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Evaluation of Antioxidant Properties of *Elaeagnus angustifolia* Flowers

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In the present work, ethanol extract of oleaster was evaluated by employing various *in vitro* antioxidant assay such as total antioxidant activity determination by ferric thiocyanate, total reducing ability determination by Fe^{3+}-Fe^{2+} transformation method, 2,2-diphenyl-1-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging, superoxide anion radical scavenging by riboflavin/methionine/illuminate system. Ethanol extract of oleaster inhibited lipid peroxidation of linoleic acid emulsion, scavenging free radical (DPPH) and superoxide anion radical. The obtained results about ethanol extract of oleaster was compared with the standard antioxidant compounds such as butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol at the same conditions.

Key Words: Antioxidant activity, Oleaster, *Elaeagnus angustifolia*, Radical scavenging effect.

INTRODUCTION

Elaeagnus angustifolia (Russian Silverberry, Oleaster, or Russian-olive, olive tree) is a species of Elaeagnus, native to western and central Asia, from southern Russia and Kazakhstan to Turkey. *Elaeagnus angustifolia*, which grows abundantly in almost all parts of Turkey and Palestine, especially about central, eastern and northern part of Turkey (Black sea region). It has a fine hard wood and yields inferior oil, but it has no relationship to the olive, which, however, it resembles in general appearance. The fruit is edible and sweet, though with a dryish mealy texture. Like all Elaeagnus species, it can fix nitrogen in its roots, enabling it to grow on bare, mineral substrates[1].

First cultivated in Germany in 1736, it is now widely grown across southern and central Europe as an ornamental plant for its scented flowers, edible fruit and attractive silver foliage and black bark. It was introduced into North America in the late 1800s and subsequently naturalized into the wild. Some people consider Russian olive to be an invasive species. It often grows in riparian vegetation where over story cottonwoods have died. It provides a plentiful source of edible fruit for birds (and is marketed in many areas as a wildlife attracting plant). Establishment and reproduction is primarily by seed, although some vegetative propagation also occurs. The fruit is readily eaten and disseminated by many species of birds. The plants begin to flower and fruit from three years old. The leaves and flowers of this plant

are well-known for their use as diuretic and antipyretic in folk medicine and also the fruits are eaten as an appetiser in Turkey[2]. Sakamura and Suga[3] identified glucose and fructose as the major sugars in oleaster fruits during ripening. Kusova *et al*.[4] isolated isorhamnetin, caffeic acid and isorhamnetin-3-O-β-galactopyranoside from mature *E. angustifolia* fruit. In the three forms of *E. Angustifolia* fruits, linoleic (C 18:0) and palmitic (C 16:0) acids in seeds and palmitoleic (C 16:1) acid in pericarps were abundantly isolated[5]. The fatty acid composition of phospholipids and glycolipids in *E. angustifolia* fruits were also reported by Goncharova *et al*.[6] Potter[7] isolated 84 peaks of floral volaties by GC-MS from the steam distillate of *E. umbellata* Thunb. fruit. The principal constituents were palmitic acid (16.9 %), eugenol (11.1 %) and methyl palmitate (10.5 %). From the "purge and trap" analysis, 47 peaks were detected, 37 of which were assigned structures. Among the headspace volatiles, the most abundant compounds were 4-methyl anisole (33.0-42.7 %) and 4-methyl phenol (10.9-13.3 %). An essential oil obtained from the flowers is used in perfumery. A gum from the plant is used in the textile industry in calico printing[8,9].

In the last decades, oxidation mechanisms and free radical role in living systems have gained increased attention[10]. Oxygen uptake inherent to cell metabolism produces reactive oxygen species (ROS). The reaction of this species with lipid molecules originates peroxyl radicals and their interaction with nucleic acids and proteins conduces to certain alterations and therefore, functional modifications[11]. ROS are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. ROS, which include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals ($OH^\bullet$) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are various forms of activated oxygen[12,13].

