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ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITIES OF ETHANOLIC EXTRACT OF FLOWERS, LEAVES, AND STEMS OF *HYSSOPUS OFFICINALIS* L. VAR. *ANGUSTIFOLIUS*

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In this study, antioxidant and antihemolytic activities of ethanolic extract of flowers, leaves, and stems of* Hyssopus officinalis *L. Var.* angustifolius *were investigated employing different* in vitro *assay systems. Extracts showed good antioxidant activity. IC_{50} for 1,1-diphenyl-2-picryl hydrazyl radical-scavenging activity were 148.8 ± 4.31 μg mL^{-1} for flowers, 79.9 ± 2.63 μg mL^{-1} for stems, and 208.2 ± 6.45 μg mL^{-1} for leaves. All extracts showed moderate iron (II) chelating ability. Extracts exhibited good antioxidant activity in the hemoglobin-induced linoleic acid model and also they were capable of scavenging hydrogen peroxide in a concentration dependent manner. Extracts showed good antihemolytic activity againts hydrogen peroxide-induced hemolysis (IC_{50} were 48.51 ± 2.27 μg mL^{-1} for flowers, 19.47 ± 0.73 μg mL^{-1} for leaves, and 63.1 ± 2.65 μg mL^{-1} for stems). The total amount of phenolic compounds in the extracts was determined as gallic acid equivalents and total flavonoid content was calculated as quercetin equivalents from a calibration curve.

Keywords: ***Antihemolytic, Antioxidant activity, Flavonoid,*** **Hyssopus officinalis** ***L. Var.*** **angustifolius*****, Nitric oxide.***

INTRODUCTION

Reactive oxygen species (ROS) have been found to play an important role in the initiation and/or progression of various diseases, such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease.[1] Antioxidants can protect the human body from damage caused by ROS and the concomitant lipid peroxidation, protein damage, and DNA strand breaking[2] and, thus, can prevent the above-mentioned diseases. The human body has multiple mechanism antioxidant systems to protect against ROS-induced

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damage.[3] But mainly it is not enough for protection against oxidative stress induced by ROS. Therefore, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in the human body. On the other hand, many side effects of synthetic antioxidants, such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), have been reported.[4] For example, previously pneumotoxicity, hepatotoxicity, and renotoxicity of butylated hydroxytoluene were reported.[5] Also, the carcinogenic effect of BHT and BHA was reported.[4] Hence, compounds especially from natural sources capable of protecting the human body from ROS-induced damage may have potential application in prevention and/or curing of diseases. *Hyssopus officinalis* L. Var. *angustifolius* (*Lamiaceae*) is one of the most important medicinal plants that is extensively cultivated in central and south European countries, such as Russia, Spain, France, and Italy.[6] It is used in tea blends for cough relief, for antispasmodic effects, and to relieve catarrh.[7] This plant is a typical xerophyte and is well adapted to drought and low input conditions. Naturally it is found in high altitude mountains and grows in natural and alkaline soil.[7] To the best of our knowledge, there is negligible scientific information reported about antioxidant and antihemolytic activities of ethanolic extract of flowers, leaves, and stem of *Hyssopus officinalis* L. Var. *angustifolius.*

EXPERIMENTAL PROCEDURES

Chemicals

Disodium salt of 3-(2-pyridil)-5,6-bis (4-phenyl-sulfonic 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. (St. Louis, MO, USA). Sodium nitropruside, ammonium thiocyanate, ascorbic acid, gallic acid, quercetin, Butylated hydroxyanisole (BHA), Vitamin C, EDTA, and ferric chloride were purchased from Merck (Damstadt, Germany). All other chemicals were of analytical grade or purer.

Plant Materials

Hyssopus officinalis L. Var. *angustifolius* was collected from the Veresk area (Latitude: 35° 54′ N; Longitude: 52° 59′ E; Altitude: 1900 m above sea level), Elburz Mountains, Mazandaran, Iran and identified by Dr. Bahman Eslami, Assistance Professor of Plant Systematic and Ecology, Department of Biology, Islamic Azad University, Ghaemshahr Branch, Iran, where a voucher specimen (No. 975) was deposited.

