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Composition, Antimicrobial and Antioxidant Properties of Endemic Species *Ferulago macedonica* Micevski & E. Mayer

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Abstract: *Ferulago macedonica* Micevski & E. Mayer (Apiaceae), an endemic species from Macedonia, was evaluated for its essential oil composition and biological activity for the first time. The essential oil composition from inflorescence and aerial parts, analyzed by gas chromatography and gas chromatography–mass spectrometry (GC and GC-MS), indicated α-pinene (43.10% and 22.78%) and sabinene (26.73% and 15.46%) as the main components. The antioxidant activities of extracts was determinated spectrophotometrically using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The strongest activity had methanol extracts of inflorescence and aerial parts (IC_{50}=490 mg/mL and IC_{50}=630 mg/mL of solution, respectively). According to 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) test, the most effective were ethanol and methanol extracts of *F. macedonica* inflorescence (1.69 and 1.63 mg Vit.C/g dw). Total phenolic and flavonoid contents evaluation indicated that their amounts were ranged between 34.12-59.68 mg gallic acid (GA)/g dw for phenols and 9.08 - 23.73 mg quercetin hydrate (QE)/g dw for flavonoids. Antimicrobial activity of extracts and essential oils was investigated by micro-well dilution assay. Minimum inhibitory (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) were defined. The methanol extract of inflorescence has shown to be the strongest in both, antibacterial (MICs=4-8 mg/mL; MBCs=9-13 mg/mL) and antifungal activity (MICs=2-12 mg/mL; MFCs=6-18 mg/mL). Essential oil from inflorescence and aerial parts of *F. macedonica* manifested stronger inhibition on growth of pathogenic bacteria and fungy compared to tested extracts and positive standards.

Keywords: *Ferulago macedonica*; Apiaceae; essential oil; antioxidant activity; antimicrobial activity.

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1. Introduction

The genus *Ferulago* (Apiaceae) includes about 50 species widespread around the Mediterranean Sea, Iran and Turkestan [1] with two-, three- or four-pinnate leaves, well developed bracts and bracteoles, yellow petals and dorsally compressed fruits [2]. *Ferulago macedonica* Micevski & E. Mayer is rare and endemic species, distributed only in central part of Republic of Macedonia, the driest steppe area of the country. This species is a part of very old relict steppe flora, existing and developing in this area since the end of the Tertiary [3]. The plant is herbaceous, glabrous and perennial with thick roots and upright steam. Compound umbels consist of 8-15 umbellules with glabrous pedicels and 10-17 flowers with yellow petals [4].

Ferulago species are used as spices and flavorings in folk medicine for their sedative, tonic, digestive and antiparasitic effects. Root extracts of some *Ferulago* spp. were used as aphrodisiac, as well as those of *Ferula* spp. [5]. Moreover, species of this genus have been employed against ulcers, snake bites, as well as headache and diseases of the spleen [6].

Recently, it was reported that essential oil of aerial parts of *F. angulata* subsp. *carduchorum* from Iran, as the dominant compound contains *cis*-ocimene. Essential oil from the seeds of this plant was reach in α-pinene [5]. Comparative analysis of *F. isaurica* essential oils, endemic species from Turkey, showed α-pinene, limonene and myrcene dominant in fruit oil, while root oil was characterized by presence of terpinolene and myrcene. Fruit oil of *F. syriaca* was characterized by myrcene and 4,6-guaiadiene and in root oil the most abundant were bornyl acetate and terpinolene [7]. Bornyl-acetate and 2,3,4-trimethylbenzaldehyde were the main volatile oil constituents of *F. macrocarpa* [8].

Ruberto et al. (1999) reported that *F. nodosa* essential oil obtained by steam distillation extraction (SDE) contains 2,3,4-trimethylbenzaldehyde and α-pinene as the main constituents, while oil obtained by supercritical fluid extraction (SFE), showed domination of α-pinene and myrcene [9]. Essential oil composition of *F. campestris* (Syn. *F. galbanifera*) has also been investigated [2, 10-12]. Reported chemical analyzes from samples of *F. campestris*, occurring in central Italy showed domination of α-pinene, myrcene and γ-terpinene [10], which were completely lacking in Turkish fruit oil samples [13, 14]. Riela et al. (2011) compared essential oil composition of *F. campestris* from Sicily with those from central Italy and Turkey [12-14]. The major compounds were 2,4,5-trimethylbenzaldehyde and 2,4,6-trimethylbenzaldehyde which percentage was dependent on the type of extraction (MAHD or HD). 2,4,5-Timethylbenzaldehyde was the most abundant component in Turkish oils, while it did not occur in samples from Italy [11, 14].

The antioxidant activity of *F. campestris* oil, obtained from fruits and roots evaluated by DPPH radical-scavenging and three additional test systems showed good radical scavenging potential of the oil [12]. Also, *F. campestris* oil was tested against a panel of human pathogenic bacteria and fungi. The oil manifested moderately activity on growth of *Streptococcus mutans* and the yeast *Candida albicans* [11]. Significant antimicrobial activity of *F. galbanifera* essential oil from Turkey was observed against *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris* and *Candida albicans* [13], while essential oil of *F. bernardii* possessed generally weak antimicrobial activity [6].

This is the first report about biological activity of any *Ferulago* species from this region and thereby about this rare and relict species. Since many representatives of Apiaceae family are known for their pharmaceutical and dietary application, this study provides new data with the purpose of demonstrating of *F. macedonica* antimicrobial and preservative features.

