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ORIGINAL RESEARCH

Antibiofilm Activity of Methanol Extract of *Rumex dentatus* Against *Pseudomonas aeruginosa*

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ABSTRACT

Biofilm formation of *Pseudomonas aeruginosa* makes up a sizeable proportion of hospital-acquired infections because bacteria in biofilms can resist antibiotic treatment. The extracellular polymeric substance of *P. aeruginosa* biofilm is an imprecise collection of extracellular polysaccharides, proteins and microbial cells. *Rumex dentatus* belongs to the family Polygonaceae. This family can be found in Middle East. The aim of this present study was to assess the effect of various concentrations of methanol extract of *Rumex dentatus* on biofilm formation of *P. aeruginosa* after 48 h and 72 h. In this experimental study we collected *R. dentatus* from Khoramabad, Iran. The working extracts were 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 and 0.48 mg/ml. We used microtiter plate method to grow *P. aeruginosa* biofilm and assess the antibiofilm activity of plant extract. The composition of methanol extract obtained from *R. dentatus* was studied by gas chromatography. The minimum biofilm inhibitory concentration (MBIC) for *P. aeruginosa* found to be 250 mg/ml. GC-MS analyses indicated that these fractions contained a variety of compounds including Bicyclo (3.1.1) heptan- 3 -one, 2, 6, 6-trimethyl, Bicyclo (3.1.1) heptan, 6, 6- dimethyl and Eucalyptol. There were consequential correlations between antibiofilm activity and the concentration of extracts after 48 and 72 h.

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Introduction

Medicinal plants products provide a diverse array of chemical structures and known to possess biological activities. Although hundreds of plant species have been tested for antimicrobial properties, the vast majority of them have not been adequately evaluated (Nascimento et al., 2000).

Rumex species are widely distributed worldwide. *Rumex* belongs to the family Polygonaceae; it includes 150 species around the world (Taj et al., 2014). This plant is used locally as a vegetable in the Middle East. It is traditionally applied as bactericidal, anti-inflammatory, anti-tumor, astringent, anti-dermatitis, diuretic, cholagogue, tonic and laxative agent (Nisa, 2013). Previous studies have shown that *Rumex dentatus* has

bactericidal effects (Elzaawely & Tawata, 2012; Fatima et al., 2009; Hameed & Dastagir, 2009; Hussain et al., 2010).

Pseudomonas aeruginosa is reputed for its ability to rapidly develop resistance to multi-antibiotics. The capability of this organism to form biofilms is an important element of its pathogenicity and biofilm formation is a serious challenge for modern medicine (Lihua et al., 2013; Wadood & Sabri, 2013).

Infections due to bacteria that form biofilms are the main problem in their treatment. *P. aeruginosa* biofilms are highly structured communities for protecting against host immune system and severe environmental conditions (Azizi et al., 2015; Walker et al., 2004). This gram-negative bacillus is able to cause several diseases such as burn wound and urinary tract infections (UTIs). Several different epidemiological studies indicate that *P. aeruginosa* is a major cause of urinary tract, and systemic infections, particularly in patients with severe burns (Alikhani et al., 2014; Mittal et al., 2009; Sutter & Hurst, 1966).

To date, the antibiofilm potential of *R. dentatus* remains uninvestigated. Thus, the present study aimed to evaluate anti-biofilm effect of methanol extract of *R. dentatus* against *P. aeruginosa*.

Materials and methods

Bacterial strains

The *P. aeruginosa* strain (ATCC: 27853) was obtained from Iranian Research Organization for Science and Technology, Tehran, Iran. Clinical isolates of *P. aeruginosa* were obtained from Emam-Mosaie-Kazem hospital and Mahdiyeh Diagnosis Laboratory Center in Isfahan, Iran during July-March 2015. 60 strains were isolated from burn wound swabs and urinary tract infection. These isolates were identified by standard biochemical tests. All samples were cultured on BA (blood agar) and EMB (Eosin Methylene Blue) media (Himedia) and were incubated at 35 ℃ for 18–24 h. The isolates were evaluated for *P. aeruginosa* by performing Gram straining, biochemical tests and PCR (polymerase chain reaction) amplification of 16S rRNA gene. Universal eubacterial 16S rRNA PCR primers, PA-SS-F (5′- GGGGGATCTTCGGACCTCA -3′) and PA-SS-R (5′- TCCTTAGAGTGCCCACCCG -3′), were used for amplification of 16S rRNA gene. PCR was carried out in 25 µl reaction mixture consisting of 2.5 µl 10x pcr buffer, 2 µl of dNTP mix (concentration 10 mM)(Fermentase, Germany), 5 µl of $MgCl_2$ (concentration 25 mM), primer forward and reverse 0.5 µl (concentration 10 mM), 0.2 µl Taq DNA polymerase (Genetbio, Korea), 2 µl of DNA (150-200 ng) and 12.3 µl nuclease free water. In our previous study (Pezeshki Najafabadi et al., 2016), in accordance with this one, PCR results and images were presented.

