

Research Article

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Inhibition of Quorum Sensing Activity by Ethanol Extract of *Scutellaria baicalensis* Georgi

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Abstract

Many Gram-negative bacteria use N-acyl homoserine lactone signal molecules to monitor their own population density and coordinate gene regulation in a process called quorum sensing (QS). Because the regulation of many bacterial processes is controlled by QS systems, the finding of natural compounds acting as QS inhibitors suggest an attractive tool to control and handle detrimental infections caused by human, animal, and plant pathogens. To search for a novel quorum sensing inhibitor and analyze its inhibitory activity, we tested 13 Chinese traditional medicinal plants for their ability to inhibit QS-regulated behaviors in different bacterial species. Plant materials were extracted using water or 70% ethanol. The ethanolic extract from *Scutellaria baicalensis* Georgi was found to inhibit violacein production, a QS-regulated behavior in *Chromobacterium violaceum* CV026. In addition, the ethanolic extract was also able to inhibit QS-regulated virulence in *Pectobacterium carotovorum* subsp. *Carotovorum*. Our results indicated that *Scutellaria baicalensis* Georgi can inhibit bacterial quorum sensing.

Keywords: Quorum sensing; Chinese traditional medicinal plants; Anti-quorum sensing, *Scutellaria baicalensis* Georgi; Antimicrobial activity

Introduction

Over a 30-year period, it has become apparent that a diversity of bacterial species commonly control expression of gene circuits in a population-dependent manner via a regulatory mechanism known as quorum sensing (QS). In QS, small diffusible molecules called autoinducers mediate the ability to monitor the size of a bacterial population [1-3]. Autoinducers produced by bacteria diffuse out and accumulate in the surrounding environment, and once a threshold concentration has been reached, they diffuse back into the bacteria and regulate the transcription of specific genes. Increasing evidence indicates that bacterial QS is involved in the regulation of diverse biological processes including exopolysaccharide synthesis, virulence factor gene expression, sporulation, biofilm formation, motility, bioluminescence and antibiotic biosynthesis [4-7]. In Gram-negative bacteria, N-acyl homoserine lactones (AHL) are most commonly used as signal molecules, and the AHL-mediated QS plays an important role in regulating virulence factors, for example extracellular enzyme production in *Pectobacterium carotovorum* subsp. *Carotovorum* (*P.c.c.*), conjugation in *Agrobacterium tumefaciens* and toxin production in *Burkholderia glumae* [8,9].

Because of the importance of quorum sensing for bacterial pathogenesis, many studies have been focused on inhibiting quorum sensing. The QS system can be interfered with in a number of ways, including (1) inhibition of AHL molecule biosynthesis, (2) degradation of AHL molecules by bacterial lactonases, and (3) using small molecules to block the activation of AHL receptor protein [10]. It has been suggested that inactivating the QS system of a bacterial pathogen can result in a significant decrease in virulence factor production [11].

A number of quorum-quenching enzymes that degrade AHLs have been identified in bacteria [12]. So far, the first known anti-QS compounds from non-bacterial origin were halogenated furanones produced by the Australian macroalgae *Delisea pulchra* [13]. The natural furanone compounds and the synthesized derivatives have been found to effectively inhibit QS *in vitro* [14] and *in vivo* [15]. However, these furanone compounds consist of halogens that give

them limitations for human use. More recently, various species of plants—including pea seedlings, garlic, vanilla, and L-canavanine, which is made by *Medicago sativa*—have also been found to be able to interfere with bacterial QS [16-18].

In this study, we screened 13 Chinese medicinal plants for their QS-inhibitory activity. The data showed that the ethanolic extracts from *Scutellaria baicalensis* Georgi inhibited violacein production, which is a phenotype that is regulated by QS in *Chromobacterium violaceum* CV026. *Chromobacterium violaceum* CV026 is a violacein-negative, double mini-Tn5 mutant from *C. violaceum* ATCC 31532. *Chromobacterium violaceum* CV026 is deficient in the autoinducer synthase *cvil* (acylhomoserine lactone synthetase) and therefore, requires exogenous addition of N-hexanoyl homoserine lactone (C6-HSL) to undergo QS and produce a natural antibiotic called violacein, which is a water-insoluble purple pigment with antibacterial activity. These extracts also inhibited QS-controlled soft rot disease caused by plant pathogen *P.c.c.*. Quorum sensing signaling in *P.c.c.* is regulated by N-acylhomoserine lactone (AHL)-based systems (3-oxo-C6-HSL) [19, 20] as well as systems that depend on autoinducer-2 (AI-2) [21].

