Analysis of Essential Oil Composition of *Thymbra spicata* var. *spicata*: Antifungal, Antibacterial and Antimycobacterial Activities

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The fresh leaves and brine of leaves of *Thymbra spicata* var. *spicata* (KARAKIZ™) were analyzed by hydrodistillation, headspace and GC/MS techniques. The main components were determined as carvacrol, *p*-cymene, β-myrcene, γ-terpinene, α-terpinene and *trans*-caryophyllene. The essential oil and the main compounds, carvacrol and *trans*-caryophyllene, have been tested against *E. coli*, *S. epidermidis*, *B. subtilis*, *S. aureus*, *S. typhimurium*, *K. pneumoniae*, *P. aeruginosa*, *E. faecalis* and *C. albicans*. While the essential oil and carvacrol showed strong activity against all microorganisms, except *P. aeruginosa, trans*-caryophyllene showed activity only against *C. albicans*. The essential oil and carvacrol also showed strong antimycobacterial activity.

Key words: Thymbra spicata, Carvacrol, Antimicrobial and Antimycobacterial Activity

Introduction

Two species and four taxa from the *Thymbra* species in Turkey are reported (Davis, 1982). The species have been used as an antiseptic, stimulant, against common cold and as herbal tea and brine in breakfast and on salads especially in the western part of Turkey (Muller-Riebau *et al.*, 1997; Tümen *et al.*, 1994).

The first report concerning antimicrobials in the *Thymbra* species in Turkey was on 6-hydroxyflavones (Miski *et al.*, 1983). The essential oil composition and antibacterial and antifungal activities of species were reported by various groups (Tümen *et al.*, 1994; Baser *et al.*, 1996; Yegen *et al.*, 1992), and one study reported antioxidant activity of Turkish *Thymbra spicata* (Kosar *et al.*, 2003). Insecticidal activity has been reported against *Sitophilus oryzae* adults and the last instars of *Ephestia kuehniella* (Sarac and Tunc, 1995a, b).

In this study, the essential oil obtained from *T. spicata* was analyzed for its chemical compostion, and then antifungal and antibacterial activities including antimycobacterial activity were investigated. Commercial thyme brine (KARAKIZ™) was also analyzed. This is the first report on antimycobacterial activity of *Thymbra spicata* oil and its main components.

Experimental

Plant material

Thymbra spicata var. *spicata* was collected from Torbalı-İzmir on May 15, 2004. The plant was identified by Professor G. Tumen of Balıkesir University, Turkey. A voucher specimen was deposited in the Herbarium of the Department of Biology, Faculty of Arts and Science, Balıkesir University. Brine of *T. spicata* (KARAKIZ™) was purchased from local markets in İstanbul.

Chemical analysis

170 g of fresh leaves of *Thymbra spicata* var. *spicata* were subjected to a Clevenger type apparatus for 3 h. 5.3 mL, a yield of 3.1% (v/w), of essential oil were obtained. It was dried over anhydrous $CaCl_2$ and stored at +4 °C.

GC/MS and headspace conditions

GC/MS was carried out on a Thermo Electron Trace 2000 GC model gas chromatograph and Thermo Electron DSQ quadrupole mass spectrometer. A non-polar Phenomenex DB-5 fused silica column (60 m × 0.25 mm i.d. with 0.5 μm film thickness) was used with helium at 1 mL/min (0.14 MPa) as a carrier gas and a polar Innowax FSC column (60 m × 0.25 mm i.d. with 0.5 μm film thickness) was also used. The GC oven tempera-

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ture was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min and then kept constant at 220 °C for 15 min. The split ratio was adjusted to 1:20, the injection volume was 0.1 μL. EI/MS spectra were recorded at 70 eV ionization energy. Mass range was *m/z* 35–500 amu. Alkanes were used as reference in the calculation of Kovats indices (KI). The identification of the compounds was based on the comparison of their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as the literature data (Adams, 1995). A Thermo AC 2000 headspace instrument was used for headspace analysis, the program and conditions of which were as follows: The vial oven temperature was 120 °C for each analysis and injection volume was 10 μL. Details are as given in our previous work (Goren *et al*., 2004).

Antibacterial and antifungal activity

The essential oil of *T. spicata*, α-pinene, β-pinene, carvacrol and *trans*-caryophyllene were tested against standard bacterial strains such as *E. coli* ATCC 29995, *S. epidermidis* ATCC 12228, *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538P, *S. typhimurium*, *K. pneumoniae* CCM 2318, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, and the yeast *C. albicans* ATCC 10239. The agar diffusion method was used to determine the inhibition zones of the tested compounds and esssential oil against standard bacterial strains. Essential oil and the compounds with inhibition zones higher than 7 mm were selected to determine the antimicrobial activity quantitatively as minimum inhibition concentration (MIC). The broth microdilution method was applied for this purpose (Goren *et al.*, 2003, 2004; Kılıç *et al.*, 2005; NCCLS, 1990). The antibacterial and antifungal activity tests were done as three replicates for each organism and RSD % value was less than 1.5.

