The Comparison of Composition and Biological Activities in Wild and Cultivated of *Thymus kotschyanus* Essential Oils and Methanolic Extracts From East Azarbayjan, Iran

Haedeh Mobaiyen¹*, Gholamreza Dehghan², Faranak Elmi³, Amir Hossein Talebpour⁴

### Abstract

**Objective:** The gathering of wild type *Thymus kotschyanus* as medicinal plant is common in Iran because of their several biological properties which caused destruction of them. The aim of this research was to compare the chemical composition, antioxidant and antibacterial properties of wild and cultivated type of *T. kotschyanus* collected from East Azarbayjan, Iran.

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

**Results:** Thirty-five components were identified representing more than 90% of the total oil constituents. The oils were dominated by monoterpene hydrocarbons (50.2%) for wild type and oxygenated monoterpenes (63.38%) for cultivated type. The major components in the oils of the wild type were; thymol (29.96%), p-cymene (21.35%) and α-pinene (12.72%) and for the cultivated type were; thymol (47.48%) and α-pinene (5.49%). The MIC values of bacterial strains, which were sensitive to the EO of *T. kotschyanus*, were in the range of 2-128 µg/mL in wild type and 2-16 µg/mL in cultivated type.

**Conclusion:** Our data shows that, cultivation significantly affects the EOs’ chemical composition and antioxidant potential of *T. kotschyanus*. They signify a reasonable source of natural antibacterial substances that exhibited potential as a drug for use in pathogenic bacteria.

**Keywords:** Antimicrobial activity, Cultivation, Essential oil composition, *Thymus kotschyanus*

### Introduction

During the past decade not only emergence of multi-drug resistant (MDR) gram-negative and gram-positive bacteria is increased in community and hospital setting (1) but also the spread of mobile carbapenemases like *bla*<sub>NDM-1</sub> resulting in superbugs phenotypes which leaving very few treatment options available for clinicians (2). So this problem recommended to investigate the new drugs with antibacterial and bioactivity effects. The plants especially medicinal plants is a 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 especially prevalent in the Mediterranean area and recognized as medicinal plants by showing several biological and pharmacological properties (4). Essential oils (EOs) of all species of *Thymus* genus contain phenolic compounds which are with antibacterial potential (5). The biosynthesis of these compounds controlled not only genetically but also by environmental factors like locality and population of them (6).

The main picking off medicinal plants methods could be destructed wild type of them which led 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 create this hypothesis to beg the difference in biological compound and their activity. The aim of the present study was to compare the chemical composition of the EOs of *T. kotschyanus* plants collected from wild and cultivated populations in East Azarbayjan, Iran; also evaluation of the antimicrobial and antioxidant activates of EOs and methanol extracts (MEs) of wild and cultivated samples.

### Materials and Methods

Fresh aerial parts of wild *T. kotschyanus* were collected in May-April 2013 at full flowering stage from 1300 m alti-
tude 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. It typically consists of pale brown, calcareous, moderately alkaline fine sandy loam. The collected part 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 mixed with 500 mL of distilled water and subjected to hydro-distillation, using clevenger-type apparatus for 3 hours until total recovery of EOs. 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 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) were used for analyzing of 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 column was regulated by 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 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 selected from 40 to 300 amu.

The retention indices and mass spectral data of wild and cultivated T. kotschyanus EOs were compared with standard compounds for identifying of 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 monitoring at 517 nm (A_517). The absorbance of 2 mL DPPH solution was determined at 517 nm (A_517) as a blank control. The percentage of radical scavenging activity (RSA %) was calculated according equation 1:

\[
RSA\% = \frac{100 (A_517 - A_517')} {A_517} \tag{1}
\]

To quantify the results IG_m value, the effective concentration that could scavenge 50% of the DPPH radicals, were calculated (12). Ferric reducing antioxidant power (FRAP) method were done according to the method of Benzie and Strain by some modifications (13). The antioxidant activity of a sample 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 were regulated by using different concentrations of FeSO_4.7H_2O and FRAP values were determined for studied EOs.

