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DRUG BIOCHEMISTRY

BIOCHEMICAL ACTIVITIES OF ACETONE EXTRACTS OF *HYSSOPUS ANGUSTIFOLIUS*

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Abstract: Antioxidant and antihemolytic activities of acetone extracts of *Hyssopus angustifolius* flowers, leaf and stems were investigated employing different *in vitro* and *ex vivo* assay systems. IC_{50} for 1,1-diphenyl-2-picryl hydrazyl ($DPPH^{\cdot}$) radical-scavenging activity were 239.4 ± 8.4 μg/mL for flowers, 357.8 ± 11.1 μg/mL for stems and 182.5 ± 7.5 μg/mL for leaf. All extracts showed moderate nitric oxide scavenging activity. The leaf extract exhibited better hydrogen peroxide scavenging and Fe^{2+} chelating activity than the others (IC_{50} were 261.0 ± 6.2 μg/mL for hydrogen peroxide and 534.0 ± 9.9 μg/mL for Fe^{2+} chelating activity). The extracts exhibited good antioxidant activity in linoleic acid peroxidation system and weak reducing power ability. The leaf extract showed better antihemolytic activity than the flower and stem (IC_{50}= 65.7 ± 1.8 μg/mL).

Keywords: antioxidant activity, chelating activity, *Hyssopus angustifolius*, linoleic acid

The autooxidation of lipids is an important problem because of decline in the foods quality in which they are included and the reduction in their nutritional value (1, 2). In addition, some of the diseases such as cardiovascular diseases are caused by oxidation of polyunsaturated fatty acids in cell membranes. Antioxidants can protect body against oxidation by quenching of reactive oxygen species. Synthetic phenols, such as, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are used to decelerate oxidation process; however, there are numerous questions about their safety (3). Therefore, attention is focused on natural antioxidants. Polyphenolic compounds are one of the most largest group of natural originated antioxidants (4, 5). Plants are accessible and highly rich sources of polyphenols. *Hyssopus angustifolius* is a member of large family, *Lamiaceae*, widely used as medicinal plant in traditional medicine, cultivated in central and south European countries such as Russia, Spain, France and Italy (6). In traditional medicine, it is used in tea blends for reduction of coughing, to reduce catarrh and has anticonvulsant activity (7). To the best of authors knowledge there are negligible scientific results reported about antioxidant and antihemolytic activities of acetone extracts of different parts of *Hyssopus angustifolius*. In order to scientifically evaluate of some ethnomedical uses of *Hyssopus angustifolius*, its antioxidant activity and protective effect against hemolysis were investigated.

EXPERIMENTAL

Chemicals

Disodium salt of 3-(2-pyridil)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine), linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide and hydrogen peroxide were purchased from Sigma Chemicals Co. (USA). Sodium nitroprusside, ammonium thiocyanate, ascorbic acid, gallic acid, quercetin, butylated hydroxyanisole (BHA), vitamin C, EDTA and ferric chloride were pur-

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chased from Merck (Germany). All other chemicals were of analytical grade or purer.

Plant materials

Whole plants of *Hyssopus angustifolius* were collected from Veresk area (Latitude: 35°54' N, Longitude: 52°59' E, altitude: 1900 above sea level), Elburz Mountains, Iran, in spring 2010 and identified by Dr. Bahman Eslami, assistant professor of plant systematics and ecology, Department of Biology, Islamic Azad University, branch of Ghaemshahr, Iran, where a voucher specimen (No. 975) at the Herbarium of Department of Biology in University of Mazandaran was deposited.

Extraction procedure

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

Phytochemical contents

Total phenolic content

Total phenolic content was determined by the Folin-Ciocalteau method (8). Extracts samples (0.5 mL of 1.6 mg/mL) were mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent at room temperature for 5 min and 2.0 mL of 75 g/L sodium carbonate were then added. The absorbance of reaction mixtures was measured at 760 nm with a double beam spectrophotometer (UV–Visible EZ201, Perkin Elmer USA) after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents.

Total flavonoid content

Total flavonoid content was estimated using the method of Ebrahimzadeh et al. (8). Briefly, 0.5 mL solutions of plant extracts were 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 min. 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

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

Samples (25–400 µg/mL) were added, at an equal volume, to 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 for three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration, which is scavenging 50% of free radicals (8).

Reducing power

The reducing power of extracts was determined using the method of Ebrahimzadeh et al (8). Aqueous solutions of samples (25–400 µg/mL, 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 were 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 its absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated an increased reducing power. Vitamin C was used as a positive control.

Metal chelating

The activity of extracts to chelate of iron ions was estimated by the method of Dinis et al. (9). Each sample (25–400 µg/mL, 1 mL) was added to a solution of $FeCl_2$ (2 mM, 0.05 mL). For initiation of reaction, 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 solution was then measured at 562 nm. Na_2EDTA was used as a 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), 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 a positive control (8).

