

Evaluation of the Inhibitory Effect of Nano-Chitosan Loaded with *Allium ursinum* Extract on *Leishmania major* under *In Vitro* Conditions

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Abstract

Background: *Leishmania major* is one of the main causative agents of cutaneous leishmaniasis in many world regions, including Iran. Common treatments for this disease include pentavalent antimonial compounds, amphotericin B, and paromomycin-based formulations. However, these treatments face challenges such as drug resistance, high toxicity, and high costs. Consequently, there is a growing need for alternative therapeutic approaches.

Objectives: The present study evaluates the inhibitory effect of nano-chitosan loaded with *Allium ursinum* (*A. ursinum*) extract against *L. major* under *in vitro* conditions.

Methods: Initially, chitosan/extract nanoparticles (NChi/Ex) were synthesized by loading *A. ursinum* extract onto chitosan, and their physicochemical properties were analyzed using Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), and field emission scanning electron microscopy (FESEM). Subsequently, *Leishmania major* parasites were cultured in RPMI 1640 medium, and the effect of NChi/Ex on the parasites was assessed using the MTT assay.

Results: The results showed that the average size of the synthesized nanoparticles was 145.3 nm, with a polydispersity index (PI) of 0.277, indicating a relatively uniform particle distribution. The FTIR spectrum confirmed the successful loading of *A. ursinum* extract onto chitosan, as characteristic peaks corresponding to the functional groups of both components were observed. The NChi/Ex nanoparticles exhibited a dose-dependent inhibitory effect, with the highest inhibition occurring at a concentration of 50 µg/ml after 48 hours, achieving 100% inhibition of *Leishmania major*.

Conclusion: The results demonstrated that NChi/Ex exhibited greater efficacy than chitosan or *A. ursinum* extract alone. This study underscores the potential of chitosan nanoparticles loaded with *A. ursinum* extract as a promising therapeutic strategy against *Leishmania major*, with potential applications in laboratory research and possibly in animal models.

Keywords: Nanostructure, Extract, Leishmaniasis, Protozoa

1. Background

Leishmaniasis is a widespread zoonotic parasitic disease transmitted to humans by biting sandflies from the *Phlebotomus* and *Lutzomyia* genera.¹ This disease presents in three clinical forms: cutaneous, mucocutaneous, and visceral, with cutaneous leishmaniasis being the most common.² Globally, an estimated 600,000 to 1 million new cases are reported annually.³ A large population is at risk of contracting leishmaniasis, particularly in regions of Asia, Africa, and the Americas.

Current treatments for leishmaniasis primarily involve pentavalent antimonial compounds, which are associated with severe side effects, including liver and kidney disorders. Furthermore, the emergence of parasite resistance has intensified the need for novel therapeutic approaches.⁴

The use of herbal medicines has gained considerable attention due to their lower toxicity, affordability, and accessibility. Plants from the *Allium* family, particularly garlic and onion, possess medicinal and anti-leishmanial properties, making them promising therapeutic agents.⁵ *Allium ursinum* (*A. ursinum*) is a medicinal and edible plant with various health benefits. Its leaves are rich in vitamin C and potent antioxidants, which contribute to immune system enhancement. Moreover, *A. ursinum* exhibits anti-inflammatory properties that may help reduce inflammation. Due to its antibacterial and antiparasitic effects, it holds potential for combating infections. However, to enhance its efficacy, nano technology-based drug delivery systems can be employed to improve bioavailability and targeted therapeutic action.^{6,7}

Nanoparticles, particularly chitosan-based formulations, are emerging as novel therapeutic agents in medicine. Chitosan, a natural polysaccharide with antiparasitic and antifungal properties, holds great potential for improving drug delivery and therapeutic strategies. Additionally, it can enhance immune system function and serve as an effective agent in tissue regeneration and wound healing.^{8,9} The chemical structure of chitosan includes amino groups at the C2 carbon, formed after the deacetylation of chitin.

