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Article

Evaluation of the Essential Oil Composition of Five *Thymus* Species Native to Greece

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Abstract: The genus *Thymus* encompasses a wide array of taxa, many of which remain underexplored in terms of their phytochemical profile. In this study, we investigated the phytochemical composition of volatile compounds of five *Thymus* species native to Greece using gas chromatography combined with mass spectrometry. Two samples of *T. parnassicus* collected from Mts Parnitha and Parnassos were studied. The predominant compounds in the Parnitha sample were α-cadinol (13.53%), *E*-caryophyllene (11.83%) and selin-11-en-4α-ol (7.29%). The sample from Mt. Parnassos exhibited a high concentration of *E*-caryophyllene (35.20%) followed by β-bisabolene (10.41%). Additionally, two species, namely *T. leucotrichus* subsp. *leucotrichus* and *T. atticus*, were collected on Mt. Chelmos (Peloponnese). The essential oil of *T. leucotrichus* was rich in elemol (35.56%), α-eudesmol (11.15%) and β-eudesmol (6.11%). *Thymus atticus* exhibited a high concentration in linalool (63.04%) and *p*-cymene (25.63%). In addition, two samples of *T. holosericeus* collected from Kefalonia Island were both rich in geraniol (89.9% and 87.7%, respectively). We also examined the volatile profile of *T. laconicus*, a local endemic species of SE Peloponnese (Lakonia area), which remains unexplored. Carvacrol (32.7%) and *p*-cymene (29.7%) were identified as the dominant compounds. Our study contributes valuable insights into the chemical profile of *Thymus* spp. and sheds further light on the well-known chemical polymorphism within this genus.

Keywords: chemotype; GC-MS; *Thymbropsis*; *Subbracteati*; volatile profile

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1. Introduction

Thymus L. (Lamiaceae) consists of 250 taxa (214 species and 36 sub-species divided into eight sections primarily distributed across the Mediterranean Basin but also in Asia and Africa [1]. Greece hosts 31 taxa (24 species and 7 subspecies), 10 of them endemic to the country [2].

Thymus spp. are valued for their medicinal and aromatic properties. They are recognized for their antimicrobial and antioxidant properties which make them beneficial in addressing respiratory, gastrointestinal disorders and various other conditions [3–5]. The essential oils extracted from *Thymus* spp. are used in the cosmetics and food industry for their aroma or for their antioxidant and/or antimicrobial properties [6–8].

Thymus vulgaris is the most studied species of the genus. Its phytochemistry (volatile and non-volatile metabolites) has been studied by numerous researchers [9–14], and its biological activity, attributed to its rich chemical profile, has been documented [4,5,15,16]. The chemical analysis of its volatile profile has revealed a chemotypic diversity. In France alone, six chemotypes have been reported, prominently featuring monoterpenes such as geraniol, linalool, α-terpineol, 4-thujanol, thymol and carvacrol [9]. Other researchers identified a total of 20 distinct chemotypes among 85 *T. vulgaris* samples, with a majority aligning with the thymol chemotype [11]. Besides *T. vulgaris*, other *Thymus* species have

also been examined for their essential oil composition [17–20]. Monoterpenes, such as thymol, *p*-cymene, carvacrol and γ-terpinene, commonly emerge as major constituents of *Thymus* spp. essential oil [21] and pure or mixed chemotypes at different or even within the same species, have been identified. However, presently, only the thymol chemotype holds official recognition within the European Pharmacopoeia [22].

Essential oils are typically extracted from aerial plant parts, particularly from the inflorescences and leaves. Their complex chemical composition, mainly comprising terpenoids and phenylpropanoids, along with substantial interspecific variability in both quality and quantity of phytochemicals, renders essential oil blends intriguing for diverse industrial applications. The chemical variability of the essential oils is not solely dependent on the species but is significantly linked to environmental, genetic and developmental factors as well as soil conditions [23,24]. *Thymus* spp. are often covered by dense indumentum, especially on their leaves, providing resistance to cold, hot or dry conditions. They have been adapted to a wide array of environmental conditions, and as a result, different metabolites are produced affecting their chemotypes. Thymol and carvacrol commonly emerge as the dominant metabolites of *Thymus* spp, lending them their characteristic aroma [25–27].

This study investigated the essential oil composition of five *Thymus* species native to Greece: *T. parnassicum* from two distinct geographical areas (Mts. Parnitha and Parnassos), *T. atticus*, *T. leucotrichus* subsp. *leucotrichus*, *T. laconicus* and *T. holosericeus* from different regions in Kefalonia Island (Mts. Roudi and Enos). All species belong to *Thymus* sect. *Hyphodromi* with *T. holosericeus* and *T. laconicus* classified under subsect. *Thymbropsis* and *T. atticus*, *T. parnassicus*, *T. leucotrichus* subsp. *leucotrichus* under subsect. *Subbracteati*. *Thymus holosericeus* and *T. laconicus* are Greek endemics restricted to the Ionian Islands and the Peloponnese, respectively. *Thymus atticus*, *T. parnassicus* and *T. leucotrichus* are distributed in the Balkans (including Greece) and Anatolia [28]. Notably essential oil documentation for *T. laconicus* is absent, while only a single study references the volatile constituents of *T. holosericeus* [29]. Similarly, research on the essential oil composition of *T. atticus* and *T. parnassicus* is especially scarce [30]. Our results contribute to the study of the genus *Thymus* by presenting new data that elucidate its chemical polymorphism.

2. Materials and Methods

2.1. Chemicals

Standard compounds, namely *p*-cymene, 3-octanol, aromadendrene, caryophyllene oxide, linalool, α-pinene, terpinene-4-ol, γ-terpinene, α-terpineol, neryl acetate and α-terpinene were purchased from Sigma Aldrich (Darmstadt, Germany), decanal was purchased from ThermoFisher Scientific (Loughborough, Leicestershire), and β-eudesmol, α-copaene, α-cubebene, and terpinolene were purchased from Fluka (Buchs, Switzerland). Anhydrous sodium sulfate was purchased from Acros organics (Morris Plains, NJ, USA) and diethyl ether was purchased from Chem-Lab (Zedelgem, Belgium).

