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Abstract: In this work,the chemical constituents of the aerial parts of Thymus munbyanus subsp. coloratus were studied. Plant is used as culinary ingredient and as traditional medicine in Algeria. The apolar constituents were analysed by headspace solid phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS), whereas the solid residues obtained from extraction with solvents at different polarity were analyzed by 1H-NMR and by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MSn). Fourty-five apolar constituents were identified, mainly oxygenated monoterpenes (65.8%), sesquiterpene hydrocarbons and monoterpene hydrocarbons (18.6 and 14.5%, respectively). HPLC-MSn and 1H-NMR analyses revealed the presence of aglyconic and glycosilated flavonoids, phenylpropanoid derivatives and triterpenoid acids mainly in the methanol, dichloromethane and hexane extracts. Overall, these data indicate that Thymus munbyanus subsp. coloratus could be a potential source of bioactive compounds, and our results represent a starting point for further research on this plant species.

Total phytochemical analysis of *Thymus munbyanus* subsp. *coloratus* from Algeria by HS-SPME-GC-MS, NMR and HPLC-MS^n studies

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Highlights

- 45 apolar constituents were identified by HS- SPME-GC-MS.
- The most abundant were sesquiterpenes, monoterpenes and oxygenated monoterpenes.
- Extracts with different solvents were obtained and analysed by NMR and HPLC-MS^n.
- Secondary metabolites in MeOH extract comprise flavonoids, phenylpropanoids and terpenoids

Total phytochemical analysis of *Thymus munbyanus* subsp. *coloratus* from Algeria by HS-SPME-GC-MS, NMR and HPLC-MS^n studies

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Abstract

In this work, we report a comprehensive study on the chemical constituents from the aerial parts of *Thymus munbyanus* subsp. *coloratus*, a shrub that is used as culinary ingredient and as traditional medicine in Algeria, mainly to treat respiratory and gastrointestinal disorders and endocrine diseases. The apolar constituents were analysed by headspace solid phase microextraction (HS- SPME) coupled with gas chromatography- mass spectrometry (GC-MS), whereas the solid residues obtained from extraction with solvents at different polarity were analyzed by ^{1}H- NMR and by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS^{n}). Fourty-five apolar constituents were identified, mainly oxygenated monoterpenes (65.8%), sesquiterpene hydrocarbons and monoterpene hydrocarbons (18.6 and 14.5%, respectively). On the other hand, HPLC-MS^{n} and ^{1}H- NMR analyses revealed the presence of aglyconic and glycosilated flavonoids, phenylpropanoid derivatives and triterpenoid acids related to oleanolic acid, mainly in the methanol, dichloromethane and hexane extracts. Overall, these data indicate that *Thymus munbyanus* subsp. *coloratus* could be a potential source of antioxidants and bioactive compounds, and our results represent a starting point for further research on this plant species.

Keywords

Thymus; apolar constituents; secondary metabolites; NMR; GC-MS; LC-MS

1. Introduction

The Thyme (*Thymu*s L.), with about 215 species distributed throughout Europe, Asia, and North Africa (Benchabane et al., 2012), is one of the largest genera into the Lamiaceae family (Cronquist, 1988). In Algeria, 20 species are recorded (Quezel and Santa, 1962; Bousmaha-Marroki, 2007), among which *T. munbyanus* Boiss. & Reut. is considered as endemic to North Africa (Benchabane et al., 2012). According to The Plant List database (http://www.theplantlist.org), this species is divided into two subspecies, namely *T. munbyanus* subsp. *coloratus* (Boiss. & Reut.) Greuter & Burdet (TMC) (synonym of *Thymus coloratus* Boiss. & Reut.), and *T. munbyanus* subsp. *munbyanus* (TMM).

TMC is a small shrub up to 30 cm tall, with tender, simple small leaves and branched and prostrated stems (Reddy et al., 1998), with flowers not exceeding 7 to 8 mm. It is made up of small floral leaves, roughly purple stained at least at the base. The stem is generally tetragonal, branched and woody in its lower part. This subspecies grows around the Mediterranean region and in northern Algeria, where it is found in lawns, rockeries and mountainous regions (Quezel and Santa, 1962).

In general, the leaves and blooming aerial parts of *Thymus* plants are widely employed as culinary ingredients, and are also used as a food preservative because of their antioxidant capacity (Stahl-Biskup and Saez, 2002). In the traditional medicine, aerial parts of *Thymus* species have been used extensively for their tonic, antiseptic, antitussive and carminative properties, and in the treatment of colds, coughs, sore throats, cystitis, insomnia, bronchitis, and indigestion (Stahl-Biskup and Saez, 2002). In Algeria, *T. munbyanus* is used in the treatment of respiratory and gastrointestinal disorders and endocrine diseases (Miara et al., 2013).

Several studies have been focused on the characterization of the essential oils and secondary metabolites from *Thymus* spp., reporting volatile components (e.g., phenolic monoterpenes such as thymol and carvacrol), phenolic acids (e.g. rosmarinic acid, caffeic acid, etc.), triterpenes (ursolic and oleanolic acids) and flavonoids (e.g., luteolin derivatives) as major constituents (Nabavi et al., 2015;

Zheng & Wang, 2001; Ikeda, Murakami, & Ohigashi, 2008). Recently, essential oils and apolar constituents from carbon dioxide supercritical fluid extracts and pressurized liquid extracts of *T*. *munbyanus* subsp. *coloratus* were characterized and investigated for biological activities (Bendif et al., 2017, 2018). However, to the best of our knowledge, there are no published reports on the aroma profile and the non-volatile polar constituents of this subspecies, which could be of interest as a source of antioxidant and bioactive compounds. Therefore, in the present work we examined the aroma profile and the non-volatile polar constituents of crude extracts of *T*. *munbyanus* subsp. *coloratus* aerial parts by using different approaches, namely headspace solid phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS), ^{1}H-NMR analysis, and high performance liquid chromatography with tandem mass spectrometric detection (HPLC-MS^{n}).

