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Antioxidant Activity of Cauliflower (*Brassica oleracea* L.)

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Abstract: Recently, a number of studies on the health benefits associated with fruits, vegetables, herbs and spices demonstrated that they possess potent antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic activity. The potential antioxidant activity of water and ethanol extracts of cauliflower (*Brassica oleracea* L.) were investigated to evaluate their potential value as a natural ingredient for foods or cosmetic application. In this study antioxidant activity was measured by 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging, N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) radical scavenging, superoxide anion ($O_2^{\bullet -}$) radical scavenging, total antioxidant activity, reducing activity using Fe^{+3}-Fe^{+2} transformation and CUPRAC assays, hydrogen peroxide (H_2O_2) scavenging, and ferrous metal chelating activity assays. The water extract of cauliflower (WEC) and ethanol extract of cauliflower (EEC), as antioxidants, neutralized the activity of radicals and inhibited the peroxidation reactions of linoleic acid emulsion. Total antioxidant activity was measured according to the ferric thiocyanate method. α-Tocopherol and trolox, a water-soluble analogue of tocopherol, were used as the reference antioxidant compounds. WEC and EEC showed 88.6% and 80.1% inhibition of lipid peroxidation of linoleic acid emulsion, respectively, at the concentration of 30 μg ml^{-1}. On the other hand, at the same concentration, the standard antioxidants α-tocopherol and trolox exhibited 68.1.4% and 81.3% inhibition of peroxidation of linoleic acid emulsion, respectively. In addition, WEC and EEC had effective $DPPH^{\bullet}$, $ABTS^{\bullet +}$, $DMPD^{\bullet +}$, and superoxide anion radical scavenging, hydrogen peroxide scavenging, total reducing power, and metal chelating of ferrous ion activity. Also, those various antioxidant activities were compared to α-tocopherol and trolox as references antioxidants.

Key Words: Cauliflower, *Brassica oleracea*; antioxidant activity, radical scavenging

Karnabaharın (*Brassica oleracea* L.) Antioxidan Aktivitesi

Özet: Son zamanlarda meyvelerin, sebzelerin, bitkilerin, otların ve baharatların sahip olduğu kuvvetli antioksidan, antienflamatuar, antimutajenik ve antikarsinojenik aktivitelerinin sağlık ile ilgi faydaları üzerinde birçok çalışma yapılmıştır. Karnabaharın (*Brassica oleracea* L.) su ve etanol ekstrelerinin antioksidan aktiviteleri gıda ve kozmetik alandaki uygulamaları için araştırıldı. Bu çalışmada karnabaharın (*Brassica oleracea* L.) antioksidant aktivitesi 2,2'-azino-bis(3-etilbenztiyoazolin-6-sülfonik asit) (ABTS) radikal giderme, 1,1-difenil-2-pikril-hidrazil serbest radikal ($DPPH^{\bullet}$) giderme, N,N-dimetil-p-fenilendiamin dihidroklorit (DMPD) radikal giderme, süperoksit anyon ($O_2^{\bullet -}$) radikal giderme, total antioksidan aktivite, Fe^{+3}-Fe^{+2} dönüşüm ve Cuprac metotlarına göre indirgeme aktivitesi, hidrojen peroksit (H_2O_2) giderme ve ferröz iyonları (Fe^{+2}) kelatlama aktivitesi metotları ile ölçüldü. Birer antioksidan kaynağı olarak karnabaharın su (WEC) ve etanol (EEC) ekstraktları radikalleri nötralize ve linoleik asit emülsiyonunun peroksidasyonunu ise inhibe ettiği gözlendi. Total antioksidan aktivite ferrik tiyosiyanat metoduna göre yapıldı. α-Tokoferol ve α-tokoferolün suda çözünen bir analoğu olan troloks referans antioksidan maddeler olarak kullanıldı. WEC ve EEC 30 μg ml^{-1} konsantrasyonunda, linoleik asit emülsiyonunun peroksidasyonunu sırasıyla %88.6 ve %80.1 inhibe ettiği belirlendi. Bunun yanısıra WEC ve EEC etkili bir şekilde $DPPH^{\bullet}$, $ABTS^{\bullet +}$, $DMPD^{\bullet +}$ ve süperoksit anyon ($O_2^{\bullet -}$) radikal giderme, hidrojen peroksit giderme, indirgeme kuvveti ve ferröz iyonlarını (Fe^{+2}) kelatlama aktivitesine de sahip olduğu belirlendi. Ayrıca bu farklı antioksidan aktiviteler birer referans antioksidan olarak α-tokoferol ve troloks ile karşılaştırıldı.

Anahtar Sözcükler: Karnabahar, *Brassica oleracea*, antioksidan aktivite, radikal giderme

