ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITIES OF *MENTHA PIPERITA*

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Summary

Antihemolytic and antioxidant activities of *Mentha piperita* aerial part were investigated employing different in vitro assay systems. The extract showed moderate antioxidant activity in some models. IC_{50} for DPPH radical-scavenging activity was 129.3 ± 4.5 µg/ ml. The extract showed potent nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1}. The extract showed good Fe^{2+} chelating ability. IC_{50} was 698.3± 18.8 µg/ ml. The extract also exhibited low antioxidant activity in the linoleic acid model but were capable of scavenging hydrogen peroxide in a concentration dependent manner. The total amount of phenolic compounds in each extract was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Biological effects may be attributed, at least in part, to the presence of phenols and flavonoids in the extract.

Key words: Antioxidant activity, Antihemolytic activity, *Mentha piperita*.

Introduction

Antioxidants are compounds that can delay or inhibit the oxidation damage of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (1). The antioxidant activity of phenolic compounds such as flavonoids is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (2). In general, there are two basic groups of antioxidants, natural and synthetic. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects (3).

Herbs have been used for a large range of purposes including medicine, nutrition, flavorings, beverages, dyeing, repellents, fragrances, cosmetics, charms, smoking, and industrial uses. Since prehistoric times, herbs were the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century. Today, herbs are still found in 40% of prescription drugs (4). Culinary herbs have been grown and used for hundreds of years, and they are becoming increasingly popular in the United States for their ability to enhance and complement the flavors of a wide variety of foods (5). Even though a variety of herbs are known to be good sources of phenolic compounds, their compositional data are insufficient (6). Moreover, various herbs along with vegetables and fruits contain numerous phytochemicals in addition to phenolic compounds, such as nitrogen compounds, carotenoids and ascorbic acid (1,7). Many of these phytochemicals possess significant antioxidant capacities that are associated with lower incidence and lower mortality rates of cancer in several human cohorts (1).

In the world, a variety of plants are commonly used as remedies for the treatment of many diseases such as neurodegenerative disease, cancer, inflammation. Many studies have shown that some plants have beneficial effects (8-13). *Mentha piperita* (Family *Labiatae*; genus *Mentha*) is one of the most widely used plants in Iran (14) and is commonly used as vegetable. Previously some biological and pharmacological activities of this plant have been reported (15). The aim of this work was to determine the antioxidant and antihemolytic activities of aerial parts extract of *Mentha piperita* in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

Materials and methods

Plant materials and preparation of freeze-dried extract: Aerial parts of *Mentha piperita* was collected from panbe chule village, north of sari, Iran, in summer 2009. Sample identified by Dr. Bahman Eslami. Sample was dried at room temperature and coarsely ground before extraction. A known amount of sample was extracted at room temperature by percolation method using Ethanol/water (70:30). The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

Chemicals: Trichloroacetic acid (TCA), 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Potassium ferricyanide and Hydrogen peroxide H_2O_2 were purchased from Sigma Chemicals Co. (USA). Butylated hydroxyanisole (BHA), Vitamin C, Sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, Ethylenediaminetetraacetic acid (EDTA) and Ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Determination of total phenolic compounds and flavonoid content: Total phenolic compound contents were determined by the Folin-Ciocalteau method (16,17). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g/ l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated as previously described (18,19). Briefly, 0.5 mL solution of extract in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with

a double beam spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

Antioxidant activity

DPPH radical-scavenging activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the extract (20). Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and Quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Determination of metal chelating activity: The ability of the *M. piperita* extract to chelate ferrous ions was estimated by our recently published paper (21). Briefly, different concentrations of extract were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixtures was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solutions was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_1)/A_0]$ x100, where A_0 was the absorbance of the control, and A_1 of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

Assay of nitric oxide-scavenging activity: The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of extract dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (22,23).

