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In vitro and *in vivo* effects of conventional and chitosan nanoparticles encapsulated miltefosine drug for treatment of cutaneous leishmaniasis

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Abstract: This study aimed to formulate polymer-based chitosan nanoparticles as a drug (miltefosine) delivery system for treating leishmaniasis. Miltefosine-loaded chitosan nanoparticles (MLCNPs) have been synthesized and then characterized by the use of UV-Visible spectroscopy, Scanning electron Microscopy (SEM), Transmission electron microscopy (TEM), zeta potential, drug loading content (DLC), encapsulation efficacy (EE) and dynamic light scattering technique (DLS). Further, the *in vitro* anti-leishmanial activity of the characterized chitosan nanoparticles was assessed by Microculture Tetrazolium (MTT) assay, and *in vivo* efficacy was evaluated in infected BALB/c mice. The lesion healing was statistically analyzed using Wilcoxon signed-rank and Mann–Whitney tests. The MLCNPs were spherical shaped (97.5 nm), which presented efficient encapsulation (97.56%), drug loading content (91.5 µg/mL), and positive surface charge (+1.04 mV). MLCNPs were less hemolytic (6%) when compared to conventional miltefosine. MLCNPs (50 µg/ml) showed potential antileishmanial effect (mean viability; 10±0.3%) on promastigotes in comparison to conventional miltefosine (mean viability; 18±1.3%). The IC50 value for MLCNPs and miltefosine was 0.0218 µg/mL and 0.3548 µg/mL, respectively. *In vivo* study proved that lesions of mice treated with oral and intralesional-injected MLCNPs heal significantly (P = 0.01). MLCNPs have a significant antileishmanial effect and could be utilized as an alternative treatment for CL.

Keywords: Cutaneous leishmaniasis; Nanotherapy; Animal model; MTT assay; Hemolysis assay

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1. Introduction

Leishmaniasis is a vector-born infectious disease transmitted by protozoan parasites and is both anthroponotic and zoonotic. After malaria, leishmaniasis is considered the second leading parasitic cause of death [1].The female sandflies are intermittently infected by the parasite that feeds on the blood of infected animals or humans and acquires the parasites from their blood [2].

Leishmaniasis is classified into three types based on signs and symptoms, which are cutaneous, mucocutaneous, and visceral leishmaniasis [3]. CL is more susceptible to lesions caused by *Leishmania tropica*, *Leishmania major*, *Leishmania braziliensis*, *Leishmania Mexicana*, and *Leishmania amazonensis*, which arise at the site of an insect bite [4]. The CL is ubiquitous worldwide but is more prevalent in underdeveloped and developing countries [5]. Treatment decisions and clinical description rely on the species engaged with causing leishmaniasis. The available drugs can cure the disease for the time being, but due to the unavailability of vaccines, complete control is impossible. Amongst the

available drugs, the first and second-line drugs are partially effective, need a longer treatment period, and have severe side effects [6].

Miltefosine is an anti-leishmanial drug in the alkyl-phosphocholine class and is effective against tumor and fungal infections [7]. This drug has zwitterionic amphiphilic properties due to the positively charged amine and negatively charged phosphoryl group. The complete mode of action of miltefosine is still not fully understood. However, some studies demonstrate that it disturbs the lipid-dependent cell-signaling pathways. Besides their efficacy, miltefosine had severe side effects like genitourinary and hematological complications [8].

Nanomedicine is a new and alternative way to treat several life-threatening infections. Along with their direct activity, nanoparticles are also used as an efficient drug delivery system, especially for drugs having severe toxicity [9]. These drug delivery systems enhance the number of medications to infected tissues and elevate the treatment performance compared to conventional therapy [10]. Chitosan nanoparticles are polymer-based cationic molecules and interact efficiently with the plasma membrane of macrophages [11]. Furthermore, it can carry the drugs inside the macrophages and reduce adverse effects related to drug administration and pro-inflammatory cytokine secretion [12]. Therefore, in the current study, we aimed to synthesize miltefosine-loaded chitosan nanoparticles (MLCNPs) as an alternative therapeutic option for the treatment of cutaneous leishmaniasis to achieve a higher efficacy rate and lower toxicity compared to conventional miltefosine.

