

# Evaluation of the Antileishmanial Activity of a Novel Chitosan based on Second-Generation Dendrimers against *Leishmania major* Strain MRHO/IR/75/ER *In Vitro*

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## Abstract

**Background:** Cutaneous leishmaniasis (CL) is one of the most significant parasitic diseases in Iran and other endemic regions. Existing standard treatments face challenges, including drug toxicity, difficulty in administration, and parasite resistance.

**Objectives:** In this study, nano chitosan based on dendrimers (NChi), which has antibacterial and antiparasitic effects, was used to target *Leishmania major* *in vitro*.

**Methods:** NChi was synthesized first. Fourier transform infrared spectroscopy (FTIR) and dynamic light scattering (DLS) were used to confirm the synthesis and measure the size, while scanning electron microscopy (SEM) was employed to examine the nanostructure's morphology. After assessing the cytotoxicity of the NChi on the macrophage cell line, the antileishmanial effects of NChi were evaluated after 24 and 48 hours. Finally, the data were statistically analyzed using the ANOVA test in SPSS software.

**Results:** The results demonstrated that NChi, with a size of 128.5 nm, is suitable for drug delivery. FTIR analysis confirmed the successful synthesis of the nanostructure by indicating the attachment of chitosan to the dendrimer. The MTT assay on macrophage cells showed no cytotoxicity of the synthesized nanocomposite at the applied concentration. Moreover, after 48 hours, the NChi exhibited significant antileishmanial activity, achieving 100% growth inhibition at a concentration of 40 µg/ml.

**Conclusion:** This study highlights NChi as a more effective alternative to conventional chitosan for leishmaniasis due to its optimal size, stability, and potent anti-promastigote activity, positioning it as a promising substitute for chemical drugs like glucantime.

**Keywords:** Nano Chitosan, Leishmaniasis, Dendrimer, *In vitro*

## 1. Background

Leishmaniasis is an endemic tropical parasitic disease in Iran, transmitted through the bite of sandflies belonging to the *Phlebotomus* and *Lutzomyia* genera.<sup>1</sup> It is caused by various species of obligate intracellular flagellated parasites of the genus *Leishmania*, which is further classified into two subgenera: *Leishmania* and *Viannia*.<sup>2,3</sup>

Leishmaniasis, with an estimated 700,000 to 1.2 million cases annually, is a significant infectious disease recently identified by the World Health Organization (WHO) as one requiring special attention.<sup>4,5</sup> The WHO has emphasized the need for monitoring the disease's progression and establishing robust surveillance systems in countries where it is prevalent. The disease is divided into three main types, each with its distinct symptoms: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL),

and mucocutaneous leishmaniasis (MCL).<sup>6</sup> Among these, our research focuses on CL, which is the most common form of the disease, with 600,000 to 1 million new cases reported annually. Of the 88 countries where CL is endemic, 90% of the cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria.<sup>7</sup> CL is a skin disease characterized by sores or wart-like plaques on exposed areas of the body that are vulnerable to bites from infected female sandflies. These sores are sometimes accompanied by smaller lesions nearby or inflammation of the lymph nodes.<sup>8</sup>

Pentavalent antimony compounds, such as sodium stibogluconate (Pentostam) and meglumine antimoniate (glucantime), have been the standard treatment for CL for the past 70 years. However, these drugs have limitations, including difficulties in administration, drug toxicity, and

variable sensitivity among different *Leishmania* species.<sup>9</sup> Two Cochrane analyses have clearly highlighted the clinical shortcomings of most available drugs. Therefore, there is an urgent need for new treatments that can completely eliminate the parasites, accelerate the healing process, and be safe, reliable, and adaptable to different healthcare system conditions.<sup>10</sup>

Chitosan is a biodegradable, biocompatible, low-toxicity biopolymer that is produced through the deacetylation of chitin. This compound dissolves in weak acidic solvents, such as acetic acid, where its amino groups are protonated. Numerous reports have pointed to the antimicrobial activity of chitosan, though its precise mechanism of action has not been fully elucidated. However, three direct mechanisms have been proposed: Interaction of the protonated NH<sub>3</sub><sup>+</sup> groups of chitosan with the negatively charged microbial cell membranes. This interaction alters the permeability of the cell membrane and leads to microbial death due to osmotic imbalance. Binding of chitosan to microbial DNA and inhibition of DNA transcription. This hypothesis is based on the assumption that chitosan crosses the microbial cell membrane and accesses DNA. Chelating metals and binding to essential nutrients required for microbial growth by chitosan.<sup>11</sup>

