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Title: “Activity of Thymus capitellatus volatile extract, 1,8-cineole and borneol against Leishmania species.”

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ABSTRACT

In the search for new leishmanicidal agents, *Thymus capitellatus* Hoffmanns. & Link (family Lamiaceae) volatile extract and its major compounds, 1,8-cineole and borneol, were tested against *Leishmania infantum*, *L. tropica* and *L. major*. Plant volatile extract (essential oil) was analysed by GC and GC-MS and the activity of essential oil on *Leishmania* promastigotes viability was assessed using tetrazolium-dye colorimetric method (MTT). The MTT test was also used to assess the cytotoxicity of essential oil on macrophages and bovine aortic endothelial cells. Effects on parasites were also analyzed by flow cytometry in order to assess mitochondrial transmembrane electrochemical gradient (JC-1), analyze phosphatidylserine externalization ( annexin V–FITC, propidium iodide) and evaluate cell cycle (DNase-free, RNase, PI). Morphological and ultrastructural studies were performed by light, scanning and transmission electron microscopy. *T. capitellatus* volatile extract exhibited anti-parasite activity on *Leishmania* species, with IC<sub>50</sub> values ranging from 35 to 62 µg/ml. However, major compounds 1,8-cineole and borneol did not showed biological activity suggesting that these monoterpenes are not responsible for the antileishmanial activity of *T. capitellatus* essential oil. Appearance of aberrant-shaped cells, mitochondrial swelling and autophagosomal structures were some of the ultrastructural alterations exhibited among treated promastigote cells. *T. capitellatus* promoted leishmanicidal effect by triggering a programmed cell death as evidenced by externalization of phosphatidylserine, loss of mitochondrial membrane potential, and cell-cycle arrest at the G(0)/G(1) phase. The volatile extract did not induced cytotoxic effects on mammalian cells. Taken together, these results suggest that *T. capitellatus* may represent a valuable source for therapeutic control of leishmaniasis in humans and animals.

Key Words: leishmaniasis, essential oil, monoterpenes; protozoa, infectious diseases, drug discovery, action mechanisms, ultrastructure, apoptosis
1. INTRODUCTION

Leishmaniasis is an important sand fly transmitted protozoan disease of humans and dogs that is endemic in the Mediterranean areas of Europe, including Portugal, the Middle East and many tropical and subtropical areas of the world (World Health Organization, 2011; Cortes et al., 2012). Global epidemiological surveillance shows that infection with *Leishmania* spp. was found in more than 12 million people worldwide. Overall, the disease has been reported from 88 developing countries, particularly found among people living in poor conditions; 350 million people are considered as “high risk” and some 2 million new cases occur yearly in the endemic zones of Latin America, Africa, the Indian subcontinent, the Middle East and the Mediterranean region (World Health Organization, 2011).

Canine visceral leishmaniasis is one of the major zoonoses responsible for severe fatal disease in dogs. Infection in cats, wild canids and horses has also been reported in areas where disease is common in dogs (Baneth et al., 2008). In southern Europe, the causative species is *Leishmania infantum* (syn. *L. chagasi* in the New World) and the vectors are plebotomine sand flies. In northern Europe, infection is mainly restricted to dogs that have travelled to and/or from endemic areas of the Mediterranean region during periods when there is high sand fly exposure (Solano-Gallego et al., 2009; Maia et al., 2010; Postigo, 2010). It has been estimated that at least 2.5 million dogs are infected in southwestern Europe alone. The number of infected dogs in South America is also estimated in millions, and there are high infection rates in some areas of Venezuela and Brazil, where a high prevalence of canine infection is associated with high risk of human disease (Fraga et al., 2012).

The canine leishmaniasis is a public health problem and, therefore, it is necessary to control the infection. In canine leishmaniasis foci, where dogs are the unique domestic reservoir, a reduction in *Leishmania* transmission would be expected if we could combine
an effective mass treatment of infected dogs with a protection of both healthy and infected dogs from the sand fly bites.

Currently, various treatment options are available for canine leishmaniasis, namely pentavalent antimonials, including meglumine antimoniate and sodium stibogluconate, allopurinol and the combination of meglumine antimoniate and allopurinol (Baneth & Shaw, 2002; Alvar et al., 2004; Noli & Auxilia, 2005). However, treatment with these drugs does not promote parasitological cure in infected dogs, leading to frequent relapses, serious side effects and resistance to parasites. Besides, the treatment with pentavalent antimonials, the main class of drugs used to treat leishmaniasis visceral in humans and dogs, is both poorly tolerated and expensive and need continuous administration. These observations imply a urgent developing of new therapeutic strategies for canine leishmaniasis that could enable parasite clearance (Miró et al., 2008; Solano-Gallego et al., 2009; World Health Organization, 2010).

