC12472

Inhibition of violacein production in the presence of plant extracts was tested using *C. violaceum* ATCC12472 and quantified as previously described method with modification[12]. For this, the dried EAF was re-suspended in dimethyl sulfoxide and added to LB broth (10 mL) at concentrations of 0.25-1.0 mg/mL. Solvent controls were prepared similarly and all the tubes were inoculated with 100 μL of *C. violaceum* ATCC12472. The inoculated tubes were incubated at 32 °C for 24 h in a shaking incubator. After incubation, 1 mL culture was centrifuged at 8 000 r/min for 10 min to precipitate the insoluble violacein. The

culture supernatant was discarded and 1 mL of water saturated *n*-butanol was added to the pellet. The solution was vortexed vigorously for 30 seconds to completely solubilize violacein and centrifuged at 8 000 r/min for 10 min to remove the cells. The violacein was quantified spectrophotometrically at OD_{585} (UV-1800, Shimadzu, Japan). To test the effect of plant extracts on bacterial growth, culture grown in the presence of active fraction was serially diluted and plated on LB agar medium. After incubation at 32 °C for 24 h, bacterial number was enumerated using the colony counter.

2.6. Effect on modulation of AHL synthesis and its activity

The effect of plant extracts on AHL synthesis and AHL activity was determined using a C_6-AHL overproducing strain, *C. violaceum* ATCC31532 and its mutant *C. violaceum* CV026[13]. *C. violaceum* ATCC31532 was cultured in the presence of EAF at a concentration of 0.25-1.0 mg/mL under conditions as described earlier and violacein produced was quantified spectrophotometrically after 24 h incubation. Culture medium inoculated with dimethyl sulfoxide was included as solvent control. AHL was extracted from the cell free supernatant (8 mL) using dichloromethane (3:1 v/v) and evaporated under a thin stream of nitrogen gas. For determining the AHL activity, the dried AHL fractions were re-suspended in 70% methanol (20 μL) and added to fresh 10 mL LB medium inoculated with biosensor strain *C. violaceum* CV026 which responded to exogenous AHL by producing violacein. Induction of violacein by the AHL fractions in *C. violaceum* CV026 was measured spectrophotometrically after incubation at 32 °C for 24 h as described earlier.

2.7. Thin layer chromatography (TLC) with biosensor overlay

The active fraction of plant extracts were analyzed by silica gel TLC using biosensor overlay to detect the migration of anti-QS compounds. EAF (10 μL) was spotted on a silica TLC plate and chromatographed using chloroform:methanol (8:2) solvent system. After elution, the plate was dried and overlaid with sterile LB medium containing exogenous C_6-AHL inoculated with an overnight culture of *C. violaceum* CV026 biosensor strain. The TLC overlay was incubated at 32 °C for 24 h and anti-QS activity was detected by the presence of a turbid zone in a purple background.

2.8. Data analysis

All the experiments were conducted in quadruplicates and one way analysis of variance (ANOVA) was used to analyze the differences between the treatments. $P<0.01$ was considered as significant unless specified.

3. Results

Biosensor bioassay of ethanol extracts of *S. cumini* and *P. dioica* showed turbid zone of violacein inhibition around the discs indicating anti-QS activity of plant extracts. A strong anti-QS activity was evident for the tested plant extracts in the concentration of 3 mg/disc. However, a weak QS inhibition zone was observed at concentrations between 0.5-1.0 mg/disc and no activity was found at lower concentrations of plant extracts.

In an effort to identify anti-QS compounds, the crude ethanol extract was subjected to bioassay guided fractionation. Biosensor bioassay of different fractions (hexane, aqueous and EAF) using *C. violaceum* CV026 revealed strong anti-QS activity for the EAF. Hexane and aqueous fractions did not show anti-QS activity against *C. violaceum* CV026. The purified EAF showed strong anti-QS activity at 0.5-1.0 mg/disc concentration with a zone of inhibition of (24.0±1.2) mm.

