Asian Journal of Chemistry Vol. 18, No. 3 (2006), 2127–2135

Evaluation of Essential Oil and Methanol Extracts of *Thymus kotschyanus* for Chemical Compounds and Antibacterial Activity

SUZAN OZTURK* and AVNI OZTURK†
The Ministry of Health of Turkey
Erzurum Regional Hygiene Institute, Erzurum 25100, Turkey
Fax: (90)(442)2342888; Tel: (90)(442)2342354
E-mail: suzanoz25@hotmail.com

The essential oils obtained from the aerial parts of *Thymus kotschyanus* were analyzed for chemical compounds by using GC and GC-MS. In addition, following the methanol extraction of aerial parts of *Thymus kotschyanus*, soluble fraction was tested for its antibacterial activity by using disc diffusion assay. Twenty-three components accounting for 98.34% of oil were identified. The main compounds were thymol (40.39%), carvacrol (28.15%), *p*-cymene (10.76%) and γ-terpinene (8.13%). The antibacterial activity results showed that methanol extract of *Thymus kotschyanus* had a great potential of antibacterial activity against 38 of 52 bacteria species, in particular, *Arthrobacter atrocyaneus*, *Bacillus cereus*, *Bacillus flexus*, *Bacillus lentimorbis*, *Brevibacillus brevis*, *Erwinia chrysanthemi*, *Kocuria rosea*, *Micrococcus lylae*, *Paenibacillus macerans*, *Pantoea agglomerans*, *Pseudomonas syringae syringae*, *Staphylococcus cohnii cohnii*, *Xanthomonas arboricola carylina* and methanol extract inhibited growth of these bacteria at the concentration of 15.60–500 μg/mL. The results suggest that *Thymus kotschyanus* represents an inexpensive source of natural mixtures of antibacterial compounds that exhibit potentials for use in food systems to prevent the growth of food-borne bacteria and extends the shelf life of the processed food.

Key Words: Antibacterial screening, Crude extract, *Thymus kotschyanus*, Essential oils.

INTRODUCTION

Herbal medicine represents one of the most important fields of traditional medicine in Turkey, especially in rural areas. Thus, phytotherapy is practised by a large proportion of Turkey population for the treatment of several physical, physiological, mental and social ailments[1]. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs it is essential

†Department of Biology, Faculty of Arts and Sciences, Yuzuncu Yil University, Van 65080, Turkey. Fax: (90)(442)2342888; Tel: (90)(442)2342354; E-mail: avniozturk2002@yahoo.com

to study medicinal plants, which have folklore reputation in a more intensified way[2]. Plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth (antibiotic activity). Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties[3, 4]. The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs. Recently, the acceptance of traditional medicine as an alternative form for healthcare and the development of microbial resistance to the available antibiotics have led authors to investigate the antimicrobial activity of medicinal plants[5, 6].

Turkey is recognized as one of the main diversity centres of *Thymus*. *Thymus* (Labiatae) is a polymorphic genus with 60 taxa belonging to 39 species in Turkey. The ratio of endemism is 45%. The studies have indicated that 49% of all *Thymus* taxa are considered as oil-rich (> 1%). The survey has also revealed that 24 *Thymus* taxa contained thymol and 11 taxa contained carvacrol as main constituents[7]. Most of *Thymus* species are traditionally used by Turkish people as folk remedies as tonic, carminative, digestive, antispasmodic, antiinflammatory, antitussive, expectorant and for the treatment of cold[1].

Recent studies have shown that *Thymus* species have strong antibacterial, antifungal, antiviral, antiparasitic and antioxidant activities[8–12]. Moreover, in Eastern part of Turkey, it is added in a special cheese, namely 'herby cheese'. About 25 kinds of herbs can be used to make herby cheese, *e.g.*, *Falcaria vulgaris*, *Allium* spp., *Thymus* spp., *Ferula* spp., *Anthriscus nemorosa*, etc. From these herbs, single or mixture of some herbs can be added. The rate of the addition of herbs changes between 0.5 and 2 kg per curd obtained from 100 L of milk[13].

Available literature indicates that no previous studies have been carried out on the antibacterial properties of *Thymus kotschyanus*. Most of the illnesses and complaints against which *Thymus kotschyanus* is traditionally used are infectious noxes. Antibacterial properties of the plant were therefore assumed. Thus, it was the aim of this study to investigate the essential oil composition and determine the antibacterial activities of methanol extract of *Thymus kotschyanus*.

