Original Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

TARGETING VISCERAL LEISHMANIASIS BY MANNOSYLATED LIPOSOME INCORPORATED PITC-2 ISOLATED FROM THE PLANT *PLUCHEA INDICA* (L) LESS.

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ABSTRACT

Leishmaniasis is a vector borne disease, endemic in large populated areas. PITC-2 is a natural compound, isolated from evergreen herb *Pluchea indica*(L.) Less. Animal studies have revealed that PITC-2 is non-toxic. The primary objective of this study was to demonstrate the possible increase in efficacy of mannosylated derivatives of PITC-2 in leishmania infected hamsters. The efficiencies of intercalated and mannose grafted liposomes of PITC-2 were found to be 37.24% and 31.25%, respectively, with the molar ratio of phosphatidylcholine, cholesterol and PITC-2 being 9:1:1. In *in vitro* studies liposomes in excised rat skin indicated slow release and penetration of PITC-2 liposomes. PITC-2 showed a significant anti-leishmanial activity against promastigotes (extracellular) of parasite *Leishmania donovani in vitro*. The IC_{50} of PITC-2 was 1.095 µg. The efficacies o f PITC-2 liposomes were tested against experimental leishmaniasis in mice and hamster models in three different forms: free PITC-2, Liposome intercalated and mannose grafted liposome of PITC-2. Efficacy of PITC-2 was significantly improved with mannose grafted liposome, as demonstrated by 88.30 % (in mice model) and 84.27% (in hamster model) reduction of splenic parasitic burden, respectively and 74.81% (in mice model) and 80.21% (in hamster model) reduction of liver parasitic burden, respectively. Histology of spleen and selected liver function tests (Alkaline phosphatase, SGPT and SGOT in blood plasma) indicated that the toxicity of PITC-2 was comparable to controls in mannosylated liposome incorporated PITC-2 animals. Although the drug release profile did not differ much the mannose grafted PITC-2 encapsulated liposomes were found to be effective in significantly lowering splenic and liver parasitic loads without toxicity.

KEYWORDS: **Leishmaniasis, Parasite burden, Mannosylated liposome, Drug release.**

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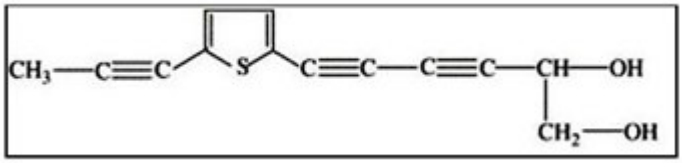
Received on: 06-03-2017
Revised and Accepted on: 24-04-2017
DOI: http://dx.doi.org/10.22376/ijpbs.2017.8.2.p396-408

INTRODUCTION

Leishmaniasis is a disease with diverse clinical manifestations that depends on both infecting species of Leishmania and the immune response of the host. This vector-borne disease is an important parasitic problem and endemic in large areas of tropics, subtropics and the Mediterranean basin.[1] Several syndromes are subsumed under the term leishmaniasis: most notably visceral, cutaneous and mucosal leishmaniasis.[2] This protozoal pathogen *Leishmania donovani* is the causative agent of visceral leishmaniasis (Kala-azar) which has a digenic life cycle. The flagellated promastigotes or the vector forms are converted to the aflagellated amastigotes, which reside and multiply within the host cell (i.e macrophages of the liver and spleen).[3] The first-choice treatment for the several forms of leishmaniasis is pentavalent antimonials which are potentially toxic and often ineffective. Second line compounds used in treatment of unresponsive cases generally include pentamidine and amphotericin B, which may be very toxic to the living system.[4-6] The plant *Pluchea indica* (L.) less is an evergreen shrub found abundantly in salt marshes and mangrove swamps in Sunderbans, Bangladesh, Myanmar, China, Malaysia, Tropical Asia and Australia.[7]Recently, one new compound PITC-2 has been isolated, purified and identified from root extract of tissue cultured *Pluchea indica*.[8]The isolated PITC-2 has the molecular formula of 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene.[9] It has wound healing, anti-oxidant, anti-cancer, anti-inflammatory, anti-hyperglycemic, anti-ulcer and anti-bacterial activities.[10-12]Patent has already been applied for PITC-2 (Application No: 2124/KOL/2008 A, Application filed on : 08/12/2008, Published on : 2010-06-11, International Classification No: A61K31/00). PITC-2 was tested against the protozoan parasite *Leishmani adonovani* and was found highly effective at killing those parasites *in vitro*. Substitution of pentavalent antimonials by Amphotericin B does not show effective results without any toxic syndromes. So these facts have raised alarm for developing new delivery systems with a view towards vectoring drugs to reticuloendothelial system (RES) without drug toxicity.[13]



R/J/3, 2-(prop-1-ynyl)-5(5,6-dihydroxyhexa-1, 3-diynyl)-thiophene.

Figure 1
Structure of PITC-2

Thus, efficient delivery systems suitable for site-specific delivery are being sought. As such, liposomes have been designed that circulate longer to bypass RES.[14]With anti-leishmanial therapy, the rapid accumulation of liposomes in the RES seems to be an added advantage, since leishmaniasis is a disease primarily associated with the fixed macrophages of RES. Efforts have been made to promote a higher uptake of liposomes by the RES through modifying liposomal surface and incorporating and grafting various ligands onto the liposomal surface, so that they are easily recognized by the various receptors located on the macrophages. When injected into the blood stream, the superior efficacy of a liposomal drug compared to a free drug is mainly due to the accumulation of liposomes in the liver and spleen. For leishmaniasis therapy, the antimonials are the drugs of choice, In addition to visceral leishmaniasis, New, Chance, and Health [14] extended their observations to show that liposomes also enhance the activity of sodium stibogluconate against experimental cutaneous leishmaniasis, when the parasites are located in the macrophages in peripheral tissues, rather than in the liver. Recently a classical drug urea-stibamine was used in the liposomal and in the mannose-grafted liposomal forms to combat experimental leishmaniasis in a hamster model. The mannose-grafted liposomal form was judged more efficient in transporting the drug to the specific site[15]The efficacy and toxicity of this drug have also been critically analysed and compared using different sugar bearing liposomes[16]Mannose-bearing liposomes have proved more efficient in the transportation of drugs compared to those bearing glucose or non-bearing liposomes (ordinary liposomes). The present study was performed to demonstrate the possible increase in the efficacy of the antileishmanial drug PITC-2 in a mannosylated liposomal drug delivery system against *in vivo Leishmania donovani* infected hamsters and mice model of leishmaniasis and to explore the possibility of utilizing a potent anti-leishmanial compound to combat visceral leishmaniasis.

