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Antifungal Compounds Isolated from *Smyrnium olusatrum* L. Essential Oil, Growing Wild in Cephalonia, Greece. Chemical Analysis and Structure Elucidation

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Abstract: The essential oils (EOs) from the leaves and the flowers of *Smyrnium olusatrum* L., growing wild in the island of Cephalonia (Greece), were analyzed by GC-FID and GC-MS. Fifty nine constituents, which accounted for 90.3% (fl) and 97.1% (lvs) of the oils, were identified. Furanodiene, germacrone and furanoeremophil-1-one were the major constituents in both essential oils; they were also isolated from the flowers essential oil and identified using spectroscopic methods, ie. 1D and 2D NMR, GC-MS. In addition β-myrcene (11.7%) and β-phellandrene (5.2%) were main constituents in the essential oil of the leaves. The essential oils and the pure isolates were evaluated for antifungal activity against *Aspergillus fumigatus*, *A. versicolor*, *A. ochraceus*, *A. niger*, *Trichoderma viride*, *Penicillium funiculosum*, *P. ochrochloron*, *P. verucosum* var. *cyclopium* by using the microdilution method and proved to possess significant antifungal effect. Among them, (+) furanoeremophil-1-one was particularly active with MIC values in the range of 0.0008-0.125 mg/mL and MFC values of 0.025-0.050 mg/mL and proved more effective than the commercial mycotics ketoconazole and bifonazole used as positive controls.

Keywords: *Smyrnium olusatrum*; essential oil; isolation; (+) furanoeremophil-1-one; furanodiene; (-) germacrone, antifungal activity.

1. Introduction

Smyrnium olusatrum L. is a biennial plant belonging to the Apiaceae family. The species distribution in Europe ranges from countries bordering the Mediterranean basin to its northern limit in Scotland. Its establishment on islands of the Mediterranean can be attributed to human agencies as the fresh seeds do not float [1]. Traditionally almost all parts (leaves, fruits and roots) of *Smyrnium* were

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consumed as vegetable because of its characteristic aromatic flavour, until Middle Ages when it was substituted by celery (*Apium graveolens* L.) [2].

Moreover a plethora of medicinal properties have been attributed to *Smyrnium* during antiquity by the Greeks and the Romans [2]. Leaves were used as an antiscorbutic, the fruit as a stomachic and antiasthmatic, and the juice of the root for its aromatic, appetite stimulant, diuretic and laxative properties [3]. The stems were blanched in Britain and eaten because the plant was regarded as having mild purging qualities. In veterinary practice, the plant was used in Isle of Man to treat mouth sores in cattle scurvy. In Scotland, seafarers used it to clean the blood and prevent scurvy [4].

Concerning the chemical profile of the species, previous investigations have been carried out on its essential oil content [2]. The furanosesquiterpenoids are the main constituents of the essential oil and to these compounds is attributed the distinct odor of the plant [5, 6]. Furanodiene is the most remarkable constituent due to its important biological properties, such as cytotoxicity in human cancer cell lines, antiproliferative, antioxidant and antibacterial activities [7]. In addition, several sesquiterpene lactones [8-11], acetylenic derivatives [12] and flavonoids [13] have been isolated from *S. olusatrum*.

Owing to the continuing development of microbial resistance in medicine and agriculture the discovery of new antifungal substances is an important, if not urgent, research objective. In addition, the desire for safer products with less environmental and mammalian toxicity is of major concern. Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by different modes of action than existing antifungal agents and, consequently, lack cross-resistance to chemicals currently used. This forgotten vegetable attracted the scientific interest during the last couple of years, being its study an important bridge for the passage from the ancient and folk medicine to the documentation and the assessment of the past information and its use and perspectives in the light of bioactive natural products based on experimental bioassays. The above data prompted us initially to investigate the chemical composition of the essential oil of *S. olusatrum*, also called wild celery, growing wild in Greece and then to evaluate its possible antifungal activity against a panel of micromycetes, which are plant, animal and human pathogens, food spoilage, food contaminators and mycotoxin producers. Furthermore, the main constituents of the essential oil were isolated, in order to correlate the antifungal activity of the essential oil to certain compounds.

2. Materials and Methods

2.1. Plant material

Leaves and flowers of *S. olusatrum* L. were collected separately during flowering period in 2011 at Minies (Cephalonia Island). The plant material was authenticated by Prof. Yiannis Samaras and deposited in the herbarium of the Department of Organic Farming and Food Technology under the number 727. The voucher specimen of the essential oil was deposited in the Institute' s Bank of essential oils, Technological Educational Institute of Ionian Islands, Cephalonia, Greece with number 539.