The antibacterial activity of EOs and MEs were tested against the following bacteria: Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC25923), Staphylococcus aureus (ATCC33591), Streptococcus sanguis (PTCC1449), Enterobacter aerogenes (ATCC13048), Klebsiella pneumoniae (ATCC700603), Proteus mirabilis (ATCC43071), and E. coli O157:H7 (Razi Institute, Tehran, Iran). These strains kept at -70°C in tryptic soy broth (TSB) with 20% of glycerol. They were inoculated in blood agar (BA) and incubated 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 (10^6 CFU/mL) for tests and antimicrobial activity of the EOs was determined with agar disc diffusion method (15). Briefly, a suspension of tested microorganisms (10^6 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. kotschyanus and placed on the surface of inoculated plates. For antibacterial effect of MEs, various concentrations of wild and cultivated T. kotschyanus (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, impregnated on paper discs as well as described above. Tetracycline (30 µg/disk) and kanamycin (30 µg/disk) (HiMedia) were used as control. The Petri dishes incubated at 37°C for 24 hours. The tests were done 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. kotschyanus were determined by using agar dilution method as recommended by the Clinical & Laboratory Standards Institute (CLSI) guidelines (16). Briefly, the twofold serial broth dilution of MEs and EOs were prepared. Petri dishes containing MHA plates (Merck, Germany) in 45°C and EOs or MEs of wild and cultivated T. kotschyanus achieved concentration 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 standardized suspension of studied bacteria at 0.5 on the McFarland scale (10 CFU/mL). The bacterial suspension equal 10^6 CFU/mL was inoculated in each plate (12 plates for each series) similar 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 2 types of plant oils. Our data show that cultivation did not significant effect in EO production. Hydro distillation of dried and ground aerial parts of wild and cultivated T. kotschyanus yielded 1.55% and 1.70% of V/W of a greenish yellow color oil. Analysis of the oils led to the identification of 35 compounds (Table 1) which accounted for 99.57% and 99.67% of the total oils. The majority of compounds in oils were monoterpene hydrocarbons (50.2%) for wild type and oxygenated monoterpenes (63.38%) for cultivated type. The chemical analyses carried out revealed the richness and variability of wild and cultivated T. kotschyanus EOs. Thymol was found as a predominant component in both of the EOs, together with p-cymene and a-pinene for wild type and with a-pinene for cultivated T. kotschyanus. In the oil obtained from cultivated T. kotschyanus, we observed a decrease in the percentage of a-pinene and m-cymene against increase in thymol (Table 1).

The antioxidant activity of wild and cultivated T. kotschyanus 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 wild and cultivated Thymus showed antioxidant activity in both assays. The cultivation of our studied medicinal plant were affected the antioxidant activity of the T. kotschyanus. For the DPPH assay, the most potent oil was obtained from wild type (IC50: 2.74 µg/mL) and highest ferric reducing capacity resided with cultivated type (IC50: 1.56 µg/mL). The antioxidant capacity of the extracts was better than that of 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. kotschyanus are summarized in Table 3. Both EOs obtained from wild and cultivated T. kotschyanus showed inhibitory activity against most of microorganisms except Streptococcus sanguis ATCC1449, Staphylococcus aureus ATCC33591 and S. aureus ATCC29213.

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

Discussion
There are some reports about high variability on biological activity and chemical composition of EOs of wild type thyme species depending on gathering site, harvesting period and the studied population. For example, Nejad Ebrahemi and 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). 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. kotschyanus from Firouzkouh, Iran 10 compounds and thymol (89.08%) as main constituents were obtained (19). The chemical compositions of T. kotschyanus EOs of East-Azerbaijan were compared with other reports about Thymus species.
Table 2. Radical Scavenging and FRAP Values (mM Fe²⁺/mg Extract) of Wild and Cultivated T. kotschyanus