Violacein production in *C. violaceum* ATCC12472 was inhibited in a concentration dependent manner by EAF of *S. cumini* and *P. dioica* leaves (Figure 1). The purified EAF extracted from *S. cumini* and *P. dioica* showed more than 80% inhibition at 0.5 mg/mL and at 1.0 mg/mL, it showed complete inhibition of violacein production. It is interesting to note that, the growth of *C. violaceum* ATCC12472 in the presence of plant extracts was not inhibited as the cell counts showed no significant difference compared to the control (Figure 1). The violacein production and cell counts in the solvent controls were similar to the control.

Inhibition of AHL activity was seen by the inhibition of violacein production in *C. violaceum* ATCC31532, but not in *C. violaceum* CV026. *S. cumini* and *P. dioica* at concentrations between 0.25-1.0 mg/mL of EAF inhibited the QS mediated violacein production in *C. violaceum* ATCC31532 in a concentration dependent manner (Figure 2). However, AHL extracted from these culture supernatants were able to induce the violacein production in the mutant *C. violaceum* CV026 without any significant difference between the treatments. The solvent did not interfere with either violacein production or AHL synthesis in *C. violaceum* ATCC31532 and was similar to the control. Hence, it was clear that AHL synthesis was not inhibited in *C. violaceum* ATCC31532 by the active fraction, but AHL mediated violacein production was inhibited.

TLC biosensor overlay profile of EAF of *S. cumini* and

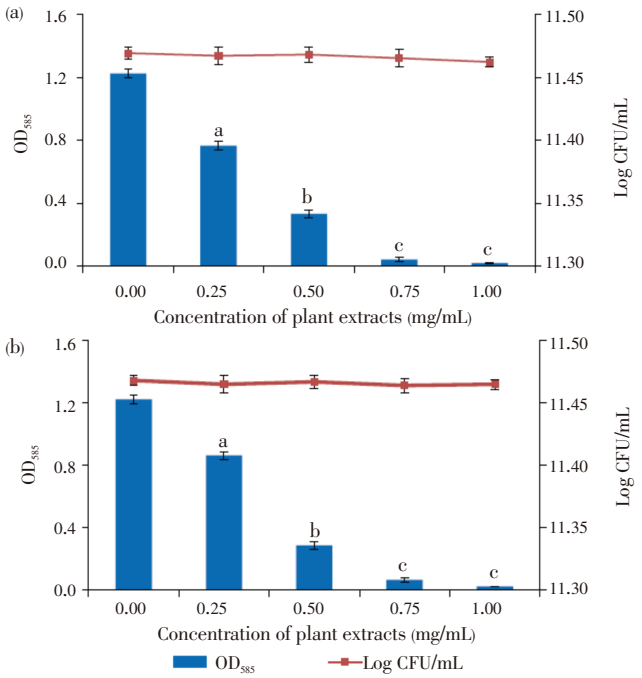

Figure 1. Concentration of violacein produced (OD_{585}) and cell counts (log CFU/mL) in *C. violaceum* ATCC12472 cultures grown in the presence of the active fraction of (a) *P. dioica* and (b) *S. cumini*.
Data points are mean±SD (n=4). $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$.

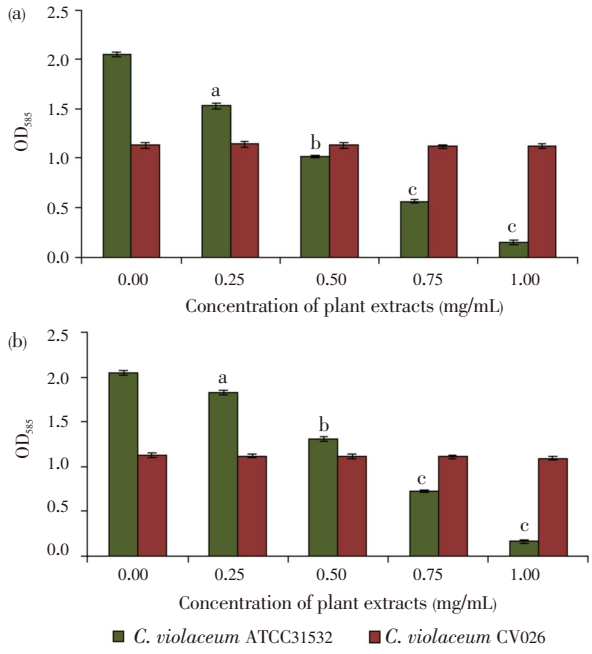

Figure 2. Effect of active fractions of (a) *P. dioica* and (b) *S. cumini* on AHL synthesis in C_6-AHL overproducing strain *C. violaceum* ATCC31532 and its activity in *C. violaceum* CV026.
Data points are mean±SD (n=4). $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$.

