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ORIGINAL ARTICLE

Antioxidant and antibacterial activity of flavonoid-, polyphenol- and anthocyanin-rich extracts from *Thymus kotschyanus* boiss & hohen aerial parts

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Abstract The total phenolic and flavonoid contents, antioxidant and antibacterial properties of flavonoid- (water, ethyl acetate and hexane fractions), polyphenol- and anthocyanin-rich extracts of *Thymus kotschyanus* aerial parts were investigated. All the extracts showed significant amounts of phenolic and flavonoid compounds and exhibited strong antioxidant activity. Among the extracts, water fraction contained the highest phenolic and flavonoid contents (881.06±16.52 mg GAE/g of extract and 74.60±3.05 mg QE/g of extract, respectively). It also presented the highest $DPPH^{\bullet}$ scavenging activity with an IC_{50} of 14.21±0.53 μg mL^{-1}, and the highest reducing power at 400 μg mL^{-1} by A_{700}=2.46±0.04. The extracts were found to exert moderate antibacterial activity against both Gram-negative and Gram-positive bacteria. These findings highlighted a scientific basis to the traditional usage of *T. kotschyanus*, also showed its potential as a rich source of natural antioxidant and antibacterial compounds.

Keywords *Thymus kotschyanus* · Total phenolic content · Flavonoid-rich · Antibacterial activity

Introduction

Consumers desire to reduce the risk for chronic diseases or to manage a specific health condition through improved diet. Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. In order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. However, the commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are restricted by legislative rules, related to their potential health risks and toxicity (Imaida et al. 1983; Kahl and Kappus 1993). Therefore, scientists are now searching for naturally occurring antioxidants in plant sources for food or medicinal materials as alternative for synthetic antioxidants (Proestos et al. 2005; D'Abrosca et al. 2007; Annegowda et al. 2013). Natural antioxidants such as phenolic compounds, flavonoids, anthocyanins and other phytochemicals can function as free radical scavengers, reducing agents, chelators of prooxidant metals, or as quenchers of singlet oxygen and thus delay the lipid oxidation process in food products (Rice-Evans et al. 1997; Kahkonen et al. 1999; Miraliakbari and Shahidi 2008; Sun et al. 2009; Amarowicz et al. 2010). A number of antioxidant sources based on natural origin have been explored so far, but still there exists need for the search of newer sources, which may be safer, more economical and preferably from dietary sources.

The genus *Thymus* L., known as "Avishan" in Persian, is a well known aromatic perennial herb originated from Mediterranean region. Among 215 species of this genus grown in the world, 14 species are distributed in Iranian flora (Jalas 1982; Stahl-Biskup and Saez 2002). *Thymus* species are well known as medicinal plants due to their biological and pharmacological properties including antispasmodic, antiviral, antibacterial, antifungal, anti-parasite, and antioxidant activities (Stahl-Biskup and Saez 2002). In traditional medicine, leaves and flowering parts of *Thymus* species are widely used as tonic and herbal tea, antiseptic, antitussive and carminative as well as treating colds (Zargari 1990).

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The chemical composition and biological activities of *Thymus kotschyanus* have been previously investigated (Rasooli and Mirmostafa 2003; Bagci and Baser 2005; Nickavar et al. 2005; Morteza-Semnani et al. 2007; Mohammed and Al-Bayati 2009). Moreover, hydroalcoholic extract of this whole plant has exhibited strong antioxidant activity (Nickavar and Esbati 2012). Thus, the aim of the present work is to evaluate the total phenolic and flavonid contents, antioxidant and antibacterial activities of flavonoid-, polyphenol- and anthocyanin-rich extracts from *T. kotschyanus* aerial parts for their possible use as source of antioxidants and also as antibacterial agents that can be used to prevent food spoilage.

Materials and methods

Chemicals

Trichloroacetic acid (TCA), Folin–Ciocalteau, gallic acid, quercetin, ascorbic acid, butylated hydroxytoluene (BHT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Chemical Company. Other chemicals and reagents were of analytical grade.