2.2. Plant Material

Plant material was collected during the flowering period from mature individuals of wild populations from different localities in Greece. The specimens were deposited at the herbarium of Agricultural University of Athens (ACA). Information regarding the geographic locations of the collected plant material is given in Table 1.

Table 1. Collection data of the studied *Thymus* species.

Taxon	Collection Site	Latitude	Longitude	Elevation (m)
T.parnassicus	Mt. Parnitha	38°10′23″	23°43′41″ E	1300
T.parnassicus	Mt. Parnassos	38°33′39″	22°34′26″ E	1700
T. atticus	Mt. Chelmos	38°05′14″	22°10′30″	950
T. leucotrichus subsp. *leucotrichus*	Mt. Chelmos	37°59′20″	22°11′25″	2150

Table 1. *Cont.*

Taxon	Collection Site	Latitude	Longitude	Elevation (m)
T. holosericeus	Kephalonia (Mt. Roudi)	38°10′31″	20°36′41″	870
T. holosericeus	Kephalonia (Mt. Enos)	38°07′35″	20°42′04″	1110
T. laconicus	Peloponnese (Geraki Lakonias)	36°58′53″	22°44′26″	340

2.3. Isolation of the Essential Oil

Air-dried parts (flowers and leaves) of the collected plant material underwent hydrodistillation using a Clevenger type apparatus for 3 h. *Tymus laconicus*, essential oil, was directly collected from the Clevenger apparatus, while for the other samples, liquid–liquid extraction followed using diethyl ether, for collecting the volatile compounds. The organic phase was then dried using anhydrous sodium sulfate, filtered and stored at −20 °C at a fixed volume, until further analysis.

2.4. GC-MS Analysis Conditions

Chromatographic analysis was performed using a Bruker chromatograph 436-GC coupled to a mass spectrometer. A capillary column Rxi-5Sil MS (30 m, 0.25 mm ID, 0.25 μm) was used for the separation of compounds. The injector and detector temperature was set to 220 °C and 230 °C, respectively. Helium was the carrier gas at a flow rate of 1.0 mL/min. The oven temperature was held at 60 °C for 3 min and then increased to 250 °C with a rate of 3 °C/min. Injection volume was 1 μL at the splitless mode. MS detector was operated in EI mode at 70 eV. Mass range was set to 45–400 m/z.

Identification was based on authentic standard solutions and/or on comparison of compounds' mass spectrum and Relative Retention Index (Arithmetic index, A.I) with those from NIST and ADAMS libraries. The standard compounds, namely *p*-cymene, 3-octanol, decanal, neryl acetate, *β*-eudesmol, *α*-copaene, *α*-cubebene, aromadendrene, caryophyllene oxide, terpinolene, linalool, *α*-pinene, terpinene-4-ol, *γ*-terpinene, *α*-terpineol and *α*-terpinene, were analyzed under the same experimental conditions. A mixture of n-alkanes solution (C8–C24, Sigma Aldrich) was also analyzed under the same experimental conditions for the calculation of the Relative Retention Index, according to Equation (1). Compound concentration was calculated as % content (Tables 2 and 3). Principal Component Analysis (PCA) of the data was implemented using GraphPad prism ver. 9.5.1 and cluster analysis (CA) using IBM SPSS statistics ver. 23. The variables used to perform the analysis are highlighted in bold (Tables 2 and 3) [31].

$$\text{A.Ix} = 100\text{n} + 100(\text{tx} - \text{tn})/(\text{tn} + 1 - \text{tn}) \quad (1)$$

where tn and tn + 1 are retention time of n-alkanes that elute before and after the unknown compound (x) and tx is the retention time of the unknown compound.

Table 2. Chemical composition of *Thymus* species (subsect. *Subbracteati*).

Subsect. *Subbracteati*								
					% Composition			
No	**Classification [a]**	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. parnassicus* (Mt. Parnitha)**	***T. parnassicus* (Mt. Parnassos)**	***T. atticus* (Mt. Chelmos)**	***T. leucotrichus* subsp. *leucotrichus* (Mt. Chelmos)**
1	MH	α-pinene [b]	931	932	0.63	1.89	- [c]	-
2	MH	camphene	947	946	-	0.08	-	-
3	others	benzaldehyde	955	952	-	-	-	0.22
4	AL	1-octen-3-ol	974	974	0.13	0.42	0.68	0.72
5	AL	6-methyl-5-hepten-2-one	979	981	tr. *	-	-	-
6	MH	myrcene	986	988	0.11	tr.	-	-
7	AL	3-octanol [b]	991	988	tr.	0.26	tr.	-
8	AL	(2E, 4E)-Heptadienal	1006	1005	-	-	-	0.11
9	MH	α-terpinene [b]	1015	1014	-	tr.	0.25	-
10	MH	***p*-cymene [b] [d]**	1022	1020	0.15	tr.	25.63	-
11	MH	limonene	1027	1024	0.22	1.10	0.08	-
12	OM	eucalyptol	1029	1026	0.84	3.91	-	-
13	others	benzylalcohol	1034	1026	-	-	-	0.44
14	others	benzene acetaldehyde	1038	1036	tr.	0.08	-	2.22
15	MH	(E)-β-ocimene	1042	1044	tr.	--	-	-
16	MH	**γ-terpinene [b]**	1055	1054	0.21	0.21	1.36	-
17	OM	*cis*-sabinene hydrate	1065	1064	-	-	0.30	-
18	OM	*cis*-linalool oxide	1067	1067	tr.	tr.	0.39	-
19	OM	*trans*-linalool oxide	1083	1084	tr.	-	0.37	-
20	OM	**linalool [b]**	1097	1095	3.11	5.30	63.04	4.04
21	AL	1-octen-1-ol, acetate	1105	1112	0.10	tr.	-	-
22	others	2,6-dimethyl-cyclohexanol	1108	-	-	-	-	0.15
23	PHP	phenyl ethyl alcohol	1114	1106	-	-	-	0.15
24	OM	camphor	1143	1141	2.02	3.53	-	0.08
25	OM	lavandulol	1163	1165	-	-	0.13	-
26	OM	**borneol**	1168	1165	2.73	2.80	-	-
27	OM	terpinen-4-ol [b]	1177	1174	0.69	2.52	0.30	0.28
28	OM	α-terpineol [b]	1191	1186	2.06	1.74	0.19	0.22