2. Materials and Methods

2.1. Solvents and chemical reagents

All solvents and chemicals were of analytical grade. Organic solvents were procured from Zorka pharma, Šabac, Serbia. Gallic acid (with purity degree of 98%), 3-tert-butyl-4-hydroxytoluene (BHT) (≥99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (≥99%), Folin–Ciocalteu phenol reagent,

potassium acetate ($C_2H_3KO_2$) (≥99%), and aluminum trinitrate nonahydrate ($Al(NO_3)_3x9H_2O$) (≥98%) were obtained from Sigma - Aldrich Co., St Louis, MO, USA. Sodium carbonate anhydrous (Na_2CO_3) (analytical grade) was purchased from Centrohem d.o.o., Stara Pazova, Serbia. Potassium peroxidisulphate ($K_2O_8S_2$) (≥99%) and L(+)-Ascorbic acid (Vitamin C) (analytical grade) were obtained from Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK. ABTS (≥98%) and quercetin hydrate (≥98%) were purchased from TCI Europe NV, Boerenveldsweg, Belgium.

2.2. Plant material

Plant material was collected in July 2011, in surrounding of Negotino city in Macedonia and determined as *Ferulago macedonica* Micevski & Mayer by Prof. V. Matevski. A voucher specimen for *F. macedonica* (BEOU 16656), has been deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

2.3. Essential oil isolation

The air dried plant material (200g) was subjected to hydrodistilation for 3h using a Clevenger type apparatus [15]. The oil was preserved in a sealed vials at 4 ºC prior to the further analysis. The yield of the oil was 0.6 % for herbal parts and 0.02 % for inflorescences (w/w-dry bases).

2.4. Preparation of plant extracts

Plant material was air dried in the dark at room temperature and pulverized into a powder. Each plant powder (10 g) was extracted with 200 mL of different solvents (methanol, ethanol, water) for 24 hours. The mixtures were exposed to ultrasound for the first and the last hour of extraction. After filtration with Whatman filter paper (No 1), the solvents were evaporated under reduce pressure by rotary evaporator at maximum temperature of 60 ºC, while the aqueous extracts were frozen and later lyophilized. After evaporation, the crude extracts were measured, packed in glass and plastic bottles and stored at 4 ºC until subjection to subsequent analysis. Obtained yields of *F. macedonica* inflorescence extracts were 3.75 g (methanol), 2.15 g (ethanol), 2.02 g (aqueous), 2.57 g (aqueous extract pretreated with chloroform). The yields of extracts of aerial parts of the plant were 1.33 g, (methanol), 1.17 g (ethanol), 1.32 g (aqueous), 1.35 g (aqueous extract pretreated with chloroform).

2.5. Gas chromatography (GC) and gas chromatography- mass spectrometry (GC-MS)

Qualitative and quantitative analyses of the essential oils were performed using GC and GC-MS. The GC analysis of the oil was carried out on a GC HP-5890 II apparatus, equipped with split-splitless injector, attached to HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to FID. Carrier gas flow rate (H_2) was 1 mL/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while column temperature was linearly programmed from 40-240 °C (at rate of 4 °/min). The same analytical conditions were employed for GC-MS analysis, where HP G 1800C Series II GCD system equipped with HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness) was used. Transfer line was heated at 260 °C. Mass spectra were acquired in EI mode (70 eV), in *m/z* range 40-400. An identification of the individual essential oil components was accomplished by comparison of retention times with standard substances and by matching mass spectral data with those held in Wiley 275 library of mass spectra. Confirmation was performed using AMDIS software and literature [16]. For the purpose of quantitative analysis area percents obtained by FID were used as a base.

2.6. Antioxidant activity

2.6.1. Determination of DPPH free radical scavenging activity

Free radical scavenging activity of plant extracts was assessed by DPPH (2,2-diphenyl-1-picrylhydrazil) method described by Blois (1958) [17]. A JENWAY 6306 UV/Vis spectrophotometer was used to evaluate the quantity of the solution of extracts needed to reduce 50% of the initial DPPH concentration.

Briefly, a series of extract solutions with varying concentrations (0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL and 1 mg/mL) were obtained with serial dilution technique by dissolving dried extracts in appropriate solvents. Serial dilutions were carried out with stock solution (10 mg/mL) of the extract. 0.2 mL of each extract dilution was added to 1.8 mL of DPPH solution (DPPH dissolved in methanol with concentration of 0.04 mg/mL). Methanol was used as blank test, while butylated hydroxytoluene (BHT) was used as a reference standard. After 30 min. of dark incubation at room temperature, the absorbance was recorded at 517 nm. The corresponding percentage of inhibitions of each extract was calculated from obtained absorbance values by using following equation:

$$\text{Percentage (\%) of inhibition} = (Ac\text{-}As) / Ac \times 100$$

The extracts concentrations which decrease absorption of DPPH solution for 50% (IC_{50}) were obtained from the curve dependence of absorption of DPPH solution on 517 nm from concentration for each tested extract and used standard [17].

2.6.2. Determination of ABTS radical scavenging activity

For determination of *in vitro* ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical- scavenging, the procedure of Miller and Rice-Evans (1997) [18] was followed, with some modifications. Stock solution included mixture of 5 mL of 2.46 mM potassium persulfate and 19.2 mg of ABTS, which was allowed to react for 12-16 h at room temperature in the dark before use. Then, 1 mL of $ABTS^{\cdot+}$ solution was diluted with 100-110 mL distilled water to adjust an absorbance of 0.7 ± 0.02 units at 734 nm. To determine the scavenging activity, 2 mL of diluted $ABTS^{\cdot+}$ solution was added to 50 μL of each plant extract, and the mixture was incubated for 30 minutes at 30 ºC. The absorbance was recorded at 734 nm (JENWAY 6306 UV/Vis), using water as a blank. For every experiment, fresh $ABTS^{\cdot+}$ solution was prepared.

The results were expressed from Vitamin C calibration curve (0-2 mg/L) in mg of Vitamin C equivalents/g of dry extract. Tests were carried out in triplicate and all measurements were expressed as average of three analyses ± standard deviation.