Plants have evolved to overcome competitive disadvantage by producing diverse and complex secondary metabolites that are valuable for screening biological activities (Anthony et al., 2005; Sen et al., 2010). In some endemic foci of parasitism, plants and their extracts are the only readily available forms of treatment, and this knowledge must be preserved and scientifically examined for potentially novel drugs. Most research effort into the effects of plants on parasite infections has been undertaken using aqueous or alcoholic extractions. In addition, plant essential oils may present advantages to treat parasite infections.

Volatile extracts (essential oils) obtained by hydrodistillation contains a huge diversity of small hydrophobic molecules (Lipinski et al., 1997). Such molecules easily diffuse across cell membranes and consequently gaining access to intracellular targets (Edris, 2007).
Thymus capitellatus Hoffmanns & Link (family Lamiaceae; local name ‘tomilho do mato’) is an endemic aromatic plant from Portugal, which grows in the estuaries and downriver parts of Tejo and Sado basins (Estremadura, Ribatejo and Alentejo provinces). In some localities of Estremadura, T. capitellatus is regarded as an antiseptic and is usually used in the treatment of cutaneous infections and in vitro studies have demonstrated its antifungal properties (Salgueiro et al., 2006)

However, there are few reports on the effects of essential oils on old world endemic Leishmania species responsible for cutaneous and visceral leishmaniasis. So, the present work focused on the leishmanicidal activity of Thymus capitellatus and its major compounds, 1,8-cineole and borneol on three old world Leishmania species, namely L. infantum, L. tropica and L. major. Additionally, we undertake other essays to demonstrate the safety of essential oil and to elucidate the action mechanisms of its anti-Leishmania activity.

2. MATERIAL AND METHODS

2.1. Plant material

2.1.1. Origin. Aerial parts of the plant were collected at the flowering stage from Ribatejo, south region of Portugal. Voucher specimens were deposited at the Herbarium of the Department of Botany of the University of Coimbra (COI), under Accession Nos LS 220–222.

2.1.2. Essential Oil. The essential oil from the aerial parts of T. capitellatus was isolated by water distillation for 3 h from air dried material, using a Clevenger-type apparatus, following the procedure described in the European Pharmacopoeia (2004).

2.1.3. Essentials Oils Analysis. Analysis was carried out by gas chromatography (GC) and by gas chromatography-mass spectroscopy (GC/MS). Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single
injector and two flame ionization detection (FID) systems. 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 and SupelcoWax-10. GC-MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column, interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. Components of each essential oil were identified by their retention indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C₈–C₂₃ of n-alkanes, were compared with those of authentic samples included in our own laboratory database. Acquired mass spectra were compared with reference spectra from our own database, Wiley/NIST database, and literature data (Joulain & Konig 1998; Adams, 2004). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

2.2. Parasites and Cultures. Promastigote forms of L. infantum Nicolle (zymodeme MON-1), L. tropica (ATCC 50129) and L. major BCN were maintained at 26°C by weekly transfers in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% inactivated fetal bovine serum (FBS). These cells were used to study the effects of essential oils on Leishmania promastigotes viability.

2.3. Viability assays. Essential oil and major compounds (1,8-cineole and borneol) were initially diluted in dimethyl sulfoxide (DMSO; Sigma Chemical) at 100 mg.mL⁻¹ and then in culture medium in order to get a range of concentrations from 10 to 400 μg.mL⁻¹. Log phase promastigotes of L. infantum, L. tropica and L. major (10⁶ cells/ml) were incubated at 26 °C in 96-well tissue culture plates in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% inactivated FBS in the presence of different concentrations of essential
oil and compounds or DMSO (vehicle control). Effects on viability were estimated by MTT colorimetric method, on the basis of the reduction of the tetrazolium-dye to insoluble formazan by the mitochondrial enzymes (Denizot & Lang, 1986). Briefly, 25μl of MTT (5 mg.ml⁻¹) was added to each well, incubated for 2h at 37 ºC and centrifuged at 3000rpm for 5 min. The supernatant was removed, the cells were washed in PBS, and the precipitated formazan was dissolved in DMSO (250μl). Cell viability was measured by absorbance at 530 nm on an ELISA plate reader (Synergy HT, Bio-TEK), and calculated using the following formula: \[\left(\frac{L2}{L1}\right)\times100\], where L1 is the absorbance of control cells and L2 is the absorbance of treated cells. Amphotericin (Sigma Chemical Co., St. Louis, USA) and miltefosine (Sigma Chemical Co., St. Louis, USA) were used as reference drugs (positive controls). The concentration that inhibited viability by 50% (IC₅₀) was determined after 24 h for \textit{L. infantum} and \textit{L. tropica} and after 48 h for \textit{L. major} using dose-response regression analysis (GraphPad Prism 5). The time of incubation was previously determined on base of growth of the different species (not shown).