Table 2. *Cont.*

Subsect. *Subbracteati*								
					% Composition			
No	**Classification** [a]	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. parnassicus* (Mt. Parnitha)**	***T. parnassicus* (Mt. Parnassos)**	***T. atticus* (Mt. Chelmos)**	***T. leucotrichus* subsp. *leucotrichus* (Mt. Chelmos)**
29	OM	*cis*-dihydrocarvone	1194	1191	-	0.20	-	-
30	OM	*trans*-dihydrocarvone	1200	1200	-	0.31	-	-
31	AL	decanal	1203	1201	tr.	tr.	-	-
32	OM	*trans*-carveol	1215	1215	-	tr.	-	-
33	others	2,3-dihydro-benzofuran	1218	-	-	-	-	0.60
34	OM	citronellol	1222	1223	tr.	-	-	-
35	OM	nerol	1225	1227	tr.	-	-	tr.
36	OM	thymol methyl ether	1226	1232	tr.	tr.	-	-
37	OM	pulegone	1234	1233	-	tr.	-	-
38	OM	carvone	1239	1239	tr.	1.09	-	-
39	OM	thymoquinone	1242	1248	-	-	0.26	-
40	OM	**geraniol**	1247	1249	0.38	-	-	2.45
41	OM	geranial	1264	1264	tr.	-	-	0.09
42	OM	bornyl acetate	1280	1284	0.64	1.99	-	-
43	OM	**thymol**	1287	1289	0.42	-	0.11	0.82
44	OM	**carvacrol**	1295	1298	0.21	0.11	5.82	3.27
45	OM	2-methoxy-4-vinylphenol	1305	1309	tr.	-	-	0.77
46	SEH	*δ*-elemene	1329	1335	tr.	-	-	-
47	SEH	*α*-cubebene	1344	1348	0.12	tr.	-	-
48	OM	eugenol	1356	1356	-	-	-	0.05
49	OM	neryl acetate	1355	1359	0.32	tr.	-	-
50	OM	carvacrol acetate	1366	1370	-	-	0.62	-
51	SEH	*α*-copaene	1372	1374	0.98	0.10	-	-
52	OM	geranyl acetate	1374	1379	0.92	0.17	-	-
53	SEH	*β*-bourbonene	1379	1387	0.22	0.16	-	-
54	SEH	*β*-elemene	1385	1389	0.86	0.17	-	-
55	SEH	(Z)-caryophyllene	1400	1408	tr.	0.10	-	-
56	SEH	*α*-gurjunene	1403	1409	tr.	-	-	-

Table 2. *Cont.*

Subsect. *Subbracteati*								
					% Composition			
No	**Classification [a]**	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. parnassicus* (Mt. Parnitha)**	***T. parnassicus* (Mt. Parnassos)**	***T. atticus* (Mt. Chelmos)**	***T. leucotrichus* subsp. *leucotrichus* (Mt. Chelmos)**
57	SEH	***E*-caryophyllene [b]**	1415	1417	11.83	35.20	0.16	-
58	SEH	*β*-copaene	1425	1430	0.07	tr.	-	-
59	SEH	*trans*-*α*-bergamotene	1429	1432	tr.	tr.	-	-
60	SEH	aromadendrene	1433	1439	tr.	-	-	-
61	SEH	*cis*-*β*-farnesene	1437	1440	tr.	-	-	-
62	SEH	*α*-humulene	1450	1452	1.09	1.85	-	-
63	OM	E-geranylacetone	1443	1453	-	0.09	-	-
64	SEH	allo-aromadendrene	1455	1458	0.61	-	-	-
65	SEH	Dauca-5,8-diene	1467	1471	0.16	-	-	-
66	SEH	*γ*-muurolene	1470	1478	0.24	tr.	-	-
67	SEH	*δ*-germacrene	1475	1480	2.08	1.20	-	-
68	SEH	*β*-selinene	1483	1489	tr.	-	-	-
69	SEH	*γ*-amorphene	1485	1495	0.45	-	-	-
70	OM	5,6-epoxy-*β*-ionone	1490	-	-	-	-	0.12
71	SEH	bicyclogermacrene	1490	1500	1.09	-	-	-
72	SEH	*α*-muurolene	1494	1500	0.66	-	-	-
73	SEH	***β*-bisabolene**	1504	1505	4.77	10.41	-	-
74	SEH	*γ*-cadinene	1507	1513	0.64	-	-	-
75	SEH	*δ*-cadinene	1513	1522	6.54	0.24	-	-
76	SEH	zonarene	1518	1528	0.21	-	-	-
77	SEH	*β*-sesquiphellandrene	1519	1521	-	0.14	-	-
78	SEH	*α*-cadinene	1531	1537	0.13	-	-	-
79	SEH	*α*-calacorene	1535	1544	0.09	-	-	-
80	others	5,6,7,7*α*-tetrahydro-4,4,7*α*-trimethyl-2(4H)-benzo-furanone	1538	-	-	-	-	0.18
81	SEO	**elemol**	1543	1548	2.36	6.92	-	35.56
82	SEO	(E)-nerolidol	1557	1561	0.12	1.90	-	-

