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Research Article

Determination of Optimum Ethanolic Extraction Conditions and Phenolic Profiles of Thyme, Mint, Uckun, Grape Seeds and Green Tea Waste Fiber

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Abstract. The objective of this study was to investigate the effect of different ethanol ratios in extraction solvent as well as the antioxidant properties of five plants. Thyme, mint, uckun, grape seeds and green tea waste fiber was analyzed to determine total phenolic content (TPC) and antioxidant activity by ABTS and DPPH radical scavenging activity assays. Individual phenolic components were analyzed with reverse phase high performance liquid chromatography (HPLC). TPC varied significantly from 2.00±0.27 to 172.68±0.19 mg GAE g^{-1} dw depending on the plant type and ethanol ratio of the solvent. The effect of ethanol ratio also varied among different plants. HPLC analysis was performed for the extracts showing highest antioxidant activity, and green tea waste fiber (699.89 mg 100 g^{-1} dw) had the highest concentration of phenolic compounds overall, while mint (173.67 mg 100 g^{-1} dw) had the lowest amount. Correlations between TPC and antioxidant activity was significant which is comparable to the previous report.

Kekik, Nane, Uçkun, Üzüm Çekirdeği ve Yeşil Çay Çöpü Lifinde Optimum Etanolik Ekstraksiyon Şartlarının ve Fenolik Profillerinin Belirlenmesi

Anahtar kelimeler:
Antioksidan, antioksidan aktivite, fenolik bileşenler, etanolik ekstraksiyon

Özet. Bu çalışmanın amacı beş bitkinin antioksidan özelliklerini ve farklı etanol oranlarının ektraksiyona etkisini belirlemektir. Kekik, nane, uçkun, üzüm çekirdeği ve yeşil çay çöpü lifinin toplam fenolik madde içeriği (TPC) ve ABTS ve DPPH radikal giderme aktivitesi testleri kullanılarak antioksidan aktiviteleri ölçülmüştür. Bireysel fenolik maddeler ters faz yüksek performanslı sıvı kromotografisi (HPLC) kullanılarak analiz edildi. TPC bitki türü ve çözücü etanol oranına bağlı olarak 2.00±0.27 ile 172.68±0.19 mg GAE g^{-1} dw arasında değişti. Etanol oranının etkisi farklı bitkiler için değişiklik gösterdi. En yüksek antioksidan aktiviteye sahip ekstraktların HPLC analizleri en yüksek fenolik bileşen konsantrasyonu yeşil çay çöpü lifinde (699.89 mg 100 g^{-1} dw), en düşük ise nanede (173.67 g 100 g^{-1} dw) bulundu. Toplam fenolik madde içeriği ve antioksidan aktivite arasında daha önceki çalışmalarda da gösterildiği gibi kayda değer bir korelasyon belirlendi.

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INTRODUCTION

Many plant species have been studied for their medicinal and health-promoting properties due to their antioxidant activity, anti-inflammatory, antimicrobial, hypolipidemic, and antimutagenic effects and anticarcinogenic potential. Therefore, medicinal plants have long been a focus of interest, and polyphenolic compounds are believed to prevent certain cancer types, cardiovascular diseases, and some other chronic diseases (Wojdylo *et al*., 2007).

Phenolic compounds have three main classes including phenolic acids, polyphenols and flavonoids with over 4000 compounds found in nature. These secondary metabolites protect plants from oxidative damage, and their protective properties have been utilized by humans as well. Phenolic compounds are found in different parts of the plants such as fruits, leaves, seeds (Roby *et al*., 2013).

In the last couple of decades, there has been an increasing interest for natural antioxidants, since the synthetic versions have negative impact in the consumers' eyes due to their possible carcinogenic and toxicologic effects (Altmann *et al*., 1986; Kumar *et al*., 2015). Although synthetic antioxidants are cheap and widely available with high antioxidant activity, there is a discussion in terms of possible adverse effects, so natural sources for antioxidants have been preferred more in food applications recently (Puangpronpitag and Sittiwet, 2009).

Plants have been used in traditional medicines for thousands of years as a source of health-promoting compounds. Moreover, many research studies on the health benefits of natural antioxidant sources have been released recently making plants popular for the health-conscious consumers. Therefore, there is a great deal of effort to identify natural antioxidant from different plant materials (Krishnaiah *et al*., 2011).

Extraction of antioxidant compounds from the plants is the first step in their analysis and different solvents have been investigated and reported in the literature (Barizão *et al*., 2014). However, there is no standard technique to extract phenolic compounds from plant material, and the efficiency of extraction is dependent on the method and solvent (Goli *et al*., 2005). Since individual phenolic compounds may have different chemical characteristics and polarities, the efficiency of different methods and solvents changes accordingly (Turkmen *et al*., 2006). The most commonly used extraction solvents are water, ethanol, methanol, acetone and ethyl acetate. Among those, ethanol has been known as an efficient solvent for phenolic compounds and the safety of human consumption is also another advantage of using ethanol rather than other solvents (Dai and Mumper, 2010).

