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RADICAL SCAVENGING AND ANTIMICROBIAL ACTIVITY OF ESSENTIAL OIL AND EXTRACTS OF *ECHINOPHORA SIBTHORPIANA* GUSS. FROM MACEDONIA

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Abstract - This study was undertaken to determine the antioxidant and antimicrobial effect of essential oil and extracts of *Echinophora sibthorpiana* Guss. (fam. Apiaceae) collected in Macedonia. The chemical composition of *E. sibthorpiana* essential oil was characterized by the presence of methyl eugenol (60.40%), *p*-cymene (11.18%) and α-phellandrene (10.23%). The free radical scavenging activity of extracts and essential oil was evaluated by DPPH and ABTS assays. The aqueous extract of aerial parts exhibited the strongest scavenging activity (IC_{50}=1.67 mg/ml); results of the ABTS test showed that the most effective was the ethanol extract of aerial parts (1.11 mg vit. C/g). The essential oil showed stronger antioxidant activity compared to hydroxyanisole, ascorbic acid and quercetin that were used in the DPPH and ABTS tests, respectively. The total phenolic and flavonoid concentrations in the extracts ranged between 38.65-60.72 mg GA/g, and 3.15-19.00 mg Qu/g, respectively. The antimicrobial properties of the extracts and essential oil were investigated using a micro-well dilution technique against human pathogenic strains. The results were comparable with the effects of the positive controls, streptomycin and fluconazole. These findings indicate that *E. sibthorpiana* extracts and oil can be used in preventive treatments and as an alternative for synthetic preservatives.

Key words: *Echinophora sibthorpiana,* Apiaceae, essential oil, extracts, DPPH, ABTS, phenols, flavonoids, antimicrobial activity

INTRODUCTION

In Europe, the genus *Echinophora* (fam. Apiaceae=Umbelliferae) is represented by two species (*E. spinosa* L. and *E. tenuifolia* L.), distributed from the Mediterranean region eastwards to Crete and Crimea. *E. tenuifolia* consists of two subspecies (subsp. *tenuifolia* and subsp. *sibthorpiana* (Guss.) Tutin) (Tutin, 1968). According to more recent literature data, later subspecies should be treated as separate species – *Echinophora sibthorpiana* Guss. (Micevski and Matevski, 2005). It is a perennial herbaceous plant, up to 60 cm tall, densely hairy, with yellow petals, short trichomes, long and strong roots (Micevski and Matevski, 2005).

E. tenuifolia L. subsp. *sibthorpiana* is traditionally used in Turkey as an antispasmodic and digestive herb (Cakilcioglu and Turkoglu, 2010). Since ancient times the seeds and root of *E. tenuifolia* are efficient in the treatment of epilepsy (Eadie, 2004). Fresh or dried herbs are used as local fungicidal medicaments,

in folk medicine for wound healing and gastric ulcers, and as flavoring agents for some foods such as meats, pickles, soups and dairy products (Baser et al., 1998, Agkul and Chialva, 1989, Kivanc, 1988). The leaves of *Echinophora sibthorpiana* (tarhana herb) are used as a spice in tarhana in various regions in Turkey (Agkul, 1993, Agkul and Kivanc, 1990). Tarhana is a fermented cereal food and one of the oldest traditional Turkish soups (Deghirmencioghlu et al., 2005, Ozdemira et al. (2007), Gurbuza et al. (2010). Tarhana herb has a pleasant flavor and it stimulates some microorganisms such as lactic acid bacteria and yeast *Saccharomyces cerevisiae* (Deghirmencioghlu et al., 2005).

Several studies of the chemical composition of *Echinophora sibthorpiana* essential oil have been done (Baser et al., 1998, Agkul and Chialva, 1989, Gokbulut et al., 2013, Ahmad et al., 1999, Baser et al., 1994). Results showed that α-phellandrene and methyl eugenol were the most abundant constituents of the oil. Gokbulut et al. (2013) acknowledged δ-3-carene as the main component of *E. tenuifolia* oil.

