Asian Journal of Chemistry Vol. 22, No. 6 (2010), 4629-4636

Total Phenolics and Antioxidant Activity of Horsemint (*Mentha Longifolia*)

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Phytochemicals are receiving a great interest due to their unique antioxidant property. Plants are the rich source of phytochemicals which are important from antioxidant point of view. Due to their natural source, these antioxidants are thought to be safer. The present research work was designed to investigate the antioxidant activity of *Mentha longifolia* (horsemint). Horsemint showed the total phenolic contents range from 2.95 ± 07-5.16 ± 0.15 gallic acid equivalent (GAE) g/100 g, total flavonoids, 2.00 ± 0.06-14.94 ± 0.35 CE g/100 g, percentage inhibition of peroxidation was (93.1 ± 1.11-97 ± 1.64 % and low IC_{50} values (12.46 ± 0.37-22.09 ± 0.66 µg/mL) for DPPH free radial scavenging. The antioxidant activity of horsemint extracts showed significant ($p < 0.05$) differences with in different solvent concentration. From the results it can be concluded that horsemint could be an efficient source of antioxidants.

Key Words: Antioxidant, Phenolics, Flavonoids, Mint, IC_{50}.

INTRODUCTION

In recent years, there has been growing interest in understanding the role of free radicals. Free radicals have been considered as the fundamental cause of different ailments such as stroke, ageing, inflammation, coronary heart diseases, liver disorder, diabetes mellitus, renal failure and cancer[1-3]. Antioxidants are the substances that interfere with the production of free radicals and also play a significant role to inactivate them[4,5]. Tocopherols, ascorbates, carotenoides and polyphenols are regarded as strong natural antioxidants. Most of the people are showing much interest in biological effects of phenolics because they provide protection against various aliments such as cardiovascular diseases and cancer[6,7].

Several investigators have shown that foods containing phytochemicals with antioxidant property are very important from medicinal point of view alongwith energy source or as diet[8]. Plant derived chemicals are gaining a lot of interest in the treatment of some severe cases of various diseases. There are several foods and medicinal plants known for their antioxidant properties. These plants mostly contain polyphenols, flavonoids and some vitamins as well[9-11]. Peoples living in small towns of Pakistan are using wild plants as a medicinal treatment for over centuries and have been noticed more resistible against these diseases due to the antioxidant property of the phytochemicals in these plants.

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Mentha longifolia (horsemint) is an aromatic perennial herb belonging to the family Labiatae. It is a perennial herb having white creeping rhizomes, with a strong aromatic odour. It is commonly known as horsemint. In Gilgat/Baltistan, it is growing on marshy places by sides of streams, spring and water channels. Horsemint, like many other members of this genus, is often used as a domestic herbal remedy, being valued especially for its stimulant nature, antiseptic properties and its beneficial effect on the digestion. Leaves-raw or cooked peppermint-scented, are used as flavouring in salads, chutneys and cooked foods. The infusion of leaves is taken as a cooling medicine. It is believed to be best remedy for headaches. An essential oil obtained from the leaves and flowering tops is used as food flavouring in sweets.

The aim of the present study is to determine the total phenolic contents and antifree radical activities of ethanolic extracts of horsemint for its potential applications in food and medical uses.

EXPERIMENTAL

Mentha longifolia (horsemint) was purchased from the local market and specimen identification was from Taxonomist, Department of Botany, University of Agriculture, Faisalabad.

Folin-Ciocalteu reagent, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), linoleic acid, trichloro-acetic acid, gallic acid and catechin were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Ethanol, ascorbic acid, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, hydrochloric acid, ferrous chloride, ammonium thiocyanate, sodium nitrite, aluminium chloride were purchased from Merck (Darmstadt, Germany). All chemicals and solvents used were of analytical grade.

Preparation of horsemint extract: The dried horsemint sample was ground to pass 80 mesh size using a commercial blender (TSK-949, Westpoint, France). The ground plant material (15 g) was extracted with 150 mL of each of the solvent system (30 % ethanol, 50 % ethanol, 70 % ethanol and absolute ethanol) in 250 mL conical flask and was shaken for 8 h at room temperature in an orbital shaker (Gallenkamp, UK). The extracts were separated from the residues by filtering through Whatman No. 1 filter paper. The residues were extracted twice with the same fresh solvent and extracts were combined. The combined extracts were concentrated and freed of solvent under reduced pressure at 45 °C, using a rotary evaporator (EYELA, SB-651, Rikakikai Co. Ltd. Tokyo, Japan). The dried, crude concentrated extracts were weighed to calculate the yield and stored in a refrigerator (-4 °C) until used for further analyses.

