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Antimicrobial activity, cytotoxicity and intracellular growth inhibition of Portuguese *Thymus* essential oils

Susana A. Dandlen,[1] A. Sofia Lima,[2] Marta D. Mendes,[2] M. Graça Miguel,[2,3] M. Leonor Faleiro,[*,4] M. João Sousa,[5] Luís G. Pedro,[2] José G. Barroso,[2] A. Cristina Figueiredo[2]

[1]CDCTPV, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Portugal,
[2]Centro Biotecnologia Vegetal, Instituto de Biotecnologia e Bioengenharia, Faculdade Ciências Lisboa, Universidade de Lisboa, Portugal,
[3]Departamento de Química e Farmácia, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Portugal,
[4]Centro de Biomedicina Molecular e Estrutural, Instituto de Biotecnologia e Bioengenharia, Faculdade de Ciências e Tecnologia, Universidade do Algarve, Portugal,
[5]Departamento de Biologia e Biotecnologia, Escola Superior Agrária, Instituto Politécnico de Bragança, Portugal.

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Abstract: Thyme essential oils are well recognized by their excellent biological activities and the antimicrobial activity of Portuguese thyme essential oils has been investigated with promising results, particularly against food borne pathogens. In this study the potential antimicrobial activity of the essential oils of five species of *Thymus* (Lamiaceae), namely *Th. caespititius* Brot., *Th. camphoratus* Hoffmanns. & Link, *Th. capitellatus* Hoffmanns. & Link., *Th. carnosus* Boiss. and *Th. zygis* L. was evaluated against *Candida albicans, Haemophilus influenza, Helicobacter pylori, Listeria monocytogenes, Salmonella enterica* and *Streptococcus pneumoniae. H. pylori* strains were the most susceptible bacteria, particularly to the essential oils of *Th. caespititius* (Planalto Central), *Th. zygis* (Rebordãos) and *Th. caespititius* (Pico) which minimum inhibitory concentration (MIC) values ranged from 0.05 to 0.08 $mg.mL^{-1}$. *Th. caespititius* essential oil from Planalto Central or its main component, carvacrol significantly ($p<0.05$) inhibited the intracellular growth of *H. pylori*, and showed no citotoxicity to the gastric cell line. Our results suggest the potential of this essential oil and its main component as a promising tool as anti-*Helicobacter* agent potentiating the eradication of this important gastroduodenal pathogen.

Introduction

The genus *Thymus* L. is a polymorphic taxon, both chemically and morphologically. Species of *Thymus* are small perennial herbs native from Europe and Asia (Morales, 2002; Sáez and Stahl-Biskup, 2002; Figueiredo et al., 2008; Howath et al., 2008).

In Portugal, eleven species of *Thymus* (Lamiaceae), totalizing fourteen taxa and five sections can be found: Sect. *Mastichina* (Mill.) Benth., Sect. *Micantes* Velen., Sect. *Pseudothymbra* Benth., Sect. *Serpyllum* (Mill.) Benth. [subsect. Alternantes Klover and subsect. *Pseudomarginati* (H. Braun & Borbás) Jalas] and Sect. *Thymus* [subsect. *Thymus* and subsect. *Thymastra* R. Morales]. *Thymbra capitata* (L.) Cav. [=*Thymus capitatus* (L.) Hoffmanns. & Link, *Thymus creticus* DC., *Corydothymus capitatus* Rechenb. f., *Satureja capitata* L.] belongs to the genus *Thymbra*, nevertheless Franco included it in the genus *Thymus* owing to the similarities between these species (Franco, 1984).

Several aspects of Portuguese thyme species including botany, taxonomy, ethnobotany, phytochemistry, pharmacology, molecular biology, among others, were recently reviewed (Figueiredo et al., 2008; Trindade et al., 2009).

In the present work the evaluation of the antimicrobial activity of several Portuguese thyme essential oils was extended to essential oils from diverse populations from different locations, both on mainland Potugal and on the Azores archipelago. One of the tested essential oils showed a very promising activity against the gastroduodenal pathogen, *Helicobacter pylori* leading to the determination of the effect of the

essential oil on the intracellular viability of *H. pylori* in a human gastric adenocarcinoma cell type.

Materials and Methods

Plant material

Plants of the different *Thymus* species (*Th. caespititius* Brot., *Th. camphoratus* Hoffmanns. & Link, *Th. capitellatus* Hoffmanns. & Link., *Th. carnosus* Boiss. and *Th. zygis* L.), family Lamiaceae, were collected, during the flowering period (May-July, 2007-2009), on mainland Portugal and on the Azores islands Pico, S. Jorge and Terceira, totalizing 28 population samples. After collection, the plant material was kept at -20 °C until extraction. For each species, voucher specimens have been deposited in the Herbarium of the Museu, Laboratório e Jardim Botânico de Lisboa (*Th. caespititius* LISU 173798; *Th. camphoratus* LISU 237718; *Th. capitellatus* LISU 237722; *Th. carnosus* LISU 237727 and *Th. zygis* subsp. *sylvestris* LISU 237738) and in the Herbarium of the Departamento de Biologia e Biotecnologia, Escola Superior Agrária de Bragança (*Th. zygis* subsp. *zygis* BREZA, number not yet assigned).

Isolation of the essential oils

The essential oils were isolated from the aerial part of the plants by hydrodistillation during three hours, using a Clevenger-type apparatus according to the European Pharmacopoeia method (Council of Europe, 2007) in order to estimate oil yield and to obtain pure essential oils. The essential oils were stored at -20 °C in the dark prior to analysis. For the antimicrobial activity determination, the pure isolated essential oils were solved in 2-propanol (1:5, v/v).

Chemical composition of the essential oils

Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph equipped with two flame ionization detectors (FID), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m x 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 [(50% phenyl)-methylpolysiloxane, 30 m x 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.min^{-1} up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures, 280 °C and 300 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm.s^{-1}. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a pentane-oil solution (1:1). 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.

The gas chromatography-mass spectrometry unit consisted on a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature,

Table 1. Microorganisms used.

Microorganism	Origin	Source
Helicobacter pylori J99	Intestine of a patient with duodenal ulcer	Instituto Ricardo Jorge, Lisbon, Portugal
Helicobacter pylori 26695	Stomach of a patient with gastritis	Instituto Ricardo Jorge, Lisbon, Portugal
Staphylococcus aureus CFSA2	Environmental	Universidade do Algarve, IBB-CBME, Portugal
Listeria monocytogenes T8	Cheese processing environment	Chambel et al. (2007)
Listeria monocytogenes C882	Portuguese cheese	INETI-DTIA, Lisbon, Portugal, Faleiro et al. 2003
Listeria monocytogenes EGD	Clinical	Dept. Inf. Immun. & Inflammation, University of Leicester, UK
Salmonella enterica subspecies *enterica serovar* Thyphimurium ATCC 14028	Animal tissue (chicken heart and liver, 4 weeks old)	American Type Culture Collection
Haemophilus influenza ATCC 49247	Sputum of a patient with pneumonia	German Collection of Microorganisms and Cell Cultures
Streptococcus pneumoniae D39	Clinical	Dept. Inf. Immun. & Inflammation, University of Leicester, UK
Candida albicans ATCC90028	Blood	American Type Culture Collection
Candida albicans YP0048	Isolated from hospitalised patients	Faculty of Medicine (Coimbra University, Portugal)

INETI-DTIA: Instituto Nacional de Engenharia e Tecnologia Industrial-Dept.Tecnologia das Industrias Alimentares.

280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 $cm.s^{-1}$; split ratio, 1:40; ionization energy, 70 eV; 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_{22} *n*-alkane indices and GC-MS spectra from a home-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercial available standards.

