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Comparison of Total Phenolic Contents and Antioxidant Activities of Different Parts of the Endemic Plant *Geranium ibericum* subsp. *jubatum*

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ABSTRACT: This paper includes the results of the first study on the phenolic content and antioxidant capacity of endemic species *Geranium ibericum* subsp. *jubatum* found in Turkey. In this study, the methanol extracts of different parts (leaf, stem, flower, and root) of the *Geranium ibericum* subsp. *jubatum* exhibited different free radical scavenging activity, total phenolic content, and antioxidant activity. The synthetic antioxidant BHT and the natural antioxidant α-tocopherol were evaluated as a positive control and compared with methanol extracts of the plant parts. There was a positive correlation between the total phenolic content and the free radical scavenging activity in different parts of *Geranium ibericum.* It has been found that the highest phenolic content and antioxidant activity were found in the roots. The total phenolic content and antioxidant activity (ABTS, DPPH) showed statistically significant differences among the different parts of the *G. ibericum* subsp. *jubatum* ($p<0.05$).

Keyword: *Geranium ibericum* subsp. *jubatum,* ABTS, DPPH, antioxidant activity, total phenolic content.

Endemik Tür *Geranium ibericum* subsp. *jubatum'*un Farklı Kısımlarının Toplam Fenolik İçerikleri ve Antioksidan Aktivitelerinin Karşılaştırılması

ÖZET: Bu çalışma, endemik tür *Geranium ibericum* subsp. *jubatum*'un fenolik içeriği ve antioksidan kapasitesi üzerine yapılmış ilk çalışmanın sonuçlarını içermektedir. Çalışmada, Türkiye için endemik tür olan *G.ibericum* subsp. *jubatum*'un farklı kısımlarının (yaprak, gövde, çiçek ve kök) metanol özütlerinin toplam fenolik içerikleri ve toplam antioksidan aktiviteleri belirlenmiş ve karşılaştırma yapılmıştır. *G. ibericum* subsp. *jubatum*'un farklı kısımlarına ait metanol özütlerinin antioksidan kapasiteleri sentetik bir antioksidan olan BHT ve doğal bir antioksidan olan α-tokoferol ile karşılaştırılmış ve sonuçlar değerlendirilmiştir. Toplam fenolik içerik ile toplam antioksidan kapasiteleri arasında pozitif bir ilişki bulunmaktadır. En yüksek fenolik içerik ve antioksidan kapasitesinin kök kısmının metanol özütüne ait olduğu bulunmuştur. Yapılan istatiksel değerlendirilmeler sonucunda *G. ibericum* subsp. *jubatum*'un farklı kısımları arasında toplam fenolik içerik ve toplam antioksidan aktiviteleri (DPPH, ABTS) yönünden önemli farklılıklar görülmüştür ($p<0.05$).

Anahtar Kelimeler: *Geranium ibericum* subsp. *jubatum,* ABTS, DPPH, antioksidan aktivitesi, toplam fenolik içerik.

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INTRODUCTION

The compounds found in the biological systems are divided into two broad sections: primary and secondary. Primary metabolites are chemical compounds such as carbohydrates, amino acids, proteins, and fats which are involved in the growth and development of the cell. Secondary metabolites help plants to overcome environmental challenges of survival and interaction to defeat their rivals and fend off pathogens (Ahmed et al., 2017; Siddiqui et al., 2017). These compounds protect plants, fruits, and vegetables from oxidative damage and have been used as antioxidants by people. Antioxidants protect cells against the effects of harmful free radicals (Mahdi-Pour et al., 2012; Kasangana et al., 2015).

Antioxidants are inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body (Kumar, 2011). There are scientific studies showing that the imbalance between excessive free radical production and antioxidant defenses in the human body may lead to the occurrence of diseases such as cancer, aging, inflammatory disorders, strokes, and diabetes. Many studies have shown that antioxidants may play an important role in preventing diseases caused by free radicals (Dai and Mumper, 2010).

