Food Sci. Biotechnol. 24(2): 673-679 (2015)
DOI 10.1007/s10068-015-0088-x

RESEARCH ARTICLE

Antioxidant Properties of *Elaeagnus umbellata* Berry Solvent Extracts against Lipid Peroxidation in Mice Brain and Liver Tissues

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Received July 12, 2014; revised October 5, 2014; accepted November 5, 2014; published online April 30, 2015

Abstract *Elaeagnus umbellata* is a plant that grows in the hilly areas of Pakistan. Free radical scavenging activities and anti-lipid peroxidative properties of hot water, methanol, *n*-hexane, and acetone extracts of *E. umbellata* berries were evaluated. Extracts showed inhibition against thio-barbituric acid reactive species (TBARS) induced using the pro-oxidants iron (10 μM $FeSO_4$) and sodium nitroprusside (5 μM) in brain and liver homogenates of mice. Extracts also showed metal chelating activities of IC_{50} (extract concentration that causes 50% scavenging)=40-43 μg/mL and DPPH radical scavenging activities of IC_{50}=45.4-49 μg/mL. HPLC analysis revealed the presence of gallic, vanillic, coumaric, ferulic, sinapic, and caffeic acids in berries. The fruit of *E. umbellata* is a potential source of antioxidants with therapeutic importance.

Keywords: *Elaeagnus umbellate*, lipid peroxidation, DPPH radical scavenging activity, pro-oxidant, iron chelation

Introduction

Reactive oxygen species (ROS) are spontaneously generated during cell metabolism and are implicated in the etiology of heart disease, stroke, rheumatoid arthritis, diabetes, and cancer (1,2). A number of studies have shown that use of polyphenolic compounds found in tea, fruits, and vegetables is associated with a low risk of these diseases (3). Consequently, there is a great deal of interest in edible plants that contain antioxidants and health-promoting phytochemicals as potential therapeutic agents. One such plant is autumn olive (*Elaeagnus umbellata* Thunb), a member of the Elaeagnacea family with a high medicinal value that is native to Southern Europe and Central Asia and was introduced to the USA in 1830 as an ornamental plant (4). It is a common medicinal shrub found in the wild at elevation of 1,300-1,800 m above sea level in Azad, Kashmir, Pakistan (5).

E. umbellata is a large spreading, spiny-branched shrub of 3.5 to 5.5 m height and 3.5 to 5.5 m width. The foliage is light green on top and a silvery green on the bottom (4). The fruit is fleshy, sub-globose to broadly ellipsoid, and 6 to 8 mm long. Individual fruits are 1.25 to 1.5 cm in size and start as spotted light green in mid-summer, turning red in the autumn (4). A mature plant can produce 0.9 to 3.4 kg of fruit per year with the number of seeds ranging from 20,000 to 54,000 (6).

The *E. umbellata* berry is an excellent source of vitamins and minerals, especially vitamins A, C, and E, flavonoids and other bioactive compounds, and is also a good source of essential fatty acids (7) and minerals (8). Proximate analysis showed that 100 g of *E. umbellata* fruit contained 69.4 g of moisture, 14.5 g of total soluble solids, 1.15 g of organic acids, 8.34 g of total sugar, 8.13 g of reducing sugars, 0.23 g of non-reducing sugars, and 12.04 mg/100 g of vitamin C. The total mineral content of the fruit, as represented by the ash content, was 1.045% (4). The fruit has lycopene, β-carotene, lutein, phytofuluene, and phytoene. The lycopene ranged from 10.09 to 53.96 mg/100 g in fresh fruit from wild plants and 17.87 to 47.33 mg/100 g in cultivars with red-pigmented fruit. In contrast, fresh tomato fruit, which is the major dietary source of lycopene, contains 0.88 to 4.20 mg/100 g. The fruit is thus newly identified source of lycopene and acts as dietary alternative

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of tomato (9,10). Different studies have shown that lycopene protects against myocardial infarction and types of cancers, including prostate cancer (9).

