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Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link.

S. Bounatirou [a,*], S. Smiti [a], M.G. Miguel [b], L. Faleiro [b], M.N. Rejeb [c], M. Neffati [d], M.M. Costa [e], A.C. Figueiredo [e], J.G. Barroso [e], L.G. Pedro [e]

[a] *Faculté des Sciences de Tunis, Université Tunis el Manar, Campus Universitaire, 2092 Tunis, Tunisia*
[b] *Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal*
[c] *Institut National de Recherche en Génie Rural, Eaux et Forêts, 2080 Tunis, Tunisia*
[d] *Institut des Régions Arides, 4119 Mednine, Tunisia*
[e] *Universidade de Lisboa, Faculdade de Ciências de Lisboa, DBV, Centro de Biotecnologia Vegetal, C2, Campo Grande, 1749-016 Lisbon, Portugal*

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Abstract

The chemical composition, antioxidant and antibacterial activities of essential oils isolated by hydrodistillation from the aerial parts of Tunisian *Thymus capitatus* Hoff. et Link. during the different phases of the plant development, and from different locations, were evaluated. The chemical composition was analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). The main components of the essential oils were carvacrol (62–83%), *p*-cymene (5–17%), γ-terpinene (2–14%) and β-caryophyllene (1–4%). The antioxidant activity of the oils (100–1000 mg l^{-1}) was assessed by measurement of metal chelating activity, the reductive potential, the free radical scavenging (DPPH) and by the TBARS assay. The antioxidant activity was compared with that of synthetic antioxidants: butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Both the essential oils and BHA and BHT showed no metal chelating activity. Although with the other methodologies, there was a general increase in the antioxidant activity, with increasing oil concentration, maxima being obtained in the range of 500 and 1000 mg l^{-1} for flowering and post-flowering phase oils. Major differences were obtained according to the methodology of antioxidant capacity evaluation. Antibacterial ability of *Th. capitatus* essential oils was tested by disc agar diffusion against *Bacillus cereus*, *Salmonella* sp., *Listeria innocua*, four different strains of *Staphylococus aureus* (C15, ATCC25923, CFSA-2) and a multi-resistant form of *S. aureus* (MRSA-2). Antibacterial properties were compared to synthetic antibiotics. Higher antibacterial activity was observed with the flowering and the post-flowering phase essential oils.

Keywords: *Thymus capitatus*; Lamiaceae; Essential oils; Antioxidant activity; Metal chelating measurement; Reductive potential; DPPH; TBARS; Antibacterial activity; *Listeria innocua*; *Bacillus cereus*; *Salmonella* sp.; *Staphylococus aureus*

1. Introduction

In Tunisia, the genus *Thymus* (Lamiaceae) is mainly represented by *Thymus capitatus* Hoff. et Link. [=*Coridothymus capitatus* (L.) Rchb.f., *Satureja capitata* L., *Thymbra capitata* (L.) Cav.], a perennial, herbaceous shrub commonly used as a spicy herb and locally known under the common name "zaâtar". Until recently, *Thymus* essential oils have been studied, mostly from the viewpoint of their flavour and fragrance chemistry, only for flavouring foods. Nowadays, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use (Ormancey, Sisalli, & Coutiere, 2001; Sawamura, 2000).

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious

* Corresponding author. Tel.: +216 97402499; fax: +216 71871666.
E-mail address: saida_behi@yahoo.com (S. Bounatirou).

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effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is some preference for antioxidants from natural rather than from synthetic sources (Abdalla & Roozen, 1999). There is therefore, a parallel increase in the use of methods for estimating the efficiency of such substances as antioxidants (Sanchez-Moreno, 2002; Schwarz et al., 2001).

Many authors have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties of essential oils. Thyme essential oils were reported to have antimicrobial activities (Bhaskara, Angers, Gosselin, & Arul, 1998), most of which are mediated by thymol and carvacrol, as the phenolic components of the oil. Spasmolytic as well as antioxidant activities (Miguel et al., 2004; Sacchetti et al., 2005) were also reported for the phenolic oil extract of the plant. There is some evidence that minor components have a critical part to play in biological activities, possibly by producing a synergistic effect between other components. Several studies have focused on the antimicrobial activity of the essential oils of thyme in order to identify the responsible compounds. Phenols seem to play an outstanding role. These terpene phenols join to the amine and hydroxylamine groups of the proteins of the bacterial membrane altering their permeability and resulting in the death of the bacteria (Juven, Kanner, Schved, & Weisslovicz, 1994).

The aim of this work was to evaluate the chemical composition of Tunisian *Th. capitatus* essential oils, isolated by hydrodistillation from the aerial parts of plants collected during different phases and from three different localities, and to determine in which way this would affect the corresponding oils antioxidant and antibacterial activities.

