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Essential oils (EOs), pressurized liquid extracts (PLE) and carbon dioxide supercritical fluid extracts (SFE-CO_2) from Algerian *Thymus munbyanus* as valuable sources of antioxidants to be used on an industrial level

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ABSTRACT

The aim of this study was to demonstrate the potential of extracts from Algerian *Thymus munbyanus* as a valuable source of antioxidants for use on an industrial level. To this end, a study was conducted on the composition and antioxidant activities of essential oils (EOs), pressurized liquid extracts (PLE) and supercritical fluid extracts (SFE-CO_2) obtained from *Thymus munbyanus* subsp. *coloratus* (TMC) and subsp. *munbyanus* (TMM). EOs and SFE-CO_2 extracts were analysed by GC-FID and GC × GC-TOFMS revealing significant differences. A successive extraction of the solid SFE-CO_2 residue by PLE extraction with solvents of increasing polarity such as acetone, ethanol and water, was carried out. The extracts were evaluated for total phenolic content by Folin–Ciocalteu assay, while the antioxidant power was assessed by DPPH, FRAP, and ORAC assays. SFE-CO_2 extracts were also analysed for their tocopherol content. The antioxidant activity of PLE extracts was found to be higher than that of SFE-CO_2 extracts, and this increased with solvent polarity (water > ethanol > acetone). Overall, these results support the use of *T. munbyanus* as a valuable source of substances to be used on an industrial level as preservative agents.

1. Introduction

Thymus (*Thymus* L.) is a large genus of the Lamiaceae family encompassing about 215 medicinal and aromatic species (Cronquist, 1988). Twenty species have been reported in Algeria (Quezel & Santa, 1963; Bousmaha-Marroki, Atik-Bekkara, Tomi & Joseph, 2007), including *T. munbyanus* Boiss. & Reut. which is considered as an endemic species in North Africa (Benchabane, Hazzit, Baaliouamer & Mouhouche, 2012). *T. munbyanus* is a small shrub, up to 30 cm tall, with tender, simple and small leaves, and branched and prostrated stems (Reddy, Angers, Gosselin & Arul, 1998). 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) (Fig. 1).

TMC is a subshrub, with flowers not exceeding 7–8 mm in length, and small floral bracts, purple-stained 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. It is found in lawns, rockeries and mountainous regions (Quezel & Santa, 1963).

Abbreviations: TMC, *Thymus munbyanus* subsp. *Coloratus*; TMM, *Thymus munbyanus* subsp. *Munbyanus*; AAPH, 2,2-azobis-(2-amidino-propane) dihydrochloride; AA, antioxidant activity; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; DWP, dry weight of plant material; DWE, Dry weight of extract; GAE, Gallic acid equivalents; H-ORAC, Hydrophilic-oxygen radical absorbance capacity; L-ORAC, Lipophilic-oxygen radical absorbance capacity; EOs, essential oils; PLE, Pressurized liquid extraction; ASE, accelerated solvent extraction; SFE-CO_2, Supercritical fluid extraction; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; TPC, Total phenolic content; GC-FID, Gas chromatography-Flame ionization detector; FRAP, Ferric reducing antioxidant power; HPLC, high performance liquid chromatography; GC × GC-TOFMS, Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry; AC, acetone; ET, ethanol; W, water

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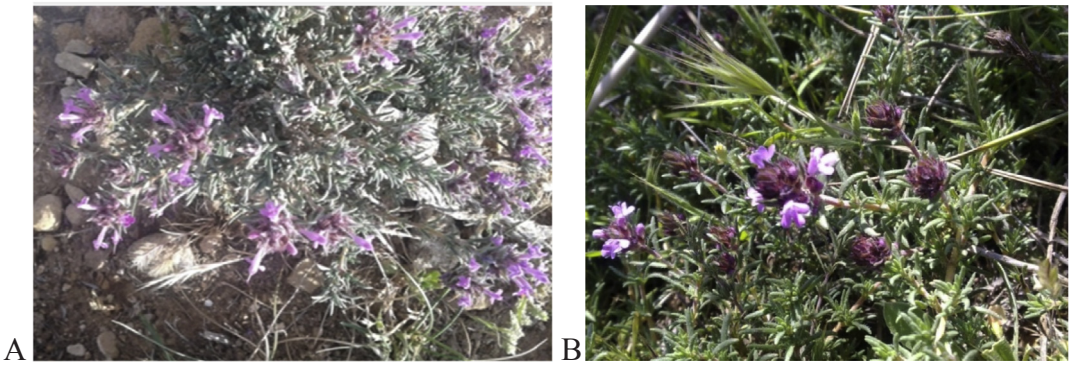

Fig. 1. A Thymus *munbyanus* subsp. *coloratus* (TMC); B *Thymus munbyanus* subsp. *munbyanus* (TMM).

TMM, locally known as 'Zaatar', is a fragrant subshrub, with flowers 16–20 mm long, pooled in false whorls; leaves are more or less contracted, with their accompanying flowers being morphologically different from those inserted on the stem, which are generally wider at the base (Miara, Ait Hammou & Hadjadj Aoul, 2013).

The *Thymus* genus is a reservoir of aromatic species endowed with important biological activities, namely antimicrobial, antioxidant, digestive, anti-inflammatory, expectorant and anticancer effects (Nabavi et al., 2015). The leaves and blooming aerial parts of thyme are widely employed as culinary ingredients, as well as in the brewing of herbal teas drunk to treat colds, coughs, sore throats, cystitis, insomnia, bronchitis and indigestion (Stahl-Biskup & Saez, 2002). Thyme is also used as a food preservative because of its strong antioxidant capacity documented by many studies. The main chemical constituents of thyme are 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) (Nabavi et al., 2015; Zheng & Wang, 2001; Ikeda, Murakami, & Ohigashi, 2008). In Algeria, *T. munbyanus* is used in the treatment of respiratory and gastrointestinal disorders and endocrine diseases (Miara et al., 2013). The composition of the essential oils (EOs) obtained by hydrodistillation (HD) from *T. munbyanus* has already been evaluated, and its antioxidant (Hazzit, Baaliouamer, Faleiro & Miguel, 2006; Benchabane et al., 2012) and antibacterial (Bousmaha-Marroki et al., 2007; Khadir et al., 2013) effects have been reported. However, to the best of our knowledge, there are no studies on the chemical composition and biological effects of other kinds of extracts from this species, for instance those prepared by pressurized liquid extraction (PLE) and supercritical fluid extraction with carbon dioxide (SFE-CO_2).

Yield, composition and biological effects of plant extracts are generally affected by the choice of solvent and extraction conditions employed (Goli, Barzegar & Sahari, 2004). SFE-CO_2 is a procedure industrially applied to recover lipophilic compounds (e.g. volatile oils) from plant matrices, avoiding thermal degradation and solvent residues. CO_2 is normally employed in SFE because of its safety, ease of use, chemical stability, availability and inexpensiveness. Thus, the aroma of the SFE-CO_2 extract is more closely related to the aroma of the botanical raw material than that of any classical extract (Oszagyh, Simhdi & Sawinsky, 1996).

