NPC Natural Product Communications

2009
Vol. 4
No. 1
109 - 114

GC-MS Analysis and Antimicrobial Activity of Essential Oil of *Stachys cretica* subsp. *smyrnaea*

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Received: August 20th, 2008; Accepted: November 15th, 2008

The essential oil from the aerial parts of *Stachys cretica* L. subsp. *smyrnaea* Rech. fil. (Lamiaceae), endemic to Turkey, was investigated by using GC and GC-MS. Thirty-four of 37 components, represented 99.7% of the total oil, were identified. The major components of the essential oil were *trans*-β-caryophyllene (51.0%), germacrene-D (32.8%), *α*-humulene (3.1%), δ-cadinene (2.1%) and δ-elemene (2.1%). The antimicrobial activity of the essential oil, *trans*-β-caryophyllene and five different extracts of the aerial parts of *S. cretica* L. subsp. *smyrnaea* were investigated by the standard disc diffusion method. The essential oil and *trans*-β-caryophyllene exhibited antibacterial and antifungal activities. The activity increased with increasing concentrations of the essential oil and the extracts. The essential oil showed antimicrobial activity, particularly against *Pseudomonas aeruginosa* and *Bacillus subtilis*. The extracts exhibited either moderate or no activity.

Keywords: *Stachys cretica* subsp. *smyrnaea*, essential oil, *trans*-β-caryophyllene, germacrene-D, antimicrobial activity.

The genus *Stachys* L., comprising more than 270 species, is one of the largest genera of the Lamiaceae family [1]. Forty-five of 81 species growing in Turkey are endemic [2]. In Anatolia, *Stachys* species are known as "dağ çayı", "çay otu", or "tokalı çay" and are used as tonics and stomachics [3]. In traditional medicine, these species have been used to treat genital tumors, sclerosis of the spleen, inflammatory tumors and cancerous ulcers [4,5]. Since they possess sedative, antispasmodic, diuretic and emmenagogue activities, either the whole plant or leaves have been consumed as a tea in phytotherapy [6]. In addition, the extracts or components of *Stachys* species possess significant antibacterial [5,7], anti-inflammatory [8], antitoxic [8], and antianoxia effects [9].

S. cretica L. subsp. *smyrnaea* Rech. fil. is an endemic medicinal plant which is distributed in north-west, west and south Anatolia. This subspecies is readily distinguishable from the others by its lax, sparsely villous-tomentose indumentum with glandular and eglandular hairs, broader leaves and ovate, glandular calyx teeth [2].

Chemical studies have been reported for some *Stachys* species [10-14], as well as various biological properties, such as radical scavenging [15], antioxidant due to polyphenol content [16,17], anxiolytic [18], anticandidal [19,20], anti-inflammatory [10], antimycobacterial [21] and antimutagenic [22] activities. A literature survey showed that germacrene-D, α-pinene, β-pinene, β-caryophyllene, caryophyllene oxide, δ-cadinene, myrtenyl acetate, dehydroabietane, pimaradiene, α-copaene, *E*-nerolidol, abietatriene, linalool, spathulenol and methyl linoleate were the most encountered essential oil components of *Stachys* species [1,23-30]. The essential oils and/or the activity of nineteen *Stachys* species growing in Turkey were investigated previously [1,17,20-22,25-32].

The aim of this study was to determine the chemical composition of the essential oil of *S. cretica* L. subsp. *smyrnaea*, and the antimicrobial activity of its oil, its major component, and five different extracts of the plant. Thirty-four components were identified by a library search (NIST, WILEY, 2005). This is the first report of the essential oil of this species and of its antimicrobial activity.

The essential oil, which was a greenish yellow color, was obtained by hydrodistillation (0.15%, v/w) of the dried aerial parts of the plant. The physical properties of the essential oil and its main compound are given in Table 1, and the chemical constitution of the essential oil in Table 2. The essential oil was analyzed by GC and GC-MS and resulted in the identification of 34 components representing 99.7% of the total oil. The major components of the oil were *trans*-β-caryophyllene (51.0%), germacrene-D (32.8%), α-humulene (3.1%), β-elemene (2.1%) and δ-cadinene (2.1%). Caryophyllene oxide was also determined as a constituent of the oil, with a yield of 1.4% (Table 2).

Table 1: The physical properties of the essential oil of *S. cretica* subsp. *smyrnaea* and *trans*-β-caryophyllene.

