

Evaluation of excretory–secretory products of *Lucilia sericata* larvae loaded onto chitosan nanoparticles as a drug candidate against *Leishmania major*

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ABSTRACT

Objective(s): Zoonotic cutaneous leishmaniasis is an infectious disease caused by the protozoan parasite *Leishmania major* and characterized by chronic skin lesions. Treatment is often prolonged and associated with adverse effects. Chitosan, due to its favorable biomedical properties, including biocompatibility, biodegradability, non-toxicity, and wound-healing potential, has attracted considerable attention for therapeutic applications. This study aimed to evaluate the anti-leishmanial effects of excretory–secretory (ES) products of *Lucilia sericata* larvae loaded onto chitosan nanoparticles against *L. major* cells.

Materials and Methods: The ES was collected, filter-sterilized, and loaded onto chitosan nanoparticles (CN). The ES-loaded CN was applied at different concentrations under both *in vitro* and *in vivo* conditions to leishmanial cells, and its cytotoxicity was compared with that of the standalone CN, crude ES, and Glucantime as the first-line drug against cutaneous leishmaniasis caused by *L. major*.

Results: ES-loaded chitosan nanoparticles were more effective in healing leishmanial lesions than the crude ES and chitosan nanoparticles applied separately. The ES-loaded CN exhibited stronger inhibitory effects against both intracellular and extracellular forms of the parasite than its components did when used alone.

Conclusion: The results indicated that ES-loaded chitosan nanoparticles exerted antileishmanial and immunomodulatory effects on lesions. The formulation may therefore represent a promising alternative remedy for treating leishmanial lesions. Due to their biocompatibility, antimicrobial activity, and controlled-release capability, ES-loaded CN showed effective performance in wound healing and treatment.

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Introduction

Cutaneous leishmaniasis is considered one of the major public health challenges in tropical and subtropical regions, including Iran (1-3). The disease is caused by *Leishmania major* in rural areas and manifests as chronic cutaneous lesions often associated with psychosocial complications and permanent scarring (4, 5). Conventional treatments using pentavalent antimonial compounds, amphotericin B, and miltefosine face multiple limitations, including drug resistance, systemic toxicity, painful administration routes, and high treatment costs (6-9). These challenges highlight the urgent need to develop novel therapeutic strategies with improved efficacy and safety. In recent years, maggot therapy using *Lucilia sericata* larvae has been introduced as an effective biological treatment for chronic wounds (10-12). The therapeutic properties of these larvae are attributed to their excretory–secretory (ES) products, which contain various bioactive molecules such as lucifensin, antimicrobial peptides, proteolytic enzymes, and allantoin (13-15). These compounds exhibit antibacterial,

debridement, and wound-healing properties (16, 17). However, the clinical application of ES products is limited by challenges related to stability and targeted delivery. Nanotechnology has provided innovative solutions for drug delivery through the development of advanced carrier systems (18, 19). Chitosan nanoparticles (CN), derived from the natural polysaccharide chitin, have gained significant attention due to their excellent biocompatibility, biodegradability, mucoadhesive properties, and intrinsic antimicrobial activity (20, 21). Their positive surface charge enables efficient encapsulation of bioactive compounds and facilitates controlled release and enhanced cellular uptake (22, 23). The combination of chitosan nanoparticles with therapeutic bioactive molecules represents a promising approach to improving treatment outcomes in infectious diseases (24, 25). Although previous studies have independently demonstrated the wound-healing potential of *L. sericata* ES products and chitosan nanoparticles, their combined application against parasitic infections has not been fully

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investigated. Therefore, the present study aimed to evaluate the antileishmanial effects of ES products of *L. sericata* larvae loaded onto chitosan nanoparticles against *L. major* cells under both *in vitro* and *in vivo* conditions.

Materials and Methods

Rearing of *Lucilia sericata* and preparation of larval excretion/secretion (ES)

L. sericata flies were reared under insectarium conditions (temperature: 28 °C; relative humidity: 50±10%; photoperiodicity: 12:12 L/D) at Tarbiat Modares University. Fresh chicken liver was used to feed the larvae, while adult flies were provided with sugar water supplemented with a mixture of powdered milk and honey.

