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Turning Organic Waste into a Valuable Alternative: Evaluation of Total Phenolic Content, Radical and Antioxidant Activity of Medlar Seed Extracts

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Abstract

In this study, the potential of medlar seeds, which have no known usage and are considered organic waste, as a natural antioxidant source was investigated. For this purpose, total phenolic, total flavonoid, and anthocyanin contents, which are important in antioxidant activity, were found in aqueous and ethanol extracts of medlar seeds. When the antioxidant activities were examined, the ethanol extract showed 92.38 ± 0.51% activity in 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) scavenging at a concentration of 25 µg/mL, slightly better than the synthetic antioxidant butylated hydroxyanisole (BHA) at the same concentration. Also, ethanol extract had 23.30 ± 1.78% of chelating activity, while aqueous extract had 1.04 ± 0.44%. In N,N-dimethyl-p-phenylenediamine ($DMPD^{+\cdot}$) scavenging activity, aqueous extract had 36.06 ± 0.89% at 200 µg/mL, while BHA had 41.25 ± 0.1%. In the β-carotene bleaching test, the aqueous extract (1.04 ± 0.02) had slightly better activity than BHA, and the ethanol extract (0.88 ± 0.002) had a somewhat similar activity to BHA. In general, both extracts had considerable antioxidant activity. When the results are examined, it is thought that medlar seeds, which are considered waste, can be made more valuable by using them as a good antioxidant source.

Keywords

radical scavenging, DPPH, ultrasound-assisted extraction, *Mespilus germanica*

1 Introduction

An antioxidant is a chemical that, when present in low quantities compared to an oxidizable substrate, considerably slows or stops the substrate's oxidation [1]. With the development of people's living standards, especially because of the potential toxicity of synthetic antioxidants, people have begun to increasingly prefer to use natural antioxidants to prevent oxidative stress or damage. Natural antioxidants are widely used in the food industry, such as polyphenols, which are widely found in the human diet and have various biological activities [2]. Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been restricted from use in foods by the Food and Drug Administration due to their suspected carcinogenicity. In addition, the fact that synthetic antioxidants cause safety concerns has increased the demand for natural resources [3]. Since plants have been used for a long time by humans for remedies, health benefits, and as a rich source of biological compounds, including antioxidants, this has led to an increasing interest in the development of natural antioxidants from plant sources in the food industry and preventive medicine [4–6]. Additionally, antioxidants can be used as additives in industries such as lubricants, oils, fuel, and rubber products [7, 8].

Medlar (*Mespilus germanica* L.) is a member of the Rosaceae family that thrives in frost-free environments, rocks, and poor soils. It is abundant in Türkiye, particularly in the northern and western Anatolia and Marmara regions [9]. Medlar is a very rich source of bioactive compounds such as phenolic compounds, anthocyanins, organic acids, minerals, and different fatty acids. Chlorogenic, caffeic, syringic, coumaric, ferulic, gallic, citric, malic, oxalic, succinic, and tartaric acids, catechin, epicatechin, rutin, fructose, glucose, sucrose, phellandrene, γ-terpinene, terpinolene, vitamin C, and potassium were given in the literature as some components of the medlar [10–17]. Bioactive compounds of medlar, their characterization, and their use in functional foods are among the main targets of recent research. Medlar is also

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a rich natural antioxidant source. It could be utilized in the manufacture of foods and dietary supplements [17, 18].

Organic waste can be used as a new material source to increase sustainability. For this reason, new ways are being developed to obtain these materials from organic waste like peels, leaves, seeds, and oils. Using these wastes also creates new value-added products. Obtaining new materials from organic waste also has potential benefits, such as low economic cost. One of the materials that can be obtained from this waste is phenolic compounds, which have antioxidant properties [19]. As the demand for natural goods and extracts has increased in recent years, suitable extraction techniques or alternative extraction techniques have been developed to reduce the use of organic solvents, raw materials, time, and expenses [20]. Since particle disruption causes a much larger contact surface area between the solid and liquid phases, ultrasound-assisted extraction (UAE) has been assessed as a simpler and more efficient substitute for traditional extraction techniques for the successful isolation of significant substances from plant tissue [21]. Therefore, medlar seeds, which can be considered as organic waste since they have no known application, could be used to extract antioxidant compounds with the simple and effective UAE. To the best of our knowledge, there is no study involving medlar seeds' antioxidant capacity.

