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Anti-quorum sensing and anti-biofilm formation activities of plant extracts from South Korea

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ABSTRACT

Objective: To investigate anti-quorum sensing (anti-QS) and anti-biofilm formation (anti-BF) activities of the ethanol extracts of 388 plants. **Methods:** The anti-QS activity of the plant extracts was evaluated by disc-diffusion assays using the bio-reporter strain, *Chromobacterium violaceum* CV017. *Pseudomonas aeruginosa* PAO1, *Yersinia enterocolitica* ATCC 9610, and *Agrobacterium tumefaciens* C58, which possess QS systems, were used to evaluate the anti-BF activity of the plant extracts. **Results:** Among 388 plant extracts, the *Cornus controversa* (*C. controversa*) and *Cynanchum wilfordii* extracts exhibited the strongest anti-QS activity. The *C. controversa* extract exhibited anti-BF activity against *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Agrobacterium tumefaciens*, whereas the *Cynanchum wilfordii* extract exhibited no anti-BF activity against *Pseudomonas aeruginosa*. In addition, the *C. controversa* extract suppressed soft rot of cabbage. **Conclusions:** The *C. controversa* extract inhibits bacterial QS and BF, and is capable of controlling soft rot. Therefore, this extract has potential for the prevention and treatment of bacterial infections and for the development of alternatives to antibiotics.

1. Introduction

Quorum sensing (QS) is a chemical mechanism by which bacteria respond to external environmental changes promptly and effectively by using their chemical languages[1-4]. Upon reaching a threshold bacterial population density, diffusible signal molecules trigger the expression of genes involved in biofilm formation (BF), virulence factor production, motility, bioluminescence, antibiotic production,

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sporulation, and nitrogen fixation[1-6]. Both gram-negative and gram-positive bacteria are known to have QS mechanism, but there are differences between them. Many gram-negative bacteria produce N-acyl homoserine lactone as a signaling molecule. In general, N-acyl homoserine lactones bind directly to the transcription factor to regulate gene expression[1-6]. Gram-positive bacteria produce autoinducing peptide as a QS signal molecule. When gram-positive bacteria sense a threshold autoinducing peptide, autoinducing peptides bind to the receptor protein and activate two-component system[1-6].

Bacterial QS plays a role in BF[7-9]. A biofilm is defined as a sessile community of microorganisms on the surface enveloped within a polymer complex composed of DNA, proteins, and exopolysaccharides[7-9]. Microorganisms form biofilms upon perceiving attachment sites, nutrient depletion, and/or certain antibiotics[10,11]. According to the National Institutes of Health, around 80% of all bacterial infections (*e.g.*, bacterial vaginosis, urinary tract infection, otitis media, tooth decay, and endocarditis) involve biofilm[12-15].

Over the past 50 years, antibiotics have significantly reduced the rate of mortality caused by bacterial infections. However, abuse of antibiotics has led to the emergence of antibiotic-resistant microorganisms. Therefore, the development of alternatives to antibiotics is urgently required[16]. Recently, non-bactericidal treatment modalities that inhibit bacterial activity, such as suppression of QS, have been developed[17,18]. We investigated the anti-QS activity of plant extracts. QS-disturbing phytocompounds are reportedly present in the extracts of medicinal plants, marine algae, essential oils, and edible fruits[19-23]. These compounds represent a new approach to the treatment of bacterial infections[19].

In the future, combination therapies comprising an antibiotics and anti-QS and/or anti-BF agent may prove useful[24-28]. Indeed, as QS is closely related to pathogenicity, anti-QS agents are currently under development[29,30].

