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Polyphenol contents and in vitro antioxidant activities of lyophilised aqueous extract of kiwifruit (*Actinidia deliciosa*)

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ABSTRACT

The aim of this study was to determine the antioxidant potency and total phenolic and flavonoid contents of kiwifruit (*Actinidia deliciosa*) in vitro by analysing the radical scavenging activity of lyophilised water extract from kiwifruit (LEK) for 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), N,N-dimethyl-p-phenylenediamine (DMPD), and superoxide anion radical ($O_2^{\bullet-}$) as well as the total reducing power by FRAP and CUPRAC assays and the metal chelating activities. LEK showed efficient radical scavenging activity with DPPH, ABTS, DMPD, and $O_2^{\bullet-}$ radicals; ferric (Fe^{3+}) and cupric (Cu^{2+}) ion reducing power and metal chelating activities. Moreover, the amounts of phenolic compounds, such as caffeic acid, ferulic acid, syringic acid, ellagic acid, catechol, pyrogallol, p-hydroxy benzoic acid, vanillin, p-coumaric acid, gallic acid, quercetin, α-tocopherol and ascorbic acid, in LEK were quantified by LC-MS-MS. The results show that pyrogallol (2070.0 mg/kg LEK) is the main phenolic compound responsible for the antioxidant and radical scavenging activities of LEK. Finally, total phenolic and flavonoid contents were determined as gallic acid (GAE) and quercetin equivalents (QE). The GAE and QE values in LEK were 16.67 ± 2.83 μg GAE/mg and 12.95 ± 0.52 μg QE/mg, respectively. The results suggest that consumption of kiwifruit (*A. deliciosa*) can be beneficial effects due to its antioxidant properties.

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1. Introduction

Free radicals are naturally present in living systems; however, high amounts of free radicals can oxidise biomolecules, leading to tissue damage, cell death or degenerative processes, including aspects of ageing, cancer, cardiovascular diseases, arteriosclerosis, neural disorders, skin irritations and inflammation. Also, free radicals and lipid peroxides play an important role in oxidative stress (Gülçin, 2006a; Ak & Gülçin, 2008). There is a balance between prooxidant and antioxidant activities. A shift in this balance towards prooxidant activity leads to the damage of functional tissues. Lipid peroxidation reactions involve free radical attack on polyunsaturated fatty acids. Antioxidants inhibit lipid peroxidation chain reactions that can produce free radicals and lipid peroxides. Free radicals can change the structures of proteins, lipids, carbohydrates, and DNA (Gülçin, Beydemir, Şat, & Küfrevioğlu, 2005; Gülçin, Berashvili, & Gepdiremen, 2005; Köksal, Gülçin, Ozturk Sarıkaya, & Bursal, 2009).

Free radicals can be deactivated and scavenged by antioxidant compounds, which can inhibit the effect of oxidants by donating a hydrogen atom or by chelating metals (Gülçin, Mshvildadze, Gepdiremen, & Elias, 2006; Prakash, Upadhyay, Singh, & Singh, 2007). Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgalate (PG) and *tert*-butyl hydroquinone (TBHQ), are used as food additives. Natural antioxidants exist in the leaves, seeds, roots and fruits of most plants (Elmastas et al., 2006; Elmastaş, Gülçin, Beydemir, Küfrevioğlu, & Aboul-Enein, 2006). Plants are the main source of vitamin E (α-tocopherol), vitamin C (ascorbate) and phenolic compounds. These are the most effective free radical scavengers in living organisms. Recently, investigating natural antioxidant sources has become important because of these compounds, which are safer and more efficient than synthetic compounds (Gülçin, 2009; Viuda-Martos, Navajas, Zapata, Fernandez-Lopez, & Perez-Alvarez, 2010).

Many fruits and vegetables contain high amounts of compounds that are beneficial effects for health, including polyphenols, ascorbic acid, carotenoids and tocopherols. The intake of fruits and vegetables can reduce the risk of chronic diseases such as cardiovascular disease and cancer (Du, Li, Ma, & Liang, 2009). Kiwifruit grows naturally in various regions. It has been primarily cultivated in the tropics and subtropics in countries such as New Zealand, Chile, France and Japan. Kiwifruit has been eaten stewed and in jams, jellies, and juices (Motohashi et al., 2001). It contains vitamin C, vitamin A, vitamin E, folic acid, and various phytochemicals. Also, kiwifruit may reduce the amount of carcinogens (Rush et al., 2006). Some nutritional values and chemical contents of the lyophilised extract of kiwifruit (*Actinidia deliciosa*) are shown in Table 1.

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Table 1
Nutritional values and chemical contents per 100 g lyophilised extract of kiwifruit (*A. deliciosa*).[a]

Kiwifruit contents	Amount
Yield	7.3[b]
Water	83.5 g
Carbohydrates	14.9 g
Protein	0.99 g
Vitamin C	105 mg
Vitamin B	0.2 mg
Fat	0.44 g
Potassium	332 mg
Chlorine	50 mg
Phosphor	40 mg
Calcium	35 mg
Magnesium	28 mg
Sulphur	16 mg
Sodium	4 mg
Selenium	0.6 mg
Iron	0.5 mg
Boron	0.2 mg
Zinc	0.16 mg
Copper	0.1 mg

[a] From the reference of Bursal (2009).
[b] The percentage amount (%) of lyophilised aqueous extract obtained from 100 mg kiwifruits.