MATERIALS AND METHODS

Chemicals and drugs

Phosphatidylcholine, cholesterols, P-aminophenyl-a-D-mannoside, and Glutaraldehyde were purchased from Sigma Chemicals (St. Louis,MO). The PITC-2 was isolated from root extract of tissue cultured medicinal plant *Pluchea indica*(L.)Less.

Animals

A four- to 6-week old of Balb/c mice (irrespective of sex) weighing 20 g, Male Wister rats weighing 180-200 g and golden hamsters (Mesocristatusauratus) weighing 100 g originally obtained from Haffkins Research Institute (Bombay, India) and maintained in Indian Institute of Chemical Biology (Kolkata,India), were used in this study. The animals were housed in an appropriate animal house facility under natural light and dark conditions. They were fed a standard pellet diet and water. The work was approved by the Institute of Animal Ethics Committee (Registration no: 147/1999/CPCSEA)

Tissue culture

P. indica (L.) Less. plants were collected from the Diamond Harbour region of West Bengal, during their flowering stage and was identified by Dr. N. Paria (Department of Botany, University of Calcutta) and Sri Saibal Basu (Botanical Survey of India, Shibpur, India). The explants of authenticated *P. indica* (roots, stems with internodes) were excised, cleaned and were treated with 0.1% w/v Mercuric chloride solution followed by repeated rinsing with sterile distilled water to ensure no trace of the sterilant. The roots were then aseptically cut into 0.5 ± 0.2 sq. cm pieces and placed with their dorsal side on the agar. The shoots were cut into approximately 1 cm with 1 - 2 nodes possessing axillary buds and embedded erect in the medium. The MS [17]basal medium was supplemented with different concentrations of NAA (Naphthalene acetic acid), IAA (Indole acetic acid), 2,4-D (2,4-Dichlorophenoxyacetic acid), and BAP (6-Benzylaminopurine). All media were adjusted to pH 5.8 before autoclaving at 121° C for 15 min. Sterilized explants (root, stems with internodes) were placed in MS media with various hormone combinations. Cultures were maintained properly. After 8 months, during flowering, the whole tissue-cultured plant was uprooted and the roots were separated, washed, treated with 1% sodium benzoate solution and dried at 55° C up to 15% moisture content. The root of tissue cultured *Pluchea indica* was separated, washed, oven dried at 60° C, powdered and sieved through 100 meshes. Fibres and unwanted materials were rejected after sieving. The powder was preserved in an airtight container for further use.

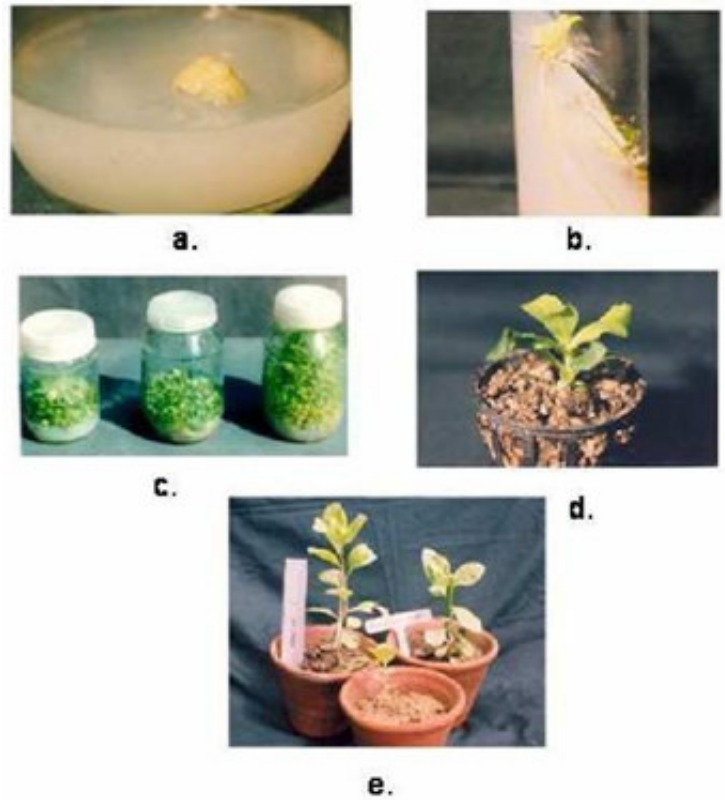


Figure 2

Tissue culture preparation of *P. indica* Photographs a-e.
a. Callus initiation b. Direct root generation c. Comparative growth of *P. indica* shoots d. Plantlets established in vermiculite. e. Potted tissue cultured plants of *P. indica.*

Figure 3

Maximum number of shoots was induced in Medium after the second subculture from nodal explants.