Many studies have investigated analytical methods to determine antioxidant activity in different plant materials. Among these different methods, $ABTS^{\cdot+}$ [2,2′-azino-bis(3ethylbenzothiazoline-6-sulfonic acid)] and $DPPH^{\cdot}$ (2,2 diphenyl-1-picrylhydrazyl) scavenging activity assays are the most common methods due to their relative stability and reproducibility (Mariutti *et al*., 2008; Bahadori, 2018). $DPPH^{\cdot}$ is a stable free radical that can be reduced by the presence of a reducing agent i.e. antioxidant causing the loss of its violet color, while ABTS assay measures the antioxidant activity by reacting oxidant radical $ABTS^{\cdot}$ with a reducing agent i.e. antioxidant causing the loss of its blue-green color (Sridhar and Charles, 2009). While there is a great deal of research on plant antioxidants, antioxidant properties of plants have been usually studied using total phenolic content (TPC) analysis with lack of individual phenolic compound content. Moreover, there are limited studies investigating different plant families and the relationship between structure and antioxidant activity of phenolic compounds. Therefore, the objective of this study was to investigate the effect of ethanol ratio on the extraction of phenolic components and the antioxidant activity of different plant materials (thyme, mint, uçkun, grape seeds, and green tea waste) using total phenolic content (TPC) method and two different radical scavenging activity assays (ABTS and DPPH). After selecting the best extraction method in terms of antioxidant activity, the extracts were evaluated by high performance liquid chromatography (HPLC) to identify and quantify the phenolic compounds of target plants.

MATERIAL AND METHOD

Chemicals

All solvents used in the experiments (methanol, ethanol, hydrochloric acid) were purchased from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis (3- ethyl-benzothiazoline-6-sulphonate) (ABTS), Folin Ciocalteu, potassium persulphate, sodium acetate, sodium carbonate, ascorbic acid, Trolox, and gallic acid are all analytical grade and purchased from Sigma Aldrich Co (St. Louis, MO, USA).

Plant Material

The different plant extracts used in the current study were obtained as follows: Thyme (*Thymus vulgaris* L.) was collected from Suveren region in Iğdır (Latitude: 39°48´46.496´´N, Longitude: 44°4´43.13´´E, Turkey) in May 2017, and whole plant was used in extraction. Mint (*Mentha piperita* L., Turkey) was purchased from a local market in Van (Latitude: 38°29′40″ N, Longitude: 43°22′59″ E, Turkey), and whole plant was used in extraction. Uckun (*Rheum ribes L.*) was collected from Kağızman region in Kars (Latitude: 40°09′24″ N, Longitude: 43°08′03″ E, Turkey), and whole plant was used in extraction. Grape seeds (*Vitis vinifera subsp vinifera*, Gamay type) was obtained from Tekirdağ Viticulture Research Institute in Tekirdağ (Latitude: 40°58′44″ N, Longitude: 27°30′54″ E, Turkey). Finally, green tea waste fiber (*Camellia sinensis*) was obtained from Caykur, Directorate General of Tea Enterprise in Rize (Latitude: 41°1′33″ N, Longitude: 40°31′8″ E, Turkey). Green tea waste fiber is a waste product of green tea packaging facility including all parts of the plant. The fresh plants were dried at room temperature under laboratory conditions.

Extraction Method

After drying the plants at room temperature, the dried plants and grape seeds were milled using a grinder. The phenolic compound extraction was performed according to Brito et al. (2014)' s method with some modifications. One gram of dry plant was extracted with 100 ml of ethanol: water solution in varying ethanol concentrations from 0, 50, 60, 70, 80 and 100% (v:v). To acidify the solution, 0.02 ml of HCl (≥ 99%) was added and stirred using a shaker (Shaking, HZQ-X300, China) at 25 °C for 3 hours. Then, the solution was centrifuged at 3500 xg at 20 °C for 20 min (Heal Force, Neofuge 23 R, China). The supernatant was collected and filtered with Whatman No. 4 filter paper (Whatman International, UK). The remaining plant material on the paper was mixed with 10 mL of the same solvent and left to stand for 15 minutes. It was combined with the previous obtained supernatant. Ethanol evaporation was carried out in the rotary evaporator (Heidoph, Germany) operating at 35°C. The extracts were dried in the lyophilizator (Christ Alpha 1-2 LD Plus, 2015, Germany) to remove water. The freeze-dried samples were collected and stored at -80 C until use. The dried extract samples for analysis were dissolved in ethanol:water (v:v) mixture in varying concentrations (0.25 g dried extract/25 ml of solvent), and stored in the dark at -20°C until analysis.