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Introduction

Oxygen, an element indispensable for life, can, under certain circumstances, adversely affect the human body. Oxidation processes are very important to living organisms. Most of the potentially harmful effects of oxygen are due to the formation of reactive oxygen species (ROS). The uncontrolled production of ROS and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. ROS are a class of highly reactive molecules formed during aerobic life in living organisms and include superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and non free-radical species, such as H_2O_2 and singlet oxygen ($^1O_2$) (Halliwell and Gutteridge, 1989; Gülçin et al., 2002a; 2002b). There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. When there is imbalance between ROS and antioxidant defense mechanisms, ROS lead to oxidative modification in cellular membranes or intracellular molecules (Duh et al., 1999; Büyükokuroglu et al., 2001; Gülçin et al., 2003). In addition, under pathological conditions or oxidative stress, ROS are overproduced and result in peroxidation of membrane lipids, leading to the accumulation of lipid peroxides; however, they are removed by antioxidant defense mechanisms. Antioxidants are considered as possible protective agents, reducing oxidative damage from ROS in the human body and retarding the progress of many chronic diseases, as well as lipid peroxidation (Pryor, 1991; Kinsella et al., 1993; Lai et al., 2001; Gülçin et al., 2003a). Therefore, there is a growing interest in substances that exhibit antioxidant properties, which are supplied to humans and animals as food components or as specific pharmaceuticals. Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and *tert*-butylhydroquinone are the most commonly used antioxidants at the present time; however, their safety has recently been questioned due to their toxicity and possible carcinogenicity (Wichi, 1988; Sherwin, 1990; Sun and Fukuhara, 1997). Hence, in recent years, the restricted use of synthetic antioxidants, such as BHA and BHT, has caused increased interest in natural antioxidant substances (Baardseth, 1989; Gülçin et al., 2005a). Thus, development of safer natural antioxidants from extracts of spices and other plant materials that can replace synthetic antioxidants is of interest (Liyana-Pathirana and Shahidi, 2006). Plant tissues synthesize a wide variety of phenolic compounds (Loliger, 1991). Many kinds of antioxidative components that contain polyphenolic compounds, chlorophylls, carotenoids, tocopherol derivatives, lignan, and related isoprenoids have been isolated from different kinds of plants, such as oilseeds, cereal crop, vegetables, leaves, roots, spices, herbs, and seaweeds, for use as antioxidants (Wettasinghe and Shahidi, 1999, Gülçin et al., 2006b).

Nowadays, natural antioxidants have become a major area of scientific research (Demo et al., 1998; Sanchez-Moreno et al., 1999); therefore, the importance of searching for and exploiting natural antioxidants, especially those of plant origin, has increased greatly in recent years. There is a growing interest in natural additives as potential antioxidants (Grice, 1986; Moure et al., 2001; Gülçin et al., 2002a; 2006a; Oktay et al., 2003). Natural antioxidants are known to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic, and vasodilatory activity. In fact, a fundamental property important for life is antioxidant activity and this property may give rise to anti-carcinogenicity, anti-mutagenicity, and anti-aging activity, among others (Cook and Samman, 1996; Liyana-Pathirana and Shahidi, 2006).

The main objectives of the present study were to assess the antioxidant potential of water and ethanol extracts of cauliflower (*Brassica oleracea* L.) (WEC and EEC, respectively) with in vitro antioxidant assays, including DPPH$^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging, $DMPD^{\bullet+}$ scavenging, superoxide anion radical scavenging, total antioxidant activity by ferric thiocyanate method, reducing power by Fe^{+3}-Fe^{+2} transformation and CUPRAC assays, hydrogen peroxide scavenging, and metal chelating effect on ferrous ions (Fe^{+2}).

Materials and Methods

Chemicals

N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD), 2,9-dimethyl-1,10-phenanthroline (neocuproine), 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), riboflavin, methionine, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), linoleic acid, 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine®), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ethylenediaminetetraacetic acid (EDTA), α-tocopherol, polyoxyethylene sorbitan monolaurate (Tween-20), $CuCl_2$, and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant materials and extraction procedure

Cauliflower (*Brassica oleracea* L.) buds were obtained from a local market in Erzurum, Turkey. Whole cauliflower (*Brassica oleracea* L.) buds were cut into small pieces and left to dry on a bench under ambient temperature for over 1 week, after which they were ground in a blender. For WEC, 100 g of dried cauliflower (*Brassica oleracea* L.) buds was ground into a fine powder in a mill and mixed with 400 ml of boiling water with a magnetic stirrer for 15 min. Then, the extract was filtered over Whatman No. 1 paper. The filtrates were frozen and lyophilized in a lyophilizator at a pressure of 5 mm Hg at –50 °C (Labconco FreeZone 1L).

For EEC, 100 g of dried cauliflower (*Brassica oleracea* L.) buds was ground into a fine powder in a mill and mixed with ethanol. The residue was re-extracted under some conditions until extraction solvents became colorless (final volume 600 ml). The obtained extracts were filtered over Whatman No. 1 paper and the filtrate was collected. Then, ethanol was removed using a rotary evaporator (RE 100 Bibby, Stone Staffordshire England) at 50 °C to obtain dry extract. Both extracts were placed in a dark plastic bottle and stored at –20 °C until used.

Determination of total phenolic compounds

Total phenolic content of WEC and EEC was determined with Folin-Ciocalteu reagent, according to the method of Slinkard and Singleton (1977), as previously described (Gülçin et al., 2003a; 2004e). Total phenolic content in WEC and EEC was determined as micrograms of gallic acid equivalent using an equation obtained from the standard gallic acid graph (R^2: 0.9217):

Absorbance (λ_{760}) = 0.014 × [Phenols (µg)]

Total antioxidant activity-ferric thiocyanate method

The antioxidant activity of WEC, EEC, and standards was determined according to the ferric thiocyanate method in linoleic acid emulsion (Mitsuda et al., 1996), as described in a previous study (Gülçin et al., 2005b). With this method peroxide formation occurred during the oxidation of linoleic acid oxidation. These compounds oxidized Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. The percent inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - \left(\frac{A_S}{A_C} \times 100\right)$$

where A_C is the absorbance of the control reaction and A_S is the absorbance in the presence of the sample of WEC, EEC, or other test compounds. In the control, the sample was replaced with an equal volume of ethanol (Gülçin et al., 2004a; Gülçin, 2006b).

