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Antioxidant and antihemolytic activities of methanol extract of *Hyssopus angustifolius*

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Summary

This study was designed to evaluate antioxidant and antihemolytic activities of *Hyssopus angustifolius* flower, stem and leaf methanol extracts by employing various *in vitro* assays. The leaf extract showed the best activity in DPPH (63.2 ± 2.3 µg mL^{-1}) and H_2O_2 (55.6 ± 2.6 µg mL^{-1}) models compared to the other extracts. However, flower extract exhibited the highest Fe^{2+} chelating activity (131.4 ± 4.4 µg mL^{-1}). The extracts exhibited good antioxidant activity in linoleic acid peroxidation and reducing power assays, but were not comparable to vitamin C. The stem (23.58 ± 0.7 µg mL^{-1}) and leaf (26.21 ± 1 µg mL^{-1}) extracts showed highest level of antihemolytic activity than the flower extract.

Introduction

There are increasing evidences that the consumption of polyphenolic compounds from natural sources may lower the risk of serious oxidative injuries such as atherosclerosis, inflammatory processes, cancer and cardiovascular diseases as a result of their antioxidant activity (Surh, 2002).

Chelation therapy is one of the most known and reported therapeutic usages of antioxidants (Grazul and Budzisz, 2009). Chelation therapy reduces iron-related complications and improves survival time in patients with various diseases such as thalassemia major, cancer, HIV and Wilson's disease (Grazul and Budzisz, 2009; Hebbel et al., 1990). Numerous studies have demonstrated that chelators, such as desferrioxamine, have anti-proliferative effects against different type of cancers (Richardson, 2002). Moreover, different synthetic chelators usage may have different side effects and therefore it remains an urgent need to identify new natural chelators with reduced side effects (Porter, 1997).

Previous studies demonstrate the direct role of nitric oxide radical in pathogenesis of different diseases, such as cancer, inflammation, burn, and etc. (Sreejayan and Rao, 1997). The antioxidant may have the property to quench NO formation and prevent illnesses induced by excessive NO.

Due to the safety and limitations of synthetic antioxidants, naturally originated antioxidants provide an interesting alternative to minimize the oxidative damage caused by NO and other oxidant agents (Ghasemzadeh et al., 2010). *Hyssopus angustifolius* is one of the most famous medicinal plants from the Lamiaceae family and is widely cultivated in several European countries such as Russia, Spain, France and Italy. It is used in tea blends for its antitussive and antispasmodic properties, and, to relieve catarrh (Omidbaigi, 2000). It appears that there is yet no scientific report about antioxidant and antihemolytic activities of methanol extracts of different parts of *H. angustifolius.* Thus, this study was carried out to determine antioxidant and antihemolytic activities of methanol extracts of *H. angustifolius* leaf, stem and flower, in order to better understand the medicinal uses of this plant.

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Materials and methods

Chemicals

Trichloroacetic acid (TCA), Folin Ciocalteau, 1, 1-diphenyl-2-picryl hydrazyl (DPPH), hydrochloric acid, linoleic acid, ferrozine, butylated hydroxyanisole (BHA), quercetin, ascorbic acid, hydrogen peroxide, gallic acid, Sodium nitroprusside and methanol were purchased from Sigma-Aldrich Chemical Company, USA. Sodium carbonate, potassium acetate, aluminium chloride ($AlCl_3$), potassium ferricyanide, sulfanilamide, N-(1-naphthyl) ethylenediamine di-hydrochloride, EDTA and ferric chloride ($FeCl_3$) were purchased from Merck Compagny, Germany. Other chemicals were purchased at analytical grade or purer.

Plant material

Aerial parts of *Hyssopus angustifolius* M. Bieb. has been collected from Veresk area (central Elburz Mountains Latitude: 35° 54' N, Longitude: 52° 59' E, altitude: 1900 above sea level,), Iran through spring 2010. The plant was authenticated at the Herbarium of Department of Biology in the University of Mazandaran (voucher specimen No 975).