In this study, we investigated the ferric ion (Fe^{3+}) reducing power (FRAP) and cupric ion (Cu^{2+}) reducing power (CUPRAC method) as well as the DPPH· scavenging, $ABTS^{\bullet +}$ scavenging, $DMPD^{\bullet +}$ scavenging, $O_2^{\bullet -}$ scavenging and ferrous ion (Fe^{2+}) chelating activities of LEK. Analysis of these activities is recommended to measure the antioxidant properties of food or pharmacological materials and investigate their potential protective effects. Furthermore, another significant goal of this study was to determine the polyphenol contents of LEK.

2. Materials and methods

2.1. Plant material

Kiwifruit (*A. deliciosa*) were cultivated on the Black Sea coast of Northeast Turkey and harvested in mid-August 2008. This region is suitable for the growth of kiwifruit because of its microclimate environment. Kiwifruit (*A. deliciosa*) were purchased from a local market in Erzurum, Turkey. The kiwifruit materials were identified by botanist Dr. Yusuf Kaya, Ataturk University, Faculty of Sciences, Department of Biology. The plant materials were stored at −80 °C until used.

Table 2
LC-MS-MS parameters and amount of selected antioxidant compounds in LEK (LEK: lyophilised water extract of kiwifruit (*A. deliciosa*)).

No	Compounds	Parent ion	Daughter ion	Collision energy (V)	LEK (mg/kg)[a]
S	Curcumin[b]	367	216.4	10	–
1	Caffeic acid	179	134.0	11	1.3
2	Ferulic acid	193	177.5	10	2.1
3	Syringic acid	197	181.6	10	1.1
4	Ellagic acid	301	150.0	10	1.0
5	Quercetin	301	178.6	10	3.2
6	α-Tocopherol	429	162.6	20	–
7	Catechol	109	64.8	35	–
8	Pyrogallol	125	78.7	20	2070.0
9	p-Hydroxybenzoic acid	137	92.7	10	1.1
10	Vanillin	181	135.5	10	2.1
11	p-Coumaric acid	163	118.7	10	4.3
12	Gallic acid	169	124.6	10	3.2
13	Ascorbic acid	175	114.0	12	217.1

[a] These values are below the limits of quantification.
[b] Internal standard.

2.2. Chemicals

The following compounds were used as standards for analysis by LC-MS/MS: 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) (Fig. 1). Stock solutions were prepared at 5 mg/L in ethanol, except for catechol and ascorbic acid, which were prepared at 50 mg/L 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. Dilutions were performed using automatic pipettes and glass

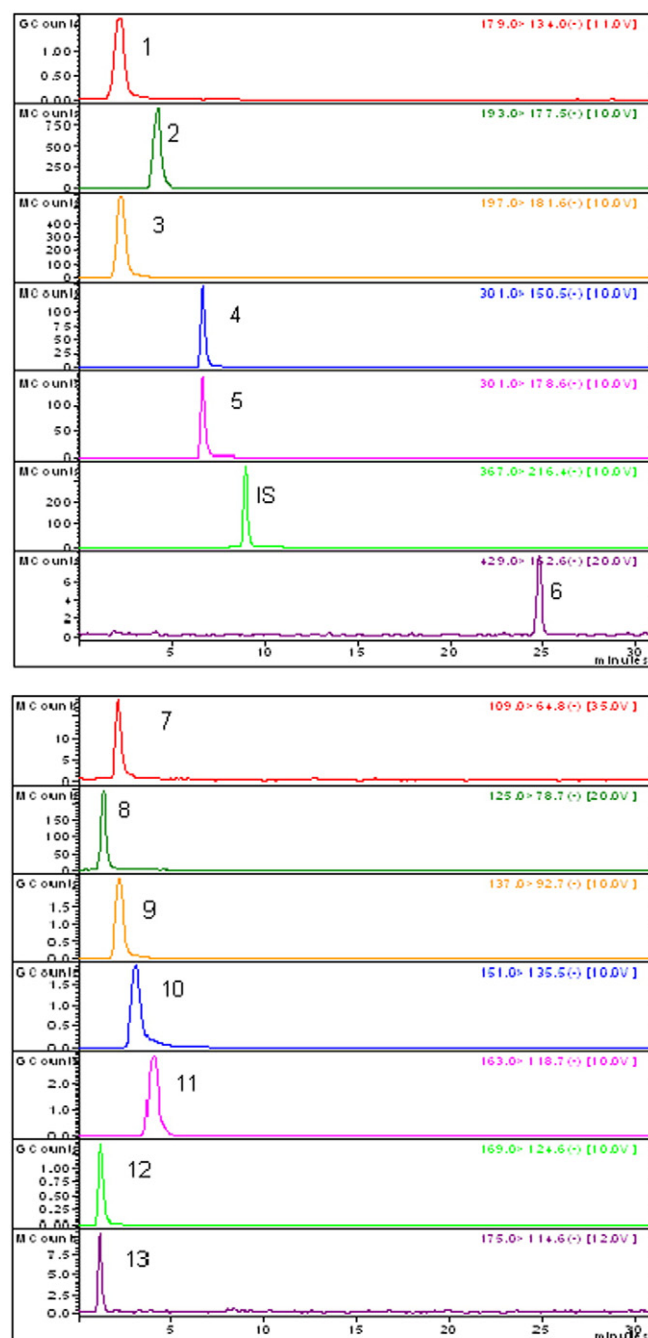

Fig. 1. Standard chromatogram of antioxidant phenolic acids by LC-MS-MS.