Determination of total phenolic compounds: Amount of total phenolic contents in the samples of horsemint was determined with Folin-Ciocalteu reagent[12]. Briefly, 50 mg of crude extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL of deionized water. The mixture was kept at room temperature for 10 min and then 1.5 mL of sodium carbonate (2 %) solution was added. The mixture was heated in a water bath at 40 ºC for 20 min and then cooled in an ice bath; absorbance was

measured at 755 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Amounts of total phenolic contents were calculated using gallic acid calibration curve within range of 10-100 mg/L ($R^2 = 0.995$). The results were expressed as gallic acid equivalents (GAE) g/100 g of dry plant material.

Determination of total flavonoids: The total flavonoids of horsemint were determined by a previously reported method[13]. Briefly, plant extract of each material (1 mL containing 0.1 mg/mL) was diluted with 4 mL water in a 10 mL volumetric flask. Initially, 0.3 mL of 5 % $NaNO_2$ was added to each volumetric flask, at 5 min, 0.3 mL of 10 % $AlCl_3$ was added and at 6 min, 2 mL of 1 M NaOH was added. Water (2.4 mL) was then added to the reaction flask and mixed well. Absorbance of the reaction mixture was measured at 510 nm. Total flavonoids were determined as catechin equivalents (g/100 g of dry plant material).

Scavenging activity on DPPH free radical: To evaluate the free radical scavenging activity, the horsemint extract was allowed to react with a stable free radical, DPPH. The assay was performed as reported by Bozin *et al.*[14]. The samples (from 0.2-500.0 µg/mL) were mixed with 1 mL of 90 µM DPPH solution and filled up with 95 % methanol, to a final volume of 4 mL. The absorbance of the resulting solutions and the blank were recorded after 1 h at room temperature. Butylated hydroxytoluene (BHT) was used as a positive control. For each sample, three replicates were recorded. The disappearance of DPPH was read spectrophotometrically at 515 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Inhibition of free radical by DPPH in per cent (%) was calculated in the following way:

$$\text{Antiradical activity (\%)} = 100 - (A_{blank} - A_{sample})/A_{blank}$$

where A_{blank} is the absorbance of the control reaction mixture excluding the test compounds and A_{sample} is the absorbance of the test compounds. Extract concentration providing 50 % inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

Antioxidant activity determination in linoleic acid system: Antioxidant activity of horsemint extracts was determined in terms of measurements of % inhibition peroxidation in linoleic acid system using thiocyanate assay[15]. Extract solution (0.1 mL) in 95 % ethanol was mixed with 2.5 mL of linoleic acid emulsion (0.2 M, pH 7) and 2 mL of phosphate buffer (0.2 M, pH 7). The reaction mixture was incubated at 37 °C to accelerate the oxidation process and used each 24 h for assessing antioxidant activity. The mixture without added extract was used as control. The mixture (0.1 mL) was taken and mixed with 5 mL of 75 % ethanol, 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL ferrous chloride ($FeCl_2$) solution (20 mM in 3.5 % HCl) being added sequentially. After 3 min of stirring, the absorbance values of mixtures measured with spectrophotometer (Hitachi U-2001 model 121-0032 Japan) at 500 nm were taken as peroxide contents. Butylated hydroxytoluene (BHT) and ascorbic acid (200 ppm) were used as positive control. Per cent inhibition of peroxidation of linoleic acid was calculated by the following formula:

$$IP\ (\%) = 100 - [(\text{Increase in } A_{sample} \text{ at 360 h/Increase in } A_{blank} \text{ at 360 h}) \times 100]$$

where A_{sample} is absorbance of sample and A_{blank} is absorbance of blank.

Statistical analysis: All the experiments were conducted in triplicate and results are reported as mean ± SD. The data was presented as mean values at 95 % confidence interval. Analysis of variance was performed using ANOVA procedures. Significant differences between means ($p < 0.05$) was determined by Minitab Software.

