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Analytical Methods

GC/MS analysis and *in vitro* antioxidant activity of essential oil and methanol extracts of *Thymus caramanicus* Jalas and its main constituent carvacrol

Javad Safaei-Ghomi [a,*], Abdolrasoul H. Ebrahimabadi [a], Zahra Djafari-Bidgoli [a], Hossein Batooli [b]

[a] Essential Oils Research Center, University of Kashan, Post Code: 87317-51167 Kashan, Iran
[b] Isfahan Research Center of Natural Sources, Kashan Station, Kashan, Iran

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ABSTRACT

Chemical composition of the essential oil, antioxidant activity (DPPH and β-carotene/linoleic acid assays), and total phenolic content (Folin–Ciocalteu assay) of aerial parts of *Thymus caramanicus* were determined. The highest radical-scavenging activity (DPPH test) was shown by the polar subfraction of the methanol extract (IC_{50} = 43.0 μg/ml) which was also higher than that of butylated hydroxytoluene (BHT, IC_{50} = 19.7 μg/ml). However, it was the nonpolar subfraction of the methanol extract that showed the highest inhibition (84.4%), as assessed by the β-carotene/linoleic acid assay, which was only slightly lower than that shown by BHT (93.3%). The antioxidant activities of the essential oil main component (carvacrol) were also evaluated for comparison. Total phenolic content of the polar subfraction, as gallic acid equivalents, was 124.3 μg/mg. Essential oil extracted from the aerial parts by hydrodistillation was analysed by GC and GC/MS. Fifteen constituents, representing 99.3% of the oil, were identified, of which the major ones, carvacrol (85.9%), thymol (3.3%), *p*-cymene (3.2%), γ-terpinene (1.8%) and borneol (1.3%), accounted for 95.6% of the oil.

1. Introduction

Antioxidants are compounds that neutralize chemically active products of metabolism, such as free radicals which can damage the body. Plant phenols and polyphenols, with their potential to act as antioxidants, play a major role in the prevention of various pathological conditions such as cancer, cardiovascular and neurodegenerative diseases believed to be associated with oxidative stress (Losso, Shahidi, & Bagchi, 2007). Plant phenols also exhibit significant antioxidant, antitumoral, antiviral, and antibiotic properties (Apak et al., 2007). Safety and efficacy of the synthetic antioxidants used in the food industry are frequently questioned because such antioxidants are unstable and highly volatile (Sokmen et al., 2004), therefore, interest in finding naturally occurring antioxidants that have the potential to protect human beings from damage induced by oxidative stress has intensified (Scalbert, Manach, Morand, & Remesy, 2005).

Genus *Thymus* comprises about 400 species of hardy, perennial, and aromatic evergreen or semi-evergreen herbaceous plants found mainly in the northern temperate region. *Thymus* is the ancient Greek name for these aromatic plants, which are commonly known as thyme (Bhattacharjee, 2005). They are native to southern Europe and Asia and well-known as medicinal plants because of several biological and pharmacological properties (Tepe et al., 2005; Zargari, 1990). The plants of *Thymus* genus are among the most popular plants throughout the world, commonly used as herbal teas, flavouring agents (condiment and spice), aromatic, and medicinal plants (Stahl-Biskup & Saez, 2002). These species have also been used as carminative, diuretic, urinary disinfectant and vermifuge (Matta et al., 2007). The antioxidant and antimicrobial activity of members of the genus *Thymus*, including the well-known specie, *Thymus vulgaris*, has been widely reported (Dob, Dahmane, Benabdelkader, & Chelghoum, 2006; Marino, Bersani, & Comi, 1999; Ryman, 1992; Schwarz, Ernst, & Ternes, 1996). The essential oils and extracts of many *Thymus* species are widely used in pharmaceutical, cosmetic and perfume industry, and for flavouring and preservation of several food products (Bauer, Garbe, & Surburg, 1997).