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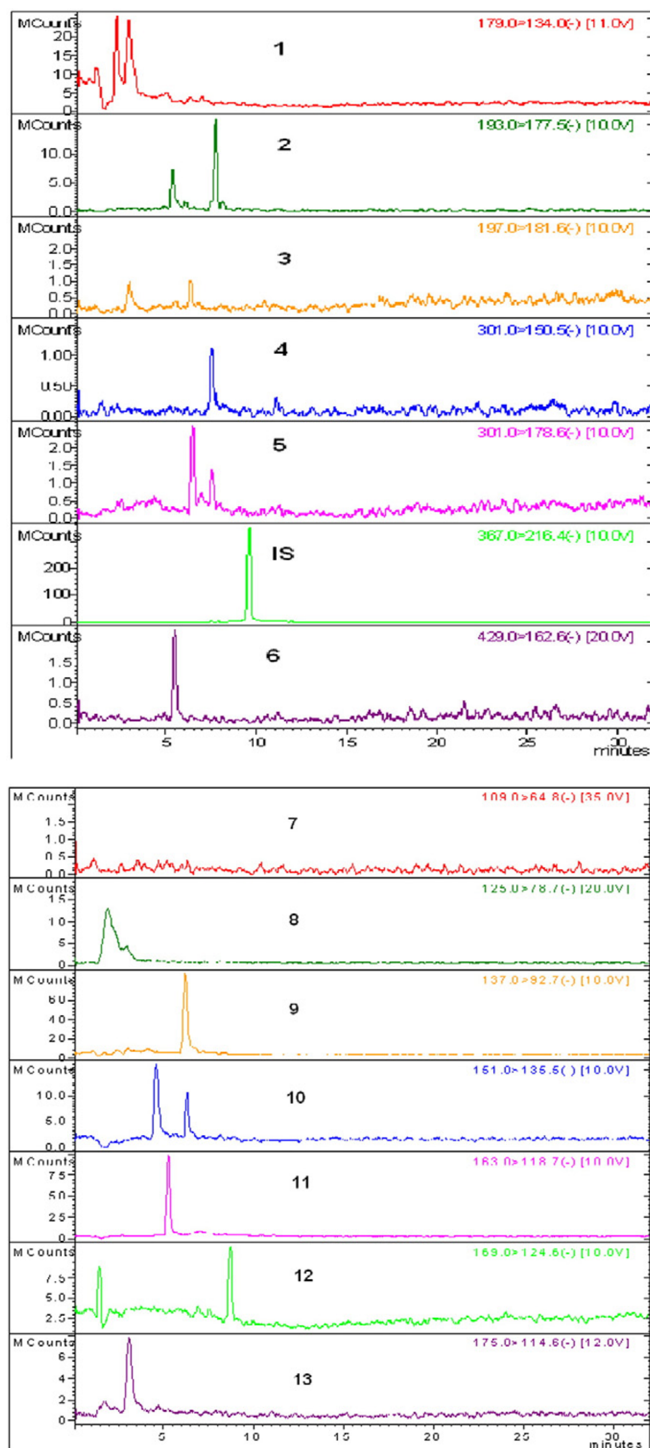

Fig. 2. Chromatogram of antioxidants of LEK determined by LC-MS-MS (diluted sample chromatogram for the correct determination of 3–8 in the linear range of LEK analysis (LEK: lyophilised water extract of kiwifruit (*A. deliciosa*).

volumetric flasks (A class), which were stored at −20 °C in glass containers. Curcumin solutions (1 mg/L) were freshly prepared; from which 100 μL was used as an internal standard (IS) in all LC-MS-MS experiments.

ABTS (2,2-azinobis 3-ethylbenzthiazoline-6-sulfonic acid), DPPH (1,1-diphenyl-2-picryl-hydrazyl), DMPD (*N*,*N*-dimethyl-*p*-phenylenediamine), 2,2′-bipyridine, 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), Folin–Ciocalteu's reagent, gallic acid, quercetin, linoleic acid, methionine, neocuproine (2,9-dimethyl-1,10-phenanthroline), NBT (nitroblue tetrazolium), polyoxyethylene-sorbitan monolaurate (Tween-20), riboflavin, α-tocopherol and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). The other chemicals were obtained from Merck.

2.3. Preparation of lyophilised aqueous extracts

To prepare the lyophilised water extracts, 100 g of whole kiwifruit (*A. deliciosa*) was ground into a fine powder in a mill and added to 250 mL distilled water. This mixture was stirred by a magnetic stirrer for 1 day at the temperature of 25 °C, and the extract was filtered with filter paper (Whatman No.1). The filtrates were frozen and lyophilised in a lyophiliser (Labconco, Freezone 1 L) at 5 mmHg at −50 °C. The lyophilised powder was stored at −30 °C until using for analysis (Elmastas et al., 2006).

2.4. Determination of total phenolic contents

The total phenolic content of the LEK was determined according to the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventõs, 1999) as described previously (Gülçin, Büyükokuroğlu, Oktay, & Küfrevioğlu, 2003; Gülçin, Tel, & Kirecci, 2008). First, we prepared a stock solution of LEK at 1 mg/mL concentration. Then, 1 mL of this solution was transferred into a test tube, and distilled water was adjusted to a total volume of 25 mL. Folin–Ciocalteu's reagent (0.5 mL) and sodium carbonate (2%, 1.5 mL) were added 3 min later. The samples were vortexed and left at room temperature for 30 min at which time the absorbance at 760 nm was measured. Distilled water was used as a blank and for control. A calibration curve of gallic acid was prepared, and phenolic contents were determined from the linear regression equation of this curve. The results are reported as gallic acid equivalents per mg extract.

