

# The Synergistic Effect of *Zataria multiflora* Extract on the Reduction of Ciprofloxacin Antibiotic Resistance through the Efflux Pump in *Pseudomonas aeruginosa*

## Abstract

**Background and Objective:** The huge costs of *Pseudomonas aeruginosa* strains' antibiotic resistance, especially in immunocompromised are very important. The plant extracts such as the *Zataria multiflora* with their compounds can have antibacterial effects against most *Pseudomonas* group bacteria and reduce their resistance. The current study aimed to investigate the synergetic effect of *Zataria multiflora* extract on reducing the resistance to Ciprofloxacin antibiotic in the *Pseudomonas aeruginosa*

**Method:** The clinical strains have been collected and Province and identified after purification by biochemical methods. Evaluation of the *Pseudomonas aeruginosa* resistance to ciprofloxacin was determined the presence and activity of the efflux pump in the bacteria in line with the antibiotic resistance.

**Findings:** It can be concluded from the current study that zattaria multiflura extract alone has an anti-microbial effect. Based on the results, it can be said that the ciprofloxacin-resistant *Pseudomonas aeruginosa* strains showed an acceptable reduction in drug resistance when the antibiotic was used simultaneously with the *Zataria multiflora*.

**Conclusion:** Based on the results, it can be said that the *Zataria multiflora* extract alone can have an anti-microbial effect. when used simultaneously with the ciprofloxacin, it can increase the bacteria's sensitivity to the antibiotic by affecting the function in the *Pseudomonas aeruginosa*, reducing the treatment duration, and decreasing the number of antibiotics needed for treatment significantly.

**Keywords:** *Pseudomonas aeruginosae*, *efflux pump*, *ciprofloxacin*, *Zataria multiflora*

Ahmad Saheb Al-Zamani1\*, Maryam Sadrnia1,2\*, Majid Akbari1,3\*\*, Sasan Saki4

1Department of Biology, Arak Branch, Islamic Azad University, Arak, Iran. sahabzamaniahmad@gmail.com

2Department of Biology, Payame Noor University (PNU), Tehran, Iran. msadrnia@pnu.ac.ir and Department of Biology, Arak Branch, Islamic Azad University, Arak, Iran And

3Infectious Diseases Research Center (IDRC), Arak University of Medical Sciences, Arak, Iran. majakbari@yahoo.com

4Department of Medical Laboratory Sciences, Arak Branch, Islamic Azad University, Arak, Iran

\*\*Corresponding author: Department of Biology, Arak Branch, Islamic Azad University, Arak, Iran And Infectious Diseases Research Center (IDRC), Arak University of Medical Sciences, Arak, Iran. majakbari@yahoo.com

Department of Biology, Arak Branch, Islamic Azad University, Arak, Iran .

\*The first two authors contributed to this study and have equal role

## Introduction

The spread of infectious diseases and consequent increase in drug resistance is one of the most serious threats to successful healing and society's health. Currently, *Pseudomonas aeruginosa* plays an important role in different infections worldwide, such as hospital-acquired infections (1). This bacterium has been found in many environments, grows in aerobic conditions, and causes disease with different virulence factors (2). *Pseudomonas* is one of the problematic bacteria causing septicemia, pneumonia, endocarditis, and skin, ear, and eye infections and is considered a very serious threat to hospitalized patients in hospitals around the world. Treatment of infections caused by *Pseudomonas aeruginosae* in patients with severe burns is very difficult since this bacterium is highly resistant to most common antibiotics(3). This bacterium plays a very important role in chronic lung infections in cystic fibrosis patients hospitalized in burn and intensive care units.

This bacterium is intrinsically resistant to many antibiotics because of the low permeability of the outer membrane and the efflux pump, and the secretion of enzymes that inactivate anti-microbial agents, making it very difficult to treat(4).

Fluoroquinolones, especially ciprofloxacin, is one of the widespread medications for treating *Pseudomonas aeruginosa*, which has faced concerns with the emergence of resistant

strains(4). One of the ways to confront the phenomenon of antibiotic resistance is to use plant extracts along with antibiotics. Also, the advantage of using plant extract is fewer side effects and the reduced possibility of resistance to these compounds. Many researchers have investigated the therapeutic and efficient effects of medicinal plants and their derivatives on bacteria(5).

Thyme is a type of mint plant of the Lamiales family and includes three genera; *Zataria*, a species that exists in the south of Iran and is known as Shirazi or broad-leaved thyme, the second type of the *Zizyphora* genus, of which the narrow-leaved thyme is a species, and the *Thymus* genus, which are very diverse, with 27 species of it reported among which 24 belong to Iran. Among the compounds found in the thyme, is phenolic compounds. Carvacrol and thymol are derivatives of Shirazi thyme plant extract, which by disrupting the efficiency of the efflux pump in *Pseudomonas aeruginosa* bacteria, reduce the bacteria's resistance to some antibiotics such as quinolones and Ciprofloxacin(6).

