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Full Length Research Paper

Essential oil, phenolic compounds and antioxidant activity of *Thymus daenensis* Celak. at different harvest times

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***Thymus daenensis* Celak. is an important medicinal plant grows in different climatic regions of Iran. A pot experiment was conducted to evaluate the essential oil yield and oil composition of thyme as affected by harvest time during 2010. Isolate the essential oil of the plant samples were determined by hydro-distillation in Clevenger Apparatus with three hours. Composition of the essential oils was determined by Gas chromatography mass spectrometry (GC-MS). Twenty six compounds, representing 98.5% of the essential oil were identified. The major compounds were thymol, p-cymene, γ-terpinene, β-caryophyllene and carvacrol. The results showed that thymol was the most abundant compound in thyme oil that a significant difference in its content due to harvest time was observed. The higher concentration of thymol was detected at full-flowering stage. In contrast, the higher concentration of γ-terpinene, a precursor of p-cymene, and p-cymene, a precursor of thymol was achieved at pre-flowering stage. Also, obtained results illustrated that phenolic compounds was higher in plant samples harvested at full-flowering stage. In addition, a significant increase in antioxidant activity of thyme was found to be due to harvest at full-flowering stage.**

Key words: *Thymus daenensis* Celak., essential oil, hydro-distillation, gas chromatography mass spectrometry (GC-MS), harvest time.

INTRODUCTION

Thyme is a perennial plant belonging to the Lamiaceae family and important aromatic plants includes nearly 215 herbal and small shrub species in the world that synthesize remarkable amount of volatile compounds that have biological and pharmacological properties. Among 215 species of this genus grown in the world, 14 species are distributed in Iranian flora (Jalas, 1982; Bruneton, 1995; Stahl-Biskup and Saez, 2002), which *Thymus daenensis* Celak. is endemic. The Persian and local name of *T. daenensis* is ‘‘Avishan-e-denaee’’ (Ghasemi Pirbalouti, 2009). The green part of thyme plant constitutes the most popular herbal medicine, spice and flavoring agents, used in all developing countries. *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 widely used as tonic and herbal tea, antiseptic, antitussive and carminative, treating colds as well as for perfume industry, flavoring and preservation of several food products (Amin, 2005; Ghasemi Pirbalouti, 2009; Zargari, 1990; Morteza-Semnani et al., 2006). *Thymus daenensis* is widely used as these mentioned purposes in Iran. Recent studies have shown that Thymus species have strong antibacterial, antifungal, antiviral, antiparasitic, spasmolytic and antioxidant activities. These properties of Thyme depend greatly on their chemical compositions and are mainly attributed to their contents of carvacrol and thymol (Stahl-Biskup and Saez, 2002).

Variation in chemical composition of essential oils of medicinal plants may be observed due to the origin and environmental conditions. It is also reported that climatic conditions during vegetative and reproductive periods have major effect on essential oil accumulation and

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composition in medicinal and aromatic plants. So, researchers pointed out that essential oil accumulation may be depended on developmental phase of the plants (Basu et al., 2009; Sangwan et al., 2001). Verma et al. (2010) stated that harvest time has a close relationship with yield and qualitative traits of essential oil and oil composition varies from place to place and genotype to genotype.

Ozguven and Tansi (1998) indicated that harvesting time affect the yield and components of thyme oil. They also reported that dry matter of this medicinal plant from before flowering to after flowering due to effect of day length, temperature and radiation increased significantly. Omidbaigi and Rezaei Nejad (2000) found out that the highest amount of oil yield of thyme was obtained from harvest after flowering and fruit set. Also, major volatile constituents obtained from the aerial parts of the plant were geranial, linalool, carvacrol, thymol and trans-thujan- 4-ol/terpinen-4-ol.

Hudaib et al. (2002) evaluated thyme essential oil and found that this medicinal plant was rich in the active monoterpene phenols (thymol and carvacrol) and their corresponding terpenic hydrocarbons precursors (p-cymene and -terpinene), which collectively showed synchronized patterns of variation during the different collection periods and in different season.

Pavel et al. (2010) estimated chemical composition of *Thymus pulegioides* and *Thymus glabrescens* were grown in Romania and obtained a yield of 0.7 to 1% of essential oil and reported that main components were carvacrol (50.5 to 62.6%), γ-terpinene (9.8 to 9.9%) and p-cymene (5.8 to 7.1%). The essential oil of *T. glabrescens* was obtained in a yield of 0.7% and the main components were geraniol (55.5%), neryl acetate (11.1%) and β-bisabolene (6.7%).

