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Effect of *Althaea officinalis* Extract on Growth and Biofilm Formation in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is an important opportunistic pathogen that typically produces the biofilm. Biofilm- producing bacteria are inherently resistant to chemical treatment. This study examines the effect of *Althaea officinalis* extract on the biofilm formation by *Pseudomonas aeruginosa*. Antibiotic susceptibility test of *Pseudomonas aeruginosa* was performed by disk diffusion method and the effect of alcoholic extract of herb marshmallow (*Althaea officinalis*) was studied on bacteria. Hock extract collected from Northwest of Iran (Kurdistan) was prepared using soxhlet extractor. Antimicrobial properties of the extract were evaluated using well plates. Minimum inhibitory concentration (MIC) and minimal lethal concentration (MBC) of plant extracts and antibiotics were determined using CLSI standard plate. Biofilm formation was performed using a modified micro-titer plates. The MIC and MBC (62.5 mg D ml) showed significant effects of studied plants on the bacteria. Different concentrations of *Althaea officinalis* extract significantly reduced the biofilm produced by *Pseudomonas aeruginosa* in comparison with the positive control (P value = 0.0124). According to our findings, significant effect of this natural substance can be a promising agent in the treatment of *Pseudomonas aeruginosa* infection; and also an inhibitor of biofilm formation.**

Keywords: *Althaea officinalis* extract, biofilm formation, *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa, as a gram-negative aerobic or facultative anaerobic bacillus, is an opportunistic human pathogen. This bacterium is one of four nosocomial pathogens responsible for ten percent of hospital-acquired infections[1]. People with Cystic Fibrosis, immunocompromised patients such as cancer patients, AIDS patients, and patients with severe burns are susceptible to *P. aeruginosa* with mortality rate of 50%[2]. Different bacterial pathogens has created bacterial resistant to most antibiotics. Biofilms in comparison to unattached or planktonic cells are so resistant to antimicrobial agents and cause biocide resistance so high concentration of antibiotic are required[3].

Multiple resistant to antibiotics has been created in *P. aeruginosa* following the indiscriminate use of antibiotics and high resistance to commonly used antibiotics, and because of the side effects of these medications and its cost, developed countries began to think of using natural resources such as medicinal plants like marshmallow. *Althaea officinalis*, as a marshmallow's latin name, is a 3 foot genus of perennial herb with large pink, white, red or purple flowers in a heart shape that bloom in August and September. This plant usually produces a lot of

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flowers, but flowers last for very low. It quickly forms a large root mass[4-5].

The root contains sucrose, pectin, asparagines, albumin, lecithin, tannins, minerals and phosphates and also starch (37%), dioxybenzoic acid and cyaniding. Of course, one of the most effective substances produced by this plant is mucilage (11%) that hydrolysis to galactose, arabinose, glucose, rhamnose and galacturonic acid[6-7].

Recently, studies on the antimicrobial properties of the Malvaceae family have proved its antimicrobial activity against many human pathogens[8]. In this study we assayed the effect of Althaea officinalis extract on growth and biofilm formation in Pseudomonas aeruginosa.

METHODS

Bacteria and Media

All experiments have been done on *P. aeruginosa* ATCC: 27853 from collection center of Iranian Research Organization for Science and Technology. It was cultivated in Mueller - Hinton agar (MHA) medium (Merck, Germany) and should be maintained in nutrient broth (NB) medium (Merck, Germany) with 15% glycerol in 80p C.

Antibiotic susceptibility tests

Qualitative antibacterial susceptibility of the microorganism was determined according to the standard disk diffusion (Kirby-Bauer) method[9] using paper disk including Azithromycin (15µg); Amikacin (30 µg); Gentamycin (10 µg); Ciprofloxacin (5µg) purchased from Mast Co (Liverpool, UK).

106 colony forming units (CFU/ml) of *P. aeruginosa* Microbial suspension in NB medium were cultivated on MHA medium[9]. The plates were incubated at 37°C for 24 h in aerobic condition and examined for the inhibition zones diameter appearing around each antibiotic disc. It was carried out thrice for each antibiotic. Inhibitory zone diameters were measured and compared to the standards provided by the National Committee for Clinical Laboratory Standards (NCCLS)[10].

Preparation of the alcoholic plant extract

The plant was collected from the north-western of Iran, identified and approved by Department of Agricultural Research. Herbarium specimen of the plant was prepared and then used for further studies.