2. Objectives

Considering the therapeutic properties of both chitosan and *A. ursinum*, loading chitosan nanoparticles with *A. ursinum* extract could offer a novel and effective strategy for reducing side effects and enhancing therapeutic efficacy against leishmaniasis. These approaches are not only cost-effective and widely accessible but may also help strengthen the patient's immune system. Furthermore, the development of innovative treatments could alleviate the economic and social burden of leishmaniasis in endemic regions. Ultimately, this research aims to contribute to public health improvements and reduce the incidence of new leishmaniasis cases. Therefore, this study investigates the effect of chitosan nanoparticles loaded with *A. ursinum* extract (NChi/Ex) on *Leishmania major* under laboratory conditions.

3. Methods

3.1. Plant Collection and Extraction

The *A. ursinum* plant was collected from the highlands of Alborz province and dried in a dark place for three days. Due to the high humidity in the environment, complete drying was achieved using a drying oven equipped with a fan at temperatures ranging from 30 to 40 °C. After drying, the plant material was crushed, and extraction was performed using a methanol-to-water ratio of 4:1. The mixture was stirred for 30 minutes using an electric shaker and then incubated in a dark environment for 48 hours. The resulting extract was filtered through filter paper, and the solvent was evaporated using a rotary evaporator.⁶

3.2. Gas Chromatography-Mass Spectrometry Analysis

Gas chromatography-mass spectrometry (GC-MS) was employed to identify the active compounds with the highest concentrations in the *A. ursinum* plant composition.

3.3. Preparation of Chitosan/Extract Nanoparticles

A total of 100 mg of chitosan was added to 20 ml of 1% acetic acid. Then, 100 mg of *A. ursinum* extract was added to the solution. The pH of the solution was adjusted to 5.8, and the mixture was stirred at 200 rpm using a magnetic stirrer for 24 hours. Subsequently, tripolyphosphate (TPP) at a concentration of 2 mg/ml was added dropwise to the mixture, and stirring continued for

an additional 6 hours. Finally, the formed nanoparticles were separated by centrifugation at 10,000 rpm for 15 minutes. Different concentrations (50, 25, 12.5, and 6.25 µg/ml) of the resulting nanoparticles were prepared and stored at 5 °C in a refrigerator for future use.¹⁰

3.4. Confirmation and Characterization of Synthesized Nanostructures

Fourier Transform Infrared Spectroscopy (FTIR) was employed to confirm the synthesis of the nanostructures. The structural characteristics of the nanoparticles, including size and dispersity index, were evaluated using Dynamic Light Scattering (DLS).

3.5. Culturing Leishmania Parasites

RPMI 1640 medium was supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin. *Leishmania major* parasites (MRHO/IR/75/ER), obtained from the Pasteur Institute of Tehran, were transferred into the prepared culture medium for bulk cultivation. The culture was incubated at 24 °C with 5% CO₂.¹¹

3.6. Evaluation of Anti-leishmanial Effect of the Synthesized Nanocomposite

Leishmania major promastigotes (2×10⁶), cultured in the medium, were transferred to a 96-well microplate. Then, 100 µl of 50 µg/ml NChi/Ex was added to the first well. Serial dilutions of the synthesized nano composite were added from the second to the eighth wells. Wells nine to twelve served as controls and received 50 µg/ml chitosan, 100 µg/ml plant extract, 50 µg/ml glucantime (positive control), and PBS (negative control), respectively. After incubation at 24 °C with 5% CO₂ for 24 and 48 hours, 20 µl of MTT solution was added to each well (The plate was shaken to dissolve Formazan crystals). Following incubation, the supernatant was removed, and DMSO was added. Absorbance was measured at 570 nm using a microplate reader (BIOTEK ELX800TS, USA).

3.7. Statistical Analysis

The data obtained were entered into SPSS software version 22, and the Kolmogorov-Smirnov test was used to assess the normality of the data distribution. After normalizing the data, ANOVA and the Tukey post-hoc test were applied. A *P*-value of <0.05 was considered statistically significant. Non-linear regression was employed to calculate the IC₅₀ value.