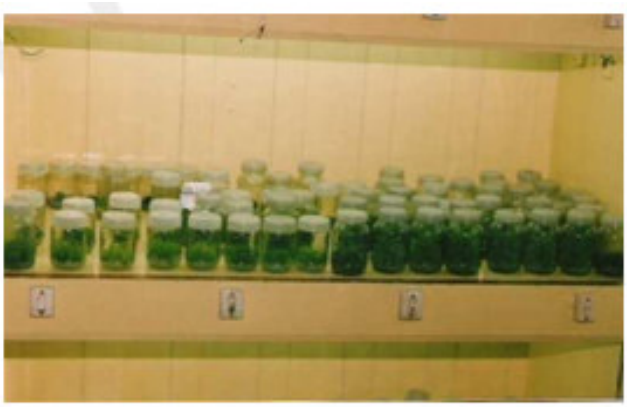


Figure 4

Plantlets of *P. indica* maintained under aseptic condition in the Laminar Air Flow.

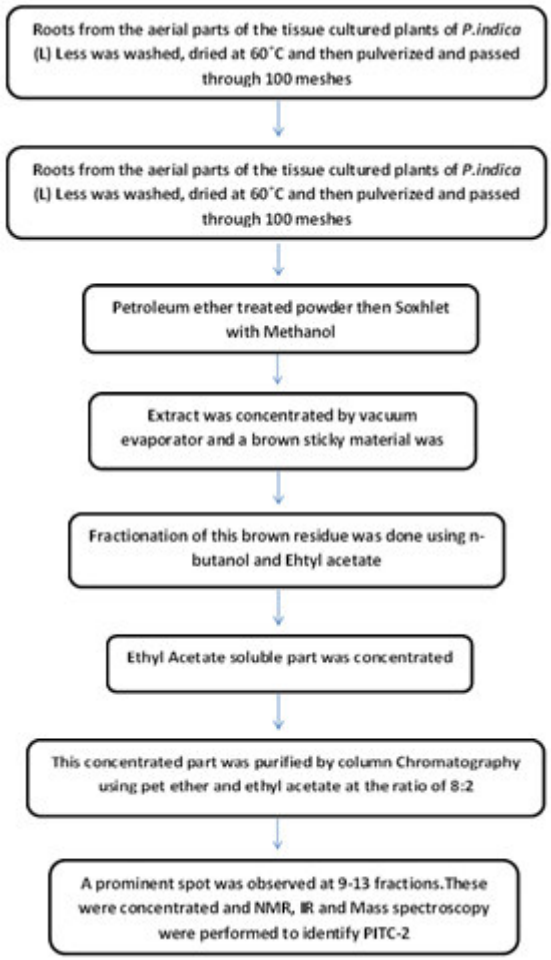


Figure 5
Schematic diagram of PITC-2 Isolation.

Preparation of liposome of PITC-2

PITC-2 was incorporated in the liposome by the modified thin-film hydration method. Phosphatidylcholine, Cholesterol and PITC-2 (9:1:1) mixture was first dissolved in chloroform and methanol (1:2) and dried in a rotary evaporator (SUPERFIT Display Cat No: SUPERVAC Model R-180) at 40˚C. The thin film layer formed was flushed with nitrogen gas for 5 minutes and maintained overnight under vacuum to remove traces of chloroform and methanol. The thin film was re-suspended in Phosphate buffer saline (PBS,pH 7.2) containing PEG 400 (6% v/v) and Tween 80 (4% v/v) by rotating the flask at 250 rpm and completely hydrated. After this the suspension was sonicated for 20 min. Then the liposome dispersions was serially passed through 1.2,0.4 and finally 0.2 µm pore size filters under nitrogen gas using an extruder. Unentrapped PITC-2 was removed from the liposome dispersion by centrifuging at 50,000 rpm for 30 minutes. Supernatant was discarded and the liposome pallet was washed two times with PBS. Liposome particles were then suspended in distilled water containing cryoprotectant 1mM EDTA and were freeze dried (Instrumentation of India Laboratory Freeze dryer). The final liposome

powders were stored in a tight container at 4º C for further use.

Covalent coupling of p-aminophenyl-a-D-mannoside with liposomal PITC-2

The PITC-2 intercalated in mutilamellar liposome was covalently coupled with p-aminophenyl-a-D-mannoside according to a method described by Torchillin *et al*. Liposome of PITC-2 (1 ml) suspension (30 mg lipid/ml 0.025M sodium phosphate buffer pH 7.2 containing 0.15 M Nacl) was mixed with 20 mg of p-aminophenyl-a-D-mannoside.

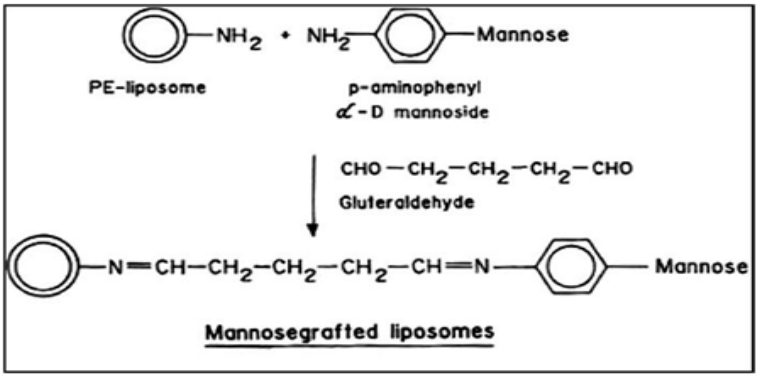


Figure 6
Coupling of p-aminophenyl-a-D-mannoside with liposome.

Glutaraldehyde was added slowly to the liposome suspension upto 15 mM final concentration and the mixture incubated for 5 min. at 20ºC. The NH_2 group of liposomes was coupled with p-aminophenyl-a-D-mannoside using glutaraldehyde as coupling agent. Uncoupled sugar derivatives and glutaraldehyde were removed by dialysis against the same buffer.

