Journal of Applied Biological Sciences 6 (3): 13-18, 2012
ISSN: 1307-1130, E-ISSN: 2146-0108, www.nobel.gen.tr

Chemical Composition, Antimicrobial and Antioxidant Properties of *Thymus haussknechtii* Velen. Essential Oil

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Received: March 28, 2012
Accepted: May 02, 2012

Abstract

The chemical composition, the antimicrobial and antioxidant activities of essential oil of *Thymus haussknechtii* has been investigated. *T. haussknechtii* collected from Erzincan was subjected to hydrodistillation to yield essential oil which was subsequently analyzed by Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC/MS). The main components were 1,8-cineole (23.6 %), trans-verbenol (6.6 %), camphor (6.12 %) and caryophyllene oxide (6 %). The antimicrobial activity of the essential oil was assessed by both the disc diffusion and microbroth dilution methods. All tested microorganisms were inhibited by essential oil sample. The essential oil is also active on test fungi. *Aspergillus flavus* and *A. niger* were sensitive to the investigated oil with MIC values of 500 µg/mL. The antioxidant activity of the essential oil (100-1000 µg/mL) was determined by means of the DPPH radical-scavenging method. At 1000 µg/mL concentration of the essential oil *T. haussknechtii,* 35.11 ± 0.22 % DPPH was scavenging.

Key words: *Thymus haussknechtii,* essential oil, chemical composition, antimicrobial and antioxidant activities.

INTRODUCTION

Lamiaceae family, with about 220 genera, the genus *Thymus* is one of the eight most important genera with regard to the number of species included [1]. Thymus is very large genus, with more than 300 species. All of them are well-known aromatic and medical plants and also the oil of different species are used against various diseases [2]. This genus is represented in Turkey flora by 38 species, the ratio of endemism in the genus is 47 % [3,4]. Thymus species are commonly used for medicinal, herbal tea, flavouring agents in Turkey [5]. The previous studies have shown that thymus essential oils have strong antibacterial, antifungal, antiviral, antiparasitic and antioxidant activities [6,7,8]. *Thymus haussknechtii* Velen. is a dwarf shrub forming large cushions, growing wild on rocky slopes. *T. haussknechtii* is an endemic species in Anatolia of Turkey [3]. In the present study, the antibacterial, antifungal and antioxidant activities of the essential oil from *T. haussknechtii* were examined. The chemical composition of the essential oil was evaluated by using GC and GC/MS analysis.

MATERIALS AND METHODS

Plant material

A sample of *Thymus haussknechtii* was collected from Erzincan of Turkey during the vegetative phase. Collection locality, date and essential oil yield are given in Table 1. A voucher specimen was placed at the Herbarium of Balikesir University in Balikesir, Turkey.

Isolation of the essential oil

The dried plant sample was subjected to water distillation using a Clevenger-type apparatus for 3h according to the *European Pharmacopoeia* [9]. The percentage yield (%) of the oil calculated on a moisture-free basis is shown in Table 1. The essential oil obtained was dried over anhydrous sodium sulfate and stored in dark glass vials with Teflon-sealed caps at +4 °C before analyses.

GC and GC/MS analysis conditions

GC analysis was performed on an Agilent Technologies 6890N Network system gas chromatograph equipped with a FID and HP-Innowax column (60m x 0.25 mm i.d., 0.25 mm film thickness). Injector and detector temperature were set at 250 °C. The oven temperature was kept at 60 °C for 10 min and increased up to 220 °C at a rate of 4 °C min and then kept constant at 220 °C for 10 min and increased up to 240 °C at a rate of 1 °C and then kept constant at 240 °C for 10 min. Helium was the carrier gas, at a flow rate of 1.7 mL/min.

GC/MS analysis of the essential oil was performed under the conditions with GC (column, oven, temperature, flow rate of the carrier gas) using an Agilent Technologies 6890N Network system gas chromatograph equipped with an Agilent Technologies 5973 inert Mass Selective Detector (Agilent G3180B Two-Ways Splitters with Make up gas) in the electron impact mode (70eV). The mass range was between m/z 10 and 425.

