Comparison of the antioxidant capacity of methanolic and ethanolic extracts of the two species of Lamiaceae *(Thymus migricus* L. and *Origanum vulgar)*

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Abstract

Lamiaceae family is one of the largest herbal families, which has about 200 genera and more than four thousand species. This family contains important medicinal herbs. *Thymus migricus* and *Origanum vulgar* are two important species of Lamiaceae family that have antioxidant and antiradical properties. In this study, shoots of mature *Thymus migricus* and *Origanum vulgar* plants were collected from the mountainous area of Dalanper Mountain in West Azerbaijan province of Iran, and their extracts with 100 ml of different solvents namely; methanol, ethanol, methanol (50%), and ethanol (50%) were assayed for flovonoid contents and antioxidant activities. The maximum levels of flavonoids, reduction power, and inhibitory power of lipid peroxidation, free radical scavenging capacity, and total antioxidant activities were recorded with methanolic extract of *Origanum vulgar*. This extract was found to have a high potential to mitigate the damaging effects of oxidants on plant tissues.

Keywords: antioxidants, free radicals, Origanum vulgar, phenolic compounds, Thymus migricus

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Introduction

Free radicals play an important role in the emergence and continuation of life. For example, oxygenated radicals play a key role in signal transduction, gene expression, and regulation of guanylate cyclase activity in cells. On the other hand, free radicals and other related species oxidize biomolecules such as proteins, amino acids, lipids, and nucleic acids, which can cause cell damage and death. Reactive oxygen species strongly affect the physicochemical and immunological properties of superoxide dismutase and increase oxidative damage in cells (Bektas et al., 2005). Therefore, the need for strong antioxidants with less toxicity and greater efficacy is an inevitable necessity. Antioxidants are compounds that help to inhibit many of the oxidation reactions induced by free radicals, thereby inhibiting or delaying damage to cells and tissues and reducing spontaneous oxidation waste in biological systems. The most common antioxidants are phenolic compounds that are abundant in nature. Natural antioxidants, mainly found in medicinal plants, fruits, and vegetables, can protect cells from oxidative damage (Kumaran, 2006). Reports suggest an inverse

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association between antioxidant-rich diets and the incidence of human disease (Nychas, 1995). So they seem to be important in preventing many diseases. Among the benefits of natural antioxidants and their use in place of synthetic antioxidants are their beneficial effects on human health. Besides, synthetic antioxidants may lead to negative effects on the biological system with no normal or minimal complications.

Lamiaceae family is one of the largest herbaceous families. This family contains important medicinal plants, and *Thymus migricus* L. and *Origanum vulgar* are two important species of this family with antioxidant properties. These two species of dicotyledonous plants are distributed throughout the world. The plants of this family include about 200 genera and more than 4000 species, of which 14 species of Thymus are distributed in Iran (Mozaffarian, 1998). Their origin is in the Mediterranean region (Gahraman, 1994) but they are spread in many countries of the world. In Iran they are mostly seen in the mountainous areas of the northwest. Essential oils of the aerial parts of Azerbaijan thyme and Origanum vulgar contain important compounds such as thymol, carvacrol, borneol, and methyl carvacrol. These plants also contain tannins, flavonoids, saponins, and bitter substances (Askari et al., 1982).

These two plants are of nutritional and medicinal significance with applications in food, health, and cosmetics industries. Thyme speeds up digestion in addition to improving the taste of food and their consumption has been common since ancient times (Mozaffariyan et al., 1996). Usually their twigs and dried leaves are used for medicinal purposes. Their flowering branches are used through brewing and decoction as an anti-bloating, food digestive, antispasmodic, anti-cough, and sputum treatment (Zargari., 1994) and there are reports on their antibacterial and antifungal properties of these two plants. This study compares the antioxidant capacity of ethanolic and methanolic extracts of the two species of mint, Azeri thyme and mint, in an attempt to introduce and identify useful plant species with antioxidant properties.

