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ORIGINAL ARTICLE

Anti-quorum sensing activity of flavonoid-rich fraction from *Centella asiatica* L. against *Pseudomonas aeruginosa* PAO1

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KEYWORDS
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Background/Purpose: Inhibition of quorum sensing (QS), a cell-density dependent regulation of gene expression in bacteria by autoinducers is an attractive strategy for the development of antipathogenic agents.
Methods: In this study, the anti-QS activity of the ethanolic extract of the traditional herb *Centella asiatica* was investigated by the biosensor bioassay using *Chromobacterium violaceum* CV026. The effect of ethyl acetate fraction (CEA) from the bioassay-guided fractionation of ethanol extract on QS-regulated violacein production in *C. violaceum* ATCC12472 and pyocyanin production, proteolytic and elastolytic activities, swarming motility, and biofilm formation in *Pseudomonas aeruginosa* PAO1 were evaluated. Possible mechanism of QS-inhibitory action on autoinducer activity was determined by measuring the acyl homoserine lactone using *C. violaceum* ATCC31532. Anti-QS compounds in the CEA fraction were identified using thin layer chromatography biosensor overlay assay.
Results: Ethanol extract of *C. asiatica* showed QS inhibition in *C. violaceum* CV026. Bioassay-guided fractionation of ethanol extract revealed that CEA was four times more active than the ethanol extract. CEA, at 400 μg/mL, completely inhibited violacein production in *C. violaceum* ATCC12472 without significantly affecting growth. CEA also showed inhibition of QS-regulated phenotypes, namely, pyocyanin production, elastolytic and proteolytic activities, swarming motility, and biofilm formation in *P. aeruginosa* PAO1 in a concentration-dependent manner. Thin layer chromatography of CEA with biosensor overlay showed anti-QS spot with an R_f value that corresponded with that of standard kaempferol.
Conclusion: The anti-QS nature of *C. asiatica* herb can be further exploited for the formulation of drugs targeting bacterial infections where pathogenicity is mediated through QS.

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Introduction

Bacterial populations coordinate communal behavior through a process of cell-to-cell signaling mediated by diffusible signal molecules.[1] This process, termed quorum sensing (QS), is known to control gene expression responsible for diverse physiological functions including virulence, antibiotic production, and biofilm formation.[2] Gram-negative bacteria use a QS system mediated by diffusible signaling molecules of the *N*-acyl homoserine lactones (AHL) family.[3] Many pathogenic bacteria use a QS system to regulate genes required for the expression of virulence, thus, inhibition of the QS system is considered as a novel strategy for development of antipathogenic agents, especially for combating bacterial infections caused by antibiotic-resistant strains.[4]

In the past few years, inhibition of QS has become an intense area of research because of its applications in medicine, industry, and biotechnology. In the quest for QS inhibitors, studies have demonstrated that many eukaryotes, particularly plants, and even bacteria themselves produce anti-QS substances.[5,6] Ajoene from garlic, catechin from *Combretum albiflorum*, and iberin from horseradish specifically inhibit QS in reporter strains.[7,8]

As an adaptive evolution, many plant species produce metabolites that can control the growth of microbes and have traditionally been used to treat human diseases, particularly microbial infections. *Centella asiatica* (L.) Urban is used as a medicinal herb in Ayurvedic medicine, traditional African medicine, and traditional Chinese medicine. *C. asiatica* is one of the chief herbs for treating skin problems, healing wounds, as well as being an antibacterial and antiviral agent.[9] The therapeutic substances in *C. asiatica* are saponin-containing triterpene acids and their sugar esters, of which asiatic acid, madecassic acid, and asiaticosides are considered to be the most important.[10] In this study, anti-QS potential of *C. asiatica* was investigated using *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PAO1. In *C. violaceum*, the LuxR homolog, CviR regulates the production of a purple pigment violacein.[11] *P. aeruginosa* PAO1, an opportunistic pathogen, utilizes two interrelated QS systems, LasI/R regulates the production of LasB elastase, LasA protease, Apr alkaline protease, and exotoxin A through 3-oxo-C_{12}-homoserine lactone and RhlI/R which regulates pyocyanin production, rhamnolipids, hydrogen cyanide, and cytotoxic lectins through C_4-homoserine lactone.[3]

