Anti-quorum sensing potential of *Adenanthera pavonina*

Halkare Suryanarayana Vasavi, Ananthapadmanabha Bhagwath Arun, Punchappady-Devasya Rekha

Yenepoya Research Center, Yenepoya University, University Road, Deralakatte, Mangalore, Karnataka, India

Submitted: 06-03-2014 Revised: 26-04-2014 Published: 17-12-2014

**INTRODUCTION**

Many pathogenic Gram-negative bacteria rely on quorum sensing (QS) circuits as central regulators of virulence expression. QS is the regulation of gene expression in accordance with population density through chemical signal molecules.\[1\] QS is well-established in *Chromobacterium violaceum* where violacein production is under the control of N-acyl homoserine lactone (AHL)-mediated QS system.\[2\] Similarly, in *Pseudomonas aeruginosa*, the production of virulence factors and formation of biofilms are mediated by QS-regulated gene expression.\[3\] Therefore, QS has been suggested as an ideal target for the development of novel anti-infective drugs, which would function to efficiently interfere with QS signal molecules, and thereby be capable of inducing chemical attenuation of pathogens. As QS is not directly involved in processes essential for growth of the bacteria, inhibition of QS does not impose harsh selective pressure for development of resistance as with antibiotics.\[4\]

Importance of QS system has been demonstrated in various infections like cystic fibrosis, burn wound, respiratory tract infections, microbial keratitis and urinary tract infection caused by *P. aeruginosa*.\[5\] To orchestrate synchronous production of virulence factors and biofilm formation, *P. aeruginosa* relies on two major LuxI/R quorum-sensing systems, the Las and Rhl systems, which are arranged in a hierarchical manner such that the las system activates the rhl system. Virulence factors of *P. aeruginosa* namely pyocyanin, proteases, elastases, exotoxin A and rhamnolipids are QS-dependent.\[6-7\]

Plants have evolved numerous chemical strategies for deterring pathogen attack, including the production of bactericidal and anti-infective compounds, leading to their use as medicines. *Adenanthera pavonina* L. (Family: Fabaceae) known as red bead tree, found in the Western Ghats region of India has been part of the Indian Ayurvedic medicine. Various parts of this plant have been used in the treatment of diarrhea, gout, inflammations, tumors and ulcers, and as a tonic.\[8\] Previous phytochemical studies on this plant
revealed the presence of secondary metabolites, mainly flavonoids, steroids, saponins, tannins and triterpenoids.\textsuperscript{9,10} As a part of the screening studies for identifying the potential sources for anti-QS activity, \textit{A. pavonina} leaf extract was tested and it showed positive for anti-QS activity. Hence, detailed study using the solvent extract of \textit{A. pavonina} was undertaken to demonstrate the anti-QS activity.

**MATERIALS AND METHODS**

**Bacterial strains, media and culture conditions**

\textit{C. violaceum} ATCC12472, a mini-Tn5 mutant \textit{C. violaceum} CV026 and \textit{P. aeruginosa} PAO1 were used to study the anti-QS potential of \textit{A. pavonina}. All the bacterial strains were grown in LB (Luria-Bertani) medium by incubating at 32°C except \textit{P. aeruginosa} PAO1, which was incubated at 37°C for 24 h. \textit{C. violaceum} CV026 is a mutant that produces violacein pigment only in the presence of exogenous C\textsubscript{6}-AHL. For all the experiments, inoculum was prepared by growing the bacteria in 10 ml LB broth under shaking (130 rpm) for 24 h. The cell density was measured spectrophotometrically (UV-1800, Shimadzu, Japan).

**Collection of plant materials and extract preparation**

The fresh leaves of \textit{A. pavonina} were collected from the foothills of Western Ghat region in Karnataka, India, washed in sterile water and air dried. The dried leaves were pulverized in an analytical mill (IKA, Germany) to fine powder. For the preparation of ethanolic extract, 100 g of powdered leaves of \textit{A. pavonina} was repeatedly extracted with 90% ethanol in the soxhlet extractor at 70°C for 16 h. The extract was concentrated to dryness and stored at 4°C until further use.

