Anti-Quorum Sensing Activity of *Forsythia suspense* on *Chromobacterium violaeum* and *Pseudomonas aeruginosa*

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**ABSTRACT**

**Background:** Quorum sensing (QS) plays an important role in the production of virulence factors and pathogenicity in *Pseudomonas aeruginosa*, and the interruption of QS will be a hopeful pathway to combat bacterial infection.

**Objective:** In this study, we selected *Forsythia suspense* (Thunb.) Vahl from traditional Chinese herbal medicines for its anti-QS activity. 

**Materials and Methods:** Anti-QS of *F. suspense* extracts (FSE) was monitored using the *Chromobacterium violaeum* 12472 bioassay. Standard methods were used to investigate the effects of FSE on QS-controlled virulence factors production, swimming motility, and biofilm establishment in *P. aeruginosa* PAO1.

**Results:** FSE could obviously inhibit the violacein production in *C. violaeum* 12472 and also could inhibit quorum sensing-regulated virulence factors production and biofilm formation in *P. aeruginosa* in a concentration-dependent manner. The elastase activity and pyocyanin production were inhibited at a maximum of 40.97 and 4758% when *P. aeruginosa* was grown in the presence of 0.25 g/mL FSE, which can also inhibit swimming motility of *P. aeruginosa*. The biofilm formation ability was decreased about 72.45% when in PAO1 cultured with the 0.25 g/mL FSE. The results suggested that FSE may be used as an alternative drug to control and handle harmful infections caused by bacterial pathogens based on QS inhibition.

**Key words:** *Pseudomonas aeruginosa*, *Forsythia suspense*, quorum sensing, virulence factors, biofilm

**SUMMARY**

- *Forsythia suspense* water extract could obviously inhibit the purple pigment production in *C. violaeum* 12472.
- *Forsythia suspense* water extract could inhibit QS-regulated virulence factors production and biofilm formation in *P. aeruginosa*.

**INTRODUCTION**

Antibiotics have been considered to be the most efficacious drug for curing bacterial infectious diseases. As the quantity of antibiotics has increased over the past decades applied in human clinical and animal husbandry, numerous multiple drug-resistant bacterial strains have been isolated. Now researchers are trying their best to find alternative approaches to prevent and treat bacterial infections. Quorum sensing (QS) is a pattern of bacterial communication used to detect the density and control collectively behaviors. This process depends on the production, release, and group-wide detection of signal molecules named autoinducers (AIs), which are typically N-acyl-homoserine-lactones (AHLs) produced in Gram-negative bacteria. QS plays an important role in the production of virulence factors and pathogenicity in PAO1. Many researchers show that disrupting the QS system can decrease the secretion of virulence factors and biofilm formation, and it will be a hopeful pathway to inhibit bacterial infectious disease.

Many researchers have explored QS inhibitors from natural organisms with the potential inhibition of the QS system of various pathogens. QS inhibitors were found from medicinal herbs, synthetic chemicals and microbes. Okusa, et al. found that extracts of *Cordia gilletii* de wild (Boraginaceae) quench the QS of *Pseudomonas aeruginosa* PAO1. The extract from root barks was found to quench the production of pyocyanin and reduce biofilm formation. Moreover, this extract is able to inhibit the expression of several QS-regulated genes, such as *lasB*, *rhlA*, *lasI*, *lasR*, *rhlI*, and *rhlR*. Mihalik et al. results showed *Camellia sinensis* (green tea, GT) extracts inhibited the expression of QS-controlled virulence factors in *P. aeruginosa*. They tested the possible mechanism of inhibiting QS at the same time. In that study, extract of *F. suspense* was used to test for its activity to inhibit pigment production in *C. violaeum* 12472, biofilm formation, and virulent factors production in *P. aeruginosa*.

