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Antioxidant activity of *Smilax excelsa* L. leaf extracts

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Abstract

Smilax excelsa L. leaves are used widely in the Black Sea region of Turkey for consumption in the daily diet and in folk medicine for their medicinal properties. In the present study, different antioxidant tests were employed in order to evaluate the antioxidant activities of water, infusion, ethanol and ethyl acetate extracts of *S. excelsa* leaves. In addition, the results were compared with natural and synthetic antioxidants. The levels of total phenolics, total flavonoids and anthocyanins of the extracts were also determined. The extracts were found to have different levels of antioxidant properties in the test models used. All extracts had good total phenolic and flavonoid contents, inhibited lipid peroxidation, showed radical scavenging and iron-chelating activities. Therefore, the leaves of the plant could be considered as a significant natural antioxidant source.
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Keywords: *Smilax excelsa*; Antioxidant activity; Free radical; Scavenging activity

1. Introduction

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, oxygen-centred free radicals and other reactive oxygen species (ROS), which are continuously produced *in vivo*, result in cell death and tissue damage. The role of oxygen radicals has been implicated in several diseases, including cancer, diabetes and cardiovascular diseases, ageing, etc. (Halliwell & Gutteridge, 1999). Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress. There is an increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage (Silva, Ferreres, Malva, & Dias, 2005). Polyphenols possess ideal structural chemistry for free radical scavenging activity, and they have been shown to be more effective antioxidants *in vitro* than tocopherols and ascorbate. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilise and delocalise the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of the Fenton reaction) (Rice-Evans, Miller, & Paganga, 1997).

The genus *Smilax* (sarsaparilla, Liliaceae), native to tropical and temperate parts of the world, is a brambled woody vine with paired tendrils for climbing (Baytop, 1999). It is well-known that *Smilax* rhizomes have various pharmacological activities (Ban et al., 2006), such as immunomodulatory (Jiang & Xu, 2003), antibacterial, antifungal, antioxidant (Navarro et al., 2003) and hepatoprotective (Chen et al., 1999).

Recent studies confirm the presence of antioxidant activity in rhizomes of some species of sarsaparilla. Extracts of *Smilax china* roots were reported to have high levels of DPPH radical scavenging activity, to inhibit lipid peroxidation and enhance the effects of various antioxidant enzymes (Lee, Ju, & Kim, 2001). Research has mainly focused on plant rhizomes, and there is a little information about the antioxidant effect of *Smilax* leaves which are used widely

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in the Black Sea region of Turkey for consumption in the daily diet and in folk medicine (Baytop, 1999). The only relatively recent publication on the antioxidant activity of the aqueous extract prepared from *Smilax glyciphylla* leaves was by Cox, Jayasinghe, and Markham (2005).

To evaluate the antioxidant potential of the extracts from *Smilax excelsa* leaves, inhibition of lipid peroxidation, metal ion chelating, reducing and radical scavenging abilities against DPPH, superoxide, hydroxyl radicals and hydrogen peroxide were measured. The extraction method of antioxidants affects the total phenolic contents and antioxidant capacities (Lee, Kim, Lee, & Lee, 2003). Water, ethanol and ethyl acetate are commonly used to extract plants. Further on, ethyl acetate is often used as an extraction solvent for phenolic compounds (Matthäus, 2002). In the present study the efficiency of the extraction method is evaluated, by comparing the antioxidant activities of the water, infusion, ethanol and ethyl acetate extracts from leaves of *S. excelsa*, based on the amount of polyphenolic compounds.

2. Materials and methods

2.1. Chemicals

Nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide reduced (β-NADH), 2-deoxy-D-ribose, linoleic acid, ammonium thiocyanate, β-carotene, butylated hydroxyanisole (BHA), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) and catechin were purchased from Fluka Chemical Co. (Buchs, Switzerland). Phenazine methosulphate (PMS), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA), α-tocopherol and gallic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium ferricyanide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ferrous and ferric chloride were obtained from Merck. All other reagents were of analytical grade.

2.2. Plant material

Smilax excelsa L. leaves were collected in September from Istanbul in Turkey and identified by Prof. Dr. Kerim Alpinar from the Faculty of Pharmacy, Istanbul University. The leaves were separated from the other parts and dried at room temperature. Voucher specimens are deposited in the herbarium of the Faculty of Pharmacy, Istanbul University (ISTE); herbarium code number: ISTE 81928. The dried leaves were manually ground to a fine powder.

2.3. Preparation of extracts

Plant extracts were prepared as a 4% (w/v) infusion with distilled water or extracted with distilled water under reflux. For aqueous infusion, 8 g of ground dried leaves were extracted with boiling water (200 ml) for 15 min while stirring. For reflux method (water extract), 8 g of ground dried leaves powder were refluxed with 200 ml water for 2 h. For ethanol and ethyl acetate extractions, the powdered leaves (8 g) were extracted in a Soxhlet apparatus with 200 ml ethanol or ethyl acetate for 4 h. The extracts were filtered and evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator, then weighed to determine the total extractable compounds (EC). The crude extracts were then transferred to vials and kept at −20 °C. These crude extracts were dissolved in water or solvents and used for the assessment of antioxidant activity.

Ultraviolet–visible (UV–Vis) spectra and λ-maximum values were obtained for *S. excelsa* extracts, using a Varian Cary 1E UV–Vis spectrophotometer (Varian Australia, Melbourne, Australia).

2.4. Determination of total phenolic compounds

Total soluble phenolics in the water, infusion, ethanol and ethyl acetate extracts of *S. excelsa* leaves were determined with Folin–Ciocalteu reagent, according to the method of Slinkard and Singleton (1977) with some modifications. Aliquots (0.1 ml) of the extracts (0.625–5 mg/ml) were transferred into test tubes and their volumes made up to 4.6 ml with distilled water. After addition of 0.1 ml Folin–Ciocalteu reagent (previously diluted 3-fold with distilled water) and 0.3 ml 2% Na_2CO_3 solution, tubes were vortexed and the absorbance of the mixture was recorded after 2 h at 760 nm, using a Shimadzu 1208 UV–Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan), against a blank containing 0.1 ml of extraction solvent. The amount of total phenolic compounds was calculated as mg of gallic acid equivalents (GAE) from the calibration curve of gallic acid standard solution (covering the concentration range between 0.05 mg/ml and 0.4 mg/ml) and expressed as mg gallic acid/g dry weight (DW) of the plant material. The data were presented as the average of triplicate analyses.