EXPERIMENTAL

The plant (aerial parts) used for the present study was collected locally in Erzurum region of Turkey. Plant materials were further identified by senior taxonomists, Avni Ozturk, Department of Botany, Yuzuncu Yil University, Van, Turkey and voucher specimen was deposited in the herbarium of the Horticulture Department, Agricultural Faculty, Ataturk University, Erzurum, Turkey. The dried and powdered plant materials (400 g) were extracted successively in a Soxhlet with methanol (MeOH) at 72 h at a temperature not exceeding the boiling point of the solvent[14]. The extracts were filtered using Whatmann filter paper (No. 1) and then concentrated *in vacuo* at 40°C using a rotary evaporator. The residues obtained were stored in a freezer at –80°C until further tests.

Essential oil distillation

Aerial parts of the plants were subjected to hydrodistillation for 3 h using a

Clevenger-type apparatus (ILDAM Ltd., Ankara, Turkey) to produce essential oil (0.44% yield w/w).

GC and GC-MS Analysis

The GC analyses were accomplished using a HP-5890 Series II gas chromatography equipped with a FID and HP-WAX and HP-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness) working with the following temperature program: 60°C for 10 min, rising at 5°C/min to 220°C; injector and detector temperatures 250°C; carrier gas, nitrogen (2 mL/min); detector, dual FID; split ratio, 1 : 30). The percentage composition was obtained from electronic integration measurements using flame ionization detection. Alkanes were used as reference points in the calculation of relative retention indices (RRI). GS-MC analyses were performed under the same conditions with GC using a Hewlett-Packard 5890 II gas chromatography equipped with a Hewlett-Packard 5972 mass selective detector. Analytic conditions: injector and transfer line temperatures 220 and 240°C, respectively; oven temperature programmed from 60–240°C at 3°C/min; carrier gas: helium at 1 mL/min; injection of 0.2 μL (10% hexane solution); split ratio: 1 : 30. Identification of the constituents was based on comparison of their retention time and mass spectra with those of authentic samples, NBS75K library data of the GC-MS system and literature data.

Biological materials

Total 100 bacterial strains belonging to 52 bacteria species which are listed in Table-1 were used in this study. The bacteria, from herby cheese collected from retail markets in Van and maintained on nutrient agar (Merck, Darmstadt, Germany) were supplied by Microbiology Laboratory of Faculty of Agriculture, Ataturk University, Erzurum, Turkey. Identity of the bacteria used in this study was confirmed by Microbial Identification System in Biotechnology Application and Research Center at Ataturk University.

Antibacterial activity

The antibacterial activity of the extracts was carried out by disc diffusion test[15] using 100 μL of suspension containing 10^8 CFU/mL of bacteria spread on nutrient agar (NA) medium. Sterile 6 mm diameter filter paper discs were impregnated with 300 μg of the sterile test material and placed on to nutrient agar. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. Ofloxacin (5 μg/disc), sulbactam (30 μg) + cefoperazona (75 μg) (105 μg/disc) and/or netilmicin (30 μg/disc) were used as positive reference standards to determine the sensitivity of one strain in each bacterial species tested. The inoculated plates with food-associated bacteria were incubated at 27°C for 24 h. The antibacterial activity was measured as the diameter (mm) of clear zone of growth inhibition. Five discs per plate and three plates were used and each test was run in triplicate[16].

Microdilution assays

The minimal inhibition concentration (MIC) values were also studied for the bacteria which were determined as sensitive to the extracts in disc diffusion assay. The inocula of bacteria were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. *Thymus kotschyanus* extracts dissolved in 0.5% dimethyl sulfoxide (DMSO) were first diluted to the highest concentration (500 μg/mL) to be tested and then serial two-fold dilutions were made in a concentration range from 7.80–500 μg/mL in 10 mL sterile test tubes containing nutrient broth. MIC values of radish extracts against bacterial strains were determined based on a micro-well dilution method. The 96-well plates were prepared by dispensing into each well 95 μL of nutrient broth and 5 μL of the inoculum. 100 μL from *Thymus kotschyanus* extracts initially prepared at the concentration of 500 μg/mL was added into the first wells. Then, 100 μL each from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μL of nutrient broth without compound and 5 μL of the inoculum on each strip was used as negative control. The final volume in each well was 200 μL. Maxipime (Bristol-Myers Squibb) at the concentration range of 500–7.8 μg/mL was prepared in nutrient broth and used as standard drug for positive control. Contents of each well were mixed on a plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by absorbance at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument Inc., Highland Park, Vermont, USA) and confirmed by plating 5 μL samples from clear wells on nutrient agar medium. The extract tested in this study was screened two times against each organism. The MIC of each extract was taken as the lowest concentration that showed no growth[17].