Determination of Total Phenol Content Assay

Total phenolic content of the extracts was determined using the Folin-Ciocalteu method (Oboh *et al*., 2017) with slight modifications. Folin-Ciocalteu reagent was prepared in distilled water (10%), and then 2 ml of the Folin-Ciocalteu solution was added to 0.4 ml of the plant extract. The resulting mixture was gently shaken, and 1.6 ml of sodium carbonate (Na_2CO_3) (7.5%) was added and the mixture was vortexed. The reaction was kept in the dark for 30 min. Then, the absorbance of samples was measured using a spectrophotometer (Thermo Scientific Orion Aquamate 8000 Uv-Vıs) at 760 nm against a reagent blank (methanol for standard curve and ethanol: water mixture for samples). A standard curve was conducted using different concentrations (0-100 ppm) of gallic acid using the equation (1) with an R^2 =0.997, and total phenolic content of the extracts was calculated and reported as mg gallic acid equivalent (mg GAE) per 1 g dry weight (dw) of plant.

$$Y = 0.008X + 0.276 \quad (1)$$

1,1-Diphenyl–2 picrylhydrazyl (DPPH) Free Radical Scavenging Ability

Free radical scavenging activity of the plant samples were determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by Blois (1958) with minor modifications. In the assay, $5x10^{-4}$ mM DPPH was prepared in methanol, kept in the dark overnight in the refrigerator. 100 µL of dissolved sample was added to a mixture of 2600 µl methanol and 300 µL of DPPH ($5x10^{-4}$ mM). After mixing, the samples were kept in the dark for 30 minutes before measuring the absorbance at 515 nm by a spectrophotometer (AquaMate 8000 UV-Vis Spectrophotometer, Thermo Scientific) using methanol as blank. The inhibition concentration (IC50) values of each analytical sample were calculated by plotting inhibition ratios against six different concentrations of the sample to obtain the regression line. Using the regression equation (y=ax+b), the concentration level related to the 50% inhibition rate i.e. IC50 (mg L^{-1}) values were calculated for each sample. Both ascorbic acid and Trolox standard solutions at different final levels (0-0.001 mM) were prepared daily. The DPPH radical scavenging activity for each sample was calculated as Trolox equivalent (TE) and ascorbic acid equivalent (AAE), and the inhibition percentages were calculated according to the equation (2) below where AS is sample reading, ABK is blank reading, Ac= MetOH reading:

$$DPHH\ inhibition\ (\%)\ =\ [(ABK\ -(AS-AC))/ABK]\ x\ 100\% \quad (2)$$

Results were expressed as mean ± SD of TE and AAE antioxidant capacity for three replicates (mg equivalent g^{-1} dry weight).

ABTS [2,2′-Azinobis (3-Ethylbenzothiazoline-6-Sulphonic Acid)] Free Radical Scavenging Activity Assay

ABTS radical scavenging activities were measured as described previously by Ozgen *et al*. (2006) with some modifications. ABTS (7 mM) stock solution was prepared with 0.0384 g ABTS and 2 ml of 12.25 mM potassium persulfate ($K_2S_2O_8$) and final volume was adjusted to 10 ml with 20 mM sodium acetate buffer (pH 4.5). Then, the solution was kept in the dark at the room temperature for 12-16 h to produce ABTS radical cation ($ABTS^{\cdot}+$) before use. Before performing the measurements, ABTS solution was diluted with 20 mM sodium acetate (CH_3COONa) (pH 4.5) to adjust the absorbance at 0.70± 0.01 at 734 nm. Diluted $ABTS^{\cdot}+$ solution (990µL) and dissolved extracts or standard solution (10µL) was mixed and absorbance was recorded at 734 nm by a spectrophotometer (Thermo Scientific, AQ 8000, USA) exactly after 6 min of initial mixing. A Trolox and ascorbic acid calibration curves were fitted for a concentration range of 2.5-20 µM. The results were reported as AAE (mg ascorbic acid g^{-1} dw) and TE (mg Trolox g^{-1} dw). The equation for Trolox standard curves (3) has an R^2 of 0.992, and the equation for ascorbic acid (4) has an R^2 of 0.994. The IC50 of each plant sample was also calculated similarly as previously explained in the DPPH method.

$$\mathrm{Y} = 4.789\mathrm{X} - 0.575 \quad (3)$$

$$\mathrm{Y} = 3.785\mathrm{X} - 1.469 \quad (4)$$

Quantification of Phenolic Compounds by HPLC-DAD

For HPLC analysis, the samples showing best antioxidant activity with the lowest IC50 values were selected and analyzed for identification of the major phenolic compounds. Reverse phase HPLC analyses were carried out using an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump (G1311B), an autosampler (G1329B), and Diode Array Detector (DAD). Chromatographic separation was performed using Agilent Poroshell 120 EC-C18 column (4.6 mm × 150 mm with an 2.7 µm particle size). The method described by Brito *et al*. (2014) and Oboh *et al*. (2017) were followed with slight modifications. Samples (10 mg extract) were prepared in 2 ml of acetonitrile: water solution (25:75) and filtered through 0.45 µm Nylon syringe filters (Millipore) into the HPLC vials. Standards were separately prepared in acetonitrile: water solution (50:50) and various concentration of standards were prepared for obtaining standard curve. Mobile phases consisted of 1% phosphoric acid in water (A) and acetonitrile (B). Gradient elution method was used in two different methods. The first method was for gallic acid, catechin, o- coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, and quercetin, with a mobile phase composition of 17, 17, 30, 40 and 17% of solvent B until 0, 1, 2, 4, 10 min, respectively, at a flow rate of 0.8 mL/min. The second method was applied for chlorogenic acid, syringic acid with a mobile phase composition of 17% B in an 8 min run time at a flow rate of 0.1 mL min^{-1}. Injected amount of sample was 20 µL for both methods. Chromatography peaks were confirmed by comparing retention times with those of reference standards and by DAD (300;200, 500;100 nm) at fixed column temperature (20ºC) [17]. The analyses were performed in triplicates, and the results were reported as mean ± standard deviation (SD) in mg 100 g^{-1} dw.

