Total Phenolic Content and Antioxidant Activity of the Methanolic Extracts of Three *Thymus* Cultivars Grown in Iran

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Summary

Total phenolic content and radical scavenging activity of leaf extracts from three thyme cultivars grown in North of Iran was investigated using ferric reducing antioxidant power (FRAP) and dihydroxy phenyl radical (DPPH°). The results showed that total phenol content (mg GAE/100 g dw) was 39.9, 44.2 and 46.8 for extracts of spring samples of Thymus *Caucasicus*, Thymus *Kotchyanus* and Thymus *Caucasicus* repectively. On the other hand, a similar difference was observed in their antioxidant activity (mMol TEAC/100 g dw) measured by DPPH and FRAP assays. It was also observed that both total phenolic content and antioxidant activity raised about 20% in extracts of summer samples. It could be concluded that the total phenolic content is significantly related to antioxidant activity in summer and spring samples from different thymus species and that hot, humid conditions of September in Gilan province caused an oxidative stress in leaves of all three thymus species.

Keywords: Thymus *Kotchyanus*, Thy. *Pubescens* and Thy. *Caucasicus*; Antioxidant activity; DPPH; ABTS.

Introduction

Antioxidants are phytochemicals, vitamins and other nutrients that protect our cells from damage caused by free radicals. Damage mediated by free radicals results disruption of membrane fluidity, oxidative DNA, protein denaturation and alteration of platelet functions; which have generally been considered to be linked with many chronic health problems such as cancers, inflammation, aging and atherosclerosis. They are natural or synthetic substances capable of absorption of reactive oxygen species produced during various metabolic reactions. Antioxidants will, therefore, prevent many pathological conditions such as cardiovascular diseases, cancer and neurological destruction. Plant phenols and polyphenols have been found to have antioxidant effects and could be used as plant derived drugs.

The potential toxicity of synthetic antioxidants (Figure 1) has necessitated the increased demand to search for antioxidants from natural sources such as herbs, spices, and vegetables. It is known that many herbs, especially those from the Lamiaceae family such as sage, oregano, and thyme, show strong antioxidant activity. It has been demonstrated that consumption of herbs and vegetables could lead to the prevention and protection from several degenerative diseases in humans, mainly because of their antioxidants that prevent free-radical damage (1, 2). It is believed that the phenolic compounds in natural resources are responsible for most of the antioxidant activity of them (3). A significant number of studies have shown that the content of phenolic compounds in fruits, herbs and vegetables varies considerably among different species, and also may depend on the season of cultivation (3-5). However, it is well established that the type and season cultivar play a major role in controlling the polyphenol composition in apples ((4, 6). The antioxidant activity (AOA) of some fruits and vegetables has been measured by different assays. The methods based on the scavenging of free radicals such as ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH [1,1-diphenyl-2-picrylhydrazyl], and the measurement of the ferric reducing power (FRAP), are some of the most employed techniques, however, there is no agreement regarding the standard methodology and the consensus is that different methods should be employed to assess the antioxidant capacity of the natural fruits, vegetables and herbs (7). Therefore, the objective of this study was to compare the total content of phenolics and the AOA measured by three different methods, in leaf extracts of *Thymus kotschyanus*, *T. caucasicus* and *T. pubescence* (Figure 1). The relationship between AOA and polyphenolic contents was also examined.

Thymus is an aromatic plant of the Mediterranean flora with different species. Various Thymus species are commonly used as spices and as traditional medicine remedies (7). They also possess some biological effects such as antispasmodic (8), antibacterial (9), antiviral, expectorating (10) and antioxidant activities (11). It has been found that the chloroform extracts of some *Thymus willdenowii* Boiss and *Thymus broussonettii* Boiss could show topical anti-inflammatory activity (12). *Thymus satureioides* (Labiatae), trivial name “azukni,” is a North African species typical of arid habitats used in the Moroccan folk medicine in form of infuse and decoctions to treat whooping cough, bronchitis and

