A novel effective therapeutic approach for treatment of Leishmania tropica through Miltefosine Loaded Chitosan Nanoparticles

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Research article

Keywords: Neglected tropical diseases, chitosan, miltefosine, Leishmaniasis, Leishmania tropica

Posted Date: March 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-18178/v1

Abstract

Objectives The aim of the present study was to develop miltefosine loaded chitosan nanoparticles (MLCNPs) to enhance the therapeutic efficacy of conventional miltefosine drug and to compare the in vitro activities of both forms of the drug against Leishmania tropica.

Methods MLCNPs were synthesized through ionic gelation with sodium tripolyphosphate (TPP). For the stabilization of nanoparticles, a controlled oxidation of MLCNPs was done with hydrogen peroxide (H2O2). The anti-leishmanial activities of MLCNP were determined by MTT cell viability colorimetric assay. The in vitro hemolysis assay was performed for assessment of toxicity in the human blood.

Results The MLCNPS displayed spherical shape with irregular surface morphology, had a mean size of 200nm and a zeta potential of +10.9 ± 4 mV. A high encapsulation efficacy and drug loading capacity of miltefosine loaded chitosan nanoparticles was also observed. The anti-leishmanial activities of MLCNP were determined by MTT cell viability colorimetric assay. The IC50 value of MLCNPs against promastigote and amastigote were recorded as 0.85µg/ml and 0.92µg/ml respectively. MLCNPs proved to be more effective as compared to conventional miltefosine. In terms of toxicity the MLCNPs caused only 2.25% hemolysis.

Conclusions The engineered MLCNPs could be potential alternative for the treatment of cutaneous leishmaniasis.

1. Introduction

Leishmaniasis is vector-borne infectious disease caused by a protozoan parasite belonging to the genus Leishmania. Both anthroponotic and zoonotictransmission of leishmaniasis have been reported. Leishmaniasis is basically a neglected and more likely to be a fatal disease that generally affects under developed and developing countries (Gkolfinopoulou et al., 2013). Leishmaniasis is classified into three basic kinds on the basis of signs and symptoms: cutaneous, mucocutaneous and visceral leishmaniasis (Kheirandish et al., 2013). Each form of the disease is caused by a different species of Leishmania parasite (McGwire and Satoskar, 2013). Leishmaniasis is prevalent worldwide, common in more than 88 countries and it has been evaluated that 0.35 billion individuals are constantly in danger of acquiring the disease (mostly cutaneous and mucocutaneous leishmaniasis) with an expected predominance of 12 million cases and a yearly incidence of 1.5 million cases (Alvar et al., 2012).

Leishmaniasis is the only tropical disease which is treated by non-leishmanial drugs (N. Singh et al., 2012). Treatment choice and clinical representation depends on the type of specie involved in causing Leishmaniasis (Minodier et al., 2007; Murray, 2012). However, treatment procedure and the type of therapy plays a vital role in speeding up the healing process and preventing relapse (Kevric et al., 2015).In the classical leishmaniasis therapy the challenges include approachability of very few drugs, resistance to the accessible drugs, toxicity and lack of cost–effectiveness (De Souza et al., 2010; Desjeux et al., 2004; Launois et al., 2008; Murray et al., 2005).

Miltefosine belongs to the alkyl-phosphorcholine drugs,that was basically an oral antitumor drug but showed activity against Leishmania as well (Varela et al., 2012). It was registered in India as an effective treatment for visceral and cutaneous leishmaniasis after successful clinical trials (Tiuman et al., 2011). The drug is effective against L. donovani with 97% healing rate (Freitas-Junior et al., 2012), but more recent reports show a decrease in its efficacy (Soong et al., 2012). For the treatment of leishmaniasis, the dose recommended is 2.5 mg/kg/day for four weeks (Chrusciak et al., 2011).The conventional miltefosine is linked with some of the adverse effects such as stomach disturbance, intestinal abnormalities including vomiting and nausea. Hepatotoxicity, renal damage and increase in creatinine are some commonly observed side effects. The drug is contraindicated in pregnancy due to its teratogenicity (Khademvatan et al., 2011).