2.5. Determination of the total flavonoid content

Total flavonoid content was determined by using a method described by Sakanaka, Tachibana, and Okada (2005). Briefly, 0.25 ml of the extracts (0.625–5 mg/ml) or (+)-catechin standard solution (15–250 μg/ml) was mixed with 1.25 ml of distilled water in a test tube, followed by addition of 75 μl of a 5% (w/v) sodium nitrite solution. After 6 min, 150 μl of a 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for a further 5 min before 0.5 ml of M NaOH was added. The mixture was made up to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The mean (±SD) results of triplicate analyses were expressed as mg of (+)-catechin equivalents of total extractable compounds.

2.6. Determination of the anthocyanin content

Anthocyanin content of *S. excelsa* leaves was analysed according to Padmavati, Sakthivel, Thara, and Reddy (1997) modified by Chung, Chen, Hsu, Chang, and Chou (2005). The leaves (1 g) were extracted with 25 mg/ml of acidified methanol (1% HCl) for 2 h at room temperature in the dark, and then centrifuged at 1000*g* for 15 min. Anthocyanin levels were estimated from the methanolic extract as $A_{530}-(0.24 \times A_{653})$ (Gould, Markham, Smith, & Goris, 2000). The content of anthocyanins was calculated as mg cyanidin 3-glucoside, using an extinction coefficient of 26,900 l mol^{-1} cm^{-1} at 530 nm and a molar mass of 449.2 g mol^{-1} (Giusti & Wrolstad, 2000).

2.7. Antioxidant activity determined in linoleic acid system

2.7.1. Ferric thiocyanate (FTC) method

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. The method was followed of Chang, Yen, Huang, and Duh (2002). Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (0.28 mg) and Tween 20 (0.28 mg) in phosphate buffer (50 ml, 0.05 M, pH 7.4). A reaction solution, containing extracts (0.2 ml, 0.16–2.5 mg/ml), linoleic acid emulsion (2.5 ml), and phosphate buffer (2.3 ml, 0.2 M, pH 7.0) was placed in a glass vial with a screw cap and mixed with a vortex mixer. The reaction mixture was incubated at 37 °C in the dark and the degree of oxidation was measured according to the thiocyanate method. To 0.1 ml of reaction mixture, 4.7 ml of 75% ethanol and 0.1 ml 30% ammonium thiocyanate were added. Exactly 3 min after the addition of 0.1 ml of 0.02 M $FeCl_2$ in 3.5% HCl, the peroxide value was determined by recording the absorbance at 500 nm every 24 h until the absorbance of the control reached a maximum. The positive and negative controls were subjected to the same procedures as the sample, except that for the negative control, only the solvent was added, and for the positive control, sample was replaced with α-tocopherol. The inhibition percent of linoleic acid peroxidation was calculated as:

$$\text{Inhibition}(\%) = (1 - \text{Absorbance of sample at 500 nm} / \text{Absorbance of control at 500 nm}) \times 100.$$

All tests were run in duplicate, and analysis of all samples was done in triplicate and averaged.

2.8. Antioxidant activity determined by β-carotene bleaching method

The antioxidant activity of extracts from *S. excelsa* leaves was evaluated using β-carotene-linoleate model system, as described by Sun and Ho (2005). Two milligrams of β-carotene were dissolved in 10 ml chloroform and 1 ml β-carotene solution was mixed with 20 mg of purified linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was then evaporated under a gentle stream of nitrogen and the resulting mixture was immediately diluted with 50 ml of distilled water. To an aliquot of 5 ml of this emulsion, 0.2 ml of *S. excelsa* extracts (0.625–5 mg/ml) or the reference antioxidants (gallic acid and BHA) were added and mixed well. The absorbance at 470 nm, which was regarded as t_0, was measured, immediately, against a blank consisting of the emulsion without β-carotene. The capped tubes were placed in a water bath at 50 °C and the absorbance was measured every 15 min up to 120 min. For the positive control, sample was replaced with gallic acid or BHA. A negative control consisted of 0.2 ml distilled water or solvent instead of extract or reference antioxidants. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of β-carotene by using the following equation: $\text{AA} = \left[1 - \left(A_0 - A_t/A_0^0 - A_t^0\right)\right] \times 100$, where A_0 and A_0^0 were the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t^0 were the absorbance values measured in the test sample and control, respectively, after incubation for 120 min. The results were expressed on a % basis of preventing bleaching of β-carotene (Abdille, Singh, Jayaprakasha, & Jena, 2005).

2.9. DPPH radical scavenging activity

The DPPH radical scavenging activity of the extracts from *S. excelsa* leaves was measured according to the procedure described by Brand-Williams, Cuvelier, and Berset (1995). A 0.1 ml aliquot of each extract (0.0625–5 mg/ml), gallic acid (0.016–0.125 mg/ml) and BHA (0.016–0.5 mg/ml) in methanol was added to 3.9 ml of 6×10^{-5} M methanolic solution of DPPH. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. The decrease in absorbance of the resulting solution was then measured spectrophotometrically at 517 nm against methanol. All measurements were made in triplicate and averaged. Two controls were used for this test, a negative control (containing all reagents except the test sample) and positive controls (using the reference antioxidants). The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity}(\%) = (1 - \text{Absorbance of sample at 517 nm} / \text{Absorbance of control at 517 nm}) \times 100.$$

2.10. Superoxide radical scavenging activity

The effects of the extracts from *S. excelsa* leaves, α-tocopherol and gallic acid on generation of superoxide radicals were determined by the nitroblue tetrazolium reduction method (Nishikimi, Rao, & Yagi, 1972). One millilitre of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4),

1 ml NADH solution (468 μM NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of the extracts (0.125–5 mg/ml), gallic acid or α-tocopherol (0.016–2.5 mg/ml) were mixed. The reaction was started by adding 100 μl of PMS solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples, containing all the reagents except the PMS. The positive and negative controls were subjected to the same procedures as the sample, except that for the negative control, only the solvent was added, and for the positive control, sample was replaced with gallic acid or α-tocopherol. All measurements were made in triplicate and averaged. The abilities to scavenge the superoxide radical were calculated using the following equation:

$$\text{Superoxide radical scavenging activity}(\%) = (1 - \text{Absorbance of sample at 560 nm} / \text{Absorbance of control at 560 nm}) \times 100.$$