Methods

Bacterial strains, media, and culture conditions

Bacterial strains used in this study were *C. violaceum* ATCC12472, *C. violaceum* ATCC31532, a mini-Tn5 mutant *C. violaceum* CV026, and *P. aeruginosa* PAO1. All the bacterial strains were grown in Luria—Bertani (LB) medium at 32°C for 24 hours. When required, the medium for *C. violaceum* CV026 was supplemented with hexanoyl homoserine lactone (C_6-HSL; Sigma—Aldrich, St Louis, MO, USA). For pyocyanin assay, *P. aeruginosa* PAO1 was grown in glycerol alanine minimal medium. Tryptone broth was used for biofilm assays. For all the experiments, inocula were prepared by growing the bacteria in 10 mL LB broth under shaking (130 rpm) for 24 hours, and the cell density was measured spectrophotometrically (UV-1800; Shimadzu, Kyoto, Japan).

Plant materials and extract preparation

Fresh plants of *C. asiatica* were collected from the areas around the paddy farms of Mangalore, Karnataka, India. Samples were authenticated in the Department of Botany, Mangalore University, Mangalore, India. Leaves were washed in sterile water, shade dried, and powdered using an analytical mill (IKA, Staufen, Germany). For the preparation of ethanol extract, 100 g powdered leaves was extracted with 1 L 90% ethanol in a soxhlet extractor at 70°C for 16 hours and concentrated by vacuum evaporation to obtain a viscous residue.

Biosensor bioassay for the detection of anti-QS activity

Anti-QS activity of *Centella asiatica* extract was detected by bioassay using the reporter strain *Chromobacterium violaceum* CV026.[12] Different concentrations of ethanol extract of *Centella asiatica* (100—1000 μg/disc) were loaded onto 6-mm sterile discs (Himedia, Mumbai, India) and placed on the surface of *C. violaceum* CV026-inoculated LB agar plates supplemented with 50 μL 1 μg/mL C_6-HSL and incubated for 24 hours. Discs loaded with ethanol were included as negative controls. Inhibition of QS was detected by the presence of a zone of colorless but viable cells around the disc.

Bioassay guided fractionation

The ethanol extract of *Centella asiatica* was exhaustively treated with *n*-hexane. The hexane layer was recovered and the remaining extract was simultaneously treated with water, mixed well to resolve into water-soluble and water-insoluble parts. The water-soluble fraction was then exhaustively partitioned with ethyl acetate and the final ethyl acetate fraction was separated from the water-soluble fraction. The ethyl acetate fraction was concentrated by vacuum evaporation and the remaining aqueous portion was lyophilized and collected as an aqueous fraction. All the fractions were concentrated and subsequently tested for anti-QS activity by *Chromobacterium violaceum* CV026 biosensor bioassay. Ethyl acetate fraction showing strong anti-QS activity (CEA) was used for further experiments at the concentration of 25—400 μg/mL and its effect on bacterial growth was also tested by standard plate count method.

Quantitative QS inhibition assay

The effect of CEA on the QS-controlled violacein production in *C. violaceum* ATCC12472 was determined as described previously with some modifications.[13] Briefly, 10 mL LB broth containing different concentrations of CEA was inoculated with 100 μL *C. violaceum* ATCC12472 (10^6 CFU/mL). Solvent control was prepared similarly and all the

tubes were incubated for 24 hours in an orbital shaking incubator (130 rpm). Violacein was extracted in water-soluble *n*-butanol from the cells and was quantified spectrophotometrically at optical density $(OD)_{585}$ (UV-1800; Shimadzu). Simultaneously, to test the effect of plant extract on bacterial growth, culture grown in presence of extract was subjected to serial dilution, and viable counts were measured by standard plate count method.

