Effect of Heat Treatment on Chemical Composition and Antioxidant Property of *Thymus daenensis* Essential Oil

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Abstract

Background: *Thymus daenensis* Celak. is one of the medicinal plants which cultivated in Iran. This plant involve high amount of polyphenols including thymol and carvacrol, which were reported to possess the high antioxidant activity.

Objective: Antioxidative activity (AOA) and chemical constituents of *Thymus daenensis* essential oil (TDEO) were examined. Also, the effect of thermal treatments (80,120 and 180 ^{o}C) for 1, 2 and 3 hours on the AOA and chemical constituents of TDEO was studied.

Methods: The chemical constituents of *Thymus daenensis* essential oil was analyzed by GC/MS. Antioxidant activity (AOA) of TDEO was determined by two complementary test methods, namely DPPH free radical-scavenging and β-carotene bleaching.

Results: TDEO had different chemical components, including thymol (54.7%), carvacrol (5.0%), linalool (1.9%), 1-octene-3-ol (1.6%), *p*-cymene (11.3%), terpin-4-ol (1.5%), γ-terpinene (12.9%), α-terpinene (2.0%), myrcene (1.1%), β-caryophelene (2.6%) and borneol (1.1%). The IC_{50} of TDEO was compared with synthetic antioxidant (BHT). Antioxidant activities of TDEO at different concentrations (0.1- 3.1 mg/ml) were determined by β-carotene bleaching method. Antioxidant activity of TDEO at these range of concentration were 20 - 96.0 %. After heating up to 180^{o}C, essential oil showed a significantly higher free radical-scavenger activity and evident changes in its chemical composition.

Conclusion: Owing to this property, the studies can be further extended to exploit not only the phenolic extracts but also the residual phenolic constituents associated with this herbal medicine as health supplement and nutraceutical.

Keywords: Antioxidant, *Thymus daenensis*, Phenolic Compound, GC/MS, DPPH

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Introduction

Phenolic compounds are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers [1, 2]. Consumption of fruits and vegetables with high content of antioxidative phytochemicals such as phenolic compounds may reduce the risk of cancer, cardiovascular disease and many other diseases [3]. Therefore, the interest in naturally occurring antioxidants has increased considerably for use in food and pharmaceutical products [4]. In recent years, there is a wide interest in finding natural compounds that could replace synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) because of its possible toxicity and due to a suspected action as promoters of carcinogenesis [5].

Thymus daenensis is an ancient herb used in medicine by the Greeks, the Egyptians and the Romans. Essential oils of various types of thymus containing high amount of thymol and carvacrol were reported to possess the highest antioxidant activity [6]. Thymus involve high amount of polyphenols. During the last decades many studies have demonstrated that polyphenols, which are the most abundant natural antioxidants in our diet, prevent oxidative alterations due to free radicals and other reactive species [7] and oxidation of low-density lipoproteins [8]. The nutrition community has recognized the importance of dietary polyphenols as health-promoting agents, based on accumulating literature data that demonstrate the association of polyphenols intake with lower risk of coronary heart disease.

In Iran, it is predominantly found in the north of the country. It is used as a food ingredient, as a tea, as an herbal drug for its reputed medicinal properties. Thyme oil strengthens the nerves, aids memory and concentration, can help with the feeling of exhaustion and combats depression, while it the lungs and helps with colds, coughs asthma, laryngitis, sinusitis, whooping cough, sore throats and tonsillitis.

Oxidation of lipids, which occurs during raw material storage, processing, heat treatment and further storage of final products, is one of the basic processes causing rancidity in food products, leading to their deterioration [9]. Oxidative deterioration can result in alterations of organoleptic characteristics, e.g., taste and aroma, in the finished products, making them unacceptable to the consumer. In addition, oxidized lipids may have undesiderable effects on the human organism [10]. Evaluation of the total antioxidant capacity of fruits, vegetables, and other plant products cannot be performed accurately by any single method due to the complex nature of phytochemicals [11].

