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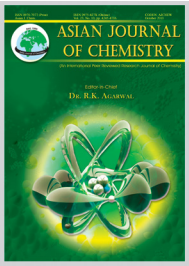

Investigation of Antioxidant Activity and Total Anthocyanins from Blackberry (*Rubus hirtus* Waldst. and Kit) and Cherry Laurel (*Laurocerasus officinalis* Roem)

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In present study, water and ethanol extracts of blackberry (*Rubus hirtus* Waldst. and Kit) and cherry laurel (*Laurocerasus officinalis* Roem.) was studied for antioxidant properties. We investigated the antioxidant properties of blackberry and cherry laurel extracts by employing several *in vitro* antioxidant assays such as total antioxidant activity, reducing abilities, DPPH free radical, superoxide anion radical and hydrogen peroxide scavenging and ferrous ions chelating activities. In addition, total phenolic and total flavonoid contents in the both extracts of fruits were determined as gallic acid equivalent. Butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol were used as references antioxidant compounds. At the concentration of 50 µg/mL, water extracts of *Rubus hirtus* (WERH) and *Laurocerasus officinalis* (WELO) showed 75.18 and 80.96 % inhibition on peroxidation of linoleic acid emulsion, respectively. At the same concentration, ethanol extracts of *Rubus hirtus* (EERH) and *Laurocerasus officinalis* (EELO) exhibited 82.86 and 74.51 % inhibition on peroxidation of linoleic acid emulsion, respectively. On the other hand, total anthocyanins were investigated in both extracts as µg/mL cyanidin-3-glucoside (cyd-3-glu). A relationship has been found between antioxidant activity of extracts and total anthocyanins. The results acquired in the present study indicate that both extracts have more effective antioxidant capacity than synthetic and natural standard compounds.

Key Words: Antioxidant activity, DPPH radical scavenging activity, Total phenol, Total flavonoid, Anthocyanin, *Rubus hirtus* Waldst. and Kit, *Laurocerasus officinalis* Roem.

INTRODUCTION

Oxidation is essential to many living organisms' production of energy-to-fuel biological processes. However, oxygen-centered free radicals and other reactive oxygen species (ROS), which are continuously, produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by free radicals may be related to aging and diseases, such as arteriosclerosis, diabetes, cancer and cirrhosis[1]. In living organisms various reactive oxygen species can be formed by different ways. These ways are classified endogenous and exogenous sources[2]. Most living species have an efficient defense system to protect themselves against the oxidative stress induced by reactive oxygen species[3]. Reactive oxygen species, including superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2) are physiological metabolites[4,5]. Also, excessive generation of reactive oxygen species induced the antioxidant capacity of the organism leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity, cancer and aging. Those primary derivates of oxygen play an important role in mediating reactive oxygen species-related effects[6].

Reactive oxygen species is also formed in aerobic cells due to electron leakage from the electron transport chain. Superoxide anion radicals are also formed by activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils and the production of superoxide anion radicals is an important factor in the killing of bacteria by phagocytes. In living organisms, superoxide anion radical is removed by the superoxide dismutases (SOD)[7,8]. Reactive oxygen species can easily initiate the peroxidation of the membrane lipids. The peroxidation products by themselves and their secondary oxidation products, such as malondialdehyde and 4-hyroxy-nonenal which react with biological protein, amines and deoxyribonucleic acid are highly reactive[5,6].

There are a number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine are the main factors for the observed efficacy of these foods in reducing the incidence of chronic diseases including heart disease and some cancers. The free radical scavenging activity of antioxidants in foods has been substantially investigated and reported in the literature[9]. Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers[10]. Recently, interest has increased considerably in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity[11].

Plants respond to periodic changes in resource availability in term of their growth, morphology and reproduction[12]. *Rubus* species (Rosaceae) have been traditionally used for therapeutic purposes. For instance, extracts of leaves and roots of this genus have been used for the treatment of diabetes mellitus, rheumatism, sore throat, hemorrhoid, diarrhea and similar enteric disorders[13]. Different parts of *Rubus* species are used in folk medicine for the treatment of numerous diseases such as diabetes mellitus, inflammatory disorders, diarrhea, hemorrhoids and ulcers[14-16].

Laurocerasus officinalis Roem. belongs to the Rosaceae family and is a popular fruit, mainly distributed in the coasts of the Black Sea region of Turkey[17]. It is locally called Taflan or Karayemis. This fruit is commonly used as vegetables and medicinal fruits and an important fruit in Turkey. It is mostly consumed as fresh fruit in local markets but may also be dried, pickled and processed into pekmez, jam, marmalade and fruit juice products. Besides its use for food, both fruit and seeds of cherry laurel are well known as traditional medicines in Turkey and have been used for many years for the treatment of stomach ulcers, digestive system complaints, bronchitis, eczemas, haemorrhoids and as a diuretic agent, among others[18]. Different parts of this plant have been utilized for various purposes. For example, almond flavouring, anti-spazmodics, narcotics and sedative chemicals are obtained from its leaves. The fruits and leaves have also been widely used in the perfumery and dye industry[19]. And also these species have antioxidant properties[20].

The aim of this study was to investigate antioxidant activity of water and ethanol extracts of fruits (RH and LO) by using different antioxidant tests such as total antioxidant activity, reducing power, superoxide anion radical scavenging, hydrogen peroxide scavenging, free radical scavenging and metal chelating activities. And, we measured the content of total phenols, flavonoids and anthocyanins in fruit extracts. The scope of the current paper is to investigate antioxidant properties of RH and LO that are traditionally used for medicinal purposes in Turkey.

