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SHORT COMMUNICATION

Screening of traditional Chinese medicinal plants for quorum-sensing inhibitors activity

Khee Hoon Koh, Foong-Yee Tham*

Department of Natural Sciences and Science Education, Nanyang Technological University, Singapore, Singapore

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The misuse of antibiotics has contributed to widespread development of antimicrobial resistance among clinically significant bacterial species. Alternative approaches other than those using antibiotics are needed in the fight against infectious diseases. Quorum sensing (QS) is an intercellular signaling and gene regulatory mechanism, which is used by a number of opportunistic pathogenic bacteria in determining virulence gene expression. The study of QS may yield another strategy for disease control by interference with QS signals. Scientific research on complementary therapies such as traditional Chinese medicine (TCM) has focused mainly on its antibacterial properties. To test for anti-QS activity, 10 TCM herbs were screened using two biomonitor strains, *Chromobacterium violaceum* CV026 and *Pseudomonas aeruginosa* PA01. Interference with violacein (purple pigment) production in CV026 (exogenously supplied with homoserine lactone signals), and swarming in PA01, both QS-regulated phenomena, was used as indication of anti-QS activity. Eight of the selected TCM (80%) yielded QS inhibitors: *Prunus armeniaca*, *Prunella vulgaris*, *Nelumbo nucifera*, *Panax notoginseng* (root and flower), *Punica granatum*, *Areca catechu*, and *Imperata cylindrica*. Compounds that interfere with QS are present in TCM herbs and these medicines may be a rich source of compounds to combat pathogenic bacteria and reduce the development of antibiotic resistance.

Introduction

When the body's normal defense cannot overcome a disease, it is often treated with chemotherapeutic agents such as antibiotics. Antibiotics have reduced the death rate from infectious diseases, but its misuse has also contributed to rapid evolution of drug resistance among clinically significant bacterial species.[1] To meet the challenges of

* Corresponding author. Department of Natural Sciences and Science Education, Nanyang Technological University, 1, Nanyang Walk, Singapore 637616, Singapore.
E-mail address: foongyee.tham@nie.edu.sg (F.-Y. Tham).

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a reducing pool of efficacious antibiotics, assessing alternatives to address the issue of drug resistance is needed. Generally, large numbers of pathogens are needed to overwhelm host defenses to cause disease as a single individual pathogen is more easily destroyed. Many bacteria release low-molecular weight metabolic intermediates as signals for cooperation in activities such as colony formation and movement.[2] This mechanism, known as quorum sensing (QS), is an intercellular signaling and gene regulatory mechanism used by bacteria to respond to their population density. It was first observed in *Vibrio fischeri*, a marine symbiotic bacterium, in controlling luminescence. QS has also been identified in bacteria such as *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, and *Enterobacter agglomerans* for control of diverse functions such as chemiluminescence, competence activities, transformation, conjugation, and sporulation.[3] The signaling molecules or autoinducers (AIs) are either peptides, homoserine lactones, or γ-butyrolactones.[4] Concentration of AI is a key factor in determining virulence gene expression in several pathogenic bacteria. Hence, the study of QS can provide a strategy for disease control by controlling production or elimination of AIs.[5]

The QS circuit consisting of homologous LuxR/I systems is used by gram-negative bacteria and the AI is acyl-homoserine lactone (AHL). Aryl-homoserine lactone is also recently identified as a QS signal.[6] Different bacteria may produce AHLs varying in carbon chain and *N*-acyl chain.[7] AHLs bind to receptor proteins and induce QS target genes.[2] Hence, gram-negative bacteria can be rendered nonpathogenic by inhibition of their QS circuit through inhibition of AHL signal generation, dissemination, or reception.[8] Compounds that disrupt QS may provide an alternative treatment for infections other than antibiotics. Interestingly, QS inhibitors can be produced by higher organisms such as marine animals and plants.[9] These eukaryotes secrete substances that mimic bacterial AHL signals and affect QS-regulated behaviors in bacteria. One example is the red macroalga, *Delisea pulchra*, which produces halogenated furanone QS inhibitors that compete with bacterial AHLs for binding sites on receptors.[10] QS inhibitors were also reported from South Florida medicinal plants[11] and dietary components.[12–14]

