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Helichrysum italicum (Roth) G. Don subsp. *italicum* from Herzegovina: Volatile Composition, Variations during Seasons, Total Polyphenols, Acetylcholinesterase Inhibition and Antioxidant Activity

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Abstract: The chemical composition of essential oils isolated from immortelle (*Helicrysum italicum* subsp. *italicum*) collected in Herzegovina during five different periods, was investigated by GC/MS analysis. The main compounds were *α*-pinene (15.7 %) and γ-curcumene (12.8 %), followed by 4,6,9-trimethyldec-8-en-3,5-dione (8.7 %), neryl acetate (6.9 %), limonene (6.4 %) and β-selinene (5.3 %). In total, 69 components were identified whose share changed over the vegetative cycle. Antioxidant activity of methanolic extracts of immortelle were determined according to DPPH (IC_{50} = 23–34 μg/mL) and FRAP (29 μg/mL is equivalent to 1.1–2.2 mM Fe^{2+}) methods. Acetylcholinesterase inhibitory potential, investigated by modified Ellman's assay and determined as IC_{50} values, were 340–440 μg/mL for methanol extracts and 135 μg/mL for essential oil. Metanolic extracts showed strong antioxidant activity and potential to inhibit AChE. Essential oil possesses complex chemical composition, inhibition activity of AChE and weak antioxidant capacity.

Keywords: *Helichrysum italicum* subsp. *italicum*, Asteraceae, essential oil composition, α-pinene, γ-curcumene, antioxidant activity.

INTRODUCTION

HELICHRYSUM italicum subsp. *italicum* (Asteraceae), also called immortelle or everlasting plant, grows in sunny, rocky areas of Herzegovina. Various extracts can be prepared from this plant, among which polar extract and essential oil (EO) have the largest application. *H. italicum* essential oil is used for various skin conditions such as inflammations, scars and allergies.[1] Its commercial importance is based on its application in perfume and cosmetics industry. It has a complex chemical composition which is hard to produce synthetically. Essential oil and extracts are also used in traditional medicine for choleretic, diuretic and inflammatory conditions related to respiratory tract.[2] Scientific studies reported various biological activity of immortelle EO and extracts, such as antimicrobial, anti-inflammatory, antioxidant and antiviral.[3–12]

Recently, cultivation of immortelle in Herzegovina region is expanding rapidly due to economic value of its EO. Several scientific studies have been conducted in the last decades addressing the composition of immortelle EOs from Mediterranean countries. These studies have showed remarkable diversity in the composition of immortelle EOs which is highly influenced by environmental factors, geographic origin and seasonal variations. Therefore, it is not surprising that several chemotypes of subsp. *italicum* have been described so far.[10] Also, data about its pharmacological activities were reported.[13]

The lack of knowledge regarding chemical com–position and pharmacological potential of medicinal and aromatic plants in Herzegovina belongs to the main threats to their sustainable use.[14] Therefore, the aim of this study was to chemically characterize, for the first time, EOs obtained from *H. italicum* subsp. *italicum* growing widely in Herzegovina. Analysis was performed for plants material collected at different growing seasons as well as for different areal parts of plant harvested in flowering period. Chemical analysis was followed by the research on

antioxidant capacity and inhibition of acetylcholinesterase of methanol (MeOH) extracts and EOs.

EXPERIMENTAL SECTION

Chemicals

Acetylcholinesterase (AChE) from electric eel (type VI-S), acetylthiocholine iodide (ATChI), galanthamine hydrobromide 2.2'-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), 2,4,6-tripyridil-*s*-triazine (TPTZ), Folin-Ciocalteu reagent, sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \times H_2O$), disodium hydrogen phosphate (Na_2HPO_4), n-pentane and sodium sulphate (Na_2SO_4) were purchased from Sigma-Aldrich (Germany). 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) was purchased from Zwijndrecht (Belgium). All reagents used in the study were of analytical grade.