Keeping in view the high medicinal and nutritional value of autumn olive berries, the antioxidant activities of berries have been extensively studied. Different extracts obtained from berries of *E. umbellata* have shown *in vitro* antioxidant activities and high phenolic contents (11). Moreover, there is limited information on potential use of autumn olive in the management/prevention of diseases arising from oxidative stress. The antioxidant activities of these plants can vary greatly depending on the pro-oxidant used. In this study, the effects of aqueous, methanol, acetone, and *n*-hexane extracts were determined against neurotoxic and hepatotoxic agents (iron and sodium nitroprusside) in brain and liver tissues of mice. Hence, the aim of this study was to determine the phenolic profile, *in vitro* antioxidant activities, and inhibitory effects of extracts against iron and sodium nitroprusside induced lipid peroxidation in an animal model.

Materials and Methods

Chemicals Thiobarbituric acid (TBA), malonaldehyde-bis-dimethyl acetal (MDA), DPPH, quercetin, gallic acid, vanillic, coumaric, ferulic, sinapic, caffeic acids, and 1,10-phenanthroline were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium nitroprusside (SNP) was obtained from Merck (Darmstadt, Germany), and ferrous sulfate was obtained from Biochemicals (Lahore, Pakistan). All chemicals used were of analytical grade.

Preparation of fruit extracts Mature berries of autumn olive (Fig. 1) were harvested in August, 2012 at Rawalakot, Azad, Kashmir and transported to the laboratory in polyethylene bags. Plant specimens were collected close to the campus of the University of Poonch, Rawalakot and identified by Professor Irfan Hussain. A voucher specimen (EMS 113) was deposited in the University of Poonch, Department of Eastern Medicine and Surgery. Fruits were dried in an oven (TFO-1; Cascade Technical Sciences Inc., Hillsboro, OR, USA) at 45°C and ground in a blender to a mesh size of 30 mm. For hot water extraction, ground fruit (10 g) was soaked in hot water (250 mL) for 30 minutes and filtered using Whatman filter paper (18.5 cm). Obtained residues were subjected to extraction twice, then concentrated using a rotary evaporator at 50°C. For solvent extraction, ground material (10 g) was subjected to extraction using 250 mL of methanol, acetone, and *n*-hexane for 3 days in a magnetic stirrer (Somatco, Riyadh, Saudi Arabia) at room temperature, followed by filtration through Whatman filter paper (pore size 11 μm). Extracts obtained after evaporation of organic solvents were weighed to determine the extraction yield (Table 1) and stored in refrigerator until further use. Serial dilutions of these extracts were prepared to obtain the desired concentration of plant extract for experimental use.

Fig. 1. Photograph showing ripe berries of *Elaeagnus umbellate*.

Table 1. Extract yields and total phenolic and flavonoid contents of autumn olive berries

Extracts	Yield (%)	Total phenolics (mg/g)	Total flavonoids (mg/g)
Aqueous	10.1[a1)]	20.0±2.1[a]	3.8±0.30[a]
Methanol	12.5[b]	18.6±1.5[b]	3.2±0.21[b]
Acetone	5.1[c]	18.2±1.3[c]	1.5±0.15[c]
n-Hexane	6.2[d]	16.3±0.8[d]	3.4±0.14[d]

[1)]Values followed by different letters are significantly ($p<0.05$) different from each other.

All animal studies were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Thirty male BALB/c mice (2.0-2.5 months and 24-30 g) were purchased from the National Institute of Health in Islamabad, Pakistan and used for *in vitro* studies. Animals were kept in separate cages with continuous access to food and water in a room with a controlled temperature of 22±3°C and on a 12 h light/dark cycle with lights turned on at 7:00 a.m.

Production of thiobarbituric acid reactive species from brain and liver tissues Production of thiobarbituric acid reactive species (TBARS) was determined as described by Ohkawa *et al.* (12) with modifications. Mice were anaesthetized using chloroform, sacrificed by decapitation, and tissues were quickly removed and placed on ice. One g of brain and liver tissues was homogenized in a tissue homogenizer (Omni, Kennesaw, GA, USA) in cold 100 mM Tris buffer pH 7.4 (1:10 w/v) and centrifuged (Thermo Fisher Scientific, Waltham, MA,USA) (15,000×*g* for 10 min). Homogenates (100 μL) were incubated with

and without 50 μL of freshly prepared oxidant (iron and sodium nitroprusside) and different concentrations (25-200 μg/mL) of plant extracts together with an appropriate volume of deionized water to achieve a total volume of 300 μL at 37°C for 1 h. A color reaction was carried out by addition of 200, 500, and 500 μL of 8.1% sodium dodecyl sulphate (SDS), acetic acid (pH 3.4), and 0.6% TBA. Reaction mixtures, including serial dilutions of a 0.03 mM standard MDA, were incubated at 97°C for 1 h. The absorbance value was read at 532 nm using a spectrophotometer (Spectronic D-20; Thermo Scientific, West Yorkshire, UK) after cooling (tubes were cooled in open air).

