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Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey

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ABSTRACT

Propolis, an extremely complex resinous material, exhibits valuable pharmacological and biological properties attributed to the presence of polyphenols. In this study, we determined the antioxidant properties of lyophilized aqueous extract of propolis (LAEP) from Erzurum province of Turkey and correlated the values with total levels of polyphenolic compounds. In order to estimate the capacity of LAEP to act as antioxidants, we studied its 1,1-diphenyl-2-picryl-hydrazyl radicals (DPPH$^{\cdot}$), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radicals (ABTS$^{\cdot+}$), N,N-dimethyl-*p*-phenylenediamine radicals (DMPD$^{\cdot+}$), superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) scavenging activity, total antioxidant activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing ability, ferrous ions (Fe^{2+}) chelating activity. LAEP inhibited 93.2% lipid peroxidation of a linoleic acid emulsion at 30 μg/mL concentration. On the other hand, BHA, BHT, α-tocopherol and trolox displayed 83.3%, 82.1%, 68.1% and 81.3% inhibition of peroxidation at the same concentration, respectively. Quantitative amounts 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 with regard to the antioxidant properties of LAEP.

1. Introduction

Propolis has been much popular as an agent in traditional medicine and food supplementary material for human health in the world (Pereira et al., 2008; Bankova et al., 2000). It is a commercial resinous product and contains phenols and many other preventive agents. Propolis is produced by honeybees using collected extracts from leaves, buds and exudates of various plant floras. Propolis is used as a building material in order to strengthen the borders of combs and as a chemical weapon against the pathogen microorganisms (Wollenweber et al., 1990). Its antibacterial, antiviral, antitumor, anti-inflammatory, anticancer and immunomodulatory effects have been reported (Banskota et al., 2002; Kimoto et al., 2001; Murad et al., 2002; Sforcin et al., 2000; Zhou et al., 2009).

Complexity and variety of the chemical composition of propolis has been reported in the literature. From different botanical and geographical origins of world, more than 300 compounds including volatile organic compounds, flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones, sesquiterpenes, quinones, coumarins, steroids, aminoacids were reported to have been isolated from propolis (Bankova et al., 2000; Marcucci et al., 2000; Ahn et al., 2007). Most of the compounds from isolated propolis include the phenolics with important protective effect against oxidation reactions such as those with regular agents like BHT and hydroquinone. Flavones, coumarines, and many other phenolic compounds have reducing activity, hydrogen-donors and metal chelating properties. They are also used as radical quenchers in organic solutions and biological fluids (Parr and Bolwell, 2000; Çikrikci et al., 2008). All of these compounds are responsible for its biological and pharmacological activities. Therefore, propolis has been used in traditional medicine, cosmetics and food industry from the Europe to East Asia (Banskota et al., 2001; Chaillou and Nazareno, 2009).

The chemical composition of propolis is affected by climate conditions and the type of bee flora. As a result, various biological

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activities of propolis are directly influenced by botanical and chemical diversity (Chaillou and Nazareno, 2009; Han and Park, 1995; Tosi et al., 2007).

Reactive oxygen species (ROS) and free radicals are by-products of normal cellular metabolism in aerobic life where molecular oxygen is ubiquitous. ROS are generated during irradiation by UV light, by X-rays and by gamma rays; are products of metal-catalyzed reactions; are present as pollutants in the atmosphere; are produced by neutrophils and macrophages during inflammation; and are by-products of mitochondria-catalyzed electron transport reactions and other mechanisms (Cadenas, 1989). It was reported that ROS may contribute to oxidative damage of lipids, protein, and nucleic acids in living cells. It is well known that free radicals or reactive oxygen species play important roles in the development of many chronic diseases, such as the aging process, heart disease and cancer (Gülçin et al., 2006a). It is significant for health to look for efficient ways to decrease or depress yielding of free radicals in the body. Due to the disturbance in the equilibrium state of pro-oxidant–antioxidant reaction, ROS are overproduced to induce oxidative stress that inhibits normal functions of cellular lipids, proteins, DNA and RNA. Thus, increasing attention has been directed toward finding some natural antioxidant compounds that could be isolated from herbal medicine and efficiently clear free radicals. Antioxidants serve as a defensive factor against free radicals' effects in the body. They act by inhibiting the initiation and propagation steps, leading to the termination of the reaction and delaying the oxidation process. The mechanism of antioxidants may involve the scavenging of free radicals (Gülçin, 2006a). At present, a variety of synthetic antioxidants are commonly used. However, the use of these compounds has been restricted by legislation due to doubts over their toxic and carcinogenic effects. Plant foods are potential sources of natural antioxidants, such as vitamin C, tocopherol, carotenoids, flavonoid, and phenolic acids which prevent free radical damage. Hence, there is a growing interest in natural and safer antioxidants for food applications, and a growing trend in consumer preferences towards natural antioxidants, all of which have given impetus to the attempts to explore natural sources of antioxidants (Gülçin, 2006b, 2007, 2010).

In this study, we investigated the inhibition of lipid peroxidation in linoleic acid system, ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP), cupric ions (Cu^{2+}) reducing antioxidant power assay (CUPRAC method), DPPH· scavenging, $ABTS^{\cdot+}$ scavenging, $DMPD^{\cdot+}$ scavenging, $O_2^{\cdot-}$ scavenging, H_2O_2 scavenging and ferrous ions (Fe^{2+}) chelating activities of LAEP. These multiple methods are recommended to measure antioxidant properties of food or pharmacological materials that better reflect their potential protective effects. Furthermore, another significant goal of this study was to clarify the polyphenol contents of LAEP.

2. Materials and methods

2.1. Chemicals

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), pyragallol (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, except for catechol and ascorbic acid, which were prepared as 50 and 25 mg/L, respectively, 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 using automatic pipettes and glass volumetric flasks (A class), which were stored at −20 °C in glass containers. Thousand micrograms per litre curcumin solution was freshly prepared, from which 100 μL was used as an Internal Standard (IS) in all LC–MS/MS experiments.

The compounds used for antioxidant activity such as neocuproine (2,9-dimethyl-1,10-phenanthroline), N,N-dimethyl-*p*-phenylenediamine (DMPD), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), riboflavin, methionine, linoleic acid, α-tocopherol, polyoxyethylenesorbitan monolaurate (Tween-20) and trichloroacetic acid (TCA) were obtained from Sigma (Sigma–Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck. All other chemicals used were of analytical grade and obtained from either Sigma–Aldrich or Merck.

2.2. Preparation of aqueous extract of propolis samples (LAEP)

Propolis samples were collected from Erzurum province (Eastern Anatolia) of Turkey and were then kept and dried in the dark until processing.

Extraction was carried out as described previously (Gülçin, 2005; Gülçin et al., 2008). For water extraction, 25 g of air-dried propolis was ground into a fine powder in a blender and mixed with 400 mL boiling water by magnetic stirrer for 15 min. Then the aqueous extract was filtered over cheese-cloth and Whatman No. 1 paper, respectively. The filtrates were frozen at −84 °C in ultra low temperature freezer (Sanyo, Japan) and lyophilized in a lyophilizator under 5 mm-Hg pressure at −50 °C (Labconco, Freezone, Japan).

2.3. Determination of total phenolic content

The total phenol contents of LAEP were determined by using the method of Gülçin et al. (2005) based on the procedure described by Singleton and Rossi (1965). The appropriate dilutions of propolis (0.5 mL) were oxidized with Folin–Ciocalteu's reagent (0.5 mL) in a volumetric flask. The reaction was neutralized with saturated sodium carbonate solution (1.5 mL), followed by adjusting the volume to 23 mL with distilled water. The contents in the tubes were thoroughly mixed and allowed to stand at ambient temperature for 2 h until the characteristic blue colour developed. Absorbance of the clear supernatants was measured at 725 nm using a spectrophotometer. The content of total phenolic in LAEP was calculated based on a standard curve prepared using gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of sample. Standard calibration was made from 100 to 500 μg gallic acid (r^2: 0.971).

$$\text{Absorbance}(\lambda_{725}) = 0.0012 \times [\text{GAE}] + 0.065$$

The content of total phenolic in LAEP was calculated by employing a standard curve as above prepared using gallic acid and expressed as micrograms of gallic acid equivalents (GAE).

