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Biological activities of flavonoid-rich fraction of *Eryngium caucasicum Trautv*

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Abstract. – OBJECTIVES, *Eryngium (E.) caucasicum* was found as a new cultivated vegetable plant in northern Iran and used in several local foods. Little information is available on biological properties of *E. caucasicum*. In this work antioxidant activity of flavonoid-rich fraction of this plant was investigated by eight *in vitro* assay systems.

MATERIALS AND METHODS, 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), nitric oxide and hydrogen peroxide scavenging activities, Fe^{2+} chelating ability, reducing power and hemoglobin-induced linoleic acid peroxidation were used to evaluate antioxidant activities. Antihemolytic activities were evaluated against CuOOH and H_2O_2 induced hemolysis in rat erythrocyte.

RESULTS, IC_{50} for DPPH scavenging activity was 391.2 ± 14.9, 706.6 ± 22.3 and 779.7 ± 16.7 μg ml-1 for aqueous (AQ), ethyl acetate (EA) and n-hexane (HE) fractions, respectively. There was no difference between reducing power of AQ fraction activity and vitamin C ($p > 0.05$). IC_{50} for NO radical-scavenging activity was in order of AQ (133.5 ± 6.2 μg ml^{-1}) > EA (350.1 ± 14.8 μg ml^{-1}) > and HE (639.9 ± 21.7 μg ml^{-1}) fractions, respectively. Extracts showed weak Fe^{2+} chelating ability. HE fraction showed better activity (173.5 ± 9.6 μg ml^{-1}). Extracts exhibited weak hydrogen peroxide scavenging activity but exhibited very good antioxidant activity against the hemoglobin-induced linoleic acid peroxidation. There was no significant difference between AQ fraction and vitamin C ($p > 0.01$). Fractions delayed the onset of the CuOOH induced hemolysis. AQ fraction showed very high total phenol and flavonoid contents which was higher than other fractions. High phenol and flavonoid content of AQ fraction may lead to its better antioxidant activity.

CONCLUSIONS, *E. caucasicum* fractions exhibited different levels of antioxidant and antihemolytic activities in all tested models. These results can be useful as a starting point of view for further applications of *E. caucasicum* aerial parts or its constituents in pharmaceutical preparations.

Key Words:

Eryngium caucasicum, Flavonoid, Cumene hydroperoxyde, Iron chelation.

Introduction

Living tissues that undertake aerobic metabolism as a source of energy are under constant threat of damage by reactive oxygen derivatives. These free radicals are usually short-lived species but possess a single unpaired electron, rendering them highly reactive against biologically important macromolecules including DNA, proteins and membrane lipids. To counteract this threat to their integrity, cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidant species, and on antioxidant enzymes. A high proportion of the antioxidant systems of the human body are dependent on dietary constituents. Today it is believed that regular consumption of dietary antioxidants may reduce the risk of several serious diseases[1]. In the family Umbelliferae (Apiaceae) 117 cultivated species excluding ornamentals have been recorded until now, which primarily used as medicinal plants (41%); vegetables, salad plants and tuberous starch crops (23.1%); spice plants (19.7%), as well as fodder plants (11.1%); essential oil plants (4.3%) and hedge plants (0.8%)[2], including numerous neglected and underutilized crops with great potential for prospective evaluation. Literature on possible activities of *Eryngium (E.)* species only highlights anti-inflammatory and antinociceptive properties[3]. *E. maritimum* has numerous medicinal uses as a diaphoretic, diuretic, stimulant, cystotonic, urethritis remedy, stone inhibitor, aphrodisiac, expectorant and as anthelmintic[4,5]. Weak radical scavenging activity and antimicrobial activities from *E. maritimum*[6], good antimicrobial activity from *E. foetidium* [7] and essential oil composition of the different parts of *E. corniculatum*[8] have been reported. A new umbelliferous crop *E. caucasicum* Trautv (Caucasian eryngo, Subfam, Saniculoideae) founds in cultivation in northern of Iran and has been reported recently[9]. This taxon

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was not included in the last edition of the most comprehensive catalogue in the subject, the Mansfeld's Encyclopedia[10]. *E. caucasicum* was found as a new cultivated vegetable plant in home gardens in northern Iran. Young leaves are used as a cooked vegetable and for flavoring in the preparation of several local foods[9]. A good antioxidant activity of *E. caucasicum* leaves at non-flowering stage has been reported recently by our group[11]. In addition its leaves and inflorescence at flowering stage showed very good activity[12]. Flavonoids form a ubiquitous group of polyphenolic substances typically produced by plants. They exert antibacterial, anti-inflammatory, anti-viral, anti-allergic, anti-mutagenic, anti-neoplastic and anti-thrombotic effect[13]. Epidemiological studies have shown an inverse relationship between dietary flavonoid intake and cardiovascular diseases[14]. Because of high flavonoids contents of leaves of this native plant, antioxidant activity of its flavonoid-rich fraction were examined by eight different *in vitro* assay systems in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

Materials and Methods

Materials

E. caucasicum leaves were collected from Panbe chule, Mazandaran, Iran in April 2009 and identified by Dr. B. Eslami. A voucher specimen (No 1442) has been deposited at the Herbarium of Department of Biology, University of Mazandaran, Babolsar, Iran. Ferrozine, linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide, cumene hydroperoxide and hydrogen peroxide were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Gallic acid, quercetin, butylated hydroxyanisole (BHA), vitamin C, 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.