EXPERIMENTAL

Ammonium thiocyanate, ferrous chloride, potassium chloride, potassium hexacyanoferrate(III), ferric chloride and, butylated hydroxy toluene (BHT) sodium acetate were purchased from E. Merck. Linoleic acid, *p*-nitro-blue tetrazolium chloride (NBT), 5-methylphenazinium methyl sulfate (PMS), nicotinamide adenine dinucleotide (NADH), absolute ethanol, hydrogen peroxide, 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$), 3-(2-pyridyl)-5,6-*bis*-(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), Folin and Ciocalteu' s phenol reagent (Folin C), HCl, α-tocopherol, butylated hydroxyanisole (BHA), gallic acid and trichloracetic acid (TCA) polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals were analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of fruit materials and extraction: RH fruit was collected from Giresun and LO fruit was collected from Samsun in Turkey, in June-August (2010). Then, fruits were left in drying oven at 40 ºC. The dried samples were chopped into small parts with a blender. They were subjected to extraction using soxhlett extractor, with an absolute ethanol or doubly-distilled water. Then the extracts were filtered over Whatman No. 1 paper. Ethanol extracts were evaporated to dryness. The filtrates were frozen and lyophilized in a lyophilizator (Christ Alpha 1-2 LD Plus) at 10 µm Hg pressure at -50 ºC. The fruit extracts were placed in a plastic flask and then kept at -30 ºC until used.

Total antioxidant activity determination: Total antioxidant activities of extracts were determined using the ferric thiocyanate (FTC) method[21]. For stock solutions, 10 mg of the lyophilized water extracts were dissolved in 10 mL doubly-distilled water. 10 mg of the lyophilized ethanol extracts were dissolved in 10 mL absolute ethanol. Extract and standard solutions (50 µg/mL) were prepared in potassium phosphate buffer (0.04 M, pH = 7.0) from stock solutions. 2.5 mL extracts or standard materials was added to 2.5 mL linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH = 7.0). A 100 mL linoleic acid emulsion contained 0.31 mL linoleic acid, 0.35 g Tween-20 and potassium phosphate buffer (0.04 M, pH = 7.0). For the control reaction, 2.5 mL linoleic acid emulsion and 2.5 mL potassium phosphate buffer (0.04 M, pH = 7.0) were mixed. Each solution was then incubated at 37 ºC in a glass flask in the dark. After that, 0.1 mL of each solution was diluted in ethanol (9.7 mL, 99 %), NH_4SCN (0.1 mL, 10 %) and $FeCl_2$ (0.1 mL, 0.02 M). The peroxide value was measured at 500 nm (Unicam UV2-100) and the percentage of inhibition was determined. After the linoleic acid oxidation, peroxides oxidize Fe^{2+} to Fe^{3+}. The latter ions form a complex with SCN^- and, which has a maximum absorbance at 500 nm. This step was repeated every 10 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid emulsion oxidation. All tests were run triplicate and an analysis of all samples was done in triplicate and averaged. The inhibition of lipid peroxidation in percent was calculated by the following equation:

$$\text{Percentage inhibition lipid peroxidation} = [1 - (A_s/A_c)] \times 100$$

where, A_c was the absorbance of control reaction, which contains only linoleic acid emulsion and phosphate buffer, A_s was the absorbance of samples (extracts) or the standard compounds[22].

Ferric ions (Fe^{3+}) reducing antioxidant power assay: The Fe^{3+} reducing power capacity of extracts were determined according to the method of Oyaizu[23]. The FRAP method is based on a redox reaction in which an easily reduced oxidant (Fe^{3+}) is used in stoichiometric excess and antioxidants act as reductants[24]. The different concentrations (50-250 µg/mL) of extracts in 1 mL doubly-distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $[K_3Fe(CN)_6]$ (2.5 mL, 1 %), then the mixture was incubated at 50 ºC for 20 min. Afterwards, TCA (2.5 mL, 10 %) was added to the mixture, which was then centrifugated at 3000 rpm for 15 min (MSE Mistral 2000, UK). The supernatant (1.0 mL) was mixed with 1 mL of doubly-distilled water and $FeCl_3$ (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm (Unicam UV2-100). The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the $[Fe(CN)_6]^{3-}$ complex to the $[Fe(CN)_6]^{4-}$. Therefore, Fe^{2+} can

be monitored by measuring the formation of Perl's Prussian Blue ($Fe_4[Fe(CN)_6]_3$) at 700 nm[25]. Higher absorbance of the reaction mixture indicated increased reducing power. The percentage inhibition of FRAP was calculated using the following formula:

$$\text{Ferric ions reducing power (\%)} = (A_s/A_c) \times 100$$

where, A_c was the absorbance of control (L-ascorbic acid), A_s was the absorbance of samples (extracts) or the standard compounds.

Superoxide anion radical scavenging activity: Superoxide anion ($O_2^{\bullet-}$) scavenging activities of extracts were determined according to the Nishimiki slightly modified[26]. Superoxide radicals are produced in phenazinemethosulphate (PMS)-nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiments, the superoxide radicals were produced in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (156 µM in 0.1 M phosphate buffer, pH = 7.4) solution, 1 mL NADH (468 µM in 0.1 M phosphate buffer, pH = 7.4) solution and 1 mL sample extract (50 µg/mL) were mixed. The reaction was started by adding 0.1 mL of PMS solution (60 µM PMS in 0.1 M phosphate buffer, pH = 7.4) to the mixtures. The reaction mixtures were incubated at 25 ºC for 5 min and the absorbance was measured at 560 nm in a spectrophotometer (Unicam UV2-100. Decrease in absorbance values of the reaction mixtures indicated increased superoxide anion scavenging activities. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\text{Superoxide anion scavenging effect (\%)} = [1 - (A_s/A_c)] \times 100$$

where, A_c is the absorbance of the control and A_s is the absorbance of samples (extracts) or standards[27].