RESULTS AND DISCUSSION

Percentage yield: The extraction of various phytochemicals from the plant materials depends on the nature of the solvent used. As most of the contents of plant material are organic and polar in nature, generally solvents having some polarity are used for the extraction purposes. To get high percentage yield of the plant extract in ethanol was used due to its polarity. In this study, total phenolics, antioxidant capacity and antiradical activity of the ethanolic extracts of *Mentha logifolia* were studied. The percentage yields obtained from different ethanol concentration are shown are Fig. 1. The percentage yield ranges from 12.9 ± 0.39-16.11 ± 0.4 g/100 g. Maximum percentage yield (16.11 ± 0.4 g/100 g) was obtained from 70 % ethanol and lowest from 30 % ethanol. So, 70 % ethanol found to be good solvent for the extraction of antioxidant components from horsemint. The results showed that extraction yield of antioxidant components depends on solvent concentration. Effect of solvent concentration on the extract yield was significant ($p < 0.05$). Efficiency of extracts is an important factor for the comparison of antioxidant activity. Therefore an efficient, appropriate and adequate concentration of the solvent should be used to extract maximum antioxidant compounds from any plant material. As the antioxidant compounds are organic in nature and organic compounds mostly dissolve in organic solvents. So ethanol is very much effective in the extraction of antioxidant compounds due to its organic nature. Ethanol is generally employed for the extraction of antioxidant components from plant materials due to its polarity, good solubility and lower toxicity[16,17]. Bushra *et al.*[18] used different solvent systems to extract total phenolics and total flavonoids from the barks of *A. indica*, *T. arjuna*, *A. nilotica* and *E. jambolana*. The highest amounts of total phenolics and flavonoids were extracted with 80 % ethanol from *A. indica* and *T. arjuna*.

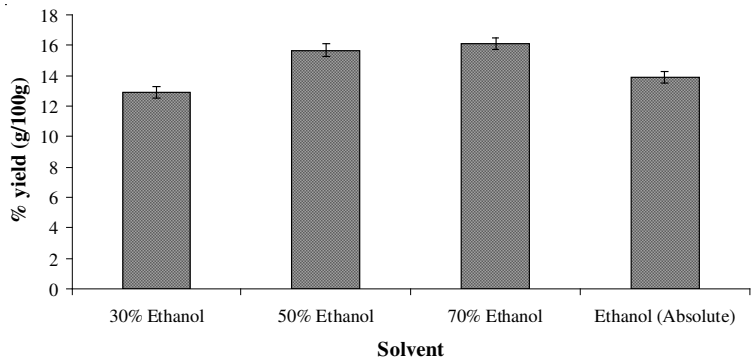

Fig. 1. Percentage yield of horsemint extracts with different ethanol concentration

Total phenolic contents: Total phenolics of *Mentha logifolia* extracts were determined by Folin-Ciocalteu method which was selected due to its sensitivity, lower interference and quickness to quantify the phenolics as compared to other competitive tests. There has been growing interests in the search of phenolic compounds due their prominent free radical scavenging activity[19]. The total phenolics of the extracts obtained from different ethanolic extract range from 2.95 ± 07-5.16 ± 0.15 GAE g/100 g (Fig. 2). Maximum amount of phenolic components (5.16 ± 0.15 GAE g/100 g) was observed with 70 % ethanolic extract. The effect of ethanol concentration was significant ($p < 0.05$). Bajpai *et al.*[20] investigated the total phenolic contents and antioxidant activity of some food and medicinal plants. The leaves and fruits of *Phyllanthus emblica* and seeds of *Syzygium cumini* were found to have high total phenolic contents (7.2-16.72 g/100 g). Similar the 80 % ethanolic extracts of barks of *A. indica*, *T. arjuna*, *A. nilotica* and *E. jambolana* were found to contain 12 ± 0.36, 12.8 ± 0.26, 16.5 ± 0.66 and 9.00 ± 0.45 g/100 g of phenolics, respectively[18]. Present results clearly indicated that the most affective antioxidant compounds were extracted with 70 % ethanolic extract. It is considered that phenolic compounds contribute directly to overall antioxidant activities of horsemint. Polyphenols are known bioactive molecules that are ubiquitously distributed in plant species, influencing their morphology, growth, reproduction as well as their resistance to parasites.