Hydrogen peroxide scavenging

Sample (2 mL of each, 0.1–1 mg/mL) 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 (8).

Hemoglobin-induced linoleic acid system

Reaction mixture (2 mL) containing 100 µL of each extracts (25–400 µg/mL), linoleic acid emulsion (1 mmol/L), phosphate buffer (40 mmol/L, pH 6.5), and hemoglobin suspension (0.0016% v/w in PBS pH 7.4), 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 $FeCl_2$ (0.02 mol/L, 100 µL) and of ammonium thiocyanate (0.3 g/mL, 50 µL). Vitamin C was used as a positive control (8).

Antihemolytic activity

Preparation of rat erythrocytes

Male rats of 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 laboratory animal feed, manufactured by the Pasteur Institute, Tehran, Iran. Water was provided *ad libitum*. 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 Ebrahimzadeh et al. (8).

Protection against H_2O_2 induced hemolysis

Antihemolytic activity of the extracts was determined by method of Ebrahimzadeh et al. (8). 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 extend of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

Statistical analysis

Experimental results are expressed as the means ± SD. All measurements were made 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 by linear regression analysis.

RESULTS AND DISCUSION

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 contents of acetone extract of flowers, leaf and stems were 144.4 ± 12.6, 155.7 ± 13.6 and 140.2 ± 12.0 mg gallic acid equivalent/g of extract, respectively. Also, total flavonoid contents of acetone extract of flowers, leaf and stems were in order of: 92.8 ± 5.6, 114.0 ± 5.9 and 48.0 ± 2.5 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). Phenolic compounds are widely found in natural products, and they have been shown to possess biological activities (10). IC_{50} for DPPH radical scavenging activity of acetone extracts was in order of: leaf (182.5 ± 7.5 µg/mL) > flowers (239.4 ± 8.4 µg/mL) > stems (357.8 ± 11.1 µg/mL), respectively. The 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, respectively. Phenol and flavonoid content in the extracts may lead to their very potent DPPH radical scavenging activity. Phenol and flavonoid can scavenge DPPH radical by either the process of hydrogen or electron donation and changes it color from violet to yellow. Substances which are able to perform this reaction can be considered as DPPH radical scavengers (8). In the reducing power ability, the presence of electron donor in the sample would result in the reducing of Fe^{3+} to Fe^{2+}. Amount of Fe^{2+} complex can be then monitored by measuring the formation of Perl's Prussian blue at 700 nm (8). Increasing absorbance at 700 nm indicates an increase in reducing power ability. Figure 1 shows the dose response curves for the reducing power of extracts. Vitamin C shows better activity than other samples. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be determined using Griess reagent. Nitric oxide scavengers contest with oxygen, leading to reduced production of nitrite ions. IC_{50} for nitric oxide radical scavenging activity of extracts were in order of: leaf (391.1 ± 12.0 µg/mL) > flowers (452.3 ± 17.0 µg/mL) > stems (534.7 ± 13.7 µg/mL). Quercetin show very good nitric oxide scavenging activity (IC_{50} = 17.01 ± 0.03 µg/mL). Although quercetin showed very good nitric oxide radical scavenging, but carcinogenic activity of it has been reported, previously (11). Nitric oxide scavenging model is based on the principle that sodium nitroprusside at physiological conditions generates nitric oxide that interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Nitric oxide scavenger compound compete with oxygen. This competition leads to

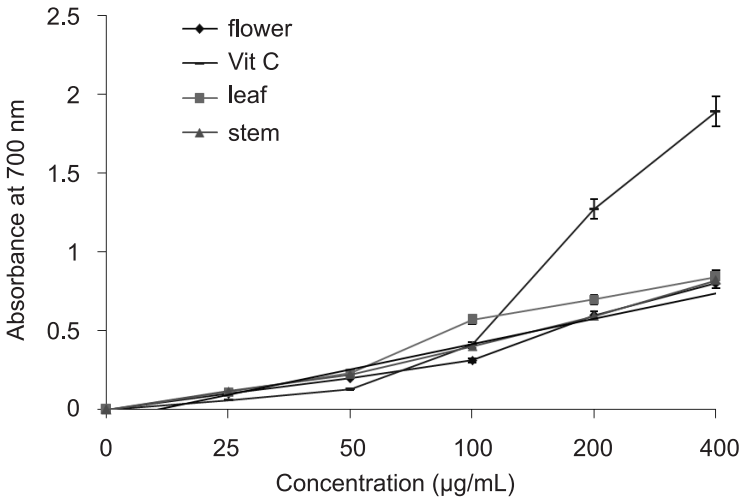

Figure 1. Reducing power of acetone extract of different parts of *Hyssopus angustifolius*

