

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

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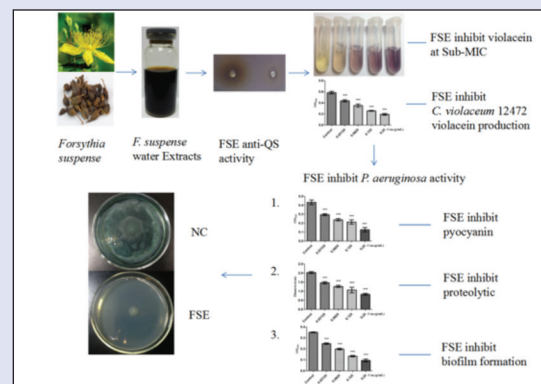
## ABSTRACT

**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 violaceum* 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. violaceum* 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 47.58% 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. violaceum* 12472
- *Forsythia suspense* water extract could inhibit QS-regulated virulence factors production and biofilm formation in *P. aeruginosa*.



**Abbreviations used:** QS: Quorum sensing, *Pseudomonas aeruginosa* *P. aeruginosa*, *Forsythia suspense* *F. suspense*, FSE: *F. suspense* extracts, *Chromobacterium violaceum* 12472 *C. violaceum* 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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## 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.<sup>[1]</sup> 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.<sup>[2]</sup> QS plays an important role in the production of virulence factors and pathogenicity in PAO1. Many researchers show that disrupt 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.<sup>[3]</sup>

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.<sup>[4]</sup> Okusa, *et al.* found that extracts of *Cordia gillettii* 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*.<sup>[5]</sup> Mihalik *et al.*<sup>[6]</sup> 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. violaceum* 12472, biofilm formation, and virulent factors production in *P. aeruginosa*.

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*F. suspense* (Thunb.) Vahl (Oleaceae.) is a notable herb that has long been used as 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.<sup>[7]</sup> 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.<sup>[8]</sup> *P. aeruginosa*, a common Gram-negative rod-shaped opportunistic pathogen, has two QS systems, RhlI/R and LasI/R, which control the proteolytic activity, pyocyanin production, biofilm formation, and swimming motility.<sup>[9]</sup>

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. *P. aeruginosa* and *C. violaceum* were grown at 37 and 30°C, respectively, with 120 rpm agitation in a shaking incubator overnight unless otherwise specified. The bacterial density was detected by recording OD 600 spectrophotometrically.

### Crude extracts preparation

The extraction of *F. suspense* powder was carried out by the method of Perera *et al.*<sup>[10]</sup> 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 10,000g 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.*<sup>[11]</sup> The MIC was defined as the lowest concentration at which there is no visible bacteria 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 QSI activity using *C. violaceum* 12472

*C. violaceum* biosensor method was used in this study.<sup>[11]</sup> 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).<sup>[12]</sup>

### Quantification of secreted 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 18 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.*<sup>[13]</sup> 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.<sup>[14]</sup> 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.*<sup>[15]</sup> 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,<sup>[16]</sup> 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.<sup>[17]</sup> 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.<sup>[18]</sup>

### 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

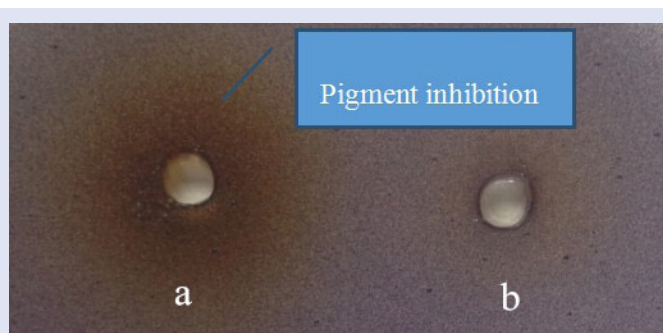
of FSE showed a significant drop in violacein content, the maximum QS inhibitory (69.28%) effect was seen at the highest concentration tested (0.25 g/mL) [Figure 2]. Bacterial cell count performed on LB agar plates at 24 h incubation showed no significant difference in the number of colony-forming units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

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

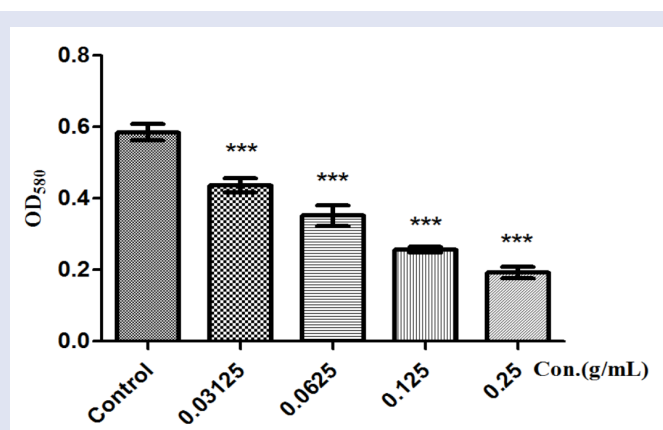
In most cases, virulence is a required as a prior condition for infection, but it is not required for bacterial survival. Therefore, antivirulence strategies that demilitarize pathogens rather than killing them may apply moderate evolutionary pressure that does not lean toward the development of resistance.

