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INVESTIGATION OF ANTIOXIDANT ACTIVITY OF *Sorbus domestica* L. EXTRACTS AND DETERMINATION OF PHENOLIC CONTENTS BY LC-MS/MS

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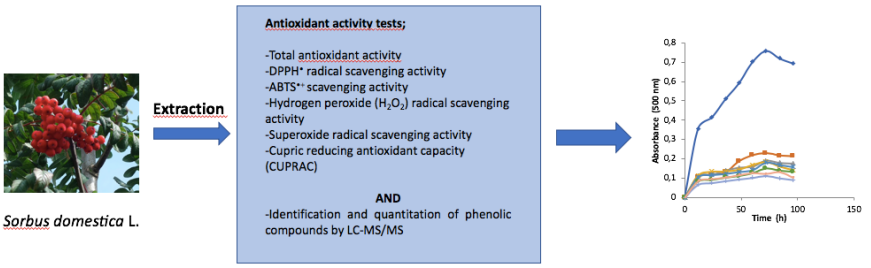
In this work, antioxidant activity of the extract *Sorbus domestica* L. was investigated using different methods. Phenolic contents were assessed using liquid chromatography-tandem mass spectroscopy (LC-MS/MS) technique. Antioxidant activity of *S. domestica* L. for different radical and compound was assessed. As a result, mountain *S. domestica* L. water extract showed the best antioxidant effect among other plant extracts tested. According to total antioxidant determination by thiocyanate method, water extract has the highest total antioxidant activity (23.54 mg ascorbic acid g^{-1} extract) compared to other extracts. The concentrations of the extracts providing 50% inhibition (IC_{50} value) of 2,2'-azino-bis(3-ethylbenzothioazoline-6-sulphoic acid) diammonium salt ($ABTS^{\bullet+}$) radical scavenging activity are between 9.14±0.50 and 10.97±0.25 µg mL^{-1}. It was also seen that extracts exhibit good antioxidant activity; therefore, it can be claimed that *S. domestica* L. can be used as natural antioxidant source.



INTRODUCTION

The oxygen molecule, which is an extremely important element for aerobic organisms, can be toxic to these organisms when it is highly concentrated. Reactive oxygen species that contain the oxygen molecule are chemically highly reactive molecules due to their unpaired electrons. Free radicals are very active molecules with one or more unpaired electrons, highly unstable, short-lived, low molecular weight, endogenous and exogenous in the cell.[1] Antioxidants are defined as substances that prevent oxidation caused by free radicals and can capture and balance free radicals. Antioxidants react with free radicals and prevent them from damaging cells.[2] Under normal conditions, while the living metabolism is healthy, antioxidants and free radicals are in balance. However, when this balance changes in favor of free radicals, susceptibility to oxidative stress-induced diseases is observed. Oxidative stress, by disrupting the active oxygen-antioxidant balance, which is necessary for the continuation of normal metabolic activities, in favor of active oxygen; it causes damage to deoxyribonucleic acid (DNA), protein, carbohydrates and lipids and causes many

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diseases. Epidemiological studies have shown that fruits and vegetables rich in natural antioxidants are protective.[3]

With the increase of free radicals, endogenous antioxidants may be insufficient, and this may require the intake of exogenous antioxidants. Antioxidants in the human body are either produced naturally by the body or taken as an external supplement. In terms of human health, the substances that come to the fore with their antioxidant functions are vitamins E and C, carotenoids and phenolic substances. Among these food, fruits and vegetables are rich in natural antioxidants. The compounds with the highest antioxidant capacity are flavones, isoflavones, flavonoids, anthocyanins, coumarin lignans, catechins, isocatechins, while natural antioxidants alcohols, stilbenes, tocopherols have rich antioxidant content and are of plant origin.[4]

Plant-based antioxidants have started to gain a very important place especially in this period when environmental awareness is increased and the effects of synthesis substances are tried to be avoided. Substances such as synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) can also be used to destroy reactive oxygen species, but the use of these molecules is risky. Therefore, in recent years, restrictions on the use of synthetic antioxidants have been introduced in many countries. Therefore, interest in natural antioxidants has increased and research on natural antioxidants has accelerated.