The current study aimed to answer the following question: Is the Shirazi thyme plant extract effective in reducing ciprofloxacin antibiotic resistance in *Pseudomonas aeruginosa* by the reduction in the efflux pump function?

## Methods and Materials:

### Collection of *Pseudomonas aeruginosa* Isolates:

In this descriptive-analytical study, one hundred and eight strains of *Pseudomonas aeruginosa* were collected from different medical centers in Markazi Province, including the clinical laboratories of different hospital departments and the respiratory samples and various clinical sources, from February 2019 to August 2020. The samples were prepared in pure form and identified by biochemical methods. Bacteria collected were cultured, colored, and heated in Mueller Hinton agar and blood agar media. During the biochemical evaluation, including fermentation of sugars in TSI and OF media and growth at 24- and 42-degrees Simmons citrate, the urease positive, indole negative, pigment pyocyanin, and oxidase positive were identified and isolated(7). They were kept in Luria Bertani Broth (LB) medium with the short-time method(8).

#### **Preparation of *Zataria Multiflora* and Extract:**

Shirazi thyme is obtained from reliable sources and is used after the approval of Arak University Medicinal Plant Research Center(8). The plant is washed twice with ordinary water and dried at a temperature of about 23 degrees. After drying, it was powdered by a crusher, and extraction was done using the obtained powder and double-distilled water. 330 cc of water is added to every 30 grams of the plant. In the end, a reddish-brown liquid with a pleasant and pungent smell is obtained, which is determined by gas chromatography of the compounds(9). Carvacrol and thymol paracymentene are the most important components of the methanolic extract of Shirazi thyme. *Zataria Multiflora* extracts prepared for use were used to affect *Pseudomonas* strains(10).

#### **Method:**

*Pseudomonas aeruginosa*, confirmed by the Kirby-Bauer method, is tested for susceptibility using the CLSI2020 standards by preparing McFarland turbidity concerning ciprofloxacin disc (5 µg per unit) antibiotic (Padtan Teb Co.)(11) in Mueller Hinton agar plates in the form of disc diffusion and incubation for 24 hours at a temperature of 35 degrees, and to ensure the results of the antibiogram, the standard strain *Pseudomonas Aeruginosa* ATCC 27853 was used as an indicator(12).

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growth at 24- and 42-degrees Simmons citrate, the urease positive, indole negative, pigment pyocyanin, and oxidase positive were identified and isolated(7). They were kept in Luria Bertani Broth (LB) medium with the short-time method(8).

*Zataria multiflora* is obtained from reliable sources and is used after the approval of Arak University Medicinal Plant Research Center(8). The plant is washed twice with ordinary water and dried at a temperature of about 23 degrees. After drying, it was powdered by a mill, and extraction was done using the obtained powder and double-distilled water. 330 cc of water is added to every 30 grams of the plant. In the end, a reddish-brown liquid with a pleasant and pungent smell is obtained, which is determined by gas chromatography of the compounds(9). Carvacrol and thymol paracymentene were the most important components of the methanolic extract of *Zataria multiflora*. *Zataria Multiflora* extracts prepared for use were used to affect *Pseudomonas* strains(10).

For drug sensitivity testing, the discs diffusion method recommended by the standard CLSI 2020, M100, 30 ed., used. In this method, the overnight culture of bacteria was suspended in physiological serum with a turbidity of 0.5 McFarland, and an antibiogram test was performed using Mueller Hinton agar medium and 5 µg Spirofloxacine disc (Padtan Teb Co.). Minimum inhibitor concentration for selected bacteria, by the microbroth dilution method in 96-well microtiter plates and with medium CAMHA and based on the standard CLSI 2018, M07, 11 th ed. Done. And then Minimum Bactericidal Concentration (MBC) test by using MTT was done. All experiments were performed in duplicate and with the quality control strain(*Pseudomonas aeruginosa* 25873)(11 & 12). To determine the minimum inhibitory concentration of thyme, the method recommended by Saidi et al. (16) was used.)

#### **Findings:**

In the test performed, Kirby-Bauer and disk diffusion methods evaluated the resistance of one hundred and eight *Pseudomonas aeruginosa* to ciprofloxacin. And based on the CLSI2020 tables, if the created zone of inhibition is less than 18 mm, the bacteria are resistant, and if it is above 25 mm(12), it is reported as susceptible. If the zone of inhibition is between these two values, the bacteria are intermediate and based on this

Among the one hundred and eight strains identified, 7.3% are intermediate, 77.1% are susceptible, and 14.7% are resistant to 5µg ciprofloxacin.

