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Chemical Composition and Bioactivity of Essential Oils of Seed and Leaf from *Foeniculum vulgare* Mill cultivated in Southeast of Morocco

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Abstract

Analysis of essential oils obtained from the seeds and leaves of *Foeniculum vulgare* Mill. cultivated in southeast of Morocco revealed that trans-anethole was the major constituent (54.67% in seed oil and 50.60% in leaf oil), followed by estragol (35.33% in seed oil and 30.15% in leaf oil). Other components present in leaf oil included fenchone, linalool and α-pinene, while seed oil contained fenchone, limonene and γ-terpinen. Besides, minor constituents like camphene, camphor, myrcene, pahllandrene, fenchyl acetate and β-bisabolene were obtained from seeds and leaves.

The essential oils of the seeds and leaves of *Foeniculum vulgare* were assayed for their antioxidant and antimicrobial activity. The seed oil show remarkable antioxidant effect, while the leaf oil exhibits a strong broad spectrum antibacterial activity against all test organisms.

Keywords: *F. vulgare*, essential oil, antibacterial, antioxidant, chemical composition.

1. Introduction

Foeniculum vulgare Mill. (Umbelliferaceae). commonly known as fennel, is a small genus of annual, biennial or perennial herbs. It is widely cultivated for its aromatic fruits, which are used as culinary spices. Herbal drugs and essential oil of fennel have antispasmodic, diuretic, anti-inflammatory, analgesic and antioxidant effect (Ebbed et al., 2010, Choi & Hwang, 2004; Misharima & polshkov, 2005; parejo *et al.*, 2002, Pardhan et al., 2008). The volatile oil showed antioxidant, antimicrobial and hepatoprotective (Toma et al., 2008; Ozbek et al., 2003).

In this study, essential oil of *Foeniculum vulgare* seeds and leaves were assayed for their antioxidant properties and antimicrobial activity.

The chemical compositions were studied by GC/SM analysis. The antioxidant capacity was quantified using 2,2-diphenyl-1-picryhydrazyl (DPPH) radical, chelating effect on Fe^{2+} ions and reducing actions assays and the β-carotene bleaching test. The antibacterial activity was determined by the disk diffusion and agar dilution assays.

2. Material and methods

2.1. Plant material

The seeds and leaves of *Foeniculum vulgare* were collected from Errachidia region (southeast of Morocco). The voucher specimens have been deposited at the Biochemistry of Natural Products Laboratory, Department of Biology, Faculty of Sciences & Techniques, Errachidia, Morocco.

2.2. Hydrodistillation apparatus and procedure

The essential oils were obtained by hydrodistillation method from fresh leaves and seeds collected from the plants grown in Errachidia region during juin 2013.

The yield of essential oil obtained from the seeds (SO) and leaves (LO) was found to be 2.8% and 2% respective. The obtained essential oil was dried over anhydrous sodium sulfate and after filtration, stored at + 4°C until tested and analyzed.

2.3. Essential oil analysis

Components were identified on the basis of gas chromatography-mass spectrometry (GC-MS) library and confirmed by calculation of retention indices from GC-FID. GC-MS was performed on a GC 6890 Agilent equipped with an HP-INNOWAX capillary column (50m x 0.2 mm; film thickness 0.5 µm). Carrier gas: Helium 1.6 ml/min, split 1/100; injector temperature: 280°C; oven temperature: 60°C (2 min isothermal) then 3°C/min to 180°C, then 8°C/min to 245°C (10 min isothermal). MS 5973 N Agilent; source temperature: 230°C; mass range: 35 to 350 amu; scan speed: 1 scan/sec. GC-FID: Fast GC HP- 6850 equipped with a DB-WAX capillary column (20 m, 0.1 mm, 0.2 µm). Carrier gas H2 at 0.7 ml/min, split 60 ml/ min. Injector temperature 275°C; Detector temperature 275°C; oven temperature 60°C (2 min isothermal) then 12°C/min to 248°C (5min

isothermal). Detected compounds concentrations are relative percentages (ISO7609), with a threshold of 0.05%.

2.4. Antibacterial activity

Microorganisms

The antimicrobial activity was evaluated by paper disc diffusion and dilution methods against four selected Gram-positive and Gram-negative species: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus cereus* (ATCC 29213). Microorganisms were obtained from the culture collection of the Institute of Hygiene (Rabat).

