Evaluation of Anti-Quorum Sensing Activity of 97 Indigenous Plant Extracts From Korea through Bioreporter Bacterial Strains Chromobacterium violaceum and Pseudomonas aeruginosa

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Abstract

Quorum sensing (QS) is a recently discovered chemical communication system that enhances survival of bacteria, as a group allowing resident bacteria to assume specialized roles vital for intra- and inter bacterial gene regulation, and for keeping bacterial colonies intact. Furthermore, with the continuing emergence and spread of multidrug-resistant bacteria, antipathogenic strategy to combat bacterial infections through the interruption of quorum sensing controlled virulence factors had been shown to receive increased attention. With this prospect in the current study, we attempt to screen anti-quorum sensing activity of 97 indigenous plant extracts from Korea, through biomonitor bacterial strains, Chromobacterium violaceum (CV12472) and Pseudomonas aeruginosa (PAO1). Standard disc-diffusion assays were used to detect anti-quorum sensing activity (ring of colorless, but viable cells around the disk), of the plant extracts for CV12472. A special swarm media that allow swarming motility growth of POA1 was used to conduct inhibition of swarming motility assay. Minimum Inhibitory Concentration (MIC) test for the 97 plant extracts against bioreporter strains (CV12472 and PAO1) revealed antibacterial activity of three plant extracts (Potentilla cryptotaeniae, Viburnum carlesii and Prunus ammenica var. ansu). Out of the 97 plant extracts, significant inhibition of pigment production were detected by six plant extracts in CV12472, while 16 plant extracts had shown inhibition of swarming motility in POA1. In conclusion, a total of 18 plant extracts were screened for their anti-quorum sensing activity by the two bioreporter strains. Of the 18 plant extracts, four had shown anti-quorum activities in both bioreporter strains.

Keywords: Quorum sensing; 97 plant extracts; Chromobacterium violaceum; Pseudomonas aeruginosa

Introduction

The rising challenge paused by infectious bacterial drug resistance could easily be recognized from the loss of susceptibility observed in about 70% of the bacteria, that cause infections to a minimum of one drug among the routinely used treatment [1]. For example E. coli isolate in US showed an increase from 3 to 17.1% in resistance from 2000 to 2010 for ciprofloxacin [2]. Furthermore, some organisms are resistant to all approved antibiotics, because the environmental antibiotics pressure activates the evolutionary mechanisms that select for resistant strains.

Quorum sensing is a recently discovered chemical communication system that enhances survival of bacteria, as a group allowing resident bacteria to assume specialized roles vital for intra- and inter bacterial gene regulation, and for keeping bacterial colonies intact. These involve several processes, such as specific signaling molecules that bind to and activate receptors that transduce the quorum-sensing signal into intracellular second messenger responses, in a similar fashion to ligand-receptor interaction [3]. This similarity opens a novel alternative that should be looked for in combating drug resistance by the infectious bacteria, with inhibitor drugs that could be designed using current standard pharmacologic principles [3,4]. Therefore, quorum-sensing inhibition offers new hope in combating resistant bacteria with inhibitor drugs that might have novel mechanisms of action, and could, therefore, be more effective against antibiotic-resistant strains of bacteria. In this aspect, although the already known quorum sensing inhibitor halogenated furanones from Delisea pulchra failed to pass for clinical therapeutic use due to its toxicity, and still there are no such drug inhibitors that are currently used clinically, the attempt is an indicative of the potential of the alternative option in combating antibiotic resistance and good example of proving this hypothesis [5]. Other attempts in identifying candidate anti-quorum sensing activities by extracts of garlic, vanilla and essential oil had been reported previously [6,7]. In the current study, we aim to screen anti-quorum sensing activity of 97 indigenous plant extracts from Korea through biomonitor bacterial strains, Chromobacterium violaceum and Pseudomonas aeruginosa.

Materials and Methods

Collection of plant material

One hundred methanol plant extracts (Table 1) from 97 plant species collection were prepared and provided by Korea Research Institute of Bioscience and Biotechnology (KRIBB), for this study. Plant extract selection was simply by availability.