2.2. Essential oil distillation

The collected plant materials were subjected separately to hydro distillation in a Clevenger-type apparatus according to the Hellenic Pharmacopoeia [14]. The obtained essential oils were of yellowish color and characteristic odor and deposited in vials at -20°C before further chemical and biological analyses.

2.3. GC analysis

Quantification was performed using gas-chromatography coupled with flame ionization detection (GC–FID). Analysis was carried out on a Perkin Elmer Clarus 500 gas chromatograph with FID, fitted with a fused silica Rtx-5 MS capillary column (30m x 0.25mm (i.d.), film thickness: 0.25μm). The column temperature was programmed from 60°C to 250°C at a rate of 3°C/min. The injector and detector

temperatures were programmed at 230°C and 280°C, respectively. 5μL of each sample were diluted in 10 μL GC grade n-pentane and 2 μL of the obtained solution was further injected in the GC apparatus. Identification of constituents was achieved by calculating arithmetic indices relative to linear alkanes from C_9–C_{23} and comparing with data from GC–MS identifications.

2.4. GC-MS analysis

GC-MS analyses were performed on a Hewlett-Packard 5973-6890 system operating in EI mode (70eV) equipped with a split/splitless injector (220 °C), a split ratio 1/10, using a fused silica HP-5 MS capillary column (30m x 0.25mm (i.d.), film thickness: 0.25μm) The temperature program for HP-5 MS column was from 60 °C (5min) to 280 °C at a rate of 4 °C /min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Injection volume for all samples, diluted as previously described, was 2 μL.

2.5. Essential oil fractionation

In order to obtain pure compounds, the essential oil of the flowers (EO) was selected for further fractionation, as the main constituents were quantified in higher amounts compared to the essential oil of the leaves. The EO was subjected to column chromatography over silica gel (Merck, Art. 9385) (15 x 3cm) applying gradient elution with mixtures of n-heptane-cyclohexane-dichloromethane (100:0:0-0:0:100). Eighty five fractions of 10 mL were collected. Based on the obtained chromatographic results, in combination with their ^{1}H-NMR spectra, the obtained fractions were further combined to twenty four groups (A-Z). Group A was eluted with n-heptane 100%, groups B-C were eluted with n-heptane-cyclohexane 97:3. Group D (60.2 mg, eluted with n-heptane-cyclohexane 95:5) was identified as furanodiene (**2**). Groups E-F were eluted with n-heptane-cyclohexane 90:10, groups G-H with n-heptane-cyclohexane 80:20, groups I-J with n-heptane-cyclohexane 70:30, groups K-L with n-heptane - cyclohexane 50:50, group M was eluted with cyclohexane 100%. Group N (1.2 mg, eluted with cyclohexane-dichloromethane 65:35) was identified as (+) furanoeremophil-1-one (**1**). Groups O-P were eluted with cyclohexane-dichloromethane 40:60, group Q with cyclohexane-dichloromethane 35:65, group R with cyclohexane-dichloromethane 30:70, group S with cyclohexane-dichloromethane 25:75, while group T (25.5 mg, eluted with cyclohexane-dichloromethane 20:80) was identified as (-) germacrone (**3**). Finally, groups U-W were eluted with cyclohexane-dichloromethane 10:90 and groups X-Z with dichloromethane 100%.

The optical rotation of the isolated compounds was measured in a Perkin Elmer 341 polarimeter. The $[\alpha]_D$ values were obtained in $CHCl_3$ at 20 ºC, λ 589 nm. The isolated compounds were again injected in GC-MS under the same conditions as the total essential oils to verify their retention times. Fractions monitoring to follow separation was performed by TLC on silica gel 60 F254 (Merck, Art. 5554). Compounds were detected using UV absorbance (λ 254 and λ 365 nm). Vanillin (Merck, Art. No. S26047 841) /sulphuric acid reagent (vanillin 5% in H_2SO_4/MeOH) [15] was used for the detection of the compounds. Analytical solvents were obtained from Panreac Quimica SA (Barcelone, Spain, Italy), while deuterated solvents were purchased from Merck, KGaA (Darmstadt, Germany).

