Evaluation of three plant extracts against biofilm formation and expression of quorum sensing regulated virulence factors in *Pseudomonas aeruginosa*

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Abstract: Following the increasing antibiotic resistance of pathogenic bacteria, the use of medicinal herbs as antibacterial agents has attracted growing attention. *Pseudomonas aeruginosa* is a human opportunistic pathogen that uses quorum sensing for regulating virulence gene expression (pyocyanin, protease, and elastase production and biofilm formation). This study examined the anti-quorum sensing activity of *Quercus infectoria*, *Zataria multiflora* and *Trachyspermum copticum* extracts on standard *P. aeruginosa* strain. The minimum inhibitory concentration (MIC) of *Q. infectoria, Z. multiflora* and *T. copticum* extracts for standard *P. aeruginosa* strain was determined through micro dilution. Microtiter plates were used to evaluate the anti-quorum sensing effects of the three extracts (at a sub-MIC concentration) on pyocyanin, protease, and elastase production and biofilm formation. The acetone extract of *Q. infectoria* showed the highest anti-quorum sensing activity and reduced the pyocyanin, protease, and elastase production and biofilm formation by 89.1%, 78%, 73.3%, and 70.1%, respectively. The corresponding values were 88.2%, 72.1%, 69%, and 61.1% for the methanol extract of Z. multiflora and 70.6%, 63.42%, 60.1%, and 59.1% for the methanol extract of *T. copticum*. Considering the high anti-quorum sensing activity of the studied extracts, especially the acetone extract of *Q. infectoria,* these herbs can be used as antipathogenic drugs.

Keywords: Anti-quorum sensing, *Pseudomonas aeruginosa*, biofilm.

INTRODUCTION

Pseudomonas aeruginosa is one of the most common nosocomial pathogens. Following its increasing resistance to antimicrobial agents, treatment of *P. aeruginosa* infections has turned into a major challenge for health systems throughout the world. In quorum sensing, bacteria secrete particular signaling molecules to communicate with each another and to trigger the expression of certain types of genes, including virulence genes (Caetano *et al.,* 2010). Since quorum sensing depends on population density of bacteria, signals are secreted as the population of bacteria reaches a critical limit. These signals are then received by specific transcription-regulatory proteins (receptors) which in turn activate the expression of target genes through DNA binding (Reading 2006 and Bhardwaj *et al*., 2013). A wide range of virulence factors of *P. aeruginosa*, e.g. biofilm formation and production of pyocyanin, protease, and elastase, are coordinated by quorum sensing (Ben Haj *et al.*, 2011). Pyocyanin plays an important role in bacterial colonization and interferes with the ciliary beat, immunoglobulin release from B-lymphocytes, and epithelial cell growth. Elastase and protease are also involved in the degradation of human lung elastin (Hoge *et al.*, 2010). Moreover, biofilm formation protects bacteria against the host's immune system and causes antibiotic resistance (Høiby *et al.,* 2010). Biotic factors (e.g. bees) can cause the development of abnormal outgrowths of plant tissue, called galls, on different parts of *Quercus infectoria* (Vermani 2009). Galls not only serve as food reservoirs for their hosts, but also protect their hosts (Cornell 1983). Galls produced by the asexual activity of *Andricus strenlichti* bees (from the family Cynipidae) on the lateral and terminal buds of *Quercus infectoria* contain phenolic compounds, such as tannic acid and gallic acid, which possess physiological effects and antioxidant, anti-bacterial, anti-inflammatory, and anti-fungal properties (Basri 2005). Acetone, methanol, and ethanol extracts of these galls are also known to have high antibacterial activity (Karbasizade *et al.,* 2014). *Zataria multiflora* (belonging to the Labiatae family) is one of the most valuable plants in traditional medicine. Carvacrol and thymol, the major components of *Zataria multiflora* (thyme), have been reported to possess antioxidant, antibacterial, and anti-fungal properties (Ben Arfa *et al.,* 2006 and Malekinejad *et al.*, 2012). *Trachyspermum copticum* is an herb in the Apiaceae family with proven antimicrobial activity against different microorganisms. The seeds and oils of this plant are commonly used and its essential oils generally contain thymol (37.2%), carvacrol, terpenes (27.3%), and p-cymene (32.3%) (Mahboubi 2011). Despite the

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antibacterial properties of the above-mentioned herbs and their wide use in Iranian traditional medicine, no study has evaluated the anti-quorum sensing properties of their extracts. Therefore, this study examined the anti-quorum sensing effects of traditional medicinal extracts of *Quercus infectoria, Zataria multiflora* and *Trachyspermum copticum* on virulence factors associated with quorum sensing in *P. aeruginosa*. This study examined the anti-quorum sensing activity of *Quercus infectoria, Zataria multiflora* and *Trachyspermum copticum* extracts on standard *P. aeruginosa* strain.

