ANTIOXIDANT AND ANTIHEMOLYTIC POTENTIALS OF *PHYSOSPERMUM CORNOBIENSE* (L.) DC.

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Summary

The antioxidative and antihemolytic potentials of hydroalcoholic extract of *Physospermum cornobiense* was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide, hydrogen peroxide, ferrous ion chelating, reducing power, hemoglobin induced linoleic acid and hydrogen peroxide induced hemolysis assays. The hydroalcoholic extract showed good activity in all antioxidant assays and contained a high level of total phenolic and flavonoid contents. IC_{50} values were 665.9 ± 33, 157.5 ± 6 and 156.4 ± 5.31 μg ml^{-1} in DPPH, hydrogen peroxide and nitric oxide radical inhibition, respectively. The extract also exhibited significant activity in nitric oxide scavenging, attributed to the high amount of phenolics. Moreover, *Physospermum cornobiense* extract showed strong reducing power and a notable capacity to suppress hemolysis.

Key words: Antioxidant activity, *Physospermum cornobiense*, DPPH, phenol

Introduction

Reactive oxygen species (ROS), which consist of free radicals such as superoxide anion ($O_2\cdot^-$) and hydroxyl ($HO\cdot$) radicals and non-free radical species such as H_2O_2 and singled oxygen ($\cdot O_2$), are different forms of activated oxygen [1, 2, 3, 4]. ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. Thus, ample generation of ROS proceed to a variety of pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity [5]. Therefore, living organisms possess a number of protective mechanisms against the oxidative stress and toxic effects of ROS. Antioxidants regulate various oxidative reactions naturally occurring in tissues and are evaluated as potential anti-aging agents. Hence, antioxidants can terminate or retard the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as electron donors. Antioxidants have been widely used as food additives to provide protection from oxidative degradation of foods and oils. Hence, antioxidants are used to protect food quality mainly by the prevention of oxidative deterioration of constituents of lipids.

The most extensively used synthetic antioxidants are propylgallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ). However BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis [7, 8]. Natural antioxidants are able to protect from ROS as well as other free radicals and retard the progress of many chronic diseases and lipid oxidative rancidity in foods [9, 10, 11 and 12]. Polyphenols are widely distributed in plants and phenolic antioxidants have been found to act as free radical scavengers as well as metal chelators [13, 14, and 15]. *Physospermum* genus is member of *umbelliferae* family and has only one species in Iran [16]. Cytotoxic activity and inhibitory effect on nitric oxide production of triterpene saponins from *Physospermum verticillatum* reported previously [17]. To best of our knowledge only essential oil composition of *Physospermum cornobiense* reported [18] and its biological activity is obscure.

Materials and methods

Chemicals: Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide, H_2O_2 were purchased from Sigma Chemicals Co. (USA). Butylated hydroxyanisole (BHA), ascorbic acid, 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 *Physospermum cornobiense* were collected from Bashm area, Iran, in spring 2008. After identification of the plant by Dr. Bahman Eslami (Assistance Professor of plant systematic, Islamic azad university of ghaemshahr, Iran) a voucher (No. 203) 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, which was then freeze-dried for complete solvent removal.

Determination of total phenolic compounds and flavonoid content

Total phenol contents were determined by Folin Ciocalteu reagent [19]. The extract sample (0.5 ml of different dilutions) or gallic acid (standard phenolic compound) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent (Sigma–Aldrich) 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. The standard curve was prepared by 0, 50, 100, 150, 200 and 250 mg ml^{-1} solutions of gallic acid in methanol: water (50:50, v/v). Results were expressed in terms of gallic acid equivalents which is a common reference compound. Colorimetric aluminum chloride method was used for flavonoid determination [19]. 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). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml^{-1} in methanol.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract [20]. Different concentrations of each extracts were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamine C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power determination

Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action [21]. The reducing power of *Physospermum cornobiense* was determined according to the method of ebrahimzadeh et al. [22]. 2.5 ml of extract (25-800 μg ml^{-1}) in water were 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 3000 rpm 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 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. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts 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 [22].

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 [23]. These processes can be delayed by iron chelation and deactivation .The chelating of ferrous ions by *Physospermum cornobiense* Extract was estimated by the method of our recent paper. (20). 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 [21].

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 [22]. 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 [22].

Antioxidant activity in a hemoglobin-induced linoleic acid system

The antioxidant activity of extract was determined by a modified photometry assay [24]. 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. [25]. 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 (*Physospermum cornobiense* 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 *Physospermum cornobiense* was 36.17 ± 1.44 mg gallic acid equivalent g^{-1} of extract. The total flavonoid contents of *Physospermum cornobiense* was 16.56 ± 0.62 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 [26]. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases [27].

DPPH radical-scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [28]. DPPH 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 which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [29]. IC_{50} for DPPH radical-scavenging activity was 665.9 ± 33 µg ml^{-1}. The IC_{50} values for Ascorbic acid, quercetin and BHA were 1.26 ± 0.11, 1.32 ± 0.07 and 13.49 ± 1.04 µg ml^{-1}, respectively.

