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In vitro antioxidant and antiradical properties of *Hippophae rhamnoides* L.

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ABSTRACT

Hippophae rhamnoides L. is a member of the Elaeagnaceae family, different parts of it, especially juice and oil of the fruits, have been used for the treatment of several diseases in traditional medicine in various countries. In the present study we investigated the possible antiradical and antioxidant activities of the hexanoic extract obtained from ripe fruit of *H. rhamnoides* L. (HRE) in in vitro conditions using different antioxidant tests: ferric thiocyanate method, reducing power, metal chelating activities and 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) free radical scavenging. Results of experiment revealed that HRE has a concentration-dependent antioxidant effect. Total antioxidant activity was measured according to ferric thiocyanate method. At the concentration of 10, 20 and 50 µg/mL, the inhibitory effect of HRE on peroxidation of linoleic acid emulsion was found to be 36.8, 50.8 and 68.7 %, respectively. On the other hand, percentage inhibition of α-tocopherol was found to be 8.9 %. In addition, HRE was also effective in reducing power, metal chelating activities and DPPH· free radical scavenging experiments. These various antioxidant activities were compared to α-tocopherol, EDTA or trolox which references antioxidants. The results obtained from present study clearly showed thad HRE had marked in vitro antioxidant and antiradical activites.

Keywords: Antioxidant activity, *Hippophae rhamnoides* L., Metal chelating, Radical scavenging, Reducing power.

INTRODUCTION

In recent years, the importance of reactive oxygen species and free radicals has attracted increasing attention. Reactive oxygen species (ROS), which include superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH·) and H_2O_2 and singlet oxygen ($^1O_2$), are exacerbating factors in cellular injury and aging process (1). ROS are continuously produced during normal physiologic events and they are removed by antioxidant defence mechanisms. There is a crucial balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms.

Under pathological conditions, excessive production of ROS cause oxidative stress and macromolecular damage including protein oxidation and lipid peroxidation (2,3).

Many synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone have been shown to have some advers effects including liver damage and carcinogenesis (4,5). Therefore, there is a growing interest on natural additives as potential antioxidant in reducing free radical-induced tissue injury. Numerous plant products have been screened and shown to have antioxidant activity due to presence of antioxidant vitamins, flavonoids, and polyphenolic compounds.

Hippophae rhamnoides L. (Family-Elaeagnaceae), also known as sea buckthorn, is a perennial plant native to European and Asian countries such as Italy, Spain, India, Tibet and Turkey (6). It is found growing more on riversides of mountains, sandy and gravel grounds at an altitude of 900–1850 m in the fields of northeastern Turkey. Different parts of *H. rhamnoides* L., especially juice and oil of the fruits, have been used for the treatment of several diseases in traditional medicine in various countries in the world. In northern and southwestern China, it has long been used for relieving cough, aiding digestion and pain killer since ancient time (6). Due to anti-inflammatory effects, it's fruit is used in pulmonary, gastrointestinal, cardiac and metabolic disorders in Indian and Tibetian medicine (7,8). As a medicinal plant, fruit of *H. rhamnoides* L. has been used to treat constipation, skin wounds and influenza infections in Turkish traditional medicine (9).

H. rhamnoides L. contains a lot of chemical compounds including water- and fat-soluble vitamins, sterols, flavonoids, lipids and tannins (10). The ripe fruit has been reported to be a rich source of vitamins A, C, E and K, carotenoids, and organic acids (10,11). The flavonoids in *H. rhamnoides* L. have been identified to be responsible for most of its pharmacological activities (12). These compounds possess biological and therapeutic activities including antioxidant, antitumoral, hepato-protective and immunumodulatory properties. For these reason, it is often used in traditional medicine.

Currently, the importance of *H. rhamnoides* L. berries has been increased in the world. Berry products are among popular foods in some countries. On the other hand, industrial utilizations of the *H. rhamnoides* L. caused to increase the necessity of content and it's biological activity informations for food and drug industries. Thus, in the present study, we investigated possible antioxidative and antiradical activities of hexanoic extract obtained from ripe fruit of *H. rhamnoides* L. (HRE) in in vitro conditions.

