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In vitro antioxidant and antihemolytic effects of the essential oil and methanolic extract of *Allium rotundum L.*

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Abstract. – OBJECTIVE: A plethora of scientific evidence showed that several plant species from the genus *Allium* (Alliaceae) possess multiple therapeutic effects. Present paper aimed to examine the antioxidant and antihemolytic activities of the essential oil and methanol extract *Allium rotundum L.* through different *in vitro* assays.

MATERIALS AND METHODS: 1,1-diphenyl-2-picryl hydroxyl radical (DPPH), nitric oxide as well as hydrogen peroxide scavenging, Fe^{2+} chelating, reducing power and also hemoglobin-induced linoleic acid peroxidation assay systems have been utilized to examine antioxidant effects of these samples. Total amounts of phenolic and flavonoid contents were calculated. The antihemolytic effect was investigated against hemolysis induced by hydrogen peroxide in rat erythrocytes. Also, mineral contents of plant were evaluated by atomic absorption spectroscopy.

RESULTS: IC_{50} for DPPH radical-scavenging activity were 284 ± 11.64 for methanol extract and 1264 ± 45.60 µg ml^{-1} for essential oil, respectively. The extract has shown better reducing effects versus essential oil. The extract also demonstrated better activity in nitric oxide-scavenging activity. IC_{50} were 464 ± 19.68 for extract and 1093 ± 38.25 µg ml^{-1} for essential oil. The extract shows better activity than essential oil in Fe^{2+} chelating system. IC_{50} were 100 ± 3.75 for extract and 1223 ± 36.25 µg ml^{-1} for essential oil. The *A. rotundum* extract and essential oil showed significant H_2O_2 scavenging effects at dose-dependent manners. IC_{50} was 786 ± 29.08 mg ml^{-1} for essential oil. The amounts of eight elements were determined. The concentrations of elements were in the order: Mn> Fe> Zn> Cu> Ni> Cd.

CONCLUSIONS: The extract showed a higher antioxidant effect in all tested models including DPPH, nitric oxide, reducing power as well as iron chelating and antihemolytic activities than essential oil. The latter showed more potent antioxidant activity in scavenging H_2O_2 and lipid peroxidation model. Antioxidant activities of extract may be attributed at least in part, due to its phenolic and flavonoid contents.

Key Words:
Allium rotundum, Antioxidant activity, Atomic absorption, DPPH, Mineral contents.

Introduction

A growing body of literature showed that oxidative stress plays a crucial role in the pathogenesis of multiple chronic diseases[1-3]. Reactive oxygen and nitrogen species cause damage to DNA and other macromolecules and cause aging, coronary heart disease and cancer[4]. Minimizing oxidative damage is known as one of the most important way to mitigate these chronic diseases, since antioxidants suppress oxidative and nitrosative stress. Antioxidants donate electrons to free radicals and convert these compounds to the harmless molecule. Also, they may inhibit radical generation, chain propagation as well as oxidative damage. During past two decades, much attention has been paid to fruits and vegetables as rich sources of natural antioxidants. Therefore, current global health problems have been focused on diets to improve lifestyle. A plethora of evidences showed that micronutrients play a key role in multiple physiological processes and consumption of these compounds lead to prevention of different human diseases.

It has been reported that malnutrition is one of the most important and common problems among different developing countries. For exam-

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ple, iron-deficiency anemia affects more than 33% of the world population[5]. There is a negligible scientific report regarding adverse effects of fruits and vegetables. Respect to this, they can be served as safe and rich sources of minerals. Nowadays, much attention has been paid to edible medicinal plants due to their high amounts of dietary phytochemicals with multiple therapeutic effects and negligible adverse reactions.

The genus *Allium* is one of the best well-known genous belonging to the *Alliaceae* family and includes near to 600-700 species; however, there are only a few species which have been used as vegetables, spices and/or ornamental plant species. Antimicrobial of *A. rotundum* has been reported previously[6]. We have recently published some reports about antioxidant activities of *Allium* spp.[7-10]. Scolicidal effect of *A. sativum* also has been reported by our group[11]. The present paper aimed to determination of antioxidant and antihemolytic potentials of the essential oil and methanol extract *A. rotundum* to provide a broad spectrum from this plant.

Materials and Methods

Plant Material and Preparation of Freeze-Dried Extract

A. rotundum L. was collected from Caspian sea side, Mazandaran, Iran and identified by Dr. B. Eslami. Aerial parts of the plant were dried at room temperature (r. t.) and ground (2-3 mm) before extraction. Materials were extracted by maceration using methanol for 24 hours. The extract was then separated from the sample residue by filtration through filter paper. This procedure was repeated three times. The resultant extracts were concentrated under vacuum until a crude solid extract was obtained which was then freeze-dried for complete solvent removal (9.3%).

