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ORIGINAL ARTICLE

Chemical composition, anti-inflammatory activity and cytotoxicity of *Thymus zygis* L. subsp. *sylvestris* (Hoffmanns. & Link) Cout. essential oil and its main compounds

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Thymus zygis subsp. *sylvestris*;
Essential oil;
Cytotoxicity;
Anti-inflammatory activity

Abstract *Thymus* L. is one of the most aromatic and medicinal plants used worldwide, mainly due to its essential oils. Several species of *Thymus* are currently used in herbal medicine for the prevention and treatment of various diseases, including disorders of the respiratory, gastrointestinal and nervous system. Moreover, they are widely used by their flavor as condiment and food preservatives, and a wide range of biological and therapeutic properties have been reported for this genus.

The aim of the present research was to evaluate the anti-inflammatory activity of *Thymus zygis* L. subsp. *sylvestris* (Hoffmanns. & Link) Cout. oil and its major compounds and, of utmost importance, assess their safety profile in several mammalian cell types at concentrations presenting strong bioactivity.

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This oil is characterized by high percentage of two phenolic compounds (thymol 19.5% and carvacrol 16.3%) and their biochemical precursor (*p*-cymene 22.0%). Interestingly, this is the first paper reporting high amounts of those phenols in the same sample of *T. zygis* subsp. *sylvestris* from Portugal.

The anti-inflammatory potential was investigated in lipopolysaccharide (LPS)-triggered nitric oxide (NO) production by macrophages and microglia concomitantly treated with *T. zygis* subsp. *sylvestris* essential oil. Our results demonstrated a significant decrease of LPS-induced NO production at concentrations up to 0.32 and 0.16 μL/mL, respectively, without affecting cell viability.

These results confirm the safety of *T. zygis* subsp. *sylvestris* oil and thus its suitability to be used as condiment and food preservative. Furthermore, the anti-inflammatory activity attributable to the essential oil supports further studies envisaging its putative use in pharmaceutical formulations for inhalation, topical application or oral administration.

1. Introduction

The word thyme is a general name for more than three hundred *Thymus* species, hybrids, varieties and ecotypes, all of which being small perennial herbs native to Europe and Asia. The genus *Thymus* L. (Lamiaceae) is a taxonomically complex group of aromatic plants used for a long time as spices or drugs, and it is among the most widely used medicinal herbs in the world, partly due to its essential oils (Stahl-Biskup and Sáez, 2002; Miguel, 2010).

In addition to the medicinal uses, thyme commercial products include the fresh or dried herb, different plant extracts and landscape plants (Figueiredo et al., 2008). Among these products essential oils are particularly relevant, namely those from *T. vulgaris*, *T. zygis*, *T. mastichina* and *Thymbra capitata*. Thyme oils are also used in flavor and food industries, mainly in the manufacture of perfumes and cosmetics, or for flavoring chocolates, toothpastes, mouthwashes, and cough medicines (Kruger, 1992; Bremnes, 1993; Morales, 2002; Zarzuelo and Crespo, 2002).

In *Thymus* the intraspecific variability concerning the essential oil composition was examined by several authors, as can be seen from the important exhaustive review performed by Stahl-Biskup and Sáez in 2002. Some chemotypes of various Portuguese *Thymus* sp. have been previously identified by our team (Salgueiro, 2007; Figueiredo et al., 2008; Vale-Silva et al., 2010; Zuzarte et al., 2013). One of the most used and polymorphic taxon in Portugal is *T. zygis* subsp. *sylvestris*.

In Portugal, *Thymus zygis* L. subsp. *sylvestris* (Hoffmanns. & Link) Cout. is widespread in the Central part of Portugal and it is traditionally used as condiment in cheese, fish, meat, salads, sauces and as food preservative, as well as digestive tonic against cold and sore throat, and in the treatment of small wounds (Ribeiro et al., 2000; Proença da Cunha et al., 2011). Externally, the essential oil is used in the treatment of skin infections, oropharynges disorders, rheumatic pain and sinusitis (Proença da Cunha et al., 2007).

