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Antioxidant activity and polyphenol content of cherry stem (*Cerasus avium* L.) determined by LC–MS/MS

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ABSTRACT

In this study, the antioxidant and antiradical properties of cherry stem (*Cerasus avium* L.) were examined. The ferric thiocyanate method, ferric ion (Fe^{3+}) and cupric ion (Cu^{2+}) reducing assays and ferrous ions (Fe^{3+}) chelating assay were used in order to measure the antioxidant activity of the plant. Also, its antiradical activity was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) radical scavenging activity. Additionally, total phenolic and flavonoid contents of the plant were determined. It was indicated that, both the water extract of cherry stem (WECS) and ethanol extract of cherry stem (EECS) have both antioxidant and antiradical properties, and there is a correlation between these properties and the phenolic and flavonoid contents. Quantities of caffeic acid, ferulic acid, syringic acid, ellagic acid, quercetin, α -tocopherol, pyrogallol, *p*-hydroxybenzoic acid, vanillin, *p*-coumaric acid, gallic acid and ascorbic acid were detected by high performance liquid chromatography and tandem mass spectrometry (LC–MS/MS). This study will bring an innovation for further studies conducted on the antioxidant properties of WECS and EECS.

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1. Introduction

Oxygen (O_2) is a considerably important element for aerobic living. Life on earth is inconceivable without oxygen, but at a higher concentration of this vital element is toxic to aerobes. Reactive oxygen species (ROS), which contain oxygen, are chemically reactive molecules due to the presence of unpaired electrons. They may occur in the human body during environmental stress and can be very dangerous. This may result in significant damage to cell structures (Gülçin, 2012). Most of the damaging effects of oxygen are due to ROS, which include superoxide anion radicals ($\text{O}_2^{\bullet-}$), hydroxyl radicals (OH^{\bullet}), hydroperoxyl radicals (HOO^{\bullet}), peroxy (ROO^{\bullet}) and non-free radical species such as hydrogen peroxide (H_2O_2), ozone (O_3), and singlet oxygen ($^1\text{O}_2$) (Balaydin, Gülçin, Menzek, Göksu, & Şahin, 2010; Halliwell & Gutteridge, 1989). ROS can lead to many diseases as atherosclerosis, coronary heart diseases, aging and cancer (Li, Wong, Cheng, & Chen, 2008). These diseases arise from the uncontrolled production of ROS and unbalanced mechanism of antioxidant protection system (Gülçin, Elias, Gepdiremen, Taoubi, & Köksal, 2009). In order to decrease harmful effects of ROS, the natural antioxidants obtained from plants can be used and there are also synthetic antioxidants such as BHA and BHT. However, the use of these molecules has certain risks (Sun & Fukuhara, 1997). Therefore, in recent years, the use of synthetic antioxidants has been

restricted in many countries and the interest towards natural antioxidants has increased more and more. Hence, natural antioxidants are preferred over synthetic antioxidants by most consumers (Gülçin, 2010; Köksal & Gülçin, 2008). One of the most important natural sources of antioxidants is the medicinal plant, on which many studies have been conducted so far (Gülçin et al., 2009). Medicinal plants have rich phenolic content. The main sources of natural antioxidants in the human diet are cereals, plants and fruits (Pokorny, 2007; Vijaya Kumar Reddy et al., 2010). Natural antioxidants, which are available in these sources, protect the human body against free radicals and oxidative stress. These antioxidants play a very important role in human health (Serbetçi Tohma & Gülçin, 2010).

In addition, other parts of some plants such as the stem and root are frequently used in alternative medicine (Baytop, 1999). In recent years, cherry (*Cerasus avium* L.) stems have been widely used in folk medicine in Anatolia. After having been dried and boiled cherry (*Cerasus avium* L.) stem is used for treatment. Many species of cherry plants are extensively cultivated in Turkey for their fruits (Baytop, 1999).

In this study, for the determination of antioxidant activity of the stems of the cherry (*Cerasus avium* L.); total antioxidant activity determination by ferric thiocyanate method, radical scavenging activity, reducing powers and total phenolic and total flavonoid compounds in plants were determined. Another significant goal of this study was to clarify the quantities of polyphenol contents such as caffeic acid, ferulic acid, syringic acid, ellagic acid, quercetin, α -tocopherol, pyrogallol, *p*-hydroxybenzoic acid, vanillin, *p*-coumaric acid, gallic acid

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and ascorbic acid in WECS and EECS using high performance liquid chromatography and tandem mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Chemicals

In this study, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), linoleic acid, 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ethylenediaminetetraacetic acid (EDTA), polyoxyethylene sorbitan monolaurate (Tween-20), 2'-bipyridine and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany) and ammonium thiocyanate was purchased from Merck.

The following compounds were used as standards in LC–MS/MS analysis: caffeic acid (98%, Sigma-Aldrich), ferulic acid (98% Sigma-Aldrich), syringic acid (97%, Fluka), ellagic acid (95%, Fluka), quercetin (98%, Sigma-Aldrich), α -tocopherol (98%, Fluka), catechol (99% Sigma-Aldrich), pyrogallol (98%, Sigma-Aldrich), p-hydroxybenzoic acid (99%, Merck), vanillin (99% Merck), p-coumaric acid (98%, Sigma-Aldrich), gallic acid (98%, Sigma-Aldrich) and ascorbic acid (99%, Sigma-Aldrich). Stock solutions were prepared as 5 mg/L in ethanol, with the exception of catechol and ascorbic acid, which were prepared as 50 mg/L and 25 mg/L respectively, although in the same solvent. Curcumin (97%) and HPLC grade methanol were purchased from Merck (Darmstadt, Germany). Calibration solutions were prepared in ethanol–water (50:50, v/v) in a linear range (Table 1). Dilutions were performed through automatic pipettes and glass volumetric flasks, which were stored in $-20\text{ }^{\circ}\text{C}$ in glass containers. 1 mg/L curcumin solution was freshly prepared and 0.1 mL of this solution was used as an Internal Standard (IS) in all LC–MS/MS experiments.