2.11. Hydroxyl radical scavenging activity

The effect of extracts on hydroxyl radicals was assayed by using the deoxyribose method (Nagai, Myoda, & Nagashima, 2005). 2-Deoxyribose is degraded on exposure to hydroxyl radicals generated by Fenton reaction. Ethanol extract and BHA were prepared in ethanol/water, and ethyl acetate extract in ethyl acetate/water mixture (3:7; v/v). The reaction mixture contained 450 μl of 0.2 M sodium phosphate buffer (pH 7.0), 150 μl of 10 mM 2-deoxyribose, 150 μl of 10 mM $FeSO_4$-EDTA, 150 μl of 10 mM H_2O_2, 525 μl of H_2O, and 75 μl of sample solution (2.5–20 mg/ml water or infusion extracts; 0.01–5 mg/ml ethanol or ethyl acetate extracts). The reaction was started by the addition of H_2O_2. After incubation at 37 °C for 4 h, the reaction was stopped by adding 750 μl of 2.8% trichloroacetic acid and 750 μl of 1% TBA in 50 mM NaOH, the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Gallic acid (2.5–10 mg/ml) and BHA (0.01–5 mg/ml) were used as positive controls. The ability to scavenge the hydroxyl radicals was calculated using the following equation:

$$\text{Hydroxyl radical scavenging activity}(\%) = (1 - \text{Absorbance of sample at 520 nm} / \text{Absorbance of control at 520 nm}) \times 100.$$

2.12. Hydrogen peroxide-scavenging activity

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch, Cheng, and Klaunig (1989). A solution of hydrogen peroxide (2 mM) was prepared in 50 mM phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm absorption using the molar extinction coefficient for H_2O_2 of 81 mol^{-1} cm^{-1}. Aliquots (0.1 ml) of water (0.625–5 mg/ml), infusion (0.625–5 mg/ml), ethanol (0.31–3.75 mg/ml) and ethyl acetate (1.25–5 mg/ml) extracts, gallic acid or BHA (0.31–1.25 mg/ml) were transferred into the test tubes and their volumes were made up to 0.4 ml with 50 mM phosphate buffer (pH 7.4) or solvents. After addition of 0.6 ml hydrogen peroxide solution, tubes were vortexed and absorbance of the hydrogen peroxide at 230 nm was determined after 10 min, against a blank solution containing 50 mM phosphate buffer without hydrogen peroxide. The abilities to scavenge the hydrogen peroxide were calculated using the following equation:

$$\text{Hydrogen peroxide scavenging activity}(\%) = (1 - \text{Absorbance of sample at 230 nm} / \text{Absorbance of control at 230 nm}) \times 100.$$

2.13. Reducing power

The reducing powers of the extracts from *S. excelsa* leaves, BHA and gallic acid were determined according to the method described by Chung et al. (2005). A 0.1 ml aliquot of each extract (0.125–20 mg/ml), BHA or gallic acid (0.008–0.5 mg/ml) were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide, and then incubated at 50 °C for 20 min. 0.25 ml of 1% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 2790*g* for 10 min. The supernatant (0.25 ml) was mixed with 0.25 ml distilled water and 0.1% $FeCl_3$ (0.5 ml) and then the absorbance was measured at 700 nm. The reducing powers of the tested samples increased with the absorbance values.

2.14. Chelating activity on Fe^{2+}

The extracts were assessed for their ability to compete with ferrozine for iron (II) ions in free solution. The chelating ability of ferrous ions by the extracts from *S. excelsa* leaves was estimated by the method of Dinis, Madeira, and Almeida (1994). Extracts (12.5–1200 μg/ml, 2.5 ml) were added to a solution of 2 mM $FeCl_2 \cdot 4H_2O$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured at 562 nm against the blank performed in the same way using $FeCl_2$ and water. EDTA (0.625–5 μg/ml) served as the positive control, and a sample without extract or EDTA served as the negative control. All tests were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula:

Chelating activity(%)
= (1 − Absorbance of sample at 562 nm
/Absorbance of control at 562 nm) × 100.

2.15. Statistical analysis

Results were expressed as mean ± standard error. Statistical comparisons were performed with Student's *t*-test. Differences were considered significant at $p < 0.05$. The correlation coefficient (r^2) between the parameters tested was established by regression analysis.

3. Results and discussion

3.1. Extract yield (amount of total extractable compounds)

The amount of extractable compounds ranged from 106.1 to 344.6 mg/g dry plant material and was 309.6, 339.9, 344.6 and 106.1 mg/g for water, infusion, ethanol and ethyl acetate extracts, respectively. So the extractions with water and ethanol resulted in the highest amount of total extractable compounds. As the results presented in Table 1 show, the extraction ability of water and ethanol were very similar to one another, whereas the extraction yield with ethyl acetate was only small in comparison with that of the other solvents.

3.2. Extractable total phenols, flavonoids and anthocyanins content

The content of extractable phenolic compounds in extracts, determined from regression equation of calibration curve ($y = 1.6x$–0.0403) and expressed in gallic acid equivalents (GAE), varied between 8.8 mg and 35.7 mg/g DW. These amounts were comparable with results described in the literature for other extracts of plant products (Abdille et al., 2005; Chung et al., 2005; Matthäus, 2002; Sakanaka et al., 2005). The results given in Table 1 showed that the highest content of total phenolic compounds was found in extracts obtained with water (30.6 ± 0.21 mg/g DW in water extract; 35.7 ± 1.40 in infusion) and ethanol (30.1 ± 1.55 mg/g DW), whereas the contents obtained with ethyl acetate were much smaller (8.8 ± 0.70 mg/g DW), which is in agreement with the report of Matthäus (2002).

As shown in Table 1, the ratio of total phenolic compounds to the total extractable compounds was about 10% for all the extracts. Although the extraction yield of total extractable compounds with ethyl acetate was the smallest (8.3%), it was comparable with the proportion of total phenolic compounds found for extracts obtained with water (9.9%) and ethanol (8.7%).

The content of flavonoids (mg/g, DW) in the total extractable compounds of the extracts, determined from regression equation of calibration curve ($y = 3.2x$) and expressed in catechin equivalents, varied from 0.61 to 22.9 mg/g DW. The results given in Table 1 showed that water, infusion, ethanol and ethyl acetate extracts contained 22.9 ± 0.81, 25.1 ± 1.07, 28.7 ± 2.41 and 0.61 ± 0.05 mg/g DW, respectively, indicating high correlation between total phenolics and flavonoids ($r^2 = 0.9930$, 0.9935, 0.9910 and 0.9998 for water, infusion, ethanol and ethyl acetate, respectively).

It was observed that leaves contained 0.32 ± 0.015 mg/g DW anthocyanins determined as mg cyanidin 3-glucoside/g dry plant material (Table 1). It is to be expected that several activities might be related to a possible antioxidant action from anthocyanosides. It was reported that some pharmacological activities of polyphenol compounds, like anthocyanosides, may be attributed to their antioxidant properties (Martin-Aragon, Basabe, Benedi, & Villar, 1998).