2.4. Transmission and scanning electron microscopy. \textit{L. infantum} promastigotes were exposed to essential oil at concentrations that inhibit viability by 50% (IC₅₀) and the morphological alterations were investigated by electronic microscopy. For ultrastructural studies with transmission electronic microscopy, the samples were treated as reported previously (Sousa et al. 2001). Briefly, cell were fixed with glutaraldehyde in sodium cacodylate buffer, post fixed in osmium tetroxide and uranyl acetate, dehydrated in ethanol and in propylene oxide and embedded in Epon 812 (TAAB 812 resin). Ultrathin sections were stained with lead citrate and uranyl acetate. For scanning electronic microscopy, the samples were fixed and postfixed as described for transmission, dehydrated in ethanol, critical point dried using CO₂ and sputter-coat with gold. The specimens were examined in
JEOL JEM-100 SX transmission electron microscopy (TEM) at 80 kV and in JEOL JSM-5400 scanning electron microscope (SEM) at 15 kV.

2.5. Flow cytometry

2.5.1. Cell cycle analysis. For analysis of DNA content, exponentially grown *L. infantum* promastigote cells (10⁶) were treated with *T. capitellatus* essential oil at IC₅₀ concentrations for 24 h at 26ºC. Promastigote suspension was then fixed in 200 µl of 70% ethanol for 30 min. at 4ºC. Next, cells were washed in PBS, and resuspended in 500 µl of PI solution (PI/Rnase, Immunostep) for 15 min. at room temperature (Darzynkiewicz *et al.*, 2001). Cells were then analyzed by flow cytometry (FacsCalibur-Beckton-Dickinson). Results were treated using ModFitLT V 2.0 programme.

2.5.2. Analysis of phosphatidylserine externalization. Double staining for annexin V–FITC and propidium iodide (PI) was performed as described previously (Vermes *et al.*, 1995). Briefly, *L. infantum* promastigotes (10⁶ cells) were exposed to essential oil at IC₅₀ concentrations for 24 h at 26ºC. Cells were then washed with PBS and resuspended in binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 NaCl, 2.5 mM CaCl₂). To 100 µl of this suspension were added 5 µl of Annexin V FITC and 5 µl of PI (AnnexinV-FITC Apoptosis detection Kit, Immunostep). After 15 min incubation in the dark at room temperature, it was added 400 µl binding buffer and cells were then analyzed by flow cytometry (FacsCalibur-Beckton-Dickinson). Data analysis was carried out using the program Paint-a-gate, and values are expressed as a percentage of positive cells for a given marker, relatively to the number of cells analyzed.

2.5.3. Measurement of Mitochondrial Membrane Potential. To assess mitochondrial membrane potential (ΔΨₘ), a cell-permeable cationic and lipophilic dye, JC-1 (5,5',6,6'-tetrachloro-1, 1',3,3'-tetraethylbenzimidazolcarbocyanine iodide), was used as previously described (Cossarizza *et al.*, 1993). This probe aggregates within mitochondria and fluoresces red (590 nm) at higher ΔΨₘ. However, at lower ΔΨₘ, JC-1 cannot accumulate...
within the mitochondria and instead remains in the cytosol as monomers, which fluoresce green (490 nm). Therefore, the ratio of red to green fluorescence gives a measure of the transmembrane electrochemical gradient. *L. infantum* promastigotes (10⁶ cells) were exposed to essential oil IC₅₀ concentrations for 24 h at 26°C. Promastigotes were then incubated JC-1 (5 µg/ml) (Molecular Probes, Invitrogen) in the dark for 15 min at room temperature. Then, cells were washed in PBS, suspended in 400 µl of PBS and analyzed by flow cytometry. Data analysis was carried out using the program Paint-a-gate.

2.6. Mammalian cell cytotoxicity assay. For cytotoxicity assays, log phase of macrophages (ATCC, RAW 264.7 cell line) and bovine aortic endothelial cells were trypsinized and incubated at 37 °C in 24-well tissue culture plates in RPMI 1640 medium (macrophages) and DMEM medium (endothelial cells) supplemented with 10% FBS under microaerophilic condition. When the monolayers reached confluence, the medium was removed and the cells were incubated with fresh medium plus essential oil at IC₅₀ concentrations for 24 h. The cells viability was evaluated by MTT test and by morphological observation by optical microscopy.