2.6.3. Total phenolic content (TPC)

Total phenolics of all extracts were detected spectrophotometrically by Folin-Ciocalteu reagent and gallic acid as a standard, according to the method described by Singleton et al. (1999) [19] with some modifications. 200 μL of tested sample (1 mg/mL) was added to 1000 μL of 10% Folin-Ciocalteau reagent. After 6 minutes incubation in the dark and adding 800 μL of 7.5% sodium carbonate solution, the mixture was allowed to stand for 2 h at room temperature under condition of darkness. The absorbance was measured at 736 nm on JENWAY 6305 UV/Vis spectrophotometer versus blank sample. Total phenols were calculated from gallic acid (GA) calibration curve (10-100 mg/L). Data were expressed as milligrams of gallic acid equivalents per gram of dry plant extract. The values were presented as means of triplicate analysis.

2.6.4. Total flavonoid content (TFC)

Measurement of total flavonoid concentrations in extracts was based on the method described by Park et al. (1997) [20] with slight modifications. Briefly, an aliquot of each sample (1 mL) was mixed with 80% C_2H_5OH, 10% $Al(NO_3)_3$ x 9 H_2O and 1M $C_2H_3KO_2$. Absorption readings at 415 nm, using JENWAY 6305 UV/Vis spectrophotometer, were taken after 40 minutes against blank sample consisting of a 0.5 mL 96% C_2H_5OH instead of tested extract. The total flavonoid content was determined from quercetin hydrate standard curve (Qu) (10-100 mg/L). Results were expressed as mg of quercetin hydrate equivalents (Qu)/g of dry extract. Measurements were done in triplicates.

2.7. Antimicrobial activity

2.7.1. Preparation of stock solutions of plant extracts

Stock solutions of respective plant extracts were prepared by dissolving dry plant extracts in 5% dimethylsulphoxide (DMSO) at concentration of 20 mg/mL, except of some aqueous extracts which were prepared at concentration of 60 mg/mL. Different concentrations of stock extract solutions were tested against different microorganisms.

2.7.2. Microbial cultures treated isolates

The antimicrobial activity of all investigated samples was tested using pure control strains obtained from Mycological laboratory, Department of Plant Physiology, Institute for Biologycal Research "Siniša Stanković" in Belgrade, Serbia. The microorganisms included four Gram negative (-) bacterial strains: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311) and *Enterobacter cloacae* (human isolate). Tested Gram positive (+) bacteria were: *Bacillus cereus* (human isolate), *Listeria monocytogenes* (NCTC 7973), *Staphylococcus aureus* (ATCC 6538) and *Micrococcus flavus* (ATCC 10240). The following micromicetes were used: yeast *Candida albicans* (ATCC 10231), *Trichoderma viride* (IAM 5061), *Penicillium ochrochloron (*ATCC 9112), *Penicillium funiculosum* (ATCC 10509), *Aspergillus ochraceus (*ATCC 12066), *Aspergillus fumigatus* (ATCC 9197), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC6275). Dilutions of bacterial inocula were cultured on solid Hilton Miller (MH) medium, while micromycetes were maintained on solid malt agar (MA) medium. The cultures were subcultured once a month and stored at +4 °C for further use [21].

2.7.3. Micro-well dilution assay

Modified microdilution technique [22] was employed for determination of antimicrobial activity of *Ferulago* extracts and oils. Assays were performed by sterile 96-well microtiter plates, by making serial dilutions of tested extracts in 5% DMSO and adding them to corresponding medium (Tryptic Soy Broth (TSB) and Malt Agar (MA), for bacteria and fungi, respectively).

To obtain the concentration of 1.0 x 10^8 CFU/mL for bacterial strains, 100 µL of overnight culture was added to eppendorf with 900 µL of medium (containing approximately 1.0 x 10^9 colony forming units (CFU)/mL). Fungal inoculum was prepared by washing spores with sterile 0.85% saline solution (which contains 0.1% Tween 80 (v/v)). The microbial cell suspensions were adjusted with sterile saline to a concentration of approximately 1.0 x 10^6 (for bacteria) and 1.0 x 10^5 (for fungi) in a final volume of 100 µL per well.

The microplates were incubated for 24 h at 37 °C for bacteria and for 72 h at 28 °C, for fungi. The lowest concentrations of samples without visible growth (at the binocular microscope) of strains which completely inhibited strains growth were defined as minimum inhibitory concentrations (MICs). The minimum bactericidal/fungicidal concentrations (MBCs, MFCs) were determined as the lowest concentration with no visible growth after serial subcultivation, indicating 99.5% killing of the

original inoculums.Two replicates were done for each sample. In addition, bacterial growth was determined by a colorimetric microbial viability assay, based on reduction of an 0.2% p-iodonitrotetrazolium violet color (INT) aqueous solution (I 8377-Sigma Aldrich, St. Louis, MQ, USA) and compared with positive control for each bacterial strain [23].

Two standards were included as positive controls: streptomycin with concentration of 1 mg/mL 5% DMSO solution (Sigma Aldrich, St. Louis, MQ, USA) for bacteria and fluconazole (antimycotic Diflucan containing 50 mg fluconazole) used at concentration of 2 mg/mL 5% DMSO solution (Pfizer PGM, Pocesur - Cisse, France) for fungi. Sterilized distillated water containing 0.02% Tween 80 and 5% DMSO were used as negative control.