The dominant compound of *E. platyloba* essential oil from Iran was reported to be *trans*-β-ocimene (Rahimi-Nasrabadi et al., 2010, Asghari et al., 2003, Saei-Dehkordi et al., 2012, Gholivand et al., 2011). There are data about the *in vitro* antioxidant activity of the essential oil and methanol extracts of *Echinophora platyloba* (Saei-Dehkordi et al., 2012, Gholivand et al., 2011), as well as other biological activities (Avijgan et al., 2006, Entezari et al., 2009, Sharafati-chaleshtori et al., 2012, Delaram et al., 2011, Youse et al., 2012, Shahneh et al., 2013, Avijgan et al., 2012, Mirghazanfari et al., 2012, Delaram and Sadeghiyan, 2010). *E. tenuifolia* was also investigated for its biological activity (Deghirmencioghlu et al, 2005, Gokbulut et al., 2013, Evergetis et al., 2013).

The objectives of this study were to define the chemical composition of *E. sibthorpiana* oil, to determine the antioxidant capacity, total phenolic and flavonoid contents and antimicrobial activity of the essential oil and various extracts.

MATERIALS AND METHODS

Solvents and chemical reagents

All solvents and chemicals were of analytical grade. Organic solvents were procured from "Zorka Pharma" Šabac, Serbia. Gallic acid, 3-tert-butyl-4-hydroxyanisole (BHA), 2,2-dyphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent, potassium acetate ($C_2H_3KO_2$) and aluminum trinitrate nonahydrate ($Al(NO_3)_3x9H_2O$) were obtained from Sigma-Aldrich Co., St Louis, MO, USA. Sodium carbonate anhydrous (Na_2CO_3) was purchased from Centrohem d.o.o., Stara Pazova, Serbia. Potassium peroxidisulphate ($K_2O_8S_2$) and L(+)- ascorbic acid (Vitamin C) were obtained from Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK. ABTS and quercetin hydrate were purchased from TCI Europe NV, Boerenveldsweg, Belgium.

Plant material

Plant material was collected in July 2011, in the surrounds of Štip in the Republic of Macedonia and determined as *Echinophora sibthorpiana* Guss. by one of the authors (V.S.M.). A voucher specimen for *E. sibthorpiana* (BEOU 16657) is deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

Essential oil isolation

The air-dried plant material (200 g) was subjected to hydrodistilation for 3 h using a Clevenger type apparatus (European Pharmacopoeia, 2005). The oil was preserved in sealed vials at 4°C prior to the further analysis. The yield of the oil was 0.43% for the herbal part (w/w-dry bases).

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 h. The mix-

tures were exposed to ultrasound for the first and the last hour of extraction. After standing in the dark, the extracts were filtered through Whatman No.1 paper. The solvents were evaporated under reduced pressure at a maximum temperature of 40°C, while the aqueous extracts were frozen and later lyophilized. After evaporation, the crude extracts were packed in glass and plastic bottles and stored at 4°C until use for subsequent analysis.

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

Qualitative and quantitative analyses of the essential oil 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 a split-splitless injector attached to an HP-5 column (25 m × 0.32 mm, 0.52 µm film thickness) and fitted to FID. The carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30; the injector temperature was 250°C, detector temperature 300°C; the 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 an HP G 1800C Series II GCD system equipped with an HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness) was used. The transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), at an *m/z* range 40-400. Identification of the individual EO components was accomplished by comparison of the retention times with standard substances and by matching mass spectral data with those held in the Wiley 275 library of mass spectra. Confirmation was performed using AMDIS software and literature (Adams, 2007). For the purpose of quantitative analysis, area percents obtained by FID were used as a base.

Determination of DPPH free radical scavenging activity

The free radical scavenging activity of plant extracts was assessed by the DPPH (2,2-diphenyl-1-picrylhydrazil) method described by Blois (1958). 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 solutions with varying concentrations (0.00625-0.05 mg/ml for essential oil and 1-3.5 mg/ml for extracts) were obtained by serial dilution technique in appropriate solvents. 0.2 ml of each dilution was added to 1.8 ml of DPPH solution (DPPH dissolved in methanol with a concentration of 0.04 mg/ml). Methanol was used as the blank test, while BHA and ascorbic acid were used as reference standards. After 30 min of dark incubation at room temperature, the absorbance was recorded at 517 nm. The corresponding percentage of inhibition of each extract was calculated from the obtained absorbance values using the following equation:

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

Concentrations of the essential oil and extracts which decreased the absorption of the DPPH solution by 50% (IC_{50}) were obtained from the curve of absorption of the DPPH solution at 517 nm (Blois, 1958).