2.5. Determination of total flavonoid contents

Flavonoids are the most common group of polyphenolic compounds in the human diet and exist ubiquitously in plants. They are most commonly known because of their antioxidant activity. The total flavonoid content in LEK was estimated by a colorimetric assay based on the procedure of Park, Koo, Ikegaki, and Contado (1997) as described previously (Köksal and Gülçin, 2008). Total flavonoids in LEK were determined as follows: the LEK solution (1 mg) was diluted with 4.3 mL of 80% aqueous ethanol containing 0.1 mL of aluminium nitrate (10%) and 0.1 mL of aqueous potassium acetate (1 M). After incubation for 40 min at room temperature, the absorbance was measured at 415 nm. Distilled water was used as a blank and control. A calibration curve of quercetin was prepared, and flavonoid contents were determined from the linear regression equation of the calibration curve. The results are reported as quercetin equivalents per mg extract.

2.6. LC-MS-MS analysis of LEK

The phenolic compounds in LEK were quantified by LC-MS-MS given in Fig. 2 (Gören, Çıkrıkçı, Çergel, & Bilsel 2009; Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010). Stock solutions of standards were prepared at 5 mg/L in ethanol, except for catechol and ascorbic acid, which were prepared at 0.45×10^{-3} M and 0.14×10^{-3} M, respectively. Curcumin solutions (2.72×10^{-6} M) were freshly prepared and used as an internal standard (IS) in all experiments.

In a volumetric flask, 100 mg of lyophilised LEK was dissolved in 5 mL of ethanol-water (50:50 v/v). One millilitre of this extract was transferred into another volumetric flask, and the volume was increased to 5 mL. Then, 100 μL of curcumin was added and diluted with ethanol-water (50:50 v/v). From the final solution, 1.5 mL was transferred into a capped autosampler vial, and 10 μL of sample was injected for analysis

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by LC. The samples in the autosampler were kept at 15 °C during the experiment.

Experiments were performed on 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, contains 5% formic acid; 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 5 μL.

The method was verified, and the uncertainties were determined as described in our previous study (Gülçin, Bursal, et al., 2010). The linearity ranges were assigned as 0–0.005 mg/mL for compounds 2, 3, 4, 5, 8, 9, 11 and 12, for compound 1, 0–2.5 mg/mL for compound 6, 1–25 mg/mL for compound 7 and 0.1–10 mg/mL for compound 13. LOD and LOQ values of the method for the reported components were determined as 0.5–50 μg/L. The limits of quantification (LOQs) were determined to be 3 times larger than LOD The relative uncertainties were in the range of 1.6% and 7.8% at 95% confidence levels (k: 2) (Gülçin, Bursal, et al., 2010).

2.7. DPPH• scavenging activity

The DPPH free radical scavenging activity of LEK was evaluated by the method of Blois (1958) as previously described by Gülçin (2005). Briefly, different concentrations (10–30 μg/mL) of LEK were prepared and diluted to 3 mL with ethanol. Then, 1 mL of ethanolic DPPH solution (0.1 mM) was added to the samples. These samples were vortexed and incubated in the dark at 30 °C for 30 min. The absorbance was measured at 517 nm against blank samples. A decrease in absorbance indicates DPPH free radical scavenging activity.

2.8. $ABTS^{\bullet+}$ scavenging activity

The $ABTS^{\bullet+}$ radical scavenging activity of LEK was evaluated according to the method of Re et al. (1999) with minor modifications (Gülçin, 2010). $ABTS^{\bullet+}$ is blue–green in colour with a characteristic absorbance at 734 nm. $ABTS^{\bullet+}$ cation radical was produced by reacting ABTS (2 mM) in H_2O and potassium persulphate (2.45 mM) at room temperature for 12 h. The $ABTS^{\bullet+}$ solution was diluted with phosphate buffer (0.1 M, pH 7.4) to achieve an absorbance of 0.750 ± 0.025 at 734 nm. Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of extract solution in methanol at different concentrations (10–30 μg/mL) of LEK. These samples were vortexed and incubated in the dark for 30 min. After 30 min, the absorbance at 734 nm was measured for each concentration relative to a blank. Decreased absorbance of the samples indicates $ABTS^{\bullet+}$ cation radical scavenging activity.

2.9. $DMPD^{\bullet+}$ scavenging activity

The DMPD radical scavenging ability of LEK was determined by the method of Fogliano, Verde, Randazzo, and Ritieni (1999) with slight modifications by Gülçin (2008). This assay is based on the capacity of the extract to inhibit $DMPD^{\bullet+}$ cation radical formation. Briefly, 105 mg of DMPD was dissolved in 5 mL of distilled water. Then, 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.3). $DMPD^{\bullet+}$ was produced by adding 0.3 mL ferric chloride (0.05 M) to this solution. Different concentrations of standard antioxidants or LEK (10–40 μg/mL) were added, and the total volume was adjusted to 0.5 mL with distilled water. One millilitre of the $DMPD^{\bullet+}$ solution was directly added to the reaction mixture. The reaction mixtures were vortexed and incubated in the dark for 15 min. The absorbance was measured at 505 nm.