Screening for antimycobacterial activity

Mycobacterium smegmatis ATCC 14468, from freshly grown cultures, was used to obtain suspensions of 0.5 McFarland turbidity. A cotton swab was wetted with the suspension and the microorganism was spread over Mueller Hinton agar plates. Wells, 6 mm in diameter, were punched into the agar, and 20 μl of samples were applied into these wells. Pure DMSO and sterile water were used as controls. The plates were incubated at 37 °C for 3 d until a growth was clearly observed. The inhibition zones around the wells were measured and photographed (Kılıç *et al.*, 2005; NCCLS, 1990). The antimycobacterial activity tests were done as three replicates for each organism and RSD % value was less than 1.5.

Determination of MIC values for selected species of Mycobacteria

Suspensions of 0.5 McFarland turbidity were prepared from freshly grown cultures of *M. smegmatis* ATCC 14468, *M. terrae* ATCC 15755, *M. intracellulare* ATCC 139450 and *M. tuberculosis* H37Ra ATCC 25177 in Tween 80 containing water. The components were diluted using Middlebrook 7H9 broth and mycobacteria were inoculated. The tubes were incubated at 37 °C and checked daily until growth was observed. The lowest concentration of drug that inhibited the growth was considered as MIC value.

Results and Discussion

5.3 mL essential oil were obtained from 170 g of dry *T. spicata* var. *spicata* leaves in a yield of 3.1% and its density was d^{22} = 0.898 g/mL. Commercial thyme brine "KARAKIZ™" was used for the analysis of brine of *Thymbra spicata* which was placed in brine in 2002 and 2003. Essential oil of fresh leaves of the species was analyzed by GC/MS, while the composition of thyme brine was determined by headspace GC/MS. Twenty – nine components were identified representing about 97.8% of the oil. The main compounds were identified as carvacrol (34.9%), γ-terpinene (25.6%), *p*-cymene (9.1%), α-terpinene (6.9%), thujene (5.2%), *trans*-caryophyllene (5.1%) and β-myrcene (4.8%) (Table I). Analysis of the dried leaves and brine of *T. spicata* by headspace GC/MS indicated the presence of the same main compounds as observed in the essential oil (Table I). According to these results, we concluded that the brine of *Thymbra spicata* preserves its active composition at least two years.

The essential oil of fresh leaves of *T. spicata* and and pure compounds; α-pinene, β-pinene, carvacrol and *trans*-caryophyllene were tested against standard bacterial strains (Table II). The essential oil showed activity against all the tested bacteria and fungi. MIC values of essential oil were deter-

Table I. Composition of essential oil of *Thymbra spicata* var. *spicata* and its brine.

Compound	KI*	KI†	a %	b %	c %	d %	Identification‡
Methyl isovalerate	789	885	0.2	0.3	0.1	t§	MS
Thujene	935	1031	5.2	4.8	t	t	MS
α-Pinene	941	1030	1.5	3.1	0.4	4.6	MS, Co, KI
Camphene	954	1074	0.2	0.4	0.7	1.0	MS, Co, KI
Sabinene	978	1132	t	t	t	t	MS, KI
β-Pinene	981	1120	0.4	0.7	t	t	MS, Co, KI
β-Myrcene	994	1175	4.8	7.7	5.7	6.7	MS, Co, KI
α-Phellandrene	1007	1177	0.8	1.2	0.4	1.1	MS, KI
Δ^3-Carene	1015	1168	t	t	t	t	MS, KI
α-Terpinene	1020	1188	6.9	10.1	6.2	9.9	MS, KI
p-Cymene	1028	1281	9.1	12.3	26.0	21.0	MS, Co, KI
DL-Limonene	1031	1204	t	t	–	t	MS, Co, KI
β-Phellandrene	1032	1218	0.8	1.0	0.3	t	MS, KI
1,8-Cineole	1035	1214	t	t	t	t	MS, Co, KI
γ-Terpinene	1062	1255	25.6	30.1	31.2	30.1	MS, Co, KI
α-Terpinolone	1091	1291	0.3	0.2	1.4	0.7	MS, Co, KI
1,3,5-*p*-Menthatriene	1125	1105	t	t	t	t	MS, KI
Terpinen-4-ol	1179	1607	0.9	0.1	0.4	0.4	MS, KI
Thymol	1294	2205	0.2	t	t	t	MS, Co, KI
Carvacrol	1300	2246	34.9	23.4	25.6	20.1	MS, Co, KI
trans-Caryophyllene	1420	1613	5.1	0.6	0.8	1.0	MS, Co, KI
Aromadendrene	1444	1628	t	t	t	t	MS, KI
α-Humulene	1458	1686	0.3	t	t	t	MS, KI
γ-Muurolone	1478	1711	0.2	t	t	t	MS, KI
β-Bisabolene	1513	1741	t	t	t	t	MS, KI
Ledene	1518	1708	0.1	t	t	t	MS, KI
δ-Cadinene	1525	1770	0.1	t	t	t	MS, KI
Spathulenol	1581	2120	0.1	0.1	0.2	t	MS, KI
Caryophyllene oxide	1585	1994	0.1	t	t	t	MS, Co, KI
Total			97.8	96.1	99.4	96.6	

GC/MS analyses were replicated three times (mean RSD % value is 0.1).
* Phenomenex DB-5 column. † Innowax FSC column. ‡ MS, mass spectrometry. Co, co-injection. KI, Kovats indices. §t, trace (less than 0.1%).
a, Essential oil of *Thymbra spicata* var. *spicata*; b, leaves, collected in May 2005, analyzed by headspace GC/MS; c, KARAKIZ™ brine, produced in 2002, analyzed by headspace GC/MS; d, KARAKIZ™ brine, produced in 2003, analyzed by headspace GC/MS.