PLE, also known as accelerated solvent extraction (ASE), is an extraction technique recently introduced for increasing the yield of analytes from solid materials. This technique also carries the advantages of reducing the time of extraction and decreasing the consumption of solvent.

In this study we focused on: (i) the study of the chemical composition of SFE-CO_2 extracts compared with EOs from the two different subspecies of *T. munbyanus*, i.e. TMC and TMM, and (ii) the evaluation of the influence of the extraction technique (i.e. SFE-CO_2 *vs* PLE) and solvent polarity on the antioxidant capacity of extracts and residue material obtained, which was assessed by DPPH, FRAP and ORAC methods. In our study, lipophilic fractions were isolated by SFE-CO_2, with the residues being further re-extracted with solvents of increasing polarity such as acetone, ethanol and water, by PLE. The antioxidant capacity of extracted products was evaluated by DPPH, FRAP and ORAC assays. Our work aimed to demonstrate the potential of *T. munbyanus* extracts on an industrial level as a valuable source of antioxidants.

2. Materials and methods

2.1. Solvents and chemicals

$FeCl_3{\cdot}6H_2O$ (> 99%) and sodium acetate (> 99%) were obtained from Acros Organics (Geel, Belgium); Tripyridyl-s-Triazine (TPTZ) and fluorescein (FL) were from Fluka Analytical (Bornem, Belgium); NaCl, KCl, Na_2HPO_4 and $K_2S_2O_8$ were bought from Merck (Darmstadt, Germany); KH_2PO_4 was purchased from Jansen Chimica (Beerse, Belgium); Na_2CO_3 (98%, anhydrous) was obtained from RPL (Grauwmeen, Belgium). Randomly methylated beta-cyclodextrin (RMCD) (Trappsol, pharmacy grade) was purchased from CTD Holdings, Inc. (High Springs, FL, USA); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azobis-(2-amidino-propane) dihydrochloride (AAPH), Folin-Ciocalteu phenol reagent (2 M), 3,4,5-trihydroxybenzoic acid (gallic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA); carbon dioxide (99.9%) was obtained from Gaschema (Jonava r., Lithuania); HPLC grade solvents for chromatographic analyses were bought from Sigma-Aldrich (Steinheim, Germany); analytical grade extractant solvent and HPLC grade solvents for chromatographic analysis were purchased from Chempur (Piekary Śląskie, Poland, Poland). A mixture of C_7–C_{30} *n*-alkanes (Supelco, Bellefonte, PA) was used for calculation of linear retention indices (RIs); pentane (> 99%) was bought from J.T. Baker, Avantor Performance Materials Inc., USA.

2.2. Plant material

Aerial parts (stems and leaves) of TMC growing in Bordj Bou Arreridj (North-East Algeria, N 36°06′; E 04°76′, collection: April 2014) and TMM growing in Tiaret (S35°.23′; W 1°.20′, collection: April 2015) were washed with water, then dried at ~22 °C in the dark for one week. Botanical determination was performed by Dr. M.D. Miara using available literature (Quezel & Santa, 1963). Dry material was ground by an ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using a sieve with 0.2 mm hole size.

2.3. Extraction

2.3.1. Supercritical fluid extraction (SFE-CO_2)

SFE-CO_2 was carried out under 99.9% carbon dioxide in a Helix system (Applied Separation, PA, USA) using 65 g of plant powder, which was loaded into a 50 cm^3 cylindrical extractor, of 14 mm inner diameter and 320 mm length. The analytical conditions employed were as follows: time 210 min (including 30 min of static extraction time), pressure 45 MPa, temperature 70 °C, and flow rate of CO_2 2 L/min. These parameters had been found to ensure a higher extraction yield, as per our previous investigation (Kemzūraitė, Venskutonis, Baranauskiene & Navikiene, 2014). The SFE-CO_2 extracts were collected in glass vials, weighed, transferred into opaque bottles and kept refrigerated until chemical analysis and antioxidant assays could be carried out.

2.3.2. Pressurized liquid extraction (PLE)

PLE was carried out in a Dionex ASE 350 system (Dionex, Sunnyvale, CA, USA) from the residues remaining after the SFE-CO_2 process. Twenty g of plant powder were mixed with 5 g of diatomaceous earth (4:1) in stainless–steel cells. A range of solvents of increasing polarity, namely acetone (AC), ethanol (ET, 96%) and water (W), was used for extraction. Extraction was performed at 70 °C with AC and ET, and 120 °C with W, keeping the pressure at 10.3 MPa for a total time of 15 min. Solvents were removed in a rotary evaporator under vacuum at 40 °C, and the extract was subsequently freeze dried. After solvent evaporation, the extracts were stored in dark vials under nitrogen flow at −18 °C for 20 min.

2.3.3. Hydrodistillation

Powders of TMC and TMM (200 g in each case) were subjected to hydrodistillation in a Clevenger-type apparatus using 2.5 L of deionized water. The obtained EOs were decanted, separated from the water and dehydrated with anhydrous sodium sulphate. Hydrodistillation was performed twice for each subspecies. The EOs were kept at −20 °C before use. The EO yields were calculated on a dry weight basis (w/w).

2.4. Gas chromatographic analyses

2.4.1. GC-FID

The SFE-CO_2 extracts of TMC and TMM and the hydrodistilled essential oils were diluted in pentane (5 mg/mL) and analysed on a PerkinElmer Clarus 500 Gas chromatograph (Shelton, USA) equipped with a flame ionization detector (FID) and an Elite-5 (5% diphenyl, 95% dimethylpolysiloxane) fused silica capillary column, 30 m length, 0.25 mm i.d., 0.25 μm film thickness (Perkin Elmer, Shelton, USA). The carrier gas was helium with an inlet pressure of 15 psi at 50 °C, which was equivalent to a 1.3 mL/min volumetric flow. The detector temperature was 300 °C. The temperature programme was as follows: from 50 °C (2 min) to 280 °C (10 min) at a rate of 5 °C/min. A split/splitless injector was used at 260 °C in split mode at a ratio of 1:10; the injection volume was 1 μL. Quantitative data were obtained by peak area normalization without using correction factors. The mean values were calculated from quadruplicate injections.