2.10. Superoxide anion radical scavenging activity

The superoxide radical scavenging activity of LEK was determined by the riboflavin/methionine/illuminate assay (Talaz, Gülçin, Göksu, & Saracoglu, 2009). This assay is based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The total volume of the reaction mixture was 3 mL, and the concentration of kiwifruit water extract was 30 μg/mL. The concentrations of riboflavin, methionine and NBT were 1.33×10^{-5} M, 4.46×10^{-5} M and 8.15×10^{-8} M, respectively. The photo-induced reactions were performed using fluorescent lamps (20 W). The reaction mixture was illuminated for 40 min at 25 °C. The photochemically reduced riboflavin generates $O_2^{\bullet-}$, which reduces NBT to form blue formazan. The absorbance was measured at 560 nm. A decrease in absorbance indicates increased superoxide anion scavenging activity. The un-illuminated reaction mixture was used as a blank.

2.11. Fe^{3+} reducing activity

The reducing power of LEK was measured according to the method of Oyaizu (1986) with slight modifications (Gülçin, 2006b). According to this method, the reduction of Fe^{3+} to Fe^{2+} is determined by measuring the absorbance of Perl's Prussian blue complex. Briefly, different concentrations (10–30 μg/mL) of LEK in distilled water (0.75 mL) were mixed with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1 mL (1%) of potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50 °C for 20 min. Afterwards, the reaction mixture was acidified with 1 mL of trichloroacetic acid (10%). Finally, 0.25 mL of iron (III) chloride (0.1%) was added to this solution. The absorbance of the mixture at 700 nm was measured. A decrease in absorbance indicates increased ferric reducing power.

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

The cupric ion (Cu^{2+}) reducing power of LEK was determined by the method proposed by Apak, Güçlü, Özyürek, and Karademir (2004) with minor modifications (Balaydın, Gülçin, Menzek, Göksu, & Şahin, 2010). Briefly, 0.25 mL of 10 mM copper (II) chloride solution, 0.25 mL of 7.5 mM ethanolic neocuproine solution and 0.25 mL of 1.0 M ammonium acetate buffer solution were added to a test tube and mixed with 0.25 mL of different concentrations (10–30 μg/mL) of LEK. The total volume was adjusted to 2 mL with distilled water, and the reaction was mixed well. The tubes were kept at room temperature. After 30 min of incubation, the absorbance was measured at 450 nm against a blank. Increased absorbance indicates increased $Cu^{2+}-Cu^{+}$ reduction.

2.13. Ferrous ion (Fe^{2+}) chelating activity

The ferrous ion (Fe^{2+}) chelating activity of LEK was measured according to the method of Re et al. (1999) as previously explained (Şerbetçi Tohma & Gülçin, 2010). Briefly, different concentrations (10–30 μg/mL) of LEK in 0.25 mL methanol were added to 0.25 mL of ferrous sulphate (0.2 mM). The reaction was initiated by the addition of 1 mL Tris–HCl buffer (pH 7.4) and 1 mL 2,2′-bipyridine (0.2% in 0.2 M HCl) dissolved in 2.5 mL methanol. The total volume was adjusted to 6 mL with distilled water. The mixture was shaken vigorously and kept at room temperature for 10 min. Then absorbance was measured at 522 nm.

2.14. Statistical analysis

Each experiment was performed in triplicate. The data are reported as the mean ± standard deviation and were analysed by SPSS (version 17.0 SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between the means were determined by Duncan's Multiple Range tests. $P < 0.05$ was considered significant.

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3. Results and discussion

3.1. Determination of total phenolic and flavonoid compounds

Phenolic compounds act as reducing agents and antioxidants. Plant phenols have been shown to inhibit the formation of superoxide anion radicals generated by various enzymes (Taubert et al., 2003; Gülçin, Kirecci, Akkemik, Topal, & Hisar, 2010). Also, the phenolic contents in plants have been shown to possess antioxidant properties (Viuda-Martos et al., 2010).

Phenolic compounds in LEK were determined by LC-MS-MS. Curcumin was used as an internal standard. The amounts of phenolic compounds in LEK are summarised in Table 2. The amounts of pyrogallol and ascorbic acid were higher than the other compounds. Pyrogallol is a benzenetriol and has powerful reducing agent properties. Also, ascorbic acid is known for its antioxidant properties. High amounts of pyrogallol and ascorbic acid are consistent with the antioxidant and antiradical activity was determined in the present study.

The total phenolic content of LEK was determined according to the Folin–Ciocalteu method. The total phenolic amounts were obtained from the linear regression equation (absorbance = 0.002 × [GAE], $r^2 = 0.970$) as gallic acid equivalents per 1 mg of extract. The total phenolic content in 1 mg of LEK was determined to be 16.67 ± 2.83 μg GAE. This value is the average of three experiments. The high levels of phenolic compounds indicate high antioxidant capabilities. The levels of phenolic compounds in other plants were found to be 43.4 μg pyrocatechol equivalent for turpentine of *Pinus nigra* (Gülçin, Büyükokuroğlu, et al., 2003), 30 μg GAE for the lyophilised water extract of anise (Gülçin, Oktay, Kireççi, & Küfrevioğlu, 2003), 179.8 μg GAE for the lyophilised water extract of clove buds, 153.92 μg GAE for the lyophilised water extract of lavender (Gülçin et al., 2004), 54.3 μg GAE for the lyophilised water extract of black paper (Gülçin, 2005), and 22.0 μg GAE for the lyophilised water extract of duckweed (Gülçin, Elias, Gepdiremen, Chea, & Topal, 2010). In a recent study, the phenolic compounds of 1 mg of aerial parts and roots of Turkish liquorice ranged from 75.7 to 185.7 μg QE (Şerbetçi Tohma & Gülçin, 2010). The total phenolic content in 1 mg of the lyophilised water extract of various fruits and vegetables ranged from 20 to 200 μg GAE.

