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Essential oil composition and antibacterial activity of *Thymus caramanicus* at different phenological stages

S. Nejad Ebrahimi [a,*], J. Hadian [b], M.H. Mirjalili [b], A. Sonboli [c], M. Yousefzadi [d]

[a] Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, Tehran, Iran
[b] Department of Agriculture, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, Tehran, Iran
[c] Department of Biology, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, Tehran, Iran
[d] Department of Biology, Faculty of Science, Tarbiat Modaress University, Tehran, Iran

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ABSTRACT

Thymus species are well known as medicinal plants because of their biological and pharmacological properties. *Thymus caramanicus* is an endemic species grown in Iran. Variation in the quantity and quality of the essential oil of wild population of *T. caramanicus* at different phenological stages including vegetative, floral budding, flowering and seed set are reported. The oils of air-dried samples were obtained by hydrodistillation. The yields of oils (w/w%) at different stages were in the order of: flowering (2.5%), floral budding (2.1%), seed set (2.0%) and vegetative (1.9%). The oils were analyzed by GC and GC–MS. In total 37, 37, 29 and 35 components were identified and quantified in vegetative, floral budding, full flowering and seed set, representing 99.3, 98.6, 99.2 and 97.8% of the oil, respectively. Carvacrol was the major compound in all samples. The ranges of major constituents were as follow: carvacrol (58.9–68.9%), *p*-cymene (3.0–8.9%), γ-terpinene (4.3–8.0%), thymol (2.4–6.0%) and borneol (2.3–4.0%). Antibacterial activity of the oils and their main compounds were tested against seven Gram-positive and Gram-negative bacteria by disc diffusion method and determining their minimum inhibitory concentration (MIC) values. The inhibition zones (IZ) and MIC values for bacterial strains, which were sensitive to the essential oil of *T. caramanicus*, were in the range of 15–36 mm and 0.5–15.0 mg/ml, respectively. The oils of various phenological stages showed high activity against all tested bacteria, of which *Bacillus subtilis* and *Pseudomonas aeruginosa* were the most sensitive and resistant strains, respectively. Thus, they represent an inexpensive source of natural antibacterial substances that exhibited potential for use in pathogenic systems.

1. Introduction

Global interest in biopreservation of food systems has recently been increased because of great economic costs of deterioration and poisoning of food products by food pathogens. Essential oils and extracts of various species of edible and medicinal plants, herbs, and spices constitute of very potent natural biologically active agents (Nychas, Tassou, & Skandamis, 2003). Use of essential oils as antimicrobial agents in food systems may be considered as an additional intrinsic determinant to increase the safety and shelf life of foods (Koutsoumanis, Taoukis, Tassou, & Nychas, 1998; Skandamis, & Nychas, 2000; Tassou, Drosinos, & Nychas, 1995).

Variation in chemical composition of essential oils, in particular, and extracts of medicinal plants may be observed due to the origin, the environmental conditions, and the developmental stage of collected plant materials. Antimicrobial activity of an essential oil is attributed mainly to its major components, although the synergistic or antagonistic effect of one compound in minor percentage of mixture has to be considered (Burt, 2004). Therefore, antimicrobial, antioxidant, and other biological activities may vary, based on the variations in the chemical composition (Chorianopoulos et al., 2004; Leung & Foster, 1996).

The genus *Thymus* L., known as "Avishan" in Persian, is a well known aromatic perennial herb originated from Mediterranean region. Among 215 species of this genus grown in the world, 14 species are distributed in Iranian flora (Jalas, 1982; Stahl-Biskup & Saez, 2002). *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties. In traditional medicine, leaves and flowering parts of *Thymus* species are

* Corresponding author. Tel./fax: +98 21 22431783.
E-mail address: s_ebrahimi@sbu.ac.ir (S. Nejad Ebrahimi).

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widely used as tonic and herbal tea, antiseptic, antitussive and carminative as well as treating colds (Amin, 2005; Zargari, 1990). *Thymus* oils and extracts are widely used in pharmaceutical, cosmetic and perfume industry also for flavoring and preservation of several food products (Bauer, Garbe, & Surburg, 1997).

Compared to reported essential oil compositions of different *Thymus* species, investigations on their biological activities are still scarce. Table 1 shows the essential oil compositions of some Iranian *Thymus* species. The antibacterial and antifungal activity of *T. revolutus* oil from Turkey has been reported (Karaman et al., 2001)). The antibacterial activities of the oils of *T. pubescens* and *T. serpyllum* have been studied and the oils were found to possess bactericidal activities (Rasooli & Mirmostafa, 2002). Bacterial susceptibility and chemical composition of the oils of *T. kotschyanus* and *T. persicus* have been studied (Rasooli & Mirmostafa, 2003). The composition and antioxidant activities of the oils of *T. caespititius*, *T. camphorates* and *T. mastichina* from Portugal have been reported (Miguel et al., 2004).

