EXPERIMENTAL PAPER

Antibacterial activity of *Zataria multiflora* essential oil and its main components against *Pseudomonas aeruginosa*

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Summary

Introduction: In Iranian traditional medicine, *Zataria multiflora* Boiss (*Lamiaceae* family) is reputed due to its antiseptic effects.

Objective: The purpose of this study was to evaluate the antibacterial and biofilm killing effects of *Z. multiflora* essential oil and main components against *Pseudomonas aeruginosa*.

Methods: The main components of essential oil were identified by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). The antibacterial properties of *Z. multiflora* oil and main components were determined by assessing the MIC and MBC values, and their inhibition percent of biofilm killing effects were determined by the evaluation of optical density. The role of each main component in these activities was determined according to the chemical profiles of essential oil.

Results: Thymol (38.7%), carvacrol (30.6%), and *p*-cymene (8.3%) were main components of twenty five components of essential oil. Carvacrol had the higher role in antibacterial activity against *P. aeruginosa*, followed by thymol. *P*-cymene enhanced the antibacterial activities of thymol and carvacrol against *P. aeruginosa*. Carvacrol showed the weak role in biofilm killing effect. In spite of the low antibacterial activity of *p*-cymene against *P. aeruginosa*, it can enhance the antibacterial activity of thymol or carvacrol.

Conclusion: *Z. multiflora* essential oil can be used for the management of *P. aeruginosa* infections. Determining the precise role of each components needs investigating in their behavior in different media.

Key words: *Zataria multiflora*, thymol, carvacrol, biofilm, *p*-cymene, pyocyanin

INTRODUCTION

Pseudomonas aeruginosa, as an opportunistic Gram-negative bacteria with high intrinsic antibiotic resistance and adaptability [1], is the major risk factor of many infections of soft tissues, urinary tract and respiratory systems, otitis externa, diabetic patients and gives rise to infections such as chronic obstructive pulmonary diseases, cystic fibrosis and bacteremia in humans [1]. The high mortality ranging from 18 to 61% is reported for *P. aeruginosa* infections [2].

Multitude of virulence factors such as flagella, type IV pili, LPS, proteases, lipase, pyocyanin, quorum sensing, alginate and forming the biofilm structure are responsible for *P. aeruginosa* infections [3]. Alginate production and biofilm formation are recognized as the most important virulence factor in *P. aeruginosa* related chronic infections. The complex structures of biofilms in *P. aeruginosa* as well as the signaling systems enable it to withstand against immune defense and antibiotics [4]. A blue green pigment of pyocyanin can interfere with some pathways such as host cell electron transport system and redox cycle in humans [1]. These mechanisms along with its intrinsic and acquired drug resistance to many classes of antibiotics make *P. aeruginosa* infections so difficult to treat [5].

Many natural or synthetic products have been subject of studies on overcoming the antibiotic resistance of *P. aeruginosa* infections [6]. Among them, essential oils with high phenolic content have exhibited the acceptable antibacterial activities against *P. aeruginosa* infections [7].

Zataria multiflora Boiss (*Lamiaceae*) is a plant with high total phenolic content and different preparations are been used in Iranian Traditional Medicine as anti-infectious agent for treatment of infections [8]. The antimicrobial activities of *Z. multiflora* essential oil was confirmed against different kinds of microorganisms (*Staphylococcus aureus*, *S. saprophyticus*, *S. epidermidis*, *Bacillus subtilis*, *B. cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, *S. flexneri*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Enterobacter aerogenes*, *Vibrio cholerae* Inaba, *Pseudomonas aeruginosa*, *Aspergillus niger*, *A. flavus*, *Candida albicans*) from vaginal tract, oral cavities, skin infections [9]. The antimicrobial activity of *Z. multiflora* essential oil against *P. aeruginosa* was the subject of some studies. The antibacterial effects of *Z. multiflora* methanol extract against IMP-type metallo-beta-lactamase

(MBL)-producing *P. aeruginosa* [10] was confirmed. The results of different studies showed that *P. aeruginosa* had been less sensitive to *Z. multiflora* essential oil than other microorganisms [11–13]. The MIC₅₀ of *Z. multiflora* essential oil was reported as 31 µg/ml against *P. aeruginosa* [11]. *Z. multiflora* essential oil with carvacrol (37%), *p*-cymene (15%), thymol (3.3%) had the inhibition zone of 10.7 mm and MIC and MBC values of 64, 128 µg/ml [13]. *Z. multiflora* essential oil had the inhibition zone diameters of 11.7±0.6 mm and MIC, MBC values of 1, 2 µl/ml [12].

The aim of this study was to evaluate the antibacterial and anti-biofilm activities of *Z. multiflora* essential oil and main components against *P. aeruginosa* in *in vitro* conditions. For the determination of the role of each main component of *Z. multiflora* essential oil, the chemical profiles of its essential oil was determined by GC and GC-MS.

