Evaluation of antioxidant and antibacterial activities of methanolic extract of medlar (Mespilus germanica L.) leaves

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ABSTRACT
The present study evaluated the antioxidant and antibacterial activities of methanolic extract of medlar (Mespilus germanica L.) leaves. The antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl radical assay and the antibacterial activity was evaluated by the disc diffusion method and determination of minimum inhibitory concentration. The methanolic extract showed high antioxidant activity (69.43%) and had antibacterial activity against both Gram positive and Gram negative bacteria. Higher inhibition zone was detected against S. aureus (30.83 mm). The extract of this plant showed high phenolic, flavonoids and carotenoids contents and it can be concluded that these compounds may be responsible for antibacterial and antioxidant activity. The results indicated that the methanolic extract of medlar, M. germanica L., leaves possessed strong antibacterial and antioxidant properties and could be an important source of natural compounds for development of new drugs.

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Introduction
Natural products from medicinal plants play a considerable role in the discovery and development of new drugs [1]. About 25% of drugs contain compounds obtained from higher plants [2]. According to the World Health Organization, about 65–80% of the world’s population which lives in developing countries depends essentially on plants for primary health care [3]. In recent years, the antimicrobial and antioxidant actions of medicinal plants have received much attention [4].

Medicinal plants have been investigated for their antioxidant properties [5]. It has been determined that the antioxidant effect of these plants products is mainly attributed to phenolic compounds such as flavonoids and phenolic acids, ascorbic acid, vitamin E and different carotenoids [6]. These natural antioxidants are very effective to prevent the destructive processes caused by oxidative stress induced by free radicals [7]. Free radicals or reactive oxygen species (ROS) have been implicated in the pathology of many diseases, including cancer, coronary artery diseases, hypertension, diabetes and neurodegenerative disorders, in addition to aging [8,9]. Many synthetic antioxidants have been shown to possess toxic and/or mutagenic effects, and this finding has promoted research into the properties of naturally occurring antioxidants [5].

Today, on the other hand, the resistance of bacteria to the available synthetic and semi-synthetic antibacterial agents is growing rapidly [10]. The available antibiotics also cause various adverse drug reactions such as hypersensitivity and immuno-suppression [11]. Due to these negative effects, and the constant development of bacterial resistance, in the pharmaceutical industry there is an urgent need for development of newer antimicrobial agents effective against microorganisms and less harmful to the host. One of the most significant natural sources of antimicrobial agents is medicinal plants [12]. Therefore, it is very important to discover new sources of safe, and inexpensive antioxidants and antimicrobial compounds from natural sources.

Medicinal plants, especially the endemic and edible plants of a region, due to their ability to produce natural compounds with antioxidant capacity and antimicrobial properties and due to their health benefits, are particularly important for the development of new drugs. Medlar, Mespilus germanica L., is a member of Rosaceae, known by the local names ‘Conos’ or
‘Condos’ in the north of Iran. The fruit is brown, ranging from 1.5 to 3 cm in diameter and the leaves are dark green, large, simple, elliptic-oblong, 8–15 cm long and 3–4 cm wide [13]. The native form of medlar is widely found at forests in Guilan, Golestan and Mazandaran Provinces of Iran. The Iranian medlar fruits and leaves have several medical benefits such as elimination of kidney and bladder stones, constipation therapy and as a diuretic, in diseases associated with high blood pressure, cardiac tonus, heart rate, for mouth and throat infections and treatment for diarrhoea [14,15].

Despite the medical benefits and significant therapeutic effects of medlar, *M. germanica*, there are only a few reports about the antimicrobial and antioxidant properties of this medicinal herb [7,16]. To our knowledge, there are no systematic studies on the in vitro antioxidant activity of medlar leaf extract so far, and in previous studies the antimicrobial properties of the leaf extract have been less widely considered. Therefore, the main objective of the present study was to determine the total phenolic and flavonoid content and to evaluate the antibacterial and antioxidant activity of a methanolic extract of medlar (*M. germanica*) leaves.

**Materials and methods**

**Plant material**

The fresh leaves of medlar, *M. germanica*, were collected from the Lalam village, Some’e Sara, Guilan province, North of Iran. These leaves were used to prepare the extract after identification and confirmation of the genus and species of this plant at the Herbarium of faculty of science, University of Guilan, Iran.

**Extraction procedure**

The fresh leaves of the medlar were collected and dried in the open air and then under shade at room temperature. Leaves were placed at 40°C in an oven for 18 h to remove the excess moisture. The dried plant material was then chopped and ground to a powder using a mechanical blender. Dried leaf powder was extracted with methanol (95%) in a Soxhlet apparatus. The extract was filtered through Whatman filter paper grade 1 and the collected filtrate was placed in a rotary evaporator under reduced pressure and controlled temperature (45–50°C) for evaporation to dryness to remove the solvent. The extract was preserved and stored at 4°C in storage vials for further study.