2.6. Identification of components

Retention indices for all compounds were determined according to the Van der Dool approach [16] with reference to a homologous series of normal n-alkanes from C_9 to C_{23}. The identification of the components was based on comparison of their mass spectra with those of Wiley and NBS Libraries [17] and those described by Adams [18], as well as by comparison of their retention indices with literature data [18]. Component relative percentages were calculated based on GC-FID peak areas without using correction factors. The 1D and 2D NMR spectra of all pure isolated substances, as well as of a mixture containing furanodiene and elemane derivatives were recorded in $CDCl_3$ on Bruker DRX 400 and Bruker AC 200 (50.3 MHz for ^{13}C NMR) instruments at 295K. Chemical shift are reported in ppm (δ) using the residual solvent signal (δ_H 7.24 in ^{1}H and δ_C 77.0 in ^{13}C, $CDCl_3$) as reference. COSY and HSQC

experiments were performed using standard Bruker microprograms. The obtained NMR spectra were also compared with those reported in the literature [2], [19- 24].

Optical rotation was recorded on a Perkin Elmer 341 polarimeter. The $[\alpha]_D$ values were obtained in $CHCl_3$ at 20 ºC, λ 589 nm. The isolated compounds were again injected in GC-MS under the same conditions as the total essential oils to verify their retention times.

2.7. Antifungal activity

For the antifungal bioassays, eight fungi were used: *Aspergillus fumigatus* (plant isolate), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112) and *P. verucosum* var. *cyclopium* (food isolate). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The micromycetes were maintained on malt agar and the cultures stored at 4 °C and sub-cultured once a month [25]. In order to investigate the antifungal activity of the samples, a modified microdilution technique was used [26-28]. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. MIC (Minimum inhibitory concentration) determinations were performed by a serial dilution technique using 96-well microtiter plates. The samples were dissolved in DMSO (1 mg/mL) and added in broth Malt medium with inoculum. The microplates were incubated for 72 h at 28 °C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μL into microtiter plates containing 100 μL of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls (1 - 3000 μg/mL). All experiments were performed in duplicate and repeated three times.

3. Results and Discussion

3.1. Chemical analysis and structure elucidation

The essential oils consisted of a complex mixture of fifty nine different substances, with sesquiterpenes, as the dominating constituents (Table 1). The furanosesquitepenoids, furanodiene (36.9% fl; 21.5% lvs), furanoeremophil-1-one (12.8% fl; 26.0% lvs) and germacrone (14.4% fl; 11.4% lvs) are the main constituents in both essential oils. Moreover, the essential oil of the leaves is characterized by the presence of high amounts of β-myrcene (11.7%) and β-phellandrene (5.2%).

The predominance of furanodiene in the present samples was confirmed by the GC-FID analysis, as well as by its NMR data. Moreover, the NMR data of the obtained fractions revealed characteristic peaks of compounds belonging to the sub group of elemane derivatives and particularly of curzurene, which was identified in significant lower concentrations compared to furanodiene and in mixtures with this latter constituent (see Supporting information). It has been previously sustained the assumption that curzurene is an artefact derived from the extraction procedure or the GC-MS thermal program that caused the thermal Cope rearrangement of furanodiene to curzurene [6], [19]. Based to this evidence, the more accurate quantification of furanodiene arises when we sum together the percentages of both compounds. Comparing the present results with literature data, in many studies the ratio between furanodiene and curzurene is presented opposite [29-31], while in other [32] furanodiene is not reported at all and curzurene is considered one of the major components of *S. olusatrum* essential oil. It is noteworthy that

apart from the heat sensitive furanodiene, the essential oils are consisted of some other compounds also sensitive in high temperatures. According to Yang et al. [33], germacrone, germacrene B and furanoeremophil-1-one degrade into trans-β-elemenone, γ-elemene and furano-4(15)-eudesmen-1-one, respectively and this should be taken in consideration regarding the percentages of the first two compounds (Table 1).

Compound **1 (**+) Furanoeremophil-1-one was isolated as white crystals. Its ^{1}H-NMR spectrum showed the characteristic chemical shift of the α-furan proton H-12 as a singlet at δ 6.99 typical for a trisubstituted furan ring; three signals at δ 1.85 (3H, d, J= 1.5 Hz, CH_3-13), 0.99 (3H, d, J= 6.7 Hz, CH_3-15), 0.58 (3H, s, CH_3-14) corresponding to the three methyl groups of the molecule. A furaneudesmane skeleton was confirmed by the ^{13}C-NMR spectrum, which gave evidence of a carbonyl group resonating at δ 211.1 (C-1), three methyl groups at δ 14.8 (C-15), 11.8 (C-14) and 8.0 (C-13) and four methylenes at δ 42.1 (C-2), 34.9 (C-3), 34.0 (C-6), 20.2 (C-9). In addition, the chemical shifts at δ 148.1 (C-8), 137.3 (C-12), 119.7 (C-11), 115.3 (C-7) revealed the presence of a trisubstituted furan ring. A detailed study on the interpretation of the ^{13}C NMR, COSY and HSQC spectra allowed us to revise the chemical shifts of C-14 (11.8 vs 14.8) and C-15 (14.8 vs 11.7) of the methyl moieties compared to those published [2].

