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COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *Helichrysum italicum* ESSENTIAL OIL AND ITS TERPENE AND TERPENOID FRACTIONS

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The essential oil of Helichrysum italicum *(Roth) G. Don from Croatia has been fractionated into terpene and terpenoid fractions and analyzed using GC/MS. Fifty-two compounds were identified. The main hydrocarbons of the oil were α-pinene (10.2%), α-cedrene (9.6%) aromadendrene (4.4%), β-caryophyllene (4.2%), and limonene (3.8%), while the main oxygen-containing compounds were neryl acetate (11.5%), 2-methylcyclohexyl pentanoate (8.3%), 2-methylcyclohexyl octanoate (4.8%), and geranyl acetate (4.7%). The essential oil and its terpene and terpenoid fractions were evaluated for antibacterial and antifungal activities. The screening of antimicrobial activity was conducted by a disc diffusion test and the minimum inhibitory concentration was determined against* Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, *and* Candida albicans. *The essential oil and its terpenoid fraction exhibited higher antimicrobial activity with respect to the terpene fraction. The antimicrobial activities of the oil and its terpenoid fraction were more pronounced against* Staphylococcus aureus *and* Candida albicans.

Key words: *Helichrysum italicum;* essential oil; terpene and terpenoid fractions; GC/MS; antimicrobial activity.

Helichrysum italicum (Roth) G. Don (everlasting) is a typically Mediterranean plant. It is a small aromatic shrub with yellow flowers, up to 40–50 cm high, growing on dry cliffs and sandy soil. It is widespread along the East coast and on the islands of the Adriatic sea. The essential oil is present in all green parts of the plant. Everlasting and its extracts are used in popular medicine in the Mediterranean region. This plant is known for its antiinflammatory, antiallergic, and antimicrobial activity. *H. italicum* ether extract has an inhibitory effect on *Staphylococcus aureus* strains, reducing both their growth and some of the enzymes such as coagulase, DNAse, thermonuclease, and lipase [1]. Six acetophenone, three flavonoid, and one g-pyrone derivatives were isolated and identified from the plant antiinflammatory extract [2]. The antiinflammatory and antioxidant activity of these nonvolatile compounds were researched in detail afterwards [3–5]. Everlasting essential oil is very valuable in the perfume industry. It has a very complex chemical composition with numerous monoterpene and sesquiterpene compounds with complicated structures. The chemical composition and antimicrobial activity of *H. italicum* essential oil of Greek origin were investigated in [6–8]. The oils chemical composition is variable depending on the origin and the cycle of vegetation [9]. Season and locality variations of everlasting oil from Croatia were investigated earlier [10]. The main components of this essential oil were α-pinene, neryl acetate, α-cedrene, nerol, α-curcumene, γ-curcumene, and geranyl acetate.

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TABLE 1a. Identified Constituents of the Essential Oil and Its Fractions Isolated from *Helichrysum italicum* (Roth) G. Don (Hydrocarbons)

Compounds	Terpene fraction	Oil	Identified with
	%		
α-Pinene	20.6	10.2	I_1, I_2, MS
β-Pinene	1.3	0.6	I_1, I_2, MS
α-Terpinene	0.6	0.3	I_1, I_2, MS
Limonene	8.1	3.8	I_1, I_2, MS
γ-Terpinene	1.5	0.7	I_1, I_2, MS
p-Cymene	0.4	0.2	-, I_2, MS
Terpinolene	0.6	0.3	I_1, I_2, MS
Tridecane	0.9	0.4	-, I_2, MS
Tetradecane	0.4	0.2	-, I_2, MS
α-Copaene	0.5	0.2	I_1, I_2, MS
2,3,4,7,8,8a-Hexahydro-1H-3a,7-methanoazulene	6.3	3.0	-, I_2, MS
α-Bergamotene	1.5	0.7	I_1, I_2, MS
β-Caryophyllene	8.9	4.2	I_1, I_2, MS
Alloaromadendrene	0.4	0.2	I_1, I_2, MS
α-Humulene	3.4	1.6	I_1, I_2, MS
β-Selinene	3.4	1.6	I_1, I_2, MS
α-Cedrene	20.5	9.6	I_1, I_2, MS
Aromadendrene	9.4	4.4	I_1, I_2, MS
α-Selinene	0.9	0.4	-, I_2, MS
δ-Cadinene	1.0	0.5	I_1, I_2, MS
ar-Curcumene	4.9	2.3	I_1, -, MS
α-Amorphene	Tr.	Tr.	-, I_2, MS
α-Patchoulene	1.1	0.5	I_1, -, MS
γ-Gurjunene	2.1	1.0	I_1, I_2, MS
Identified terpenes	98.7	46.9	
Unidentified	1.3		

I_1 = retention indices on HP-20M; I_2 = retention indices on HP-101; MS = mass spectra; Tr. = trace < 0.1%; - = not identified.