Among the various biological properties reported for thyme, some are very well established, such as antioxidant, insecticidal, antibacterial, antifungal and antiviral activities (Figueiredo et al., 2008; Pina-Vaz et al., 2004; Vale-Silva et al., 2010), as well as the stimulatory effect of gastric secretions (Salgueiro, 2007). All these activities are related to the high content of phenolic compounds, with special emphasis in thymol and carvacrol (Karaman et al., 2001; Rasooli and Mirmostafa, 2003; Rota et al., 2008; Silveira e Sá et al., 2014).

Previously, we have evaluated the antifungal activity of four chemotypes (thymol, carvacrol, geraniol/geranyl acetate and linalool) of *T. zygis* subsp. *sylvestris* from Portugal, being the oil rich in carvacrol the most active (Gonçalves et al., 2010).

As far as we know, there are no previous papers concerning the evaluation of the anti-inflammatory activity of *T. zygis* subsp. *sylvestris* essential oil. Only, Ocaña and Reglero (2012) evaluated the effect of the extracts of *Thymus vulgaris*, *T. zygis* and *T. hyemalis* (obtained by CO_2 supercritical fluid extraction) in the production and gene expression of the inflammatory mediators TNF-α, IL-1β, IL-6, and IL-10, in a monocyte cell line.

Although *T. zygis* subsp. *sylvestris* has a variety of applications, there is lack of scientific studies assessing safe bioactive doses of its essential oil in mammalian cells, namely alveolar epithelial cells, keratinocytes and hepatocytes. This evaluation is of utmost importance to confirm its safety profile as condiment and food preservative, as well as its suitability for inclusion in pharmaceutical formulations for inhalation, topical application and oral administration.

Therefore, the aim of the herein presented research was to characterize *T. zygis* subsp. *sylvestris* essential oil from plants collected in Serras de Aire e Candeeiros (Central Portugal), where the plant is extensively used for medicinal and culinary purposes, and to unveil safe bioactive concentrations of the oil and its main compounds in several mammalian cell lines. Concomitantly, this research aimed to disclose the anti-inflammatory activity on cell lines representative of peripheral and central nervous system inflammatory cells.

2. Materials and methods

2.1. Plant material

Aerial parts of *T. zygis* subsp. *sylvestris* were collected in flowering stage in the Parque Natural das Serras de Aire e Candeeiros (Central Portugal). After harvesting, the plants were dried in the shade for 4 days before essential oil isolation. The voucher specimen was identified by a plant taxonomist and deposited in the Herbarium of Medicinal Plants, Faculty of Pharmacy, University of Coimbra.

2.2. Essential oil isolation

Essential oil was isolated by hydrodistillation for 3 h using a Clevenger-type apparatus, according to the procedure

described in the European Pharmacopoeia (Council of Europe, 1997) and stored in appropriate vials at 4 °C.

2.3. Gas chromatography (GC)

Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm, film thickness 0.20 μm), and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm, film thickness 0.20 μm); oven temperature program: 70–220 °C (3 °C/min), 220 °C (15 min); injector temperature: 250 °C; carrier gas: helium, adjusted to a linear velocity of 30 cm s^{-1}; splitting ratio: 1:40; and detectors temperature: 250 °C.

2.4. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm, film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described above are interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; and scans per second: 4.51.

2.5. Qualitative and quantitative analyses

Components of the volatile oils were identified by their retention indices on both SPB-1 and SupelcoWax-10 columns, calculated by linear interpolation relative to retention times of C_8–C_{24} of *n*-alkanes and compared with those of reference compounds included in CEF laboratory database or literature data (Adams, 2007), and by their mass spectra by matching with reference spectra from the CEF laboratory own spectral database, Wiley/NIST database or literature data (Joulain and Konig, 1998; Adams, 2007; Wiley Registry, 2006). Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

2.6. Anti-inflammatory activity evaluation

2.6.1. Cell culture

The macrophage cell line (Raw 264.7), obtained from the American Type Culture Collection (TIB-71) was kindly supplied by Dr. Otília Vieira (Center for Neuroscience and Cell Biology, University of Coimbra, Portugal). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) non-inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 μg/mL streptomycin and 100 U/mL penicillin at 37 in a humidified atmosphere of 95% air and 5% CO_2.