2.7. Statistical analysis. All experiments were performed in triplicate and in three independent assays (n=9). Values were expressed as mean±SEM and the means were statistically compared using student t and ANOVA test, with a Dunnett’s post-test. The significance level was *p < 0.05, **p < 0.01 and ***p < 0.001.

3. RESULTS

3.1. Essential oils analysis. *Thymus capitellatus* essential oil is composed by monoterpenes hydrocarbons (18.3%) and oxygen-containing monoterpenes (78.7%), sesquiterpenes hydrocarbons (0.5%) and oxygen-contain sesquiterpenes (0.6%).
main compounds from *T. capitellatus* were 1,8-cineole (58.6%) and borneol (10.1%), two oxygen-containing monoterpenes (Table 1).

3.2. Anti-protozoa activity. *T. capitellatus* essential oil induced loss of viability on *L. infantum*, *L. tropica* and *L. major* (Figure 1). All three strains of *Leishmania* were susceptible to essential oil, showing a marked effect on *L. infantum* (IC$_{50}$=37µg/ml), *L. tropica* (IC$_{50}$=35µg/ml) and *L. major* (IC$_{50}$= 62µg/ml) promastigotes viability (Table 2). Major compounds, 1,8-cineole and borneal, did not reveal any effect on promastigotes at the tested concentrations. The activity of miltefosine and amphotericin B (positive controls) was evaluated under the same conditions and IC$_{50}$ values were similar to those described in the literature, i.e., 6.6-7.7 µM to miltefosine and from 0.032 to 0.25 µg/ml to amphotericin B (not shown).

3.3. Ultrastructural effects. In order to investigate the ultrastructural effects, *L. infantum* promastigotes was chosen as cellular model. The cells was incubated in the presence or absence of the *T. capitellatus* essential oil, and then observed by scanning (Figure 2) and transmission (Figure 3) electron microscopy. Untreated promastigotes (control) observed by scanning electron microscopy presented a typical elongated body shape and anterior flagella (Figure 2A). Essential oil treated promastigotes showed round and aberrant forms (Figure 2 B, C, D,F) with septation of the cell body (Figure 2B, E, F) and irregular surface with blebs formation. Flagella are also impaired presenting membrane disruption, with loss of intracellular content (Figure 2 B, D), blebs formation (Figure 2 D, E) and double flagella cells (Figure 2 B).

Control parasites, observed by transmission electron microscopy, presented normal nucleus, kinetoplast, mitochondria and flagellar pocket (Figure 3 A). The most flagrant ultrastructural effect observed in promastigotes treated with *T. capitellatus* was
cytoplasmatic organelles disorganization (Figure 3 B-D), besides an increase in
cytoplasmatic clearing. There was an increase in the number of autophagosomal
structures, characterized by intense cytoplasmic vacuolization (Figure 3B, D). Treated
parasites also presented swelling of cell body (Figure 3C, D) and mitochondria (Figure 3B-
D). The swelling of the unique and highly branched mitochondria resulted in an inner
mitochondrial membrane disorganization, displaying several and complex invaginations
and forming concentric membranous structures (Figure 3B-D), and finally, mitochondria
clearing (Figure 3C, D). It was also noted the presence of myelin-like figures as
multilamellar bodies (Figure 3B). Other commonalteration was nuclear chromatin
organization, resembling the nucleus of apoptotic cells (Figure 3B, C). Large amounts of
cytoplasmatic vesicles could be seen on many treated cells (Figure 3B, D) and cells with
double nucleus (Figure 3C).

3.4. Cell-cycle arrest at the G(0)/G(1) phase. Cell cycle analysis was performed by flow
cytometry after PI staining of the promastigotes incubated with essential oil for 24 h at
IC_{50} concentrations. Figure 4 shows a representative distribution of cell DNA trough cell
cycle of *L. infantum* in the absence and presence of essential oil. After 24 h of incubation,
the majority of treated parasite cells were arrested on G0/G1 phase of cell cycle (70 %),
opposite to what occurs in not treated cells (36 %).