Various nanomedicine formulations such as polymeric nanoparticles, liposomes, nanocapsules and nanoemulsions have been produced by pharmaceutical scientist in order to address the toxicity related issues. Polymeric nanoparticles are one of the extensively used drug delivery systems due to their chemical versatility, additional biocompatibility and simple preparation technique (Chen et al., 2011). They consist of different biodegradable materials like natural or synthetic polymers, metals, or lipids. Nanoparticles are taken up more conveniently by cells than larger micro molecules so they can be used as effective transport and delivery mechanism (Yasinzai et al., 2013). This project was designed to synthesize miltefosine loaded chitosan nanoparticles to enhance its therapeutic efficacy against Leishmania tropica.

2. Material And Methods

2.1 Materials

The chemicals and media used in this research were purchased from different manufacturer and suppliers. The material used in this study include, 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 (PenStrep soln, Lot # 01161018, Caisson), Trypan blue (Invitrogen, Lot # 1844453),Chitosan (Sigma-Aldrich), Miltefosine (gifted by local pharmaceutical company), Tripolyphosphate (TPP), D-Trehalose, Sodium hydroxide (Scharlau Chemie S.A), Phosphate Buffered Saline (PBS tabs, Oxoid, Thermo Fisher Scientific), BD Ultra-Fine Insulin Syringes, Geimsa stain, Methanol, Ethanol, Dimethyl Sulfoxide (DMSO).

2.2. Synthesis of Miltefosine loaded Chitosan Nanoparticles

Low molecular weight chitosan was interacted ionically with TPP to synthesize the nanoparticles. Nanoparticles were synthesized according to the literature as reported by Bernkop-Schnürch et al., 2006. In brief, chitosan polymer (0.5% w/v) was dissolved in (1% v/v) acetic acid solution. TPP powder (0.5% w/v) was dissolved in distilled water. The pH was adjusted to 5 and miltefosine (3mg/ml) was also added to the TPP solution. TPP solution containing miltefosine was added to chitosan solution drop wise

to synthesize ionically cross-linked nanoparticles. H_2O_2 solution (0.5% v/v) was then added to nanoparticles for partial oxidization. The reaction mixture was incubated for one hour under constant stirring at room temperature. To avoid aggregation, D-Trehalose (3% w/v) was consequently added to the nanoparticles solution and centrifuged for 10 min at 13,400 rpm to accumulate the nanoparticles in pellet.

2.3. Characterization of miltefosine loaded Chitosan Nanoparticles

Morphology and size of miltefosine loaded chitosan nanoparticles was determined through scanning electron microscopy (SEM). The magnification used was between 20,000xs to 45,000xs with accelerating voltage of 20 kV. Encapsulation efficacy and drug loading contents were determined by colorimetric assay based on the complexation of the zwitterionic miltefosine with the anionic ammonium ferrithiocyanate (NH4Fe[NCS]4) dye developed (Dorlo, Eggelte *et al.,* 2012). The test was derived from an assay developed for the phosphatidylcholine lecithin, in which it forms a coloured complex with ammonium ferrithiocyanate, which can then be extracted inorganic solvents (Dorlo, Hillebrand *et al.,* 2008). This procedure was modified for miltefosine and 1, 2-dichloroethane was used for extraction of the brown-red colored complex. The results were visually and spectrophotometrically assessed at 460nm. The drug loading of miltefosine in the chitosan nanoparticles was evaluated by determining the difference between the concentration of miltefosine before preparing the nanoparticles and the non-incorporated miltefosine. The following formula was used for drug loading contents in chitosan nanoparticles

Drug loading % = (Mass of the drug in NP / Mass of NP recovered) X 100

The zeta potential of miltefosine loaded chitosan nanoparticles dissolved in deionized water was determined by a PSS Nicomp 380 ZLS zetasizer.

2.4 Antileishmanial Activities of Miltifosine loaded Chitosan Nanoparticles

MTT colorimetric assay was used to determine the activity of mitefosine alone and MLCNPs by a method as reported earlier (Dutta *et al.,* 2012). The reagent enters the live cells and goes into their mitochondria where it is reduced to an insoluble formazan product, thus a dark purple color is obtained in the wells. No change in color is seen if the cell density is non-viable. Stock solutions of Miltefosine loaded chitosan nanoparticles and conventional miltefosine were prepared. Preparation procedure was same for both compounds in a way that 1 mg of drug (compound) was dissolved in 1 ml of distilled water to get the final concentration of 1000 µg/1000 µl. MTT reagent is soluble in water therefore the solution is prepared by dissolving 1 mg reagent in 1 mL distilled water. Cultured media containing 1×10^7 parasites per ml was used to carry out the assay.

