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ANTI-QUORUM SENSING ACTIVITY OF SOME MEDICINAL PLANTS

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## Abstract

**Background:** Quorum sensing is the key regulator of virulence factors of *Pseudomonas aeruginosa* such as biofilm formation, motility, productions of proteases, hemolysin, pyocyanin, and toxins. The aim of this study was to explore the effect of the extracts from some medicinal plants on quorum sensing and related virulence factors of *P. aeruginosa*.

**Material and Methods:** Quorum sensing inhibitory (OSI) effect of the alcohol extracts of 20 medicinal plants was evaluated by *Chromobacterium violaceum* reporter using agar cup diffusion method. The efficient QSI extracts were tested for their activity against biofilm synthesis, motility, and synthesis of pyocyanin from *P. aeruginosa* PA14

**Results:** The extracts of *Citrus sinensis*, *Laurus nobilis*, *Elettaria cardamomum*, *Allium cepa*, and *Coriandrum sativum* exhibited potent quorum quenching effect. On the other hand, *Psidium guajava* and *Mentha longifolia* extracts showed lower QSI activity. These extracts exhibited significant elimination of pyocyanin formation and biofilm development of *Pseudomonas aeruginosa* PA14. In addition, they significantly inhibited twitching and swimming motilities of *P. aeruginosa* PA14.

**Conclusion:** This study illustrated, for the first time, the importance of *C. sinensis*, *L. nobilis*, *E. cardamomum*, *A. cepa*, and *C. sativum* as quorum sensing inhibitors and virulence suppressors of *P. aeruginosa*. Thus, these plants could provide a natural source for the elimination of *Pseudomonas* pathogenesis.

**Key words:** Quorum sensing inhibitory activity, *P. aeruginosa*, *Chromobacterium violaceum*, virulence factors

## Introduction

Most medical applications of plants focused on their antimicrobial and antioxidant effects with low attention towards anti-pathogenic effects (Wallace, 2004). Recently, research efforts are focused on controlling bacterial infection through developing anti-pathogenic agents which manage bacterial diseases by inhibiting bacterial communication process called bacteria quorum sensing (QS). Quorum communication system regulates the release of *Pseudomonas* virulence factors such as protease, elastase, pyocyanin, alginate, biofilm formation, bacterial motility, and toxins production (Zhang and Dong 2004). Quorum sensing in *P. aeruginosa* is regulated by signaling molecules named *N*-acylated homoserine lactones (AHLs).

The concentration of these auto-inducers increases in relation to the increase in bacterial population till threshold, those signaling molecules return back to the bacteria to control bacterial pathogenicity (Fuqua and Greenberg, 2002). Therefore, elimination of QS represents an imperative advance to manage bacterial virulence and antimicrobial resistance (Hong et al., 2012). Plants are considered as a rich natural resource of quorum quenching agents (Mohamed et al., 2014; Koh and Tham 2011; Choo et al., 2006). Therefore, the current study investigated the QSI effect of some medicinal plants using the reporter *Chromobacterium violaceum*. Plant extracts that showed QSI activity were investigated for anti-pathogenic potential against *P. aeruginosa* PA14. For this instance, their influence on the virulence of *P. aeruginosa* was examined, including biofilm formation, pyocyanin production, and motility.

## Materials and Methods

### Collection of Plant Materials and Preparation of the Extracts

The fresh materials of *Mentha longifolia*, *Senna italica*, *Valantia hispida*, *Tephrosia purpurea*, *Teucrium polium*, *Tribulus arabicus*, and *Commophora molmol* were collected from Abyar Al-Mashy and Gabal Al-aquiq, Al Madinah Al Munawwarah, Saudi Arabia in March 2015. The other plant materials were purchased at the markets in Al-Madinah Al-Munawwarah, Saudi Arabia (Table 1). Authentication of the plant samples was established by Prof. Dr. A. Fayed (Professor of Plant Taxonomy, Faculty of Science, Assiut University, Egypt). The plants were shade dried for 7 days and ground to powder, using mortar and grinder of the electric mixer. Voucher specimens have been deposited at the Department of Pharmacognosy and Pharmaceutical Chemistry, Faculty of Pharmacy, Taibah University, Al Madinah Al Munawwarah, Saudi Arabia. Powdered samples (100 g each) were separately extracted using 95% methanol (5 × 250 mL). The extracts were concentrated under reduced pressure using rotary

evaporator (Heidolph, Schwabach, Germany) and re-dissolved in dimethyl sulfoxide (DMSO 2.5%) for the assay of QSI activity. The solvent DMSO (1.5%) would not inhibit growth of the microorganisms (Zgoda and Porter 2001).

