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Changes in anti-oxidant activity of *Thymus transcaspicus* (Klokov) during growth and developmental stages

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Abstract

Antioxidant activities protect the cell against oxidative agents that are constant metabolic by-products. The aim of this study was to investigate the relationship between harvesting time of *Thymus transcaspicus* and its antioxidant activities. The plant samples were harvested 5 times in different growth phases from 17 April to 22 July 2008, and its antioxidant activity was studied using the ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, and β-carotene bleaching (BCB) assays. The results of FRAP assay indicated that the reduction activity of the plant was in the highest level in stage 5 of sampling. The result of DPPH assay showed that the crude extract of the plant was more capable of DPPH radical scavenging in stage 2. The highest level of gallic acid and quercetin in the crude extract of *T. transcaspicus* was determined as 85.29 ± 6.22 mg and 18.88 ± 0.9 mg in stage 2, respectively. Therefore, stage 2 was the optimum time to harvest the *T. transcaspicus*.

Keywords**:** *Thymus transcaspicus,* antioxidant capacity, growth stages, total phenolic content, total flavonoid content

Introduction

The genus *Thymus* L. (*Lamiaceae*), an aromatic and medicinal plant, includes numerous species with quite different botanical characteristics. This genus consists of about 215 species of herbaceous perennials and sub shrubs that grow naturally in the Mediterranean region, and is represented in the Iranian flora by 14 species (Javadi et al., 2009). Leaves and flowers of *Thymus* species are commonly used as herbal tea, spice, flavoring agents and medicinal plants. Furthermore, *Thymus* oils and extracts are widely used in pharmaceutical, cosmetic and perfume industries and are also used for flavoring and preserving food products. It has been reported that *Thymus* species have strong antibacterial, antifungal, antiviral, anti-parasitic, spasmolytic and antioxidant activities (Imelouane et al., 2009). *Thymus transcaspicus* (Klokov; Lamiaceae family), is an endemic species that exists in the Northern regions of Iran.

Epidemiological studies have indicated possible roles of fruits and vegetables in preventing numerous diseases such as cancer, cardiovascular disorders, cataract, neuro-degenerative diseases, atherosclerosis and inflammation. The preservative effects of many plant spices are attributed to the large amounts of antioxidative constituents in their tissues (Deepa et al., 2007). Antioxidant compounds, are important in protecting plants against harmful chemical compounds including free radicals and reactive oxygen species (ROS) that are constantly produced by the cell metabolism and their concentration increases under stress situations (Ferreyra et al., 2007). Although free radicals and ROS can rapidly attack all types of biomolecules and lead to lipid peroxidation, plasma membrane injury and proteins and deoxyribonucleic acid damages that finally end in cell death (Berlett and Stadtman, 1997), antioxidants are able to scavenge or deactivate free radicals before they attack plant cells. The antioxidant systems consist of enzymatic (superoxide dismutase, catalase, peroxidase, etc.) and non-enzymatic components (carotenoids, phenolic compounds e.g. catechins, anthocyanins and vitamins A, C, and E). The level and mode of antioxidant activity depends on genotype, maturity stages, and conditions of plant growth (Chirinos et al., 2007). Changes in antioxidant activity or antioxidant compounds during plant growth and developmental stages appear to vary among fruits, vegetables and herbs, even though there is no consistent report. Prior et al. (1998) found an increase in antioxidant activity and total phenols

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and anthocyanins contents in ripe fruit of industrial berry varieties. Also, Howard et al. (2000) observed that upon *Capsicum* cultivars growth and maturation, the concentration of antioxidant constituents was increased. Wang and Lin (2000) found that blackberries and strawberries had the highest antioxidant values during the green stages, whereas red raspberries had the highest antioxidant activity at the ripe stage. Moreover, it has been reported that cherry tomatoes harvested at full ripeness exhibited the highest level of carotenoids and antioxidant activity in the water-insoluble fraction (Raffo et al., 2002). Huang et al., (2007) observed that the activity of oxygen-scavenging enzymes (SOD, CAT and G-POD) was decreased with ripening and maturation of fruit in orange pulp. Furthermore, the highest antioxidant activity was observed at the stage of flower opening of the plant (Fu et al., 2009). However, a decreasing trend of antioxidant activity in blueberry (*Vaccinium corymbosum* L.) fruit during ripening was observed by Castrejón et al. (2008). Alteration in antioxidant activity and related compounds during growth and maturation affects functional properties in plants for their narrow harvest window (Fu et al., 2008).

