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RESEARCH ARTICLE

Efficacy of pentamidine-loaded chitosan nanoparticles as a novel drug delivery system for *Leishmania tropica*

Khan, R.U.[1*], Khan, M.[1], Sohail, A.[1], Ullah, R.[2], Iqbal, A.[3], Ahmad, B.[4], Khan, I.U.[3], Tariq, A.[5], Ahmad, M.[6], Said, A.[7], Ullah, S.[8], Ali, A.[1], Rahman, M.U.[1], Zaman, A.[3], Bilal, H.[9*]

[1]Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar, Pakistan
[2]Department of Dairy Technology, Faculty of Veterinary and Animal Sciences, University of Agriculture Dera Ismail Khan, Pakistan
[3]Faculty of Veterinary and Animal Sciences, Gomal University, Dera Ismail Khan, Pakistan
[4]Department of Animal Sciences, Faculty of Veterinary and Animal Sciences, MNS University of Agriculture Multan, Punjab, 25000, Pakistan
[5]Directorate of Livestock and Dairy Development Department, Khyber Pakhtunkhwa, Pakistan
[6]Department of Animal Sciences, Quaid-E Azam University Islamabad, Pakistan
[7]Department of Parasitology, University of Agriculture Faisalabad, Pakistan
[8]Department of Biochemistry, Hazara University Mansehra, Pakistan
[9]Second Affiliated Hospital of Shantou University Medical College, Shantou, China
*Corresponding author: Rahatullahkhan@gu.edu.pk (Khan, R.U.); bilal.microbiologist@yahoo.com (Bilal, H.)

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ABSTRACT

The present study compares the *in vitro* effects of nanoparticles loaded pentamidine drug and conventional pentamidine on *Leishmania tropica*. Herein, pentamidine-loaded chitosan nanoparticles (PTN-CNPs) have been synthesized through an ionic gelation method with sodium tripolyphosphate (TPP). Next, the physical characteristics of PTN-CNPs were determined through the surface texture, zeta potential, *in vitro* drug release, drug loading content (DLC), and encapsulation efficacy (EE) and compared its efficacy with free pentamidine (PTN) drug against promastigotes and axenic amastigotes forms of *L. tropica in vitro*. The PTN-CNPs displayed a spherical shape having a size of 88 nm, an almost negative surface charge (-3.09 mV), EE for PTN entrapment of 86%, and *in vitro* drug release of 92% after 36 h. *In vitro* antileishmanial activity of PTN-CNPs and free PTN was performed against *Leishmania tropica* KWH23 promastigote and axenic amastigote using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyletetrazolium bromide (MTT) assay. It was observed that the effect of PTN-CNPs and free PTN on both forms of the parasite was dose and time dependent. Free PTN presented low efficacy even at higher dose (40 µg/ml) with 25.6 ± 1.3 and 26.5 ±1.4 mean viability rate of the promastigotes and axenic amastigotes, respectively after 72 hrs incubation. While PTN-CNPs showed strong antileishmanial effects on both forms of parasite with 16 ± 0.4 and 19 ± 0.7 mean viability rate at the same higher concentration (40 µg/ml) after 72 hrs incubation. Half maximal inhibitory concentration (IC50) values of PTN-CNPs toward promastigotes and amastigotes were obtained as 0.1375 µg/ml and 0.1910 µg/ml, respectively. In conclusion, PTN-CNPs effectively inhibited both forms of the *L. tropica*; however, its effect was more salient on promastigotes. This data indicates that the PTN-CNPs act as a target drug delivery system. However, further research is needed to support its efficacy in animal and human CL.

Keywords: *L. tropica*; Chitosan nanoparticles; Pentamidine; MTT assay; hemolysis assay.

INTRODUCTION

Leishmaniasis is a neglected vector-borne disease resulting from a protozoan parasite that belongs to the Trypanosomatidae family. The parasite is transmitted by a female phlebotomine sandfly taking blood as a meal (Garrido-Jareño *et al*., 2020; Elaagip *et al*., 2020). The disease exists in various clinical forms, such as cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL). Among these, CL is the most predominant (Khokhar *et al*., 2022).

CL is restricted to the skin and caused by more than ten species of *Leishmania* parasite. *L. amazonensis*, *L. guyanensis*, *L. brazilliensis*, and *L. maxicana* caused CL in the new world while *L. tropica*, *L. major*, and *L. aethiopica* cause old-world cutaneous leishmaniasis (OWCL) (Sohail *et al*., 2021). CL caused by *L. tropica* and *L. major* is a significant health issue worldwide, with an annual incidence rate of 1.5 million. (Eddaikra *et al*., 2018). About 90% of CL cases are reported from Saudi Arabia, Pakistan, Afghanistan, Iran, Iraq, Peru, and Algeria (Khazaei *et al*., 2015; Khan *et al*., 2016).

