



# Evaluation of the Antibacterial Effect of Ethanol and Ethyl Acetate Extracts of *Myrtus communis* Against Antibiotic-Resistant *Pseudomonas aeruginosa*

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## Abstract

**Background:** *Pseudomonas aeruginosa* is a human and plant pathogen. The aim of this study was to investigate the antibacterial effects of ethanol and ethyl acetate extracts of *Myrtus communis* against antibiotic-resistant *P. aeruginosa* isolates.

**Methods:** The plant was collected from the plains of Kerman province, and its ethanol and ethyl acetate extracts were prepared using a rotary machine. *Pseudomonas aeruginosa* strains were isolated from urine and blood samples of patients in Zabol, Iran. Antibiotic resistance pattern was determined by the agar diffusion method. Finally, the minimum inhibitory concentration (MIC) and minimum trace concentration (MTC) were determined by the microdilution method.

**Results:** The antibiotic-resistance patterns of the standard and clinical strains showed that *P. aeruginosa* was resistant to all antibiotics at the following rates: Azithromycin (25%), ampicillin (12.5%), gentamycin (0%), amoxi-clav (12.5%), cefazolin (12%), and amikacin (12.5%). The study of the effect of ethanol extract on clinical and standard *P. aeruginosa* strains showed that the MIC of the ethanolic extract against the standard strain of *P. aeruginosa* was 25 ppm.

**Conclusions:** The results of this study showed good antimicrobial effects of the plant extracts against antibiotic-resistant *P. aeruginosa*, which can be used to treat *Pseudomonas* infections.

**Keywords:** *Pseudomonas aeruginosa*, Antibiotic Resistance, Zabol, Ethanol Extract, *Myrtus communis*

## 1. Background

The use of medicinal plants in the treatment of diseases has been steadily growing in recent years. The excessive use of chemical drugs for the treatment of diseases has led to the increasing emergence of microbial-resistant isolates (1), leading researchers to attempt to find new antimicrobial agents.

Plants and their essential oils and extracts can be potential substitutes for chemical drugs (2). The side effects of these compounds are lower compared to chemical drugs (3). Studies showed that most essential oils produced from plants have insecticidal, antifungal, antiparasitic, antibacterial, antiviral and antioxidant activities (4).

*Myrtus communis* is an evergreen shrub that is native to the Mediterranean and Asian countries, including Iran. This plant is grown as an ornamental plant due to its evergreen leaves and beautiful flowers. Since the ancient times, essential oil of this plant has been used as an antiseptic (5).

It is used locally as an astringent and for the treatment

of respiratory and urinary tract diseases.

*Pseudomonas aeruginosa* is a Gram-negative and pathogenic bacterium and is a major contributor to severe infections in burns patients (6). *Pseudomonas aeruginosa* is also a plant pathogen. *Pseudomonas aeruginosa* produces a green fluorescent colorant with a smell of amyl alcohol. Due to its specific internal systems, including its specific internal system and the antibiotic resistance transfer system, the bacterium becomes rapidly resistant to various antibiotics and causes the spread of septicemia in the patient's body (7).

## 2. Objectives

The aim of this study was to investigate the antimicrobial effects of the ethanolic and ethyl acetate extracts of *M. communis* against antibiotic-resistant *P. aeruginosa* isolates.

### 3. Methods

#### 3.1. Plant Materials

The plant was collected from the city of Kerman and dried. To prepare the ethanol and ethyl acetate extracts, 10 g of dried powder was placed inside half-liter erlens containing 100 mL of 96% ethanol (to prepare ethanolic extract). The contents of the erlens were stirred at room temperature for 24 hours by means of a Pars Azma machine (Iran) at 130 rpm, and then filtered with Watten No. 2 paper. Solvent separation from the extract was carried out by a rotary machine (Heidolph, Germany) and by using a vacuum pump (vacuum distillation). The extract was weighed and then solved in dimethyl sulfoxide (DMSO) solvent. The extract was stored in a refrigerator at 4°C until use for antimicrobial experiments.

#### 3.2. Isolation of *Pseudomonas aeruginosa*

Eight strains of *P. aeruginosa* were isolated from blood and urine specimens of hospitalized patients in Zabol city. For the identification of *P. aeruginosa*, the G-Catalase-Oxidase staining, the triple sugar iron agar (TSI) and oxidative-fermentative (OF) tests were used and antibiotic resistance patterns were determined using the Kirby-Bauer method (8).

