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Original Article

Identification of Essential Oil Components, Total Phenolic Content and Antioxidant Properties of *Mentha aquatica* in Southern Khorasan Province, Iran

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ABSTRACT

Many medicinal plants have natural antioxidant properties. In this study, the chemical composition of *Mentha aquatica* L. essential oil was determined and antioxidant properties of essential oils and methanolic extracts, ethanolic extracts, acetonic extracts and aqueous extracts were studied. Also, Phenolics, flavonoids and tannins contents of all the mentioned extracts were determined. The essential oil of the dried flowering aerial part of wild water mint is extracted by hydrodistillation. The essential oil was analyzed by capillary GC and GC-MS. The Folin–Ciocalteu method was adopted to determine the total phenols content while flavonoids were estimated according to the aluminum chloride colorimetric method. To evaluate tannins content, vanillin and HCl were added to the extracts. The antioxidant potential was measured by 1, 1-diphenyl-2-picrylhydrazyl radical scavenging and the inhibition of β-carotene bleaching assays. The main components of essential oil were pipritenone oxide (37.80%), 1, 8-cineole (24.13%), α-gurjunene (11.96%) and pulegone (4.64%). The antioxidant properties of the essential oil were more considerable than all the other extracts. The antioxidant properties and total phenolics, flavonoids and tannins contents of methanolic extracts, ethanolic extracts and acetonic extracts were larger than the aqueous extract. These results show that essential oils, methanolic extracts, ethanolic extracts and acetonic extracts of *M. aquatica* from Southern Khorasan of Iran have a great potential of polyphenols which can be used as a natural food preservative and antioxidant source.

INTRODUCTION

Mentha aquatic L. belongs to the Lamiaceae family used as fresh or dried in herbal drinks and condiments for their special fragrance [1]. The essential oil extracted from plants of this family is used in pharmaceutical, food and cosmetics industries. Mentha genus has been considered for pharmaceutical properties such as the easing of maldigestion and relieving muscular cramps [2]. Water mint grows wild in damp lands in the periphery of rivers and springs and is odiferous due to its possession of essential oils. The essential oil is produced in the capillary glands on the surface of the plants' aerial parts [3].

Mohsenpour *et al*. [4] identified the ingredients of wild *M. aquatica* essence in Mazandaran Province and reported menthoforan, trans-caryophyllene, eucalyptol and germacron as the main ingredients of its essence. Bozin *et al* [5], Getahun *et al*. [6] and Sutour *et al*. [7] also confirmed the existence of menthofuran, 1, 8-cineole and trans-caryophyllene in the essence of *M. aquatica*. Andro *et al*. [8] investigated the essence building blocks of *M. aquatica* and identified menthofuran, limonene, iedol and caryophyllene. Chaker *et al*. [9] recognized such building blocks as α-pinene, sabinene, myrcene, limonene, α-terpineol, terpinen-4-ol and pulegone in the essences of *M. aquatica* plants from Algeria. Zamfirache *et al*. [10] found limonene, α-pinene, sabinene, borneol and myrcene in the essence extracted from *M. aquatica*.

Antioxidants are compounds that can effectively prevent the oxidation of macromolecules such as lipids, proteins and nucleic acids, hence preventing the damage and programmed death of the cells as well as cardiovascular diseases and cancers [11]. The use of chemical antioxidants most frequently used in the food industry has been limited for carcinogenic and negative effects on human health [12]. Natural antioxidants that are abundant in fruits

and vegetables have been regarded as efficacious in disease prevention. A number of these compounds are made with antioxidant properties as secondary metabolites by the wild plants amongst which such compounds as phenols, flavonoids, terpenoids and steroids can be pointed out [13]. Dhifi *et al*. [14] and Getahun *et al*. [6] reported that the essence of *M. aquatica* grown in Tunis and Ethiopia feature high antioxidant activities. Water mint grows wild in many parts of Iran including the southern Khorasan Province. Climatic conditions of southern Khorasan Province are different from other habitats of the water mint. This area is dry and the days are hot and the nights are cool in the summer. Water mint is a main medicinal plant in this region and it is also used to repel pests and harmful rodents. To date, the chemical composition of water mint essential oil has not been identified in this area. The present study aims at the identification of chemical compounds and investigation of antioxidant activities of *M. aquatica* essential oil and aqueous extract and alcoholic extracts, and of the phytochemical properties of the all extracts in southern Khorasan Province, Iran.