Drug encapsulation efficiency

The entrapment efficiency is defined as the ratio of the amount of the PITC-2 encapsulated in the liposome to that of the total PITC-2 in liposome suspension. For estimation of intercalated anti-leishmanial drug PITC-2 in liposomes, the liposomal pellet was dissolved in 1 ml of 10% Triton-X, and an aliquot of 100 µl was taken and diluted in PBS to make to make 1 ml. PITC-2 entrapment in liposome was done by JASCO V-650 UV/Vis spectrometer. The analysis was performed at 325 nm. Encapsulation efficiency was calculated from the equation:-

$$\text{Encapsulation efficiency} = \frac{\text{Amount of the PITC-2 encapsulated in the liposome (µg)}}{\text{Amount of the total PITC-2 in liposomal suspension (mg)}} \times 100\%$$

The maximum drug entrapment of PITC-2 encapsulated into liposome was 37.24% with the molar ratio of phosphatidylcholine, cholesterol and PITC-2= 9:1:1 .The same procedure was followed in the case of mannose grafted Liposome formulation and its encapsulation efficiency was 31.25 %.

Preparation of calibration curve of PITC-2

Stock solution of PITC-2 was prepared at 0.1mg/ml in Methanol. Serial dilution of the stock solution using Methanol was performed to prepare standard working solution of 4.5,2.2, 1.125, 0.562 and 0.281 µg/ml. Absorbance of these solutions was measured at 325 nm using blank methanol in JASCO V-650 UV/Vis spectrometer. The values of absorbance were plotted against respective concentrations to plot the calibration curve (Figure 7).

Preparation of Rat skin

Male Wister rats weighing 180-200 g were anesthetized with brief ether inhalation and killed by cervical dislocation. The abdominal skin was carefully excised after the hair was removed with an electrical clipper. The adhering fat and debris were carefully removed from the skin samples and kept in -80˚C deepfreeze until used in the diffusion studies. The skin samples were soaked in isotonic saline solution for 30 min. before starting diffusion experiments.

In vitro Permeation studies

In vitro permeation studies of liposome and MG liposome were performed using a modified keshary-chien diffusion cell with a capacity of 100 ml apparatus using rat skin membrane as diffusion barrier. One donor medium contains 13.43 mg liposome containing 5 mg PITC-2 and other donor contains same amount of mannose grafted liposome formulation.The receptor medium was 100 ml of isotonic phosphate buffer at pH 7.4. The receptor phase was maintained at 37± 0.5˚C by using circulated water bath with stirring modules at 100 rpm. The aliquots of 5 ml were withdrawn periodically and replaced with the same volume of receptor fluid during 48 hrs (0.5, 1,2,3,4,5,6,7,8,9,10,11,12,24,36 hrs) for *in vitro* studies. The withdrawn aliquots were immediately analysed for drug concentration spectrophotometrically at 325 nm directly.

Parasites

L. donovani strain AG83 (MHOM/IN/1983/AG83) was maintained *in vitro* in M-199 containing 10% fetal calf serum (FCS). Amastigotes were prepared from the spleens of AG83-infected mice on a discontinuous Percoll gradient as described by Hart *et al.*[18]

Transformation of amastigotes to promastigotes

Infected spleens were cultured at 22ºC for 5 to 7 days in M199 medium, supplemented with 10% FCS for synchronized transformation of amastigotes to

promastigotes. Promastigotes were seen after 5 days, and thereafter the parasites were routinely subcultured.

Medium

RPMI 1640 medium (GIBCO), pH 7.2-7.4, buffered with HEPES (SIGMA) $NaHCO_3$(SIGMA) and supplemented with 10% fetal Calf serum (FCS,GIBCO) 100 µg/ml Penicillin (GIBCO) and100 µg/ml Streptomycin (GIBCO) was used for macrophage culture. Medium 199 (GIBCO), pH 7.2 buffered with HEPES (SIGMA) $NaHCO_3$(SIGMA) and supplemented with 10% FCS, 100 µg/ml Penicillin (GI BCO), 100 µg/ml Streptomycin (GIBCO) was used for liquid culture of parasites.

Evaluation of in vitro growth kinetics

Leishmanial promastigotes were aseptically centrifuged at 3000 rpm for 10 min, counted with the help of improved Neubauer chamber under the microscope and diluted with the fresh M199 medium to a final concentration of 10^6parasite/ml. In a 96 well microtiter plate, 100 µl of the parasite culture (10^5 parasite/100 µl) was added in different wells in which 20 µl (co ntain 100 µg PITC-2) of the experimental compound was added in culture and serially diluted (100,50,25,12.5,6.25,3.125,1.5625,0.78 µg/ml) so that minimum concentration of the compound was 0.78µg/ml. Negative control contained 100 µl (10^6 cells/ml) parasite in medium only. The DMSO control contained 20 µl of DMSO in place of drug. The plate was incubated between 21-22ºC for 72 hrs. The culture was examined microscopically on an improved Neubauer chamber and IC_{50} values of compound possessing anti-leishmanial activity were counted.

Drug Preparation

PITC-2, a thiophene derivative was used for Anti-leishmanial study by dissolving it in 1% DMSO and the concentrations used was 1mg/ml. Liposome formulation of PITC-2 and mannose grafted liposome of PITC-2 were dissolved in sterile PBS at a concentration of 1mg/ml and were administered at a doses of 2.5 mg/kg body weight respectively .

In vivo *infection and treatment in mice and Hamsters*

Four to 6-week old BALB/c mice and golden hamster were used in this experiment. Mice and hamster were infected with $1x10^7$*L.donovani* strain AG83 promastigote through intracardiac route. The treatments were initiated two months post-infection. Two days before administration of treatments the mice and hamster were randomly divided into four groups of three each respectively. The animals of group-I were left untreated and considered as the infected control, group-II was treated daily with PITC-2 (5 mg/kg body weight) through subcutaneous route, animals of group III and IV was treated one day interval with liposome encapsulated PITC-2 (2.5 mg/kg body weight, s.c) respectively. The untreated group received daily 50 µl of PBS. Drugs treatment was continued by subcutaneous route for 10 days.

