BIOLOGICAL ACTIVITIES OF *MENTHA SPICATA* L.

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Summary

The antioxidant activity of *Mentha spicata* L. aerial part was investigated employing eight in vitro assay systems. The extract showed moderate antioxidant activity in some models. IC_{50} for DPPH radical-scavenging activity was 105.8 ± 3.98 μg/ml. The extract showed potent nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1}. The extracts showed weak Fe^{2+} chelating ability. IC_{50} were 757.4 ± 29.5 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 to the presence of phenols and flavonoids in the extract.

Introduction

Living tissues that derive energy from aerobic metabolism are under constant threat of damage by reactive oxygen derivatives. Such free radicals are usually short-lived species but they possess a single unpaired electron, rendering them highly reactive against biologically important macromolecules including DNA, proteins and membrane lipids [1]. To counteract this threat to their integrity, cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidant species, and on antioxidant enzymes [1]. A high proportion of the antioxidant systems of the human body are dependent on dietary constituents [1]. Consequently, the search for natural antioxidants, especially of plant origin, has notably increased in recent years [1]. *Mentha spicata* L. (*Labiatae*) is commonly known as spearmint and in Iran as "pooneh sonbolei" [2]. Spearmint oils are used in the food confectioneries and pharmaceuticals [3]. This plant is known to be endowed with a variety of biological and pharmacological properties due to the high content of secondary metabolites [4]. Previously anti-allergic [5], anti-oxidant [6], anti-platelet [3], anti-proliferative [7], chemo-preventive [8], besides exerting H_2O_2 scavenging activities [9] and anti-mutagenic activity [10] of this plant have been reported. In the present study, the antihemolytic and antioxidant activities of *Mentha spicata* were investigated by deferent in vitro assay systems.

Materials and methods

Plant materials: *Mentha spicata* was collected from Panbeh chuleh, near the Caspian Sea side, Mazandaran, Iran and identified by Dr. B. Eslami, assistance professor of plant systematic and ecology, Department of biology, Islamic Azad University, branch of Ghaemshahr, Iran, where a voucher specimen (No 567) 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 (14.5%), which was then freeze-dried for complete solvent removal.

Determination of total phenolic and flavonoid contents: Total phenolic content was determined by the Folin-Ciocalteau method [11]. The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau 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. [11]. 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 [12]. 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 our recently published paper [13]. 2.5 ml of sample (25-800 μg ml^{-1}) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, 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.

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 [13].

Metal chelating activity: The chelating of ferrous ions by the extract was estimated by the method of Ebrahimzadeh et al. [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 publisher paper [15]. 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 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 A_1 was the absorbance in the presence of the sample of extract and standard [15].

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: The antioxidant activity of extract was determined by a modified photometry assay [15]. 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 ºC 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 $FeCl_2$ 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. [16]. Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4 ºC, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5min) 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 ºC and used within 6 h for further studies.

Antihemolytic activity of extract against H_2O_2 induced hemolysis: Antihemolytic activity of the extract was assessed as described by Ebrahimzadeh et al. [17]. 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 H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 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. 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 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 ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic contents of was 153.3± 5.1 mg gallic acid equivalent/g of extract, respectively. The total flavonoid contents was 11.0± 0.31 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 activities [18]. Previous Studies have shown that using of flavonoids in the diet could decrease certain human diseases [18].

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- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [19]. IC_{50} for DPPH radical-scavenging activity was 105.8± 3.98 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.

Reducing power of extract: In this model, the presence of reductants (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. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose–response curve for the reducing power of the extract. According to results it was found that the reducing powers increase with the increase of their concentrations. There were significant differences ($p< 0.05$) among the extract and with vitamin C

