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ARAŞTIRMA MAKALESİ RESEARCH PAPER

LC–MS/MS and RP–HPLC–UV Analysis and Antioxidant Activities of *Arum italicum* Miller Edible and Nonedible Tuber Parts

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Abstract: In the study, the phenolic components and antioxidant activities of edible and nonedible parts of tuber of *Arum italicum* plant were carried out to determine in methanolic and water extracts. In addition, the antioxidant potential of these parts of the plant was associated with their phenolic composition. Total phenolic content, FRAP, CUPRAC and DPPH tests as radical scavenging activity were performed to determine the antioxidant activity of the plant. The phenolic profiles in the two parts of the tuber were determined in the prepared methanolic extracts by RP–HPLC–UV and LC–MS/MS. The highest values of the total phenolic content, FRAP, CUPRAC and % DPPH were measured in methanolic extracts of nonedible parts of the tuber as 164 µg GAE/mL, 878 TEAC (µM), 0.064 TEAC (µM) and 19.41, respectively. Ferulic acid was determined as the main phenolic compound in methanolic extracts of both the tuber parts by LC/MS-MS. However, luteolin and rutin phenolics were measured as the major compounds in the edible and nonedible parts of the plant tuber with analysis based on RP-HPLC-UV, respectively.

Keywords: Antioxidant activity, *Arum italicum*, LC–MS/MS, Phenolic, RP–HPLC–UV.

Arum italicum Miller Bitkisinin Yenilebilir ve Yenmeyen Yumru Parçalarının LC–MS/MS ve RP–HPLC–UV Analizi ve Antioksidan Aktiviteleri

***Sorumlu yazar:**
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Öz: Bu çalışmada, *Arum italicum* bitkisine ait yumrunun yenilebilir ve yenmeyen kısımlarından elde edilen metanolik ve sulu ekstraktların, fenolik içerikleri ve antioksidan aktiviteleri belirlenmiştir. Ek olarak, bitkinin bu kısımlarının antioksidan potansiyeli, fenolik kompoziyonu ile ilişkilendirilmiştir. Bitkinin antioksidan aktivitesini belirlemek için Toplam Fenolik Madde Miktarı, FRAP, CUPRAC ve radikal süpürme aktiviteyi belirleyen DPPH antioksidan aktivite testleri yapılmıştır. Bitki yumrusunun her iki kısmındaki fenolik profiller hazırlanan metanolik ekstraktlarında RP– HPLC–UV ve LC–MS/ MS cihazları kullanılarak belirlenmiştir. En yüksek toplam fenolik içerik, FRAP, CUPRAC ve % DPPH değerleri, yumrunun yenmeyen kısmının metanolik ekstraktında sırasıyla 164 µg GAE/mL, 878 TEAC(µM), 0.064 TEAC(µM) ve 19.41 olarak ölçülmüştür LC/MS-MS ölçümlerinde her iki yumru kısmının metanolik ekstraktlarında ferulik asit, ana fenolik bileşik olarak belirlendi. Bununla birlikte RP–HPLC–UV ölçümlerinde ise, bitki yumrusunun yenilebilir ve yenilmeyen kısımlarında sırasıyla luteolin ve rutin fenolikleri ana bileşikler olarak ölçülmüştür.

Anahtar kelimeler: Antioksidan aktivite, *Arum italicum*, Fenolik, LC–MS/MS, RP– HPLC–UV.