### Pyocyanin production and proteolytic activities

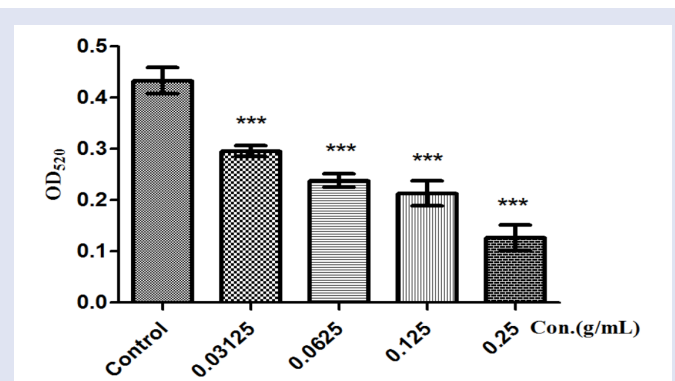
The synthesis of pyocyanin is controlled by QS-system.<sup>[19]</sup> Pyocyanin was produced when the cell of *P. aeruginosa* PAO1 reached high density, and pyocyanin was in limited production [Figure 3] when cultured with drugs that do not affect *P. aeruginosa* PAO1 growth but disrupt the QS systems. 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.



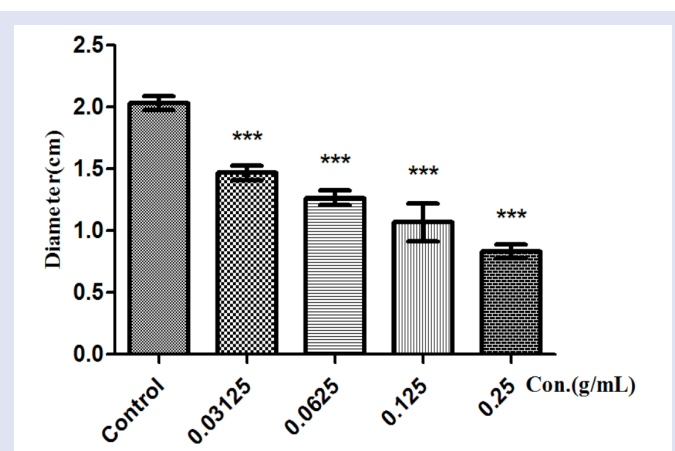
**Figure 1:** Anti-QS properties of *F. suspense* extract. (a) 0.25 g/mL *F. suspense* extract; (b) water. The bacteria around the positive control well was able to grow without producing violacein



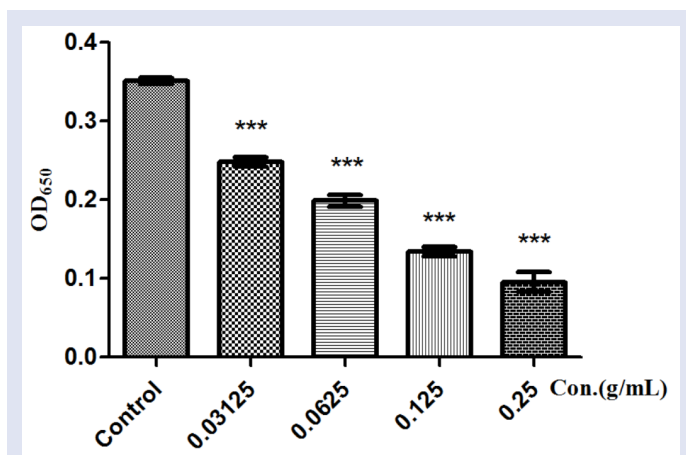
**Figure 2:** FSE inhibits *C. violaceum* 12472 violacein production in a concentration-dependent manner. The violacein production was measured spectrophotometrically and quantified by reading the OD values of the solution at 580 nm. Values are presented as mean  $\pm$  SD,  $n = 3$



**Figure 3:** Pyocyanin inhibition was performed with *P. aeruginosa* PAO1 in triplicate with *F. suspense*. Error bars represent SD of three replicates



**Figure 4:** Effect of *F. suspense* extract on *P. aeruginosa* PAO1 proteolytic activities. The total proteolytic activity on skim milk plate. Mean values of triplicate independent experiments and SD are shown



**Figure 5:** Biofilm formation of *P. aeruginosa* in the presence of *F. suspense*. Total biofilm amount at different *F. suspense* extract concentrations has distinguished difference. Values are presented as mean  $\pm$  SD,  $n = 3$

## 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.<sup>[20]</sup> 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.<sup>[21]</sup> 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 *rhlI/rhlR* mutant strain, and the *lasI/lasR* mutants completely diminished swimming ability.<sup>[22]</sup> 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.<sup>[23]</sup> 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 potentially capable of attenuating the pathogenicity without killing the bacteria. This method makes its success, QS inhibitory substance 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.<sup>[24]</sup>

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



**Figure 6:** Impairment of motility by a sub-MIC level of water extracts. swimming motility, the diameter of *F. suspense*-treated *P. aeruginosa* PAO1 cultures is significantly shorter than that of NC. The numbers associated with indicating concentration (0.25 g/mL for FSE) NC FSE

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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Nil

## Conflicts of interest

There are no conflicts of interest.

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