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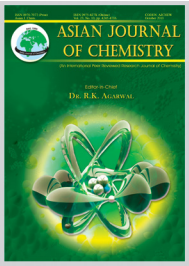

Composition and *in vitro* Antimicrobial and Antioxidant Activities of the Essential Oils of Four *Thymus* Species in Turkey

A.D. AZAZ and S. CELEN*

Faculty of Science and Letters, Department of Biology, Balikesir University, Çagis Campus, 10145 Balikesir, Turkey

*Corresponding author: Tel: +90 266 6121000, Fax: +90 266 6121215, E-mail: scelen@balikesir.edu.tr

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The genus *Thymus* (Lamiaceae) is represented in Turkey by 38 species. Aerial parts of *Thymus canoviridis* Jalas (I and II), *T. cilicicus* Boiss. & Bal., *T. comptus* Friv. and *T. revolutus* Celak. collected from different localities of Turkey were subjected to hydro distillation to yield essential oils and analyzed by GC and GC/MS. Thymol was found as the main component in the oils of *T. canoviridis* (I) (60.44 %), *T. canoviridis* (II) (64.79 %), *T. cilicicus* (34.03 %), *T. comptus* (55.14 %) and *T. revolutus* (66.96 %). All test bacteria and *Candida albicans* were inhibited by all the essential oils. The essential oils showed weak antifungal activity against all microfungi tested. Antioxidant activity was tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method.

Key Words: *Thymus sp.*, Essential oil, GC/MS analysis, Antimicrobial activity, Antioxidant activity, Thymol.

INTRODUCTION

The genus *Thymus L.* (Lamiaceae) consists of about 215 species of herbaceous perennials and sub shrubs. The Mediterranean region can be described as the centre of the genus[1]. This genus is represented in Turkey flora by 38 species (64 taxa). 24 of which are endemic[2,3]. Thymus species growing in Turkey are extensively used in folk medicine, herbal tea, flavouring agents (condiment and spice)[4]. The infraspecific variability of the essential oils of the genus *Thymus* is widely accepted. Thymol and carvacrol are the major compounds in most of the *Thymus* essential oils, while non-aromatic terpenes may also be present as main constituents[1,5]. The antimicrobial and antioxidant properties of essential oils have been known for a long time and a number of investigations have been conducted on their antimicrobial activities, using bacteria, viruses and fungi[6-8]. Recent studies have showed that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolicalytic and antioxidant activities[1,9,10]. This aim of this study is to evaluate the chemical composition, antibacterial, antifungal and antioxidant activities of the essential oils of *Thymus canoviridis* Jalas (I and II), *T. cilicicus* Boiss. & Bal., *T. comptus* Friv. and *T. revolutus* Celak. collected from different localities of Turkey.

EXPERIMENTAL

Information on the plant material used in this study is given in Table-1. The voucher specimens have been deposited at the Department of Biology, Balikesir University, Balikesir, Turkey.

Extraction of the essential oil: Air-dried aerial parts of plants were hydrodistilled for 3 h using a Clevenger-type apparatus. The percentage yields (%) of the oils calculated on moisture-free basis are given in Table-1.

Gas chromatography: The GC analyses were carried out using Hewlett-Packard 6890 GC with FID. A HP-5 MS capillary column (30 m × 0.25 mm i.d. 0.25 µm film thickness) was used. Helium was used as a carrier gas (1.4 mL/min). The column was temperature programmed as follow: 5 min at 45 ºC; then at 3 ºC/min to 220 ºC and held for 10 min. The injector and detector temperatures were to 220 and 250 ºC, respectively. Injection was carried out automatic mode. Samples [0.5 µL of the solution in hexane (1:100)] were injected by split less technique into helium carrier gas. The percentages were obtained from electronic integrator (EI) using flame ionization detection (FID, 220 ºC).

Gas chromatography-mass spectrometry: GC/MS analyses of the essential oils were carried out on Hewlett Packard 5970 A mass selective detector (MSD), directly coupled to a HP 6890 GC. The column, temperature programme and injection were performed as described above. Injection was carried out automatic mode. Library search was carried out using Wiley Library. El mass spectra were measured at 70 eV ionization voltages over mass range 10-400 µ.

