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PITC-2 Loaded Solid Lipid Nanoparticle: Design, Preparation, Characterization and Therapeutic Comparison with Free Phytochemical PITC-2 Isolated from Tissue Cultured Plant *Pluchea Indica*

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Abstract

Aim: The objective of the study is to design, preparation and characterization of PITC-2 loaded Solid Lipid Nanoparticle for i.v. administration is performed.
Methods: Phytochemical PITC-2 is isolated from methanolic root extract of medicinal plant *Pluchea indica* which has a potent cytotoxic effect. PITC-2 loaded solid lipid nanoparticles were prepared by emulsion evaporation method. Formulation characterization was done using different technique and then pharmacological estimation of prepared SLNs was performed by comparing effects of PITC-2 SLNs with free phytochemical PITC-2 on ehrlich ascites carcinoma (EAC) cell in mice. **Results:** PITC-2 SLNs formed are smooth spherical particles observed in cryo-FESEM with less than 200nm in size. 52% encapsulation efficacy is found with a stable zeta potential of -35nV. DSC and PXRD studies indicate complete encapsulation of drug within the nanoparticle matrix in amorphous form. This formulation shows a sustained and prolonged drug release. Treatment with PITC-2 SLNs decreases tumor volume and increases lifespan of cancer bearing mice in comparison to phytochemical PITC-2. The histopathological examination also indicates that PITC-2 SLNs have promising apoptotic activity on tumor cells. **Conclusion:** The developed PITC-2 loaded SLNs can be used as drug carrier for sustained and prolonged drug release with improve therapeutic activity and bioavailability.

Keywords
Solid Lipid Nanoparticle, Cytotoxicity, Apoptosis, Cell viability, PITC-2, Ehrlich Ascites Carcinoma (EAC).

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Introduction:
Cancers are considered to be the most killer disease all over the world. Therapy of cancer is based on surgery, radiotherapy and on systemic chemotherapy. Cancer therapies are remarkably consistent for last 50 years. Cytotoxic drugs play a

major role in chemotherapy for cancer except for a few cancer types where hormonal therapy and immunotherapy is used. Various categories of cytotoxic drugs are available which acts primarily by being toxic to the cancer cells that are rapidly growing and dividing. Cytotoxic drugs are earlier administered typically by free drug solutions as intravenous bolus or infusion (1).Drug carrier system plays a great role in therapeutic effectiveness and safety profile for this conventional form of this cancer therapies. Different type of drug carrier systems was used previously like colloidal drug delivery system which includes lipid emulsion, liposome and polymeric nanoparticles. But from a long time, trials are done to utilize solid lipid nanoparticle (SLN) as an alternative drug carrier system. SLN is advantageous over other colloidal drug delivery system as it improves the bioavailability of drugs (2) and obtains sustained release of lipophilic drugs (3).Moreover production of SLN are simple and in large scale with low toxicity (4). SLN consists of an amphiphilic outer core of surfactants and a biocompatible lipid inner core. The physically stable and biocompatible lipid core can incorporate lipophilic drug by emulsification of lipid molten matrix and subsequent recrystallization of the dispersed phase. SLN also provide a good protection of drugs against chemical degradation, a good drug release profile modulation avoidance of organic solvents and combination of a wide range of lipid/surfactant which results in high drug loading (5).

Although different type of carrier systems had developed for the delivery of cytotoxic drugs yet clinical success of these treatments reached a plateau (6).Moreover one main problem is its nonspecific toxicity due to their bio distribution throughout the body, which requires the administration of a large total dose to achieve high local concentrations in a tumor. Another problem in cancer chemotherapy is drug resistance. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents. Recent interest focused on medicinal properties of the secondary metabolites of these medicinal plants (7).

In this project tissue cultured medicinal plant *Pluchea indica* was used. The plant was tissue cultured with the purpose to obtain more secondary metabolites (8). *Pluchea indica* (L.) Less is a flowering plant in the family Asteraceae. The terminal flowers are rose-purple and grow in rather loose few-flowered heads. It is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia (9). From the methanolic root extract of tissue cultured medicinal plant *Pluchea indica* PITC 2 was isolated. It is a Thiophen derivative which is [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene].