This study was aimed at checking whether medlar seeds can be a new natural antioxidant source by examining the antioxidant properties, which have no generally known use and are evaluated as waste. For this reason, UAE was used to obtain aqueous and ethanol extracts of the seeds, and total phenolic, flavonoid, and anthocyanin contents were found. After that, antioxidant properties were evaluated and compared to synthetic antioxidants with different methods because it is difficult to assess antioxidant capacity with only a few methods.

2 Materials and methods

2.1 Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and linoleic acid were procured from Sigma-Aldrich, Germany. N,N-dimethyl-p-phenylenediamine (DMPD), trichloroacetic acid and Folin-Ciocalteau reagent obtained from Merck, Germany. Butylated hydroxyanisole (BHA) and Ferrozine were obtained from Fluka, Switzerland. Every chemical utilized was of analytical quality and did not require any additional processing. All solutions were prepared freshly before the experimentation.

2.2 Extract preparation

Medlar fruit was bought locally from different markets. Seeds were separated from fruit and washed thoroughly with distilled water until no fruit material was left and dried at 30 °C. The dried seeds were ground into powder and then sieved with a 1 mm sieve. UAE at 25 °C was done with ethanol and distilled water as solvents to prepare extracts. An ultrasonic bath (Bandelin RK 100 H, 35 kHz, p 80/320 W, Germany) was used. 0.5 g of powder was put in a centrifuge tube, and 5 mL of solvent was added, and the mixture was sonicated for 5 min at 1-min intervals to prevent overheating. After 5 min, the solids were separated by centrifuge for 10 min at 6000 rpm, and the solvent was evaporated to obtain the extracts. The extracts were kept at 4 °C. From these extracts, mg/mL extract solutions were prepared for use in experimental studies. All experiments were conducted in triplicate, and results were reported as mean ± standard deviation. Standard deviations were calculated with Microsoft Office 365 Excel software [22].

2.3 Evaluation of antioxidant activity

2.3.1 Determination of total phenolic content

Following Slinkard and Singleton's protocol [23], the sample (1 mg/mL) was subjected to spectrophotometric analysis using the Folin-Ciocalteau reagent to determine its total phenolic content. Pyrocatechol was the standard phenolic compound, and a standard curve was obtained. Total phenolic content was defined as μg pyrocatechol equivalent/mg extract.

2.3.2 Determination of total flavonoid content

Total flavonoid content was determined by the method of Zhishen et al. [24]. Different concentrations of (+)-catechin were used as a standard solution, and a calibration curve was obtained. Extract concentrations were 1 mg/mL. The results were given as μg (+)-catechin equivalent/mg extract.

2.3.3 Anthocyanin determination

Determination of anthocyanin content was done according to the modified method of Padmavati et al. [25]. A 25 mg/mL seed solution was prepared using 1% HCl/methanol and kept in the dark at 4 °C for 24 h. Then it was centrifuged at 6000 rpm for 15 min. Spectrophotometric measurements (Perkin Elmer Lambda 25, USA) of the anthocyanin content in the supernatant at 530 and 657 nm were made, and the resulting absorbance values were denoted as A_{530} and A_{657}. The concentration was calculated using Eq. (1):

$$\text{Anthocyanin concentration}\left(\frac{\text{mmol}}{\text{g}}\right) = \frac{A_{530} - 0.33 \times A_{657}}{31.6 \times l} \times \frac{\text{volume}\ (\text{mL})}{\text{mass}\ (\text{g})}, \quad (1)$$

The absorbance values were converted to anthocyanin concentration using an extinction coefficient of 31.6 1/(M × cm). 0.33 was used as a correction factor for chlorophyll interference, and the path length of the cuvette (l) was 1 cm.

