

BIOLOGICAL ACTIVITY OF *MENTHA AQUATICA* L.,

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Summary

In present study antioxidant and antihemolytic activities of *Mentha aquatica* L. aerial part were examined employing different in vitro assay systems. The extract showed moderate antioxidant activity in some models. In DPPH radical-scavenging model, extract show potent activity (IC_{50} was $46.05 \pm 1.7 \mu\text{g ml}^{-1}$). The extract showed moderate nitric oxide-scavenging activity between 0.2 and 0.8 mg ml⁻¹. EDTA showed very strong activity with $IC_{50} = 18 \mu\text{gml}^{-1}$. The extract exhibited weak antioxidant activity in peroxidation inhibition through linoleic acid emulsion system. Also, the extract show weak antihemolytic activity. The total amount of phenolic and flavonoid content of extract were determined by employing Folin-Ciocalteu and aluminum chloride colorimetric assays.

Key words: Antioxidant activity, Antihemolytic activity, *Mentha aquatica*, DPPH, Fe²⁺ chelating ability.

Introduction

According to previous studies free radicals have been shown to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease [1]. Antioxidant can prevent these effects by quenching of free radicals and so can protects body against above mention diseases. On the other hand numerous studies demonstrate that synthetic Antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are suspected to be carcinogenic [2]. Also many of studies focused on great number of medicinal and aromatic herbs, as well as fruits and leaves of some berry plants biosynthesize

phytochemicals possessing antioxidant activity against various diseases induced by free radicals [1, 2]. *Mentha aquatica* L. (*Labiatae*) is commonly known as water mint or fish mint and in Iran as "poone abi". Nomads of Northern Province of Iran call this plant 'ouji' [3]. 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. Previously anti-allergic, anti-oxidant, anti-platelet, Anti-proliferative, chemo-preventive, besides exerting H₂O₂ scavenging activities and anti-mutagenic activity of this genus have been reported [1,2]. In the present study, the antihemolytic and antioxidant effects of *Mentha aquatica* were investigated by deferent in vitro assay systems.

Materials and methods

Plant materials: *Mentha aquatica* L. aerial parts were 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 569) was deposited.

Chemicals: Ferrozine, Linoleic acid, Trichloroacetic acid (TCA), 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Potassium ferricyanide 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. 100 gram of aerial parts powder 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-Ciocalteu method [4]. Briefly, 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 Ebrahimzadeh et al. [4]. 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 content was 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

[5,6]. 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. [7]. Briefly, 2.5 ml of sample (25-800 $\mu\text{g ml}^{-1}$) in water was 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 [8, 9].

Metal chelating activity: The chelating of ferrous ions by the extract was determined by the method of Ebrahimzadeh et al. [10]. 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.

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: The antioxidant activity of extract was determined by a modified photometry assay [11]. 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 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. [12]. 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 by Ebrahimzadeh et al. [13]. 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% of hemolysis of blood cells after 240 min. After incubation the reaction mixtures 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 [14].

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 IC₅₀ 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 content of extract was 156.15 ± 6.4 mg gallic acid equivalent/g of extract, respectively. The total flavonoid content of extract was 17.05 ± 0.63 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). 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 [15]. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases [15].

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 [16]. 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 [17]. IC₅₀ for DPPH radical-scavenging activity was $46.05 \pm 1.7 \mu\text{g ml}^{-1}$. The IC₅₀ values for Ascorbic acid, quercetin and BHA were 1.26 ± 0.11 , 1.32 ± 0.07 and $13.49 \pm 1.04 \mu\text{g ml}^{-1}$, respectively.

Reducing power: In the reducing power assay, the presence of reductants (antioxidants) in the sample 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. It was found that the reducing power of extract also increased with the increase of its concentration. Vitamin C used as a standard antioxidant [18]. There was no significant difference among the extract and vitamin C in reducing power ($p > 0.05$).

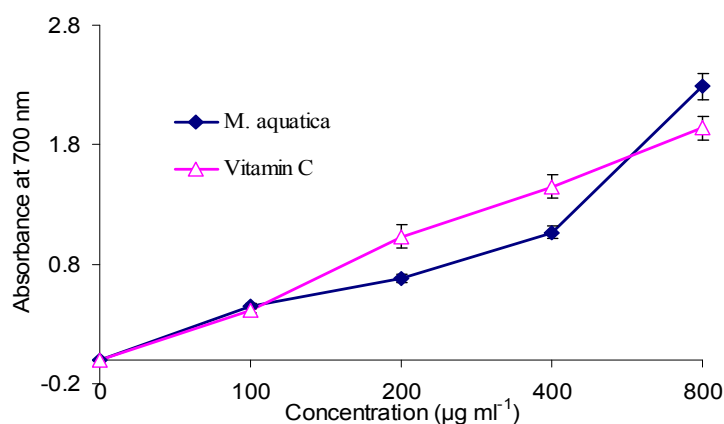


Fig 1. Reducing power of *Mentha aquatica* extract.

