KSÜ Tarım ve Doğa Derg 25 (6): 1225-1233, 2022
KSU J. Agric Nat 25 (6): 1225-1233, 2022
https://doi.org/10.18016/ksutarimdoga.vi. 926519

Antioxidant Activity of Lyophilized Water Extract of Aerial Parts of Italian Bugloss (*Anchusa azurea* Mill.)

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ABSTRACT

In many studies, an increase in fruit and vegetable consumption has been shown to reduce the death rate from age-related diseases such as coronary heart disease and cancer. For this reason, given the potential health benefits of natural antioxidants from plants, investigations on them have increased. Italian bugloss (*Anchusa azurea* Mill.), in some regions of Turkey is a plant consumed as a vegetable and used in traditional medicine therefore it is important to evaluate the biological activity. In this study, the antioxidant properties of Italian bugloss (*Anchusa azurea* Mill.) were investigated. The antioxidant capacity of water-based lyophilized extract obtained from air parts was evaluated with ferric ions (Fe^{3+}) reducing, CUPRAC, FRAP, DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ scavenging antioxidant methods. In addition, total phenol and flavonoid contents were determined. This investigation could be a basis for further phytochemical investigations of Italian bugloss (*Anchusa azurea* Mill.).

Biyokimya

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 23.04.2021
Kabul Tarihi : 27.03.2022

Anahtar Kelimeler
Anchusa azurea
Antioxidant activity
Reducing power
Radical scavenging
Total phenolic content

İtalyan Bugloss (*Anchusa azurea* Mill.) Toprak Üstü Kısımlarının Liyofilize Su Ekstresinin Antioksidan Aktivitesi

ÖZET

Birçok çalışmada meyve ve sebze tüketimindeki artışın koroner kalp hastalığı ve kanser gibi yaşa bağlı hastalıklardan ölüm oranını azalttığı gösterilmiştir. Bu nedenle, bitkilerden elde edilen doğal antioksidanların potansiyel sağlık yararları göz önüne alındığında, bunlarla ilgili araştırmalar artmıştır. İtalyan bugloss (*Anchusa azurea* Mill.), Türkiye'nin bazı bölgelerinde sebze olarak tüketilen ve geleneksel tıpta kullanılan bir bitkidir, bu nedenle biyolojik aktivitesini değerlendirmek önem taşımaktadır. Bu çalışmada, İtalyan bugloss'un antioksidan özellikleri araştırılmıştır. Toprak üstü kısımlarından elde edilen liyofilize su ekstresinin antioksidan kapasitesi demir iyonlarını (Fe^{3+}) indirgeyici, CUPRAC, FRAP, DPPH$^{\cdot}$ ve ABTS$^{\cdot+}$ süpürücü antioksidan yöntemleri ile değerlendirildi. Ayrıca toplam fenol ve flavonoid içerikleri de belirlendi. Bu araştırma, İtalyan bugloss'un (*Anchusa azurea* Mill.) daha ileri fitokimyasal incelemeleri için bir temel oluşturabilir.

Biochemistry

Research Article

Article History
Received : 23.04.2021
Accepted : 27.03.2022

Keywords
Anchusa azurea
Antioksidan aktivite
İndirgeme gücü
Radikal giderme
Toplam fenol içeriği

Atıf Şekli: Kızıltaş H 2022. İtalyan Bugloss (*Anchusa azurea* Mill.) Toprak Üstü Kısımlarının Liyofilize Su Ekstresinin Antioksidan Aktivitesi. KSÜ Tarım ve Doğa Derg 25 (6): 1225-1233. https://doi.org/10.18016/ksutarimdoga.vi. 926519

To Cite : Kızıltaş H 2022. Antioxidant Activity of Lyophilized Water Extract of Aerial Parts of Italian Bugloss (*Anchusa azurea* Mill.) KSU J. Agric Nat 25 (6): 1225-1233. https://doi.org/10.18016/ksutarimdoga.vi.926519

