

Original Article

Evaluation of the Antioxidant Properties of Five *Mentha* Species

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Abstract

The aim of the study was to evaluate the antioxidant and free radical scavenging properties and to determine the phenolic content of the ethanol extract from five *Mentha* species [*M. longifolia* (L.) Huds., *M. piperita* L., *M. pulegium* L., *M. rotundifolia* (L.) Huds., and *M. spicata* L.]. The antioxidant activities of the extracts were investigated with two different methods, 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH[•]) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS^{•+}). *M. piperita* exhibited the strongest activity as a DPPH[•] scavenger. On the other hand, all the extracts were active in the ABTS^{•+} assay and no significant difference was observed in this assay. The total phenolic content (TPC) of the extracts was determined by Folin-Ciocalteu method and *M. piperita* showed the highest TPC. A high correlation was found between the DPPH[•] scavenging potency and the total phenolic content of the extracts ($r^2 > 0.989$).

Keywords: Antioxidant activity; Labiate; *Mentha* species; Total phenolic content.

Introduction

Body functions rely on oxidation reactions which in turn produce reactive oxygen species (ROS) as by products or intermediates during normal metabolism. ROS is a collective term used to include both the oxygen radicals and some nonracial reactive derivatives of oxygen. A balance between formation and removal of these molecules is required to maintain normal physiological functions (1, 2). However, prolonged high level production of the ROS can cause problems because they can react with biological molecules including DNA, proteins and lipids, leading to cell and tissue injuries and pathological events. The role of free radicals and reactive oxygen species in the etiology of many

chronic diseases such as atherosclerosis, cancer, inflammation, and neurodegenerative disorders like Parkinson's and Alzheimer's diseases, has been documented (3, 4). One effective way to eliminate ROS is with the help of antioxidant compounds. Therefore, attention is being focused on the use of antioxidants to protect the cells from biological damages due to free radicals (5, 6). Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods and to extent their shelf-life (7).

Antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules and/or activators of antioxidative defense enzyme systems to suppress the radical damages in biological systems (8, 9). Most of the antioxidants in use commercially [e.g., butylated hydroxytoluene (BHT) and butylated

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hydroxyanisole (BHA)] are synthetic and some of them have been suspected of causing or promoting negative health effects; therefore, some restrictions are placed on their applications and there is a trend to substitute them with naturally occurring antioxidants (2, 3). In recent years the search for natural antioxidants especially from plants has greatly increased and in the last few years some medicinal plants have been extensively studied for antioxidant activities (5, 10, 11).

A great number of aromatic, spicy, and medicinal plants contain chemical compounds, with antioxidant properties. Several studies that have been carried out on some of these plants have resulted in the development of natural antioxidant formulations for food, cosmetic, and other applications (7, 10). For example, *Salvia officinalis* L. and *Rosmarinus officinalis* L. (Labiatae) are well known for their antioxidant properties (12). Among the natural compounds, phenolic compounds constitute one of the major groups of herbal compounds acting as radical scavengers and antioxidants (13, 14).

The Labiatae family includes about 220 genera and 3300 species which are widely used for various purposes worldwide (15). Plants belonging to the Labiatae family are rich in polyphenolic compounds and a large number of them are well known for their antioxidant properties (1, 12). The genus *Mentha* is one of the important members of the family and represents by about 6 species in the flora of Iran (16). *Mentha* species are generally known under the name "na'na" in Persian and commonly used as herbal tea, flavoring agent, and medicinal plant. Infusion, decoction, and distillated water of the aerial parts of *Mentha* species have been used for centuries as tonics, carminative, digestive, stomachic, antispasmodic, and anti-inflammatory agents in Iranian Traditional Medicine (17, 18). The literature data on the antioxidant activities of *Mentha* species is frequently scattered throughout the papers and the data available is often difficult to compare because of the differences in the methodologies. (1, 5, 14, 19-28). Thus, comparison of the antioxidant activities of the species, using a similar approach is necessary.

The objectives of the research were (1) to determine and compare the antioxidant activity

and total phenolic content (TPC) of five *Mentha* species [*M. longifolia* (L.) Huds., *M. piperita* L., *M. pulegium* L., *M. rotundifolia* (L.) Huds., and *M. spicata* L.] by two different methods [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS^{•+}) and 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH[•]) assays]; (2) to investigate a possible correlation between activity and phenolic content.

Experimental

Chemicals

All chemicals were purchased from Sigma (USA), Fluka Chemie (Switzerland) and Merck (Germany) companies.