The aims of this work were: (i) to determine the chemical composition of TDEO by using GC/MS, (ii) to evaluate the antioxidant activity (AOA) of TDEO by using the 2, 2′-diphenyl 1-picrylhydrazyl ($DPPH^{o}$) radical scavenging and β-carotene bleaching (BCB) methods, (iii) and to study the effect of heat treatment (80,120 and 180 ^{o}C) for 1, 2 and 3 hours on AOA (IC_{50}) and chemical composition of TDEO.

Materials and Methods

Essential oil extraction

Essential oil was obtained from 50g Thymus. *daenensis* with 600 ml distilled water by hydrodistillation during 3 hours using a Clevenger-type [12].

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Gas chromatography

Samples of 0.1 µl were injected to analysis by capillary gas chromatography. A Hewlett-Packard 5890 gas chromatograph (GC) equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm HP-5 column with 0.25 µm film thicknesses (Hewlett-Packard, CA, USA), was used for this study. The FID and the injector were maintained at 280 and 250 °C, respectively. Helium was used as carrier gas, the flow through the column was 1 ml/min, and the split ratio was set to 5:1. The column was maintained at 60 °C, increased its temperature at a rate of 5 °C/min to 250 °C. For the identification of the compounds, retention time and retention index were confirmed with commercially available standard compounds by external standard method.

Mass spectrometry analysis

Gas chromatography–mass spectrometry (GC/MS) was used for the identification of volatile components in TDEO. A Hewlett-Packard 5890 gas chromatograph (GC), equipped with a 30 m × 0.25 mm HP-5 column with 0.25 µm film thickness, was used. GC/MS analysis was performed on a GC mentioned above coupled with an Agilent Technologies 5973 Mass system. The other operating conditions were the same conditions as mentioned above, mass spectra were taken at 70 eV. Mass range was from 35–375 amu, emission current 150 mA. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the essential oils were identified by comparison of their mass spectra and retention indices with those published in the literature and presented in the MS computer library.

Determination of total phenolic content

Total phenolic content (TPC) of TDEO was determined according to the method described by Makkar et al. [13]. Four hundred µl of sample was taken in the test tube; then, 1.0 ml of Folin–Ciocalteu reagent (diluted 10-fold with distilled water) and 0.8 ml of 7.5 % sodium carbonate were added. After vortexing the reaction mixture, the tube was placed in a dark place for 40 min and the absorption at 765 nm was measured against a blank. The total phenolic content was expressed as gallic acid equivalents in mg/g of methanol extract.

DPPH^o assay

The purple colored DPPH^o has a strong characteristic absorption at 515 nm and can undergo reactions with hydrogen donating antioxidant compounds to yield the stable yellow DPPH–H molecule easily monitored with UV spectroscopy [14]. The DPPH^o scavenging capacity assay was therefore used in this study to evaluate the free radical scavenging capacity of the extract (IC_{50}). The decrease in absorbance at 515 nm was determined continuously at every minute with a Perkin- Elmer UV/Vis model Lambda 2s spectrophotometer until the reaction reached a plateau. The percentage of remained DPPH^o (% DPPH^o-R) was calculated according to the following equation:

$$DPPH^o\text{-}R\ (\%) = [(DPPH^o)_t / (DPPH^o)_{t=0}] \times 100$$

$(DPPH^o)_{t=0}$ and $(DPPH^o)_t$ are concentrations of DPPH^o at t= 0 and t, respectively. The percentage of remaining DPPH^o against the sample/standard ratio was plotted to obtain the amount of antioxidant necessary to reduce the initial concentration of DPPH^o by 50 % (IC_{50}).

The ß-carotene bleaching method

First, 0.5 mg of *β* –carotene was dissolved in 1 ml of chloroform (HPLC grade), then 25 µl of linoleic acid and 200 mg of Tween 40 were added. The chloroform was removed and 100 ml of oxygenated distilled water was added slowly to the semi-solid residue with vigorous agitation, to form an emulsion.