Total reduction activity by Fe^{3+}-Fe^{2+} transformation

The samples prepared for the ferric thiocyanate method were used for this and the other antioxidant assays. The reducing activity of WEC and EEC was determined by the method of Oyaizu (1986), as previously described (Gülçin et al., 2004b). The capacity of WEC and EEC to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. Increased absorbance of the reaction mixture indicates greater reduction capability (Elmastas et al., 2006b; Gülçin et al., 2007a).

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

For determination of the reducing ability of WEC and EEC, the cupric ions (Cu^{2+}) reducing power method was also used (Apak et al., 2006), with slight modification. To test tubes containing different concentrations of WEC and

EEC (10-30 μg ml^{-1}), 0.25 ml of $CuCl_2$ solution (0.01 M), 0.25 ml of ethanolic neocuproine solution (7.5×10^{-3} M), and 0.25 ml of NH_4Ac buffer solution (1 M) were added, consecutively. The total volume was then adjusted with distillated water to 2 ml and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min.

Metal chelating activity on ferrous ions (Fe^{2+})

Ferrous ion (Fe^{2+}) chelation by WEC, EEC, and the standards, including EDTA, was estimated by the Ferrozine® assay (Dinis et al., 1994), as explained by Gülçin et al. (2005c). The results were expressed as a percentage of inhibition of the ferrozine-Fe^{2+} complex formation. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Ferrous ion chelating effect (\%)} = \left(\frac{A_C - A_S}{A_C} \times 100\right)$$

where A_C is the absorbance of the ferrozine-Fe^{2+} complex and A_S is the absorbance in the presence of the sample of WEC, EEC, or the standards (Gülçin et al., 2004b; Elmastas et al., 2006).

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging ability of WEC and EEC was determined according to the method of Ruch et al. (1989). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). WEC and EEC, at the 30 μg ml^{-1} concentration in 3.4 ml of phosphate buffer, were added to an H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. A blank solution contained the phosphate buffer without H_2O_2. The hydrogen peroxide scavenging capacity of WEC and EEC was calculated from the calibration curve determined by linear regression (r^2: 0.9831):

$$\text{Absorbance } (\lambda_{230}) = 0.0497 \times [H_2O_2]$$

The percentage of H_2O_2 scavenging of WEC, EEC, and the standard compounds was calculated as:

$$H_2O_2 \text{ scavenging effect (\%)} = \left(\frac{A_C - A_S}{A_C} \times 100\right)$$

where A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample of WEC, EEC, or the standards (Elmastas et al., 2005; Gülçin, 2006a).

ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activity was performed according to Re et al. (1999), as described in our previous study (Gülçin et al., 2006b). The $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve determined by linear regression (R^2: 0.9922):

$$\text{Absorbance } (\lambda_{734\ nm}) = 2.744 \times [ABTS^{\bullet+}]$$

The capability to scavenge the $ABTS^{\bullet+}$ radical was calculated using the following equation:

$$ABTS^{\bullet+} \text{ scavenging effect (\%)} = \left(\frac{A_C - A_S}{A_C} \times 100\right)$$

where A_C is the initial concentration of $ABTS^{\bullet+}$ and A_S is the absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of scavengers (Gülçin et al., 2006b; Gülçin, 2006c).

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of WEC and EEC was measured by bleaching the purple-colored ethanol solution of the stable DPPH radical, according to the Blois method (1958). This method was described previously in our studies (Gülçin et al., 2004c; Gülçin 2006b). The DPPH· concentration (mM) in the reaction medium was calculated from the following calibration curve determined by linear regression (R^2: 0.9974):

$$\text{Absorbance } (\lambda_{517\ nm}) = 5.869 \times 10^{-4}\ [DPPH^{\bullet}] + 0.0134$$

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

$$DPPH^{\bullet} \text{ scavenging effect (\%)} = \left(\frac{A_C - A_S}{A_C} \times 100\right)$$

where A_C is the initial concentration of the stable DPPH· radical without the test compound and A_S is the absorbance of the remaining concentration of DPPH· in the presence of WEC and EEC (Gülçin et al., 2004c, 2007a; Cristiane de Souza et al., 2004).

Preparation of $DMPD^{\bullet+}$ radical cation and measurement of $DMPD^{\bullet+}$ scavenging

The $DMPD^{\bullet+}$ radical scavenging activity of WEC and EEC was determined according to the method described by Fogliano et al. (1999). Different concentrations of WEC and EEC samples (10-30 µg ml^{-1}) were added to a spectrophotometric cuvette and total volumes of these samples were adjusted to 1 ml with distilled water. Then, 1 ml of $DMPD^{\bullet+}$ radical solution was directly transferred to the quartz cuvette. After 10-min incubation the absorbance of the test samples was measured at 505 nm. The $DMPD^{\bullet+}$ scavenging capacity for each tested sample was calculated from the calibration curve determined by linear regression (R^2: 0.9831):

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

The percentage of $DMPD^{\bullet+}$ scavenging capability of WEC and EEC was calculated using the following equation:

$$DMPD^{\bullet+} \text{ scavenging effect (\%)} = \left(1 - \frac{A_S}{A_C} \times 100\right)$$

where A_C is the absorbance of the control, which contains 1 ml of control reaction (containing $DMPD^{\bullet+}$ solution, except the WEC and EEC), and A_S is the absorbance in the presence of WEC and EEC. $DMPD^{\bullet+}$ decreases significantly upon exposure to radical scavengers (Fogliano et al., 1999).

