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Chemical composition and biological activities of essential oils and methanolic extracts obtained from wild and cultivated types of Thymus *fedtschenkoi* from East Azarbayjan, Iran

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ABSTRACT

Background and aims: The aim of this research was to compare the chemical composition and the antioxidant and antibacterial properties of wild and cultivated types of *T. fedtschenkoi* collected from East Azarbayjan, Iran.

Methods: The essential oils (EOs) from aerial parts of wild and cultivated *T. fedtschenkoi* were investigated by gas chromatography/mass spectrometry (GC/MS). Antibacterial activities of EOs and methanol extracts were tested against bacteria by disc diffusion method and determining their minimum inhibitory concentration (MIC) values by agar dilution method and antioxidant activity by DPPH and FRAP assays.

Results The major components in the oils of the wild type were: thymol (49.21%), p-cymene (15.20%) and Carvacrol (10.20%) and for the cultivated type were: thymol (62.33%) and carvacrol (7.12%). The MIC values of bacterial strains, which were sensitive to the EO of *T. fedtschenkoi*, were in the range of 2-128 µg/mL in wild type and 2-16 µg/mL in cultivated type. For the DPPH assay, the most potent oil was obtained from the cultivated type (IC50; 3.24 µg/mL) and the highest ferric reducing capacity resided with the wild type (IC50; 1.83 µg/mL).

Conclusion: Our data show that cultivation significantly affects the EOs' chemical composition and antioxidant potential of *T. fedtschenkoi*. They signify a reasonable source of natural antibacterial substances that proved to be potential as a drug for use in pathogenic bacteria. The antibacterial activity by the presence of inhibition zone appeared from EOs in the cultivated type on all tested microorganisms significantly higher than that in the wild type, which can be attributed to the presence of high concentration of thymol (47.48%) against the wild type (29.96%).

Keywords: Biological activity, Cultivation, Essential oil composition, Thymus fedtschenkoi

Original article

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INTRODUCTION

During the past decade not only the emergence of multi-drug resistant (MDR) gram-negative and gram-positive bacteria has increased in community and hospital settings (1) but also the spread of mobile carbapenemases like bla_{NDM-1} has resulted in superbugs phenotypes which leave very few treatment options available for clinicians (2). To solve this problem, new drugs with antibacterial and bioactivity effects should be investigated. The plants, especially medicinal ones, are the main alternative compounds because of their effective biological activity (3). On the other hand, the plant of Thymus genus is one of the most popular plants throughout the world, which is especially prevalent in the Mediterranean area and recognized as a medicinal plant by showing several biological and pharmacological properties (4). Essential oils (EOs) of all species of Thymus genus contain phenolic compounds that have antibacterial potential (5). The biosynthesis of these compounds is controlled not only genetically but also by environmental factors like locality and population of them (6). The main methods of picking off medicinal plants could destruct the wild type of them, which leads to their subtraction and demolition (7,8), but the cultivation of these plants could be as an attractive ideal to surmount their high exploitation from the wild environment (7,9). This strategy is widely accepted in Iran for many medicinal and aromatic plants, which creates this hypothesis to seek out the difference in biological compounds and their activities. The aim of the present study was to compare the chemical composition of the EOs of *T. fedtschenkoi* plant collected from wild and cultivated populations in East Azarbayjan, Iran. In addition, it was also attempted to evaluate the antimicrobial and antioxidant activations of EOs and methanol extracts (MEs) of the wild and cultivated samples.

METHODS

Fresh aerial parts of the wild *T. fedtschenkoi* were collected in May-April 2017 at full flowering stage from 1300 maltitude in Arasbaran mountains, East Azarbayjan, and the cultivated samples were obtained from Saeed Abad station of agricultural research center (37° 58´ N and 46° 33´ E, average height: 1880-1900 m), East Azarbaijan province, Iran. Texture of the soil was nearly level and gently sloping. The collected parts of plants were air-dried at room temperature (~25°C) in the shade and powdered using a clean laboratory blender. Fifty grams of each sample was mixed with 500 mL of distilled water and subjected to hydro-distillation, which was done by using the clevenger-type apparatus for 3 hours until the total recovery of Eos was made. The preparation of EOs was performed two times and oils obtained were dried with sodium sulfate, weighed and stored at 4°C until use. Plant material (100 g) of each sample