rheumatism (13) and, generally, for its anti-inflammatory properties after topical or oral administration. There are at least 400 species of Thymus family known to date. In Iran, the plant is predominantly found in the North and is represented in Iranian flora by 14 species. The Persian name of the genus is 'Azorbeh' and/or 'Avishan'(14). Most of the Thymus oils and extracts are used in pharmaceutical, cosmetic and perfume industry and also for flavoring and preservation of foodstuff (15). According to traditional medicine, flowering parts and leaves of Thymus species have been extensively used as herbal tea, digestive, carminative, tonic, expectorant, anti-tussive, anti-inflammatory and antispasmodic, as well as for the treatment of colds (16).

Figure 1. (A) *T.caucasicus*, (B) *T.pubescens* and (C) *T.kotschyanus*

Materials and methods

Thymus cultivars

Three Thymus species (one kilogram each), *Thymus kotschyanus*, *T. caucasicus* and *T. pubescence* (Figure 1), were collected from Amarloo Mountains in Gilan Province in North of Iran 15-25th May as well as 1-10th September 2010. The atmospheric conditions during the two growing season of 2010 in the Province of Gilan are summarized in Table 1.

Table 1. The usual atmospheric conditions in Amarloo of Gilan.

Growing season	Mean monthly temperature (°C)	Mean amount of rainfall (mm)	Relative humidity (%)
15-25th May	18.61	62	85
1-10th September	32.23	42	89

All cultivars were grown under the same geographical conditions and with the same applied agronomic practices.

Chemical reagents

Folin-Ciocalteu's phenol reagent, (+)-catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid, citric acid, potassium peroxydisulfate ($K_2S_2O_8$), potassium chloride (KCl), sodium carbonate (Na_2Co_3) and sodium acetate trihydrate were obtained from Vetec (Rio de Janeiro, RJ, Brazil). Hydrochloric acid (HCl), anhydrous ferric chloride ($FeCl_3$), methanol, ethanol and acetone were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade and all water used was deionized.

Plant samples

Samples from all three species of thymus were collected from Gilan province, Amarloo Mountains during May and September 2010 (Fig 2). Immediately after harvest the leaf samples were washed with deionized water, towel dried, cut into small pieces, dried overnight in an air dryer at 40 °C, ground to a particle size of 25 mesh by using a grinder and stored at −20 °C in an airtight container until used.

Extraction procedure

Dried thymus powder (5.0 ± 0.05 g dry basis) was extracted by stirring with 50 ml of ethanol and water (3:1, v/v) at 40 °C for 1 h. Each extract was then filtered through filter paper (595 1/2 folded filters, ø125 mm, Ref. No. 10311644, Schleicher and Schuell GmbH, Germany);

the filtrate was collected, the volume measured and reduced to 25% by applying 40 °C for 12 hours, filled in a plastic bottle and stored at −20 °C until used. These samples were then regarded as stock extracts.

Total phenolic (TP) content

The TP content was measured using a modified Folin–Ciocalteu method (17). Sample extract (100 μL) was mixed with 2.5 mL of water in a 10 mL volumetric flask. Folin–Ciocalteu reagent (0.5 mL) was added and allowed to react for 5 min. Then, 1.5 mL Na_2CO_3 solution (20 g/100 mL) was added and the mixture was made up to 10 mL with water. The incubation was carried out at room temperature for at least 100 min, the absorbance at 765 nm was read using a UV-visible spectrophotometer model HP 8452A. TP was expressed as mg gallic acid equivalent/100 g dried weight (mg GAE/100 g dw).

Antioxidant activity (AOA)

Trolox was used for calibration of the standard curve and the results were expressed as μMol Trolox equivalent antioxidant capacity/100 g fresh leaves (mMol TEAC/100 g dw). In order to be able to make a direct comparison, the antioxidant activity determined by both DPPH and FRAP methods were all expressed in the same unit.