Superoxide anion radical scavenging activity

Superoxide radicals were generated according to the method of Beauchamp and Fridovich (1971), as described by Zhishen et al. (1999), with slight modification. Superoxide radicals were generated in riboflavin and methionine, illuminated (20 W for 5 min), and assayed by the reduction of NBT to form blue formazan. This method was previously described (Gülçin, 2006c). The percentage of superoxide anion scavenged was calculated using the following formula:

$$O_2^{\bullet-} \text{ scavenging effect (\%)} = \left(1 - \frac{A_S}{A_C} \times 100\right)$$

where A_C is the absorbance of the control and A_S is the absorbance in the presence of WEC, EEC, or the standards (Gülçin et al., 2003, 2004d).

Statistical Analysis

All the analyses of total antioxidant activity were performed in duplicate. The other analyses were performed in triplicate. The data were recorded as means ± standard deviation and analyzed using SPSS v.11.5 for Windows (SPSS Inc., Chicago). One-way analysis of variance (ANOVA) was performed. Significant differences between means were determined by LSD tests. P values < 0.05 were regarded as significant and P values < 0.01 very significant.

Results and Discussion

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Parr and Bolwell, 2000). Phenolic compounds are very important plant constituents because of their scavenging ability, which is due to their hydroxyl groups (Hatano et al. 1989). In addition, it was reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993). As can be seen in Table 1, 78.6 and 175.7 mg of gallic acid equivalent of phenols was detected in 1 mg of WEC and EEC. Phenolic compounds appear to be responsible for the antioxidant activity of WEC and EEC; however, which components are responsible for the antioxidative activity of both extracts remains unclear. Therefore, it is suggested that further work should be performed on the isolation and identification of the antioxidant constituents of WEC and EEC.

Table 1. The yield of crude extract, total phenolic content, hydrogen peroxide scavenging, and superoxide anion radical scavenging of WEC and EEC.

	WEC	EEC
Yield (%)		
Total phenolics (µg mg^{-1} extract)[a]	78.6	175.7
Hydrogen peroxide scavenging (%)	51.2	41.4
Superoxide anion scavenging (%)	57.5	43.9

[a] Determined as gallic acid equivalent(GAE).

For determining total phenolic content, calibration curves were obtained using known quantities of standard gallic acid. Among the 2 extracts, EEC possessed more phenolic compounds. It was reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993, Gülçin, 2005). According to another report, a very positive relationship between total phenols and antioxidant activity was found in many plant species (Velioglu et al., 1998). As seen in Figures 1 and 2 and Table 1, the results indicate that there wasn't a positive correlation between total antioxidant activity and total phenolic content of WEC and EEC; however, some authors did find a correlation between phenolic content and antioxidant activity (Yen et al., 1993).

In the present study the antioxidant activity of WEC and EEC were compared to α-tocopherol and its water-soluble analogue, trolox. The antioxidant activity of WEC, EEC, α-tocopherol, and trolox was evaluated in a series of in vitro tests: DPPH· free radical scavenging, DMPD radical scavenging, ABTS radical scavenging, superoxide anion radicals scavenging, total antioxidant activity by ferric thiocyanate method, reducing activities, hydrogen peroxide scavenging activity, and metal chelating activity.

The ferric thiocyanate method measures the amount of peroxides produced during the initial stages of oxidation, which are the primary products of oxidation. The total antioxidant activity of WEC, EEC, and the standard compounds was determined by the ferric thiocyanate method in a linoleic acid system. WEC and EEC had strong antioxidant activity. The effects of various concentrations of WEC and EEC (from 10-30 μg ml^{-1}) on lipid peroxidation of linoleic acid emulsion are shown in Figures 1 and 2. At the 30 μg ml^{-1} concentration, WEC and EEC exhibited 88.6% and 80.1% lipid peroxidation of linoleic acid emulsion, respectively. On the other hand, at the same concentration, α-tocopherol and trolox showed 68.1% and 81.3% inhibition of peroxidation of linoleic acid emulsion, respectively. The results clearly showed that WEC and EEC had more total antioxidant activity than trolox, similar to α-tocopherol at the same concentration (30 μg ml^{-1}).

The CUPRAC method was developed as an antioxidant capacity assay. 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 may be more efficiently extended to the possible in vivo reactions of antioxidants. The CUPRAC chromogenic redox reaction is carried out at a pH 7.0, close to the physiological pH (Apak et al., 2004). This method is capable of measuring thiol-type antioxidants,

