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RESEARCH

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Comparative efficacy of amphotericin B-loaded chitosan nanoparticles and free amphotericin B drug against *Leishmania tropica*

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Abstract

Background: The preparation of an effective drug delivery formulation is an urgent need to treat cutaneous leishmaniasis (CL). Pentavalent antimonials and Amphotericin B (AmB) are considered to treat leishmaniasis; however, their clinical usage is hampered by poor solubility, high cost, toxicity, and the emergence of drug-resistant *Leishmania* spp. The drug delivery systems (DDS) could be used as an alternative treatment option for the treatment of CL to circumvent these problems. We tested the antileishmanial efficacies of free AmB and amphotericin B-loaded chitosan nanoparticles (AmB-CNPs) under in vitro conditions.

Results: Chitosan nanoparticles (CNPs) were synthesized using the ionic gelation method with negatively charged tripolyphosphate (TPP). During the synthesis of CNPs, AmB was incorporated into the nanoparticles (NPs). The NPs were characterized for their size, surface morphology, encapsulation efficacy (EE), drug loading content (DLC), and surface charge using different techniques. Their efficacy was evaluated against promastigotes and axenic amastigotes forms of *Leishmania tropica* using MTT assay. The synthesized AmB-CNPs displayed a spherical shape with a mean particle size of 118 nm, a positive zeta potential of ($+6.21 \pm 2.02$ mV), and an encapsulation efficacy of 88%. Dynamic light scattering technique (DLS) shows that the average size of prepared AmB-CNPs was 95.5 nm. Free AmB presented very low efficacy (only 65% and 67% inhibition of the promastigotes and axenic amastigotes parasite load), whereas AmB-CNPs exhibited 90% and 84% parasite inhibition after 72 h incubation. The AmB-CNPs exhibited significantly higher efficacy than free AmB in terms of reduction in parasite viability. Half-maximal inhibitory concentration (IC50) measured values of the AmB-CNPs were significant lowers than free AmB.

Conclusions: The present data indicated that AmB-CNPs exhibited vigorous anti-leishmanial activity than free AmB by dose and time-dependent manner. This formulation can be used for local therapy of CL after in vivo efficacy conformational studies.

Keywords: Amphotericin B, Antileishmanial activity, Cutaneous leishmaniasis, Chitosan nanoparticles, Drug delivery, MTT assay

Background

Leishmaniasis is one of the most common neglected tropical parasitic diseases caused by the genus *Leishmania* disseminated by female phlebotomine sandflies (Bennai et al. 2018). According to the world health organization (WHO) report in 2017, this disease threatened more than one billion people worldwide, including