In a 96-well plate under sterile culture conditions, axenically grown 100 µl promastigotes were plated. Each of synthesized nanoparticles and conventional drug solution was added up to the volume of 100 µl to initial well and serially diluted by transferring 100 µl of solution up to last well. 100µl was discarded

from last well, to keep the balance. 10 µl of MTT reagent was added to each well after 72 hours of incubation at 24 ^{0}C. As MTT is light sensitive so the plate was wrapped in aluminium foil and further incubated for 4 hours at 24 ^{0}C after adding the reagent and then centrifuged at 3000 rpm for 3 minutes. The supernatant was discarded from wells and the pellet was diluted with 100 µl of DMSO to stop the enzymatic reaction. The wells were incubated again for 1 hour in shaking incubator at 24 ^{0}C. Absorbance was checked at an optical density of 570 nm by a microplate reader. As a positive control, media containing parasites was added to wells without any drug. And for negative control only media without parasites and drug was added to wells. The experiment in duplicate was performed for the observation of sensitivity to each drug. The same procedure was performed amastigote with two modification i.e. the pH of the medium was reduced to 5 and then grown on 37 ^{0}C .The data obtained from microplate reader was then subjected to Graphpad prism version 5 software for statistical analysis.

2.5. In vitro toxicity study of Miltifosine loaded Chitosan Nanoparticles

In vitro Hemolysis assay was performed to check the toxicity of drug in the blood. It evaluates hemoglobin discharge in the plasma due to certain release of drug, which serves as an indicator of red blood cell lyses. First 3 mL of blood was collected from healthy volunteer to perform hemolysis assay. The blood was immediately centrifuged at 1500 rpm for 15 minutes to prevent clotting. To attain erythrocytes, plasma and the white buffy layer in the form of supernatant was discarded carefully by aspiration with a pipette. The pellet containing erythrocytes were then washed for three times with 1X PBS for 5 minutes. The erythrocytes suspension was prepared by mixing 11 mL of 1X PBS into 3 mL of centrifuged erythrocytes. The drug solutions of synthesized nanoparticles and conventional drug were prepared in distilled water (100 µg/ mL each). 100 µl of erythrocytes suspension was added to 1 mL of the nanoparticles and conventional drug solution each. Both the reaction mixtures were incubated for 4 hours at 37 0C. Lastly Eppendorf tubes were centrifuged at 13,000 rpm for 15 minutes and the remaining hemoglobin in supernatant was measured by spectrophotometer at 570 nm. 1 mL of PBS 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 percentage hemolysis was calculated as follows

$$\text{Hemolysis}(\%) = \frac{\text{OD at 576 nm in the SNEDDS solution} - \text{OD a at 576 nm in HBBS}}{\text{OD at 576 nm in 0.1\% Triton X-100 - OD at 576 nm in HBBS}} \times 100$$

3. Results

3.1 Synthesis and accumulation of Miltifosine loaded Chitosan Nanoparticles

Miltefosine loaded thiolated nanoparticles were prepared according to the literature previously described (Bernkop-Schnurch *et al.,* 2006). At the end of the experiment milky color solution of NPs were observed (Fig.1) and by centrifugation at 13400rpm for 10 minutes the miltefosine loaded chitosan nanoparticles

were collected in the pellet form and then by the help of lyophilizer the NPs were lyophilized at -30 ^{o}C and 0.01mbar pressure for further use in the experimrnt.

3.2 Characterization of Miltifosine loaded Chitosan Nanoparticles

The size and surface morphology of miltefosine loaded thiolated nanoparticles were determined using scanning electron microscopy. The diameters of nanoparticles were ranging from 200 to 300nm but average diameter 250nm. The miltefosine loaded chitosan nanoparticles displayed spherical shape with irregular surface morphology (Fig. 2) and no aggregation was observed. Drug loading content of miltefosine in nanoparticles were determined was 91.5µg/ml (Table 1).The miltefosine loaded thiolated nanoparticles possessed a zeta potential of (positive) +10.9 ± 4 mV (Table 1).