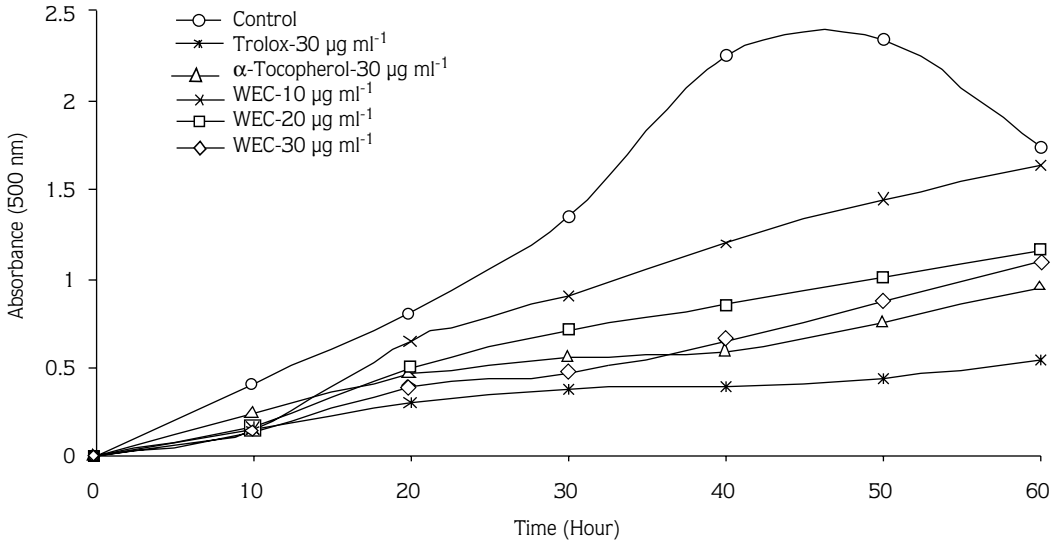

Figure 1. Total antioxidant activity of WEC at different concentrations (10-30 μg ml^{-1}), and α-tocopherol and trolox (30 μg ml^{-1}).

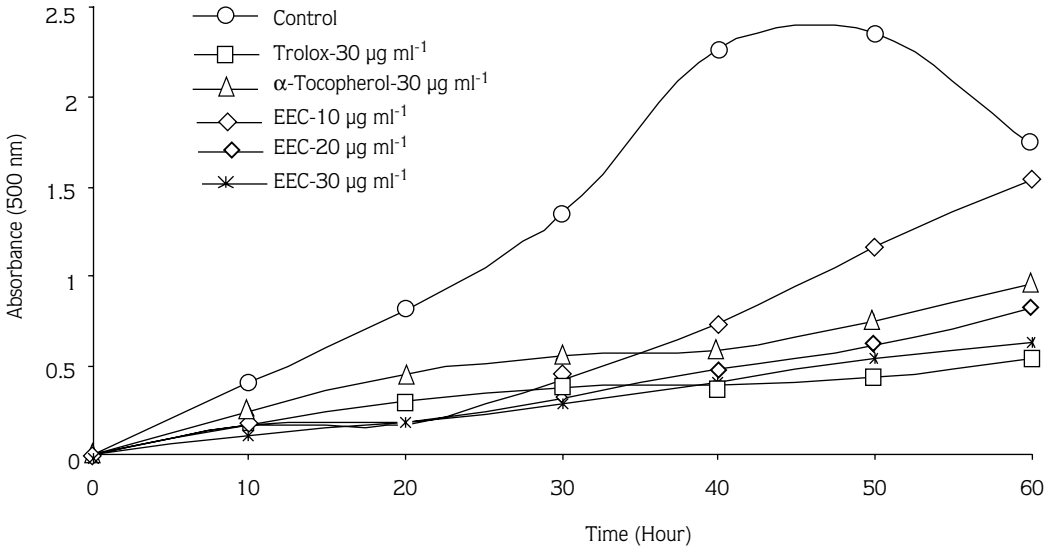

Figure 2. Total antioxidant activity of EEC at different concentrations (10-30 µg ml^{-1}), and α-tocopherol and trolox (30 µg ml^{-1}).

such as glutathione and non-protein thiol, unlike the widely applied FRAP test, which is non-responsive to -SH group antioxidants. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Gülçin 2006b, 2006c). In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of the antioxidant sample (Chung et al., 2002; Gülçin and Daştan, 2007).

Figure 3 depicts the reducing activity of WEC, EEC, and the standards (α-tocopherol and trolox) using the potassium ferricyanide reduction method. For measuring reductive activity, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of WEC and EEC, using the method of Oyaizu (1986). The reducing activity of WEC, EEC, α-tocopherol, and trolox increased with increasing sample concentration. As can be seen in Figure 3, WEC and EEC showed greater effective reducing activity than

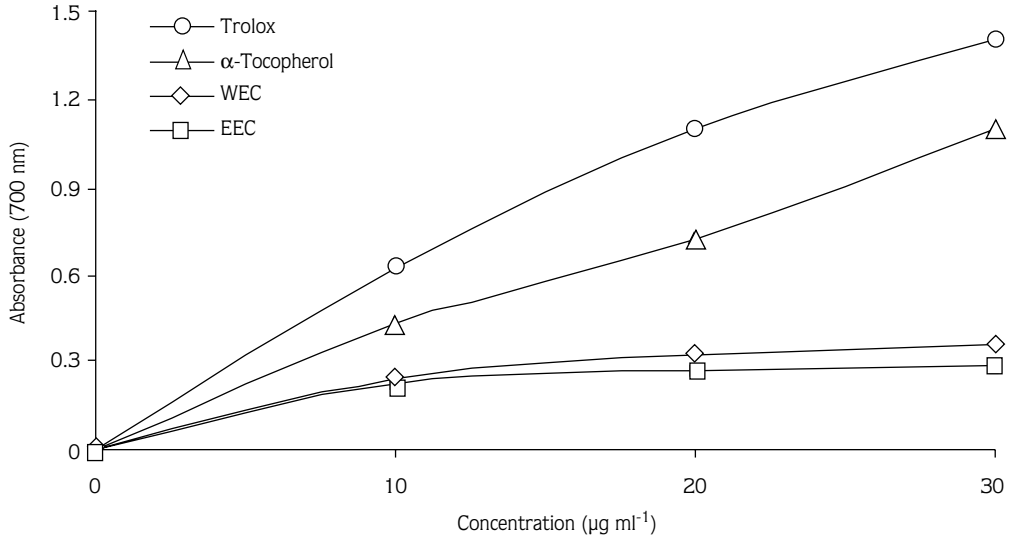

Figure 3. The Fe^{3+}-Fe^{2+} reducing activity of different concentrations (10-30 µg ml^{-1}) of WEC, EEC, α-tocopherol, and trolox.

the control, at different concentrations (R^2: 8608, R^2: 7202). These differences were statistically significant ($P < 0.01$). The reducing power of WEC, EEC, and the standard compounds exhibited the following order: trolox > α-tocopherol > WEC ≈ EEC.

