Essential oil composition and antinociceptive activity of *Thymus capitatus*

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**ABSTRACT**

**Context:** The essential oil (EO) from *Thymus capitatus* Hoff. et Link. (Lamiaceae) has been traditionally used for its medicinal properties, such as anti-inflammatory, analgesic, antioxidant and antimicrobial properties.

**Objective:** Characterize the constituents from *T. capitatus* EO and further evaluate the antinociceptive activity by *in vivo* and *in vitro* procedures.

**Materials and methods:** Gas chromatography–mass spectrometry was used to identify and quantify the constituents of the *T. capitatus* EO. The antinociceptive activity was evaluated *in vivo* by the glutamate-induced nociception model in male Swiss mice (25 g), at doses of 3, 6 and 12 mg/kg, 1 h before evaluation of the licking time response (0–15 min). The mechanism of *T. capitatus* EO (1–500 μg/mL) on the isolated nerve excitability of Wistar rat (300 g) was assessed by the single sucrose technique.

**Results and discussion:** The EO of *T. capitatus* presented 33 components, mainly monoterpenes and sesquiterpenes, carvacrol (ca. 80%) was its major constituent. *T. capitatus* EO induced antinociception in orally treated mice (3, 6, and 12 mg/kg) reducing the licking time from control (100.3 ± 11.9 s) to 84.8 ± 12.2, 62.7 ± 9.9, and 41.5 ± 12.7 s, respectively (n = 8; p < 0.05). Additionally, we have demonstrated that *T. capitatus* EO (500 μg/mL) decreased the compound action potential amplitude (V\(_{CAP}\)) of about 80.0 ± 4.3% from control recordings (n = 4; p < 0.05). Such activity was presumably mediated through a voltage-gated Na\(^+\) channels.

**Conclusions:** The present study demonstrated the antinociceptive activity of *Thymus capitatus* essential oil, which acts via peripheral nervous excitability blockade.

**Introduction**

The essential oils (EO) are volatile molecules generated by the secondary metabolism of higher plants and are produced by specialized secretory structures, such as glandular hairs and differentiated parenchyma cells (Lane et al. 2010). EO constituents belong mainly to two chemical groups: terpenoids (monoterpenes and sesquiterpenes of low molecular weight) and, to a lesser extent, phenylpropanoids (Regnault-Roger et al. 2012). These EO also have shown many biological activities, such as analgesic, anticonvulsant, anxiolytic, antioxidant, and antimicrobial properties (Faturi et al. 2010; Pergolizzi et al. 2010; Almeida et al. 2011).

*Thymus* (Lamiaceae) comprises over 300 species of hardy perennial herbaceous plants and subshrubs, which are native to southern Europe, Asia and North Africa (Tepe et al. 2004). Thyme is a distinguished condiment that is used in traditional medicine for several purposes, for example, as an anti-inflammatory and analgesic agent (Alamger et al. 2015; Amirghofran et al. 2016). The pharmacological activity of *Thymus capitatus* Hoff. et Link. EO have been demonstrated, including antiseptic, antioxidant, and antimicrobial properties (Consentino et al. 1999; Al-Mustafa & Al-Thunibat 2008; Mkaddem et al. 2010; Russo et al. 2013; Džamić et al. 2015), however, the antinociceptive and analgesic properties have not yet been evaluated. Therefore, the present study was designed to determine the major constituents from the *T. capitatus* EO and to evaluate its antinociceptive effects by *in vivo* and *in vitro* procedures.

**Materials and methods**

**Chemicals**

Pure standards for GC-MS analysis were purchased from Sigma-Aldrich (St. Louis, MO) and Extrasynthese (Lyon, France). Glutamate, morphine, Tween 80 (polyoxymethylene sorbitan monoo- late), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). All salts used in physiological solution, sucrose, glucose and NaOH, were purchased from Vetec (Rio de Janeiro, Brazil). HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was acquired from Amresco (Solon, OH).

**Plant processing**

Wild Thyme sample was collected in the middle of Sicily, in 2006, at bloom stage and dried at room temperature until no
constant weight loss was observed at the collection point, Calascibetta lands (Enna province, Italy), 37° 59' 83” (N), 14° 25' 26” (E). The plant collection and identification was made by the Regional Unit UOT Enna, as described by Napoli et al. (2010), in which herbarium voucher specimen was deposited. Dried aerial part of the sample (100 g) was subjected to hydrodistillation in accordance with current European Pharmacopoeia 6.0 (2008) until there was no significant increase in the volume of oil collected (3h). The essential oil obtained was dried over anhydrous sodium sulfate and stored under N2 in a sealed vial until required.