Hydrogen peroxide scavenging activity: The ability of the ethanol and water extracts to scavenge hydrogen peroxide was carried out following the procedure of Ruch *et al*.[28]. For this aim, a solution of H_2O_2 (40 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). Hydrogen peroxide concentration was determined from absorption at 230 nm in a spectrophotometer (Unicam UV2-100). 3.4 mL extract solutions at 50 µg/mL concentration in phosphate buffer were added to 0.6 mL hydrogen peroxide solution. Absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing in phosphate buffer (pH 7.4) without hydrogen peroxide. The percentage of scavenging H_2O_2 of extracts and standard compounds were estimated using the following equation:

$$H_2O_2 \text{ scavenging effect (\%)} = [1-(A_s/A_c)] \times 100$$

where, A_c is the absorbance of the control and A_s is the absorbance in the presence of the samples (extracts) or standards. Triplicate samples were run for each set and averaged.

DPPH$^\bullet$ free radical scavenging activity: The free radical scavenging activity of extracts was measured by 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^\bullet$) using the method of Shimada *et al*.[29]. Solution of DPPH$^\bullet$ (0.2 mM) in ethanol was prepared. Then, 1 mL of this solution was added to 3 mL of extract solutions at 250 µg/mL concentration. The mixtures were shook forcefully and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer (Unicam UV2-100). The absorbance of the DPPH$^\bullet$ control (containing no sample) was also noted like these of samples. Lower absorbance values of the reaction mixtures indicated higher free radical scavenging activities. The DPPH$^\bullet$ concentration (µM) was calculated using calibration curve (R^2 = 0.9895). Triplicate samples were run for each set and averaged[30].

$$\text{Absorbance} = 15.531 \times [\text{DPPH}^\bullet] - 0.0612$$

Ferrous metal ions chelating activity: Metal chelating activity of ferrous ions by the extracts and standards were estimated by the method of Dinis *et al*.[31]. 1 mL $FeCl_2$ solution (2 mM) was added to 5 mL of extract solutions at different concentrations (50-250 µg/mL). The reaction was started by the addition of 0.2 mL ferrozine (5 mM) and the mixtures were shook forcefully and left standing at room temperature for 10 min. After the mixtures had reached equilibrium, the absorbance of the solution was then measured at 562 nm in a spectrophotometer (Unicam UV2-100). The control contains only $FeCl_2$ and ferrozine, complex formation molecules.

The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following equation:

$$Fe^{2+} \text{ chelating effect (\%)} = [1 - (A_s/A_c)] \times 100$$

where, A_c is the absorbance of the control, A_s is the absorbance in the presence of the samples (extracts) and standards. Triplicate samples were run for each set and averaged.

Determination of total phenolic compounds: Total phenolic compounds in the extracts were determined using gallic acid as a standard phenolic compound[32]. Fruit extract solutions (1000 µg/mL) were prepared. Then, each solution was diluted with 46 mL distilled water. 1 mL of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of Na_2CO_3 (2 %) was added and then was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer (Unicam UV2-100). The total concentration of phenolic compounds in the extracts determined as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph (R^2 = 0.9908).

$$\text{Absorbance} = 0.0464 \times \text{total phenols [gallic acid equivalent (µg)]} - 0.0144$$

Determination of total flavonoid contents: The total flavonoid was determined according to colourimetric method[33]. Firstly, each powder product (0.1 g) derived from lyophilized fruit extracts were dissolved in 0.1 mL ethanol or water. This solution (0.1 mL) was placed in a 10 mL volumetric flask. dd H_2O was added to make 5 mL and 0.3 mL 20 % $NaNO_2$ were added. 3 mL 10 % $AlCl_3.6H_2O$ was added 5 min later. After 6 min, 2 mL NaOH (1 M) was added and the total volume was made up to 10 mL with double distilled water. The solution was mixed well again and the absorbance was measured at 510 nm in a spectrophotometer (Unicam UV2-100). Gallic acid was chosen as the standard. Using standard curve, the levels of total flavonoid contents in sample extract were determined in triplicate. The total concentration of flavonoid contents in the extracts determined as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph (R^2 = 0.9968).

$$\text{Absorbance} = 0.0596 \times \text{total flavonoid [gallic acid equivalent (µg)]} - 0.0424$$

Determination of total anthocyanin contents: The total anthocyanin was determined according to reported

methods[34,35]. The dried extract samples (1 g) mixed in 50 mL HCl (1.0 %) and then were centrifugated at 3000 rpm for 10 min (MSE Mistral 2000, UK). Two supernatants tubes (0.2 mL) were prepared with buffer solutions, one with pH 1 buffer and the other with pH 4.5 buffer. The solutions were measured at 520 and 700 nm in a spectrophotometer (Unicam UV2-100). Buffer solutions were used as blank tubes in this experiment.

pH 1 buffer (potassium chloride, 0.025 M): 1.86 g KCl into a beaker and add 980 mL doubly-distilled water. Measure the pH and adjust pH to 1 (± 0.05) with HCl. Transfer to a 1 L volumetric flask and dilute to volume with doubly-distilled water.

pH 4.5 buffer (sodium acetate, 0.4 M): 54.43 g $CH_3COONa \cdot 3H_2O$ in a beaker and add 960 mL doubly-distilled water. Measure the pH and adjust pH to 4.5 (± 0.05) with HCl. Transfer to a 1 L volumetric flask and dilute to volume with doubly-distilled water.