Statistical Analysis

Data analysis was performed using SPSS for Windows (Version 25). Three replicates were used for all assays performed and results expressed as mean ± SD was reported. One-way analysis of variance (ANOVA) was used to determine any significant differences ($p<0.05$) between groups extracted with different ethanol ratio in the solvents. Duncan's post hoc test was performed for individual differences between groups. Pearson's correlation coefficients were used to evaluate the relationship between antioxidant activity and total phenolic assays as well as the correlation between different antioxidant activity methods.

RESULTS AND DISCUSSION

Total Phenolics

The amount of total phenolics in different plants varied significantly from 2.00±0.27 to 172.68±0.19 mg GAE g^{-1} dw. depending on the plant type and ethanol ratio of the solvent (Table 1). Among extracts of each plant, the extracts obtained by 100% water gave the highest amount of total phenolics for mint and thyme, however, the lowest amount of total phenolics was found in uckun, green tea waste fiber and grape seeds extracted with 100% water. For mint Bahadori *et al.* (2018) also reported a higher phenolic content in water extraction than methanol extraction. However, Haraguchi *et al.* (2019) reported that ethanolic extracts of *Thymus vulgaris* showed higher content of phenolics than aqueous extracts.

Moreover, TPC in grape seeds were found to be in higher amounts when the extraction solvent is a mixture of water and ethanol compared to pure water extraction (Dalia and Hala, 2006) which is consistent with our results for grape seeds. Extraction efficiency increased up to 50% ethanol and then decreased with further increase of ethanol ratio in solvent, which is consistent with another study evaluating the effect of ethanol on the TPC of grape seed meal (Shi *et al*., 2003). For green tea waste fiber, we found highest total phenolic amount in 70% ethanol extraction. Similarly, Nadiah and Uthumporn (2005) reported 50% ethanol extraction revealed higher TPC than pure water extraction. The results are consistent in terms of comparing pure water and ethanol: water mixture, although researchers only compared 50% ethanol with 100% water.

Although it was reported that phenolic compounds were usually more soluble in organic solvents compared to water (Turkmen *et al*., 2006), the efficiency of different solvents was found to be changed for each plant. For thyme, mint, uckun, green tea waste fiber and grape seeds, 0% (49.67±0.11 mg GAE g^{-1} dw), 0% (37.88±0.06 mg GAE g^{-1} dw), 70% (78.58±0.38 mg GAE g^{-1} dw), 70% (172.68±0.19 mg GAE g^{-1} dw), and 60% (159.31±1.73 mg GAE g^{-1} dw) ethanol gave the highest amount of total phenolics, respectively. When statistical analysis performed, all the results for TPC using different ratio of solvent was significantly different from each other for thyme, uckun, green tea waste fiber, and grape seeds. For mint, only 80% and 70% ethanolic extracts was not significantly different from each other, but remaining samples were statistically different. Therefore, selection of appropriate ethanol ratio in the extraction solvent can be made individually for each plant according to the highest levels obtained for each plant. The results were comparable to the literature when similar conditions used as in our study in terms of TPC in some plants. For example, Roby *et al.* [23] found 7.30±1.47 mg GAE g^{-1} dw of total phenolics in thyme while we obtained 7.01±0.13 mg GAE g^{-1} dw using 100% ethanol extraction. For green tea waste fiber, 50% ethanol extraction revealed 186.83 mg GAE g^{-1} dw, while we found 98.59 mg GAE g^{-1} dw using same ratio of ethanol in the extraction solvent. The difference might be due to the plant part used which was green tea waste fiber in our study versus whole green tea (Nadiah and Uthumporn, 2015).