Materials and Methods

Plant materials and extraction

Thirteen Chinese medicinal plant species were purchased from a local medicine store in Shijiazhuang, China, including *Clerodendron cyrtophyllum*, *Gentiana scabra*, *Lonicera japonica* (stem, *Liriope*

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spicata, *Andrographis paniculata*, *Forsythia suspense*, *Senecio scandens*, *Taraxacum mongolicum*, *Isatis tinctoria*, *Pulsatilla chinensis*, *Sophora flavescens*, *Lonicera japonica* (flower), *Scutellaria baicalensis*. All the plant materials have been dried in an oven at 35°C for 72 h, and grounded to a fine powder. The dried powder was extracted using water or ethanol respectively. For ethanolic extracts, the powder was added to 70% (v/v) ethanol (200 g dry weight per litre) and allowed to stand for 48 h at room temperature before vacuum filtration with filter paper to remove particulate matter. An aliquot was removed test for anti-QS activity and the rest was evaporated to dryness and stored at -20°C. In addition, the dried plant material was added to sterile water at 200 g dry weight per litre, and boiled for 5 min. An aliquot was removed for testing and the remainder of the decoction was freeze-dried and the lyophilized water extracts were stored at -20°C. Water extracts were filtered by 0.45 µm-membrane filter (MEMBRANA, German) and collected by autoclaved vials to ensure sterility of the samples.

Bacterial strains and culture conditions

Chromobacterium violaceum CV026 was used to determine the anti-QS activity. N-hexanoyl homoserine lactone (C6-HSL) was purchased from Sigma-Aldrich (Denver, Colo. USA) and supplemented in *C. violaceum* CV026 cultures to induce violacein production. *C. violaceum* CV026 and *P.c.c.* was cultivated in Luria-Bertani (LB, 5 g yeast extract, 10 g tryptone, 5 g NaCl, 1 L water) medium at 30°C aerobically, overnight. *Escherichia coli* (*E. coli*) was grown in LB media at 37°C with 180-rpm agitation in a shaking incubator, overnight.

Screening for the effective plants

The disc-diffusion assay was used to detect anti-QS activity of the medicinal plant extracts by means of the double layer culture plates. 15 ml LB medium (1.5% agar) was overlaid with 15 ml LB (0.5% agar) containing 50 µl *C. violaceum* CV026 supplemented with 20 µg ml⁻¹ kanamycin and 50 ng ml⁻¹ C6-AHL. 20 µL of each extract was loaded onto sterile disks (8 mm diameter) and placed on the agar. The plates were then incubated overnight at 30°C and QS inhibition was detected as a colourless zone around the disk, where viable cells, indicative of growth but QS-inhibition, were also present. Measurements were made from the outer edge of the discs to the edge of the zones of anti-QS inhibition. Organic solvents were used as negative controls. Another control azithromycin was included to compare the antibiotic effect with anti-QS activity.

Anti-bacterial assay

The effective extract (20 µl) was added to 3 ml LB containing OD_{600nm} = 0.1 (approximately 1×10⁸ CFU ml⁻¹) *P.c.c.* and *E. coli* in the tubes, respectively. The tubes were further incubated for 12 h at 30°C, and the amount of bacteria was then counted with a spectrophotometer.

Assay for attenuation of soft rot disease on potato slices

Potato was surface sterilized with 10% Clorox which includes 0.6% sodium hypochlorite for 2 min, rinsed with tap water, air-dried and cut into slices (80×60 mm) before use. In the middle of each slice, one puncture wound (6 mm in diameter and 3 mm deep) were made. After wounding, potato slices were inoculated with 20 µl *P.c.c.* (1×10⁸ CFU ml⁻¹) and 20 µl different extracts. The control wounds were inoculated with 20 µl *P.c.c.* (1×10⁸ CFU ml⁻¹) alone and 20 µl *P.c.c.* (1×10⁸ CFU ml⁻¹) together with 70% ethanol. The potato slices were incubated at 37°C. There were 3

slices per replicate and three replicates per treatment.

