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# The effect of Niosomes-Loaded Rapamycin against Leishmania major: An in vitro and in vivo study

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Figures 3 to 5 is avilable in supplementray files section.

**The effect of Niosomes-Loaded Rapamycin against *Leishmania major*: An *in vitro* and *in vivo* study**


## Abstract

This study aimed to characterize Niosomes-Loaded Rapamycin as a potential drug delivery system and to assess its effects on *Leishmania major* model *in vitro* & *in vivo*. Niosomes-Loaded Rapamycin were successfully prepared using ethanol injection method with optimized physicochemical properties, including particle size of approximately 220 nm, a low polydispersity index (PDI < 0.3), spherical bilayer morphology, and a high encapsulation efficiency (EE%) of ≥ 99.99%. These characteristics suggested a stable, uniform, and efficient nanocarrier system suitable for topical route of administration. Physical stability studies over 90 days at different temperatures confirmed the robustness of the formulation, showing no significant changes in particle size, phase separation, or drug crystallization, underscoring its potential shelf-life.

*In vitro* drug susceptibility assays demonstrated that both free and Niosomes-Loaded Rapamycin exhibited activity against *Leishmania major* promastigote and amastigote forms. Notably, Niosomes-Loaded Rapamycin showed comparable efficacy to Glucantime against the intracellular amastigote stage ($IC_{50}$ ≈ 14-15 µg/mL), which is the pathogenic form residing within host macrophages. This suggests that encapsulation of rapamycin in niosomes may enhance its apparent therapeutic performance compared with the free drug, bringing its activity close to the standard reference treatment. However, Glucantime was significantly more potent against the extracellular promastigote stage. While niosomal formulation offered a slight, non-statistically significant numerical improvement over free rapamycin for promastigotes, its main advantage might lie in drug stability and controlled release rather than enhancing *in vitro* potency alone.

The *in vivo* study showed the efficacy of topical Niosomes-Loaded Rapamycin against lesions induced by *Leishmania major major* infection in murine model. At all measured time points (Weeks 1-4), no statistically significant difference in lesion size was observed between the Niosomes-Loaded Rapamycin or free rapamycin group and the untreated control group. This indicates that the topical formulation, as applied, was ineffective to treat the lesion. In contrast, the intraperitoneal administration of Glucantime demonstrated a consistent and significant reduction in the lesion size compared to the control group, confirming its established efficacy *via* a systemic route.

The findings highlight a critical disparity, while rapamycin exhibits *in vitro* anti-amastigote activity, its topical formulation failed to translate this into *in vivo* therapeutic efficacy, likely due to insufficient drug penetration or concentration at the site of infection. This contrasts sharply with the proven effectiveness of systemically administered Glucantime. The study underscores the profound impact of drug formulation and route of administration on therapeutic outcomes, suggesting that for leishmaniasis, systemic delivery remains the gold standard, while topical

therapies face significant challenges in achieving adequate drug concentrations to combat deep-seated parasites effectively.

**Key words:** Rapamycin, Niosomes, Drug Delivery System, Leishmaniasis, Topical Formulation

## Introduction

Cutaneous leishmaniasis (CL) is a neglected tropical disease caused by protozoan parasites of the genus. *Leishmania*, representing a significant public health challenge [1, 2]. CL manifests as a spectrum of skin lesions, ranging from self-healing ulcers to chronic, disfiguring scars that can lead to significant social stigma and psychological morbidity [1].

Current therapeutic options for CL remain largely unsatisfactory. Pentavalent antimonials, the first-line treatment for decades, are associated with considerable drawbacks including cardiac, hepatic, and renal toxicity, parenteral administration requiring multiple injections, increasing parasite resistance, and poor patient compliance [3, 4]. Although alternative agents such as amphotericin B, paromomycin, and miltefosine have been introduced, they are limited by variable efficacy depending on the *Leishmania* species, high cost, systemic toxicity, and the emergence of drug-resistant strains [5]. Topical formulations have been explored as a strategy to circumvent systemic toxicity, yet their efficacy is frequently compromised by poor drug penetration through the stratum corneum and inadequate retention within the dermal macrophages that harbor the parasite [6].

The host-parasite interaction in leishmaniasis is critically dependent on the immune status of the infected individual. A protective immune response against *Leishmania* requires the development of a T-helper 1 (Th1)-polarized response characterized by the production of interferon-gamma (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor-alpha (TNF-α), which activate macrophages to produce nitric oxide and reactive oxygen species for parasite killing [7]. Conversely, a Th2-type response with elevated levels of IL-4, IL-10, and IL-13 promotes disease progression and parasite survival [8]. *Leishmania* parasites have evolved sophisticated mechanisms to subvert host immune responses, including the manipulation of key signaling pathways within macrophages [9].

Among these pathways, the mammalian target of rapamycin (mTOR) has emerged as a critical regulator of macrophage function and immune polarization during Leishmaniasis [10]. mTOR is a serine/threonine kinase that forms two distinct complexes, mTORC1 and mTORC2, which integrate signals from nutrients, growth factors, and cellular energy status to control protein synthesis, cell growth, proliferation, and metabolism [11]. Importantly, *Leishmania* parasites have been shown to actively manipulate the mTOR signaling pathway to establish a permissive environment for their intracellular survival [12]. Jaramillo et al. (2011) demonstrated that *Leishmania* GP63 protease cleaves mTOR, leading to inhibition of mTORC1 and subsequent activation of the translational repressor 4E-BP1 [13]. This cleavage event promotes parasite proliferation, revealing that mTOR suppression is a parasite-driven survival strategy [13].

