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Inhibitory Effects of Plant Extracts on *Pseudomonas aeruginosa* Biofilm Formation

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Abstract

Background: *Pseudomonas aeruginosa* is opportunistic bacteria that cause diseases in human beings, animals and plants. It is one of the most important factors of the nosocomial infections in a wide range of patients with immunodeficiency including patients with malignant diseases, cystic fibrosis and burns. Biofilm production is one of the pathogenic factors of these bacteria.
Objectives: The current study aimed to investigate the effects of a few plant extracts on in-vitro formation of *Pseudomonas aeruginosa* biofilm.
Materials and Methods: Using the rotary system, the extracts of various plants were concentrated and prepared. Standard bacterial strains were provided and growth and biofilm formation of strains were determined using the microtiter plate method.
Results: Results showed desirable antimicrobial effects of the silver nanoparticles and plant extract on the formation of the *Pseudomonas aeruginosa* biofilm and the highest effect was observed in silver nanoparticles and the lowest inhibitory effects were observed in Bucks beard and *Prangos ferulaceae*.
Conclusions: Medicinal plants used in the study decreased the rate of biofilm formation. Since the biofilm formation is one of the pathogenic factors in mucoid strains, further studies will probably help to control the infections using the findings of the current study.

Keywords: Biofilm, Plant Extract, Inhibitory Effects, *Pseudomonas aeruginosa*

1. Background

Populations composed of one or more species of bacteria, stuck together, or stuck to the living and non-living surfaces of extracellular polysaccharides in the soil are called biofilm. Biofilm development is a complicated process that requires the collective behavior of bacteria and is useful for the bacteria compared to their single life.

Microorganisms are found in a wide range of ecosystems as highly structured multi-species communities and termed biofilms (1, 2). The downside of the microbial biofilms is associated with their involvement in major problems associated with industry, medicine and everyday life (3-6).

Biofilm bacteria have a greatly enhanced tolerance to the stress and antimicrobial agents. Thus, biofilm bacteria are different from planktonic bacteria in terms of gene expression and cellular physiology. Genetic studies including various Gram-negative bacteria have identified genes involved in the biofilm formation and development (7).

Biofilm formation comprises the following stages: 1- Reversible connection of the planktonic cells to the surfaces; 2- Irreversible connection of cells and the micro colony formation; 3-Production of the extracellular polymeric substances and 4- the final maturation of biofilm (8).

Pseudomonas aeruginosa is a Gram-negative aerobic bacilli and the natural flora of the skin and intestinal tract that is also found in water and soil (9). It is an opportunistic pathogen and one of the main causes of nosocomial infections in a wide range of the immunocompromised patients, including patients with malignancy, cystic fibrosis, burns, etc. Due to possession of a high number of pathogenic factors, this bacterium shows high resistance to the most common antibiotics. Glycocalyx in *P. aeruginosa* facilitates bonding between bacteria and host cells, thereby forming micro colonies. In addition, it con-

tributes to the biofilm formation, thereby, protecting the bacteria from the phagocyte system and antimicrobial materials (10, 11). Multiple resistance of this bacterium is increasing and this ascending trend is proved in different treatment centers as well as Iran (12).

Biofilms are not simply removed with controlling factors such as heating, drying, cleaners and detergents and remain on the surfaces, especially in hospitals, and cause pollution and transmission of diseases (13).

A study by Fonseca showed that sub-minimum inhibitory concentrations (MIC) of piperacillin/ticarcillin reduce the biofilm production (14).

Another study by Warren showed that sub-MIC concentrations of tobramycin/gentamicin reduce the level of proteases in *Pseudomonas* spp. (15).

A study by Jafari, on the effect of *Chlorella vulgaris* microalgae on proliferation and formation of biofilms by *Streptococcus* mutans and its toxicity, showed that the minimum bactericidal concentrations of the extract were at 25 and 50 mg/mL. It was also effective in preventing the biofilm formation at a concentration of 50 mg/mL and evaluating the toxicity at a concentration of 100 mg/mL (16).