Extraction Procedure

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

Determination of Total Phenolic and Flavonoid Contents

Total phenolic content was determined by the Folin-Ciocalteau method.[8] Extract samples (0.5 mL of 1.6 mg mL^{-1}) were mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 mL of 75 g L^{-1} sodium carbonate were then added. The

absorbance of the reactions was measured at 760 nm with a double beam spectrophotometer (UV–Visible EZ201, Perkin Elmer, Wellesley, MA, USA) after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoid content was estimated using the method of Nabavi et al.[4] 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

DPPH radical-scavenging activity. The stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical was used for determination of free radical-scavenging activity of the extracts.[9,10] Different concentrations of the sample were added, at an equal volume, to ethanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was 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 of the sample, which is required to scavenge 50% of DPPH free radical.

Reducing power determination. The reducing power of extracts was determined according to the method of Dehpour et al.[11] An aliquot of 2.5 mL of the sample (25–800 μg mL^{-1}) in water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, 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 $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as the positive control.

Metal chelating activity. The chelating of ferrous ions by the extracts was estimated by the method of Dinis et al.[12] Briefly, 1 mL of each sample (0.2–3.2 mg mL^{-1}) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). Reaction was initiated by the addition of 5 mM ferrozine (0.2 mL); the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solutions was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control and A_s was the absorbance of the extract/standard. Na_2EDTA was used as the positive control.

Assay of nitric oxide-scavenging activity. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extract, dissolved in wáter, and incubated at room temperature for 150 min. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as the positive control.[9]

Scavenging of hydrogen peroxide. Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Then, 2 mL of each sample

(0.1–1 mg mL^{-1}) in distilled water was added to a hydrogen peroxide solution (0.6 mL, 40 mM). The absorbance of samples at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and extracts. The percentage of hydrogen peroxide scavenging by the extracts and standard was calculated as follows:

$$\% \text{ scavenged } [H_2O_2] = [(A_o - A_1)/A_o] \times 100,$$

where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the samples of extract and standard.[4]

Antioxidant activity in a hemoglobin-induced linoleic acid system. The antioxidant activity of extracts was determined by a modified photometry assay.[13] Reaction mixture (200 mL) containing 10 ml of each extract (10–400 mg mL^{-1}), 1 mmol L^{-1} of linoleic acid emulsion, 40 mmol L^{-1} of phosphate buffer (pH 6.5), and 0.0016% hemoglobin suspension was incubated at 37°C for 45 min. After the incubation, 2.5 mL of 0.6% HCl in ethanol 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 100 mL of 0.02 mol L^{-1} of $FeCl_2$ and 50 mL of ammonium thiocyanate (0.3 g mL^{-1}). Vitamin C was used as the positive control.

Antihemolytic Activity of Extracts

Preparation of rat erythrocytes. All the animal experiments were carried out with the approval of the institutional animal ethical committee of the University of Mazandaran, Babolsar. 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 a standard diet. The animals were sacrificed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan et al.[14] Briefly, blood samples collected were centrifuged (1000 g, 10 min) at 4°C, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1000 g, 5 min) in 10 volumes of 10-mM phosphate buffered saline (pH 7.4). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes were stored at 4°C and used within 6 h for further studies.

Antihemolytic activity of extract against H_2O_2 induced hemolysis. Antihemolytic activity of the extracts was assessed as described by Nabavi et al.[4] Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. Then 0.5 mL of different concentrations of the each extract was added to 2 mL of erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of hydrogen peroxide solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of hydrogen peroxide in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation, 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 ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means were separated by Duncan's multiple range tests. The IC_{50} values were calculated from linear regression analysis.

