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ORIGINAL ARTICLE

Phytochemical, Antibacterial, Antifungal and Antioxidant Properties of *Agastache foeniculum* Essential Oil

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KEYWORDS

Agastache foeniculum; Essential oil; Phytochemicals

ABSTRACT: Phytochemicals are good sources of natural antioxidants and have beneficial effects on human health. Many of phenolic phytochemicals have shown antimicrobial and antifungal activity. *Anise hyssop* (*Agastache foeniculum [Pursh] Kuntze*) is a perennial aromatic plant of the Lamiaceae family with antimicrobial and antifungal properties and useful for gastrointestinal problems. In this investigation, the antioxidant activity of extracts, their antimicrobial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *B. subtilis*, *Salmonella thyphimurium*, *S. enteritidis*, *Escherichia coli* strains and their antifungal activity against *Aspergillus niger* and *A. flavus* was evaluated**.** Seven components were identified, representing 95.4% of the oils including methyl chavicol (83.1%), limonene (3.4%), spathulenol (3.1%) and caryophyllene oxide (3.1%). Agar disk diffusion and broth micro-well dilution assays showed that *B. subtilis* was the most resistant strain against both of the EOs and *E. coli* was the most sensitive bacteria. Results of both disc diffusion and MIC showed that the EO was more effective against *A. flavus* than *A. niger*. Antioxidant activity of *A. foeniculum* by DPPH and ABTS assays revealed remarkable antioxidant activities of this EO comparing with BHT. Results of current study indicated that *A. foeniculum* EOs can be used as a food preservative in having antibacterial, antifungal and antioxidant activity for the control of food deterioration.

INTRODUCTION

Essential oils are known as natural, volatile compounds having extreme odor. Aromatic plants produce these compounds and they have recently attracted much attention due to their use in food

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preservation and shelf life prolongation of foods. Their secondary metabolites such as flavonoids, alkaloids and terpenoids are the main reason of their biological properties.

The composition of EOs' secondary metabolites which cause different antimicrobial, antioxidant and other biological properties could be affected by many factors such as genotype, environmental and climate conditions, geographic location, cultivation and harvest time, vegetative stage or plant maturity, growing season, distillation method and storage condition[1, 2]. Besides, the concentrates from various parts of the plants, for example the flowers and the leaves are used to get the EOs. Such EOs have different components with various usefulness and properties [3, 4].

Anise hyssop (*Agastachefoeniculum [Pursh] Kuntze*) is a perennial aromatic plant of the Lamiaceae family grown for its pharmaceutical, culinary and ornamental values [5]. It is native to the Great Plains of the United States and Canada [6] and is cultivated in the Mediterranean region, north and central Europe. It has an anise-like scented essential oil essentially biosynthesized in the leaves and blossoms of the ornamental plant [7]. The leaves and the flowers of this plant are used in herbal tea, cakes, sweets, salads and desserts; the leaves are used in treating cardiac problems, chest pain, inducing sweating to reduce fever and fight against cold [6]. Anise hyssop has valuable source of nectar for honeybee forage. The essential oils have antimicrobial and antifungal properties and they are used for gastrointestinal problems [8].

The aim of this study was to investigate the antioxidant and antimicrobial properties of *A. foeniculum* EO.

MATERIALS AND METHODS

Plant material and essential oil extraction

The areal parts of Anise hyssop were harvested at the flowering stage from plants grown in the experimental farm at the Department of Horticulture, Faculty of Agriculture, Urmia University, Urmia, Iran, and were air-dried in the shade at room temperature. The essential oils in dried and powdered samples (25gr) were isolated by hydro-distillation for 3 h using a glass Clevenger type apparatus. The extracted yellow-colored essential oils were dried over anhydrous sodium sulfate and were kept at refrigerator (4ºC) in sealed dark glass vials until analysis.

Essential oil analysis

The essential oils constituent was determined by GC and GC/MS analysis. GC analysis was carried out on a Shimadzu 9A gas chromatograph, equipped with a Ph-5 column (30 m × 0.1 mm, film thickness 0.25 μm). Oven temperature was held at 60 ºC for 5 min, then programmed to 210 ºC at a rate of 3 ºC/min and finally was increased with the rate of 20 ºC/min. to 240 ºC and was kept constant at 240 ºC for 8.5 min. The injector and detector's(FID) temperature were 280 ºC and helium (with 99.999% purity) was used as carrier gas with a linear velocity of 31.5 cm/s, split ratio 1/60, ionization energy 70 eV.