P. dioica revealed two spots with anti-QS zone in both *S. cumini* (R_f of 0.16 and 0.87) and *P. dioica* (R_f of 0.16 and 0.81) (Figure 3).

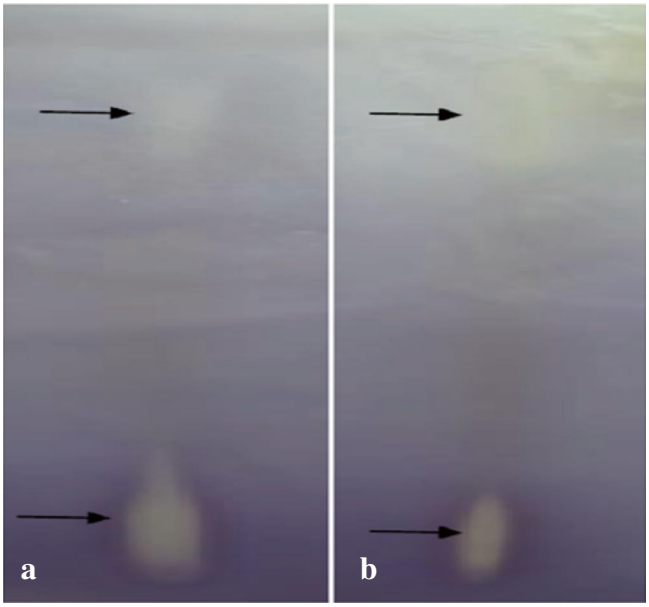

Figure 3. TLC biosensor overlay profile of active fraction of (a) *P. dioica* and (b) *S. cumini*.
Spots with anti-QS zone at different R_f values were indicated by arrows. The experiments were repeated twice.

4. Discussion

The present study demonstrates the anti-QS properties of *S. cumini* and *P. dioica* plants against *C. violaceum* CV026 grown in the presence of C_6-AHL. Inhibition of QS regulated violacein production in *C. violaceum* is commonly used for anti-QS screening studies. The AHL synthesized by different strains of *C. violaceum* vary in their acyl chain length or substitution, and the two wild type strains used in this study though produce two different major AHL molecules[11], the tested plant extracts were able to inhibit the violacein synthesis in both. Other reports have also found natural plant extracts, such as that of *Myristica cinnamomea*, showing anti-QS activity at 3 mg/mL against *C. violaceum* CV026[14].

S. cumini is an ancient medicinal plant and has a long tradition in alternative medicine. All parts of the plant have been used for a wide variety of ailments, including cough, diabetes, dysentery, inflammation and ringworm[15]. *P. dioica*, also known as allspice, is traditionally used as analgesic, antimicrobial, antioxidant, stimulant, carminative and muscle relaxative[16]. Most of the studies on these plants have been conducted using crude preparation of the plant without pointing out their chemical profile.

The mechanism of action of anti-QS compounds on QS system is a complex phenomenon. In this study, all the tested plant extracts inhibited AHL mediated violacein production in *C. violaceum*. However, synthesis of AHL in *C. violaceum* was not inhibited by the plant extracts as AHL extracted from the culture could induce the violacein production in the mutant. The anti-QS compounds are known to include molecules that mimic autoinducer structure and/or function and compounds that are antagonistic to the

autoinducer molecules[17]. In addition, these compounds could potentially target other components of QS system, such as interfering with the stability and function of the regulator protein or autoinducer synthase[18,19].