RESULTS

Total phenolic content of extracts are shown in Table 1. The maximum of total phenolic content was recorded in stems extract with 374.60 ± 15.7 mg gallic acid equivalent/g of dry extract, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). Also, maximum total flavonoid content was recorded in flowers extract with 167.8 ± 7.8 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). IC_{50} of extracts, Vitamin C, quercetin, and BHA for DPPH radical-scavenging activity exists in Table 1. Stems extract showed better DPPH radical scavenging activity than others ($IC_{50} = 79.9 \pm 2.63$ μg mL^{-1}). In iron (II) chelating activity, among the different extracts, stems extract showed better activity than the others ($IC_{50} = 129.6 \pm 6.2$ μg mL^{-1}). The % inhibition of nitric oxide radical increased by increasing the concentration of the extracts. The leaf extract showed better nitric oxide-scavenging activity than others ($IC_{50} = 145.5 \pm 4.96$ μg mL^{-1} versus quercetin 20 ± 0.01 μg mL^{-1}). Althouth quercetin showed very potent nitric oxide radical scavenging, its carcinogenic activity has been reported.[15] The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. Results are shown in Table 1. Stems extract showed better activity than others (IC_{50} was 32.38 ± 1.52 μg mL^{-1}). The IC_{50} values for vitamin C and BHA were 21.4 ± 1.1 and 52 ± 2.6 μg mL^{-1}, respectively. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose dependent reducing powers of the extracts. There were no significant differences ($p > 0.05$). Tested extracts show good activity in the hemoglobin-induced linoleic acid system (Figure 2). There were no significant differences between extracts ($p > 0.05$). The effects of extracts were tested and found that they did not show any side effects on erythrocytes. Results have been shown in Table 1. Leaf extract showed better antihemolytic activity than others (IC_{50} was 19.47 ± 0.73 versus 235 ± 9.1 μg mL^{-1} for vitamin C).

DISCUSSION

Polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown potent antioxidant and antihemolytic activities.[4] $DPPH^{\cdot}$ is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron-donation. Substances that are able to perform this reaction can be considered as free radical scavengers and, therefore, antioxidants.[4,16] Phenolic compounds of this plant seem to have direct roles for its good free radical scavenging activity. Iron (II) chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in humans and thereby improves quality of life and overall survival in some diseases, such as thalassemia major, cancer, HIV, or Wilson's disease.[17,18] In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease

Table 1 Phenol and flavonoid contents and antioxidant activities of flowers, stems, and leaves of *Hyssopus officinalis* L. Var. *Angustifolius*.

Extract	Extraction yield (%)	Total phenol contents (mg g^{-1})[a]	Total flavonoid contents (mg g^{-1})[b]	DPPH free radical scavenging, IC_{50} (μg ml^{-1})	Nitric oxide scavenging, IC_{50} (μg ml^{-1})	H_2O_2 scavenging activity, IC_{50} (μg ml^{-1})	Fe^{2+} chelating ability IC_{50} (μg ml^{-1})	Antihemolytic activity (μg ml^{-1})
Flower	17.2	337.30 ± 15	167.8 ± 7.8	148.8 ± 4.31	232.6 ± 8.01	200 ± 8.2	154.38 ± 6.48	48.51 ± 2.27
Leaf	18	348.01 ± 12.8	92.43 ± 3.4	208.2 ± 6.45	145.5 ± 4.96	120 ± 4.4	204.4 ± 8.5	19.47 ± 0.73
Stem	15.5	374.60 ± 15.7	84.59 ± 3.8	79.9 ± 2.63	879.0 ± 14.07	32.38 ± 1.52	129.6 ± 6.2	63.1 ± 2.65
BHA	—	—	—	53.96 ± 3.1	—	—	—	—
Vitamin C	—	—	—	5.05 ± 0.1	—	21.4 ± 1.1	—	235 ± 9
EDTA	—	—	—	—	—	—	18 ± 1.5	—
Quercetin	—	—	—	5.28 ± 0.2	20 ± 0.1	52 ± 2.6	—	—

[a]Milligram gallic acid equivalent/g dry of extract.
[b]Milligram quercetin equivalent/g of dry extract powder.

