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Antioxidant and antihemolytic activities of flavonoid rich fractions of *Artemisia tschernieviana* Besser

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Abstract. – OBJECTIVES, ***Artemisia*** **(Asteraceae) contains more than 400 species. Many of the plants belonging to this genus are known to possess biological properties. In this study, antioxidant and antihemolytic activities of flavonoid rich fractions of *A. tschernieviana* Besser were evaluated.**

MATERIAL AND METHODS, Plant aerial parts were extracted with 60% acetone. Extract was fractionated sequentially with hexane (HE), ethyl acetate (EA) and water (AQ). Antioxidant and antihemolytic activities of these fractions were assessed. Their antihemolytic activity was determined by H_2O_2 and cumene hydroperoxide induced hemolysis models.

RESULTS, AQ fraction showed very powerful activity in 1,1-disphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity with IC_{50} = 0.12 ± 0.01 µg ml-1 that was better than controls (vitamin C, quercetin and butylated hydroxyanisole (BHA)). This fraction showed very powerful reducing power assay that was better than vitamin C ($p < 0.01$). EA fraction showed higher activity in scavenging nitric oxide with IC_{50} = 0.14 ± 0.04 μg ml^{-1}. In iron chelating activity HE fraction showed the best activity (IC_{50} = 162.2 ± 8.7 μg ml^{-1}). In scavenging of hydrogen peroxide, AQ fraction showed better activity than control group. This fraction had higher phenol and flavonoid contents. EA fraction showed higher antihemolytic activity with IC_{50} = 728.8 ± 29 μg ml^{-1}.

CONCLUSIONS, The fractions show very good activities in studied models. Aqueous fractions showed better activity than the others in nearly all tested models. These results can be useful as a starting point of view for further applications of *A. tschernieviana* aerial parts or its constituents in pharmaceutical preparations after performing clinical *in vivo* researches.

Key Words:

Antioxidant activity, *Artemisia tschernieviana*, Flavonoid, Cumene hydroperoxid, Antihemolytic activity.

Introduction

Antioxidants play vital role in protection against tissue damage associated with various human diseases[1]. Antioxidant capacity is widely used as a parameter to characterize nutritional health food or plants and their bioactive components. 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[2]. *Artemisia* (Asteraceae) is one of the largest and most widely distributed genera of the approximately 60 genera in the Anthemideae tribe. This genus comprises of more than 400 species, and is predominantly distributed in the northern temperate region of the world. Thirty-four of them have been reported in Iran and some are endemic[3]. Previously, antimalarial, antiulcerogenic, antitumoral, antihemorrhagic, antipyretic, anticoagulant, antioxidant, antiviral, antihepatitis, antispasmodic, antidepressant, anti complementary and interferon inducing activities of some substances from this genus have been reported[4,5]. *Artemisia austriaca* and *A. spicigera* are odorous herbs used as antiseptics and stomachic in folk medicine[6]. *A. vestita* is an herb that has been widely used in traditional Tibetan and Chinese medicine for treating inflammatory diseases such as rheumatoid arthritis[7]. *A. dracunculus* has been used orally as an antiepileptic in those whose anticonvulsant potential has been assessed[8]. *A. tschernieviana* is member of this genus, its Persian name is "Dermane-Shendust" and it is only distributed in the Caspian Sea coastal sands[3,9]. Moreover, the species has a limited distribution over Eastern Europe, Iran, Caucasus, C Asia[9]. Its antimicrobial activity has been

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reported[10]. To the best of our knowledge, there is no scientific report of antioxidant and antihemolytic effects of this plant.

Materials and Methods

Chemicals

Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1, 1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide, cumene hydroperoxide (CuOOH) and hydrogen peroxide were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Gallic acid, quercetin, butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

Plant Material and Preparation of Freeze-dried Extract

A. tschernieviana aerial parts were collected from Caspian Sea coastal sand dune, Babolsar, Iran and identified by Dr. Alireza Naqinezhad. A voucher specimen (No 629) was deposited in the School of Pharmacy Herbarium. The materials were dried at room temperature (for 2 weeks) and then in oven (35°C, for 2 days). Dried materials were coarsely grounded (2-3 mm) before extraction. 10 g of aerial parts powder was defatted twice with 100 ml of $CHCl_3$ and extracted twice with 100 ml of 60% acetone for 12 hours at room temperature. The solvent in the combined filtrates was removed using a rotary evaporator, leaving the crude acetone extract. After preparation of 10% (W/W) methanol slurry, the crude acetone extract was fractionated sequentially with 300 ml of n-hexane (HE), ethyl acetate (EA) and water (AQ), respectively. The HE, EA and AQ fractions were used for the determinations of bioactivity. The yields of HE, EA, and AQ fractions obtained from 10 g of aerial parts were 0.042, 0.31 and 1.97 g, respectively.