was soaked in methanol for two days at room temperature. The mixture was filtered through a membrane and the solvent was separated from samples with vacuum evaporator to obtain MEs. A gas chromatograph mass spectrometer (Shimadzu-17A-QP505, Japan) was used for analyzing the EOs. The gas chromatography column was a super CP-Sil 5CB capillary column (50 m × 0.32 mm ID, 0.25 µm film thickness). The temperature of the column was regulated by the following methods: 1) for 1 minute at 70°C with a rate of 1.5°C/min, up to 100°C, 2) at a rate of 4°C/min, up to 180°C and left for 1 minute, 3) at a rate of 10°C/min, up to 200°C, and 4) at a rate of 2.5°C/min, up to 250°C and left for 5 minutes. The 280°C and 300°C were applied as injection and detection temperatures, respectively. The ionization energy was scanned for 1 second, with 70 eV. The mass range was selected from 40 to 300 amu. The retention indices and mass spectral data of wild and cultivated *T. fedtschenkoi* EOs were compared with standard compounds for the purpose of identifying their available compounds. The results of identification were matched by NIST NBS54K Library in computer, and fragmentation patterns of mass spectra were compared by other reports (10,11). DPPH radical scavenging capacity was assessed according to Wettasinghe et al. (12), with some modification as the report of Dehghan et al (13). Different concentrations of extracts and EOs were added to 2 mL of DPPH solution (0.1 mM in methanol), and reduction of DPPH absorbance was followed by doing monitoring at 517 nm (AS). The absorbance of 2 mL DPPH solution was determined at 517 nm (AC) as a blank control. The percentage of radical scavenging activity (*RSA* %) was calculated according to equation 1:

(1)

$$RAS = \frac{100(\mathrm{Ac} - \mathrm{As})}{\mathrm{Ac}}$$

To quantify the results of IC50 value, the effective concentration that could scavenge 50% of the DPPH radicals was calculated (12). Ferric-reducing antioxidant power (FRAP) method was done in accordance with the method of Benzie and Strain with some modifications (13). The antioxidant activity of a sample was measured at 593 nm in spectrophotometer because of reducing ferric (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) in this method. The incubation temperature was selected 37°C during the monitoring period. The standard calibration curve was regulated by using different concentrations of FeSO4. 7H2O and FRAP values were determined for studied EOs.

Table 1. Chemic al Composition of EOs From Aerial Part of Wild and Cultivated *T. fedtschenkoi*

N.P	compound	RI[a]	Wild(%)	Cultivated(%)
1	α-Thujone	926	1.28	0.36
2	α-Pinene	930	2.79	1.23
3	Camphene	941	2.08	1.0
4	β-Pinene	964	0.53	0.42
5	Myrcene	970	0.50	0.10
6	*α*-Terpineol	979	1.68	1.72
7	p-Cymene	993	15.20	t
8	1,8-Cineol	997	2.85	2.98
9	*γ*-Terpinene	1008	1.47	1.81
10	Linalool	1011	0.46	-
11	Borneol	1013	3.46	3.33
12	Terpinen-4-ol	1023	0.21	-
13	*α*-Terpineol	1047	1.08	0.92
14	MethylThymol	1060	1.64	1.55
15	Methyl carvacrol	1076	t	0.92
16	Geraniol	1078	-	1.32
17	Thymol	1121	49.21	63.33
18	Carvacrol	1126	11.20	9.12
19	Caryophyllene	1128	1.63	-
20	Geranyl acetate	1138	0.91	-
21	*α*-Terepinene	1160	-	1.10
22	Thymoquinone	1161	1.48	1.23
	Total		99.20	92.44

A) Retention indexes measured relative to n-alkanes (C-9 to C-24) on the non-polar HP-5 column. t, traces (<0.1%). B) Compound not detected in the oil

Table 2. Radical Scavenging and FRAP Values (mM F^{2+}/mg Extract) of Wild and Cultivated *T. fedtschenkoi*

Test	**EO**		**ME**	
	wild	cultivated	wild	cultivated
DPPH (IC_{50} µg/mL)	2.74	3.24	2.36	1.82
FRAP value (mM Fe^{2+}/mg extract)	1.83	1.56	1.27	1.53

Abbreviations: EO: essential oil; ME, methanol extract.