2. Materials and Methods

2.1. Material

The reagents used were: Roswell Park Memorial Institute Medium-1640 (RPMI, Lot # 1868632, GIBCO, USA), Medium 199 (M199, Lot # CP17-1058, Capricorn Scientific). Heat inactivated Fetal Bovine Serum (hiFBS, Lot # 10270, GIBCO), Penicillin-Streptomycin solution (PenStrepsoln, Lot # 01161018, Caisson), Trypan blue (Invitrogen, Lot # 1844453), Chitosan (Sigma-Aldrich), Miltefosine (Sigma-Aldrich), Tripolyphosphate (TPP), D-Trehalose, Sodium hydroxide (Scharlau Chemie SA), Phosphate Buffered Saline (Thermo Fisher Scientific), and Dimethyl Sulfoxide (DMSO).

2.2. Parasites and animals

The *L. tropica* strain was collected from the infectious disease laboratory at Quaid-i-Azam University, Islamabad, Pakistan, for in vitro experiment. *In vivo* study was carried out using *Leishmania* parasites isolated from CL patients and cultured in NNN media. BALB/c mice were purchased from the National Institute of Health, Islamabad, Pakistan.

2.3. Promastigotes culturing

The RPMI 1640 was prepared by adding heat-inactivated FBS (10 mL) to RPMI 1640 (90 mL) in a sterile flask [13]. After culturing, promastigotes were observed under a light microscope at 40X and 10X.

2.4. Synthesis of MLCNPs

Chitosan polymer encapsulated miltefosine nanoparticles were synthesized following the previous method [14]. Briefly, chitosan polymer (0.5% w/v) was dissolved in (1% v/v) acetic acid solution. TPP powder (0.5% w/v) and miltefosine drug (3 mg/mL) were dissolved in deionized water, and the pH was adjusted to 5. TPP solution containing miltefosine was added to the chitosan solution dropwise to synthesize ionically cross-linked nanoparticles and the reaction mixture was incubated for one hour under constant stirring at room temperature, and centrifuged for 10 min at 13,400 rpm to accumulate the nanoparticles in the pellet.

2.5. Characterization of MLCNPs

UV- Visible Spectrophotometer (Schimadzu UV-visible 1800) was used to confirm the presence of MLCNPs by obtaining UV-visible spectra at 250 nm absorption rate. Scanning electron microscopy (SEM) was used to study the size and surface morphology of MLCNPs with a magnification of 20 to 45 k-X with an accelerating voltage of 20 kV. TEM was used to assess the morphology of the MLCNPs. TEM samples were dilute in Milli-Q water (10×, *v*/*v*), and then placed (10 μL) onto specimen grids and negatively stained with uranyl acetate solution (2% (*w*/*v*), Sigma-Aldrich, St.Louis, MO, USA). The size distribution and zeta potential of MLCNPs were measured using a DLS zeta sizer (PSS Nicomp 380).

The absorbance value for the EE and DLC of MLCNPS was calculated using the following equations: % EE= [(A-B)/A] × 100; A is the total volume of miltefosine utilized to synthesize nanoparticles (mg), B is equivalent to the free miltefosine calculated in the supernatant in mg.% DLC= [(A-B)/C] × 100; A is the total volume of miltefosine utilized to synthesize nanoparticles (mg), B is equivalent to the volume of free miltefosine calculated in the supernatant in mg, while C is the mass of nanoparticles in the supernatant

2.6. In vitro drug release assay

As mentioned earlier, a drug release study was performed using the dialysis bag technique [15]. Briefly, the nanoparticles suspension and drug solution (10 mg each) were dissolved in a beaker containing 5 mL Tris-HCL buffer solution and placed in a dialysis bag.The dialysis bags were maintained in the medium under stirring and maintained in a temperature-constant water bath (37 °C). Afterward, the suspension was stirred magnetically at 100 rpm for 48 h at room temperature. At specific periods, samples were centrifuged at 15,000 g for 20 min at 14 °C. The concentration of miltefosine release from the NPs was assessed using UV/Vis spectrophotometer at 270 nm.

