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Chemophenetic Analysis of Ten Wild-Growing East Adriatic *Helichrysum italicum* (Roth) G. Don (Gnaphalieae, Asteraceae) Populations

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ABSTRACT

The essential oil (EO) and phenolic composition of *Helichrysum italicum* (Roth) G. Don from natural populations in east Adriatic were analyzed using gas and liquid chromatography coupled with mass spectrometry. In EOs of the studied populations, 146 compounds were identified, representing, on average, 97.3% of the total oil composition. The essential oils were dominated by sesquiterpenes. γ -Curcumen was among the most abundant compounds in all samples. Twelve compounds were present in concentrations greater than 5% in at least one of the ten populations. In the extracts, 73 phenolic compounds were identified, belonging to four classes. Compound classes included phenolic acids, flavonoid glycosides, flavonoid aglycones, pyranones, and related compounds. Arzanol was the most prominent peak across all samples, and pyranones and related compounds were the most abundant. No correlations between EO and flavonoid aglycone composition and the studied environmental parameters were found, whereas phenolic acids and glycosides showed correlations with temperature and precipitation.

1 | Introduction

The genus *Helichrysum* Mill. (Asteraceae family) includes approximately 600 accepted species distributed worldwide [1, 2]. *Helichrysum italicum* (Roth) G. Don, commonly known as immortelle, is a typical Mediterranean plant that grows in arid areas of Southern Europe on alkaline, dry, sandy, and poor soils [3]. It is an aromatic shrub, 30–70 cm high [4], with elongated, linear leaves. The underside of leaves is silvery due to tomentose and glandular hairs [5]. The capitula is composed of numerous bright yellow flowers with a strong, curry-like aroma [6]. Their essential oil (EO) is located in glandular hairs on flower petals, sepals, bracts, and stem leaves [7].

According to the Plants of the World database (<https://powo.science.kew.org/>), five subspecies were previously recognized within *H. italicum*: *H. italicum* subsp. *italicum* (Roth) G. Don (widespread in Italy, Croatia, eastern Mediterranean coast of France and Corsica, Bosnia and Herzegovina, Greece—mainly Aegean islands and Cyprus), subsp. *microphyllum* (Willd.) Nyman (endemic to Crete), subsp. *siculum* (Jord. & Fourr.) Galbany, L. Sáez & Benedí (endemic to Sicily) [8], subsp. *ericoideum* (Fiori) Georgiadi and subsp. *pseudolitorum* (Fiori) Bacch., Brullo & Mossa. Herrando-Moraira et al. [9]. provided a taxonomic revision of the whole *H. italicum* complex. The Majorcan Mountain populations of *H. italicum* subsp. *microphyllum* is now considered as *H. massanellianum* Herrando, J. M. Blanco, L.

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Sáez & Galbany; *H. italicum* subsp. *tyrrhenicum* (Bacch., Brullo & Giusso) Herrando, J.M. Blanco, L. Sáez & Galbany is now sixth subspecies and it comprises populations of *H. italicum* subsp. *microphyllum* from Corsica, Sardinia, Majorca coastline, and Dragonera islet, while subsp. *microphyllum* is restricted to the island of Crete.

Numerous scientific studies, summarized in Ninčević et al. [3], confirm the health-promoting properties of immortelle. Due to the large number of subspecies and genotypes, along with the influence of environmental conditions (e.g., climate and soil type), there is significant variation in EO quality [10, 11]. A wide range of variations in the composition of the EO of immortelle has been identified [12–15]. Although its EO is highly valued, there is no official quality assessment standard. However, there are two main types of *H. italicum* EO on the market: Corsican (rich in oxygenated monoterpenes, especially neryl acetate) and Balkan (with dominant sesquiterpenes γ -curcumene and *ar*-curcumene, and monoterpene α -pinene) [16]. Researchers who studied the chemical composition of *H. italicum* natural populations in Croatia found significant variability in the EO. This diversity is attributed to the influence of geographical origin [17], environmental factors, growth stage [18], genetic factors [19], and processing methods [20]. Among phenolic compounds, numerous chemical subgroups have been identified in *H. italicum*, including flavonoids, acetophenones, coumarins, phenolic acids, and their derivatives [21]. Several previous studies have shown that flavonoids are the major part of phenolic compounds present in the extracts of *H. italicum* [22, 23].

Most of the studies regarding phytochemistry of *H. italicum* are focused on bioactivity, while variability was rarely directly studied. Our aim was to investigate variability in the composition of EOs and extracts from different populations from Croatia and Montenegro. Additionally, environmental parameters that could potentially influence the variability of the studied specialized metabolites were also analyzed.

2 | Results and Discussion

2.1 | Essential Oil Composition and Variability

The composition of EOs of the studied populations is shown in Table 1. In EOs of the studied populations, 149 compounds were detected, of which 146 were identified, representing an average of 97.3% of the total oil composition. An average of 64 compounds were detected per population, of which 62 were identified. The EOs were dominated by sesquiterpenes (56.0%–75.1%). A high percentage of sesquiterpene hydrocarbons characterized the EOs of populations HI1, HI2, HI4, HI5, HI7, and HI8, while EOs of HI6, HI9, and HI10 were dominated by oxygenated sesquiterpenes. In EOs of population HI3, percentages of sesquiterpene hydrocarbons and oxygenated sesquiterpenes were about equal. The most abundant components were α -pinene (HI1), γ -curcumene (HI2, HI8, and HI9), neryl acetate (HI3), β -selinene (HI4, HI5, and HI7), ledol (HI6), and 5-*epi*-7-*epi*- α -eudesmol (HI10). γ -Curcumene was among the most abundant compounds in all samples (5.4%–17.2%). Twelve compounds were present in concentrations greater than 5% in at least one of the ten populations. The most abundant compounds were α -pinene, γ -curcumene, *ar*-curcumene,

(*E,Z*)-2,6-dodecadial-1-ol, β -selinene, α -selinene, neryl acetate, ledol, rosifolol, 5-*epi*-7-*epi*- α -eudesmol, selin-11-en-4- α -ol, and (*E*)-nerolidyl isobutyrate.

Principal component analysis (PCA) separated all samples into two groups (Figure 1a). The first two eigenvectors accounted for 58.1% of the total variability. Most of the variability is attributed to four components: ledol, α -pinene, γ -curcumene, and 5-*epi*-7-*epi*- α -eudesmol. On the first axis, population AC_Stoja (HI6) is clearly separated from the other populations, characterized by a high abundance of ledol and a lower percentage of γ -curcumene and α -pinene. Due to differences in percentages of 5-*epi*-7-*epi*- α -eudesmol, β -selinene, and neryl acetate, the rest of the populations formed three clusters on the second axis. Populations Orjen (HI1), Kukljica (HI2), and Pula (HI8) differed from the rest in the lowest percentages of 5-*epi*-7-*epi*- α -eudesmol and the highest percentages of γ -curcumene and α -pinene. On the other hand, populations Brsač and Kali had the highest levels of 5-*epi*-7-*epi*- α -eudesmol, positioning them on the opposite end of the second axis.