Table 2. *Cont.*

Subsect. *Subbracteati*								
					% Composition			
No	**Classification [a]**	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. parnassicus* (Mt. Parnitha)**	***T. parnassicus* (Mt. Parnassos)**	***T. atticus* (Mt. Chelmos)**	***T. leucotrichus* subsp. *leucotrichus* (Mt. Chelmos)**
83	AL	dodecanoic acid	1574	1565	-	-	-	0.55
84	SEO	palustrol	1563	1567	0.12	-	-	-
85	SEO	spathulenol	1570	1577	0.94	-	-	0.77
86	SEO	caryophyllene oxide [b]	1575	1582	3.21	1.72	0.17	0.59
87	SEO	viridiflorol	1587	1592	0.23	-	-	-
88	SEO	1,10-di-epi-Cubenol	1621	1618	-	-	-	0.25
89	SEO	1-epi-cubenol	1621	1627	0.71	-	-	
90	SEO	γ-eudesmol	1625	1630	0.77	1.32	-	2.42
91	SEO	caryophylla-4(12),8(13)-dien-5-ol	1632	1639	0.49	-	-	-
92	SEO	epi-α-muurolol	1637	1640	6.49	-	-	-
93	SEO	α-muurolol	1640	1644	0.94	-	-	-
94	SEO	**β-eudesmol**	1648	1649	-	2.87	-	6.11
95	SEO	α-cadinol	1649	1652	13.53	-	-	5.82
96	SEO	**selin-11-en-4α-ol**	1656	1658	7.29	-	-	-
97	SEO	**α-eudesmol**	1666	1652	-	-	-	11.15
98	SEO	α-bisabolol	1679	1685	-	tr.	-	-
99	SEO	shyobunol	1703	1688	-	-	-	3.16
100	SEO	(2E, 6Z)-farnesal	1703	1713	1.02	0.10	-	-
101	SEO	(2E, 6Z)-farnesol	1714	1714	3.34	2.16	-	-
102	SEO	(2Z, 6E)-farnesol	1725	1722	-	-	-	0.26
103	SEO	(2E, 6E)-farnesal	1730	1740	1.8	0.47	-	-
104	others	benzyl benzoate	1756	1759	-	tr.	-	-
105	AL	tetradecanoic acid	1769	-	-	-	-	0.92
106	SEO	cryptomeridiol	1820	1813	-	-	-	0.27
107	SEO	(2Z, 6E)-farnesyl acetate	1828	1821	2.13	-	-	-

Table 2. *Cont.*

Subsect. *Subbracteati*								
					% Composition			
No	**Classification [a]**	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. parnassicus* (Mt. Parnitha)**	***T. parnassicus* (Mt. Parnassos)**	***T. atticus* (Mt. Chelmos)**	***T. leucotrichus* subsp. *leucotrichus* (Mt. Chelmos)**
108	AL	hexadecanoic acid	1975	1959	-	-	-	4.13
				%Yield (mL/100 g of dry plant material)	0.35	0.32	0.3	0.5
			AL	Aliphatic compounds	0.23	0.68	0.68	6.43
			MH	Monoterpene hydrocarbons	1.32	3.28	27.32	-
			OM	Oxygenated monoterpenes	14.34	23.76	71.53	12.19
			SEH	Sesquiterpenes hydrocarbons	32.84	49.57	0.16	-
			SEO	Sesquiterpenes oxygenated	46.43	17.46	0.17	66.36
			others		-	0.08	-	3.96
				TOTAL	95.51	94.83	99.24	88.94

* tr.: traces (% concentration $\leq$ 0.06%); [a] AL: aliphatic compounds; MH: monoterpene hydrocarbons; OM: oxygenated monoterpenes; SEH: sesquiterpene hydrocarbons; SEO: oxygenated sesquiterpenes; [b] identification based on standard compounds; [c] not detected; [d] The variables used to perform PCA and CA are highlighted in bold.

Table 3. Chemical composition of *Thymus* species (subsect. *Thymbropsis*).

Subsect. *Thymbropsis*							
					% Composition		
No	**Classification [a]**	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. holosericeus* (Mt. Roudi)**	***T.holosericeus* (Mt. Enos)**	***T. laconicus* Peloponnesse (Geraki Laconias)**
1	MH	α-thujene	925	924	- [c]	-	0.3
2	MH	α-pinene [b]	932	932	-	-	1.1
3	MH	camphene	948	946	-	-	2.0
4	AL	1-octen-3-ol	976	974	-	-	1.1
5	MH	myrcene	989	988	-	0.1	0.4
6	MH	α-phellandrene	1005	1002	-	-	tr.*

Table 3. *Cont.*

Subsect. *Thymbropsis*							
					% Composition		
No	**Classification** [a]	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. holosericeus* (Mt. Roudi)**	***T.holosericeus* (Mt. Enos)**	***T. laconicus* Peloponnesse (Geraki Laconias)**
7	MH	*α*-terpinene	1017	1014	-	-	0.7
8	MH	***p*-cymene** [b][d]	1029	1020	-	-	29.7
9	others	benzene acetaldehyde	1037	1036	tr.	-	-
10	MH	(E)-β-ocimene	1049	1044	-	tr.	-
11	MH	**γ-terpinene** [b]	1060	1054	-	-	6.8
12	OM	*cis*-linalool oxide	1070	1067	0.1	-	-
13	OM	*trans*-linalool oxide	1083	1084	tr.	-	-
14	MH	terpinolene	1089	1086	-	-	tr.
15	MH	*p*-cymenene	1089	1089	-	-	0.3
16	OM	**Linalool** [b]	1100	1095	0.4	0.5	1.0
17	OM	camphor	1145	1141	-	-	0.1
18	OM	**borneol**	1171	1165	1.2	0.2	8.2
19	OM	terpinen-4-ol [b]	1181	1174	-	tr.	2.1
20	OM	*p*-cymen-8-ol	1185	1179	-	-	0.2
21	OM	*α*-terpineol [b]	1192	1186	-	-	0.1
22	OM	verbenone	1207	1204	0.1	-	-
23	OM	nerol	1227	1227	0.5	0.4	-
24	OM	neral	1238	1235	tr.	0.6	-
25	OM	**geraniol**	1247	1249	89.9	87.7	-
26	OM	geranial	1275	1264	1.1	-	-
27	OM	bornyl acetate	1287	1284	-	-	0.3
28	OM	lavandulyl acetate	1299	1288	-	0.1	-
29	OM	**thymol**	1303	1289	tr.	1.7	7.9
30	OM	**carvacrol**	1311	1298	-	0.4	32.7
31	OM	ethyl nerolate	1361	1351	-	tr.	-
32	OM	carvacrol acetate	1366	1370	-	-	tr.
33	OM	geranyl acetate	1385	1379	0.7	0.2	-
34	SEH	*β*-bourbonene	1389	1387	0.2	-	-
35	SEH	***E*-caryophyllene** [b]	1424	1417	0.7	2.0	2.8
36	SEH	*α*-humulene	1460	1452	-	0.1	tr.