Akbarinia and Mirza (2008) identified essential oil components of *T. daenesis* Celak. in Qazvin, Iran and reported that the ratio of aromatic components to its dry weight was 2.8 ± 0.1% (w/w). They found twenty four components and main constituents of the essential oil were thymol (74.61%), p-cymene (4.6%), γ-terpinene (4.48%) carvacrol methyl ether (4.27%), 1.8 cineol (1.64%), borneol (1.61%), and carvacerol (1.40%).

The purpose of this experiment was to evaluate the essential oil, phenolic compounds and antioxidant character of *T. daenensis* Celak in response to harvest time.

MATERIALS AND METHODS

This experiment was conducted at a greenhouse in Dena, Iran, during 2010 to 2011. Thyme seeds were sown at 6 November 2010 in a culture bed containing 1/3 sand, 1/3 clay and 1/3 peat (v/v). 8 weeks after sowing the plants that were in 4 leaf stage were transplanted to pots (20 × 20 × 20). Experiment was carried out using a randomized complete block design (RCBD) with three replications. Each replicate contained 10 pots. The study was performed in two harvest time as: at pre-flowering (27 March, 2010) and at full-flowering (2 May, 2010).

In each harvest time, above ground biomass of each treatment collected and their fresh weight measured. Then, plant materials were dried at room temperature at 30°C and after 10 days dry weight also recorded. To determine the essential oil in dried materials from each harvest, essential oils were isolated by hydro-distillation method utilizing Clevenger-type apparatus over 3 h. The oils were collected, dehydrated with anhydrous sodium sulphate, measured and stored in tightly closed dark vials at 4°C prior to chromatographic analysis. Essential oil content was calculated as volume (ml) of essential oil per 100 g of plant dry matter. A Hewllet-Packard series 6890 gas chromatograph (GC) equipped with a flame ionization detector and DB5 column (30 m × 0.25 mm; 0.25 µm film thicknesses) was used for this study. The oven temperature was fixed at 60°C for 3 min, after which it was increased to 240°C for 3 min and then held at this temperature for 45 min. Injection volume was 1 µl split 1:50. The injector and detector temperatures were kept at 240 and 250°C, respectively. Helium used as a carrier gas. Then, samples were subjected to analysis by gas chromatography. There were three replications for each specimen. Chemical composition of essential oil was determined by Gas chromatography mass spectrometry (GC/MS) analysis. GC-MS analyses were carried out on Hewllet-Packard series 6890 with ionization energy of 70 eV and DB5 column (30 m × 0.25 mm; 0.25 µm film thicknesses). Also, splitting ratio was 1:50. The same temperature program and carrier gas were used for GC analysis. The samples were injected at the injector temperature of 240°C. The constituents of the essential oils were identified by comparing linear Kovats indices and their retention times with corresponding data of components from reference oils.

Total phenols were determined by Folin Ciocalteu reagent. A dilute extract of each plant extract (50 µl of 1:10 g/ml) mixed with Folin Ciocalteu reagent (2.5 ml, 1:10 diluted with distilled water) and aqueous Ca_2CO_3 7.5% (2 ml). The mixtures were allowed to stand for 10 min in water 45°C and the total phenols were determined by spectrophotometer apparatus at 765 nm. Phenolic contents of the sample were calculated on the basis of the standard curve for Gallic acid. The results were expressed as gallic acid equivalents (GAE)/g DW of the plant materials. In this study, antioxidant activity of thyme volatile compounds measured with respect to radical scavenging ability using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). In this experiment we used four different concentrations of DPPH and sample. Firstly, DPPH added to test tube and each two minutes Trolox and quercetin and stock solutions of the essential oil added to test tube and then, allowed to stand at 37^{o} C for 30 minutes. Absorbance measurements commenced immediately. The reduction of the DPPH free radical was measured by reading the absorbance at 517nm with a UV-Vis (double-beam) spectrophotometer and related to the absorbance of the control without the herbal drugs. Experimental results were expressed as mean ± standard deviation. Then, all data were subjected to analysis of variance (ANOVA) using SAS software and means were separated by Duncan multiple range test at $P < 0.05$ significant level.

RESULTS AND DISCUSSION

All measurements were replicated three times. The oils isolated by hydro-distillation from the aerial parts of *T. daenensis* Celak at pre-flowering and full-flowering stages were found to be essential oil contents of 1.37 and 1.52% (v/w), based on dry weights, respectively (Table 3). It is clear that essential oil percentage of thyme was higher due to harvest at full-flowering stage compared to pre-flowering stage; however, the difference between them was not statically significant (Table 1). Nickavar et

Table 1. Analysis of variance for measured parameters of thyme as affected by harvest time.