Additionally, an indirect mechanism has been proposed that is linked to the known pro-inflammatory effects of chitosan on macrophages. This effect involves the stimulation of the production of TNF- $\alpha$ , IL-6, nitric oxide (NO), reactive oxygen species (ROS), and IFN- $\gamma$ , which play a crucial role in the pro-inflammatory response against intracellular microbes by increasing the production of microbicidal nitrogen species.<sup>11,12</sup> Since *Leishmania major* is an intracellular parasite, various studies have shown that Th1 cells and NK cells produce cytokines such as interferon-gamma (IFN- $\gamma$ ), which stimulate the production of nitric oxide (NO) and reactive oxygen species (ROS).<sup>7</sup> These compounds, along with other antimicrobial lysosomal activities, are responsible for eliminating *Leishmania* parasites. Furthermore, chitosan activates polymorphonuclear leukocytes, macrophages, and fibroblasts, which accelerates the wound healing process.<sup>12</sup>

Given the antibacterial and anti-inflammatory properties of chitosan and its enhancement of the Th1 system, which is the primary mechanism for eliminating *L. major* parasites, chitosan was utilized in the present study to target the parasite in culture media. This was aimed at achieving both anti-parasitic effects and preventing secondary infections in leishmanial lesions.<sup>13</sup> However, due to the low solubility of chitosan in water, its application has been limited. Therefore, in the present study, dendrimers were employed to enhance the solubility of chitosan in water.<sup>11</sup>

The use of dendrimer nanocarriers as an innovative

technology in the treatment of diseases such as CL has gained attention in recent years due to their unique properties, including a three-dimensional branched structure at the nanoscale. This technology has been applied to improve drug efficacy, reduce side effects, and overcome therapeutic challenges associated with leishmaniasis.<sup>14</sup>

The spaces between the branches of these molecules are suitable for encapsulating drugs. A biocompatible and biodegradable dendrimer made from citric acid–polyethylene glycol–citric acid (CPEGC) is a suitable candidate among these types of dendrimers. Generations 1–3 of this dendrimer were synthesized as drug delivery systems by Namazi and Adeli (2005), and controlled release of drugs such as mefenamic acid and diclofenac using these carriers has been previously investigated.<sup>15</sup> These dendrimers are biocompatible and have a high potential as drug carriers. When combined with hydrophobic drugs, they enhance the solubility of the drugs.

Since *L. major* is an intracellular parasite residing in macrophages, dendrimers can serve as targeted drug delivery systems. Their functional groups facilitate direct drug delivery to macrophages, enhancing therapeutic efficacy at the infection site. Dendrimers release the drug gradually, helping maintain an effective concentration at the infection site. This feature helps reduce the need for repeated doses and minimizes side effects. Because dendrimers have a branched structure with numerous functional groups, they can simultaneously carry multiple drugs, making them suitable for combination therapy. Combination therapy is crucial in CL, as in addition to the presence of the parasite at the wound site, secondary bacterial infections can complicate treatment. Therefore, drug loading that possesses both anti-parasitic and anti-bacterial properties could help address the challenges in treating CL.<sup>16,17</sup>

Given the challenges mentioned in the treatment of CL, such as parasite resistance to common drugs like pentavalent antimonial, low solubility of drugs like amphotericin B, fluconazole, and natural compounds like chitosan in water, toxicity of some drugs that increases with dose, and the contamination of wounds with secondary bacterial infections, the present study aims to achieve a new nanostructure for treating CL. This involves using a water-soluble NChi that possesses both antileishmanial and antibacterial effects, thus preventing the worsening of wounds by opportunistic and pathogenic agents.<sup>17</sup>

## 2. Objectives

In this study, nano chitosan based on dendrimers (NChi), which has antibacterial and antiparasitic effects, was used to target *L. major in vitro*.

## 3. Methods

### 3.1. Synthesis of Second-Generation Dendrimer

The G2 nano dendrimer, used as the base nanocarrier in

this study, was synthesized using a modified method by Namazi et al.<sup>18</sup> First, 2 ml of polyethylene glycol (PEG) was taken and 5 ml of DMSO was added to it. The solution was then stirred using a magnetic stirrer until thoroughly mixed. Once completely solubilized, 0.5 g of DCC and 0.5 g of citric acid were added to the reaction, and after 15 minutes of stirring, an additional 0.7 g of citric acid was added. The reaction mixture was continuously stirred for one week under controlled conditions. The reaction was then stopped by adding 5 ml of distilled water, and to remove the DCC precipitate from the mixture, a paper filter was used. To remove impurities from citric acid and DMSO, the solution was dialyzed in a 1000-500 cut-off dialysis bag for 17 hours in distilled water. For further purification, the obtained solution was re-purified using a Sephadex G-75 column. For this, 0.6 g of Sephadex powder was dissolved in 20 ml of distilled water and left at room temperature for 24 hours before being transferred to the column. Initially, the column was washed once with distilled water. Then, the dendrimer solution was added to the column, and the purified solution was collected from the bottom of the column (this step was repeated several times). Finally, the purified solution was lyophilized.<sup>19</sup>

