ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 5:3 (2011) 158-175

records of natural products

Polyphenol Contents and Antioxidant Properties of Medlar (*Mespilus germanica* L.)

İlhami Gülçin[1,2*], Fevzi Topal[1], S. Beyza Öztürk Sarıkaya[3], Ercan Bursal[4], Gökhan Bilsel[5] and Ahmet C. Gören[5]

[1]*Atatürk University, Faculty of Sciences, Department of Chemistry, 25240-Erzurum-Türkiye*
[2]*Agri Ibrahim Cecen University, School of Health Services, TR-04100-Agri- Türkiye*
[3]*Gumushane University, Faculty of Engineering, Department of Food Engineering, 29100-Gumushane- Türkiye*
[4]*Mus Alparslan University, Faculty of Arts and Sciences, Department of Chemistry, 49100-Mus, Türkiye*
[5]*TUBITAK UME, Chemistry Group Laboratories, P.O. Box: 54, 41470-Gebze-Kocaeli, Türkiye*

(Received December 30, 2010; Revised February 21, 2011; Accepted March 1, 2011)

Abstract: Medlar is the fruit of *Mespilus germanica* L. in the family of Rosaceae. The present study outlines that the native medlar (*Mespilus germanica* L.) fruits an extremely rich source of antioxidants. In this study, antioxidant and antiradical property of medlar fruits were evaluated. Total phenolics and flavonoids amounts in lyophilized extract of medlar (LEM) fruits were calculated as gallic acid and quercetin equivalents, respectively. Antioxidant and radical scavenging activity of LEM were investigated using different in vitro assays including 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), N,N-dimethyl-p-phenylenediamine ($DMPD^{\bullet+}$), and superoxide anion radicals ($O_2^{\bullet-}$) scavenging activity, hydrogen peroxide (H_2O_2), ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing ability, Fe^{3+}-TPTZ reducing ability, ferrous ions (Fe^{2+}) chelating activity as trolox equivalent. In addition, quantitative amounts of caffeic acid, ferulic acid, syringic acid, ellagic acid, quercetin, α-tocopherol, pyrogallol, p-hydroxybenzoic acid, vanillin, p-coumaric acid, gallic acid and ascorbic acid in LEM were detected by high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS). The presence of these antioxidant compounds can be considered as a quality parameter for edible medlar fruits.

Keywords: Medlar; *Mespilus germanica;* antioxidant activity; radical scavenging; trolox equivalent

1. Introduction

The medlar (*Mespilus germanica* L.) fruit is brown, sometimes reddish tinged, pear and appleshaped fruits ranging from 1.5 to 3 cm in diameter and weighing from very small (about 10 g) to large (more than 80 g) [1,2]. It grows poorly in frost-free areas, and on rocks and in poor soils. Medlar has elongated leaves and the leaves and flowers are similar to that of apple. The medlar is a typical climacteric fruit which has gained a value in human consumption and commercial importance in

* Corresponding author:E-Mail: igulcin@atauni.edu.tr

The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/RNP © Published 03 /15/2011 EISSN:1307-6167

recent years. In general, ripening occurs late in medlar. The fruits of medlar are used as a nutrition material by the local customer and are consumed by the local people as marmalade. The medlar fruit is also used as treatment of constipation, diuretic and to rid the kidney and bladder of stones [3-5]. Contents of sugars such as fructose and glucose [5,6], linoleic acid and palmitic acid [7], organic acids such as malic acid and citric acid [5,6], aspartate and glutamate [6], potassium [8], amino acids [5] and volatile components [9] were determined in high levels in the mature medlar fruits. Medlar fruits are widely consumed in Turkey and especially in northeast Anatolia where it is one of the unique places where the people grow the wild and alternative cultivars to consume their fruits in different ways. A long list of recipes utilizing medlar fruits such as in jams and jellies are well known [2,4]. The astringency of the fruit is well known and it has been reported that bletted pulp or syrup of the fruit was a popular remedy against enteritis and has many human healing properties. Bletting or blet is a process certain fleshy fruits undergo when, beyond ripening, they have started to decay and ferment [2,4]. Medlar fruits are harvested through October and November storing part of the crop in cold, dark, and aerated places, to induce the fruit to soften. However, the fruits are not appropriate for market sale and home uses [7]. Several common varieties of medlar plant are well-known throughout Europe and Asia [10].

Fruits and vegetables are primary food sources providing essential nutrients for sustaining life. They also contain a variety of phytochemicals such as phenolics and flavonoids, which provide important health benefits [11]. Hence, regular consumption of fruits and vegetables is associated with reduced risks of chronic diseases, such as cancers and cardiovascular disease [12]. A free radical is any atom or molecule that has a single unpaired electron in an outer shell. Free radical-induced oxidative stress has been associated with several toxic cellular processes including oxidation damage to protein and DNA, membrane lipid oxidation, enzyme inactivation, and gene mutation that may lead to carcinogenesis [13]. Antioxidants are reducing agents, and limit oxidative damage to biological structures by passivating free radicals. They are compounds that when added to lipids and lipid-containing foods, can increase their shelf-life by retarding the process of lipid peroxidation. Also, they have been widely used as food additives to avoid food degradation, and they play an important role in preventing many lifestyle-related diseases and aging, being closely related to the formation of reactive oxygen species (ROS) and to lipid peroxidation [14]. Production of ROS during normal cell metabolism is a normal and necessary process that provides important physiological functions. An imbalance between ROS production and antioxidant defences results in oxidative stress which has been recognized as playing a prominent role in the causation of several age-related and chronic diseases, neurodegenerative and cardiovascular diseases. Intake of sufficient amounts of antioxidants is necessary to prevent free radical-induced oxidative stress. It has been reported that most of the antioxidant capacity of fruits and vegetables may come from total phenolics, anthocyanins, and flavonoids [15,16].

Recently, attention has more been focused on assessing the distribution on biologically active compounds among different cultivars [17]. In addition, increasing recent interest in nutraceuticals and functional foods has led plant breeders to initiate selection of crops with higher-than-normal phenolic antioxidant contents [15]. Medlar fruits are very rich sources of bioactive compounds such as phenolics, anthocyanins, organic acids and minerals. Bioactive compounds of medlar fruits, their characterization and utilization in functional foods, and clinical assessment for human health are among the major targets of contemporary research. There is currently heightened interest focused on medlar as major sources of antioxidants may protect against a wide variety of human diseases [18-20]. Previously, health-promoting content of medlar have been reported [5,6]. According to our knowledge, the detailed information about the health-promoting components of medlar is needed to better understand their use as functional foods and as ingredients in pharmaceuticals, nutraceuticals and in medicine.

In this study, we investigated the ferric ions (Fe^{3+}) reducing antioxidant power assay, cupric ions (Cu^{2+}) reducing antioxidant power assay (Cuprac method), DPPH· radical scavenging, $DMPD^{\bullet+}$ radical scavenging, superoxide anion radical scavenging, hydrogen peroxide (H_2O_2) scavenging and

ferrous ions (Fe^{2+}) chelating activities of LEM. These multiple methods are recommended to measure antioxidant properties of food materials that better reflect their potential protective effects.

2. Materials and Methods

2.1. Chemicals

The compounds used for antioxidant activity such as neocuproine (2,9-dimethyl-1,10-phenanthroline), N,N-dimethyl-p-phenylenediamine (DMPD), 2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), nitroblue tetrazolium (NBT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), riboflavin and methionine were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

The following compounds were used as standards in LC-MS/MS analysis: caffeic acid (98%, Sigma-Aldrich), ferulic acid (98% Sigma-Aldrich), syringic acid (97%, Fluka), ellagic acid (95%, Fluka), quercetin (98%, Sigma-Aldrich), α-tocopherol (98%, Fluka), catechol (99% Sigma-Aldrich), pyrogallol (98%, Sigma-Aldrich), p-hydroxybenzoic acid (99%, Merck), vanillin (99% Merck), p-coumaric acid (98%, Sigma-Aldrich), gallic acid (98%, Sigma-Aldrich) and ascorbic acid (99%, Sigma-Aldrich). Stock solutions were prepared as 5 mg/L in ethanol, except for catechol and ascorbic acid, which were prepared as 50 mg/L and 25 mg/L, respectively, in the same solvent. Curcumin (97%) and HPLC grade methanol were purchased from Merck (Darmstadt, Germany). Calibration solutions were prepared in ethanol-water (50:50, v/v) in a linear range (Table 1). Dilutions were performed using automatic pipettes and glass volumetric flasks (A class), which were stored at -20^{o}C in glass containers. 1000 µg/L curcumin solution was freshly prepared, from which 100 µL was used as an Internal Standard (IS) in all LC-MS/MS experiments.