Determination of total phenolic and flavonoid contents

Total phenolic content was determined by the Folin-Ciocalteau method[11]. Briefly, the samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagents 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 as gallic acid equivalents. Colorimetric aluminum chloride method was used for total flavonoid determination[12]. 0.5 ml solution of samples 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 (UV-Visible EZ201, Perkin Elmer, LAMBDA, Well2582, Waltham, MA, USA). Total flavonoid content was 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 samples[13,14]. Different concentrations of sample were added, at an equal volume, to ethanol solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm (UV-Visible EZ201, Perkin Elmer, LAMBDA, Well2582, Waltham, MA, USA). 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 fractions was determined according to our recently published paper[15]. Briefly, 2.5 ml of sample (25-800 μg ml^{-1}) in water was 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 (UV- Visible EZ201, Perkin Elmer, LAMBDA, Well2582, Waltham, MA, USA). 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 sample dissolved

in water and incubated at room temperature for 150 min. The same reaction mixture, without samples, but with an equivalent amount of water, was served as control. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm (UV-Visible EZ201, Perkin Elmer, LAMBDA, Well2582, Waltham, MA, USA). Quercetin was used as positive control[16,17].

Metal Chelating Activity

The chelating of ferrous ions by the samples was determined by our recently published papers[18,19]. Briefly, the sample (0.2-3.2 mg ml^{-1}) 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 (UV- Visible EZ201, Perkin Elmer, LAMBDA, Well2582, Waltham, MA, USA). The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \leftrightarrow 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the tested samples/standard. Na_2EDTA was used as positive control.

Scavenging of Hydrogen Peroxide

Briefly, a solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Sample (0.1-1 mg ml^{-1}) in distilled water was added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance of sample at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2 (UV-Visible EZ201, Perkin Elmer, LAMBDA, Well2582, Waltham, MA, USA). The percentage of H_2O_2 scavenging by the samples and standard 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 samples or standard[20].

Antioxidant Activity in a Hemoglobin-Induced Linoleic Acid Peroxidation Test

The antioxidant activity of fractions was determined by a modified photometry assay[21]. Reaction mixture (200 ml) containing 10 ml of each samples (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, was 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^{-1} of $FeCl_2$ and 50 ml of ammonium thiocyanate (0.3 g ml^{-1}). Vitamin C was used as positive control.

Antihemolytic Activity of Samples Against H_2O_2 induced Hemolysis

Antihemolytic activity of the samples was assessed according to our recently published paper[21,22]. Erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 1g of samples/ml of saline buffer was added to 2 ml of erythrocyte suspension and the volume was made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% of hemolysis of blood cells after 240 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and then extends of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

CuOOH-Induced Hemolysis

Red blood cells (RBC) were isolated from male Wistar rats and suspended in balanced phosphate buffered saline (PBS) to obtain a 1% RBC suspension[21]. Aliquots (3.5 ml) were incubated at 37 °C for 210 min in the presence of 50 □M CuOOH (dissolved in ethanol) and the cellular integrity was determined turbidimetrically at 710 nm at 30 min intervals[23]. The samples (dissolved in EtOH; final concentrations 0.5 μg ml^{-1}) were preincubated for 30 min with RBC before the addition of CuOOH [blanks were RBC added with ethanol, at a final concentration always less than 0.1% (v/v)]. Percentages of hemolysis were determined, setting as a 100% hemolysis the absorbance value determined in RBC suspensions sonicated for 5 s at 50% power (mean values of 4 determinations were used for the calculation).

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 IC_{50} values were calculated from linear regression analysis.

Results

Total phenol compounds, as determined by Folin Ciocalteau 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 AQ, EA and HE fractions were 50.73 ± 3.3, 44.67 ± 3.2 and 35.52 ± 2.7 mg gallic acid equivalent/g of extract, respectively. The total flavonoid contents of AQ, EA and HE fractions were 145.8 ± 13.1, 76.28 ± 5.4 and 33.23 ± 1.9 mg quercetin equivalent/g of extract, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). AQ showed higher level of total phenol and flavonoid contents than others.

IC_{50} for DPPH radical scavenging activity was in order to: AQ fraction (0.12 ± 0.01 µg ml^{-1}) > EA (6.45 ± 0.31 µg ml^{-1}) > HE (250.9 ± 12.8 µg ml^{-1}), respectively. The IC_{50} values for ascorbic acid, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1 µg ml^{-1}, respectively. AQ fraction showed very powerful scavenging activity that was better than the most powerful standard used, vitamin C ($p < 0.01$).