Results

Anti-QS activity of the ethanolic extract of *Scutellaria baicalensis* Georgi

The biosensor strain, *C. violaceum* CV026, is a mutant of the wild type strain and is unable to produce its own AHL signal, but responds to exogenous active signal molecules to produce a purple pigment, violacein. Loss of purple pigment in *C. violaceum* CV026 cultured with exogenous AHL is indicative of QS inhibition by the plant extracts tested. No inhibitory effect was observed with the water extracts of all the plants tested in this study. Among the 13 medicinal plants, a strong anti-QS activity was only detected in the ethanolic extract of *Scutellaria baicalensis* Georgi. This inhibitory effect was relative to the amount of extract added (Figure 1).

The zone of inhibition was opaque, as a result of inhibition of QS and not inhibition of bacterial cell growth. Azithromycin, an antibiotic active against the indicator strain, was used as a control and gave a transparent zone of growth inhibition. This indicated that whilst the ethanolic extract of *Scutellaria baicalensis* Georgi could inhibit QS, it did not affect cell growth; the ethanol control had no effect on either QS or cell growth (Figure 1).

Antibacterial activity of the ethanolic extract of *Scutellaria baicalensis* Georgi

To further investigate the antibacterial activity of the ethanol extract of *Scutellaria baicalensis* Georgi, we cultivated the cells of plant pathogenic bacteria, *P.c.c.* and *E. coli* with or without the extract respectively for 12 hours. The absorbance was read with a Spectrophotometer at a wavelength of 600 nm (Figure 2). The result showed that no significant difference was observed in the number of bacterial cells between the treatments, further indicating that the

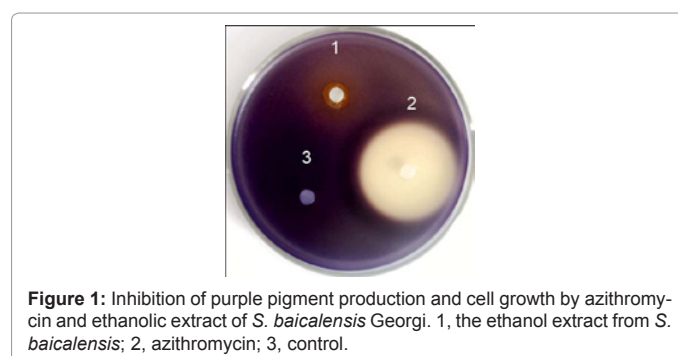


Figure 1: Inhibition of purple pigment production and cell growth by azithromycin and ethanolic extract of *S. baicalensis* Georgi. 1, the ethanol extract from *S. baicalensis*; 2, azithromycin; 3, control.

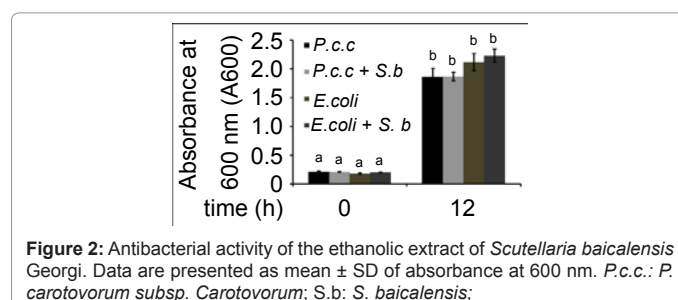


Figure 2: Antibacterial activity of the ethanolic extract of *Scutellaria baicalensis* Georgi. Data are presented as mean ± SD of absorbance at 600 nm. *P.c.c.*: *P. carotovorum* subsp. *Carotovorum*; *S.b.*: *S. baicalensis*;