### Bacterial Strains and Growth Conditions

The anti-quorum sensing assay was performed using *Chromobacterium violaceum* ATCC 12472. It was grown in Luria-Bertani (LB) media (1% peptone, 0.5% yeast extract (Bactoagar, BD Difco), and 1% NaCl (pH 7.4) solidified using 0.5 or 1.5% agar) (Bertani, 2004) and incubated at 28 °C for 48 h. *C. violaceum* ATCC 12472 was cultivated every 7 days on fresh LB agar slant and kept at 28 °C. *P. aeruginosa* PA14 was cultivated in LB media, incubated at 37 °C, and preserved as glycerol stocks at -20 °C.

### Reporter Strain Assay of QSI Potential of Plant Extracts

Quorum sensing inhibitory effect of the tested extracts was estimated by agar cup diffusion assay using *C. violaceum* strain ATCC 12472 (McClellan et al., 1997). Cultures were prepared by cultivating the bacteria in LB broth for 24 h at 28 °C. Luria-Bertani agar plates (1.5% agar) were prepared 15 mL/plate. *C. violaceum* was inoculated (100 µL/plate) in LB soft agar (0.5% agar) and poured on the solidified LB plates. Cups were made in LB agar medium of 10 mm diameter. A volume of 100 µL of each extract was placed in the corresponding cup and the assay plates were incubated at 28 °C for 48 h. Inhibition of quorum sensing was calculated using the equation ( $r_2 - r_1$ ) in mm; where  $r_2$  is the total growth and the QS inhibition zone radius and  $r_1$  is the clear zone radius. Quorum sensing inhibition zone <10 mm was considered moderate activity and when QSI zone >10 mm designated potent effect (Zaki et al., 2013).

### Assay of Some Virulence Factors of *P. aeruginosa* PA14

#### Assay of Pyocyanin

Quantification of pyocyanin was carried out in triplicate via King A broth medium (peptone 2%, K<sub>2</sub>SO<sub>4</sub> 1.0%, and MgCl<sub>2</sub> 0.14%). An overnight *P. aeruginosa* PA14 culture (500 µl) was inoculated into 5 mL of King A media with and without plant extracts (200 µL) and incubated for 24-48 h at 37°C (Essar et al., 1990). Pyocyanin was extracted using CHCl<sub>3</sub> (3 mL), 1 mL HCl 0.2 N was added to the CHCl<sub>3</sub> extract to have pink color and OD<sub>520</sub> nm of the solution was measured. The concentration of pyocyanin was expressed as µg/mL (OD<sub>520</sub> × 17.072) (Ra'boof and Latif, 2010).

### Formation of Biofilm

The activity of various plant extracts on biofilm assembly by *P. aeruginosa* PA14 was measured using tube assay method (Christensen et al., 1982). Overnight culture of *P. aeruginosa* PA14 (500 µL) was inoculated into fresh LB broth (5 mL) with and without plant extracts (200 µL), then tubes were incubated overnight at 37 °C. Free unbound cells were discarded and biofilm layer was washed 3-4 times with water. The formed biofilm was stained by crystal violet (5% w/v) for 10 min, unbound stain was discarded. The tubes were washed and dried in opposite position. The formed biofilm on sides and bottom of the tubes was assigned.

### Motility Assay

*P. aeruginosa* is capable of swimming on soft surfaces, twitching on hard surfaces, and swarming on semi-solid surfaces. The tested plants extracts were added to the motility plates. An overnight culture of PA14 was diluted to 0.1-0.2 nm at OD<sub>600</sub>. Twitching motility was performed through stab-inoculation 1% LB plates with and without plant extracts using 2 µL of the diluted *Pseudomonas* culture and inoculated at 37 °C for 48 h. The diameter of *Pseudomonas* twitching at the interface between plastic and agar was measured (Murray et al., 2010). Flagellum-dependent swimming was performed using swimming media (1% tryptone, 0.5% NaCl, and 0.5% agar). The plates were inoculated with 2 µL of diluted PA14 and incubated for 18 h at 37 °C. The diameter of the turbid zone (mm) was measured (Murray et al., 2010). Swarming motility was assayed using swarming media (0.5% agar, 0.5% peptone, 0.2% yeast extract, and 1.0 % glucose). Two micro-liters of the diluted PA14 culture were inoculated at the middle of the plate and the plates were incubated for 18 h at 37 °C (Krishnan et al., 2012). Motility assay was performed in triplicate and the mean of the diameter was assigned.

### Statistical Analysis

The mean of three separate experiments ± standard deviation was calculated using Excel data sheet. Statistical analysis was estimated using GraphPad Instate software package (version3.05), Tukey-Kramer multiple-comparison test and the significant difference was assigned when P <0.05.

