

## Bioactive component analysis and *in vitro* antioxidant activities of *Plantago Major L.*

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### Keywords

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*P. major*

**Abstract:** *Plantago major L. (P. major)* is a plant used by people for medicinal purposes. This study prepared ethanol extract after species identification of *P. major* plant growing in Varto district of Muş. Phenolic content and amount were determined by high pressure liquid chromatography (HPLC). Four different methods were used for antioxidant activities. As a result of the chemical content analysis of the plant, the highest amount of trans-p-cumaric acid (89.0 ng/µL), abscisic acid (11.4 ng/µL) and salicylic acid (8.20 ng/µL) were detected. When the antioxidant results were evaluated, it was observed that the extract scavenged DPPH and ABTS radicals with a power close to that of standard antioxidants. At the same time, the extract significantly reduced Fe<sup>3+</sup> and Cu<sup>2+</sup> ions. It was found that the activity of the samples generally increased with increasing absorbance. The fact that there are few studies on the antioxidant activity of *P. major* in the literature makes this study even more important. We hope that this study will shed light on future research on this species.

## *Plantago Major L.*'nin biyoaktif bileşen analizi ve *in vitro* antioksidan aktiviteleri

### Anahtar Kelimeler

Antioksidan,  
Serbest Radikal,  
HPLC,  
Fenolik,  
*P. major*

**Öz:** *Plantago major L. (P. major)* halk arasında tıbbi amaçlarla kullanılan bir bitkidir. Bu çalışmada, Muş'un Varto ilçesinde yetişen *P. major* bitkisinin tür tanımlaması yapıldıktan sonra etanol ekstraktı hazırlanmıştır. Fenolik içeriği ve miktarı HPLC ile belirlendi. Antioksidan aktiviteler için dört farklı yöntem kullanıldı. Bitkinin kimyasal içerik analizi sonucunda sırasıyla en yüksek miktarda Trans-p kumarik asit (89.0 ng/µL), Absisik asit (11.4 ng/µL), Salisilik asit (8.20 ng/µL) tespit edildi. Antioksidan sonuçlar değerlendirildiğinde, ekstraktın DPPH ve ABTS radikallerini standart antioksidanlara yakın bir güçle temizlediği gözlemlenmiştir. Aynı zamanda ekstrakt Fe<sup>3+</sup> ve Cu<sup>2+</sup> iyonlarını önemli ölçüde azaltmıştır. Örneklerin aktivitesinin genel olarak absorbans artışıyla birlikte arttığı tespit edilmiştir. Literatürde *P. major*'un antioksidan aktivitesi üzerine sınırlı sayıda çalışma olması bu çalışmayı daha da önemli kılmaktadır. Bu çalışmanın, bu türle ilgili gelecekte yapılacak araştırmalara ışık tutacağını umuyoruz.

## 1. INTRODUCTION

Molecules with unoccupied electrons in their terminal orbitals are defined as free radicals. An excess of these radicals, which are constantly produced in the body, causes oxidative damage. As a result of oxidative damage, various diseases occur in the body, including cancer, cardiovascular diseases, neurodegenerative diseases, Alzheimer's and immune diseases. Therefore, free radicals must be neutralised by antioxidants [1,2].

Enzymes, the glutathione molecule and hormones, which are endogenous antioxidants, are the molecules

responsible for establishing the basic antioxidant balance. However, in addition to these molecules, exogenous molecules may also need to be ingested to scavenge free radicals. Plants, which contain important bioactive components, are the most important members of the exogenous antioxidants [3-5].

*Plantago*, which belongs to the Plantaginaceae family, is a plant used by the public for medicinal purposes. It is known that this plant, whose leaves, roots, seeds and aerial parts are consumed, is used as a wound healer and in the treatment of skin diseases. This genus, which is represented by about 275 species worldwide, has been

reported to have 22 species in Turkey, two of which are endemic [6,7].

*P. major* L., whose benefits as a folk medicine have been recognised worldwide for many years, is known to contain a variety of bioactive constituents, including phenolic acid derivatives, flavonoids, terpenoids fatty acids, and, alkaloids, which contribute to its specific healing sides [8]. Various studies have reported that *P. Major* has antioxidant, antibacterial, anti-inflammatory and wound healing properties [9-11]. In this study, we determined the phenolic content of *P. Major* using high pressure liquid chromatography (HPLC). In this research we determined the phenolics of *P. major* using high pressure liquid chromatography (HPLC). We used various *in vitro* methods to determine its antioxidant properties. We believe that this study will guide future studies and make a significant contribution to the literature.