3.2 Antileishmanial Activities of Miltifosine loaded chitosan Nanoparticles

In-vitro anti-leishmanial activity was carried out in 96 well microtitter plates as previously described. Anti-promastigote and anti-amastigote activities were performed against *Leishmania tropica* by MTT colorimetric method. The IC_{50} was evaluated by a software Graph pad Prism version 5.04 and found that the activity of miltefosine loaded chitosan nanoparticles was higher than the conventional miltefosine. The anti-promastigote activity (IC_{50} value) of conventional miltefosine, chitosanand miltefosine loaded chitosan nanoparticles (MLCNPs) were 0.15µg/ml, 0.32µg/ml and 0.07µg/ml respectively (Table 2).The anti-amastigote activity in terms of IC_{50} value of conventional miltefosine, chitosan and MLCNPs were 0.18µg/ml, 0.381µg/ml and 0.09µg/ml.

3.4 Toxicity studies of Miltifosine loaded chitosan Nanoparticles

Conventional miltefosine showed more hemolysis than MLTNPs, as the light pink color of its supernatant was observed in the tube. PBS was used as negative control and transparent supernatant was clearly seen in the tube which showed that no hemolysis occurred there. The last tube containing Triton-X 100 (positive control) showed dark red color due to complete breakdown of erythrocytes.It was observed that less hemolysis was induced by synthesized nanoparticle (2.25 %) as compared to conventional miltefosine (4.76 %). PBS shows 0 % hemolysis where as Triton-X 100 implies potent hemolytic activity with 100% hemolysis (Table 3).

4. Discussions

The aim of this research project was to synthesize Miltefosine loaded Chitosan Nanoparticles in order to increase the therapeutic potential of mitifosine in order to minimize its toxicities and increase their anti-leishmanial activities. These MLCNPs were synthesized by ionic gelation with TPP as reported earlier by Tiyaboonchai in 2003. Chitosan based nanoparticles are broadly synthesized using the same technique (Tiyaboonchai, 2003). The first reported Chitosan nanoparticles preparation by ionic gelation method was done in 1997 in Spain (Calvo *et al.,* 1997). Result of our study revealed the average size of MLCNPs is 250 nm with spherical shape, irregular surface morphology and no aggregation. Studies showed that

chitosan nanoparticles synthesized by this method mentioned above are small in size ranging from 200 nm to 300 nm in diameter (Liu *et al.,* 2008; Makhlof *et al.,* 2010). The drug loading content of MLTNPs was found to be 91.5µg/ml. This high amount of DLC was noticed because of the zwitterionic and amphiphilic nature of the drug used in the experiment (Thagele *et al.,* 2011; Dorlo *et al.,* 2012). The surface charge is another essential feature for the nanoparticles attachment to cell surfaces. Positive charge zeta potential of nanoparticles when interacts with negative charge mucus layer results in strong electrostatic interaction. The MLCNPs showed a zeta potential of +10.9 ± 4 mV. In 2016, the study was done on preparation of liposomal nanoparticles, the zeta potential observed was −8.2 ± 3.50 mV. It indicated that zeta potential of liposomal formulation is slightly less than MLCNPs (Ribeiro *et al.,* 2016).

In vitro, MTT colorimetric assay was performed for synthesized nanoparticles against *L. tropica*. In this study biological activity of MLTNPs and conventional miltefosine was assessed against leishmaniasis. The IC_{50} value calculated for MLCNPs promastigotes of *Leishmania tropica* was 0.07µg/ ml which is significantly lower than un modified miltefosine (IC_{50} = 0.15 µg/ ml. In 2012, a study carried out in Iraq to calculate the IC_{50} value of miltefosine showed to be 0.19 µg/ ml which means that IC_{50} value calculated in ours experiment was almost the same as their study (Ali, 2012). It is indicated clearly that the efficacy of MLCNPs was higher than the conventional miltefosine. The satisfying result of MLCNPs could be due to the antimicrobial characteristic of chitosan as we also observed anti-leishmanial activity of chitosan alone which s IC_{50} 0.32µg/ ml. It may be considered a synergistic effect of both the drug (miltefosine) and polymer (chitosan) which turned out into successful outcome. It has been reported that chitosan acquires not only antiparasitic property but has good activity against bacteria and fungi, which is why this polymer has been the center of attention for many researchers (Hafdani and Sadeghinia, 2011). In 2017, a study carried out in Brazil revealed the antileishmanial activity of chitosan which opened a path for drug development. Scientific researchers could use chitosan polymers as a pharamacological tool for treating leishmaniasis that lessen the required dose as well as the side effects caused by conventional drug (Lima *et al.,* 2017)