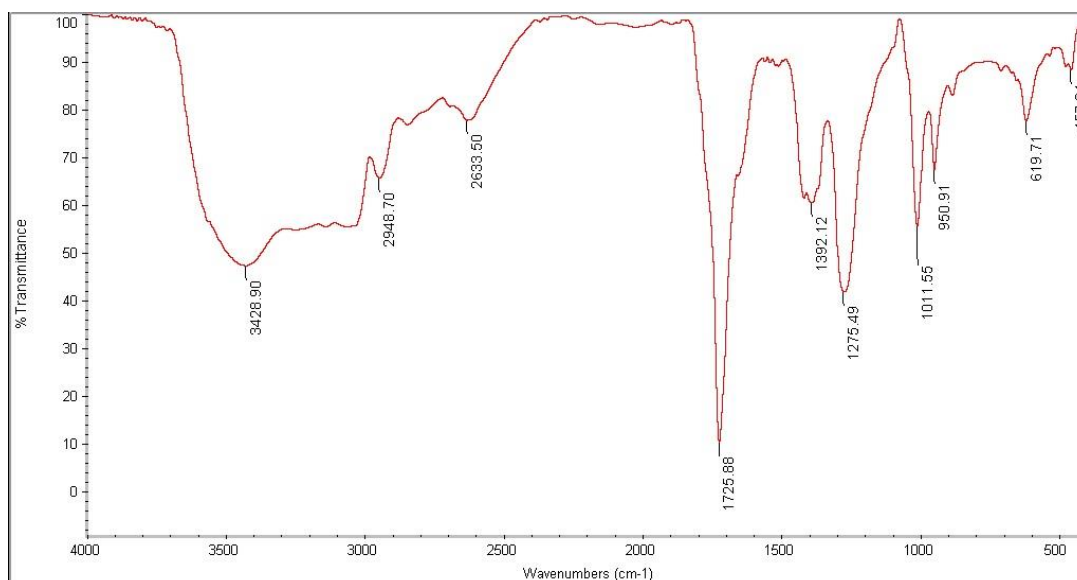
4. Results

4.1. Gas Chromatography-Mass Spectrometry Finding

GC-MS analysis was performed to identify the volatile compounds present in the *A. ursinum* extract. The results revealed that the primary compounds in the sample were primarily allyl sulfur compounds, which are responsible

Table 1. The Most Phytochemical Constituents Identified in the Hydro-alcoholic Extract of *Allium ursinum* Using Gas Chromatography-Mass Spectrometry (in % of total ion current)

| No. | Compounds | % Of total |
|-----|-------------------------------------|------------|
| 1 | Diallyl disulphide | 7.1 |
| 2 | Dimethyl tetrasulphide | 0.2 |
| 3 | Methyl-2-propenyl disulfide | 3.2 |
| 4 | Propenyl propyl disulfide | 0.9 |
| 5 | Allicin | 3.2 |
| 6 | 2-hexenal | 0.2 |
| 7 | Propylene sulfide | 1.4 |
| 8 | Decanal | 2.3 |
| 9 | Heptacosane | 1.1 |
| 10 | Tricosane | 0.5 |
| 11 | Furfural | 0.2 |
| 12 | Hexacosane | 0.8 |
| 13 | Benzaldehyde | 3.3 |
| 14 | Diallyl trisulphide | 5.1 |
| 15 | Ethene,1-(ethylthio)-2-(methylthio) | 0.1 |
| 16 | Cysteine sulfoxides | 0.6 |
| 17 | Isoalliin | 0.8 |
| 18 | Di-2-Propyl trisulfide | 3.9 |
| 19 | Vinyl-4H-1,3-dithiine | 1.2 |
| 20 | Di-2-Propenyl trisulfide | 2.7 |

**Figure 1.** FTIR Spectrum of Synthesized Nano Chitosan/Extract.

for the biological properties and activities of the extract. The most abundant compounds identified were Diallyl disulfide (7.1%), Diallyl trisulfide (5.1%), Di-2-Propyl trisulfide (3.9%), and Allicin (3.2%), all of which are significant sulfur-containing compounds with notable antioxidant, anti-inflammatory, antimicrobial, and medicinal properties (Table 1).

4.2. Findings Obtained from Fourier Transform Infrared Spectroscopy

FTIR spectra were used to examine the changes in functional groups after the *A. ursinum* extract was bound to chitosan. The results revealed distinct changes in the regions corresponding to the main functional groups, indicating an interaction between the extract components

and the chitosan structure. In the NChi/Ex spectrum, a broad peak at 3428 cm^{-1} was observed, corresponding to the stretching vibration of hydroxyl and amine groups, which indicates the formation of hydrogen bonds between the phenolic compounds in the extract and the functional groups of chitosan (Figure 1). A distinct band at 1725 cm^{-1} , associated with type I amide in chitosan, suggests a possible interaction between the organic compounds of the extract and the amide groups of chitosan.

