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Lavandula Luisieri and *Lavandula Viridis* Essential Oils as Upcoming Anti-Protozoal Agents: A Key Focus on Leishmaniasis

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Abstract: *Background and objectives: Leishmania* species is the causative agent of leishmaniasis, a broad-spectrum clinical condition that can even be life-threatening when neglected. Current therapeutic strategies, despite beings highly cost-effective, have been increasingly associated with the appearance of drug-resistant microorganisms. Thus, an increasing number of thorough studies are needed towards upcoming drug discovery. This study aims to reveal the anti-protozoa activity of *Lavandula luisieri* and *Lavandula viridis* essential oils (EO) and their main components (1,8-cineole, linalool, and borneol). *Materials and Methods: L. luisieri* and *L. viridis* EO and their main components' leishmanicidal effects were tested in vitro against *Leishmania infantum, Leishmania major,* and *Leishmania tropica* strains. Cell viability effects were estimated by using the tetrazolium-dye (MTT) colorimetric method, morphological changes were assessed by scanning electron microscopy (SEM) and ultrastructural investigation by transmission electronic microscopy (TEM). Phosphatidylserine externalization, mitochondrial membrane potential (MMP), and cathepsin D activity assessment were also carried out. Finally, cytotoxic activity of the studied matrices was also determined in mammalian cells. *Results:* Plant-studied EO exhibited prominent anti-*Leishmania* effects (IC_{50} = 31–263 µg/mL), with *L. luisieri* being the most active one. At concentrations corresponding to IC_{50} values, EO-exposed *L. infantum* promastigotes suffered marked ultrastructural modifications. The presence of aberrant-shaped cells, mitochondrial and kinetoplast swelling, and autophagosomal structures were the most common evidenced changes. *L. luisieri* EO exerted its leishmanicidal activity through different mechanisms, but mainly through unleashing apoptosis. Phosphatidylserine externalization, mitochondrial membrane potential loss, and cell-cycle arrest at G(0)/G(1) phase were the most remarkable apoptosis-mediated aspects. Inhibition of cathepsin D activity was also observed. No toxic effects were found on macrophage cells. *Conclusions: L. luisieri* seems to be an upcoming source of bioactive molecules for leishmaniasis control and to find leading molecules for new drugs formulation against *Leishmania* infections.

Keywords: anti-Leishmania activity; *Lavandula* spp., essential oil; flow cytometry; drug development

1. Introduction

Leishmania species is the causative agent of leishmaniasis. This unicellular trypanosomatid induces a broad-spectrum disease, varying from localized, self-healing, and cutaneous lesions to disfiguring forms of mucocutaneous leishmaniasis, possibly even lethal visceral forms, when neglected [1–3]. Chemotherapy treatment is considered the most cost-effective intervention on parasitic diseases. Nevertheless, given the ineffectiveness of the current therapeutic approaches, the appearance of drug-resistant microorganisms, the rate of incidence of parasitic diseases, linked with the associated toxicity, bioavailability, and care costs, more intense studies are needed towards effective drugs discovery [4].

Plant extracts, characterized by their rich diversity and complexity of secondary metabolites, involved on the expression of evolutionary strategies to overcome competitive disadvantages of plants, comprise valuable collections of compounds to screen bioactive effects [5,6]. In some endemic foci of parasitism, plants and their corresponding extracts are the only readily available treatment forms, and therefore, this knowledge must be preserved and scientifically examined towards upcoming drugs formulation. Aqueous and alcoholic extractions are the most commonly selected extraction methods used to assess the antimicrobial activity of plant matrices; however, the use of purified essential oils (EO) of a plant may represent a plus. In fact, EO composes a pool of hydrophobic molecules that easily diffuse across cell membranes [7] and, consequently, gain advantage and interact with intracellular targets [8]. They can be conceived as a promisor approach on parasite infections control and even treatment.

Lavandula genus gives valuable EO for food, perfume, and cosmetic industries. Moreover, its EO has been reported to have prominent biological activities, namely sedative and antispasmodic, as well as, acaricidal, antibacterial, antifungal, and antioxidant effects [9]. Among *Lavandula* genus, *Lavandula viridis* L'He'r. and *Lavandula luisieri* Rivas-Martinez, both endemic aromatic shrubs from the Iberian Peninsula, have been widely used for medical purposes [10]. For example, it has been reported that *L. luisieri* EO possesses remarkable antifeedant, antimicrobial, and antioxidant effects, in addition of being a source of biologically active molecules against yeast, dermatophyte, and *Aspergillus* strains, responsible for food contamination and human infections [11]. However, there are only few reports available investigating EO effects on *Leishmania* species, and, specifically, no previous reports were stated on *L. luisieri* and *L. viridis*.