For further characterisation of the phenolic compounds, the UV–Vis absorption spectra of the extracts were assessed. Phenolic compounds exhibit two major absorption bands in the ultraviolet/visible region: a first band in the range between 320 and 380 nm and a second band in the 250 to 285 nm range (Matthäus, 2002). As can be seen from Fig. 1, water (λ_{max} 322 nm), infusion (λ_{max} 322 nm) and ethanol (λ_{max} 323 nm and 415 nm) extracts showed maximum absorption in the range between 320 and 380 nm. Absorption maxima of the ethanol extract at 415 nm may be due to the presence of carotenoids (Guerra, Melo, & Filho, 2005). Two distinctive bands of absorption, one in the UV-region (260–280 nm) and another in the visible region (490–550 nm) were shown by all anthocyanins (Giusti & Wrolstad, 2000). Absorption maxima of the ethyl acetate extract at 220 nm may be due to the presence of

Table 1
Total extractable compounds (EC), total phenolic compounds (PC) (as gallic acid equivalents), total flavonoids (as catechin equivalents) and total anthocyanins in water, infusion, ethanol and ethyl acetate extracts from *Smilax excelsa* leaves

Extract	EC (mg/g DW)	PC (mg/g DW)	PC/EC (%)	Flavonoids (mg/g DW)	Anthocyanin (mg/g DW)
Water	309.6[a]	30.6 ± 0.21[a]	9.9	22.9 ± 0.81[a]	
Infusion	339.9[a,d]	35.7 ± 1.40[b]	10.5	25.1 ± 1.07[b]	
Ethanol	344.6[b,d]	30.1 ± 1.55[a,b]	8.7	28.7 ± 2.41[c]	
Ethyl acetate	106.1[c]	8.8 ± 0.70[c]	8.3	0.61 ± 0.05[d]	
Overall experiment (dry leaves)					0.32 ± 0.015

Values were the means of three replicates ± standard deviation (SD).
Values with different letters in the same column were significantly ($p < 0.05$) different.

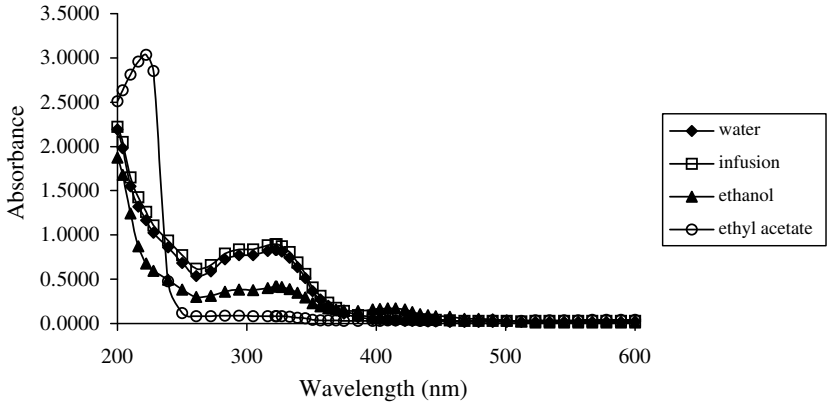

Fig. 1. UV–Vis spectra of water, infusion, ethanol and ethyl acetate extracts from *Smilax excelsa* leaves.

flavone/flavonol derivatives (Chang et al., 2002) or anthocyanins. Small proanthocyanidins are very soluble in ethyl acetate, whereas especially di- and oligomeric proanthocyanidins remain in the water phase (Matthäus, 2002) and they may contribute to the radical scavenging activity and reducing power of the extracts.

3.3. Antioxidant activity determined in linoleic acid system

The inhibitory effect of the extracts from *S. excelsa* leaves on the peroxidation of linoleic acid at concentrations within the range of 0.16–2.5 mg/ml, in comparison to α-tocopherol as positive control, was measured using the ferric thiocyanate (FTC) method.

The absorbance data of linoleic acid peroxidation, determined by the FTC method at 37 °C, after addition of 0.625 mg of the water, infusion, ethanol and ethyl acetate extracts from *S. excelsa* leaves were plotted in Fig. 2.

When different concentrations of the extracts (0.16–2.5 mg/ml) were added, we observed a significant concentration-dependent inhibition of lipid peroxidation. The autooxidation of linoleic acid without added herbal extracts was accompanied by a rapid increase of peroxide value at day 1 of testing, reached maximum levels on day 4 and dropped on day 5. Each extract showed strong antioxidant activity in inhibition of linoleic acid peroxidation at a concentration of 0.625 mg/ml, as compared to the control ($p < 0.05$), and significantly prolonged the induction period of autooxidation of linoleic acid. From the FTC results, the percentage inhibition of peroxidation in linoleic acid system by 0.625 mg of water, infusion, ethyl acetate and ethanol extracts was found to be $83.38 \pm 0.80\%$, $82.70 \pm 1.23\%$, $97.5 \pm 1.15\%$ and $99.2 \pm 0.35\%$, respectively at the fourth day of testing. These values were significantly ($p < 0.05$) higher than that exhibited by 0.625 mg of α-tocopherol (14.9%). No significant differences ($p > 0.05$) in antioxidant activities were found among water, infusion, ethanol and ethyl acetate extracts, indicating that extracts with different solvents displayed a similarly strong antioxidant activity. At concentrations above 0.625 mg/ml the water, infusion, ethyl acetate and ethanol extracts almost completely inhibited lipid peroxidation. A similar report (Cox et al., 2005) found that the water extract of *Smilax glyciphylla* leaves strongly inhibits lipid peroxidation.

3.4. Antioxidant activity determined by β-carotene bleaching method

We have also evaluated the antioxidant potential of the extracts from *S. excelsa* leaves by the β-carotene bleaching

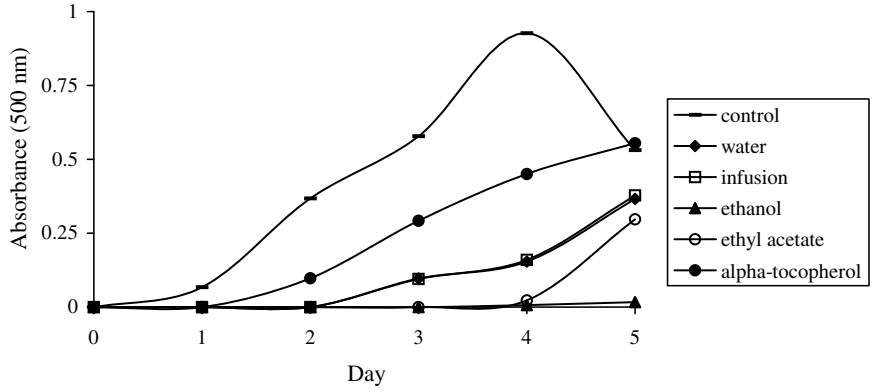

Fig. 2. Absorbance values of extracts at 0.625 mg/ml concentration using FTC method. Results are of duplicate measurements.