Plant Material, Oil and Methanol Extract Isolation

The samples of Herzegovinas *H. italicum* subsp. *italicum* were collected in west Herzegovina (43°23´14,7´´N; 17°36´22´´E), in five different periods of the year (February, May, June, August and October 2016). The species were identified by Dr. Anđelka Lasić, Assistant professor of Botany at the Department of Biology, Faculty of Science and Education, University of Mostar (Voucher no. FPMOZ-SB-10-2016). The plant material was air-dried at ambient temperature (25 ± 2 °C) without exposure to direct sunlight. The air-dried samples (100 g) of each plant were submitted to hydro-distillation for 1.5 hours using Clevenger type apparatus according to European Pharmacopoeia. Aerial parts of the plant (stems with leaves and flower) were used for the analysis of essential oils depending on the season. The sample from June was also subjected to the distillation of flowers and leaves separately. The collected oils were dried over anhydrous sodium sulphate and the essential oil yields were 0.17–0.41 % (percentages from dry weight).

Five grams of ground dry plant material (sample from June) and 50 mL of methanol (95 %) were subjected to ultrasonic extraction (35 kHz, 60 min, 30 °C). After extraction the mixture was filtered under vacuum followed by evaporation of the filtrate under reduced pressure. The experiments were performed in triplicate.

Gas Chromatography - Mass Spectrometry

The analysis of the oils was carried out using Shimadzu GC/MS QP2010 system equipped with an AOC–20i autosampler, using two fused silica capillary columns with different polarity. The non-polar column was Inert Cap (5 % diphenyl – 95 % dimethylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm) and the polar column was Rtx–Wax (polyethylene glycol, 30 m × 0.25 mm × 0.25 µm). The operating conditions for non-polar column were as follows: injection volume: 1.0 µL of solution diluted 1:500 *v/v* in pentane; injection mode: splitless; injection temperature: 250 °C; carrier gas: helium, 1.15 mL/min; oven temperature program: 70 °C (1.5 min), 70–120 °C (5 °C/min), 120–240 °C (4 °C min), 240 °C (2 min). For polar column, the operating conditions were as follows: flow rate of carrier gas: 1.21 mL/min; oven temperature program: 60 °C (2 min), 60–240 °C (3 °C/min), 240 °C (10 min). MS conditions: ion source temperature: 250 °C, ionization voltage: 70 eV, mass range: *m/z* 40–400 u. GCMSSolution 2.5 (Shimadzu) was used to handle data.

Identification of oil components was based on (a) retention indices on a polar and non-polar column relative to a homologous series of *n*-alkanes (C_8–C_{40}) (b) on the comparison of their mass spectra and retention indices with the NIST and Wiley spectra library and with those reported in the literature.[15–21]

Determination of Total Polyphenols

The total phenolic content was determined spectrophotometrically, as described by Hernandez et al. in methanolic extract of *H. italicum* subsp. *italicum*.[22] Briefly, 100 µL of the methanol solution of extract (1 mg/mL) was mixed with 4.5 mL of distilled water; then 100 µL of Folin-Ciocalteu reagent was added and the contents of the flask were mixed thoroughly. After 3 minutes, 300 µL of sodium carbonate (20 % Na_2CO_3) was added. The mixture was incubated at room temperature for 2 hours and the absorbance was measured at 765 nm. Gallic acid was used as a reference standard for plotting calibration curve (A = 0.1075 GAE + 0.0089; R^2 = 0.997). Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract. The data is presented as the average ±SD of triplicate analyses.

DPPH Radical Scavenging Assay

Antioxidant activity of the essential oils and methanolic extracts was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, 2,2'-diphenyl-1-picrylhydrazyl (DPPH).[23] The 50 µL methanolic solutions of the essentials oils (2.0–30.0 mg/mL), or 50 µL methanolic solutions of the plant extracts(0.05–1.0 mg/mL), were placed in a cuvette and 1 mL (6×10^{-5} M) of methanolic solution of DPPH was added. The mixture was incubated for 30 minutes at room temperature. The absorbance was measured at 517 nm. Synthetic antioxidant butylated hydroxytoluene (BHT) was used as a positive control (0.1–30.0 mg/mL). All determinations were performed in triplicate. The concentrations of essentials oils, methanolic extracts and BHT were expressed as a final

concentrations. Inhibition of DPPH expressed in percentage was calculated according to equation 1:

$$\text{Inhibition (\%)} = ((A_{C(0)} - A_{A(t)}) / A_{C(0)}) \times 100 \quad (1)$$

were $A_{C(0)}$ is the absorbance of the control at t = 0 min, and $A_{A(t)}$ is the absorbance of the antioxidant at t = 30 min. The IC_{50} values were obtained from dose-effect curves.

