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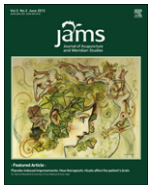

RESEARCH ARTICLE

Evaluation of the Antioxidant Capacity and Phenolic Content of Three *Thymus* Species

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Abstract
Thymus species are known to have significant amounts of phenolic and flavonoid compounds and exhibit strong antioxidant activities. This work was designed to evaluate the antioxidant activities of three endemic Iranian *Thymus* species (including *T. daenensis*, *T. kotschyanus*, and *T. pubescens*) in different test systems [namely $DPPH^{\bullet}$ (2,2′-diphenyl-1-picrylhydrazyl), $ABTS^{\bullet+}$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid], and linoleic acid/β-carotene bleaching assays] to determine the total phenolic and flavonoid contents of the species (assayed by colorimetric techniques) and to study the possible composition-activity relationship. All the tested plants exhibited concentration-dependent antioxidant and free radical scavenging activities. *T. pubescens* showed the highest free radical scavenging activities in both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ methods, while *T. daenensis* and *T. kotschyanus* were the most active species in the β-carotene bleaching inhibition test. Alternatively, *T. pubescens* exhibited a significantly higher level of the total flavonoid content compared with those of the other species, while no significant statistically differences were found among the tested plants regarding the total phenolic content. In addition, significant correlations were found between the flavonoid content and $DPPH^{\bullet}$/$ABTS^{\bullet+}$ radical scavenging activities, but not between the β-carotene bleaching inhibition system and the flavonoid content.

1. Introduction

Plants are good sources of active natural products that differ widely in terms of structure and biological properties so; they can be used for various applications, especially as food additives and health promoting ingredients. For the reason, during last few decades, they have been become a subject for study of bioactive compounds [1].

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A growing amount of evidence has shown that the oxidative stress and free radicals play the important roles in the etiology of some chronic diseases. Epidemiological studies have revealed that the consumption of antioxidants is positively associated with the reduced risk of developing chronic and ageing related diseases. On the other hand, synthetic antioxidants have been shown to be potentially toxic. Therefore, there is a growing interest in searching for antioxidants naturally present in plants [2]. Numerous crude extracts and pure natural compounds have been reported to have antioxidant and radical scavenging activities [3]. Among the various kinds of plant constituents, phenolic compounds and flavonoids have received particular attention as potential natural antioxidants [4,5]. They can delay or inhibit the oxidation of molecules by inhibiting the initiation or propagation of oxidative chain reactions [6].

A great number of aromatic and spicy plants, especially from Labiatae family, contain chemical compounds with antioxidant properties. For example, rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) have been reported to be rich in phenolic compounds and possess antioxidant properties comparable with synthetic antioxidants [7]. Among the Labiatae members, various *Thymus* species are among the most popular plants throughout the world. They are commonly used as herbal teas, flavoring agents, and aromatic and medicinal plants. These species have been used as carminative, antitussive, and expectorant agents. They are also reported to possess several and potent biological and pharmacological properties such as anti-inflammatory, antispasmodic, among others. Recent studies on some *Thymus* species indicate that they possess antioxidative and hepatoprotective activities [8].

The genus of *Thymus* consists of about 215 species which particularly prevalent in the Mediterranean area [8]. The genus is represented in the flora of Iran by 14 species which some of them are endemic such as *T. daenensis*, *T. pubescencs,* among others [9]. They are widely used as herbal tea, condiment, spice, and digestive and to treat whooping cough, bronchitis, inflammation and rheumatism in Iranian folk medicine [10—12]. Despite the considerable antioxidant activities shown by a lot of *Thymus* species investigated, there are few studies that evaluate Iranian *Thymus* species. However, these studies have been focused on the essential oils obtained from the plants. According to the study of Alavi and colleagues [13], the essential oil from *T. daenensis* was found to be active in some of antioxidant assays. Asbaghian and colleagues [14] have shown that the essential oil of *T. kotschyanus* was an effective scavenger of the DPPH$^{\bullet}$ free radicals. Based on the study of Nazemiyeh and his co-workers [15], the essential oil of *T. pubescens* had an anti-2,2'—diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) activity. The aim of the present work was to evaluate and compare the antioxidant activities of the hydroalcoholic extracts obtained from three Iranian *Thymus* species (including *T. pubescence* Boiss. & Kotschy ex Celak, *T. daenensis* Celak, and *T. kotschyanus* Boiss. & Hohen) by different methods. Because of the important roles of the phenolics and flavonoids as potent antioxidants, the total amounts of the compounds were also determined.