Antioxidant activity based on DPPH radical scavenging The antioxidant activity of fruit extracts was measured based on scavenging of stable DPPH radicals following the method of Hatano *et al*. (13). Briefly, a 0.25 mM solution of the DPPH radical (0.5 mL) was added to a fruit extract sample solution in ethanol (1 mL) at concentrations from 25-200 μg/mL. The mixture was shaken vigorously and left to stand for 30 min in the dark, after which the absorbance was measured (Spectronic D-20; Thermo Scientific) at 517 nm. The capacity to scavenge the DPPH radical was calculated as:

$$\text{DPPH radical scavenging (\%)}=[(Ao-A_1)/Ao)]\times 100$$

where Ao is the absorbance of the control reaction and A_1 is the absorbance of the sample reaction. IC_{50} values (extract concentration that causes 50% scavenging) were determined from a graph of the scavenging effect percentage plotted against the extract concentration. All determinations were carried out in triplicate.

Metal chelating activity The Fe (II) chelating activities of extracts were determined using a modified method of Puntel *et al*. (14). Briefly, 150 μL of freshly prepared 2 mM $FeSO_4 \cdot 7H_2O$ was added to a reaction mixture containing 168 μL of 0.1 M Tris-HCl (pH 7.4), 218 μL of saline, and plant extracts at concentrations of 25-200 μL. The reaction mixture was incubated for 5 min before addition of 13 μL of 0.25% 1,10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm using a spectrophotometer (Spectronic D-20; Thermo Scientific).

Determination of total phenolic content The total phenolic content as gallic acid equivalents was determined as described by Singleton *et al*. (15). Extracts (0.5 mL) were added to 2.5 mL of 10% Folin-Ciocalteu's reagent (v/v) and 2 mL of 7.5% sodium carbonate. The reaction mixture was incubated at 45°C for 40 minutes and the absorbance was measured at 765 nm using a spectrophotometer (Spectronic D-20; Thermo Scientific). Gallic acid was used as a standard phenol. The total phenolic content, expressed as mg of gallic acid equivalents/g of extract, was calculated using the following linear equation based on a calibration curve:

$$Y=0.0063x+0.0396\ (r^2=0.99)$$

where Y is the absorbance at 765 nm and x is the total phenolic content of autumn olive extracts.

Determination of total flavonoid content The total flavonoid content as quercetin equivalents was determined following the method of Kosalec *et al*. (16). Quercetin was used for preparation of a calibration curve (0.04, 0.02, 0.0025, and 0.00125 mg/mL in 80% (v/v) ethanol). Standard solutions or extracts (0.5 mL) were mixed with 1.5 mL of 95% (v/v) ethanol, 0.1 mL of 10% (w/v) aluminum chloride, 0.1 mL of 1 mol/L sodium acetate, and 2.8 mL of water. The same volume of distilled water was substituted for 10% aluminum chloride in a blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer (Spectronic, D-20; Thermo Scientific). The total flavonoid content, expressed as mg of quercetin equivalents/g of extract, was calculated using the following linear equation based on a calibration curve:

$$Y=0.0026x+0.0114\ (r^2=0.996)$$

where Y is the absorbance at 765 nm and x is the total flavonoid content of autumn olive extracts.

Extraction of phenolic acids For extraction of phenolic acids, 500 mL of acidified methanol containing 10 % (v/v) HCl was added to 5 g of berries in a refluxing flask. Then, 5 mL of HCl (1.2 M) was added and the mixture was stirred at 90°C under reflux (Cold finger, Cole-Parmer, UK) for 2 h. The extract was cooled to room temperature and the upper layer was obtained.