INTRODUCTION

Free radicals are formed in cells during usual metabolic activity and also various environmental factors such as air pollution, some chemicals, additives, and artificial nutrition are effective in their formation. They damage cells by breaking hydrogen atoms. However, molecules called antioxidant substances stop or minimize the effects of free radicals in the organism and prevent the formation of chain reactions which can cause the occurrence of various diseases. Thus, a balance between free radicals and antioxidants is obligation for cells and tissues to maintain their usual physiological activities. It is known that free radicals increase in the body due to the decline of the body antioxidant protective system. This causes many diseases, mainly cancer (Uttara et al., 2009; Valko et al., 2007). In order to maintain this system in a balanced way it is very important to consume natural compounds with antioxidant activity. Many plants showed high antioxidant, anti-inflammatory, antimicrobial activities due to their phytochemical compound content (Djeridane et al., 2006; Erez et al., 2019; Hirasa & Takemasa, 1998). Although these compounds called secondary metabolites do not have a direct relationship with the basic vital functions of the plant, they are chemical substances that are at least as important as primary metabolites such as protein, fat, carbohydrate. Plants include compounds having antioxidant activities such as carotenoids, lycopenes, coenzyme-Q, antioxidant vitamins, and phenolics (phenolic acids, flavonols, flavonoids, anthocyanins, lignins and tannins) (Cai et al., 2004; Mau et al., 2002; Ng et al., 2000; Xiao et al., 2000). Plant antioxidants have an important role in reducing of oxidative stress on organisms associated with industrialization and technology. Turkey, which has a rich flora, has great potential for natural antioxidant compounds. Consumed as food *Arum italicum* Miller in the family Araceae grows naturally in the northern regions of Turkey. Also, it is used as traditional herbal medicines to control some illness such as hemorrhoid, eczema, cancer, blepharitis, stye, abscess, muscle pain and antifebrile (Akbulut & Özkan, 2014; Bozyel et al., 2020). Generally, the tuber part of the plant is consumed as food boiled. However, the all of tuber part is not consumed as food by local people in Black Sea Region of Turkey. Edible part of the tuber is more delicious than the other part. It is the close to stem of plant and is separated from the nonedible part by a node. The nonedible part of the tuber is not consumed as food due to the unpleasant. Tubers of the plant are also used industrially due to the abundance of starch in the tubers of the plant (Ahmed et al., 2018). There are various antioxidant activity determination methods to detect the antioxidant activities of natural and synthetic components (Gidik et al., 2019; Tosun et al., 2015). These methods can be classified according to the type of measured antioxidants substance (hydrophilic or lipophilic, enzymatic or nonenzymatic), solvent character (organic or aqueous), reactive type (radical or non-radical) and reaction mechanism (electron transfer and hydrogen atom transfer) (Gülcin, 2012; Huang et al., 2005).

In the study, it was aimed to determine the antioxidant activity of methanolic and water extracts of both edible and nonedible parts of tuber of *Arum italicum* plant according to four antioxidant activity methods, which are widely used in the literature and have different reaction mechanisms. These methods are % DPPH Radical Scavenging Activity (Brand-Williams, 1995). Total Phenolic Method with Folin-Ciocalteu Separator (FCR), Iron (III) Reduction/Antioxidant Power (FRAP) Determination (Benzie & Strain, 1996). Copper (II) Reducing Antioxidant Activity (CUPRAC) (Apak et al., 2004). In addition, the phenolic contents of the methanolic extracts of both parts were determined with RP–HPLC–UV and LC–MS/MS devices comparatively.

MATERIALS AND METHODS

Preparation of Plant Extraction: The tuber parts of the *A. italicum* plant were collected in March 2017 from the Derecik district of Trabzon-Akçaabat province of Turkey. The edible and the nonedible of the tuber, were separated from each other (Table 1). After these parts were completely dried, they were and grounded into powder with blender. Then, edible and nonedible parts were extracted both water and methanol for two hours in a magnetic stirrer. Filtration of the extracts was performed with passing filter papers and through a 0.45 syringe filter respectively. Finally, the prepared solutions were separated for antioxidant analysis and RP-HPLC-UV phenolic analysis.

For LC/MS-MS, 1 g of each of the samples was added to 10 mL solvent (75% methanol + 25% dichloromethane) and the solutions were extracted on the shaker for 2 hours. Then, extracts were filtered and injected into the device with passing through a 0.45µm syringe filter.