Figure 1 shows the dose-response curves for the reducing power of fractions. It was found that the reducing power of sample also increased with the increase of its concentration. AQ fraction showed better activity than vitamin C ($p < 0.01$).

IC_{50} was 0.40 ± 0.01 mg ml^{-1} for AQ and 0.14 ± 0.04 µg ml^{-1} for EA fraction. HE fraction showed very weak activity with only (10% inhibition at 1.6 mg ml^{-1}). IC_{50} for quercetin was 17.01 ± 0.03 $\mu g ml^{-1}$.

HE fraction showed better activity with IC_{50} = 162.2 ± 8.7 μg ml^{-1}. AQ fraction showed very low activity with IC_{50} = 1214.9 ± 75 μg ml^{-1}. EA fraction did not show any activity with only 21% inhibition at 800 μg ml^{-1}. EDTA showed very powerful activity (IC_{50}= 18 ± 0.5 μg ml^{-1}).

Extracts were capable of scavenging H_2O_2 in a concentration dependent manner. IC_{50} of H_2O_2 scavenging activity was 8.05 ± 0.3 for AQ, 24.13 ± 1.6 for EA and 27.93 ± 1.6 µg ml^{-1} for HE fraction. The IC_{50} for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 µg ml^{-1}, respectively.

Tested fractions show good activity in inhibition of hemoglobin-induced linoleic acid peroxidation, but there have been significant differences between samples and vitamin C ($p < 0.05$) (Figure 2).

The effect of *A. tschernieviana* fractions were tested and found that they did not show any harmful effects on erythrocytes. IC_{50} of antihemolytic activity of fractions was 479.5 ± 16 for AQ, 728.8 ± 29 for EA and 817.8 ± 27 µg ml^{-1} for HE fraction, respectively (*vs*. 235 ± 9 μg ml^{-1} for vitamin C). Fractions delayed the onset of the CuOOH-induced hemolysis; at 150 min hemolysis was inhibited by 27.5 % for HE, 21.9% for AQ and 24.7 % for EA fraction, respectively (as compared with control group).

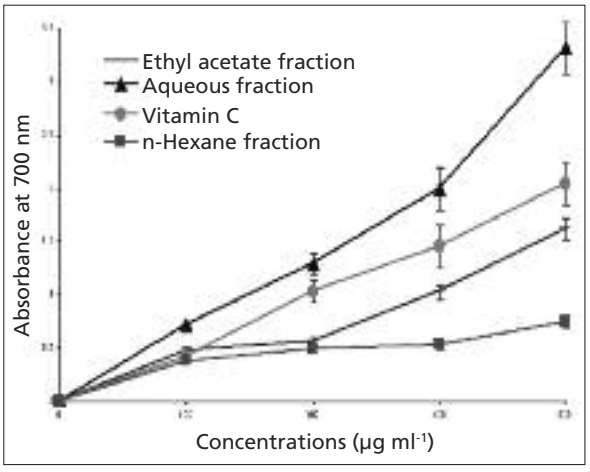

Figure 1. Reducing power of different fraction of *A. tschernieviana*.

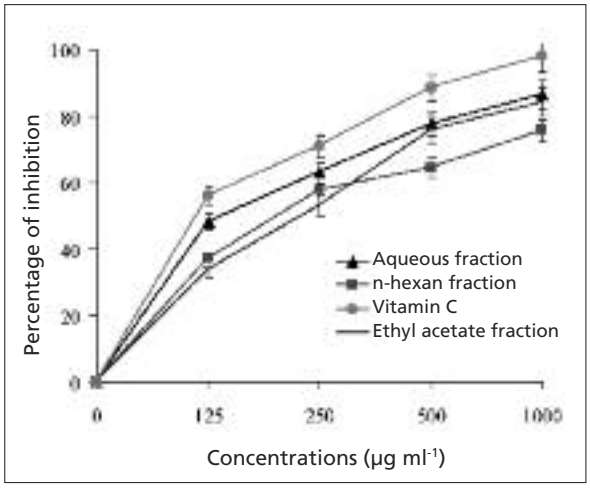

Figure 2. Antioxidant activities of *A. tschernieviana* fractions against linoleic acid peroxidation test. Each value is expressed as mean of 3 standard deviations. Vitamin C used as positive control.

Discussion

AQ showed higher level of total phenol and flavonoid contents than other extracts. 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[24].