2.2. Preparation of LEM

LEM extraction procedure was carried out as described previously [21,22]. For water extraction, 130 g of each medlar sample was ground into a fine powder in a blender and mixed with 200 mL distilled water by magnetic stirrer for 15 min. Then the aqueous extract was filtered over cheese-cloth and Whatman No. 1 paper, respectively. The filtrates were frozen at -84°C in ultra low temperature freezer (Sanyo, Japan) and lyophilized in a lyophilizator under 5 mm-Hg pressures at -50°C (Labconco, Freezone, Japan).

2.3. Determination of total phenolic content in LEM

The total phenol contents in LEM were determined by using the method of Gülçin et al. [23] based on the procedure described by Singleton and Rossi [24]. The appropriate dilutions of LEM (0.5 mL) were oxidized with Folin-Ciocalteu's reagent (0.5 mL) in a volumetric flask. The reaction was neutralized with saturated sodium carbonate solution (1.5 mL), followed by adjusting the volume to 23 mL with distilled water. The contents in the tubes were thoroughly mixed and allowed to stand at ambient temperature for 2 h until the characteristic blue color developed. Absorbance of the clear supernatants was measured at 725 nm using a spectrophotometer. Quantification was done with respect to the standard of gallic acid. The content of total phenolic in LEM was calculated based on a standard curve prepared using gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of sample. Standard calibration was made from 100 to 500 µg gallic acid (r^2: 0.9711).

Absorbance = 0.0012 x [Phenolics] + 0.0069

The content of total phenolic in LEM was calculated by employing a standard curve as above prepared using gallic acid and expressed as micrograms of gallic acid equivalents [25,26].

2.4. Determination of total flavonoids contents in LEM

The total flavonoid contents in lyophilized aqueous extract of medlar fruits were determined using the aluminum chloride colorimetric method of Köksal and Gülçin [27] based on the method of Adedapo and others [28]. The appropriate dilution of extracts (0.5 mL) were mixed with 1.5 mL methanol (95%), followed by 0.1 mL of $AlCl_3$ (10%), 0.1 mL of CH_3COOK (1 M) and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The flavonoid content was calculated using a standard calibration of quercetin solution and expressed as micrograms of quercetin equivalent (QE) per gram of sample.

Absorbance = 0.0141 x [Flavonoids] + 0.0042

The content of total flavonoids in LEM was calculated from the above standard curve prepared using quercetin and expressed as micrograms of quercetin equivalents (QE). Standard calibration was made from 10 to 50 µg quercetin (r^2: 0.9939).

2.5. Preparation of test solution for LC-MS-MS

One hundred mg of LEM was dissolved in 5 mL of ethanol-water (50:50 v/v) in a volumetric flask, from which 1 mL was transferred into another 5 mL of volumetric flask. Then, 100 µL of curcumin was added and diluted to the volume with ethanol-water (50:50 v/v). From the final solution 1.5 mL of aliquot was transferred into a capped autosampler vial and 10 µL of sample was injected to LC. The samples in the auto-sampler were kept at 15°C during the experiment [15].

2.5.1. Instruments and chromatographic conditions

Experiments were performed by a Zivak® HPLC and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometer equipped with a Macherey-Nagel Nucleoder C18 Gravity column (125 x 2 mm i.d., 5 µm particle size). The mobile phase was composed of methanol (A, 0.5% formic acid) in water (B, 0.5% formic acid), the gradient which was 0-1.00 minute 50% A and 50% B, 1.01-30.00 minutes 100% A and finally 30.01-35.00 50% A and 50% B. The flow rate of the mobile phase was 0.3 mL/min, and the column temperature was set to 30°C. The injection volume was 10 µL [15].

2.5.2. LC-MS/MS analysis

The optimum mobile phase solution was determined to be a gradient of acidified methanol and water system and good ionization has been obtained by ESI source using triple quadrupole mass spectrometry system, details of experimental parameters are also given in our previous studies [15,29]. The LOD and LOQ of method have been determined in the range of 0.5- 50µg/L to 5.0-500 µg/L, respectively. The whole validation procedure and uncertainty assessment of the method were reported in the literature [15].

The concentration of the compounds in the plant extract, expressed in µg/L within the linear range, was obtained from the linear regression equation of each compound [15]. If the quantity of compounds above the linear range, the samples were diluted with the mobile phase.

$$Amount\ \ (mg/kg) = \frac{C_a \text{ x } V_{final}}{m \text{ x } V_{initial}} x1000$$

where C_a is the concentration of compound (in µg/L); V_{final} is the final diluted volume, m is amount of extract as gram, $V_{initial}$ is the initial sample volume.

2.6. Fe^{3+} reducing power assay

Reducing power of LEM was measured by the direct reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$, and was determined by measuring absorbance resulting from the formation of the Perl's Prussian Blue complex followed the addition of excess ferric ions (Fe^{3+}). For this reason, the ferric reducing antioxidant power method of Oyaizu (30) with slight modification was used to measure the reducing capacity of LEM [31]. This method is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants [32]. In this method, higher absorbance values indicate greater reducing capacity of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. Different concentrations of LEM (20 µg/mL) in 0.75 mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%). The mixture was incubated at 50°C for 20 min. After 20 min of incubation, the reaction mixture was acidified with 1.25 mL of trichloroacetic acid (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was added to this solution, and the absorbance was measured at 700 nm in a spectrophotometer. Moreover, blanks were also run in parallel with their absorbance values subtracted from those of the samples. The ferric ions (Fe^{3+}) reducing power of LEM was determined as trolox equivalent [33].

2.7. Cupric ions (Cu^{2+}) reducing-CUPRAC assay

In order to determine the cupric ions (Cu^{2+}) reducing ability of LEM, the method proposed by Apak et al. [34] was also used with slight modification [35]. For this reason, 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution ($7.5x10^{-3}$ M) and 0.25 mL CH_3COONH_4 buffer solution (1 m) were added to a test tube, followed by mixing with different concentrations of LEM (20 µg/mL). Then, total volume was adjusted to 2 mL with distilled water, and mixed well. The tubes were stoppered and kept at room temperature. Absorbance was measured at 450 nm against a reagent blank after a waiting period of 30 minutes. Increased absorbance of the reaction mixture indicates increased reduction capability. The Cupric ions (Cu^{2+}) reducing power of LEM was determined as trolox equivalent

2.8. FRAP assay

Another reducing power assay is ferric reducing antioxidant power, which is based upon reduction of Fe^{3+}-TPTZ complex under acidic conditions. In this method, increase in absorbance of blue-colored ferrous form (Fe^{2+}-TPTZ complex) is measured at 593 nm. FRAP reagent was freshly prepared by mixing 25 mL of acetate buffer (0.3 M, pH 3.6), 2.25 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.25 mL of FeCl3 (20 mM) in water solution. 100 µL of LEM dissolved in appropriate solvent were adjusted to 5 mL with acetate buffer. The reaction mixture was incubated at 37°C. The solution was stirred and incubated for 30 min. Finally; the absorbance of mixture was measured at 593 nm [36].