Table 1. Abbreviations for solution extracts of tuber of *A. italicum.*

1S	Edible part of the tuber water extract
1M	Edible part of the tuber methanol extract
2S	Nonedible part of the tuber water extract
2M	Nonedible part of the tuber methanol extract

Determination of Antioxidant Activity

Total Phenolic Content (TPC): The total phenolic content of the edible part and the nonedible part of the *A. italicum* plant tuber was determined by using

Folin-Ciocalteu reagent, modified according to Slinkard and Singleton (1977) method. Firstly, 50 µL of sample solution was diluted with 2.5 mL of distilled water and 250 µl 0.2 N Folin–Ciocalteu reagent was added. Then 750 µl of Na_2CO_3 (7.5%) was put in the mixture and vortexed. The tubes were incubated at room temperature for 2 hours and the absorbance values at 765 nm were measured. The amounts of phenolic compounds in the samples were calculated in terms of gallic acid equivalent (GAE µg/mL) with using the function of the line obtained from the standard calibration graph of gallic acid prepared at six different concentrations (starting at 1000 µg/mL).

***Determination of Iron (III) Reduction/Antioxidant Power (FRAP)*:** Edible part and non-edible of the plant tuber were studied at a concentration of 100 mg/mL based on the FRAP method developed by Benzie and Strain (1996). As in determination of TPC, Trolox antioxidant standard was studied in six different concentrations (starting at 1000 µM). Samples were pipetted as triplicate together with a sample and reagent blank. After 20 minutes, the absorbance values were read at 595 nm. The results were calculated as µM TEAC comparing with the standard antioxidant substance Trolox.

***Copper (II) Reducing Antioxidant Activity (CUPRAC)*:** The method developed by Apak et al (2004) was modified and applied in this study. Cu (II) chloride and neocuproine solution, ammonium acetate buffer (pH = 7) and analysis solutions were added in equal volumes respectively. The volume of final solution was adjusted to 4.1 mL After 30 minutes, absorbance values were measured at 450 nm. As in FRAP, the antioxidant capacities of the samples in terms of Trolox equivalent were calculated as µM TEAC with using values obtained from the standard antioxidant Trolox graph studied at six different concentrations (starting from 1000 µM).

***DPPH Radical Scavenging Activity*:** In DPPH scavenging activity, 100 µM methanolic solution of DPPH radical was used to determine the activity of edible and non-edible parts of the tuber of the plant. The antioxidant standard and the extracts of the tuber parts of *A. italicum* were conducted in triplicate. In addition, a sample and a reagent blank were studied for each concentration of the samples. After the incubation period (50 min), the absorbance values of the solutions mixed with DPPH reagent were read at 517 nm and the % inhibition (DPPH• scavenging) values were calculated (Huang et al., 2005).
% inhibition (DPPH• cleaning) values were calculated using the following formula.

% Inhibition (radical cleaning power) = $[(A_{DPPH} - A_{Sample}) / A_{DPPH}] \times 100$

A_{DPPH}: Absorbance value of the DPPH solution

A_{Sample}: Absorbance value of the sample extract

Determination of Phenolic Components by HPLC–UV

RP–HPLC–UV Conditions: Analysis of phenolic compounds was carried out on HPLC (Elite LaChrom Hitachi Japan) device. The analysis were performed with using a reverse phase C18 (150 mm x4.6mm, 5µm; Fortis) column. In this process, the gradient program was applied containing 2% acetic acid (pure water) in a reservoir and 70% acetonitrile (pure water) in B reservoir (Can et al., 2015). In addition, it is optimized that the injection volumes of the standards and samples to 20 µL, the flow of mobile phase to 1.0 mL/min, and temperature of the column to 30 °C were fixed. In addition, for the gradient program the optimization was performed with passing through reservoir A as follows: 95% for 0-3 minutes, 85% for 5-8 minutes, 80% for 8-10 minutes, 75% for 10-12 minutes, 60% for 12-20 minutes, 20% for 20-30 minutes and 95% for 35-50 (Can et al., 2015).