3. Results and Discussion

3.1. Essential oil composition

Essential oils from inflorescences and aerial parts of *F. macedonica* were analyzed separately by GC and GC-MS techniques. A total of ninety-four different compounds in both oils were identified as shown in Table 1. Thirty-eight components were identified, representing 99.89% of the inflorescence. Monoterpene hydrocarbons were dominant group (86.97%), followed by oxygenated monoterpenes (10.77%). Oxygenated sesquiterpenes reached 1.15%, and sesquiterpene hydrocarbons 1.00% of the total oil. The oil was characterized by high content of α-pinene (43.10%) and sabinene (26.73%), followed by limonene (6.53%), myrcene (4.52%), terpinen-4-ol (3.97%), *p*-cymene (2.95%), *cis*–chrysanthenyl acetate (2.78%) and β-pinene (1.49%). Other components were present in amounts less than 1%, as listed in Table 1.

In essential oil from aerial parts fifty-six compounds were identified, representing 98.80% of total oil. The essential oil was dominated by monoterpene hydrocarbons (55.94%) and oxygenated monoterpens (36.81%), followed by oxygenated sesquiterpenes (3.16%) and sesquiterpene hydrocarbons (2.90%). The most abundant components were α-pinene (22.78%), sabinene (15.46%), terpinen-4-ol (9.60%), *cis*-chrysanthenyl acetate (9.46%) and *p*-cymene (9.08%). In the second group with less present compounds limonene (3.88%), *p*-cimen-8-ol (2.39%), *trans*-verbenol (2.35%), α-thujene (1.84%), *trans*-pinocarveol (1.68%), verbenon (1.35%), myrtenol (1.27%), myrcene (1.15%) and caryophyllene oxide (1.13%) were detected. The other constituents were identified in very small amounts (>1%) (Table 1).

Both oil compositions of *F. macedonica* were chemically similar in terms of amount of different groups of compounds and the most dominant constituents. Still, some differences were observed like in total number of compounds and the presence or the abundance of some components in one of the tested oils. *p*-Cymen-8-ol (2.39%) and *trans*-pinocarveol (1.68%) were present in essential oil of aerial parts but completely absent in essential oil from inflorescences of the investigated samples. In both analyzed oils, the most abundant group of compounds were monoterpene hydrocarbons (86.97% and 55.94% in inflorescence and aerial parts, respectively), while sesquiterpene hydrocarbons were present in the smallest amounts (1.00% and 2.90%). α-Pinene (43.10% and 22.78% for inflorescences and aerial parts, respectively) and sabinene (26.73% and 15.46% for inflorescences and aerial parts, respectively were the most dominant components in both analyzed oils).

According to available data there is no report about essential oil composition of *F. macedonica* to date. Several *Ferulago* species from Turkey and Greece were studied for their essential oil compositions [7, 13,14, 24, 25, 26].

Essential oil of *F. bernardii* was characterized by large amount of oxygenated monoterpenes (46.4%) and monoterpene hydrocarbons (23.7%). The main components were 2,4,5-trimethyl-benzaldehyde (21.2%) and α-pinene (17.0%) [6]. Similar compounds, 2,3,6-trimethyl-benzaldehyde (29.0%) and α-pinene (17.0%) were detected as dominant constituents of *F. longistylis* fruits oil by Ozkan et al. (2008) [27].

Rustaiyan et al. (2010) found that Iranian *F. contracta* oil was rich in β-phellandrene, α-phellandrene, β-eudesmol, (E)-β-ocimene. When comparing these results with results obtained in our study, it is obvious that significant difference exists in essential oil composition of those two species, since all main components of *F. contracta* were absent in *F. macedonica,* probably due to different ecological factors [29]. From provided data it can be seen that α-pinene was present in high quantity in most of analyzed essential oil of species from *Ferulago* genus.

3.2. Antioxidant activity

The extracts of *F. macedonica* were screened for their antioxidant activity by two *in vitro* test systems (DPPH and ABTS radical-scavenging). Four extracts along with the reference chemicals, gallic acid and quercetin hydrate, were further analyzed to determine their total phenolic content by Folin-Ciocalteau and total flavonoid content by Park et al. (1997) method. Some studies were made for determination of antioxidant activity and effect on lipid peroxidation of some *Ferulago* species, but no earlier reports are available regarding antioxidant activity of *F. macedonica* extracts. Earlier, the antioxidant activities of *F. campestris* fruits and roots essential oils were published [12], so as of *F. sandrasica* essential oil [30]. Also, the beneficial effect on inhibition of lipid peroxidation of the hydroalcoholic *F. angulata* extract was observed by Rafieian-Kopaei et al. (2014) [31].

Table 1. Chemical composition of *F. macedonica* essential oil of inflorescence and aerial parts

