Effects of Sub-inhibitory Concentrations of Essential Oils of *Mentha spicata* and *Cumminum cyminum* on Virulence Factors of *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is one of important opportunistic pathogen, that cases serious infections. It produces many virulence factors, and this bacterium usually is resistance against antimicrobial agents.

Objective: The aim of this study was evaluate the effects of sub-MICs of essential oils of *Mentha spicata* and *Cumminum cyminum* on alginate production, biofilm formation, swimming, twitching and adhesion in *P. aeruginosa* 8821M.

Method: Minimal inhibitory concentrations (MIC) of essential oils of *Mentha spicata* and *Cumminum cyminum* were determined by macrodilution method. Alginate production, biofilm formation, swimming, twitching and adhesion in the present of sub-MICs (1/2, 1/4 and 1/8 MIC) of essential oils were determined in mucoid *P. aeruginosa* 8821M and compared with controls.

Results: The MICs of essential oils against *P. aeruginosa* for *M. spicata* and *C. cyminum* oils were obtained 16 and 32 µg/ml respectively. The results show that all oils at 1/2 and 1/4 MICs were significantly reduced all tested virulence factors. At 1/8 MICs, *M. spicata* had effect just on adhesion but *C. cyminum* had effect on Alginate production, biofilm formation, swimming and twitching.

Conclusion: This study showed that sub-MIC levels of *M. spicata* and *C. cyminum* essential oils affected alginate production, biofilm formation, swimming, twitching and adhesion in *P. aeruginosa* 8821M and it is probable to use of these medicinal plants for treating.

Keywords: Alginate, Biofilm, *Cumminum cyminum*, *Mentha spicata*, *Pseudomonas aeruginosa*

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Introduction

The opportunistic pathogen *Pseudomonas aeruginosa*, a ubiquitous environmental bacterium showing great adaptability and metabolic versatility, is the leading cause of morbidity and premature mortality in patients with cystic fibrosis. *Pseudomonas aeruginosa* is remarkable in that it can cause both very acute and very chronic infections. Progress in understanding the pathogenesis of acute *P. aeruginosa* infections has implicated virulence factors. This opportunistic pathogen produces a number of unique virulence factors. Extracellular toxins, proteases, hemolysins, and exopolysaccharides are some of the types of virulence factors that have been implicated in the pathogenicity of *P. aeruginosa* [1, 2]. Fimbriation, host defense interaction, adhesion and biofilm formation are of number of cell associated virulence or survival factors that contribute to its pathological effects [3]. Some antimicrobial agents at sub-inhibitory concentrations could influence factors like adherence [4]. Treatment with sub-inhibitory concentrations (sub-MIC) of some antimicrobial agents may influence bacterial virulence factors, such as adherence [4], cell surface hydrophobicity [5], biofilm formation [6], sensitivity to oxidative stress and motility [7]. A motility type in aqueous environments is brought about by polar flagellum in association with cell surface structures like type IV Pilli that act as a virulence factor. These, being responsible for surface translocation or twitching motility, facilitate bacterial adhesion to biotic and abiotic surfaces [8]. Some plant-derived compounds inhibit peptidoglycan synthesis [9], damage microbial membrane structures [10], modify bacterial membrane surface hydrophobicity [11], and modulate quorum sensing [12], all of which could influence biofilm formation. Terrestrial plants also support populations of surface-attached bacteria and could potentially produce phytochemicals that attenuate biofilm development through specific mechanisms [13]. However, many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth [14]. To date, there is no information in the literature concerning the influence of sub-MIC essential oils on *P. aeruginosa* virulence factors. The present study describes specific inhibition of virulence factors production by activity of *Cumminum cyminum* and *Mentha spicata* essential oils against *P. aeruginosa*.

Methods

Bacterial strain

Mucoid *P. aeruginosa* 8821M was kindly donated by Dr. Isabel Sa-Corria, Instituto Superior Tecnico, Lisboa, Portugal [15]. The strain was maintained in 10% skimmed milk (Difco Laboratories, Ditroit, MI) at -80ºC and was subcultured on muller-Hinton agar (Difco Laboratories, Ditroit, MI).

