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A medicinal herb *Cassia alata* attenuates quorum sensing in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*

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Running head: *Casia alata* attenuates QS in *C. violaceum* and *P. aeruginosa*

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Significance and impact of the study

Present study demonstrates the quorum sensing inhibitory activity of metabolites from *Cassia alata*, important medicinal herb a commonly used worldwide in the treatment of infections caused by microorganisms. An extract prepared from the leaves of the plant showed activity

against quorum sensing in *Chromobacterium violaceum* and was also effective against attenuating the quorum sensing controlled virulence factors in *Pseudomonas aeruginosa*. Activity is attributed to the rich flavanoid composition of the plant. Results of the present investigation throw an insight into the possibility of developing drug formulations using the isolated compounds against infections caused by quorum sensing-mediated pathogenicity of bacteria.

Abstract

Quorum sensing (QS) has been shown to play a crucial role in the pathogenesis in many bacteria and attenuation of QS is one of the targets of antimicrobial therapy with particular interest in combating drug resistance. This study reports the QS inhibitory activity of metabolites from *Cassia alata* L. (*Ca. alata*), an important medicinal herb widely used in the treatment of microbial infections. For investigating the QS inhibition (QSI) potential of *Ca. alata* L., initially, metabolites of the leaves extracted using ethanol were tested against biosensor strain *Chromobacterium violaceum* CV026 and *C. violaceum* wild type strains. Further, a purified fraction rich in flavonoids (F-AF) was used for establishing QSI activity by studying the inhibition of violacein production in *C. violaceum*, and QS controlled virulence and biofilm formation in *P. aeruginosa* PAO1. The study results showed 50% inhibition of violacein production in *C. violaceum* at 0.05 mg mL^{-1} concentration of F-AF. In *P. aeruginosa* PAO1, it inhibited the tested virulence factors and biofilm formation significantly. The F-AF contained major flavanoids namely, quercetin, quercetrin and kaempferol displaying QSI activity individually against the test organisms.

Keywords

Quorum sensing inhibition, Pseudomonas aeruginosa, virulence, biofilm, plant metabolite

Introduction

Quorum sensing (QS) has attracted significant research attention due to its role in controlling virulence factors in pathogenic bacteria and this has led to investigations on the development of new class of antibiotics to target the expression of virulence factors leading to a new paradigm of antimicrobial therapy (Clatworthy *et al.* 2007;Chang *et al.* 2014). The N-acyl homoserine lactone (AHL)-based QS has been well studied due to its important role in the regulation of proteobacterial virulence determinants (Ng *et al.* 2009). Opportunistic human pathogens such as *Pseudomonas aeruginosa, Chromobacterium violaceum, Serratia marcescens, Yersinia pseudotuberculosis* and *Aeromonas hydrophila* use AHL mediated QS systems to regulate some key physiological processes (Camara *et al.* 2002).

P. aeruginosa is an important nosocomial pathogen, affecting patients with cystic fibrosis and other lung diseases, traumatized cornea, burns, long-term intubated patients, immune-compromised and elderly individuals (Lee and Zhang 2015). It has been well established by several research studies that in *P. aeruginosa,* QS plays a significant role in the pathogenesis. Further, co-ordination of biofilm mode of growth has been strongly associated with QS in this bacterium. *P. aeruginosa* possesses four QS systems: two LuxI/LuxR-type QS systems that function in series to control expression of virulence factors as well as a third, non LuxI/LuxR-type system called the Pseudomonas quinolone signal (PQS) system (Siehnel *et al.* 2010). Recently, the fourth QS system called the integrated QS system (IQS) was identified having control on PQS and LuxI/R signals in coordinating the expression of pyocyanin, rhamnolipids and elastase (Lee *et al.* 2013).

Inhibition of QS is an important therapeutic target to attenuate bacterial virulence and control infection by interrupting the signal molecules interaction with LuxR homologous protein (Dong *et al.* 2007). Among the natural sources of anti QS compounds, plants are one of the potential sources of novel metabolites to target QS activity (Park *et al.* 2008; Vasavi *et al.* 2015). *Cassia alata* L. (*Ca. alata*) (Fam: Caesalpiniaceae) (synonym *Senna alata* (L.) Roxb) commonly known as guajava is a shrubby leguminous plant, widely distributed having high medicinal value in traditional practice (Khan *et al.* 2001; Reezal *et al.* 2002). Among the different plant parts, the leaves have higher use in traditional medicine compared to other parts. Existing reports suggest its potential activity against fungi however, its activity against bacteria was ambiguous with varying MIC values (Somchit *et al.* 2003; Adedayo *et al.* 2001; Ibrahim and Osman 1995; El-Mahmood and Doughari 2008). In this study anti-QS activity of *Ca. alata* was demonstrated for the first time using *C. violaceum* and *P. aeruginosa* test systems.