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Aliquots (3 ml) of the β-carotene/linoleic acid emulsion were mixed with 200 µl of the TDEO and BHT samples dissolved in solvent, and incubated in a water bath at 50 °C for 60 min. Oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Control samples contained 200 µl of solvent instead of TDEO. All determinations were performed in triplicate and results were averaged. The percentage of inhibition was calculated using the following equation:

$AOA\ (\%) = (A_t - A'_t) / (A'_0 - A'_t) \times 100$

A_t is the absorbance measured in the test sample after incubation for 30 min. A'_0, A'_t are the absorbance of control measured at t= 0 and 30 minutes, respectively.

Heat treatment of samples

Samples (300µl) of essential oil were put in a glass tube with screw cap and incubated for 1, 2 and 3 hours at different temperatures (80, 120 and180 ^{o}C). At the end of the incubation, the samples were cooled in an ice bath and immediately used to determine their AOA and chemical compositions. All experiments were carried out in triplicate.

Chemicals

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), 2, 2`-diphenyl-1-picrylhydrazyl (DPPH^{o}), linoleic acid, chloroform, methanol, ethanol and Tween 40 were obtained from Fluka Chemical Co. (Buchs, Switzerland) with highest purity available without further purification. The plant of TDEO was obtained from a research farm of the Institute of Medicinal Plants and Natural Products Research, Golestan, Iran. Essential oil was extracted from the aerial parts and flower heads, respectively, by hydrodistillation method (Clevenger-type apparatus) for 3 hours [12]. The oils were dried over anhydrous sodium sulphate and kept at -4 °C until it was used.

Statistical analysis

Data were analyzed statistically using analysis of variance (ANOVA) and differences among the means were determined for significance at $p< 0.001$ using least significant differences (LSD) test (by SAS software). The data are presented as mean ± standard deviation of the three determinations.

Results

Chemical composition and total phenolic content of TDEO at room temperature

Chemical composition of TDEO at room temperature was determined by GC/MS (Table 1). Forty compounds were identified, representing 94.0 % of the total TDEO. The chemical composition of TDEO has been presented in Table 1 (the identified components present with a concentration >1 % in the analyzed essential oils are listed in Table 1). Thymol (54.7%), carvacrol (5.0%), linalool (1.9%), 1-octene-3-ol (1.6%), *p*-cymene (11.3%), terpin-4-ol (1.5%), γ-terpinene (12.9%), α-terpinene (2.0%), myrcene (1.1%), β-caryophelene (2.6%) and borneol (1.1%) were the main components of TDEO.

Total phenolic component of Thymus *daenensis* was 140.0 ± 0.1 ppm. Total phenolic content of Psammogeton *canescens* were determined in Iran. These data were showed that total phenolic content of TDEO is more than Psammogeton *canescens* [15]. Total phenolic content of some selected culinary herbs and spices were determined in Finland. These data were showed that total phenolic content of TDEO is more than culinary herbs and spices [16].

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The antioxidant activity (AOA) of TDEO determined by ß-carotene bleaching method

Antioxidant activity of the TDEO in comparison with synthetic antioxidant, namely BHT was evaluated. Fig. 1 shows the AOA of TDEO over the range 0.1- 3.1 mg/ml and BHT (0.1 and 0.2 mg/ml). AOA of the TDEO at a concentration of 2.5 mg/ml was similar to BHT at a concentration of 0.2 mg/ml ($p < 0.001$).