Materials and Methods

Complete and mature plants of Azeri thyme and mint species were collected from the mountainous area of Dalanper Mountain in West Azerbaijan province of Iran and were identified in herbarium of Urmia Agricultural Research Center. Samples were freshly stored in a freezer at 80 ℃ until extraction for biochemical studies. To extract, their aerial parts were first washed with distilled water and dried at room temperature, then powdered with an industrial mill. For extraction, 15 g of the dried samples were mixed individually with 100 ml of methanol, ethanol, methanol (50%), and ethanol (50%) as solvent. The samples were extracted at room temperature and in the dark for 3 hours on a magnetic shaker. The resulting solutions were filtered through Whatman No. 1 filter paper and kept in a refrigerator at 4 ℃ for 30 min after centrifugation (Wijeratneet et al., 2006). The total phenolic content of the extracts was determined using Folin-Ciocalteu reagent and modified according to Horwits (1984) method. A spectrophotometric device (Biowave, WPA S2100, UK) with a wavelength of 750 nm was used to measure the absorption of the solution.

The flavonoid content of the extracts was determined according to the method of Bonvehi et al. (2001) with little modification. Aluminum chloride reagent was also used which was expressed using the standard catechin curve. The amount of DPPH stable radical scavenging (2,2-diphenyl-1-picrylhydrazyl) was determined according to the Burits and Bucar (2000) and the percentage of the scavenging activity of the extracts was calculated according to the following formula:

$$\text{Inhibition \%} = (A_{C\,(0)} - A_{A\,(t)}) / (A_{A\,(t)}) \times 100\%]$$

where $A_{C\,(0)}$ is the control uptake at t = 0 min and $A_{A\,(t)}$ is the uptake of the antioxidant extract at t = 1hr.

The ability of the extract to collect hydrogen peroxide (H_2O_2) was measured by the method of Ruch et al. (1989) and nitric oxide radical scavenging was calculated using the Illosvoy Griess

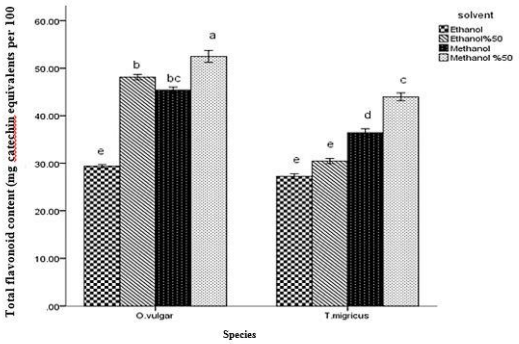

Fig. I. Total flavonoid contents of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study

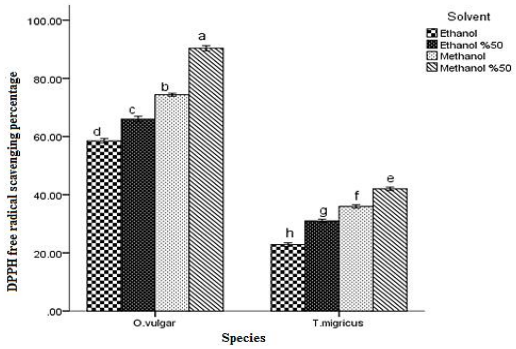

Fig. II. DPPH scavenging percentage of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study (three repetitions ± SE, p≤0.05)

reaction (Garrat, 1964). Also thiobarbituric method was used to calculate the lipid peroxidation rate.

Statistical analysis was performed for all experiments with three replications. The data was shown as means ± standard deviation (SD). One-way ANOVA was performed to compare the values at each point with the control values. $p<0.05$ was considered as the significance level.

Results

Total flavonoid content

Total flavonoid content of ethanolic, methanolic, ethanol (50%), and methanol (50%) extracts of two species of mint and thyme were studied. The results showed a significant difference in total flavonoid contents of ethanol, methanol, ethanol (50%), and methanol (50%) extracts of the plants under study. The maximum flavonoid contents (466.154 ± 52.2 mg catechin equivalents per 100 g dry weight) were obtained from the methanolic extract (%50) of *O. vulgar* while the minimum level of flavonoids were recorded in the ethanol (96%) extract of *T. migricus L.* (27.26 ± 0.929 mg catechin equivalents per 100 g dry weight). Intermediate levels of flavonoids were recorded for the other extracts of both species (Fig. I).

