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Nano scale injection for determination of chemical composition and antioxidant activity of the essential oils and methanolic extracts from *Foeniculum vulgare Mill.* cultivated in central Iran

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The essential oils and extracts from leaves and fruits of *Foeniculum vulgare* from Kashan area was investigated for its chemical composition by nano scale injection, antioxidant activities by DPPH and β-carotene-linoleic acid assays, and total phenolic content by Folin-Ciocalteu reagent and gallic acid as standard. The essential oils obtained by hydrodistillation, were analyzed by GC and GC/MS. Trans-anethole and limonene were the major components in leaf (78.13%, 12.88%) and fruit (74.12%, 11.81%) oils, respectively. In the DPPH assay, the strongest activity was exhibited by the leaf methanolic extract with an IC_{50} value of 127.13 ± 0.33 μg/mL. In the β-carotene-linoleic acid system, fruit methanolic extract exhibited the highest inhibition (79.97% ± 0.0568) against linoleic acid oxidation. Also, the amount of total phenolics of leaf methanolic extract was higher than fruit ones. In both systems, antioxidant capacities of BHT were also determined in parallel experiments.

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Keywords: Foeniculum vulgare, essential oil composition, trans-Anethole, antioxidant activity, total phenolic content.

1. Introduction

Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, appear to be very important in the prevention of many diseases [1]. Synthetic antioxidants (butylated hydroxyanisol and butylated hydroxytoluene) are being restricted because of their side effects such as carcinogenicity [2]. Fresh and dried aromatic plants are among the most important targets to search for natural antioxidants due to many active substances such as tocopherols, flavanoids, terpenoids, etc. [3,4].

Fennel (*Foeniculum vulgare*) is a plant belonging to the Apiaceae family, native to Europe and the Mediterranean area [5]. This plant has a long history of medicinal use. Typically, fennel and its preparations are used to cure various disorders, acting as a carminative, inflammatory, digestive, expectorant, diuretic agent, nervous disturbances, constipation, dysentery, analgesic, diarrhea, spasmodic and bronchitis [6].

Because of the various uses of *F. vulgare* we have investigated the essential oils and extracts which were separated from dried leaves and fruits of *F. vulgare* from Kashan area and report herein its antioxidant activities by DPPH (2,2-diphenyl-1-picrylhydrazyl) and β-carotene-linoleic acid assay methods. Also, total phenolic content of the extracts were determined as gallic acid equivalent.

2. Experimental

2.1. Plant materials

Leaves and fruits of *F. vulgare* were collected during the fruition period, cultivated in Kashan botany garden (province of Isfahan, Iran), in December 2008. The voucher specimens of the plant were deposited in the herbarium (Voucher No.KBGH 1170) of Research Institute of Forests and Rangelands, Kashan, Iran.

2.2. Preparation of the extracts

A portion (20g) of air-dried and ground leaves and fruits were Soxhlet-extracted, with 350 mL of methanol for 8 h [7]. The extracts were concentrated using a rotary evaporator at a maximum temperature of 45ºC, and dried extracts stored at low temperature (4ºC) until analysis. The yields of dried methanolic extracts for leaf and fruits were 31.1% and 7.49%, respectively.

2.3. Extraction of the essential oils

Dried and ground leaves (50g) and fruits (50g) of *F. vulgare* were subjected to separate hydrodistillation for 3.5 h using a Clevenger-type apparatus [8]. After decanting and drying over anhydrous sodium sulfate, the corresponding yellowish colored oils were recovered from

the leaves and fruits in yields of 0.42% and 0.82% (v/w), respectively.

2.4. GC-MS analysis conditions

2.4.1. GC

GC analysis of the oils was performed on an Agilent HP-6890 gas chromatograph equipped with flame ionization detector (FID) and an HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness, 0.25 µm). The oven temperature was programmed as follows: 50ºC (3 min), 50-130ºC (3ºC min^{-1}), 130ºC (2 min), 130-200ºC (2ºC min^{-1}), 200ºC (3 min) and 200-280ºC (8ºC min^{-1}). Injector and detector temperatures were maintained at 220ºC and 290ºC, respectively. The amount of the sample injected was 1.0 nL (diluted 1.0 µL of sample in 1000 ml of *n*-pentane, v/v) in the splitless mode. Helium was used as carrier gas with a flow rate of 1 mL min^{-1}.

2.4.2. GC-MS

GC-MS analysis of the oils were performed on a Agilent HP-5973 mass selective detector coupled with a Agilent HP-6890 gas chromatograph, equipped with a cross-linked 5% PH ME siloxane HP-5MS capillary column (30 m × 0.25 mm i.d, film thickness, 0.25 µm) and operating under the same conditions as above was described. The flow rate of helium as carrier gas was 1 mL min^{-1}. The MS operating parameters were as follows: ionization potential, 70 eV; ionization current, 2 A; ion source temperature, 200ºC; resolution, 1000.