The antibacterial activities of Eos and MEs were tested against the following bacteria: *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC25952), *Staphylococcus aureus* (ATCC33591), *Staphylococcus aureus* (ATCC29213), *Streptococcus sanguis* (PTCC1449), *Enterobacter aerogenes* (ATCC13048), *Klebsiella pneumoniae* (ATCC700603), *Proteus mirabilis* (ATCC43071), and *E. coli* O157:H7 (Razi Institute, Tehran, Iran). These strains were kept at -70°C in tryptic soy broth (TSB) with 20% of glycerol. They were inoculated in blood agar (BA) and incubated overnight at 35°C. Subsequently, one colony from each culture was inoculated in TSB and incubated at 35°C for 24 hours with shaking (100 rpm) in order to obtain freshly cultured microbial suspension (108 CFU/ mL) for tests, and antimicrobial activity of the EOs was determined with agar disc diffusion method (15). Briefly, a suspension of tested microorganisms (108 CFU/mL-1) in log phase was spread on Mueller Hinton agar (MHA) using sterile cotton swabs. Subsequently, filter paper discs (6 mm in diameter, Mast) were impregnated with 10 µL of EOs of each wild and cultivated *T. fedtschenkoi* and placed on the surface of inoculated plates. For antibacterial effect of MEs, various concentrations of wild and cultivated *T. fedtschenkoi* (0.05 g/mL, 0.1 g/ mL, 0.2 g/ mL and 0.4 g/ mL) were prepared. They were filtered by 0.22 µm sterile filters and 10 µL of each was impregnated on paper discs as described above. Tetracycline (30 µg/disk) and kanamycin (30 µg/disk) (HiMedia) were used as the control. The Petri dishes were incubated at 37°C for 24 hours. The tests were done in duplicate for each strain. Antibacterial activity was evaluated by measuring the diameter of inhibition zone to the nearest millimeter. Determination of minimum inhibitory concentration (MIC) of EOs and MEs of wild and cultivated *T. fedtschenkoi* was done by using agar dilution method as recommended by the guidelines of the Clinical & Laboratory Standards Institute (CLSI) guidelines (16). Briefly, the twofold serial broth dilution of MEs and EOs was prepared. Petri dishes containing MHA plates (Merck, Germany) in 45°C and EOs or MEs of wild and cultivated *T. fedtschenkoi* achieved concentrations ranging from 512 µg/mL to 0.5 µg/mL for EOs and 2048 µg/mL to 2 µg/mL for MEs. The bacterial strains were inoculated in TBS and incubated for 24 hours in 37°C, and the standardized suspension of the studied bacteria was done at 0.5 on the McFarland scale (108 CFU/mL). The bacterial suspension equal to 106 CFU/mL was inoculated in each plate (12 plates for each series) similar to that in a plate without EOs and MEs as a positive growth control. They were incubated at 35°C for 24 hours. The microorganisms that are sensitive to the concentration of MEs or EOs contained in any given agar do not produce a circle of growth at the inoculum site where those that are resistant appeared as circular colonies.

RESULTS

Chemical compositions of the EOs yield state of the distinct variation between the 2 types of plant oils. Our data show that cultivation did not have significant effects on EO production. Hydro distillation of dried and ground aerial parts of wild and cultivated *T. fedtschenkoi* yielded 1.55% and 1.70% of V/W of a greenish yellow color oil. Analysis of the oils led to the identification of 22 compounds (Table 1) which accounted for 99.20% and 92.44% of the total oils. Thymol was found as a predominant component in both of the EOs, together with p-cymene and Carvacrol for the wild type and with Carvacrol for the cultivated *T. fedtschenkoi*. In the oil obtained from the cultivated *T. fedtschenkoi*, we observed a decrease in the percentage of p-cymene against an increase in thymol (Table 1).