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) was followed, with some modifications. The stock solution included a 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. 1 ml of $ABTS^+$ solution was diluted with 100-110 ml distilled water to adjust the absorbance of 0.7±0.02 units at 734 nm. To determine the scavenging activity, 2 ml of diluted $ABTS^+$ solution was added to 50 µl of each tested solution, and the mixtures were incubated for 30 mn at 30°C. The absorbances were recorded at 734 nm (JENWAY 6306 UV/Vis), using water as a blank. For every experiment, fresh $ABTS^+$ solution was prepared.

The results were expressed from a 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 the average of three analyses ± standard deviation.

Total phenolic content (TPC)

The total phenolic content in all extracts was detected spectrophotometrically using the Folin-Ciocalteu reagent and gallic acid as a standard, according to the method described by Singleton et al. (1999), with some modifications. Two hundred µl of the tested extract solution (1 mg/ml) was added to 1 ml of 10% Folin-Ciocalteu reagent. After 6 min incubation in the dark and addition of 800 µl of 7.5% sodium carbonate solution, the mixture was allowed to stand for 2 h at room temperature in the dark. The absorbance was measured at 736 nm on a JENWAY 6305 UV/Vis spectrophotometer versus blank sample. Total phenols were calculated from the 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.

Total flavonoid content (TFC)

The measurement of the total flavonoid concentrations in the extracts was based on the method described by Park et al. (1997) with slight modifications. Briefly, an aliquot of each sample (1 ml) was mixed with 80% C_2H_5OH, 10% $Al(NO_3)_3x9H_2O$ and 1M $C_2H_3KO_2$. Absorption readings at 415 nm, using the JENWAY 6305 UV/Vis spectrophotometer, were taken after 40 min against the blank sample consisting of 0.5 ml 96% C_2H_5OH instead of the tested extract. The total flavonoid content was determined from a 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 triplicate.

Preparation of stock solutions of plant extracts

Stock solutions of the respective plant extracts were prepared by dissolving dry plant extracts in 5% dimethyl sulfoxide (DMSO) at a concentration of 30 mg/ml, except for some aqueous extracts which were prepared at a concentration of 60 mg/ml. Different concentrations of stock extract solutions were tested against different microorganisms.

Microbial cultures treated isolates

The antimicrobial activity of all investigated samples was tested using pure control strains obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" in Belgrade, Serbia. The microorganisms included four Gram-positive bacterial strains: *Bacillus cereus* (human isolate), *Listeria monocytogenes* (NCTC 7973), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538). The tested Gram-negative bacteria were *Enterobacter cloacae* (human isolate), *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhimurium* (ATCC 13311). The following micromycetes were used: *Aspergillus fumigatus* (ATCC 9197), *Aspergillus niger* (ATCC6275) *Aspergillus ochraceus (*ATCC 12066), *Aspergillus versicolor* (ATCC 11730)*, Candida albicans* (ATCC 10231), *Penicillium funiculosum* (ATCC 10509), *Penicillium ochrochloron (*ATCC 9112) and *Trichoderma viride* (IAM 5061). 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 (Booth, 1971).

Micro-well dilution assay

A modified microdilution technique (Hanel and Raether, 1998) was employed for the determination of the antimicrobial activity of *Echinophora* oil and extracts. The assay was performed using 96-well microtiter plates by adding dilutions of the tested extracts (in 5% DMSO) into the corresponding medium (Tryptic Soy Broth (TSB) and Malt Agar (MA), for bacteria and fungi, respectively).

To obtain the concentration of $1.0x10^8$ CFU/ml for bacterial strains, 100 µl of overnight cultures

were added to Eppendorf tubes containg with 900 µl of medium (containing approximately $1.0x10^9$ colony forming units (CFU)/ml). Fungal inocula were prepared by washing spores with a sterile 0.85% saline solution that contained 0.1% Tween 80 (v/v). The microbial cell suspensions were adjusted with sterile saline to a concentration of approximately $1.0x10^6$ (for bacteria) and $1.0x10^5$ (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 (viewed through a binocular microscope) of strains which completely inhibited the growth were defined as the 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 inocula (Hanel and Raether, 1998). Two replicates were used for each sample. In addition, bacterial growth was determined by a colorimetric microbial viability assay, based on the reduction of an 0.2% p-iodonitrotetrazolium violet color (INT) aqueous solution (I 8377–Sigma Aldrich, St. Louis, MQ, USA) and compared with a positive control for each bacterial strain (Tsukatani et al., 2012).