2.4.3. Compound identification

Essential oils were analyzed by GC and GC/MS systems using a non-polar column and identification of components in the oil was based on retention indices (RI) relative to *n*-alkanes and computer matching with the WILEY 275.L library, as well as by comparison of the fragmentation pattern of the mass spectra with data published in the literature [9,10]. The percentage composition of the samples was computed from the GC-FID peak areas without the use of correction factors.

2.5. Antioxidant activity

2.5.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH, 95%), β-carotene, linoleic acid, 2,6-di-tert-butyl-4-methylphenol (butylated hydroxyl toluene, BHT) and gallic acid were from Sigma–Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol and dimethyl sulfoxide (DMSO), HPLC grade chloroform, standard Folin–Ciocalteu's phenol reagent, anhydrous sodium sulfate, Na_2CO_3 and Tween 40 were from Merck (Darmstadt, Germany). Ultra pure water was used for the experiments.

2.5.2. DPPH assay

The hydrogen atom or electron donation ability of the essential oil and methanolic extract was measured using stable free radical 2,2-diphenylpicrylhydrazyl (DPPH) by a published DPPH radical scavenging activity assay method [11] with minor modifications. It is a widely used reaction based on the ability of antioxidant molecule to donate hydrogen to DPPH which consequently turns into an inactive form. Briefly, stock solutions (10 mg mL^{-1}) of the essential oil, extract and synthetic standard antioxidant butylated hydroxyl toluene (BHT) in methanol were prepared. Dilutions are made to obtain concentrations ranging from 2 to 5×10^{-4} ng mL^{-1}. Two mililiters of various concentrations of each sample in methanol were added to 2 mL of a freshly prepared 80 µg mL^{-1} DPPH methanol solution. After a 30 min incubation period at room temperature, the absorbencies were read against a blank at 517 nm. Inhibition of free radical DPPH in percent (I %) was calculated in the following way:

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

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated form the graph plotted from inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.5.3. β-Carotene/linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Tepe et al. [12] was used with slight modifications A stock solution of β-Carotene/linoleic acid mixture was prepared as follows: 0.5 mg of β-Carotene was dissolved in 1 mL of chloroform (HPLC grade), 25 µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 mL of distilled water saturated with oxygen (30 min, 100 mL min^{-1}) were added with vigorous shaking. The samples (2 g L^{-1}) were dissolved in DMSO and 350 µL of each sample solution was added to 2.5 mL of the previous mixture in test tubes and the emulsion system was incubated in hot water (50°C) for 2 h. The same procedure was repeated with the synthetic antioxidant butylated hydroxytoluene (BHT) as positive control and a blank. After this incubation period, absorbencies of the mixtures were measured at 470 nm. Antioxidant capacities (Inhibition percentages, I %) of the tested solutions were calculated using the following equation:

$$I\% = (\beta\text{-carotene content after 2 h assay/initial } \beta\text{-carotene content}) \times 100$$

Tests were carried out in triplicate. Percent inhibitions of the samples were compared with that of positive and negative standards.

2.5.4. Assay for total phenolics

The total phenolic compounds content in the methanolic extract of *C. iberica* was determined by employing the methods given in the literature [13,14] involving Folin-Ciocalteu reagent and gallic acid as standard. An aliquot (0.1 mL) of extract solution containing 1000 µg extract was added to a volumetric flask, 46 mL distilled water and 1 mL Folin-Ciocalteu reagent was added and the flask was shaken thoroughly. After 3 min, a 3 mL solution of Na_2CO_3 (2% w/v) was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760 nm. The same procedure was repeated for all standard gallic acid solutions (0–1000 mg 0.1 mL^{-1}) and a standard curve was obtained with the equation given below:

Absorbance = 0.0012 × Gallic acid (µg) + 0.0033

Total phenolic constituent of each extract as gallic acid equivalent was determined by putting its measured absorbance at 760 nm in this standard curve and equation. Tests were carried out in triplicate.

3. Results and discussion

3.1. Chemical composition of oils

Table 1 shows the percentage essential oil composition from leaves and fruits of *F. vulgare*. In the leaf oil, 10 components were identified, which made up 99.98% of the total oil. The most abundant constituents were (E)-anethole (78.13%), limonene (12.88%) and estragol (2.67%). On the contrary, 24 components (99.99%) were identified in the oil from the fruit, with anethole (74.12%), limonene (11.81%), fenchone (4.74%) and estragol (2.63%) as major components.

Previous studies have reported the chemical composition of *F. vulgare* fruit essential oils from different localities: India [15], Podgorica region (central south Montenegro) [16], Yugoslavia [17], China [18] and Iran (Tehran) [19]. In general, the profile obtained from the GC analysis of the essential oil from fruits used in this experiment was similar to those described by other authors, although the trans-anethole and fenchone content was slightly different.

These quantitative differences for the constituents of the oil from fruits of *F. vulgare* may be attributed to the differences in environmental conditions, the harvest time and the type of processing followed. Finally, volatile constituents of the essential oils from different parts of *F. vulgare* in different areas by various methods extraction have previously been investigated and often trans-anethole was found as the main component [20,21].

