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Composition and antioxidant activities of the essential oils of *Thymus caespititius*, *Thymus camphoratus* and *Thymus mastichina*

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Abstract

The essential oils, isolated by hydrodistillation, from the aerial parts of *Thymus caespititius*, *Thymus camphoratus* and *Thymus mastichina*, collected during the vegetative phase, were analysed by gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC–MS). The antioxidant property of these oils was tested, with and without peroxidation inducer, by the egg yolk-based thiobarbituric acid-reactive substances assay, in the concentrations of 62.5, 125, 250 and 500 mg l^{-1}. α-Terpineol (32%) dominated *Th. caespititius* essential oil and 1,8-cineole (58%) that of *Th. mastichina*. Linalool (17%), linalyl acetate (15%) and 1,8-cineole (11%) were the main components of *Th. camphoratus* oil. The oils demonstrated antioxidant capacity in the absence of radical inducer 2,2′-azobis-(2-amidinopropane) dihydrochloride (ABAP), mainly that of *Th. caespititius* at 250 and 500 mg l^{-1}, comparable in some cases to that of α-tocopherol and butylated hydroxytoluene (BHT). The presence of ABAP diminished the antioxidant ability of all essential oils tested, *Th. caespititius* oil still showing the highest antioxidant capacity at 500 mg l^{-1}. At 250 and 500 mg l^{-1}, for BHA, and 500 mg l^{-1}, for α-tocopherol, the antioxidant capacity significantly increased in the presence of ABAP.
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Keywords: *Thymus caespititius*; *Thymus camphoratus*; *Thymus mastichina*; Essential oils; GC–MS; Antioxidant activity; TBARS assay

1. Introduction

Eleven *Thymus* species have been recorded for Portugal included in five sections of this genus (Figueiredo et al., 2001), among which are the north-western Iberian Peninsula and Madeiran and Azorean archipelagos endemism *Thymus caespititius*, the Iberian Peninsula endemic *Thymus mastichina* and the southern and southwestern Portuguese coasts endemism *Thymus camphoratus*.

Portuguese *Thymus* essential oils have been a subject of study by several researchers, as can be seen from the exhaustive review of Stahl-Biskup (2002) and recent studies (Figueiredo et al., 2001; Pereira et al., 2000, 2003). Nevertheless, studies on their biological activity capacity, either antimicrobial or antioxidant, are still rather scarce (Faleiro et al., 2003; Miguel et al., 2003).

Two main types of assays can be performed to measure the antioxidant ability of natural substances: (a) assays for radical scavenging ability and (b) assays measuring the ability to inhibit lipid oxidation. As all methods are based on different chemical and physical principles of oxidation monitoring, the antioxidant activities may vary according to the assay used (Puertas-Mejía, Hillebrand, Stashenko, & Winterhalter, 2002) particularly in matrices rich in oxidisable lipids (lard, cholesterol, chicken fat, linoleic acid, vegetable oils, lecithin emulsion, egg yolk, rat liver and active oxygen radical, among others) (Madsen & Bertelsen, 1995). In addition, the comparison of the results of different experiments is often complicated by the fact that the antioxidant activity of an essential oil varies according to its chemical composition, which is also

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dependent on several factors (Figueiredo, Barroso, Pedro, & Scheffer, 1997).

As part of our studies on essential oil-bearing plants from Portugal, we now report the antioxidant capacity and composition of the essential oils isolated from the aerial parts of *Th. caespititius* Brot., *Th. mastichina* L. (L.) subsp. *mastichina* and *Th. camphoratus* Hoffmanns & Link, collected during the vegetative phase. A modified thiobarbituric acid-reactive species (TBARS) assay was used to measure the potential antioxidant capacity of the essential oils, using egg yolk as a a lipid-rich medium.

2. Materials and methods

2.1. Plant material

The aerial parts of *Th. mastichina* and *Th. caespititius* were collected, during the vegetative phase (January 2002), in Trás-os-Montes (N.E. Portugal), and *Th. camphoratus* was collected in Algarve (S. Portugal). For each species, the collective sample was constituted of a mixture of 10–15 individual plants.

2.2. Isolation procedure

The essential oils of each collective sample were isolated from fresh plant material (30 g) by hydrodistillation, for 4 h, using a Clevenger-type apparatus.

2.3. Gas chromatography

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

2.4. Gas chromatography-mass spectrometry

The gas chromatography-mass spectrometry unit consisted of a Carlo Erba 6000 Vega gas chromatograph, equipped with a DB-1 fused-silica column (30 m $\times$ 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc.) and interfaced with a Finnigan MAT 800 Ion Trap Detector (ITD; software version 4.1). Oven temperature was as above; transfer line temperature was 280 °C; ion trap temperature, 220 °C; carrier gas was helium, adjusted to a linear velocity of 30 cm s^{-1}; splitting ratio, 1:40; ionisation energy, 70 eV; ionisation current, 60 μA; scan range, 40–300 u and scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{17} *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.

