Phytochemical contents and enzyme inhibitory and antioxidant properties of *Anethum graveolens* L. (dill) samples cultivated under organic and conventional agricultural conditions

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A B S T R A C T

Inhibitory effect of the *n*-hexane, dichloromethane, ethyl acetate, and ethanol extracts from *Anethum graveolens* L. (dill) cultivated under organic (AG-O) and conventional (AG-C) conditions was tested against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase at 200 μg mL^{-1}. Their antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), *N,N*-dimethyl-*p*-phenylendiamine (DMPD), and nitric oxide (NO) radical scavenging assays as well as ferric ion-chelation capacity, ferric-(FRAP), and phosphomolybdenum-reducing antioxidant power (PRAP). The phytochemical analyses have been performed on both of the plant samples. GC–MS analysis pointed out that α-phellandrene was the main component in both of the essential oils in varying amounts (47.75% for AG-O and 27.94% for AG-C), while oleic acid was the dominant in the fruit oils of two samples (36.39% for AG-O and 53.87% for AG-C). HPLC analysis showed that both of the extracts contained rosmarinic acid as the major phenolic acid. The extracts inhibited BChE at moderate level, while the ethanol extracts exerted remarkable NO scavenging effect. The results emphasize that cultivation conditions may have effect on bioactivity and phytochemical content on plant samples.

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1. Introduction

Anethum graveolens L. (dill) (Apiaceae) is one of the most popular culinary herbs in the world. There have been records on use of this plant for medicinal and edible purposes dating back to the Greek and Egyptian civilizations (Hemphill, 2006). Dill seeds are widely used to give flavor to cakes and pastries, soups, salads, potatoes, meats, and pickles. The leading producers of dill herb are India and Pakistan, while the principal producers of the dill essential oil are USA, Canada, Hungary, and Bulgaria (Small, 2006).

Various pharmacological actions of *A. graveolens* such as antimicrobial, antispasmodic, antidiabetic, antihypercholesteromic, and anti-inflammatory have been reported (Heamalatha et al., 2011). Dill is also cultivated widely in Turkey and consumed in soups and salads and has been used for carminative and stomachic purposes in Anatolian folk medicine (Ugulu et al., 2009). On the other hand, *A. graveolens* was mentioned as "brain tonic" in 17th century in Europe (Stannard, 1982). In our ongoing studies to discover new cholinesterase inhibitors from food or crop plants, we previously reported potent inhibitory effect of the essential oil of *A. graveolens* sample cultivated using organic fertilizer against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Orhan et al., 2008) that are linked to Alzheimer's disease (Orhan et al., 2006). Taking the historical evidence about dill as brain tonic into account, we have herein designed the present study to investigate inhibitory activity of the *n*-hexane, dichloromethane, ethyl acetate, and ethanol extracts prepared from the aerial parts of *A. graveolens* (AG) cultivated under organic (AG-O) and conventional (AG-C) agricultural conditions against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as well as tyrosinase related to Parkinson's disease. Antioxidant activity of the extracts was also assessed by a number of *in vitro* test systems. In this study, we also aimed to compare phytochemical content of two dill samples grown according to organic and conventional agriculture by gas chromatography–mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Plant materials

The sample of *A. graveolens* cultivated under organic conditions (AG-O) was kindly provided by "Nar Organic Agriculture Farm" located in Kocaeli Province (Turkey) in October, 2010. According to the organic agricultural conditions, the field composed of clay soil with half-shade was irrigated only once after the seeds were sown and was not irrigated again and no fertilizer was applied to the field. Organic agriculture conditions and soil analysis of this company were certificated by the Turkish Ministry of Agriculture and Rural Affairs. The other plant sample cultivated using conventional agricultural method (AG-C) was obtained from the Experimental Farm of Faculty of Agriculture, Selcuk University, Konya (Turkey), in August, 2010. In the conventional agriculture method to grow *A. graveolens*, the soil with 18.3% of clay was fertilized with organic manure 15 days before the seeds were sown. In addition to the organic manure, the field was also exposed to inorganic (nitrogenous) manure just before flowering period.

2.2. Extraction procedure

The air-dried and powdered aerial parts of AG-O and AG-C were extracted sequentially with *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and ethanol (EtOH). The extracts were prepared by macerating 5 g of each plant in 100 mL of the mentioned solvents each for 2 days, respectively. The macerates were evaporated *in vacuo* until dryness and kept in freezer until the experiments were performed.

2.3. Isolation of the essential oils

The air-dried aerial parts of AG-O and AG-C were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus to produce the essential oils with the yields of 0.07% and 0.23%, respectively.

2.4. Isolation and derivatization of the fruit oils

The fruits of AG-O and AG-C were independently subjected to continuous extraction technique with *n*-hexane for 8 h using Soxhlet apparatus. The organic phases were evaporated *in vacuo* until dryness to give the fatty oils with the yields of (w/w) 3.79% and 5.01%, respectively. Prior to GC–MS analysis, *trans*-methyl derivatives of the fatty oils were prepared. For that purpose, the oils were separately weighed to 50 mL of volumetric flasks, saponified by addition of 0.5 N methanolic sodium hydroxide (NaOH) (12 mL) to the mixture and heated on a steam bath. Then, 20 mL of borontrifluoride (BF_3)/methanol (Sigma Aldrich, St. Louis, MO, USA) were added into the each flask and the mixtures were boiled for 2 min. After cooling down, the oils were completed up to 50 mL with saturated sodium chloride (NaCl) solution. These mixtures were, then, transferred to separation funnels independently and extracted with 30 mL of *n*-hexane for each. The *n*-hexane phases were taken and evaporated on a water bath at 60 °C. Fatty acid profiles were determined as fatty acid methyl esters (FAMEs) and the FAMEs were dissolved in *n*-hexane for injection and analyzed by GC–MS.