Sample Preparation

The collected plant material was dried at room temperature (r.t.) and coarsely ground before extraction. 100 g of dried sample 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 extract was then separated from the sample residue by filtration through Whatman No.1 filter paper. The solvent in the combined filtrates was removed at low temperature (35°C) using a rotary evaporator, leaving the crude acetone extract (22 g). After preparation of 10% methanol slurry, the crude acetone extract was fractionated sequentially with 300 ml of each *n*-hexane, ethyl acetate, and water. The *n*-hexane, ethyl acetate, and aqueous fractions and crude acetone extract were used for the determinations of bioactivity. The yields of *n*-hexane (HE), ethyl acetate (EA), and aqueous (AQ) fractions obtained from 100 g of *E. caucasicum* leaves were 0.51, 0.3 and 20.8 g, respectively.

Determination of Total Phenolic and Flavonoid Contents

Total phenolic content was determined by the Folin-Ciocalteau method[15]. Briefly, the samples (0.5 ml) were 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 spectrophotometrically (UV- Visible EZ201, Perkin Elmer: Norwalk, CA, USA) at 760 nm after 2 h of incubation at r.t. Results were expressed as gallic acid equivalents. Total flavonoid content was estimated using our recently published paper[15]. Briefly, 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. 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[16]. Different concentrations of sample were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at r.t. 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.

Determination of Reducing Power

The reducing power of fractions was determined according to the method of Yen and Chen[17]. 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. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of Nitric Oxide (NO) 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, 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. Quercetin was used as positive control[18].

Determination of Metal Chelating Activity

The ability of extract to chelate ferrous ions was estimated by a method described in our recently published papers[19,20]. 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. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0T - A_s)/A_s] \leftrightarrow 100$, 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

The ability of the extract to scavenge hydrogen peroxide was determined as previously described[21]. 1.4 ml of extract (0.1-3.2 mg ml^{-1}) in distilled water was added to a solution of H_2O_2 (0.6 ml, 40 mM in phosphate buffer, pH 7.4). The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging by the extract or 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. Ascorbic acid and BHA were used as standards

Antioxidant Activity in a Hemoglobin- Induced Linoleic Acid Peroxidation Test

The antioxidant activity of the extract was determined by a modified photometry assay[16]. Reaction mixtures (200 µl) containing 40 µl of extract (10-400 mg ml^{-1} in ethanol), 40 µl of linoleic acid emulsion (1 mmol l^{-1}), 70 µl of phosphate buffer (pH 6.5), and 50 µl hemoglobin (0.0016%), were incubated at 37°C 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 in the test solution 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 (300 mg ml^{-1}). Vitamin C was used as a positive control.

Antihemolytic Activity of the Extract Against H_2O_2 Induced Hemolysis

The inhibition of rat erythrocyte hemolysis by extracts was evaluated as previously described[22]. Hemolysis was induced using H_2O_2 as free radical initiator. To 100 µl of a 5% (v/v) suspension of washed erythrocytes in phosphate buffered saline (PBS), aliquots of 50 µl containing different amounts of extract (50-250 µg ml^{-1} in PBS pH 7.4), which corresponds to 100-3200 µg of extract, was added. To this, 100 µl of 100 µM H_2O_2 (in PBS pH 7.4) was added. The reaction mixtures were shaken gently during incubation at 37°C 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 supernatant was measured at 540 nm by spectrophotometer to determine the extent of hemolysis. Likewise, the erythrocytes were treated with 100 µM of H_2O_2 without plant extract to obtain complete hemolysis. The absorbance of the supernatants was measured under the same conditions. The inhibitory effect of the extract was compared with that of the standard antioxidant vitamin C. To evaluate the hemolysis induced by the extract, erythrocytes were pre- incubated with 50 µl of extract for 1 h and the hemolysis was deter-

mined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 μM H_2O_2 as 100%. The IC_{50} values were determined graphically as the antioxidant concentration required to produce a 50% inhibition of hemolysis.