Sorbus L. genus is in the Rosaceae family. There are 12 species and 17 taxa naturally distributed in our country. The most important ones are *S. domestica* L., *Sorbus torminalis* L. and *Sorbus aucupari* L. *Sorbus* species grown in our country are called by different names (such as eyvaz, ivaz, övez) in different regions. *S. domestica* L. is generally 5-10 m high, deciduous in winter and blooming white flowers in May-June. In Turkey, it grows wild in the western part of North Anatolia, the North of Central Anatolia, the Marmara region and Thrace, and it is cultivated in many regions for its fruits.[5] There are tannis, essential oils, organic acids, color pigments, phenolic acids and their derivatives in service tree fruits. Service tree is a nutritious fruit and has traditionally been used as an anti-diabetic agent. Since its dried fruits are good for the symptoms of diabetes, it is used as an anti-diabetic (hypoglycemic) agent in type-2 diabetes. Various parts of the mountain service tree are widely used in modern medicine and alternative medicine.[6] Its fruits and leaves are used in infusion due to their constipation effects, and its leaves are used in infusion against diabetes. In this study, the antioxidant activities of acetone and water extracts of *S. domestica* L., which can be an alternative to synthetic antioxidants used in removing free radicals, were investigated using different methods and their phytochemical profile was determined by LC-MS/MS.

RESULTS AND DISCUSSION

Extraction yields and recovery percent

In the present study, the percent yield obtained from extracts of *S. domestica* L. are shown in Table 1. The extraction was carried out with two different solvents, including water-fruit, acetone-flower and acetone-fruit to obtain extracts from fresh plant material, which will be used in all assays. The extraction with water-fruit resulted in the highest amount of total compounds. The extraction yields were found to be 327.36, 61.708 and 148.37 mg/g of fresh plant materials for the water-fruit, acetone-flower and acetone-fruit extracts, respectively. These extraction yields indicated that the solvents used for extract preparation from *S. domestica* L. fresh plant materials showed different capacities to extract the leaf compounds and probably different compositions of the extracts.

Table 1

Extraction yields and antioxidant effect (IC_{50}) of 1,1-diphenyl-2-picrylhyrazyl ($DPPH^{\bullet}$) radicals and $ABTS^{\bullet+}$ of *S. domestica* L. extracts

Extract	Extraction yield (%)*	Scavenging ability on $DPPH^{\bullet}$ radicals (IC_{50} µg/mL)	Scavenging ability on $ABTS^{\bullet+}$ radicals (IC_{50} µg/mL)
Water-fruit	32.73	9.86	10.97
Acetone-fruit	6.17	10.66	10.73
Acetone-flower	14.83	14.08	9.14