Instrumentation and Analytical Procedures

The properly dried and ground plant sample was ash-dried overnight at 400-420°C in a Vitreosil crucible. Temperature not to exceed 450°C to avoid losses of zinc. This procedure destroys organic matters, leaving an inorganic residue that was kept in a desiccator until needed for analysis. Two g of ash were dissolved in a mixture of HCl and HNO_3 (1:3)[5] diluted to 50 ml with double distilled water and used for analysis by means of an atomic absorption spectrometer Perkin Elmer AAS 100 (Wellesley, MA, USA).

Isolation of the Essential Oil

Briefly, 100 g of air-dried plant material were subjected to hydrodistillation for 3 h using a Clevenger apparatus. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4°C until further analysis with a yield of 0.8% (w/w).

Determination of Total Phenolic Compounds and Flavonoid Content

In this study, Folin-Ciocalteau method has been used for determination of the total phenolic content of tested extract[12,13]. Briefly, 1 ml of a metanolic solution of the extract was mixed with 5 ml of Folin-Ciocalteau solution (0.2 N) and after 5 min and 4 ml of sodium carbonate (75 g l^{-1}) was added to the reaction mixture. After 2 hrs incubation, the absorbance of the reaction mixture has been recorded at 760 nm.

Also, the total flavonoid content of extract has been measured by aluminum chloride assay [9,10]. In this assay, metanolic solution of tested extract (1 ml) was mixed with methanol (3 ml), and thereafter, 10% aluminum chloride (0.2 ml), 1 M potassium acetate (0.2 ml), as well as distilled water (5.6 ml) have been added to reaction mixture and eventually reaction mixture incubated for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (UV, Visible EZ201, Perkin Elmer, Waltham, MA, USA). Total flavonoid content was calculated as quercetin equivalents (QE) from a calibration curve.

DPPH Radical-Scavenging Activity

1,1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical-scavenging activity of the samples[12,13]. One ml of different concentrations of samples were added, to 1 ml of a methanol solution of DPPH (100 µM). After 15 min at r. t., the absorbance was recorded at 517 nm (UV, Visible EZ201, Perkin Elmer, Waltham, MA, USA). The experiment was repeated for three times. Quercetin, BHA and vitamin C were used as controls. IC_{50} values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals.

Determination of Reducing Power

The reducing power of essential oil and methanol extract were determined according to recently published papers[12,13]. Briefly, 2.5 ml of samples (25-800 $mgml^{-1}$) in water were mixed

with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. Then 2.5 ml of trichloroacetic acid (10%) was added to the mixture to stop the reaction. The mixture was centrifuged at 3500 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of double distilled water and 0.5 ml of $FeCl_3$ (0.1%), and the absorbance was recorded at 700 nm (UV, Visible EZ201, Perkin Elmer, Waltham, MA, USA). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as control.

Assay of Nitric Oxide-Scavenging Activity

For this experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline (PBS), was mixed with different concentrations of each sample (in water and methanol as a co-solvent) and incubated at r. t. for 150 min. The same reaction mixture, without extract or essential oil but with an equivalent amount of solvent, used as control. After the incubation period, 0.5 ml of Griess reagent was added and then absorbance was measured at 546 nm. Quercetin was used as positive control[14].

Metal Chelating Activity

The chelating of ferrous ions by essential oil and methanol extract were estimated by Wang et al[15]. Sample (0.2-3.2 mg ml^{-1}) was added to a solution of 2 mM $FeCl_2$ (50 µl). The reaction was initiated by the addition of 5 mM ferrozine (200 µl), the mixture was shaken vigorously and left standing at r. t. for 10 min. Then, absorbance was then read spectrophotometrically at 562 nm (UV, Visible EZ201, Perkin Elmer, Waltham, MA, USA). The percentage inhibition of 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.

Scavenging of Hydrogen Peroxide

The ability of the extracts to scavenge H_2O_2 was determined according to our Elmasta et al[16]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Samples (0.1-1 mg ml^{-1}) in double distilled water and methanol as a co-solvent, were added to 0.6 ml of H_2O_2 solution (40 mM). The absorbance of H_2O_2 at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without H_2O_2. The percentage of H_2O_2 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 standard and extract samples.

Antioxidant Activity in a Hemoglobin-Induced Linoleic Acid Peroxidation Test

The antioxidant activity of the essential oil and methanol extract were determined by a photometry assay[17,18]. Briefly, 200 ml of reaction mixtures containing 10 ml of extracts (10-400 mg), 1 mmol/l of the linoleic acid emulsion, 40 mmol/l of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, were incubated at 37 ºC 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%). Vitamin C was used as control.