The antioxidant activity of TDEO before and after heat treatment using DPPH^o method

In this study, antioxidant activity of TDEO was measured by the model of scavenging the stable DPPH^o radical which is a widely used method to evaluate antioxidant activity in a relatively short time compared the other methods should be pointed out [17]. Antioxidant activity of TDEO tested by the DPPH^o model system and the antioxidant activity of TDEO was IC_{50} = 0.80 ± 0.02 µg/ml. BHT showed higher antioxidant activity (IC_{50} = 0.04 ± 0.01 µg/ml) than TDEO. The radical scavenging effect of the studied essential oil (by DPPH^o method) increased with increasing its concentration. The addition of essential oil to the DPPH^o

Table 1- Chemical composition of TDEO identified by GC/MS

Component	Amount (%)	K. I.	t_R
1-octene-3-ol	1.6	993	10.34
Myrcene	1.1	1008	10.73
α-terpinene	2.0	1038	11.58
p-cymene	11.3	1047	11.83
γ-terpinene	12.9	1082	12.79
Linalool	1.9	1117	13.78
Borneol	1.1	1193	15.92
Terpin-4-ol	1.5	1203	16.22
Thymol	54.7	1319	19.39
Carvacrol	5.0	1325	19.57
ß-caryophyllene	2.6	1461	23.16

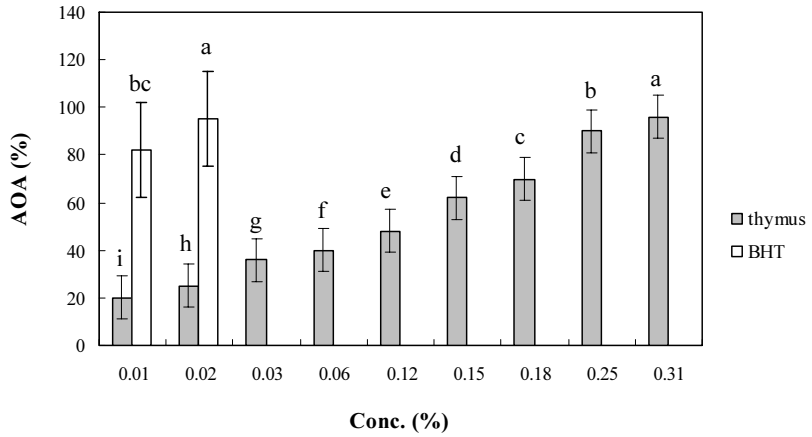


Fig 1- The antioxidant activity of TDEO as measured by ß-carotene bleaching method

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solution caused a rapid decrease in the absorption at 517 nm. The degrees of discoloration indicate the radical scavenging capacity of the essential oil. IC_{50} value of the TDEO has been compared with other essential oils and BHT in Table 2. As seen from Table 2, radical scavenging power of TDEO is higher than activities reported in other papers [18].

The antioxidant activity of TDEO were examined using DPPH^{o} free radical-scavenging method after heating essential oil at different temperatures (80, 120 and 180 ^{o}C) for 1, 2 and 3 hours. As seen from Table 3, by increasing heating time (from 1 to 3 hours), AOA of TDEO has been increased significantly at three tested temperatures ($p < 0.001$). Also, by increasing temperature from 80 to 120 ^{o}C, AOA of TDEO has been increased and at higher temperature, a decreasing trend was observed. For studying this behavior, the chemical compositions of heat treated TDEO have been determined by GC/MS. This behavior can be due to decrease of amounts ß-caryophyllene and carvacrol and increase of thymol (Table 4). For simplicity,

Table 2- Comparison of DPPH radical scavenging capacity (IC_{50} value) of TDEO with some natural and synthetic antioxidants

Sample	IC_{50}	Reference
TDEO	0.80 ± 0.02 µgmL^{-1}	This work
Petroselinum crispum	80.21 ± 3.41 (mg mL^{-1})	[31]
Thymus sipyleus subsp. *sipyleus* var. *sipyleus*	2670 ± 0.5 (µgmL^{-1})	[24]
BHT	0.04 ± 0.01 (µg mL^{-1})	This work

Table 3- The changes of AOA (%) of heated TDEO determined by DPPH^{o} method[a]

Temperature (^{o}C) / Time (h)	80	120	180
1	22.2±0.2^{g}	28.3±0.5^{f}	12.6±0.5^{h}
2	33.4±0.5^{d}	36.7±0.8^{c}	21.9±0.5^{g}
3	41.1±0.2^{b}	62.1±0.7^{a}	32.8±0.1^{e}

a Data are expressed as means ± s.d. and different letters in the table show significant difference between them at $p < 0.001$.

