http://www.cjmb.org
doi 10.34172/cjmb.2022.29
Open Access Original Article
Crescent Journal of Medical and Biological Sciences
Vol. 9, No. 3, July 2022, 173–183
eISSN 2148-9696

The Effect of *Zataria multiflora* Boiss Leaves Essential Oil on Some Pathogenic Bacteria as an Alternative for Conventional Antibiotics

Abdollah Dadazadeh[1], **Hassan Nourafcan**[1*]

Abstract

Objectives: *Zataria multiflora* Boiss has been used as a conventional medicinal plant for treating infections in traditional medicine. Therefore, the aim of this work was to reveal the composition and antibacterial effect of *Zataria multiflora* leaves essential oil.

Materials and Methods: *Z. multiflora* essential oil was isolated through two different methods: hydro isolation and steam isolation. Essential oil was analyzed by gas chromatography–mass spectrometry (GC/MS) and its composition was determined. The antibacterial effect of *Z. multiflora* essential oil was investigated on gram-positive (*Staphylococcus aureus, Streptococcus mutants, Staphylococcus epidermidis*) and gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium)* bacteria compared to that of 12 antibiotics.

Results: The essential oil yields in hydro isolation and steam isolation methods were 2.33% and 0.33% respectively. Thirty-seven compounds were identified in the essential oil using GC/MS, among which carvacrol, thymol, linalool, *p*-cymene, decane, β-caryophyllene and γ-terpinene were the dominant compounds.

Conclusions: *Z. multiflora* essential oil, at the concentration of 80 µL/mL, had a strong or similar antibacterial effect on *S. aureus* and *S. typhimurium* compared to the antibacterial effect of some of the antibiotics in the study.

Keywords: Antibacterial, Antibiotic, Herbal medicine, Phytomedicine, Thyme

Introduction

Zataria multiflora Boiss is one of the most important medicinal plants in traditional medicine, which is commonly used for treating infectious diseases. *Z. multiflora* essential oil is among the top 10 essential oils of the world's medicinal plants (1). The presence of the active constituents of carvacrol, thymol, and *p*-cymene in *Z. multiflora* has led it to be considered as an antimicrobial drug (2).

Since early 2000s, the use of antibiotics has significantly increased (3) ledwhich caused 40% of the strains of Streptococcus pneumoniae and 61% of *Enterococcus faecalis* become resistant to penicillin and vancomycin, respectively (4). Research showed that the leading causes of death before the discovery of antibiotics in the United States were tuberculosis, pneumonia, and gastrointestinal infections, which accounted for the 30% of all deaths (4). With the emergence of bacterial resistance and the ineffectiveness of antibiotics, there is a serious concern about approaching the pre-antibiotics era, so that the World Health Organization (WHO), because of the global importance, declared 2011 the year of "microbial resistance poses a serious threat to life" (5); that is why the use of medicinal plants is developing due to the increase in antibiotic-resistant pathogenic bacteria (6). Therefore, the aim of this study was to identify the bioactive compounds of *Z. multiflora* medicinal plant that can be used as an alternative or supplement to antibiotics for pathogenic bacteria. For this purpose, essential oils of thyme leaves were extracted by two methods of distillation and the optimal method was determined. The composition of the essential oils was identified by gas chromatography–mass spectrometry (GC-MS) device, and the antibacterial effects of these essential oils at different concentrations were investigated on different bacterial species. Finally, the results were compared to the results of the antibacterial test of 12 antibiotics and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined.

Material and Methods

Material

Chemical Material

Ethanol (96%) was purchased from Zakaria Jahrom Company, sodium sulfate from Chem Lab (Belgium), hexane and dimethyl sulfoxide (DMSO) solvent from Merck (Germany).

The standard microbial susceptibility strains of *Escherichia coli* (American Type Culture Collection (ATCC) 25922)), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginos*a (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC

Received 23 August 2021, **Accepted** 10 February 2022, **Available online** 29 July 2022

[1]Department of Medicinal Plants, Miyaneh Branch, Islamic Azad University, Miyaneh, Iran.
*Corresponding Author: Hassan Nourafcan, Tel: +98 914 414 53 39, Email: nourafcan@m-iau.ac.ir

Key Messages

- *Zataria multiflora* Boiss is used to treat infections in traditional medicine.
- The essential oil of this plant was extracted through different methods.
- The essential oil yields were obtained and analyzed by GC-MS.
- The antibacterial effect of *Z. multiflora* essential oils was compared to 12 antibiotics.
- The antibacterial effect of *Z. multiflora* essential oils showed suitable antibiotic properties.

12228) and *Streptococcus mutans* (ATCC 35668) were purchased from Bahar Afshan Company. Antibiogram and Blank discs were purchased from Padtan Teb Company.

The culture media of Mueller-Hinton Agar (MHA), Tryptone Soya Broth (TSB), Spain's Scharlau brand, Mueller-Hinton Broth (MHB) and Brain Heart Infusion Broth (BHI Broth), of Ibresco brand, as well as Serum ringer tablet of Merck brand were purchased. The required distilled water was prepared using the German GFL (Gesellschaft für Labortechnik) water distiller in the laboratory.

Plant Material

Five kilograms of *Z. multiflora*, a plant located in the N35 29, E3752, and 1778 m above the sea level, and harvested in Shiraz, Iran, was bought from the medicinal plant market in Tabriz, Iran and identified by Dr. Nasser Mohabelipour (an academic member of the Medicinal Plants Department of Islamic Azad University, Miyaneh Branch). The plant's name was checked with the Plant List website, and it was verified (Record numb10er: 215611). It was also identified as registered in the East Azerbaijan's Agricultural Research Education and Extension Organization herbarium with the voucher specimen number of 7249. Its impurities were separated, and the plant was dried afterwards. Then the essential oil was extracted in triplicate (Figure 1).

Methods

Hydro Isolation Method (HD)

Isolation of the essential oil from *Z. multiflora* with a ratio of 120 g dried leaves to 1200 mL water was done in triplicate using a Clevenger device particular for hydro isolation method following to the method described by Khalili et al (7) and Mahboubi et al (8). In this method, the dry leaves were put in the flask containing water and then the mixture were boiled. Essential oil with steams evaporated and then condensed in the condenser and collected in the receiver. The isolated essential oil was dehydrated using anhydrous sodium sulfate. Essential oil isolation was performed within 3 hours. Afterwards, it was stored at 1°C in refrigerator until the experiments were conducted (9, 10).

Steam Distillation Method (SD)

Isolation of the *Z. multiflora* essential oil with a ratio of 120 g dried leaves to 1200 mL water were performed in triplicate. To obtain the essential oil by steam isolation, the leaves was put into a separate container and placed on top of a flask containing boiling water. The plant did not come into contact with water but was exposed to produce steam from water. The steam then passes through the leaves mass. The hot steam separated the essential oil of the leaves and entered the condenser and turned into receiver part (11). The isolated essential oil was dehydrated using anhydrous sodium sulfate. Essential oil isolation was performed within 3 hours. Afterwards, it was stored at 4°C in refrigerator until the experiments were conducted (9, 10).

Figure 1. Dry Leaves of. *Zataria multiflora* Boiss.

