Appraisal of Quorum sensing inhibitory activity of Some Medicinal Plants

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المستخلص العربى:

تكشف هذه الدراسة تاثير بعض المستخلصات النباتية على الاتصال الخلوى البكتيرى (احساس النصاب) حيث تم اختبار تاثير ٢١ مستخلص لنباتات غذائية كمثبطات لاحساس النصاب باستعمال بكتريا *Chromobacterium violaceum* strain ATCC 12472 وبينت النتائج ان احسن فاعلية لتعطيل احساس النصاب كانت من مستخلصات النباتات أوراق اللاورى، بذور البرتقال، الاوراق الحرشفية الخارجية للبصل الاصفر، بذور الحبهان، ثمار الكزبرة ولوحظ ايضا فاعلية منخفضة لخلاصة نباتى أوراق الجوافة و عشب الحبق.

ولان احساس النصاب يتحكم فى عوامل الضراوة لبكتريا السودوموناس اريجينوزا مثل انتاج الايلاستياز والبروتياز والهيموليزين والبيوسيانين وتكون الأغشية الحيوية والحركة البكتيرية و انتاج السموم. فقد تم اختبار تاثير الخمسة مستخلصات المانعة لاحساس النصاب على تكون الاغشية البكتيرية و افراز البيوسيانين والحركة البكتيرية لبكتريا السودوموناس اريجينوزا ب ا ١٤ .وعند زراعة بكتريا السودوموناس اريجينوزا ب ا ١٤ فى وجود الستخلصات النباتية اظهرت النتائج منعا ملحوظا فى انتاج والبيوسيانين وانخفاضا فى تكون الاغشية البكتيرية. بالاضافة للتأثير مستخلصات النباتات أوراق اللاورى، بذور البرتقال، الاوراق الحرشفية الخارجية للبصل الاصفر على الحركة البكتيرية.

ونستنتج من هذة الدراسة ولاول مرة اهمية نباتات أوراق اللاورى، بذور البرتقال، الاوراق الحرشفية الخارجية للبصل الاصفر، بذور الحبهان، ثمار الكزبرة كمثبطات للاتصات البكتيرى ولعوامل الضراوة لبكتريا السودوموناس اريجينوزا. المزيد من الدراسة متطلبة للتعرف على التركيب الكيميائى للمركبات و ألية عملها كمثبطات للاتصال الخلوى البكتيرى.

I- Abstract

Out work investigated the influence of some plant extracts on bacterial communication system, expressed as quorum sensing (QS) activity. Quorum sensing inhibition effect of alcohol extracts of 21 medicinal plants were screened using *Chromobacterium violaceum* reporter assay using agar cup diffusion method. Five extracts named *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), *Elettaria cardamomum* (seeds), *Allium cepa* (outer scales), and *Coriandrum sativum* (fruits) exhibited potent quorum quenching effect. While, *Psidium guajava* and *Mentha longifolia* showed lower QS inhibition activity. Virulence factors of *Pseudomonas aeruginosa* are controlled by QS such as biofilm formation, motility, productions of proteases, hemolysin, pyocyanin, and toxins. The five efficient anti-quorum sensing plant extracts were tested for their activity against biofilm synthesis, motility and synthesis of pyocyanin from *P. aeruginosa* PA14. Cultivation of PA14 under the influence of those extracts showed significant elimination of formation of pyocyanin and development of *Pseudomonas* biofilm. In addition, twitching and swimming motilities of *P. aeruginosa* PA14 in the presence of *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), and *Allium cepa* (outer scales) extracts were significantly inhibited. In conclusion, this study illustrated for the first time the importance of *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), *Elettaria cardamomum* (seeds), *Allium cepa* (outer scales), and *Coriandrum sativum* (fruits) as quorum sensing inhibitors and virulence suppressor of *P. aeruginosa*.

II. Introduction

Traditional study and use of medicinal plants have focused on their antibacterial potential (Cowan, 1999; Wallace, 2004). However, the anti-pathogenic potential facts of plants are not completely explored yet.