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For treating leishmaniasis, the first-line antimonial drugs, including Glucantime and Pentostame, have been the principal therapy over the last 70 years. These drugs have many disadvantages, such as painful parenteral administration, limited clinical efficacy, drug resistance, and severe side effects in the form of hepatotoxicity, cardiotoxicity, hypotension, and pancreatitis (Yesilova *et al*., 2016; Erber *et al*., 2020). Due to high toxicity and cost of the first line of drugs, amphotericin B (AmB), pentamidine, and paromomycin are recommended as a second-line treatment. However, these drugs remained unsatisfactory because of their high cost, drug- resistance, adverse reactions, and teratogenic effects (Tahir *et al*., 2019).

An aromatic diamidine compound PTN was primarily used to treat the acute phase of sleeping sickness since the 1940s (Babokhov *et al*., 2013). In Suramine and French Guiana, PTN is still used as the first line of drug to treat leishmaniasis caused by *L. guyanensis* (Diro *et al*., 2019). Its mechanism of action is unknown, but it appears to inhibit the parasite's DNA, RNA and protein synthesis (Basselin *et al*., 2002). However, *Trypanosoma* parasite developed resistance against PTN due to changes in the intracellular concentration of polyamine and arginine (Reigada *et al*., 2017). Therefore, safe, inexpensive, and effective drug delivery formulation is needed for their curing. Nanotechnology is an important field of cutting-edge technology dealing with synthesizing particles ranging from 1 to 100 nm in size. Nano-drug delivery systems (NDDSs) have been emerging as novel approaches to effectively treat intracellular parasites. The NDDSs are superior to traditional treatments as these nanocarriers can safeguard therapeutic drugs against oxidoreduction and enzymatic reactions and increase bioavailability through the sustained release of loaded drugs (Ullah *et al*., 2021; Raj *et al*., 2022). In various DDS, polymeric NPs have gained importance in treating intracellular pathogens (Oliveira *et al*., 2021). Chitosan is a biodegradable, non-toxic, biocompatible, and positively charged mucoadhesive polymer obtained by deacetylation of chitin shells of crustaceans with sodium hydroxide (Vichare *et al*., 2020). The proposed mechanism of action is that chitosan binds to the negatively charged microbial DNA and inhibits DNA transcription (Loiseau *et al*., 2020; Riezk *et al*., 2020).

In the present study, we described the synthesis, characterization, and anti-leishmanial effect of PTN-CNPs on *L. tropica*.

MATERIALS AND METHODS

Ethical approval

Ethical approval was given by the Ethics committee of Institute of Basic Medical Sciences Khyber Medical University, Pakistan (Bioethics approval No. Dir/KMU-EB/IV/000512).

Materials

The chemicals used in the present study were: RPMI 1640 and M199 media (Sigma- Aldrich, ST. Louis, U.S.A.), TPP, acetic acid, and low molecular weight chitosan polymer (MW, 120 kDa) (Merck, Germany), PTN isethionate, AmB, and MTT dye (Sigma Aldrich, St. Louis Missouri, USA), temperature deactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Massachusetts, USA), Penicillin/ streptomycin (Scharlau, Barcelona, Spain). All the solvents used in the present study were research grade and procured locally.

Leishmania Parasites and culturing

L. tropica promastigotes (KWH23) were obtained from the Department of Zoology, University of Peshawar, Pakistan. RPMI-1640 medium was prepared by adding 10 mL of FBS to 90 mL liquid RPMI 1640 media inside the sterile culture flask. The promastigotes were cultured at RPMI 1640 supplemented with penicillin (200 IU/mL), streptomycin (100 µg/mL), and 10 % heat-inactivated FBS (Krobthong *et al*., 2022). Axenic amastigotes were cultured at 37°C in M199, with acidic pH (5.5), in a 5% CO_2 incubator (Chanmol *et al*., 2019).

Synthesis of PTN-CNPs

The NPs were formed by using the ionic- gelation method as mentioned earlier (Shafiei *et al*., 2019). Briefly, chitosan polymer was measured and dissolved in a diluted aqueous solution of acetic acid 1% (w/v), stirred magnetically, and kept overnight at 25°C. The pH of the solution was maintained at 5.5 by adding a minimal solution of 0.5-M NaOH. After this, aqueous solution of TPP at a concentration of 0.5mg/mL was prepared by dissolving 0.5% (w/v) TPP in ultra-pure water. This solution was kept in a fridge for 5 h at 4°C. The NPs were synthesized spontaneously by adding 0.5% TPP solution to the NPs solution with a chitosan and TPP ratio of 6:1 (w/w). Concurrently, PTN was added to the NPs solution dropwise at a 3mg/mL concentration. The mixture was stirred magnetically for 60 min at 24°C. The NPs were separated from precipitants by centrifugation at 15000g for 1hr. D-Trehalose (3% w/v) was mixed into the reaction mixture to prevent particle aggregation. In the end, nanoparticles were stored at 5°C for characterization.