## Results and Discussions

### Quorum Sensing Inhibitory Effect

Assay of QSI activity of the alcohol extracts of 20 medicinal plants was performed using *Chromobacterium violaceum* ATCC12472 reporter strain (McClellan et al., 1997). Most of the tested extracts exhibited potent antibacterial activity against the reporter strain. Inhibition of purple pigmentation of *C. violaceum* provided a readily and easily observable phenotype that simplified and facilitated screening for QSI effect. In the following work, seven of the screened extracts demonstrated QS antagonistic activity. The extracts of *C. sinensis*, *L. nobilis*, *E. cardamomum*, *A. cepa*, and *C. sativum* showed a pronounced quorum quenching effect with violacein inhibitory actions (Table 1). They showed clearly visible white halo in the violacin bioassay (20, 10, 10, 20, and 10 mm),

respectively compared to garlic extract (*A. sativa*) (positive control). It was reported that *A. sativa* inhibited LuxR-based QSI in *P. aeruginosa* due to the presence of ajoene a sulfur-rich molecule (Jakobsen et al., 2012), while the extracts of *P. guajava* and *M. longifolia* showed moderate QSI effect.

**Table 1:** QSI potential of the tested plant extracts.

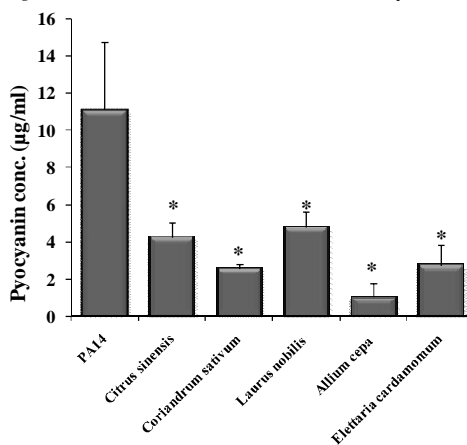
Plant	Part used	Anti-QS zone (mm)	Anti- QS potential
<i>Anethum graveolens</i>	Fruits	-	-
<i>Cucumis melo</i>	Seeds	-	-
<i>Carum carvi</i>	Fruits	-	-
<i>Citrus sinensis</i>	Seeds	20	++
<i>Pimpinella anisum</i>	Fruits	-	-
<i>Foeniculum vulgare</i>	Fruits	-	-
<i>Trigonella foenum graecum</i>	Seeds	-	-
<i>Coriandrum sativum</i>	Fruits	10	++
<i>Laurus nobilis</i>	Leaves	10	++
<i>Psidium guajava</i>	Leaves	3	+
<i>Allium cepa</i>	Outer scales	20	++
<i>Eugenia aromatic</i>	Flowers	-	-
<i>Mentha longifolia</i>	Aerial part	5	+
<i>Elettaria cardamomum</i>	Seeds	10	++
<i>Senna italic</i>	Aerial part	-	-
<i>Valantia hispida</i>	Aerial part	-	-
<i>Tephrosia purpurea</i>	Aerial part	-	-
<i>Teucrium polium</i>	Aerial part	-	-
<i>Commophora molmol</i>	Bark	-	-
<i>Tribulus arabicus</i>	Aerial part	-	-
<i>Allium sativa</i> (positive control)	Bulbs	10	++

+: Moderate anti-quorum sensing activity.

++: Potent anti-quorum sensing activity

### Inhibition of Pyocyanin

*P. aeruginosa* produces green characterized pigment called pyocyanin after 24-48 h of growth. The disappearance of the green coloring of the *P. aeruginosa* PA14 culture indicated the lower produced levels of pyocyanin or no pyocyanin is present in the supernatant. The effect of *C. sinensis*, *L. nobilis*, *E. cardamomum*, *A. cepa*, and *C. sativum* extracts on the production pyocyanin was performed. In this study, pyocyanin level in *Pseudomonas* culture treated with these five plant extracts was significantly reduced ( $P < 0.05$ ) without affecting bacterial growth in contrast to the green pigment of untreated cultures (Figure 1). This could be explicated as quorum-control of pyocyanin pigment production (Dietrich et al., 2006). Moreover, quorum quenching agents have a great impact on pyocyanin release from *P. aeruginosa* (Morkunas et al., 2012; El-Mowafy et al., 2014).

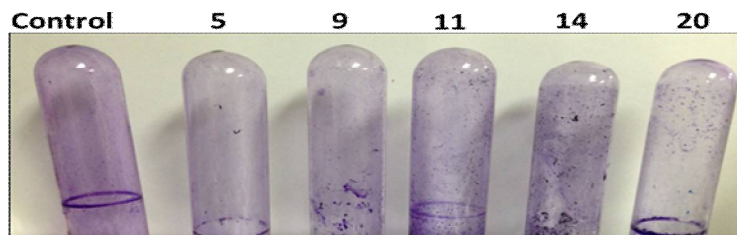


**Figure 1:** Influence of plant extracts on pyocyanin production by PA14: Extracts of *C. sinensis*, *C. sativum*, *L. nobilis*, *A. cepa*, and *E. cardamomum* significantly (\*;  $P < 0.05$ ) inhibited pyocyanin compared to control.