Oil analysis

Gas chromatographic (GC) analyses were run on a Shimadzu gas chromatograph, Model 17-A, equipped with a flame ionization detector (FID) and with an operating software Class VP Chromatography Data System, version 4.3 (Shimadzu Corporation, Kyoto, Japan). Analytical conditions were SPB-5 capillary column (15 m × 0.10 mm × 0.15 μm) and helium as the carrier gas (1 mL/min). Injection was done in split mode (1:200), injected volume was 1 μL (4% essential oil/CH2Cl2 v/v), and the injector and detector temperatures were 250°C and 280°C, respectively. Linear velocity in column 19 was cm/s. The oven temperature was held at 60°C for 1 min, then programed from 60 to 280°C at 10°C/min then 280°C for 1 min. Percentages of compounds were determined from their peak areas in the GC-FID profiles.

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out in the fast mode on a Shimadzu GC-MS model GCMS-QP5050A, with the same column and the same operative conditions used for the analytical GC and the operating software GCMS solution version 1.02 (Shimadzu Corporation, Kyoto, Japan). We adjusted the ionization voltage at 70 eV, the electron multiplier at 900 V and the ion source temperature at 180°C. Mass spectra data were acquired in the scan mode in an m/z range of 40–400. The same oil solutions (1 μL) were injected with the split mode (1:96).

The compounds were identified based on their GC retention index (relative to C9-C22 n-alkanes on the SPB-5 column), computer matching of spectral MS data with those from NIST MS 107 and NIST 21 libraries (National Institute of Standard and Technology 1998), comparison of the fragmentation patterns with those reported in the literature (Adams 2001) and, whenever possible, co-injections with authentic samples.

Animals

Adult male Swiss mice and Wistar rats, weighing around 25 and 300 g, respectively, were randomly housed in appropriate cages at 24 ± 2°C on a 12 h light cycle with free access to food (Purina, São Paulo, Brazil). All procedures in this study were carried out in accordance with Institutional Animal Care and Use Committee (CEEA-UFPJ and CEPA-UFPB, Brazil).

Glutamate-induced nociception

The procedure used was similar to that previously described by Beirith et al. (2002). In brief, a volume of 20 μL of glutamate (10 μM/paw) was injected intraplantarly in the ventral surface of the mice’s right hind paw. Animals were divided into five groups (n = 8), orally treated with vehicle (Tween 80 0.1% in distilled water) in the control group, T. capitatus EO (3, 6, and 12 mg/kg) and morphine (5 mg/kg), 1 h before experimentation. After drug treatment, the licking time (0–15 min) of the glutamate-injected paw was recorded and considered as indicative of nociception (Donato et al. 2015).

Electrophysiological experiments

The single sucrose gap technique was used as previously described by Gonçalves et al. (2010). Briefly, rat sciatic nerves (n = 4) were carefully removed and immediately positioned in an experimental chamber composed of five compartments (I–V), electrically isolated by solid baseline. All compartments were filled with a physiological solution, composed of (in mM): NaCl 150; KCl 4; CaCl2 2; MgCl2 1; glucose 10 and HEPES 10. The pH was adjusted to 7.4 with NaOH. Compartments I and II of the chamber were used to apply supramaximal stimulation (6–10 V/100 ms), manually triggered at 5 min intervals (CF Palmer, London, UK). The compartment III was super fused by isotonic sucrose (290 mM; 1 mL/min) to electrically isolate the neighbouring recording compartments. Compartments IV and V were used for drug perfusion (1 mL/min) and data recording, acquired by a microcomputer-based 12-bit A/D converter (10.5 kHz) and later analyzed by proper software (Lynx, São Paulo, Brazil).

T. capitatus EO was diluted into a vehicle (0.1% Tween 80 in physiological solution) at concentrations of 1, 100 and 500 μg/mL. Drug incubation was performed for 30 min followed by nerve washout with physiological solution for additional 30 min. To quantify drug effects, we analyzed the compound action potential (CAP) amplitude (VCAP, in mV) by measuring the difference between the baseline and the maximal voltage achieved. The CAP depolarization velocity (DV CAP, in V/s) was the rate between VCAP and the time required to reach the CAP peak. The time constant of repolarization (trep in ms) was calculated by the equation: V = V0 exp(–t/trep), as described by Alves et al. (2010).

Statistical analysis

Data are presented as the mean ± S.E.M. of independent experiments. To assess the significance level, analysis of variance (ANOVA) followed by Dunnet’s test for in vivo data (n = 8) and the two-tailed Student’s t-test for in vitro data (n = 4) were used. Differences between experimental and control groups were considered significant when p < 0.05.