MATERIAL AND METHODS

Plant material, essential oil extraction and its analysis by GC and GC-MS

Z. multiflora full flowering aerial parts were collected from Barij Essence Research Farm (Kashan, Iran) in May 2015. The identified herbarium sample was kept under voucher number 189 at the Herbarium Center of Agriculture Department, Research Center of Barij, Kashan, Iran.

For the extraction of the essential oil, dried aerial parts (100 g) of plant were subjected to hydro-distillation method by Clevenger type apparatus for 4 h. The essential oil was extracted and dried using anhydrous sodium sulfate. The essential oil was stored in tightly closed dark vial until the analysis.

The chemical composition of essential oil was identified using GC and GC/MS apparatuses by Agilent technology (HP) 6890 system, with HP-5MS capillary (60 m – 0.25 mm i.d., film thickness 0.25 µm). The oven temperature program was initiated at 40°C (1 min), then increased to 230°C (rate of 3°C/min) and held for 10 min. Helium was the carrier gas at a flow rate 1.0 ml/min. The detector and injector temperatures were 250 and 230°C, respectively. The compounds of the essential oil were identified by comparison of their retention indices (RI) and mass spectra fragmentation with those on the stored Wiley 7n.1 mass computer library, and National Institute of Standards and Technology [14].

Antibacterial evaluation of *Z. multiflora* essential oil and its main components against *P. aeruginosa*

Pseudomonas aeruginosa ATCC 27853 was used as standard bacteria in this investigation. Antibacterial evaluations were performed by micro-broth dilution assay. Thymol (T), carvacrol (C), *p*-cymene (P) (Sigma-Aldrich Co. LLC.) and *Z. multiflora* essential oil (ZM) were used for all antibacterial screenings. The quota of each component (T, C, P) and their combination forms (CP, TP, TCP, TC) in essential oil were estimated based on chemical profile of analyzed essential oil. The stock solutions of these compounds (64 µl/ml) were prepared in dimethyl sulfoxide (DMSO).

For the determination of MIC and MBC values, micro-broth dilution assay was used. The stock solutions for compounds were diluted twofold serially in Muller Hinton Broth (8–0.125 µl/ml). After shaking, 100 µl of diluted components was added separately to each well of 96-well micro titer plates. Bacterial suspension were diluted to 10⁶ CFU/ml and then 100 µl of bacterial suspension was added to each well and incubated at 37°C for 24 h. MIC values were defined as the lowest concentration of compound that inhibit the bacteria growth after 24 h. MBC values were determined using the first well showing no growth on nutrient agar [15]. The growth Inhibitory percent (I%) of *Z. multiflora* essential oil and components were determined by evaluating the OD₆₀₀ of each sub-inhibitory concentration via this equation:

$$[(OD_c - OD_w)/OD_c] \times 100 = IP\%$$

where OD_c is the OD of control, OD_w is OD of each well at sub-inhibitory concentration.

Biofilm killing effects of *Z. multiflora* essential oil and its main components

The bacterial biofilms were established by inoculating the bacterial suspension (10⁶ CFU/ml) into the wells of micro titer plates and incubating at 37°C for 24 h. After that, the culture media were removed and the wells were washed with distilled water to remove the planktonic cells. The diluted compounds (1–0.125 µl/ml) were added to wells and they were incubated at 37°C for 24 h, again. The biofilm staining with crystal violet and estimating the biofilm killing effects was performed as below [16]. For staining the biofilms, crystal violet (0.1%) was

poured into the dried wells of plates and incubated for 10 min. The crystal violet was removed, rinsed and well dried. After inserting acetic acid (30%) into wells the biofilm killing effects of each compounds were estimated by determining the OD₅₅₀ of each well in comparison with control wells (bacterial wells without essential oil).

Statistical analysis

All experiments were performed in triplicate and means of triplicate were used for drawing all diagrams. For the determination, the role of each main component in anti-pseudomonal effect of *Z. multiflora* essential oil, the results of OD₆₀₀ related to bacterial growth in different concentration of compound were statistically analyzed by SPSS software (v. 17, Chicago, IL, USA). One-way ANOVA test was used to compare the difference between compounds and the *p*_{value} were calculated. The results were significant at level 0.05.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

The yield of essential oil was 2.3% (w/v). Twenty five components were identified in *Z. multiflora* essential oil that represents 99.7% of total oil composition. Thymol (38.7%), carvacrol (30.6%), and *p*-cymene (8.3%) were main components of essential oil, followed by *γ*-terpinene (2.8%), trans-caryophyllene (2.1%) and *β*-myrcene (1.7%), respectively (tab. 1).