2. Experimental Section

2.1.Plant Material

Mixture of inflorescences, stems and leaves of *T*. *munbyanus* subsp. *coloratu*s, growing in Mechta Fatima, Province of Bordj Bou Arreridj (North-East Algeria, N 36°060; E 04°760, 820 m) was harvested in March 2016. Botanical determination was performed by Dr. Miara M.D.J., using available literature (Quézel and Santa, 1962). A voucher specimen was deposited in the Herbarium Universitatis Camerinensis (CAME, included in the online edition of Index Herbariorum c/o School of Biosciences and Veterinary Medicine, University of Camerino, Italy), under the codex CAME 27741; it was also archived in the anArchive System for Botanical Data (http://www.anarchive.it). Before undergoing extraction, plant material was washed in running water and dried in the shadow at r.t. for 7 days.

2.2.HS-SPME-GC-MS analysis

For HS-SPME, the method previously described by Ascrizzi et al. (2017) was used. Briefly, a Supelco SPME device coated with polydimethylsiloxane (PDMS, 100 μm) was used. After the equilibration time, the fibre was exposed to the headspace for 30 min, and sampling was accomplished in an air-conditioned room (22 ± 1 °C) to guarantee a stable temperature. Once sampling was finished, the fibre was withdrawn into the needle and transferred to the injection port of the GC-MS system.

GC-EI- MS analyses were performed with a Varian CP- 3800 gas- chromatograph equipped with a DB- 5 capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. The analytical conditions were the following: injector and transfer line temp. 220 and 240°C, respectively; oven temp. programmed from 60 to 240°C at 3°C/min; carrier gas helium at 1 ml/min; splitless mode. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices (LRI) relative to the C_6 – C_{28} series of *n*- hydrocarbons, and on computer matching against commercial (Adams, 2007 and NIST, 2008) and home- made library mass spectra built up from pure substances.

2.3.Preparation of extracts for NMR and HPLC-MS^n analyses

Sample preparation was performed accordingly to previously published methods (Dall'Acqua et al. 2010; Bendif et al., 2017a), with some modifications. Thirty g of the dried vegetative parts (stems and leaves) were ground with a blender, to obtain a fine powder. The powder was suspended in 150 mL of methanol and then sonicated for 10 min. The supernatant was removed after centrifugation and the residue was then re-extracted with further 50 ml of the same solvent, for two more times. Supernatants were collected in a round bottom flask and the extract was concentrated under reduced pressure at 35°C with a rotary evaporator, yielding 2.9 g of crude extract (yield 9.6%, w/w).

The obtained dry extract was stored in dark glass vials at -20 °C before chemical characterization. A part of the extract (150 mg) was dissolved in deuterated methanol and used for NMR analysis. The

remaining extract was dissolved in methanol/water (1:9) mixture (50 ml) and sonicated. The obtained solution was partitioned using 20 mL of solvents at increasing polarity, namely hexane, dichloromethane (DCM) and ethyl acetate (EA). For each solvent, extraction was repeated three times. Solvents were dried under vacuum and the residues were dissolved in deuterated methanol for NMR analysis.

2.4.NMR Analysis

NMR spectra were acquired on a Bruker Avance III 400 MHz spectrometer, using standard pulse sequences. ^{1}H-NMR spectra were acquired for all the obtained fractions, and ^{1}H, HSQC-DEPT, HMBC, COSY and TOCSY spectra were acquired for the DCM extract.

2.5.HPLC-MS^{n} analysis of methanol extract from stems and leaves

Polar constituents were tentatively identifed in the methanol extract of *T*. *Munbyanus* subsp. *coloratus* vegetative parts by HPLC-MS^{n}, comparing the fragmentation patterns with literature data and with standard compounds, when available. Before analysis, the sample was dissolved in methanol at a concentration of 5 mg/ml and the solution was filtered through a 0.45 μm Millipore filter. The HPLC system was composed by a Varian 212 binary pump equipped with a Varian Prostar 430 autosampler, coupled to a Varian 500 Ion Trap mass detector (MS). Electrospray Ionisation (ESI) was employed as ion source, operating in negative ion mode. The stationary phase was composed by an Agilent Eclipse plus C18 column (2.1x150 mm, 3.5 μm). The mobile phase was a mixture of acetonitrile (A) and 0.1% formic acid in water (B), and the gradient was set as follows: 0 min, 10% A; 20 min, 54% A; 23 min, 100% A and isocratic up to 32 min. Re-equilibration time was 8 min. Flow rate was 0.2 ml/min. ESI parameters were: needle voltage, 4500 V; capillary voltage, 70 V; RF loading, 100%; nebulizing gas pressure, 20 psi (nitrogen); drying gas pressure, 15 psi; drying gas temperature, 350 °C. *m*/*z* range was

50 – 2000. Fragmentation patterns of eluted compounds were obtained using the turbo detection data scanning (TDDS) function of the instrument, setting n = 3 levels of fragmentation.