MATERIALS AND METHODS

Chemicals

Linoleic acid, α-tocopherol, a stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), polyoxyethylenesorbitan monolaurate (Tween-20) and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck (Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant material

The ripe fresh fruit of *H. rhamnoides* L. were collected from Erzurum-Tortum (altitude of 1600 m) in December 2003. The plant was identified by the Botanical Institutes, Atatürk University, Erzurum, Turkey, where a voucher specimen is kept.

Extraction and preparation of test sample

Fruits of *H. rhamnoides* L. were removed from the branches and washed with tap water and dried. Fruits were crushed in a mortar and mixed. Fruit mash (500 g) was placed in a glass jar and hexane was added in equal volume. 48 h later, juice was obtained from the mixture by squeezing and centrifuging at 1000xg for 15 min; clear supernatant was removed by a drip. Hexane was evaporated from liquid by evaporator (Büchi, Rotavapor, R 110, Switzerland), yielding 43.5 g (8.7%) of oily material, and this extract was called HRE.

Total antioxidant activity determination by ferric thiocyanate method (FTC)

The antioxidant activity of HRE and standards was determined according to the ferric thiocyanate method (13). For stock solutions, 10 mg of HRE was dissolved in 10 mL ethanol. Then, a solution, which contains different amounts of stock HRE solution (50, 100 and 250 µg) or standard sample (250 µg) 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). Final concentrations were 10, 20 and 50 µg/mL for HRE, and 50 µg/mL for α-tocopherol. Five mL linoleic acid emulsion contained 17.5 µg Tween-20 and 15.5 µL linoleic acid, in 0.04 M potassium phosphate buffer (pH 7.0). A solution without added HRE was used as a blank sample. The mixed solution (5 mL) was incubated at 37°C in a glass flask. The peroxide level

was determined by reading the absorbance at 500 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland) after reaction with $FeCl_2$ and thiocyanate at intervals during incubation time. During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{+2} to Fe^{+3}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. This step was repeated every 5 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid emulsion oxidation. All data on total antioxidant activity are the average of duplicate analyses. The percentage inhibition of lipid peroxidation in linoleleic acid emulsion was calculated by the following equation:

Inhibition of lipid peroxidation (%) = $100 - (A_1A_o)\times100$

where A_o is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample (HRE or standard compound).

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

The reducing power of HRE was determined by the method of Oyaizu (14). Different amounts of HRE or α-tocopherol (50 and 100 µg) were mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 mL) and potassium ferricyanide [$K_3Fe(CN)_6$] (1%, 2.5 mL). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10%) were added to the mixture. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.1%, 0.5 mL), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing power. All test and analyses were run in triplicate and averaged.

Ferrous metal ions chelating activity

The chelating of ferrous ions by the HRE and standards was estimated by the method of Dinis (15). Briefly, 20 µg dose of HRE in 0.4 mL ethanol was added to a 50 µL solution of $FeCl_2$ (2 mM). Total volume was adjusted to 4 mL ethanol. Then, the reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Then the mixture was shaken vigorously and left standing at room temperature for 10 minutes. Absorbance of the solution was then measured spectrophotometrically at 562 nm. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given bellow:

Metal chelating effect (%) = $[(A_o - A_1)/A_o] \times 100$

where A_o is the absorbance of the control, and A_1 is the absorbance in the presence of the sample of HRE or standards. The control only contains $FeCl_2$ and ferrozine, which are molecules of complex formation (16).

Antiradical activity

The free radical scavenging activity of HRE was measured by the following method described by Blois (17). Wherein the bleaching rate of a stable free radical, 1,1-Diphenyl-2-picryl-hydrazil (DPPH·) is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH· absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH· in ethanol was prepared and 1 ml of DPPH· solution was added to 4 mL of ethanol contains HRE at different amounts (50–250 µg) and α-tocopherol (250 µg). Final concentrations were 10, 20 and 50 µg/mL for HRE, and 50 µg/mL for α-tocopherol. Thirty minutes later, the absorbance was measured at 517 nm. All test and analyses were run in triplicate and averaged. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH · concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2: 0.999):

Absorbance = $9.2872 \times [DPPH\cdot] + 0.097$

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

DPPH · Scavenging effect (%) = $[(A_o - A_1 / A_o) \times100]$

wherein A_o is the absorbance of the control reaction and A_1 is the absorbance in the presence of HRE (18, 19).