Table 1
Validation and uncertainty parameters for antioxidant phenolic acids.

No.	Compound 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	Pyragallol	$y = 0.411x - 0.0107$	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

2.4. Determination of total flavonoids contents

The total flavonoid contents of LAEP were determined using the aluminum chloride colorimetric method of Köksal and Gülçin (2008) based on the method of Chang et al. (2002). The appropriate dilution of extracts (0.5 mL) were mixed with 1.5 mL ethanol (95%), followed by 0.1 mL of aluminum chloride (10%), 0.1 mL of potassium acetate (1 M) and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The flavonoid content was calculated using a standard calibration of quercetin solution and expressed as micrograms of quercetin equivalent (QE) per gram of sample.

$$\text{Absorbance}(\lambda_{415}) = 0.0141 \times [\text{QE}] + 0.429$$

The content of total flavonoids in LAEP was calculated from the above standard curve prepared using quercetin and expressed as micrograms of quercetin equivalents (QE). Standard calibration was made from 10 to 50 μg quercetin (r^2: 0.994).

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

Hundred milligrams of LAEP was dissolved in 5 mL of ethanol–water (50:50 v/v) in a volumetric flask, from which 1 mL was transferred into another 5 mL of volumetric flask. Then, 100 μL of curcumin was added and diluted to the volume with ethanol–water (50:50 v/v). From the final solution 1.5 mL of aliquot was transferred into a capped auto-sampler vial and 10 μL of sample was injected to LC. The samples in the auto-sampler were kept at 15 °C during the experiment (Figs. 1 and 2).

2.6. Instruments and chromatographic conditions

Experiments were performed by a 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 programme 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.

2.7. Optimization of HPLC Methods and LC–MS/MS Procedure

One of the best mobile phase solutions was determined to be a gradient of acidified methanol and water system after the serial experiments. Such a mobile phase was shown to be satisfactory for the ionization abundance and separation of the compounds. The good ionization of small and relatively polar antioxidants was obtained by the ESI source instead of APCI source. Ionization technique and collision energies of the experiments are the most important parameters in quantitative mass spectrometry analyses. Triple quadrupole mass spectrometry system is commonly used for its fragmented ion stability (Gören et al., 2009). Therefore, triple quadrupole mass spectrometry was decided to be used for 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. Detailed information on experiment parameters is given in Table 2.

2.8. Validation

In validation experiments of all of the compounds, curcumin was used as an internal standard. The validation parameters were determined to be linearity, recovery, repeatability, LOD and LOQ experiments.

2.8.1. Linearity

The linearity of the method for the reported compounds was assayed by analyzing the standard solutions. The linearity ranges of each compound are given 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.

2.8.2. Recovery, repeatability and precision

The recovery of the experiments was 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 given in Table 1

$$\text{Recovery}(100\%) = \left(\frac{\text{Measured concentration} - \text{Endogenous concentration}}{\text{Spiked concentration}}\right) \times 100$$

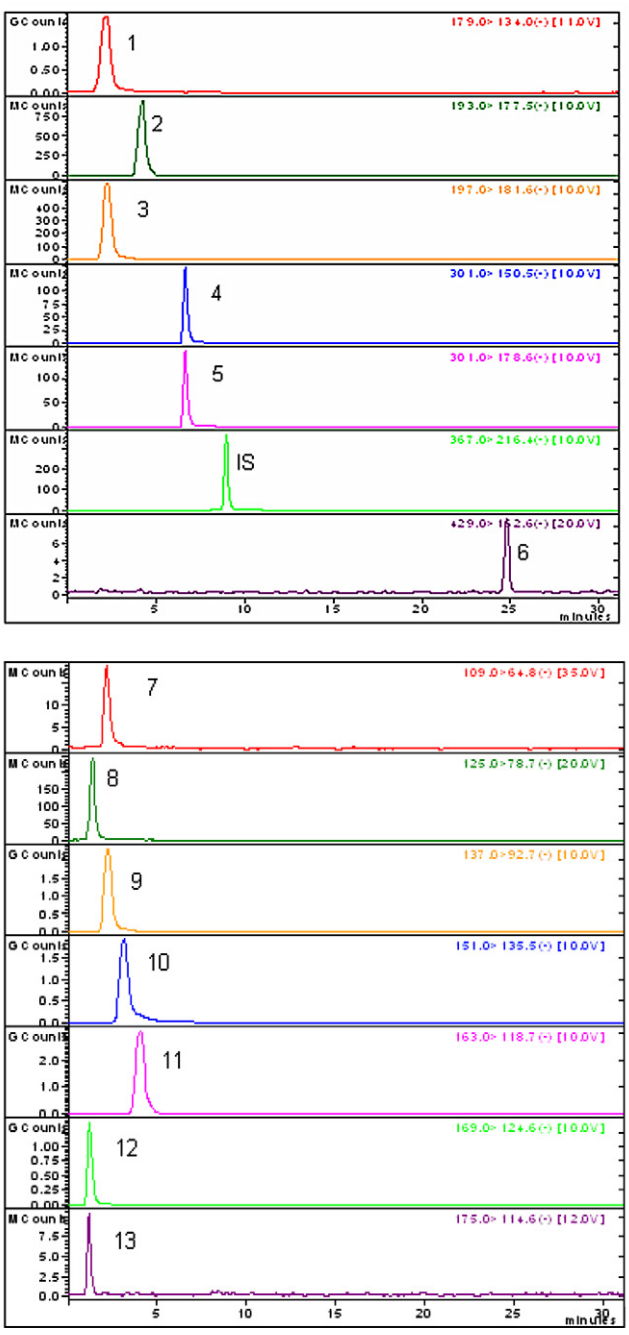

Fig. 1. Standard chromatogram of antioxidant phenolic acids by LC–MS/MS (1 mg/mL).

Precision of the method was evaluated by repeating the measurements at three concentrations for each compound. A good precision was determined and the results were implemented to the uncertainty budget.

2.8.3. 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× the S/N for the above concentrations (Table 1).

2.9. Estimation of uncertainty

2.9.1. Identification of uncertainty sources

The analyte concentration in the sample solution was expressed in μg/L within the linear range. Calculated concentration of the compounds in solution by the calibration curve was converted to mg/kg of crude sample by 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