### 3.2. Preparation of Chitosan

Chitosan was dissolved in a 37% acetic acid solution and a 1:1 mixture of water/ethanol. Ethyl acrylate and triethylamine were added to the solution while stirring on a magnetic stirrer. The reaction product was precipitated by adding acetone and sodium bicarbonate. The resulting precipitate was dispersed in sodium bicarbonate-saturated water. The mixture was dialyzed in distilled water and lyophilized to obtain N-carboxyethyl chitosan ethyl ester. The product was then placed in a 0.1 M sodium hydroxide solution until the pH reached approximately 10, causing the ethyl ester to convert into sodium salt. To remove salts and purify the N-carboxyethyl chitosan, dialysis was performed for 48 hours. Finally, a suspension of N-carboxyethyl chitosan in methanol was prepared.<sup>20</sup>

### 3.3. Loading Chitosan onto Dendrimer

To load chitosan onto the dendrimer, 1 g of dendrimer was first dissolved in methanol, and 500 mg of N-carboxyethyl chitosan was added. Then, 500 mg of EDC powder was added to the mixture and stirred for 7 days at 37 °C, with daily checks. Afterward, solvents were removed using a rotary evaporator, and 30 ml of distilled water was added and stirred until the chitosan conjugate dissolved in water and free chitosan with EDC precipitated. The solution was filtered using filter paper, followed by purification through a Sephadex G-75 column. Finally, the purified solution was lyophilized.<sup>21</sup>

### 3.4. Characterization of Size and Shape of Synthesized

### Products

To determine the size and distribution of particle size, Dynamic Light Scattering (DLS) was used.<sup>22</sup> DLS is a common method for measuring particle sizes in the nanometer scale. The method is based on the scattering of laser light by suspended particles in a liquid medium, followed by analysis of the changes in the intensity of scattered light due to the Brownian motion of the particles. Initially, the nanoparticles were dispersed in methanol and filtered to remove insoluble impurities. The measurements were taken at a scattering angle of 90 degrees, a temperature of 25.2 °C, and a dispersion medium viscosity of 0.892 mPa·s. The results were presented as a particle size distribution chart, providing information about the average particle size and its scattering. To examine the surface and three-dimensional structure of the synthesized particles, and also to confirm the size recorded by DLS, Scanning Electron Microscopy (SEM) was used. In this technique, electrons interact with the surface of the sample, generating high-resolution images.

### 3.5. Confirmation of Nanostructure Synthesis

Fourier-transform infrared (FTIR) spectroscopy was employed to identify functional groups present in chitosan and confirm the successful loading process of chitosan onto the dendrimer.<sup>23</sup> In FTIR, the absorption of infrared energy by various chemical bonds in the synthesized nanostructures was examined in the range of 400-4000 cm<sup>-1</sup>, and their absorption spectra were recorded. Since each functional group has a distinct absorption region, the bond between the dendrimer and chitosan was investigated using FTIR, and the results were reported in the form of spectra.

### 3.6. Determination of Chitosan Loading onto Dendrimer

To determine the amount of chitosan loaded onto the dendrimer G2, a standard curve for chitosan was used. The absorption of chitosan was measured in the wavelength range of 200-800 nm, and it was found that chitosan exhibited the highest absorption at 560 nm.<sup>23</sup> Various dilutions of chitosan were prepared and measured at this wavelength using a Nanodrop<sup>TM</sup> 2000/2000 C device (Thermo, USA). The absorption of the NChi at a concentration of 500 µg/ml was recorded. Based on the standard curve, the amount of chitosan present was calculated using the following formula:

$$\text{Loading percentage (\%)} = \frac{\text{Initial drug amount} - \text{Amount of drug in solution}}{\text{Initial drug amount}} \times 100$$

### 3.7. Solubility Evaluation of the Synthesized Nanocomposite in Water

To assess the solubility enhancement of chitosan in water, spectrophotometry was employed. 5 mg of chitosan and the synthesized NChi were separately dissolved in 10 ml water and centrifuged for one minute at 13,000 rpm. the

precipitate was separated and dissolved in acetic acid. The OD value measured at a wavelength of 560 nm was compared with the standard curve to calculate the percentage of precipitated chitosan.

### 3.8. Cellular Toxicity Assessment

To assess the cytotoxic effects of NChi and chitosan, the J774A.1 mouse macrophage cell line was exposed to different concentrations of the prepared formulations (5, 10, 20, and 40 µg/ml of NChi and chitosan). After a 48-hour incubation period, the medium containing the formulations was replaced with fresh culture medium. Following this, 10 µl of MTT solution (5 mg/ml) was added to the wells, and the cells were incubated at 37 °C for 4 hours. Afterward, the medium was carefully removed, and 100 µl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The absorbance at 545 nm was then measured to evaluate cell viability.<sup>24</sup>