Hierarchical cluster analysis (Euclidean distances, UPGMA) supports the separation of AC_Stoja (HI6) from all other samples due to the high presence of ledol, and Orjen (HI1) from all others due to a high presence of α -pinene. In contrast, all other populations are separated into distinct clades due to differences in the abundance of several components (Figure 1b).

Multivariate tests of EO composition revealed high variability within the sample, separating geographically proximate populations (Figure 1). For example, two geographically proximate populations, Kukljica (HI2) and Kali (HI3), exhibited different primary components, and their EO profiles differed substantially. The same can be said for samples from Istria (HI6–HI9), which showed even greater variability in a small geographic area. The differences in EO profiles cannot be attributed solely to environmental factors, suggesting the presence of putative chemotypes in the studied area. Since each sample is a pooled sample of individuals from a single locality, one may assume that differences between closely spaced sampling locations could be due to differences in chemotype ratios within each sample.

Results of literature search on immortelle EO composition are presented in Table 2. The classification of *H. italicum* chemotypes was generated by Aćimović et al. [15]. Depending on the main constituents, the authors identified ten chemotypes of *H. italicum*, namely (1) high neryl acetate chemotype (50.5%–83.4%), (2) moderate neryl acetate chemotype (19.5%–48%), (3) neryl acetate + *ar*-curcumene (3.9%–20.3% and 0.8%–14.5%, respectively), (4) *ar*-curcumene + γ -curcumene (17.9%–28.6% and 12%–22%, respectively), (5) γ -curcumene (13.6%–27.7%), (6) high α -pinene chemotype (25.2%–53.5%), (7) moderate α -pinene (5.6%–20%), (8) juniper camphor (25.3%–45.1%), (9) β -selinene (11.6%–38%) and (10) italidiones chemotype.

According to this classification, HI1 belongs to a moderate α -pinene chemotype, which was also recorded in Croatia [5] and Bosnia and Herzegovina [12, 25]. Sample HI2 can be assigned to the γ -curcumene chemotype, which was also present in samples from Montenegro and Portugal, as well as in some samples from Serbia [22, 34, 35]. Sample HI7 belongs to the β -selinene chemo-

TABLE 1 | Chemical composition of the immortelle essential oils.

No.	Compound	LRI	HI1	HI2	HI3	HI4	HI5	HI6	HI7	HI8	HI9	HI10
1	3,4-Hexanedione	835	0.2	0.4	0.4	1.0	1.64	—	—	0.4	0.4	0.9
2	α -Pinene	933	16.5	1.6	1.9	1.6	1.60	0.6	0.6	8.0	1.8	1.7
3	α -Fenchene	944	0.7	tr	0.1	—	—	—	—	0.2	—	0.2
4	Camphene	946	0.7	0.1	0.2	—	—	—	—	0.3	—	0.2
5	3-Octanone	957	0.5	1.0	2.2	2.2	2.27	2.8	1.7	2.1	1.0	1.7
6	β -Pinene	975	0.2	—	0.2	—	—	—	—	—	—	—
7	<i>trans</i> -Dehydroxylinalool oxide	991	—	—	0.1	—	0.3	—	—	—	—	—
8	<i>n</i> -Decane	1000	—	0.3	0.4	0.2	1.0	0.5	0.3	0.6	0.3	0.4
9	<i>p</i> -Cymene	1021	—	—	—	—	—	—	—	—	—	—
10	Limonene	1027	3.44	0.5	1.1	0.7	0.8	0.6	0.8	1.7	0.6	1.1
11	Eucalyptol	1029	—	—	0.4	—	—	—	—	—	—	—
12	Isobutyl angelate	1047	—	—	—	—	—	—	—	—	—	—
13	γ -Terpinene	1057	0.3	—	—	—	—	—	—	—	—	—
14	Terpinolene	1088	0.4	—	—	—	—	—	—	—	—	—
15	Undecane	1099	0.2	0.5	0.7	0.8	0.9	0.6	0.8	0.2	0.4	0.5
16	<i>trans</i> -Sabinenehydrate	1100	—	—	0.5	0.4	—	—	—	—	—	—
17	Linalool	1100	0.9	0.4	—	—	0.7	0.6	0.4	0.6	0.5	0.4
18	<i>endo</i> -Fenchol	1113	0.3	0.1	0.1	—	—	—	—	0.2	—	—
19	Isoamyl tiglate	1152	0.4	—	0.2	—	—	—	—	—	—	—
20	Nerol oxide	1153	—	—	—	—	0.3	—	—	—	—	—
21	Borneol	1164	0.6	0.5	0.4	0.3	0.3	—	0.2	0.7	—	0.3
22	Terpinen-4-ol	1176	0.5	0.1	—	—	—	—	—	0.3	—	—
23	3-Decanone	1186	0.8	3.0	2.5	2.5	4.0	1.4	2.4	1.3	1.5	2.5
24	α -Terpineol	1190	1.2	1.2	1.4	0.7	1.4	0.7	1.3	1.7	1.0	1.2
25	<i>n</i> -Decanal	1205	—	—	—	—	—	0.4	—	—	—	—
26	Nerol	1228	0.4	1.2	2.6	1.2	3.2	0.7	2.0	2.9	2.4	3.1
27	(2Z)-2-Hexenyl isovalerate	1231	—	—	0.5	0.4	0.8	—	—	—	—	0.4
28	(2E)-2-hexenyl isovalerate	1232	—	0.5	—	—	—	—	0.4	0.3	—	—
29	6-Undecanol	1287	0.3	0.2	0.2	—	—	—	—	—	—	—
30	2-Methoxy-4-vinylphenol	1308	—	—	0.2	—	0.4	0.4	—	—	—	—
31	Neryl acetate	1365	3.5	3.3	11.0	3.4	5.4	1.7	2.9	7.3	2.2	4.3
32	Decanoic acid	1369	—	—	—	—	—	0.8	—	—	1.5	—
33	α -Ylangenol	1371	0.5	—	—	—	—	—	—	0.3	—	—
34	2- <i>epi</i> - α -Funebrene	1376	—	—	—	—	—	—	—	—	—	0.3
35	α -Copaene	1376	1.0	0.8	0.6	0.8	0.5	1.3	0.6	0.3	0.3	—
36	<i>iso</i> -Italicene	1396	—	—	—	—	—	—	—	—	—	—
37	Italicene	1403	2.1	1.1	1.0	1.2	1.3	0.6	1.1	0.8	0.6	1.2
38	Dodecanal	1408	—	—	0.2	—	—	0.4	—	—	—	—
39	α - <i>cis</i> -Bergamotene	1415	0.5	0.5	—	—	0.3	—	0.5	—	0.7	0.2
40	(<i>E</i>)-Caryophyllene	1420	5.6	1.5	1.0	1.9	1.3	2.2	2.1	2.7	1.7	1.4
41	α - <i>trans</i> -Bergamotene	1436	0.6	0.6	0.2	—	0.5	0.5	0.6	—	1.0	0.3
42	6,9-Guaiadiene	1443	—	—	—	—	—	0.4	—	0.4	—	—
43	(<i>E,Z</i>)-2,6-Dodecadienal-1-al	1443	6.7	2.8	4.6	6.7	1.5	0.7	1.7	2.0	1.3	3.8

