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Antiproliferative Effect of Liposome Encapsulated PITC-2 on Cancer Cell Line (U937, K562 and HL60) *in Vitro*

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ABSTRACT

PITC-2, a new thiophene derivative was encapsulated *in vitro* in liposomes composed of phosphatidylcholine, cholesterol and PITC-2 at a molecular ratio of 9: 1: 1. Permeation studies were carried out with a Keshary-chien diffusion cell apparatus (with a capacity of 100 ml), using rat skin membrane as the diffusion barrier. *In vitro* studies showed that the liposome formulations provided an increasing drug release profile, up to 24 hrs. After 24 hrs it gradually decreased and followed a steady-state pattern up to 48 hrs. *Ex vivo* studies with excised rat skin revealed that liposome formulation was released and penetrated slowly into rat skin. This result is consistent with the fact that hydrophobic thiophene derivative is effective on the percutaneous absorption for sustained release dosage form. The anticancer property and cytotoxic effect of PITC-2, liposome-encapsulated PITC-2 and tissue cultured *Pluchea indica* root extract (TCPIRE) was compared against human leukemic cell lines U937, K562 and HL60. Liposome-encapsulated PITC-2 significantly inhibited the cell proliferation (in the case of U937 - 93.01%, K562 - 78% and HL60 - 89.525%) and showed a higher cytotoxic effect than free PITC-2 and tissue-cultured *P. indica* root extract (TCPIRE) *in vitro*. An MTT assay showed that the growth of metabolically active cells was inhibited by treatment with the drugs. Result revealed that liposome formulation of PITC-2 possesses potent antiproliferative activity *in vitro*. The PITC-2 liposome formulation can show great promise against leukemia in the future.

Keywords: cytotoxicity, leukemic cell lines, percutaneous, TCPIRE

INTRODUCTION

Chemotherapeutic treatment of neoplastic diseases is often restricted by their severe adverse systemic toxicity which limits the dose of drugs. Administration of these drugs has some complications like drug resistance, inability to access the target site and drug metabolism (Park 2002a). Liposome are promising drug delivery systems which are used to target specific cell by attaching antibodies, proteins or appropriate molecules that target specific receptor sites. Liposomes are synthetic phospholipids vesicles that are used as drug carriers. Depending on their size and phospholipids composition, they have the potential ability to circulate in the body for a certain period of time and to serve as a depot for encapsulated drugs (Rutenfranz *et al.* 1990).

PITC-2 is a new thiophene derivative isolated from root extract of tissue-cultured *Pluchea indica* (L.) Less. (Patent application no: 1990/KOL/2008). Because of its poor hydrophobic property, PITC-2 is being encapsulated in liposome to enhance its therapeutic effects and reduce the toxicities. In our earlier study, it was found that tissue-cultured *P. indica* root extract (TCPIRE) inhibited Ehrlich Ascites Carcinoma (EAC) in mice (Pramanik *et al.* 2008). In the present study we have investigated the methanolic root extract of tissue cultured *P. indica* and PITC-2 and its liposomal formulation for their anticancer property and cytotoxic effect on different cell line (U937, a human leukemic cell line, K562, an erythroleukemic cell line and HL60, a human leukemic cell line). It is well known that tumor promoters recruit inflammatory cells to the relevant site and aggravation of inflammation in the tissue causes cancer enlargement and *vice versa* (Ghosh *et al.* 2006). So it may be concluded that anti-inflammatory agents may possess anticancer activity. We have already reported in our earlier studies that *Pluchea indica* root extract has potent anti-inflammatory and anti-oxidant properties (Ghosh *et al.* 2008). In the present study we have investigated *in vitro* permeation and stability of PITC-2, encapsulated into liposome to exert an antiproliferative effect on cancer cell lines (U937, K562 and HL60).

MATERIALS AND METHODS

Chemicals

Phosphatidyl choline (PC), cholesterol, glutaraldehyde, RPMI 1640 medium, fetal bovine serum, streptomycin and penicillin, L-glutamine, HEPES, Ara-c,3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, MO). PITC-2 was isolated from root extract of tissue cultured medicinal plant *P. indica* and all other reagents like chloroform, methanol, EDTA, Triton X-100, etc. of high purity grade were purchased from a local manufacturer.

Preparation of liposome

PITC-2 was incorporated in the liposome by the modified thin-film hydration method (Yang *et al.* 2007). The hydrophobic excipients, e.g. PITC-2 and lipids (in a molecular ratio phosphatidylcholine, cholesterol and PITC-2 at a 9: 1: 1) were first dissolved in chloroform and methanol (chloroform: methanol with the ratio of 1: 2) and then dried in a rotary evaporator (HAHNVAPOR model no: HS-2005 V) under an aspirate vacuum maintaining the temperature at 40°C. The thin film layer thus formed was flushed with nitrogen gas for 5 min and kept overnight under vacuum to remove traces of chloroform and methanol. The thin film was resuspended 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 until the lipid film was completely hydrated (Joshi and Misra 2001). After this, the suspension was sonicated for 20 min. Then

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Original Research Paper

Table 1 Drug encapsulation estimation result of PITC-2 in liposome.

