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Biochemical composition of essential oil of Corsican *Helichrysum italicum* (Roth) G. Don, introduced and cultivated in South Bulgaria

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Abstract

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This study provided GC-FID and GC-MS analysis of the volatile constituents of *Helichrysum italicum* (Roth) G. Don, introduced from Corsica (France), cultivated in South Bulgaria, harvested in different phenological stages – floral budding period and flowering period, and compared its quality to the quality of the species of native origin. Similarities in qualitative, but with differences in quantitative composition of their essential oils were indicated, because of the different environmental conditions. 41 components were identified, representing 89.25-95.63% of the total essential oil composition. In the essential oil of *H. italicum*, harvested in the floral budding period, the content of sesquiterpenes and oxygenated monoterpens were higher. The essential oil had a good, balanced content of monoterpenes, sesquiterpenes and their oxidized derivatives, like α- and γ-curcumene, neryl acetate, α-pinene, α-copaene, limonene, *cis*- and *trans*-α-bergamotene, β-caryophyllene, eudesm-5-en-11-ol and selina-4,11-diene. Cultivating ability of these plant species opens up new opportunities for the food, pharmaceutical and cosmetic industries.

Keywords: Helichrysum italicum; essential oil; neryl acetate; GC-FID; GC-MS

Introduction

Genus *Helichrysum* (from the Greek *helios* – sun and *chrysos* – gold) belongs to the family *Asteraceae* and includes over 600 species distributed preeminently in the Mediterranean area, at sea level up to 1700 m and growing preferably on sandy or loamy soils (Perrini et al., 2009). One of the most common species in the genus is *Helichrysum italicum*. Viegas et al. (2014) reviewed a number of reports on the traditional use of *H. italicum* (immortelle) in European countries, mainly Italy, Spain, Portugal and Bosnia and Herzegovina. From its aerial parts were extracted by various techniques and liquid phases a lot of different biologically active substances. To immortelle extracts, were attributed anti-microbial (Nostro et al., 2001; Taglialatela-Scafati et al., 2012; Viegas et al., 2014; Cui et al., 2015), anti-inflammatory (Sala et al., 2002; Appendino et al., 2007) and anti-oxidant properties (Facino et al., 1990; Politeo et al., 2006; Mastelić et al., 2005, 2008). The extract by a hydro-distillation technique, named also essential oil (EO), found application as a food supplement (Mari et al., 2014), cosmetic and pharmaceutical industries (Viegas et al., 2014; Perrini et al., 2009). EO can be used for aid skin regeneration and wound healing (Voinchet and Giraud-Rober, 2007). A few ethnobotanical

data reported its use as parasite repellent for animal use (Barber et al., 2005; Rivera et al., 2008) but not as insecticidal or insect repellent (Viegas et al., 2014).

Because of the widespread applications of the immortelle EO, the species begin nowadays to be cultivated. The geographic location and climate of Bulgaria are suitable for growing of essential oil plants, including *H. italicum.* The country is third in the world and leader in Europe in the production and export of herbs (http://www.bta.bg/bg/c/BO/id/1385863) and immortelle is the new hit.

The aim of this study was to determine the biochemical composition and quality of EO of a cultivated in South Bulgaria *H. italicum* and to compare it to the quality of species of native origin.

Materials and Methods

Plant material and EO isolation procedure

The object of this study was plant material from the aerial parts of *Helichrysum italicum* (Roth) G. Don, introduced species from Corsica (France). The plant was cultivated in Turia village, municipality of Pavel Banya, Stara Zagora region (42° 34′ 0″ N, 25° 11′ 0″ E; 475 m a.s.l.). The ecological conditions of the region were: continental Mediterranean climate and eroded Luvisols. The conditions were suitable for cultivation of *H. italicum* (Perrini et al., 2009). For the purpose of the study, aerial parts of the herb were harvested during floral budding period (just before flowering) and full flowering period (June and July 2017, respectively). For the essential oil analysis were used the aerial parts from four batches in each phenological stage and prepared two parallel samples for each batch (EO laboratory samples). Plant material was air dried in shadow at room temperature and grounded in a mechanical grinder (final powder size less than 400 μm). The samples were stored in the dark and cool rooms at 16-18°C prior to EO extraction. 100 g of each sample were extracted by hydro-distillation for 4 hours with a Clevenger-type apparatus according to the European Pharmacopoeia 7th edition. The organic layer obtained on top of the aqueous distillate was separated. After drying with anhydrous sodium sulfate (Na_2SO_4) EO was kept in sealed airtight glass vials at 4 °C until used.