Calculate total anthocyanin contents in the extracts determined as mg/L of cyanidin-3-glucoside (cyd-3-glu) equivalent using the following equation:

Anthocyanin pigment (cyd-3-glu equivalent, mg/L) =

$$\frac{A \times MW_{cyd-3-glu} \times DF \times 10^3}{\varepsilon \times l}$$

where, A = $(A_{520\,nm} - A_{700\,nm})$ pH1.0 - $(A_{520\,nm} - A_{700\,nm})$ pH 4.5, $MW_{cyd-3-glu}$ (molecular weight for cyanidin-3-glucoside) = 449.2 g/mol, DF = dilution factor, l = path length in cm, ε = 26900 molar extinction coefficient for cyd-3-glu ($L \times mol^{-1} \times cm^{-1}$) and 10^3 = factor for conversion from g to mg.

Statistical analysis: Experimental results were given as mean ± S.D. of the three parallel measurements. 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. Both operations were done with SPSS 15.0 for Windows.

RESULTS AND DISCUSSION

Total antioxidant activity: Water and ethanol extracts of blackberry (*Rubus hirtus*) (RH) and herry laurel (*Laurocerasus officinalis*) (LO) fruits demonstrated antioxidant activity. Antioxidant activities were determined by ferric thiocyanate (FTC) method. This method measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of lipid oxidation. In this assay, hydroperoxide produced by linoleic acid added to the reaction mixture, which had oxidized by air during the experimental period, was indirectly measured. Ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate by means of hydroperoxide[36].

The results of activity assays of extracts of fruits after 100 h incubation with linoleic acid emulsion are also summarized as inhibition % in Fig. 1. The percentage inhibition values were calculated at 60 h (Fig. 2). The oxidation of linoleic acid was inhibited by the tested extracts except for extracts in comparison with the control. The different concentration of extracts showed higher antioxidant activities than butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol. Ethanolic extract of *Rubus hirtus* has the most effective inhibition in this part (82.86 %). The other ethanolic extracts of LO exhibited potent antioxidant activity with 74.51 % inhibition. The water extracts of RH and LO demonstrated antioxidant activities with 75.18 % and 80.96 % inhibition, respectively. However, the same concentration of butylated hydroxyanisole, butylated hydroxy toluene and α-tocopherol inhibited lipid peroxidation up to 72.93, 70.93 and 65.46 %, respectively.

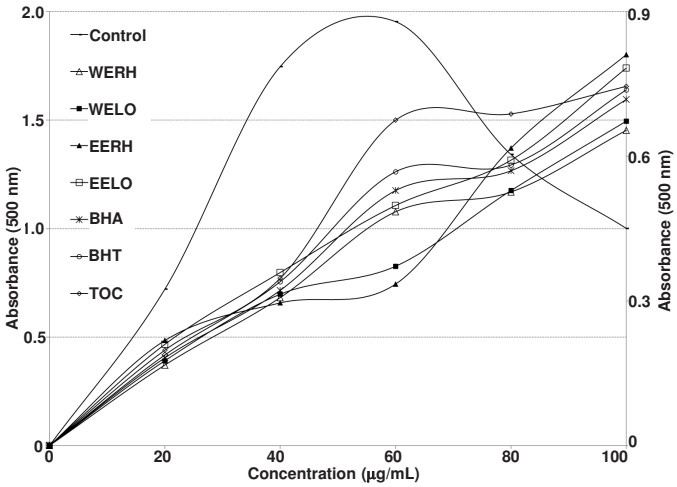

Fig. 1. Total antioxidant activities of extracts and BHA, BHT, α-TOC in the linoleic acid emulsion system by FTC method at the same concentration (50 µg/mL). WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus* officinalis water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract) BHA (butylated hydroxyanisole), BHT: (butylated hydroxytoluene), TOC: (α-tocopherol). Left axis for control and right axis for samples and standards

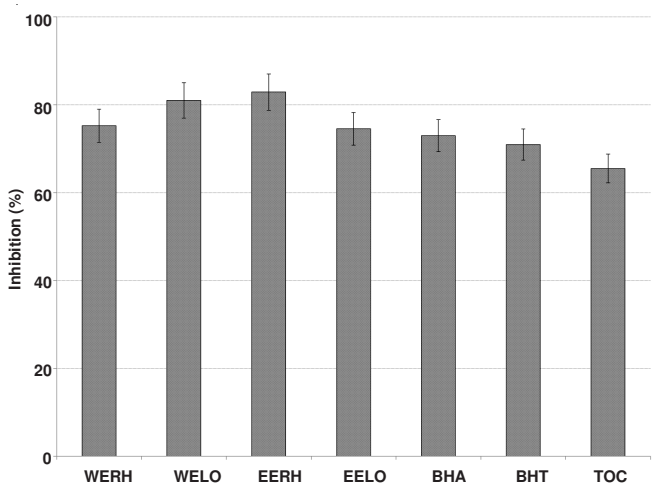

Fig. 2. Inhibition (%) of lipid peroxidation by extracts and BHA, BHT, α-TOC at the same concentration (50 µg/mL) at 60 h. WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus officinalis* water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract) BHA (butylated hydroxyanisole), BHT: (butylated hydroxytoluene), TOC: (α-tocopherol)