Diffusion method

The qualitative antimicrobial essay of the volatile fraction of *Foeniculum vulgare* was carried out by the disc diffusion method (NCCLS, 1999). It was performed using culture growth at 37°C for 18h and adjusted to approximately 10^8 colony forming unit per milliliter (CFU/ml). The culture medium used for the bacteria was Mueller Hinton Agar (MHA). Five hundred microliters of the inoculums were spread over plates containing MHA and a Whatman paper disc (6 mm) impregnated with 5, 10, 15µl of the volatile fraction was placed on the surface of the media. The plates were left 30min at room temperature to allow the diffusion of the oil. They were incubated 24h at 37°C for the bacteria. After incubation period, the inhibition zone obtained around the disc was measured. Two controls were also included in the test, the first was involving the presence of microorganisms without test material and the second was standard antibiotic: Ampicillin used to control the sensitivity of the tested bacteria. The experiments were run in triplicate, and the developing inhibition zones were compared with those of reference discs.

Dilution method

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of tested volatile fractions were determined using the Mueller Hinton broth (MHB) dilution method (NCCLS, 1999). All tests were performed in MHB supplemented with Tween 80 (1%). Bacterial strains were cultured overnight in MHB at 37°C. Tubes of MHB containing various concentrations of volatile fractions were inoculated with 10 µl of 10^8 CFU/ml of standardized microorganism's suspensions. Control tubes without tested samples were essayed simultaneously. All samples were tested in triplicate. The MIC was defined as the lowest concentration preventing visible growth (May et al., 2000; Burt, 2004).

2.5. Antioxidant activity

The antioxidant activity was assessed by 2,2-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene bleaching method systems. Data collected for each assay was an average of three experiments.

Free radical-scavenging assay

The method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant. DPPH solutions show a strong absorption band at 517 nm with a deep violet color. The absorption vanishes and the resulting discoloration is stochiometric with respect to degree of reduction. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant (Shahidi, 2001). 50µL of the extracted oil dilutions in ethanol was added to 1mL of 100 µM solution of DPPH. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517nm (Jenway UV/Vis 6000). Inhibition of DPPH free radical in percent (I %) was calculated as follows:

$$I(\%) = (1 - Asample/Ablank) \times 100$$

- Ablank is the absorbance of the control reaction (containing all reagents except the test compound);
- Asample is the absorbance of the test compound. Extract concentration providing 50% inhibition;
- IC50 was calculated from the graph plotting inhibition percentage against extract concentration.

All tests were carried out in triplicate. The BHT was used as positives control.

β-Carotene bleaching assay

The β -carotene method was carried out according Shahidi (2001), two milliliters of β -carotene solution (0.2 mg ml^{-1} in chloroform) were pipetted into a round-bottomed flask containing 20 µl linoleic acid and 200 µl Tween 20. The mixture was then evaporated at 40°C for 10min to remove the solvent, the addition of distilled water (100 ml) followed immediately. After agitating the mixture, 1.5ml aliquot of the resulting emulsion was transferred into test tubes containing 150µl of extract and the absorbance was measured at 470 nm against a blank consisting of an emulsion without β -carotene. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored by measuring absorbance at 470 nm after two hours using spectrophotometry. The same procedure was repeated with the synthetic antioxidant, butylated hydroxytoluene (BHT) as positive control. The antioxidant capacity (AA%) of the solutions tested was calculated via the following formula:

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

2.6. Statistical analysis

Results are presented as mean ± SD of three independent tests. All tests were carried out in an identical

condition.

3. Results and discussion

Chemical Composition of the Essential Oil

The yellowish oils isolated by hydrodistillation from the leaves and the seeds of *F. vulgare* were obtained in yield of 2% and 2.8% (w/w) respectively. The essential oil was analysed by means of GC-MS. The components of the oil, the retention times (RT) the percentage constituent (%) are summarized in Table 1. The number of identified compounds was 24 in the seeds and 26 in the leaves, representing 97.05% and 98.16% of the total composition of the two organs. Trans anethole (50.60%), estragole (30.15%), fenchone (4.32%), linalool (2.83%), α-pinene (2.56%) and γ-terpinene (2.02%) were the main components for the leaf oil, comprising 92.48% of the oil. Trans anethole (54.67%), estragole (35.33%) and α-pinene (2.01%) were the main components for the seed oil, comprising 92.01% of the oil. The components present in the essential oil obtained from seeds and leaves of fennel cultivated in southeast of Morocco are similar to those reported for sweet fennel but the relative percentage of compounds such as anethole, estragole and fenchone differed (Chowdhury et al., 2009; Ebeed et al., 2010, Raal et al., 2012). It may be attributed to different factors such as geographical environment, growth season and physiological age of the plant besides the method of oil isolation.

Table 1. Essential oil Composition of *Foeniculum vulgare* Mill. cultivated in southeast of Morocco.