Analyzed were also two parallel samples for each batch in each phenological stage, obtained by steam hydro-distillation in a distillery located in the town of Kazanlak (EO manufacturing samples). As raw materials in the distillery were used grounded plant aerial parts and the process of distillation lasted for 4 hours. The samples are presented in Table 1.

Table 1. Sample identification by groups

№	EO preparation	Phenological stage
1	laboratory	floral budding
2	laboratory	full flowering
3	manufacture	floral budding
4	manufacture	full flowering

Chemical analysis

The samples were analysed further by Gas Chromatography techniques: GC-FID for quantification of neryl acetate (NA) by external calibration method and GC-MS for identification and relative quantification of other EO components.

The GC-FID analysis was performed with a capillary column HP-5 Agilent Technologies, CA, USA (30 m x 0.32 mm i. d., film thickness 0.25 μm) on Agilent 7890 gas chromatograph (Agilent Technologies, CA, USA) with a flame ionization detector (FID). Nitrogen was the carrier gas (1ml.min^{-1}). The samples (1 μl) were injected in split mode 100:1. The injector temperature was kept at 260°C. The start column temperature was 40°C. After 2 min isothermal mode the column temperature was set up to 80°C at a rate of 2°C.min^{-1} and finally up to 240°C at a rate of 5°C.min^{-1}. FID was operated at 280°C.

NA was quantificated by the external standard method. The calibration was carried out using five concentration levels (0.015, 0.050, 0.10, 0.50 and 1.00 mg. l^{-1}) of reference material neryl acetate (min 97%, GC), purchased from Sigma-Aldrich (St. Louis, MO). Each calibration standard was run in triplicates. The squared correlation coefficient (r^2 = 0.9991) obtained by linear regression demonstrated an excellent relationship between peak area and concentration according to the International Conference on Harmonization guidelines (1997). Each sample solution was run in triplicate and the results were expressed as mean values ± standard deviation (SD).

The determination of other EO components was carried out by using Agilent 7890 gas chromatograph with Agilent 5975 Inert MS quadrupole detection with electron capture ionization (70 eV). The chromatography separation was performed on a capillary column HP-5-MS (5% phenyl methyl siloxane, 30 m x 0.25 mm i.d., film thickness 0.25 μm) purchased from Agilent Technologies, CA, USA. The column temperature program was identical to the other one, applied by GC-FID method. The scan time was 2 s and m/z was in the range from 50 to 550. The carrier gas phase was helium (1 ml.min^{-1}). The samples (1 μl) were injected in split mode 40:1. Before injecting EO, was diluted with methanol in ratio 1:20 (v/v).

The EO components were identified by comparing registered mass spectrums with those of the NIST 08 database (National Institute of Standardization and Technology, Gaithersburg, MD, USA) or by comparison of their mass spectra and retention indices with those reported in literature (Kováts, 1958; Adams, 2001).

Results and Discussion

Samples from four batches of the aerial parts of *H. italicum*, introduced from Corsica (France) and cultivated in South Bulgaria, were harvested in different phenological stages – in the floral budding period and in full flowering period. By the hydro-distillation technique were prepared laboratory and manufacturing essential oil samples (Table 1). The amount of EO extracted in manufacturing conditions by steam hydro-distillation, was generally quite low, 0.19 and 0.26% (v/w) in contrast to the laboratory EO yields – 0.4 and 0.5% (v/w); respectively (Table 3). Maksimović et al. (2017) reviewed a lot of studies, reporting hydro-distillation of *H. italicum* in a Clevenger-type apparatus from 1 to 5 h. According to the literature data, the obtained yields were in the range of 0.02 to 0.78%. So, the yielded EOs in this study felt within the specified limits. The yields of EOs of plant material collected at full flowering period were definitely higher, than these obtained from plant material collected in budding period. But the manufacturing process was not so effective and needs to be optimized.

For immortelle EO qualifying the recommended approach is the determination of the monoterpene – neryl acetate (NA), which amount should not be below 30% (v/w) (Rottenburg, 2015) and the most common methods for analysis and identification of essential oil components were GC-FID and GC-MS. Voinchet and Giraud-Robert (2007) highlighted, that NA is the main component of the EO, contributed to pain relief. They also attributed the observed effects to the occurrence of italidiones in *H. italicum* EO. To this class of molecules attributed anti-haematoma properties, so that *H. italicum* EO was often called the "super arnica" of aromatherapy (Guinoiseau et al., 2013) and applied in cases of couperose skin, haematoma, thrombosis and the prevention of bruises. When *H. italicum* EO is mixed with some other specific EOs, the mixtures are thought to be anti-allergenic. So, these aromatherapy prescriptions could be helpful in cases of asthma, hay fever or eczem (Rhind, 2012). The neryl acetate content in the present study was determined by GC-FID analysis and the values were evaluated by external standard method (Table 2). The results were presented as mean value ± SD of the tested eight samples (two parallel samples from each batch).