Paradoxically, pharmacological inhibition of mTOR with rapamycin has yielded conflicting results in the context of leishmaniasis. While the study suggested that mTOR cleavage promotes infection, Cheekatla et al. (2012) provided evidence that mTOR inhibition by rapamycin reverses *Leishmania Donovani*-induced modulation of the IL-12/IL-10 axis in macrophages [10]. Their work demonstrated that rapamycin treatment blocked the parasite-induced phosphorylation of P70S6K (a downstream effector of mTOR) and restored the balance between pro-inflammatory IL-12 and anti-inflammatory IL-10, suggesting that mTOR inhibitors may serve as immunomodulatory agents to redirect the host immune response toward a protective Th1 phenotype [10]. This immunomodulatory potential is particularly relevant for L. tropica, which is known to induce a mixed Th1/Th2 response and often requires systemic therapy due to inadequate response to topical treatments [10].

Nanotechnology-based drug delivery systems offer innovative solutions to overcome the limitations of conventional topical formulations for CL treatment. Liposomal and niosomal carriers have garnered significant attention due to their ability to encapsulate both hydrophilic and lipophilic drugs, protect them from degradation, enhance skin penetration, and target drugs to macrophages—the primary host cells for *Leishmania* [14-16]. The structural similarity between lipid-based vesicles and biological membranes facilitates fusion with the stratum corneum and delivery of encapsulated agents into the viable epidermis and dermis [17]. Furthermore, nanoparticles are efficiently phagocytosed by macrophages, enabling targeted delivery of therapeutic agents directly to the site of parasite residence [18].

Recent advances in liposomal technology for CL have demonstrated the promise of this approach. Santos et al. (2025) developed PEGylated liposomes co-encapsulating amphotericin B and retinoic acid, achieving 99% reduction in parasite burden in *L. major*-infected mice and promoting a Th1-skewed immune response with increased IFN-γ/IL-10 ratio [19]. Similarly, Alharthi et al. (2024) reported that PEGylated liposomal miltefosine significantly reduced lesion size (7.8-fold compared to free drug) and parasite burden in murine CL models, with improved sustained release profiles [20]. Silva et al. (2024) demonstrated the efficacy of liposomal chloroaluminum phthalocyanine combined with oral fexinidazole for topical photodynamic therapy of CL [21].

Niosomes, which are vesicles composed of non-ionic surfactants and cholesterol, offer several advantages over liposomes, including greater chemical stability, lower cost, and ease of large-scale manufacturing [22, 23]. They possess similar bilayer structures that can encapsulate drugs and deliver them to the skin based on the similarity of their lipid organization to natural membranes. Niosomes have been successfully employed to enhance the topical delivery of various anti-leishmanial agents, improving drug retention in the skin and targeting dermal macrophages [24, 25].

Rapamycin (sirolimus) is a lipophilic macrolide compound with well-established immunosuppressive and antiproliferative properties mediated through mTOR inhibition [26]. Given its ability to modulate immune responses and its paradoxical roles in Leishmaniasis,

rapamycin presents an intriguing candidate for repurposing in CL therapy. The immunomodulatory effects of rapamycin, particularly its capacity to enhance Th1 responses while suppressing Th2-type cytokines, align with the requirements for protective immunity against *Leishmania* [27]. However, the clinical application of rapamycin is limited by poor aqueous solubility, low oral bioavailability, and systemic immunosuppressive effects that could predispose patients to opportunistic infections [28].

The encapsulation of rapamycin within niosomes for topical delivery offers a strategic approach to harness its immunomodulatory potential while circumventing systemic toxicity. Topical application would concentrate the drug at the site of infection, within the dermal microenvironment where parasite-laden macrophages reside. The incorporation of rapamycin into a gel-based niosomal formulation could provide several therapeutic advantages: (i) enhanced drug penetration through the stratum corneum, (ii) targeted delivery to infected macrophages *via* phagocytic uptake, (iii) controlled release of rapamycin at the site of action, (iv) modulation of the local immune response toward a protective Th1 phenotype, and (v) minimal systemic exposure and immunosuppression.

Despite the biological rationale supporting the immunomodulatory role of mTOR inhibition in leishmaniasis and the established benefits of nanocarrier-mediated topical delivery, the therapeutic potential of Rapamycin-Loaded Niosomes base against *L. Major* infection has not been systematically investigated. Given the refractory nature of *L. Major* to conventional treatments and the species-specific differences in drug susceptibility, evaluating this novel formulation specifically against *L. Major* is of considerable clinical relevance.

We hypothesize that topical application of Rapamycin-Loaded Niosomes will enhance drug delivery to the dermis, target infected macrophages, and modulate the local immune response to promote parasite clearance in *L. Major* cutaneous leishmaniasis. Therefore, the present study aims to develop and characterize a Rapamycin-Loaded Niosomes formulation, evaluate its *in vitro* antileishmaniasis activity against *L. Major* promastigotes and amastigotes, and assess its therapeutic efficacy and immunomodulatory effects in an experimental model of *L. Major* infection.

## Materials & Methods

### Materials

Rapamycin was a gift from Zahravi Pharmaceuticals (Tabriz, Iran). Span 20® were supplied from Sigma-Aldrich (Darmstadt, Germany). Polysorbate 80 and Ethanol were supplied by Kimyagaran Emrooz and Dr Mojalali, respectively (Tehran-Iran). All other chemicals and solvents were of analytical grade.

The authors used an AI language model (ChatGPT, OpenAI) to assist in drafting and editing parts of the manuscript. All content was carefully reviewed, revised, and verified by the authors, who take full responsibility for the final version of the manuscript.

