Composition and the *in vitro* Antimicrobial Activities of the Essential Oils of some *Thymus* Species

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The genus *Thymus* (Lamiaceae) is represented by 38 species (64 taxa) in Turkey, and 24 of which are endemic to Turkey. Aerial parts of *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*, *T. zygioides* var. *lycaonicus*, *T. longicaulis* subsp. *longicaulis* var. *subisophyllus* and *T. pulvinatus* collected from three different localities in Balikesir province were subjected to hydrodistillation to yield essential oils which were subsequently analysed by GC and GC/MS. The main constituents of the oils were identified, and antimicrobial bioassay was applied. Thymol (56.6%, 42.8%, 36.9%) was the main component in the oils of *T. longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype I), *T. longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype II) and *T. zygioides* var. *lycaonicus* respectively. The oil of *T. longicaulis* subsp. *longicaulis* var. *subisophyllus* contained carvacrol (60.0%) and the oil of *T. pulvinatus* borneol (27.9%) as main constituents.

Key words: Thymus sp., Essential Oil, Antimicrobial Activity

Introduction

Among the aromatic plants belonging to the family Lamiaceae, the genus *Thymus* is noteworthy for the numerous species and varieties of wild-growing plants. Many of these species are typical for the Mediterranean area (Consentino *et al.*, 1999).

The genus *Thymus* is represented in Turkey by 38 species, and the ratio of endemism in the genus is 53% (Tümen *et al.*, 1998). Several *Thymus* species are locally known as “kekik” or “tas kekik” and their dried herbal parts are used in herbal tea, condiment and folk medicine. The essential oils of some *Thymus* spp. are characterized by the presence of high concentration of the isomeric phenolic monoterpenes thymol and/or carvacrol (Baser, 1995).

The genus *Thymus* has numerous species and varieties, and their essential oil composition has been studied earlier (Papageorgio, 1980; Baser *et al.*, 1992, 1998; Guillen and Manzanos, 1998). However, antimicrobial activity of the *Thymus* species has rarely been studied (Vardar-Ünlü *et al.*, 2002; Consentino *et al.*, 1999; Karaman *et al.*, 2001).

This paper reports the results of GC and GC/MS analysis of the major constituents of *Thymus longicaulis* C. Presl. subsp. *chaubardii* (Boiss. et Heldr. ex Reichb. fil) Jalas var. *chaubardii*, *T. zygioides* Griseb. var. *lycaonicus* (Celak) Ronniger, *T. longicaulis* C. Presl. subsp. *longicaulis* var. *subisophyllus* (Borbas) Jalas and *T. pulvinatus* Celak and their antibacterial and antifungal properties against common pathogenic bacteria, *Candida albicans* and saprophytic microfungi.

Experimental

Plant material and isolation of the oils

Information on the plant material used in this study is given in Table I. Air dried aerial parts were hydrodistilled for 3 h using a Clevenger-type apparatus. Yields (in percent) of oils calculated on moisture free basis are also indicated in Table I. The voucher specimens were deposited in the herbarium of the Faculty of Pharmacy, Anadolu University (ESSE). They are shown in Tables I and III.

Gas chromatography

GC analysis was carried out using a Shimadzu GC-9A with CR4-A integrator. Thermon 600T

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FSC column (50 m × 0.25 mm i.d., 0.2 μm film thickness) was used with nitrogen as carrier gas. Oven temperature was kept at 70 °C for 10 min and programmed to 180 °C at a rate of 2 °C/min, and then kept constant at 180 °C for 30 min. Split ratio was adjusted at 60:1. The injector and FID detector temperatures were 250 °C.