MATERIALS AND METHODS

Due to the *in-vitro* experimental design of the current study, it did not require sampling or sample size determination. Standard *P. aeruginosa* strains (PTCC 1430) were purchased from the Persian Type Culture Collection (PTCC). The obtained strains were capable of pyocyanin, elastase, and protease synthesis.

Preparation of herbal extracts

Quercus infectoria and *Zataria multiflora* were obtained from Khor and Biabanak, Iran. *Trachyspermum copticum* was collected from Sistan and Baluchestan Province, Iran. Maceration was used to prepare the acetone extract of *Quercus infectoria* and methanol extracts of *Zataria multiflora* and *Trachyspermum copticum.*

In order to prepare the *Quercus infectoria* extract, 50 g of *Quercus infectoria* powder was allowed to macerate in 250 ml of acetone at room temperature for 24 hours. Afterward, the solution was passed through Whatman filter papers. The remaining solid was again solved in 100 ml of acetone, let stand for 24 hours, and finally filtered. The extract was poured into sterile plates under sterile conditions and dried at room temperature (Basri 2005). To prepare the methanol extracts of *Zataria multiflora* and *Trachyspermum copticum*, 50g of each plant's powder was added to 250ml of 85% methanol in an Erlenmeyer flask. The flasks were then covered with aluminum foil and placed on a shaker for 24 hours. Afterward, the solution was passed through Whatman filter papers and the extract was poured into sterile plates and oven dried for 48 hours (Samsam Shariat 1992).

The extracts were diluted to obtain a dilution series of 10 mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.3125mg/ml, 0.1562mg/ml, 0.0781mg/ml, 0.039 mg/ml, and 0.0195mg/ml. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each extract was determined through micro dilution (Pelzb *et al.,* 2007).

Evaluation of the anti-quorum sensing activity of the extracts

Pyocyanin assay

In this method, 250µl of the extracts were added to sterile tubes and a concentration of 0.5MIC was reached. In the next stage, 100µl of the microbial suspension with a turbidity of 1.5×10^7 CFU/ml were added to each tube. Similar tubes containing only the microbial suspension were also prepared and considered as controls. The tubes were incubated at 37°C for 24 hours and their content was centrifuged at 8000 rounds per minute (rpm) for 10 minutes at 4°C. The supernatant was transferred to a sterile tube and added with 3ml of chloroform. The optical absorption of the solutions (both control and the extract-treated samples) was measured using a spectrophotometer (UNIC-UV-2100, USA) at a wavelength of 690 nm (Krishnan *et al.,* 2012).

Total proteolytic activity

In order to prepar (e a 0.5% azocasein solution, 100 ml of 50mM Tris buffer containing 2mM calcium chloride were added to 0.5g of azocasein. After centrifugation of the mixture, the supernatant was transferred to a sterile tube and maintained at 4°C for future use. Afterward, 250µl of the extract (at a sub-MIC concentration) and 100µl of the microbial suspension were poured into a sterile tube. Another tube containing the microbial suspension and no extract was also prepared to serve as the control. The tubes were incubated at 37°C for 24 hours. The contents of the tubes were then centrifuged at 8000 rpm for 10 minutes at 4°C. Protease activity was determined through Lowry assay (Lowry *et al.,* 1951). In brief, 100µl of the supernatant from the control and extract-treated tubes were separately added to 900µl of 0.5% azocasein. After maintaining the samples at 37°C for 30 minutes, 250µl of 15% trichloroacetic acid were added to the solution. Five minutes later, the solutions were centrifuged at 8000 rpm for 10 minutes at 4°C. The transparent supernatant of both the control and extract-treated samples was transferred to a cuvette and its optical absorption was measured using a spectrophotometer at a wavelength of 440 nm (Huerta *et al.,* 2008).