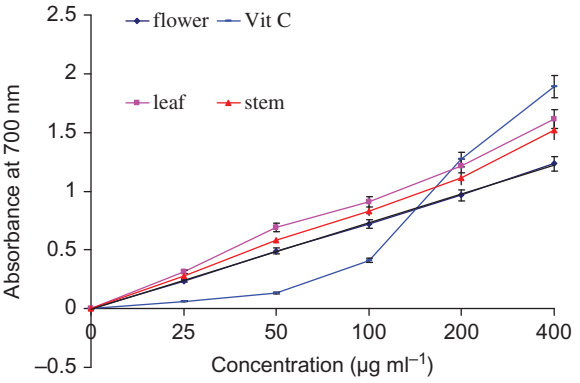

Figure 1 Reducing power of extracts. Vitamin C used as positive control. (Color figure available online.)

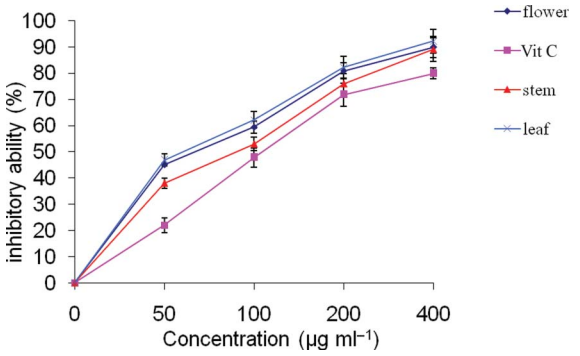

Figure 2 Antioxidant activity of extracts against hemoglobin-induced lipid peroxidation. Vitamin C used as positive control. (Color figure available online.)

(AD) pathology, and so iron chelation could be considered a rational therapeutic strategy for AD.[19] Foods are often contaminated with transition metal ions, which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry.[20] These processes can be delayed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease.[9] Because iron (II) also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing iron (II) concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extracts was estimated according to a recent paper.[13] Ferrozine can quantitatively form complexes with iron (II). In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, extracts and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has metal chelating activity and captures ferrous ion before ferrozine. The nitric oxide method is based on the principle that sodium nitroprusside in aqueous solution at physiological conditions spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen,

leading to reduced production of nitrite ions. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cáncer, and other pathological conditions.[15,21] A number of disease states, including sepsis and hepatic damage, are characterized by abnormally high nitric oxide production and removing the excess nitric oxide could have beneficial effects.[22] Natural products may have the property to counteract the activity of nitric oxide formation and in turn may be an important role in preventing the ill effects of excessive nitric oxide generation in the human body. Further, the scavenging effect may also help to inhibit the chain of reactions initiated by excess production of nitric oxide that are detrimental to human health. Scavenging of hydrogen peroxide by extracts may be originated from their polyphenolics compounds, and other active components, which have the electron donating ability, that can neutralize hydrogen peroxide to water.[8] Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing hydrogen peroxide is very important throughout food systems. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation.[9] In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of iron (III) to iron (II) by donating an electron. The amount of iron (II) complex can be then be monitored by measuring the formation of Perl's Prussian Blue at 700 nm.[13] Polyphenolic contents of extracts appear to activity as good electron and hydrogen atom donors and, therefore, should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic compounds in terms of dose dependent reducing power activity have been reported for several plant extracts.[4] Erythrocytes are considered as prime targets for reactive oxygen species attacks owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the oxygen transport associated with redox active hemoglobin molecules, which are potent promoters of reactive oxygen species. Especially, linoleic acid and arachidonic acid are targets of lipid peroxidation.[8] The elimination of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical.[13] Hydroxyl radicals inhibited hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Previously, antihemolytic activity of some of the flavonoids, such as quercetin, has been reported and good activity of tested extracts may be the result of high flavonoid content, especially quercetin.[4]

CONCLUSIONS

In conclusion, the remarkably strong *Hyssopus officinalis* L. Var. *angustifolius* extracts can be used as a powerful herbal antioxidant. Results demonstrate that all of the extracts showed good DPPH, nitric oxide radical, hydrogen peroxide scavenging, reducing power, and metal chelating ability as well as antihemolytic activity compared with standard substances. It may be due to the presence of polyphenolic compounds. These results may explain some of the therapeutic effects of this plant since the excessive production of reactive oxygen species are involved in initiation or progression of several diseases. Further investigation of individual compounds, their *in vivo* antioxidant activities, and in different antioxidant mechanisms is needed.

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