Assessment of parasitic load

After 10 days of chemotherapy and 2 resting day, the hamsters and mice were killed by cervical dislocation. The number of parasite amastigotes per hamster and mice spleen and liver nucleus were counted respectively by examining the Giemsa stained spleen smears through light microscope and the total number of amastigotes in the hamster and mice's spleen and liver were calculated as Stauber's formula and results were expressed as a mean parasite number ±SEM. Total no of parasites= (no. of parasites/1000 monocytes)x (weight of organ in mg) x ($2x\ 10^5$).[19]

Estimation of drug toxicity

Specific serum enzyme levels and spleeny tissue histology were assessed to find out the drug toxicity. Hepatotoxicity of the drug was assessed by measuring the serum Alkaline Phosphatase (AP) and serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT). Further Serum Urea, Total protein, Cholesterol and Triglyceride levels were analysed to find out the nephrotoxicity and hepatotoxicity of the drug using a standard kit.

Histopathology

The histoarchitecture of spleen was studied by microscopic examination of Haematoxylin-Eosin stained sections.

Statistical Analysis

The data were subjected to one way ANOVA (Graph Pad Prism Ver 4.0) followed by Dunnett's test and the values of $P \leq 0.001$ were considered statistically significant.

RESULT AND DISCUSSION

Antimonials, dangerous for their toxic nature, have remained in the list as a first group of therapeutic agents for the treatment of intracellular parasitic disease leishmaniasis. But in many cases clinical resistant to antimonials has been reported. Pentamidine and Amphotericin B, the second line drugs have been applied for the treatment of antimony-resistant leishmaniasis although these drugs are too toxic to be used in general. Recently one new drug PITC-2 has been isolated, purified and identified from root extract of tissue cultured *Pluchea indica*, which is a thiophene derivative.

Table 1
Calibration curve data of PITC-2 at different concentration.

Sl no	Concentration	Absorbance
1	4.5	0.3065
2	2.25	0.1744
3	1.225	0.1182
4	0.562	0.0797
5	0.281	0.0634

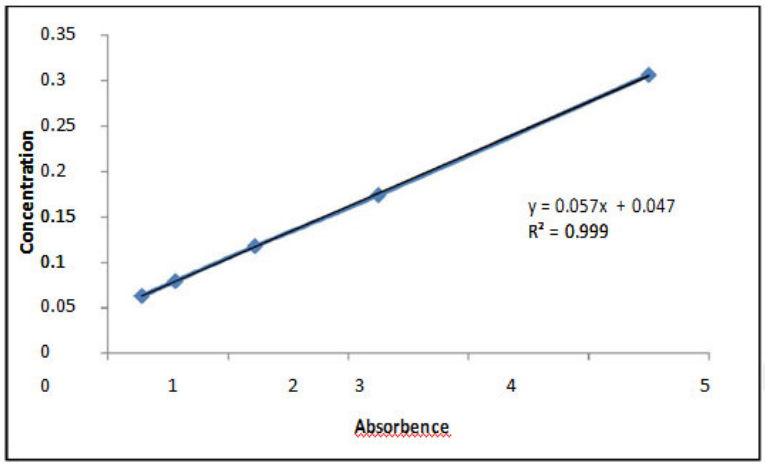


Figure 7
Standard Calibration Curve of PITC-2.

The intercalation efficiency of PITC-2 was found to be 37.24% in case of liposome and 31.25% in case of mannose grafted liposome, with the molar ratio of phosphatidylcholine, cholesterol and PITC-2 = 9:1:1 in MLV liposome. *In vitro* studies with excised rat skin revealed that the liposome formulation was released with slow penetration into rat skin slowly. This result is consistent with the fact that hydrophobic thiophene derivative was effective on the percutaneous absorption for sustain release dosage form. Figure 8 shows that liposome and mannose grafted liposome followed almost same kind of release pattern.

Table 2
***In vitro* Drug release study of both liposomal PITC-2 and mannose grafted liposomal (Liposome MG) PITC-2.**

Time In Hours	Cumulative drug Release of liposome	Cumulative drug Release of liposome MG	% of Cumulative drug Release of liposome MG	Of Cumulative drug Release of liposome MG
0.5	1048.6	976	5.631578947	6.2464
1	1461.53	1236	7.84924812	7.9104
2	1517.35	1364	8.149033298	8.7296
3	1945.34	1764	10.44758324	11.2896
4	2758.05	2565	14.8122986	16.416
5	2860.82	2787	15.36423201	17.8368
6	3122.08	2957	16.76734694	18.9248
7	3214.68	3183	17.26466165	20.3712
8	2610.27	3316	14.01863588	21.2224
9	2475.85	3207	13.29672395	20.5248
10	2672.65	3097	14.35365199	19.8208
11	2719.65	2854	14.60606874	18.2656
12	2681.59	2697	14.40166488	17.2608
24	2662.6	2601	14.29967777	16.6464
36	2693.75	2583	14.466971	16.5312

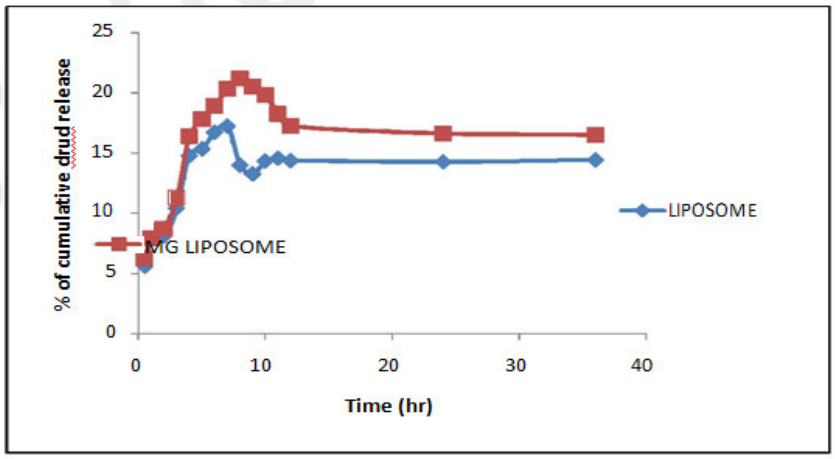


Figure 8
***In vitro* drug release study of both liposomal PITC-2 and Mannose-grafted liposomal PITC-2.**

PITC-2 showed a significant Anti-leishmanial activity against promastigotes (extracellular) of parasite *Leishmanial donovani in vitro* and the IC_{50} (drug concentration needed to reduce the parasite by 50%) value of PITC-2 was 1.095 µg. The growth kinetics curve against promastigotes of *Leishmania donovani* was presented exponentially (Table 3, Fig 9).