2.9. Chelating activity on ferrous ions (Fe^{2+})

Ferrous ions (Fe^{2+}) chelating activity was measured by inhibiting the formation of Fe^{2+}-ferrozine complex after treatment of test material with Fe^{2+}, following the method of Dinis et al. [37]. Fe^{2+}-chelating ability of LEM was monitored by the absorbance of the ferrous iron-ferrozine complex

at 562 nm. Briefly, different concentrations of LEM (10-30 µg/mL) in 0.4 mL methanol were added to a solution of 0.6 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.1 mL) dissolved in methanol. Then, the mixture was shaken vigorously and left at room temperature for ten minutes. Absorbance of the solution was then measured spectrophotometrically at 562 nm [38]. The ability of each antioxidant to inhibit the formation of the ferrous iron-ferrozine complex, expressed as its Fe^{2+} chelating activity. The control contains only $FeCl_2$ and ferrozine [39]. The ferrous ions (Fe^{2+}) chelating effect of LEM was determined as trolox equivalent

2.10. DPPH• scavenging activity

The total radical scavenging capacity of LEM was determined and compared to that of BHA, BHT, α-tocopherol and trolox by using the DPPH·, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging methods. DPPH· radical scavenging activity is an often used means of comparing the antioxidant capacity of differing compounds. The DPPH· solution has a deep violet color, radical scavenging activity of antioxidant compounds can be measured spectrophotometrically at 517 nm by the loss of the absorbance as the pale yellow non-radical form (DPPH-H) is produced [40]. The hydrogen atom or electron donation abilities of some pure compounds were measured by the bleaching of a purple colored methanol solution of the stable DPPH radical. The method of Blois [41], previously described by Elmastas et al. [42] was used with slight modifications [43], in order to assess the DPPH· free radical scavenging capacity of LEM. The DPPH radical shows absorbance at 517 nm, but its absorption decreases upon reduction by an antioxidant or a radical. When a hydrogen atom or electron was transferred to the odd electron in DPPH·, the absorbance at 517 nm decreased proportionally to the increases of non-radical forms of DPPH [44]. Briefly, 0.1 mM solution of DPPH· was prepared in ethanol and, 0.5 mL of this solution was added to 1.5 mL of LEM solution in ethanol at different concentrations (10-30 µg/mL). These solutions were vortexed thoroughly, and incubated in dark for 30 min. 30 min later, the absorbance was measured at 517 nm against blank samples lacking scavenger [45-50]. Results were expressed as trolox equivalent DPPH· scavenging activity.

2.11. Superoxide radical scavenging activity

Superoxide radicals were generated by method described by Zhishen and others [51] with slight modification [52]. Superoxide radicals were generated in riboflavin, methionine, illuminate and assayed by the reduction of NBT to form blue formazan. All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The concentration of LEM in the reaction mixture was 20 µg/mL. The total volume of the reaction mixture was 3 mL, and the concentrations of the riboflavin, methionine and NBT were $1.33x10^{-5}$, $4.46x10^{-5}$ and $8.15x10^{-8}$ M, respectively. The reaction mixture was illuminated at 25°C for 40 min. The photochemically reduced riboflavin generated $O_2^{\bullet-}$ which reduced NBT to form blue formazan [53]. The un-illuminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. LEM was added to the reaction mixture, in which $O_2^{\bullet-}$ was scavenged, thereby inhibited the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity superoxide anion radicals scavenging activity of LEM was determined as trolox equivalent [54].

2.12. $DMPD^{\bullet+}$ scavenging activity

Finally, antiradical capacity was analyzed by $DMPD^{\bullet+}$ assay. DMPD radical scavenging ability of LEM was performed according to Fogliano and co-workers [55] with slight modification [56]. In the presence of Fe^{3+}, a colored DMPD radical cation is generated; antioxidant compounds able

to transfer a hydrogen atom to $DMPD^{\bullet+}$ cause a decolouration of the solution measured by the decrease in absorbance at 505 nm. DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 mL of deionized water, and 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.25), and the colored radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 mL of a solution of 0.05 M $FeCl_3$. The absorbance of this solution, which is freshly prepared daily, is constant up to 12 h at room temperature. Different concentrations of standard antioxidants or LEM (10-30 µg/mL) were added in test tubes, and the total volumes were adjusted to 0.5 mL with distilled water. Ten min later, the absorbance was measured at 505 nm. One milliliter of $DMPD^{\bullet+}$ solution was directly added to the reaction mixture, and its absorbance was measured at 505 nm. The buffer solution was used as a blank sample [31,55]. Results were expressed as trolox equivalent $DMPD^{\bullet+}$ scavenging activity.

2.13. Statistical Analysis

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance ANOVA was performed by following the procedures. Significant differences between means were determined by Duncan's Multiple Range tests, and $p<0.05$ was regarded as significant, and $p<0.01$ was very significant.

3. Results and Discussion

The recent focus of interest on phenolic acids stems from their potential protective role, through ingestion of fruits and vegetables, against oxidative damage diseases such as coronary heart disease, stroke, and cancers, cardiovascular diseases and inflammation. Also, researchers and food manufacturers are interested in phenolic acids because of their strong antioxidant properties, abundance in the human diet, and their probable role in the prevention of various diseases associated with oxidative stress [15]. Phenolic acids are regarded as one of the functional food components in fruits, and are thought to contribute to the health effects of plant-derived products by scavenging free radical species, inhibiting free radical formation, and preventing oxidative damage to DNA [10,15]. Our study provides valuable information on the antioxidant capacity of medlar.

Polyphenols, the large group of phytochemicals, are known to act as antioxidants. The content of phenolic compounds (mg/g) in LEM was expressed as milligram of gallic acid equivalents (GAE) (Table 1). The total phenolic compounds in LEM were found as 25.08 mg GAE. Phenolic compounds are likely to contribute to the radical scavenging activity of these plant extracts. The content of flavonoid compounds in LEM was determined as milligram quercetin equivalent (Table 1). The total flavonoids in LEM were found as 2.39 mg QE.

Table 1. Total phenolics, flavonoids and yield determination of LEM (LEM: Lyophilized aqueous extract of medlar fruits).

	LEM
Extraction yield[*]	4.71
Total phenolics[§]	25.08
Total flavonoids [ψ]	2.39

[*]: It was expressed as percentage
[§]: It was calculated as gallic acid equivalent
[ψ]: It was calculated as quercetin equivalent

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. The recent focus of interest on phenolic acids stems from their potential protective role, through ingestion of

fruits and vegetables, against oxidative damage diseases such as coronary heart disease, stroke, and cancers. The profile of phenolic acids in LEM was analyzed by HPLC-MS-MS. Referring to Table 2 and Figures 1 and 2; it is clearly shown that caffeic acid and p-coumaric acid are the predominant phenolic compounds identified in free form in LEM. Among phenolic compounds catechol, p-hydroxybenzoic acid, vanillin and gallic acid could not detected in LEM. On the other hand, 13.4 mg of α-tocopherol and 184.6 mg of ascorbic acid were detected in LEM by LC-MS/MS (Table 2).