Results and discussion

Effect of *Ca. alata* extracts on violacein production in *C. violaceum*

Preliminary screening of the *Ca. alata* extracts prepared in different solvents, showed activity in ethanol, methanol and ethyl acetate extracts while, extracts in hexane and chloroform did not show activity against QS. Among the extracts showing activity, the ethanol extract (0.25 mg per disc) showed the highest zone of violacein inhibition in CV026 reporter plate assay (Fig. 1A). In subsequent experiments, inhibition zone was also observed in the wild type strain *C. violaceum* ATCC12472 (Fig 1B). Bioassay guided purification of the ethanol extract resulted in a flavanoid containing ethyl acetate fraction (F-AF) with violacein inhibition zone of 24 mm at 0.10 mg per disc concentration against *C. violaceum* ATCC12472 (Fig. 1C).

Quantification of the QS inhibitory activity of F-AF using *C. violaceum* ATCC12472 showed a concentration-dependent increase in the activity with 50% inhibition at 0.05 mg mL^{-1} concentration (Fig.1D). However, the cell viability at the highest tested concentration was similar to the control without significant decrease (Fig. 1E).

The QS inhibitory activity of *Ca. alata* metabolites has not been reported earlier; however, previous research on methanol extract of *Ca. alata* leaves against *P. aeruginosa* and *E. coli* revealed the bactericidal activity at concentrations of 200 and 150 mg mL^{-1} (El-Mahmood and Doughari 2008). This clearly indicates that *Ca. alata* contain multiple metabolites having anti-infective properties against certain gram negative bacteria. Metabolites from medicinal plants have potential anti-QS activity and bactericidal activity (Bacha *et al.* 2016). Differences in the activity of different plant extracts are mainly due to the presence of major plant metabolites responsible for QSI activity and in cases presence of multiple metabolites also contribute to the same activity synergistically.

Attenuation of QS controlled virulence in *P. aeruginosa* PAO1 by *Ca. alata* metabolites

The F-AF was tested for against growth and major QS controlled virulence factors such as swarming motility, pyocyanin production, elastolytic and proteolytic activities in *P. aeruginosa* PAO1. Growth details of *P. aeruginosa* in the broth culture supplemented with F-AF is shown Fig. 2A. Swarming motility in agar medium containing F-AF was significantly reduced (5-12 mm) compared to untreated control (64 mm). At these concentrations the bacteria were able to form colony but did not show swarming motility (Fig. 2B & C). Similarly, pyocyanin production was significantly attenuated by F-AF at the concentrations (Fig. 3A). Pyocyanin production plays an important role in the pathogenicity of *P. aeruginosa* by interfering with a number of cellular functions mainly due to redox active

properties of pyocyanin (Ran *et al.* 2003). Production of pyocyanin is a multi step process initiating with synthesis of primary QS molecule followed by a series of QS controlled expression of *phzA-G* operons (Mavrodi *et al.* 2001). It also has an indirect role in the biofilm formation (Das *et al*. 2015).

Other virulence factors namely, elastase and protease were attenuated by more than 90% at 0.2 mg mL^{-1} concentration F-AF (Fig. 3B & C). Elastase and protease synthesis are interactively mediated through LasR-LasI and RhlR-RhlI regulated QS circuits in *P. aeruginosa* PAO1 (Brint and Ohman 1995). These degradative enzymes have significant role in the invasiveness of the pathogen and hence become important targets for studies on anti-QS compounds. Significant reduction in these enzymes by F-AF clearly demonstrates its QSI activity against *P. aeruginosa* PAO1.

Biofilm formation, one of the hallmark traits of *P. aeruginosa*, is associated with AHL mediated QS. Many compounds with QS inhibitory activity have the ability to interfere with biofilm formation. In this study significant reduction in biofilm formation was achieved in presence of F-AF at 0.4 mg mL^{-1} concentration (Fig. 3D). Biofilm has clinical significance as it plays a key role in the ability of organisms to resist antibiotics and persist in long term infections such as cystic fibrosis and indwelling medical devices (Singh *et al.* 2000; Donlan 2001; Popat *et al.* 2012). Molecules with QS inhibitory activity are of special interest against *P. aeruginosa* due to the burgeoning antibiotic resistance and unfavorable outcomes of inflammatory injury after the infection (Luo *et al.* 2016).