The plant was dried in shade and grounded in appropriate temperature. Five gram of the sample were solved in 100 ml of ethanol as solvent extraction and extracted using Soxhlet extractor. Extracts were filtered and sterilized using a 0.45 micron, and collected in sterile dark containers at 4p C Dimethyl sulfoxide 5% (DMSO) was used for serial dilution[11-12].

Quality Control of the extract:

The pH, an important factor in the quality of the extracts, was measured with pH meter. It was 7.4 alternatively**.**Ten ml of the extract was poured in the flask, weight and using the formula d = m / v, the density of the extract was determined as 12/1[13].

***In-vitro* inhibitory effect of plant extract and Antibiotics**

Agar Well Plate Method

1.5×108 CFU/ml *of P. Aeruginosa* suspension (equivalent to the 0.5 McFarland) should be cultivated on MHA gar medium (Merck, Germany) with sterile swab. 100 µl of different concentration of extracts (50, 250 and 500 mg/ml) were inoculated into the wells with a diameter of 6 mm. 100 µl of the solvent DMSO as a negative control were inoculated to one of the wells. Plates were incubated for 24 h at 37p C. Results from the presence or absence of inhibition zone diameters were measured with calipers and the test was repeated three times[14]

Determination of minimum inhibitory concentrations (MICs)

The MICs were determined by broth microdilution assay according to the procedures recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards 2006). Serial dilutions of antibiotics (Azithromycin, Ciprofloxacin, Amikacin or Gentamicin), in the range of 0.125 µ g/ml to 256 µ g/ml, and Serial dilutions of the plant extract in the range of 0.062 D to 125 D were prepared in Mueller Hinton broth. Each plate has positive and negative controls 100 µl of bacterial inoculum 5 × 105 CFU/ml was added to each well containing 100 µl of the two fold serial dilutions of the antibiotics. The final volume of each well was 200 µl. The plates were incubated at 37°C for 24 h. The MIC was defined as

the lowest concentration of antibiotic which can inhibited visible growth of microorganism[10, 15]. 100 µl of the suspension from each well, without visible growth on MHA, was used for determination of MBC and then incubated at 37°C for 48-72 hrs. Finally the lowest concentration of antimicrobial agent being able to reduce 99.9% of the bacteria was assessed as MBC. Experiments were done in triplicate[16].

Effect of plant extract on *Pseudomonas aeruginosa* biofilms

A modified Microtiter plate method was used for assessment of *Pseudomonas Aeruginosa* Biofilm Formation[17]. The bacteria were cultured on Trypticase soybean agar (TSA) medium plus 0.2 % glucose (Merck, Germany), and were incubated at 37 p C for 24 h**.** Isolated colonies of bacteria were inoculated to the Trypticase soybean broth (TSB) + 0.2% glucose medium (Merck, Germany), obtained a suspension with absorbance of 0.1 at 625 nm.

100 µL of each antimicrobial dilution were added to each well of microtiter plates. 100 µL of bacterial suspension and media culture was added to each well. Concentration range of the plant extract was 1.95 mg/ml - 500mg / ml (using Dimethyl

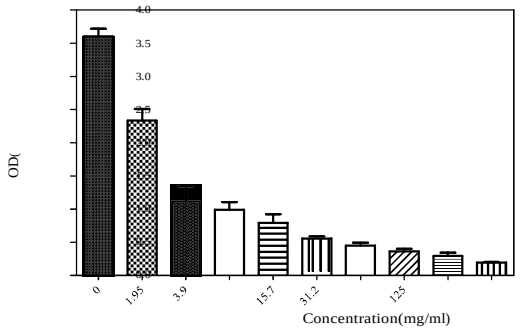

Fig. 1. Effect of hibiscus extract on biofilm formation in *P. aeruginosa*

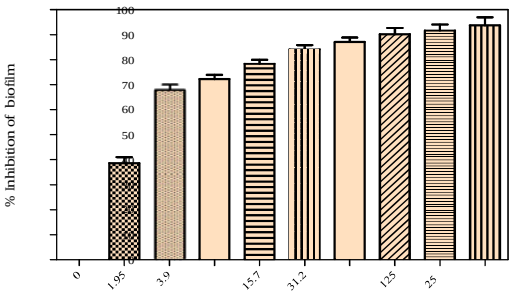

Fig. 2. Diagram of inhibition percent of biofilm formation in Pseudomonas aeruginosa by marshmallow extract