2.7. Hemolysis assay

For the hemolysis assay, 3 mL of blood was collected from a healthy volunteer in EDTA tubes. The blood was centrifuged at 1500 rpm for 15 minutes to prevent clotting. The pellet containing erythrocytes was washed three times with 1X PBS. The erythrocytes suspension was prepared by mixing 11 mL of 1X PBS into 3 mL of centrifuged erythrocytes. The NPs and drug concentrations were prepared by adding (150 μg, 200 μg, and 250 μg) MLCNPs and conventional miltefosine in 1 ml ddH_2O. The reaction mixtures of MLCNPs and conventional miltefosine with erythrocytes suspension were incubated for 4 h at 37 ^{0}C for hemolysis. After, Eppendorf tubes were centrifuged at 13,000 rpm for 15 min, and the remaining hemoglobin in the supernatant was measured by spectrophotometer at 570 nm. The PBS (1 mL) was used as the negative control with 0% hemolysis, and 1 mL Triton-X 100 was used as the positive control with approximately 100% hemolysis. The experiment was performed in triplicate. The hemolysis (%) was calculated as; hemolysis (%) = [(OD at 570 nm in the drug solution – OD at 570 nm in PBS) ÷ (OD at 570 nm in 0.1% Triton X-100 – OD at 570 nm in PBS)] × 100.

2.8. Anti-promastigotes assay

In vitro activity of MLCNPs on *L. tropica* (promastigotes) was performed as described elsewhere [16]. Briefly, in a 96-well plate, 100 μL promastigotes (culture media containing 10^7 CFU) were added. In each well, the L. tropica were incubated with 100 μL of MLCNPs and conventional miltefosine at different concentrations (50, 40, 30, 20, 10, and 5 μg/mL). After 72 h of incubation at 24 °C, 10 μL of MTT reagent was added to each well, wrapped in aluminum foil, and further incubated for 4 h at 24 °C. The cultured media were centrifuged at 3000 rpm for 3 min, the supernatant was discarded, and the pellet was diluted with 100 μL of DMSO to stop the enzymatic reaction. The wells were incubated for 1 hour in a shaking incubator at 24 °C. Absorbance was checked at an optical density of 570 nm

by a microplate reader. The data obtained from the microplate reader was then subjected to GraphPad prism *v.8.0.2* software for statistical analysis.

2.9. In vivo experiment

For in vivo experiment, 6-8 weeks old, 28 g female BALB/c mice (n = 16) were used. BALB/c mice were shaved from the tail base using a shaving machine and were injected intradermally using 1CC Ultra-Fine insulin syringes with promastigotes (1×10^7) [17]. The mice were examined every two days for 28 days to assess the appearance of lesions. Infection was well established after three to four weeks, and noticeably visible lesions were seen at the site of inoculation. Lesions were tested for positive evidence of CL by determining the presence of amastigotes with Giemsa-stained smear under a light microscope at 100X objective lens. Lesion size was calculated with a vernier caliper at right angles in two dimensions (D and d mm) to each other, and the equation determined the lesion size (S) = (D × d)/2 mm^2. The mean of the two measured diameters was calculated and further used for statistical analysis. The 16 mice were divided into four groups (4 in each) to determine the treatment efficacy of MLCNPs and conventional miltefosine for 14 consecutive days.

Group 1: Mice have injected synthesized nanoparticles intralesional once a day.

Group 2: Mice were treated orally with synthesized nanoparticles once a day.

Group 3: Mice were treated orally with miltefosine once a day.

Group 4: Mice were given PBS orally once daily, serving as the placebo group.

Groups 1 and 2 were treated at a dose of 89 μg/28 g/day in ddH_20 up to a final volume of 0.1 mL. Group 3, containing the conventional miltefosine, was also used at a similar dose of 89 μg/28 g/day (2.5 mg/kg/day). For group 4, sterilized PBS solution (0.1 mL) was given orally as a placebo. The reduction of lesion sizes was observed daily, and the final measurements were performed after two weeks using a vernier caliper.