# Methods

## Niosomes Loaded Rapamycin Preparation

Niosomes loaded rapamycin were prepared using the ethanol injection. For this purpose, accurately weighed amounts of rapamycin (180 mg), cholesterol (180 mg), Polysorbate 80 (786 mg) and Span 20 (104 mg) were dissolved in 4 mL of absolute ethanol in a sealed glass container. The mixture was gently stirred or vortexed until a clear, homogeneous solution was obtained. The use of both hydrophilic (Polysorbate 80, HLB = 15.0) and lipophilic (Span 20, HLB = 8.6) surfactants ensures the formation of stable niosomal bilayers. In another beaker, 25 mL of phosphate-buffered saline, PBS, was heated to 60°C while placed on a magnetic stirrer. The temperature was maintained at 60°C throughout the injection process, as this temperature is above the phase transition temperature of the surfactants and facilitates proper vesicle formation [1, 5]. The organic phase containing all components (rapamycin, cholesterol, and surfactants dissolved in ethanol) was slowly injected into the heated aqueous phase using a manually operated syringe fitted with a fine needle (e.g., 21-23 gauge). The injection was performed at a controlled rate of approximately 1 mL/min while the aqueous phase was continuously stirred at 400-600 rpm using a magnetic stirrer. Upon injection, ethanol rapidly diffuses into the aqueous phase, causing the surfactants and cholesterol to self-assemble into bilayer vesicles, encapsulating the lipophilic rapamycin within the membrane. The mixture immediately turns opalescent, indicating niosome formation.

After injection, the dispersion was stirred for an additional 30-60 minutes at 60°C to allow for complete vesicle formation and stabilization and ethanol evaporation.

## Characterization of Niosomes Loaded Rapamycin

### Size & Morphology

The mean droplet size (Z-average), polydispersity index (PDI), and zeta potential were measured by DLS using a Zetasizer Nano ZS (Malvern Panalytical, UK). For size determination, the niosomal dispersion was diluted appropriately with distilled water to achieve a suitable scattering intensity (typically 200 μg/mL lipid concentration). The diluted sample was placed in a disposable polystyrene cuvette and equilibrated at 25°C for 2-3 minutes before measurement. Measurements were performed in triplicate at a scattering angle of 173°. For zeta potential measurement, the diluted sample was injected into a folded capillary zeta cell ensuring no air bubbles.

The morphology of the Niosomes-Loaded Rapamycin was assessed by transmission electron microscopy (TEM; Zeiss EM10C, Germany). A sample was negatively stained with 2% phosphotungstic acid and placed on a copper grid for TEM imaging.

### Encapsulation Efficiency (EE%)

The Niosomes-Loaded Rapamycin dispersion was centrifuged at 15,000-20,000 rpm for 30-60 minutes at 4°C using a cooling ultracentrifuge (Sigma, 3-30k). The supernatant containing unentrapped rapamycin was collected. High-Performance Liquid Chromatography (HPLC) system (KNAUER) with UV detector was for this purpose HPLC column, C18 reverse-phase column (e.g., 250 mm × 4.6 mm, 5 µm) at 50º C was used. The mobile phase contains Methanol: Water (80:20 v/v) and flow rate was 1.0 mL/min. Detection wavelength was set at 278 nm (for rapamycin) with injection volume of 20 µL.

$$EE\% = \frac{\text{Amount of drug encapsulated in niosomes}}{\text{Total amount of drug added}} \times 100$$

### Physical Stability Study

The Niosomes-Loaded Rapamycin dispersion was stored in sealed glass vials at refrigerated conditions (4-8°C) and room temperature: 25 ± 2°C. At predetermined time intervals (0, 15, 30 and 60, 90), samples were analyzed for particle size and PDI and visual inspection for aggregation, precipitation, or phase separation.

## Promastigote Drug Susceptibility Assay

### Promastigote Culture

*Leishmania major* promastigotes (strain MRHO/IR/75/ER) were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, USA). Cultures were maintained at 24 ± 1°C in an incubator and sub-cultured every 3-4 days. For experiments, parasites were harvested during the logarithmic phase of growth (day 3-4) by centrifugation at 2000 × g for 10 minutes at 4°C (Eppendorf 5810 R centrifuge). The pellet was washed twice with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in fresh complete medium. Parasite density was determined using a hemocytometer (Improved Neubauer chamber) and were seeded into each well of a sterile 96-well flat-bottom microplate that was adjusted to $2 \times 10^6$ parasites/mL [1, 3].

### Leishmania major Drug Preparation and Treatment

The anti-*Leishmania* activity of free rapamycin, Niosomes-Loaded Rapamycin, and the reference drug (Glucantime-Sanofi Aventis) were evaluated against *Leishmania major major* promastigotes using a colorimetric assay based on metabolic activity [1, 2].

Stock solutions of free rapamycin and Glucantime were prepared in sterile DMSO (final DMSO concentration < 0.1% v/v). Niosomes-Loaded Rapamycin and empty niosomes (control) were prepared as described previously. All test compounds were serially diluted in complete RPMI-

1640 medium to achieve final concentrations ranging from 2.5 to 30 µg/mL [2, 4]. The plates were incubated at 24°C for 72 hours in a humidified atmosphere. After 72 hours of incubation, the supernatant was carefully aspirated using a multichannel pipette without disturbing the parasite pellet. 100 µL of dimethyl sulfoxide (DMSO) (Merck, Germany) was added to each well to dissolve the parasite pellets. The plates were gently shaken on an orbital shaker for 15-20 minutes at room temperature to ensure complete solubilization. The optical density (OD) of each well was measured at 540 nm using a microplate reader (MicroPlate Reader BioTek ELx800). The wavelength of 540 nm is appropriate for direct cellular density measurement.

The percentage of 50% inhibition was calculated using the following formula:

$$IC50\% = \frac{(\text{Control} - \text{Sample})}{\text{Control}} \times 100$$

The $IC_{50}$ value was subsequently obtained by fitting the data to a nonlinear regression model using GraphPad Prism.

All experiments were performed in triplicate and repeated independently three times (n=3). Results were expressed as mean ± standard detection (SD).

## Intracellular Amastigote Drug Susceptibility Assay

The efficacy of the formulations against the clinically relevant intracellular amastigote stage of *L. major* was evaluated using murine peritoneal macrophages infected with promastigotes harvested at stationary-phase.