Physical Property	Essential Oil	*trans*-β-Caryophyllene
d_{20}	0.8990	0.9052
$[\alpha]^{D}_{20}$	-61.64	-15.00
n^{0}_{20}	1.5001	1.487

d_{20}: Density (g/mL) 20˚C, n^{0}_{20}: Refractive index at 20˚C, $[\alpha]_{D}^{20}$: Specific rotation at 20˚C.

Sesquiterpene hydrocarbons represented 92.3% of the essential oil, monoterpene hydrocarbons 0.4%, oxygenated monoterpenoids 0.20%, oxygenated sesquiterpenoids 2.9%, and the remaining percentage (4.1%) consisted of aliphatic alcohols, aldehydes, esters, hydrocarbons and ketones.

Table 2: Essential oil oonstituents of *Stachys cretica* subsp. *Smyrnaea*.

Peak No	Compound	RI[a]	RI[b]	Content (%)	Identification Method
1	α-Pinene	932	936	0.2	MS,Co-GC,RI
2	2-Methyl-2-Heptene	935		0.04	MS
3	**1-Octen-3-ol**	**963**	**977**	**1.5**	MS,Co-GC,RI
4	β-Pinene	975	978	0.04	MS,Co-GC,RI
5	Limonene	1014	1027	0.2	MS,Co-GC,RI
6	Phenyl acetaldehyde	1025	1041	0.1	MS
7	Linalool	1060	1098	0.1	MS,Co-GC,RI
8	*n*-Nonanal	1061	1102	0.09	MS,Co-GC,RI
9	Phenylethylacetate	1098		0.03	MS
10	*n*-Nonanol	1100	1071	0.03	MS,Co-GC,RI
11	Terpinen-4-ol	1119	1175	0.06	MS,Co-GC,RI
12	Decanal	1125	1203	0.4	MS,Co-GC,RI
13	2-Hidroxymethylbenzoate	1129		0.05	MS
14	(*E*)-2-Decenal	1156	1260	0.05	MS,RI
15	*cis*-Verbenol	1160		0.1	MS
16	(2*E*, 4*Z*)-2,4-decadienal	1185	1291	0.1	MS,RI
17	(*E*)-2-Undecanal	1205		0.04	MS
18	α–Copaene	1208	1375	0.1	MS,Co-GC,RI
19	β–Bourbonene	1215	1382	0.9	MS,Co-GC,RI
20	β–Cubebene	1223	1388	0.06	MS,Co-GC,RI
21	**β-Elemene**	**1229**	**1391**	**2.1**	MS,Co-GC,RI
22	***trans*-β-Caryophyllene**	**1238**	**1418**	**51.0**	MS,Co-GC,RI,NMR
23	**α-Humulene**	**1247**	**1452**	**3.1**	MS,Co-GC,RI
24	(*E*)-β-Farnesene	1255	1457	0.06	MS,Co-GC,RI
25	*allo*-Aromadendrene	1260	1458	0.05	MS,Co-GC,RI
26	**Germacrene-D**	**1263**	**1480**	**32.8**	MS,Co-GC,RI
27	**δ-Cadinene**	**1276**	**1524**	**2.1**	MS,Co-GC,RI
28	Spathulenol	1290	1577	0.1	MS,Co-GC,RI
29	β-Copaen-4-α-ol	1303	1579	0.05	MS,RI
30	**Caryophyllene oxide**	**1306**	**1581**	**1.4**	MS,Co-GC,RI
31	$C_{15}H_{24}O$	1320	---	0.2	MS
32	Globulol	1324	1608	0.1	MS, RI
33	α-Muurolol	1327	1641	0.1	MS,RI
34	8-α-Acetoxyelemol	1335		0.9	MS
35	Unidentified	1362	---	0.07	---
36	Unidentified	1376	---	0.04	---
37	***trans*-Phytol**	**1430**	**1950**	**1.5**	MS, RI
	Total identified:				
	Monoterpene hydrocarbons:			0.4	
	Oxygenated monoterpenoids:			0.2	
	Sesquiterpene hydrocarbons:			92.3	
	Oxygenated sesquiterpenoids:			2.9	
	Others:			4.1	

[a]**:** Kovats index on ZEBRON-5 fused silica column; [b]**:** Kovats index on HP-5 fused silica column [23,24]; **Co-GC:** Co-injection with authentic compounds; **NMR:** ^{1}H-NMR and ^{13}C-NMR; **RI:** Retention Index literature comparison.

trans-β-Caryophyllene was the major component of *S. aleurites* and *S. balansae*. The principal components of the essential oil of *Stachys* species previously studied showed chemical variations that could be chemotaxonomically important for the genus *Stachys* [24].

