ANTIOXIDANT AND ANTIHEMOLYTIC ACTIVITIES OF *CRUCIANELLA SINTENISII*

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Summary

In this study antioxidant and antihemolytic effect of hydroalcoholic extract of *Crucianella sintenisii* was evaluated using nitric oxide and hydrogen peroxide radicals scavenging, ferrous ion chelating, hemoglobin induced linoleic acid and hydrogen peroxide induced hemolysis assays. The hydroalcoholic extract showed different activity in all antioxidant assays and contained a high level of total phenolic and flavonoid contents. IC_{50} values were 885.8 ± 39.29 and 386.2 ± 18.7 μg ml^{-1} in hydrogen peroxide and nitric oxide radicals inhibition, respectively. The extract also exhibited weak activity in ferrous ion chelating.

Key words: Antioxidant activity, *Crucianella sintenisii*, flavonoids, nitric oxide

Introduction

Free radicals and reactive oxygen species (ROS) are well known inducers of cellular and tissue pathogenesis leading to several human diseases such as cancer, inflammatory disorders, as well as in aging processes [1]. Much research into free radicals has confirmed that foods or plants rich in antioxidants play an essential role in the prevention of free radical related diseases [2, 3]. Wide range Synthetic antioxidants such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) are used to decelerate these processes [4]. However, due to their unstable and highly volatile nature, they have frequently brought some questions about their safety and efficiency ever since their first introduction to the food industry [5]. Hence, nowadays the search for natural antioxidants source is gaining much importance. The high antioxidant potential observed in many tropical plants is obviously part of their natural defence mechanism against noxious events causing oxidant damage, e.g. microbial infections. *Crucianella sintenisii* genus is member of *Rubiaceae* family and has 10 species in Iran [7]. Several biological and pharmacological activities of some genuses of *Rubiaceae* family reported previously [8].

Coumarin and irodoids from this genus reported previously [9]. The objective of this study was examination the antioxidant and antihemolytic activities of *Crucianella sintenisii* with employing five various assay systems.

Materials and methods

Chemicals: Ferrozine, Linoleic acid and H_2O_2 were purchased from Sigma Chemicals Co. (USA). Butylated hydroxyanisole (BHA), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA) and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Plant material and preparation of freeze-dried extract: Aerial parts of *Crucianella sintenisii* were collected from golestanak area, north of Iran, in summer 2008. After identification of the plant by Dr. Bahman Eslami (Assistance Professor of plant systematic, Islamic azad university of ghaemshahr, Iran) a voucher (No. 216) has been deposited in the Faculty of Pharmacy herbarium. Aerial parts were dried at room temperature and coarsely ground before extraction. A known amount of each part was extracted at room temperature by percolation method using ethanol/water (70/30 v/v). The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained.

Determination of total phenolic compounds and flavonoid content: Total phenol contents were determined by Folin Ciocalteu reagent [10, 11]. The extract sample (0.5 ml of different dilutions) was 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 reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed in terms of gallic acid equivalents which is a common reference compound. Colorimetric aluminum chloride method was used for flavonoid determination [11, 12]. 0.5 ml of sample in methanol was 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. The extract remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA).

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. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of extract dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control [13, 14, 15, and 16].

Metal chelating activity: 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 [17, 18]. These processes can be delayed by iron chelation and deactivation .The chelating of ferrous ions by *Crucianella sintenisii* Extract was estimated by the method of our recent paper (14). briefly, the extract (0.2–3.2 mg/ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The 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 solution 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 positive control [15].

Scavenging of hydrogen peroxide: The ability of the extract to scavenge hydrogen peroxide was determined according to the method adapted from our recently published paper [16]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extract (0.1-1 mg ml^{-1}) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows:

% 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 sample of extract and standard [16].

Antioxidant activity in a hemoglobin-induced linoleic acid system: The antioxidant activity of extract was determined by a modified photometry assay [19]. Reaction mixtures (200 ml) containing 10 ml extract (10-400 mg), 1 mmol l^{-1} of linoleic acid emulsion, 40 mmol l^{-1} of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, were incubated at 37ºC 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 480nm after coloring with 100 ml of 0.02 mol l^{-1} of $FeCl_2$ and 50 ml of ammonium thiocyanate (30 g/100 ml). Vitamin C was used as positive control.

Antihemolytic activity

Preparation of rat erythrocytes: All the animal experiments were carried out with the approval of institutional animal ethical committee. Male wistar rats in the body weight range of 180–220g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with 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 Ebrahimzadeh et al. [20]. Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4 ºC, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and Buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4 ºC and used within 6 h for further studies.

In vitro assay inhibition of rat erythrocyte hemolysis: The inhibition of rat erythrocyte hemolysis by the extract was evaluated according to the procedure described by Ebrahimzadeh et al. [25]. The rat erythrocyte hemolysis was performed with H_2O_2 as free radical initiator. To 100 µl of 5% (v/v) suspension of erythrocytes in PBS, 50 µl of extract with different concentrations (5–25 µg in PBS pH 7.4), which corresponds to 100– 3200 µg of fresh extract, was added. To this, 100 µl of 100 lM H_2O_2 (in PBS pH 7.4) was added. The reaction mixture was shaken gently while being incubated at 37 ºC for 3 h. The reaction mixture was diluted with 8 ml of PBS and centrifuged at 2000×g for 10 min. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer to determine the hemolysis. Likewise, the erythrocytes were treated with 100 µM H_2O_2 and without inhibitors (*Crucianella sintenisii* Extract) to obtain a complete hemolysis. The absorbance of the supernatant was measured at the same condition. The inhibitory effect of the extract was compared with standard antioxidant Vitamin C. To evaluate the hemolysis induced by leaves extract, erythrocytes were preincubated with 50 µl of extract corresponding to 25 µg extract for 1 h and the hemolysis was determined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 µM H_2O_2 as 100%. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

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

Results and discussion

Total phenol and flavonoid contents: 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 *Crucianella sintenisii* was 99.10 ± 4.26 mg gallic acid equivalent g^{-1} of extract. The total flavonoid contents of *Crucianella sintenisii* was 25.36 ± 0.93 mg quercetin equivalent g^{-1} of extract powder, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [21]. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases [22].