### Isolation of Peritoneal Macrophages

Female BALB/c mice (6-8 weeks old, 20-25 g) were purchased from Pasteur Institute of Iran. All animal procedures were approved by the Institutional Animal Ethics Committee (IAEC) IR.TUMS.AEC.1404.174 and followed international guidelines for the care and use of laboratory animals. Mice were injected into intraperitoneally with 2 mL of sterile 3% thioglycolate broth (Sigma-Aldrich). After 72-96 hours, mice were euthanized by cervical dislocation under anesthesia. The peritoneal cavity was lavage with 5-8 mL of cold sterile PBS (containing 100 U/mL penicillin and 100 µg/mL streptomycin). The peritoneal fluid was collected using a sterile syringe and transferred to a 15 mL centrifuge tube on ice

The cell suspension was centrifuged at 250 X g for 10 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in RPMI-1640 complete medium (supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin). Cells were counted using a hemocytometer, and viability was assessed using Trypan blue exclusion (>95% viability required). The cell density was adjusted to 2 X $10^6$ macrophages/mL.

## Macrophage Seeding and Infection

100 µL of the macrophage suspension (containing $1 \times 10^5$ cells) was added to each well of a 16-well plate. The plates were incubated overnight (16-18 hours) at 37°C with 5% $CO_2$ in a humidified incubator to allow macrophage adherence.

## Preparation for Infective Promastigotes

*L. major* promastigotes were cultured to stationary phase (day 5-6), which is enriched for metacyclic (infective) forms. Parasites were harvested by centrifugation at 2,000 × g for 10 minutes, washed twice with PBS, and resuspended in RPMI-1640 complete medium. The parasite density was adjusted to $1 \times 10^7$ promastigotes/mL to achieve the desired multiplicity of infection (MOI).

## Infection

The medium from the adhered macrophages was gently aspirated. 100 µL of the stationary-phase promastigote suspension ($1 \times 10^6$ parasites) was added to each well, achieving 10:1 parasite:macrophage. The plates were incubated for 24 hours at 37°C, 5% $CO_2$ to allow phagocytosis of the parasites.

## Treatment of Infected Macrophages

After 24 hours of infection, the medium containing non-internalized promastigotes was carefully aspirated. Each well was gently washed 3 times with pre-warmed sterile PBS (37°C) to remove all extracellular parasites and non-adherent cells. Fresh complete RPMI-1640 medium containing various concentrations of the test compounds (rapamycin solution, Niosomes-Loaded Rapamycin, Glucantime) was added to the designated wells. The concentrations ranged from 2.5 to 30 µg/mL, prepared by serial dilution. The slides were incubated for an additional 72 hours at 37°C, 5% $CO_2$.

## Determination of Amastigote Burden

After 72 hours of treatment, the medium was aspirated, and the plates were washed gently with PBS. The adhered cells were fixed by immersing the slides in absolute methanol for 5-10 minutes. The slides were air-dried and then stained with 10% Giemsa stain (Merck, Germany) in phosphate buffer (pH 7.2) for 30-45 minutes. The slides were rinsed gently with distilled water to remove excess stain and air-dried completely.

The chamber walls were removed according to the manufacturer's instructions. Stained slides were examined under a light microscope (Olympus BX53, Japan) using a 100× oil immersion objective. For each well, at least 200 macrophages were counted in randomly selected fields. (Fig. 1)

**Fig. 1.**

The percentage of 50% inhibition was calculated using the following formula:

$$IC50\% = \frac{(\text{Control} - \text{Sample})}{\text{Control}} \times 100$$

The $IC_{50}$ value was subsequently obtained by fitting the data to a nonlinear regression model using GraphPad Prism.

## *In Vivo* Study Design

### Animals

To evaluate the therapeutic efficacy of topical Rapamycin Loaded Niosomes formulation compared to conventional treatments in a murine model of leishmaniasis caused by *Leishmania major major*, female BALB/c mice (6-8 weeks old) were used in this study. BALB/c mice are highly susceptible to infection with *Leishmania major* and are the standard model for leishmaniasis studies [1, 2]. Mice weighed 20-30 grams at the beginning of the experiment. Animals were housed under standard conditions:

Temperature: 22 ± 2°C

Humidity: 50-60%

Light/dark cycle: 12 hours light/12 hours dark

Food and water: Provided

Mice were acclimatized for 7 days before the start of the experiment. All animal procedures were approved by the Institutional Animal Ethics Committee (IAEC) IR.TUMS.AEC.1404.174 and conducted in accordance with the guidelines for the care and use of laboratory animals.

### Parasite Preparation

*Leishmania major* promastigotes (strain:MRHO/IR/75/ER) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 24 ± 1°C. Stationary-phase promastigotes (day 5-6 of culture, enriched for metacyclic forms) were harvested by centrifugation at 2,000 × g for 10 minutes at 4°C. The pellet was washed twice with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS at a concentration of $2 \times 10^7$ promastigotes/mL.

### Inoculation

Mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). A volume of 100 µL of the parasite suspension containing $2 \times 10^6$ stationary-phase *L. Major* promastigotes were injected subcutaneously into the right hind footpad of each mouse using a 27-gauge needle. The left hind footpad remained untreated as a negative control.

### Lesion Development

The infection was allowed to progress for approximately 4 weeks (28 days) post-inoculation to allow for the development of measurable lesions. During this period, lesion development was monitored weekly by measuring footpad thickness using a digital caliper (Mitutoyo, Japan).

### Efficacy of Topical Rapamycin Loaded Niosomes Against Cutaneous Leishmaniasis

After 4 weeks of infection, when lesions were well-established, mice were randomly divided into 4 experimental groups, each containing 5 mice (n = 5 per group); Rapamycin-Loaded Niosomal gel (topical, applied directly to the lesion surface every 8 hours for 28 days). Meglumine antimoniate (Glucantime -Positive Control) (Intraperitoneal (IP) injection with standard dose (e.g., 20 mg $Sb^{5+}$/kg body weight), Once daily for 28 days), Empty niosomes (without rapamycin) in gel base (topical, applied directly to the lesion surface every 8 hours for 28 days). Free Rapamycin (Drug Control) (topical, applied directly to the lesion surface every 8 hours for 28 days). Throughout the 28-day treatment period and for a follow-up period, the following parameters were assessed through lesion size measurement and clinical observations. Footpad thickness was measured every 7 days using a digital caliper (Mitutoyo, Japan). The thickness of the infected footpad was compared to the uninfected contralateral footpad.