Formulation No.	Formulation code	Lipid and drug composition (PC: CH: PITC-2) Unit (mol)	Average vesicles diameters (µm)	Percent encapsulation efficiency
1	LF-1	1: 1: 1	200 ± 0.86	7.90 ± 0.28
2	LF-2	1.5: 1: 1	182 ± 0.55	10.32 ± 0.25
3	LF-2	2: 1: 1	210 ± 2.50	11.25 ± 0.33
4	LF-4	3: 1: 1	170 ± 1.50	13.10 ± 0.25
5	LF-5	5: 1: 1	200 ± 1.60	18.21 ± 0.23
6	LF-6	7.5: 1: 1	220 ± 1.30	20.15 ± 2.50
7	LF-7	9: 1: 1	200 ± 0.50	37.24 ± 0.52

PC, Phosphatidyl choline; CH, cholesterol

the liposome dispersion was serially passed through 1.2, 0.4 and finally 0.2 µm pore sized filter (Precise Filter) using nitrogen gas as extruder. Un-entrapped PITC-2 was removed from the liposome dispersion by centrifuging at 50,000 rpm for 30 min. Supernatant was discarded, and the liposome pellet was washed with PBS twice. Liposome particles were then suspended in distilled water containing cryoprotectant with molar ratio of 1.8 to lipid content and 1 mM EDTA before freeze-drying (Instrumentation India Laboratory FREEZE DRYER). The final liposome powders thus obtained were stored in a tight container at 4°C for further experiments.

Particle size analysis

The liposome sample (5-10 µl) was placed on carbon coated copper grid (400 mesh carbon coated copper grid; Sigma-Aldrich, USA) and maintained for 1 min. The excess sample was soaked by blotting paper and 2% uranyl acetate was then put on the grid for 30 sec. Excess acetate was soaked by filter paper and observed under transmission electron microscope (TEM) [Tecnai Spirit (FFI), Japan] at 60 kb. The picture was recorded on a 2/2 CCD camera (Mega view, soft Imaging system, Tecnai Spirit (FFI)) (**Fig. 1**).

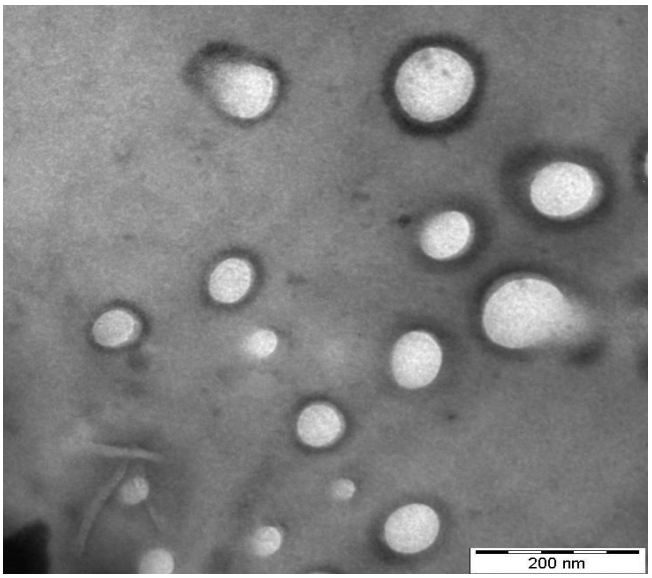

Fig. 1 TEM Photograph of liposome encapsulated PITC-2.

Drug encapsulation efficiency

The entrapment efficiency (EE) 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. Briefly, aliquots (0.1 ml each) of liposome suspensions diluted to 1.1 ml by PBS (pH 7.2) immediately after preparation were centrifuged at 1,000 rpm for 10 min to remove any PITC-2 particle already released from the liposome. Then 1.0 ml of liposome supernatant was precipitated by ultracentrifugation at 50,000 rpm for 30 min. After removing the supernatant by decantation, the precipitate (i.e., liposome pellet) was washed twice with phosphate buffered saline (PBS, pH = 7.2). The liposome pellets were then dissolved in 6 ml of 10% methanolic Triton X-100 and estimated for PITC-2 content by HPLC. PITC-2 entrapment in liposome was studied by HPLC instrument. The HPLC system (JASCO PU- 2089, UV-2075, and Column Watrex - 250 × 4 mm SN-6820 ODS2 5 µm) was operated in a quaternary gradient pump with an intelligent UV/VIS detector. The analysis was performed at 325 nm with a reverse phase column C18, 250 mm 4 mm, 5-µm (Column: Watrex W102007 250 × 4 mm Column, Pump: PU: 2089 Plus Quaternary gradient pump) column maintained at 25°C (column oven) using a mobile phase of acetonitrile: 10 mMol of phosphate buffer (50: 50) flow rate 1 ml/min with a loop capacity 20 µl.

EE was calculated as follows:

$$EE\ \% = (W_1 - W_2)\ X\ 100 / W_1$$

where W1 = weight of suspended PITC-2 in the solution and W2 = weight of PITC-2 in the wash solution.

EE and content were determined in three sets separately prepared liposome suspensions and were expressed as the mean ± standard deviation (**Fig. 2, Table 1**).