Thymus caramanicus Jalas is an endemic species to Iran. In Iranian folk medicine leaves of this plant is used in treatment of Rheumatism, skin disorders and as an antibacterial agent (Zargari, 1990). Our preliminary study showed that this species is a rich source of phenolic monoterpenes and carvacrol. Considering that carvacrol rich essential oils are gaining increasing importance for their considerable antimicrobial and antioxidant activity (Pank, Pfefferkorn, & Kruger, 2004), the aim of this research was to study the essential oil composition of this species at different developmental stages and its coherence with antibacterial activity. These results can be used to investigate the optimal harvesting time of this plant for relevant industries.

2. Experimental

2.1. Plant material and isolation procedure

The aerial parts of *T. caramanicus* were collected at different developmental stages (vegetative, floral budding, full flowering and seed set) from its wild habitat in Baft, Kerman province, at an altitude of 2300 m. Voucher specimen was deposited at the Herbarium of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

The essential oil of all air-dried samples (100 g) was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in British Pharmacopoeia (British Pharmacopoeia, 1988). The distillated oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 °C until analysis. The oils were yellow in color and had distinct sharp odor.

2.2. Oil analysis procedure

GC analysis was performed using a Thermoquest gas chromatograph with a flame ionization detector (FID). The analysis was carried out on fused silica capillary DB-1 column (30 m × 0.25 mm i.d.; film thickness 0.25 μm). The injector and detector temperatures were kept at 250 °C and 300 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 1.1 ml/min; oven temperature program was 60–250 °C at the rate of 4 °C/min and finally held isothermally for 10 min; split ratio was 1:50.

GC–MS analysis was carried out by use of Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-1 column (60 m × 0.25 mm i.d.; film thickness 0.25 μm) coupled with a TRACE mass (Manchester, UK). Helium was used as carrier gas with ionization voltage of 70 eV. Ion source and interface temperatures were 200 °C and 250 °C, respectively. Mass range was from 35 to 456 amu. Oven temperature program was the same given above for the GC.

2.3. Identification of compounds

The constituents of the essential oils were identified by calculation of their retention indices under temperature-programmed conditions for *n-alkanes* (C_6–C_{24}) and the oil on a DB-1 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those of reported in the literature (Adams, 2001). For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

2.4. Antibacterial activity

In vitro antibacterial activity of the essential oils was evaluated by disc diffusion method using Mueller-Hinton Agar with determination of inhibition zones (IZ). Three Gram-negative and four Gram-positive bacteria were used as follows: *Bacillus subtilis* ATCC 9372, *Enterococcus faecalis* ATCC 15753, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27852, *Klebsiella pneumoniae* ATCC 3583. Tetracycline and Gentamicine were used as positive standards in order to control the sensitivity of the microorganisms. The incubation conditions used were 24 h at 37 °C. Minimum inhibitory concentration (MIC), defined as the lowest concentration of the oil that resulted in a complete inhibition of visible growth in the broth, was measured by microdilution broth susceptibility assay recommended by NCCLS (1999). The

Table 1
The chemical composition of essential oil of *Thymus* species

Species	Components	References
T. kotschyanus	Thymol (38.0%), carvacrol (14.2%)	Rustaiyan et al. (2000)
T. pubescens	Thymol (37.9%), carvacrol (14.1%)	
T. carmanicus	Thymol (40.8%), carvacrol (24.8%)	Mojab and Nickavar (2006)
T. carnosus	Thymol (27.0%) before flowering Thymol (36.1%) at full flowering	Sefidkon, Askari, and Mirmostafa (2001)
T. eriocalyx	Thymol (42.8%) before flowering Thymol (43.1%) at full flowering	Kalvandi, Sefidkon, Atri, and Mirza (2004)
T. pubescens	Carvacrol (64.8%) before flowering Carvacrol (48.8%) at full flowering	Sefidkon, Askari, and Ghorbanli (2002a)
T. persicus	Carvacrol (39.0%) before flowering Carvacrol (27.1%) at full flowering	Sefidkon et al. (2002a), Sefidkon, Dabiri, and Mirmostafa (2002b)
T. fedtschenkoi	Thymol (31.8%), carvacrol (24.3%)	Abousaber, Hadjakhoondi, and Shafiiee (2002)
T. pubescens	Thymol (63.4%), -terpineol (19.2%)	
T. transcaspicus	Thymol (56.4%)	Miri, Ramezani, Javidnia, and Ahmadi (2002)
T. daenensis subsp. lancifolius	Thymol (73.9%), carvacrol (6.7%)	Sadjadi and Khatamsaz (2003)

essential oils (5 μl) and the pure constituent (5 μl of 70% solution of Carvacrol in MeOH) was applied on the paper discs (the disc diameter was 6 mm). Then disc papers were placed in the inoculated plates. After 24 h of incubation at 37 °C the diameter of growth inhibition zones were measured.

