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PII: S0304-4017(13)00638-9
DOI: http://dx.doi.org/doi:10.1016/j.vetpar.2013.11.016
Reference: VETPAR 7037

To appear in: *Veterinary Parasitology*

Received date: 14-12-2012
Revised date: 1-11-2013
Accepted date: 18-11-2013

Please cite this article as: Machado, M., Dinis, A.M., Santos-Rosa, M., Alves, V., Salgueiro, L., Cavaleiro, C., Sousa, M.C.,"Activity of Thymus capitellatus volatile extract, 1,8-cineole and borneol against Leishmania species.", *Veterinary Parasitology* (2013), http://dx.doi.org/10.1016/j.vetpar.2013.11.016

TITLE PAGE

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_{50} 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 leishmaniais 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 C_8–C_{23} 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 $\mu g.mL^{-1}$. Log phase promastigotes of *L. infantum*, *L. tropica* and *L. major* (10^6 cells/ml) were incubated at 26 ºC 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

158 oil and compounds or DMSO (vehicle control). Effects on viability were estimated by MTT
159 colorimetric method, on the basis of the reduction of the tetrazolium-dye to insoluble
160 formazan by the mitochondrial enzymes (Denizot & Lang, 1986). Briefly, 25µl of MTT (5
161 $mg.ml^{-1}$) was added to each well, incubated for 2h at 37 ºC and centrifuged at 3000rpm for
162 5 min. The supernatant was removed, the cells were washed in PBS, and the precipitated
163 formazan was dissolved in DMSO (250µl). Cell viability was measured by absorbance at
164 530 nm on an ELISA plate reader (Synergy HT, Bio-TEK), and calculated using the
165 following formula: [(L2/L1)x100], where L1 is the absorbance of control cells and L2 is the
166 absorbance of treated cells. Amphotericin (Sigma Chemical Co., St. Louis, USA) and
167 miltefosine (Sigma Chemical Co., St. Louis, USA) were used as reference drugs (positive
168 controls). The concentration that inhibited viability by 50% (IC_{50}) was determined after 24 h
169 for *L. infantum* and *L. tropica* and after 48 h for *L. major* using dose-response regression
170 analysis (GraphPad Prism 5). The time of incubation was previously determined on base
171 of growth of the different species (not shown).
172
173 ***2.4. Transmission and scanning electron microscopy.*** *L. infantum* promastigotes were
174 exposed to essential oil at concentrations that inhibit viability by 50% (IC_{50}) and the
175 morphological alterations were investigated by electronic microscopy. For ultrastructural
176 studies with transmission electronic microscopy, the samples were treated as reported
177 previously (Sousa *et al.* 2001). Briefly, cell were fixed with glutaraldehyde in sodium
178 cacodylate buffer, post fixed in osmium tetroxide and uranyl acetate, dehydrated in ethanol
179 and in propylene oxide and embedded in Epon 812 (TAAB 812 resin). Ultrathin sections
180 were stained with lead citrate and uranyl acetate. For scanning electronic microscopy, the
181 samples were fixed and postfixed as described for transmission, dehydrated in ethanol,
182 critical point dried using CO_2 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 (10^6) were treated with *T. capitellatus* essential oil at IC_{50} 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 (10^6 cells) were exposed to essential oil at IC_{50} concentrations for 24 h at 26ºC. Cells were then washed with PBS and ressuspended in binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 NaC1, 2.5 mM $CaCl_2$). To 100 µl of this suspension were added 5 µl of Annexin V FITC and 5 µl of PI (AnnexinV-FITC Apoptosis detection Kit, Immmunostep). 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 ($\Delta\Psi_m$), 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 $\Delta\Psi_m$. However, at lower $\Delta\Psi_m$, 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 (10^6 cells) were exposed to essential oil IC_{50} concentrations for 24 h at 26ºC. 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 ºC 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 IC_{50} 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%). The

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 multilamelar 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-

