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Chemical composition and antimicrobial effect of the essential oil of *Zataria multiflora* Boiss endemic in Khorasan-Iran

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PEER REVIEW

Peer reviewer

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Comments

This study more or less confirms previous researches on *Z. multiflora* Boiss essential oil in Iran (Table 1) and shows that the endemic species in Khorasan has similar composition to other species in Iran and emphasizes on the antimicrobial effects of this essential oil. The results are interesting and suggest that the essential oil has pharmaceutical application and can be as an additive for its ability to inhibit fungi (especially *A. niger*).
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ABSTRACT

Objective: To determine the composition and antimicrobial effect of *Zataria multiflora* Boiss. essential oil in "*in vitro*" condition.
Methods: The chemical composition of the essential oil obtained by hydro-distillation was examined by GC/MS and the antimicrobial effect was studied on the growth of seven microbial species including *Bacillus cereus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Saccharomyces cereviciae*, *Candida utilis*, *Penicillium digitatum* and *Aspergillus niger* using micro-dilution method. The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration were determined.
Results: Chemical composition analysis identified a total of 43 compounds in which the main components were thymol (42.46%), carvacrol (16.85%), p-cymene (10.62%), γ-terpinene (7.26%) and α-pinene (3.00%) representing 80.19% of the total oil. Other separated components accounted for less than 19.81% of the oil. Results of antimicrobial analysis showed that *Bacillus cereus* (MIC=50 and minimum bactericidal concentration=200 μg/mL) was more resistant than two other bacterial species. Among the tested yeasts, *Saccharomyces cereviciae* (MIC=200 and minimum fungicidal concentration=1 600 μg/mL) was more resistant than *Candida utilis*, while among the fungal species, growth of *Penicillium digitatum* and *Aspergillus niger* inhibited at the same concentration of oil.
Conclusions: The results of the present study indicated that *Zataria multiflora* Boiss. essential oil had significant ($P<0.05$) antimicrobial activity.

KEYWORDS

Zataria multiflora Boiss., Antimicrobial activity, Essential oil composition

1. Introduction

One of the more alarming recent trends in infectious diseases has been the increasing frequency of antimicrobial resistance among microbial pathogens causing nosocomial and community-acquired infections[1]. In the recent years, efforts have been devoted to find new antimicrobial materials from natural resources for food preservation[2]. Reports indicated that many extracts and essential oils of edible plants had properties to prevent against a wide range of fungal contamination of foods[3-5].

Essential oils have long been applied as flavoring agents in foods, and they have shown a wide spectrum of antimicrobial activity on food borne pathogens and spoilage bacteria[6]. There are more than 1 300 plants with defined antimicrobial compounds, but characterization of preservative properties is available for only few essential oils[7].

Zataria multiflora Boiss (*Z. multiflora*), is a thyme-like plant belonging to the Lamiaceae family that geographically grows wild only in Central and Southern Iran, Pakistan and Afghanistan. This plant known as Avishan-e-Shirazi in Iran (Avishan meaning thyme in the Persian language and

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Shirazi being the name of a city in Southern Iran), is used as a flavor agent in a variety of foods in Iran, especially in yoghurt flavoring and has different effects such as anti-nociceptive, antimicrobial, spasmolytic and anti-inflammatory effects. Currently, some pharmaceutical forms of this plant, such as syrups, oral drops, soft capsules and vaginal creams are sold as treatments for various diseases. In addition to modern medicinal uses, *Z. multiflora* is still used in folk remedies[8].

Regarding to *Z. multiflora* Boiss., many studies have focused on chemical and functional characteristics of the plant purchased from local stores where the origin of the plant was unknown[9]. So the aim of present study was to evaluate the composition and potential antimicrobial activities of essential oil of another variety of *Z. multiflora* Boiss. (collected from Khorasan-Iran) on the growth of some bacteria, yeasts and fungi which had not been studied.

2. Materials and methods

2.1. Plant material and extraction of essential oil

Aerial parts of the *Z. multiflora* Boiss. plant were collected in 2013 from Khorasan-Razavi Province (the northeast of Iran). The plant was confirmed by Medicinal Plants Institute, Ferdowsi University, Mashhad, Iran. The essential oil of aerial parts of the *Z. multiflora* Boiss. was extracted with water steam distillation using a Clevenger apparatus according to the method of British Pharmacopoeia. The distilled essential oils were dried with anhydrous sodium sulfate and stored in the sterilized vial at 4 °C until use[5].