3. Results and discussion

3.1.HS-SPME-GC-MS analysis

SPME is a relatively recent and easy to automate technique that allows the sampling of the volatile compounds. It is a solvent-free sample preparation technique used for extracting volatile and semivolatile analytes in a fast and easy way for analysis by GC. The volatiles emitted by the aerial parts of *T*. *munbyanus* subsp. *coloratus* are summarized in Table 1. A total of 45 volatile components were identified, accounting for 99.2% of the total composition. The chemical profile of volatiles extracted by SPME from aerial parts powder of *T*. *munbyanus* subsp. *coloratus* growing in Algeria was qualitatively and quantitatively different from the one of the essential oil of the same population already reported in literature (Bendif et al., 2017). Nevertheless, it should be considered that the results of SPME and essential oil analyses are not comparable, due to completely different sampling methods. Overall, the apolar fraction of the powder from the aerial parts was dominated by oxygenated monoterpenes (65.8% of the total composition), followed by sesquiterpene hydrocarbons and monoterpene hydrocarbons (18.6 and 14.5%, respectively). Oxygenated sesquiterpenes and non-terpene derivatives were considerably less represented (0.2 and 0.1%, respectively). The characterizing compounds were camphor (26.4%), pulegone (10.6%), camphene (9.4%) and terpinen-4-ol (9.1%). Other important components were 1,8-cineole (6.3%), borneol (6.1%) and germacrene D (5.0%).

Table 1: Aroma profile of *Thymus munbyanus* subsp. *coloratus* aerial parts.

N.	Constituents	*LRI*[a]	*Lit. RI*[b]	[%]
1.	tricyclene	928	926	0.2

2.	α-pinene	941	939	1.3
3.	camphene	955	954	9.4
4.	β-pinene	982	979	0.6
5.	myrcene	993	990	1.9
6.	α-terpinene	1020	1017	0.1
7.	1,8-cineole	1034	1031	6.3
8.	*(E)*-β-ocimene	1052	1050	0.4
9.	γ-terpinene	1063	1059	0.3
10.	*cis*-sabinene hydrate	1070	1067	0.2
11.	terpinolene	1090	1088	0.3
12.	linalool	1101	1096	1.1
13.	α-campholenal	1128	1126	0.3
14.	camphor	1145	1146	26.4
15.	pinocarvone	1164	1161	0.1
16.	borneol	1168	1165	6.1
17.	terpinen-4-ol	1179	1177	9.1
18.	α-terpineol	1191	1188	2.8
19.	verbenone	1207	1204	0.9
20.	isobornyl formate	1232	1235	0.3
21.	pulegone	1239	1237	10.6
22.	*(E)*-2-decenal	1263	1261	0.1
23.	bornyl acetate	1287	1288	1.4
24.	piperitenone	1342	1340	0.2
25.	α-cubebene	1352	1348	0.2
26.	α-copaene	1377	1376	1.2
27.	β-bourbonene	1385	1387	1.6
28.	β-cubebene	1390	1388	0.5
29.	β-elemene	1392	1389	0.4
30.	α-gurjunene	1410	1409	2.8
31.	β-caryophyllene	1419	1417	1.7
32.	β-copaene	1430	1432	0.6
33.	aromadendrene	1440	1439	0.3

34.	α-humulene	1456	1454	0.3
35.	alloaromadendrene	1461	1458	0.5
36.	*cis*-muurola-4(14),5-diene	1463	1465	1.1
37.	γ-muurolene	1479	1479	0.1
38.	germacrene D	1481	1485	5.0
39.	*trans*-muurola-4(14),5-diene	1492	1493	0.6
40.	bicyclogermacrene	1497	1500	0.3
41.	α-muurolene	1499	1500	0.1
42.	β-bisabolene	1508	1505	0.1
43.	*trans*-γ-cadinene	1514	1513	0.2
44.	δ-cadinene	1524	1523	1.0
45.	caryophyllene oxide	1582	1583	0.2
	Grouped compounds [%]			
	Monoterpene hydrocarbons			14.5
	Oxygenated monoterpenes			65.8
	Sesquiterpene hydrocarbons			18.6
	Oxygenated sesquiterpenes			0.2
	Non-terpene derivatives			0.1
	Total identified			99.2
	Number of identified compounds			45

[a] LRI relative to C_6-C_{28} n-alkanes on the DB-5 column. [b] LRI taken from Adams, 2007.

3.2.NMR and HPLC-MS^n analysis of secondary metabolites

The analysis of the chemical composition of *T. munbyanus* subsp. *coloratus* aerial parts was performed by extraction with solvents at different polarity. The extracts, after solvent removal, were dissolved in deuterated solvent and analyzed by ^{1}H-NMR for obtaining a preliminary phytochemical profile. Methanol extract presents signals that support the extraction of different classes of phytochemicals (Figure 1). A summary of preliminary assignments of the detected phytoconstituents is reported in Table 2. Starting from the aromatic region of the spectrum, signals ascribable to phenylpropanoid derivatives and flavonoid moieties are observed. Furthermore, signals ascribable to anomeric proton

signals as well as to other sugar residues are detected in the range of 4.80-5.10 ppm and 3.00-4-13 ppm, respectively. The aliphatic region shows clear signals ascribable to methyl groups (both secondary and quaternary), as well as superimposed multiplets suggesting the presence of CH and CH_2 that can indicate the presence of triterpenoids. The spectra from hexane (Figure 2) and DCM (Figure 3) extracts present mostly signals ascribable to fatty acids and lipids, as well as the signlets that suggest the presence of triterpenoids (Table 2). On the other hand, the use of sequential extraction allowed to obtain another lipophylic fraction of EA that presents purified triterpenoid fraction, being its spectrum charactherized by almost the absence of signals ascribable to lipids (Figure 4). Thus, preliminary NMR analysis allowed the observation of the presence of phenolic constituents containing phenylpropanoids and flavonoid units, glycosidic derivatives and triterpenoids. Detailed observation of ^{1}H-NMR signals in the different extracts suggests the presence of rosmarinic acid as well as triterpenoid acid related to oleanolic acid (Table 2).