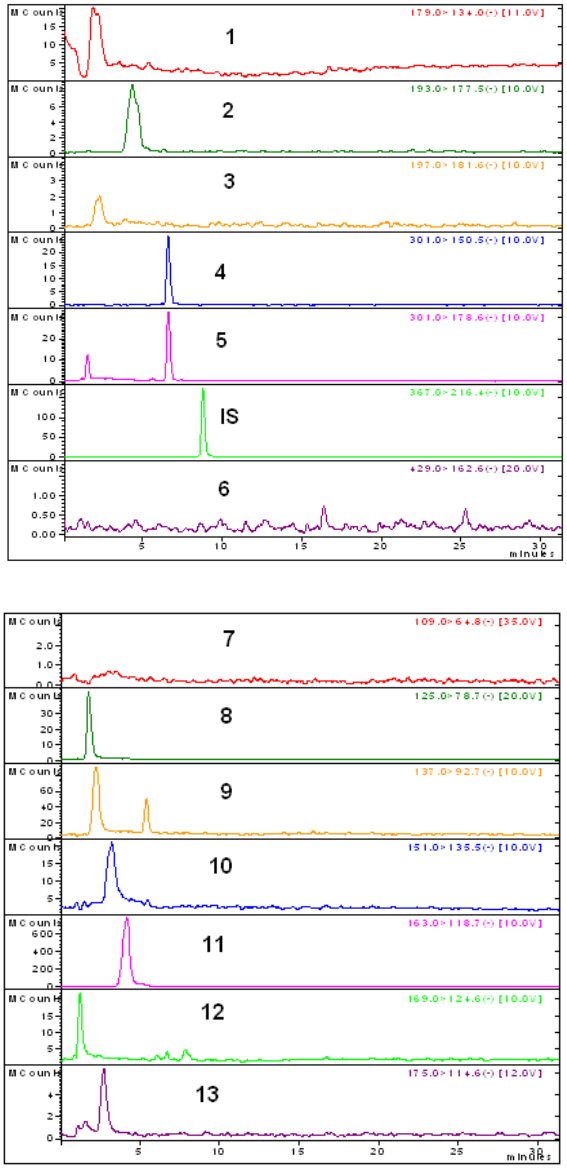

Figure 1. LC-MS/MS Chromatogram of antioxidants

Table 2. LC-MS/MS parameters of selected compounds and amount of antioxidants in LEM in mg/kg concentration (LEM: Lyophilized aqueous extract of medlar fruits).

No	Compounds	Parent ion	Daughter ion	Collision energy (V)	Amount of phenolic compounds in LEM (mg/kg)
S	Curcumin *	367	216.4	10	-
1	Caffeic acid	179	134.0	11	4.9
2	Ferulic acid	193	177.5	10	2.4
3	Syringic acid [a]	197	181.6	10	-
4	Ellagic acid	301	150.0	10	0.2
5	Quercetin	301	178.6	10	2.4
6	α-Tocopherol	429	162.6	20	13.4
7	Catechol [a]	109	64.8	35	-
8	Pyrogallol	125	78.7	20	3.6
9	p-Hydroxybenzoic acid [a]	137	92.7	10	-
10	Vanillin [a]	181	135.5	10	-
11	p-Coumaric acid	163	118.7	10	2.4
12	Gallic acid [a]	169	124.6	10	-
13	Ascorbic acid	175	114.0	12	184.6

*: internal standard,

[a]: (-)These values are below the limits of the quantification

Antioxidants such as phenolics and flavonoids, present in fruits and vegetables, have been positively correlated to the reduced incidence of heart disease, some cancers, and age-related degenerative diseases. Medlar fruits investigated in this study were shown to be a novel rich source of polyphenolic and antioxidant compounds. This study demonstrated that medlar has high potential value for fruit growers as well as the food manufacturer because of their high polyphenolic contents. The polyphenolics are a structurally diverse class of plant secondary metabolites. Generally they possess an aromatic ring bearing one or more hydroxyl substituent [15,57].

Different antioxidant compounds may act through different mechanisms; consequently, one method alone cannot be utilized to fully evaluate the antioxidant capacity of foods and does not reflect antioxidant capacity of pure compounds. For this reason, different antioxidant capacity tests with different approaches and mechanisms have been carried out [58]. In present study, we have demonstrated the antioxidant and radical scavenging mechanism of LEM by using different in vitro bioanalytical methodologies. As it has been reported in many studies, the activities of natural antioxidants in influencing diseases are closely related to their ability to reduce DNA damage, mutagenesis, carcinogenesis and inhibition of pathogenic bacterial growth [59]. Antioxidant activity is widely used as a parameter for food and medicinal bioactive components. In this study, the antioxidant and radical scavenging activities of LEM were evaluated as trolox equivalent. These comparisons were made using a series of in vitro tests including DPPH· free radical scavenging, $DMPD^{\bullet+}$ scavenging, total antioxidant activity by the ferric thiocyanate method, reducing power by two methods (Fe^{3+}-Fe^{2+} transformation and Cuprac assays), superoxide anion radical scavenging generated, hydrogen peroxide scavenging and metal chelating on ferrous ions (Fe^{3+}) activities.

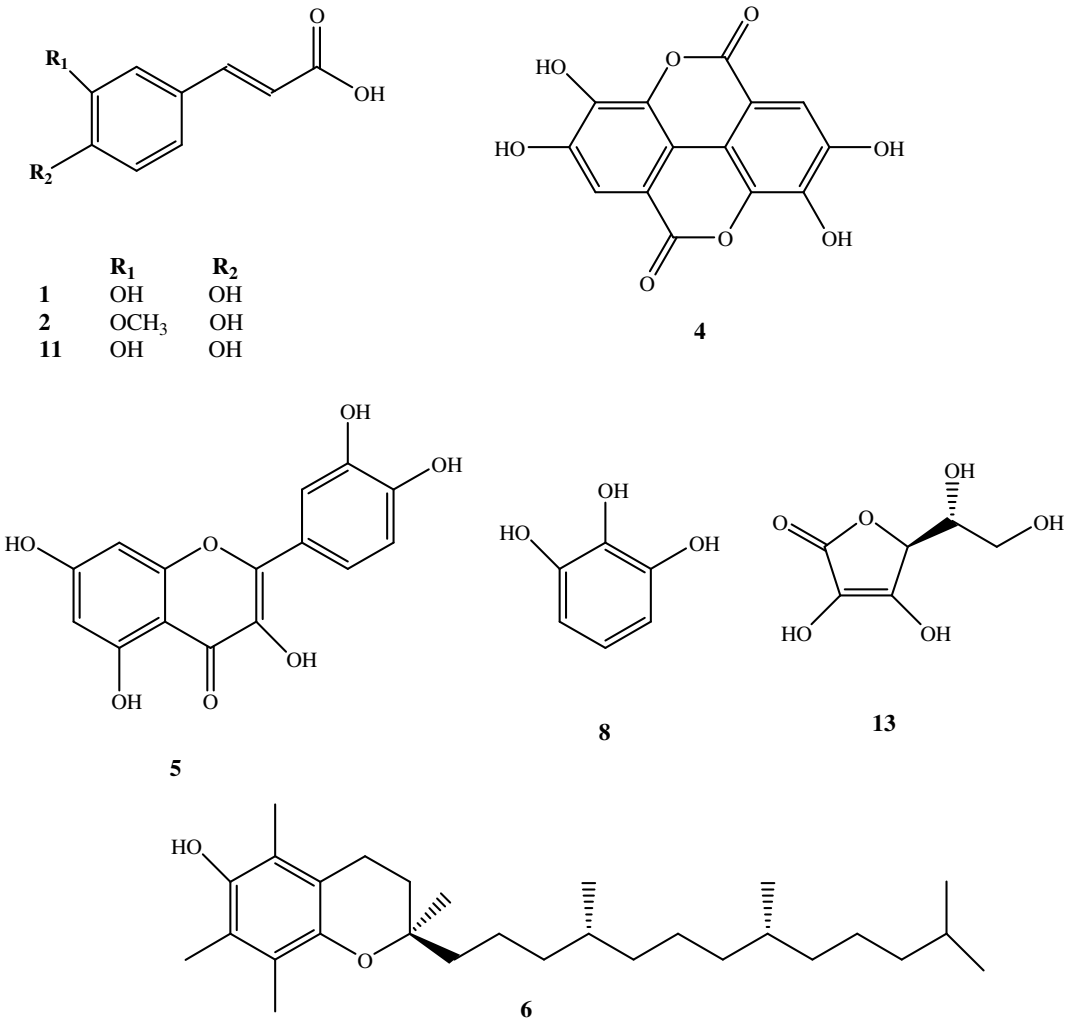

Figure 2. Identified compounds from *Mespilus germanica* L.