Effect of active fraction CEA on AHL activity in *C. violaceum*

Effect of the active fraction namely, CEA, on AHL activity was determined using an AHL-overproducing strain, *C. violaceum* ATCC31532 and its mutant *C. violaceum* CV026.[14] LB medium (10 mL) supplemented with different concentrations of plant extract was inoculated with *C. violaceum* 31532 (100 μL). After 24 hours incubation, violacein produced was quantified spectrophotometrically and viable counts were measured by standard plate count method. The QS signal molecule AHL was extracted from the culture supernatant (9 mL) using dichloromethane (3:1 v/v). The collected fraction was passed through an anhydrous $MgSO_4$ column and dried under a thin stream of nitrogen gas. AHL fractions were resuspended in 20 μL 70% methanol and added to 10 mL fresh LB medium inoculated with *C. violaceum* CV026. Violacein induction by the exogenous AHL in *C. violaceum* CV026 was measured after incubation for 24 hours spectrophotometrically.

Effect of CEA on production of QS-controlled virulence factors in *P. aeruginosa* PAO1

The effect of CEA on pyocyanin pigment production in *P. aeruginosa* PAO1 was determined as described previously.[15] In brief, *P. aeruginosa* PAO1 was grown in glycerol alanine minimal medium supplemented with different concentrations of CEA and incubated for 24 hours. Pyocyanin was extracted from the cell-free supernatant with chloroform, acidified with 0.2 M HCl, and quantified by recording OD_{520} spectrophotometrically. Swarming assay was performed in LB semisolid (0.5% agar) medium supplemented with CEA. LB agar plates inoculated with *P. aeruginosa* PAO1 were incubated for 24 hours and the extent of swarming was determined by measuring the swarming diameter.[14]

Inhibition of elastolytic and proteolytic activities was assessed as previously described.[16,17] *P. aeruginosa* PAO1 was grown in LB medium supplemented with different concentrations of CEA and incubated for 16 hours. For elastolytic activity, culture supernatant (100 μL) was added to 900 μL elastin congo red (ECR) buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg ECR (Sigma) and incubated for 3 hours at 37°C. Absorbance of the supernatant after removing the insoluble ECR by centrifugation was measured spectrophotometrically at OD_{495}. For proteolytic activity, 100 μL culture supernatant was added to 900 μL ECR buffer containing 3 mg azocasein (Sigma) and incubated for 30 minutes at 37°C. Trichloroacetic acid (10%, 100 μL) was added to each reaction tube. After 30 minutes, the tubes were centrifuged and absorbance of the supernatant was determined by reading OD_{440}.

Biofilm studies were carried out as described previously.[18] Briefly, 50 μL overnight grown *P. aeruginosa* PAO1 culture (10^6 CFU/mL) was diluted to 3 mL with fresh tryptone broth containing CEA and incubated statically for 18 hours. The biofilm formed was assessed by crystal violet staining method by recording OD_{590} spectrophotometrically.

Thin layer chromatography overlay assay and phytochemical screening

The active fraction (CEA) showing anti-QS activity was tested for major phytochemicals and also spotted on silica gel thin layer chromatography (TLC). Chromatograms were developed using chloroform:ethyl acetate (8:2) and after drying, the TLC plate was overlaid with LB semisolid medium seeded with *C. violaceum* CV026 and C_6-HSL. After incubation for 24 hours, the anti-QS activity was spotted by the absence of purple pigment, and R_f value was recorded. Quercetin, kaempferol, and asiatic acid (Sigma) were used as reference compounds.

Results

Inhibition of QS by Centella asiatica extracts

Ethanol extract of *Centella asiatica* (CE) showed anti-QS activity in *Chromobacterium violaceum* CV026 biosensor bioassay. At the concentration of 80 μg/disc, CE showed a pigmentless zone (20 mm) indicative of violacein inhibition around the discs. Bacteria in this zone were alive, but lost their QS ability. CE did not show any clear zone of antibacterial activity even at 1000 μg/disc. At 50 μg/disc, it showed < 10 mm anti-QS zone and <100 μg/disc and did not show violacein inhibition. Bioassay-guided fractionation of CE resulted in a CEA fraction with strong anti-QS activity, with a turbid halo zone of 22 mm diameter around the discs at 20 μg/disc (Fig. 1). However, hexane and aqueous fractions did not show any anti-QS activity. CEA showed no significant effect on bacterial growth up to 2 mg/mL (Table 1).