Bacterial strains and culture medium

Bio-reporter strain C. violaceum (CV12472), a wild strain,
| No. | Species Name                          | Family name | Parts extracts prepared |
|-----|--------------------------------------|-------------|-------------------------|
| 1   | Morus bombycis for. kase             | Moraceae    | Leaf, Stem              |
| 2   | Quercus aliena                       | Fagaceae    | Leaf, Stem              |
| 3   | Forsythia koenae                     | Oleaceae    | Leaf, Stem              |
| 4   | Rumex longifolius                    | Polygonaceae| Whole plant             |
| 5   | Papaver rhoes                       | Papaveraceae| Whole plant             |
| 6   | Boehmeria tricuspis                  | Urticaceae  | Whole plant             |
| 7   | Philadelphia schrenckii              | Saxifragaceae| Leaf, Stem, Flower     |
| 8   | Aci pumt vari. mono                  | Acraceae    | Leaf, Stem              |
| 9   | Rubus phoenicolasius                 | Rosaceae    | Leaf, Stem, Flower      |
| 10  | Securinega sulphurella               | Euphorbiaceae| Leaf, Stem             |
| 11  | Carex neurocarpa                     | Cyperaceae  | Whole plant             |
| 12  | Angelica dahurica                    | Umbellifera | Root                    |
| 13  | Lonicera vesicaria                   | Caprifoliaceae| Leaf, Stem           |
| 14  | Prunus padus                         | Rosaceae    | Leaf, Stem, Flower      |
| 15  | Beta vulgaris var. cicla             | Chenopodiaceae| Whole plant         |
| 16  | It is saviatre                        | Iridaceae   | Whole plant             |
| 17  | Sciadopitys verticillata             | Taxodiaceae | Leaf                    |
| 18  | Calendula arvensis                   | Composite   | Whole plant             |
| 19  | Draba nemorosa var. hebecarpa        | Cruciferae  | Whole plant             |
| 20  | Silene aemoria                       | Caryophyllaceae| Whole plant       |
| 21  | Junipenis rigida                     | Cupressaceae| Leaf, Stem              |
| 22  | Celastrus orbiculatus                | Celastraceae| Leaf, Stem              |
| 23  | Clerodendrum trichotomum             | Verbenaceae | Leaf, Stem              |
| 24  | Ulmus davidiana var. japonica        | Ulmaceae    | Leaf, Stem              |
| 25  | Ambrosia trifida                     | Composite   | Whole plant             |
| 26  | Rheum undulatum                      | Polygonaceae| Whole plant             |
| 27  | Cocculus trilobus                    | Menispermacae| Whole plant         |
| 28  | Rodgeriales podophylla               | Saxifragaceae| Underground         |
| 29  | Aerial continentalis                 | Araliaceae  | Whole plant             |
| 30  | Sambucus williams vari. coreana      | Caprifoliaceae| Leaf                    |
| 31  | Sedum oregifolium                    | Crassulaceae| Whole plant             |
| 32  | Grapnhamillum affine                 | Compositae  | Whole plant             |
| 33  | Allium sativum for. pekinense        | Liliaceae   | Whole plant             |
| 34  | Tagetes patula                       | Compositae  | Whole plant             |
| 35  | Sambucus sieboldiana var. pendula    | Caprifoliaceae| Leaf, Seed           |
| 36  | Persicaria seneciosa                 | Polygonaceae| Ground                 |
| 37  | Persicaria perforlata                | Polygonaceae| Whole plant             |
| 38  | Chenopodium album var. centrorubrum  | Chenopodiaceae| Leaf, Stem          |
| 39  | Potentilla cryptolataeae             | Rosaceae    | Whole plant             |
| 40  | Epilobium pyrroclophum              | Onagraceae  | Whole plant             |
| 41  | Pinus densiflora for. multicaulis    | Pinaceae    | Stem                    |
| 42  | Dictamus dasycarpus                  | Rutaceae    | Whole plant             |
| 43  | Pinus bungeana                       | Pinaceae    | Stem                    |
| 44  | Lotus corniculatus var. japonicus    | Leguminosae | Whole plant             |
| 45  | Carex parviflora var. macroglosa     | Cyperaceae  | Whole plant             |
| 46  | Eulaeagnus umbellate                 | Eulaeagnaceae| Leaf, Seed            |
| 47  | Viburnum carlesi                     | Caprifoliaceae| Leaf, Stem, Flower  |