2. Materials and methods

2.1. Plant material

Collective samples (approximately 30 g dry weight) of the aerial parts from *Thymus. capitatus* Hoff. et Link. growing wild in Tunisia, were collected during the vegetative (January, 2005), the flowering and the post-flowering phases (July–August, 2005) from three different localities: Jendouba (interior north), Haouaria (littoral north) and Aîn Tounine (littoral south). Depending on the amount of the plant material available, the results are the mean values of one sample to three replicas of plant material that was collected at each stage. The material was dried in the dark at room temperature before extraction.

2.2. Essential oil extraction

Depending on the amount of the plant material available, essential oil was obtained from a total of 16 samples (Table 1), by hydrodistillation during 3 h using a Clevenger-type apparatus (European Pharmacopoeia, 1996). The essential oils were kept at −4 °C until analysis.

2.3. Essential oils analysis

2.3.1. Gas chromatography

Gas chromatographic analyses were performed using a Perkin–Elmer 8700 gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 μm; J&W Scientific Inc.). Oven temperature was programmed, 45–175 °C, at 3 °C/min, subsequently at 15 °C/min up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures, 280 °C and 290 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using correction factors.

2.3.2. Gas chromatography–mass spectrometry

The GC–MS unit consisted on a Perkin–Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific, Inc.), and interfaced with Perkin–Elmer Turbomass mass spectrometer (software version 4.1). Injector and oven temperatures were as above; transfer line temperature, 280 °C; ion trap temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40–300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_9–C_{16} *n*-alkanes, and GC–MS spectra with corresponding data of components of reference oils, laboratory-synthesized components and commercially available standards from a home-made library.

2.3.3. Cluster analysis

The percentage composition of the essential oil samples was used to determine the relationship between the different samples of *Th. capitatus* by cluster analysis using the NTSYS-pc software (version 2.02, Exeter Software, Setauket, New York) (Rohlf, 1998). Correlation coefficient was selected as a measure of similarity among the accessions, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition. Experimental units were the same as for essential oil analysis. The degree of correlation was evaluated according to Pestana and Gageiro (2000) in: very high if correlation ranged between 0.9 and 1, high, between 0.7 and 0.89, moderate, between 0.4 and 0.69, low, between 0.2 and 0.3 and very low if <0.2.

Table 1
Percentage composition of the essential oils isolated from the aerial parts of *Th. capitatus*, collected from three different locations in Tunisia [Jendouba (J), Aîn Tounine (AT) and Haouaria (H)], during the vegetative, the flowering and the post-flowering phases