Results

Table 1 lists the composition of the T. capitatus essential oil. In total, 33 components were fully identified, covering more than 96% of the total composition. Monoterpenes, both hydrocarbons and oxygenated, were the most highly represented classes, while sesquiterpenes and other classes were the least represented.

We stated that the oxygenated monoterpenone carvacrol (ca. 80%) strongly characterizes the T. capitatus EO being its major constituent. The two monoterpane hydrocarbons, namely p-cymene (ca. 4.4%) and γ-terpinene (ca. 3.7%) biosynthetically correlated to carvacrol, were the other two main components, although in lower concentrations (Table 1). Finally, among the other components, only the sesquiterpene caryophyllene reaches an appreciable amount (ca. 2.3%).

During the test of glutamate nociception, we observe that animals orally treated with T. capitatus EO at doses of 3, 6, and...
12 mg/kg (Figure 1), reduced the licking time from control (100.3 ± 11.9 s) to 84.8 ± 12.2, 62.7 ± 9.9 and 41.5 ± 12.7 s (p < 0.05), respectively. As expected, the standard drug morphine (5 mg/kg) decreased the nociceptive response to 10.3 ± 3.4 s from control (p < 0.05).

Testing the T. capitatus EO in the peripheral nerve excitability, we showed that 500 μg/mL was sufficient to completely block $V_{\text{CAP}}$ after 30 min of incubation and this effect was latter reverted by 30 min of drug removal (Figure 2(A)). In fact, 500 μg/mL of T. capitatus EO practically reached maximum effect with only 15 min of incubation (Figure 2(B)). At 100 μg/mL of T. capitatus EO, we observed that $V_{\text{CAP}}$ was reduced to 10.6 ± 3.6, 31.4 ± 11.3, and 58.1 ± 16.8% from control, after 5, 15, and 30 min of incubation, respectively (Figure 2(B)). Vehicle alone did not induce any significant change in the CAP characteristics (data not shown).

We also evaluated the effect of T. capitatus EO on $D_{V_{\text{CAP}}}$ and $\tau_{\text{rep}}$ parameters in order to assess for alterations in the depolarization and/or repolarization phases of CAP recordings. Taken together, our results showed that T. capitatus EO (100 μg/mL) decreased $D_{V_{\text{CAP}}}$ from 48.4 ± 7.6 V/s (control) to 18.1 ± 9.1 V/s (p < 0.05) after 30 min of incubation. Such effect was reversed to 26.1 ± 10.2 V/s after drug washout (Figure 3(A)). Meanwhile, no differences were observed on the CAP repolarization phase, measured by $\tau_{\text{rep}}$ (Figure 3(B)).

Discussion

The aforementioned aromatic profile is similar to Sicilian Thyme, as recently reported in a study on 30 samples collected throughout the Sicilian island, suggesting that Thymus capitatus [syn. Coridithymus capitatus (L.) Rchb.f., Satureja capitata L., Thy Thymbra capitata (L.) Cav.] is the most widespread wild species in the Sicilian area (Napoli et al. 2010). In the present study, we analyzed the EO composition of T. capitatus, demonstrating that it is composed of 33 different molecules, including monoterpenes and sesquiterpenes, and other compounds like phenols, alcohols, organic acids, aldehydes and ketones. The monoterpene carvacrol was found as the main component, corresponding to ~80% of the whole oil (Table 1).

Recently, several studies have reported the antinociceptive activity of Thymus sp. extracts and their major constituents (Mahmoudi et al. 2011; Taherian et al. 2009; Cavalcante Melo et al. 2012). In this way, we evaluated whether T. capitatus EO also induces analgesy in vivo. For that, the glutamate-induced nociception model was performed in mice pretreated with T. capitatus EO by the oral tract, in order to imply that the drug was absorbed in vivo. Our data demonstrated significant analgesic activity and dose-dependency of T. capitatus EO (Figure 1).

At the peripheral level, the glutamate is involved in nociceptive transmission through primary afferent fibres, as well as in the development and maintenance of the pain response (Osiowicz et al. 2013; Bardi 2013). This amino acid acts on ionotropic receptors such as N-methyl-D-aspartate (NMDA), increasing cation influx, like Na⁺ or Ca²⁺, through plasma membrane, activating post-synaptic transmission and deflagrating action potentials in nociceptive fibres, leading to pain sensation (Beirith et al. 2002; Srebro et al. 2015). Therefore, it is plausible to suggest that the constituents of T. capitatus EO might also interfere with the excitability of peripheral nerves.

