IMPROVEMENT OF LIVER ANTIOXIDANT STATUS IN HYPERCHOLESTEROLAMIC RATS TREATED WITH ANETHUM GRAVEOLENS EXTRACTS

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Summary

The present study was designed to investigate the effects of the crude extract of Anethum graveolens L. (Umbilliferae) on liver antioxidant status in rats fed a high-fat diet (HFD). Feeding the animals by a HFD for 7 consecutive days resulted in elevation of the serum total cholesterol (TC), triglyceride (TG) and low density lipoprotein-cholesterol (LDL-C) levels. Treatment of hyperlipidaemic rats with the crude extract (with a single daily dose of 1 ml, equivalent to 500 mg plant powder) for 30 days reversed the serum lipid levels of the treated rats by varying extents. To evaluate the mechanism(s) of action, we studied the antioxidative potential of Anethum graveolens crude extract (AGE) in terms of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities along with the level of reduced glutathione (GSH) in the livers. In addition, hepatic tissue malondialdehyde (MDA) level was also determined. Furthermore, Antioxidant activity of the crude extract was evaluated using different in vitro assay systems such as 2′-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS) radical cation and ferric reducing antioxidant power (FRAP). Pretreatment of rats with AGE significantly decreased the activities of liver GR, GPx and MDA levels while the activities of SOD, CAT and GSH content were elevated. In vitro, the extract presented radical scavenging ability for ABTS radical cations as well as reducing activity for ferric ions. In conclusion, the present study demonstrates that AGE, besides of having strong anti-hyperlipidaemic effects, can improve the biological antioxidant status by reducing lipid peroxidation in liver and modulating the activities of antioxidant enzymes in rats fed HFD.

Keywords: Anethum graveolens; Antioxidant activity; Lipid peroxidation; Reduced glutathione
Introduction

Oxidative stress, which results from an imbalance between radical-generating and radical scavenging systems, is currently suggested as a mechanism underlying atherosclerotic lesions [1-2]. This event increases the production of free radical leading to accelerated lipid peroxidation (LPO) or reduced activity of antioxidant defenses or both [3]. Most living species have efficient defense systems to protect themselves against the oxidative stress induced by reactive oxygen species (ROS) [4]. These include anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) and non-enzymatic antioxidants, such as glutathione (GSH), vitamin C and vitamin E [5]. The efficiency of the antioxidant defense system is altered under different pathological conditions and therefore, there is growing interest in natural antioxidants present in medicinal and dietary plants that might help attenuate oxidative damages. Recently, it has been shown that the intake of medicinal plants with high polyphenolic content has resulted in an increase in the antioxidant enzyme activities, the HDL-C levels and a decrease in malondialdehyde (MDA) production [6]. *Anethum graveolens* L. (Umbiliferae), known as dill, is an annual herb growing in the Mediterranean region, Europe, central and southern Asia. Nowadays, it is widely cultured in south eastern region of Iran. The plant is used both medicinally and as an aromatic herb and spice and cookery. Dill has been used traditionally for gastrointestinal ailments such as flatulence, indigestion, stomachache and colic to tract intestinal gas. The presence of flavonoids, other phenolic compounds and essential oil has been reported in *Anethum graveolens* [7-10]. Some pharmacological effects have also been reported for the plant such as anti microbial [11], antispasmodic [12], anti secretary and mucosal protective effects [13]. The anti-hypercholesterolaemic and anti-hyperlipidaemic activities (TC, TG) of the crude extract have previously been reported [14]. The present study was designed for further details about activities of *A. graveolens* extract (AGE) on hepatic antioxidant status in liver homogenates of rats fed high-fat diet. Furthermore, Antioxidant activity of the crude extract was evaluated using different in vitro assay systems such as 2′-azinobis 3-ethylbenothiazoline-6-sulfonate (ABTS) radical cation and ferric reducing antioxidant power (FRAP).
Materials and methods

**Reagents:**
Reduced glutathione (GSH) and oxidized glutathione (GSSG) were obtained from Fluka (Buchs, Switzerland). Glutathione reductase (GR), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2, 2′-azinobis 3-ethylbenzothiazoline-6-sulfonate (ABTS) and EDTA were obtained from Sigma-Aldrich Chemical Co. Ltd. (England). Nitroblue tetrazolium (NBT), 5, 5′-dithiobisnitrobenzoic acid (DTNB), thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Merck Co (Germany). All other chemicals used were analytical grades.

