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Antibiofilm and Antibacterial Activity of *Urginea maritima* Against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract

Background: One of the most important therapeutic challenges is fighting against infectious diseases and developing their bacteria high antibiotic resistance. Bacterial resistance is one of the reasons for the growing uses of herbs in treating bacterial infections.
Objectives: The purpose of this study was investigating the antibacterial and anti-biofilm properties of the bulb of *Urginea maritima* against *Pseudomonas aeruginosa* (ATCC: 27853, ATCC: 9027) and *Staphylococcus aureus* (ATCC: 25923, ATCC: 9144).
Methods: Methanol and acetone extracts of the bulb were prepared by the Soxhlet method. The antibacterial properties of the extracts were evaluated by the agar well diffusion method, and the minimum inhibitory concentration was evaluated by the microdilution method. The anti-biofilm effect of the extracts was also measured by the microplate technique and by crystal violet dying. Determining the compounds of the *U. maritima* bulb was done by the GC/MS device.
Results: Results showed that acetone extract of *U. maritima* bulb prevents the growth of standard and clinical strains of *P. aeruginosa* and *S. aureus*. The highest antibiofilm effect was related to the methanol extract of the bulb against *S. aureus*, with the reducing of biofilm production of about 94%. Most of the extracted compounds of the *U. maritima* bulb include xylene, pentane, and phenyl ethane.
Conclusions: The data of this study suggest that methanol and acetone extracts of the bulbs of *U. maritima* have potential activity as an antibacterial and antibiofilm agent, especially against *S. aureus*.

Keywords: Anti-Bacterial Agent, Biofilms, Plant Extract, *Urginea maritima*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*

1. Background

Excessive use of antimicrobial medications has led to increasing medicinal resistance in most bacteria against various antibiotics. Thus, using effective herbal materials has been considered as low-risk, available, and as natural materials in treating bacterial infections. Antibiotic resistance also increases the risk of spreading the biofilms producing bacteria. Treating the infections due to the bacteria producing biofilms is difficult, causing various physicians to encounter with extensive problems. Researchers try to find an appropriate approach for restraining and preventing the formation of biofilms (1, 2). Regarding the role of effective herbal materials in eliminating bacteria and prohibition of the formation process of biofilms in them, analysis of medical properties of native herbs of each region is quite an important subject to be considered (3).

Urginea maritima is a species of flowering plants in *Liliaceae*. This plant is a native to the south of Europe, west of Asia, and north of Africa, and is growing in the western Iranian mountainous regions. *U. maritima* grows from a white and large bulb with the weight of almost 1 kg. Each bulb produces about 10 leaves with a rosette form and length of one meter, in spring seasons. The leaves are dark gray and have leather-like tissue. Mainly, *U. maritima* grows in rainy mountainous areas (4, 5). Pharmacologic research on the compounds of European varieties of *U. maritima* has indicated the existence of glucose scillaren A, proscillaridin A, scillaren A, and glucoside (6). *U. maritima* had shown anti-insects activity against larvae and adults of different insects (7).

Antimicrobial properties of methanol and acetone extracts of the bulbs of other strains of Urginea, from various geographic regions, against different microorganisms, are reported (8). Researchers had shown that methanol and acetone extracts of the bulbs of *U. sanguinea* had appropriate antibacterial activity against Gram-negative bacteria, especially *P. aeruginosa*, while the extracts showed no considerable antiparasitic properties (9). The research showed that fruit extracts of *U. maritima* exhibited antimicrobial and anticancer activities (10).

The evaluating antimicrobial properties of aqueous, methanol, acetone, and chloroform extracts of the bulbs of *U. indica*, showed that Gram-positive bacteria were more sensitive than Gram-negative bacteria to those extracts. Most of the antibacterial activities of the methanol extract of the bulbs of *U. indica* were found against *Bacillus cereus* and *Staphylococcus epidermidis*, while acetone and chloroform extracts showed their greatest antibacterial activities against *P. aeruginosa* and *Klebsiella pneumoniae*. Acetone extract of the bulbs of this plant had considerable antifungal activity against *Candida albicans* and *Aspergillus niger* (11).

Imipenem is an antibacterial agent of the carbapenems that is effective against Gram-positive, Gram-negative, and even multidrug-resistant strains. Imipenem is a broad-spectrum antibiotic used for severe bacterial infections caused by *P. aeruginosa* and *S. aureus*. Unfortunately, due to the excessive consumption, expansion of metallo-beta lactamases and other methods of antibiotic resistance, the rate of *P. aeruginosa* and *S. aureus* carbapenems resistance is increasing.