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 (Jakobsen *et al*., 2012). Bacteria use quorum sensing to multiply and once reached threshold start producing virulence factors. Quorum sensing controls bacterial pathogenicity so interfering with cell-cell communication reduces bacterial pathogenicity (Wu *et al*., 2004).

Quorum sensing circuits are exploited in most of bacteria especially *Pseudomonas aeruginosa. Pseudomonas* infection is associated with cystic fibrosis, urinary tract infection, wound, burn infection and nosocomial infections especially in immune-compromised patients (Hoiby, 1994; Lieberman, 2003.). This organism produces numerous virulence factors such as protease, elastase, pyocyanin, alginate, biofilm formation, bacterial motility, and toxins production (Lyczak *et al*., 2003; Zhang and Dong, 2004). Quorum sensing controls the release of these virulence factors (Donabedian, 2003; Schauder and Bassler, 2001). Quorum sensing of *P. aeruginosa* is regulated by signaling molecules named *N*-acylated homoserine lactone (AHL). The concentration of these auto-inducers increases proportionally with the increase in bacterial population till reaches certain point those signaling molecules diffuse back into the bacteria to control bacterial pathogenicity.

In this study, a number of commonly used medicinal plants: *Allium cepa* (outer scales), *Allium sativa* (bulbs), *Anethum graveolens* (fruits), *Cuminum cyminum* (fruits), *Foeniculum vulgrae* (fruits), *Cinnamomum cassia* (bark), *Carum carvi* (fruits), *Citrus sinensis* (seeds), *Coriandrum sativum* (seeds), *Cucumis melo* L. (seeds), *Capsicum annuum* (fruits), *Elettaria cardamomum* (seeds), *Eugina aromatic* (flowers), *Laurus nobilis* (leaves), *Mentha longifolia* (herbs), *Nigella sativa* (seeds), *Psidium guajava* (leaves), *Thymus vulgaris* (herbs), *Pimpinella anisum* (fruits), *Trigonella foenum gracum* (seeds), and *Piper nigrum* (fruits) were studied for QSI effect with reporter strain assay of *Chromobacterium violaceum*. Plant extracts which showed quorum sensing modulating activity, were investigated for anti-pathogenic potential against *P. aeruginosa* PA14. For this instance, their influence on virulence of *P. aeruginosa* was examined including biofilm formation, pyocyanin production, and motility behavior.

III-Methodology

A-Plant material

A total of 21 plant materials were purchased from commercial sources in Al-Madinah Al-Munawwarah, KSA (Table 1). The selection of plants was based on availability and medicinal use. All plant samples were air-dried at room temperature and finely crushed.

B- Preparation of plant extracts

The air-dried powdered materials (100 g each) were separately extracted at room temperature with 95% methanol (5 × 250 mL). The extracts were filtered with filter paper. They were evaporated under reduced pressure on rotary evaporator, lyophilized, and kept in dry form till use. One milligram concentration was used for anti-quorum sensing activity testing.

Table 1: List of plants used for screening quorum sensing inhibition activity

No.	plant	Part used
1	*Carum carvi*	Fruit
2	*Cucumis melo*	Seed
3	*Anethum graveolens*	Fruit
4	*Cuminum cyminum*	Fruit
5	*Citrus sinensis*	Seed
6	*Pimpinella anisum*	Fruit
7	*Foeniculum vulgrae*	Fruit
8	*Trigonella foenum gracum*	Seed
9	*Coriandrum sativum*	Fruit
10	*Nigella sativa*	Seed
11	*Laurus nobilis*	Leaves
12	*Psidium guajava*	Leaves
13	*Thymus vulgaris*	Herbs
14	*Allium cepa*	Outer scales
15	*Capsicum annuum*	Fruit
16	*Eugina aromatic*	Flowers
17	*Piper nigrum*	Fruit
18	*Cinnamomum cassia*	Bark
19	*Mentha longifolia*	Herbs
20	*Elettaria cardamomum*	Seeds
21	*Allium sativa*	Bulbs

C-Bacterial strains and growth conditions

Reporter assay was performed using *Chromobacterium violaceum* ATCC 12472 (the strain was provided from R.J.C. (Bob) McLean Dept. Biology, Texas State University, San Marcos, TX 7866, USA) were grown at 28 ºC in Luria-Bertani (LB) broth (1% peptone,

0.5% yeast extract, 1%NaCl pH7.4) solidified with 0.5 or 1.5% agar as required (Bertani, 2004). *Chromobacterium violaceum* ATCC 12472 was cultivated every 7 days on fresh LB agar slant and kept at room temperature (26Cº). *Pseudomonas aeruginosa* PA14 was cultivated in (LB) broth and incubated at 37 ºC. It was preserved as glycerol stocks at -20Cº.