Table 4 - Effect of heat treatment on chemical composition of TDEO (main components (%))[a]

Heating Temperature / Component	Heating time								
	1h			2h			3h		
	80 ^{o}C	120 ^{o}C	180 ^{o}C	80 ^{o}C	120 ^{o}C	180 ^{o}C	80 ^{o}C	120 ^{o}C	180 ^{o}C
p-cymene	11.6±0.2^{a}	8.5±0.3^{e}	8.4±0.3^{e}	10.9± 0.1^{b}	8.1± 0.2^{e}	6.7±0.1^{de}	9.6±0.6^{b}	7.1±0.1^{d}	6.2±0.1^{e}
γ-terpinene	13.5±0.3^{c}	13.8±0.2^{c}	13.8± 0.4^{c}	13.4±0.2^{c}	14.6± 0.3^{b}	16.4±0.1^{a}	11.2±0.1^{e}	14.9± 0.3^{b}	13.8± 0.2^{c}
Thymol	56.1±6^{c}	53.3±0.2^{c}	53.2± 0.1^{e}	57.8±0.2^{b}	56.3± 0.1^{cd}	56.3±0.3^{cd}	55.9±0.3^{d}	56.0±0.4^{cd}	60.7±0.3^{a}
Carvacrol	3.3±0.2^{a}	3.4±0.3^{a}	3.4±0.3^{a}	3.2±0.1^{a}	2.5± 0.1^{b}	3.6±0.2^{a}	3.5±0.2^{a}	1.8±0.3^{c}	3.6±0.2^{b}
β-caryophyllene	2.4± 0.1^{a}	2.5±0.8^{a}	2.5±0.2^{a}	1.9±0.2^{a}	2.1± 0.2^{a}	2.1±0.2^{a}	2.2±0.4^{a}	2.0±0.1^{a}	2.2±0.1^{a}

a Data are expressed as means ± s. d. of three experiments and were analyzed by the Student's t-test for paired data ($p < 0.001$). Data with different letters in the same row are significantly different at $p < 0.001$.

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we have chosen five major components namely p-cymene, γ-terpinene, thymol, carvacrol and ß-caryophyllene (Table 4). The amount of thymol in heated TDEO at 80 ^{o}C ((56.1 %), *p*-cymene (11.6%), γ-terpinene (13.2%) is more than TDEO at room temperature (thymol (54.7 %) and *p*-cymene (11.3%), γ-terpinene (12.9%)).

Discussion

The chemical composition of Thymus *capitatus* Hoff. et Link grown in Portugal [19]; Thymus *capitatus* was grown in Portugal contains carvacrol (62-83%), *p*-cymene (5-17%), γ -terpinene (2-14%) and β-caryophyllene (1-4%) as major components [19]. Thymus *caramanicus Jalas* composed carvacrol (85.9%), thymol (3.3%), p-cymene (3.2%), γ-terpinene (1.8%) and borneol (1.3%). In the present study, the relative amount of thymol was found to be (54.7%), p-cymene (11.3%), γ-terpinene (12.9%), borneol (1.1%) and β-carvacrol (5.0%). Difference among chemical compositions of the essential oils widely depends on production conditions such as climate, soil, harvest date, storage time, variety and cultivar factors [20]. Generally, TDEO characterized by the presence of aliphatic terpenoids, oxygenated terpenoids and phenolic compounds. Among them, the phenolic compounds and oxygenated terpenoids are the main portion of investigated essential oil (such as thymol (54.7%), γ-terpinene (12.9%)). Oxygenated monoterpenes and sesquiterpenes shown to be the main group of terpenoids among chemical composition of the TDEO.