The microglia cell line (BV2) was purchased from Biological and Cell Banking Factory Centro di Risorse Biologiche, Genova. Cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% (v/v) non-inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 μg/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.6.2. Nitric oxide (NO) measurement

The anti-inflammatory potential of the essential oil of *T. zygis* subsp. *sylvestris* and its main compounds was tested separately: *p*-cymene (The British Drug House LTD), thymol (The British Drug House LTD) and carvacrol (Eastman Organic Chemicals) were evaluated in the macrophage cell line (RAW 264.7) and microglia (BV2).

The stock solutions of the essential oil and its major compounds were prepared in dimethyl sulfoxide (DMSO) in a proportion of 1:1 of essential oil per DMSO. The different concentrations of the essential oil and its major compounds (0.08–0.64 μL/mL) were then prepared by serial dilution with the respective medium of the each cell line tested. DMSO never exceeds 0.1% in the final solutions.

The macrophage cells (RAW 264.7) and microglia (BV2) (0.6×10^6/0.3×10^6 respectively cells/well) were cultured in 48-well microplates, allowed to stabilize for 12 h, and then incubated with culture medium (control), or stimulated with 1 μg/mL LPS, or with 1 μg/mL LPS in the presence of different concentrations of the essential oil and major compounds during 24 h (600 μL medium – control; 588 μL medium + 12 μL – each dilution of essential oil or major compound).

The production of NO was measured by the accumulation of nitrites in the culture supernatants, by the colorimetric reaction with the Griess reagent (Cruz et al., 2001; Green et al., 1982). Briefly, 170 μL of medium was diluted with equal volumes of the Griess reagent [0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide containing 5% (w/v) H_3PO_4 (v/v)] and maintained during 30 min, in the dark. The absorbance at 550 nm was measured in an automated plate reader.

Three independent experiments were performed with the essential oil of *T. zygis* subsp. *sylvestris* and their main compounds. The results were expressed as a percentage of the nitrite production by cells cultured with LPS.

2.7. In vitro cytotoxicity evaluation

2.7.1. Cell culture and materials

The human keratinocytes cell line (HaCaT), obtained from DKFZ (Heidelberg), was kindly supplied by Dr. Eugenia Carvalho (Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal). Keratinocytes were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% (v/v) inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 μg/mL streptomycin and 100 U/mL penicillin, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

The human hepatocyte cell line (HepG2) was purchased from ATCC (number: 77400) and kindly provided by Dr. Conceição Pedroso Lima. Hepatocytes were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% (v/v) inactivated fetal bovine serum, with 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 mg/mL streptomycin, and kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

The human alveolar epithelial cell line (A549) was purchased from ATCC (CCL-185 number). The alveolar epithelial cells were cultured in the same medium for keratinocytes and above.

The method for culturing the cell lines of microglia (BV2) and macrophages (RAW 264.7) has been described previously.

2.7.2. MTT assay for cell viability

Cell viability was assessed for the essential oil of *T. zygis* subsp. *sylvestris* and its main compounds in the cell lines: macrophages (Raw 264.7), microglia (BV2), keratinocytes (HaCaT), hepatocytes (HepG2) and alveolar epithelial (A549).

Evaluation of cell viability was performed by a colorimetric assay using the MTT reduction colorimetric assay, as previously reported (Mosmann, 1983). The macrophages (RAW 264.7), microglia (BV2), keratinocytes (HaCaT), hepatocytes (HepG2) and alveolar epithelial cells (A549) were cultured at densities of 0.6×10^6; 0.3×10^6; 0.2×10^6; 0.2×10^6; and 0.2×10^6 cells/well. Cells were cultured in 48-well microplates in a final volume of 600 μL for 12 h and were further cultured with different concentrations of the essential oil and its major compounds (600 μL medium – control; 588 μL medium + 12 μL each dilution of essential oil or major compound).