2. Materials and methods

2.1. Plant materials

The aerial parts of *T. daenensis* and *T. kotschyanus* were collected in Hamedan province and *T. pubescens* was collected in Tehran province, Hamedan and Tehran, Iran, during their flowering period in June 2008. The species were identified by Dr. Amin at Tehran University of Medical Sciences and Eng. Kamalinejad at Shahid Beheshti University of Medical Sciences. The voucher specimens have been deposited at the Herbarium of School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.2. Chemicals and instruments

All of the chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (France) and/or Merck Company (Germany). A Shimadzu Multispect-1501 spectrophotometer (Kyoto, Japan) was used for absorbance measurements.

2.3. Preparation of extracts

The dried and ground aerial parts of thyme samples (200 g) were extracted with 90% EtOH (2000 mL) at 25 °C for 72 hours. The extracts were filtered and concentrated under reduced pressure at 35 °C. The dried extracts were kept in the dark at 4 °C until tested.

2.4. Antioxidant assays

2.4.1. DPPH$^{\bullet}$ assay

The free radical scavenging abilities of the samples were measured using the stable radical DPPH$^{\bullet}$ [5]. A total of 1 mL of DPPH$^{\bullet}$ solution (0.3 mM) was added to 2.5 mL of each sample (at concentrations 800, 400, 200, 100, 50, 25, 12.5, and 6.25 μg/mL in 90% ethanol). The tubes were incubated at room temperature for 30 minutes, then the absorbance values were determined at 518 nm. Rutin and gallic acid were used as positive controls. Inhibition percentage of DPPH$^{\bullet}$ ($I_{\text{DPPH}^{\bullet}}$ %) was calculated by the following formula:

$$I_{\text{DPPH}^{\bullet}}(\%) = 100 \cdot \left[\frac{A_{\text{control}} - \left(A_{\text{sample}} - A_{\text{blank}}\right)}{A_{\text{control}}}\right],$$

where A_{sample}, A_{blank} and A_{control} were the absorbance of sample, blank sample, and control, respectively.

2.4.2. ABTS$^{\bullet+}$ assay

The antioxidant capacities of the samples were evaluated by a method based on the decolonization of radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) [4]. The ABTS$^{\bullet+}$ radical cation was prepared by the reaction of 7 mM ABTS with 2.54 mM potassium persulfate, after incubation at room temperature for 12—16 hours. Prior the assay, the ABTS$^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. A total of 5 mL of the diluted ABTS$^{\bullet+}$ solution was added to 50 μL of each sample (at concentrations 800, 400, 200, 100, 50, 25, 12.5, and 6.25 μg/mL in 90% ethanol). The

reaction mixture was allowed to stand at room temperature for 10 minutes and absorbance at 734 nm was immediately recorded. Rutin and gallic acid were used as positive controls. Inhibition percentage of $ABTS^{\bullet+}$ ($I_{ABTS^{\bullet+}}$ %) was calculated by the following formula:

$$I_{ABTS^{\bullet+}}(\%) = 100 \cdot \left[\frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}}\right],$$

where A_{sample}, A_{blank} and $A_{control}$ were the absorbance of sample, blank sample, and control, respectively.

2.4.3. Linoleic acid/β-carotene bleaching assay

The anti-lipid peroxidation activities of the samples were determined by the linoleic acid/β-carotene system [16]. A stock solution of β-carotene and linoleic acid was prepared with 2 mg of β-carotene in 10 mL chloroform, 45 μL linoleic acid, and 400 mg Tween 40. The chloroform was evaporated under vacuum and 100 mL of aerated distilled water was then added to the residue. A total of 0.5 mL of each sample (at concentrations 800, 400, 200, 100, 50, 25, 12.5, and 6.25 μg/mL in 90% ethanol) was added to 4.5 mL of the above mixture in the separated test tubes. As soon as the sample was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. Then, the tubes were incubated in a hot water bath at 50 °C. After 2 hours, the absorbance values were determined again at 470 nm. Gallic acid was used a positive control. A blank, devoid of β-carotene, was prepared for background subtraction. Inhibition percentage of bleaching ($I_{bleaching}$ %) was calculated using the following equation:

$$I_{bleaching}(\%) = (\text{Absorbance after 2 h of assay/Initial absorbance}) \times 100.$$