2. Materials and methods

2.1. Plant extracts

The plant extracts were obtained from the National Institute of Horticultural & Herbal Science, National Institute of Horticultural Science, South Korea. Plant parts used for extraction are mainly used in oriental medicine. Plant samples were extracted with 80% ethanol for 24 h followed by filtering with Whatman No. 1 filter papers (Advantec, Tokyo, Japan). The supernatants were vacuum-concentrated under reduced pressure. The ethanol extracts of 446 samples (including plant parts of leaf, stem, root, flower, bark, and/or twig) from 388 plants were diluted to 100 mg/mL in 100% ethanol and stored at -20 ℃.

2.2. Bacterial strains and culture conditions

Chromobacterium violaceum (*C. violaceum*) CV017 was used for anti-QS assays and *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1, *Yersinia enterocolitica* (*Y. enterocolitica*) ATCC 9610, and *Agrobacterium tumefaciens* (*A. tumefaciens*) C58 were used for anti-BF assays. *C. violaceum* CV017 (derived from ATCC 31532) and *Y. enterocolitica* ATCC 9610 were obtained from the American Type Culture Collection, Rockville, MD. *A. tumefaciens* C58 and *P. aeruginosa* PAO1were kindly provided by C. Fuqua, Indiana University, Bloomington, IN. The bacteria were cultured on Luria-Bertani (LB) broth or agar at 28 ℃. For biofilm assays, the bacteria were cultured in Agrobacterium (AB) broth at 28 ℃.

2.3. Anti-QS activity assays

C. violaceum has been extensively studied in QS-mediated violet pigment violacein. To evaluate the anti-QS activity of the extracts, disc-diffusion assays using *C. violaceum* CV017 were performed; those with such an activity inhibited pigment production, resulting in the formation of a zone of clearance around the disc, as in antibacterial susceptibility tests[31]. Disc-diffusion assays were performed as described previously with some modifications[19]. An overnight culture of *C. violaceum* CV017 (1/100 ratio) was embedded in LB agar, mixed thoroughly, and decanted into Petri dishes. Sterile paper discs (6 mm diameter) were placed on the LB agar and loaded with the plant extracts (20 μL). After incubation at 28 ℃ for 24 h, anti-QS activity was evaluated by measuring the diameter of the zone of clearance. As a control, 100% ethanol (20 μL) was used instead of plant extracts. Antibacterial activity was determined by confirming bacterial growth around the zone of clearance showing anti-QS effect. All experiments were carried out in triplicate.

2.4. Anti-BF activity of plant extracts

BF assays were performed as described previously[32] using *P. aeruginosa* PAO1, *Y. enterocolitica* ATCC 9610, and *A. tumefaciens* C58. The bacteria were shake-cultured in AB broth at 28 ℃ overnight, and diluted in AB broth to an optical density at 600 nm (OD_{600}) of 0.05. Next, 150 μL of AB medium plus 0.1%-2% ethanol extracts were added to each of three wells of a 96-well plate. The negative control was treated with ethanol instead of the extract. The plates were placed in a plastic box with saturated paper towels and incubated at room temperature for 48 h. The medium was decanted, the wells were rinsed with water, and adherent biomass in the wells

was stained by adding 0.1% (w/v) crystal violet for 5 min. Next, the wells were rinsed three times with water and allowed to air dry. Adsorbed crystal violet was solubilized by adding 150 μL 33% acetic acid and the OD_{600} of the resulting solution was determined using a microplate reader (Bio-Rad). Biofilm assays involved three independent experiments, each comprising three technical replicates. Anti-BF rate (%) was calculated according to previous report[33]: [(Control BF – Treatment BF)/Control BF] × 100.

2.5. Anti-QS and anti-BF activities of Cornus controversa (C. controversa) twig and leaf extracts

Due to *C. controversa* extracts used for anti-QS and anti-BF assays were a mixture of twigs and leaves, they were extracted separately from twigs and leaves according to the above-mentioned methods in order to see whether they have higher activity. Anti-QS and anti-BF activity assays of extracts of the twig and leaves of *C. controversa* were performed according to the above-mentioned methods. For anti-QS activity assays, each ethanol extract (50 μL) was loaded onto a paper disc. As a negative control, ethanol (50 μL) was used instead of plant extracts.