In recent years, our research group focused on EO antimicrobial effects evaluation; thus, based on previous promising results obtained to *Lavandula* species EO and its main chemotypes (1,8-cineole, 1,8-cineole/borneol, and 1,8-cineole/linalyl acetate/linalool) on different fungal strains [1–4], we also started evaluating their anti-protozoa activity, mainly against *Giardia lamblia* and *Leishmania* spp. [5–10].

In this sense, this study aimed to access the *L. luisieri*, *L. viridis*, and its major compounds' (α-pinene, 1,8-cineole, linalool, and borneol) leishmanicidal effects against three *Leishmania* species, namely *L. infantum*, *L. major*, and *L. tropica*. Other assays were also undertaken to extend our knowledge on EO safety and to assess the effective modes of action underlining its biological potential.

2. Materials and Methods

2.1. Plant Material

2.1.1. Origin

Lavandula luisieri L. inflorescences were identified and collected in the centre of Portugal (Beira Alta), and a voucher specimen was prepared and deposited in the Faculty of Pharmacy from the University of Coimbra (FFUC) herbarium (accession number: Zuzarte 02010). *Lavandula viridis* L'Hér aerial parts were collected from field-growing flowering plant parts in the south of Portugal (Algarve), being voucher specimens deposited at FFUC herbarium (accession number: Zuzarte 0206).

2.1.2. Essential Oil Preparation

The essential oil from the *L. luisieri* and *L. viridis* aerial parts 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 (Council of Europe, 1997). Please see the Supplementary Materials for the Composition of the essential oil of *Lavandula viridis* and *Lavandula luisieri*.

2.1.3. Essential Oils Analysis

Gas chromatography (GC) and gas chromatography–mass spectroscopy (GC/MS) were used to perform EO analysis. GC was carried out using a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, as previously described by Machado et al. (2012). GC/MS was performed using a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column, interfaced with a Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00 [9]. Acquired mass spectra were compared with reference spectra from Wiley/NIST database [11] and literature data [12,13]. Individual components were determined based on GC peak areas without flame ionization detection (FID) response factor correction.

2.2. Parasites and Cultures

L. infantum Nicolle (zymodeme MON-1), *L. major* BCN, and *L. tropica* (ATCC 50129) promastigotes were maintained at 26 °C by weekly transfers in HEPES (25 mM)-buffered RPMI 1640 medium, enriched with 10% inactivated fetal bovine serum (FBS). Then, cells were used to investigate EO effects on *Leishmania* strains growth.

2.3. Viability Assays

EO and its major compounds (α-pinene, 1,8-cineol, borneol, and linalool) were formerly diluted in 100 mg/mL dimethyl sulfoxide (DMSO; Sigma Chemical) and then in a culture medium to get concentrations ranging from 10–300 μg/mL.

L. infantum, *L. major*, and *L. tropica* (106 cells/mL) log phase promastigotes were incubated as previously described [9] in the presence of different EO concentrations and compounds or DMSO (vehicle control) at 26 °C. Then, viability effects were estimated by tetrazolium-dye (MTT) colorimetric method [14]. Concentration inhibiting 50% (IC_{50}) cells viability was reached 24 h after for *L. infantum* and *L. tropica*, and for *L. major* at 48 h.

2.4. Transmission and Scanning Electron Microscopy

L. infantum promastigotes were exposed to essential oils at concentrations that inhibit viability by 50% (IC_{50}) and the morphological alterations were investigated by electronic microscopy. For ultrastructural studies with transmission electronic microscopy, samples were treated as reported previously (Sousa et al., 2001). Briefly, the cell was 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 post-fixed as described for transmission, dehydrated in ethanol, critical point dried using CO_2, and sputter-coated 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 flow cytometry analysis of DNA content, exponentially grown *L. infantum* promastigote cells (106) were treated with essential oil at IC50 concentrations for 24 h at 26 °C. Promastigote suspension was then fixed in 200 μL of 70% ethanol for 30 min at 4 °C. Next, cells were washed in PBS and resuspended in 500 μL of propidium iodide (PI) solution (PI/Rnase, Immunostep) for 15 min at room temperature (Darzynkiewicz et al., 2001). Cells were then analyzed by flow cytometry (Facs Calibur-Beckton-Dickinson). Results were treated using the ModFit LT V 2.0 program.

