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In vitro Antileishmanial Activity of *Lavandula angustifolia* Essential Oil on *Leishmania infantum* Parasites

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ABSTRACT

Objective: Leishmaniasis is an endemic tropical disease that is disseminated through the bite of a sandfly infected with *Leishmania* parasites. Conventional antileishmanial drugs are mainly toxic and can be ineffective; therefore, there is a need for new natural drug candidates. This study investigated the antileishmanial effects of *Lavandula angustifolia* (LA) essential oil on *Leishmania infantum (L. infantum)* parasites, and the safety features were tested on RAW264.7 murine macrophages.

Materials and Methods: LA essential oil was produced through the process of hydro-distillation, and its phytochemical content was determined using the gas chromatography-mass spectrometry (GC-MS) analysis. The antileishmanial effects of LA (0.063 to1 µL/mL) on *L. infantum* parasites as well as their safety features were assessed on RAW264.7 murine macrophages.

Results: The composition of LA essential oil was detected using GC-MS analysis, including linalool, pinene, 1, 8-cineole, linalyl acetate, and lavandulol. Concentrations at and above 0.5 µL/mL LA indicated a significant reduction (71% decrease) in the parasite proliferation, and caused a slight reduction in macrophage viability to 70% at 72 h.

Conclusions: The findings revealed the antileishmanial effect of LA on *L. infantum* parasites with relatively less toxicity on macrophages. The promising antileishmanial efficacy highlights the potential for further *in vivo* studies.

Keywords: Antileishmanial therapy, *Lavandula angustifolia*, *Leishmania infantum*, macrophages

INTRODUCTION

Leishmaniasis is a parasitic disease widespread in tropical regions, caused by protozoan parasites classified under the genus *Leishmania*. It significantly affects millions of individuals globally, particularly in developing countries, and is transmitted via the bite of phlebotomine sandflies. More than 20 different *Leishmania* species exhibit the ability to survive as either promastigotes (extracellular form) within sandflies or amastigotes (intracellular form) inside mammalian macrophages (1). According to a report by the World Health Organization in 2018, leishmaniasis is widespread in 98 tropical countries, thereby posing a significant threat to more than 600,000 individuals (2). There is an annual occurrence of around 1.3 million new cases of leishmaniasis, which manifest in three clinically different forms, including visceral Leishmaniasis, which is generally known as "Kala Azar" and can lead to mortality, if not treated.

The current treatment options available for Leishmaniasis have been reported to contain pentavalent antimonials, conventional amphotericin B (AmpB) deoxycholate, miltefosine, paromomycin, AmpB micelles (Fungizone), and liposomal AmpB (AmBisome) (3–9). However, therapeutic interventions have certain limitations associated with these therapies, such as restricted efficacy against parasites (10), numerous adverse effects owing to low therapeutic

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index (11), the need for cautious and gradual intravenous administration (e.g. AmpB) (12), and the emergence of drug resistance against the therapy (3, 5, 7, 13– 17). Therefore, there is a need for new drugs with fewer side effects and higher efficacy.

In recent years, researchers have turned their attention to natural products as potential candidates for new drugs (18–24). The plant Lavandula, commonly referred to as lavender, has been utilized for its medicinal properties for centuries. Previous studies demonstrated that lavender exhibits diverse biological activities, including anti-protozoal effects against Leishmaniasis and other protozoan infections (19, 25, 26). Besides, the essential oils of *Lavandula angustifolia* (LA) and *Lavandula intermedia* were assessed for their anti-parasitic properties against protozoans, namely *Giardia duodenalis, and Trichomonas vaginalis*, as reported in a previous study (26). In another study, the antileishmanial effects of LA and *Rosmarinus (R.) officinalis* essential oils, as well as their nanoemulsions against *Leishmania major*, were examined by Shokri et al. (2017) (19). Upon treatment of *Leishmania* parasites with different species of lavender, several compounds were investigated in the composition of lavender, such as linalool and linalyl acetate, which displayed a potent antileishmanial activity. This has led to increased interest in exploring lavender as a potential source of new drugs for leishmaniasis.