The cupric ion (Cu^{2+}) reducing ability (CUPRAC method) of WEC and EEC is shown in Figure 4. A correlation between the cupric ion (Cu^{2+}) reducing ability and concentrations was observed. The cupric ion (Cu^{2+}) reducing capability of WEC and EEC, as determined by the CUPRAC method, was concentration dependent (10-30 μg ml^{-1}). The cupric ion (Cu^{2+}) reducing power of WEC, EEC and the standard compounds at the same concentration (30 μg ml^{-1}) exhibited the following order: α-tocopherol > trolox > EEC ≈ WEC.

The ferrous ion chelating activity of WEC, EEC, α-tocopherol, trolox, and EDTA are shown in Figure 5. The ferrous ion chelating activity of WEC, EEC, and the

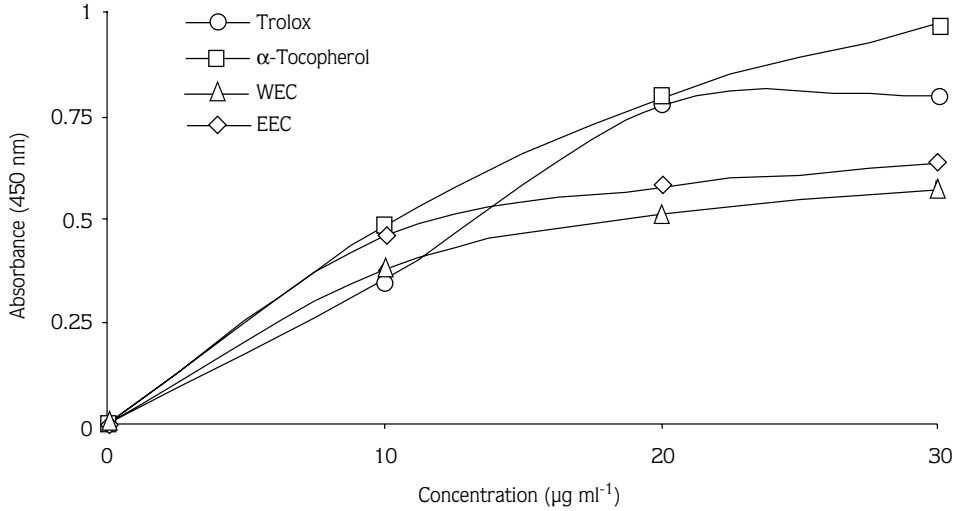

Figure 4. The Cu^{2+}-Cu^{+} reducing activity of WEC, EEC, α-tocopherol, and trolox at different concentrations (10-30 μg ml^{-1}).

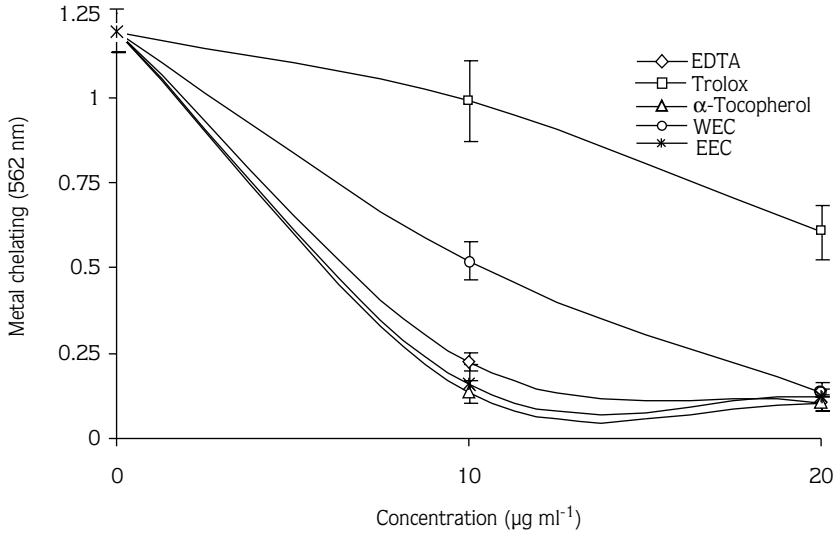

Figure 5. The ferrous ion (Fe^{2+}) chelating activity of WEC, EEC, α-tocopherol, trolox, EDTA at different concentrations (10-20 μg ml^{-1}).

standards was determined according to 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. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$). Fe^{3+} ions also produce radicals from peroxides, although the rate is 10-fold less than that of Fe^{2+} ions (Miller, 1996). Fe^{2+} ions are the most powerful pro-oxidant among the various species of metal ions (Halliwell and Gutteridge, 1984; Gülçin, 2007).

