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Investigation of antioxidant activity of some fruit stem barks in the Eastern Black Sea Region

Doğu Karadeniz Bölgesi' ndeki bazı meyve ağaçlarının antioksidan aktivitelerinin karşılaştırılması

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Abstract
Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Antioxidant activity can be investigated by using different methods such as total antioxidant activity, hydrogen peroxide and DPPH free radical scavenging activities, metal-chelating activity, total phenolic and flavonoid contents and others. In this study, antioxidant activity of the ethanol-water extracts of three stem barks, Kiwi (*Actinidia chinensis* Planch.) (AC), lemon (*Citrus limon* (L.) Burm. f.) (CL) and chery laurel (*Laurocerasus officinalis* Roem.) (LO) has been designated. According to FTC method, the total antioxidant activities (%) of AC, CL and LO have been determined as 73.35, 67.59 and 61.62, respectively. The DPPH radical scavenging activities of AC, CL, LO, BHA, RUT and TRO in terms of SC_{50} values (µg/mL) were found as 50.52, 56.56, 98.18, 8.58, 17.01, 26.84, respectively. Total phenolic and total flavonoid contents in AC, CL and LO ranged from 850.71 to 457.79 µg gallic acid equivalent/g and 58.77 to 22.91 µg of catechin equivalents/g, respectively. In conclusion, the extracts of AC showed higher antioxidant activity than the other samples so needs further exploration for its effective use in pharmaceutical and medicine sectors.

Özet
Besinlerde bulunan antioksidanlar sağlığımızı koruyucu faktör olarak önemli rol oynarlar. Bilimsel kanıtların önerdiği gibi antioksidanlar kanser ve kalp hastalıklarını içeren kronik hastalıklar için riski azaltırlar. Doğal yollarla oluşan antioksidanların başlıca kaynakları tohumlar, meyveler ve sebzelerdir. Antioksidan aktivite, toplam antioksidan aktivite, hidrojen peroksit ve DPPH radikal giderme aktiviteleri, metal-şelatlama aktivitesi, toplam fenolik ve flavonoid içerikleri ve diğer metotlar kullanılarak incelenebilir. Bu çalışmada, üç ağaç kabuğunun (kivi (*Actinidia chinensis* Planch.) (AC), limon (*Citrus limon* (L.) Burm. f.) (CL) and taflan (*Laurocerasus officinalis* Roem.) (LO)) etanol-su sistemi ektraktlarının antioksidan aktiviteleri belirlendi. FTC metoduna göre, AC, CL ve LO' nun toplam antioksidan aktiviteleri (%) sırasıyla 73.35, 67.59 ve 61.62 olarak belirlendi. AC, CL, LO, BHA, RUT ve TRO' nun DPPH radikal giderme aktiviteleri SC_{50} değerleri (µg/mL) cinsinden sırasıyla 50.52, 56.56, 98.18, 8.58, 17.01, 26.84 olarak bulundu. AC, CL ve LO' nun toplam fenolik ve toplam flavonoid içerikleri sırasıyla 850.71 - 457.79 µg gallik asit ekivalent/g ve 58.77 - 22.91 µg of kateşin ekivalent/g aralığında değişmektedir. Sonuç olarak, AC ekstraktı diğer örneklerden daha yüksek antioksidan aktivite göstermiştir. Bu nedenle eczacılık ve tıp sektöründe daha etkili kullanmak için ileri araştırmalar gerekmektedir.

