

Original Article

Antileishmanial Activity of *Lantana camara* L. Leaf Extract in Niosomes

João Pedro Bastos¹, Ari Sérgio Lemos^{1*}, Ana Luiza Freitas¹, Tatielle Nascimento², Rodrigo Luiz Fabri³, Alessandra Valverde⁴, Eduardo Ricci-Junior², Luciana Maria Antinarelli¹, Elaine Coimbra^{1,*}

¹ Laboratory of Parasitology, Department of Parasitology, Microbiology and Immunology, Federal University of Juiz de Fora (UFJF), Juiz de Fora, MG, Brazil. joaopedro.reis@estudante.ufjf.br

² Laboratory of Pharmaceutical Nanotechnology, Faculty of Pharmacy, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil. eduardoriccijunior@gmail.com

³ Laboratory of Bioactive Natural Products - Bioassays, Department of Biochemistry, Institute of Biological Sciences, Federal University of Juiz de Fora (UFJF), Juiz de Fora, MG, Brazil. rodrigo.fabri@ufjf.edu.br

⁴ Fluminense Federal University, Institute of Chemistry, Department of Organic Chemistry, Postgraduate Program in Chemistry, Rio de Janeiro, Brazil. alessandravalverde@id.uff.br

* Correspondence: ariufjf@gmail.com, elaine.coimbra@ufjf.br

Abstract: Leishmaniasis is a disease caused by more than 20 *Leishmania* species and comprises a complex group of clinical manifestations, including Cutaneous leishmaniasis (CL). CL is a neglected tropical disease affecting skin and mucous membranes, with treatment options restricted to a limited number of drugs, many of which are associated with significant toxicity. *Lantana camara* L. is traditionally used to treat dermatological ailments, with studies suggesting its antileishmanial potential due to triterpenes. Nanotechnology, including niosomes (NIOS), has shown promise in the field of medicine. In this context, this study aimed to evaluate the ethanolic extract of *L. camara* (LCE) and nanoencapsulated in niosomes (LCE-NIOS) against promastigotes and amastigotes of *Leishmania amazonensis*, as well their toxic effect on mammalian cells. The chemical composition and immunomodulatory effect of LCE was also evaluated. LCE was obtained by static maceration with ethanol, while nanoencapsulation was performed via lipid film hydration. Cytotoxicity was assessed in BALB/C mouse peritoneal macrophages, while antileishmanial activity was tested against *L. amazonensis* promastigotes, both using MTT method. The effect against intracellular amastigotes was after Giemsa staining. Nitric oxide (NO) induction was evaluated by Griess assay. Phytochemical analysis of LCE showed high content of terpenes (477.51 mg/g) with low levels of phenolic compounds (34.80 mg/g). Although the selectivity index (SI = 14.33) was lower than that of pure LCE (SI = 30.64), nanoencapsulation (LCE-NIOS) markedly enhanced antileishmanial potency, achieving IC₅₀ values of 0.1431 ± 0.0002 µg/mL for promastigotes and 0.833 ± 0.037 µg/mL for intracellular amastigotes of *L. amazonensis*, representing approximately a 14-fold improvement over the crude extract. LCE also significantly induced nitric oxide (NO) production in a dose-dependent manner in *L. amazonensis*-infected macrophages, suggesting a possible immunomodulatory mechanism contributing to parasite clearance. These results highlight the pivotal role of terpenoids in LCE's antileishmanial activity and demonstrate that nanoencapsulation can substantially enhance its efficacy. Further *in vivo* studies are warranted to confirm its therapeutic potential.

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1. Introduction

Leishmaniasis comprises a complex group of infectious diseases caused by more than 20 species of protozoan parasites of the genus *Leishmania* spp., transmitted by phlebotomine sandflies of the genera *Phlebotomus* spp. and *Lutzomyia* spp. [1]. These diseases are endemic in approximately 90 countries across tropical and subtropical regions—including Asia, Africa, the Middle East, and Central and South America—and threaten more than one billion people living in affected areas [2,3,4]. The geographic expansion of leishmaniasis has been linked to several contributing factors, including forced migration, poverty, malnutrition, limited access to healthcare, and environmental and climate changes [1].