### 3.9. Cultivation of *Leishmania major* Parasite

In this experimental study, the standard strain of *L. major* (MRHO/IR/75/ER) was obtained from the Pasteur Institute of Tehran and inoculated into a biphasic NNN medium under sterile conditions. To prepare the NNN medium, 1.4 g of agar and 0.6 g of NaCl were dissolved in 90 milliliters of deionized water, and the resulting mixture was sterilized in an autoclave. Then, 15% sheep blood without fibrin was added to provide essential nutrients for parasite growth. After adding 250 units per milliliter of penicillin and 250 µg/ml of streptomycin, 10 milliliters of this mixture were added to Falcon tubes, and the medium was allowed to form the biphasic structure. Once the biphasic medium was formed, the standard *Leishmania* strain was added to the NNN medium for cultivation and incubated at 24 °C. After the parasite grew in the NNN medium and reached a concentration of  $10^6 \times 3$  parasites per milliliter, it was transferred to RPMI medium containing 10% FBS, 100 µg/ml of streptomycin, and 100 units per milliliter of penicillin, and incubated at

24 °C. Passage was performed every 3 days, as needed.

### 3.10. Evaluation of Antileishmanial Effect of Nano-chitosan

To evaluate the effect of the synthesized NChi on the growth of *L. major* parasite, 100 µl of diluted RPMI culture medium containing  $2 \times 10^6$  promastigote parasites was added to wells 1 to 11 of a 96-well ELISA plate. Then, 50 µl of NChi concentrations of 40, 20, 10, and 5 µg/ml were added to wells 1 to 4; 50 µl of chitosan concentrations of 40, 20, 10, and 5 µg/ml were added to wells 5 to 8; 50 µl of 100 µg/ml glucantime (as a positive control) was added to well 9; 50 µl of culture medium (as a negative control) was added to well 10; and 50 µl of dendrimer at a concentration of 40 µg/ml was added to well 11. Well 12 contained only 100 µl of culture medium without parasites. After 24 and 48 hours, the number of parasites in each well was counted.

### 3.11. Statistical Analysis

The data were entered into SPSS version 22 for statistical analysis. Initially, the normality of the data was tested using the Kolmogorov-Smirnov test, and the normally distributed data were analyzed using one-way analysis of variance (ANOVA) and the Tukey post hoc test. To calculate the dose of the drug that caused a 50% improvement in the tested mice, nonlinear regression was used.

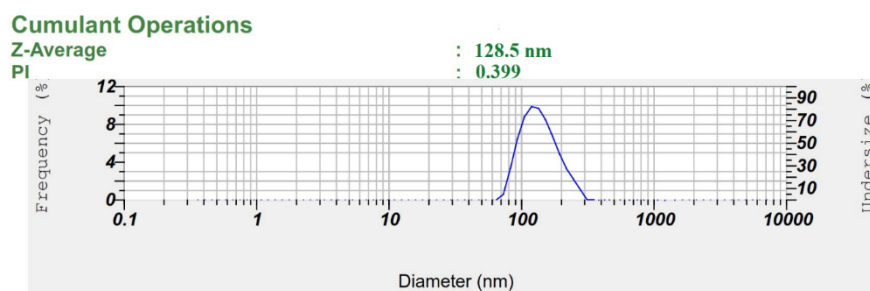
## 4. Results

### 4.1. Synthesis and Physical Properties of Chitosan Nanoparticles

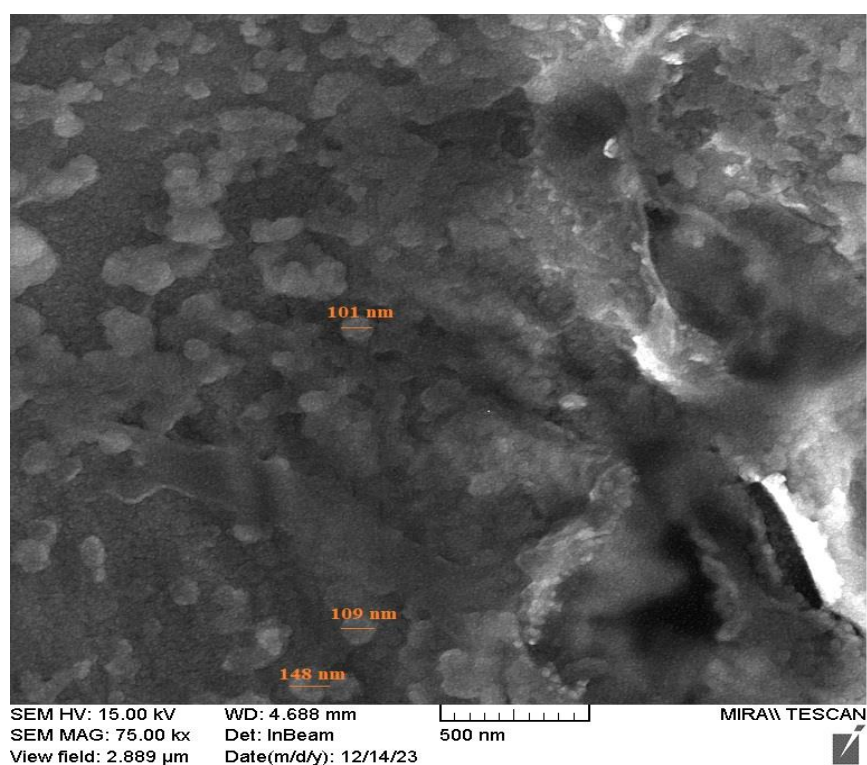
In this study, NChi were synthesized, and their physical properties were investigated (Table 1). The Dynamic Light Scattering (DLS) analysis determined the average hydrodynamic diameter of NChi to be 128.5 nm. The polydispersity index (PDI) was determined to be 0.399, indicating a relatively uniform size distribution of the synthesized nanoparticles (Figure 1).