FRAP Assay

The total antioxidant potential of essential oils, methanolic extracts and BHT was determined using the ferric reducing ability of plasma (FRAP) assay of Benzie and Strain as a measure of »antioxidant power«.[24] The FRAP reagent was prepared by mixing 10 parts of acetate buffer (300 mM, pH 3.6) with 1 part of TPTZ (2,4,6-tripyridil-*s*-triazine) (10 mM in 40 mM hydrochloride acid) and with 1 volume of ferric chloride (20 mM). All solutions were used on the day of preparation. A linear calibration graph for $FeSO_4 \times 7\ H_2O$ in the concentration range over 0.1–5.0 mM was prepared. The corresponding regression calibration equation was A = 0,6152c + 0.0606 (R^2 = 0.9998); where A is absorbance at 593 nm, c is concentration of $FeSO_4 \times 7\ H_2O$ in mM. The procedure for the preparation of calibration graph was as follows. The reaction mixture consisted of 150 µL of deionized water, 1.5 mL of FRAP reagent and 50 µL solution of $FeSO_4 \times 7H_2O$. The FRAP reagent (1.5 mL) was warmed to 37 ºC and a reagent blank reading was taken at 593 nm (B1 reading). The same procedure was used for spectrophotometric measurements with solutions of essentials oils and methanolic extracts. Methanolic solution of essentials oils (2.0–30.0 mg/mL) or methanolic solution of plant extracts (0.1–1.0 mg/mL) were added instead of 50 µL solution of Fe^{2+}. All reaction mixtures were incubated at 37 ºC throughout the monitoring period. The change in absorbance between the final reading (4-min reading) and B1 reading was selected for the calculation of FRAP values. The BHT was used as positive control (0.1–1.5 mg/mL). In the FRAP assay, the antioxidant efficiency of the antioxidant tested was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, representing a one-electron exchange reaction. The concentrations of essentials oils, methanolic extracts and BHT were expressed as a final concentrations. All determinations were performed in triplicate.

Acetylcholinesterase (AChE) Inhibition Assay

The AChE inhibitory activities of the *H. italicum* subsp. *italicum* essential oils and their methanolic extracts were determined using modified Ellman's method described by Wszelaki et al.[25,26] Briefly, 180 µL (0.1 M, pH 8.0) sodium phosphate buffer, 10 µL of AChE (0.45 U/mL) and 10 µL tested solution (0.5–3.0 mg/mL essential oils and 2.0–10.0 mg/mL methanolic extracts) were mixed and pre-incubated for 15 minutes at 4 °C. Essential oils for analysis were dissolved in 86 % ethanol. The reaction was then initiated with the addition of 10 µL of DTNB (0.6 mM) and 10 µL of ATChI (10 mM). AChE activity was measured using a 96-well microplate reader (IRE 96, SFRI Medical Diagnostics) at 405 nm over a period of 30 minutes at 37 °C. Galanthamine (0.01–0.5 mg/mL) was used as a positive control. The experiment was run in triplicate. The concentrations of essentials oils and methanolic extracts were expressed as a final concentrations. Percentage enzyme inhibition was calculated according to equation 2:

$$\text{Inhibition (\%)} = ((A_C - A_T) / A_C) \times 100 \quad (2)$$

where A_C is the activity of enzyme without test sample and A_T is the activity of enzyme with test sample. The IC_{50} values were obtained from dose-effect curves. The graphical presentation of results, data processing and determination of IC_{50} values were performed using the Microsoft Excel software for Windows, version 10.0.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oil and Seasonal Variability

The chemical composition of immortelle EOs collected during five different periods of year at the same locality, were characterized by GC/MS analysis and the results are reported in Table 1. Samples were collected in February (vegetative period), May (pre-flowering period), June (flowering period), August (post-flowering period) and October (vegetative period).