D-Reporter strain assay of QSI potential of plant extracts

Quorum sensing inhibition activities of the plant extracts were determined by the agar cup diffusion assay using *Chromobacterium violaceum* strain ATCC 12472 (Zahin *et al*. 2010). Cultures were prepared by cultivating the bacteria in LB broth for 24 hrs at 28°C. Luria Bertani agar plates (1.5% agar) were prepared 20mL/plate. *Chromobacterium violaceum* was inoculated (100µl/plate) in LB agar soft agar (0.5% agar) and solidified. Cups were made in LB agar media of 10 mm diameter. A volume of 200µL of each extract was transferred to the cups and assay plates were incubated at 28°C for 24-48 hrs. Inhibition of quorum sensing was calculated using the equation (r2-r1) in mm; where r2 is the total growth-inhibition zone radius and r1 is the clear zone radius. Quorum sensing inhibition zone <10 mm was considered moderate activity and when QSI zone >10 mm designated potent effect (Trivedi *et al*., 2011).

E- Assay of some virulence factor of *P. aeruginosa* PA14

1-Assay of Pyocyanin

Pyocyanin quantification assay was performed using King A broth media (Peptone 2%, K_2SO_4 1.0%, and $MgCl_2$ 0.14%). Overnight PAO1 culture (500µl) was inoculated into 5ml of King A media with plant extract (200 µL), and incubated 24-48 hrs at 37°C (Essar *et al*., 1990).

Pyocyanin was extracted using chloroform (3mL), 1 mL HCl 0.2 N was added to chloroform extract to have pink color and OD_{520} of solution was measured. Concentration of pyocyanin was expressed as µg/mL (OD_{520} × 17.072) (Ra'oof and Latif, 2010).

2- Formation of Biofilm

The activity of various plant extracts on formation of biofilm by *P. aeruginosa* PA14 was measured by tube assay method (Christensen *et al*., 1982). Overnight culture of *P. aeruginosa* PA14 (500 µL) was inoculated in fresh LB broth (5 mL) with or without plant extract (200 µL), then tubes were incubated overnight at 37°C. Free unbound cells were removed and biofilm layer was washed 3-4 times with water. The formed biofilm was stained

by crystal violet (0.1% w/v) for 10 mins, unbound stain was discarded, and tubes were washed, dried in opposite position and the formed biofilm on sides and bottom of the tubes was assigned.

3- Motility assay

P. aeruginosa is capable of swimming in aqueous environments (low agar conditions), twitching on dry environments (high agar conditions), and swarming on semi-solid surfaces (Yeung *et al*., 2009). The tested plants were added to the motility plates. Overnight culture of PA14 was diluted to 0.1-0.2 nm at OD_{600} (Chow *et al*., 2011).

Pili-dependent twitching was performed through stab-inoculation of 1% LB plates with 2 µL of the diluted *Pseudomonas* culture and inoculated at 37°C for 48 hrs. The twitching zone diameter at interface between plastic and agar was measured (Murray *et al*., 2010).

Flagellum-dependent swimming was performed according Murray *et al*., 2010. Swimming plates (1% tryptone, 0.5% NaCl, and 0.5% agar) were inoculated with 2µL of diluted PA14 and plates were incubated 18 hrs at 37°C. The diameter of the turbid zone (mm) around the inoculation point was measured.

Swarming motility was assayed using swarming media (agar (0.5%), peptone (0.5%), and yeast extract (0.2%) and glucose (1.0 %)) (Kinscherf, 1999). Two micro-liters of the diluted PA14 culture were inoculated in the center of plate`s surface and incubated 18 hrs at 37°C (Krishnan *et al*., 2012).