289 negative) and live cells (PI- and annexin V negative). In untreated *L. infantum*
290 promastigotes, the degree of binding of annexin V for 24 h was 3.3 % (Table 2). After the
291 treatment with *T. capitellatus* essential oil the percentage of annexin V-positive cells
292 increased to 16 % at 24 h. The percentage of PI-stained control cells was1.1 % and in the
293 presence of *T. capitellatus* it increased to 4 %.
294
295 **3.6. Depolarization of Mitochondrial Membrane Potential***.* Maintenance of the
296 mitochondrial transmembrane potential is essential for parasite survival, as *Leishmania*
297 has a single mitochondrion. *T. capitellatus* essential oil induced a decrease on $\Delta\Psi_m$. Data
298 indicate that essential oil caused a sustained decrease on $\Delta\Psi_m$ (Figure 5). At 24h,
299 incubation with *T. capitellatus* exhibited a higher number of cells (20%) with low $\Delta\Psi_m$
300 compared to control (4%).
301
302 **3.7. Mammalian cell cytotoxicity assay.** The cytotoxicity of *T. capitellatus* essential oil
303 was evaluated in cultures of bovine aortic endothelial cells (primary culture) and
304 macrophages cell line using the MTT test. Results showed that this essential oil did not
305 induced toxicity against mammalian cells (Figure 6).
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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_{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_{50} values from 25 to 296 µg/ml) (Machado et al., 2010a,b), *L. tropica* (IC_{50} value of 52 µg/ml) (Machado et al., 2012) and *L. major* (IC_{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_{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 *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, *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 correlaction between mithocondrial membrane permeabilization and induction of apoptosis on *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 *T. capitellatus* essential oil. This effect was confirmed by the arrest of *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 *Artemisina annua* (Radulovic´et al., 2013), *Croton argyropylloides* (de França-Neto et al., 2012), *Ligustim chuanxiong* (Zhang et al., 2012) and *Menta villosa* (Da Silva et al., 2012) are not toxic in animal models. In addition, the present work has

418 also demonstrated that *T. capitellatus* essential oil has anti-*Leishmania* activity without
419 significant cytotoxicity against mammalian cell lines (macrophages and endothelial cells).
420 Therefore, our overall results strongly suggest that *Thymus capitellatus* oil may represent
421 a valuable source for the development of drugs against *Leishmania* infections.
422

423 **ACKNOWLEDGEMENTS**

424 Authors are grateful to Prof. Jorge Paiva for help in plant taxonomy, Correia da Costa from
425 Centro de Imunologia e Biologia Parasitária, Instituto Nacional Dr. Ricardo Jorge, Porto for
426 supplying the *Leishmania infantum* Nicolle (zymodeme MON-1), António Osuna,
427 Departamento de Parasitología, Facultad de Ciencias, Instituto de Biotecnología,
428 Universidad de Granada, for suplly *Leishmania major* BCN.
429 *Funding***:** This work was supported by "Programa Operacional Ciência e Inovacão 2010
430 (POCI)/FEDER" da Fundação para a Ciência e Tecnologia (FCT).
431
432 *Competing interests*: None declared.
433
434 *Ethical approval*: Not required.
435