INTRODUCTION

The *Anchusa* genus belongs to the family Boraginaceae (Chamberlain, 1978; Ceramella et al., 2019). *Anchusa azurea* (*A. azurea*), also known as 'Hamham' in some regions, is widely distributed in the Mediterranean region, especially in Algeria and Morocco, as well as in Southern Europe and North Africa (Baghiani et al., 2013; Ceramella et al., 2019). *Anchusa* species are used in the treatment of arthritis, stomach diseases, rheumatism, antitussive, open wounds and cuts, as well as a diuretic agent, in traditional medicine (Uz et al., 2010; 2012; Baghiani et al., 2013; Ceramella et al., 2019). Furthermore *A. azurea* is used as a pomade to treat burns (Uz et al.,

2012; Baghiani et al., 2013). Antioxidant, anticancer, antibacterial and antifungal activity has been observed in studies conducted on some *Anchusa* species (Uz et al., 2012; Hu et al., 2020). In some study, it was reported that *A. azurea* have both protective and therapeutic effects on ulcers (Uz et al., 2010; 2012). *Anchusa* genus is represented by 15 species in the flora of Turkey (Chamberlain, 1978). *A. azurea*, popularly known as 'tort', is also known as 'ballık otu' and 'sığırdili' in different regions, In Turkey. The leaves of *A. azurea* are consumed as a vegetable in many regions of Turkey. The leaves are boiled and roasted with onions, especially in spring and summer, and consumed as food, used in pastries. The root bark is grinded together with wheat and used in the treatment of inflamed wounds. It is useful against wild plant poisoning (Murathan & Özdinç, 2018).

Since ancient times, people primarily benefited from plants in order to obtain nutrients and eliminate health problems (Gulcin et al., 2006; Köksal & Gulcin, 2008; Tohma et al., 2016a). Especially Anatolian people used plants for food and medicinal purposes (Murathan & Özdinç, 2018). In recent years, new herbal compounds have been studied extensively for their potential to treat many diseases without any side effects (Hussain et al., 2019). Experimental animal studies have shown that plant extracts could have lower toxicity than synthetic drugs. This is one of the most important advantages of herbal medicines (Uz et al., 2012).

Oxidation is essential for many living organisms to generate energy to fuel biological processes (Gulcin, 2012). But, uncontrolled production of oxygen-induced free radicals plays a role in the formation of many diseases such as cardiovascular disorders, cancer, Alzheimer's disease, Parkinson's disease, nervous disorders, alcohol-related liver disease, ulcerative colitis, atherosclerosis and degenerative processes associated with aging (Wong et al., 2006; Baghiani et al., 2013; Aliyazıcıoğlu et al., 2018). A nutritious diet which is rich in antioxidant compounds for health is important (Tohma et al., 2016b; Altay et al., 2018) because of their roles in preventing many neurodegenerative diseases, cancer, Parkinson's, Alzheimer's diseases and cardiovascular diseases (Baghiani et al., 2013; Bursal et al., 2019). Various synthetic antioxidants are currently widely used. However, there are doubts that these compounds have toxic and carcinogenic effects (Gulcin et al., 2010a). which has accelerated the attempts to discover natural and safer sources of antioxidants that will constitute an alternative to synthetic antioxidants in foods (Gulcin, 2006; 2007; Huo et al., 2011).

In this study, the antioxidant capacities of lyophilized water extract of aerial part of Italian bugloss (*Anchusa azurea* Mill.) (WEAA) were determined by 5 different in vitro antioxidant activity methods as; ferric ions (Fe^{3+}) reducing, cupric ions (Cu^{2+}) reducing capacity (CUPRAC), Fe^{3+}-TPTZ reducing capacity (FRAP), DPPH and ABTS radical scavenging methods. Furthermore, total phenol and flavonoid contents were also investigated.

MATERIALS and METHODS

Chemicals

Compounds which are used for antioxidant activity suchlike neocuproine (2,9-dimethyl-1,10-phenanthroline), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid, 2,2-azino-bis3-ethylbenzthiazoline-6-sulfonic acid (ABTS), BHT (butylated hydroxytoluene), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), α-tocopherol and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate and all other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

Collection and Identification of the Plant Material

Italian bugloss (*Anchusa azurea* Mill. var. azurea) was collected from Beytüşşebap, Şırnak, in July 2019 (Location: 37°36'31.0"N 43°13'14.3"E; Herbarium code: M.P 16520). The plant was identified by botanist Dr. Süleyman Mesut PINAR, Van Yüzüncü Yıl University, Faculty of Health Sciences, Department of Nutrition and Dietetics. Plant samples were deposited at Van Yüzüncü Yıl University, Faculty of Science, Herbarium of the Biology Department (VANF), Van, Turkey.