Fe^{3+} reducing power capacity: The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[37]. Fig. 3 shows the reductive capability of extracts compared to butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[37]. The reducing power of extracts increased

with increasing amount of sample. All extracts demonstrated efficient reducing power than butylated hydroxy toluene and α-tocopherol. Butylated hydroxyanisole demonsrated higher reductive activity than WELO but this difference was statistically insignificant ($P < 0.01$). Reducing power of extracts and standards exhibited the following order: BHA≈WELO > EELO > WERH > EERH > BHT > α-tocopherol. There are a number of assays designed to measure overall antioxidant activity or reducing potential, as an indication of host's total capacity to withstand free radical stress[38]. FRAP assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant[24]. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

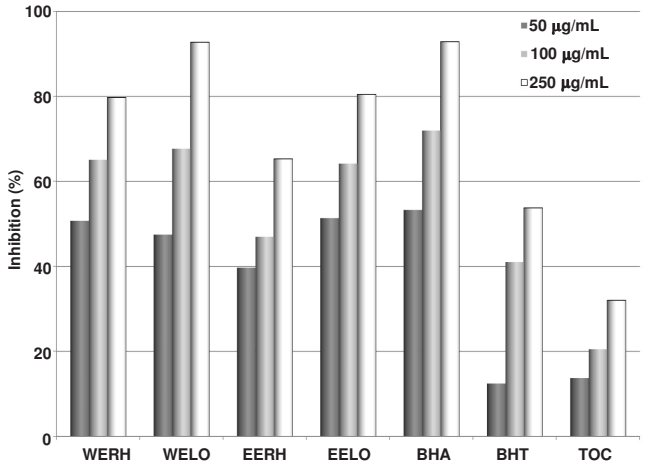

Fig. 3. Reducing power of extracts and BHA, BHT, α-TOC by Oyaizu method on different concentrations (50-250 µg/mL). WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus officinalis* water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract) BHA (butylated hydroxyanisole), BHT: (butylated hydroxytoluene), TOC: (α-tocopherol)

Superoxide anion radical scavenging activity: The primary free radical in most biological systems is superoxide anion radical ($O_2^{\bullet-}$). Although $O_2^{\bullet-}$ itself is quite unreactive compared to the other radicals, the biological systems convert it into more reactive species[39]. $O_2^{\bullet-}$, which is a reduced form of O_2, has been implicated in the initiating oxidation reactions associated with aging[40]. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. Fig. 4 presented the superoxide anion radical scavenging activity of extracts and was compared with the same dose of known antioxidants butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol. All of the extracts had strong superoxide anion radical scavenging activity than standard antioxidants ($P < 0.05$). Superoxide radical scavenging activity of 50 µg/mL concentration of those samples followed the order: WELO > WERH > EERH > EELO > BHA > BHT > α-tocopherol. Inhibition values are found 94.50, 93.33, 88.00, 85.17, 83.33, 81.33 and 65.50%, respectively.

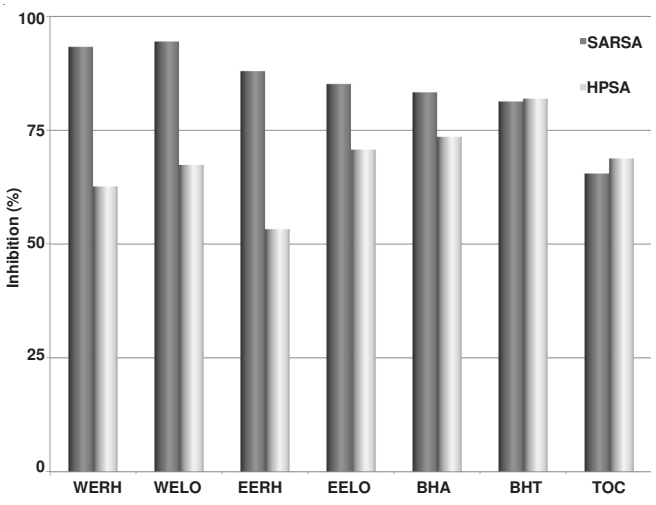

Fig. 4. Superoxide anion radical scavenging activities (SARSA) and H2O2 scavenging activities (HPSA) of extracts and BHA, BHT and α-TOC at the same concentration (50 µg/mL). WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus officinalis* water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract) BHA (butylated hydroxyanisole), BHT: (butylated hydroxytoluene), TOC: (α-tocopherol)

Hydrogen peroxide scavenging activity: Hydrogen peroxide is also produced in biological systems and it is not very reactive. However, it can be toxic to cells sometimes because it may give rise to $OH^{\bullet}$ within the cells. Addition of hydrogen peroxide to cells in culture can lead to transition metal ion-dependent $OH^{\bullet}$ mediating oxidative DNA damage. Thus, removing hydrogen peroxide as well as superoxide anion, is very important for protection of pharmaceuticals and food systems[41]. The scavenging ability of extracts on H_2O_2 is shown in Fig. 4 and compared with that of butylated hydroxyanisole, butylated hydroxy toluene and α-tocopherol as standards. Extracts were capable of scavenging H_2O_2 in a concentration-dependent manner. These results showed that both extracts had stronger H_2O_2 scavenging activity. On the other hand, extracts are not effective as standards exclusive of EELO. The H_2O_2 scavenging activity of 50 µg/mL concentration of the both extracts and standards decreased in the order of BHT (81.95) > BHA (73.57) > EELO (70.75) > α-tocopherol (68.80) > WELO (67.38) > WERH (62.67) > EERH (53.27). H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to $OH^{\bullet}$ in the cells[42].