2.2. Analysis of the essential oil

The chemical composition of the essential oil was analyzed using GC-MS technique. The mass spectrometer was Agilent 6890N GC/5973 MSD-SCAN (Agilent Technologies, Palo Alto, CA, USA) in the electron impact ionization mode (70 eV) and HP-5MS (bonded and cross-linked 5% phenyl-methylpolysiloxane, 30-0.25 mm, coating thickness 0.25 mm) capillary column (Restek, Bellefonte, PA). Injector and detector temperatures were set at 220 °C. The oven temperature was held at 50 °C for 30 min, then programmed to 240 °C at rate of 3 °C/min. Helium (99.99%) was the carrier gas at a flow rate of 1 mL/min. Diluted samples (1/100 in hexane, v/v) of 1.0 were injected manually. The identification of the components was based on the comparison of their retention times and mass spectra with the data given in the literature, National Institute of Standard and Technology, Wiley and our own created library[10].

2.3. Organisms and inoculation conditions

The test organisms used in this study included *Bacillus cereus* (*B. cereus*) PTCC 1023, *Pseudomonas aeruginosa* (*P. aeruginosa*) PTCC 1310, *Proteus vulgaris* (*P. vulgaris*) PTCC 1449, two yeasts [*Saccharomyces cereviciae* (*S. cereviciae*) PTCC 24860, *Candida* (*C. utilis*) *utilis* PTCC 5052] and two fungi species [*Penicillium digitatum* (*P. digitatum*) ATCC 201167 and *Aspergillus niger* (*A. niger*) PTCC 5011] which were obtained from Persian Type Culture Collection (PTCC), Iran and American Type Culture Collection (ATCC).

To prepare microbial suspension, bacterial species were cultivated on nutrient agar (Merck, Germany) slant at 37 °C for 24 h while yeasts and fungal species were cultivated on PDA (Merck, Germany) slants and incubated at 25 °C for 48 h. Finally, suspensions were adjusted to 0.5 McFarland standard turbidity[11]. The yeasts and fungal suspensions were adjusted to make a conidial or spores concentrations of 10^6 cell or spore/mL via counting with a hemacytometer[4,12]. Bacterial suspensions were standardized to concentrations of 1.5×10^8 CFU/mL.

2.4. Minimum inhibitory concentration (MIC) test

Z. multiflora Boiss. essential oil dissolved at 5% dimethyl sulfoxide (Aplichem, Germany) and then, it diluted to the highest concentration (25 600 μg/mL), and then serial twofold dilutions were made in a concentration range from 12.5 to 6 400 μg/mL.

MIC values of essential oil against microbial strains were determined based on a Microwell dilution method. About 95 μL of Muller-Hinton broth (Merck, Germany) was dispended into each 96 wells. A total of 100 μL of stock solution of *Z. multiflora* Boiss. essential oil was added into the first well. Then 100 μL from their serial dilutions was transferred into other consecutive wells except the well number 11 as positive control. Then 5 μL of the microbial suspension was added to each well except well number 12 as negative control. Contents of each well were mixed on a plate shaker at 300 r/min for 20 seconds and then incubated at 25 °C for 48 h for yeasts and fungi and 37 °C for 24 h for bacterial strains. Microbial growth was determined by detecting the absorbance at 630 nm using the ELx808 Elisa reader (Biotek Instrument Inc, USA). The MIC of essential oil was taken as the lowest concentration that showed no growth[11].

2.5. Minimum fungicidal concentration (MFC) test

The MFC or minimum bactericidal concentration (MBC) were determined with sub-culturing 10 μL aliquot from all MIC wells showing no visible growth on the Muller-Hinton agar plates[5].

2.6. Statistical analysis

All data obtained from the trial were analyzed as a completely randomized design using the procedure of the general linear model of SPSS 19 software (SPSS Inc., Chicago, IL, USA). The mean values were compared using Duncan's new multiple range test at 5% probability level of significance.

3. Results

3.1. Chemical composition of Z. multiflora Boiss. essential oil

Chemical composition analysis of the essential oil identified

Table 1
The main components of *Z. multiflora* essential oil in other studies.