Scavenging of hydrogen peroxide: The ability of the extract to scavenge H_2O_2 was determined according to the method of Dehpour et al. (24). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of H_2O_2 was determined by absorption at 230 nm using a spectrophotometer. The Extract (0.1-3.2 mg/ ml) in distilled water were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging by the extract and standard compounds was calculated as follows: % Scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$ where A_o was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

Reducing power determination: The reducing power of extract was determined according to our recently published paper (25). 2.5 ml of extract (25-800 μg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop

the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Hemoglobin-induced linoleic acid peroxidation test: The antioxidant activity of extract was determined by a modified photometry assay (26). Reaction mixtures (200 ml) containing 10 ml of extract (10–400 mg), 1 mmol/ l of linoleic acid emulsion, 40 mmol/ l of phosphate buffer (pH 6.5) and 0.0016% hemoglobin, were incubated at 37ºC for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480 nm after colouring with 100 ml of 0.02 mol/l of $FeCl_2$ and 50 ml of ammonium thiocyanate (30 g/100 ml). Vitamin C was used as positive control.

Antihemolytic activity

Preparation of rat erythrocytes: Experiments were carried out with the approval of institutional animal ethical committee. Male Wistar rats in the body weight range of 180–220g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were sacrificed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored (27). Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4ºC, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4ºC and used within 6 h for further studies.

Antihemolytic activity of extract against H_2O_2 induced hemolysis: The inhibition of rat erythrocyte hemolysis was evaluated according to the procedure described by Ebrahimzadeh et al. (28). The rat erythrocyte hemolysis was performed with H_2O_2 as free radical initiator. To 100 µl of 5% (v/v) suspension of erythrocytes in PBS, 50 µl of extract with different concentrations (5–25 µg in PBS pH 7.4), which corresponds to 100– 3200 µg of extract, was added. To this, 100 µl of l M H_2O_2 (in PBS pH 7.4) was added. The reaction mixtures were shaken gently while being incubated at 37ºC for 3 h. The reaction mixtures were diluted with 8 ml of PBS and centrifuged at 2000×g for 10 min. The absorbance of the resulting supernatants was measured at 540 nm by spectrophotometer to determine the hemolysis. Likewise, the erythrocytes were treated with 100 µM H_2O_2 and without inhibitors (plant extract) to obtain a complete hemolysis. The absorbance of the supernatants was measured at the same condition. The inhibitory effect of the extract was compared with standard antioxidant Vitamin C. To evaluate the hemolysis induced by extract, erythrocytes were preincubated with 50 µl of extract corresponding to 25 µg extract for 1 h and the hemolysis was determined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 µM H_2O_2 as 100%. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

Statistical analysis: Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range tests. The EC_{50} values were calculated from linear regression analysis.

Results and discussion

Total phenol and flavonoid contents: Total phenol compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve (y = 0.0063x, r^2 =0.987). The total phenolic content of extract was 13.4 ± 0.3 mg gallic acid equivalent/g of extract powder, respectively. The total flavonoid content was 122.3 ± 4.1 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve (y= 0.0067x+ 0.0132, r^2= 0.999). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (29). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (30).

DPPH radical-scavenging activity: The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (31,32). DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (26). It was found that the radical-scavenging activity of extract increased with increasing concentrations. IC_{50} for DPPH radical-scavenging activity was 129.3± 4.5 µg/ml. The IC_{50} values for Ascorbic acid, quercetin and BHA were 1.26± 0.11, 1.32± 0.07 and 13.49± 1.04 µg/ml, respectively.

Metal chelating activity: In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex was decreased dose dependently, i.e. the activity was increased on increasing concentrations from 0.2 to 3.2 mg ml^{-1}. Metal chelating capacity was significant since the extract reduced the concentrations of the catalyzing transition metal in lipid peroxidation (21). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (33). *M. piperita* extract showed weak Fe^{2+} chelating ability. IC_{50} were 698.3 ± 18.8 µg/ ml. EDTA showed very strong activity (IC_{50} = 18 µg ml^{-1}).

Nitric oxide-scavenging activity: The extract showed potent nitric oxide-scavenging activity between 50 and 800 µg ml^{-1}. The % inhibition was increased with increasing concentrations of the extract. IC_{50} was 170.7 ± 6.9 µg/ ml. IC_{50} for quercetin was 17.01 ± 0.03 µg/ ml. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (20, 35). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

Scavenging of hydrogen peroxide: This effect may be attributed to its phenolics, which can donate electrons to H_2O_2 thus neutralizing it to water. Extract was capable of scavenging H_2O_2 in a concentration dependent manner. IC_{50} was 564.3± 16.3

µg/ml. The IC_{50} values for ascorbic acid and BHA were 21.4 and 52.0 µg ml^{-1}, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems (28).