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622
623
624

625 **CAPTIONS TO FIGURES**

626 **Figure 1- Effect of *Thymus capitellatus* essential oil on *Leishmania* promastigotes**
627 **viability.** Cultures of log-phase promastigotes (10^6) were incubated at 26°C for 24h (*L.*
628 *infantum, L. tropica*) or 48h (*L. major*), as function of essential oil concentration. Values are
629 expressed as means and SEM.
630
631 **Figure 2- Scanning electron micrographs of *Leishmania infantum* promastigotes**
632 **exposed to *Thymus capitellatus* essential oil. A,** Untreated cells showing the typical
633 elongated shape showing parasite body and anterior flagella; **B-F,** Treated promastigotas.
634 Note round and aberrant forms (B-F) with cell body septation (B, E, F). Note the irregular
635 surface (asterisks) and membrane disruption (arrows) A-F. Bars=5 µm.
636
637 **Figure 3- Transmission electron micrographs of *Leishmania infantum***
638 **promastigotas exposed to *Thymus capitellatus* essential oil. A**, Control parasites; **B-**
639 **D,** parasites treated with essential oil. Note mitochondrial swelling (MS), gross alterations
640 in the organization of cytoplasm (*). N, nucleus; K, kinetoplast, F, flagellum, FP, flagelar
641 pocket, MB, multilamelar bodies, A, autophagicveshicles, V, cytoplasm veshicles.Bars, 2
642 µm.
643
644 **Figure 4- Cell cycle histograms of *Leishmania infantum* promastigotes.** *L. infantum*
645 promastigotas were incubated at 26 ºC for 24 hours in the absence (A) or presence of *T.*
646 *capitellatus* essential oil at IC_{50} concentrations (B). Propidium iodide staining was
647 performed and samples were analyzed by flow cytometry.
648
649 **Figure 5- Representative dot plots showing JC-1 staining of *Leishmania infantum.***
650 Promastigotas were incubated at 26 ºC for 24 hours in the absence (A-3h, B-24h) or
651 presence of *T. capitellatus* essential oil (C-3h, D-24h) at IC_{50} concentrations. JC-1 staining
652 was performed and samples were analyzed by flow cytometry. J-aggregates (blue) reflect
653 functioning mitochondria and monomers are indicative of compromised mitochondria
654 (pink).
655
656 **Figure 6- Cytotoxicity of *Thymus capitellatus* essential oil on mammalian cells.** Log
657 phase of macrophages (ATCC, RAW 264.7 cell line) and bovine aortic endothelial cells
658 were incubated with essential oil (IC_{50}) for 24 h at 37ºC and the viability was evaluated by
659 MTT test. Values are expressed as means and SEM.
660

660 **Table 1. Composition of *Thymus capitellatus* essential oil.**

661

RI[a]	RI[b]	Compound	%
921	1016	Tricyclene	0.3
923	1028	α-Thujene	0.1
930	1028	α-Pinene	4.5
943	1074	Camphene	6.4
964	1125	Sabinene	3.0
970	1115	β-Pinene	2.0
980	1160	Myrcene	0.2
1012	1271	*p*-Cymene	1.0
1020	1204	Limonene	0.8*
1020	1215	1,8-Cineole	58.6*
1050	1457	*trans*-Sabinene hydrate	0.3
1055	1442	*cis*-Linalool oxide	0.1
1082	1540	Linalool	1.7
1117	1511	Camphor	3.0
1120	1645	*trans*-Pinocarveol	0.4
1125	1645	*cis*-Verbenol	0.5
1134	1561	Pinocarvone	0.2
1146	1695	Borneol	10.1
1157	1594	Terpinene-4-ol	1.0
1165	1620	Myrtenal	0.3
1168	1691	α-Terpineol	1.1
1175	1695	Verbenone	0.4
1264	1573	Bornyl acetate	0.7
1328	1688	α-Terpinyl acetate	0.3
1471	1707	β-Selinene	0.3
1487	1688	Ledene	0.2
1557	1965	Caryophyllene oxide	0.4
1569	2067	Viridiflorol	0.2
Monoterpene hydrocarbons			18.3
Oxygen containing monoterpenes			78.7
Sesquiterpene hydrocarbons			0.5
Oxygen containing sesquiterpenes			0.6
Total identified			**98.1**

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

663 [a] Retention indices on the SPB-1 column relative to C8–C22 *n*-alkanes.

664 [b] Retention indices on the relative to C8 to C22 *n*-alkanes.

665 * Quantification based on SupelcoWax-10 column chromatogram.

666

667

668

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.