4.3. Dynamic Light Scattering Analysis

Particle size analysis using DLS was performed to assess the particle size distribution. The results from the DLS spectrum revealed that the particles were distributed in the range of 90 to 200 nm. The average particle size (Z-

average) was found to be 145.3 nm, representing the predominant particle size in the suspension. The Polydispersity Index (PI) was 0.277, indicating a relatively uniform particle size distribution and suggesting a narrow

size distribution within the system. The particle size distribution graph showed that the maximum frequency on the y-axis was 22.5%, indicating the highest frequency of particles near the average size (Figure 2).

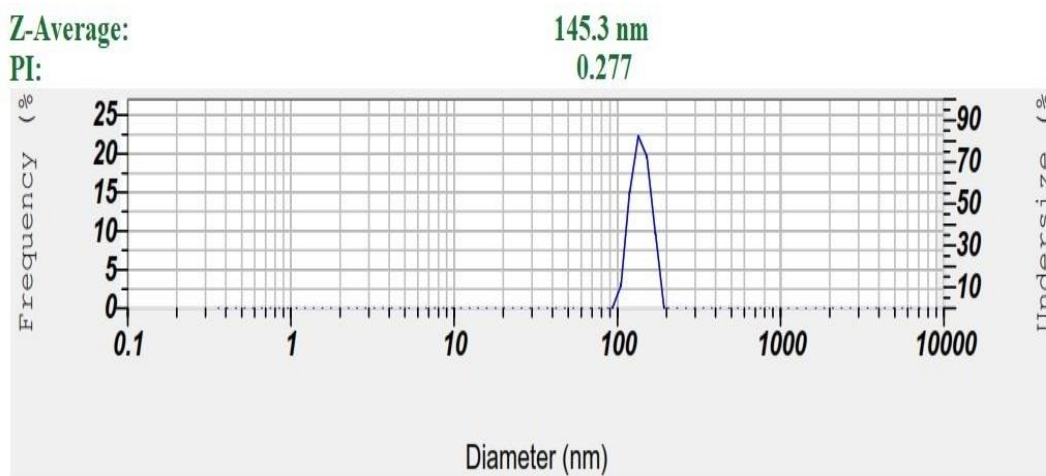


Figure 2. DLS Graph of Synthesized Nano Chitosan/Extract (Average particle size and Polydispersity Index [PI]).

4.4. Anti-leishmanial Effect of Chitosan/Extract Nanoparticles

The results revealed that NChi/Ex at concentrations of 50, 25, and 0.7 $\mu\text{g/ml}$ exhibited inhibitory effects on *Leishmania* growth after 24 hours, with inhibition rates of 96.33%, 66.33%, and 0%, respectively. After 48 hours, the inhibition rates were 100%, 98.33%, and 5.3%, respectively. In comparison, empty chitosan at 50 $\mu\text{g/ml}$ showed 76.66% inhibition after 24 hours and 83.66%

inhibition after 48 hours. The plant extract alone at 100 $\mu\text{g/ml}$ showed a 48.66% inhibitory effect after 48 hours. These results indicate that the combined formulation is more effective than the individual components, with the differences being statistically significant ($P < 0.05$) (Table 2). Non-linear regression analysis showed that the synthesized nanocomposite at concentrations of 13.26 and 3.03 $\mu\text{g/ml}$ could prevent 50% of promastigote growth in culture after 24 and 48 hours, respectively (Figure 3).