Table 3. *Cont.*

Subsect. *Thymbropsis*							
					% Composition		
No	**Classification** [a]	**Compound Identification**	**A.I Experimental**	**A.I Literature**	***T. holosericeus* (Mt. Roudi)**	***T.holosericeus* (Mt. Enos)**	***T. laconicus* Peloponnesse (Geraki Laconias)**
37	OM	geranyl propanoate	1474	1476	-	0.1	-
38	SEH	δ-germacrene	1487	1480	0.2	tr.	-
39	SEH	bicyclogermacrene	1502	1500	0.4	0.1	-
40	SEH	δ-Cadinene	1527	1522	0.1	--	-
41	OM	geranyl butanoate	1562	1562	-	1.2	-
42	SEO	spathulenol	1586	1577	0.8	0.1	-
43	SEO	caryophyllene oxide [b]	1592	1582	1.0	1.1	1.4
44	OM	geranyl isovalerate	1603	1606	-	0.5	-
45	SEO	caryophylla-4(12),8(13)-dien-5-ol	1645	1639	-	0.1	tr.
46	OM	geranyl valerate	1656	1655	-	tr.	-
47	SEO	epi-β-bisabolol	1672	1670	0.4	-	-
				% Yield (mL/100 g of dry plant material)	2	3.3	2
			AL	Aliphatic compounds	-	-	1.1
			MH	Monoterpene hydrocarbons	-	0.1	41.3
			OM	Oxygenated monoterpene	94	93.6	52.6
			SEH	Sesquiterpenes hydrocarbons	1.6	2.2	2.8
			SEO	Sesquiterpenes oxygenated	2.2	1.3	1.4
				TOTAL	97.8	97.2	99.2

* tr.: traces (% concentration $\leq$ 0.06%); [a] AL: aliphatic compounds; MH: monoterpene hydrocarbons; OM: oxygenated monoterpenes; PHP: phenylpropanoid compounds; SEH: sesquiterpene hydrocarbons; SEO: oxygenated sesquiterpenes; [b] identification based on standard solutions; [c] not detected; [d] The variables used to perform PCA and CA are highlighted in bold.

3. Results

The yield (% v/w) and chemical composition of the essential oils extracted from the aerial parts of the studied *Thymus* species are presented in Table 2 (subsect. *Subbracteati*) and Table 3 (subsect. *Thymbropsis*). The compounds in both tables are arranged according to their Relative Retention Index (R.R.I.) values. Seventy-six compounds were identified in the essential oil of *T. parnassicus* (Mt. Parnitha), representing the 94.22% of the oil and 54 in *T. parnassicus* (Mt. Parnassos), representing 94.83% of the oil. For *T. atticus*, 19 compounds accounting for 99.24% of the oil were detected. Additionally, 35 compounds were identified in the essential oil of *T. leucotrichus* subsp. *leucotrichus*, representing 88.94% of the essential oil. Within subsect. *Thymbropsis*, 20 compounds were identified in the oil of *T. holosericeus* (Mt. Roudi), constituting 97.8% of the oil and 24 compounds accounting for 97.2% were detected in the plants from Mt. Enos. Finally, for *T. laconicus*, 99.2% of the oil's composition was identified comprising 25 compounds. GC-MS chromatograms of the studied species are given as Supplementary Material (Figures S1–S7).

More precisely, the major compound found in the sample of *T. parnassicus* from Mt. Parnitha is *α*-cadinol (13.53%), followed by *E*-caryophyllene (11.83%). In the sample from Mt. Parnassos, *E*-caryophyllene is in abundance (35.20%), followed by *β*-bisabolene (10.41%). *Thymus atticus* is characterized by the prevalence of linalool (63.04%). For *T. leucotrichus* subsp. *leucotrichus*, the primary compound is elemol (35.56%), followed by *α*-eudesmol (11.15%) (Table 2). Concerning the species of subsect. *Thymbropsis*, both samples of *T. holosericeus* from Kefalonia Island are rich in geraniol (89.9% from Mt. Roudi and 87.7% from Mt. Enos). For *T. laconicus*, the dominant compounds are carvacrol (32.7%), and *p*-cymene (29.7%) (Table 3).

Two principal components were chosen for PCA analysis which explain the 56.72% of the total variance (Figure 1).

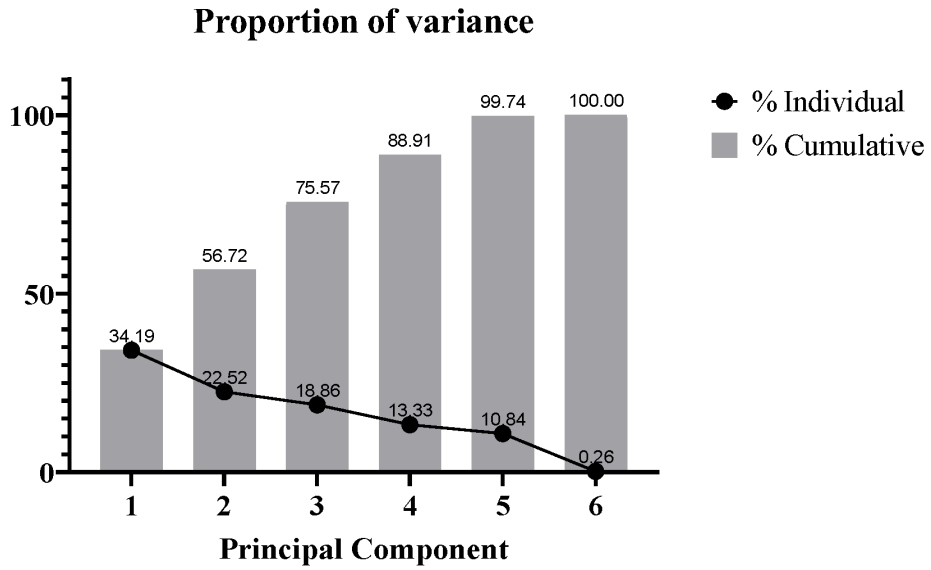

Figure 1. Proportion of variance explained by each principal component.