*: $W_{fresh\ fruit}$ / $W_{dry\ extract}$

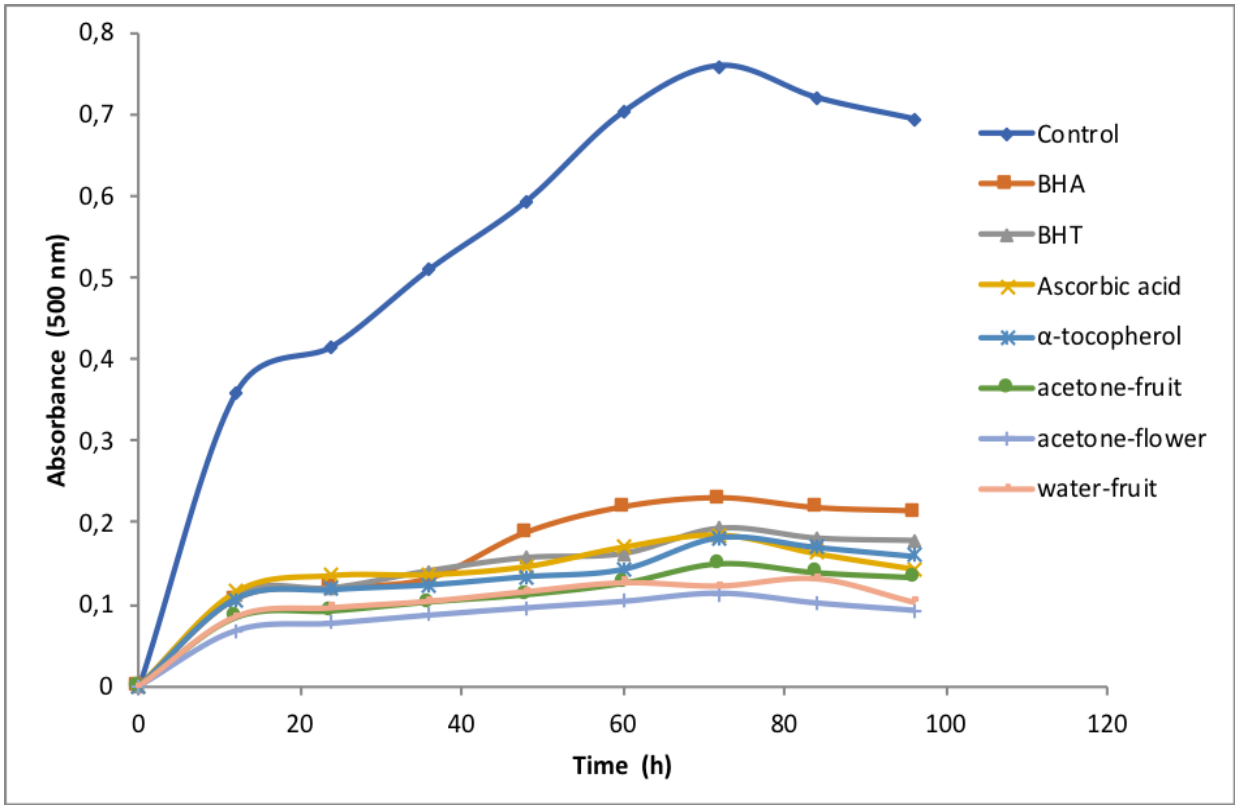

Fig. 1 – Inhibitory effect of the extracts from *S. domestica* L. on lipid peroxidation. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n=3).

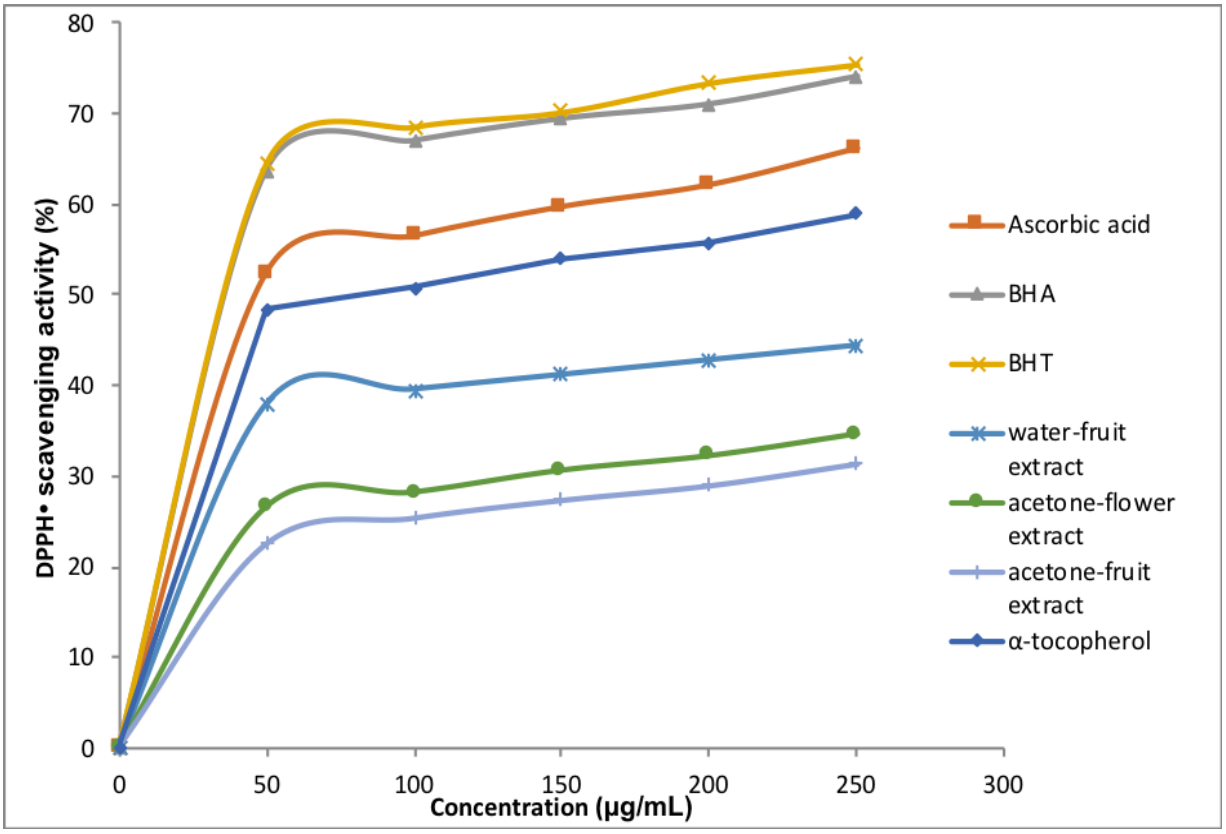

Fig. 2 – DPPH• radical scavenging activity of the extracts from *S. domestica* L. BHA, BHT, ascorbic acid and α-tocopherol were used as refence antioxidants. Values are means ± SD (n = 3).