2.5.2. Phosphatidylserine Externalization Analysis

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 26 °C. Cells were then washed with PBS and resuspended in binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 NaC1, 2.5 mM CaCI2). 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, 400 μL binding buffer was added and cells were then analyzed by flow cytometry (Facs Calibur-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, relative to the number of cells analyzed.

2.5.3. Assessment of Mitochondrial Membrane Potential (MMP)

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, 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 (106 cells) were exposed to essential oil IC_{50} 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. Cathepsin D Activity Assay

To evaluate the effect on protozoa aspartic proteases activity, axenic cultures of protozoa parasites were used. The enzymatic activity of cathepsin D was determined using parasites suspensions and following the instructions kit (Cathepsin D Activity Assay Kit, Abcam). The cleavage of cathepsin D substrate was monitored in an ELISA plate reader using an excitation wavelength of 328 nm and an emission wavelength of 460 nm.

2.7. Cytotoxicity Evaluation in Mammalian Cells

For cytotoxicity assays, log phase of macrophages cells (ATCC, RAW 264.7 cell line) were trypsinized and incubated at 37 °C in 24-well tissue culture plates in RPMI 1640 medium 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 100 μg/mL for 14 h. Cell viability was evaluated by MTT test and by morphological observation by optical microscopy.

2.8. Statistical Analysis

Data were expressed as mean ± SEM. T-tests and ANOVA with a Dunnett's post-test were used to compare means. Significance level was * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. All samples were analyzed in triplicate and repeated in three different occasions.

3. Results

3.1. Analysis of EO

Major compounds found in both plant EO and their relative proportions are shown in Table 1. The most abundant components in *L. luisieri* right after necrodane derivatives (5/36%) were α-pinene (2.3%), linalool (3.1%), 1,8-cineol (18.9%); from *L. viridis*, the main compounds were α-pinene (9.2%), camphene (2.7%), 1,8-cineol (29.7%), linalool (9.0%), camphor (10.0%), borneol (2.7%), among others.

Table 1. Major composition of *Lavandula luisieri* and *Lavandula viridis* essential oils.

Compounds	*Lavandula luisieri* (%)	*Lavandula viridis* (%)
Monoterpene hydrocarbons	4.7	17.3
Oxygen containing monoterpenes	75.7	58.3
Sesquiterpene hydrocarbons	2.4	18.6
Oxygen containing sesquiterpenes	3.0	-
Others	1.2	1.9
Total identified	(49) 86.9	(38) 96.1
Major compounds (>2.0%)	α-Pinene (2.3) 1,8-cineole (18.9) Linalool (3.1) Necrodane derivatives (36.0) Lavandulyl acetate (7.2)	α-Pinene (9.2) Camphene (2.7) 1,8-cineole (29.7) Linalool (9.0) Camphor (10.0) Borneol (2.7) Z-α-bisabolene (6.3) Selina-3,7(11)-diene (6.6)

3.2. Evaluation of Leishmanicidal Activity

All studied *Leishmania* strains were susceptible to *L. luisieri* EO, and we observed a more prominent effect on *L. infantum* (IC_{50}/24 h = 63 µg/mL), followed by *L. tropica* (IC_{50}/24 h = 38 µg/mL) and *L. major* (IC_{50}/48 h = 31 µg/mL) viability. *L. viridis* essential oil displayed less activity against *L. infantum* (IC_{50}/24 h = 263 µg/mL) (Table 2).

Table 2. *Lavandula luisieri* and *Lavandula viridis* essential oils activity and major compounds on *Leishmania* promastigotes viability.

	L. infantum IC50 µg/mL (CI) *	*L. tropica* IC50 µg/mL (CI) *	*L. major* IC50 µg/mL (CI) *
L. luisieri	63 (52–77)	38 (33–45)	31 (25–38)
L. viridis	263 (248–279)	>400	>400
α-pinene	161 (149–175)	>400	>400
1,8-cineole	>400	>400	>400
Borneol	>400	>400	>400
Linalool	>400	>400	>400

Cultures of log-phase promastigotes (10^6) were incubated at 26 °C for 24 h (*L. infantum*, *L. tropica*) or 48 h (*L. major*), in function of essential oil concentration; * CI, Confidence Intervals; n.d., not determined.