The antimicrobial activity against species known to cause infections in humans was determined of the essential oil, *trans*-β-caryophyllene (the main component of the essential oil), and five extracts. *In vitro* evaluation was conducted against three Gram-positive bacteria (*B. subtilis*, *S. aureus*, *S. mutans*), two Gram-negative bacteria (*E. coli*, *P. aeruginosa*), and one yeast (*C. albicans*).

As seen in Table 3, *trans*-β-caryophyllene and the essential oil exhibited antimicrobial activity against microorganisms, particularly *B. subtilis*. The essential oil showed the highest activity of the tested samples. At low quantities (10 μL), the essential oil caused growth inhibition in bacteria and *C. albicans* with zone diameters between 10-17 mm. The essential oil, when applied at 25 μL produced a greater effect than when10 μL was applied. The essential oil exhibited high activity against *P. aeruginosa*. On the other hand, *trans*-β-caryophyllene exhibited high activity, especially against *S. aureus* and *E. coli*. None of the extracts, essential oil, and *trans*-ß-caryophyllene showed activity against *S. mutans* (thus not mentioned in Table 3).

In the present study, the essential oil exhibited antimicrobial activity, especially against *P. aeruginosa* and *B. subtilis*. The antimicrobial activity of *trans*-β-caryophyllene, and its derivatives has been observed [33], and thus the activity of the oil might be related to the presence of *trans*-β-caryophyllene and caryophyllene oxide. However, further studies are needed to understand the origin of the activity. In particularly, other minor and major components of the oil need to be tested for their antimicrobial activity, both individually and for possible synergistic effects. Moreover, MIC and MCB assays are needed to evaluate the antimicrobial activity.

Table 3: Antimicrobial activity of *S. cretica* essential oil.

Samples	Concentration (μL/disc)	Inhibition zone diameter (mm)				
		Microorganisms				
		B. subtilis ATCC 6633	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923	*C. albicans* ATCC 10239
Essential Oil	10	13	10	17	15	14
	25	20	14	25	19	19
trans-β-Caryophyllene	10	15	17	12	19	15
	25	NT	NT	NT	NT	NT
Petroleum ether	10	-	-	-	-	-
	25	10	-	-	-	-
Chloroform	10	-	-	-	-	-
	25	-	6	7	-	-
Ethyl acetate	10	-	-	-	-	-
	25	-	6	-	-	-
Butanol	10	8	-	8	-	-
	25	14	6	14	-	-
Water	10	-	7	-	-	-
	25	-	11	-	-	-
Reference antibiotics						
Penicillin	10 U	10	17	NT	29	NT
Gentamicin	10 μg	NT	NT	16	NT	NT
Nystatin	100 U	NT	NT	NT	NT	17

NT: not tested

Experimental

Plant material: The aerial parts of *Stachys cretica* L. subsp. *smyrnaea* Rech. fil. were collected at the flowering stage in May 2005 from the Yaraş-Muğla region of Turkey by one of us (Mehmet Öztürk) and identified by Dr Tuncay Dirmenci. A voucher specimen (No: S-106) has been deposited in the Department of Chemistry, Faculty of Arts and Sciences, Muğla University, Muğla Turkey.

Preparation of the extracts: Air dried and powdered aerial parts (90.6 g) were extracted with acetone by using a Soxhlet apparatus, then the solvent was evaporated to dryness under vacuum. The crude acetone extract (4.10 g) was dissolved in a small amount of water, and was then extracted with light petroleum, chloroform, ethyl acetate and *n*-butanol, respectively in a separating funnel. The light petroleum (0.40 g), chloroform (1.98 g), ethyl acetate (0.37 g), and *n*-butanol extracts (0.30 g), as well as the remaining aqueous part (1.01 g) were tested for antimicrobial activity.