Traditional Chinese herbs are used in the treatment of many ailments in the Far East.[15] Scientific research on traditional Chinese medicine (TCM) has focused mainly on their effectiveness in treatment of diseases ranging from hypertension to diabetes, hepatitis, and pneumonia as well as laboratory, biochemical, and microbiological work, in particular on their antibacterial properties.[16] Further research on determining their QS inhibition (QSI) ability is worth exploring. The objective of this study is to screen some commonly used TCM for QS inhibitory properties using two bacteria reporter species. The first is CV026, a mutant of *C violaceum*. Wild type of *C violaceum* produces violacein, a purple pigment, when AHL reaches a threshold level.[17] CV026 lacks the ability to produce violacein unless exogenous AHLs are supplied. In the presence of a mixture of exogenous AHLs and QSIs, transcription from the AHL-induced promoter is diminished and violacein production is abolished, indicating the presence of QSI(s). The other reporter used is wild type *P aeruginosa*, PA01. In the presence of a QSI compound, the swarming effect of PA01, a swarming bacterial species, will be limited.[18]

Methods

TCM plants

Ten plant parts from different herbs used in TCM were selected for screening for QSI (Table 1). They were selected based on the following criteria: (1) ethnobotanical efficacy as cures for acute inflammation, and treatment of fevers, coughs, sore throat, diarrhea, and dysentery;[15] and (2) general usage among the Chinese population. TCM herbs were purchased from Chinese medical halls in Singapore and voucher specimens stored in Microbiology Lab, Natural Sciences and Science Education Department, Nanyang Technological University, Singapore. Dried ground plant material was soaked in 1:1 acetone and water on a shaker overnight (O/N) and vacuum-filtrated with Whatman No. 1 filter paper to remove particulate matter (plant material weight to solvent volume ratio was 1/6). The filtrate was evaporated to dryness using a Heidolph rotary evaporator (Laborota 4000, Schwabach, Germany) and stored at 4°C. Extracts were reconstituted in deionized water to obtain desired dilutions for testing. Extracts were filter-sterilized using 0.22 μm (pore size) Iwaki filter disks before application.

Bacterial strains

CV026, a *C violaceum* mutant deficient in production of *N*-hexanoyl-L-homoserine lactone (C6HSL), which can produce purple pigmentation on external application of C6HSL, was used as a reporter organism to test for QSI. Wild type *P aeruginosa* (PA01) was also used as an additional reporter to test for QSI as it uses QS signals to activate several genes for swarming. *P aeruginosa* is an opportunistic pathogen responsible for a number of nosocomial infections. O/N CV026 cultures were grown in Luria Bertani (LB) broth at 30°C with shaking.[17] O/N PA01 cultures were grown on LB agar (LBA) plates at 37°C.

Bioassay for QSI activity using CV026

Five milliliters of warm molten Soft Top Agar (1.3 g agar, 2.0 g tryptone, 1.0 g sodium chloride, 200 mL deionized water) was seeded with 100 μL of an O/N CV026 culture, and 20 μL of 100 μg/mL C6HSL was added as exogenous AHL source. This was gently mixed and poured immediately over the surface of a solidified LBA plate as an overlay. Wells of 5 mm in diameter were made on each plate after the overlay had solidified. Each well was filled with 50 μL of filter-sterilized herb extract. The positive control well was filled with 5 μL of 100 μg/mL C10HSL (*N*-decanoyl-L-homoserine lactone) and 45 μL of sterile LB broth. A white or cream-colored halo around this well against a purple lawn of activated CV026 bacteria was an indication of QSI (Fig. 1). A clear halo indicated antimicrobial (AM) activity. The limit of detection of activity was also determined by applying serial dilutions of the extracts (1:1 to 1:16, using LB broth as diluent). Endpoints were estimated as the lowest dilution of