	***Leishmania* intracellular entities (% of cells)**											
	Anexine				**PI**				**Anexine/PI**			
	3h	**5h**	**7h**	**24h**	**3h**	**5h**	**7h**	**24h**	**3h**	**5h**	**7h**	**24h**
T. capitellatus	4	3	2	16	1	1	2	4	1,0	2,1	0,8	10
Control	1.3	1.6	1.3	3.3	0.4	0.2	0.3	1.1	0.5	0.3	0.3	1.6

Figure1

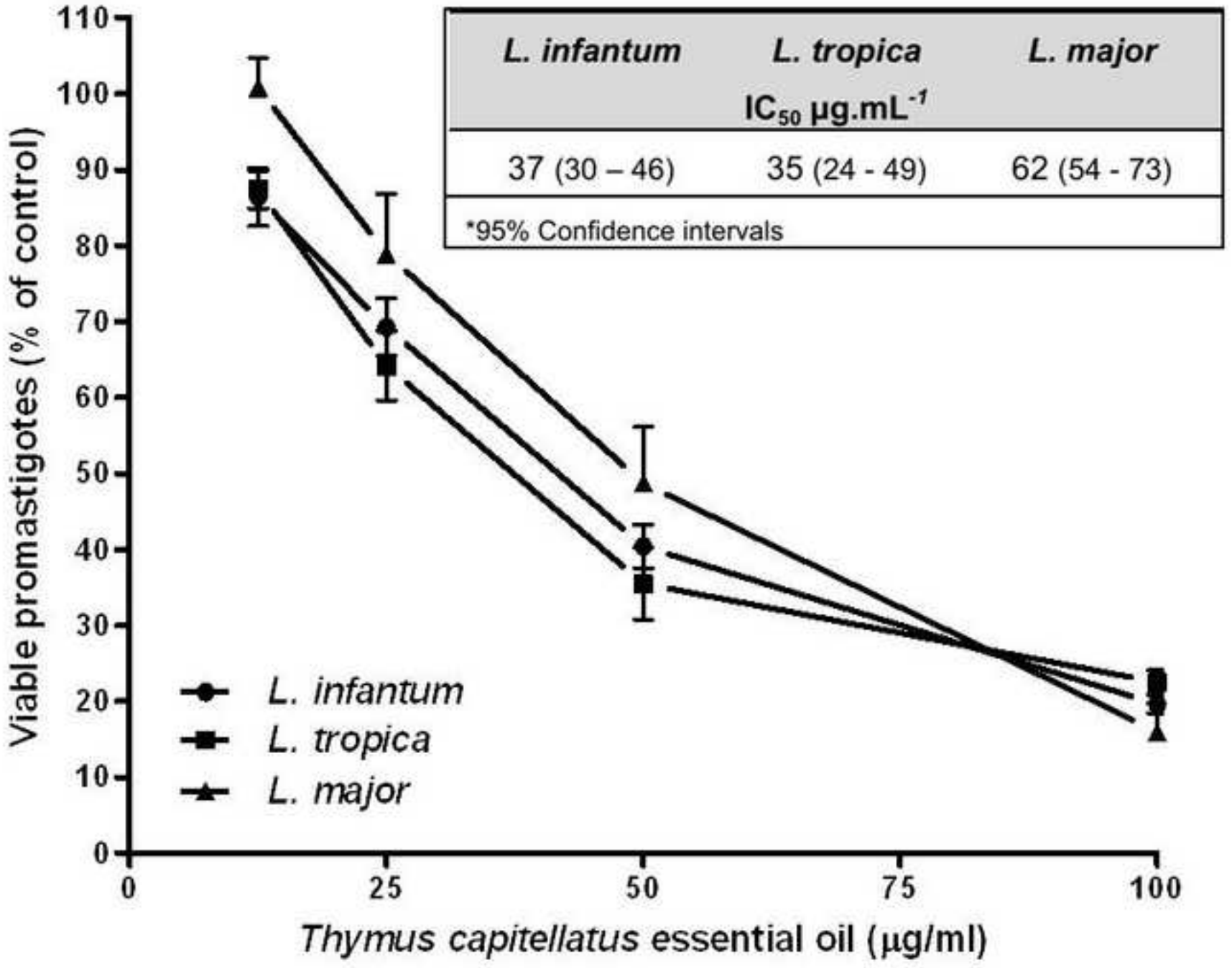
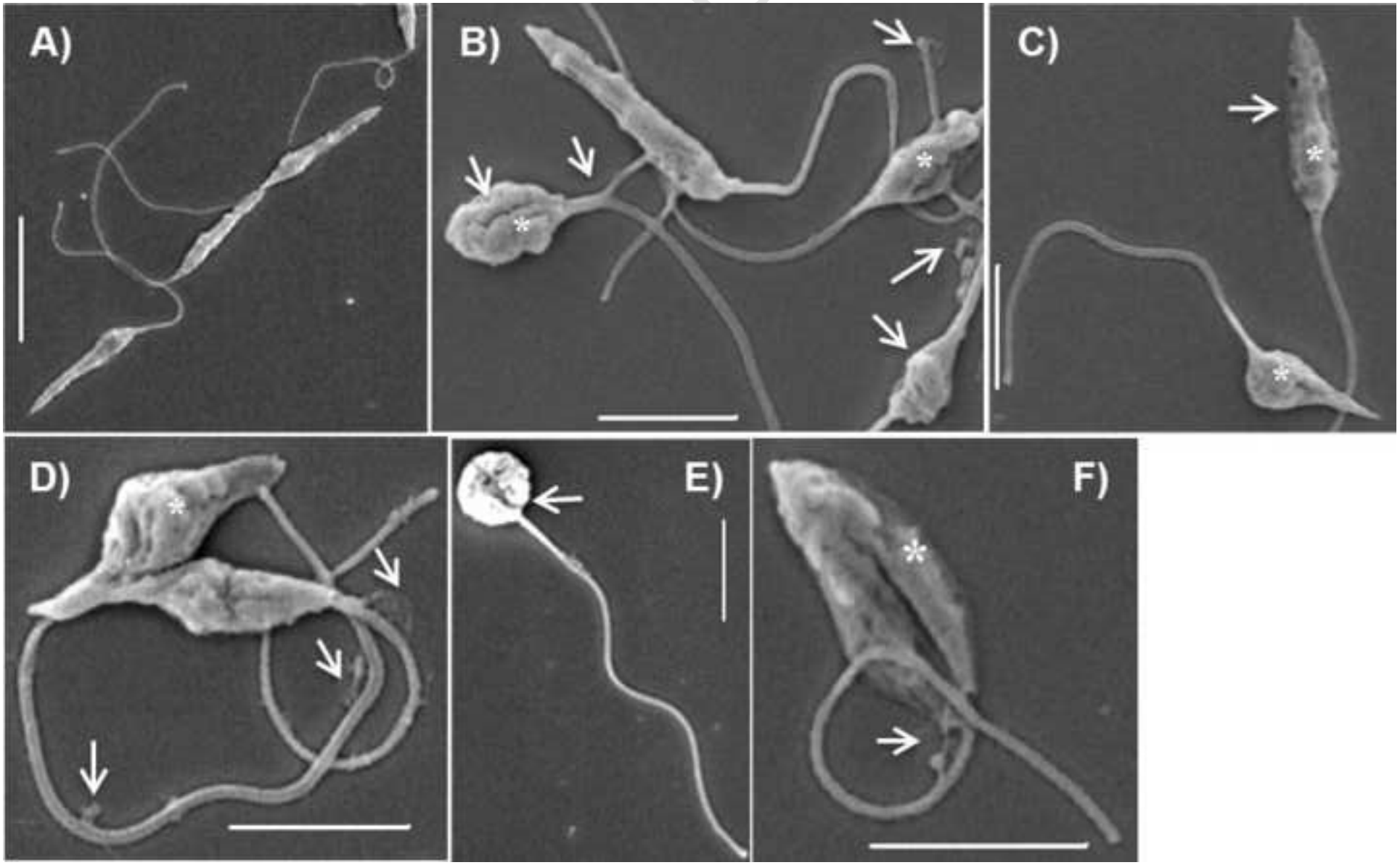