**Determination of total phenolic content**

Total phenolic content of methanolic extracts of the medlar, *M. germanica*, was determined using Folin–Ciocalteu reagent according to the method of Singleton et al. [17]. Briefly, 100 µL of this extract solution was diluted to 3 mL distilled water and then mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent. After 3 min, 2 mL of 20% (w/v) of sodium carbonate was added and the contents were mixed thoroughly. Finally, the mixture was kept at room temperature in darkness and the absorbance of the reaction mixture was measured at 760 nm after 2 h with a UV-VIS spectrophotometer (APEL, PD-303, USA). Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract.

**Determination of total flavonoid content**

Total flavonoid content was determined using the aluminium chloride colorimetric method as described earlier [18]. Briefly, 0.5 mL solutions of extract in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 mol/L potassium acetate and 2.8 mL of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a UV/visible spectrophotometer (APEL, PD-303, USA). Total flavonoid content was calculated as quercetin from a calibration curve. Quercetin was used as standard and the flavonoid content is expressed in terms of milligrams of quercetin equivalents (QE) per gram of dry extract.

**Determination of total carotenoid content**

Total carotenoid content was determined by the method reported by Lichtenthaler and Welburn [19]. Briefly, 1 g of fresh medlar leaves was homogenized with a glass mortar in 50 mL acetone (80% Merck) and the homogenate was then centrifuged at 10,000 rpm (11,536×g) for 15 min at 4°C (UNIVERSAL 320R, Hettich, Germany). The absorbance values of the centrifuged samples were measured at 470 nm with a UV/visible spectrophotometer (APEL, PD-303, USA).

**Antioxidant activity by DPPH assay**

The antioxidant activity of the extracts was determined based on radical scavenging activity by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) according to Kulkarni and Aradhya [20]. A 0.1 mL aliquot of the extract solution (1 mg/mL) was mixed with 0.9 mL of 100 mmol/L Tris–HCl buffer (pH 7.4) to which 1 mL of
DPPH (500 µmol/L in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm using a UV–visible spectrophotometer (APEL, PD-303, USA). The reaction mixture without DPPH was used for background correction. The antioxidant activity was calculated using the following equation:

Antioxidant activity(%) = \( \frac{1 - [A_{\text{sample}}/A_{\text{control}}]}{A_{\text{control}}} \times 100 \)

**Antibacterial activity**

**Preparation of bacterial strains**

Different American-Type Cell Culture (ATCC) reference bacterial strains, including: *Staphylococcus aureus* (ATCC 33591), *Staphylococcus epidermidis* (ATCC 12228), *Salmonella typhi* (ATCC 19430), *Salmonella paratyphi* (ATCC 11511), *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 9997), *Pseudomonas aeruginosa* (ATCC 10662), *Streptococcus pyogenes* (ATCC 10403), *Enterococcus faecalis* (ATCC 29212), *Yersinia enterocolitica* (ATCC 23715), *Seratia marcescens* (ATCC 13880), *Shigella dysenteriae* (ATCC 13313) and *Citrobacter freundii* (ATCC 12453), were obtained from Iranian microbial collections, Pasteur Institute of Iran. Bacterial strains were inoculated on nutrient broth and incubated at 37°C for 24 h. For the antibacterial assay, all strains of bacteria were adjusted to 0.5 Mc-Farland standard by the optical density (OD) method at 620 nm (10^8 cfu/mL) as described earlier [21].

**Antibacterial bioassay**

The antibacterial activity of methanolic extracts of the medlar, *M. germanica*, was assayed by agar disc diffusion method [22]. Briefly, different concentrations of extract (62.5, 125, 250, 500 and 1000 mg/mL) were prepared in dimethyl sulfoxide (DMSO). Bacterial suspensions that were adjusted to 10^8 CFU/mL were spread in Muller–Hinton agar plates using sterile swabs. Then, sterilized paper discs (6 mm Ø; Whatman #1) impregnated with 20 µL of different concentrations of extract were placed on the surface of Muller–Hinton agar plates. After storage at 4°C for 2 h to allow diffusion of the active compounds in the medium, all of the bacterial plates were incubated at 37°C for 24 h. Discs treated with 20 µL DMSO were used as negative controls and gentamicin (10 µg/disk) and ciprofloxacin (5 µg/disk) were used as positive controls. The antibacterial activity was determined by measuring the diameter of the inhibition zone to the nearest millimeter.