### Effect on Biofilm Development

Furthermore, QS controls bacterial adhesion, biofilm formation, and production of alginates and polysaccharides required for biofilm development (Schuster and Greenberg, 2007). The first discovered natural products inhibited QS and biofilm maturation in Gram-negative bacteria are the halogenated furanones from *Delisea pulchra* (Givskov et al., 1997) and other QSI compounds such as cyclic sulfur derivatives obtained from garlic (Persson et al., 2005) and patulin produced by *Penicillium sp.* (O'Loughlin et al., 2013).

The extracts of *C. sinensis*, *L. nobilis*, *E. cardamomum*, *A. cepa*, and *C. sativum* were tested for *Pseudomonas* biofilm formation using tube assay method. All these plants extracts showed discriminative effect on biofilm formed by *P. aeruginosa* PA14 compared to the untreated control (Figure 2).



**Figure 2:** Effect of plant extracts on biofilm formation by PA14; *Citrus sinensis*, *Coriandrum sativum*, *Laurus nobilis*, *Allium cepa*, and *Elettaria cardamomum* (5, 9, 11, 14, and 20, respectively) compared to control untreated *Pseudomonas* PA14.

### Motility Assay

Normal bacterial motility is important for proliferation of burn and colonization of wounds (Arora et al., 2005). *P. aeruginosa* has three types of bacterial motility: swimming, twitching, and swarming which are propagated by flagella and pili IV. The extracts of *C. sinensis*, *L. nobilis*, *E. cardamomum*, *A. cepa*, and *C. sativum* were tested for their influence on the motility of *P. aeruginosa* PA14. They manifested a distinct influence on swarming and twitching motility (Table 2). *C. sinensis*, *L. nobilis*, and *A. cepa* extracts reduced twitching in PA14 by 88, 77, and 85%, respectively ( $P < 0.01$ ). Previous studies verified that QS systems control *Pseudomonas* motilities and inhibition of QS signals affects the motilities (Glessner et al., 1999; Juhas et al., 2005). Although *C. sativum* and *E. cardamomum* extracts substantially inhibited biofilm and pyocyanin production in PA14, it was noticed that they did not significantly affect bacteria motility. It appears that these extracts contain compounds; some of them are agonist of the motility, while others may affect the different signaling pathways in *P. aeruginosa* PA14. Motility is a complex phenotype that involves various regulatory components (Overhage et al., 2008). Elimination of *Pseudomonas* motility confirmed the potential effect of *C. sinensis*, *L. nobilis*, and *A. cepa* on biofilm formation as modulation of bacterial motilities is associated with thin and dispersed biofilm (Shrout et al., 2006).

**Table 2:** Effect of the tested plant extracts on *Pseudomonas* motility.

Plant	Twitching diameter (mm)	Swimming diameter (mm)	Swarming diameter (mm)
Control/no plant extract	45 ± 0.01	41 ± 0.01	50 ± 0.1
<i>Citrus sinensis</i>	10 ± 0.02	15 ± 0.00	15 ± 0.00
<i>Coriandrum sativum</i>	45 ± 0.01	33 ± 0.02	05 ± 0.00
<i>Laurus nobilis</i>	15 ± 0.01	05 ± 0.01	05 ± 0.03
<i>Allium cepa</i>	07 ± 0.00	06 ± 0.01	01 ± 0.00
<i>Elettaria cardamomum</i>	45 ± 0.01	35 ± 0.01	04 ± 0.00

### Conclusion

*P. aeruginosa* is an opportunistic pathogen that causes serious human infections. The study of QSI activity of some medicinal plants and inhibition of various virulence factors among *P. aeruginosa* could play an important role in eliminating its pathogenicity. From current research, quorum inhibition appears to be a potential mode of action of some of tested extracts to control bacterial pathogenicity. Anti-QS could offer an alternative mode of action against opportunistic pathogenic bacteria. The extracts of *C. sinensis*, *L. nobilis*, *E. cardamomum*, *A. cepa*, and *C. sativum* exhibited strong anti-QS activity. Our findings highlight the importance of these medicinal plants as a rich source of compounds able to inhibit QS and QS-related virulence processes. These medicinal plants could manage *Pseudomonas* pathogenesis and hinder its dissemination. Further investigation of the nature of these QS inhibitor compounds and mechanism of action are still required.

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