| 48  | Oryza sativa var. terrestris         | Gramineae   | Whole plant             |
| 49  | Prunus ammeniaca var. ansu           | Rosaceae    | Leaf, Stem              |
| 50  | Orika japonica                       | Rutaceae    | Leaf, Stem, Flower      |
| 51  | Taraxacum officinale                 | Compositae  | Whole plant             |
| 52  | Stachys riederi var. japonica        | Labiatae    | Whole plant             |
| 53  | Phnomis umbrosa                      | Labiatae    | Whole plant             |
| 54  | Senecio integrifolius var. spathalathus| Compositae   | Whole plant             |
| 55  | Potentilla discolor                  | Rosaceae    | Whole plant             |
| 56  | Centaurea cyanus                     | Compositae  | Whole plant             |
| 57  | Nymphaea tetragona var. angusta      | Nymphaeaceae| Underground             |
| 58  | Astariea koreana                     | Saxifragaceae| Whole plant         |
| 59  | Eudora daniieli                      | Rutaceae    | Stem                    |
| 60  | Passiflora coerulea                  | Passifloraceae| Ground              |
| 61  | Spianica oleara                      | Chenopodiaceae| Whole plant        |
| 62  | Ixiris dentata                       | Compositae  | Whole plant             |
| 63  | Robinia pseudo-acacia                | Leguminosae | Leaf, Stem             |
| 64  | Rumex acetocefa                      | Polygonaceae| Whole plant             |
| 65  | Crataegus maximowsicizi              | Rosaceae    | Leaf, Stem, Flower      |
| 66  | Prunus salicina var. columnatis      | Rosaceae    | Leaf, Stem, Flower      |
| 67  | Mallotus japonicus                   | Euphorbiaceae| Leaf, Stem             |
| 68  | Paulownia coreana                    | Scrophulariaceae| Stem-Bark       |
| 69  | Cucumis sativus                      | Cucurbitaceae| Ground                 |
| 70  | Vitis amurensis                      | Vitaceae    | Leaf, Stem              |
| 71  | Acer saccharinum                     | Aceraceae   | Leaf, Stem              |
| 72  | Potentilla nivea                      | Rosaceae    | Whole plant             |
| 73  | Magnoliob obovata                    | Magnoliaceae| Leaf, Stem             |
| 74  | Ardisia japonica                     | Myrsinaceae | Whole plant             |
| 75  | Hydrocharis dubia                    | Hydrocharitaceae| Whole plant    |
| 76  | Callicarpa japonica                  | Verbeanaeae | Leaf, Stem             |
| 77  | Mellotus suaveolens                  | Leguminosae | Ground                 |
| 78  | Cephalonclos setegum                 | Compositae  | Whole plant             |
| 79  | Boheheria scipica                    | Urticaceae  | Leaf, Stem              |
| 80  | Carex pumila                         | Cyperaceae  | Whole plant             |
| 81  | Ligurium obtusifolium                | Oleaceae    | Leaf, stem              |
| 82  | Rosa multiflora                      | Rosaceae    | Leaf, Stem              |
| 83  | Lilium lancifolium                   | Lilaceae    | Whole plant             |
| 84  | Pimpinella brachycarpa               | Umbelliferae| Whole plant             |
| 85  | Paulownia tomentosa                  | Scrophulariaceae| Leaf               |
| 86  | Aster scaber                         | Compositae  | Whole plant             |
| 87  | Portulaica grandiflora               | Portulacaceae| Whole plant         |
| 88  | Cornus controversa                   | Cornaceae   | Leaf, Stem              |
| 89  | Lysmachia cletroides                 | Primulaceae | Whole plant             |
| 90  | Disporum viridescens                 | Liliaceae   | Whole plant             |
| 91  | Achilea sibirica                     | Compositae  | Whole plant             |
| 92  | Phyma leptostachya var. asiatica     | Phymaceae   | Whole plant             |
| 93  | Dianthus sinensis                    | Caryophyllaceae| Whole plant    |
| 94  | Vitis vinifera                       | Vitaceae    | Ground                 |
| 95  | Cleome spinosa                       | Capparidaceae| Ground                 |
| 96  | Humulus japonicus                    | Cannabisaceae| Ground                |
| 97  | Viola patrina                        | Violaceae   | Whole plant             |