Table 2. ^{1}H-NMR signals obtained from the analysis of the MeOH, ethyl acetate, DCM and hexane fractions of the *T. munbyanus* subsp. *coloratus* extract

Signal ^{1}H-NMR Resonance	Assignment	Extract
7.51 d (J=16.8)	H-7 of caffeic acid unit	MeOH
6.29 d (J=16.8)	H-8 of caffeic acid unit	MeOH
7.05 d (J=1.8)	H-2, H-7 of caffeic acid unit	MeOH
6.94 dd (J=1.8, 7.8)	H-6 of caffeic acid unit	MeOH
6.78 d (J=7.8)	H-5 of caffeic acid unit	MeOH

6.79 d (J=1.9)	Aromatic signal of trisubstitued ring	MeOH
6.67-6.66 dd and d partially overlapped	Aromatic signal of trisubstitued ring	MeOH
6.16 d (J=2.0)-6.48 d (J=1.8)	Signals ascribable to protons 6-8 of flavonol glycosides	MeOH
5.41-5.25-5.11-5.09	Anomeric signal of glycosidic sugar residues	MeOH
3.00-4.13 multiplets	Signals ascribable to sugar residues	MeOH
3.17 dd	CHOH of position 3 of triterpenoids	DCM, EA
2.27-2.33-2.10-1.93-1.16 multiplets	Signals ascribable to CH_2 and aliphatic CH	MeOH, DCM, EA
1.17-0.96-0.90-0.92-0.82-0.70 singlets	Quaternary methyl groups	MeOH, Hexane, DCM, EA
0.88 d	Secondary methyl group	MeOH, Hexane, DCM, EA
0.92 m	Terminal methyl unit of fatty acid chains	Hexane, DCM
1.31-1.35	Aliphatic CH_2 of fatty acids	Hexane, DCM
1.43-1.61-2.08-2.28-2.83	Deshielded aliphatic CH_2 of fatty acid vicinal to sp2 carbon or to carbonyl function	Hexane, DCM

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188 For the analysis of the secondary metabolites from the aerial parts of *T*. *munbyanus* subsp. *coloratus*,
189 the methanolic extract was analyzed by HPLC-MS^n in negative ionization mode. Only the negative
190 mode was used due to higher sensitivity in the detection of phenolic compounds and terpenes. The
191 method allowed the detection of 34 constituents, among which 24 where tentatively identified on the
192 basis of their fragmentation patterns and by comparison with literature data (Table 3). Overall, the
193 identified phenolic compounds of the methanolic extract from *T*. *munbyanus* subsp. *coloratus* include
194 rosmarinic acid (in accordance with the identification data from NMR) and other common phenolic
195 acids, namely ferulic, quinic and caffeic acids. Several derivatives of common flavonoids were also
196 identified, including derivatives of luteolin bonded to different sugar moieties, eriodictyol-7-O-
197 hexoside, gallocatechin and derivatives of quercetin and isorhamnetin (Table 3). Many glycosidic
198 derivatives of eriodictyol have been previously described in the genus *Thymus*, namely eriodictyol-7-
199 O-glucoside (Fecka and Turek, 2008), eriodictyol-7-O-rutinoside (Wang et al., 1998), eriodictyol-7-O-
200 glucuronide (Justesen, 2000), and eriodictyol-di-O-hexoside (Pereira et al., 2013). Also rosmarinic acid
201 and luteolin have been already reported as main phenolic compounds in thyme plants. Rosmarinic acid
202 has been identified as the main phenolic acid in several spp., among which *T*. *pulegioides* (Loziene et
203 al., 2007), *T*. *mastichina* (Delgado et al., 2014) and *T*. *x*. *citriodorus* (Pereira et al., 2013), while
204 luteolin and its glucoside and glucuronide derivatives have been identified as abundant flavonoids in *T*.
205 *x*. *citriodorus* (Pereira et al., 2013) and *T*. *herba* (Fecka and Turek, 2008).
206 Finally, the two triterpenes oleanolic and ursolic acids were also detected, thus confirming the
207 preliminary NMR observation that showed signals ascribable to their methyl groups and unsaturations.
208 Previous works on *Thymus* spp. have reported the identification and isolation of different triterpenes
209 related to oleanolic and ursolic acids, as for example in *T*. *alternans* from Slovakia, from which six
210 triterpenoids were isolated after extracxtion of aerial parts with DCM and were further assayed on a
211 panel of human cancer cell lines, showing a potent citotoxic activity (Dall'Acqua et al., 2017).

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214 **Table 3.** Identification of secondary metabolites from the methanol extract of *Thymus munbyanus*
215 subsp. *coloratus* by HPLC-MS^n in negative ion mode. Compounds are sorted by mass.

216

N.	R.T. (min)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	Tentative compound identification	References
1.	11.42	191	173 127 111 85		Quinic acid*	Gouveia and Castilho, 2011
2.	29.30	194	179 149		Ferulic acid*	Jianping et al., 2007
3.	12.59	305	225	224 207 182 181 165 163 135 133	Gallocatechin	Ozarowski et al., 2013
4.	26.87	311	293 275 235 133		15,16-dihydroxy-9,12 -Octadecadienoic acid	Llorent-Martinez et al., 2015
5.	27.00	325.5	185 183 170		Unknown	--
6.	20.1	327	291 229 211 193 171	211	Hydroxy-trimethoxyflavone	Desta et al., 2017
7.	21.4	329	229 211	211 209	Trihydroxyoctadecenoic acid	Llorent-Martínez et al., 2015
8.	25.43	343	328 313	285 298	Dihydroxy trimethoxy flavonol isomer	El-Sayed et al., 2014,
9.	12.02	355	263 225 197		Unknown	Ozarowski et al., 2013
10.	14.90	359	161	133 115 105	Rosmarinic acid#	Kontogianni et al., 2013 Ozarowski et al., 2013
11.	13.90	371	249		Caffeic acid derivative	Ozarowski et al., 2013
12.	12.72	387	207 163		Medioresinol	Ozarowski et al., 2013
13.	12.80	434	359 387 313 271 227		Unknown	--
14.	13.68	447	285	241 199 175	Luteolin-7-O-hexoside	Gouveia and Castilho, 2012 Kang et al., 2016
15.	11.95	449	287		Eriodictyol-7-O-hexoside	Gouveia and Castilho, 2011
16.	32.0	455.2	407 391 377 363		Ursolic acid#	Sut et al., 2018
17.	32.3	455.3	407 391 377 363		Oleanolic acid#	Sut et al., 2018
18.	14.73	461	285	267 257 243 241 217 213 199 197 175 151 133	Luteolin 7-O-glucuronide	Pereira et al., 2015
19.	29.46	471.5	452 424 361 293		Corosolic acid	Lee et al., 2017