In micro-broth dilution assay, *Z. multiflora* essential oil, thymol, carvacrol, thymol+carvacrol, carvacrol+*p*-cymene, thymol+*p*-cymene, and thymol+carvacrol+*p*-cymene had the same MIC and MBC values (2, 4 µl/ml) against *P. aeruginosa*. Among different compounds, only *p*-cymene had the higher MIC and MBC values than the other compounds (4, 8 µl/ml). Therefore, *p*-cymene alone as third main component of *Z. multiflora* has played a little important role in its antibacterial activity against *P. aeruginosa*. In micro-broth dilution assay, inserting the *p*-cymene into thymol, carvacrol and thymol+carvacrol did not change the MIC and MBC values of related compounds (carvacrol+*p*-cymene, thymol+*p*-cymene, and thymol+carvacrol+*p*-cymene) (tab. 2).

Table 1.Chemical composition of *Zataria multiflora* essential oil

Compound	Retention Index	Percent
α -Thujone	916	0.9
α -Pinene	925	1.2
β -Pinene	961	1.2
3-Octanone	968	0.3
β -Myrcene	980	1.7
Phellandrene	987	0.4
α -Terpinene	1001	0.6
<i>p</i>-Cymene	1013	8.3
Limonene	1017	0.5
1,8-Cineole	1023	0.9
γ -Terpinene	1050	2.8
α -Terpinolene	1066	0.2
Linalool	1077	1.3
2-mMethoxy-4-methyl-1-(1-methylethyl)-benzene	1192	1.5
Carvacrol methyl ether	1202	1.3
Thymol	1282	38.7
Carvacrol	1299	30.6
Thymol acetate	1393	1.0
Carvacryl acetate	1346	0.8
trans Caryophyllene	1392	2.1
Aromadendrene	1403	1.3
β -Selinene	1405	0.1
α -Humulene	1411	0.2
Ledene	1440	0.8
Caryophyllene oxide	1505	1.0

Table 2.Antibacterial activity of *Zataria multiflora* essential oil and main components against *Pseudomonas aeruginosa*

Components	Broth dilution assay (μ l/ml)	
	MIC	MBC
Thymol (38.7%)	2	4
Carvacrol (30.6%)	2	4
<i>p</i> -Cymene (8.3%)	4	8
Thymol, carvacrol (38.7%, 30.6%)	2	4
Thymol, <i>p</i> -cymene (38.7%:8.3%)	2	4
Carvacrol, <i>p</i> -cymene (30.6%:8.3%)	2	4
Thymol, carvacrol, <i>p</i> -cymene (38.7%:30.6%:8.7%)	2	4
<i>Z. multiflora</i> essential oil	2	4

Determining the growth inhibitory percent (I%) at sub-inhibitory concentration showed –among different compounds – that *p*-cymene with higher MIC and MBC values had the lower growth inhibitory

percent than other combinations and *Z. multiflora* essential oil and the difference was statistically significant ($p < 0.05$). Carvacrol had a significant difference with carvacrol+*p*-cymene and thymol+carvacrol+*p*-cymene ($p < 0.05$), while there was no significant difference between thymol and thymol+*p*-cymene ($p > 0.05$). In fact, *p*-cymene increased the antibacterial activity of carvacrol against *P. aeruginosa*, while this increasing effect was not observed for combination thymol+*p*-cymene in contrast to thymol alone. Also, in this graph, the growth inhibitory effects of 38% thymol (corresponding amount in essential oil) and 30% carvacrol was equal against *P. aeruginosa*. In other word, lower concentrations of carvacrol had higher growth inhibitory effects than that of thymol in *Z. multiflora* essential oil against *P. aeruginosa*. Thymol+carvacrol, thymol, and thymol+*p*-cymene had the weaker effects on bacterial growth than that of thymol+carvacrol+*p*-cymene ($p < 0.05$). It means that *p*-cymene increased the antibacterial activity of thymol+carvacrol. There was a significant difference between the antibacterial activity of *Z. multiflora* essential oil and thymol+carvacrol+*p*-cymene ($p < 0.05$). Therefore, thymol+carvacrol+*p*-cymene had the stronger antibacterial activity against bacterial growth than that of *Z. multiflora* essential oil (fig. 1).

Figure 1.

The effects of sub-inhibitory concentrations of *Zataria multiflora* essential oil and corresponding components on the growth of *Pseudomonas aeruginosa*; C – carvacrol, T – thymol, P – *p*-cymene, ZM – *Z. multiflora* essential oil

In evaluating the biofilm killing effects of *Z. multiflora* essential oil and main components were shown than thymol, *Z. multiflora* essential oil and thymol+carvacrol+*p*-cymene had the best biofilm killing effects than the others. The biofilm killing effects of *p*-cymene and carvacrol+*p*-cymene was lower than the others (fig. 2).

Figure 2.