IV- Result

A- Anti-quorum sensing potential of the plant extracts;

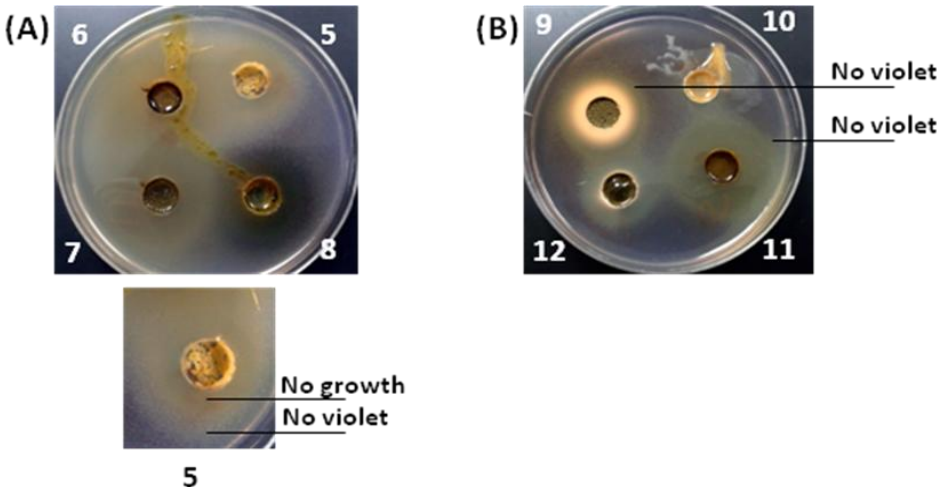
Total 21 plant extracts were tested for their QSI activity using reporter strain *Chromobacterium violaceum* strain ATCC 12472. Most of tested extracts exhibited potent antibacterial activity against the reporter strain. Seven of the tested extracts: *Citrus sinensis*, *Laurus nobilis*, *Elettaria cardamomum*, *Allium cepa*, *Coriandrum sativum*, *Psidium guajava*, and *Mentha longifolia* showed anti-quorum sensing potential indicated as disappearance of the violet color of the reporter strain (Fig. 1). The five plant extracts *Citrus sinensis*, *Coriandrum sativum*, *Laurus nobilis*, *Allium cepa*, and *Elettaria cardamomum* exhibited strong anti-quorum sensing activity/AHL-mediated violacein inhibition activities (20, 10, 10, 20, and 10 mm) radius, respectively. While extracts of *Psidium guajava* and *Mentha*

longifolia showed moderate anti-quorum sensing activity of 6-9 mm radius. Garlic extract was performed as positive control.

Table 2: QSI potential of plant extracts.

No.	Plant	Anti-QS zone (mm)	Anti- QS potential
1	*Carum carvi*	-	-
2	*Cucumis melo*	-	-
3	*Anethum graveolens*	-	-
4	*Cuminum cyminum*	-	-
5	*Citrus sinensis*	**20**	++
6	*Pimpinella anisum*	-	-
7	*Foeniculum vulgrae*	-	-
8	*Trigonella foenum gracum*	-	-
9	*Coriandrum sativum*	**10**	++
10	*Nigella sativa*	-	-
11	*Laurus nobilis*	**10**	++
12	*Psidium guajava*	**3**	+
13	*Thymus vulgaris*	-	-
14	*Allium cepa*	**20**	++
15	*Capsicum annuum*	-	-
16	*Eugina aromatic*	-	-
17	*Piper nigrum*	-	-
18	*Cinnamomum cassia*	-	-
19	*Mentha longifolia*	**5**	+
20	*Elettaria cardamomum*	**10**	++
21	*Allium sativa*	**10**	++