MATERIALS AND METHODS

Plant Materials

The aerial flower-bearing parts of wild *M. aquatica* L. were collected from the periphery of an aqueduct in Ghaisar Village of Khosf city in southern Khorasan Province during the summer, 2016. The plant materials were dried in the shade at room temperature for ten days. The voucher specimen of this plant is SPNH-2045 (Sari Payame Noor University Herbarium).

Essential oil was extracted and the ingredients were identified in the laboratory of Research Institute of Forests and Rangelands. In addition, other phytochemical properties were delineated in the laboratory of Islamic Azad University of Birjand.

Essential Oil Extraction

Essential oil extraction was carried out based on hydrodistillation method in the Clevenger Device. First, 100 g of the plant was used for essential oil extraction and the duration of essential oil extraction was 3 hours. The extracted essential oil was kept in a cold and dark environment in a refrigerator before being subjected to analysis [4].

Essential Oil Analysis

To isolate and identify essential oil ingredients, a gas chromatography device and gas chromatograph attached to a mass spectrometer (GC/MS) were employed. Spectra detection was carried out based on their Kovats indices and by the injection of normal hydrocarbons (C7-C25) under identical conditions and essential oil injection using a computer program in basic language. Moreover, comparisons were made with various study sources [15-17] using a mass spectrum of standard compounds and information existent in GC/MS Device's library. Thermo-UFM gas chromatograph equipped with an FID detector was used and the data processor in Chrom-Card 2006 Software was employed for a Ph-5 column (10 m in length, 0.1mm in internal annulus and 0.4 micron in the thickness of the resident phase layer) that had been thermally programmed from 60 °C to 285 °C with a 3 °C increase per minute. The carrier gas was helium and the injection section temperature and detector temperature were set at 280 °C. The gas chromatography device was connected to the Varian 3400 Model Mass spectrometer. Saturn II was employed for analysis and the same column was utilized in the GC device. The gas pressure was 35 Psi in the column head and ionization energy was equivalent to 70 eV. The column's thermal programming was the same as that used for the GC device [4].

Preparation of Solvent Extracts

A portion (20 g) of the powdered plant material was Soxhlet-extracted with water, ethanol, acetonic and methanol for 8 h, at a temperature not exceeding the boiling point of the solvent. The extracts were concentrated using a rotary evaporator at 50 °C to obtain crude extracts. All the extracts were kept in the dark at 4 °C prior to use [18].

Determination of total phenolic content

Total phenol content was measured based on Folin-ciocaltue colorimetric method using gallic acid as a standard [19]. Then, about 1.8 ml of the distilled water and 0.2 ml of dilute folin-ciocaltue (1:15 v/v) reagent were added to 2 ml of plant extract or standard solution (Concentrations of 0-100 mg/ml gallic acid in the distilled water). After 5 minutes, 3 ml of 7% sodium carbonate solution was added to the reaction mixture, and the reaction mixture increased with the distilled water to 5 ml. After 90 minutes of storage at laboratory temperature, their absorption at 750 nm was examined by a spectrophotometer (Jenway, 6305) and the amount of total phenol was determined from the standard curve in terms of mg/GAE g DW.

Determination of Total Flavonoid Content

Total flavonoid content was measured by aluminum chloride colorimetric method using quercetin as

standard [20]. First, standard solutions with concentrations of 0-100 mg/L quercetin in absolute methanol were prepared. Then, 2 ml of the plant extract or standard solution of aluminum chloride and 1 ml of aqueous acetic acid were added and mixed well. Finally, the reaction mixture was increased to 90 ml with 90% ethanol and the tubes were stored at room temperature for 30 minutes. Their light absorption was read at 414 nm by the spectrophotometer (Jenway, 6305) and total flavonoids were obtained using a standard curvature in terms of mg/g DW.