Table 2. The Effect of Synthesized Nano Chitosan/Extract on *Leishmania major* Promastigotes *In vitro*

| Groups | Concentration ($\mu\text{g/ml}$) | Growth inhibition % (24 hours) | Growth inhibition % (48 hours) | P-value |
|---------------------------------|------------------------------------|--------------------------------|--------------------------------|---------------------|
| Nano Chitosan/Extract (NChi/Ex) | 50 | 96.33 \pm 2.08 | 100 | 0.035 ^a |
| | 25 | 66.33 \pm 4.72 | 98.33 \pm 1.52 | |
| | 12.5 | 49.33 \pm 3.21 | 83.66 \pm 3.78 | |
| | 6.25 | 23.66 \pm 3.78 | 66.33 \pm 1.52 | |
| | 3.12 | 15.33 \pm 1.52 | 52.66 \pm 2.51 | |
| | 1.56 | 2.33 \pm 2.08 | 36.33 \pm 4.50 | |
| | 0.78 | 0 | 5.3 \pm 2.64 | |
| | 0.39 | 0 | 2.66 \pm 1.52 | |
| Chitosan | 50 | 76.66 \pm 4.72 | 83.66 \pm 5.13 | <0.001 ^b |
| Extract | 100 | 44.66 \pm 3.05 | 48.66 \pm 2.08 | <0.001 ^c |
| Positive Control | 100 | 97.66 \pm 2.08 | 100 | 0.601 ^d |
| Negative Control | - | 0 | 0 | |

a: Comparison of the effect of the NChi/Ex at 24 hours and 48 hours; b: Comparison of the synthesized NChi/Ex with chitosan alone; c: Comparison of the synthesized NChi/Ex with the extract alone; d: Comparison of the synthesized NChi/Ex with the positive control (Glucantime); (The $P < 0.05$ indicates a significant difference between the two groups).

5. Discussion

GC-MS analysis identified the volatile compounds present in the *A. ursinum* extract and revealed that allyl sulfur compounds such as Diallyl disulfide, Diallyl trisulfide, Di-2-Propyl trisulfide, and Allicin were the predominant compounds. These compounds possess valuable biological properties, including antioxidant, anti-inflammatory, and antimicrobial activities. Similar

sulfur-containing compounds have been reported in other species of garlic and *A. ursinum* in previous studies. For instance, Ashraf et al. (2019) reported high levels of Diallyl disulfide and Diallyl trisulfide in *Allium sativum*, which were associated with similar biological activities. These findings suggest that allyl sulfur compounds play an important role in the medicinal properties of these plants.¹²

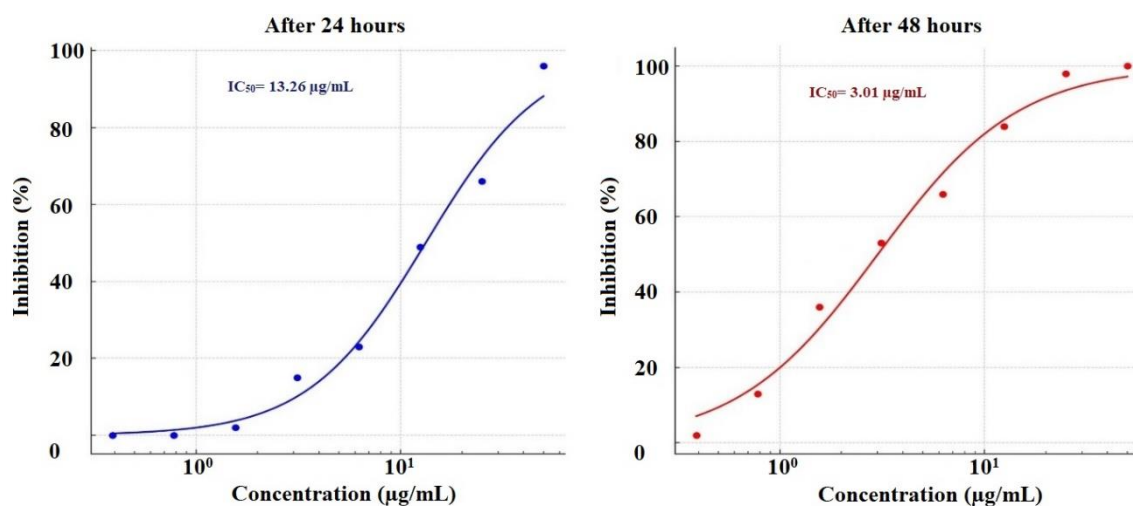


Figure 3. Calculation of IC₅₀ of the Synthesized Nano Chitosan/Extract Using Nonlinear Regression.