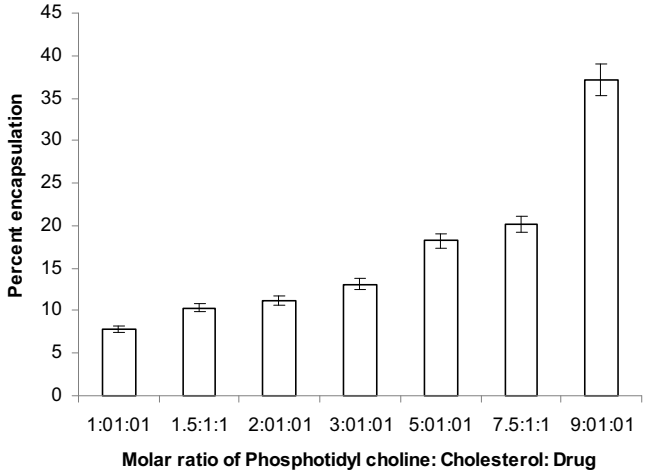

Fig. 2 Drug encapsulation efficiency was determined with 10 µl of each sample, where lipid and drug composition is PC: CH: PITC-2 Unit (mol). Statistical significance: 0.0477 (*$P < 0.05$)

Drug-excipients interaction study

The pure drug, PITC-2 and a mixture of it with cholesterol, and phosphatidylcholine and another mixture lipids (without PITC-2) were mixed separately with IR grade KBr in a 100: 1 ratio and the pellets were prepared by applying 5.5 metric tons of pressure in a hydraulic press. These pellets were scanned over a wave number range of 4000 to 400 cm^{-1} in a Magna IR 750 series II FTIR instrument (Nicolet, Madison, Wis) (**Fig. 3A-C**).

Preparation of rat skin

Male Wistar rats weighing 180-200 g obtained from local supplier m/s Reeta Ghosh, Kolkata were anesthetized with brief ether inhalation and sacrificed by cervical dislocation. The abdominal skin was carefully excised after the removal of hair with an electrical