Agar disc diffusion method: The agar disc diffusion method was employed for the determination of antimicrobial

TABLE-1
INFORMATION ON *Thymus sp*. AND ESSENTIAL OILS COMPOSITION

Species		*Thymus canoviridis* (I)	*Thymus canoviridis* (II)	*Thymus cilicicus*	*Thymus comptus*.	*Thymus revolutus*
Collector number		B.Y.15832a	B.Y.15832b	T.D.3075	B.Y.16204	T.D.3072
Locality and		Erzincan:Otlukbeli mountain, at 1700 m	Erzincan:Otlukbeli mountain, at 1700 m	Mugla: between Mugla and Köycegiz at 5 km 100 m	Çanakkale: between Gelibolu and Kesan at 38 km 15 m	Antalya: between Manavgat and Serik at 19 km 20 m
Collecting date		08.08.2004	08.08.2004	13.06.2005	21.06.2005	13.06.2005
Yield of the oil (%)		0.33	0.61	0.71	1.20	1.50
Compounds	RRI					
α-Thujene	1060	0.37	0.49	1.25	0.57	1.10
α-Pinene	1068	1.92	0.78	1.45	1.09	1.84
Camphene	1088	1.15	0.38	5.48	1.13	2.50
β-Pinene	1124	0.75	0.27	2.65	0.41	0.33
Myrcene	1152	0.43	0.28	0.36	0.73	0.45
α-Terpinene	1188	-	0.53	1.37	0.83	0.77
p-Cymene	1213	-	1.89	0.84	9.84	2.35
1,8-Cineole	1217	1.66	2.03	6.54	4.59	5.64
trans-β-Ocimene	1248	-	-	0.45	0.94	-
γ-Terpinene	1259	0.41	1.22	2.58	7.14	8.13
Linalool	1370	3.49	2.40	2.85	0.09	-
Camphor	1447	0.33	2.46	7.57	0.24	-
Terpinen-4-ol	1549	-	-	0.59	-	1.00
Terpinolen	1581	0.88	-	8.29	0.32	-
Borneol	1665	0.49	-	0.35	0.08	0.67
Linalyl acetate	1754	-	3.51	0.65	-	-
Geraniol	1875	2.97	3.46	-	-	-
Thymol	1911	60.44	64.79	34.03	55.14	66.96
Carvacrol	1916	0.88	0.22	12.11	6.44	10.12
Geranyl acetate	1985	5.32	1.60	-	-	0.22
β-Bourbonene	2001	0.52	-	-	0.27	-
β-Caryophyllene	2014	8.49	6.58	1.70	0.46	5.35
β-Cubebene	2016	0.37	-	-	0.21	-
Allo aromadendrene	2019	0.41	0.41	0.97	0.19	0.56
α-Humulene	2024	-	0.29	-	-	0.18
α-Amorfen	2137	0.35	0.21	0.55	0.15	-
Germacrene-D	2142	0.82	0.53	0.55	1.92	0.25
β-Bisabolen	2209	4.39	2.63	-	6.74	-
Δ-Cadinene	2265	0.51	0.68	1.83	0.48	1.25
Geranyl butyrate	2273	0.33	0.58	-	-	-
Spathulenol	2295	0.83	0.84	2.83	-	-
Caryophyllene oxide	2306	1.49	0.94	2.17	-	0.45

TABLE-2
INHIBITION ZONES OF *THYMUS* ESSENTIAL OILS ACCORDING TO THE AGAR DISC DIFFUSION METHOD (mm)

Microorganisms	Stock solution					
	Diameter of inhibition zone (mm)					
	A	B	C	D	E	Control
Enterobacter aerogenes NRRL 3567	10	10	11	11	10	22^{C}
Escherichia coli ATCC 25292	10	10	11	10	10	22^{C}
Listeria monocytogenes ATCC 7644	9	9	9	9	8	24^{C}
Pseudomonas aeruginosa ATCC 27853	10	10	10	11	9	23^{C}
Proteus vulgaris NRRL 123	9	9	8	7	9	24^{C}
Serratia marcescens (clinic isolate)	11	11	11	10	11	24^{C}
Staphylococcus aureus ATCC 6538	9	9	9	7	8	22^{C}
Candida albicans (clinic isolate)	9	8	7	6.5	7	27^{K}