Plant materials

Aerial parts of *Mentha longifolia* (L.) Huds., *M. piperita* L., *M. pulegium* L., *M. rotundifolia* (L.) Huds., and *M. spicata* L. were collected in Tehran province during the flowering period in summer 2006 and authenticated by M. Kamalinejad. A voucher specimen for each plant was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Shahid Beheshti University (M. C.), Tehran, Iran. The plants were dried in the drying room with active ventilation at ambient temperature.

Preparation of the extracts

Dried and powdered aerial parts (100 g) from each species were macerated in ethanol at room temperature for 48 h. After filtration, each macerate (extract) was concentrated under reduced pressure. The dried extracts were dissolved in ethanol to a final concentration of 500 µg/ml (sample stock solution).

Measurement of antioxidant activity

DPPH[•] assay

One ml of 0.3 mM DPPH[•] solution in ethanol was added to 2.5 ml of each sample at different concentrations (500, 250, 100, 50, 25, 10, 5, 2.5 µg/ml). Test tubes were vortexed and incubated for 30 min at room temperature in the dark; then, the absorbance values were determined at 518 nm in a Shimadzu Multispect-1501 spectrophotometer (Kyoto, Japan). The inhibition percentage of DPPH radical was assessed by the

following formula:

$$I_{DPPH\cdot\%} = \left[\frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \right] \times 100$$

Ethanol (1 ml) plus each sample solution (2.5 ml) was used as a blank. DPPH[·] solution (1 ml) plus ethanol (2.5 ml) was used as a negative control. Also, rutin solution (at the concentrations of 200, 100, 50, 20, 10, 5, 2.5, 1 µg/ml) was used as a positive control (11).

The $I_{DPPH\cdot\%}$ was plotted against the sample concentration and a logarithmic regression curve established in order to calculate the IC_{50} value (inhibitory concentration), which is the concentration of sample (µg/ml) necessary to decrease by 50% the absorbance of DPPH[·].

ABTS^{·+} assay

The ABTS radical cation, was generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate solution and the mixture was kept in the dark at room temperature for 12-16 h before use. Prior the assay, the ABTS^{·+} solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. An aliquot (50 µl) of each sample at different concentrations (500, 250, 100, 50, 25, 10, 5 µg/ml) was added to 5 ml of diluted ABTS^{·+} solution. After 10 min, the absorbance was measured at 734 nm. The Inhibition of ABTS radical cation in percent was calculated by the following formula:

$$I_{ABTS\cdot+\%} = \left[\frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \right] \times 100$$

Ethanol (5 ml) plus each sample solution (50 µl) was used as a blank. ABTS^{·+} solution (5 ml) plus ethanol (50 µl) was used as a negative control. Also, rutin solution (at the concentrations of 200, 100, 50, 20, 10, 5 µg/ml) was used as a positive control (29).

The $I_{ABTS\cdot+\%}$ was plotted against the sample concentration and a logarithmic regression curve established in order to calculate the IC_{50} value (inhibitory concentration), which means the concentration of sample (µg/ml) necessary to decrease the absorbance of ABTS^{·+} in 50%.

Determination of total phenolic content

The total phenolic content (TPC) of the samples was measured by Folin-Ciocalteau reagent using rutin as the reference compound (10). One ml of rutin solution in ethanol, (at the concentrations of 150, 100, 75, 50, 25 µg/ml) was mixed with 5 ml Folin-Ciocalteau reagent (diluted 1/10) and incubated at room temperature. After 10 min, 4 ml of sodium carbonate solution (75 mg/ml) was added. The final solution was mixed thoroughly and allowed to remain for 30 min in a dark place. The absorbance was measured at 765 nm with a spectrophotometer. Then a linear calibration curve (absorbance versus concentration) was developed. The plot was found to be linear across the ranged assay (150-25 µg/ml, $r^2>0.99$). The same procedure was carried out with 1 ml of each sample in ethanol instead of rutin. The TPC for each extract [as µg rutin equivalent (RE)/mg of extract] was determined on the basis of the standard curve.

Statistical analysis

All the experiments were carried out in triplicate. The IC_{50} were presented by their respective 95% confidence limits. The TPC (µg/mg) were shown as mean±SEM. One-way analysis of variance (ANOVA) followed by Tukey's post test was used to assess significant differences ($p<0.05$) between extracts. All the statistical analysis were accomplished using the computer software GraphPad Prism 3.02 for Windows (GraphPad Software, San Diego, CA, USA).