LC–MS/MS Analysis: Phenolic component analysis was performed with LC–MS/MS Thermo Scientific/Dionex Ultimate 3000–TSQ Quantum device. LC–MS/MS analyzes were carried out by Hitit University Scientific Technical Application and Research Center. The analyses were performed using ODS Hypersil 4.6 * 250 mm, 5µm column and applying a gradient program including formic acid, water and methanol. Gradient program with 0.1% formic acid (pure water) in reservoir A and 100% methanol in reservoir B was applied. In addition, it is optimized that it is optimized that the injection volumes of the standards and samples to 20 µL, the flow of mobile phase to 0.7 mL/min, and temperature of the column to 30 °C were fixed. The gradient program was optimized by passing through the reservoir 100% A for 0-1 minutes, 5% A for 3 minutes in 22 minutes, and 100% B for 8 minutes in 26 minutes.

RESULTS AND DISCUSSION

Antioxidant Activity of A. italicum Extracts: There are many antioxidant activity determination methods. In the study, to determine the antioxidant activity of the plant tuber was used such as total phenolic content, FRAP, CUPRAC and % DPPH scavenging activity tests.

Total Phenolic Content: Polyphenols, flavonoids and phenolic compounds found in plants are natural antioxidants substances which prevent the harmful effects of oxidative stress induce by ROS. In the study, the total phenolic content of the edible and nonedible parts of the tuber of *Arum italicum* was determined using different solvents (water and methanol). Total phenolic content of methanolic extracts were determined higher than water extracts. While the 2M sample showed highest phenolic content it was followed by 1M, 2S and 1S, respectively (Figure 1).

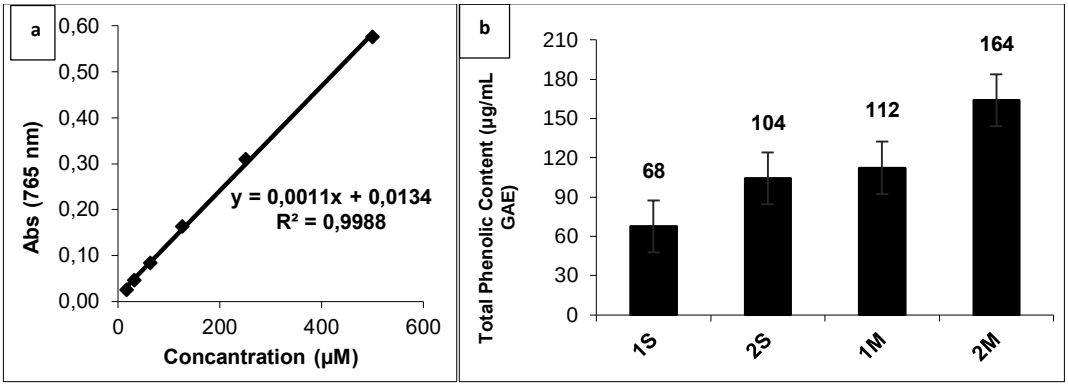

Figure 1. Calibration graphic of the gallic acid standard studied at different concentrations (a) and GAE (µg/mL) values of the Total Phenolic Content of the water and methanolic extracts of the two parts of the tuber (b).

Karahan et al. (2006) conducted experiments the TPC and TFC activity in the leaves of the *Arum dioscoridis* Sm. with using different organic solvents (acetone, ethanol, methanol and water). They report that the highest total phenolic and flavonoid contents were in the ethanol and methanol extracts respectively. In our study, also the total phenolic content was found to be higher in both methanolic extracts of edible and nonedible parts. The highest total phenolic content was determined as 164 µg GAE/mL in sample 2M, 112 µg GAE/mL in 1M sample, 104 µg GAE/mL in 2S sample, 68 µg GAE/mL in sample 1S.

***Iron (III) Reduction/Antioxidant Power and Copper (II) Reducing Antioxidant Activity Analysis*:** The reducing power of bioactive compounds is an indicator of the electron donating ability and this situation is related to the antioxidant activity of the compounds (Arabshahi-Delouee & Urooj, 2007). The antioxidant activity of the plant was determined with methods of FRAP and CUPRAC. In the FRAP test, antioxidant activity values were measured as higher in both water and methanolic extracts of the nonedible tuber part than the edible part (Figure 2).