DPPH radical scavenging

In evaluating the DPPH radical scavenging capacity, the potentials of ethanol, methanol, ethanol (50%), and methanol (50%) extracts of the two species of *O. vulgar* and *T. migricus* in hydrogen or electron donation to DPPH and therefore, transforming DPPH to neutral DPPH-H compounds were investigated. All extracts under study showed a change in color from purple (radical) to yellow (neutral). This anti-radical activity was highest in methanol extract (50%) of *O. vulgar* (90.33 ± 1.52%) and the lowest in ethanol extract (96%) of T. migricus (22.83 ± 1.06%). Extracts of other species showed intermediate levels (Fig. II).

Scavenging hydrogen peroxide radicals (%)

The percentages of H2O2 radical accumulation in ethanol, methanol, ethanol (50%), and methanol (50%) extracts of shoots of the two species are shown in Fig. III. As noticed, there was a significant difference between shoot extracts of the two species in percentage of inhibition. The best solvent for this assay was methanol. The highest inhibition percentage was recorded in *O. vulgar* methanol extract (64.1 ± 1.31%), and the lowest percentage was observed in ethanol (50%) extract of *T. migricus* (9.84 ± 0.255). The other extracts of both species had intermediate levels of hydrogen peroxide (Fig. III).

Scavenging nitric oxide radicals

Nitric oxide was obtained from sodium nitroprusside, which reacts with oxygen to form nitrite. Nitrite radicals were obtained through the Griesslllosovy reaction (Garrat., 1964). The inhibitory effects of ethanol, methanol, ethanol

(50%), and methanol (50%) extracts of *O. vulgar* and *T. migricus* under study are shown in Fig. IV. The best solvent for this experiment was methanol. The highest percentage of scavenging was observed in the methanolic extract of *O. volgar* (%76.22 ± 2.12) while the lowest percentage was recorded in ethanol (%50) extract of *T. migricus* (%34.73 ± 0.998) with the other shoot extracts falling within this range (Fig. IV).

Inhibitors of lipid peroxidation

Figure (V) shows the inhibition percentage of MAD and inhibition of lipid peroxidation in ethanolic, methanolic, ethanol (50%), and methanol (50%) extracts of shoots of two species of the mint family. Lipid peroxidation potential was calculated by calculating lipid peroxidation activity based on the amount of MAD collected. The higher the MAD, the lower the antioxidant activity, and the lower the MAD in the environment, the higher the inhibition percentage. There was a statistically significant difference between the two species in terms of MAD. The best solvent for this parameter was methanol. The methanolic extract of *O. vulgar* had the highest inhibition of lipid peroxidation (%48.72 ± 0.291) while the lowest lipid peroxidation inhibition (12.52 ± 0.489) was observed in the ethanol (50%) extract of *T. migricus* species. Other extracts of the two species showed fell within this range (Fig. V).

Determination of reduction capacity

Figure (VI) presents reduction power of the four solvent extracts of *O. vulgar* and *T. migricus* species. A significant difference was observed between the two species in terms of reduction power. Specifically, the reduction power of the methanol extract of *O. vulgar* (0.955 ± 0.011) was the highest, whereas in ethanol (%50) extract of *T. migricus* the lowest value (0.556 ± 0.006) was recorded and the other extract fell somewhere along the line between these values (Fig. VI).

Total antioxidant activity

For this experiment, we used the method of Psotova et al. (2001) based on the measurement of phosphomolybdenum and reduction of molybdenum VI to molybdenum V, which is a

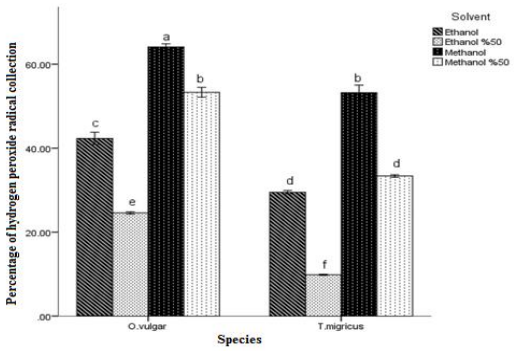

Fig. III. Hydrogen peroxide scavenging percentage of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study (three repetitions ± SE, p≤0.05)