Table 1. Percentage composition of the essential oils from Leaves and Fruits of F. vulgare

Compounds[a]	**RI** [b]	**Leaf oil (%)**	**Fruit oil (%)**
α-Pinene	928	-	1.24
Sabinene	968	-	0.28
Myrcene	998	-	0.52
α-Phellandrene	1000	1.28	0.43
Limonene	1026	12. 88	11. 81
(Z)-β-Ocimene	1035	-	0.36
γ-Terpinene	1054	-	0.18
Fenchone	1084	0.54	4.74
Allo-Ocimene	1127	0.53	1.20
Camphor	1139	-	0.14
Estragole	1196	2.67	2.63
Endo-Fenchyl	1217	-	0.10
acetate	1231	1.90	0.47
Exo-Fenchyl acetate	1252	0.76	0.42
(Z)-Anethole	1294	78.13	74.12
(E)-Anethole	1372	-	0.13
α- Copaene	1386	-	0.06
β-Cubebene	1412	-	0.17
β- Ylangene	1421	-	0.10
β-Copaene	1429	-	0.28
(+)-Aromadendrene	1453	0.29	-
(E)- Nerone	1472	-	0.17
Germacrene D	1517	-	0.09
δ-Cadinene	1576	-	0.18
Globulol	1622	-	0.17
Diphenyl methanone	1836	0.56	-
Neophytadiene			
Total identified		**99.98**	**99.99**

[a] Compounds listed in order of those RI.
[b] RI, (retention index) measured relative to n-alkanes (c_8-c_{32}) on the non-polar HP-5MS column.
[c] %, Relative percentage obtained from peak area

3.2. Antioxidant activity

In this work, two classical antioxidant tests namely DPPH and *β*-carotene/linoleic acid tests were carried out alongside with Folin-Ciocalteu test, which evaluate total phenolic content of the plant extracts. The results of these tests are presented in Table 2. From Table 2, it can be observed that the free radical scavenging activity of the leaf methanolic extract (IC_{50} = 127.13±0.33 µg/mL) was superior to fruit methanolic extract (470.23 ±1.3 µg/mL), but not as well as comprise to BHT (IC_{50} = 19.72 ± 0.8 µg/mL).

In the case of inhibition of linoleic acid assay, leaf and fruit methanolic extracts of the plant were showed an inhibition percentages 73.56% ± 0.028 and 79.97% ± 0.056, respectively, comparable to that of synthetic standard BHT (98.13% ± 0.026), since, leaf and fruit essential oils were indicated an inhibition percentages (3.78% ± 0.032, 10.25 ± 0.074).

Table 2. Antioxidant activity of the essential oils and methanol extracts of F. vulgare and BHT in DPPH free radical-scavenging activity and the β-carotene/linoleic acid bleaching assay methods.

Sample	$IC_{50,}$ [µg/mL]	β-carotene/linoleic acid inhabitation (%)
Leaf extract	127.13 ± 0.33	73.56 ± 0.028
Fruit extract	470.23 ± 1.3	79.97 ± 0.056
Leaf Essential oil	ND [a]	3.78 ± 0.032
Fruit Essential oil	ND [b]	10.25 ± 0.074
BHT	19.72±0.8	98.13 ± 0.026
Negative control	NA[c]	6.25 ± 0.044

a Less than 8% inhibition at 10mg/mL (ND = Not Determined).

b Less than 6% inhibition at 10mg/mL (ND = Not Determined).

C Not applicable.

Peroxy radicals usually initiate lipid peroxidation by abstraction of an allylic or benzylic hydrogen atom from the molecule under oxidation [22]. Thus, the possible presences of allylic and/or benzylic hydrogen containing secondary metabolites in the above mentioned extracts, establish considerable antioxidant activity in the *β*-carotene/linoleic acid test.

The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports [23], thus, total phenolic compounds of methanolic extracts were determined as gallic acid equivalent, results are given in Table 3. These results indicated that low total phenolic constituent of the leaf and fruit methanolic extracts (27.31±0.78, 11.36 ±1.23 µg/mg, respectively), presented relatively low antioxidant activity these extracts in DPPH assay, when compared to BHT, a synthetic antioxidant.

Table 3. Amounts of total phenolic compounds in F. vulgare extracts.

Extracts	Gallic acid equivalents (µg/mg)
Leaf methanolic extract	27.31 [a] ± 0.78 [b]
Fruit methanolic extract	11.36 [a] ± 1.23 [b]

Result is given as mean ± standard deviation of three different experiments.
[a] Value are the means of three replicates
[b] Standard deviation

Literature survey indicated that there are many reports on the antioxidant activity and total phenolic content of *F. vulgare* [24-27]. But no report on the chemical composition and antioxidant activity of the *F. vulgare* from Kashan area and this is the first one.

4. Conclusions

Growing tendency for replacing synthetic additives by natural ones has emerged great interest on the evaluation of antioxidant properties of plants products in both academia and the food industry. The antioxidant results observed in this investigation indicated that the leaf and fruit methanolic extracts of this plant have potent antioxidant property on DPPH and inhibition of linoleic acid assays, respectively, and may have potential as a natural additive in food and pharmaceutical industry.

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