No.	Compounds	KIE	KIL	%[a]	%[b]	Classes
1	*α*-Thujene	924.1	924	0.56	1.84	
2	*α*-Pinene	931.0	932	**43.10**	**22.78**	
3	Camphene	943.5	946	0.20	0.14	
4	Thuja-2,4(10)-diene	950.0	953	0.12	0.39	
5	Sabinene	970.6	969	26.73	15.46	
6	*β*-Pinene	972.7	974	1.49	0.96	
7	Myrcene	990.0	988	4.52	1.15	**A**
8	Mesitylene	993.2	994	-	0.11	
9	*α*-Terpinene	1014.2	1014	0.06	-	
10	*p*-Cymene	1022.2	1020	2.95	9.08	
11	Limonene	1025.8	1024	6.53	3.88	
12	*cis*-*β*-Ocimene	1037.7	1032	0.23	-	
13	*trans*-*β*-Ocimene	1047.8	1044	0.28	-	
14	*γ*-Terpinene	1056.3	1054	0.20	0.15	
15	*cis*-Sabinene hydrate	1066.6	1065	0.20	0.35	
16	6-Camphenone	1093.3	1095	0.32	0.45	
17	*trans*-Sabinene hydrate	1097.9	1098	0.15	0.63	
18	Linalool	1102.2	1095	-	0.14	
19	*β*-Thujone	1114.8	1112	-	0.09	
20	*cis*-*p*-Menth-2-en-1-ol	1120.9	1118	0.22	0.55	
21	*α*-Campholenal	1124.2	1122	0.11	0.71	
22	*trans*-Pinocarveol	1137.3	1135	-	1.68	
23	*cis*-Verbenol	1139.8	1137	0.47	-	
24	*trans*-Verbenol	1144.5	1140	0.66	2.35	
25	Sabina ketone	1156.6	1154	-	0.78	
26	Pinocarvone	1160.0	1160	0.09	-	
27	*cis*-Chrysanthenol	1165.1	1160	0.11	0.85	
28	*cis*-Sabinol	1171.5	1170	-	0.41	**B**
29	Terpinen-4-ol	1176.0	1174	3.97	9.60	
30	*p*-Cymen-8-ol	1188.1	1179	-	2.39	
31	*α* -Terpineol	1191.8	1186	0.30	-	
32	Myrtenal	1194.3	1195	0.15	0.40	
33	Myrtenol	1197.3	1194	0.28	1.27	
34	Verbenon	1207.7	1204	0.33	1.35	
35	*trans*-Carveol	1221.7	1215	-	0.78	
36	*cis*-Carveol	1225.1	1226	0.21	-	
37	Carvone	1244.7	1239	-	0.32	
38	*cis*-Chrysanthenyl acetate	1259.6	1261	2.78	9.46	
39	*iso*-3-Thujanol-acetate	1269.7	1267	-	0.06	
40	*trans*-Ascaridole glycol	1273.9	1273	0.18	0.27	
41	*α*-Terpinen-7-al	1285.4	1283	-	0.16	
42	*p*-Cymen-7-ol	1296.5	1289	-	0.93	
43	*cis*-Pinocarvyl acetate	1303.1	1311	0.24	0.07	

44	Carvacrol	1313.3	1298	-	0.76	
45	*α*-Copaene	1371.9	1374	0.15	0.16	
46	*β*-Bourbonene	1380.5	1387	-	0.06	
47	*β*-Elemene	1389.1	1389	-	0.11	
48	Methyl eugenol	1407.4	1403	-	0.61	
49	*β*-Caryophyllene	1414.8	1417	0.37	0.20	
50	6,9-Guaiadiene	1452.2	1442	-	0.13	**C**
51	*trans*-*β*-Farnesene	1455.2	1454	0.25	0.46	
52	Germacrene D	1477.3	1484	0.23	-	
53	*β*-Selinene	1482.0	1489	-	0.69	
54	Valencene	1489.2	1496	-	0.34	
55	*α*-Muurolene	1496.9	1500	-	0.14	
56	Myristicin	1524.9	1517	0.48	0.44	
57	Salviadienol	1553.5	1545	-	0.27	
58	Spathulenol	1576.0	1577	-	0.55	
59	Caryophyllene oxyde	1579.6	1582	0.52	1.13	
60	Humulene epoxide II	1605.8	1608	-	0.36	**D**
61	*β*-Biotol	1611.4	1612	-	0.11	
62	Muurola-4,10(14)-dien-1- β -ol	1629.6	1630	0.15	-	
63	Eudesma-4(15),7-dien-1 *β*-ol	1685.5	1687	-	0.13	
64	Octyl benzoate	1788.0	1792	-	0.17	
	Groups of components					
	A - monoterpene hydrocarbons (%)			86.97	55.94	
	B - oxygenated monoterpens (%)			10.77	36.81	
	C - sesqiterpene hydrocarbons (%)			1.00	2.90	
	D - oxygenated sesquiterpenes (%)			1.15	3.16	
	Total identified (%)			99.89	98.80	

KIE = Kovats (retention) index experimentally determined (AMDIS) KIL = Kovats (retention) index - literature data (Adams, 2007)
[a] Inflorescence
[b] Aerial parts
- Not identified

3.2.1. DPPH scavenging activity

In the present study, radical scavenging activities of tested extracts were dose-dependent; activity increased at higher extract concentrations, as shown in Table 2. The obtained results showed that the strongest radical scavenging capacity have methanol extracts of inflorescence and aerial parts, with values of IC_{50}=490 μg/mL and IC_{50}=630 μg/mL of solution, respectively. Other extracts demonstrated moderate radical scavenging capacity with IC_{50} values of 730-1810 μg/mL of solution. When compared with synthetic antioxidant BHT (IC_{50}=140 mg/mL), all of the investigated extracts showed lower antioxidant activity.

Table 2. DPPH and ABTS results, total phenolic (TPC) and flavonoid contents (TFC) of F. *macedonica* extracts (C=1 mg/mL)

	Extracts	DPPH IC_{50} (µg/mL)	ABTS (mg VitC/g of dry extract)	Total phenols (mg GE/g of dry extract)	Total flavonoids (mg QE/g of dry extract)
Methanol	Inflorescence	490	1.63 ± 0.008	47.90 ± 0.001	20.90 ± 0.004
	Aerial parts	630	0.23 ± 0.005	44.81 ± 0.009	10.56 ± 0.011
Ethanol	Inflorescence	730	1.69 ± 0.002	59.68 ± 0.023	23.73 ± 0.013
	Aerial parts	1100	1.47 ± 0.002	34.12 ± 0.017	23.50 ± 0.004
Aqueous	Inflorescence	1170	0.81 ± 0.009	50.59 ± 0.011	14.92 ± 0.011
	Aerial parts	1810	0.60 ± 0.005	49.41 ± 0.010	11.05 ± 0.003
Aqueous [h]	Inflorescence	1180	0.99 ± 0.004	58.99 ± 0.000	14.50 ± 0.041
	Aerial parts	1230	0.30 ± 0.000	35.11 ± 0.006	9.08 ± 0.016
Standard		BHT	Quercetin (1mg/mL)	/	/
		140	2.749 ± 0.004		

Each value in the table was obtained by calculating the average of three analyses (± standard deviation)
[h] Aqueous extracts pretreated with chloroform

3.2.2. ABTS scavenging activity

As summarized in Table 2, extracts (1 mg/mL) revealed radical-scavenging effects on $ABTS^{+}$ in range of 0.23-1.69 mg/L. Activities of ethanol and methanol extracts of inflorescence of the plant were the highest with values of 1.69 ± 0.002 and 1.63 ± 0.008 mg VitC/g of dry extracts, respectively. The lowest activity was identified for the methanol extract of the aerial parts (0.23 ± 0.005 mg VitC/g of dry extract). The extracts evinced moderate to low antioxidant capacity compared to quercetin hydrate (Table 2).