**Plant material:**
The aerial sections 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 was deposited in the central herbarium of University of Tehran. Then, the collected materials were dried at room temperature away from sunlight. The dried leaves were pulverized and kept at 8 °C for further use.

**Crude extract preparation:**
The *Anethum graveolens* powder (200 g) was defatted with hexane at room temperature. Then, the defatted residue was extracted four times with ethanol (70 %, v/v) at room temperature. The combined extracts were concentrated under reduced pressure and the volume was adjusted to 400 ml (equivalent to 500 mg plant powder). Aliquots were kept at -10 °C for further investigation.

**Determination of total phenolic and total flavonoid contents:**
The total phenolic content of AGE was determined with the Folin–Ciocalteu’s reagent (FCR) according to a published method [15]. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract). The total flavonoid content of AGE was evaluated by colorimetric methods as described in the literature [16]. Results were expressed as catechin equivalents (mg catechin/g dried extract).
**ABTS radical-scavenging activity:**
The ABTS radical-scavenging activity was determined according to Re et al [17]. This method is based on the ability of an antioxidant to quench the long-lived ABTS radical cation, in comparison to that of trolox, a water-soluble vitamin E analogue. The decrease in the absorption at 734 nm was used for calculating the inhibition values relative to the control.

**Ferric reducing antioxidant power (FRAP) assay:**
This method has been frequently used for rapid evaluations of the total antioxidant capacity of various food and beverages [18]. The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain [19]. The FRAP value was defined as the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 µM FeSO₄.

**Experimental animal and diet protocols:**
Male N-Mary rats initially weighing 200-250 g were allocated in groups of six per cage and maintained on a 10 h light/14 h dark cycle, temperature 22 ± 2 °C and relative humidity of 60 ± 5%. The animal had free access to water and normal or high-fat diet *ad libitum*. Chow, with high-fat content, was made from normal pulverized chow (Protein, vitamin, carbohydrate) (47%), cholesterol (1%), sodium cholate (1%), dextrose (40%), olive oil (10%), and water (15%). The cake was cut into pieces and dried at room temperature for 3 days before feeding to rats. After one week of adaptations, the rats were randomly divided into three groups each containing 6 of animals by the following:
Group 1: received normal diet (NC), Group 2: received high-fat diet (HFD) and Group 3: received HFD + *Anethum graveolens* extract (HFD+AGE) (1 ml equivalent to 500 mg plant powder). At the end of the study, the animals were fasted overnight and then sacrificed under diethyl ether anesthesia according to the guidelines for the care and use of experimental animals and approved by state veterinary administration of University of Tehran. Blood samples and liver tissues were taken from the animals of all groups. Each liver tissue was immediately washed with saline, blotted on filter paper, weighted and was frozen until use. The serum samples were centrifuged at 3000 rpm, 4 °C for 15 min and were stored at -70 °C for biochemical analyses.
Preparation of liver homogenates:
Prior to biochemical analyses, the liver samples were cut into small pieces and homogenized in Tris-HCl buffer (0.025 M, pH 7.5) with a homogenizer to give a 10% (w/v) liver homogenate. The homogenates were then centrifuged at 12000 rpm for 15 min at 4 °C (Beckman). The supernatant obtained was used for biochemical analyses. The protein concentration of each extract was determined by the method of Lowry et al [20] using bovine serum albumin as the standard.