GC/MS Analysis

GC/MS (GC Agilent USA 6890N, MS Agilent USA 5973N) with HP-5MS 19091S-433 column (0.25×30 m in 0.25 µm) was used to analyze the composition of essential oils according to the method by Khalili et al (7) with some modifications (8). Carrier gas helium with a rate of 1 mL/min was used. Fifty microliters of pure *Z. multiflora* essential oil was dissolved in 450 µL of hexane solvent, obtaining 10% essential oil. Afterwards, 1 µL of the solution was injected to the device to identify the components. The temperature of the column was 60°C at the beginning of the process, and it maintained this temperature for one minute. After one minute, the temperature rose to 140°C at the rate of 2.30°C per minute. After this sequence, the device was programmed to raise the temperature up to 240°C at a rate of 25°C per minute and keep it at 240°C for one minute. A 70eV electrical source was used for ionization. The data were processed using Chemstation plus Wiley 7.1 software. Standard mixture (Sigma-Aldrich, Missouri, USA) was used to determine the essential oil composition retention index (RI). This standard was aliphatic hydrocarbons mixture of ranging from C8 to C32, solved in *n*-hexane. Due to exact determination of the analyzed compounds, the literature retention index (LRI) was also obtained from NIST website, in accordance with the column type used in GC/MS device (12-14). In addition to this, co-injection with the available authentic sample of identified

compounds to GC or GC/MS was done. Quantification was done by external standard method using calibration curves generated by GC analysis of available compounds (decane, *p*–cymene, linalool, γ-terpinene, carvacrol, thymol, and β-caryophyllene).

Antibacterial Activities

This study was done *in vitro* and antibacterial activities were checked and determined as follows:

Disc diffusion method: Sensitivity test was done by DIFFUSION DISc method recommended by the Clinical and Laboratory Standards Institute (CLSI) 2011. Standard 0.5 McFarland (1.5×10^8 CFU/mL) solution was also prepared according to the 2011 CLSI instructions. The steps of the CLSI 2011 standard method are briefly described below:

Bacterial suspension preparation: 2 mL of BHI Broth culture medium was injected into a bacterial lyophilized ampoule under sterile conditions, and it was incubated at 37°C for 4 hours. Afterwards, it was cultured in BHI Agar medium. Then, 3-5 colonies were picked off and added to a tube containing Ringer's serum, and the tube's suspension was adjusted in front of a paper with black and white stripes so that its turbidity was equal to the standard turbidity of 0.5 McFarland (1.5×10^8 CFU/mL) (15).

Bacterial inoculation in plates containing MHA culture medium: A sterile swab was dipped into the bacterial suspension. Thereafter, streaking method procedure was carried out to bacterial inoculation.

Disc placement, incubation and recording results: At first, DMSO solvent was passed through a bacteriological filter. Blank sterile discs with a diameter of 6 mm were impregnated with various concentrations of the essential oils diluted with DMSO, viz. 5, 10, 20, 40, and 80 µL/mL and placed in the culture medium. For the homogeneous absorption of the essential oils in the culture medium and prevention of the evaporation of the essential oils, they were kept at 4°C for 2 hours in the refrigerator and then transferred into an incubator to be kept there at 35 ± 2°C for 24 hours. Subsequently, the diameter of the inhibition zone was measured and recorded (7).

Determination of Minimum Inhibitory Concentration

The MIC was determined based on the 2012 CLSI guideline with some modifications to the method proposed by Vagalas et al (16). From the MHB culture medium, 100 µL was poured into each well. Four milliliters of the essential oil was dissolved in 1 mL of 10% DMSO, and 100 µL of the diluted essential oil was transferred to the first well. Subsequently, 100 µL of the first well was drawn up and transferred to the second one, then 100 µL of the second well was drawn up and transferred to the third well. This sequence went on until the ninth well and the 100 µL drawn up from the ninth well was discarded. Then, a bacterial suspension with the concentration equal to 0.5 McFarland (1.5×10^8 CFU/ mL) was prepared and 5 µL of the bacterial suspension was inoculated to each of the aforementioned wells. The remaining three tubes in the row were control wells, which were prepared as follows: the tenth well contained only culture medium (negative control), the 11th well contained culture medium and DMSO solvent (negative control), and the 12th well contained culture medium and bacterium (positive control). The inoculated plate (containing clear liquid culture medium) was incubated at 36°C for 18 hours, and then the wells' turbidity was examined (16). In tubes containing culture medium with the given essential oil concentration, bacteria growth was inhibited and tubes were remained clear. The bacteria did not grow, indicating that this concentration of essential oil could inhibit the growth of bacteria; therefore, it was reported as the "growth inhibitory concentration". If the tubes with culture medium and the essential oil went turbid after incubation, it indicated that this concentration of essential oil was not able to inhibit bacterial growth. Turbid tubes were removed from the experiment, because that concentration did not inhibit bacterial growth. The normal saline solution was used as the solvent for producing 10% DMSO (17). The turbid wells were ignored and the clear wells were reported as the MIC.

Determination of Minimum Bactericidal Concentration

The samples from the wells in which no bacterial growth was inspected during the MIC stage were taken for the MBC test. The clear wells (100 µL) were inoculated and cultured in the plates containing MHA culture medium and then incubated at 37°C for 24 hours. The plates in which there were no bacterial growth and had the lowest concentration of the essential oils were reported as the MBC (18-20).

Statistical Analysis

The factorial experiment was performed in a Completely Randomized Design using SPSS statistics software. The data (efficiency of distillation methods, value of the components in the essential oil and antibacterial effect) were gathered through 3 replications, and their mean values and deviations were calculated. The mean values of the data were compared using Duncan's new multiple range test ($P < 0.05$).

Results and Discussion

Efficiency of Different Isolation Methods

In this study, essential oil isolation was performed by two methods under the same conditions, including hydro isolation and steam isolation. Hydro isolation was the most efficient method with a 2.56 g (2.13%) (efficiency rate, while steam isolation with 0.36 g (0.30%) had a lower efficiency (Table 1).

Several previous studies examined the effect of different methods on the efficiency, which confirms the results of this study. Sadjia et al (21) reported that isolation yield

Table 1. Efficiency of *Z. multiflora* Boiss Essential Oil Via Different Isolation Methods

Method & Efficiency					
Distillation method	**Dry Plant(g)**	**Essential Oil Content (g)**		**Essential Oil Yield (W/W %)**	
Hydro distillation	120	2.56[a*]		2.13	
Steam distillation	120	0.36[c]		0.30	
Tests of Between-Subjects Effects					
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between groups	9.108	2	4.554	93.600	0.000
Within groups	0.292	6	0.049		
Total	9.400	8			

* Different small letters show significant differences ($P<0.05$) between data obtained with different distillation methods.

Thymus pallecens essential oil by hydro isolation was higher than steam isolation method. Salehi et al (22) reported that the efficiency of thyme essential oil extracted through hydro isolation method was 2.42%, which is similar to our results (22). In another study, the extraction of *Thymus vulgaris* essential oil was done using hydro isolation and microwave extraction methods. In measuring the amount of carvacrol in each method, it was shown that carvacrol made up 64.4% of the essential oil hydro isolation method, while it made up 44.7% of the essential oil isolated by microwave method, indicating a great deal of difference in efficiency value (23). In a similar study that compared the separation efficiency of basil essential oil through hydro distillation and steam distillation methods, it was shown that the yield efficiency with steam was higher than water, which is different from the results of our study (24).

