Antioxidant and Free Radical Scavenging Activities of Different Fractions of *Anethum Graveolens* Leaves Using in Vitro Models

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Summary

The protective effects of fruits and vegetables against chronic diseases have been in part attributed to the presence of antioxidants in these foods. *Anethum graveolens L*. (dill) [Umbelliferae] is used in Iranian folk medicine as an anti-hypercholesterolaemic plant. To clarify the mode of action, primarily the antioxidant potency of the leaf crude extract and its different fractions (diethyl ether, ethyl acetate and water) were investigated, employing various established in vitro systems such as the ferric reducing antioxidant power (FRAP) and 2, 2′-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS) assays, 1,1-diphenyl-2-picrylhydrazyl (DPPH) /nitric oxide radical scavenging, iron ion chelating activity and inhibitory effect on Fe^{2+}/ascorbate induced lipid peroxidation in rat liver homogenate. In the above assays, crude extract and all the fractions showed antioxidant potential to varying degrees. Among these fractions, ethyl acetate fraction exhibited higher antioxidant potency compared to the other fractions. The ethyl acetate fraction also exhibited the highest anti-hyperlipidaemic effects in animal models. These data clearly indicate that *A. graveolens* especially its ethyl acetate fraction possess potent antioxidant property, and this might account for its anti-hyperlipidaemic and anti-hypercholesterolaemic effects.

Kew words: *Anethum graveolens*, Antioxidant potency, Ferric reducing antioxidant power, Lipid peroxidation, Nitric oxide radical scavenging.

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Introduction

Oxidative stress has been defined as an imbalance in the production of free radicals within the body and the biochemical antioxidant defense mechanisms to combat them. In aerobic organisms, one of the major targets of free radicals is the cellular biomembranes, where they induce lipid peroxidation. Under this process, not only the membrane structure and its function are affected, but also some oxidation reaction products, for example, malondialdehyde (MDA), can react with biomolecules and exert cytotoxic and genotoxic effects. Therefore, high levels of free radicals especially lipid peroxides play a crucial role in the pathogenesis of several human diseases such as cancer, diabetes, aging, coronary heart diseases, various neurodegenerative and pulmonary diseases [1]. Although living organisms' combat free radicals using different antioxidant systems such as antioxidant enzymes and a variety of indigenous antioxidant agents [2], however, under serious oxidative stress these systems will not be capable of challenging the free radical levels. This situation usually leads to irreversible oxidative damages to the biological systems [3]. Regarding these facts, there is emerging interest in the use of naturally occurring antioxidants for the prevention of various biological damages associated with oxidative stress. Natural antioxidants, mainly in fruits and vegetables, have gained increasing interest among consumers because epidemiological studies have indicated that frequent consumption of vegetables and fruits is associated with a lower risk of cardiovascular disease and cancer [4]. Phenolic or polyphenolic compounds constitute one of the largest groups of plant secondary metabolites which are therefore an integral part of the diet with significant amounts being reported in vegetables, fruits and beverages [5]. The antioxidant property of polyphenols is mainly due to their redox properties. They mainly act as free radical terminators, hydrogen donors and metal chelators [6]. *Anethum graveolens* Linn., Umbelliferae, known as dill, is an annual herb growing in the Mediterranean region, Europe, central and southern Asia. Nowadays it's widely cultured in south eastern region of Iran. Dill has been used traditionally by a cross section of people of the region as for gastrointestinal ailments such as flatulence, indigestion, stomachache and colic to tract intestinal gas. Some pharmacological effects have also been reported for the plant such as anti microbial [7], antispasmodic [8], anti secretary, mucosal protective effects [9] and anti-hyperlipidaemic [10]. The presence of flavonoids and other phenolic compounds has already been reported in different extracts of *Anethum graveolens* [11, 12, 13]. Our earlier studies have confirmed the antioxidative potential of an ethanolic extract of *A. graveolens* using the livers of rats fed high-fat diets [14].