Components	RI[a]	Vegetative			Flowering									Post-flowering			
		Leaves			Leaves			Flower buds			Flowers			Leaves		Post-flowers	
		J	AT	H	J	AT	H	J	AT	H	J	AT	H	J	AT	J	AT
α-Thujene	924	1.0	0.2	0.8	0.2	0.4	0.4	0.8	0.4	0.3	1.0	2.1	0.8	1.1	0.9	1.3	1.1
α-Pinene	930	1.0	0.2	0.4	0.2	0.3	0.3	0.6	0.4	0.2	0.6	1.1	0.4	0.9	0.9	1.0	0.8
Camphene	938	0.7	0.1	0.3	t	0.1	0.2	0.1	0.1	0.1	0.1	0.3	0.1	0.7	0.6	0.3	0.2
1-Octen-3-ol	961	0.1	t	0.1	t	t	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
β-Pinene	963	0.1	t	0.1	t	t	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Myrcene	975	0.5	0.1	1.3	0.3	0.6	1.1	1.1	1.0	1.1	1.2	1.8	1.4	1.9	1.5	1.4	1.8
α-Phellandrene	995	0.2	t	0.2	0.2	0.2	0.3	0.5	0.4	0.3	0.4	0.8	0.4	0.2	0.3	0.4	0.3
δ-3-Carene	1000	t	t	0.1	t	t	0.1	0.2	0.2	0.1	0.1	0.2	0.1	0.1	t	0.1	t
α-Terpinene	1002	0.4	0.2	0.8	0.6	0.9	1.4	1.8	1.9	1.4	1.2	2.2	1.2	1.7	1.4	1.8	1.7
p-Cymene	1003	*16.7*	*5.5*	*8.1*	*5.4*	*7.3*	*8.0*	*8.9*	*8.2*	*5.9*	*5.3*	*8.6*	*4.8*	*13.7*	*15.4*	*9.1*	*10.8*
β-Phellandrene	1005	0.2	t	0.2	0.4	0.2	0.2	0.4	0.6	0.2	0.3	0.5	0.3	0.3	0.3	0.3	0.3
Limonene	1009	0.3	t	0.4	0.1	0.3	0.4	0.4	0.1	0.4	t	0.6	0.3	0.6	0.5	0.5	0.5
cis-β-Ocimene	1017	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
trans-β-Ocimene	1027	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
γ-Terpinene	1035	*3.5*	*2.0*	*4.5*	*5.3*	*6.3*	*10.9*	*11.0*	*13.7*	*10.5*	*6.1*	*12.3*	*6.0*	*8.9*	*7.5*	*6.5*	*8.0*
trans-Sabinene hydrate	1037	t	0.2	0.5	t	0.1	0.2	t	t	0.2	0.1	t	0.2	0.3	0.1	0.3	0.3
Terpinolene	1064	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.1
cis-Sabinene hydrate	1066	t	t	t	t	t	t	t	t	0.1	t	t	t	t	t	t	t
Linalool	1074	0.3	0.6	1.7	0.1	0.5	0.7	t	0.1	0.6	0.2	0.1	0.5	1.0	0.9	0.9	1.0
Borneol	1134	1.8	1.2	0.9	0.5	0.3	0.4	0.2	0.3	0.2	0.2	0.2	0.2	1.6	1.3	0.4	0.4
Terpinen-4-ol	1148	t	0.3	0.7	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.5	0.6	0.6	0.5	0.5
Thymol	1275	0.3	t	0.3	0.2	0.4	0.3	t	t	0.4	0.1	t	0.3	0.4	0.5	0.3	0.4
Carvacrol	1286	*65.9*	*83.0*	*71.3*	*80.7*	*77.4*	*70.6*	*69.5*	*68.1*	*72.9*	*78.6*	*65.6*	*78.0*	*61.6*	*63.0*	*70.7*	*68.5*
Eugenol	1327	t	t	0.1	t	t	t	t	t	t	t	t	t	0.1	t	t	t
Carvacrol acetate	1348	0.4	1.0	2.1	0.2	t	t	t	t	0.4	t	t	t	t	t	t	t
β-Caryophyllene	1414	*4.3*	*2.8*	*3.4*	*3.2*	*1.9*	*2.2*	*2.2*	*1.9*	*2.9*	*2.4*	*1.8*	*2.9*	*1.9*	*1.8*	*1.0*	*1.5*
Aromadendrene	1428	0.1	0.2	0.1	0.1	t	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.3	0.1	0.2
α-Humulene	1447	0.1	0.1	0.1	0.2	t	t	t	t	t	t	t	t	0.1	0.2	t	t
Viridiflorene	1487	t	0.1	0.1	t	t	0.1	t	t	0.1	t	t	t	t	t	t	t
β-Bisabolene	1494	0.1	0.5	0.1	0.2	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1
Spathulenol	1551	t	t	0.1	t	t	t	t	t	t	t	t	t	t	t	t	t
β-Caryophyllene oxide	1561	0.9	1.0	0.5	0.3	0.7	0.7	0.3	0.2	0.5	0.4	0.2	0.3	0.6	0.8	0.3	0.3
% *Identification*		98.6	99.4	99.0	98.9	98.9	99.4	98.9	98.3	99.5	99.1	99.4	99.5	99.1	99.2	97.9	99.1
Grouped components																	
Monoterpene hydrocarbons		24.5	8.4	17.0	12.7	16.7	23.2	26.0	27.1	20.6	16.4	30.8	16.1	30.5	29.4	23.0	25.9
Oxygen-containing monoterpenes		68.6	86.3	77.4	82.1	79.2	72.6	70.1	68.9	75.2	79.7	66.2	79.9	65.5	66.3	73.2	70.9
Sesquiterpene hydrocarbons		4.5	3.7	3.9	3.7	2.3	2.7	2.4	2.1	3.1	2.6	2.1	3.1	2.3	2.5	1.2	1.8
Oxygen-containing sesquiterpenes		0.9	1.0	0.6	0.3	0.8	0.7	0.3	0.2	0.5	0.4	0.2	0.3	0.6	0.9	0.3	0.3
Phenylpropanoids		t	t	0.1	t	t	t	t	t	t	t	t	t	0.1	t	t	t
Others		0.1	t	0.1	t	t	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Oil yield (%, *w*/*w*)		1.7	1.8	1.2	3.4	2.6	2.8	5.3	5.4	3.6	5.4	5.1	4.1	3.1	3.6	5.6	4.1

t = traces (<0.05%).
[a] RI is the Retention Index relative to C_9–C_{16} *n*-alkanes on the DB-1 column.

2.4. Antioxidant activity evaluation

From each sample, different concentrations of essential oils were prepared in methanol: 100, 250, 500, 750 and 1000 mg l^{-1}. The antioxidant activity of *Th. capitatus* essential oils was carried out using four different methods: metal chelating, reductive potential, free radical-scavenging activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) and by the TBARS (thiobarbituric acid reactive substances) assay.

2.4.1. Metal chelating measurements

The Fe^{2+}-chelating ability of *Th. capitatus* essential oils was determined according to the method of Carter (Carter, 1971). 100 μl of the essential oils was incubated with 100 μl of $FeCl_2$ and 3.7 ml of methanol. The reaction was initiated by the addition of 100 μl of ferrozine and, after the mixture had reached equilibrium (10 min), the absorbance at 562 nm was read.

The percentage of inhibition of the complex ferrozine–Fe^{2+} was calculated using the following equation: $Inhibition\% = [(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control (without essential oil) and A_1 was the absorbance in presence of the essential oil.

2.4.2. Reductive potential

The reductive potential was determined according to the method of Oyaizu (Oyaizu, 1986). The different concentrations of *Th. capitatus* essential oils were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer.