2.10. Statistical analysis

Statistical analysis was performed using SPSS *v.22* software. Differences between each group pair (before and after treatment of the same group) were analyzed using Wilcoxon signed-rank test. At the same time, the significance between the two groups was assessed by the Mann–Whitney test. The difference was considered significant with the P value < 0.05.

3. Results

3.1. Synthesis and characterization of NPs

The MLCNPs were formed as a milky color solution, and their powder form was attained by centrifugation. The synthesis of chitosan nanoparticles was confirmed through UV-Visible spectroscopy, as shown in figure 1. The reported UV-Visible spectrum showed an absorption peak at 434 nm. The SEM analysis revealed that MLCNPs displayed a spherical shape and irregular surface morphology (Figure 2), while DLS showed a mean particle size was 97.5 nm (Figure 3). The TEM micrograph of the nanoparticles is shown in Figure 4. It is clear from the TEM image that MLCNPs are spherical with a smooth surface. The synthesized particles possessed a substantial zeta potential of +1.04 mV (Figure 5). The percentage of drug-loaded chitosan was DLC of 91.5 μg/mL, and the encapsulation efficiency was 97.56%.

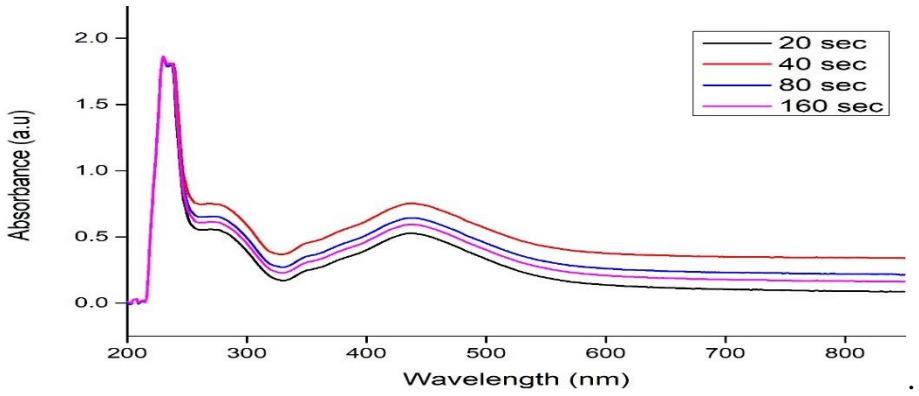


Figure 1. UV -Visible spectrum of chitosan nanoparticles.

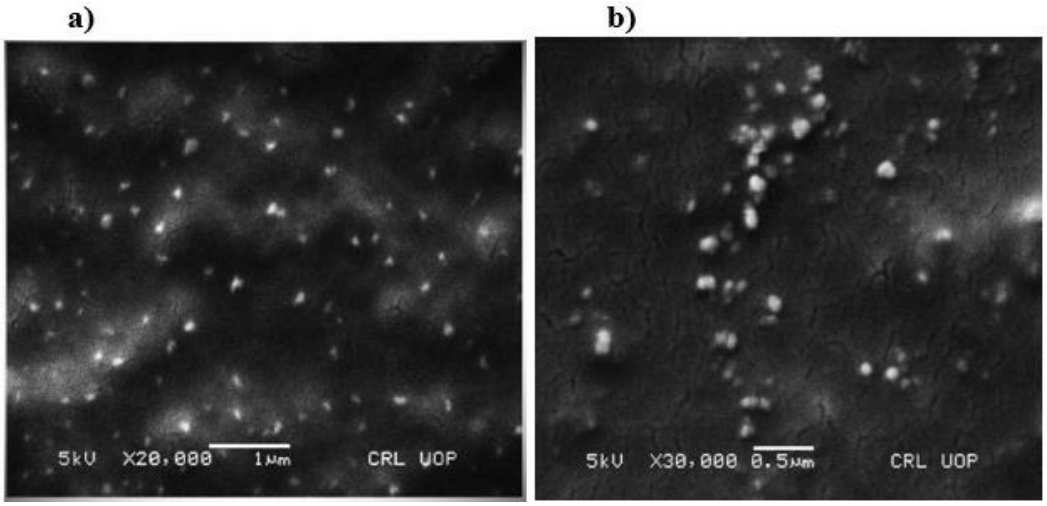


Figure 2. SEM images of the MLCNPs with spherical morphology magnified 20,000X and 30,000X.