Although reports on the essential oils composition of different *Thymus* species are relatively common, investigations on their biological activities are still scarce. *Thymus caramanicus* Jalas (Persian name: avishan kermani) is one of the fourteen species of *Thymus* that grows in the wild in different regions of Iran. In Iranian folk medicine, leaves of this plant are used in the treatment of rheumatism, skin disorders and as an antibacterial agent, and its aerial parts are frequently used in traditional Iranian dishes (Zargari, 1990). In the present work, the results of a study to determine: (1) the chemical composition of a hydrodistilled essential oil from aerial parts of the plant growing wild in Kashan, (2) the antioxidant activity of its essential oil and carvacrol as the main constituent of

* Corresponding author. Tel.: +98 3615555333; fax: +98 3615552930.
E-mail address: safaei@kashanu.ac.ir (J. Safaei-Ghomi).

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the plant essential oil and polar and nonpolar subfractions of the methanol extract using two complementary assay methods: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical activity assay and β-carotene/linoleic acid bleaching assay and (3) total phenolic content of plant extract polar subfraction are reported.

2. Experimental

2.1. Materials

2.1.1. Plant material

Plant materials for extraction were aerial parts (leaves and flowers/inflorescences) of *T. caramanicus* collected during summer 2007 from Kashan area (Isfahan Province, Iran) at an altitude of ca. 2100 m. An authenticated specimen of the plant was deposited in the herbarium of the Kashan Research Botanical Garden, Research Institute of Forests and Rangelands, Kashan, Iran.

2.1.2. Solvents and chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH, 95%), β-carotene, linoleic acid, 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene, BHT), and gallic acid were procured from Sigma–Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol, and dimethyl sulphoxide (DMSO), HPLC grade chloroform, standard Folin–Ciocalteu's phenol reagent, anhydrous sodium sulphate, sodium carbonate, carvacrol, and Tween 40 were obtained from Merck (Darmstadt, Germany). Ultra pure water was used for the experiments.

2.2. Preparation of the extracts

2.2.1. Isolation of the essential oil

Air-dried and ground plant material (125 g) was submitted to water distillation for 3.5 h using an all-glass Clevenger-type apparatus as recommended by European Pharmacopoeia (Anonymous, 1996). The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored in an amber vial at low temperature (4 °C) prior to analysis.

2.2.2. Preparation of methanol extracts

Twenty grams of the powdered aerial parts of the plant were Soxhlet-extracted with 400 ml methanol for 8 h. Solvent removal by rotary evaporator and drying the residue in vacuum oven at 50 °C was yielded 2.3 g (11.5%) of dried extract. This extract was suspended in water and extracted with chloroform (4 × 100 ml) to obtain 1.2 g (6.1%) polar and 1.1 g (5.3%) nonpolar extracts. Extracts were concentrated, dried and kept in the dark at 4 °C until tested.

2.3. Chromatographic analysis

2.3.1. Gas chromatography (GC) analysis

Oil obtained from aerial parts of *T. caramanicus* was analysed using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Restek, Bellefonte, PA) equipped with an FID detector. Oven temperature was maintained at 40 °C for 3 min initially, and then raised at the rate of 3 °C/min to 280 °C. Injector and detector temperatures were set at 220 °C and 290 °C, respectively. Helium was used as carrier gas at a flow rate of 1 ml/min, and diluted samples (1/1000 in *n*-pentane, v/v) of 1.0 μl were injected manually in the splitless mode. Peaks area percents were used for obtaining quantitative data.

2.3.2. Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis of the oil was carried out on an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness; Restek, Bellefonte, PA) equipped with an Agilent HP-5973 mass selective detector in the electron impact mode (Ionization energy: 70 eV) operating under the same conditions as described above. Retention indices were calculated for all components using a homologous series of *n*-alkanes injected in conditions equal to samples ones. Identification of components of essential oil was based on retention indices (RI) relative to *n*-alkanes and computer matching with the Wiley275.L and Wiley7n.L libraries, as well as comparisons of the fragmentation pattern of the mass spectra with data published in the literature (Adams, 2001).