2.2. Plant samples

The samples of cherry plants (*Cerasus avium* L.) were collected in May at a village of Uluköy, Erzincan, Turkey and identified by Dr. Mustafa Korkmaz (Erzincan University, Faculty of Sciences and Arts, Department of Biology, Erzincan, Turkey). The stems and fruit parts of the collected plants were separated and washed with distilled water and then dried in the shade at room temperature.

2.3. Preparation of the extracts

The extraction process was carried out as described previously (Gülçin, Tel & Kirecci, 2008). WECS was prepared with 25 g dried cherry (*Cerasus avium* L.) stem, which was ground in a mill and then mixed with 100 mL of distilled water. This mixture was boiled on a magnetic stirrer for 20 min. The extracts were filtered, and filtrates were frozen

and lyophilized in lyophilizer under $5\text{ }\mu\text{m Hg}$ pressure at $-50\text{ }^{\circ}\text{C}$ (Labconco, Freezone).

As for EECS, 25 g dried cherry (*Cerasus avium* L.) stem was ground in a mill, and the powdery cherry (*Cerasus avium* L.) stem was mixed with 100 mL ethanol on a magnetic stirrer for 1 h. The extracts were filtered and then filtrates were collected. The ethanol was removed by a rotary evaporator (RE 100 Bibby, Stone Staffordshire England) at $50\text{ }^{\circ}\text{C}$. All the extracts were put in a dark plastic bottle and stored at $-20\text{ }^{\circ}\text{C}$ until they were used for experimental studies (Gülçin, Oktay, Şerbetcı, Beydemir, & Küfrevioğlu, 2008a, Gülçin et al., 2008b).

2.4. Determination of the total phenolic content by Folin–Ciocalteu assay

The total phenolic contents in cherry (*Cerasus avium* L.) stems were estimated by a colorimetric assay based on the procedure described by Singleton, Orthofer, and Lamuela-Raventós (1999). 1 mg EECS or WECS was added into test tube and the final volume reached 23 mL with the addition of distilled water. Afterwards, 0.5 mL of Folin–Ciocalteu reagent and 1.5 mL of Na_2CO_3 (2%) were added. The samples were vortexed and then were kept at room temperature for 30 min. The absorbance measurements were recorded at 760 nm. The results were reported as μg gallic acid equivalents (GAE) per mg extract.

2.5. Determination of the total flavonoid content

Flavonoids are the group of polyphenolic compounds, that are commonly available in the human diet, and they are found ubiquitously in plants. The total flavonoid contents in EECS and WECS were estimated by a colorimetric assay based on the procedure described by Gülçin et al. (2011). 1 mg EECS or WECS sample was added into a test tube. Then 0.1 mL CH_3COOK (1 M) and 0.1 mL of 10% $\text{Al}(\text{NO}_3)_3$ in 4.3 mL ethanol were then added and the samples were vortexed. Then the vortexed samples were kept at room temperature for 40 min. The absorbance measurements were recorded at 415 nm. The results were reported as μg quercetin equivalents (QE) per mg extract.

2.6. Preparation of test solution for LC–MS/MS

One hundred milligram of WECS and EECS was dissolved in 5 mL of ethanol–water (50:50 v/v) in a volumetric flask, 1 mL of which was transferred into another volumetric flask of 5 mL. 100 μL of curcumin was then added and diluted to the volume with ethanol–water (50:50 v/v). 1.5 mL of aliquot out of the final solution was transferred into a capped autosampler vial and 10 μL of sample was injected to LC. The samples in the autosampler were kept at $15\text{ }^{\circ}\text{C}$ during the experiment (Figs. 2 and 3, Gülçin, Bursal, Şehitoğlu, Bilsel & Gören, 2010).

Table 1
Validation and uncertainty parameters for antioxidant phenolic acids.

No	Compounds name	Linear regression equation	r^2	Linear range (ppm)	LOD/LOQ (ppb)	Recovery (%)	U_{95} (%)
1	Caffeic acid	$y: 4.1981x + 0.0831$	0.995	0–0.5	0.6/2.3	90.0	7.76
2	Ferulic acid	$y: 2.483x - 0.0347$	0.996	0–1	0.2/0.8	94.1	3.97
3	Syringic acid	$y: 1.599x - 0.0131$	0.997	0–1	0.3/1.5	94.7	3.10
4	Ellagic acid	$y: 0.2358x + 0.0003$	0.992	0–1	0.2/1	99.2	2.53
5	Quercetin	$y: 0.245x - 0.0001$	0.992	0–1	1.2/4.2	100.1	1.64
6	α -Tocopherol	$y: 0.0743x - 0.0079$	0.986	0–2.5	10/50	104	3.43
7	Catechol	$y: 0.0246x + 0.0154$	0.991	1–25	7.5/25	98.2	2.40
8	Pyrogallol	$y: 0.411x - 0.0107$	0.993	0–1	1.4/5	101.5	2.06
9	p-Hydroxybenzoic acid	$y: 5.664x - 0.0436$	0.998	0–1	0.5/2	100.7	1.84
10	Vanillin	$y: 5.516x - 0.0732$	0.997	0–1.0	0.6/2	99	1.93
11	p-Coumaric acid	$y: 10.976x - 0.1661$	0.996	0–1	0.2/1	93.6	3.98
12	Gallic acid	$y: 2.236x - 0.046$	0.996	0–1	0.4/1.4	101.3	1.87
13	Ascorbic acid	$y: 0.0171x - 0.00114$	0.995	0.1–10	15/50	108.0	2.28

2.7. Instruments and chromatographic conditions

Experiments were performed with Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometer equipped with a Macherey-Nagel Nucleoder C18 Gravity column (125×2 mm i.d., 5 µm particle size). The mobile phase was composed of methanol (A, 0.5% formic acid) in water (B, 0.5% formic acid), the gradient program of which was 0–1.00 min 50% A and 50% B, 1.01–30.00 min 100% A and finally 30.01–35.00 50% A and 50% B. The flow rate of the mobile phase was 0.3 mL/min, and the column temperature was set to 30 °C. The injection volume was 10 µL (Gülçin et al., 2011).