(Continues)

TABLE 1 | (Continued)

No.	Compound	LRI	HI1	HI2	HI3	HI4	HI5	HI6	HI7	HI8	HI9	HI10
44	<i>cis</i> -Muurolo-3,5-diene	1449	—	—	—	—	—	—	—	0.2	—	—
45	Aromadendrene	1450	0.4	—	—	—	—	—	—	—	—	—
46	Neryl propanoate	1454	—	1.5	1.4	1.4	3.3	0.9	1.8	1.6	2.7	2.2
47	α -Humulene	1455	1.0	—	—	—	—	—	—	—	—	—
48	α -Acoradiene	1465	—	0.2	—	—	—	—	—	—	—	—
49	β -Acoradiene	1468	—	0.2	—	—	—	—	—	—	—	—
50	4,5-di- <i>epi</i> -Aristolochene	1470	—	0.2	—	—	—	—	—	—	—	—
51	γ -Gurjunene	1477	1.3	2.5	1.4	1.6	1.4	—	3.5	1.5	1.4	0.9
52	γ -Muurolole	1477	—	—	—	—	—	1.2	—	—	—	—
53	γ -Curcumene	1483	13.3	17.2	5.8	6.5	6.5	6.4	11.4	11.4	9.8	5.4
54	<i>ar</i> -Curcumene	1486	7.2	7.7	4.2	4.9	3.8	2.6	4.9	3.2	3.3	3.9
55	Neryl isobutanoate	1488	—	—	—	—	—	—	—	—	—	—
56	β -Selinene	1489	6.0	10.1	7.6	8.9	6.6	2.8	12.8	7.1	5.9	5.2
57	Valencene	1490	4.3	4.8	1.7	2.5	1.0	—	—	1.0	0.6	1.7
58	α -Selinene	1498	3.4	6.8	3.7	4.7	4.2	2.2	9.5	4.5	4.1	3.0
59	α -Muurolole	1501	—	1.0	0.2	0.9	0.4	1.6	0.6	—	0.5	—
60	δ -Amorphene	1508	—	—	—	—	—	0.8	—	1.0	—	—
61	(<i>E,E</i>)- α -Farnesene	1509	—	—	—	—	—	—	—	—	0.8	—
62	β -Himachalene	1509	0.7	—	—	—	—	—	—	—	—	—
63	β -Bisabolene	1510	—	0.5	—	—	—	—	0.5	—	—	—
64	β -Curcumene	1513	1.2	2.2	0.9	—	1.1	—	0.6	0.5	1.1	0.6
65	γ -Cadinene	1516	—	—	—	1.5	0.4	1.4	0.6	0.3	—	—
66	δ -Cadinene	1525	1.1	1.8	0.9	2.6	0.8	4.2	1.1	0.5	1.0	0.3
67	Zonarene	1526	—	—	—	—	0.7	0.4	—	—	—	—
68	<i>trans</i> -Cadina-1,4-diene	1533	—	0.3	—	—	—	0.4	—	—	—	—
69	10- <i>epi</i> -Italicene ether	1535	—	—	0.3	0.5	—	—	—	—	—	—
70	Italicene ether	1535	0.3	0.3	—	—	0.9	—	0.3	0.4	0.4	0.4
71	<i>cis</i> -Nerolidol	1536	—	—	—	—	—	—	—	—	—	—
72	α -Cadinene	1539	—	0.2	—	—	—	0.3	—	—	—	—
73	Selina-3,7(11)-diene	1544	—	0.3	—	—	—	—	0.5	—	—	—
74	α -Calacorene	1544	0.6	—	—	0.7	0.9	1.8	—	1.0	0.8	0.4
75	Longicamphenylone	1552	—	—	—	0.3	—	—	—	—	—	—
76	<i>trans</i> -Nerolidol	1564	0.8	0.8	—	—	—	0.8	0.3	3.0	—	—
77	Dodecanoic acid	1568	0.8	—	1.9	3.8	4.5	—	2.7	—	4.6	2.8
78	Caryophyllenyl alcohol	1570	0.5	0.3	0.5	1.4	0.8	3.3	1.9	0.8	0.7	0.9
79	<i>n</i> -Tridecanol	1573	—	—	—	—	—	—	—	—	—	—
80	Unknown #1 (57,69,41,96)	1576	0.7	1.7	0.8	1.1	1.0	—	—	0.9	0.7	1.4
81	Unknown #2 (57,96,125,41)	1581	1.0	0.4	0.8	3.4	0.7	—	—	—	—	1.0
82	1-Hexadecene	1582	1.3	3.3	1.2	—	—	—	—	—	—	—
83	Caryophyllene oxide	1583	—	—	—	—	—	—	—	0.6	—	—
84	Neryl isovalerate	1584	—	—	—	—	1.5	—	0.5	—	1.3	1.5
85	Gleenol	1585	—	—	—	—	—	0.6	—	—	—	—
86	Globulol	1593	—	—	—	—	—	—	—	—	1.0	—

(Continues)

TABLE 1 | (Continued)