Main components					Origins of plant	References
Thymol (42.50%)	Carvacrol (16.85%)	p-Cymene (10.62%)	α-Pinene (3.00%)	γ-Terpinene (7.25%)	Iran, Khorasan	Current research
Thymol (27.05%)	Borneol (7.10%)	p-Cymene (9.49%)	cis-Sabinene hydrate (6.12%)	Linalool (5.63%)	Iran, Poldokhtar	[27]
Thymol (64.87%)	Carvacrol (4.65%)	p-Cymene (5.63%)	β-Caryophyllene (3.41%)	γ-Terpinene (9.11%)	Iran, Najafabad	[27]
Thymol (40.94%)	Carvacrol (22.39%)	p-Cymene (7.73%)	β-Caryophyllene (3.95%)	γ-Terpinene (5.43%)	Iran, Yazd	[27]
Thymol (46.61%)	Carvacrol (17.26%)	p-Cymene (11.51%)	g-Terpinene (4.01%)	β-Caryophyllene (2.91%)	Iran, Farashband	[27]
Thymol (47.46%)	Carvacrol (9.64%)	p-Cymene (13.16%)	Linalool (7.92%)	γ-Terpinene (2.72%)	Iran, Hajiabad	[27]
Thymol (44.60%)	Carvacrol (2.35%)	p-Cymene (13.70%)	β-Caryophyllene (2.20%)	γ-Terpinene (21.5%)	Iran, Tehran	[19]
Thymol (52.40%)	Carvacrol (6.10%)	p-Cymene (13.20%)	Terpinenyl acetate (5.40%)	γ-Terpinene (17.1%)	Iran, Shiraz	[28]
Thymol (37.59%)	Carvacrol (33.65%)	p-Cymene (7.72%)	β-caryophyllene (2.06%)	γ-Terpinene (3.88%)	Iran, Firoozabad	[17]

a total of 43 compounds. The main components of essential oil were thymol (42.46%), carvacrol (16.85%), p-cymene (10.62%), γ-terpinene (7.26%) and α-pinene (3.00%) representing 80.19% of the total oil. Other separated components accounted for less than 19.81% of the oil. Different studies have been done in other regions on chemical composition of the essential oil of different species of *Z. multiflora* Boiss. Table 1 shows and compares the main components of *Z. multiflora* essential oil in current research and other studies.

3.2. Effect of essential oil of Z. multiflora Boiss. on microbial species

Antimicrobial activity of essential oil of *Z. multiflora* Boiss. was determined via the Microwell dilution method at 10 concentrations against three bacteria, two yeasts and two fungi species. The results of *in vitro* antimicrobial activity assay showed that the essential oil possessed broad antimicrobial activity against the microorganisms tested.

The antimicrobial effect of essential oil against the microorganisms is shown in Table 2. Results obtained from the microdilution method, followed by measurements of MIC and MBC indicated that essential oil of *Z. multiflora* Boiss. exhibited significant ($P<0.05$) antibacterial activity against tested bacteria and the sensitivity was as follows: *P. vulgaris*=*P. aeruginosa*>*B. cereus*. Among the tested yeasts and fungi, the most sensitive yeast was *C. utilis* while resistance of *A. niger* and *P. digitatum* was the same and more than *S. cereviciae*.

Table 2
Minimum inhibitory concentration and minimum fungicidal or bactericidal concentration of essential oil of *Z. multiflora* Boiss.

Microorganisms	MIC (μg/mL)	MBC or MFC (μg/mL)
B. cereus	50	200
P. aeruginosa	25	100
P. vulgaris	25	100
S. cereviciae	200	1 600
C. utilis	100	1 600
P. digitatum	200	6 400
A. niger	200	6 400

The values in the table are an average of 3 experiments.

3.3. Comparing the effect of essential oil of Z. multiflora Boiss. on the growth of tested microorganisms

Comparison results of ten essential oil concentrations on the growth of *B. cereus*, *P. aeruginosa* and *P. vulgaris* are presented in Figure 1. According to the Figure 1, the inhibitory effect of the essential oil on the growth of all microbial species increased significantly ($P<0.05$) as oil concentration increased.