Classified among the seven most significant neglected tropical diseases, leishmaniasis represents a major global public health concern [5]. Their clinical manifestations are diverse, resulting from infections with different *Leishmania* species. Depending on complex host–parasite interactions within mammalian hosts, the disease may present as cutaneous leishmaniasis (CL) or the more severe visceral leishmaniasis (VL), the latter being potentially fatal if untreated [3].

Clinically, CL is the most prevalent form of disease, and although likely underreported, the global incidence of CL is estimated at 700,000 to 1.2 million new cases annually.^[2,3] In the Americas, Brazil accounts for the highest number of cases, with reports from all federal units and particularly elevated incidence rates in the northern region⁶. *Leishmania* (L.) *amazonensis* is among the species implicated in CL. Clinically, CL typically presents as ulcerative skin lesions on exposed areas of the body, most commonly at the site of the sandfly bite. These lesions often result in permanent scarring and may lead to profound social and psychological impacts due to the associated stigma [6]. Moreover, CL can manifest in several clinical variants, including localized (single or multiple) cutaneous, disseminated cutaneous, mucocutaneous, and diffuse cutaneous forms.

Current antileishmanial chemotherapy relies mainly on pentavalent antimonials, such as Glucantime and Pentostan. These drugs have been used over a century, but are associated with several toxic effects, including hepatic, cardiac and renal issues, as well as myalgia and injection site pain. Intralesional therapy with antimonials has been recommended by the World Health Organization (WHO) and the Pan American Health Organization (PAHO) to treat CL, but this approach is effective and safe in at maximum of four lesions, especially without face localization. Other drugs, such as amphotericin B (amphotericin B deoxycholate or lipid formulations), paromomycin, and pentamidine isethionate are also recommended as second line but their use is also related to side effects. Miltefosine, the first oral agent introduced into this therapeutic arsenal, also has limitations, including teratogenicity and variable efficacy against New World *Leishmania* species. Thermotherapy, cryotherapy and lasers are also cited in the literature, but with discrepancy among the cure rates, needing more studies [7].

To address these shortcomings, researchers have explored drug combinations and novel delivery systems, such as nanoformulations designed to reduce toxicity and improve solubility, bioavailability, and the pharmacological profile of therapeutic agents. Examples include nanoparticles, liposomes, and niosomes. Niosomes (NIOS) are innovative vesicular carriers composed primarily of hydrated amphiphilic molecules, particularly non-ionic surfactants. NIOS are being investigated for their potential in controlled and targeted drug delivery against several bacteria, fungi, and protozoa, such as *Plasmodium* spp. and *Leishmania* spp [8]. Among emerging therapeutic strategies, natural products are particularly attractive as accessible and cost-effective alternatives, especially in resource-limited settings [9]. *Lantana camara* L., a medicinal plant widely used in traditional medicine, possesses a broad spectrum of biological activities. Various parts of the plant, including its leaves, roots, and flowers—have been traditionally employed to treat ailments such as malaria, tuberculosis, lymphadenitis, mumps, gastrointestinal disorders, rheumatism, and bone pain [10]. Although no ethnopharmacological data currently supports its use against leishmaniasis, organic extracts of *L. camara* have

demonstrated leishmanicidal activity against *L. amazonensis* and *L. mexicana* in experimental models [11]. Preliminary investigations conducted by our research group have revealed promising antileishmanial effects of the ethanolic leaf extract of *L. camara* (LCE). Building on these findings, we have focused our efforts on enhancing the efficacy of LCE against both amastigote and promastigote forms of *L. amazonensis*, while minimizing mammalian cytotoxicity through nanoencapsulation in niosomes (LCE-NIOS), in addition to investigating the immunomodulation of LCE in infected macrophages and the content of substances related to antileishmanial activity.

2. Results

The total terpene and phenolic contents of the *L. camara* ethanolic extract (LCE) were evaluated to elucidate the chemical composition and solubility profile of the extract. Quantitative analysis revealed that LCE is rich in terpenes (477.51 ± 26.41 mg/g of extract) while exhibiting relatively low levels of phenolic compounds (34.80 ± 2.71 mg/g) (Table 1).