The NA amounts in the EOs, extracted from the cultivated *H. italicum* harvested in the full flowering period were higher than these, extracted from the cultivated *H. italicum* harvested in the floral budding period (Table 2). Because of the low EO yield, extracted in manufacturing condition, the NA content in the manufacturing samples was small (from 9.5 ± 0.9% to 12.1 ± 1.2%). Compared to the NA content determined in EOs, extracted from *H. italicum* species from native origin, the obtained results are similar: the obtained amounts in EOs from immortelle, harvested in Croatia, ranged from 8.1% (Zeljković et al., 2015) to 10.4-23.2% (Mastelić et al., 2008; Viegas et al., 2014). In contrast, *H. italicum* from Corsician coast (France) was extremely rich in neryl acetate – from 15.8% to 42.5% (Bianchini et al., 2001). Ornano et al. (2015) investigated the content of immortelle EO from Sardinia (Italia) and determined the major compound was the monoterpene ester neryl acetate (18.2%). Leonardi et al. (2013) conducted a large-scale study and found a content of 5.6% to 45.9% in EOs from *H. italicum,* harvested in Tuscany (Italy) in different locations and seasons. So, the NA amounts were depended on the soil and climatic conditions and also on the phenological phases.

Table 2. Neryl acetate content in EO samples from *H. italicum*, evaluated by GC-FID analysis (n = 8)

№	Neryl acetate content	
	mg/100g DM*	C, % (v/w)
1	56.8 ± 5.6	15.9 ± 1.7
2	80.1 ± 7.9	17.8 ± 1.8
3	30.4 ± 0.3	9.5 ± 0.9
4	27.6 ± 2.7	12.1 ± 1.2

*DM – dry (plant) material

Table 3. Compound content of EO of *H. italicum* evaluated by GC-MS analysis (n = 8)

№	Compounds	RI[a]	Group No 1	Group No 2	Group No 3	Group No 4
			%[b]	%[b]	%[b]	%[b]
1	2	3	4	5	6	7
1	α-Pinene	927.6	5.59	8.77	19.52	7.03
2	Camphene	941.1	0.22	0.32	0.61	0.26
3	β-Pinene	970.6	0.15	0.20	0.65	0.26
4	Limonene	1028.4	1.94	2.86	4.45	1.72
5	1,8-cineole	1030.9	0.06	0.06	0.08	tr
6	γ-Terpinene	1061.9	0.23	0.56	0.08	0.21

Table 3. (Continued)

1	2	3	4	5	6	7
8	Linalool	1114.0	0.87	1.89	1.11	0.51
9	Terpenen-4-ol	1183.3	0.58	1.17	0.66	0.61
10	α-Terpineol	1195.3	2.78	2.72	0.37	0.28
11	Nerol	1229.5	2.32	2.05	0.63	0.21
12	Neryl acetate	1365.8	17.13	20.60	12.02	18.01
13	α-Ylangene	1370.1	2.39	2.07	2.75	3.69
14	α-Copaene	1376.8	6.29	5.42	6.23	12.83
15	*iso*-Italicene	1403.0	0.77	0.55	0.83	0.98
16	*cis*-α-Bergamotene	1412.2				
17	β-Caryophyllene	1420.0	4.86	4.78	3.07	3.29
18	*trans*-α-Bergamotene	1433.2				
19	Neryl propionate	1451.7	tr	tr	tr	tr
20	α-Humulene	1457.2	tr	tr	tr	tr
21	Alloaromadendrene	1461.6	tr	tr	tr	tr
22	α-Acoradiene	1466.0	tr	tr	tr	tr
23	β-Acoradiene	1469.9	tr	tr	tr	tr
24	Selina-4,11-diene	1476.4	2.58	2.54	2.92	2.72
25	Citronellol isobutanoate	1482.1				
26	γ-Curcumene	1485.7	18.11	12.46	25.76	12.99
27	α-Curcumene	1488.1				
28	β-Eudesmene	1496.1	3.07	5.57	5.06	3.08
29	α-Selinene	1502.7	1.00	2.70	0.46	0.28
30	*δ*-Amorphene	1507.3	3.47	0.59	1.76	0.43
31	β-Curcumene	1513.5	1.45	0.26	0.84	1.06
32	γ-Cadinene	1517.4	0.76	0.21	0.33	0.57
33	δ-Cadinene	1522.1	0.68	0.50	0.25	0.29
34	(E)-Nerolidol	1563.8	0.56	0.61	0.83	0.85
35	Guaiol	1598.7	0.25	0.46	0.66	1.73
36	Eudesm-5-en-11-ol	1617.9	5.73	4.96	1.92	8.14
37	α-Eudesmol	1660.5	1.61	1.19	0.34	1.34
38	Juniper camphor	1664.4	2.56	2.54	0.56	3.40
39	Bulnesol	1669.6	0.75	0.64	0.32	0.96
40	β-Bisabolol	1673.7	0.3	0.19	0.13	0.34
41	Longibornyl acetate	1689.8	0.93	0.59	0.22	1.12
Total compounds			41	41	41	41
Total identified, %			90.16	89.75	95.63	89.25
Yield, % (v/w)			0.4	0.5	0.19	0.26
Monoterpenes (M), %			8.36	12.99	25.60	9.55
Oxygenated monoterpenes (MO), %			23.68	28.43	14.79	19.61
Sesquiterpenes (S), %			45.43	37.15	50.26	42.21
Oxygenated sesquiterpenes (SO), %			12.69	11.18	4.98	17.88
Ketones and β-Diketones			18.11	12.46	25.76	12.99