The data was calculated by electronic integration of FID peak area without using any response correction factor. GC/MS analysis was also carried out on a Varian 3400 GC/MS system, equipped with a DB-5 fused silica column (30 m × 0.25 mm, film thickness 0.25 μm). The oven temperature was 50 – 280 °C with the rate of 4 °C/min. The mass spectrums obtained were compared to those of the database (Wiley 229), and the Kovats retention index (KI) calculated for each peak was compared to the values described earlier [9, 10].

Evaluation of antibacterial properties

Preparation of Bacterial strains

The *A. foeniculum* essential oil was assessed against seven standard strains of pathogenic bacteria among which 4 Gram-positive and 3 Gram-negative bacteria have been identified. The bacte-

ria were set up as lyophilized cultures utilizing the culture collection of Food Hygiene Department, Faculty of Veterinary Medicine, University of Urmia, and Urmia, Iran. The microorganisms selected for this study were as follows; *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 19118, *Bacillus cereus* BC 6830, *B. subtilis* ATCC 6633, *Salmonella typhimurium* ATCC 13311, *S. enteritidis* ATCC 13076 and *Escherichia coli* ATCC 43894 strains. The density of the bacterial suspensions was set spectrophotometrically over 10^8 cells.mL^{-1}, equivalent to 0.5 of McFarland' scale.

Disc diffusion method

Sterile paper disks (6mm diameter) were impregnated with 20 µL of two concentrations (10 and 15 mg.ml^{-1}) of *foeniculum* and ofloxacin and were placed on the surface of the nutrient agar plates inoculated with 0.1 ml of the bacterial cultures (1.5×10^6 cfu.ml^{-1}) under aseptic condition. Plates were incubated at 37 °C for 24 h and the diameters of inhibition zones were measured by making use of a caliper [11]. Vancomycin and Ampicillin antibiotic disks were used as control positives as well.

Microdilution method

The use of micro-well dilution assay leads to the determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the EOs against the tested bacterial strains. The preparation of the bacterial suspensions in the log phase conformed to the 0.5 McFarland standard turbidity and it was serially diluted (1:10) to achieve the desired concentration (1.5×106 cfu.ml^{-1}). The essential oils were dissolved in 10% dimethyl sulfoxide. Then, the solutions were diluted to the highest concentration (40 mg.ml^{-1}) as a stock solution and later serial two-fold dilutions were made in a concentration range from 0.62 to 40 mg.ml-1 in a nutrient broth. Briefly, 160 µL of the nutrient broth, 20 µL of the inoculums and 20 µL of the EOs were added into one of the 12 wells. The wells with no bacteria were considered negative and wells with no EOs were considered as positive controls. The microplates were mixed gently and were incubated at 37 °C for 24 h. The final volume in each well was 200 µl, the final concentrations of EOs were in a range between 0.062 to 4 mg.ml^{-1} and the final bacterial suspensions in the wells were approximately 1.5×105 cfu.ml^{-1}. The lowest concentration with no visible bacterial growth was regarded as MIC values of the EOs. The MBC values were determined by inoculating 10 µL of none turbid wells on BHI agar and the lowest concentration with no visible bacterial growth on the agar was regarded as MBC values of the EOs [12].

Evaluation of antifungal properties

Preparation of fugal suspensions

A solution containing 0.5% (v/v) Tween 80 was used to wash the fungal spores from the surface of PDA plates slowly. A sterile saline solution including 0.5% (v/v) Tween 80 adjusted the spores, counting by Neubauer slides, and the suspensions to a concentration of approximately 10^6 spore.ml^{-1}. To verify the validity of each inoculum, dilutions of the inocula were cultured on PDA medium [11].

Disc-diffusion assay

The essential oils were dissolved in methanol to a final concentration of 1, 2.5, 5 and 10 mg.ml^{-1} and subsequently were sterilized by 0.45 µm Millipore filters. Then 100 µl of each fungal suspension (10^6 spore.ml^{-1}) was spread on a PDA plate. The discs (6 mm in diameter) were impregnated with 10 µl of each essential oil concentrations (10, 25, 50 and 100 µg/disc) and were placed on the inoculated PDA plates. Negative controls were prepared using methanol without the essential oil. In order to determine the sensitivity of each fugal species tested, the inoculated plates were incubated at

28˚C for 72 h and Netilmicin (30 µg/disc) was used as positive control. Antimicrobial activity was evaluated via measuring the inhibition zone against the tested organisms [13].