Characterization of NPs

PTN-CNPs were characterized for their size, morphology, surface charge, *in vitro* drug release, EE, and DLC.

UV- Visible spectroscopy

To confirm NPs synthesis, UV-visible spectra were obtained using a spectrophotometer (Shimadzu UV-visible 1800) at 320 nm.

Analysis of surface morphology and size

The surface morphology and size of PTN-CNPs were analyzed through high resolution scanning electron microscope (SEM) (KYKY-EM 3200 Shanghai China). An appropriate amount of freeze-dried NPs dispersion (10µl) was mounted on stabs and coated with colloidal gold under vacuum using JFC-1100. In the end, SEM images were obtained at the magnification of 30,000 to 60,000 X.

Determination of surface charge

The surface charge analysis of the PTN-CNPs was carried out using Nano Zetasizer (Malvern Instruments, UK) based on a dynamic light scattering technique. The freeze-dried NPs samples were prepared by dissolving 2mg of formulated NPs in 4mL of Milli-Q water. The stability of the NPs was assessed by determining the surface charge through a scattering angle of 90 at 25°C. The results were obtained by repeating the experiment in triplicate. The data obtained were expressed as the mean value.

***In vitro* drug release study**

The *in vitro* release study of PTN-CNPs was performed through the dialysis bag diffusion technique. For *in vitro* release of PTN from CNPs, both the nanoparticles suspension and drug solution were taken in the same quantity (10mg). Nanoparticles equal to 10 mg of the drug were mixed in Tris-HCL PBS (pH 6.4), placed in a dialysis bag with the addition of small dissolution media, and then sealed from both sides. This suspension was stirred on a magnetic stirrer at 100 rpm for 48 h at 37°C. At specified periods, samples were centrifuged at 13000 rpm for 15 min at 10°C. Supernatant was discarded, the samples were removed, and the same volume was replaced with an equal volume of fresh buffer medium. The samples were assessed by UV spectrophotometer at a wavelength of 270 nm.

EE and DLC analysis

The synthesized formulation was centrifuged at 20,000 g for 30 min at 15°C to determine the EE and DLC. The supernatant was collected, and the EE of PTN was calculated as follows:

$$\% EE = [(A-B)/A] \times 100$$

Where A is the total volume of PTN utilized to synthesize NPs (mg), B is equivalent to the volume of free PTN calculated in the supernatant in mg.

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

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

In vitro hemolysis test

Fresh venous blood was collected from five healthy donors, and a hemolysis assay of PTN-CNPs on human erythrocytes was performed as described elsewhere (Lima *et al*., 2017). Ethical approval was given by the Ethics committee of the Institute of Basic Medical Sciencesat Khyber Medical University, Pakistan (Bioethics approval No. Dir/KMU-EB/IV/000512). The blood was centrifuged at 800 x g for 15 minutes. Plasma was discarded, and erythrocytes were washed three times with 1X phosphate buffer at pH 7.4. To prepare RBCs stock suspension, 1 mL of the erythrocytes was mixed on 49 mL of phosphate buffer (pH=7.4), obtaining a 1:50 stock suspension. A hemolysis test was performed in 2 mL conical bottom tubes with varying concentrations of PTN-CNPs (12, 24, and 48 µg/ml). The tubes were incubated at 37°C for 2 h and centrifugation at 800 X g for 10 min. In the end, 100 µl of the supernatant was transferred to the ELISA plate, and the optical density (OD) was obtained at 540 nm. The experiment was adjusted to a final volume of 1000 µl. TritonX-100 and PBS were used as positive and negative controls, respectively.