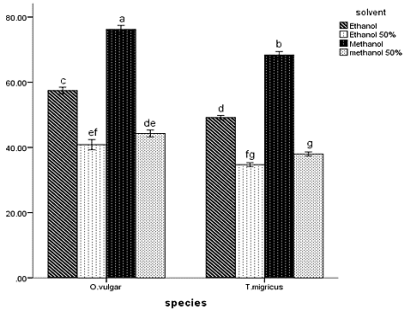

Fig. IV. Nitric oxide scavenging percentage of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study (three repetitions ± SE, p≤0.05)

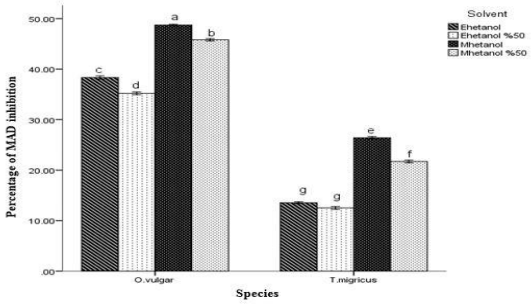

Fig. V. MAD inhibition percentage of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study (three repetitions ± SE, p≤0.05)

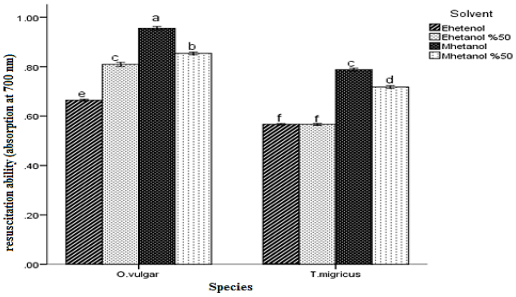

Fig. VI. Reduction power (absorption in 100 nm) of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study (three repetitions ± SE, p≤0.05)

quantitative method for calculating antioxidant capacity. Herbal extracts may act as radical chain fragments because they show the ability to donate electrons. Therefore, they transform free radicals into more sustainable products. The antioxidant activity of ethanolic, methanolic, 50% ethanol and 50% methanol extract of aerial parts of *O. vulgar* and *T. migricus* species is shown in Fig. (VII). As can be seen from the graph, the highest antioxidant activity was recorded in the ethanolic extract of *O. vulgar* (0.221 ± 0.0005 mg alpha-tocopherol per g dry weight) and the lowest activity was recorded with the 50% methanol extract of *T. migricus* (0.110. ± 0.0015 mg α-tocopherol per g dry weight) (Fig. VII).

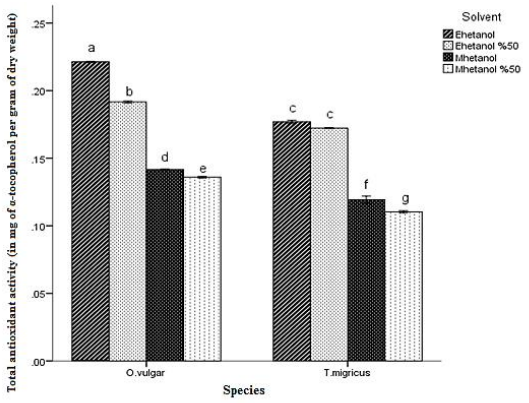

Fig. VII. Antioxidant activity (mg α-tocopherol per g dry weight) of the of *O. vulgar* and *T. migricus* plant extracts with four extraction solvents under study (three repetitions ± SE, p≤0.05)

IC_{50} test

IC_{50} test is commonly used for a sound comparison of the antioxidant activity of different extracts. By definition, IC_{50} is a concentration of the extract in which 50% of the free DPPH radicals present in the reaction medium are inhibited. The lower this concentration indicates that the desired extract has higher antiradical activity (Ismail, 2002).

The IC_{50} values of the four solvent extracts were evaluated for DPPH and the results showed that *T. migricus* had the highest level of IC_{50}. Also, among the extracts under investigation, ethanol extract of *T. migricus* with the lowest phenolic content, had the highest IC_{50} level (0.839 g/ml) (r^2 = 0.9978). Finally, ethanol (%50) extract of *O. vulgar* with the highest phenolic content showed the lowest IC_{50} level (0.1303 g/ml), (r^2 = 944) (Table 1).

Discussion

Studies show that the antioxidant properties of flavonoids are due to the presence of phenolic hydroxyl groups in their structure. Trapping and removing free radicals are among the important roles of the antioxidant activity of these compounds.