Table 1 – Quantification of total terpene and phenolic contents in the ethanolic extracts of *L. camara*.

Extract	Content of terpene equivalents in lupeol (mg/g extract)	Content of phenolic substances equivalent to tannic acid (mg/g extract)
LCE	477.51 ± 26.41	34.80 ± 2.71

Legend: LCE_ Ethanolic extract of *L. camara* leaves. One way ANOVA followed by Boneferroni's post hoc test. a_ means statistical difference in relation to the other extracts.

Following the preparation of LCE and LCE-loaded niosomes (LCE-NIOS), both formulations were evaluated against promastigote and amastigote forms of *L. amazonensis* (Table 2). Additionally, the cytotoxicity of the extracts was assessed on peritoneal macrophages from BALB/c mice. LCE-NIOS demonstrated higher activity than the free extract, showing promising antileishmanial activity against promastigotes and amastigotes with IC_{50} values of 0.1431 ± 0.0002 μ g/mL and 0.833 ± 0.037 μ g/mL, respectively. However, moderate cytotoxicity and selectivity were also observed ($CC_{50} = 11.94 \pm 2.57$ μ g/mL, $SI = 14.33$). In contrast, LCE exhibited promising antileishmanial activity with IC_{50} values of 12.20 ± 0.12 μ g/mL and 7.29 ± 1.11 μ g/mL for promastigote and amastigote forms, respectively, accompanied by low cytotoxicity ($CC_{50} = 223.40 \pm 47.02$ μ g/mL) and high selectivity ($SI = 30.64$).

Table 2 – Evaluation of anti-leishmanial activity and cytotoxicity of LCE and LCE-NIOS.

Sample	Promastigote IC_{50} (μ g/mL)	CC_{50} (μ g/mL)	Amastigote IC_{50} (μ g/mL)	Selectivity Index (SI) for Amastigotes
LCE-NIOS	0.1431 ± 0.0002^a	11.94 ± 2.57^a	0.833 ± 0.037^a	14.33
LCE	12.20 ± 0.12	223.40 ± 47.02	7.29 ± 1.11	30.64
Miltefosina	8.96 ± 0.5	67.93 ± 4.29	5.10 ± 1.79	13.31

Legend_ LCE_ Ethanolic extract of *L. camara* leaves. LCE-NIOS_ Ethanolic extract of *L. camara* leaves L nanoencapsulated with niosome. a_ Indicates statistical difference. Results expressed as mean $\pm$ standard deviation of three independent experiments. One way ANOVA followed by Bonferroni's post hoc test. CI_{50} _ 50% inhibitory concentration; CC_{50} _ 50% cellular cytotoxicity; IS _ selectivity index = CC_{50}/IC_{50} amastigotes.

To elucidate the mechanism of action of LCE on amastigote forms, the induction of nitric oxide (NO) production was assessed in macrophages infected with *L. amazonensis* parasites and treated with varying concentrations of LCE (6.25 to 50 µg/mL). After 72 hours of treatment, supernatants were collected for indirect NO quantification by Griess method. As can be noted in Figure 1, the results demonstrated that LCE induced a dose-dependent response of NO levels in *L. amazonensis*-infected macrophages. Also, LCE significantly induced NO production in 137.5; 118.2 and 97.3% at concentrations of 50, 25, and 12.5 µg/mL respectively (Figure 1) compared to the untreated infected macrophage control group. As expected, *L. amazonensis*-infected macrophages treated with a combination of lipopolysaccharide (LPS) and interferon-gamma IFN- γ , used as positive control, was able to induce the NO levels (762.5 %).