2. Objectives

Due to the fact that so far there have not been any studies about the anti-biofilm properties of *U. maritima*, the aim of this research was evaluating the antibacterial and anti-biofilm effects of methanol and acetone extracts of the bulbs of *U. maritima* against some of the *P. aeruginosa* and *S. aureus*.

3. Methods

3.1. Plant Material and Extraction

The fresh bulbs of *U. maritima* were collected from areas around the city of Izeh (31°50'14" North, 49°52'10" East), Khuzestan province, Iran. *U. maritima* was identified in the Ministry of Agriculture Jihad herbarium in Izeh city. Fresh bulbs were peeled and dried in a suitable ventilated condition and in a dark place. After complete drying, powder of the bulbs was prepared.

3.2. Soxhlet Extraction

The dried bulbs powder was extracted by the Soxhlet procedure for five to six hours. Methanol and acetone extracts (40 gr bulb powder in 200 mL acetone or methanol) were prepared. The extracts were kept in darkness at 4°C. To prepare different dilutions of methanol extract, 2 g of dried methanol extract mixed with 2 mL of 50% dimethyl sulfoxide (DMSO), concentration of 1000 mg/mL was provided and serial dilutions of 500, 250, and 125 mg/mL were prepared. Different concentration of acetone extracts was prepared the same way.

3.3. Sample Preparation for Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis is best used to make an effective chemical analysis for bioactive chemicals. The phytochemical screening of the bulbs of *U. maritima* was done by Headspace method in Meyar-Danesh-Pars Co. In this method, the soft powder of the plant was used for injection into the machine.

3.4. GC-MS Analysis and Data Preprocessing

The GC-MS system consisted of a gas chromatograph mass detector Aglient 5975C equipped with an electron ionization system fused with silica capillary column HP-5MS (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness). The injector and ion source of mass detector temperatures were 280°C and 150°C. The quadruple and transfer line temperature were 230°C and 280°C. Using computer searches on data library and comparing the spectrum obtained through GC-MS compounds present in the plants sample were identified.

3.5. Bacterial Strains

The strain of *S. aureus* (ATCC: 25923), *S. aureus* (ATCC: 9144), *P. aeruginosa* (ATCC: 9027), and *P. aeruginosa* (ATCC: 27853) were purchased from the Iranian Organizations for Science and Technology (IROST). One clinical strain of *S. aureus* and *P. aeruginosa* were also available. Bacterial inoculum was prepared in concentration of 1.5×10^8 cfu/mL, which was standardized by adjusting the optical density of the bacterial suspension at 0.08 - 0.1 at 620 nm spectrophotometrically. The bacterial suspension was diluted to 1.5×10^7 cfu/mL.

3.6. Antibacterial Assay

Antibacterial assay was done by the agar well diffusion method. The bacterial inoculum (100 mL) were poured on Muller Hinton agar (MHA) media. The wells (6 mm diameter) were made in each of the plates by a sterile cork-borer. About 100 μL of different concentrations of methanol and acetone extracts were separately added into the wells and allowed to diffuse at room temperature. One hundred microliters of DMSO 50% was served as a negative control and 100 μL of imipenem (10 μg/mL) was added to another well as a positive control. The plates were incubated for 24 h at 37°C and the diameter of clear zone of growth inhibition was recorded (12, 13).

Determination of the MIC was carried out using the microtiter plate's method. A total of 100 microliters of different concentrations of methanol and acetone extracts were separately added into each well. Bacterial inoculum (1.5×10^7 cfu/mL) were prepared and 100 μL of them were added

into the wells. The plates were incubated at 37°C for 24 h. After incubation, the optical density (OD) of each well was measured at a wavelength of 630 nm by ELISA. At the end of incubation, the lowest concentration with no visible growth was defined as the MIC. The MBC was determined by plating 10 μL of samples from wells, where no indicator color change was recorded, on nutrient agar. The lowest concentration with no visible growth on plates is MBC (14).