Antioxidant activities in all plants are directly related to the amount of phenolic and flavonoid compounds. In fact, plants with higher phenolic and flavonoid compounds have been reported to show higher antioxidant activity. For example, peppermint extract contains high phenolic and flavonoid compounds and consequently, high antioxidant activity (Jamshidi et al., 2010). Aquil et al. (2006) studied antioxidant activity and free radical scavenging in 12 plants used in traditional Indian medicine. They found the highest flavonoid content in mango (Mangiferaindica L.). Many genes encode flavonoid biosynthesis and produce abnormal amounts of flavonoid under abiotic stresses such as drought and food deprivation

Table 1
IC_{50} values for *O. vulgar* and *T. migricus*

Species	IC50 (g/ml) g/ml /06-/3 DPPH			
	ethanol, 96%	methanol, 96%	ethanol, 50%	methanol, 50%
O.vulgar	0.416 (g/ml)	0.1392(g/ml)	0.1120(g/ml)	0.1303(g/ml)
	R^2=0. 9977	R^2=0. 9854	R^2=0. 9972	R^2=0. 9445
T.migricus	0.839(g/ml)	0.276(g/ml)	0.193(g/ml)	0.338(g/ml)
	R^2=0. 9978	R^2=0. 9796	R^2=0. 9976	R^2=0. 9924

(Winkel-Shirley, 2002). In the present study, *O. vulgar* had more flavonoid contents than *T. migricus*.

In this study, a direct relationship was found between phenolic contents of ethanolic, methanolic, ethanol (50%), and methanol (50%) extracts on the one hand and free radical scavenging of DPPH on the other. The higher the phenol content, the greater the potential for DPPH radical scavenging. Yildirimet al. (2001) reported that ascorbic acid, cysteine, tocopherol, glutathione, tannins, flavonoids, and aromatic amines are capable of reduction and discoloration of the DPPH solution. The study by Mirzaii et al. (2011) on the antioxidant activity of stems and flowers of 5 medicinal plants also confirmed a direct relationship between phenol content and free radical scavenging of DPPH. LeBlanc et al. (2009) obtained similar results to our results and reported that methanolic extracts of Artemisia seeds had a stronger scavenging potential than ethanol, acetone, and water extracts. Nickavar et al. (2008) in a review of antioxidant activities of 5 species of mint family in Iran also reported a direct relationship between antioxidant activity and DPPH inhibition. In our study, the relationship between phenol and hydrogen peroxide radicals was positive and direct, suggesting that the higher the phenol content in a plant extract, the higher the specificity of the extract for the percentage of hydrogen peroxide accumulation.

Although NO is a gaseous signaling molecule, it is an exceptional radical gas. Mineral nitrate also behaves in the same way as nitric oxide. Nitric oxide (NO *) and nitrogen dioxide (NO2 *) molecules are free radicals because of unpaired electrons. This free radical is a chemical intermediary produced by endothelial cells, macrophages, neutrons, and is involved in regulating many physiological processes. Increased concentrations of NO lead to several diseases. Oxygen reacts with available nitric oxide to produce nitrite anion and peroxynitrite which act as free radicals (Sainani et al., 1997). The extracts studied in this experiment inhibit directly the formation of nitrite in competition with oxygen to react with nitric oxide and also to inhibit its synthesis. Scavenging competition of nitric oxide with oxygen leads to the production of reduction products of nitric oxide. Active plant compounds have the potential to counteract the effects caused by the presence of these radicals. Therefore, the relationship between phenolic contents and nitric oxide radical scavenging capacity is direct, i.e. with an increase in phenolic contents the inhibitory capacity also increases. This relationship indicates that the ethanolic, methanolic, and 50% ethanolic plus 50% methanolic extracts of shoots of the two species of mint and thyme in this study contained phenolic compounds capable of radical scavenging. In their study on *Prunus domestica*, (Morrabi and Jamei (2014) have also reported a very high correlation between phenolic contents and nitric oxide free radical scavenging.