DPPH free radical scavenging activity: The effect of antioxidants on $DPPH^{\bullet}$ radical scavenging is thought to be due to their hydrogen or electron donating abilities. $DPPH^{\bullet}$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[43]. $DPPH^{\bullet}$ 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. When a hydrogen atom or electron was transferred to the odd electron in $DPPH^{\bullet}$, the absorbance at 517 nm decreased proportionally to the increases of non-radical forms of DPPH[44]. Fig. 5 illustrates a significant ($P < 0.05$) decrease in the concentration of $DPPH^{\bullet}$ due to the scavenging ability of extracts and standards. The scavenging effects of extracts and standards on the $DPPH^{\bullet}$ decreased in that order:

BHA > BHT > WELO > WERH > EELO > EERH > α-tocopherol, which were 94.11, 88.73, 88.28, 86.92, 85.82, 83.61 and 77.07 %, respectively. DPPH• scavenging activity also increased with increasing concentration.

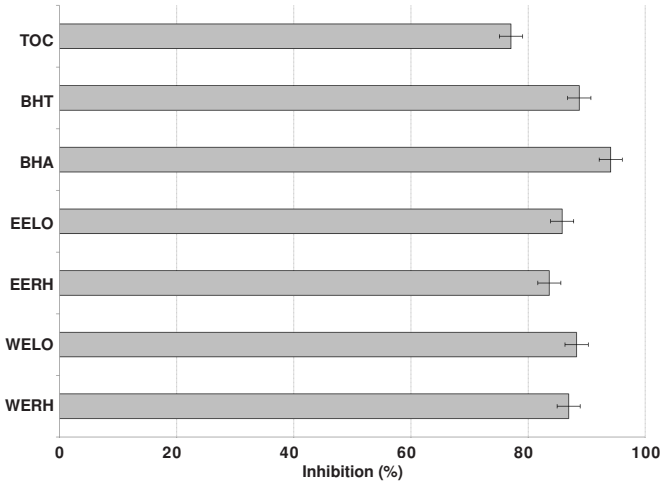

Fig. 5. Comparison of DPPH radical scavenging activities of extracts and BHA, BHT and α-TOC at the same concentration (250 µg/mL). WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus officinalis* water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract) BHA (butylated hydroxyanisole), BHT: (butylated hydroxytoluene), TOC: (α-tocopherol)

Ferrous ions chelating activity: Metal chelating capacity is important since it reduced the concentration of the catalyzing transition metal in lipid peroxidation[22]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents the complex 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. Lower absorbance indicates higher metal chelating activity. Metal chelation is an important antioxidant property[45]. In this assay, fruit extracts interfered with the formation of ferrous and ferrozine complex suggesting that they have chelating activity and are capable of capturing ferrous ion before ferrozine. As shown in Fig. 6, the formation of the ferrous and ferrozine complex is not complete in the presence of extracts indicating both extracts chelate with the Fe^{2+}. The difference between 100 and 250 µg/mL was statistically insignificant ($P > 0.05$). The metal chelating activity of both extracts and standards decreased in the order of α-tocopherol > BHA > WELO > WERH > EERH > EELO > BHT.

Total phenolic contents: Phenolic compounds are very important plant constituents because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, free radicals and hydroxyl radicals[46,47]. In our investigation, 161.63 to 261.13 µg gallic acid equivalent of phenols was detected in 1 mg of dried extracts. Table-1 shows total phenols as gallic acid equivalent in both extracts.

These results indicate that there is no correlation between antioxidant activity and total phenolic content. However, different results were reported on this aspect. Some authors found correlation between phenolic content and antioxidant activity, whereas the others found no such relationship, since other compounds are responsible for the antioxidant activity[48,49]. The phenolic compounds may contribute directly to antioxidative action[22]. The high antioxidant activity was not correlated with the phenol content, probably other factors played major roles as antioxidants[50].

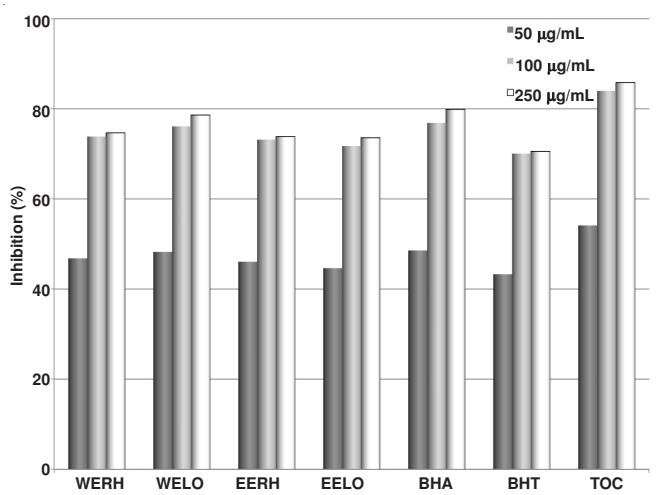

Fig. 6. Metal chelating activities of extracts and BHA, BHT and α-TOC on ferrous ions at different concentrations (50-250 µg/mL). WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus officinalis* water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract) BHA (butylated hydroxyanisole), BHT: (butylated hydroxytoluene), TOC: (α-tocopherol)

Total flavonoid contents: Flavonoids are a group of interesting compounds that not only give fruits and vegetables various red, blue, or violet colours, but also are related to the group of bioactive compounds called stilbenes[51]. Flavonoids are very important plant constituents because of active hydroxyl groups and show antioxidant activity[52]. The contents of flavonoid in the extracts, determined from the regression equation of the calibration curve and expressed in gallic acid equivalents, varied from 34.54 to 55.77 µg gallic acid equivalent of flavonoid was detected in 1 mg of dried extracts (Table -1).