Antioxidant parameters assay:
Mean GPx and GR activities (nmol NADPH oxidized to NADP+ /min/mg protein) were assayed according to the method of Jollow et al. [21] and Paglia and Valentine [22], respectively. CAT activity was measured according to the method of Aebi [23] and was expressed as k/mg protein, where k is the rate constant of the first order reaction of CAT. SOD activity was measured based on inhibition of the formation of amino blue tetrazolium formazan in nicotinamide adenine dinucleotide, phenazine methosulfate and nitroblue tetrazolium (NADH-PMS-NBT) system, according to method of Kakkar et al. [24]. One unit of enzyme activity was expressed as 50% inhibition of NBT reduction / (min mg protein). The mean MDA levels (nmol/mg protein), an index of LPO, were measured by the double heating method of Draper and Hadley [25]. The method is based on spectrometric measurement of the purple color generated by the reaction of thiobarbituric acid (TBA) with MDA. The mean GSH concentration (µg/mg protein) in the liver homogenate was assayed by the method of Jollow et al. [21] and was expressed as µg/mg of protein.

Biochemical analysis:
TG, TC, HDL-C and LDL-C in serum were determined using enzymatic kits (Pars Azmoon, Iran). Atherogenic index was calculated in term of the LDL/HDL cholesterol ratio.

Statistical analysis:
All values are expressed as mean ± S.D. The significance of differences between the means of the treated and untreated groups has been calculated by unpaired Student’s t-test and P < 0.05 was considered significant.
Results

In vitro Antioxidant activity of *A. graveolens* extract:
The total phenolic and flavonoid contents of AGE were determined and expressed in terms of gallic acid and catechin equivalents. The results, as presented in Table 1, show that the total phenol and total flavonoid contents of the AGE are 105.2 mg gallic acid equivalents/g dried extract and 58.2 mg catechin equivalents/g dried extract, respectively. Among the various methods used to evaluate the total antioxidant activity of vegetables or other plants, the ABTS radical cation scavenging and FRAP assay are the most common applied methods. The ABTS assay is based on the inhibition of the absorbance of the ABTS radical cation which has a characteristic long wavelength absorption spectrum. As shown in Table 1, AGE and Trolox, as a positive control, exhibited potent scavenging activity for ABTS radical cation in a concentration dependent manner (not data shown) with EC₅₀ of 38.2 and 6.5 µg/ml, respectively. In still another approach, FRAP method was used to measure the reducing capability of AGE. Based on results, both the crude extract and trolox have the ability of reducing Fe³⁺ to Fe²⁺ by FRAP value of 0.909 mM/mg dried extract and 1.450 mM/mg trolox, respectively (Table 1).

Table 1. Total phenol, total flavonoid, FRAP (EC) value and ABTS radical cation scavenging activity of the crude extract of *A. graveolens* and Trolox.

<table>
<thead>
<tr>
<th>Samples</th>
<th>a Total phenol (mg/g)</th>
<th>b Total flavonoid (mg/g)</th>
<th>c EC value (mM/mg)</th>
<th>ABTS (EC50) (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. graveolens</em></td>
<td>105.20 ± 3.20</td>
<td>58.21 ± 2.80</td>
<td>0.909 ± 0.05</td>
<td>38.2</td>
</tr>
<tr>
<td>extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trolox</td>
<td>-</td>
<td>-</td>
<td>1.450 ± 0.09</td>
<td>6.5</td>
</tr>
</tbody>
</table>

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.
c EC expressed concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM FeSO₄.
Effects of *A. graveolens* extract on lipid parameters:

Feeding HFD for 7 days increased the serum levels of TC, TG and LDL-C among the rats of group II compared to the rats fed normal diet, while the serum level of HDL-C decreased (p < 0.01). As shown in Table 2, administration of AGE (at a single daily dose of 1 ml, equivalent to 500 mg plant powder) to rats for 30 consecutive days reduced the levels of TC, TG and LDL-C by 25.8%, 40.4%, and 31.56 %, respectively, compared to rats fed HFD (p < 0.05). Whereas the level of serum HDL-C increased by 10.9 %.