Inhibition of QS-regulated violacein production in C. violaceum ATCC12472

Inhibition of QS-regulated production of violacein pigment in *C. violaceum* ATCC12472 was observed at concentrations between 100 μg/mL and 400 μg/mL of CEA in a concentration-dependent manner (Fig. 2). At 100 μg/mL, > 50% inhibition of violacein production was observed and complete inhibition of violacein production was observed at 300 μg/mL CEA. However, CE did not inhibit the growth of *C. violaceum* at concentrations up to 4 mg/mL. The CEA fraction was four times more effective than the crude extract CE.

Effect of CEA on AHL activity in *C. violaceum*

The effect of CEA on AHL synthesis or activity was determined by using C_6-HSL-overproducing wild-type strain *C.*

Figure 1. Biosensor bioassay of CEA showing inhibition of C_6-HSL-mediated violacein production in bioreporter *Chromobacterium violaceum* CV026. C_6-HSL = hexanoyl homoserine lactone; CEA = active fraction (CEA) of *Centella asiatica*.

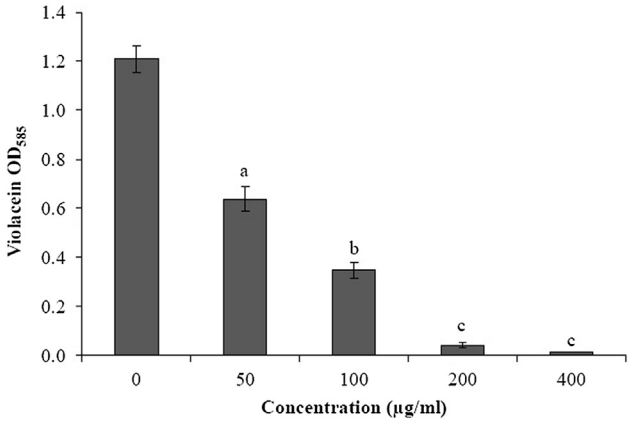

Figure 2. Inhibition of violacein production in *Chromobacterium violaceum* ATCC12472 by different concentrations of CEA. Data are presented as mean ± standard deviation, $n = 4$. Same letters in the columns are not significantly different ($p < 0.05$). CEA = active fraction of *Centella asiatica*.

violaceum 31532 and its mutant *C. violaceum* CV026, which responds to exogenous AHL. At 100—400 μg/mL concentrations, CEA was able to inhibit AHL-mediated violacein production in *C. violaceum* ATCC31532 significantly in a concentration-dependent manner, but the induction of violacein production in the mutant by AHL extracted from the culture supernatant of *C. violaceum* ATCC31532 treated with CEA was not significantly different from that of the control (Fig. 3).

Effect of CEA on pyocyanin production in *P. aeruginosa* PAO1

Different concentrations of CEA were tested on pyocyanin production in *P. aeruginosa* PAO1. As shown in Fig. 4A, concentrations of 100—400 μg/mL had a significant negative impact on pyocyanin production without affecting *P. aeruginosa* PAO1 growth. At 300 μg/mL, there was a 75% decrease in pyocyanin production and at 400 μg/mL, almost complete inhibition of pyocyanin production was observed.

Effect of CEA on elastolytic and proteolytic activity in *P. aeruginosa* PAO1

The CEA fraction inhibited elastolytic and proteolytic activities significantly at 100—400 μg/mL concentrations (Fig. 4B). At 200 μg/mL, > 50% inhibition of proteolytic and elastolytic activities was observed, and almost complete inhibition of proteolytic and elastolytic activities was evident at 400 μg/mL CEA.

Inhibition of swarming motility in *P. aeruginosa* PAO1

Complete inhibition of swarming motility in *P. aeruginosa* PAO1 was observed at concentrations as low as 50 μg/mL CEA (Fig. 5). *P. aeruginosa* PAO1 exhibited swarming motility on LB agar plates at the point of inoculation with a total swarming diameter of 55 mm. In the presence of CEA,

Table 1 Effect of CEA on viability of bacterial cells in culture medium, as estimated by the standard plate count method after 24 hours of incubation