TABLE-1
TOTAL PHENOL, FLAVONOID AND ANTHOCYANINS CONTENTS

	Total phenols[a]	Total flavonoids[a]	Total anthocyanins[b]
WERH	238.19 ± 8.02	44.15 ± 1.13	14.36 ± 0.93
WELO	261.13 ± 6.58	55.17 ± 2.25	14.7 ± 0.88
EERH	180.58 ± 5.04	34.54 ± 1.08	16.36 ± 0.91
EELO	161.63 ± 4.10	37.10 ± 1.29	13.19 ± 0.82

[a]Microgram/milligram gallic acid equivalent; [b]Milligram/L cyanidin-3-glucoside equivalent; WERH: (*Rubus hirtus* water extract), WELO: (*Laurocerasus officinalis* water extract), EERH: (*Rubus hirtus* ethanolic extract), EELO: (*Laurocerasus officinalis* ethanolic extract)

Total anthocyanin contents: The anthocyanins constitute a major flavonoid group that is responsible for cyanic colours ranging from salmon pink through red and violet to dark blue of most flowers, fruits and leaves of angiosperms. Regular consumption of anthocyanins and other polyphenols in fruits, vegetables, wines, jams and preserves is associated with probable reduced risks of chronic diseases such as cancer, cardiovascular diseases, virus inhibition and Alzheimer's disease.

Anthocyanins and other flavonoids are regarded as important nutraceuticals mainly due to their antioxidant effects, which give them a potential role in prevention of the various diseases associated with oxidative stress. However, flavonoids have further been recognized to modulate the activity of a wide range of enzymes and cell receptors[53].

Total anthocyanin contents in extracts ranged from 13.19 to 16.36 mg/L as cyanidin 3-glucoside equivalents (Table-1). It is to be expected that several activities might be related to a possible antioxidant action from anthocyanosides like polyphenol compounds[54]. Polyphenols have been found to be one of the most effective antioxidant constituents in plant foods, including fruits, vegetables and grains[50].

Conclusion

We can confirm that our results clearly show that both extracts had strong antioxidant activity, reducing power, superoxide anion radical scavenging, hydrogen peroxide scavenging, free radical scavenging and metal chelating activities when compared with natural and synthetic standard antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene and α-tocopherol. The various antioxidant mechanisms of RH and LO may be attributed to effective hydrogen or electron donating abilities, a metal chelating ability and their effectiveness as good scavengers of hydrogen peroxide, superoxide and free radicals. In addition, phenolic compounds appear to be responsible for the antioxidant activities of RH and LO. Especially the reducing power of RH and LO were excellent and increased steadily with the increasing concentration. In general, the greater reducing power and metal chelating activity of one antioxidant compound may relate to its marked antioxidant activity. These results obtained from this study show that RH and LO can be used as easy accessible source of natural antioxidants as a possible food supplement or in pharmaceutical and medical industry. However, the components responsible for the antioxidant activity of extracts are currently unclear. Therefore, it is suggested that further work could be done on the isolation and identification of the antioxidative components especially anthocyanins in RH and LO.