The main objective of the study is to prepare SLN formulation of PITC-2 where lipid used is glycerol monostearate (GMS), poloxamer as a surfactant and soya lecithin as emulsifier. Then characterization of this prepared formulation and evaluation of in-vivo antitumor activity of the phytochemical PITC 2 against SLN PITC-2 formulation on ehrlich ascites carcinoma (EAC) cell in Swiss albino mice.

MATERIAL AND METHODOLOGY:

Materials:

Methanol (Sisco Research Laboratories Pvt. Ltd., boiling range 64 – 65°C), Petroleum ether (Marck Limited, boiling range 60-80°C), Ethyl acetate (Marck Limited), 1-Butanol (n-butyl alcohol for synthesis, Marck Limited), Silica gel (60-120 mesh, Sisco Research Laboratories Pvt. Ltd.), Lecithin from eggs, 30% from Himedia, Pluronic F 127 NF PrillPoloxamer 407 fromBASF**,** Glycerol monostearate (GMS) from Marck Limited,PLL hydrobromide (Mrs 30,000-70,000) and (4, 5-Dimethutthiazol-2-yl) -2, 5-dipheneyltetrazolim bromide(MTT) from Sigma Aldrich. Haematoxylin and Eosin stain, Papanicalaou stain from Qualigene (Mumbai, India). EDTA and Trypan blue from HiMedia. All chemicals are analytical grade (AR).

Animals and their maintenance:

An anticancer study in Swiss albino mice of either sex weighing 20 to 30 gram was conducted according to the OECD 407 guideline (OECD, 2008). The maintenance of animal handling and care were performed under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA). Swiss albino mice were selected for the anticancer evaluation animals of both sexes were designated within the same weight range. Precautions were taken to ensure that the selected female animals were nulliparous and non-pregnant. The animals were acclimatized under the standard controlled conditions, (temperature, 25 ± 5 C; relative humidity, 55± 10%) with light and dark cycle of 14 h and 10 h, respectively. A period of 7-10 days were provided to the animals to acclimatize under stipulated laboratory conditions prior to the initiation of experimentation. Throughout the experimentation period, animals were housed in

polypropylene cages provided with standard pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum* (10). All experiments were conducted as per guidelines cleared by the Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India (Registration number: 147/1999/CPCSEA).

Extraction and Isolation:

The roots after separating from the whole plant were dried, grounded and defatted with petroleum ether (60-80°C for synthesis). Then it was extracted in a soxhlet extractor with methanol (AR Grade, Purity 99.8%). After methanol distillation a sticky dark brown residue was obtained which is 8.9% (w/w). Then the methanolic root extract was partitioned in a mixture of n-Butanol and water. From this the n-butanol fraction was taken. Then it was shacked with ethyl acetate to distinguish the ethyl acetate soluble and insoluble part. Finally ethyl acetated soluble part is concentrated by a rotary vacuum evaporator and dried to obtain crude residue which is further go through column chromatography technique with a mixture of ethyl acetate and petroleum ether to obtained pure photochemical PITC 2 (11). A prominent spot on TLC plate (Mobile Phase: pet ether and ethyl acetate are in a ratio 8:2) denoted the presence of PITC-2 and it was found from the fraction 8 to15 which is the desired phytochemical PITC-2 [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene] of a yield of 0.016%(w/w). Then this compound is structurally elucidated by FT-IR, NMR and Mass spectroscopy.