## 2. MATERIAL AND METHOD

### 2.1. Plant collection and species identification

*P. Major* L. was collected in East Anatolia, Muş, Varto district, and the species was determined by Murat Aydın ŞANDA of the Molecular Biology and Genetics Department of Muş Alparslan University. The chemical processes were carried out in the laboratories of Muş Alparslan University.

### 2.2. Preparation of Extracts

The plants were collected during the growing season (May, 2024) and dried. After species identification, the ethanol extract of the plant was obtained using a Soxhlet apparatus. For the extracts, about 50 g of plant samples were dissolved in 300 mL of ethanol. The extracts were filtered and lyophilised in the laboratories of the Molecular Biology and Genetics Department of Muş Alparslan University.

### 2.3. Phenolic Compound Analysis by HPLC

Agilent Technologies 1260 Infinity II HPLC device (Agilent, USA) was used to determine the phenolic substance content. Ascorbic acid, gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, trans-p-coumaric acid, myricetin, abscisic acid, quercetin, apigenin, kaempferol, curcumin, catechol, vanillin, caffeic acid, cinnamic acid, rosmarinic acid and salicylic acid (Dr. Ehrenstofer GmbH, Germany) standards were used. The final concentrations of the standards were weighed to be 10 mg/mL and placed in 50 mL volumetric flasks. The prepared standards were prepared by adding 1% acetic acid and 1/9 acetonitrile to the prepared standards. The stock solution required to dissolve the standards was prepared by adding 1/1 methanol to the solution. Stock standards were prepared in 5 different dilutions (100 mM, 75 mM, 50 mM, 25 mM and 10 mM) and loaded onto the HPLC device [12]. For HPLC analysis, 1 mL of the previously prepared ethanol extract was taken, 4 mL of stock solution was added and

vortexed. The mixture was passed through filters with a pore diameter of 0.45 µm and approximately 0.5 mL was transferred into vials. Samples were loaded onto the HPLC device to determine the phenolics in the resulting mixture. As mobile phases, 1% acetic acid was used for solvent A and acetonitrile was used for B. The HPLC configuration consists of 1260 DAD WR detector (272, 280 and 310 nm), 1260 Quat Pump VL pump (1 mL/min flow rate), 1260 Vialsampler (20 µl injected) and G7130A column oven (28 °C). The analytical column used for analysis is ACE 5 C18 (250x4.6 mm id). The necessary procedure and information to load the homogenates we prepared before into HPLC were performed according to our previous study [13].

## 2.4. Antioxidant Assays

### 2.4.1. Reducing Power of Fe<sup>3+</sup> Ions

The reducing power of Fe<sup>3+</sup> ions to Fe<sup>2+</sup> ions was determined by Oyaizu method [14]. The volume of the samples (25, 50 and 100 µg/mL) taken in tubes was completed to 200 µL with distilled water. After adding 500 µL each of buffer solution (pH: 6.6) and K<sub>3</sub>Fe(CN)<sub>6</sub> to each tube, it was incubated for twenty minutes (50 °C). Then 500 µL of trichloroacetic acid (TCA) was added to the mixture. After centrifugation, 500 µL of the supernatant was taken and the same amount of distilled water and 100 µL of FeCl<sub>3</sub> were added. Absorbances were measured at 700 nm in a micro-volume spectrophotometer.

### 2.4.2. Cu<sup>2+</sup> Ions Reduction Power By CUPRAC Method

After taking different concentrations of extracts and standards into test tubes, their total volumes were made up to 1 mL with distilled water. 0.25 mL each of CuCl<sub>2</sub>, ethanolic neocuprin and acetate buffer were added. After thirty minutes of incubation, the absorbance values of the samples at 450 nm were recorded [15].

### 2.4.3. DPPH Radical Scavenging Assay

Ethanol was added to the extracts (25 µg/µL, 50 µg/µL and 100 µg/µL) in test tubes to a final volume of 600 µL. After 200 µL of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution was added, the reaction was allowed to incubate for about 30 minutes to complete the reaction. Absorbance values at 517 nm were measured with the help of a spectrophotometer [16].