Flavonoids are the most common group of polyphenolic compounds in the human diet. They are abundant in plants. Flavonoids have many antioxidant, antiviral, and antimutagenic effects. Quercetin is a well-known plant-derived flavonoid that may have anti-inflammatory and antioxidant properties (Davis, Murphy, Carmichael, & Davis, 2009). Flavonoids are capable of chelating Fe^{3+}, Fe^{2+} and Cu^{2+} cations. Chelates of flavonoids have been shown to be more potent superoxide radical scavengers than uncomplexed flavonoids (Moridani, Pourahmad, Bui, Siraki, & O'Brien, 2003).

The amount of total flavonoids in LEK was determined using the linear regression equation obtained from the standard quercetin curve (absorbance = 0.006 x [QE], $r^2 = 0.9865$) as quercetin equivalents per 1 mg of extract (QE/mg extract). The total flavonoid content in 1 mg of LEK was determined to be 12.95 ± 0.52 μg QE from the average of three experiments. In different studies, various amounts of flavonoids have been detected in fruits and vegetables. For example, 8.15 μg QE was detected in the lyophilised water extract of propolis (Gülçin, Bursal, et al., 2010), and 4.5 μg QE was detected in the lyophilised water extract of duckweed (Gülçin, Elias, et al., 2010). This value ranged from 2 to 5 μg GAE for the aerial parts and roots of Turkish liquorice (Şerbetçi Tohma & Gülçin, 2010).

Antioxidant compounds play important roles in the scavenging and inhibition of free radicals. Therefore, the investigation and discovery of new sources of antioxidants have become important. The antiradical potential of LEK was analysed by DPPH· scavenging, $ABTS^{\bullet+}$ scavenging, $DMPD^{\bullet+}$ scavenging, $O_2^{\bullet-}$ scavenging, Fe^{3+}–Fe^{2+} transformation, cupric ion (Cu^{2+}) reducing and ferrous ion (Fe^{2+}) chelating assays.

3.2. Radical scavenging activity

Many diseases such as inflammation, reperfusion injury, atherosclerosis, Alzheimer's disease, and Parkinson's disease have been linked to ROS-mediated damage of biological macromolecules, which arises from an imbalance between radical-generating and radical-scavenging systems (Taubert et al., 2003). DPPH and ABTS assays are widely used to determine the radical scavenging ability of various samples (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Jang et al., 2007). The radical scavenging activity of LEK was determined by DPPH·, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ scavenging assays in the present study. The percentage of radical scavenging capability was calculated using the following equation (Gülçin, Elias, et al., 2010):

$$\text{Radical scavenging capability}\,(\%) = \left(1 - \frac{A_{\text{Sample}}}{A_{\text{Control}}}\right) \times 100$$

The antiradical activity of LEK or standard compounds can be determined by assessing the scavenging activity on DPPH radicals. This method has been widely used to evaluate the free radical scavenging activity of various antioxidant substances (Jacob, Hakimuddin, Paliyath, & Fisher, 2008; Danino, Gottlieb, Grossman, & Bergman, 2009). This method is based on the reduction of DPPH in alcoholic solutions in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction (Gülçin, 2007). The dark colour of the DPPH radical becomes lighter due to the antioxidant compound, causing a decrease in the absorbance at 517 nm. DPPH free radical scavenging activity of LEK and standard antioxidants (α-tocopherol and trolox) were investigated.

The free radical inhibition of LEK and standard antioxidants decreased in the following order: α-tocopherol (84.5%) > trolox (47.2%) > LEK (16.7%) in presence of same concentration of test sample (30 μg/mL). The DPPH radical scavenging percentages of different concentrations (10–30 μg/mL) of LEK were 4.9, 14.8 and 16.7% respectively. LEK effectively inhibited DPPH free radical scavenging activity (Fig. 3A and Table 3). Ascorbic acid appears the main component of LEK; however, it has not strong effect as other putative antioxidants such as BHA, BHT, α-tocopherol and trolox.

Another effective method to measure radical scavenging activity is the ABTS radical cation decolourisation assay, which showed similar results to those obtained in the DPPH reaction. The ABTS radical scavenging by LEK and standard antioxidants decreased in the following order: trolox (99.8%) > α-tocopherol (98.7%) > BHA (98.6%) > BHT

Table 3
EC_{50} values (for radical scavenging assays) of LEK and standard antioxidants (LEK: lyophilised water extract of kiwifruit (*A. deliciosa*), BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

EC_{50} (μg/mL)					
	LEK	BHA	BHT	α-Tocopherol	Trolox
$ABTS^{\bullet+}$ scavenging	28.4 ± 2.14	13.4 ± 1.33	7.8 ± 0.55	12.8 ± 1.13	10.9 ± 1.33
DPPH• scavenging	83.4 ± 4.67	–	–	17.2 ± 2.18	29.8 ± 2.17
$DMPD^{\bullet+}$ scavenging	40.0 ± 3.48	66.0 ± 7.13	–	–	14.2 ± 3.11
$O_2^{\bullet-}$ scavenging	38.4 ± 4.21	–	–	36.8 ± 5.21	46.7 ± 5.21

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(98.2%) > LEK (62.9%) at a concentration of 30 μg/mL test sample. Also, the percentages of ABTS cation radical scavenging activity of different concentrations (10, 20 and 30 μg/mL) of LEK were found to be 7.7, 19.4 and 62.5%, respectively. These results show that LEK has effective ABTS cation radical scavenging activity at higher concentration (Fig. 3B and Table 3). However, these values are lower than those of standard antioxidants.