Based on the structure of (**1**), the ^{1}H-NMR spectrum of furanodiene (**2**) showed also the typical singlet at δ 7.06 of the olefinic H-12 of the trisubstituted furan ring, as well as the characteristic doublets at δ 3.53 (1H, d, J=15.8 Hz, H-9a), 3.41 (1H, d, J= 15.8 Hz, H-9b) representing the AB system of the methylene protons of position 9. The geminal protons at C-2 appeared as multiplet in the range of δ 2.12-2.06 (2H, m, H-2a, H-2b), showing ^{1}H-^{1}H COSY correlations with the vicinal protons of C- 3 at δ 2.23 (1H, dt, J=11.9, 3.5 Hz, H-3a) and 1.77 (1H, m, H-3b). HSQC spectrum indicated the presence of the typical carbon signal at δ 135.5 (C-12) of the furan ring. Moreover, the signals of the olefinic carbons at δ 128.6 (C-1), 127.1 (C-5), as well as the methylene carbons at δ 26.9 (C-2), 39.3 (C-3), 24.7 (C-6), 40.6 (C-9) completed the central skeleton. The three methyl groups resonated at δ 8.7 (C-13), 16.3 (C-14), 15.9 (C-15).

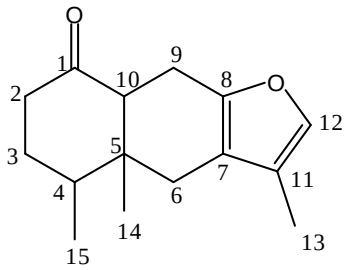
The ^{1}H-NMR spectrum of germacrone (**3**) indicated the presence of four methyl groups at δ 1.75 (3H, s, CH_3-12), 1.69 (3H, s, CH_3-13), 1.60 (3H, s, CH_3-15), 1.41 (3H, s, CH_3-14), four methylene groups at δ 3.38 (1H, d, J=10.2 Hz, H-9b), 2.91 (1H, d, J=10.2 Hz, H-9a), 2.80-2.90 (2H, m, H-6a, H-6b), 2.03-2.30 (m, H-2a,b, H-3a,b) and two olefinic protons at δ 4.95 (1H, d, J= 11.9 Hz, H-1), 4.68 (1H, dd, J= 2.8, 11.9 Hz, H-5). ^{1}H-^{1}H COSY correlations enabled us to confirm the structure of **3**, which was in complete accordance with the literature data [21, 23].

3.2. Spectral data

(+) *furanoeremophil-1-one* (**1**): White crystals; $[\alpha]_D$ +2.47 (c 0.81, $CHCl_3$); Rt 42.45; MS (EI, 70 eV): *m/z* (%) = 232 [M+] (100), 199 (70). ^{1}H NMR (400Hz, $CDCl_3$) δ, ppm, J/Hz: 6.99 (1H, brs, H-12); 2.65(1H, m, H-10); 2.67 (1H, m, H-9a); 2.56-2.45 (1H, m, H-9b); 2.37- 2.23 (2H, m, H-2a, H-2b); 1.86 (1H, m, H-4); 1.85 (3H, d, J= 1.5 Hz, CH_3-13); 1.84-1.80 (1H, m, H-3a); 1.67-1.56 (1H, m, H-3b); 0.99 (3H, d, J= 6.7 Hz, CH_3-15); 0.58 (3H, s, CH_3-14). ^{13}C NMR (50.3 Hz, $CDCl_3$) δ, ppm: 211.1 (C-1), 148.1 (C-8), 137.3 (C-12), 119.7 (C-11), 115.3 (C-7), 54.2 (C-5), 42.1 (C-2), 41.4 (C-4), 37.0 (C-10), 34.9 (C-3), 34.0 (C-6), 20.2 (C-9), 14.8 (C-15), 11.8 (C-14), 8.0 (C-13).

