

ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITIES OF *MENTHA LONGIFOLIA*

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Summary

The antioxidant and antihemolytic activities of *Mentha longifolia* L. aerial part was investigated employing seven in vitro assay systems. The extract showed moderate antioxidant activity in some models. IC₅₀ for DPPH radical-scavenging activity was 12.6 ± 1.6 µg/ml. The extract showed potent nitric oxide-scavenging activity between 100 and 800 µg/ml. The extracts showed good Fe²⁺ chelating ability. IC₅₀ were 766.6 ± 13.7 µg/ml. The extract exhibited low antioxidant activity in the linoleic acid model but were capable of scavenging hydrogen peroxide in a concentration dependent manner. Also, the extract show good reducing power and antihemolytic activity. 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.

Key words: Antioxidant activity, Antihemolytic activity, *Mentha longifolia*, nitric oxide-scavenging activity, Fe²⁺ chelating ability.

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 (1). 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 (1,3). 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 (4).

Even though a variety of herbs are known to be good sources of phenolic compounds, their compositional data are insufficient (2). Moreover, various vegetables and fruits contain numerous phytochemicals in addition to phenolic compounds, such as nitrogen compounds, carotenoids, and ascorbic acid (5). 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). *Mentha longifolia* L. (*Labiatae*) is commonly known as Horsemint or Brookmint and in Iran as "pooneh" or "poondeh" (2). The genus of *Mentha* is known to be endowed with a variety of biological and pharmacological properties due to the high content of secondary metabolites (2). Previously anti-allergic, anti-oxidant, anti-platelet, Anti-proliferative, chemo-preventive, besides exerting H₂O₂ scavenging activities and anti-mutagenic activity (2) of this genus have been reported. In the present study, the antihemolytic and antioxidant effects of *M. longifolia* were investigated by deferent in vitro assay systems.

Materials and methods

Plant materials: *Mentha longifolia* was collected from Panbeh chuleh village, near the Caspian Sea, Mazandaran, Iran and identified by Dr. Bahman Eslami, assistance professor of plant systematic and ecology, Department of biology, Islamic Azad University, branch of Ghaemshahr, Iran, where a voucher specimen (No 568) was deposited.

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

Freeze-dried extract: Aerial parts of plant were dried at room temperature and coarsely ground before extraction. A known amount of aerial parts of plant was extracted at room temperature by percolation method using ethanol-water (70-30 v/v). The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained (12.1%), which was then freeze-dried for complete solvent removal.

Determination of total phenolic and flavonoid contents: Total phenolic content was determined by the Folin-Ciocalteu method (6). The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g l⁻¹ 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 using the method of Nabavi et al. (7). Briefly, 0.5 ml solution of plant extract was 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 samples (8). Different concentrations of sample were added, at an equal volume, to ethanolic 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.

Reducing power determination: The reducing power of extract was determined according to the method of Nabavi *et al.* (9,10). 2.5 ml of sample (25-800 $\mu\text{g ml}^{-1}$) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) (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.

Assay of nitric oxide-scavenging activity: For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of sample dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract, but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (11, 12).

Metal chelating activity: The chelating of ferrous ions by the extract was estimated according to our recently published papers (13, 14). Briefly, the sample (0.2-3.2 mg ml^{-1}) was 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), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine- Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/standard. Na_2EDTA was used as positive control.

Scavenging of hydrogen peroxide: The ability of the extract to scavenge hydrogen peroxide was determined according to our recently published papers (15, 16). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Sample (0.1-1 mg ml^{-1}) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of sample at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extract and standard was calculated as follows: % Scavenged (H_2O_2) = $((A_0 - A_1)/A_0) \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard.

Antioxidant activity in hemoglobin-induced linoleic acid peroxidation test: The antioxidant activity of extract was determined by a modified photometry assay (17). Reaction mixture (200 ml) containing 10 ml extract (10–400 mg), 1 mmol/l of linoleic acid emulsion, 40 mmol/l of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, was incubated at 37 °C 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 coloring with 100 ml of 0.02 mol/l of FeCl₂ and 50 ml of ammonium thiocyanate (30 g/100 ml). Vitamin C was used as positive control.

Antihemolytic activity of extract

Preparation of rat erythrocytes: All the animal experiments were carried out with the approval of institutional animal ethical committee. Male 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 according to the method described by Ebrahimzadeh et al. (18,19). Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4 °C, 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 4 °C and used within 6 h for further studies.

Antihemolytic activity of extract against H₂O₂ induced hemolysis: Antihemolytic activity of the extract was assessed as described recently (20). Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH = 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 1g of samples/ml of saline buffer was added to 2 ml of erythrocyte suspension and the volume was made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H₂O₂ solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H₂O₂ in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation, the reaction mixture was centrifuged at 1500 rpm for 10 min and extend of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

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 EC₅₀ 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 ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic contents of was 164.0 ± 6.72 mg gallic acid equivalent/g of extract, respectively. The total flavonoid contents was 15.4 ± 0.70 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). Polyphenolic compounds,

that derived from natural sources have been shown to poses significant antioxidant activity (21). Previous Studies have shown that using of flavonoids in the diet could decrease certain human diseases (21, 22).

DPPH radical-scavenging activity: 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/electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (23). IC₅₀ for DPPH radical-scavenging activity was $12.6 \pm 1.6 \mu\text{g/ml}$. The IC₅₀ values for Ascorbic acid, quercetin and BHA were 1.26 ± 0.11 , 1.32 ± 0.07 and $13.49 \pm 1.04 \mu\text{g/ml}$, respectively.

Reducing power: The presence of reductants (antioxidants) in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. 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. According to results it was found that the reducing power of the extract also increased with the increase of their concentrations. There was no significant difference ($p > 0.05$) among the extract and vitamin C in reducing power.