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

RESULTS

Essential oil composition

The chemical composition of *E. sibthorpiana* oil from Macedonia is summarized in Table 1. Overall, twenty-six constituents, representing 99.33% of the total oil composition, were identified by combined GC and GC-MS analyses. Methyl eugenol was the predominant component (60.40%), followed by *p*-cymene (11.18%) and *α*-phellandrene (10.23%). *α*-Phellandrene epoxide (6.87%), *β*-phellandrene (2.92%) and carvacrol (1.74%) were found in significant amounts. The oil was richest in oxygenated monoterpenes (42.32%) and monoterpene hydrocarbons (38.46%). Our results showed that the oil contained 11.54% of phenylpropanoids, one oxygenated sesquiterpene and one diterpene (3.85% of each).

DPPH scavenging activity

The results of the assessment of the antioxidant activity by the DPPH test are presented in Tables 2 and 3. All extracts exhibited lower free radical scavenging activities compared to the used references. The strongest effect among the tested extracts was that of the aqueous extract of the aerial parts of the plant, while the lowest effectiveness was that of the aqueous extract of the roots. In addition, extracts from the aerial parts expressed much stronger free radical scavenging than extracts of *E. sibthorpiana* (Table 2) roots. On the other hand, the essential oil had stronger activity (IC_{50}=0.02 mg/ml) than both BHA and vitamin C. Therefore, it is obvious that *E. sibthorpiana* oil possesses high antioxidant activity (Table 3).

ABTS scavenging activity

The results of the ABTS assay presented in Table 2 indicate that the ethanol extract of aerial parts (1.11±0.016 mg/L vit. C equivalents) and aqueous extract of aerial parts (1.02±0.07 mg/L vit. C equivalents) possess the highest antioxidant capacity. It can be seen that all root extracts were less effective than the extracts from the aerial-parts. As shown in Table 3, the essential oil expressed high antioxidant activity that was several fold stronger than the reference, flavonol quercetin.

Total phenolic concentrations (TPC)

The highest phenolic content was found for methanol (60.72±0.012 mg gallic acid/g of dry extract),

Table 1. Chemical composition of essential oil from aerial parts of *E. sibthorpiana*.

No	Compounds	KIE	KIL	%
1.	α-Thujene	923.5	924	0.22
2.	α-Pinene	929.0	932	0.44
3.	Sabinene	969.9	969	0.42
4.	β-Pinene	972.0	974	0.07
5.	Myrcene	990.5	988	0.39
6.	α-Phellandrene	1002.5	1002	**10.23**
7.	*p*-Cymene	1023.3	1020	**11.18**
8.	β-Phellandrene	1026.2	1025	**2.92**
9.	γ-Terpinene	1056.7	1054	0.21
10.	α-Terpinolene	1086.3	1086	0.15
11.	*cis-p*-Menth-2-en-1-ol	1120.6	1118	0.39
12.	*trans-p*-Menth-2-en-1-ol	1139.5	1136	0.25
13.	Terpinen-4-ol	1175.9	1174	0.15
14.	Cryptone	1185.4	1183	0.78
15.	*cis*-Piperitol	1195.2	1195	0.08
16.	α-Phellandrene epoxide	1203.6	1202	**6.87**
17.	*trans*-Piperitol	1208.7	1207	0.38
18.	*cis*-Carvotanacetol	1232.2	n/a	0.08
19.	Cumin aldehyde	1241.5	1238	0.67
20.	Carvotanacetone	1246.8	1244	0.21
21.	*cis*-Chrysanthenyl acetate	1259.3	1261	0.10
22.	Carvacrol	1315.1	1298	**1.74**
23.	Methyl eugenol	1414.9	1403	**60.40**
24.	Myristicin	1525.5	1517	0.52
25.	Caryophyllene oxide	1579.6	1582	0.38
26.	Neophytadiene (isomer II)	1835.7	1830	0.10
	Total			99.33%
	Yield			0.43%
	Number of constituents			26
	Monoterpene hydrocarbons			38.46%
	Oxygenated monoterpens			42.32%
	Oxygenated sesquiterpenes			3.85%
	Phenylpropanoids			11.54%
	Diterpene			3.85%

KIE=Kovats (retention) index experimentally determined (AMDIS)
KIL=Kovats (retention) index - literature data (Adams, 2007)
n/a=not available

aqueous and ethanol extracts of the aerial parts of *E. sibthorpiana,* (Table 2). The range of TPC values was between 38.65±0.003 and 60.72±0.012 mg gallic acid/g of dry extract.