In this study, we evaluated the antioxidative activities of different organic fractions of the crude extract hoping to assist the purification process of the active constituent(s). we examined the antioxidant activity of different fractions of *A. graveolens* extract, employing various in vitro assay systems, such as the 2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS), ferric reducing ability potential (FRAP) assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH)/nitric oxide (NO) radical scavenging, chelating abilities on ferrous ions and inhibitory effect on Fe^{2+}/ascorbate induced lipid peroxidation in rat liver homogenate.

Materials and Methods

I. Chemicals

DPPH was obtained from Fluka (Buchs, Switzerland). ABTS, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), Potassium ferric cyanide and ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma-Aldrich Chemical Co. Ltd. (England). 2, 4, 6-tripyridyl-S-triazine (TPTZ), was obtained from BDH chemicals Ltd (England). Gallic acid, ascorbic acid and ferric chloride were obtained from Sigma (St. Louis, MO, USA). Butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), ferrozine and potassium persulfate (di-potassium peroxodisulfate) were obtained from Merck Co. All other chemicals used were analytical grade. Glass double distilled water was used in all experiments.

II. Plant material

The leaves of *Anethum graveolens L*. were collected from suburbs of Aleshtar city (Lorestan province, Iran) at the end of May 2006 and identified by Dr. F. Attar (Department of Biology, Faculty of Sciences, University of Tehran) and a voucher specimen (No. 11018) was deposited in the central herbarium of University of Tehran. The collected materials were dried at room temperature away from sun light. The dried leaves were pulverized and kept at 8 ^{o}C for further use.

III. Crude extract preparation and fractionation

Three hundred grams of the plant powder was extracted four times (4×24 h) with ethanol (70%, v/v) at room temperature. The extracts were filtrated, concentrated using a rotary evaporator and then dried to a residue by lyophilization. The average yield of the extracts was 26%. The residue re-dissolved in water and divided into two aliquots. One aliquot was kept at -20 ^{o}C and the other aliquot was subjected to fractionation processes. The aliquot was extracted first with diethyl ether for four times at room temperature. The extracted liquid phase was then re-extracted with ethyl acetate for four times.

The resulting three fractions (diethyl ether, ethyl acetate and water) were evaporated under vacuum to dryness to give the diethyl ether, ethyl acetate, and water fractions, respectively. They were quantitatively re-dissolved in ethanol to a 10 mg/ml concentration.

IV. Methodology:

1. Determination of total phenolic content

Total phenolic contents were determined with the Folin–Ciocalteu's reagent (FCR) according to a published method [15]. Each sample (0.5 ml) was mixed with 2.5 ml FCR (diluted 1:10, v/v) followed by 2 ml of Na_2CO_3 (7.5%, v/v) solution. The absorbance was then measured at 765 nm after incubation at 30 ^oC for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

2. Determination of total flavonoid content

The total flavonoid contents were evaluated by colorimetric methods as described in the literature [16]. Each sample (0.5 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a $NaNO_2$ solution (15%). After 6 min, 0.15 ml of an $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus water blank. Results were expressed as catechin equivalents (mg catechin/g dried extract).

3. Non-enzymatic lipid peroxidation induced by Fe^{2+}/ascorbate

Male N-Mary rats, weighting 200-250 g and purchased from Pasteur institute (Tehran, Iran), were housed under conventional conditions and were allowed free access to food and water, ad libitum. The rats were anesthetized using diethyl ether and then the abdomen was opened and the liver was quickly removed. Liver was immediately rinsed with saline, blotted on filter paper, weighed and homogenized in phosphate buffer (50 mM, pH 7.4) to give a 10 % (w/v) liver homogenate. The homogenates were then centrifuged at 5000 g for 15 min at 4 ^oC (Beckman). The supernatant was obtained and the protein concentration was determined by the method of Lowry et al. [17] using crystalline bovine serum albumin as the standard. The extent of lipid peroxidation was evaluated by measuring the product of thiobarbituric acid reactive substances (TBARS) in the rat liver homogenate using the modified method previously described [18]. The reaction mixture was composed of tissue homogenate 0.5 ml, phosphate buffer (50 mM, pH 7.4) 0.9 ml, $FeSO_4$ (0.01 mM) 0.25 ml, ascorbic acid (0.1 mM) 0.25 ml, and 0.1 ml of different