After 24 h, 60 μL of a MTT solution (5 mg/mL in PBS) was added per well to keratinocytes, hepatocytes and alveolar epithelial cells; and 43 μL of MTT solution per well to macrophages and microglia. Cells were further incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Keratinocytes were incubated for 30 min; microglial cells, macrophages and hepatocytes were incubated for 1 h and the alveolar epithelial cells for 2 h and 30 min. After this time of incubation with MTT, the supernatants were discarded and 300 μL of acidic isopropanol (0.04 N HCl in isopropanol) was added to each well. Quantification of formazan crystals was performed using an ELISA microplate reader at 570 nm with a reference wavelength of 620 nm.

Three independent experiments were performed with the essential oil of *T. zygis* subsp. *sylvestris* and with its main compounds. The results were expressed as a percentage of MTT reduction and compared with control.

2.8. Data analysis

All the experiments were performed in duplicate, being the results expressed as mean ± SEM of three independent experiments, performed in duplicate. The means were statistically compared using one-way ANOVA, with a Dunnett's multiple comparison test. The differences between the means were considered significant for values of $p < 0.05$. The statistical tests were applied using GraphPad Prism, version 6.00 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Essential oil composition

The essential oil was obtained with yield of 1.2%. The qualitative and quantitative composition of the oil is shown in Table 1, where the compounds are listed by order of their elution on a polydimethylsiloxane column.

Table 1 Chemical composition of *Thymus zygis* subsp. *sylvestris* essential oil from Portugal.

Compound[A]	RI SPB-1[a]	RI SW 10[b]	%
α-Thujene	922	1029	1.9
α-Pinene	930	1030	1.8
Camphene	943	1077	0.8
Oct-1-en-3-ol	959	1447	0.1
Sabinene	964	1128	0.1
β-Pinene	970	1118	0.2
Myrcene	980	1161	1.1
α-Phellandrene	997	1171	0.1
α-Terpinene	1010	1187	t
p-Cymene	1011	1275	22.0
1,8-Cineole	1019	1214	1.0
Limonene	1020	1206	0.8
β-Phellandrene	1020	1215	0.3
cis-Verbenol	1122	1648	t
Z-β-Ocimene	1025	1235	t
γ-Terpinene	1046	1249	7.4
trans-Sabinene hydrate	1050	1459	0.4
Terpinolene	1076	1288	t
Linalool	1082	1543	5.5
Camphor	1118	1515	2.1
trans-Pinocarveol	1119	1647	0.1
trans-Verbenol	1126	1664	0.1
Borneol	1144	1695	3.4
Terpinene-4-ol	1158	1597	2.2
Bornyl acetate	1266	1578	0.1
α-Terpineol	1169	1692	0.8
Geraniol	1233	1842	0.1
Linalyl acetate	1240	1555	t
Geranial	1242	1730	0.1
Thymol	1268	2183	19.5
Carvacrol	1275	2212	16.3
Geranyl acetate	1359	1755	2.0
β-Elemene	1382	1585	t
E-Caryophyllene	1408	1590	1.1
α-Humulene	1442	1665	t
Germacrene-D	1466	1699	0.1
β-Bisabolene	1495	1723	0.1
Spathulenol	1553	2112	0.1
Caryophyllene oxide	1557	1968	1.2
Viridiflorol	1569	2072	t
α-Cadinol	1628	2218	0.1
Monoterpene hydrocarbons			37.0
Oxygen containing monoterpenes			53.5
Sesquiterpene hydrocarbons			1.4
Oxygen containing sesquiterpenes			1.4
Others			0.1
Total identified			93.4

t = traces (⩽0.05%).
RI[a] = Retention indices on the SPB-1 column relative to C_8–C_{24} *n*-alkanes.
RI[b] = Retention indices on the SupelcoWax-10 column relative to C_8–C_{24} *n*-alkanes.
[A] Compounds listed in order of their elution on the SPB-1 column.