Table 2. Changes in the serum lipid profile of rats fed normal diet (NC), high fat diet (HFD) and high-fat diet + extract (HFD+AGE).

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HFD</th>
<th>HFD+AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>92.01 ± 5.52</td>
<td>136.80 ± 10.01*</td>
<td>101.53 ± 9.50**</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>83.11 ± 8.70</td>
<td>125.30 ± 9.31*</td>
<td>74.73 ± 5.40**</td>
</tr>
<tr>
<td>LDL(mg/dl)</td>
<td>37.36 ± 4.61</td>
<td>59.33 ± 4.30*</td>
<td>40.61 ± 5.42**</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>25.30 ± 3.50</td>
<td>22.26 ± 1.11*</td>
<td>24.70 ± 1.32**</td>
</tr>
</tbody>
</table>

Values are mean ± SD, for 6 rats.
*Significantly different from Group I (P< 0.01).
**Significantly different from Group II (P< 0.05).

Effects of *A. graveolens* extract on liver antioxidant defense system:

In order to explore the effect of the crude extract on the liver function of the hypercholesterolaemic rats, lipid peroxidation and antioxidant defense system capabilities were evaluated according to methods presented in materials and methods. Fig. 1 shows that MDA level of the liver tissue was significantly decreased upon crude extract therapy in Group III (by 36.0 %), whereas HFD rats (Group
II) showed enhanced levels of lipid peroxidation. As shown in Fig. 2, GSH levels were lower in HFD rats (Group II). However, treatment of the HFD rats with AGE significantly increased the GSH content by 48.1 % (p < 0.01). Table 3 shows the CAT and SOD activities of the liver tissues. CAT and SOD activities of the liver tissues have significantly decreased relative to control group after 5 weeks of high fat diet administration. However, AGE administration resulted in a significant increase in both CAT and SOD activities by 36.4 and 37.5 %, respectively. Table 3 shows the changes in hepatic GPx and GR activities after 5 weeks of HFD consumption. Activities of GPx and GR had significantly decreased compared to rats of the control group. Treatment of rats in group III with AGE resulted in elevation of hepatic GPx and GR activity levels by 42.2 and 29.8 %, respectively.

Table 3. Changes on antioxidant status of liver of rats fed normal diet (NC), high fat diet (HFD) or high-fat diet + extract (HFD+AGE)

<table>
<thead>
<tr>
<th>Antioxidant enzymes</th>
<th>NC</th>
<th>HFD</th>
<th>HFD + AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD (U/mg Protein)</td>
<td>4.74 ± 0.41</td>
<td>3.47 ± 0.31*</td>
<td>4.77 ± 0.32**</td>
</tr>
<tr>
<td>CAT (k/mg Protein)</td>
<td>0.29 ± 0.02</td>
<td>0.22 ± 0.01*</td>
<td>0.30 ± 0.03**</td>
</tr>
<tr>
<td>GPx (nmol NADPH/min/mg)</td>
<td>2.28 ± 0.17</td>
<td>1.54 ± 0.24*</td>
<td>2.19 ± 0.17**</td>
</tr>
<tr>
<td>GR (nmol NADPH/min/mg)</td>
<td>4.64 ± 0.21</td>
<td>3.55 ± 0.33*</td>
<td>4.61 ± 0.10**</td>
</tr>
</tbody>
</table>

Values are mean ± SD, for 6 rats.
*Significantly different from Group I (P< 0.01).
**Significantly different from Group II (P< 0.01).
Figure 1. Effect of *Anethum graveolens* extract (AGE) on hepatic level of MDA in rats fed high-fat diet. Normal diet (NC), high fat diet (HFD) and high-fat diet + extract (HFD+AGE). Values are mean ± SD of 6 rats. *Significantly different from Group I (P< 0.05). **Significantly different from Group II (P < 0.01).