Microorganisms

The microorganisms used in this study are listed in Table 1. The microbial cultures were maintained at -80 °C until use. The recovery and maintenance of the microbial strains were as previously described (Faleiro et al., 2003; Faleiro et al., 2005; Hazzit et al. 2009).

Antimicrobial activity

The antimicrobial activity of the tested essential oils was evaluated by agar diffusion as previously described (Faleiro et al., 2003; Faleiro et al., 2005, Hazzit et al. 2009). The antibiotic chloramphenicol (30 µg/disc) and amphotericin B (10 µg/disc) were used as positive reference antimicrobial agents and 2-propanol as negative. The assays were done in triplicate.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the essential oils of *Th. caespititius* from Pico, *Th. caespititius* from Planalto Central and *Th. zygis* from Rebordãos for *Helicobacter pylori* 26695 was determined. Each essential oil, at 0.03 $mg.mL^{-1}$, 0.05 $mg.mL^{-1}$ and 0 08 $mg.mL^{-1}$, was added to Columbia agar medium (supplemented with 10%, v/v blood). The bacterial viability was determined as described by Miguel et al. (2008). The assay was done in triplicate.

Cell line

The cell line 23132/87 DSMZ nº ACC201, human gastric adenocarcinoma cell type, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). ACC201 cells were grown in RPMI 1640 medium (Gibco-Invitrogen, USA) supplemented with 10% Fetal bovine serum (Gibco-Invitrogen, USA) Penicillium- Streptomycin Solution (100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Gibco-Invitrogen, USA) at 37 ºC in the presence of 5% (v/v) CO_2. The cells were grown until they reached 90% of confluence and were removed by trypsin/EDTA 0.25 % (Sigma, Madrid, Spain).

Cytotoxicity assay

The cytotoxicity of the essential oil of *Th. caespititius* from Planalto Central against cell line 23132/87 DSMZ nº ACC201 was determined by the MTT method (Vibrant MTT Cell Proliferation assay kit, Molecular Probes inc., Invitrogen US) using 96-well microplate (Greiner Bio-One GmbH). Four essential oil concentrations were tested (0.08 $mg.mL^{-1}$, 0.50 $mg.mL^{-1}$, 1.00 $mg.mL^{-1}$ and 2.00 $mg.mL^{-1}$). The tested essential oils concentrations were selected according the obtained MIC value for *H. pylori* and the higher values in the base of the IC_{50} value that corresponds to different antioxidant activities (Dandlen et al., 2010). All determinations were done in triplicate and, for control, the RPMI 1640 culture medium with no essential oil added, supplemented with 2- propanol and 5% H_2O_2 (v/v) (cytotoxic agent, positive control), was used. Carvacrol was tested at 61.9% (v/v) concentration, which corresponds to its percentage amount in the pure essential oil. The cell line viability was determined by absorbance (A_{540} nm) using a microplate reader (TECAN Infinite M200) after 30 min, 1, 4 and 8 h after essential oil contact.

The effect of the essential oil on intracellular Helicobacter pylori viability

Intracellular viability of *H. pylori* 26695 in the presence of the essential oil was determined on the human gastric adenocarcinoma cell line 23132/87 (DSMZ nº ACC201) following the procedure described by Fahey et al. (2002). The cells were cultured in RPMI 1640 medium and after trypsinization the cells were washed with Hank's balanced salt solution. A cell suspension of 8 x 10^5 cell mL^{-1} was used to inoculate each well of a 96-well microplate. The plates were incubated overnight at 37 °C in the presence of 5% CO_2. The culture monolayers were inoculated with *H. pylori* 26695 at 8 x 10^7. The incubation of the inoculated cell monolayers occurred during 12 h at 37 °C. The cells were then washed 6x with Hank's solution to eliminate nonadherent bacteria and incubated with 100 $\mu g.mL^{-1}$ of gentamicin during 1.5 h to kill extracellular bacteria. Before addition of the essential oil and control compounds the monolayers were washed 6x with Hank's solution. The monolayers were subjected to addition of 1) culture medium with no agents added 2) culture medium with 30 $\mu g.mL^{-1}$ chloramphenicol (MIC value for *H. pylori*), 3) culture medium supplemented with 0.08 $mg.mL^{-1}$ of *Th. caespititius* (Planalto Central) essential oil and 4) 61.9% of carvacrol (percentage of the compound in the essential oil sample). The microplates were incubated at 37 °C. The culture medium with no supplement was used as a negative control. To

determine the intracellular bacterial viability the cell line was washed 6x with Hank's solution and lysed with distilled water. The recovered bacteria were inoculated in Columbia agar supplemented with 10% (v/v) blood.

Data analysis

The percentage composition of the essential oils was used to determine the relationship between the different Portuguese thyme samples by principal components analysis (PCA) using the NTSYS-pc software (Rohlf, 1992). The results on the antimicrobial activity were used to perform a cluster analysis. A dendrogram was generated to illustrate the overall relationships between the tested essential oils. The dendrogram was constructed using UPGMA clustering using again the NTSYS software.

Results and Discussion

The identified compounds in the 28 essentials oils from Portuguese *Thymus* species analysed, from mainland Portugal and three Azores islands, are indicated in Table 2, as well as their essential oil yields. All essential oils are clearly dominated by monoterpenes (64-99%) with oxygen-containing monoterpenes (40-82%) being the major group in almost all samples (23) whereas monoterpene hydrocarbons (8-59%) appeared as the major group only in five samples. The essential oil yield (0.4-3.6% $v.w^{-1}$) shows some variation even within each species: *Th. caespititius* (0.4-2.3%), *Th. camphoratus* (0.7-1.9%), *Th. capitellatus* (1.7-3.6%), *Th. carnosus* (0.6-1.2%) and *Th. zygis* (0.5-1.0%).

In *Th. caespititius* essential oils, the five major components are carvacrol (t-62%), α-terpineol (4-50%), thymol (t-35%), carvacryl acetate (n.d.-19%) and *p*-cymene (2-14%). The variation of the major components in each oil allows to define three chemotypes: α-terpineol type (all samples from plants collected on mainland Portugal), carvacrol type (two samples from Pico island) and thymol type (samples from S. Jorge and Terceira islands). These results are according to previous studies on *Th caespititius* collected in the Azorean archipelago (Pereira et al., 2000; Pereira et al., 2003; Santos et al., 2005)

The five major components in the essential oils isolated from *Th. camphoratus* are 1,8-cineole (1-47%), borneol (1-23%), camphor (0.4-19%), α-pinene (4-12%) and terpinen-4-ol (0.4-10%). According to the dominant component in each sample, three are 1,8-cineole-rich essential oils whereas the fourth is borneol-rich. In *Th. capitellatus* essential oils, the five major components are 1,8-cineole (7-35%), borneol (16-22%), camphene (11-18%), camphor (5-18%) and α-pinene (9-14%). All the analysed essential oils from *Th. carnosus* are dominated by borneol (20-31%) and camphene (15-23%), being terpinen-4-ol (8-14%), α-pinene (4-10%) and bornyl acetate (4-10%) the other three major components.

In the case of *Th. zygis*, *p*-cymene (24-40%) is present in considerable amounts in all essential oil samples, whereas carvacrol (1-35%) dominates in three samples, one of which is from subsp. *zygis*, and thymol (1-24%) appears as the second major component in the other two samples. γ-Terpinene (5-11%) and camphene (2-6%) are the other two major components in these essential oils.

The scatter plot, obtained by PCA (Figure 1), showed four groups of individuals (A, B, C and D), along axis PC1 (44% of explained variance) and PC2 (18% of explained variance), according to their major volatile components. Borneol and camphene were the main compounds in the essential oils from the individuals in group A, the same for 1,8-cineole and borneol in group B and for carvacrol in group C. For individuals in group D, α-terpineol or thymol were the major compounds in their essential oils.