The main compounds, 1,8-cineole, borneol, and linalool did not reveal any effect on *L. infantum* promastigotes at tested concentrations, while α-pinene revealed some activity against *L. infantum* (IC_{50}/24 h = 161 µg/mL).

3.3. Ultrastructural Effects

SEM (Figure 1) and TEM (Figure 2) imaging were used to assess the occurrence of ultrastructural changes in *L. infantum* promastigotes after 7 h incubation in presence or absence of *L. luisieri* EO.

Regarding SEM findings, untreated cells (control) displayed elongated body shape and anterior flagella (Figure 1A,B), while EO-treated ones (Figure 1C–F) displayed round (Figure 1D,E) and aberrant forms (Figure 1C), with cell body septation (Figure 1 D). Note the uneven bubble-forming surface of all treated parasites. On the other hand, considering TEM imaging, control parasites evidenced normal nucleus, kinetoplast, mitochondria, and flagellar pocket (Figure 2A). Cytoplasmic organelles disorganization (Figure 2B,C,F), in addition, an increase in cytoplasmic clearing (Figure 2B,F) was the most prominent ultrastructural effect observed in *L. luisieri*-treated cells. A marked raise was also found in a number of autophagosomal structures, featured by intense cytoplasmic vacuolization (Figure 2F). EO-treated parasites also evidenced cell body (Figure 2F) and mitochondria (Figure 3B,D,F) swelling. Indeed, single and highly branched mitochondria swelling led to inner mitochondrial membrane disorganization, as shown by the presence of complex invaginations and concentric membranous structures formation (Figure 2B,D,F), and, lastly, mitochondria clearing (Figure 2B,D). Another common alteration was kinetoplast swelling and clearing (Figure 3B,D,E).
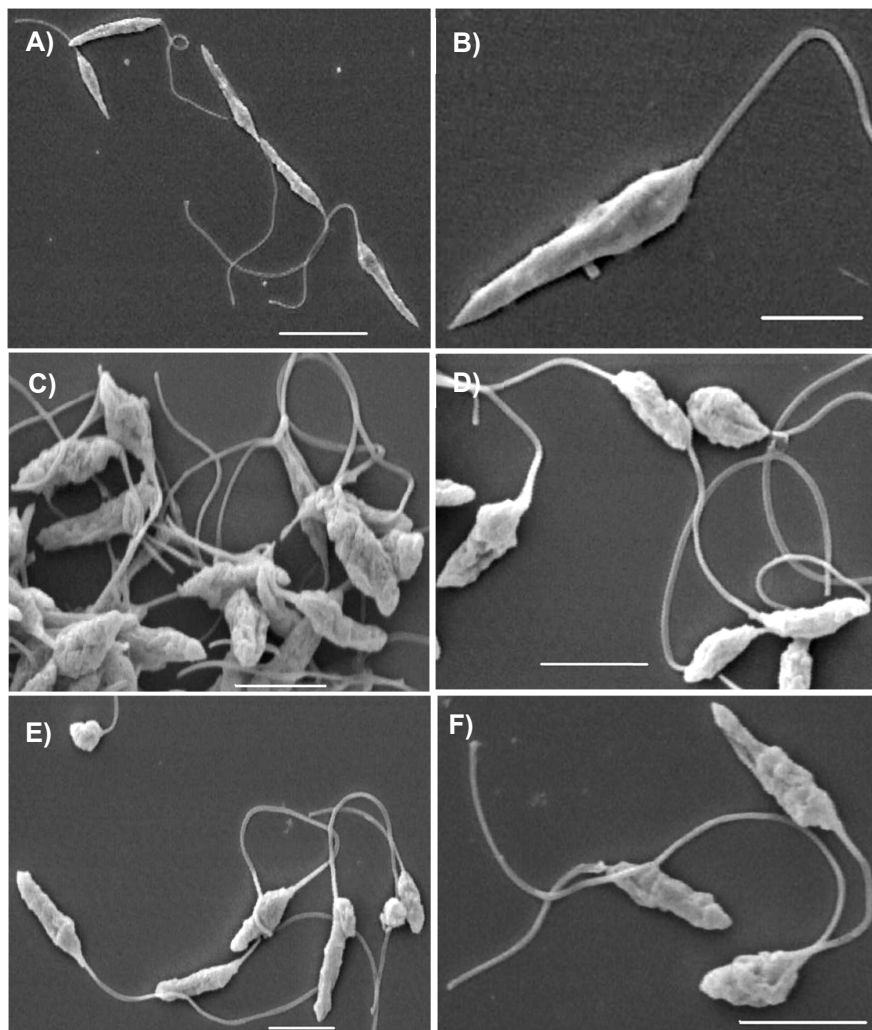