The PCR reaction was carried out in a thermal cycler (Bio Rad CO) with an initial denaturation at 95 ℃for 3 minutes, followed by 35 cycles of denaturation at 94 ℃for 20 seconds, annealing at 58 ˚C for 30 seconds, extension at 72 ℃for 40 seconds, with a final extension at 72 ℃ for 5 minutes. Amplicons were detected by electrophoresis on 1.0 % (w/v) agarose ethidium bromide gel.

Determination of MIC and MBC

The ability of *R. dentatus* leaf extract to inhibit bacterial growth was determined using a standard procedure of the Minimum Inhibitory Concentration (MIC). This was defined as the lowest concentration of the extract that causes inhibition of visible growth of *P. aeruginosa* cells. MIC was determined by micro-dilution method as described by the Clinical and Laboratory Standards Institute (CLSI) method. The Minimum Bactericidal Concentrations (MBC) determination applied to assess if the inhibitory effect observed in MIC determinations. Samples (20 µl) were removed from the wells of the MIC microtitre plates that showed no turbidity and were inoculated onto BHI agar. The MBC was the lowest concentration of the test reagent in the well of the microtitre tray that in which all bacteria were killed (Wiegand et al., 2008).

Preparation of *R. dentatus* extract

Healthy and fresh leaves of *R. dentatus* were collected from Gahar Lake, Lorestan, Iran in May 2013. These plants were identified and deposited at the herbarium of the Lorestan Agricultural and Natural Resources Research Center, Khoramabad, Iran. Collected leaves were washed in water followed by successive washing in distilled water. The leaves were shade dried at room temperature. Dried materials were chopped into powder using an

electric grinder. The dried powder of *R. dentatus* leaves (50 gr) were extracted with 500 ml methanol 80% for 5 h in soxhelet apparatus. The extracts were filtered through filter paper (Whatman no. 1) and excessive solvent was dried using Rotary evaporator. These extracts were stored at 4° C. Dried extracts were dissolved in dimethyl sulphoxide 10% (Merck, Germany) and serial dilutions were prepared to final concentration of 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.97 and 0.48 mg/ml (Abou Elfotoh et al., 2013).

Gas chromatography-mass spectrometry (GC-MS) analysis

1. *R. dentatus* methanol extract was subjected to detailed GC-MS analysis using an Agilent model 7890. Helium was used as the carrier gas. The chemical components from the methanol extract were identified by comparing the retention times of chromatographic peaks with those of authentic compounds using the NIST05sLIB.

Inhibition of biofilm formation

The microtitre plate method was used to determine the inhibition of biofilm production. The bacterial suspension was adjusted with the same Muller Hinton Broth (Merck, Germany) to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08 - 0.1 at 630 nm) in a spectrophotometer, corresponding to approximately 10^8 CFU/ml. The methanol extracts of *R. dentatus* were then tested for their potential to prevent biofilm formation of *P. aeruginosa* strain. Sterile 96-wells flat-bottomed polystyrene microtitre trays were inoculated with 100 µl of bacterial suspension and 100 µl of each methanol extract concentrations. Muller Hinton Broth containing 1% glucose plus bacterial suspension was employed as a positive control and 200 µl of pure Muller Hinton Broth was used as the blank control. The microtitre trays were incubated at 35 ℃ for 48 h and 72 h. After cultivating, the supernatant was removed and wells were rinsed with distilled water. The remaining attached bacteria were fixed with 50 µl of 96% ethanol per well, and after 15 min, plates were made empty and left to dry. Each well stained for 5 min with 200 µl of 2% crystal violet (CV Gram stain, Merck, Germany). After 15 min, stain was raised off by placing the plates under running tap water. After drying, biofilms were visible as purple rings formed on the sides of each well (Pitts et al., 2003). Then optical density of each well was measured at 495 nm by an ELISA (enzyme-linked immunosorbent assay) tray reader (Statfax-2100, Awareness Technology, USA).