Table 1 Traditional Chinese medicines screened for antiquorum-sensing activity

Species (family)	Common name (Chinese name)	Plant part	Medicinal use[b]
Polygonatum odoratum (Liliaceae)[a]	Solomon's seal (Yu Zhu)	Underground stem	Treatment of fever, hacking cough, common cold, sore throat, rheumatism
Prunus armeniaca (Rosaceae)[a]	Common apricot (Bei Xing)	Kernel of seed	Treatment of common cold, coughs, bronchial asthma, rheumatism, constipation
Prunella vulgaris (Labiatae)	Heal-all (Xia Ku Cao)	Whole plant (stem, leaves, flower, fruit)	Treatment of fever, tuberculosis of the lymphatic glands, headache, inflammation of the eyes, dizziness
Nelumbo nucifera (Nymphaeaceae)	Lotus (Lian Ye)	Leaves	Treatment of sunstroke, diarrhea, dysentery, fever, dizziness, vomiting of blood
Lycium chinense (Solanaceae)	Chinese Wolfberry (Gou Qi)	Fruit	Treatment of anemia, back ache, vision problems, chronic cough, circulation, impotence, dizziness, tinnitus
Panax notoginseng (Araliaceae)	Notoginseng (Tian Qi Hua)	Flower	Treatment of dizziness, vertigo
P notoginseng (Araliaceae)	Notoginseng (Tian Qi)	Root	Treatment of nosebleed, injuries, fractures, heart problems; stops bleeding, reduces swelling and pain
Punica granatum (Punicaceae)	Pomegranate (Shi Liu Gen)	Bark	Treatment of piles, vaginal discharge, sore throat, bad breath, nosebleed
Areca catechu (Palmae)	Betel Nut (Bing Lang)	Seed	Treatment of diarrhea, indigestion, lumbago, urinary problems; expels tapeworms & roundworm.
Imperata cylindrica (Gramineae)	Lalang (Bai Mao Gen)	Underground stem	Stop bleeding, fever, acute inflammation of kidney, cough with phlegm

[a] Extracts remained in sticky or oily liquid form that could not be evaporated to dryness and were used directly in bioassays.
[b] Ref. 15; traditional Chinese medicine dispensers.