2.4.2. GC × GC-TOFMS analysis

The composition of EOs and SFE-CO_2 extracts was analysed on a Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry (GC × GC-TOFMS) LECO Pegasus 4D system, consisting of an Agilent 7890A GC system, a GERSTEL Multipurpose Sampler MPS (Gerstel GmbH, Mulheim an der Ruhr, Germany), a high-speed TOFMS detector (LECO, St. Joseph, MI, USA) and a four jet cryogenic modulator (Zoex, Houston, TX) by comparing the 1D first dimension linear temperature programmed retention index, with the peak identities provided by a mass spectral similarity search. The chromatographic system was made up of a primary column BPX-5 (30 m, 0.25 mm i.d., 0.25 μm film thickness) (SGE Analytical Science, Australia) linked with a secondary column, BPX-50 (2.0 m, 0.10 mm i.d., 0.1 μm film thickness). The primary oven temperature programme was as follows: 50 °C (2 min) then ramped to 280 °C at 5 °C/min (hold 10 min); the secondary programme was the following: 65 °C (2 min) then ramped to 295 °C at 5 °C/min. The transfer line temperature was 250 °C. The GC injector port was kept at 280 °C with a desorption time of 5 min. The TOFMS acquisition rate was 10 spectra s, the mass range used for identification was from 30 to 550 *m/z* units. Detector voltage was set at 1550 V and ion source temperature of 250 °C. Data from the GC × GC-TOFMS system was collected by ChromaTOF software v.4.22 (LECO) after a solvent peak delay of 360 s, split ratio was set 1:20; mass spectrum assignment was based on matching against Adams, NIST, MainLib, and Replib mass spectral libraries; signal-to-noise threshold was set at 50 with the minimum similarity accepted was 750. The identity of the components was assigned by comparison of their linear retention indices (as Kovats indices, KIs) relative to C_7–C_{30} *n*-alkanes, obtained on an Elite-5 capillary column, with those reported in Adams library (Adams, 2007) and by comparing their MS with those of Adams, NIST (NIST 08, 2008), Mainlib, and Replib mass spectral libraries. Furthermore, a home-made spectral library was used as well.

2.5. Determination of tocopherols content

Quantitative determination of tocopherols in the SFE-CO_2 extracts (1% in acetonitrile:methanol:dichloromethane) was achieved by following an HPLC method developed by Jolanta and Jerzy (2007) with minor changes. A Perkin Elmer Series 200 HPLC system was equipped with a C30 reverse-phase column and thermostatted at 30 °C (particle size 5 μm, 250 × 4.6 mm) applying an isocratic elution with acetonitrile:methanol:dichloromethane (110/360/30). The injection volume was 25 μL and flow rate was 1 mL/min. Tocopherols were analysed using a fluorescence detector at 290 nm excitation and 330 nm emission. The analytes eluted in 20 min: α-tocopherol at 12.2 min, β-tocopherol at 10.6 min, γ-tocopherol at 10.2 min and δ-tocopherol at 8.9 min. Tocopherols were identified by comparison of retention time of peaks with those of analytical standards whose solutions were prepared at different concentrations (0–10 μg/mL) using a mobile phase. Their quantitative determination was obtained from the calibration curves. Analyses were repeated three times.

2.6. Assessment of in vitro antioxidant activity

The majority of antioxidant assays are based on electron or hydrogen atom transfer. DPPH, TPC assay with Folin–Ciocalteu reagent, and FRAP assays are based on a single electron transfer, though the quenching of $DPPH^{\cdot}$ to form DPPH-H is also possible. These assays were performed for evaluating the antioxidant activities of extracts. Three replicate measurements were carried out for each of the three samples (n = 3) of each extract. All data were expressed as means ± standard deviations (SD). TEAC was expressed in mg of trolox equivalents per g dry weight plant material (DWP) and extract (DWE). These values refer to the activity of extracts obtained by using different solvents, and to the potential of the whole plant material, respectively.

2.6.1. Total phenolic content (TPC)

The TPC was determined in the SFE-CO_2 and PLE extracts of TMC and TMM using a method established by Singleton and Rossi (1965) with slight modifications. Thirty μL of samples (0.1%), water (blank) or gallic acid solutions (standard) were transferred to a 96-well microplate. Then, 150 μL of 1:10 diluted Folin-Ciocalteau reagent (2 M) were mixed thoroughly with distilled water. After 3 min of reaction, 120 μL of Na_2CO_3 (7.5%) were added. The absorbance was measured at 765 nm using a FLUOstar Omega (BMG LABTECH GmbH, Ortenberg, Germany) microplate reader across 90 min. The TPC was expressed as mg GAE/g DWE and mg GAE/g DWP, obtained from the standard curve

Table 1
Yields and total phenolic compounds in PLE and SFE-CO_2 from *Thymus munbyanus* subsp. *coloratus* (TMC) and *Thymus munbyanus* subsp. *munbyanus* (TMM).

Extracts		Yield %		TPC mg GAE/g			
		TMC	TMM	TMC		TMM	
				DWE	DWP	DWE	DWP
PLE	AC	6.5 ± 0.1[a]	2.7 ± 0.0[a]	141.4 ± 0.7[a]	9.2 ± 0.0[a]	121.1 ± 0.5[a]	3.3 ± 0.0[a]
	ET	1.1 ± 0.3[b]	3.9 ± 0.2[a]	164.7 ± 0.9[b]	1.9 ± 0.0[c]	174.1 ± 0.5[b]	6.8 ± 0.0[a]
	W	21.2 ± 0.6[c]	9.3 ± 0.1[b]	160.1 ± 1.8[b]	33.9 ± 0.4[e]	157.8 ± 1.6[c]	14.7 ± 0.1[b]
SFE-CO_2	0.4 ± 0.0[b]	0.4 ± 0.0[c]	nd	nd	nd	nd	

Values represented as mean ± standard deviation (n = 3); the same letter in each column means that the values are not significantly different each other ($P < 0.05$). DWE, dry weight of extract; DWP, dry weight of plant material. nd, not determined.

based on gallic acid (y = 7.2618x + 0.0724; R^2 = 0.99).

2.6.2. DPPH˙ scavenging

DPPH˙ scavenging capacity was assessed following the procedure used by Brand-Williams, Cuvelier & Berset (1995), with minor changes. Fifty µL of the samples (0.1%) were added to 200 µL of DPPH˙ solution in a 96-well microplate. Appropriate control was prepared using the solvent in addition to the DPPH˙ reagent to get rid of any inherent solvent activity. Trolox solutions (50 µL) at various concentrations (0–100 µM/L MeOH) were used for calibration.

The absorbance of each solution was determined at 517 nm using a FLUOstar Omega (BMG LABTECH GmbH, Ortenberg, Germany) microplate reader. After 40 min, the free radical-scavenging activity of each solution was calculated as % inhibition according to the following equation: % inhibition = 100 (A_{blank}-A_{sample})/A_{blank}. Results were expressed in mg TE/g (DWE and DWP). The concentration of the extract resulting in 50% inhibition (IC_{50}) was calculated using the graph by plotting inhibition percentage against extract concentration. $TEAC_{DPPH}$ was calculated by a dose–response curve for Trolox (y = 0.6011x + 2.302; R^2 = 0.99).

2.6.3. Ferric reducing antioxidant power (FRAP) assay

The total antioxidant activity of the extracts was measured using a FRAP assay (Benzie & Strain, 1996). FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mM; pH 3.6), 2.5 mL TPTZ solution (10 mM TPTZ (Fluka) in 40 mmol/L HCl) and 2.5 mL of 20 mM $FeCl_3$ water solution, in the ratio of 10:1:1 (v:v:v) mixed at time of use. The FRAP reagent was warmed to 37 °C before use. Fifty µL of each sample (0.1%), blank or standard were added to 200 µL of FRAP reagent for each well of the 96-well microplate. Absorbance was measured using a plate reader (595 nm) over 40 min. Various concentrations of trolox (0–500 µM/L) were used for calibration. Results were expressed in mg TE/g DWE and DWP. $TEAC_{FRAP}$ was calculated by a dose–response curve for Trolox (y = 0.0066x + 0.0275; R^2 = 0.99).