CuOOH-Induced Hemolysis

Red blood cells (RBC) were isolated from male Wistar rats and suspended in balanced phosphate buffered saline to obtain a 1% RBC suspension. Aliquots (3.5 ml) were incubated at 37°C for 210 min in the presence of 50 μM CuOOH (dissolved in ethanol) and the cellular integrity determined turbidimetrically at 710 nm at 30 min intervals[15,23]. The samples (dissolved in ethanol; 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 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 Mean ± 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 and Discussion

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 of *E. caucasicum* were 214.18 ± 11.5, 140.57 ± 6.5 and 29.06 ± 1.8 mg gallic acid equivalent/g of extract, respectively. The total flavonoid contents of AQ, ET and HE fractions were in order of 75.36 ± 3.6, 31.51 ± 1.2 and 97.37 ± 4.9 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). AQ fraction showed higher total phenol and flavonoid contents than others. 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]. IC_{50} for DPPH radical-scavenging activity was in order of: AQ (391.2 ± 14.9 μg ml^{-1}) > EA (706.6 ± 22.3 μg ml^{-1}) > and HE (779.7 ± 16.7 μg ml^{-1}) extracts, 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 higher scavenging activity than other fractions. High phenol and flavonoid content in of aqueous 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 it color from violet to yellow. Substances which are able to perform this reaction can be considered as antioxidants, and therefore, radical scavengers[25]. Reducing power is another mechanism for determination of electron donating ability of fractions. In this assay the presence of electron donor in the sample would result in the reducing of Fe^{3+} to Fe^{2+}. Amount of Fe^{2+} complex can be then be 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 shows the dose-response curves for the reducing power of fractions. It was found that the reducing power of sample increased with the increase of its concentration. There was no difference between AQ fraction activity and vitamin C ($p > 0.05$). The NO scavenging assay 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 NO compete with oxygen, leading to reduced production of nitrite ions. The % inhibition was increased with increasing concentration of samples. IC_{50} for NO radical-scavenging activity was in order of AQ (133.5 ± 6.2 μg ml^{-1}) > EA (350.1 ± 14.8 μg ml^{-1}) > and HE (639.9 ± 21.7 μg ml^{-1}) fractions, respectively. Quercetin showed very powerful activity (IC_{50} was 17.01 ± 0.03 μg ml^{-1}). Although quercetin showed very potent NO radical scavenging but its carcinogenic activity has been reported[27]. In addition to reactive oxygen species (ROS) NO is also implicated in inflammation, cancer and other pathological conditions[18]. 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

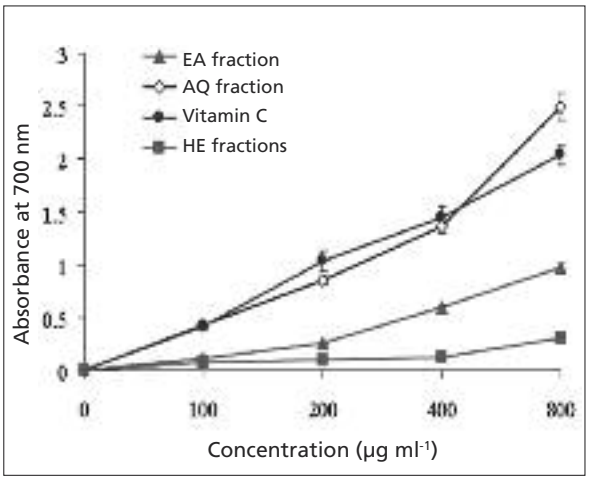

Figure 1. Reducing power of different fraction of *E. caucasicum*. EA, ethyl acetate; AQ, aqueous and HE, *n*-hexane fractions. Vitamin C used as positive control.