Identification and quantification of essential oil constituents

Retention indices were calculated by using retention times of *n*-alkanes (C_7 – C_{29}) homologous series that were injected after the essential oil at the same chromatographic conditions according to Van den Dool method [10]. Identification of individual components of the essential oil was performed by computerized matching of the acquired mass spectra with those stored NIST 05/ Wiley 7n/Adams (comparison quality > 90%) mass spectral library of the GC/MS data system and/or by confirmed with the aid of retention indices from published sources [11]. The relative concentration of each compound in essential oil was quantified according to the peak area integrated by the analysis program. The individual compounds identified in the essential oil are given in Table 1

Antibacterial and anticandidal activities

The essential oil was also subjected to screening for their antimicrobial activity by using agar disc diffusion assay, microdilution broth assay [12,13]. The following bacteria and yeasts were tested: *Campylobacter jejuni* (ATCC 3291), *Enterobacter aerogenes* (NRRL 3567), *Escherichia coli* (ATCC 25292), *Listeria monocytogenes* (ATCC 7644), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus vulgaris* (NRRL 123), *Staphylococcus aureus* (ATCC 6538), *Serratia marcescens* (clinical isolate), *Shigella sonnei* (ATCC 25931), *Klebsiella pneumoniae* (clinical isolate), *Candida albicans* (ATCC 10231), and *Candida albicans* (clinical isolate).

The agar disc diffusion method was employed for the determination of antimicrobial activities of essential oil [12]. A suspension of the tested bacteria and yeasts (10^8 CFU/mL) was spread on the solid media plates. Stock solution of essential oil was prepared in dimethylsulfoxide (DMSO). Then filter paper discs (6 mm in diameter) were soaked with 20 µL of the stock solution and placed on the inoculated plates. After keeping at 2 °C for 2 h, they were incubated 37 °C for 24 h bacteria and *Candida albicans* (*Campylobacter jejuni* was incubated at 42 °C, microaerophilic conditions for 48 h). The diameters of the inhibition zones were measured in millimeters (Table 2).

Microdilution broth susceptibility assay was used determination of minimum inhibitory concentration (MIC) [13]. Stock solution of essential oil was prepared in dimethylsulphoxide (DMSO). Serial dilution of essential oil was prepared in sterile distilled water in 96-well microtitre plates. Freshly grown bacterial suspension was standardized to 10^8 CFU/mL (McFarland no. 0.5) in double-strength Mueller-Hinton broth (*Listeria monocytogenes* in Buffered Listeria Enrichment Broth and yeast suspension of *Candida albicans* in Saboraud Dextrose Broth). Sterile distilled water served as growth control. 100 µL of each microbial suspension were then added to each well. The last row containing only the serial dilutions of antibacterial agent without microorganism was used as negative control. After incubation at 37 °C for 24 h. (*Campylobacter jejuni* was incubated at 42 °C, microaerophilic conditions for 48 h) the first well without turbidity was determined as the minimal inhibitory concentration (Table 2). Chloramphenicol (1000µg/mL) and Ketoconazole (1000µg/mL) served as positive controls.

Antifungal activity

Screening for antifungal activity of the stock solution of the essential oil was performed qualitatively using the disc diffusion method and microdilution broth assay (Table 3) against saprophytic fungi namely *Alternaria alternata*, *Aspergillus flavus, Aspergillus niger, Penicillum expansum* and *Penicillium lanosum*. In order to obtain conidia, the fungi were cultured on Czapex Dox Agar (Merck) and Malt Extract Agar medium (Merck) in 9 cm petri dishes at 25 °C, for 7-10 days. Harvesting was carried out by suspending the conidia in a 1% (w/v) sodium chloride solution containing 5% (w/v) DMSO. The spore suspension was then filtered and transferred into tubes and stored at -20 °C, accordingly to Hadecek and Greger 2000 [14]. The spore suspension was adjusted with 1% (w/v) sodium chloride solution containing 5% (w/v) DMSO to concentration of approximately 10^4 CFU/mL.

Antifungal activity testing was carried out using standard disc diffusion assays. Czapex Dox Agar and Malt Extract Agar medium were used for the culture maintenance and the bioassays. Then 0.5 mL of the prepared inoculum was spread on plates. For preparing the test discs, 20 µL of the stock solution of essential oil was pipetted onto 6 mm filter paper discs, which were carefully transferred onto the surface of seeded agar plate. Ketoconazole (1000µg/mL) was used as a positive control. After incubation at 25 °C for 72 h. and the diameters of the inhibition zones were measured in millimetres (Table 3).

Minimum inhibitory concentration (MIC) determination was performed by microdilution broth susceptibility assay. Stock solution of essential oil was prepared in dimethylsulphoxide (DMSO). Serial dilutions of essential oil were prepared in Malt Extract Broth in 96-well microtitre plates. 100 µL of each spore suspension were then added to each well after the microplates were incubated for 72 h at 25 °C. The lowest concentration without any visible growth was defined as MIC (Table 3).