Table II. Antibacterial and antifungal activity of essential oil of *T. spicata* var. *spicata* and its pure compounds[a].

Compound	*E. coli*	*S. epidermidis*	*B. subtilis*	*S. aureus*	*S. typhimurium*	*K. pneumoniae*	*P. aeruginosa*	*E. faecalis*	*C. albicans*
T. spicata var. *spicata*	< 0.47	0.94	< 0.47	0.94	< 0.47	0.94	NA[b]	< 0.47	3.75
α-Pinene	0.94	0.94	7.5	0.47	0.94	7.5	NA	0.94	7.5
β-Pinene	NA	NA	NA	NA	NA	NA	NA	NA	NA
Carvacrol	< 0.47	< 0.47	< 0.47	< 0.47	< 0.47	< 0.47	1.88	< 0.47	0.94
trans-Caryophyllene	0.94	NT[c]	1.88	0.94	NT	NA	NA	NA	1.88
Gentamycin[d]	0.97	7.8	0.97	0.48	0.48	0.48	0.97	3.1	NT
Fluconazole[d]	NT	NT	NT	NT	NT	NT	NT	NA	15.6

[a] MIC values are given as mg/L. [b] NA, non-active. [c] NT, not tested. [d] Gentamycin and fluconazole are used as positive controls and results are given as μg/mL.

mined as < 0.47 mg/L against *E. coli, B. subtilis, S. typhimurium* and *E. faecalis*, 0.94 mg/L against *S. epidermidis, S. aureus, K. pneumoniae*, and 3.75 mg/L against *C. albicans*. The essential oil of *T. spicata* var. *spicata* did not show activity against *P. aeruginosa* (Table II).

The most active compound was carvacrol with a MIC value less than 0.47 mg/L, except for *P. aeru-*

Table III. Antimycobacterial activity of essential oil of *T. spicata* var. *spicata* and its pure components[a].

Tested material	*M. smegmatis*	*M. terrae*	*M. intracellulare*	*M. tuberculosis*
Essential oil of *T. spicata*	256	128	512	NT[c]
α-Pinene	1024 (7.51)	256 (1.88)	256 (1.88)	128 (0.94)
β-Pinene	NA[b]	NA	NA	NT
trans-Caryophyllene	512 (2.5)	1024 (5.0)	NA	512 (5.0)
Carvacrol	64 (0.42)	128 (0.85)	128 (0.85)	64 (0.42)
Rifampicin[d]	NT	NT	NT	0.5 (6.1×10^{-4})

[a] MIC values are given as μg/mL (mM). [b] NA, non-active. [c] NT, not tested. [d] Rifampicin is used as positive control and results are given as μg/mL.

ginosa (1.88 mg/L) and *C. albicans* (0.94 mg/L). α-Pinene showed activity against the tested bacteria with the following MIC values: 0.47 mg/L against *S. aureus*, 0.94 mg/L against *E. coli, S. epidermidis*, *S. typhimurium* and *E. faecalis* and 7.5 mg/L against *K. pneumoniae*, *B. subtilis* and *C. albicans*. However, in contrast to α-pinene, β-pinene did not show activity against all tested bacteria and fungi. Moreover, *trans*-caryophyllene showed activity only against *E. coli*, *S. aureus*, *B. subtilis* and *C. albicans*, with MIC values of 0.94, 0.94, 1.88, 1.88 mg/L, respectively. Gentamycin and fluconazole were used as positive controls.

The antimycobacterial activity of essential oil of *T. spicata* and its components α-pinene, β-pinene, *trans*-caryophyllene and carvacrol was tested against *Mycobacterium smegmatis, M. terrae*, *M. intracellulare* and *M. tuberculosis*. The MIC values for the essential oil were found to be 256, 128, 512 μg/mL, respectively (Table III). The essential oil was not tested against *M. tuberculosis*. The pure compound α-pinene showed activity against all mycobacteria. The MIC values were found to be 1024, 256, 256 and 128 μg/mL, respectively. However, β-pinene did not show activity against all mycobacteria. The main compound carvacrol showed activity against *M. smegmatis*, *M. terrae*, *M. intracellulare* and *M. tuberculosis* with MIC values of 64, 128, 128 and 64 μg/mL, respectively. *trans*-Caryophyllene showed activity only against *M. smegmatis* (512 μg/mL) and *M. terrae* (1024 μg/mL) (Table III).

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