The first principal component (PC1) accounts for 34.19% of the total variance, displayed positive associations with *γ*-terpinene, carvacrol, *p*-cymene, thymol and borneol. The second component (PC2) contributes 22.5% of the total variance, positively correlated *δ*-cadinene, selin-11-en-4*α*-ol, *α*-cadinol, *β*-bisabolene and *E*-caryophyllene, while it was negatively correlated to *α*-eudesmol, *β*-eudesmol and elemol (Figure 2).

Therefore, the studied samples can be grouped as follows: Group 1 consists of *T. holosericeus* rich in geraniol, an oxygenated monoterpene, while Group 2 is divided into four sub-groups (Figure 3). *Thymus leucotrichus* subsp. *leucotrichus*, *T. laconicus* and *T. atticus*,

each form a distinct cluster. Both samples of *T. parnassicus* were clustered together probably because of their high sesquiterpene content.

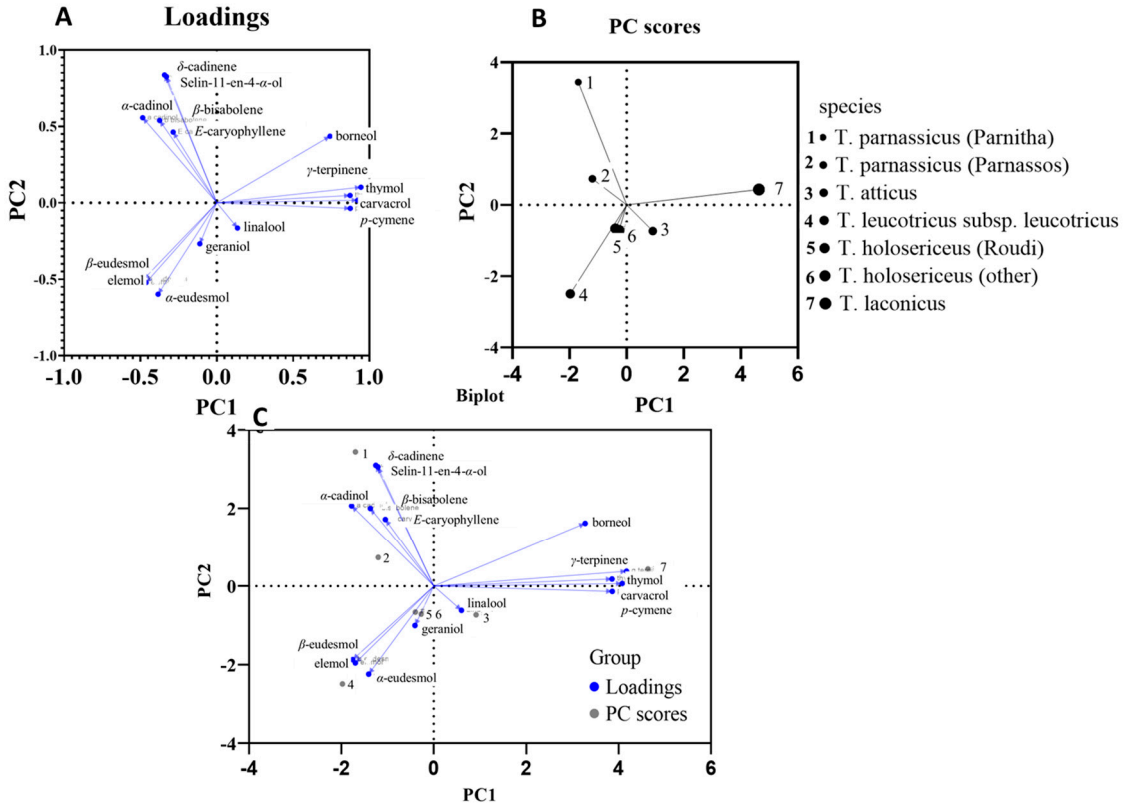

Figure 2. (**A**) Correlation between the variables and the principal components; (**B**) Dimension reduction achieved by PCA; (**C**) Biplot of PCA analysis. Eigenvalues greater than one were chosen to perform the analysis.

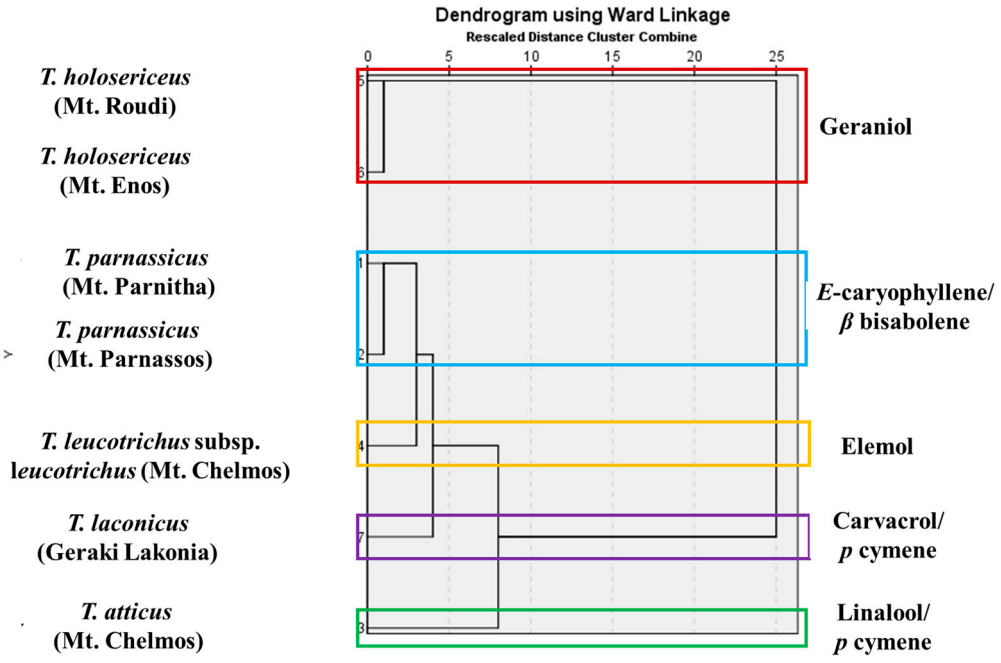

Figure 3. Chemical polymorphism of *Thymus* species represented by a dendrogram obtained by cluster analysis using Ward's method.