3.5. Phosphatidylserine externalization. During early apoptosis, plasma membrane
loses asymmetry causing PS to be translocated from the cytoplasmic face of the plasma
membrane to the external face which can be detected using Annexin V. To distinguish
apoptotic cell death from necrotic cell death, cells were counterstained with PI, a non-
permeable stain with an affinity for nucleic acids, as it selectively enters necrotic cells.
Therefore, co-staining of annexin V and PI can differentiate between cells undergoing
early apoptosis (annexin V-positive, PI-negative), necrosis (PI-positive, annexin V-
negative) and live cells (PI- and annexin V negative). In untreated *L. infantum*
promastigotes, the degree of binding of annexin V for 24 h was 3.3 % (Table 2). After the
treatment with *T. capitellatus* essential oil the percentage of annexin V-positive cells
increased to 16 % at 24 h. The percentage of PI-stained control cells was 1.1 % and in the
presence of *T. capitellatus* it increased to 4 %.

3.6. Depolarization of Mitochondrial Membrane Potential. Maintenance of the
mitochondrial transmembrane potential is essential for parasite survival, as *Leishmania*
has a single mitochondrion. *T. capitellatus* essential oil induced a decrease on \( \Delta \Psi_m \). Data
indicate that essential oil caused a sustained decrease on \( \Delta \Psi_m \) (Figure 5). At 24h,
incubation with *T. capitellatus* exhibited a higher number of cells (20%) with low \( \Delta \Psi_m \)
compared to control (4%).

3.7. Mammalian cell cytotoxicity assay. The cytotoxicity of *T. capitellatus* essential oil
was evaluated in cultures of bovine aortic endothelial cells (primary culture) and
macrophages cell line using the MTT test. Results showed that this essential oil did not
induced toxicity against mammalian cells (Figure 6).
4. DISCUSSION

The increased resistance of parasites to conventional therapy, low efficacy, serious adverse effects and high cost have prioritized the development of new anti-parasitic agents (Leandro & Campino, 2003; Croft et al., 2006; Natera et al., 2007, World Health Organization, 2010). The low density of aromatic plant essential oils and their rapid diffusion across cell membranes can enhance the activities of active components within essential oils for control of endoparasites (Bakkali et al., 2008). In the present study, we have, therefore, evaluated the activity of Thymus capitellatus essential oil and its major compounds on three Leishmania species.

Thymus capitellatus oil is mainly composed of oxygen-containing monoterpenes (78.7%), with its major components being 1,8-cineole (58.6%) and borneol (10.1%). To our knowledge, no previous reports have appeared on the antileishmanial activity of T. capitellatus essential oil. In this study, the oil was shown to cause the death of promastigotes of Leishmania infantum, L. tropica and L. major with values of IC\text{50} between 35 and 62 μg/mL. These values are considered promising in relation to the development of new drugs from natural sources (Simões et al., 2009).

The interpretation of results and comparisons between studies need to take into account the Leishmania species and the parasite model cells, i.e., promastigotes or amastigotes. There are only a few reports on the activity of essential oils against L. infantum (IC\text{50} values from 25 to 296 μg/ml) (Machado et al., 2010a,b), L. tropica (IC\text{50} value of 52 μg/ml) (Machado et al., 2012) and L. major (IC\text{50} values from 29.1 to >640 μg/ml) (Mikus et al., 2000; Monzote et al., 2010; Machado et al., 2012; Sanchez-Suarez et al., 2013). Comparing these with the present data, we see that Thymus capitellatus oil shows antileishmanial activity that is similar or higher than that described for the other essential oils on these three Old World species. Various essential oils were tested against Leishmania species of the New World: L. amazonensis (IC\text{50} values from 1.7 to 135 μg/ml)
(Ueda-Nakamura et al., 2006; Monzote et al., 2006; Santin et al., 2009; Santos et al., 2010; Moura do Carmo et al., 2012); *L. braziliensis* (IC$_{50}$ values from 52.1 to 204.36 µg/ml) (Monzote et al., 2010; Sanchez-Suarez et al., 2013); *L. chagasi* (IC$_{50}$ values from 4.4 to 181 µg/ml) (Oliveira et al., 2009; Escobar et al., 2010; Rondon et al., 2012); *L. donovani* (IC$_{50}$ values from 4.45 to 156 µg/ml) (Monzote et al., 2007; Zheljazkov et al., 2008; Monzote et al., 2010; Parreira et al., 2010;); *L. guyanensis* (IC$_{50}$ values from 15.2 to 315.55 µg/ml) (Moura do Carmo et al., 2012; Sanchez-Suarez et al., 2013); *L. mexicana* (IC$_{50}$ 63.3µg/ml) (Monzote et al., 2010); *L. panamensis* (IC$_{50}$ values from 42.23 to 427.95 µg/ml) (Sanchez-Suarez et al., 2013). The oils showed variable levels of activity against *Leishmania*, which depended on the species used in the assays.