2.4. Antioxidant activity

2.4.1. DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay usually involves hydrogen atom transfer reaction but, based on kinetic data, an electron transfer mechanism has also been suggested for this assay (Foti, Daquino, & Geraci, 2004; Huang, Ou, & Prior, 2005). Radical-scavenging activity (RSA) of *T. caramanicus* essential oil and extracts was determined using a published DPPH radical-scavenging activity assay method (Sarker, Latif, & Gray, 2006) with minor modifications. Briefly, stock solutions (10 mg/ml each) of the essential oil, carvacrol, extracts and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions are made to obtain concentrations ranging from 1 to 5×10^{-10} mg/ml. Diluted solutions (2 ml each) were mixed with 2 ml of freshly prepared 80 μg/ml DPPH methanol solution and allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Ultraviolet (UV) absorbencies of these solutions were recorded on a spectrometer (Cintra 6, GBC, Australia) at 517 nm using a blank containing the same concentration of oils or extracts or BHT without DPPH. Inhibition of free radical DPPH in percent (I%) was calculated as follow:

$$I\% = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

2.4.2. β-Carotene/linoleic acid bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Miraliakbari and Shahidi (2008) was used with slight modifications. A stock solution of β-carotene and linoleic acid was prepared with 0.5 mg of β-carotene in 1 ml chloroform, 25 μl of linoleic, acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of aerated distiled water was then added to the residue. The samples (2 g/l) were dissolved in DMSO and 350 μl of each sample solution was added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h, together with two blanks, one contained the antioxidant BHT as a positive control and the other contained the same volume of DMSO instead of the extracts. The test tube with BHT maintained its yellow colour during the incubation period. The absorbencies were measured at 470 nm on an ultraviolet spectrometer (Cintra 6, GBC, Australia). Antioxidant activities

(inhibition percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after 2h assay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$$

where $A_{\beta\text{carotene after 2 h assay}}$ is the absorbance of β-carotene after 2 h assay remaining in the samples and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

2.4.3. Assay for total phenolics

Total phenolics constituent of the polar subfraction of methanol extract of *T. caramanicus* was determined by literature methods involving Folin–Ciocalteu reagent and gallic acid standard (Slinkard & Singleton, 1997). Solution of the extract (0.1 ml) containing 1000 μg of the extract was taken in a volumetric flask, 46 ml of distiled water and 1 ml Folin–Ciocalteu reagent were added, and the flask was thoroughly shaken. After 3 min, 3 ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (0–1000 mg/0.1 ml) and a standard curve obtained with the following equation:

$$\text{Absorbance} = 0.0012 \times \text{gallic acid}(\mu g) + 0.0033$$

Total phenols of the extract, as gallic acid equivalent, was determined by using the absorbance of the extract measured at 760 nm as input to the standard curve and the equation. All tests were carried out in triplicate and gallic acid equivalent values were reported as means ± SD of triplicates.