**Biosensor bioassay**

For the detection of anti-QS activity of the extract, disc diffusion agar plate assay was carried out using the reporter strain \textit{C. violaceum} CV026 as described earlier.\textsuperscript{12} Different concentrations of ethanol extract of \textit{A. pavonina} (10 \(\mu\)l) loaded onto 6 mm sterile discs (Himedia, India) were placed on the LB agar plates that were seeded with 100 \(\mu\)l of \textit{C. violaceum} CV026 and supplemented with 100 \(\mu\)l of 5 \(\mu\)g/ml of C\textsubscript{6}-AHL. Inhibition of QS was detected after 24 h incubation by the presence of a zone of pigmentless but viable cells around the disc. The experiment was also repeated with wild type strain \textit{C. violaceum} ATCC12472.

**Bioassay-guided fractionation of ethanol extract**

The ethanolic extract was fractionated into hexane fraction (HF), water soluble (WF) and water insoluble (WI) fractions. The WF fraction was repeatedly re-extracted with ethyl acetate to obtain ethyl acetate fraction (AEF). All the fractions were concentrated, redissolved in dimethyl sulfoxide and subjected to biosensor bioassay using 10 \(\mu\)l of the concentrated fractions in sterile discs. The most active fraction, AEF, was used for further studies.

**Quantification of inhibition of violacein production in \textit{C. violaceum} 12472**

Inhibition of violacein production in the presence of solvent extracts was quantified using previously described method with modification.\textsuperscript{11} Briefly, 10 ml LB broth containing AEF (0.25-1 mg/ml) was inoculated with 100 \(\mu\)l of \textit{C. violaceum} ATCC12472 and incubated at 32°C for 24 h under shaking (130 rpm). After incubation, culture (1 ml) was centrifuged and the cell pellet was mixed with equal volume of water saturated n-butanol and centrifuged again. The supernatant containing violacein was quantified spectrophotometrically at OD\textsubscript{585}. The cell viability in the culture medium was tested by standard plate count method.

**Effect of AEF on QS-controlled virulence factors production in \textit{P. aeruginosa} PAO1**

Effect of active fraction, AEF, on widely studied QS-controlled virulence factors such as pyocyanin, proteolytic, elastolytic activity, swarming motility and biofilm formation was tested in \textit{P. aeruginosa} PAO1. To determine the effect of plant extract on pyocyanin production, \textit{P. aeruginosa} PAO1 was grown in glycerol alanine minimal medium in the presence or absence of AEF for 24 h at 37°C.\textsuperscript{13} Pyocyanin from the cell-free supernatant (5 ml) was extracted with 3 ml of chloroform and subsequently, the chloroform layer was acidified with HCl. The pyocyanin-rich acid layer was quantified by recording OD\textsubscript{520} spectrophotometrically. Swarming assay was performed in LB semisolid (0.5% agar) medium supplemented with AEF. LB plates were point inoculated with \textit{P. aeruginosa} PAO1 and incubated at 37°C for 24 h. The extent of swarming was determined by measuring the swarming diameter.\textsuperscript{13}

Inhibition of proteolytic and elastolytic activities was assessed according to previously described methods.\textsuperscript{14,15} Briefly, \textit{P. aeruginosa} PAO1 was grown in LB medium supplemented with different concentrations of AEF and incubated at 37°C for 16 h. Culture supernatant (100 \(\mu\)l) was added to 900 \(\mu\)l of elastin congo red (ECR) buffer (100 mM Tris, 1 mM CaCl\textsubscript{2}, pH 7.5) containing 20 mg ECR (Sigma) and incubated for 3 h at 37°C. Insoluble ECR was removed by centrifugation, and the absorbance of the supernatant was measured at 495 nm. For proteolytic activity, 100 \(\mu\)l culture supernatant was added to 900 \(\mu\)l of ECR buffer containing 3 mg of azocasein (Sigma) and were incubated at 37°C for 30 min. Trichloroacetic acid (10%, 100 \(\mu\)l) was added to each reaction tube. After 30 min, the tubes were centrifuged and
absorbance of the supernatant was determined at 440 nm. Cell-free LB medium alone and with extract were used as negative controls for both the assays.

Biofilm studies were carried out as described by Vandeputte et al. Briefly, 50 μl of 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 h. The biofilm formed was assessed by crystal violet staining method by recording OD_590 spectrophotometrically.

Separation of anti-QS compounds by thin layer chromatography
The active fraction AEF was spotted on a silica gel TLC plate and chromatographed using chloroform: methanol (80:20) solvent system. After elution, the plate was dried and overlaid with sterile LB medium containing exogenous C_6-AHL inoculated with C. violaceum CV026 biosensor strain. The TLC overlay was incubated at 32ºC for 24 h and anti-QS activity was detected by the presence of turbid halo region in purple background.