3. Result and discussion

3.1. Chemical composition of the essential oil

The oils were obtained by hydrodistillation of air-dried samples. The yield of essential oil (w/w%) in different stages was in order of: Flowering (2.5%) > floral budding (2.1%) > seed set (2.0%) > vegetative (1.9%). The essential oils were analyzed by GC and GC–MS. In total, 37, 37, 31 and 36 constituents were identified and quantified in the subsequent stages, respectively (Table 2). Results showed that oxygenated monoterpenes are the major portion of all samples (Table 2). The highest content of carvacrol as major component (68.9%) was observed in full flowering stage. The lowest content of carvacrol was observed in vegetative stage but during flower development the amount is increased (66.9%) and at seed setting stage is decreased again. During the flowering stage, the highest and the lowest amounts of phenolic compounds (carvacrol + thymol) and their precursors (*p*-cymene + γ-terpinene) were observed, respectively. At vegetative and seed set stages the amount of phenolic portion was decreased, but the amount of their precursors increased (Table 2).

3.2. Antibacterial activity

The antibacterial activity of *T. caramanicus* essential oils against microorganisms which are considered in this study was assessed by evaluating the presence of IZ and MIC values. Results (Table 3), showed that the essential oils of *T. caramanicus* have great potential of antibacterial activity against all of the seven bacteria tested. The IZ and MIC values for bacterial strains, which were sensitive to the essential oils of *T. caramanicus*, were in the range of 14–37 mm and 0.45–14.4 mg/ml. Screening of pure standard carvacrol on the same microorganisms, under identical conditions, exhibited strong antibacterial activity with IZ ranged 11–40 mm and MIC value of 0.22–14.4 mg/ml. No considerable differences between antibacterial activity of carvacrol and various oils of plant in phenological stages were observed which could be attributed for high amount of this component in all of the oils. Compared to

Table 2
Composition of essential oil of *Thymus carmanicus* at different developmental stages

Compounds	RI	Vegetative stage	Floral budding	Flowering stage	Seed set	Identification methods
Hepten-3-one	863	t	0.1	0.2	0.1	RI, MS
α-Thujene	926	1.1	0.6	1.0	0.9	RI, MS
α-Pinene	935	1.1	0.5	0.6	0.8	RI, MS, CoI
Camphene	949	0.7	0.3	0.6	0.5	RI, MS
Octan-3-one	961	–	t	0.8	0.8	RI, MS
Sabinene	965	0.9	0.7	–	–	RI, MS
β-Pinene	976	0.3	0.1	0.1	0.3	RI, MS, CoI
Octan-3-ol	979	0.2	0.2	t	0.4	RI, MS
Myrcene	981	1.8	1.2	0.9	1.3	RI, MS
α-Phellandrene	1001	0.4	0.2	0.2	0.3	RI, MS
Δ^3-Carene	1008	0.1	t	t	0.1	RI, MS
α-Terpinene	1012	3.0	1.7	–	–	RI, MS
p-Cymene	1014	4.6	3.0	6.0	8.9	RI, MS
1,8-Cineole	1024	1.6	1.2	1.0	2.0	RI, MS, CoI
(Z)-β-Ocimene	1037	0.2	t	–	0.1	RI, MS
γ-Terpinene	1052	8.0	6.4	4.6	6.7	RI, MS, CoI
cis-Sabinen hydrate	1056	0.7	0.5	0.9	0.8	RI, MS
Nonan-3-one	1065	t	t	0.1	0.2	RI, MS
Linalool	1083	1.7	1.3	0.7	2.0	RI, MS, CoI
trans-Sabinen hydrate	1098	0.2	–	0.2	0.1	RI, MS
trans-Pinocarveol	1126	–	0.2	–	t	RI, MS
Borneol	1153	2.8	2.3	4.0	2.4	RI, MS, CoI
Terpine-4-ol	1164	2.0	1.4	0.9	2.4	RI, MS
α-Terpineol	1173	0.2	0.1	0.3	t	RI, MS
cis-Dihydrocarvone	1195	0.4	0.3	0.1	0.1	RI, MS
trans-Dihydrocarvone	1186	t	t	–	0.2	RI, MS
thymol methyl ether	1213	t	0.1	–	–	RI, MS
Bornyl formate	1214	0.1	–	–	0.2	RI, MS
Carvacrol methyl ether	1224	0.6	0.3	0.7	0.6	RI, MS
Carvone	1226	0.2	t	–	0.1	RI, MS, CoI
Thymol	1263	4.6	6.0	5.3	2.4	RI, MS, CoI
Carvacrol	1282	58.9	66.9	68.9	60.2	RI, MS, CoI
Bornyl acetate	1284	t	t	0.1	–	RI, MS
β-Caryophyllene	1419	1.3	1.3	0.5	1.1	RI, MS
β-Bisabolene	1499	0.2	t	–	–	RI, MS
γ-Cadinene	1509	0.1	t	–	t	RI, MS
δ-Cadinene	1523	0.1	–	–	t	RI, MS
cis-α-Bisabolene	1530	1.2	1.7	0.5	1.6	RI, MS
Caryophylene oxide	1583	–	t	t	0.2	RI, MS, CoI
Monoterpene hydrocarbons		13.5	20.7	15.7	15.2	
Oxygenated monoterpenes		82.2	72.9	82.1	80.1	
Sesquiterpene hydrocarbons		1.8	2.7	1.0	3.0	
Oxygenated sesquiterpenes		–	0.2	–	–	
Others		1.4	1.5	1.0	0.3	
Total		99.3	98.6	99.2	97.8	
Oil yield (w/w%)		1.9	2.1	2.5	2.0	