To our knowledge, there is no available information on the variations in antioxidant properties of *T. transcaspicus* during different growth and developmental stages. Therefore, the aim of this research is to study the antioxidant activity of *T. transcaspicus* at different developmental stages, in order to determine the best harvesting time of this plant.

Materials and Methods

Collection and preparation of plant samples

Fresh aerial parts of *T. transcaspicus* were collected from Sar-Aliabad with a longitude of 54° 33' 11" N. and a latitude of 36° 40' 0" E. and an altitude of 2339 ± 10.62 meter around Gorgan city of Iran, during the early and late of growth (early of flowering), mid (with 70% flowering) and late (early of seed formation) of flowering, and seed formation (100%) stages with 24 days intervals (17 Apr – 22 Jul, 2008).

One part of the samples were frozen with liquid nitrogen for ferric reducing antioxidant power (FRAP) assay, and the ascorbic acid content was then stored in -70°C (up to 3 days). Other samples were dried at room temperature and powdered. One gram of each powder sample was extracted in 50 ml of 80% methanol by maceration (48 h). The extract was then filtered and the pellet was dissolved in 80% methanol and remained for 24 hours re-extracted. Finally, both filtered solutions were mixed and concentrated using rotary evaporator at 40°C, and the crude extract was stored at 4°C until usage (up to 10 days).

Total phenolic and flavonoid contents

Total phenolic content of the crude extracts of *T. transcaspicus* was determined by Folin–Ciocalteu reagent (Ercisli and Orhan, 2007), and expressed as milligram gallic acid equivalents (GAE)/$g^{-1}dw^{-1}$. The total flavonoid content of the extracts was determined using the aluminum chloride colorimetric method described by Chang, et al., (2002). The same procedure was repeated for all standard quercetin solutions (20–200 mg/l) to obtain a curve. The data were expressed as milligram quercetin equivalents (QE)/$g^{-1}dw^{-1}$.

Ascorbic acid content

Ascorbic acid (ASA) content was determined by 2, 6-dichlorophenolindophenol-dye method as described by Deepa et al. (2007), and the results were expressed as mg ASA/100 g fw.

Antioxidant activity assays

FRAP assay

The FRAP assay of the extracts was assessed on the basis of the ferric to ferrous ion reduction (Benzie and Strain 1996). The standard curve was obtained based on the absorbance of several concentrations of freshly prepared ammonium ferrous sulphate (100–1000 µM ferrous ion). Tests were carried out in triplicates and expressed as mmol Fe^{2+} g^{-1} fw.

1, 1-diphenyl-2-picrylhydrazyl free radical scavenging activity

The ability of corresponding extracts and some pure compounds in donating a hydrogen atom or electron was determined by bleaching purple-colored methanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. In this spectrophotometric assay, the stable DPPH radical is used as the reagent according to a procedure described by Cuendet et al., (1997). Tests were carried out in triplicates and results were expressed as inhibition of free radical by DPPH in percent (%I), and calculated by the following:

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

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 sample. $IC_{50,}$ which denotes the amount (mg) of plant extract that inhibits DPPH radicals by 50%, was calculated from the plotted graph of inhibition

percentage against extract concentration. The ascorbic acid (AA) was used as a standard and results were expressed as ascorbic acid equivalent antioxidant activity (AEAC) using the following equation:

$$AEAC\ (mg\ AA/gdw) = IC_{50}\ (ascorbate)/IC_{50}\ (sample) \times 100$$

β-carotene bleaching (BCB) assay

BCB assay determines antioxidant capacity by measuring the inhibition of volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius, et al., 1998). The degradation rate (DR) of β-carotene was calculated as ln ($A_{initial}$ / A_{60})/60 and the percentage of antioxidant activity (AOA%) was calculated as:

$$[(DR_{control} - DR_{sample})/DR_{control}] \times 100$$

Results

Ascorbic acid content

As observed in table 1, ascorbic acid content significantly varied among samples harvested during different stages and it ranged from 2.01 ± 0.29 mg/100 g fw in stage 2 to 5.81 ± 0.35 mg/100 g fw in stage 5.