**Table 1.** Size and Weight of the Synthesized Dendrimer G2 and Nano Chitosan (NChi)

Compounds	Size distribution (nm)	PI	Molecular weight (kDa)	Zeta potential (mV)
Nano chitosan (NChi)	128.5	0.39±0.12	42.7±6.3	2.7±3.04
Dendrimer G2	68	0.23±0.08	21.71±3.6	-3.6±2.5



**Figure 1.** DLS Showing the Size Distribution of Nano Chitosan based on Second-Generation Dendrimers. Polydispersity index (PI) is related to the distribution of the nanocomposite. The PI average was 0.399 (the PI value which is close to 0 shows the homogeneous nanoparticle solution while the PI value above 0.5 indicates the heterogeneous nanoparticle solution).



**Figure 2.** SEM Image of Nano Chitosan based on Second-Generation Dendrimers Showing the Surface Morphology and Particle Size Distribution (SEM image of nano chitosan based on second-generation dendrimers at 75kx magnification).

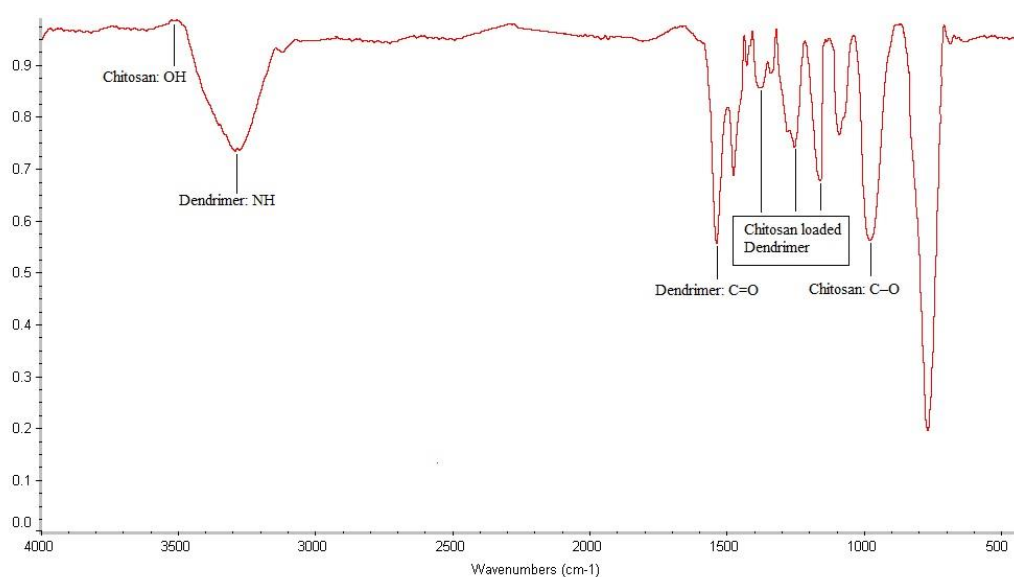
#### 4.2. Scanning Electron Microscopy (SEM) Analysis

The scanning electron microscopy (SEM) results also confirmed the particle size, where most particles had sizes ranging from approximately 101 to 148 nanometers. (Figure 2). The SEM results corroborated the DLS measurements, confirming the stability and uniformity of the nanoparticles.

#### 4.3. FTIR

The FTIR results indicated that chitosan was successfully

attached to the dendrimer as expected. As shown in Figure 3, the peaks in the regions of 900 to 1400  $\text{cm}^{-1}$  indicate the combined groups, and the C=O stretching caused by the amide bond due to the attachment of chitosan to the dendrimer. The functional groups NH and C=O related to the dendrimer were observed in the ranges of 1500–1700  $\text{cm}^{-1}$  and 3200–3500  $\text{cm}^{-1}$ , respectively. The peaks in the regions of 3500–3700  $\text{cm}^{-1}$  and 800–1000  $\text{cm}^{-1}$  correspond to OH and C-O of chitosan, respectively, and appear only when chitosan is present in the composition.



**Figure 3.** FTIR spectrum of nano chitosan based on G2 Dendrimers (highlighting characteristic peaks for amide bonds [C=O stretching] and functional groups [NH and OH]).