2.6. Effect of extracts on soft rot

The plant extracts (10-20 μL) were loaded onto Chinese cabbage leaves and dried at room temperature. Next, a *Pectobacterium carotovorum* (*P. carotovorum*) suspension [10 μL; 7.0×10^7 colony-forming unit (CFU)/mL] was placed at the same position as the plant extracts. The inoculated cabbage leaves were placed in a plastic box with saturated paper towels and incubated for 52 h. Disease progression was examined after 20, 28, 42, or 52 h. All experiments were carried out in triplicate.

3. Results

3.1. Anti-QS activity assays

Of the 388 plant extracts, 21 (Table 1) inhibited pigment production by *C. violaceum* CV017 (Figure 1). The *C. controversa* and *Cynanchum wilfordii* (*C. wilfordii*) extracts significantly inhibited pigment production by *C. violaceum* CV017 (Figure 2). Antibacterial activity was determined by confirming bacterial growth around the clear zone, showing the inhibition of QS-mediated violet violacein production. Most of the plant extracts showing the inhibition of violet pigment production exhibited antibacterial activity, however the four of them (*Alnus sibirica*, *Caryopteris incana*, *C. wilfordii*, and *C. controversa*) showed no antibacterial activity against *C. violaceum* (Figure 2). The *C. controversa* and *C. wilfordii* extracts were selected for further anti-BF assays and soft rot tests.

Table 1

Plant extracts showing anti-QS activity.

No.[a]	Scientific name	Family	Part(s) used
22	*Sedum middendorfianum* Max.	Crassulaceae	aerial part
57	*Impatiens balsamina* L.	Balsaminaceae	aerial part
59	*Psoralea corylifolia* L.	Leguminosae	aerial part
62	*Pulsatilla koreana* Nakai	Ranunculaceae	aerial part
139,140	*Aruncus dioicus* var. *kamtschaticus* Hara	Rosaceae	aerial part
186	*Ruta grabeolens* L.	Rutaceae	aerial part
217	*Pharbitis nil* Chosy	Convolvulaceae	aerial part
220	*Artemisia iwayomogi* Kitamura	Compositae	aerial part
232	*Ixeridium dentatum* (Thunb. ex Mori)	Compositae	root
272	*Cynanchum wilfordii* Hemsl.	Asclepiadaceae	root
325	*Alnus sibirica* Fisch. ex Turcz.	Betulaceae	leaf
326	*Alnus sibirica* Fisch. ex Turcz.	Betulaceae	twig
328	*Cornus controversa* Hemsl. ex Prain	Cornaceae	leaf/twig
348	*Althaea rosea* Cav.	Malvaceae	root
361	*Caryopteris incana* (Thunb.) Miq.	Verbenaceae	stem
362–364	*Caryopteris incana* (Thunb.) Miq.	Verbenaceae	leaf
365	*Caryopteris incana* (Thunb.) Miq.	Verbenaceae	root
366	*Caryopteris incana* (Thunb.) Miq.	Verbenaceae	flower

[a]Numbers of plant extracts coincide with those in 388 plant extracts.

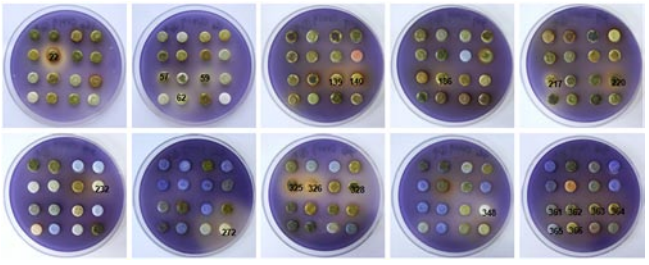

Figure 1. Anti-QS activity of plant extracts against *C. violaceum* CV017. Each ethanol extract (20 μL) was loaded onto a paper disc. As a negative control, ethanol (20 μL) was used instead of plant extracts. Numbers of plant extracts coincide with those in Table 1.