Concentration of CEA (μg/mL)	*Chromobacterium violaceum* 12472	*Chromobacterium violaceum* 31532	*Pseudomonas aeruginosa* PAO1
	10^8 CFU/mL		
Control	2.48 ± 0.02	2.15 ± 0.03	2.33 ± 0.04
Solvent control	2.28 ± 0.03	2.29 ± 0.02	2.37 ± 0.05
50	2.45 ± 0.03	2.38 ± 0.05	2.10 ± 0.02
100	2.23 ± 0.02	2.17 ± 0.02	2.25 ± 0.04
200	2.34 ± 0.02	2.09 ± 0.03	2.32 ± 0.02
400	2.02 ± 0.03	2.15 ± 0.01	2.12 ± 0.01

Data are presented as mean ± standard deviation; $p < 0.05$, $n = 4$.
CEA = active fraction of *Centella asiatica*.

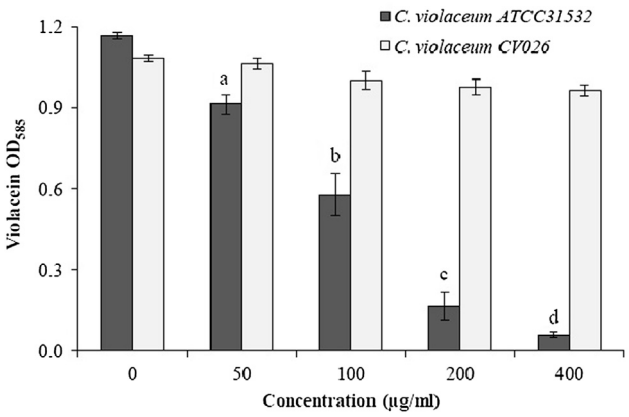

Figure 3. Effect of CEA on AHL activity of *Chromobacterium violaceum* ATCC31532. CEA inhibited violacein production in *C. violaceum* ATCC31532 in a concentration-dependent manner. However, AHL extracted from the culture supernatants induced violacein production in mutant *C. violaceum* CV026. Data are presented as mean ± standard deviation, $n = 4$. Same letters in the columns are not significantly different ($p < 0.05$). AHL = *N*-acyl homoserine lactone; CEA = active fraction of *Centella asiatica*.

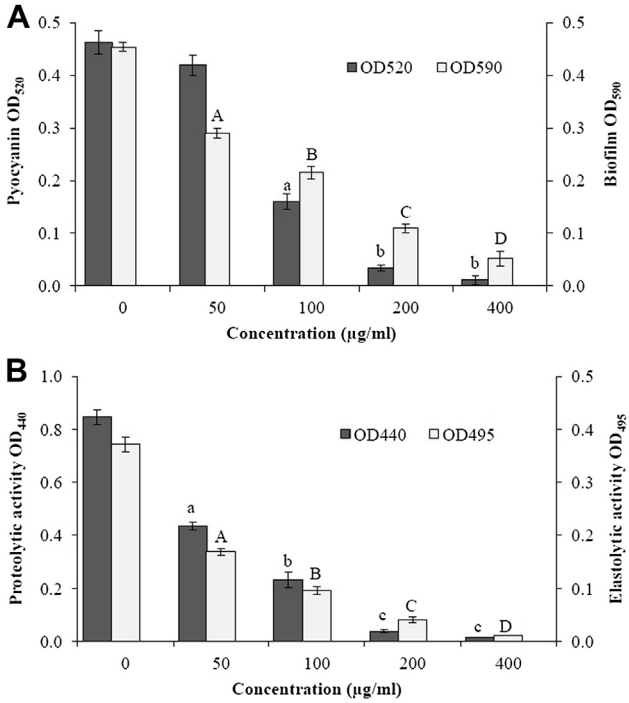

Figure 4. Effect of CEA on QS-regulated virulence factor production in *Pseudomonas aeruginosa* PAO1. (A) Effect of CEA on pyocyanin production and biofilm formation. (B) Effect of CEA on proteolytic and elastolytic activities. Data are presented as mean ± standard deviation, $n = 4$. Same letters in the columns are not significantly different ($p < 0.05$). CEA = active fraction of *Centella asiatica*; QS = quorum sensing.

the bacteria were able to grow and form a colony in the center with a diameter not exceeding 12 mm, and tendril formation or other features indicative of swarming motility were not observed.