#### 3.3. Bacterial Strains and Culture Condition

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

#### 3.4. Determining the Sensitivity of Bacteria to Conventional Antibiotics

The susceptibility rates of the eight bacterial strains to azithromycin (AZM), ampicillin (AM), gentamycin (GM), amoxi-clav (AMC), cefazolin (CZ), and amikacin (AN) antibiotics (antibody medicine, Iran) were evaluated using the standard Kirby-Bauer test. To this end, all the bacterial strains were prepared at a concentration of 0.5 McFarland in a Mueller-Hinton broiler medium and cultured on Mueller-Hinton agar. Antibiotic discs were placed at a proper distance from each other. The plates were incubated for 24 h at 37°C and the diameters of the inhibitory halos were measured to determine the resistance and sensitivity of the strains to the antibiotic drugs.

#### 3.5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Susceptibility of bacterial isolates to the plant extracts was determined using the serial dilution method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). All tests were performed in Mueller Hinton Broth supplemented with Tween 80 at a final concentration of 0.5% (v/v). Briefly, serial doubling dilutions of the extract were prepared in a 96-well microtiter plate ranged from 6.25 ppm to 100 ppm. To each well, 10 µL of indicator solution (prepared by dissolving a 10 mg extract in 2 mL of DMSO) and 10 µL of Mueller Hinton Broth were added. Finally, 10 µL of bacterial suspension ( $10^6$  CFU/mL) was added to each well to achieve a concentration of  $10^4$  CFU/mL. The plates were wrapped loosely with cling film to ensure that the bacteria did not get dehydrated. The plates were prepared in triplicates, and then they were placed in an incubator at 37°C for 18 - 24 hours. The color change was then assessed visually. The lowest concentration at which the color change occurred was taken as the MIC value. The average of 3 values was calculated providing the MIC and MBC values for the tested extract. The MIC is defined as the lowest concentration of the extract at which the microorganisms does not demonstrate the visible growth. The microorganisms growth was indicated by turbidity. The MBC was defined as the lowest concentration of the extract at which the incubated microorganisms was completely killed. ELISA test was performed for absorbance study.

To prepare the disks of ethanol and ethyl acetate extracts, the extracts were inoculated onto 25 blank discs at a concentration of 25 µL. The disks were placed in an incubator at 37°C for 1 hour to dry. Then, the effects of the disks containing the ethanol and ethyl acetate extracts against *P. aeruginosa* strains were investigated.

#### 3.6. Statistical Analyses

Growth was compared in each experiment using repeated measures analysis of variance (ANOVA) in SPSS version 16.0. P value less than 0.01 was considered significant.

### 4. Results

The results of antibiotic resistance patterns of the standard and clinical strains showed that *P. aeruginosa* was resistant to all antibiotics at the following rates: AZM (25%), AM (12.5%), GM (0%), AMC (12.5%), CZ (12.5%), and AN (12.5%) (Table 1).

The results of this study showed that the lowest MIC of the ethanolic extract was 6.25 ppm, two of the strains were inhibited at this concentration, while the highest MIC was

**Table 1.** Susceptibility Rates of *Pseudomonas aeruginosa* Strains to Antibiotics

	AZM	Am	Gm	AMC	CZ	AN
S	37.5	12.5	50	25	25	25
I	37.5	75	50	6.25	6.25	6.25
R	25	12.5	0	12.5	12.5	12.5

Abbreviations: AM, ampicillin; AMC, amoxi-clav; AN, amikacin; AZM, azithromycin; CZ, cefazolin; GM, gentamycin.

**Table 2.** Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Ethanol and Ethyl Acetate Extracts on Standard and Clinical *Pseudomonas aeruginosa* Strains (ppm)

Bacterium Sample	MIC/MBC Ethanol Extract	MIC/MBC Ethyl Acetate Extract
1	6.25/12.5	25/50
2	25/50	25/50
3	6.25/12.5	12.5/25
4	12.5/25	25/50
5	25/50	12.5/25
6	12.5/25	12.5/25
7	12.5/25	12.5/25
8	12.5/25	12.5/25
Standard sample	25/50	50/100

**Table 3.** The Absorption Rate of Each Well in Different Concentrations of the Ethanol Extract (ppm)

Bacterium	Concentrations (ppm)					
	100	50	25	12.5	6.25	3.1
1	0.176	0.183	0.214	0.411	0.434	0.828
2	0.156	0.172	0.177	0.391	0.672	1.029
3	0.192	0.200	0.206	0.421	0.602	0.760
4	0.163	0.175	0.183	0.390	0.543	0.821
5	0.153	0.178	0.203	0.422	0.575	1.015
6	0.143	0.157	0.204	0.441	0.474	0.850
7	0.154	0.200	0.209	0.412	0.678	0.784
8	0.194	0.219	0.336	0.380	0.656	0.807
<i>Pseudomonas</i> standard sample	0.172	0.215	0.305	0.513	0.562	0.580

25 ppm, three of the strains were inhibited at this concentration. The highest MBC value of the ethanolic extract was 50 ppm, which completely eliminated three strains (Table 2).