In total, 41 compounds were identified accounting for 93.4%. The oil is characterized by high percentage of monoterpene hydrocarbons (37.0%) and oxygen containing monoterpenes (53.5%), with the phenolic compounds thymol (19.5%)

and carvacrol (16.3%) as main constituents and their biochemical precursor *p*-cymene (22.0%).

3.2. Anti-inflammatory activity

In this study we evaluated the anti-inflammatory effect (inhibition of NO production) of *T. zygis* subsp. *sylvestris* and its main compounds tested separately, *p*-cymene, carvacrol and thymol.

3.2.1. Effect of the essential oil and its main compounds on nitric oxide production induced by LPS in macrophages

The effect of the oil on NO production was analyzed by measuring the accumulation of nitrites in the culture medium of macrophages stimulated with LPS. As shown in Fig. 1, a dose dependence inhibition of NO triggered by LPS was achieved in the presence of four concentrations of the essential oil: 42.67 ± 12.24 (0.64 μL/mL), 62.67 ± 10.84 (0.32 μL/mL), 91.67 ± 2.03 (0.16 μL/mL) and 88.00 ± 5.57 (0.08 μL/mL).

The production of nitric oxide induced by LPS in the presence of the main compounds of the essential oil (*p*-cymene, thymol and carvacrol) is also shown in Fig. 1. All the concentrations tested decreased NO production evoked by LPS with a stronger inhibitory activity attributable to thymol and carvacrol. Indeed, *p*-cymene was without effect at concentrations 0.16 μL/mL and 0.08 μL/mL.

3.2.2. Effect of the essential oil and main compounds on nitric oxide production induced by LPS in microglia

After stimulation with LPS in the presence of four concentrations of the essential oil (Fig. 2), the nitric oxide production induced in microglia by LPS was strongly inhibited to: 22.00 ± 1.03 (0.64 μL/mL), 24.34 ± 0.96 (0.32 μL/mL), 26.71 ± 1.25 (0.16 μL/mL) and 38.94 ± 1.48 (0.08 μL/mL).

The production of nitric oxide induced by LPS in the presence of the main compounds of the essential oil is also shown in Fig. 2. The inhibitory profile triggered by the main compounds was more pronounced in microglia relative to macrophages. As depicted in Fig. 2, all the concentrations of *p*-cymene, thymol and carvacrol tested decreased NO production evoked by LPS to values quite similar to those obtained in the presence of the essential oil.

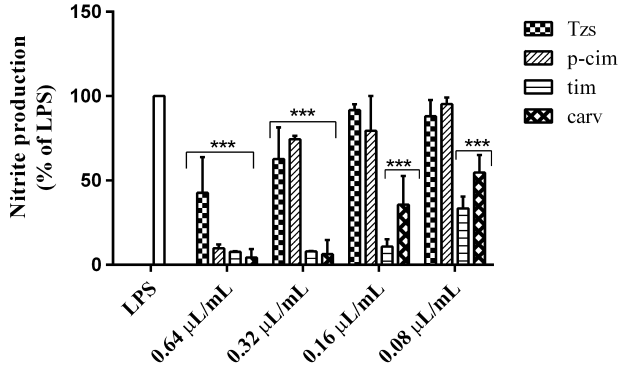

Figure 1 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on NO production in macrophages (Raw 264.7). Results are expressed as a percentage of the nitrite production by cells cultured in the presence of LPS ($^{***}p < 0.001$, compared to LPS).