Total phenolic and flavonoid contents

As it is summarized in table 1, the stage of harvesting affected the total phenolic and flavonoid contents of plant samples. The total phenol content in samples varied from 35.75 ± 3.15 mg GAE/g dw, to 85.29 ± 6.22 mg GAE/g dw, and the highest content of the total phenol was determined in stage 2 of sampling. The content of flavonoids ranged from 7.54 mg ± 0.43 QE/g dw in stage 4 to 18.88 ± 0.9 mg QE/g dw in stage 2.

Antioxidant activity

Since different antioxidant compounds may act through different mechanisms *in vivo*, no single method can fully evaluate the antioxidant activity of bio-samples. Therefore, three methods, FRAP, DPPH, and BCB assays, were used in the present study to evaluate the antioxidant activity of plant extracts. FRAP, DPPH and BCB assays evaluate the reducing activities (Benzie and Strain, 1996), hydrogen atom-or-electron donation ability (Cuendet et al., (1997), and the inhibition lipid peroxidation (Lim and Quah, 2007), respectively.

The results of FRAP and BCB revealed that the crude extract of *T. transcaspicus* in stage 5 of growth had the highest anti-oxidant activity (figures 1 and 2). However, DPPH assay indicated that in stage 2 of harvesting, the highest antioxidant activity was observed (figure 3). Based on these three methods, the lowest anti-oxidant activity has been determined in stages 1, 4 and 3 (figures 1, 2 and 3). Our results have also indicated that the ascorbic acid content in *T. transcaspicus* was correlated with ferric reducing antioxidant activity (figure 4. a, R^2 = 0.67). Present findings showed that the phenol and flavonoid content in *T. transcaspicus* were lineary correlated with DPPH free radical scavenging activity (R^2 = 0.69, R^2 =0.80, respectively), (figures 4b and 4c).

Table 1. Total phenolic and flavonoid contents of *T. transcaspicus* in different stages of sampling.

Stage of sampling	Date	Phenology	Total phenol (mgGAE/gdw)	Total flavonoid (mgQE/gdw)	Ascorbic acid content (mg/100gfw)
1	2008/4/17	Early growth (two weeks after growth)	35.96 ± 4.97 ^{c}	10.71 ± 0.89 ^{b}	3.39 ± 0.34 ^{c}
2	2008/5/12	Late growth (early flowering)	85.29 ± 6.22 ^{a}	18.88 ± 0.9 ^{a}	2.01 ± 0.29 ^{e}
3	2008/6/5	Mid flowering (with 70% flowering)	68.31 ± 4.99 ^{b}	10.34 ± 0.33 ^{b}	3.88 ± 0.32 ^{b}
4	2008/6/29	Late flowering (early seed formation)	35.75 ± 3.15 ^{c}	7.54 ± 0.43 ^{d}	2.85 ± 0.40 ^{d}
5	2008/7/22	Seed formation (100%)	37.03 ± 2.14 ^{c}	8.91 ± 0.74 ^{c}	5.81 ± 0.35 ^{a}

Data are expressed as means ± S.D. (n=8). Similar upper case letters indicate no significant differences (Duncan test, P<0.05).

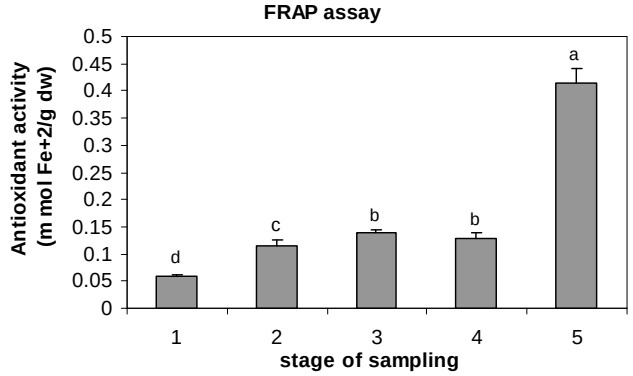

Figure 1. Antioxidant activity of *T.transcaspicus* extract during different stages of sampling by FRAP assay. Data are expressed as means ± S.D. (n=8). The same letters above the bars indicate no significant differences (Duncan test, P<0.05).