20.	13.59	477	301	283 255 229 211 201 165 135	Quercetin 3-O-glucuronide	Pereira et al., 2015
21.	18.50	493	313 295		Salvianolic acid A	Yang et al., 2015
22.	14.17	537	313 295		Lithospermic acid isomer	Yang et al., 2015
23.	16.05	555	509 493 359		Unknown	--
24.	14.25	597.5	329 311		Unknown	--
25.	18.71	607	299 285		Methylkaempferol-O-rutinoside	Sonmezdag et al., 2016
26.	13.51	623	337 285	161	Luteolin-O-hexoside-O-glucuronide	Pereira et al, 2015
27.	15.75	639	315 301		Isorhamnetin dihexoside	Barros et al., 2012
28.	30.16	648	601 568 559 513 419		Unknown	--
29.	16.97	658	616 551 548 432		Unknown	--
30.	19.43	705	525 507	463	Unknown	--
31.	14.21	717	519 359	339 295 267	Pinoresinol dihexoside	Whitehill et al., 2012
32.	27.36	721.5	675 662		Unknown	--
33.	18.67	726	627 519 359		Unknown	--
34.	15.34	735	537 519		Salvianolic acid isomer*	Hauck et al., 2014

*Compounds identified on the basis of comparison with standard available in the lab.
Confirmed by NMR data.

Conclusions

Our study represents, to the best of our knowledge, the first comprehensive characterization of both apolar and polar constituents from *Thymus munbyanus* subsp. *coloratus*, a species widely used as culinary ingredient and in the traditional medicine of Algeria, that could be an evaluable natural source of antioxidants and bioactive compounds. A multi-technique approach was used to cover the analysis of a broad spectrum of compounds. HS-SPME coupled to GC-MS allowed to identify 45 components of the apolar fraction of *T. munbyanus* subsp. *coloratus*, among which camphor, pulegone, camphene, terpinen-4-ol, 1,8-cineole, borneol and germacrene D are the most abundant, representing almost the

73% of the fraction. NMR analysis of methanol, hexane, DCM and EA extracts allowed the rapid determination of phenolic constituents containing phenylpropanoids and flavonoid units, glycosidic derivatives and triterpenoids. Finally, the qualitative HPLC-MS^n analysis of the methanol extract allowed to detect 34 secondary metabolites, among which several ones were identified as derivatives of common flavonoids (luteolin, eriodictyol, quercetin and isorhamnetin), typically encountered in *Thymus* spp., and as the triterpenes oleanolic and ursolic acids, as a confirmation of the NMR data. Overall, the results here presented represent a starting point for further research on this plant species.