2.5. Antioxidant activity measurement

Two sets of experiments based on a modified TBARS assay were used to measure the antioxidant ability of the sample (essential oils or tested substances), without (1) and with (2) a lipid peroxidation inducer. In both cases, egg yolk homogenates were used as lipid-rich media, obtained as described by Dorman, Deans, Noble, and Surai (1995); i.e., 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. For set (1) of TBARS assay, 500 μl 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) TBA in 1.1% (w/v) sodium dodecyl sulphate (SDS). Each essential oil and tested substance was assayed at the concentrations of 62.5, 125, 250 and 500 mg l^{-1}. This mixture was stirred in a vortex, and heated at 95 °C for 60 min. 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%), whereby the control is completely peroxidized and each oil and tested substance demonstrated a comparative percentage of antioxidant protection. The AI% was calculated using the formula: $(1 - t/c) \times 100$, c being the absorbance value of the fully oxidized control and t, the absorbance of the test sample (Baratta et al., 1998a).

For set (2) of the TBARS assay, 50 μl of 2,2′-azobis-(2-amidinopropane) dihydrochloride (ABAP) (0.07M) was added to induce lipid peroxidation, soon after the addition of sample, the remaining procedure being as reported above.

2.6. Statistical analysis

The analytical values of the antioxidant activity measurement represent means of three replicates, done

Table 1
Oil yields and percentage composition of the essential oils isolated from the aerial parts of *Th. caespititius*, *Th. camphoratus* and *Th. mastichina*, collected during the vegetative phase

Components	RI[a]	*Th. caespititius*	*Th. camphoratus*	*Th. mastichina*
Tricyclene	921	0.1	0.1	0.1
α-Thujene	924	1.2	0.1	0.1
α-Pinene	930	0.8	2.1	2.2
Camphene	938	1.2	1.8	0.8
Sabinene	958	2.8	0.4	1.9
Octen-3-ol	961	0.2		
β-Pinene	963	0.7	0.3	3.3
Dehydro-1,8-cineole	973	0.1		0.2
*iso*Limonene	974		0.4	
Myrcene	975	2.1	1.2	0.8
α-Phellandrene	995	0.2		0.1
δ-3-Carene	1000	t		
α-Terpinene	1002	1.1		0.2
p-Cymene	1003	9.2	0.3	0.5
β-Phellandrene	1005	0.3		
1,8-Cineole	1005		**10.8**	**57.8**
Limonene	1009	1.6	0.7	**10.8**
cis-β-Ocimene	1017	0.1	0.6	0.2
trans-β-Ocimene	1027	0.6	2.2	0.8
γ-Terpinene	1035	6.6	0.1	0.5
trans-Sabinene hydrate	1037			0.5
cis-Linalool oxide	1045		1.4	
trans-Linalool oxide	1059		0.9	
Terpinolene	1064	0.3	0.3	0.2
cis- Sabinene hydrate	1066	0.1		
Linalool	1074	0.3	**16.6**	1.8
Octen-3-yl acetate	1086	0.2		0.1
Campholenal	1088		t	0.1
trans-*p*-Menth-2-en-1-ol	1095	0.1		
Camphor	1095		1.4	0.2
trans-Pinocarveol	1106			0.2
cis-*p*-2- Menth-2-en-1-ol	1110	0.1		
trans-Verbenol	1114		0.5	0.1
Pinocarvone	1121		0.2	0.2
Nerol oxide	1127		0.9	
Borneol	1134	1.9	2.9	2.1
δ-Terpineol	1134			t
Terpinen-4-ol	1148	1.3	0.4	1.1
Myrtenal	1153			0.1
α-Terpineol	1159	**32.1**	3.4	1.7
Myrtenol	1168			0.2
trans-Carveol	1189	t		0.1
Decanal	1180		0.3	
Bornyl formate	1199			0.1
Nerol	1206		0.7	
Carvacrol methyl ether	1224	0.5		
Geraniol	1236		1.3	0.1
Linalyl acetate	1245		**15.2**	
Decanol	1259		0.5	
Bornyl acetate	1265	0.8	2.0	0.8
Thymol	1275	0.2		
α-Terpinyl acetate	1334		0.3	2.4
Geranyl acetate	1370		1.6	
α-Copaene	1375	0.1		
β-Bourbonene	1379	0.2		
β-Elemene	1388	0.1	0.3	
α-Gurjunene	1400	0.2		
β-Caryophyllene	1414	1.7	0.8	0.3
α-Humulene	1447	0.3		
allo-Aromadendrene	1454		0.3	0.7
Geranyl propionate	1461			0.1
Dodecanol	1468		0.3	

