In Vitro Antioxidant and Antihemolytic Activities of Grain Bran

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Summary

The aim of this study was to determine the antioxidant and antihemolytic activities and total phenols and flavonoids of the hydro alcoholic extract of Grain Bran. The antioxidant and antihemolytic activities of the extract were investigated with different in vitro methods i.e. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), nitric oxide and hydrogen peroxide radicals scavenging, reducing power and hemoglobin-induced linoleic acid peroxidation test. Antihemolytic activity was determined by inhibition of H_2O_2 induced hemolysis in rat erythrocytes. Extract exhibited weak activity in some model such as DPPH scavenging. The total phenolic content was determined by Folin-Ciocalteu method and was 27.8 ± 1.1 mg gallic acid equivalent/g of extract and total flavonoid content was determined by colorimetric method and was 20.0 ± 0.5 mg quercetin equivalent/g of extract powder. The total phenolic content of the extracts was determined by Folin-Ciocalteu method.

Key words: Antioxidant activity, Grain Bran, DPPH, Antihemolytic activity.

Introduction

Epidemiological studies have consistently shown the role of Reactive oxygen species (ROS) in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease [1]. So consumption of fruits and vegetables with high antioxidant capacity can be useful to prevention from above mention diseases [2]. Previous studies have been shown that the protective effect of these foods is attributed to the presence of phytochemical compounds such as carotenoids, tocopherols and polyphenols [3]. Thus, recent studies have focused in to searching for food materials and dietary supplements with potent antioxidant potential. Among the various medicinal plants, some culinary and edible species are of particular interest [4]. Cereals make up a major part of most people's diets. During the refining of cereal grains, much of the outer parts of the grain (kernel) are usually removed.

Previous studies shown that consumption of whole grains (the bran, germ, and endosperm) may reduce the risk of several diseases, including various types of cancer, heart attacks, diabetes and strokes [5]. Some biological activities of grain bran such as antioxidant [6,7] and anti-inflammatory activity [8] have been reported.

Materials and methods

Chemicals: Trichloroacetic acid (TCA), 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Potassium ferricyanide and Hydrogen peroxide H_2O_2 were purchased from Sigma Chemicals Co. (USA). Butylated hydroxyanisole (BHA), Vitamin C, Sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, and Ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Sample preparation: Grain Bran was purchase from local markets in Sari city, north of Iran. A known amount of sample was extracted by percolation method using Ethanol/water (70: 30). The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

Determination of total phenolic compounds and flavonoid content: Total phenolic compound contents were determined by the Folin-Ciocalteau method [9, 10]. The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagents 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 as previously described [11, 12]. Briefly, 0.5 ml solution of extract 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 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.

Antioxidant activity

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 [13-15]. Different concentrations of extract 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. 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.

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 the 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 [16, 17].

Scavenging of hydrogen peroxide: The ability of the extract to scavenge hydrogen peroxide was determined according to published method [18,19]. 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. The Extract (0.1-3.2 mg/ ml) 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 extract 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.

Reducing power determination: The reducing power of extract was determined according to the method of Yen and Chen [20,21]. 2.5 ml of extract (25-800 μg/ ml) in water were mixed with phosphate buffer (2.5 ml, 0.2M, 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.

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: The antioxidant activity of extract was determined by a modified photometry assay [22]. Reaction mixtures (200 ml) containing 10 ml of extract (10–400 mg), 1 mmol/ l of linoleic acid emulsion, 40 mmol/ l 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 480 nm after coloring with 100 ml of 0.02 mol/l 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 180–220 g 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 [23, 24]. Briefly blood samples collected were centrifuged (1500 ×g, 10 min) at 4ºC, 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 4ºC and used within 6 h for further studies.

Antihemolytic activity of extract against H_2O_2 induced hemolysis: The inhibition of rat erythrocyte hemolysis by the extract was evaluated according to the procedure described by Ebrahimzadeh et al. [25, 26]. 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 extract, was added. To this, 100 µl of 100 lM H_2O_2 (in PBS pH 7.4) was added. The reaction mixtures were shaken gently while being incubated at 37ºC for 3 h. The reaction mixtures were diluted with 8 ml of PBS and centrifuged at 2000×g for 10 min. The absorbance of the resulting supernatants 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 (plant extract) to obtain a complete hemolysis. The absorbance of the supernatants 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 extract, erythrocytes were preincubated with 50 µl of 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% of 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: The total phenolic content, was determined by Folin Ciocalteu method and was 27.8 ± 1.1 mg gallic acid equivalent/g of extract by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total flavonoid contents was 20.0 ± 0.5 mg quercetin equivalent/g 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 [2]. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases [2].

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 [14]. 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 [21]. IC_{50} for DPPH radical-scavenging activity was 621.1 ± 15.2 µg/ml. 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, respectively.

Reducing power: In the reducing power assay, the presence of reductants (antioxidants) in the sample 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 power of the extract according to results it was found that the reducing power of the extract also increased with the increase of their concentrations. There were significant differences ($p< 0.01$) among the extract and with vitamin C in reducing power.