Figure 1. Scanning electron micrographs of *Leishmania infantum* promastigotes exposed to *Lavandula luisieri* essential oil. (**A**,**B**) Untreated cells showing the typical elongated shape, parasite body and anterior flagella; (**C**–**F**), Treated promastigotes. Note round forms and aberrant forms (**C**–**F**) with cell body septation (**D**). Note the irregular surface. (**A**–**F**) Bars = 5 μm.
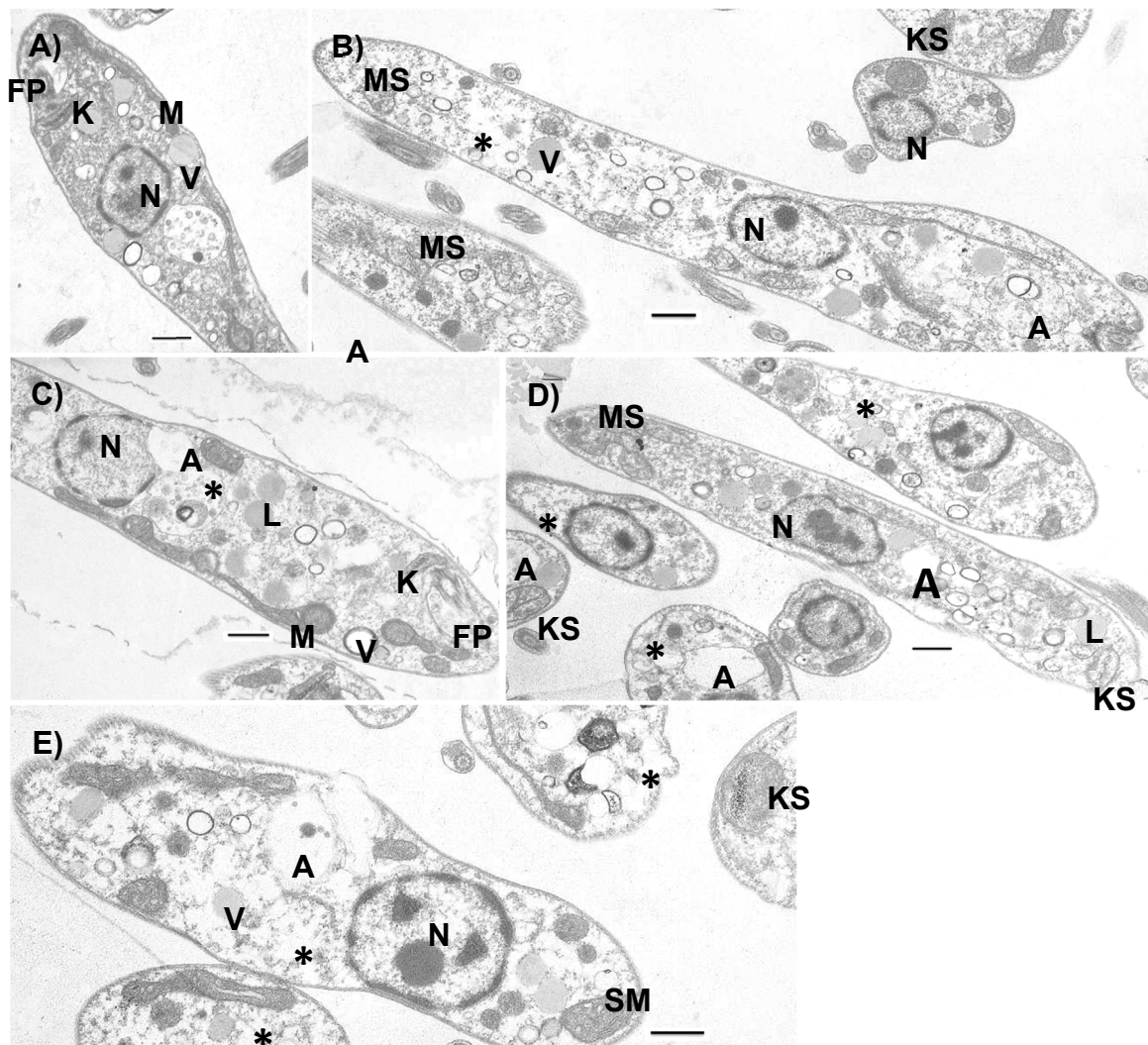
Figure 2. Transmission electron micrographs of *Leishmania infantum* promastigotes exposed to *Lavandula luisieri* essential oil. (**A**) Control parasites; (**B–E**) parasites treated with essential oil. Note mitochondrial swelling (MS) (**B,D,E**) and kinetoplast swelling (**B,D,E**), gross alterations in the organization of cytoplasm (*) (**B–E**). N, nucleus; K, kinetoplast; F, flagellum; FP, flagellar pocket; A, autophagic vesicles; V, cytoplasm vesicles. Bars, 2 μm.