In addition to being active against promastigote cells, *T. capitellatus* essential oil is also expected to exhibit activity on amastigotes forms, as is generally found among natural extracts and synthetic drugs. In almost all reports, the antiparasitic activity is higher against amastigotes (Dutta et al., 2007; Lakshmi et al., 2007; Nakayama et al., 2007; Santin et al., 2009; Rondon et al., 2012), but in others the activity is similar (Monzote et al., 2007; Santos et al., 2010; Moura do Carmo et al., 2012) although it has been found to be lower in some cases (Rondon et al., 2011). This discrepancy is generally related to differences in the ability of the compounds to cross the membranes of the macrophage and the parasitophorous vacuole. In addition, it has been shown that activity of antileishmanial drugs in intracellular amastigotes was host cell dependent (Seifert et al., 2010). The data obtained by the use of mousse peritoneal macrophages, mousse bone marrow-derived macrophage, human peripheral blood monocyte-derived macrophages, and differentiated THP-1 cells were quite different. If all the considerations about the models used for screening anti-leishmanial activity are taken into account, we feel that it is necessary and urgent for these drug activity assays to be standardized.
The major compounds of Thymus capitellatus essential oil, 1,8-cineole and borneol, do not show anti-leishmanial activity and do not seem to be responsible for the essential oil activity. We must, however, consider the possibility that the activity of natural extracts could result from the interaction between their constituents. Therefore, further phytochemical work and drug association studies are needed to identify the compound(s) responsible for the effects of Thymus capitellatus essential oil.

In drug discovery, the potential toxicity of the compounds towards mammalian cells must be considered. We have found that T. capitellatus oil is not cytotoxic toward the macrophage cell line and bovine aortic endothelial cells, suggesting that toxicity of this essential oil is not significant for mammalian cells. Therefore, T. capitellatus oil is promising candidate for leishmaniasis therapy.

In addition to the anti-Leishmania activity of essential oils, they have also shown insecticidal and repellence activity (Bakkali et al., 2008), which is extremely relevant in the prevention and treatment of canine leishmaniasis.

Our results have demonstrated that Thymus capitellatus essential oil promoted phosphatidylserine exposure, depolarization of mitochondrial potential and arrested G0/G1 cell cycle phase on L. infantum promastigotes. These characteristics have been reported to play a key role in drug-induced death in protists such as Leishmania (Sen et al., 2004). Data obtained by scanning and transmission microscopy suggest that some of the triggers of the events described above could be associated with morphological alterations induced by T. capitellatus oil. The cells treated with essential oil did not show intact an kinetoplast, and we observed an increase in cell and organelle volume, cytoplasm clearing and disorganization. Some of these features have already been observed in studies with other drugs and are reported to contribute to cell death mechanisms (Santoro et al., 2007; Oliveira et al., 2009). The rapid diffusion across cell membranes (owing to their lipid solubility) and low density of essential oils allow their accumulation in the organelles of
parasites and membrane destabilization (Lipinski et al., 1997). In addition, the presence of myelin-like figures suggests that there may be an autophagic process, with the formation of structures known as autophagosomes. These structures are probably involved in the breakdown and recycling of abnormal membrane structures, suggesting an intense process of remodeling of intracellular organelles promoted by the \textit{T. capitellatus} essential oil. These alterations have previously been observed in Kinetoplastids treated with drugs such as ketoconazole and terbinafine (Lazardi et al., 1990; Lorente et al., 2004).

Along with above findings, \textit{T. capitellatus} essential oil also induced ultrastructural alterations in the mitochondria, mainly on the mitochondrial matrix, with the appearance of complex structures and swelling of the mitochondrion. The effects observed may be related with the depolarization of mitochondrial membrane potential, and could promote cell death, as suggested by anexin-V values. Previous work has, in fact, reported a correlation between mitochondrial membrane permeabilization and induction of apoptosis on \textit{L. major} (Arnoult et al., 2002).

The presence of cells with double flagella or/and with double nucleus, suggesting an incapacity to conclude the process of division may also be related to the cell death mechanisms induced by \textit{T. capitellatus} essential oil. This effect was confirmed by the arrest of \textit{Leishmania} cells in the G0/G1 cycle phase, with a decrease in the number of promastigotes occurring on phase S and G2/M. The decrease on mitochondrial membrane potential may contribute to this event since it is required for the production of cellular energy.