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97 endemic countries, due to the absence of a vaccine and effective chemotherapy (Serban 2019). In terms of morbidity and mortality, leishmaniasis is the third most common parasitic disease among neglected tropical diseases (Reguera et al. 2019). The disease appears in three main infectious types cutaneous, mucocutaneous, and visceral leishmaniasis; among these, the cutaneous type is predominant (Oliveira et al. 2021). About 15 Leishmania species cause CL, mainly infecting the human body's exposed parts (Rather et al. 2021). Approximately 0.7 to 1.2 million cases of CL have been reported annually around the globe, with more than 90% of cases of CL are prevalent in Afghanistan, Algeria, Iran, Iraq, India, Pakistan, Saudi Arabia, Syria, Brazil, and Peru (Soltani et al. 2019). CL appears in different clinical appearances, including small self-healing lesions but scarring skin and even gross lesions leading to considerable disfigurements, such as diffused mucocutaneous leishmaniasis (DMCL) and mucocutaneous leishmaniasis (MCL) (Ballart et al. 2021). In Pakistan, CL caused by *L. tropica* is a significant health concern, especially in Khyber Pakhtunkhwa (KPK) province (Kämink et al. 2021). Until now, anti-leishmanial vaccines are not available, and chemotherapy is the only option to combat the disease (Ikeogu et al. 2020). The currently recommended anti-leishmanial drugs such as antimonials compounds, miltefosine, amphotericin B, pentamidine, and paromomycin have been associated with considerable disadvantages, including high costs outlay, toxicity, poor bioavailability, and the emergence of drug resistance *Leishmania* spp in recent years. Hence, there is an urgent need for safe, efficient, and low-cost novel alternative treatment options to cope with leishmaniasis (Garrido-Jareño et al. 2020). Nanomedicine is applying nano-scale medicine, which has recently attracted the attention of researchers to treat infectious diseases (Bezerra-Souza et al. 2021). Nano-drug delivery systems (NDDS) have been widely used in various fields as a vehicle for the targeted delivery of loaded drugs to minimize their toxicity and increase the therapeutic potential of the drugs. Drug delivery systems may also reduce the required therapeutic dosages of the loaded drugs to eradicate the intracellular parasites residing within the macrophages (Patra et al. 2018). Up till now, several drug-nanoparticle formulations have been investigated against leishmaniasis both in vitro and in vivo (Riaz et al. 2020; Rebouças-Silva et al. 2020). Polymeric nanoparticles synthesized by the ionic cross-linking technique are of great concern as NDDS because of their low cost, environmental and easy preparation, and long-term stability at room temperature (Krishnamurthy et al. 2015). Among various NDDS, Chitosan NPs have gained significant attention in biomedical sciences as they are non-toxic, biodegradable, and biocompatible (Saeed et al. 2020). The Food and Drug Administration (FDA) approved the chitosan polymer for wound dressing (Matica et al. 2019). The antibacterial, antiviral, and anti-leishmanial activity of the CNPs prepared by the ionic gelation method have been investigated previously. The antimicrobial activity of the chitosan and chitosan NPs is due to the interactions between the positively charged ammonia groups of the chitosan and the negatively charged cell membrane of microorganisms (Alqahtani et al. 2020; Ke et al. 2021).

Amphotericin B deoxycholate is used as a second-line anti-leishmanial agent for the treatment of leishmaniasis. Studies showed that AmB interferes with the synthesis of ergosterol present in the cell membranes of the *Leishmania* spp, resulting in membrane damage (Riezk et al. 2020). However, the use of AmB deoxycholate is restricted due to severe side effects, including nephrotoxicity, liver damage, hemolysis, nausea, and poor solubility. In order to overcome the toxicity and emergence of drug resistance issues associated with conventional AmB administration, a liposomal formulation was also prepared to cope with the event of drug intolerance or toxicity. The liposomal form of the AmB is widely used for the treatment of VL and is also reported to have good efficacy in CL patients. However, high cost, the requirement of the cold chain, and a change in drug content upon storage limited its clinical use (Silva-Carvalho et al. 2020).

In the present study, we report the synthesis, characterization, and in vitro antileishmanial effect of chitosan-coated AmB formulation. We demonstrated substantial anti-leishmanial activity of AmB-CNPs than free AmB against promastigotes and axenic amastigotes forms of *L. tropica* in terms of in vitro parasite viability reduction.

Materials

Culture media and compound

RPMI 1640 and M199 media were acquired from Sigma-Aldrich, USA. Acetic acid, TPP, and low molecular weight chitosan polymer (MW, 120 kDa) were supplied by Merck, Germany. Amphotericin B, Dimethyl sulfoxide (DMSO), and MTT dye were from Sigma Aldrich, USA. Temperature deactivated fetal bovine serum (FBS) was attained from Thermo Fisher Scientific, US. The antibiotics penicillin/streptomycin was received from Scharlau, Spain. All the other solvents used were of analytical grade (AG) and purchased locally.

Methods

Culturing of parasites

The experimental culture of *L. tropica* was obtained from the Department of Biotechnology, Quaid e Azam University, Pakistan. Promastigotes of *L. tropica* were cultured

in RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin (100 U/mL), streptomycin (100 mg/mL) solution in 20 cm2 culture flasks at 24 ℃ for 7 days (Siripattanapipong et al. 2019).

