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Retardation of biofilm formation with reduced productivity of alginate as a result of *Pseudomonas aeruginosa* exposure to *Matricaria chamomilla* essential oil

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ABSTRACT - Bacterial adhesion to solid surfaces and biofilm development are ubiquitous phenomena with several deleterious medical and economic consequences. The establishment of biofilms by alginate-producing *P. aeruginosa* strains is the most common mode of growth in cystic fibrosis patients with chronic lung infections, with the biofilms providing a protected environment against the host immune system and a number of antibiotics. Selected natural products that originate in plants can influence microbial biofilm formation. In this paper specific inhibition of alginate production by and antibiofilm activity of *Matricaria chamomilla* essential oil against *Pseudomonas aeruginosa* are studied. Disk diffusion method employed to evaluate *P. aeruginosa* inhibition exposed to the essential oil concentrations of *Matricaria chamomilla* L. did not show antimicrobial property. The oil composition analyzed by GC and GC/MS led to identification of 18 components of which the major ones were: guaiazulene (25.6%), (E)-β-faransens (20.1%), chamazulene (12.4%), α-bisabolol oxide B (7.3%), α-bisabolol (7.3%), and hexadecanole (5.6%). Biofilm formation was studied using saffranin stain employing plate reader. Alginate production was quantified in absence and in presence of the essential oil concentrations. The alginate production reduced significantly as the oil concentration increased. At the oil concentration of 0.5 μg/ml both biofilm and alginate reduced restoring alginate/biofilm ratio to normal level with decreased biofilm/alginate ratio. Use of natural agents capable of reducing biofilm formation would be useful in control of microbial pathogenicity leading to the prevention of infections of numerous biofilm producing bacterial species.

KEYWORDS: Biofilm; Alginate; *Pseudomonas aeruginosa*; *Matricaria chamomilla* L.; Essential oil

INTRODUCTION

Bacterial adhesion to solid surfaces and biofilm development are ubiquitous phenomena with several deleterious medical and economic consequences. *Pseudomonas aeruginosa* infections are difficult to eliminate due to their propensity to form biofilms (1) and their inherent resistance to antibiotics. The opportunistic pathogen *P.aeruginosa*, a ubiquitous environmental bacterium showing great adaptability and metabolic versatility (2), is the leading cause of morbidity and premature mortality in patients with cystic fibrosis (3, 4). *Pseudomonas aeruginosa* is remarkable in that it can cause both very acute and very chronic infections (5). Progress in understanding the pathogenesis of acute *P. aeruginosa* infections has implicated virulence factors including exotoxin A and type III secreted exotoxins (6, 7). The establishment of biofilms by alginate-producing *P. aeruginosa* strains is the most common mode of growth in cystic fibrosis patients with chronic lung infections, with the biofilms providing a protected environment against the host immune system and a number of antibiotics (8). The presence of *P. aeruginosa* strains with the mucoid phenotype in cystic fibrosis patients with chronic lung infections is a marker of a poor prognosis (9). Although it is not possible to eradicate *P. aeruginosa*, antibiotic therapy contributes to the maintenance of lung function for decades (10, 11). Therefore, the development of antibiotic resistance in *P. aeruginosa* is of great therapeutic concern (12). Antipseudomonal β-lactam antibiotics are widely used for the treatment of lung infections in cystic fibrosis patients. Various industries are now looking into sources of alternative,

more natural and environmentally friendly antimicrobials, antibiotics and antioxidants. Hundreds of new natural substances are being isolated and identified every year, but data concerning their biological activities are known for only some. Chamomile is one of the most widely used and well-documented medicinal plants in the world (13). It is included in the pharmacopoeia of 26 countries (14). The possibility of utilizing volatile oils is now being investigated as, although their biological activity has been known for centuries, their mode and range of action were not fully understood. Selected natural products that originate in plants can influence microbial biofilm formation. Some plant-derived compounds inhibit peptidoglycan synthesis (15), damage microbial membrane structures (16), modify bacterial membrane surface hydrophobicity (17), and modulate quorum sensing (18), all of which could influence biofilm formation. Terrestrial plants also support populations of surface-attached bacteria (19, 20) and could potentially produce phytochemicals that attenuate biofilm development through specific mechanisms. However, many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth (21-23). Owing to its glycocalyx, *Pseudomonas aeruginosa* adhesion to surfaces is facilitated while resistance to many antibiotics enables the bacterium survive easily. Chamomile contains a diverse array of secondary products including sesquiterpenes, polyacetylenes, flavonoids and coumarins. Coumarins, the group of phenypropanoid metabolites, showed antimicrobial (24) and anti-inflamatory effects (25). Successful clinical applications of *Matricaria chamomilla* have been studied (26). The present study describes specific inhibition of alginate production by and antibiofilm activity of *Matricaria chamomilla* essential oil against *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Microbial strain and growth media

Pseudomonas aeruginosa 8821M was kindly supplied by Dr. Isabel Sa-corria of Instituto Superior Tecnico, Lisboa-Portugal.