**Abbreviations used:** QS: Quorum sensing, *Pseudomonas aeruginosa* *P. aeruginosa*, *Forsythia suspense* FSE: *F. suspense* extracts, *Chromobacterium violaeum* 12472, AIs: autoinducers, AHLs: N-acyl-homoserine-lactones, LB: Luria-Bertani, MICs: Minimum inhibitory concentrations, CFU: Colony-Forming Units, ATCC: American Type Culture Collection, PBS: phosphate buffered saline

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F. suspense (Thunb.) Vahl (Oleaceae) is a notable herb that has long been used as an antibacterial, antiviral, antipyretic, and anti-inflammatory agent in traditional Chinese medicine. It is widely distributed in Asia and Europe. F. suspense is widely grown mainly in Hebei, Shanxi, and Shanxi Province in China. Dried fruit of F. suspense is the only drug source. However, little is known about its mechanism to prevent bacterial infection. Our results have suggested that the extract could obviously inhibit the purple pigment production in C. violaceum 12472 and could also inhibit QS-regulated virulence factors production and biofilm formation in P. aeruginosa. These results suggest that F. suspense extract, or some efficacious compounds, can be used as an alternative drug to control and cope with harmful infections caused by bacterial pathogens based on QS inhibition.

MATERIALS AND METHODS

Strains and culture conditions

C. violaceum ATCC 12472 was used as a report strain to detect QS inhibiting ability. The purple pigment, violacein production was underregulated by QS system, so it is used as a biosensor strain to detect possible QS inhibitors. P. aeruginosa, a common Gram-negative rod-shaped opportunistic pathogenic, has two QS systems, RhlI/R and LasI/R, which control the proteolytic activity, pyocyanin production, biofilm formation, and swimming motility. Both strains were grown in Luria-Bertani broth (LB, 0.5% yeast extract, 1.0% tryptone, 0.5% NaCl, pH 7.4) with or without antibiotics.

Crude extracts preparation

The extraction of F. suspense powder was carried out by the method of Perera et al. Briefly, 100 g of F. suspense powder (the Tong Ren Tang Pharmaceutical Store, Nanjing, China) was decocted in water (2 L) for 2 h (two times). The extract was centrifuged at 10,000 g for 10 min to remove all plant debris, and then the supernatant was filtered through a Whatman filter paper No. 1 paper filter. The filtrate was then evaporated in a rotary vacuum evaporator. The crude extract was re-dissolved into 50 mL water to a concentration of 2 g of raw herbal material per mL (2 g/mL). Filtration of the water extracts using a 0.22 μm filters and tested for microbial contamination. The extracts were stored at -20°C before application.

Effect of FSE on bacterial growth

The minimum inhibitory concentrations (MICs) of FSE for P. aeruginosa and C. violaceum 12472 were determined by two-fold macrodilution in LB broth with an inoculum of 106 CFU/mL. The technique as described by Chu et al. The MIC was defined as the lowest concentration at which there is no visible bacterial growth. A range of concentrations below MIC value (sub-MIC) of the drug was taken to evaluate their effect on the QS of P. aeruginosa.

Bioassay for QS inhibition activity using C. violaceum 12472

C. violaceum biosensor method was used in this study. Six milliliter of warm molten soft agar (LB with 0.7% agar) was seeded with 20 μL of 18-h cultured C. violaceum ATCC 12472 and mixed. Then mixed culture was poured over the surface of a solidified LB plate to form the overlay. Subsequently, 50-mm wells were punched through the agar after the overlay had solidified and 100 μL FSE at sub-MIC were loaded onto it. The assay plates were then cultured at 30°C for 18 h. The absence of the purple violacein pigmentation of the bacterial lawn surrounding the well indicated QS inhibition activity. Disks loaded with sterilized water were considered as controls.

Effect of FSE on C. violaceum 12472 violacein production

For quantitative QS inhibition assay, violacein was extracted from the wells by water-saturated butanol according to the method of Blosser and Gray (2000) and quantified spectrophotometrically at optical density OD 580 (UV-1800; Shimadzu).