The majority of the antioxidants found in foods are phenolic compounds. It is also known that antioxidants are added to foods in order to prevent off-flavors, off-odors and discolorations. Most antioxidants added to foods such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), and Propyl Gallate (PG) are synthetic. Some studies revealed toxic effects of synthetic antioxidants (Kulisic et al., 2004). For this reason, researchers began to intensify their studies on plant based natural antioxidants (Aksoy et al., 2013).

An interest in functional foods and nutraceuticals has been increased day by day. The interest in functional foods and nutroceuticals requires new and healthy antioxidants produced from natural sources. The first techniques used to detect antioxidant compounds in plants are the screening of phytochemicals (Do et al., 2014).

There are many techniques used to isolate antioxidants from plants, such as soxhlet extraction, maceration, supercritical fluid extraction, subcritical water extraction, and ultrasound assisted extraction. In addition, the extraction efficiency and antioxidant activity depend not only on the method used, but also on the solvent used in the extraction (Azwanida, 2015). Polar solvents such as water, ethanol, methanol, acetone, and ethyl acetate are frequently used to extract polyphenols from plant parts. Methanol has been found to be effective in obtaining low molecular weight polyphenols. Also, the water-acetone mixture was found to be effective in obtaining high molecular weight flavanols. Phenolic compounds can be obtained from dried or fresh plants. The sample is subjected to milling before extraction to make it homogenous. The choice of the drying method is determined to affect the total phenolic content. Freeze-drying keeps the phenolic content in higher level in the plant samples compared with air drying (Mojzer et al., 2016).

The Geraniaceae family is 15-40 cm in length, with a long and soft hairy body. It is known that *Geranium* L. genus is the largest of the Geraniaceae family. Some species of this genus are used as medicinal plants in traditional medicine, and some species are used for tanning and dyeing (Serkedjieva, 1996; Kahriman et al., 2010; Zeljković et al., 2017). The chemical content of the genus *Geranium* spp. was investigated and it was found that the flavonoids and phenolic acids were clearly dominant (Harborne and Williams, 2002).

Geranium ibericum subsp. *jubatum* which endemic to Turkey is cultivated the North-West and North-East from Turkey to the Caucasus and Iran. It is known as turnagagas among local people (Hüseyinoğlu et al., 2017).

Although there are many studies on the chemical content of other geranium species (Leucusta et al., 2005), no studies have been done with regard to the chemical content of the *Geranium ibericum* subsp. *jubatum*. This work represents the first analysis of the phenolic contents and antioxidant activities of this endemic species.

In this study, the total phenolic content and antioxidant capacity of the different parts of the endemic *G. ibericum* subsp. *jubatum* samples obtained by methanol extraction were evaluated.

MATERIALS AND METHODS

Plant Material

Geranium ibericum subsp. jubatum samples were collected from Eğribel Pass, Giresun-Turkey on June 2018 (40° 27′ 59″ 38° 41′ 55″ 2509.53m) and stored at the Department of Biology, Ondokuz Mayıs University, Samsun.

Chemicals

Foline-Ciocalteu reagent (FCR), methanol and ethanol were obtained from Merck (Darmstadt, Germany), sodium carbonate anhydrous, gallic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azinobis-(3-ethylbenzothiaziline-6-sulfonate) and BHT (Butylated Hydroxytoluene) and α-tocopherol were purchased from Sigma-Aldrich GmbH (Sternheim, Germany) $K_2S_2O_8$ (potasium persulfate) was purchased from Carlo Erba.

Preparation of Extracts of Plant Parts

Flower, leaf, stem and root of the plant samples were separated, cleaned and dried in a hot-air oven at 40 °C for 48 h. MeOH was used as the solvent for the soxhlet extraction process. For soxhlet extraction, 10 g samples (leaf, stem, flower and root) of different plant sections were milled in the blender and placed in the soxhlet cartridge and 250 mL MeOH was added to the apparatus and the system was operated. After this process, the solvent mixture was filtered through and then the solvent was removed by evaporator at 40 ^oC, 175 mbar. The extract was stored for further analysis (Chou et al., 2009; Proestos et al., 2013; Arumugam et al., 2019).