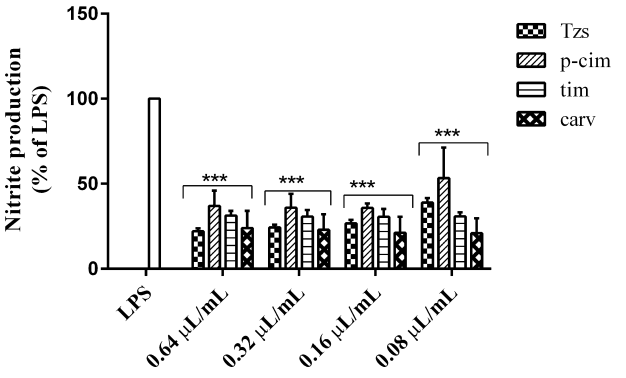

Figure 2 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on NO production in microglia cells (BV2). Results are expressed as a percentage of the nitrite production by cells cultured in the presence of LPS ($^{***}p < 0.001$, compared to LPS).

3.3. Evaluation of cell viability

The safety of the essential oil and its main compounds was assessed by the MTT assay in macrophages (RAW 264.7), microglia (BV2), keratinocytes (HaCaT), hepatocytes (HepG2) and alveolar epithelial cells (A549).

3.3.1. Effect of the essential oil and its main compounds on macrophages viability

As shown in Fig. 3, the essential oil showed no significant cytotoxicity in macrophages (Raw 264.7) for concentrations up to 0.32 μL/mL. However, the main compounds of the essential oil (*p*-cymene, thymol and carvacrol) showed cytotoxicity in all tested concentrations.

3.3.2. Effect of the essential oil and its main compounds on microglia viability

As shown in Fig. 4, the essential oil and *p*-cymene showed no significant cytotoxicity in microglial cells (BV2) for concentrations up to 0.16 μL/mL.

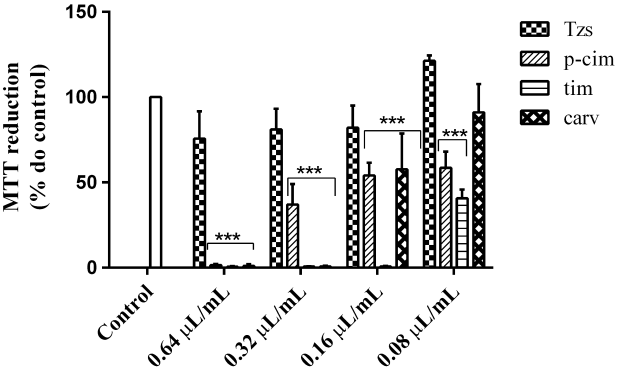

Figure 3 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on macrophages (Raw 264.7) viability by the MTT assay. Results are expressed as a percentage of MTT reduction by control cells ($^{***}p < 0.001$, compared to control).

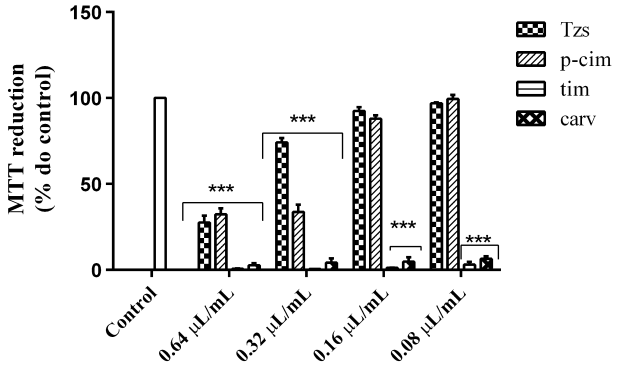

Figure 4 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on microglia viability (BV2) by MTT assay. Results are expressed as a percentage of MTT reduction by control cells ($^{***}p < 0.001$, compared to control).