3.4. Cell-cycle Arrest at G(0)/G(1) Phase

Figure 3 shows cell DNA distribution through parasite cell cycle, in absence and presence of EO. After 24 h incubation, almost all treated cells were on cell cycle G0/G1 phase (91%) arrest, in opposite to what occurs in non-treated cells (36%).

3.5. Phosphatidylserine Externalization

As shown in Table 3, in non-treated cells, the annexin V lashing degree at 24 h was 3%. After *L. luisieri* EO-treatment, annexin V-positive cells percentage raised to 17.5%. PI-stained control cells percentage was 1.1%, while in presence of *L. luisieri* EO raised to 4%.
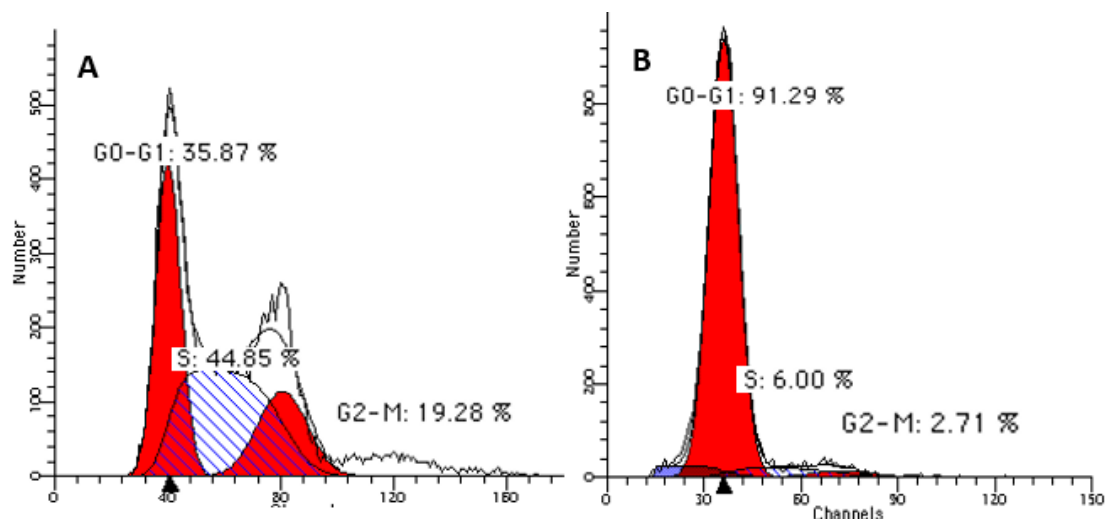
Figure 3. Cell cycle histograms of *Leishmania infantum* promastigotes exposed to *Lavandula luisieri* essential oil. *L. infantum* promastigotas were incubated at 26 °C for 24 h in the absence (**A**) or presence (**B**) of *L. luisieri* essential oil at IC$_{50}$ concentrations. Propidium iodide staining was performed and samples were analyzed by flow cytometry.

Table 3. Flow cytometry analysis of *Leishmania infantum* promastigotes treated with *Lavandula luisieri* essential oil, showing the percentage of propidium iodide (PI) and annexin-V positive cells.

	Leishmania **Promastigotes (% of Cells) 24 h**		
	Anexine	**PI**	**Anexine/PI**
L. luisieri	17.5	4	10
Control	3.3	1.1	4.8

3.6. Mitochondrial Membrane Potential (MMP) Depolarization

MMP maintenance is crucial for cell survival, including for *Leishmania* species, as they have a single mitochondrion. *L. luisieri* EO induced a sustained decrease in $\Delta\Psi_m$ (Figure 4). At first hour's incubation (3 h), a significant difference was already observed between control and treated cells with low mitochondrial transmembrane potential, being, respectively, 13% and 40%. At 24 h *L. luisieri* incubation, 29.2% of cells displayed low $\Delta\Psi_m$ when compared to control (3.6%).