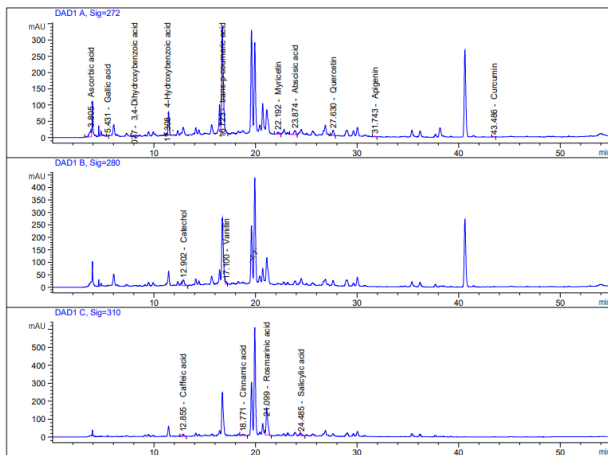
### 2.4.4. ABTS Radical Scavenging Assay

(2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution was diluted with phosphate buffer until the absorbance of the solution reached 0.750 ± 0.025 at 734 nm. After the total volume of the samples was made up to 200 mL with water, 1 mL of ABTS<sup>+</sup> solution was added. After 30 minutes of incubation, the absorbance values of the extracts were measured at 734 nm [17].

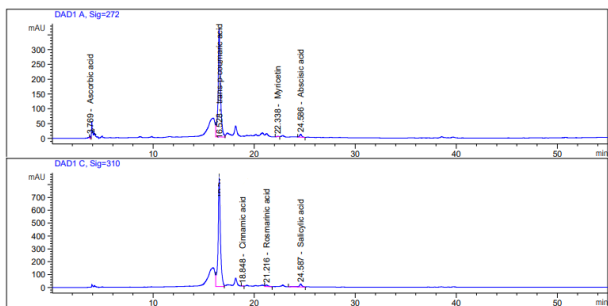
### 3. RESULTS

#### 3.1. Phenolic Substance Analysis

The phenolic compound composition of *P. major* analysed by HPLC device was evaluated with the phenolics introduced as standard. The results showed that the extract contained significant amounts of phenolic compounds (**Table 1**). According to the results, the highest amounts of trans-p-coumaric acid (89.0ng/ $\mu$ L), abscisic acid (11.4 ng/ $\mu$ L), salicylic acid (8.20 ng/ $\mu$ L), rosemarinic acid (3.32 ng/ $\mu$ L), ascorbic acid (2.48 ng/ $\mu$ L), myricetin (2.05ng/ $\mu$ L) and cinnamic acid (0.79 ng/ $\mu$ L) were detected in the extract. Chromatograms of the standards and ethanol extract used in HPLC analysis are shown in **Figure 1** and **Figure 2**.



**Figure 1.** Chromatogram curves of the standards used in HPLC analysis



**Figure 2.** Chromatogram curves of the *P. major* ethanol extract used in HPLC analysis

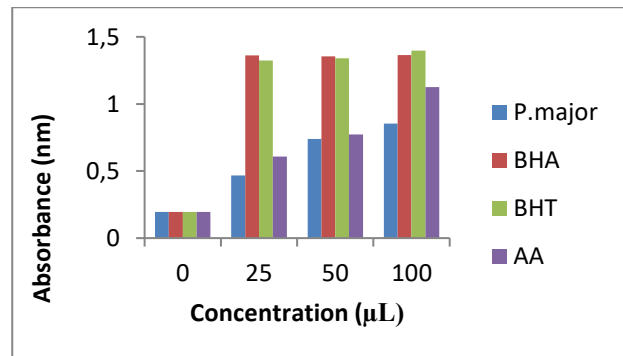
**Table 1.** Phenolic substance amounts (ng/ $\mu$ L) by the HPLC analysis results of *P. major* ethanol extract

Phenolics	Amounts
Ascorbic acid	2,483
Gallic acid	-
3, 4-dihydroxybenzoic acid	-
4- hydroxybenzoic acid	-
Trans-p coumaric acid	<b>89.085</b>
Myricetin	2.058
Abscisic acid	<b>11.416</b>
Quercetin	-
Apigenin	-
Kaemferol	-
Curcumin	-
Catechol	-
Vanillin	-
Caffeic acid	-
Cinnamic acid	0,791
Rosemarinic acid	3.322
Salicylic acid	<b>8.209</b>

#### 3.2. Antioxidant Activities

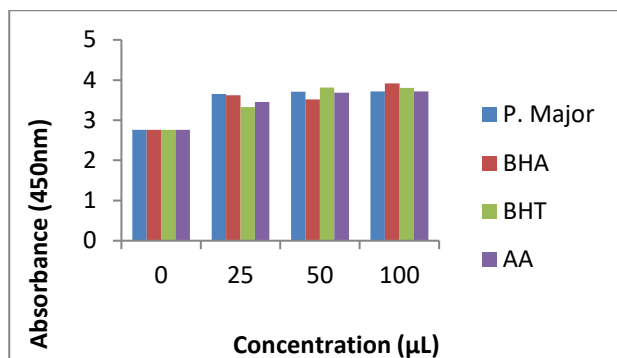
Four different *in vitro* methods were used to determine the antioxidant properties of the extracts. The data obtained as a result of the research showed that *P. major* has strong antioxidant properties. The % activities of extract and standard antioxidants at the highest concentration, FRAP, CUPRAC, DPPH and ABTS are shown in **Table 2**. The FRAP method was preferred to determine the reducing power of ferric ions. According to the experimental results, the extract was found to have lower activity than standard antioxidants (BHA, BHT and AA). However, it was observed that the extract had significant activity and its activities increased as the concentration of the samples increased (**Figure 3**).