3.7. Microtiter Plate Biofilm Assay

A modified crystal violet assay was employed in this research (15). One hundred bacterial suspensions were added in to the 96 well microtiter plates. One hundred microliter of different concentrations of methanol (500, 250, 125, 62.5, and 31.12 mg/mL) and acetone (250, 125, 62.5, 31.12 and 15.62 mg/mL) extracts were added separately in to the wells. Negative control well consisted of 200 μL of TSB media and positive control well filled with 100 μL of bacteria suspension and 100 μL of TSB media. Microtiter plates were incubated at 37°C for 72 h. Then, the wells were inverted to remove the planktonic bacteria and 250 μL of 95% ethanol was added into the wells for 15 minutes. After drying, 200 μL of 2% aqueous solution of crystal violet was added. After five minutes, the microplates were inverted and washed then tapped on paper towels to remove the crystal violet and air-dried. Then, 100 μL of 33% glacial acetic acid (Sigma Chemical Co.) were added to wells and the microplates incubated at 37°C for 30 minutes. Finally, optical density of each microplate was measured at a wavelength of 405 nm (16, 17). The percent of biofilm formation reduction was calculated by the following formula:

$$Reduction\ percent\ = RP = \left\{ \frac{(C - B) - (T - B)}{C - B} \right\} \times 100 \quad (1)$$

C, OD of positive controls; B, OD of negative controls; T, the average OD of wells with repeating 3 times.

3.8. Statistical Analysis

All experiments were carried out in triplicate. Data were analyzed using SPSS (Ver.17) significant differences between groups were assessed by Mann-Whithney and Cruskal-Wallis tests.

4. Results

The results of phytochemical screening of *U. maritima*bulbs was shown in Table 1.

The methanol and acetone extracts of *U. maritima* bulb were tested against standard and clinical isolates of *S. aureus* and *P. aeruginosa*. Their activities of these extracts were qualitatively and quantitatively evaluated by the presence or the absence of inhibition zone, MICs, and MBCs values. These results are given in Tables 2 and 3.

In this study, methanol bulbs extract was effective against standard and clinical isolated strains of *S. aureus*. The extract has a low inhibitory effect against standard strains of *P. aeruginosa*. The clinical isolated strain of *P. aeruginosa* was resistant to the methanol bulb extract. Based on the results of the Mann-Whitney test at concentration 1000 mg/mL, diameter of inhibition zone in *S. aureus* was significantly higher than *P. aeruginosa* ($P < 0.05$). At the concentration of 500 mg/mL, the diameter of inhibition zone was only observed for *S. aureus* (ATCC: 25923) and was significantly higher than *P. aeruginosa*. In other concentrations, the diameter of the inhibition zone was not observed for any of the two bacteria.

In reporting the results of a Mann-Whitney test, inhibition zone of all concentrations of antibiotic and acetone extract in standard strains of *S. aureus* and *P. aeruginosa* was significantly higher than clinical isolated strains ($P < 0.05$).

The MIC and MBC values of the methanol and acetone extracts of *U. maritima* bulbs against *S. aureus* and *P. aeruginosa* are shown in Table 4.

According to the results, methanol and acetone extracts of *U. maritime* bulbs inhibited biofilm formation by *S. aureus* sand *P. aeruginosa* at sub-lethal concentration of these extracts. Anti-biofilm activity of acetone extract was better than methanol extract. On the other hand, anti-biofilm effect of methanol and acetone extracts of *U. maritima* bulbs against *S. aureus* was higher than *P. aeruginosa* (Figure 1).

5. Discussion

The results showed that methanol and acetone extracts of *U. maritima* bulbs prevent the growth and production of the biofilms of standard and clinical strains of *P. aeruginosa* and *S. aureus*.

According to the present study, standard and clinical strains of *P. aeruginosa* were more resistant against the methanol extract of *U. maritima* bulbs. Hence, it is possible that the different effectiveness of the methanol extract of *U. maritima* bulbs was relative to the differences of the cell wall of these bacteria.

Studies by other researchers such as Sparg showed that the 50 mg/mL density of the ethanol extract of the bulbs of *Scilla natalensis* had most antibacterial effect against *S. aureus* (18). Pandey and Gupta also reported that 150 mg/mL of methanol extract of *U. indica* bulbs has similar antibacterial effects on the standard strains of *S. aureus* and *P. aeruginosa*, and the diameters of the zone of inhibition were ob-