A: *T. canoviridis* I; B: *T. canoviridis* II; C: *T. cilicicus*; D: *T. comptus;* E: *T. revolutus;* ^{C}: chloramphenicol; ^{K}: ketoconazole; Stock solution : 4 mg essential oil + 2mL DMSO

activities of essential oils[11]. A suspension of the tested microorganism (10^{8} CFU/mL) was spread on the solid media plates. Stock solutions of essential oils were prepared in dimethyl sulfoxide (DMSO). Than Filter paper discs (6 mm in diameter) were soaked with 20 µL of the stock solutions and placed on the inoculated plates. After keeping at 2 ºC for 2 h, they were incubated 37 ºC for 24 h bacteria and *Candida albicans*. The diameters of the inhibition zones were measured in millimetres (Table-2).

TABLE-3
MINIMUM INHIBITORY CONCENTRATION (μg/mL) OF *THYMUS* ESSENTIAL OILS

Microorganisms	Sources	A	B	C	D	E	Control
Enterobacter aerogenes	NRRL 3567	250	250	125	125	250	-[C]
Escherichia coli	ATCC 25292	250	250	250	250	250	-[C]
Listeria monocytogenes	ATCC 7644	1000	1000	500	500	1000	-[C]
Pseudomonas aeruginosa	ATCC 27853	250	250	250	250	250	-[C]
Proteus vulgaris	NRRL 123	500	250	500	500	500	-[C]
Serratia marcescens	Clinic isolate	125	125	125	125	125	-[C]
Staphylococcus aureus	ATCC 6538	500	250	500	500	500	-[C]
Candida albicans	Clinic isolate	500	500	500	1000	500	-[K]

A: *T. canoviridis* I; B: *T. canoviridis* II; C: *T. cilicicus* ; D: *T. comptus;* E: *T. revolutus;* [C] : chloramphenicol; [K] : ketoconazole - : No turbidirty

Determination of minimum inhibitory concentration (MIC): Microdilution broth susceptibility assay was used[12]. Stock solutions of essential oils were prepared in dimethyl sulphoxide (DMSO). Serial dilutions of essential oils were prepared in sterile distilled water in 96-well microtitre plates. Freshly grown bacterial suspension in double strength Mueller Hinton Broth (Merck) but *Listeria monocytogenes* in Buffered Listeria enrichment broth (Oxoid) and yeast suspension of *Candida albicans* in Saboraud dextrose broth were standardized to 10^8 CFU/mL (McFarland no. 0.5). Sterile distille 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 ºC for 24 h. The first well without turbidirty was determined as the minimal inhibitory (Table-3). Chloramphenicol and ketoconazole served as positive controls.

Antifungal studies: Screening for antifungal activities of the stock solution of the essential oils were performed qualitatively using the disc diffusion method (Table-4) against saprophytic fungi namely *Alternaria brassicola, Aspergillus flavus, Aspergillus niger* and *Penicillum expansum* cultured on Czapex Dox Agar (Merck) and Malt extract medium (Oxoid). 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 placed on the fungi spores and incubated at 25 ºC 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[13].

$$\text{Inhibition \%} = 100 \times [(\text{C-T})/\text{C}]$$

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 complex have been compared with the activity of standard antifungicide ketoconazole.

DPPH radical scavenging assay: An essential oil solution (1 μg/mL) was prepared by dissolving the essential oil in methanol. Radical scavenging activity (RSA) of *Thymus* essential oils against stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was determined by a slightly modified DPPH radical scavenging assay[14]. 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 daily. Briefly, 1 mL of a 1 mM solution of DPPH radical methanol was mixed with 3 mL of essential oil solution (final concentration of essential oil: 100-750 μg/mL) and left for 0.5 h (incubation period) in the dark at room temperature the absorbance was read against a blank at 515 nm. This activity is given as % DPPH radical-scavenging calculated according to the equation:

$$\text{\% DPPH radical-scavenging} = [(A_0 - A_S)/(A_0)] \times 100$$

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 $P < 0.05$.