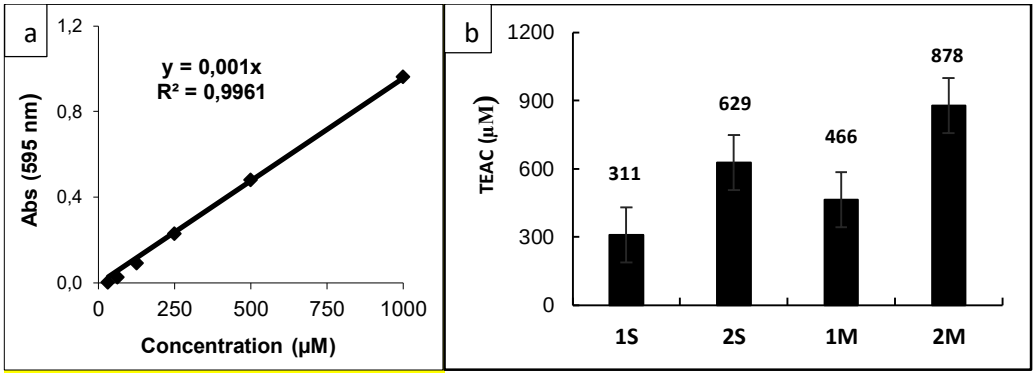

Figure 2. Calibration graphic of the Trolox standard studied at different concentrations (a) and FRAP values (µM TEAC) of water and methanolic extracts of the two parts of the tuber (b).

In CUPRAC test, Cu^{+2} reduction activity of methanolic extracts was measured higher than the water extracts. In addition, as with FRAP, the highest values of water and methanolic extracts were measured for the nonedible parts in CUPRAC test (Figure 3). FRAP results were determined as 878 TEAC (µM) in sample 2M, 629 TEAC (µM) in sample 2S, 466 TEAC (µM) in sample 1M, and 311 TEAC (µM) in sample 1S. The CUPRAC results were measured as 0.064 TEAC (µM) in sample 2M, 0.036 TEAC (µM) in sample 1M, 0.024 TEAC (µM) in sample 2S, 0.018 TEAC (µM) in sample 1S. The CUPRAC results are consistent with the results for the total phenolic content.

Antioxidant Activity Analysis with % DPPH Method: To determine the antioxidant activities, DPPH free radical scavenging test was applied to water and methanol extracts of both parts of the plant tuber. The DPPH radical scavenging method is commonly used in measuring the antioxidant activity of the phenolic compounds or plant extracts due to its easy to operate, rapid and sensitive nature (Uguzlar et al., 2012). Many plant extracts have antioxidant properties because they contain phytocomponents such as phenolic acids and flavonoids (Chu et al., 2000). When DPPH activity results were examined for the all the parts of the plant tuber , % DPPH scavenging activity the methanol extracts was higher than that of the water extracts. In addition, as in all the tests, the highest activity values of % DPPH in both methanolic and water extracts were found in the nonedible parts of the tuber. The % DPPH scavenging activity was determined as 19.41 in sample 2M, 19.02 in sample 1M, 18.23 in sample 2S, 14.78 in sample 1S, respectively (Figure 4).

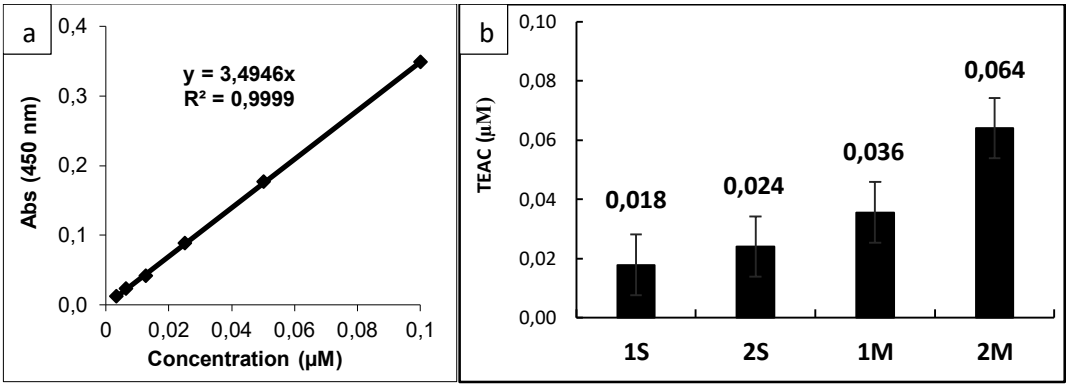

Figure 3. Calibration graphic of the Trolox standard studied at different concentrations (a) and CUPRAC values (µM TEAC) of water and methanolic extracts of the two parts of the tuber (b).