Although the pharmacological application of essential oils against mammalian parasitic infections remains unclear due the potential risk of toxicity, it has recently been reported that essential oils from \textit{Artemisina annua} (Radulovic’ et al., 2013), \textit{Croton argyropylloides} (de França-Neto et al., 2012), \textit{Ligustim chuanxiong} (Zhang et al., 2012) and \textit{Menta villosa} (Da Silva et al., 2012) are not toxic in animal models. In addition, the present work has
also demonstrated that *T. capitellatus* essential oil has anti-*Leishmania* activity without significant cytotoxicity against mammalian cell lines (macrophages and endothelial cells). Therefore, our overall results strongly suggest that *Thymus capitellatus* oil may represent a valuable source for the development of drugs against *Leishmania* infections.

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CAPTIONS TO FIGURES

Figure 1- Effect of *Thymus capitellatus* essential oil on *Leishmania* promastigotes viability. Cultures of log-phase promastigotes ($10^6$) were incubated at 26°C for 24h (*L. infantum*, *L. tropica*) or 48h (*L. major*), as function of essential oil concentration. Values are expressed as means and SEM.

Figure 2- Scanning electron micrographs of *Leishmania infantum* promastigotes exposed to *Thymus capitellatus* essential oil. A, Untreated cells showing the typical elongated shape showing parasite body and anterior flagella; B-F, Treated promastigotes. Note round and aberrant forms (B-F) with cell body septation (B, E, F). Note the irregular surface (asterisks) and membrane disruption (arrows) A-F. Bars=5 µm.

Figure 3- Transmission electron micrographs of *Leishmania infantum* promastigotes exposed to *Thymus capitellatus* essential oil. A, Control parasites; B-D, parasites treated with essential oil. Note mitochondrial swelling (MS), gross alterations in the organization of cytoplasm (*). N, nucleus; K, kinetoplast, F, flagellum, FP, flagellar pocket, MB, multilamelar bodies, A, autophagicveshicles, V, cytoplasm vesicles.Bars, 2 µm.

Figure 4- Cell cycle histograms of *Leishmania infantum* promastigotes. *L. infantum* promastigotes were incubated at 26 ºC for 24 hours in the absence (A) or presence of *T. capitellatus* essential oil at IC$_{50}$ concentrations (B). Propidium iodide staining was performed and samples were analyzed by flow cytometry.

Figure 5- Representative dot plots showing JC-1 staining of *Leishmania infantum*. Promastigotes were incubated at 26 ºC for 24 hours in the absence (A-3h, B-24h) or presence of *T. capitellatus* essential oil (C-3h, D-24h) at IC$_{50}$ concentrations. JC-1 staining was performed and samples were analyzed by flow cytometry. J-aggregates (blue) reflect functioning mitochondria and monomers are indicative of compromised mitochondria (pink).

Figure 6- Cytotoxicity of *Thymus capitellatus* essential oil on mammalian cells. Log phase of macrophages (ATCC, RAW 264.7 cell line) and bovine aortic endothelial cells were incubated with essential oil (IC$_{50}$) for 24 h at 37ºC and the viability was evaluated by MTT test. Values are expressed as means and SEM.
Table 1. Composition of *Thymus capitellatus* essential oil.