Immortelle essential oil showed expected complexity in chemical composition with numerous terpene and non-terpene compounds. Total of 69 compounds were indentified representing 96.1–98.8 % of the chemical composition. The oil extracted from flowering period is characterized by high concentration of hydrocarbon terpenes as a sum of sesquiterpenes (34.8 %) and monoterpenes (24.4 %). Appreciable amount of compounds belong to the chemical class of β-diketones (18.7 %), while percentages of oxygenated monoterpenes and sesquiterpenes were quite low (11.8 % and 6.0 %, respectively). The major compound present in flowering plant was *α*-pinene (15.7 %) followed by γ-curcumene (12.8 %), 4,6,9-trimethyldec-8-en-3,5-dione (8.7 %), neryl acetate (6.9 %), limonene (6.4 %) and β-selinene (5.3 %). Other compounds present in noticeable amounts were *trans*-cariophyllene (3.9 %), α-selinene (3.6 %), γ-selinene (3.1%) and β-diketones.

From the blooming samples, different parts of plant were investigated and the main components of flowers and

Table 1. Essential oil composition (%) of *H. italicum* subsp. *italicum* from Herzegovina

Name	RI[a]	RI[p]	February	May	June	August	October
α-Pinene	935	1095	14.2	22.7	15.7	21.3	26.1
β-Pinene	979	1126	0.2	0.3	0.2	0.0	0.2
β-Myrcene	986	1166	0.2	0.3	1.3	0.0	0.1
α-Terpinene	1000	1178	0.4	0.3	0.0	0.0	0.2
Limonene	1030	1195	9.1	6.4	6.4	1.4	4.9
Isobutyl 2-methyl-2-butenoate	1045	1282	0.3	0.4	0.0	4.6	0.3
γ-Terpinene	1055	1238	0.2	0.2	0.5	0.0	0.5
α-Terpinolene	1087	1272	0.2	0.2	0.3	0.2	0.2
2-Nonanone	1090	1444	0.4	0.2	0.2	0.2	0.1
Linalool	1099	1542	1.3	2.8	1.8	0.2	0.9
2-Methylbutyl 2-methyl-butanoate	1103	1274	0.3	0.0	0.0	1.8	0.0
D-Fenchyl alcohol	1119	1570	0.3	0.1	0.0	0.3	0.1
trans-Pinocarveol	1141	1635	0.4	0.4	0.0	0.5	0.1
Pentyl 3-methyl-2-butenoate	1149	1387	2.1	1.8	1.3	8.8	2.1
Borneol	1172	1683	0.5	0.2	0.1	0.7	0.1
4,6-Dimethyl-3,5-octandione	1180	1588	5.0	2.5	2.2	3.6	1.4
α-Terpineol	1195	1682	2.2	1.5	1.0	1.8	0.3
Nerol	1222	1789	0.8	1.1	1.0	0.3	0.6
Hexyl 2-methylbutanoate	1248	1454	0.3	0.2	0.1	0.7	0.3
Hexyl senecioate	1282	1532	1.3	1.4	0.8	0.3	0.9
2-Undecanone	1291	1588	0.3	0.1	0.1	0.0	0.1
Neryl acetate	1357	1718	5.9	10.6	6.9	3.9	6.0
α-Ylangene	1369	1474	0.2	0.1	0.2	0.3	0.3
α-Copaene	1376	1480	0.6	0.5	1.2	1.7	2.4
Isoitalicene	1376	1527	0.8	0.7	0.0	0.0	1.0
Italicene	1406	1524	2.6	2.2	1.8	1.2	4.5
α-Bergamotene	1413	1556	0.1	0.3	0.7	0.0	0.9
trans-Caryophyllene	1420	1577	1.8	3.5	3.9	3.8	4.5
4,6,9-Trimethyldec-8-en-3,5-dione	1433	1873	7.4	8.5	8.7	5.3	3.3
Neryl propionate	1446	1778	0.8	0.5	1.0	1.1	0.4
β-Farnesene	1451	1669	0.0	0.1	0.2	0.1	tr
α-Humulene	1455	1646	0.1	0.2	0.2	0.2	0.3
Aromadendrene	1459	1654	0.1	0.1	0.1	0.1	0.2
α-Acoradiene	1462	1654	0.2	0.3	0.2	0.2	0.8
β-Acoradiene	1466	1654	0.2	0.0	0.0	0.0	0.0
γ-Selinene	1473	1657	1.9	1.1	3.1	0.0	4.5
2,4,6,9-Tetramethyldec-8-en-3,5-dione A*	1476	1868	7.9	3.1	2.8	2.4	3.4
γ-Curcumene	1477	1679	1.4	3.3	12.8	3.2	7.2
2,4,6,9-Tetramethyldec-8-en-3,5-dione B*	1480	1868	7.2	2.1	2.5	3.9	0.7
ar-Curcumene	1481	1758	0.7	1.3	0.9	2.4	3.3
β-Selinene	1495	1696	1.5	3.9	5.3	0.4	5.2
α-Selinene	1497	1702	0.9	2.5	3.6	0.2	3.4
5,7,10-Trimethylundec-9-en-4,6-dione	1503	1868	0.8	0.7	0.4	0.5	0.4
β-Bisabolene	1507	1710	0.0	tr	0.1	0.0	0.1
β-Curcumene	1509	1727	0.0	0.1	0.2	0.1	0.4
γ-Cadinene	1512	1741	0.0	0.3	0.0	0.0	0.6