4. Discussion

The genus *Thymus* comprises a significant number of taxa that largely remain unexplored in terms of their phytochemistry. One such example is *T. laconicus*, for which we present the volatile profile data for the first time. In addition to this unexplored species, others like *T. parnassicus*, *T. holosericeus* and *T. atticus* have received limited research attention and have also been included in our study. Chemical polymorphism, a common trait in many medicinal and aromatic plants, has also been reported in various *Thymus* species [32–34]. These diverse profiles of both main and secondary metabolites highlight the need for further investigation of their chemical composition. Each species may contribute "uniquely" potentially offering distinct modes of action and therefore differing biological activities.

Plants synthesize secondary metabolites in response to external stimuli and their production is affected by multiple factors [35]. Biosynthetic pathways as well as specific enzymes responsible for their production may vary between plant species and they also depend on environmental factors and developmental stage of the plant. Therefore, it is necessary to understand how metabolism pathways and related enzymes vary in response to different exogenous and endogenous factors, to which a plant has been adapted.

As depicted in Tables 2 and 3, the investigated species exhibit distinct chemotypes. Their qualitative and quantitative differences confirm the already-known patterns of chemical polymorphism within *Thymus*, a genus rich in secondary metabolites. Our results demonstrate that nearly all studied *Thymus* species, excluding *T. holosericeus*, display a mixed chemotype. Both thymol and carvacrol are present in almost all species; however, their proportions vary considerably among them. Especially noteworthy is the high chemical variability observed in *T. parnassicus* collected from two distinct geographical areas. The plants from Mt. Parnitha are rich in oxygenated sesquiterpenes followed by sesquiterpenes hydrocarbons. They present a mixed chemotype of α-cadinol (13.53%), *E*-caryophyllene (11.83%), selin-11-en-4α-ol (7.29%), δ-cadinene (6.54%), epi-α-muurolol (6.49%) and β-bisabolene (4.77%). These compounds constitute 50.45% of the total essential oil. One study was found to examine the chemical composition of *T. parnassicus* collected from three distinct geographic areas, including Mt. Parnitha [31]. The main compounds identified in this study were *E*-caryophyllene (8.5%), linalool acetate (8.2%), γ-terpinene (8.0%) and myrcene (5.3%). The chemical composition of *T. parnassicus* collected from Mt. Parnassos reveals a mixed chemotype primarily comprised *E*-caryophyllene (35.20%), β-bisabolene (10.41%) and elemol (6.92%) accounting 52.53% of the total constituents. In this sample, sesquiterpene hydrocarbons prevail, followed by oxygenated monoterpenes. The observation by Tzakou and Constantinidis [30] indicating the stability of the volatile profile of *T. parnassicus* across different origins where sesquiterpene hydrocarbons and oxygenated sesquiterpenes dominate with consistent quantities is not fully supported by our results. Our findings are partially consistent with the results of Tzakou and Constantinidis [30], since *T. parnassicus* from Mt. Parnitha has a similar chemical profile, with sesquiterpenes (hydrocarbons and oxygenated) being in abundance. However, significant % quantitative differences were observed among these groups of compounds (32.84% for sesquiterpenes hydrocarbons and 45.49% for oxygenated sesquiterpenes). On the other hand, the sample collected from Mt. Parnassos, albeit rich in sesquiterpenes hydrocarbons (49.57%), deviates from this trend since the second most abundant group is oxygenated monoterpenes accounting for 23.76%. Nevertheless, the combined quantity of sesquiterpenes outweighs that of monoterpenes. For a more precise characterization of the *T. parnassicus* samples included in this study, a comparison of their overall fingerprint is essential. Apart from α-cadinol, selin-11-en-4α-ol and epi-α-muurolol exclusively present in the Parnitha sample, other marker compounds, despite their minor % concentration, warrant attention. These include the compounds 2, 9, 29, 30, 32, 37, 62, 77, 94, 98 and 104 (Table 2) that are present only in the Parnassos sample. These differences, qualitative and quantitative, should not be neglected, as they contribute to the chemical polymorphism of *T. parnassicus*.

Regarding *T. leucotrichus* subsp. *leucotrichus*, elemol is the characteristic compound (35.56%), followed by α-eudesmol (11.15%) and β-eudesmol (6.11%), indicating a mixed chemotype for this taxon. Interestingly, a different chemotype was identified in [36], which examined *T. leucotrichus* collected in Bulgaria. The examined sample exhibited mixed chemotype, predominantly featuring β-caryophyllene (23.10%) and elemol (9.8%). β-caryophyllene was not among the compounds detected in our study. Greek samples of this species have been assessed in previous studies [37,38]. The initial study [37] did not specify identification to the infraspecific rank, whereas the subsequent study [38] focused on examining *T. leucotrichus* var. *creticus*. Diverse chemotypes were identified among the studied samples, namely β-caryophyllene/1.8 cineole and β-caryophyllene/linalool in the former study, while the latter revealed a *p*-cymene/γ-terpinene chemotype. Likewise, *T. leucotricus* of unidentified subspecies collected in Turkey showed a high percentage of *p*-cymene (21.55%) and thymol (31.01%) with monoterpenes being in abundance. Indeed, their % quantity far exceeded that of sesquiterpenes [39]. This is opposed to our results, since we observed the quantity of oxygenated sesquiterpenes to be five-fold that of oxygenated monoterpenes. According to our knowledge, studies regarding *T. leucotricus* are few. Our results, alongside the studies mentioned herein, demonstrate the importance of the accurate description of the plant, which contributes important information regarding its phytochemistry [40]. Nevertheless, all these results strengthen previous evidence of the enormous chemical variability within *Thymus* spp.

For *T. atticus*, the abundance in linalool (63.04%) and *p*-cymene (25.63%) detected in our samples significantly differs from previously reported values by [30], [36] and [41]. Both *p*-cymene and linalool were identified in all studies; however, in our study, these compounds were found in significantly higher quantities. According to [42], *p*-cymene production is favored by soil conditions, specifically a soil rich in carbohydrates, rather than environmental factors. Furthermore, δ-germacrene and (*E*)-nerolidol, which were detected in considerable quantities by [30], were not found in our study.