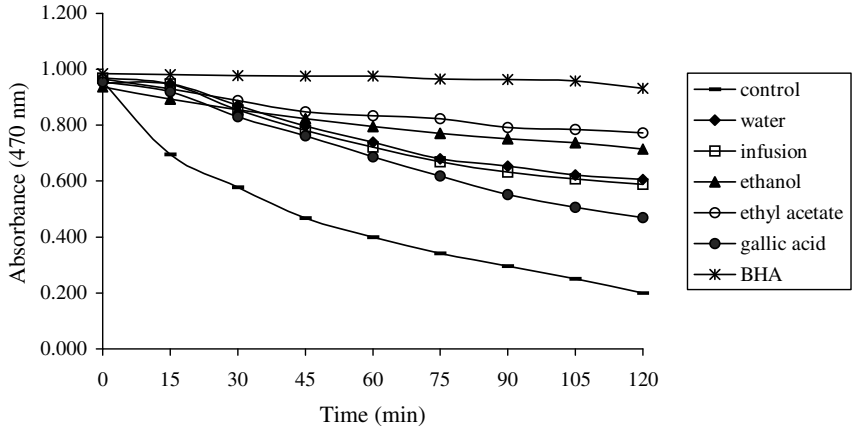

Fig. 3. Antioxidant activities of water, infusion, ethanol and ethyl acetate extracts (2.5 mg/ml) from *Smilax excelsa* leaves measured by β-carotene bleaching method. Gallic acid and BHA were used as reference antioxidants. Values are means ± SD ($n = 3$).

method. Fig. 3 shows the decrease in absorbance of β-carotene in the presence of 2.5 mg/ml of the extracts or reference antioxidants (BHA and gallic acid).

The addition of *Smilax* leaves extracts and BHA at 2.5 mg were markedly effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β-carotene, in comparison with the control ($p < 0.05$), which contained no antioxidant component. Most effective were the ethanolic (71.5 ± 0.91%) and ethyl acetate (79.1 ± 2.75%) extracts, whereas the effects of water (53.5 ± 2.54%) and infusion (48.9 ± 1.90%) extracts were not as strong. All the extracts were less effective in comparison with BHA (93.0 ± 2.37%), but showed greater inhibitory activity than gallic acid (34.7 ± 1.68%) at the same concentration. The results indicated that the extracts had acted as an effective antioxidant in a β-carotene linoleic acid model system.

The antioxidant activities measured in the linoleic acid system by FTC assay correlated well with those found in the β-carotene linoleic acid system. The correlation coefficients (r^2) between FTC assay and β-carotene linoleic acid system were 0.9181, 0.8006, 0.9926, 0.9669 for water, infusion, ethanol and ethyl acetate extracts, respectively, an indication that the antioxidant activities measured in the two model systems complemented each other.

The antioxidative effectiveness in natural sources has been reported to be mostly due to phenolic compounds. Some authors have found a correlation between the phenolic content and the antioxidant activity (Duh & Yen, 1997), while others have found no such relationship (Sun & Ho, 2005).

In this study the correlation between antioxidant activity determined by FTC method and total phenolic content was significant; $r^2 = 0.9377$, 0.9999, 0.9944 and 0.9987 for water, infusion, ethanol and ethyl acetate extracts, respectively, indicating that phenolic compounds may contribute directly to antioxidative effect of the extracts. There was also a correlation found between the antioxidant activity values tested by the β-carotene bleaching method and total phenolics content ($r^2 = 0.8562$, 0.9806, 0.8787, 0.8969 for water, infusion, ethanol and ethyl acetate extract, respectively).

This observation is in agreement with other reports (Turkoglu, Duru, Mercan, Kivrik, & Gezer, 2007), in extracts which a high polyphenolic content was significantly associated with antioxidant activity.

3.5. DPPH radical scavenging activity

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants (Sánchez-Moreno, 2002). All the extracts were capable of scavenging DPPH radicals in a concentration-dependent manner. Fig. 4 shows the dose-response curves of DPPH radical scavenging activities of the extracts from *S. excelsa* leaves. The scavenging effect of water, infusion, ethanol and ethyl acetate extracts on DPPH radicals increased from 0.0625 to 5 mg/ml and were 88.0 ± 0.05%, 86.1 ± 0.35%, 79.9 ± 2.89% and 56.2 ± 1.26% at a concentration of 2.5 mg/ml, respectively, indicating that the water, infusion and ethanol extracts showed similar DPPH radical scavenging activity, while ethyl acetate extract was a considerably less effective DPPH radicals scavenger (56.2 ± 1.26%) ($p < 0.05$). After addition of 5 mg of this extract, a DPPH radical scavenging activity of 83.9 ± 1.28% was obtained. The scavenging effect of water, infusion and ethanol extracts at a concentration of 2.5 mg/ml and ethyl acetate at a concentration of 5 mg/ml was nearly equal to that of 0.125 mg/ml gallic acid (87.1 ± 0.77%) and 0.5 mg/ml BHA (89.1 ± 0.35%).

The antioxidant activity in DPPH assay correlated well with phenolic content ($r^2 = 0.7432$, 0.6701, 0.9645, 0.9995 for water, infusion, ethanol and ethyl acetate extracts, respectively).

From the effective concentration (EC_{50}: the effective concentration at which the DPPH radicals were scavenged by 50%) of the extracts, it was seen that water (1.19 ± 0.015 mg/ml) and infusion (1.24 ± 0.015 mg/ml) extracts had the highest DPPH radical scavenging activity, as

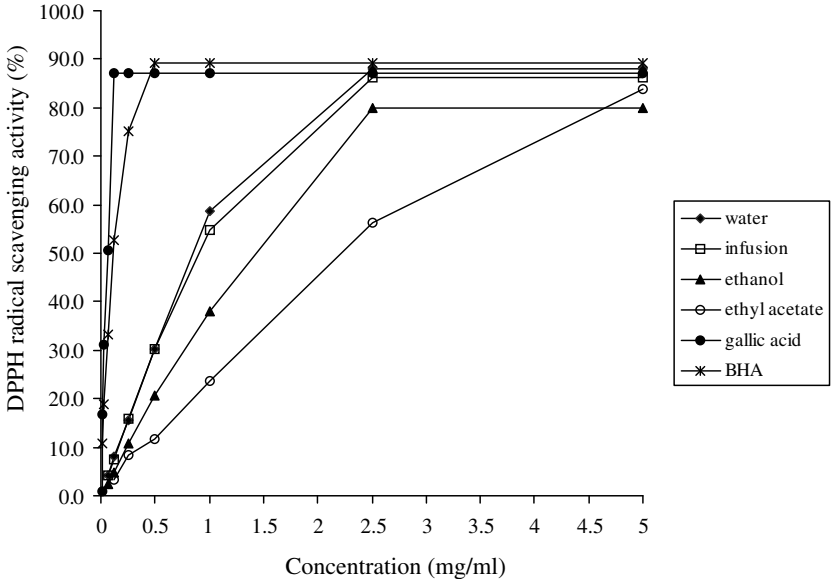

Fig. 4. DPPH radical scavenging activity of the extracts from *Smilax excelsa* leaves. Gallic acid and BHA were used as reference antioxidants. Values are means ± SD ($n = 3$).

shown by the lowest value of EC_{50}, followed by the ethanol extract (1.49 ± 0.061 mg/ml), while the ethyl acetate extract had the least activity (2.66 ± 0.03 mg/ml). A higher DPPH radical scavenging activity is associated with a lower EC_{50} value. It was evident that the extracts did show the hydrogen donating ability to act as antioxidants. EC_{50} values, in scavenging abilities on DPPH radicals, were significantly different ($p < 0.05$) from the EC_{50} values obtained for gallic acid (64 μg/ml) and BHA (114 μg/ml).