Plant material

The aerial parts of *T. kotschyanus* were collected from the central Alborz Mountains, Veresk area, north of Iran, in June 2012. The plant was identified and authenticated by Dr. A. Naqinezhad at Department of Biology, University of Mazandran, Babolsar, Iran. A specimen is deposited in the Herbarium of this Institution, under the voucher number 4026.

Preparation of flavonoid-rich extracts (water, ethyl acetate and hexane fractions)

Separation of the flavonoid-rich fractions was done as described previously by Nabavi et al. (2012). Accordingly, 100 g of dried sample was defatted twice with 100 mL of $CHCl_3$ and extracted twice with 100 mL of 60 % acetone for 12 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper. The solvent in the combined filtrates was removed at 35 °C using a rotary evaporator, leaving the crude acetone extract (12 g). After preparation of 10 % methanol slurry, the acetone extract was separately fractionated sequentially with hexane, ethyl acetate, and finally with water. All the extracts were dried at 40 °C using a rotary evaporator. The percentage yield of extracts was expressed in terms of dried weight of plant material.

Extraction of polyphenols and anthocyanins

Polyphenols and anthocyanins were extracted from crushed aerial part tissues (100 g) of *T. kotschyanus*, according to the modified method of Zhang et al. (2000). The extraction was performed twice at 20 °C in a shaking incubator (ZHWY-200B, Zhicheng Analytical Co., Shanghai, China). Extracting time was 30 min, and extracting solvents were 100 mL of methanol/acetone/water (3.5:3.5:3, v/v/v) containing 1 % formic acid. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper. The combined filtrates were evaporated under vacuum at 40 °C to remove methanol and acetone. Lipophilic pigments were then eliminated from the aqueous phase by two successive extractions in a separatory funnel with a twofold volume of petroleum ether. The aqueous phase was collected and further extracted three times by ethyl acetate (ethyl acetate: aqueous phase=1:1, *v/v*) in the separatory funnel. Three ethyl acetate phases were collected, evaporated and dried under vacuum at 35 °C to obtain polyphenol sample. The aqueous phase was also dried under vacuum at 50 °C and used as an anthocyanin sample. All samples were stored at −20 °C prior to further assays.

Determination of total phenolic content (TPC)

Total phenolic content was determined by the Folin-Ciocalteau method (Meda et al. 2005). Accordingly, aliquots of 4 mg lyophilized powder of extracts were separately dissolved in 50 mL deionized water. Folin-Ciocalteau reagent (0.2 N, 2.5 mL) for 5 min and then 2.0 mL of 75 g/L sodium carbonate were added to extract solution (0.5 mL). The absorbance of reaction was measured spectrophotometrically (UV-Visible EZ201, Perkin Elmer: Norwalk, CA, USA) at 760 nm after 30 min of incubation at room temperature. The results were expressed as gallic acid equivalent (GAE)/g of the dry extract.

Determination of total flavonoid content (TFC)

The total flavonoids content was evaluated by the aluminum chloride colorimetric method (Willet 2002). Accordingly, aliquots of 4 mg lyophilized powder of extracts were separately dissolved in 50 mL deionized water. This solution (0.5 mL) was mixed with 1.5 mL of methanol, 0.1 mL of 10 % aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm on a spectrophotometer (UV-Visible EZ201, Perkin Elmer: Norwalk, CA, USA). Total flavonoid content was calculated as quercetin equivalent (QE)/g of the dry extract.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (Brand-Williams et al. 1995). Two milliliters of different concentrations of extracts (5–80 μg mL^{-1}, in water) were added to 2 mL of methanol solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Ascorbic acid and BHT were used as standard controls for comparison. IC_{50} values denote the concentration of sample, which is required to scavenge 50 % of DPPH free radicals.

Reducing power

The reducing power of fractions was determined according to the method of Yen and Chen (1995). Accordingly, 2.5 mL of sample with different concentrations (25–400 μg mL^{-1}, in water) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10 %) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbance was measured at 700 nm. Ascorbic acid was used as positive control.