2.8. Optimisation of the HPLC method and LC–MS/MS procedure

It was determined after a series of experiments that one of the best mobile phase solutions was the gradient of acidified methanol and water system. Such a mobile phase was found to be satisfactory for the ionization abundance and the separation of the compounds. Proper ionization of small and relatively polar antioxidants was ensured by the ESI source instead of the APCI source. The ionization technique and collision energies of the experiments constitute the most important parameters in quantitative mass spectrometry analyses. Triple quadrupole mass spectrometry system has always been widely applied by researchers due to its fragmented ion stability (Gören, Çiknıç, Çergel & Bilsel, 2009). Therefore, triple quadrupole mass spectrometry was selected as the instrument of analysis in the experiment. The optimum ESI parameters were determined as 2.40 mTorr CID gas pressure, 5000 V ESI needle voltage, 600 V ESI shield voltage, 300 °C drying gas temperature, 50 °C API housing temperature, 55 psi Nebulizer gas pressure and 40 psi drying gas pressure (Gülçin et al., 2010). Detailed information regarding the experiment parameters is presented in Table 2.

2.9. Validation

In validation experiments of all the compounds, curcumin was used as an internal standard. Linearity, recovery, repeatability, LOD and LOQ were used as validation parameters of experiments.

Table 2

LC–MS/MS parameters of selected compounds and amounts of antioxidants in WECS and EECS in mg/kg concentration [WECS: water extract cherry (*Cerasus avium* L.) stem, EECS: ethanol extract cherry (*Cerasus avium* L.) stem].

No	Compounds	Parent ion	Daughter ion	Collision energy (V)	Amount of antioxidants in the extracts (mg/kg) ^{a,b}	
					WECS	EECS
	Curcumin	367	216.4	10	– ^{a,c}	– ^{a,c}
1	Caffeic acid	179	134	11	11.4	91.3
2	Ferulic acid	193	177.5	10	231.5	220.4
3	Syringic acid	197	181.6	10	– ^c	– ^c
4	Ellagic acid	301	150	10	– ^c	71.1
5	Quercetin	301	178.6	10	– ^c	116.0
6	α-Tocopherol	429	162.6	20	– ^c	– ^c
7	Catechol	109	64.8	35	– ^c	– ^c
8	Pyrogallol	125	78.7	20	262.0	261.1
9	p-Hydroxybenzoic acid	137	92.7	10	11.1	20.0
10	Vanillin	181	135.5	10	79.2	– ^c
11	p-Coumaric acid	163	118.7	10	38.2	161.1
12	Gallic acid	169	124.6	10	50.3	41.1
13	Ascorbic acid	175	114	12	31.1	30.2

^a It was used for the internal standard.

^b The uncertainty of the results should be calculated in line with Table 1.

^c These values are below the limits of the quantification.

2.9.1. Linearity

The linearity of the method for compounds under the examination was assayed by analyzing the standard solutions. The linearity ranges of the compounds are given separately in Table 1. The correlation coefficients (r^2) were found to be ≥ 0.99 . Linear regression equations of the reported compounds are also presented in Table 1, where y is the peak area and x is the concentration.

2.9.2. Recovery, repeatability and precision

The recoveries of the experiments were determined by three fortification levels (0.25, 0.5 and 1 mg/L for compounds 1–6 and 8–10, and 1, 5 and 10 mg/L for compounds 7 and 13, respectively). The unspiked plant extracts were also analyzed to determine the selectivity of curcumin (IS) in blank sample, for which no peak was found. The recoveries of the reported compounds were evaluated for each fortification level employing the following formula and the recoveries of experiments are presented in Table 1.

$$\text{Recovery (\%)} = \left(\frac{\text{MC} - \text{EC}}{\text{SC}} \right) \times 100$$

where MC is the measured concentration; EC is endogenous concentration and SC is the spiked concentration. Precision of the method was evaluated by repeating the measurements at three concentrations for each compound. Good precision was determined and the results were implemented to the uncertainty budget.

2.9.3. LOD and LOQ

LOD and LOQ of the LC–MS/MS methods for the reported compounds were found to be 0.5–50 µg/L. The limits of the quantification (LOQs) were determined to be 10 times higher than the S/N in terms of the above concentrations (Table 1).

2.10. Estimation of uncertainty

2.10.1. Identification of uncertainty sources

The analyte concentration in the sample solution was expressed as µg/L within the linear range. Concentrations of the compounds in the solution calculated by the calibration curve were converted to units of mg/kg of crude sample with the equation below. To determine the quantity of compounds above the linear range, the samples were diluted with the mobile phase to obtain satisfactory results.

$$A = \left(\frac{C_a \times V_{\text{Final}}}{m \times V_{\text{Initial}}} \right) \times 1000$$

where A is amount (mg/kg); C_a is the analyte concentration calculated by calibration curve (in µg/L); V_{final} is the final diluted volume before the analysis; m is amount of extract as gram; and V_{initial} is the initial sample volume (Gülçin et al., 2010).

2.10.2. Identification of standard uncertainties

The sources and the quantification of uncertainty for the applied method were evaluated and calculated by using EURACHEM/CITAC Guide, 2000, and below equation, respectively (EURACHEM/CITAC 2000). It was determined that the sources of uncertainty for LC–MS/MS experiments were the impurity of reference standard, the sample weighing, calibration curve and dilution of the solutions. For all analytes, the maximum contribution comes from calibration. Detailed procedures of uncertainty evaluation have been previously addressed in the literature Gülçin et al. (2011).

$$U_{\text{rel}}(\text{Con}) = \sqrt{u^2 \text{rel}(C_a) + u^2 \text{rel}(m_a) + u^2 \text{rel}(m_{\text{is}}) + u^2 \text{rel}(V_{\text{Final}}) + u^2 \text{rel}(V_{\text{Initial}})}$$

where C_a is the uncertainty from the calibration curve, V_{Final} is the final volume of the sample, V_{Initial} is the initial volumes of internal standard (IS), sample weighing of m_a is the weighing of analyte and m_{is} is the weighing of internal standard. The percent relative uncertainties [$U_{95}(\%)$] of the reported compounds were found to be within the range of 1.64% and 7.76% at 95% confidence level ($k:2$) (Table 1) (Gülçin et al., 2010).