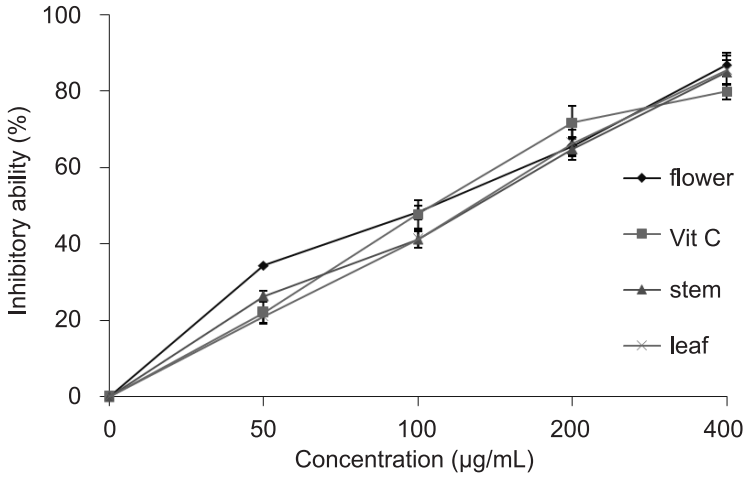

Figure 2. Antioxidant activities of acetone extract of different parts of *Hyssopus angustifolius* against linoleic acid peroxidation induced by hemoglobin

nitrite ions reduction. Many reports demonstrate that nitric oxide has important role in initiation or progression of inflammation, cancer, burn inflammatory, toxicant intoxication and other pathological conditions (12, 13). Extremely high production of NO radicals appears in numerous diseases such as sepsis and hepatic failure. This abnormal increases in NO production may lead to intensive damage to tissue, so removing the excess NO could have beneficial effects (14). Also scavenger compound can help to arrest the chain of reactions initiated by excessive generation of NO that is detrimental to human health (13). Further, the scavenging activity may also help to stop the chain of reactions initiated by extra generation of nitric oxide that is harmful for human body. Leaf extract showed better iron chelating activity than other extracts. Iron ion chelating activity of extracts was in order of: leaf (534.0 ± 9.9 µg/mL) > flowers (702.4 ± 13.1 µg/mL) > stems (876.6 ± 15.7 µg/mL), respectively. EDTA showed

very good activity (IC_{50} = 18 ± 0.5 µg/mL). Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapies reduce iron related complications in human and thereby ameliorate life quality and overall survival in some diseases such as thalassemia major (15). The use of iron chelators can improve the symptoms of iron overload and ameliorate life quality and overall survival rate for sufferers. In recent years, search in natural products for finding iron chelators with lower side effect has been increased (16). On the other hand, previous studies showed that iron chelators and hydroxyl radical scavengers protect against acute renal failure, especially aminoglycoside antibiotic-mediated nephrotoxicity (17). These procedures can be suppressed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be involved in cardiovascular disease (8). So, removal of extra Fe^{2+} affords protection against oxidative stress. The chelating of ferrous ions was determined according to Dinis et al. (9). 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 the extracts and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} of hydrogen peroxide scavenging activity of the extracts were in order of: leaf (261.0 ± 6.2 µg/mL) > flower (297.5 ± 7.1 µg/mL) > stems (307.6 ± 8.4 µg/mL), respectively. The IC_{50} values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 µg/mL, respectively. Scavenging of hydrogen peroxide by samples may be attributed to their phenolic compounds and/or other active components which can donate electrons to hydrogen peroxide, thus neutralizing it to water (18). Although hydrogen peroxide itself is not very reactive, but it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell (8). Membrane lipids are rich in unsaturated fatty acids that are the most susceptible to oxidative processes. The extracts tested showed good activities in hemoglobin-induced linoleic acid emulsion (Figure 2). Erythrocytes are considered as major targets for free radicals 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 reactive oxygen species. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation (19). The inhibition of lipid peroxidation by samples may be due to their free radical scavenging activities. Superoxides indirectly initiate lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (8). Hydroxyl radicals remove hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The antihemolytic activity of extracts were tested and found that they did not show any harmful effects to erythrocytes. IC_{50} of antihemolytic activity of extracts was in order of: leaf (65.7 ± 1.8 µg/mL) > stems (108.7 ± 3.3 µg/mL) > flowers (120.9 ± 3.7 µg/mL) *vs.* vitamin C (235 ± 9 µg/mL), respectively. Antihemolytic activity of flavonoids was previously reported and good activity of extracts maybe a result of high flavonoid content (20).

This study shows the antioxidant and antihemolytic activities of acetone extract of flowers, leaf and stems of *Hyssopus angustifolius*. All the extracts show different activity in studied models. Future studies on *in vivo* antioxidant activity and/or using this plant in clinical study are needed. Also, these results can be useful as a starting point of view for further applications of this plant or its constituents in pharmaceutical formulations.

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