Total flavonoid content (TFC)

Total flavonoid concentrations were higher in the extracts of the herbal parts than in the root extracts, with values ranging from 3.15±0.003-19±0.010 mg of quercetin/g of dry extract (Table 2). The highest flavonoid content was recorded in the ethanol and methanol extracts of *E. sibthorpiana* aerial parts (19±0.010 and 17.46±0.011 mg quercetin/g of dry extract, respectively).

Antibacterial activity

The tested extracts exhibited moderate to strong antibacterial activity against pathogenic bacteria (Table

Table 2. DPPH and ABTS results, total phenol and flavonoid content of *E. sibthorpiana* extracts (C=1-3.5 mg/ml)

Extracts		DPPH IC_{50} (mg/ml)	ABTS (1 mg/ml) (mg vit. C/g of dry extract)	Total phenols (1 mg/ml) (mg GE/g of dry extract)	Total flavonoids (1 mg/ml) (mg Qu/g of dry extract)
Methanol extracts	Aerial parts	1.73	0.80±0.008	60.72±0.012	17.46±0.010
	Roots	5.44	0.38±0.006	42.19±0.011	3.92±0.007
Ethanol extracts	Aerial parts	2.89	1.11±0.016	51.67±0.003	19.00±0.000
	Roots	6.58	0.33±0.003	38.65±0.003	3.15±0.003
Aqueous extracts	Aerial parts	1.67	1.02±0.007	57.34±0.001	16.81±0.005
	Roots	6.81	0.3 ±0.007	50.06±0.021	7.51±0.006
Standards		BHA 0.13 Vit. C 0.034	Quercetin 2.749±0.004		

Each value in the table was obtained by calculating the average of three analyses (± standard deviation)

Table 3. Antioxidant activity of *E. sibthorpiana* essential oil using DPPH and ABTS methods.

	DPPH IC_{50} (mg/ml)	ABTS (0.1 mg/ml) (mg vit. C/ml of essential oil)
E. sibthorpiana essential oil	0.02	2.02±0.008
Standards	BHA 0.13 Vit. C 0.034	Quercetin (1 mg/ml) 2.749±0.004

4). The MIC values were in the range of 0.45-12 mg/ml, while MBC values were from 0.75 to 21 mg/ml. The most resistant strains were *M. flavus* and *E. coli*, as presented in Table 4. The strongest antibacterial effect was that of the ethanol extract of roots (MIC=0.45-4.5 mg/ml; MBC=0.5-9 mg/ml) and methanol extract of aerial parts (MIC=3-4.5 mg/ml; MBC=4.5-6 mg/ml). The lowest antibacterial activity was determined for the aqueous extracts. Compared with streptomycin, all extracts showed stronger antibacterial effect against *L. monocytogenes* and *E. cloacae*.

The essential oil of *E. sibthorpiana* had stronger antibacterial activity than the positive control against all bacterial strains except *M. flavus* and *E. coli* with MIC values from 0.67 to 2.70 and MBC values from 1.35 to 16.17 mg/ml. The most sensitive bacteria were *B. cereus* and *S. typhimurium* (MICs=0.67 mg/ml; MBCs=1.35 mg/ml, for both strains).

Antifungal activity

It can be seen in Table 5 that the ethanol extract of the aerial parts had the strongest antifungal activity (MIC=1.5-15 mg/ml, MFC=2.5-17.5 mg/ml). The lowest antifungal activity was detected in aqueous extracts. The most resistant fungus was *A. niger*. Generally, extracts of aerial parts expressed a stronger effect against the tested fungi than the root extracts used in the experiment

E. sibthorpiana oil showed strong antifungal activity. MIC values for the oil ranged from 0.17-2.70 mg/ml, and MFC values from 0.34-10.78 mg/ml (Table 5). The most sensitive fungi were *A. ochraceus* and *P. ochrochloron* (MICs=0.17 mg/ml; MFCs=0.34 mg/ml for both strains), while the most resistant strain was *C. albicans* (MIC=2.70 mg/ml; MFC=10.78 mg/ml). Plant oil exhibited similar or stronger antifungal

Table 4. Antibacterial activity of *E. sibthorpiana* extracts and essential oil in terms of MICs and MBCs (mg/ml).