Determination of Total Phenolic Compounds Content

The amount of the total phenolic compound found in the methanolic extracts of the different parts of *G. ibericum* subsp. *jubatum* was determined by Folin-Ciocalteu reagent (FCR) method. Gallic acid was used as the standard phenolic component. Gallic acid solutions were prepared at 20-40-60-80-100 μg mL^{-1}. 5 mL of each of these solutions were taken. 0.5 mL of Folin-Ciocalteu reagent and 5 mL of 7% Na_2CO_3 solution were added to the gallic acid solution, respectively. The prepared mixture was stirred for 1 hour at room temperature in a shaker, then the absorbance of the spectrophotometer was measured at 760 nm against a blank sample of MeOH in place of the gallic acid. 0.2 mg mL^{-1} stock solutions were prepared from the methanolic extracts of the different parts of *Geranium ibericum*. As in the preparation of the standard graphic, 5 mL of methanolic extract was taken into the test tubes. 0.5 mL of Folin-Ciocalteu reagent and 5 mL of 7% Na_2CO_3 solution were added, respectively. After mixing of the mixtures in the shaker for 1 hour, the readings of the absorbances against the blank solution at 760 nm were carried out. The results were expressed as mg gallic acid/g dried sample (dry weight: dw) (Kähkönen et al.,1999; Gezen, 2018).

Determination of Antioxidant Activity Using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Method

DPPH free radical scavenging activity was performed according to the method of Zhang et al. (Zhang et al., 2009). BHT and α-tocopherol were used as standard. 1 mM DPPH solution was used for analysis. The methanolic extracts of plant parts solutions prepared at 20-40-60-80-100-120-140 µg mL^{-1} were transferred to the test tubes with a total volume of 4 mL. 1 mL of a DPPH solution was added. The mixture was stirred for 30 minutes at room temperature in a shaker and readings of absorbances against the blank solution containing MeOH instead of extract solution at 517 nm in spectrophotometer were performed. Each assay was carried out in triplicate. The percentage of the inhibition of DPPH radical was calculated using the following **formula 1**:

$$\% \text{ Inhibition} = [(A_{control} - A_{test}) / A_{control}] \times 100 \quad \textbf{(Formula 1.)}$$

$A_{control}$: The absorbance value of the control value without extract sample.

A_{test}: Absorbance value of sample that contains extract.

Antioxidant activities of test compounds or extracts were expressed as IC_{50}, defined as the concentration of the test material required to decrease the initial DPPH concentration by 50% (Erkan et al., 2008; El-Hashash et al., 2010; Akar et al., 2017).

Determination of Antioxidant Activity Using the ABTS Free Radical Scavenging Method

ABTS free radical scavenging activity was performed according to the method of Re et al. (Re et al., 1999). BHT and α-toc were used as standard. 7 mM ABTS stock solution was used for analysis. The methanolic extracts of plant parts solutions prepared at 20-40-60-80-100-120-140 µg mL^{-1} were transferred to the test tubes with a total volume of 4 mL. 1 mL of a ABTS solution was added. The mixture was stirred for 30 minutes at room temperature in a shaker and readings of the absorbances against the blank solution using MeOH instead of sample at 734 nm in spectrophotometer were performed. The percentage of inhibition of $ABTS^{\bullet+}$ was calculated using the following **formula 1**:

$$\% \text{ Inhibition} = [(A_{control} - A_{test}) / A_{control}] \times 100 \quad \textbf{(Formula 1.)}$$

$A_{control}$: The absorbance value of the control without extract sample.

A_{test}: Absorbance value of the sample with extract.

Antioxidant activities of test compounds or extracts were expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial $ABTS^{\bullet+}$ concentration.