There are a number of assays designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to withstand free radical stress [60]. Reducing power reflects the electron donating capacity of bioactive compounds, is associated with antioxidant activity. The reducing capacity of a compound can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN^-)_6]_3$, which has a strong absorbance at 700 nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. The ferric ion reducing antioxidant power assay takes advantage of an electron transfer reaction in which a ferric salt is used as an oxidant [61]. In this assay, the yellow color 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.

LEM had effective reducing power determined by using the potassium ferricyanide $[Fe^{3+}(CN^-)_6]$ reduction and cupric ions (Cu^{2+}) reducing methods when compared to the standards. Such an assay may indicate just how easily a given antioxidant donates electrons to reactive free radicals species,

thus promoting the termination of free radical chain reactions. Electron donation is an important means by which antioxidants promote the formation of less reactive species, and may be assessed using the reducing power assay. For the measurements of the reductive ability of LEM, Fe^{3+}-Fe^{2+} transformation was investigated in the presence of LEM using the method of Oyaizu [30]. As can be seen from Table 3, LEM demonstrated powerful Fe^{3+} reducing ability as trolox equivalent. The reducing power of medlar was found as 0.69 µg TE. These results demonstrated that LEM had marked ferric ions (Fe^{3+}) reducing ability and had electron donor properties for neutralizing free radicals by forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

In the present study, we use the Cuprac assay which is based on reduction of Cu^{2+} to Cu^{+} by antioxidants. This method is simultaneously cost-effective, rapid, robust, selective and suitable for a variety of antioxidants regardless of chemical type or hydrophilicity [15,62]. Moreover, it was reported that the results obtained from in vitro cupric ion (Cu^{2+}) reducing measurements might be more efficiently extended to the possible in vivo reactions of antioxidants. Cuprac chromogenic redox reaction is carried out at a pH (7.0) close to the physiological pH, and the method is capable of measuring thiol-type antioxidants such as glutathione and non-protein thiols unlike the widely applied FRAP test, which is non-responsive to -SH group antioxidants [62]. On the other hand, cupric ion (Cu^{2+}) reducing ability of LEM was determined as trolox equivalent and shown in Table 3. Cu^{2+} reducing powers of LEM at the same concentration (30 µg/mL) were found as 0.43 µg TE.

Table 3. DPPH·, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging, ferrous ion (Fe^{2+}) chelating, Fe^{3+}-Fe^{2+} reducing ability, Cu^{2+}-Cu^{+} reducing ability, FRAP reducing ability of liyophlised extract of medlar fruits (LEM) as trolox equivalent. (LEM: Liyophlised extract of medlar fruits, FRAP: Ferric reducin antioxidant power, DPPH·: 1,1-diphenyl-2-picryl-hydrazyl radicals, $DMPD^{\bullet+}$: N,N-dimethyl-p-phenylenediamine eadicals, $O_2^{\bullet-}$: superoxide anion radicals radicals)

Antioxidant assays	Trolox equivalent antioxidant activity
DPPH· scavenging	0.62
$DMPD^{\bullet+}$ scavenging	0.81
$O_2^{\bullet-}$scavenging	1.41
Fe^{2+} chelating	2.76
Fe^{3+}-Fe^{2+} reducing	0.69
Cu^{2+}-Cu^{+} reducing	0.43
FRAP reducing assay	0.36

The FRAP assay is based on the measurement of the ability of the substance to reduce Fe^{3+} to Fe^{2+} and was initially proposed to measure the total antioxidant capacity. It can be used to measure the total reducing capability of antioxidants. Fe^{2+} is measured spectrophotometrically via determination of its colored complex with TPTZ, which has a high absorbance at 593 nm. Since the antioxidant activity of a substance is usually correlated directly to its reducing capacity, the FRAP assay provides a reliable method to study the antioxidant activity of various compounds [61]. We selected the FRAP assay to evaluate the antioxidant activities of LEM for following reasons. Firstly, the FRAP assay treats the antioxidants in the samples as reductants in a redox-linked colorimetric reaction. Secondly, the procedure of FRAP assay is relatively simple and easy to be standardized. This method has been frequently used for a rapid evaluation of the total antioxidant capacity of various food and beverages and also different plant extracts [19,63]. Furthermore, it has been applied to measure the antioxidant activity of dietary polyphenols and a limited number of flavonoids in vitro [64]. One possible disadvantage of this assay is the fact that this assay does not react fast with some antioxidants, such as glutathione. However, we consider that FRAP assay is still suitable for assessment of antioxidant

activity of fruit samples because only limited amounts of plant glutathione are absorbed by humans [65].

Iron is essential for life because it is required for oxygen transport, respiration, and activity of many enzymes. In complex systems, such as food and food preparations, various different mechanisms may contribute to oxidative processes, such as Fenton reaction, where transition metal ions play a vital role. Different reactive oxygen species might be generated and various target structures such as lipids, proteins, and carbohydrates, can be affected. If they undergo the Fenton reaction, these reduced metals may form highly reactive hydroxyl radicals, and thereby, contribute to oxidative stress [66]. The resulting oxy radicals cause damage to cellular lipids, nucleic acids, proteins, and carbohydrates, and lead to cellular impairment. Since ferrous ions are the most effective pro-oxidants in food systems, the good chelating effect would be beneficial, and removal of free iron ion from circulation could be a promising approach to prevent oxidative stress-induced diseases. When iron ion is chelated, it may lose pro-oxidant properties. Iron, in nature, can be found as either ferrous (Fe^{2+}) or ferric ion (Fe^{3+}), with the latter form predominant in foods. Ferrous chelation may render important antioxidative effects by retarding metal-catalyzed oxidation [34].

Ferrous ion chelating activities of LEM are shown in Table 3. The chelation of ferrous ions by LEM was determined according to the method of Dinis et al. [37]. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. An effective ferrous ion chelator affords protection against oxidative damage by removing iron that may otherwise participate in HO· generation via the Fenton type reactions. Ferric ions (Fe^{3+}) also produce radicals from peroxides although the rate is ten-fold less than that of ferrous ion. Ferrous ions (Fe^{2+}) are the most powerful pro-oxidant among the various species of metal ions [67,68]. Minimizing ferrous ion may afford protection against oxidative damage by inhibiting production of ROS and molecular damage. Ferrozine can quantitatively form complexes with Fe^{2+} in this method. In the presence of chelating agents, complex formation is disrupted, resulting in a reduction in the red color of the complex. Measurement of color reduction therefore allows estimation of the metal chelating activity of the coexisting chelator. Lower absorbance indicates higher metal chelating activity. Metal chelation is an important antioxidant property [69] and hence LEM was assessed for its ability to compete with ferrozine for ferrous ion in the solution.

One measurement of the metal-chelating activity of an antioxidant is based on the absorbance measurement of Fe^{2+}-ferrozine complex after prior treatment of a ferrous ion solution with test material. Ferrozine forms a complex with free Fe^{2+}, but not with Fe^{2+} bound to other chelators; thus, a decrease in the amount of ferrozine-Fe^{2+} complex formed after treatment indicates the presence of antioxidant chelators. The ferrozine-Fe^{2+} complex produced a red chromophore with absorbance that can be measured at λ_{562} nm. A significant drawback of this complexation reaction in measuring the presence of antioxidant chelator is that the reaction is affected by both the antioxidant-Fe^{2+} and ferrozine-Fe^{2+} complex formation constants, and the competition between the two chelators for binding to iron. Thus, a weak antioxidant iron chelator would be seriously underestimated in quantitative determination. From a nutritional point of view, it is not yet possible to access the role of a weak antioxidant iron chelator in preventing the Fenton reaction in vivo. Nonetheless, this reaction serves as a convenient assay to access iron chelating activity of antioxidant [15]

The metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential. EDTA is a strong metal chelator; hence, it was used as standard metal chelator agent in this study. The data obtained from Table 3 reveal that LEM possesses a marked capacity for iron binding, suggesting that its main action as a peroxidation inhibitor may be related to its iron binding capacity. In this assay, LEM interfered with the formation of the ferrous-ferrozine complex. It suggests that LEM has chelating activity and is able to capture ferrous ion before ferrozine. The present study demonstrated that LEM bound ferrous ions (Fe^{2+}).