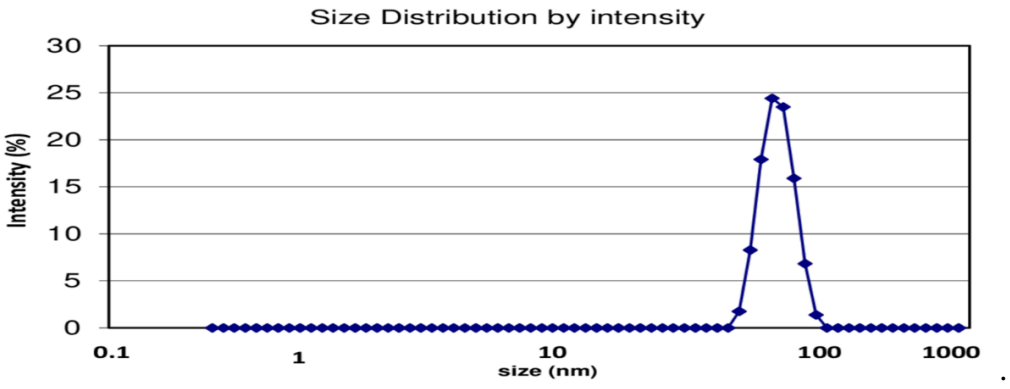
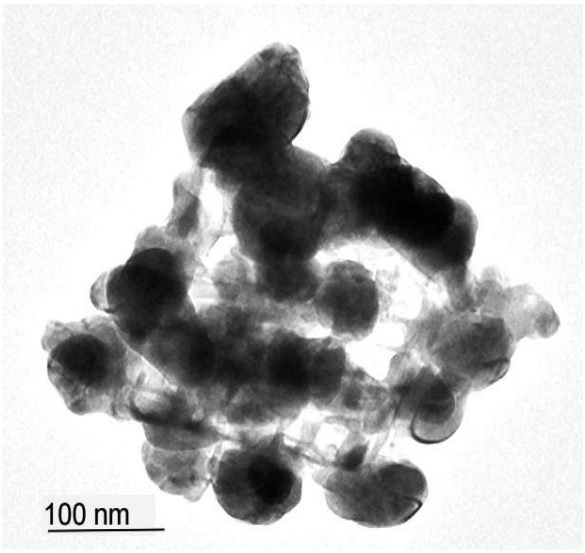


Fig.3. Average size distribution intensity of MLCNPs using dynamic light scattering technique.



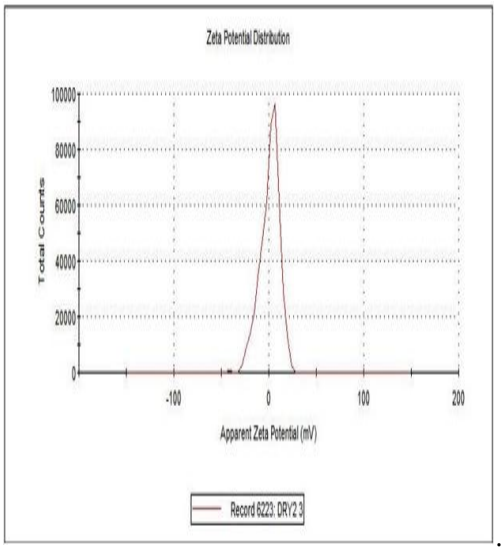


Figure 4. TEM image of the MLCNPs. Scale bar- 100 nm.

Fig.5. Zeta potential of MLCNPs.