RESULTS
Inhibition of QS by A. pavonina extracts
The ethanol extract of A. pavonina showed anti-QS activity in C. violaceum CV026 biosensor bioassay. After 24 h incubation, clear turbid halo zone of 12 mm diameter of violacein inhibition was observed at the concentration of 200 μg/disc. The antimicrobial activity was ruled out as the sample from the halo zone region showed the growth of bacterial cells on LB agar medium, which further confirms the inhibition of violacein production by A. pavonina. Bioassay-guided fractionation resulted in AEF with more pronounced anti-QS activity with 24 mm diameter of violacein inhibition zone at the concentration of 100 μg/disc [Figure 1a]. HF, WI did not show anti-QS activity in bioassay.

Inhibition of violacein production in C. violaceum ATCC12472
The active fraction, AEF, inhibited violacein production in a concentration-dependent manner as evidenced by the quantitative assay conducted using C. violaceum ATCC12472. The tested concentrations of AEF (0.25–1.0 mg/ml) showed a significant drop in violacein content [Figure 2] without interfering with the bacterial growth [Table 1].

Effect of AEF of A. pavonina on virulence factor production in P. aeruginosa PAO1
The AEF fraction significantly inhibited the production of pyocyanin in P. aeruginosa PAO1 [Figure 3a]. At 0.25 mg/ml of AEF, 50% inhibition of pyocyanin production was obtained and complete inhibition of pyocyanin production was attained at 1.0 mg/ml concentration of AEF. The swarming motility was completely inhibited in P. aeruginosa PAO1 at a very low concentration of 100 μg/ml of AEF [Table 2]. Under control conditions, the swarming diameter was 65 mm and in the presence of AEF, the bacteria were able to grow and form a colony in the center with diameter not exceeding 12 mm, and tendril formation or other features indicative of swarming motility were not observed. When P. aeruginosa PAO1 was grown in the presence of AEF, significant decrease in the elastolytic and proteolytic activities was observed in a concentration-dependent manner [Figure 3b]. At 1.0 mg/ml concentration, AEF showed complete

Figure 1: Anti-QS activity of active fraction (AEF) of A. pavonina. (a) Biosensor bioassay of AEF showing inhibition of C_6-AHL-mediated violacein production in bioreporter C. violaceum CV026 (b) TLC overlay assay of AEF illustrating the inhibition of C_6-AHL-mediated violacein production in C. violaceum CV026. Areas of pigment clearing (indicated by arrow) show region of AEF compounds that inhibit AHL-regulated violacein production in C. violaceum CV026

Figure 2: Inhibition of violacein production in C. violaceum ATCC12472 by different concentrations of active fraction (AEF) of A. pavonina. Values are expressed as mean ± SD, n = 4. Same letters in the columns (bar) are not significantly different (P < 0.001)
inhibition of elastolytic and proteolytic activities in
P. aeruginosa PAO1. Since biofilm formation is partially
controlled by QS mechanisms, the effect of AEF on
biofilm formation in P. aeruginosa PAO1 was assessed after
18 h of growth. At 1.0 mg/ml concentration of AEF,
the biofilm formation was decreased by 81% [Figure 3a].

Phytochemical screening of anti-QS compounds separated by TLC
The AEF fraction was spotted onto a silica gel TLC plate and eluted with chloroform: Methanol, after
which the TLC plate was cast into agar containing
C. violaceum CV026 biosensor strain. After incubation, a
band showing anti-QS zone was observed with R_f value
0.63 [Figure 1b].