2.4.3. Free radical-scavenging activity

Radical scavenging using DPPH radicals is the main mechanism by which antioxidants act in food. The DPPH method as summarized below was introduced nearly 50 years ago by Blois (1958).

The free radical-scavenging activity of extracts were measured by 2,2-diphenyl-2-picrylhydrazyl (DPPH). A solution of DPPH in methanol (24 μg/ml) was prepared and 2 ml of this solution was added to 50 μl of extracts solution in methanol at different concentrations (100, 250, 500, 750 and 1000 mg l^{-1}). Then, the absorbance was measured at 517 nm in a spectrophotometer Schimadzu 160-UV. In the original method, a reaction time of 30 min was recommended. Shorter times have also been used, such as 5 min (Lebeau et al., 2000), or 10 min (Schwarz et al., 2001). In our experience, we used 5 min, necessary time for the end of the reaction. Radical-scavenging activity was calculated using the following equation: $Scavenging\ effect\% = [(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control sample (without essential oil) and A_1 was the absorbance in the presence of the sample ($t = 5$ min).

2.4.4. TBARS assay

A modified thiobarbituric acid-reactive substances (TBARS) assay (Wong, Hashimoto, & Shibamoto, 1995) was also used to measure the potential antioxidant capacity of *Th. capitatus* essential oils. Egg yolk homogenate was used as lipid-rich media, an aliquot of yolk material was made up to a concentration of 10% (w/v) in KCl (1.15%, w/v). The yolk was then homogenized for 30 s, followed by ultrasonication for a further 5 min. Five hundred microliters of 10% (w/v) homogenate and 100 μl of sample, solubilized in methanol, were added to a test tube and made up to 1 ml with distilled water, followed by addition of 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) 2-thiobabituric acid (TBA) in 1.1% (w/v) sodium dodecyl sulphate (SDS). Each essential oil and tested substance was assayed at the concentrations of 100, 250, 500, 750 and 1000 mg l^{-1}. This mixture was stirred in a vortex, and heated at 95 °C for 1 h. After cooling, at room temperature, 5 ml butan-1-ol was added to each tube, stirred and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer Schimadzu 160-UV. All the values were expressed as antioxidant index (AI%), calculated by the formula: $Antioxidant\ index\% = [(A_0 - A_1)/A_0] \times 100$, where A_0 being the absorbance value of the fully oxidized control and A_1, the absorbance of the test sample.

2.5. Antibacterial activity determination

The microorganisms used in this study were *Bacillus cereus* strain C1060 (a food isolate), *Salmonella* sp. (an environmental isolate), *Listeria innocua* (a food isolate), four different strains of *Staphylococus aureus* (C15 a food isolate ATCC 25923, a clinical isolate, CFSA-2-an environmental isolate) and a methicillin-resistant strain of *S. aureus* (MRSA-2, a clinical isolate). *B. cereus* and *S. aureus* strains C15 and ATCC 25923 were a gift from Instituto Nacional de Engenharia e Tecnologia Industrial, Departamento de Tecnologias de Industrias Alimentares (INETI/DTIA). The methicillin-resistant strain of *S. aureus* (MRSA-2) was a gift from the Centro Hospitalar do Barlavento Algarvio. *Listeria innocua* and CFSA-2 belong to the Microbiology Laboratory of the Faculty of Engineering and Natural Resources of the University of the Algarve. The antimicrobial activity of essential oils was tested by the disc agar diffusion method as described by Burt and Reinders (2003) and Faleiro et al. (2003), Faleiro et al. (2005). Original cultures were kept at −80 °C during the study. The bacterial strains were maintained in tryptic soy agar (TSA) except the *S. aureus* strains that were maintained in brain heart infusion (BHI). Prior to the assay, the bacterial cultures were plated in fresh TSA or BHI plates and grown for 24 h at 37 °C. From each plate a loop was used to inoculate

10 ml of BHI and left for about 2 h at 37 °C until the cultures reach the exponential phase. From the above exponential culture 100 μl were used to inoculate brain heart infusion (BHI) agar plates. Sterile filter paper discs (6 mm, Oxoid), containing 4 μl of essential oil diluted in 2-propanol (0.8 μl/disc), were distributed in the agar surface. Sterile water was used as the negative control whereas the antibiotic chloramphenicol disc was used as positive control (30 μg). Inhibition zones were determined after an incubation period of 24 h at 37 °C.

2.5.1. Statistical analysis

The obtained antioxidant and antimicrobial results were stated in mean ± standard deviation. Analysis of variance was performed by ANOVA procedures (SPSS 14.0 for Windows). Significant differences between means were determined by Tukey post hoc tests. p values inferior to 0.05 were regarded as significant.

3. Results and discussion

3.1. Essential oil yield and chemical composition

The essential oils extracted by hydrodistillation from the dried aerial parts of *Th. capitatus*, collected in three diverse locations at different developmental stages, ranged from 1% to 6% (w/w) with a maximum obtained during the post-flowering phase (Table 1). These results are in agreement with previous data for the same species (Hedhili, Romdhane, Abderrabba, Planche, & Cherif, 2002).