Effect of CEA on biofilm formation in *P. aeruginosa* PAO1

P. aeruginosa PAO1 can switch to a biofilm mode of growth. Biofilm formation is partially controlled by QS mechanisms, therefore, the effect of CEA on biofilm formation in *P. aeruginosa* PAO1 was assessed after 18 hours growth and interestingly, at 400 µg/mL CEA, the biofilm formation was decreased by > 80% (Fig. 4A).

Identification of anti-QS compounds by phytochemical screening

Phytochemical screening showed that CEA contained abundant levels of flavonoids and TLC biosensor overlay with *Chromobacterium violaceum* CV026 showed two spots with anti-QS activity at different R_f values (0.81 and 0.59; Fig. 6). The major constituents of *Centella asiatica*, that is, quercetin, kaempferol, and asiatic acid, were used as reference compounds and the R_f value 0.59 of CEA corresponded with that of standard kaempferol.

Discussion

This study reports that *C. asiatica* contains constituents that can disrupt AHL mediated QS-controlled systems in *C. violaceum* and *P. aeruginosa* PAO1. Compounds that interfere with QS have been documented from natural sources such as bacteria, fungi, algae, and higher plants, as well as synthetic compounds with some structures resembling AHL signal molecules.[18] Some plant extracts that have antibacterial activity with higher minimum inhibitory concentration values have demonstrated anti-QS activity at lower concentrations, for example, garlic, honey, and *Terminalia catappa* extracts.[19] In the present study, the growth rate of *C. violaceum* was not affected at the tested concentrations of ethyl acetate extract of *C. asiatica*. The effective concentration of CEA for 50% reduction in the QS activity was approximately 100 µg/mL, which can be designated as the minimum QS inhibitory concentration against *C. violaceum*.

P. aeruginosa PAO1 has known QS systems, LasI/R and RhlI/R, to control several genes involved in biofilm formation and virulence factor production such as, LasB elastase, LasA protease, pyocyanin, rhamnolipids, and exotoxins.[20] Pyocyanin, a phenazine toxic metabolite produced by *P. aeruginosa*, is one of the major virulence determinants of *P. aeruginosa* PAO1.[21] In the present study, CEA decreased PAO1 pyocyanin production by 80% when administered at 300 µg/mL concentration. By comparison, malabaricone C from *Myristica cinnamomea* inhibited 50% pyocyanin production at 1 mg/mL.[22] Over 75% of clinical isolates of *P. aeruginosa* secrete elastase B (LasB), an elastolytic metalloproteinase that is encoded by the *lasB* gene. *In vitro* studies have demonstrated that LasB degrades several

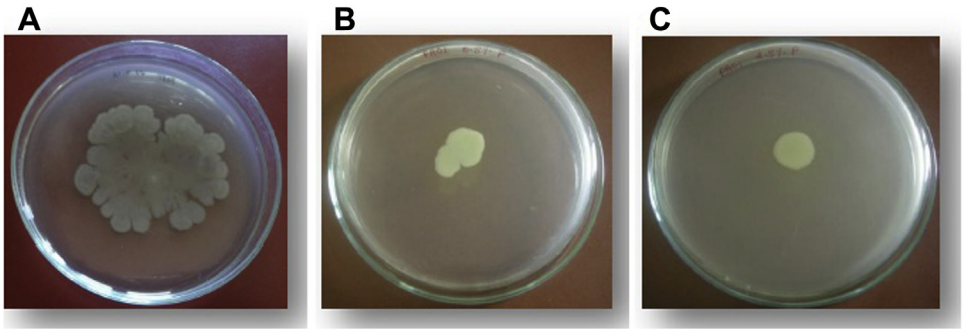

Figure 5. Inhibition of swarming motility in *Pseudomonas aeruginosa* PAO1 by different concentrations of CEA. (A) Control; (B) 50 μg/mL CEA; and (C) 100 μg/mL CEA. CEA = active fraction of *Centella asiatica*.

components in both the innate and adaptive immune systems.[23] The contribution of *P. aeruginosa* proteases to the pathogenesis of acute infections is also well documented. In this study, the tested CEA fraction of *C. asiatica* showed inhibition of some of these functions, including biofilm formation at 100—400 μg/mL, indicating the property of CEA as an anti-QS agent.

