Leaf Extracts of Selected Gardening Trees Can Attenuate Quorum Sensing and Pathogenicity of *Pseudomonas aeruginosa* PAO1

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Abstract An increasing concern on resistance to multiple-antibiotics has led to the discovery of novel agents and the establishment of new precaution strategy. Numerous plant sources have been widely studied to reduce virulence of pathogenic bacteria by interfering cell-to-cell based communication called quorum sensing (QS). Leaf extracts of 17 gardening trees were collected and investigated for their anti-QS effects using a sensor strain *Chromobacterium violaceum* CV026. Methanolic extracts of K4 (*Acer palmatum*), K9 (*Acer pseudosieboldianum*) and K13 (*Cercis chinensis*) leaves were selected for further experiments based on their antagonism effect on QS without inhibiting *C. violaceum* CV026 growth. Subsequently, the leaf extracts on QS-mediated virulence of *Pseudomonas aeruginosa* PAO1 involved in biofilm formation, motility, bioluminescence, pyocyanin production, QS molecules production, and *Caenorhabditis elegans* killing activity

were evaluated. The biofilm formation ability and swarming motility of *P. aeruginosa* PAO1 were decreased approximately 50% in the presence of these leaf extracts at a concentration of 1 mg/mL. The expression level of *lecA::lux* of *P. aeruginosa* PAO1 and pyocyanin production were also reduced. The three leaf extracts also decreased autoinducer (AI) production in *P. aeruginosa* PAO1 without direct degradation, suggesting that AI synthesis might have been suppressed by these extracts. The three leaf extracts also showed anti-infection activity in *C. elegans* model. Taken together, these results suggest that methanolic leaf extracts of K4, K9 and K13 have the potential to attenuate the virulence of *P. aeruginosa* PAO1.

Keywords Autoinducer · *C. elegans* · Gardening trees · *P. aeruginosa* PAO1 · Pathogenicity · Quorum sensing

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Introduction

Rapid emergence of multidrug resistant (MDR) bacteria has rendered many antibiotics less effective. It poses a major public health concern [1]. Therefore, new methods and smarter strategies are urgently needed to fight against MDR infection. Quorum sensing (QS) has been found to be able to regulate a wide variety of virulent genes with potential to develop strategies to disrupt signaling pathways involving pathogenesis [2–9]. QS is a communication mechanism used by individual cells to produce, release, detect, and respond to signal molecules [10]. Two general diffusible signals, oligopeptides and *N*-acyl-homoserine lactones (AHLs) often termed as autoinducers, are involved in Gram-positive bacteria and proteobacteria, respectively [11]. Gram negative bacteria generally use LuxI/LuxR QS system to control the production of virulent factors [12].

Pseudomonas aeruginosa has homologous QS circuits, LasI/LasR and RhlI/RhlR while *Chromobacterium violaceum* has CviI/CviR for QS [13–15]. *P. aeruginosa*, a Gram-negative bacteria, is an opportunistic pathogen that causes severe disease such as cystic fibrosis (CF), bronchiectasis and chronic obstructive pulmonary disease in immunosuppressed patients [16]. In order to develop new approaches for treating *P. aeruginosa* infection, many treatment strategies have been attempted, including QS inhibition [16], lectin inhibitors [17, 18], iron chelation [19, 20], anti-resistance targeting efflux pumps [21], anti-sense oligomer [22], and cell lysis using bacteriophage [23].

Phytochemicals have attracted attention with possible application in controlling QS-based pathogenicity [24]. These molecules include GABA, pyrogallol, curcumin, quercetin, taxifolin, salicylic acid, chlorogenic acid, volatile organic compounds, and others [25]. Many plants have been reported to be able to disrupt microbial signaling pathway and release signal-degrading enzymes, signal blockers, or signal analogues [26]. Different plant parts such as bud, bulbs, seed, stem, root, fruits, and leaves contain massive natural active compounds with anti-QS function [27]. Hence, plant-based molecules can be used as potential therapeutic agents to control bacterial infection.

Gardening trees show both ornamental and pharmacognosy [28–30] values because of their appearance and ability to produce a number of aromatic compounds. Although the anti-QS effects of many medicinal plants have been studied, the property of gardening trees has been reported in few studies. The curiosity to explore gardening trees as anti-QS resources have led to this work.