Table 1 (*continued*)

Components	RI[a]	*Th. caespititius*	*Th. camphoratus*	*Th. mastichina*
γ-Muurulene	1469	0.5		
Germacrene D	1474	1.1		0.3
Valencene	1477		0.4	
Bicyclogermacrene	1487		0.3	0.2
trans-Dihydroagarofuran	1489	2.6		
γ-Cadinene	1500	2.2	1.1	
δ-Cadinene	1505	1.1	0.4	
Kessane*	1517	1.4		
α-Cadinene	1529	0.1		
Elemol	1530	0.7		0.6
Spathulenol	1551			t
Germacrene D-4-ol	1557	0.3		
β-Caryophyllene oxide	1561	0.3	1.2	0.2
Viridiflorol	1569	1.0	0.5	0.4
Ledol	1580		0.3	0.2
Geranyl isovalerate	1590			0.2
epi-Cubenol	1600	0.3		
T-Cadinol	1616		1.4	0.2
δ-Cadinol	1618	4.5	1.4	
β-Eudesmol	1620	0.6		0.3
α-Cadinol	1626	1.5		
Intermedeol	1629		6.2	
α-Eudesmol	1634			0.4
Identified components (%)		87.6	86.8	96.1
Grouped components				
Monoterpene hydrocarbons		28.9	10.6	22.5
Oxygen-containing monoterpenes		37.5	60.5	69.8
Sesquiterpene hydrocarbons		9.0	3.6	1.6
Oxygen-containing sesquiterpenes		11.8	11.0	2.1
Others		0.4	1.1	0.1
Oil yield (v/w)		0.5	1.0	1.3

t, trace (<0.05%).
* Identification based on mass spectra only.
[a] Retention index, relative to C_9–C_{17} *n*-alkanes on the DB-1 column.

in two different experiments. Data obtained were subjected to one-way analysis of variance and Tuckey's test analysis. Significance was assumed at $P < 0.05$.

3. Results and discussion

The oil yields obtained from the different species varied considerably (Table 1). The highest oil yield was obtained from *Th. mastichina* (1.3%, v/w), while the lowest one was obtained from *Th. caespititius* (0.5%), *Th. camphoratus* (in between) yielding 1.0%. These yields were relatively lower than the average oil yields reported by Salgueiro (1994) from the aerial parts collected during the flowering phase (1.1% for *Th. caespititius*, 2.1% for *Th. mastichina* and 1.4% for *Th. camphoratus*). The different harvesting period of the samples could partly be responsible for these differences because both the oil yield and the proportions of the several constituents ofan essential oil may vary greatly according to the developmental phase of the plant (Bruneton, 1999).

Forty eight to fifty two components could be identified, representing 87–96% of the total oils, which are listed in Table 1 in order of their elution on a DB-1 column. Although monoterpenes were dominant in all oils (66–92%), the importance of the oxygen-containing- or monoterpene hydrocarbons varied. The essential oils of *Th. camphoratus* and *Th. mastichina* were dominated by oxygen-containing monoterpenes (61% and 70%, respectively), whereas in *Th. caespititius*, the oxygen-containing monoterpenes (38%) and the monoterpene hydrocarbons (29%) were present in more approximate amounts.

α-Terpineol (32%) was the dominant component in *Th. caespititius* essential oil, which is in accordance with the report by Salgueiro (1994) for the mainland oils, although different from most of the Portuguese islands chemotypes (Pereira et al., 2000, 2003; Salgueiro et al., 1997). Linalool (17%), linalyl acetate (15%) and 1,8-cineole (11%) dominated *Th. camphoratus* oil, showing some similarities to some previously studied populations of this species (Salgueiro, 1994). 1,8-Cineole (58%) was

the main component of *Th. mastichina* oil, which is also in agreement with previous reports for this species (Salgueiro, 1994).

All the essential oils showed some antioxidant capacity, in the absence of the radical inducer, increasing over the concentration range tested, (Table 2). *Th. caespititius* oil showed, at 250 and 500 mg l^{-1}, the highest antioxidant index, comparable to or higher than that of α-tocopherol and BHA, respectively, but still lower than that of BHT, at the same concentrations. The essential oils of *Th. camphoratus* and *Th. mastichina* showed much lower antioxidant indices than that of *Th. caespititius* or that the other tested substances. The decrease in concentration of the oils produced a drastic reduction in their activity, and at the lowest concentration (62.5 mg l^{-1}) the oils were scarcely active.