Bacterial strains	Methanol extracts				Ethanol extracts				Aqueous extracts				Essential oil		Streptomycin	
	Aerial parts		Roots		Aerial parts		Roots		Aerial parts		Roots					
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	3.00	4.50	4.50	9.00	3.00	4.50	0.45	0.75	12.00	15.00	12.00	15.00	0.67	1.35	1.50	2.50
Enterobacter cloacae	3.00	4.50	6.00	9.00	6.00	7.50	1.50	3.00	12.00	15.00	12.00	15.00	0.67	2.70	10.00	20.00
Escherichia coli	4.50	6.00	4.50	9.00	3.00	7.50	1.50	3.00	8.00	21.00	12.00	19.50	1.35	10.78	2.50	5.00
Listeria monocytogenes	3.00	4.50	1.50	3.00	6.00	9.00	4.50	9.00	12.00	15.00	12.00	15.00	2.70	16.17	15.00	20.00
Micrococcus flavus	4.50	6.00	6.00	9.00	1.50	6.00	1.50	3.00	12.00	18.00	12.00	21.00	1.35	10.78	2.50	5.00
Pseudomonas aeruginosa	4.50	6.00	4.50	9.00	3.00	6.00	1.50	3.00	12.00	15.00	12.00	15.00	0.34	2.70	2.50	5.00
Salmonella typhimurium	3.00	6.00	4.50	9.00	3.00	7.50	1.50	3.00	12.00	15.00	12.00	15.00	0.34	1.35	2.50	5.00
Staphylococcus aureus	3.00	4.50	4.50	9.00	4.50	9.00	3.00	6.00	12.00	15.00	12.00	18.00	0.67	2.70	2.50	5.00

Table 5. Antifungal activity of *E. sibthorpiana* extracts and essential oil in terms of MICs and MFCs (mg/ml).

Fungal strains	Methanol extracts				Ethanol extracts				Aqueous extracts				Essential oil		Fluconazole	
	Aerial parts		Roots		Aerial parts		Roots		Aerial parts		Roots					
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus fumigatus	10.00	15.00	17.50	20.00	5.00	7.50	10.00	15.00	20.00	23.75	17.50	20.00	0.34	1.35	0.50	1.00
Aspergillus niger	15.00	20.00	17.50	22.50	15.00	17.50	15.00	21.00	20.00	23.75	40.00	>54.00	1.35	2.70	0.25	1.00
Aspergillus ochraceus	15.00	17.50	15.00	20.00	2.50	7.50	5.00	10.00	17.50	20.00	40.00	>54.00	0.34	1.35	0.50	1.00
Aspergillus versicolor	7.50	15.00	15.00	20.00	1.25	2.50	5.00	10.00	15.00	17.50	15.00	17.50	0.17	0.34	0.13	0.50
Candida albicans	5.00	7.50	5.00	7.50	10.00	12.50	15.00	21.00	15.00	17.50	17.50	20.00	2.70	10.78	0.02	0.03
Penicillium funiculosum	7.50	15.00	15.00	20.00	5.00	7.50	7.50	10.00	17.50	20.00	15.00	20.00	0.17	0.67	0.25	0,50
Penicillium ochrochloron	12.50	17.50	15.00	20.00	5.00	7.50	7.50	10.00	12.50	17.50	17.50	20.00	0.17	0.34	1.00	1.50
Trichoderma viride	7.50	15.00	10.00	12.50	2.50	5.00	4.50	9.00	12.50	15.00	20.00	21.25	0.34	0.67	1.00	1.50

activity than fluconazole against all fungi except *C. albicans* and *A. niger*.

DISCUSSION

While several reports about the oil composition of this species have been published previously, no data have been published on the chemical composition of the essential oil from Macedonia. It was reported by Telci and Hisil (2008) that the best period for plant material collecting is from rosette to pre-flowering. After that period, the leaf content decreases and, therefore, the plants have no economic value as a spice or herbal tea (Telci and Hisil, 2008). In their study, methyl eugenol (41.80% to 62.90%), δ-3-carene (3.30% to 5.70%), *p*-cymene (7.80% to 9.10%) and α-phellandrene (30.40% in one sample) were the dominant components (Telci and Hisil, 2008).