The aim of this study was to determine the antimicrobial activity of the overall *H. italicum* oil and to compare it with its fractions of hydrocarbons (terpenes) and oxygen-containing compounds (terpenoids), since the antimicrobial activity of everlasting oil from Croatia has not yet been investigated. The yield of the essential oil obtained by the hydrodistillation of fresh plant material was 0.12% (w/w). The chromatograms of the oil had numerous peaks and many peaks were overlapping. In order to improve the peak separation and better estimate the antimicrobial activity, the oil was fractionated on a silica gel column. One fraction of terpene hydrocarbons and one fraction of oxygen-containing compounds (terpenoids) were obtained by liquid–solid chromatography. Both fractions, as well as the oil, were analyzed by gas chromatography-mass spectrometry (GC/MS) on two columns. More than 60 components were separated and 52 of them were identified. The mass content of terpene hydrocarbons in the essential oil was 46.9%, and the content of terpenoids was 43.7%. The mass of both fractions was almost equal to the mass of the used oil. The chemical composition of the essential oil was almost equal to the composition calculated from both fractions. Artefacts were not present in the fractions. Unidentified compounds (9.4% of the essential oil, 13.3% of terpenoid fraction) are mainly monoterpene esters. The chemical composition of the essential oil calculated from the composition and content of fractions is given in Table 1.

TABLE 1b. Identified Constituents of the Essential Oil and Its Fractions Isolated from *Helichrysum italicum* (Roth) G. Don (Oxygen containing compounds)

Compounds	Terpenoid fraction	Oil	Identified with
	%		
1,8-Cineole	0.7	0.3	-, I_2, MS
trans-2-Methyl-2-butenoic acid	0.2	0.1	I_1, -, MS
cis-3-Hexen-1-ol	Tr.	Tr.	-, I_2, MS
6-Methyl-3-heptanone	0.5	0.2	I_1, -, MS
Nerol oxide	0.2	0.1	I_1, -, MS
Linalool	3.2	1.4	I_1, -, MS
Fenchol	0.3	0.2	I_1, I_2, MS
trans-Pinocarveol	0.5	0.2	I_1, I_2, MS
α-Terpineol	3.2	1.4	I_1, I_2, MS
Neryl acetate	23.2	11.5	I_1, I_2, MS
Geranyl acetate	9.7	4.7	I_1, I_2, MS
Nerol	0.5	0.2	I_1, I_2, MS
Geraniol	0.5	0.2	I_1, I_2, MS
2-Methylcyclohexyl pentanoate	12.9	8.3	I_1, I_2, MS
2-Methylcyclohexyl octanoate	7.9	4.8	I_1, -, MS
Unknown	0.7	0.3	I_1, I_2, -
Unknown	5.5	2.4	I_1, I_2, -
trans-Nerolidol	1.4	0.6	I_1, I_2, MS
Guaiol	1.4	0.6	I_1, I_2, MS
Phenylethyl tiglate	2.1	0.9	I_1, I_2, MS
Thymol	2.5	1.1	I_1, I_2, MS
Torreiol	2.5	1.1	I_1, I_2, MS
α-Bisabolol	1.8	0.8	I_1, I_2, MS
β-Eudesmol	1.1	0.5	-, -, MS
Decanoic acid	1.4	0.6	-, I_2, MS
Undecanoic acid	Tr.	Tr.	I_1, -, MS
Dodecanoic acid	1.6	0.7	I_1, -, MS
β-Costol	Tr.	Tr.	I_1, I_2, MS
Tridecanoic acid	0.5	0.2	I_1, I_2, MS
Tetradecanoic acid	0.7	0.3	
Identified terpenoids	86.7	43.7	
Unidentified	13.3	9.4	

I_1 = retention indices on HP-20M; I_2 = retention indices on HP-101; MS = mass spectra; Tr. = trace < 0.1%; - = not identified.