In this study, LA essential oil (found in Turkish flora) was first obtained by hydro-distillation, and further composition of LA was detected by gas chromatography-mass spectrometry (GC-MS) analysis. Following the investigation of the plant composition, the antileishmanial effect of LA on the growth of *Leishmania infantum (L. infantum)* parasites was evaluated together with its safety properties on healthy murine RAW264.7 macrophages at different ranges of concentrations (0.063-1 µL/mL). Cell culture studies, including cell viability, and morphology were conducted to evaluate the antileishmanial efficacy of LA essential oil.

MATERIALS AND METHODS

Materials

Resazurin sodium salt (R7017 - 1G) was purchased from Sigma-Aldrich, Germany. The flowering aerial tops of LA were collected from Yalova, Turkiye, in May 2022. The plant material was identified by Prof. Dr. Fikrettin Sahin.

The Process of Obtaining LA Essential Oil Through Hydro-Distillation

A quantity of 200 grams of herbal material that had been dried and crushed was subjected to distillation with 2 liters of pure water for a duration of 3 h, utilizing a Clevenger-type apparatus.

GC-MS Analysis

The carrier gas in the experiment was helium, which was maintained at a consistent flow rate (1 mL/min). 1 µL sample was introduced into the system via injection. The temperature program for the GC was established in the following manner: a hold at 50°C for 5 minutes, subsequently a ramp to 250°C at a rate of 5°C /minute, and a subsequent hold for 10 minutes. The transfer line for the mass spectrometer was maintained at 220°C. The mass range of 50 to 650 m/z was used in scan mode, and as a column, DB-Wax 60 m x 0.25mm ID x 0.25 µm was utilized.

Parasite Culture

The *L. infantum* (MHOM/MA/67/ITMAP-263) strain was obtained from Dr. Ana M. Tomás at the University of Porto, Portugal. The cultivation of *L. infantum* promastigotes was performed in RPMI 1640 Glutamax medium, which was supplemented with 10% (v/v) inactivated fetal bovine serum (iFBS), 50 U/mL penicillin, 50 µg/mL streptomycin, and 20 mM HEPES sodium salt at 25°C. The collection of promastigotes was carried out through centrifugation of the pellet at 3000 × g for 10 minutes.

Macrophage Culture

The RAW 264.7 murine macrophage cells (American Type Culture Collection TIB-71) were cultured in DMEM that was supplemented with 10% iFBS, 2 mM L-glutamine, and 100 Units/mL of penicillin and 100 µg/mL of streptomycin. The culture was maintained at a temperature of 37°C in a 5% CO_2. The cells were cultured through regular sub-passaging at 3-day intervals.

The Effect of LA on the Growth of *L. infantum* Promastigotes

The antileishmanial properties of LA essential oil against *L. infantum* were implemented using a resazurin assay, as introduced in Islek et al. (27). Briefly, promastigotes were cultured in 96-well plates at $3x10^5$ cells per well in a completed RPMI medium. The cells were subsequently treated with 0.063-1 µL/mL of LA essential oil and incubated for 24, 48, and 72 h. Following incubations, the resazurin solution (2.5 mM) was put into each well at a concentration of 10% (v/v), and luminescence intensity was assessed using a UV-Vis spectrophotometer with

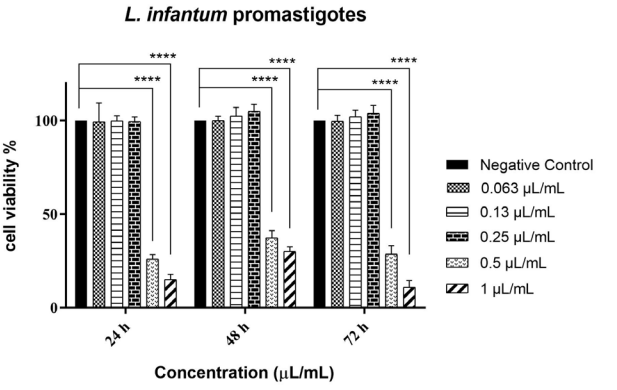

Figure 1. Effect of LA on the growth of *L. infantum* promastigotes. Viability of parasites following 24, 48, and 72 h of treatment with LA essential oil at various concentrations (0.063 to 1 µL/mL).