Total antioxidant activity determination

Total antioxidant activity of *S. domestica* L. extracts was determined by the thiocyanate method. *S. domestica* L. extracts (water-fruit, acetone-fruit, acetone-flower) exhibited effective antioxidant activity. The effects of the same amounts of water-fruit, acetone-fruit and acetone-flower extracts of *S. domestica* L. (250 μg/mL) on peroxidation of linoleic acid emulsion are shown in Fig. 1. The effects on lipid peroxidation of the linoleic acid emulsion of all extracts and standards decreased in that order: acetone-flower extract > water-fruit extract > acetone-fruit extract > α-tocopherol > BHT > ascorbic acid >BHA. The total antioxidant activity of plant extract may be attributed to their chemical composition and phenolic acid content demonstrated that some bioactive compounds present in citrus possessed high total antioxidant activity, which was due to the presence of phenolics, carotenoids and flavonoids.[14]

DPPH• 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.[15] The model of scavenging the stable DPPH• radical is a widely used method to evaluate antioxidant activities in relatively short time as compared to other methods. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.

The DPPH• radical scavenging activities of all the extracts of *S. domestica* L. increased with increasing concentration. About the *S. domestica* L. extracts, Fig. 2 illustrates that the sequence for DPPH• radical-scavenging ability was BHT > BHA > ascorbic acid > α-tocopherol > water-fruit > acetone-flower > acetone-fruit. At 200 μg/mL, the scavenging abilities on DPPH• radicals were 73.38±0.70, 70.79±0.77, 62.09±0.67, 55.64±0.51, 42.74±0.19, 32.25±0.80 and 29.03±0.22% for the BHT, BHA, ascorbic acid, α-tocopherol, water-fruit extracts, acetone-flower extracts and acetone-fruit extracts, respectively.

DPPH• scavenging activity is best presented by the IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH• present in the test solution (Table 1). A higher DPPH• radical scavenging activity was associated with a lower IC_{50} value. IC_{50} values for water-fruit extract, acetone-flower extract, acetone-fruit extract, BHT, BHA, ascorbic acid and α-tocopherol on DPPH• radical scavenging activity were found as 9.86, 10.66, 14.08, 16.86, 19.55, 17.53 and 15.71μg/mL. *S. domestica* L. extracts showed similar DPPH• radical scavenging activities compared to the DPPH• radical scavenging activity of the standards.

$ABTS^{\bullet+}$ scavenging activity

The $ABTS^{\bullet+}$ method is widely employed for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plants extracts.[16] The $ABTS^{\bullet+}$ scavenging activity (%) of the water-fruit, acetone-flower and acetone-fruit extracts of *S. domestica* L. compared to standards are shown in Fig. 3. They increased with increasing concentration, reaching 34.49, 35.97 and 36.95%, respectively at the concentration of 50 μg/mL and these values were comparable to those of the positive controls, ascorbic acid (19.34%), α-tocopherol (16.38%), BHA (19.71%) and BHT (17.62%) at the same concentration, respectively.

$ABTS^{\bullet+}$ scavenging activity is best presented by the IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of $ABTS^{\bullet+}$ present in the test solution (Table 1). A higher $ABTS^{\bullet+}$ radical scavenging activity was associated with a lower IC_{50} value. IC_{50} values for water-fruit extract, acetone-flower extract, acetone-fruit extract, BHT, BHA, ascorbic acid and α-tocopherol on $ABTS^{\bullet+}$ radical scavenging activity were found as 10.97, 9.14, 10.73, 5.07, 6.48, 6.35 and 5.65 μg/mL. *S. domestica* L. extracts showed similar $ABTS^{\bullet+}$ radical scavenging activities compared to the $ABTS^{\bullet+}$ radical scavenging activity of the standards.

Hydrogen peroxide (H_2O_2) radical scavenging activity

H_2O_2 itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell.[17] Extracts from *S. domestica* L. were capable of scavenging H_2O_2 in a concentration dependent manner (50-250 μg/mL). The H_2O_2 scavenging activities of water-fruit extract (26.62±0.37%), acetone-flower extract (23.67±0.58%) and acetone-fruit extract

(29.86±0.89%) were lower than that of BHA (17.19±0.21%), BHT (16.91±0.03%), α-tocopherol (14.37±0.61%) and ascorbic acid (15.50±0.44%) at 100 μg/mL (Fig. 4). The scavenging abilities on H_2O_2 were in descending order of acetone-fruit extract > water-fruit extract > acetone-flower extract > BHA > BHT > ascorbic acid > α-tocopherol.