Table 3
***In vitro* Anti-leishmaniasis activity of PITC-2**

Groups	Dose of drugs (PITC-2)	Parasitic burden
Control	-	$3.6x10^5$
DMSO control	20 µl	$2.6x10^5$
	0.78 µg	$2.0x10^5$
	1.56 µg	$1.4x10^5$
	3.12 µg	$1.4x10^5$
Drug PITC-2	6.25 µg	$1.0x10^5$
	12.5 µg	$0.6x10^5$
	25.0 µg	$0.3x10^5$
	50.0 µg	Nil
	100 µg	Nil

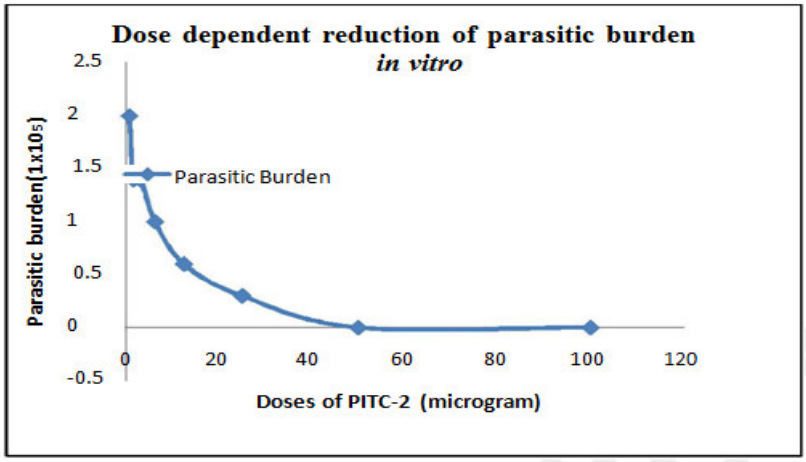


Figure 9
Dose dependant reduction of parasitic burden *in vitro* (parasitic burden x 10^5)

The anti-leishmanial activity of the PITC-2 was tested *in vivo* against experimental leishmaniasis in both mice and hamster model (Table 4,5,6,7; Figure 10, 11). The results presented reveal that the efficacy of PITC-2 tested against experimental leishmaniasis in both mice and hamster model in three different forms: free PITC-2, Liposome intercalated and mannose grafted liposome of PITC-2 forms. Highly improved efficacy was observed using mannose grafted liposome of PITC-2, causing 88.30 %(in mice model) and 84.27%(in hamster model) reduction of splenic parasitic burden. 74.81% (in mice model) and 80.21% reduction of liver parasitic burden were observed using grafted liposome of PITC-2.

Table 4
Effect of Mannose grafted liposome encapsulated PITC-2 on spleen in the 10 days infected mice model undergoing experimental leishmaniasis

Group	Spleen weight in gram	Parasite load in the spleen x 10^7	Percent suppression of spleen parasite load
Infected Control	0.226±0.10	1.97±0.008	-
Free drug PITC-2	0.073±0.001	0.67±0.110*	65.98
Liposome encapsulated PITC-2	0.100±0.001	0.35±0.120*	82.23
Mannose grafted liposome of PITC-2	0.047±0.003	0.23±0.034*	88.30

The values are expressed as mean±SEM (n=3). *P= < 0.01 when compared to infected control.

Table 5
Effect of Mannose grafted liposome encapsulated PITC-2 on spleen in the 10 days infected hamster model undergoing experimental leishmaniasis

Group	Spleen weight in gram	Parasite load in the spleen x 10^7	Percent suppression of spleen parasite load
Infected Control	0.928±0.002	11.00±2.80	-
Free drug PITC-2	0.920±0.001	5.26±0.110*	48.90
Liposome encapsulated PITC-2	0.926±0.003	3.31±0.031**	69.90
Mannose grafted liposome of PITC-2	0.922±0.002	1.73v0.023**	84.27

*The values are expressed as Mean±SEM (n=3). *P= < 0.05, **P=< 0.01 when compared to infected control*

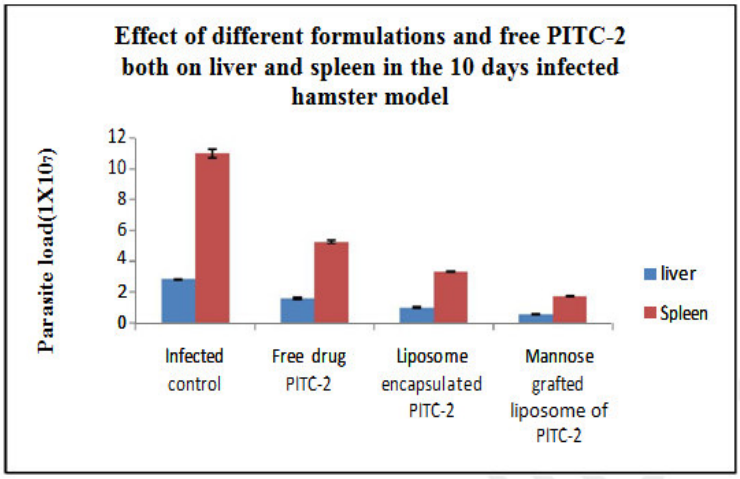


Figure 10
Effect of different formulations and free PITC-2 both on liver and spleen in the 10 days infected hamster model.