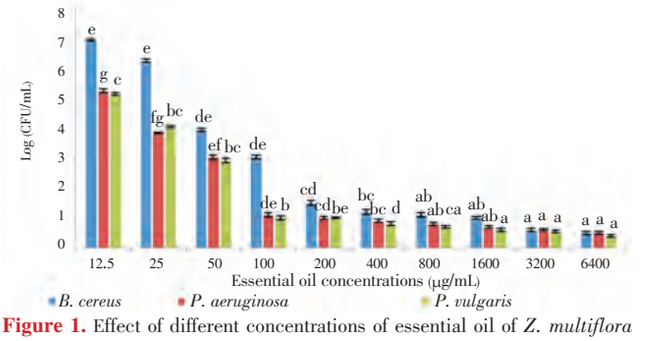

Figure 1. Effect of different concentrations of essential oil of *Z. multiflora* Boiss. on the growth of *B. cereus*, *P. aeruginosa* and *P. vulgaris* ($n=3$).
Data with the same letter for each essential oil concentration are not significantly different ($P<0.05$) according to Duncan's multiple range test.

Growth of all the three bacterial strains was the same at the two initial concentrations of 6400 and 3200 μg/mL but at lower concentrations, growth of *B. cereus* was more than two other bacteria.

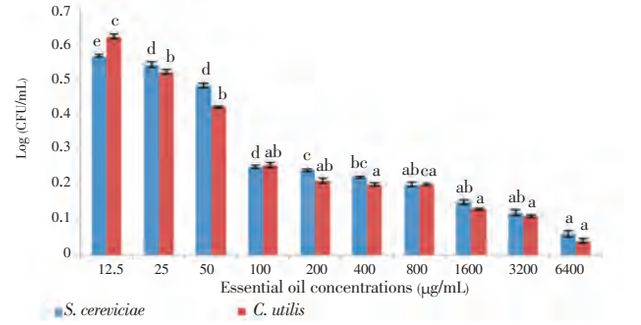

Figure 2. Effect of different concentrations of essential oil of *Z. multiflora* Boiss. on the growth of *S. cereviciae* and *C. utilis* ($n=3$).
Data with the same letter for each essential oil concentration are not significantly different ($P<0.05$) according to Duncan's multiple range test.

According to Figure 2, growth of yeasts decreased with increasing concentration of essential oil and the mount of growth decreased substantially at the concentration of 100 μg/mL.

According to Figure 3, growth of fungus including *P. digitatum* and *A. niger* decreased with increasing concentration of essential oil and the mount of growth decreased substantially at the concentration of 200 μg/mL. Growth of both genus inhibited at the same concentration of the essential oil.

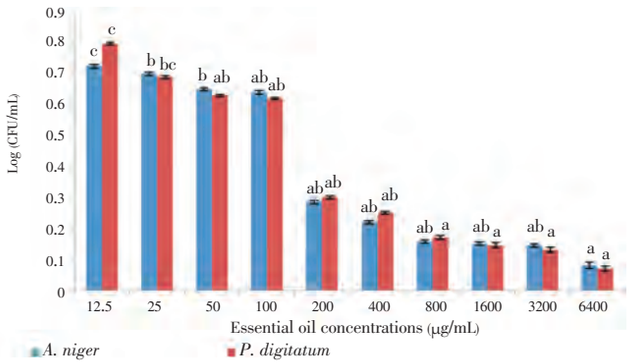

Figure 3. Effect of different concentrations of essential oil of *Z. multiflora* Boiss. on the growth of *P. digitatum* and *A. niger* (n=3).

Data with the same letter for each essential oil concentration are not significantly different (P<0.05) according to Duncan's multiple range test.

4. Discussion

It has been accepted that the anti-microbial activity of most essential oils is related to their phenolic monoterpenes[13]. According to Table 1, *Z. multiflora* Boiss. essential oil is a good source of oxygenated mono-terpenes, in particular thymol and carvacrol which have significant anti-microbial properties. Although due to the differences in the test methods, bacterial strains, plant source, genetic constitution and harvest season making a direct comparison of the findings from different studies is difficult, we can have a view on the composition of the essential oil in different environmental positions and conditions.

In contrast to the present study, Misaghi and Basti (2007) reported carvacrol as the main constituent (71.12%) of the plant. They also indentified 12 components corresponding to 91.9% of total oil composition[14]. In addition, Sharififar *et al.* (2007), detected nearly 25 compounds, representing 99.78% of the total oil in which the thymol (37.59%) was the major compound followed by carvacrol (33.65%)[15].