The experiment was repeated three times, and the hemolysis percentages were calculated from the following formula:

$$\text{Percentage hemolysis} = \frac{\text{OD at 540 nm in the drug solution} - \text{OD at 540 nm in PBS}}{\text{OD at 540 nm in 0.1\% Triton X-100} - \text{OD at 540 nm in PBS}} \times 100$$

In vitro Anti-leishmanial assay

The promastigotes and amastigotes viability efficacy of free PTN and PTN-CNPs was determined by MTT assay (Lima *et al*., 2017). Briefly, 100µl of the RPMI 1640 medium containing logarithmic phase promastigotes suspension (1×10^6) was added to the 96 well culture plate. Then, the promastigotes were exposed to varying concentrations (40, 30, 20, 10, 5, and 2.5µg/ml) of free PTN and PTN-CNPs at 24°C. The treated cells were incubated for 24 h, 48 h, and 72 h. After incubation, 10 µl MTT dye (5mg/ml) was added per well, and the plate was kept at 24°C for 4 h in a dark place. MTT dye was removed by centrifugation of the plate at 3000 g for 3 min. The supernatant was discarded, and 200µl DMSO (dimethyl sulfoxide) was transferred to each well to dissolve the purple-color formazan crystals. The optical density (OD) was measured at 570 nm. Three wells with only 100µl of the culture medium were considered blank wells. Promastigotes with the culture medium without any treatment with PTN and PTN-CNPs were considered a control group. The amastigotes assay was also performed according to the same protocol described above, but the incubation was carried out at 37°C rather than at 24°C in a 5% CO2 incubator. The data are expressed as mean ± SD and the experiments were performed in triplicate. The viability percentage was calculated by using the following formula:

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

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.

Statistical analysis

The IC50 values were determined using sigmoid dose-response curves using the software GraphPad Prism version 9.0 for Windows (GraphPad Software, USA). The IC50 value of PTN-CNPs was lower than the free PTN drug.

RESULTS

Synthesis and characterization of PTN-CNPs

For the synthesis of PTN-CNPs, chitosan polymer, TPP solution, and PTN drug were mixed using ionotropic gelation. In short, the aqueous solution of PTN was mixed with chitosan and 1% acetic acid solution, and TPP was added dropwise to synthesized PTN-CNPs. This reaction results in the light yellow color solution of NPs.

Scanning electron microscopy

SEM was used to determine the surface morphology and size of the PTN-CNPs. The SEM micrograph (Figure 1) indicates that the synthesized drug-loaded NPs were spherical and found to have a size of 88 nm. The PTN-CNPs also displayed smooth surface morphology that may contribute to the sustained release of the loaded drug.

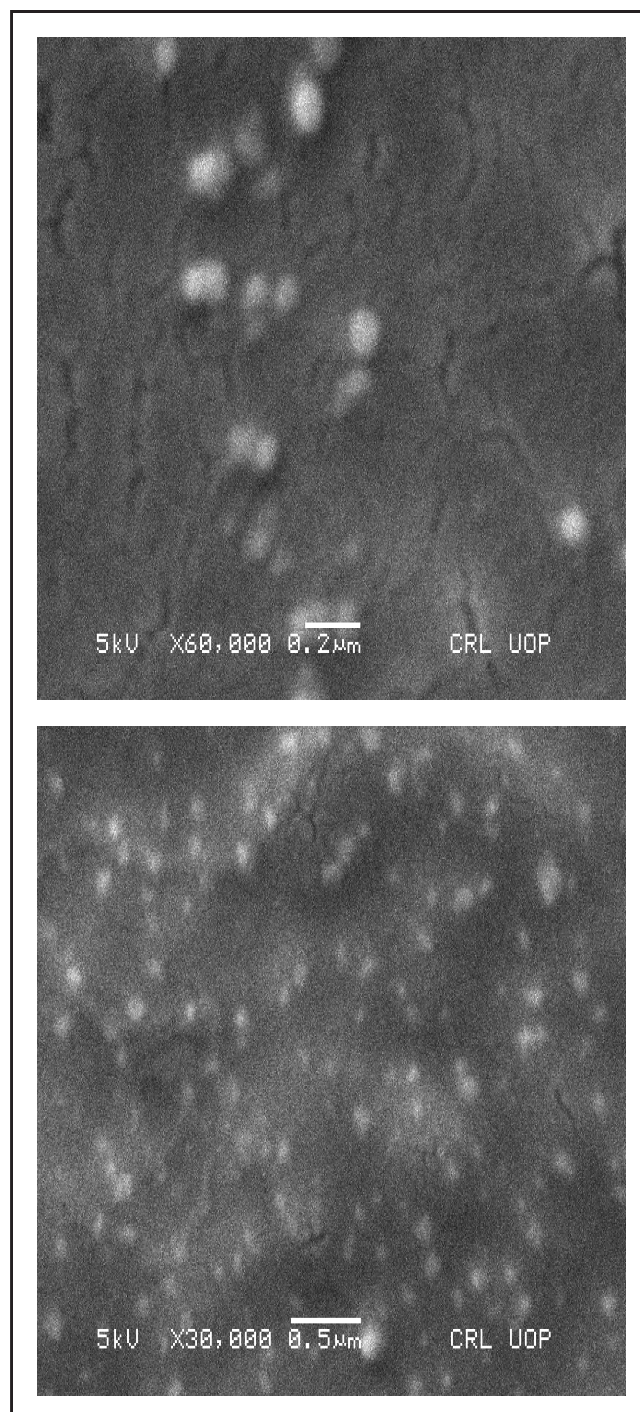

Figure 1. SEM micrograph of the PTN-CNPs with spherical morphology magnified 60,000X and 30,000X.