2.6.4. Hydrophilic-oxygen radical absorbance capacity (H-ORAC) assay

A slightly modified ORAC method (Prior et al., 2003) was performed. Twenty-five µL of sample (0.1%), PBS (blank) or standard (Trolox: at various concentrations 0–500 µM/L PBS) for calibration, were added with 150 µL of FL solution to the 96-well black opaque microplates using a multichannel pipette, and preincubated at 37 °C for 15 min, followed by a rapid addition of 25 µL of AAPH solution (240 mmol/L). After the adjustment of the blank for a few min, the microplate was immediately placed in the FLUOstar Omega reader (BMG Labtech, Offenburg, Germany), and the fluorescence recorded every min for 90 min using a microplate reader at 485–520 nm. Raw data were exported from the Fluostar Omega software to an Excel sheet for further calculations. Antioxidant curves (fluorescence versus time) were first normalised, and from these normalised curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + sum(f1/f0 + f2/f0 + f3/f0 + f4/f0 + f5/f0 + f6/f0 + f7/f0 + \cdots + fi/f0)$$

Where AUC: area under the FL decay curve (X). F0: fluorescence reading at cycle 0 (initial fluorescence), Fi: fluorescence reading at cycle i,

Net AUC (standard + sample): AUC-AUC blank, Net AUC: net area under the FL decay curve (X). This was obtained by subtracting the AUC of the blank from that of a sample.

$TEAC_{H\text{-}ORAC}$ values were expressed as µM TE/g of DWE and DWP.

$TEAC_{H\text{-}ORAC}$ was calculated by means of a dose–response curve for Trolox (y = 0.2573x − 1.0861; R^2 = 0.99).

2.7. Statistical analysis

All measurements were repeated three times and means ± SD obtained using GraphPad Prism 6. To examine the differences between groups, one-way ANOVA and Tukey's multiple comparisons test were carried out. A p value < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Extraction yields

The effects of solvents on extract yield are reported in many studies (El Hadj Ali, Bahri, Chaouachi, Boussaïd, & Harzallah-Skhiri, 2014). In order to test the optimum extraction conditions for TMC and TMM, consecutive applications of solvents with increasing polarity, such as AC, ET, and W, were used for PLE. The yields (%, w/w) of different extracts obtained from TMC and TMM through both SFE-CO_2 and PLE methods are presented in Table 1. In PLE the yield was highly dependent on the solvent used. The efficiency of SFE-CO_2 was lower than that of the other method. For PLE, the W extract showed the highest yields, with 21.2 and 9.3% for TMC and TMM, respectively, followed by ET and AC. The yields of SFE-CO_2 extracts in our study were very low (0.35 and 0.43%, respectively) probably due to the precipitation of waxes in the first separator at 45 MPa and 70 °C. Thus, there was a significant difference in the yields obtained using different extraction methods. In general, yields of TMC extracts were higher than those of TMM. Statistically, all differences detected were significant ($p < 0.05$).

3.2. Comparison of EOs and SFE-CO_2 extracts

The chemical profiles of EOs and SFE-CO_2 extracts of TMC and TMM were determined using GC-FID and GC × GC-TOFMS (Table 2). Hydrodistillation of the arial parts of TMC and TMM gave yields of 0.11 and 0.09%, respectively. These values are lower than those previously reported for flowering aerial parts of *T. munbyanus* from Algeria (1.8–3%) (Hazzit et al., 2006; Tefiani et al., 2015).

In total, 119 and 69 constituents representing 95.3 and 94.4% of the

Table 2
Chemical composition of EOs and SFE-CO_2 from *Thymus munbyanus* subsp. *coloratus* (TMC) and *Thymus munbyanus* subsp. *munbyanus* (TMM).