Elastase activity

During this procedure, 250 µl of the extract (at a sub-MIC concentration) was prepared and mixed with 100µl of the microbial suspension. Another tube containing the microbial suspension alone was considered as the control. The tubes were incubated at 37°C for 24 hours. Their content was then centrifuged at 8000 rpm for 10 minutes at 4°C. Afterward, 200µl of the supernatants were transferred to a sterile tube. A 0.5% solution of elastin Congo red in phosphate-buffered saline was then prepared and 1 ml of the solution was added to the mentioned supernatants. The tubes were maintained in a water bath at 37°C for 3 hours. The samples were then centrifuged at 1200 rpm for 10 minutes at 10°C. The transparent supernatant of the samples was transferred to a cuvette and the optical absorption was measured using a spectrophotometer at a wavelength of 494 nm (Huerta *et al.,* 2008).

Table1: The minimum inhibitory concentration (MIC) of different extracts against the *P. aeruginosa*

Extract	MIC (mg/ml)	MBC (mg/ml)
QAE	0.312	0.625
ZME	5	10
TME	2.5	5

QAE, *Q*uercus *infectoria* aceton extract ZME *Zataria multiflora* methanol extract TME, *Trachyspermum copticum* methanol extract.

Biofilm formation

The bacteria were cultured in the Mueller Hinton Broth for 24 hours. The optical absorption of the microbial suspension was then set at 0.4 using a spectrophotometer at a wavelength of 600nm. In the next stage, 1ml of the herbal extracts (at a sub-MIC concentration) was prepared and mixed with 100µl of the microbial suspension. Afterward, 250µl of the mixture was transferred to the wells of a micro plate. Wells containing the culture medium and microbial suspension were considered as the positive control. Moreover, wells containing the culture medium and the extracts were considered as the negative control. After maintaining the micro plate at 30°C for 16 hours, 200µl of the supernatants were extracted from the wells and each well was washed twice by adding 300µl of phosphate-buffered saline. Then, 200µl of a 0.2% crystal violet solution was added to each well. Fifteen minutes later, the content of the wells were removed and the wells were washed with distilled water using a micropipette until the blue color disappeared. After adding 150µl of 96% ethanol to each well, the wells were left untouched for 15 minutes until the stained cells were stabilized. The optical absorption of the wells was read using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 650 nm (Abraham *et al.,* 2011).

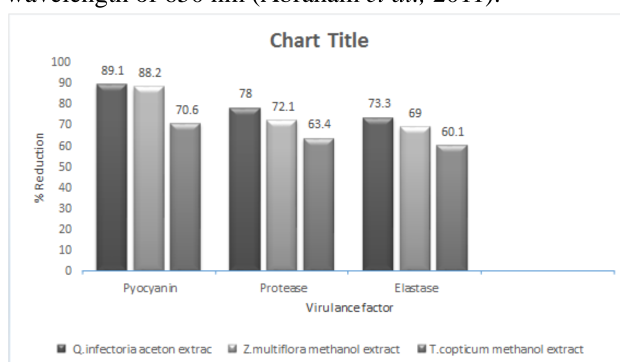

Fig. 1: Effect of sub-inhibitory concentration of extracts on quorum sensing dependent pyocyanin pigment formation, elastase, proteolytic enzymes production in *P. aeruginosa*

Each test was performed in triplicates and the mean percentage reduction in virulence factors production was calculated using the following equation:

Percentage reduction in virulence factors production = optical absorption of the control sample - optical absorption of the extract-treated sample / optical absorption of the control sample × 100

Data analysis

The least significant difference (LSD) test in MSTAT-C software package (Michigan State University, USA) was used to compare the mean values of the groups.

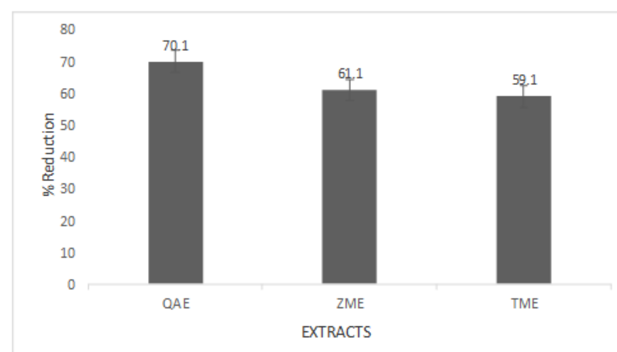

Fig. 2: Inhibition of biofilm formation in sub- MIC concentrations of extracts in *P. aeruginosa.* QAE, *Q. infectoria aceton* extract; ZME*, Z. multiflora* methanol extract; TME, *T. copticum* methanol extract

RESULTS

The MIC and MBC for all the three extracts were determined through three replicates of micro dilution method. The maximum antibacterial activity was seen in case of the acetone extract of *Quercus infectoria* and then the methanol extract of *Trachyspermum copticum* (table 1).