Scavenging abilities on DPPH radicals were in descending order:

water > infusion > ethanol > ethyl acetate

It has been reported that *Smilax china* root extract showed a relatively high DPPH radical scavenging effect with an average IC_{50} value of 7.4 μg/ml (Lee et al., 2001). It was evident that *Smilax china* root extract had a stronger effect on the DPPH radicals compared to *S. excelsa* leaves extracts. Antioxidant activity of *Smilax* species rhizomes was attributed to different compounds like proanthocyanidins (Rugna et al., 2003), *trans*-resveratrol, naringenin, 1-*O*-*trans*-*p*-coumaroylglycerol, etc. (Ivanova, Marinova, Toneva, Kostova, & Yanishlieva, 2006). In the only study reported for leaf extracts, Cox et al. (2005) attributed the antioxidant activity of *S. glyciphylla* leaves to a phenolic constituent named glyciphyllin.

3.6. Superoxide radical scavenging activity

Superoxide radical is known to be very harmful to cellular components as a precursor of the more reactive oxygen species, contributing to tissue damage and various diseases (Halliwell & Gutteridge, 1999). The scavenging activities of the extracts on superoxide radicals are shown

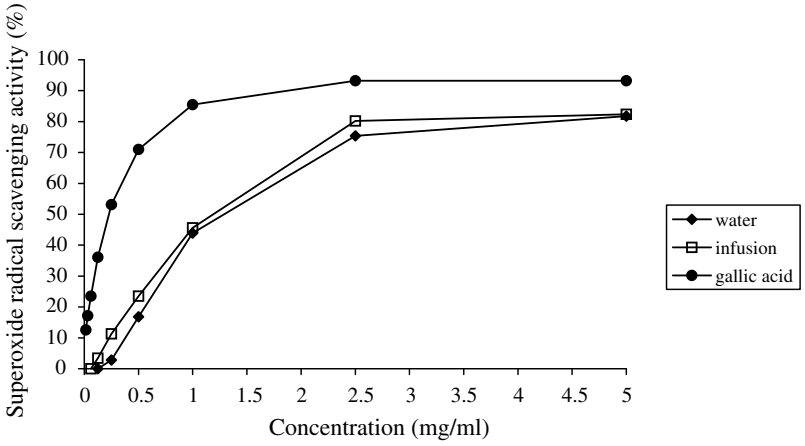

Fig. 5. Superoxide radical scavenging activity of the extracts from *Smilax excelsa* leaves. Gallic acid was used as reference antioxidant. Values are means ± SD ($n = 3$).

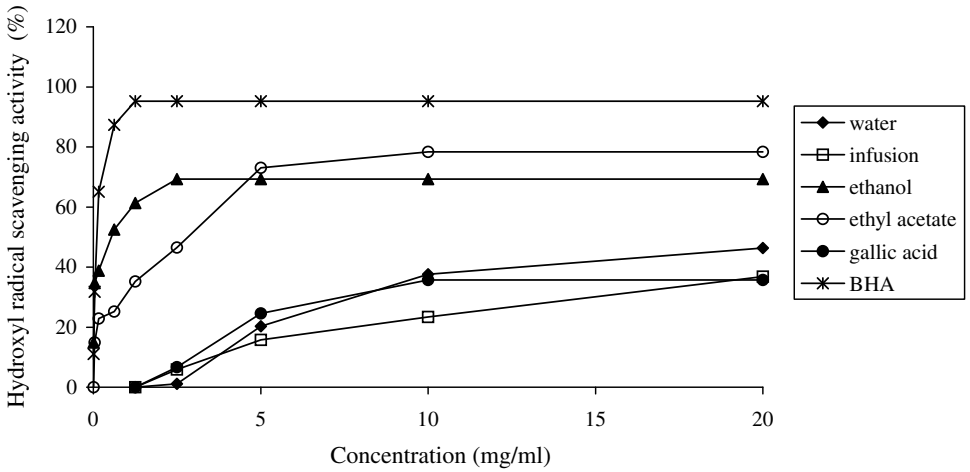

Fig. 6. Hydroxyl radical scavenging activity of the extracts from *Smilax excelsa* leaves. Gallic acid and BHA were used as reference antioxidants. Values are means ± SD ($n = 3$).

in Fig. 5. It was found that the superoxide-scavenging activities of the extracts increased with the increase of their concentrations. Water and infusion extracts at 2.5 mg/ml exhibited 75.4 ± 1.35% and 80.2 ± 1.17% superoxide radical scavenging activity, respectively. These values were lower than that of the same dose of gallic acid (93.2 ± 0.66%). Ethanol and ethyl acetate extracts and α-tocopherol showed negligible superoxide radical scavenging activity, and therefore these values were not shown in Fig. 5.

EC_{50} values, in scavenging abilities on superoxide radicals, were comparable for water (1.47 ± 0.03 mg/ml) and infusion (1.37 ± 0.01 mg/ml) extracts but significantly different ($p < 0.05$) from the EC_{50} values obtained for gallic acid (0.574 ± 0.009 mg/ml).

These results imply that water and infusion extracts are superoxide scavengers and their capacity to scavenge superoxide may contribute to their antioxidant activity. This result is similar to the work of Cox et al. (2005), who reported that the aqueous extract of *Smilax glyciphylla* leaves also quenched chemically-generated superoxide anion ($IC_{50} = 50$ μg/ml).