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FIGURES

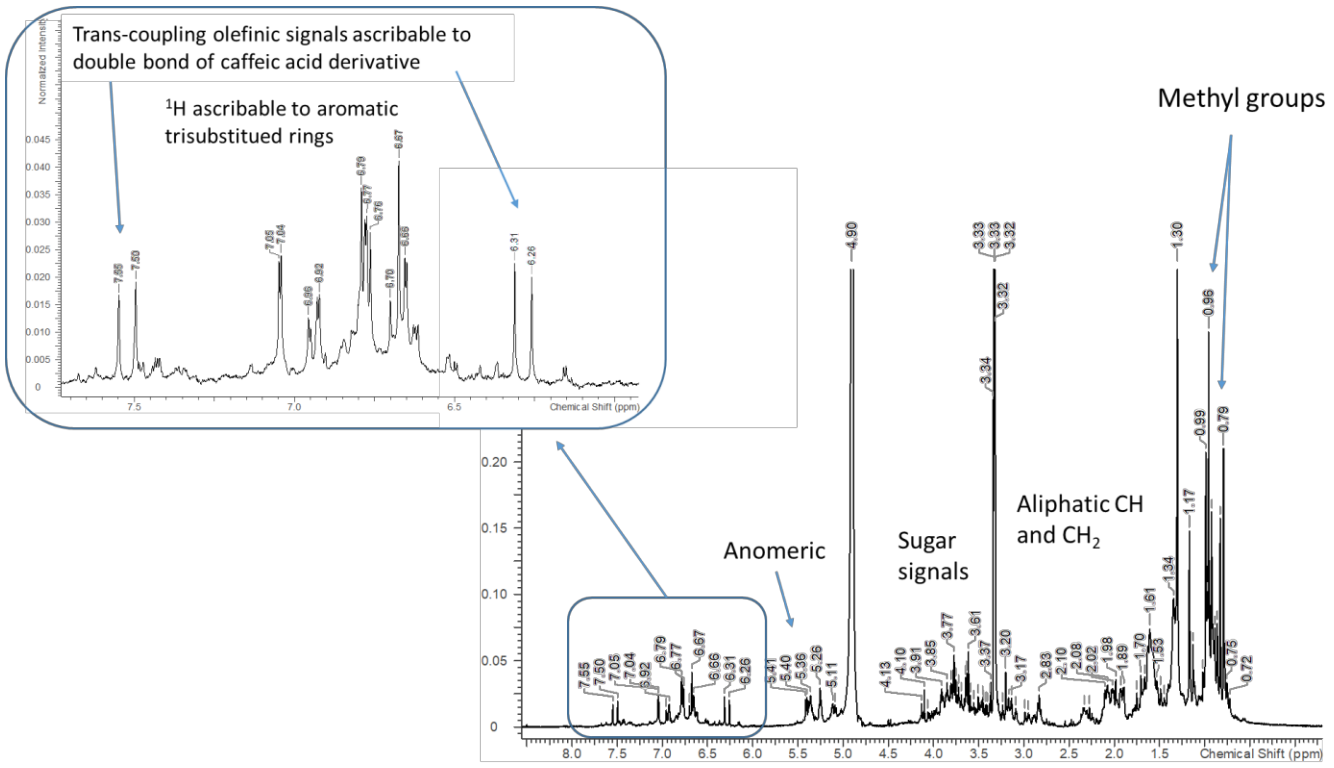


Figure 1. [1]H-NMR spectrum obtained from the analysis of the methanol extract of *Thymus munbyanus* subsp. *Coloratus*.

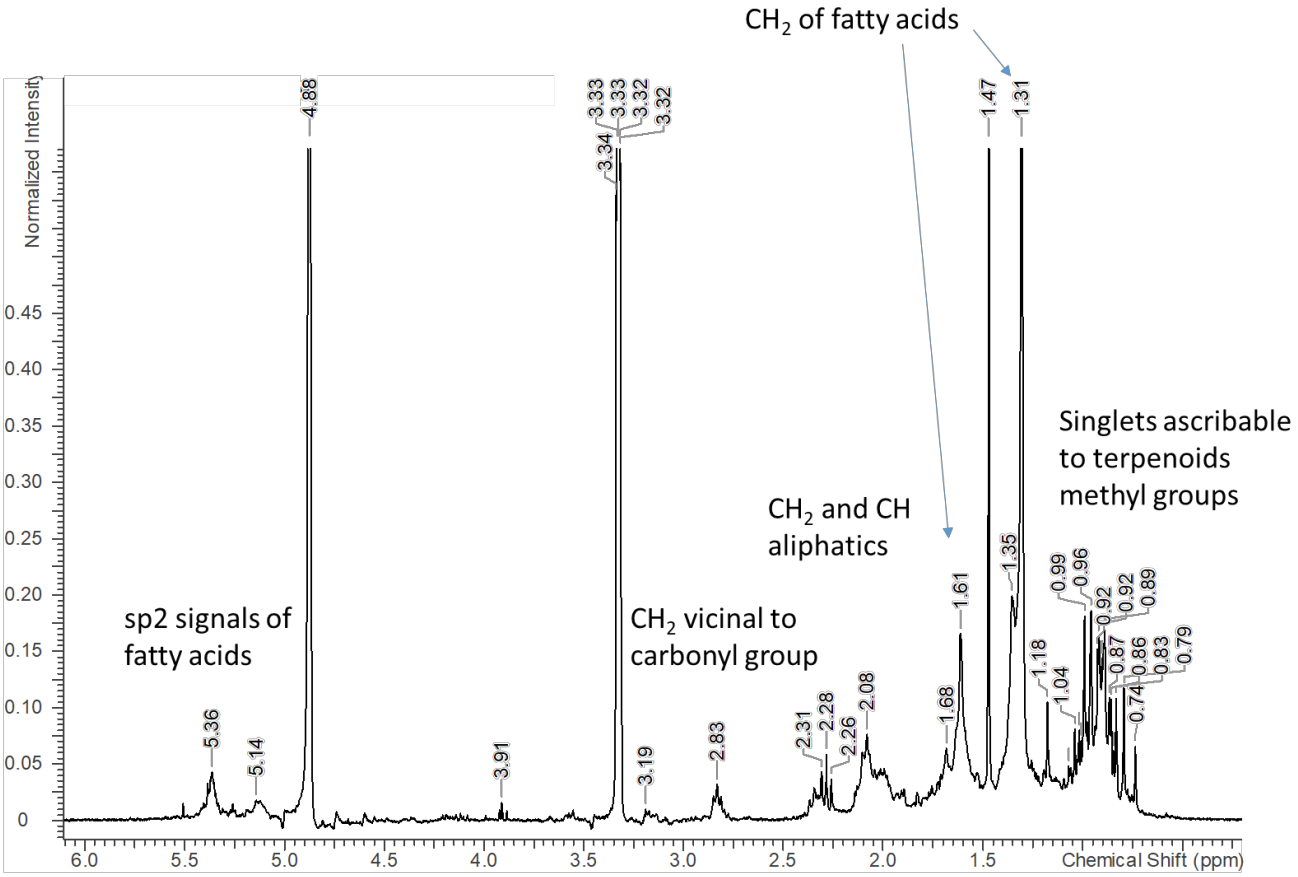


Figure 2. ^{1}H-NMR spectrum obtained from the analysis of the hexane extract of *Thymus munbyanus* subsp. *Coloratus*.