No.	Compound	LRI	HI1	HI2	HI3	HI4	HI5	HI6	HI7	HI8	HI9	HI10
87	Guaiol	1599	—	—	1.6	—	1.3	—	0.5	0.4	2.3	2.1
88	Ledol	1605	0.4	2.4	1.4	4.8	0.4	18.9	1.3	—	0.5	—
89	Rosifoliol	1608	—	—	—	—	6.3	—	—	—	3.5	—
90	5- <i>epi</i> -7- <i>epi</i> - α -Eudesmol	1608	0.3	—	9.2	—	—	—	3.4	0.8	—	9.2
91	<i>epi</i> -Cedrol	1614	0.3	1.0	0.1	0.6	0.8	0.7	0.2	0.7	0.2	0.3
92	Junenol	1614	—	—	0.7	1.3	1.0	—	1.4	0.9	0.9	0.7
93	di- <i>epi</i> -1,10-Cubenol	1616	—	—	—	—	—	0.5	—	—	—	—
94	2-(7Z)-Bisaboladien-4-ol	1618	0.4	0.2	—	—	—	—	—	—	—	—
95	10- <i>epi</i> - γ -Eudesmol	1626	—	—	—	—	—	1.1	—	1.2	—	—
96	(<i>E</i>)-Sesquilandulol	1627	—	0.4	—	—	1.2	—	1.0	—	1.4	0.6
97	1- <i>epi</i> -Cubenol	1629	—	0.2	—	—	—	1.9	—	—	—	—
98	Muurola-4,10(14)-dien-1 β -ol	1629	—	—	0.5	1.4	0.3	—	—	0.4	—	—
99	Eremoligenol	1630	—	—	—	—	—	—	—	—	0.4	—
100	<i>cis</i> -Cadin-4-en-7-ol	1632	0.4	—	—	0.7	1.1	3.8	—	2.9	1.8	1.3
101	γ -Eudesmol	1633	—	—	1.7	—	—	—	0.3	—	2.0	1.6
102	10- <i>epi</i> - α -Muurolool	1641	—	—	—	1.1	—	—	—	—	—	—
103	α -Muurolool	1641	—	0.5	0.6	—	—	1.0	—	—	—	—
104	τ -Cadinol	1642	—	—	—	—	—	3.2	0.5	0.3	—	—
105	β -Eudesmol	1651	—	0.2	—	0.3	0.5	1.1	0.4	0.7	2.5	1.8
106	α -Eudesmol	1651	0.3	—	1.6	—	0.9	—	0.6	—	1.2	1.6
107	α -Cadinol	1653	—	—	—	—	—	1.7	—	1.0	—	—
108	Pogostol	1656	—	3.7	—	—	—	—	—	—	—	—
109	Selin-11-en-4- α -ol	1656	2.0	—	5.6	6.3	6.0	2.1	6.4	3.6	5.5	4.5
110	<i>neo</i> -Intermedeol	1659	—	—	—	—	—	—	—	0.9	—	—
111	Tridecanoic acid	1663	—	—	—	—	—	0.6	—	—	—	—
112	Bulnesol	1669	—	—	0.7	—	0.3	—	0.2	—	0.8	0.8
113	<i>epi</i> - β -Bisabolol	1670	tr	1.3	0.6	1.1	0.6	0.7	0.4	1.1	0.5	0.4
114	β -Bisabolol	1671	—	—	—	—	0.5	0.4	0.3	—	0.8	0.4
115	Cadalene	1676	—	—	—	—	—	0.4	—	—	—	—
116	α -Bisabolol	1684	—	0.4	0.4	0.9	0.6	0.2	0.4	0.8	0.7	0.2
117	Geranyl tiglate	1694	—	0.2	—	—	—	—	—	—	1.4	—
118	(2Z,6Z)-Farnesol	1694	—	—	—	—	—	—	0.3	0.2	—	0.3
119	14-Hydroxy- α -humulene	1712	—	0.5	0.6	—	—	0.3	0.3	0.3	0.4	—
120	Methyl tetradecanoate	1725	—	—	—	—	—	0.34	—	—	—	—
121	(2 <i>E</i> ,6 <i>E</i>)-Farnesal	1730	—	—	—	—	—	—	—	—	—	1.5
122	(<i>Z</i>)-Sesquilandulyl acetate	1730	0.2	—	—	—	—	—	—	—	—	—
123	(2Z,6 <i>E</i>)-Farnesol	1731	—	1.1	2.8	0.2	0.2	—	1.4	1.7	4.1	—
124	(2 <i>E</i> ,6 <i>E</i>)-Farnesol	1738	—	—	—	0.7	—	—	0.3	1.0	0.4	—
125	(<i>E</i>)-Sesquilandulyl acetate	1739	—	0.3	0.5	—	—	—	—	—	—	—
126	Xanthorrhizol	1752	—	—	—	0.4	0.5	0.4	—	—	0.6	0.4
127	Tetradecanoic acid	1757	—	—	—	—	—	—	—	—	—	—
128	Myristic acid	1764	0.9	—	1.3	3.3	2.1	2.9	2.7	1.8	2.7	2.0
129	β -Costol	1769	—	0.6	0.6	1.0	0.2	—	—	0.5	—	0.6

(Continues)

TABLE 1 | (Continued)

No.	Compound	LRI	HI1	HI2	HI3	HI4	HI5	HI6	HI7	HI8	HI9	HI10
130	14-Hydroxy- α -muurolene	1779	—	—	—	—	—	0.4	—	—	—	—
131	8-Cedren-13-ol acetate	1787	—	0.2	—	—	—	—	—	—	—	—
132	(Z)-Nerolidyl isobutyrate	1800	—	—	—	—	0.2	—	—	0.3	0.7	0.3
133	Octadecane	1800	—	0.3	—	—	—	—	—	—	—	—
134	<i>epi</i> - α -Bisabolol acetate	1804	—	—	—	—	—	—	—	—	—	—
135	(E)-Nerolidyl isobutyrate	1827	—	—	—	—	—	—	—	—	0.5	7.7
136	Isoamyl dodecanoate	1844	—	—	0.3	0.4	0.6	0.7	0.2	0.4	0.5	0.5
137	Pentadecanoic acid	1861	—	—	—	—	—	0.4	—	—	—	—
138	(Z,Z)-Farnesyl acetone	1861	—	—	—	—	—	—	—	0.4	—	—
139	(5E,9Z)-Farnesyl acetone	1885	—	—	—	—	—	—	—	—	—	1.7
140	(5E,9E)-Farnesyl acetone	1924	—	0.1	—	—	—	—	0.4	0.3	0.9	0.7
141	Hexadecanoic acid	1964	—	—	—	—	2.6	—	1.0	1.9	—	—
142	(E,E)-Geranyl linalool	2028	—	—	—	—	—	0.7	—	—	—	—
143	Heneicosane (<i>n</i> -C21)	2100	—	—	—	—	—	0.4	—	—	—	—
144	Unknown #3 (93,69,41,80,121)	2124	—	—	—	—	—	—	0.3	—	0.3	0.2
145	Unknown #4 (200,145,160, 185)	2203	—	—	—	—	—	1.0	—	—	—	—
146	Pendacosane (<i>n</i> -C25)	2500	0.2	—	0.3	0.6	—	1.8	0.6	0.4	1.1	0.5
147	Heptacosane (<i>n</i> -C27)	2700	—	—	0.2	0.5	—	0.8	0.3	0.3	0.5	0.2
148	Nonacosane (<i>n</i> -C29)	2900	—	—	0.4	0.7	0.7	1.3	0.3	0.7	0.5	0.3
149	Untriacontane (<i>n</i> -C31)	3100	—	—	tr	—	—	—	—	—	—	—
Total monoterpenes			29.5	9.1	20.2	8.2	13.8	4.8	8.3	23.8	8.4	12.3
<i>Monoterpene hydrocarbons</i>			22.2	2.3	3.6	2.3	2.4	1.2	1.5	10.2	2.4	3.1
<i>Oxygenated monoterpenes</i>			7.3	6.9	16.6	6.0	11.5	3.6	6.8	13.6	6.1	9.1
Total sesquiterpenes			56.5	75.1	59.2	61.4	56.0	74.5	73.2	61.3	68.2	56.8
<i>Sesquiterpene hydrocarbons</i>			49.3	58.2	29.1	38.6	31.0	31.3	50.8	36.6	32.8	24.6
<i>Oxygenated sesquiterpenes</i>			7.2	17.0	30.1	22.9	25.0	43.2	22.4	24.7	35.4	32.2
Diterpenes			0.0	0.0	0.0	0.0	0.0	1.6	0.3	0.0	0.3	0.2
Other			12.3	13.7	18.9	24.4	27.8	17.9	17.3	14.0	21.4	28.0
Unknown			1.7	2.0	1.6	4.5	1.8	0.0	0.0	0.9	0.7	2.3
No. compounds			57	65	67	53	65	66	62	69	67	66
TOTAL			99.9	100.0	99.8	98.6	99.5	98.8	99.2	100.0	99.1	99.6

tr—compounds present in traces (0.05<x<0.1).

type, which is also found in a sample from Montenegro [33]. However, some samples did not fit into any of the previously described chemotypes. For example, HI3 and HI10 had dominant 5-*epi*-7-*epi*- α -eudesmol; HI4 and HI5 β -selinene (but lower than 11.6%); HI6 had ledol as a dominant compound, while HI8 and HI9 had γ -curcumene as a dominant compound (but lower than 13.6%). 5-*epi*-7-*epi*- α -Eudesmol and ledol, while previously detected, were never dominant compounds in *H. italicum* EO [30, 32].