Monoterpene and sesquiterpene hydrocarbons and oxygen-containing compounds were identified. Fifty-four compounds were separated and 52 identified, representing about 90% of the total oil. The major identified monoterpene and sesquiterpene hydrocarbons of the terpene fraction and the oil were: α-pinene (20.6–10.2%), α-cedrene (20.5–9.6%), aromadendrene (9.4–4.4%), β-caryophyllene (8.9–4.2%), limonene (8.1–3.8%), 2,3,4,7,8,8α-hexahydro-1H-3a,7-methanoazulene (6.3–3.0%), and *ar*-curcumene (4.9–2.3%). The most abundant oxygenated compounds of the terpenoid fraction and the oil were: neryl acetate (23.2–11.5%), 2-methylcyclohexylpentanoate (12.9–8.3%), geranyl acetate (9.7–4.7%), and 2-methylcyclohexyl-octanoate (7.9–4.8%). Other terpene compounds were present with smaller percentages, such as α-humulene, linalool, β-selinene, and α-terpineol. Numerous other monoterpenes, sesquiterpenes and nonterpene compounds were present in small amounts and are listed in Table 1. The chemical composition of everlasting essential oils from Greece and Croatia are quite different [7], while the oil from Croatia is similar to the oil of Italian origin [11]. The antimicrobial activity of the oil of Croatian origin and its fractions has been evaluated for the first time. The results obtained in the antimicrobial assay are shown in Table 2 and Table 3.

TABLE 2. Antimicrobial Activity of *Helichrysum italicum* Essential Oil and Its Terpene and Terpenoid Fractions Using Disc Diffusion Method

Microorganism	Diameter of inhibitory zones, mm		
	Terpene fraction	Terpenoid fraction	Oil
	Gram-positive		
Staphylococcus aureus	6	9	10
	Gram-negative		
Echerichia coli	66	66	66
Pseudomonas aeruginosa			
	Fungi		
Candida albicans	6	10	9

TABLE 3. MIC (μL/mL) for the Essential Oil, Terpene, and Terpenoid Fractions Using the Agar Method

Microorganism	Terpene fraction	Terpenoid fraction	Oil
	Gram-positive		
Staphylococcus aureus	>8.0	5	5
	Gram-negative		
Echerichia coli	7.0	8.0	7.0
Pseudomonas aeruginosa	>8.0	>8.0	>8.0
	Fungi		
Candida albicans	8	5	5

The hydrocarbon fraction of the essential oil showed a weak antimicrobial activity against all the test strains. Similar to this, the terpenoid fraction and the essential oil had weak antimicrobial activity against *Pseudomonas aeruginosa* and *Escherichia coli* (Gram-negative strains). Higher growth reductions of the medically important pathogen such as *Staphylococcus aureus* was noted for the essential oil (inhibition zone 10 mm) and its terpenoid fraction (inhibition zone 9 mm). The essential oil has been shown to inhibit the growth of *Candida albicans* (inhibition zone 9 mm), and the terpenoid fraction showed a higher inhibition zone (10 mm), Table 2. *S. aureus* and *C. albicans* were inhibited at a minimal concentration of 5 μg/mL by the essential oil and its terpenoid fraction. *E. coli* was inhibited at a concentration of 7 μg/mL by the oil and its terpene fraction. *Pseudomonas* was not inhibited, Table 3. Oxygen-containing compounds are probably responsible for the antimicrobial activity of the essential oil of *H. italicum.* This activity can originate from monoterpene oxygen-containing compounds such as neryl acetate, geranyl acetate, geraniol, and nerol [12] that are the main components of this oil. However, the possible synergism of the minor components on the antimicrobial activity should be considered. Further studies of antimicrobial activity require a larger number of strains and further fractionation of the oxygenated compounds.

EXPERIMENTAL

Plant Material. *Helichrysum italicum* (Roth) G. Don. (everlasting) was collected in June 2002 near Split (south Croatia) during the flowering period. Fresh plant material was used (stems *ca.* 15 cm with leaves and flower heads) for this research. A voucher specimen is deposited at the Department of Organic Chemistry and Natural Products, Faculty of Chemical Technology, University of Split.

Isolation of the Essential Oil. Plant material (100 g) and water (500 mL) were placed in a Clevenger type apparatus. The essential oil was isolated by hydrodistillation for 3 hours. The obtained essential oil was separated, dried over anhydrous sodium sulfate, and stored under argon in a sealed vial at 4°C until required. The essential oil yield was determined by the gravimetric method.