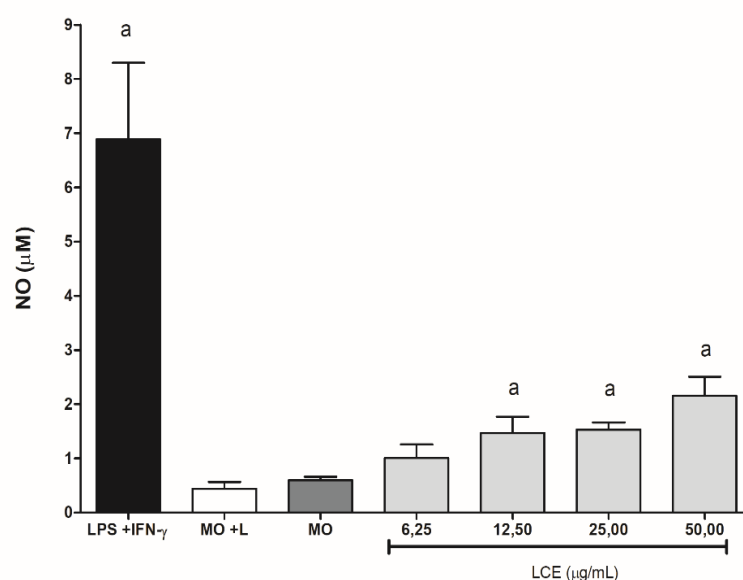


Figure 1 – Evaluation of nitric oxide (NO) induction in macrophages infected with *L. amazonensis* protozoan treated with varying concentrations of the ethanolic leaf extract of *L. camara*. Results are expressed as mean $\pm$ standard deviation of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. a indicates a statistically significant difference compared to the untreated infected control group. LPS + IFN- γ : Lipopolysaccharide + interferon-gamma were used as a positive control. MO+L: Murine peritoneal macrophages infected with amastigote forms of *L. amazonensis* without treatment; MO: Murine peritoneal macrophages without infection; LCE: Ethanolic leaf extract of *L. camara*.

3. Discussion

In pharmaceutical chemistry, nanoformulations have been highlighted as a promising strategy to address the limitations associated with free drugs [12]. In this context, the main objective was to explore the antileishmanial activity of *L. camara* extract nanoencapsulated in niosomes (LCE-NIOS) compared with the free extract. In our analysis we primally observed a high content of terpenes in LCE. Generally, terpenes exert antileishmanial effects by directly targeting the parasites or indirectly via modulation of the host immune response. In the latter mechanism, terpenes promote a pro-inflammatory profile and activate microbicidal pathways, leading to enhanced production of reactive oxygen species (ROS) and consequent reduction of parasitic burden. Terpenes can also compromise parasite viability by damaging plasma membranes or inhibiting DNA topoisomerase activity [13]. In *Lantana camara*, the terpenes commonly found have ursane and oleanane skeletons. Therefore, the elevated terpene content in LCE likely underpins its observed bioactivity.

In other moment, the investigation of antileishmanial potential of LCE and LCE-NIOS was performed. Our results demonstrated that LCE-NIOS exhibits promising

antileishmanial activity, being 85.25 and 8.75 times more active against promastigotes and amastigotes of *L. amazonensis*, respectively, compared with LCE. Moreover, LCE-NIOS was approximately 10 times more toxic to intracellular forms than for macrophages, the host cells. The SI, a critical parameter in the *in vitro* screening of novel drugs, reflects the differential toxicity of a compound towards the parasite relative to the host mammalian cell. According to several authors, compounds with an SI greater than 1 are considered selective for *Leishmania* spp. parasites over host cells and thus represent promising candidates for leishmaniasis treatment [14]. Furthermore, high SI values suggest reduced cytotoxicity to host cells, encouraging further investigation of these compounds and subsequent *in vivo* testing [15]. Based on the findings of this study, both LCE-NIOS and free LCE exhibited high selectivity, underscoring their potential as antileishmanial agents.

Niosomes, on their part, are nanocarriers structurally analogous to liposomes, characterized by cost-effectiveness, stability, and enhanced bioavailability. Their bilayer configuration enables regulated drug release. The amphiphilic nature of niosomes—attributable to their composition of lipids and non-ionic surfactants—renders them highly efficient for the nanoencapsulation of both hydrophobic and hydrophilic substances. Moreover, the incorporation of cholesterol within the niosomal membrane enhances stability and mitigates gelation processes upon contact with aqueous environments [7].

