

IN VITRO ANTIOXIDANT ACTIVITY OF *PHYTOLACCA AMERICANA* BERRIES

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Summary

Phytolacca americana grows extensively in northwestern parts of Iran, mainly in the coastal areas and forest. Antioxidant activity of *P. americana* berries was investigated employing six in vitro assay systems. It showed a very powerful antioxidant activity in DPPH radical-scavenging. IC₅₀ was 62.0± 2.1 µgml⁻¹. It also showed a very high activity in reducing power assay. The activity was significantly higher than Vitamin C (p< 0.01). The *P. americana* berries extract showed moderately good nitric oxide-scavenging activity between 0.2 and 3.2 mg ml⁻¹. The percentage of inhibitions was increased with increasing concentration of the extract. IC₅₀ was calculated as 0.89± 0.02 mg ml⁻¹. Extract showed very weak Fe²⁺ chelating ability and very low antioxidant activity in peroxidation inhibition. Extract showed a good scavenging activity of H₂O₂. IC₅₀ was 245.3± 13.78 µg ml⁻¹. The total amount of phenolic compounds in each extracts was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Extract had high total phenolic contents. The powerful activity of *P. Americana* in DPPH radical-scavenging activity and reducing power assay suggests that hydrogen/ electron donation maybe a possible mechanism for antioxidant activity of this plant.

Key words: Antioxidant activity, DPPH, *Eryngium caucasicum*, Free radical scavenging activity, *Froriepia subpinnata*

Introduction

Reactive oxygen species 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). Recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals (2). Among the various medicinal and culinary plants, some endemic species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (3). Additionally, it has been determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (4).

Recently, interest has considerably increased in finding naturally occurring antioxidant to replace synthetic antioxidants, which were restricted due to their side effects such as carcinogenesis (5). *Phytolacca americana* (Pokeweed, *Phytolaccaceae*), is a perennial plant native to North America. The boiled leaves are used in a popular salad (called grandmother salad) in the American diet (6-8). *P. americana* grows widely in northwestern parts of Iran, mainly in the coastal areas and forest (9, 10). It is well known for several medicinal properties in despite of its toxicity, especially hepatotoxicity (11). *P. americana* has been most commonly used as laxative. It has been shown to possess pain relieving, anti inflammatory, anti rheumatism and antiarthritic activities, also it is suitable for treatment of various skin diseases (12). Nowadays, pokeweed is still used cautiously by some herbalists to treat above mentioned conditions. 150 species of *Phytolacca* dried flowers are available on North America, other parts of the world and northwestern areas of Iran that used to relieve pain and reduction of fever (12-15). They are used single or mixed. In addition, these species or their isolated constituent known to possess antifungal activity (16), Mitogenic (17), antiviral (18), translational inhibitory activity (19), antimicrobial (20) and neurotrophic activities (21). Antioxidant activity has been reported in *P. americana*, by DPPH method (22). To the best of the author's knowledge, antioxidant activity of berries of *P. americana* has not reported yet. Therefore, the aim of the present work is to determine the antioxidant activity of berries of *P. americana* by employing six various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, scavenging of hydrogen peroxide, linoleic acid and iron ion chelating power, in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

Materials and methods

Chemicals: Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide were purchased from Sigma Chemicals Co. (USA). Gallic acid, quercetin, Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, 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: *P. americana* berries were collected from Mazandaran forest and identified by Dr. Bahman Eslami. A voucher (No. 991) has been deposited in the Sari School of Pharmacy herbarium. Materials dried at room temperature and coarsely ground before extraction. It was extracted by percolation method using methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained.

Determination of Total Phenolic Compounds and Flavonoid Contents: Total phenolic compound contents were determined by the Folin-Ciocalteu reagent according to the recently published method (23). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g/l 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 as gallic acid equivalents. Total flavonoids were estimated according to the recently published method (23). Briefly, 0.5 ml solution of each plant extracts in methanol 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 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

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 (24). Different concentrations of each extracts were added, at an equal volume, to methanol 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. Vitamin 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: The reducing power of extracts was determined according to our recently publish paper (25). Different amounts of each extracts (25-800 $\mu\text{g ml}^{-1}$) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (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: 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. 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 positive control (26).