Reducing power: In this assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (28,36). Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose-response curves for the reducing power of the extract. Reducing power of extract increased with the increase of its concentrations (Fig 1).

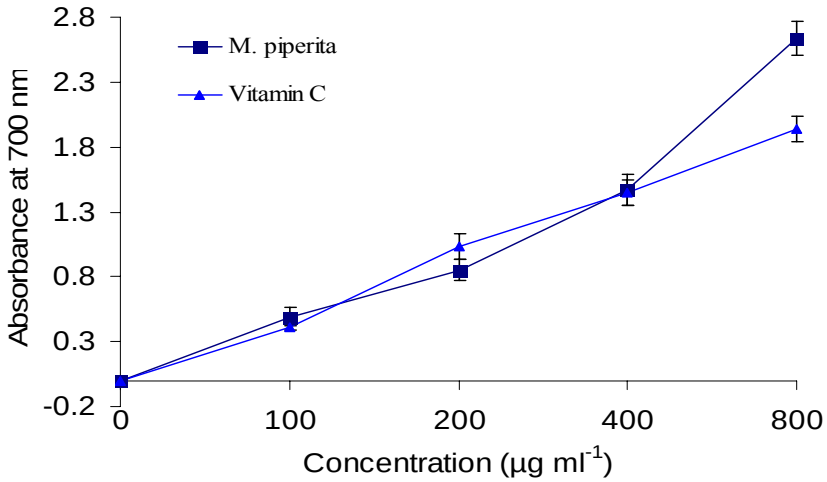

Fig 1. Reducing power of *Mentha piperita* extract.

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (26,37). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested extract show good activity in this test. There was significant difference between extract and control ($p < 0.001$) (Fig 2).

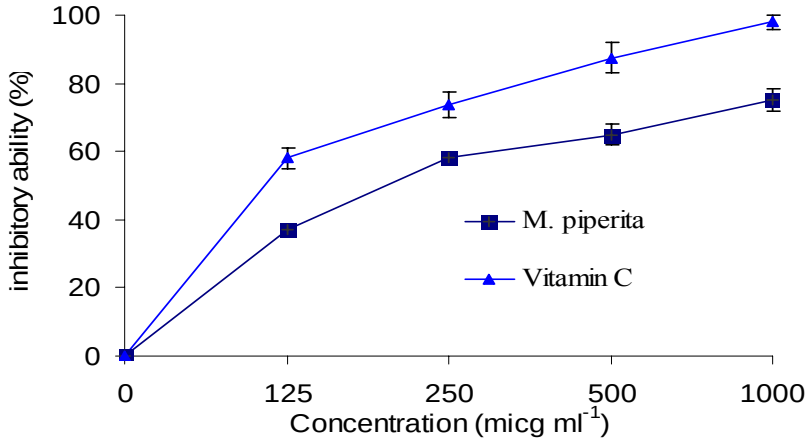

Fig 2. Antioxidant activities of *M. piperita* extract against linoleic acid peroxidation induced by hemoglobin.

Antihemolytic activity of extract against H_2O_2 induced hemolysis: In this study, we used a biological test based on Hydrogen peroxide-induced hemolysis in rat erythrocyte. This assay is useful either for screening studies on various antihemolytic drugs, especially on the one hand drugs having an antioxidizing activity and on the other hand drug having a long-term action (38). Lipid oxidation of rat blood erythrocyte membrane mediated by H_2O_2 induces membrane damage and subsequently hemolysis. The extract showed good inhibiting activity. (IC_{50}= 836.4± 29.2).

Conclusions

Our studies indicate that the hydroalcoholic extract of *M. piperita* has good antioxidant and antihemolytic activates that maybe result of its high phenol and flavonoid contents. It is very promising for further phytopharmacological studies, which will be focused on evaluating other biological effects or improving mechanism of these effects.

Acknowledgements

This paper dedicated to Seyed Maryam Nabavi, Seyed Ali Asghar Nabavi and Seyed Morteza Nabavi.

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