Preparation of Lyophilized Water Extract

To prepare WEAA, 25 g aerial parts were taken from the shade-dried Italian bugloss *(Anchusa azurea* Mill.) and finely powdered in a grinder, boiling with 400 mL distilled water for 15 minutes in a magnetic stirrer then, it is lyophilized in a (Labconco, Freezone 1L) brand lyophilizer at -50°C at of 5 mm-Hg a pressure setting, the yield of the extract was found as 1.08 % and stored at –20 °C until use. (Gulcin 2005; Gulcin et al., 2008).

Radical Scavenging Methods

DPPH· scavenging activity

For evaluating the DPPH· scavenging potential of the extract, the concentration of WEAA and standard antioxidants were prepared as 10-30 µg mL^{-1} and 1 mL of DPPH· (0.1 mM) was added to each sample. After 30 minutes of incubation, absorbance was recorded at 517 nm (Blois, 1958; Taslimi et al., 2020).

$ABTS^{\cdot+}$ scavenging activity

The method in a previous study was used to

determine the $ABTS^{\cdot+}$ scavenging effects of WEAA (Bingöl & Bursal, 2018). First, 2.45 mM persulfate solution was added to 2 mM ABTS solution to generate ABTS radicals. The absorbance of the $ABTS^{\cdot+}$ radical control solution was adjusted to 0.750 ± 0.025 nm at 734 nm with a phosphate buffer of 0.1 M and pH 7.4. Then 1 mL of $ABTS^{\cdot+}$ solution was added to different WEAA concentrations and after 30 minutes incubation absorbances were recorded at 734 nm (Gulcin et al., 2005; Gulcin et al., 2011; Huyut et al., 2017).

Reducing Ability Assay

Fe^{3+} Reducing Ability

The Fe^{3+} reducing ability of WEAA was carried out by the $Fe^{3+}(CN^{-})_6$–$Fe^{2+}(CN^{-})_6$ reduction procedure. In this method, the reducing capacity of a bioactive compound can be measured by direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. As a result, the Prussian blue complex of Perl, which has a strong absorbance at 700 nm, leads to the formation of $Fe_4[Fe(CN^{-})_6]_3$ (Gulcin, 2015).

$Fe(CN)_6^{3-} + AH \rightarrow Fe(CN)_6^{4-} + A\cdot + H^+$,

$Fe(CN)_6^{4-} + + Fe^{3+} \rightarrow Fe_4[Fe(CN)_6{}^{4-}]_3 + A\cdot + H^+$ (Gulcin, 2020).

Briefly, different concentrations of WEAA (10-30 lg/mL) in 0.75 mL of deionized water were added to 1mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The mixture was incubated at 50 °C for 20 min. After the incubation period, TCA was added (1 mL, 10%). Last, a portion of $FeCl_3$ (0.5 mL, 0.1%) was transferred to this mixture and the absorbance value was spectrophotometrically recorded at 700 nm. A decrease in absorbance indicates increased ferric reducing power (Gulcin et al., 2003; Köksal & Gulcin, 2008; Gulcin et al., 2010b; Bursal & Gulcin, 2011).

CUPRAC Test

To determine the CUPRAC of WEAA, Apak et al. (2006) method was applied with some changes. Briefly, 0.25 mL of 10 mM copper (II) chloride solution, 0.25 mL of 7.5 mM ethanolic neocuproine solution and 0.25 mL of 1.0 M ammonium acetate buffer solutions were added to a test tube and mixed with 0.25 mL of different concentrations (10-30 µg/mL) of WEAA. The total volume was completed with distilled water to 2 mL followed by vigorously shaking. The tubes were stoppered and kept at room temperature. Absorbance was measured at 450 nm against a reagent blank after 30 min of incubation. Increased absorbance indicates increased Cu^{2+}–Cu^{+} reduction (Ak & Gulcin, 2008; Erkan et al., 2008).