Metal Chelating Activity: The chelating of ferrous ions by extracts was estimated by our recently published paper (27). 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.

Determination of Antioxidant Activity by the FTC Method: The inhibitory capacity of extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki (23, 26). Twenty mg/ml of samples dissolved in 4 ml of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 ml), 0.05 M phosphate buffer pH 7.0 (8 ml), and distilled water (3.9 ml) and kept in screwcap containers at 40°C in the dark. To 0.1 ml of this solution was then added 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as: (%) inhibition = $100 - [(\text{absorbance increase of the sample}/\text{absorbance increase of the control}) \times 100]$. All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged. Vitamin C and BHA used as positive control.

Scavenging of Hydrogen Peroxide: The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch (26). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer.

Extracts (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 (H₂O₂) = [(A₀ - A₁)/A₀] × 100 where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the sample of extract and standard.

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 test. The EC₅₀ values were calculated from linear regression analysis.

Results and discussion

Total Phenol and Flavonoid Contents: Total phenol compounds are reported as gallic acid equivalents by reference to standard curve (y = 0.0054x ± 0.0628). The total phenolic contents were 102.11 ± 6.37 mg gallic acid equivalent/g of extract powder. The total flavonoid contents was 24.7 ± 1.24 mg quercetin equivalent/g of extract powder, by reference to standard curve (y = 0.0063x). *P. americana* berries extract had high total phenol contents. Phenols and polyphenolic compounds are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (28).

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 (29). 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 (30). It was found that the radical- scavenging activities of extract increased with increasing concentration. It showed a very powerful activity. IC₅₀ for DPPH radical-scavenging activity was 62.0 ± 2.1 µgml⁻¹. The IC₅₀ values for Ascorbic acid, quercetin and BHA were 5.05 ± 0.12, 5.28 ± 0.43 and 53.96 ± 2.13 µgml⁻¹, respectively. The powerful activity of *P. Americana* suggests that hydrogen donation may be a possible mechanism for antioxidant activity of this plant.

Reducing Power: Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action (31). In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ 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. It was found that the reducing powers of extract also increased with the increase of their concentrations. Extract showed a very high activity. There were significant differences (p < 0.01) between extract and Vitamin C in reducing power. Because of significant reductive abilities of extract, it was evident that *P. americana* could serve as electron donors, terminating the radical chain reaction.

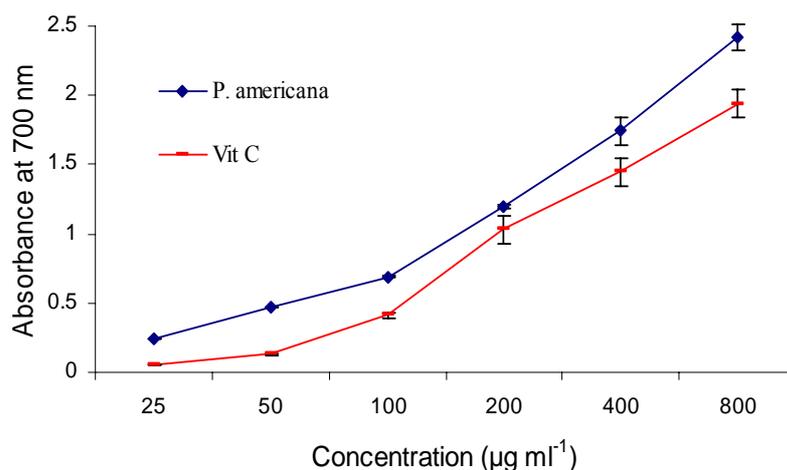


Fig.1. Reducing power of *P. americana* berries. Vitamin C used as control.