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

This was a modified method originally described by Brand-Williams (18). In brief, 0.1 mM solution of DPPH in methanol was freshly prepared every day. The absorbance of this solution was measured at 515 nm using a glass cuvette at time $t = 0$ min (t_0). 0.1 mL of plant sample was then added and the mixture was shaken vigorously and kept in the dark at room temperature for 30 min (t_{30}) and the absorbance at 515 nm was then measured. The reduction of DPPH in percent was calculated as: (100 − [Absorbance t_{30}/Absorbance t_0] × 100).

Ferric reducing/antioxidant power (FRAP) assay

Ferric reducing/antioxidant power was measured using a modified method of Arnous (19). 0.1 mL of Thymus extracts was added to 0.1 mL of $FeCl_3$ (3 mM in 5 mM citric acid) and the resulting solution was mixed in a 1.5-mL Eppendorf tube and incubated for 30 min in a water bath at 37 °C. The solution was then added to 0.9 mL of 1 mM TPTZ solution in 50 mM HCl and vortexed. The mixture was left at room temperature for exactly 10 min and absorbance of the mixture was read at 620 nm.

Statistical analysis

All data were reported as mean ± standard deviation (S.D.) of three replicates and analyzed using STATISTICA 7.0 software (Statsoft Inc., Tulsa, OK, USA). Differences between the means were established using one-way analysis of variance (ANOVA) followed by Tukey's test. To evaluate the relationship between the antioxidant activity and polyphenolic contents, linear regression analysis was performed. Differences at the 5% level ($p < 0.05$) were considered statistically significant.

Results and discussion

Total phenolic contents

The total phenolic contents (TP) and antioxidant activity in all cases were approximately 20% higher in cultivars collected at September as compared to may growing seasons (Table 2). It is worth indicating that the temperature and, therefore, humidity is quite high during August-September in this part of Iran. The stress of high temperature could be the reason for increase in the oxidation reactions leading to the high antioxidant activity. Although similar research on thyme species have not been reported, but some reports on other plant material and fruits support our findings. It has been reported that Golden Delicious apple has a lower content of TP in the during spring than hot summer days (20). Table 2 also shows the difference in antioxidant activity of the three thyme species used. This finding is in agreement with the results obtained comparing various types of apple grown in different regions (6). It was demonstrated that the growing region influences the nature and content of

phenolic compounds in apples, but the amount of the difference was highly cultivar-dependent (6). However, in this research we concluded that since all three thymus species studied were grown in the same location using similar horticultural practices, the variation in total phenolics indicate that both cultivar and atmospheric conditions (high temperature stress) could end to the differences in the biosynthesis of secondary metabolites in these herbs.

Antioxidant activity (AOA)

The TEAC values found for various thymus leaf extracts are presented in Table 2. In this case, also, the September samples showed significantly higher TEAC values than the May leaf extracts for all three cultivars and a great variability in TEAC, measured by both DPPH and FRAP methods. Significant differences were also observed between the different cultivars ($p < 0.05$). In the September samples, the AOA values measured by DPPH ranged from 0.623 to 0.766; and by FRAP from 0.282 to 0. 374 TEAC. The September extracts of T.*Caucasicus* showed the highest TEAC obtained by all methods, whereas the lowest value was found in May samples of T.*Pubescens*. Although similar research on Thymus were not found in the literature to be used for comparison, but our results are consistent with those of some research on different plants and fruits. It has been reported that AOA measured by the DPPH were around 2.5 times higher in apple peel compared to the flesh (4, 21). It is also found that the antioxidant activity is dependent on apple cultivars and the parts of the plant used for extracts (4). The differences between our results compared to researches elsewhere may be attributed to growth period, growing season, extraction methods, geographical difference and the cultivar variation.

Table 2. Total phenolic content (mg GAE/100 g dw) and antioxidant activity (mMol TEAC/100 g dw) measured by DPPH and FRAP assays in the leaf extracts of three Thymus species.