+: Moderate antiquorum sensing activity
++: Potent antiquorum sensing activity



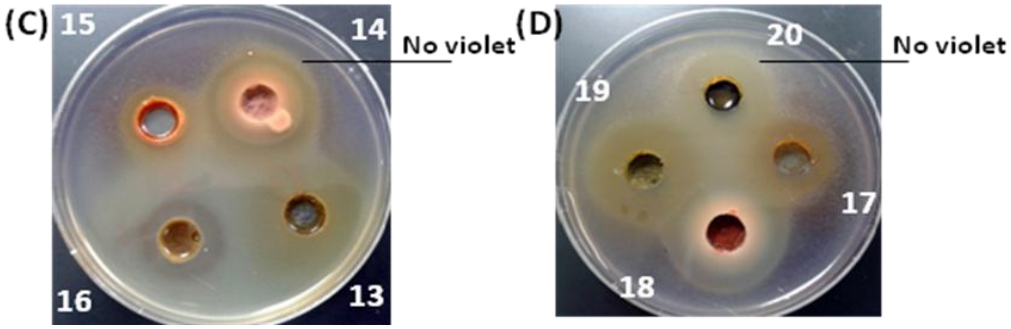


Fig. 1: Inhibition of quorum sensing activity of the tested extracts using reporter strain *Chromobacterium violaceum* strain ATCC 12472. Clear zone indicated antimicrobial activity of the extracts. Inhibition of violet color characterized QSI activity of *Citrus sinensis*, *Coriandrum sativum*, *Laurus nobilis*, *Allium cepa*, and *Elettaria cardamomum* (5, 9, 11, 14, and 20, respectively).

b- Assay of pyocyanin

Pyocyanin assay for the plant extracts *Citrus sinensis*, *Laurus nobilis*, *Elettaria cardamomum*, *Allium cepa*, and *Coriandrum sativum* was performed using King A broth media showed significant QSI. We investigated the effect of those extracts on the production pyocyanin. *P. aeruginosa* produces green characterized pigment called pyocyanin after 24-28 hrs of growth. Disappearance of the green coloring of the *P. aeruginosa* PA14 culture indicated lower produced levels of pyocyanin, or no pyocyanin is present in the supernatant. Treated *Pseudomonas* culture with the five plant extracts showed decrease in the green *Pseudomonas* pigment Fig. 2 in contrast to the green pigment of untreated cultures.

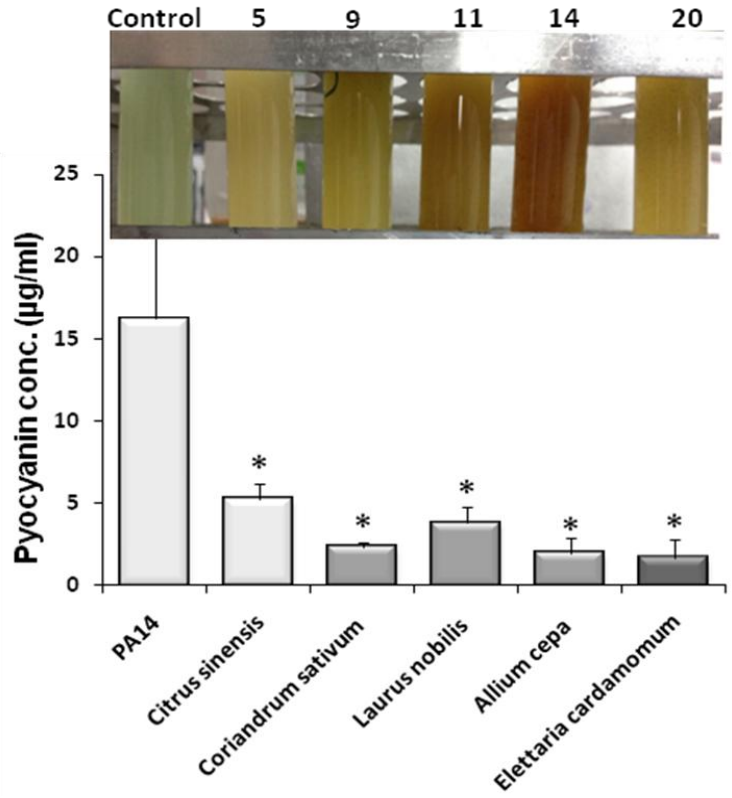


Fig. 2: Influence of plant extracts on pyocyanin production by PA14; Extracts of *Citrus sinensis*, *Coriandrum sativum*, *Laurus nobilis*, *Allium cepa*, and *Elettaria cardamomum* (5, 9, 11, 14, and 20, respectively) significantly inhibited pyocyanin compared to control**.**

c- Effect on biofilm development:

Extracts of *Citrus sinensis*, *Laurus nobilis*, *Elettaria cardamomum*, *Allium cepa*, and *Coriandrum sativum* were tested for *Pseudomonas* biofilm formation using tube assay method. All these plants extracts showed discriminative effect on biofilm formation compared to the control untreated PA14 Fig. 3.