3.2. In vitro drug release assay

The drug release concentration of miltefosine drug from the nanoparticles was divided into two phases. In the initial stage, 30 % drug was rapidly released from the NPs after 6 h at pH 7.4. In the second stage, the drug was constantly released from the nanoparticles, resulting in around 96 % of the loaded drug up to 48 h (Figure 6).

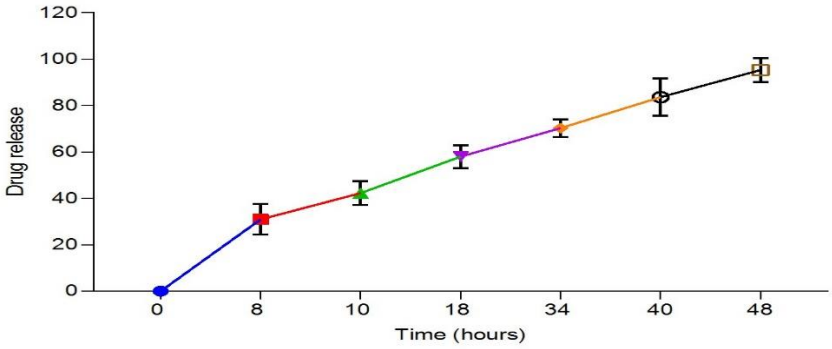


Figure 6. In vitro miltefosine release pattern from chitosan nanoparticles at pH 7.4.

3.3. Hemolysis assay

The hemolysis activity of MLCNPs and conventional miltefosine were compared. The result showed that MLCNPs have 6% less hemolytic activity than conventional miltefosine. The OD values and hemolysis (%) are presented in table 1.

Table 1. Hemolysis assay of MLCNPs and conventional miltefosine drug.

	Scheme 570.	**Concentrations**	**OD at 570 nm (Mean ±SD)**	**Hemolysis (%)**
MLCNPs		150 µg/mL	0.032 ±0.0025	1.60%
		200 µg/mL	0.043 ±0.0015	2.45%
		250 µg/mL	0.051 ±0.0010	3.20%

	150 µg/mL	0.062 ±0.0020	5.96%
Con MFS	200 µg/mL	0.075 ±0.0015	6.92%
	250 µg/mL	0.082 ±0.0030	7.40%
PBS	-	0.003 ±0.0005	0%
Triton 100X	-	1.223 ±0.0251	100%

3.4. MTT assay

The cytotoxicity of conventional miltefosine and MLCNPs was assessed at six different concentrations (50, 40, 30, 20, 10, 5 µg/mL) for 24, 48, and 72 h. The mean viability percentage of promastigotes exposed to different MLCNPs and conventional miltefosine concentrations is illustrated in table 2. The IC50 value of MLCNPs (0.0218 µg/mL) was higher than the conventional miltefosine (0.3548 µg/mL).

Table 2. Mean promastigotes viability percentages exposed to different concentrations of MLCNPs and conventional.

Miltefosine. Data are expressed as the mean± SD (n=3).

Concentrations	**24 h**		**48 h**		**72 h**	
	MLCNPs	**Con MFS**	**MLCNPs**	**Con MFS**	**MLCNPs**	**Con MFS**
50 µg/mL	16 ± 4.2	28 ± 1.1	14 ± 0.7	21 ± 0.7	10 ± 0.3	18 ± 1.3
40 µg/mL	17 ± 2.5	30 ± 2.05	16 ± 0.3	25 ± 1.8	12 ± 0.8	20 ± 2.0
30 µg/mL	20.5 ± 4.5	33 ± 3.08	18 ± 1.1	30 ± 0.9	15 ± 1.8	25 ± 1.5
20 µg/mL	24 ± 4.1	44 ± 1.8	21 ± 1.5	40 ± 1.5	23 ± 2.5	30 ± 0.5
10 µg/mL	31.5 ± 3.4	52 ± 1.6	26 ± 1.6	45 ± 1.4	25 ± 2.1	41 ± 1.3
05 µg/mL	35 ± 3.8	60 ± 1.4	30 ± 1.3	52 ± 0.9	29± 1.6	44 ± 1.5

3.5. In vivo study

At four weeks post-inoculation, when lesions were established, the mice were treated for two weeks, and a visible reduction in lesion size was noted, as shown in figure 7. The mean and SD of each group's pre- and post-treatment lesion sizes were determined, and *P*-values were determined as presented in table 3. All the groups exhibited decreased lesion size after drug therapy except the placebo group. Among the groups, the orally administrated MLCNPs group was considered significant ($P = 0.01$).