Microdilution assay

The effect of MIC and MFC was determined against *Aspergillus niger* and *A. flavus* using a broth micro-well dilution method [14, 15]. The aim of the serial two-fold dilutions of the essential oils was to get to a concentration ranging from 50 to 1600 µg.ml-1. Aliquots of 20 µL of spore suspension (10^6 spore.ml^{-1}), 160 µL of PD broth were distributed into the 96-well micro plates. In order to reach a final volume (200 µL per well), equal amount (20 µL) of the both of the concentration of EOs was added to the wells. The final concentration of spore suspensions and the essential oils were about 10^5 spore.ml^{-1} and 25 to 800 µg.ml^{-1} respectively. Both positive controls (180 µl of PD broth + 20 µl of inoculums) and negative controls (180 µl of the uninoculated PD broth + 20 µl of EOs) were included in the last wells. When the incubation period (72 h at 28 °C) was over, the definition of the lowest concentrations with no visible growth was the concentrations that completely inhibited fungal growth (MIC values). The MFC values were determined by serial subculturing of 5 µL of the wells without any visible growth into PDA plates and further incubation was done for 72 h at 28°C. MFC values are the lowest concentrations without any visible growth on PDA plates.

Evaluation of antioxidant properties

DPPH assay

The bleaching of the purple methanolic solution of DPPH was a measure for the electron donation ability and hydrogen atoms of the EOs. 50µl of EOs in methanol (1, 2.5, 5 and 10 mg.ml^{-1}) was added to 2 ml of methanolic solution of DPPH (24µg.ml^{-1}). After 1 h of incubation at room temperature, the absorbance was read against a blank at 517 nm using a spectrophotometer (LKB Novaspec II; Pharmacia, Uppsala, Sweden) and radical scavenging activity (RSA) of the EOs was calculated using the following equation:

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

A blank is the absorbance of the control reaction (including all reagents except the test compound) and A sample is the absorbance of the test compound (EOs). Different concentrations of butylated hyroxytoluene (BHT) were used as positive control of reference synthetic antioxidant [14].

ABTS

ABTS+ radical cation decolorization assay is used to measure the total antioxidant activity of Eos [16]. Potassium persulphate solution (2.45 mmol.L^{-1}) and ABTS solution (7 mmol.L^{-1}) in distilled water were separately prepared and reacted together. The mixture was kept in the dark at room temperature for 16 h, while the ABTS+ solution was diluted with phosphate buffer saline (PBS) to an absorbance of 0.70 at 734 nm and 30 °C. Aliquots of 200 µL of various concentrations (1, 2.5, 5 and10 mg.ml^{-1}) of the EOs in methanol along with the reference antioxidants (ascorbic acid and BHT) were added to 2 ml of ABTS+ solution and were mixed severely. Tubes were incubated for 6 min at room temperature and then read at 734 nm and the percentage of inhibition was calculated as follows:

$$\text{Inhibition } (\%) = [(A_{Control} - A_{Extract})/A_{Control}] \times 100$$

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS, Version 18.0 (Chicago, IL, USA). Turkey's test was used to compare the differences among mean values ($P< 0.05$). All experiments were carried out in triplicate.

RESULTS

Chemical composition of the EO

The identified essential oil components and their percentage are shown in Table 1. In total, seven components were identified, representing 95.4% of the oils. The main constituents of the essential oils were methyl chavicol (83.1%), limonene (3.4%), spathulenol (3.1%) and caryophyllene oxide (3.1%).

Table 1. Constituents of the essential oil of *A. foeniculum* by GC/MS analysis.

Compound	RI	(%)
Methyl chavicol	1119	83.1
Limonene	1029	3.4
Spathulenol	1577	3.1
Caryophyllene oxide	1581	3.1
β-gurjunene	1434	1.7
n-decanal	1270	0.3
1-octen-3-ol	979	0.7
Total identified		95.4

Antibacterial properties

The results of in vitro antibacterial activity of *A. foeniculum* and *Ofloxacin* EOs against the tested food-borne bacteria strains were assessed by agar disk diffusion and broth micro-well dilution assays; shown in Tables 2 and 3 respectively. As it can be seen in Table 2, *B. subtilis* was the most resistant strain against both of the EOs (*A. foeniculum* and *Ofloxacin*) and the highest sensitivity to the tested concentrations of the EOs is pertaining to *E. coli*.

MIC and MBC values of the EOs against tested bacterial strains are shown in Table 3. The highest MIC and MBC values of *A. foeniculum* EO were observed against *E.coli* with values (MIC: 500(µg/mL), MBC: 1000(µg/mL)). *E. coli* was the most resistant bacteria to the *Ofloxacin* with similar MIC and MBC values (250 µg/mL).