3.2.3. Total phenolic concentrations

Phenolic concentrations of *F. macedonica* extracts (1 mg/mL) were determined spectrophotometrically. Results presented in Table 2, expressed as gallic acid equivalents per g of dry plant extract, ranged between 34.12 ± 0.017 (for ethanol extract of steams and leaves) as the lowest content to 59.68 ± 0.023 mg (for ethanol extract of inflorescence) GA/g of plant extract, as the highest value.

3.2.4. Total flavonoid contents

Table 2 summarizes that total flavonoid content in extract solutions (1 mg/mL) varied widely, ranging from 9.08 ± 0.016 to 23.73 ± 0.013 mg/g expressed as quercetin equivalents (QE) per gram of dry extract. The highest flavonoid content was identified in ethanol extracts of inflorescence (23.73 ± 0.013 mg QE/g of dry extract) and aerial parts of the plant (23.50 ± 0.004mg QE/g of dry extract), followed by methanol extract of inflorescence. The aqueous extracts showed lower flavonoid concentrations.

In general, there was not strong relationship between total phenolic and flavonoid contents and anti-ABTS and DPPH radical activities. These results can be explained since the antioxidant property of a plant extract is generally considered as the result of the combined activity of a wide range of

compounds including, not only phenolics, but also peptides, organic acids and other components [32]. Both, phenols and flavonoids were present in higher amounts in *F. macedonica* extracts of inflorescence.

3.3. Antimicrobial activity

Micro-well dilution technique was used to determine *in vitro* antimicrobial activity in order to allow screening of effect *F. macedonica* extracts on various bacterial and fungal strains.

3.3.1. Antibacterial activity

In vitro activity of essential oils and various extracts on some bacterial strains is presented in Tables 3 and 4. Minimum inhibitory and minimum bactericidal concentrations were defined (mg/mL). According to the results (Table 3), extracts of tested species showed lower effectiveness against a set of eight bacteria compared to streptomycin, standard antibiotic that was used as positive control. Both tested essential oils exhibited strong antibacterial activity, according to the results listed in Table 4.

Inhibitory effects of plant extracts were moderate against selected human pathogenic bacteria with MIC values ranging between 4-16 mg/mL, while minimum bactericidal concentrations were from 8-28 mg/mL, for all extracts. The results indicate that Gram-positive bacteria were more sensitive (MBCs 8-17 mg/mL) than Gram-negative strains (MBCs=9-28 mg/mL). When comparing the effectiveness of extract of inflorescence (MICs=4-12 mg/mL; MBCs=8-17 mg/mL) and aerial (MICs=4-16 mg/mL; MBCs=8-28 mg/mL) parts separately, the slightly lower activity was observed for aerial parts (the lowest had methanol extract of aboveground parts on *E. coli* (MIC=16 mg/mL; MBC=28 mg/mL).

The strongest potency had methanol extract of inflorescence (MICs=4-8 mg/mL; MBCs=9-13 mg/mL), while both types of aqueous extracts showed the lowest effects (MICs=4-12 mg/mL; MBCs =8-17 mg/mL). It can be seen in Table 3 that the most susceptible strains were *B. cereus* and *L. monocytogenes*. These findings could be of great importance since strains of *Bacillus cereus* species are producing two kinds of strong enterotoxins causing different clinical and epidemic forms of food poisoning, while bacteria *Listeria monocytogenes* can cause meningitis, encephalitis or septicemia at newborns [29]. The most resistant strains on tested *F. macedonica* extracts concentrations were *E. coli* and *P. aeruginosa*. Compared to streptomycin (MBCs=20 mg/mL), all extracts showed stronger effect on *L. monocytogenes* (MBC=8-14 mg/mL) and *E. cloacae* (MBC=10-14 mg/mL).

Table 3. Results of antibacterial activity of *F. macedonica* various extracts in terms of MICs and MBCs (mg/mL)

Tested bacterial strains	Methanol				Ethanol				Aqueous				Aqueous[h]				Streptomycin	
	Inflorescence		Aerial parts		Inflorescence		Aerial parts		Inflorescence		Aerial parts		Inflorescence		Aerial parts			
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram (-) bacteria																		
Escherichia coli	8.0	13.0	16.0	28.0	10.0	12.0	8.0	10.0	12.0	14.0	12.0	13.0	10.0	15.0	10.0	16.0	2.5	5.0
Pseudomonas aeruginosa	8.0	11.0	8.0	12.0	10.0	14.0	10.0	14.0	8.0	12.0	8.0	12.0	10.0	12.0	10.0	12.0	2.5	5.0
Salmonella typhimurium	8.0	10.0	8.0	9.0	10.0	11.0	10.0	12.0	8.0	12.0	8.0	12.0	10.0	12.0	10.0	12.0	2.5	5.0
Enterobacter cloace	6.0	11.0	4.0	10.0	10.0	14.0	10.0	14.0	8.0	12.0	8.0	12.0	10.0	12.0	10.0	12.0	10.0	20.0
Gram (+) bacteria																		
Bacillus cereus	5.0	9.0	6.0	10.0	10.0	12.0	8.0	12.0	4.0	8.0	8.0	10.0	10.0	12.0	10.0	12.0	1.5	2.5
Listeria monocytogenes	4.0	9.0	5.0	8.0	8.0	10.0	8.0	9.0	8.0	12.0	8.0	12.0	10.0	12.0	10.0	12.0	15.0	20.0
Staphylococcus aureus	8.0	10.0	6.0	9.0	10.0	11.0	9.0	12.0	8.0	12.0	8.0	12.0	10.0	12.0	10.0	12.0	2.5	5.0
Micrococcus flavus	8.0	13.0	8.0	12.0	12.0	14.0	12.0	14.0	12.0	17.0	12.0	13.0	10.0	15.0	8.0	11.0	2.5	5.0