Table 6
Effect of Mannose grafted liposome encapsulated PITC-2 on liver parasite load in the 10 days infected mice model undergoing experimental leishmaniasis

Group	Liver weight in gram	Parasite load in the liver x 10^7	Percent suppression of liver parasite load
Infected Control	1296±0.57	13.5±0.23	-
Free drug PITC-2	919±0.57	6.93±0.09*	48.66
Liposome encapsulated PITC-2	1121±1.73	4.43±0.46*	67.18
Mannose grafted liposome of PITC-2	1273.66±2.33	3.40±0.11*	74.81

*The values are expressed as Mean±SEM (n=3). *P= < 0.01 when compared to infected control.*

Table 7
Effect of Mannose grafted liposome encapsulated PITC-2 on liver parasite load in the 10 days infected hamster model undergoing experimental leishmaniasis

Group	Liver weight in gram	Parasite load in the liver x 10^7	Percent suppression of liver parasite load
Infected Control	4.11±0.009	2.83±0.03	-
Free drug PITC-2	4.40±0.005	1.60±0.05*	43.46
Liposome encapsulated PITC-2	3.92±0.014	1.0±0.05*	64.66
Mannose grafted liposome of PITC-2	3.80±0.005	0.56±0.03*	80.21

*The values are expressed as Mean±SEM (n=3). *P= < 0.01 when compared to infected control.*

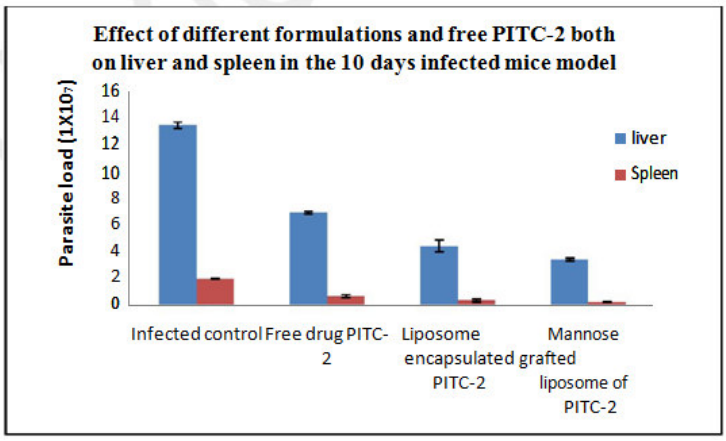


Figure 11

Effect of different formulations and free PITC-2 both on liver and spleen in the 10 days Infected mice model.

The amount of alkaline phosphatase (AP) and glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT) in serum were measured in different PITC-2 treatment conditions (Table 8).For the leishmania infected control group AP, SGPT and SGOT increased compared to normal animals. A slight increase in AP, SGPT and SGOT value was noticed when free PITC-2 (2.5 mg/kg body weight 5 times in 1 day intervals) was injected (s.c) to infected animals. But a substantial decrease in AP, SGPT and SGOT level was observed when identical amounts of PITC-2 in liposome or mannose grafted liposomes were injected into infected animals, indicating lack of hepatotoxicity. Similarly, the amount of urea, total protein, cholesterol and triglyceride in serum was measured in different PITC-2 treatment conditions (Table 8).In the Leishmania infected control and free drug treated (PITC-2) groups, Urea, T.P, Cholesterol, and Triglyceride levels were also increased in comparison to normal animals. A decrease in all the biochemical parameters were noticed when mannose grafted liposome of PITC-2 or liposomal PITC-2 (2.5 mg/kg body weight 5 times in 1 day intervals) was injected in infected animals. It is very interesting to note that a substantial decrease in both biochemical parameters levels, were observed when an identical amounts of PITC-2 in mannose grafted liposome were injected into infected animals. Levels of serum urea and Cholesterol were found higher as compared to normal animals and the elevated levels of urea and cholesterol were also observed in free drug treated animals and found to be near normal in animals treated with either liposomal PITC-2 or mannose grafted liposomal PITC-2, indicating a non-nephrotoxic effect.

Table 8
Effect of PITC 2 on enzyme levels related to normal liver function in serum hamster undergoing experimental leishmaniasis.

Groups	SGOT (IU/L)	SGPT (IU/L)	Alkaline Phosphatase (IU/L)	Total Protein (mg/dl)	Urea (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Infected Control	57.33 ±2.60	81.00±2. 64	130.00±1.15	5.83±0.03	22.00± 1.52	6 5.00± 1.73	168.3±5.23
PITC-2 Treated	74.00 ±2.08	75.66±1. 02	122.33±7.21	4.26±0.12	17.33± 0.21	88.66± 3.75	179.00±4.5 8
Liposome encapsulated with PITC-2	67.33 ±1.20	39.00±3. 21	90.00±6.42	5.26±0.29	20.90± 1.05	85.33± 1.85	138.00±8.0 2
Mannose grafted liposome of PITC-2	61.23 ±2.08	38.96±1. 56	84.66±14.11	3.90±0.14	17.00± 0.11	76.66± 2.40	126.00±1.8 5