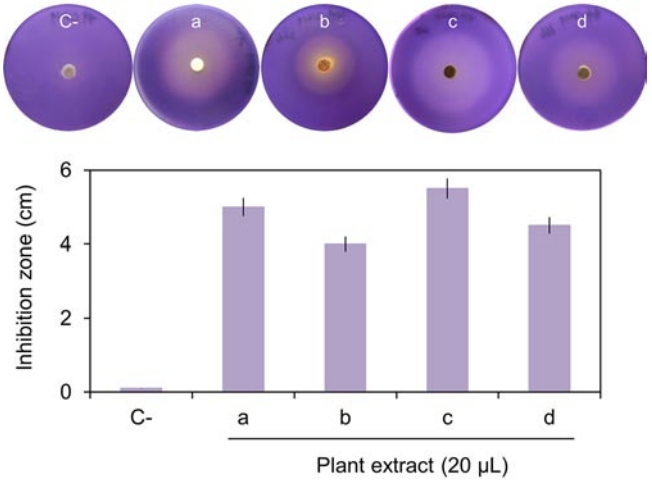

Figure 2. Anti-QS activity of four plant extracts against *C. violaceum* CV017. a, *C. wilfordii*; b, *Alnus sibirica*; c, *C. controversa*; and d, *Caryopteris incana*. Ethanol (C–) was used as the negative control. Means ± SD are shown.

3.2. Anti-BF activity of the plant extracts

Next, we evaluated the anti-BF activities of the *C. controversa* and *C. wilfordii* extracts against *P. aeruginosa* PAO1, *Y. enterocolitica* ATCC 9610, and *A. tumefaciens* C58. Ethanol extract of *C. controversa* inhibited BF against all three bacterial strains (Figure 3). The *C. wilfordii* ethanol extract exhibited significant anti-BF activity against *Y. enterocolitica*, although the anti-*P. aeruginosa* BF activity was low (Figure 4). In addition, the *C. wilfordii* extract showed a higher anti-BF activity on *Y. enterocolitica* than the *C. controversa* extract (Figure 3 and 4).