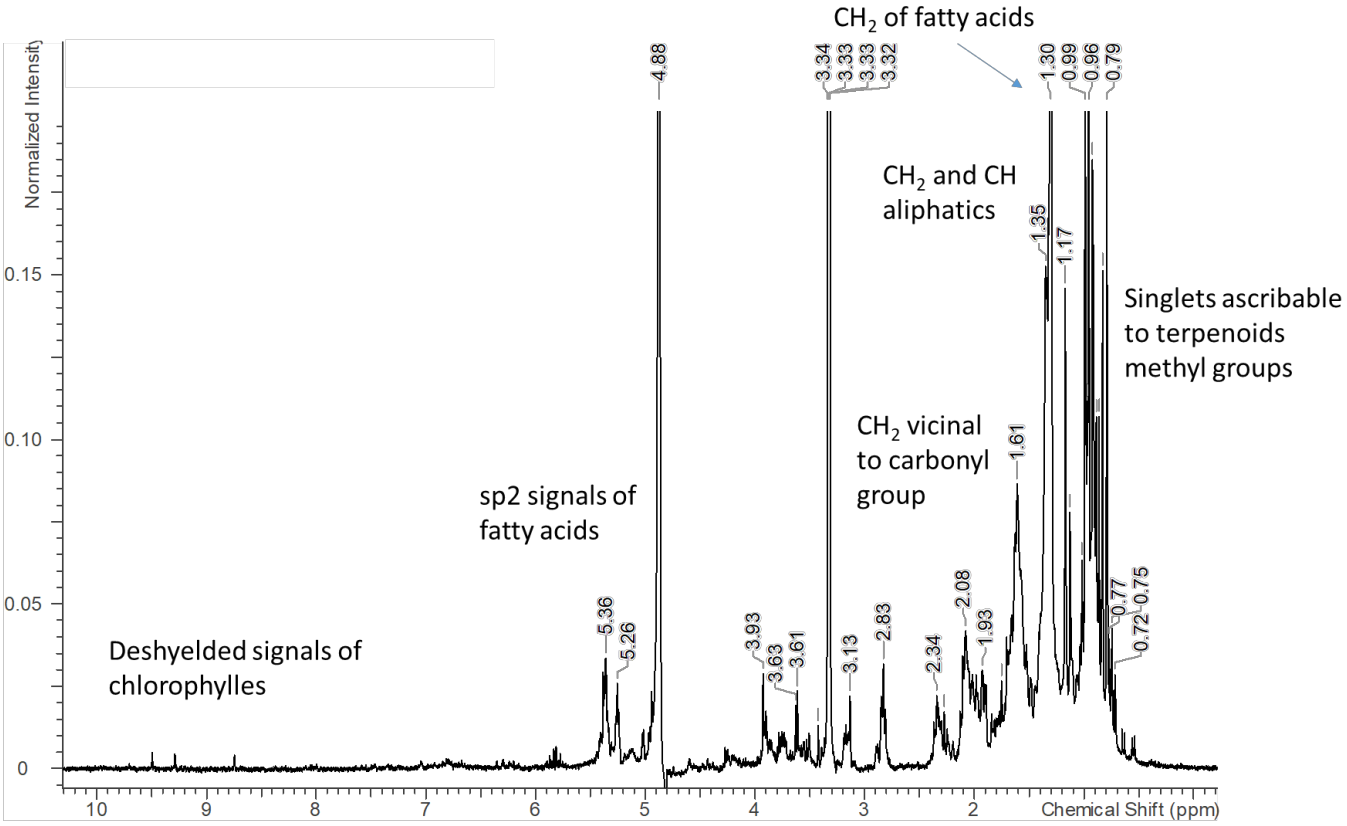


Figure 3. ^{1}H-NMR spectrum obtained from the analysis of the dichloromethane extract of *Thymus munbyanus* subsp. *Coloratus*.