Excess iron, one of the most important metals for the organism, can cause several undesirable side effects. Excess  $Fe^{2+}$  ions in the environment can be converted to hydroxyl radical, a very dangerous free radical, by the Fenton reaction [21]. Therefore, FRAP is one of the most preferred methods for researchers. In our research, we found that studies on the reducing power of the genus *Plantago* were quite limited. Beara et al. found that extracts of *P. altissima* and *P. lanceolata* strongly reduced ferric ions [18]. Huan et al. reported that polysaccharides from *Plantago asiatica* L. have significant reducing power, but lower than the antioxidant power of BHT [22]. In another study, *P. albicans* ethanol extract was found to have stronger reducing activity than hexane, dichloromethane and acetone extracts and weaker reducing activity than ascorbic acid (AA) [23]. The results of this study generally support previous studies.



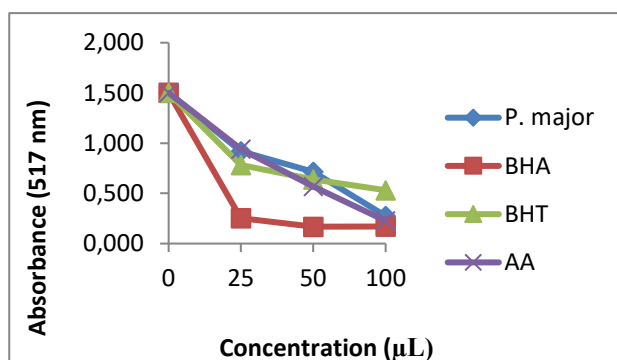
**Figure 3.** Comparison of ferric ion reduction capacities of ethanol extract of *P. major* with standard antioxidants (BHA, BHT and AA)

According to the CUPRAC results of this study, the extract strongly reduced  $Cu^{2+}$  ions and had similar performance with standard antioxidants. The activities of all samples were found to increase with increasing concentration (**Figure 4**). In previous studies, no study was found on the reducing power of cupric ions in this plant genus. Therefore, this study may be the first in this area.



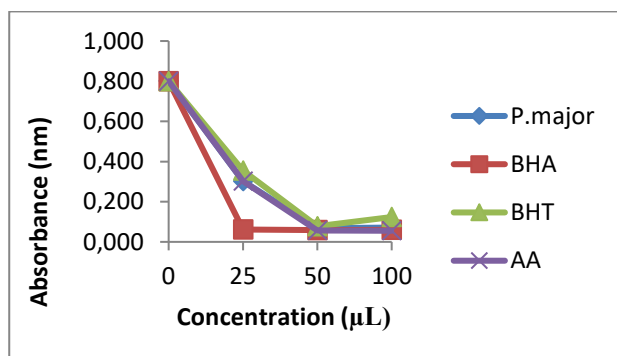
**Figure 4.** Comparison of cupric ion reduction capacities of ethanol extract of *P. major* with standard antioxidants (BHA, BHT and AA)

According to the results of DPPH radical scavenging activity, it was observed that the extract scavenged radicals very strongly. At the highest concentration (100µg/mL), the DPPH radical scavenging percentages of the samples were as follows: BHA (88.57%) > AA (84.89%) > *P. major* (81.72%) > BHT (64.61%). Furthermore, the activities of the samples increased with increasing concentration (**Figure 5**).



**Figure 5.** Comparison of DPPH radical scavenging activity of *P. major* extract with standard antioxidants

The ABTS radical scavenging results showed similar data to the DPPH results. It was observed that the activities generally increased with increasing concentration (**Figure 6**). The ABTS radical scavenging percentages of the samples at the highest concentration were ranked as follows: AA (93.10) > BHA (92.50) > *P. major* (91.05) > BHT (84.52).