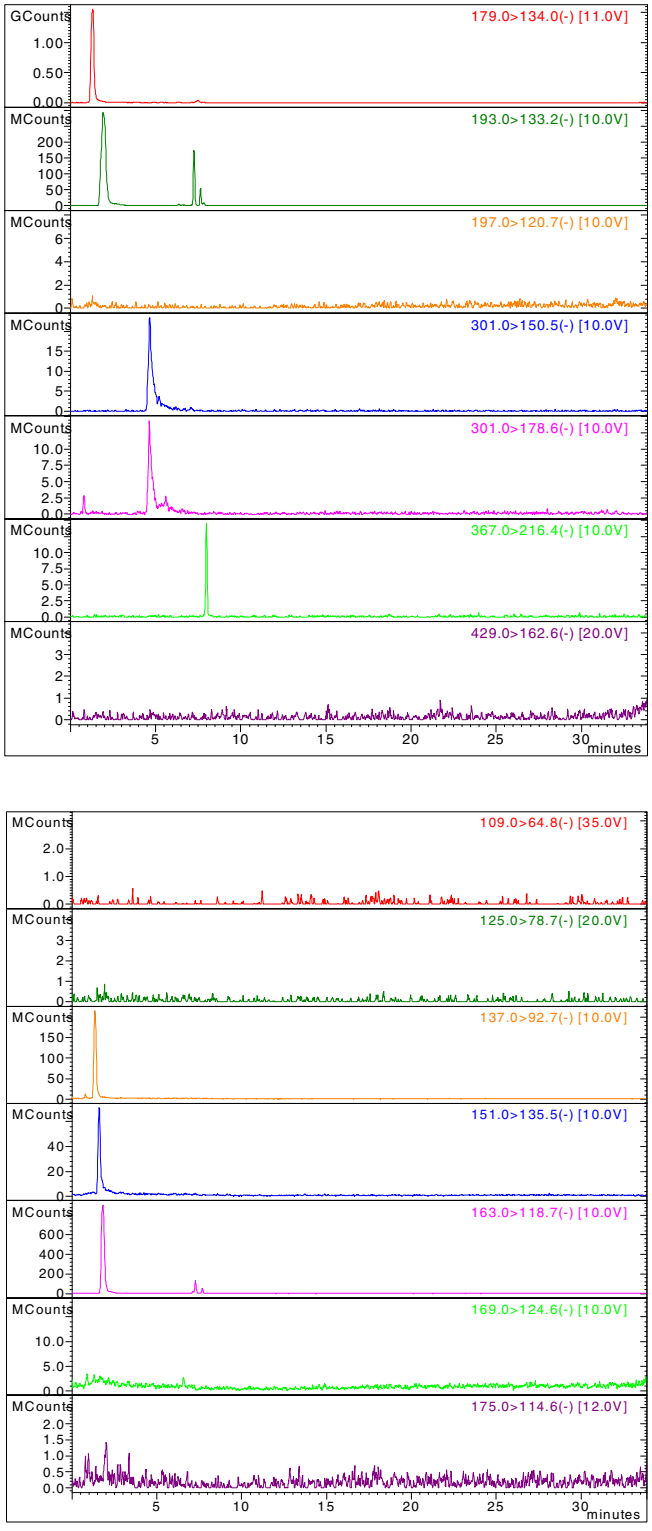

Fig. 2. Chromatogram of antioxidants by LC–MS/MS (diluted sample chromatogram for the correct determination of 3–8 in the linear range of LAEP analysis).

$$\text{Amount}(\text{mg/kg}) = \left(\frac{C_a \times V_{\text{Final}}}{m \times V_{\text{Final}}}\right) \times 1000$$

where C_a is the analyte concentration obtained 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.

Table 2
LC–MS/MS parameters of selected compounds and amount of antioxidants in LAEP in mg/kg concentration (LAEP: lyophilized aqueous extract of propolis).

No.	Compounds	Parent ion	Daughter ion	Collision energy (V)	Amount of antioxidants in the plant extracts (mg/kg)[c]
	Curcumin[a]	367	216.4	10	–
1	Caffeic acid	179	134	11	5033.8
2	Ferulic acid	193	177.5	10	5261.8
3	Syringic acid	197	181.6	10	12.8
4	Ellagic acid	301	150	10	4202.7
5	Quercetin	301	178.6	10	3851.4
6	α-Tocopherol	429	162.6	20	265.5
7	Catechol[b]	109	64.8	35	–
8	Pyragallol	125	78.7	20	45.3
9	*p*-Hydroxy benzoic acid	137	92.7	10	1370.9
10	Vanillin	181	135.5	10	400.7
11	*p*-Coumaric acid	163	118.7	10	1908.8
12	Gallic acid	169	124.6	10	419.1
13	Ascorbic acid	175	114	12	1098.0

[a] It was used for internal standard.
[b] These values are below the limits of the quantification.
[c] The uncertainty of results should be calculated according to Table 1.

2.9.2. Identification of standard uncertainties

The sources and quantification of the uncertainty for the applied method were evaluated and calculated by using EURACHEM/CITAC Guide, 2000, and Eq. (3), respectively (EURACHEM/CITAC, 2000). The sources of uncertainty for LC–MS/MS experiments were identified to be 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 has been previously reported in the literature (Gören et al., 2007)

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

where C_a is the uncertainty from the calibration curve, V_{final} is the final volume of the sample, V_{init} is the initial volumes of 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 range between 1.64% and 7.76% at 95% confidence level (k: 2) (Table 1).

2.10. Total antioxidant activity determination

The ferric thiocyanate method was used to evaluate the effect of LAEP on the prevention of peroxidation of linoleic acid emulsion, as described previously (Gülçin and Daştan, 2007; Gülçin, 2006b). A stock solution containing 10 mg of LAEP was dissolved in 10 mL distilled water. LAEP (30 μg/mL) was prepared by diluting the stock solution in 2.5 mL of sodium phosphate buffer (0.04 M, pH 7.0) and these were added to 2.5 mL of linoleic acid emulsion in sodium phosphate buffer (0.04 M, pH 7.0). The linoleic acid emulsion was prepared by homogenizing 15.5 μL of linoleic acid, 17.5 mg of Tween-20 as emulsifier, and 5 mL phosphate buffer (pH 7.0). The control was composed of 2.5 mL of linoleic acid emulsion and 2.5 mL 0.04 M sodium phosphate buffer (pH 7.0). The reaction mixtures (5 mL) were incubated at 37 °C in polyethylene flasks. The peroxide levels were determined by reading the absorbance at 500 nm in a spectrophotometer (Shimadzu, UV-1208 UV–VIS Spectrophotometer, Japan) after reactions with $FeCl_2$ and thiocyanate at intervals during incubation (Köksal and Gülçin, 2008; Ak and Gülçin, 2008). The peroxides formed during linoleic acid peroxidation oxidize Fe^{2+} to Fe^{3+}, and Fe^{3+}forms a complex with thiocyanate that has a maximum absorbance at 500 nm. The assay steps were repeated every 10 h until the maximum was reached. The percentage of inhibition was calculated at this point (50 h). The solution without LAEP was used as the blank sample. Linoleic acid mixture without the addition of sample was used as control. The percentage of inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation}(\%) = \left(1 - \frac{\lambda_{500\text{-S}}}{\lambda_{500\text{-C}}}\right) \times 100$$

where $\lambda_{500\text{-C}}$ is the absorbance of the control reaction, which contains only linoleic acid emulsion and sodium phosphate buffer. $\lambda_{500\text{-S}}$ is the absorbance of sample in the presence LAEP or other test compounds (Gülçin, 2006a,b).

2.11. Fe^{3+} reducing power assay

Ferric-reducing antioxidant power was measured by the direct reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ and was determined by measuring absorbance resulting from the formation of the Perl's Prussian Blue complex following the addition of excess ferric ions (Fe^{3+}). Thus, the ferric-reducing antioxidant power (FRAP) method of Oyaizu (1986) with slight modifications was used to measure the reducing capacity of LAEP (Ak and Gülçin; 2008). This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants (Gülçin, 2007). Different concentrations of LAEP (10–30 μg/mL) in 0.75 mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The mixture was incubated at 50 °C for 20 min. After 20 min of incubation, the reaction mixture was acidified with 1.25 mL of trichloroacetic acid (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was added to this solution, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates grater reduction capability (Büyükokuroğlu et al., 2001).

2.12. Cupric ions (Cu^{2+}) reducing-CUPRAC assay

In order to determine the cupric ions (Cu^{2+}) reducing antioxidant capacity of propolis, the method proposed by Apak et al. (2006) and Karaman et al. (2010) was used with slight modifications. To this end, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5×10^{-3} M) and 0.25 mL CH_3COONH_4 buffer solution (1 M) were added to a test tube, followed by mixing with different concentrations of LAEP (10–30 μg/mL). Then, total volume was adjusted to 2 mL with distilled water, and thoroughly mixed. The tubes were stoppered and kept at room temperature. Absorbance was measured at 450 nm against a reagent blank 30 min later. Increased absorbance of the reaction mixture indicates increased reduction capability (Talaz et al., 2009).

2.13. Chelating activity on ferrous ions (Fe^{2+})

Ferrous ions (Fe^{2+}) chelating activity was measured by inhibiting the formation of Fe^{2+}–ferrozine complex after treatment of test material with Fe^{2+}, following the method of Dinis and co-workers (1994). Fe^{2+}-chelating ability of LAEP was monitored by the absorbance of the ferrous iron–ferrozine complex at 562 nm. Briefly, different concentrations of LAEP (10–20 μg/mL) in 0.4 mL methanol were added to a solution of 0.6 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.1 mL) dissolved in methanol. Then, the mixture was shaken vigorously and left at room temperature for ten minutes. Absorbance of the solution was then measured spectrophotometrically at 562 nm (Gülçin et al., 2006b). The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated by using the formula given below:

$$\text{Bounded ferrous ions}(\%) = \left(1 - \frac{\lambda_{562\text{-}S}}{\lambda_{562\text{-}C}}\right) \times 100$$

where $\lambda_{562\text{-}C}$ is the absorbance of control and $\lambda_{562\text{-}S}$ is the absorbance in the presence of LAEP or standards. The control contains only $FeCl_2$ and ferrozine (Benzie and Strain, 1996; Gülçin, 2010).