Thirty-two components were identified in the 16 *Th. capitatus* essential oil samples analyzed, amounting to 98–100% of the total oils, which are listed in Table 1 in order to their elution from a DB-1 column. All of the oils analyzed were dominated by the monoterpene fraction (93–97%), obtained during both the flowering and the post-flowering phases, the oxygen-containing monoterpene being the most representative group (66–86%) of this fraction and in all the oils.

Sesquiterpenes attained 3%, in the oil samples collected during both the flowering and the post-flowering phases, which was lower than the relative amounts recorded for the oils collected during the vegetative phase (5%).

Carvacrol was the main component of all the essential oils attaining in average 73%, 74% and 66% in the vegetative, flowering and post-flowering phases, respectively, which is in concordance with the carvacrol chemotype growing in Tunisia previously reported by Hedhili, Romdhane, Planche, and Abderrabba (2005). In addition, the oil was characterized by high levels of p-cymene (5–17%), γ-terpinene (2–14%) and β-caryophyllene (1–4%). p-Cymene showed a maximum when carvacrol was at its minimum, which is in agreement with the literature, reporting that p-cymene is the precursor of carvacrol (Vernet, Gouyon, & Valdeyron, 1986).

Cluster analysis of the chemical composition of all *Th. capitatus* essential oils, Fig. 1, showed that all the samples were very highly correlated ($S_{corr} = 0.99$), indicating a strong oil homogeneity independent of the plant part analyzed (leaves, flower buds, flowers and post-flowers), of the location of collection of the plant material and of the corresponding different climatic conditions (interior and littoral north and littoral south).

3.2. Antioxidant activity

All the essential oil samples showed antioxidant activity, but significantly, statistic differences were only observed when they were compared by the developmental stage criteria. Since no major differences were found between the antioxidant activities of the oils collected at different locations, the results were grouped in three batches, which are the mean values of samples collected during the vegetative, the flowering and the post-flowering stages.

The antioxidant activity may be due to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction, free radical scavenging, reducing capacity, and binding of transition metal ion catalysts (Mao, Pan, Que, & Fang, 2006). It is thus important that for evaluating the effectiveness of antioxidants, several analytical methods and different substrates are used.

3.2.1. Chelating effect

Transition metal ions can stimulate lipid peroxidation by two mechanisms, namely by participating in the generation of initiating species and by accelerating peroxidation decomposing lipid hydroperoxides into other components which are able to abstract hydrogen, perpetuating the chain of reaction of lipid peroxidation (Deshpande, Deshpande, & Salunkhe, 1995).

Both the essential oils and BHA and BHT showed no metal chelating activity. Previous studies (Chung, Chang, Chao, Lin, & Chou, 2002) showed that BHT presented no detectable Fe^{2+}-chelating effect which is in agreement with our results. In fact, BHT as carvacrol, which is the main component of *Th. capitatus* essential oils, are both mono-hydroxylated compounds which are not able to form a complex with Fe^{2+} justifying the obtained results. This result is in contrast to that reported by some authors (Gülçin, Şat, Beydemir, Elmastaş, & Küfrevioğlu, 2004) in which BHT and BHA exhibited metal chelating ability (72% and 59%, respectively).

3.2.2. Reductive potential

The reductive potential measures the ability of a sample to act as electron donor and, therefore, reacts with free radicals converting them to more stable products and thereby terminates radical chain reactions.

Generally, the reductive potential of all *Th. capitatus* essential oil samples increased steadily with increasing essential oils concentration (Table 2), the maximum being obtained within the range of 500–1000 mg l^{-1} for *Th. capitatus* essential oils. The essential oils extracted during the