Metal-binding capacity was investigated by assessing the ability of the antioxidants to compete with the indicator ferrozine to complex with ferrous ions (Fe^{2+}) in solution. LEM had strong chelating effect on ferrous ions (Fe^{2+}). The difference between different concentrations of LEM (10-20 µg/mL) and the control values were statistically significant ($p<0.01$). In addition, LEM exhibited effective chelation of ferrous ion. As can be seen in Table 3, the ferrous ion chelating effect of LEM was found to be 2.76 µg trolox equivalents.

The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reactions, and improve the quality and stability of food products [70]. Assays based upon the use of DPPH· and $DMPD^{\bullet+}$ radicals are among the most popular spectrophotometric methods for determination of the antioxidant capacity of foods, beverages and vegetable extracts. Both chromogens and radical compounds can directly react with antioxidants. Additionally, DPPH· and $ABTS^{\bullet+}$ scavenging methods have been used to evaluate the antioxidant activity of compounds due to the simple, rapid, sensitive, and reproducible procedures [71].

In this study, three radical scavenging methods were used to assess the determination of potential radical scavenging activities of LEM, namely DPPH scavenging, $DMPD^{\bullet+}$ scavenging and superoxide anion radical scavenging activity. With this method, it was possible to determine the antioxidant power of an antioxidant by measuring a decrease in the absorbance of DPPH· at 517 nm. [72].

Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies, and offers a rapid technique for screening the radical scavenging activity of specific compounds. The antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby form a stable end-product which does not initiate or propagate further oxidation of lipid [73].

In DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine. This method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants [74]. Based on the results in Table 3, there is a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of LEM and the reference compounds. DPPH radical scavenging effect of medlar was found as 0.62 trolox equivalent. The inhibition of superoxide anion radical generation by 30 µg/mL concentration of LEM was found 1.41 trolox equivalents. Based on these results, LEM had marked superoxide anion radical scavenging activity.

The principle of the $DMPD^{\bullet+}$ assay is that, DMPD can form a stable and colored radical cation ($DMPD^{\bullet+}$) at acidic pH and in the presence of a suitable oxidant solution. The UV-visible spectrum of $DMPD^{\bullet+}$ shows a maximum absorbance at 505 nm. Antioxidant compounds, which are able to transfer a hydrogen atom to $DMPD^{\bullet+}$, quench the color and produce a decoloration of the solution. This reaction is rapid, and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Therefore, this assay reflects the ability of radical hydrogen-donors to scavenge the single electron from $DMPD^{\bullet+}$ [48,50].

Preliminary experiments show that the choice of oxidant solution and the ratio between the concentration of $DMPD^{\bullet+}$ and the concentration of the oxidative compound are crucial for the effectiveness of the method. In fact, formation of radical cation is very slow and results in a continuous increase of the absorbance. The best results were obtained with $FeCl_3$, which gives a stable colored solution up to a final concentration of 0.1 mM. Moreover, this method ensures low cost and highly reproducible analysis [34].

DMPD assay is particularly suitable for hydrophilic antioxidants, but is less sensitive to hydrophobic bioactive compounds [55], the opposite for the other two tests. In contrast to the ABTS

procedure, the $DMPD^{\bullet+}$ method guarantees a very stable end point. This is particularly important when a large-scale screening is required. It was reported that the main drawback of the $DMPD^{\bullet+}$ method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-tocopherol or BHT were used. Hence, these standard antioxidant compounds were not used in this antiradical assay. As shown in Table 3, LEM had effective $DMPD^{\bullet+}$ radical scavenger. LEM demonstrated 0.81 trolox equivalent. Similarly LEM exhibited effective $DMPD^{\bullet+}$ radical scavenging. [15]. Superoxide is an oxygen-cantered radical with selective reactivity. Although a relatively weak oxidant, superoxide exhibits limited chemical reactivity, but can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids [75]. These species are produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complexes such as cytochrome c. Superoxide anions are a precursor to active free radicals that have potential for reacting with biological macromolecules, and thereby, inducing tissue damage. Superoxide is easily formed by radiolysis of water in the presence of oxygen, which allows accurate reaction rate constants to be measured [68]. It has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical. Also, superoxide has been observed to directly initiate lipid peroxidation [76]. It has also been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical [77]. Superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of free radicals and oxidizing agents [50]. Superoxide anions derived from dissolved oxygen by the riboflavin / methionine / illuminate system will reduce NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm [78]. Antioxidants inhibit the blue NBT formation [79]. The test implements two principal reactions [80]:

$$2NBTH\cdot \rightarrow NBT + NBTH_2 \quad \text{(Formazan)} \qquad \text{(a)}$$

$$NBTH\cdot + O_2 \leftrightarrow NBT + O_2^{\bullet-} \quad \text{(A quasi eq.)} \qquad \text{(b)}$$

When the riboflavine is photochemically activated, it reacts with the NBT to give NBTH· [79] that leads to formazan according to the reaction (a). In presence of oxygen, concentrations of radical species are controlled by the quasi equilibrium (b). Thus, superoxide anions appear indirectly when the test is performed under aerobic conditions. In the presence of an antioxidant molecule that can donate an electron to NBT, the purple color typical of the formazan decays, a change that can be followed spectrophotometrically at 560 nm. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table 4 shows the inhibition of superoxide radical generation by LEM as trolox equivalent.

The superoxide radicals are a highly toxic species that are generated by numerous biological and photochemical reactions via the Haber-Weiss reaction. It can generate the hydroxyl radical, which reacts with DNA bases, amino acids, proteins, and polyunsaturated fatty acids, and produces toxic effects. LEM was the most powerful in inhibiting the superoxide radical generation in the sample used and was found to be 1.41 trolox equivalents (Table 3). Based on these results, it is concluded that LEM had higher superoxide anion radical scavenging activity.

In conclusion, LEM was found to be an effective antioxidant in several in vitro assays including: ferric ions (Fe^{3+}), cupric ions (Cu^{2+}) and Fe^{3+}-TPTZ reducing abilities, DPPH·, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging, H_2O_2 scavenging and metal chelating activities as trolox equivalent.

Acknowledgments

The authors would like to express their gratitude to the Research Fund of the Atatürk University for the funding of Project (BAP-2008/75).

References

[1] K. Browicz and L. Mespilus (1972). In Davis PH. (Ed.). Flora of Turkey and the East Aegean Islands, Vol. 4, pp. 128-129, Edinburgh: Edinburgh University Press.

[2] C. Bignami (2000). Il nespolo comune. *L'Informace Agrario,* **25**, 43-46.

[3] R. Baird and JW. (1989). Thieret The medlar (*Mespilus germanica*, Rosaceae) from antiquity to obscurity. Economic Botany 3; Bassi E 1932. Frutticoltura. In Nuova Enciclopedia Agraria Italiana, Parte quinta. Unione Tipografica Torinese, Torino.

[4] T. Baytop (1999). Therapy with medicinal plants in Turkey-past and present, 2nd, Nobel Tip Basimevi, p. 299, Çapa-Istanbul.

[5] R.H. Glew, F.A. Ayaz, C. Sanz, D.J. VanderJagt, H.S. Huang, L.T. Chuang and M. Strnad (2003). Changes in sugars, organic acids and amino acids in medlar (*Mespilus germanica* L.) during fruit development, *Food Chem.* **83,** 363-369.

[6] R.H. Glew, F.A. Ayaz, C Sanz, D.J. VanderJagt, H.S. Huang, L.T. Chuang and S. Miroslav (2003). Effect of postharvest period on sugar, organic acid and fatty acid compositions in commercially sold medlar (*Mespilus germanica* 'Dutch') fruit, *Eur. Food Sci. Technol.* **216**, 390-394.