The harmful action of the free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism[14]. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage[15]. Antioxidants can protect the human body from free radicals and ROS effects. They retard the progress of many chronic diseases as well as lipid peroxidation[16,17]. Hence a need for identifying alternative natural and safe sources of food antioxidants has been created and the search for natural antioxidants, especially of plant origin, has notably increased in recent years. Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods[18-21]. At the present time, the most commonly used antioxidants are BHA, BHT, propyl gallate and *t*-butyl hydroquinone. Besides that BHA and BHT have suspected of being responsible for liver damage and carcinogenesis[22]. Therefore, there is a growing interest on natural and safer antioxidants[23].

No detailed studies on the antioxidant capacity of *Elaeagnus angustifolia* flowers have previously been reported. The aim of this study is to investigate the antioxidant activity of flowers of *Elaeagnus angustifolia*.

EXPERIMENTAL

Riboflavin, methionine linoleic acid, α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), the stable free radical 2,2-diphenyl-1-picryl hydrazyl (DPPH$^{\bullet}$), 3-(2-pyridyl)-5,6-*bis*-(4-phenyl sulfonic acid)-1,2,4-triazine (ferrozine), ammonium thiocyanate, trichloroacetic acid (TCA) and polyoxyethylenesorbitan monolaurate (Tween-20) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant materials and extraction procedures: Oleaster (*Elaeagnus angustifolia*) flower was obtained from local market in Malatya province in Turkey. For ethanol extraction, 25 g powder of oleaster ground into a fine powder in a mill and were mixed five times with 100 mL ethanol. Extraction continued until the extraction solvents became colourless (total solvent volume is 400 mL). For obtained crude extracts were filtered on Whatman No.1 paper and the filtrate was collected, then ethanol was removed by a rotary evaporator at 50 ºC. This crude extract was used for antioxidant activity tests.

Antioxidant activity

Total antioxidant activity determination by ferric thiocyanate method (FTC): The antioxidant activity of crude extract of oleaster and standards were determined according to the ferric thiocyanate method[24]. For preparation of stock solutions, 10 mg of crude extract of oleaster was dissolved in 10 mL of ethanol. Then, the solution of crude extract of oleaster (50 µg/mL) or standard samples (50 µg/mL) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution (5 mL) was incubated at 37 ºC in a glass flask. During incubation the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{2+}-Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. On the other hand, 5 mL control was composed of 2.5 mL linoleic acid emulsion and 2.5 mL, 0.04 M potassium phosphate buffer (pH 7.0). This step was repeated every 5 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid emulsion oxidation. The solutions without added extract or standard were used as control sample. All data on total antioxidant activity are the average of duplicate analyses. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

$$\text{Inhibition of lipid peroxidation}\,(\%) = 100 - \left(\frac{A_{\text{Sample}}}{A_{\text{Control}}} \times 100\right)$$

Herein $A_{Control}$ is the absorbance of the control reaction and A_{Sample} is the absorbance in the presence of the sample (extract of oleaster) or standard compounds[13].

Total reductive capability (FRAP): The samples prepared for ferric cyanate method were used for this and the other assays. The reducing power of extract was determined by the method of Oyaizu[25]. Different concentrations of essential oils (20-200 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferric cyanide [$K_3Fe(CN)_6$] (2.5 mL, 1 %). The mixture was incubated at 50 ºC for 20 min. Aliquots (2.5 mL) of trichloro acetic acid (10 %) were added to the mixture, which was then centrifuged for 10 min at 1000 g. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm in a spectrophotometer (Jasco V-530 UV/VIS spectrophotometer). Increased absorbance of the reaction mixture indicates increased reducing power.