The FTIR spectroscopy results indicated interactions between the *A. ursinum* extract and chitosan. Changes in functional groups suggested that hydrogen bonds were formed between the phenolic compounds in the extract and the functional groups of chitosan. Additionally, the presence of the 1725 cm⁻¹ band, which is associated with amide type I, indicated the interaction between the organic compounds in the extract and the chitosan structure. Previous studies on chitosan compounds have shown that this material can form hydrogen bonds with polyphenols and other bioactive compounds.¹³⁻¹⁵ These results suggest that chitosan could serve as an effective carrier for the bioactive compounds in *A. ursinum*.

Various studies have reported chitosan nanoparticles in the approximate range of 100 to 300 nanometers,^{16,17} which aligns with the results of this study. The appropriate size of nanoparticles directly affects their stability and bioavailability, and the DLS results in this study indicate favorable characteristics for biological applications. Several studies have explored the anti-leishmanial effects of different compounds, including biopolymers and plant extracts. Compared to previous studies, the results of this investigation show that the NChi/Ex exhibits a greater inhibitory effect than the individual components. The IC₅₀ of the NChi/Ex was calculated as 13.26 µg/ml after 24 hours and 3.01 µg/ml after 48 hours.

In the study by Haddad et al., the effect of chitosan/curcumin nanoparticles on the growth of *Leishmania major* was investigated. The results showed that the IC₅₀ of the nanoparticles against the promastigote stage of *Leishmania major* was 3.13 µg/ml, which is consistent with the findings of the present study.¹⁸ This suggests that chitosan, as a biological carrier, has its own anti-leishmanial activity and, when combined with other plant or chemical bioactive compounds, can demonstrate enhanced effects.

In the current study, chitosan alone at a concentration

of 50 µg/ml inhibited only 83.66% of parasite growth in culture. However, when combined with *A. ursinum* extract, the new formulation exhibited a 100% inhibitory effect, similar to that of glucantime. This difference was statistically significant ($P < 0.001$).

Studies investigating the effects of plant extracts on *Leishmania* spp. have shown that different extracts typically exhibit inhibitory effects ranging from 10% to 60%. For example, in the 2019 study by Hesami et al., the *Pistacia atlantica* extract induced apoptosis in 10% of *Leishmania* promastigotes.¹⁹ Moreover, a study by Bagherin et al. found that a 4.6 µg/ml concentration of garlic extract could inhibit 50% of parasite growth in culture,²⁰ which is close to the results of the present study. This is because garlic and *A. ursinum* belong to the same plant family and contain sulfur compounds like allicin, sulfides, and thiosulfates, which have antimicrobial, anticancer, and antioxidant effects.²¹⁻²³

This comparison highlights that the combination of chitosan with plant extracts is an effective strategy for enhancing anti-leishmanial activity and could serve as a potential therapeutic option for further investigations.

6. Conclusion

The study showed that the chitosan nanoparticle formulation loaded with *A. ursinum* extract exhibited a stronger inhibitory effect on the growth of *Leishmania major* compared to the individual components (chitosan or plant extract alone). This suggests a synergistic interaction between chitosan and *A. ursinum* extract. Additionally, the inhibitory effect of this formulation increased over a longer period (48 hours), leading to complete inhibition of parasite growth at higher concentrations. These findings indicate that chitosan nanoparticles, as a drug delivery system, can enhance the bioavailability and stability of the active compounds from *A. ursinum* extract, thereby improving its efficacy against *L. major*.

Research Highlights

What Is Already Known?

The treatment challenges of leishmaniasis include drug resistance, side effects, high cost, long treatment duration, and limitations in alternative therapies.

What Does This Study Add?

This study provided new insights into the anti-leishmanial effects of nano-chitosan loaded with *A. ursinum* extract in *in vitro* conditions. It showed the synergistic effects of nanotechnology and herbal medicine as an alternative treatment for *Leishmania major*, aiming to improve efficacy, reduce toxicity, and enhance drug delivery.

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Author Contributions

Authors contributed equally to this work.

Conflict of Interest Disclosures

All authors declared that they have no conflict of interest.

Ethical Approval

Ethics approval for this study was obtained from the Ethics Committee of Islamic Azad University, Babol, with the approval code IR.IAU.BABOL.REC.1403.094.

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