Preparation of AmB-CNPs

Chitosan NPs were formed by the ionic gelation method (Shafiei et al. 2019). Different concentrations of chitosan polymer (1, 2, 3 mg/mL) were dissolved in 1% *v*/*v* acetic acid solution to synthesize CNPs. NPs were formed by adding TPP solution (in the concentration of 0.75 mg/mL) dropwise with a pipette to chitosan solution, stirred on a magnetic stirrer, and kept overnight at 25 ℃, followed by sonication for 15 min. AmB-CNPs were prepared spontaneously upon dropwise adding TPP solution to chitosan solution containing 4 mg/mL AmB drug, under magnetic stirring at room temperature. The solution was mechanically stirred for 4 h. The resulting NPs suspensions were centrifuged at 15,000 g for 1 h. In the end, nanoparticles were washed with ultrapure- water and dried.

Physicochemical characterization

Scanning electron microscopy

The morphological analysis and size of solid-state AmB-CNPs were performed using a high-resolution scanning electron microscope (SEM) (TESCAN VEGA- 3, New York, USA). The samples of dried particles were covered with a carbon coating, with an accelerating voltage of 20 kV and a counting time of 1 min to assess the morphology of AmB-CNPs. Then, the particle images were observed at 10,000 to 40,000× magnification power.

Determination of average size and surface charge

The dynamic light scattering (DLS) method was employed to determine the average size of AmB-CNPs. About 300 µL of nanoparticles suspension was filled directly into the cuvette of the Zetasizer instrument by placing it in the device. The zeta potential of the nanoparticles was assessed by using a Zetasizer Nano ZS apparatus (ZEN0040, Malvern Instruments, UK). Operating conditions during the experiment were as follows: temperature 25 ℃, with a scattering angle of 90°.

In vitro release of AmB from CNPs

Synthesized AmB-CNPs were examined for in vitro drug release activity. Prepared AmB-CNPs were dissolved in a beaker containing 5 mL Tris–HCL buffer solution. The sample was sonicated at 100 rpm for 72 h at room temperature. At predetermined time periods, 0, 0.5, 1, 2, 4, 6, 12, 20, 24, and 48 h samples were centrifuged at 15,000 g for 40 min and the temperature was adjusted to 14 °C. The supernatant was discarded and replaced with an equal amount of fresh PBS solution. In the end, the amount of AmB released was observed by a UV–Vis spectrophotometer at 270 nm. The release concentration of the drug at each time interval was calculated using the cumulative calibration curve method.

Encapsulation efficacy and drug loading content

To observe the percentage of encapsulation efficacy (EE) and drug loading content (DLC) of AmB-CNPs, the NPs were centrifuged at 20,000 g for 30 min at a controlled temperature of 4 ℃. The experiment was performed in triplicate. The EE and DLC of the synthesized nano-formulation were then calculated by using the equations as follows:

$$\% \text{ EE} = [(A - B)/A] \times 100 \tag{1}$$

where*A* is the total volume of AmB utilized to synthesize nanoparticles (mg) and *B* is equivalent to the free AmB calculated in the supernatant in mg.

$$\% \text{ DLC} = [(A - B)/C] \times 100 \tag{2}$$

where *A* is the total volume of AmB utilized to synthesize nanoparticles (mg) and *B* is equivalent to the volume of free AmB calculated in the supernatant in mg, while *C* is the mass of nanoparticles in the supernatant.