Variation among the amounts of main components of *E. tenuifolia* subsp. *sibthorpiana* essential oil is affected by the harvest year conditions (methyl eugenol (24.99% to 90.16%), δ-3-carene (2.57% to 34.80 %) and *p*-cymene (1.23% to 9.81%)) (Chalchat et al., 2011). Another study of the composition of the oil from *E. tenuifolia* (subsp. *sibthorpiana*) oil composition showed different results, β-phellandrene and α-pinene being the major constituents (Evergetis et al., 2013). GC and GC-MS analyses of the essential oil composition of *E. sibthorpiana* from Greece showed that oil in the flowering period was dominated by the presence of α-phellandrene (43.8%), followed by methyl eugenol (28.6%) (Georgiou et al., 2010).

There is variability among *E. tenuifolia* ssp. *sibthorpiana* oil yield, composition and main component amounts in different studies. According to the available literature data, the dominant oil constituents in this taxon were α-phellandrene, methyl eugenol or δ-3-carene, depending on the origin of the sample (geographic area), ontogenetic stage of development (collection time) and harvest years (climatic conditions) (Chalchat et al., 2011, Telci and Hisil, 2008). The essential oils of Greek *E. tenuifolia* and of two Turkish samples were richer in phellandrenes (α and β) (Agkul and Chialva, 1989, Baser et al., 1994, Evergetis et al., 2013, Georgiou et al., 2010), while the oil that we tested was richer in methyl eugenol, as in samples from Turkey and oil from Iran (Baser et al., 1998, Ahmad et al., 1999, Chalchat et al., 2011; Telci and Hisil, 2008). It is interesting that only Evergetis et al. (2013) did not identify methyl eugenol in *E. tenuifolia* ssp. *sibthorpiana* essential oil. In general, *E. tenuifolia* oil is characterized by very small amounts of sesquiterpenes, but in our study, as in previous data (Agkul and Chialva, 1989, Georgiou et al., 2010), the absence of sesquiterpenes was notable. In *E. tenuifolia* oil from Turkey and Iran, δ-3-carene appears in high amounts (even as a dominant compound) (Gokbulut et al., 2013, Ahmad et al., 1999, Chalchat et al., 2011, Telci and Hisil, 2008), while the presence of this compound was not detected in Greek oil (Georgiou et al., 2010) or in our sample.

Glamočlija et al. (2011) reported chemical analyses of the essential oil of *E. spinosa* from Montenegro. The authors described δ-3-carene (60.86%) as the main constituent. Similar results (48.1% of δ-3-carene) were found for related species – *E. lamondiana* from Turkey (Baser et al., 2000). As previously mentioned, there are some studies about the essential oil composition of *E. platyloba* from Iran and it was shown that *trans*-β-ocimene was the most abundant volatile constituent in all studies (20.89% to 67.9%) (Rahimi-Nasrabadi et al., 2010, Asghari et al., 2003, Saei-Dehkordi et al., 2012, Gholivand et al., 2011). It was concluded that climatic, seasonal conditions, geographic area and vegetation stage affect the variations in chemical oil composition.

It has been shown that methyl eugenol possesses relaxant and antispasmodic activity. This compound exhibited a slightly greater relaxant potency in the isolated intestinal smooth muscles than in combination with its parent oil (Magalhaes et al., 1998). In addition, it was established that phellandrene and methyl eugenol were responsible for a strong, toxic and larvicidal activity against the larvae of *Culex pipiens* biotype *molestus*, suggesting that there was no strong synergistic effect among them (Evergetis, 2013).

Tan and Nishida (2012) have reviewed the various roles of methyl eugenol in nature, especially in relation to the chemical defenses of plants, such as antifungal, antibacterial, antinematodal or toxicant roles against pathogens and insect herbivores, as well as its function in pollination. Methyl eugenol may have positive effects on human health by reducing cerebral ischemic injury through the suppression of oxidative injury and inflammation (Choi et al., 2010).

Recently, the total phenol content and antioxidant activity of *E. tenuifolia* subsp. *sibthorpiana* oil (Gokbulut et al., 2013) and *Echinophora tenuifolia* extract (mixture of 90% methanol, 9% water and 1% acetic acid) (Ozcan and Al Juhaimi, 2011) were evaluated by Folin-Ciocalteu, DPPH and ABTS tests, but no data were found on the determination of antioxidant activity of ethanol and aqueous extracts of this species. In our work, the essential oil showed very strong antioxidant activity, which corroborates previous study (Gokbulut et al., 2013).