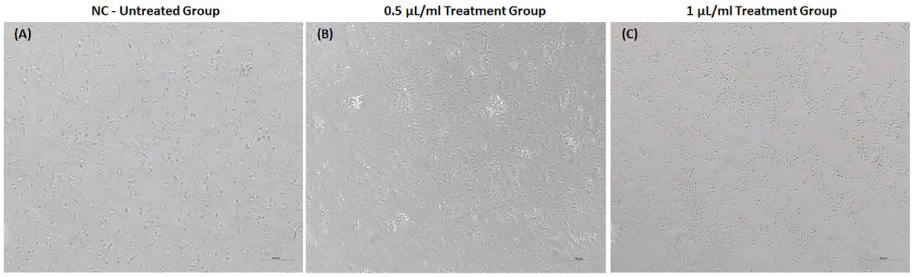

Figure 2. Phase-contrast microscopy images of **(A)** negative control (NC - untreated *L. infantum* promastigotes), **(B)** 0.5 µL/mL, and **(C)** 1 µL/mL LA essential oil treatment groups after 24 h of incubation. Bars correspond to 50 µm.

an excitation (560 nm) and an emission wavelength (590 nm). The viabilities (%) were determined relative to the negative control group, which was not subjected to any treatment. The data underwent analysis through the utilization of GraphPad Prism 8.01 software, following which the values for the 50% inhibitory concentration (IC_{50}) were determined.

Cell Viability Assay on Macrophages

RAW264.7 macrophages were treated with LA essential oil at varying concentrations (0.063 to 1 µL/mL), and viabilities were conducted through the utilization of the resazurin assay. Accordingly, the RAW264.7 cells were harvested in 96-well plates at 10,000 cells per well. Following 24h, the cells were incubated with various concentrations (0.063-1 µL/mL) of LA essential oil for 24, 48, and 72 h. After treatments, the cells were subjected to a 2-h incubation period in a 2.5 mM resazurin solution with a concentration of 10% (v/v), and then the luminescence intensity was assessed by a ultraviolet-visible (UV-Vis) spectrophotometer. The cell viability (%) was determined according to the negative control group. The data underwent analysis using GraphPad Prism 8.01 software.

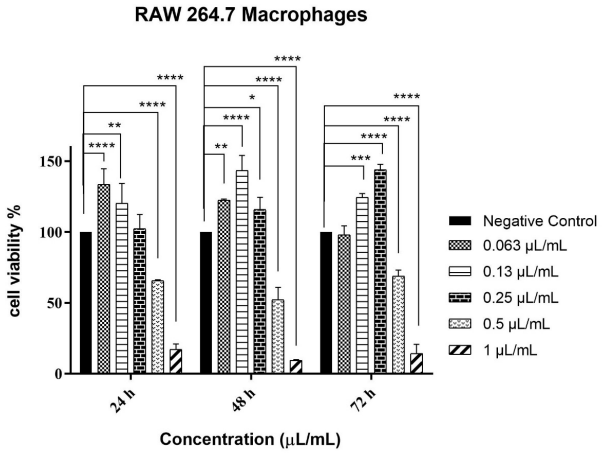

Figure 3. The viability analysis of RAW264.7 murine macrophages treated with LA essential oil at different concentrations (0.063-1 µL/mL) after 24, 48, and 72 h, compared to negative control.

Statistical Analysis

The statistical analysis was conducted utilizing GraphPad Prism Software (version 8.01). The study used statistical analysis by conducting a comparison of data sets using a two-way ANOVA, followed by Tukey's multiple comparison test. The statistical significance of the findings were assessed at different levels of probability, *i.e.* (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

RESULTS

GC-MS Analysis

The GC-MS technique was utilized to determine the phytochemical composition of LA essential oil. As presented in Table 1, linalool was found to be the predominant constituent of the lavender oil at 37%, and it was consistent with the European Pharmacopoeia, in which linalool content should range from 20.0% to 45.0%. In addition, 1,8-Cineole, camphor, and limonene were detected in the lavender oil as 13.8%, 12.7%, and 2.4%, respectively (Table 1), which was found above the criteria of European Pharmacopoeia, whereas 7.7% linalyl acetate was detected as below the criteria. However, the percentage of the 4-terpinol content was found at 3.7%, which was consistent with the value within the range from 1.0 to 8.0% given in the European Pharmacopoeia. Likewise, the content of lavandulol and α-terpineol in lavender oil was detected at 0.5% and 1.5%, respectively, which confirms the quality criteria of obtained lavender oil considering the European Pharmacopoeia (the criteria for lavandulol: min. 0.1%; α-terpineol: max. 2.5%). The chemical quality of the oil utilized was found to be above the specified limitation regarding to the high linalool content (*i.e.,* 37%) (Table 1).