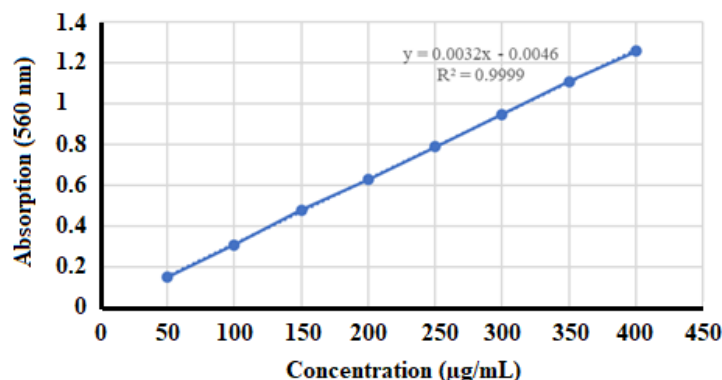


Figure 4. Standard Curve for Chitosan.

#### 4.4. Amount of Loaded Drug

To determine the amount of chitosan loaded onto the dendrimer, a standard chitosan curve was used. The absorbance of a 400 µg/ml concentration of the synthesized NChi in acetic acid at a wavelength of 560 nm was measured to be 0.533. Using a standard calibration curve ( $y = 0.0032x - 0.0046$ ), the chitosan loading efficiency was calculated to be 42% of the total nanocomposite mass (Figure 4).

#### 4.5. Solubility in Water

Free chitosan exhibited low solubility in water, with a large portion precipitating upon addition to water. To confirm this, the OD values of both free chitosan and NChi were compared. Spectrophotometric analysis demonstrated a 54% increase in chitosan solubility upon conjugation with the dendrimer, indicating improved aqueous dispersibility.

#### 4.6. Drug Release

The present study on chitosan release from the nano dendrimer over a period of 3 to 48 hours showed that the release of chitosan began at a moderate rate in the early hours and continued toward full release (100%). The release amount was approximately 5% in the first 3 hours

and reached 100% after 48 hours, indicating a controlled release pattern (Figure 5).

#### 4.7. Cytotoxicity Activity

The MTT assay evaluated the cytotoxicity of NChi on J774A.1 macrophage cells (Figure 6). The results indicated an 11% reduction in toxicity compared to free chitosan, with no significant cytotoxic effects at the tested concentrations.

#### 4.8. Anti-Promastigote Activity of Chitosan Nanoparticles

In the evaluation of the anti-promastigote effects of NChi and chitosan at different concentrations, the following results were observed:

At concentrations of 5, 10, 20, and 40 µg/ml, NChi after 24 h showed anti-promastigote effects of 12%, 37%, 58%, and 86% reduction in the number of promastigotes, respectively. Chitosan, at the same concentrations, exhibited lower anti-promastigote effects compared to NChi with only a 67% inhibitory effect on *L. major* promastigotes at 40 µg/ml (Figure 7). In the positive control group, the glucantime-treated group showed an 87% reduction in the number of promastigotes, whereas in the negative control group (PBS), no significant change in the parasite count was observed ( $P > 0.05$ ).

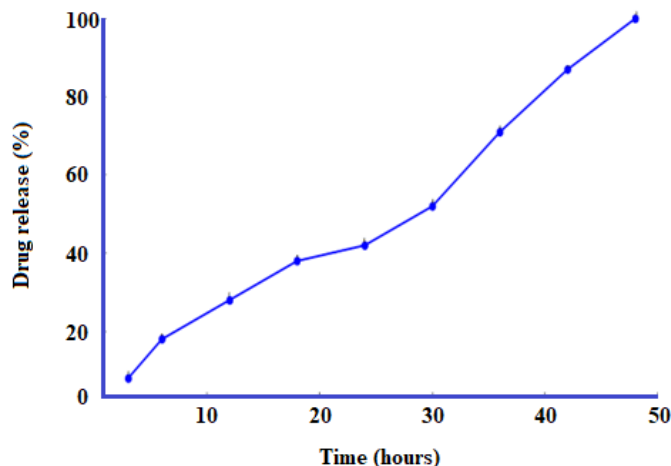


Figure 5. The Cumulative Release Curve of Nano Chitosan from Dendrimer G2 Shows that the Drug Release Pattern from Nanoparticles Follows a Slow-Release Pattern, as Indicated by the Graph.

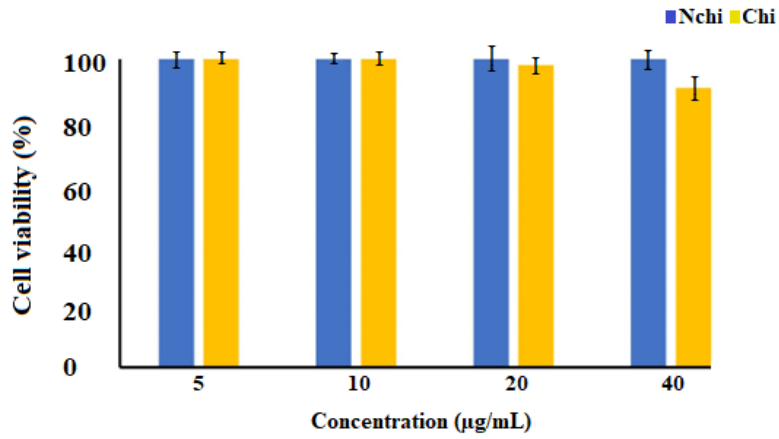


Figure 6. Percentage of Survival of Macrophages Cells Exposed to Synthesized Nano Chitosan Compared to Chitosan Within 48 h.