Mode of action of F-AF on AHL activity in *C. violaceum*

In *C. violaceum* ATCC31532 (major autoinducer is C_6-HSL), pigment violacein was inhibited significantly in presence of F-AF at the tested concentrations (0.1, 0.2 and 0.4 mg mL^{-1}), however, the AHL extracted from these cultures could induce the synthesis of

violacein in the mutant *C. violaceum* CV026 (Fig. 4). The violacein extracted from *C. violaceum* CV026 grown in presence of AHL isolated from the wild strain *C. violaceum* ATCC31532 treated with F-AF were not significantly different ($p< 0.01$) from control. This observation indicates the production of AHL (autoinducer) by the bacteria in presence of the QS inhibitor (F-AF). In QS mechanism, at an adequate population density the accumulated autoinducers bind to specific regulators and trigger the expression of target genes. Inhibition of QS is generally through either degradation of signal molecule (e.g., AHL) or by the production of mimicking compounds (Tay and Yew 2013). In this study, F-AF did not degrade the AHL, as the AHL isolated from the cultures of *C. violaceum* ATCC31532 grown with F-AF could induce violacein production in the mutant CV026. Alternatively, the compounds in F-AF can block the binding of AHL to its receptor thereby inhibiting the expression of downstream signaling. Addition of exogenous AHL can restore the pigment production under circumstances where the inhibitor has blocked the AHL synthesis or degraded the AHL.

Phytocompounds in F-AF and their QSI activity

Thin layer chromatography (TLC) of F-AF showed a single spot (R_f 0.59) with a clear QSI activity in TLC-overlay with *C. violaceum* ATCC12472. In HPLC, this spot showed four peaks with retention times at 3.82, 5.92, 10.41 and 27.95 min that corresponded in MS with *m/z* 317, 447, 285 and 301 respectively (Fig. S1). These compounds were identified based on these *m/z* values as myricetrin (*m/z* 317), quercetrin (*m/z* 447), kaempferol (*m/z* 285), and quercetin (*m/z* 301). *Ca. alata* leaves are a rich source of flavanoids and these include compounds such as kaempferol, quercetin and their glycosides having diverse biological activities (Saito *et al*. 2012). Among these, quercetin, and kaempferol have been reported for the QS inhibitory activity against *C. violaceum* ATCC12472 (Vasavi *et al*. 2014). However, myricetrin did not show activity in the biosensor agar plate assay. Further, we tested these

compounds against *P. aeruginosa* PAO1, wherein, quercetin, kaempferol and quercetrin induced significant reduction in pyocyanin production, elastolytic and proteolytic activities in a dose dependent manner (Fig. 5). Quercetin at 0.025 mg mL^{-1} concentration inhibited all the tested activities in *P. aeruginosa* PAO1 by 50%, while, kaempferol showed lower activity with 50% inhibition at 0.05 mg mL^{-1}. Quercetrin showed comparatively lower activity than quercetin and kaempferol.

Flavanoids are important class of plant metabolites with proven benefits on health, initially attributed to its radical scavenging activities (Nijveldt *et al.* 2001). Kaempferol, quercetin and quercetrin also have multifunctional activities that are increasingly being explored for the use as important therapeutics (Choiprasert *et al.* 2010). Also, these compounds have immune-modulatory properties particularly of special interest for *P. aeruginosa* infection due to the inflammatory response it elicits. The F-AF contained multiple active metabolites exerting synergistic activity against QS and this synergism offers advantages on the use of plants as phyto-therapeutics without elaborate downstream processing. Translation of these research efforts for developing effective and safe molecules to target QS will offer several advantages for combating antibiotic resistance in *P. aeruginosa*, a pathogen of serious concern.

Material and Methods

Bacterial strains and culture conditions

For evaluating QS inhibitory activity, bacterial strains namely, *C. violaceum* ATCC12472, *C. violaceum* CV026 (a mini-Tn5 mutant), *C. violaceum* ATCC31532 and *P. aeruginosa* PAO1 were used.