No	Compound[a]	RI Calc.[b]	RI Lit.[c]	EO (%)[d]		SFE-CO_2 (%)[d]		ID[e]
				TMC	TMM	TMC	TMM	
1.	1-Penten-3-ol	673	689	0.1				RI,MS
2.	1-Hexen-3-ol	767	776		0.1			RI,MS
3.	α-Thujene	927	930		0.1			RI,MS
4.	α-Pinene	934	939	1.0	2.7			RI,MS
5.	Camphene	949	954	0.1	1.2			RI,MS
6.	Thuja-2,4(10)-diene	955	960	0.5	0.1	0.1		RI,MS
7.	Sabinene	964	975	0.1				RI,MS
8.	1-Octen-3-ol	973	979	tr[b]	0.3			RI,MS
9.	β-Pinene	977	979	0.4	1.0			RI,MS
10.	2-Pentyl furan	987	988	0.1				RI,MS
11.	3-Octanol	994	991	0.1	0.1			RI,MS
12.	Myrcene	990	991	1.2	1.4			RI,MS
13.	α-Phellandrene	1002	1002	0.1				RI,MS
14.	δ-3-Carene	1010	1011	tr				RI,MS
15.	α-Terpinene	1015	1017	0.3	0.4			RI,MS
16.	*p*-Cymene	1024	1024	0.6	1.1			RI,MS
17.	Limonene	1028	1029	0.6	3.2			RI,MS
18.	1,8-Cineole	1032	1031	0.6	1.2			RI,MS
19.	Benzene acetaldehyde	1047	1042	0.3				RI,MS
20.	(*E*)-β-ocimene	1050	1050	0.1	0.7			RI,MS
21.	γ-terpinene	1059	1059	0.5	1.0			RI,MS
22.	*cis*-Sabinene hydrate	1068	1070	0.6				RI,MS
23.	*cis*-Linalool oxide	1073	1072	0.8				RI,MS
24.	*trans*-Linalool oxide	1084	1086	0.5				RI,MS
25.	Terpinolene	1087	1088	0.7	0.2			RI,MS
26.	*p*-Cymenene	1089	1091	0.5				RI,MS
27.	Linalool	1097	1096	2.7	4.0	0.1		RI,MS
28.	*trans*-sabinene hydrate	1100	1098	0.1	0.1			RI,MS
29.	*n*-Nonanal	1103	1100	0.2				RI,MS
30.	6-Methyl-3,5-heptadien-2-one	1107	1110	0.3		0.6		RI,MS
31.	1,3,8-*p*-menthatriene	1111	1110	0.1				RI,MS
32.	6-Camphenol	1115	1113	0.1				RI,MS
33.	*trans*-*p*-Mentha-2,8-dien-1-ol	1122	1122	0.6	0.9			RI,MS
34.	α-Campholenal	1120	1126	1.4	0.6	0.1		RI,MS
35.	*cis*-*p*-Mentha-2,8-dien-1-ol	1132	1137	tr	0.3			RI,MS
36.	*trans*-Pinocarveol	1141	1139	1.8	0.5			RI,MS
37.	*trans*-Verbenol	1137	1140	0.3				RI,MS
38.	(*E*)-Sabinol	1139	1142	3.5	1.5	0.3		RI,MS
39.	Camphor	1149	1146	11.7	7.6	1.1		RI,MS
40.	*neoiso*-3-Thujanol	1152	1151	0.6				RI,MS
41.	Sabina ketone	1159	1159	0.1				RI,MS
42.	*p*-vinyl Anisole	1155	1160	0.2				RI,MS
43.	*trans*-Pinocamphone	1163	1162	0.1				RI,MS
44.	Pinocarvone	1166	1164	1.4	0.3			RI,MS
45.	Borneol	1169	1169	3.0	5.6			RI,MS
46.	*p*-Mentha-1,5-dien-8-ol	1176	1170	0.1				RI,MS
47.	Terpinen-4-ol	1015	1174	1.5	10.6	0.2		RI,MS
48.	*p*-methyl-Acetophenone	1181	1182	0.1				RI,MS
49.	*p*-cymen-8-ol	1186	1182	0.8	0.8	0.3		RI,MS
50.	Cryptone	1188	1185	0.2				RI,MS
51.	α-Terpineol	1191	1188	1.3	3.7		0.2	RI,MS
52.	Myrtenal	1193	1195	0.2	0.1			RI,MS
53.	Myrtenol	1194	1195			0.3		RI,MS
54.	*p*-Cymen-9-ol	1197	1205	2.8	0.9	0.5		RI,MS
55.	Verbenone	1211	1205	1.8	0.6	0.3		RI,MS
56.	(*E*)-Piperitol	1205	1208	0.1	0.2			RI,MS
57.	*trans*-Carveol	1218	1216	1.9	1.5	0.3		RI,MS
58.	Linalool formate	1223	1216	0.1				RI,MS
59.	*cis*-Carveol	1229	1229	0.1	0.1			RI,MS
60.	Neral	1233	1229	0.2				RI,MS
61.	Thymol methyl ether	1231	1235				0.1	RI,MS
62.	Cumin aldehyde	1242	1241	0.2				RI,MS
63.	Carvone	1247	1243	0.7	0.8			RI,MS
64.	Geraniol	1256	1252	7.5	3.8	1.4	0.4	RI,MS
65.	Geranial	1271	1267	0.3	0.2	0.2		RI,MS
66.	*iso*-3-Thujanol acetate	1278	1270	0.1	0.2			RI,MS
67.	Perilla aldehyde	1276	1271	tr				RI,MS
68.	*neoiso*-3-Thujanol acetate	1287	1283	0.3				RI,MS
69.	Thymol	1294	1290	0.9	0.5	1.5	0.3	RI,MS
70.	Perilla alcohol	1300	1295	0.2				RI,MS
71.	Carvacrol	1302	1299	0.3		0.3		RI,MS
72.	Myrtenyl acetate	1325	1326	0.2				RI,MS

(*continued on next page*)

Table 2 (*continued*)

No	Compound[a]	RI Calc.[b]	RI Lit.[c]	EO (%)[d]		SFE-CO_2 (%)[d]		ID[e]
				TMC	TMM	TMC	TMM	
73.	*p*-Mentha-1,4-dien-7-ol	1332	1327	tr				RI,MS
74.	Verbenol acetate	1346	1343	0.2				RI,MS
75.	β-Terpinyl acetate	1344	1349	5.1	4.8	0.5	0.2	RI,MS
76.	Neryl acetate	1362	1361	0.1		0.1		RI,MS
77.	(*E*)-*p*-Menth-6-en-2,8-diol	1375	1374			1.0		RI,MS
78.	α-Copaene	1378	1376	0.5	0.1		0.2	RI,MS
79.	Geranyl acetate	1381	1381	6.3	2.4	1.9	0.4	RI,MS
80.	β-Bourbonene	1387	1388	0.6	0.2	0.2	0.4	RI,MS
81.	(*Z*)-caryophyllene	1408	1408	0.2				RI,MS
82.	α-Gurjunene	1412	1409	0.1	0.1			RI,MS
83.	(*E*)-Caryophyllene	1423	1419	0.5	0.4		11.2	RI,MS
84.	β-Ylangene	1420	1420	0.1				RI,MS
85.	Carvone hydrate	1424	1423			1.0	0.2	RI,MS
86.	β-Copaene	1433	1432	0.1			0.1	RI,MS
87.	γ-Elemene	1436	1436	tr				RI,MS
88.	Aromadendrene	1449	1441	0.1			0.1	RI,MS
89.	α-Humulene	1455	1454	0.1		0.9	2.0	RI,MS
90.	Geranyl acetone	1454	1455	0.1				RI,MS
91.	*allo*-Aromadendrene	1466	1460	0.2	0.1		0.3	RI,MS
92.	γ-Muurolene	1479	1479	0.1			0.2	RI,MS
93.	Germacrene D	1485	1484	0.9	0.5		3.1	RI,MS
94.	α-Amorphene	1486	1484			0.1		RI,MS
95.	(*E*)-Muurola-4(14),5-diene	1492	1493				0.2	RI,MS
96.	α-Selinene	1501	1498	0.1				RI,MS
97.	α-Muurolene	1494	1500				0.5	RI,MS
98.	β-Bisabolene	1507	1505	0.1				RI,MS
99.	δ-Amorphene	1517	1512	0.3				RI,MS
100.	*endo*-1-Bourbanol	1518	1520	0.2		1.3		RI,MS
101.	(*E*)-Calamenene	1509	1522	0.3	0.2		0.4	RI,MS
102.	Dihydroactinidiolide	1520	1522			0.2	0.3	RI,MS
103.	δ-Cadinene	1518	1523		0.4	0.3	0.5	RI,MS
104.	γ-Cadinene	1524	1523	0.2				RI,MS
105.	(*Z*)-Nerolidol	1539	1532			0.3		RI,MS
106.	α-Cadinene	1532	1538				0.5	RI,MS
107.	α-Calacorene	1548	1545	0.3				RI,MS
108.	(*E*)-Nerolidol	1562	1563	3.8	13.7	2.0	3.0	RI,MS
109.	1-nor-Bourbanone	1569	1563	0.8	0.4	0.6	0.2	RI,MS
110.	8-Acetoxy-carvotanacetone	1567	1565		0.5	1.8	0.3	RI,MS
111.	Palustrol	1574	1568	0.5				RI,MS
112.	Spathulenol	1577	1578	1.5	0.4	0.9	1.3	RI,MS
113.	Caryophyllene oxide	1591	1583	4.5	2.4	1.9	4.1	RI,MS
114.	Eudesm-7(11)-en-4-ol	1659	1589		0.2			RI,MS
115.	β-Copaen-4-α-ol	1595	1590	0.1				RI,MS
116.	Viridiflorol	1597	1592	0.2			0.2	RI,MS
117.	*n*-Hexadecane	1600	1600	0.3		0.2	0.1	RI,MS
118.	Ledol	1611	1602	0.3	0.1			RI,MS
119.	Tetradecanal	1609	1608			0.6	0.2	RI,MS
120.	Humulene epoxide II	1617	1608	0.8			0.9	RI,MS
121.	1,10-*di*-*epi*-Cubenol	1621	1619		0.5			RI,MS
122.	Caryophylla-4(12),8(13)-dien-5-ol	1636	1640	tr				RI,MS
123.	*epi*-α-Muurolol	1647	1640	0.9	3.0			RI,MS
124.	*allo*-Aromadendrene epoxide	1641	1641	0.1		0.1		RI,MS
125.	tau-Muurolol	1651	1642			0.5		RI,MS
126.	α-Muurolol	1638	1646			0.5	1.7	RI,MS
127.	β-Eudesmol	1659	1650	0.9	0.5	1.4	0.9	RI,MS
128.	α-Cadinol	1664	1654	0.4	0.6	0.1	0.1	RI,MS
129.	Intermedeol	1664	1666	1.5	1.0	0.8	0.1	RI,MS
130.	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	1665	1668		0.1			RI,MS
131.	(*E*)-Calamenen-10-ol	1675	1669	tr				RI,MS
132.	Cadalene	1678	1676	0.6	0.2		0.1	RI,MS
133.	Mustakone	1682	1677	0.2		0.5		RI,MS
134.	Germacra-4(15),5,10(14)-trien-1-α-ol	1695	1686	0.2	0.1	0.2		RI,MS
135.	*n*-Heptadecane	1700	1700				0.2	RI,MS
136.	Eudesm-7(11)-en-4-ol	1700	1700	0.2				RI,MS
137.	Cedroxyde	1720	1713			0.2		RI,MS
138.	Cryptomerione	1724	1724		0.1			RI,MS
139.	Oplopanone	1739	1740			1.5	0.2	RI,MS
140.	Mint sulfide	1738	1741				0.3	RI,MS
141.	Cyclocolorenone	1770	1760	0.1				RI,MS
142.	β-Bisabolenal	1769	1769			0.2		RI,MS
143.	*n*-Pentadecanol	1772	1774			0.6		RI,MS
144.	14-Hydroxy-α-muurolene	1777	1780	0.2	0.1	1.1	0.4	RI,MS
145.	β-Bisabolenol	1786	1789		0.9		0.1	RI,MS