2.11. Total antioxidant activity determination by ferric thiocyanate method

The total antioxidant activities of WECS, EECS and standard antioxidants were determined by ferric thiocyanate method in linoleic acid emulsion (Mitsuda, Yuasumoto, & Iwami, 1996). The different concentrations of WECS and EECS (10–20 $\mu\text{g}/\text{mL}$) were prepared in 2.5 mL of potassium phosphate buffer solution (0.04 M, pH 7.0) and then 2.5 mL of linoleic acid emulsion was added into potassium phosphate buffer solution (0.04 M, pH 7.0). The mixture was incubated at 37 °C. During the incubation period, a 0.1 mL aliquot of the mixture was diluted with 3.7 mL of ethanol and then added to the mixture of 0.1 mL of ammonium thiocyanate (30%) and 0.1 mL of ferrous chloride (20 mM) in hydrochloric acid (3.5%). The absorbance was measured at 500 nm for the determination of the peroxide levels. The peroxides formed during linoleic acid oxidation oxidized Fe^{2+} to Fe^{3+} and the latter ions formed a complex with thiocyanate. The complex had a maximum absorbance at 500 nm. The process was repeated every 8 h until the maximum absorbance value was achieved in the control. The amounts of inhibition were calculated by the following equation:

$$A(\%) = 100 - \left(\frac{A_s}{A_c} \times 100 \right)$$

where, A is inhibition of lipid peroxidation, A_s is the absorbance value of the control reaction and A_c is the absorbance value of working samples and standards.

2.12. Fe^{3+} reducing power assay

The reducing activities of WECS and EECS were examined through the method of Oyaizu (1986). The reducing capacity levels of WECS and EECS, which mean to reduce the ferric–ferricyanide complex to the ferrous–ferricyanide complex of Prussian blue, were measured by reading the absorbance at 700 nm. Shortly, different concentrations of WECS and EECS (10–30 $\mu\text{g}/\text{mL}$) in 1 mL of distilled water were mixed with a phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. Then, 2.5 mL trichloroacetic acid (10%) was added to the mixture. Finally, 0.5 mL of FeCl_3 (0.1%) was added to this solution, and the absorbance was measured at 700 nm. An increasing level of absorbance indicates greater reduction capability.

2.13. Cu^{2+} reducing power assay

The cupric ion (Cu^{2+}) reducing method was used with slight modification (Ak & Gülçin, 2008) to determine the reducing ability of WECS and EECS. Briefly, 0.25 mL of CuCl_2 solution (0.01 M), 0.25 mL of ethanolic neocuproine solution (7.5×10^{-3} M) and 0.25 mL of $\text{CH}_3\text{COONH}_4$ buffer solution (1 M) were placed into a test tube and this solution was mixed with different concentrations of WECS and EECS (10–30 $\mu\text{g}/\text{mL}$). At the end of these mixtures, the final volume increased to 2 mL together with distilled water. The absorbance was read at 450 nm after 30 min. An increasing level of absorbance indicates a greater reduction capability.

2.14. Chelating activity on ferrous ion (Fe^{2+})

Ferrous ion (Fe^{2+}) chelating activities of EECS and WECS were measured according to the method of Re et al. (1999). In line with this method, the different concentrations (10–30 $\mu\text{g}/\text{mL}$) of EECS or WECS in 0.25 mL ethanol, 0.25 mL FeSO_4 solution (2 mM), 1 mL Tris–HCl buffer solution (pH 7.4), 1 mL 2,2'-bipyridine solution (0.2% in 0.2 M HCl) and 2.5 mL ethanol solution were placed into a test tube, respectively. The total volume was adjusted to 6 mL with distilled water and the mixture was stirred vigorously. The absorbance was measured at 562 nm. In addition to the existing standards, EDTA was also used as a standard metal chelator.

2.15. DPPH free radical scavenging activity

DPPH free radical scavenging activities for WECS and EECS were measured according to the method of Blois (1958). In this method, a 0.1 mM ethanolic solution of DPPH• was prepared on a daily basis. Then, 1 mL of this solution was added to 3 mL of WECS and EECS solution in ethanol at different concentrations (10–30 $\mu\text{g}/\text{mL}$). After half an hour, the absorbance level was measured at 517 nm for each sample. The DPPH• concentration (mM) in the reaction medium was calculated through the following calibration curve determined by linear regression ($r^2: 0.9974$):

$$\text{Absorbance} = 5.869 \times 10^{-4} [\text{DPPH}\cdot] + 0.0134.$$

The capability to scavenge the DPPH• radical was calculated using the following equation:

$$\text{DPPH}\cdot \text{scavenging effect}(\%) = \left(\frac{A_c - A_s}{A_c} \right) \times 100$$

where A_c is the initial concentration of the stable DPPH free radical and A_s is the absorbance of the concentration of vestigial DPPH• in the presence of WECS and EECS (Köksal & Gülçin, 2008).

2.16. Statistical analysis

All the analyses with regards to the total antioxidant activity were carried out in duplicate analysis. However, the other analyses were carried out in triplicate. The data was recorded as mean \pm standard deviation and analyzed by SPSS (version 11.5 for Windows 98, SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. The significant differences between the means were determined by LSD tests. $P < 0.05$ was accepted as significant while $p < 0.01$ was regarded as being substantially significant.

3. Result and discussion

Lipid peroxidation can have hazardous effects on foods by forming complex mixture of secondary breakdown products of lipid peroxides. The further intake of these foods can lead to a number of adverse effects including toxicity in the mammalian cells. Lipid peroxidation is thought to proceed via radical mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids. Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation such as lipid peroxidation (Roginsky & Lissi, 2005).

Natural antioxidants have biofunctionalities such as the reduction of chronic diseases, DNA damage, mutagenesis, carcinogenesis, etc. and inhibitions of pathogenic bacteria growth, which are usually associated with the termination of free radical propagation in biological systems. Therefore, antioxidant capacity is widely used as a parameter for medicinal bioactive components. A number of assays have been introduced to measure the total antioxidant activity of pure compounds so far (Miller, Castelluccio, Tijburg, & Rice-Evans, 1996).