In this study, sensor strain *C. violaceum* CV026, a violacein-negative, mini-Tn5 mutant of *C. violaceum* [15], was used to screen the anti-QS effects of extracts of different gardening trees. Subsequently, their effects in attenuating QS-based virulence of *P. aeruginosa* PAO1 were determined.

Materials and Methods

Preparation of Leaf Extracts

Plant leaves were collected from the campus of Konkuk University (Seoul, Korea) in October, 2015. The common and scientific names of collected leaves were investigated and presented in Table 1. These leaves were washed with distilled water and dried naturally at room temperature. They were then smashed to powder state using a grinder (DA5000, Healthmic, Korea) and stored at -20°C . Leaf powders were extracted with 99% methanol in a ratio of

1:10 (w/v) at 4°C for 72 h. Supernatants were collected after centrifugation (12,000 rpm, 4°C , 10 min) and filtered with Whatman No. 1 filter paper. After a further filtration with syringe filter (pore size of $0.45\ \mu\text{m}$; Sartorius, Germany), the solvent was removed with a vacuum evaporator RE111 (BÜCHI, Germany). The stock concentration was adjusted to 100 mg/mL using methanol or diluted 10 times with sterile distilled water prior to use.

Sensors and Culture Conditions

The AI sensor strain *C. violaceum* CV026 was obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB). *P. aeruginosa* PAO1 was obtained from Gyeonggi Institute of Health Environment. A bioluminescence reporter strain *P. aeruginosa* PAO1/*lecA::lux* was obtained from Dr. Kok-Gan Chan, University of Malaya, Malaysia. The three strains were maintained in Luria Bertani (LB) broth (BD, New Jersey, USA). *C. violaceum* CV026 was cultured at 30°C for 24 h. *P. aeruginosa* PAO1 was cultured at 37°C for 16 h.

Antibacterial Activity

Antibacterial activities of all prepared leaf extracts were determined using automated microtiter assay [31, 32] with some modifications to exclude false anti-QS effect. Briefly, overnight grown biosensor strain was inoculated into fresh and sterile LB broth to an OD of 0.1 at 600 nm. Subsequently, 180 μL of pathogen culture was loaded into the well of a 96-well plate (20 μL of leaf extract per well). In this assay, methanol (10%, v/v) was used as control. The absorbance of well was measured every hour at wavelength of 595 nm for a total of 30 h using a multi-mode reader (synergy2, BioTek, USA) at 37°C .

Anti-quorum Sensing Effect on Sensor Strain *C. violaceum* CV026

The anti-QS effects of leaf extracts were determined using published method [32] with some modifications. Briefly, sensor strain *C. violaceum* CV026 was cultured at 30°C for 16 h. The prepared culture was evenly spread onto LB agar plates using sterilized cotton bud. Then 6 mm diameter of holes was made using sterilized Pasteur pipette. Each prepared leaf extract (50 μL) with 50 μL of 100 μM *N*-acyl homoserine lactone (AHL, Sigma) dissolved in DMSO (99%, Georgiachem) was added into the hole. Diluted methanol (10 $\times$) was used as control. QS inhibitory effect can be evaluated based on the level of purple color formed around the hole after 20 h of incubation at 30°C .

Table 1 Common and scientific names of plant leaves used in this study

Sample no.	Used leaves	
	Common name	Scientific name
K1	Rattan	<i>Wisteria floribunda</i>
K2	Yew	<i>Taxus cuspidata</i>
K3	Cherry	<i>Prunus serrulata</i> var. <i>spontanea</i>
K4	Maple	<i>Acer palmatum</i> Thunb.
K5	Painted maple	<i>Acer pictum</i> subsp. <i>mono</i>
K6	Donarium cherry	<i>Prunus donarium</i> Sieb
K7	Magnolia	<i>Magnolia Kobus</i>
K8	Zelkova	<i>Zelkova serrata</i>
K9	Purple bloom maple	<i>Acer pseudosieboldianum</i> (Pax) Kom.
K10	<i>C. obtusa</i> ‘Nana Aurea’	<i>Chamaecyparis obtusa</i>
K11	Horse chestnut	<i>Aesculus turbinata</i>
K12	Juniper	<i>Juniperus chinensis</i>
K13	Redbud	<i>Cercis chinensis</i>
K14	Comelian cherry	<i>Cornus officinalis</i>
K15	Golden-wave	<i>Coreopsis drummondii</i> L.
K16	Ginkgo	<i>Ginkgo biloba</i>
K17	Hydrangea	<i>Hydrangea macrophylla</i> for. <i>otaksa</i>