S.O.V	df	Phenol	Antioxidant	P-cymene	γ-terpinene	Thymol	Essential oil content	Essential oil efficiency
Replication	2	0.11^{ns}	0.04^{ns}	0.48^{ns}	2.49^{ns}	0.82^{ns}	0.00^{ns}	13.87^{ns}
Treatment	1	0.03^{ns}	0.08*	3.85^{ns}	1.75^{ns}	35.54*	0.03^{ns}	2460.0^{ns}
Error	2	0.02	0.003	0.25	0.19	0.82	0.004	24.35
CV (%)		0.83	0.65	7.94	11.74	1.31	4.79	5.11

Ns= Non significant; * and ** = Significant at 5 and 1% probability, respectively.

Table 2. Effect of harvest time on oil yield, oil efficiency and oil composition of thyme.

Treatment	Phenol (mg G/g D.W.)	Antioxidant (mg/ml)	P-cymene (%)	γ-terpinene (%)	Thymol (%)	Oil content (%)	Oil efficiency (mg/plant)
Pre-flowering	18.82 a	8.46 b	7.13 a	4.3 a	66.62 a	1.37 a	90.1 a
Full-flowering	18.97 a	8.23 a	5.53 a	3.22 a	71.49 b	1.53 a	102.9 a

Means followed by same letter in each column are not significantly different at the 5% level.

al. (2005) reported that essential oil content of *T. daenensis* Celak. was 2.4%. Toncer and Kizil (2005) reported that the highest essential oil content of thyme (*Thymbra spicata* L. var. spicata) recorded at the full-flowering stages. This may be due to higher biomass yield and content of oil at this stage (Inan et al., 2011). This increase not only depended on flower development, but also temperature, relative humidity and duration of sunshine, air movement and rainfall can affect this parameter (Kastner, 1969). In the other hand, in most aromatic plants, the essential oil preferentially accumulates during the flowering stage, probably due to its ecological role in attracting pollinators and as an antifungal defense mechanism (Verma et al., 2010). Obtained results presented in Table 2 illustrated that oil efficiency of samples that harvested at full-flowering stage with mean of 102.9 mg/plant was higher than those harvested at pre-flowering stage (90.1 mg/plant). The higher oil efficiency observed in full-flowering stage may be due to increase of dry matter from pre-flowering to full-flowering stages, because of available day-length, temperature and sunlight (Abou-Zied, 1973).

The chemical composition of the essential oil of thyme can be seen in Table 3. The components are listed in order of their elution on the DB-5 column. Twenty six compounds were identified in the essential oil of *T. daenensis* Celak. representing 98.5% of the total essential oil. The main abundant components of oil were thymol (66.62 ± 0.79%), p-cymene (7.12 ± 0.17%), γ-terpinene (4.30 ± 1.40%), β-caryophyllene (3.91 ± 0.04%) and carvacrol (2.64 ± 0.07%). Similarly, Nickavar et al. (2005) reported that the essential oil of the aerial parts of *T. daenensis* had twenty six components, which represented about 99.7% of the total detected constituents. In their study the major compounds were: thymol (74.7%), p-cymene (6.5%), b-caryophyllene (3.8%) and methyl carvacrol (3.6%).

Harvest at full-flowering stage increased the amount of thymol in the essential oil of thyme. Thymol content with mean of 71.49% at full-flowering stage was higher than pre-flowering stage (66.62%). Whereas, γ-terpinene content at pre-flowering stage with mean of 4.3% was higher than full-flowering stage (3.22%). However, the difference between treatments in the case of this trait was not statically significant (Table 1). These results are in agreement with the findings of Inan et al. (2011) in *T. spicata* L. var. spicata. Also, Hudaib et al. (2002) stated that the highest level of monoterpene phenols (mainly thymol) and the lowest amount of γ-terpinene of thyme (*T. vulgaris*) were observed from full flowering-beginning of fruit maturation. Furthermore, p-cymene percentage at pre-flowering stage (7.13%) was higher compared to full-flowering stage (5.53%).

The correlation coefficients among concentrations of related components (Table 4) showed that a negative correlation existed between thymol and p-cymene. It is clear that the increase of thymol content was strongly correlated with p-cymene content ($R^2 = 0.92$) so that, with increase of thymol at full-flowering stage, p-cymene decreased and with increase of p-cymene at pre-flowering stage, thymol content decreased. Nejad Ebrahimi et al. (2008) found a similar result in the case of *Thymus caramanicus*. Harvest time caused a change in the phenolic compounds of *T. daenensis* Celak., so that the highest amount of phenolic compounds (18.97 mg Gallic acid/g D.W.) was obtained from the full-flowering stage and the lowest one (18.82 mg Gallic acid/g D.W.) was recorded from the pre-flowering stage, however, this difference was not statically significant. It is clearly obvious from results that Thymol and carvacrol constituted the main monoterpene phenols of thyme oil. So, the increase of phenolic compounds at full-flowering

Table 3. Amounts of the chemical components of thyme oil at two harvest time.