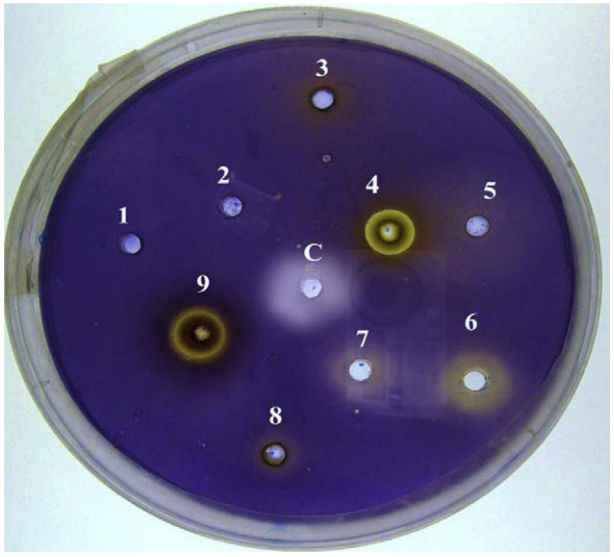

Figure 1. Bioassay using *Chromobacterium violaceum* CV026 as the reporter strain. A cream-colored halo is produced around the wells when quorum-sensing inhibition (QSI) compounds are present and prevents CV026 from responding to the exogenously applied C6HSL. The cream-colored CV026 bacteria are able to grow around the wells, but are unable to produce purple pigment. Positive control, C, with 100 μg/mL C10HSL, a QSI of CV026. The bacteria around the positive control well was able to grow, but did not produce violacein. The TCM extracts shown are: (1) *Polygonatum odoratum*, (2) *Prunus armeniaca*, (3) *Prunella vulgaris*, (4) *Nelumbo nucifera*, (5) *Lycium chinense*, (6) *Panax notoginseng* (flower), (7) *P notoginseng* (root), (8) *Punica granatum*, (9) *Areca catechu*. TCM 3, 4, 6, 7, 8, and 9 showed varying degrees of QSI (creamish to yellowish, violacein-absent zones around wells). Clear zones showing antibiotic activity of the extracts are most obvious in TCM 4 and 9.

extract giving discernible inhibition of violacein synthesis. Each experiment was repeated and the assay plates were incubated at 30°C for 3 days.

Anti-swarming in PA01

A loopful of O/N PA01 culture on LBA was resuspended in 4 mL of sterile LB broth. Fifty microliters of sterile herb extract was mixed with 3 mL of molten Soft Top Agar and poured immediately over a plate. Five microliters of the PA01 suspension was inoculated in the center of the plate when the agar had solidified, and the surface was air-dried for 10—15 minutes. All experiments were repeated and the plates were incubated at 37°C for 3 days. Extent of swarming was determined by measuring the area of the colony in square millimetre using graph paper.

Results and discussion

With CV026 as a reporter strain, seven out of ten TCM plant parts (i.e. 70%) displayed QS antagonistic activity as indicated in Table 2. *Polygonatum odoratum* and *Lycium chinense* which were also screened in the present study did

Table 2 Traditional Chinese medicinal plants that exhibit QSI and AM activity as detected *by Chromobacterium violaceum*, CV026 and *Pseudomonas aeruginosa*, PA01

TCM plant species and plant part	CV026		PA01
	AM[a]	QSI[b]	QSI[c]
Prunus armeniaca (kernel of seed)	No	No	Yes (29%)
Prunella vulgaris (whole plant)	Yes (7)	Yes (15.5)	No*
Nelumbo nucifera (leaf)	Yes (11)	Yes (16)	No*
Panax notoginseng (flower)	Yes (8)	Yes (20)	Yes (32%)
P notoginseng (root)	No	Yes (24)	Yes (50%)
Punica granatum (bark)	Yes (12)	Yes (15)	No
Areca catechu (seed)	Yes (14.5)	Yes (18)	Yes (79%)
Imperata cylindrica (underground stem)	Yes (12)	Yes (20)	No*

[a] Presence of antimicrobial inhibition observed as a clear halo. Diameter of zone of inhibition in millimeter in parentheses.
[b] Diameter of zone of quorum-sensing inhibition (cream-white halo) in millimeter in parentheses.
[c] Percentage reduction of area of colony (indicating inhibition of swarming) compared with control in parentheses. The percentage of reduction was calculated by comparing the area of the swarming colonies on medium supplemented with TCM with reference to that of the control on medium without TCM. Extracts marked with an asterisk increased the area of the colony (promoted swarming) compared with the control.
AM = antimicrobial; QSI = quorum-sensing inhibition; TCM = traditional Chinese medicine.

not show distinct QSI or AM activity with the two reporters used. *Panax notoginseng* extracts had among the greatest QSI zones with CV026. The percentage of plants producing QSI compounds here is much higher than reported from other ethnobotanical screening[11] and could be because of the criteria used for selection of TCM herbs. The herbs selected have had a long history of use by the Chinese as remedies for illness caused by bacterial infections, such as diarrhea, eye infection, and inflammation. The results appear to provide some support for the validity of the use of these traditional herbs.

The seven extracts with QSI activity also exhibited dilution-dependent inhibitory results on CV026. The lowest dilution of extract that gave visually detectable inhibition of violacein synthesis ranged from 1:4 to 1:8. *P notoginseng* (flower) and *Prunella vulgaris* showed inhibition even at 1:8 dilution whereas in *Imperata cylindrica*, inhibition disappeared at 1:2 dilution. The other TCM continued to exhibit inhibition up to 1:4 dilution.