FRAP Test

FRAP is based on the reduction of a colorless Fe^{3+}-TPTZ complex to intense blue Fe^{2+}-TPTZ when interacting with a potential antioxidant. This method is useful for comparing the effectiveness of antioxidant capacities of different compounds (Spiegel et al., 2020). TPTZ solution (2.25 ml, 10 mM TPTZ in 40mM HCl) was freshly prepared and transferred to $FeCl_3$ (2.25 ml, 20 mM) and acetate buffer (25 ml, 0.3 M, pH 3,6) solution (Bursal et al., 2019). Different concentrations (10–30 lg/mL) of WEAA were dissolved in 5 mL of appropriate buffer, vortexed and incubated for 30 min. At 37°C. The increased absorbance was measured at 593 nm (Köse et al., 2015; Aksu et al., 2016).

Determination of Total Phenolics and Flavonoids Concentration

The total amount of phenolic found in WEAA was performed as previously described by Köksal et al. (2008). 1 mg of WEAA was diluted with 23 ml of distilled water. 0.5 ml of Folin-Ciocalteau reagent was added to the volumetric flask, followed by 1.5 ml of 2% Na_2CO_3 solution after 3 minutes. The mixture was then allowed to stand for 2 hours with intermittent shaking. The absorbance of the samples was read at 760 nm. Transactions were carried out in triplicate. Distilled water was used instead of sample for control (Köksal et al., 2008). Gallic acid was used as a standard and the amount was determined as gallic acid equivalent (GAE) (Gulcin et al., 2004).

The determination of total amount of flavonoids found in WEAA was made according to the method applied by Park et al. (1997). 1000 µg of extract was diluted with 4.3 ml of 80% aqueous ethanol solution containing 0.1 ml (1 M) CH_3COOK and 0.1 ml (10%) Al $(NO_3)_3$ solutions. After 40 minutes incubation at room temperature, their absorbance at 415 nm was recorded. Quercetin is used as a standard and quantity was determined as microgram quercetin equivalent (QE) from the equation obtained from the standard quercetin plot (Eruygur et al., 2019).

Statistical Analyses

The results of the experiments were determined by averaging the triplicate analyses. For statistical analysis one-way analysis of variance (ANOVA) test was used. Differences between groups were made with Duncan's correction. Statistical significance level was taken as $p<0.05$; $p<0.001$ was very significant and SPSS statistical software version 19.0 (SPSS Inc, Chicago, III, USA) package was used for analysis.

RESULTS and DISCUSSION

According to the methods of DPPH radical scavenging activity, the antioxidants reduce the $DPPH^{\cdot}$, and the specific purple color of the radical solution turns yellow, which can be observed with a decrease in absorbance (Gadow et al., 1997; Gulcin, 2020). $DPPH^{\cdot}$ scavenging activities of WEAA and positive controls

were investigated. Also, IC_{50} values of both extract and standard antioxidants were determined. The IC_{50} (µg/mL) values WEAA and standard antioxidants demonstrated in following order: Ascorbic acid (17.325 ± 0.004, r^2: 0.9509) < α-Tocopherol (30.130 ± 0.043, r^2: 0.9578) < BHT (38.500 ± 0.023, r^2: 0.9858) < WEAA (231.0 ± 0.059, r^2: 0.9926) (Table 1 and Figure 1a). The lower IC_{50} values demonstrate an effective DPPH˙ scavenging activity. Ascorbic acid was found to have the most effective DPPH˙ scavenging activity when considering other samples. When we examine the results of previous studies, DPPH radical scavenging activity of the extracts prepared from *A. azurea* aerial parts with distilled water was determined in only one study and the IC_{50} has been found as 88.65 mg mL^{-1} (Uz et al., 2010). In the study of Conforti et al (2011), 70% aqueous ethanol extract of the flowers of *A. azurea* was used and the IC_{50} of the DPPH˙ scavenging activity found as 84 ± 1.3 µg mL^{-1}. When we evaluate all the results together with the WEAA results, we see that the IC_{50} values are quite high in all studies in general. The results of this study consistent with the literature and it was determined that the Italian bugloss (*Anchusa azurea* Mill.) water extract has a low effective DPPH˙ scavenging activity.