Cytotoxicity study on promastigotes

Cytotoxicity of AmB-CNPs on *L. tropica* promastigotes was investigated using the tetrazolium dye assay (MTT) as described earlier (Lima et al. 2017). Stationary-phase promastigotes (1 × 107 cells/mL) were seeded in 96 well plates and incubated with six different concentrations (50, 40, 30, 20, 10, and 5 µg/mL) of AmB-CNPs for 24 h, 48 h, and 72 h. Later on, 20 µL of MTT dye (5 mg/mL) was added to each well and incubated the plate for a further 4 h at 37 ℃. After incubation, the purple-colored formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO) and centrifuged at 3000 g for 5 min. Optical density (OD) was evaluated at 570 nm using an ELISA reader (Thermo Scientific Microplate Reader). Miltefosine was used as a positive control. The IC50 values of AmB-CNPs and free AmB were calculated by ELISA machine and compared using the GraphPad Prism software (version 5.0). The experiments were performed in triplicate, and data are expressed as mean ± SD.

The percentage of viability was calculated using the following formula:

$$\text{Viable cells \% : } [(\text{AT} - \text{AB})/(\text{AC} - \text{AB})] \times 100.$$

where AB is the absorbance of the blank sample, AC is the absorbance of the negative control, and AT is the absorbance of the treated samples.

Cytotoxicity study on axenic amastigotes

In vitro, antileishmanial activities of AmB-CNPs and free AmB were assessed against axenic amastigotes of *L. tropica* (Dias-Lopes et al. 2021). Initially, the promastigotes were incubated at 37 ℃ in a 5% CO_2 incubator. This results in the conversion of promastigotes form into axenic amastigotes type. Afterward, the amastigotes were transferred into an ELISA plate and then exposed to the same concentrations of AmB-CNPs and free AmB as used for promastigotes.

Statistical analysis

The IC50 values were determined using sigmoid dose–response curves using GraphPad Prism version 8.0 for Windows (GraphPad Software, USA). The IC50 value of AmB-CNPs was lower than the free AmB drug.

Results

Preparation of nanoparticles

The synthesis of AmB-CNPs, chitosan polymer, TPP solution, and AmB drug was mixed using an ionotropic gelation method. Briefly, the aqueous solution of AmB was mixed with chitosan and 1% acetic acid solution; in last, the TPP was added dropwise to synthesized AmB-CNPs. This reaction results in a light yellow color solution of nanoparticles (Fig. 1a, b). The solution was then centrifuged, and D-Trehalose was added to the solution.

Characterization of AmB-CNPs

The physicochemical characterization of AmB-CNPs was evaluated using different methods, involving UV–visible spectroscopy, SEM, DLS, zeta potential, DLC, and encapsulation efficacy. The synthesis of NPs was demonstrated through UV–visible spectroscopy, which is the easiest method to confirm nanoparticles synthesis. The absorbance spectra were recorded at 320 nm. A broad absorption band intensity was examined for chitosan polymer, whereas CNPs show sharp intensity spectra as depicted in Fig. 2.

For the morphological studies of AmB-CNPs, SEM illustrations of the prepared NPS revealed spherical primary particles with a mean size of 118 nm, as shown in Fig. 3. The average size of NPs determined by DLS technique is shown in Fig. 4. Zeta potential or surface charge plays a vital role in the physical stability and interaction of nanoparticles with biological surfaces. AmB-CNPs possessed a stable positive surface charge of ($+6.21 \pm 2.02$ mV) as represented in Fig. 5. The intense interaction between positively charged chitosan nanoparticles and negatively charged microbial membranes may be due to this surface charge.

The cumulative release concentration of AmB drug from the CNPs was divided into two stages (Fig. 6). In the first phase, at a pH of 7.4, the drug was immediately released from the nanoparticles in the first 6 h. This results in a 42% release of the drug. In the second stage, AmB released slowly from the NPs up to 48 h resulting in 68% of the drug.

DLC means an appropriate amount of drug present in a defined quantity of the NPs. At the same time, EE is the amount of drug in percentage that has been encapsulated into the NPs. EE can be computed by (initial amount of drug added-free non encapsulated drug) divided by the initial amount of drug added. The DLC and EE of the AmB-CNPs were 48% and 88%, respectively.