Quantification of Individual Phenolic Compounds

Since total phenolic content analysis does not give information on quantity of individual phenolic compounds, individual phenolic compounds were also investigated using reverse phase HPLC method and results were reported as mg phenolic compound in g dry weight (dw) in Table 2. Different species showed different profile and concentrations of phenolic compounds. Green tea waste fiber (699.89 mg 100 g^{-1} dw) was found to have the highest concentration of phenolic compounds overall, and mint (173.67 mg 100 g^{-1} dw) had the lowest amount. The plants tested in this study was found to have different profile of phenolic acids and flavonoids (Table 2). Mint had trans-ferulic acid, catechin, syringic acid, chlorogenic acid, caffeic acid, caffeic acid, gallic acid, o- coumaric acid, p-coumaric acid and quercetin in a decreasing order of amount. Thyme had chlorogenic acid, gallic acid, trans-ferulic acid in high amounts while quercetin, p-coumaric acid, caffeic acid, catechin, o-coumaric acid amounts were very low. Grape seeds had very high amount of catechin (431,19±0,01 mg g^{-1} dw) and other phenolic compounds were syringic acid, p-coumaric acid, gallic acid, caffeic acid and chlorogenic acid in decreasing order; however, no trans-ferulic acid, o-coumaric acid and quercetin could be detected. Chlorogenic acid was highest in uckun (151.03±0.04 mg g^{-1} dw), and other phenolic compounds were trans-ferulic acid, catechin, gallic acid, p-coumaric acid, and syringic acid in decreasing order of amount. Green tea waste fiber had high amounts of catechin (198.40±0.60 mg g^{-1} dw), syringic acid (186.53±0.53 mg g^{-1} dw) and chlorogenic acid (148.60±0.60 mg g^{-1} dw), while lower amounts of gallic acid, p-coumaric acid, caffeic acid and very low amounts of quercetin and o-coumaric acid. Among the selected major phenolic compounds, uckun did not have any caffeic acid, o- coumaric acid, and quercetin, while grape seed was lack

Table 1. Total phenolic content and antioxidant activity measured by two different assays in five plants.
Çizelge 1. Beş bitkinin toplam fenolik madde içeriği ve iki farklı yöntem kullanılarak ölçülmüş antioksidan aktivitesi.