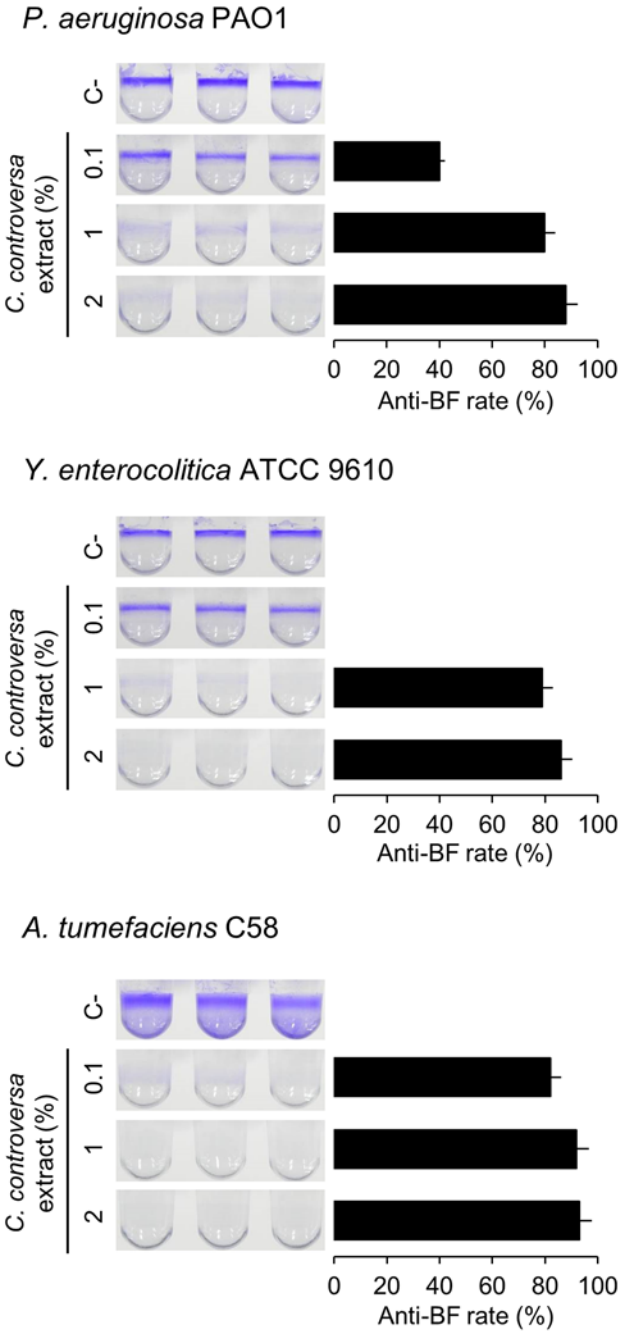

Figure 3. Anti-BF activity of *C. controversa* extract against *P. aeruginosa* PAO1, *Y. enterocolitica* ATCC 9610, and *A. tumefaciens* C58.

Ethanol (C-) was used as the negative control. Means ± SD are shown.

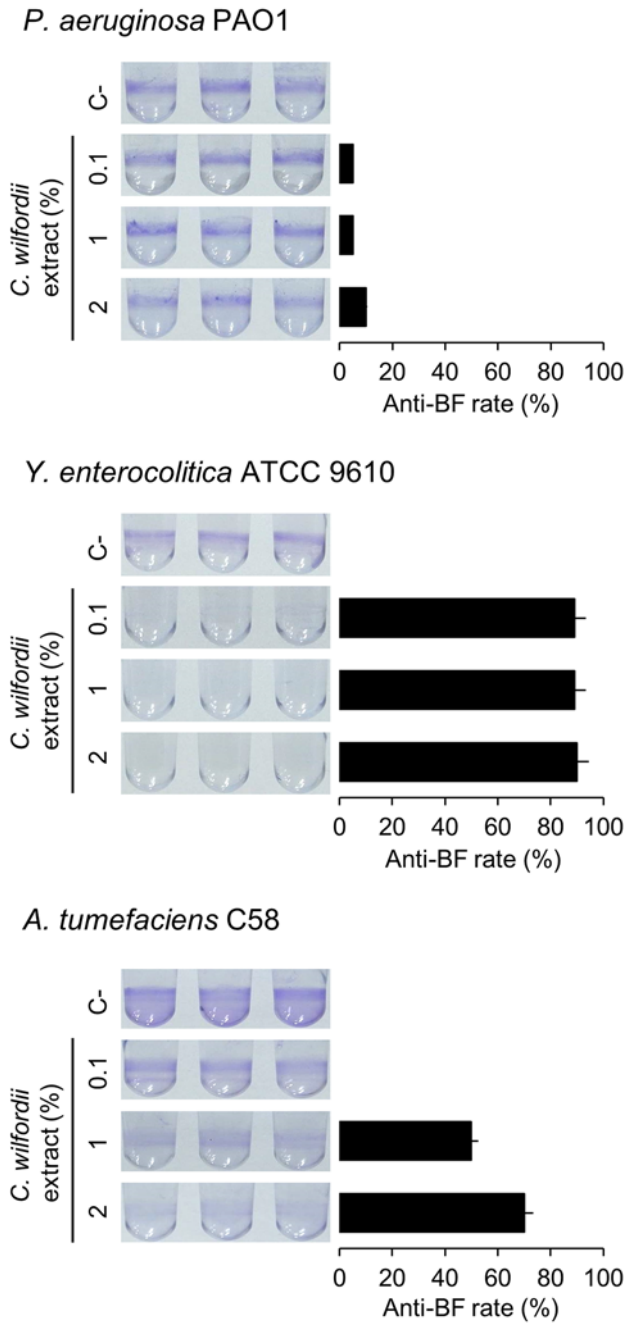

Figure 4. Anti-BF activity of the *C. wilfordii* extract against *P. aeruginosa* PAO1, *Y. enterocolitica* ATCC 9610, and *A. tumefaciens* C58.