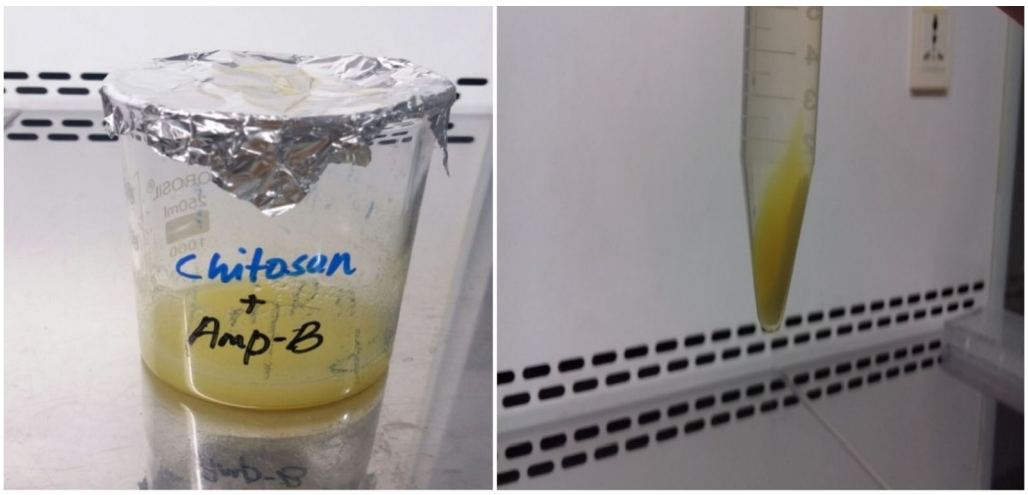

Fig. 1 **a** AmB-CNPs in solution form, **b** AmB-CNPs in pellet form

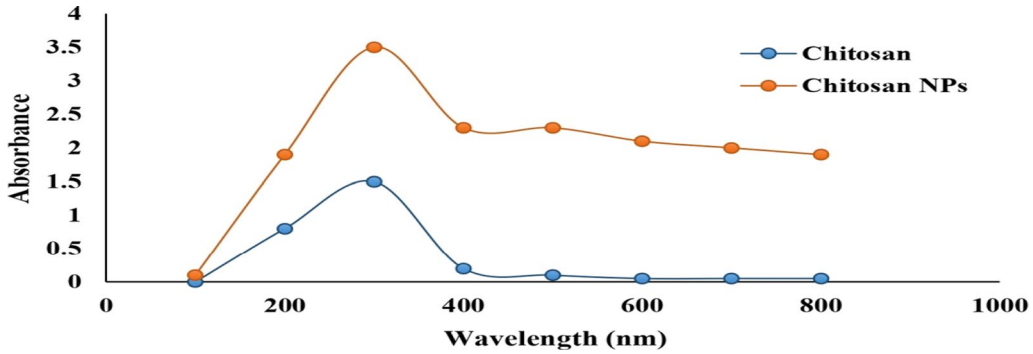

Fig. 2 UV–visible spectra of chitosan and CNPs

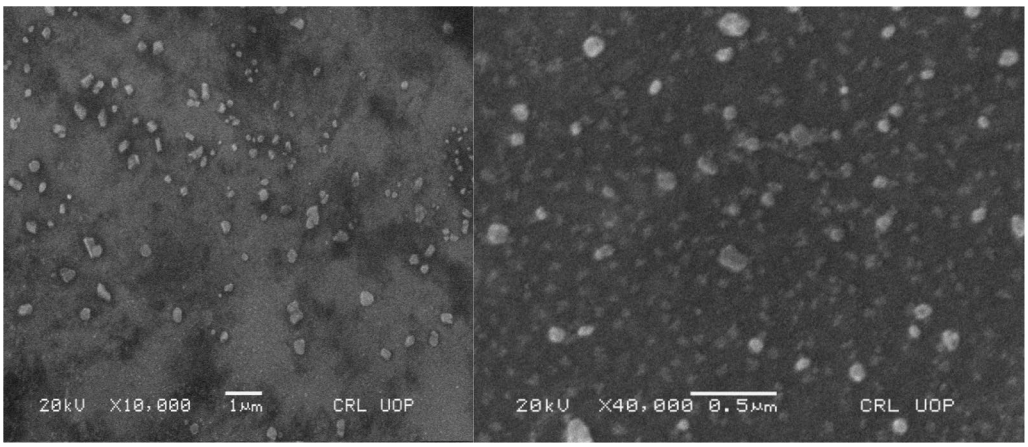

Fig. 3 SEM images of AmB-CNPs at ×10,000 and ×40,000 magnifications

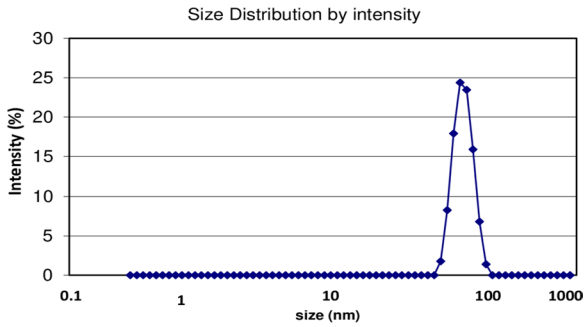

Fig. 4 Average size distribution by intensity of AmB-CNPs by using dynamic light scattering technique

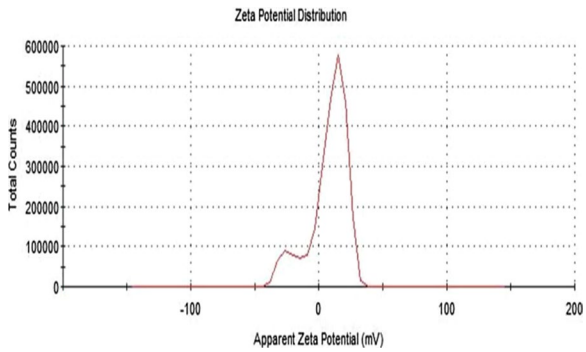

Fig. 5 Zeta potential distribution of amphotericin B-loaded chitosan nanoparticles (AmB-CNPs)

Anti-promastigotes cytotoxicity by MTT assay

The promastigotes viability assay was evaluated by using MTT colorimetric assay. Briefly, *L. tropica* promastigotes were incubated with six concentrations (50, 40, 30, 20, 10, and 5 µg/mL) of both AmB-CNPs and free AmB at 24, 48, and 72 h, respectively. Both the loaded and free drugs have time and dose-dependent parasite viability