Table 1. (continued)

Name	RI[a]	RI[p]	February	May	June	August	October
δ-Cadinene	1517	1742	0.0	0.1	0.2	0.6	0.9
Italicene ether	1533	-	0.6	0.3	0.2	0.2	0.2
α-Calacorene	1538	-	0.0	0.1	0.1	0.0	0.2
Nerolidol d	1560	2029	0.1	0.1	0.1	0.1	0.1
3,5,7,10-Tetramethylundec-9-en-4,6-dione A*	1564	1949	1.9	1.2	1.0	2.0	0.9
3,5,7,10-Tetramethylundec-9-en-4,6-dione B*	1569	1949	1.7	1.1	1.1	1.9	0.9
Caryophyllene oxide	1580	1964	0.2	0.3	0.1	0.7	0.1
Widdrol	1585	-	0.2	0.1	0.0	0.1	0.0
Guaiol	1595	2067	0.6	0.2	0.3	1.4	0.2
Geranyl isovalerate	1604	-	0.1	0.1	0.5	0.0	0.1
Viridiflorol	1608	2160	0.7	0.6	0.6	1.9	0.4
10-epi-γ-Eudesmol	1612	2093	1.9	1.1	0.8	2.5	0.4
1,10-diepi-Cubenol	1616	2108	0.2	0.3	0.3	0.1	0.2
Cubenol	1626	2036	0.1	0.2	0.1	0.2	0.0
γ-Eudesmol	1631	2142	0.3	0.2	0.1	1.1	0.0
β-Eudesmol	1635	2198	0.3	0.4	0.4	1.6	0.2
τ-Cadinol	1641	2146	0.3	0.0	0.0	0.1	0.0
δ-Cadinol	1645	2157	0.1	0.0	0.0	0.1	0.0
α-Eudesmol	1654	2188	2.0	0.7	0.8	tr	0.3
Juniper camphor	1657	2219	0.8	1.0	0.9	5.2	0.2
Bulnesol	1663	2184	0.1	0.1	0.2	0.3	0.0
β-Bisabolol	1667	2130	0.5	0.4	0.3	0.5	0.1
α-Bisabolol	1684	2193	0.3	0.1	0.3	0.2	0.1
TOTAL			96.1	97.8	98.2	98.5	98.8
Monoterpene hydrocarbons			24.5	30.4	24.4	22.8	32.2
Oxygenated monoterpenes			12.0	17.2	11.8	8.7	8.5
Non-terpenic ketones			0.7	0.3	0.3	0.2	0.2
Non-terpenic esters			4.4	3.8	2.2	16.3	3.6
β-Diketones			31.9	19.2	18.7	19.4	11.0
Sesquiterpene hydrocarbons			13.1	20.7	34.8	14.3	40.7
Oxygenated sesquiterpenes			9.4	6.2	6.0	15.6	2.6

Note: RI[a] = Retention index on apolar column; RI[p] = Retention index on polar column; tr = traces. Percentages and order of elution are given on the apolar column.
*A and B are diastereomers.[15,16]

leaves are shown in Table 2. Neryl acetate and α-pinene are present in greater quantity in the leaves, while there are more of 4,6,9-trimethyldec-8-en-3,5-dione, γ-curcumene and 2,4,6,9-tetramethyldec-8-en-3,5-dione B in flowers. Other major components occur in similar proportions in the flowers and in the leaves (Table 2).