Table 1. Composition of Methanol Extract of *U. maritima* Bulbs

Compounds	Possibility of Presence, %	Kovats Index	Retention Time, min
Pentan-1-ol	13.21	766	4.191
3,4-Hexanedione	10.57	793	4.429
Hexamethyl-2-P-nitrophenyl-oxadiazol	1.29	822	5.262
Cyclotrisiloxane	1.64	837	5.588
Phenylethane	10.07	869	6.308
3-Methylbutyl acetat	7.96	876	6.464
Alpha-pinene	3.13	924	7.969
Alpha-pinene(2,4,6-Octatriene)	7.19	925	8.008
o-xylene(1,3-dimethyltrisulfane-benzaldehyde)	1.04	961	8.387
Pentana	1.45	935	8.412
2-Pentoxyethyl trifluoroacetate	1.30	1051	10.418
2,2,5,5-Tetramethylhexane	1.09	1052	10.447
2,3,3-Trimethylbicyclo[2.2.1]heptan-2-ol	0.56	1150	16.995
cyclohexasiloxane	0.82	1321	21.309
4-Allyl-2-methoxyphenol	1.79	1356	22.292
Pentasiloxane	2.04	1497	24.653

Table 2. Antibacterial Activity (Diameter Zone of Inhibition) of the Methanol Bulbs Extract of *U. maritima* (mm) against *S. aureus* and *P. aeruginosa*

	Concentration, mg/mL				Antibiotic (IPM)	DMSO
	125	250	500	1000		
S. aureus						
ATCC: 9144	-	-	-	15.67 ± 1.15	32.33 ± 3.21	-
ATCC: 25923	-	-	12.33 ± 0.58	17.33 ± 1.53	30.33 ± 2.52	-
Clinical isolate	-	-	-	13.67 ± 1.15	29.33 ± 1.53	-
P value[a]	-	-	0.08	0.017	0.148	-
P. aeruginosa						
ATCC: 27853	-	-	-	-	31.00 ± 2.00	-
ATTC: 9027	-	-	-	13.00 ± 1.00	30.67 ± 2.08	-
Clinical isolate	-	-	-	-	14.33 ± 1.53	-
P value[a]	-	-	-	0.047	0.010	-
P value[b]	-	-	0.033	0.001	0.175	-

[a] Comparison between standard and clinical species.
[b] Comparison between two bacteria.

tained as 12.33 and 13.13 mm, respectively (11). In the current study, the inhibition zone diameters of methanol extract of *U. maritima* bulbs against standard strains of *S. aureus* and *P. aeruginosa* were obtained as 15.67 and 13.00 mm, respectively. Thus, the results of the present study are in conformity with the results obtained by other researchers.

The results obtained from the antibacterial activity of acetone extract of *U. indica* bulbs showed that the diameter of zone of inhibition in the density of 150 mg/mL against *P. aeruginosa* (16.26 mm) was more than *S. aureus* (11.20 mm) (11). However, the density of 500 mg/mL of the acetone extract of *U. maritima* bulbs was similarly effective on *S. aureus* (24.00 mm) and *P. aeruginosa* (25.00 mm), and the diameter zone of inhibition of the acetone extract of *U. maritima* bulbs is more than that in the *U. indica* bulbs, which is due to the high density of the acetone extract of *U. mar-*

Table 3. Antibacterial Activity (Diameter Zone of Inhibition) of the Acetone Bulbs Extract of *U. maritima* mm) Against *S. aureus* and *P. aeruginosa*

	Concentration, mg/mL				Antibiotic (IPM)	DMSO
	125	250	500	1000		
S. aureus						
ATCC: 9144	-	14.33 ± 0.58	20.33 ± 2.31	24.00 ± 1.73	31.67 ± 3.06	-
ATCC: 25923	-	-	12.67 ± 1.15	15.33 ± 1.53	30.33 ± 1.53	-
Clinical isolates	-	-	11.67 ± 2.52	14.67 ± 1.15	27.33 ± 1.53	-
P value[a]	-	0.010	0.047	0.010	0.120	-
P. aeruginosa						
ATCC: 27853	-	-	20.00 ± 2.00	25.00 ± 2.00	31.67 ± 2.52	-
ATCC: 9027	-	-	11.00 ± 1.73	14.33 ± 1.15	30.33 ± 1.53	-
Clinical isolates	-	-	-	13.67 ± 0.58	12.67 ± 2.08	-
P value[a]	-	-	0.009	0.032	0.010	
P value[b]	-	0.500	0.124	0.200	0.212	

[a] Comparison between standard and clinical species.
[b] Comparison between two bacteria.