To assess the effects of T. capitatus EO on the isolated nerve excitability, we performed the single sucrose technique. This method enables the study of bioactive compounds on the peripheral nerve by measuring the CAP evoked by external electrodes (Alves et al. 2010; Gonçalves et al. 2010). According to the presented data, we have demonstrated that T. capitatus EO impede nervous transmission by blocking the CAP amplitude, in a concentration- and time-dependent manner (Figure 2(A) and (B)). In addition, such effect was reverted by washing the drug out. These data also suggest that T. capitatus EO did not damage the nerve fibres functionality during the experimentation period.

The CAP recording is considered the algebraic sum of the action potential from all individual fibres of the nerve. Their

| Table 1. Chemical composition of T. capitatus essential oil (EO). |
|---|---|---|
| # | Ki* | Compound | Area(b) (%) |
| 1 | 778 | 2-Methyl-butyanoic acid methyl ester | 0.1 |
| 2 | 932 | α-Thujene | 0.7 |
| 3 | 939 | α-Pinene | 0.6 |
| 4 | 954 | Camphene | 0.2 |
| 5 | 982 | β-Pinene | 0.4 |
| 6 | 993 | β-Mycene | 1.7 |
| 7 | 1008 | α-Phe-landrone | 0.2 |
| 8 | 1015 | p-Menth-1-(7),8-diene | 0.1 |
| 9 | 1021 | α-Terpine | 1.2 |
| 10 | 1033 | p-Cymene | 4.4 |
| 11 | 1035 | Limonene | 0.4 |
| 12 | 1052 | trans-β-Ocimene | 0.1 |
| 13 | 1063 | γ-Terpine | 3.7 |
| 14 | 1072 | Sabinene Hydrate | 0.2 |
| 15 | 1091 | α-Terpineolene | 0.2 |
| 16 | 1100 | Linalool | 0.9 |
| 17 | 1126 | α-Campholenal | 0.1 |
| 18 | 1172 | Bornol | 0.4 |
| 19 | 1181 | Terpinen-4-ol | 0.8 |
| 20 | 1193 | α-Terpineol | 0.2 |
| 21 | 1248 | Nerol | 0.1 |
| 22 | 1255 | Neral | 0.1 |
| 23 | 1264 | Carvone | 0.1 |
| 24 | 1273 | Geraniol | 0.1 |
| 25 | 1290 | Thymol | 0.3 |
| 26 | 1313 | Carvacrol | 79.9 |
| 27 | 1362 | Thymol acetate | 0.1 |
| 28 | 1425 | Caryophyllene | 2.3 |
| 29 | 1446 | Aromadendrene | 0.1 |
| 30 | 1460 | α-Humulene | 0.1 |
| 31 | 1511 | β-Bisabol | 0.2 |
| 32 | 1545 | α-Cadinene | 0.2 |
| 33 | 1589 | Caryophyllene Oxide | 0.2 |

*K*: Kovat’s indices; RT: retention time.

aThe numbering refers to elution order, and values (relative peak area percent) represent averages of 3 determinations.

\( **p < 0.01, **p < 0.001 \) vs. control (ANOVA followed by Dunnett’s test).
shapes and properties indicate a single-action potential and therefore it can be a useful tool for an initial searching of neuroactive drug candidates. It has also been shown that inhibition of neuronal excitability, observed as a decrease in the CAP amplitude, might be associated with the blockade of the voltage-gated Na⁺ channels (Naᵥ), which are responsible for the rising phase of the action potential in neurons (Araújo et al. 2011). The present study demonstrated that 100 μg/mL of *T. capitatus* EO delayed the CAP depolarization phase (DV_CAP) after 30 min-incubation in rat sciatic nerves. Thus, we can suggest a Naᵥ blocking-like effect promoted by *T. capitatus* EO in a reversible manner, since this effect was recovered by drug washout (Figure 3(A)). In fact, recent findings have demonstrated the ability of carvacrol, the major constituent of *T. capitatus* EO, to induce Naᵥ blockade in DRG neurons (Joca et al. 2012, 2015). Those findings corroborate our results and strongly suggest that carvacrol is the main active molecule behind the antinociceptive effects of *T. capitatus*.

On the other hand, the voltage-gated K⁺ channels are believed to be an important factor in the repolarization phase of peripheral nerves, especially the delayed rectifier potassium channel (Kᵥ). Some studies have demonstrated that the time constant of depolarization (τ_rep) of the CAP can be used as a valuable tool for the investigation of potential Kᵥ blockers (Alves et al. 2010). Hence, it is unlikely that *T. capitatus* EO is related to this channel variety since no change in τ_rep was observed (Figure 3(B)).

In conclusion, our study reported that the carvacrol-rich essential oil from *T. capitatus* exerts its antinociceptive activity through peripheral nervous excitability blockade.

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**Disclosure statement**

The authors report no declarations of interest.
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