Gas chromatography/mass spectrometry

A Shimadzu GCMS-QP5050A system, with CP-Sil 5CB column (25 m × 0.25 mm i.d. 0.4 μm film thickness) was used with helium as carrier gas. GC oven temperature was kept at 60 °C and programmed to 260 °C at a rate of 5 °C/min, and then kept constant at 260 °C for 40 min. Split flow was adjusted at 50 ml/min. The injector temperature was 250 °C. MS were taken at 70 eV. Mass range was between *m/z* 30 to 425. Library search was carried out using the in-house "BASER Library of Essential Oil Constituents". Relative percentage amounts of the separated compounds were calculated as reference points in the calculation of relative retention indices (RRI). The components identified in the oils are listed in Table I.

Antimicrobial screening

The agar disc diffusion method was employed for the determination of antimicrobial activities of the essential oils in questions (NCCLS, 1997). Suspension of the tested microorganisms (10^5 CFU/μl.) was spread on the solid media plates. Serial dilutions of essential oils were prepared in sterile distilled water in tubes. Filter paper discs (5 mm in diameter) were soaked with 10 μl of the oils and placed on the inoculated plates. After keeping at 2 °C for 2 h, they were incubated at 37 °C for 3 days for bacteria. The diameters of the inhibition zones were measured in millimetres.

Determination of minimum inhibitory concentration (MIC)

Microdilution broth susceptibility assay was used (Koneman *et al.*, 1997). Stock solutions of essential oils were prepared in dimethylsulfoxide (DMSO). Serial dilutions of essential oils were prepared in sterile distilled water in 96-well microtiter plates. Freshly grown bacterial suspension in double strength Mueller Hinton Broth (Merck) and yeast suspension of *Candida albicans* in yeast medium were standardised 10^8 CFU/ml (McFarland No:0.5). 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 the first well without turbidity was determined as the minimal inhibitory concentration (Table II).

Fungal spore inhibition assay

In order to obtain conidia, the fungi were cultured on Czapex Dox Agar 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). The 1 ml spore suspension was taken, diluted in a loop drop until one spore could be captured (Hasenekoğlu, 1990). One loop drop from the spore suspension was applied onto the centre of the petri dish containing Czapex Dox Agar and Malt Extract Agar. 10 μl of each essential oil was applied onto sterile paper discs (5 mm in diameter) and placed in the petri dishes and incubated at 25 °C for 72 h. Spore germination during the incubation period was followed using a microscope (Olympus BX51) in 8 h intervals. The fungi *Mucor hiemalis* (BUB Malt.163), *P. clavigerum* (BUB Czp.181) and *Absidia glauca* ATCC 22752 were used for this assay and deposited in Balikesir University, Faculty of Science and Letters, Department of Biology (BUB), Balikesir, Turkey.

Result and Discussion

Aerial parts of *T. zygioides* var. *lycaonicus*, *T. pulvinatus*, *T. longicaulis* subsp. *longicaulis* var. *subisophyllus*, *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype I and II) collected from different localities in Balikesir province were water distilled. The resulting main components of the essential oils are shown in Table I along with other collections and yield information.

The analyses showed that thymol (36.9%–56.6%) was the main component in the oils of *T. zygioides* var. *lycaonicus, Thymus longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype I and II) and carvacrol (60%) was the main component in the oils of *T. longicaulis* subsp. *longicaulis* var. *subisophyllus.* Other major components were identified as *p*-cymene (4.1%–14.3%), γ-terpinene (1.4%–

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Table I. Information on collection of *Thymus* sp., their oil yields and essential oil compositions.