GC/MS Analysis of the Essential Oils Isolated by Both Methods

Thirty-seven components of *Z. multiflora* essential oil were identified (Figure 2; Table 2). The most predominant components were carvacrol, thymol, linalool, *p*-cymene, decane, β-caryophyllene and γ-terpinene, in order of prevalence (Figure 3). Quantification of components with higher level in the essential oils showed that carvacrol 37.77-18.26% (0.657-0.337 mg g^{-1}), thymol 13.97-10.75% (0.243-0.130 mg g^{-1}) and linalool 8.29-10.40% (0.144-0.122 mg g^{-1}), had the highest amounts, respectively (Table 2).

Studying 3 ecotypes of *Z. multiflora*, Zomorodian et al (25) identified 36 essential components, seven of which (thymol, carvacrol, linalool, *p*-cymene, caryophyllene oxide, limonene and γ-terpinene) were the most dominant components in the essential oil. The results of this study were similar to our results. Yahyaraeyat et al (26) identified 29 essential components, including carvacrol (61.29%), thymol (25.18%), linalool (1.96%), p-cymene (1.90%), β-caryophyllene (1.82%) and β-phellandrene (1.82%) through analyzing *Z. multiflora*, respectively. The aforementioned components constitute 92.5% of the essential oil which is similar to the results of this study (26). Mahmoudvand et al (27) identified 32 essential oil components in *Z. multiflora* viz., including thymol (40.8%), carvacrol (27.8%), pinene (8.4%), γ-terpinene (4%), β-caryophyllene (2%), linalool (1.7%), α-terpinolene (1.3%), thymol methyl ether (1.3%) and α-terpinol (1.1%), which constitute 88.4% of the total essential oil content (27). In general, comparing different studies showed that carvacrol, thymol, linalool, *p*-cymene, caryophyllene and its

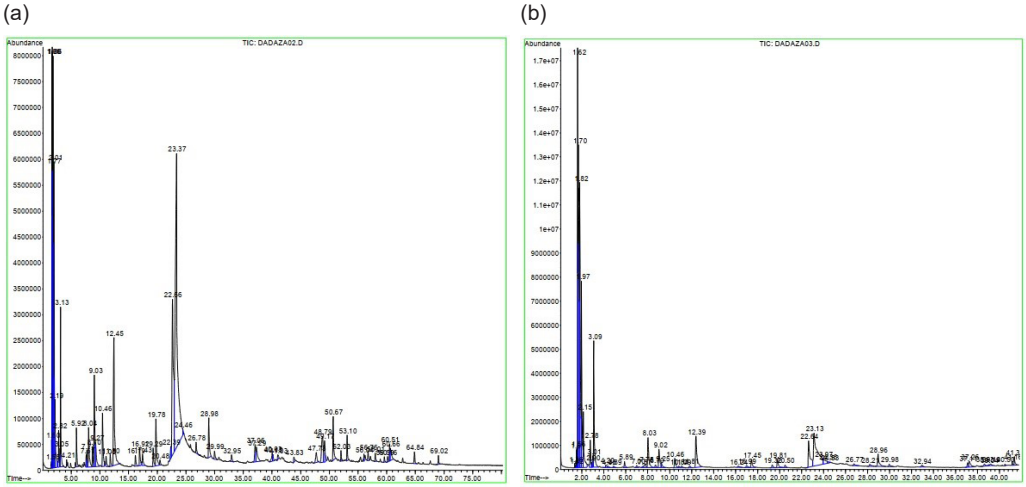


Figure 2. (a) *Z. multiflora* essential oil chromatogram isolated by hydro distillation. (b) *Z. multiflora* essential oil chromatogram isolated by stem distillation.

Table 2. Components of *Z. multiflora* Boiss Via Various Isolation Methods

No.	Component	RT (min)	RIs	LRI	HD % (mg g^{-1})	SD % (mg g^{-1})
1	α-Thujene	4.89	930	927	0^{a**}	0^{a}
2	α-Pinene	5.03	938	936	1.25^{b}	1.36^{a}
3	β-Pinene	6.51	981	977	0^{a}	0^{a}
4	n-Octanone-3	7.61	986	984	1.96^{a}	0^{b}
5	β-Myrcene	7.74	991	989	0.68^{b}	1.23^{a}
6	Decane*	8.04	1003	1000	**2.04^{b} (0.035)**	**8.31^{a} (0.097)**
7	α-Terpinene	8.70	1018	1017	0.84^{a}	0.72^{b}
8	*p*-Cymene	9.03	1027	1024	**5.47^{b} (0.095)**	**6.44^{a} (0.075)**
9	γ-Terpinene	10.46	1060	1059	**2.5^{b} (0.043)**	**2.69^{a} (0.031)**
10	Cis-4-thujanol	10.48	1069	1066	0^{b}	0.44^{a}
11	Linalool oxide cis	11.07	1078	1075	0.58^{a}	0.6^{a}
12	Linalool oxide trans	11.80	1091	1089	0.55^{a}	0.57^{a}
13	Linalool	12.45	1101	1099	**8.29^{b} (0.144)**	**10.40^{a} (0.122)**
14	Terpineneol-1	16.19	1137	1136	0.57^{a}	0.54^{a}
15	α-Terpineol	16.62	1194	1189	0.95^{a}	0.76^{b}
16	Dodecane	17.43	1201	1200	0.64^{b}	2.31^{a}
17	Thymol methyl ether	19.29	1237	1234	0.87^{a}	0.82^{a}
18	Carvacrol methyl ether	19.77	1248	1243	2.54^{b}	2.7^{a}
19	Linalyl acetate	20.50	1258	1255	0.25^{b}	0.69^{a}
20	Bornyl acetate	22.00	1285	1283	0^{a}	0^{a}
21	Thymol	22.67	1295	1290	**13.97^{a} (0.243)**	**10.75^{b} (0.130)**
22	Carvacrol	23.37	1302	1300	**37.77^{a} (0.657)**	**28.62^{b} (0.337)**
23	*p*-Thymol	26.77	1337	1334	0.14^{b}	3.51^{a}
24	Thymol acetate	26.77	1360	1356	0.51^{b}	0.73^{a}
25	Carvacrol acetate	26.82	1376	1373	0^{b}	0^{a}
26	Tetradecane	28.20	1418	1416	0^{b}	0.82^{a}
27	β-Caryophyllene	28.98	1421	1420	**2.07^{b} (0.036)**	**4.25^{a} (0.049)**
28	Aromadendrene	29.99	1443	1440	0.45^{b}	0.92^{a}
29	α-Humulene	30.80	1462	1458	0^{a}	0^{a}
30	1H-Cycloprop[e] azulene	32.94	1502	1498	0.38^{b}	0.7^{a}
31	Spathulenol	37.11	1580	1576	1.10^{a}	1.11^{a}
32	Caryophyllene oxide	37.29	1583	1580	1.35^{b}	2.69^{a}
33	Hexadecane	37.93	1602	1600	0^{a}	0^{a}
34	Isospathulenol	40.02	1636	1633	0.5^{a}	0^{b}
35	Valencene	40.16	1677	1671	0.32^{b}	0.64^{a}
36	Benzoic acid	43.83	1764	1761	0.29^{a}	0^{b}
37	Phthalic acid	50.66	2550	2546	2^{a}	0^{b}
	Total				90.83^{b}	95.32^{a}
Hydrocarbon monoterpenes (1,2,3,5,7,8,9) * **					10.74^{b}	12.44^{a}
Oxygenated monoterpenes (10,11,12,13,14,15,17,18,19,20,21,22,23,24,25)					66.99^{a}	61.13^{b}
Hydrocarbon sesquiterpenes (27,28,29,30,35)					3.22^{b}	6.51^{a}
Oxygenated sesquiterpenes (28,29,30,31,32,34)					2.95^{b}	3.80^{a}
Carboxylic acids (6,16,26,33,36,37)					4.97^{b}	11.44^{a}
Ketones (4)					1.96^{a}	0^{b}
	Total				90.83^{b}	95.32^{a}

RT, Retention time; RIS, Sample retention indices; LRI, Literature retention index reported in the literature on HP-5MS column; HD, Hydro distillation method; SD, Steam distillation method.