Table 1: List of plant species and their parts extracts prepared for anti-quorum sensing activity.

Producing quorum sensing controlled purple pigment violacein, that produces and responds to the cognate C₃ and C₆ Acyl Homoserine Lactones (AHls), an important intercellular signaling molecules used by bacteria to monitor their population density, and P. aeruginosa (PAO1), a pathogenic strain with many traits controlled by quorum sensing signaling, including swarming motility were used in the study. A stock culture of Chromobacterium violaceum (C. violaceum) was purchased from Sigma stock. Chromobacterium violaceum (C. violaceum) was provided by Professor Stephen K Farrand, University of Illinois, USA. Unless and otherwise stated, all strains were grown in LB broth and Agar (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) media, with temperature ranging 30-37°C.
**Determination of MIC**

MIC of the 97 plant extracts was determined against biosensor strains *C. violaceum* (CV12472), and *P. aeruginosa* (PA01) by broth macrodilution method. MIC was defined as the minimum concentration of plant extracts at which there was no visible growth of test strain.

**Bioassay for anti-quorum sensing**

Disk diffusion assay, swarming motility assay and flask incubation assay were selected for their simplicity, and ability to easily and inexpensively investigating large number of biological materials, for their potential anti-quorum sensing activity. The flask incubation assay has also an advantage on to quantify the production of the violacein production by *C. violaceum* (CV12472).

**Disk diffusion assay**

The Disk diffusion assay is an assay used to evaluate anti-QS activity by evaluating zones of inhibition around the disk, in a similar fashion to a standard disk diffusion assay used for antibacterial activity test. Standard disc-diffusion assays were used to detect anti-QS activity of the plant extracts, as previously described [8]. Briefly, each extract (50 µL) was loaded onto sterile disks (6 mm diameter), placed onto prepared LB plates spread with overnight culture (100 µL) of *C. violaceum* (CV12472). Plates were incubated at 30°C overnight and anti-QS activity was detected by a ring of colorless, but viable cells around the disk. Measurements were made from the outer edge of the disks to the edge of the zones of anti-QS activity. Purified halogenated furanon (100 µg) was used as a positive control for anti-QS activity, and methanol (20 µL) as a negative control. The methanol was allowed to evaporate from the control and sample discs before testing, to eliminate toxicity. A third control (Orbifloxacin 10 µg per disc) was included to compare antibiotic effect with anti-QS activity.

**Swarming motility assay (P. aeruginosa PA01)**

The Swarming motility assay were conducted with LB media, consisted of 0.5% (wt/vol) Difco bacto-agar, to which 5 g/liter glucose was added. Swarm plates (small size Petri dish (30×10)) were typically allowed to dry at room temperature overnight, before being used. Swarm plates prepared with 50 µL extracts were inoculated with bacteria from an overnight culture in LB agar, and incubated for 24 hr at 30°C. Levels of the swarming were determined by measuring diameters of the swarms and comparing it with the control.

**Flask incubation assay for quantification of violacein production**

The flask incubation assay used to quantify anti QS activity was determined, as described elsewhere [7]. Briefly, *Chromobacterium violaceum* (CV12472) was incubated for 16-18 h and inoculated to OD₅₆₅nm=0.1 in Erlenmeyer flasks containing 20 mL LB, LB supplemented with Furanon, and LB supplemented with extracts at concentrations of 1% plant extracts and 1 µg/mL of Furanon. The flasks were incubated at 30°C, with 150 rev min⁻¹ agitation for 24 h in a shaking incubator. Then after from each flask, 1 mL of culture sample was transferred to 1.5 mL e-tube and centrifuged at 13 000 rev min⁻¹ for 10 min to precipitate the insoluble violacein. After discarding the culture supernatant, 1 mL of DMSO was added to the pellet and vortexed vigorously for 30 s, to completely solubilize violacein and centrifuged at 13 000 rev min⁻¹ for 10 min to remove the cells. From the violacein-containing supernatants, 200 µL were transferred to four wells of 96-well flat bottomed microplates (SPL Life science, Gyeonggi-do, Korea), per each extract and control samples, and the absorbance was read with a microplate reader (Versa max Molecular Devices, Sunnyvale, CA, USA), at a wavelength of 585 nm. To confirm any antibacterial activity by the extracts in each flask, 100 µL of culture was collected and serially diluted to factors of 10⁻¹⁻¹⁸, and 100 µL of the diluted cultures were spread on LB-agar plates from each flask. The plates were incubated at 30°C for 24 h, and bacterial colony counts were compared with control.