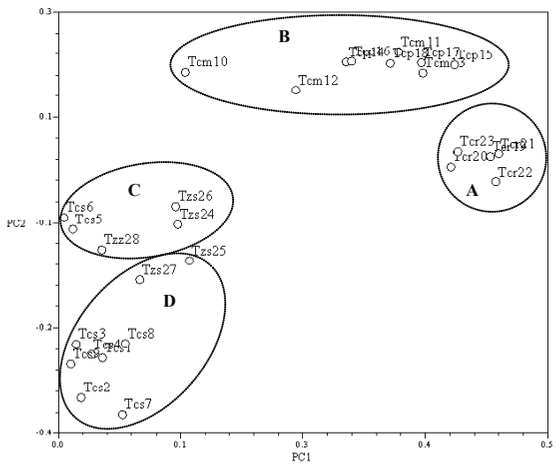

Figure 1. Principal components analysis of the twenty-eigth essential oils from five Portuguese thyme species, tested for antimicrobial activity. For the analysis, the complete composition (Table 2) of every essential oil sample, was considered. A. Borneol/camphene-rich oils, B. 1,8-Cineole/borneol-rich oils, C. Carvacrol-rich oils, D. Thymol or α-terpineol-rich oils.

The results obtained on the chemical characterization of the essential oils from the Portuguese thyme species used in this study, are according to the large chemical polymorphism for the majority of Portuguese *Thymus* taxa (Figueiredo et al., 2008).

The inhibitory activities of the tested *Thymus* essential oils are indicated in Table 3. The different microorganisms tested showed different susceptibilities to the diverse *Thymus* essential oils. The most

Table 2. Percentage composition of the essential oils of *Thymus* from Portugal.

Components	RI♦		*Thymus caespititius*									*Thymus camphoratus*				*Thymus capitellatus*					*Thymus carnosus*					*Thymus zygis* ssp. *sylvestris*			*T. zygis* ssp. *zygis*	
	DB-1	DB-17HT	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Tricyclene	921	1027	0.2	t	t	0.1	t	t	t	t	t	t	0.6	0.1	1.1	0.6	1.0	0.6	0.9	0.7	0.6	0.5	0.9	0.7	0.9	0.1	0.2	0.2	0.1	t
α-Thujene	924	1031	1.6	0.4	1.9	1.7	1.3	0.9	1.8	0.9	1.7	t	t	0.1	t	0.1	0.2	0.1	0.2	0.2	1.7	1.2	1.8	2.8	1.3	1.0	1.2	0.8	1.2	1.5
α-Pinene	930	1034	2.1	0.5	1.1	1.3	0.5	0.3	0.6	0.3	0.9	4.3	12.3	10.0	7.1	12.4	12.4	13.6	9.4	11.1	8.4	4.2	10.0	5.0	6.5	2.6	3.0	2.4	1.5	1.1
Camphene	938	1042	-	0.4	-	-	-	-	-	-	-	0.1	11.6	2.5	17.2	11.6	18.2	11.2	16.9	12.9	19.0	14.6	22.5	19.4	22.4	4.3	6.2	5.3	2.1	1.2
Thuja-2.4(10)-diene*	940	1055	3.2	t	0.5	1.5	t	t	t	0.1	0.6	0.2	0.4	1.1	0.5	0.3	0.5	0.6	0.3	0.3	0.4	0.4	0.5	0.5	0.5	t	0.1	t	t	-
Sabinene	958	1062	0.6	0.2	0.6	1.1	t	t	0.2	0.1	0.5	1.5	2.2	2.8	0.2	1.0	0.9	1.6	2.8	1.4	1.2	0.4	0.6	0.9	0.5	t	t	t	t	-
1-Octen-3-ol	961	na	t	0.2	t	t	t	t	0.2	0.1	t	t	t	t	t	-	-	-	-	-	t	t	t	t	t	t	t	t	t	0.2
β-Pinene	963	1064	0.5	0.4	0.5	0.5	0.1	0.1	0.1	0.1	0.2	1.1	1.6	0.7	0.7	1.9	1.3	1.9	1.5	2.0	3.1	1.9	2.5	2.5	2.3	0.7	0.7	0.7	0.2	-
Dehydro-1.8-cineole	973	na	t	t	t	0.3	t	t	t	t	t	0.1	0.1	0.4	0.1	0.1	0.1	0.2	0.1	0.1	t	t	t	0.1	t	-	-	-	-	-
3-Octanol	974	1073	1.0	0.9	1.0	1.3	t	t	t	t	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	t	t	t	0.9
β-Myrcene	975	1073	1.0	0.9	1.0	1.3	0.1	0.1	t	0.2	1.0	-	-	-	-	t	t	t	t	0.3	t	0.1	t	0.2	0.1	0.1	0.8	t	1.2	0.9
α-Phellandrene	995	1082	t	t	t	0.1	0.1	0.1	t	0.1	t	0.1	t	0.2	0.2	t	t	t	t	0.1	t	t	t	t	t	t	0.1	t	t	0.2
α-Terpinene	1002	1091	0.4	0.3	0.6	0.7	0.7	0.5	0.9	0.7	0.5	t	0.2	2.4	0.2	0.1	0.3	0.2	0.2	0.3	2.4	1.9	1.8	3.6	1.6	0.6	0.5	0.3	0.6	1.3
p-Cymene	1003	1120	13.4	13.6	13.9	13.8	4.4	2.3	11.5	8.2	13.2	0.2	1.0	3.6	0.7	0.5	0.8	0.5	1.0	0.6	1.9	2.8	2.9	2.0	3.5	23.5	35.9	24.6	39.5	28.3
β-Phellandrene	1005	1105	t	0.1	0.1	t	0.2	0.1	t	0.1	t	-	-	-	-	-	-	-	-	-	t	0.2	0.1	0.3	0.2	1.6	0.2	1.3	t	-
1,8-Cineole	1005	1116	-	-	-	-	-	-	-	-	-	46.6	26.5	36.7	0.8	35.1	6.9	33.8	21.1	25.8	-	-	-	-	-	-	-	-	-	0.3
Limonene	1009	1097	2.1	1.2	2.4	2.0	1.7	1.1	0.9	1.2	1.8	0.7	1.4	0.8	t	1.5	2.0	2.3	1.2	1.5	2.9	1.5	2.2	1.3	1.3	2.8	0.9	2.3	1.3	0.4
trans-β-Ocimene	1027	na	t	t	t	t	t	t	t	t	t	0.7	t	t	t	t	t	t	t	0.3	1.0	1.2	1.0	2.1	0.4	t	t	t	t	-
γ-Terpinene	1035	1132	5.6	5.9	6.1	8.7	3.4	2.0	9.6	3.8	6.2	0.1	0.7	4.1	0.3	0.3	0.5	0.3	0.5	0.6	5.1	4.5	3.7	6.7	3.2	7.7	7.1	4.6	10.7	18.4
trans-Sabinene hydrate	1037	na	t	t	t	t	t	t	0.2	0.2	t	t	0.7	1.3	0.8	0.3	0.6	0.3	0.6	0.2	1.5	0.5	1.2	0.7	0.5	1.1	0.1	1.0	0.2	0.4
cis-Linalool oxide	1045	1159	-	-	-	-	-	-	-	-	-	2.2	0.2	0.4	2.0	0.1	0.1	0.2	0.1	t	-	-	-	-	-	-	-	-	-	t
trans-Linalool oxide	1059	na	-	-	-	-	-	-	-	-	-	t	0.1	0.3	1.3	0.1	0.3	0.2	0.1	0.4	-	-	-	-	-	t	t	t	t	-
Terpinolene	1064	1163	0.1	0.1	0.3	0.2	1.0	0.7	0.1	0.8	0.2	1.2	t	0.9	0.2	0.1	0.1	t	0.1	0.2	0.6	0.8	0.5	1.2	0.5	t	0.1	t	0.1	0.1
cis-Sabinene hydrate	1066	1163	t	t	t	t	t	t	t	t	t	t	0.1	0.5	0.1	-	-	-	-	-	1.9	0.3	1.5	1.0	0.8	0.1	t	0.4	t	-
Linalool	1074	1191	t	t	t	t	t	t	t	t	t	12.2	1.1	0.7	9.5	1.1	2.6	2.2	1.8	5.8	t	t	t	t	t	4.1	3.5	3.7	4.3	3.1
α-Campholenal	1088	na	-	-	-	-	-	-	-	-	-	t	0.1	0.6	0.2	0.2	0.3	0.2	0.2	0.2	t	t	t	t	t	t	t	t	t	-
trans-p-2-Menthen-1-ol	1095	1207	t	t	t	t	t	t	t	t	t	0.1	t	0.6	t	t	t	t	t	t	0.5	0.5	0.3	0.6	0.3	-	-	-	-	-
Camphor	1095	1281	-	-	-	-	-	-	-	-	-	0.4	11.1	2.2	19.1	7.2	17.8	5.0	10.5	4.8	2.6	4.2	2.8	2.0	4.0	2.0	3.1	2.4	1.2	0.2