2.5. Content determination

2.5.1. Total phenolic content

The total phenolic contents (TPCs) of the extracts were determined spectrophotometrically with Folin-Ciocalteu reagent and calculated as rutin equivalents [4]. For the preparation of the calibration curve, 1 mL of the rutin solution in ethanol (at the concentrations 25, 50, 75, 100, 150, and 200 μg/mL) was thoroughly mixed with 5 mL Folin-Ciocalteu reagent (diluted 1/10). After 10 minutes, 4 mL of the sodium carbonate solution (75 mg/mL) was added, and the mixture was allowed to stand for 30 minutes. The absorbance was measured at 765 nm on a UV-Vis spectrophotometer. Then, a linear calibration curve (absorbance versus concentration) was developed. The plot was found to be linear across the ranged assay (25—200 μg/mL, $r^2 > 0.99$). The same procedure was carried out with 1 mL of the samples (at the concentration 400 μg/mL) instead of rutin. The TPC for each extract (as μg rutin equivalents/mg of the extract) was calculated according to the following equation:

$$TPC(\mu g/mg) = \frac{C \times V}{M},$$

where C, V, and M were the concentration of rutin established from the calibration curve (μg/mL), the volume of the extract (mL), and the weight of the extract (mg), respectively.

2.5.2. Total flavonoid content

The total flavonoid contents (TFCs) of the extracts were measured colorimetrically using $AlCl_3$ reagent and calculated as rutin equivalents [5]. The rutin calibration curve was prepared by mixing 2.5 mL of the rutin solution in ethanol (at the concentrations 25, 50, 75, 100, and 150 μg/mL) with

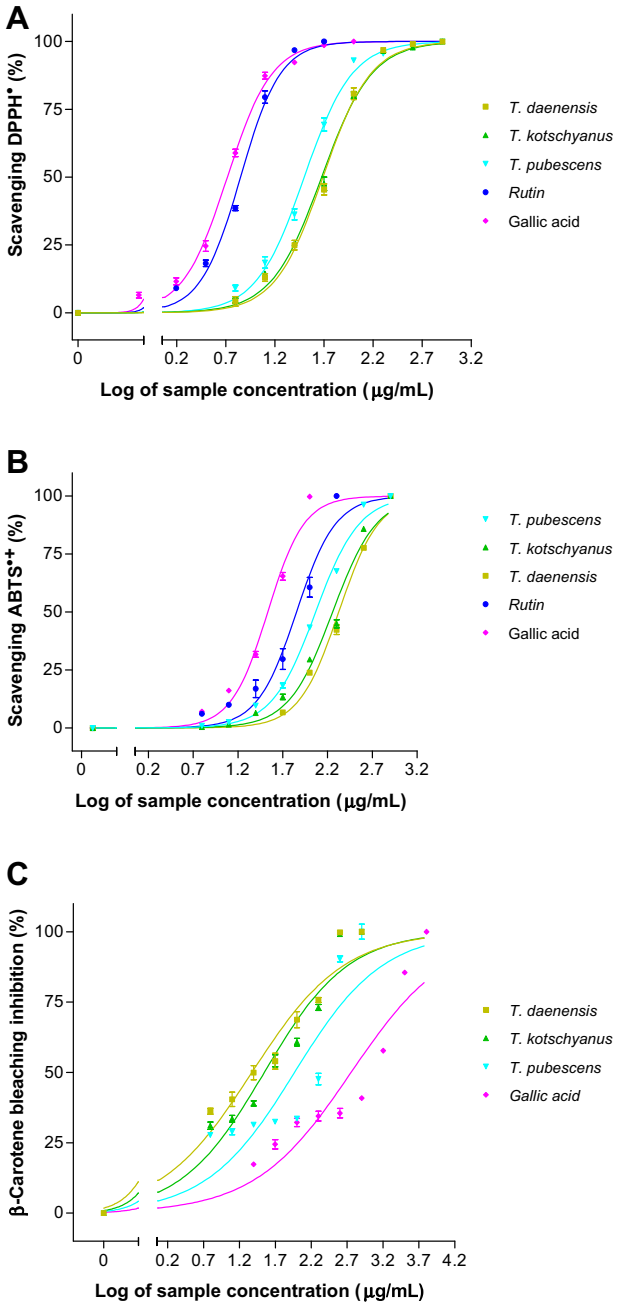

Figure 1 Dose-dependent antioxidant activities of the studied *Thymus* extracts measured by using (A) the $DPPH^{\bullet}$ assay; (B) the $ABTS^{\bullet+}$ assay; and (C) Linoleic acid/β-carotene bleaching assay. Each point represents the mean of three experiments and the vertical bars represent the standard error of measurement.