<table>
<thead>
<tr>
<th>Test</th>
<th>EO</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild</td>
<td>Cultivated</td>
</tr>
<tr>
<td>DPPH (IC₅₀ μg/mL)</td>
<td>2.74</td>
<td>3.24</td>
</tr>
<tr>
<td>FRAP value (mM Fe²⁺/mg extract)</td>
<td>1.83</td>
<td>1.56</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Test microorganisms</th>
<th>Inhibition Zone Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EOs</td>
</tr>
<tr>
<td></td>
<td>Wild</td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td>32.5±3.5</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 25952</td>
<td>27.5±3.53</td>
</tr>
<tr>
<td>Streptococcus sanguis PTCC 1449</td>
<td>43.0±1.4</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 33591</td>
<td>29.0±1.4</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 29213</td>
<td>38.0±2.8</td>
</tr>
<tr>
<td>Enterobacter aerogenes ATCC13048</td>
<td>26.5±2.1</td>
</tr>
<tr>
<td>Klebsiella pneumoniae ATCC 700603</td>
<td>18.0±2.8</td>
</tr>
<tr>
<td>Esherichia coli ATCC 25922</td>
<td>18.0±2.8</td>
</tr>
<tr>
<td>Proteus mirabilis ATCC43071</td>
<td>37.0±1.4</td>
</tr>
<tr>
<td>E coli O157:H7</td>
<td>29.5±0.7</td>
</tr>
</tbody>
</table>

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

In Iran. There are shown 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 collected from Markazi province of Iran, before flowering stage. and 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 in Lorestan province on T. eriocalyx from Iran is reported thymol and 1-borneol (66.3% and 10.5%), respectively (4).

According to El Bouzidi et al, the major compounds of wild and cultivated types of T. broussonetii, T. maroccanus and T. satureioides were determined 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 wild and cultivated types (4). The similarity in quantity of thymol has appeared from EOs in cultivated type on all tested microorganisms significantly higher than wild type which can be attributed to the presence of high concentration of thymol (47.48%) against wild type (29.96%), one of oxygenated monoterpenes with well documented antibacterial and antifungal potential (23-27). T. kotschyanus EOs and MEs showed strong antibacterial activity against S. sanguis which made them as alternative compound in maintenance oral health system. Antibacterial activity of different species of thyme were 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), in thymol rich EO, Ebrahimi et al, (17) and El Bouzidi et al, (8) by carvacrol rich of T. masticina, T. caramanicus and Moroccan thyme species, respectively. Anti-Bacillus cereus (8), EOs of T. pallescens, T. algeriensis and T. dreatensis in Algeria (18), T. vulgaris EOs (25,28).
In comparing wild and cultivated *Thymus* species oils, it appears the cultivated type exhibit strong antimicrobial activity, but in extracts obtained from the same plant did not observe the same results. In MEs poor antimicrobial activity were shown on streptococci and staphylococci species. The results of our study were marked difference with the report of El Bouzidi et al, in which domestication had not effect on antimicrobial activity of studies of *Thymus* species in which EOs obtained from wild and cultivated thyme species showed an inhibitory activity (MIC) the same on all the microorganisms tested (8). The MIC of *T. vulgaris* EO ranged from 75-1100 µg/mL (29), 0.12-1.78 mg/mL of *T. broussonetii*, *T. maroccanus* and *T. satureoides* EOs, 15.6->500 µg/mL of *T. kotschyanus* EOs in Iraq for tested bacteria (30). The MIC values of *T. kotschyanus* in Mohammed et al (30) were similar with wild type EOs of Iranian thyme while by increasing in thymol concentration in cultivated type, reduced MIC’s values were seen for all tested organisms. In Ahmadi et al, the MIC of *T. kotschyanus* EOs prepared from Estahban was lower than our study but the zone of inhibition was similar (31).

In conclusion, our results demonstrated that the variations in the qualitative and quantitative composition in the obtained oils from the wild and cultivated *Thymus* species. The environmental condition, geographic distribution or genotyping of medicinal plants can be explained these variation. Generally, the EOs of medicinal plants which contain a high percentage of phenolic compounds such as carvacrol and thymol possessing antibacterial activity is against pathogens. The results obtained in this study support that 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 (methylcin-resistant *S. aureus*, MRSA) and *S. sanguis* PTCC 1449 (normal flora of oral) is conferred by high thymol content. High antimicrobial effect of EOs of cultivated type had straight relation to thymol content. Therefore this study suggest that the possibility of using the oils of this *Thymus* species as natural antimicrobial component in toothpaste because of anti-streptococcal activity of that and as a food additive accompanied by antibiotics in life threaten infection such as ESBLs producing gram negative bacteria and MRSA associated infections. Present results show that the cultivation significantly affected the biological activity and may constitute an alternative solution for maintaining this medicinal plant.

**Ethical Issues**
This study was approved by ethical research committee, Tabriz Branch, Islamic Azad University.

**Conflict of Interests**
The authors declare no conflict of interests.

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**References**