trans-Pinocarveol	1106	na	-	-	-	-	-	-	-	-	-	t	0.4	0.7	0.3	0.3	0.3	0.4	0.3	0.3	0.2	0.3	0.2	0.2	0.2	-	-	-	-	-
cis-Verbenol	1110	1247	t	t	t	t	t	t	t	t	t	-	-	-	-	0.2	0.2	0.2	0.1	t	0.2	0.2	0.2	0.2	0.2	-	-	-	-	-
cis-p-2-Menthen-1-ol	1110	na	t	t	t	t	t	t	t	t	t	t	t	t	t	-	-	-	-	-	0.2	0.2	0.2	0.2	0.2	-	-	-	-	-
trans-Verbenol	1114	1263	-	-	-	-	-	-	-	-	-	0.7	1.9	2.6	0.8	1.0	1.0	1.0	0.9	0.5	1.5	1.2	1.3	1.1	1.3	-	-	-	-	-
Pinocarvone	1121	na	-	-	-	-	-	-	-	-	-	0.1	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.1	t	t	t	t	t	-	-	-	-	-
Isoborneol	1132	na	-	-	-	-	-	-	-	-	-	-	-	-	-	t	0.2	t	0.2	t	-	-	-	-	-	-	-	-	-	-
Borneol	1134	1275	3.2	2.3	0.6	2.4	t	t	t	t	0.8	0.8	14.9	4.3	23.2	16.2	20.4	16.9	22.4	21.0	24.6	31.1	20.1	20.3	28.6	6.4	6.4	9.7	2.2	1.4
Terpinen-4-ol	1148	1279	1.5	1.3	1.1	1.3	1.2	0.8	0.5	0.5	0.8	0.4	2.1	9.7	1.1	0.9	1.4	1.2	1.5	1.5	11.7	11.5	8.3	13.6	7.5	3.1	0.7	1.8	0.5	0.4
p-Cymen-8-ol	1148	na	0.2	0.2	0.1	0.3	t	t	t	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1
Myrtenal	1153	na	-	-	-	-	-	-	-	-	-	0.1	0.1	0.5	0.1	0.2	0.3	0.3	0.2	0.2	-	-	-	-	-	-	-	-	-	-
α-Terpineol	1159	1307	41.0	35.6	49.8	40.7	3.6	4.8	19.1	4.3	42.4	3.6	0.4	1.2	1.4	1.0	0.5	1.0	0.6	1.7	0.4	0.6	0.3	0.4	0.2	0.2	t	0.8	1.4	t
Verbenone	1164	na	-	-	-	-	-	-	-	-	-	0.2	0.1	0.8	0.2	-	-	-	-	-	t	0.1	t	0.1	0.1	-	-	-	-	-
Myrtenol	1168	na	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.5	0.4	0.3	0.1	-	-	-	-	-	-	-	-	-	-
Hexyl butanoate	1173	na	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.3	0.4	0.3	0.3	-	-	-	-	-	-	-	-	-	t
Bornyl formate	1199	1341	-	-	-	-	-	t	t	t	t	t	0.2	0.1	0.4	0.5	0.7	0.4	0.5	0.4	0.2	0.4	0.3	0.2	0.4	0.2	0.4	0.4	0.1	0.1
Carvacryl methyl ether	1224	na	0.1	0.5	0.2	0.3	0.3	t	t	0.2	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geraniol	1236	1467	-	-	-	-	-	-	-	-	-	-	-	-	-	t	t	t	t	t	-	-	-	-	-	t	0.8	t	t	-
Linalyl acetate	1245	1356	-	-	-	-	-	-	-	-	-	8.8	t	t	3.1	t	1.3	t	t	0.9	-	-	-	-	-	-	-	-	-	-
Thymyl formate	1262	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	0.1	t	0.1	t
Bornyl acetate	1265	1409	1.6	0.3	0.2	0.5	-	-	-	-	0.1	-	0.6	1.2	1.1	0.7	1.0	0.3	0.6	0.7	3.7	10.0	10.2	7.9	9.0	-	0.1	-	0.1	-
Thymol	1275	1463	t	t	t	t	3.2	2.4	24.9	34.8	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.0	24.2	0.9	21.7	0.4
Carvacrol	1286	1479	t	0.2	t	t	48.3	61.7	1.7	12.7	t	t	t	t	t	t	t	t	t	t	-	-	-	-	-	31.8	1.7	34.5	1.1	36.7
Thymyl acetate	1330	1548	-	-	-	-	1.2	0.9	2.1	7.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
δ-Elemene	1332	na	-	-	-	-	0.6	0.9	t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Terpinyl acetate	1334	na	-	-	-	-	-	-	-	-	-	t	t	0.1	t	-	-	-	-	-	t	t	0.1	0.1	0.1	-	-	-	-	-
Carvacryl acetate	1348	1567	-	-	-	-	19.4	11.4	t	2.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geranyl acetate	1370	na	-	-	-	-	-	-	-	-	-	0.6	t	t	t	t	t	t	t	t	-	-	-	-	-	t	t	t	t	-
β-Bourbonene	1379	na	0.3	0.4	t	0.1	t	t	t	0.2	0.1	-	-	-	-	t	t	t	t	t	t	t	t	t	t	-	-	-	-	-
β-Cubebene	1385	na	t	t	t	0.1	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Elemene	1388	na	t	0.1	t	0.2	t	t	t	t	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bornyl isobutyrate	1398	na	-	-	-	-	-	-	-	-	-	0.2	0.1	t	0.2	t	0.2	t	t	0.1	-	-	-	-	-	-	-	-	-	-
α-Gurjunene	1400	na	0.1	0.1	t	0.1	t	t	t	t	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t	t	t	t	t