Overall results are presented in the following table:

psudo	Disk	psudo	Disk	psudo	Disk	psudo	Disk	psudo	Disk
1	15	20	8	40	33.00	61	58.00	81	45.00
2	35.00	21	39.00	41	39.00	62	70.00	82	52.00
3	26.00	22	35.00	42	37.00	63	45.00	83	33.00
4	25.00	23	33.00	43	20.00	64	11	84	32.00
5	40.00	24	9	44	25.00	65	29.00	85	11
6	10	25	19	45	45.00	66	20	86	56.00
7	33.00	26	30.00	46	26.00	67	18	87	18.00
8	22	27	44.00	47	24.00	68	6	88	42.00
9	50.00	28	56.00	48	30.00	69	25.00	89	29.00
10	35.00	29	4	49	33.00	70	30.00	90	20.00
11	33.00	30	24.00	50	3	71	39.00	91	27.00
12	19	31	26.00	51	35.00	72	40.00	92	26.00
13	27.00	32	42.00	52	46.00	73	19	93	25.00
14	30.00	33	36.00	53	5	74	38.00	94	23.00
15	8	34	33.00	54	46.00	75	32.00	95	22.00
16	39.00	35	45.00	55	26.00	76	39.00	96	42.00
17	35.00	36	55.00	56	30.00	77	41.00	97	29.00
18	40.00	37	48.00	57	18	78	36.00	98	30.00
19	33.00	38	41.00	58	30.00	79	37.00	99	7
20	8	39	5	59	33.00	80	60.00	100	30.00
				60	29.00	81	45.00	101	46.00
								102	8
								103	41.00

**Table 1** Over all disk diffiotion result

the results obtained from the SPSS are as follows:

Susceptible to Ciprofloxacin

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1	.9	.9	.9
I	8	7.3	7.3	8.3
R	16	14.7	14.7	22.9
S	84	77.1	77.1	100.0
Total	109	100.0	100.0	

**Table 2** susceptible to ciprofloxacin by SPSS

$$volum(ml) = \frac{Weight(mg) \times Potency(\frac{\mu g}{mg})}{Concentration(\frac{\mu g}{mg})}$$

Based on the CLSI2020 tables, the zone of inhibition in terms of *Pseudomonas aeruginosa*'s resistance to ciprofloxacin is resistant for  $\leq 18$ mm, susceptible for  $\geq 25$ mm, and intermediate for values between 18 and 25mm. Eighty-five strains were susceptible, and 15 were resistant. Then, the resistant strains' MIC was evaluated by the macro and microdilution methods. The results are presented in the table(12).

To perform MIC, first, ten times the concentration of ciprofloxacin was prepared according to the formula:

Then, using the formula  $n_1v_1 = n_2v_2$ , the required concentration and serial dilutions in sterile 96 plates were prepared, and the results were compared according to the table below.

**Table 2- *Pseudomonas aeruginosa*'s MIC and MBC to ciprofloxacin**

Resistant strains	Zone of inhibition created in disk diffusion	Sensitivity per CLSI2020	Ciprofloxacin MBC	Ciprofloxacin MIC
Strain No.1	15mm	R	128	64
Strain No.2	10mm	R	64	32
Strain No.3	22mm	I	1	0.5
Strain No.4	18mm	R	8	4
Strain No.5	11mm	R	2	1
Strain No.6	8mm	R	512	256
Strain No.7	20mm	I	4	2
Strain No.8	6mm	R	256	128
Strain No.9	5mm	R	512	256
ATCC27853	27mm	S	0.5	0.25
Strain No.10	19mm	I	1	0.5

To evaluate the *Pseudomonas aeruginosa*'s sensitivity to the concentrations of Zattaria thyme(15), the concentration of the effective material in the extract is determined, and ten

times the concentration is prepared according to the formula. Then, it was performed for the effects of the extract on the pseudomonas MIC(16). The results are as follows :

Table 3: *Pseudomonas aeruginosa*'s MIC to Zataria multiflor

Resistant strains of pseudomonas aeruginosae	Zattaria multiflora extract MBC	Zattaria multiflora extract MIC
Strain No.1	64	32
Strain No.2	512	256
Strain No.3	16	8
Strain No.4	16	8
Strain No.5	128	64
Strain No.6	64	32
Strain No.7	16	8
Strain No.8	512	256
ATCC27853	32	16
Strain No.9	1024	512
Strain No.10	128	64

**Discussion:**

Today, *Pseudomonas aeruginosa* plays an important

Today, *Pseudomonas aeruginosa* plays an important role in hospital infections, and quinolones are one of the important

antibiotics in treating this bacterium. Regarding the increasing emergence of resistance to this series of antibiotics by *Pseudomonas aeruginosa*, finding effective methods to treat and reduce bacterial resistance is very important.