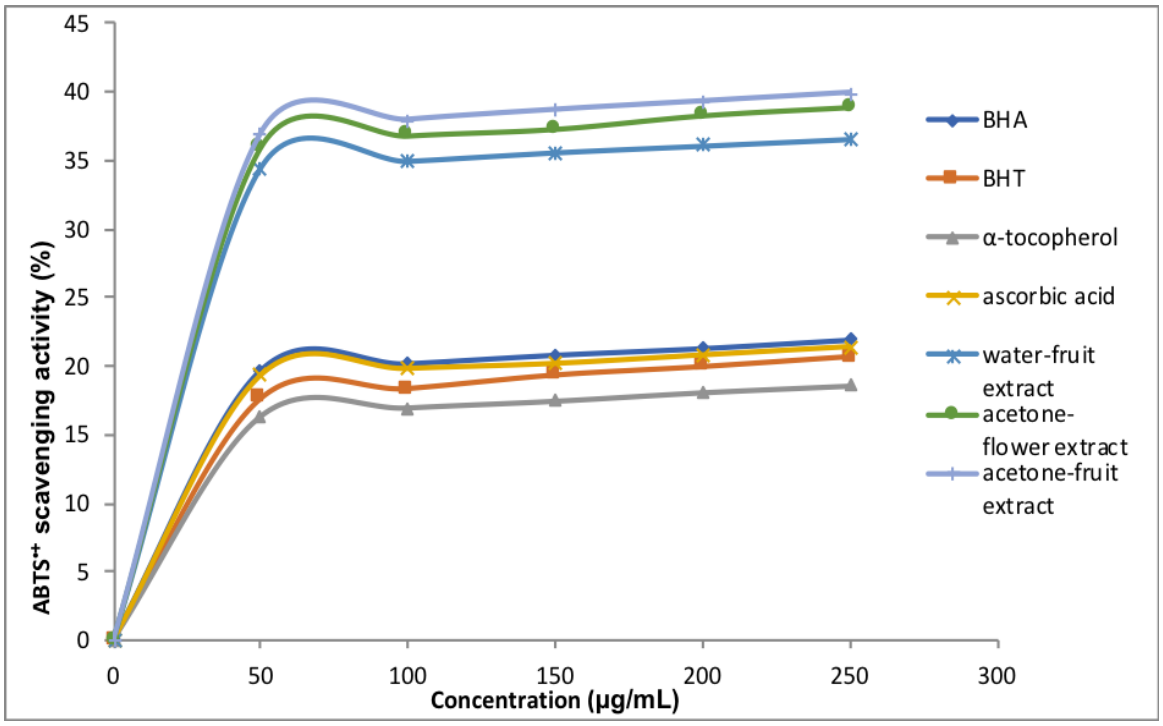

Fig. 3 – $ABTS^{\bullet+}$ radical scavenging activity of the extracts from *S. domestica* L. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3).