(*continued on next page*)

Table 2 (*continued*)

No	Compound[a]	RI Calc.[b]	RI Lit.[c]	EO (%)[d]		SFE-CO_2 (%)[d]		ID[e]
				TMC	TMM	TMC	TMM	
146.	*n*-Octadecane	1800	1800				0.1	RI,MS
147.	Hexadecanol	1862	1875				0.8	RI,MS
148.	Methyl hexadecanoate	1914	1921				0.1	RI,MS
149.	Manool oxide	1994	1987	0.2		0.3	0.1	RI,MS
150.	*n*-Eicosane	2002	2000			0.1		RI,MS
151.	Abietatriene	2057	2056	0.1		0.2	0.1	RI,MS
152.	*n*-Octadecanol	2083	2077				0.1	RI,MS
153.	*trans*-Phytol	2104	2103	0.2	0.1	1.6	3.9	RI,MS
154.	α-Linolenic acid	2147	2143				0.2	RI,MS
155.	*n*-Docosane	2200	2200			0.7		RI,MS
156.	(*E*)-Phytol acetate	2221	2218			0.2	0.3	RI,MS
157.	*Z*-9-Octadecenamide	2392	2397		0.1			RI,MS
158.	*n*-Tetracosane	2400	2400			0.3	0.2	RI,MS
159.	*n*-Hexacosane	2600	2600			0.4	0.9	RI,MS
160.	*n*-Heptacosane	2700	2700				2.5	RI,MS
161.	*n*-Heptacosane	2700	2700			1.0		RI,MS
162.	*n*-Octacosane	2800	2800			3.5	3.5	RI,MS
163.	Squalene	2853	2847			10.8	11.4	RI,MS
164.	*n*-Nonacosane	2900	2900			1.2	0.2	RI,MS
165.	*n*-Triacontane	3000	3000			6.5	8.9	RI,MS
166.	*n*-Untriacontane	3100	3100			1.0		RI,MS
	Total identified (%)			95.3	94.4	58.9	69.5	
	Yield (%)			0.11	0.09	0.35	0.43	
	Identified compounds			119	69	61	57	
	Grouped compounds (%)							
	Monoterpene hydrocarbons			6.8	13.1	0.1	0	
	Oxygenated monoterpenes			63.5	53.8	11.4	1.8	
	Sesquiterpene hydrocarbons			5.4	2.2	1.5	19.8	
	Oxygenated sesquiterpenes			17.4	24.6	16.1	13.8	
	Diterpenes			0.5	0.1	2.3	4.4	
	Triterpenes					10.8	11.4	
	Alkanes			0.3	0	14.9	16.6	
	Alcohols			0.2	0.5	0.6	0.9	
	Aldehydes and ketones			0.2	0	0.6	0.2	
	Aromatics			0.6	0	0	0	
	Others			0.4	0.1	0.6	0.6	

[a] Compounds are listed in order of their elution from an Elite-5 capillary column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes.
[c] Linear retention index taken from Adams (2007).
[d] Relative percentage values are means of three determinations with a RSD% in all cases below 18%.
[e] Identification methods: MS, based on comparison with NIST, Mainlib, Replib and Adams libraries; RI, based on comparison of calculated RI with those reported in ADAMS and NIST.

integrated peaks were identified in the oils of TMC and TMM, respectively. These numbers were generally higher than those reported in previous studies on *T. munbyanus* from Algeria, where the number of constituents identified ranged from 45 to 78 (Hazzit et al., 2006; Khadir et al., 2013). We assume that a more sensitive chromatographic system (GC × GC-TOFMS) allowed the detection of a higher number of peaks, together with constitutive differences of the samples related to genetics and environmental factors.

The EOs of TMC and TMM were characterized by a high content of oxygenated monoterpenes (63.5 and 53.8%, respectively) followed by oxygenated sesquiterpenes (17.4 and 24.6%, respectively) and monoterpene hydrocarbons (6.8 and 13.1%, respectively). On the other hand, sesquiterpene hydrocarbons (5.4 and 2.2%, respectively) formed a minor contribution in both subspecies.