3.7. Hydroxyl radical scavenging activity

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka et al., 2005). Fig. 6 shows the dose-response curves of radical scavenging activities of the extracts and reference antioxidants (gallic acid and BHA) on the hydroxyl radicals. Among the aqueous extracts there were no significant differences ($p > 0.05$) in their activities in scavenging hydroxyl radicals. Water and infusion extracts scavenged hydroxyl radicals by 20.3 ± 3.46% and 15.8 ± 2.89% at 5 mg/ml, respectively, and 46.3 ± 0.56% and 36.8 ± 3.60% at 20 mg/ml, respectively. The scavenging effect of gallic acid (35.7 ± 1.76%) was nearly equal to that of the water and infusion extracts and the maximum of its activity was reached with a smaller quantity (10 mg/ml). Ethyl acetate and ethanol extracts exhib-

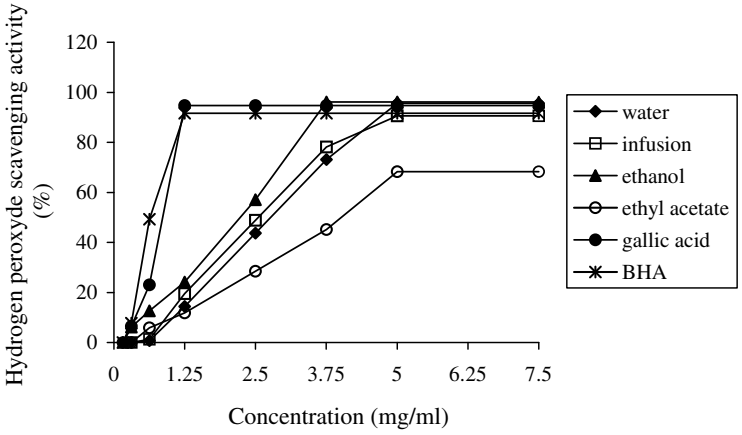

Fig. 7. Hydrogen peroxide-scavenging activity of the extracts from *Smilax excelsa* leaves. Gallic acid and BHA were used as reference antioxidants. Values are means ± SD ($n = 3$).

ited good scavenging activity of 73.1 ± 0.91% at a concentration of 5 mg/ml and 69.3 ± 0.85% at a concentration of 2.5 mg/ml, respectively. However, those values were significantly lower than the value of the positive control BHA (95.3 ± 0.37% at 1.25 mg/ml).

The EC_{50} values obtained for the ethanol (1.20 ± 0.16 mg/ml) and ethyl acetate (2.77 ± 0.17 mg/ml) extracts were significantly different ($p < 0.05$) from the EC_{50} values obtained for the water (21.12 ± 0.89 mg/ml) and infusion (24.44 ± 1.52 mg/ml) extracts, which were comparable. As compared by their EC_{50} values, ethanol and ethyl acetate extracts showed less hydroxyl radical scavenging activity than BHA (0.115 ± 0.004 mg/ml), but much more than gallic acid (14.21 ± 0.77 mg/ml).

The scavenging abilities on hydroxyl radicals were in descending order:

ethanol > ethyl acetate > water > infusion

It was reported (Cox et al., 2005) that the water extract of *Smilax glyciphylla* also inhibited deoxyribose degradation.

3.8. Hydrogen peroxide scavenging activity

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell (Halliwell, 1991). Extracts from *S. excelsa* leaves were capable of scavenging hydrogen peroxide in a concentration-dependent manner (0.625–5 mg/ml). The scavenging effect of the extracts on hydrogen peroxide is shown in Fig. 7. There were no significant differences ($p > 0.05$) in the hydrogen peroxide-scavenging activities of the water, infusion and ethanolic extracts, showing these extracts to be equally potent in scavenging hydrogen peroxide, by 95.6 ± 1.21%, 90.6 ± 1.33% and 96.1 ± 3.17% at a concentration of 5 mg/ml, respectively, while ethyl acetate extract was a considerably ($p < 0.05$) less effective hydrogen peroxide scavenger (68.3 ± 4.07%) at the same concentration. The scavenging effect of water, infusion and ethanol extracts was nearly equal to that of gallic acid and BHA, which showed the strongest scavenging activity of 94.8 ± 0.75% and 96.6 ± 0.37%, respectively on hydrogen peroxide at a dose of 1.25 mg/ml. These results showed that *S. excelsa* leaves extracts had high hydrogen peroxide-scavenging activities.

As compared with the EC_{50} values, the hydrogen peroxide-scavenging activities of water (2.94 ± 0.076 mg/ml), infusion (2.88 ± 0.005 mg/ml) and ethanol (3.23 ± 0.066 mg/ml) extracts were comparable, and more effective ($p < 0.05$) than that of ethyl acetate (4.03 ± 0.14 mg/ml). EC_{50} values of all the extracts, in scavenging abilities on hydrogen peroxide, were significantly different ($p < 0.05$) from the EC_{50} values obtained for gallic acid (0.823 ± 0.013 mg/ml) and BHA (0.724 ± 0.018 mg/ml).

The scavenging abilities on hydrogen peroxide were in descending order:

infusion > water > ethanol > ethyl acetate

3.9. Reducing power

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003). Fig. 8 shows the dose-response curves for the reducing powers of the extracts from *S. excelsa* leaves. The reducing power of the water and infusion extract increased from 0.07 ± 0.056 and 0.09 ± 0.005 at 0.25 mg/ml, respectively to 1.58 ± 0.050 and 1.57 ± 0.002 at 10 mg/ml, respectively. The reducing power of ethanol and ethyl acetate extracts increased from 0.07 ± 0.006 and 0.10 ± 0.009, respectively at 0.125 mg/ml to 1.63 ± 0.029 at 5 mg/ml and 1.58 ± 0.009 at 20 mg/ml, respectively. At a dosage of 5–20 mg/ml extracts showed high reducing values of 1.58–1.63, almost equal to that of gallic acid (1.78 ± 0.013) and BHA (1.70 ± 0.06) at a concentration of 0.5–1 mg/ml.

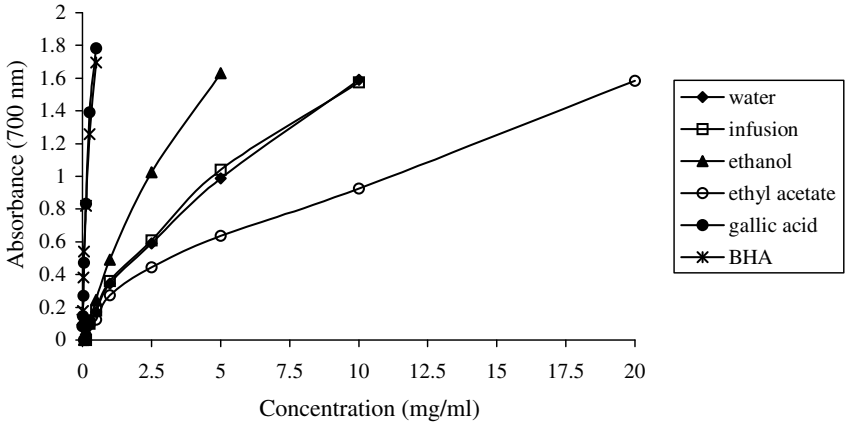

Fig. 8. Reducing power of the extracts from *Smilax excelsa* leaves. Gallic acid and BHA were used as reference antioxidants. Values are means ± SD ($n = 3$).