RESULTS AND DISCUSSION

The aromatic and medicinal properties of the genus *Thymus* have made it one of the most popular plants throughout the world. The present study was conducted to study the *in vitro* antibacterial activity of aerial parts of *Thymus kotschyanus* used by Turkish people for medicinal purposes to show that the therapeutic properties of this species used in traditional medicine coincide with laboratory findings.

Water-distilled essential oil from aerial parts of *Thymus kotschyanus* was analyzed by GC-MS and results shown in Table-1. In the oil of *Thymus kotschyanus*, twenty-one components were identified, which represented about 98.34% of the total detected constituents. The main compounds were thymol 40.39, carvacrol 28.15, *p*-cymene 10.76 and γ-terpinene 8.13%. The other components were present in amount less than 2% (Table-1). In particular, monoterpene phenols were the most abundant compound group of the oil. In regard to the previously reported contents of the essential oil of *Thymus kotschyanus*[18, 19], it is interesting that there are no important qualitative differences between the present work and those studies but there are some quantitative differences indicating that environmental factors strongly influence its chemical composition.

TABLE-1
COMPOSITION OF THE ESSENTIAL OIL OF *THYMUS KOTSCHYANUS*

Compounds	RRI	Relative (%)
Tricyclene	968	0.53
α-Thujene	971	0.33
α-Pinene	975	0.32
β-Pinene	983	0.18
Myrcene	988	1.49
α-Phallandrene	993	0.24
α-Terpinene	997	1.71
p-Cymene	1013	10.76
Limonene	1028	0.68
γ-Terpinene	1037	8.13
Thymol	1107	40.39
Carvacrol	1129	28.15
β-Caryophyllene	1145	0.88
Germacrene-D	1263	0.70
Bicyclogermacrene	1272	0.16
β-Bisabolene	1345	0.88
γ-Cadinene	1367	0.20
Elemol	1382	0.15
Spathulenol	1412	0.13
Caryophyllene oxide	1423	0.23
α-Cadinol	1433	1.10
Total		98.34

RRI, relative retention indices calculated against *n*-alkanes.
%, calculated from FID.

The highest antibacterial activity of this plant was determined against *Arthrobacter atrocyaneus*, *Bacillus cereus*, *Bacillus flexus*, *Bacillus lentimorbis*, *Brevibacillus brevis*, *Erwinia chrysanthemi*, *Kocuria rosea*, *Micrococcus lylae*, *Paenibacillus macerans*, *Pantoea agglomerans*, *Pseudomonas syringae syringae*, *Staphylococcus cohnii cohnii* and *Xanthomonas arboricola carylina* (Table-2).

Based on MIC values, MICs of the tested extract show bacteriostatic activity between 15.60 and 500 μg/mL against 37 out of 52 bacteria species. Although the MICs obtained with the methanol extracts are high compared with those of maxipime in general between 7.81–250 μg/mL (Table-3), these results are of interest since they have been obtained with methanol extracts and are not a pure product and it could be considered to have a good potency level. With phytochemical studies, *Thyms kotschyanus* was found to be rich in monoterpene, phenols, especially thymol, carvacrol, γ-terpinene and *p*-cymene (Table-1). Thus, the obtained results might be considered sufficient to explain possible synergism among extract components for their antibacterial activity. Based on these results, it is possible to conclude that aerial parts of *T. kotschyanus* have stronger and broader spectrum of antibacterial activity against many food-borne bacteria. This is the first study to provide data that the extracts of *Thymus kotsyanus* evaluated against a wide range of bacteria possess potential antibacterial activities. Therefore, the results also suggest that *T. kotschyanus* represents an inexpensive source of natural mixtures of antibacterial compounds that exhibit a potential for use in food systems to prevent the growth of food-borne bacteria and extend the shelf life of the processed food.