[7] F.A. Ayaz, O. Demir, H. Torun, Y. Kolcuoglu and A. Colak (2008). Characterization of polyphenoloxidase (PPO) and total phenolic contents in medlar (*Mespilus germanica* L.) fruit during ripening and over ripening, *Food Chem.* **106**, 291-298.

[8] R.H. Glew, F.A. Ayaz, D.J. VanderJagt, M. Millson, R. Dris and R. Niskanen (2003). A Research note mineral composition of medlar (*Mespilus germanica*) fruit at different stages of maturity, *J. Food Qual.* **26**, 441-447.

[9] S.M. Pourmortazavia, M. Ghadirib, S.S. (2005). Hajimirsadegh Supercritical fluid extraction of volatile components from *Bunium persicum* Boiss. (Black cumin) and *Mespilus germanica* L. (medlar) seeds, *J. Food Comp. Anal.* **18**, 439-446.

[10] H. Hacıseferogulları, M. Ozcan, M.H. Sonmete and O Ozbek. (2005). Some physical and chemical parameters of wild medlar (*Mespilus germanica* L.) fruit grown in Turkey, *J. Food Eng*. **69**, 1-7.

[11] M. Oktay, İ. Gülçin and Ö.İ. Küfrevioğlu, (2003). Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts, *Lebensm.Wissen Technol.* **36**, 263-271.

[12] L.O. Dragsted, M. Strube and J.C. Larsen (1993). Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacol. Toxicol.* **72**, 116-135

[13] H.E. Poulsen, H. Prieme, S. Loft (1998). Role of oxidative DNA damage in cancer initiation and promotion, *Eur. J. Cancer. Prevent*. **7**, 9-16.

[14] İ. Gülçin, V. Mshvildadze, A. Gepdiremen and R. Elias, (2004). Antioxidant activity of saponins isolated from ivy: a-Hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside F, *Planta Med.* **70**, 561-563.

[15] İ. Gülçin, E. Bursal, H.M. Şehitoğlu, M. Bilsel and A.C. Gören (2010). Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey, *Food Chem. Toxicol.* **48**, 2227-2238.

[16] U.Ş. Harput, Y. Genç, N. Khan and İ Saracoglu, (2011). Radical scavenging effects of different Veronica species, *Rec. Nat. Prod.* **5** 100-107.

[17] G.E. Pantelidis, M. Vasilakakis, G.A. Manganaris and G. Diamantidis (2007). Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and cornelian cherries, *Food Chem.* **102**, 777-783.

[18] N. Deighton, R. Brennan, C. Finn and HV. Davies (2000). Antioxidant properties of domesticated and wild *Rubus species, J. Sci. Food Agric.* **80**, 1307-1313.

[19] R.A. Moyer, K.E. Hummer, C.E. Finn, B. Frei and R.E. Wrolstad (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: vaccinium, rubus, and ribes, *J. Agric. Food Chem.* **50**, 519-525.

[20] D. Stewart, G.J. McDougall, J. Sungurtas, S. Verrall, J. Graham and I. Martinussen (2007). Metabolomic approach to identifying bioactive compounds in berries: Advances toward fruit nutritional enhancement, *Mol. Nutr. Food Res*. **51**, 645-6451.

[21] İ. Gülçin, Ş. Beydemir, İ.G. Şat and Ö.İ. Küfrevioğlu (2005). Evaluation of antioxidant activity of cornelian cherry (*Cornus mas* L.), *Acta Aliment. Hung.* **34**, 193-202.

[22] İ. Gülçin, A.Z. Tel and E. Kirecci (2008). Antioxidant, antimicrobial, antifungal and antiradical activities of *Cyclotrichium niveum* (Boiss.) Manden and Scheng, *Int. J. Food Propert.* **11**: 450-471.
[23] İ. Gülçin, İ.G. Şat, Ş. Beydemir, M. Elmastaş and Ö.İ. Küfrevioğlu (2004). Comparison of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandula stoechas* L.), *Food Chem.* **87**, 393-400.
[24] V.L. Singleton and J.L.Rossi (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents, *Am. J. Enol. Viticult.* **16**, 144-148.
[25] M. Elmastaş, İ. Gülçin, Ş. Beydemir, Ö.İ. Küfrevioğlu and H.Y. Aboul-Enein (2006). A study on the in vitro antioxidant activity of juniper (*Juniperus communis* L.) seeds extracts, *Anal. Lett.* **39**, 47-65.
[26] H. Serbetci Tohma and İ. Gülçin (2010). Antioxidant and radical scavenging activity of aerial parts and roots of Turkish liquorice (*Glycyrrhiza glabra* L.), *Int. J. Food Propert.* **13**, 657-671.
[27] E. Köksal, İ. Gülçin, S.B. Öztürk Sarıkaya and E. Bursal (2008).On the in vitro antioxidant activity of silymarin, *J. Enzym. Inhib. Med. Chem.* **24**, 395-405.
[28] A.A. Adedapo, F.O. Jimoh, A.J. Afolayan and P.J. Masika (2009). Antioxidant properties of the methanol extracts of the leaves and stems of *Celtis Africana, Rec. Nat. Prod.* **3**, 23-31.
[29] A.C. Gören, S. Çıkrıkçı, M. Çergel and G. Bilsel (2009). Rapid quantitation of curcumin in turmeric via NMR and LC-tandem mass spectrometry, *Food Chem.* **113**, 1239-1242.
[30] M. Oyaizu (1986). Studies on product of browning reaction prepared from glucose amine, *Jpn. J. Nut.* **44**,307-315.
[31] T. Ak and İ. Gülçin (2008). Antioxidant and radical scavenging properties of curcumin, *Chem. Biol. Interact.* **174**, 27-37.
[32] G. Zengin, A. Aktumsek, G.O. Guler, Y.S. Cakmak and E. Yildiztugay (2011). Antioxidant properties of methanolic extract and fatty acid composition of *Centaurea urvillei* DC. subsp. *hayekiana* Wagenitz, *Rec. Nat. Prod.* **5**, 123-132.
[33] M.E. Büyükokuroğlu, İ. Gülçin, M. Oktay and Ö.İ. Kufrevioğlu (2001). *In vitro* antioxidant properties of dantrolene sodium, *Pharmacol. Res*. **44**, 491-495.
[34] H.T. Balaydın, İ. Gülçin, A. Menzek, S. Göksu and E. Şahin (2010). Synthesis and antioxidant properties of diphenylmethane derivative bromophenols including a natural product, *J. Enzym. Inhib. Med. Chem.* **25**, 685-695.
[35] R. Apak, K. Güçlü, M. Özyürek and S.E. Karademir (2004). A novel total antioxidant capacity index for dietary polyphenols, vitamin C and E, using their cupric ion reducing capability in the presence of neocuproine: The CUPRAC method, *J. Agric. Food Chem.* **52**, 7970-7981.
[36] R. Apak, K. Güçlü, M. Özyürek, S.E. Karademir and E. Erçal (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas, *Int. J. Food Sci. Nut.* **57**, 292-304.
[37] T.C.P. Dinis, V.M.C Madeira and L.M. (1994). Almeida Action of phenolic derivates (acetoaminophen, salycilate, and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers, *Arch. Biochem. Biophy*. **315**, 161-169.
[38] İ. Gülçin, R. Elias, A. Gepdiremen and L. Boyer (2006). Antioxidant activity of lignans from fringe tree (*Chionanthus virginicus* L.), *Eur. Food Res. Technol*. **223**, 759-767.
[39] İ. Gülçin (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid), *Toxicology* **217**, 213-220.
[40] İ. Gülçin (2007). Comparison of in vitro antioxidant and antiradical activities of L-tyrosine and L-Dopa, *Amino Acids* **32**, 431-438.
[41] M.S. Blois (1958). Antioxidant determinations by the use of a stable free radical, *Nature* **26**, 1199-1200.
[42] M. Elmastas, İ. Türkekul, L. Öztürk, İ. Gülçin, Ö. Işıldak and H.Y. Aboul-Enein (2006). The antioxidant activity of two wild edible mushrooms (*Morchella vulgaris* and *Morchella esculanta*), *Comb. Chem. High T. Scr.* **9**: 443-448.
[43] İ.Gülçin (2006). Antioxidant and antiradical activities of L-Carnitine, *Life Sci.* **78**, 803-811.
[44] İ. Gülçin, E. Kirecci, E. Akkemik, F. Topal and O. Hisar (2010). Antioxidant and antimicrobial activities of an aquatic plant: Duckweed (*Lemna minor* L.), *Turk. J. Biol.* **34:** 175-188.
[45] İ. Gülçin, D. Berashvili and A. Gepdiremen (2005). Antiradical and antioxidant activity of total anthocyanins from *Perilla pankinensis* decne, *J. Ethnopharmacol.* **101**, 287-293.
[46] İ. Gülçin, M. Elmastas and H.Y. Aboul-Enein (2007). Determination of antioxidant and radical scavenging activity of basil (*Ocimum basilicum*) assayed by different methodologies, *Phytother. Res.* **21**, 354-361.
[47] İ. Gülçin, (2009). Antioxidant activity of L-Adrenaline: An activity-structure insight. *Chem. Biol. Interact.* **179**, 71-80.