concentration of samples and the standard sample. The reaction mixture was incubated at 37 ^{o}C for 30 min and the reaction was then terminated by adding BHT (2% w/v in 95% v/v ethanol), followed by addition of 1 ml of TCA (20% w/v) to the mixture. After centrifugation at 3000g for 15 min, the supernatant was incubated with 1 ml of TBA (0.67%) at 100 ^{o}C for 15 min. Catechin was used as the positive control. The color of the complex of TBARS with TBA was detected at 532 nm. The absorbance value was subtracted from that of the test values. The amount TBARS formed was calculated using the absorption coefficient of 1.56×10^5 cm $^{-1}$ M $^{-1}$ [19].

4. Metal chelating activity

The chelating of ferrous ions was estimated by the method of Dinis et al [20]. Briefly, different concentrations (200–500 μg/ml) of the crude extract and each of its different fractions were separately 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 $[(A_0-A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the samples. EDTA was used as a positive control.

5. Assay of NO-scavenging activity

The procedure 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. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of the samples dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the samples but with an equivalent amount of water, served as the 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. Catechin was used as the positive control [21].

6. DPPH radical scavenging activity

Radical scavenging activities of the crude extract and its various fractions were measured according to the method of Blois [22]. Briefly, 1 ml each of sample with variable concentrations (25-400 μg/ml in ethanol) was added to 1 ml of a DPPH solution (0.2 mM in ethanol) as the free radical source and kept for 30 min at room temperature. The decrease in the solution

absorbance, due to proton donating activity of *A. graveolens* component(s), was measured at 517 nm. Vitamin C was used as the positive control. The DPPH radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $[(A_0 - A_1 / A_0) \times 100]$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of test or the standard sample.

7. ABTS assay

The ABTS radical-scavenging activity was determined according to Re et al. [23]. The ABTS radical cation was generated through the reaction of an aqueous solution of ABTS (7.4 mM) with potassium persulfate (2.6 mM, final concentration) followed by incubation at 25 ^{o}C for 12-16 h in the dark. The solution was diluted in methanol to an absorbance of 0.70 (± 0.020) at 734 nm before use. Aliquots (with different concentrations) of trolox or the sample in water (200 µl) were added to 2.8 ml of this diluted solution and the absorbance at 734 nm was determined at 30 ^{o}C, exactly 6 min after initial mixing. The extent of decolorization is calculated as percentage reduction of absorbance.

8. Measuring the antioxidant power (FRAP assay)

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain [24]. Working FRAP reagent was prepared as required by mixing 25 ml of 300 mM acetate buffer, pH 3.6 (3.1 g sodium acetate and 16 ml glacial acetic acid per liter of buffer solution) with 2.5 ml of 10 mM TPTZ solution (0.031 g of TPTZ in 10 ml of 40 mM HCl) and 2.5 ml of 20 mM $FeCl_3$-$6H_2O$ solution (3.24 g of ferric chloride in l liter distilled water). Freshly prepared reagent warmed at 37 ^{o}C. The test samples (200µl), with appropriate concentrations, were added to 2.8 ml of FRAP reagent and the reaction mixture was incubated for 30 min in the dark. The increase in absorbance at 593 nm was measured after 30 min. Aqueous solutions of known $FeSO_4$ concentrations, in the range of 20–500 µM, were used for obtaining the calibration graph. The reducing power was expressed as equivalent concentration (EC). This parameter was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

9. Statistical analysis

All analyses were performed in triplicate and data were reported as means ± SD. Statistical analyses were performed using student t-test and $P<0.05$ was considered to be statistically significant. The EC_{50} values were calculated from linear regression analyses. Results were processed using Excel and

Statistic soft wares.

Results

Total phenolic and flavonoid contents

The Total phenolic and flavonoid contents of crude extract and its different fractions were determined and expressed in terms of gallic acid and catechin equivalents (Table 1). Among these fractions ethyl acetate fraction showed the highest phenolic and flavonoid contents by 179.2 mg gallic acid equivalents/g dried fraction/extract and 135.2 mg catechin equivalents/g dried fraction/extract, respectively.