**Figure 6.** Comparison of ABTS radical scavenging activity of *P. major* extract with standard antioxidants

**Table 2.** Antioxidant activities of extract and standart antioxidants at 100µL

Samples (100µL)	Ferric ion reduction capacities (%)	Cupric ion reduction capacities (%)	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)
<i>P. major</i>	77.01±0.3	25.61±0.2	81.72±0.1	91.05±0.4
BHA	85.63±0.11	29.36±0.5	88.57±0.3	92.50±0.1
BHT	85.96±0.7	27.43±0.2	64.61±0.3	84.52±0.2
AA	82.60±0.2	25.62±0.8	84.90±0.1	93.10±0.2

#### 4. DISCUSSION AND CONCLUSION

In the literature review, it was observed that the genus *Plantago* contains various bioactive components such as phenolics, flavonoids, terpenes, alkaloids and polysaccharides. According to the research results of Beara et al. it was found that *P. altissima* and *P. lanceolata* extracts were rich in phenolic acids [18]. Significant amounts of p-hydroxybenzoic acid, gallic acid, chlorogenic acid, vanillic acid, luteolin, cinnamic acid and apigenin were detected. Another study reported that *P. major* leaf and seed extracts contained significant amounts of phenolic and flavonoids [19]. Kartini et al. reported that the methanol extract of *P. major* contained significant amounts of chlorogenic acid, caffeic acid, vanillin and p-coumaric acid [20]. Although this study is similar to studies in the literature, there are differences in the content and amounts of phenolic compounds. This could be due to different species, geographical differences, climate change and different solvent usage.

Excess iron, one of the most important metals for the organism, can cause several undesirable side effects. Excess Fe<sup>2+</sup> ions in the environment can be converted to hydroxyl radical, a very dangerous free radical, by the Fenton reaction [21]. Therefore, FRAP is one of the most preferred methods for researchers. In our research, we found that studies on the reducing power of the genus *Plantago* were quite limited. Beara et al. found that extracts of *P. altissima* and *P. lanceolata* strongly reduced ferric ions [18]. Huan et al. reported that polysaccharides from *Plantago asiatica* L. have significant reducing power, but lower than the antioxidant power of BHT [22]. In another study, *P. albicans* ethanol extract was found to have stronger reducing activity than hexane, dichloromethane and acetone extracts and weaker reducing activity than ascorbic acid (AA) [23]. The results of this study generally support previous studies.

Several studies on the DPPH radical scavenging activity of the genus *Plantago* were found in the literature. Kadri reported that *P. albicans* ethanol extract scavenged DPPH radicals more than other extracts and standard antioxidants. The results of the study were as follows: Ethanol (IC<sub>50</sub>: 19.17) > Acetone (IC<sub>50</sub>: 21.33) > Aqueous (IC<sub>50</sub>: 64.33) > Ascorbic acid (IC<sub>50</sub>: 102.00) > Dichloromethane (IC<sub>50</sub>: 169.00) > Hexane (IC<sub>50</sub>: 515.33) [21]. In another study it was reported that *P. ovata* Forssk polysaccharides were very effective in scavenging DPPH radicals [24]. According to the results of the study by Kartini et al., methanol extract of *P. major* scavenged DPPH radicals very strongly [20]. In another study, Bahadori et al. found that methanol extract of *P.*



*lanceolata* scavenged DPPH radicals strongly according to the results of their study [6]. When the results of this study are compared with previous studies, they are found to be mutually supportive.

In the study by Huan et al. the ABTS radical scavenging activity of *P. asiatica* L. polysaccharides was found to increase with increasing concentration [22]. In another study, water and ethanol extracts of *P. albicans* were found to be more effective in scavenging ABTS radicals than hexane, dichloromethane and acetone extracts [23]. There is no study on the ABTS radical scavenging activity of *P. major*. However, our study is in parallel with studies on other plant species.

Plants rich in phenolic compounds, which are commonly used for medicinal purposes, are antioxidant organisms that act as electron donors to scavenge free radicals. In this study, ethanol extract of *P. major* collected in Muş province was prepared after species identification. Phenolic content and amount were determined by HPLC apparatus. Four different *in vitro* methods were used to determine the antioxidant capacity of the extract. As a result of the chemical content analysis of the plant, the highest amount of trans-p-cumaric acid (89.0 ng/μL), abscisic acid (11.4 ng/μL) and salicylic acid (8.20 ng/μL) were detected. When the antioxidant results were evaluated, it was observed that the extract scavenged DPPH and ABTS radicals with a power close to that of standard antioxidants. At the same time, the extract significantly reduced Fe<sup>3+</sup> and Cu<sup>2+</sup> ions. It was found that the activity of the samples generally increased with increasing absorbance. The fact that there are few studies on the antioxidant activity of *P. major* in the literature makes this study even more important. We hope that this study will shed light on future research on this species.

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