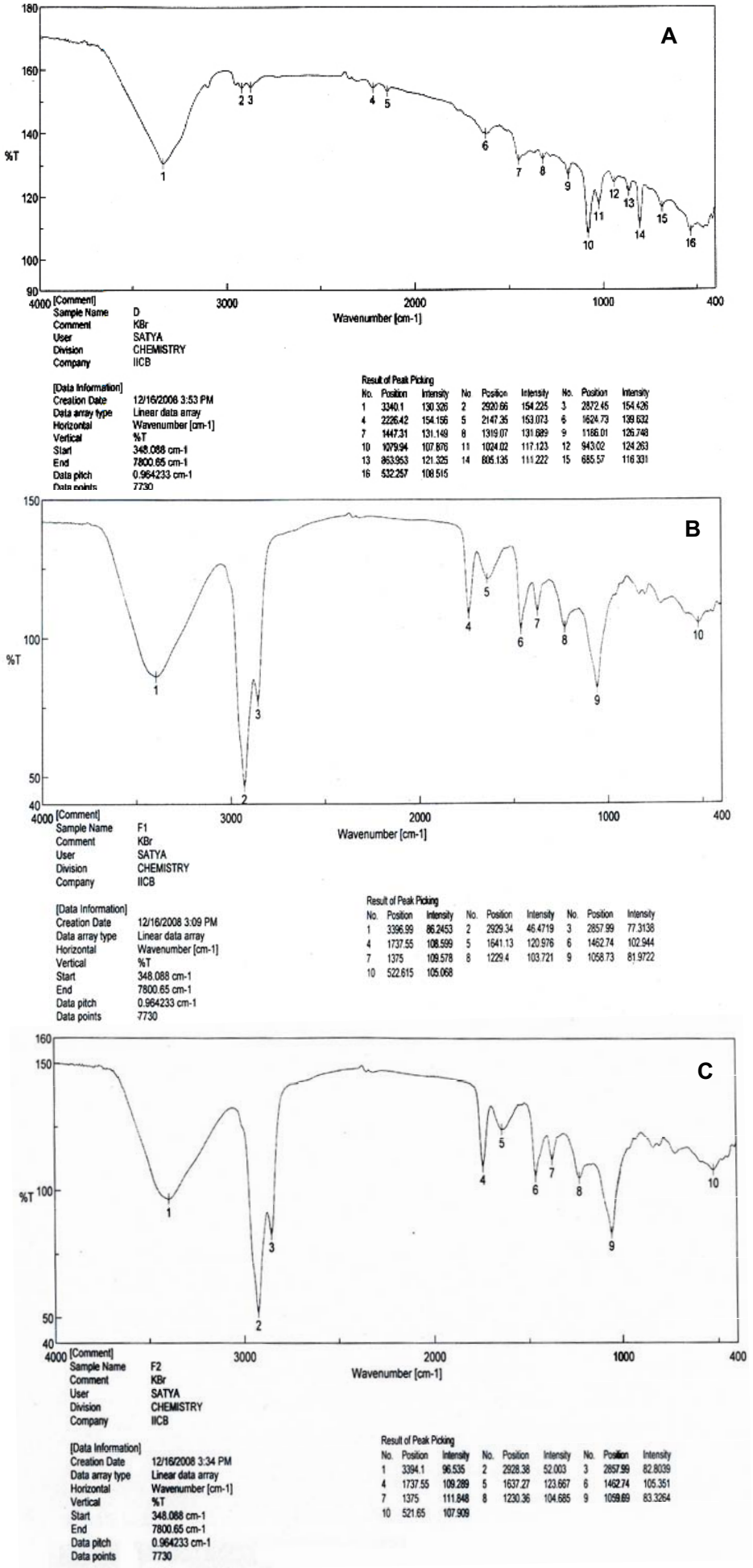

Fig. 3 IR spectra of (**A**) PITC-2 (pure drug), (**B**) drug (PITC-2) + cholesterol + phosphatidylcholine, (**C**) cholesterol + phosphatidylcholine.

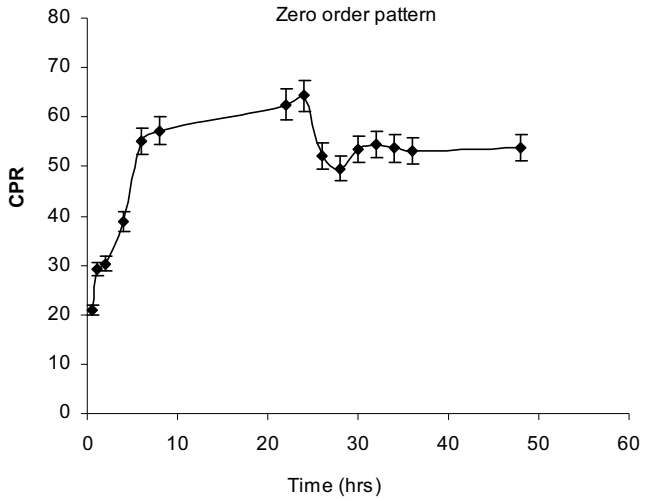

Fig. 4 Permeation study of PITC-2-encapsulated liposome during 48 hrs. CPR = cumulative percentage of drug-release.

Table 2 Thermal degradation study of best formulation.

Liposome formulation of maximum drug entrapment	Incubation temperature (°C)	Incubation time (min)	Drug entrapment efficacy (%)
LF-7	25	10	37.24 ± 0.21
LF-7	25	20	37.10 ± 2.50
LF-7	25	30	35.25 ± 1.80
LF-7	50	10	35.80 ± 2.60
LF-7	50	20	31.20 ± 1.25
LF-7	50	30	29.00 ± 2.75

Stability study of lyophilized and suspended liposome for 3 months at 2-8°C.

clipper. The adhering fat and debris were carefully removed from the skin samples and kept in a -80°C deep freeze until used in the diffusion studies. The skin samples were soaked in isotonic saline solution for 30 min before starting the diffusion experiments.

In vitro permeation studies of liposome-encapsulated PITC-2

In vitro permeation studies of liposome encapsulated PITC-2 were performed using the modified Keshary-Chien diffusion cell with a capacity of 100 ml apparatus using rat skin membrane as the diffusion barrier. The donor medium contained 5 mg PITC-2 in 13.43 mg of liposome and 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 constant 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, 4, 6, 8, 10, 22, 24, 26, 28, 30, 32, 34, 36, 48 hrs) for *in vitro* studies (**Fig. 4**). The withdrawn aliquots were immediately analyzed for drug concentration spectrophotometrically at 230 nm directly. This dilution of the receiver contained was taken into account when calculating the penetration data (**Table 2**).

Leukemic cell lines

Human leukemic cell lines U937, K562 and HL60 were purchased from the National Facility for Animal Tissue and Cell Culture, Pune, India. Cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated FCS. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. In all the experiments untreated leukemic cells were termed as control group.

Preparation of TCPIRE, PITC-2 and liposome of PITC-2

The TCPIR (8 months matured), based on the protocol by Pramanik *et al.* (2007), were separated, washed, oven-dried at 60°C, powdered by a micro pulverizer and sieved through 100 meshes (0.0254 cm diameter). The plant was taxonomically identified and authenticated by Botanical Survey of India (BSI), Shibpur, India. The pulverized powdered root (500 g) was soaked overnight with petroleum ether (60-80°C for synthesis, Merck) dried it at room temperature and extracted with methanol (AR Grade, Purity 99.8%, Sisco Research Laboratories Pvt. Ltd.) using a Soxhlet extractor to obtain the methanolic TCPIR. The solvent was then evaporated under reduced pressure using a rotary evaporator (HAHNVAPOR Model no: HS-2005V) to obtain a semisolid residue. The yield of the extract was 8.7% (w/w). TCPIRE was dissolved in normal saline at a concentration of 100 µg/ml and doses of 10, 20 and 30 µg were used after standardization. A portion (43.5 g) of the crude methanolic extract was partitioned between *n*-butanol and water. The butanol and water fractions were separately evaporated. The butanol fraction was then shaken with ethyl acetate to obtain an ethyl acetate soluble part and ethyl acetate insoluble part. The ethyl acetate soluble part was concentrated in the rotary vacuum evaporator and then dried to obtain a crude residue (10 g) which was subjected to column chromatography over silica gel (60-20 mesh, 300 g) as an adsorbent, using petroleum ether and ethyl acetate mixtures (with increasing polarity of the eluants). Fractions of 10 ml were collected and mixed on the basis of their TLC behavior. Elution with pet-ether: ethyl acetate mixture (6: 4, 4: 6) afforded one fraction 'A' (1.5 g) with trace material in the TLC chamber I (benzene: chloroform: ethyl acetate = 6: 3: 1). Fraction A was further chromatographed over silica gel (45 g) yielding a yellowish solid 'B' (0.8 g) in the chamber I, when eluted with pet-ether: ethyl acetate (8: 2, 7: 3). This B fraction was further chromatographed over silica gel (24 g) by elution with pet-ether: ethyl acetate (8.5: 1.5, 8: 2) to yield single prominent spot which was further analyzed by NMR, IR, and MASS spectra. Finally purified PITC-2, a light yellow solid (80 mg) was obtained. PITC-2, the thiophene derivative, was then used for anticancer study by dissolving it in 1% DMSO with different concentrations (10, 20 and 30 µg/ml). Liposome formulation of PITC-2 was dissolved in normal saline at a concentration of 1mg/ml and different doses of 10, 20 and 30 µg/ml were prepared after standardization for experiment.