2.5 mL $AlCl_3$ reagent (20 mg/mL). After 40 minutes, the absorbance was measured at 415 nm on a UV-Vis spectrophotometer, and then a standard curve (absorbance versus concentration) was developed. The plot was found to be linear across the ranged assay (25–150 μg/mL, $r^2 > 0.99$). The same procedure was performed with 2.5 mL of the samples (at the concentration 400 μg/mL) instead of rutin. The TFC for each extract (as μg rutin equivalents/mg of extract) was calculated by the following formula:

$$TFC(\mu g/mg) = \frac{C \times V}{M},$$

where *C*, *V*, and *M* were the concentration of rutin established from the calibration curve (μg/mL), the volume of the extract (mL), and the weight of the extract (mg), respectively.

2.6. Statistical analysis

All the experiments were carried out in triplicate. IC_{50} values [inhibitory concentration (μg/mL)] were calculated from logarithmic regression curve (*I*% against sample concentration) and presented by their respective 95% confidence limits. The one-way analysis of variance followed by Tukey's post-test was used for comparisons. A *p* value of < 0.05 was considered to denote a statistically significant difference. All the statistical analyses were accomplished using the computer software GraphPad prism 3.02 for Microsoft Windows (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. DPPH$^{\bullet}$ scavenging capacity

DPPH$^{\bullet}$ is a stable free radical and it is commonly employed to assess the radical scavenging activity of plant extracts. Antioxidant molecules can quench DPPH$^{\bullet}$ radicals (by providing hydrogen atom or electron donation) and convert them to a colorless product [17]. Fig. 1A illustrates a decrease in the concentration of DPPH$^{\bullet}$ due to the scavenging ability of the tested *Thymus* extracts. The IC_{50} values (the concentration with scavenging activity of 50%) were found to be 31.47 (29.67–33.38), 47.22 (44.35–50.28), and 48.68 (45.37–52.22) μg/mL for *T. pubescens*, *T. kotschyanus*, and *T. daenensis*, respectively (Table 1). Among the extract, *T. pubescens* extract showed the most potent activity ($p < 0.001$). The extracts of *T. daenensis* and *T. kotschyanus* species revealed moderate scavenging activities. However, the results showed no significant difference between activities of the two extracts ($p > 0.05$).

Table 2 Total phenolic and flavonoid contents of the studied *Thymus* species.

Plant species	Phenolic content (μg rutin/mg extract)*	Flavonoid content (μg rutin/mg extract)*
T. daenensis	$295.93 \pm 34.07^{\dagger}$	$35.21 \pm 2.51^{\dagger}$
T. kotschyanus	$337.00 \pm 8.31^{\dagger}$	$37.11 \pm 3.36^{\dagger}$
T. pubescens	$295.57 \pm 1.91^{\dagger}$	$50.39 \pm 0.75^{\ddagger}$

†,‡ Indicate significant difference in the same column ($p < 0.05$) (samples connected by the same symbol are not statistically different).

* Values are expressed as mean ± standard error of mean of three measurements ($n = 3$).

3.2. ABTS$^{\bullet+}$ scavenging capacity

ABTS$^{\bullet+}$ decolorization assay is a rapid and reliable method that is widely used in the total radical scavenging measurement of the plant extracts [18]. Fig. 1B shows the ABTS$^{\bullet+}$ scavenging activity of the extracts at different concentrations. All the studied extracts exhibited concentration-dependent activity. The IC_{50} values, as indicated in Table 1, were found to be 116.60 (109.80–123.80), 180.40 (164.30–198.10), and 210.90 (194.60–228.60) μg/mL, for *T. pubescens*, *T. kotschyanus*, and *T. daenensis*, respectively. When the IC_{50} values were compared for the three tested extracts, *T. pubescens* showed the highest activity among the extracts ($p < 0.001$). The extracts of *T. kotschyanus* and *T. daenensis* exhibited lower scavenging activity with no significant difference between them ($p > 0.05$).

3.3. Antioxidant capacity in linoleic acid/β-carotene system

The β-carotene bleaching assay determines the capacity of the antioxidants to inhibit lipid peroxidation in the phase of initiation as well as in the phase of propagation [19]. As shown in Fig. 1C, all the extracts showed potent activity in a concentration-dependent manner. They significantly inhibited the bleaching of β-carotene in comparison with control (gallic acid). The highest activities were found in *T. daenensis* [$IC_{50} = 23.68$ (18.42–30.44) μg/mL] and *T. kotschyanus* [$IC_{50} = 35.17$ (27.96–44.25) μg/mL] with no

Table 1 Antioxidant potency of the studied *Thymus* species.