2.2 | Phenolic Composition and Variability

LC-MS analyses identified 73 phenolic compounds belonging to four classes: phenolic acids, flavonoid glycosides, flavonoid

aglycones, and pyranones and related compounds. Since there are no available standards for most compounds, only the total areas under the curves were used in the analysis, which do not correspond to quantities. However, within the same chemical class, the relative ratios of compounds could approximate the relative abundances of their components. Arzanol (**56**) was the most dominant peak in all samples. Of the eight identified phenolic acids, dicaffeoylquinic acid (**7**) was the highest peak in all samples. Nine flavonoid glycosides were identified, and the most dominant were based on quercetin and kaempferol. Of the 15 flavonoid aglycones found, three were the most prominent in all samples: isorhamnetin (**23**), hispidulin (**28**), and acacetin (**32**). The most numerous were pyranones and related compounds (cf. Table S1).

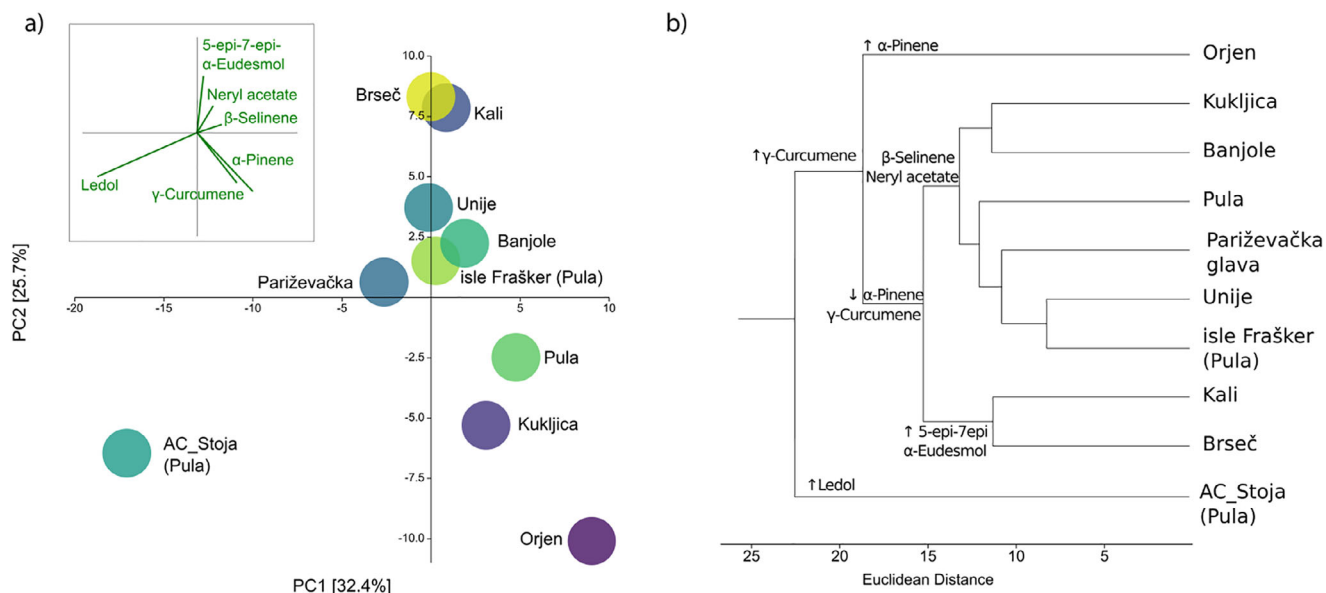


FIGURE 1 | (a) Principal component analysis scatter plot based on the 29 essential oil (EO) components of ten *Helichrysum italicum* populations from the east Adriatic coast; the most.

TABLE 2 | Main compounds in essential oils of *Helichrysum italicum* L. obtained from natural populations or commercial essential oils.

Origin	Three main compounds (%)	Ref.
Algeria	Limonene (6.1%), nerol (5%), α-curcumene (11.4%)	[24]
Bosnia and Herzegovina ^a	α-Pinene (16%–26.1%), neryl acetate (6%–10.7%), γ-curcumene (7.2%–20.6%)	[12, 25, 26]
Bulgaria ^a	Neryl acetate (9.3%–18%), α-copaene (2.1%–12.8%), γ-curcumene (1.6%–13%)	[14, 27]
Croatia	Neryl acetate (4.5%–21.4%), α-curcumene (0%–10.7%), γ-curcumene (0%–21.6%)	[5, 20, 28, 29]
France ^{a,b}	Neryl acetate (30.4%–46.2%), γ-curcumene (10.1%–11.7%), α-pinene (1.8%–12.5%)	[26, 30, 31]
Italy ^{a, b}	Neryl acetate (8%–44.5%), neryl propionate (1.7%–16.4%), γ-Curcumene (2.5%–13.7%)	[13, 32]
Montenegro ^b	Neryl acetate (10.7%–29.2%), β-selinene (1.6%–21.7%), γ-curcumene (0%–18.8%)	[22, 33]
Portugal	γ-Curcumene (16%), italicene (12.5%), neryl acetate (11.5%)	[34]
Serbia ^a	γ-Curcumene (11.3%–20%), neryl acetate (0.2%–15.6%), α-curcumene (1.1%–11.7%)	[15, 35]
Slovenia ^a	Rosifoliol (15.3%), γ-eudesmol (10.8%), guaial (9.4%)	[19]

^aAll or some samples grown *ex situ*.

^bSome of the EOs are obtained commercially.

For multivariate analysis of phenolics from ethyl acetate extracts, data were log-transformed ($\log(x+1)$). PCA scatter showed separation of samples into several groups. The first two eigenvectors account for 40.7% of the total variability, with eight compounds contributing the most. Interestingly, four of these compounds were flavonoids (**11**, **14**, **15**, and **20**), while the remaining were pyranones and related compounds (Figure 2a). What is more interesting, the class of compounds with the lowest areas in the sample (**11**, **14**, and **15**) contributed the most to the separation of the samples. Two flavonoids (compounds **14** and **20**) separate populations into three groups. Samples Pariževačka and Banjole had the highest abundance of **14**, and populations Kali, Brseč, and Pula had the highest amounts of compound **20**. The rest fell somewhere between these two groups.

On the other hand, Unije separated from the rest based on the highest amounts of **15**, while AC Stoja separated on the highest abundances of **11** and several pyrananes (**52**, **59**, **63**, and **65**). Since the flavonoid glycosides contributed most to sample variability, PCA using only the flavonoid glycosides was also performed (Figure 2b). The first two eigenvectors explain 71.6% of the total variability. Three groups could be distinguished: i) Orjen and Kali; ii) Pula and Brseč, which had the lowest values for flavonoid glycosides, and iii) the rest, which had the highest values. Unije is separated from the third cluster by the highest value of **14**.