Lesion size was calculated as:

Lesion size = (Thickness of infected footpad) - (Thickness of uninfected footpad) [mm]

Further, mice were observed daily for signs of distress, toxicity, or adverse reactions to treatments.

### Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Statistical comparisons between treatment groups were performed using One-way ANOVA.

Differences were considered statistically significant at $p < 0.05$. Significance levels were denoted as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

## Results & Discussion

### Characterization of Niosomes-Loaded Rapamycin

The successful characterization of rapamycin-loaded niosomes, meeting the specified criteria of ~220 nm size, PDI < 0.3, spherical bilayer morphology, and EE% ≥ 99.99%, confirms their potential as a highly efficient drug delivery system. These optimized physicochemical properties lay the foundation for further *in vitro* and *in vivo* studies to evaluate the therapeutic efficacy and pharmacokinetic profile of rapamycin delivered *via* this advanced nanocarrier system.

This size range is often considered ideal for various administration routes, including topical, oral, and parental, as it can facilitate cellular uptake and improve drug circulation time. A low PDI ensures the uniformity of the drug delivery system, leading to more consistent and reliable therapeutic outcomes. It minimizes the risk of aggregation and improves physical stability. The

bilayer morphology confirms the proper formation of the niosomal vesicles, essential for encapsulating the drug within a stable lipophilic structure and controlling its release. The high drug loading efficiency signifies a highly effective encapsulation process, maximizing the amount of drug delivered per vesicle and potentially reducing the required dosage. It suggests minimal drug loss during formulation and a robust system for drug retention (Fig. 1).

Fig 1

The rapamycin-loaded niosomal formulation exhibited remarkable physical stability over a 90-day period at both 4-8°C and ambient temperature. The consistent particle size below 300 nm, coupled with the absence of phase separation and drug crystallization/exudation, underscores the robustness and suitability of this niosomal system for storing and delivering rapamycin. These findings provide strong evidence for the formulation's shelf-life potential and its reliability as a drug delivery vehicle. The absence of phase separation signifies the stability of the niosomal dispersion and the integrity of the surfactant-bilayer structure, which prevents the components from separating into distinct phases. Furthermore, no crystalline formations of rapamycin were detected, either as precipitates at the bottom of the containers or as turbidity within the colloidal suspension. This finding is highly significant, as it confirms the high encapsulation efficiency and the strong retention capability of the niosomal system for rapamycin. The absence of crystalline drug indicates that rapamycin is molecularly dispersed within the niosomal bilayers or matrix, rather than existing as solid particles.

## Promastigote & Amastigote Drug Susceptibility Assay

The data provided from *in vitro* studies demonstrate the anti-*Leishmania* activity of rapamycin, both in its free form and encapsulated within niosomes, compared to the standard drug, Glucantime. The studies were conducted on both promastigote (extracellular) and amastigote (intracellular) forms of *Leishmania*.

Rapamycin, in both free and niosomal forms, demonstrates inhibitory effects against *Leishmania* in both promastigote and amastigote stages, as evidenced by the decreasing OD values with increasing drug concentrations.

The most striking finding is the comparable efficacy of rapamycin (both free and niosomal) to Glucantime against the intracellular amastigotes (Fig. 2).

Fig. 2

The $IC_{50}$ values are very close (around 14-15 µg/mL), and the statistical analysis shows no significant difference compared to Glucantime ($p > 0.05$). This is particularly important because amastigotes are the pathogenic form residing within host macrophages, and effective treatment requires targeting these intracellular parasites. The results suggest that rapamycin could be a viable alternative or adjunct therapy for leishmaniasis, especially considering its potential to overcome resistance mechanisms that may affect existing drugs. Against the extracellular promastigotes, Glucantime demonstrates significantly higher potency (lower $IC_{50}$ values) compared to both free and niosomal rapamycin (16 µg/mL vs. 25 µg/mL, respectively). While niosomal rapamycin shows a slight numerical improvement in $IC_{50}$ (25 µg/mL) over free rapamycin (29 µg/mL), this difference is not statistically significant (Fig 3).

Fig. 3

This suggests that while the niosomal formulation might offer some benefits, it does not fully overcome the lower intrinsic potency of rapamycin compared to Glucantime for the promastigote stage. Against amastigotes, the $IC_{50}$ values for niosomal rapamycin were very similar to free rapamycin and comparable to Glucantime, with no statistically significant difference observed when compared to Glucantime. The primary benefit of niosomes might lie in other aspects not fully captured by $IC_{50}$ alone, such as improved drug stability, controlled release kinetics *etc*. The high loading efficiency (99.99%) mentioned previously also contributes to the formulation's potential.

These findings suggest that rapamycin, particularly when delivered *via* niosomes, holds promise as an anti-leishmanial agent, especially for targeting the intracellular amastigote stage. Its comparable efficacy to Glucantime against amastigotes is a key highlight.