In this study, the antioxidant activities of the WECS and EECS were compared to BHA, BHT, α -tocopherol and its water-soluble analog trolox. The antioxidant activities of the WECS, EECS and standards were measured using the total antioxidant activity by ferric thiocyanate method, DPPH free radical scavenging activity, metal chelating activity, Fe^{3+} and Cu^{2+} reducing activity. In addition the total phenolic and flavonoid contents of these samples were determined.

3.1. Total antioxidant activity – ferric thiocyanate method

The ferric thiocyanate method determines the amount of peroxide produced during the initial stages of oxidation. The protective effects of WECS and EECS on lipid peroxidation of linoleic acid emulsion at the same concentration (20 $\mu\text{g}/\text{mL}$) are presented in Fig. 1. The inhibition effects of WECS and EECS on linoleic acid peroxidation were found to be 51.8 and 47.3%, respectively. On the other hand, at the above-mentioned concentration, α -tocopherol, trolox, BHA, and BHT displayed 61.5, 29.8, 74.4, and 71.2% inhibition on peroxidation of linoleic acid emulsion, respectively. These results clearly showed that WECS and EECS had significantly potent antioxidant activities in the ferric thiocyanate assays (Fig. 1 and Table 4).

3.2. Reducing power

Reducing powers of WECS, EECS and standards (BHT, α -tocopherol and trolox) were determined by ferric ions (Fe^{2+}) and cupric ion (Cu^{2+}) reducing methods. In the measurements of the reductive activity, the Fe^{3+} – Fe^{2+} transformation was examined in the presence of WECS and EECS using the method of Oyaizu (1986). The reducing activities of WECS (r^2 : 958), EECS (r^2 : 975) and standard compounds increased with increasing concentrations. Reducing powers of samples decreased as follows: BHA (1.334 ± 0.060) > BHT (0.974 ± 0.124) > EECS (0.709 ± 0.061) > Trolox (0.570 ± 0.110) \geq α -Tocopherol (0.532 ± 0.032) \approx WECS (0.523 ± 0.049) (Table 4). These results emphasized that EECS had notable ferric ions (Fe^{3+}) reducing ability and electron donor properties for neutralizing free radicals. On the other hand, the Cu^{2+} reducing ability (CUPRAC method) is frequently used so as to determine the reducing powers of antioxidant compounds and herbal extracts. Cu^{2+} reducing capabilities of WECS and EECS were measured by Cuprac method and were found to be concentration dependent (Table 4). A correlation was also found between the cupric ion reducing abilities and concentrations of the samples. Decreases observed in the results in terms of the reducing abilities of the samples were as follows: WECS (0.523 ± 0.007 , r^2 : 947) > BHA (0.399 ± 0.056) > BHT (0.357 ± 0.093) > EECS (0.269 ± 0.041 , r^2 : 892) \approx Trolox (0.265 ± 0.021) > α -Tocopherol (0.235 ± 0.032).

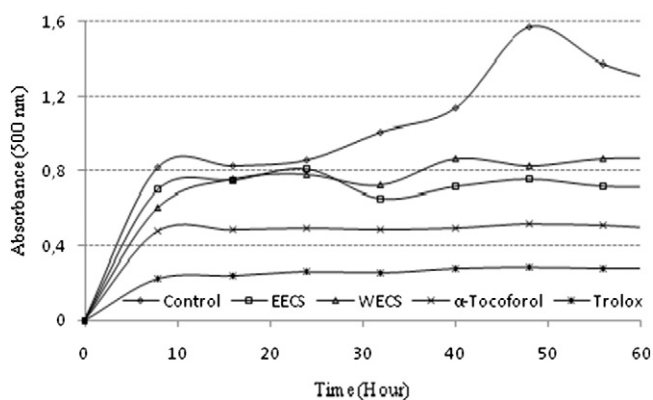


Fig. 1. Total antioxidant activities of WECS and EECS [WECS: water extract cherry (*Cerasus avium* L.) stem, EECS: ethanol extract cherry (*Cerasus avium* L.) stem].

3.3. Chelating capacity

Metal ions can cause lipid peroxidation, which can induce the production of free radicals and lipid peroxides. Therefore, metal chelating activity indicates antioxidant and antiradical properties. In this study, 2,2'-bipyridine was used as a metal chelating agent. A decreasing level of absorbance in the reaction mixture indicates a higher metal chelating capability. According to the results, EECS and WECS indicated higher metal chelating levels when compared to that of standard antioxidants. The results obtained from ferrous ion (Fe^{2+}) chelating methods clearly showed that EECS and WECS were found the most effective ferrous ion metal chelating effect (Table 4). IC_{50} values for the metal chelating capacity of EECS and WECS were found to be 11.59 $\mu\text{g}/\text{mL}$ (r^2 : 879) and 24.90 $\mu\text{g}/\text{mL}$ (r^2 : 842), respectively. Also, the ferrous ion chelating effects of EECS and WECS were compared to those of BHA, BHT, α -tocopherol and trolox. On the other hand, IC_{50} values for BHA, BHT, α -tocopherol and trolox were found to be 42.17, 53.36, 43.19 and 30.71 $\mu\text{g}/\text{mL}$, respectively. These results show that the ferrous ion chelating effect of EECS and WECS was higher than those of EDTA, BHA, BHT, α -tocopherol and trolox ($p > 0.05$).

3.4. Radical scavenging activity

Radical scavenging activity is extremely important due to the deleterious role of free radicals in nourishment and living systems. The excessive production of free radicals accelerates the oxidation of lipids in nourishments and reduces their quality. DPPH radicals (DPPH•) have been extensively used to measure the radical scavenging abilities of various antioxidant substances (Gülçin, 2009). DPPH• scavenging assay was used in this study for the primary screening of WECS and EECS free radical scavenging activity. This is because of the fact that this assay can accommodate a large number of samples in a short period and is sensitive enough to detect natural compounds at low concentrations. Also, DPPH• scavenging method provides information on the reactivity of test compounds with a stable free radical. Furthermore, this method is simple and fast (Gülçin et al., 2009).