Extraction procedures

Plant powder (100 g) was placed in a soxhlet extractor and extracted with methanol (3 liter) 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 content

Briefly, 0.5 mL of sample (1.6 mg mL^{-1}) was incubated with 2.5 mL of Folin-Ciocalteau reagent (0.2 N) for 5 min and then 2.0 mL aqueous solution of sodium carbonate (75 mg mL^{-1}) was added. The absorbance of reactions mixture was measured at 760 nm after 2 h of incubation at room temperature. Total phenol content was calculated using gallic acid standard curve (Akillioglu and Karakaya, 2010).

Determination of flavonoid content

Briefly, extract (0.5 mL of a solution at 1.6 mg mL^{-1}) was incubated with methanol (1.5 mL), aluminum chloride (0.1 mL, 10%), potassium acetate (0.1 mL, 1 M) and distilled water (2.8 ml) at room temperature for 30 min. Then, absorbance of sample was recorded at 415 nm by spectrophotometer (Akillioglu and Karakaya, 2010). Quercetin was used for calibration curve preparation.

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

Equal volumes of sample (25-400 µg mL^{-1}) were added to methanol solution of DPPH (100 µM). Absorbance of reaction mixture was recorded at 517 nm after 15 min incubating at room temperature. The

experiment was performed triplicate. Vitamin C, BHA and quercetin were used as standard antioxidants (Villaño et al., 2007).

Reducing power

For determination of the reducing power ability of extracts, 2.5 mL of sample (25-400 µg mL^{-1}) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%), and, were incubated for 20 min (50°C). Then, 2.5 mL of trichloroacetic acid (10%) was added to the sample in order to stop the reaction. Reaction mixture was the centrifuged (10 min, 1000 g). The upper layer of reaction mixture (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%). Finally, absorbance of the sample was recorded at 700 nm (Ferreira et al., 2007).

Metal chelating

Iron ions chelating by the extracts was examined according to the method of El and Karakaya (2004). Sample (25-400 µg mL^{-1}, 1 mL) was mixed with 0.05 mL of $FeCl_2$ (2 mM) and 0.2 mL of ferrozine (5 mM). After 10 min incubation at room temperature, absorbance of the sample was recorded at 562 nm.

Nitric oxide scavenging

Extract (25-400 µg mL^{-1}) was mixed with sodium nitroprusside (10 mM) and incubated for 150 min at 25°C. Then, 0.5 mL of Griess reagent (1% sulfanilamide, 2% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added to the reaction mixture. Finally, absorbance of the sample was recorded at 546 nm (Kumaran and Karunakaran, 2006).

Hydrogen peroxide scavenging

Extract (2 mL, 0.1-1 mg mL^{-1}) was mixed with 0.6 mL of 40 mM of hydrogen peroxide solution in phosphate buffer (pH 7.4). The absorbance of reaction mixture was recorded at 230 nm against a blank (Gülçin et al., 2010).

Hemoglobin-induced linoleic acid model

Reaction mixture (2 mL) containing extract (25-400 µg mL^{-1}), phosphate buffer (40 mmol L^{-1}, pH 6.5), hemoglobin suspension (0.0016%) and linoleic acid emulsion (1 mmol L^{-1}). Reaction mixture was incubated for 45 min (37°C). Then, 2.5 mL of ethanolic solution of hydrochloric acid (0.6%) was added to the sample for stopping lipid peroxidation process. The level of peroxidation was evaluated via thiocyanate method by recording the absorbance at 480 nm after adding 100 µl of $FeCl_2$ (0.02 mol/L) and 50 µl of ammonium thiocyanate (0.3 g mL^{-1}) (Bae and Suh, 2007).

Preparation of rat erythrocytes

Male Wistar rats (180-220 g) were purchased from Pasteur Institute of Iran, Amol research center. The animals were anesthetized via intraperitoneally administration of ketamine (60 mg/kg) and xylazine (5 mg/kg). Blood samples were collected through cardiac puncture. Erythrocytes of the blood samples were isolated and further stored at -60°C.