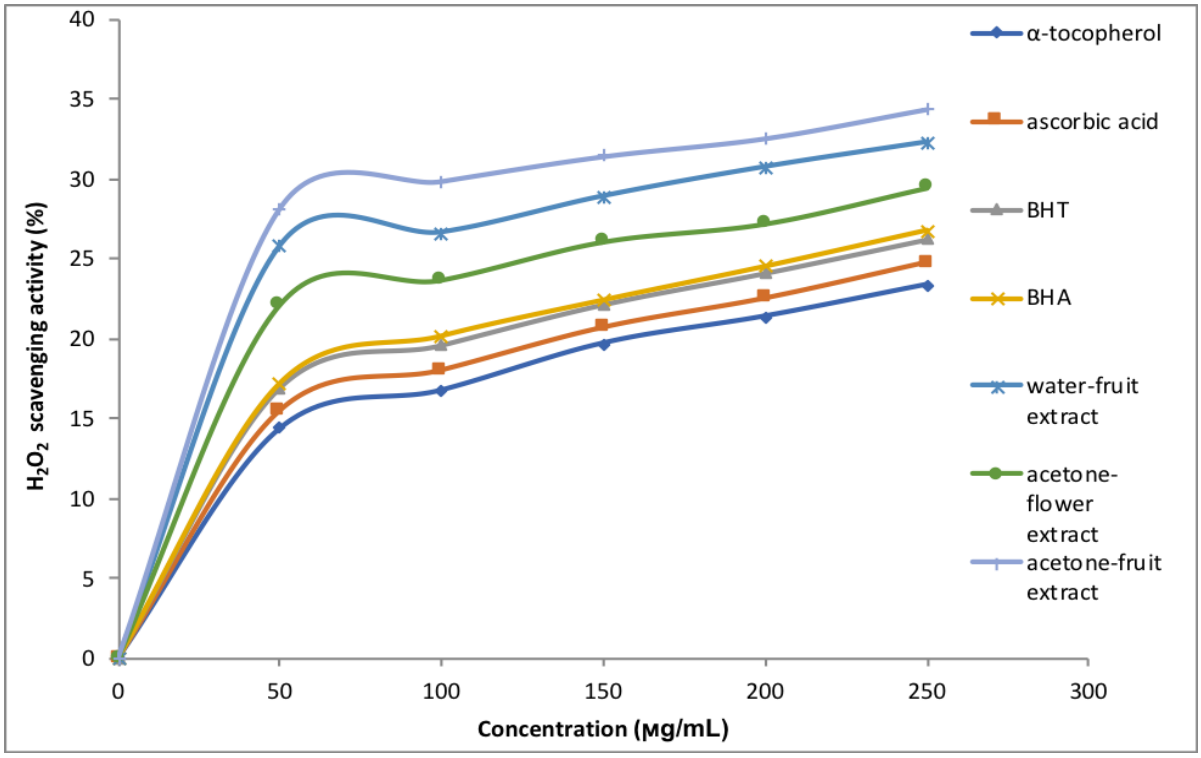

Fig. 4 – H_2O_2 scavenging activity of *S. domestica* L. extracts. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3).

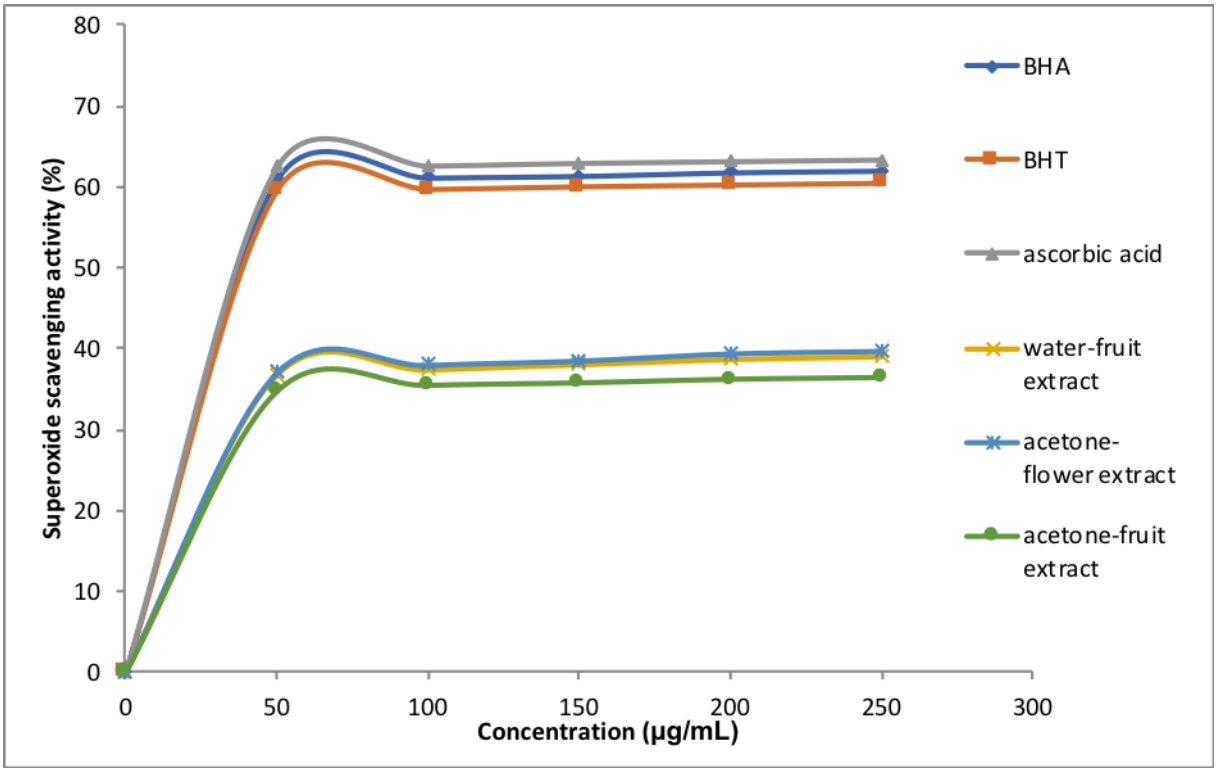

Fig. 5 – Superoxide anion radical scavenging activity of *S. domestica* L. extracts. BHA, BHT and ascorbic acid were used as reference antioxidants. Values are means ± SD (n = 3).