## *In vivo* study

At all measured time points (W1-W3), there is no statistically significant difference in lesion size between the topical Rapamycin Loaded Niosomes group and the untreated control group. This indicates that the topical Rapamycin Loaded Niosomes, as applied in this study, was not effective in reducing lesion size. However, based on the trend of decreasing lesion sizes in the Glucantime group and increasing sizes in the control group, and knowing Glucantime's established efficacy, we can infer from Week 2 onwards, the Glucantime group's average lesion size is consistently lower than the control group. The fact that the lesions in the control group are growing while Glucantime-treated lesions are shrinking strongly suggests that the differences would become statistically significant, particularly at later points like W3 and W4 ($p < 0.05$) (Fig. 4)

Fig. 4

The data unequivocally demonstrates the efficacy of Glucantime when administered *via* intraperitoneal injection. This systemic route ensures adequate drug levels at the parasite's intracellular location, leading to significant lesion healing. This aligns with established clinical and experimental findings for Glucantime in leishmaniasis. The inclusion of "niosomal" in the formulation for Rapamycin highlights an attempt to enhance its topical delivery. However, the *in vivo* results indicate that this strategy was unsuccessful in achieving therapeutic efficacy against lesion induced by *Leishmania major*. The P-values clearly show no significant difference compared to the control. This suggests that either the drug did not penetrate deeply enough into the infected tissue, or the concentration at the site of infection was insufficient to exert a significant anti-parasitic or immunomodulatory effect, or rapamycin itself, even at therapeutic concentrations, does not have significant direct anti-leishmanial activity or beneficial immunomodulatory effects in this specific *in vivo* context when delivered topically. This comparison starkly illustrates the importance of both the route of administration and the drug formulation. Systemic delivery (IP injection of Glucantime) proved highly effective, as it allowed the drug to reach the parasites wherever they were located. Topical delivery, despite being an advanced formulation designed to improve local drug concentration, failed to deliver therapeutic levels to combat the infection effectively. This is a crucial finding, especially if rapamycin or similar mTOR inhibitors are being considered for topical leishmaniasis treatment.

This reinforces that while advanced formulations like niosomes can improve drug delivery, the choice of drug, its intrinsic activity against the parasite or host response, and achieving adequate therapeutic concentrations at the site of infection *via* the chosen route are paramount for *in vivo* efficacy. For *Leishmania*, systemic treatment remains the gold standard, and topical therapies face significant challenges in delivering sufficient drug to the deep-seated parasites.

Given the limitations observed with topical Niosomes-Loaded Rapamycin *in vivo*, further research should investigate strategies to enhance drug penetration and achieve therapeutic concentrations at the site of infection. This could include exploring alternative niosomal compositions, incorporating penetration enhancers, or investigating different topical delivery vehicles. Beyond topical application, the potential of systemic administration of Niosomes-Loaded Rapamycin (e.g., parenteral) warrants investigation. This could leverage the improved stability and controlled release offered by the niosomal formulation to achieve effective systemic drug levels.

## Conclusion

This study successfully formulated and characterized Niosomes-Loaded Rapamycin with optimized physicochemical properties (~220 nm size, PDI < 0.3, spherical bilayer morphology, and EE% ≥ 99.99%), indicating their high potential as a drug delivery system. These niosomes demonstrated remarkable physical stability over 90 days under various storage conditions, suggesting a favorable shelf-life. *In vitro* assays revealed that both free and niosomal rapamycin possess anti-*Leishmania* activity. Notably, niosomal rapamycin exhibited comparable efficacy to

the standard drug, Glucantime, against the intracellular amastigote stage, which is critical for therapeutic success. While rapamycin showed less potency against the extracellular promastigote stage compared to Glucantime, the niosomal formulation did not significantly alter this observation, suggesting its primary benefits may lie in stability and controlled release rather than enhanced intrinsic potency for this stage. However, the *in vivo* study indicated that topical application of Niosomes-Loaded Rapamycin was ineffective in reducing lesion size in the model used, with no statistically significant difference observed compared to the untreated control group. This contrasts sharply with the significant efficacy of intraperitoneally administered Glucantime, highlighting the critical importance of the route of administration and achieving adequate drug concentrations at the site of infection. The failure of the topical niosomal formulation suggests potential issues with drug penetration, insufficient local concentration, or the intrinsic activity of rapamycin in this specific *in vivo* context.

In summary, while Niosomes-Loaded Rapamycin show promises *in vitro* due to their stability and efficacy against intracellular *Leishmania* amastigotes, their topical application requires further investigation and optimization to achieve *in vivo* therapeutic outcomes. Systemic delivery remains the gold standard for treating leishmaniasis, and overcoming the challenges associated with topical drug delivery to deep-seated parasites is paramount for the development of effective topical treatments.

## Funding Declaration

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## References

1. de Vries, H.J. and H.D. Schallig, *Cutaneous Leishmaniasis: A 2022 Updated Narrative Review into Diagnosis and Management Developments: HJC de Vries, HD Schallig.* American journal of clinical dermatology, 2022. **23**(6): p. 823–840.
2. Kyari, S., *Epidemiology of leishmaniasis*, in *Leishmania Parasites-Epidemiology, Immunopathology and Hosts*. 2024, IntechOpen.
3. Frézard, F. and C. Demicheli, *New delivery strategies for the old pentavalent antimonial drugs.* Expert opinion on drug delivery, 2010. **7**(12): p. 1343–1358.
4. Razavi, M.R., et al., *Human cutaneous leishmaniosis in Iran, up to date-2019.* Journal of Arthropod-Borne Diseases, 2021. **15**(2): p. 143.
5. Abbasi, E., *A perspective on human leishmaniasis and novel therapeutic methods for diagnosis, prevention and treatment.* The Pediatric infectious disease journal, 2025. **44**(10): p. e369–e373.
6. Van Bocxlaer, K., *Cutaneous Leishmaniasis-Skin Barrier Properties And Drug Delivery Strategies*. 2015, UCL (University College London).
7. dos Santos Meira, C. and L. Gedamu, *Protective or detrimental? understanding the role of host immunity in leishmaniasis.* Microorganisms, 2019. **7**(12): p. 695.