inhibition at different time intervals. However, AmB-CNPs show a maximum reduction of promastigotes viability rate (90%) at 50 µg/mL after 72 h of incubation (Table 1). The viability of promastigotes exposed to various

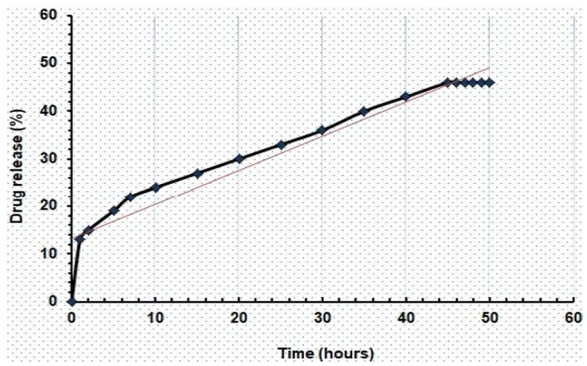

Fig. 6 Amphotericin B release pattern from chitosan nanoparticles (chitosan 3 mg/mL, TPP 2 mg/mL, pH of the medium 7.4)

Table 1 Promastigotes viability percentage after treatment with AmB-CNPs

AmB-CNPs concentration (µg/mL)	Promastigotes percentage (%) viability		
	24 h	48 h	72 h
50	48	32	10
0	55	48	18
30	64	51	22
20	66	55	25
10	69	57	29
05	78	61	35

Table 2 Promastigotes viability percentage after treatment with conventional AmB drug

AmB concentration (µg/mL)	Promastigotes percentage (%) viability		
	24 h	48 h	72 h
50	65	47	35
40	69	55	38
30	73	58	41
20	78	61	44
10	80	66	52
05	84	68	62

dilutions of free AmB is also shown in Table 2. The IC50 values of AmB-CNPs and free AmB are shown in Table 3.

