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# New insights on the anti-inflammatory potential and safety profile of *Thymus carnosus* and *Thymus camphoratus* essential oils and their main compounds

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ABSTRACT

*Ethnopharmacological relevance:* *Thymus camphoratus* and *T. carnosus* are widely used in Portugal for the treatment of inflammatory-related conditions, such as inflammation of the respiratory tract, being the later also used as an antitussive.

*Aim of the study:* Bearing in mind the lack of scientific studies focused on the pharmacological activity of *Thymus camphoratus* and *T. carnosus*, this work was designed to validate the anti-inflammatory properties ascribed to these traditional species and concomitantly to unveil both the putative molecular mechanisms behind their bioactivity as well as the safety profile of their essential oils and major compounds.

*Materials and methods:* The chemical composition of the essential oils was assessed by gas chromatography (GC) and gas chromatography – mass spectroscopy (GC/MS). The nitric oxide (NO) scavenging potential of the oils was tested using S-nitroso-N-acetyl-D,L-penicillamine (SNAP) as NO donor. The anti-inflammatory potential of the essential oils and their major compounds was evaluated by measuring the nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophages as well as the expression of the pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Importantly, and in an attempt to assess the safety profile of the oils and respective major compounds, their effect on macrophages and hepatocytes viability was also determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

*Results:* *T. carnosus* essential oil was characterized by high amounts of borneol and camphene whereas *T. camphoratus* oil was rich in 1,8-cineole and borneol. The later presented higher pharmacological activity showing inhibitory effects towards NO production at lower concentrations (0.16 μL/mL) and concomitantly inhibiting the expression of two crucial pro-inflammatory proteins, iNOS and COX-2 (at 0.32 μL/mL). Since no NO scavenging activity was achieved, it is reasonable to conclude that the anti-inflammatory activity of the essential oils occurs upstream of iNOS expression, probably through inhibition of relevant pro-inflammatory signal transduction pathways. Importantly, at bioactive concentrations, the essential oils were devoid of toxicity towards macrophages and hepatocytes. The activity of the isolated compounds was far from that observed for the essential oils, thus suggesting that the anti-inflammatory activity is due to a synergic effect between several compounds in the mixture.

*Conclusion:* Overall, the results herein presented sustain and strengthen the anti-inflammatory properties traditionally ascribed to *T. carnosus* and *T. camphoratus*. Additionally, the molecular mechanisms associated to their pharmacological activity were highlighted, opening new avenues for the development of effective anti-inflammatory herbal medicinal products.