**Results**

**Antibacterial activity against test organisms**

MIC test for the 97 plant extracts against bio-reporter strains (CV12472 and PA01) revealed no inhibition (≥ 8.4% v/v), except three plants (*Potentilla cryptantha varia, Viburnum carlesii* and *Prunus armeniaca var. ansu*), which have showed antibacterial activity at 8.4% (v/v) concentration for *P. aeruginosa* and one (*Viburnum carlesii*) for *C. violaceum* at the same concentration.

**Anti-quorum sensing activities against C. violaceum and P. aeruginosa**

Production of purple colored violacein pigmentation by quorum sensing chemical communications in *C. violaceum* provides a naturally occurring, readily observable phenotype, without the need for additional substrate, and eases the evaluation of quorum sensing inhibition of compounds in this bacterium [9]. Using this bacterium, 97 plant extracts (Table 1) were screened for anti-quorum sensing activities, out of which significant inhibition of pigment production were detected by six plant extracts (Table 2). Except one, all the remaining five plant extracts did not show antibacterial activity. This might be an important trait, as quorum sensing inhibition is focused on the interference of bacterial signaling. Our result did not show any growth inhibition zones of test organism, except *Viburnum carlesii* extract (Figure 1). Furthermore, besides the MIC test, additional experiments of disc diffusion assay at higher doses by all the 97 plant extract shown inhibition zones, only by the three plant extracts similar to the MIC test, indicating the tested plant extracts have less antibacterial effect on the growth of *C. violaceum*.

In case of *P. aeruginosa*, flagellar motility dependent swarming are under regulation of quorum sensing related gene expressions with other virulence characters, such as biofilm formation and proteolytic activity. This surface translocation on the surface of agar, 0.45% or more in concentration, supports a swarming motility has been considered as quorum sensing inhibitor indicator [6,10]. From the 97 plant extracts we investigated for quorum sensing inhibition of *P. aeruginosa*, as measured by the inhibition of swarming motility, 16 plant extracts had shown inhibition of quorum sensing activity against *P. aeruginosa* (Table 2). The plant extracts show inhibition, ranging from 33.33-71.42% compared to the control (Figure 2). From the 16 plant extracts, four of them have also shown inhibition of pigment production in *C. violaceum*. Furthermore, the inhibitory activity of the six plant extracts against bacterial quorum sensing was determined using violacein production. As indicated in figure 2 (Left panel), significant reduction in violcin production compared to the normal control in *C. violaceum* CV12472 was observed by all the six plant extracts (P<0.05), while bacterial cell count performed on LB Agar plates at 24 h incubation from the same experimental flask of the inhibition experiments revealed no significant difference in the number of colony forming units (CFU) (Figure 2 (Right panel)).
Table 2: Result summary for screening of 97 plant extracts anti-quorum sensing activity with C. violaceum and P. aeruginosa.