Quantification of secreted of virulence factors and static biofilms of PAO1

P. aeruginosa PAO1 was cultured in LB medium containing a series sub-MIC concentrations (0–0.25 g/mL) of extract for 1 h at 30°C and then centrifuged (10,000g, 5 min, 4°C); standard protocols were then used to detect the virulence factors in cell-free supernatants.

Pyocyanin assay

Pyocyanin was detected by growing the bacteria in LB medium for 18 h. Pyocyanin secreted by P. aeruginosa PAO1 in the supernatant was measured by methods as Reimmann et al. Two milliliter of cell-free supernatants was extracted with 1.5 mL chloroform and vortexed immediately. After centrifugation (10,000g, 3 min), the chloroform layer was transferred to a new tube and 1.5 mL 0.2 M HCl was added to the tube and the color of the liquid changed to pink. The absorbance was detected at OD 520.

Proteolytic assays

The proteolytic activity was tested by a skim milk plate assay. Briefly, 50 μL of cell-free supernatant of FSE-treated and FSE-untreated P. aeruginosa was separately added in LB solid medium containing 5% skim milk have holes and incubated at 30°C for 18 h. The circle of casein hydrolysis was detected.

Biofilm formation assays

Biofilm formation experiments were performed as previously described by Agarwala et al. in the presence or absence of extract. Briefly, 10 μL of 18 h cultured P. aeruginosa PAO1 (106 CFU/mL) was diluted with 2 mL of LB liquid medium containing a series of sub-MIC concentrations of FSE and then incubated for 18 h without shaking. The mixture was discarded after incubation, and the tube was washed thrice with phosphate buffered saline (PBS) and set in formaldehyde (10%) for 10 min. The solutions were removed and air dried at room temperature. Crystal violet (0.1% in ethanol) was added to stain the biofilm for 15 min. Deionized water was used to remove the unbound dye for three times and absorbed dye was eluted with ethanol. Then the OD 650 was recorded.

Swimming assay

For swimming assays, PAO1 (106 CFU/mL) was point inoculated on LB plates containing 0.3% agar mixed with 0.25 g/mL FSE extract and incubated at 37°C for 18 h. The swimming diameter was detected by the ruler.

Analytical methods

Analytical Methods

All experiments were conducted in quadruplicates at least thrice. ANOVA was used to analyze the differences between the treatments using STATISTICA. P less than 0.01 was considered as significant unless otherwise specified and was represented by "**"; P less than 0.05 was represented by "***".
RESULTS AND DISCUSSION

Biosensor assays

The FSE demonstrated QS inhibitory activity in *C. violaceum* 12472. At the concentration of 0.25 g/mL, a colorless muddy zone of purple pigment inhibition diameter was reached to 18 mm (6 mm hole) [Figure 1]. Similarly, Vasavi *et al.* used *C. violaceum* 12472 as a biosensor bioassay and found that *Psidium guajava* L. flavonoids inhibit violacein product.[17] Selected from the Traditional Chinese Herbs, Priya *et al.* found that *Phyllanthus amarus* showed a distinguished colorless area on the lawn of *C. violaceum* 026 indicating a QS inhibitory activity.[18]

MIC assays

Tests on antibacterial activity of the *F. suspense* extracts have revealed MIC values for *P. aeruginosa* relatively. For example, water extracts of *F. suspense* showed visible of 0.25 g/mL against *P. aeruginosa*, but invisible of 0.5 g/mL. So, MIC of FSE against *P. aeruginosa* was 0.5 g/mL. The MIC of FSE against *C. violaceum* 12472 was 0.5 g/mL.

Inhibition of violacein production in *C. violaceum* 12472 by FSE

Concentration-dependent inhibition of violacein production in *C. violaceum* 12472 by FSE was observed. Although all tested concentrations of FSE showed a significant drop in violacein content, the maximum QS inhibitory (69.28%) effect was seen at the highest concentration tested (0.25 g/mL) [Figure 2]. Bacterial cell count performed on LB agar plates at 24 h incubation showed no significant difference in the number of colony-forming units (CFUs) of the FSE concentrations tested (data not shown).