RI, retention indices relative to C_6–C_{24} *n*-alkanes on the DB-1 column; t, trace <0.1%; MS, mass spectrum; CoI, coinjection with an authentic sample.

Table 3
Antibacterial activity of the essential oil of *T. caramanicus* and its main component

Microorganism	Essential oil								Main compound		Standards[d]	
	Vegetative stage		Floral budding		Flowering stage		Seed set		Carvacrol (5 μl/disk)[c]		Tet (30 μg/disk)	Gen (30 μg/disk)
	DD[a]	MIC[b]	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	DD
B. subtilis	35	0.45	37	0.45	36	0.45	34	0.45	37	0.22	21	na
S. aureus	28	1.8	27	1.8	28	1.8	27	1.8	35	0.45	20	na
S. epidermidis	32	1.8	35	0.9	35	0.9	30	1.8	40	0.22	34	na
E. faecalis	17	7.2	16	7.2	16	7.2	18	7.2	20	0.9	9	na
K. pneumoniae	23	1.8	24	1.8	25	1.8	22	3.6	25	0.9	na	20
E. coli	31	0.9	31	0.9	30	0.9	30	0.90	34	0.45	na	23
P. aeruginosa	14	14.4	15	14.4	15	14.4	14	14.4	11	>14.4	na	12

[a] Diameter of inhibition zones (mm) including diameter of sterile disk (6 mm), Essential oil was tested at 5 μl for bacteria.
[b] Minimum Inhibitory Concentration, values as mg/ml.
[c] 5 μl 70% solution of carvacrol in MeOH.
[d] Tet, Tetracycline; Gen, Gentamicine (7–14) moderately active; (>14) highly active; na, not active.

the positive antibacterial standards, the essential oils of *T. caramanicus* and their main component have a stronger antibacterial activity.

Essential oils rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity (Baydar, Sagdic, Ozkan, & Karadogan, 2004). Several studies have focused on the antimicrobial activity of the essential oils of thyme in order to identify the responsible compounds (Burt, 2004; Crespo, Jimenez, Gomis, & Navarro, 1990; Nelson, 1997). Carvacrol, which is the main component of *T. caramanicus* essential oils, has been considered as a biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Helander et al., 1998; Juven, Kanner, Schued, & Weisslowicz, 1994; Ultee, Kets, & Smid, 1999). The effect of carvacrol on *Staphylococus* was investigated by Knowles, Roller, Murray, and Naidu (2005). However, it was also considered that minor components, as well as a possible interaction between the substances could also affect the antimicrobial activities. In fact, other constituents, such as γ-terpinene, have been considered to display relatively good activity due to their possible synergistic or antagonistic effects (Didry, Dubreuil, & Pinkas, 1993; Vardar-Unlu et al., 2003) which is in agreement with our results showing that low amounts of γ-terpinene during the flowering phase may justify the low antimicrobial activity during this period.

4. Conclusion

The data presented confirm the antibacterial potential of *T. caramanicus* essential oil. The essential oils tested represent an inexpensive source of natural antibacterial substances for use in pathogenic systems to prevent the growth of bacteria and extend the shelf life of the processed food. However, further research is needed to evaluate the effectiveness of *T. caramanicus* essential oils in food ecosystems to establish their utility as natural antimicrobial agents in food preservation and safety.

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