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REFERENCES

1. B. Halliwell and J.M.C. Gutteridge, *Biochem. J.*, **219**, 1 (1984).
2. P.A. Lachance, Z. Nakat, and W.S. Jeong, *Nutrition*, **17**, 835 (2001).
3. M. Sato, N. Ramarathnam, Y. Suzuki, T. Ohkubo, M. Takeuchi and H. Ochi, *J. Agric. Food Chem.*, **44**, 37 (1996).
4. B. Halliwell and J.M.C. Gutteridge, Free Radicals in Biology and Medicine, Clarendon Press, Oxford, p.23 (1989).
5. M.E. Büyükokuroglu, I. Gülçin, M. Oktay and Ö.I. Küfrevioglu, *Pharmacol. Res.*, **44**, 491 (2001).
6. J. Kehrer, *Crit. Rev. Toxicol.*, **23**, 21 (1993).
7. L. Packer and A. Glazer, Methods in Enzymology: Oxygen Radicals in Biological Systems, Part B: Oxygen Radicals and Antioxidants, Academic Press, New York, vol. 186, 371 (1990).
8. M. Wettasinghe and F. Shahidi, *Food Chem.*, **70**, 17 (2000).
9. H.E. Miller, F. Rigelhof, L. Marquart, A. Prakash and M. Kanter, *J. Am. College Nutr.*, **19**, 312 (2000).
10. G.C. Yen and P.D. Duh, *J. Agric. Food Chem.*, **42**, 629 (1994).
11. I.M.C. Brighente, M. Dias, L.G. Verdi and M.G. Pizzolatti, *Pharm. Biol.*, **45**, 156 (2007).
12. A. Gazda, J. Szwagrzyk, H. Nybom and G. Werlemark, *Pol. J. Ecol.*, **55**, 49 (2007).
13. I. Pesin, U. Koca, H. Keles and E.K. Akkol, *Evidence Based Compl. Alt. Med.*, **2011**, 1 (2011).
14. H. Jouad, M. Maghrani and M. Eddouks, *J. Ethnopharmacol.*, **81**, 351 (2002).
15. M.A. Marquina, G.M. Corao, L. Araujo, D. Buitrago and M. Sosa, *Fitoterapia*, **73**, 727 (2002).
16. L. Panizzi, C. Caponi, S. Catalano, P.L. Cioni and I. Morelli, *J. Ethnopharmacol.*, **79**, 165 (2002).
17. C. Alasalvar, M. Al-Farsi and F. Shahidi, *J. Food Sci.*, **70**, 47 (2005).
18. T. Baytop, Therapy with Plants in Turkey, Istanbul University Publ., Istanbul, pp. 226-227 (1984).
19. F. Akdeniz and M. Gündogdu, *Energ. Convers. Manage.*, **48**, 189 (2007).
20. C.M. Liyana-Pathirana, F. Shahidi and C. Alasalvar, *Food Chem.,* **99**, 121 (2006).
21. P.D. Duh and G.C. Yen, *Food Chem.*, **60**, 639 (1997).
22. P.D. Duh, Y.Y. Tu and G.C. Yen, *LWT-Food Sci. Technol.*, **32**, 269 (1999).
23. M. Oyaizu, *Jap. J. Nutr.*, **44**, 307 (1986).
24. I.F.F. Benzie and J.J. Strain, *Anal. Biochem.*, **239**, 70 (1996).
25. Y.C. Chung, C.T. Chang, W.W. Chao, C.F. Lin and S.T. Chou, *J. Agric. Food Chem.*, **50**, 2454 (2002).
26. I. Gülçin, M. Oktay, Ö.I. Küfrevioglu and A. Aslan, *J. Ethnopharmacol.*, **79**, 325 (2002).
27. X.Y. Ye, H.X. Wang, F. Liu and T.B. Ng, *Int. J. Biochem. Cell Biol.*, **32**, 235 (2000).
28. R.J. Ruch, S. Cheng and J.E. Klaunig, *Carcinogenesis*, **10**, 1003 (1989).
29. K. Shimada, K. Fujikawa, K. Yahara and T. Nakamura, *J. Agr. Food Chem.*, **40**, 945 (1992).
30. M. Oktay, I. Gülçin and Ö.I. Küfrevioglu, *LWT-Food Sci. Technol*., **36**, 263 (2003).
31. T.C.P. Dinis, V.M.C. Madeira and L.M. Almeida, *Arch. Biochem. Biophys.*, **315**, 161 (1994).
32. K. Slinkard and V.L. Singleton, *Am. J. Enol. Viticult.*, **28**, 49 (1977).
33. J. Zhishen, T. Mengcheng and W. Jianming, *Food Chem.*, **64**, 555 (1999).
34. T. Fuleki and F.J. Francis, *J. Food Sci.*, **33**, 72 (1968).
35. J. Lee, R.W. Durst and R.E. Wrolstad, *J. AOAC Int.*, **88**, 1269 (2005).
36. R. Inatani, N. Nakatani and H. Fuwa, *Agric. Biol. Chem.*, **47**, 521 (1983).
37. S. Meir, J. Kanner, B. Akiri and S.P. Hadas, *J. Agric. Food Chem.*, **43**, 1813 (1995).
38. L.G. Wood, P.G. Gibson and M.L. Garg, *J. Sci. Food Agric.*, **86**, 2057 (2006).
39. C.C. Winterbourn and A.J. Kettle, *Biochem. Biophys. Res. Commun.*, **305**, 729 (2003).
40. P. Cos, L.Y. Ying, M. Calomme, J.H. Hu, K. Cimanga, B. Van Poel, L. Pieters, A.J. Vlietinck and D.V. Berghe, *J. Nat. Prod.*, **61**, 71 (1998).
41. I. Gülçin, *Innov. Food Sci. Emerg.*, **11**, 210 (2010).
42. B. Halliwell, *Am. J. Med.*, **91**, 14 (1991).
43. J.R. Soares, T.C.P. Dinis, A.P. Cunha and L.M. Almeida, *Free Radic. Res.*, **26**, 469 (1997).
44. J. Ancerewicz, E. Migliavacca, P.A. Carrrupt, B. Testa, F. Bree, R. Zini, J.P. Tillement, S. Labidelle, D. Guyot, A.M. Chauvet-Monges, A. Crevat and A. Le Ridant, *Free Radic. Biol. Med.*, **25**, 113 (1998).
45. J.P. Kehrer, *Toxicology*, **149**, 43 (2000).
46. C.A. Hall and S.L. Cuppett, in eds.: O.I. Auroma and S.L. Cuppett, Structure-activities of natural antioxidants. Antioxidant Methodology *in vivo* and *in vitro* Concepts, AOCS Press, Champaign, pp. 141-170 (1997).
47. T. Hatano, R. Edamatsu, A. Mori, Y. Fujita and E. Yasuhara, *Chem. Pharm. Bull.*, **37**, 2016 (1989).
48. J.H. Yang, H.C. Lin and J.L. Mau, *Food Chem.*, **77**, 229 (2002).
49. M.N. Maillard and C. Berset, *J. Agric. Food Chem.*, **43**, 1789 (1995).
50. Y.S. Velioglu, G. Mazza, L. Gao and B.D. Oomah, *J. Agric. Food Chem.*, **46**, 4113 (1998).
51. Y.H. Hui, Food Biochemistry and Food Processing, Blackwell Publ., Iowa, USA, pp.16-17 (2006).
52. P.S. Kumar, S. Sucheta, V.S. Deepa, P. Selvamani and S. Latha, *Afr. J. Biotechnol.*, **7**, 1826 (2008).
53. Ø.M. Andersen and K.R. Markham, Flavonoids: Chemistry, Biochemistry and Applications, CRC Press, Broken Sound Parkway, NW, pp. 472-473 (2006).
54. L.S. Einbond, K.A. Reynertson, X.D. Luo, M.J. Basile and E.J. Kennelly, *Food Chem.*, **84**, 23 (2004).