With the exception of the oil of *Th. camphoratus*, and in comparison with the previous experiment, the remaining essential oils showed a decrease in their antioxidant capacities in the presence of the radical inducer, ABAP (Table 3). Again the highest degree of activity of the tested oils was detected at 500 mg l^{-1}, for the oil of *Th. caespititius*, but this was still lower than that of the synthetic antioxidants and α-tocopherol. Further work is needed to clarify the pro-oxidant behaviour of *Th. caespititius* (62.5 mg l^{-1}) and *Th. mastichina* (62.5, 125 and 250 mg l^{-1}).

The different behaviour of the synthetic antioxidants and α-tocopherol in the absence/presence of ABAP suggests that BHA and α-tocopherol are able to operate as good antioxidants when the levels of peroxyl radicals are relatively high, whereas BHT and the essential oils tested do not seem to possess such ability. According to some authors (Baratta et al., 1998a; Baratta, Dorman, Deans, Biondi, & Ruberto, 1998b; Ruberto, Baratta, Deans, & Dorman, 2000), this behaviour may have a structural explanation. BHA and α-tocopherol have a lower steric hindrance around the phenol function than BHT, that possesses two *tert*-butyl groups in the *ortho*-positions. BHT would, therefore, prevent hydrogen bonding occurring in water solutions. The highest hydrogen bonding of water molecules to the phenol function in α-tocopherol and BHA, due to their reduced steric hindrance, may make them less reactive toward radicals in the reaction system, decreasing the antioxidant capacity. Consequently, the capacity of BHA and α-tocopherol to sequester peroxyl radicals increases with the increase in their production.

Further work will be required to fully characterize the potential of essential oils as protectors of highly unsaturated lipids. The fact that *Th. caespititus* oil, which has no phenolic compounds, showed the highest antioxidant activity demonstrates that the presence of this type of compound is not obligatory for the antioxidant activity.

Table 2
Antioxidant index (%) of the essential oils, α-tocopherol, BHT and BHA, in different concentrations (mg l^{-1}), using TBARS assay without ABAP

Oil/Substance	Concentration, mean ± SE (mg l^{-1})			
	62.5	125	250	500
α-Tocopherol	70.2 ± 3.8^{bc}	71.4 ± 4.1^{d}	75.0 ± 1.7^{d}	74.2 ± 1.3^{d}
BHA	60.3 ± 3.8^{b}	65.8 ± 4.1^{cd}	65.1 ± 1.7^{c}	65.6 ± 1.3^{c}
BHT	73.5 ± 3.8^{c}	79.3 ± 4.1^{d}	82.1 ± 1.7^{e}	87.7 ± 1.3^{e}
Th. caespititius	21.1 ± 3.8^{a}	43.3 ± 4.1^{b}	73.0 ± 1.7^{d}	76.3 ± 1.3^{d}
Th. camphoratus	14.1 ± 3.8^{a}	16.0 ± 4.1^{a}	38.3 ± 1.7^{b}	51.6 ± 1.3^{b}
Th. mastichina	9.6 ± 3.8^{a}	17.0 ± 4.1^{a}	31.5 ± 1.7^{a}	38.9 ± 1.3^{a}

SE, standard error.
Means with different superscript letters are significantly different ($P < 0.05$).

Table 3
Antioxidant index (%) of the essential oils, α-tocopherol, BHT and BHA in different concentrations (mg l^{-1}) using TBARS assay with ABAP

Oil/Substance	Concentration, mean ± SE (mg l^{-1})			
	62.5	125	250	500
α-Tocopherol	50.6 ± 5.2^{c}	66.8 ± 6.7^{c}	76.1 ± 4.0^{c}	82.1 ± 3.6^{e}
BHA	54.8 ± 5.2^{c}	63.7×6.7^{c}	74.2 ± 4.0^{c}	82.8 ± 3.6^{e}
BHT	41.9 ± 5.2^{c}	50.8 ± 6.7^{c}	65.6 ± 4.0^{c}	69.4 ± 3.6^{d}
Th. caespititius	-35.7 ± 5.2^{a}	1.6 ± 6.7^{ab}	26.3 ± 4.0^{b}	42.0 ± 3.6^{c}
Th. camphoratus	7.4 ± 5.2^{b}	17.1 ± 6.7^{b}	34.4 ± 4.0^{b}	37.4 ± 3.6^{b}
Th. mastichina	-19.5 ± 5.2^{a}	-14.7 ± 6.7^{a}	-9.6 ± 4.0^{a}	16.0 ± 3.6^{a}

SE, standard error.
Means with different superscript letters are significantly different ($P < 0.05$).

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