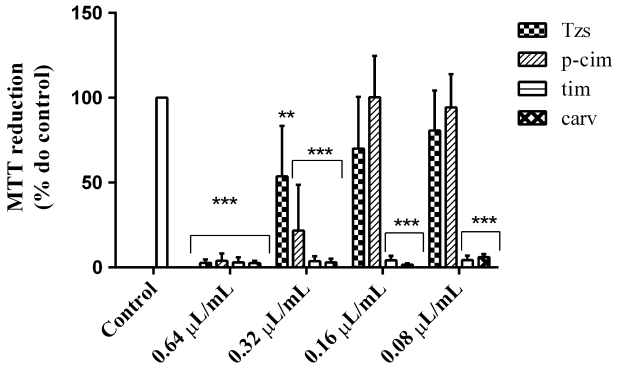

Figure 5 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on keratinocytes (HaCaT) viability by MTT assay. Results are expressed as a percentage of MTT reduction by control cells ($^{***}p < 0.001$, $^{**}p < 0.01$ compared to control).

Thymol and carvacrol showed cytotoxicity in cells of microglia (BV2) for all concentrations tested.

3.3.3. Effect of the essential oil and its main compounds on keratinocytes viability

As shown in Fig. 5, the essential oil showed no significant cytotoxicity in keratinocytes (HaCaT) for concentrations up to 0.32 µL/mL.

Thymol and carvacrol showed cytotoxicity in keratinocytes (HaCaT) for all concentrations tested. However, *p*-cymene showed no significant cytotoxicity for concentrations up to 0.16 µL/mL.

3.3.4. Effect of the essential oil and its main compounds on hepatocytes viability

As shown in Fig. 6, the essential oil and *p*-cymene showed no significant cytotoxicity in hepatocytes (Hep G2) for concentrations up to 0.16 µL/mL. Thymol and carvacrol showed cytotoxicity in all concentrations tested.

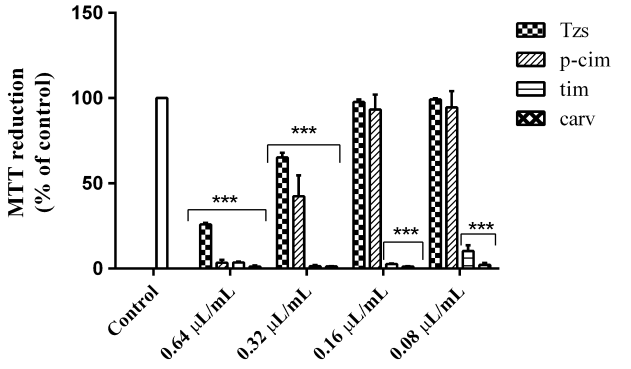

Figure 6 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on hepatocytes (HepG2) viability by MTT assay. Results are expressed as a percentage of MTT reduction by control cells ($^{***}p < 0.001$, compared to control).

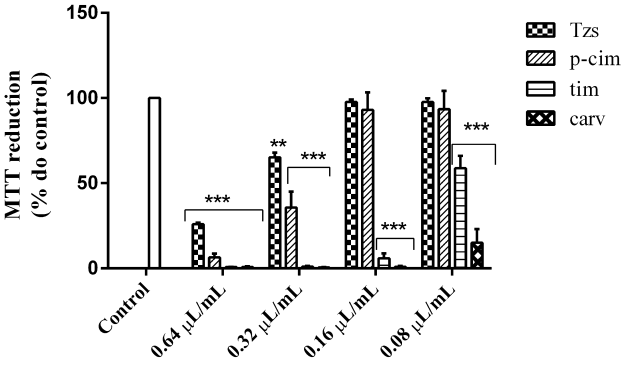

Figure 7 Effect of essential oil of *T. zygis* subsp. *sylvestris* (**Tzs**) and its main compounds: *p*-cymene (**p-cim**), thymol (**tim**) and carvacrol (**carv**) on alveolar epithelial cells (A549) viability by MTT assay. Results are expressed as a percentage of MTT reduction by control cells ($^{***}p < 0.001$, compared to control).