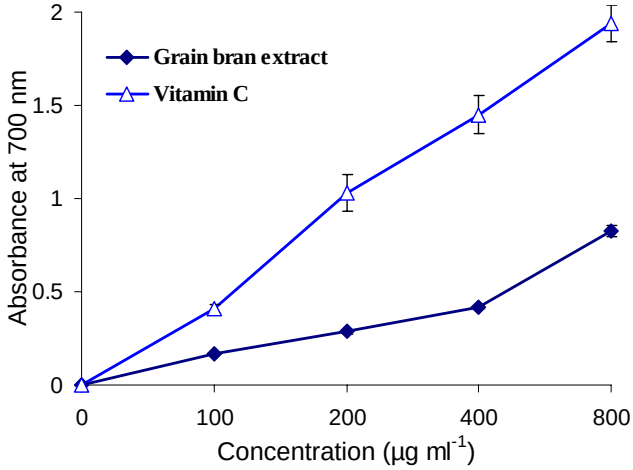

Fig. 1. Reducing power of grain bran extract.

Nitric oxide radical scavenging: The extract showed weak activity in nitric oxide-scavenging. Inhibition was only 27 % at 1.6 mg ml^{-1}. The % inhibition was increased with increasing concentration of the extract. IC_{50}= 0.20 ± 0.01 mg ml^{-1} for quercetin. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [12]. 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.

Antioxidant activity in a hemoglobin-induced linoleic acid peroxidation test: Membrane lipids are rich in unsaturated fatty acids which are most susceptible to oxidative processes. Specifically, linoleic acid and arachidonic acid are targets of lipid peroxidation [27]. Since polyphenolics contents appear to function as good electron and hydrogen atom donors and therefore be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products, the reducing potential of the extract may be attributed to this mode of activity. A similar observation has been reported for several plant extracts [11]. Hemoglobin-induced linoleic acid peroxidation test could evaluate the results with only 1 h for oxidation time. Generally antioxidant assays with linoleic acid need more auto-oxidation for 5-6 days [2]. Extract showed good reducing activity in hemoglobin-induced linoleic acid system. Inhibition was 23% at 0.125 mg/ml. Highest inhibitory ability was 68% (at 1 mg/ml) (Fig. 2). There were significant differences among the extract and vitamin C in hemoglobin-induced linoleic acid system ($p< 0.01$).

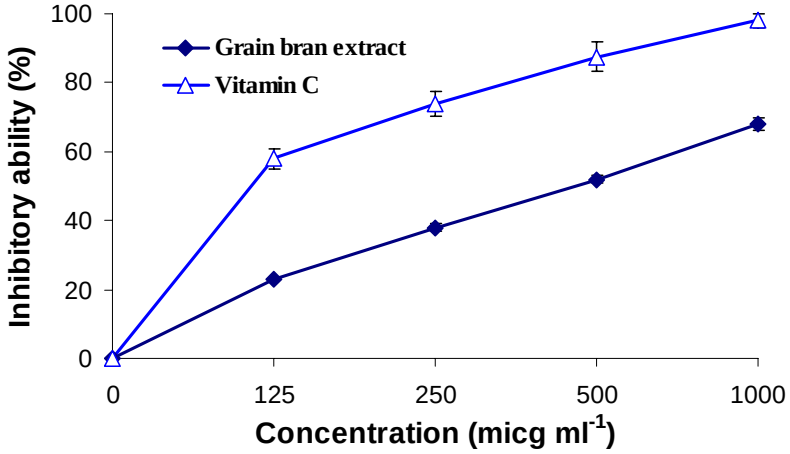

Fig. 2. Antioxidant activity of grain bran extract against hemoglobin induced linoleic acid peroxidation test.

H_2O_2 radical scavenging: The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. IC_{50} for H_2O_2 scavenging activity was 1.01 ± 0.04 mg/ml. The IC_{50} values for ascorbic acid and BHA were 21.4 ± 0.81 and 52.0 ± 2.02 μg/ml, 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 [18].

Antihemolytic activity: Hemolysis has a long history of use in measuring free radical damage and its inhibition by antioxidants but only few studies have been performed with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocytes lyses in rat blood. Lipid oxidation of rat blood erythrocyte membrane mediated by H_2O_2 induces membrane damage and subsequently hemolysis. The extracts showed very weak inhibiting activity. IC_{50} were 1.32 ± 0.07 mg/ml for extract and 235 ± 9.1 μg ml^{-1} for vitamin C. The antihemolytic activity of quercetin and other flavonoid have been previously reported [22]. Good activity of some extracts maybe result in high flavonoid content especially quercetin [22]. Flavonoids interactions with cell membranes, which generally serve as targets for lipid peroxidation (LP), constitute an important area of research [28]. Various model membrane systems like LDL and red blood cell (RBC) membrane comprising physiologically important membrane protein components offer a physiologically relevant and a relatively simple system for studying LP [29]. RBC has been chosen as an in vitro model to study the oxidant/ antioxidant interaction since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible to peroxidation [28]. During recent years, a few interesting studies have been reported, indicating the protective effects of some plants extracts against oxidative damage in intact RBC membranes [22-26].

Conclusions

The extract exhibited different levels of antioxidant activity and antihemolytic activity in models studied. Future investigations of the chemical composition and in vivo models of antioxidant activity are needed.

Acknowledgements

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