Another assay used to measure free radical scavenging activity involves the decolourisation of the $DMPD^{•+}$ cation radical, similar to the ABTS cation radical decolourisation assay. $DMPD^{•+}$ has a maximum absorbance at 505 nm. Antioxidant compounds or radical scavengers, which can act as hydrogen donors for $DMPD^{•+}$, decrease the absorbance at 505 nm of $DMPD^{•+}$ (Gülçin, Elias, Gepdiremen, Taoubi, & Köksal, 2009).

As seen in Fig. 3C and Table 3, $DMPD^{•+}$ radical inhibition by LEK and standard antioxidants decreased in the following order: trolox (67.2%) > LEK (48.6%) > BHA (25.4%) in the presence of 30 μg/mL test sample. The percentages of $DMPD^{•+}$ cation radical scavenging activity at different concentrations (10–40 μg/mL) of LEK were 22.2, 32.4, 48.6 and 50.0%, respectively.

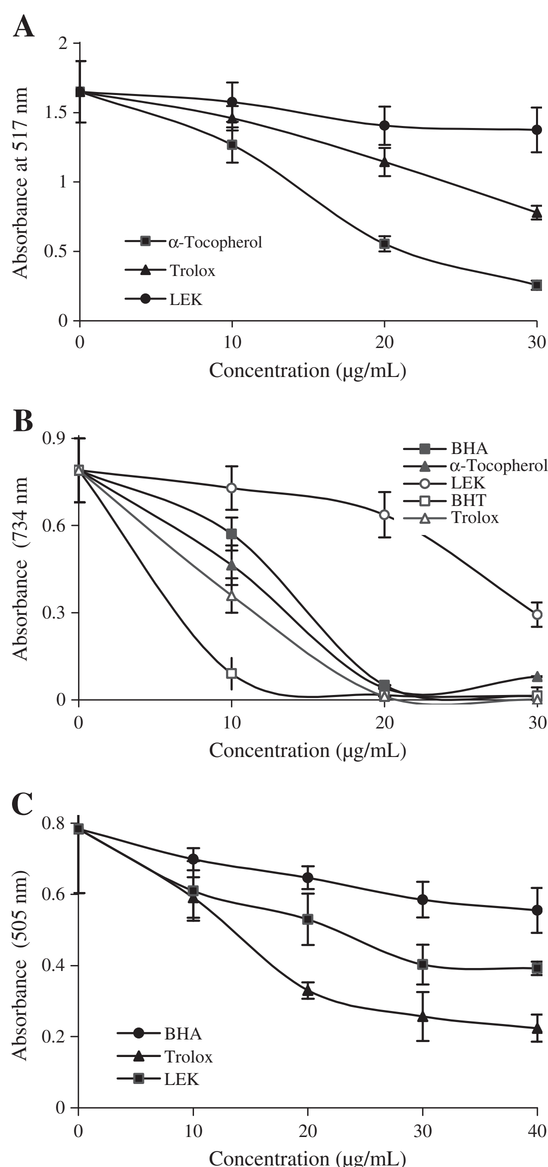

Fig. 3. DPPH•, $ABTS^{•+}$ and $DMPD^{•+}$ radical scavenging activity of LEK. A. DPPH· radical scavenging activity. B. $ABTS^{•+}$ radical scavenging activity. C. $DMPD^{•+}$ radical scavenging activity.

Superoxide anion radicals ($O_2^{•-}$) are generated by one electron transfer to molecular oxygen. This process can generate other more harmful ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) (Taubert et al., 2003; Gülçin, Elias, Gepdiremen, Boyer, & Köksal, 2007). Two different in vitro superoxide-generating systems are commonly used; the first is a xanthine oxidase/hypoxanthine system, and the other is a riboflavin/methionine/illuminate system. In this study, we used riboflavin/methionine/illuminate system for generation of superoxide anion radicals.

In this system, the riboflavin/ methionine/ illuminate system was used to generate superoxide anion radicals ($O_2^{•-}$). Then, superoxide anion radicals reduce NBT^{2+} to produce formazan, which is blue in colour. Antioxidants inhibit the formation of blue NBT (Parejo et al., 2002). The two principal reactions are involved in this assay are (Liochev & Fridovich, 1995):

$$2NBTH\cdot \rightarrow NBT + NBTH_2(\text{Formazan}) \quad (a)$$

$$NBTH\cdot + O_2 \leftrightarrow NBT + O_2^{•-}(\text{Aquasi equilibrium}) \quad (b)$$

When riboflavin is photochemically activated, it reacts with NBT to generate NBTH· (Beauchamp & Fridovich, 1971), which leads to formazan according to reaction (a). In the presence of oxygen, radical species are controlled by a quasi equilibrium (b). Thus, superoxide anion radicals appear indirectly when the assay is performed under aerobic conditions. In the presence of an antioxidant molecule that can donate an electron to NBT, the typical purple colour of formazan decays, which can be followed spectrophotometrically at 560 nm. Antioxidants are able to inhibit the formation of NBT and scavenge superoxide anion radicals. The decrease in absorbance at 560 nm in the presence of antioxidants indicates the scavenging of superoxide anions in the reaction mixture (Gülçin, Oktay, et al., 2003).