Table 1. Determination of IC_{50} (µg/mL) values for DPPH˙ and $ABTS^{\cdot+}$ scavenging activity
Çizelge 1. DPPH˙ ve $ABTS^{\cdot+}$ giderme aktivitesi için IC_{50} (µg/mL) değerlerinin belirlenmesi

Antioxidants	DPPH˙ scavenging		$ABTS^{\cdot+}$ scavenging	
	IC_{50} (µg/mL)	r^2	IC_{50} (µg/mL)	r^2
Ascorbic acid	17.325 ± 0.004	0.9509	7.533 ± 0.037	0.9519
BHT	38.500 ± 0.023	0.9858	5.824 ± 0.011	0.9539
α-Tocopherol	30.130 ± 0.043	0.9578	8.058 ± 0.008	0.9606
WEAA	231.0 ± 0.059	0.9926	16.500 ± 0.005	0.9710

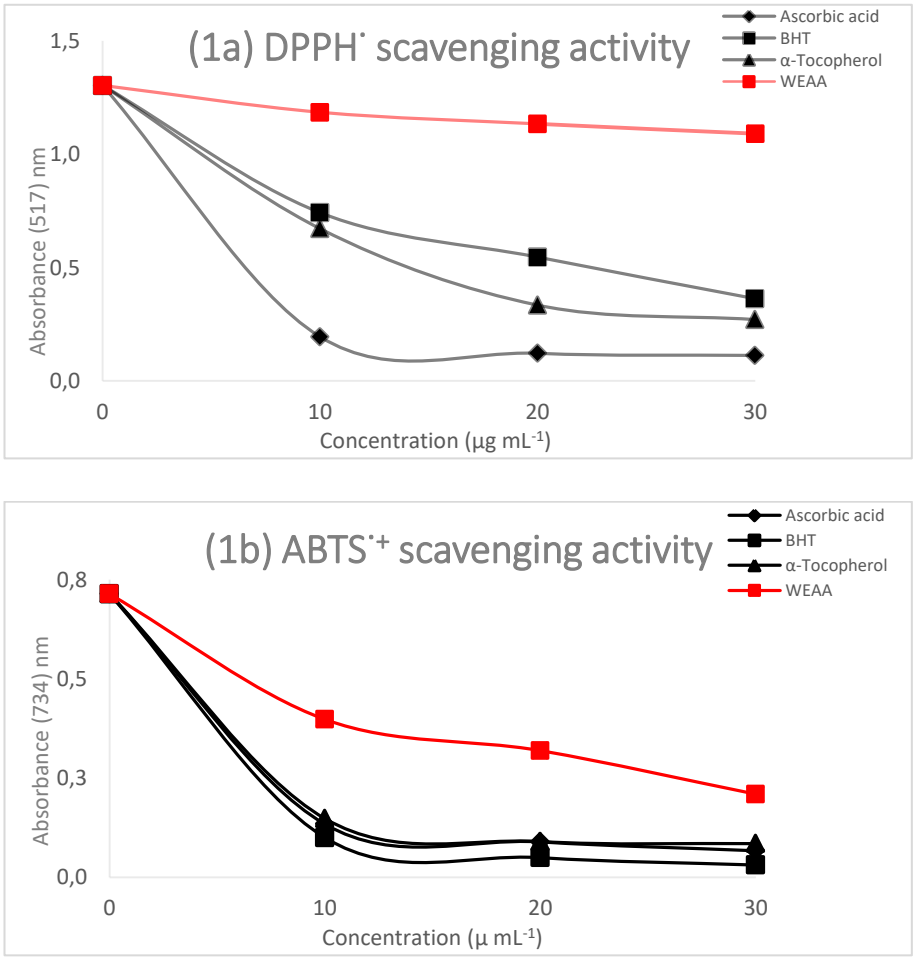

Figure 1. Radical scavenging activity of WEAA (DPPH free radical and ABTS cation radical scavenging methods)
Şekil 1. WEAA'nın radikal giderme aktivitesi (DPPH serbest radikal ve ABTS katyon radikal giderme yöntemleri)