**Determination of minimum inhibitory concentration (MIC)**

Minimum inhibitory concentration of active crude extracts was determined by broth microdilution method [23]. The test was performed using polystyrene 96-well plates. Two-fold serial dilutions of the extract were prepared in Cation-Adjusted Muller-Hinton Broth ranging from 1000 to 62.5 mg/mL. Each inoculum was prepared with 50 µL Muller-Hinton Broth and 50 µL of the different concentrations of the extract which were mixed together. Test bacterial inoculum of 50 µL was added to each well of the plate. The plates with bacteria were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of the extract at which the microorganisms showed no visible growth.

**Statistical analysis**

All the experiments were done in triplicate. The SPSS software version 20 was used for data analysis. The results are expressed as mean values with standard deviation (±SD) from three experiments. The experimental data obtained were analyzed for multiple comparisons using one-way analysis of variance (ANOVA) and when the results were significant, Duncan's test was also used.

**Results and discussion**

Medicinal plant tissues are commonly rich in secondary metabolites including phenolic compounds, flavonoids and carotenoids, which exhibit antioxidant and antibacterial activities due to their redox properties and chemical structures. In this study we determined the antioxidant and antibacterial activity of medlar, *M. germanica*, leaf methanolic extract. The results of the antioxidant activity estimated by the DPPH scavenging activity, total phenolic content, total flavonoid content and total carotenoid content of this plant are presented in Table 1.

**Phenolic and flavonoid content**

Phenolic compounds are the major class of natural antioxidants present in plants and are usually quantified using the Folin–Ciocalteu method. In this study, the total phenolic content in the methanolic extract of medlar leaves was 380.58 mg GAE/g dry extract (Table 1). The total flavonoid content of the extract was 75.169 mg QE/g dry extract using the aluminium chloride colorimetric method (Table 1). This result indicated that the methanolic extract of medlar leaves has higher total phenolic and flavonoid content in
Comparison to extracts from this and other plants that have been reported in recent years [18,24,25]. A few studies have reported on the total phenolic and flavonoid content of medlar and the antioxidant capacity of its extracts. For example, Ercisli et al. [26] reported that the average total phenolic content of a methanolic extract of medlar fruits was 194 mg GAE/g; however, our results showed that the total phenolic contents from medlar leaves were more than that of fruits. Also, the phenolic and flavonoid content from medlar found in this work was higher than that reported by Nabavi et al. [18]. Phenolic compounds and flavonoids – due to their property to scavenge free radicals and suppress reactive oxygen formation – are important plant constituents and could be used as a basis for rapid screening of antioxidant activity [27].

### Carotenoid content

As shown in Table 1, the total carotenoid content of the *M. germanica*, leaf methanolic extract was 3.43 mg/g dry extract based on the Lichtenthaler and Welburn method [19]. To our knowledge, this is the first report of the total carotenoid content of *M. germanica* leaves. The antioxidant activity of carotenoids is well documented [28]. The results from this study indicated that methanol can be a suitable solvent for the extraction of phenolic, flavonoid and carotenoid compounds. This result is in accordance with previous findings [29]. Also, it has been observed that methanolic extracts of medicinal plants exhibit higher phenolic, flavonoid and carotenoid content as well as antioxidant activities than aqueous, ethyl acetate, chloroform and acetone extracts [30,31]. Thus, it has been well noted that medicinal plants with high amounts of phenols, flavonoids and carotenoids have potential antioxidant actions [32]. This suggested that the methanolic extract of *M. germanica* leaves could show potent antioxidant activity.

### Antioxidant activity

The antioxidant activity of the *M. germanica* extract was evaluated by DPPH radical scavenging. DPPH is a free radical compound that has widely been used to test the free-radical scavenging abilities of various types of samples [12]. The DPPH radical scavenging test is a sensitive antioxidant assay and depends on substrate polarity, as well as on hydrogen donation and/or radical scavenging activity. Although the DPPH radical has little relevance to biological systems and living organisms, this method is widely regarded as indicative of the capacity of plant extracts to scavenge free radicals, and will refer to hydrogen atom or electron donation ability, independently of any enzymatic activity [33].

The use of the DPPH radical scavenging assay is advantageous in evaluating antioxidant effectiveness because this radical is more stable than hydroxyl or superoxide radicals. The DPPH assay usually involves a hydrogen atom transfer reaction [34]. The decrease in absorbance of DPPH caused by antioxidants is due to the reaction between antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation. This is visualized as a discoloration from purple to yellow.