Plant species/standard	$IC_{50\ (DPPH^{\bullet})}$* (μg/mL)	$IC_{50\ (ABTS^{\bullet+})}$* (μg/mL)	$IC_{50\ (\beta\text{-carotene bleaching})}$* (μg/mL)
T. daenensis	48.68 (45.37–52.22)†	210.90 (194.60–228.60)†	23.68 (18.42–30.44)†
T. kotschyanus	47.22 (44.35–50.28)†	180.40 (164.30–198.10)†	35.17 (27.96–44.25)†
T. pubescens	31.47 (29.67–33.38)‡	116.60 (109.80–123.80)‡	92.87 (61.50–140.2)‡
Rutin	7.06 (6.61–7.54)§	71.31 (62.11–81.87)§	—
Gallic acid	5.18 (4.93–5.45)$^{\|\|}$	34.00 (31.17–37.08)$^{\|\|}$	570.90 (417.20–781.40)§

$^{\dagger,\ddagger,\S,\|\|}$Significant difference in the same column ($p < 0.001$) (samples connected by the same symbol are not statistically different).

* IC_{50} values are presented with their respective 95% confidence limits.

significant difference between them ($p > 0.05$). Although the activity of *T. pubescens* [$IC_{50} = 92.87$ (61.50—140.20) μg/mL] was lower than that of *T. daenensis* and/or *T. kotschyanus* ($p < 0.001$), it was much higher than that of gallic acid, used as a positive control. Rutin (the other reference compound) was very poor in activity (Table 1).

3.4. Total contents of phenolics and flavonoids

Phenolic compounds contribute to the overall antioxidant activities of plants. Therefore, the first approach to the characterization of each extract was to determine its amount of phenolic content [20,21]. The phenolic content was calculated from rutin calibration curve ($y = 0.00457x + 0.0825$, $r^2 = 0.996$) and expressed as μg of rutin equivalents in mg of the dry matter of the extract. Table 2 presents the total phenolic content determined for each extract. They ranged from 295.57—337.00 μg/mg. The distribution of phenolic compounds in the three extracts demonstrated that the differences among them were not statistically significant.

For a further specification of the extracts, their total flavonoid contents were determined [20,21]. The flavonoid content was calculated from rutin calibration curve ($y = 0.00966x—0.00468$, $r^2 = 0.993$) and expressed as μg of rutin equivalents in mg of the dry matter of the extract. As can be seen from the Table 2, *T. pubescens* was found to have the highest flavonoid content (50.39 ± 0.75 μg/mg) among the other species evaluated ($p < 0.001$). This is followed by *T. kotschyanus* (37.11 ± 3.36 μg/mg) and *T. daenensis* (35.21 ± 2.51 μg/mg) with no significant difference between them ($p > 0.05$). As it could be expected, the extracts contained smaller proportion of flavonoids than phenols.

3.5. Relation between measured antioxidant capacity and phenolic composition

In this study, strong correlations between both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacities (expressed as the reciprocal of the calculated IC_{50} values) with total flavonoid contents were found ($r^2 = 0.996$ and $r^2 = 0.993$ for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively). This is shown in Fig. 2A and B. The correlation suggests that flavonoids are likely contributed to the radical scavenging activities of the extracts. Several studies have shown a positive correlation between flavonoid content and $DPPH^{\bullet}$ and/or $ABTS^{\bullet+}$ scavenging potency in plants. Both methods are used the alcoholic $DPPH^{\bullet}$ and/or $ABTS^{\bullet+}$ solution so, the technique are independent of the substrate polarity. In other words, hydro-/lipophilicity of a sample does not affect the radical scavenging activities [18,22—24].