Table 4. The MIC and MBC Values of Bulbs Extract of *U. maritima* (mg/mL)

Bacteria	Methanol		Acetone	
	MIC	MBC	MIC	MBC
S. aureus				
ATCC: 9144	500	1000	125	500
ATCC: 25923	500	1000	125	500
Clinical isolates	500	1000	125	250
P. aeruginosa				
ATCC: 9027	1000	1000	125	250
ATCC: 27853	1000	1000	250	500
Clinical isolates	500	1000	500	500

itima bulbs and existence of extensive amount of effective materials in that density. Generally, it was shown in the present study that the diameter zone of inhibition of the acetone extract of *U. maritima* bulbs had no significant difference against *S. aureus* and *P. aeruginosa*. Naidoo studied the antibacterial effects of methanol and acetone extracts of bulbs of *U. sanguinea* on four types of bacteria. The bulb extracts showed more antibacterial effects against Gram-negative bacteria, especially *P. aeruginosa* (19). However, in the present study, the antibacterial effects of methanol and acetone extracts were more against *S. aureus*, and the existence of different released compounds on methanol and acetone solvents for the two species of *U. sanguinea* and *U. maritima* can be a reason for different results of the two studies in that respect.

Comparing the imipenem effect on the diameter zone of inhibition of *S. aureus* and *P. aeruginosa*, which was done by the Mann-Whitney test, there was no significant difference between the two bacteria ($P < 0.05$). On the other hand, in the standard and clinical strain of *S. aureus*, the diameter zone of inhibition was only observed on 1000 mg/mL methanol extract of bulb, which was significantly less than imipenem ($P < 0.05$). The diameter zone of inhibition was observed on 500 mg/mL acetone extract of *U. maritima* bulb and showed significantly less than Imipenem ($P < 0.05$).

Ceylan had some studies on anti-biofilm properties of ethanol extract of the above ground parts and bulbs of *Allium orientale* plant of *Liliaceae* family on some Gram-positive and Gram-negative bacteria. The results showed that ethanol extract of the above ground parts of *A. orientale* had the most anti-biofilm effects on *E. coli* bacteria, with the reducing rate of biofilm production 68.51%. The rates of biofilm production reduction of the ethanol extract of the above ground parts and the bulbs on *S. aureus* (ATCC: 25923) were 81.13% and 44.12%, respectively, and on *P. aeruginosa* (ATCC: 27853) were 63.45% and 39.11%, respectively (20). The reducing rate of biofilm production of methanol extract of *U. maritima* bulbs against *S. aureus* (ATCC: 25923) was 94%, and that against *P. aeruginosa* (ATCC: 27853) was equal to 87% in the present study. Comparing the results of other research and the present study indicates that the anti-biofilm effect of methanol extract of *U. maritima* bulbs is more than that in *A. orientale*, and different active compounds in both species can be the reason for the higher rate of activities in that regard.