HPLC analysis of phenolic acids An Agilent 1100-series HPLC system equipped with a quaternary pump (G1311A Quat pump; Aglient, Santa Clara, CA, USA), vacuum degasser, auto-sampler/auto-injector, column compartment, and DAD (Shimadzu, Tokyo, Japan). Data processing used using Agilent Chem Station data handling software. A Hibar® RP-C18 column (250 mm×4.6 mm, 5-μm particle size; Merck KGaA, Darmstadt, Germany) thermostat controlled at 25°C was used for separation. The mobile phase consisted of 50% trifluoroacetic acid (0.3%), 30% acetonitrile, and 20% methanol delivered at a flow rate of 1.0 mL/min. Phenolic acids were detected at 254 nm. Chromatography peaks were confirmed by comparison of retention times with reference standards based on DAD spectra.

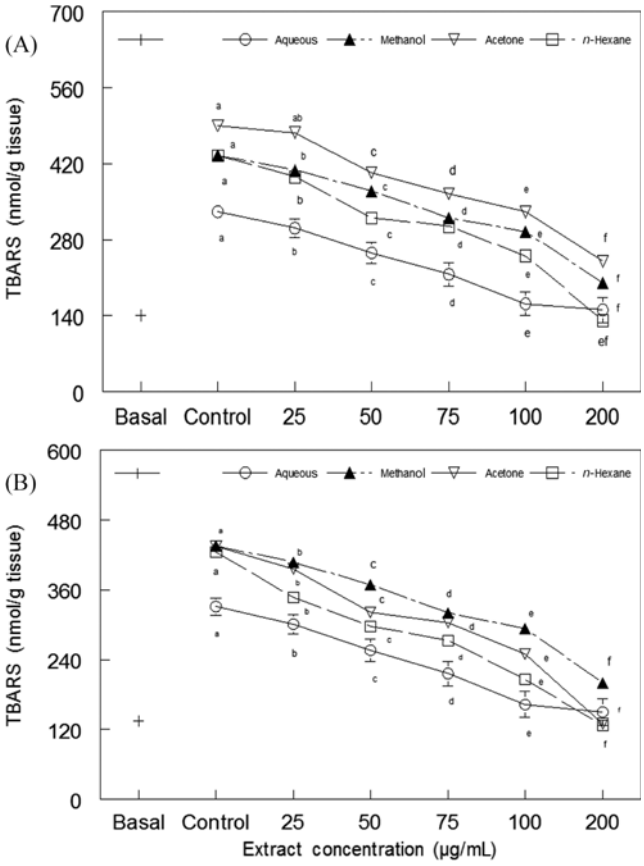

Fig. 2. Inhibitory effect of aqueous, methanol, acetone, and *n*-hexane extracts against lipid peroxidation in mice brain tissues (A) and against iron sulfate induced lipid peroxidation (B). Antioxidant activities of extracts against sodium nitroprusside sulfate induced lipid peroxidation; Data are reported as mean±SD of 3 separate experiments; Values followed by different letters are significantly (p<0.05) different from each other.