INTRODUCTION

Many fruit kinds are grown in Eastern Black Sea Region in Turkey, but kiwi, lemon and chery laurel are commonly known. The genus *Actinidia*, commonly known as kiwifruit, includes several economically important horticultural species such as *A. chinensis* Planch., *A. deliciosa*, *A. arguta* and *A. Eriantha* (Chat et al. 2004). Nearly, 54 species and 75 taxa were identified in *Actinidia* (Li et al. 2007). The kiwi fruit is known as "the king of fruits" by reason of its crucially high vitamin C content and other benefits for health. Many studies in kiwifruits metabolites and its antioxidant activity have been performed previously (Cassano et al. 2006; Kaya et al. 2010; Singletary 2012). The origin centre of kiwifruit is in the mountains and ranges of southwestern China. Although kiwifruit has short history of enculturation, it was started in New Zealand in the early 20th century by introducing its seeds. By way of decades of enculturation and substantial efforts for selection from wild kiwifruits, a lot of varieties have been developed so it has been seen as an important fresh fruit worldwide (Huang et al. 2013).

Citrus limon (L.) Burm. f. (Lemon) is an crucial medicinal plant of Rutaceae family. Especially, it is valuable with alkaloids in it. Because they have important biological activities such as anticancer, antioxidant and antibacterial activities in crude extracts (Kawaii et al. 2000; Akhila et al. 2009; Campelo et al. 2011).

Laurocerasus officinalis Roem. which belongs to the Rosaceae family is a popular fruit and mainly distributed in the coasts of the Black Sea region of Turkey. It is locally called Taflan or Karayemis. This fruit is commonly used as vegetables and medicinal fruits and an important fruit in Turkey. Its fruits are usually consumed as fresh by the community but sometimes can be dried, pickled and processed as pekmez, jam, marmalade and fruit juice. In addition, its fruit and seeds have been used for the treatment of ailments such as stomach ulcers, digestive system complaints, bronchitis, eczemas, haemorrhoids and diuretic agent in Turkey for many years. Different parts of this plant have been utilized for various purposes for example its leaves are known as anti-spazmodics, narcotics and sedative chemicals. Also, fruits and leaves have been used in the cosmetic and dye sectors. And also these species have antioxidant properties (Kolaylı et al. 2003; Güder and Korkmaz 2012).

The aim of this paper is to determine the antioxidant activities of stem bark extracts (AC, CL and LO) by using different antioxidant tests. Six different antioxidant activity assays were used in the experiments. Inhibition of the lipid peroxidation measurement, ferric thiocyanate (FTC) method has been performed. Hydrogen peroxide and radical scavenging activities are carried out by widely used methods in the literature. Metal chelating activities were applied, as excess free metals have been implicated in the induction and formation of free radicals. Finally, phenolic and flavonoid compounds are widespread in plantae where they act as antioxidants and free radical scavengers. The results can guide to medicinal purposes as natural source.

MATERIAL and METHODS

Plant material and extraction

Plant materials were collected in 2015 from Giresun (Centrum) and authenticated by Assist. Prof. Dr. İlginç KIZILPINAR TEMİZER from Giresun University. Localities were presented in Table 1. Stem barks were left on a bench to dry. The dried samples were carved into small parts via blender. All samples were extracted using by magnetic stirrer in ethanol and double-distilled water system (1:1, 3x) at 40 ^{o}C. Then, the extracts were filtered over Whatman No.1 paper. The filtrates were combined, frozen and lyophilized by using a lyophilizator. The final extracts were put into plastic vessel, and stored at -20^{o}C.

Table 1. Localities of collected fruit stem barks

Species	Locality Name
Actinidia chinensis Planch	Giresun Center, Gedikkaya, 200 m
Citrus limon (L.) Burm. f	Giresun, Espiye District, 50 m
Laurocerasus officinalis Roem	Giresun Center, Güre, 40 m

Investigation of inhibition of lipid peroxidation

Total antioxidant activities of extracts were investigated using ferric thiocyanate (FTC) method described by Mitsuda et al. (Mitsuda et al. 1996). 2.5 mL extract and standard solutions (50 µg/mL) in potassium phosphate buffer (PBS) (0.04 M, pH = 7.0) was mixed to 2.5 mL linoleic acid emulsion in PBS (0.04 M, pH = 7.0). 2.5 mL linoleic acid emulsion and 2.5 mL PBS mixture was used as control tube. All mixtures were incubated at 37 ºC 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 and the percentage of inhibition was determined. Lipid peroxidation inhibition values were calculated by the following equation:

$$Percentage\ inhibition\ lipid\ peroxidation = [1 - (A_{sample(or\ standard)}/A_{control})] \times 100$$