The lowest MIC of the ethyl acetate extract against *P. aeruginosa* was 12.5 ppm, which inhibited five strains and the highest MIC value was 50 ppm, which inhibited one strain. The highest MBC value of the ethyl acetate extract was 100 ppm, completely eliminating one strain (Table 3).

The results of the absorption study by ELISA showed that by increasing optical density, absorption decreased, that is, the rate of turbidity of the bacterial growth was reduced (Tables 4 and 5).

The results of this study showed that the lowest inhibitory halo diameter at 25 ppm of ethanolic extract was 1 mm, while the lowest inhibitory zone diameters at 50 and

100 ppm were 2 and 6 mm, respectively. The greatest inhibitory halo diameter at the 100-ppm concentration of ethanolic extract was 24 mm (Table 5).

The inhibitory zone diameter of the ethyl acetate extract at the concentration of 25 ppm was 1 mm, and five bacterial strains had this inhibitory halo diameter, while the lowest inhibitory halo diameters at the 50 and 100 ppm concentrations were equal to 4 and 8 mm, respectively. The largest inhibitory halo diameter at 100 ppm concentration was 18 mm (Table 6).

## 5. Discussion

The results of this study showed that the lowest MIC value of the ethanolic extract was 6.25 ppm, which inhibited two stains, while its highest MIC value was 25 ppm, inhibiting three bacterial strains. The highest MBC value of the ethanolic extract was 50 ppm, which completely eliminated three strains.

The lowest MIC value of the ethyl acetate extract against *P. aeruginosa* was 12.5 ppm, inhibiting five bacterial strains and the highest MIC value was 50 ppm, where one strain was inhibited. The highest MBC value of the ethyl acetate extract was 100 ppm, which completely eliminated one strain.

In a study by Saeedi et al., the results showed that the MIC of *Myrtus communis* L. extract was 5 mg/mL, while the minimum trace concentration (MTC) of the extract was 10 mg/mL (9).

The results reported by Salvagnini et al. revealed that the ethanolic extract of *M. communis* had antimicrobial activity against *Staphylococcus aureus* (10). The methanolic and ethanolic extracts of leaves of *Myrtus communis* had antimicrobial activity against *Listeria monocytogenes*, *P. aeruginosa* and *S. aureus*, and the inhibition zone diameters of the ethanolic extract for these bacteria were 30, 23 and 37 mm, respectively, and the MTC for *S. aureus* was less than 0.75 mg/mL (11). In a previous study in Iran, the positive effect of the alcoholic extract of *Myrtus communis* on *Escherichia coli* was reported. Also, according to Ghasemi et al., methanolic extract of *M. communis* showed a significant effect on intrusive propagation activity, but the MBC for *E. coli* was higher than 10 mg/mL (12, 13).

In a study of Bouzabata, the results showed that compositions of *M. communis* were  $\alpha$ -pinene, linalool, and linalyl acetate (14).

Ben Hsouna et al. evaluated the in vitro antibacterial and antifungal properties of the *M. communis* essential oil. They showed that of the inhibition zones and MICs of the plant's essential oil were within the ranges of 16 - 28 mm and 0.078 - 2.5 mg/mL, respectively (15).