Determination of cell proliferation *in vitro*

Log phase cells (U937, K562 and HL60) were taken at a concentration of 10^6/ml in RPMI 1640 (with 10% FCS) in multiple well sterile plastic plate. TCPIRE, PITC-2 and Liposome encapsulated PITC-2 (10, 20, 30 µg/ml) were added to the neoplastic cells and viable cells were counted by Trypan blue exclusion principle (Sur *et al.* 2000) after 24 hours and values were presented graphically (**Figs. 5, 6, 7**).

Cytotoxic effect of TCPIRE, PITC-2 and liposome formulation of PITC-2 on U937 K562 and HL60

Cytotoxicity was evaluated after 24 hrs by means of MTT assay (Kawada *et al.* 1999). This method estimates the capacity of living cells to reduce the tetrazolium salt – MTT by means of mitochondrial dehydrogenase enzyme. The amount of the blue dye production is proportional to the number of living cells in the culture. Cells in logarithmic phase of proliferation were seeded in 96-well micro titer plates. Control conditions comprised of (1) cells with medium alone and (2) cells with medium and vehicle. Cells were cultured both under normoxia and hypoxia conditions in a final volume of 100 µL in replicates of 4 wells per condition. Control of the O_2 tension in an atmosphere of 5% CO_2 in air was achieved using N_2. 20% O_2 (termed as normoxia) and 3% O_2 (termed as hypoxia) were the test atmospheres. Plates were assayed at 72 hrs after initiation of compound exposure. Next, 10 µL of stock MTT solution were added to each well (0.5 mg/ml) for an additional 4 hrs incubation (37°C, 5% CO_2). After 4 hrs incubation, 100 µL of DMSO is added to each well and the optical density was measured at 570 nm. The IC_{50} values were calculated using the Prism Pad computer program (GraphPad Software).

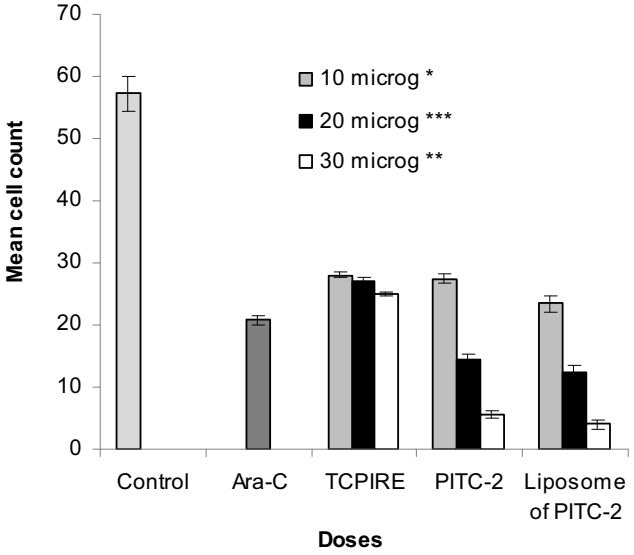

Fig. 5 Effect of TCPIRE, PITC-2 and liposome of PITC-2 on cell count of U937. Values are expressed as mean ± SEM from at least four independent experiments in case of tumor cells and percentage survivability in case of normal cells taking untreated as 100%. **P* > 0.05, ***P* < 0.05, ****P* < 0.01 when same doses of TCPIRE, PITC-2 and Liposome of PITC-2 compared to the standard drug Ara-C.