Plant	Parts used	E:W (v:v)	TPC	ABTS			DPPH		
				AAE	TE	IC50	AAE	TE	IC50
Thyme	Leaves	100:0	7.01 ± 0.13 ^{y}	0.02 ± 0.00 ^{o}	0.03 ± 0.00 ^{p}	3.65 ± 0.05 ^{p}	0.01 ± 0.00 ^{l}	0.02 ± 0.00 ^{n}	0.73 ± 0.04 ^{i}
		80:20	18.34 ± 0.43 ^{t}	0.08 ± 0.00 ^{o}	0.10 ± 0.00 ^{nop}	0.82 ± 0.02 ^{j}	0.04 ± 0.01 ^{j}	0.06 ± 0.01^{l}	0.74 ± 0.01 ^{i}
		70:30	15.50 ± 0.31 ^{v}	0.06 ± 0.00 ^{o}	0.08 ± 0.00 ^{nop}	2.08 ± 0.02 ^{m}	0.04 ± 0.00 ^{ij}	0.07 ± 0.02 ^{k}	0.31 ± 0.04 ^{ef}
		60:40	9.53 ± 0.00 ^{x}	0.08 ± 0.00 ^{o}	0.09 ± 0.00 ^{nop}	2.87 ± 0.02 ^{o}	0.04 ± 0.01 ^{ij}	0.07 ± 0.01^{jk}	0.45 ± 0.01 ^{g}
		50:50	2.00 ± 0.27 ^{z}	0.08 ± 0.00 ^{o}	0.10 ± 0.00 ^{nop}	8.21 ± 0.01 ^{q}	0.05 ± 0.00 ^{fghi}	0.09 ± 0.01 ^{ijk}	0.98 ± 0.00 ^{j}
		0:100	49.67 ± 0.10 ^{n}	0.04 ± 0.00 ^{o}	0.05 ± 0.00 ^{nop}	11.15± 0.05 ^{t}	0.05 ± 0.00 ^{ghi}	0.08 ± 0.00 ^{ijk}	2.43 ± 0.02 ^{l}
Mint	Leaves	100:0	11.64 ± 0.11 ^{w}	0.06 ± 0.00 ^{o}	0.07 ± 0.00 ^{op}	2.40 ± 0.00 ^{n}	0.02 ± 0.00 ^{kl}	0.03 ± 0.00 ^{m}	0.42 ± 0.00 ^{g}
		80:20	23.90 ± 0.09 ^{r}	0.30 ± 0.00 ^{m}	0.15 ± 0.20 ^{mno}	0.56 ± 0.01 ^{g}	0.04 ± 0.00 ^{ij}	0.07 ± 0.01 ^{k}	0.56 ± 0.01 ^{h}
		70:30	24.66 ± 0.09 ^{r}	0.15 ± 0.01 ^{n}	0.19 ± 0.01 ^{mn}	0.63 ± 0.00 ^{h}	0.05 ± 0.01 ^{hi}	0.08 ± 0.01 ^{jk}	0.53 ± 0.30 ^{h}
		60:40	22.93 ± 0.21 ^{s}	0.19 ± 0.00 ^{n}	0.24 ± 0.00 ^{m}	0.71 ± 0.00 ^{i}	0.05 ± 0.01 ^{ij}	0.08 ± 0.01^{jk}	0.52 ± 0.10 ^{h}
		50:50	17.09 ± 0.21 ^{u}	0.20 ± 0.01 ^{n}	0.26 ± 0.00 ^{m}	1.32 ± 0.01 ^{l}	0.05 ± 000 ^{fghi}	0.09 ± 0.01 ^{hij}	0.12 ± 0.01 ^{cd}
		0:100	37.87 ± 0.06 ^{p}	0.03 ± 0.00 ^{o}	0.04 ± 0.00 ^{op}	9.47 ± 0.02 ^{r}	0.02 ± 0.00 ^{k}	0.04 ± 0.00 ^{m}	3.06 ± 0.01 ^{m}
Uckun	Leaves	100:0	60.54 ± 0.21 ^{k}	0.82 ± 0.00 ^{j}	1.04 ± 0.02 ^{i}	0.64 ± 0.01 ^{h}	0.06 ± 0.00 ^{def}	0.10 ± 0.01 ^{efg}	0.34 ± 0.01 ^{f}
		80:20	55.20 ± 0.00 ^{l}	0.72 ± 0.01 ^{j}	0.91 ± 0.00 ^{j}	0.40 ± 0.00 ^{ce}	0.10 ± 0.01 ^{a}	0.16 ± 0.01 ^{a}	0.42 ± 0.01 ^{g}
		70:30	78.58 ± 0.38 ^{i}	0.52 ± 0.01 ^{l}	0.66 ± 0.00 ^{k}	0.50 ± 0.01 ^{fg}	0.10 ± 0.00 ^{a}	0.16 ± 0.01 ^{a}	0.25 ± 0.01 ^{e}
		60:40	44.76 ± 0.29 ^{o}	0.48 ± 0.00 ^{l}	0.61 ± 0.00 ^{k}	0.45 ± 0.01 ^{ef}	0.08 ±0.01 ^{b}	0.14 ± 0.01^{bc}	0.15 ± 0.01 ^{d}
		50:50	52.62 ± 1.87 ^{m}	0.47 ± 0.00 ^{l}	0.60 ± 0.00 ^{k}	1.06 ± 0.04 ^{k}	0.10 ± 0.00 ^{a}	0.17 ± 0.00 ^{a}	0.06 ± 0.00^{abc}
		0:100	30.66 ± 0.12 ^{q}	0.04 ± 0.00 ^{o}	0.05 ± 0.00 ^{op}	9.60 ± 0.13 ^{s}	0.06 ± 0.01 ^{efgh}	0.09 ± 0.00 ^{ghi}	1.12 ± 0.00 ^{k}
Green tea	Waste fiber	100:0	45.62 ± 0.08 ^{o}	1.16 ± 0.06 ^{i}	1.46 ± 0.04 ^{h}	0.15 ± 0.01^{b}	0.02 ± 0.00 ^{kl}	0.03 ± 0.00 ^{m}	0.03 ± 0.00 ^{a}
		80:20	126.88 ± 1.13 ^{f}	4.42 ± 0.02 ^{d}	5.59 ± 0.01 ^{d}	0.10 ± 0.01 ^{ab}	0.09 ± 0.01^{a}	0.16 ± 0.00 ^{a}	0.12 ± 0.00 ^{cd}
		70:30	172.69 ± 0.19 ^{a}	6.12 ± 0.08 ^{a}	7.74 ± 0.14 ^{a}	0.10 ± 0.00 ^{ab}	0.10 ± 0.01 ^{a}	0.16 ± 0.01 ^{a}	0.04 ± 0.00 ^{b}
		60:40	81.85 ± 0.16 ^{h}	5.45 ± 0.13 ^{b}	6.89 ± 0.22 ^{b}	0.07 ± 0.01 ^{a}	0.07 ± 0.01 ^{cd}	0.11 ± 0.01 ^{de}	0.05 ± 0.00^{abc}
		50:50	98.59 ± 0.00 ^{g}	0.3 ± 0.01 ^{m}	0.37 ± 0.00 ^{l}	0.53 ± 0.03 ^{g}	0.08 ± 000 ^{b}	0.14 ± 0.00 ^{b}	0.08 ± 0.00^{abcd}
		0:100	6.08 ± 0.00 ^{y}	1.32 ± 0.02 ^{h}	1.68 ± 0.00 ^{g}	0.37 ± 0.01^{c}	0.05 ±0.01 ^{fghi}	0.09 ± 0.00 ^{ijk}	0.12 ± 0.00^{cd}
Grape	Seeds	100:0	129.99 ± 1.07 ^{e}	0.79 ± 0.00 ^{j}	1.00 ± 0.06 ^{ij}	0.09 ± 0.01 ^{ab}	0.07 ± 0.00 ^{cd}	0.11 ± 0.01 ^{de}	0.11 ±0.00 ^{bcd}
		80:20	157.53 ± 0.49 ^{b}	3.63 ± 0.01 ^{e}	4.60 ± 0.00 ^{e}	0.08 ± 0.03 ^{ab}	0.07 ± 0.01 ^{cd}	0.11 ± 0.01 ^{ef}	0.08 ±0.01^{abcd}
		70:30	137.29 ± 0.26 ^{c}	4.80 ± 0.00 ^{c}	6.07 ± 0.04 ^{c}	0.08 ± 0.05 ^{ab}	0.07 ± 0.01 ^{bc}	0.12 ± 0.00 ^{cd}	0.03 ±0.00 ^{a}
		60:40	159.31± 1.73 ^{b}	3.65 ± 0.02 ^{e}	4.62 ± 0.00 ^{e}	0.09 ± 0.03 ^{ab}	0.06 ± 0.00 ^{defg}	0.10 ± 0.01 ^{fgh}	0.12 ±0.01^{cd}
		50:50	82.55 ± 0.22 ^{h}	2.21 ± 0.00 ^{f}	2.79 ± 0.00 ^{f}	0.06 ± 0.10 ^{a}	0.06 ± 0.00 ^{cde}	0.11 ± 0.01 ^{ef}	0.06 ±0.00^{abc}
		0:100	49.28 ± 0.25 ^{n}	1.4 ± 0.00 ^{g}	1.77 ± 0.00 ^{g}	0.12 ± 0.00 ^{ab}	0.05 ± 0.00 ^{hi}	0.08 ± 0.00 ^{ijk}	0.25 ±0.01^{e}