Preparation of PITC-2 loaded solid lipid nanoparticles:

PITC-2 loaded solid lipid nanoparticles were prepared by emulsion evaporation method (12). Approximately PITC-2 (10 mg), soya lecithin (100 mg) and glycerol monostearate GMS (200 mg) were mixed in a beaker containing 10 ml of ethyl acetate. Then this mixture is sonicated which is the organic phase. In another beaker 40 mg of poloxamer was mixed with ultra-pure water (20 ml) and heated at around 75 °C and starred at 1000 r.p.m. (aqueous phase). Then slowly organic phase was injected into the aqueous one and the temperature was maintained at 75°C with 1000 r.p.m. By this way, the organic phase was evaporated and simultaneously the aqueous phase was also reduced. Then added ice cold ultra-pure water and maintained temperature at around 3°C with 650 r.p.m. Then it was sonicated and centrifuged at 30,000 r.p.m. to remove free drug contains in it. After centrifugation the precipitate was separated and was lyophilized to obtain the final PITC-2 loaded SLN powdered formulation. In the present study phytochemical PITC-2 have a melting point (106 – 108°C) more than the melting point of the lipid used (57 – 65 °C). Hence upon cooling the hot emulsion it is expected that the lipid will solidify faster than the drug and the drug will form a lipid core (13). And thus the formulation will be a PITC-2 enriched core SLNs formulation.

Characterization of prepared SLNs

Determination of drug content and Entrapment efficiency:

By measuring the concentration of free drug in the dispersion medium, entrapment efficiency of the drug in the formulation can be determined. The samples were subjected to centrifugation for 30 minutes at 4°C and 20,000 rpm using an ultracentrifuge. The encapsulated amount of drug in the supernatant was analyzed using UV Spectrum. The amount of drug loading (DL) in the lipid phase and entrapment efficacy (EE) was also estimated by the given equation below (14).

$$\text{Entrapment efficiency (EE) \%} = \frac{\text{Total mass of PITC2 - Mass of PITC2 in suspension}}{\text{Total mass of PITC2}} \times 100$$

$$\text{Drug loading (DL) \%} = \frac{\text{Total mass of PITC2 - Mass of PITC2 in suspension}}{\text{Total mass of Lipid}} \times 100$$

Particle side and polydispersity index:

Particle size (z-average diameter) and polydispersity index (PI) were measured using Malvern Zetasizer Nano ZS (Malvern 90) by photon correlation spectroscopy at 25°C. Before measurement, nanoparticles are dispersed in Milli-Q water (Milli-Q, Merck Millipore, Billerica, MA, USA). The measurement was conducted at 90° detection angle.

Zeta Potential:

An electronic change on the particle size was identifies by Zeta potential (ZP). To determine the physical stability of a colloidal system, ZP is used. It measures the electrophoretic mobility Malvern Zetasizer Nano ZS with a field strength 20V cm^{-1}. Equation used for ZP conversion

$$\zeta = \frac{EM \times 4\pi\eta}{\varepsilon}$$

Where ζ is zeta potential, EM is electrophoretic mobility, η is viscosity of the dispersion medium, and ε is dielectric constant (15). Before analysis weighed quantity of the experimental sample was dispersed in Milli-Q water (Milli-Q, Merck Millipore, Billerica,

MA, USA) by vortexing and sonication and then placed in a cuvette for zeta potential measurement.

Differential scanning calorimetry (DSC):

DSC was performed to investigate the melting point and crystalline behavior of crystalline materials. Samples were placed in aluminum pans and empty pans were used as reference. A heating rate of 10 °C / min was employed in the range of 30–300°C. Analysis was performed under nitrogen purge (50ml/min).

Powder X-ray diffractometry (PXRD):

PXRD was performed on the samples by exposing them in CuKα radiation at 40Kv, 30mA. The results were scanned from 2° to 70° using diffraction angle 2ϑ at a step size of 0.045° and step time 0.5 s.

Cryogenic field emission scanning electron microscopy (Cryo-FESEM):

Cryo-FESEM is used to determine the surface morphology and shape of SLNs. 2-3 drops of dispersed SLNs were placed on a copper stub and freeze in liquid nitrogen at -196°C. Samples were then stored in liquid nitrogen and placed into cryopreservation chamber. Then it was fractured and sublimed at -95°C for 30s. Then the samples are coated with platinum and introduced into specimen stage of the FESEM at -140°C and examined at an excitation voltage of 5kV.