ES products were collected from second- and third-instar larvae of *L. sericata* under sterile conditions. Approximately 150 larvae were selected from a laboratory colony and starved for 6 hr to allow complete evacuation of the digestive tract. The larvae were then surface sterilized using 0.5% sodium hypochlorite for 2 min, followed by 1% formaldehyde for 2 min, and finally washed twice with sterile saline solution for 1 min. After sterilization, the larvae were transferred into sterile 50 ml Falcon tubes containing phosphate-buffered saline (PBS). The tubes were covered with aluminum foil to prevent evaporation and incubated at 37 °C for 2 hr to allow the release of larval excretory-secretory products. Following incubation, the ES-containing supernatant was carefully collected using sterile pipettes, transferred to sterile microtubes, and filtered through a sterile syringe filter to remove debris. The collected ES products were stored at -20 °C until further use.

Collection of larval excretion/secretion products

Larvae were first surface-sterilized using 0.5% sodium hypochlorite (2 min), 1% formaldehyde (2 min), and sterile saline (twice, 1 min each). To collect *L. sericata* excretion/secretion (ES) products, second- and third-instar larvae were starved for 12 hr and then incubated in phosphate-buffered saline (PBS) for 2 hr at 37 °C. ES products were aseptically collected from batches of 100 larvae and stored in 1.5 ml microtubes. The collected supernatant was passed through a syringe filter (13, 26, 27). Protein concentration of the crude ES extract was determined using the Bradford assay with bovine serum albumin (BSA) as the standard, and samples were stored at -20 °C until use (28, 29).

The total protein concentration of the ES products was determined using the Bradford protein assay. A standard curve was prepared using serial dilutions of bovine serum albumin (BSA) at concentrations ranging from 0 to 500 µg/ml. ES samples were mixed with Bradford reagent in microplate wells and incubated for 5 min at 25 °C in the dark to allow color development. Absorbance was measured at 595 nm using a microplate reader. All samples were analyzed in triplicate, and the mean absorbance values were used to quantify protein. The protein concentration of the samples was calculated using the linear regression equation obtained from the BSA standard curve and expressed in µg/ml.

Synthesis and characterization of chitosan nanoparticles

Chitosan nanoparticles were prepared according to the ionic gelation method. The chitosan (1% w/v) was dissolved in 1% acetic acid, and 0.5% sodium tripolyphosphate (TPP) was then added dropwise under magnetic stirring for 1 hr at room temperature. Nanoparticles were purified by centrifugation (12,000 rpm, 15 min) and washed three times with distilled water. To prepare ES-loaded chitosan

nanoparticles, ES solution (2 mg/ml) was added during the gelation process (30, 31). Characterization was performed using dynamic light scattering (DLS) and Fourier-transform infrared (FTIR) spectroscopy (32).

Chitosan nanoparticles (CSNPs) were prepared using the ionic gelation method. Briefly, 1 g of high-molecular-weight chitosan was dissolved in 100 ml of distilled water under magnetic stirring. To facilitate dissolution, glacial acetic acid was added dropwise until the pH reached 4.5. Separately, a sodium tripolyphosphate (TPP) solution was prepared by dissolving 0.5 g of TPP in 100 ml of distilled water. To initiate nanoparticle formation, 50 ml of the TPP solution was added dropwise to 50 ml of the chitosan solution under vigorous magnetic stirring at room temperature. The mixture was stirred for 1 hr to ensure complete cross-linking. The resulting nanoparticle suspension was centrifuged at 12,000 ×g for 15 min, and the supernatant containing unreacted compounds was discarded. The pellet was washed three times with sterile distilled water, followed by centrifugation, to obtain purified plain CSNPs.

Preparation of ES-loaded chitosan nanoparticles (ES-CSNPs)

To prepare ES product-loaded chitosan nanoparticles (ES-CSNPs), 50 ml of the crude ES solution (2 mg/ml) was gently mixed with 50 ml of the chitosan solution under magnetic stirring. Subsequently, 50 ml of the TPP solution (0.5% w/v) was added dropwise to the mixture while stirring. The suspension was stirred for 1 hr until the encapsulation process was completed. The ES-CSNPs were collected by centrifugation at 12,000 ×g for 15 min, washed three times with sterile distilled water, and stored at 4 °C for further characterization and biological assays. All preparation steps were carried out under sterile conditions.

Physicochemical characterization

The physicochemical characteristics of the synthesized nanoparticles were determined using DLS and FTIR. Before analysis, the nanoparticle suspensions were subjected to ultrasonication for 30 min to ensure uniform dispersion. DLS analysis (Malvern Zetasizer) revealed that the plain CSNPs had a mean size of 150–200 nm, a polydispersity index (PDI) of <0.3, and a positive zeta potential of +30 to +35 mV, indicating good colloidal stability.