Thymus sp.	Collection site and date	ESSE[a]	Oil yields	Main components	Mass fraction (%)[b]
Thymus longicaulis subsp. *chaubardii* var. *chaubardii* (chemotype II)	Balikesir/Kaz Dagı/ Eybek Kulesi 15.06.2002 (with purple flowers)	14182	1.8	thymol	42.8
				p-cymene	14.3
				γ-terpinene	11.3
				borneol	7.2
				1,8-cineole	3.9
				camphene	2.3
				carvacrol	2.2
				β-caryophyllene	2,1
				α-pinene	2.1
				α-terpenine	1.6
				β-bisabolene	1.3
				myrcene	1.1
				α-terpineol	0.4
Thymus longicaulis subsp. *chaubardii* var. *chaubardii* (chemotype I)	Balikesir/Kaz Dagı/ Eybek Kulesi 15.06.2002 (with white flowers)	14185	1.9	thymol	56.6
				p-cymene	9.8
				γ-terpinene	7.1
				borneol	4.9
				1,8-cineole	4.4
				carvacrol	2.5
				β-caryophyllene	2.0
				β-bisabolene	1.1
				α-terpinene	1.0
				α-terpineol	0.4
Thymus zygioides var. *lycaonicus*	Balikesir/Ikizce Tepe Baraji 25.05.2002	14183	0.7	thymol	36.9
				geraniol	22.8
				borneol	6.4
				p-cymene	4.1
				β-bisabolene	4,1
				trans-sabinene hydrate	2.3
				carvacrol	2.3
				γ-terpinene	1.9
				β-caryophyllene	1,8
				1,8-cineole	1.4
				terpinen-4-ol	1.2
				camphor	1.2
				1-octen-3-ol	1.0
				α-terpineol	0.4
Thymus longicaulis subsp. *longicaulis* var. *subisophyllus*	Balikesir/Susurluk 26.05.2002	14179	0.6	carvacrol	60.0
				thymol	7.0
				β-bisabolene	4.8
				borneol	4.7
				p-cymene	4.2
				1,8-cineole	2.3
				trans-sabinene hydrate	1.8
				γ-terpinene	1.4
				linalool	1.3
				β-caryophyllene	1.3
				1-octen-3-ol	1.2
				camphor	1.0
				α-terpineol	0.3

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Table I. (cont.)

Thymus sp.	Collection site and date	ESSE[a]	Oil yields	Main components	Mass fraction (%)[b]
Thymus pulvinatus	Balikesir/Kaz Dagı/ Eybek Kulesi 15.06.2002	14189	0.6	borneol	27.9
				camphene	9.3
				camphor	8.7
				1,8-cineole	6.7
				α-pinene	3.1
				thymol	2.7
				trans-verbenol	2.5
				linalyl acetate	2.3
				terpinen-4-ol	2.2
				linalool	2.2
				bornyl acetate	1.8
				(*E*)-nerolidol	1.8
				p-cymene	1.5
				trans-sabinene hydrate	1.4
				γ-terpinene	1.2
				α-terpineol	0.7
				carvacrol	0.5

[a] Acronym of the Herbarium of the Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey.
[b] Relative percentage from FID.

11.3%) and borneol (4.7%–7.2%) besides other components. In contrast, *T. zygioides* var. *lycaonicus* contained geraniol (22.8%) as a major component. On the other hand borneol (27.9%) was the main component in the oils of *T. pulvinatus* (Table I).

In an earlier study, essential oil of *T. longicaulis* subsp. *longicaulis* var. *subisophyllus* was reported to contain thymol (3.0%), borneol (16.0%), *p*-cymene (15.0%) as main constituents (Baser *et al.*, 1992). The essential oil of *T. zygioides* var. *lycaonicus* was reported to contain thymol (42.0%–57.0%) and γ-terpinene (19.5%) (Baser *et al.*, 1996). The essential oil of *T. longicaulis* subsp. *longicaulis* was reported to contain geraniol (69.0%) and thymol (53.0%). The essential oil of *T. pulvinatus* contained borneol (29.0%–31.0%) and in the essential oil of *T. longicaulis* subsp. *chaubardii* thymol (45.0%) and *p*-cymene (14.0%) were the main constituent (Tümen *et al.*, 1995).

In this present study, using the microdilution broth assay (Koneman *et al.*, 1997), the essential oil of *Thymus zygioides* var. *lycaonicus* and *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype I) showed a minimal inhibitory concentration value of 31.25 μg/ml against the pathogenic yeast *Candida albicans*. *Proteus vulgaris* was inhibited by all oils except for *Thymus zygioides* var. *lycaonicus*. *Staphylococcus aureus* was inhibited best by *Thymus longicaulis* subsp. *longicaulis* var. *subisophyllus* and the other oils tested also showed inhibitory activities. As a general result, all the bacteria assayed showed inhibition when tested against the *Thymus* oils (Table II).