Determination of DPPH Free Radical Scavenging Activity

The extract solutions (5-100 µg/mL) were prepared with ethyl alcohol. DPPH free radical scavenging activities were examined with reference to Blois method (1958). 3.0 mL sample solutions and 1.0 mL DPPH$^{\bullet}$ (0.1 mM) prepared in absolute ethanol was mixed and then vortexed. It was kept at RT in a dark environment. 30 min later, the absorbance values were measured at 517 nm. Free radical scavenging activities were calculated by using absorbance estimations 30 min later and compared with standard antioxidants. The decrease in absorbance estimation is a demonstration of high rate of free radical scavenging activity. The free radical scavenging activity of the sample is expressed as SC_{50} (µg/mL).

Hydrogen Peroxide Scavenging Activity Determination

The hydrogen peroxide scavenging activities of the samples used in the study were done according to Ruch

method (1989). In this experiment, 3.4 mL was taken from the sample (5-100 µg/mL), and 0.6 mL 40 mM H_2O_2 (prepared with 0.04 M phosphate buffer (pH =7.4)) was added. After 10 min the absorbance of the mixture was measured at 230 nm compared to a blank sample. Phosphate buffer (0.04 M, pH=7.4) which does not include hydrogen peroxide solution was used as blank sample. Decreasing in the absorbance value shows that hydrogen peroxide scavenging activity of this sample is high. Hydrogen peroxide scavenging activity of sample is expressed as SC_{50} values (µg/mL) and the obtained result compared with standart antioxidants.

Determination of Metal Chelating Activity

The metal chelating activity of the solution of extracts and the standard antioxidant materials prepared with absolute ethanol was determined according to the Dinis Method (1994). 0.4 mL was taken from both the solution of extract and each of the standard antioxidant materials. The samples were let sit for 10 min with 0.05 mL $FeCl_2$ (2 mM). Then they were filled to 4 mL by adding 0.2 mL ferrozine (5 mM) and absolute ethanol. These mixtures were kept at room temperature for 10 min. They were shaken forcefully and the absorbance of the mixtures was measured at 562 nm. A decrease in absorbance estimation demonstrates a high level of metal chelating activity of extracts and standard antioxidant materials. The metal chelating activities of the extract and the standard antioxidant material were calculated according to the formula below:

Ferrous Ions Chelating Activity (%) = [1 − (A_s/A_c)] × 100

A_c: control's absorbance values; A_S : extract's or standard material's absorbance values.

The measurement of total phenolic content

Amount of total phenolic compound in the extracts were determined according to the Slinkard and Singleton method (1977) using Folin-Ciocalteu reactive. A sample was taken in ethyl alcohol solution (1 mg/mL, 0.5 mL) and then deionized water (7.0 mL). 0.5 mL Folin C reagent was added and the content of the tube mixed thoroughly. After 3 minutes, Na_2CO_3 (2.0%, 2.0 mL) was added and the sample was let sit at room temperature and was shaken occasionally for 2 h. The absorbance of mixtures was measured at 760 nm. Total phenolic contents of the samples were calculated with the gallic acid calibration curve (R^2:0.9998).

The measurement of total flavonoid content

Total flavonoid contents of extracts were measured according to the aluminium chloride colorimetric method (Chung *et al.* 2002). Ethyl alcohol solution of the samples (1 mg/mL, 0.5 mL) were taken and deionised water (1.5 mL) was added. Then, $A1C1_3.6H_2O$ (10.0%, 0.1 mL) and 1 M potassium acetate (0.1 mL) were added and was diluted using 2.8 mL deionized water. After it was incubated at room temperature for 30 min, its absorbance was immediately measured at 415 nm. The sample's total flavonoid contents were calculated with the the catechin calibration (R^2:0.9976).