L. infantum
L. tropica
L. major
IC50 µg.mL-1
37 (30 – 46)
35 (24 - 49)
62 (54 - 73)
*95% Confidence intervals
Viable promastigotes (% of control)
Thymus capitellatus essential oil (µg/ml)
0
10
20
30
40
50
60
70
80
90
100
110
0
25
50
75
100

Figure2



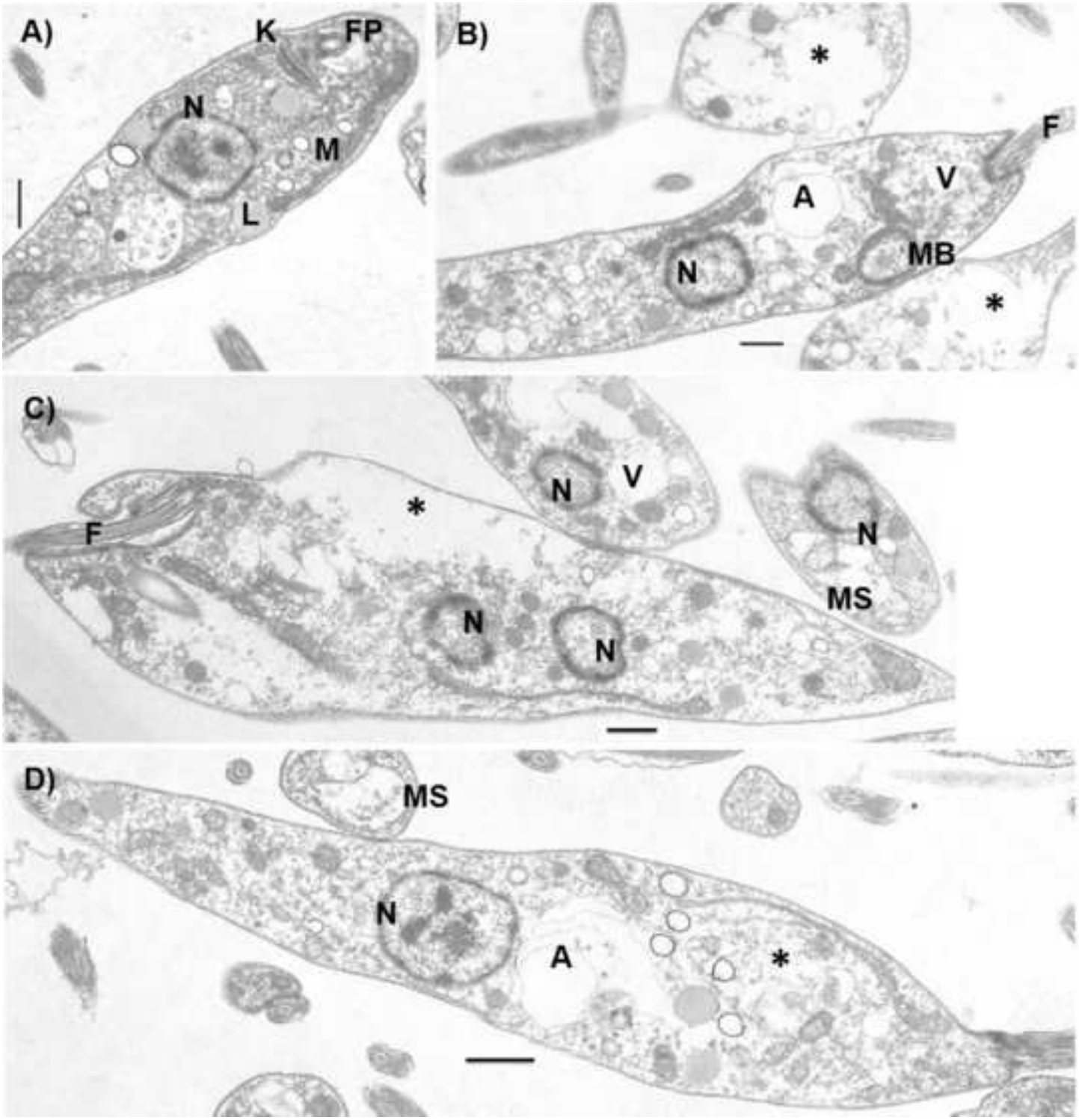