Antioxidants react with DPPH•, which is a free radical, and convert it to 1,1-diphenyl-2-picryl hydrazine. In the meantime, the discoloration degree at the test tube indicates the radical-scavenging capability of the antioxidant. In this study, the antioxidant activities of WECS, EECS and standards were measured. DPPH• provides a strong absorption at 517 nm because of its odd electron. As this electron becomes paired off in the existence of a free radical scavenger, the absorption vanishes (Talaz, Gülçin, Göksoy, & Saracoglu, 2009). Consequently, especially WECS (r^2 : 965), EECS (r^2 : 893) in particular exhibits a remarkable DPPH free radical scavenging activity. BHA and BHT were used as the reference radical scavengers in this study. As can be seen in Table 3, the scavenging effects of WECS, EECS and standards on the DPPH radical decreased as such: BHA (9.13 $\mu\text{g}/\text{mL}$) \geq Trolox (10.68 $\mu\text{g}/\text{mL}$) \approx α -Tocopherol (11.13 $\mu\text{g}/\text{mL}$) > BHT (13.54 $\mu\text{g}/\text{mL}$) > EECS (17.36 $\mu\text{g}/\text{mL}$) > WECS (23.38 $\mu\text{g}/\text{mL}$). Lower IC_{50} value indicates higher radical scavenging activity.

As shown in Table 4, WECS and EECS were effective DPPH• radical scavenging in concentration-dependent manner (10–30 $\mu\text{g}/\text{mL}$). EC_{50}

Table 3

Total phenolic and total flavonoid contents of WECS and EECS [WECS: water extract cherry (*Cerasus avium* L.) stem, EECS: ethanol extract cherry (*Cerasus avium* L.) stem].

	WECS	EECS
Total phenolic content ^a	118 \pm 9.12	146.5 \pm 13.10
Total flavonoid content ^b	16.3 \pm 3.31	20.5 \pm 4.62

^a Determined as gallic μg of acid equivalent (GAE) in mg extracts.

^b Determined as μg of quercetin equivalent (QE) in mg extracts.

Table 4

Total antioxidant activity by thiocyanate method, reducing ability by Fe³⁺–Fe²⁺ transformation method and Cu²⁺ reducing ability by Cuprac method of WECS, EECS and standard compounds such as BHA, BHT, α-tocopherol and trolox [DPPH•: 1,1-diphenyl-2-picryl-hydrazyl free radical, DMPD•⁺: N,N-dimethyl-p-phenylenediamine radical, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene, WECS: water extract cherry (*Cerasus avium* L.) stem, EECS: ethanol extract cherry (*Cerasus avium* L.) stem].

BHA	Total antioxidant activity ^a	Fe ³⁺ reducing activity [*]	Cu ²⁺ reducing activity [*]	DPPH scavenging activity ^{**}	DMPD ⁺ scavenging activity ^{**}	Fe ²⁺ chelating activity ^{**}
BHA	74.4	1.334 ± 0.060	0.399 ± 0.056	9.13	11.18	42.17
BHT	71.2	0.974 ± 0.124	0.357 ± 0.093	13.54	9.25	53.36
Trolox	29.8	0.570 ± 0.110	0.265 ± 0.021	10.68	9.29	30.71
α-Tocopherol	61.5	0.532 ± 0.032	0.235 ± 0.032	11.13	9.78	43.19
WECS	51.8	0.523 ± 0.049	0.523 ± 0.007	23.38	14.43	11.59
EECS	47.3	0.709 ± 0.061	0.269 ± 0.041	17.36	15.44	24.90

^a Percentage inhibition effect of 20 µg/mL concentration of WECS, EECS and standard compounds such as BHA, BHT, α-Tocopherol and trolox on linoleic acid emulsion peroxidation determined by thiocyanate method.

^{*} The values were expressed as absorbance and belonging to 10 µg/mL concentration. High absorbance indicates high reducing power ability. The difference between indicated concentration of WECS and EECS and the control values were statistically significant (p < 0.01).

^{**} The values were expressed as IC₅₀. Lower IC₅₀ value indicates higher radical scavenging or metal chelating ability.

for WECS and EECS were 14.43 µg/mL and 15.44 µg/mL, respectively. This value was found as 11.18 µg/mL for BHA, 9.25 µg/mL for BHT, 9.78 µg/mL for α-Tocopherol and 9.29 µg/mL for trolox. There is a significant decrease (p < 0.01) in the concentration of DMPD•⁺ due to the scavenging capacity at all WECS and EECS concentrations. Reportedly, the main drawback of the DMPD•⁺ method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-Tocopherol or BHT were used (Gülçin, 2012).

3.5. Total phenolic content

Phenolic compounds are secondary metabolites widely found in fruits, mostly represented by flavonoids and phenolic acids. The growing interest in these substances is mainly due to their antioxidant potential and the association between their consumption and the prevention of certain diseases. The health benefits of these

phytochemicals are directly linked to a regular intake and their bio-availability. Studies have shown the importance of the regular consumption of fruits, especially for preventing diseases associated with oxidative stress. Phenolic compounds have an aromatic ring bearing one or more hydroxyl groups and their structure may vary from that of a simple phenolic molecule to that of a complex high-molecular mass polymer (Balasundram, Sundram, & Samman, 2006). They interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. Therefore, they act as reducing agents and antioxidants. Many studies have shown that the phenolic contents of plants display antioxidant properties. These antioxidant compounds donate an electron to the free radical and convert it into an innocuous molecule (Gülçin, Mshvildadze, Gepdiremen & Elias, 2006).