Table 1. Total phenolic and flavonoid contents of the crude extract and different fractions of *A. graveolens*.

Sample	Total phenolic content (mg/g)[a]	Total flavonoid content (mg/g)[b]
Crude extract	105.2± 3.2	58.2 ± 2.8
Diethyl ether fraction	63.2 ± 2.9	38.2 ± 2.6
Ethyl acetate fraction	179.2 ± 4.9	135.2 ± 3.6
Water fraction	42.2 ± 2.7	30.2 ± 2.1

Each value represents the mean ± SD (n=3).
[a] Total phenolic content was expressed as mg gallic acid equivalents/g dried extract.
[b] Total flavonoid content was expressed as mg catechin equivalents/g dried extract.

Lipid peroxidation assay

The addition of $FeSo_4$-ascorbic acid to the liver homogenate for 30 min significantly increased the extent of TBARS formation, compared to the control sample (4.46 nmol/mg protein versus 0.27 nmol/mg protein). However, adding crude extract and each of its fractions to rat liver homogenate significantly and dose-dependently reduced TBARS formation in the liver homogenate (data not shown). Based on EC_{50} values, the order of inhibition percentage of crude extract and different fractions was found to be: ethyl acetate fraction > crude extract > diethyl ether fraction > water fraction (Table 2).

Metal chelating activity and NO-scavenging activity

Table 2 shows the chelating effects of the crude extract and its three fractions relative to EDTA as the standard. EC_{50} values for chelating effects were 512.5, 601.8, 391.7 and 655.2 µg/ml for the crude extract, diethyl ether, ethyl acetate and water fractions, respectively, which are significantly lower than that of EDTA (EC_{50} Value of 5.2 µg/ml). In addition, the crude extract and the different fractions of *A. graveolens* also showed a moderate NO-scavenging activity relative to catechin. Based on EC_{50} values the maximum and minimum NO-scavenging activity was observed for ethyl acetate fraction and water fraction, respectively (Table 2).

Table 2. EC_{50} values of the crude extract and different fractions of *A. graveolens* leaves on NO radical scavenging activity, Iron chelating activity and Inhibition of TBARS formation.

Samples	NO radical scavenging activity	Iron chelating activity	Inhibition of TBARS formation
		EC_{50} (µg/ml)	
Crude extract	535.2	512.5	141.2
Diethyl ether fraction	700.2	601.8	183.3
Ethyl acetate fraction	440.9	391.7	55.5
Water fraction	771.2	655.2	197.2
Catechin	400.2	-	20
EDTA	-	5.2	-

The EC_{50} values for the crude extract and each of the fractions of *A. graveolens* were calculated from the plots of the antioxidant concentration versus above mentioned activities. The values for Catechin and EDTA were calculated based on data obtained from similar experiments.

DPPH and ABTS radicals scavenging activities

The effective concentrations of crude extract, each of its fractions and the reference compound required to scavenge 50% of DPPH radicals, the EC_{50} values; are presented in Table 3. It can be seen that the different fractions exhibited varying degrees of scavenging capacities. Among these fractions,

ethyl acetate fraction showed the highest scavenging activity with an EC_{50} value of 75.6 µg/ml which is lower than that of ascorbic acid ($P < 0.05$). The other two fractions, diethyl ether and water, showed significantly weaker scavenging potency than that of ethyl acetate fraction ($P< 0.05$), with the EC_{50} values of 124.1 and 152.2 µg/ml, respectively. In addition, Table 3 depicts a decrease in the absorbance of ABTS radical cation at 734 nm in the presence of the crude extract and its different fractions. Both the fractions and the crude extract of *A. graveolens* exhibited a potent scavenging activity for ABTS radical cation (Table 3). Maximum inhibition was observed with the ethyl acetate fraction which is less active than trolox (EC_{50}: 6.5 µg/ml). Based on EC_{50} values, the order of scavenging activity of different fractions was found to be: ethyl acetate fraction > crude extract > diethyl ether fraction > water fraction.