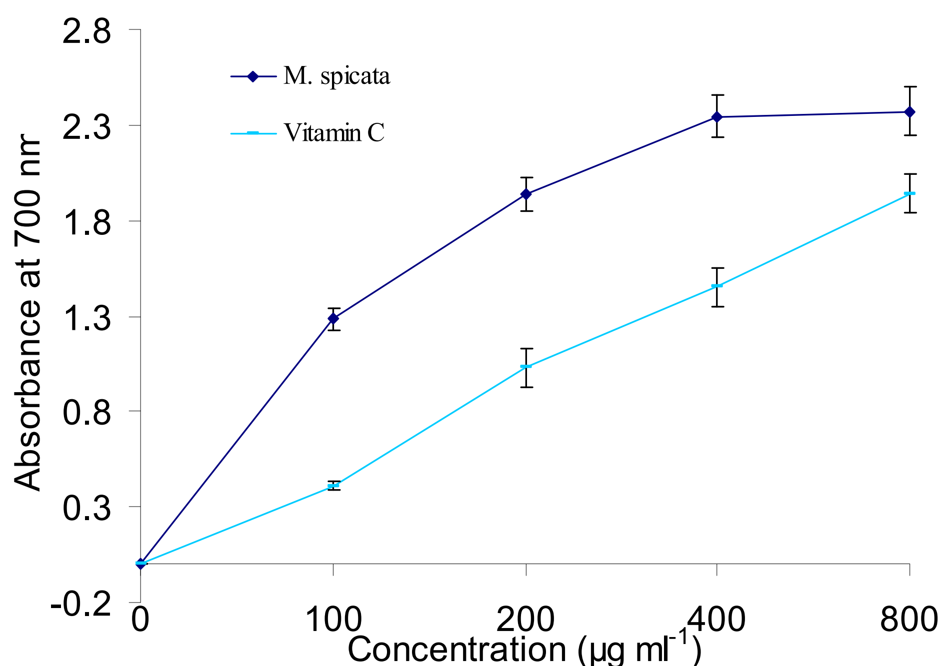

Fig 1. Reducing power of *Mentha spicata* L.extract.

Nitric oxide radical scavenger potential: The extract also showed good activity in nitric oxide-scavenging. IC_{50} was 210.6± 7.7 µg/ml. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [1]. 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 [12].

Metal chelating activity: The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease [20]. 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. [21]. 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 colour of the complexes decreases. In this assay, extract and essential oil 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 [21]. The extract show weak Fe^{2+} chelating activity. IC_{50} was 757.4± 29.5 µg/ml. EDTA showed very strong activity with IC_{50} = 18 ± 0.9 µg/ml.

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: Extract showed good reducing activity in hemoglobin-induced linoleic acid system. (27%) at 125 µg/ml and high inhibitory ability (71%) at 1000 µg/ml (Fig. 2). There were significant differences among the extract and vitamin C (p< 0.01).

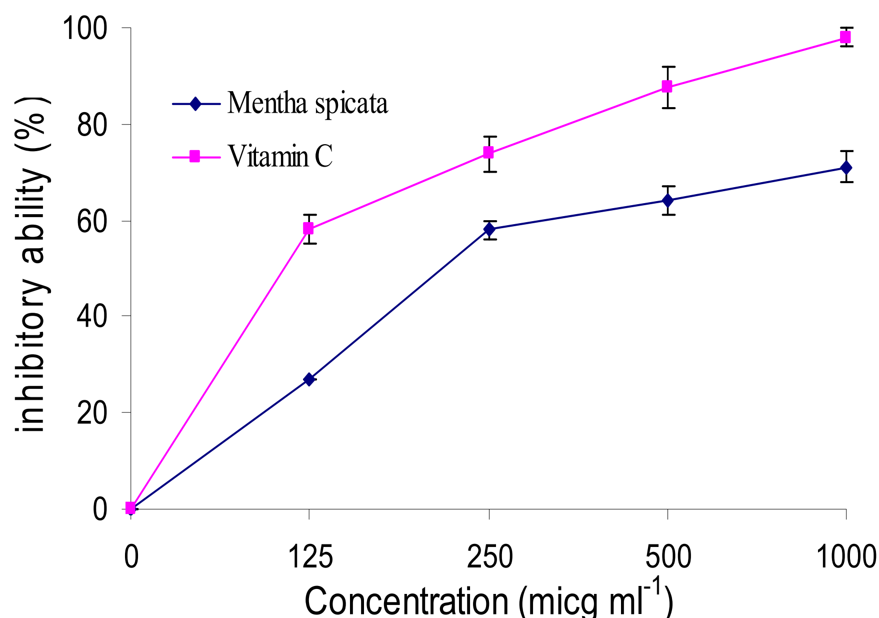

Fig 2. Antioxidant activities of *Mentha spicata* extract against linoleic acid peroxidation induced by hemoglobin

Scavenging of H_2O_2: Previous studies showed that scavenging of H_2O_2 usually attributed to poly phenolics compounds which can act as electron donator and with reducing H_2O_2, neutralizing it to water [22]. The *Mentha spicata* extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} for H_2O_2 scavenging activity was 631.1 ± 26.0 µg/ml. The IC_{50} values for vitamin C and BHA were 21.4± 0.81 and 52.0± 2.02 µg/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].

Antihemolytic activity: 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 H_2O_2 induces membrane damage and subsequently hemolysis. The extract showed weak inhibiting activity. IC_{50} were 1250.7± 46.1 and 235± 9.1 µg/ml for extract and vitamin C, respectively. The antihemolytic activity of quercetin and other flavonoid have been previously reported and good activity of the extracts maybe result in high flavonoid content especially quercetin [13].

Conclusions

The extract of *Mentha spicata* exhibited good but different levels of antioxidant activity in all the models studied.

Acknowledgements

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