E. platyloba oil from Iran showed a remarkable antioxidant activity (IC_{50}=49.7 μg/ml) (Saei-Dehkordi et al., 2012) which was similar to our results for *E. sibthorpiana* obtained by DPPH test (IC_{50}=0.02 mg/ml). It has been shown by Sharafati-Chaleshtori et al. (2012) that the aqueous extract of *E. platyloba* had a higher antioxidant capacity than the ethanol extract, but still lower than the reference butylated hydroxytoluene (BHT). In our investigation, reference BHA was also stronger than both *E. sibthorpiana* aqueous and ethanol extracts (which had similar antioxidant potency).

It was recently published that the total phenolic content was highest in the aqueous extract of *E. platyloba* while the highest flavonoid concentrations were in the ethanol extract (Sharafati-Chaleshtori et al., 2012), as in our analyses.

The essential oil and extracts of *E. platyloba* from Iran were examined by three test systems for their free radical-scavenging capacity. Total phenol concentrations were determined for polar and non-polar subfractions of the methanol extract and for the oil (Gholivand et al., 2011). The results by the DPPH test showed that the highest radical-scavenging activity was provided by the polar sub-fraction of the methanol extract. It was found that the essential oil was the strongest in relative inhibition capacity (Gholivand et al., 2011).

Several articles on the antimicrobial activity of some *Echinophora* species have been published (Eadie, 2004, Saei-Dehkordi et al., 2012, Avijgan et al., 2006, Entezari et al., 2009, Sharafati-Chaleshtori et al., 2012, Glamočlija et al., 2011, Ozcan and Al Juhaimi, 2011). In a recent study, *E. tenuifolia* essential oil showed very strong antimicrobial activity against *B. cereus* and *Staphylococcus* spp. detected by the broth dilution method (Gokbulut et al., 2013), which is in compliance with the previously mentioned results; *E. sibthorpiana* oil showed a remarkable activity against *S. typhimurium* (Table 4).

E. platyloba oil was effective against some tested Gram-positive bacteria, such as *L. monocytogenes*, *S. aureus* and yeasts like *R. mucilaginosa, R. rubra*, while *E. spinosa* was the most effective against Gram-negative strains *E. coli*, *P. aeruginosa* and fungus *T. viride* (Saei-Dehkordi et al., 2012, Glamočlija et al., 2011). *L. monocytogenes* and *E. coli* were the most resistant strains to the effectiveness of *E. sibthorpiana* oil.

Strong antibacterial effects of the *E. platyloba* ethanol extract on *Alcaligenes faecalis,* and the aqueous extract on *Listeria monocytogenes* have been reported (Sharafati-Chaleshtori et al., 2012). The methanol extract of the same species inhibited the growth of *S. aureus* and *P. aeruginosa* growth (Entezari et al., 2009).

The *E. tenuifolia* extract from Turkey was screened for antifungal activity by the paper disc method. The extract was most effective on mycelia growth of *A. alternate*. *A. niger* and *A. parasiticus* were less sensitive, but at higher concentrations, the extract showed high fungitoxic activity (Ozcan and Al Juhaimi, 2011). *A. niger* in our study was the most resistant micromycete to the tested extracts.

Avijgan et al. (2006) studied the antifungal activity of the *E. platyloba* ethanol extract on *C. albicans* growth and concluded that it has an inhibitory effect at concentrations above 2 mg/ml. It can be seen from Table 5 that the ethanol extract of *E. tenuifolia* was effective against the same strain at concentrations of 10-15 mg/ml.

According to the obtained data, it can be concluded that the antimicrobial effectiveness decreased with the polarity of the extracts, and that the extracts showed stronger antibacterial than an antifungal activity. In general, roots have less effective antioxidant and antimicrobial activities than the aerial part. This could be related to the higher concentrations of phenols and flavonoids in the aerial parts. The essential oil exhibited the highest potency.

CONCLUSIONS

The essential oil of *E. sibthorpiana* contains methyl eugenol as the main constituent. Our experiments also show that in view of the high antioxidant activity, the oil has a potential use as a natural antioxidant for food supplementation and pharmaceutical applications. In addition, the essential oil is effective in the inhibition of different pathogens, so that it can be used as an antimicrobial agent. Further research is needed to examine these activities in more detail *in vivo*, and to determine the correlation between the chemical composition and activity.

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