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Effects of Extracts and an Essential Oil from Some Medicinal Plants against Biofilm Formation of *Pseudomonas aeruginosa*

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Biofilm of *Pseudomonas aeruginosa*, an opportunistic pathogen, can cause serious health problems, such as chronic infections, especially in immunocompromised patients. Many studies have suggested administration of new generation of antibiotics, as *P. aeruginosa* biofilms have developed high resistance to antimicrobial drugs. This study reports the inhibitory effect of three medicinal plant extracts and an essential oil on biofilm formation by a clinical isolate of *P. aeruginosa*. In this study biofilm formation of *P. aeruginosa* strain 214 was determined in presence of three plant extracts, *Cyclamen coum*, *Dianthus orieltalis* and *Origanum majorana*, and *Zataria multiflora* Bio essential oil. Minimum Biofilm Inhibitory Concentrations (MBICs) were determined by microdilution techniques and XTT assay. The *C. coam* extract and *Z. multiflora* Bio essential oil inhibited biofilm formation completely at concentrations<0.062 mg/ml and 4 µl/ml, respectively. The *D. orientalis* and *O.* majorana extracts did not inhibit biofilm formation at the used concentrations (0.003 – 8 mg/ml). The results of this study indicate that some plant extracts at low concentrations may provide a complementary medication for biofilm-associated infections. Further evaluations are required to validate the antibiofilm effect of these medicinal plants.

Keywords: *Pseudomonas aeruginosa*, *biofilm*, *plant extract*, *drug resistance.*

INTRODUCTION

Few bacteria live as free floating cells in nutrient rich mediums, and nearly majority of them depend on other microorganisms for energy, carbon and other nutrients and live in microecosystems filled with hundreds of other microorganisms. it has estimated that in the natural world more than 99% of all bacteria exist as biofilms [1]. When bacteria form biofilms, they become more resistant to many harmful environmental factors such as fluctuation of nutrients and oxygen, alteration of pH, and antibiotics effects. One of the reasons for the chronic nature of some infections caused by the opportunistic pathogen *Pseudomonas aeruginosa* is the ability of this bacterium to form biofilms in which the bacteria are protected from host defenses and killing by antibiotics [2]. Medicinal plants have been used as traditional treatments for numerous human infections for thousands of years worldwide [3]. while biofilms of bacteria are more resistant to antimicrobial agents, most studies with plant-based antimicrobial studies have been focused on the planktonic forms [4].

In this study we report antibiofilm effects of *Dianthus orientalis*, *Origanom majorana*, and *Cyclamen coam* extracts, and essential oil of *Zataria multiflora* on *P. aeruginosa* 214. Antibacterial and antifungal effects of these medicinal plants have already been studied by some authors [5-7], but no report on antibiofilm activity of these plants is available.

MATERIALS AND METHODS

Aerial parts of *D. orientali,* leaves of *O. majorana* and *Z. multiflora*, and tubers Of *C. coam* were used in this study.

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Tubers of *C. coam* were collected from Gorgan region, Golestan Province, Iran, and air-dried. Ten grams of the powdered tubers was defatted in 120 ml of petroleum ether for 1 h at 40°C by Soxhlet extractor. After removing the solvent, the sample was extracted with 70% and 100% EtOH for 2 h. The combined EtOH extract was gradually concentrated and eventually dried. The residue was resuspended in distilled water and partitioned with diethyl ether (10×3) and n-BuOH (10×3), successively. The n-BuOH extracts were combined and concentrated under reduced pressure and then the residue suspended in DMSO with final concentration of 2 mg/ml for antimicrobial assay.

O. majorana plant was obtained from Barij Essence Pharmaceutical Co. Some 30g of the powdered leaves and flowers (30 g) was used for extraction in 120 ml petroleum ether (60-80ºC) and 120 ml diethylether for 2 h in a Soxhlet extractor. The defatted sample powder was then extracted with 80% MeOH for 18-20 h; the extract was concentrated under vacuum condition and was resuspended in distilled water to final concentration of 8 mg/ml. The extract was stored at 4°C until used. Aerial parts, leaves and stems of *D. orientalis* were collected at the late July, 2010 from Lavasan region, Tehran province. *Z. multiflora* essential oil was purchased from Barij Essence Pharmaceutical Company.

P. aeruginosa 214, a clinical strain, was chosen due to its strong biofilm production ability assessed in previous studies [8].

Serial dilutions of plant extracts were prepared in TSB (Tryptic Soy Broth) plus 0.2% glucose in 96 well microtiter plates. Bacterial suspension was prepared in sterile PBS buffer (pH 7.2) and the turbidity adjusted to 0.5 McFarland standards to achieve 10^8 CFU/ml. An amount of 100 µl of plant extract and 100 µl of bacterial suspension were added to each well. For each extract negative and positive controls were included; negative control wells contained 100 µl plant extract and 100 µl PBS buffer (without bacteria) and positive controls contained 100 µl bacterial suspension and 100 µl PBS buffer (without plant extract). The microtiter plates were then incubated at 37ºC for 24 h.

A semi-quantitative measurement of biofilm formation was performed using the commercial XTT kit (Roche, Germany) [9]. After a 24 h incubation period, the contents of the wells were aspirated carefully and wells were rinsed three times with sterile PBS and fixed by drying for 1 h at 37ºC. Once the wells were fully dried, 200 µl of XTT solution was added to the wells. The plates were covered with aluminum foil to protect XTT from light and incubated at 37ºC for 5 h. After the incubation period, the well contents were transferred to a new plate and Color changes were measured as optical density (OD) with a microtitration plate spectrophotometric reader at 450 nm.