Zeta potential

Zeta potential of NPs plays a significant role in drug design concerning their stabilization and attachment to the biological surfaces. This surface charge is due to the solid electrostatic interaction of positively charged chitosan NPs with negatively charged microbial surfaces. PTN-CNPs exhibited a surface charge of (-3.09 mV), as shown in Figure 2.

PTN drug *in vitro* release

The cumulative release pattern of PTN from the nanoparticles was biphasic, as shown in Figure 3. Initially, at pH 7.4, 50% of the loaded drug is rapidly released in 9 h. In the second stage, the drug was constantly released from the nanoparticles, and around 92% was released from the nanoparticles from 24 h up to 36 h.

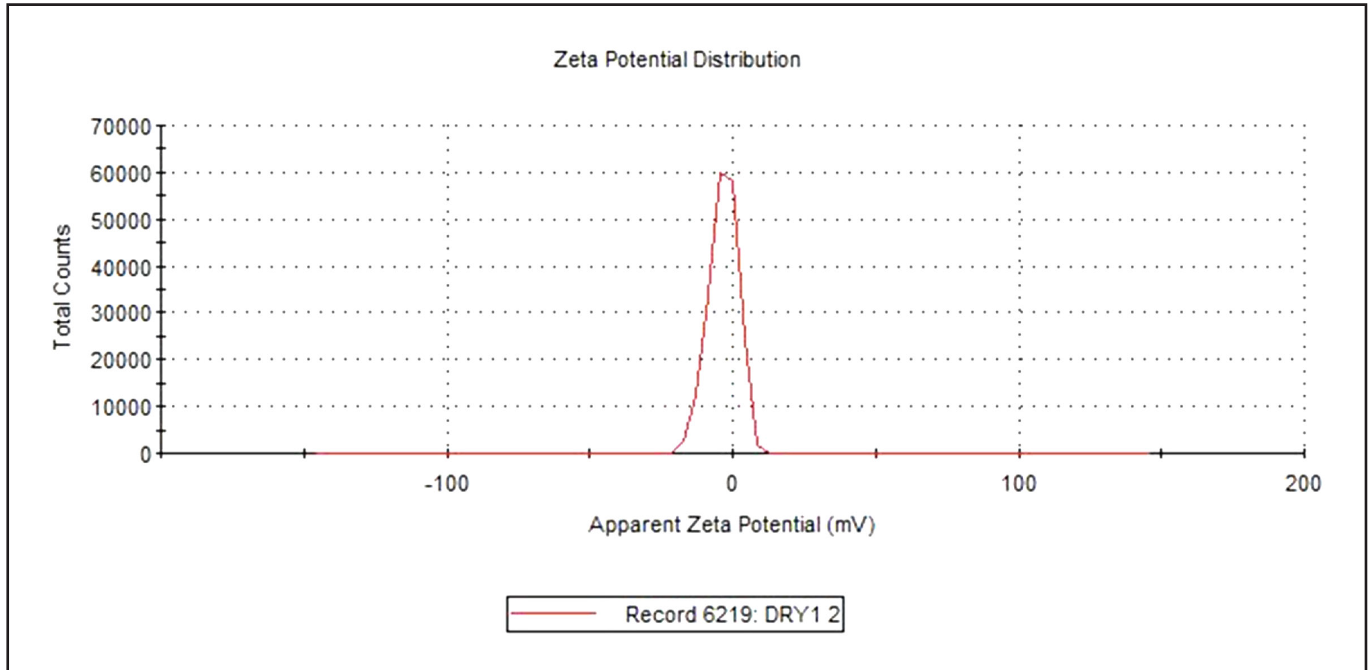

Figure 2. Zeta potential measurement of PTN-CNPs.