2.14. H_2O_2 scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch and co-workers (1989). The principle of this method is that there is a decrease in the absorbance of H_2O_2 upon oxidation of H_2O_2. A solution of 40 mM H_2O_2 was prepared in 0.1 M phosphate buffer (pH 7.4). Then, 30 μg/mL of LAEP, which is in 3.4 mL phosphate buffer, was added to 0.6 mL of H_2O_2 solution (40 mM), and absorbance of the reaction mixture was recorded at 230 nm. A blank solution contained the sodium phosphate buffer without H_2O_2 (Elmastaş et al., 2006a). The concentration of H_2O_2 (mM) in the assay medium was determined using a standard curve (r^2:0.9956):

$$\text{Absorbance}(\lambda_{230}) = 0.505 \times [H_2O_2]$$

The percentage of H_2O_2 scavenging by LAEP and standard compounds was calculated using the following equation:

$$\text{Scavenged}H_2O_2(\%) = \left(1 - \frac{\lambda_{230\text{-}S}}{\lambda_{230\text{-}C}}\right) \times 100$$

where $\lambda_{230\text{-}C}$ is the absorbance of the control and $\lambda_{230\text{-}S}$ is the absorbance in the presence of LAEP or other scavengers (Gülçin et al., 2004a; Elmastaş et al., 2006b).

2.15. DPPH· scavenging activity

The total radical scavenging capacity of LAEP was determined and compared to that of BHA, BHT, α-tocopherol and trolox by using the DPPH·, $ABTS^{\cdot+}$, $DMPD^{\cdot+}$ and $O_2^{\cdot-}$ radical scavenging methods.

The DPPH· solution has a deep violet colour and radical scavenging activity of antioxidant compounds can be measured spectrophotometrically at 517 nm by the loss of absorbance as the pale yellow non-radical form (DPPH-H) is produced. The hydrogen atom or electron donation abilities of some pure compounds were measured by bleaching a purple-coloured methanol solution of the stable DPPH radical. The method of Blois (1958), previously described by Gülçin (2006a), was used with slight modifications in order to assess the DPPH· free radical scavenging capacity of LAEP. The DPPH radical shows absorbance at 517 nm, but its absorption decreases upon reduction by an antioxidant or a radical. When a hydrogen atom or electron was transferred to the odd electron in DPPH·, the absorbance at 517 nm decreased proportionally to the increases in non-radical forms of DPPH (Ak and Gülçin, 2008). Briefly, 0.1 mM solution of DPPH· was prepared in ethanol and, 0.5 mL of this solution was added to 1.5 mL of LAEP solution in ethanol at different concentrations (10–30 μg/mL). These solutions were vortexed thoroughly, and incubated in dark for 30 min. Thirty minutes later, the absorbance was measured at 517 nm against blank samples lacking scavenger. A standard curve was prepared using different concentrations of DPPH·. The DPPH· scavenging capacity was expressed as mM in the reaction medium, and calculated from the calibration curve determined by linear regression (r^2: 0.9974):

$$\text{Absorbance}(\lambda_{517}) = 0.5869 \times [\text{DPPH}\cdot] \times 10^{-4}\text{M} + 0.0134$$

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

$$\text{Scavenged DPPH}\cdot(\%) = (1 - \frac{\lambda_{517\text{-}S}}{\lambda_{517\text{-}C}}) \times 100$$

where $\lambda_{517\text{-}C}$ is the absorbance at 517 nm of the control reaction (containing all reagents except the test compound) and $\lambda_{517\text{-}S}$ is the absorbance at 517 nm containing the test compound. The concentration of LAEP providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against LAEP concentration (μg/mL, Blois, 1958; Gülçin et al., 2004a). DPPH significantly decreases upon exposure to radical scavengers (Gülçin et al., 2007).

2.16. $ABTS^{\cdot+}$ scavenging activity

ABTS radical cation decolourization test is widely used to assess the antioxidant activity of various substances. The experiment was carried out using an improved ABTS decolourization assay (Re et al., 1999). In this method, an antioxidant is added to a pre-formed ABTS radical solution, and after a fixed time period, the remaining $ABTS^{\cdot+}$ is quantified spectrophotometrically at 734 nm (Gülçin et al., 2010). The $ABTS^{\cdot+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for 6 h. The $ABTS^{\cdot+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 1 mL of $ABTS^{\cdot+}$ solution was added to 3 mL of LAEP solution in ethanol at different concentrations (10–30 μg/mL). The absorbance was recorded 30 min after mixing, and the percentage of radical scavenging was calculated for each concentration relative to a blank lacking scavenger. The extent of decolourization is calculated as percentage reduction of absorbance. For preparation of a standard curve, different concentrations of $ABTS^{\cdot+}$ (0.033–0.33 mM) were used. The $ABTS^{\cdot+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r^2: 0.9899):

$$\text{Absorbance}(\lambda_{734}) = 2.5905 \times [\text{ABTS}^{\cdot+}]$$

The scavenging capability of test compounds was calculated using the following equation:

$$\text{ABTS}^{\cdot+}\text{scavenging effect}(\%) = \left(1 - \frac{\lambda_{734\text{-}S}}{\lambda_{734\text{-}C}}\right) \times 100$$

where $\lambda_{734\text{-}C}$ is the absorbance of a control lacking any radical scavenger and $\lambda_{734\text{-}S}$ is absorbance of the remaining $ABTS^{\cdot+}$ in the presence of scavenger (Köksal et al., 2009).

2.17. $O_2^{\cdot-}$ scavenging activity

Superoxide anion radicals were generated by using a method described by Zhishen et al. (1999) with slight modifications (Köksal et al., 2009). Superoxide radicals were generated in riboflavin, methionine, illuminated and assayed by the reduction of NBT to form blue formazan. All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The total volume of the reaction mixture was 3 mL, and the concentrations of the riboflavin, methionine and NBT were 1.33×10^{-5}, 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The reaction mixture was illuminated at 25 °C for 40 min. The photochemically reduced riboflavin generated $O_2^{\cdot-}$ which reduced NBT to form blue formazan. The un-illuminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. LAEP was added to the reaction mixture, in which $O_2^{\cdot-}$ was scavenged, thereby inhibited the NBT reduction. Decreased absorbance of the reaction mixture indicates increased $O_2^{\cdot-}$ scavenging activity. The percentage of scavenged $O_2^{\cdot-}$ was calculated by using the following formula:

$$\text{Scavenged superoxide radicals}(\%) = \left(1 - \frac{\lambda_{560\text{-}S}}{\lambda_{560\text{-}C}}\right) \times 100$$

where $\lambda_{560\text{-}C}$ is the absorbance of the control and $\lambda_{560\text{-}S}$ is the absorbance in the presence of LAEP or standards (Gülçin et al., 2005b, 2008).

2.18. DMPD·+ scavenging activity

Finally, antiradical capacity was analyzed by DMPD·+ assay. DMPD radical scavenging ability of LAEP was performed following Fogliano and co-workers (1999) with slight modifications (Gülçin, 2008). In the presence of Fe^{3+}, antioxidant compounds are able to transfer a hydrogen atom to DMPD·+, resulting in a decolourization of the solution measured by the decrease in absorbance at 505 nm. DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 mL of deionised water, and 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.25), and the coloured radical cation (DMPD·+) was obtained by adding 0.2 mL of a solution of 0.05 M ferric chloride ($FeCl_3$). The absorbance of this solution, which is freshly prepared daily, is constant up to 12 h at room temperature. Different concentrations of standard antioxidants or LAEP (10–30 μg/mL) were added in test tubes, and the total volumes were adjusted to 0.5 mL with distilled water. Ten minutes later, the absorbance was measured at 505 nm. One millilitre of DMPD·+ solution was directly added to the reaction mixture, and its absorbance was measured at 505 nm. The buffer solution was used as a blank sample. The DMPD·+ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r^2: 0.9993):

$$\text{Absorbance}(\lambda_{505}) = 0.0088 \times [\text{DMPD}^{\cdot+}]$$

The scavenging capability of ABTS·+ radical was calculated using the following equation:

$$\text{DMPD}^{\cdot+}\text{scavenging effect}(\%) = \left(1 - \frac{\lambda_{505\text{-}S}}{\lambda_{505\text{-}C}}\right) \times 100$$

where in $\lambda_{505\text{-}C}$ is the initial concentration of the DMPD·+ and $\lambda_{505\text{-}S}$ is absorbance of the remaining concentration of DMPD·+ in the presence of LAEP (Fogliano et al., 1999; Gülçin, 2010).