TABLE-2
ANTIBACTERIAL ACTIVITY OF *THYMUS KOTSCHYANUS* EXTRACTS AGAINST THE BACTERIA

Bacterial species	Number of strains/ origins	Inhibition zone in diameter (nm/sensitive strains)*		Positive controls (mm)† Standard antibiotic disc
		Thymus kotschyamus extracts (300 μg/disc) (mm)	Negative control MeOH	
Acidovorax facilis	1	12	—	28 (OFX)
Arthrobacter agilis	2	—	—	31 (SCF)
Arthrobacter atrocyaneus	3	9–16	—	15 (OFX)
Arthrobacter ilicis	4	—	—	20 (OFX)
Arthrobacter protophormiae	2	—	—	21 (NET)
Bacillus cereus	3	12–15	—	21 (OFX)
Bacillus dipsauri	1	11	—	26 (OFX)
Bacillus flexus	1	13	—	27 (SCF)
Bacillus lentimorbus	4	10–15	—	30 (OFX)
Bacillus lichemiformis	4	—	—	29 (OFX)
Bacillus marinus	4	—	—	14 (OFX)
Bacillus megaterium	2	—	—	26 (OFX)
Bacillus psychrosaccharolyticus	2	—	—	15 (OFX)
Bacillus pumilus	1	—	—	24 (SCF)
Bacillus sphaericus	1	15	—	21 (OFX)
Bacillus spp	7	10–13	—	20 (SCF)
Bacillus subtilis	1	10	—	29 (OFX)
Brevibacillus agri	5	15	—	27 (OFX)
Brevibacillus brevis	6	13–14	—	32 (NET)
Brevibacterium linens	2	—	—	22 (SCF)
Chryseomonas luteola	4	7–15	—	30 (OFX)
Citrobacter amalonaticus	2	10	—	23 (NET)
Corynebacterium ammoniagenes	5	9–13	—	20 (OFX)

Bacterial species	Number of strains/ origins	Inhibition zone in diameter (nm/sensitive strains)*		Positive controls (mm)† Standard antibiotic disc
		Thymus kotschyamus extracts (300 μg/disc) (mm)	Negative control MeOH	
Corynebacterium cystitidis	2	—	—	18 (OFX)
Corynebacterium flavescens	1	11	—	24 (OFX)
Enterococcus faecalis	3	—	—	10 (SCF)
Enterobacter hormaechei	5	9–15	—	22 (OFX)
Enterobacter intermedius	4	8–10	—	16 (SCF)
Enterobacter sakazakii	1	—	—	21 (NET)
Erwinia carotovora	4	8–13	—	20 (NET)
Erwinia chrysanthemi	4	16–20	—	17 (SCF)
Exiguobacterium acetylicum	1	12	—	20 (OFX)
Flavimonas oryzihabitans	3	8–18	—	30 (OFX)
Kocuria kristinae	2	10	—	24 (NET)
Kocuria rosea	1	14	—	15 (OFX)
Micrococcus luteus	1	8	—	28 (OFX)
Micrococcus lylae	3	9–21	—	30 (OFX)
Moraxella catarrhalis	1	19	—	18 (OFX)
Neisseria subflava	1	11	—	24 (OFX)
Paenibacillus apiarius	3	11–16	—	30 (OFX)
Paenibacillus macerans	4	13–18	—	30 (OFX)
Paenibacillus polymyxa	1	—	—	10 (OFX)
Pantoea agglomerans	1	14	—	30 (OFX)
Proteus vulgaris	1	10	—	20 (OFX)
Pseudomonas putida	3	8–12	—	17 (OFX)
Pseudomonas syringae syringae	2	18	—	15 (OFX)
Psychrobacter immobilis	1	—	—	20 (OFX)
Salmonella typhimuirum	1	—	—	28 (OFX)
Serratia liquefaciens	3	10–12	—	30 (OFX)
Shigella dysenteriae	1	9	—	21 (NET)
Staphylococcus cohnii-cohnii	1	13	—	12 (OFX)
Xanthomonas arboricola corylina	4	10–14	—	22 (OFX)
Total 52 bacterial species	130			

*MeOH, methanol extract.

†OFX, ofloxacin (5 μg/disc); SCF, sulbactam (30 μg) + cefoperazona (75 μg) (105 μg/disc); NET, netilmicin (30 μg/disc) were used as positive reference standards antibiotic discs (oxoid).