furanodiene (**2**): Yellow oil; racemic mixture; Rt 27.90; MS (EI, 70 eV): *m/z* (%) = 216 [M+] (20), 108 (100); ^{1}H NMR (400Hz, $CDCl_3$) δ, ppm, J/Hz: 7.06 (1H, brs, H-12); 4.92 (1H, m, H-1); 4.72 (1H, t, J=7.2 Hz, H-5); 3.53(1H, d J=15.8 Hz, H-9a); 3.41 (1H, d, J= 15.8 Hz, H-9b); 3.06 (2H, d, J=7.5 Hz, H-6); 2.12-2.06 (2H, m, H-2a, H-2b); 2.23 (1H, dt, J=11.9, 3.5 Hz, H-3a); 1.77 (1H, m, H-3b); 1.26 (3H, s, CH_3-15); 1.59 (3H, s, CH_3-14); 1.91 (3H, d, J=1.2 Hz, CH_3-13). ^{13}C-NMR (50.3Hz, $CDCl_3$) δ, ppm: 135.5 (C-12), 128.6 (C-1), 127.1 (C-5), 40.6 (C-9), 26.9 (C-2), 39.3 (C-3), 24.7 (C-6), 8.7 (C-13), 16.3 (C-14), 15.9 (C-15)

(-) germacrone (**3**): White crystals; $[\alpha]_D$ -4.95 (c 0.18, $CHCl_3$); R_t 35.57; MS (EI, 70 eV): *m/z* (%) = 218 [M^+] (20), 107 (100). ^{1}H NMR (400Hz, $CDCl_3$) δ, ppm, J/Hz: 4.95 (1H, d, J= 11.9 Hz, H-1); 4.68 (1H, dd, J= 2.8, 11.9 Hz, H-5); 3.38 (1H, d, J=10.2 Hz, H-9b); 2.91 (1H, d, J=10.2 Hz, H-9a); 2.80-2.90 (2H, m, H-6a, H-6b); 2.03-2.3 (m, H-2a,b, H-3a,b); 1.75 (3H, s, CH_3-12); 1.69 (3H, s, CH_3-13); 1.60 (3H, s, CH_3-15); 1.41 (3H, s, CH_3-14).



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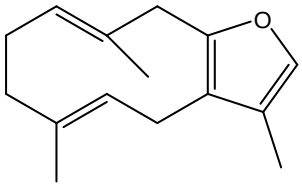

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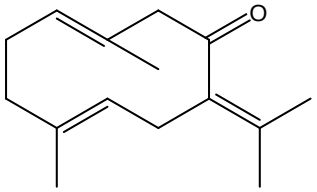

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Figure 1. Structures of the isolated compounds **1-3**.

Table 1. Qualitative and quantitative composition (% v/v) of volatile compounds in the flowers and leaves of *Smyrnium olusatrum* L.

Components	RI[a]	Flowers	Leaves
α-Thujene	925	-	0.1
α-Pinene	933	-	1.2
Camphene	947	-	0.1
Sabinene	967	0.1	0.2
β-Pinene	973	-	0.2
β-Myrcene	990	3.0	11.7
α-Phellandrene	1003	-	0.3
δ-3-Carene	1005	0.1	0.8
α-Terpinene	1011	-	0.1
p-Cymene	1018	-	0.2
Limonene	1022	0.4	-
β-Phellandrene	1026	0.5	5.2
(*E*)-β-Ocimene	1038	0.2	1.0
γ-Terpinene	1049	0.2	0.6
Terpinolene	1076	0.1	0.1
Linalool	1091	0.3	0.3
Camphor	1136	-	0.1
Borneol	1160	0.1	0.2
Terpinen-4-ol	1168	0.1	0.1
α-Terpineol	1183	0.1	0.1
β-Fenchyl alcohol	1185	-	0.1
Estragole	1186	0.2	0.2
Verbenone	1198	tr	0.1
(*E*)-Octanyl acetate	1207	0.1	0.1
Citronellol	1214	0.1	0.1
(*E*)-Anethole	1272	0.1	0.1
Carvacrol	1286	0.8	0.4
(*Z*)-Pinocarvyl acetate	1307	0.1	-
Citronellyl acetate	1344	0.5	0.9
Neryl acetate	1359	0.6	1.1
β-Elemene	1371	1.7	0.8
Methyl eugenol	1388	0.5	0.1
β-Caryohyllene	1398	0.4	0.5
γ-Elemene	1408	1.2	0.6