Preparation of *Ca. alata* extract

Fresh leaves of *Ca. alata* plant authenticated by a taxonomist were collected from areas around Yenepoya University campus and were washed with MilliQ water (Direct Q5, Millipore, India), air dried and finely powdered in an analytical mill (IKA, Germany). 10 g of sample was extracted with different solvents of different polarity: hexane, chloroform, ethyl acetate, ethanol and methanol for 16 h. Based on the activity further extractions were made in the most suitable solvent. For this, ethanol extract of the dried powder (100 g) was prepared by soxhlet extraction (16 h). Concentrated extract (yield 21.6 g) was subsequently subjected to bioassay guided fractionation using n-hexane and water (3:1 v/v), and ethyl acetate. The fraction showing activity was labeled as F-AF, concentrated and used for quantitative anti-QS assays.

AHL reporter plate bioassay for the detection of QS inhibition

All the extracts prepared were used for screening anti-QS activity using *C. violaceum* CV026 reporter plate bioassay as previously described (McClean *et al*. 2007). Sterile discs (Himedia, India) loaded with extracts were placed on the LB agar plates (supplemented with 50 µl of 1 µg mL^{-1} C_6-HSL) seeded with *C. violaceum* CV026 and incubated for 24 h. Zone of violacein inhibition around the discs was used to measure the QS inhibitory activity. Subsequently, the solvent extracts showing activity were tested against *C. violaceum* ATCC12472.

Quantitative assay for violacein inhibition and auto inducer activity

QS inhibitory activity of the F-AF was quantified using *C. violaceum* ATCC12472 according to Choo *et al*. (2006) at concentrations ranging between 0.05 and 0.40 mg mL^{-1}. LB broth (10 mL) inoculated with 100 µl (10^6 CFU mL^{-1}) of *C. violaceum* was incubated under shaking (130 rpm) for 24 h and violacein was extracted in water saturated n-butanol (1 mL) from the cells separated by centrifugation (8000 rpm, 10 min). Intensity of violacein was quantified as

OD_{585} spectrophotometrically. Controls without extract and with/without solvent were used for comparison. Viability of bacteria in the experimental cultures was measured by reading OD_{600} spectrophotometrically.

To test whether synthesis of AHL was inhibited by F-AF, *C. violaceum* ATCC31532 was cultured for 24 h in LB broth supplemented with 0.1, 0.2 and 0.4 mg mL^{-1} F-AF. Intensity of violacein produced was quantified as described earlier. Simultaneously, AHL from the spent culture supernatants was collected and concentrated (Shaw *et al.* 1997). It was re-suspended in 20 µl methanol (70%) and added to LB broth (10 mL) inoculated with *C. violaceum* CV026 (10^6 CFU mL^{-1}). After incubation for 24 h, induction of violacein in *C. violaceum* CV026 was quantified as described above. Suitable controls were included for comparison.

Activity against QS controlled virulence factors in *P. aeruginosa* PAO1

To study the effect of F-AF on the growth, *P. aeruginosa* was inoculated to LB broth containing different concentrations of F-AF and incubated at 37 ºC for 24 h. Following the incubation, OD_{600} values were measured using a spectrophotometer. Effect of F-AF on swarming motility was assayed in LB semisolid agar plates (0.5% agar, w/v). For this, agar plates were point inoculated with *P. aeruginosa* PAO1, incubated for 24 at 37 ºC and diameter of motility swarms was compared with respect to control to establish the inhibition of swarming (Ren *et al.* 2001). Pyocyanin production was measured by growing *P. aeruginosa* PAO1 in glycerol alanine minimal medium (1% v/v glycerol, 6 g L-alanine, 2 g $MgSO_4$, 0.1 g K_2HPO_4, 0.018 g $FeSO_4$ in 1000 mL) in presence F-AF for 24 h at 37 ^oC. Pyocyanin was extracted from cell-free culture supernatants with chloroform, acidified with 0.2 M HCl and quantified spectrophotometrically as OD_{520} (Essar *et al.* 1990). For the assessment of LasB elastase and proteolytic activities, *P. aeruginosa* PAO1 was grown in LB medium in presence F-AF and incubated for 16 h. An aliquot of culture supernatant (100 µl) was added to 900 µl of elastin congo red (ECR) buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5)