Microtiter Plate Biofilm-Inhibiting Assay

Biofilm inhibiting effect of leaf extracts was determined using microtiter plate biofilm production assay described previously [33]. Briefly, the optical density of overnight culture of sensor strain was adjusted to 0.1 at 570 nm. Culture solution (180 μ L) with 20 μ L of leaf extracts was added to the wells of 96-well plate. Methanol (10%, v/v) was used as control. Plates were cultured at 37 °C for 20 h. The culture solution was discarded and washed with sterile distilled water several times. Each well was stained using 25 μ L of 1% (w/v) crystal violet solution in pure water for 15 min. After the staining, wells were washed with sterile distilled water several times. Then 200 μ L of 99% ethanol was added to the well for de-staining. The solution (150 μ L) was transferred to a new plate and the optical density was measured with multi-mode microplate reader at wavelength of 570 nm. The experiment was conducted with three replicates.

Analysis of Motility

Swimming Motility

Leaf extracts (10 mg/mL) were added into LB swimming agar plates in a ratio of 1: 200. The agar plate was made with LB broth powder (2.5%, w/v) and Bacto agar (0.3%, w/v). Methanol (10%, v/v) was used as control. *P. aeruginosa* PAO1 was inoculated into 5 mL of fresh and sterile LB broth, and incubated at 37 °C for 16 h. Then 3 μ L of

the prepared culture (0.1 at 570 nm) was pipetted onto the center of LB swimming agar plates. The plates were incubated at 37 °C for 20 h. Attenuated swimming motility of *P. aeruginosa* PAO1 indicated that leaf extracts possessed anti-QS activity. The experiments were conducted with three replicates.

Swarming Motility

LB swarming agar plate was prepared with LB broth powder (2.5%, w/v), Bacto agar (0.5%, w/v), and glucose (0.8%, w/v). Other steps were the same as described above for swimming motility.

Pyocyanin Production

Pyocyanin production was evaluated using the method described in the study of Priya *et al.* [34]. Briefly, an overnight culture of wild-type *P. aeruginosa* PAO1 was inoculated into 5 mL of LB broth and the optical density was adjusted to 0.1 at 520 nm. Leaf extract (250 μ L) was added into the culture and then incubated at 37 °C overnight. Bacterial cells were removed by centrifugation (4 °C, 12,000 rpm, 10 min) and the supernatant was mixed with 3 mL of chloroform followed by addition of 1 mL of 0.2 M HCl to the collected chloroform layer. After mixing well and centrifugation (28 °C, 8000 rpm, 10 min), the absorbance of removed HCl layer was measured at wavelength of 520 nm using a spectrophotometer (Synergy 2, Bio Tek, USA).

Bioluminescence Assay

The bioluminescence assay was performed using a reporter strain of *P. aeruginosa* PAO1/*lecA::lux* as described in the study of Krishnan [35]. This *P. aeruginosa* PAO1-based reporter contains a single, chromosomally located *lecA::luxCDABE* Km^r fusion (*lecA::lux*) [36]. *P. aeruginosa* PAO1/*lecA::lux* was inoculated into sterile LB broth (5 mL) and incubated at 37 °C for 16 h. Overnight grown cells were transferred into fresh and sterile LB broth (2%, v/v). The resulting diluted culture (180 µL) was transferred to wells of microtiter plate loaded with 20 µL of leaf extracts per well. The optical density of the plate was measured at wavelength of 595 nm every hour on the micro-plate reader (Synergy 2, Bio Tek, USA). Bioluminescence was expressed as relative light unit (RLU) per unit of optical density to indicate the influence of increased growth on total bioluminescence. Experiments were conducted with three replicates.