Reducing power

In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose– response curves for the reducing powers of the extract. The extract show moderate reducing power that was not comparable with Vit C ($p< 0.01$). Because the reductive ability of extract was significantly comparable to Vit C, it was evident that *Physospermum cornobiense* did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

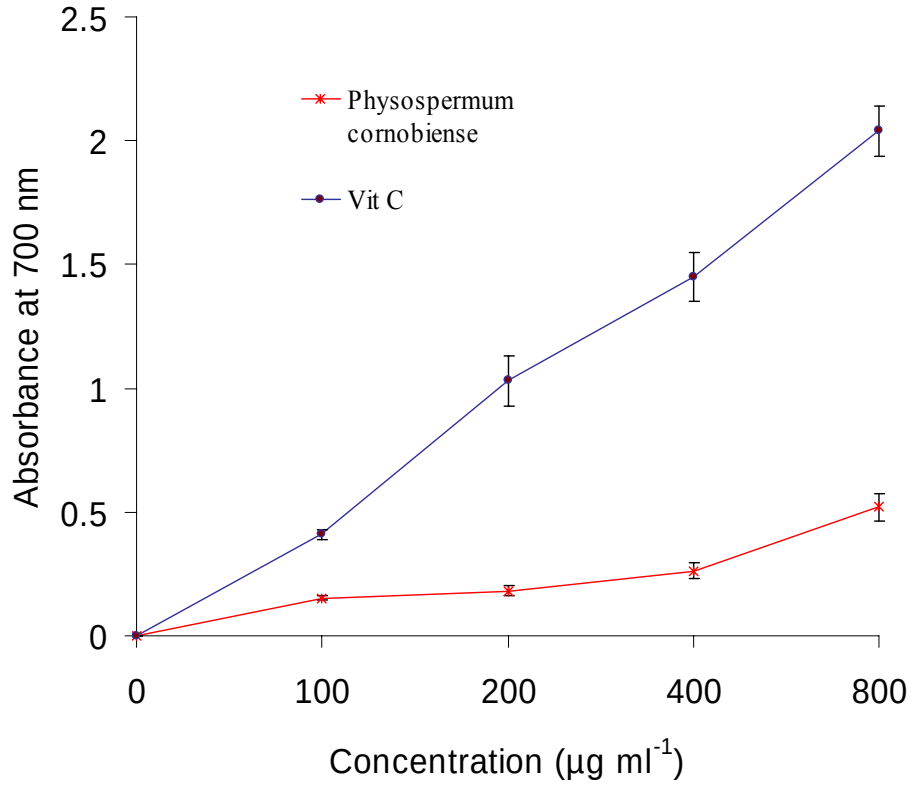

FIG.1

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 156.4 ± 5.31 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 [30, 31, 32]. 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 [27, 28]. 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., [33]. 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 [29]. The extract showed weak Fe^{2+} chelating ability (IC_{50} was 1.123 ± 0.047 mg ml^{-1}). EDTA showed very strong activity (IC_{50} = 18 µg ml^{-1}).

Scavenging H_2O_2

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 [14]. 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 [14]. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner (IC_{50} = 157.5 ± 6 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[34].

Antioxidant activity in a hemoglobin-induced linoleic acid system

The extract showed moderate inhibitory ability on lipid oxidation (49%) at 250 µg ml^{-1} and high inhibitory ability (71%) at 1000 µg ml^{-1} (Fig. 2). extract show moderate activity in hemoglobin-induced linoleic acid system that was not comparable with vitamin C ($p < 0.001$) (Fig. 2).

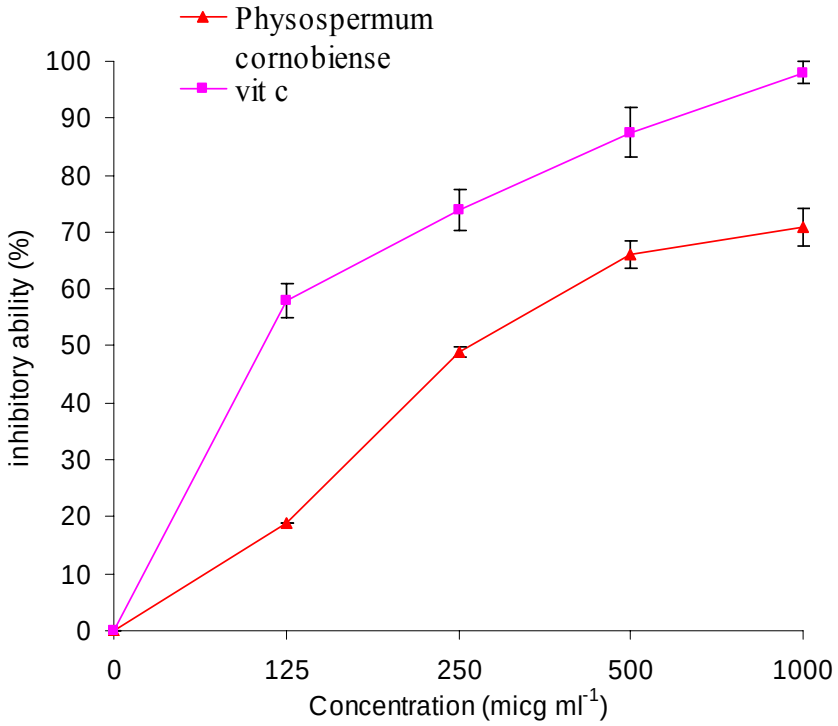

FIG. 2

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}= 347.3 ± 13.2µg ml^{-1}). The Vitamin C exhibited with an IC_{50}value of 235 ± 9 µg ml^{-1} that is comparable to that of extract.

Conclusions

The extract of *Physospermum cornobiense* exhibited good but different levels of antioxidant activity in all the models studied. The extract had good reducing power and nitric oxide scavenging activity. 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.

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