Determination of Total Tannins Content

Total tannins were determined according to Rebaya *et al*. [21] with slight modifications. A volume of 12.5 mL of the extract was added to 750 mL of vanillin and 375 mL of HCl. The mixture was then shaken and incubated at room temperature for 15 min. The absorbance was measured at 500 nm by the spectrophotometer (Jenway, 6305) and the tannin content was expressed as milligrams of catechin equivalent per gram of dry weight (mg CE/g DW).

Antioxidant Properties Investigation

DPPH Radical Scavenging Assay

A total of 50 microliters of various concentrations of the essential oil and extracts in methanol was admixed with 5 ml of 0.004 %2, and 2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. The specimens were placed in ambient temperature in a dark room for 30 minutes. Then, their light absorption was read in room temperature at 517 nm. Next, the following formula was utilized to calculate the percentage of the inhibition of DPPH free radicals [14]:

Inhibition percentage = (light absorption of the evidence specimen without essential oil and extract-amount of the light absorption of various concentrations of essential oil and extract / light absorption of the evidence specimen without essential oil and extract) × 100.

Then, the concentration of water mint essential oil with 50% radical inhibition (IC_{50}) was calculated by the linear regression. Obviously, smaller values mean greater antioxidant power or free radical scavenging. In this test, synthetized 2, 6-di-tertbutyl-4-methylphenol (butylated hydroxyl toluene, BHT) antioxidant was used as positive control. All the experiments were repeated three times.

Carotene/Linoleic Acid Assay

A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 20 mL of chloroform. This solution (2 mL) was mixed with 20 mg linoleic acid and 200 mg Tween 40. After the chloroform was removed at 40 °C under vacuum, 50 mL of oxygenated ultra-pure water was added, and then the emulsion was vigorously shaken. Aliquots (750 mL) of this emulsion were transferred into test tubes containing different concentrations of extracts (50 mL). After the addition of the emulsion to each tube, zero-time absorbance of the control, containing methanol instead of extract, was measured at 470 nm by the spectrophotometer (Jenway, 6305). The test samples were then incubated in a water bath at 50 °C for 120 min, when the absorbance was measured again. The β-carotene bleaching inhibition was calculated using the following equation: Inhibition (%) = [(At – C / (C0 − Ct)] × 100

Where, At and Ct are the absorbance values measured for the test sample and control, respectively, after incubation for 120 min, and C0 is the absorbance value for the control measured at zero time during the incubation. The results are expressed as IC_{50} values, the concentration required to cause a 50% β-carotene bleaching inhibition. BHT was used as a positive control [22].

RESULTS

Essential oil Analysis

Table (1) provides a list of the constituents of *M. aquatica* essential oil and their values in percentage. The natural ingredients accounted for 96.97% (13 compounds) of the *M. aquatica*'s essential oil volume. Pipritenone oxide (37.80%), 1, 8-cineole (24.13%), α-gurjunene (11.96%) and pulegone (4.64%), respectively, were the basic compounds in *M. aquatica*'s essential oil. Such ingredients as limonene (3.76%), myrcene (3.48%), sabinene (2.93%), perilla aldehyde (2.52%), piperitone (2.07%) and α-pinene (1.96%) were found in lesser amounts.

Monoterpenes oxygenated 72.88%, monoterpenes hydrocarbons 12.13%, and sesquiterpenes hydrocarbons 11.96% were also accounted for in water mint essential oil.

Antioxidant Properties

In DPPH assay IC_{50}, the values of essential oil and BHT were 3.24 and 4.78 µg/mL respectively. The essential oil had significantly stronger antioxidant properties than methanolic extracts (IC_{50}= 25.4 µg/mL), ethanolic extracts (IC_{50}= 26.13 µg/mL), acetonic extracts (IC_{50}= 25.45 µg/mL) and aqueous extracts (IC_{50}= 51.11 µg/mL) and there was no significant difference with BHT. IC_{50} value of all

extracts was significantly higher than BHT, so the antioxidant properties were less than the positive control. Aqueous extract had the highest IC_{50} value and therefore the lowest antioxidant properties.