Isolation of the essential oil: The essential oil of the air-dried aerial parts of *S. cretica* subsp. *smyrnaea* (1600g) was obtained by hydrodistillation for 4h by using a Clevenger type apparatus, according to the recommendation of the European Pharmacopoeia [34]. The essential oil was dried by treatment with anhydrous sodium sulfate, and was then stored under nitrogen in a sealed vial until required.

Isolation of the main component: The essential oil of *S. cretica* subsp. *smyrnaea* was subjected to column chromatography, using silica gel 60 F_{254} (70-230 mesh) and eluting with *n*-hexane containing 1% increasing amounts of diethyl ether. The main component of the essential oil, *trans*-β-caryophyllene, was obtained from the *n*-hexane:diethyl ether fractions (80:20, v/v).

Gas chromatography: GC analyses of the essential oil were performed using a Shimadzu GC-17 AAF, V3, 230V LV Series (Kyoto, Japan) gas chromatography, equipped with a FID and a Optima-5 fused silica column [30m x 0.25 mm (i.d.), film thickness 0.25 μm]; the oven temperature was held at 40°C for 15 min., then programmed to 220°C at 3°C/min and held isothermal for 15 min; injector and detector temperatures were 250°C and 270°C respectively; carrier gas was He at a flow rate of 1.3 mL/min; Sample size, 1.0 μL; split ratio, 50:1. The percentage composition of the essential oil was determined with a Class-GC 10 computer program.

Gas chromatography-mass spectrometry: The analysis of the essential oil was performed using a Varian Saturn 2100 (Old York Rd., Ringoes, NJ, USA), E.I Quadrapole machine, equipped with a ZEBRON–5 MS fused silica capillary column [60 m x 0.25 mm (i.d.), film thickness 0.25 μm]. For GC–MS detection, an electron ionization system with an ionization energy of 70eV was used. The carrier gas was helium (20 psi) at a flow rate of 1.7 mL/min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The oven temperature was held at 40°C for 5 min, then increased up to 220°C with 2°C/min increments and held at this temperature for 10 min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 μL were injected manually in the splitless mode. The relative percentages of the oil constituents were expressed as percentages.

Identification of components: Identification of components of the essential oil was based on GC retention indices and computer matching with the Wiley and NIST, 2005 Library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature, and when possible, by co-injection with authentic samples. The identity of the main component of the essential oil was also assigned by ^{1}H-NMR spectroscopy at 300 MHz, on a Varian-300 Spectrometer, using $CDCl_3$ as solvent and TMS as internal standard. The NMR spectroscopic data of *trans*-β-caryophyllene were in agreement with data given in the literature [35].

Antimicrobial activity

Microorganisms and cultivation conditions: Human pathogens *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 27607 and *Candida albicans* ATCC 10239 were used. The above mentioned bacteria were cultured in Nutrient Broth (NB) (Difco) at 37±0.1°C; *S. mutans* was cultured in Brain Heart Infusion Broth (BHIB) (Difco) at 37±0.1°C; and *C. albicans* in Sabouraud Dextrose Broth (SDB) (Difco) at 28±0.1°C. Inocula, prepared by adjusting the turbidity of the medium to match the 0.5 McFarland Standard Dilutions of this suspension in 0.1% peptone (w/v) solution in sterile water, were inoculated on NB, BHIB, and SDB to check the viability of the preparation. The cultures of bacteria were maintained on their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Antimicrobial assays: The antimicrobial activity of the essential oil was determined by using the standard disc diffusion method [36]. The oil was injected into sterilized discs of 6 mm diameter (Schleicher & Schuell). Mueller Hinton Agar (MHA) (Difco) and Sabouraud Dextrose Agar (SDA) (Difco) sterilized in a flask and cooled to 45-50°C were distributed into sterilized Petri dishes with a diameter of 9 cm (15 mL), after injecting cultures (0.1 mL) of bacteria and yeast and distributing the medium in Petri dishes homogeneously. Dishes injected with the above mentioned materials were located on the solid agar medium by pressing slightly. Petri dishes were kept at 4°C for 2 h; plaques injected with yeast were incubated at 28°C for 48 h, and the bacteria were incubated at 37°C for 24 h. On each plate, an appropriate reference antibiotic disc was applied, depending on the test microorganism. At the end of the period, inhibition zones formed on the MHA and SDA were evaluated in mm. Studies were performed in triplicate, and the developing inhibition zones were compared with those of reference discs.