Visualization of Autoinducer Production

P. aeruginosa PAO1 was cultured in LB broth (5 mL) at 37 °C for 16 h. Then 1% of bacterial seed was inoculated into 50 mL of sterile and fresh LB broth added with 100 µL of each leaf extract or control (10% methanol) followed by incubated at 37 °C for 20 h. Bacterial cells were removed after centrifugation (12,000 rpm, 4 °C, 10 min) and the supernatant was filtered (Pore size of 0.45 µm; Sartorius, Germany). Cell-free supernatant was mixed with ethyl acetate at a ratio of 1:1 and the upper layer was separated and collected in a separation funnel. After removing the solvent using a vacuum evaporator (BÜCHI, RE111, Switzerland), it was re-dissolved in 1 mL of ethyl acetate. Subsequently, the ethyl acetate solution was transferred to a 15-mL microtube and completely dried with the vacuum evaporator. Finally, 500 times concentrated sample was prepared by adding 100 µL of methanol.

Visualization of AI production was performed using TLC method in conjunction with *C. violaceum* CV026 as described previously [15]. Briefly, 6 µL of AHL extract was spotted onto TLC plate for 3 times (Each 2 µL) and dried. The same amount of 100 µM of *N*-heptanoyl-L-homoserine lactone (HHL, Sigma) was loaded as control. Then 100 mL of solvent (60%, v/v methanol in water) was used as loading solution. The plate was visualized by overlay with a thin layer of top agar (0.8%, w/v) seeded with *C. violaceum* CV026.

C. elegans Killing Assay

L4-stage *Caenorhabditis elegans* were prepared by the following steps. NGM plates containing *C. elegans* were

washed with 4 mL of sterile distilled water and poured into a 50 mL conical tube. Then, 100 µL of 12% sodium hypochlorite (NaClO) and 1 mL of 10% (v/v) sodium hydroxide (NaOH) were added into the tube. To kill all adult worms except eggs, the tube was shaken (100 rpm) at 25 °C for 10 min. Then 45 mL of sterile distilled water was added to the tube and centrifuged (4 °C, 3000 rpm, 15 min). The supernatant was discarded. After adding 50 mL of K medium broth (NaCl, 3.075 g; KCl, 2.42 g; Distilled water, 1L) to the tube, it was cultured overnight in a shaking incubator (25 °C, 100 rpm). After the incubation, the supernatant was separated by centrifugation (4 °C, 3000 rpm, 15 min) and discarded. The L1 stage *C. elegans* were moved to NGM plates (8 g peptone, others same) [37] inoculated with *E. coli* OP50 and incubated at 16 °C until L4 stage.

The inhibitory activity of leaf extracts against *P. aeruginosa* PAO1 was determined by *C. elegans* slow-killing assay with some modifications [38]. Briefly, 100 µL of *P. aeruginosa* PAO1 culture was spread onto NGM plates with the addition of leaf extracts (0.5%, v/v). The plates were then seeded with the prepared *C. elegans* (10–20 worms). The plates were incubated at 16 °C and monitored for 7 days. Worms were regarded as dead if they showed no response to touch.

Statistical Analysis

Data were analyzed using IBM SPSS statistics 24 for window system (IBM, New York, US) and presented as mean ± standard deviation (SD) from triplicate trials. Significant differences were determined using Duncan's multiple range test. Statistical significance was considered at ($P < 0.05$).

Results and Discussion

Preliminary Screening

Various natural resources have abundant of bioactive compounds as anti-QS agents to control the emergence of MDR bacteria. Recently, scientists are working on interfering QS-regulated bacteria communication to attenuate the virulence of pathogens [39, 40]. In general, anti-QS effects of traditional medical plants have been widely reported [27]. In this study, 17 leaves of gardening trees were collected from the campus of Konkuk University and their methanolic extracts were screened for anti-QS effect using *C. violaceum* CV026. A double Tn5 mutant *C. violaceum* CV026 could not produce purple pigment violacein without external supplement of short chain AHL [15]. Antibacterial activities of leaf extracts were first conducted