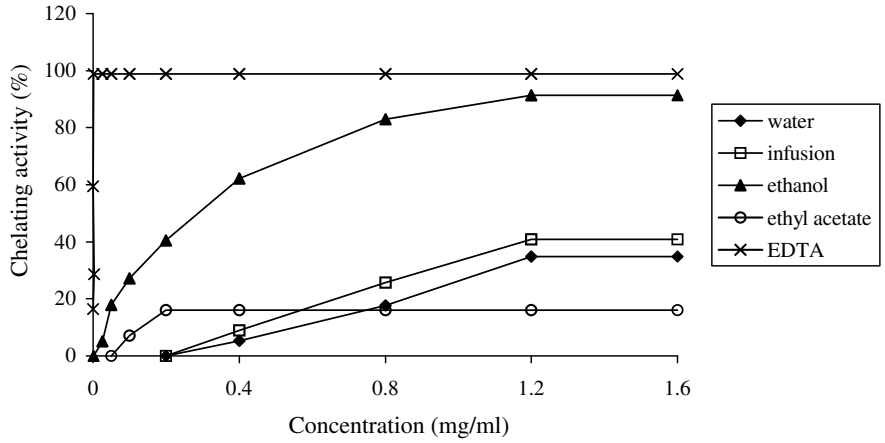

Fig. 9. Chelating activity of the extracts from *Smilax excelsa* leaves. EDTA was used as the positive control. Values are means ± SD ($n = 3$).

The EC_{50} value (the effective concentration at which the absorbance was 0.5) was very high for the ethyl acetate extract (3.63 ± 0.16 mg/ml), compared with the water, infusion and ethanol extracts, of which the EC_{50} values were 1.60 ± 0.045, 1.44 ± 0.037 and 1.18 ± 0.039 mg/ml, respectively. EC_{50} values of the extracts in reducing power were significantly different ($p < 0.05$) from the EC_{50} values obtained for gallic acid (0.07 ± 0.003 mg/ml) and BHA (0.061 ± 0.0025 mg/ml).

Effectiveness in reducing power inversely correlated with EC_{50} values and was in descending order:

ethanol > infusion > water > ethyl acetate

A high correlation was observed between reducing power and antioxidant activity determined by linoleic acid system ($r^2 = 0.9989$, 0.9822, 0.8639, 0.9840 for water, infusion, ethanol and ethyl acetate extracts, respectively), β-carotene bleaching system ($r^2 = 0.9201$, 0.9999, 0.9407, 0.9310 for water, infusion, ethanol and ethyl acetate extracts respectively), and DPPH ($r^2 = 0.9958$, 0.9997, 0.9997, 0.9918 for water, infusion, ethanol and ethyl acetate extracts respectively). Our results were in accordance with other investigators who have also reported that antioxidant properties are concomitant with the development of reducing power (Chung et al., 2005; Duh & Yen, 1997; Kanatt, Chander, & Sharma, 2007).

3.10. Chelation activity on Fe^{2+}

Water, infusion, ethanol and ethyl acetate extracts were assessed for their ability to compete with ferrozine for iron (II) ions in free solution. All the extracts demonstrated an ability to chelate iron(II) ions in a dose-dependent manner (Fig. 9). Water and infusion extracts chelated ferrous ions by $34.8 \pm 1.27\%$ and $40.9 \pm 1.87\%$ at 1.2 mg/ml, whereas ethanol extract showed an excellent chelating ability of $91.3 \pm 0.070\%$ at the same concentration. The chelating effect of the ethyl acetate extract on ferrous ions was low (16%) and insignificant at 1.2 mg/ml. None of the extracts appeared to be better chelators of iron (II) ions than the positive control EDTA in this assay system. EDTA showed excellent chelating ability of $99.8 \pm 0.51\%$ at a concentration as low as 0.01 mg/ml.

EC_{50} value (the effective concentration at which ferrous ions were chelated by 50%) of ethanolic extract (0.36 ± 0.02 mg/ml) was significantly higher ($p < 0.05$) than that of water (1.55 ± 0.06 mg/ml) and infusion (1.48 ± 0.03 mg/ml) extracts, which were comparable.

The chelating abilities on ferrous ions were in descending order:

ethanol > infusion > water > ethyl acetate

Natural antioxidants present in plants are closely related in their medicinal and beneficial properties. Thus antioxidant capacity is a widely used parameter for assessing the bioavailability of foodstuffs as medicinal plants. The antioxidant properties of plant extracts should be evaluated in a variety of model systems using several indices to ensure the effectiveness of such antioxidant materials. In this study 7 antioxidant activity methods and 4 extraction systems of different polarities, i.e., water, ethanol and ethyl acetate were compared. To our knowledge this is the first record on the antioxidant potential of *S. excelsa* leaves.

4. Conclusion

We have demonstrated that extracts of *S. excelsa* leaves contain high levels of total phenolic compounds and were capable of inhibiting lipid peroxidation, directly quenching free radicals to terminate the radical chain reaction, acting as reducing agents, and chelating transition metals to suppress the initiation of radical formation. It is well-known that phenolic compounds present in the plant kingdom are mainly responsible for the antioxidant potential of plants. Accordingly in this study, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. To explore the suitability of different extracting solvents with

different polarity, we have compared the yield, total phenolic content and antioxidant properties of water, infusion, ethanol and ethyl acetate extracts. Water and infusion extracts showed the highest scavenging activities against DPPH, superoxide radicals and hydrogen peroxide. Ethyl acetate extract showed the highest activity only in β-carotene linoleic acid model system and was found to be the least active radical scavenger and metal chelator. Ethanol extract showed the highest inhibition of linoleic acid peroxidation, reducing power, hydroxyl radical scavenging and chelation activities. The data on extraction procedures and antioxidant activity assessment obtained in these experiments single out ethanol and water as the most promising sources for the isolation of natural antioxidative compounds from the leaves of *S. excelsa*. Although the antioxidant activity found in an *in vitro* experiment is only indicative of the potential health benefit, these results remain important as the first step in screening antioxidant activity of *S. excelsa* leaves. It can be concluded that, water extracts of *S. excelsa* leaves, in the way which they are consumed as a foodstuff in the Black Sea region of Turkey, can be used as an accessible source of natural antioxidants with consequent health benefits. Further scientific work in our laboratory is in progress to ensure the medicinal properties of the plant *in vivo* correlate with its antioxidant activity.

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