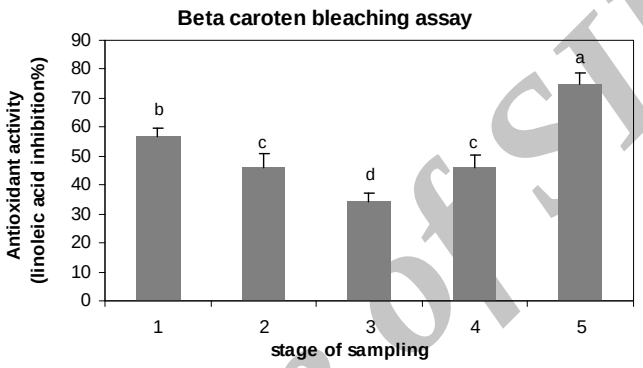

Figure 2. Antioxidant activity of *T.transcaspicus* extract during different stages of sampling by BCB assay. Data are expressed as means ± S.D. (n=8). The same letters above the bars indicate no significant differences (Duncan test, P<0.05).

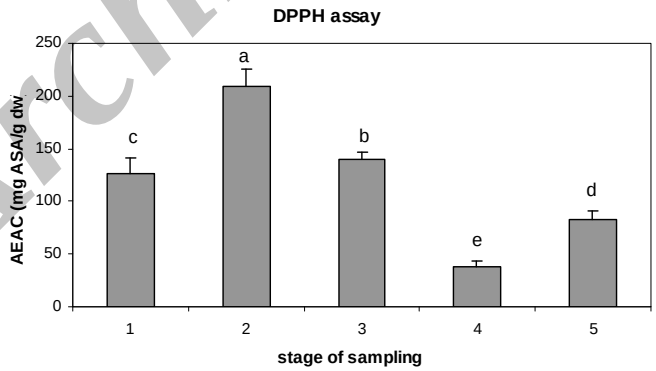

Figure 3. Antioxidant activity of *T.transcaspicus* extract during different stages of sampling by DPPH assay. Data are expressed as means ± S.D. (n=8). The same letters above the bars indicate no significant differences (Duncan test, P<0.05).