2.3.4 DPPH$^{\bullet}$ scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging activity of the medlar seed extract was measured with the method of Brand-Williams et al. [26], with slight modification. 1.5 mL of 20 mg/L DPPH$^{\bullet}$ solution was added to 0.75 mL extract solutions prepared at a concentration of 5–25 µg/mL. After 30 min incubation in the dark and at room temperature, the absorbance A_{Sample} was measured at 517 nm. BHA was used as a standard, and the control reaction was done with solvents instead of extract solutions. Its absorbance is labelled as A_{Control}. Radical scavenging activity was calculated with Eq. (2):

$$\text{Radical scavenging activity}\ (\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100. \quad (2)$$

2.3.5 DMPD$^{+\bullet}$ scavenging activity

Fogliano et al.'s method [27] was used to assess the N,N-dimethyl-p-phenylenediamine radical (DMPD$^{+\bullet}$) scavenging activity. DMPD$^{+\bullet}$ solution was prepared by adding 1 mL of 100 mM DMPD solution to 100 mL of 0.1 M pH 5.3 acetate buffer. After this, 0.2 mL of 0.05 M ferric chloride was added, and a colored radical solution was obtained. 1 mL of this solution was added to 0.5 mL of the sample (50–200 µg/mL), and after 10 min incubation at room temperature, the absorbance was measured at 505 nm. Eq. (2) was used to calculate the scavenging activity.

2.3.6 ABTS$^{+\bullet}$ scavenging activity

To evaluate the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS$^{+\bullet}$) scavenging activity of the extracts, Arnao et al.'s method was used [28]. Equal amounts of 7.4 mM ABTS and 2.6 mM potassium persulfate solutions were mixed and kept at room temperature in the dark for 12 h. The incubated solution was diluted with methanol to obtain 1.1 ± 0.02 absorbance units at 734 nm. After this, 150 µL of the extract or standard at 25–100 µg/mL was mixed with 2850 µL of ABTS$^{+\bullet}$ solution. The mixture was kept in the dark for 2 h. Then the absorbance was measured at 734 nm. BHA was used as a standard with the same concentration of the extracts. A control reaction was done with solvents instead of the extracts. Radical scavenging activity was calculated by Eq. (2).

2.3.7 Reducing power

To analyze the reducing power of the extracts, the method of Oyaizu was used [29]. Different concentrations (10–25 µg/mL) of the extracts or standard (BHA) were prepared in 1 mL of distilled water, then 2.5 mL of pH 6.6, 0.2 M phosphate buffer was added. After this, 2.5 mL of 1% (w/v) potassium ferricyanide was added, and then the mixture was incubated at 50 °C for 30 min. After incubation, 10% (w/v) trichloroacetic acid was added and centrifuged for 10 min at 6000 rpm. 2.5 mL of the upper layer solution was taken, and 2.5 mL of distilled water was added. Absorbance was measured at 700 nm after the addition of 0.5 mL of 0.1% (w/v) $FeCl_3$. Higher absorbance means better reducing power.

2.3.8 Chelating activity

The chelating activity of the extracts was measured by the method of Decker and Welch [30]. 1 mL of extract solution at 1 mg/mL concentration was added to a tube containing 3.7 mL of distilled water. To this solution, 0.1 mL of 2 mM $FeCl_2$ was added and incubated for 30 min. At the end of the incubation, the reaction was started by adding 0.2 mL of 5 mM ferrozine. After 10 min, the absorbance was measured at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as a standard at 0.037 mg/mL. A control was tested without a sample. The chelating activity was calculated with Eq. (3):

$$\text{Chelating activity}\ (\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100. \quad (3)$$

2.3.9 β-carotene bleaching test

The β-carotene bleaching test was done according to Bruni et al. [31]. A 1 mg/mL β-carotene solution in chloroform was prepared. 0.2 mL of β-carotene solution, 20 mg linoleic acid, and 200 mg Tween 40 were put in a container, and the chloroform was evaporated. 50 mL of distilled water was added to the container and vigorously shaken. 5 mL of this emulsion was added to a tube containing 0.2 mL of 1 mg/mL extracts or standard and incubated for 120 min at 50 °C. The absorbance of the mixture was

measured after 60 min and 120 min at 470 nm. BHA was used as a positive control. The relative antioxidant activity (RAA) was calculated by dividing the absorbance of the sample by the absorbance of BHA.