In comparison to other chemotypes described, chemical composition of Herzegovina's immortelle EO showed some similarity to those described for ex-Yugoslavia which contained α-pinene and γ-curcumene as dominant components.[27–29] However, Herzegovina's oil contained 4,6,9-trimethyldec-8-en-3,5-dione within the main composition which makes it different from all known chemotypes. It is particularly different from oils characterized by the prevalence of neryl acetate such as those of Corsica, North America, Montenegro, Calabria and Sardinia.[10,15,16,30–32] Samples collected in Tuscany as well as oils from South Croatia also had α-pinene in the highest percentage, but other main constituents distinguished our oil from those.[4,17,20] γ-Selinene represents a characteristic component, not detected in other EOs reported in literature.

Although composition of the essential oils varies with the seasonal changes, α-pinene was found to be the major compound through the whole season. During post-flowering period, a significant reduction in concentration of

Table 2. The main constituents of essential oils from *H. italicum* subsp. *italicum* flowers and leaves

Name	RI[a]	RI[p]	Flowers	Leaves
α-Pinene	935	1095	15.1	19.5
Limonene	1030	1195	6.5	6.1
Linalool	1099	1542	2.3	2.0
Pentyl 3-methyl-2-butenoate	1149	1387	1.2	2.3
4,6-Dimethyl-3,5-octandione	1180	1588	1.8	3.0
α-Terpineol	1195	1682	1.4	1.6
Neryl acetate	1357	1718	5.5	11.4
Italicene	1406	1524	1.2	2.3
trans-Caryophyllene	1420	1577	2.9	3.7
4,6,9-Trimethyldec-8-en-3,5-dione	1433	1873	12.7	8.5
γ-Selinene	1473	1657	3.7	3.6
2,4,6,9-Tetramethyldec-8-en-3,5-dione A*	1476	1868	4.4	3.0
γ-Curcumene	1477	1679	9.3	4.0
2,4,6,9-Tetramethyldec-8-en-3,5-dione B*	1480	1868	4.2	1.4
ar-Curcumene	1481	1758	1.5	0.9
β-Selinene	1495	1696	5.0	4.5
α-Selinene	1497	1702	3.1	2.8
3,5,7,10-Tetramethylundec-9-en-4,6-dione A*	1564	1949	1.2	1.2
3,5,7,10-Tetramethylundec-9-en-4,6-dione B*	1569	1949	1.2	1.1

Note: RI[a] = Retention index on apolar column; RI[p] = Retention index on polar column; tr = traces. Percentages and order of elution are given on the apolar column.
*A and B are diastereomers.[15,16]

limonene, α, β and γ-selinene, γ-curcumene and neryl acetate occurred, followed by increase in amount of esters. This notable amount of non-terpenic esters and sesquiterpenoides was typical only for August samples, since their content was quite low during other parts of season. Hydrocarbon terpenes were a dominant class of compounds during the whole season, while among compounds that contain oxygen, contribution of non-terpenic oxygenated compounds was higher than that of terpenoides. Monoterpenes were dominant in May and August. Important class of compounds were β-diketones whose level reached the maximum of 31.9 % in winter (February), followed by decrease during season until their minimum in mid-autumn (October, 11.0 %). Increase in β-diketone share is followed by decrease in sesquiterpenes.

The Total Polyphenols, Antioxidant Activity and Acetylcholinesterase Inhibition

The content of total polyphenols was determined in methanolic extracts of *H. italicum* subsp. *italicum*. The highest total phenol content was measured for the whole plant (271.36 ± 5.3 mg GAE/g dry extract), then the flowers (221.04 ± 6.7 mg GAE/g d.e.) and the leaves (153.12 ± 4.6 mg GAE/g d.e.).

Several previous studies indicated that flavonoids were the major fraction of phenolic compounds present in polar extracts of *H. italicum*.[10,13] Albayrak et al. reported that the total phenolic contents of the methanolic extracts from *Helichrysum* species ranged from 66.74 to 160.63 mg GAE/g d.e.[33] In ethanol/aqueous extract (45 % *w*/*w*) total phenolic was 31.97 mg GAE/g d.e.[10] The differences in the total phenolic contents of *Helichrysum* species may be due to the difference in their collection time, collection area, season, part of the plant and type of extraction.[32,33]

The antioxidant activity of MeOH extracts and EOs of *H. italicum* subsp. *italicum* was quantified using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing ability of plasma assay (FRAP). Examined EOs did not achieve 50% of DPPH radical inhibition (Figure 1).