Radical scavenging activity

2,2-Diphenyl-1-picryl-hydrazil (DPPH) free radical scavenging activity: The free-radical scavenging capacity of essential oils was evaluated with the DPPH$^\bullet$ stable radical following the methodology described by Blois[26]. This method is described extensively elsewhere[17]. Wherein the bleaching rate of a stable free radical, DPPH$^\bullet$ is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH$^\bullet$ absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases[27]. Briefly, 0.1 mM solution of DPPH$^\bullet$ in ethanol was prepared and 1 mL of this solution was added 3 mL of essential oils solution in water at different concentrations (25-75 µg/mL). After 0.5 h, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

The capability to scavenge the DPPH$^\bullet$ radical was calculated using the following equation:

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

wherein $A_{Control}$ is the absorbance of the control reaction and A_{Sample} is the absorbance in the presence of extract of oleaster[14].

Superoxide anion radical scavenging activity: Measurement of superoxide anion scavenging activity of extract of oleaster was based on the method described by Beacuhamp and Fridovich[28]. All solutions were 0.05 M in phosphate buffer (pH 7.8). The photo-induced reactions were performed in an aluminium foil-lined box with fluorescent lamps. The distance between reactant and lamp was adjustment until the intensity of illumination reached 4000 lx. The total volume of reactant was 5 mL and the concentrations of riboflavin, methionine and nitro blue tetrazolium (NBT) were 3×10^{-6}, 1×10^{-2} and 1×10^{-4} M, respectively. The reaction was performed at room temperature for 25 min. During this period riboflavin generated O_2^{-}. which reduced NBT to form formazan. The absorbance was read at 560 nm. Essential oils or BHT, α-tocopherol as a standard were added to the reaction mixture, in which of

O_2^- was scavenged, thereby inhibiting the NBT reduction. The unilluminated reaction mixture was used as a blank. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Scavenging (\%)} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance of extract of oleaster or standards[18].

Statistical analysis: All data on total antioxidant activity are the average of duplicate analyses. The other analyses were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 9.0 for Windows 98, SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's Multiple Range tests. p values < 0.05 were regarded as significant and p values < 0.01 very significant.

RESULTS AND DISCUSSION

Antioxidant capacity is widely used as a parameter to characterize food or medicinal plants and their bioactive components. In this study, the antioxidant activity of the ethanol extract of oleaster flower, BHA, BHT and α-tocopherol have been evaluated in a series of *in vitro* test: 2,2-diphenyl-1-picryl-hydrazyl free radical scavenging, ferric thiocyanate method, reducing power, scavenging of superoxide anion radical-generated non-enzymatic system.

Total antioxidant activity determination in linoleic acid emulsion system by ferric thiocyanate method: Lipid peroxidation leads to rapid development of rancid and stale flavours and is considered as a primary mechanism of quality deterioration in lipid foods and oils[21]. Total antioxidant activity of ethanol extract of oleaster flower and the reference compounds such BHA, BHT and α-tocopherol were determined by the ferric thiocyanate method. Etanol extract of oleaster flower and standards compounds exhibited effective antioxidant activity. At the 50 mg/mL concentration, etanol extract of oleaster flower, BHA, α-tocopherol and BHT on lipid peroxidation of linoleic acid emulsion are shown in Fig. 1. The percentage inhibition of BHT, BHA, ethanol extract of oleaster flower and α-tocopherol in linoleic acid system was 71.8, 66.5, 63.1 and 51.5 %, respectively at the same concentration.

Total reductive capability by Fe^{3+}-Fe^{2+} transformation: In this assay, the yellow colour of the test solution changes to various shades of green and blue colour depending upon the reducing power of each antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidants substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian

blue at 700 nm[22]. Fig. 2 depicts the reducing power of ethanol extract of oleaster flower and standards (BHT, BHA and α-tocopherol) using the potassium ferric cyanide reduction method. For the measurements of the reductive ability, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of essential oil using the method of Oyaizu[23]. Like the antioxidant activity, the reducing power of ethanol extract of oleaster flower, BHT, BHA and α-tocopherol increased with increasing concentration. Reducing power of ethanol extract of oleaster flower and standard compounds exhibited the following order: BHA > BHT > α-tocopherol > ethanol extract of oleaster flower.