The inhibition of fungal growths expressed in percentage terms determined the tested filamentous fungi cultured on Czapex Dox Agar and Malt Extract Agar medium. The fungi spores were inoculated onto the centre of the petri dishes via a pin, then 20 µL stock solutions was applied to sterile paper discs (6 mm in diameter) and latter placed on the fungi spores and finally incubated at 25 °C for 72 h. The inhibition of fungal growths expressed in percentage terms was determined on the growth in test plates compared to the respective control plates as given % inhibition [15] (Table 3).

Table 1 Chemical composition of the essential oil of *Thymus haussknechtii*

			T. haussknechtii		
Collector number			BY 16828		
Locality and Collecting date			Erzincan: Kemaliye-Arapkir way, Fırat Valley 900-1000m, july 2008		
Yield of the oil (%)			0.34%		
Compound	**RRI**	**Composition (%)**	**Compound**	**RRI**	**Composition (%)**
Tricyclene	1008	1.20	*cis*-p-menth-2-en-1-ol	1677	0.24
α-Pinene	1021	4.89	α-Thujenal	1686	1.02
α-Thujene	1024	1.30	Alloaromadendrene	1699	0.12
Camphene	1070	4.24	cis-Verbenol	1704	1.20
β-Pinene	1119	0.84	trans-Pinocarveol	1705	1.00
Sabinene	1136	0.45	cis-p-Menth-2,8-dienol	1714	0.24
Myrcene	1195	0.30	δ-Terpineol	1715	0.62
α-Terpinene	1213	0.60	trans-verbenol	1723	6.60
Limonene	1238	0.70	1,8-Menthadien-4-ol	1728	0.24
1,8-Cineole	1247	23.6	α-Terpineol	1737	1.00
β- Phellandrene	1249	0.29	Borneol	1743	4.20
γ- Terpinene	1297	1.02	Verbenone	1750	2.28
E-β-Ocimene	1306	0.07	trans-p-Menth-2-ene-1,8-diol	1759	0.97
p-Cymene	1328	2.00	Carvone	1772	0.60
Delta-carene	1339	0.30	cis-piperitol	1773	0.08
3-Octenyl acetate	1446	0.10	Geranylacetate	1780	0.12
3-Octanol	1459	0.10	Myrtenol	1812	0.48
trans-Linalool oxide	1510	0.70	E-β-Damascenone	1835	0.06
1-Octen-3-ol	1516	0.14	trans-Carveol	1842	0.98
trans-Sabinene Hydrate	1531	2.26	Geraniol	1847	0.93
cis-Linalool oxide	1538	0.47	p-Cymene-8-ol	1852	0.50
Nerol oxide	1540	0.08	Isocaryophyllene oxide	1947	0.40
α-Campholene aldehyde	1561	1.10	Caryophyllene oxide	1961	6.00
trans-Crysanthemal	1577	0.20	E-Nerolidol	1980	0.42
Camphor	1586	6.12	Germacrene-D-4-ol	1996	0.18
Linalool	1608	1.22	Elemol	2012	0.90
cis-Sabinene hyrate	1609	0.66	Spathulenol	2043	0.42
Linalyl acetate	1618	1.20	Cumin alcohol	2056	0.30
trans-p-menth-2-en-1-ol	1622	0.42	a-Eudesmol	2111	0.14
Pinocarvone	1633	0.60	β-Eudesmol	2115	0.16
Bornyl acetate	1639	0.24	Diisobutyl phthalate	2329	2.06
Endobornyl acetate	1642	0.28	Monoterpene hydrocarbons		18.20
6-Methyl-3,5-Heptadien-2-one	1652	0.06	Oxygenated monoterpenes		64.41
Terpinen-4-ol	1658	3.26	Sesquiterpenes		9.04
			Others		3.82
			Total identified		95.47

RRI, relative retention indices; tr, trace (< 0.05 %)

Table 2 The antibacterial and anticandidal activities of the essential oil of *T. haussknechtii*