As a consequence, we may state that the samples containing high amounts of the monoterpene phenols thus influence the antibacterial activity (Crippa and Bruno, 1989; Sivropoulou *et al.*, 1996). As seen in Table I, phenols and oxygenated monoterpenes dominate within all samples with high percentages.

When the fungal spore inhibition assay was applied to the oils, observations during the three day incubated period show that *Mucor hiemalis* spores were strongly inhibited with the oil of *T. zygioides* var. *lycaonicus* (125 μg/ml). The molecular groups with the strongest antibacterial action are also active on fungi. However, treatment must be continued over a longer period. Fundamental studies have revealed the anti-fungal activity of alcohols and sesquiterpenic lactones. While germination of *Penicillium clavigerum* and *Absidia glauca* ATCC 22752 were not inhibited by the tested samples.

According to agar disc diffusion method, all tested bacteria were inhibited by essential oils used in this study (Table III). If the light area measures between 2 and 3 millimetres the essential oil has a good bactericidal action on the tested germs. If the light area is more than 3 millimetres across it is very effective and if there is no light area the essential oil has no activity on the analysed germ,

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Table II. Minimum inhibitory concentration [μg/ml] of *Thymus* essential oils.

Microorganism	Sources	A	B	C	D	E	Standard
Escherichia coli	ATCC 25292	125	125	125	125	125	–[C]
Staphylococcus aureus	ATCC 6538	125	62.5	125	125	125	–[C]
Pseudomonas aeruginosa	ATCC 27853	125	125	125	125	62.5	–[C]
Enterobacter aerogenes	NRRL 3567	125	125	125	125	125	–[C]
Proteus vulgaris	NRRL 123	125	62.5	62.5	62.5	62.5	–[C]
Candida albicans	OGU	31.25	62.5	31.25	62.5	62.5	–[K]

A: *Thymus zygioides* var. *lycaonicus*.
B: *Thymus longicaulis* subsp. *longicaulis* var. *subisophyllus*.
C: *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype I).
D: *Thymus longicaulis* subsp. *chaubardii* var. *chaubardii* (chemotype II).
E: *Thymus pulvinatus*.
[C]: Chloramphenicol.
[K]: Ketoconazole.
–: No turbidity.

Table III. Inhibition zones according to agar disc diffusion method [mm]. As the same concentration of each essential oil from each of the five ESSE revealed close zone values for each microorganism tested, only one ESSE per microorganism was used.

Microorganisms	Serial dilution (100 μl Stock + μl H_2O)					
	ESSE number	Stock solution	100	200	300	C[a]
Staphylococcus aureus	14183[b]	12	8	7	6	
ATCC 6538	Chloramphenicol					16
	Streptomycin					12
Pseudomonas aeruginosa	14183	10	8	7	6	
ATCC 27853	Chloramphenicol					24
	Streptomycin					15
Enterobacter aerogenes	14183	9	8	7	6	
NRRL 3567	Chloramphenicol					21
	Streptomycin					15
Proteus vulgaris	14183	11	9	8	6	
NRRL 123	Chloramphenicol					21
	Streptomycin					20
Escherichia coli	14183	8	7	6	6	
ATCC 25292	Chloramphenicol					23
	Streptomycin					17

[a] Control.
[b] *Thymus zygioides* var. *lycaonicus*.
[c] 4 mg essential oil + 2 ml DMSO.

and will not be retained for treatment (Dominique, 2003).

The main oxygenated monoterpenes thymol and carvacrol, appear to contribute significantly to the microbial activity of the *Thymus* oils examined. The possibility that the other minor components may possess some antimicrobial power or synergistic effect still remains.

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