Acknowledgments – The authors would like to thank to Dr Tuncay Dirmenci, Department of Biology, Faculty of Necatibey Education, Balikesir University, for the identification of the plant sample. GC and GC-MS spectra were performed at the Department of Chemistry, Faculty of Arts and Science and, University of Muğla.

References

[1] Duman H, Kartal M, Altun L, Demirci B, Baser KHC. (**2005**) The essential oil of *Stachys laetivirens* Kotschy & Boiss. ex Rech. fil., endemic in Turkey. *Flavour and Fragrance Journal*, ***20***, 48-50.

[2] Bhattacharjee R. (**1982**) *Stachys* L. In: *Flora of Turkey and the East Aegean Islands*, Davis PH. (Ed.). Edinburgh University Press, Edinburgh, 199–262.

[3] Baytop T. (**1995**) *Therapy with Medicinal Plants in Turkey*. Nobel Tıp Publication Press, Istanbul, 193.

[4] Hartwell JL. (**1982**) *Plants Used Against Cancer*. A Survey. Quarterman Publications, Lawrence, MA, 274-275.

[5] Skaltsa HD, Lazari DM, Chinou IB, Loukis AE. (**1999**) Composition and antibacterial activity of the essential oils of *Stachys candida* and *S. chrysantha* from Southern Greece. *Planta Medica*, ***65***, 255–256.

[6] Duke JA. (**1986**) *Handbook of Medicinal Herbs*. CRC Press, FL, 457.

[7] Giorgio P, Mario C, Paola M, Stefania Z, Antonella D, Bruno T. (**2006**) Chemical composition and antimicrobial activities of essential oil of *Stachys glutinosa* L. from Sardinia. *Natural Product Communications*, ***1***, 1133-1136.

[8] Maleki N, Garjani A, Nazemiyeh H, Nilfouroushan N, Sadat ATE, Allameh Z, Hasannia N. (**2001**) Potent anti-inflammatory activities of hydroalcoholic extract from aerial parts of *Stachys inflata* on rats. *Journal of Ethnopharmacology*, ***75***, 213-218.

[9] Yamahara J, Kitani T, Kobayashi H, Kawahara Y. (**1990**) Studies on *Stachys sieboldii* Miq .2. Anti-anoxia action and the active constituents. *Yakugaku Zasshi-Journal of the Pharmaceutical Society of Japan,* ***110***, 932–935.

[10] Khanavi M, Sharifzadeh M, Hadjiakhoondi A, Shafiee A. (**2005**) Phytochemical investigation and anti-inflammatory activity of aerial parts of *Stachys byzanthina* C. Koch. *Journal of Ethnopharmacology*, ***97***, 463-468.

[11] Delazar A, Celik S, Gokturk RS, Unal O, Nahar L, Sarker SD. (**2005**) Two acylated flavonoid glycosides from *Stachys bombycina*, and their free radical scavenging activity. *Pharmazie,* ***60***, 878-880.

[12] Takeda Y, Zhang HJ, Masuda T, Honda G, Otsuka H, Sezik E, Yesilada E, Sun HD. (**1997**) Megastigmane glucosides from *Stachys byzantine. Phytochemistry*, ***44***, 1335-1337.

[13] Ahmad VU, Arshad S, Bader S, Ahmed A, Iqbal S, Tareen RB. (**2006**) New phenethyl alcohol glycosides from *Stachys parviflora*. *Journal of Asian Natural Products Research*, ***8***, 105-111.

[14] Basaran AA, Calis I, Anklin C, Nishibe S, Sticher O. (**1988**) Lavandulifolioside - A new phenylpropanoid glycoside from *Stachys lavandulifolia*. *Helvetica Chimica Acta,* ***71***, 1483-1490.

[15] Puertas-Mejia M, Hillebrand S, Stashenko E, Winterhalter P. (**2002**) *In vitro* radical scavenging activity of essential oils from Columbian plants and fractions from oregano (*Origanum vulgare* L.) essential oil. *Flavour and Fragrance Journal*, ***14***, 380-384.

[16] Vundac VB, Brantner AH, Plazibat M. (2007) Content of polyphenolic constituents and antioxidant activity of some *Stachys* taxa. *Food Chemistry*, ***104***, 1277-1281.

[17] Erdemoglu N, Turan NN, Cakici I, Sener B, Aydin A. (**2006**) Antioxidant activities of some Lamiaceae plant extracts. *Phytotherapy Research*, ***20***, 9-13.