RESULTS AND DISCUSSION

Aerial parts of *Thymus canoviridis* Jalas (I and II), *T. cilicicus* Boiss. & Bal., *T. comptus* Friv. and *T. revolutus* Celak. collected from different localities of Turkey (Table-1), were hydrodistilled and the essential oils were analized by GC and GC/MS. The resulting components of the essential oils are shown in Table-1 along with yield information. The GC-GC/MS showed that the essential oils of *Thymus canoviridis* (I-II) were mainly represented by thymol (60.44 %-64.79 %), β-caryophyllene (8.49 %-6.58 %) while thymol (34.03 %) and carvacrol (12.11 %) were found major components of *Thymus cilicicus* essential oil. Thymol (55.14 %), *p*-cymene (9.84 %) and γ-terpinene (7.14 %) were the main components of *Thymus comptus* essential oil while *Thymus revolutus* essential oil was rich in thymol (66.96 %), carvacrol (10.12 %), γ-terpinene (8.13 %). From present results, thymol was found to be a main component of the essential oils of *Thymus canoviridis* (I-II), *Thymus cilicicus, Thymus comptus, Thymus revolutus* (Table-1). Generally, Thymus oils, which is characterized by the high percentage of thymol, carvacrol, linalool, *p*-cymene, γ-terpinene, borneol, 1,8-cineole and geraniol[5]. In earlier study, essential oil of *Thymus canoviridis* was reported to contain carvacrol (29.51 %), geraniol (13.25 %), thymol (9.49 %) as main components[15]. The essential oil of *Thymus cilicicus* was reported to contain α-pinene (16.74 %) and 1,8 cineole (10.39 %)[16]. The essential oil of *Thymus comptus* contained thymol (36 %-50 %) and in the essential oil of *Thymus revolutus p*-cymene (39 %) and borneol (12 %) were the main constituent[4].

The composition of the essential oil depends on plant type, geographical location and collection season[17]. In the present

TABLE-4
ANTIFUNGAL ACTIVITY OF *THYMUS* ESSENTIAL OILS (INHIBITION %)

Microorganisms	A	B	C	D	E	Ketoconazole
Alternaria brassicola	8	6	5	0	10	78.00
Aspergillus flavus	7	16	0	0	0	83.63
Aspergillus niger	30	28	8	25	15	40.00
Penicillum expansum	0	0	0	9	0	65.00

A: *T. canoviridis* I; B: *T. canoviridis* II; C: *T. cilicicus;* D: *T. comptus;* E: *T. revolutus*

TABLE-5
DPPH RADICAL-SCAVENGING ACTIVITY OF ESSENTIAL OILS

Concentrations (µg/mL)	DPPH Scavenging ability (%. mean ± SD)*					
	T. canoviridis (I)	*T. canoviridis* (II)	*T. cilicicus*	*T. comptus*	*T. revolutus*	BHA
100	19.16 ± 0.22 a	23.68 ± 0.32 a	14.76 ± 0.47 a	20.39 ± 0.27 a	29.54 ± 0.59 a	93.79 ± 0.75 a
125	24.06 ± 0.25 b	29.35 ± 0.20 b	18.87 ± 0.13 b	26.42 ± 0.29 b	37.33 ± 0.31 b	95.15 ± 0.33 a
250	33.21 ± 0.54 c	37.40 ± 0.35 c	25.97 ± 0.48 c	35.45 ± 0.24 c	46.82 ± 0.51 c	-
375	43.29 ± 0.20 d	48.33 ± 0.38 d	34.18 ± 0.26 d	44.94 ± 0.39 d	56.56 ± 0.25 d	-
500	52.26 ± 0.27 e	57.19 ± 0.49 e	42.95 ± 0.36 e	54.20 ± 0.36 e	64.85 ± 0.43 e	-
625	61.03 ± 0.35 f	66.18 ± 0.37 f	51.02 ± 0.28 f	62.79 ± 0.30 f	72.75 ± 0.52 f	-
750	70.42 ± 0.26 g	74.93 ± 0.20 g	60.09 ± 0.83 g	72.35 ± 0.21 g	82.69 ± 0.54 g	-