Statistical Analysis

All data on total antioxidant activity are the average of duplicate analyses. The other analyses were performed in triplicate. All values were expressed as mean ± S.D. Statistical analysis of data was performed using a one-way analysis of variance (ANOVA) and Tukey's post test. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

It was demonstrated that antioxidants in plants are closely related with their biofunctionalities such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis, which are often associated with the termination of free radical propagation in biological systems (20). Because of this, 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 HRE been evaluated in a series of in vitro test: ferric thiocyanate method, reducing

power, metal chelating activities and 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging.

Total antioxidant activity determination in linoleic acid emulsion system by ferric thiocyanate method

The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of oxidation (21). Therefore, total antioxidant activity of HRE and α-tocopherol was determined by the ferric thiocyanate method and the data are shown in Figure I. HRE and standard compound α-tocopherol, exhibited effective antioxidant activity. The percentage inhibition of peroxidation in linoleic acid system by HRE solutions at concentrations of 10, 20, and 50 µg/mL was found to be 36.8, 50.8 and 68.7 %, respectively. However, 50 µg/mL of α-tocopherol exhibited 8.9 % inhibition of peroxidation in the linoleic acid emulsion system. Effect of HRE was dose-dependent and the most effective dose of HRE was found to be 50 µg/mL. Again, all doses of HRE were more potent than that of α-tocopherol.

Total reductive capability using the potassium ferricyanide reduction method

The yellow colour of the test solution changes to various shades of green and blue, depending upon the reducing power of each antioxidant sample in this method. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances 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).

As can be seen in Figure II, HRE and a standard (α-tocopherol) showed the effective reducing power using by the potassium ferricyanide reduction method. For the determination of the reductive ability, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of HRE, using the method of Oyaizu (14). Like the antioxidant activity, the reducing power of HRE and α-tocopherol increased with increasing concentrations of sample.

Ferrous ions chelating capacity

Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (23,24). Ferrous ion (Fe^{2+}) chelating activities of HRE, α-tocopherol, trolox and EDTA (a strong chelator), are shown in Figure III. The chelating of ferrous ions by the HRE and standards was determined according to the method of Dinis et al. (15). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex

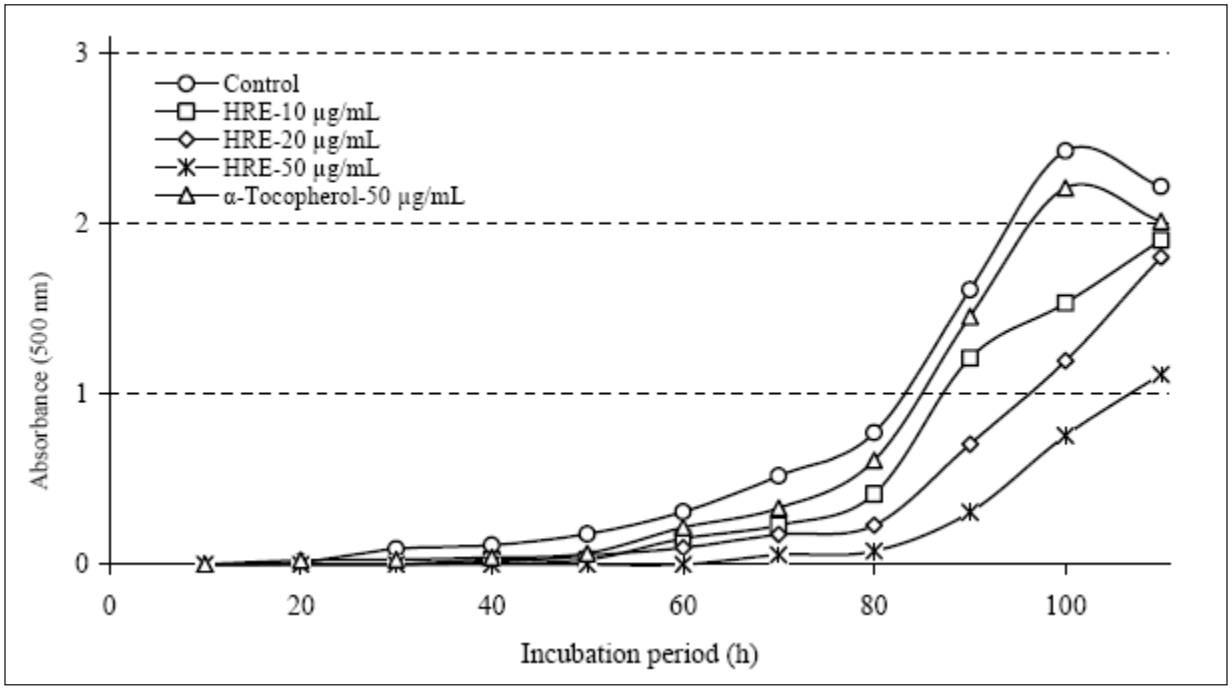

Figure 1: Total antioxidant activities of different amounts of HRE (10–50 µg/mL) and of α-tocopherol (50 µg/mL) as standard antioxidants were determined by ferric thiocyanate method (HRE: Hexanoic extract of *Hippophae rhamnoides* L.; n=2).

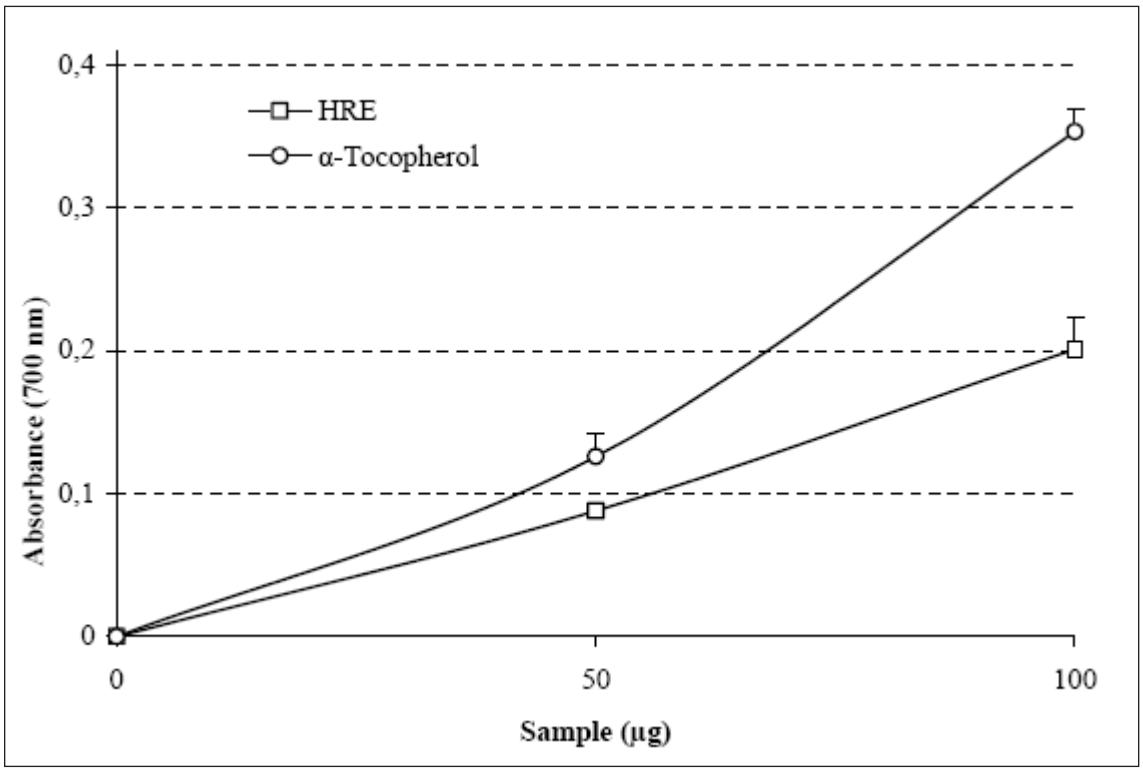

Figure II: Total reductive potential of different concentrations of HRE and α-tocopherol using spectrophotometric detection of the Fe^{+3}-Fe^{+2} transformations (HRE: Hexanoic extract of *Hippophae rhamnoides* L., n=3).