Table 3. EC_{50} values of the crude extract and different fractions of *A. graveolens* leaves on DPPH and ABTS radical scavenging.

Samples	DPPH radical scavenging activity	ABTS radical scavenging activity
	EC_{50} (µg/ml)	
Crude extract	105.6	39.8
Diethyl ether fraction	124.1	35.55
Ethyl acetate fraction	75.6	11.28
Water fraction	152.2	37.2
Vitamin C	3.5	-
Trolox	-	6.5

Each value represents the mean ± SD (n=3).
The EC_{50} values for the crude extract and each of the fractions of *A. graveolens* were calculated from the plots of the antioxidant concentration versus free radical scavenging activity. The values for Vitamin C and Trolox were calculated based on data obtained from similar experiments

Ferric ion reducing activity

The reducing ability of polyphenols correlates well with their free radical scavenging capacities. In the present study, the FRAP value for the crude extract and each of the organic fractions and trolox, as a positive control,

were determined using the calibration graph for $FeSO_4$ (Fig. 1). Based on these data, the crude extract and each of the organic fractions have the ability of reducing Fe^{3+} to Fe^{2+} to varying degrees. The highest EC values were obtained for the ethyl acetate fraction and the minimum extent of inhibition was observed for the water fraction. Based on the EC values, the order of the reducing activity of different fractions was found to be: ethyl acetate fraction > crude extract > diethyl ether fraction > water fraction.

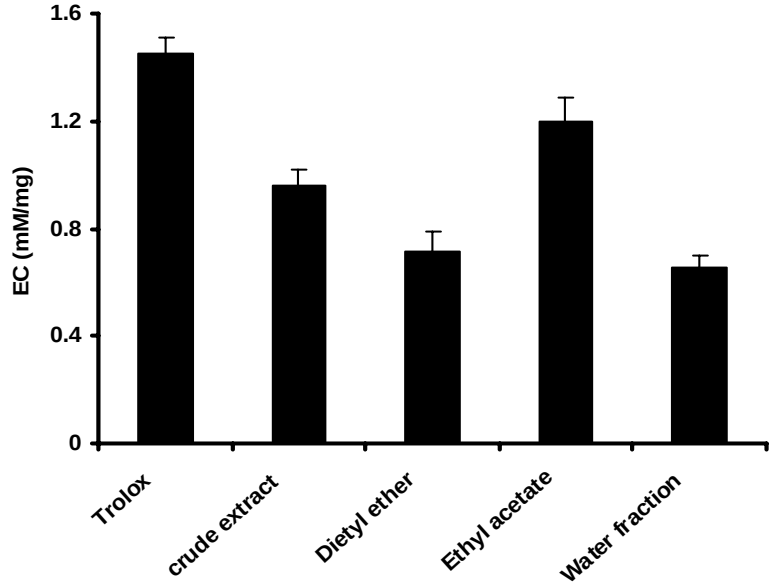

Figure 1. Antioxidant activity of the crude extract and different fractions *A. graveolens* of at various concentrations using FRAP assay.
Each value represents the mean ± SD (n=3).
[a] EC expressed concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

Discussion

Reactive oxygen species (ROS) and their likely involvement in some human physiopathologies have been the center of focus in many health oriented fields over the last few decades. Destructive and irreversible damages to cellular components including proteins, lipids, lipoproteins and DNA are imparted by ROS due to their high chemical reactivates [25]. Overproduction of ROS, referred to as oxidative stress, is currently suggested as a mechanism underlying hypercholesterolaemia, a major

known risk factor in coronary artery diseases [26]. This event increases the production of free radicals leading to accelerated lipid peroxidation or reduced activity of antioxidant defense systems or both. *A. graveolens* is a medicinal plant widely used in Iran principally as an anti-hyperlipidaemic herb. Our previous studies have documented the anti-hypercholesterolaemic and anti-hyperlipidaemic activities of the crude extract of *A. graveolens* [10]. To set up a correlation between the anti-hypercholesterolaemic activity of *A. graveolens* extract and antioxidant activity, studied were also extended to in vitro. In the present study, we measured the total phenolic and flavonoid contents of the plant extract and each of its fractions. As shown in Table 1, the highest amount of phenolic and flavonoid contents were found in the ethyl acetate fraction which might be responsible for the enhanced antioxidative activity compared to the other fractions.