Folin–Ciocalteu reagent was used for determination of total phenolic contents in WECS and EECS. The standard graph of gallic acid was

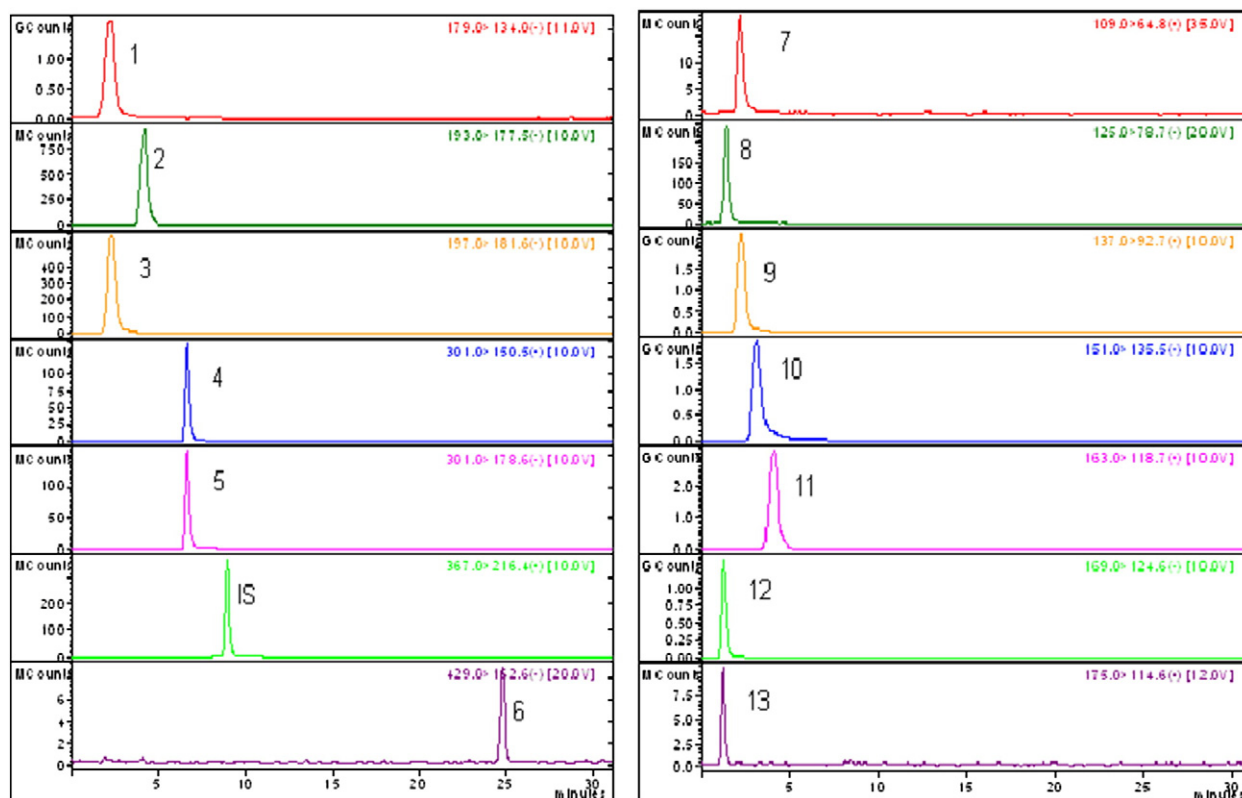


Fig. 2. Standard chromatogram of antioxidant phenolic compounds by LC-MS/MS (mg/mL).

drawn (r^2 : 0.994). The amount of total phenolic was determined from the standard graph equation as gallic acid equivalents per 1 mg of extract (GAE/mg extract). As can be seen in Table 3, 146.5 ± 13.10 and $118.0 \pm 9.12 \mu\text{g}$ of gallic acid equivalents of phenolic content were calculated from 1 mg of EECS and WECS, respectively. A correlation

was found between the antioxidant capacities of WECS, EECS and gallic acid. The correlation coefficient in the graph of the antioxidant capacities obtained from ferric ions reducing antioxidant powers for EECS and WECS was 0.991 and 0.993, respectively while the correlation coefficient in the graph of gallic acid was 0.994. Therefore, it can be