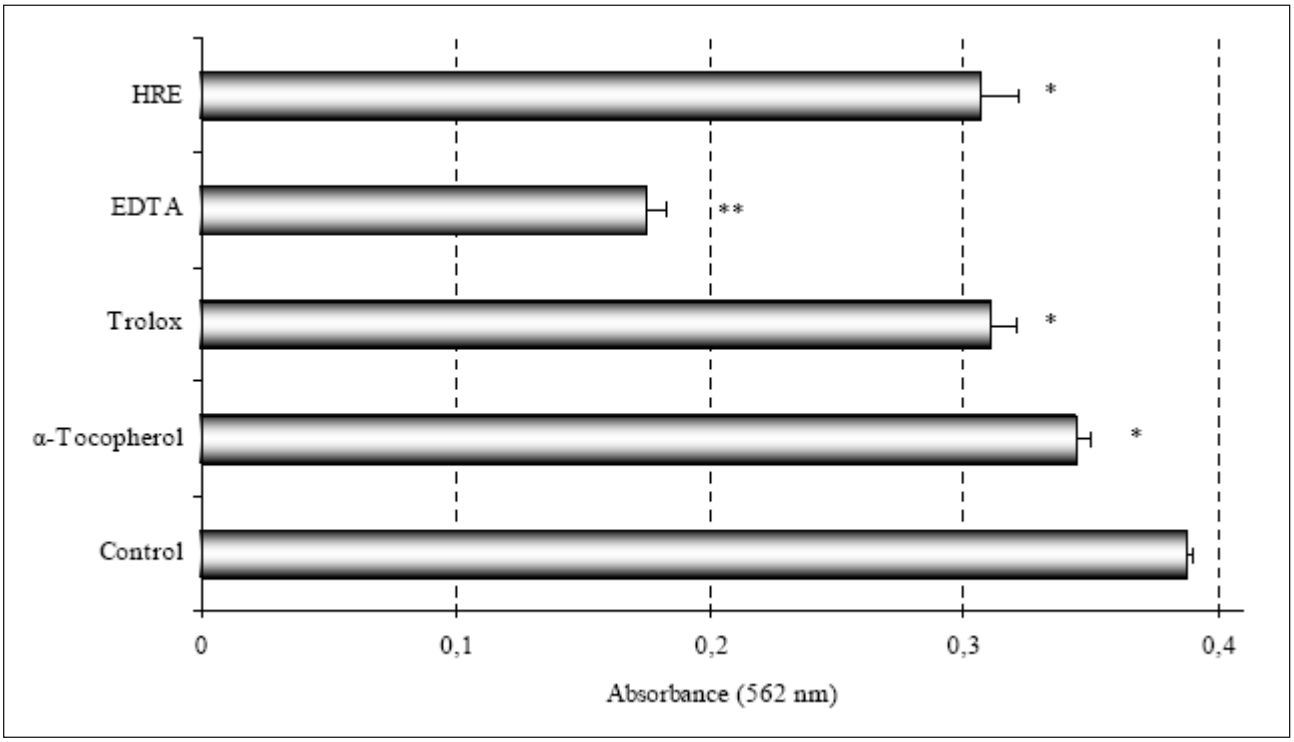

Figure III: Ferrous ions (Fe^{2+}) chelating effect of of HRE, α-tocopherol, trolox and EDTA at the same dose (20 µg) (HRE: Hexanoic extract of *Hippophae rhamnoides* L.; EDTA: Ethylenediaminetetraacetic acid; n=3). * p< 0.01; ** p<0.001, with respect to control, by ANOVA (Tukey's post test).

formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelator. In this assay, HRE and three standard compounds are interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine. EDTA is a strong metal chelator; hence, it is used as standard metal chelator agent in this study.

Metal chelating capacity was significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (25). The data obtained from Figure III reveal that HRE demonstrate a marked capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity. The percentages of metal scavenging capacity of 20 µg of HRE, α-tocopherol, trolox and EDTA were found as 20.9, 13.4, 19.9 and 54.9%, respectively. The metal scavenging effect of these samples decreased in the order of EDTA > HRE ≈ α trolox > α-tocopherol.

Radical scavenging activity

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 (26). In this study, antioxidant activities of HRE and standard antioxidant α-tocopherol 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 (27, 28).