According to ABTS cation radical scavenging activity methods, a stable form of the radical is produced in the experiment and form blue-green $ABTS^{\bullet+}$ by reacting with an antioxidant, and decolorization indicates the rate of $ABTS^{\bullet+}$ inhibition (MacDonald-Wicks et al., 2006). The IC_{50} (µg/mL) values of $ABTS^{\bullet+}$ scavenging for WEAA and standard antioxidants were determined in following order: BHT (5.824 ± 0.011, r^2: 0.9539) < Ascorbic acid (7.533 ± 0.037, r^2: 0.9519) < α-Tocopherol (8.058 ± 0.008, r^2: 0.9606) < WEAA (16.500 ± 0.005, r^2: 0.9710) (Table 1 and Figure 1b). It was found that WEAA has an efficient $ABTS^{\bullet+}$ scavenging activity in a concentration-based way (10–30 µg/mL) and differences have been found statistically significant ($p<0.001$). In the literature search, no publication was found in which $ABTS^{\bullet+}$ scavenging activity was measured in *A. azurea* extracts, this work provides a reference.

The antioxidant profile of lyophilized water extract of Italian bugloss *(Anchusa azurea* Mill.) has been characterized using the ferric ions (Fe^{3+}) reducing, CUPRAC and FRAP assays, as shown in Table 2 and Figure 2. Reduction capacity is an important factor in determining whether a molecule has antioxidant activity (Meir et al., 1995). According to the study, the concentrations of positive controls and WEAA were increased steadily (10-30 µg mL^{-1}). The reducing power of WEAA and positive controls were as follows: Ascorbic acid (1.540 ± 0.014, r^2: 0.9998) > BHT (1.198 ± 0.038, r^2: 0.9997) > α-Tocopherol (0.939 ± 0.083, r^2: 0.9984) > WEAA (0.150 ± 0.017, r^2: 0.9787) (Table 2 and Figure 2a). The results demonstrated that lyophilized water extract of *A. azurea* had marked ferric ions (Fe^{3+}) reducing ability ($p<0.001$) (Table 2). However, this reducing power was lower than the standard antioxidants. The CUPRAC of 30 mg/mL concentration of WEAA and positive controls are demonstrated in Table 2 and Figure 2b.

The CUPRAC of WEAA and positive controls were measured depending on the concentration of (10-30 µg mL^{-1}) and were as follows: BHT (1.003 ± 0.151, r^2: 0.9987) > α-Tocopherol (0.780 ± 0064, r^2: 0.9938)> Ascorbic acid (0.496 ± 0.029, r^2: 09861) > WEAA (0.211 ± 0.029, r^2: 0.9820). The last method studied in this regard is the FRAP method. The FRAP of WEAA and standard antioxidants decreased in the following order: Ascorbic acid (1.221 ± 0.054, r^2: 0.9891) > BHT (0.820 ± 0.028, r^2: 0.9908) > α-Tocopherol (0.700 ± 0.023, r^2: 0.9816) > WEAA (0.376 ± 0.014, r^2: 0.9327) (Table 2 and Figure 2c). According to the method, higher absorbance values represent the higher reduction ability of the Fe^{3+}-TPTZ complex. Furthermore, WEAA demonstrates effective FRAP reduction ability ($p<0.001$) (Table 2). In the literature search we came across only one study that includes the reducing abilities of *A. azurea*. In this study which conducted by Morales et al. (2014), the result of Fe^{3+}-Fe^{2+} reducing power was found as (EC_{50}: 0.01 mg mL^{-1}) in the methanol extract of *A. azurea*. In conclusion, it has been determined that evaluated by using different antioxidant activity assays, the lyophilized water extract of Italian bugloss's *(Anchusa azurea* Mill.) aerial part had a value of antioxidant activity which is close to the standard antioxidants. On the other hand, standard antioxidants have showed the best results.