C. asiatica is a well-studied herb with uses in traditional and alternative medicine due to a wide spectrum of pharmacological activities associated with the compounds present in this plant.[24] The phytochemical screening of active fraction, CEA, revealed that *C. asiatica* contains an abundant level of flavonoids. The flavonoids chiefly present in *C. asiatica* are kaempferol, quercetin, apigenin, rutin, and naringin.[25] In TLC, CEA showed an R_f value similar to that of kaempferol and hence the anti-QS activity might have contributed to the presence of kaempferol. However, synergistic activity by the other flavonoids can also be expected. Quercetin and kaempferol showed anti-QS activity against *C. violaceum* and *P. aeruginosa* PAO1 at 100 μg/mL in our previous studies; however, the major constituent like triterpenes, namely, asiatic acid, did not show anti-QS activity.

Figure 6. Thin layer chromatography overlay assay of CEA, illustrating inhibition of C_6-HSL-mediated violacein production in *Chromobacterium violaceum* CV026. Areas of pigment clearing (indicated by arrow) show the region of CEA compounds that inhibit AHL-regulated violacein production in *Chromobacterium violaceum* CV026. AHL = *N*-acyl homoserine lactone; C_6-HSL = hexanoyl homoserine lactone; CEA = active fraction (CEA) of *Centella asiatica*.

Some known mechanisms of QS inhibition include competitive binding of signal-like molecules to cognate receptors, as in the case of furanones,[26] and enzymatic degradation of QS signals, as in the case of AHL acylases.[27] In bioassays involving *C. violaceum* CV026, CEA is unlikely to interfere with the synthesis of AHL because it has a defective *luxI* synthase gene. AHL synthesis was not inhibited by CEA in *C. violaceum* ATCC31532, but AHL-mediated violacein production was inhibited, indicating that CEA might have modulated the interaction of AHL with the CviR receptor.

Some QS inhibitors exhibit specificity towards the LuxIR QS system compared to the LasIR and RhlIR QS systems operating in *P. aeruginosa*, for example, vanilla, blueberry, basil, turmeric, and strawberry extracts show anti-QS activity against *C. violaceum* but not against *P. aeruginosa* and *Escherichia coli*.[8,13,14] Production of multiple AHL molecules is reported within the strains of the same species as well in different species. *C. violaceum* ATCC12472, produces 3-hydroxy-C_{10}-HSL, whereas, *C. violaceum* ATCC31532 produces C_6-HSL as the major AHL.[11] *P. aeruginosa* PAO1 uses two interrelated QS systems mediated through C_4-HSL and 3-oxo-C_{12}-HSL to control the production of virulence factors, and *Serratia marcescens* also produces multiple AHLs.[28] Compounds such as alkylamine-modified cyclodextrins show anti-QS activity against *C. violaceum*, *P. aeruginosa*, and *S. marcescens*.[29] Similarly, *N*-acyl cyclopentylamides (C_{10}), intervened with the *las* and *rhl* systems in *P. aeruginosa* by inhibiting interaction between their response regulators and autoinducers.[30] In the present study, CEA showed anti-QS activity against *C. violaceum* and *P. aeruginosa* PAO1, indicating probable broad spectrum activity. Further research is warranted to understand the exact

mechanism of action by the active phytocompounds present in *C. asiatica*.

QS inhibitors have widespread application in diverse fields. QS inhibitor compounds could work synergistically with antibiotics for enhancement of treating QS-dependent infections.[31] One of the most recommended approaches in inhibition of biofouling (marine biofouling and membrane biofouling) is via anti-biofouling compounds, where QS inhibitors inhibit the QS mechanism of bacteria, thereby causing low bacterial attachment or recruitment and inhibiting the formation of biofilm.[32] As an alternative to antibiotics that can lead to the prevalence of antibiotic-resistant strains, QS inhibitors have been used in aquaculture to eliminate pathogens and in agriculture as biocontrol agents against phytopathogens.[18] In conclusion, considering the vast applications of anti-QS agents, the present study highlights the anti-QS property of *Centella asiatica* as well as its possible mechanism of inhibition.

Conflict of interest

No conflict of interest disclosed.

Acknowledgments

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