The antioxidant activity of the wild and cultivated *T. fedtschenkoi* EOs and MEs were screened by DPPH free radical scavenging assay and ferric-reducing antioxidant potential (FRAP). As shown in Table 2, both EOs and MEs obtained from the wild and cultivated Thymus showed antioxidant activities in both assays. The cultivation of our studied medicinal plant was affected by the antioxidant activity of the *T. fedtschenkoi*. The EO amount for the wild and cultivated types in the DPPH assay (IC50µg / mL) were 2.74 and 3.24, and in the FRAP test (mM Fe^{2+} / mg extract) were 1.83 and 1.56, respectively. Also in the DPPH, the ME values for the wild and cultivated types were 2.36 and 1.82, and for FRAP were 1.27 and 1.53, respectively. The antioxidant capacity of the extracts was better than that of the EOs in two assay systems as shown in Table 2.

The results of the antimicrobial assays by disc diffusion method with EOs and MEs of *T. fedtschenkoi* are summarized in Table 3. Both EOs obtained from the wild and cultivated studies of Thymus showed an inhibitory activity on the tested microorganisms, but the cases of ME obtained in this regard had not such an activity against most of the microorganisms, except for *Streptococcus sanguis* PTCC1449, *Staphylococcus aureus* ATCC33591, and *S. aureus* ATCC29213.

The MIC of EOs and MEs of the studied species are shown in Table 4. In the present study, the MIC of *T. fedtschenkoi* EOs ranged from 2-128 µg/mL in the wild type and from 2-16 µg/mL in the cultivated type for the tested bacteria.

DISCUSSION

There are some reports about high variability on the biological activity and chemical composition of EOs of the wild type thyme species depending on gathering site, harvesting period and the studied population**.** For example, Nejad Ebrahemi et al. identified 31-37 constituents in different parts of *T. caramanicus'* oil from Kerman, Iran, which oxygenated monoterpenes are the major portion of all samples (17).

Table 3. Antimicrobial Activity of the Studied EOs and MEs Using Disk Diffusion Agar

Test microorganisms	Inhibition Zone Diameter(mm)					
	EO		MEs			
	wild	cultivated	wild	cultivated	CP[a]	K[b]
Enterococcus faecalis ATCC 29212	32.5 ± 3.5	36.0 ± 5.6	-	-	22.5 ± 2.1	19.5 ± 2.1
Staphylococcus aureus ATCC 25952	27.5 ± 3.53	33.0 ± 2.8	-	-	23.5 ± 2.1	19.0 ± 1.4
Streptococcus sanguis PTCC 1449	45.0 ± 1.4	49.0 ± 1.4	19.0 ± 1.4	19.0 ± 1.4	27.5 ± 0.7	19.0 ± 1.4
Staphylococcus aureus ATCC 33591	29.0 ± 1.4	31.0 ± 1.4	18.0 ± 2.8	18.5 ± 2.1	22.5 ± 3.5	16.0 ± 2.8
Staphylococcus aureus ATCC 29213	38.0 ± 2.8	42.0 ± 2.8	18.5 ± 2.1	21.0 ± 1.4	25.5 ± 0.7	16.0 ± 2.8
Enterobacter aerogenes ATCC13048	26.5 ± 2.1	33.5 ± 0.7	-	-	22.0 ± 2.8	17.5 ± 3.5
Klebsiella pneumoniae ATCC 700603	18.0 ± 2.8	17.5 ± 3.5	-	-	19.0 ± 1.4	12.0 ± 2.8
Esherichia coli ATCC 25922	18.0 ± 2.8	25.5 ± 0.7	-	-	28.0 ± 2.8	18.0 ± 2.8
Proteus mirabilis ATCC43071	37.0 ± 1.4	38.5 ± 3.5			27.5 ± 3.5	19.0 ± 2.8
E coli O157:H7	29.5 ± 0.7	31.0 ± 1.4			26.0 ± 5.6	19.0 ± 1.4

Abbreviations: EO: essential oil; ME, methanol extract a Ciprofloxacin, b Kanamycin.