The drugs contain chemical compounds that may induce a hemolytic effect and many drugs have severe adverse effects on erythrocytes. In this experimentation, hemolysis assay was performed to examine the hemolytic activity of synthesized nanoparticles. It was observed that less hemolysis was induced by synthesized nanoparticle (2.25 %) as compared to conventional miltefosine (4.76 %). In 2016, a similar study was carried out where hemolytic activity of miltefosine was checked, considerable hemolysis of 5.4% was observed (Alonso and Alonso, 2016) which is similar to our results. Hence, less synthesized nanoparticle showed less hemolysis as compared to conventional miltefosine. Synthesized nanoparticles did not show significant hemolysis so they could be utilized and evaluated for leishmaniasis therapy in animal models (Chávez-Fumagalli *et al.,* 2015).

5. Conclusions

Ant-leishmanial activities of the miltefosine can be enhanced significantly by modifying and incorporating this drug into chitosan nanoparticles. Miltefosne loaded chitosan nanoparticles may

provide better treatment regime compared to conventional unmodified miltefosine.

6. Declarations

6. **Acknowledgements**

We are thankful to the office of research innovation and commercialization (ORIC) Khyber Medical University, Peshawar for partially sponsoring the research project.

7. **Conflict of Interest**

All the authors contributed in this manuscript has no financial or academic conflict of interest.

8. **Funding Source**

The office of research innovation and commercialization (ORIC), Khyber Medical University, Peshawar provided partially funding for accomplishment of this research project.

9. **Ethical Approval**

This study has been approved by the bioethical committee of the Department of Microbiology, Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, KPK, Pakistan.

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Tables

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Figures

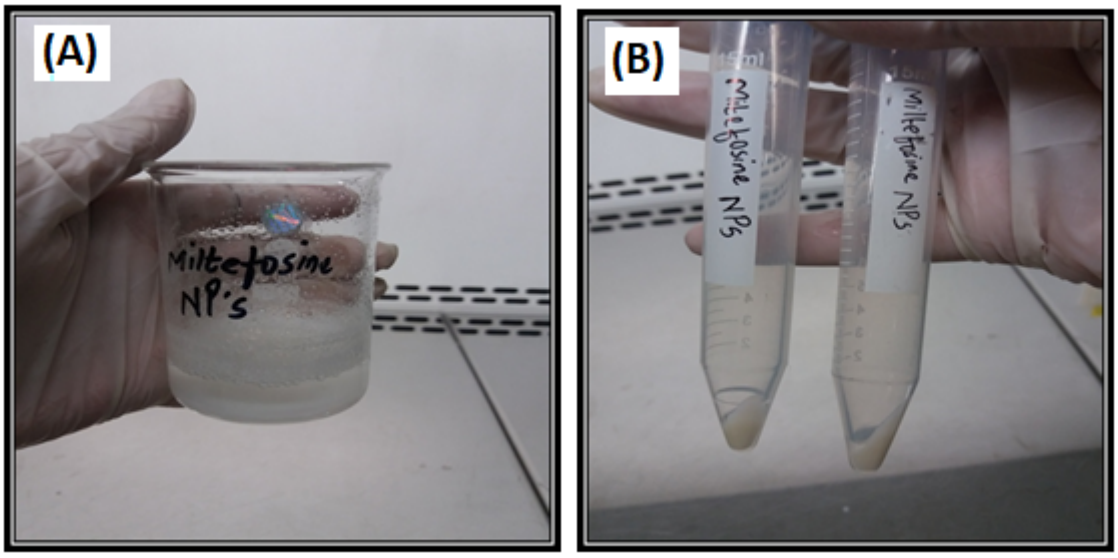

Figure 1

The milky solution and pellet form of MLCNPs. The chitosan polymer, TPP powder and miltefosine solutions were mixed for formation of ionically cross-linked nanoparticles. H2O2 and D-Trehalose were added before centrifugation. The resulting milky solution (A) and pellet (B) MLCNPs, after centrifugation are shown.