*Compounds are listed in order of their elution; [a]Linear retention time index on HP-5MS column, experimentally determined using homologous series of C6-C36 alkanes; [b]Percentage values are average of determinations of eight samples with a RSD% not more than 10%; trace, tr ≤ 0.1%

The chemical composition of the essential oils from samples of *Helichrysum italicum* subsp. *microphyllum* (Willd.) Nyman, introduced species from Corsica (France) and cultivated in South Bulgaria, was determined in the present study by GC-MS. The obtained results were given as mean value of 8 tested samples (two parallel samples of four batches) from each phenological stage (Table 3).

In total, 41 components were identified in the EOs, representing 89.25-95.63% of the essential oil composition. The essential oil was rich in sesquiterpenes (S), which was 37.15-50.26% of the total EO composition, and their oxygenated derivates (SO) were contained in smallest quantities – from 3.06% to 11.01% (Fig. 1). The major compounds of the tested EOs were α- and γ-curcumene (12.46-25.76%), neryl acetate (12.02-20.6%), α-pinene (5.59-19.52%), α-copaene (5.42–12.83%), limonene (1.72-4.45%), α-*cis*- and α-*trans*-bergamoten (3.07-5.57%), β-caryophyllene (3.07-5.57%), *δ*-amorphene (0.43-3.47%), eudesm-5-en-11-ol (1.92-8.14%), selina-4,11-diene (2.54-2.92%).

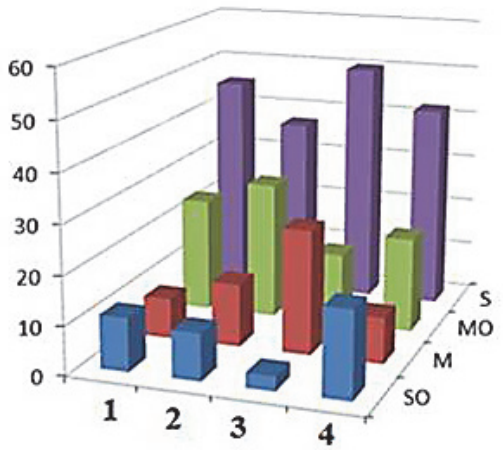

Fig. 1. Distribution of terpenes of EO from *H. italicum,* evaluated by GC-MS analysis: S – sesquiterpenes; MO – oxygenated monoterpenes; M – Monoterpenes; SO – oxygenated sesquiterpenes

In the EOs of *H. italicum*, harvested in the floral budding period the content of sesquiterpenes and oxygenated monoterpenes were higher. The amount of monoterpenes and their oxygenated derivates in the full flowering period were increased at the expense of sesquiterpenes and their oxygenated derivates, which were decreased.