Cell Culture Studies

In vitro Antileishmanial Effect of the LA Essential Oil on *L. infantum* Parasites

The treatment groups of 0.5 and 1 µL/mL exhibited a dose and time-dependent inhibition of parasite proliferation. Treatment with 0.5 µL/mL LA essential oil significantly decreased promastigote viability to 26% ($p \leq 0.0001$) at 24 h, and became nearly 38% and

Table 1. Phytochemical composition and retention time (min) of the hydro-distilled LA essential oil using GC-MS analysis.

Retention index	*Retention time (min)*	*Compounds*	*Area%*
1027	7.98	α-pinene	1.5
1113	10.06	β-pinene	1.1
1212	12.63	limonene	2.4
1224	13.05	1,8-Cineole	13.8
1498	21.98	camphor	12.7
1548	22.51	linalool	37.0
1569	22.61	linalyl acetate	7.7
1593	24.00	4-terpineol	3.7
1679	25.65	lavandulol	0.5
1694	26.24	α-terpineol	1.5

29% at 48, and 72 h ($p \leq 0.0001$), respectively. Upon an increase in the concentration to 1 µL/mL, the viability of promastigotes exhibited a significant decrease to 15% (*i.e.*, 15.11%) after 24 h ($p \leq 0.0001$), and became nearly 30% (*i.e.*, 30.09%) and 11% (*i.e.*, 11.01%) at 48 h and 72 h, respectively, as depicted in Figure 1 ($p \leq 0.0001$)**.** However, there was no significant further alteration in the inhibition of the promastigote proliferation at concentrations lower than 0.5 µL/mL.

The IC_{50} values were determined for LA essential oil following 24, 48, and 72 h of incubation, as 0.43 ± 0.089 µL/mL, 0.46 ± 0.053 µL/mL, and 0.47 ± 0.033 µL/mL, respectively. The findings demonstrated that 24, 48, and 72 h-incubation of LA essential oil exhibited efficacy against *L. infantum* promastigotes.

Effects of LA Essential Oil on Morphology of *L. infantum* Parasites

In parallel to the parasite viability, phase-contrast microscopy images suggested that treatments with 0.5 and 1 µL/mL concentrations of LA essential oil indicated a dose-dependent reduction in the number of the parasite and transformation from elongated-shape to round-shaped morphologies at 24 h of incubation (Figure 2).

Cytotoxicity of LA Essential Oil on Macrophages

After conducting an *in vitro* assessment of the antileishmanial efficacy of LA essential oil on *L. infantum* promastigotes, the cytotoxicity was assessed on healthy murine RAW264.7 macrophages (Figure 3). As shown in Figure 3, when murine macrophages were treated with LA essential oil at concentrations ≤ 0.25 µL/mL, cell viability of macrophages remained above 100%, and LA essential oil did not lead to toxicity on macrophages for 24, 48, and 72 h. Besides, the viability was increased to approximately 100%, 116% ($p \leq 0.05$), and 144% ($p \leq 0.0001$) following treatment with 0.25 µL/mL LA essential oil for 24, 48, and 72 h, respectively. Conversely, upon increasing concentration to 0.5 µL/mL, the viability was decreased to 66% at 24 h, which became 52% and 70% at 48, and 72 h of incubation, compared to negative control ($p \leq 0.0001$; Figure 3). A minor recovery in cell viability was detected among 48- and 72-h of treatment as ranging from 52% to 70% of cell viability. However, when the macrophages were treated with 1 µL/mL LA essential oil, the macrophage viability was decreased to below 17%, 10%, and 15% at 24, 48, and 72 h, respectively ($p \leq 0.0001$).

The results revealed that 0.5 µL/mL concentration of LA essential oil indicated a significant antileishmanial effect on *L. infantum* parasite proliferation with relatively less toxicity on macrophages.