Figure 7. L. tropica infected BALB/c mice, a) lesion development after four weeks of inoculation, b) reduction of lesion size after two weeks of MLCNPs treatment.

Table 3. Lesion sizes (mean ± SD) comparison before and after treatment of each group.

Groups	**N**	**Before treatment**	**After treatment**	***P*-value**
Con MFS	4	6.9 ± 0.26 mm	6.6 (± 0.23) mm	0.09
MLCNPs (oral)	4	6.9 (± 0.46) mm	5.9 (± 0.33) mm	0.01
MLCNPs (IL)	4	7.2 (± 0.50) mm	6.4 (± 0.45) mm	0.06

Placebo	4	6.8 (± 0.50) mm	7.2 (± 0.29) mm	0.24

The Mann-Whitney test was performed for lesion size comparison between the two groups. Post-treatment lesion comparisons of conventional miltefosine and MLCNPs (oral) treated groups were statistically significant ($P = 0.019$). The *P*-values differences between pre-and post-treated groups were also statistically significant ($P = 0.020$), as shown in table 4. Similarly, the amastigote count on the last day of treatment was significantly less under the light microscope than the pre-treated parasite burden. The comparison between conventional miltefosine and intralesional injected MLCNPs treated groups was also performed but detected as less effective. The P- value difference was 0.065 and considered insignificant (Table 5).

Table 4. Lesion size comparison of the conventional miltefosine group and MLCNPs (oral) group.

Lesion Size	Groups	Mean rank	Sum of ranks	*P*-value	*P*-value difference
Before (Trt)	Con MFS	4.63	18.50	0.885	0.020
	MLCNPs (oral)	4.38	17.50		
After (Trt)	Con MFS	6.50	26.00	0.019	
	MLCNPs (oral)	2.50	10.00		

Table 5. Lesion size comparison of conventional miltefosine group and MLCNPs (intralesional) group.

Lesion Size	Groups	Mean rank	Sum of ranks	*P*-value	*P*-value difference
Before (Trt)	Con MFS	4.63	18.50	0.885	0.065
	MLCNPs (IL)	4.38	17.50		
After (Trt)	Con MFS	4.88	19.50	0.661	
	MLCNPs (IL)	4.13	16.50		

Furthermore, comparisons based on the route of administration of MLCNPs were performed. The difference in the *P*-value between the pre-and post-treated groups was 0.019, as calculated in table 6. The results revealed that the oral route of administration for MLCNPs is much more significant than the intralesional route.

Table 6. Lesion size comparison of MLCNPs (oral) and MLCNPs (intralesional) groups.

Lesion Size	Groups	Mean rank	Sum of ranks	*P*-value	*P*-value difference
Before (Trt)	MLCNPs (oral)	4.25	17.00	0.770	0.019
	MLCNPs (IL)	4.75	19.00		
After (Trt)	MLCNPs (oral)	3.13	12.50	0.108	
	MLCNPs (IL)	5.88	23.50		

4. Discussion

The high toxicity and emergence of resistance against available drugs (antimonial) for leishmaniasis is a worldwide grave concern. Recently, various methods have been tried to develop alternative medicine against Leishmania species. Amphotericin B has been prepared among the novel approaches, but these drugs remain limited due to their high cost [18]. A nanoparticle-based drug delivery system is considered an essential tool for treating leishmaniasis due to its direct interaction with residing Leishmania parasites. Among the NPs-based drug delivery system, chitosan polymers are considered the best option due to their ideal characteristics like enhanced permeation, high uptake of drug intracellularly, sustained release, increased biocompatibility, and stability [19].