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## 1. Introduction

Several industries are actively searching for bioactive natural products, biodegradable and without toxicity to humans and animals (Bakkali et al., 2008). In the last years, aromatic plants and their essential oils have drawn increasing attention in the scientific and industrial fields. Indeed, these natural products are pleiotropic agents that can be driven to multiple cellular and molecular targets, thus forming an attractive source of molecules for multi-targeting therapeutic strategies. Essential oils are biodegradable extracts of volatile compounds, many of them listed by the Food and Drug Administration as food additives and with a GRAS (Generally Recognized as Safe) status (Milovanovic et al., 2015). Essential oils are isolated from aromatic plants (Gandhi et al., 2015), being the *Lamiaceae* one of the most representative families (Raja, 2012). In this family, *Thymus* is widely used in traditional medicine, being considered one of the eight most relevant genus comprising several essential oil producing species very relevant to the food, cosmetic, perfumery and pharmaceutical industries (Bakkali et al., 2008; Edris, 2007; Morales Valverde, 1997; Pauli, 2006; Petrović et al., 2016). This genus is widely distributed in the Iberian Peninsula with several endemic species. For instance, in Portugal, eleven thyme species, totalizing fourteen *taxa* are found (Franco, 1984; Morales Valverde, 1997). Despite the high industrial and therapeutic demand for these plants, only two species, *T. vulgaris* and *T. zygis*, have a community herbal monograph (EMA/HMPC/131901/2009 (HMPC/EMA, 2010)) and an ISO monograph (ISO/TC 54 - 14715: 2010) and due to their commercial interest, these species attain a production higher than 1000 t / year (Franz and Novak, 2009). Importantly, several scientific studies have shown the huge potential of other thyme essential oils as antioxidant, antimicrobial and anti-inflammatory agents (Dandlen et al., 2011a; Figueiredo et al., 2008; Miguel, 2010), but the main concern is that scientific studies focused on their mechanism of action and respective safety and toxicity profiles are clearly lacking, despite their use in traditional medicine. For instance, plants from the genus *Thymus* are widely used for the treatment of several ailments, such as coughs associated with cold, laryngitis, bronchitis, catarrh and inflammations of the mouth, as well as diseases of the digestive system. In addition, these plants are also used as antibacterial, antiseptic, anthelmintic agents and as appetite stimulants (Heinrich and Jager, 2015; Hosseinzadeh et al., 2015; Jarić et al., 2015). In Portugal, several thyme species are traditionally valued, namely *Thymus zygis*, *T. mastichina*, *T. camphoratus*, *T.carnosus* and *T. caespititius*. These species are used for the treatment of inflammatory problems, mainly inflammation of mouth, pharynx and skin, as well as rheumatism (Carapeto, 2006; Proença da Cunha et al., 2007; Rivera and Obón, 1995; Silva et al., 2011). Particularly, *T. camphoratus* is widely used for the treatment of respiratory tract inflammation associated with cough in combination with *Lithodora diffusa* (Camejo Rodrigues, 2007) and *T. carnosus* is also used as an antitussive (dos Santos, 2004). Furthermore, these species are used as culinary herbs and as fragrance agents in soaps and perfumes (Camejo Rodrigues, 2007; dos Santos, 2004; Figueiredo et al., 2008; Fonseca, 2015). Several scientific studies have pointed out the bioactive potential of *T. carnosus* and *T. camphoratus*, with highlights on their antioxidant effect (Albano et al., 2012; Dandlen et al., 2010; Miguel et al., 2004, 2007, 2003; Miguel et al., 2005a, b), anti-acetylcholinesterase activity (Albano et al., 2012; Dandlen et al., 2011b) and antimicrobial properties (Dandlen et al., 2011a). Nevertheless, studies on the anti-inflammatory effects and cytotoxicity of these essential oils are lacking, despite their use in traditional medicine as anti-inflammatory agents. The gap in this knowledge was the starting point for the herein presented study focused on the evaluation of the anti-inflammatory activity of *T. carnosus* and *T. camphoratus* essential oils using both *in chemico* and *in vitro* approaches, namely the nitric oxide (NO) scavenging potential of the oils and the inhibition of NO production in lipopolysaccharide (LPS)-stimulated macrophages, respectively. Also, the main compounds were assessed, either isolated or in combination, according to their proportion in the total essential oil. Of relevance, the mechanism of action of the essential oils was elucidated by assessing their effect on the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), two key pro-inflammatory enzymes. Concomitantly, the cytotoxicity of the essential oils was screened on macrophages and hepatocytes to disclose both bioactive and safe concentrations. Overall, results achieved in this work validate the traditional uses ascribed to these species and bring new insights on their anti-inflammatory potential paving the way for future industrial exploitation in the health/food sector.

## 2. Material and methods

### 2.1. Plant Material

Flowering aerial parts of *Thymus carnosus* Boiss ("tomilho-das-praias" in Portuguese) were collected in Praia da Manta Rota, Algarve, Portugal (N 37° 9′ 49″, W 7° 31′ 7″) and flowering aerial parts of *T. camphoratus* Hoffmanns. & Link ("tomilho-do-mar" in Portuguese) were collected in Praia de Odemira, Alentejo, Portugal (N 37° 38' 16.07", O 8° 48' 28.57"). Voucher specimens were included in the Herbarium of the University of Coimbra (COI), with accession numbers L. Salgueiro 54 and L. Salgueiro 33 for *T. camphoratus* and *T. carnosus*, respectively. Species authenticity was confirmed by Dr. Jorge Paiva, a taxonomist at the University of Coimbra and plant names checked with http://www.theplantlist.org.

### 2.2. Pure compounds

The following synthetic compounds were purchased: 1,8-cineole (Merck, extra pure), borneol (Fluka, pure) and camphene (Fluka, pure).

### 2.3. Essential oil isolation and analyses

Essential oils were isolated by hydrodistillation during 3 h, from the aerial parts of plants, using a *Clevenger*-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 2010).

The essential oils were analyzed by gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC/MS). The GC analysis was carried out on with an Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with a flame ionization detector and a HPGC ChemStation Rev. A.05.04 data handling processor. 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 i.d., film thickness 0.20 μm), and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm). The oven temperature was programmed at 70–220 °C (3 °C·min$^{-1}$), 220 °C (15 min); injector temperature: 250 °C; the carrier gas was helium, adjusted to a linear velocity of 30 cm·s$^{-1}$; splitting ratio 1:40; detectors temperature: 250 °C.

GC/MS analysis was performed on an Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm) directly coupled 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; 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; scans·s$^{-1}$: 4.51.

The volatile compounds were identified by their GC retention indices on both SPB-1 and SupelcoWax-10 columns and by their mass spectra. Retention indices, calculated by linear interpolation relative to the retention times of $C_8$–$C_{23}$ n-alkanes, were compared with those of