Thymus species	Total phenol content		DPPH assay		FRAP assay	
	May	September	May	September	May	September
Pubescens	39.9±1.4	47.52±1.5	0.623±0.21	0.743±0.31	0.282±0.02	0.342±0.02
Kotchyanus	44.25±0.5	53.21±0.5	0.632±0.15	0.752±0.21	0.309±0.02	0.369±0.03
Caucasicus	46.83±1.2	56.12±1.4	0.649±0.11	0.766±0.12	0.336±0.03	0.401±0.03

Results as mean ± SD from three replicates.

[a–h]Different superscript letters between cultivars denote significant differences (Tukey's test, $p < 0.05$).

Correlation analysis

Contribution of polyphenols to the antioxidant properties of all three thymus species, was measured using the linear regression between AOA and TP contents (Fig. 1).

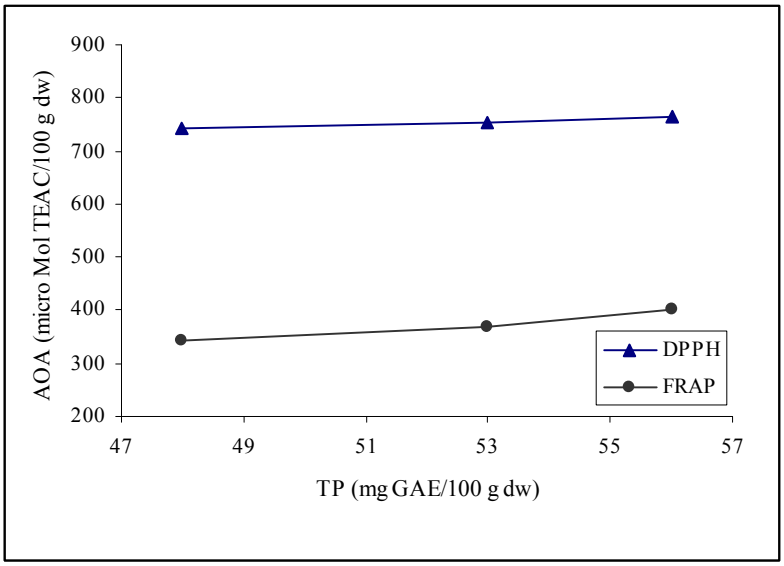

Figure 1. The relationship between total phenolic (TP) content and antioxidant activity (AOA) measured by DPPH and FRAP assay in the (a) Thymus *Kotchyanus*, (b) Thymus *Pubescens* and (c) Thymus *Caucasicus*. The data are extracted from only one growing (September) season. Similar relationship existed between TP and AOA for samples of May with approximately 20% difference.

There was a significant relationship ($p < 0.05$) between total phenol content and AOA measured by DPPH and FRAP methods for spring and summer samples; however, this was slightly weaker in the summer samples compared to the spring ones (Fig. 1). In contrast to our results, Eberhardt (22) and Wolfe (23) did not find any relation between the antioxidant activity and total phenol content in fruit tissues.

Conclusions

The results of this study indicate that the polyphenolic content significantly contributes to the antioxidant activity of thymus leaf extracts. Both polyphenolic content and antioxidant activity are dependent on the cultivar and sampling season. In all conditions the highest total phenolic content and antioxidant activity measured by three methods is related to Thymus *Caucasicus*, whereas the lowest value is shown by Thymus *pubescens* extracts. The significant differences between Thymus samples could confirm that the cultivar is the main factor determining the production of secondary metabolites and contribution of bioactive compounds in plant leaves. In addition, the contribution of phenolics to the antioxidant activity in all samples confirms their important role in the bioactivity of the plant material.

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References

1. Boyer J, Liu RH. Apple phytochemicals and their health benefits. Nutrition Journal 2004; 3:517S-520S.
2. Di Pietro PF, Medeiros NI, Vieira FGK, Fausto MA, Belló-Klein A. Breast cancer in southern Brazil: association with past dietary intake. Nutrition in Hospital 2007; 22:565-572.
3. Tsao R, Yang R, Xie S, Sockovie E, Khanizadeh S. Which polyphenolic compounds contribute to the total antioxidant activities of apple? Journal of Agriculture and Food Chemistry 2005; 53:4989-4995.
4. Khanizadeh S, Tsao R, Rekika D, Yang R, Charles MT, Rupasinghe HPV. Polyphenol composition and total antioxidant capacity of selected apple genotypes for processing, Journal of Food Composition and Analysis 2008; 21:396-401.