*Bold items are the dominant compounds found in each method.

** Different small letters show significant differences (P<0.05) between data obtained with different isolation methods.

*** Numbers inside parentheses show the compounds in each row.

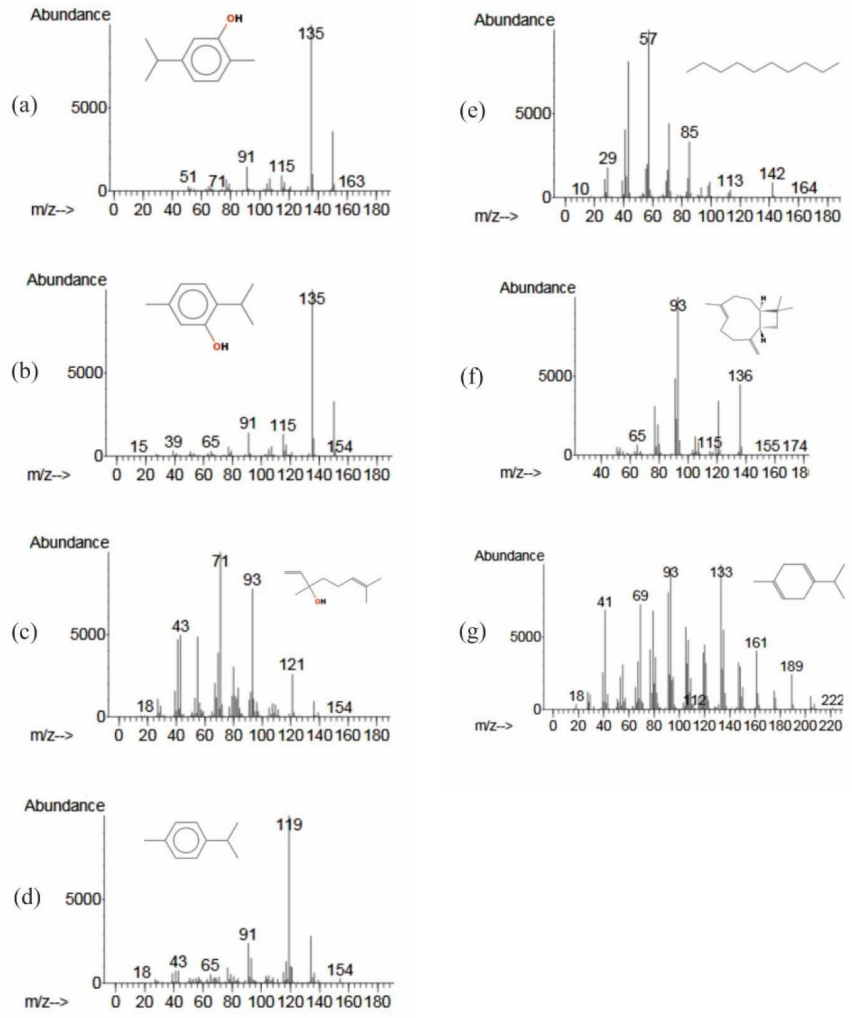


Figure 3. Molecular Structure and Spectrum (Courtesy of NIST Chemistry WebBook) of Essential oil Components: (a) Carvacrol, (b) Thymol, (c) Linalool, (d) p-Cymene, (e) Decan, (f) β-Caryophyllene, (g) γ-Terpinene.

derivatives (caryophyllene oxide, β-caryophyllene), and terpinene and its derivatives (γ-terpinene, α-terpinene, etc) constitute at least 88% of the total thyme essential oil. Thus, it can be concluded that the biochemical, antimicrobial and pharmacological properties of this essential oil are related to the mentioned components. Carvacrol is the first predominant component that constitutes the highest proportion of the essential oil in all the two isolation methods; its value was 37.77% in hydro isolation method and 28.62% in steam isolation method. The second predominant component in all the two isolation methods was thymol, making up 13.97% of the total essential oil in hydro isolation method and 10.75% in steam isolation method.

According to other studies, carvacrol with 61.29% and thymol with 25.18% were the first and second predominant components of thyme (23), which is consistent with the results of this study, but other studies reported thymol as the first and carvacrol as the second predominant essential component (25,27). The proportions of carvacrol and thymol in the essential oil of dry *Z. multiflora* were 63.3% and 25.1%, respectively, while they were 12.6% and 48.4% respectively, in the essential oil of fresh *Z. multiflora* (8).

Some components such as isospathulenol, n-octanone-3, benzoic acid and phthalic acid were only sighted in the essential oil extracted through hydro isolation method and some components such as α-thujene, borneol, bornyl acetate, carvacrol acetate, α-humulene and hexadecane were only sighted in the essential oil extracted through steam isolation method. Tetradecane was the only component obtained through steam isolation method. Considering the effect of isolation method on the quality of *Z. multiflora* essential oil, it can be noticed that in all the two isolation methods, oxygen monoterpenes and hydrocarbon monoterpenes were the most dominant essential oil groups, respectively. The third dominant group of essential oils in steam isolation method was related to fatty acids, and in hydro isolation methods, it was related to hydrocarbon sesquiterpenes. These three main groups were different in terms of quantity in the two isolation methods. The predominant compounds of oxygen monoterpene were carvacrol, thymol and linalool,

all of which were slightly different from each other, so that the highest amounts of carvacrol and thymol were related to hydro isolation methods.

The effect of the two isolation methods on the quality of *Z. multiflora* essential oil has not been studied so far. However, in several discrete studies, extraction of essential oils through hydro or steam methods was performed and the components were studied. Barkhori-Mehni *et al*. (28) prepared *Z. multiflora* essential oil by hydro isolation method, analyzed it by GC-MS, and reported that carvacrol and thymol levels were the most dominant constituents of *Z. multiflora* essential oil.

Z. multiflora essential oil was extracted by hydro isolation method, which introduced 25 essential oil components after analysis, the most dominant of which were carvacrol and thymol. Ziaei *et al*. (29) also prepared thyme essential oil by water isolation method and reported the amount of carvacrol and thymol as the first and second essential components, respectively. Aida *et al*. (30) prepared *Z. multiflora* essential oil by steam isolation, identified 43 components of its essential oil, and introduced thymol and carvacrol as the predominant components in terms of amount, respectively. Generally, these reports are consistent with the results of this study.

Due to the lack of sufficient studies on the preparation of essential oils from *Z. multiflora* by different distillation methods, Sadjia *et al*. (21) investigated essential oil isolation from similar species, which showed that the most predominant essential component was carvacrol. They demonstrated that the order of dominance regarding the amount of the essential oil component was related to hydro isolation and steam isolation, respectively (21). Generally, this report is consistent with the findings of the present research.