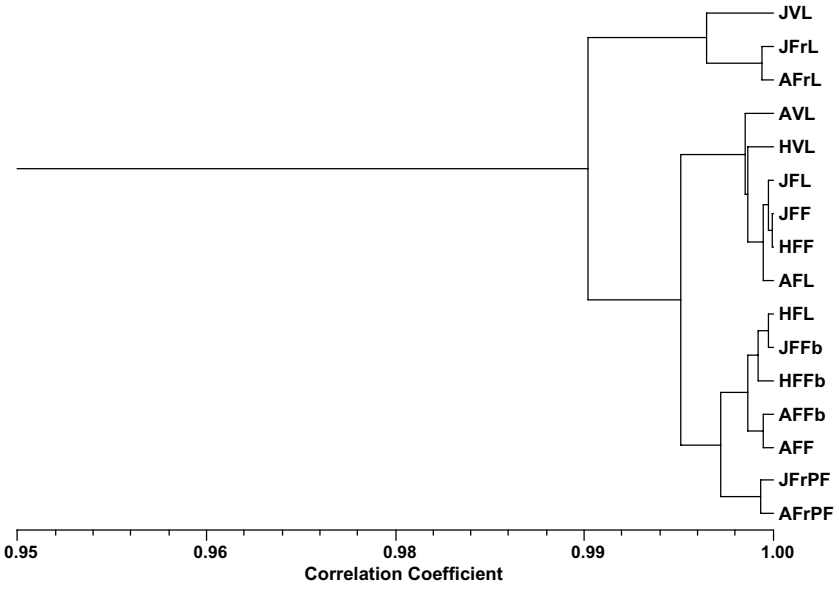

Fig. 1. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the *Thymus capitatus* populations examined, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). J, A and H for Jendouba, Aîn Tounine and Haouaria, respectively. VL, vegetative leaves; FL, flowering leaves; FFb, flowering flower buds; FF, flowering flowers; FrL, post-flowering leaves; FrPF, post-flowering post-flowers.

Table 2
Reductive potential ability of *Thymus capitatus* essential oils isolated during vegetative, flowering and post-flowering stages and from BHA and BHT

Concentrations (mg l^{-1})	*Thymus capitatus* essential oils			Synthetic antioxidants	
	Vegetative	Flowering	Post-flowering	BHA	BHT
100	0.2 ± 0.0c	0.0 ± 0.0c	0.6 ± 0.1b	1.4 ± 0.1a	1.3 ± 0.1a
250	0.4 ± 0.0c	0.1 ± 0.0d	1.2 ± 0.1b	1.7 ± 0.2a	1.6 ± 0.2a
500	0.6 ± 0.0c	0.2 ± 0.0d	2.0 ± 0.1a	1.6 ± 0.1b	1.7 ± 0.1b
750	0.8 ± 0.0b	0.3 ± 0.1b	2.2 ± 0.0a	1.9 ± 0.0a	1.8 ± 0.1a
1000	1.0 ± 0.0b	0.4 ± 0.1c	2.1 ± 0.1a	1.7 ± 0.1a	1.7 ± 0.1a

Values represent mean ± standard deviation of four replicates. Values followed by the same letter under the same row, are not significantly different ($p > 0.05$).

post-flowering phase showed a reductive potential similar to that of BHA and BHT (all in the range of a mean absorbance of 2, Table 2), and higher than those extracted during the vegetative (mean absorbance of 1, Table 2) and the flowering (mean value of absorbance of 0.3, Table 2) stages, these differences being statistically significant. The samples extracted during the flowering phase showed the lowest reductive ability when evaluated by this methodology.

Th. capitatus essential oils seem to act as electron donors. Similar results were obtained with cinnamon bark extracts where the reductive potential also reached an absorbance value of 3 at 1000 mg l^{-1} (Mathew & Abraham, 2006). The reductive potential, measured by the absorbance at 700 nm, may be due to the di- and mono-hydroxyl substitutions in the aromatic ring which possess potent hydrogen donating abilities as described by Shimada, Fujikawa, Yahara, and Nakamura (1992). Nevertheless our results seem to reveal the existence of some components not determined, other than carvacrol, the main phenol compound present in these oils, responsible for the significant reductive ability of the oils extracted during the post-flowering stage.

3.2.3. Free radical scavenging

The reduction ability of DPPH radicals' formation was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable dimagnetic molecule (Soares, Dinis, Cunha, & Almeida, 1997).

The scavenging ability of all *Th. capitatus* essential oils extracted during vegetative, flowering and post-flowering stages increased steadily with increasing oil concentration (Table 3). The oils isolated during both the flowering and the post-flowering period showed higher scavenging values than those isolated during the vegetative period with statistically significant differences ($p < 0.05$). The oil samples extracted during the flowering phase at concentrations of 750 and 1000 mg ml^{-1} possessed the most effective capacity

Table 3
DPPH scavenging activity of *Thymus capitatus* essential oils isolated during vegetative, flowering and post-flowering stages and from BHA and BHT

Concentrations (mg l^{-1})	*Thymus capitatus* essential oils			Synthetic antioxidants	
	Vegetative	Flowering	Post-flowering	BHA	BHT
100	5.7 ± 1.0c	17.1 ± 1.7b	15.2 ± 1.5bc	–	17.2 ± 2.2b
250	7.6 ± 2.2c	46.6 ± 5.5b	43.5 ± 2.9b	–	30.8 ± 1.7bc
500	11.3 ± 0.9c	82.7 ± 4.7b	70.1 ± 1.7b	–	76.0 ± 6.8b
750	15.1 ± 1.8b	–	84.8 ± 3.9a	–	80.0 ± 4.9a
1000	21.1 ± 2.7b	–	93.1 ± 2.2a	–	80.1 ± 2.7a

Values represent mean ± standard deviation of four replicates. Values followed by the same letter under the same row, are not significantly different ($p > 0.05$). – for explanation see text.

for free radical scavenging because such concentrations showed an almost immediate change of the solution colour from purple to yellow, affecting the correct measurements and revealing a relative high antioxidant capacity. The same happened with BHA for concentrations higher than 100 mg ml^{-1}, therefore for these concentrations the results of BHA were not considered.