Nanoencapsulation in niosomes is widely reported to enhance the solubility and bioavailability of hydrophobic compounds [16]. Although the superior antileishmanial activity observed for LCE-NIOS, this study did not directly assess changes in the solubility or other properties related to nanoencapsulation. Therefore, the improved antileishmanial activity observed here may be partially attributed to increased solubility, which remains a hypothesis to be confirmed in future investigations.

Nonetheless, the observed increase in cytotoxicity might be linked to the presence of surfactants, which are known to interact directly with biological membranes. In order to overcome this limitation, future studies conducted on other surfactants, as well as analyses of the cytotoxic profile of these components prior to the production of niosomes, are necessary. In addition, we observed a significant increase in NO in macrophages infected and treated with LCE. NO quantification revealed that after 72 hours of treatment with LCE, concentrations of 50.0, 25.0, and 12.5 µg/mL induced a significant increase in NO production compared to infected macrophages without treatment. NO radical is produced via nitric oxide synthase (NOS) in macrophages upon activation by IFN-γ and TNF-α, leading to the elimination of intracellular parasites [17]. These results suggest that NO production induced by LCE may be one of the mechanisms involved in the elimination of intracellular forms of the parasite.

4. Materials and Methods

4.1 Plant Material

Leaves of *L. camara* were collected in Juiz de Fora, Minas Gerais, Brazil, on January 17, 2020. A voucher specimen of the species (CESJ 30671) was deposited in the Leopoldo Krieger Herbarium at the Federal University of Juiz de Fora. The collection was carried out in accordance with Brazilian regulations under license number A18AB08 SISGEN/BRASIL.

4.2 Preparation of *L. camara* Leaf Extracts

The ethanolic extract of *L. camara* leaves was obtained by static maceration of 90 g of dried and previously pulverized leaf material in ethanol. The extraction process was carried out in three successive steps using 500 mL of ethanol each time (3 × 500 mL). The combined extract was concentrated under reduced pressure using a rotary evaporator (Heidolph – Laborota 4000). The resulting ethanolic leaf extract (LCE) was weighed, and the extraction yield was calculated as 8.39% (7.55g). The obtained extract was presented

as a fine powder with a characteristic green coloration. The LCE was stored under refrigeration until further use in biological assays and niosome formulation.

4.3 Determination of Total Phenolic Content

The total phenolic content was determined by visible-range spectrophotometry using the Folin–Ciocalteu method, with slight modifications [18,19]. Tannic acid served as the standard for calibration. A standard curve was prepared using a methanolic solution of tannic acid (500 µg/mL), with concentrations ranging from 7.5 to 350 µg/mL. In 96-well microplates, 120 µL of 20% Folin–Ciocalteu reagent, 30 µL of the sample extract, and 100 µL of 4% sodium carbonate solution were added. After 30 minutes of incubation in the absence of light, absorbance was measured at 770 nm using a spectrophotometer (Multiskan EX, Thermo Electron Corporation, Vantaa, Finland). Sample stock solutions were prepared at a concentration of 500 µg/mL in either methanol or water. Wells containing only samples and methanol were used as blanks. All assays were performed in triplicate. The total phenolic content was calculated based on the standard calibration curve and expressed as milligrams of tannic acid equivalents per gram of plant extract (mg TAE/g extract). Results are presented as mean ± standard deviation of three independent experiments.

4.4 Determination of Total Terpene Content

The quantification of terpenes followed the method described by Pedrosa et al [20]. Triterpene content was determined using a standard calibration curve prepared with lupeol as the reference compound. A stock solution of lupeol (100 µg/mL in ethanol) was used to prepare a series of test tubes containing 50, 100, 200, 400, 600, 800, and 1000 µL of the standard. These tubes were placed in a water bath at 85 °C until complete solvent evaporation. Subsequently, 250 µL of vanillin solution (50 mg/mL) and 500 µL of sulfuric acid (99.5%) were added to each tube. The mixtures were heated in a water bath at 60 ± 1 °C for 30 minutes, followed by cooling in an ice bath. After cooling, 2500 µL of acetic acid (99.7%) was added to each tube. The resulting solutions were kept under refrigeration for 20 minutes and then allowed to stand at room temperature for an additional 20 minutes. The final lupeol concentrations ranged from 1.5 to 31 µg/mL. The same procedure was applied to 1000 µL of plant extract solutions (100 µg/mL in ethanol). A blank solution was prepared using the same protocol. Absorbance was measured at 548 nm. The total terpene content was expressed as milligrams of lupeol equivalents per gram of plant extract (mg LE/g extract).