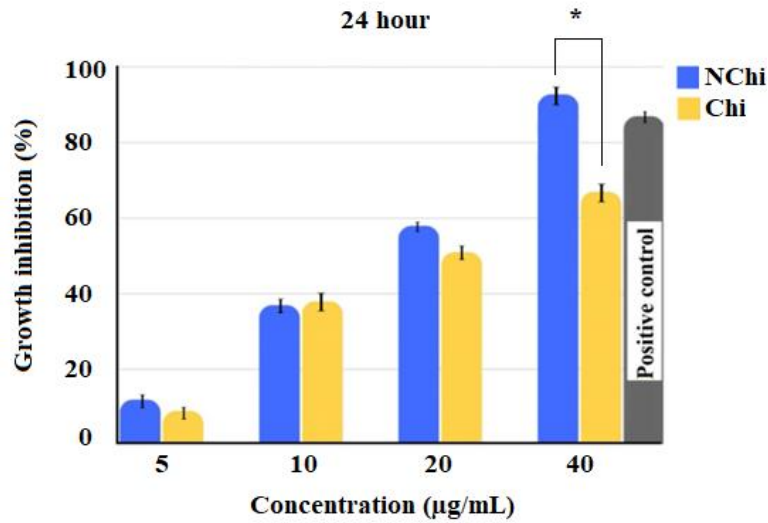


Figure 7. Percentage Inhibition of Nano Chitosan based on Second-Generation Dendrimers Growth after 24 Hours. \* Indicates a significant difference between the two groups ( $P < 0.05$ ).

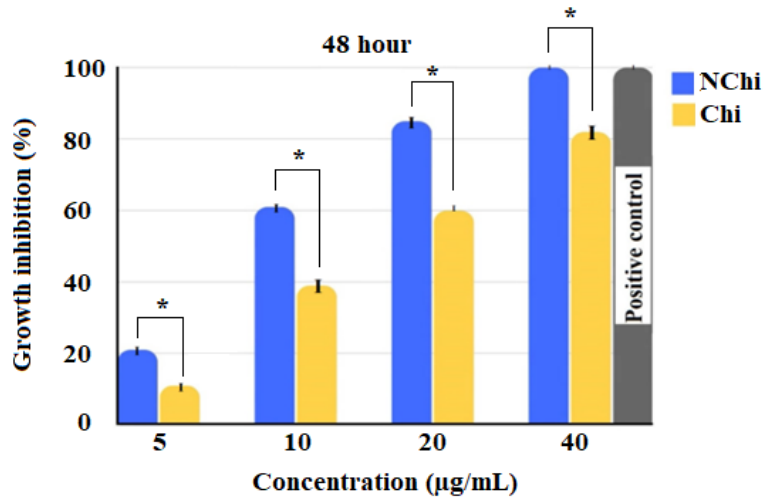


Figure 8. Percentage Inhibition of Nano Chitosan based on Second-Generation Dendrimers Growth after 48 Hours. \* Indicates a significant difference between the two groups ( $P < 0.05$ ).

The anti-promastigote effects of NChi and chitosan increased over 48 hours, with NChi showing anti-promastigote effects of 21%, 61%, 85%, and 100% at concentrations of 5, 10, 20, and 40 µg/ml, respectively.

Chitosan, at 40 µg/ml, was able to inhibit 82% of promastigote growth in the culture medium (Figure 8).

Statistical analysis (ANOVA,  $P < 0.05$ ) confirmed the superior efficacy of NChi over chitosan in inhibiting

promastigote proliferation, with a dose-dependent increase in activity over 24 and 48 hours.

## 5. Discussion

In this study, NChi were synthesized, and their physical characteristics and promastigote inhibitory effects were evaluated. The results indicated that NChi, with an average size of 128.5 nm and a particle size distribution index (PI) of 0.399, exhibited satisfactory stability and homogeneity. SEM images confirmed these sizes, which were in good agreement with DLS measurements. Particle size is particularly critical in biological and pharmaceutical applications, as smaller nanoparticles tend to enhance bioavailability and reduce toxicity compared to larger particles. These properties position NChi as a promising drug delivery system for treating parasitic diseases.