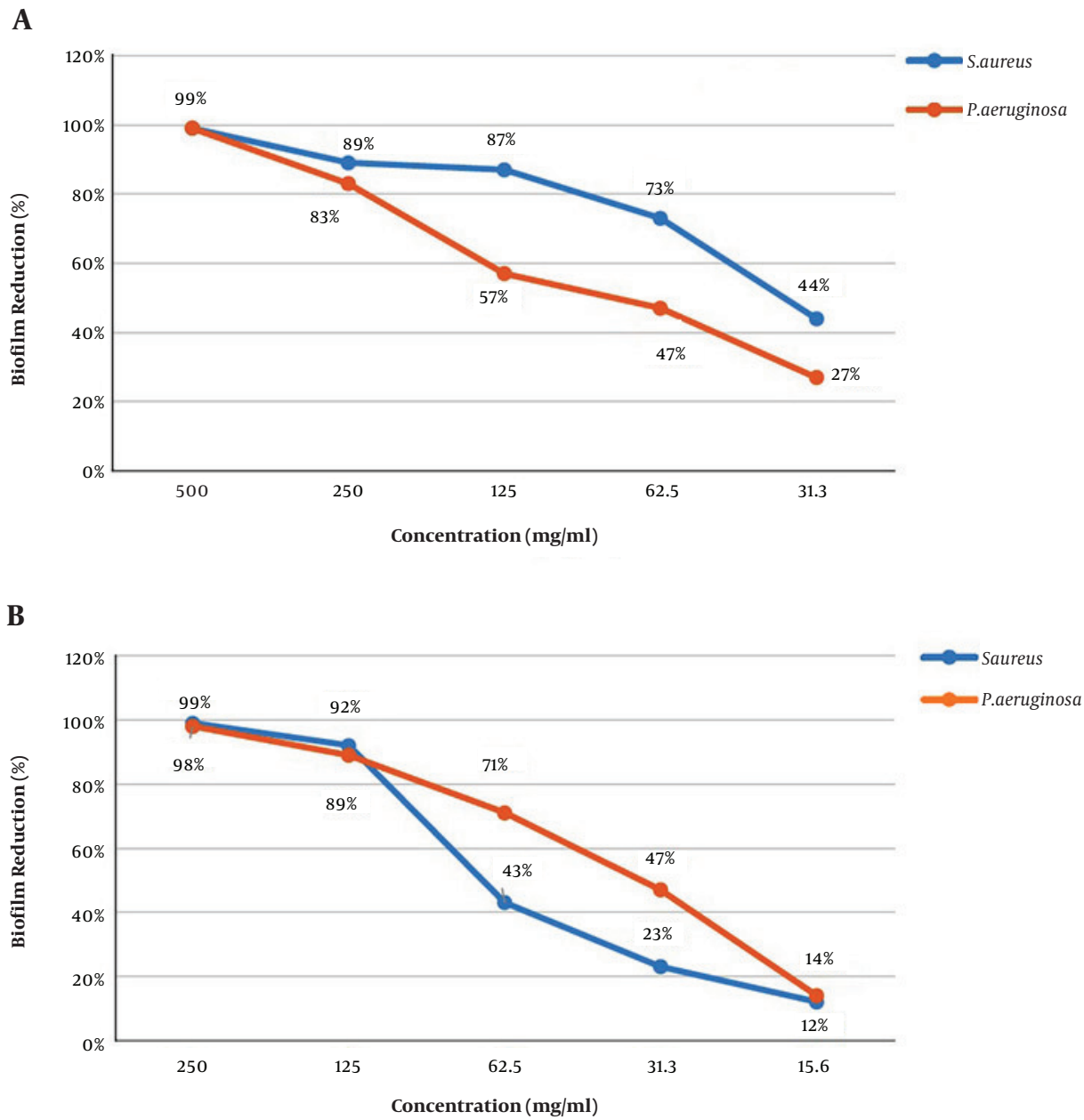

Figure 1. Reduction of biofilm formation of *U. maritima* bulbs against *S. aureus* and *P. aeruginosa* (A: methanol extract, B: acetone extract).

According to research by Belhaddad et al., which is completely consistent with our results, the bulb of *U. maritima* contains wide range of phytochemical constituents such as flavonoids, glycosides, tannins, reducing compounds, triterpenes, and steroids, which exhibited antioxidant and antibacterial activity (6).

Results have shown that methanol and acetone extracts of *U. maritima* bulbs have considerable anti-biofilm properties on standard and clinical strains of *S. aureus* and *P. aeruginosa*, and by increasing the density of the extracts, the rate of decreasing biofilm production would increase. According to the results, the anti-biofilm effect of methanol extract of *U. maritima* bulbs on standard strains of *S. aureus* (ATCC: 9144) is more than that in the bulbs acetone extract, while the anti-biofilm effect of the bulbs acetone extract is more than that in the bulbs methanol

extract in the standard strains of *S. aureus* (ATCC: 25923). Comparison of the antimicrobial effect and anti-biofilm effect of *U. maritima* bulb extracts show that the antimicrobial effect of acetone extract is more than that in the methanol extract in general, in the standard strains of *S. aureus* and *P. aeruginosa*, while the anti-biofilm effect of methanol extract is more than that in the acetone extract. The antimicrobial effects of methanol and acetone of the bulbs are similar in clinical strains of *S. aureus*, while, the anti-biofilm effect of the bulbs acetone extract is more than that in the bulbs of methanol extract. The antimicrobial and anti-biofilm effects of acetone extract of the bulbs are more than that in the of bulbs methanol extract, and it can be said that the antimicrobial effect of the methanol extract is negligible.

5.1. Conclusions

Our results showed that the methanol and acetone extracts of the bulbs of *U. maritima* species have potential antibacterial and anti-biofilm properties. It is necessary to have more extensive analyses in vitro conditions, for the effective densities of the essences and extracts against the considered bacteria and other bacteria, cytotoxic effects, as well as their precise formulations to be evaluated, and investigations to be done in vivo.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

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