Agar Disc Diffusion Test (The Kirby-Bauer Test)

In the present study, the essential oils extracted from *Z. multiflora* through different distillation methods showed different antibacterial effects (Tables 3 and 4; Figure 4). Among the seven dominant components of the essential oils, carvacrol and thymol had acceptable antibacterial effects. Based on previous studies, thymol can control the growth or cause bacterial death by activating changes in the mitochondria and plasma membranes of the bacteria and by inactivating the enzymes involved in inhibiting energy production (9). Carvacrol and thymol have a good synergistic antimicrobial effect on each other (26). The antibacterial effect of *Z. multiflora* essential oil is a function of the amounts of the two phenolic terpenes, thymol and isomeric phenol thymol called carvacrol and the two metabolic precursors of thymol called γ-terpinene and *p*-cymene. Overall, the amount of carvacrol in essential oil by hydro isolation method was 37.77% and the amount of thymol was 13.97%. Meanwhile, the amount of carvacrol was 28.62% and thymol was 10.75% by steam isolation method, indicating that with the increase of these essential components, the antimicrobial properties practically increase. The antibacterial properties of *Z. multiflora* essential oil also depend on the concentration. The reason for this statement is that the essential oil concentrations of 5 and 10 µL/mL of both methods either did not have an antibacterial effect or had a very little effect, while higher concentrations showed better antibacterial effects in both methods, among which the highest antibacterial effect was related to 40 and 80 µL/mL concentrations.

Antibacterial Test of the Essential Oils on *Staphylococcus aureus*

The essential oils isolated by both methods had very little or no effect on *S. aureus* at concentrations of 5, 10, and 20 µL/mL (Table 3). Hydro distillation had a higher antibacterial effect on *S. aureus*. The strongest antibacterial effect was related to the essential oil extracted through hydro isolation at a concentration of 80

Table 3. Antibacterial Activity of *Z. multiflora* Boiss Essential Oil Isoleted by Different Methods

Methods	Concentration (µL/mL)	Bacterium					
		Gram-Positive			Gram-Negative		
		S. aureus	*S. epidermidis*	*S. mutans*	*P. aeruginosa*	*E. coli*	*Salmonella typhi*
Hydro isolation	5	0^{h*}	0^{i}	$7.2^{i}\pm0.2$	0^{d}	$9.2^{e}\pm0.2$	0^{g}
	10	$7.3^{g}\pm0.2$**	$8.2^{g}\pm0.2$	$10.3^{h}\pm0.2$	0^{d}	$10^{d}\pm0.4$	$7.3^{f}\pm0.2$
	20	$8.2^{g}\pm0.2$	$17.3^{d}\pm0.2$	$21.2^{d}\pm0.2$	0^{d}	$11.3^{c}\pm0.2$	$11.2^{d}\pm0.2$
	40	$30^{b}\pm0.4$	$18.2^{c}\pm0.2$	$23.2^{c}\pm0.2$	$8^{c}\pm0.4$	$16.2^{b}\pm0.2$	$16.3^{c}\pm0.2$
	80	$31^{a}\pm1$	$20^{b}\pm0.4$	$26.3^{b}\pm0.2$	$11.3^{a}\pm0.2$	$19.2^{a}\pm0.2$	$19^{a}\pm0.4$
Steam isolation	5	0^{h}	0^{i}	$7.2^{i}\pm0.2$	0^{d}	0^{g}	0^{g}
	10	$7.3^{g}\pm0.2$	0^{i}	$8.8^{i}\pm0.2$	0^{d}	0^{g}	0^{g}
	20	$8.2^{e}\pm0.2$	0^{i}	$12.2^{g}\pm0.2$	0^{d}	0^{g}	$8.2^{e}\pm0.2$
	40	$20.5^{c}\pm0.4$	$8^{g}\pm0.4$	$16.2^{e}\pm0.2$	0^{d}	0^{g}	$11.2^{d}\pm0.2$
	80	$30^{b}\pm0.4$	$9^{f}\pm0.4$	$21.2^{d}\pm0.2$	0^{d}	0^{g}	$16.2^{c}\pm0.2$

Note: Different small letters show significant differences ($P<0.05$) between data obtained with different isolation methods.
Data reported in millimeters.

Table 4. Antibacterial Activity of Different Antibiotics

Antibiotics	Bacterium					
	Gram-Positive			Gram-Negative		
	S. aureus	*S. epidermidis*	*S. mutans*	*P. aeruginosa*	*E. coli*	*S. typhi*
Tobramycin	39.8^{a}±0.2*	34.7^{a}±0.2	21.7^{f}±0.7	23.2^{b}±0.2	15^{d}±0.4	10.7^{g}±0.2
Cephalexin	27^{e}±0.4**	35.8^{a}±0.2	30.2^{c}±0.6	0^{g}	20^{c}±0.4	16.2^{d}±0.2
Gentamicin	33.5^{c}±0.4	35^{a}±0.4	31.2^{bc}±0.8	20.8^{c}±0.6	19.7^{c}±1.2	12^{f}±1
Ceftriaxone	29.7^{d}±0.5	29.7^{c}±0.8	21.7^{f}±0.8	20^{c}±0.4	29.7^{a}±0.5	22.8^{a}±0.6
Ampicillin	40.8^{a}±1.3	29.7^{c}±0.5	31.3^{bc}±0.6	10.2^{e}±0.6	13.5^{d}±0.4	16.5^{d}±0.4
Ciprofloxacin	31.3^{d}±06	34.5^{a}±0.4	31.8^{b}±0.2	26.8^{a}±1	24.2^{b}±0.6	13.8^{e}±0.2
Tetracycline	37.5^{b}±0.4	31.3^{b}±0.9	26^{e}±0.4	10^{e}±0.4	23.8^{b}±0.2	17.8^{c}±0.6
Vancomycin	27.8^{e}±0.2	24.3^{d}±0.2	19^{g}±0.8	7.2^{f}±0.2	0^{f}	19.5^{b}±0.4
Cloxacillin	34.8^{c}±1.3	23.7^{d}±1.2	0^{h}	0^{g}	0^{f}	0^{h}
Penicillin	29.1^{d}±0.6	23.7^{d}±1	34.5^{a}±0.4	0^{g}	8.2^{e}±0.2	20^{b}±0.4
Erythromycin	37.5^{b}±0.4	29.3^{c}±0.6	20.2^{fg}±1	12^{d}±0.4	0^{f}	0^{h}
Amikacin	27.5^{e}±0.4	29.8^{bc}±0.6	28^{d}±0.4	20.8^{c}±0.2	19.8^{c}±0.6	22.3±0.5

Note: Different small letters show significant differences ($P<0.05$) between data obtained with different isolation methods.
Data reported in millimeters.