2.19. Statistical analysis

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance ANOVA was performed by following the procedures. Significant differences between means were determined by Duncan's Multiple Range tests and $p < 0.05$ was regarded as significant and $p < 0.01$ as very significant.

3. Results

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. The recent focus of interest on phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases (coronary heart disease, stroke, and cancers). It was reported that the main compounds found in propolis include phenolic acids such as caffeic, ferulic, *p*-coumaric and cinnamic acids. Various pharmacological activities of propolis are attributed to phenolics such as the flavonoids it contains (Kumazawa et al., 2004). The content of phenolic compounds (mg/g) in LAEP determined using regression equation of calibration curve (y: $0.0141x + 0.429$, r^2: 0.994) and expressed in gallic acid equivalents (GAE) was found to be 124.3 μg (GAE)/g(LAEP). Phenolic compounds are likely to contribute to the radical scavenging activity of these plant extracts.

Flavonoids are the most common group of polyphenolic compounds in human diet and are found ubiquitously in plants. They can prevent coronary heart disease and have antioxidant properties. The content of flavonoid compounds (mg/g) in LAEP was determined using the regression equation of calibration curve (y: $0.0012x + 0.065$, r^2: 0.971). The content of total flavonoids in LAEP was determined spectrophotometrically as 8.15 quercetin equivalents. These results clearly demonstrated that there is a positive correlation between the total flavonoid content in LAEP and antioxidant activity.

Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation like lipid peroxidation (Roginsky and Lissi, 2005). The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which are the primary products of oxidation. LAEP exhibited effective antioxidant activity in the linoleic acid emulsion system. The effect of 30 μg/mL LAEP on lipid peroxidation of a linoleic acid emulsion is shown in Table 3 and Fig. 3, and was found to be 93.2%. On the other hand, BHA, BHT, α-tocopherol and trolox exhibited 83.3%, 82.1%, 68.1% and 81.3% peroxidation of linoleic acid emulsion at the same concentration, respectively. Peroxidation of linoleic acid emulsion without LAEP or standard compounds was accompanied by a rapid increase in peroxides. Consequently, these results clearly indicate that LAEP had effective and potent antioxidant activity in the ferric thiocyanate assays.

Furthermore, LAEP had effective reducing power determined by using the potassium ferricyanide reduction and cupric ions (Cu^{2+}) reducing methods when compared to the standards. To measure the reductive ability of LAEP, Fe^{3+}–Fe^{2+} transformation was investigated in the presence of LAEP using the method of Oyaizu (1986). As can be seen in Table 3, LAEP (r^2: 0.918) demonstrated powerful Fe^{3+} reducing ability with statistically significant differences ($p < 0.01$). The reducing power of LAEP, BHA, BHT, α-tocopherol and trolox increased steadily with increasing concentration of samples. The reducing power of LAEP and the standard compounds were as follows: BHA > BHT ≈ α-tocopherol > trolox > LAEP. The results demonstrated that LAEP had marked ferric ions (Fe^{3+}) reducing ability and electron donor properties for neutralizing free radicals by forming stable products. However, this reducing power was lower than that of the standard antioxidants used. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

The putative CUPRAC method developed by Apak and co-workers (2006) was used to determine the antioxidant capacity of LAEP by the Cu^{2+}-neocuproine (Cu^{2+}-Nc) reagent as the chromogenic oxidizing agent. A cupric ion (Cu^{2+}) reducing ability of LAEP is shown in Table 3, and a correlation was observed between the cupric ions reducing ability and LAEP concentrations (r^2: 0.933). Cu^{2+} reducing capability of LAEP measured by Cuprac method was found to be concentration-dependent (10–30 μg/mL). Cu^{2+} ions reducing power of LAEP and standard compounds at the same concentration (30 μg/mL) were as follows: trolox < α-tocopherol < BHA < LAEP < BHT. However, the differences were not found to be statistically significant.

From a compilation of biochemical, animal and human data, links have been proposed between increased levels of iron in the body and an enhanced risk of a variety of diseases such as vascular disease, cancer and certain neurological conditions (Berg et al., 2001; Siah et al., 2005). Iron-mediated formation of ROS leading to DNA and lipid damage appears to result from an exaggeration of the normal function of iron, which is to transport oxygen to tissues. Iron-induced free radical damage to DNA appears to be important for the development of cancer and cancer cells are known to grow rapidly in response to iron (Ullen et al. 1997; Valko et al. 2006). LAEP had a strong chelating effect on ferrous ions (Fe^{2+}). The difference between different concentrations of LAEP (10–20 μg/mL) and the control values were statistically significant ($p < 0.01$). IC_{50} value for the metal chelating capacity of LAEP was found to be 12.04 μg/mL (r^2: 0.945). Also, the ferrous ion chelating effect of LAEP was compared to those of BHA, BHT, α-tocopherol, trolox and EDTA. On the other hand, IC_{50} values for BHA, BHT, α-tocopherol, trolox and EDTA were found to be 5.89 μg/mL (r^2: 0.973), 7.16 μg/mL (r^2: 0.987), 4.95 μg/mL (r^2: 0.873), 21.61 μg/mL (r^2: 0.962) and 5.31 μg/mL (r^2: 0.948), respectively. These results show that the ferrous ion chelating effect of LAEP was lower than those of EDTA, BHA, BHT and α-tocopherol ($p > 0.05$) but higher than that of trolox ($p < 0.01$).

Table 3
Total antioxidant activity by thiocyanate method, reducing ability by Fe^{3+}–Fe^{2+} transformation method and Cu^{2+} reducing ability by Cuprac method of LAEP and standard compounds such as BHA, BHT, α-tocopherol and trolox at the same concentration (10 μg/mL; LAEP: lyophilized aqueous extract of propolis, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

	Total antioxidant activity		Fe^{3+}–Fe^{2+} reducing ability[a]		Cu^{2+}–Cu^{+} reducing ability	
	λ_{500}[a]	r^2	λ_{700}[b]	r^2	λ_{450}[b]	r^2
BHA	83.3	–	1.027	0.899	0.856	0.941
BHT	82.1	–	0.772	0.915	0.722	0.918
Trolox	81.3	–	0.671	0.971	0.354	0.986
α-Tocopherol	68.1	–	0.727	0.896	0.486	0.958
LAEP	93.2	–	0.568	0.918	0.814	0.933

[a] Percentage inhibition effect of 10 μg/mL concentration of LAEP on linoleic acid emulsion peroxidation determined by thiocyanate method.
[b] The values were expressed as absorbance. High absorbance indicates high reducing power ability.