2.5. Enzyme inhibition assays

2.5.1. Cholinesterase inhibition

AChE and BChE inhibitory activity was measured by slightly modified spectrophotometric method of Ellman et al. (1961). Electric eel AChE (Type-VI-S; EC 3.1.1.7, Sigma, St. Louis, MO, USA) and horse serum BChE (EC 3.1.1.8, Sigma, St. Louis, MO, USA) were the enzyme sources used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as the substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic)acid (DTNB; Sigma, St. Louis, MO, USA) was used for the measurement of the anticholinesterase activity. All reagents and conditions were same as described in our previous publication (Orhan et al., 2009). Briefly, in this method, 140 μL of sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of test solution and 20 μL of AChE/BChE solution were added by multichannel automatic pipette (Gilson pipetman, Paris, France) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 μL of acetylthiocholine iodide/butyrylthiocholine chloride. Hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, Sunnyvale, CA, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software (Sunnyvale, CA, USA). Percentage of inhibition of AChE/BChE was determined by comparison of reaction rates of the samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula $(E–S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were run in six parallel sets. Galanthamine (Sigma, St. Louis, MO, USA), the anticholinesterase alkaloid-type of drug obtained from the bulbs of snowdrop (*Galanthus* sp.), was used as the reference.

2.5.2. Tyrosinase inhibition

Inhibition of tyrosinase (TYRO) (EC 1.14.1.8.1; 30 U, mushroom tyrosinase, Sigma) was determined using the modified dopachrome method with *L*-DOPA as substrate (Masuda et al., 2005). The assays were conducted in 96-well microplate using ELISA microplate reader (VersaMax Molecular Devices, USA) to measure absorbance at 475 nm. An aliquot of the extracts dissolved in DMSO with 80 μL of phosphate buffer (pH 6.8), 40 μL of tyrosinase, and 40 μL of *L*-DOPA were put in each well. Results were compared with control (DMSO). α-Kojic acid (Sigma, St. Louis, MO, USA) was used as the reference. The percentage tyrosinase inhibition (*I*%) was calculated as follows:

$$I\% = (\text{Absorbance}_{control} - \text{Absorbance}_{sample})/\text{Absorbance}_{control} \times 100$$

2.6. Antioxidant activity assays

2.6.1. DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA) was determined by the method of Blois (1958). The samples (30 μL) dissolved in ethanol (75%) were mixed with 2700 μL of DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA). Gallic acid (Sigma, St. Louis, MO, USA) was employed as the reference. Inhibition of DPPH expressed as RSA in percent (*I*%) was calculated as given below:

$$RSA\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/reference. Experiments were run in six parallel sets and the results were expressed as average values with SEM.

2.6.2. DMPD radical scavenging activity

The assay is based on reduction of the purple-colored radical DMPD+ (*N,N*-dimethyl-*p*-phenylendiamine). According to the method (Schlesier et al., 2002), a reagent comprising of 100 mM DMPD, 0.1 M acetate buffer (pH = 5.25), and 0.05 M ferric chloride solution, which led to formation of DMPD radical, was freshly prepared and the reagent was equilibrated to an absorbance of 0.900 ± 0.100 at 505 nm. Then, the reagent was mixed up with 50 μL of the extract dilutions and absorbance was taken at 505 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA). Quercetin was employed as the reference and the experiments were done as six replicates. The results were calculated according to the same formula given for DPPH radical scavenging test and expressed as average values with SEM.

2.6.3. Nitric oxide (NO) radical scavenging activity

The scavenging activity of the extracts against NO was assessed by the method of Marcocci et al. (1994). Briefly, the extract dilutions were mixed with 5 mM sodium nitroprusside and left to incubation for 2 h at 29 °C. An aliquot of the solution was removed and diluted with Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the occurred chromophore was measured at 550 nm using a Unico 4802 UV–visible double beam spectrophotometer (USA). Inhibition of NO radical expressed as RSA in percent (*I*%) was calculated as given below:

$$RSA\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts. Experiments were run in six replicates and the results were expressed as average values with SEM. (Standard error of the mean). Quercetin was the reference in this test.

2.6.4. Ferric ion-chelating capacity

The ferric ion-chelating capacity of the samples was estimated by the method of Chua et al. (2008). Briefly, dilutions of the extracts were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ, USA). The ratio of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing only $FeCl_2$ and ferrozine), and A_{sample} is the absorbance of the extracts/reference. Experiments were run in six replicates and the results were expressed as average values with SEM. The reference was employed as ethylenediamine tetraacetic acid (EDTA) in this assay.

2.6.5. Ferric-reducing antioxidant power (FRAP) assay

FRAP of the samples was tested using the assay of Oyaizu (1986). Different concentrations of the extracts were mixed with 2500 μL of phosphate buffer (pH 6.6) and 2500 μL of potassium ferricyanide. Later, the mixture was incubated at 50 °C