3 Results and discussion

3.1 Determination of total phenolic, flavonoid, and anthocyanin contents

Phenolics have exceptional antioxidant effects due to their redox characteristics. These compounds may effectively adsorb and neutralize free radicals, as well as chelate iron and copper cations [32]. The total phenolic contents of the extracts of the medlar seeds were determined by the Folin-Ciocalteau method. Ethanol extract had 73.76 ± 0.73 µg pyrocatechol equivalent/mg extract, and aqueous extract had 12.88 ± 0.04 µg pyrocatechol equivalent/mg of the extract. The total flavonoid content results show that the ethanol extract had 100.8 ± 1.67 µg catechin equivalent/mg extract and the aqueous extract 23.86 ± 0.86 µg catechin equivalent/mg extract. The anthocyanin content of medlar seeds was found to be 0.17 ± 0.01 mmol/g seed.

Nabavi et al. [33] reported that methanol and water extracts of wild medlar stem bark, leaf, and fruit contained between 7.26 ± 0.4 and 457.07 ± 22.3 mg gallic acid equivalent (GAE)/g, while aqueous bark extract was the highest, and aqueous fruit extract was the lowest. Total flavonoid content, expressed as mg quercetin equivalent (QE)/g extract, were between 14.08 ± 1.1 for the aqueous fruit extract and 59.92 ± 3.6 for the methanol leaf extract. Isbilir et al. [34] reported that the total phenol contents of ethanol extracts of leaf, flower bud, and fruit were 60.3 ± 1.69, 50.3 ± 0.51, and 16.5 ± 3.53 mg GAE/g extract, respectively. The total flavonoid contents of the same extracts were 14.77 ± 1.15, 6.54 ± 0.08, and 1.99 ± 0.02 mg GAE/g extract, respectively. Ercisli et al. [9] found that 11 genotypes of medlar fruits had an average of 194 mg GAE/100 g fresh fruit. Gülçin et al. [18] reported that the lyophilized aqueous extract of medlar (LEM) fruits had 25.08 mg GAE/g total phenolics and 2.39 mg QE/g total flavonoids. Wani et al. [35] studied 80% ethanol extracts of seeds of different varieties of pears (*Pyrus communis* L., Rosaceae) and found that they contained 4.30 ± 0.18 – 6.00 ± 0.02 mg GAE/g total phenolics and 1.30 ± 0.01 – 1.97 ± 0.02 mg QE/g total flavonoid. In Yang et al.'s study [36], it was reported that seed kernels of Siberian apricot (*Prunus sibirica*, Rosaceae) from different provinces had an average of 215.37 mg GAE/100 g total phenolics and 63.08 mg rutin equivalent/100 g total flavonoid content.

While there are no studies in the literature about the total phenolic amounts of medlar seeds, there are studies of medlar fruits, leaves, and bark. Even though it is difficult to compare because of the difference in reporting the results, our results are somewhat comparable to the literature. It can be said that medlar in general contains a good amount of phenolics.

3.2 DPPH• scavenging activity

DPPH• scavenging activity is one of the well-known methods to determine the antioxidative potential of a substance. In this test, antioxidants react with the stable DPPH• and as a result, the purple-colored DPPH• solution loses its color and turns yellow. This color loss can be measured spectrophotometrically, and the antioxidant activity of the substance can be found [37]. DPPH• scavenging activity of the extracts between 5–25 µg/mL was measured. At the highest concentration, ethanol extract showed very high activity at 92.38 ± 0.51%, and aqueous extract had 21.15 ± 1.6% scavenging activity. The standard antioxidant BHA had an activity of 89.97 ± 0.83%. This result shows that the ethanolic extract of the medlar seeds has even better activity than one of the highest activity standards, BHA, and has a very high capacity to scavenge DPPH•. Results are given in Fig. 1.