The predominant volatile components in both subspecies were oxygenated monoterpenoids such as camphor (11.7%) and geraniol (7.5%) in TMC, and sesquiterpenoids and monoterpenoids such as (*E*)-nerolidol (13.7%), terpinen-4-ol (10.6%) and camphor (7.6%) in TMM. Other quantitatively important compounds were geranyl acetate (6.3%), β-terpinyl acetate (5.1%), and caryophyllene oxide (5.1%) in TMC, and borneol (5.6%), β-terpinyl acetate (4.8%) and linalool (4%) in TMM. These chemical profiles are rather different to those previously reported for TM (subspecies not specified), based on the populations growing in Algeria (Hazzit et al., 2006; Benchabane et al., 2012; Khadir et al., 2013; Tefiani et al., 2015). In these studies, carvacrol (35.2–80%), thymol (37.7–52%), and camphor (21–83%) were detected as the major volatile components.

The chemical profiles of TMC and TMM SFE-CO_2 extracts, consisting of 61 and 47 components, respectively, and representing 58.9–69.5% of the total GC peak area, were quite similar (Table 2). They were rich in long chain hydrocarbons such as squalene (10.8 and 11.4%, respectively) and *n*-triacontane (6.5 and 8.9%, respectively). It may be assumed that the volatile compounds in SFE-CO_2 extracts were diluted by the higher boiling point lipophilic constituents, with some volatiles being lost with the CO_2 gases in depressurizing the extractor (Šulniūtė, Baranauskienė, Ragažinskienė, & Venskutonis, 2017).

3.3. Tocopherol contents

Tocopherols are lipophilic molecules synthesized by plant cells and stored in leaves and seeds, and are endowed with antioxidant functions. They have multiple beneficial healthy effects, such as the prevention of cardiovascular diseases and cancer. In nature, there are four main forms, namely α-, β-, γ-, and δ-tocopherol. The principal analytical method used for their quantitative determination in plants, foods and vegetables is normal phase HPLC coupled with fluorescence detection

Table 3
Tocopherol content (μg/g DWE) in SFE-CO_2 from *Thymus munbyanus* subsp. *coloratus* (TMC) and *Thymus munbyanus* subsp. *munbyanus* (TMM).

Compound	TMC	TMM
α-tocopherol	1580 ± 0.04	780 ± 0.06
β-tocopherol	170 ± 0.07	140 ± 0.05
γ-tocopherol	220 ± 0.03	120 ± 0.01
δ-tocopherol	160 ± 0.01	130 ± 0.01

Values represented as mean ± standard deviation (n = 3), ($P < 0.05$).

(Jolanta and Jerzy, 2007). Using this method, the tocopherol content in SFE-CO_2 extracts of TMC and TMM was measured and results are shown in Table 3. The major tocopherol form occurring in both TMC and TMM extracts was α-tocopherol, with concentrations of 1580 and 780 μg/g DWE respectively, while δ-tocopherol and γ-tocopherol were at remarkably lower concentrations in TMC and TMM extracts. Thus, SFE-CO_2 extracts of TMC and TMM are potential sources of tocopherols, with a total content ranging from 120 to 1580 μg/g DWE.

3.4. Antioxidant activity of extracts

There are many *in vitro* antioxidant activity assays, however most of them are based on the transferral of a single electron or hydrogen atom (SET or HAT). Well-known experts in this area (Huang, Ou, & Prior, 2005) concluded that ORAC, TPC and one of the SET or HAT assays should be recommended for the representative evaluation of the antioxidant properties of biomaterials. Following this recommendation TPC, DPPH·, ABTS·+ scavenging assays and ORAC were used for the evaluation of TMC and TMM extracts obtained by different methods and solvents. It should also be noted that the mechanism of ORAC is based on the inhibition of the peroxyl-radical-induced oxidation and this use of a biologically relevant radical source can be considered an advantage (Prior et al., 2003). In addition, the DPPH assay is more applicable for hydrophilic compounds, while ABTS·+ is equally suitable both for lipophilic (better soluble in acetone) and hydrophilic (better soluble in water) antioxidants.

3.4.1. Total phenolic content (TPC)

The antioxidant activities of plants, foods and vegetables are strongly related to their polyphenol content. Therefore, it is important to consider the effects of the chosen extraction solvent on the TPC of fruit and vegetables. By studying the absorbance values of the extracts reacted with Folin-Ciocalteu reagent and comparing them with standard solutions of gallic acid, the TPC of TMC and TMM extracts were examined and variations in TPC values are shown in Table 1. The highest values of TPC in W extracts were 33.95 and 14.70 mg GAE/g DWP for TMC and TMM respectively, while AC extracts exhibited significantly lower TPC values.

Similar findings were reported for other botanicals such as *T. vulgaris* L., *Salvia officinalis* L. and *Origanum majorana* L. TPC for other *Thymus* species were as follows: *T. numidicus* Poir. (98.66 mg GAE/g DW of leaf methanolic extract) (El Hadj Ali et al., 2014), *T. carmanicus* Jalas (124.30 μg/mg) (Safaei-Ghomi, Ebrahimabadi, Djafari-Bidgoli & Batooli, 2009), *T. spathulifolius* Hausskn. & Velen. (141 μg/mg of the polar sub fraction of a methanol extract) (Atalay et al., 2004), *T. algeriensis* Boiss. & Reut. (18.73 mg/g DW of 70% ET extract) (Bakchiche, Gherib, Smail, Custódia, & Graca, 2013). In general, higher polarity solvents are more effective in recovering polyphenolic compounds: W > ET > AC. In addition, it should be noted that the TPC assay was not applied to SFE-CO_2 extracts due to the poor solubility of lipophilic compounds in reaction media. Most likely, SFE-CO_2 should be recommended for separating the lipophilic fraction from the herb before applying higher polarity solvents.

3.4.2. DPPH˙ scavenging assay

All tested TMC and TMM extracts showed antioxidant activities (Table 4). The DPPH scavenging capacity of plant extracts was dependent on solvent polarity and extraction method. Thus, W extracts of TMC and TMM showed the highest DPPH˙ scavenging activity, with an IC_{50} of 0.06 and 0.05 mg/mL, respectively: this was 5–6 times higher than that of the synthetic antioxidant Trolox (IC_{50} = 0.01 mg/mL). Lipophilic SFE-CO_2 extracts were remarkably weaker antioxidants with IC_{50} of 0.55 and 0.41 mg/mL for TMC and TMM, respectively. The data were also expressed as $TEAC_{DPPH}$ in mg TE/g DWP and DWE (Table 4). In general, the differences in $TEAC_{DPPH}$ values for ET, W and AC extracts were significant ($P < 0.05$) although not considerable, for all extracts in the range of 28.43–33.31 mg TE/g. The SFE-CO_2 extracts yielded $TEAC_{DPPH}$ values almost twofold lower. However, due to extraction yields (Table 1), the polyphenols recovered from DWP fell across a wide range: from 0.08 mg TE/g (SFE-CO_2 extract) to 6.33 mg TE/g (W extract). The differences observed in the $TEAC_{DPPH}$ assay were in agreement with the IC_{50} values.

Consequently, the radical scavenging activity of extracts may be ranked in the following order: W > AC ~ ET > SFE-CO_2; however there were no significant differences between the two subspecies (Table 4). TMC and TMM extracts exhibited higher DPPH˙ scavenging activity than in results found by Kholkhal, et al.. (2013) on *T. ciliatus* Lam. subsp. *coloratus*, and similar results to those found for *T. carmanicus* (Safaei-Ghomi et al., 2009), *T. quinquecostatus* Celak. *T. vulgaris* L., and *T. numidicus* Poir. (El Hadj Ali et al., 2014).