Table 2 Anti-QS and antibacterial activities of leaf extracts

Samples	QS inhibition		Antibacterial activity <i>C. violaceum</i> CV026
	HHL	OHHL	
K1	—	—	—
K2	—	—	—
K3	—	—	—
K4	+	+	—
K5	—	—	—
K6	—	—	—
K7	—	—	—
K8	—	—	—
K9	++	++	—
K10	—	—	—
K11	—	—	—
K12	—	—	—
K13	+	—	—
K14	—	—	—
K15	—	—	—
K16	—	—	—
K17	—	—	—

QS, inhibition; ++, Strong activity; +, Weak activity; —, No activity

to prevent false anti-QS effect. Only maple species K4 (*Acer palmatum*), K9 (*Acer pseudosieboldianum*), and redbud species K13 (*Cercis chinensis*) leaf extracts showed anti-QS effects in the presence of synthetic autoinducers (AIs), namely HHL (*N*-hexanoyl-L-homoserine lactone) and OHHL (*N*-(3-oxohexanoyl-L-homoserine lactone), without antibacterial activity (Table 2).

Therefore, the three leaf extracts were selected and evaluated for their abilities in attenuating QS-mediated virulence of *P. aeruginosa* PAO1 in the following tests. In addition, we verified the growth of pathogenic strain *P. aeruginosa* PAO1 was not inhibited by the three leaf extracts even after incubation for 30 h (Fig. 1). K13 resulted in less growth of *P. aeruginosa* PAO1 at the stationary phase compared to K4 and K9. However, no lag pattern during exponential phase was observed, indicating that K13 might have very little inhibition on the growth of *P. aeruginosa* PAO1.

Inhibition of Biofilm Formation

Inhibition of biofilm formation is considered as another principal ability for selecting potential substances. In comparison with the control, the three leaf extracts reduced biofilm formation of *P. aeruginosa* PAO1 by the leaf extracts of K4 (47%), K9 (55%), and K13 (35%) as shown in Fig. 2. Biofilm can help pathogen survive at high concentration of antibiotics, leading to infections recurrence [41]. Formation of biofilm is regulated by cyclic

Fig. 1 Growth curve of *P. aeruginosa* PAO1 in the presence of leaf extracts. The experiments were conducted with 3 replicates and the mean $\pm$ SD was presented

Fig. 2 Inhibitory effect of leaf extracts on biofilm formation of *P. aeruginosa* PAO1. The experiments were conducted with 3 replicates and the mean $\pm$ SD was presented. Different letters indicate statistically significant difference by Duncan's multiple test at $P < 0.05$

diguanosine-5'-monophosphate (c-di-GMP), small RNAs and quorum sensing [42]. In our study, methanolic extracts of two maple species (K4, K9) showed significant reduction on biofilm formation. This is consistent with the result in a previous study using phenolic-rich maple syrup extract (PRMSE) for the inhibition of biofilm formation and increased the susceptibility of bacterial biofilms to antibiotics [30]. Phenolic extract of *Rubus rosaefolius* [39] and other natural products [43] also presented inhibitory effects on biofilm formation of *P. aeruginosa* PAO1. It has been reported that the PRMSE is rich in phenolic compounds such as gallic acid, 3-hydroxybenzoic acid, syringaldehyde, and others, in particular, catechol. These compounds can increase outer-membrane permeability of bacteria and attenuate efflux pump activity [30]. The PRMSE and catechol can also downregulate the gene expression of *P.*

aeruginosa associated with biofilm formation, namely *lasB* and *cupA1*. It has been reported that *C. chinensis* (K13) has anti-biofilm ability due to its enrichment of active compounds such as quercetin [44] and resveratrol [29]. Quercetin has been reported to be able to reduce the biofilm formation of *S. aureus* [45]. Resveratrol also exhibits strong inhibitory capability on the biofilm formation of *E. coli* O157:H7 [46].