Rt [b]	Compounds [a]	Leaf oil	Seed oil
4,08	α-thujone	0,21	0,21
8,49	**α-pinene**	**2,56**	**2,01**
9,95	camphene	0,2	0,32
10,56	sabinene	0,29	0,29
10,95	β-pinene	0,3	-
11,68	mycerene	0,03	0,06
11,82	Δ-3-carene	0,61	0,61
12,25	α-terpinene	0,4	0,06
12,92	p-cymene	0,22	0,23
13,04	**limonene**	**1,2**	**0,4**
13,84	**γ-terpinene**	**2,02**	**1,2**
13,95	**fenchone**	**4,32**	**0,06**
14,46	**linalool**	**2,83**	**1,09**
15,9	camphor	0,27	0,98
16,04	terpinen-4-ol	0,3	0,03
16,84	methyl chavicol	0,02	0,27
17,15	fenchyl acetate	0,13	0,03
18,01	**estragole**	**30,15**	**35,33**
21,05	**trans anethole**	**50,6**	**54,67**
23,83	thymol	0,02	0,03
24,81	α-copaene	0,21	0,05
27,09	β-caryophyllene	0,06	0,16
27,71	α-phellandrene	0,07	-
28,36	β-bisabolene	0,03	0,07
	totale	**97,05%**	**98,16%**

[a] Compounds listed in order of elution.
[b] Retention time (as minutes).

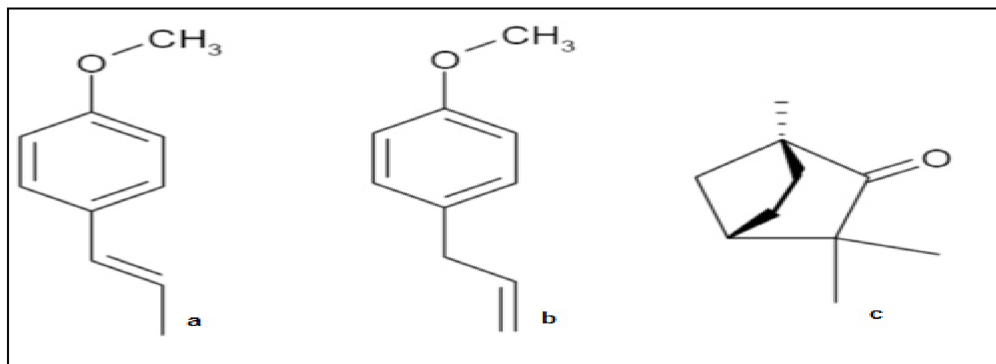

Figure1. The molecular Structures of the major bioactive essential oil components of *Foeniculum vulgare*: trans-anethole (a), estragol (b) and fenchone(c).

Antibacterial activity

The seed and leaf essential oil of *F. vulgare* exhibited a strong broad-spectrum antibacterial activity against all test organisms using the disk diffusion method (table 2). All tested Gram-positive bacteria species are sensitive to *F. vulgare* leaves and seeds essential oil with the inhibition zones ranging from 24.0±1.5 to 26.2±0.4 mm for *B. cereus* and 28.0±1.5 to 30.9±0.4 mm for *S. aureus*, respectively at least concentration (5µl/disc). The strongest activity was observed against *B. cereus*, followed by *S. aureus*. The weakest activity was observed against *P. aeruginosa*.

According to the results of dilution method, MIC and MBC values of *F. vulgare* seeds and leaves essential oil on different kinds of bacteria summarized in Tables 3, The seeds oil showed strongest antibacterial activity against tested bacteria than leaves oil. The most sensitive bacteria to essential oil of *F. vulgare* were *B. cereus* and *S. aureus*, MIC values of leaves oil against *B. cereus* and *S. aureus* was 0.039 mg/ml. The minimum inhibitory concentration of seeds oil against *S. aureus* and *B. cereus* was lower than 0.039 mg/ml.

Most studies concerning the antimicrobial mode of action of essential oil constituents have been performed on bacteria, gram-negative bacteria are generally less susceptible than gram-positive bacteria. The outer membrane of gram-negative bacteria contains hydrophilic lipopolysaccharides (LPS), which create a barrier toward macromolecules and hydrophobic compounds, providing gram-negative bacteria with higher tolerance toward hydrophobic antimicrobial compounds like those found in essential oils (Celikel et al., 2008; Joshi, 2013) .

Results of this study suggested that F. vulgare essential oil have potential effects as antibacterial agents. Main components of essential oil and extract, such as anethole, estragole (methyl chavicol), fenchone and α-pinene have been previously reported to have antibacterial activity (Ebeed et al., 2010; choudhury et al., 2009).

Table 2. Antibacterial activity of *Foeniculum vulgare* essential oil against the bacterial strains based on disc diffusion method.