3.4.3. FRAP assay results

The FRAP assay gives a measure of the reducing ability of a substance in the reaction medium. Antioxidant potentials of TMC and TMM extracts were estimated for their ability to reduce the TPTZ-Fe (III) complex to a TPTZ-Fe (II) complex and expressed as $TEAC_{FRAP}$ in mg TE/g in DWP and DWE (Table 5). The $TEAC_{FRAP}$ values were in the following range: 30.93–66.77 mg TE/g DWE for TMC extracts, and 38.09–63.89 mg TE/g DWE for TMM extracts. Conversely, the DPPH assay estimated the highest $TEAC_{FRAP}$ for the SFE-CO_2 extracts, followed by AC, ET and W obtained by PLE from TMC, and W, ET, and AC

Table 4
DPPH· scavenging activities of *Thymus munbyanus* subsp. *coloratus* (TMC) and *Thymus munbyanus* subsp. *munbyanus* (TMM) extracts.

Extracts		$TEAC_{DPPH}$, mg TE/g				IC_{50}, mg/mL	
		TMC		TMM		TMC	TMM
		DWE	DWP	DWE	DWP		
PLE	AC	30.55 ± 0.11[a]	1.99 ± 0.01[a]	28.43 ± 0.17[a]	0.78 ± 0.00[a]	0.32	0.29
	ET	33.31 ± 0.04[b]	0.38 ± 0.00[b]	32.78 ± 0.04[b]	1.27 ± 0.00[b]	0.29	0.22
	W	29.84 ± 0.05[c]	6.33 ± 0.01[c]	31.23 ± 0.1[c]	2.91 ± 0.01[c]	0.06	0.05
SFE-CO_2		18.76 ± 0.21[d]	0.07 ± 0.00[d]	18.05 ± 0.05[d]	0.08 ± 0.00[d]	0.55	0.41

Values represented as mean ± standard deviation (n = 3); the same character in each column separately means that the values are not significantly different ($P < 0.05$). DWE, dry weight extract; DWP, dry weight plant material. IC_{50} of trolox = 0.01 mg/mL.

Table 5
FRAP and ORAC of *Thymus munbyanus* subsp. *coloratus* (TMC) and *Thymus munbyanus* subsp. *munbyanus* (TMM) extracts.

Extracts		TEAC $_{FRAP}$, mg TE/g				TEAC $_{H\text{-}ORAC}$, μM TE/g			
		TMC		TMM		TMC		TMM	
		DWE	DWP	DWE	DWP	DWE	DWP	DWE	DWP
PLE	AC	49.65 ± 0.00^{a}	3.24 ± 0.00^{a}	38.09 ± 1.81^{a}	1.04 ± 0.3^{a}	723.1 ± 42.4^{a}	47.2 ± 2.8^{a}	746.3 ± 8.2^{a}	20.4 ± 0.2^{a}
	ET	43.48 ± 0.98^{a}	0.5 ± 0.01^{b}	39.53 ± 0.28^{a}	1.54 ± 0.01^{b}	991.5 ± 23^{b}	11.4 ± 0.3^{b}	937.8 ± 26.8^{b}	36.5 ± 1.00^{b}
	W	30.93 ± 0.12^{b}	6.56 ± 0.03^{c}	50.07 ± 0.22^{b}	4.66 ± 0.02^{c}	871.9 ± 14.7^{c}	184.8 ± 3.1^{c}	958.3 ± 42.2^{b}	89.2 ± 3.9^{c}
SCE-CO_2		66.77 ± 0.21^{c}	0.23 ± 0.00^{d}	63.89 ± 1.94^{c}	0.27 ± 0.05^{d}	787.4 ± 16.8^{a}	2.8 ± 0.1^{d}	584.1 ± 2.6^{c}	2.5 ± 0.00^{d}

Values represented as mean ± standard deviation (n = 3); the same character in each column separately means that the values are not significantly different ($P < 0.05$).

from TMM. Lipophilic antioxidants (e.g., tocopherols, squalene) may be more effective in the FRAP assay, while polyphenolics are better at scavenging DPPH˙. Again, due to remarkably higher yields of W extracts, the latter was the most effective solvent for recovery of reducing substances in the FRAP assay when the values were expressed as mg TE/g DWP. Thus, the recalculated $TEAC_{FRAP}$ values for W extracts were 6.56 and 4.66 mg TE/g DWP, respectively, whereas low yield SFE-CO_2 extracts yielded $TEAC_{FRAP}$ values of 0.23 and 0.27 TE/g DWP, respectively). No significant differences were found in $TEAC_{FRAP}$ values between TMC and TMM extracts. These results confirm the link between the solvent polarity and the recovery of antioxidants.

3.4.4. H-ORAC assay results

The ORAC method, which is widely used for assessing the antioxidant activity of plant extracts, biological samples and foods, relies on the inhibition of peroxyl-radical-induced oxidation initiated by thermal decomposition of AAPH (Prior et al., 2003). As shown in Table 5, the highest $TEAC_{H\text{-}ORAC}$ values were found for ET extracts from TMC and TMM (991.5 and 958.3 μM TE/g DWE, respectively) whereas they were lower for the AC extract from TMC and SFE-CO_2 extract from TMM (723.1 and 584.1 μM TE/g DWE, respectively). Water was the most effective solvent for recovering the highest amounts of TE from DWP from both subspecies, as was the case for DPPH and FRAP assays, whereas the SFE-CO_2 gave the lowest values (Table 5). Our extracts exhibited higher $TEAC_{H\text{-}ORAC}$ values than found for extracts of *T. longicaulis* C. Presl which ranged between 414.14 and 776 μM Trolox Equivalents (Silvia et al., 2014).

4. Conclusions

Systematic studies of two medicinal and aromatic plants of Algeria, namely *T. munbyanus* subsp. *coloratus* and *T. munbyanus* subsp. *munbyanus* are reported here for the first time. Traditional hydrodistillation and supercritical fluid extraction revealed remarkable differences in the composition of volatile oil and lipiphilic SFE-CO_2 extracts; the former was mostly composed of typical terpenoids, whereas the latter contained higher amounts of long chain hydrocarbons and tocopherols. Pressurized liquid extraction (PLE) with solvents of increasing polarity, namely acetone, ethanol and water, produced extracts demonstrating various total phenolic content and antioxidant activities in different assays, though the differences were not too remarkable, up to 38% in a FRAP assay. However, due to remarkable differences in the total yield, water was the most effective in the recovery of antioxidants from the dried plant material, e.g. up to 89.2 mM TE/g in ORAC assay. These results support the need for further studies leading to wider industrial processing and application of the species studied, for use as natural preservatives and other functional ingredients.

Conflicts of interest

The authors declare no conflict of interest.

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