The DPPH scavenging activity of the essential oils isolated from post-flowering samples were statistically similar ($p > 0.05$) to that of the synthetic antioxidant BHT for the majority of the concentrations tested.

High values for DPPH radical-scavenging activity were achieved for flowering phase oils but these same oils were characterized by a low reducing power. In contrast the vegetative oil samples were characterized by a lower DPPH radical-scavenging value and a higher reducing power (Tables 2 and 3). These data may be due to the fact that radical-scavenging capacity is directly related to the hydrogen atom donating ability of a compound and not correlated to the redox potentials alone, as observed by Lucarini et al. (1999) when studying the antioxidant capacity of phenothiazine and other related compounds.

3.2.4. TBARS assay

Thiobarbituric acid-reactive substances (TBARS) assay spectrophotometrically measures, at 532 nm, the pink pigment produced through reaction of thiobarbituric acid (TBA) with malonaldehyde (MDA), which is one of the secondary lipid peroxidation products. Other secondary lipid peroxidation compounds can also react with TBA.

When evaluated by the TBARS assay, the antioxidant activity of *Th. capitatus* essential oils was dose-dependent (Table 4). The best activities were observed at high concentrations. The antioxidant indices of *Th. capitatus* essential oils isolated during the vegetative phase were significantly different ($p < 0.05$) in comparison with both samples extracted during the flowering and the post-flowering phases. Those essential oils were less effective than the remaining oils to prevent lipid peroxidation, mainly at lower concentrations (100–500 mg l^{-1}). Maximums of activity were observed with the essential oils from post-flowering phase.

The difference found in the antioxidant index within the development stage can be partly explained by the diverse relative amounts of the minor compounds present in the essential oils. Ruberto, Biondi, and Piatelli (2000) studied the antioxidant activity using TBARS method with pure compounds and showed that both α- and γ-terpinene had a high antioxidant index reaching 72% at 1000 mg l^{-1}, whereas β-caryophyllene showed a low antioxidant index of 9% at 1000 mg l^{-1}. Although this data was not under evaluation in the present study, they seem to be in agreement with our results. In fact α- and γ-terpinene showed lower amounts in the essential oils isolated during the vegetative phase comparing to the other phases whereas β-caryophyllene was present in higher relative amounts.

In conclusion, to the best of our knowledge, this is the first report on the chelating effect, reductive potential and free radical scavenging of *Th. capitatus* essential oils. Previous studies on the antioxidant activity of Portuguese *Thymbra capitata* essential oils isolated during the flowering phase and evaluated by the TBARS methodology (Faleiro et al., 2005), showed in average, an antioxidant index of 80% which is within the range of the average value found in the present study (74%) for the same range of oil concentrations.

The differences found with the different methodologies can be to a certain extent explained by the diverse relative amounts of minor compounds in the oils but that can have a major impact in the final oil antioxidant effect. Further work is needed to fully understand the variables that can affect the evaluation of the antioxidant capacity by different methodologies.

3.3. Antibacterial activity

As for the antioxidant evaluation, no major differences were found between the antibacterial activities of the oils collected at different locations, so the results were grouped in three batches which are the mean values of all samples collected during the vegetative, the flowering and the post-flowering stages, respectively. Data is summarized in Table 5.

The essential oils isolated during the vegetative phase showed no activity in contrast to flowering and post-flowering stages.

S. aureus ATCC 25923 was the most susceptible strain to the flowering stage essential oils, whereas surprisingly the MRSA-2 (16.7 ± 3.9 mm) strain was not the most resistant but *B. cereus* (10.1 ± 1.2 mm) *Listeria innocua*

Table 4
Antioxidant index of *Thymus capitatus* essential oils isolated during vegetative, flowering and post-flowering stages and from BHA and BHT

Concentrations (mg l^{-1})	*Thymus capitatus* essential oils			Synthetic antioxidants	
	Vegetative	Flowering	Post-flowering	BHA	BHT
100	28.0 ± 10.5c	61.1 ± 9.6b	54.0 ± 5.6b	84.3 ± 6.7a	63.6 ± 4.9b
250	48.0 ± 13.0c	68.3 ± 5.7ab	68.5 ± 11.5ab	87.1 ± 4.6a	68.1 ± 3.2b
500	55.0 ± 4.5c	75.8 ± 5.8b	79.5 ± 9.5ab	94.4 ± 5.4a	66.6 ± 8.7bc
750	64.8 ± 1.0b	78.9 ± 3.4ab	84.9 ± 5.9a	91.1 ± 2.5a	77.3 ± 1.7ab
1000	68.9 ± 5.1b	79.1 ± 4.1ab	88.4 ± 7.5a	86.8 ± 4.0a	77.7 ± 5.2ab

Values represent mean ± standard deviation of four replicates. Values followed by the same letter under the same row, are not significantly different ($p > 0.05$).