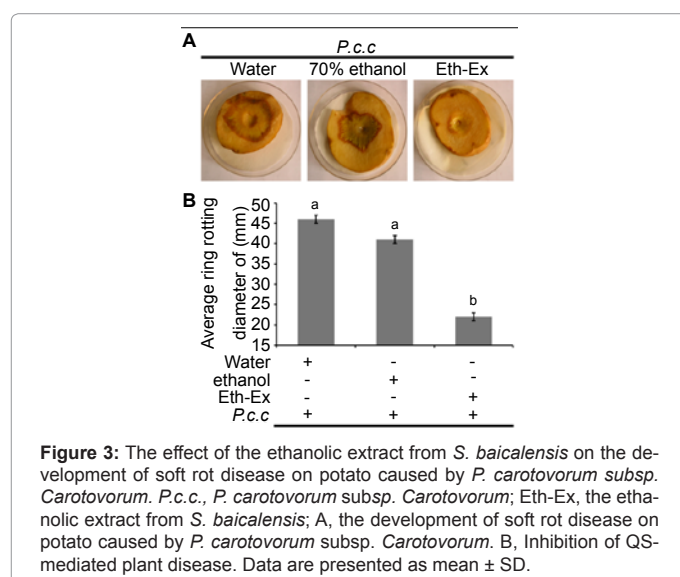


Figure 3: The effect of the ethanolic extract from *S. baicalensis* on the development of soft rot disease on potato caused by *P. carotovorum* subsp. *Carotovorum*. P.c.c., *P. carotovorum* subsp. *Carotovorum*; Eth-Ex, the ethanolic extract from *S. baicalensis*; A, the development of soft rot disease on potato caused by *P. carotovorum* subsp. *Carotovorum*. B, Inhibition of QS-mediated plant disease. Data are presented as mean \pm SD.

ethanolic extract of *Scutellaria baicalensis* Georgi has no antibacterial activity.

Inhibition of QS-mediated plant bacterial pathogenicity by the ethanolic extract of *Scutellaria baicalensis* Georgi

The ability of the ethanol extract from *S. baicalensis* to attenuate the pathogenicity of *P.c.c.* was tested. The surface of potato slices were inoculated with a cell suspension of *P.c.c.*, together with the ethanol extract from *S. baicalensis*. These were incubated (37°C, 24 h) and the diameter of maceration was measured. The results showed that the presence of the ethanolic extract from *S. baicalensis* significantly reduced the symptoms of soft rot. No obvious difference in the diameter of the maceration was detected between inoculating with *P.c.c.* alone and together with 70% ethanol controls (Figure 3).

Discussion

Scutellaria baicalensis Georgi, also named Huang-Qin, has been used since ancient time in China to treat allergic and inflammatory diseases by the mechanisms known as “cleansing heat”, “drying moisture”, and “removing toxins” [19]. It possesses various medicinal effects including antiviral, antibacterial antitumor, sedative, anti-pyretic, antihypertensive, diuretic, and haemostatic.

In this study, we found that *S. baicalensis* Georgi also possessed anti-QS activity. The production of violacein pigment in *C. violaceum* CV026, which is mediated by quorum sensing, was inhibited by the ethanolic extracts from *S. baicalensis* Georgi (Figure S1B). Further results showed that addition of the ethanolic extracts from *S. baicalensis* Georgi into incubation media did not affect the cell growth of *C. violaceum* CV026, *E. coli*, and *P.c.c.* (Figure S1, Figure 2). These data uncovered a potential mode of action for *S. baicalensis* Georgi to combat bacterial infection in humans in addition to those traditionally-known and further validated its continued use in traditional Chinese medicine.

In plants, certain pathogenic bacteria employ QS systems to control the expression of multiple virulence factors such as extracellular plant cell wall degradative enzymes. The results revealed that the development of soft rot disease caused by *P.c.c.* on potato slices was significantly inhibited by the ethanolic extracts from *S. baicalensis* Georgi, implicating the use of the extract as a biocontrol

agent for this particular QS-mediated bacterial disease in plants, but the exact quorum sensing signal inhibited by *S. baicalensis* Georgi is still unknown. Further study has to be conducted to investigate the mechanism.

Adonizio et al. [22] screened 6 species having anti-QS activity from 50 medicinal plants from southern Florida, USA, and found anti-QS activity present in both water and ethanol extracts of these plants, with the exception of *Chamaecybe hypericifolia* where anti-QS activity was only detected with its ethanol extract [20]. In this study, from the 13 medicinal plants tested, only one ethanol extract was found to possess anti-QS activity. No anti-QS activity was associated with the water extract of any of the 13 plants.

To our knowledge, this is the first report of QS inhibition by *S. baicalensis* Georgi. Inhibition of QS offers a new hope in combating with multi antibiotic-resistant bacteria. Inhibition of bacterial QS systems, rather than bactericidal or bacteriostatic strategies, may find application in many different fields, such as medicine, agriculture, and food technology. This approach is highly attractive because it does not impose harsh selective pressure for the development of resistance as with antibiotics because QS is not directly involved in processes essential for growth of bacteria. Furthermore, QS-inhibitory compounds are not expected to eliminate beneficial bacteria existent in the host. Therefore, natural nontoxic extracts, such as *S. baicalensis*, could have a greater advantage in many fields than toxic halogenated furanones.

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