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SHORT COMMUNICATION

Biological activities of ethyl acetate extract of different parts of *Hyssopus angustifolius*

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Abstract

Context: Hyssopus angustifolius M. Bieb. (Lamiaceae) is one of the most important medicinal plants in Iranian traditional medicine for the treatment of lung inflammation, laryngitis and cough relief. Much attention has been paid to this medicinal plant because of its traditional uses.

Objective: The present study examined the antioxidant and antihemolytic activities of ethyl acetate extract of stems, leaf and flowers of *Hyssopus angustifolius*.

Materials and methods: Antioxidant activity of extracts was evaluated by employing six different models, i.e., DPPH, nitric oxide and hydrogen peroxide scavenging, metal chelating and reducing power activities and hemoglobin-induced linoleic acid system. Also, antihemolytic activity was evaluated against hydrogen peroxide-induced hemolysis.

Results: Flowers extract showed the better activity than leaf and stems extracts in DPPH radical scavenging activity (IC_{50} was 275.4 ± 7.6 μg mL^{-1}). Leaf, stems and flowers extracts showed good nitric oxide scavenging activity (IC_{50} were 376.6 ± 11.4 μg mL^{-1} for flowers, 297.6 ± 9.6 μg mL^{-1} μg mL^{-1} for leaves and 837.8 ± 19.2 μg mL^{-1} for stems). The leaf extract exhibited better hydrogen peroxide scavenging and Fe^{2+} chelating activity than stems and flowers extracts. In hemoglobin-induced linoleic acid system, all of the extracts exhibited very good activity. Also, extracts show weak reducing power activity. The ethyl acetate extract of leaf showed better antihemolytic activity than the flower and stems (IC_{50} was 94.0 ± 2.4 μg mL^{-1}).

Discussion and conclusion: These findings give a scientific basis to the traditional usage of *Hyssopus angustifolius*, also showing its potential as rich sources of natural antioxidant compounds.

Keywords: Free radicals, antihemolytic, linoleic acid

Introduction

Recently, there has been interest in the use of antioxidative nutritional substances (Gigante et al., 2007; Simpore et al., 2006). Numerous studies showed that using of some vitamins, minerals, and other food constituents with antioxidant activity can protect our body against some diseases caused by oxidative imbalance and free radicals such as cardiovascular disease, cancer, and the aging process (Wu et al., 2005; Hsia et al., 2007; Luchsinger et al., 2007; Marcason, 2007). Antioxidants can decrease the chance of getting the above-mentioned diseases via inhibition of reactive oxygen species that originated from aerobic metabolism (Abdel-Wahhab et al., 2005). Natural products are very rich sources of natural antioxidant agents.

Hyssopus angustifolius M. Bieb. (Lamiaceae) is a medicinal plant that used in Iranian folk medicine and cultivated in central and south area of Europe (Omidbaigi, 2000). In Iranian folk medicine, this species used in tea blends to diminish coughing and induce sedative effects,

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and to reduce catarrh (Khazaie et al., 2008). To the best of our knowledge there are no reports about biological activities of ethyl acetate extract of different parts of *Hyssopus angustifolius*.

Materials and methods

Chemicals

Ferrozine, linoleic acid, trichloroacetic acid, 1,1-diphenyl-2-picryl hydrazyl, potassium ferricyanide, sodium nitrite, and hydrogen peroxide were purchased from Sigma Chemical Co. (USA). Gallic acid, quercetin, butylated hydroxyanisole, vitamin C, sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck Co. (Germany). All other chemicals were of analytical grade or purer.

Plant materials

Whole plants of *Hyssopus angustifolius* were collected from Elburz Mountains, Iran, in spring 2010. The plant was identified by Dr. Bahman Eslami (Assistant Professor of Plant Systematic and Ecology, Department of Biology, Islamic Azad University, Branch of Ghaemshahr, Iran) and authenticated at the Herbarium of Department of Biology in the University of Mazandaran (a voucher specimen No 975).

Extraction

Approximately 100 g of the samples powder were placed in a Soxhlet extractor and extracted with ethyl acetate for 8 h. The solvent was recovered by distillation *in vacuo*, and the residue, stored in the desiccator, was used for subsequent experiments.