Statistical Analysis

The experiments were carried out in triplicate and results are given as the mean ± standard deviation. Statistical analyses were conducted with SPSS 22.0 version (IBM Corp. 2013). The differences among plant parts for total phenolic contents DPPH and ABTS free radical scavenging activities (IC_{50}) capacity were determined by one-way ANOVA test.

RESULTS AND DISCUSSION

Total Phenolic Content

The amount of the total phenolic compound in the plant extracts was calculated as the Gallic acid equivalent (GAE) with the formula obtained from the standard graph (R2: 0.9996). The Gallic acid standard graphic prepared for this purpose is shown in Figure 1.

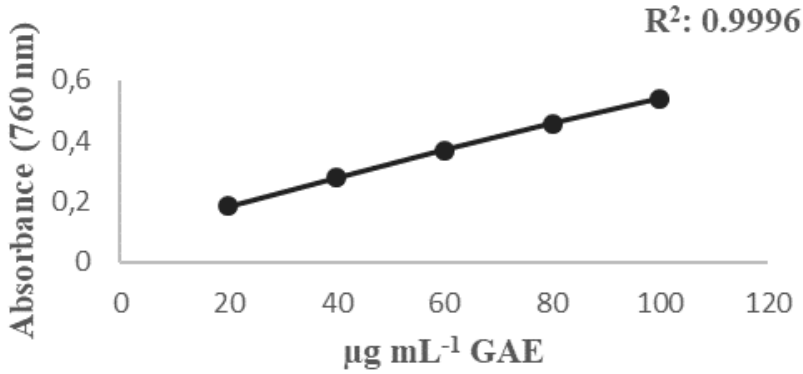

Figure 1: Standard curve of Gallic acid

The Gallic acid equivalents of *G. ibericum* subsp. *jubatum* parts extracts prepared by soxhlet extraction to determine the total phenolic content are shown below in Table 1. The highest phenolic content was observed for the root with 229.09 ±0.40 mg GAE g^{-1}dw.

Table 1. Statistically interpretation of the total phenolic contents by the one-way ANOVA test. Different letters indicate the significant differences among the parts of *G.ibericum* at a 0.05 level using Tukey's HSD test.

Plant parts	Stem	Root	Flower	Leaf
Total Phenolic Contents (mg GAE g^{-1}dw)	98.00 ±4.78a	229.09 ±1.35c	193.84 ±0.20c	226.30 ±0.90b
Significance	0.000	0.000	0.000	0.000

According to the total phenolic results, the total phenolic content of the extracts of the four different parts were determined as follows: root>leaf>flower >stem with 229.09±0.40, 226.38±1.25, 193.96 ±0.80, 100.72 ±1.44 mg GAE g^{-1}dw respectively (Table 1).

Free Radical Scavenging Activity

The results of the DPPH and ABTS radical scavenging activity values were converted to IC_{50} and then evaluated (Figure 2.) (El-Hashash et al., 2010; Akar et al., 2017).