Plant and oil isolation

The plant origin was of Yasooj region of Iran collected during May-June 2005. The plant was identified at the department of Botany, Shahed university-Tehran as *Matricaria chamomilla* L. The shadow dried flowers were hydro distilled for 90 minutes in full glass apparatus. The oil was isolated using a Clevenger type asssssspparatus. The extraction was carried out for 2 hours after 4-hour maceration in 500 ml of water. The oil so extracted had a specific gravity of 0.95 at 20^oC, and refractive index 1.48-1.505 at 25^oC was stored in dark glass bottles in a refrigerator until they were used.

Oil analysis

GC analysis was performed by GC (9-A-Shimadzu) gas chromatograph equipped with a flame ionization detector. Quantitation was carried out on Euro Chrom 2000 from KNAUER by area normalization method. The analysis was carried out using a DB-5 fused-silica column (30 m×0.25mm, film thickness 0.25µm) using a temperature program of 40–250^OC at a rate of 4^oC/min, injector temperature 250 ^OC, detector temperature 265^OC, carrier gas: helium (99.99%). The GC/MS unit consisted of Varian-3400 gas chromatograph coupled to a Saturn II ion trap detector. The column was same as of the GC under the same conditions stated above. The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention indices with those of authentic compounds or with literature data.

Oil dilution

1 ml of oil weighing 1mg was diluted to the final volume of 2ml with DMSO (Dimethyl sufoxide). This served as the stock solution. Mueller Hinton broth containing 0.5, 0.35 and 0.2 µg/ml of oil concentrations were made from the stock solution by adding appropriate amounts of the stock oil solution to medium.

Antibacterial analysis

Unless otherwise stated, all the procedures were carried out under aseptic conditions. Disk diffusion method was employed to assess anti bacterial properties of the solvent, DMSO. Bacterial suspension equivalent to 0.5 McFarland standard units was streaked on Mueller Hinton agar plates using sterile cotton swabs. 10µl of DMSO loaded on three sterile blank disks were placed on the agar plates and were then incubated at 37^oC for 24 hours. 0.2, 0.35 and 0.5 µg/ml of *Matricaria chamomilla* essential oil was added to each sterile blank disk. These concentrations were chosen on the basis of our experience and in order to avoid interference of chamomile oil colour when needed to take spectrophometric readings. The disks were placed on Mueller Hinton agar plates freshly streaked with 24 hour old bacterial suspension of *Pseudomonas aeruginosa*. The plate containing solvent on the disks served as control. Zones of inhibitions

were measured using a vernier clipper. The tests were carried out in triplicate.

Biofilm formation and adherence assays

The assays of biofilm formation and adherence were performed in 96-well polystyrene microplates. 190 µl of Mueller Hinton broth containing 0.5, 0.35 and 0.2 µg/ml of oil concentrations were added to each of the wells in triplicate. Bacterial cells from overnight cultures grown at 37°C were collected by centrifugation and resuspended at OD_{620}=0.01 in Mueller Hinton broth. 10 µl of the bacterial suspension was added to each well. The wells containing Mueller Hinton broth without oil, inoculated with 10 µl of the bacterial suspension served as positive control. The wells containing only Mueller Hinton broth without oil or bacteria served as negative control. The microplate was incubated at 37°C for 24 hours. The contents of the wells were drained at the end of incubation period and were then rinsed with sterile distilled deionized water (six rinses). 200 µl of 0.025% saffranin solution was added to each well for 2 minutes. The saffranin contents were drained and the wells were rinsed with sterile distilled deionized water (three rinses). 200 µl of ethanol:acetone (50:50 vol/vol) solution was added to each well and were allowed to stand for 15 minutes. The absorbance of the well contents, were then measured using plate reader at OD_{492}. Biofilm production was determined by using the following formula : Biofilm= $(B\text{-}S)/G$, where *B* is the amount of biofilm formed, *S* is the amount of saffranin that adhered to the polystyrene tubes due to abiotic factors, and *G* is the optical density of cells grown in suspended culture (27). At least three replicate experiments were performed for each concentration of chemical that was tested.