3.7. Cathepsin D Activity Assay

The effect of *L. luisieri* essential oil on cathepsin D enzymatic activity was explored by applying a constant concentration (100 µg/mL) related to their IC$_{50}$ value and evaluating changes over time (2.5, 4, and 7.5 h). Data obtained demonstrated any inhibition of cathepsin D enzymatic activity around 20% (data not shown).

3.8. Cytotoxicity Evaluation in Mammalian Cells

L. luisieri EO cytotoxic activity was assessed in macrophages cell lines using MTT test. Data obtained show that this EO did not induce macrophages toxicity at the tested dose (CC50 > 200 µg/mL; SI > 3.17).

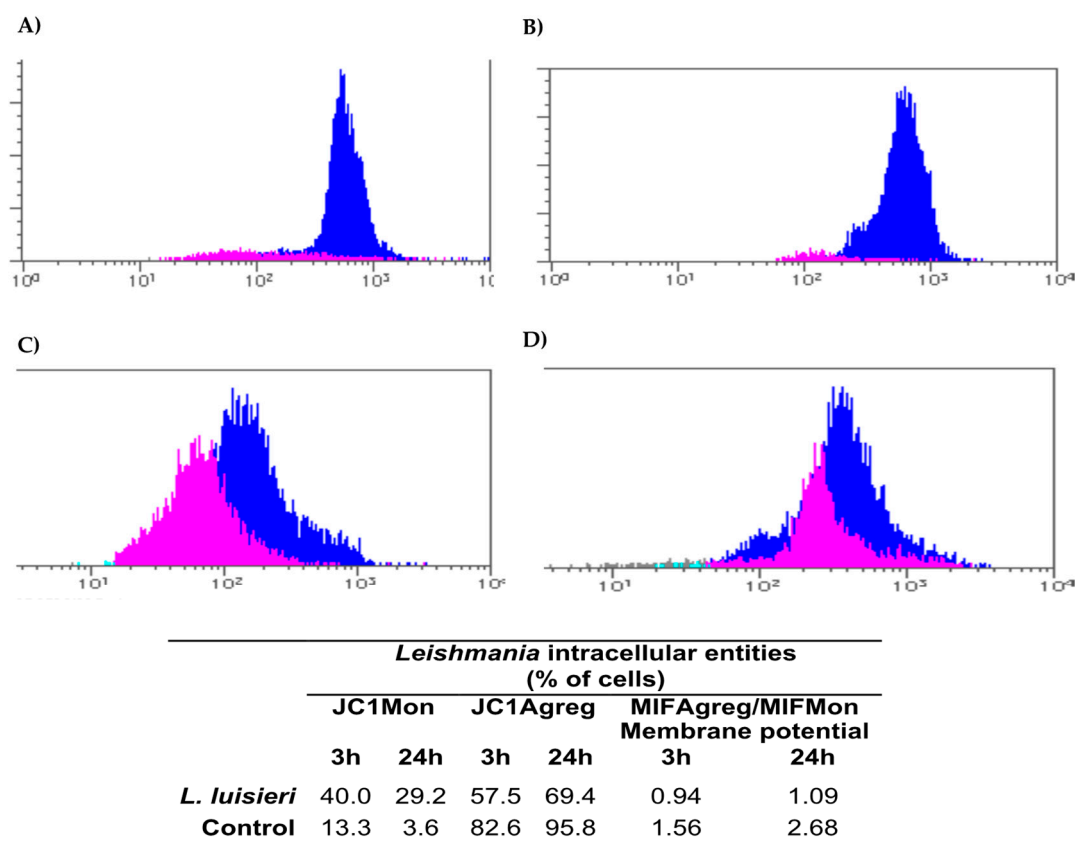|	Leishmania intracellular entities (% of cells)					
	JC1Mon		JC1Agreg		MIFAgreg/MIFMon Membrane potential	
	3h	24h	3h	24h	3h	24h
L. luisieri	40.0	29.2	57.5	69.4	0.94	1.09
Control	13.3	3.6	82.6	95.8	1.56	2.68

Figure 4. Representative dot plots showing JC-1 staining of *Leishmania infantum* promastigotes exposed to *Lavandula luisieri* essential oil. *L. infantum* promastigotas were incubated at 26 °C for 24 h in the absence ((**A**) 3 h; (**B**) 24 h) or presence of *L. luisieri* essential oil ((**C**) 3 h; (**D**) 24 h) 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).