A comparison of this study with previous research on chitosan-nanoparticles and their derivatives for treating parasitic diseases like leishmaniasis reveals notable similarities in particle size and efficacy, alongside some differences. Typically, chitosan nanoparticles designed to target *Leishmania* are synthesized within the 50 to 200 nm range, indicating a broad size range for effective application. For example, previous studies have reported that chitosan nanoparticles with an average size of 94 nm were effective in treating CL,<sup>25</sup> which aligns well with the 128.5 nm size observed in this study, supporting the efficacy of nanoparticles in this size range for leishmaniasis treatment. Similarly, a study demonstrated that chitosan nanoparticles with an average size of 102 nm successfully inhibited *Leishmania* growth, further supporting the notion that nanoparticles within this size range can exhibit strong antileishmanial effects.<sup>20</sup>

The 128.5 nm size of NChi in this study falls within the optimal range for efficient penetration and interaction with promastigote cells. In contrast, the study by Bahraminegad et al.<sup>21</sup> reported that chitosan-nanofibers containing amphotericin B, with a size of approximately 431 nm, exhibited reduced particle stability and a higher tendency to aggregate in biological systems. This comparison underscores the advantage of NChi smaller size (128.5 nm), which not only enhances stability but also facilitates more effective cellular penetration.

The anti-promastigote results at various concentrations demonstrated that NChi effectively inhibited the growth of *L. major* promastigotes compared to free chitosan. At a concentration of 40 µg/ml, NChi achieved an 86% reduction in promastigote numbers, which was significantly higher than the 67% reduction observed with free chitosan ( $P < 0.05$ ), indicating the superior efficacy of NChi.

The findings of this study are consistent with previous research demonstrating the effectiveness of NChi in inhibiting promastigote growth. For instance, Riezk et al.<sup>26</sup> reported that chitosan nanoparticles inhibited promastigote

growth at comparable concentrations, although their efficacy was lower than that of chemical drugs like glucantime. These observations suggest that both chitosan and NChi have potential as complementary or alternative treatments for leishmaniasis.

A key finding of this study is the comparison between NChi and glucantime, a standard chemical drug. In the positive control group treated with glucantime, an 87% reduction in the number of promastigotes was observed. Similarly, NChi at a concentration of 40 µg/ml achieved an 86% reduction. These results suggest that the newly formulated NChi is nearly as effective as glucantime, highlighting their potential as a promising alternative treatment for leishmaniasis in the future.

When compared with previous studies on nanoparticles for leishmaniasis treatment, the findings of this study are consistent with earlier results.<sup>27</sup> For example, it was demonstrated that Ag-NPs significantly affected *L. major* promastigotes and amastigotes, inducing apoptosis in promastigotes with an IC<sub>50</sub> value of 2.35 ppm after 72 hours. Additionally, research on nanoparticles such as curcumin, gold, and silver has shown their notable antileishmanial properties. However, nano chitosan stands out due to its distinct advantages, including higher biocompatibility and lower production costs. Moreover, in this study, the anti-promastigote effects of NChi increased over 48 hours, achieving complete inhibition (100%) at a concentration of 40 µg/ml. This progressive enhancement in efficacy highlights the potential of NChi for long-term therapeutic applications.

Previous studies have suggested that chitosan nanoparticles induce cell death in *Leishmania* promastigotes via several mechanisms, including disruption of the cell membrane, generation of reactive oxygen species (ROS), and interference with the parasite's metabolic pathways, leading to apoptosis.<sup>28</sup> Therefore, one of the reasons for the inhibition of parasite growth in the present study could be this factor, which requires further investigation.

## 6. Conclusion

The findings of this study suggest that NChi, as a drug delivery system, exhibits greater efficacy than chitosan alone in the treatment of leishmaniasis. Its advantageous properties, including optimal size, stability, and significant anti-promastigote effects, especially when compared to chemical drugs like glucantime, underscore its potential as a promising candidate for the development of novel treatments for parasitic diseases. Further investigations into its precise mechanisms of action, along with clinical trials in animal and human models, are crucial to validate these outcomes and advance the development of nanoparticle-based therapeutic approaches.

## Acknowledgments

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## Research Highlights

**What Is Already Known?**

*Leishmania major* is one of the main causative agents of cutaneous leishmaniasis, which is particularly prevalent in tropical and subtropical regions. Common treatments include antimonial compounds, amphotericin B, and other drugs, which are often associated with side effects.

**What Does This Study Add?**

The results of this study showed that dendrimers are reliable drug delivery systems for pharmaceutical applications. Additionally, considering the antileishmanial properties of the chitosan-dendrimer combination, this nanostructure could also be tested as an antiparasitic candidate for other parasitic infections.

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**Author Contributions**

TE and MTG: Supervision, project administration, conceptualization, methodology, data curation, formal analysis, writing—review, and editing. HF and AKH: Methodology, resources, investigation, data analysis, writing—review, and editing.

**Conflict of Interest Disclosures**

All authors declared that they have no conflict of interest.

**Ethical Approval**

The study protocol was reviewed and approved by the ethics committee of the Shahrood branch, Islamic Azad University (IR.IAU.SHAHROOD.REC.1402.010).

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