However, the β-carotene bleaching inhibitory potency ($1/IC_{50}$) and flavonoid content was inversely related (slope = -0.00180, $r^2 = 0.89$). This is shown in Fig. 2C. At the first glance, this negative correlation suggests that flavonoids are not contributed to the bleaching inhibitory activity. The β-carotene bleaching technique employs an emulsified system, so the activity depends on the substrate polarity. Apolar antioxidants can exhibit stronger antioxidative properties in emulsions because they concentrate

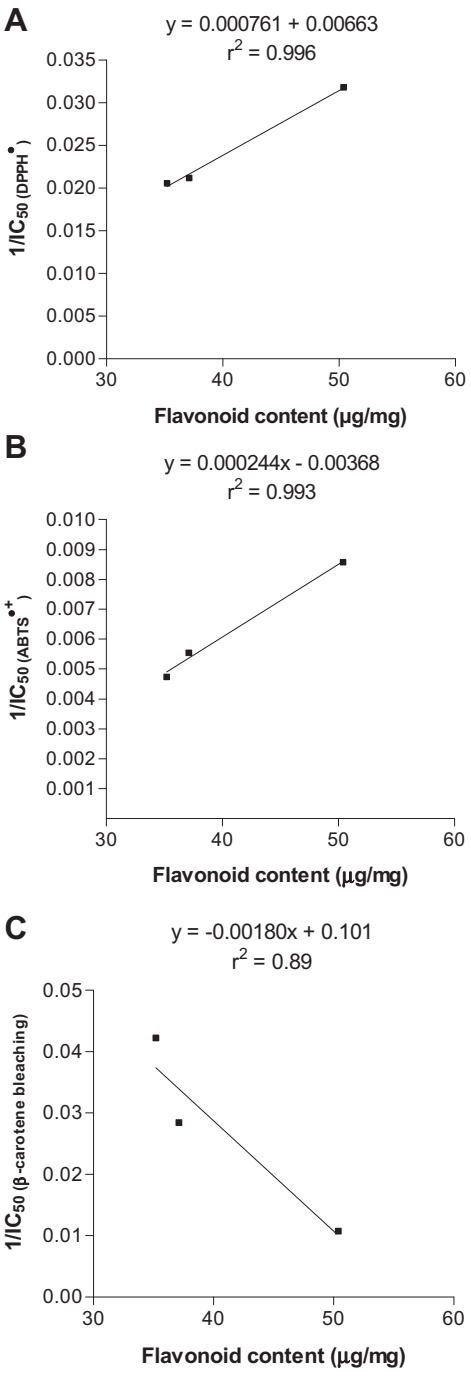

Figure 2 Relationship between the antioxidant capacities of the studied *Thymus* extracts measured by using (A) the $DPPH^{\bullet}$ assay; (B) the $ABTS^{\bullet+}$ assay; and (C) Linoleic acid/β-carotene bleaching assay and their total flavonoid contents.

at the lipid phase. On the other hand, polar antioxidants remain in the aqueous phase and are thus less effective in the lipid protecting. The phenomenon can be explained why the extracts with higher flavonoid content showed lower inhibitory activity. The established weaker abilities of the control compounds (gallic acid and/or rutin) in the β-carotene bleaching inhibition compared to those of the extracts agree with these results. Various studies have also shown such a correlation [22,25]. Furthermore, other

factors such as structural features of the antioxidants and the complex composition of the extracts can play important roles in the antioxidant activities of plant materials [21,26,27].

Literature review shows the presence of the two main groups of secondary metabolites in the genus of *Thymus*, volatile terpenoids and polyphenolic compounds. Both of them are mainly responsible for the biological effects of the genus, particularly antioxidant activities [8]. The monoterpenoid phenols (such as thymol and carvacrol) constitute the main compounds of the essential oils obtained from different *Thymus* species (including *T. daenensis*, *T. kotschyanus*, and *T. pubescens*) [8,10,14,28–34]. On the other hand, the presence of various polyphenolic constituents, especially flavonoids and phenolic acids, is well known in *Thymus* plants. Among the flavonoids, the most widespread skeletons are flavones (mainly luteolin, apigenin, and scutellarin) and then flavanones (mainly eriodictyol and naringin). Besides, different phenolic acids have been reported in the genus. Caffeic and rosmarinic acids have been more frequently found [8]. However, to the best of our knowledge, there is no previous report on the polyphenolic content of the three *Thymus* species studied in this investigation.

4. Conclusion

In this study, three *Thymus* species (including *T. daenensis*, *T. kotschyanus*, and *T. pubescens*) were evaluated for the antioxidant capacity and phenolic content. The results showed that all the samples possessed the potent free radical scavenging and antioxidant activities in different assays. They were also rich in phenolic and flavonoid contents. Besides, there was a significant correlation between flavonoid contents and free radical scavenging activities. The strong observed activities may be attributed, at least in part, to the present of flavonoids. Therefore, the tested *Thymus* species might be valuable antioxidant natural sources and seem to be applicable in both healthy medicine and food industry. However, the in vivo safety and identification of active compounds needs to be thoroughly investigated in prior to their possible application.

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