[18] Rabbani M, Sajjadi SE, Zarei HR. (**2003**) Anxiolytic effects of *Stachys lavandulifolia* Vahl on the elevated plus-maze model of anxiety in mice. *Journal of Ethnopharmacology*, ***89***, 271-276.

[19] Duarte MCT, Figueira GM, Sartoratto A, Rehder VLG, Delarmelina C. (**2005**) Anti-candida activity of Brazilian medicinal plants. *Journal of Ethnopharmacology*, ***97***, 305-311.

[20] Digrak M, Alma MH, Ilcim A. (**2001**) Antibacterial and antifungal activities of Turkish medicinal plants. *Pharmaceutical Biology*, ***39***, 346-350.

[21] Tosun F, Kizilay CA, Sener B, Vural M, Palittapongarnpim P. (**2004**) Antimycobacterial screening of some Turkish plants. *Journal of Ethnopharmacology*, ***95***, 273-275.

[22] Karakaya S, Kavas A. (**1999**) Antimutagenic activities of some foods. *Journal of the Science of Food and Agriculture*, ***79***, 237-242.

[23] Skaltsa HD, Demetzos C, Lazari D, Sokovic M. (**2003**) Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece. *Phytochemistry*, ***64***, 743-752.

[24] Skaltsa HD, Mavrommati A, Constantinidis T. (**2001**) A chemotaxonomic investigation of volatile constituents in *Stachys* subsect. Swainsonianeae (Labiatae). *Phytochemistry*, ***57***, 235-244.

[25] Cakir A, Duru ME, Harmandar M, Izumi S, Hirata T. (**1997**) The volatile constituents of *Stachys recta* L. and *Stachys balansae* L. from Turkey. *Flavour Fragrance Journal*, ***12***, 215-218.

[26] Kaya A, Demirci B, Baser KHC. (**2001**) The composition of the essential oil of *Stachys iberica* subsp. *stenostachya* growing in Turkey. *Chemistry of Natural Compounds*, ***37***, 326-328.

[27] Harmandar M, Duru ME, Cakir A, Hirata T, Izumi S. (**1997**) Volatile constituents of *Stachys obliqua* L. (Lamiaceae) from Turkey. *Flavour and Fragrance Journal*, ***12***, 211-213.

[28] Özkan G, Göktürk RS, Ünal O, Çelik S. (**2006**) Determination of the volatile constituents and total phenolic contents of some endemic *Stachys* taxa from Turkey. *Chemistry of Natural Compounds*, ***42***, 172-174.

[29] Duru ME, Cakir A, Harmandar M, Izumi S, Hirata T. (**1999**) The volatile constituents of *Stachys athorecalyx* C. Koch. from Turkey. *Flavour and Fragrance Journal*, ***14***, 12-14.

[30] Flamini G, Cioni PL, Morelli I, Celik S, Gokturk RS, Unal O. (**2005**) Essential oil of *Stachys aleurites* from Turkey. *Biochemical Systematics and Ecology*, ***33***, 61-66.

[31] Dulger B, Uğurlu E, Aki C, Suerdem TB, Çamdeviren A, Tazeler G. (**2005**) Evaluation of antimicrobial activity of some endemic *Verbascum*, *Sideritis*, and *Stachys* species from Turkey. *Pharmaceutical Biology*, ***43***, 270-274.

[32] Dulger B, Gönüz A. (**2004**) Antimicrobial activity of some endemic *Verbascum*, *Salvia*, and *Stachys* species. *Pharmaceutical Biology*, ***42***, 301-304.

[33] Kılıç T. (**2006**). Analysis of essential oil composition of *Thymbra spicata* var. *spicata*: Antifungal, antibacterial and antimycobacterial activities. *Zeitschrift fur Naturforschung C*, ***61***, 324-328.

[34] European Pharmacopoeia 5th edition (**2004**) Council of Europe: Strasbourg Cedex, France 2.8.12, 217-218.

[35] Swigar, A. (**1981**) Monoterpenes. In *Infrared, Mass, ^{1}H NMR and ^{13}C NMR Spectra, and Kovats Indices*. Silverstein RM (Ed). Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

[36] Collins CH. (**1995**) Microbiological Methods. In: Lyne PM, Grange JM, (Ed). Butterworths, London, p. 493.