Plants and oil isolation

The plants (*Mentha spicata* and *Cumminum cyminum*) were identified and provided by Research Institute of Medicinal Plants (Tehran, Iran). The shadow dried plants were hydro-distilled for 90 minutes in full glass apparatus. The oil was isolated using a Clevenger type apparatus. The extraction was carried out for 2 hours after 4-hours maceration in 500 ml of water. The essential oils were dissolved in dimethylsulfoxide (with volume ratio of 1:1) and sterilized by filtration through a 0.45µm membrane filter.

The oils so extracted were stored in dark glass bottles in a refrigerator until they were used.

Antimicrobial susceptibility

Determination of MICs was carried out using a macrobroth dilution method according to the NCCLS procedure. Mueller -Hinton

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broth (Merck) incorporated with double dilutions of an initial antibiotic solution was inoculated with 1.0×10^5 CFU/ml test organism and incubated overnight at 37°C. Highest dilution or lowest concentration that inhibited visible growth was regarded as MIC [16].

Adhesion assay

In the adhesion assay, bacterium was grown overnight (37°C, 150 rpm) in Luria–Bertani (LB) broth (Difco) in the presence or absence of oils at a subinhibitory concentration (1/2, 1/4 and 1/8 MIC). Then bacteria were washed with PBS, pH 7.4, twice and centrifuged at 1000g followed by resuspension in PBS to give an approximate inoculums size of 10^7 CFU/ml. The concentration was further confirmed by culturing the serial dilutions followed by colony count. Sterile 96-well polystyrene microtitre plate wells containing medium with or without oils were inoculated with 200µl of the inoculums and incubated at 37°C/1hour. The plates were then double rinsed with PBS to remove non-adhereing bacteria and were air dried in inverted position. Safranin solution (0.1%) was added to the wells for 30s. The wells were rinsed to remove the excess dye. Acetone-ethanol solution was used to extract the bound dye. OD_{492} was taken to measure the absorbance [17].

Motility assays

Assays were performed on bacterium grown overnight with 1/2, 1/4 and 1/8 MIC oils and inoculated on to plates with medium containing 1/2, 1/4 and 1/8 MIC oils. Control plates contained no oils.

Swimming- A medium composed of 1% tryptone (Difco), 0.5% NaCl and 0.3% agar was used for swimming assay. Sterile toothpicks were used to inoculate the plates in the centre and incubated at 25°C/16h. The turbid zone indicating bacterial migration away from the centre was measured to assess the motility [18].

Twitching- A thin (approximately 3 mm) layer of 1% LB agar was inoculated by stabbing with a toothpick to the bottom of the Petri dish and incubated at 30°C/24-48h until a hazy zone of bacterial growth was visible. The agar layer was then removed and the attached cells were washed out by running water. The attached cells were stained with a 1% (w/v) crystal violet solution to determine the bacterial ability to twitch strongly on polystyrene surface of petri dish [18].

Biofilm-formation assay

Biofilm formation was examined in microtitre plates. Briefly, bacteria was grown overnight in LB broth (37 ºC, 150 rpm.) oils to an approximate inoculums size of 10^7 CFU/ml in the presence or absence of 1/2, 1/4 and 1/8 MIC oils as described for the adhesion assay. Bacteria were quantified after an incubation period of 16h at 37°C with 0.025% safranin solution employing the method described for the adhesion assay [17].

Alginate production assay

500 µl of bacterial suspension corresponding to 0.5 McFarland standard solution was added to each of 50ml flasks each containing 20ml sterile LB broth. Test flasks contained 1/2, 1/4 and 1/8 MIC of essential oil while flasks without oil served as control. The flasks were then placed on an incubator shaker for 24 hours at 37ºC. The samples were subjected to quantitative assay of alginate. Alginate production was estimated as follows: 70 µl of the sample was slowly added to 600 µl of boric acid- H_2SO_4 solution in a test tube placed in an ice bath. The mixture was vortexed for about 4 seconds and was placed

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back in the ice bath. 20 µl of 0.2% carbazole solution in ethanol was added to the test tube and was then immediately vortexed for about 4 seconds. The mixture was placed in a water bath at 55ºC for 30 minutes. The absorbance was measured spectrophotometrically at 530 nm [19].