Protective role of extracts against H_2O_2 induced hemolysis

Protective role of the extracts against H_2O_2 induced hemolysis was evaluated according to the method of Ajila and Prasada Rao (2008). Different concentrations of extracts (0.5 mL, 10-100 µg·mL^{-1}) were mixed with 2mL of erythrocyte suspension (4%) and the volume of reaction mixture was made up to 5 mL with phosphate buffered saline. After 5 min incubation at 25°C, 0.5 mL of H_2O_2 solution was added to the reaction mixtures. After 240 min incubation at 25°C, reaction mixtures were centrifuged (2500 g, 10 min). Absorbance of the reaction mixture was recorded at 540 nm.

Statistical analysis of the data

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 separated by Duncan's multiple range tests. The IC_{50} values were calculated from linear regression analysis.

Results and discussion

The total phenolic content of the methanol extract of flowers, leaves and stems were respectively of 445 ± 22.1, 663.9 ± 33.1 and 349.6 ± 18.7 mg gallic acid equivalent g^{-1} of extract. Also, total flavonoid contents of methanol extract of flowers, leaves and stems were in the order of 122 ± 6.1, 131.7 ± 6.5 and 88.4 ± 4.3 mg quercetin equivalent·g^{-1} of extract powder, respectively. Leaf extract revealed better DPPH radical scavenging activity (IC_{50} = 63.20 ± 2.32 µg mL^{-1}) than others extracts tested, while in Fe^{2+} chelating activity, flower extract displayed the highest activity (IC_{50} = 131.40 ± 4.43 µg mL^{-1}). The inhibition percentage of nitric oxide radical increased concomitantly with the concentration. In this assay, the leaf extract showed the highest nitric oxide-scavenging activity (IC_{50} = 194.13 ± 4.12 µg mL^{-1} vs. quercetin 20 ± 0.01 µg mL^{-1}). Quercetin showed good NO radical scavenging activity but it has carcinogenic activity (Dunnick and Hailey 1992), therefore search for new natural NO quenching substances has been increased recently. The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner (Tab. 1).

Tab. 1: Antioxidant activities of flowers, stems and leaves extracts of *Hyssopus angustifolius*

Samples	DPPH free radical scavenging	Nitric oxide scavenging	H_2O_2 scavenging activity	Fe^{2+} chelating ability	Antihemolytic activity
flower	149.6 ± 5.5	196 ± 6.4	177.9 ± 7.8	131.4 ± 4.4	65.3 ± 2.5
leaf	63.2 ± 2.3	194.1 ± 4.1	55.6 ± 2.6	154.6 ± 7.1	26.2 ± 1
stem	197.3 ± 6	259.7 ± 7.9	123.3 ± 3.9	211 ± 9.7	23.6 ± 0.7
Vitamin C	5.05 ± 0.1	-	21.4 ± 1.1	-	235 ± 9
EDTA	-	-	-	18 ± 1.5	-
Quercetin	5.3 ± 0.2	20 ± 0.1	52 ± 2.6	-	-

There is no significant difference between quercetin and leaf extract in H_2O_2 scavenging models ($p > 0.05$) and between leaf and stem extract in antihemolytic activity ($p > 0.05$). IC_{50} (µg/mL)

Vitamin C shows better activity than others. In reducing power assay, increasing absorbance of reaction mixture at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose dependent reducing power of the extracts, however no significant differences ($p > 0.05$) were observed.

Tested extracts showed good activity in hemoglobin-induced linoleic acid system (Fig. 2) with no significant differences among them ($p > 0.05$). In addition, extracts did not show any side effects on erythrocytes. The best antihemolytic activity was observed while assessing the leaf and stem extracts (Tab. 1).