Table 2

CUPRAC test assay of the three extracts and standard ascorbic acid*

Extracts	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL	250 µg/mL
Water-fruit	0.397±0.065	0.556±0.081	0.612±0.066	0.778±0.070	0.885±0.016
Acetone-fruit	0.344±0.038	0.488±0.021	0.532±0.030	0.718±0.009	0.853±0.050
Acetone-flower	0.360±0.039	0.491±0.072	0.544±0.040	0.720±0.020	0.867±0.071
Ascorbic acid	0.558±0.078	0.698±0.013	0.936±0.055	1.126±0.090	1.448±0.080

*Values are given as mean and standard deviation of three parallel measurements

Table 3

The constituents of *S. domestica* L. fruits extract

	Extracts	
Phenolic Compounds (ng analyte/g)	**Water-fruit**	**Acetone-fruit**
Gallic acid	33.33	33.56
Protocatectuic acid	3110.13	3105.07
Salicylic acid	472.56	485.08
Syringic acid	468.58	416.02
Rutin	158.23	58.43
Absisic acid	1832.33	1997.42
Jasmonic acid	5.18	6.64
GSH	3.28	3.01
GSSG	7.45	5.97

Superoxide radical scavenging activity

Superoxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. It was, therefore, proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical.[18] *S. domestica* L. extracts exhibited excellent superoxide scavenging activities of water-fruit extract, acetone-flower extract, acetone-fruit extract, BHA, BHT, and ascorbic acid were 39.06±0.42, 39.71±0.73, 36.45±0.26, 62.02±0.39,

60.50±0.05 and 63.43±0.85, respectively, with ascorbic acid > BHA > BHT > acetone-flower extract > water-fruit extract > acetone-fruit extract (Fig. 5).

Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC antioxidant determination method was studied at five different concentrations (50, 100, 150, 200, 250 μg/mL) (Table 2). In this method, the water-fruit extract was found to be more active than the acetone-flower extract. However, it was determined that water-fruit extract showed lower activity than the standard ascorbic acid.

Identification and quantitation of phenolic compounds by LC-MS/MS

LC-MS/MS qualitative and quantitative analysis results allowed us the detection of 9 compounds; out of 2 are antioxidants glutathione (GSH) and glutathione disulfide (GSSG), the rest of them being phenolics, particularly phenolic acids. Protocatechuic acid was found to be the highest phenolic compound (3110.13 ng analyte/g) in the LC-MS/MS analysis of the water-fruit extract (Table 3).

EXPERIMENTAL

Plant material and extraction procedures

Fresh service tree (*S. domestica* L.) flowers and fruits were collected from Kırklareli (Kırklareli, Turkey). Since it will be used with fresh material, no drying process was performed and it was stored in the deep freezer until used. Fresh plant material taken from the deep freezer was homogenized by grinding with the help of a blender. Although several solvent systems were examined for obtaining the extracts from fruits and flowers, extraction was successfully carried out using water and acetone. For this purpose extracts were prepared using acetone and water solvents. For the preparation of the water extract, 25 g of fruit was mixed in 500 mL of water for 30 minutes by heating in a magnetic stirrer. The resulting water extracts were filtered through filter paper and the filtrate was lyophilized. For the preparation of acetone extracts, 25 g of fruit and flowers were incubated in 500 mL solvent at room temperature in a shaking water bath at 100-150 rpm for 3 hours. The obtained extracts were filtered through filter paper and the solvents of the filtrates were evaporated at 40-50ºC in the rotary evaporator (Buchi R-200, Switzerland). All the extracts were kept at -20ºC and were dissolved in water or solvent before use.

Total antioxidant activity assay

The total antioxidant activity of the *S. domestica* L. extracts were determined according to the thiocyanate method described by Mitsuda *et al.*[7] For stock solutions, 10 mg of *S. domestica* L. extracts were dissolved in 10 mL of deionized water. The solution, which contains the same concentration of *S. domestica* L. extracts or standard samples (150 μg/mL) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Fifty milliliters of linoleic acid emulsion contained 175 μg polyoxyethylenesorbitan monolaurate (Tween-20), 155 μL linoleic acid emulsion and 0.04 M potassium phosphate buffer (pH 7.0). 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). The mixed solution (5 mL) was incubated at 37ºC in a glass flask. At regular intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 3.7 mL of solvent (ethanol or methanol), followed by the addition of 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer. This step was repeated every 10 h until the control reached its maximum absorbance value. Therefore, high absorbance indicated high linoleic acid oxidation. All data on total antioxidant activity are the average of triplicate experiments. The percent inhibition of lipid peroxidation in the linoleic acid emulsion was calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

DPPH$^{\bullet}$ radical scavenging activity assay

The free radical scavenging activity of *S. domestica* L. extracts was determined by the DPPH$^{\bullet}$. The activity was measured by following the methodology described by Shimada *et al*.[8] Where in the bleaching rate of a stable free radical, DPPH$^{\bullet}$ is monitored at a characteristic wavelength in the presence of the sample. Briefly, 0.1 mM solution of DPPH$^{\bullet}$ in ethanol (or methanol) was prepared and 1 mL of this solution was added to 3 mL of *S. domestica* L. extracts solution in water or acetone at different concentrations (50-250 μg/mL). 0.5 h later, the absorbance was measured at 517 nm. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The capability to scavenge the DPPH$^{\bullet}$ radical was calculated using the following equation:

$$\text{DPPH}^{\bullet}\text{ scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