Phytochemical screening

Total phenolic and flavonoid contents

Total phenolic content was determined by employing the Folin-Ciocalteau method (Ebrahimzadeh et al., 2010). The sample (0.5 mL) at 1.6 mg mL^{-1} was mixed with Folin-Ciocalteau reagent (0.2 N, 2.5 mL) for 5 min and sodium carbonate (75 g L^{-1}, 2.0 mL) was then added. The reactions mixture was incubate for 2 h at room temperature and then measured at 760 nm. Results were expressed as gallic acid equivalents. Total flavonoid content was measured by employing the method of Ebrahimzadeh et al., (2010). 0.5 mL of sample at 1.6 mg mL^{-1} was separately mixed with 1.5 mL of methanol, aluminum chloride (10%, 0.1 mL), potassium acetate (1 M, 0.1 mL) and distilled water (2.8 mL) and incubated for 30 min at room temperature. The reaction mixture was measured at 415 nm. Total flavonoid content was calculated as quercetin from a calibration curve.

Antioxidant activity

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging

Samples (25–400 μg mL^{-1}) were added, at an equal volume, to a solution of DPPH (100 μM in methanol). Reactions were incubating for 15 min at room temperature and then were recorded at 517 nm. The experiment was repeated three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration, which is scavenging 50% of free radical (Nabavi et al., 2010).

Reducing power

The reducing power of extracts was determined using the method of (Pulido et al., 2000). Aqueous solutions of samples (25–400 μg mL^{-1}, 2.5 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). Reaction mixtures were incubated at 50°C for 20 min. To stop the reaction, trichloroacetic acid (10%, 2.5 mL) was added to the mixtures, which was then centrifuged at 1000*g* for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.1%, 0.5 mL), and measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Metal chelating

The activity of extracts to chelate iron ions was estimated by the method of Dinis et al. (1994). Each sample (25–400 μg mL^{-1}, 1 mL) was added to a solution of $FeCl_2$ (2 mM, 0.05 mL). For initiated the reactions, ferrozine (5 mM, 0.2 mL) was added and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solutions was then measured at 562 nm. Na_2EDTA was used as positive control.

Nitric oxide scavenging

Sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extract (25–400 μg mL^{-1}), dissolved in water and incubated at room temperature for 150 min. After the incubation period, 0.5 mL of Griess reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance was measured at 546 nm. Quercetin was used as positive control (Sreejayan & Rao, 1997).

Hydrogen peroxide scavenging

Each sample (2 mL) (0.1-1 mg mL^{-1}) in distilled water was added to a hydrogen peroxide solution (0.6 mL, 40 mM) in phosphate buffer (pH 7.4). The absorbances of samples at 230 nm were measured after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and extracts. Vitamin C and quercetin were used as standards (Elmastas et al., 2006).

Hemoglobin-induced linoleic acid system

A reaction mixture (2 mL) containing 100 μL of each extract (25–400 μg mL^{-1}), linoleic acid emulsion (1 mmol L^{-1}), phosphate buffer (40 mmol L^{-1}, pH 6.5), and hemoglobin suspension (0.0016%), was incubated at 37°C for 45 min. After the incubation, hydrochloric acid in ethanol (0.6%, 2.5 mL) 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 of $FeCl_2$ (0.02 mol L^{-1}, 100 µl) and of ammonium thiocyanate (0.3 g mL^{-1}, 50 µl). Vitamin C was used as positive control (Nabavi et al., 2010).

Antihemolytic activity

Preparation of rat erythrocytes

Male rats in the body weight range of 180–220 g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) given intraperitoneally. Blood samples were collected via retro-orbital puncture in plain plastic tubes. Erythrocytes were isolated and stored according to the method described by Nabavi et al. (2010).

Protection against H_2O_2 induced hemolysis

Antihemolytic activity of the extracts was determined by the method of Ebrahimzadeh et al. (2010).

Different concentrations of the each extracts (0.5 mL) were added to erythrocyte suspension (4%, 2 mL) and the volume was made up to 5 mL with saline buffer. Reaction mixtures were incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce hemolysis. After incubation (240 min) at room temperature, the reaction mixture was centrifuged at 250*g* for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

Statistical analysis

Experimental results are expressed as means ± S.D. All measurements were in triplicate. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range tests. The IC_{50} values were calculated from linear regression analysis.

Results and discussion

Total phenolic content of extracts are reported as gallic acid equivalents by reference to a standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic content of the ethyl acetate extract of flowers, leaves and stems were 134.4 ± 8.0, 122.6 ± 7.9 and 97.0 ± 6.4 mg gallic acid equivalent/g of extract, respectively. Also, total flavonoid contents of ethyl acetate extract of flowers, leaves and stems were in the order of 67.2 ± 2.0, 76.1 ± 3.1 and 44.1 ± 1.3 mg quercetin equivalent/g of extract powder, respectively, by reference to a standard curve ($y = 0.0063x$, $r^2 = 0.999$). Phenol and polyphenolic contents in natural products have significant biological activities (van Acker et al., 1996).