Microorganism	Inhibition zone [mm]		MIC[µg/mL]	
	T. haussknechtii	standard	*T. haussknechtii*	standard
Campylobacter jejuni ATCC 33291	9	25[C]	250	-[C]
Enterobacter aerogenes NRRL 3567	9	22[C]	250	-[C]
Escherichia coli ATCC 25292	9	22[C]	250	-[C]
Listeria monocytogenes ATCC 7644	9	24[C]	250	-[C]
Pseudomonas aeruginosa ATCC 27853	9	23[C]	250	-[C]
Proteus vulgaris NRRL 123	9	24[C]	250	-[C]
Staphylococcus aureus ATCC 6538	9	22[C]	250	-[C]
Serratia marcescens (clinical isolate)	8	24[C]	500	-[C]
Shigella sonnei ATCC 25931	8	25[C]	500	-[C]
Klebsiella pneumoniae (clinical isolate)	8	22[C]	500	-[C]
Candida albicans ATCC 10231	9	24[K]	250	-[K]
Candida albicans (clinical isolate)	9	27[K]	250	-[K]

[C] : Chloramphenicol
[K] : Ketoconazole
-: negative (not grow)

Table 3 The antifungal activity of essential oil of *T. haussknechtii* (MIC and % Inhibition)

Microfungi	Inhibition zone [mm]		MIC[µg/mL]		% inhibition	
	T. haussknechtii	ketoconazole	*T. haussknechtii*	ketoconazole	*T. haussknechtii*	ketoconazole
Aspergillus flavus	8	22	500	-	25	84
Aspergillus niger	8	13	500	-	14	40
Penicillium expansum	8	16	1000	-	9	65
Penicillium lanosum	7	14	1000	-	15	54
Alternaria alternata	7	18	1000	-	8	82

-:negative (not grow)

Table 4. DPPH radical- scavenging activity of *T. haussknechtii* **essential oil.**

concentration (µg/mL)	DPPH scavenging ability (%, mean ± SD)*	
	T. haussknechtii	BHA
100	6.90 ± 0.15 a	93.79 ± 0.75 a
200	9.04 ± 0.14 b	-
300	11.03 ± 0.19 c	-
400	13.66 ± 0.32 d	-
500	17.68 ± 0.14 e	-
600	22.23 ± 0.33 f	-
700	24.79 ± 0.17 g	-
800	28.30 ± 0.32 h	-
900	31.57 ± 0.29 i	-
1000	35.11 ± 0.22 j	-

*Each represents the mean of three replicates
Numbers in columns (a-j) followed by the same letter are not significantly different (P>0.05).
BHA: Butylated Hydroxyanisole
SD: Standard Deviations

Inhibition % = 100X($C - T$) / C

where C is the diameter of fungal growth on the control, T is the diameter of fungal growth on the test plate. The activities of the essential oil have been compared with the activity of standard antifungicide Ketoconazole.

DPPH radical scavenging assay

The essential oil solution (1 µg/mL) was prepared by dissolving the essential oil in methanol. Radical scavenging activity (RSA) of *Thymus* essential oil against stable 2,2-diphenyl-1 –picrylhydrazyl radical (DPPH) was determined by a slightly modified DPPH radical scavenging assay [16] (Table 4). It is widely used reaction based on the ability of antioxidant molecule to donate hydrogen to DPPH; which consequently turns into an inactive form. The solution of DPPH was prepared freshly and daily. Briefly, 1mL of a 1mM solution of DPPH radical methanol was mixed with 3 mL of essential oil solution (final concentration of essential oil: 100-1000 µg/mL), and left for 30 min (incubation period) in the dark at room temperature the absorbance was scanned against a blank at 515 nm. This activity is given as % DPPH radical-scavenging calculated according to the equation:

%DPPH radical-scavenging = $[(A_0 - A_S)/ (A_0)]$ X 100

where A_0 is the absorbance of the control (containing all

reagents except the test compound), and A_S is the absorbance of the tested sample. Test were carried out in triplicate and butylated hydroxyanisole (BHA) was used as positive control.

Statistical analysis: Means were compared one-way analysis of variance (ANOVA) and subsequently, means were separated using Tukey's Honestly Significant Difference (HSD) post hoc test. A statistical software program (SPSS, version 15.0 for Windows, SPSS Science, Chicago, IL) was used for data analysis. Results were considered statistically significant when $P < 0.05$.

RESULT

The essential oil isolated by hydrodistillation from the aerial parts of *T. haussknechtii*, and yield 0.34 % (v/w), based on dry weights. The essential oil of *T. haussknechtii* was analyzed by GC and GC-MS. The chemical composition of the oil can be seen in Table 1. Sixty-five components were identified in the essential oil of *T. haussknechtii*, the main components were found to be 1,8-cineole (23.6%), trans-verbenol (6.6%), camphor (6.12%) and caryophyllene oxide (6.0%).