Among the eight identified phenolic acids, six (**3**–**8**) have been previously reported for *H. italicum* [36–38]. Compounds **1** and **2** were reported so far only for *Helichrysum arenarium* (L.) Moench [39].

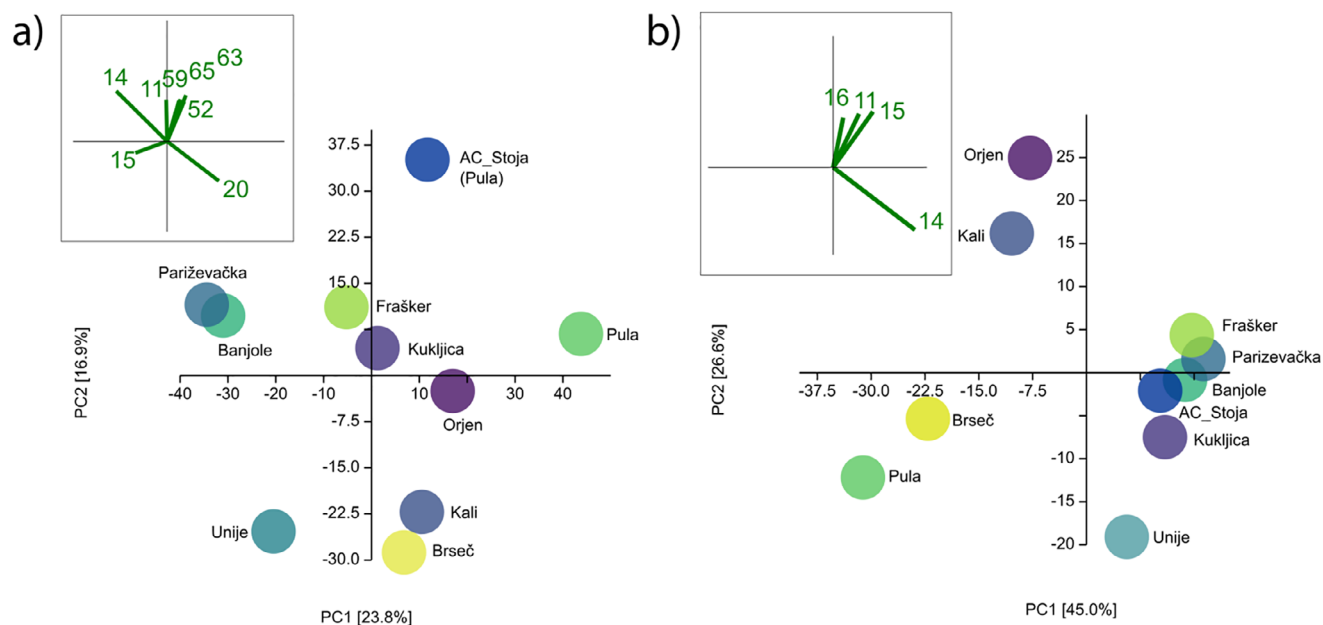


FIGURE 2 | (a) PCA scatter plot of phenolics from immortelle; (b) PCA scatter plot of flavonoid glycosides of immortelle. 11 – Quercetin 3-O-(6"-acetyl)-hexoside; 14 – Dihydrokaempferol 5-O-hexoside (Heliciodide A); 15 – Kaempferol 3-O-(6"-acetyl)-hexoside; 16 – Quercetin 3-O-(6"-p-coumaroyl)-hexoside (Helichryside); 20 – Luteolin; 59 – Italipyrone; for 52, 62, 63 cf. Table 2.

Nine flavonoid glycosides have been identified in our study. Six compounds were reported in *H. italicum* [37, 40, 41], though in these reports, they were specified as glucosides, while we were only able to identify them as hexoses. The remaining compounds are reported in other members of *Helichrysum* [42]. Among fifteen flavonoid aglycones detected, eleven were previously found in *H. italicum* [21, 36]. Other compounds were found in different members of the *Helichrysum* genus [43–46].

In this study, we identified forty-one pyranones and related compounds. Among these, twelve compounds were previously found in *H. italicum* [21, 36, 37]. Compounds 34–41, 43–45, 47–49, 51–54, 60–63, 65–70, and 72 were reported in other *Helichrysum* species [43, 47–52].

2.3 | Correlation Between Environmental Parameters and Phytochemistry of Immortelle

PCA analysis of 19 bioclimatic parameters (Figure S1) shows a clear separation of localities: Orjen and Brseč on one side and Pariževačka glavica on the other, from the rest. On the first axis, separation was primarily driven by the mean temperature of the coldest month (BIO6). The highest winter temperatures were in Unije, while the lowest were in Orjen and Pariževačka glavica. On the second axis, populations Unije, Brseč, and Orjen were separated from all others due to higher values of bioclimatic parameters related to precipitation (BIO12–BIO19). Köppen-Geiger climate types show three distinct climates, roughly corresponding to the bioclimatic parameter-based separation. Populations HI2–HI5 have a hot-summer Mediterranean climate type (Csa), while population Orjen (HI1) has a warm-summer Mediterranean climate type (Csb). The remaining populations have a humid subtropical climate (Cfa).

Multivariate analysis of bioclimatic parameters revealed groupings of localities that differed from the EO composition, thereby confirming the greater influence of genetics in determining the EO profile. To analyze the correlation of the EO profile with bioclimatic and geographic proximity, Mantel and partial Mantel tests were performed. Tests did not reveal any statistically significant correlation between the EO profile and bioclimatic parameters, even after accounting for geographical proximity. However, a moderate but unsupported correlation ($R = 0.3$, $p = 0.17$) was found between the EO profile and geographic proximity. This suggests that geographically closer populations tend to have more similar EO profiles. Given the pollination system of this taxon, the EO profile may reflect the genetic structure of the studied populations. On the other hand, the sampling strategy and the EO analysis limit the ability to verify such a claim. Additionally, since EOs primarily mediate short-term ecological interactions (e.g., pollinator attraction), and since these interactions can vary sharply across microclimates and local habitats, the volatile profile is oftentimes highly sensitive to local ecological factors and its dynamics. This makes EOs excellent markers for understanding localized intraspecific variability, and signals the chemophenetic value of EOs. Sharp differences in the EO profiles, as indicated by dominant compounds, suggest that the genetic makeup of the individuals is more responsible for the EO profile differences, than just short-term interaction with the environment. Considering this plant is perennial, a multi-year study of the same populations/individuals would give a better understanding of the EO variability, and how much of it is attributable to the environment, and how much to the genotype of the individuals.