Table 1. The mean of diameter of inhibition zone in *P. aeruginosa* with different concentrations (mg/ml) of marshmallow alcoholic extracts and antibiotic discs (µg / disc)

Antimicrobial Agents	Amikacin (30 µg)	Azithromycin (15 µg)	Ciprofloxacin (5 µg)	Gentamycin (10 µg)	Negative control	Concentration of PlantExtract (D ml)		
						50	250	500
Pseudomonas aeruginosa ATCC: 27853	17.5± 0.7	-	28±0.57	17.3± 0.57	6±00	6±00	6±00	10.5±0.7

-DMSO 5% as a negative control
- The results are reported as(X ± SD). Diameter of well is 6 mm.

Table 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of antimicrobial agents in *P.aeruginosa* PTCC: 1430 a

Antimicrobial agent MIC & MBC	Plant extract(D)a	Antibiotics(µg) Amikacin (30)	Gentamicin (10)	Ciprofloxacin (5)	Azithromycin (15)
MIC	*62.5*	8	1	0.25	128
MBC	62.5	16	4	0.5	_

a Plant extract dissolved in Muller Hinton Broth. All determinations were done in triplicate

sulfoxide, DMSO, as a diluent). 200 µl TSB medium + 0.2 % glucose were added to the negative control wells, and the positive control wells were included by 200µl of microbial suspension and media culture. Total volume of each well was 200 µl.

Plates were incubated for 24 h at 35p C. After the incubation period, the contents of the wells were carefully aspirated by sterile samplers. Each well was washed 2-3 times with 200 µl sterile Phosphate-buffered saline (PBS) to remove unattached bacteria. Then 150 µl absolute methanol (Merck, Germany) was added to each well and after 10 minutes rinsed with methanol. 200 µl of crystal violet 1% was added to each well. After 20 minutes excess dye was removed and plates were washed under running tap water. After air drying the plate, 150 µl Glacial acetic acid 33% (Merck, Germany) was added to each well and the absorbance of each well was read at 650 nm by ELISA reader (statfax-2100, Awareness Technology Inc., USA). The percentage of biofilm inhibition was calculated using the following equation[17].

Percent growth inhibition= 100- [(OD650 of Biocide/ OD650 of positive control) ×100]

Analysis methods

Paired t-test and analysis of variance (ANOVA) was used to compare means and data correlation. Significant differences were evaluated with 0.05= á. Graph Pad Prism software version 5 (Graph pad Software In, San Diego, USA) was used for all statistical studies in different groups.

Results and Discussion

The pattern of antibiotic susceptibility showed that *P. aeruginosa* ATCC 27853 was sensitive to Ciprofloxacin, Amikacin, and Gentamicin but resistant to Azithromycin. 500 mg/ ml of marshmallow alcoholic extract had an inhibitory effect on the *P. aeruginosa* bacteria using Agar Well Plate Method. The results of measuring the diameter of inhibition zone for different concentrations of plant extracts and antibiotics are given in table1. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of marshmallow ext**ra**ct against *P. aeruginosa* is equal to 62.5 mg per liter (Table2).

Biofilm formation in the presence of plant extracts

The mean values and standard deviations were obtained for each dilution by the results of the ELISA reader and the curves were plotted. Concentration of positive control was zero. The results showed that the extract of marshmallow had a significant effect on biofilm formation. Biofilm formation was strikingly reduced in the presence of milligram levels of this extract and it was quite significant in comparison to control (solvent DMSO).

Increasing the concentration of extract significantly increased the inhibition efficiency. Inhibition percent of biofilm formation in the presence of MIC and the highest concentration of plant extract was 87.3 and 93.7 respectively. The concentrations less than MIC of the extract dramatically reduce the amount of biofilm formation (Fig 1& Fig 2).

CONCLUSION

P. aeruginosa is an opportunistic pathogen which has become as multi-resistant bacteria and the increasing resistance of bacteria have been encountered depressive problems in patients[18-19]. According to the importance of biofilms in the virulence and antibiotic resistance of *P. aeruginosa*, it is necessary to find new antimicrobial compounds that lower concentration can inhibit growth of bacteria in biofilms[3]. The use of medicinal plants appears to be an appropriate option. The plants inhibit bacterial growth with different antimicrobial mechanisms, even different from antibiotics, so the more comprehensive research in the medicinal plant area is needed.