The quantitative analysis of biofilm production was performed by adding 200 µl of 33% (v/v) glacial acetic acid (Merck, Germany) per well. Then optical density of the stain was measured at 495 nm by an ELISA tray reader (Stepanovic et al., 2000). Reduction percent of biofilms was obtained by using the following formula:

$$\text{Reduction percent} = \text{R.P} = \{\frac{(C-B)-(T-B)}{C-B}\} \times 100$$

C = mean of OD of control wells
B = mean of negative controls
T = mean of test wells.

Statistical analysis

All experiments were performed in triplicate. Data (Fig. 2 a,b) were analyzed using the Statistical Package for Social Sciences (SPSS-Ver.22). Data were analyzed using one-way analysis of variance (ANOVA). A p-value less than 0.05 were considered significant.

Results

Table 1 indicates that GC-MS analysis of the methanol extract of *R. dentatus* revealed major components with different concentrations. Total ion chromatograms of the methanol extract of *R. dentatus* are shown in Fig. 1.

MIC and MBC values of the methanol extract of *R. dentatus* were 250 mg/ml and 500 mg/ml against *P. aeruginosa*, respectively. MIC and MBC values were the same for *P. aeruginosa* isolated from burn wound swabs and urinary tract infection. The capability of methanol extract of *R. dentatus* to attenuate biofilm formation is shown in Fig. 2.

Table 1. Major components identified in the methanol extract of *R. dentatus*.

Retention time (min)	Compounds identified	Kovats index *KI*
7.423	Sabinene Bicyclo (3.1.0) hexane	973
7.484	Alpha-Pinene	933
11.335	Bicyclo (3.1.1) heptan,6,6- dimethyl	978
8.685	Eucalyptol	1,033
11.601	Biocyclo (3.1.1) heptan-3- one,2,6,6- trimethyl	1,173
11.267	Hydrazine	1,159
15.369	Alpha-Bourbonene	1,389

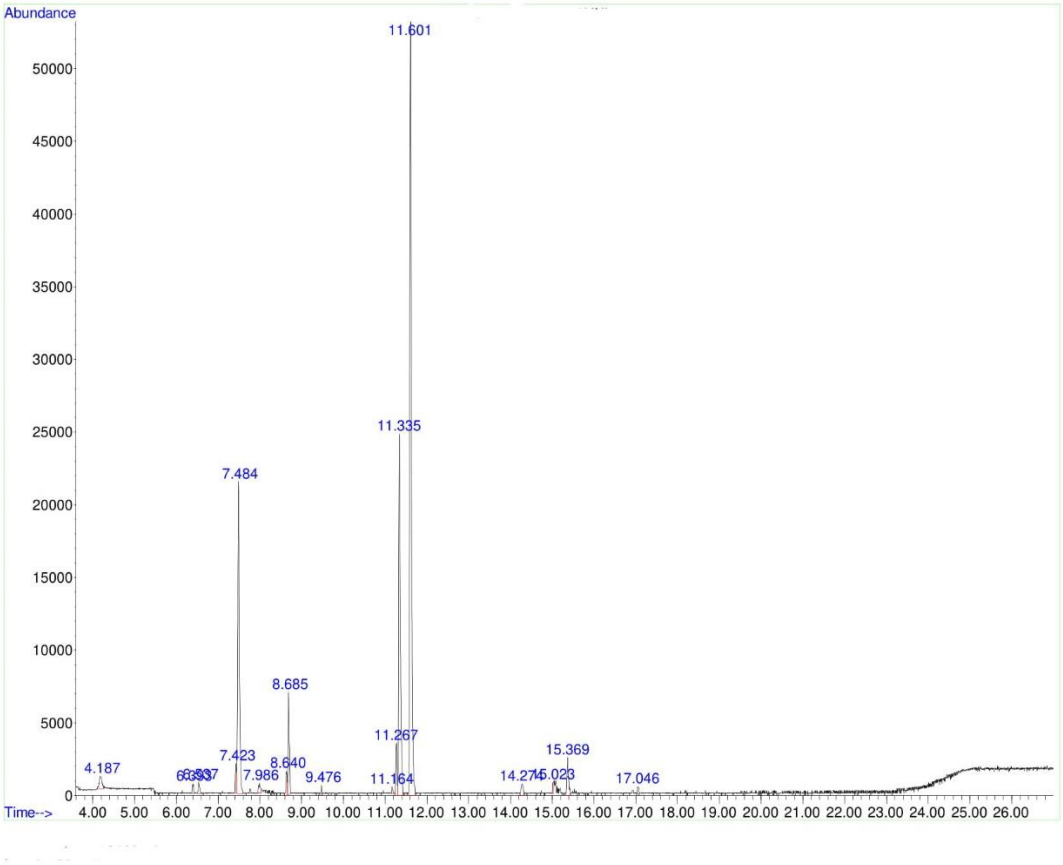

Fig. 1. Total ion chromatograms (GC/MS) of the methanol extract of *R. dentatus*.