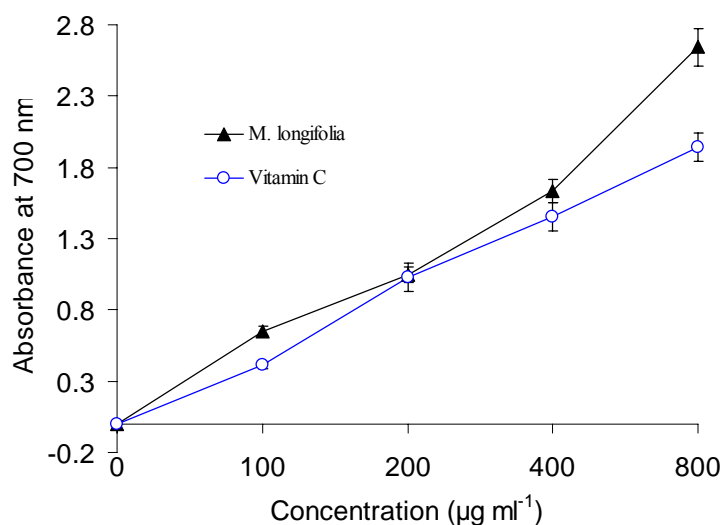


Fig 1. Reducing power of *Mentha longifolia* extract.

Nitric oxide radical scavenger potential: The extract also showed good activity in nitric oxide-scavenging. IC₅₀ was $229.5 \pm 7.11 \mu\text{g/ml}$. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (24). The plant/plant products may have the property to counteract the effect of Nitric oxide formation and in turn may be of considerable interest in preventing the ill effects of excessive Nitric oxide generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of nitric oxide that are detrimental to human health.

Iron chelating: The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease

(25). Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of Ebrahimzadeh *et al.* (26). Ferrozine can quantitatively form complexes with Fe^{2+} . In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, 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 concentration from 0.1 to 1.6 mg ml^{-1} . 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 (26). IC_{50} was $766.6 \pm 13.7 \text{ }\mu\text{g/ml}$. EDTA showed very strong activity ($\text{IC}_{50} = 18 \pm 0.9 \text{ }\mu\text{g/ml}$).

Antioxidant activity in hemoglobin-induced linoleic acid peroxidation test:

Extract showed moderate antioxidant activity in hemoglobin-induced linoleic acid system. (20%) at $125 \text{ }\mu\text{g/ml}$ and high inhibitory ability (68%) at $1000 \text{ }\mu\text{g/ml}$ (Fig. 2). There were significant differences ($p < 0.01$) among the extract and vitamin C.

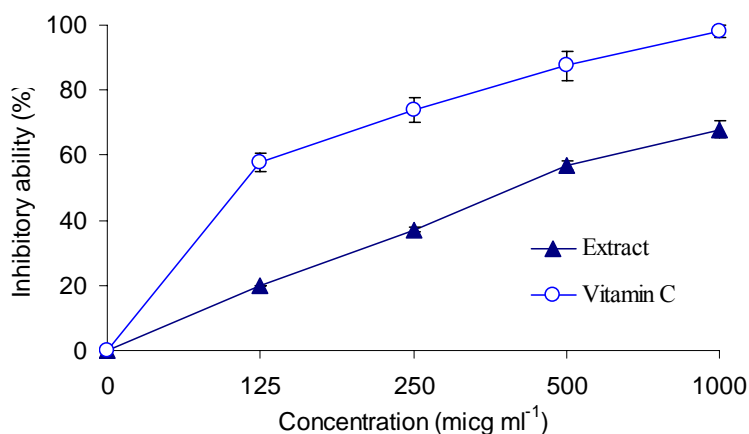


Fig 2. Antioxidant activity of *Mentha longifolia* extract against hemoglobin induced linoleic acid peroxidation test.

H_2O_2 scavenging: Previously studies showed that scavenging of H_2O_2 usually attributed to polyphenolic compounds which can act as electron donator and with reducing H_2O_2 , neutralizing it to water (26). The *M. longifolia* extract was capable of scavenging H_2O_2 in a concentration dependent manner. IC_{50} for H_2O_2 scavenging activity was $215.7 \pm 9.2 \text{ }\mu\text{g/ml}$. The IC_{50} values for ascorbic acid and BHA were 21.4 ± 0.81 and $52.0 \pm 2.02 \text{ }\mu\text{g/ml}$, 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 (23).

Testing for antihemolytic activity: Flavonoids interactions with cell membranes which generally serve as targets for lipid peroxidation (LP), constitute an important

area of research (27). Various model membrane systems like LDL and red blood cell (RBC) membrane comprising physiologically important membrane protein components offer a physiologically relevant and a relatively simple system for studying LP (28). RBC has been chosen as an in vitro model to study the oxidant/antioxidant interaction since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible to peroxidation (27). During recent years, a few interesting studies have been reported, indicating the protective effects of some plants extracts against oxidative damage in intact RBC membranes (14-16,19). Hemolysis has a long history of use in measuring free radical damage and its inhibition by antioxidants but only few studies have been performed with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocytes lysis in rat blood. Lipid oxidation of rat blood erythrocyte membrane mediated by Hydrogen peroxide induces membrane damage and subsequently hemolysis. The extract showed weak inhibiting activity. IC_{50} was $951.4 \pm 36 \mu\text{g/ml}$. IC_{50} for vitamin C was $235 \pm 9.1 \mu\text{g/ml}$. The antihemolytic activity of flavonoids has been previously reported and activity of the extract maybe results in high flavonoid content (20).

Conclusions

The extract of *Mentha longifolia* exhibited good but different levels of antioxidant capacity in all the studied models. Future investigations of the chemical composition and in vivo models of antioxidant activity are needed.

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