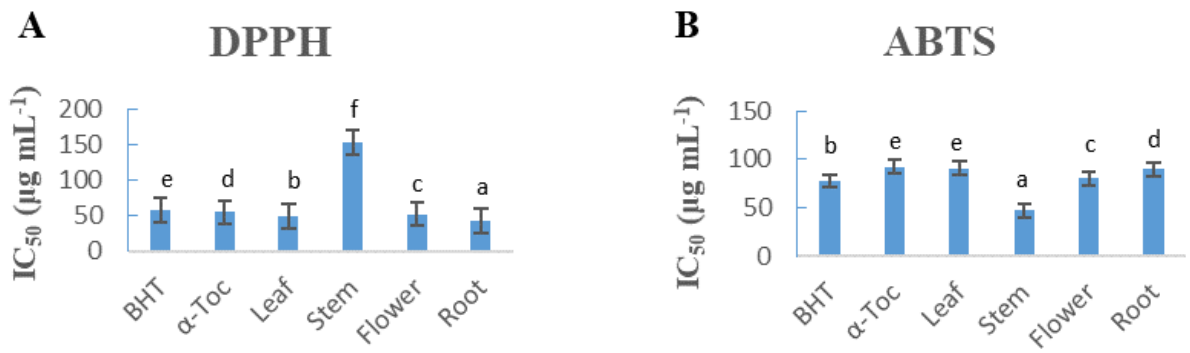

Figure 2. A-) Comparison of DPPH free radical scavenging activities (IC_{50}) of different parts of *G.ibericum* with BHT and α-tocopherol. **B-)** Comparison of ABTS free radical scavenging activities (IC_{50}) of different parts of *G. ibericum* with BHT and α-tocopherol

According to the Figure 2, the total antioxidant activities of the extracts of the four different parts were as follows: root>leaf >flower >stem with IC_{50} 43.33, 49.86, 52.58 and 152.44 µg mL^{-1}, respectively. For the DPPH analysis, when the results were evaluated, the antioxidant activities of the root, leaf, and flower parts were higher than those of the BHT and α-tocopherol (Figure 2 and Table 2).

According to the ABTS analysis, the total antioxidant capacity of the extracts of the four different parts was as follows: stem>flower>root>leaf with IC_{50} 47.24, 80.19, 89.47, and 90.93 µg mL^{-1}, respectively. For the ABTS analysis, the antioxidant activities of the stem and flower parts were higher than the synthetic antioxidants BHT and α-tocopherol (Figure 2 and Table 2).

Table 2. Free radical scavenging activity and statistically significant differences among the different parts of *G. ibericum*, α-Toc and BHT values using one-way ANOVA. Different letters indicate the significant differences among the parts of *G.ibericum* at a 0.05 level using Tukey's HSD test.

	Plant Parts						
	Stem	**Flower**	**Root**	**Leaf**	**BHT**	**α-Toc**	**Significance**
DPPH FRSA IC_{50} (µg mL^{-1})	152.44±0.00f	52.02±1.06c	43.33±0.00a	49.081±0.79b	57.00±0.82e	55.00±0.48d	0.000
ABTS FRSA IC_{50} (µg mL^{-1})	47.18±0.10a	80.44±0.45c	88.84±1.09d	90.58±0.60e	77.55±0.37b	92.10±0.00e	0.000

FRSA: Free Radical Scavenging Activity

The methods of ABTS and DPPH, which are used in this study, are the most popular two methods based on the colorimetric method for the determination of the antioxidant capacity of plant extracts. Different methods were used in different analysis to evaluate the total antioxidant capacity. There are differences in the antioxidant capacity of the *G.ibericum* subsp. *jubatum* extracts between the ABTS and DPPH methods. According to Shalaby and Shanab (2013), it can be said that one of the reasons is the solvent used in the extraction. As the polarity of the solvent changes, so does the content of the extract. The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds (Shalaby and Shanab, 2013). According to this information, it can be concluded that if the extraction solvent is changed, different results will be obtained in terms of antioxidant capacity of *Geranium ibericum* parts.

In light of the results, *G. ibericum* subsp. *jubatum* has been determined to be a very high source of antioxidant (Figure 2). It was collected from 2509.53 meters at Eğribel Pass. Due to the thinning of the atmosphere as the sea level increases, the plant is exposed to more ultraviolet light and produces more antioxidants in the defense mechanism (Martz et al., 2010). There are many studies that support the increase of total phenolic content in proportion to altitude (Wang et al., 2017). In a study published by Taremi et al. in 2015, the phenolic content of extracts of *Marrubium astracanicum* L. collected from different altitude increased as the altitude increased (Taremi et al., 2015).

CONCLUSION

It can be said that the phenolic compounds are higher in the roots than other parts, and this may be related to larger stiffness, low tissue flexibility and consequently lignification and resistance structures which constitute physical obstacles to soil pathogens (Sakihama et al., 2002; Dores et.al, 2014).

The present study is the first work performed on *G. ibericum* subsp. *jubatum* to evaluate the antioxidant capacity of different parts of plant.

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