The antioxidants were able to reduce the stable radical DPPH to the yellow-coloured diphenyl-picrylhydrazine, in the DPPH assay. 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. Figure IV illustrates an effective decrease in the concentration of DPPH radical due to the scavenging ability of HRE with dependent on its increased amount. In this study, α-tocopherol was used as reference radical scavenger. The scavenging effect of all of HRE doses was higher than that of α-tocopherol.

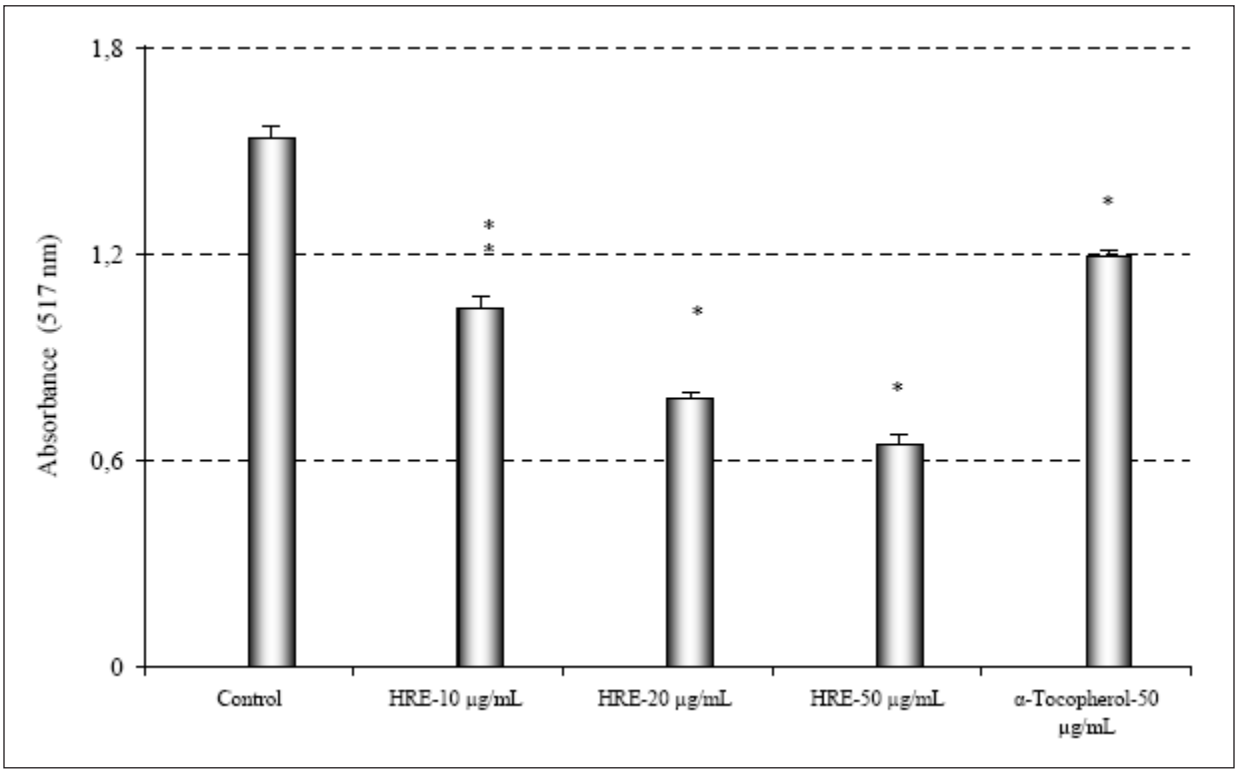

Figure IV: DPPH· free radical scavenging activity of different concentrations of HRE (10–50 µg/mL) and α-tocopherol (50 µg/mL) was spectrophotometrically measured at 517 nm using the DPPH radical assay (HRE: Hexanoic extract of *Hippophae rhamnoides* L.; DPPH: 1,1-diphenyl-2-picryl-hydrazyl; n=3). * p< 0.001, with respect to control, by ANOVA (Tukey's post test).

In conclusion, according to the results obtained from present study clearly indicated that HRE has effective antioxidant activity, reducing power, DPPH radical and metal chelating activities when compared to standard antioxidant compounds such as a natural antioxidant α-tocopherol, and trolox which is water-soluble analogue of tocopherol. Also, the results of this study show that of HRE can be use easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. The components responsible for the antioxidant activity could be performed on further detailed studies.

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