4.5 Development of LCE-NIOS Formulation

Niosomes were prepared using the thin-film hydration method, employing the components listed in Table 3. The nonionic surfactants (Poloxamer 184 and Tween 80®), solubilized LCE (5 mg in dimethyl sulfoxide [DMSO]), and cholesterol were dissolved in dichloromethane and transferred to a 2-liter round-bottom flask. The solvent was evaporated using a rotary evaporator for 2 hours and 30 minutes at 760 mmHg to form a thin lipid film. The resulting lipid film was then hydrated with TRIS buffer (pH 6.8), prepared by dissolving 0.484 g of tris(hydroxymethyl)aminomethane and 0.2 g of methylparaben in distilled water to a final volume of 200 mL, with pH adjusted using 3 M HCl. The hydration process was carried out for 1 hour using the rotation function of the rotary evaporator under atmospheric pressure. Following hydration, the niosomal formulations were sonicated using an ultrasonic processor for 5 minutes at 60% amplitude. The obtained formulation was presented as a green-colored liquid with low viscosity.

Table 3- Components used in the development of niosomes formulations.

Poloxamer 184 (g)	Tween 80® (g)	LCE (mg)	DMSO (μL)	Cholesterol (g)	Dichloromethane (mL)	TRIS buffer (mL)
0,6	0,3	5	100	0,008	5	10

4.6 Parasite Culture

In this study, a wild-type strain of *L. amazonensis* (IFLA/BR/67/PH8) was used. The parasites were cultured in Warren medium (BHI-Bovine Heart and Brain Infusion Medium (M210) (Himedia, Mumbai, Maharashtra, India) -added with hemin (Sigma-Aldrich Louis, Missouri, USA) and folic acid (Sigma-Aldrich Louis, Missouri, USA)] supplemented with 10% fetal bovine serum (FBS) (Cultilab, SP, Brazil) and 0.1% penicillin solution 10. 000 I.U./mL penicillin solution and 10 mg/mL streptomycin (Sigma-Aldrich Louis, Missouri, USA) and maintained in a BOD (Biochemical Oxygen Demand) incubator at 25 °C.

4.7 Evaluation of the Cytotoxicity of Extracts on *L. amazonensis* Promastigotes

The cytotoxic activity of LCE and LCE-NIOS against promastigote forms of *L. amazonensis* was assessed using the colorimetric MTT assay, based on the method developed by Mosmann [21]. This assay relies on the conversion of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO, USA) into insoluble purple formazan crystals by cellular oxidoreductase enzymes, such as dehydrogenases, oxidases, and peroxidases, using NADH, NADPH, succinate, or pyruvate as cofactors. Promastigotes in the logarithmic growth phase were counted using a Neubauer chamber and adjusted to a final concentration of 2×10^6 parasites/mL in their respective culture medium. They were seeded into 96-well plates (. LCE and LCE-NIOS were serially diluted to yield final concentrations of 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.09 μg/mL, and were added to the wells. Miltefosine (Cayman Chemical Company, Denver, CO, USA) was used as the reference drug. All treatments were performed in duplicate, and the results were derived from three independent experiments. Wells without extract addition served as negative controls. After 72 hours of incubation at 25 °C in a BOD (Biochemical Oxygen Demand) incubator, 10 μL of MTT solution (5 mg/mL) ; (Sigma Chemical Co., St. Louis, MO, USA) was added to each well, followed by an additional 4-hour incubation at 25 °C. The reaction was then halted by adding 100 μL of acidified isopropanol (0.7% HCl in 10 N solution) to each well. Absorbance was measured at 570 nm using a spectrophotometer (Multiskan EX, Thermo Electron Corporation, Vantaa, Finland). The absorbance values were converted into percentage inhibition of promastigote growth relative to the untreated control. The half-maximal inhibitory concentration (IC₅₀)—the concentration required to inhibit 50% of parasite growth—was calculated for each extract using GraFit 5 software (Erithacus Software Ltd., Horley, U.K.). Miltefosine (Cayman Chemical Company, Denver, CO, USA) was used as the reference antileishmanial drug. Results are presented as mean ± standard deviation of three independent experiments performed in duplicate.