trans-β-Caryophyllene	1414	1500	1.3	2.3	0.9	2.8	t	0.1	0.1	t	3.7	-	-	-	-	0.1	0.2	0.1	t	0.1	0.7	0.2	0.4	0.3	0.2	0.6	0.8	0.7	0.8	1.4
β-Copaene	1426	na	t	t	0.1	t	t	0.1	0.2	0.1	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Humulene	1447	1549	0.2	t	t	0.1	0.1	t	t	t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t
Bornyl butyrate	1451	na	-	-	-	-	-	-	-	-	-	t	0.1	0.1	0.2	t	0.1	t	t	0.1	-	-	-	-	-	-	-	-	-	-	-
allo-	1456	na	t	0.6	0.1	t	0.1	0.2	0.8	0.3	t	t	0.1	0.1	0.2	t	0.1	t	t	0.2	0.2	0.1	0.2	0.1	t	-	-	-	-	-
γ-Muurolene	1469	1578	t	0.1	t	t	t	t	t	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-Muurola-4(14),5-diene*	1453	na	t	0.3	0.1	t	t	t	t	t	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Germacrene-D	1474	na	0.6	1.0	0.1	0.5	0.1	t	0.1	0.2	1.7	t	t	t	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t
β-Selinene	1476	na	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.4	t	0.2	0.2	-	-	-	-	-	-	-	-	-	-
trans-Muurola-4(14),5-diene*	1479	na	0.3	-	0.5	0.1	t	t	t	t	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Viridiflorene	1487	na	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	t	t	t	-	-	-	-	-	-	-	-	-	-
trans-β-Dihydroagarofuran*	1489	1627	1.8	3.0	0.5	0.9	0.9	0.4	2.0	1.6	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Muurolene	1494	na	0.1	0.2	0.3	0.3	0.2	0.1	0.2	0.4	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bornyl 2-methyl butyrate	1495	na	-	-	-	-	-	-	-	-	-	0.4	0.2	0.1	0.4	0.2	0.3	0.1	-	0.3	-	-	-	-	-	-	-	-	-	-
γ-Cadinene	1500	1660	1.7	2.6	2.1	0.9	0.5	0.5	2.0	1.3	0.9	0.2	0.2	0.1	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
trans-Calamenene	1505	na	0.1	0.3	0.6	t	0.1	0.1	0.2	0.3	t	-	-	-	-	0.1	0.1	t	t	0.1	-	-	-	-	-	-	-	-	-	-
γ-Cadinene	1505	na	0.4	1.0	0.7	0.9	0.4	0.4	0.6	0.2	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	t
Kessane	1517	na	1.0	1.0	t	0.1	0.5	0.2	0.9	0.6	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Calacorene	1525	na	t	t	t	t	-	-	-	-	t	-	-	-	-	0.1	0.1	0.1	0.1	t	-	-	-	-	-	-	-	-	-	-
α-Cadinene	1529	na	t	0.1	0.1	t	t	0.4	0.1	0.1	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Elemol	1530	na	0.3	0.6	0.4	0.6	0.1	t	0.5	0.1	1.0	-	-	-	-	-	-	-	-	-	0.4	0.8	0.4	0.7	0.4	-	-	-	-	-
Spathulenol	1551	na	t	0.1	t	t	t	0.1	t	t	0.2	t	t	0.2	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Caryophyllene oxide	1561	1751	t	-	0.1	0.1	-	-	-	-	0.1	1.5	1.3	1.5	0.7	0.7	0.8	0.5	0.3	0.2	-	-	-	-	-	0.3	0.9	0.9	0.6	0.3
Globulol	1566	na	0.1	t	1.0	1.3	t	t	0.2	t	0.1	0.3	0.4	0.4	0.1	t	t	0.1	0.1	t	-	-	-	-	-	-	-	-	-	-
Viridiflorol	1569	1738	-	-	-	-	-	-	-	-	-	-	-	-	-	1.5	1.2	0.8	0.8	0.3	0.4	0.3	0.3	t	0.1	-	-	-	-	-
Ledol	1580	na	-	-	-	-	-	-	-	-	-	0.2	0.2	0.4	0.1	0.2	0.2	0.3	0.3	0.1	-	-	-	-	-	-	-	-	-	-
n.i. *Th.caespititius* A	1597	1758	1.1	1.9	0.2	0.6	0.4	0.2	1.1	0.7	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-*epi*-Cubenol	1600	na	1.4	2.8	0.7	0.9	-	-	-	-	1.2	-	-	-	-	0.3	0.3	0.1	0.2	0.2	-	-	-	-	-	-	-	-	-	-
n.i. *Th.caespititius* B	1609	1766	1.4	2.8	0.7	0.9	1.6	1.2	4.4	2.7	1.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
o-Amorphene	1605	na	0.1	0.2	t	0.2	t	t	t	t	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

γ-Eudesmol	1609	na	0.5	0.9	0.4	0.9	0.2	0.3	0.4	0.1	1.7	-	-	-	-	-	-	-	-	-	t	0.2	t	0.1	t	-	-	-	-	-
τ-Cadinol	1616	1808	5.3	7.6	6.0	2.7	1.5	1.9	6.6	4.7	3.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Muurolol	1618	1809	0.5	0.6	0.4	0.6	0.1	0.2	0.3	t	1.2	2.0	1.9	0.1	1.3	t	t	t	t	t	-	-	-	-	-	-	-	-	-	-	-
β-Eudesmol	1620	na	0.5	0.6	0.4	0.6	0.1	0.2	0.3	t	1.2	-	-	-	-	-	-	-	-	-	t	0.2	t	0.1	0.1	-	-	-	-	-
α-Cadinol	1626	1850	-	t	-	-	-	-	-	-	-	3.8	0.4	t	0.4	0.1	0.1	0.2	0.2	0.2	-	-	-	-	-	-	-	-	-	-	t
α-Eudesmol	1634	1845	0.8	1.2	1.3	1.7	0.9	1.0	1.4	3.3	3.0	-	-	-	-	-	-	-	-	-	t	0.1	t	0.1	t	-	-	-	-	-
n.i. *Th.caespititius* C	1648	1854	0.6	1.8	0.1	0.5	0.4	0.3	1.4	1.0	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
epi-α-Bisabolol	1658	na	t	t	0.3	0.3	-	-	-	-	t	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
n.i. *Th.caespititius* D	1662	1865	-	0.1	-	-	0.1	0.2	1.1	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Abietatriene	2027	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	t	0.1	-	-	-	-	-
% Identification			96.8	93.2	99.1	97.1	97.1	97.3	91.3	93.4	95.9	95.7	97.7	97.4	97.9	99.9	99.3	99.9	99.7	99.6	99.4	99.3	99.4	99.3	99.5	99.9	99.8	99.7	92.8	99.3
Grouped Components																														
Monoterpene hydrocarbons			30.8	24.0	29.0	33.0	13.5	8.2	25.7	16.6	26.8	10.2	32.0	29.3	28.4	30.4	38.2	32.9	35.0	32.5	48.3	36.2	51.0	49.2	45.2	45.0	57.0	42.5	58.5	53.4
Oxygen-containing monoterpenes			47.6	40.4	52.0	45.8	77.2	82.0	48.5	63.1	44.5	77.5	61.1	65.2	66.5	65.8	57.1	64.4	62.2	65.1	49.2	61.1	47.0	48.7	53.4	54.0	41.1	55.6	32.9	43.1
Sesquiterpene hydrocarbons			5.1	9.1	5.6	6.1	2.1	2.8	4.3	3.2	9.3	0.2	0.4	0.3	0.4	0.6	1.1	0.2	0.3	0.7	1.0	0.3	0.6	0.4	0.2	0.6	0.8	0.7	0.8	1.4
Oxygen-containing sesquiterpenes			12.3	18.6	11.5	10.9	4.3	4.3	12.6	10.4	14.3	7.8	4.2	2.6	2.6	2.8	2.6	2.0	1.9	1.0	0.8	1.6	0.7	1.0	0.6	0.3	0.9	0.9	0.6	0.3
Diterpenes																					0.1	0.1	0.1	0.0	0.1					
Others			1.0	1.1	1.0	1.3	0.0	0.0	0.2	0.1	1.0	0.0	0.0	0.0	0.0	0.3	0.3	0.4	0.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.1
Oil yield (vw^{-1})			0.8	1.0	2.3	0.8	0.4	0.6	0.7	0.6	0.6	0.9	1.1	0.7	1.9	2.2	1.7	2.6	2.0	3.6	0.6	1.1	1.2	0.9	1.2	1.0	0.7	1.0	0.5	0.5

*Based on mass spectra only; n.i.: not identified; •Retention index relative to C_9-C_{21} *n*-alkanes; na: not available; t: trace (<0.05%); -: not detected; MP: mainland Portugal.