Components	RI[a]	Flowers	Leaves
Dihydro-β-ionone	1412	0.2	-
(*Z*)-β- Farnesene	1440	0.1	-
α-Humulene	1450	0.1	0.1
Germacrene D	1458	5.3	4.2
Furanodiene	1477	36.9	21.5
Curzurene	1480	0.4	0.2
Germacrene A	1483	-	0.2
(*Z*)-Eudesma-6,11-diene	1486	0.3	-
β-Selinene	1490	0.1	-
δ-Cadinene	1491	0.2	0.1
Elemicin	1521	0.1	0.1
Germacrene B	1531	3.5	1.8
α-Amorphene	1548	0.2	0.1
(*E*)-β-Elemenone	1573	2.7	2.3
Longiborneol	1593	0.2	0.2
Khushimone	1610	0.3	0.1
Selina-3, 11-dien-6-α-ol	1633	0.4	0.2
Atractylone	1637	-	0.2
Selin-11-en-4-α-ol	1645	0.3	-
Germacrone	1688	14.4	11.4
Furanoeremophil-1-one	1856	12.8	26.0
Alexandrofuran	1879	0.1	-
Heinecosane	2100	0.1	0.4
Docosane	2200	0.1	0.1
Tricosane	2300	-	0.2
Terpenoids		**89.2**	**96.1**
Monoterpene hydrocarbons		4.6	21.8
Oxygenated monoterpenes		3.0	3.8
Sesquiterpene hydrocarbons		50.4	30.1
Oxygenated sesquiterpenes		31.2	40.4
Phenylpropanoids		**0.6**	**0.2**
Compounds with 13 carbons		**0.2**	**-**
Aliphatic (Alkanes, aliphatic esters)		**0.3**	**0.8**
Total		**90.3**	**97.1**

[a]Retention indices were calculated against C_9-C_{23} *n*-alkanes on the HP 5MS capillary column.

3.3. Antifungal activity

The essential oil of the flowers (EO) and pure compounds **1**-**3** were assayed in vitro for their antifungal activity against eight micromycetes which are plant, animal and human pathogens, food spoilage, food contaminators and mycotoxin producers.

The results are presented in Table 2. It is obvious that all samples showed antifungal effect. MIC of EO is in range of 0.6-9.4 mg/mL and MFC in range of 9.4-18.8 mg/mL. Furanoeremophil-1-one (**1**) possessed inhibitory effect on fungi in the range of 0.0008-0.125 mg/mL, whereas fungicidal effect was achieved at 0.025-0.05 mg/mL. Furanodiene (**2**) showed MIC at 0.004-0.5 mg/mL and MFC at 0.25-1.0 mg/mL, while germacrone (**3**) exhibited fungistatic potential at 0.0325-0.125 mg/mL and fungicidal effect at 0.125-0.25 mg/mL. It is noteworthy that pure isolates are more active than the essential oil, since their antifungal potential could be presented as follows: furanoeremophil-1-one > germacrone > furanodiene > EO. Essential oil and compounds tested showed the best activity against *Aspergillus versicolor*, while *A.*

niger was the most resistant species. The commercial antifungal agent, bifonazole, showed MIC at 0.10-0.20 mg/mL and MFC at 0.20-0.25 mg/mL. Ketoconazole showed fungistatic activity at 0.2-2.50 mg/mL and fungicidal effect at 0.30-3.50 mg/mL. It is noteworthy that furanoeremophil-1-one (**1**) was more effective than both mycotics against all fungi tested. Germacrone (**3**) possessed higher antifungal potential towards all fungi tested with the exception of *A. niger*, where this compound showed lower fungicidal potential than commercial mycotics. Furanodiene (**2**) showed higher antifungal capacity than bifonazole and ketoconazole with exception towards *A. fumigatus* and *P. verucosum* var. *cyclopium*. Regarding the antifungal activity of the total essential oil, the present results are consistent with the previously reported, where it was revealed active against a series of several other fungi [31, 32]. It is worth noticing that previous study investigating the fungicidal and fungistatic effects of the essential oil and extracts from several plants including *S. olusatrum* L. concluded that the latter showed the most potent inhibition on the pathogen fungus *Phytopthora capsici leon* with MIC values 0.250 μg/mL [34]. Moreover, the important outcome of the present study is that the antifungal activity was attributed to the isolated components, being tested separately.

In conclusion the above data indicate that much attention should be given in plant derived substances with almost equal activity as synthetic commercial mycotics and can be found in nature in abundance, while are less possible to cause hazards to the environment.

Table 2. Minimal Inhibitory (MIC) and Minimal Fungicidal Concentrations (MFC) of flower Essential Oil (EO) and isolated compounds **1-3 (**mg/mL).