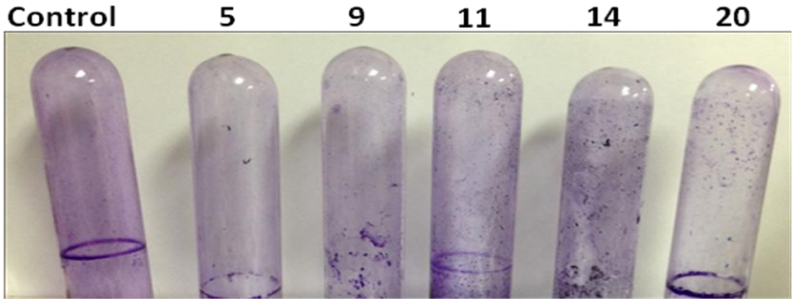


Fig. 3: Effect of plant extracts on biofilm formation by PA14; *Citrus sinensis*, *Coriandrum sativum*, *Laurus nobilis*, *Allium cepa*, and *Elettaria cardamomum* (5, 9, 11, 14, and 20, respectively) compared to control untreated *Pseudomonas* PA14.

d- Motility assay

Pseudomonas aeruginosa has three kinds of bacterial motility; swimming twitching and swarming which are propagated by flagella and pili IV (Rashid and Kornberg, 2000).

Plant extracts *Citrus sinensis*, *Laurus nobilis*, *Elettaria cardamomum*, *Allium cepa*, and *Coriandrum sativum* were tested for their influence on the motility of *P. aeruginosa* PA14. Three extracts *Citrus sinensis*, *Laurus nobilis* and *Allium cepa* showed marked variation in the *Pseudomonas* motility compared to control *Pseudomonas* (Table 3).

Table 3: Effect of the tested medicinal plants extract on *Pseudomonas* motility.

Plant	Twitching diameter (mm)	Swimming diameter (mm)	Swarming diameter (mm)
Control/no plant extract	45	41	50
Citrus sinensis	10	15	15
Coriandrum sativum	45	33	05
Laurus nobilis	15	05	05
Allium cepa	07	06	01
Elettaria cardamomum	45	35	04

Discussion

Research efforts have focused recently upon developing anti-pathogenic agents to attenuate bacterial infection by inhibiting the communication between bacteria. Bacteria communication is attained through chemical signaling molecules known as auto-inducers (Siehnela *et al*., 2010). Most of the pathogenic microbes use QS circuits to induce the production of their virulence factors. Thus, inhibition of QS is considered as an imperative approach to manage bacterial virulence and antimicrobial resistance (Hong *et al*., 2012). Plants are an enriched resource of quorum sensing inhibitors as *Panax notoginseng*, *Areca catechu*, *Prunus armeniaca*, *Prunella vulgari*s, *Nelumbo nucifera*, *Punica granatum*, and *P. ginseng* (Koh and Tham, 2011; Song *et al*., 2010), *Vanilla planifolia* (Choo *et al*., 2006). Therefore, current study investigated the influence of extracts of some medicinal plants on QS.

Assay of QSI activity of the plant extracts was performed using *Chromobacterium violaceum* ATCC12472 reporter strain ((McClean *et al*., 1997; Zahin *et al.,* 2010*;* Zaki *et al.,* 2013*)*. Inhibition of purple pigmentation of *C. violaceum* provided a readily and easily observable phenotype that simplified and facilitated screening for QSI. In the following work, using reporter strain assay, seven out of the screened plant extracts (21) demonstrated QS antagonistic activity (Table 1). Five extracts named *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), *Elettaria cardamomum* (seeds), *Allium cepa* (outer scales), and *Coriandrum sativum* (fruits) showed clearly visible white halo in the violacin bioassay (Table 2; Fig. 1). Extracts from *Citrus sinensis* (seeds) and *Allium cepa* (outer scales) showed the strongest QSI