Qualitative phytochemical screening of anti-QS spot showed positive for flavonoids in *S. cumini* and *P. dioica* showed positive for phenols. The bioactivity of *S. cumini* leaves are mainly attributed to flavonoids such as quercetin, myricetin, myricitin and triterpenoids[15]. *P. dioica* plants are rich in polyphenols such as phenolic acids, flavonoids, catechins, diterpenes and lupeol[16]. Anti-QS activity of some polyphenols (epigallocatechin gallate, ellagic acid, tannic acid), flavonoids (naringin, neohesperidin, quercetin and hesperidin), alkaloids and essential oils (cinnamaldehyde and its derivatives) has earlier been demonstrated against *C. violaceum*[20,21]. Further, studies are needed to identify the major compound responsible for anti-QS activity present in the tested plants.

Efficacy of plant compounds *in vivo* has earlier been described against QS targets in *Pseudomonas aeruginosa* (*P. aeruginosa*) infections using animal model systems. Fresh garlic extract having strong anti-QS activity, injected subcutaneously for 7 d [1.5% of the mass of the mouse (20 g)] promoted rapid clearing of pulmonary *P. aeruginosa* infections[8]. Oral treatment with a fresh garlic extract for 14 d significantly lowered renal tissue destruction by anti-QS activity against *P. aeruginosa* infections in the mouse urinary tract infection model[22]. Similarly, furanone C-30, a synthetic analogue of a natural furanone derived from the red alga, administered subcutaneously for 3 d (0.7 μg/g body weight) to *P. aeruginosa* infected mice reduced the pulmonary infection by targeting the QS in *P. aeruginosa* and promoted their clearance by the mouse immune response[23].

Compounds that can inhibit QS have an enormous scope for developing therapeutics for countering the antibiotic use in some infections. Anti-QS drugs such as anti-QS based antiseptic ointments, drops for ear infections, tablets for stomach ulcers and mouthwash for oral infections have been emerged. Similarly, functional foods for controlling infections in immunocompromized individuals can also be developed from the plant products rich in compounds with anti-QS activity[24].

Due to the clinical, environmental and industrial application of the QS inhibitors, search for potential compounds with anti-QS activity has increased over the last few years[25]. Efforts towards the development of anti-QS compounds should provide a means of treating bacterial infections without the overuse of antibiotics that unavoidably develop resistant organisms. The present study is an effort towards exploring the anti-QS property of some medicinal plants and with further studies, identification of active compounds and understanding the mechanism can be achieved.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

QS is a generic phenomenon in Gram negative bacteria and is linked to virulence. Treatment of infections caused by such bacteria poses a challenge as they are often multidrug resistance. Hence anti-QS compounds seem to hold a promise for development of novel non-antibiotic agents against the pathogens.

Research frontiers

The present research work investigated the anti-QS activity of ethanol extract of *S. cumini* and *P. dioica* against *C. violaceum* ATCC12427. The study has also demonstrated the presence of anti-QS activity in different concentrations.

Related reports

The studies on *S. cumini* and *P. dioica* have been conducted using crude preparation of the plant without pointing out their chemical profile and anti-QS activity of these herbs has not been reported earlier. Other reports have found natural plant extracts of *Myristica cinnamomea*, showing anti-QS activity against *C. violaceum* CV026.

Innovations and breakthroughs

S. cumini is an ancient medicinal plant and all parts of the plant have been used for a wide variety of ailments, including cough, diabetes, dysentery, inflammation and ringworm. *P. dioica*, also known as allspice, is traditionally used as analgesic, antimicrobial, antioxidant, stimulant, carminative and muscle relaxative. This work has validated the traditional use of tested plants for bacterial infections by inhibiting the QS activity in *C. violaceum*.

Applications

Compounds that can inhibit QS have an enormous scope

for developing novel non-antibiotic, anti-pathogenic therapeutic agents, which interfere with bacterial cell to cell communication and render them less virulent and more susceptible to biocide treatment. The active ingredients of these herbs may be utilized to formulate new antiseptic and anti-infective drugs.

Peer review

This valuable scientific work has demonstrated the anti-QS activity of two medicinal herbs, *S. cumini* and *P. dioica* using *C. violaceum* in which QS-regulated violacein production was inhibited. The presence of anti-QS compound was confirmed on TLC biosensor overlay. The purified compound may be explored further to be a potential anti-infective agent for bacterial infections.

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