Assay of nitric oxide-scavenging activity: The extract showed moderate nitric oxide-scavenging activity between 0.2 and 0.8 mg ml⁻¹. The % inhibition was increased with increasing concentration of the extract. IC₅₀ was $525.6 \pm 22 \mu\text{g ml}^{-1}$. IC₅₀ = $0.20 \pm 0.01 \text{ mg ml}^{-1}$ for quercetin. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [19]. 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.

Fe²⁺ 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 Fe²⁺ also has been shown to cause the production of oxy-radicals and lipid peroxidation, minimizing Fe²⁺ concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by our recently published paper [10]. 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, 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 concentration from 0.1 to 1.6 mg ml^{-1} . Metal chelating capacity was significant since the extract 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, thereby stabilizing the oxidized form of the metal ion [21]. The extract showed moderate Fe^{2+} chelating activity. IC_{50} was $540.4 \pm 21 \mu\text{g ml}^{-1}$. EDTA showed very strong activity ($\text{IC}_{50} = 18 \mu\text{g ml}^{-1}$).

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: Membrane lipids are rich in unsaturated fatty acids which are most susceptible to oxidative processes. Specifically, linoleic acid and arachidonic acid are targets of lipid peroxidation [22]. Since polyphenolics contents appear to function as good electron and hydrogen atom donors and therefore be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products, the reducing potential of the extract may be attributed to this mode of activity. A similar observation has been reported for several plant extracts [5]. Hemoglobin-induced linoleic acid peroxidation test could evaluate the results with only 1 h for oxidation time. Generally antioxidant assays with linoleic acid need more auto-oxidation for 5-6 days [15]. The extract showed weak inhibitory ability on lipid oxidation. There was significant difference between extract and vitamin C ($p < 0.01$) (Figure 2). Activity in metal chelating test indicates some compounds in extract are electron donors and can react with free radicals to convert them into more stable products and to terminate radical chain reactions.

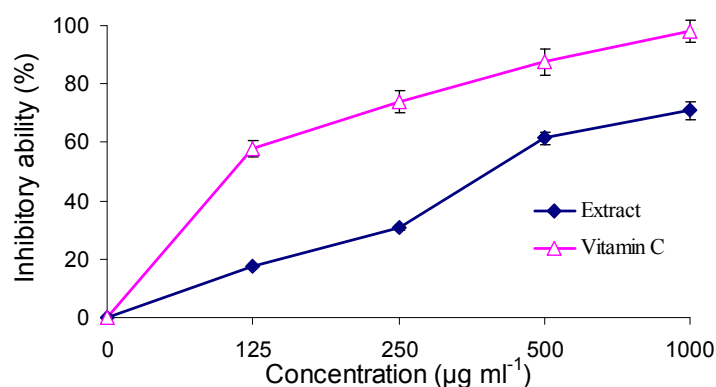


Fig 2. Antioxidant activities of *M. aquatica* extract in hemoglobin-induced linoleic acid peroxidation test.

Antihemolytic activity: Flavonoids interactions with cell membranes, which generally serve as targets for lipid peroxidation (LP), constitute an important area of research [23]. 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 [24]. 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 [23]. 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 [2,11,13,25,26]. 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 lyses in rat blood. This assay is useful either for screening studies on various molecules and their metabolites, especially those having an oxidizing or antioxidizing activity and molecules having a long-term action [27]. Lipid oxidation of rat RBC membrane mediated by H₂O₂ induces membrane damage and subsequently hemolysis. The effect of extract was tested and found that they did not show any harmful effects on erythrocytes in fact, exhibited weak antihaemolytic activity with IC₅₀ = 1.2 ± 0.12 mg ml⁻¹ compared with 235 ± 9 µg ml⁻¹ for vitamin C which served as positive control. Antihaemolytic activity and the relationship between iron ion chelating activity and protective activity against oxidative damage to erythrocyte membrane by quercetin have been reported previously [23,28]. It seems high total phenol and flavonoid contents, especially rutin in extract lead to its good antihaemolytic activity. There are some reports that improve our hypothesis [23, 25, 29].

Conclusions

The extract of *Mentha aquatica* L. exhibited different levels of antioxidant and antihemolytic activity in all the studied models. Future investigations of the chemical composition and in vivo models of antioxidant activity are needed.

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