TABLE-3
MIC VALUES (μg/mL) OF *THYMUS KOTSCHYANUS* EXTRACTS AGAINST MICROORGANISMS IN MICRODILUTION ASSAY

Bacterial species	No. of strains	*T. kotschyanus* extracts (μg/mL)	Standard drug (maxipime) (μg/mL)
Acidovorax facilis	1	250	250
Arthrobacter atrocyaneus	1	250	62.50
Bacillus spp.	1	31.25	7.81
Bacillus cereus	1	125	7.81
Bacillus dipsauri	1	250	250
Bacillus flexus	1	250	125
Bacillus lentimorbus	1	31.25	7.81
Bacillus sphaericus	1	500	31.25
Bacillus subtilis	1	62.50	7.81
Brevibacillus agri	1	62.50	500
Brevibacillus brevis	1	62.50	7.81
Citrobacter amalonaticus	1	31.25	7.81
Corynebacterium ammoniagenes	1	250	7.81
Corynebacterium flavescens	1	250	7.81
Chryseamanas luteola	1	125	31.25
Enterobacter harmaechei	1	125	7.81
Enterobacter intermedius	2	250	31.25
Erwinia carotovora	1	250	31.25
Erwinia chrysanthemi	1	250	15.60
Exiguobacterium acetylicum	1	125	7.81
Flavimonas oryzihabitans	1	250	15.60
Kocuria kristinae	1	250	15.60
Kocuria rosea	1	31.25	31.25
Micrococcus luteus	1	125	62.50
Micrococcus lylea	1	250	7.81
Moraxella catarrhalis	1	15.60	7.81
Neisseria subflava	1	250	7.81
Paenibacillus apiarius	1	125	250
Paenibacillus macerans	1	250	62.50
Pantoea agglomerans	1	500	7.81
Proteus vulgaris	1	250	62.50
Pseudomonas putida	1	250	250
Pseudomonas syringae syringae	2	500	250
Serratia liquefaciens	1	250	62.50
Shigella dysenteriae	1	500	7.81
Staphylococcus cohnii cohnii	1	15.60	7.81
Xanthomonas arboricola corylina	1	250	250

REFERENCES

1. T. Baytop, Türkiye'de Bitkiler ile Tedavi, I.U. Yayinlari No. 3255, Eczacilik Fak. No. 40, 444s (1984) (in Turkish).
2. R.A.A. Mothana and U. Lindequist, *J. Ethnopharmacology*, **78**, 267 (2004).
3. R.N. Chopra, S.L. Nayer and I.C. Chopra, Glossary of Indian Medicinal Plants, 3rd Edn., Council of Scientific and Industrial Research, New Delhi, pp. 7–246 (1992).
4. J. Bruneton, Pharmacognosy, Phytochemistry, Medicinal Plants, Lavoisier Publishing Co., France, pp. 265–380 (1995).
5. D. Srinivasan, S. Nathan, T. Suresh and O. Perumalsamy, *J. Ethnopharmacology*, **74**, 217 (2001).
6. Y. Kumarasamy, P. Cox, M. Jaspars, L. Nahar and S. Sarker, *J. Ethnopharmacology*, **83**, 73 (2002).
7. K.H.C. Baser, *Pure Appl. Chem.*, **74**, 527 (2002).
8. S. Cosentino, C.I.G. Tuberoso, B. Pisano, M. Satta, V. Mascia, E. Arzedi and F. Palmas, *Lett. Appl. Microbiol.*, **29**, 130 (1999).
9. S. Karaman, M. Digrak, U. Ravid and A. Ilcim, *J. Ethnopharmacology*, **76**, 183 (2001).
10. I. Rasooli and S.A. Mirmostafa, *Fitoterapia*, **73**, 244 (2002).
11. E. Stahl-Biskup and F. Saez, Thyme, Taylor & Francis, London (2002).
12. G. Vardar-Unlu, F. Candan, A. Sokmen, D. Daferera, M. Polissiou, M. Sokmen, E. Donmez and B. Tepe, *J. Agric. Food Chem.*, **51**, 63 (2003).
13. Z. Tarakci, H. Coskun and Y. Tuncturk, *Food Technol. Biotechnol.*, **42**, 47 (2004.)
14. J. Lin, A.R. Opoku, M. Geheeb-Keller, A.D. Hutchings, S.E. Terblanche, A.K. Jager and J. van Staden, *J. Ethnopharmacology*, **68**, 267 (1999).
15. J. Kim, M.R. Marshall and C. Wie, *J. Agric. Food Chem.*, **43**, 2839 (1995).
16. C.D. Djipa, M. Delmee and P. Quetin-Leclercq, *J. Ethnopharmacology*, **71**, 307 (2000).
17. F. Sahin, I. Karaman, M. Gulluce, H. Ogutcu, M. Sengul, A. Adiguzel, S. Ozturk and R. Kotan, *J. Ethnopharmacology*, **87**, 61 (2003).
18. S.Y. Guseinov, K.M. Kagramanova, F.Y. Kasumov and R.A. Akhundov, *Farmakologiya i Toksikoloiya*, **50**, 73 (1987).
19. F. Sefidkon and M. Dabiri, *Flav. Fragr. J.*, **14**, 405 (1999).

(*Received*: 23 September; *Accepted*: 3 March 2006) AJC-4694