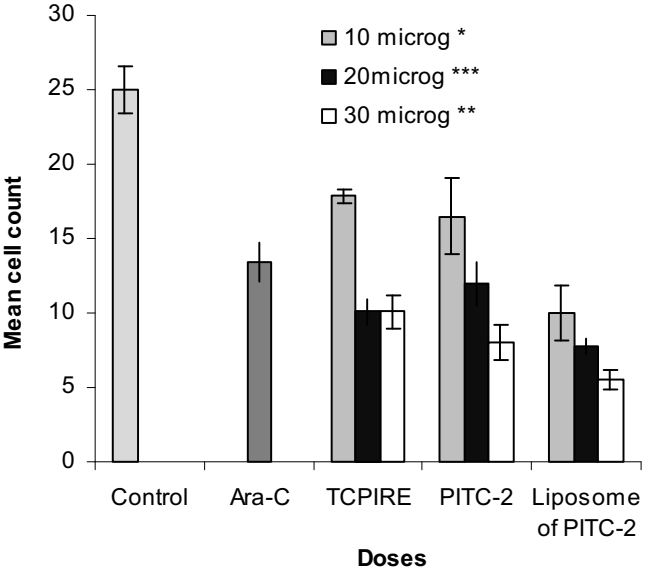

Fig. 6 Effect of TCPIRE, PITC-2 and liposome of PITC-2 on cell count of K562. Values are express as mean ± SEM from at least four independent experiments in case of tumour cells and percentage survivability in case of normal cells taking untreated as 100%. **P* > 0.05, ***P* < 0.05, ****P* < 0.01 when same doses of TCPIRE, PITC-2 and Liposome of PITC-2 compared to the standard drug Ara-C.

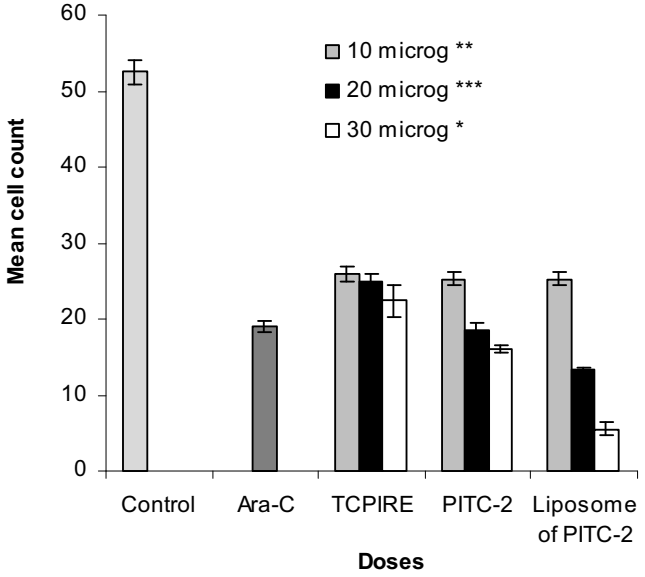

Fig. 7 Effect of TCPIRE, PITC-2 and liposome of PITC-2 on cell count of HL60. Values are expressed as mean ± SEM from at least four independent experiments in case of tumour cells and percentage survivability in case of normal cells taking untreated as 100%. **P* > 0.05, ** *P* < 0.05, *** *P* < 0.01 when same doses of TCPIRE, PITC-2 and Liposome of PITC-2 compared to the standard drug Ara-C.

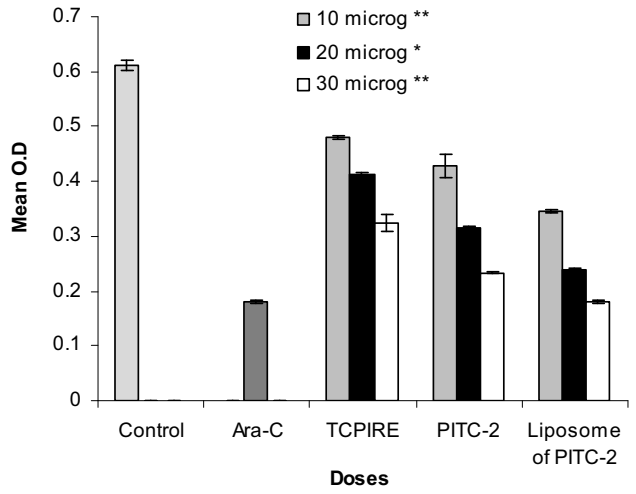

Fig. 8 Cytotoxic effect of TCPIRE, PITC-2 and liposome of PITC-2 on U937. Values are expressed as mean ± SEM from at least four independent experiments in case of tumour cells and percentage survivability in case of normal cells taking untreated as 100%. **P* > 0.05, ** *P* < 0.05 when same doses of TCPIRE, PITC-2 and Liposome of PITC-2 compared to the standard drug Ara-C.

Statistical analysis

The data were subjected to one way ANOVA followed by Dunnett's test (GraphPad Prism software) and values at $P \leq 0.001$ were considered to be statistically significant.