Ethanol (C-) was used as the negative control. Means ± SD are shown.

3.3. Anti-QS and anti-BF activities of C. controversa twig and leaf extracts

We also assessed the anti-QS and anti-BF activities of *C. controversa* twig and leaf extracts. There was no significant difference between twig and leaf extracts (Figure 5), and thus twig extracts were used in suppression of soft rot.

3.4. Effect of the extracts on soft rot

P. carotovorum has been extensively studied in QS-mediated

virulence factors expression. We determined the effects of *C. controversa* and *C. wilfordii* extracts on soft rot of Chinese cabbage, which is caused by *P. carotovorum*. *C. controversa* extract suppressed the development of soft rot (Figure 6), resulting in a greenish stain. After incubation for 52 h, soft rot symptoms were evident in ethanol control and *C. wilfordii* extract treatment, but not in *C. controversa* extract treatment.

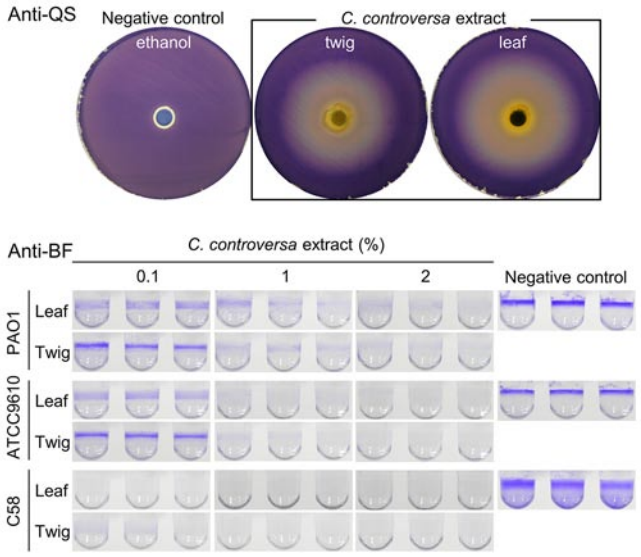

Figure 5. Anti-QS (*C. violaceum* CV017) and anti-BF (*P. aeruginosa* PAO1, *Y. enterocolitica* ATCC 9610 and *A. tumefaciens* C58) activities of extracts of twig and leaves of *C. controversa*.

For anti-QS activity assays, each ethanol extract (50 μL) was loaded onto a paper disc. As a negative control, ethanol (50 μL) was used instead of plant extracts.

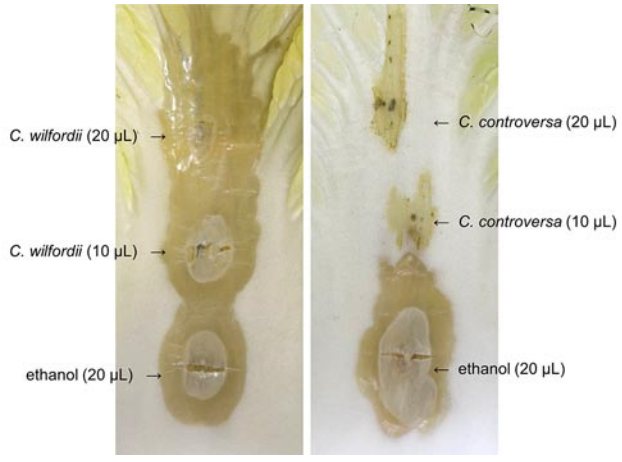

Figure 6. Effect of the *C. controversa* extract against soft rot.