Table 2. Results of the antibacterial tests of *A. foeniculum* essential oil by disc diffusion test

Bacterial strains	Mean diameter ± SD of inhibition hole in mm	
	A. foeniculum	**Ofloxacin**
S. aureus $^{(a)}$	16.33 ± 0.88^{cdefg}	18.67 ± 1.2^{g}
E. coli $^{(b)}$	9 ± 0.58^{defg}	10.33 ± 1.2^{g}
B. cereus $^{(c)}$	14.67 ± 0.33^{adefg}	13.33 ± 0.88^{efg}
B. subtilis $^{(d)}$	19.33 ± 1.85^{abc}	24 ± 1.53^{g}
S. enteritidis $^{(e)}$	12.67 ± 1.2^{abc}	13.67 ± 0.33^{c}
S. typhimurium $^{(f)}$	11 ± 1.15^{abc}	11.33 ± 0.67^{c}
L. monocytogenes *(g)*	15.67 ± 1.2^{abc}	20.33 ± 0.88^{abcd}

Lowercase letters in table are significant difference (< 0.05) between mean diameters for bacterial strains.

Table 3. Determination of MIC and MBC value (µg/mL) of *A. foeniculum* essential oil against pathogenic bacterial strains

Bacterial strains	MIC (µg/mL)		MBC (µg/mL)	
	A. foeniculum	Ofloxacin	*A.foeniculum*	Ofloxacin
S. aureus	125	31.25	125	31.25
E. coli	500	250	1000	250
B. cereus	125	125	250	125
B. subtilis	62.5	31.25	125	62.5
S. enteritidis	250	62.5	250	125
S. typhimurium	250	125	500	250
L. monocytogenes	125	31.25	250	31.25

Antifungal properties

The antifungal properties of EO were evaluated by the disc diffusion assay in parallel with the broth micro-dilution technique against two food-borne fungal strains (*A. flavus* and *A. niger*). The results presented in Table 4 indicate that the EO extracted from the flower part of *A. foeniculum* exhibited variable degrees of antifungal activity.

The MIC values of EO against *A. niger* and *A. flavus* were 400 and 800 ppm respectively (Table 5). According to MFC values, the *A. foeniculum* EO showed a notable fungicidal activity with MFC values between 400 to 800 ppm. The disc diffusion results of EO against fungal strains have shown a close agreement with MIC results and it has revealed that the EO was more effective against *A. flavus* than *A. niger*. The diameter of inhibition zone against *A. niger* and *A. flavus* was dose-dependent and had improved from 12 and 13.2 mm to 22.67and 25.33 mm respectively for the EO, following the concentration increment from 10 µg/disc to 100 µg/disc. According to MFC values, *A. flavus* is more resistant than *A. niger*.

Table 4. Antifungal properties of *A. foeniculum* EO using agar disc diffusion method

Essential oil	Fungus/concentration	1	2.5	5	10
A. foeniculum	***A. flavus***	13,2±1	0.88±18/67	0.67±21/33	25,33±1,2
	A.niger	12±0.78	15.3±0.3	19.67±1,2	22.67±0.67

Table 5. Antifungal properties of *A. foeniculum* EO using microdilution assay

Essentional oil	Fungus	MIC(ppm)	MFC(ppm)
A. foeniculum	***A. flavus***	200	400
	A.niger	400	800

Antioxidant properties

DPPH assay was used to indicate and compare antioxidant activity of EO extracted from the flowers of *A. foeniculum*. The EO has followed a dose dependent pattern for DPPH radicals scavenging. Although this EO had a notable scavenging ability especially at higher concentrations, it was significantly lower than BHT (P> 0.05). As shown in Table 6, scavenging ability of DPPH radicals was: BHT > EO and the values at highest concentration (10 $mg.ml^{-1}$) were 99.9 and 93.5% respectively.

The EO was compared for its antioxidant potency by ABTS+ decolorization assay as well. Similar to DPPH assay results, the percentage of inhibition (%) of ABTS radicals for the flower oils was lower than BHT (Table 7). The ABTS results for BHT

and the EO at highest concentration (10 mg.ml^{-1}) were 99.4 and 92.1% respectively (P> 0.05).The findings of this study are in agreement with the remarkable antioxidant activities of this EO.

Table 6. DPPH results of *A. foeniculum* EO.

EO/concntration	1	2.5	5	10
A.foeniculum	30.8±3.4	43.7±3.6	81.4±4.5	93.5±4.5
BHT	89.3±0.24	93.5±0.02	97.7±0.05	99.9±0.01

Table 7. ABTS results of *A. foeniculum* EO.