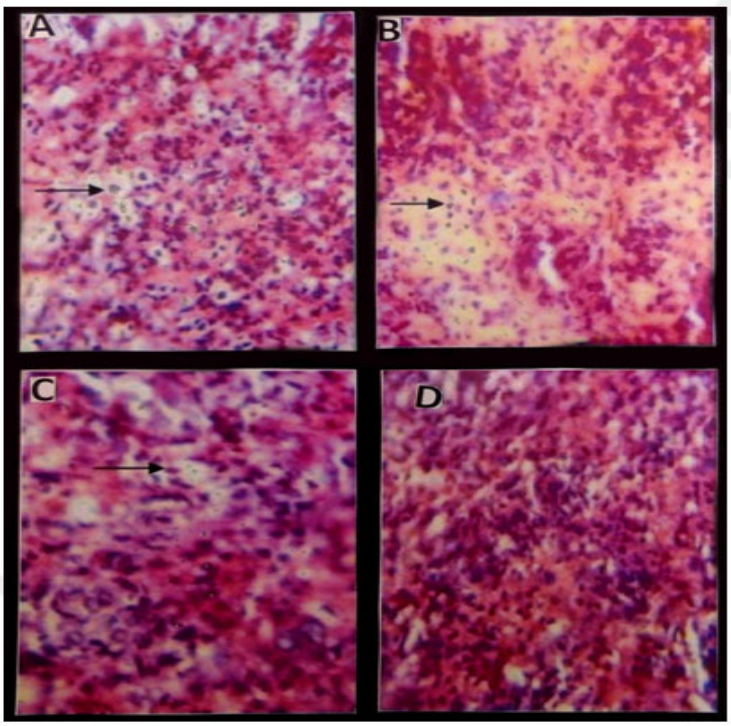


Figure 12

Histopathological examination of eosin-hematoxylin stained splenic sections of infected untreated control and other treated experimental hamsters with magnification x 100. (A) infected control, (B) PITC-2 treated, (C) Liposome intercalated PITC-2 treated, (D) Mannose grafted liposome intercalated PITC-2 treated.

Histology was done with light microscopy of Eosin-Haematoxylin stained splenic sections. Positive changes were observed in comparison with normal animals (Figure 12). In untreated infected controls, there was atrophy of the white pulp region indicating granuloma of lymphocytes, macrophages epithelial cells and giant cells. *Leishmania donovani* parasites were visible in a fair number of macrophages. Red pulp shows dilation and congestion of the sinuses and reticuloendothelial cells hyperplasia. Depletion of peri-arteriolar lymphatic sheaths were also noticed (Figure 12A). In free drug treatment mild hyperactivity of reticuloendothelial cells were noticed and there were few ill-defined granuloma formations in small numbers of lymphocytes, macrophages, epithelial cells and giant cells. Red pulp shows dilation and congestion of the sinuses and reticuloendothelial cells shows mild hyperactivity (Figure 12B). In liposomal drug treated animals, granuloma formation in lymphocyte, macrophages, epithelial cells and giant cells were seen and only in a few of the macrophages seen consisting of fewer number of parasites, indicating the antiprotozoal activity of the drug (Figure 12C).In mannose grafted liposome intercalated drug treated animals, a normal and healthy appearance of red and white pulp regions with intact peri-arteriolar lymphatic sheath were visible. There was no appearance of monocyte migration, confirming maximum reduction of infection (Figure 12D). Thus an attempt was made to search for a suitable delivery system, which would not only eliminate the toxicity of PITC-2 but also would be able to increase drug efficacy. Amphotericin B coupled with palmitoyl mannose was used to elevate immuno-adjuvant properties of Amphotericin B against fungal infections.[20]Liposomes are well recognized drug delivery devices for drug treatments and would likely be effective in leishmaniasis incorporated in phospholipid vesicles. The leishmania parasites are taken up by the RES system and it thus it creates an ideal situation for a high degree of drug parasite interaction.[16]Moreover, if appropriate ligands are covalently attached to liposomes, so that they could be easily recognised by the macrophage receptors, then these modified liposomes could possibly be used effectively as vesicles for site specific delivery.[21]Drug targeting using liposomes can be achieved by passive or active means. The uptakes of liposomes by the macrophage cells represent an excellent example of passive targeting. Regular liposomes have been used as carrier to introduce various components into the cells both *in vivo* and *in vitro*. Most of the drugs currently used in leishmaniasis have significant side effects for the host. But when these drugs are liposome encapsulated, their side effects are minimized and are better tolerated by the host.[22,23]The efficacies of a series of liposome entrapped antimonial drugs in the treatment of both cutaneous and visceral leishmaniasis were reported. [24] In this study the drug release pattern and the efficacy of the thiophene derivative of *Pluchea Indica* i.e PITC-2 was tested against reversible visceral leishmaniasis in the hamster and mice model with free, liposome encapsulated and mannose grafted liposome encapsulated forms. The mannose grafted liposome encapsulated PITC-2 was found to be the best choice in lowering of splenic and liver parasitic load without any significant side effects. It is a possibility that mannose grafted liposomes with PITC-2 could surface bind with the mannosyl receptors specifically in macrophages.[25]Based on the presence of MFR on the

macrophage surface; mannose bearing liposomal delivery system were recommended and used previously for the treatment of leishmaniasis. [26]In addition, liposomal PITC-2 was non-toxic to the host. Recommendations are being made from observations in this study for the possible use of mannosylatedPITC-2 liposomes for visceral leishmaniasis. Additional clinical trials are needed with this dosage form for possible efficacy in humans.

CONCLUSION

Naturally occurring substance PITC-2 was isolated from evergreen herb *Pluchea indica (L.) Less*. The present study showed leishmanicidal activities of both liposomal and mannose grafted liposomal formulations of PITC-2 which represents an exciting advance in the search for novel anti protozoal agents at a time when there is an urgent need for new innovative drug leads. In terms of lowering splenic and liver parasitic load, Mannose grafted liposome was found to be more efficacious compared to ordinary liposome bearing PITC-2. So it can be concluded that this new formulation can be used clinically to combat leishmaniasis in near future.

FUNDING/ACKNOWLEDGEMENT

We acknowledge the resources and financial support for the study provided by the Department of Science and Technology, West Bengal at Department of Pharmaceutical Technology, Jadavpur University, Kolkata. DST Project Memo No.775(Sanc.)/ST/P/S & T/1G-27/2014 dated 22/12/2015).

CONFLICT OF INTEREST

Conflict of interest declared none.

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We sincerely thank the above reviewers for peer reviewing the manuscript