| Species Name                  | Family name          | Parts extracts prepared | Zone of inhibition |
|------------------------------|----------------------|-------------------------|--------------------|
| Rumex longifolious           | Polygonaceae         | Ground                  | 14 (66.67)         |
| Rubus phoenicolasius         | Rosaceae             | Leaf, Stem              | 15 (71.42)         |
| Carex neurocarpa             | Cyperaceae           | Whole plant             | 11 (52.38)         |
| Securinega suffruticosa      | Euphorbiaceae        | Leaf, Stem              | 3                  |
| Angelica dahurica            | Umbelliferae         | Root                    | 4                  |
| Prunus Padus                 | Rosaceae             | Leaf, Stem, Flower      | 11 (52.38)         |
| Calendula arvensis           | Compositae           | Whole plant             | 13 (61.9)          |
| Silene armeria               | Caryophylliaceae     | Whole plant             | 13 (61.9)          |
| Rodgersia podophylla         | Saxifragaceae        | Underground             | 6                  |
| Potentilla cryploaenae       | Rosaceae             | Whole plant             | 13 (61.9)          |
| Pinus bungeana               | Pinaceae             | Stem                    | 14 (66.67)         |
| Viburnum carlesii            | Caprifoliaceae       | Leaf, Stem, Flower      | 6 (71.42)          |
| Prunus armeniaca var. ansu   | Rosaceae             | Stem                    | 13 (61.9)          |
| Centaurea cyanus             | Compositae           | Whole plant             | 13 (61.9)          |
| Nymphaea tetragona var. angusts | Nymphaceae           | Underground             | 12 (71.43)         |
| Malotus japonicus            | Euphorbiaceae        | Leaf, Stem              | 7 (33.33)          |
| Pimpinella brachycarpa       | Umbelliferae         | Whole plant             | 9 (42.85)          |
| Disporum viridescens         | Liliaceae            | Whole plant             | 14 (66.67)         |
| Control                      |                      |                         |                    |
| Furanon                      | Positive control     |                         | 5 (80.95)          |
| Orbifloxacin                 | Antibiotic           |                         | 2 (9.52)           |

*Zones are in mm beyond the sample disk** Zones are the diameters untreated swarming movements minus the treated swarming movement in mm

Figure 1: Anti-quorum sensing activity of (A) six plant extracts against C. violaceum with disc diffusion assay and (B) a selected representative extracts anti-quorum sensing activities against P. aeruginosa swarming motility. Methanol (Met) and Orbifloxacin (Orb).

Figure 2: Inhibition of violacein production and bacterial cell count by flask incubation assay. Violacein production inhibition (Left panel) and bacterial count from the same experiment (Right panel) by the six plant extract as measured spectrophotometrically, as described in the materials and methods. Data are presented as mean ± SD of absorbance at 585 nm for violacein production, and as the logarithm of mean CFU ± SD for bacterial count (*P>0.05).
Discussion

Recently, because of continuing emergence and spread of multidrug-resistant bacteria, antipathogenic strategy to combat bacterial infections through the interruption of quorum sensing controlled virulence factors has received increased attention [11]. In the present study, the most known cases of quorum sensing system regulated phenotypes, the pigment production by C. violaceum bio-reporter strain and the swarming motility of P. aeruginosa potential were utilized to screen 97 plant extracts for their quorum sensing inhibition potentials. These plant extracts investigated for their anti-quorum sensing potential were selected from indigenous and other Korean plants of bio-sources. Our findings indicated the potentials of the indigenous and other plants as a source of anti-quorum sensing compounds, and highlight the importance of evaluating the unexplored diversity of indigenous and other plant bio-sources, besides the usual evaluation of traditionally used medicinal plant sources for such activity. Besides, this is the first report on anti-quorum sensing activity form indigenous plants bio-sources of Korea. However, others have reported anti-quorum sensing activity of medicinal plant, essential oil and edible plants and fruits extracts of phytochemicals [4,6,8,12,13]. Contrary to our observation in previous studies, most of the plant extracts in this study have not shown any antibacterial activity, even at their higher concentrations. This might be due to the effects of the extract compounds in limited molecular target areas, that might involve only in quorum sensing signaling of the bacteria [4,6,8].

While four plant extracts were inhibiting quorum sensing of both C. violaceum pigment production and the swarming motility of P. aeruginosa, 2 and 12 other plant extracts were observed, inhibiting quorum sensing and the swarming motility of C. violaceum and P. aeruginosa, respectively. This is suggestive of the responsible compounds of the four plant extracts have a broad spectrum effect in inhibiting, both the signaling involved in pigment production of C. violaceum, and the swarming motility of P. aeruginosa. On the other hand, the higher numbers of plant extracts observed inhibiting the swarming motility of P. aeruginosa might be due to many down and upstream signaling involved the swarming motility of P. aeruginosa, creating multiple targets of interaction for inhibitions by compounds of many plant extracts.

The observation of anti-quorum sensing activities of plant extracts from more than one plant species from one family, such as Euphorbiaceae, Rosaceae, Compositae suggests further studies of other plants species in this families.

In summary, the selected indigenous and other Korean plants of bio-sources may have a potential, and serve as important as medicinal plants species in this families.

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