Provides the mean percentage reduction in the production of the three virulence factors (i.e. pyocyanin, protease, and elastase) at sub-MIC concentrations of the three extracts (fig. 1).

According to the LSD test, the mean percentage reductions in pyocyanin produced by the acetone extract of *Quercus infectoria* and the methanol extract of *Zataria multiflora* were not significantly different (P>0.05). However, there was a significant difference between the above-mentioned extracts and *Trachyspermum copticum* extract in this regard (LSD value = 1.796).

The three extracts had significant differences in terms of the mean percentage reduction in protease production at sub-MIC concentrations. The acetone extract of *Quercus infectoria* showed the highest anti-quorum sensing activity in protease production (LSD value = 1.189).

Likewise, there were significant differences between the three extracts in terms of the mean percentage reduction in elastase production. In fact, the acetone extract of *Quercus infectoria* caused the greatest percentage reduction in elastase production (LSD value = 0.3991).
Finally, among all three extracts, the acetone extract of *Quercus infectoria* had the highest inhibitory effects on biofilm formation (LSD value =0.4701). The second most effective extract in this regard was the methanol extract of *Zataria multiflora* (fig. 2).

DISCUSSION

Based on the results of this study, all three extracts showed anti-quorum sensing activity. In a similar study, Balakumar *et al*. reported the anti-quorum sensing effects of the aqueous and ethanol extracts of *Quercus infectoria* on pyocyanin production in *P. aeruginosa* as 100% and 72%, respectively (Balakumar and Jeyanthi, 2010). Differences in the types and active components of extracts, as well as the types of gall wasps, could have been responsible for the observed inconsistencies between different studies. The inhibitory effects of the acetone extract of *Quercus infectoria* on protease activity were higher in the present study than in previous research. Balakumar *et al*. found the aqueous and ethanol extracts of *Quercus infectoria* to reduce protease activity by 75% and 72%, respectively.

In the current study, the acetone extract of *Quercus infectoria* reduced elastase activity by 73.52%. According to Balakumar *et al*., the aqueous and ethanol extracts of the same herb (the type of gall wasp is unknown) could inhibit elastase activity by 75% and 69%, respectively. Apparently, our findings were in line with the results of previous studies.

In our study, a 70.1% reduction in biofilm formation was observed when *P. aeruginosa* was growth occurred in the presence of the acetone extract of *Quercus infectoria.* Likewise, the above-mentioned study reported the inhibitory effects of the aqueous and ethanol extract of *Quercus infectoria* on biofilm formation as 72% and 66%, respectively.

No previous research has evaluated the anti-quorum sensing activity of *Zataria multiflora* and *Trachyspermum copticum* extracts. However, the anti-quorum sensing activity of these herbal extracts is not surprising as both plants contain similar chemical phenolic and antibacterial compounds including carvacrol and thymol. Such compounds are known to act like the analogs of bacterial signals and suppress the expression of virulence factors (Nagy 2010). All the three studied herbs, i.e. *Quercus infectoria, Zataria multiflora* and *Trachyspermum copticum*, contain phenolic or flavonoid compounds which can disturb and shut down the quorum sensing system by impairing the synthesis, secretion, and transmission of signals, performing antagonistic activity (producing signals resembling bacterial signals), and receptor binding inhibition (Nazzaro and Coppola,2013). Moreover, flavonoid compounds have strong antibacterial effects. In fact, they cause changes in acidity gradient by affecting respiratory enzymes, binding to adhesions, having reactions with sulfhydryl groups and forming a complex with cell walls through non-specific reactions with microbial proteins. The consequent electrical potential ultimately impairs the energy system of microorganisms (Policegoudra *et al.,* 2010). This study was the first time to confirm the efficacy of *Quercus infectoria, Zataria multiflora* and *Trachyspermum copticum* extracts in reducing the virulence factors of *P. aeruginosa*. Therefore, these three medicinal herbs, especially *Quercus infectoria*, can be potentially used as anti-pathogenic agents to inhibit quorum sensing. Considering the increasing antibiotic resistance of *P. aeruginosa*, active biological compounds of these extracts can be appropriate alternatives for antibiotics in the future.

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