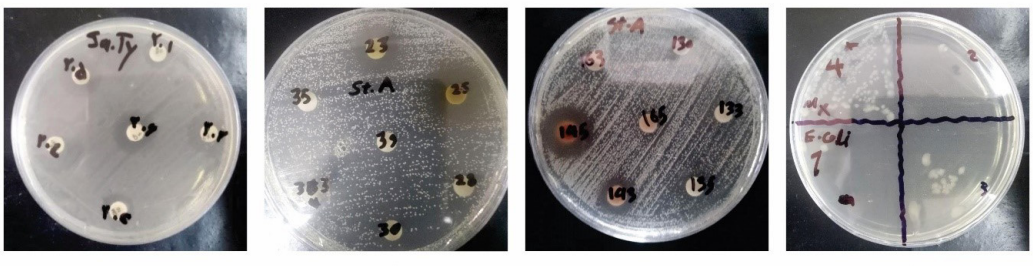
µL/mL with an inhibition zone diameter (IZD) of 31 mm. Compared with standard antibiotics such as penicillin G (IZD: 29.1 mm) and vancomycin (IZD: 27.8 mm), it had a stronger effect. It was also stronger than other antibiotics, including cephalexin (IZD: 27 mm), ceftriaxone (IZD: 29.7 mm) and amikacin IZD (27.5 mm). Also, it had a similar effect compared to ciprofloxacin (IZD: 31.3 mm), but a relatively weak effect compared to gentamicin (IZD: 33.5 mm). It was also weaker than tobramycin (IZD: 39.8 mm), tetracycline (IZD: 37.5 mm), ampicillin (IZD: 40.8 mm), cloxacillin (IZD: 34.8 mm) and erythromycin (IZD: 37.5 mm). In another study on other species of Thyme, the effect of *Thymus algeriensis genuinus* on *S. aureus* was investigated which showed that it had good antibacterial properties (31). Moreover, *Z. multiflora* essential oil at the concentration of 80 µL/mL inhibited a zone with a diameter of 24 mm on *S. aureus* (ATCC 25923) after 24 hours of incubation, which indicates good antibacterial properties of this plant (17). *Z. multiflora* essential oil inactivates the production of type C toxin in *S. aureus* (ATCC 6538) by preventing the transcription of the toxin-producing gene. Considering that the toxin of this bacterium plays a key role in food poisoning, *Z. multiflora* can be recommended as a food preservative (31).

Antibacterial Test of the Essential Oils on Staphylococcus epidermidis

The essential oils isolated by the two methods at the concentrations of 5 and 10 µL/mL had very weak and insignificant antibacterial effects on *S. epidermidis*. The great antibacterial effect was related to the essential oils isolated through hydro isolation at 80 µL/mL concentration with the IZD of 20 mm. Compared with 12 antibiotics with IZDs between 19 and 34.5 mm, *Z. multiflora* had a minimal effect on *S. epidermidis*.

Antibacterial Test of the Essential Oils on Streptococcus mutans

Examination of the essential oils isolated by the two methods on *S. mutans* showed that the essential oils of 5 and 10 µL/mL concentrations had very little antibacterial effects, but the concentration of 80 µL/mL had a positive and significant effect on the bacteria. Comparison of the antibacterial effect of the essential oil hydro isolated at



(a) (b) (c) (d)

Figure 4. Antibiogram test of essential oils obtained from different methods of *Zataria multiflora* Boiss distillation and common antibiotics; (a) Antibacterial effect of some common antibiotics on *Salmonella typhimurium,* (b) Antibacterial effect of different concentrations of *Z. multiflora* essential oil extracted by hydro distillation on *Staphylococcus aureus,* (c) Antibacterial effect of different concentrations of *Z. multiflora* essential oil extracted by steam distillation on *Staphylococcus aureus,* (d) Determination of Minimum Bactericidal Concentration of *Z. multiflora* essential oil on *E.coli*

a concentration of 80 µL/mL with the IZD of 26.3 had a stronger effect than the steam isolation method. The essential oil hydro isolated at a concentration of 80 µL/mL with the IZD of 23.3 mm, which had the strongest antibacterial effect on *S. mutans*, was compared with 12 antibiotics. The results indicated that the mentioned essential oil had a stronger antibacterial effect than tobramycin (IZD: 21.7 mm), ceftriaxone (IZD: 21.7 mm), vancomycin (IZD: 19 mm), cloxacillin (IZD: 0 mm), and erythromycin (IZD: 20.2 mm), but it was weaker than other antibiotics. Comparison of the antibacterial effect of *Th. vulgaris* essential oil with chlorhexidine digluconate and triclosan was investigated by Gonçalves et al (32). It was reported that the toothpaste composed of 1% *T. vulgaris* essential oil not only had a longer shelf life, but also had a better effect on *S. mutans* than the other two substances.

Antibacterial Test of the Essential Oils on Pseudomonas aeruginosa

Examination of the antibacterial effect of the essential oils isolated by the two methods indicated that none of the essential oils had antibacterial effects on *P. aeruginosa* at concentrations of 5, 10 and 20 µL/mL. Even the essential oils extracted through steam isolation at concentrations of 40 and 80 µL/mL were ineffective or had little effect on *P. aeruginosa*. The strongest antibacterial effect was related to the essential oils hydro isolated at the 80 µL/mL concentration which created an inhibition zone with a diameter of 11.3 mm. Compared to antibiotics, including cefalexin (IZD: 0 mm), ampicillin (IZD: 10.2 mm), tetracycline (IZD: 10 mm), vancomycin (IZD: 7.2 mm), cloxacillin (IZD: 0 mm), and penicillin (IZD: 0 mm), *Z. multiflora* had a stronger antibacterial effect. Moreover, it had a similar effect to that of erythromycin (IZD: 12 mm). It also had a weaker antibacterial effect than tobramycin (IZD: 23.2 mm), gentamicin (IZD: 20.8 mm), ceftriaxone (IZD: 20 mm), ciprofloxacin (IZD: 26.8 mm) and amikacin (IZD: 20.8 mm). Gavanji et al (11) investigated the antibacterial effect of *Z. multiflora* essential oil on *P. aeruginosa* (Persian Type Culture Collection (PTCC) 1310)) and showed that after 24 hours of incubation, the inhibition zone created at a concentration of 80 µL/mL was 17.20 mm and at a concentration of 100 µL/mL it was 19.90 mm. This study suggested that the antibacterial effect had a direct relation to the dose of the essential oil (11).

Antibacterial Test of the Essential Oils on Escherichia coli

Studying the antibacterial effect of the essential oils isolated by the two methods on *E. coli* showed that the essential oil extracted by steam isolation had no or very little antibacterial effect and only the essential oil extracted by hydro isolation was effective against *E. coli*, as all the concentrations of 5, 10, 20, 40, and 80 µL/mL had antibacterial effects. The strongest antibacterial effect was related to the concentration of 80 µL/mL with the IZD of 19.2 mm. Compared with antibiotics, including tobramycin (IZD: 15 mm), ampicillin (IZD: 13.5 mm), vancomycin (IZD: 0 mm), cloxacillin (IZD: 0 mm), penicillin (IZD: 8.2 mm) and erythromycin (IZD: 0 mm), it had a stronger antibacterial effect. Compared to antibiotics such as cephalexin (IZD: 20 mm), gentamicin (IZD: 19.7 mm), and amikacin (IZD: 19.8 mm), it had a similar effect; but it had a weaker antibacterial effect compared to ceftriaxone (IZD: 29.7 mm), ciprofloxacin (IZD: 24.2 mm) and tetracycline (IZD: 23.8 mm). In another study on the antibacterial effect of *T. vulgaris* essential oil, it was reported that the essential oil created an inhibition zone with a diameter of 40 mm on *E. coli* (type S22/12), an inhibition zone with a diameter of 30 mm on *E. coli* (S77/15) and an inhibition zone with a diameter of 37 mm on *E. coli* (ATCC 25922) (33).