In DPPH radical scavenging activity model, ethyl acetate extracts showed lower activity than standard compound. IC_{50} values of the samples was in the order of flowers (275.4 ± 7.6 µg mL^{-1}) > leaves (297.0 ± 9.6 µg mL^{-1}) > stems (396.4 ± 10.4 µg mL^{-1}), respectively. Also IC_{50} values for ascorbic acid, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1 µg mL^{-1}, respectively. The polyphenolic content in the extracts may correlate to their good DPPH radical scavenging activity. It has been assumed that phenol and flavonoid can quench DPPH radical via hydrogen- or electron-donation mechanism and changes its color from violet to yellow. Antioxidant is defined as any substances which are able to perform this reaction.

In the reducing power model, electron donor compound in the extracts can reduce Fe^{3+} to Fe^{2+}. Perl's Prussian blue color form by Fe^{2+} complex can be monitored by measuring of absorbance of sample at 700 nm (Ebrahimzadeh et al., 2010). Increasing in absorbance at 700 nm indicates an increase in reducing ability. Dose response curves for the reducing power of extracts are shown in Figure 1. Vitamin C has better electron donating activity than tested samples ($p < 0.001$).

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen leading to the production of nitrite ions that can be monitored using griess reagent. Nitric oxide scavengers challenge with oxygen, leading to reduced production of nitrite ions. The IC_{50} values for nitric oxide radical scavenging activity of extracts were in order to: leaves

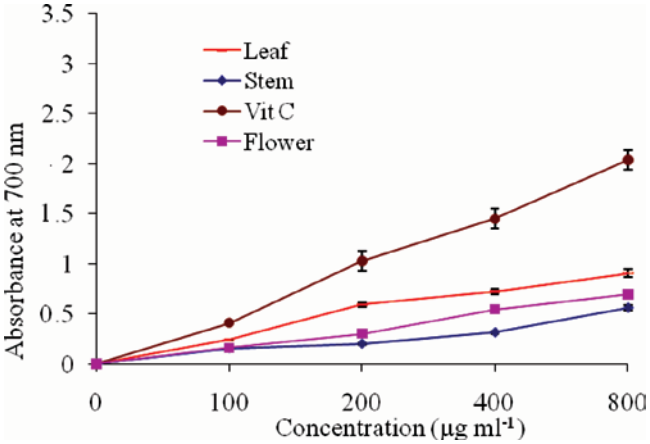

Figure 1. Reducing power of extracts. Vitamin C used as positive control.

(297.6 ± 9.6 μg mL^{-1}) > flowers (376.6 ± 11.4 μg mL^{-1}) > stems (837.8 ± 19.2 μg mL^{-1}). Although quercetin showed better nitric oxide scavenging activity than extracts (IC_{50} = 17.01 ± 0.03 μg mL^{-1}, $p < 0.001$), there are numerous reports on its carcinogenic activity (Dunnick & Hailey, 1992). Nitric oxide has an important role in the initiation or progression of many diseases such as inflammation, cancer and other pathological conditions (Nabavi et al., 2010). Natural products with nitric oxide scavenging ability may be interest in preventing the above-mentioned diseases.

Leaf extract showed better iron chelating activity than others. Iron ion chelating activity of extracts was in the order of leaves (603.5 ± 11.2 μg mL^{-1}) > flowers (776.0 ± 12.4 μg mL^{-1}) > stems (942.2 ± 16.0 μg mL^{-1}), respectively. EDTA showed potent activity that was better than extracts (IC_{50}= 18 ± 0.5 μg mL^{-1}, $p < 0.001$). Iron chelators can remove tissue iron via forming soluble, stable complexes that can excreted in the feces and/or urine. Chelation therapy is one of the most common ways to reduce iron related complications in human and so improve life quality and overall survival in many diseases such as thalassemia major (Hebbel et al., 1990). Deferoxamine is an iron chelator drug used for chelation therapy in treatment of iron overload in thalassemia patients. Overdose of deferoxamine may cause some adverse effects (Porter, 1997). Another drug, deferiprone, is orally absorbed bi-dentate iron chelator that can reduce iron via increase in urinary iron excretion, improve negative iron balance and reduce hepatic iron levels. But arthritis, nausea and agranulocytosis are common side effects of this drug. For the above-mentioned reason urgent needs to find an active chelator regimen that is as effective as deferoxamine and deferiprone and has a lower side effect remain (Porter, 1997).