Stability study:

Stability of the prepared SLNs were evaluated for 3 months. PITC-2 loaded SLNs optimized formulations were stored at 25°C and 4°C for 3 months and then average size, poly dispersity index, zeta potential and entrapment efficacy were determined (15).

In vitro release kinetics of PITC-2 from SLN:

In vitro drug release studies of the selected optimized formulation were performed by using dialysis bag method (16). The molecular weight cut off of the used Dialysis membrane was 12,000-14,000. Before use of the membrane for drug release dialysis membrane was soaked for 12 hours. Each Dialysis bag was filled by 2 ml of formulation and the release media was 100 ml of phosphate buffer consist of pH 7.4. At fixed intervals of 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 22, 24, 28, 32, 36, 44, 46 and 48 h, 1ml of sample was withdrawn and replaced with fresh buffer from release media and analyzed by UV at 325 nm.

Anticancer activity of prepared SLN by liquid tumor model

Ascites tumor induction by EAC cell line:

EAC cells were preserved *in vivo* by intraperitoneal inoculation of cells in the peritoneal cavity of mice with sterile normal saline. EAC cells were taken from those inoculated mice and injected (0.2 ml of 2 x 10^6 cells/mouse) to all the group's intraperitoneally except the normal group (17). From day 1 after inoculation with EAC cells treatment was given for 14 days (18) (19). From each group 6 mice were sacrificed and other 6 were kept for calculation of Mean survival time**.**

Experimental Design for anticancer study:

SLNs formulation of PITC-2 was dissolved in water for injection before administration in required concentration. For experimental purpose animals were divided into 4 groups each containing 12 mice.

Group 1: Normal animals (negative control).

Group 2: EAC cancer animals + PITC 2 (5mg/kg/day given i.p)

Group 3: EAC cancer animals + PITC2 formulation (5mg/kg/day given i.v.).

Group 4: EAC cancer animals (Standard) + 5FU (20mg/kg in alternative day given i.p).

Collection of ascetic fluid and determination of viable and non-viable cell count:

After 14 days of drug treatment, on 15th day animals were sacrificed. 2ml of normal saline water was injected (i.p.) in the abdominal region and then tumor cells along with ascetic fluid was collected in 15ml centrifuge tubes. Then they are centrifuged for 10 min at 3000 r.p.m at 4°C. Ascites tumor volume was then calculated by subtracting injected saline volume from the collected whole ascetic fluid and packed cell volume was calculated (20). The viability and non-viability of the cells were checked by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take the dye were viable and those who took the dye were non-viable. Viability percentage is calculated by (No. of viable/ No. of viable and non-viable cells) x 100 (21).

Percentage cell growth inhibition was calculated by the formula = $1 - \frac{Tw}{Cw} \times 100$

Where Tw is mean of number of tumor cells of the treated group of mice and Cw is mean of number of tumor cells of the control group of mice (22).

Determination of mean survival time (MST) and percentage increase in lifespan(%ILS):

Mean survival time and percentage increase in life span (%ILS) were calculated by the following formulas (18).

$$\textbf{Mean survival time (MST)} = \frac{\sum \textbf{Survival time(day) of each mice in a group}}{\textbf{total number of mice}}$$

$$\%ILS = \frac{\text{MST of treated mice}}{\text{MST of control mice}} \times 100$$

Hematological Parameters:

Hematological study includes hemoglobin content, red blood cell (RBC) and white blood cells (WBC) counts. After 14 days of treatment animals were sacrificed by cervical dislocation and blood samples were collected from heart. WBC leukocyte counts were also carried out including neutrophils, lymphocytes and monocytes from leishaman stained blood smears of all groups of animals.

Biochemical Parameters:

After 24 hours of last dose and 18 hours of fasting the blood samples were collected from heart. Biochemical parameters include serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase and serum protein to identify alteration in liver function and creatinine was measured as renal function parameter.