EO/concentration	1	2.5	5	10
A.foeniculum	44.3±2.2	52.3±1.8	83.7±1.2	92.1±0.02
BHT	85.34±0.1	89.6±0.06	95.1±0.02	99.4±0.01

DISCUSSION

Seven components of *A. foeniculum* essential oils were identified, with the principal compounds being methyl chavicol (83.1%), limonene (3.4%), spathulenol (3.1%) and caryophyllene oxide (3.1%). Mazza et al. detected over 50 compounds in the oils, which only ten constituents, however, accounted for more than 0.1% and methyl chavicol constituted 95-98% of the essential oil. Other essential oil compositions of *A. foeniculum* were camphen, p-cymene, β -pinene and α –guaiene [17]. In another study, four major constituents of *A. foeniculum* essential oil were methyl chavicol (94.003%) 1,8-cineole (3.334%), 1-octen-3-ol (0.461%) and germacrene D (0.430%)[18]. Both studies showed similar results with current investigation and identified methyl chavicol as the main constituent.

Studies have demonstrated some medicinal effects of herba *Agastache* such as antimicrobial, antiviral, anti-mutagenic, antiproliferative, antiatherogenic, anticancer, anti-inflammatory and antioxidant properties [19, 20]. In the present study, through agar-well diffusion and broth microdilution methods, *A. foeniculum* essential oils had in vitro antifungal activity against *A. niger* and *A. flavus*. The disc diffusion results of EO against fungal strains showed a close agreement with MIC results and revealed that EO was more effective against *A. flavus* than *A. niger*. Blaszczyk et al. screened the extracts of 56 widely used dried Chinese medical plants or their parts (TCD) for their antimycotic properties against pathological phyla of *A. fumigatus*, *Candida albicans*, *Geotrichum-candidum* and *Rhodotorularubra*. The highest activity against *Geotrichum candidum* was shown by *Agastache rugose*, another species of *Agastachea* family [21]. Ownagh et al. studied the growth inhibition of *Thyme*, *Agastache* and *Satureja* against *A. fumigatus*, *A. flavus* and *Fusarum solani* through disc diffusion method. Their results showed that all three examined essential oils, had antifungal effects against three fungi species [22]. The antimicrobial activity of the essential oils is dependent upon their major components [23]. Cell leakage induced by methyl chavicol and linalool was far greater as compared to that induced by AmB, a commonly used antifungal drug [24].

The results of in vitro antibacterial activity of *A. foeniculum* EO against tested food borne bacterial strains were also assessed by agar disk diffusion and broth micro-well dilution assays in the current study. *B. subtilis* was the most sensitive strain against *A. foeniculum* while the lowest sensitivity to the tested concentrations of the EO belonged to *E. coli*. Results of MIC and MBC values also showed that *E.coli* was the most resistant bacteria against the EO .

As far as we are aware, there have been no previous studies on the potential antibacterial effect of

A. foeniculum, therefore, it is not possible to make any comparisons with other similar studies. Depo et al. evaluated the antibacterial activity of *Agastache rugoa*, another species of *Agastachea* family, against 16 strains of skin bacteria in which the EO showed poor inhibition effect [25].

DPPH and ABTS assays were used in order to determine antioxidant activity of *A. foeniculum*. The percentage of inhibition (%) of ABTS radicals for the flower oils was lower than BHT and was 99.4 and 92.1% in highest concentration of EOs (10 mg.ml-1) respectively. Of the dried culinary herbs tested by Dragland et al., which assessed the contribution of culinary and medicinal herbs to the total intake of dietary antioxidants, *A. foeniculum* contained lower concentrations of the antioxidants (i.e., 34.4 mmol/100 g) in comparison with other herbs [26]. Dapkevicius et al. examined the various potentialities of some aromatic herbs grown in Lithuania as a source of natural antioxidants by the b-carotene bleaching test. Among the herbs, thyme and sage acetone oleoresins showed higher antioxidant activity than other herbs such as *A. foeniculum*[27]. Methyl chavicol, as major component was the important source of antioxidative [28] and antibacterial properties of *A. foeniculum* in this study.

CONCLUSIONS

The essential oil of *A. foeniculum* aerial parts and its main constituent compound; methyl chavicol, could be regarded as a promising preservative in food industry in terms of having antibacterial, antifungal and antioxidant activity for food deterioration control. However, in vivo studies must be performed to confirm this efficacy in vitro.

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Conflict of interest

None.

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