ABTS and DPPH radical scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of plant components. These chromogens (the violet DPPH radical and the blue green ABTS radical cation) have high sensitivity and allow for rapid analyses of the antioxidant activity of a larger number of samples. $DPPH^{\bullet}$ is a stable free radical which accepts an electron or a hydrogen radical to become a stable diamagnetic molecule. Based on our results, ethyl acetate fraction showed the highest DPPH radical scavenging activity. *A. graveolens* crude extract has an IC_{50} value of 105.6 μg/ml, which is much lower than the IC_{50} of *P. ferulacea (*242 μg/ml), *C. macropodum* (623 μg/ml) and *H. persicum* (483 μg/ml) other species of Umbelliferae family [27]. Re et al. [23] reported that the decolorization of the ABTS radical cation also reflects the capacity of an antioxidant agent in donating electrons or hydrogen atoms to inactivate the ABTS radical cation. Similar to DPPH radical scavenging activity, the ethyl acetate fraction exhibited higher ABTS radical cation scavenging activity relative to other fractions. The potent radical scavenging activity of the ethyl acetate fraction might be due to the presence of polyphenolics and flavonoids in the fraction. Independence studies have demonstrated that free radicals cause auto oxidation of unsaturated lipids of membranes and other lipid constituents present in the biological systems which will lead to high level of MDA production. That might lead to various pathological consequences [28]. Our data clearly indicated that ethyl acetate fraction of *A. graveolens* extract is capable of quenching the extent of lipid peroxidation in a rat liver homogenate caused by Fe^{2+}-ascorbate system. This inhibition is either due to chelation of Fe ions or due to trapping of the free radicals produced by Fe^{2+}/ascorbate in the reaction system. In either case, the inhibitory effect of the fraction might be due to phenolic hydroxyl groups with the ability to trap electrons. It is also

likely that inhibition of the iron-dependent (Fe^{+2}-acsorbate) lipid peroxidation may result from metal chelation. To verify this, we measured the iron chelating ability of *A. graveolens* crude extract and its different fractions. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal ions in both initiation and propagation of lipid peroxidation [29]. It has been reported that plant extracts enriched in phenolic compounds are capable of forming stable complexes with the transition metal ions, rendering them unable to participate in metal ion-catalyzed initiation and hydroperoxide decomposition reactions [30]. High Fe^{2+}-chelating capacity of *A. graveolens* especially its ethyl acetate fraction suggests that active constituent(s) in the fraction are capable of forming these complexes. Regarding the direct correlation between antioxidant activity and reducing power of certain plant extracts, we measured the reducing ability of *A. graveolens* crude extract and its different fractions. Reductants inhibit lipid peroxidation by donating hydrogen atoms and thereby terminating the free radical chain reactions [31]. Regarding these facts, the FRAP assay provides a reliable method to study the antioxidant activity of various compounds [24]. In the present study, ethyl acetate fraction showed the highest reducing power, as has been recorded in DPPH and ABTS methods.

In addition to ROS, NO is also implicated in inflammation, cancer and some other pathological conditions [32]. 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 adverse effects of excessive NO generation in the biological systems. Based on our results, the ethyl acetate fraction of *A. graveolens* is also capable of scavenging NO species. This capability might be due to its phenolic/ flavonoid content.

Conclusion

In conclusion, regarding the results presented in this report, it can be concluded that the leaves of *A. graveolens* contain a number of antioxidants which can effectively scavenge various reactive free radicals under in vitro conditions. They also have mild metal chelation properties as well as NO-radical scavenging. The observed properties, besides of confirming the traditional use of *A. graveolens*, necessitates extra effort for isolation and structural elucidation of the active constituents.

Acknowledgments

The authors appreciate the joint financial support of this investigation by the research Council of University of Tehran and Iran National Science Foundation.

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