**Table 4.** The Absorbance of Each Well in Different Concentrations of Ethyl Acetate (ppm)

Bacterium	Concentrations (ppm)					
	100	50	25	12.5	6.25	3.1
1	0.171	0.185	0.210	0.356	0.761	0.873
2	0.099	0.177	0.194	0.509	0.827	0.843
3	0.160	0.179	0.186	0.558	0.563	0.627
4	0.200	0.223	0.389	0.743	0.809	0.883
5	0.177	0.186	0.188	0.801	0.824	0.927
6	0.174	0.179	0.179	0.692	0.917	0.934
7	0.177	0.197	0.200	0.584	0.748	0.828
8	0.186	0.212	0.213	0.676	0.826	0.850
<i>Pseudomonas aeruginosa</i> standard sample	0.172	0.175	0.585	0.796	0.825	1.040

**Table 5.** Inhibition Range of Different Concentrations of Ethanolic Extract of the Plant (ppm)

Bacterium Sample	Concentrations (ppm)		
	100	50	25
1	9	2	0
2	24	10	4
3	6	2	0
4	15	6	0
5	24	8	2
6	21	6	1
7	14	8	0
8	12	5	3

**Table 6.** Inhibition Range of Different Concentrations of the Ethyl Acetate Extract (ppm)

Bacterium Sample	Concentrations (ppm)		
	100	50	25
1	15	4	1
2	18	8	1
3	3	0	0
4	9	4	1
5	15	6	1
6	8	8	1
7	10	8	3
8	12	5	3

Barac explored the antifungal activity of *M. communis* essential oil against *Malassezia* spp. isolated from the skin of patients with pityriasis versicolor. The results showed the antimicrobial activity of *M. communis* essential oil against of *Malassezia* (16).

In a study by Anwar, *M. communis* essential oil was assessed for its antimicrobial activity, the results demonstrated the antibacterial and antifungal activities of this essential oil against *Bacillus subtilis*, *S. aureus* and *Candida albicans*. The oil moderately reduced the radical diphenylpicryl-hydrazyl ( $IC_{50} = 4.2 \mu\text{L/mL}$  or  $4.1 \text{ mg/mL}$ ) (17).

Aleksic determined antimicrobial activity of *M. communis* L. essential oil against multidrug resistant (MDR) *A. baumannii* isolated from infected wound (18).

The study of Pirbalouti revealed that the essential oil

of *M. communis* had strong antibacterial activity against *E. rhusiopathiae*. The inhibition zones and MIC values for the bacteria that were sensitive to *M. communis* essential oil were within the ranges of 14.7 - 27.0 mm and 0.031 - 0.25 mg/mL, respectively (19).

In the study *P. aeruginosa* were resistant to all antibiotics at the following rates: AZM (25%), Am (12.5%), GM (0%), AMC (12.5%), CZ (12.5%), and AN (12.5%).

Mardaneh et al. (20) conducted a study on 111 *P. aeruginosa* strains isolated from hospitalized patients. Clinical specimens were cultured on microbiological media. Subsequently, drug susceptibility test was performed using the disc diffusion method according to CLSI recommendations. Their results indicated that most *P. aeruginosa* strains were from wound specimens (48.6%). In antimicrobial susceptibility testing, colistin exhibited the greatest anti-*Pseudomonas* activity (78.3%). Isolates demonstrated resistance to beta-lactam antimicrobials such as antipseudomonal penicillins, including piperacillin and carbenicillin.

Mohageri (21) examined antibiotic susceptibility and resistance of *Pseudomonas aeruginosa* strains in Kerman-shah, Iran. The results showed that resistance was 38% to amikacin, 72% to carbonyl, 50% to ceftazidime, 38% to ciprofloxacin, 52% to gentamicin, 100% to imipenem, 98% to mesosylin, 90% to ticarsillin, and 46% to tobramycin.

Ruiz-Roldán et al. reported low antimicrobial resistance levels as follows: Ceftazidime (8%), cefepime (7%), aztreonam (7%), gentamicin (3%), ciprofloxacin (1%), and imipenem (1%). Four MDR strains were found in that study (22).

Igbalajobi et al. investigated the prevalence of acquired MDR of *P. aeruginosa* among clinical samples obtained from patients attending Ekiti State University Teaching Hospital, Ado Ekiti, Ekiti State, Nigeria. The results show that 80.95% of the isolates were resistant to ceftriaxone and ceftizoxime, 76.2% to augmentin, 73.8% to ceftazidime, 71.4% to nitrofurantoin, 47.6% to ofloxacin, 45.23% to gentamicin. The lowest resistance was to

ciprofloxacin (42.86%) (23).

### 5.1. Conclusions

The results showed that the antimicrobial effects of the extracts were enhanced by increasing concentration.

### Footnotes

**Authors' Contribution:** All authors had equal role in study design, work, statistical analysis and manuscript writing.

**Conflict of Interests:** The authors declare no conflict of interest.

**Ethical Considerations:** The code of ethics was P10-95-734. The written informed consent was obtained.

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