Antimicrobial activity (disk diffusion assay)

In vitro antibacterial activity of the extracts was evaluated by disc diffusion method using Mueller-Hinton Agar with determination of inhibition zones (IZ) (Kirby-Bauer Method; Drago et al. 1999). The Gram-negative and Gram-positive bacteria were as follows: *Escherichia coli* PTCC 1330, *Pseudomonas aeruginosa* PTCC 1074, *Staphylococcus aureus* ATCC 35923 and *Bacillus subtilis* PTCC 1023. 20 μL of the extracts (20 mg/ml in DMSO) was applied on the paper discs (the disc diameter was 6 mm). The paper discs were placed on nutrient agar plates that had been previously seeded with inoculum containing indicator microorganisms. After incubation of the plates at 37 °C for 24 h, the diameter of growth inhibition zones were measured. The antibacterial activity of the extracts was compared with known antibiotics gentamicin (10 μg/disc) and chloramphenicol (30 μg/disc) as positive controls and DMSO (20 μL/disc) as negative control.

Statistical analyses

Experimental results were mean±kS.D. of three parallel measurements and analyzed by SPSS 18. Differences between means were determined using ANOVA and Tukey multiple comparisons and least significant difference (LSD). Correlations were obtained by Pearson correlation coefficient in bivariate correlations. p values≤0.05 were regarded significant.

Results and discussion

Extraction yields, total phenolic and flavonoid contents

Content of phenolic and flavonoid compounds, and extraction yields of *T. kotschyanus* aerial parts were listed in Table 1. Yields varied from 0.43 to 7.52 % among the extracts. The highest yield was obtained for water fraction (7.52 %) followed by anthocyanin fraction (6.41 %), polyphenols fraction (2.57 %), ethyl acetate fraction (0.58 %), and hexane fraction (0.43 %), respectively; differences being significant ($p<0.05$) among them.

The TPC of extracts was determined spectrophotometrically by Folin-Ciocalteu reagent and calculated in terms of gallic acid equivalent with reference to standard curve ($y=0.0054x+0.0628$, $R^2=0.987$). The high phenolic content was observed in all extracts which varied over a wide range, 309.31–881.06 mg GAE/g of dry extract (Table 1). The results revealed that water fraction possessed the highest phenolic content (881.06±16.52) mg GAE/g followed by ethyl acetate fraction (751.56±6.91) mg GAE/g, hexane fraction (555.72 ±5.23) mg GAE/g, polyphenols fraction (391.14±10.82) mg GAE/g, and anthocyanin fraction (309.31±15.84) mg GAE/g; with significant difference between them ($p<0.05$).

The total flavonoid contents of various extracts were recorded spectrophotometrically using quercetin as calibration standard ($y=0.0063x$, $R^2=0.999$). The TFC ranged from 32.04 to 74.60 mg QE/g of dry extract (Table 1). Highest TFC was recorded for water extract, while the lowest was for the anthocyanin extract; differences again being significant for TFC among the extracts ($p<0.05$). Variations in the TPC and TFC of *T. kotschyanus* extracts might be attributed to the different polarities of the extraction solvents as well as the chemical nature of the endogenous extractable compounds (Jaffery et al. 2003).

Free-radical scavenging activity

The DPPH radical-scavenging assay is a widely used and comparably facile method to evaluate antioxidant activity. Antioxidant molecules scavenge DPPH radicals by the process of either hydrogen or electron donation and the purple color from the $DPPH^{\bullet}$ assay solution becomes light yellow which can be quantified by its decrease of absorbance at a wavelength 517 nm (Blois 1985). Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation.