Antioxidant Activity Analysis with % DPPH Method: To determine the antioxidant activities, DPPH free radical scavenging test was applied to water and methanol extracts of both parts of the plant tuber. The DPPH radical scavenging method is commonly used in measuring the antioxidant activity of the phenolic compounds or plant extracts due to its easy to operate, rapid and sensitive nature (Uguzlar et al., 2012). Many plant extracts have antioxidant properties because they contain phytocomponents such as phenolic acids and flavonoids (Chu et al., 2000). When DPPH activity results were examined for the all the parts of the plant tuber , % DPPH scavenging activity the methanol extracts was higher than that of the water extracts. In addition, as in all the tests, the highest activity values of % DPPH in both methanolic and water extracts were found in the nonedible parts of the tuber. The % DPPH scavenging activity was determined as 19.41 in sample 2M, 19.02 in sample 1M, 18.23 in sample 2S, 14.78 in sample 1S, respectively (Figure 4).

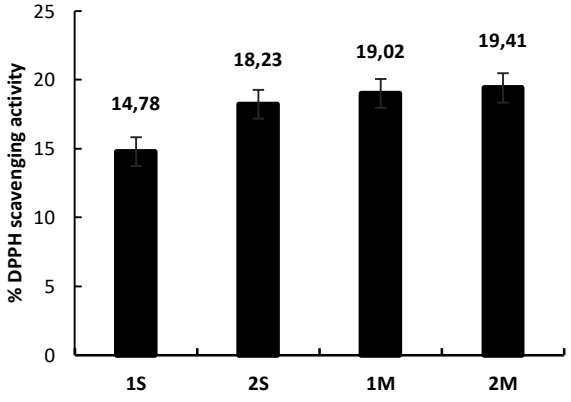

Figure 4. % DPPH radical scavenging activity of water and methanolic extracts of the two parts of the tuber.

***Phenolic Component Analysis by HPLC–UV and LC–MS/MS*:** The plants are rich source of secondary metabolite diversity. One of the most important groups of this metabolite diversity is phenolic compounds (Michalak, 2006). Phenolic compounds are present in plant parts such as fruit, leaves and stems and available in almost all plant parts. As their potential antioxidant properties and their possible role in preventing oxidative stress related diseases such as cancer, they have been attracted much attention (Odbayar et al., 2006).

Phenolic acids consist of two subgroups which are hydroxybenzoic acid and hydrocinnamic acid. In the study, the phenolic compositions of the methanolic extracts of the tuber were determined using both RP–HPLC–UV devices. In addition, methanol and dichloromethane extracts were prepared in LC–MS/MS devices.

The phenolic content in the samples was analyzed using 19 phenolic acid standards in RP–HPLC–UV device Table 2. More phenolic components were determined in the methanolic extracts of the nonedible parts than the edible part. In this case, the phenolic content is consistent with those of the antioxidant activity determination tests which are higher in nonedible part extract for all the tests.

Table 2. RP–HPLC–UV Phenolic Compounds.