Fungi	**EO** **MIC** **MFC**	**1** **MIC** **MFC**	**2** **MIC** **MFC**	**3** **MIC** **MFC**	**Bifonazole** **MIC** **MFC**	**Ketonazole** **MIC** **MFC**
Aspergillus fumigatus	2.35 18.8	0.0125 0.025	0.5 1.0	0.0625 0.125	0.15 0.20	0.20 0.50
Aspergillus versicolor	0.6 9.4	0.0008 0.025	0.004 0.25	0.0325 0.125	0.10 0.20	0.20 0.50
Aspergillus ochraceus	2.35 18.8	0.0008 0.025	0.004 0.25	0.0625 0.125	0.15 0.20	1.50 2.0
Aspergillus niger	9.4 18.8	0.125 0.05	0.015 0.50	0.125 0.25	0.15 0.20	0.20 0.50
Trichoderma viride	9.4 18.8	0.0625 0.025	0.004 0.25	0.0625 0.125	0.15 0.20	1.0 1.0
Penicillium funiculosum	4.7 18.8	0.0125 0.025	0.004 0.25	0.0625 0.125	0.20 0.25	0.20 0.50
Penicillium ochrochloron	2.35 18.8	0.0125 0.025	0.125 0.25	0.09 0.125	0.20 0.25	2.5 3.5
Penicillium verucosum var. *cyclopium*	4.7 18.8	0.0125 0.025	0.25 0.5	0.09 0.125	0.10 0.20	0.20 0.30

References

[1] R.E. Randall (2003). *Smyrnium olusatrum* L, *J. Ecol.* **91**, 325-340

[2] U. Mölleken, V. Sinnwell and K.H. Kubeczka (1998). Essential oil composition of *Smyrnium olusatrum*, *Phytochemistry* **49**, 1709-1714.

[3] P. Fournier (1947). Le Livre des Plantes Médicinales de France. Tome II, Lechevalier, P. (Ed.), Paris, p. 466.

[4] D.A. Allen, G. Hatfield (2004). Medicinal plants in folk tradition. An ethnobotany of Britain and Ireland. Timber Press, Inc., Portland, Oregon, USA.

[5] L.O. Hanuš, T. Rezanka, V.M. Dembitsky and A. Moussaieff (2005). *Myrrh Commiphora* Chemistry, *Biomedical Papers* **149**, 3–28.

[6] F. Maggi, L. Barboni, F. Papa, G. Caprioli, M. Ricciutelli, G. Sagratini and S. Vittori (2012). A forgotten vegetable (*Smyrnium olusatrum* L., Apiaceae) as a rich source of isofuranodiene, *Food Chem.* **135**, 2852-2862.

[7] L. Quassinti, M. Bramucci, G. Lupidi, L. Barboni, M. Ricciutelli, G. Sagratini, F. Papa, G. Caprioli, D. Petrelli, L.A. Vitali and F. Maggi (2013). In vitro biological activity of essential oils and isolated furanosesquiterpenes from the neglected vegetable *Smyrnium olusatrum* L. (Apiaceae), *Food Chem.* **138**, 808-813.

[8] A. El-Gamal (2001). Sesquiterpene lactones from *Smyrnium olusatrum*, *Phytochemistry* **57**, 1197–1200.

[9] A. Ulubelen, S. Ö ksüz, Z. Samek and Y. Holub (1971). Sesquiterpenic lactones from *Smyrnium olusatrum* L. roots, *Tetrahedron Lett.* **12**, 4455-4456.

[10] A. Ulubelen, S. Ö ksüz,, J.D. Korp, I. Bernal, D.A. Gage, J. Gershenzon and T.J. Mabry (1983). Relative configuration of glechomafuran isolated from the fruits of *Smyrnium olusatrum, J. Nat. Prod.* **46**, 490-492.

[11] A. Ulubelen, S. Öksüz, N. Gören, T. J. Mabry and F. Bohlmann (1985). A furanoeudesmane from the fruits of *Smyrnium olusatrum*, *Phytochemistry* **24**, 1105-l 106.

[12] Bohlmann and C. Zdero (1973). Über die Inhaltsstoffe von *Smyrnium olusatrum,* L. Chem. Ber. **106**, 3614–3620.

[13] R. Bencheraiet, M. Jay, A. Kabouche and Z. Kabouche (2011). Flavonoids of *Smyrnium olusatrum* from Algeria, *Chem. Nat. Comp*. **47**, 812-813.

[14] Hellenic Pharmacopoeia. (2002). Edition V, National Organization for Medicines of Greece, Chapter 28.12, Athens.

[15] E. Stahl (1969). Thin-Layer Chromatography. Springer Verlag, Berlin-Heidelberg-N. York. p. 904.

[16] H. Van den Dool and P.D. Kratz (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography, *J. Chromatogr. A.* **11**, 463-471.