Leishmanicidal effects on axenic amastigotes

The in vitro axenic amastigotes effects of AmB-CNPs and free AmB were also evaluated against *L. tropica* at 37 ℃ in a 5% CO2 incubator. The axenic amastigotes culture was incubated with six dilutions (50, 40, 30, 20, 10, and 5 µg/mL) of AmB-CNPs and free AmB at 24, 48, and 72 h

Table 3 Mean IC50 values of amphotericin B-loaded chitosan nanoparticles (AmB-CNPs)

Treatments	Promastigotes (µg/mL)	Amastigotes (µg/mL)
AmB-CNPs	0.1275 ± 0.08	0.3810 ± 0.18
Free AmB	0.5427 ± 0.37	0.6024 ± 0.41

Data are expressed as mean ± SD ($n = 3$)

Table 4 Amastigotes viability percentage after treatment with AmB-CNPs

AmB-CNPs concentration (µg/mL)	Amastigotes percentage (%) viability		
	24 h	48 h	72 h
40	52	35	16
30	58	55	24
20	69	58	28
10	73	61	31
05	75	63	34
2.5	81	65	41

Table 5 Amastigotes viability percentage after treatment with conventional AmB drug

AmB concentration (µg/mL)	Amastigotes percentage (%) viability		
	24 h	48 h	72 h
40	67	48	33
30	72	56	40
20	74	58	44
10	80	63	45
05	82	67	54
2.5	86	69	65

at 37 ℃ with a pH of 5.5. Table 4 represents the cytotoxicity of AmB-CNPs on *L. tropica* axenic amastigotes form at different incubation periods. The maximum dose and time-dependent parasite viability reduction for AmB-CNPs against amastigotes form was 84% after 72 h incubation. On the other hand, free AmB presents anti-axenic amastigotes viability inhibition rate of only 67% (Table 5). AmB-CNPs have potent anti-axenic amastigotes activity than free AmB in terms of reduction in parasites viability percentage.

Discussion

Nanotherapy can be used as a novel alternative treatment to overcome various problems related to leishmanicidal drugs, such as low solubility, painful parenteral administration, and adverse effects (Baranwal et al. 2018). In this study, we examined for the first time the anti-leishmanial effects of chitosan nanoparticles loaded with AmB

and free AmB, one of the most potent and commercially available antileishmanial drugs on *L. tropica* promastigotes and amastigotes in vitro. Chitosan polymer, acquired from the deacetylation of chitin, is one of the most famous drug delivery carriers in nanotechnology. Previous studies have investigated its most potent antibacterial, antiviral, antileishmanial, and antifungal effects (Loiseau et al. 2020; Sudatta et al. 2020). Synthesis of CNPs was performed via the ionic gelation technique. The interaction between cationic chitosan polymer with TPP anions results in the formation of CNPs. A recent study by Hadidi et al. shown that CNPs were mostly synthesized via this technique (Hadidi et al. 2020). This method also results in high nanoparticle yields with a more potent antimicrobial efficacy (Lazaridou et al. 2020). The primary purpose of this study was to develop chitosan-based drug delivery formulation for the treatment of *L. tropica*. The structural morphology of the NPs showed that AmB-CNPs exhibit a spherical form, with a mean particle size of 118 nm. The size of nanoparticles was also assessed by DLS which was found to be in the range of 95.5 nm. In the earlier study (Bhattamisra et al. 2020), it was found that drug-loaded CNPs prepared via the ionic gelation method were 200–300 nm in size. Moreover, nanoparticles larger than 200 nm are easily bound and phagocytized by reticuloendothelial cells used as host cells by the intracellular parasites (Mosaiab et al. 2019). The EE and DLC of AmB-CNPs were observed as 88% and 48%. These data are consistent with previously published work (Ashvini et al. 2019; Shi et al. 2014).