In this study, the antioxidant activity of the methanolic extract of medlar leaves measured by the DPPH assay is shown in Table 1. The results indicated that the antioxidant activity of this plant extract was equivalent to 69.43% radical inhibition in vitro. Our result is in agreement with previous data on the antioxidant activity of *M. germanica* and showed even stronger scavenging potential than these studies [18,35]. Our results are also in accordance with those of previous works, indicating close relationship between the DPPH radical scavenging effect and antioxidant activity with total phenolic and flavonoid content [7]. The effect of the methanolic extract of medlar leaves on DPPH scavenging is thought to be due to their hydrogen donating ability.

As a rule, the biological properties of plant extracts cannot be attributed to activities of single constituents. The scavenging activity could be explained by the combined effects of different compounds [36]. Several studies have evaluated the relationship between the antioxidant activity of plant products and their total phenolic content. Substances that are able to perform DPPH scavenging can be considered as antioxidants and radical scavengers. Higher total phenol and flavonoid content lead to better DPPH scavenging activity [5,25].

### Antibacterial activity

The results from the antibacterial activity assay of the methanolic extract of *M. germanica* leaves are provided
The antibacterial effect of the methanolic extract could be observed for DMSO used as a negative control. The results showed that the methanolic extracts of medlar leaves possessed relatively higher antibacterial activity against both Gram-positive and Gram-negative bacteria at different concentrations. Higher inhibition zone was detected against S. aureus, which is one of the most common causes of bacteremia and infective endocarditis and is also responsible for food poisoning [37]. The results showed that the methanolic extracts of medlar leaves possessed relatively higher antibacterial activity against Gram-positive than against Gram-negative bacteria. This higher sensitivity of the Gram-positive bacteria than the Gram-negative ones could possibly be attributed to their differences in cell wall structure. Gram-positive bacteria contain an outer peptidoglycan layer, which is an ineffective permeability barrier, while, Gram-negative bacteria possess an outer membrane and a unique periplasmic space which is rich in lipopolysaccharide molecules, presenting a barrier for the penetration of numerous antimicrobial compounds. Similar results have been reported previously [38]. Ciprofloxacin and gentamicin, which were used as positive experimental controls against all bacterial strains assayed, produced a zone of inhibition of 11–27 and 6–40 mm, respectively, while no inhibitory effect could be observed for DMSO used as a negative control. The antibacterial effect of the methanolic extract of medlar leaves against S. aureus, S. epidermidis and E. coli was stronger than that of gentamicin, ciprofloxacin did not have any effect on S. epidermidis and E. coli. The MIC values of the leaf extract ranged from 62.5 to 125 mg/mL as shown in Table 2. Only a few works in the literature have reported on the antimicrobial activity of M. germanica. Ahmady-Asbchin et al. [16] reported antibacterial effects of methanolic and Ethanolic leaf extracts of medlar against S. aureus, E. coli and P. aeruginosa. To our knowledge, this study reports the antibacterial activity of methanolic extract of medlar leaves against some pathogenic bacteria, including S. epidermidis, S. paratyphi, E. faecalis, Y. enterocolitica, S. marcescens and C. freundii, for the first time.

The results from this study suggest that the antibacterial activity of the extract could be attributed to the high content of phenols and flavonoids. Several studies have reported the antibacterial effects of these metabolites against a wide range of bacteria [39–41]. Numerous works have also reported that carotenoids have potential antibacterial effects on various bacteria [42]. Phenolic compounds and especially flavonoids can act as antimicrobial agents via several different mechanisms, including inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function, inhibition of energy metabolism, inhibition of the attachment and biofilm formation, inhibition of the porin on the cell membrane, alteration of the membrane permeability, which can result in cell destruction, and attenuation of pathogenicity [43]. It seems that phenolic compounds are synthesized by medicinal plants in response to microbial infection; it is therefore logical that they have been found in vitro to be effective antimicrobial substances against a wide array of pathogenic microorganisms [43,44].

Conclusions

The present study demonstrated that the methanolic extract of medlar, M. germanica, and leaves possessed strong antibacterial and antioxidant properties. This
extract showed high phenolic, flavonoid and carotenoid content. It could be speculated that these compounds may be responsible for its antibacterial and antioxidant activity. As far as we know, this is the first simultaneous study of the antibacterial and antioxidant activity of metanolic medlar leaf extract and of the total carotenoid content of this plant. With regard to the results, the methanolic extract of medlar leaves could be an important source of natural compounds with antioxidant capacity and antibacterial properties for development of new drugs. Further studies are needed to isolate the bioactive compounds from the extract and to elucidate the exact mechanism of action of the free radical scavenging effect and the antibacterial activity. Also cytotoxicity to human cells and physiological relevance of different concentrations of the extract should be subject of further investigation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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