RESULTS

The result of the thermal degradation study of the best liposome formulation i.e., LF 7 (with molar ratio of phosphatedylcholine, cholesterol and PITC-2 = 9: 1: 1) revealed that the maximum drug entrapment of PITC-2 in liposome was 37.24%, when it was incubated at 25°C for 10 min (**Table 2**). The thermal degradation property of best formulation (LF-7) was studied and results are shown in **Fig. 11**. **Fig. 7** shows the percentage of drug EE of the best formulation LF-7 (phosphatidylcholine: cholesterol: PITC-2 = 9: 1: 1) at different temperatures (25 and 50°C) for different time periods (10, 20, and 30 min). Particle size was analyzed by a transmission electron microscope (TEM) and the optimum size and shape were at a molar ratio of PC: CH = 9: 1 (**Fig. 1**). On the other hand, from the results of the stability study of freeze-dried and suspended liposomes for 3 months it is concluded that the freeze-dried liposome formulation is much more stable than the suspended liposome formulation (**Fig. 12**). Drug-excipient interactions were studied using FTIR technique to understand the physical and chemical interactions among the drug and excipients. FTIR result confirmed that all the excipients were compatible with PITC-2. There were few changes in peak areas simply due to the mixing of components without any physical and chemical interaction. *In vitro* studies (**Table 3**) with excised rat skin revealed that the liposome formulation could release and penetrated into the rat skin in a slow manner. This result

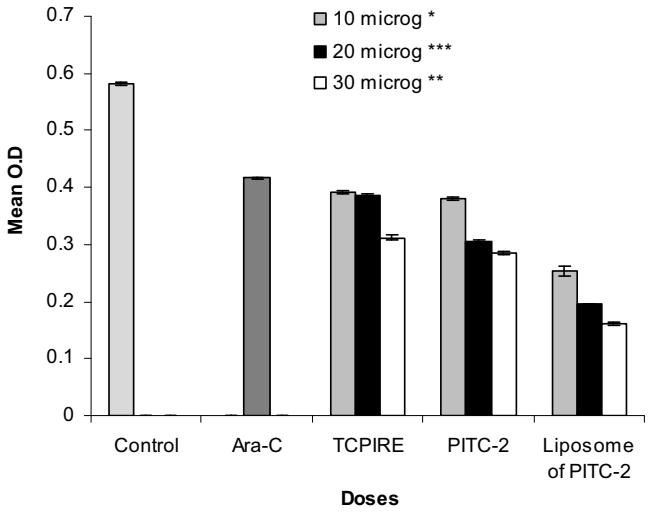

Fig. 9 Cytotoxic effect of TCPIRE, PITC-2 and liposome of PITC-2 on K562. Values are expressed as mean ± SEM from at least four independent experiments in case of tumour cells and percentage survivability in case of normal cells taking untreated as 100%. *$P > 0.05$, ** $P < 0.05$, *** $P < 0.01$, when same doses of TCPIRE, PITC-2 and Liposome of PITC-2 compared to the standard drug Ara-C.

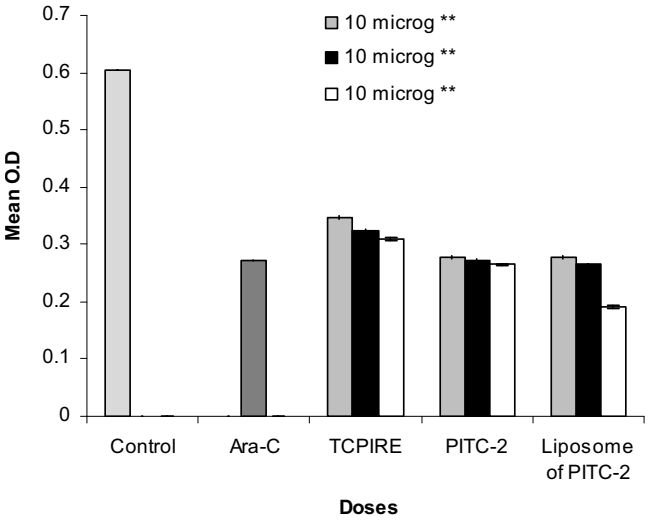

Fig. 10 Cytotoxic effect of TCPIRE, PITC-2 and liposome of PITC-2 on HL60. Values are expressed as mean ± SEM from at least four independent experiments in case of tumour cells and percentage survivability in case of normal cells taking untreated as 100%. ** $P < 0.05$, when same doses of TCPIRE, PITC-2 and Liposome of PITC-2 compared to the standard drug Ara-C.

was related to the fact that the hydrophobic thiophene derivative was effective through percutaneous absorption for sustained release dosage form. Several pure liposome preparations (without PITC-2) were tested in preliminary studies to investigate whether the test systems were influenced by phospholipid vesicles.

Antiproliferative effects of TCPIRE, free PITC-2 and liposome-encapsulated PITC-2 were tested on three cancer cell lines. We got a very positive antiproliferative activity of PITC-2 (30 µg) on U937 and HL-60 cell lines (**Fig. 5, 6, 7**). The proliferation of both cell lines was inhibited to a greater degree (in the case of U937 it was 93.03% and in the case of HL60 it was 89.52%) when PITC-2 was applied in liposomal form, whereas the growth inhibition by TCPIRE, PITC-2 and were 56.01, 90.39, 55.71 and 69.52%, respectively on U937 and HL60 cell lines after 24 hrs of incubation when they were applied at 1 mg/ml and a dose of 30 µg (**Fig. 5, 7**). The growth of K562 cells was inhibited to 57, 68 and 78% in the case of TCPIRE, PITC-2 and liposome-encapsulated PITC-2 at a dose of 30 µg (**Fig. 6**).

Table 3 *In vitro* permeability study of PITC-2-encapsulated liposome.