Standards	1M(µg phenolic/g sample)	2M(µg phenolic/g sample)
Gallic acid	-	-
Protocateuic acid	6.02	-
p-OH Benzoic acid	4.539	-
Catechin	-	-
Caffeic acid	-	26.724
Syringic acid	-	13.179
Epicatechin	-	31.284
p-Coumaric acid	-	13.264
Ferulic acid	-	14.376
Rutin	-	247.754
Myricetin	-	-
Resveratrol	-	-
Daidzein	-	-
Luteolin	8.092	-
t-Cinnamic acid	4.970	2.696
Hesperidin	-	-
Chrysin	-	-
Pinocembrin	-	-
CAPE	-	-

*-: Not Detected

Rutin and epicatechin were major flavonoid components and caffeic acid was determined as hydroxycinnamic acid derivatives in the nonedible part. At the same time, the phenolic components of the two parts of the tuber of the plant were performed using LC–MS/MS device with 20 phenolic standards (Table 3). While ferulic acid, p-coumaric acid, epicatechin, vanillin, caffeic acid, p-hydroxy benzoic acid and salicylic acid are identified as

major components, rutin, catechin and taxipholine were measured at low levels at both parts of the tuber.

Table 3. LC/MS–MS Phenolic Compounds.

Standards	1M(µg phenolic/g sample)	2M(µg phenolic/g sample)
Gallic acid	-	-
Protocateuic acid	-	-
p-OH Benzoic acid	0.179	0.189
Benzoic acid	-	-
Catechin	0.014	0.090
Caffeic acid	0.579	0.716
Syringic acid	-	-
Epicatechin	1.165	0.711
Salicylic acid	0.159	0.108
Vanillic acid	-	
p-Coumaric acid	4.391	4.629
Ferulic acid	11.131	10.800
Rutin	0.014	0.021
Taxifolin	0.003	0.007
Protocatechuic aldehyde	-	-
Vanillin	1.111	1.263
Rosmarinic acid	-	-
Ellagic acid	-	-
Oleuropein	-	-
Resveratrol	-	-

*-: Not Detected

Phenolic components defined and measured in both RP–HPLC–UV and LC–MS/MS are compatible with each other except for minor differences. Ağalar et al (2017) reported that the whole tuber part of *Arum italicum* has hydroxycinnamic acid derivatives such as ferulic, caffeic and *p*-coumaric acid in analysis with LC–MS/MS. The results obtained in both RP–HPLC–UV and LC–MS /MS are similar to the results of study by Ağalar et al (2017). Ağalar et al (2017) stated that ferulic acid and caffeic acid were present in the leaves of plants belonging to the Araceae family, also p-coumaric acid in the seeds of some species of the same family. Hydroxycinnamic acids containing ferulic acid, caffeic acid, and p-coumaric acid prevent metastasis or invasion of cancer cells (Ağalar et al., 2017). It has been pointed out that rutin exhibits many pharmacological activities such as antitumor, antimutagenic, antibacterial, anti-inflammatory, antiulcer, antidiarrheal, vasodilator, hepatoprotective activities and immunomodulator (Janbaz et al., 2002; Kamalakkannan & Prince, 2006). In our study, rutin was detected in the nonedible part of the tuber with both RP–HPLC–UV and LC–MS/MS devices. It is known that this phenolic prevent ulcer in stomach where widespread in recent years. Ferulic acid was determined as 14.376 µg phenolic/g sample in 2M part with RP–HPLC–UV, 10.800 in 2M sample and 11.131 µg phenolic/g sample in 1M sample by LC–MS/MS. It has been reported that ferulic acid can be useful in the treatment of chronic diseases with its biological activity (de Oliveira Silva & Batista, 2017). In China, herbs rich in ferulic acid have been used in the repair of blood vessel damage and in the treatment of thrombosis diseases for many years (Ou & Kwok, 2004). In addition, it has been reported that ferulic acid has very high antimicrobial activity, showed particularly strong inhibitory effects on the growth of some human gastrointestinal pathogenic microbiota including *Helicobacteria pylori* and *Shigella sonnei* (Lo & Chung, 1999; Nilsson, 1999; Tsou et al., 2000).

CONCLUSION

It has been determined that the nonedible part of the tuber showed higher antioxidant activity than the edible part consumed as food in *Arum italicum* plant. Also, the phenolic profile determined by HPLC–UV and LC–MS/MS devices is richer in the nonedible part. According to these results, although nonedible tuber part has a bad taste it can be concluded that the part can be consumed as food and used for treatment in alternative medicine.

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