<table>
<thead>
<tr>
<th>RI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Compound</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>921</td>
<td>1016</td>
<td>Tricyclene</td>
<td>0.3</td>
</tr>
<tr>
<td>923</td>
<td>1028</td>
<td>α-Thujene</td>
<td>0.1</td>
</tr>
<tr>
<td>930</td>
<td>1028</td>
<td>α-Pinene</td>
<td>4.5</td>
</tr>
<tr>
<td>943</td>
<td>1074</td>
<td>Camphene</td>
<td>6.4</td>
</tr>
<tr>
<td>964</td>
<td>1125</td>
<td>Sabinene</td>
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</tr>
<tr>
<td>970</td>
<td>1115</td>
<td>β-Pinene</td>
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<tr>
<td>980</td>
<td>1160</td>
<td>Myrcene</td>
<td>0.2</td>
</tr>
<tr>
<td>1012</td>
<td>1271</td>
<td>p-Cymene</td>
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</tr>
<tr>
<td>1020</td>
<td>1204</td>
<td>Limonene</td>
<td>0.8*</td>
</tr>
<tr>
<td>1020</td>
<td>1215</td>
<td>1,8-Cineole</td>
<td>58.6*</td>
</tr>
<tr>
<td>1050</td>
<td>1457</td>
<td><em>trans</em>-Sabinene hydrate</td>
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<tr>
<td>1055</td>
<td>1442</td>
<td>cis-Linalool oxide</td>
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<td>1082</td>
<td>1540</td>
<td>Linalool</td>
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</tr>
<tr>
<td>1117</td>
<td>1511</td>
<td>Camphor</td>
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</tr>
<tr>
<td>1120</td>
<td>1645</td>
<td><em>trans</em>-Pinocarveol</td>
<td>0.4</td>
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<tr>
<td>1125</td>
<td>1645</td>
<td>cis-Verbenol</td>
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<td>1134</td>
<td>1561</td>
<td>Pinocarvone</td>
<td>0.2</td>
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<td>1146</td>
<td>1695</td>
<td>Borneol</td>
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<tr>
<td>1157</td>
<td>1594</td>
<td>Terpinene-4-ol</td>
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<td>Myrtenal</td>
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<td>β-Selinene</td>
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<tr>
<td>1557</td>
<td>1965</td>
<td>Caryophyllene oxide</td>
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<td>2067</td>
<td>Viridiflorol</td>
<td>0.2</td>
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<tr>
<td></td>
<td></td>
<td>Monoterpene hydrocarbons</td>
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<td></td>
<td></td>
<td>Oxygen containing monoterpenes</td>
<td>78.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sesquiterpene hydrocarbons</td>
<td>0.5</td>
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<tr>
<td></td>
<td></td>
<td>Oxygen containing sesquiterpenes</td>
<td>0.6</td>
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<tr>
<td></td>
<td></td>
<td><strong>Total identified</strong></td>
<td><strong>98.1</strong></td>
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</table>

Compounds listed in order to their elution on the SPB-1 column;

<sup>a</sup> Retention indices on the SPB-1 column relative to C8–C22 *n*-alkanes.

<sup>b</sup> Retention indices on the relative to C8 to C22 *n*-alkanes.

* Quantification based on SupelcoWax-10 column chromatogram.
Table 2- Flow cytometry analysis of *Leishmania infantum* promastigotes treated with *Thymus capitellatus* essential oil showing the percentage of propidium iodide (PI) and annexin-V positive cells.

<table>
<thead>
<tr>
<th>Leishmania intracellular entities % of cells</th>
<th>Anexine</th>
<th>PI</th>
<th>Anexine/PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3h 5h 7h 24h</td>
<td>3h 5h 7h 24h</td>
<td>3h 5h 7h 24h</td>
</tr>
<tr>
<td><em>T. capitellatus</em></td>
<td>4 3 2 16</td>
<td>1 1 2 4</td>
<td>1.0 2.1 0.8 10</td>
</tr>
<tr>
<td>Control</td>
<td>1.3 1.6 1.3 3.3</td>
<td>0.4 0.2 0.3 1.1</td>
<td>0.5 0.3 0.3 1.6</td>
</tr>
</tbody>
</table>
Figure 1

![Graph showing the effect of Thymus capitellatus essential oil on the viability of different Leishmania species.](image)

**Table:**

<table>
<thead>
<tr>
<th></th>
<th>L. infantum</th>
<th>L. tropica</th>
<th>L. major</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>37 (30 - 46)</td>
<td>35 (24 - 49)</td>
<td>62 (54 - 73)</td>
</tr>
</tbody>
</table>

*95% Confidence intervals*
Figure 5

<table>
<thead>
<tr>
<th></th>
<th>Leishmania intracellular entities</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(%) of cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JC1Mon</td>
<td>3h</td>
<td>7h</td>
<td>24h</td>
</tr>
<tr>
<td>T. capitellatus</td>
<td>14</td>
<td>16</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JC1Agreg</td>
<td>3h</td>
<td>7h</td>
<td>24h</td>
</tr>
<tr>
<td>T. capitellatus</td>
<td>86</td>
<td>83</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83</td>
<td>89</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIFAgreg / MIFMon membrane potential</td>
<td>3h</td>
<td>7h</td>
<td>24h</td>
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<tr>
<td>T. capitellatus</td>
<td>1.5</td>
<td>1.4</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.6</td>
<td>2</td>
<td>2.7</td>
<td></td>
</tr>
</tbody>
</table>

The table above shows the percentage of cells with different Leishmania intracellular entities at 3h, 7h, and 24h time points for T. capitellatus and control samples. The histograms (A, B, C, D) illustrate the distribution of these entities over the time course.
Figure 6

The graph shows the viability of cells expressed as a percentage of control. The comparison includes the following groups:

- **Control**
- **Macrophage cells**
- **Endothelial cells**

The graph indicates that there is no significant difference (ns) between the viability percentages of these cell types.

Y-axis: Viable cells (% of control)

X-axis: Categories of cells