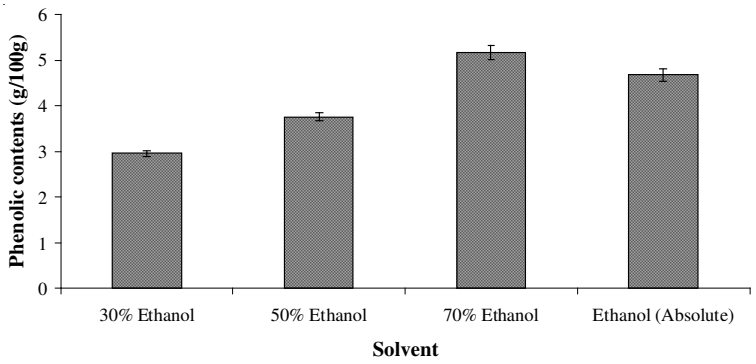

Fig. 2. Total phenolic contents of horsemint extracts with different ethanol concentration

Total flavonoids: Phenolic compounds are generally classified as simple phenols, a simple aromatic ring with at least one hydroxyl group and polyphenols with at least two phenol subunits like flavonoids and three or more phenol subunits called tannins[21]. The results of total flavonoids of different ethanolic extracts of green cardamom are shown in the Fig. 3. The flavonoids content of *Mentha logifolia* extracts obtained from different ethanol concentrations range from 2.00 ± 0.06-14.94 ± 0.35 CE g/100 g. The maximum value of flavonoids was 14.94 ± 0.35 CE g/100 g obtained from 70 % ethanolic extracts while the minimum (2.00 ± 0.06 CE g/100 g) value was recorded for 30 %. The effect of solvent concentration on the amount of flavonoids was significant ($p < 0.05$). The results obtained from the

present study were comparable to the earlier reported results[18], in which 80 % ethanol was used as solvent to extract total flavonoids contents. The results of total flavonoids content of the *Azadirecthta indica*, *Terminalia arjuna*, *Acacia nilotica* and *Eugenia jambolana* were comparable to the results obtained from the present study.

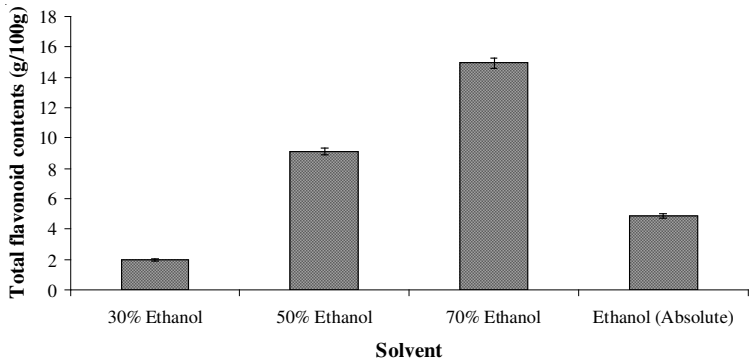

Fig. 3. Total flavonoids of horsemint extracts with different ethanol concentration

DPPH radical scavenging activity: DPPH radicals are widely used in the model systems to investigate the scavenging activities of several natural compounds. When DPPH radicals are scavenged, the colour of the reaction mixture changed from purple to yellow with decreasing of absorbance at wavelength 517 nm. DDPH is a very stable organic free radical with deep violet colour which give absorption maxima at 515-528 nm, upon receiving proton from any hydrogen donor species mainly, phenolics loses this absorption, resulting in a visually noticeable colour change from deep violet to yellow. As the concentration of phenolic compounds or degree of hydroxylation of the phenolic compounds increases DPPH scavenging activity increases, consequently, antioxidant activity increases[22]. The IC_{50} values of four extracts are shown in Fig. 4. Lower the IC_{50} value of extract, more effective it will be for the inhibition of DPPH. The IC_{50} value of 70 % ethanolic extract was the

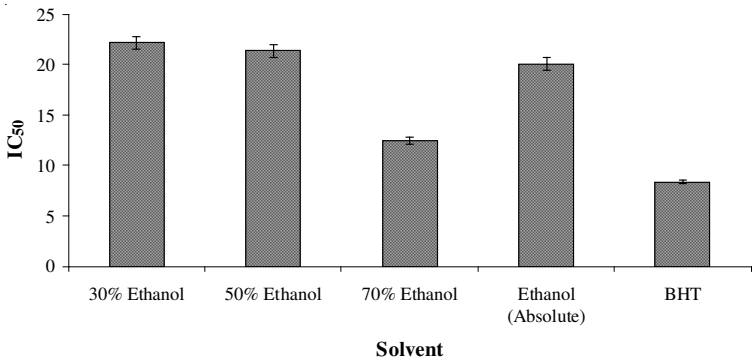

Fig. 4. DPPH free radical scavenging activity (IC_{50} μg/mL) of horsemint extracts with different ethanol concentration and BHT