Quantitative assay of alginate

500µl of bacterial suspension corresponding to 0.5 McFarland standard solution was added to each of 50ml flasks each containing 20ml sterile Luria Bertani broth. Test flasks contained 0.2, 0.35 and 0.5 µg/ml of *Matricaria chamomilla* essential oil while flasks without oil served as control. The flasks were then placed on an incubator shaker for 24 hours at 37°C. 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 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 55°C for 30 minutes. The absorbance was measured spectrophotometrically at 530nm. Standard alginate solutions were made by serial dilutions from the stock solution of 1000 µg/ml. Standard alginate curve was plotted using absorbance readings at 530nm (28).

Statistical analysis

Data obtained from the experiments were presented as mean values and the differences between control and test were analyzed using the paired t-test.

RESULTS

DMSO loaded disks showed no anti Pseudomonas activity on the plates, hence allowing use of this solvent as the oil diluent. 0.2, 0.35 and 0.5 µg/ml concentrations of *Matricaria chamomilla* essential oil were screened for the ability to inhibit the growth of *P.aeruginosa* 8821M. The essential oil concentrations had no effect on growth. Chemical analysis of the components of the oils led to identification of 18 components (Table 1). The major components of *Matricaria chamomilla* L. oil were guaiazulene (25.6%), (E)-ß-faranesens (20.1%), chamazulene (12.4%), α-bisabolol oxide B (7.3%), α-bisabolol (7.3%), and hexadecanole (5.6%). Alginate production was affected in proportionate to the increasing concentration of the oil while biofilm formation was retarded at 0.5µg/ml oil concentration (Table 2). Alginate/biofilm ratio ran in opposite directions on X axis (Fig. 1).

DISCUSSION

The results presented here show clearly that *Matricaria chamomilla* essential oil did not exert antimicrobial effect. *Matricaria chamomilla* essential oil has been reported to be antimicrobial against some bacteria at higher oil concentration (29). The difference in microbial susceptibility is attributable to the chemical composition of essential oil. The ineffectiveness of some oils might reflect the lack of antibacterial compounds in the plants against the microorganism under study. A possible explanation for this is that some of the plant extracts may have contained antibacterial constituents, but were not present in sufficient concentrations to be effective.

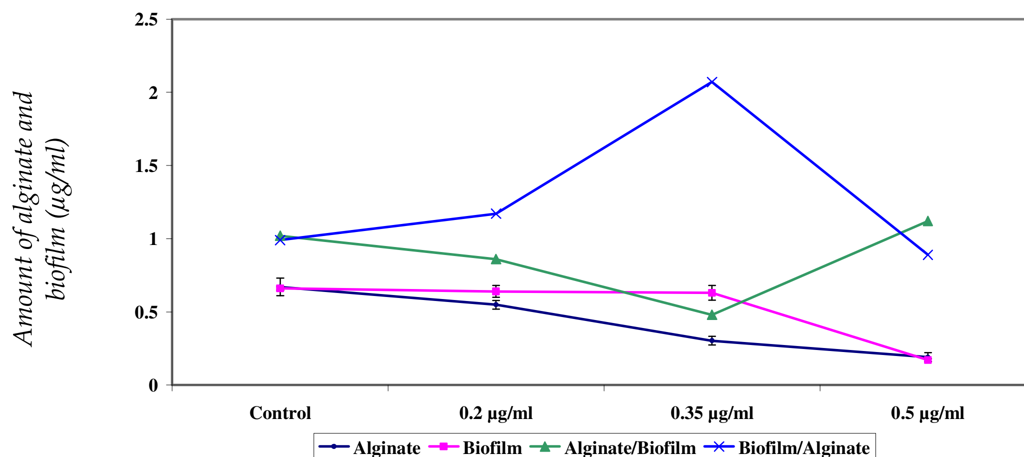
Table 1: Chemical composition of essential oil from Matricaria chamomilla L.