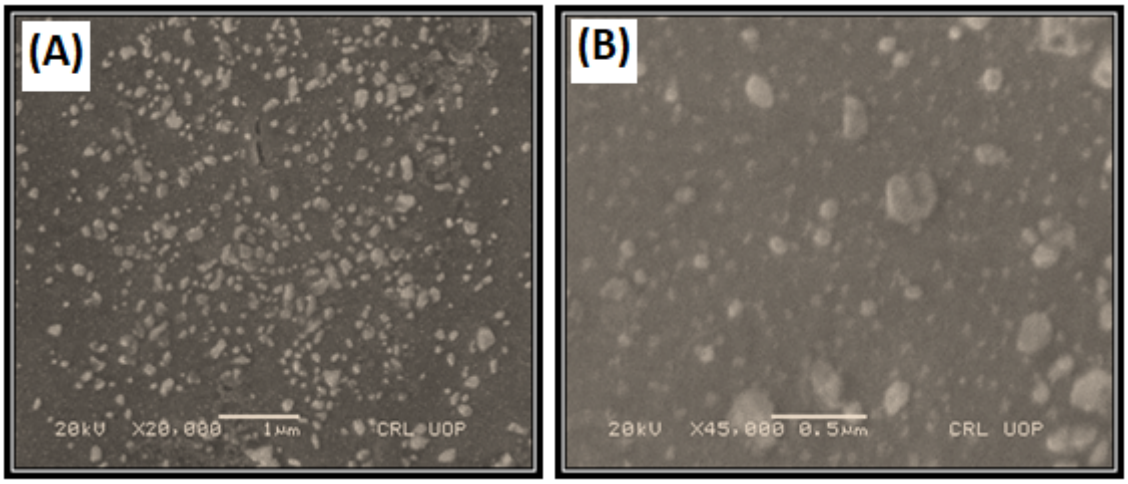

Figure 2

Morphological examination of MLCNPs. The morphology and size of Miltefosine Loaded Chitosan Canoparticles was determined through scanning electron microscopy (SEM). The magnification used was between 20,000xs (A) to 45,000xs (B) with accelerating voltage of 20 kV. The MLCNPs displayed spherical shape with irregular surface morphology.

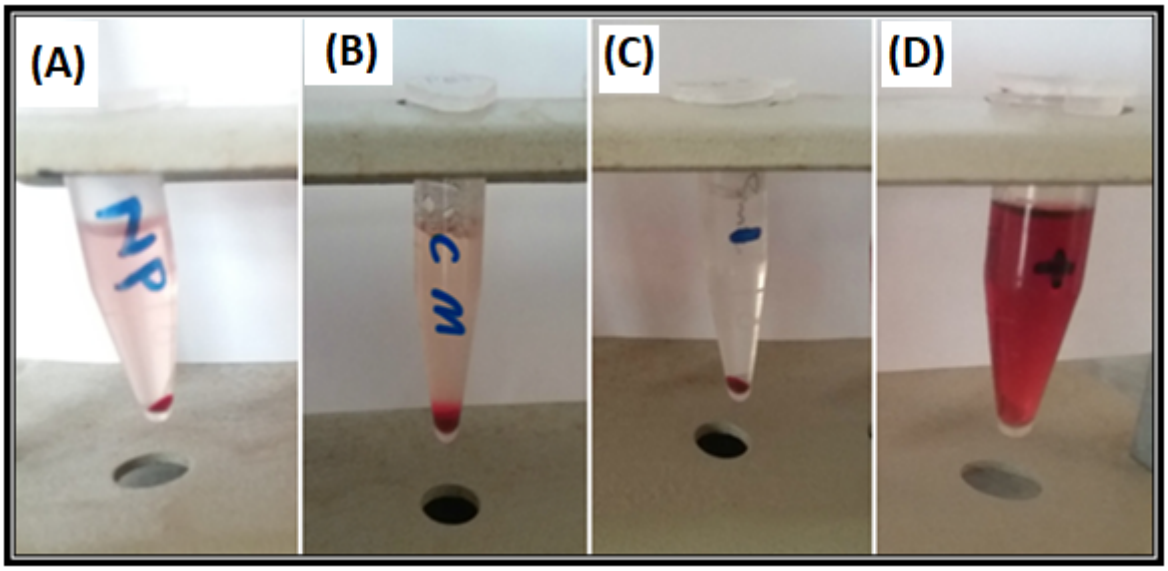

Figure 3

In vitro hemolysis assay for Mitefosine and MLCNPs. In vitro hemolysis assay was performed to check the toxicity of drug in human blood. The human blood was exposed to each drug and the remaining hemoglobin in supernatant was measured. Conventional miltefosine showed more hemolysis (B) with light pink color, than MLTNPs (A), the negative control PBS solution (C) is transparent (no hemolysis) and the positive control Triton-X 100 solution (D) showed dark red color due to erythrocytes lysis.

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