5. Lata B, Trampczynska A, Paczesna J. Cultivar variation in apple peel and whole fruit phenolic composition Sci. Hortic 2009; 121:176-181.
6. McGhie TK, Hunt M, Barnet LE. Cultivar and growing region determine the antioxidant polyphenolic concentration and composition of apples grown in New Zealand. Journal of Agriculture and Fod Chemistry 2005; 53:3065-3070.
7. Stahl-Biskup E, Saez F. Thyme. London: Taylor & Francis 2002.
8. Meister A, Bernhardt G, Christoffel V, Buschauer A. Antispasmodic activity of *Thymus vulgaris* extract on the isolated guinea-pig trachea: discrimination between drug and ethanol effects. Planta Medica 1999; 65:512-516.
9. Essawi T, Srour M. Screening of some Palestinian medicinal plants for antibacterial activity. Journal of Ethnopharmacology 2000; 70:343-349.
10. Brasseur T. Etudes botaniques, phytochimiques et pharmacologiques consacrees au Thym. Journal de Pharmacie de Belgique 1983; 38**:**261-272.
11. Miura K, Nakatami N. Antioxidative activity of flavonoids from thyme (*Thymus vulgaris* L.). Agricultural Biological Chemistry 1983; 53:3043-3045.
12. Ismaili H, Sosa S, Brkic D, Fkih-Tetouani S, Ilidrissi A, Touati D, Aquino R, Tubaro, A. Topical anti-inflammatory activity of extracts and compounds from *Thymus broussonettii*. Journal of Pharmacy & Pharmacology 2002; 54:1137-1140.
13. Bellakhadar J, Claiss R, Fleurentin J Younos C. Repertory of standard herbal drugs in the Moroccan pharmacopoea. Journal of Ethnopharmacology 1991; 35:123-143.
14. Mozaffarian V. A dictionary of Iranian plants names. Tehran: Farhang Moaser Publishers. 1998; 547-548.
15. Safaei-ghomi J, Meshkatalsadat MH, Shamai S, Hasheminejad M, Hassani A. Chemical characterization of bioactive volatile molecules of four thymus species using nanoscale injection method. Journal of Nanomaterials and Biostructures 2009; 4:835- 841.
16. Amin G. Popular medicinal plants of Iran. Tehran: Research Deputy of Health Ministry 1991; 1:39.
17. Budini R, Tonelli D, Girotti S. Analysis of total phenols using the Prussian blue method. Journal of Agriculture and Food Chemistry 1980; 28:1236-1238.
18. Brand-Williams W, Culivier ME, Berset C. Use of free radical method to evaluation of antioxidant activity. Lebensm Wiss Technol. 1995; 28:25-30.
19. Arnous A, Markis D, Kefalas P. Correlation of pigment and flavonol content with antioxidant properties in selected aged regional wines from Greece. Journal of Food Composition Analysis 2002; 15:655-665.
20. Leccese A, Bartolini S, Viti R. Antioxidant properties of peel and flesh in Goldrush and Florina scab-resistant apple (*Malus domestica*) cultivars. New Zealand J. Crop Hortic. Sci. 2009; 37:71-78.
21. Vieira FGK, Borges GSC, Copetti C, Gonzaga LV. Nunes and R. Fett, Activity and contents of polyphenolic antioxidants in the whole fruit, flesh and peel of three apple cultivars. Arch Latinoam Nutr. 2009; 59:101-106.
22. Eberhardt MV, Lee CY, Liu RH. Antioxidant activity of fresh apples. Nature 2000; 405:903-904.
23. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. Journal of Agriculture and Food Chemistry 2003; 51:609-614.