The antibacterial and antifungal activities of *T. haussknechtii* essential oil has been tested in vitro against 10 pathogenic bacteria, 5 filamentous fungi and 2 yeast by the Agar Disc Diffusion Method, Microdilution Broth Susceptibility Assay (Table 2 and 3). All tested microorganisms were inhibited by essential oil sample. The essential oil exhibited activity against all bacteria and yeast tested, with the inhibition zone values ranging from 8 to 9 mm. The results of antibacterial activity according to the Minimum Inhibitory Concentration, *Shigella sonnei, Klebsiella pneumoniae, Serratia marcescens* (MIC values of 500 µg/mL) displayed lower sensitivity than the other microorganisms to tested essential oil. The essential oil also exhibited activity against all *Candida albicans* strains with MIC values of 250 µg/mL (Table 2).

The result of testing the antifungal activity of *T. haussknechtii* essential oil is shown in Table 3. The essential oil is also active on test fungi. *Aspergillus niger* and *A. flavus,* were more sensitive to the investigated oil with MIC values of 500 µg/mL*, Alternaria alternata, Penicillium expansum, P. lanosum* showed similar susceptibility to the investigated oil with MIC values of 1000 µg/mL (Table 3). The inhibition of growths on fungi expressed in percentage terms was determined on growth in test plates. *A. flavus* was more sensitive (25%) against *T. haussknechtii* essential oil compared with other filamentous fungi.

It is shown in Table 4 that the essential oil was capable of varying degrees of scavenging action against DPPH. The % DPPH radical scavenging activity values of the essential oil *T. haussknechtii* was determined as 6.90 ± 0.15 % at 100 µg/mL concentration. At the 1000 µg/mL the essential oil concentration of *T. haussknechtii* 35.11 ± 0.22 % DPPH was scavenging.

DISCUSSION

Several reports have been represented the composition and the biological properties of *Thymus* essential oils [7,17-20]. These studies have indicated the existence of marked chemical differences among oils extracted from different species or varieties. These variations are likely to influence the antimicrobial and antioxidant activity of the oil and are generally a function of three factors: genetically determined properties, the age of the plant and the environment.

We now report the antimicrobial and antioxidant capacity and chemical composition of the essential oil isolated from the aerial parts of *T. haussknechtii* collected during the vegetative phase. *T. haussknechtii* essential oil revealed an abundance of monoterpene hydrocarbons (18.20%), oxygenated monoterpenes (64.41%), sesquiterpenes (9.04%) and others (3.82 %).

In the oils the monoterpenes usually make up more than 90 percent. Sesquiterpenes are always present, but with only few exceptions in minor percents [21].

In the previous studies; Bagcı and Baser 1,8-cineole (21.5%) reported to be the main constituents of the volatile oil of *T. haussknechtii* collected from Elazığ, Harput-Ankuzubaba Mountain [22]. In 1992, Baser et al. reported Linalool (19.91%) and borneol (10.35%) as the major constituents of the oil of *T. haussknechtii* collected from Elazığ of Turkey [23]. These variations in the essential oil composition might have arisen from several differences (plant type, geographical location and collection season) [24]. In our study, the high chemical divergence among populations was correlated with the geographic distance and chemotypes occurred at a local scale.

All tested microorganisms was inhibited by *T. haussknechtii* essential oil. Recent studies demonstrated that the essential oils of other *Thymus* species are among the most potent essential oils with regard to antimicrobial properties [25-28]. The antimicrobial activity of the *T. haussknechtii* essential oil could be due to 1,8-cineole [29, 30]. Minor components have a critical part to play in antibacterial activity, possibly by producing a synergistic effect between other components [31].

DPPH is often used as a substrate to evaluate antioxidative activity of antioxidants [32]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [33]. The antioxidant activity of T. *haussknechtii* essential oil was lower than butylated hydroxyanisole (BHA), nevertheless the essential oils can be considered effective natural antioxidant (Table 4). The antioxidant activities of flavonoids increased with the number of hydroxyl groups. There were also some antioxidant activities in herbs that may be attributable to other unidentified substances or to synergistic interactions [34].

As can be seen from the Tables 2- 4 essential oil is the most promising for both antimicrobial and antioxidant activity. *T. haussknechtii* essential oil can be used as preservative ingredient in the food, medical industries and sources of aroma chemicals.

Acknowledgments

The authors want to thank Balikesir University Research Fund (Project no: 2009/22) for its financial support and also we would like to thank Prof. Dr. Bayram Yildiz for helps the field studies and identification of the *Thymus* species.

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