4.8 Evaluation of Extract Cytotoxicity in Peritoneal Macrophages from BALB/c Mice

The cytotoxic potential of LCE and LCE-NIOS in mammalian cells was assessed using peritoneal macrophages isolated from BALB/c mice. To obtain macrophages, the animals were pre-stimulated with 2 mL of 3% thioglycolate sodium broth (Merck Darmstadt, Germany), administered intraperitoneally 72 hours prior to peritoneal lavage. After euthanasia, the peritoneal exudate was collected, transferred to conical tubes, and centrifuged at 1000 rpm for 10 minutes (Thermo Fisher Scientific D37520 centrifuge, Waltham, Massachusetts, EUA). The supernatant was discarded, and the pellet was

resuspended in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA). Cell concentration was determined using a Neubauer chamber, and adjusted to 2×10^6 cells/mL in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil) and 0.5% penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA). The cell suspension was distributed into 96-well microplates and incubated for 16 hours at 37 °C in a 5% CO₂ atmosphere to allow macrophage adhesion. Following incubation, non-adherent cells were removed by washing with PBS, and fresh RPMI-1640 medium was added. Cells were then treated with serial dilutions of LCE and LCE-NIOS final concentrations of 300.0, 150.0, 75.0, 37.5, 18.75, 9.37 and 4.68 µg/mL. Plates were incubated for an additional 72 hours under the same conditions. After this period, 10 µL of MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 2 hours at 37 °C [22]. The reaction was stopped by adding an acidified isopropanol solution (7% HCl in 10 N solution). Absorbance was measured at 570 nm using a microplate reader (Multiskan EX, Thermo Electron Corporation, Vantaa, Finland). Cell viability was expressed as the percentage of viable macrophages in treated wells relative to the untreated control. Based on these values, the 50% cytotoxic concentration (CC₅₀)—defined as the extract concentration required to reduce macrophage viability by 50%—was calculated using GraFit 5 software (Erithacus Software Ltd., Horley, U.K.). All experiments were conducted in triplicate and in duplicate wells. Untreated wells were used as the negative control, and miltefosine was employed as the reference drug. This experimental protocol was approved by the Animal Ethics Committee of the Federal University of Juiz de Fora (CEUA No. 007/2018).

4.9 Evaluation of Cytotoxicity of Extracts on Intracellular Amastigotes of *L. amazonensis*

The anti-amastigote activity of the extracts was assessed using the *L. amazonensis*, following the protocol described by Granato et al. [23]. Peritoneal macrophages from BALB/c mice, obtained as previously described, were adjusted to a concentration of 2×10^6 cells/mL in RPMI-1640 medium (RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) and seeded in 24-well plates containing round glass coverslips. The cells were incubated for 16 hours at 33 °C in a 5% CO₂ atmosphere to promote adherence. After washing with phosphate-buffered saline (PBS), logarithmic-phase promastigotes were added at a ratio of 10:1 (parasite:macrophage), and plates were incubated for 4 hours to allow infection. Subsequently, wells were washed to remove non-phagocytosed parasites, and fresh RPMI medium supplemented was added. The LCE and LCE-NIOS were tested in duplicate at concentrations of 100.0, 50.0, 25.0, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.09 µg/mL, prepared by serial dilution. Plates were then incubated for 72 hours under the same conditions. Following treatment, the medium was removed, and the coverslips were fixed with absolute ethanol and stained with Giemsa. Dehydration was performed through graded acetone/xylene series (100% acetone to 100% xylene), and the coverslips were mounted on glass slides for analysis under a light microscope (Olympus BX41, Shinjuku, Tokyo, Japan). Antileishmanial activity was determined by counting 200 macrophages (infected and uninfected) and their intracellular amastigotes. Efficacy was expressed as the percentage inhibition of the parasite load relative to the negative control, and the IC₅₀ values were calculated using Probit analysis. Miltefosine served as the reference drug. Three independent experiments were conducted, all in duplicate. The protocol was approved by the Animal Ethics Committee of the Federal University of Juiz de Fora (CEUA No. 008/2018).