Figure 1 illustrates the percent inhibition of DPPH radical by the various extracts and the standard antioxidants at different

Table 1 Extraction yields, total phenolic and flavonoid contents of water, ethyl acetate, hexane, polyphenol, and anthocyanin extracts of *T. kotschyanus* aerial parts

Extract	Extraction yields (%)	Total phenolic content[a]	Total flavonoid content[b]
Flavonoid-rich (Water fraction)	7.52±0.16	881.06±16.52	74.60±3.05
Flavonoid-rich (Ethyl acetate fraction)	0.58±0.03	751.56±6.91	59.52±2.31
Flavonoid-rich (Hexane fraction)	0.43±0.04	555.72±5.23	42.45±3.54
Polyphenols fraction	2.57±0.09	391.14±10.82	47.61±1.92
Anthocyanin fraction	6.41±0.14	309.31±15.84	32.04±0.53

The data are expressed as mean±S.D. (n=3). ANOVA test for data in the same column indicates significant difference (p<0.05)

[a] mg GAE/g of dry extract

[b] mg QE/g of dry extract

concentrations. The descending order of IC_{50}, the effective concentration of the sample required to scavenge 50 % of DPPH$^{\bullet}$, was as follows: anthocyanin extract (IC_{50}: 41.13±5.94 μg mL^{-1})>polyphenol extract (IC_{50}: 32.66±3.16 μg mL^{-1})>hexane extract (IC_{50}: 23.71±3.68 μg mL^{-1})≈ethyl acetate extract (IC_{50}: 23.71±1.21 μg mL^{-1})>water extract (IC_{50}: 14.21±0.53 μg mL^{-1})>BHT (13.18±3.11 μg mL^{-1})>ascorbic acid (6.56±0.12 μg mL^{-1}); with significant difference between them (p<0.05). However, the tested extracts presented a remarkable capacity to scavenge DPPH-radical with IC_{50} values being found at the μg mL^{-1} level. It is interesting to mention that water fraction, which contains the highest phenolic and flavonoid contents, showed better DPPH$^{\bullet}$ scavenging activity than other extracts (p<0.05), while it exhibited a slightly greater IC_{50} value than that of ascorbic acid and BHT (p>0.05). Interestingly, according to Fig. 1, when sample concentration was above 20 μg mL^{-1}, the radical scavenging activity of water fraction was higher than that of BHT.

In the present study, DPPH radical scavenging capacity of the flavonoid- rich extracts (water, ethyl acetate and hexane fractions) of *T. kotschyanus* was found to be greater than that reported for hydroalcoholic extracts of *T. pubescens* (IC_{50}: 31.47 μg/mL), *T. kotschyanus* (IC_{50}: 47.22 μg/mL), and *T. daenensis* (IC_{50}: 48.68 μg/mL) (Nickavar and Esbati 2012).

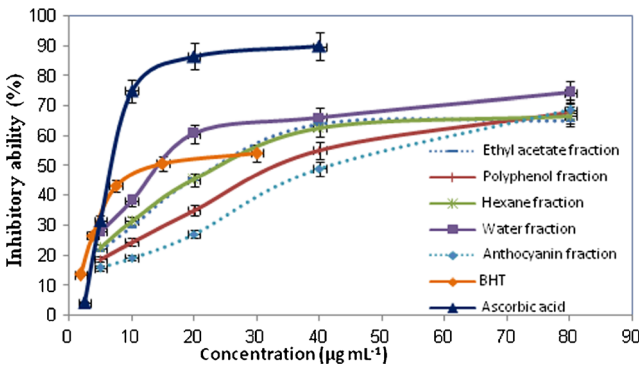

Fig. 1 DPPH radical scavenging activity of flavonoid- (water, ethyl acetate and hexane fractions), polyphenol- and anthocyanin- rich extracts of *T. kotschyanus*

Relation between measured antioxidant capacity and phenolic/flavonoid contents

Phenolic compounds are a class of antioxidant agents due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers

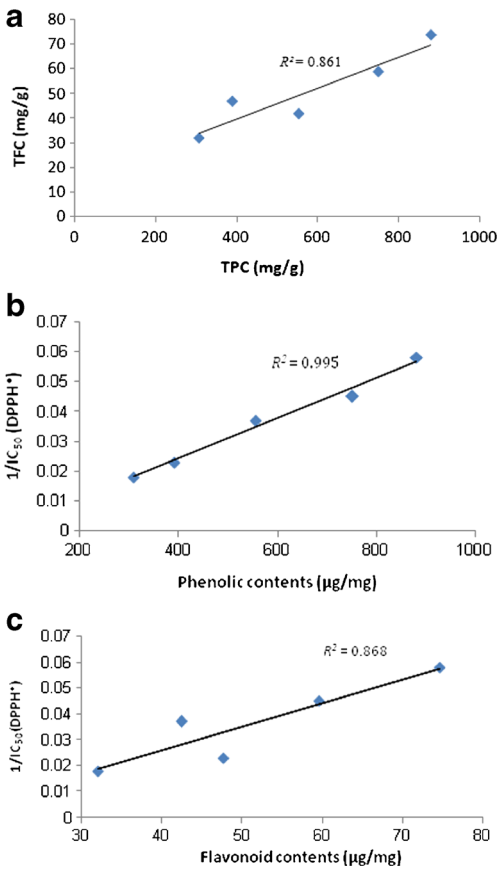

Fig. 2 **a** Relationship between the TFC and TPC of *T. kotschyanus* extracts. **b** Relationship between the antioxidant capacity of *T. kotschyanus* extracts measured using the DPPH assay and their total phenolic content. **c** Relationship between the antioxidant capacity of *T. kotschyanus* extracts measured using the DPPH assay and their total flavonoid content

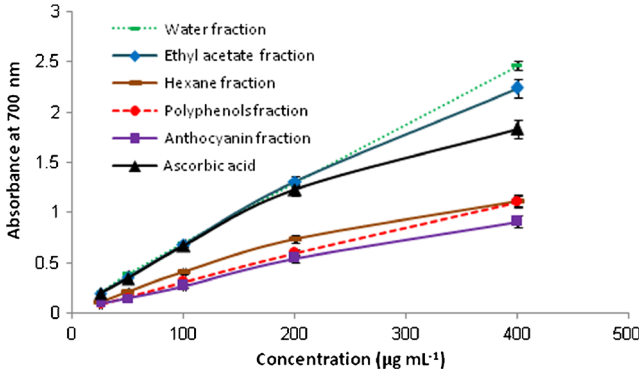

Fig. 3 Reducing power of flavonoid- (water, ethyl acetate and hexane fractions), polyphenol- and anthocyanin- rich extracts of *T. kotschyanus*

(Chang et al. 2001). Flavonoids are a large group of phenolic plant constituents, which show antioxidant activity through scavenging or chelating processes.

In our experiments, it was observed that the TFC of extracts correlated well (R^2=0.86) with the data obtained for the TPC. This indicates that flavonoids might be the major contributors towards the phenolic compounds count for *T. kotschyanus* aerial parts (Fig. 2a). In addition, a significant linear relationship was found between both phenolic and flavonoid contents, and DPPH$^{\bullet}$ scavenging capacity (expressed as the reciprocal of the calculated IC_{50} values) of different extracts (R^2=0.99, p<0.05; R^2=0.86, p<0.05, respectively) (Fig. 2b and c). The positive correlation indicates that the higher phenolic and flavonoid contents resulted in a stronger antioxidant activity. Moreover, these correlation coefficients suggest that the phenolic and flavonoid compounds of *T. kotschyanus* extracts contributed by 99 and 86 %, respectively, to their antioxidant capacities. This observation has also been reported by other researchers (Bhatt and Negi 2012; Yusri et al. 2012; Turumtay et al. 2014).

Reducing power

Reducing power is another mechanism for determination of electron donating ability of extracts. The presence of electron donor in the extract would result in the reducing of Fe^{3+} to Fe^{2+}. The amount of Fe^{2+} complex can be then monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 3 shows the dose–response curves for the reducing power of studied extracts. It is found that, the reducing power of sample increases with the increase of its concentration. Moreover, in a concentration of 400 µg mL^{-1} of tested extracts the descending order of reducing power was as follows: water fraction (A_{700}=2.46±0.04)>ethyl acetate fraction (A_{700}=2.23±0.06)>hexane fraction (A_{700}=1.11±0.03)≈polyphenols fraction (A_{700}=1.10±0.02)>anthocyanin fraction (A_{700}=0.91±0.01) with significant difference between them (P=0.000). The water and ethyl acetate fractions exhibited higher reducing power than ascorbic acid (A_{700}=1.83±0.03) (p<0.05).