Studies have shown that most of these plants have no antimicrobial effect on *P. aeruginosa*[8,20]. Hollyhocks are related to hibiscus as a medicinal plant and have numerous antimicrobial compounds and the results of studies on the antimicrobial properties of this plant suggest that it has antibacterial, antifungal and antiviral activity against many human pathogens. Previous studies have shown that use of the herb reduces cold symptoms especially in cough, inflammation of respiratory tract, urinary

tract, intestinal tract, dermatological diseases and have a treatment effect on bronchitis[21-22].

In 2004 Shanab et al investigated on clinical effect of ethanol and methanol extracts of several medicinal plants of hibiscus family on multiple Drug resistance S*taphylococcus aureus, MDR- Bacillus subtilis* and *MDR-Pseudomonas aeruginosa* using well plates and Broth Micro Dilution Method. They showed that antimicrobial activity of extracts from mixture and compounds of some selected medicinal plants will have the synergistic effect on *P. aeruginosa* and inhibit its growth while these plants individually have no antimicrobial effects[23].

In 2011Wallter et al showed effect of hexanol extract of leaves, flowers and roots of marshmallow and hibiscus plants collected from Pakistan on growing of some gram positive bacteria such as *Staphylococcus aureus* and gram negative bacteria such as *Escherichia* coli and *P. aeruginosa* using well plate. It was shown that antimicrobial effect of hibiscus against gram positive bacteria was higher than gram negative[8].

In 2012 the research on antimicrobial agents and antimicrobial effects of Garcinia indica fruit extracts was done by Sutar et al. They showed that the most potent antimicrobial compounds in the fruit including Cyaniding and Anthocyanins has a strong antimicrobial effect against variety of human pathogens, including *P. aeruginosa*[24].

The results obtained in this study demonstrated that marshmallow extract has antibacterial properties on *P. aeruginosa* due to its special compounds such as Cyaniding and Anthocyanins. However, there are differences in inhibition power of plants comparison to previous works which may be due to different types of bacterial strains, and also because of different plants collected from different places. Extraction method may also affect on the antimicrobial effect of extracts that make small differences in our work, but in general, these results are consistent with previous results.

The mean diameter of inhibition zone of the plant extract at a concentration of 500 mg/ml compared with antibiotics used in this study was significantly different at the 0.05 (P value<0.05). Although the inhibitory power of this extract is less than amikacin, gentamicin and ciprofloxacin, it should be noted that this extract contains many compounds which can neutralize the bacteria. The antimicrobial effect of this plant will increase substantially without these compounds.

In 2008 Head observed that marshmallow and several other species of hibiscus extract inhibited the growth of gram-negative bacteria involved in the urinary tract infections. He studied on the effects of a number of sugars such as D-mannos on urinary tract infections that completely prevent binding ability of *Escherichia coli* to epithelial cells so the formation of biofilms is difficult for the bacteria[25].

In 2010 Deters showed that the aqueous extract of marshmallow root stimulated the production of mucus from epithelial cells. Clinical studies have proven the association between the presence of mucilage polysaccharides (Rhamnogalacturonan) and formation of physical connection to mucin and tissue stimulation. There is no further information on this extract and the polysaccharides derived of it, that how they affect on mucus secretion and cell attachment or how they make changes in cell function to improve tissue regeneration[26].

In 2012 Chifiriuc interrogated effect of a species of hibiscus extract on yeast biofilms, Candida albicans and C.tropicalis. These fungi are one of the most important factors in biofilm production on urinary catheters. In this study they created a nanobiosystem, a catheter covered by nanoparticle layer and an extra layer of the plant extract was settled on it. The result showed that the nanobiosystem effectively inhibits adhesion and development of fungal biofilms on catheters as they were measured by CLSM, Confocal laser scanning microscopy[27].

The results of our work done in vitro showed the prevention of biofilm formation because of the presence of sugars and polysaccharides and mucilages in the plant extrat which greatly inhibit the bacterial attachment to surfaces and biofilm formation. Further researches and in vivo studies are needed to understand the mechanism of these materials and its reaction on host epithelial cells. According to the role of biofilms in pathogenicity and the results of this study, different concentrations of the plant extracts showed a high capability of inhibition in biofilm bacteria formation and it reveals the potential of *Althaea officinalis* extracts as an alternative and

complementary medicine for *P. aeruginosa* infections by inhibiting its virulence factors, so it can be used as an additive in a burn ointment.

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