The inhibitory effect of this essential oil on lipid peroxidation was determined by the β-carotene/linoleic acid bleaching test and loss of the yellow color of β-carotene due to its reaction with radicals which are formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants [21, 23].

The antioxidant activity was related to phenolic and terpenic components such as thymol, carvacrol, linalool, *p*-cymene, terpin-4-ol, γ-terpinene, α-terpinene, β-caryophelene and borneol. In addition, TDEO is a good antioxidant; It can be attributed to phenolic content (such as thymol) and OH (such as linalool, terpin-4-ol, carvacrol, borneol), terpenic compounds (such as *p*-cymene, γ-terpinene, α-terpinene, β-caryophelene) fractions in the oil. In the literature, there are many reports indicating the antioxidant potential of the thymol, carvacrol, linalool, *p*-cymene, terpin-4-ol, γ-terpinene, α-terpinene, β-caryophelene and borneol [24]. So the key role of phenolic compounds and terpenoids as scavengers of free radicals is emphasized in several reports.

Compounds containing hydrogen atoms in the allylic and/or benzylic positions may show better activity in this test because of relatively easy abstraction of hydrogen atom from these functional groups by peroxy radicals formed in the test circumstances [25]. Occurrence of the compounds with allylic and/or benzylic hydrogen's such as terpenoids and steroids were also reported in the stachys genus plants and Stachys inflata Benth from Iran [26].

Oxidation of lipids, is one of the basic processes causing rancidity in food products [27]. Oxidative deterioration can result in alterations of organoleptic characteristics, in the finished products, making them

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unacceptable to the consumer. In addition, oxidized lipids may have undesirable effects on the human organism [28]. Application of antioxidants is one of the technically simplest ways of reducing fat oxidation [29]. The antioxidant activity of TDEO were examined using DPPH^{o} free radical-scavenging method after heating essential oil at different temperatures (80, 120 and 180 ^{o}C) for 1, 2 and 3 hours. As seen from Table 4 phenolic and terpenic compounds (such as thymol and γ-terpinene) in heated TDEO have been increased in comparison to unheated TDEO. Heating (up to 180 ^{o}C.) of TDEO showed that the amount of thymol and γ-terpinene was increased. Therefore, as observed in Tables 3 and 4, TDEO showed thermal stability and high free radical-scavenging activity and heat treatment had no considerable effect on it. Effect of thermal processing on some spice essential oils at 80, 100, 120, 180 ^{o}C was studied. Heating (up to 180 ^{o}C.) of basil, cinnamon, clove, oregano and thyme oils did not influence either their antioxidant activities by DPPH^{o} method or their chemical composition. Conversely, when heated at 180 ^{o}C, nutmeg oil showed a significantly higher free radical-scavenging activity, together with a marked loss of α-pinene, ß-pinene and sabinene, and an evident increase in safrole and myristicin contents. Thus one could consider that the observed higher free radical-scavenging capacity of the nutmeg oil might be related to a heating-induced increase in the content of these two components [30]. However, due to their complex composition, the correlation between antioxidant activity and the components present in the oil is difficult to establish. Phenolic compounds show high antioxidant activity. The phenolic OH is the most preferable group for the proton loss from the one-electron oxidized species. The stability of the resultant phenoxyl radicals therefore imparts greater ability for thymol to scavenge the oxidizing free radicals. This finally resulted in a much greater ability to inhibit free radical-induced lipid peroxidation and the antioxidant activity. The resonance-stabilized radicals can undergo loss of second hydrogen from the second phenolic OH group, producing a diradical. This diradical may be converted into stable products [31].

Unlike synthetic antioxidants, TDEO can be added in larger quantities to get optimal effects (addition of synthetic antioxidants is limited under food laws and regulations). However, further investigation (such as other antioxidant testing methods, toxicological test and etc.) and the antioxidant activity mechanism are warranted. These studies can be useful as a starting point for further application of TDEO in food preparations.

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