These results are in agreement with previously published data by Hazzit et al. (18) and Sarikurkcu et al (3). In the study of Mazooji et al. on *T. fedtschenkoi* from Firouzkouh, Iran, 10 compounds and thymol (89.08%) as main constituents were obtained (19). The chemical compositions of *T. fedtschenkoi* EOs of East-Azarbayjan were compared with other reports about Thymus species in Iran. It has been shown to be some differences and similarities between these reports. For example, Kalvandi et al. showed that thymol (42.8%), linalool (11.1%), γ-terpinene (6.0%), 1,8-cineole (5.6%), borneol (3.4%), and α-terpineol (1.8%) in *T. eriocalyx* were collected from Markazi province of Iran before flowering stage. Also, in full flowering stage, these compounds were changed to 43.1%, 4.0%, 6.3%, 3.3%, 4.9%, and 7.1%, respectively (20). However, in another study on *T. eriocalyx* in

Lorestan province, Iran, there were some reports about thymol and 1-borneol (66.3% and 10.5%, respectively) (4).

According to El Bouzidi et al, the major compound of wild and cultivated types of *T. broussonetii*, *T. maroccanus*, and *T. satureioides* were determined to be carvacrol (43.4%, 60.8%, 70.1%, 71.6%, 26.5%, and 26.0%, respectively) (8). In a study by Safaei-Ghomi et al., carvacrol content of Thymus species was 85.94%. Data obtained from this study showed a noticeable difference in percentage of thymol (29.96% and 47.48%) in the oils of the wild and cultivated types (4). The similarity in quantity of thymol has previously been published by Sarikurkcu et al. from the wild type of *T. longicaulis* in Turkey (3). The percentage of carvacrol resulted in this study was comparable with that of *T. algeriensis* studied by Teixeira et al. in Portugal (21) but did not resemble the results obtained for *T. pallescens* in Algeria (18) and in *T. caramanicus* Jalas from Iran (4). In the study of Khoshsokhan et al on 10 populations of *T. fedtschenkoi*, oxygenated monoterpenes were the main group of constituents in all samples along with much difference in thymol (2.45%-78.65%), carvacrol (1.84%-49.38%), α-terpineol (1.79%-17.1%), borneol (0.68%-3.8%), linalool (0.5%-39.05%), 1,8 cineole (0.53%-8.39%), and p-cymene (0.38%-7.74%) (6). In some previous reports, it was shown to be the in vitro antioxidant activity in several Thymus species EOs (3,8,20). The excessive amount of flavonoid and phenolic compounds in MEs of medicinal plants could be included in the free-radical-scavenging activity, which is because of more polar sub-fraction of them (22). The Roby et al. and Sokmen et al. stated that a few Thymus species such as some varieties of *T. tosevii*, *T. daenensis* subsp *lancifolius*, and *T. longidens* could be used as natural antioxidant source compounds (23,24). Based on the results of this study, *T. fedtschenkoi* can also be added to the above-mentioned species which have antioxidant activities. The high content of thymol in wild and cultivated *T. fedtschenkoi* explains their antioxidant activities. The antibacterial activity by presence of inhibition zone appeared from EOs in the cultivated type on all tested microorganisms significantly higher than that in the wild type, which can be attributed to the presence of high concentration of thymol (47.48%) against that of the wild type (29.96%), which is considered one of the oxygenated monoterpenes with well documented antibacterial and antifungal potential (25-27). *T. fedtschenkoi* EOs and MEs showed strong antibacterial activity against *S. sanguis* which made them an alternative compound in the maintenance of the oral health system.