8. Dayakar, A., et al., *Cytokines: key determinants of resistance or disease progression in visceral leishmaniasis: opportunities for novel diagnostics and immunotherapy.* Frontiers in immunology, 2019. **10**: p. 670.
9. Olivier, M., D.J. Gregory, and G. Forget, *Subversion mechanisms by which Leishmania parasites can escape the host immune response: a signaling point of view.* Clinical microbiology reviews, 2005. **18**(2): p. 293–305.
10. Cheekatla, S.S., A. Aggarwal, and S. Naik, *mTOR signaling pathway regulates the IL-12/IL-10 axis in Leishmania donovani infection.* Medical microbiology and immunology, 2012. **201**(1): p. 37–46.
11. Laplante, M. and D.M. Sabatini, *mTOR signaling at a glance.* Journal of cell science, 2009. **122**(20): p. 3589–3594.
12. Rashidi, S., et al., *The host mTOR pathway and parasitic diseases pathogenesis.* Parasitology research, 2021. **120**(4): p. 1151–1166.
13. Jaramillo, M., et al., *Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection.* Cell host & microbe, 2011. **9**(4): p. 331–341.
14. Nurizan, A.N.B., et al., *Emerging niosomal formulations for skin-targeted drug delivery: strategies, applications, and limitations.* International Journal of Polymeric Materials and Polymeric Biomaterials, 2026: p. 1–13.
15. Carita, A.C., et al., *Recent advances and perspectives in liposomes for cutaneous drug delivery.* Current medicinal chemistry, 2018. **25**(5): p. 606–635.
16. Teron, C., et al., *A Comprehensive Review on Niosomes in Drug Delivery and Recent Advancements.* Journal of Drug Delivery & Therapeutics, 2024. **14**(9).
17. Sguizzato, M., E. Esposito, and R. Cortesi, *Lipid-based nanosystems as a tool to overcome skin barrier.* International journal of molecular sciences, 2021. **22**(15): p. 8319.
18. Owais, M. and C. Gupta, *Targeted drug delivery to macrophages in parasitic infections.* Current drug delivery, 2005. **2**(4): p. 311–318.
19. Santos, T.T., et al., *Long-Circulating Liposomes Codelivering Amphotericin B and Retinoic Acid for Cutaneous Leishmaniasis Treatment.* ACS omega, 2025. **10**(41): p. 48514–48530.
20. Alharthi, S., et al., *Enhancing the therapeutic landscape of cutaneous leishmaniasis: pegylated liposomal delivery of miltefosine for controlled release and improved efficacy.* Journal of Drug Delivery Science and Technology, 2024. **96**: p. 105735.
21. Silva, R.A., et al., *Combination of the Topical Photodynamic Therapy of Chloroaluminum Phthalocyanine Liposomes with Fexinidazole Oral Self-Emulsifying System as a New Strategy for Cutaneous Leishmaniasis Treatment.* Pharmaceutics, 2024. **16**(4): p. 509.
22. Abdelkader, H., A.W. Alani, and R.G. Alany, *Recent advances in non-ionic surfactant vesicles (niosomes): self-assembly, fabrication, characterization, drug delivery applications and limitations.* Drug delivery, 2014. **21**(2): p. 87–100.
23. Mostafavi, M., et al., *Niosomal formulation of amphotericin B alone and in combination with glucantime: In vitro and in vivo leishmanicidal effects.* Biomedicine & Pharmacotherapy, 2019. **116**: p. 108942.
24. Jain, N.K., V. Mishra, and N.K. Mehra, *Targeted drug delivery to macrophages.* Expert opinion on drug delivery, 2013. **10**(3): p. 353–367.
25. Jaafari, M.R., et al., *Development of a topical liposomal formulation of Amphotericin B for the treatment of cutaneous leishmaniasis.* International journal for parasitology: drugs and drug resistance, 2019. **11**: p. 156–165.

26. Panwar, V., et al., *Multifaceted role of mTOR (mammalian target of rapamycin) signaling pathway in human health and disease.* Signal transduction and targeted therapy, 2023. **8**(1): p. 375.
27. Khadir, F., et al., *Antileishmanial effect of rapamycin as an alternative approach to control Leishmania tropica infection.* Veterinary parasitology, 2019. **276**: p. 108976.
28. Lee, D.J., A.H. Kuerec, and A.B. Maier, *Targeting ageing with rapamycin and its derivatives in humans: a systematic review.* The Lancet Healthy Longevity, 2024. **5**(2): p. e152–e162.