Statistical analysis

Each assay was repeated 6 times. Data obtained from the experiments were presented as differences between controls and tests and were analyzed by the paired *t*-test.

Results

The MICs of essential oils against *Pseudomonas aeruginosa* for *Mentha spicata* and *Cumminum cyminum* oils were 16 and 32 µg/ml respectively.

The results of sub-MICs of essential oils on swimming, twitching, adhesion, alginate production and biofilm formation in *P. aeruginosa* were summarized in Table 1. The results show that all oils at 1/2 and 1/4 MICs were significantly reduced swimming, twitching, adhesion, alginate production and biofilm formation in *P. aeruginosa*. At 1/8 MICs, *M. spicata* oil had not significantly effects on swimming, twitching and biofilm formation, but had significantly effect on adhesion and alginate production. Also, 1/8 MIC of *C. cyminum* oil had significantly affected on all virulence factors except adhesion.

Discussion

Motility (Swimming and twitching), adhesion, alginate production and biofilm formation, has several roles in *P. aeruginosa* pathogenesis. Consequently, any treatment which reduces production of these virulence factors might be effective in counteracting the pathogenesis of *P. aeruginosa* especially in patients with suppressed immune system [18].

Table 1- Effects of essential oils on swimming, twitching, adhesion, alginate production and biofilm formation of *P. aeruginosa*

Test	Concentration	Essential oil	
		M. spicata	***C. cyminum***
Swimming	1/2 MIC	+	+
	1/4 MIC	+	+
	1/8 MIC	–	+
Twitching	1/2 MIC	+	+
	1/4 MIC	+	+
	1/8 MIC	–	+
Adhesion	1/2 MIC	+	+
	1/4 MIC	+	+
	1/8 MIC	+	–
Alginate	1/2 MIC	+	+
	1/4 MIC	+	+
	1/8 MIC	+	+
Biofilm	1/2 MIC	+	+
	1/4 MIC	+	+
	1/8 MIC	–	+

+: Significantly different from control, –: Not significantly different from control, MIC: Minimal inhibitory concentration; Medium without essential oil was defined as control.

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It has been shown that sub –MIC values of antibiotics, reduce the level of alginate and proteases in-vitro [20]. Fonseca et al., showed different bacterial characteristics on the effects of sub-inhibitory concentration of piperacillin/tazobactam for various *P. aeruginosa* strains. Significant changes such as decrease in adhesion values in all clinical isolates, decreased biofilm formation, remarkable hike in sensitivity to oxidative stress, hampered flagellum-mediated swimming and lowered type IV fimbriae-mediated twitching could be attributed to a change in bacterial morphology and hydrophobicity [18]. However, a few reports regarding the effects of essential oils on virulence factors production were reported. Owlia *et al*. had shown that *Matricaria chamomilla* essential oil, reduced alginate production and biofilm formation in *P. aeruginosa* [21].

An important determinant of virulence is the ability to adhere. The exposure of bacteria to sub-MICs of antibiotics generally weakens this ability [18]. We showed that sub-MICs of mentioned essential oils reduced the adherence potential of *P. aeruginosa*. The motility (swimming and twitching) has very important role in pathogenicity [18].The results indicate that some essential oils can reduce motility. The establishment of biofilms by alginate-producing *P. aeruginosa* strains is the most common mode of growth in infections, with the biofilms providing a protected environment against the host immune system and a number of antibiotics [21].

The antibacterial activity of essential oils has been consider as a substitution for antibiotics, because, resistance to antibiotics has been developing in the world and Iran [22, 23]. In this study, the sub-MIC levels of *M. spicata and C. cyminum* essential oils affected adhesion, swimming, twitching, alginate production and biofilm formation. New antimicrobial agents against *P. aeruginosa* are very valuable, especially in multi drug resistant strains. We believe that the present investigation together with previous studies provide support to the antibacterial properties of *M. spicata and C. cyminum* essential oils. It can be used as antibacterial supplement towards the development of new therapeutic agents. Additional in vivo studies and clinical trials will also be needed to justify and further evaluate the potential of this oil as an antibacterial agent in topical or oral applications.

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