4. Discussion

Currently, with the increasing rate of conventional therapy-resistant parasites, low drug efficacy, prominent adverse effects, and heavy costs, it is urgent to search for new anti-parasitic agents [15,16]. As previously highlighted, aromatic plant EO has low density and rapid diffusion across cell membranes, and this aspect may improve in targeting EO active components to endoparasites [17]. *L. luisieri* EO evidenced on its chemical composition oxygen containing monoterpenes (75.7%) in large amounts, with 1,8-cineole (18.9%) being the major compound and representing necrodane derivatives (36%). *L. viridis* presented α-pinene (9.2%), 1,8-cineole (29.7%), linalool (9.0%), and camphor (10.0%) as major compounds. The assessment of *L. luisieri* and *L. viridis* inhibitory concentration at 50% (IC$_{50}$) showed promising anti-*Leishmania* activity, both against cutaneous and visceral strains, *L. infantum, L. major*, and *L. tropica*. A more prominent effect was observed in *L. luisieri* (IC$_{5}$ = 31–63 μg/mL), while *L. viridis* showed a high IC$_{50}$ value (263 μg/mL) as well as its compound, α-pinene (IC$_{50}$ = 161 μg/mL). This fact might be related with the higher content on oxygen containing monoterpenes, and somehow, with necrodane derivatives. The other tested compounds did not reveal any activity. Therefore, it is feasible to suppose that the major compounds present in EO did not seem to be accountable for EO-stated effects.

L. luisieri EO revealed a promising potential, as it obtained data in accordance with that already described research by Simões [18]. In this study, *L. luisieri* EO also promoted phosphatidylserine exposure, MMP depolarization, and G0/G1 phase cell cycle arrest leading to cell death [18]. These features have been stated as playing a pivotal role in protists drug-induced death, such as *Leishmania* species [19]. Taking SEM and TEM imaging advantages, some of the above highlighted facts could be

linked to *L. luisieri* EO-induced morphologic changes. Indeed, among treated cells, it was not possible to identify an intact kinetoplast (swelled), only an increment in cell and organelle volume, cytoplasm clearing, and disorganization was found, as already stated in other studies [20–22]. Further, low density and rapid diffusion across cell membranes can raise EO in targeting parasites organelles, as well as inducing membrane destabilization [23]. This also suggests the existence of autophagic process with the consequent formation of autophagosomes [24], which seems to be involved in abnormal membrane structures' breakdown and recycling (supposing an intense intracellular organelles remodeling process, EO-irreversibly damaged). Similarly, other reports have already stated this change in drugs-treated kinetoplastids [25,26]. Mitochondrial changes were also stated, mainly on its matrix, arising complex structures, or otherwise, a less electrodense matrix, and mitochondrion swelling. Those alterations were also previously stated by Brenzan et al. [27] using *L. brasiliensis,* and by Rosa et al. [28] and Ueda-Nakamura et al. [29] using *L. amazonensis*. These data may be explained by the ability of inducing mitochondrial potential depolarization, which may promote apoptosis as corroborated by the anexin-V values reached [30]. Moreover, the occurrence of G0/G1 cycle phase cells arrest with the consequent reduction of cell numbers on S and G2/M phase, may be possibly explained by mitochondrial potential decrease that may trigger this event since it reduces energy available.

In addition, it is important to point out that cell toxicity risk due to EO ingestion or skin contact requires further investigation. In this sense, cytotoxic potential of the studied EO was assessed using macrophage cell lines, but no toxic effects were stated using *L. luisieri* EO. These findings are even more important as *Leishmania* amastigotes forms are intracellular. Therefore, *L. luisieri* essential oil might be effective against parasites without affecting host cell, as described before.

5. Conclusions

Overall, the present findings allow us to support the popularity of this plant as an upcoming approach to treat skin infections and, at the same time, the strengths they use on *Leishmania* infections, particularly in cutaneous forms, both in humans and animals. In any case, the upcoming use of EO for mammalian parasitic infections treatment remains unclear, although its potential for seeking new compounds is clear. Therefore, further studies are needed to expand our knowledge on this field, as well as to find the responsible phytochemicals by the observed effects.

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