In fact, as shown in Figure 5, WEC and EEC disrupted the Fe^{2+}-ferrozine complex at different concentrations (10-20 μg ml^{-1}). The difference between all WEC and EEC concentrations and the control was statistically significant ($P < 0.01$). In addition, WEC and EEC exhibited 88.6% and 89.5% chelation of ferrous ions, respectively, at the 20 μg ml^{-1} concentration. On the other hand, the percentage of metal chelating capacity of the same concentration of α-tocopherol, trolox, and EDTA were 90.8%, 49.3% and 91.2%, respectively. The metal scavenging effect of those samples decreased in the order of EDTA ≈ α-tocopherol ≈ EEC ≈ WEC > trolox. The data obtained from Figure 5 reveal that WEC and EEC demonstrated a marked capacity for iron binding, suggesting that their main action as a peroxidation protector may be related to their iron binding capacity.

The ability of WEC and EEC to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1989) (Table 1) and was compared to that of α-tocopherol and trolox as standards. WEC and EEC exhibited a 51.2% and 41.4% scavenging effect of hydrogen peroxide at the 30 μg ml^{-1} concentration, respectively. On the other hand, α-tocopherol and trolox exhibited a 39.1% and 37.7% hydrogen peroxide scavenging activity at the same concentration, respectively. These results show that WEC and EEC have greater hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of WEC, EEC, and both standards decreased in the order of WEC > EEC ≈ α-tocopherol > trolox. Hydrogen peroxide itself is not very reactive; however, it can sometimes be toxic to cells because it may give rise to hydroxyl radical in the cells.

The improved technique for the generation of $ABTS^{\bullet+}$ described herein involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. As seen in Figure 6, the $ABTS^{\bullet+}$ radical scavenging activity of WEC and EEC was concentration dependent (10-20 μg ml^{-1}). There was a significant decrease ($P < 0.01$) in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity of WEC, EEC, and the standards. In addition, the $ABTS^{\bullet+}$ scavenging effect of WEC, EEC, and the standards decreased accordingly: trolox > WEC > EEC > α-tocopherol, which was 90.7%, 79.8%, 68.3%, and 55.9% at the concentration of 20 μg ml^{-1}, respectively.

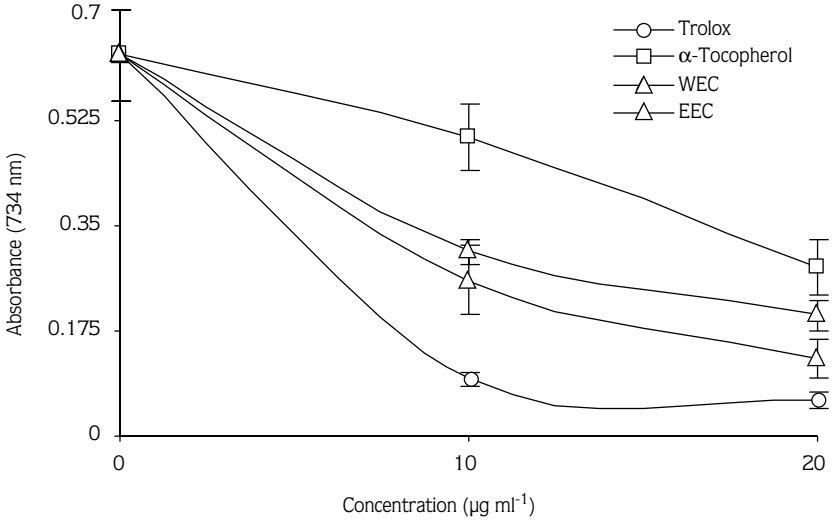

Figure 6. The stable $ABTS^{\bullet+}$ scavenging effect of WEC, EEC, α-tocopherol, and trolox at different concentrations (10-30 μg ml^{-1}).

Antioxidants react with DPPH•, which is a stable free radical, and convert it to 1,1-diphenyl-2-picryl hydrazine. The degree of discoloration indicates the radical-scavenging potential of the antioxidant (Singh et al., 2002). The antioxidant activity of WEC, EEC, and the standard antioxidants, was determined using the DPPH• method. Since the DPPH• assay can accommodate a large number of samples in a short period of time and is sensitive enough to detect natural compounds at low concentrations, it was used in the present study for the primary screening of WEC and EEC free radical-scavenging activity. WEC and EEC exhibited marked DPPH free radical scavenging activity in a concentration-dependent manner. Figure 7 illustrates a significant decrease ($P < 0.05$) in the concentration of the DPPH radical due to the scavenging ability of WEC, EEC, and the standards. α-Tocopherol and trolox were used as reference radical scavengers. The DPPH radical scavenging effect of WEC, EEC, and the standards decreased in the following order: EEC > trolox > α-tocopherol ≈ WEC, which was 64.6%, 56.5%, 51.9%, and 51.2%, at the concentration of 30 μg ml^{-1}, respectively.

The principle of the DMPD assay is that at an acidic pH and in the presence of a suitable oxidant solution DMPD can form a stable and colored radical cation ($DMPD^{\bullet+}$). $DMPD^{\bullet+}$ has a maximum absorbance at 505 nm. Inhibition of the absorbance at 505 nm was linear between 10 and 30 μg ml^{-1} of WEC and EEC. This assay is based on the extent of radical cation reduction at a fixed time point and not on the rate of reduction. This feature eliminated the complications due to the monitoring of color inhibition over time, which is present in other methods (Pryor et al., 1993; Tubaro et al., 1996), and allows the simultaneous analysis of many samples.