[48] İ. Gülçin (2010). Antioxidant properties of resveratrol: A structure-activity insight, *Innov. Food Sci. Emerg.* **11**, 210-218.
[49] İ. Gülçin and A. Daştan (2007). Synthesis of dimeric phenol derivatives and determination of in vitro antioxidant and radical scavenging activities, *J. Enzym. Inhib. Med. Chem.* **22**, 685-695.
[50] O. Talaz, İ. Gülçin, S. Göksu and N. Saracoglu (2009). Antioxidant activity of 5,10-dihydroindeno[1,2-b]indoles containing substituents on dihydroindeno part, *Bioorg. Med. Chem*. 17, 6583-6589.
[51] J Zhishen, T Mengcheng and W. Jianming (1999). The determination of flavonoid contents on mulberry and their scavenging effects on superoxide radical, *Food Chem.* **64**: 555-559.
[52] İ. Gülçin, R. Elias, A. Gepdiremen, A. Chea and F. Topal (2010). Antioxidant activity of bisbenzylisoquinoline alkaloids from *Stephania rotunda*: Cepharanthine and fangchinoline, *J. Enzym. Inhib. Med. Chem.* **25**, 44-53.
[53] E. Köksal and İ. Gülçin (2008). Antioxidant activity of cauliflower (*Brassica oleracea* L.), *Turk. J. Agric. For.* **32**, 65-78.
[54] İ. Gülçin, V. Mshvildadze, A. Gepdiremen and R. Elias (2006). Screening of antioxidant and antiradical activity of monodesmosides and crude extract from *Leontice smirnowii* Tuber, *Phytomedicine* **13**, 343-351.
[55] V. Fogliano, V. Verde, G. Randazzo and A. Ritieni (1999). Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines, *J. Agric. Food Chem.* **47**, 1035-1040.
[56] İ. Gülçin. (2008). Measurement of antioxidant ability of melatonin and serotonin by the DMPD and CUPRAC methods as trolox equivalent, *J. Enzym. Inhib. Med. Chem.* **23**, 871-876.
[57] K. Robards, P.D. Prenzler, G. Tucker, P. Swatsitang, W. Glover (1999). Phenolic compounds and their role in oxidative processes in fruits, *Food Chem.* **66**, 401-436.
[58] İ. Gülçin, R. Elias, A. Gepdiremen, K. Taoubi, and E. Köksal, (2009). Antioxidant secoiridoids from fringe tree (*Chionanthus virginicus* L.), *Wood Sci. Technol.* **43**, 195-212.
[59] V. Roginsky and E.A. Lissi (2005).Review of methods to determine chain-breaking antioxidant activity in food, *Food Chem.* **92**, 235-254.
[60] İ. Gülçin, H.A. Alici and M. Cesur, (2005). Determination of in vitro antioxidant and radical scavenging activities of propofol, *Chem. Pharm. Bull.* **53**, 281-285.
[61] İ. Gülçin, M.E. Büyükokuroğlu, M. Oktay and Ö.İ. Küfrevioğlu (2003). Antioxidant and analgesic activities of turpentine of *Pinus nigra* Arn. Subsp. pallsiana (Lamb.) Holmboe, *J. Ethnopharmacol.* **86**, 51-58.
[62] S. Karaman, E. Tütem, K.S. Baskan and R. Apak (2009). Comparison of total antioxidant capacity and phenolic composition of some apple juices with combined HPLC-CUPRAC assay, *Food Chem.* **120**, 1201-1209.
[63] A. Luximon-Ramma, T. Bahorun and A. Crozier (2003).Antioxidant actions and phenolic and vitamin C contents of common Mauritian exotic fruits, *J. Sci. Food Agric.* **83**, 496-502.
[64] R Pulido, L Bravo and F. Sauro-Calixto (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay, *J. Agr. Food Chem.* **48**, 3396-3402.
[65] F.Q. Schafer and G.R. Buettner (2001). Redox state of the cell as viewed through the glutathione disulfide/glutathione couple, *Free Radic. Biol Med.* **30**, 1191-1212.
[66] İ. Gülçin, Ş. Beydemir, H.A. Alici, M. Elmastaş and M.E. Büyükokuroğlu (2004). In vitro antioxidant properties of morphine, *Pharmacol. Res.* **49**. 59-66.
[67] İ. Gülçin, V. Mshvildadze, A. Gepdiremen and R. Elias, (2006). Antioxidant activity of a triterpenoid glycoside isolated from the berries of Hedera colchica: 3-O-(β-D-glucopyranosyl)-hederagenin, *Phytother. Res.* **20**, 130-134.
[68] B. Halliwell and J.M.C. Gutteridge (1984). Oxygen toxicology, oxygen radicals, transition metals and diseas, *Biochem. J.* **219**, 1-4.
[69] İ. Gülçin, (2005). The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds, *Int. J. Food Sci. Nut.* **56**, 491-499.
[70] P.C. Chai, L.H. Long and B. Halliwell (2003).Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines, *Biochem. Bioph. Res. Co.* **304**, 650-654.
[71] B. Özcelik, J.H. Lee and D.B. Min. (2003). Effects of light, oxygen and pH on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method to evaluate antioxidants, *J. Food Sci.* **68**, 487-490.
[72] B. Matthäus (2002).Antioxidant activity of extracts obtained from residues of different oilseeds, *J. Agric. Food Chem.* **50**, 3444-3452.

[73] R. Amarowicz, R.B. Pegg, P. Rahimi-Moghaddam, B. Barl and J.A. Weil (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies, *Food Chem.* **84**, 551-562.
[74] B. Halliwell and S. Chirico (1993). Lipid peroxidation: its mechanism, measurement, and significance, *Am. J. Clin. Nut.* **57**, 715-725.
[75] İ. Gülçin, Ö.İ. Küfrevioğlu, M. Oktay and Buyukokuroglu M.E. (2004). Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.), *J. Ethnopharmacol*. **90**, 205-215.
[76] A.P. Wickens (2001). Aging and the free radical theory, *Resp. Physiol.* **128**, 379-391.
[77] P.G. Pietta (2000). Flavonoids as antioxidants, *J. Nat. Prod.* **63**, 1035-1042.
[78] I. Parejo, F. Viladomat, J. Bastida, A. Rosas-Romero, N. Flerlage, J. Burillo and C. Codina (2002). Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled Mediterranean herbs and aromatic plants, *J. Agric. Food Chem*. 50, 6882-6890.
[79] S.I. Liochev and I. Fridovich (1995). Superoxide from glucose oxidase or from nitroblue tetrazolium, *Arch. Biochem. Biophys.* **318**, 408-410.
[80] C. Beauchamp and I. Fridovich (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels, *Anal. Biochem.* **44**, 276-287.

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