Reduction of Bioluminescence

Virulence of infectious microbes is closely related to biofilm formation and release of virulent factors such as lectin, elastase, pyocyanin, motility, rhamnolipids, and toxins [12]. It is necessary to produce diverse exoproducts for maintenance the virulence and survival of pathogens. It has been reported that LecA (PA-IL) lectin is a soluble carbohydrate-binding cytotoxic protein that can affect the function of respiratory cells and biofilm formation [47]. The *lecA::luxCDABE* reporter strain was used to determine *lecA* expression of *P. aeruginosa* PAO1. As shown in Fig. 3, bioluminescence was decreased along with the incubation of *P. aeruginosa* PAO1 in the presence of each leaf extract following the order of K9, K4, and K13. The decrease in bioluminescence indicated that *lecA* expression was reduced by methanolic leaf extracts used in this study. This is likely to be involved in the interruption of RhlR/C4-HSL QS-system or down-regulation of the expression of *rpoS* [36]. Priya et al. [34] have also reported crude methanolic extract of traditional Chinese herb, *Phyllanthus amarus* can reduce *lecA* expression in *P. aeruginosa* PAO1. However, lower bioluminescence in the presence of these methanolic leaf extracts might be due to toxicity not detected in growth curves. Further experiment using a control with constitutive expression of bioluminescence is

Fig. 3 Bioluminescence of *P. aeruginosa* PAO1 (*lecA::luxCDABE*) in the presence of the three leaf extracts. The experiments were conducted with 3 replicates and the mean $\pm$ SD was presented

needed to exclude a false positive quorum quenching effect [48].

Reduction of Pyocyanin Production

Pyocyanin is one exogenous toxin. It is a redox-active pigment that promotes autophagy in bronchial epithelial cells, thereby causing chronic airways infection [49, 50]. As shown in Fig. 4, pyocyanin produced by *P. aeruginosa* PAO1 was decreased in the presence of three leaf extracts. The decreasing ability from high to low was in the order of K4, K9, and K13. We found that maple (*Acer palmatum thumb*) species effectively reduced pyocyanin synthesis. The reduction of pyocyanin production is considered as an important anti-QS effect. Methanolic clove extract has been reported to be able to inhibit pyocyanin production [35]. Pyocyanin synthesis of *P. aeruginosa* has been reported in relation to *rhl* system [51]. This implies that extracts of maple leaves might contain *rhl* inhibitor.

Motility Analysis

Moving property has been presented as critical determinants of flagellar-mediated virulence factor. There are three types of movement according to medium viscosity, swimming (Aqueous solution), twitching (Solid surfaces) and swarming (Semisolid) motilities. As shown in Fig. 5a, only K13 leaf extract was capable of reducing the swimming motility of *P. aeruginosa* PAO1. In addition, the three leaf extracts were found to be able to reduce swarming motility of *P. aeruginosa* PAO1 (Fig. 5b). Swarming is a more complex process impacted by a variety of genes, in particular, *lasB* and *pvdQ* [52]. In our study, the three leaf extracts showed more effect on swarming

Fig. 4 Pyocyanin production of *P. aeruginosa* PAO1 in the presence of leaf extracts. The experiments were conducted with 3 replicates and the mean $\pm$ SD was presented. Different letters indicate statistically significant difference by Duncan's multiple test at $P < 0.05$

Fig. 5 Effect of leaf extracts on the motility of *P. aeruginosa* PAO1. **a** Swimming motility, **b** swarming motility. The experiments were conducted with 3 replicates and mean $\pm$ SD was presented. Different letters indicate statistically significant difference by Duncan's multiple test at $P < 0.05$

motility than on swimming motility, suggesting that some unknown compounds within these methanolic extracts might have the ability to suppress the expression of swarming-related genes.

AI Production Analysis

The effect of leaf extracts on signal molecules is shown in Fig. 6. After *C. violaceum* CV026 was spread on the agar plate, its color change was used to confirm AI production. A considerable color decrease was observed for *P. aeruginosa* PAO1 with the addition of K9 or K13 supernatant compared to that of control (Fig. 6a). In case of K4, very slight decrease was observed. Same result was found in TLC (Fig. 6b). As expected, there was no color difference for synthetic AHL in the presence of leaf extracts (Fig. 6c, d), indicating that AHL molecules were not affected or degraded by these leaf extracts.