[h] Aqueous extracts pretreated with chloroform

Both oils exhibited strong antibacterial activity. It can be explained by dominance of α-pinene which is reported as one of the most effective antimicrobial agents occuring in the essential oils [10]. Tested oils had much stronger activity than streptomycin against *Listeria monocytogenes* (MBCs = 4.5 and 4.8 mg/mL for oils of inflorescence and aerial parts, respectively; MBC=20 mg/mL for streptomicyn). *B. cereus* and *L. monocytoges* were equally sensitive to both oils, while *P. aeruginosa* and *S. tiphymurium* were more susceptible to oil from aerial parts. This can be attributed due to more complex composition of the oil from aerial parts and possible synergistic effect among its chemical constituents. The most sensitive strain was *B. cereus*, followed by *P. aeruginosa*, as shown in Table 4.

These are the first results for antimicrobial activity of *F. macedonica*, obtained using micro-well dilution assay. Our results can be compared with some previous studies about antimicrobial activitiy of essential oil of related *Ferulago* species. In research by Taran et al. (2010), antibacterial effect of *F. angulata* subsp. *carduchorum* essential oil of aerial parts and seeds was evaluated against four bacterial genera. It was found that the most sensitive were Gram-positive bacteria *S. aureus* (MIC=15 μg/mL and MIC=>$4x10^3$ μg/mL) and *L. monocytogenes* (MIC=170 μg/mL and MIC=137 μg/mL), while *E. coli* (MIC=>$1.9x10^3$ μg/mL and MIC=$4x10^3$ μg/mL) showed the lowest suscebility to both tested oils. Also it was observed that oil of aerial parts provided a higher inhibition activity against all tested microorganisms [5]. Similar results on the sensitivity of tested strains was obtained in our study.

Table 4. Antibacterial activity of *F. macedonica* essential oil of inflorescence and aerial parts (mg/mL)

Tested bacteria	F.m. [i]		F.m. [ap]		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram (+) bacteria						
Bacillus cereus	1.1	2.2	1.2	2.4	1.5	2.5
Listeria monocytogenes	0.6	4.5	0.6	4.8	15.0	20.0
Gram (-) bacteria						
Pseudomonas aeruginosa	1.1	4.5	0.6	2.4	2.5	5.0
Salmonella typhimurium	2.2	9.0	2.4	4.8	2.5	5.0

F. m. = *Ferulago macedonica*
[i] Essential oil of inflorescence
[ap] Essential oil of aerial parts

According to Khalighi-Sigaroodi et al. (2005) *F. bernardii* essential oil did not show any activity against *Pseudomonas aeruginosa* (MICs>1000 μg/mL), while antimicrobial activity was detected against *Bacillus subtilis* (MICs<125 μg/mL) and *Staphylococcus aureus* (MICs=250 μg/mL) [6]. In our investigation, both *F. macedonica* essential oils and all axtracts exhibited activity against *P. aeruginosa* as presented in Tables 3 and 4.

Maggi et al. (2009) reported that *F. campestris* essential oils of flowers and leaves from central Italy showed that they were particulary effective on *B. subtilis* and *E. coli*, but no inhibition activity was observed against *S. aureus*, unlike *F. macedonica* extracts that showed the lowest effectivenes on *E. coli* but exhibited moderate activity against *S. aureus* [10]. Research done by Cecchini et al. (2010) on *F. campestris*, showed that essential oil obtained from roots had significantly stronger activity than oil obtained from fruits regarding to tested bacterial strains. Those two oils differed quantitativilly in major compounds; α-pinene was by far the most abundant volatile of the oil of roots [12], which is also the most dominant oil constituent of both analyzed *F. macedonica* oils. For

F. longistylis [27] and *F. confusa* oils from fruits [26] both originated from Turkey, only moderate to low activity was observed against the used microorganisms. Similar conclusion was brought for *F. macedonica* extracts, while essential oils showed good antimicrobial activity.

3.3.2. Antifungal activity

F. macedonica oils and extracts were assayed for their antifungal potency using micro-well dilution technique, by calculating MIC and MFC values of tested concentrations (mg/mL). From the results given in Table 5 it can be concluded that tested extracts expressed moderate to low activity, or were unaffected against some fungi, while oils showed slightly lower activity than commercial antimycotic fluconazole (Table 6).