AQ fraction showed very powerful DPPH radical scavenging activity that was better than the most powerful standard used, vitamin C ($p < 0.01$). High phenol and flavonoid content in of AQ fraction may lead to its very potent DPPH radical scavenging activity. Phenol and flavonoid can reduce DPPH radical by either the process of hydrogen- or electron- donation and changes its color from violet to yellow. Substances which are able to perform this reaction can be considered as antioxidants and, therefore, radical scavengers[25].

In the reducing power assay the presence of electron donor in the sample would result in the reduction of Fe^{3+} to Fe^{2+}. Amount of Fe^{2+} complex can be then monitored by measuring the formation of Perl's Prussian blue at 700 nm[26]. Increasing absorbance at 700 nm indicates an increase in reductive ability (Figure 1).

The nitric oxide (NO) scavenging activity is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions. Percentage of inhibition was increased with increasing concentration of samples. In spite of potent NO radical scavenging of quercetin, some carcinogenic activity has been reported for this compound[26]. In addition to reactive oxygen species NO is also implicated in inflammation, cancer and other pathological conditions[16]. 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.

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[28]. The usage of iron chelators can ameliorate the symptoms of iron overload and improve the quality of life and overall survival rate for sufferers. Deferoxamine used as an iron chelator for treatment of iron overload in thalassemia major. It has some adverse effects[29]. Deferiprone is another chelator that promotes negative iron balance and reduces hepatic iron levels in some patients. A number of adverse effects may occur, and require cessation of therapy in up to 30% of patients. There remains an urgent need to identify another chelator that is as effective as deferoxamine and has an acceptable degree of tolerability[29]. Thus, in recent years search for natural iron chelators with lower side effects have been increased[30]. Recent study has shown iron chelators and hydroxyl-radical scavengers protect against acute renal failure especially aminoglycoside mediated nephrotoxicity[31]. Iron can generate free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular diseases[21]. Hence, extra Fe^{2+} removal affords protection against oxidative damage. 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 all of the fractions and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine.

Extracts were capable of scavenging H_2O_2 in a concentration dependent manner. Scavenging of H_2O_2 by samples may be attributed to its phenolics and other active components which can donate electrons to H_2O_2; thus, neutralizing it to water[25]. Hydrogen peroxide itself is not very reactive, but it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell[13].

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. In particular, linoleic acid and arachidonic acid are targets of lipid peroxidation[32]. Tested fractions show good activity in inhibition of hemoglobin-induced linoleic acid peroxidation, but there have been significant differences between samples and vitamin C ($p < 0.05$) (Figure 2). The inhibition of lipid peroxidation by antioxidants may be due to their free radical scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical[25]. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which

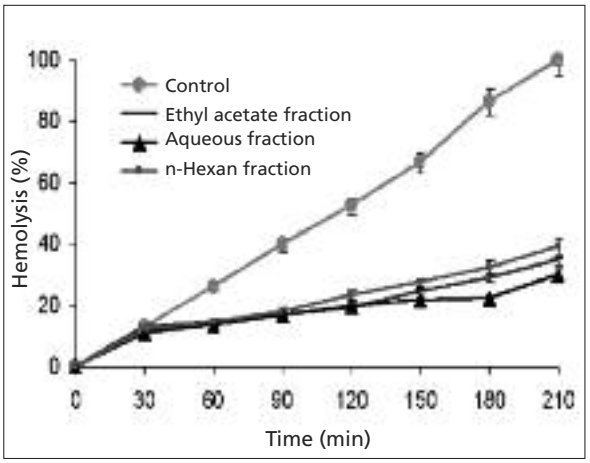

Figure 3. Protective effect of *A. tschernieviana* fractions on RBC hemolysis induced by CuOOH (50 μM). Values are the mean ± S.D. Data are from 3 independent experiments.

results in lipid peroxidation. Tested materials show good activity in inhibition of linoleic acid peroxidation test.

Antihemolytic activity of quercetin and other flavonoid previously reported and good activity of fractions maybe result of high flavonoid content especially quercetin[33]. The antioxidant activity of the samples was confirmed in rat RBC exposed to CuOOH, by measuring the erythrocyte membrane resistance to free radical-induced hemolysis. When control RBC were incubated with samples (50 μgml^{-1}) no significant hemolysis was observed within 3 h, thus to exclude any membrane-perturbing effect of the compounds. In RBC exposed to CuOOH (Figure 3), hemolysis started after 30 min incubation.

Conclusions

The fractions show very good activities in studied models. Aqueous fractions show better activity than the others in nearly all tested models. These results can be useful as a starting point of view for further applications of *A. tschernieviana* aerial parts or its constituents in pharmaceutical preparations after performing clinical *in vivo* researches.

Acknowledgements

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