The inhibition of superoxide radical formation by LEK and standard antioxidants decreased in the following order: α-tocopherol (40.8%) > LEK (39.1%) > trolox (32.1%) in presence of 30 μg/mL test sample. The percentage of superoxide radical scavenging of LEK and standards were similar (Table 3).

3.3. Reducing power determination

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species (Gülçin, Elmastas, & Aboul-Enein, 2007). The reducing power of LEK was investigated by FRAP and CUPRAC assays.

Antioxidant compounds reduce Fe^{3+}-ferricyanide complexes to the ferrous (Fe^{2+}) form. The Prussian blue coloured complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, the amount of reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm (Chung, Chang, Chao, Lin, & Chou, 2002). In this assay, the yellow colour of the test solution changes to green or blue depending on the reducing power of the antioxidant. A higher absorbance indicates higher ferric reducing power.

$$K_3Fe(CN)_6 \xrightarrow{\text{Reductant}} Fe(CN)_6^{4-}$$

$$Fe(CN)_6^{4-} + FeCl_3 \longrightarrow Fe_4[Fe(CN)_6]_3$$

As shown in Fig. 4A, the ferric reducing power of LEK increased with increasing concentration, similar to the standard antioxidants. However, these differences were found as insignificant ($p > 0.05$). The

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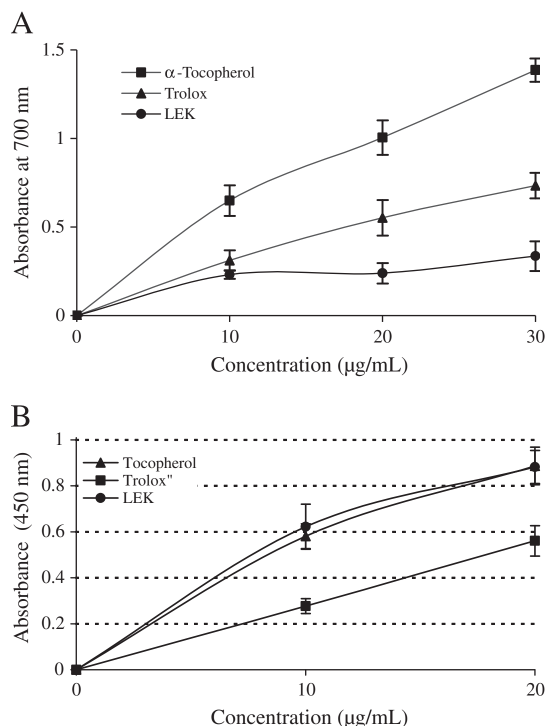

Fig. 4. Reducing power of LEK. A. Fe^{3+} reducing ability by Fe^{3+}–Fe^{2+} transformation. B. Cu^{2+}–Cu^{+} reducing ability by CUPRAC method.

reducing power of the LEK and standard antioxidants decreased in the following order: α-tocopherol>trolox>LEK with 30 μg/mL test sample. LEK had a similar ferric reducing power to standard antioxidants (α-tocopherol and trolox). Fe^{3+} reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidants (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003).

The CUPRAC method has also been used to determine the reducing power of antioxidant compounds (Apak et al., 2004). This method is based on the reduction of Cu^{2+} to Cu^{+} by antioxidants in the presence of neocuproine (Gülçin, 2008). In this assay, a higher absorbance indicates higher cupric ion (Cu^{2+}) reducing ability. Cupric ion (Cu^{2+}) reducing power of LEK and standard compounds decreased in the following order: α-tocopherol≥LEK>trolox in the presence of 20 μg/mL test sample (Fig. 4B).

Metal ions can cause lipid peroxidation, which can produce free radicals and lipid peroxides (Gülçin, Elias, et al., 2010). Therefore,

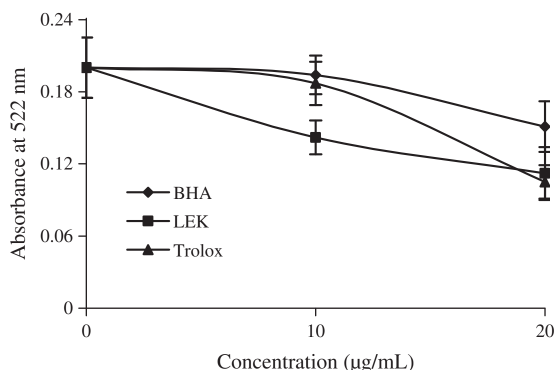

Fig. 5. Ferrous ion (Fe^{2+}) chelating activity of LEK.

metal chelating activity indicates antioxidant property. The decreased absorbance in the reaction mixture indicates higher metal chelating ability. In the present study, 2,2′-bipyridine was used as a chelating agent. According to the results, LEK has effective metal chelating abilities. Metal chelating percentage of LEK was 55.9% with 30 μg/mL test sample (Fig. 5).

4. Conclusion

In this paper, we studied the antioxidant and antiradical activities of LEK. LEK had good scavenging capabilities for DPPH·, $ABTS^{•+}$, $DMPD^{•+}$ and $O_2^{•-}$ radicals compared to standard antioxidant compounds. Also LEK shown good reducing capacity by FRAP and CUPRAC assays and good metal chelating abilities on ferrous ions (Fe^{2+}). The high antioxidant properties of LEK indicate that the fruit may be available in the diet and useful in food and pharmaceutical industries. In addition, kiwifruits can be used as an easily accessible source of natural antioxidants and possibly as a food supplement. They can also be used to stabilise food against oxidative deterioration.

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