Silver nanoparticles could be applied in various areas including biomedical, materials science and catalysis. A single silver nanoparticle interacts with light more efficiently than a particle of the same dimension composed of any known organic or inorganic chromophore6. Silver is also the only material that its plasmon resonance can be tuned to any wavelength in the visible spectrum.

2. Objectives

The current study aimed to investigate the effects of a few plant extracts on the in-vitro formation of *Pseudomonas aeruginosa* biofilm.

3. Materials and Methods

3.1. Bacterial Strains and Culture Condition

Bacterial strains were obtained from a standard laboratory. Evaluating the antibacterial activity of the plant extracts were investigated using *Pseudomonas aeruginosa* ATCC27853 (Arian Mehr Co. Iran). The bacteria were subcultured on nutrient agar and stored at 4°C until required for the study.

3.2. Plant Materials

Peganum harmala, *Teucrium polium*, *Prangos feralaceae*, *Tragopogon graminifolius*, *Eremurus persicus* were collected from a region in Chaharmahal and Bakhtiari province, Iran; to dry plants, a dark place with room temperature were used. Samples were crashed and transferred into a glass container and preserved until the extraction procedure was completed in the laboratory; silver nanoparticles were purchased (Table 1).

Table 1. Plants and the Used Parts

Number	Plant	Family	Used Part of Plant
1	*Pseudomonas harmala*	*Nitrariaceae*	Seed
2	*Teucrium polium*	*Lamiaceae*	Leaf
3	*Prangos feralaceae*	*Umbelliferae*	Leaf
4	*Tragopogon graminifolius*	*Asteraceae*	Leaf
5	*Eremurus persicus*	*Liliaceae*	Leaf

3.3. Preparation of the Extracts

Plants were properly dried and pulverized into a coarse powder. Each of the 20 g grinded powders was soaked in 60 mL ethanol 95%, separately for one day, shaking occasionally with a shaker. After a day of dissolving process, materials were filtered (Whatman No. 1 paper filter). Then the filtrates were evaporated using the rotary evaporator. Finally, 0.97 g of dried extracts were obtained and stored at 4°C in an airtight screw-cap tube.

3.4. Biofilm Formation Assay in the Presence of Biocides

To measure the strength of biofilm formation by isolated bacteria, an 18 to 24 hour culture of each isolate was provided in tryptic soy broth at 30°C. Then, 1 milliliter of the culture was added to 10 mL of the sterile TSB medium and its turbidity was set through optical absorption reading of suspension between 0.08 - 0.1 at the 625 nm. It was conducted using the spectrophotometry. The turbidity of the suspension was 0.5 McFarland and contained more than 108 bacteria. However, 250 microliter of the suspension was transferred to each well in the microplate.

All eight wells in the microplate were filled up with a certain bacterial suspension, therefore, each well contained nearly 25×10^6 cfu/well bacteria. Blank wells were sterilized. The 96-well microplates were polystyrene and the capacity of each well was 300 L. Then, the plates were covered and incubated for 24 hours at 30°C. After 24 hours, dissolved nutrients and microbial suspensions were removed from the wells and each well was washed three times by 300 L of sterile physiological saline. The plates were strongly shaken in order to remove planktonic or not-adhered cells during washing.

Then, the bacteria adhered to the wall and the well floor were fixed with 250 L of 96% ethanol. After 15 minutes,

the contents of the wells were emptied and the plates were placed in a location in the laboratory to dry. After drying, the plates were stained for five minute with 200 L of the crystal dye of 2% violet used for Gram stain. Finally, additional colors were washed by placing the plates in the path of the tap water. This color is suitable for measuring the biofilm and can be used to assess the effects of biocides on biofilm. The concentration range of the plant extract was from 1.5 to 100 ppm.