The biochemical composition of *H. italicum* EO is already known: esters 24-66%, including neryl acetate 20-62%; monoterpenes approximately 24%; ketones 15-22%, including diones 11-20% and others (Rottenburg, 2015) The unique mixture of esters, hydrocarbons and ketones makes the solid and at the same time highly effective composition of the *H. italicum* EO. The major biologically active substances determined were: neryl acetat, α-pinen, γ-curcumen, β-selinen, geraniol, *trans*-nerolidol, β-caryophyllen, linalool, limonen and 2-methyl-cyclohexylpentanoat (Mastelić et al., 2008; Tucker et al., 2009). Depending on the region of origin, soil conditions and climatic conditions, the biochemistry of Immortelle essential oil varies greatly and the activities of essential oils obtained by hydro-distillation were diverse.

Bianchini et al. (2001) investigated the chemical profile of essential oils from *Helichrysum italicum* subsp. *microphyllum* growing in Corsica (France) and found neryl acetate as predominant compound, with amounts from 15.8% (from plants in stage of early shoots) to 42.5% (in full flowering period) and determined from plants in early shoots higher amounts of ketones and β-diketones in contrast to samples harvested in the stages of flowering. The analyzed, from the authors, Corsican essential oils were rich in oxygenated compounds (neryl acetate, neryl propionate, aliphatic ketones and diketones) and low contents of hydrocarbons (limonene,γ-curcumene, curcumene). On the contrary, the cultivated in South Bulgaria Corsican immortelle contained less ketones and β-diketones and expected the amounts were higher in the floral budding period (Table 3). The tested EOs were characterized predominately by hydrocarbons – sesquiterpenes and monoterpenes (Fig. 1). The hydrocarbon content was also differed: the sesquiterpenes was the major hydrocarbon fraction in the EOs from introduced species, and the monoterpenes – the major hydrocarbon fraction in the Corsican EOs.

The authors in another study (Bianchini et al., 2003) compared the chemical composition of EOs from *Helichrysum italicum* subsp. *microphyllum* growing in Corsica (France), Tuscany and Sardinia (Italy): Corsican EOs contented more oxygenated derivates: neryl acetate (major compound), neryl propionate, nerol, acyclic ketones and *β*-diketones. Tuscan EOs were characterized by higher contents of hydrocarbons (*α*-pinene, *β*-caryophyllene, *α*- and *β*-selinenes). The comparison of essential oils hydrodistilled from Corsican and Sardinian *Helichrysum italicum* subsp. *microphyllum* showed almost similar chemical compositions. The main constituents were: neryl acetate, nerol, neryl propionate, linalool, eudesm-5-en-11-ol and *γ*-curcumene.

The chemical profile of the EOs extracted from *H. italicum* subsp. *microphyllum*, introduced and cultivated in Bulgaria, was found to be a mix of similar to the essential oil content of Corsican and Tuscanian plants of *H. italicum*. The different climate and soil conditions affected the chemical composition. Leonardi et al. (2013) investigated the impact

of the environment conditions, including the soil type, on the Immortelle EO chemical profile. Essential oils of Luvisols samples were characterized by the presence of α- and β-pinene, camphene, γ-terpinene, 1,8-cineole, aterpineol, borneol, nerol, and linalool, much of them were found in good quantities in the cultivated in South Bulgaria Immortelle EOs.

The NA content of the tested in the present study EOs did not reached 42.5% (Bianchini et al., 2001) in the full flowering period, but the EOs had a good, balanced content of monoterpenes, sesquiterpenes and their oxidized derivatives, like α- and γ-curcumene, neryl acetate, α-pinene, α-copaene, limonene, α-*cis*- and α-*trans*-bergamoten, β-caryophyllene, eudesm-5-en-11-ol and selina-4,11-diene.

Conclusions

This study provided GC-FID and GC-MS analysis of the volatile constituents of the Mediterranean species *Helichrysum italicum*, introduced and cultivated in South Bulgaria and compared its quality to the quality of the species of native origin. Similarities in qualitative, but differences in quantitative composition of their essential oils were indicated. The reason was the different environmental conditions. The contained flavoring substances, such as neryl acetate (sweet, floral, reminiscent of oranges and roses smell), pinenes (fresh pine odor), limonene (lemon-like odor), curcumenes (herbal, "curry" odor), etc. make the immortelle EO attractive and very wanted food and perfume supplement. The ability for cultivating of these plant species opens up new opportunities for the food, pharmaceutical and cosmetic industries, whose production capacities rise up, because of the world population increasing, for one side and for the other side higher getting of the requirements for the quality of the used raw materials and products. In order to achieve the maximum yield of EO and the results to show good repeatability and recovery in production conditions, care should be also taken to the technological parameters of the steam hydro-distillation.

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