Although several individual phenolics showed correlations with bioclimatic parameters, the Mantel and partial Mantel tests did not detect significant associations between the phenolic profile and bioclimatic parameters or population geographic

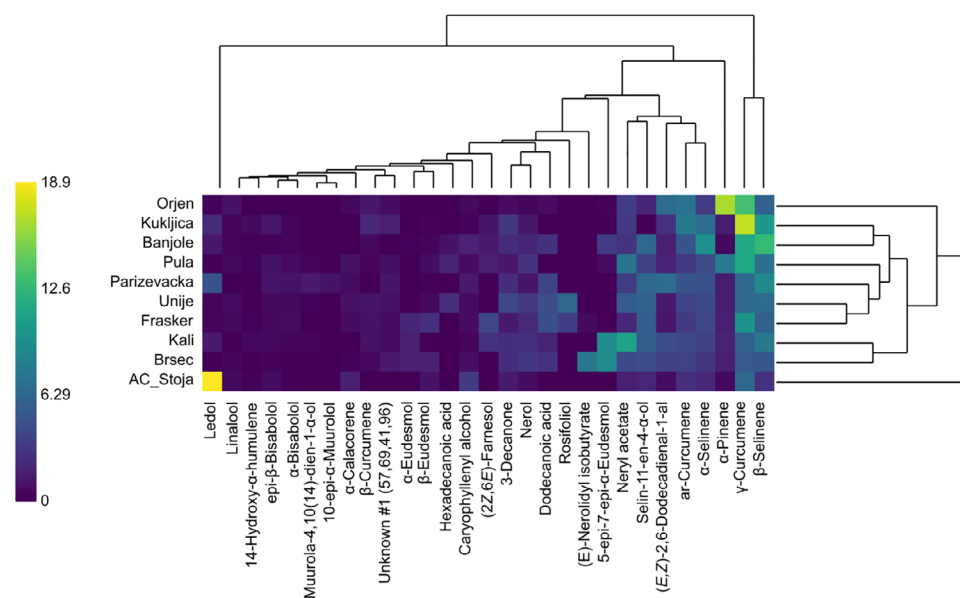


FIGURE 3 | Two-way hierarchical clustering (UPGMA, Euclidean distances) of ten analyzed EOs of immortelle from the eastern Adriatic.

location. On the other hand, the linear correlation test revealed associations between total phenolic acid and flavonoid glycoside contents and several environmental factors. For example, latitude showed a strong negative correlation with the content of these two groups ($R = -0.8$ and -0.6 , respectively, $p < 0.01$), whereas longitude showed a positive correlation ($R = 0.8$ and 0.7 , $p < 0.01$). This means that the further south and the further east the population lay, the more phenolic acids and flavonoid glycosides it contained. This was also true for the altitude ($R = 0.6$ and 0.7 , $p < 0.01$). Several bioclimatic parameters were also correlated with total phenolic acid and glycoside content. Phenolic acids were positively correlated with BIO3 (isothermality) and BIO15 (precipitation seasonality), while glycosides negatively correlated with BIO1 (annual mean temperature), BIO6 (min. temperature of coldest month), BIO10 (mean temperature of warmest quarter), and BIO11 (mean temperature of coldest quarter). Total aglycone and total pyranone contents did not correlate with any environmental parameters. This suggests that the flavonoid aglycones and possible pyranones may have taxonomic value in the study of immortelle. Moreover, pyranones are very important as attracting dispersers, defensive agents, and signaling molecules for growth, etc. their synthesis and presence are predominantly genetically determined, which highlights their chemophenetic value. At the same time, phenolic acids and flavonoid glycosides may be modified by environmental parameters, making them suitable for optimizing growing conditions to achieve higher yields of these bioactive compounds. Phenolic acids and glycosides, similar to EOs, have a very strong ecological function since they function primarily in defense against systemic abiotic stressors, such as UV radiation, drought, and soil composition. These environmental pressures tend to be stable over longer periods, so correlation with the studied environmental parameters is probably not an artifact.

Regarding soil type, most of the populations grow on either redzina or cambisols. Eutric cambisols were the most abundant soil type, and all of the putative EO chemotypes could be found growing on them. This suggests that soil type does not significantly influence EO or phenolics profile. Two-way hierar-

chical cluster analysis shows that remote populations can share similar phenolic/EO profile, while close populations can have significantly different profiles, attesting to a limited influence of the environmental factors on general composition, though a more complex relationship cannot be excluded (Figures 3 and 4).

3 | Conclusions

This study provided an integrated analysis of the EO and phenolic composition of *H. italicum* from natural populations in Croatia and Montenegro, contributing to a clearer understanding of phytochemical variability within this species. The results confirmed known chemical diversity, both in volatiles and phenolics, indicating that *H. italicum* represents a metabolically highly variable taxon, while also adding more information (i.e., additional compounds). Essential oil profiles revealed clear chemotypic differentiation among populations, whereas several samples exhibited atypical compositions, suggesting that existing chemotype classifications may not fully reflect the species' chemical complexity. Phenolic compounds also showed considerable variability; however, their distribution patterns were not always consistent with EO chemotypes. Statistical analyses showed no correlation between EO and flavonoid aglycone composition and the studied environmental parameters, although other phenolics showed correlations with temperature and precipitation. This indicates that volatile and nonvolatile metabolites of immortelle are influenced by different ecological factors. These findings are important for taxonomy and understanding intraspecific variability.

4 | Experimental Section

4.1 | Plant Material

Whole plants were collected in Croatia (nine populations) and Montenegro (one population). Plant identification was provided

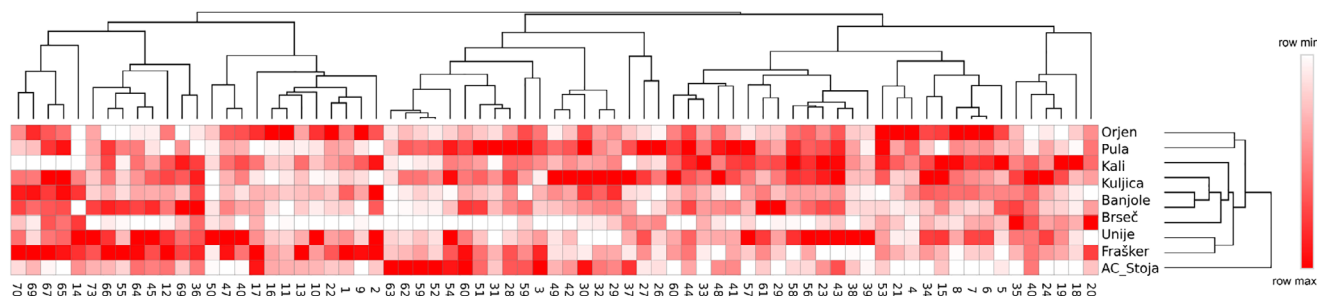


FIGURE 4 | Heatmap of the scaled data of untargeted metabolite analysis, with the samples (both columns and rows) arranged according to the HCA (Spearman method of cluster agglomeration). The intensity of the red color indicates the abundance of compounds in the samples, as shown on the color scale. Hierarchical cluster analysis (HCA) plots were constructed in Morpheus software (Broad Institute, 2025) using the Spearman method of cluster agglomeration with average linkage.

TABLE 3 | Geographic characteristics of the analyzed populations of *Helichrysum italicum* L.