Antioxidant activity determined by β-carotene/linoleic acid emulsified system capacity was estimated by inhibiting the formation of free radicals in the β-carotene from oxidized linoleic acid. It was estimated by spectrophotometrically the paling of the orange color characteristic of β-carotene, which is related to the inhibitory capacity of free radical formation. In the Carotene/linoleic acid assay method, the antioxidant properties of the essential oil (IC_{50} = 448 µg/mL) were significantly higher than methanolic extracts (IC_{50} = 665 µg/mL), ethanolic extracts (IC_{50} = 643 µg/mL), acetonic extracts (IC_{50} = 654 µg/mL) and aqueous extracts (IC_{50} = 971 µg/mL). Aqueous extract had the lowest antioxidant properties and BHT had the highest antioxidant properties**.**

Total Phenolic, Flavonoid and Tannin Contents

All extracts had noticeable phenolic contents (Table 3). The uppermost amounts were observed for methanolic extracts (45.30 mg GAE/g DW, 34.87 mg RE/g DW and 8.77 mg CE/g DW) whereas minimum amounts were noticed for aqueous extracts (20.12 mg GAE/g DW, 15.43 mg RE/g DW and 2.32 mg CE/g DW) for the total phenolic, flavonoid and tannin contents, respectively.

Total phenol, flavonoids and tannins contents in the aqueous extract was significantly lower than other extracts. But methanolic extracts, ethanolic extracts and Acetonic extracts were not significantly different.

Table 1 Chemical ingredients constituting *M. aquatica* L. essential oil

Row	Ingredient	CC	KI	KI^{lit}	Percentage
1	α-Pinene	C1	937	932	1.96
2	Sabinene	C1	973	969	2.93
3	Myrcene	C1	988	988	3.48
4	Limonene	C1	1029	1224	3.76
5	1,8-Cineole	C3	1035	1026	24.13
6	Borneol	C3	1178	1165	0.36
7	Terpinen-4-ol	C3	1165	1174	0.57
8	α-Terpineol	C3	1172	1186	0.79
9	Pulegone	C3	1235	1233	4.64
10	Piperitone	C3	1251	1249	2.07
11	Perilla aldehyde	C3	1270	1269	2.52
12	Piperitenone oxide	C3	1357	1366	37.80
13	α-Gurjunene	C2	1404	1409	11.96
-	Other compound	C4	-	-	3.03

CC—chemical class; C1—monoterpenes hydrocarbons; C2—sesquiterpenes hydrocarbons; C3—monoterpenes oxygenated; C4—unidentified compounds; KI^{lit}— Kovats indices from literature.

Table 2 Antioxidant activities of essential oil and different extract of Water mint (*M. aquatica*) and Synthetic antioxidant BHT

Antioxidant activities	Essential oil	Aqueous extract	Methanolic extract	Ethanolic extract	Acetonic extract	BHT
DPPH [IC50 (µg/mL)]	3.24±0.17 a	51.11±1.05 c	25.40±1.35 b	26.13±2.11 b	25.45±0.95 b	4.78±0.12 a
β-Carotene bleaching [IC50 (µg/mL)]	448± 0.95 b	971±0.40 d	665±0.70 c	643±0.50 c	654±0.8 c	6.5±0.25 a

Values are given as mean (n = 3). Values in each line followed by different letters are significantly different ($P < 0.05$).

Table 3 Total phenolics, flavonoids and tannins content in different extract of Water mint (*M. aquatica*)

Content	Aqueous extract	Methanolic extract	Ethanolic extract	Acetonic extract
Total phenolic contents (mg GAE/g DW)	20.12±0.58 a	45.30±0.98 b	44.95±0.90 b	45.15±1.05 b
Flavonoid contents (mg RE/g DW)	15.43±0.30 a	34.87±1.00 b	34.56±0.24 b	33.91±0.19 b
Tannin contents (mg CE/g DW)	2.32±0.45 a	8.77±0.14 b	8.45±0.22 b	8.24±0.12 b

In each line, values followed by different letters are significantly different ($P < 0.05$).