Parasite culture

L. major promastigotes were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 µg/ml) at 26 °C. The murine macrophage cell line J774A.1 was maintained in DMEM with 10% FBS at 37 °C in 5% CO₂. Both cultures were monitored regularly and passaged in the logarithmic phase of growth (33, 34).

In vitro assays

Promastigotes (1×10⁶ cells/ml) were treated with serial concentrations (0–500 µg/ml) of chitosan nanoparticles, ES-loaded nanoparticles, crude ES material, and Glucantime (positive control) in 96-well plates. Viability was assessed at 24, 48, and 72 hr using Neubauer slide counting (35, 36).

MTT cytotoxicity assay

Macrophages (1×10⁵ cells/well) were treated with different concentrations of the test compounds for 72 hr. MTT solution (5 mg/ml) was added, and the macrophages were then incubated for 4 hr. Formazan crystals were dissolved, and absorbance was measured at 570 nm. The

percentage of cell viability was calculated, and DMSO IC_{50} and CC_{50} values were determined from the dose-response curves. The selectivity index (SI) was calculated as the ratio CC_{50}/IC_{50} (37, 38).

In vivo study

Animal model: Twenty-five mice (BALB/c) were infected subcutaneously at the base of the tail with stationary-phase promastigotes (1×10^6). Lesion formation was monitored weekly until ulceration (approximately 5 weeks post-infection). The experimental groups were then divided as follows:

- Group 1: Glucantime (20 mg/kg, intraperitoneal)
- Group 2: Raw ES (350 μ g/ml, topical)
- Group 3: Chitosan nanoparticles (50 mg/kg, topical)
- Group 4: ES-loaded chitosan nanoparticles (50 mg/kg containing 350 μ g/ml ES, topical).
- Group 5: Negative control (no treatment)

Lesion size was measured weekly with digital calipers. At week 8 post-treatment, the mice were anesthetized, and spleen samples were collected. Parasite burden was determined by limiting dilution assay. Spleen homogenates were serially diluted in complete medium and monitored for promastigote growth for 10-14 days. Parasite burden was calculated as the negative logarithm of the number of parasites per gram of tissue (39, 40).

A total of 25 female BALB/c mice (4 weeks old) were obtained from the Royan Institute and used in the *in vivo* experiments. The animals were randomly assigned to five experimental groups ($n=5$ per group) and housed in separate cages under standard laboratory conditions (temperature 22 ± 2 °C, humidity $55 \pm 5\%$, and a 12 h light/dark cycle). Food and water were provided *ad libitum*. All experimental procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals and approved by the Animal Ethics Committee of Tarbiat Modares University (Ethical code: IR.MODARES.AEC.1403.051).

The progression of cutaneous lesions was monitored weekly throughout the experimental period. Lesion dimensions (length, width, and height) were measured using digital calipers, and the recorded measurements were used to evaluate the therapeutic response in different treatment groups. These measurements were performed systematically during the monitoring period to assess lesion progression and healing.

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using one-way ANOVA and Mann-Whitney tests in SPSS software (version 26). Results are presented as mean $\pm$ standard deviation, and $P < 0.05$ was considered the level of significance (41, 42).

All experiments were performed in three independent replicates. Statistical analyses were conducted using SPSS software (version 26) and Microsoft Excel. Data were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD). Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA), while pairwise comparisons were performed using the Mann-Whitney U test when appropriate. A P -value of less than 0.05 ($P < 0.05$) was considered statistically significant.

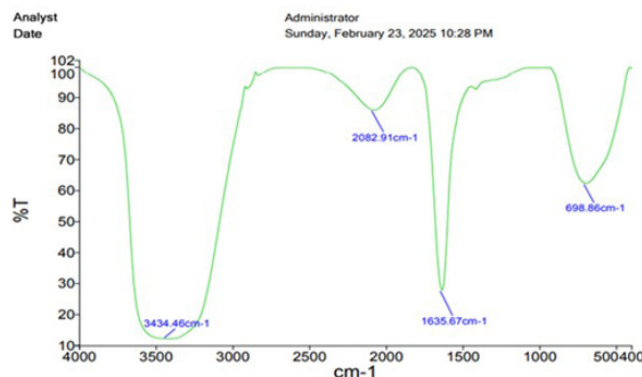


Figure 1. Fourier-transform infrared (FTIR) spectrum for characterization of chitosan nanoparticles

Results

Preparation of Larval ES products and protein concentration assay

The protein concentration of the crude ES extract was determined using a standard curve generated with BSA (50-100 mg). The results showed that the mean net absorbance of the crude ES sample at 595 nm was 1.961, corresponding to a mean protein concentration of 824 μ g/ml.