Results and Discussion

There are several methods to determine the antioxidant capacity of plant extracts. However, the chemical complexity of extracts could lead to scattered results obtained from different techniques, depending on the test employed. Therefore, an approach with multiple assays in the screening work is highly advisable (9). Among these methods, two *in vitro* DPPH[·] and ABTS^{·+} assays, are used as common and useful methods to evaluate the free radical scavenging activity and antioxidant capacity of extracts. They are excellent tools for determining the antioxidant activity of hydrogen donating and

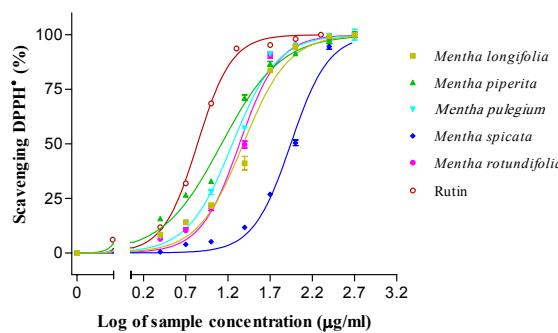


Figure 1. Radical scavenging and antioxidant activities of the studied *Mentha* species extracts using DPPH[·]. Each point represents the mean of three experiments and the vertical bars represent the SEM.

chain breaking antioxidants (30). Phenolic compounds are considered as a major group of compounds that contributed to the antioxidant activities of botanical materials because of their scavenging ability on free radicals due to their hydroxyl groups (7, 31). Therefore in this study, the antioxidant capacities (IC_{50} values) of the ethanol extracts obtained from five *Mentha* species were measured using two methods and their total phenolic contents were determined and compared with each other.

According to the DPPH[·] assay (Figure 1 and Table 1), all the extracts exhibited a noticeable concentration-dependent antiradical effect but differed in their inhibiting activities. The highest scavenging activity was observed for *M. piperita* [$IC_{50}=13.32$ (12.12-14.64) µg/ml], while lowest for *M. spicata* [$IC_{50}=87.89$ (81.66-94.59) µg/ml]. The $IC_{50(DPPH)}$ values of the extracts increased in the following order:

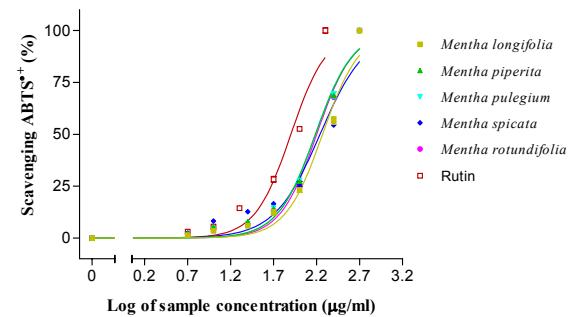


Figure 2. Radical scavenging and antioxidant activities of the studied *Mentha* species extracts using ABTS^{·+}. Each point represents the mean of three experiments and the vertical bars represent the SEM.

M. piperita < *M. pulegium* < *M. rotundifolia* < *M. longifolia* < *M. spicata*. Based on the data obtained from the ABTS^{·+} scavenging test, the extracts had a concentration-dependent activity on this radical but no significant difference was seen (Figure 2 and Table 1). On the other hand, the extracts possessed a weaker inhibition effect on the ABTS radical cation than the DPPH radical. The phenolic content of the tested extracts differed significantly and ranged from 150.91 to 433.60 µg/mg. *M. piperita* showed the highest TPC (433.60 ± 19.62 µg/mg), whereas *M. spicata* had the lowest content (150.9 ± 5.14 µg/mg). The TPC of the extracts in decreasing order was: *M. piperita* > *M. pulegium* > *M. rotundifolia* > *M. longifolia* > *M. spicata* (Figure 3 and Table 1). Therefore, it can be deduced that all the tested plants are rich in phenolic compounds and they have high antioxidant properties.

The ABTS^{·+} scavenging activity of the extracts

Table 1. IC_{50} values of DPPH[·] and ABTS^{·+} scavenging activities and total phenolic contents (TPCs) of the studied *Mentha* species extracts.

Plant species	$IC_{50(DPPH)}$ (µg/ml) ^{*‡}	$IC_{50(ABTS·+)}$ (µg/ml) ^{*‡}	TPC (µg/mg) ^{†‡}
<i>M. longifolia</i>	24.07 (21.34-27.14) ^c	185.60 (164.90-209.00) ^a	288.82 ± 11.55^c
<i>M. piperita</i>	13.32 (12.12-14.64) ^a	153.80 (139.90-169.00) ^a	433.60 ± 19.62^a
<i>M. pulegium</i>	17.92 (16.52-19.44) ^b	152.60 (139.60-166.80) ^a	358.10 ± 15.57^b
<i>M. rotundifolia</i>	21.71 (19.90-23.67) ^c	158.90 (145.40-173.60) ^a	$331.31 \pm 6.51^{b,c}$
<i>M. spicata</i>	87.89 (81.66-94.59) ^d	173.80 (146.20-206.60) ^a	150.91 ± 5.14^d

Note: The $IC_{50(DPPH)}$ and $IC_{50(ABTS·+)}$ values of the positive control, rutin, were measured as 6.90 (6.61-7.20) and 79.59 (69.73-90.86) µg/ml, respectively.