DISCUSSION

In the present study, pipritenone oxide and 1, 8-cineole were identified as the basic ingredients of *M. aquatica* essential oil grown in southern Khorasan Province which is consistent with the results obtained in the studies by Esmaeili *et al*. [23]. They also detected pipritenone oxide and 1, 8-cineole as the fundamental compounds in the essential oil of *M. aquatica* collected from the Amol region. In the present study, 1, 8-cineole was identified as major constituents of water mint essential oil. Consistent with the results of the present study, Bozin *et al*. [5] and Senatore *et al*. [24] reported 1, 8-cineole as a major constituent of *M. aquatica* essential oil. Jerkovic and Matelic [25] and Fallahian *et al*. [26] also similarly reported that 1, 8-cineole is a main oxygenated monoterpene in *M. aquatica* essential oil.

In the present study, the antioxidant properties of *M. aquatica* essential oil were stronger than all other extracts. Getahun *et al*. [6] and Dhifi *et al*. [14] also investigated the antioxidant properties of the essential oil of *M. aquatica* grown in Ethiopia and Tunis and reported high antioxidant activities for *M. aquatica* (IC_{50} values equal to 2.11 µg/ml and 8 µg/ml, in DPPH assay respectively) similar to the findings of the present study. *M. aquatica* plants used in the present study were grown in a hot and arid climate in the desert margin so their growth conditions were almost identical to those in Ethiopia, hence having high antioxidant properties akin to the plants grown in Ethiopia. Likewise, Grzeszczuk and Jadczak [27] reported antioxidant activities for various species of *Mentha* plants, especially water mint. Boumendjel *et al*. [28] also investigated the antioxidant properties of the essential oil of *M. aquatica* grown in Algeria and, similar to the findings of the present study's experiment, reported high antioxidant activities for *M. aquatica* (IC_{50} values equal to 0.69 µg/ml, in DPPH assay). A major oxygenated monoterpene which accounts for a large percentage of *M. aquatica* essential oil is 1, 8-cineole. In accordance with the present study's findings, Mimica-Dukić *et al*. [19] reported that the antioxidant properties of *M. aquatica* are predominantly the result of a large volume of 1, 8-cineol existing in the essential oil of this plant.

In the present study, we noticed that methanolic extracts, ethanolic extracts and acetonic extracts of the *M. aquatica* from Iran were rich in phenolics, flavonoids and tannins contents, thereby justifying the antioxidant ability of these extracts. Total phenolic, flavonoid and tannin contents and antioxidant activity of aqueous extract were lower than other extracts.

According to the findings of this experiment, Benabdallah *et al*. [29] reported that the antioxidant activity of different types of mint extract was correlated with total phenolic, flavonoid and tannin contents in the extracts.

The high correlation between the values of phenols concentration in plant extracts and antioxidant activity is well documented [30, 31]. In this regard, Luximun-Ramma *et al*. [32] showed a linear correlation between antioxidant activity and phenolic contents of the plant extracts.

The antioxidant activity of polyphenols is mainly due to their ability to act as hydrogen donors, reducing agents and radical scavengers [33]. This activity is generally dependent on total phenol content [34]. In fact, the methanolic extracts, ethanolic extracts and acetonic extracts of *M. aquatica*, which had the highest phenolic, flavonoid and tannin contents, showed the greatest antioxidant activities in comparison with the aqueous extract.

Furthermore, it should be taken into consideration that antioxidant capacities might be attributed to the chemical structure of compounds, as well as synergistic or antagonistic effect of compounds present in the crude extract [35].

The essential oil and all extracts of *M. aquatica* evaluated in this study showed considerable antioxidant levels correlated to the strong polyphenols content. The present study contributed much to the application of *M. aquatica* as herbal tea or additive in foods and folk medicine for the therapy of infectious diseases.

CONCLUSION

Piperitenone oxide, 1,8-cineole and α-gurjunene were the main constituents of water mint essential oil grew in southern Khorasan Province of Iran, and the essential oil of water mint can be considered as a source of natural antioxidants because it has high antioxidant activity. However, the antioxidant properties of the extracts were less than the antioxidant properties of the essential oil. Also, methanolic extracts, ethanolic extracts and acetonic extracts can be considered as sources of natural antioxidants because they had high antioxidant properties and total phenolics, flavonoids and tannins contents.

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