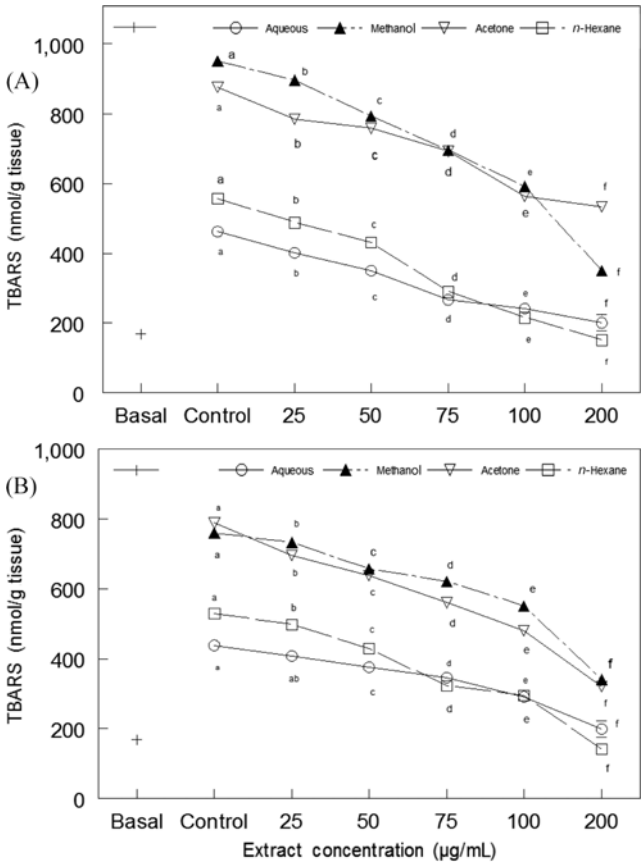

Fig. 3. Inhibitory effect of aqueous, methanol, acetone, and *n*-hexane extracts of berries against lipid peroxidation in mice liver tissues (A) and against iron sulfate induced lipid peroxidation (B). Antioxidant activities of extracts against sodium nitroprusside sulfate induced lipid peroxidation; Data are reported as mean±SD of 3 separate experiments; Values followed by different letters are significantly (p<0.05) different from each other.

Statistical analysis Data were expressed as mean± standard deviation (SD) and analyzed using a one way analysis of variance (ANOVA). Different group mean values were compared using Duncan's multiple range (DMR) test where necessary. Significance was defined as p<0.05. The correlation was studied using linear regression (R). The Statistica version 4.5 software package (Statsoft Inc., Cairo, Egypt) was used for analysis of data.

Results and Discussion

Effect of autumn olive berry extracts on lipid peroxidation induced using iron and SNP in rat brain and liver tissues Oxidative stress is now recognized to be associated with more than 200 diseases, as well as with the normal aging process (17). There is a strong correlation between TBARS as a marker of lipid peroxidation and products that reflect oxidative damage to DNA (18). It is known that metal-catalysed generation of ROS results in an attack not only on DNA and proteins, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (19). Inhibition of lipid peroxidation in rabbit brain homogenates was analogous to neuro-protection (20). Increases in the formation of TBARS in iron (II) sulfate (10 μM) induced oxidative stress indicated possible damage to tissues with an excess of iron. Free iron in the cytosol and mitochondria can cause considerable oxidative damage due to an increase in superoxide formation, which can react with Fe (III) and regenerate the Fe (II) (21).

Lipid peroxidation in mice brain and liver homogenates were induced with iron and sodium nitroprusside. Antioxidant effect of autumn olive extracts was investigated. A significant (p<0.05) increase in formation of TBARS in iron (II) sulfate (10 μM) and sodium nitroprusside (5 μM) induced lipid peroxidation, compared to the basal control level (Fig. 2, 3), was identified. Lipid peroxidation was significantly

Table 2. Phenolic composition of autumn olive berries based on HPLC

Phenolics	Peak area (mAU)	Concentration (μg/g)
Gallic acid	1628.1[a1)]	0.286±0.010[a]
Vanillic acid	410742.4[b]	0.781±0.04[b]
Coumaric acid	5208298[c]	3.00±0.07[c]
Ferulic acid	3098429[d]	0.81±0.06[d]
Sinapic acid	4552291[e]	1.24±0.1[e]
Caffeic acid	7048980[f]	3.19±0.2[f]

[1)]Values followed by different letters are significantly (p<0.05) different from each other based on DMR test.

Table 3. IC_{50} values of extracts against lipid peroxidation in mice brain tissues (unit: μg/mL)

Extract	IC_{50} iron	IC_{50} SNP
Aqueous	104.5	99.1
Methanol	158.0	185.2
Acetone	255.0	142.8
n-Hexane	137.2	161.3
Vitamin C	28.1	35.2

Table 4. IC_{50} values of extracts against lipid peroxidation in mice liver tissues (unit: μg/mL)

Extract	IC_{50} iron	IC_{50} SNP
Aqueous	98.0	181.2
Methanol	185.2	181.1
Acetone	196.0	168.6
n-Hexane	142.8	136.0
Vitamin C	33.2	42.1

(p<0.05) reduced in a dose dependent manner at a concentration ranging from 25-200 μg/mL, compared with controls (Fig. 2). In mice brain tissues, IC_{50} values of extracts followed the order of aqueous>*n*-hexane>methanol >acetone against iron (Table 3). For sodium nitroprusside induced lipid peroxidation, the IC_{50} values of the extracts followed the order aqueous>acetone *n*-hexane>methanol (Table 3).