A clear halo is produced when there is no bacterial growth around wells. This indicates that AM activity is present in the extract. Clear halos of varying diameters formed around wells for six of the TCM, indicating presence of antibiotic compounds, in addition to QSI compounds (Table 2). Of these, *Areca catechu* produced the largest zone of AM inhibition (Table 2).

Flagella motility-dependent swarming is also under-regulation of QS-related gene expression. A reduction in swarming area compared with control suggests presence of QSI compounds. Four of the TCM distinctly reduced the swarming area in the reporter PA01, suggesting inhibition of swarming motility (Table 2). *A catechu* reduced swarming the greatest (79% reduction compared with control). In contrast, three extracts increased swarming, with *I cylindrica* inducing the most swarming compared with control. *Prunus armeniaca* extract inhibited swarming in PA01 only, but did not inhibit violacein production in CV026 (Table 2).

Extracts from *P vulgaris*, *Nelumbo nucifera*, *Punica granatum*, and *I cylindrica* also had both AM and QSI activity. *P vulgaris* has been reported to have antiseptic and antibacterial effects.[19] In this study, extracts from leaves of *N nucifera* appeared to be one of the TCMs, which showed good AM and QSI activity. Lotus is an aquatic plant with large leaves exposed to water, presenting a great surface for growth of bacteria. It could be that this plant in nature has some mechanism, as the marine alga, *D pulchra*, to produce compounds that deter biofilm formation on its surface. The QSI compound, furanone, was first isolated from *D pulchra*.[10]

Interestingly, extracts from different parts of *P notoginseng*, i.e. roots and flowers, showed slightly different effects on the reporter bacteria CV026 and PA01. Roots appeared to have only QSI activity whereas flowers seemed to have both AM and QSI properties. Together with *A catechu*, these extracts were capable of inhibiting QS in both CV026 and PA01 bacteria, and seemed to have a wider spectrum of QSI activity compared with the other TCM that inhibit QS in only either of one bacteria.

In this study, reduction in swarming in PA01 was used as a reporter for presence of QSI. Reduction in swarming could be because of either inhibition of PA01 growth or quenching of QS signals. To distinguish between these effects, bacteria can be isolated from treated PA01 and grown on LBA to test for viability. Although purple pigmentation inhibition in CV026 and swarming effect in PA01 are easily observable phenotypes that facilitate screening for QSI, they do not provide deeper insight as to the precise types and numbers of active chemical compounds present. QSI can be achieved through several methods such as signal binding, degradation or direct interaction with the gene.[8] Vanilla extract is reported to inhibit QS in CV026.[13] The main components, however, do not show structural similarity to natural AIs or furanone derivatives, and is believed to be a new class of inhibitors. For future studies, thin layer chromatography or liquid chromatography-mass spectrometry could be used to identify the anti-QS compounds in TCM. Screening for QSI activity can also be improved by using multiple bacteria reporters[11] as ability to detect QSI

depends on the reporter systems used. When using a single reporter CV026, QSI was detected in 70% of the TCM in the present study. However, when using PA01 as a reporter alone, QSI was detected in only 40% of the TCM (Table 2). Thirty percent of the TCM inhibited QS-mediated phenomenon in both reporters with the rest inhibiting only QS in one reporter.

TCM is commonly used by people in the Far East as an alternative as well as supplement to mainstream health care.[15,16] There is active interest in searching for new therapeutic approaches to deal with pathogenic bacteria. In particular, alternative modes of action against opportunistic bacteria that use QS to regulate virulence expression have received special attention.[20] Eight out of ten TCM screened in the present investigation exhibited anti-QS properties. QSI compounds are thus shown to be present in TCM herbs and these medicines may be a rich source of compounds to combat pathogenic bacteria and reduce development of antibiotic resistance.

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