containing 20 mg ECR (Sigma, USA) and incubated for 3 h at 37 °C. Absorbance of the supernatant was measured at 495 nm after removing insoluble ECR by centrifugation and the elastase activity was expressed as change in OD_{495} with respect to control (Ohman *et al.* 1980). Proteolytic activity was similarly assessed by incubating 100 µl culture supernatant with 3 mg of azocasein (Sigma, USA) in 900 µl of ECR buffer at 37 ºC for 30 min. Subsequently, reaction was stopped by the addition of trichloroacetic acid (10%, 100 µl). Contents were centrifuged after 30 min and absorbance of the supernatant was determined at 400 nm (Mihalik *et al.* 2008). Effect of F-AF on biofilm formation in *P. aeruginosa* PAO1 was studied by crystal violet staining method (Stepanovic *et al.* 2008). For this, 50 µl of *P. aeruginosa* PAO1 culture (10^6 $CFUmL^{-1}$) in tryptone broth (TB) was diluted with 2950 µl of fresh TSB containing F-AF and incubated statically for 18 h. Biofilm formed was stained with crystal violet (0.1% aq) for 15 min and excess stain was washed with water, dried, solubilized using acetic acid (33%) and biofilm was quantified as OD_{590} spectrophotometrically. All the experiments were conducted at concentration of F-AF between 0.05 and 0.4 mg mL^{-1}.

Isolation of metabolites from F-AF and its activity

For isolation of metabolites, F-AF was spotted on a silica TLC plate and chromatographed using chloroform: ethyl acetate (80:20) solvent system. After elution, the dried plate was overlaid with sterile LB medium seeded with *C. violaceum* ATCC12472 and incubated for 24 h to identify spots with violacein inhibition (Shaw *et al*. 1997). From the preparative TLC plates, spots corresponding to the activity were collected and pooled based on the R_f value. The reconstituted material was subjected to LC-MS analysis for identification of compounds using BDS HYPERSIL RP-C_{18} column (4.6 mm I.D. x 250 mm, Thermo Finnigan Surveyor) with CH_3CN:H_2O (40:60) as solvent system at a flow rate of 0.5 mL min^{-1}. Compounds were identified based on the major ion peaks (*m/z*) using electrospray ionization mass spectrometer

(LCQ Deca XP MAX, Thermo, USA). Identified compounds were purchased from Sigma (USA) and tested at different concentrations (0.025 –0.20 mg mL^{-1}) for its activity as described in earlier sections.

Data Analysis

All the experiments were performed in quadruplicates and data are expressed as mean ± standard deviation. Significance of differences ($p< 0.01$) among the treatments was determined using one-way analysis of variance, followed by pair wise t-test.

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Conflict of Interest

Authors declare that they have no conflicts of interest.

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Legends to figures

Figure 1. A. Agar plate assay showing inhibition of violacein by different solvent extracts: 1, hexane; 2, chloroform; 3, methanol; 4, ethyl acetate and 5. ethanol. B & C. Inhibition of violacein by the ethanol extract and E-AF respectively in *C. violaceum* ATCC12472. D & E. Effect of F-AF on violacein production and growth in *C. violaceum* ATCC12472. Values are mean ± SD, n=4.

Figure 2 A. Growth (OD_{600}) of *P. aeruginosa* PAO1 at different concentrations of F-AF, B. Inhibition of swarming motility by F-AF with respect to control (bars with * indicate significant difference compared to control ($p < 0.01$). C. Representative images showing inhibition of swarming motility (1) Control, (2) 0.05 mg mL^{-1}, (3) 0.1 mg mL^{-1}, (4) 0.2 mg mL^{-1}.

Figure 3. Attenuation of virulence factor production in *P. aeruginosa* PAO1 by F-AF (A) pyocyanin, (B) protease activity, (C) elastase activity and (D) biofilm formation. Values are mean ± SD, n=4.

Figure 4. Induction of violacein in *C. violaceum* CV026 by the AHL extracted from the cultures of *C. violaceum* ATCC31532 grown in presence of F-AF. Values are mean ± SD, n=4.

Figure 5. Effect of quercetin, quercetrin and kaempferol on (A) pyocyanin, (B) protease and (C) elastase activity in *P. aeruginosa* PAO1. Values are mean ± SD, n=4.

Fig S1. A. TLC showing a spot corresponding to anti-QS activity; B: HPLC of the spot from TLC and C, D, E, & F are mass spectra of the major compounds corresponding respectively to RT, 3.82, 5.92, 10.41 and 27.95 min in HPLC.