Sub-MIC concentrations of *R. dentatus* methanol extracts inhibited *P. aeruginosa* biofilm formation. At a concentration of 250 mg/ml, this extract reduced *P. aeruginosa* biofilm formation by 100%. Methanol extract of *R. dentatus* dose-dependently inhibited biofilm formation of *P. aeruginosa* on polystyrene surface at both 48 h and 72 h (Fig. 2).

As shown in Fig. 2, the elimination potential of the methanol extract on *P. aeruginosa* isolated from UTIs and burn wound infections were nearly 100% after 72 h. This extract was able to inhibit biofilm formation completely. Our study leads to the result that *R. dentatus* methanol extracts are able to efficiently reduce biofilms.

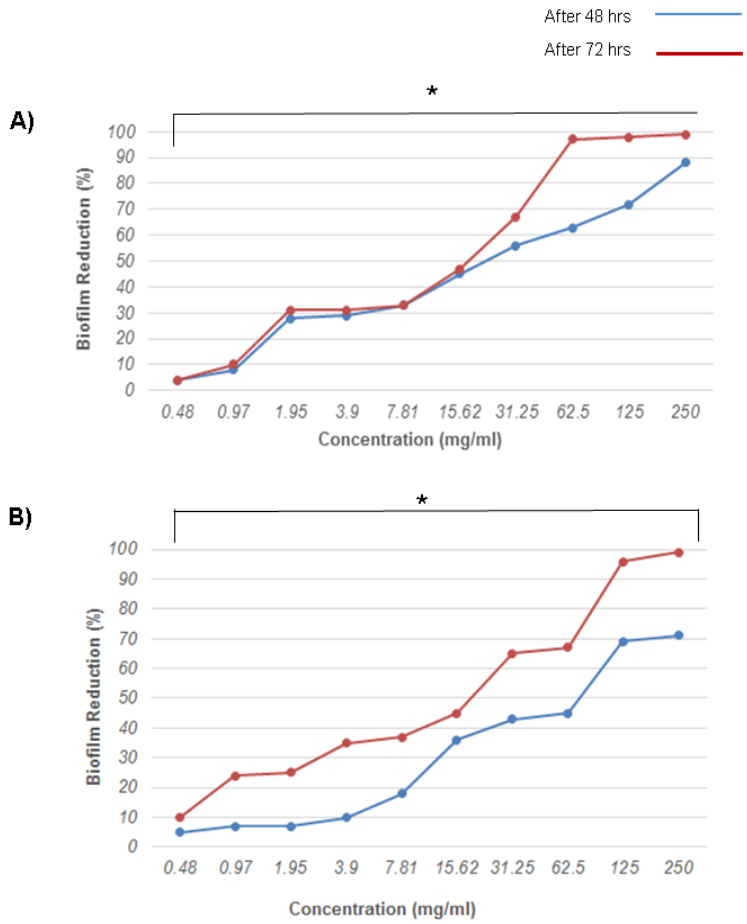

Fig. 2. Biofilm formation assessment in the presence of *R. dentatus* methanol extract (A: For UTIs isolates. B: For burn wound infections isolates. (*) in Fig. 2B shows the significance of data mean). **Notes**: In the case of concentrations (horizontal lines), 60 isolates were used and the experiment was performed three times. Means of biofilm reduction at each concentration (vertical lines) were obtained. The effect of methanolic extracts on the biofilm reduction at different concentrations is expressed in two diagrams.

Discussion

Increasing antimicrobial resistance among bacterial pathogens is one of the most distressing experiences in recent years. The prevalence of multidrug resistant *P. aeruginosa* strains is increasing both in community-acquired and hospital-acquired infections (Oskay et al., 2009). *P. aeruginosa* is one of the most important biofilm producing bacteria. Fighting the clinical complications associated with biofilm formation is very difficult and linked to a high risk of failure. This bacteria can produce several infections, such as bacteremia, respiratory infection, urinary tract infection, osteomyelitis and infections of burn and soft tissues (Yahya et al., 2014). Biofilm formation is one of the most important factors in pathogenesis of *P. aeruginosa*. Biofilm formation in this bacterium is motivated by some stresses such as some non-optimal growth conditions in the immediate environment of bacterial cells. While biofilms of bacteria are more resistant to antimicrobial agents, most studies with plant-based antimicrobial investigations have been focused on biofilm formations (Bacalso et al., 2011). In this study anti-biofilms effects of different concentrations of methanol extract of *R. dentatus* against *P. aeruginosa* had been significant. The results of this study showed that the methanol extract of *R. dentatus* has antibiofilm activity on *P. aeruginosa* strains isolated from UTIs and burn wound infections. *R. dentatus* is a medicinal plant that is extremely complicated mixture of substances. This plant contains terpens, alkenes, monoterpenoids, alpha-pinenes and flavonoids.