ABTS$^{\bullet+}$ scavenging activity

The ABTS$^{\bullet+}$ scavenging activity of the *S. domestica* L. extracts was measured according to the procedure described by Re et al.[9] ABTS$^{\bullet+}$ was produced by the reaction between 7 mM ABTS$^{\bullet+}$ in water (H_2O) and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the ABTS$^{\bullet+}$ solution was diluted to get an absorbance of 0.700±0.025 at 734 nm with phosphate buffer (0.1 M, pH 7.4). Then, 1 mL of ABTS$^{\bullet+}$ solution was added to

3 mL of *S. domestica* L. extracts at different concentrations (50-250 µg/mL). After 0.5 h, the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^{\bullet+}$ scavenging activity was calculated using the following equation:

$$ABTS^{\bullet+} \text{ radical scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

H_2O_2 scavenging activity

The ability of the *S. domestica* L. extracts to scavenge H_2O_2 was determined according to the method of Ruch *et al.*[10] A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (50-250 µg/mL) in distilled water were added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The blank solution contained the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of both *S. domestica* L. extracts and standard compounds was calculated as follows:

$$H_2O_2 \text{ scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

Superoxide anion radical scavenging assay

Measurement of superoxide anion scavenging activity of *S. domestica* L. extracts was done based on the method described by Liu *et al.*[11] with slight modification. About 1 mL of nitro blue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solution of *S. domestica* L. extracts (50-250 µg/mL) in water or acetone were mixed. The reaction started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25ºC for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. All data are an average of triplicate analyses. The percentage of inhibition of superoxide anion scavenging formation was calculated by using the formula given below:

$$\text{Inhibition (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

Cupric reducing antioxidant capacity (CUPRAC)

S. domestica L. extracts were dissolved in distilled water to prepare their stock solution at 1000 µg/mL concentration. Aliquots of 61 mL of 1.0×10^{-2} M copper (II) chloride, 61 µL of ammonium acetate (NH_4OAc) buffer (1 M, pH 7.0) and 61 µL of 7.5×10^{-3} M neocuproine solution were mixed, × µL sample solution (50-250 µg/mL) and (67 - ×) µL distilled water was added to make the final volume 250 µL. The tubes were stopped, and after 1 h, absorbance at 450 nm was measured against a reagent blank.[12]

Identification and quantitation of phenolic compounds by LC-MS/MS

A previously established and validated LC-MS/MS method was applied to determine the phenolic contents in the acetone and water extracts of *S. domestica* L. The validation parameters of this study were linearity, recovery, repeatability, limits of the detection (LOD), and limits of the quantification (LOQ). Detailed procedures of uncertainty evaluation have been previously reported in the literature.[13] Validation parameters are given in Table 3.

CONCLUSION

The extracts of *S. domestica* L. exhibited different levels of antioxidant activity in all the models studied. The results from various free radical-scavenging systems revealed that the *S. domestica* L. had significant antioxidant activity and free radical-scavenging activity. The free radical-scavenging property may be one of the mechanisms by which this drug is useful as a foodstuff as well as traditional medicine. However, further investigation of individual compounds, their *in vivo* antioxidant activities and in different antioxidant mechanisms is warranted. It was concluded that *S. domestica* L. fruit can be used as a natural antioxidant source.

Supplementary data

Supplementary data 1: Analytical parameters of LC-MS/MS method

No	Extracts	[a]RT	[b]r^2	Linearity Range (ng/mL)	[c]LOQ	[d]LOD
1	**Gallic acid**	1.70	0.9999	5-100	33.33	11.11
2	**Protocatechuic acid**	1.82	0.9995	5-100	3110.13	1036.71
3	**Salicylic acid**	3.77	0.9993	5-100	472.56	157.52
4	**Syringic acid**	3.82	0.9969	5-100	468.58	156.19
5	**Rutin**	3.97	0.9996	5-100	158.23	52.74
6	**Abscisic acid**	4.38	0.9997	5-100	1832.33	610.77
7	**Jasmonic acid**	4.47	0.9999	5-100	5.18	1.72
8	**GSH**	4.94	0.9997	10-1000	3.28	1.09
9	**GSSG**	5.03	0.9993	10-1000	7.45	2.48

[a]RT: Retention time, [b]r^2: coefficient of determination, [c]LOQ: Limit of quantification, [d]LOD: Limit of detection.

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