Figure4

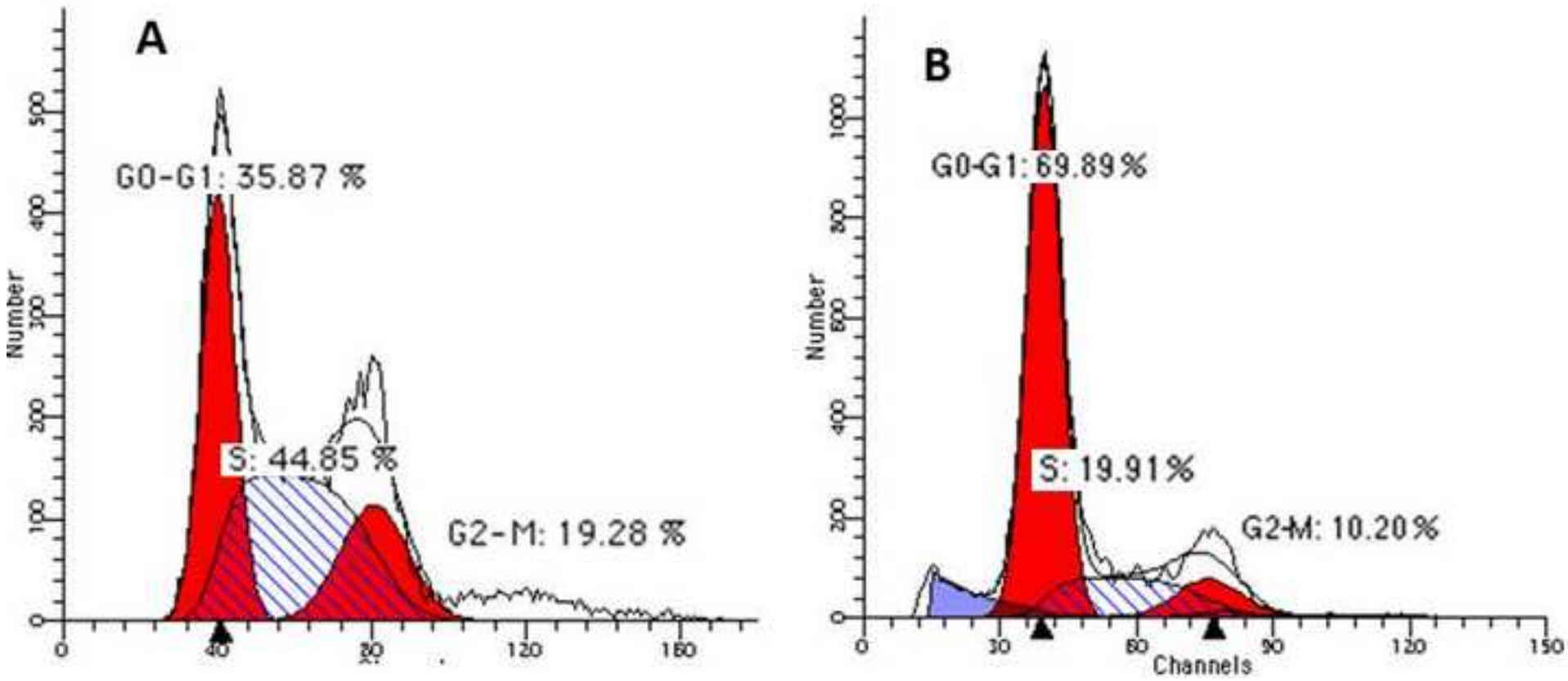

A
G0-G1: 35.87 %
S: 44.85 %
G2-M: 19.28 %
Number
0
100
200
300
400
500
0
40
80
120
160
B
G0-G1: 69.89%
S: 19.91%
G2-M: 10.20%
Number
0
200
400
600
800
1000
0
30
60
90
120
150
Channels

Figure 5

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