A similar trend in antioxidant activity was shown in mice liver tissues after treatment with different extracts (Table 3). In the liver, IC_{50} values of different extracts followed the order aqueous>*n*-hexane>methanol>acetone against iron induced lipid peroxidation (Table 4). For sodium nitroprusside induced lipid peroxidation, the IC_{50} values of extracts followed the order *n*-hexane>acetone> methanol aqueous (Table 4), comparable to results for the vitamin C standard.

Possible mechanisms of iron toxicity include free radical-mediated peroxidative reactions, which are readily catalyzed by iron. Protections provided by autumn olive fruit extracts indicated that extracts may be useful for amelioration of oxidative stress in mice. A decrease in the amount of Fe (II) induced lipid peroxidation in brain and liver homogenates in the presence of extracts was probably due to chelation of Fe (II) and/or scavenging of free radicals produced by Fe (II) catalyzed production of reactive oxygen species (ROS). Extracts showed a greater ability to reduce TBARS in mice brain tissues than in liver tissues. Aqueous and *n*-hexane extracts showed greater abilities to reduce lipid peroxidation against pro-oxidants. The higher antioxidant activity in *n*-hexane extracts was probably due to a greater ability to extract lycopene, which is a powerful antioxidant (22).

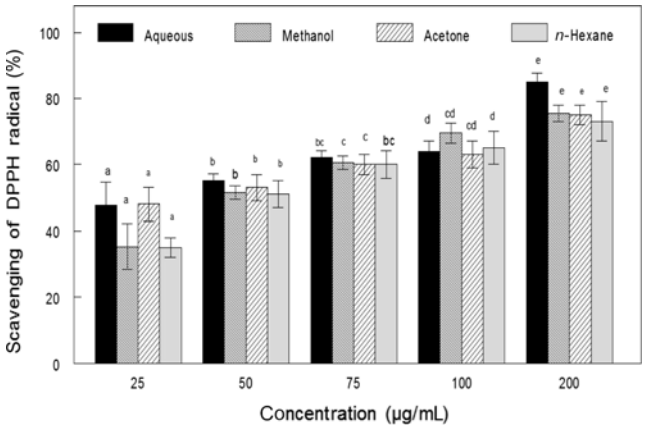

Fig. 4. DPPH radical scavenging activity of extracts of autumn olive berries. Values are reported as mean±SD (n=3); Values followed by different letters are significantly (p<0.05) different from each other based on DMR test.

Effect of autumn olive extracts on free radical scavenging activities Antioxidants are capable of neutralizing free radicals and negate negative effects. They act at different stages (prevention, interception, and repair) and by different mechanisms. Reducing agents act by donation of hydrogen, quenching singlet oxygen, and by the action of chelators and trapping of free radicals (23). DPPH$^{\bullet}$ is considered to be a model of stable lipophilic radical. Antioxidants react with DPPH$^{\bullet}$ and reduce the number of DPPH free radicals to the number of available hydroxyl groups. The absorbance at 517 nm is proportional to the amount of residual DPPH$^{\bullet}$ and can be noticed as a color change from purple to yellow.

The effect of autumn olive extracts on DPPH reduction is shown in Fig. 4. Extracts exhibited a strong antioxidant activity against DPPH radical scavenging in a dose-dependent manner (Fig. 4). Based on IC_{50} values, the ability of extracts to scavenge the DPPH radical was ranked as aqueous> acetone>methanol≈*n*-hexane (Table 5), in agreement with the report of Khattak (11) where extracts obtained from autumn olive fruit showed a high DPPH radical scavenging activity.

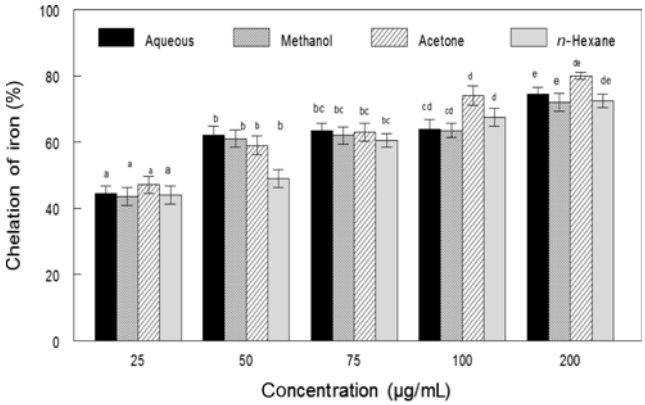

Fig. 5. The Fe(II) chelating ability of extracts of autumn olive berries measured based on reaction with 1,10-phenanthroline. Data are mean±SD (n=3); Values followed by different letters are significantly (p<0.05) different from each other based on DMR test.