In recent years, searches in natural products to there has been extensive investigate iron chelators with lower adverse effect (Grazul & Budzisz, 2009). Also, many studies showed that iron chelators and hydroxyl-radical scavengers can protect against acute renal failure especially aminoglycoside antibiotic-mediated nephrotoxicity (Nabavi et al., 2012). Iron chelators can suppress these procedures. Dinis et al., (1994) method has been used for determination of ferrous ion chelating activity. 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 color of the complexes decreases. In iron chelation assay, all of the extracts and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.

Samples show hydrogen peroxide scavenging in a concentration dependent manner. The IC_{50} values of hydrogen peroxide scavenging activity of extracts were in the order of leaves (297.5 ± 5.9 μg mL^{-1}) > stems (377.5 ± 6.4 μg mL^{-1}) > flower (425.1 ± 7.7 μg mL^{-1}), respectively. The IC_{50} values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 μg mL^{-1}, respectively. Hydrogen peroxide scavenging ability of sample may be originated from phenolic compounds and/or other active components which can donate electrons to hydrogen peroxide and neutralizing it to water (Elmastas et al., 2006). Hydrogen peroxide itself is not very reactive, but it can increase hydroxyl radical formation in the cell and cause cytotoxicity (Ebrahimzadeh, 2010).

Tested extracts show good activities in hemoglobin-induced linoleic acid emulsion (Figure 2). Erythrocytes are major targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids and the oxygen transport associated with redox active hemoglobin, which are potent promoters of free radicals. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (Yu, 2001). The inhibition of lipid peroxidation by samples may be due to their free radical scavenging activities. Lipid peroxidation indirectly initiated by superoxide because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (Ebrahimzadeh et al., 2010). Hydroxyl radicals attack to membrane lipids via removing of hydrogen atoms that causes lipid peroxidation. There were no significant differences between extracts and vitamin C ($p > 0.05$).

The antihemolytic activity of extracts were tested and it was found they did not show any harmful

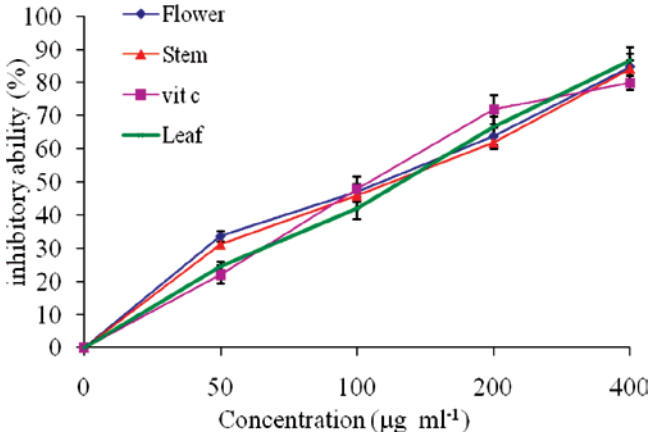

Figure 2. Antioxidant activity of extracts against hemoglobin-induced lipid peroxidation. Vitamin C was used as positive control.

effects on erythrocytes. IC_{50} of antihemolytic activity of extracts was in the order of leaves (94.0 ± 2.4 μg mL^{-1}) > stems (128.6 ± 3.1 μg mL^{-1}) > flowers (137.2 ± 3.9 μg mL^{-1}) vs. vitamin C (235 ± 9 μg mL^{-1}), respectively. All of the extract showed better activity than vitamin C ($p < 0.001$). Previous studies showed a correlation between antihemolytic activity and flavonoid content and the good activity of extracts may be the result of high flavonoid, especially the flavonol group (Chaudhuri et al., 2007).

Conclusion

The present study shows biological activities of the ethyl acetate extract of flowers, leaves and stems of *Hyssopus angustifolius*. All of the extracts show different levels of antioxidant and antihemolytic activities in the studied models. Other studies on *in vivo* biological and toxicological activities and/or using this species in clinical study are needed. The results of this study can be benefit as a starting point for further applications of this species or its bioactive natural compounds in natural pharmaceutical formulation.

Declaration of interest

The authors report no declarations of interest.

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