Any substances which can donates hydrogen or electron, can change DPPH color from violet to yellow can be considered as a DPPH radical scavengers and, therefore as antioxidant (NABAVI et al., 2012b). Previously, CHAPOVA et al. (2010) reported potent DPPH radical scavenging activity for another species of *Hyssopus* genus (*Hyssopus officinalis* L.) that was higher than the results in the present study, where *H. angustifolius* showed a moderate DPPH scavenging ability. This difference of activity may be associated with phenolic and flavonoid contents (NABAVI et al., 2012b). Another assay that is related to the electron donating ability of this plant is the reducing power assay. In this assay, phenols or other electron donor compounds in the sample can reduce Fe^{3+} to Fe^{2+}. The amount of Fe^{2+} complex monitored through reading absorbance of sample at 700 nm (FERREIRA et al., 2007). Fig. 1 showed the dose-response curves for the reducing power of extracts. They demonstrated very good activity, which was comparable to the vitamin C ($p < 0.01$). In iron ion chelating model, extracts and EDTA compete with ferrozine in capturing iron ion, and thus inhibited ferrozine-iron complex formation. Abnormally excessive NO radical production has a crucial role in the pathogenesis of numerous diseases such as sepsis and renal failure and therefore elimination of nitric oxide have therapeutic effects (SHAH et al., 2004). Also, NO scavengers inhibited chain reactions initiated by NO radicals that are harmful for human health. Hydrogen peroxide is not very reactive but it causes cytotoxicity through increasing of hydroxyl radicals in the cell. Therefore, hydrogen peroxide removal can help to protect human health (CHAUDHURI et al., 2007). Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes, mainly the linolenic and arachidonic acids, which are targets for lipid peroxidation (NABAVI et al., 2012C). Erythrocytes are known as main targets oxidative stress due to the presenting of membrane polyunsaturated fatty acids and also existence of the oxygen transport joined to hemoglobin molecules. Antihemolytic activity of flavonoids has been previously reported and the good activity of extracts may be the result of high flavonoid content (CHAUDHURI et al., 2007).

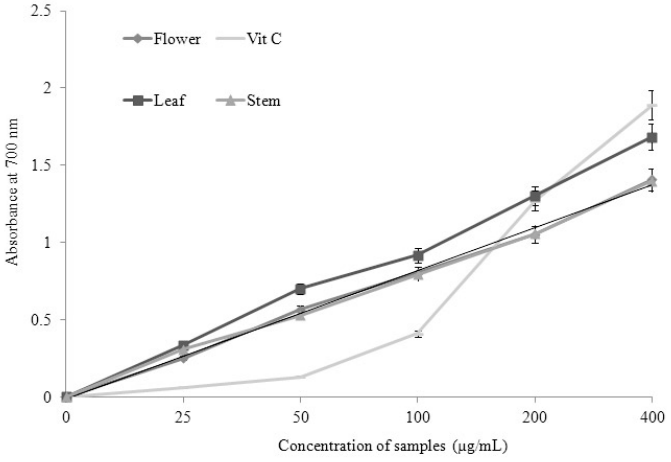

Fig. 1: Reducing power activity of methanolic extracts of leaf, stem and flowers of *Hyssopus angustifolius*. Vitamin C is used as standard.

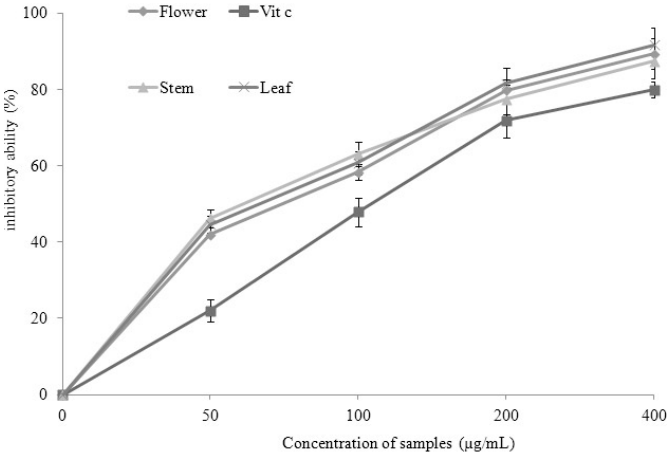

Fig. 2: Antioxidant activity of leaf, stem and flowers of *Hyssopus angustifolius* against linoleic acid peroxidation induced by hemoglobin. Vitamin C is used as standard.

Conclusion

In conclusion, *Hyssopus angustifolius* extracts can be used as a powerful herbal antioxidant. This activity may be associated with the presence of polyphenolic compounds. These results may explain some of the medicinal uses of *Hyssopus angustifolius* since the excessive production of reactive oxygen species have important role in the pathogenesis of several diseases.

Author Disclosure Statement

No competing financial interests exist.

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