Antibacterial test of the Essential Oils on Salmonella typhimurium

Examination of the antibacterial effect of the essential oils isolated by the two methods on *S. typhimurium* showed that the essential oils had no antimicrobial effect at 5 and 100 µL/mL concentrations and only the concentrations of 20, 40 and 80 µL/mL were somewhat effective. Comparison of the antibacterial effect of the essential oils isolated by the two methods in the concentration 80 µL/mL showed that the essential oil extracted by hydro isolation (IZD: 19 mm) was more effective than the essential oil extracted by steam isolation (IZD: 16.2 mm). The essential oil hydro isolated in the concentration 80 µL/mL had stronger antibacterial effect than antibiotics, including tobramycin (IZD: 10.7 mm), cefalexin (IZD: 16.2 mm), gentamicin (IZD: 12 mm), erythromycin (IZD: 0 mm), cloxacillin (IZD: 0 mm), ampicillin (IZD: 16.5 mm), ciprofloxacin (IZD: 13.8 mm) and tetracycline (IZD: 17.8 mm). Also, it had a similar antibacterial effect to that of vancomycin (IZD: 19.5 mm) and a relatively weaker effect than ceftriaxone (IZD: 22.8 mm) and amikacin (IZD: 22.3 mm).

Other studies have shown that the antibacterial properties of *Thymus fontanesii* Boiss essential oil are effective on *S. typhimurium* by creating an inhibition zone with a diameter of 27 mm, on *S. aureus* by creating an inhibition zone with a diameter of 26 mm, and on *E. coli* by creating an inhibition zone with a diameter of 23 mm, but they are ineffective against *P. aeruginosa* by the formation of an inhibition zone with a diameter of 8 mm. Considering that this species of Thyme has a high level of carvacrol, its results are somewhat comparable to those of this study (34). The antibacterial properties of *Thymus vulgaris* essential oil have also been studied in higher concentrations on *S. typhimurium*, with a reported IZD of 35 mm (33).

Determination of MIC and MBC

The MIC and MBC values and the related results are shown

Table 5. The MIC and MBC Results (µL/mL) of Essential Oils of *Zataria multiflora* Boiss Obtained by Different Isolation Methods

Bacterium		HD		SD	
		MIC	MBC	MIC	MBC
Gram-positive	*Staphylococcus aureus*	31	500	125	500
	Staphylococcus epidermidis	250	1000	2000	-
	Streptococcus mutans	500	2000	500	2000
Gram-megative	*Pseudomonas aeruginosa*	500	2000	-	-
	E. coli	250	1000	1000	-
	*Salmonella typh*i	250	500	500	1000

HI, Hydro distillation method; SD, Steam distillation method.

in Table 5. Examination of MIC and MBC of the essential oils isolated by the two methods showed that the essential oils extracted by hydro isolation had the best effect on *S. aureus* with MIC and MBC equal to 31 µL/mL and 500 µL/mL, respectively. Also, hydro isolation had the best result for *S. typhi* (125 µL/mL and 500 µL/mL, respectively) and *S. mutans* (MIC and MBC equal to 250 µL/mL and 500 µL/mL, respectively). Furthermore, the study of MIC and MBC of the essential oils isolated by all the two methods suggested that the essential oil extracted by hydro isolation had the best effect on *P. aeruginosa* with MIC and MBC equal to 500 µL/mL and 2000 µL/mL, respectively, on *E. coli* with MIC and MBC equal to 250 µL/mL and 1000 µL/mL, respectively, and on *S. typhimurium* with MIC and MBC equal to 250 µL/mL and 500 µL/mL, respectively. In another study conducted by Barkhori-Mehni et al (28) the MIC and the MBC of *Z. multiflora* essential oil for *E. coli* were reported 250 µL/mL and 500 µL/mL, respectively. Also, the MIC and the MBC of *Z. multiflora* essential oil for *Pseudomonas aeruginosa* were equal to 0.5 mg/mL and 4 mg/mL, respectively (3) (Figure 4). Imelouane et al (35) investigated the antibacterial effect of *Th. vulgaris* essential oil on *S. aureus*, *S. epidermidis* and two types of *E. coli* (type 1 and type 2), the MIC of which was 1330, 1330, 330 and 1330 µL/mL, respectively (35). These results were weaker that the MIC values of all the three *Z. multiflora* essential oils obtained by different methods in this study.

Conclusions

Different distillation methods had significant effects on the efficiency of Z. multiflora essential oil. Among the two isolation methods evaluated in this study, hydro distillation had a higher efficiency. The essential oil obtained by hydro distillation had more oxygen monoterpenes and its carvacrol and thymol levels were higher than those of steam distillation method. Z. multiflora essential oil had an effect on Gram-positive and Gram-negative bacteria, but a stronger effect on Gram-positive bacteria. The essential oil extracted by hydro distillation at a concentration of 80 µL/mL was stronger than cephalexin, ceftriaxone, vancomycin, penicillin, and amikacin and had a similar effect to Staphylococcus aureus on ciprofloxacin, gentamicin, and cloxacillin. It seems that higher levels of carvacrol and thymol as phenolic compounds in the essential oil obtained by hydro isolation had stronger antibacterial effects.

Authors' Contribution

AA: Project design, methodology, laboratory works, results and discussion, and writing the paper . HN: Supervision and study design.

Conflict of Interests

The authors declare that they have no conflict of interests.

Ethical Issues

This research has been registered in Islamic Azad University, Miyaneh Branch, Iran with the code 1525915.

Financial Support

None to be declared.

References

1. Catauro M, Bollino F, Tranquillo E, et al. Chemical analysis and anti-proliferative activity of Campania Thymus Vulgaris essential oil. Journal of Essential Oil Research. 2017;29(6):461-70. doi:10.1080/10412905.2017.1351405
2. Shomali T, Mosleh N. Zataria multiflora, broiler health and performance: a review. Iranian Journal of Veterinary Research. 2019;20(2):81.
3. Meyer E, Gastmeier P, Deja M, Schwab F. Antibiotic consumption and resistance: data from Europe and Germany. Int J Med Microbiol. 2013;303(6-7):388-95. doi:10.1016/j.ijmm.2013.04.004
4. Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. Perspect Medicin Chem. 2014;6:25-64. doi:10.4137/PMC.S14459
5. Leung E, Weil DE, Raviglione M, Nakatani H. The WHO policy package to combat antimicrobial resistance. Bull World Health Organ. 2011;89:390-2.
6. Mazandarani M, Zeinali Z, Ghafourian M. Autecology essential oil composition, antibacterial, anti candidacies and ethnopharmacological survey of *Ferula gummosa* L. as anti infection to treat of vaginal infections in traditional medicine of Razavi Khorasan province (North East of Iran). Crescent Journal of Medical and Biological Sciences. 2015;2(2):42-7.
7. Khalil R, Li Z-G. Antimicrobial activity of essential oil of *Salvia officinalis* L. collected in Syria. Afr J Biotechnol. 2011;10(42):8397-402. doi:10.5897/AJB10.2615
8. Mahboubi M, Bidgoli FG. Antistaphylococcal activity of *Zataria multiflora* essential oil and its synergy with vancomycin. Phytomedicine. 2010;17(7):548-50. doi:10.1016/j.phymed.2009.11.004.
9. Chemat S, Cherfouh R, Meklati BY, Belanteur K. Composition and microbial activity of thyme (*Thymus algeriensis genuinus*) essential oil. Journal of Essential Oil Research. 2012;24(1):5-11. doi:10.1080/10412905.2012.645303.
10. Mobaiyen H, Dehghan G, Elmi F, Talebpour AH. The Comparison of Composition and biological activities in wild and cultivated of Thymus kotschyanus essential oils and methanolic extracts from East Azarbayjan, Iran. Crescent Journal of Medical and Biological Sciences. 2017;4(1):17-22.
11. Gavanji S, Mohammadi E, Larki B, Bakhtari A. Antimicrobial and