lowest 12.46 ± 0.37 µg/mL and that of 30 % ethanolic was highest 22.9 ± 0.66 µg/ mL. The results were compared with BHT as standard (IC_{50} = 8.39 ± 0.2 µg/mL). Yadav and Bhantnagar[23] investigated antioxidant power of some Indian spices such as cloves, licorice, mace and greater cardamom and found that the cloves exhibited the highest DPPH radical scavenging activity followed by licorice, mace and cardamom. Similarly, Kikuzaki *et al*.[24] reported that ethyl acetate soluble fraction of greater cardamom showed a high radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Percentage inhibition of peroxidation in linoleic acid system: The antioxidant activity has also been assessed as ability to prevent the oxidation. Therefore, inhibition of linoleic acid oxidation was also used to assess the antioxidant activity of horsemint extracts. Antioxidant activity of different mint extracts was determined by inhibition of peroxidation in linoleic acid system by using thiocyanate method. Linoleic acid is a polyunsaturated fatty acid, upon oxidation peroxides are formed which oxidize Fe^{2+}-Fe^{3+}, the later forms complex with SCN^{-}, concentration of which is determined spectrophotometrically by measuring absorbance at 500 nm. Inhibition of linoleic acid oxidation showed significantly ($p < 0.05$) different effectiveness for analyzed extracts of horsemint (Fig. 5). The results clearly show that 70 % ethanolic extract of horsemint extracted by shaking method exhibited the highest inhibition in linoleic acid system and thus reflected the highest antioxidant activity (97 ± 1.64 %) while that of 30 % ethanolic extract has the lowest activity (93.1 ± 1.11 %). Sultana *et al*.[18] investigated the effect of different extracts of barks of *Azadirecthta indica*, *Terminalia arjuna*, *Acacia nilotica* and *Eugenia jambolana* on the inhibition of oxidation of linoleic acid and found that the different bark extracts inhibited oxidation of linoleic acid by 44-99 %.

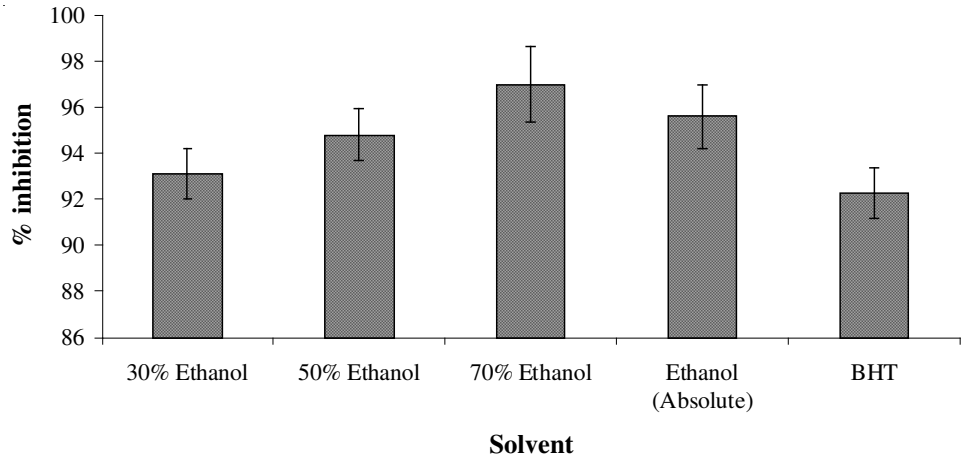

Fig. 5. Percentage inhibition of peroxidation in linoleic acid by horsemint extracts with different ethanol concentration and BHT

Conclusion

In the present study, horsemint was found to be a good source of antioxidants and hence show high antioxidant activity. This study reveals that the use of horsemint in diet not only provides flavour to the foods but also reduces the chances of their oxidation. This study showed that 70 % ethanol is a good solvent for the extraction of antioxidants due to its high polarity. Hence wild mint can also be used in the treatment of cardiovascular diseases, neurodegenerative diseases and aging process. Further work is required to isolate those compounds which showed antioxidant activity.

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(*Received*: 17 September 2009; *Accepted*: 17 February 2010) AJC-8453