RESULTS-DISCUSSION

Actinidia chinensis, Citrus limon and *Laurocerasus officinalis* are widely grown in the Eastern Blacksea region of Turkey. Their fruits are used as food, supplement, preserver for food etc because of the vitamin C in them (Selman, 1983; Kolaylı et al., 2003, Nishiyama et al., 2004). In the ferric thiocyanate (FTC) assay, hydroperoxide that was formed by using linoleic acid emulsion, which has been oxidized via air oxygen during the experimental period was indirectly determined by the reaction between ferrous chloride and thiocyanate by means of hydroperoxide (Inatani et al. 1983). The percentage inhibition values were calculated at 72 h (Fig. 1). In reference to total antioxidant activity results, AC shows higher activity than the other samples. The inhibition of lipid peroxidation activity results (%) decrease as follow BHA (81.68) > TRO (80.21) > RUT (78.22) > AC (73.35) > CL (67.59) > LO (61.62). Boni et al. (2014) found water and methanol extracts of stems bark of *Spondias mombin* as 56.21 ± 1.22 and 65.78 ± 2.00 %, respectively. In addition, Noorhajati et al. (2012) studies three different extracts of *Cassia fistula* L. and inhibition of lipid peroxidation values of acetate, methanol and n-hexane extract were found as 65.98, 58.19 and 32.66%, respectively. When obtained results compare with these values, our AC and CL results are higher than others.

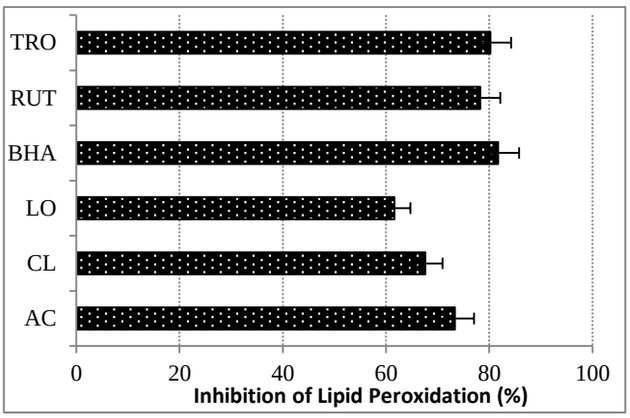


Figure 1. Comparison of antioxidant activity of samples and standards

Hydrogen peroxide is produced in biological systems. Although it is not very reactive, it can be sometimes toxic to cells due to the higher $OH^{\bullet}$ concentration. Also, increasing hydrogen peroxide levels to cells in culture can bring about oxidative DNA damage. Therefore, scavenging hydrogen peroxide is very vital for protection of pharmaceuticals and food systems (Gülçin 2010). Hydrogen peroxide scavenging activity increase depend on the concentration and obtained SC_{50} values (µg/mL) present in Table 2. Subramanian et al. (2013) found the hydrogen peroxide activity IC_{50} values of *Shorea roxburghii* stem bark for acetone and methanol extracts as 87.18 and 63.67 µg/mL, respectively. In respect to activity, these results are lower than AC, CL and LO.

Table 2. Hydrogen peroxide scavenging activities of samples and standards.

AC	CL	LO	BHA	RUT	TRO
208.36	218.36	228.14	194.92	124.05	447.55

When antioxidants and $DPPH^{\bullet}$ radical interact, antioxidant molecules give hydrogen or electron to DPPH radical so it changes stable non-radical form as DPPH-H (Soares et al. 1997). The SC_{50} values (µg/mL) were found as 50.52, 56.56, 98.18, 8.58, 17.01, 26.84 for AC, CL, LO, BHA, RUT and TRO, respectively. Boni et al. (2014) found DPPH radical scavenging activities (IC_{50} values) of water and methanol extracts of *Spondias mombin* stems bark as 10.33 ± 1.09 and 5.83 ± 0.88 µg/mL, respectively. Duganath et al. (2010) studied DPPH radical scavenging activity (IC_{50} values) of ethanolic extracts of *Filicium decipiens* stem and its activity was found 2300 µg/mL. AC, CL, LO were found to have radical scavenging activities at higher concentrations compared with standards. In addition, our samples show considerably higher activity than *Filicium decipiens* stem.