Thymus caespititius
1. Caramulo (MP)
2. Lordelo (MP)
3. Óbidos (MP)
4. Outeiro (MP)
5. Pico (Pico, Azores)
6. Planalto Central (Pico, Azores)
7. Ponta dos Rosais (S. Jorge, Azores)
8. Serra do Cume (Terceira, Azores)
9. Vilarinho das Furnas (MP)

Thymus camphoratus
10. Atalaia (MP)
11. Boca Rio (MP)
12. Cabo S. Vicente (MP)
13. Espartal (MP)

Thymus capitellatus
14. Alcácer do Sal (MP)
15. Carvalhal (MP)
16. Santiago de Cacém (MP)
17. Sines-Grândola (MP)
18. Tróia (MP)

Thymus carnosus
19. Carvalhal (MP)
20. Praia do Barril (MP)
21. Qta do Lago (MP)
22. Tróia (MP)
23. V. R. Sto. António (MP)

Thymus zygis subsp. *sylvestris*
24. Alcanena (MP)
25. Condeixa (MP)
26. Covão do Coelho (MP)
27. Duas Igrejas (MP)

Thymus zygis subsp. *zygis*
28. Rebordãos (MP)

Table 3. Inhibitory activity of *Thymus* essential oils.

	Inhibition zone (mm)*							
Essential oil	*H. pylori* J99	*H. pylori* 26695	*S. aureus* CFSA2	*L. monocytogenes* T8	*L. monocytogenes* C882	*L. monocytogenes* EGD	*S. Thyphimurium* ATCC 14028	*H. influenza* ATCC 49247
Th. caespititius Caramulo	10.67±0.58^{defg}	14.67±1.15^{hij}	7.33±0.58^{cde}	14.67±0.58^{b}	14.83±1.04^{bc}	14.67±0.58^{b}	7.67±0.58^{efg}	14.00±1.73^{efg}
Th. caespititius Lordelo	11.67±0.58^{de}	16.00±1.00^{defghi}	NIA±0.00^{ef}	8.67±0.58^{efghij}	10.17±0.76^{e}	8.67±0.58^{efghi}	8.33±0.58^{cde}	10.00±0.00^{klm}
Th. caespititius Outeiro	12.33±0.58^{d}	15.00±0.00^{ghij}	6.67±0.58^{cde}	8.50±0.87^{efghij}	9.00±0.00^{fgh}	8.50±0.87^{efghij}	8.33±1.15^{defg}	10.33±0.58^{klm}
Th. caespititius Pico	23.67±1.15^{b}	21.67±2.89^{b}	7.33±0.29^{cde}	14.67±0.58^{b}	15.67±0.58^{b}	14.67±0.58^{b}	9.67±0.58^{c}	14.50±0.87^{def}
Th. caespititius Planalto Central	16.67±0.58^{b}	22.50±4.33^{b}	9.67±0.58^{bcd}	10.33±0.58^{cd}	10.67±0.58^{e}	10.33±0.58^{cd}	9.00±0.00^{cde}	13.33±0.58^{efg}
Th. caespititius Ponta dos Rosais	17.67±1.15^{c}	18.17±0.29^{cd}	7.00±1.00^{cde}	9.67±0.58^{def}	10.83±0.76^{e}	9.67±0.58^{defg}	8.00±0.00^{defg}	13.00±3.46^{fghi}
Th. caespititius Serra do Cume	11.67±0.58^{de}	14.67±0.58^{hij}	8.33±0.58^{bcd}	8.67±2.31^{efghij}	9.83±1.26^{efg}	8.67±2.31^{efghij}	9.67±0.58^{c}	10.00±0.00^{klm}
Th. caespititius Vilarinho das Furnas	10.33±0.58^{defg}	15.00±0.00^{ghij}	NIA±0.00^{ef}	8.33±0.58^{fghij}	8.83±0.29^{ghi}	8.33±0.58^{fghij}	7.33±0.58^{efg}	10.00±0.00^{klm}
Th. caespititus Óbidos	8.67±0.58^{fghi}	17.00±0.00^{cdefgh}	6.83±1.44^{cde}	8.33±0.58^{fghij}	8.00±0.00^{hijk}	8.33±0.58^{fghij}	NIA±0.00^{fg}	17.33±0.58^{b}
Th. camphoratus Atalaia	12.3±1.15^{d}	17.67±0.58^{cdefgh}	6.67±0.58^{cde}	9.33±0.58^{def}	9.00±0.00^{fgh}	9.33±0.58^{defgh}	7.33±0.58^{efg}	10.00±0.00^{klm}
Th. camphoratus Boca Rio	10.33±0.58^{defg}	17.33±1.53^{cdefgh}	6.67±0.58^{cde}	9.33±0.29^{hijk}	10.83±0.29^{e}	9.33±0.29^{defg}	7.33±0.58^{efg}	11.33±1.15^{ijkl}
Th. camphoratus Cabo S.Vicente	10.83±0.76^{def}	15.33±0.58^{fghi}	7.00±0.00^{cde}	7.67±1.53^{ijkl}	7.83±0.29^{ijk}	7.67±1.53^{hijk}	8.00±0.00^{defg}	11.67±0.58^{hijk}
Th. camphoratus Espartal	11.50±0.87^{de}	15.33±0.58^{fghi}	6.56±0.58^{defg}	7.33±1.53^{def}	10.33±0.58^{e}	7.33±1.53^{ijkl}	7.33±0.58^{efg}	12.33±0.58^{ghij}
Th. capitellatus Alcácer do Sal	10.67±0.76^{def}	14.50±0.87^{ij}	6.67±0.58^{cde}	6.67±0.58^{kl}	7.50±0.50^{ijk}	6.67±0.58^{kl}	7.33±2.31^{efg}	9.33±0.58^{m}
Th. capitellatus Carvalhal	11.33±1.15^{de}	17.67±1.15^{cde}	7.17±0.29^{cde}	10.33±0.58^{cde}	10.00±0.00^{ef}	10.33±0.58^{cd}	8.67±0.58^{cde}	10.00±0.00^{klm}
Th. capitellatus Santiago de Cacém	8.17±2.25^{ghi}	14.50±0.87^{ij}	7.17±0.29^{def}	8.33±0.58^{fghij}	14.33±0.58^{c}	8.33±0.58^{fghij}	7.33±1.15^{efg}	10.00±0.00^{klm}
Th. capitellatus Sines - Grândola	10.83±1.44^{def}	12.67±0.58^{ij}	7.00±0.00^{cde}	8.00±0.00^{ghijk}	8.50±0.50^{hij}	8.00±0.00^{ghijk}	8.00±3.46^{defg}	10.67±0.58^{iklm}
Th. capitellatus Tróia	10.33±1.04^{defg}	12.67±0.58^{ij}	6.63±0.00^{def}	8.00±0.00^{ghijk}	14.33±0.58^{c}	8.00±0.00^{ghijk}	NIA±0.00^{g}	10.00±0.00^{klm}
Th. carnosus Carvalhal	9.67±3.21^{efghi}	14.67±0.58^{hij}	6.33±0.58^{dfg}	6.00±0.00^{l}	6.00±0.00^{l}	NIA±0.00^{l}	7.00±0.00^{fg}	10.33±0.58^{klm}
Th. carnosus Praia do Barril	10.67±0.58^{defg}	14.33±1.15^{ij}	6.67±0.58^{cde}	7.00±1.73^{jkl}	10.17±0.29^{e}	7.00±1.73^{jkl}	7.00±0.00^{fg}	9.33±0.58^{m}
Th. carnosus Quinta do Lago	10.00±0.00^{defgh}	16.33±1.15^{cdefghi}	7.00±0.00^{cde}	7.00±1.00^{kl}	9.00±0.00^{fgh}	7.00±1.00^{jkl}	7.00±0.00^{fg}	9.67±0.58^{lm}
Th. carnosus Tróia	8.00±2.08^{fghi}	15.00±0.00^{ghij}	7.33±0.58^{cde}	7.00±0.0^{jkl}	7.17±0.29^{k}	7.00±0.00^{jkl}	8.33±0.58^{cde}	10.67±1.15^{iklm}
Th. carnosus V.R.Sto. António	10.33±0.58^{defg}	15.67±0.58^{efghi}	6.67±0.58^{cde}	NIA±0.00^{l}	8.00±0.00^{hijk}	NIA±0.00^{l}	8.00±0.00^{defg}	15.33±0.58^{cde}
Th. zygis sylvestris Alcanena	7.67±2.89^{hi}	15.33±0.58^{fghi}	7.33±1.53^{cde}	9.17±0.29^{defgh}	10.00±0.00^{ef}	9.17±0.29^{defgh}	7.00±0.00^{fg}	11.33±1.15^{ijkl}
Th. zygis sylvestris Condeixa	10.00±0.00^{defgh}	18.00±0.00^{cde}	8.67±0.29^{bcd}	10.00±0.00^{de}	10.17±0.29^{e}	10.00±0.00^{def}	9.67±0.58^{c}	13.83±1.04^{efg}
Th. zygis sylvestris Covão do Coelho	10.33±0.29^{defg}	17.33±1.53^{cdefgh}	7.00±1.00^{cde}	9.67±0.29^{defgh}	9.83±0.29^{efg}	9.67±0.29^{def}	9.33±0.58^{cde}	14.67±0.58^{def}
Th. zygis sylvestris Duas Igrejas	7.33±2.31^{i}	18.67±0.58^{c}	6.67±1.15^{cde}	9.67±0.58^{def}	10.00±0.00^{ef}	9.67±0.58^{def}	7.00±0.00^{fg}	16.00±1.32^{cde}
Th. zygis zygis Rebordãos	16.00±1.73^{c}	20.83±1.44^{b}	7.67±0.29^{cde}	11.67±0.58^{b}	12.67±1.15^{d}	11.67±0.58^{c}	11.83±2.75^{b}	16.67±0.58^{bc}
Chloramphenicol/Amphotericin B	36.00±1.00^{a}	36.33±0.58^{a}	17.67±0.58^{a}	23.33±0.58^{a}	22.33±1.53^{a}	24.33±1.15^{a}	23.33±2.08^{a}	26.50±0.71^{a}