No	Component	RI	Percent at pre-flowering	Percent at full-flowering
1	α-Thujene	928	1.04 ± 0.07	0.9 ± 0.08
2	α-Pinene	934	0.8 ± 0.34	0.79 ± 0.13
3	Camphene	950	0.38 ± 0.12	0.36 ± 0.00
4	Sabinene	973	0.32 ± 0.10	0.35 ± 0.03
5	β-Pinene	978	1.12 ± 0.25	0.92 ± 0.22
6	Mircene	990	0.16 ± 0.03	0.14 ± 0.02
7	α-Phelandren	1002	0.06 ± 0.00	0.05 ± 0.00
8	α-Terpinene	1015	0.89 ± 0.18	0.69 ± 0.11
9	Para-cymene	1024	7.12 ± 0.17	5.52 ± 0.83
10	1.8 Cineole	1033	1.36 ± 0.23	1.43 ± 0.15
11	γ-Terpinene	1057	4.3 ± 1.40	3.22 ± 0.83
12	Sis Sabinen hydrate	1061	0.37 ± 0.07	0.41 ± 0.04
13	Terpinolene	1087	9.09 ± 0.00	9.09 ± 0.01
14	Linalool	1098	0.92 ± 0.08	0.88 ± 0.04
15	Borneol	1161	1.35 ± 0.49	1.22 ± 0.25
16	Timil metylether	1237	0.2 ± 0.03	0.21 ± 0.02
17	Carvacrol Methylether	1241	1.81 ± 0.05	1.22 ± 0.13
18	Thymol	1293	66.62 ± 0.79	71.49 ± 1.00
19	Carvacrol	1303	2.64 ± 0.07	2.77 ± 0.06
20	β-Caryophyllene	1417	3.91 ± 0.05	4.09 ± 0.15
21	Aromandrone	1439	0.11 ± 0.00	0.13 ± 0.01
22	Homolene	1454	0.12 ± 0.01	0.12 ± 0.00
23	Bicyclo Germacren	1496	0.26 ± 0.08	0.18 ± 0.02
24	Farnezene	1503	0.5 ± 0.22	0.54 ± 0.17
25	α-Bisabolene	1533	1.28 ± 0.42	1.14 ± 0.15
26	Caryophyllen Oxide	1581	0.77 ± 0.51	0.9 ± 0.4
	Total		98.50	99.66
	Essential oil content		1.37 ± 0.06	1.52 ± 0.03

Table 4. Correlation coefficients between some measured traits of thyme.

Trait	Phenol	Antioxidant	P-cymene	γ-terpinene	Thymol	Essential oil content	Essential oil efficiency
Phenol	1						
Antioxidant	0.71^{ns}	1					
P-cymene	0.31^{ns}	0.76^{ns}	1				
γ-terpinene	0.47^{ns}	0.16^{ns}	0.13^{ns}	1			
Thymol	0.24^{ns}	0.78^{ns}	0.92*	0.40^{ns}	1		
Essential oil content	0.09^{ns}	0.81*	0.72^{ns}	0.18^{ns}	0.86*	1	
Essential oil efficiency	0.18^{ns}	0.82*	0.80^{ns}	0.13^{ns}	0.88*	0.99**	1

Ns= Non significant; * and ** = significant at 5 and 1% probability, respectively.

stage must be related to the increase of thymol and carvacrol content. Analysis of variance (Table 1) clear that harvest time had the significant effect on antioxidant activity of thyme. Antioxidant activity of the extract of thyme was significantly higher at full-flowering stage (8.23 µg/ml) compared to pre-flowering stage (8.46 µg/ml). Medicinal plants contain a wide variety of natural antioxidants which possess antioxidant activity. Phenolic compounds are a class of antioxidant agents which can significantly delay or prevent the oxidation of easily oxidizable substances (Shahidi and Wanasundara, 1992). Many researchers found a relationship between

total phenolic content and total antioxidant activity (Safaei-Ghomi et al., 2009). It seems that the higher antioxidant activity at full-flowering stage can be explained by the higher total concentration of thymol phenolic component which also define the essential oil quality of thyme.

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