Synthesis and characterization of chitosan nanoparticles (CSNPs)

Chitosan nanoparticles were successfully synthesized via the ionic gelation method to serve as carriers for the ES extract and were also tested independently. FTIR spectroscopy was used to confirm the chemical structure of the chitosan nanoparticles. The obtained spectrum showed characteristic peaks at 3434 cm^{-1} (stretching vibrations of O-H and N-H, indicating hydroxyl and amine groups), 1635 cm^{-1} (C=O stretching vibration related to amide I, confirming the presence of chitosan amide groups), and 698 cm^{-1} (C-H bending of the glycosidic ring). A minor peak at 2082 cm^{-1} was considered insignificant and likely related to overtone vibrations (Figure 1).

DLS was used to determine the hydrodynamic size distribution of the nanoparticles. The main peak was observed at 485 nm, confirming the nanometric size of the primary particles, although some aggregation was observed (Figure 2).

In vitro anti-leishmanial activity

The inhibitory effects of crude ES, free-standing chitosan nanoparticles (CSNPs), and ES-loaded chitosan nanoparticles (ES-CSNPs) on the viability of *L. major* promastigotes over 72 hr were evaluated. The half-maximal inhibitory concentration (IC_{50}) for each treatment was calculated.

The IC_{50} value for crude ES was approximately 1050 μ g/ml, which was similar to the positive control (Glucantime, ~ 1000 μ g/ml), indicating no significant difference in inhibitory effect. For free-standing CSNPs, a reliable IC_{50} value could not be obtained due to a non-sigmoidal dose-response curve, suggesting low intrinsic anti-leishmanial activity of these nanoparticles.

In contrast, the ES-CSNPs formulation showed significantly higher inhibitory potency, with an IC_{50} of approximately 500 μ g/ml at 72 hr. This IC_{50} was significantly lower than that of the positive control ($P < 0.0001$), indicating superior inhibitory activity. Microscopic observations revealed that promastigotes treated with ES-CSNPs initially lost motility, became spherical,

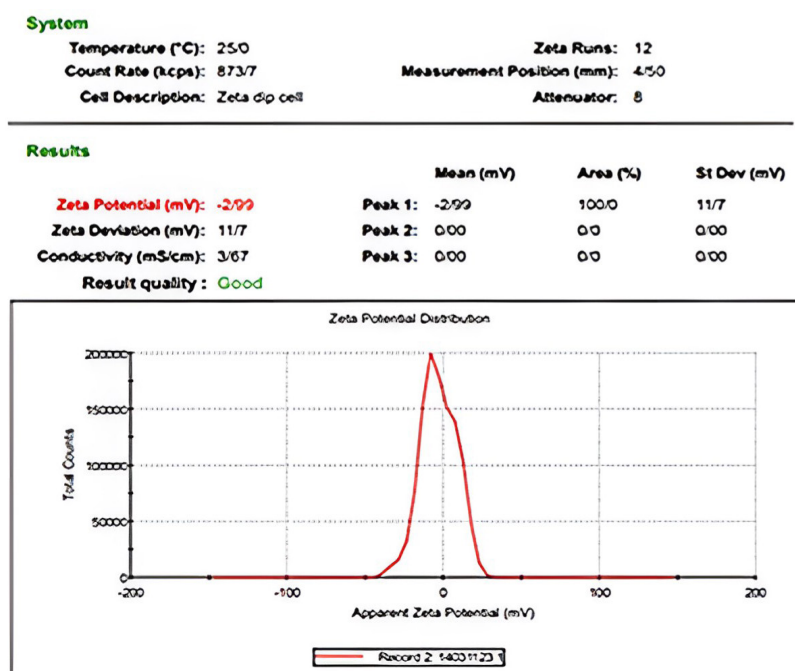


Figure 2. Outcome of dynamic light scattering (DLS) for determining the hydrodynamic size distribution of nanoparticles peaking at 485 nm

and eventually disintegrated and disappeared. The lethal effect was dose- and time-dependent.