Figure 2. Effect of *Anethum graveolens* extract (AGE) on hepatic level of GSH in rats fed high-fat diet. Normal diet (NC), high fat diet (HFD) and high-fat diet + extract (HFD+AGE). Values are mean ± SD of 6 rats. *Significantly different from Group I (P< 0.05). **Significantly different from Group II (P < 0.01).
Discussion

*Anethum graveolens* has been reported to have a variety of biological effects including antimicrobial, anti-secretory, mucosal protective and hypolipidaemic activity. For the hypolipidaemic and antioxidant effects, scientific data on their efficacy are scarce. In the present study, we examined whether the AGE is capable diminishing the extent of oxidative damage induced by a HFD in rats. Regarding the critical role of ROS in inducing tissue damages and also the initiation of ROS-induced cellular signaling, therapeutic approaches designed to enhance antioxidant defense system may lead to appropriate therapeutic strategy to lessen the incidence of ROS dependent diseases. In recent years, applications of dietary plants with antioxidative property have been the center of focus for improving the life quality of patients with hypercholesterolaemia [26-27]. Hypercholesterolaemia leads to increased production of ROS which may react with polyunsaturated fatty acids in cell membranes leading to lipid peroxidation (LPO) [28]. LPO will in turn result in the elevated production of free radicals. MDA, a secondary product of LPO, is used as an indicator of tissue damage involving a series of chain reactions [29]. An increase in MDA level in animals fed HFD has been observed in the present study. Administration of AGE decreased the rise in MDA level in the treated group, thereby suggesting that AGE may have antioxidant effect and counteract the deleterious effects of HFD to some extent. The biological effects of ROS are controlled in vivo by a wide spectrum of defense elements such as the antioxidant nutrients and enzymes. The most important hepatic detoxification elements are GPx, GR and GSH [30]. GSH is an intracellular tripeptide which constitutes the main reducing source of the cell cytoplasm [31]. GSH is known to protect the cellular system against toxic effects of LPO [32]. In our study, a dramatic rise in liver GSH level was observed among the AGE-treated rats. This probably indicates that AGE can either increase the biosynthesis of GSH or reduce the extent of oxidative stress leading to less GSH degradation, or it may have both effects. In addition, the increase in GPx and GR activities might be responsible for lowered hepatic MDA content, because GPx eliminates not only H$_2$O$_2$ but also other organic peroxide including lipid peroxides [33]. AGE treatment might primarily reduce the hepatic peroxidative activities followed by induction of activities of the GSH-related enzymes, thereby leading to restoration of GSH
content in HFD fed rats. SOD and CAT are the major enzymes dealing with ROS in most cells. Both enzymes play an important role in the elimination of ROS derived from the redox processes of xenobiotics in liver tissues [34]. Different studies have shown that HFD feeding leads to generation of peroxy radicals, (O2-); which inactivates CAT and SOD enzymes [26]. This probably explains the low activity levels of CAT and SOD among the group II rats. It is well-known that flavonoids and polyphenols are natural antioxidant capable of increasing the antioxidant defense capability in terms of CAT and SOD [35]. In rats receiving HFD and AGE (Group III), the activities of CAT and SOD were significantly higher than Group II rats, and very similar to the values noted in normal rats (Group I). The elevated levels of both SOD and CAT with AGE could be due to the influence of flavonoids and polyphenols. Based on this report, it is concluded that AGE possesses in vivo antioxidant activity through decreasing the availability of lipid substances and increasing the activity of antioxidant enzymes, in addition to its hypolipidaemic effects. The observed properties apparently validate the folk medicinal use of this herb. Further efforts are going on in our lab to isolate the bioactive compound(s) and to establish the possible mechanisms of hypolipidaemic and antioxidative activities of AGE.

Acknowledgment

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