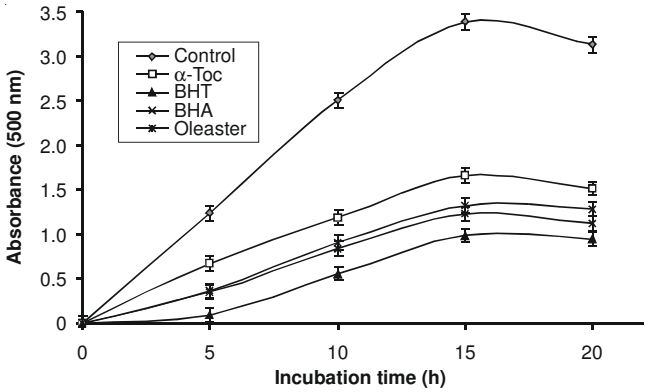

Fig. 1. Total antioxidant activities of different concentrations (5-20 µg/mL) of oleaster and standard antioxidant compounds such as BHA, BHT and α-tocopherol at the same concentrations (BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene)

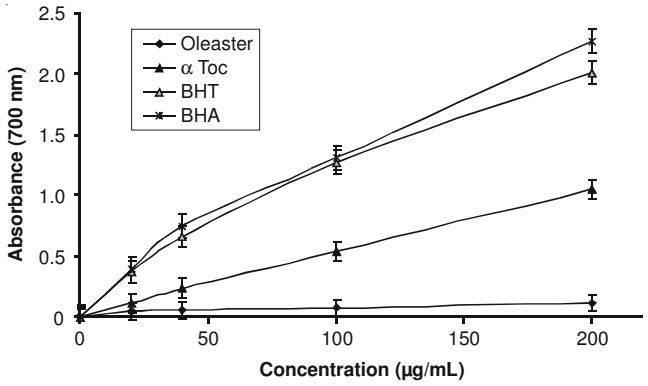

Fig. 2. Total reductive potential of different concentrations (15-200 µg/mL) of oleaster and reference antioxidants; BHA, BHT and α-tocopherol determined by FRAP method (BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene)

Radical scavenging activity: Antioxidant properties, especially radical scavenging activities, are very important due to the deleterious role of free radicals in foods and in biological systems. Excessive formation of free radicals accelerates

the oxidation of lipids in foods and decreases food quality and consumer acceptance. In this study, antioxidant activities of ethanol extract of oleaster flower and standard antioxidants were determined using a DPPH method. DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances in food systems[23-25]. DPPH free radical scavenging is an accepted mechanism by which antioxidants act in inhibiting lipid oxidation; scavenging of DPPH radical was used in this work. The method has been used extensively to predict antioxidant activities because of the relatively short time required for analysis. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow-coloured diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction[13]. Fig. 3 illustrates a significant decrease ($p < 0.05$) in the concentration of DPPH radical due to the scavenging ability of ethanol extract of oleaster flower, α-tocopherol and BHT were used as references radical scavengers. The scavenging effect of ethanol extract of oleaster flower and standards on the DPPH radical decreased in that order: α-tocopherol > BHT > ethanol extract of oleaster flower. Free radical scavenging activity of these samples also increased with increasing concentration.