Also the antimicrobial activities of different *Z. multiflora* Boiss. species have been studied by different researchers[5,14,15]. But, there is not enough data about the antimicrobial activity of essential oil of the *Z. multiflora* Boiss. on the mentioned species studied in this research. Inhibitory effects of ethanol, methanol, chloroform and hexane extracts of *Z. multiflora* were investigated against multiple drug resistant *P. aeruginosa* antagonistic effect[16]. Abou Fazeli *et al.* demonstrated the activity of the essential oil against *Candida albicans*[17]. Fakour *et al.* (2007) studied the anti-fungal properties of the essential oil on the growth inhibition of *Aspergillus parasiticus* and aflatoxin production; the essential oil demonstrated powerful inhibitory properties on fungal growth and aflatoxin production[18].

Rasouli *et al.*, (2001) found that bactericidal concentrations of hydro-distilled essential oil of *Z. multiflora* on *Staphylococcus aureus* and *Escherichia coli* were less than that of ampicillin[19]. Mahmoudabadi *et al.* (2007) investigated anti-fungal activity of three extracts of the aerial parts of *Z. multiflora* (aqueous, ethanolic and methanolic) against four *Candida* species (*Candida albicans*, *Candida tropicalis*, *Candida glabrata* and *Candida parapsilosis*)[20]. Abdollahi *et al.*, (2011) and Shokri *et al.*, (2011) reported respectively the antifungal activity of *Z. multiflora* against *Aspergillus* and *Fusarium* species[21,22].

Oxygenated mono-terpenes including thymol and carvacrol are lipophilic in nature and act on the cell membrane which cause substantial morphological damage, resulting in a change in permeability and the release of cellular contents[23].

High proportion of carvacrol present in *Z. multiflora* and its combination with phenolic monoterpens and γ-terpinene have shown to lead to a synergistic activity resulting in destabilization of the microbial membrane[24,25]. Acidic nature of the hydroxyl group of carvacrol and also involvement of the hydroxyl group during formation of hydrogen bonds may explain the highest antimicrobial activity[26].

The existence of other antimicrobial constituents such as linalool (Bassole *et al.*, 2003), p-cymene[27], 1,8-cineole, terpinen-4-ol[28] combined with other minor constitutes might be involved in improving overall antimicrobial activity of essential oils.

Z. multiflora Boiss. is a popular and medicinal plant native to Iran. During recent years, more attention has paid to this plant due to its significant antimicrobial activity in food industry. This study characterized chemical composition and antibacterial properties of *Z. multiflora* Boiss. essential oil endemic to Khorasan province in Iran. In conclusion, the results of the present work showed that *Z. multiflora* Boiss. essential oil had an antimicrobial and antifungal activity and can be used as an antimicrobial additive, especially a mold inhibitor in food systems. However, further studies are needed to evaluate the organoleptic and pharmaceutical effects and practical effectiveness of this application.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors thank Islamic Azad University, Quchan Branch, for financial support of this research (Grant No. 323/1392) .

Comments

Background

Evaluation of the chemical and antimicrobial properties of the essential oil is an applied field in pharmaceutical plant and it is recommended.

Research frontiers

In this study, the chemical composition and antimicrobial effect of the essential oil of *Z. multiflora* Boiss. endemic in Khorasan-Iran, have been studied. As an endemic variety, the results might be useful to compare the characteristic with other varietries in Iran or other regions.

Related reports

The antimicrobial activity and chemical composition of different components of *Z. multiflora* Boiss species are studied by other researchers which show different results.

Innovations & breakthroughs

The innovations include the variety of the essential oil (endemic in Khorasan), some of the microorganism tested (*P. digitatum*, *P. vulgaris*) and the way for presenting the effect of different concentrations of essential oil on the growth of microorganisms (Figures 1-3). The antifungal activity of the essential oil may be an excellent finding for its industrial application.

Applications

This study emphasizes on existence of thymol, carvacrol and p-cymene in the essential oil of *Z. multiflora* Boiss., which have well-known pharmaceutical applications. Also as mentioned before, in the case of antifungal activity, this essential oil can be used as a flavor with antifungal activity.

Peer review

This study more or less confirms previous researches on *Z. multiflora* Boiss essential oil in Iran (Table 1) and shows that the endemic species in Khorasan has similar composition to other species in Iran and emphasizes on the antimicrobial effects of this essential oil. The results are interesting and suggest that the essential oil has pharmaceutical application and can be as an additive for its ability to inhibit fungi (especially *A. niger*).

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