No.	Compound	R.I.	%
1	Limonene	1029	0.5
2	γ-terpinene	1062	0.5
3	(E)-β-faranesens	1459	20.1
4	germacrene-D	1481	3.1
5	α-muurolene	1496	0.8
6	germacrene-A	1504	0.5
7	Z-γ-bisabolene	1515	2.6
8	caryophyllene oxide	1570	1.2
9	Spathulenol	1578	1.7
10	α-bisabolol oxide B	1654	7.3
11	α-bisabolol	1685	7.3
12	Chamazulene	1729	12.4
13	α-bisabolol oxide A	1746	1.9
14	Guaiazulene	1756	25.6
15	Hexadecanole	1882	5.6
16	n-nonadecane	1891	1.4
17	Sclarene	1968	0.4
18	n-pentacosane	2500	0.5

Table 2: The amount (µg/ml) of alginate production and biofilm formation by P.aeruginosa exposed to various concentrations (0.2, 0.35 and 0.5 µg/ml) of M. chamomilla essential oil

	Control	0.2 µg/ml	0.35 µg/ml	0.5 µg/ml
Alginate	670±27	549.33±13.17	304.5±17	190.33±9.9
Biofilm	0.66±0.12	0.64±0.04	0.63±0.05	0.17±0.01
Alginate/Biofilm	1.02	0.86	0.48	1.12
Biofilm/Alginate	0.99	1.17	2.07	0.89

Figure 1: The amount of alginate production and biofilm formation by P. aeruginosa exposed to M. chamomilla essential oil 0.2, 0.35 and 0.5 µg/m



Of the major components of chamomile oil Chamazulene, α-bisabolol and flavonoids have been reported to display antifungal properties (30). The oil under study did not have sufficient quantities of these components to impart bacteriostatic or bactericidal effect on *P.aeruginosa*. On the other hand resistance of *P.aeruginosa* to some antimicrobial agents is another factor contributing to the survival of microorganism. Endobronchial chronic infections caused by the mucoid, alginate-producing phenotype of *P. aeruginosa* are impossible to eradicate with antibiotics (31). The MICs of antimicrobial agents can be increased 100- to 1,000-fold when bacteria grow in biofilms (32). Bacterial biofilms have been defined as communities of bacteria intimately associated with each other and included within an exopolymer matrix. These biological units exhibit their own properties, which are quite different from those shown by the single species in planktonic form (33). Bacterial attachment is influenced by the surface of cells and attachment media as well as by other environmental factors (34). The results (Table 2) show that alginate production reduces significantly as the oil concentration increases. However this does not affect the biofilm formation at the oil concentrations of 0.2 and 0.35 µg/ml (Fig. 1). Understanding the pathogenesis of the chronic infections caused by *P. aeruginosa* is also progressing. Current concepts propose that biofilm formation is a key factor in chronic *Pseudomonas* airway infection in cystic fibrosis and bronchiectasis and chronic urinary tract and device-related infections (35- 38). Biofilm/alginate ratio increases significantly at the oil concentration of 0.35 µg/ml (Fig. 1) only due to the decreased level of alginate. At the oil concentration of 0.5 µg/ml (Fig. 1) both biofilm and alginate are reduced restoring alginate/biofilm ratio to the initial normal level with decreased biofilm/alginate ratio (Fig. 1). This may indicate that biofilm formation although related to, but does not merely depend upon alginate production. Biofilms confer a number of survival advantages to the bacteria, including increased resistance to antimicrobial agents (38, 39). This could be the reason as to why *P.aeruginosa* resisted *M. chamomilla* oil. However, planktonic bacteria dispersed from a biofilm usually no longer demonstrate increased levels of resistance to antibiotics, suggesting that the mode of growth is of major importance (37). The switch from the nonmucoid to the mucoid phenotype may provide profound advantages to the bacteria growing in biofilms, such as increasing their levels of resistance to antibiotics (such as tobramycin) and protecting them against the host immune system (40). Alginate appears to have important roles in the host parasite relationship. Alginate in *P. aeruginosa* may confer several selective advantages on the bacterial invader, which have been reviewed and include increased resistance to phagocytosis and reduced susceptibility to antibody-dependent bactericidal mechanisms. Alginate also provides a polyanionic barrier that may exclude cationic peptide antibiotics (41). By aggressive antibacterial chemotherapy it is possible to suppress the growth of and the damage caused by *P. aeruginosa* in the lungs of chronically infected cystic fibrosis patients. Alternatively, any treatment with natural agents that can potentially reduce biofilm formation would be useful in control of microbial pathogenicity. Consequently chronic respiratory tract infections or other infections of numerous bacterial species capable of producing biofilms might be prevented.

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