Effect of FSE on *P. aeruginosa* PAO1 virulence factors production

In most cases, virulence is a required as a prior condition for infection, but it is not required for bacterial survival. Therefore, antivirulence strategies that demilitarize pathogens rather than killing them may apply moderate evolutionary pressure that does not lean toward the development of resistance.

Pyocyanin production and proteolytic activities

The synthesis of pyocyanin is controlled by QS-system.[19] Pyocyanin was produced when the cell of *P. aeruginosa* PAO1 reached high density, and pyocyanin was in limited production [Figure 3] when cultured with drugs that do not affect *P. aeruginosa* PAO1 growth but disrupt the QS systems. Similarly, significant decrease in proteases’ production was observed, with mostly inhibition at 0.25 g/mL [Figure 4]. The elastase activity and pyocyanin production was inhibited at a maximum of 40.97, 47.58% when *P. aeruginosa* was grown in the presence of 0.25 g/mL FSE.
Biofilm formation

When bacteria-acquired biofilm may readily make incursions into the human body parts, it may cause many chronic infections; but the attempt to eliminate the biofilm is very arduous.[20] QS plays a significant role in the formation of biofilm. Research work indicates that disrupt bacterial QS may lead to the restricting of biofilm formation.[21] The ability of FSE in reducing QS-related biofilm formation activity was assessed. As shown in [Figure 5], a decrease of more than 72.45% in biofilm formation was observed when in PAO1 cultured with the 0.25 g/mL FSE.

Swimming motility

Swimming motility of *P. aeruginosa* was mediated by QS systems. Some results were shown that reduced and delayed swimming in rhl/rhlR mutant strain, and the las/lasR mutants completely diminished swimming ability.[22] In this part, the extract of *F. suspense* inhibited swimming motility of *P. aeruginosa*, the swimming diameter was decreased as seen in [Figure 6] when FSE at 0.25 g/mL.

Our experimental data showed that *F. suspense* could be useful for weakening the virulence factors production in *P. aeruginosa*, thence more traditional Chinese herbal medicine should be screened for its anti-QS function, and its active ingredients might supply as hopeful antibacterial drugs. The active ingredients from herb might target the QS system by inhibiting the AHLs synthesis, degradation of the signal molecules, or inhibiting of the QS by targeting the AHLs receptor.[23] The appearance of multi-antibiotic resistance among pathogenic bacteria gives the researchers an opportunity to find out new strategies alternative to antibiotic against bacterial infection. The discoveries of the bacterial QS system provide a different treatment to conventional chemotherapy. Efforts to block the QS-based biofilms and virulence factors are important in eliminating the biofilm without killing the bacteria. This method makes its success, QS inhibitory molecules may reduce the production of virulence factors in bacterial pathogen without killing bacterial cells as antibiotics. Therefore, improved understanding of QS systems in the pathogenicity of bacteria may provide more insight into combating bacterial infection by using either antibiotics or emerging methods such as regulated degradation of QS receptor or QS signals.[24]

In conclusion, *F. suspense* extract can inhibit the QS-controlled pigment production in *C. violaceum* and reduce biofilm formation and virulence factors production, which is crucial to the pathogenicity in *P. aeruginosa*. *F. suspense* has been used as anti-infection traditional Chinese medicinal herb for a long time and is hence considered as a safe medical herb. So far, *F. suspense* presents a possibility for use as a new substance for controlling *P. aeruginosa* infections in the future. This method could be an advantage for the use of anti-QS drugs that show superior results compared with traditional antimicrobial drugs by not exerting any selective pressure on the drug-resistant mutant strains.

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Conflicts of interest

There are no conflicts of interest.

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