3.3.5. Effect of the essential oil and its main compounds on alveolar epithelial cells viability

As shown in Fig. 7, the essential oil and *p*-cymene showed no significant cytotoxicity in alveolar epithelial cells (A549), at concentrations up to 0.16 µL/mL. Thymol and carvacrol showed cytotoxicity at all concentrations studied.

4. Discussion and conclusions

T. zygis subsp. *sylvestris* is a quite variable subspecies with several chemotypes widespread in Central Portugal (Proença da Cunha and Salgueiro, 1991). The oil analyzed in the present work was from Serras de Aire e Candeeiros (Central Portugal), where the plant is extensively used for medicinal and culinary purposes. This oil is characterized by high percentage of two phenolic compounds (thymol 19.5% and carvacrol 16.3%) and their biochemical precursor (*p*-cymene 22.0%). It is the first time that high amounts of both phenols in the same sample of *T. zygis* subsp. *sylvestris* from Portugal are reported.

Previous studies with four chemotypes of *T. zygis* subsp. *sylvestris* from Portugal showed the potential of the carvacrol

chemotype against dermatophyte strains (Gonçalves et al., 2010). As far as we know, there are no previous reports on the anti-inflammatory potential of *T. zygis* subsp. *sylvestris* essential oil. Our results performed with a sample with high amounts of thymol, carvacrol and *p*-cymene on macrophages and microglia demonstrated a significant decrease of LPS-induced nitric oxide production. In macrophages, the higher dose able to be used without affecting cell viability is 0.32 μL/mL and, using this concentration, an inhibition higher than 30% was achieved. In microglia, the higher safe dose of the oil is 0.16 μL/mL being the inhibition with this concentration higher than 70%. The major compounds (thymol, carvacrol and *p*-cymene) also inhibited NO production with a similar profile to that achieved with the oil. However, the chemicals thymol and carvacrol present higher cytotoxicity relative to the oil, suggesting that their inhibitory effect on NO production is indeed due to cell death. Among all the major compounds tested, *p*-cymene is the chemical presenting lower cytotoxicity with values quite similar to that obtained with the essential oil.

The various biological properties reported for thyme, such as antioxidant, insecticidal, antibacterial, antifungal and antiviral activities (Figueiredo et al., 2008; Pina-Vaz et al., 2004; Vale-Silva et al., 2010), are usually related to the high content of phenolic compounds, with special emphasis in thymol and carvacrol (Karaman et al., 2001; Rasooli and Mirmostafa, 2003; Rota et al., 2008). However, the present study confirms that these two phenolic compounds possess high cytotoxicity for all the cell types tested.

Accordingly, we previously demonstrated relevant differences in the chemical composition of *T. zygis* subsp. *sylvestris* essential oil, corresponding to different chemotypes (Gonçalves et al., 2010). The chemotype carvacrol demonstrated higher cytotoxicity to dendritic cells than the essential oil herein presented, which probably is due to their higher concentration of carvacrol that presents *per se* some degree of toxicity. Indeed, essential oils are complex mixtures of multiple constituents, and their biological properties seem to be the result of a synergism between these compounds. Our results point out the safety profile of *T. zygis* subsp. *sylvestris* oil rather than its main compounds assessed separately. Safe concentrations of the essential oil significantly inhibited NO production elicited by LPS in macrophages and microglia thus emphasizing its strong anti-inflammatory potential. Indeed, *T. zygis* subsp. *sylvestris* essential oils do not affect cell viability in concentrations up to 0.32 μL/mL on macrophages and in concentrations up to 0.16 μL/mL on microglia, keratinocytes, hepatocytes and alveolar epithelial cells. Taken together, it is reasonable to conclude that *T. zygis* subsp. *sylvestris* essential oil presents a safety profile, thus justifying and reinforcing its suitability as condiment and food preservative. Importantly, the high yield in essential oil together with its ability of disrupting the inflammatory response strongly encouraged *T. zygis* subsp. *sylvestris* industrial exploitation, namely concerning its essential oil use in pharmaceutical formulations for inhalation, topical application or oral administration.

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