Each value is the mean of triplicate determinations ± SD; values within the same column with different letters are significantly different at $p < 0.05$, E:W : Ethanol:water (v:v), TPC: Total phenolic content (mg GAE g^{-1} dw), AAE: mg ascorbic acid equivalent g^{-1} dw, TE: mg Trolox equivalent/g dw.

of trans-ferulic acid, o- coumaric acid, and quercetin. For thyme, syringic acid could not be detected in HPLC analysis which is consistent with literature (Gedikoglu *et al*., 2019). Roby *et al.* (2013) reported that gallic acid and chlorogenic acid could not be detected by HPLC from methanolic extraction of thyme phenolics. However, in our study, we found considerable amounts of gallic acid (31.13±0.70 mg g^{-1} dw) and chlorogenic acid (124.29±0.71 mg g^{-1} dw) in thyme with 70% ethanol extraction. This difference might be due to different solvents used in extraction as well as the variety and growing location of the thyme used in our study (Igdir, Turkey). In a study conducted by Mišan *et al.* (2011), it was reported that in 80% ethanolic extraction of mint does not yield any gallic acid, ferulic acid, or syringic acid, and all other phenolic compound concentrations were very low compared to our study. The reason for the differences might be the use of 50% ethanolic extraction in our study versus 80% in Mišan's study (Mišan *et al*., 2011). The reason for different results in phenolic compounds might be the use of different type and ratio of solvents, sample preparation methods (lyophilization, conventional drying, etc.) as well as the variety and growth location of plant used in different studies (Beato *et al*., 2011).

Table 2. Quantitative analysis of major phenolic compounds identified by HPLC in five different plants.
Çizelge 2. HPLC ile tanımlanan major fenolik bileşenlerin kantitatif analizi.

Name of plant	Phenolic acids (mg 100 g^{-1} dw)							Flavonoids (mg 100 g^{-1} dw)	
	GA	**CAA**	***p*-CA**	***t*-FA**	***o*-CA**	**CGA**	**SA**	**QUE**	**CA**
Thyme	31.13±0.70	8.74±0.05	9.25± 0.25	30.76±0.58	6.49±0.39	124.29±0.71	ND	9.33±0.27	6.74±0,04
Mint	11.63±0.14	13.06±0.02	3.12±0.03	41.90±1.10	5.36±0.08	20.51±0.42	24.04±0.06	0.39±0.01	33.66±0,01
Uckun	34.61±0.22	ND	24.81± 0.19	43.01±0,11	ND	151.03±0.04	7.22±0.08	ND	39.33±0.33
GrTWF	64.67±0.68	45.21±0.81	45.76± 0.24	9.93±0,08	0.30±0.10	148.60±0.60	186.53±0.53	0.49±0.29	198.40±0.60
Grape seed	29.63±0.37	18.25±0.25	39.42±0.59	ND	ND	4.10±0.30	70.32±0.33	ND	431.19±0,01

*GA-gallic acid; CAA-caffeic acid; p-CA – p-coumaric acid; t-FA- trans-ferulic acid; o-CA -o- coumaric acid; CGA- Chlorogenic acid; SA-Syringic acid; QUE- quercetin; CA-catechin; GrTWF-Green Tea Waste Fiber ; ND-Not Detected.