Antioxidant capacity measured by the FRAP method showed the greatest reducing power of EOs at concentration 880 μg/mL (1.0–2.2 mM Fe^{2+}) (Figure 2). BHT was used as a positive control (59 μg/mL is eq. to 2.6 mM Fe^{2+}).

The MeOH extracts displayed significantly better antioxidant activity according to both methods. DPPH method revealed that MeOH extract from flowers exhibited the most effective radical scavenging ability (IC_{50} = 23 μg/mL), followed by whole plant (IC_{50} = 31 μg/mL) and leaves (IC_{50} = 34 μg/mL) (Figure 3). BHT achieved IC_{50} at 80 μg/mL. As shown in Figure 4, the greatest FRAP antioxidant

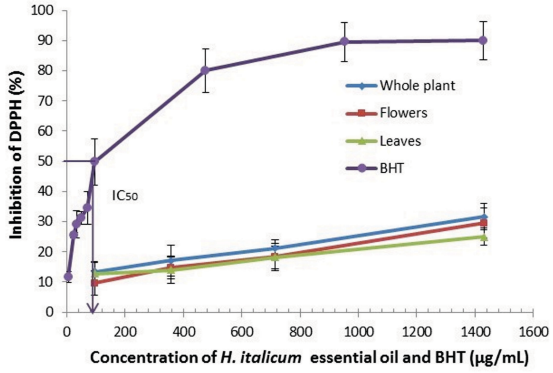

Figure 1. The inhibition of the DPPH by different concentrations of *H. italicum* subsp. *italicum* essential oils and BHT.

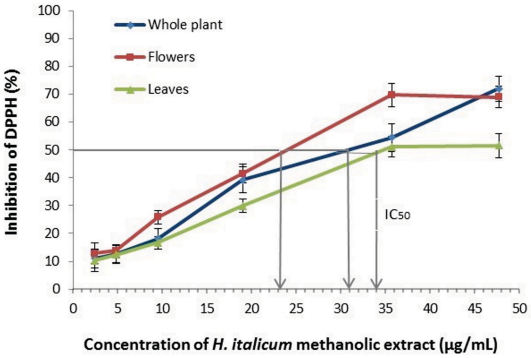

Figure 3. The inhibition of the DPPH by different concentrations of *H. italicum* subsp. *italicum* methanolic extracts.

capacity of MeOH extracts was reached at concentration 29 μg/mL (1.1–2.2 mM Fe^{2+}). Therefore, MeOH extracts of *H.italicum* subsp. *italicum* had very strong antioxidant activity in comparison with the BHT (29 μg/mL is eq. to 0.9 mM Fe^{2+}). The higher antioxidant activities of methanolic extracts may be attributed to the high phenolic concentrations in these extracts, especially flavonoids.[34,35]

Furthermore, immortelle also contains non-flavonoids such as pyrones, phloroglucinols and acetophenones, which might act as scavengers of free radicals.[10,33] Tundis et al. confirmed strong antioxidant activity of immortelle methanolic extracts from Calabria at concentrations of 50 μg/mL.[32] Kladar et al. noticed a significant difference between the radical scavenging potential of the ethanolic extract (IC_{50} = 0.99 μg/mL) and the essential oil (IC_{50} = 1.76 mg/mL).[10] Our research did not find a high activity of immortelle essential oils.

AChE inhibitory activity of immortelle EOs and MeOH extracts are presented in Figures 5 and 6. The MeOH extract and EO from leaves showed the highest AChE inhibitory activity; MeOH extract of leaves at 340 μg/mL, MeOH extract of whole plant at 440 μg/mL and EO from leaves at 135 μg/mL achieved 50.0 % inhibition. Galanthamine was used as the reference AChE inhibitor (IC_{50} = 22 μg/mL). The results indicate that the MeOH extracts and EO from immortelle can inhibit AChE. Flavonoids from polar extracts and terpenes from essential oil are considered responsible for the positive biological effects of immortelle.[13] Various plant extracts, essential oils and their components have been investigated for their effects on AChE.[36–39] It has been confirmed that the majority of AChE inhibitors identified in the essential oils are terpenoids, especially monoterpenes.[40–42] Significant inhibitory activity of EO from leaves may be related to its high content of