# Figures

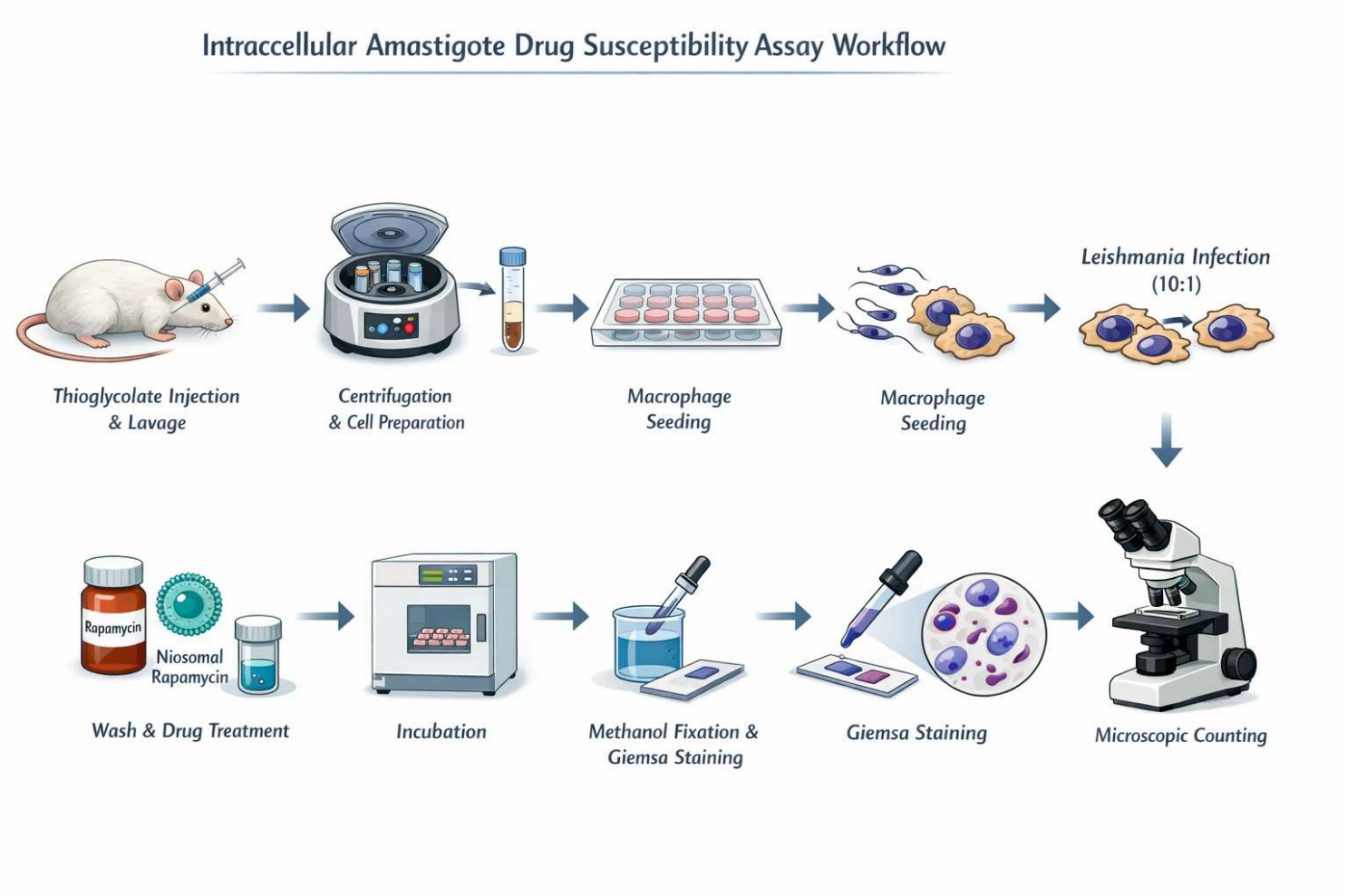



**Figure 1**

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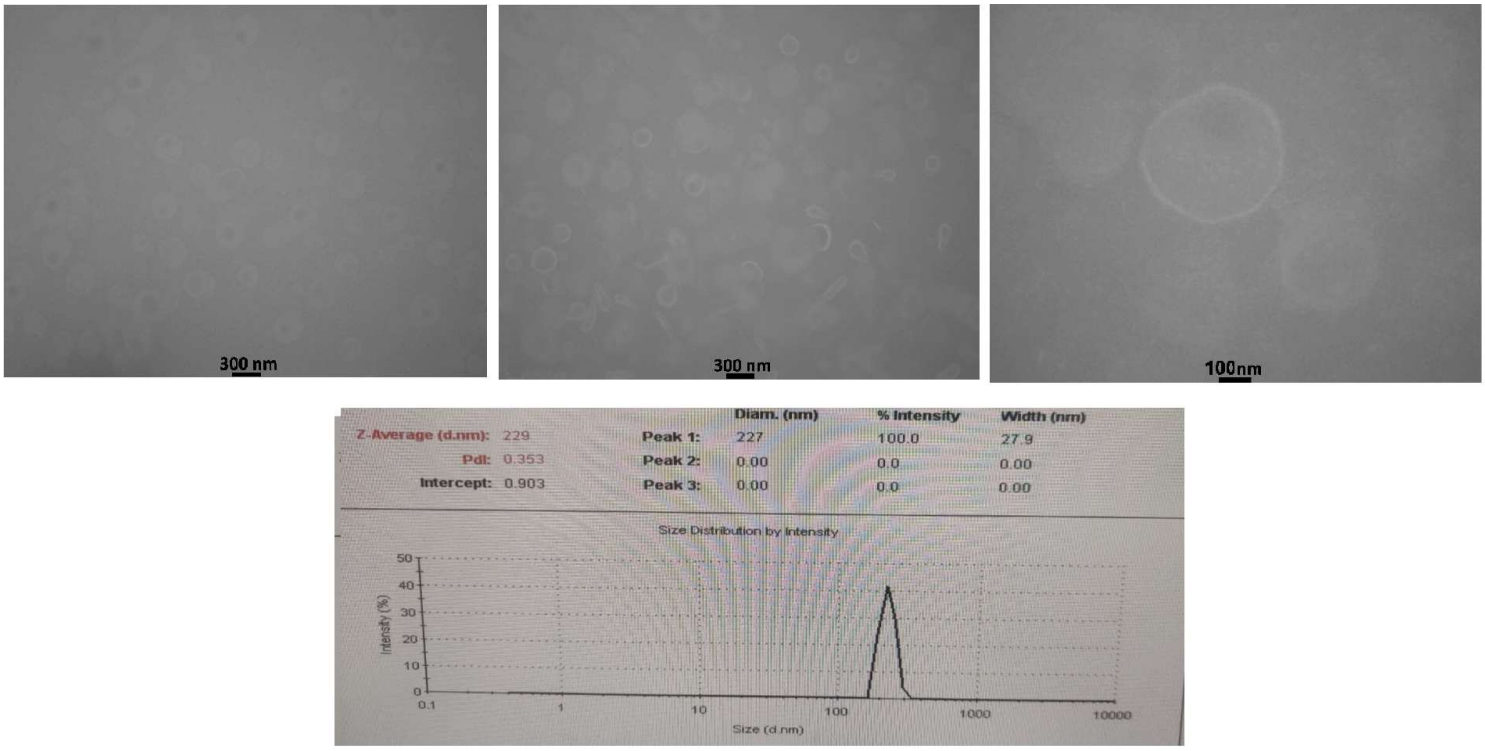



**Figure 2**

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