DISCUSSION

QS plays an important role in the regulation of cell
physiology in many Gram-negative bacteria. QS system
consisted of inducer and regulator proteins of las and rhl
components, which work interdependently in a hierarchal
manner to regulate the expression of various genes,
including virulence ones in P. aeruginosa.[17] In the present
study, A. pavonina inhibited of both las- and rhl-mediated
phenotypes in P. aeruginosa PAO1. Preliminary screening of
anti-QS activity was carried out using C. violaceum
CV026 biosensor strain. In C. violaceum CV026 plate
assay, formation of halo zone indicates that A. pavonina
is either inhibiting the C6-AHL competitively from
binding to its transcriptional regulator, CviR; degrading
the C6-AHL enzymatically, or removing the C6-AHL via
active transport.[18,19] Inhibition of violacein production was
quantified and from the results obtained in this study, it
was proven that A. pavonina reduced violacein production
significantly. In agreement to this finding, other plant
extracts of Conocarpus erectus, Quercus virginiana and other
higher plants have demonstrated the anti-QS activity against
biosensor strain C. violaceum CV026.[20]

In P. aeruginosa, the production of pyocyanin is under
the control of rhlI-rhlR QS system. Pyocyanin is highly
permeable to the biological membrane and causes
extensive cellular damage in the lungs of cystic fibrosis
patients. Secretion of elastase and protease enzymes is
also an important aspect of pathogenicity, which helps
in combating adverse conditions and tissue colonization
inside the host.[21] P. aeruginosa exhibits swarming motility,
which helps in initial attachment and later in relocation of
biofilm from one site to another.[22] In the present
study, significant reduction in the pyocyanin production,
proteolytic and elastolytic activities, swarming motility, and
biofilm formation in P. aeruginosa PAO1 were observed in
the presence of AEF of A. pavonina.

The anti-QS compound present in the active fraction of
A. pavonina was separated by TLC. Phytochemical analysis of
A. pavonina plant is still in its primitive stages; exploration of
its activity may invite further studies on the phytochemicals

---

### Table 1: Effect of active fraction of A. pavonina on the viability of the bacterial cells in the culture medium as estimated by the standard plate count method after 24 h incubation. Values are mean±SD, n=4

| Concentration of AEF (mg/ml) | C. violaceum (12472) | P. aeruginosa (PAO1) |
|-----------------------------|----------------------|----------------------|
| Control                     | 2.48±0.02            | 2.33±0.04            |
| Solvent control             | 2.28±0.03            | 2.37±0.05            |
| 0.25                        | 2.15±0.02            | 2.15±0.03            |
| 0.5                         | 2.28±0.03            | 2.18±0.02            |
| 1.0                         | 2.19±0.01            | 2.23±0.01            |
| 2.0                         | 2.07±0.02            | 2.09±0.01            |

AEF=Active fraction; SD=Standard deviation

### Table 2: Inhibition of swarming motility in Pseudomonas aeruginosa PAO1 by different concentrations of active fraction of A. pavonina. Values are mean±SD. n=4. Same letters in the columns are not significantly different (P<0.001)

| Concentration of AEF (mg/ml) | Swarming diameter (mm) |
|-----------------------------|------------------------|
| Control                     | 65.75±0.95             |
| 0.10                        | 09.25±1.50a            |
| 0.25                        | 07.50±1.29b            |
| 0.50                        | 05.00±1.08c            |
| 1.00                        | 05.00±1.05c            |

AEF=Active fraction; SD=Standard deviation

---

Figure 3: The effect of AEF on QS-regulated virulence factor production in P. aeruginosa PAO1. (a) Pyocyanin production and biofilm formation (b) Proteolytic activity and elastolytic activities. Values are expressed as mean ± SD, n = 4. Same letters in the columns (bar) are not significantly different (P < 0.001)
present. Tannin-rich fraction from *Terminalia catappa* showed inhibition of QS-mediated virulence factor production in *C. violaceum* and *P. aeruginosa* PAO1.[23] Flavonoids, catechin, catechin and naringin also been proved to inhibit QS in Gram-negative pathogens.[24] Some known mechanisms of QS inhibition include competitive binding of signal-like molecules to cognate receptors, as in the case of furanones, enzymatic degradation of QS signals, as in the case of AHL acylases. Further studies are needed to demonstrate the exact mechanism of QS inhibition by *A. pavonina* phytochemicals. It is interesting to study the important mechanism involved by the plants used in traditional phytomedicine and revealing the possible mechanism/mode of action shall aid in the invention of lead molecules for the antimicrobial drugs.

**REFERENCES**

1. Rutherford ST, Bassler BL. Bacterial quorum sensing: Its role in virulence and possibilities for its control. Cold Spring Harb Perspect Biol 2012;2:a012427.

2. McClean KH, Winson MK, Fish L, Taylor A, Chabra SR, Camara M, et al. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of N-acyliminoborine lactones. Microbiology 1997;143:3703-11.