Time (hrs)	Absorbance at 230 nm	Cumulative drug release	Cumulative percentage drug release
0.5	0.6549	1048.60	20.9
1	0.8647	1461.53	29.2
2	0.8867	1517.35	30.3
4	1.1347	1945.34	38.9
6	1.5953	2758.05	55.1
8	1.6321	2860.82	57.2
22	1.7823	3122.08	62.4
24	1.8287	3214.68	64.3
26	1.4748	2610.27	52.2
28	1.4143	2475.85	49.5
30	1.5319	2672.65	53.5
32	1.5531	2719.65	54.4
34	1.5301	2681.59	53.6
36	1.5205	2662.60	53.3
48	1.5388	2693.75	53.9

We assessed the effect of TCPIRE, PITC-2 and liposome-encapsulated PITC-2 on the proliferation of U937, K562 and HL60 cell lines using the MTT assay method. Significant dose-dependent inhibition of cell growth was observed in all three cell lines after treatment with TCPIRE, PITC-2 and liposome-encapsulated PITC-2 for 72 hrs. Liposome-encapsulated PITC-2 at 30 µg caused > 70% growth inhibition in three cell lines (**Fig. 8, 9, 10**).

DISCUSSION

A liposome-encapsulated drug delivery system lowers the toxicity of certain drugs and prolongs their half-life in the body. In the present study we evaluated that a low concentration of PITC-2 was sufficient to inhibit proliferation of U937, K562 and HL60 cells if administered in liposomal form. The mechanism for this increased action is not yet known, however a better penetration by liposome encapsulation and sustained delivery PITC-2 from liposomes may improve the effects. PITC-2, which is linked with the liposomal surface, may encourage early reactions whereas the slow release of PITC-2 during the degradation of liposomes may cause a continuous stimulus with low PITC-2 activity. It is postulated that PITC-2 actions are initiated by binding to the high affinity receptor which is constitutively articulated on various cell lines from different tissues. Thus the capacity and type of response to PITC-2 seems to be largely resolute by a post receptor level. Nevertheless, in sensitive cell lines the degree of response is proportional to the receptor-ligand interaction (Ucer *et al.* 1985).

PITC-2 and liposome-encapsulated PITC-2 infatuated much better the cytotoxic effect than TCPIRE on all three cell lines. As in our previous study, it was reported that TCPIRE possessed significant antioxidant activity (Ghosh *et al.* 2008); thus, the antioxidant activity of TCPIRE may be related with the inhibition of neoplastic cell growth.

The cytotoxic effect was also confirmed by the result of the MTT assay which showed that TCPIRE, PITC-2 and PITC-2, when encapsulated into liposomes, killed the cells in 24 hrs to a large extent (**Figs. 8, 9, 10**). The present *in vitro* study confirmed that TCPIRE, PITC-2 and PITC-2, when encapsulated into liposomes, possess potent anti-leukemic activity (Sunilson *et al.* 2009). A further study should be conducted in the future to establish the mode of actions of the compounds against leukemic cells.

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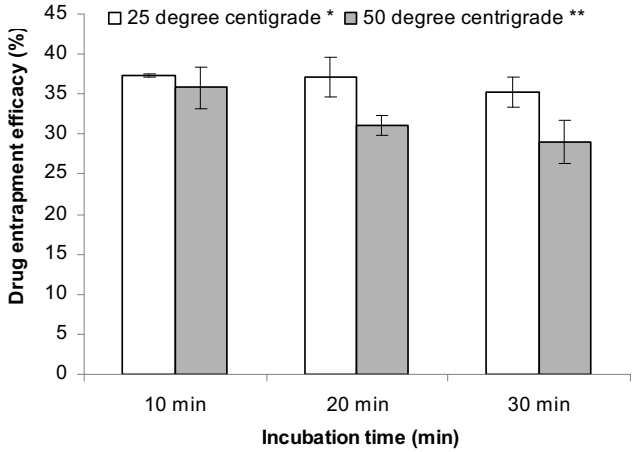

Fig. 11 Thermal degradation study of best formulation (LF-7). * $P >$ 0.05, and ** $P < 0.05$.

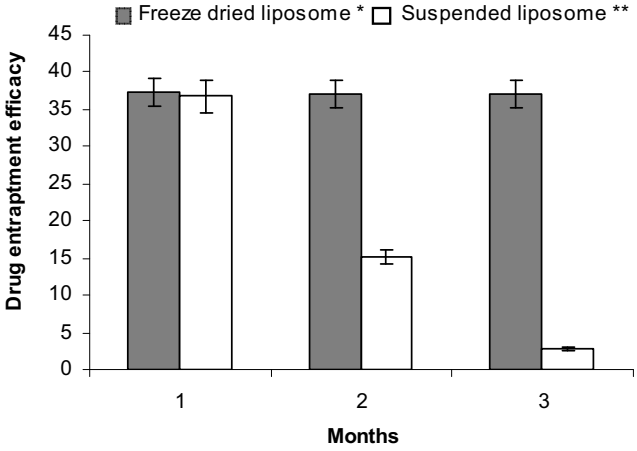

Fig. 12 Stability study of freeze-dried and suspended liposome for 3 months. * $P > 0.05$, and ** $P < 0.05$.

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