cytotoxic evaluation of some herbal essential oils in comparison with common antibiotics in bioassay condition. Integr Med Res. 2014;3(3):142-52. doi:10.1016/j.imr.2014.07.001
12. Babushok V, Linstrom P, Zenkevich I. Retention indices for frequently reported compounds of plant essential oils. J Phys Chem Ref Data. 2011;40(4):043101. doi:10.1063/1.3653552.
13. Goodner K. Practical retention index models of OV-101, DB-1, DB-5, and DB-Wax for flavor and fragrance compounds. LWT-Food Science and Technology. 2008;41(6):951-8. doi:10.1016/j.lwt.2007.07.007.
14. Rofouei MK, Kojoori SMH, Moazeni-Pourasil RS. Chemical Variation in Essential Oil Composition and Rosmarinic Acid Content in Rosemary From Iran at Different Harvesting Times During One Day. Crescent Journal of Medical and Biological Sciences. 2021;8(1):48-55.
15. Sagun E, Durmaz H, Tarakci Z, Sagdic O. Antibacterial activities of the extracts of some herbs used in Turkish herby cheese against Listeria monocytogenes serovars. International Journal of Food Properties. 2006;9(2):255-60. doi:10.1080/10942910600596365.
16. Valgas C, Souza SMd, Smânia EF, Smânia Jr A. Screening methods to determine antibacterial activity of natural products. Braz J Microbiol. 2007;38:369-80.
17. Cavas M, Beltrán D, Navarro JF. Behavioural effects of dimethyl sulfoxide (DMSO): changes in sleep architecture in rats. Toxicol Lett. 2005;157(3):221-32. doi:10.1016/j.toxlet.2005.02.003.
18. Owuama CI. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method. Afr J Microbiol Res. 2017;11(23):977-80. doi:10.5897/AJMR2017.8545.
19. Salama HM, Marraiki N. Antimicrobial activity and phytochemical analyses of *Polygonum aviculare* L. (Polygonaceae), naturally growing in Egypt. Saudi J Biol Sci. 2010;17(1):57-63. doi:10.1016/j.sjbs.2009.12.009.
20. Waites KB, Bade DJ, Bébéar C, Brown SD, Davidson MK, Duffy LB, et al. Methods for antimicrobial susceptibility testing for human mycoplasmas; Approved guideline. 2019.
21. Sadjia B, Naima S, Chahrazed B. Extraction of thyme (*Thymus pallecens* de Noé) essential oil by steam-distillation, steam-diffusion and hydro-distillation processes: optimization of operating conditions and antioxidant activity. Journal of Essential Oil Bearing Plants. 2012;15(2):336-47. doi:10.1080/0972060X.2012.10644056.
22. Salehi S, Golparvar AR, Hadipanah A. Effect of harvest time on yield and quality of Thymus vulgaris L. essential oil in Isfahan province, Iran. Agriculturae Conspectus Scientificus. 2014;79(2):115-8.
23. Benmoussa H, Elfalleh W, Farhat A, Bachoual R, Nasfi Z, Romdhane M. Effect of extraction methods on kinetic, chemical composition and antibacterial activities of Tunisian *Thymus vulgaris* L. essential oil. Separation Science and Technology. 2016;51(13):2145-52. doi:10.1080/01496395.2016.1201507
24. Wesolowska A, Grzeszczuk M, Jadczak D. Comparison of the chemical composition of essential oils isolated by water-steam distillation and hydrodistillation from garden thyme (*Thymus vulgaris* L.). Journal of Essential Oil Bearing Plants. 2016;19(4):832-42. doi:10.1080/0972060X.2015.1025296
25. Zomorodian K, Saharkhiz M, Rahimi M, Bandegi A, Shekarkhar G, Bandegani A, et al. Chemical composition and antimicrobial activities of the essential oils from three ecotypes of *Zataria multiflora*. Pharmacogn Mag. 2011;7(25):53. doi:10.4103/0973-1296.75902
26. Yahyaraeyat R, Khosravi A, Shahbazzadeh D, Khalaj V. The potential effects of *Zataria multiflora* Boiss essential oil on growth, aflatoxin production and transcription of aflatoxin biosynthesis pathway genes of toxigenic *Aspergillus parasiticus*. Braz J Microbiol. 2013;44(2):649-55.
27. Mahmoudvand H, Mirbadie SR, Sadooghian S, Harandi MF, Jahanbakhsh S, Saedi Dezaki E. Chemical composition and scolicidal activity of Zataria multiflora Boiss essential oil. Journal of Essential Oil Research. 2017;29(1):42-7. doi:10.1080/10412905.2016.1201546.
28. Barkhori-Mehni S, Khanzadi S, Hashemi M, Azizzadeh M. Antibacterial activity of *Zataria multiflora* Boiss essential oil against some fish spoilage bacteria. Journal of Human, Environment and Health Promotion. 2017;2(4):220-5.
29. Ziaee E, Razmjooei M, Shad E, Eskandari MH. Antibacterial mechanisms of *Zataria multiflora* Boiss. essential oil against *Lactobacillus curvatus*. LWT. 2018;87:406-12. doi:10.1016/j.lwt.2017.08.089.
30. Aida A, Ali MS, Behrooz MV. Chemical composition and antimicrobial effect of the essential oil of *Zataria multiflora* Boiss endemic in Khorasan-Iran. Asian Pacific Journal of Tropical Disease. 2015;5(3):181-5. doi:10.1016/S2222-1808(14)60649-6.
31. Parsaeimehr M, Akhondzadeh Basti A, Misaghi A, Gandomi H, Jebellijavan A. The Effect of *Zataria multiflora* Boiss. Essential Oil on Gene Expression of Enterotoxin C in *Staphylococcus aureus* ATCC 6538. Journal of Food Processing and Preservation. 2015;39(6):1702-9. doi:10.1111/jfpp.12401.
32. Gonçalves G, Bottaro M, Nilson A. Effect of the *Thymus vulgaris* essential oil on the growth of *Streptococcus mutans*. Revista de ciências farmacêuticas basica e aplicada. 2011;32(3).
33. Benameur Q, Gervasi T, Pellizzeri V, et al. Antibacterial activity of *Thymus vulgaris* essential oil alone and in combination with cefotaxime against bla_{ESBL} producing multidrug resistant Enterobacteriaceae isolates. Natural product research. 2019;33(18):2647-54. doi:10.1080/14786419.2018.1466124.
34. Bekhechi C, Bekkara FA, Abdelouahid DE, Tomi F, Casanova J. Composition and Antibacterial Activity of the Essential Oil of *Thymus fontanesii* Boiss. et Reut. from Algeria. Journal of Essential Oil Research. 2007;19(6):594-6. doi:10.1080/10412905.2007.9699339
35. Imelouane B, Amhamdi H, Wathelet J-P, Ankit M, Khedid K, El Bachiri A. Chemical composition and antimicrobial activity of essential oil of thyme (Thymus vulgaris) from Eastern Morocco. Int J Agric Biol. 2009;11(2):205-8.