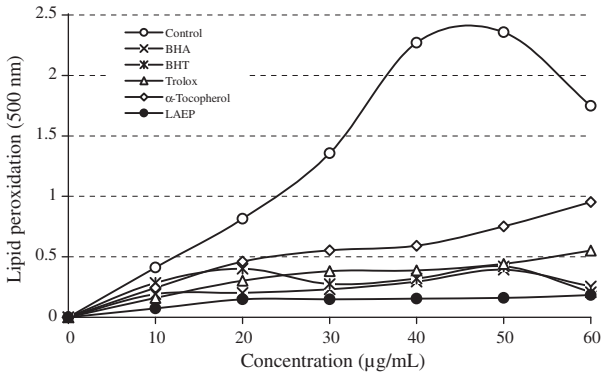

Fig. 3. The total antioxidant activity of LAEP and standard compounds such as BHA, BHT, α-tocopherol and trolox at the same concentration (30 μg/mL) was determined by ferric thiocyanate method in the linoleic acid emulsion system. In this method, peroxide formation occurred during the oxidation of linoleic acid emulsion and these compounds oxidized Fe^{2+} to Fe^{3+}, and Fe^{3+} forms a complex with SCN^- with a maximum absorbance at 500 nm. Control samples contain only linoleic acid emulsion and sodium phosphate buffer. The linoleic acid emulsion was incubated at 37 °C until control values reached to plateau (LAEP: lyophilized aqueous extract of propolis, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

Hydrogen peroxide is naturally produced in organisms as a by-product of oxygen metabolism. Almost all living things possess enzymes known as peroxidases, which harmlessly and catalytically decompose low concentrations of H_2O_2 to water and oxygen. H_2O_2 scavenging ability of LAEP is shown in Table 4, and was compared to those of BHA, BHT, α-tocopherol and trolox, which are reference compounds. IC_{50} value of H_2O_2 scavenging activity for LAEP was calculated as 6.54 μg/mL. However, BHA, BHT, α-tocopherol and trolox exhibited 10.68, 6.06, 12.78 and 13.26 μg/mL H_2O_2 scavenging activity, respectively. At this concentration, the H_2O_2 scavenging effect of LAEP and four standard compounds decreased in the following order: BHT ≈ LAEP > BHA > α-tocopherol ≈ trolox.

In DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow-coloured diphenyl-picrylhydrazine. This method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants (Oyaizu, 1986).

Table 4 illustrates a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of LAEP and the reference compounds. BHA, BHT, α-tocopherol and trolox were used as reference radical scavengers. When DPPH· reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour were measured at 517 nm. IC_{50} values for LAEP, BHA, BHT, α-tocopherol and trolox on the DPPH radical were found as 31.81 μg/mL (r^2: 0.985), 10.97 μg/mL (r^2: 0.813), 48.42 μg/mL (r^2: 0.929), 13.61 μg/mL (r^2: 0.969), and 11.45 μg/mL (r^2: 0.883), respectively. A lower EC_{50} value indicates a higher DPPH free radical scavenging activity. DPPH· radical scavenging capacity of these samples decreased in the following order: BHA ≈ trolox > α-tocopherol > LAEP > BHT.

All the tested compounds exhibited effective radical cation scavenging activity. As seen in Table 4, LAEP is an effective $ABTS^{\cdot+}$ radical scavenger in a concentration-dependent manner (10–20 μg/mL, r^2: 0.9033). EC_{50} value for LAEP in this assay was 14.29 μg/mL. There is a significant decrease ($p < 0.01$) in the concentration of $ABTS^{\cdot+}$ due to the scavenging capacity at all LAEP concentrations (10–20 μg/mL). On the other hand, EC_{50} values for BHA, BHT, α-tocopherol and trolox were found to be 4.95 μg/mL (r^2: 0.999), 6.05 μg/mL (r^2: 0.997), 16.61 μg/mL (r^2: 0.982) and 6.11 μg/mL (r^2: 0.875), respectively. The $ABTS^{\cdot+}$ scavenging effect of LAEP and standards decreased in the following order: BHA > BHT ≈ trolox > α-tocopherol ≈ LAEP.

Superoxide is biologically quite toxic and is deployed by the immune system to kill invading microorganisms. In phagocytes, it is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. LAEP was the most powerful in inhibiting the superoxide radical generation in the sample used. EC_{50} value of superoxide anion radical scavenging for LAEP was found to be 9.89 μg/mL (Table 4). On the other hand, these values were found to be 17.67, 11.06, 23.47 and 20.92 μg/mL for BHA, BHT, α-tocopherol and trolox, respectively. Based on these results, it is concluded that LAEP had higher superoxide anion radical scavenging activity than all reference compounds tested.

Table 4
Concentration required for 50% scavenging (IC_{50}) of DPPH· scavenging activity, $ABTS^{\cdot+}$ activity, $DMPD^{\cdot+}$ activity, $O_2^{\cdot-}$ scavenging activity, H_2O_2 scavenging and ferrous ions (Fe^{2+}) chelating activity of LAEP and standard compounds such as BHA, BHT, α-tocopherol and trolox (LAEP: lyophilized aqueous extract of propolis, DPPH·: 1,1-diphenyl-2-picrylhydrazyl free radical, $ABTS^{\cdot+}$: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, $DMPD^{\cdot+}$: N,N-dimethyl-p-phenylenediamine dihydrochloride radical, $O_2^{\cdot-}$: superoxide anion radicals, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene; H_2O_2: hydrogen peroxide).

Compounds	DPPH· scavenging		$ABTS^{\cdot+}$ scavenging		$DMPD^{\cdot+}$ scavenging		$O_2^{\cdot-}$ scavenging	H_2O_2 scavenging	Fe^{2+} chelating	
	IC_{50}[a]	R^2	IC_{50}[a]	R^2	IC_{50}[a]	R^2	IC_{50}[a]	IC_{50}[a]	IC_{50}[a]	R^2
BHA	10.97	0.813	4.95	0.999	40.25	0.891	17.67	10.68	5.98	0.973
BHT	48.42	0.929	6.05	0.997	–	–	11.06	6.06	7.16	0.987
Trolox	11.45	0.883	6.11	0.875	12.45	0.982	20.92	13.26	21.61	0.962
α-Tocopherol	13.61	0.969	16.61	0.982	–	–	23.47	12.78	4.95	0.873
LAEP	31.81	0.985	14.29	0.943	18.32	0.938	9.89	6.54	12.04	0.945
EDTA[b]	–	–	–	–	–	–	–		5.31	0.948

[a] The values were expressed as μg/mL concentration. Lower IC_{50} values indicate higher radical scavenging activity.
[b] EDTA was only used as a reference for metal-chelating activity.

As shown in Table 4, LAEP was an effective $DMPD^{\cdot+}$ radical scavenger in a concentration-dependent manner (10–30 μg/mL, r^2: 0.938). EC_{50} for LAEP was 18.32 μg/mL. This value was found as 40.25 μg/mL for BHA (r^2: 0.891) and 12.45 μg/mL for trolox (r^2: 0.982). There is a significant decrease ($p < 0.05$) in the concentration of $DMPD^{\cdot+}$ due to the scavenging capacity at all LAEP concentrations. Reportedly, the main drawback of the $DMPD^{\cdot+}$ method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-tocopherol or BHT were used (Köksal et al., 2009).

4. Discussion

Phenolics share the same general structure composed of an aromatic hydroxyl nucleus and exist in an approximated number of 8000 in nature (Karaman et al., 2010). So far, plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Plant polyphenols are multifunctional in the sense that they can act as reducing agents, hydrogen atom donators, and singlet oxygen scavengers. Certain polyphenols are also effective as antioxidants capable of chelating transition metal ions which may otherwise induce Fenton-type oxidation reactions in their free states (Rice-Evans et al., 1996; Karaman et al., 2010). It was reasonable to determine their total amount in the selected plant or propolis extracts. Moreover, flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. Flavonoids as a member of the polyphenol family have more than 4000 varieties in nature that exist in the leaves, flowers, and roots of plants (Heim et al., 2002). The nature of their substituents as well as the overall conjugation of the molecules gives rise to a wide range of chelating and radical scavenging activities (Heim et al., 2002). The main role of the phenolic compounds as scavengers of free radicals is emphasized in our several reports (Gülçin et al., 2004b,c, 2005a).

Recently, many studies have been carried out on green propolis, owing to its characteristic chemical composition and biological activities (Shimazawa et al., 2005; Inokuchi et al., 2006; Nakajima et al., 2007). Propolis is commercialized in different parts of the world and is recognized as an important source of compounds with pharmacological properties (Miyataka et al., 1997). In the present study, we determined 124.3 μg GAE/g LAEP. However, different results were found in the literature. Bornes samples showed the highest values of total phenols in comparison to propolis samples from other countries; high total phenol amounts were also found in Chinese samples from Hebei, 302 mg/g of GAE (Ahn et al., 2007), and Hubei, 299 mg/g of GAE (Kumazawa et al., 2004); and Korean propolis from Yeosu, with 212.7 mg/g of GAE (Choi et al., 2006). Even the total phenols obtained from Fundão samples, from the central region of Portugal, was half the value of total phenols registered in Bornes samples (151 mg/g of GAE). Its amount was higher than the propolis from Brazil, with 120 mg/g of GAE (Choi et al., 2006) and Thailand, with 31.2 mg/g of GAE (Kumazawa et al., 2004).