Antihemolytic Activity of Extract Against H_2O_2 Induced Hemolysis

In the present study, erythrocytes have been separated from male Wistar rats according to the method of Yuan et al[19]. Antihemolytic effect of samples has been evaluated through hydrogen peroxide-induced hemolysis assay system[20,21]. Briefly, erythrocytes have been diluted in the PBS (at physiological pH) to obtain 4% erythrocytes suspension. Thereafter, samples (at doses 1 g/ml) have been added to erythrocyte suspension (2 ml) and then, the volume of the reaction mixture has been increased to 5 ml through saline buffer adding. Reaction mixtures have been incubated for 5 min and finally H_2O_2 solution (0.5 ml) has been added to cause hemolysis. After 240 min incubation at room temperature, reaction mixtures have been centrifuged at 2000 rpm (10 min) and finally, the absorbance of reaction mixture have been recorded at 540 nm. The inhibitory activity of the extract was compared with that of vitamin C as a standard antioxidant. 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

Table I. Amount of trace elements in the plants by AAS analysis (µg/g of ash)[a].

Sample	Zn	Mn	Fe	Cu	Cr	Ni	Pb	Cd
A. rotundum	17	27	18	12.3	ND[b]	7	ND[b]	1

[a]For all elements, the slit width 0.2 nm. The air and acetylene flow rates were 4.0 and 0.5 L/min, respectively. [b]Not Detected. Values are averages of three independent measurements having a precision of approx ± 2.

times. The data were analyzed by an ANOVA ($p < 0.05$) and the means separated by Duncan's multiple range test. The IC_{50} values were calculated from linear regression analysis.

Results

The yield of the production of ash was 28%. Table I presents the elemental analysis in the ash of plant by AAS technique. The concentration of analyzed elements decreases in the order: Mn> Fe> Zn> Cu> Ni> Cd. Total phenol compounds are reported as gallic acid equivalents (GAE) by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic contents of was 166.66 ± 4.49 mg GAE/g of extract, respectively. The total flavonoid contents was 10.81 ± 0.37 mg QE/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). IC_{50} for DPPH radical-scavenging activity were 284 ± 11.64 for methanol extract and 1264 ± 45.60 mg ml^{-1} for essential oil, respectively. The IC_{50} values for vitamin C, quercetin and BHA were 1.26 ± 0.05, 1.32 ± 0.07 and 13.49 ± 0.8 mg ml^{-1}, respectively. Figure 1 shows the dose-response curves for the reducing powers of the extract and essential oil. The extract also showed better activity in nitric oxide-scavenging activity. IC_{50} were 464 ± 19.68 for extract and 1093 ± 38.25 µg ml^{-1} for essential oil. The absorbance of Fe^{2+}-ferrozine complex in Fe^{2+} chelator assay was decreased dose-dependently, i.e. the activity increased by increasing concentration from 0.1 to 1.6 mg ml^{-1}. IC_{50} were 100 ± 3.75 µg ml^{-1} for extract and 1223 ± 36.25 µg ml^{-1} for essential oil. EDTA showed very strong activity (IC_{50} = 18 ± 0.9 µg ml^{-1}). Extract and essential oil showed good reducing activity in the hemoglobin-induced linoleic acid system (41% for essential oil and 34% for extract) at 125 mg ml^{-1} and high inhibitory ability (78% for essential oil and 64.6% for extract) at 1000 mg ml^{-1} (Figure 2). The essential oil showed better activity than extract in hemoglobin-induced lipid peroxidation

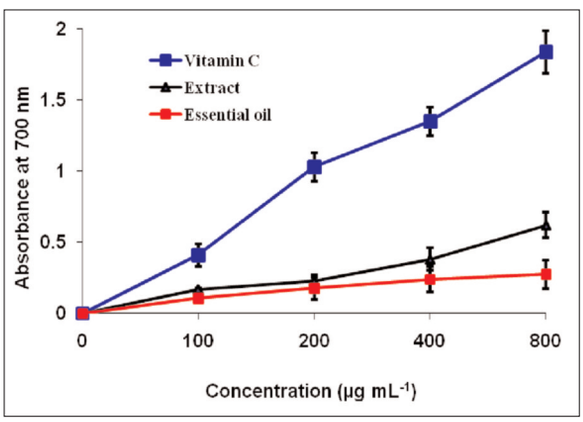

Figure 1. Reducing power of *A. rotundum* L. extract and essential oil. Vitamin C used as positive control.

but was not comparable with vitamin C ($p < 0.01$) (Figure 2). IC_{50} for H_2O_2 scavenging activity were in the order 2670 ± 109.15 for extract and 786 ± 29.08 µg ml^{-1} for essential oil. The IC_{50} values for ascorbic acid and BHA were 21.4 ± 0.81 and 52.0 ± 2.02 µg ml^{-1}, respectively. The extracts showed weak anti -hemolysis activity. The extract showed better activity than essential oil at 1 g ml^{-1}. IC_{50} were 260 ± 8.84 for extract and 755 ± 25.57 µg ml^{-1} for essential oil.