*Each represents the mean of three replicates; Numbers in columns (a-g) followed by the same letter are not significantly different (P > 0.05); BHA: Butyl Hydroxylanisole; SD: Standard deviations

study, using the agar disc diffusions method and microdilution broth susceptibility assay (Tables 2 and 3), the essential oil of *Thymus comptus* and *Thymus cilicicus* showed a minimal inhibitory concentration value of 125 (µg/mL) against *Enterobacter aerogenes. Proteus vulgaris* and *Staphylococcus aureus* was inhibited best by *Thymus canoviridis* (II) and the other oils tested also showed inhibitory activities.

The tested fungi were *Aspergillus flavus*, *A. niger, Penicillium expansum* and *Alternaria brassicola*. The results showed that *A. niger* was more sensitive (30 %, 28 %, 25 %) against the essential oils compare with other filamentous fungi (Table-4). Fundamental studies have revealed the antifungal activity of alcohols and sesquiterpenic lactones.

Table-5 shows that the essential oils were capable of varying degrees of scavenging action against DPPH. Inhibition ratio (%) against increasing essential oils concentration is shown in Fig. 1. As can be seen from the figure, free radical inhibition of the oil is correlated with its concentration, because it reaches at higher value in the presence of the highest oil concentration.

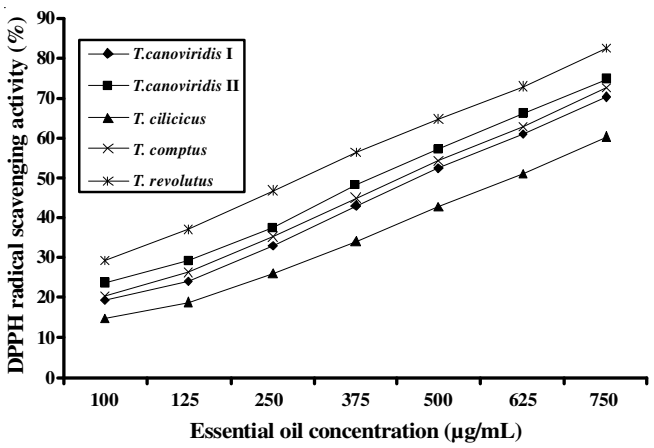

Fig. 1. DPPH radical scavenging activities of different concentrations of the essential oils

The % DPPH radical scavenging activity values of the essential oils *T. canoviridis* I, *T. canoviridis* II, *T. cilicicus*, *T. comptus* and *T. revolutus* were determined 19.16 ± 0.22, 23.68 ± 0.32, 14.76 ± 0.47, 20.39 ± 0.27 and 29.54 ± 0.59 % at 100 µg/mL concentration, respectively. At the 750 µg/mL the essential oil concentrations of *T. canoviridis* I, *T. canoviridis* II, *T. cilicicus*, *T. comptus* and *T. revolutus* 70.42 ± 0.26, 74.93 ± 0.20, 60.09 ± 0.83, 72.35 ± 0.21 and 82.69 ± 0.54 % DPPH was scavenging. DPPH is often used as a substrate to evaluate antioxidative activity of antioxidants[18]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[19]. The results showed that thymol and carvacrol were the active components of the essential oils in DPPH radical scavenging activity. The antioxidant activity of the tested essential oils were lower than butylated hydroxyanisole (BHA), nevertheless the essential oils can be considered effective natural antioxidant (Table-5). Each herb generally contained different phenolic compounds and each of these compounds possessed differing amounts of antioxidant activity. 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[20].

Conclusion

All tested microorganisms were inhibited by essential oil samples. The antioxidant activity of the tested essential oils were lower than butylated hydroxyanisole (BHA), nevertheless the essential oils can be considered effective natural antioxidant. The tested essential oils can be used as a natural preservative ingredient in the food and medical industries.

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