Table 5. IC_{50} values of extracts from DPPH radical and iron chelation assays (unit: μg/mL)

Extracts	IC_{50} for DPPH	IC_{50} for iron chelation
Aqueous	45.4	40.3
Methanol	49.0	41.0
Acetone	47.2	42.4
n-Hexane	49.0	43.0
Vitamin C	11.3	25.0

Effect of autumn olive extracts on metal chelation The effect of autumn olive extracts on iron chelation is shown in Fig. 5. Extracts exhibited strong chelating abilities in a dose-dependent manner (Fig. 5). Based on IC_{50} values, the extract ability to chelate Fe (II) was ranked as aqueous> methanol>acetone>*n*-hexane (Table 5). Foods are often contaminated with transition metal ions that may be introduced during processing. Bivalent transition metal ions catalyze the oxidative processes, resulting in the formation of hydroxyl radicals, in addition to hydroperoxide decomposition reactions, via the Fenton reaction (24). These processes can be delayed by iron chelation and deactivation. The ability of the extract to chelate iron was measured as a percentage of iron chelating. Chelating agent disrupts the complex formation with 1,10-phenathroline and iron leads to a decrease in color intensity. The highest chelating activity was shown by aqueous extracts while the lowest chelating action was shown by hexane extracts.

Total phenolic and flavonoid contents The phenolic content ranged from 16.3±0.8 to 20.0±2.1 mg/g in autumn olive fruit. The flavonoid content ranged between 1.5±0.15 to 3.8±0.3 mg/g (Table 1). The high content of phenolics and flavonoids in extracts of autumn olive berries contributed to antioxidant activities. The highest phenolic content was shown by aqueous extracts while the lowest phenolic content was indentified in hexane extracts. Water and methanol extracts showed the highest flavonoid contents while acetone extracts showed the least. Earlier studies by Wang and Fordham (24) reported phenolic and carotenoid contents in different genotypes of autumn olive ranging from 168.9 to 258.1 mg/100 g and 43.4 to 59.3 mg/100 g, respectively, on a fresh weight basis. Perkins-Veazie *et al*. (25) reported that autumn olives fruits contain high levels of phenolics (1,700 mg/kg) and suggested that the astringent flavor of the berry may be due to phenolics. Moreover, the autumn olive berry is a rich source of lycopene that contributed to the antioxidant activity, especially in the *n*-hexane extract, which is better for extraction of lycopene. IC_{50} values were correlated with phenolic (r^2=0.581) and flavonoid (r^2=0.448) contents.

HPLC analysis of phenolic acids An HPLC phenolic profile of *Elaeagnus umbellata* was acquired (Table 2). HPLC fingerprinting analysis revealed the presence of gallic (0.286 μg/g), vanillic (0.781 μg/g), coumaric (3.00 μg/g), ferulic (0.81 μg/g), sinapic (1.24 μg/g), and caffeic acids (3.19 μg/g) in methanolic berry extracts. This is the first study to date to report important phenolic acids in autumn olive berries. The exogenous antioxidants from autumn olive extracts may act directly or indirectly with internal antioxidant systems for synergetic protection effects against diseases linked to free radicals, such as heart disease, neurodisorders, and other stress related disorders.

Results of this study demonstrated the high efficacy of crude extracts of autumn olive fruits for free radical scavenging, inhibition of reactive oxygen species, and lipid peroxidation. These effects may be associated with use of autumn olive fruits for medicinal purposes and as a functional food with effectiveness for treatment of degenerative diseases. Autumn olive fruits can be considered as a source of plant antioxidants with a potential use in food, cosmetics, and pharmaceutical fields. However, more detailed *in vivo* studies are required for evaluation of the antioxidant activities and bio-availability of fruit compounds.

Disclosure The authors declare no conflict of interest.

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