Table 4. MICs of tested bacteria towards EOs and ME of *T. fedtschenkoi*

Test microorganism	**MIC(µg/ml)**			
	EO		MEs	
	wild	Cultivated	wild	cultivated
***Enterococcus faecalis* ATCC 29212**	128	16	>2048	2048
***Staphylococcus aureus* ATCC 25952**	2	2	16	32
***Streptococcus sanguis* PTCC 1449**	16	8	128	1024
***Staphylococcus aureus* ATCC 33591**	16	2	64	1024
***Staphylococcus aureus* ATCC 29213**	16	4	256	128
Enterobacter aerogenes ATCC13048	64	16	>2048	>2048
***Klebsiella pneumoniae* ATCC 700603**	256	64	>2048	>2048
***Esherichia coli* ATCC 25922**	64	16	>2048	>2048
***Proteus mirabilis* ATCC43071**	64	16	>2048	2048
***E coli* O157:H7**	64	16	>2048	>2048

Abbreviations: EO: essential oil; ME, methanol extract; MIC, Minimum inhibitory concentration

Antibacterial activities of different species of thyme documented in various studies include: anti-*E. coli* activity of *T. vulgaris* EOs in Slovak, *T. broussonetii*, *T. maroccanus* and *T. satureioides* EOs in Morocco (8), and *T. caramanicus* EOs in Iran (17). Our observations are in accordance with those reported by Teixeira et al. (21) on thymol rich EO, Ebrahimi et al. (17), and El Bouzidi et al. (8) with carvacrol rich of *T. mastichina*, *T. caramanicus*, and Moroccan thyme species, respectively. Here, anti–*Bacillus cereus* (8), EOs of *T. pallescens*, *T. algeriensis* and *T. dreatensis* in Algeria (18), *T. vulgaris* Eos can also be mentioned, to name just a few (25,28). In comparing wild and cultivated Thymus species oils, it appears that the cultivated type

exhibits strong antimicrobial activity, but in extracts obtained from the same plant, the same results were not observed. In MEs, poor antimicrobial activities were shown on streptococci and staphylococci species. The results of our study were much different from the report of El Bouzidi et al. in which domestication had not an effect on antimicrobial activity of studies of Thymus species and also EOs obtained from wild and cultivated thyme species showed the same inhibitory activity (MIC) on all tested microorganisms (8). The MIC of *T. vulgaris* EO ranged from 75- 1100 µg/mL (29), from 0.12-1.78 mg/mL of *T. broussonetii*, *T. maroccanus* and *T. satureioides* EOs , and from 15.6->500 µg/mL of *T. fedtschenkoi* EOs in Iraq for tested bacteria (30). The MIC values of *T. fedtschenkoi* in the study of Mohammed et al. (30) were similar to those of the wild type EOs of Iranian thyme, while MIC's values for all tested organisms were seen to be reduced by increasing thymol concentration in the cultivated type.. In the study of Ahmadi et al., the MIC of *T. fedtschenkoi* EOs prepared from Estahban was lower than that in our study, but the zone of inhibition was similar (31).

CONCLUSIONS

Our results demonstrated that there were some variations in the qualitative and quantitative composition in the oils obtained from the wild and cultivated Thymus species. The environmental condition, geographic distribution or genotyping of medicinal plants can explain these variations. Generally, the EOs of medicinal plants which contain a high percentage of phenolic compounds such as carvacrol and thymol possessing antibacterial activity are against pathogens. The results obtained in this study support this imagination that higher antimicrobial potential of Iranian Thymus oils, especially against *Klebsiella pneumoniae* ATCC700603 (extended-spectrum β-lactamases producer bacteria, ESBLs), *S. aureus* ATCC 33591 (methicillin-resistant *S. aureus*, MRSA), and *S. sanguis* PTCC 1449 (normal flora of oral) is conferred by high thymol content. Antimicrobial effect of EOs of the cultivated type had some relation to thymol content. The antibacterial activity, by presence of inhibition zone, appeared from EOs in the cultivated type on all tested microorganisms was significantly higher than that of the wild type, which can be attributed to the presence of high concentration of thymol (47.48%) against the wild type (29.96%). Present results show that the cultivation significantly affects the biological activity and may constitute an alternative solution for maintaining this medicinal plant.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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Authors' Contributions

Asad Kazemi and Faranak Elmi contributed in all trials, coordinated the data-analysis and contributed to the writing of the manuscript. Faranak Elmi is corresponding to the methods and experimental section and designed the research strategy and prepared the study.

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