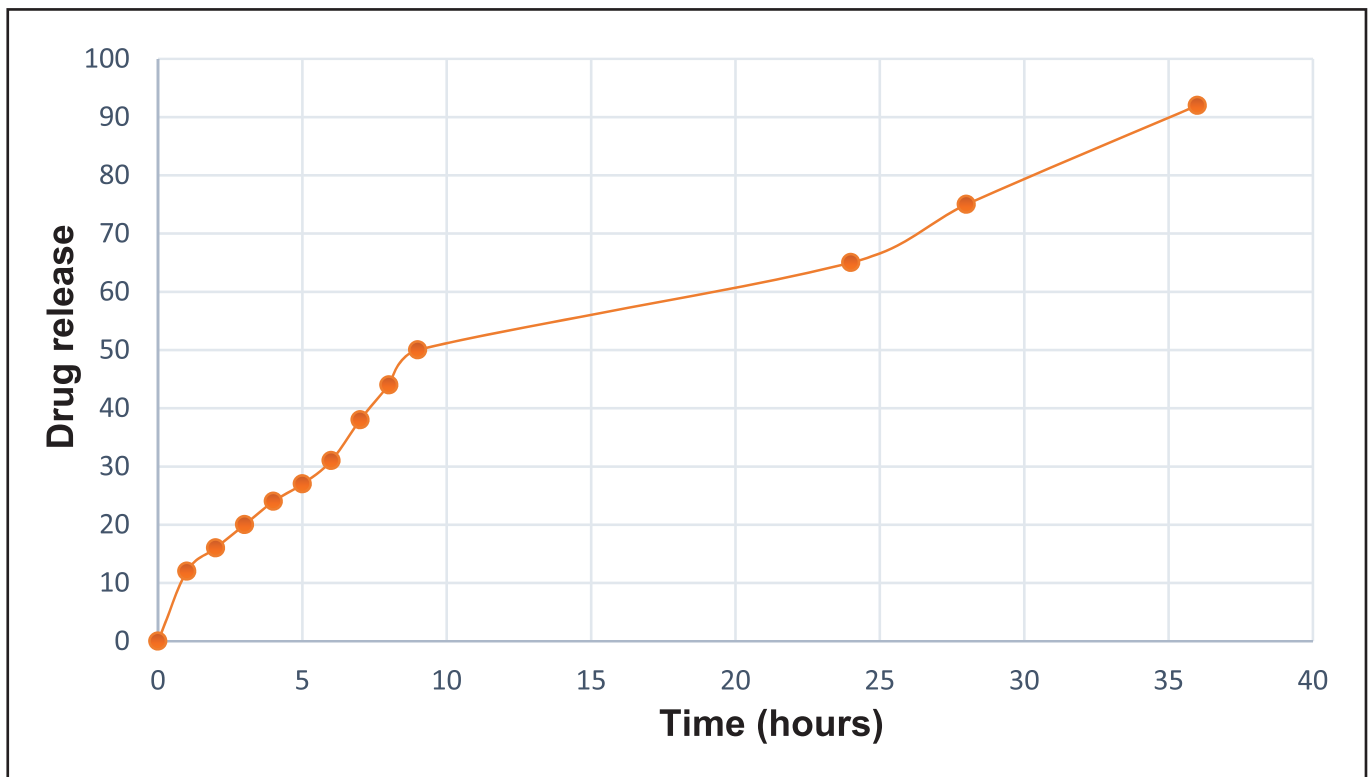

Figure 3. *In vitro* drug release pattern of PTN drug from chitosan nanoparticles at pH 7.4.

Hemolytic activity

Table 1 shows the mean ± SD values of the hemolysis assay obtained by calculating the OD of the spectrophotometer at 540 nm for PTN-CNPs at different concentrations. Our results shows that PTN-CNPs presented less hemolytic activity (1.73%, 2.82% and 4.00%) than conventional PTN (4.82%, 5.64% and 6.92%) at the same concentrations (12, 24 and 48 µg/ml). It was also noticed that the hemolytic efficacy was dose-dependent for both formulations. PBS (negative control) was non-hemolytic while TritonX-100 (positive control) showed 100% hemolysis, respectively. Clearly, for both the PTN-CNPs and conventional PTN the hemolysis caused by PTN-CNPs was much lower than free PTN.

EE and DLC

DLC means an appropriate amount of drug in a defined amount of the NPs. In contrast, EE is the amount of drug in percentage captured 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 PTN-CNPs were 44% and 86%, respectively.

In vitro cytotoxicity studies

The cytotoxic effect of PTN and PTN-CNPs on promastigotes and amastigotes of *L. tropica* was determined at six concentrations (40, 30, 20, 10, 5, and 2.5 µg/ml) for 24h, 48h, and 72h. The PTN-CNPs concentrations of 40 µg/mL after 72 h and the concentration of 2.5 µg/mL after 24h incubation showed the maximum and minimum percentage of cytotoxic effect on promastigotes Table 2.

The present study showed that the efficacy of both the loaded and free PTN drug was dose and exposure time dependent. The promastigotes percentage viability rate of free PTN is also shown in Table 3. Similarly, the viability percentage of the amastigotes forms of *L. tropica* after treatment with free PTN and PTN-CNPs are shown in Tables 4 and 5, respectively. The IC50 values for PTN-CNPs, free PTN, and AmB against promastigotes and amastigotes form of *L. tropica* are presented in Table 6.