Table 5. Antifungal activity evaluated by micro-well dilution method for tested *F. macedonica* extracts (mg/mL)

Tested fungy	Methanol				Ethanol				Aqueous				Aqueous[h]				Fluconazole	
	Inflorescence		Aerial parts		Inflorescence		Aerial parts		Inflorescence		Aerial parts		Inflorescence		Aerial Parts			
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans	2.00	6.00	8.00	16.00	10.00	12.00	4.00	6.00	12.00	16.00	6.00	10.00	12.00	16.00	16.00	17.00	0.02	0.03
Aspergillus ochraceus	10.00	14.00	10.00	14.00	6.00	10.00	6.00	10.00	16.00	>36.00	12.00	16.00	10.00	14.00	16.00	19.00	0.50	1.00
Trichoderma viride	2.00	6.00	4.00	6.00	2.00	4.00	6.00	8.00	16.00	17.00	12.00	16.00	12.00	16.00	16.00	19.00	1.00	1.50
Aspergillus versicolor	4.00	8.00	8.00	12.00	6.00	10.00	6.00	10.00	12.00	16.00	4.00	6.00	12.00	16.00	16.00	17.00	0.13	0.50
Penicillium funiculosum	8.00	12.00	10.00	14.00	12.00	16.00	12.00	16.00	16.00	19.00	12.00	16.00	12.00	16.00	16.00	19.00	0.25	0.50
Penicillium ochlochloron	2.00	4.00	2.00	4.00	6.00	8.00	3.00	4.00	12.00	14.00	2.00	4.00	12.00	16.00	16.00	17.00	1.00	1.50
Aspergillus fumigatus	8.00	12.00	8.00	12.00	6.00	10.00	8.00	10.00	12.00	14.00	12.00	14.00	14.00	16.00	16.00	17.00	0.50	1.00
Aspergillus niger	12.00	18.00	10.00	18.00	14.00	19.00	12.00	19.00	16.00	>42.00	18.00	42.00	14.00	>42.00	36.00	>54.00	0.25	1.00

[h] Aqueous extracts pretreated with chloroform

The extracts had an inhibitory effect on mycelial growth at concentrations of 2-36 mg/mL and fungicidal activity of extracts was detected in range of 4->54 mg/mL. Results indicated that *P. ochrochloron* (MICs=2-16 mg/mL, MFCs=4-17 mg/mL) and *T. viride* (MICs=2-16 mg/mL, MFCs=4-19 mg/mL) were the most sensitive fungal strains. Extracts concentrations that were effective in inhibition of growth of *A. niger* ranged from 10 to 36 mg/mL, while effective fungicidal amounts were between 18->54 mg/mL. The methanol extract of inflorescence was found to be the strongest (MICs=2-12 mg/mL; MFCs=6-18 mg/mL). Conversely, both type of aqueous extracts exhibited the least activity with MIC values of 2-36 mg/mL and MFC values from 4 to >54 mg/mL. That is considered as significantly weak inhibitory activity of extracts when compared to fluconazole (concentration of 2 mg/mL). Regarding *A. niger*, fungicidal activity of aqueous extracts was not detected even at the highest tested concentrations (MFC=>54 mg/mL). According to data from Table 5, there was no significant difference in potency between inflorescence and aerial parts of the plant extracts. However, it was noted that the most resistant strains to fluconazole, were the most sensitive to *F. macedonica* extracts.

According to this test, both oils have demonstrated strong, approximately equal effectiveness, still slightly lower compared to fluconazole (MICs=0.3-2.2 and MFCs=2.2-4.8 mg/mL for oils of inflorescence and aerial parts, respectively; MICs=0.3-1.0 and MFC=0.5-1.5 mg/mL for fluconazole). As presented in Table 6, *T. viride* and *P. funiculosum* were less sensitive than *A. fumigatus*.

Table 6. Antifungal activity of *F. macedonica* essential oil of inflorescence and aerial parts (mg/mL)

Tested fungi	**F.m.** [i]		**F.m.** [ap]		**Fluconazole**	
	MIC	MFC	MIC	MFC	MIC	MFC
Trichoderma viride	0.6	4.5	1.2	4.8	1.0	1.5
Penicillium funiculosum	1.1	4.5	1.2	4.8	0.3	0.5
Aspergillus fumigatus	0.3	2.2	2.2	2.4	0.5	1.0

F. m. = *Ferulago macedonica*
[i] Essential oil of inflorescence
[ap] Essential oil of aerial parts

In most of the available data, antifungal activities of different *Ferulago* species were assayed against various *Candida* strains [5, 6, 10, 12, 27]. Literature review shows that *F. campestris* essential oil from roots was remarkable against clinically isolated *C. albicans* (MIC values of 39-78 μg/mL), *C. tropicans* and *C. glabrata* (MIC values 78 μg/mL). This founding supports the historical use of this plant roots in gynecological affliction and may justify the use of the oil as an anti-*Candida* agent, since the clinically isolated strains are normally resistant to more than one antibiotic [12]. In another research on essential oil obtained from flowers of *F. campestris*, moderately effectiveness against yeast *C. albicans* was observed [10]. The extracts of *F. macedonica* were also tested against *C. albicans* and the inhibition activity on the yeast was low compared to used synthetic standard.

F. bernardii essential oil evaluation showed weak activity against *C. albicans* (MICs=500 μg/mL) and *A. niger* (MICs=250 μg/mL), which was similar with activity of *F. macedonica* extracts against *A. niger* (Table 5) [6]. The antimicrobial activity against *C. albicans* of *F. angulata* subsp. *carduchorum* essential oil was detected as low antifungal activity (with MICs values of $>1{,}9x10^3$ μg/mL and $>4x10^3$ μg/mL, for aerial parts and seeds oil, respectively). Still, essential oil from aerial parts of the plant had stronger effect on *C. albicans*, compared to essential oil obtained from the seeds of the plant. Better antimicrobial activity was attributed to difference in percentage of α-pinene [5].

4. Conclusion

This work represents the first report of chemical composition of essential oil and biological activity of *Ferulago macedonica*. The findings of the study revealed that essential oil and various extracts of this species possess valuable antioxidant and antimicrobial potential. In essential oils of

inflorescence and aerial parts α-pinene and sabinene were the main constituents, and both oils exhibited strong antimicrobial activity. Results of the present work indicated that methanol extract of inflorescence showed the best antioxidant potential of all tested extracts. Still, further survey and investigation to evaluate the suitability of this species is needed, so these results will aid in studying of *in vivo* biological activity and active principles of isolated compounds.

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