Table 2. The reducing abilities of WEAA and standard antioxidants at the same concentration (30 µg/mL)
(Çizelge 2. WEAA ve standart antioksidanların aynı konsantrasyondaki (30 µg/mL) indirgeme yetenekleri)

Antioxidants	Fe^{3+}-Fe^{2+} reducing*		CUPRAC*		FRAP*	
	$\lambda_{700\ (nm)}$	r^2	$\lambda_{450\ (nm)}$	r^2	$\lambda_{593\ (nm)}$	r^2
Ascorbic acid	1.540 ± 0.014a	0.9998	0.496 ± 0.029c	0.9861	1.221 ± 0.054a	0.9891
BHT	1.198 ± 0.038b	0.9997	1.003 ± 0.151a	0.9987	0.820 ± 0.028b	0.9908
α-Tocopherol	0.939 ± 0.083c	0.9984	0.780 ± 0.064b	0.9938	0.700 ± 0.023c	0.9816
WEAA	0.150 ± 0.017d	0.9787	0.211 ± 0.029d	0.9820	0.376 ± 0.014d	0.9327

*Different letters in the same column indicate statistically significant difference between the means ($p<0.001$ regarded as significant).

Many biologically active phytochemicals such as phenolic compounds and flavonoids and phenolic constituents, are the major components of plants, which show antioxidant activity (Baghiani et al., 2013; Çakmak & Gulcin, 2019). Plants which contain phenolic compounds and flavonoids are beneficial for human health due to their antiallergic, anticancer, anti-viral, antioxidant, and antimicrobial properties (Boussoualim et al., 2015; Bursal et al., 2019). Secondary metabolites can protect against oxidative reactions because of their antioxidant properties such as hydrogen donor functions, reducing activity or metal chelating activity (Bursal et al., 2019).

Flavonoids are also known to be potent inhibitors of various enzymes, including xanthine oxidase, cyclooxygenase and lipoxygenase (Lin et al., 2002; Boussoualim et al., 2015). The content of total phenolic and flavonoid compounds in WEAA was found to be 18.18 ± 0.3 µg GAE and 12.42 ± 0.5 µg QE, respectively (Table 3). It was shown that there is

a positive correlation between total phenolic and flavonoid contents in WEAA and antioxidant activities. Baghiani et al. (2013) investigated the aerial parts' water extract of *A. azurea* and they found the total phenol and flavonoid content as 32.77 ± 0.59 mg GAE gE^{-1} and 1.32 ± 0.33 mg QE gE^{-1}, respectively. In a study conducted in 2015, *A. azurea* was extracted with 70% aqueous ethanol and the amount of total flavonoid was found as 0.9 ± 0.1 mg g^{-1} (Marrelli et al., 2015). In another study, 70% aqueous ethanol extract of the flowers of *A. azurea* was used and the and the total phenolics content was found as 85.5 ± 1.3 Chlorogenic acid equivalents (mg g^{-1}) (Conforti et al., 2011). The results obtained in the previous studies mentioned above were found to be sometimes higher and sometimes lower than results of this study. The reason for this is thought to be due to differences in ecological and soil structure of the region where the plant is grown, analysis methods, solvents and extraction conditions.