3. Results and discussion

3.1. Chemical composition of the essential oil

Hydrodistillation of dried and ground aerial parts of *T. caramanicus* for 3.5 h yielded its essential oil (the yield was 0.99%, w/w). GC and GC/MS analysis of the plant's essential oil led to the identification and quantification of 15 components (Table 1), which accounted for 99.26% of the total oil. Carvacrol (85.94%), thymol (3.33%), *p*-cymene (3.16%), γ-terpinene (1.80%) and borneol (1.33%) were the most abundant components and comprised 95.56% of the oil. The major compounds obtained in this study were also found to be the major components of the essential oil obtained from different parts of *T. caramanicus* growing in Kerman (a province at the south east of Iran), at different stages (Ebrahimi, Hadian, Mirjalili, Sonboli, & Yousefzadi, 2008) and from its aerial parts at the flowering stage (Nariman et al., 2007). The main components of the oil in these two studies were carvacrol (58.9–68.9%), thymol (2.4–6.0%), *p*-cymene (3.0–8.9%), γ-terpinene (4.3–8.0%) and borneol (2.3–4.0%). The high carvacrol content (85.94%) of the plant oil seen in our study is in good agreement with its levels in other thyme species (Bounatirou et al., 2007; Juliano, Mattana, & Usai, 2000; Tepe et al., 2005) but contrary to that observed in those oils from other *Thymus* species the major components of which are geranial, α-muurolol, α-pinene and linalool (Brantner, Pfeifhofer, Ercegovac, Males, & Plazibat, 2005; Miguel et al., 2003; Tepe et al., 2005), compounds not found in the oil we analysed. However, we did find myrcene, β-caryophyllene, and 1,8-cincole in small proportions, although these were the major components in the oil from some *Thymus* species including *Thymus capitatus* (Bounatirou et al., 2007), *Thymus bracteosus* (Brantner et al., 2005), *Thymus mastichina* (Miguel et al., 2003) and *Thymus camphorates* (Miguel et al., 2004).

Table 1
Chemical composition of the essential oil of *T. caramanicus*.

No.	Compound[a]	Composition (%)	*RI*[b]	*RI*[c]
1	α-Thujene	0.16	923	930
2	Myrcene	0.39	987	991
3	α-Terpinene	0.43	1011	1017
4	*p*-Cymene	3.16	1019	1025
5	1,8-Cineole	0.34	1025	1031
6	γ-Terpinene	1.80	1054	1060
7	Borneol	1.33	1163	1169
8	Terpinene-4-ol	0.92	1173	1177
9	α-Terpineol	0.24	1191	1189
10	Thymol methyl ether	0.47	1241	1235
11	Thymol	3.33	1293	1290
12	Carvacrol	85.94	1309	1299
13	Eugenol	0.23	1357	1359
14	β-Caryophyllene	0.33	1413	1419
15	β-Bisabolene	0.19	1511	1506
	Total	99.26		

[a] Compounds listed in order of elution from HP-5MS column.
[b] Relative retention indices to C_8–C_{24} *n*-alkanes on HP-5MS column.
[c] Literature retention indices (Adams, 2001).

3.2. Amount of total phenolic constituents

Based on the measured absorbance value of the polar subfraction of the plant extract reacting with Folin–Ciocalteu reagent, and in comparison with absorbance values of gallic acid solutions in the standard curve, the amount of total phenolics in the extract was estimated at 124.30 ± 2.62 μg/mg (12.4%, w/w). This value is comparable to the values reported in the literature for other *Thymus* species such as *Thymus spathulifolius* (141 μg/mg of the polar subfraction of a methanol extract, reported by Sokmen et al. (2004)) and *Thymus Serpyllum* (113 μg/mg of an ethanol extract, reported by Mata et al. (2007)).

3.3. Antioxidant activity

Radical-scavenging activity of the subfractions of methanol extracts and essential oil from aerial parts of *T. caramanicus* and carvacrol, the main constituent of the plant essential oil, was evaluated by means of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay. The concentrations that led to 50% inhibition (IC_{50}) are given in Table 2. The antioxidant activities were compared with that of butylated hydroxytoluene (BHT), the standard commercial synthetic antioxidant (IC_{50} = 19.72 ± 0.80 μg/ml), and carvacrol (IC_{50} = 448.05 ± 3.62 μg/ml) as the main component of the essential oil. The extract polar subfraction (IC_{50} = 43.17 ± 0.65 μg/ml), with radical-scavenging activity equal to about half of that of BHT, was particularly effective and proved superior to oil and the nonpolar subfraction.