In *T. holosericeus* from Kefalonia Island, a well-defined chemotype was observed consisting of geraniol (89.9% from Mt. Roudi and 87.7% from Mt. Enos), along with its stereoisomer nerol. Neral, an oxidation product of geraniol was present in both samples, while geranial, another oxidation product was only detected in the plants collected from Mt. Roudi. A previous examination of *T. holosericeus* on the same island had identified a mixed chemotype specifically the carvacrol/geraniol chemotype [29]. Constituents, such as borneol, linalool and thymol, were present in significant quantities, similarly to our sample. Other geraniol chemotypes of *Thymus* spp. have been detected across the Baltic countries [43].

Thymus laconicus is classified as a mixed chemotype containing carvacrol (32.7%) and *p*-cymene (29.7%). To our knowledge, no previous studies have been conducted on this species. Notably, among the studied species, only in *T. laconicus* was carvacrol found in abundance. The presence of both thymol and carvacrol in the *T. laconicus* sample aligns with the biosynthetic pathway described by [44]. Terpinene-4-ol is an intermediate that converts γ-terpinene to *p*-cymene [45], which through oxidation and dehydrogenation reactions, respectively, leads to the formation of thymol and carvacrol. Similarly, in *T. atticus*, the metabolic pathway moves one step forward with the hydroxylation of thymol and carvacrol resulting in the formation of thymohydroquinone, which either spontaneously or via enzymatic reaction converts to thymoquinone, a compound found exclusively in *T. atticus* essential oil [44]. A high quantity of γ-terpinene is usually required to produce thymol or carvacrol [46]. Considering all our data, γ-terpinene is prominently present in *T. laconicus* essential oil (6.8%) and in a lower quantity in *T. atticus* (1.36%), while both *T. parnassicus* samples are poor in this compound. This observation partially explains the higher percentage of carvacrol found in *T. laconicus* compared to the other species. However, the genetic background of each species, which activates distinct enzymes in combination with variable environments conditions which can influence the expression of

specific genes, may clarify the abundance of either carvacrol or linalool in *T. laconicus* and *T. atticus*, respectively.

The relationships between the samples were investigated by PCA and CA. *Thymus parnassicus* samples were grouped together due to the abundance of sesquiterpenes and were negatively correlated with that of *T. atticus*, a sample rich in oxygenated monoterpenes, primarily linalool and secondarily *p*-cymene. *T. leucotricus* subsp. *leucotricus* was not correlated with any of the samples of subsection *Subbracteati* and formed a distinct group, due to the high percentage of elemol. Regarding plants of the *Thymbropsis* subsection, *T. holosericeus* samples were grouped together apparently due to the remarkable percentage of geraniol. *Thymus laconicus* was isolated from the other samples due to the presence of thymol and carvacrol, which far exceeded that of the other samples. A cluster analysis was performed using the same data as for the PCA analysis and revealed five distinct chemotypes. The geraniol chemotype was attributed to *T. holosericeus* plants, *E*-caryophyllene/*β*-bisabolene chemotype accounted for *T. parnassicus* plants, with the elemol chemotype for *T. leucotricus* subsp. *leucotricus*, carvacrol/*p*-cymene chemotype for *T. laconicus* and linalool/*p*-cymene chemotype for *T. atticus*.

A chemotype is defined "as same species/subspecies/varieties of an organism containing different secondary metabolites with different quantities" [47]. Each species has a unique profile which is attributed both to qualitative and quantitative differences. Compounds that dominate in the essential oil of the species, such as *p*-cymene, linalool, geraniol, *E*-caryophyllene, elemol and others, each possess a biological or other activity [48–52]. However, the presence of other compounds, even in minor quantity, may indicate possible synergistic effect. Therefore, in vitro and in vivo experiments are required to explore new perspectives for different applications of *Thymus* spp. in the food, cosmetic and pharmaceutical industry.

5. Conclusions

This study unveils the chemical composition of unexplored (*T. laconicus*) or less studied (*T. parnassicus*, *T. atticus*, *T. leucotrichus* subsp. *leucotrichus* and *T. holocericeus*) *Thymus* species native to Greece. *Thymus parnassicus* samples were rich in sesquiterpenes, both hydrocarbons and oxygenated. *Thymus atticus* presented oxygenated monoterpenes in abundance, while for *T. leucotricus* subsp. *leucotricus*, oxygenated sesquiterpenes constitute more than 50% of the total essential oil. Additionally, the members of subsection *Thymbropsis* are all rich in oxygenated monoterpenes, while for *T. laconicus*, a species endemic to Greece and studied herein for the first time, monoterpene hydrocarbons are also found in considerable quantity. According to PCA and cluster analysis, samples were classified into five groups based both on qualitative and quantitative differences of their total content in monoterpenes (samples that belong to subsect. *Thymbropsis* and the sample of *T. atticus*) and sesquiterpenes (both samples of *T. parnassicus* and *T. leucotrichus* subsp. *leucotrichus*). The determination of a plant chemotype is crucial not only for plant chemotaxonomy but also for understanding its biological activities. Research interest should extend beyond the dominant compounds of the essential oils. Even minor compounds in quantity deserve attention since they could contribute to the species identification and provenance and may also significantly contribute to the biological activity exhibited by each species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/chemosensors12010007/s1, Figure S1: GC-MS chromatogram of *T. parnassicus* (Mt. Parnitha); Figure S2: GC-MS chromatogram of *T. parnassicus* (Mt. Parnassos); Figure S3: GC-MS chromatogram of *T. atticus* (Mt. Chelmos); Figure S4: GC-MS chromatogram of *T. leucotricus* subsp. *leucotricus* (Mt. Chelmos); Figure S5: GC-MS chromatogram of *T. holosericeus* (Mt. Roudi); Figure S6: GC-MS chromatogram of *T. holosericeus* (Mt. Enos); Figure S7: GC-MS chromatogram of *T. laconicus* (Geraki Lakonias).

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E.K.; writing—review and editing, E.K., D.D., P.T. and P.A.T.; supervision, P.A.T. All authors have read and agreed to the published version of the manuscript.

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