In the current study, chitosan NPs were synthesized by the ionotropic gelation method. Chitosan-based nanoparticles were broadly synthesized using the same technique [20]. The first reported Chitosan nanoparticles preparation by ionic gelation method was done in 1997 in Spain. Studies showed that chitosan nanoparticles synthesized by this method were relatively small, ranging from 100 nm to 150 nm in diameter [21]. The UV-Visible spectrum was recorded for the determination of the optical properties of the

chitosan polymer by using UV- Visible spectroscopy. The peak observed for the chitosan was at 250 nm in the UV region. These results are in good agreement with the previous study. The DLS results of this study revealed the average size of MLCNPs to be 97.5 nm with a spherical shape and irregular surface morphology without aggregation. SEM analysis of MLCNPs showed a spherical morphology with a smooth surface. Spherical nanoparticles were shown to incorporate maximum drug accumulation [22]. TEM images of the MLCNPs showed that they were spherical and about 90-98 nm in size. The concentration of chitosan, molecular weight, and TPP significantly affected the size of the nanoparticles. By increasing the concentration of chitosan or TPP, the size of nanoparticles also increased [23]. Our TEM results align with the previously reported study [24].

The encapsulation efficiency of MLCNPs observed in our study was 97.56 %. Our results align with other antileishmanial drugs encapsulated in chitosan nanoparticles prepared through ionic gelation method[25].

Similarly, the drug loading content of MLCNPs was 91.5 μg/ml. This high amount of DLC may be due to the zwitterionic and amphiphilic nature of the drug used in this experiment [26]. The zeta potential of MLCNPs was +1.04 mV. In a previously published study, the zeta potentials of liposomal-based formulation were in the range of −8.2 ± 3.50 mV, which is less than the chitosan nanoparticles of the current study [27].

In vitro, MTT colorimetric assay was performed to analyze the biological activity of MLCNPs and conventional miltefosine against *L. tropica*. In the current study, the IC50 value of conventional miltefosine was 0.3548 μg/mL, and in another study, it was reported to be 0.19 μg/mL [28]. In contrast, the MLCNPs reported in the present study were 0.0218 μg/mL, less than conventional miltefosine, indicating the high efficacy of MCLNPs. The hemolysis assay revealed that MLCNPs induced less hemolysis and were considered safer than conventional miltefosine. Our study's hemolysis activity of conventional miltefosine was similar to an earlier reported study [29].

The effectiveness of miltefosine encapsulated in polymer nanoparticles was studied for treating CL in BALB/c mice by oral and intralesional routes of administration. The efficacy of conventional miltefosine (2.5 mg/kg/day) and our newly synthesized nanoparticles (MLCNPs) were compared. Although both the conventional miltefosine and MLCNPs were able to reduce the lesion size over time; however, the activity of MLCNPs was much better. It indicates that chitosan nanoparticles are targeted drug delivery systems, which transport miltefosine specifically to macrophages for anti-leishmanial activity. While in the case of orally administrated conventional miltefosine, some quantity of the drug is lefted-over in the body. A study reported that a high concentration of miltefosine efficiently reduced lesion size compared to a lower concentration of miltefosine [30]. Based on this, the higher efficacy of MLCNPs is because they provide a high concentration of miltefosine to infected macrophages compared to conventional miltefosine.

5. Conclusions

The current study describes a potential novel therapy for curing CL caused by *L. tropica* in the form of MLCNPs. The chitosan nanoparticles encapsulate the miltefosine efficiently. The overall results revealed that MLCNPs have higher efficacy than conventional miltefosine, which is proved both *in vitro* and *in vivo*. Regarding the route of administration, the efficacy of MLCNPs given orally was more significant than intralesional injections. Moreover, the hemolysis assay showed significantly less hemolysis activity of MLCNPs than conventional miltefosine. In summary, all these features make these newly synthesized nanoparticles an ideal drug delivery system and might be used as an alternative therapeutic option for curing leishmaniasis.

Conflicts of Interest: The authors declare no conflict of interest.

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