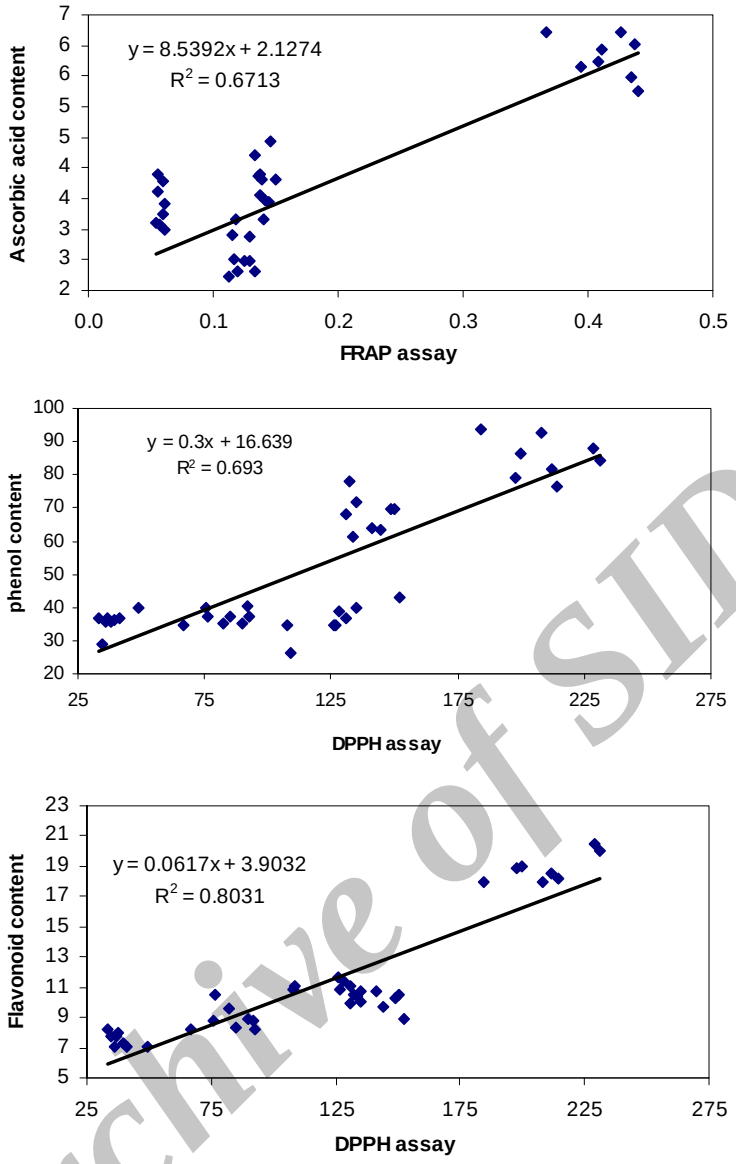

Figure 4. Correlation coefficients between antioxidant activity (FRAP and DPPH assay) and ascorbic acid (a), phenol (b) and flavonoid (c) contents.

Discussion

The mode of antioxidant action, which serves as a defensive system in all stages of plant development, depends on plant's growth, developmental stage and sample specificity (Howard et al., 1994; Deepa et al., 2007).

The significant variation in antioxidant properties, total phenols, flavonoid and ascorbic acid content between different stages of *T. transcaspicus* growth indicates that the potential efficacy of antioxidants in this plant varies considerably during maturity stages. The FRAP assay allows the overall estimation of water-soluble compounds influencing the total antioxidant activity (Deepa et al., 2007). By using this assay, our results showed an enhanced ascorbic acid level (as a compound water-soluble, R^2 = 0.67) during different stages, and also increased ferric reducing antioxidant activity of the extracts. Furthermore, we showed that the highest antioxidant activity was obtained in period 5, in which the ascorbic acid content was also the highest. In relation to our data, Proteggente et al. assessed the antioxidant activity of extracts from regularly consumed fruit and vegetables using FRAP assay, and found that the indices were well correlated with vitamin C content. The BCB assay showed that during all stages of growth, all samples were capable of inhibiting lipid peroxidation (Proteggenete et al., 2002). Current studies have been focused on a proposed role of carotenoids as lipid antioxidants, which can protect plants against destructive processes mediated by singlet oxygens and free radicals (Menichini et al., 2009). Although

the value of compounds was not assessed in this study, reports indicated that carotenoid concentration increased in the oldest leaves of acerola genotypes (Lima et al., 2005). Therefore, it can be concluded that the level of carotenoid concentration in step 5 might be responsible for enhanced antioxidant activity in the BCB assay.

In agreement with Pyo et al. (2004), our results suggested a linear correlation between DPPH radical scavenging activity and phenol and flavonoid concentrations (Figures 1b and 1c). These relationships could be due to the fact that both DPPH and the Folin–Ciocalteu methods are based on redox balances in phenols (Huang et al., 2005). The radical scavenging activity of phenolic compounds mostly depends on their molecular structure, due to the availability of phenolic hydrogens and stabilization of the resulting phenoxyl radicals formed by hydrogen donation. The changes in flavonoid composition during maturation most likely affected the antioxidant activity. It has been reported that some flavonoids such as patuletin derivatives show significant activity against DPPH radical and enhanced radical scavenging capacity, whereas in spinacetin derivatives radical scavenging capacity is reduced. Furthermore, it has been indicated that flavonoids with no, minor or high activity against free radicals varied in concentration in response to maturation (Pandjaitan et al., 2005). Therefore, the connection between the particular antioxidant agents and antioxidant activity is difficult to explain on the basis of quantitative analysis (Capecka et al., 2005). The synergism between antioxidants in mixture makes the antioxidant activity not only depend on the concentration, but also on the structure and the interaction between the antioxidants (Conforti et al., 2007). Therefore, the antioxidant activity did not always exhibit an additive or synergistic effect equal to the antioxidant content individually (Howard et al., 2000). Our results revealed that anti-oxidant behavior, as radical scavenger, oxidation inhibitor and reducing agent, differs in 5 growth stages of *T. transcaspicus*. This discrepancy may be due to different mechanisms involved in these stages of growth. Understanding the biochemical changes in plant physiology, food science, nutrition and health should stimulate interest in maximizing beneficial effects of plants in diet.

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