The present study compared the time and dose-dependent inhibitory effects of AmB-CNPs and free AmB against both forms of *L. tropica*. The obtained IC50 values for AmB-CNPs were 0.1275 µg/mL and 0.3810 µg/mL, while the IC50 values of free AmB were 0.5427 µg/mL and 0.6024 µg/mL, respectively. In the past, different studies have been conducted to evaluate drug-loaded nanoparticles against leishmaniasis with encouraging results (Unciti-Broceta et al. 2015; Valle et al. 2019). In a recent study, Mostafavi et al. loaded the AmB drug on noisome-loaded selenium nanoparticles and compared its cytotoxicity against *L. tropica* in vitro (Mostafavi et al. 2019). They observed that free AmB and AmB-loaded noisome have dose and time-dependent effects under in vitro conditions. Their results are consistent with our results. In another study, Mehrizi et al. observed the cytotoxic effects of AmB-loaded dendrimers and betulinic acid chitosan combinations on *L. tropica* (Mehrizi et al. 2019). Their results are practically in line with our results. Casa et al. investigated the inhibitory effects of AmB-loaded bovine serum albumin nanoparticles and free AmB drug in murine cutaneous leishmaniasis (Casa et al. 2018). Their findings proved that the loaded drug has more potency than the free drug against the Leishmania parasite. In a previous study, Ammar et al. also investigated the toxicity of nanoparticles loaded amphotericin b drug for the local treatment of cutaneous leishmaniasis using MTT assay (Ammar et al. 2019). They observed that NPs loaded AmB drug was more cytotoxic than free AmB drug. These results comply with our results.

Conclusion

The present study demonstrates that a chitosan-based drug delivery system is helpful for the in vitro clearance of *L. tropica*. Herein, Chitosan NPs were synthesized by the ionic gelation method, and AmB was encapsulated in CNPs during the synthesis process. The developed AmB-CNPs had a small size range of (118 nm), high encapsulation efficacy (88%), with a stable positive zeta potential of ($+6.21 \pm 2.0$ 2 mV), respectively. The average size of AmB-CNPs was 95.5 when analyzed by DLS. The synthesized nano-formulation exhibited strong antileishmanial efficacy on *L. tropica* promastigotes and axenic amastigotes. The obtained IC50 values of AmB-CNPs against promastigotes and amastigotes were significantly lower than conventional AmB and the control drug miltefosine. AmB-CNPs also presented a significant antileishmanial effect in reducing parasite viability compared to free AmB drugs. In conclusion, AmB-CNPs may be a suitable alternative treatment candidate for eradicating drug-resistant Leishmania parasites.

Abbreviations

TPP: Tripolyphosphate; NPs: Nanoparticles; AmB: Amphotericin B; SEM: Scanning electron microscopy; EE: Encapsulation efficacy; DLC: Drug loading content; AmB-CNPs: Amphotericin loaded chitosan nanoparticles; RPMI: Roswell Park Memorial Institute Medium; DMSO: Dimethyl sulfoxide; NDDS: Nano-drug delivery system; FBS: Fetal bovine serum; OD: Optical densities.

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Authors' contributions

RUK, MK, AS, MK, conducted the experiments. HB, RUK, SUK, MK wrote the draft of the manuscript. SU, MK, MK, AS, RUK, BA, MK, and AA reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All the analyzed data are included in the submitted manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests
We have no conflict of interest to disclose regarding this manuscript. As a corresponding author, I confirm that the manuscript has been read and approved for submission by all the named authors.

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