4.10 Determination of Nitric Oxide (NO) Levels in Macrophages Infected with *L. amazonensis*

Nitric oxide (NO) production was measured in the supernatants of macrophages infected with *L. amazonensis* following a 72-hour treatment with LCE at concentrations of 50.0, 25, 12.5, and 6.25 µg/mL. The Griess assay was employed to quantify nitrite, a stable metabolite of NO [24]. After 72 hours of incubation, 50 µL aliquots were collected from wells containing both uninfected and *L. amazonensis*-infected macrophages, either treated

or untreated. Lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) at 1 µg/mL combined with 1 ng/mL interferon-gamma (IFN-γ), obtained from L1210 cell supernatant, was used as a positive control. The collected supernatants were incubated with 50 µL of Griess reagent, consisting of 25 µL of 1% sulfanilamide in 2.5% phosphoric acid and 25 µL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid, for 10 minutes. Absorbance was measured at 540 nm using a spectrophotometer (Multiskan EX, Thermo Electron Corporation, Vantaa, Finland). Nitrite production was quantified by reference to a sodium nitrite (NaNO₂) standard curve. All experiments were performed in triplicate.

4.11 Determination of the Selectivity Index (SI) of the Extracts

The Selectivity Index (SI) indicates the degree to which a compound or extract is active against the parasitic form without causing harm to the host cells. It is calculated as the ratio between the CC₅₀ obtained from the cytotoxicity assay on macrophages and the IC₅₀ obtained for the amastigote forms, which in this case represents the parasitic form of interest due to its responsibility for the clinical manifestations of leishmaniasis [25].

4.12 Statistical Analysis

Statistical analysis of *in vitro* assays was performed using ANOVA followed by Bonferroni post hoc test. All analyses were conducted using GraphPad Prism 5.0 software (Erithacus Software Ltd.). Values of *p* < 0.05 were considered statistically significant. Results are presented as mean ± standard deviation.

5. Conclusions

Based on the results obtained, it can be concluded that the ethanolic extract of *L. camara* is rich in terpenes and demonstrates promising antileishmanial activity. Moreover, the efficacy against intracellular amastigote forms appears to be linked to the induction of nitric oxide production in infected macrophages, a modulatory effect that is likely associated with the high terpene content. Although the nanoencapsulation process significantly enhanced the antileishmanial activity of LCE, it was also accompanied by an increase in cytotoxicity. This elevated toxicity can be attributed to the surfactants used in the niosome formulation, substances known to interact directly with cellular membranes. Therefore, despite these findings, further investigations are necessary to comprehensively assess the therapeutic potential of LCE-NIOS in the treatment of cutaneous leishmaniasis. In this context, the nanoencapsulation of *Lantana camara* ethanolic extract in niosomes emerges as a promising strategy to enhance the antileishmanial activity of lipophilic metabolites, combining potent *in vitro* antiparasitic effects with an immunomodulatory profile linked to nitric oxide (NO) induction. Furthermore, this study is important for the investigation of naturally occurring nanoformulations that may be effective against cutaneous leishmaniasis. However, translational validation of this approach necessitates *in vivo* studies to assess therapeutic efficacy, pharmacokinetics, and safety in cutaneous leishmaniasis models, as well as its potential for topical or systemic administration. Furthermore, mechanistic investigations are essential to elucidate the molecular basis underlying the observed activity.

Supplementary Materials: not applicable

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Institutional Review Board Statement: The study was conducted according to the guidelines of Animal Ethics Committee of the Federal University of Juiz de Fora (CEUA No. 007/2018 and CEUA No. 008/2018)

Informed Consent Statement: not applicable

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