In metal chelating activity assays, ferrozine and Fe^{2+} form strong red complex. In the presence of other chelating agents, the red color intensity of complex distrupted and new complex forms between chaleting agents and Fe^{+2} (Güder and Korkmaz 2012). Metal chelating activity were changed as in Figure 3. At the 1000 µg/mL concentration, Krishnappa et al. (2014) found petroleum ether, chloroform and ethanol extracts of *Delonix elata* L. stem bark extracts as 0.47±0.03, 7.51±0.39 and 27.29±0.29, respectively. Related study results are excessive lower than AC, CL, LO' s chelating activities.

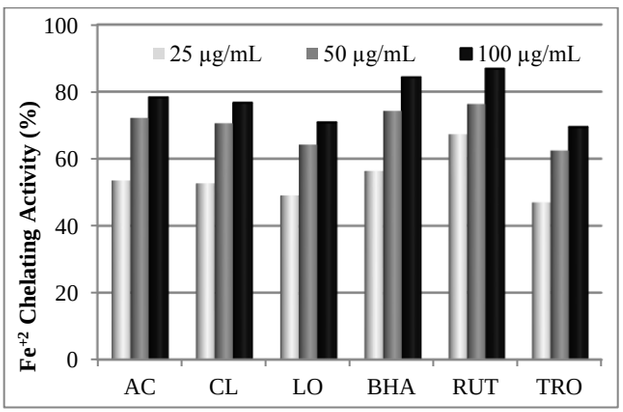


Figure 2. Metal chelating activities of samples and standards at the different concentrations.

Phenolic compounds shows antioxidant activity owing to their free radical and active oxygen species scavenging activities (Hall and Cuppett 1997). Flavonoids which are very important plant compounds have active hydroxyl groups that cause antioxidant activity (Kumar et al. 2008). According to our results, the gallic acid equivalents of total phenolic contents of extracts were found 850.71, 716.40 and 457.79 µg GAE/g for AC, CL and LO, respectively. Total flavonoid contents were found as 58.77, 43.30 and 22.91 µg of catechin equivalents/g for AC, CL and LO, respectively. Boni et al. (2014) studied total phenol and flavonoid contents of water and methanol extracts of *Spondias mombin* stems bark. Total phenol contents of water and methanol extracts ranged from 183.5±1.89 to 343.5±6.44 mg GAE/g of dry plant. Also, total flavonoid contents of water and methanol extracts were found as 7.48±0.19 and 11.28±0.45 mg QE/g of dry plant, respectively. Subramanian et al. (2013) investigated the

total phenolic contents of *Shorea roxburghii* stem bark for acetone and methanol extracts as 65.74±8.70 and 67.67±4.90 µg GAE/mL, respectively. Krishnappa et al. (2014) found total phenolic contents of petroleum ether, chloroform and ethanol extracts of *Delonix elata* L. stem bark extracts as 7.85±0.01, 45.71±0.25 and 77.75±0.05 μg GAE/mg extract, respectively. As to flavonoid contents are 0.16±0.35, 61.50±1.16 and 75.33±0.67 μg QE/mg extract, respectively.

CONCLUSION

Medicinal plants or their parts are used as primary source for the alternative medicine. They have numerous advantages because of the fewer side effects. Stem barks can be helpful for the treatment of various diseases due to the presence of antioxidant components in them. When these activities compared with the standard antioxidants, they demonsrate very efficient activity. Thereby, the obtained results show that three stem barks can be used as source of natural antioxidants in medical and food industry. In the future studies, active components could be isolated and characterized. Also, their quantities can be detected by using chromatography methods.

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