properties, resulting in a cream-coloured zone 20 mm diameter, which was comparable to that observed in the control known QS antagonist (*Allium sativa*) (Fig. 2). It was reported that *Allium sativa* inhibited LuxR-based QSI in *P. aeruginosa* due to its contents of disulphides and trisulphides (Rasmussen *et al*., 2005). While, *Psidium guajava* and *Mentha longifolia* extracts showed lower activity (3 and 5 mm, respectively). The remaining screened plants extracts did not show noticeable QSI activity with *Chromobacterium violaceum*.

Chromobacterium violaceum reporter strain is considered as model organism so assessment of QSI on pathogenic organism was required to specify activity of the extracts against virulence of pathogenic microorganisms. *P. aeruginosa* is an opportunistic organism commonly associated with nosocomial infections. Quorum sensing controls expression of virulence gene such as biofilm mauration and toxins release in *P. aeruginosa* **(**Hentzer *et al*., 2002). In our study, *P. aeruginosa* PA14 was used to study the effect of QSI potential of *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), *Elettaria cardamomum* (seeds), *Allium cepa* (outer scales), and *Coriandrum sativum* (fruits) extracts on bacterial pathogenesis.

Pyocyanin a blue pigment that appears on king A media. In this study, pyocyanin level was significantly reduced by *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), *Elettaria cardamomum* (seeds), *Allium cepa* (outer scales), and *Coriandrum sativum* (fruits) without affecting bacterial growth (Fig. 2). This could be explicated as pyocyanin is a quorum-controlled extracellular virulence pigment (Dietrich *et al*., 2006). Quorum quenching agents has great impact on pyocyanin release from *P. aeruginosa* (Morkunas *et al.,* 2012)**.**

Furthermore, QS controls bacterial adhesion and biofilm formation, production of alginates and polysaccharides required for biofilm development (Schuster and Greenberg, 2007). In the following work the five plant extracts *Citrus sinensis* (seeds), *Laurus nobilis* (leaves), *Elettaria cardamomum* (seeds), *Allium cepa* (outer scales), and *Coriandrum sativum* (fruits) exhibited anti-QS activity reduced also biofilm formed of *P. aeruginosa* PA14 (Fig. 2). Some natural products inhibited QS and biofilm maturation in gram-negative bacteria. The first discovered compounds QSI are halogenated furanone from *Delisea pulchra* (Givskov *et al*., 1997), cyclic sulfur derivatives obtained from garlic (Persson *et al*. 2005), and Patulin produced by *Penicillium sp* (Colleen *et al*., 2013).

Normal motility has also been showed to be important for the pathogen proliferation of burn and wound infections (Arora *et al.,* 2005). In the PA14 bioassay, the five extracts

manifested a distinct influence on swarming and twitching motility (Table 3). Extracts from *Citrus sinensis*, *Laurus nobilis*, and *Allium cepa* reduced twitching in PA14 by 88, 77, and 85%, respectively. Previous studies verified that QS systems contribute *Pseudomonas* motilities and inhibition of QS signals affects the motilities (Glessner *et al.,* 1999; Juhas *et al.,* 2005). Although, *Coriandrum sativum* and *Elettaria cardamomum* substantially inhibited biofilm and pyocyanin in PA14. It was noticed that they also did not significantly affect bacteria motility. It appears there may be more than one compound present in their extracts, one of which is an agonist of motility, with others that may affect a different signaling pathway 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 *Citrus sinensis*, *Laurus nobilis*, and *Allium cepa* on biofilm formation as modulation of bacterial motilities is associated with thin and disperse biofilm (Shrout *et al.,* 2006; Martin *et al*., 2008).

From current research, quorum inhibition appears to be a potential mode of action of some of these extracts to control bacterial pathogenicity. Anti-QS could offer an alternative mode of action against opportunistic pathogenic bacteria. Our findings highlight the importance of these medicinal plants as a rich source of compounds able to inhibit QS and QS- related virulence processes. Further investigation for the nature of these QS inhibitor compounds and mechanism of action are still required.

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