4. Results

The results showed that different concentrations of the plant extracts decreased the rate of the biofilm formation in *P. aeruginosa* and the silver nanoparticles at 1.5 ppm were the sole formed biofilm and other concentrations were related to the inhibitory nanoparticles of the biofilm formation. Plant extract of the Bucks beard and *P. ferulaceae* were in higher inhibitory concentrations of the biofilm formation, however, they were formed in lower biofilm concentrations. Therefore, as extract concentration increased, biofilm formation decreased (Table 2).

5. Discussion

The results showed that different concentrations of the plant extracts decreased the rate of biofilm formation by *Pseudomonas aeruginosa*; therefore, the silver nanoparticles at 1.5 ppm were the sole formed biofilm and other concentrations were related to the inhibitory nanoparticles of the biofilm formation. Plant extract of the Bucks beard and *Prangos ferulaceae* were in higher inhibitory concentrations of the biofilm formation.

Pseudomonas spp. is one of the most important factors of the nosocomial infections in a wide range of patients with immunodeficiency including patients with malignant diseases, cystic fibrosis and burns. It contains various pathogenic agents and has a high resistance to most commonly used antibiotics. Glycocalyx in these bacteria facilitates the connection of the bacteria to the host cell and micro colony formation. Moreover, it causes biofilm formation; thereby, protects the bacteria from the phagocytosis system and penetration of the antimicrobial materials (9-11).

In the first stage of the biofilm formation, the connection of the bacteria and the surfaces, a variety of factors including bacterial movement, hydrophobic bacterial surfaces, the surface material, etc., play a role (8).

A study by Sichani showed that the MIC of the oak galls extract against *Streptococus mutans* was 160 - 320 mg/mL and its minimum lethal concentration was 320 - 640 mg/mL. Aqueous extract of the oak galls showed no antimicrobial activity. Oak gall extracts inhibit biofilm formation of the *S. mutans* at concentrations above 19.5 mg/mL (17).

Hassanshahian and Mohsenipour investigated the effect of alcoholic extract of pomegranate on biofilms of the human pathogens and showed that these extracts eliminated the biofilm at least 50% and at most 95%. The highest inhibitory effect on the formation of biofilm was on *Staphylococcus aureus* (84.95%) and *P. aeruginosa* (48.51 %,), respectively. The highest inhibition of metabolic activity was observed in *Bacillus cereus* (13.77%) (18).

Another study investigated the effect of cornflower extract (*Centaurea cyanus*) on biofilms of the *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. The results showed that the inhibitory effect of extracts on the biofilm structure of bacteria depended on the type of solvent and the concentration of the extract and the highest inhibitory effect was on the formation of biofilm bacteria of the *Escherichia coli* (84.26%) and *Streptococcus pneumoniae* (83.14%), respectively (19).

The study of Krasowska reported that high hydrophobicity of the cell surfaces is a good reason for bacteria to stick to the surfaces and cause the biofilm formation with adverse consequences. According to the results of the current study, the inhibitory effects of the biofilm formation by *P. aeruginosa* (20) revealed that the concentrations of 5 and 10 mg/mL of *Capsicum annuum* L were the most resistant in the biofilm formation of the isolated plates. In a study conducted by Bokaeian et al. (21) and Sederi (22), the effect of pomegranate peel on *P. aeruginosa* biofilm formation was investigated.

The results showed no significant difference between the biofilm formation and positive control when the bacteria with no antibiotic resistance and the essence with the concentrations of the 0.2 and 0.35 mg/mL were used.

However, biofilm formation using 0.5 mg/mL of the essence showed a significant decrease compared to the positive control. A study conducted by Fonseca et al. (14) revealed that concentrations of the sub-MIC piperacillin/ticarcillin decreased the rate of biofilm formation.