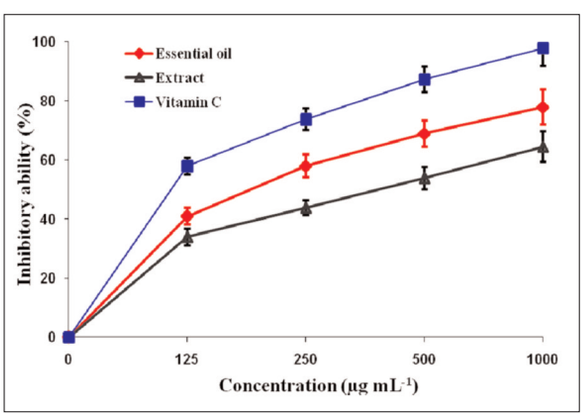

Figure 2. Antioxidant activity of *A. rotundum* L. extract and essential oil against linoleic acid peroxidation. Vitamin C used as positive control.

Discussion

The daily requirements of adults are as follows (mg/d): 10-15 Fe, 12-15 Zn and 2-3 Cu. On the contrary, the foods consumed in the third world countries population are poor in important elements such as Fe and the consumption of this vegetable could bring the amount required to meet the requirements either. This plant has collected from the Mazandaran ashore but it founds also in Iran, Turkey and Caucasia[22]. It has a good potential for human nutrition. The knowledge of the composition of elements in plants might be crucial to minimize or reduce the toxic effects of the environment pollutant heavy metals[23]. *A. rotundum* contained a high level of total phenolic flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plants, and they have been shown to have high antioxidant activities[22]. Studies have shown that increasing the content of flavonoids in the diet could decrease some human diseases[22]. Scavenging of DPPH radical is a widely used method for evaluation of the free radical scavenging ability of different samples[9]. DPPH is a stable free radical in which its violet color turns to yellow by reduction via hydrogen donors. Substances with this ability can be considered as anti-oxidants and radical scavengers[9]. In the reducing power assay, the presence of reductants in the samples would result in the reducing of ferric to ferrous ions by donating an electron. The amount of Fe^{2+} complex can be monitored by detecting the formation of Prussian blue at 700 nm. Increasing absorbance at this wavelength indicates an increase in reductive ability. Reducing powers of the extract and essential oil have increased with increasing of their concentrations. The extract has shown a better reducing power activity than essential oil but was not comparable with vitamin C ($p < 0.05$) (Figure 1). In addition to reactive oxygen species, NO is also implicated in CNS problems, inflammation, cancer and other pathological diseases[14]. Plant products with the ability of reduction of NO formation are of considerable interest in preventing the problems of excessive NO generation. Further, the scavenging activity may also help to arrest the chain reactions initiated by an excess generation of NO that is detrimental to human health[14]. The transition metal, iron, is capable of generating free radicals from peroxides *via* Fenton reactions and may be implicated in human cardiovascular disease[15]. Because Fe^{2+} also has been shown to cause lipid peroxidation and oxyradicals, minimizing Fe^{2+} concentration in Fenton reactions gives protection against oxidative damage. Chelators can inhibit Fenton reaction-mediated oxidative stress by forming chelate complexes with free iron cations[9]. Among tested samples, extract demonstrated better effect versus essential oil in Fe^{2+} chelating assay model. We also concluded that H_2O_2 scavenging effects of *A. rotundum* extract and essential oil might be due to presence polyphenolic compounds which lead to electrons donation to hydrogen peroxide[24]. The *A. rotundum* extract and essential oil were capable of scavenging H_2O_2 in a concentration dependent manner. Although H_2O_2 itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Therefore removing of H_2O_2 is important in food systems[16]. Hemolysis has a long history of use in measuring free radical damage. But until now, only few studies have been done with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocytes lysis in rat blood. This assay is useful for screening of various molecules and their metabolites, especially with oxidizing/antioxidizing activity with a long-term action[25]. Lipid oxidation of rat red blood cell membrane mediated by H_2O_2 induces membrane damage and subsequently hemolysis.

Conclusions

The *A. rotundum* L. extract was found to be more active in DPPH, and nitric oxide radical scavenging, reducing power and iron ion chelating as well as antihemolytic activity models. The essential oil was also found to be more effective in H_2O_2 and lipid peroxidation methods. The most abundant elements were Mn, Fe and Zn respectively. Further works are necessary to isolate the active constituents.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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