Antioxidant Activity Assays

The results are shown in Table 1 with mean values of three replicates and SD. Although the similar relationships were obtained in terms of highest and lowest levels of antioxidant activity in both methods, different results were obtained for ABTS and DPPH assays. Overall, DPPH assay resulted in lower antioxidant activity values than ABTS assay for both AAE and TE. There were also differences between AAE and TE values of the same sample calculated using the same assay. TE values were only slightly higher than AAE values when compared in the same assay with very close values to each other. This result is in accordance with a previous study comparing the antioxidant activity of different reference compounds. The researchers found almost equal activity of ascorbic acid and Trolox using DPPH and ABTS assays (Nenadis *et al*., 2004). For both assays, AAE and TE values were similar in terms of the highest and lowest values of antioxidant activity among different extracts when results were evaluated in the same assay. The highest antioxidant activity obtained for thyme, mint, uckun, green tea waste and grape seeds were with 50%, 80%, 100%, 70%, and 70% ethanol, respectively, in ABTS assay for both AAE and TE values. On the other hand, using DPPH assay, highest values of antioxidant activity were obtained with 50% ethanol extraction for thyme, mint, and uckun and 70% for green tea fiber waste and grape seeds. While the results from two assays were somewhat consistent in terms of highest antioxidant activity, only mint and uckun samples had different results in different assays. A possible explanation might be the structural differences of two assays and difference in the phenolic compounds of each plant (Kim *et al*., 2002). In ABTS assay, 80% ethanol extraction showed highest antioxidant activity for thyme, mint, and uckun, while 60% and 50% ethanol extraction showed highest antioxidant activity for green tea fiber waste and grape seeds, respectively. In DPPH assay, 70% ethanol extraction showed highest antioxidant activity for thyme and grape seeds, and 50% ethanol extraction showed highest antioxidant activity for mint and uckun, while green tea fiber waste showed highest antioxidant activity in pure ethanol extraction (Table). TPC determined by Folin Ciocalteu's method showed a significant correlation with antioxidant activity levels overall (Table 3). There were significant correlations (p=0.01) between TPC and AAE (R=0.789) and TE (R=0.790) determined by ABTS, and between TPC and AAE (R=0.577) and TE (R=0.582) determined by DPPH. The significant correlation values suggest that antioxidant activity of the five plants tested is mainly due to their phenolic compounds. Our results are in accordance with previous work reporting varying correlations between TPC and antioxidant activity assays (ABTS and DPPH) (Piluzza and Bullitta, 2011; Alwazeer and Dham, 2018).

The importance of correlations between TPC and antioxidant activity was shown in Table 3. High correlations were found between TPC and ABTS_AA (R= 0.789), and TPC and ABTS_TE (R= 0.790). Medium correlations were found between TPC and DPPH_AA (R= 0.577), and TPC and DPPH_TE (R= 0.582). Also, there was a significant

correlation (p=0.01) between IC50 values of ABTS and DPPH assays (R=0.868). High correlation could be explained by the similarity of the reactivity of phenolic compounds in tested plants toward both radicals (ABTS and DPPH) (Mariutti *et al*., 2008).

Table 3. Correlations between total phenolic content and antioxidant activity analysis.
Çizelge 3. Toplam fenolik madde içeriği ve antioksidan aktivite arasındaki korelasyon.

	TPC	ABTS_IC50	DPPH_IC50	ABTS_AA	ABTS_TE	DPPH_AA	DPPH_TE
TPC	1	-.372**	-.333**	.789**	.790**	.577**	.582**
ABTS_IC50		1	.868**	-.403**	-.401**	-.342**	-.345**
DPPH_IC50			1	-.398**	-.398**	-.399**	-.405**
ABTS_AA				1	1.000**	.429**	.431**
ABTS_TE					1	.430**	.431**
DPPH_AA						1	.966**
DPPH_TE							1

** Correlation is significant (p<0.01).

CONCLUSION

Our focus was investigating the effect of different ratio ethanol:water solvents on the phenolic compositions and antioxidant properties of five different antioxidant-rich plants. For TPC assay, optimum ethanol ratio was 70% for uckun and green tea fiber waste, 60% for grape seed and pure water extraction was optimum for thyme and mint. For ABTS assay, 80% ethanolic extraction showed highest antioxidant activity (lower IC50) for thyme, mint and uckun, while 60% and 50% was optimum for green tea fiber waste and grape seeds, respectively. For DPPH assay, 70% ethanolic extraction showed highest antioxidant activity (lower IC50) for thyme and grape seeds, while 50% ethanol was optimum for mint and uckun, and pure ethanol extraction was best for green tea fiber waste. For two different antioxidant activity assays, different ethanol ratios were optimum for same plant due to the structural differences of DPPH and ABTS assays and differences in the compositions of phenolic compounds of the plants. Our results showed that the plants tested in this study have varying antioxidant activity and TPC. Extracting antioxidant compounds and showing optimum extraction conditions could provide further alternative application in food industry other than traditional utilization of these plants. Additionally, grape seeds and green tea waste fiber were found to have the highest level of TPC as well as highest antioxidant activity. Since grape seeds and green tea waste fiber are the waste products of food industry, they can be utilized in antioxidant compound production to be used in fortification of any product including functional food product development.

CONFLICT OF INTEREST

The authors report that there are no conflicts of interest to declare.

DECLARATION OF AUTHOR CONTRIBUTION

Menekşe Bulut: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. Hacer Akpolat: Formal analysis, Writing - review & editing. Yusuf Tunçtürk: Conceptualization, Methodology, Resources, Supervision. Duried Alwazeer: Conceptualization, Methodology, Resources, Supervision. Ayşe Türkhan: Investigation.

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