In Nabavi et al.'s study [33], the DPPH• scavenging activities of different medlar extracts were given as 50% inhibitory concentration (IC_{50}) between 10.7 ± 0.6 and 492 ± 33.1 µg/mL. The results from the study showed that the aqueous extract of the medlar bark has the best results against DPPH•. Isbilir et al. [34] reported that the ethanol extract of medlar leaf had 41.3 ± 0.7% and 63.4 ± 2% scavenging activity at 100 and 250 µg/mL, respectively. Ercisli et al. [9] reported that 11 different genotypes of medlar fruit had an average of 46.6 µg/mL DPPH activity, while BHA

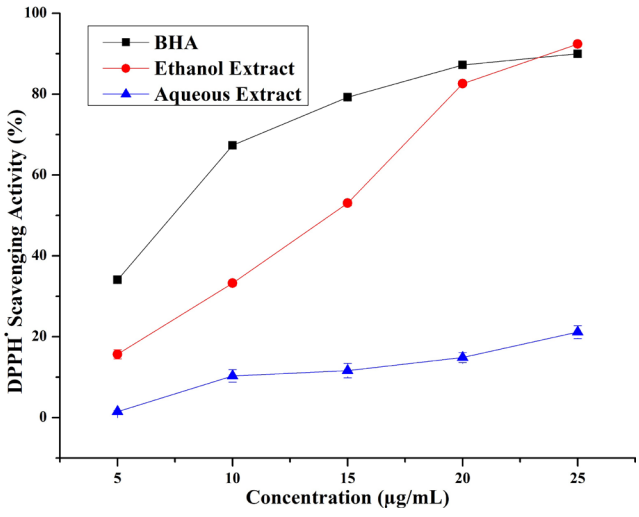

Fig. 1 DPPH• scavenging activity of the samples

had an activity of 21.24 µg/mL. DPPH scavenging activity of LEM fruits was reported as 0.62 Trolox equivalent (TE) by Gülçin et al. [18]. Wani et al.'s study [35] showed that the DPPH$^{\bullet}$ scavenging activities of different pear seeds were between 75.90 ± 0.32 and 80.00 ± 0.84%. Black raspberry seed oil (*Rubus occidentalis* L., Rosaceae) had an IC_{50} value of 3.02 ± 0.03 mg/mL [38]. In Yang et al.'s study [36] with seed kernels of the Siberian apricot from different provinces, scavenging results were reported between 18.66–70.49%. These results, compared to our study, show that it may be possible to say that medlar seeds contain more DPPH$^{\bullet}$ scavenging compounds compared to the other parts.

3.3 DMPD$^{+\bullet}$ scavenging activity

In this method, firstly, the DMPD$^{+\bullet}$ solution was created with the help of Fe^{3+} in acidic conditions. The obtained colored solution, which shows a peak at 505 nm, becomes colorless because of the ability of the extracts to donate hydrogen atoms [39]. The ability to scavenge DMPD$^{+\bullet}$ was measured at 50–200 µg/mL concentration of the extracts and BHA. At 200 µg/mL, BHA had the highest activity with 41.25 ± 0.1%, while ethanol extract had 20.17 ± 0.19% and aqueous extract had 36.06 ± 0.89% scavenging activity. Results were given in Fig. 2. The DMPD$^{+\bullet}$ scavenging activity of LEM fruits was reported as 0.81 TE by Gülçin et al. [18].

3.4 ABTS$^{+\bullet}$ scavenging activity

A blue-green colored ABTS$^{+\bullet}$ solution, which gives absorption at 734 nm, is obtained by reacting ABTS with potassium persulfate. When this solution reacts with an antioxidant, the radical gets reduced, resulting in decolorization and absorbance decrease [40]. 25–100 µg/mL concentration was used to measure the ABTS$^{+\bullet}$ scavenging activity of the samples. At 100 µg/mL, both extracts showed that they can scavenge ABTS$^{+\bullet}$. Ethanol extract had 25.52 ± 1.05% activity, aqueous extract had 8.44 ± 1.71% activity, and BHA had the highest activity with 99.62 ± 0.07%. Results were given in Fig. 3.

Krgović et al.'s study [38] reported that the IC_{50} of black raspberry seed oil for ABTS$^{+\bullet}$ scavenging activity was 1.33 ± 0.01 mg/mL.