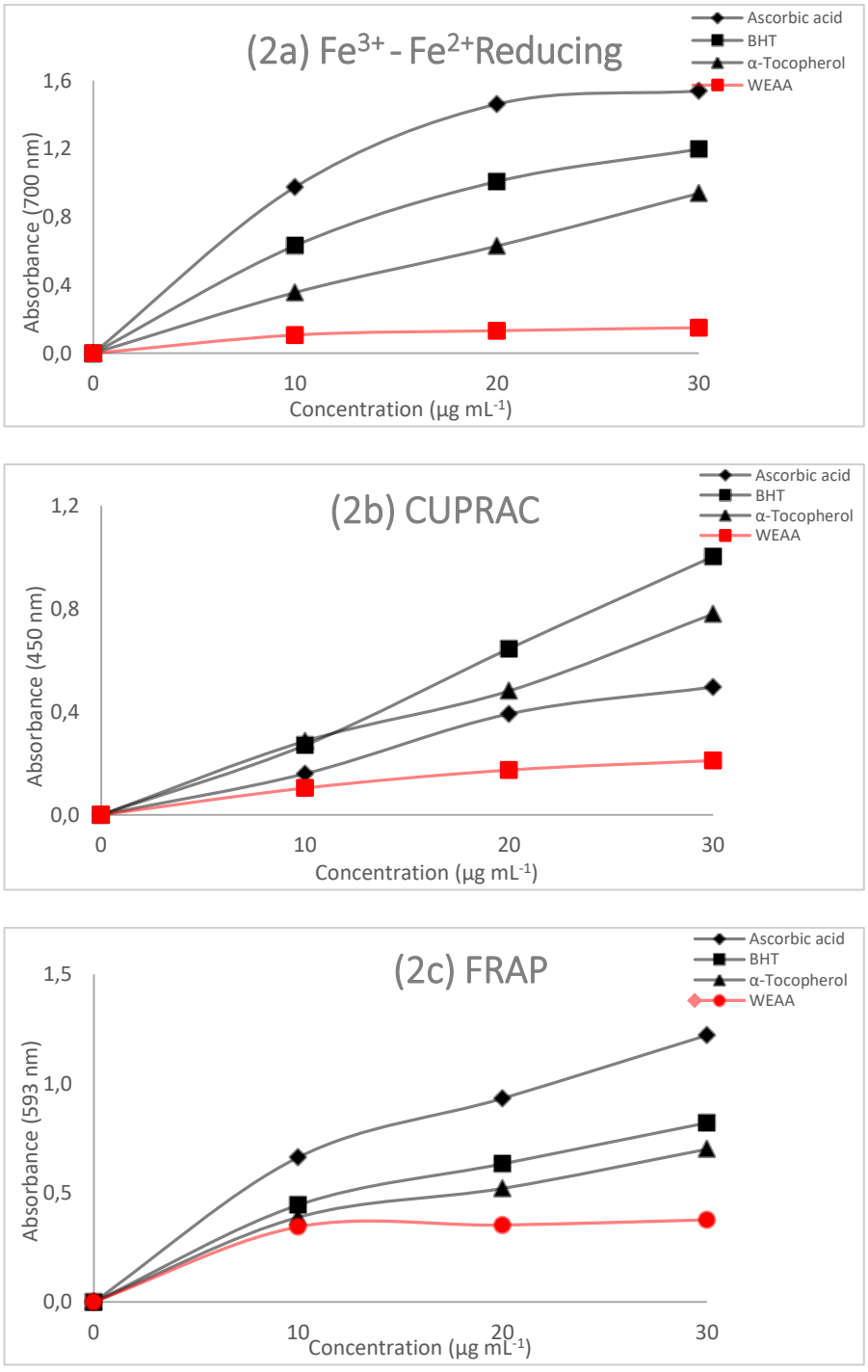

Figure 2. The reducing abilities of WEAA and standard antioxidants at the same concentration (30 µg mL^{-1})
(Şekil 2. WEAA ve standart antioksidanların aynı konsantrasyondaki (30 µg mL^{-1}) indirgeme yetenekleri)

Table 3. The total phenolic and flavonoid contents of WEAA

Çizelge 3. WEAA'nın toplam fenolik ve flavonoit içeriği

	Total phenolics (GAE µg mL^{-1} extract)	Total flavonoid (QE µg mL^{-1} extract)
WEAA	18.18 ± 0.3	12.42 ± 0.5

CONCLUSION

In conclusion, this study provides important information about the phytochemical properties and bioactivity of Italian bugloss (*Anchusa azurea* Mill.) through the determination of its antioxidant capacity and phenolic and flavonoid contents. The results showed that Italian bugloss (*Anchusa azurea* Mill.) has an effective antioxidant activity and also includes high phenolic and flavonoid content. In addition, it was observed that there was a statistically significant difference between the group averages. These results support the potential use of Italian bugloss (*Anchusa azurea* Mill.) as a natural source of antioxidants and may be a basis for further phytochemical studies.

Conflict of Interest Statement

All authors declare that there is no material or other substantive conflict of interest in their article that could affect the results or comments.

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