DISCUSSION

Leishmaniasis is a significant public health issue worldwide, especially in underdeveloped countries, including Pakistan (Hussain *et al*., 2018). For treating this ignored parasitic infection, pentavalent antimonials such as glucantime and pentostam have been used for more than 60 years. In addition to pentavalent antimonials drugs, Miltefosine, AmB, and PTN are also identified as potential anti-leishmanial agents. All these treatment options are limited by their high cost, severe toxicity, and development of parasite resistance (Ahmed *et al*., 2020; Allotey-Babington *et al*., 2020). The current

Table 1. *In vitro* hemolysis assay of PTN-CNPs and free PTN at various concentrations

Solutions	Concentrations	OD at 570 nm (Mean ± SD)	Hemolysis %
PTN-CNPs	12 µg/ml	0.021 ± 0.0018	1.73%
	24 µg/ml	0.033 ± 0.0005	2.82%
	48 µg/ml	0.046 ± 0.0015	4.00%
Free PTN	12 µg/ml	0.055 ± 0.0020	4.82%
	24 µg/ml	0.064 ± 0.0010	5.64%
	48 µg/ml	0.078 ± 0.0030	6.92%
PBS (Negative control)		0.002 ± 0.008	0%
Triton-X 100 (Positive control)		1.10 ± 0.01	100%

Table 2. Mean promastigotes viability percentage after adding different concentrations of PTN-CNPs during the incubation times Data are expressed as the mean ± SD (n=3)

PTN-CNPs Concentrations (µg/ml)	Promastigotes percentage (%) viability		
	24h	48h	72h
40	20 ± 4.3	18 ± 0.8	16 ± 0.4
30	22 ± 2.3	20 ± 0.4	18 ± 0.9
20	25 ± 4.4	23 ± 1.2	21 ± 1.7
10	28 ± 4.2	25 ± 1.4	24 ± 2.4
05	33 ± 3.5	28 ± 1.6	27 ± 2.1
2.5	38 ± 3.7	32 ± 1.3	31 ±1.7

Table 3. Mean promastigotes viability percentage after adding different concentrations of free PTN during the incubation times. Data are expressed as the mean ± SD (n=3)

Free Pentamidine (µg/ml)	Promastigotes percentage (%) viability		
	24h	48h	72h
40	30 ± 1.2	28 ± 0.6	25 ± 1.3
30	35 ± 2.0	32 ± 1.9	28 ± 2.1
20	41 ± 3.0	37 ± 0.8	32 ± 1.6
10	48 ± 1.7	43 ± 1.5	37 ± 0.5
05	55 ± 1.6	48 ± 1.3	43 ± 1.4
2.5	65 ± 1.3	55 ± 0.8	48 ± 1.5

Table 4. Mean axenic amastigotes viability percentage after adding different concentrations of PTN-CNPs during the incubation times Data are expressed as the mean ± SD (n=3)

PTN-CNPs Concentration (µg/ml)	Amastigotes percentage (%) viability		
	24h	48h	72h
40	26 ± 1.0	22 ± 0.8	19 ± 0.7
30	29 ± 0.8	25 ± 0.6	22 ± 1.6
20	32 ± 1.9	27 ± 0.9	25 ± 1.8
10	36 ± 0.5	31 ± 1.4	29 ± 0.6
05	40 ± 1.3	35 ± 1.2	33 ± 1.4
2.5	44 ± 0.7	39 ± 1.3	36 ± 0.5

Table 5. Mean axenic amastigotes viability percentage after adding different concentrations of free PTN during the incubation times Data are expressed as the mean ± SD (n=3)

Free Pentamidine (µg/ml)	Amastigotes percentage (%) viability		
	24h	48h	72h
40	32 ± 1.5	29 ± 1.6	26 ±1.4
30	38 ± 0.9	35 ± 1.8	29 ± 1.0
20	41 ± 2.5	37 ± 1.3	32 ± 1.6
10	46 ± 1.9	43 ± 1.7	36 ± 1.7
05	49 ± 1.8	46 ± 0.8	39 ± 0.8
2.5	55 ± 0.9	49 ±0.6	44 ±1.8

Table 6. IC50 values of free PTN, PTN-CNPs, and AmB against *L. tropica*

Treatments	Promastigotes	Amastigotes
PTN-CNPs	0.1375 (µg/ml)	0.1910 (µg/ml)
Free PTN	0.3527 (µg/ml)	0.4023 (µg/ml)
AmB	0.9981 (µg/ml)	1.139 (µg/ml)

chemotherapy options are further complicated by the intracellular survival of the *Leishmania* spp. in the macrophages (Liévin-Le Moal & Loiseau, 2016). 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 their adverse toxic effects (Saleem *et al*., 2019). Chitosan polymer, which is acquired from the deacetylation of chitin, is one of the most famous drug delivery carrier in nanotechnology. Previous studies have investigated its strongest antibacterial, antiviral, antileishmanial, and antifungal effects (Singh *et al*., 2016; Cabral *et al*., 2019).