The prevalence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates has increased worldwide in recent years (19). Ciprofloxacin antibiotics are used in the treatment of bacterial infections, especially *Pseudomonas aeruginosa*. The effect of this important antibiotic has decreased due to the spread of resistant bacteria, especially in the ICU department. Therefore, quick and accurate identification of resistant *Pseudomonas aeruginosa* isolates and identification of resistance mechanisms to these antibiotics in the ICU department is necessary for proper treatment and prevention of the spread of these isolates (20).

In the studies by Sharifi Far et al. in 2014, the ethanolic extract of Oak Leaf has more anti-microbial activity than its aqueous extract, which may be due to various organic compounds that can pass through the filter more often in such solvents (32).

These findings are consistent with those of the current study, and the synergistic effect is completely evident so that in strain No.2, which has a zone of inhibition of 11, it has a MIC equal to 2 µg/ml of ciprofloxacin. The MIC of thyme equals was 128 µg/ml.

In the research by Khalkhali in 2015, the mutual interaction of the thyme and antibiotic was used to test the synergistic effect. As a result, the minimum inhibitory and lethal concentration of fosfomycin was 64 µg/ml, and the minimum lethal concentration of thyme essential oil was 5%. They showed that in the treatment of PAO1 strain with all doses of fosfomycin, there is a significant difference in the amount of biofilm production compared to the case without treatment. The use of thyme essential oil and fosfomycin antibiotic, especially in lower concentrations than fosfomycin antibiotic, is a promising method to control and prevent biofilm production by PAO1 strain (39).

In the present study it was observed that the ciprofloxacin-resistant *Pseudomonas* strain has a MIC value of 128 mg/cc. The results indicated that the thyme extract has an anti-microbial effect on bacteria. It was shown that a significant reduction in drug resistance, and resistant strains have shown less resistance when exposed to the extract.

Inhibiting the efflux pump by the extract by increasing the sensitivity of the bacteria can greatly improve the effectiveness of the antibiotic and reduce the MIC of the bacterium.

Furthermore, the effect of *Zataria* extract has been shown up to a concentration of 1.16, and the resistance of the bacterium has disappeared.

Efflux pumps are very significant in creating multidrug resistance due to substrate diversity. Among Gram-negative bacteria, the MexAB-OprM efflux system is one of the important efflux pumps involved in the multidrug resistance of *Pseudomonas aeruginosa*, and it is the only efflux pump system inherent in this bacterium (21). Increased expression of the efflux pump system among clinical isolates of *Pseudomonas aeruginosa* has been reported in different reports of 57.9%-1.21% (22). Also, in the study conducted by TubA Moderris and his colleagues in Turkey in 2018, 6.47% of isolates showed increased expression of MexAB-OprM efflux (23).

In a study conducted by Ji-Young Lee and his colleagues in Korea in 2012, among 57 carbapenem-resistant isolates, 32 (56.1%) isolates increased MexAB-OprM gene expression and 54 (7.94%) isolates decreased expression. showed the OprD gene (24).

Based on the results, it can be said that *Zataria* extract has an anti-microbial effect alone; however, using it simultaneously with antibiotics that are pumped out of the cell by the efflux pump increases the efficiency of the antibiotic by affecting the activity and reducing the function of the efflux pump in bacteria.

#### **Conclusion:**

The current study results show that plant extracts contain suitable anti-microbials substances that can be used as a medicinal base or to help treat some bacteria with drug resistance, especially *Pseudomonas aeruginosa*. Based on the results, it can be said that *Zataria multiflora* extract alone has an anti-microbial effect; however, using it simultaneously with antibiotics that are pumped out of the cell by the efflux pump increases the efficiency of the antibiotic by affecting the activity and reducing the function of the efflux pump in bacteria. Medicinal extracts, while reducing the resistance of bacteria to antibiotics, do not have side effects; bacteria do not show resistance to them and can prevent the use of antibiotics. Plant extracts are available and involve less cost and processing concerning the preparation and production of antibiotics. And as a therapeutic factor when using antibiotics, especially in resistant.

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The authors declare that there are no conflicts of interest.

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Original Article  
JYOTI MEHTA\*

22-Department of Microbiology, Shoolini University of Biotechnology and Management Sciences, Bajhol 173229, Solan, Himachal Pradesh Email: yjjyoti@gmail.com , SAVITA JANDAIAK, URMILA  
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