The correlation coefficient indicated a significant direct relationship between phenolic contents and MDA inhibition. This suggests that the varieties with higher phenolic contents can effectively prevent the peroxidation of fats in the living environment. Lipid peroxidation is one of the important effects of the accumulation of ROS, which eventually leads to the degradation of biological systems. One method of measuring antioxidant activity is to calculate the rate of inhibition of lipid peroxidation in which the main substance used is TBA (Huda-Faujanet et al., 2009).

Abalaka et al. (2011) studied the antioxidant potential of the ethanol and hexane extracts of *Ziziphusmauritiana* and *Z. spinachriti* leaves, comparing these extracts with the standard ascorbic solvent. Their findings showed that ethanolic extract had more antioxidant activity than hexane-based extracts. Therefore, the type of solvent can affect the rate of extraction of active compounds such as phenols, and thus the antioxidant effects. The lower activity of the hexane extract in their study may reflect the fact that the active plant compounds in nature are polar and are not fully extracted with hexane.

Phenolic compounds are good electron donors and exhibit reduction properties. Reduction power increases with increasing phenolic content (Khanizadehet al., 2007). Evaluation of regeneration potential in extracts and essential oils is one of the mechanisms for determining the

scope of antioxidants. Generally, the rate of reduction in extracts depends on the presence of the restorers. These compounds are highly regenerative decomposition products produced by heating hexoses with alkali and are biochemically reducible.

Sweetie et al. (2007) in their study in India on the antioxidant effects of ethanolic extract of Menthaspicata showed that its 50% inhibitory concentration in free radical scavenging test was 28.5 µg / ml while the case for hydroxy toluene was 10.1 µg / ml. The effect of radical inhibition of Iranian peppermint extract is stronger than that in India. In the study of Golluce et al. (2007) in Turkey on antioxidant properties of essential oil and methanol extract of Menthalongifolia by DPPH method, the inhibitory concentration of 50% methanol extract was 74.4 µg / ml. Compared with the radical effect of essential oil of Iranian peppermint, the effect of the essential oil of the studied mint was much greater in Turkey.

32The relationship between the phenolic content of the extracts in the study and the total antioxidant activity was direct and high, which may indicate that the phenolic compounds have high antioxidant properties and perform well in reduction. It might be argued that increasing the phenol contents of the whole plant extracts make them capable of reduction by giving electrons to molybdenum VI and convert it to molybdenum V. The concentration of herbal extract in this experiment was effective on the antioxidant activity, and the higher this concentration, the higher the amount of reduction (Dorman et al., 2009).

Dalmeida-Daffodil et al. (2012) studied IC_{50} values for DPPH of various solvent of Suaedamonaica extracts including benzene, ethyl acetate, ascorbic acid, methanol, ethanol, etc. and found that among the solvent extracts, the standard ascorbic acid had the highest IC 50 for DPPH (19.24µg / ml).

On the other hand, Kenny et al. (2014) reported that plants extracted with aqueous extract showed the lowest phenolic content and the highest IC_{50}. In another study conducted by Mau and Huang (2006) on *A. absintium* L. leaves using water, methanol, and ethyl acetate solvents, the highest IC50 values were found in ethyl acetate, aqueous, and methanol extracts as 41.45 ml/ml, 7.37 µg /ml, and 6.77 µg/ml, respectively, where the extracts with the highest phenol contents showing the lowest IC_{50} values. In this study, 50% methanol extract of *O. vulgar* with the highest phenolic content (0.1303 g/ml) showed the lowest IC50 ($r^2 = 0.944$).

Conclusion

The most important plant compounds with high antioxidant properties as documented in research are phenolic compounds such as flavonoids. These effects include the ability to scavenge nitric oxide radicals, hydrogen peroxide, and superoxide in various ways. It is concluded in the study that peppermint plants are one of the rich sources of phenolic compounds with important applications in medicine and are good sources of antioxidants. The study compared various extraction solvents with *Thymus migricus* and *Origanum vulgar* plants to shed light on the most favorable solvents. In the present study, methanol (%50) extract of *O. vulgar* recorded the highest total flavonoid content, and free radical scavenging of DPPH. In fact, shoots of *O. vulgar* generally contained significantly higher phenolic compounds and free radical scavenging capacity as compared to *T. migricus* and Therefore, *Origanum vulgar* can be recommended as a rich source of phenolic compounds and a natural antioxidant for food and pharmaceutical purposes.

Acknowledgment

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