*The IC_{50} values are presented with their respective 95% confidence limits.

[†]The TPC values are means \pm SEM of three determinations.

[‡]Letters (a-d) denote homogenous subsets at $p < 0.05$ (Tukey's post test).

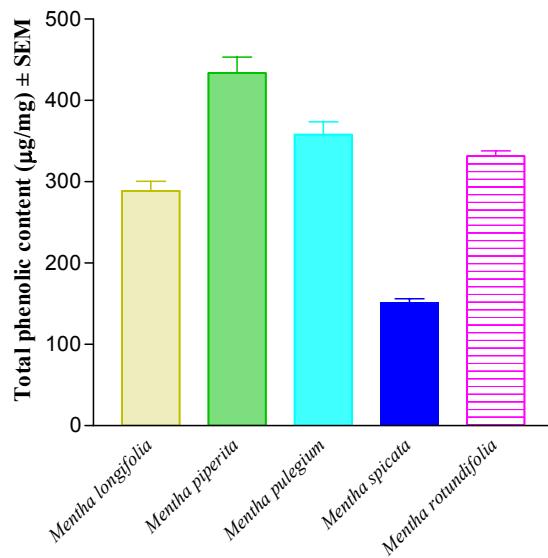


Figure 3. Total phenolic contents of the studied *Mentha* species extracts. Data are represented as mean \pm SEM (n = 3). Vertical bars represent the SEM.

was different from that DPPH[•] scavenging capacity and no correlation was found between them. The difference in the behavior of the extracts in the two assays may be explained by the different chemical mechanisms involved in the tests and the different chemical properties of the radicals. In other words, the radical system used for the antioxidant evaluation has some influences on the testing results; for example, the stoichiometry of reactions between the antioxidant compounds in the extract and ABTS⁺ and DPPH[•] is different and the electron reduction potential of ABTS⁺ and DPPH[•] with respect to the compounds in the extracts are different. Also, stereoselectivity of the radicals may affect the capacity of the extracts to react with the radicals (6, 8, 32, 33). Besides, there was a positive correlation between anti-DPPH[•] potency [expressed as 1/IC_{50(DPPH[•])}] and TPC ($r^2 > 0.989$, $p \leq 0.0005$) (Figure 4). This correlation suggests that phenolic content may be responsible for a part of the antioxidant and free radical scavenging effects of the extracts of *Mentha* species. However, no similar relationship was observed between TPC and 1/IC_{50(ABTS⁺)} values among the extracts. Total extracts are complex mixtures of many different compounds with distinct activities (3, 34). Therefore, the ABTS⁺ scavenging activity of the species tested,

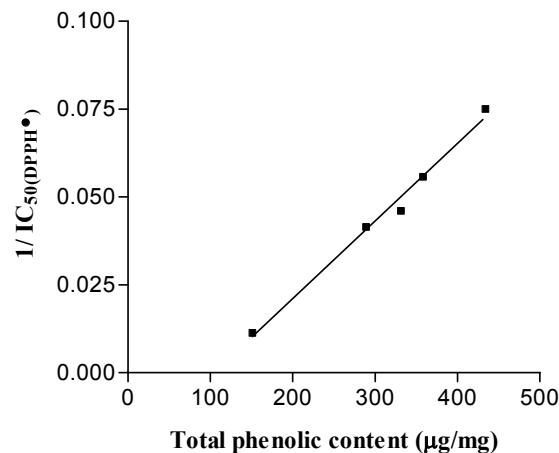


Figure 4. Correlation between anti-DPPH[•] potency [1/IC_{50(DPPH[•])}] and total phenolic content (as µg rutin equivalent/mg of extract) of the studied *Mentha* species extracts ($r^2 > 0.989$, $p \leq 0.0005$).

could be due to the composition of phenolics and other compounds in the mixture that may exert a synergistic effect. Generally, Labiate species are rich sources of terpenoids and phenolic compounds (1). Therefore, it is likely that the phenolic constituents present in the *Mentha* species are, at least in part, responsible for the antioxidant and free radical scavenging activities. However, other compounds without a phenolic structure may explain, in part, the antioxidant activity found for these plants.

The results of this study indicate that the genus *Mentha* are favorable free radical scavengers as well as primary antioxidants that may react with free radicals and limit ROS attack on biological and food systems.

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