Antibacterial activity

The antibacterial activity of *T. kotschyanus* extracts against Gram-positive (*S. aureus* and *B. subtilis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria were recorded by the disc diffusion method (see Table 2). These bacteria are commonly known to cause food poisoning or food spoilage. In addition, the finding towards inhibition of bacterial strains was compared with that of positive controls, gentamicin and chloramphenicol. As shown in Table 2, the extracts exhibited moderate to good growth inhibitory effect against the tested microorganisms. Maximum activity was observed against *P. aeruginosa*, whereas *B. subtilis* was the most resistant to

Table 2 Antibacterial activity of *T. kotschyanus* extracts using Kirby–Bauer disk diffusion method

Sample	Zone of growth inhibition (mm)			
	E. coli	*P.aeruginosa*	*S. aureus*	*B. subtilis*
Flavonoid-rich (Water fraction)	10.5±0.7	15.0±1.1	11.0±1.3	11.5±1.4
Flavonoid-rich (Ethyl acetate fraction)	11.5±0.7	13.5±0.7	14.0±1.4	16.5±0.7
Flavonoid-rich (Hexane fraction)	8.5±0.7	11.0±1.4	12.5±0.7	10.5±0.7
Polyphenols fraction	10.5±0.6	14.0±1.4	10.5±0.7	12.0±1.4
Anthocyanin fraction	13.0±1.4	12.5±0.5	11.5±0.7	10.5±0.4
Gentamicin (10 µg/disc)	19.6±1.1	15.6±0.5	20.3±1.5	26.0±1.7
Chloramphenicol (30 µg/disc)	20.7±1.5	NE [a]	21.7±0.6	22.3±1.2

The data are expressed as mean±S.D. (n=3). ANOVA test for data in the same column indicates significant difference (p=0.000)

Concentration of extract: 20 mg/ mL

[a] No Effect

these extracts. Interestingly, chloramphenicol as a standard antibacterial agent remained inactive against *P. aeruginosa.*

The antibacterial activity of the plant extracts could be attributed to the presence of bioactive compounds such as tannins, terpenoids, polyphenols, and flavonoids (Fernandez et al. 1996; Ouattara et al. 2011). 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 this genus. The various polyphenolic constituents, especially flavonoids and phenolic acids, have been reported in *Thymus* plants. Among the flavonoids, the most widespread skeletons are flavones (luteolin, apigenin, and scutellarin) and then flavanones (eriodictyol and naringin). Besides, different phenolic acids like caffeic acid and rosmarinic acid have been more frequently found in the *Thymus* species (Marin et al. 2003; Jordan et al. 2009).

Conclusions

In this study, flavonoid-, polyphenol- and anthocyanin-rich extracts of *T. kotschyanus* aerial parts were evaluated for their antioxidant capacity, phenolic and flavonoid contents, and antibacterial activity. The results indicated that all of the samples possess high potent free radical scavenging and antioxidant activity. They were also rich in phenolic and flavonoid compounds. Interestingly, the antioxidant activity of various extracts positively correlated with their phenolic and flavonoid contents. Water extract which had the highest phenolic and flavonoid content, showed an appreciable DPPH radical scavenging activity as well as reducing power as compared with other extracts. On the basis of the obtained results from the antibacterial test, the extracts contained moderate to good growth inhibitory effect against both Gram-negative and Gram-positive bacteria. Therefore, the tested *Thymus* extracts might be valuable antibacterial and antioxidant natural sources and seem to be applicable in both medicine and food industry.

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Conflict of interest The authors declare that they have no conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence the present work.

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