[17] Y. Massada (1976). Analysis of essential oil by gas chromatography and spectrometry. John Wiley & Sons, New York.

[18] R.P. Adams (2007). Identification of essential oil components by gas chromatography / quadrupole mass spectrometry. Allured Publishing Co. Carol Stream, Illinois.

[19] N. Baldovini, F. Tomi and J. Casanova (2001). Identification and quantitative determination of furanodiene, a heat-sensitive compound, in essential oil by ^{13}C-NMR, *Phytochem. Anal.* **12**, 58-63.

[20] F.Q. Yang, H.K. Wang, J.D. Chen and Z.N. Xia. (2011). Fractionation of volatile constituents from *Curcuma rhizome* by preparative gas chromatography, *J. Automat. Meth. Manag. Chem.* doi: 10.1155/2011/942467.

[21] J. Yan, G. Chen, S. Tong, Y. Feng, L. Sheng and J. Lou (2005). Preparative isolation and purification of germacrone and curdione from the essential oil of the rhizomes of *Curcuma wenyujin* by high speed counter-current chromatography, *J. Chromatogr. A.* **1070**, 207-210.

[22] A. Dekebo, E. Dagne and O. Sterner (2002). Furanosesquiterpenes from *Commiphora sphaerocarpa* and related adulterants of true myrrh, *Fitoterapia* **73**, 48–55.

[23] K. Firman, T. Kinoshita, A. Itai and U. Sankawa (1988). Terpenoids from *Curcuma heyneana*, *Phytochemistry* **27**, 3887-3891.

[24] N. Jia M. Xiao Chi, X. Xiu Lan, Ji. H. Wei and G. Dean (2008). Structural determination of two new sesquiterpenes biotransformed from germacrone by *Mucor alternata*, *Magn. Res. Chem.* **46**, 178–181.

[25] C. Booth (1971). Fungal Culture Media. In: Norris, J.R., Ribbons, D.W. (Eds.), Methods in Microbiology IV. Academic Press, London and New York, pp. 49-94.

[26] H. Hanel and W. Raether (1988). A more sophisticated method of determining the fungicidal effect of water-insoluble preparations with a cell harvester, using miconazole as an example. *Mycoses.* **31**, 148-154.

[27] R.K. Daouk, S.M. Dagher and E.J. Sattout (1995). Antifungal activity of the essential oil of *Origanum syriacum* L, *J. Food Prot.* **58**, 1147-1149.

[28] A. Espinel-Ingroff (2001). Comparation of the E-test with the NCCLS M38-P method for antifungal susceptibility testing of common and emerging pathogenic filamentous fungi, *J. Clinical Microbiol.* **39**, 1360-1367.

[29] A. Bertoli, L. Pistelli, I. Morelli, D. Fraternale, L. Giamperi and D. Ricci (2004). Volatile constituents of different parts (roots, stems and leaves) of *Smyrnium olusatrum* L., *Flavour Fragr. J.* **19**, 522–525.

[30] F. Papaioannou, A. Koutsaviti and O. Tzakou (2010). Volatile constituents of different parts of *Smyrnium olusatrum* from Greece, *Nat. Prod. Com.* **5**, 1809–1810.

[31] H. Daroui-Mokaddem, A. Kabouche, M. Bouacha, B. Soumati, A. El-Azzouny, C. Bruneau and Z. Kabouche (2010). GC/MS analysis and antimicrobial activity of the essential oil of fresh leaves of *Eucalyptus globulus*, and leaves and stems of *Smyrnium olusatrum* from Costantine (Algeria), *Nat. Prod. Comm.* **5**, 1669–1672.

[32] B. Marongiu, A .Piras, S. Porcedda, D. Falconieri, M.A. Frau, A. Maxia, M.J. Gonçalves, C. Cavaleiro and L. Salgueiro (2012). Antifungal activity and chemical composition of essential oils from *Smyrnium olusatrum* L. (Apiaceae) from Italy and Portugal, *Nat. Prod. Res.* **26**, 993-1003.

[33] F.Q. Yang, S.P. Li, J. Zhao, S.C. Lao and Y.T. Wang (2007). Optimization of GC-MS conditions based on resolution and stability of analytes for simultaneous determination of nine sesquiterpenoids in three species of *Curcuma* rhizomes, *J. Pharm. Biomed. Anal.* **43**, 73–82

[34] Ö.E. Kiran, A. Ilçim and M. Digrak. (2010). Antifungal activity against *Phytophthora capsici leon* which causes root neck burn in pepper around Kahramanmaras, *Asian J. Chem.* **22**, 468-474.