3.5 Reducing power

Reducing power was assessed as the reduction of potassium ferricyanide to potassium ferrocyanide by the samples, eventually forming a ferric ferrous complex with the addition of ferric chloride. The complex has a maximum absorption at 700 nm [41]. The reducing power of the extracts and BHA were given in Fig. 4. The absorbance results show that the ethanolic extract had the highest reducing power at 25 µg/mL with 0.23 ± 0.01, followed by the aqueous extract with 0.17 ± 0.00. The standard tested,

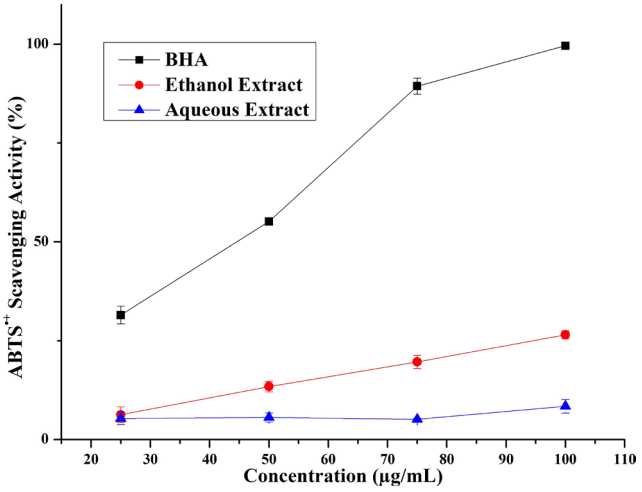

Fig. 3 ABTS$^{+\bullet}$ scavenging activity of the samples

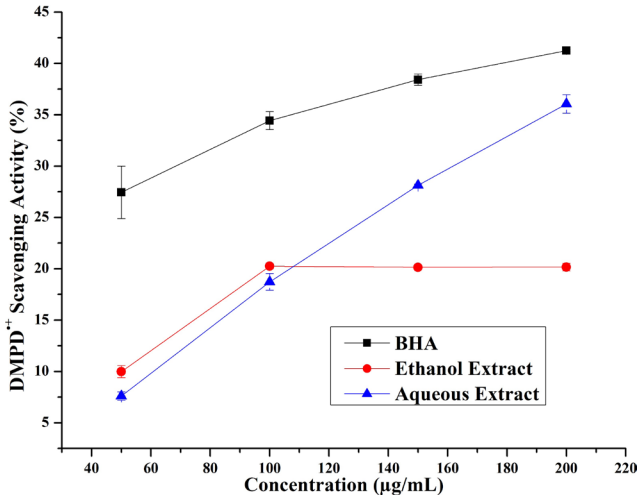

Fig. 2 DMPD$^{+\bullet}$ scavenging activity of the samples

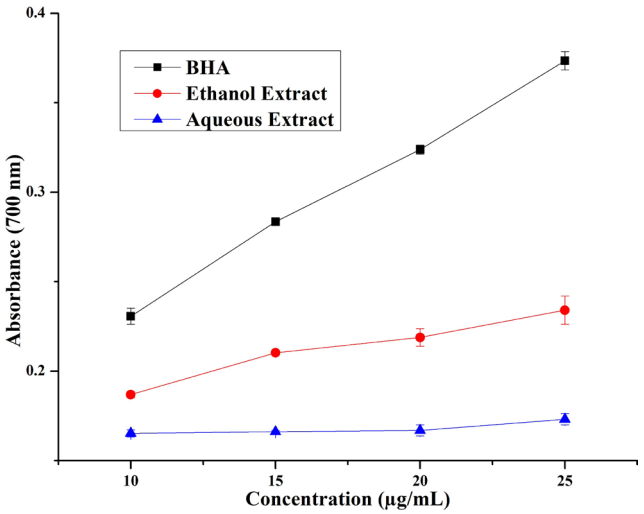

Fig. 4 Reducing power of the samples

BHA, had the highest reducing power with 0.37 ± 0.01. The reducing power of LEM was reported as 0.69 TE by Gülçin et al. [18].

3.6 Chelating activity

The chelating activity of the samples was determined by the ability of ferrozine to form complexes with ferrous ions. The complex has a red color. In the presence of samples with chelating activity, ferrous ions cannot form complexes with ferrozine, which causes a decrease in the absorbance and red color [42]. At 1 mg/mL concentration, the ethanol extract showed 23.30 ± 1.78% activity, while the aqueous extract had very low activity with 1.04 ± 0.44%. EDTA, which is generally used as a chelating agent, had 69.38 ± 0.14% activity at 0.037 mg/mL. The results were given in Fig. 5. Gülçin et al. reported that LEM had a chelating activity of 2.79 TE [18].