may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. HE fraction showed better iron chelating activity than other fractions. The activity was in order of HE (173.5 ± 9.6 μg ml^{-1})> AQ (834.4 ± 29.1 μg ml^{-1}) > and EA fractions (1039.7 ± 46.8 μg ml^{-1}), respectively. EDTA showed very powerful activity (IC_{50}= 18 ± 0.5 μg ml^{-1}). 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 and deferiprone used as iron chelators for treatment of iron overload in thalassemia major[28]. A number of adverse effects have been reported[29]. It remains an urgent need to identify active chelator regimen that is as effective as established drugs and has an acceptable degree of tolerability. So, in recent years search for natural iron chelators with lower side effect has been increased[12]. Studies showed iron chelators and hydroxyl-radical scavengers protect against acute renal failure especially aminoglycoside antibiotic mediated nephrotoxicity[30]. These processes can be suppressed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease[30]. So, 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[30]. 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. *E. caucasicum* fractions were capable of scavenging H_2O_2 in a concentration dependent manner. IC_{50} of H_2O_2 scavenging activity was in order of AQ (336.9 ± 11.3 µg ml^{-1}) > EA (482.3 ± 19.5 µg ml^{-1}) > and HE fractions (754.6 ± 14.1µg ml^{-1}), respectively. The IC_{50} values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 µg ml^{-1}, respectively. Scavenging of H_2O_2 by samples may be attributed to its phenolic contents, and other active components which can donate electrons to H_2O_2, thus neutralizing it to water[21]. H_2O_2 itself is not very reactive, but it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell[31]. 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[32]. Tested fractions showed very good activity in hemoglobin-induced linoleic acid system. There was not significant difference between AQ fraction and vitamin C ($p > 0.05$) (Figure 2). Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O_2 transport associated with redox active hemoglobin molecules, which are potent promoters of ROS. 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[30]. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested materials show good activity in hemoglobin-induced linoleic acid peroxidation. The effect of *E. caucasicum* fractions were tested and found that they did not show any harmful effects on erythrocytes. IC_{50} of antihemolytic activity of fractions was in order of EA (658.3 ± 23.3 µg ml^{-1}) > AQ (672.2 ± 27.8 µg ml^{-1}) > and HE fraction (705.7 ± 21.7 µg ml^{-1}), respectively. Vitamin C showed good activity (IC_{50} was 235 ± 9 μg ml^{-1}). Effect of fractions were tested and

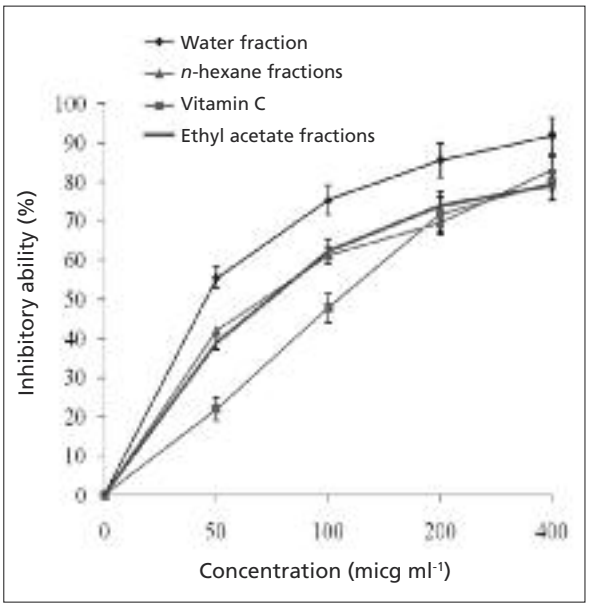

Figure 2. Antioxidant activities of *E. caucasicum* fractions against linoleic acid peroxidation induced by hemoglobin. Values are the Mean ± SD of 3 independent experiments. Vitamin C used as positive control. EA, ethyl acetate; AQ, aqueous and HE, *n*-hexane fractions

found that they did not show any harmful effects on erythrocytes. 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 erythrocytes (RBC) exposed to CuOOH, by measuring the erythrocyte membrane resistance to free radical-induced hemolysis. When control RBC were incubated with samples (50 μg ml^{-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. Fractions delays the onset of the CuOOH-induced hemolysis; at 150 min hemolysis was inhibited by 41% already for *n*-hexane, 35% for aqueous and 31% ethyl acetate fractions, respectively (compared with control group, 74%).

Conclusions

The fractions show very good activities in studied models. Aqueous fraction show better activity than others in all of studied model except iron cheation. These results can be useful as a starting point of view for further applications of *E. caucasicum* or its constituents in pharmaceutical preparations after performing clinical *in vivo* researches.

Acknowledgements

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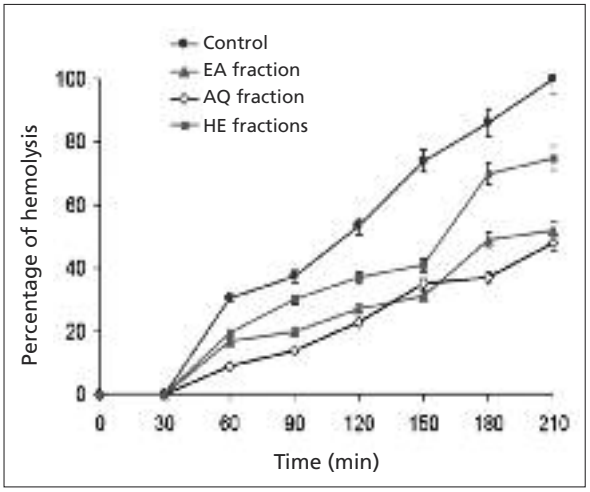

Figure 3. Protective effect of *E. caucasicum* fractions on red blood cell hemolysis induced by CuOOH (50 μM). Values are the Mean ± SD of 3 independent experiments. EA, ethyl acetate; AQ, aqueous and HE, n-hexane fractions.

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