Some other studies have also been conducted to investigate the phenolic composition of propolis samples (Marcucci and Bankova, 1999; Tazawa et al., 2000). The compounds reported by different authors include caffeic acid; *p*-coumaric acid; 3,4-dimethoxycinnamic acid; quercetin; pinobanksin 5-methyl ether; apigenin; kaempferol; pinobanksin; cinnamylideneacetic acid; chrysin; pinocembrin; galangin; pinobanksin 3-acetate; phenethyl caffeate; cinnamyl caffeate; tectochrysin; artepillin C (Marcucci and Bankova, 1999; Medic-Saric et al., 2004; Kumazawa et al., 2004).

According to LC–MS/MS experiment, the main phenolic acids were found to be ferulic acid, caffeic acid, ellagic acid in the studied LAEP, respectively. Also, quercetin is one of the main constituents of the propolis flavonoids. This constituent could be responsible for the antioxidant activity of LAEP. Furthermore, the results are similar to other literature reports (Marcucci and Bankova, 1999; Medic-Saric et al., 2004; Kumazawa et al., 2004).

On the other hand, ascorbic acid content has been determined in LAEP, showing that LAEP as a source of vitamin C will help refreshing activity in human diet. While trihydroxy phenol, pyragallol, was determined as a minor constituent, dihydroxy phenol, catechol was not determined in this experiment. Also, a limited amount of α-tocopherol has been determined in the LAEP.

In the present study, we have demonstrated the antioxidant and radical scavenging mechanism of LAEP by using different in vitro bioanalytical methodologies. As reported in many studies, the activities of natural antioxidants in influencing diseases are closely related to their ability to reduce DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacterial growth (Roginsky and Lissi, 2005). Antioxidant capacity is widely used as a parameter for medicinal bioactive components. In this study, the antioxidant and radical scavenging activities of LAEP were compared to those of BHA, BHT, α-tocopherol and its water-soluble analogue trolox. These comparisons were made using a series of in vitro tests including $DPPH^{\cdot}$ free radical scavenging, $ABTS^{\cdot+}$ scavenging, $DMPD^{\cdot+}$ scavenging, total antioxidant activity by the ferric thiocyanate method, reducing power by two methods (Fe^{3+}–Fe^{2+} transformation and Cuprac assays), superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating on ferrous ions (Fe^{3+}) activities.

Various antioxidant assay methods have been developed for food and biological samples. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences (Hochstein and Atallah, 1988). The resulting lipid hydroperoxides can affect membrane fluidity and the function of membrane proteins. In addition, lipid hydroperoxides can undergo iron-mediated, one-electron reduction and oxygenation to form epoxyallylic peroxyl radicals which trigger a chain reaction of free radical-mediated lipid peroxidation. The end-products of lipid peroxidation are reactive aldehydes, such as 4-hydroxyl nonenal and malondialdehyde, many of which are highly toxic to cells (Yu and Yang, 1996). In addition, reactive aldehydes generated by lipid peroxidation can attack other cellular targets, such as proteins and DNA; thereby propagate the initial damage in cellular membranes to other macromolecules. Because lipid hydroperoxides formed in membranes are important components of ROS generation in vivo. Their detoxification appears to be critical for the survival of an organism in oxidative stress (Dargel, 1992). Therefore, antioxidants play a vital role in inhibition of lipid peroxidation or in protection against cellular damage by free radicals.

Lipid oxidation consists of a series of free radical-mediated chain reaction processes, and is associated with several types of biological damages. The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of lipid oxidation. In this assay, indirect measurement was performed for the amount of hydroperoxides produced from linoleic acid emulsion by auto-oxidation during the experiment period. Then ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate by means of hydroperoxides (Inatani et al., 1983).

On the other hand, reducing power reflects the electron donating capacity of bioactive compounds and is associated with antioxidant activity. Antioxidants can be reductants and inactivate oxidants. The reducing capacity of a compound can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN^-)_6]_3$, which has a strong

absorbance at 700 nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. There are a number of assays designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to withstand free radical stress. The ferric ion reducing antioxidant power assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant (Gülçin, 2009). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Gülçin et al., 2010).

In the present study, we used the Cuprac assay which is based on reduction of Cu^{2+} to Cu^{+} by antioxidants. This method is simultaneously cost-effective, rapid, stable, selective and suitable for a variety of antioxidants regardless of chemical type or hydrophilicity. Moreover, it was reported that the results obtained from in vitro cupric ion (Cu^{2+}) reducing measurements might be more efficiently extended to the possible in vivo reactions of antioxidants. Cuprac chromogenic redox reaction is carried out at a pH (7.0) close to the physiological pH, and the method is capable of measuring thiol-type antioxidants such as glutathione and nonprotein thiols unlike the widely applied FRAP test, which is nonresponsive to -SH group antioxidants (Gülçin and Daştan, 2007; Karaman et al., 2010).

Elemental species, such as ferrous iron (Fe^{2+}), can facilitate the production of ROS within animal and human systems, and the ability of substances to chelate iron can be valuable for antioxidant property. Iron is an essential mineral for normal physiology, but excess can result in cellular injury. If they undergo the Fenton reaction, these reduced metals may form highly reactive hydroxyl radicals, and thereby, contribute to oxidative stress (Hippeli and Elstner, 1999). The resulting oxy radicals cause damage to cellular lipids, nucleic acids, proteins, and carbohydrates, leading to cellular impairment. Since ferrous ions are the most effective pro-oxidants in food systems, the good chelating effect would be beneficial, and removal of free iron ion from circulation could be a promising approach to prevent oxidative stress-induced diseases. When iron ion is chelated, it may lose its pro-oxidant properties. Iron, in nature, can be found as either ferrous (Fe^{2+}) or ferric ion (Fe^{3+}), with the latter form predominant in foods. Ferrous chelation may render important antioxidative effects by retarding metal-catalyzed oxidation.

Ferrous ion chelating activities of LAEP, and standard compounds are shown in Table 4. The chelation of ferrous ions by LAEP or standards was determined by following the method of Dinis et al. (1994). Among the transition metals, iron is known to be the most important lipid oxidation pro-oxidant due to its high reactivity. An effective ferrous ion chelator affords protection against oxidative damage by removing iron that may otherwise participate in $HO^{\cdot}$ generation via the Fenton type reactions. Ferric ions (Fe^{3+}) also produce radicals from peroxides although the rate is tenfold less than that of ferrous ion. Ferrous ions (Fe^{2+}) are the most powerful pro-oxidants among the various species of metal ions (Halliwell and Gutteridge, 1984; Gülçin et al., 2004d). Minimizing ferrous ion may afford protection against oxidative damage by inhibiting production of ROS and molecular damage. Ferrozine can quantitatively form complexes with Fe^{2+} in this method. In the presence of chelating agents, complex formation is disrupted, resulting in a reduction in the red colour of the complex. Measurement of colour reduction therefore allows estimation of the metal-chelating activity of the coexisting chelator. Lower absorbance indicates higher metal-chelating activity. Metal chelation is an important antioxidant property (Kehrer, 2000) and hence LAEP was assessed for its ability to compete with ferrozine for ferrous ion in the solution.

One measurement of the metal-chelating activity of an antioxidant is based on the absorbance measurement of Fe^{2+}–ferrozine complex after prior treatment of a ferrous ion solution with test material. Ferrozine forms a complex with free Fe^{2+}, but not with Fe^{2+} bound to other chelators; thus, a decrease in the amount of ferrozine–Fe^{2+} complex formed after treatment indicates the presence of antioxidant chelators. The ferrozine–Fe^{2+} complex produced a red chromophore with absorbance that can be measured at λ_{562} nm. A significant drawback of this complexation reaction in measuring the presence of antioxidant chelator is that the reaction is affected by both the antioxidant-Fe^{2+} and ferrozine–Fe^{2+} complex formation constants, and the competition between the two chelators for binding to iron. Thus, a weak antioxidant iron chelator would be seriously underestimated in quantitative determination. From a nutritional point of view, it is not yet possible to assess the role of a weak antioxidant iron chelator in preventing the Fenton reaction in vivo. Nonetheless, this reaction serves as a convenient assay to assess iron chelating activity of antioxidant.

The metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby, stabilizing the oxidized form of the metal ion. EDTA is a strong metal chelator; hence, it was used as the standard metal chelator agent in this study. The data obtained from Table 4 reveal that LAEP possesses a marked capacity for iron binding, suggesting that its main action as a peroxidation inhibitor may be related to its iron binding capacity. In this assay, LAEP interfered with the formation of the ferrous–ferrozine complex. It suggests that LAEP has chelating activity and is able to capture ferrous ion before ferrozine. Biological systems can produce H_2O_2. It is also produced from polyphenol-rich beverages under quasi-physiological conditions, and it increases in amount with longer incubation time. The generation of H_2O_2 by activated phagocytes is known to play an important part in killing several bacterial and fungal strains. Additionally, H_2O_2 is formed in vivo by a variety of enzymes, including superoxide dismutase. H_2O_2 is most generally considered as a powerful oxidizing agent. There is increasing evidence that H_2O_2, either directly or indirectly via its reduction product OH^{-}, acts as a messenger molecule in the synthesis and activation of inflammatory mediators. It can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated phagocytes. The H_2O_2 scavenging capacity of LAEP was determined using the method of Ruch and co-workers (1989), as shown in Table 4. LAEP had effective H_2O_2 scavenging activity. It is known that H_2O_2 is toxic and induces cell death in vitro. H_2O_2 can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Ak and Gülçin, 2008).

Hydrogen peroxide itself is not very reactive; however, it can sometimes be toxic to cells because it may give rise to hydroxyl radical within the cells. Its toxicity derives from its conversion to hydroxyl radical. H_2O_2 to cells in culture can lead to transition metal ion-dependent OH radicals mediating oxidative DNA damage. Levels of hydrogen peroxide at or below about 20–50 μg/cell seem to have limited cytotoxicity to many cell types. Thus, removing hydrogen peroxide as well as superoxide anion is very important for protection of pharmaceuticals and food systems (Chai et al., 2003).

The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reactions, and improve the quality and stability of food products (Soares et al., 1997). Assays based upon the use of $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and $DMPD^{\cdot+}$ radicals are among the most popular spectrophotometric methods for determination of the antioxidant

capacity of foods, beverages and vegetable extracts. Both chromogens and radical compounds can directly react with antioxidants. Additionally, DPPH· and $ABTS^{\cdot+}$ scavenging methods have been used to evaluate the antioxidant activity of compounds due to the simple, rapid, sensitive, and reproducible procedures (Özcelik et al., 2003).

In this study, three radical scavenging methods were used to assess the potential radical scavenging activities of LAEP, namely $ABTS^{\cdot+}$ scavenging, DPPH scavenging and superoxide anion radical scavenging activity. With these methods, it is possible to determine the antioxidant power of an antioxidant by measuring a decrease in the absorbance of DPPH· at 517 nm. The structure of the LAEP provides a chromophoric system which leads to interference in the DPPH· method currently using the 517 nm wavelength as described above. The absorbance is decreased when the DPPH· is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH radical molecule. In the radical form, this molecule has an absorbance at 517 nm, which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (Matthäus, 2002).

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies, and offers a rapid technique for screening the radical scavenging activity of specific compounds. The antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby form a stable end-product which does not initiate or propagate further oxidation of lipid (Amarowicz et al., 2004).

$ABTS^{\cdot+}$ is applicable for both lipophilic and hydrophilic compounds. $ABTS^{\cdot+}$ radicals are more reactive than DPPH radicals, and unlike the reactions with DPPH radical, which involve H atom transfer, the reactions with $ABTS^{\cdot+}$ radicals involve an electron transfer process. Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to measure the total antioxidant activity of pure substances, aqueous mixtures and beverages (Gülçin, 2009). A more appropriate format for the assay is a decolourization technique in which the radical is generated directly in a stable form prior to the reaction with putative antioxidants.

$ABTS^{\cdot+}$ was generated by oxidation of ABTS with potassium persulfate. This assay is based on the inhibition of the absorbance of the radical cation $ABTS^{\cdot+}$, which has a characteristic long-wavelength absorption spectrum showing absorption at 734 nm. It should also be noted that the reaction with $ABTS^{\cdot+}$ was quite fast and almost in all cases was completed in 0.25–0.5 min. Bleaching of a pre-formed solution of the blue-green radical cation $ABTS^{\cdot+}$ has been extensively used to evaluate the antioxidant capacity of complex mixtures and individual compounds. The reaction of the pre-formed radical with free-radical scavengers can be easily monitored by following the decay of the sample absorbance at 734 nm (Gülçin et al., 2009).

The principle of the $DMPD^{\cdot+}$ assay is that DMPD can form a stable and coloured radical cation ($DMPD^{\cdot+}$) at acidic pH and in the presence of a suitable oxidant solution. The UV–visible spectrum of $DMPD^{\cdot+}$ shows a maximum absorbance at 505 nm. Antioxidant compounds, which are able to transfer a hydrogen atom to $DMPD^{\cdot+}$, quench the colour and produce a decolouration of the solution. This reaction is rapid, and the end point, which is stable, is taken as a measure of antioxidative efficiency. Therefore, this assay reflects the ability of radical hydrogen-donors to scavenge the single electron from $DMPD^{\cdot+}$ (Fogliano et al., 1999; Ak and Gülçin, 2008).

Preliminary experiments show that the choice of oxidant solution and the ratio between the concentration of $DMPD^{\cdot+}$ and the concentration of the oxidative compound are crucial for the effectiveness of the method. In fact, formation of radical cation is very slow and results in a continuous increase of the absorbance. The best results were obtained with $FeCl_3$, which gives a stable coloured solution up to a final concentration of 0.1 mM. Moreover, this method ensures low cost and highly reproducible analysis (Gülçin, 2008).

DMPD assay is particularly suitable for hydrophilic antioxidants, but is less sensitive to hydrophobic bioactive compounds, while the opposite case applies for the other two tests. In contrast to the ABTS procedure, the $DMPD^{\cdot+}$ method guarantees a very stable end point. This is particularly important when a large-scale screening is required. It was reported that the main drawback of the $DMPD^{\cdot+}$ method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-tocopherol or BHT were used. Hence, these standard antioxidant compounds were not used in this antiradical assay.

Superoxide is an oxygen-cantered radical with selective reactivity. Although a relatively weak oxidant, superoxide exhibits limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals (OH·), which cause the peroxidation of lipids (Halliwell and Chirico, 1993). Superoxide anions are precursors to active free radicals that have potential for reacting with biological macromolecules, and thereby, inducing tissue damage (Halliwell and Gutteridge, 1984). Superoxide is easily formed by radiolysis of water in the presence of oxygen, which allows accurate reaction rate constants to be measured (Gülçin and Daştan, 2007). It has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical. Also, superoxide has been observed to directly initiate lipid peroxidation (Wickens, 2001). It has also been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical. $O_2^{\cdot-}$ is the precursor of H_2O_2, OH·, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA. Superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of free radicals and oxidizing agents (Pietta, 2000). Superoxide anions derived from dissolved oxygen by the riboflavin/methionine/illuminate system will reduce NBT in this system. In this method, $O_2^{\cdot-}$ reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants inhibit the blue NBT formation (Parejo et al., 2002). The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table 4 shows the inhibition of superoxide radical generation by 15 μg/mL concentration of LAEP and the other molecules tested.

In conclusion, LAEP was found to be an effective antioxidant in several in vitro assays including total antioxidant activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing ability, DPPH·, $ABTS^{\cdot+}$, $DMPD^{\cdot+}$ and $O_2^{\cdot-}$ radical scavenging, H_2O_2 scavenging and metal chelating activities.

Conflict of Interest

The authors declared that there are no conflicts of interest.

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