In a study by Warren et al. (15) demonstrated that tobramycin and gentamicin in concentrations of sub-MIC decrease the rate of protease formation in *Pseudomonas* spp. Another study that discussed the antimicrobial effect of the alcoholic extracts of the pomegranate plant on the single biofilm form of the six pathogenic bacteria showed that discs soaked with the extracts of the pomegranate peels inhibited the growth of the bacteria at least 50%. However, they had no effect on *Streptococcus pneumoniae*, *Pseu-*

Table 2. The Effects of Different Concentrations of Plant Extracts on Biofilm Formation

	Concentration, ppm						
	100	50	25	12.5	6.25	3.1	1.5
Nanosilver	0.00	0.00	0.00	0.00	0.00	0.00	0.011
P. harmala	0.00	0.00	0.00	0.00	0.005	0.007	0.009
T. polium	0.00	0.00	0.00	0.003	0.004	0.006	0.006
P. feralaceae	0.00	0.00	0.003	0.005	0.007	0.007	0.013
T. graminifolius	0.00	0.00	0.00	0.009	0.007	0.007	0.013
E. persicus	0.00	0.00	0.00	0.003	0.001	0.008	0.010

Abbreviation: ppm, part per million.

domonas aeruginosa and *Klebsiella pneumoniae*. On the contrary, in the liquid media, MIC test of these extracts successfully inhibited the growth of all bacteria (70%). These extracts removed the biofilm structures at least 50% and at most 95%. Biofilm formation had the highest inhibitory effect on the *S. aureus* (95.84%) and *P. aeruginosa* had the highest destructive effect in the treatment with the extracts of this plant (51.48%) (23), and showed that the extracts of the pomegranate peels inhibit the swarming movement in the different strains of chromo-bacteria up to 65% and inhibit quorum sensing (24).

Nanoparticles are increasingly studied for a wide range of medical applications. The advantages of nanoparticles include their high surface-to-volume ratios and their nanoscale sizes. The high surface areas of nanoparticles allow more active sites to interact with biological entities such as cells. The higher surface areas of nanoparticles compared with conventional micron-size particles also offer more sites for functionalization with other bioactive molecules such as anticancer and antibacterial drug molecules. The nanoscale sizes of nanoparticles provide valuable properties that are not available in micron particles. For example, nanoparticles (with or without drugs attached) of sizes 10 - 100 nm can penetrate tissues with tumors and kill cancerous cells while not affecting healthy cells. This effect, called enhanced permeation and retention, is attributed to the fact that the blood vessels in tissues with tumors have pore sizes ranging from 100 to 800 nm, while the vessels in healthy tissues have much smaller pore sizes, from 2 to 6 nm (25).

In the study by Sotiriou and Pratsinis, the antibacterial effect of Ag(+) ions was distinguished from that of nanosilver particles by monitoring the growth of E. coli populations in the presence and absence of Ag/SiO_2 particles. The antibacterial activity of nanosilver was dominated by Ag(+) ions when fine Ag nanoparticles (less than about 10 nm in average diameter) that release high concentrations of Ag(+) ions were employed. In contrast, when relatively larger Ag nanoparticles were used, the concentration of the released Ag(+) ions was lower. Then the antibacterial activity of the released Ag(+) ions and nanosilver particles was comparable (26).

Therefore, Ag inhibits the biofilm formation in these extracts. Results of the current study suggest that the extracts of *P. harmala*, *T. polium*, *P. feralaceae*, *T. graminifolius*, *E. persicus* and nanosilver may be useful either alone or combined with the antimicrobial agents to treat bacterial infections. The antibacterial properties of *P. harmala*, *T. polium*, *P. feralaceae*, *T. graminifolius*, *E. persicus* and nanosilver are mostly attributable to the *P. harmala*, *T. polium*, *P. feralaceae*, *T. graminifolius*, *E. persicus* and nanosilver aldehyde. Further studies are necessary to evaluate the possible toxicity of this extract and its application in the medicinal system.

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Footnote

Conflict of Interest: None declared.

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