DISCUSSION

Leishmaniasis, a neglected disease, has affected a significant number of individuals globally and continues to pose significant challenges in terms of drug development. The complexity of the disease, including the difficulty of diagnosis, coupled with limited financial incentives for pharmaceutical companies, has hindered progress in finding effective treatments. Additionally, the available drugs suffer from drawbacks such as high toxicity, prolonged treatment durations, and emerging drug resistance. In contrast to synthetic pharmaceuticals, a greater abundance of natural drug resources exists, and the pursuit of novel drug discovery from natural products and traditional herbal medicine warrants serious consideration. In the present study, the plant-based, potential antileishmanial therapy was examined, such as LA, due to its high biocompatibility, natural properties, and low toxicity in RAW264.7 cells through mitochondrial respiration. In short, LA essential oil was examined by GC-MS

analysis to detect its composition, according to the European Pharmacopoeia, and following the GC-MS analysis, the cell culture studies, including the antileishmanial activity on *L. infantum*, and safety features of the compound on healthy macrophages were examined *in vitro* models.

Accordingly, the composition of LA essential oil was detected by the GC-MS technique, which was consistent with previous reports in the literature (19, 28). Linalool was found to be the main component of lavender oil (37%), which was linked to the characteristic compounds within the composition of LA essential oil, and consistent with the quality criteria of the European Pharmacopoeia (*i.e.*, 20.0 to 45.0%; Table 1). A previous study suggested that the presence of the key ingredients in lavender essential oil, such as linalool, and terpineol can enhance its penetration to the cell membrane (29, 30). Besides that, terpineol has the ability to bind to particular G protein-coupled receptors, leading to an impact on the cAMP and Ca^{2+} concentrations, thus enhancing the activation of corresponding kinases, and ultimately facilitating the manifestation of its biological activities. Antileishmanial efficacy could be linked to the regulation of the kinase domain of calcium-dependent protein kinases (CDPKs) through calcium ions, and CDPK1 exhibits a significant association with the adhesion and invasion processes of protozoons (29).

In the present study, according to the growth inhibition on *L. infantum* promastigotes, the IC_{50} values were calculated for LA essential oil as 0.47±0.033 µL/mL at 72 h, and 0.5 and 1 µL/mL treatment groups exhibited a dose-dependent inhibition of parasite proliferation (Figure 1). Additionally, RAW264.7 macrophage cytotoxicity studies revealed relatively no observed toxicity toward the host cells at concentrations equal to the IC_{50} concentrations (Figure 3). Similarly, Yao et al. (2021) stated the antiprotozoal activity of LA essential oil against *Toxoplasma gondii* as dose-dependent within safe concentration ranges, and furthermore, the viability was not significantly reduced by LA essential oil at a 4.48 mg/ml in human foreskin fibroblasts (29). Furthermore, LA, and their nanoemulsions were found to be significantly effective on *L. major* parasites compared to *R. officinalis* essential oils, and conventional pentavalent antileishmanial chemotherapeutic, meglumine antimoniate (19). *Lavender* and *Rosemary* essential oils indicated the activity of *L. major* promastigote with IC_{50}= 0.11 µL/mL, and IC_{50}= 0.26 µL/mL, respectively, after 72 h of incubation (19). In parallel to viability studies, phase-contrast microscopy images demonstrated that 0.5 and 1 µL/mL treatments groups of LA essential oil indicated a dose-dependent reduction in the number of the parasite and transformation from elongated-shape to round-shaped morphologies at 24 h of incubation (Figure 2). The observed inhibitory effect could potentially be attributed to the impact on the morphology of *L. infantum* parasites, associated with the loss of membrane and flagellum integrity, and apoptosis. Nevertheless, the precise mechanism through which essential oils act on *L. infantum* remains uncertain and necessitates further investigation.

CONCLUSION

In short, this study is the first statement on the antileishmanial activity of LA essential oil on *L. infantum* parasites. The present investigation provides preliminary studies of the traditional use of LA for antileishmanial therapy with *in vitro* cell culture analysis. The promising antileishmanial efficacy of lavender essential oil highlights the potential therapy in future *in vivo* studies.

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Ethics Committee Approval: Ethics committee approval is not required because of no material or experimental animal that would require permission.

Authors' Contributions: Conception/Design of Study – F.S., Z.I.; Data Acquisition – F.S., Z.I.; Data Analysis/Interpretation – Z.I.; Drafting Manuscript– F.S., Z.I.; Critical Revision of Manuscript- F.S., Z.I.; Final Approval and Accountability– F.S., Z.I.

Conflicts of Interest: The authors did not disclose any potential conflicts of interest.

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