Code	Country	Location	Latitude [°N]	Longitude [°E]	K-G ^[a]	Soil type	Altitude [m]
HI1	MNG	Orjen	42.5276	18.4677	Csb	Rendzina	739
HI2	CRO	Ugljan, Kuljica	44.0412	15.2466	Csa	Rendzina	4
HI3		Ugljan, Kali	44.0392	15.2144	Csa	Limestone combined with rendzina	1
HI4		Pariževačka glavica	44.2066	15.6131	Csa	Brown on limestone	125
HI5		Unije	44.6398	14.2548	Csa	Eutric cambisol	24
HI6		Pula, AC Stoja	44.8546	13.8152	Cfa	Eutric cambisol	0
HI7		Banjole	45.1794	14.2348	Cfa	Eutric cambisol	144
HI8		Pula, Stoja	44.8602	13.8066	Cfa	Eutric cambisol	5
HI9		Pula, Frašker island	44.8221	13.8434	Cfa	Eutric cambisol	3
HI10		Brseč	44.8171	13.8954	Cfa	Eutric cambisol	1

^aKoppen-Geiger climate type: Csb- warm-summer Mediterranean climate, Csa- Hot-summer Mediterranean climate, Cfa- Humid subtropical climate.

by the authors PJ and IVK using standard identification keys [44]. Voucher specimens were deposited at the Herbarium of the University of Belgrade, Faculty of Biology and Botanical Garden “Jevremovac” (BEOU). Population details are given in Table 3.

4.2 | Isolation of Essential Oils

The EOs were obtained from dry aerial parts (5 g). The plant material was chopped and subjected to simultaneous hydrodistillation-extraction using a Lickens Nickerson-type apparatus for 2 h. The volatiles were collected in dichloromethane (VWR, Germany) and stored in amber glass vials at 4°C until GC/MS analysis.

4.3 | GC-FID and GC/MS Analyses

The GC-FID and GC/MS analyses were carried out with an Agilent 7890A apparatus equipped with a 5975C mass-selective detector (MSD), a flame ionization detector (FID), and a DB-5 MS fused-silica cap. column (30 m 0.25 mm i.d., film thickness 0.25 mm). The oven temp. was programmed linearly rising from 60° to 300° at 3°/min; injector temp., 220°; detector temp., 300°; transfer-line temp., 240°; carrier gas, He (1.0 mL/min at 210°, constant pressure mode); injection vol., 1 mL; split ratio, 10 1. Electron impact mass spectra (EI-MS; 70 eV) were acquired over the *m/z* range 30–550. Library search, mass spectral deconvolution, and extraction were performed using the software

NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) version 2.64.113.71, with the retention index (RI) calibration data analysis parameters set to the strong level and a 10% penalty for compounds without an RI. The RIs were experimentally determined using the standard method, which involved the retention times (t_R) of *n*-alkanes injected after the EO under the same chromatographic conditions. The linear RI was calculated for all compounds using the following formula: $LRI = 100 \times (t_{rs} - t_{rn}) / (t_m + 1 - t_{rn}) + 100 \times n$. The search was performed against our in-house library, which contains 4972 spectra. The relative amounts of the identified compounds were calculated from their GC peak areas.

4.4 | Extracts

Air-dried, ground aerial parts (5 g per population) were extracted with 200 mL of ethyl acetate (VWR, Germany). All the samples were ultrasonicated for 30 min in an ultrasonic bath at 25°C. Each extract was filtered through a Whatman No. 1 filter paper after 24 h. The filtrates were evaporated to dryness with a rotary vacuum evaporator and stored at 4°C until use. For metabolite profiling, all prepared extracts were dissolved in MeOH at 3 mg/mL. Extract yields are presented in Table S2.

4.5 | The LC/MS Metabolic Profile

The metabolite profile of the extracts was analyzed using LC/MS (Thermo Scientific Vanquish Core HPLC system) coupled with the Orbitrap Exploris 120 mass spectrometer (San Jose, CA, USA). The Orbitrap Exploris 120 mass spectrometer was equipped with an ESI source operating in negative ionization mode. Liquid chromatography system was equipped with a Hypersil GOLD C18 analytical column (50 × 2.1 mm, 1.9 µm particle size). The compounds of interest were eluted with ultrapure water +0.1% formic acid (A) and acetonitrile (MS grade) + 0.1% formic acid (B): 5% B in the first min; 5%–95% B from 1 to 10 min; 95% B from 10 to 12 min; 5% B until 15 min. Full scan MS were monitored from 100 to 1500 m/z with Orbitrap resolution set to 60 000 FWHM, while data-dependent MS2 experiments were conducted at an Orbitrap resolution of 15 000 FWHM with normalized collision energy of CID set to 35%. All the other chromatographic and mass spectrometric parameters were applied as previously described in the literature [45]. LC/MS data were evaluated using R Studio (version 2023.09.1, build 494) software. Peak picking was performed using *enviPick* R package and peak correspondence across samples was performed using the density method available in *xcms* R package [46]. The compounds were identified based on their chromatographic behavior and mass spectra, with those of standard compounds, and also by comparison with literature data on previously identified compounds in various *Helichrysum* species. Data acquisition was carried out using an Xcalibur data system (Thermo Fisher Scientific, San Jose, CA, USA).

4.6 | Environmental Data and Statistical Analyses

Univariate (Pearson's correlation test) and multivariate statistical analyses (Principal Components Analysis, Hierarchical Cluster Analysis, Mantel test, and partial Mantel test) were performed

using Past 5.3 [53] was used for all statistical analyses. For EO data, only components present at >0.5% were included; those that were not normally distributed or correlated with one another were excluded. For phenolics, a log transformation ($\log(x+1)$) was applied for multivariate analyses. The bioclimatic data (temperature, precipitation) for all ten studied localities were obtained from the WorldClim 2.0 set of global climate layers, with an accuracy of approximately 1 sq km [54] using the QGIS software (QGIS Development Team, 2015). Climate types from Present and future Köppen-Geiger climate classification maps with a 1-km resolution were also used [55].

Author Contributions

Milica Trajković: investigation, writing – original draft, methodology. **Ivana Vitasović Kosić:** investigation, writing – review and editing, data curation. **Milan Gavrilović:** conceptualization, supervision, writing – review and editing. **Pedja Janačković:** conceptualization, supervision, writing – review and editing, resources. **Nemanja Rajčević:** supervision, formal analysis, writing – original draft, visualization, methodology, investigation. **Uroš Gašić:** writing – review and editing, investigation.

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Consent

All authors agreed with the final version of this manuscript and with the current submission.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available from the authors upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplementary data: Table S1. LC-MS data on metabolites identified in *Helichrysum italicum*; Figure S1. Base peak chromatograms of 10 different *Helichrysum italicum* ethyl acetate extracts; Figure S2. PCA Scatter plot of 19 bioclimatic data for ten studied localities in the eastern Adriatic region; Figure S3. FID chromatograms of six chemotypes found in *Helichrysum italicum* essential oils studied in the east Adriatic coast.

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cbdv.71672>.

Supporting File: cbdv71672-sup-0001-SuppMat.pdf.