Cellular toxicity evaluation (MTT assay)

The potential toxicity of the treatments on murine macrophage J774A.1 cells was assessed after 72 hr of exposure using the MTT assay. Results showed that ES-loaded nanoparticles (ES-CSNPs) significantly affected macrophage viability ($P < 0.05$) in a dose-dependent manner. However, no statistically significant cellular toxicity was observed for crude ES ($P = 0.9706$), plain CSNPs ($P = 0.2464$), or Glucantime compared to the untreated control. These results indicate that the ES-CSNPs formulation, despite its high efficacy against the parasite, has a selective toxicity profile, meaning it is more toxic to the parasite than to host macrophages at the tested concentrations.

In vivo therapeutic efficacy in BALB/c mice

The therapeutic potential of the formulations was evaluated in a murine model of cutaneous leishmaniasis caused by *L. major*. Skin lesions began to appear in infected BALB/c mice starting in the fourth week post-infection. Lesion size was measured weekly. In the negative control group, the mean lesion size progressively increased to $9.97 \pm 1.98 \text{ mm}^2$ by the fifth week. In contrast, all treatment groups showed a gradual decrease in lesion size during therapy.

A statistically significant difference ($P < 0.0001$) in lesion size reduction was observed between the ES-CSNPs-treated group and the Glucantime-treated group, indicating the better efficacy of the nanoparticle formulation. The differences between the crude ES and plain CSNPs groups compared with Glucantime were not significant. Furthermore, during the 12-week study period, no mortality was observed in the ES-CSNPs-treated group, highlighting the safety of this formulation in the animal model (Figure 3).

Parasite burden in the spleen

Ten days after the end of the treatment period, the parasite

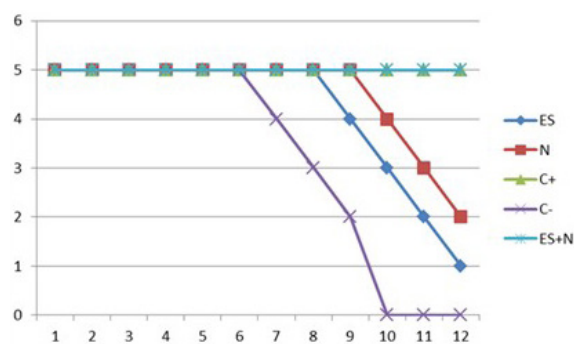


Figure 3. Survival outcomes of mice following 4 weeks of treatment and 8 weeks post-treatment

C+: Glucantime-treated positive control; C-: Untreated negative control; ES+N: Lucilia sericata excretory-secretory products loaded on chitosan nanoparticles

burden in the spleens of infected mice was measured using the limiting dilution method. A significant reduction in parasite burden was observed in all treated groups compared to the negative control. The parasite burden was significantly lower in the ES-CSNPs-treated group than in the Glucantime group, indicating a higher ability of this formulation to control the progression of systemic infection. No significant difference was found between the crude ES- and CSNP-treated groups in splenic parasite burden (Figure 4).

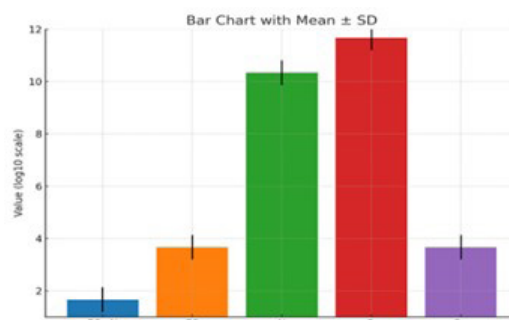


Figure 4. Parasite burden test results in the four mouse study groups

Table 1. Mean lesion diameter (mm±standard deviation) in different mouse experimental groups over time (Weeks 1-4: Treatment period. Weeks 5-8: follow-up period)

Week	PC	NC	Cr ES	CN	ES+ CN
1	6.44±2.72	7.58±2.14	6.58±2.63	6.56±2.82	6.40±2.65
2	5.94±1.65	7.87±2.16	5.89±2.12	5.87±1.98	5.35±1.43
3	4.15±1.34	8.80±1.68	4.20±1.87	5.20±1.87	4.13±1.26
4	3.82±1.65	9.23±2.16	3.90±1.76	3.90±1.76	3.67±1.19
5	2.92±1.34	9.97±1.98	3.10±1.56	3.20±1.67	2.82±1.23
6	1.95±1.65	10.28±1.68	2.10±1.56	2.20±1.58	1.87±1.18
7	1.43±1.20	11.47±1.98	1.68±1.56	1.89±1.67	1.29±1.40
8	1.53±1.40	12.56±2.16	1.77±1.48	1.83±1.54	1.10±1.10*