The potential of the plant to inhibit lipid peroxidation was evaluated using the β-carotene/linoleic acid bleaching test. This test

Table 2
Antioxidant activity of the essential oil and methanol extracts subfractions of *T. caramanicus*, carvacrol and BHT in DPPH free radical-scavenging activity and the β-carotene/linoleic acid bleaching assay methods.

Sample	DPPH IC_{50} (μg/ml)	β-carotene/linoleic acid inhibition (%)
Polar subfraction	43.17 ± 0.65	59.83 ± 0.75
Nonpolar subfraction	374.11 ± 2.37	84.55 ± 0.92
Essential oil	263.09 ± 0.62	79.03 ± 0.54
Carvacrol	448.05 ± 3.62	50.18 ± 0.34
BHT (positive control)	19.72 ± 0.80	98.26 ± 0.80
Negative control	NA	4.67 ± 0.53

NA, not applicable.

measures the plant's potential for inhibiting of conjugated diene hydroperoxides formation from linoleic acid oxidation (Tepe et al., 2005). The results of *T. caramanicus* samples and standards (BHT and carvacrol) are presented in Table 2. The essential oil and nonpolar subfraction of the extract showed 79.03% and 84.55% inhibition, respectively, values comparable to that of the synthetic standard BHT (98.26%), whereas the polar subfraction activity was about two-thirds of the activity achieved by BHT.

The plant whole essential oil was showed better antioxidant activities in comparison with its main component (carvacrol) in both DPPH and β-carotene/linoleic acid bleaching tests (Table 2). Seemingly, the presences of components other than carvacrol, even in small amounts (totally making only about 15% of the oil), inserts a synergistic effect on the total oil antioxidant potential and improves its value to about 1.6–1.7 folds in both tests. Collectively, the data obtained from the evaluation of antioxidant activity of *T. caramanicus* by both DPPH and β-carotene/linoleic acid bleaching tests in this research are comparable to the results reported on the other members of *Thymus* family (Bounatirou et al., 2007; Mata et al., 2007; Sacchetti et al., 2005; Sokmen et al., 2004; Tepe et al., 2005), highlighting the considerable potential of the plant as an antioxidant food additive.

Literature review shows the presence of different phenolic compounds such as rosmarinic acid, apigenin, luteolin, caffeic acid, ferulic acid, carnosic acid, and flavonoids in the *Thymus* family plants (Jordan, Martınez, Martınez, Monino, & Sotomayor, 2009; Loziene, Venskutonis, Sipailiene, & Labokas, 2007; Zheng & Wang, 2001). The presence of these compounds in the polar subfraction of *T. caramanicus* extract may also be the main cause of its high radical-scavenging activity and high total phenolic contents. On the other hand, the essential oils of *Thymus* species are rich sources of phenolic monoterpenes such as thymol and carvacrol (Pank, Pfefferkorn, & Kruger, 2004) and high percent of carvacrol in the essential oil of this plant has led to its moderate DPPH inhibitory potential.

Peroxidation inhibition by hydrogen atom donation is the underlying basis of β-carotene/linoleic acid bleaching test. Compounds containing hydrogen atoms in the allylic and/or benzylic positions will show better activity in this test because of relatively easy abstraction of atomic hydrogen from these functional groups by peroxy radicals formed in the test circumstances (Larson, 1997). Except 1,8-cineole and borneol (Table 1), all of the other components of the plant oil abundantly contain allylic and/or benzylic moieties offering it considerable activity in this test. High bleaching activity of the plant extracts in this test may also be a consequence of the presence of allylic and/or benzylic containing compounds in them.

In conclusion, many plant species are currently used as sources of nutritional additives because of their antioxidant properties that increase immunity to some diseases. Our study is the first report of *in vitro* antioxidant activity of the essential oil and various extracts of *T. caramanicus*. The activity was high enough for the plant to be a natural source of strongly antioxidant substances for use as a natural additive in food and pharmaceutical industries.

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