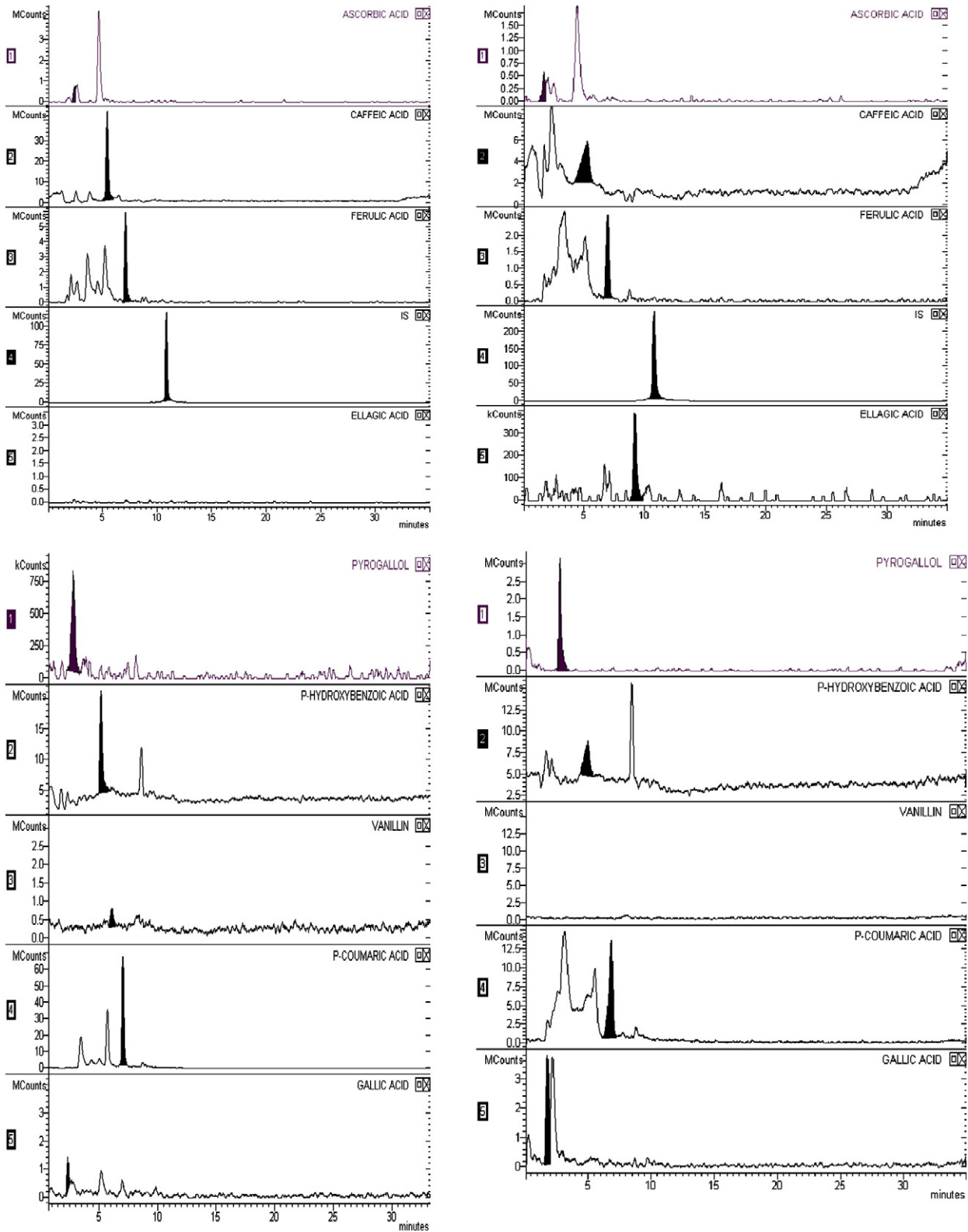


Fig. 3. Chromatogram of antioxidants by LC-MS/MS [diluted sample chromatogram for the correct determination of 3–8 in the linear range of WECS and EECS. (WECS: water extract cherry (*Cerasus avium* L.) stem, EECS: ethanol extract cherry (*Cerasus avium* L.) stem)].

concluded that high phenolic content is an important factor in the antioxidant capacity of cherry (*Cerasus avium* L.) stem. This finding means that the phenolic compounds contribute significantly to the antioxidant capacities of the stem parts of cherry (*Cerasus avium* L.) plants.

3.6. Total flavonoid content

The consumption of the flavonoid-containing fruits and vegetables has been associated with protection against cancer and heart disease. Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants. Quercetin is a well-known plant-derived flavonoid. Some studies have shown that it may have antioxidant properties (Davis, Murphy, Carmichael, & Davis, 2009). The standard graph of quercetin was drawn. The amount of total flavonoid as quercetin equivalents was determined by the equation obtained from this standard graph. The results of EECS and WECS were found to be 20.55 ± 4.62 $\mu\text{g QE/mg extract}$ and 16.3 ± 3.31 $\mu\text{g QE/mg extract}$, respectively (Table 3).

Standard chromatogram of antioxidant phenolic acids by LC–MS/MS (mg/mL) is presented in Fig. 2. Diluted sample chromatogram is also given in Fig. 3 for the correct determination of 3–8 in the linear range of WECS and EECS. According to LC–MS/MS experiment, the main phenolic acids found in WECS and EECS, which were examined, were pyrogallol and ferulic acid, respectively. Phenolic compounds, in particular, are of considerable interest to scientists, manufacturers and consumers due to their influence on food quality, and protective and preventive roles in the pathogenesis of certain types of cancer and several other chronic diseases (Shahidi & Naczk, 2004). It was known that pyrogallol had shown to possess marked antioxidant activity (Bickoff, Coppinger, Livingston, & Campbell, 1952). In addition, *p*-coumaric acid, gallic acid, ascorbic acid and *p*-hydroxybenzoic acid were significantly found in both extracts. Ferulic acid, like many phenols, is an antioxidant in vitro in the sense that it is reactive toward free radicals such as ROS. ROS and free radicals are implicated in DNA damage, cancer, and accelerated cell aging. It is considered as one of the most important phenolic acids, having many physiological functions, including antioxidant, anti-microbial, anti-inflammatory, anti-thrombosis, and anti-cancer activities (Ou & Kwok, 2004). Both animal and in vitro studies suggest that ferulic acid may have direct antitumor activity against cancer. It also protects against coronary disease, lowers cholesterol in serum and the liver and increases sperm viability (Lee, 2005; Ou & Kwok, 2004). *p*-Coumaric is another type of phenolic acid of great interest due to its chemoprotectant and antioxidant properties (Torres y Torres & Rosazza, 2001). In addition, both acids are potential precursors in the biocatalytic production of value-added aromatic natural products (Mussatto, Dragone, & Roberto, 2007). These constituents could be responsible for the antioxidant activities of WECS and EECS. Furthermore, the results show parallelism with the other literature reports (Kumazawa, Hamasaka, & Nakayama, 2004; Marcucci & Bankova, 1999; Medic-Saric, Jasprica, Mornar, Smolic-Bubalo, & Golja, 2004). In addition to the above-mentioned compounds, *p*-coumaric acid has been found in WECS and EECS as another main phenolic compound. Nevertheless, catechol, syringic acid and α -tocopherol were not determined in both extracts (WECS and EECS).

4. Conclusion

This study pointed out comparatively the potential antioxidant properties of WECS and EECS. According to the data obtained in the study, WECS and EECS were found to be effective antioxidant activities in different in vitro assays including ferric thiocyanate method, reducing power, DPPH• scavenging and metal chelating activities when compared to standard antioxidant compounds such as BHA and BHT, α -tocopherol and trolox which is the water-soluble analog of tocopherol. Furthermore, phenolic and flavonoid contents of WECS

and EECS were determined as gallic acid and quercetin equivalents, respectively. In the light of the results obtained in this study, it can be suggested that both extracts (WECS and EECS) have effective antioxidant and antiradical capabilities when compared to standard antioxidant compounds. However, neither of the extracts do not have good chelating powers. Antioxidant and antiradical activities of WECS were found to be lower than that of EECS. The inhibition of lipid peroxidation in linoleic acid emulsion of EECS was found to be higher than that of WECS. The amounts of total phenolic and flavonoid content of EECS were also found to be higher than WECS comparatively. The amounts of total phenolic were approximately six times higher than the amounts of total flavonoid in both extracts. As a conclusion, under normal circumstances, unused and discarded cherry (*Cerasus avium* L.) stems can be used as a new and inexpensive source of antioxidants.

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