RESULTS

The antibiofilm activity of extracts and essential oil was measured using the minimum inhibitory concentration (MIC) assay for biofilms. The extracts and essential oil showed various effects on the biofilm formation. One out of three extracts *C. coum* showed a strong antibiofilm effect against *P. aeruginosa* 214 biofilm with the MIC<0.062 mg/ml (Fig 1).

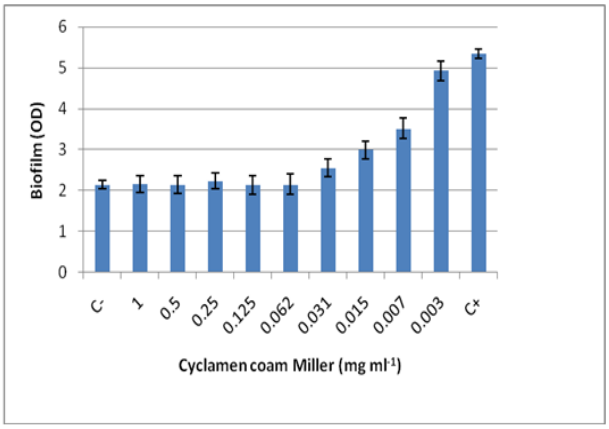

Fig. 1. Biofilm formation of *P. aeruginosa* 214 in presence of different concentrations of *C. coam* extract

Also, the essential oil of *Z. multiflora* showed a very strong antibiofilm activity with the biofilm MIC 4 µl/ml (Fig. 2).

D. orientalis and *O. majorana* extracts did not show any antibiofilm effect, and conversely enhanced biofilm formations as evidenced by the increase in optical density compared to the control biofilm formation.

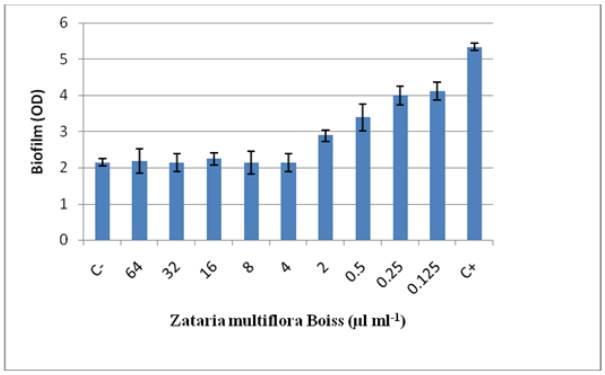

Fig.2. Biofilm formation of *P. aeruginosa* 214 in presence of different concentrations of *Z. multiflora* Boiss essential oil

DISCUSSION

Natural products derived from medicinal plants are abundant source of biologically active compounds. Many plant compounds have been used for development of new antimicrobial agents [10]. However, few plant extract have been investigated for their antibiofilm activity.

C. coam extract contains several saponins as the major antimicrobial agents. These compounds have shown strong inhibitory effect against many bacteria and fungi, but there is not much report on their antibiofilm effects [5]. Antibiofilm activity of *Cyclamen hederifolium* extract on Methicillin-Resistant *Staphylococcus aureus* (MRSA) has been reported by Quave *et al.* [11]. Considerable antibacterial activity of *Z. multiflora* essential oil on several gram positive and gram negative bacteria has been reported [6]. In this study, this essential oil showed a strong antibiofilm activity against *P. aeruginosa*. Carvacrol is one of the major constituents of *Z. multiflora* essential oil [12], which inhibits biofilm formation during the primary stages of biofilm development by preventing the initial attachment of the cells to the surfaces [13]. Thymol, another main constituent of *Z. multiflora* essential oil, is also of antifungal effect [14]. Some studies using *P. aeruginosa* have shown that antibiofilm effects of tannins is mediated via mechanisms like interfering with the bacteriostatic properties, damaging the bacterial membrane and hindering the matrix production [15]. *Conocarpus erectus*, *Bucida buceras*, and *Callistemon viminalis* extracts have shown to considerably inhibit biofilm formation of *P. aeruginosa* by interfering with quorum sensing molecules [16].

Some studies have reported antibacterial and antifungal activity of *O. majorana* and *Dianthus caryophyllus* extract, [7, 17]; however, in our study, *O. majorana* and *D. orientalis* extracts not only didn't inhibit the biofilm formation, but interestingly stimulated biofilm formation. Our results are in agreement with a study on antibiofilm effects of phenolic compounds of plants on *P. aeruginosa* PAO1, which showed that different plant phenolic compounds, at concentrations that did not or weakly suppressed bacterial growth, increased biofilm formation. They suggested that there might be a relationship between stimulation of biofilm formation and las quorum-sensing system of this bacterium [18]. Some other studies have shown that antimicrobial agents can stimulate biofilm formation by different bacteria. In an investigation on *Bacillus subtilis*, biofilm formation was increased under the effect of biocide ClO_2 , and it was interpreted that this phenomenon is a self-protective response to the presence of harmful agents in the environment at their sublethal doses [19]. Similar results have been observed in biofilm studies with aminoglycoside antibiotics [20]. In this study, increase of biofilm formation by *P. aeruginosa*, in presence of *O. majorana* and *D. orientalis* extracts, could be a protec-

tive response by bacteria to evade the antimicrobial effects of the extracts. It has been shown that some medicinal plants that have the antimicrobial activity against planktonic form of some bacteria can induce biofilm formation and consequently more resistant in other bacteria. The dosage of plant extract is another crucial factor in biofilm inhibition and using the inappropriate amount of plant extracts could yield an opposite result, i.e., more biofilm production. To determine the antibiofilm mechanisms of the plant extracts studied here, the antibiofilm compounds of each extract should be identified and studied separately.

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