Note: P.C.: Positive Control (Glucantime). N.C.: Negative Control, Cr. ES: Crude Larval Extract, C.N.: Chitosan Nanoparticles, ES+C.N.: ES-loaded Chitosan Nanoparticles

Mean diameters of progressive lesions

The progression and healing of lesions were carefully tracked by measuring the mean lesion diameter over eight weeks (Table 1). The results showed a clear therapeutic trend. Although all treatment groups showed improvement, Group 5 (treated with ES-CSNPs) consistently had the smallest lesion diameter from the third week onward. By week eight, the mean lesion diameter in this group was 1.10±1.1 mm; in the positive control (Glucantime), it was 1.53±1.4 mm, and in the negative control, it was 12.56±2.16 mm. Statistical analysis (ANOVA) confirmed that the mean lesion size in Group 5 was significantly smaller than in the other groups ($P<0.05$), wound progression was effectively halted, and fresh tissue growth was observed at the lesion site.

Discussion

The present study was conducted to investigate the anti-leishmanial effects of (ES) materials against *L. major* cells under *in vitro* and *in vivo* conditions. This study demonstrated that loading the larval ES materials of *L. sericata* on chitosan nanoparticles significantly enhanced the anti-leishmanial activity of *L. sericata* ES under both *in vitro* and *in vivo* conditions. This increase in efficacy is likely attributable to a synergistic effect between the antimicrobial peptide compounds present in the larval extract and the permeability properties and controlled-release capability of chitosan nanoparticles. The findings of this research regarding the strong inhibitory effects of larval products on leishmanial promastigotes are consistent with the results of previous studies and suggest their potential as candidates for the treatment of cutaneous leishmaniasis (43, 44).

One important aspect of the development of new drugs is their selective toxicity, meaning the drug should be lethal to the parasite while remaining safe for host cells. In this study, the larval ES of *L. sericata* exhibited strong toxicity on J774A.1 macrophage cell line. This finding aligns with previous reports by other researchers. The results of this study indicated that the ES-CSNP formulation (ES-loaded chitosan nanoparticles) was significantly more effective than the crude ES or plain nanoparticles. Lower IC_{50} values under *in vitro* conditions, improved healing of skin lesions, and a lower parasite burden in *in vivo* models indicated the higher effectiveness of the ES-CSNP formulation. Chitosan nanoparticles likely enhance this efficacy through multiple mechanisms. According to previous studies, chitosan

nanoparticles have been shown to improve drug delivery remarkably (45).

The *in vivo* results of this study showed significant improvement in skin lesions in BALB/c mice treated with ES-CSNP, which is consistent with, and superior to, the findings of studies that used live larvae or crude ES. Although precise identification of the mechanism of action requires further investigation, observations from this research and prior studies suggest several possible mechanisms. The antimicrobial peptide compounds (AMPs) present in ES, such as lucifensin, may cause parasite death by disrupting the parasite's cell membrane. These compounds may also act by inducing programmed cell death (apoptosis) in the parasite, leading to its demise. Furthermore, ES components may interfere with vital metabolic or signaling pathways, thereby killing the parasite (45-47).

Conclusion

In the present study, a single therapeutic dose was selected for each treatment formulation based on previously reported studies and experimental feasibility. Therefore, a comprehensive dose-response evaluation was not performed. Future studies are required to investigate the dose-dependent therapeutic efficacy of ES-loaded chitosan nanoparticles in experimental models of cutaneous leishmaniasis.

Although the present study demonstrates the therapeutic potential of ES-loaded chitosan nanoparticles against experimental cutaneous leishmanial cells, the efficacy of this formulation against drug-resistant strains of *L. major* was not directly evaluated. Therefore, further investigations are necessary to determine whether this formulation could be effective in drug-resistant cases.

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Ethical Considerations

This study was approved by the Ethics Committee of Tarbiat Modares University (Approval No. IR.MODARES.AEC.1403.051).

Authors' Contributions

F S contributed to conceptualization, investigation, data curation, visualization, and writing the original draft. MS D handled conceptualization, methodology, supervision, software development, and final review and editing. F G was responsible for visualization, investigation, and final writing, review, and editing. A A contributed through investigation, software, methodology, validation, and final review and editing.

Conflicts of Interest

The authors declare there are no conflicts of interest.

Declaration

An artificial intelligence (Chatgpt) tool was used solely for final English language editing and improving the clarity and readability of the manuscript.

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