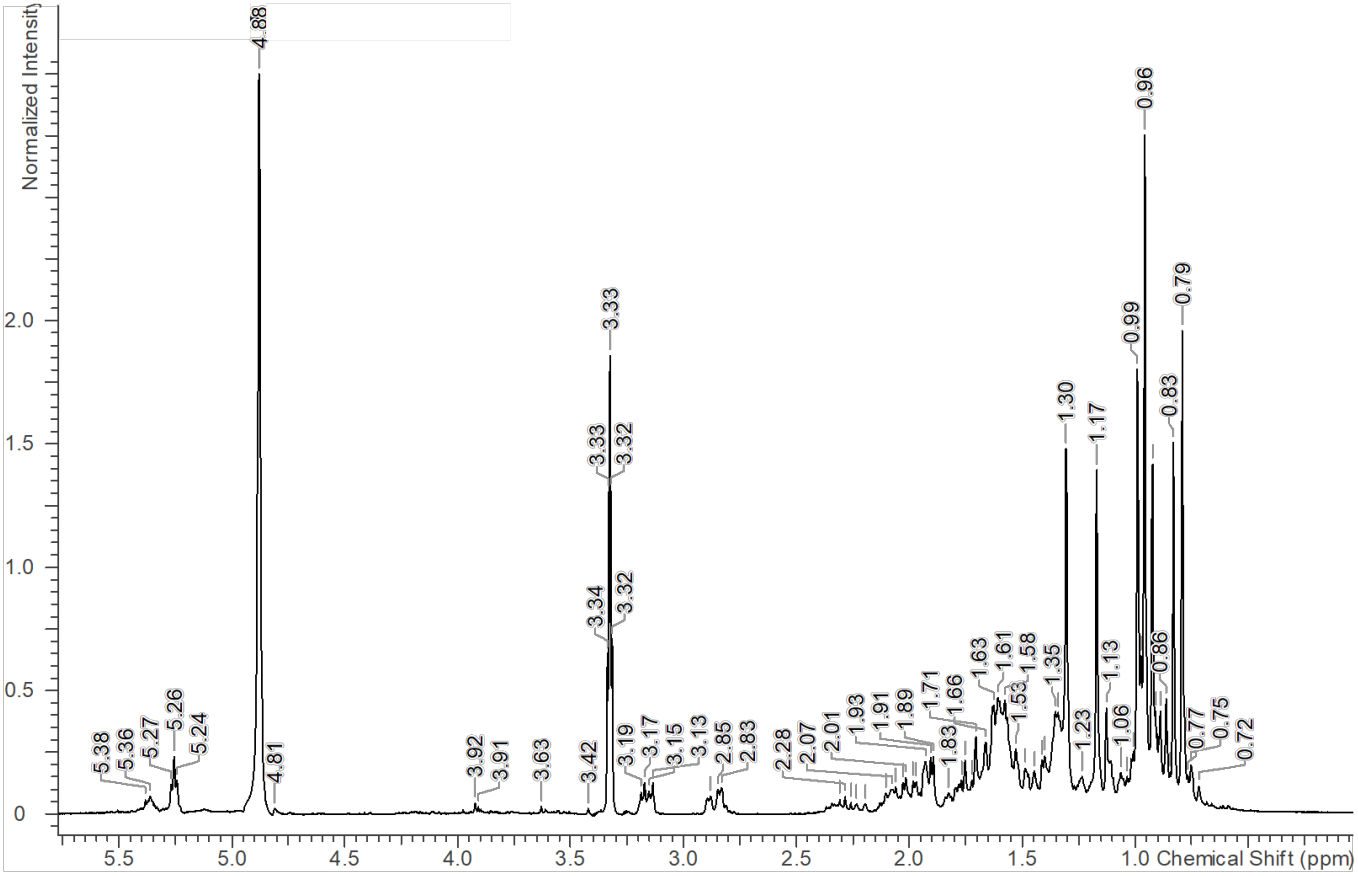
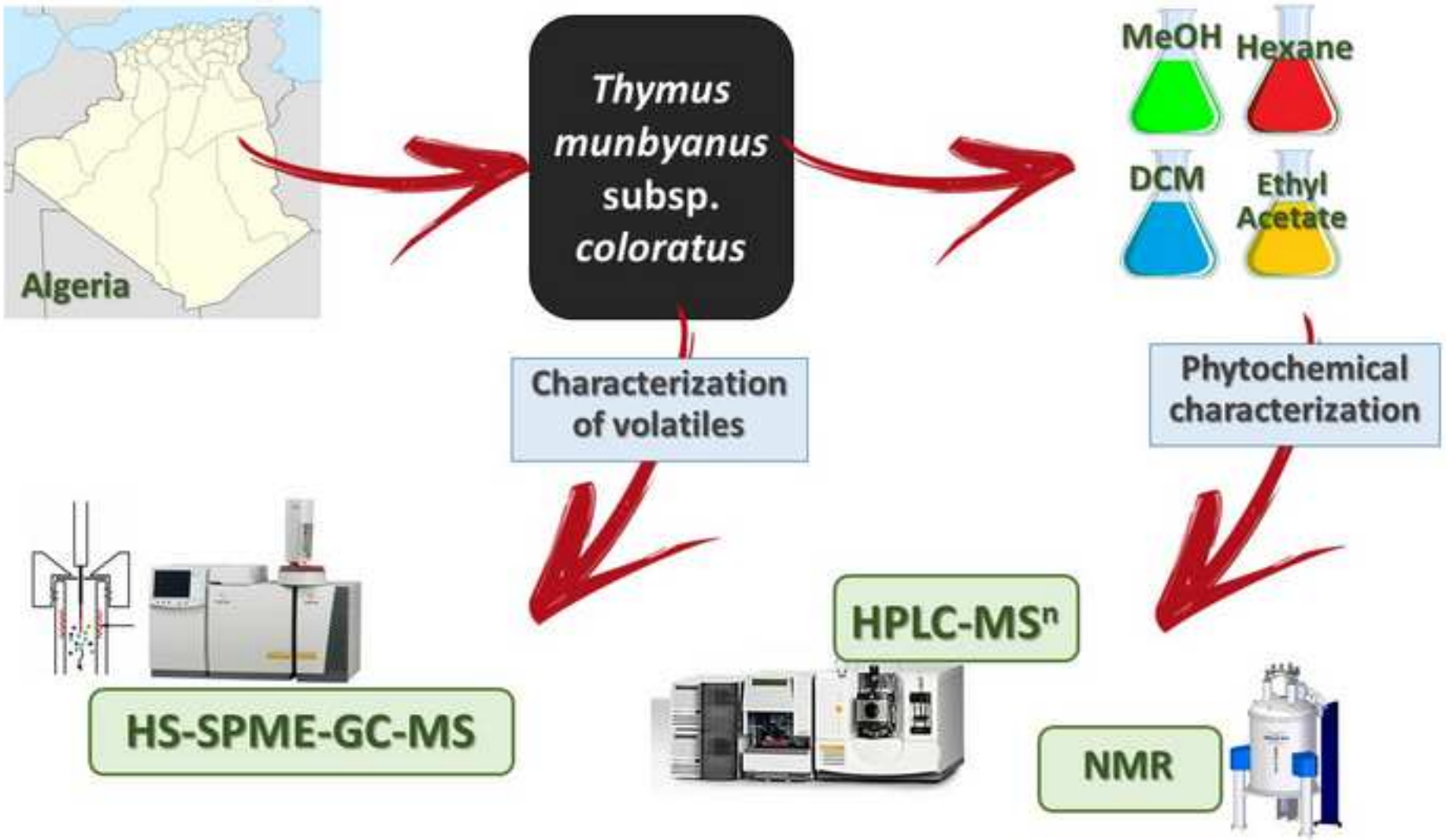


Figure 4. ^{1}H-NMR spectrum obtained from the analysis of the ethyl acetate extract of *Thymus munbyanus* subsp. *Coloratus*.



Total phytochemical analysis of *Thymus munbyanus* subsp. *coloratus* from Algeria by HS-SPME-GC-MS, NMR and HPLC-MS^n studies

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AUTHOR DECLARATION TEMPLATE

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from: stefano.dallacqua@unipd.it.

All the co-authors have read and approved this form.