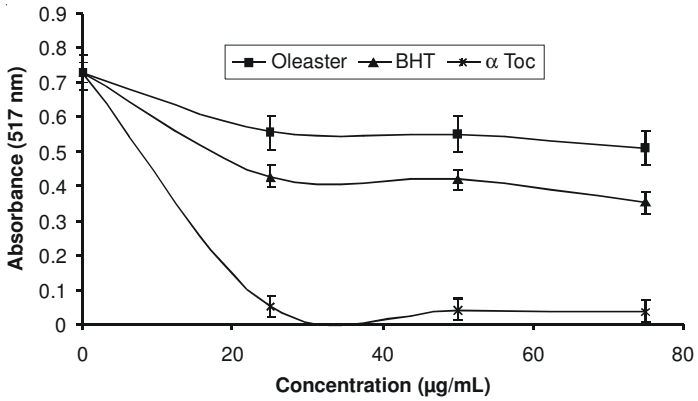

Fig. 3. DPPH free radical scavenging activity of different concentrations (25-75 µg/mL) of oleaster and reference antioxidants; BHT and α-tocopherol (BHT: Butylated hydroxytoluene; DPPH•: 2,2-diphenyl-1-picryl-hydrazyl free radical)

Superoxide anion radical scavenging activity: Superoxide anion, which is a reduced form of molecular oxygen, has been implicated in the initiating oxidation reactions associated with aging[26]. It has also been implicated in several pathophysiological processes, due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Superoxide has also been observed to directly initiate lipid peroxidation[27]. It has also been reported that antioxidant properties of some flavonoids are effective mainly *via* scavenging of superoxide anion radical[13,28]. Superoxide anion plays an important role in formation of other reactive oxygen

species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA[25]. Also, superoxide anion is an oxygen-centred radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalent reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome c.

The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture[13,17]. Fig. 4 shows the percentage inhibition of superoxide radical generation by 4-16 μg/mL concentration of ethanol extract of oleaster flower, BHA and α-tocopherol. The inhibition of superoxide radical generation results of ethanol extract of oleaster flower and standard were found similar statistically ($p > 0.05$). As can see in Fig. 4, the percentage inhibition of superoxide generation by 16 μg/mL concentration of ethanol extract of oleaster flower, while α-tocopherol and BHA were found as 30.3, 34.3 and 36.6 %, respectively. Superoxide radical scavenging activity of those samples showed the following order: ethanol extract of oleaster flower < α-tocopherol < BHA.

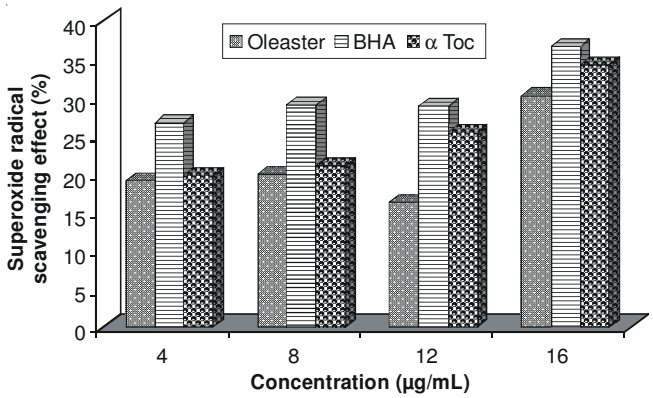

Fig. 4. Comparison of superoxide anion radical scavenging activity of 16 μg/mL concentration of oleaster BHA and α-tocopherol (α-Toc: α-tocopherol, BHA: Butylated hydroxyanisole)

Conclusion

In recent years, antioxidants have gained a lot of importance due to their potential as prophylactic and therapeutic agents in many diseases. Therefore the discovery of novel antioxidants has lead to a medical revolution that is promising a new paradigm of health care. Antioxidant properties of plant extracts should be evaluated in a variety of model systems using several different indices because the effectiveness of such antioxidant materials. The obtained present results clearly indicated that the ethanolic extracts of oleaster flower examined in this study exhibit antioxidant activity against various antioxidant systems *in vitro* and this activity is correlated with the total phenolic compounds content in the extract. Additionally this extract

provides new pharmaceutical perspective for application as easily accessible source of natural antioxidants and as a possible food supplement. It can also be used in stabilizing food against oxidative deterioration or in pharmaceutical applications.

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