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. The *P. americana* berries extract showed moderately good nitric oxide-scavenging activity between 0.2 and 3.2 mg ml⁻¹. The percentage of inhibitions was increased with increasing concentration of the extract. IC₅₀ was calculated as 0.89± 0.02 mg ml⁻¹. However, activity of quercetin was very more pronounced than that of our extract (IC₅₀= 17 µg ml⁻¹). 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. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (32).

Fe²⁺ Chelating Ability: Iron 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 human and thereby improves quality of life and overall survival in some diseases such as Thalassemia major (33). In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD (34). 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 (35). The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (36). Because Fe²⁺ causes the production of oxyradicals and lipid peroxidation, minimizing its concentration affords protection against oxidative damage. In the presence of other chelating agents, the ferrozine complex formation is disrupted with the result that the red color of the complexes decreases. The absorbance of Fe²⁺-ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2 to 3.2 mg ml⁻¹. 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 (37). *P. americana* berries exhibited very weak Fe²⁺ chelating ability. The IC₅₀ was 1.75± 0.08 mg ml⁻¹. EDTA showed very strong activity (IC₅₀ = 18 µg ml⁻¹).

FTC Method: Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (38). Figure 2 shows the time-course plots for the antioxidative activity of the plant extract using the FTC method. The peroxidation inhibition of *P. americana* berries extract exhibited values from 89 (at 24th) to 58% (at 72nd hrs). There were significant differences ($p < 0.001$) among plant and Vitamin C and BHA at different incubation times ($p < 0.001$).

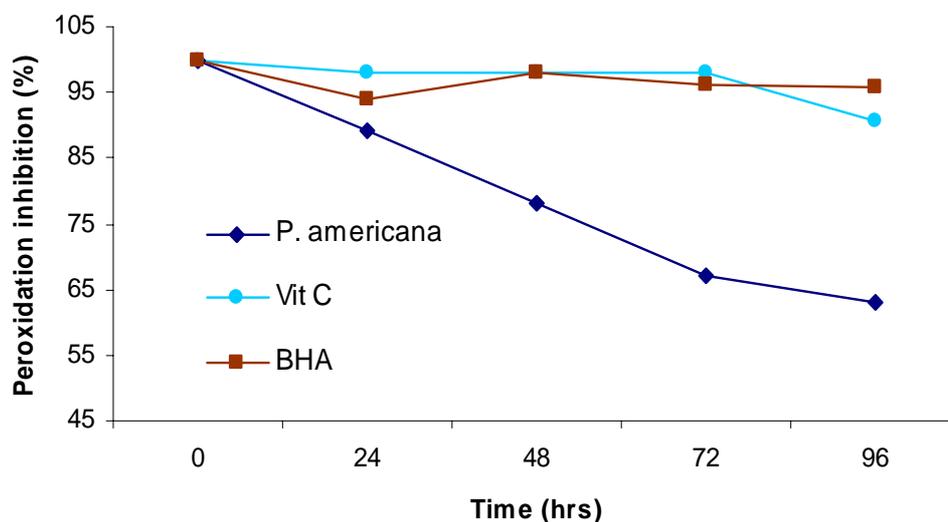


Fig. 2. Antioxidant activity of *P. americana* berries in FTC method at different incubation times (0.4 mg/ ml). Vit C and BHA used as controls (0.1 mg/ ml).

Hydrogen Peroxide Scavenging: Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2 , thus neutralizing it to water. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Extract showed a good scavenging activity. IC_{50} for scavenging of H_2O_2 was $245.3 \pm 13.78 \mu g ml^{-1}$. The IC_{50} values for ascorbic acid and quercetin were 21.4 ± 0.12 and $52.0 \pm 3.11 \mu g ml^{-1}$, 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.

P. americana berries methanol extract exhibited different levels of antioxidant activity in all the models studied. It showed good activity in reducing power, scavenging of DPPH radical and scavenging of H_2O_2 . Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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