$DMPD^{\bullet+}$ scavenging efficiency was determined according to Miller et al. (1996). As seen in Figure 8, the $DMPD^{\bullet+}$ scavenging activity of WEC and EEC was concentration dependent (10-30 μg ml^{-1}). WEC and EEC demonstrated marked $DMPD^{\bullet+}$ scavenging activity. The $DMPD^{\bullet+}$ scavenging activity of WEC, EEC, and the standards decreased in the following order: Trolox > EEC > WEC (α-tocopherol, a hydrophobic standard antioxidant, was not used as a standard in this method).

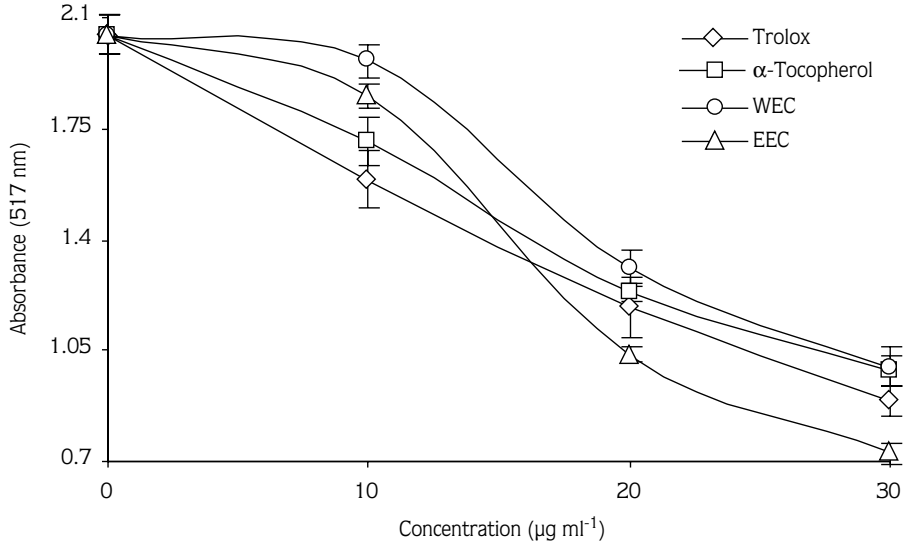

Figure 7. The $DPPH^{\bullet}$ scavenging effect of WEC, EEC, α-tocopherol, and trolox at different concentrations (10-30 μg ml^{-1}).

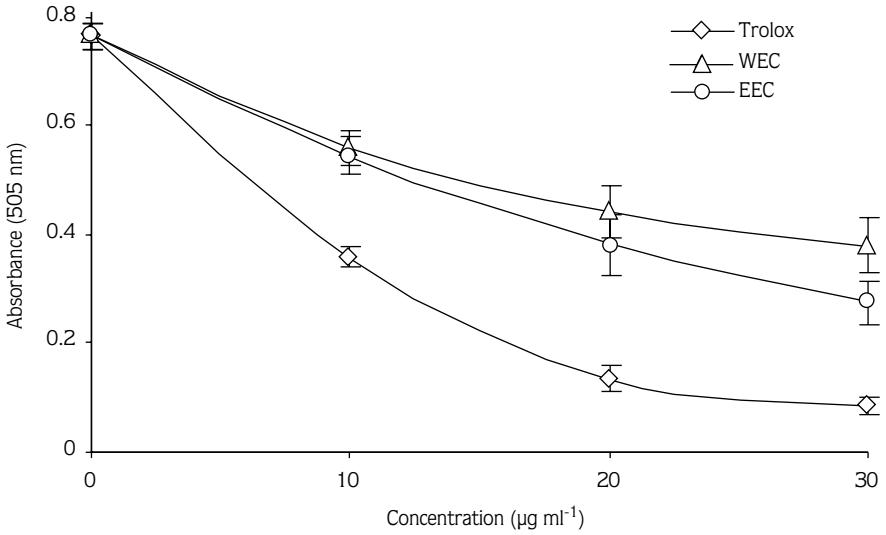

Figure 8. The $DMPD^{\bullet+}$ scavenging effect of WEC, EEC, and trolox at different concentrations (10-30 µg ml^{-1}).

The superoxide anion radical included in free radical species is a factor that can induce aging and destruct cell membranes, and it can be generated by oxidative stress. In this method, superoxide anion is derived from dissolved oxygen by riboflavin-methionine- illuminate reaction and reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit blue NBT formation (Cos et al., 1998; Parejo et al., 2002). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table 1 shows the percent inhibition of superoxide radical generation by WEC, EEC and the standards at the 30 µg ml^{-1} concentration. As seen in Table 1, the percent inhibition of superoxide anion radical generation by the 30 µg ml^{-1} concentration of WEC and EEC was 57.5% and 43.9%, respectively. On the other hand, at the same concentration, α-tocopherol and trolox exhibited 21.3% and 23.9% superoxide anion radical scavenging activity, respectively.

Conclusion

This study demonstrated the potential antioxidant properties of WEC and EEC. According to the presented data, WEC and EEC were effective antioxidants in different in vitro assays, including the ferric thiocyanate method, Fe^{3+}-Fe^{2+} and Cu^{2+}-Cu^{+} reducing power assays, DPPH• scavenging, $ABTS^{\bullet+}$ scavenging, $DMPD^{\bullet+}$ scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activity, when compared to the standard antioxidant compounds α-tocopherol (a natural antioxidant) and trolox (the water-soluble analogue of tocopherol). On the basis of the results of this study, it is clear that both plant extracts have powerful antioxidant activity against various antioxidant systems in vitro. Moreover, WEC and EEC can be used as easily accessible sources of natural antioxidants and possible food supplements, or in pharmaceutical applications.

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