Microorganism	Inhibition zone diameter						
	Leaf oil (µl/disc)			Seed oil (µl/disc)			Amp[b] (30µg/disc)
	5	10	15	5	10	15	
Gram-negative bacteria							
E. coli ATCC25922	14.2±0.3	16.2±0.5	17.4±1.2	15.2±0.8	17.5±0.5	20.2±0.4	27.7±1.5
P. aeruginosa ATCC27853	8.4±0.6	10.2±1.4	11.3±0.4	9.8±1.2	11.5±0.9	13.2±1.2	21.0±1.2
Gram-positive bacteria							
S. aureus ATCC25923	24.0±1.5	28.7±0.3	36.4±0.2	28.0±1.5	30.7±0.4	37.4±0.7	32.7±0.6
B. cerus ATCC 29213	26.2±0.4	34.8±0.5	36.7±1.4	30.9±0.4	38.5±1.3	42.7.±1.6	24.0±1.0

[a] Diameter of the zone of inhibition (mm) including disk diameter of 6 mm.
Antibiotic: Ampicillin (Amp).

Table 3. The MIC and MBC values of leaves and seeds essential oil from Foeniculum *vulgare* against the microorganism.

Microorganism	Leaf oil (mg/ml)		Seed oil (mg/ml)		Antibiotic (Amp µg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria						
E. coli ATCC25922	0.078	0.156	0,039	0,078	1.95	>3.90
P. aeruginosa ATCC27853	0.156	1.25	0,078	0,156	3.90	7.81
Gram-positive bacteria						
S. aureus ATCC25923	0.039	0.078	< 0,039	0,039	6.25	15.62
B. cerus ATCC 29213	0.039	0.078	< 0,039	0,039	3.90	7.81

MIC: Minimum Inhibitory Concentration. MBC: Minimum Bactericide Concentration; values given as mg/ml for the essential oils and as µg/ml for antibiotics.
A negative control was performed in 0 µl/disc no inhibition was not observed.

Antioxidant activity

The potential antioxidant activity of seeds and leaves essential oil from *F. vulgare* were determined by employing two complementary tests, namely DPPH free radical scavenging and β carotene/linoleic acid test systems. Total antioxidant activity of the plant extracts are recommended to carry out by employing two or more methods (Politeo et al., 2007).

Based on this recommendation, in the current work two complementary test systems were used to evaluate the antioxidant properties of the essential oil of seed and leaves of *F. vulgare*. In both test systems the essential oil of *F. vulgare*, exhibited antioxidant properties. Free radical scavenging properties and the inhibition effects on the linoleic acid oxidation of seeds and leaves essential oil of *F. vulgare* is given in Table 3. The IC_{50} value of seed oil is 14.3 ±1.8 µg/ml, which is higher than the leaves oil (22.6 ± 1.7 µg/ml). In the β-carotene/linoleic acid assay, the seeds oil and leaves oil seemed to inhibit the oxidation of linoleic acid with the value of 76.6 ± 2.6 and 82.4 ± 2.4 % inhibition respectively (Table 4). It is worth to mention that the value of the leaves oil (82.4%) is quite close to synthetic antioxidant, BHT (86.2%).

Several studies have been conducted to clarify the possible substances involved in antioxidant properties of the essential oil. Among the identified compounds in the essential oil from Fennel, Anethole, estragole and fenchone may be considered the main contributors to the antioxidant activity (Mohamad et al., 2011; Faudale et al., 2008). And there are reports that, monoterpene hydrocarbons (limonene, γ-terpinene) and oxygenated monoterpenes (linalool, and terpinen-4-ol) have shown antioxidant activity and it's likely that the activity of essential oil of *F. vulgare* is due to this compounds (Ruberto and Barta, 2000; Shahat et al., 2011).

Table 4. Antioxidant capacity of *Foeniculum vulgare* essential oil.

	Foeniculum vulgare		**Synthetic antioxidant**
	leaf oil	**seed oil**	**BHT**
DPPH (IC_{50} µg/mL DPPH solution)	**14,3 ± 1,8**	**22,6.±1,7**	**7.73±1.62**
Inhibition in linoleic acid system (%)	**82,4 ± 2,4**	**76.6±2,6**	**86.2±0.5**

Values are means ± standard deviation of three separate experiments.

In conclusion, this current study has revealed some variation in biological activities and chemical composition of essential oil from the seeds and leaves of *F. vulgare.* It was concluded that the seeds oil has strong antimicrobial activity, while the leaves oil has a strong antioxidant activity. Handed the chemical composition of these two essential oils is marked by the presence of same major compounds; anethole, estragale and fenchone with almost the same concentrations. Therefore the variations observed in biological activities can be explained by the differences reported for minority compounds between seeds oil and leaves oil.

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