With the addition of K9 or K13, QS signals were reduced, implying that some compounds in these extracts could destroy AHL synthase or disrupt its binding with cognate receptors. Although K4 exhibited good inhibition on reduction of biofilm formation, pyocyanin production, and bioluminescence, it showed little effects on the production of QS signals. In the case of K4, it might contain unknown QS signal analogues that compete to bind with receptors [53]. Plant extracts are known to contain phytochemicals and functional molecules such as catechin, naringenin, curcumin, and rosmarinic acid [54]. It has been reported that these compounds can down regulate the expression of QS related genes or reduce the production of AHL signaling molecules, thereby controlling pathogenesis [53, 55].

Pseudomonas aeruginosa PAO1 can produce AI signals C4-, C6-, C10- and C12-HSLs by *C. violaceum* 026 biosensor [56]. Only C4- and C6-HSLs could be detected on TLC from the culture supernatants extracted by ethyl acetate acidified with 0.1% acetic acid or 0.5% formic acid [56, 57]. However, only C6-HSL was extracted by ethyl acetate acidified with 10% acetic acid as shown in Fig. 6b. Such discrepancy in results might be due to differences in extraction solvents or overdeveloped TLC with C4-HSL missing.

Attenuation of Pathogenicity Based on *Caenorhabditis elegans* Killing Assay

Caenorhabditis elegans pathogenicity model is widely used for investigation of QS-based virulence. The effect of the three leaf extracts on pathogenicity of *P. aeruginosa* PAO1 was investigated by *C. elegans* killing assay. As shown in Fig. 7, Almost of *C. elegans* were survived after feeding with the K4 or K9-treated *P. aeruginosa* PAO1 compared to the control after 4 days of incubation. However, a fast decrease in survival of *C. elegans* was observed when they were fed with K13-treated *P. aeruginosa* PAO1. After 7 days, a total of 20% of *C. elegans* remained alive in the K4 and K9 treatment groups, indicating prolonged-life span. It has been reported that *P. aeruginosa* PAO1 can kill *C. elegans* by cyanide poisoning and neuromuscular paralysis [58]. The addition of leaf extracts reduced the mortality of nematode caused by *P. aeruginosa* PAO1. It has been reported that aqueous extracts of medicinal plants *Conocarpus erectus*, *Callistemon viminalis*, and *Bucida buceras* have inhibitory effect on the mortality of *C. elegans* caused by *P. aeruginosa* [59]. Salicylic acid (SA), a plant phenolic compound, has been reported to be able to attenuate the infectivity of *P. aeruginosa* in *C. elegans* model [60]. K13 showed weak inhibition effect on the killing of *C. elegans* caused by *P. aeruginosa*. However, it showed strong inhibitory effect on QS inhibition in motility

Fig. 6 Effect of leaf extracts on AI production in *P. aeruginosa* PAO1 and C6-AHL degradation using *C. violaceum* CV026. Inhibitory effect on AI production in *P. aeruginosa* PAO1 shown on agar plate (a) and TLC plate (b). Degradative effect on C6-AHL (HHL 100 μ M, sigma) compound shown on agar plate (c) and TLC plate (d). C6-AHL and methanol (10%, v/v) were used as positive and negative controls, respectively

Fig. 7 Survivability of *C. elegans* infected by *P. aeruginosa* PAO1 in the presence of leaf extracts. The experiments were conducted with 2 replicates and the mean $\pm$ SD was presented. The survival ratio of *C. elegans* on the same culture day was statistically analyzed by Duncan's multiple test at $P < 0.05$ and the small letters indicate statistically significant difference ($P = 0.001$)

and autoinducer synthesis compared to K4 and K9. The action pattern on quorum quenching of K13 in *P. aeruginosa* PAO1 might be different from that of K4 or K9. Component(s) involved in quorum quenching of these extracts need to be determined in further studies.

Conclusion

Methanolic extracts of *A. palmatum*, *A. pseudosieboldianum* and *C. chinensis* leaves exhibited activities in attenuating biofilm formation and diverse virulent factors of *P. aeruginosa* PAO1. They also reduced the

mortality of *C. elegans* without inhibiting bacterial growth.

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Compliance with Ethical Standards

Conflict of interest We have no conflicts of interest to declare.

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