CNPs were fabricated through the ionic gelation method with non-toxic TPP. We have observed that the PTN-CNPs were spherical, with an average size of 88 nm. Chitosan NPs prepared with TPP have an average size of 100–120 nm with spherical morphology (Sreekumar *et al*., 2018), our results agree with the previous studies (Rampino *et al*., 2013). The EE and DLC of PTN-CNPs were 86% and 44%, respectively. Similar results of EE and DLC of the drug-loaded NPs were reported in earlier studies (Shi *et al*., 2014; Ashvini *et al*., 2019).

In the present study, the cytotoxic effect of free PTN and PTN-CNPs against the Pakistani strain of *L. tropica* by using MTT assay. Promastigotes and amastigotes exposed to PTN-CNPs showed a lower viability rate than free PTN at different time intervals. The IC50 values of PTN-CNPs against promastigotes and amastigotes were 0.1375 µg/ml and 0.1910 µg/ml. Similarly, the IC50 values of free PTN against promastigotes and amastigotes were found as 0.3527 µg/ml and 0.4023 µg/ml, respectively. It was observed that both forms of drug exhibited significant anti-leishmanial efficacy; however, PTN-CNPs showed strong anti-promastigotes and anti-amastigotes activity than free PTN.

To our knowledge, no research has been performed on the inhibitory effect of PTN-CNPs on *L. tropica in-vitro*. However, in a previous study, Juan *et al*. synthesized PTN-CNPs and compared their efficacy with free PTN against African trypanosomiasis *in-vivo* (Unciti-Broceta *et al*., 2015). They showed a difference in the anti-trypanosomal efficacy of drug-loaded polyacrylamide NPs compared to the free drug in an animal model. However, their results could not be compared to our findings because *Leishmania* is an intracellular parasite while *T. brucei* is an extracellular pathogen. In another study, Valle *et al*. (2019) loaded PTN onto the PLGA NPs and compared the anti-leishmanial effectiveness of free and NPs loaded PTN. It was shown that PTN had an excellent cytotoxic effect in reducing parasite burden in mice (Valle *et al*., 2019). A recent study evaluated the effect of paromomycine-loaded solid lipid NPs against *L. tropica* and *L. major in-vitro*. They found that paromomycine-loaded NPs efficacy was superior compared to free paromomycine against promastigotes and amastigotes forms of the parasites (Heidari-Kharaji *et al*., 2016). These results were practically in agreement with our findings. Barazish *et al*. also used MTT colorimetric assay to evaluate meglumine antimonite-loaded albumin NPs against promastigotes form of *L. tropica* (Barazesh *et al*., 2018). They observed that meglumine antimoniate-loaded NPs strongly inhibited the promastigotes of *L. tropica*. Similar findings have been observed in the present study. Mostafavi *et al*. (2019) compared the cytotoxic effect of AmB in combination with noisome-loaded selenium against promastigotes and amastigotes forms of *L. tropica*. They investigated the dose-dependent anti-leishmanial activity of both formulations *in vitro* (Mostafavi *et al*., 2019). Their results are practically consistent with our results. Mehrizi *et al*. also investigated the inhibitory concentrations of AmB-CNPs, AmB- dendrimers, and bucolic acid chitosan combinations against *L. tropica* (Mehrizi *et al*., 2019). They showed that AmB-NPs have more potency than free Amp-B against both forms of parasites. These results are consistent with our results.

CONCLUSION

In the current study, PTN-CNPs were successfully prepared through the ionic gelation method and then characterized. The synthesized NPs were evaluated for enhanced anti-leishmanial efficacy. Compared to free PTN, PTN-CNPs showed a higher inhibitory effect on both forms of *L. tropica* in terms of parasite viability. The PTN-CNPs also showed enhanced encapsulation efficacy (86%). PTN-CNPs also showed less hemolysis than free PTN at different concentrations. The IC50 values of PTN-CNPs against promastigotes and axenic amastigotes were (0.1375 µg/mL and 0.1910 µg/mL) many folds lower than the conventional PTN drug. Search for the antileishmanial effect of PTN-CNPs against animal CL can be an interesting subject for study in the future.

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Conflict of interest

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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