Table 5
Antibacterial activity of the essential oils of *Thymus capitatus* isolated during vegetative, flowering and post-flowering stages and from antibiotic, expressed by diameter of inhibition zone (including the disc diameter, 6 mm)

Bacteria	Diameter of zone of inhibition (mm)[‡]			
	Thymus capitatus essential oils			Antibiotic (30 μg/disc)
	Vegetative	Flowering	Post-flowering	
Bacillus cereus (C1060)	NA	10.1 ± 1.2ab	7.8 ± 0.5a	20.0 ± 0.0a
Listeria innocua	NA	13.9 ± 1.6a	14.8 ± 3.1b	23.3 ± 1.2ab
Salmonella sp.	NA	9.7 ± 1.2a	7.9 ± 1.3a	21.7 ± 0.6a
Staphylococus aureus				
C15	ND	19.4 ± 1.7cd	17.3 ± 1.1b	23.3 ± 2.3ab
ATCC25923		23.4 ± 3.3d	17.4 ± 1.6b	23.3 ± 2.3ab
CFSA-2		18.1 ± 4.0bc	13.1 ± 2.4b	28.3 ± 1.5c
MRSA-2		16.7 ± 3.9bc	16.2 ± 2.2b	26.3 ± 1.2bc

Values followed by the same letter under the same column, are not significantly different ($p > 0.05$).
NA, no activity.
ND, not determined.
[‡] Including the disc diameter, 6 mm.

(13.9 ± 1.6 mm) and *Salmonella* spp. (9.7 ± 1.2 mm) that were equally the most resistant ($p > 0.05$), (Table 5). Data on the antibacterial activity of the essential oils from the post-flowering stage, indicate *B. cereus* and *Salmonella* sp. as the more resistant bacteria ($p < 0.05$) whereas all the other bacterial strains were equally susceptible ($p > 0.05$). All tested bacteria were more susceptible to the antibiotic than to the essential oils collected either at flowering or post-flowering stage (Table 5).

Carvacrol, which is the main component of *Th. capitatus* essential oils, has been considered as a biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Ultee, Kets, & Smid, 1999). The effect of carvacrol on *Staphylococus* was investigated by Knowles, Roller, Murray, and Naidu (2005). However, it was also considered that minor components, as well as a possible interaction between the substances could also affect the antimicrobial activities. In fact, other constituents, such as γ-terpinene, have been considered to display relatively good activity due to their possible synergistic or antagonistic effects (Vardar-Unlu et al., 2003) which is in agreement with our results showing that low amounts of γ-terpinene during the vegetative phase may justify the low antimicrobial activity during this period.

Bouzouita, Kachouri, Hamdi, and Chaabouni (2003) previously studied the antimicrobial activity of essential oils from Tunisian aromatic plants, against two bacteria (*Lactobacillus plantarum* and *Escherichia coli*) using a submerged broth culture method. The results obtained showed that *Th. capitatus* oils were one of the greatest inhibitors of all the strains tested. Oussalah, Caillet, Saucier, and Lacroix (2006) also evaluated the antimicrobial activity of 60 different essential oils against *Pseudomonas putida* strain of meat origin, associated with meat spoilage. The study showed that for the 60 samples tested, *Coridothymus capitatus* essential oil was the most active one showing a minimum inhibitory concentration (MIC) of 0.025%.

Th. capitatus essential oils presently studied seem to constitute an effective biocide to either combat foodborne pathogens or serious clinical pathogens such as MRSA.

As previously shown by Faleiro et al. (2005), despite the similarity in the essential oil composition, the degree of antibacterial and/or antioxidant activity of the essential oils obtained for the same species may result in differences. Faleiro et al. (2005) showed that the antibacterial activity of the essential oil isolated from *Th. capitatus* grown in Portugal was higher than that of the antibiotic against the forty *L. monocytogenes* strains tested, whereas in the present study, *Listeria innocua* was one of the most resistant strains.

Likewise, Duraffourd and Lapraz (2002) stated that the biological activity of an essential oil is related with numerous variables and is not proportional to the proposed biological activity of the main active principle alone.

In spite of the strong oil homogeneity, regardless of the plant part analyzed and of the location of collection of the plant material, the antioxidant and antimicrobial activities of *Th. capitatus* oils, significantly differed according to the harvesting period of plant material and to the methodology used in the antioxidant evaluation. The oils isolated from plants collected in the vegetative phase, independent of the collection location, generally showed a lower oil yield and lower antioxidant and antimicrobial activities. When considering from an applicability point of view, this study shows that the oils obtained from plants collected during the flowering phase attained the highest yields and best activity to prevent lipid oxidation or to act as biocide to combat bacterial pathogens. It is thus noteworthy to point out the interest in investigating the plants showing the highest biological activities. In future these plants may be under the designation of protected origin, due to their unique properties. Recent works indicated that among the *Thymus* spp. collected from different regions, largely different antibacterial and antioxidant activities could be detected (Faleiro et al., 2005; Hazzit, Baaliouamer, Faleiro, & Miguel, 2006). These studies point out the importance of comparing and exploring the variance of essential oils composition from different provenances, since this will most probably affect their potential biological activities, jeopardizing its use either in food industries or for medical purposes.

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