Fractionation of Essential Oil. The essential oil (0.5 g) was fractionated on a silica gel column (15 g; 30–60 μm) and two fractions were obtained. Pentane (100 mL) was used for the elution of apolar hydrocarbons (terpenes), and diethylether (80 mL) for the elution of oxygenated compounds (terpenoids). The separation was monitored by thin layer chromatography using Kieselgel 60 aluminum-backed sheets (Merck). The obtained fractions were concentrated by fractional distillation under diminished pressure. The mass of fractions and their ratio were determined by a gravimetric method. Both fractions without solvents as well as the essential oil were used for the investigation of antimicrobial activity.

Gas Chromatography/Mass Spectrometry. The isolated essential oil as well as its fractions were analyzed by Hewlett Packard GC/MS (model 5890 series II) with mass selective detector (model 5971A). Two columns of different polarities were used: an HP-101 column (methyl silicone fluid, Hewlett Packard; 25 m × 0.2 mm i.d., film thickness 0.2 μm) and an HP-20M column (Carbowax 20M, Hewlett Packard; 50 m × 0.2 mm i.d., film thickness 0.2 μm). The GC operating conditions were similar as those in our previous paper [13]. Oven temperature was programmed as follows: isothermal at 70°C for 4 min, then increased to 180°C at a rate of 4°C min^{-1} and subsequently held isothermal for 15 min (for HP-20M column); isothermal at 70°C for 2 min, then increased to 200°C at a rate of 3°C min^{-1} and held isothermal for 15 min (for HP-101 column). Carrier gas was helium, flow rate: 1 mL min^{-1}; injector temperature: 250°C; volume injected: 1 μL; split ratio: 1:50. MS conditions: ionization voltage: 70 eV; ion source temperature: 280°C; mass range: 30–300 mass units.

Qualitative and Quantitative Determination. Quantitative results are mean data derived from duplicate GC/MS analyses. The individual peaks were identified by comparison of their retention indices to those of authentic samples, as well as by comparing their mass spectra with the Wiley library mass spectral database and literature [14]. The percentage composition of the samples was computed from the GC peak areas.

Test Microorganisms. The *in vitro* antimicrobial activity of the oil and its terpene and terpenoid fractions were measured using a panel that included both clinical pathogens and laboratory control strains, all of them belonging to the American Type Culture Collection Maryland, USA: Gram-positive: *Staphylococcus aureus* (ATCC 25923); Gram-negative: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and fungal organism: *Candida albicans* (ATCC 10231). All were kept for long-term storage in glycerol at –20°C and for short-term storage on tryptone soya agar (bacteria) and potato dextrose agar (fungal organism) at 4°C.

Microbial Cultures. Bacteria were subcultured from nutrient agar slopes into nutrient broth and incubated at 37°C for 18 h. Subculturing was performed at least twice. The resulting bacterial broth was used as the inoculum in microbial analysis. Cell numbers of the inoculum were standardized at 10^5 cell/mL.

Disc Diffusion Method. The essential oil and its fractions were investigated for biological activity by the disc diffusion method. For this purpose we used Petri dishes (diameter 110 mm) with 20 mL Mueller-Hinton nutrient agar. Paper discs (6 mm diameter) were placed on the inoculated agar surfaces and impregnated with 5 μL of the oil or its fractions. The plates were inverted during the incubation. After aerobic incubation for 24–48 hours at 37°C (bacteria) and 25°C (fungi), the antimicrobial activity was estimated by measuring the diameter of the zone inhibition. Each test was performed in triplicate.

Minimum Inhibitory Concentration (MIC) – Agar Method. Test material was added aseptically to the sterile molten agar to give a final concentration of 1–8 μg/mL. The essential oil and its fractions were dissolved in dimethyl sulfoxide (DMSO) in the ratio 1:20 V/V. The agar solution and DMSO solution were vortexed at high speed for 1 min and immediately poured into sterile Petri dishes and left to set for 30 min. The plates were then inoculated by spotting 6 μL of a suspension of the desired microorganism from the prepared inoculum. Plates were incubated at 37°C for 24–48 h. The controls H_2O–DMSO were also established. DMSO showed no toxicity in the concentrations used for the tested microorganisms [15]. Each test was performed in triplicate. From these results the MIC of the test material was calculated as the lowest concentration at which no growth of the microorganism had occurred.

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