4. Discussion

Anti-QS is a process that prevents QS by disrupting the signal. This is accomplished by inactivating the signaling enzyme, mimicking the signal molecule, introducing a molecule that blocks the receptor, or by degrading the signal molecule itself without killing bacteria[34]. Using the principle of non-bacteriostasis may prevent the emergence of antibiotic resistant individuals. This interesting research area has led many research groups to the development of QS-disturbing phytomolecules from plant extracts. It will introduce not only a new mode of action and possible validation for traditional plant use, but also a potentially new therapeutic direction for treatment of bacterial infections. Therefore, this study was conducted to investigate plant extracts that can disturb the bacterial QS.

Adonizio *et al*.[19] reported that terrestrial medicinal plants possess anti-QS compounds. Among 50 medicinal plants from southern Florida, 6 plants inhibited QS: *Conocarpus erectus*, *Chamaecyce hypericifolia*, *Callistemon viminalis*, *Bucida burceras*, *Tetrazygia bicolor*, and *Quercus virginiana*. In India, Zahin *et al*.[25] reported that ethanol extracts of 5 plants among 24 Indian medicinal plants, namely *Hemidesmus indicus* (root), *Holarrhena antidysenterica* (bark), *Mangifera indica* (seed), *Punica granatum* (pericarp), and *Psoralea corylifolia* (seed), inhibited violacein production by *C. violaceum* and swarming by *P. aeruginosa* PAO1. In China, Priya *et al*.[27] showed that methanol extracts of the traditional Chinese herb *Phyllanthus amarus* exhibited anti-QS activity. Damte *et al*.[26] reported that 6 of 97 plant extracts inhibited pigment production by *C. violaceum* CV12472, and 16 inhibited the swarming motility of *P. aeruginosa* POA1. Han *et al*.[35] demonstrated that extracts of pine needle, green tea, and mugwort have anti-QS activity against the tobacco soft rot pathogen. The plants showed anti-QS activity was inconsistent with plants reported in previous studies[19,25,27,35].

Anti-QS activity of the *C. controversa* and *C. wilfordii* extracts inhibited the BF of *Y. enterocolitica* and *A. tumefaciens*. Correlation of anti-BF with anti-QS effect is easily predicted, but is exceptional for *A. tumefaciens*. Nevertheless, there is no data so far that have related QS to BF in agrobacteria[36-38]. It is not known how the *C. controversa* and *C. wilfordii* extracts inhibited the BF of *A. tumefaciens*. This is not due to the inhibition of bacterial density recognition, but because it inhibited the mechanism involved in BF. To our knowledge, no literature has reported that the extracts of the *C. controversa* and *C. wilfordii* have the effect of inhibiting the BF of agrobacteria. Studies related to this are considered to be needed in the future.

In *P. carotovorum*, virulence factors production is mediated by QS[39]. Anti-QS activity of *C. controversa* extract attenuated QS-mediated virulence expression of *P. carotovorum*. Ethanol extract of *C. controversa* suppressed soft rot, but extract of *C. wilfordii* did not show any inhibitory effect. Thus, the anti-QS activity of the *C. controversa* extract is more broad-spectrum than that of the *C. wilfordii* extract. In addition, the *C. wilfordii* extract showed a higher anti-BF activity on *Y. enterocolitica* than the *C. controversa* extract, suggesting the potential for use in the food contamination through inhibitory effect on BF of *Yersinia* spp.

Two plant extracts that exhibited anti-QS activity were subjected to assays of their anti-BF and anti-disease activity. *C. controversa* inhibited bacterial QS and BF, and was capable of controlling

soft rot. Therefore, this extract has potential for the prevention and treatment of bacterial infections and for the development of alternatives to antibiotics.

Conflict of interest statement

We declare that we have no conflict of interest.

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