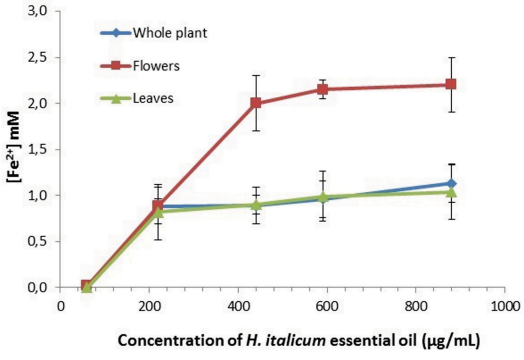

Figure 2. The antioxidant capacity in the presence of different concentrations of *H. italicum* subsp. *italicum* essential oils by the FRAP method.

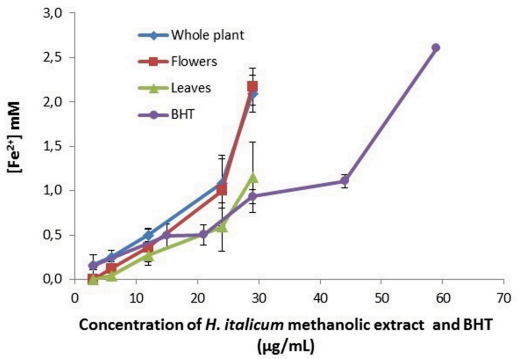

Figure 4. The antioxidant capacity in the presence of different concentrations of *H. italicum* subsp. *italicum* methanolic extracts and BHT by the FRAP method.

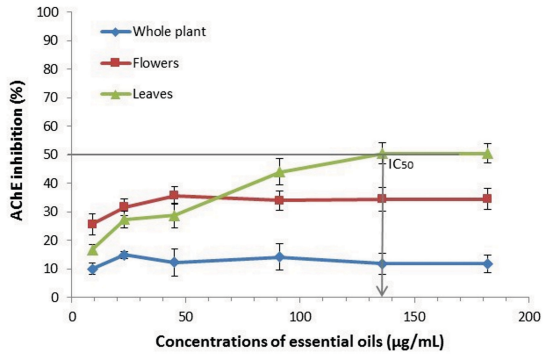

Figure 5. Acetylcholinesterase inhibition by different concentrations of *H. italicum* subsp. *italicum* essential oils.

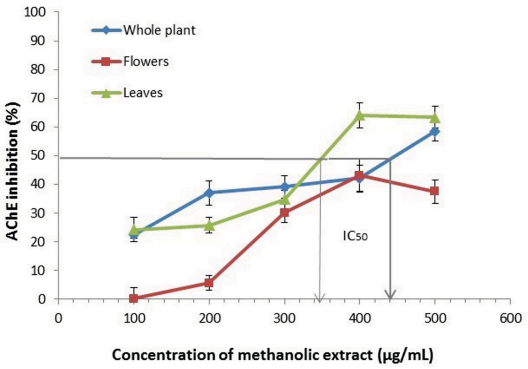

Figure 6. Acetylcholinesterase inhibition by different concentrations of *H. italicum* subsp. *italicum* methanolic extracts.

monoterpene. This oil contains a large amount of α-pinene which is a potent inhibitor of AChE.[41] The MeOH extract show moderate potential to inhibit AChE. Future similar research should be directed towards determining the active components in the MeOH extracts.

CONCLUSION

Documentation of all chemotypes of *Helichrysum italicum* subsp. *italicum* essential oil is of great importance since the use of this subspecies is due to the specific properties related to chemical composition. The results demonstrate remarkable chemical diversity observed for the *H. italicum* subsp. *italicum* EO from different localities. Thus, presented data can be valuable in chemotypes assessment. Immortelle EO from Herzegovina belongs to the oil type which is rich in hydrocarbon terpenes and with appreciable amount of β-diketones. Through vegetation cycle, a change in amount of identified compound occurs. Our results indicate that the MeOH extracts isolated from immortelle have strong antioxidant activity and that EO and MeOH have inhibitory potential to acetylcholinesterase.

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