Assay of nitric oxide-scavenging activity: The extract also showed good nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1}. The % inhibition was increased with increasing concentration of the extract. The extract show good nitric oxide scavenging (IC_{50} were 386.2 ± 18.7 µg ml^{-1} vs 0.20 ± 0.01 µg ml^{-1} for quercetin). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [23, 24, 25, 26, and 27]. The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

Fe^{2+} chelating activity: The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease [22, 23]. Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of ebrahimzadeh et al., [28]. 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 this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+}– ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2 to 3.2 mg ml^{-1}. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [24]. The extract showed weak Fe^{2+} chelating ability (IC_{50} was 1.721 ± 0.083 mg ml^{-1}). EDTA showed very strong activity (IC_{50} = 18 μg ml^{-1}).

Scavenging Hydrogen Peroxide: Scavenging of H_2O_2 by extract may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water [12]. The differences in H_2O_2 scavenging capacity between the extract may be attributed to the structural features of their active components, which determine their electron donating abilities [12]. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner (IC_{50} = 885.8 ± 39.29 μg ml^{-1}). The IC_{50} values for ascorbic acid and BHA were 21.4 and 52.0 μg ml^{-1}, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems [29].

Antioxidant activity in a hemoglobin-induced linoleic acid system: The extract showed moderate inhibitory ability on lipid oxidation (10%) at 100 μg ml^{-1} and high inhibitory ability (58%) at 800 μg ml^{-1} (Fig. 1). extract show moderate activity in hemoglobin-induced linoleic acid system that was not comparable with vitamin C ($p < 0.001$) (Fig. 1).

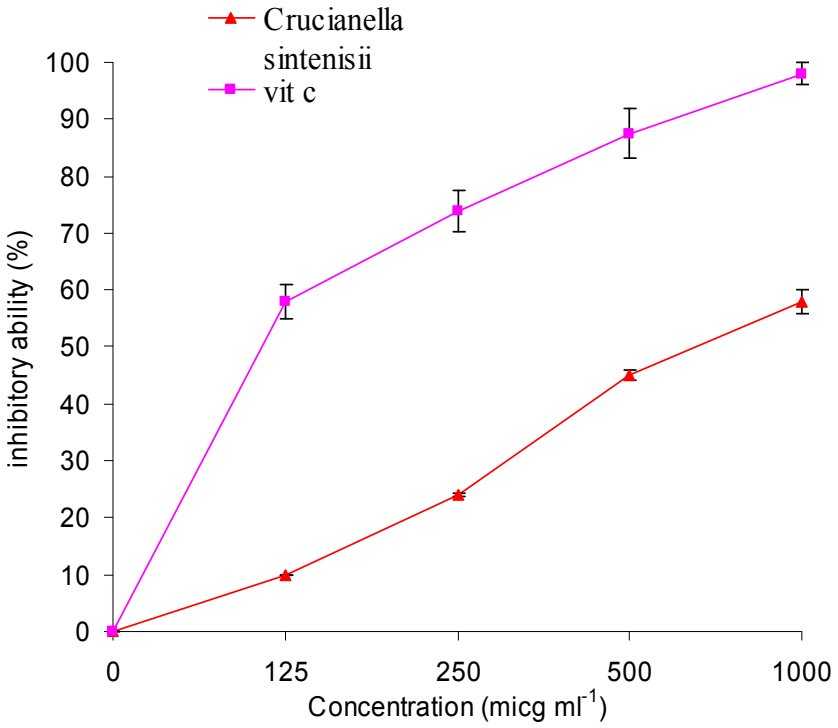

Figure 1. Antioxidant activity of extract in a hemoglobin-induced linoleic acid system

Inhibition of rat erythrocyte hemolysis: Initially, the effect of extract was tested and found that it did not show any harmful effect on erythrocytes. The extract inhibited the hemolysis of rat erythrocytes in a dose dependent manner with 88% as maximum inhibition of erythrocyte hemolysis at 3.2 mg ml^{-1}. The extract showed 50% hemolysis inhibition at concentrations ranging from 100 to 3200 µg of extract (IC_{50}= 1.108 ± 0.048). The Vitamin C exhibited with an IC_{50}value of 235 ± 9 µg ml^{-1} that is comparable to that of extract.

Conclusions

This study showed antioxidant and antihemolytic activity of *Crucianella sintenisii*. Further investigation of individual compounds, their *in vivo* antioxidant activities and in different antioxidant mechanisms is needed. Such identified potential and natural constituents could be exploited as cost effective food/feed additives for human health.

Acknowledgements

The authors wish to thank Pharmaceutical sciences research center of Mazandaran University of Medical Sciences (Sari, Iran) for the sanction of research grants to conduct the present study. This paper dedicated to Mrs. Seyed Maryam Nabavi and Mr. Seyed Ali Asghar Nabavi.

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