Chemical compositions of *R. dentatus* extract could be changed in another region because of plant allotments. Fatima et al. (2009) evaluated methanol and hexane extracts of *R. dentatus* for antibacterial,

antifungal, cytotoxicity, antitumor and allopathic potential. The methanol extracts were found to be effective against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *E. coli*, *Pseudomonas picketiiand Bordetella bronchiseptica*. They also showed that the methanol extract contained alkaloids, saponin, anthraquinones, tannins and flavonoids (Fatima et al., 2009). Hussain et al. (2010) indicated antibacterial activities of crude extract of *R. dentatus* against *E. coli*, *Salmonella typhi*, *S. aureus*, *P. aeruginosa*, *Enterobacter aerogenes*, and *Citrobacter freundii*. Elzaawely & Tawata (2012) showed that different extract of *R. dentatus* contained varying degrees of 1,1- diphenyl-2-picrylhydrazyl (DPPH) scavenging activity. Our results were in line with other studies. The methanolic extract had nearly 100% antibiofilm effects after 72 h. Harmsen et al. (2010) studied the *P. aeruginosa* biofilm lifestyle. They explained that extracellular DNA is necessary for matrix and biofilm formation which depends on nutrients, motility and quorum sensing.

Kim & Park (2013) evaluated the ability of ginger ability to inhibit *P. aeruginosa* PA14 biofilm formation. They showed that biofilm development was reduced by 39-56% when toluene extract of ginger was added, as this extract reduced extracellular polysaccharides. This extract inhibited biofilm formation in both gram negative and positive bacteria. Varposhti et al. (2013) reported the inhibitory effect of three medicinal plant extracts and an essential oil of the biofilm formation by *P. aeruginosa*. They showed that *Cyclamencoum* and *Zatariamultiflora* inhibited biofilm formation completely at 0.062 mg/ml and 4 µg/ml concentrations, other extracts did not have any biofilm formation effect. Our results revealed that inhibition of biofilm formation of methanol extract against *P. aeruginosa* had significant effects between times and concentration. Exceeding from acceptable concentration of extract had significant effects after 48 h and 72 h on these biofilm cells. The results indicated that 250 mg/ml methanol extract after 72 h was more effective in *P. aeruginosa* biofilms formation. The results showed that the methanol extract administrated significantly ($p < 0.05$) reduced the biofilm biomass. But some concentrations had no significant influence over these parameters. Our results suggested that the extract have a beneficial effect on biofilm formation reduction.

This study showed that the methanol extract of *R. dentatus* has an anti-biofilm impact on *P. aeruginosa*. The most concentration (250 mg/ml) was used in order to inhibit biofilm formation completely after 72 h. This MIC value was higher, compare to other concentrations of exposition analyzed. The effect on biofilm formation seems to be directly related to the growth inhibition of anti-biofilm activity of methanol extract (Cardoso et al., 2012). The increase in the methanol extract concentration positively regulated the biofilm formation. Exopolysaccharide production and flagella motility in pathogenic bacteria such as *E. coli*, *PAO1*, *P. mirabilis*, and *S. marcescens* play a major role in biofilm formation. It could be possible that the anti-biofilm activity could be due to inhibition of exopolysaccharide synthesis. Maybe it is because that the methanol extract impairs exopolysaccharide synthesis in *P. aeruginosa* which limits the biofilm formation (Mohanty et al., 2011).

The data presented in Fig. 2 reveal statistically significant correlations between anti-biofilm activity and concentrations of methanol extract of *R. entatus* after 48 and 72 h. This finding suggested that methanol extract of this plant could be used as the natural anti-biofilm activity on *P. aeruginosa*. To determine the anti-biofilm mechanisms of the extract, compounds of methanolic extract including Sabinene Bicyclo (3.1.0) hexane and Bicyclo (3.1.1) heptan,6,6- dimethyl (obtained according to Table 1) should be identified and studied separately.

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Conflict of interest

The authors declare no conflicts of interest.

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