3.7 *β*-carotene bleaching test

In the *β*-carotene bleaching test, the ability of the samples to prevent oxidation of *β*-carotene by radicals created during the linoleic acid peroxidation was tested. The color of the solution decreases if there is no antioxidant sample present [43]. The aqueous extract showed slightly higher activity than BHA with RAA after 60 min, 1.04 ± 0.02, and after 120 min, 1.02 ± 0.01. The ethanol extract had an RAA after 60 min, 0.88 ± 0.002, and after 120 min, 0.87 ± 0.02. *β*-carotene bleaching test results were given in Fig. 6. The average of the *β*-carotene bleaching assay of 11 genotypes of medlar fruit by Ercisli et al. [9] was 80.8%, while BHA was 94.33%.

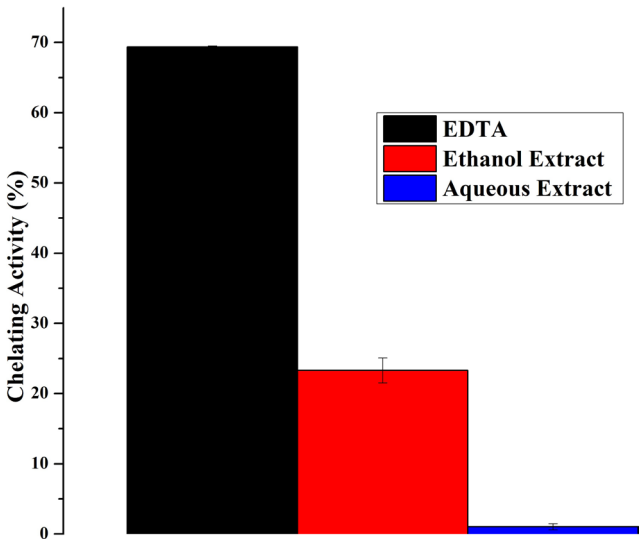

Fig. 5 Chelating activity of the samples

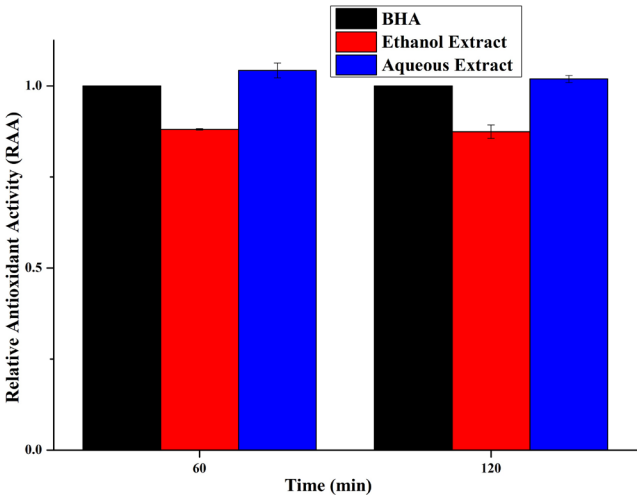

Fig. 6 β-carotene bleaching test results of the samples

4 Conclusion

In this study, the aqueous and ethanol extracts of medlar seeds were obtained with the simple and cost-effective UAE method. The obtained extracts contained good amounts of total phenolics, flavonoids, and anthocyanin content. In general, medlar seed extracts had good antioxidant activity. Especially ethanol extract had a tremendous DPPH$^{\bullet}$ scavenging activity even at a very low concentration of 25 µg/mL and a somewhat similar activity to BHA in the *β*-carotene bleaching test. While aqueous extract had slightly better activity than BHA in the *β*-carotene bleaching test, it had similar DMPD$^{+\bullet}$ scavenging activity to BHA. The results show that medlar seeds have great potential to be used as a new source of antioxidant compounds instead of synthetic antioxidants. Upon further studies, the seeds have the possibility to be used as additives in different industries or as a source for antioxidant compound isolation. This would also cause the valorization of unused medlar seeds, decreasing organic waste in the environment.

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