Table 3. (Continued)

Essential oil	Inhibition zone (mm)*		
	S. pneumoniae D39	*C. albicans* ATCC90028	*C. albicans* YP0048
Th. caespititius Caramulo	11.33±0.58^{hij}	NIA±0.00^{e}	NIA±0.00^{g}
Th. caespititius Lordelo	15.00±0.00^{cdef}	ND±NDnd	ND±NDnd
Th. caespititius Outeiro	13.33±0.58^{fghi}	NIA±0.00^{e}	NIA±0.00^{g}
Th. caespititius Pico	13.67±1.15^{efgh}	ND±NDnd	ND±NDnd
Th. caespititius Planalto Central	19.00±0.87^{b}	NIA±0.00^{e}	NIA±0.00^{g}
Th. caespititius Ponta dos Rosais	15.00±0.00^{cdef}	NIA±0.00^{e}	NIA±0.00^{g}
Th. caespititius Serra do Cume	16.83±1.61^{cde}	NIA±0.00^{e}	NIA±0.00^{g}
Th. caespititius Vilarinho das Furnas	12.33±0.58^{ghij}	NIA±0.00^{e}	NIA±0.00^{g}
Th. caespititus Óbidos	15.83±1.44^{cde}	NIA±0.00^{e}	NIA±0.00^{g}
Th. camphoratus Atalaia	14.33±0.58^{defg}	8.33±0.58^{c}	7.83±0.29^{e}
Th. camphoratus Boca Rio	16.00±0.00^{cde}	9.33±0.58nd	9.50±0.50^{d}
Th. camphoratus Cabo S.Vicente	11.33±0.29^{hij}	NIA±0.00^{e}	NIA±0.00^{g}
Th. camphoratus Espartal	16.17±0.29^{cd}	9.33±0.58^{c}	NIA±0.00^{g}
Th. capitellatus Alcácer do Sal	12.00±0.00^{ghij}	NIA±0.00^{e}	NIA±0.00^{g}
Th. capitellatus Carvalhal	14.33±1.15^{defg}	9.00±0.87^{c}	10.00±0.00^{c}
Th. capitellatus Santiago de Cacém	13.00±1.73^{fghi}	NIA±0.00^{e}	NIA±0.00^{g}
Th. capitellatus Sines - Grândola	13.00±1.73^{fghi}	NIA±0.00^{e}	NIA±0.00^{g}
Th. capitellatus Tróia	11.00±0.00^{ij}	NIA±0.00^{e}	NIA±0.00^{g}
Th. carnosus Carvalhal	13.33±0.29^{fghi}	NIA±0.00^{e}	NIA±0.00^{g}
Th. carnosus Praia do Barril	10.50±0.50^{j}	6.67±0.58^{e}	NIA±0.00^{g}
Th. carnosus Quinta do Lago	14.00±0.87^{defg}	NIA±0.00^{e}	NIA±0.00^{g}
Th. carnosus Tróia	12.33±0.29^{ghij}	NIA±0.00^{e}	NIA±0.00^{g}
Th. carnosus V.R.Sto. António	15.00±0.00^{cdef}	NIA±0.00^{e}	NIA±0.00^{g}
Th. zygis sylvestris Alcanena	15.00±0.00^{cdef}	12.17±0.29^{b}	13.17±0.29^{b}
Th. zygis sylvestris Condeixa	20.00±4.33^{c}	NIA±0.00^{e}	NIA±0.00^{g}
Th. zygis sylvestris Covão do Coelho	19.07±1.85b	ND±NDnd	ND±NDnd
Th. zygis sylvestris Duas Igrejas	19.17±0.29^{b}	NIA±0.58^{e}	7.17±0.29^{f}
Th. zygis zygis Rebordãos	15.33±0.58^{cdef}	9.50±0.50^{c}	10.33±0.58^{c}
Chloramphenicol/Amphotericin B	23.67±2.31^{a}	15.67±1.15^{a}	15.67±1.15^{a}

*Inhibition zone includes the diameter of the disc (6 mm); Data in the same column with different letters are significantly different (p<0.05); ND- Not determined, NIA- no inhibitory activity.

susceptible to the majority of *Thymus* essential oil was *H. pylori* (both strains, J99 and 26695) followed by *Streptococcus pneumoniae* D39. *S. aureus, Salmonella Thyphimurium* and *Candida albicans* showed to be resistant to the majority of the tested *Thymus* essential oils. *L. monocytogenes* strains showed an intermediate susceptibility, in particular to *Th. capitellatus* from Tróia and *Th. caespititius* from Pico (Table 3).

Besides both *H. pylori* strains could be considered the most susceptible bacteria it was evident that they display a different susceptibility to the same essential oil, namely *H. pylori* J99 showed a very low inhibition zone (8.17±2.25 mm) to the essential oil of *Th. capitellatus* (Santiago de Cacém) but the strain *H. pylori* 26695 showed a higher inhibition zone (14.50±0.87 mm). The same behaviour can be seen for the essential oils of *Th. caespititius* (Óbidos), *Th. zygis* subsp. *sylvestris* (Duas Igrejas and Alcanena) and *Th. carnosus* (Tróia). The essential oils of *Th. caespititius* (Planalto Central), *Th. zygis* subsp. *zygis* (Rebordãos) and *Th. caespititius* (Pico) produced the highest inhibition zone when tested against *H. pylori* 26695, namely 22.50±4.33 mm, 20.83±1.44 mm and 21.67±2.89 mm, respectively. The MIC value varied from 0.05 to 0.08 mg.mL^{-1} for *Th. caespititius* (Planalto Central), *Th. zygis* Bragança (Rebordãos) and *Th. caespititius* (Pico) essential oils determined for *H. pylori* 26695. Carvacrol, a phenolic monoterpene, is the main component of these essential oils.