The killing effects of *Zataria multiflora* essential oil and components on Biofilm formation; C – carvacrol, T – thymol, P – *p*-cymene, ZM – *Z. multiflora* essential oil

DISCUSSION

The antibacterial activity of *Z. multiflora* essential oil and its three main components were confirmed against *P. aeruginosa*. Although, the antibacterial activity of *Z. multiflora* essential oil against *P. aeruginosa* was the subjects of other studies [11-13] but the role of main components had not considered in other studies. The antibacterial activity of *Z. multiflora* essential oil is caused by killing effects against biofilm formation.

According to our results, the biofilm killing effects of *Z. multiflora* essential oil was related to carvacrol compound and then its thymol content. Although, enhancing the antibacterial effects of carvacrol and combination form of thymol and carvacrol in presence of *p*-cymene can't be neglected. *P*-cymene cannot considerate as an efficient antibacterial agent against *P. aeruginosa*, but it can increase the antibacterial activity of other compounds in *Z. multiflora* essential oil. The synergistic effects of *p*-cymene with carvacrol against different kinds of microorganisms have been confirmed [17, 18]. *P*-cymene with high affinity to cell membranes decreases the melting temperature of membranes [19] and perturbs the cells integrities [18].

The difference in antibacterial activities of carvacrol and thymol is maybe related to hydroxyl group position. Carvacrol, as the best antibacterial agent against *P. aeruginosa*, acts as trans membrane monovalent cations carrier of cell membranes [18, 20], and interacts with main physiological functions of bacterial cells [21, 22]. Thymol, with hydroxyl group

in Meta position had the lower antibacterial activity against *P. aeruginosa* than that of carvacrol.

Higher antibacterial activity of triple combination of thymol, carvacrol, and *p*-cymene in contrast to *Z. multiflora* essential oil showed that the other minor components of *Z. multiflora* essential oil had some role in its antibacterial activity and decreased the antibacterial activity of three main components of *Z. multiflora* essential oil.

Although, carvacrol had an important role in antibacterial activity of *Z. multiflora* essential oil against *P. aeruginosa*, but it had lower anti-biofilm activity than other components. One of probable mechanisms of this phenomenon may be non-polar structure of carvacrol and its insolubility in liquid media.

The antibacterial activity of *Z. multiflora* essential oil against *P. aeruginosa* is related to carvacrol and thymol, respectively. *P*-cymene as the third main component of this essential oil can increase the antibacterial effects of carvacrol and thymol-carvacrol combination. Due to the lower antibacterial effects of *Z. multiflora* essential oil than that of triple combination of thymol, carvacrol and *p*-cymene, the role of other minor components of essential oil in reducing the antibacterial effects of essential oil should be considered.

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Aktywność antybakteryjna olejku eterycznego z *Zataria multiflora* i jego głównych składników przeciwko *Pseudomonas aeruginosa*

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Streszczenie

Wstęp: *Zataria multiflora* Boiss (rodzina *Lamiaceae*) jest cenionym środkiem antyseptycznym w irańskiej medycynie tradycyjnej.

Cel: Celem tej pracy było określenie działania antybakteryjnego oraz niszczącego biofilm olejku eterycznego i innych podstawowych składników *Z. multiflora* przeciwko *Pseudomonas aeruginosa*.

Metody: Najważniejsze składniki olejku eterycznego zostały określone za pomocą chromatografii gazowej (GC) i chromatografii gazowej sprzężonej ze spektrometrią masową (GC-MS). Właściwości antybakteryjne olejku eterycznego i głównych składników *Z. multiflora* zostały określone przez oznaczenie wartości MIC i MBC. Efekt niszczenia biofilmu określono za pomocą gęstości optycznej. Rolę każdego z głównych składników określono zgodnie z profilami chemicznymi olejków eterycznych.

Wyniki: Olejek eteryczny *Z. multiflora* tworzy 25 składników, a głównymi są tymol (38,7%), karwakrol (30,6%) i *p*-cymen (8,3%). Karwakrol miał największy udział w działaniu antybakteryjnym przeciw *P. aeruginosa*, kolejnym był tymol. *P*-cymen wzmacniał działanie antybakteryjne tymolu i karwakrolu przeciwko *P. aeruginosa*. Karwakrol wykazywał słabe działanie przeciwko biofilmowi. Oprócz niskiego poziomu aktywności antybakteryjnej przeciwko *P. aeruginosa*, *p*-cymen może wzmacniać działanie antybakteryjne karwakrolu.

Wnioski: Olejek eteryczny z *Z. multiflora* może być stosowany w leczeniu infekcji *P. aeruginosa*. Określenie dokładnej roli każdego składnika wymaga badania ich działania na różnych podłożach.

Słowa kluczowe: *Zataria multiflora*, tymol, karwakrol, biofilm, *p*-cymen, piocyjanina