3. Roger SS, Iglweski BH. *Pseudomonas aeruginosa* quorum sensing systems and virulence. Curr Opin Microbiol 2003;6:56-60.

4. Hentzer M, Givskov M. Pharmocolological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest 2003;112:1300-7.

5. Willcox MD, Zhu H, Conibear TC, Hume EB, Givskov M, Kjelleberg S, et al. Role of quorum sensing by *Pseudomonas aeruginosa* in microbial keratitis and cystic fibrosis. Microbiology 2008;154:2184-94.

6. Williams P, Camara M, Hardman A, Swift S, Milton D, Hope VJ, et al. Quorum sensing and the population-dependent control of virulence. Philos Trans R Soc Lond Biol Sci 2000;355:667-80.

7. Schuster M, Greenberg EP. A network of networks: Quorum-sensing regulation in *Pseudomonas aeruginosa*. Annu Rev Microbiol 2001;55:675-701.

8. Adedapo AD, OsudeYO, Adedapo AA, Moody JO, Adeagbo AS, et al. Blood pressure lowering effect of *Adenantherapavonina* seed extract on normotensive rats. Records Nat Prod 2009;3:82-9.

9. Su EN, Yu SS, Pei YH. Studies on chemical constituents from stems and leaves of *Adenanthera pavonina*. Zhongguo Zhongya Zazhi 2007;32:2135-8.

10. Kothale KV, Rothe SP. Phytochemical screening of *Adenantherapavonina*. Linn. World J Sci Tech 2012;2:19-22.

11. Choo JH, Rukayadi Y, Hwang JK. Inhibition of bacterial quorum sensing by vanilla extract. Lett Appl Microbiol 2006;42:637-41.

12. Essar DW, Eberly L, Hadero A, Crawford IP. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: Inter changeability of the two anthranilate synthases and evolutionary implications. J Bacteriol 1990;172:884-900.

13. Vattem DA, Mihalik K, Crixell SH, McLean RJ. Dietary phytochemicals as quorum sensing inhibitors. Fitoterapia 2007;78:302-10.

14. Kessler E, Israel M, Landsman N, Chechik A, Blumberg S. *In vitro* inhibition of *Pseudomonas aeruginosa* aslaetase by metal-chelating peptide derivatives. Infect Immun 1982;38:716-23.

15. Mihalik K, Chung DW, Crixell SH, McLean RJ, Vattem DA. Quorum sensing modulators of *Pseudomonas aeruginosa* characterized in *Camellia sinensis*. Asian J Trad Med 2008;3:12-23.

16. Vandeputte OM, Kiendrebeogo M, Rajaonson S, Diallo B, Mol A, El Jaziri M, et al. Identification of catechin as one of the flavonoids from *Combretumalbiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. Appl Environ Microbiol 2010;76:243-53.

17. Wagner VE, Li LL, Isabella VM, Iglewski BH. Analysis of the hierarchy of quorum-sensing regulation in *Pseudomonas aeruginosa*. Anal Bioanal Chem 2007;387:469-79.

18. Martellini D, Grossmann G, Sequin U, Brandi H, Bachofen R. Effects of natural and chemically synthesized furanones on quorum sensing in *Chromobacterium violaceum*. BMC Microbiol 2004;4:25.

19. Bauer WD, Teplitzki M. Can plants manipulate bacterial quorum sensing? Aust J Plant Physiol 2001;28:913-21.

20. Adonizio AL, Downum K, Bennett BC, Mathew K. Anti-quorum sensing activity of medicinal plants in southern Florida. J Ethnopharmacol 2006;105:427-35.

21. Siehnela R, Traxlerb B, An DD, Parsek MR, Schaeferb AL, Singh PK, et al. Unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 2010;107:7916-21.

22. Taganna JC, Quanico JP, Perono RM, Amor EC, Rivera WL. Tannin-rich fraction from *Terminaliacatappa* inhibits quorum sensing (QS) in *Chromobacterium violaceum* and the QS-controlled biofilm formation and Las Astaphylolytic activity in *Pseudomonas aeruginosa*. J Ethnopharmacol 2011;134:865-71.

23. Manefield M, deNys R, Read R, Gijskens M, Steinberg P, et al. Evidence that halogenated furanones from *Combretumalbiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. Appl Environ Microbiol 2010;76:243-53.

24. Zhang HB, Wang LH, Zhang LH. Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 2002;99:4638-43.