The cluster analysis based on the antimicrobial activity data (Figure 2) apart from showing a low correlation between the diverse tested essential oils, revealed a distinctive grouping pattern from that of the PCA of the composition of the essential oils (Figure 1).

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was previously observed, in one of our studies with rich-carvacrol essential oils of *T. pallescens* from Algeria (Hazzit et al., 2009). Nevertheless and as verified in the present work, other components must have a predominant role, either alone or in combination with carvacrol on the antimicrobial activity, because other oils also with high percentages of carvacrol did not show a significant activity. The complexity of the chemical composition of the essential oils with dozen of compounds makes the process of the identification of the component responsible for the antimicrobial activity very difficult. Often the antimicrobial activity results from the synergism or antagonism between several components. In our study one of the examples is the result obtained for *T. zygis* subsp. *sylvestris* from Covão do Coelho and from Alcanena, with relative high amounts of carvacrol, comparable to that of *T. zygis* subsp. *zygis*, but not showing similar inhibitory activity. These results, suggest that, despite the high percentage of some compounds, the presence of other minor components or chiral isomers in the essential oils, providing a synergistic or antagonistic effect, can be determinant for their bioactivity.

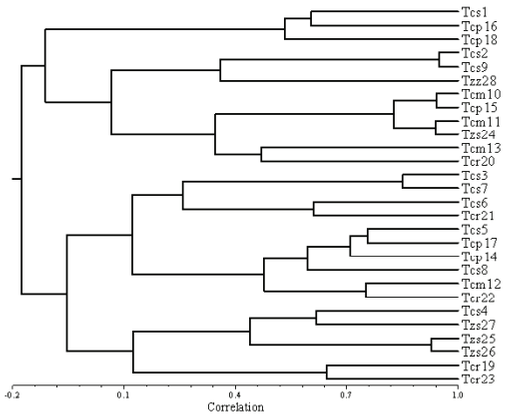

Figure 2. Dendrogram based on unweighted pair-group method with arithmetic average (UPGMA) showing the correlation among the twenty-eigth Portuguese thyme essential oils tested for their antimicrobial activity.

The cytotoxicity of *Th. caespititius* (Planalto Central) to adenocarcinoma gastric cells (ACC201) was determined and the results are presented in Figure 3. The essential oil used at the MIC value (0.08 $mg.mL^{-1}$) on the first 30 min the cell line decrease the viability to 45% but at the end of the first hour recovered the viability to 64% and until the end of the assay (8 h) the viability was maintained at 95%. Higher concentrations of the essential oil, 0.50 and 1.00 $mg.mL^{-1}$ were detrimental to the viability of the gastric cell line, namely on the first 30 min the viability decrease to 30.00±0.05 and 20.00±0.05%, respectively and maintains this low value after 8 h (Figure 3). Carvacrol (61.9%, v/v) had no effect on the gastric cell viability. The essential oil solvent, 2-propanol caused a slight decrease on the viability of the gastric cell line, the viability was between 90 and 80% during the assay period. The activity of H_2O_2 (5%, v/v) was injurious to the cell line, as predicted.

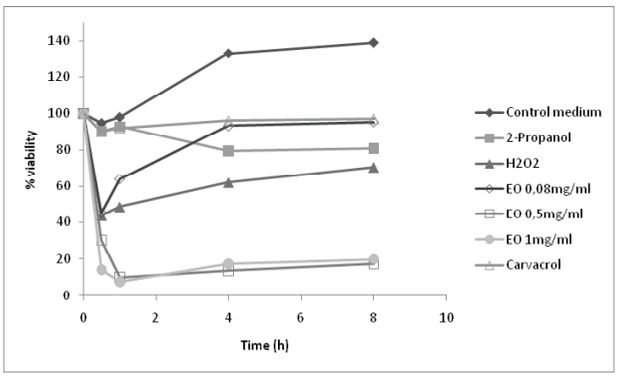

Figure 3. Citotoxicity of the essential oil (EO) of *Thymus caespititus* on the viability of the human gastric adenocarcinoma cell line 23132/87 DSMZ. The RPMI 1640 medium with no agents added and supplemented with 2-propanol or with 5% H_2O_2 (v/v) were used as control. The main component of the *Thymus caespititus* essential oil, carvacrol (61.9%, v/v) was tested. Data is the mean of three replicates. The standard error bars represent the standard deviation and are not visible as they are within the symbol area.

The intracellular antibacterial activity of the *Th. caespititius* essential oil from Planalto Central was determined in the human gastric adenocarcinoma cell line 23132/87 inoculated with *H. pylori* 26695. The results are indicated in Figure 4. The reduction on the *H. pylori* viability was significant ($p<0.05$) for all the tested agents (essential oil, carvacrol and the antibiotic chloramphenicol), but no significantly differences were observed between the activity of the essential oil and chloramphenicol ($p>0.05$). The highest viability reduction ($p<0.05$) was achieved when carvacrol was used. This component of *Th. caespititius* essential oil, when compared to control, caused an inhibition of intracellular growth of *H. pylori* of about 3 log in 8 h (Figure 4). *H. pylori* nowadays is considered an intracellular microorganism due to its ability to invade various host cells, namely macrophages, dendritic cells and epithelial cells and undergoes replication inside the autophagosome (Wang et al., 2009) and once inside the infected cells the bacterium increases its resistance to antimicrobial agents (Fahey et al., 2002; Chu et al., 2010). Our results show that both *Th. caespititus* essential oil from Planalto Central and its main component, carvacrol, are effective inhibitors of *H. pylori* 26695 intracellular growth. Our findings are in agreement with the work of Bergonzelli et al.

(2003). The use of carvacrol rich essential oils or its main component seems to have potential to be used in combination with current treatments potentiating a total eradication.

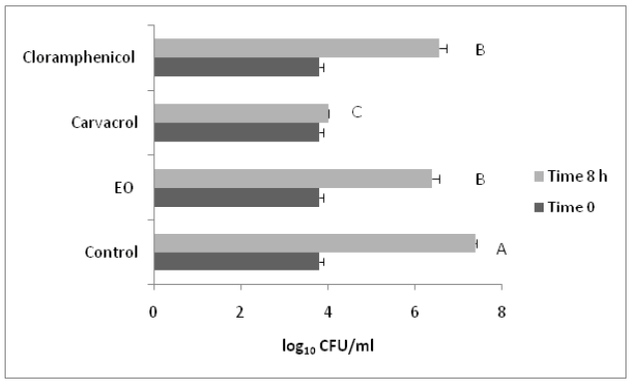

Figure 4. Intracelular viability of *H. pylori* 26695 in the human gastric adenocarcinoma cell line 23132/87. The essential oil (EO) of *Th. caespititus* at 0.08 mg/mL and carvacrol at 61,9% (v/v) were tested. The RPMI 1640 medium with no agents added or supplemented with chloramphenicol (30 µg/mL) were used as control. Data is the mean of three replicates. The standard error bars indicate the standard deviation. The bars with same letter are not significantly different (p>0.05).

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***Correspondence**

Maria Leonor Faleiro
Universidade do Algarve,FCT, Edf.8, IBB-CBME
Campus de Gambelas, 8005-139, Faro, Portugal
mfaleiro@ualg.pt
Tel.: +351 289 800 100
Fax: + 351 289 818 419