Study on change in morphology of EAC cells by staining method:

After collecting ascetic fluid from treated and control group, they were centrifuged at 3000 rpm for 10 minutes and one part of it is fixed in glass slides with neutral buffer formation. Then they were stained with hematoxylin and eosin. Another part is fixed on glass slide using 95% ethanol and stained with papanicalaou stain. Then both stained slides were mounted with Distrene Dibutyl Phthalate xylene and were examined under light microscope (40X) (23).Liver and kidney tissues were isolated from the animals and washed with saline. Then it was fixed in 10% buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin (24). Then the tissues were sectioned 5 to 6 µm, stained with hematoxylin and eosin (H&E) and examined under light microscope (40X).

Statistical analysis:

All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as mean ± standard error of the mean (SEM) and were compared by one-way analysis of variance (ANOVA) followed by Kruskal Wallis Test using SPSS statistical software of 20.0 version. $P<0.05$ was considered to be statistically significant when compared with control.

RESULT AND DISCUSSION:

Identification of PITC-2:

The present light-yellow solid compound was further confirmed by NMR spectroscopy and MASS spectroscopy (25). In mass spectroscopy a strong pick was observed at *m/z* 230 shown in Fig. 1A. Molecular weight of compound PITC-2 is 230. So, the present peak in mass spectroscopy is may be for PITC-2 which will be confirmed by NMR shown in Fig. 1 B1 and B2. In ^1H NMR δ^{TMS} ($CDCl_3$, 300MHz): 1.64 (D_2O exchangeable, OH merged with solvent H_2O), 2.04(3H, s), 3.78(^1H, dd, J=11.4, 6.3Hz, H_A of CH-CH_2OH), 3.82 (^1H, dd, J=11.4, 3.9 Hz, H_B of CH-CH_2OH), 4.69 (^1H, dd, J=6.3, 3.9 Hz, CHOH--CH_2OH), 7.04, 7.18 (^2H, m, thiophene-H). The ^1H NMR spectrum showed peaks for a methyl attached to unsaturation δ (2.04, s), a CHX-CH_2Y unit and an aromatic system. The compound finally identified is PITC-2 which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene.

Characterizations of SLNs

Particle size and Zeta Potential:

The size distribution of PITC-2 loaded SLNs were shown in Fig. 2A and Table 1. In general, PITC-2 loaded SLNs looks round, uniform sized and well dispersed. The mean size of our nanoparticles was 171.5d.nm. It is also observed that particle size of PITC – 2 loaded SLNs are smaller than drug free SLN formulation. Zeta potential and stability of nanoparticles are closely related to each other. By measuring zeta potential, the stability of a nanoparticle system can be analyzed. Due to Van Der Wall inter particle attraction if a dispersion has low zeta potential then particles will get aggregated (4). Intracellular distribution of nanoparticles depends upon its zeta potential (26). In our study the zeta potential of all the prepared formulation were negatively charged and varied from -33.2 to -37.5 mV which ensured a system with good stability as it posse's sufficient repulsion. The zeta potential with negative charge might not interfere with the absorption of the formulation (27). Zeta potential values were presented in Table1 and shown in Fig 2B.

Table 1: Particle Size, Polydispersity index and Zeta Potential of drug free SLNs and PITC-2 loaded SLNs(data represents mean ±SD).

Formulations	Particle size (d.nm)		Polydispersity index		Zeta Potential (mV)	
	Medium (n=3)	Standard deviation	Medium (n=3)	Standard deviation	Medium (n=3)	Standard deviation
Drug free SLNs	290.5	±3.21	0.212	±1.32	-35	±5.90
PITC-2 loaded SLNs	171.5	±4.87	0.101	±1.09	-36.1	±5.57

Table 2: Particle Size, Polydispersity index, Zeta Potential and Entrapment Efficacy of PITC-2 loaded SLNs (data represents mean ±SD)

Storage	Particle size(d.nm)	Polydispersity index	Zeta Potential (mV)	Entrapment efficacy (%)
Fresh	171.5 ± 4.87	0.101 ±1.09	-36.1 ±5.57	52 ±0.53
4°C for 3 months	182.43 ± 5.13	0.116 ± 2.74	-31.87 ± 4.32	48 ± 1.23
25°C for 3 months	172.21 ± 3.08	0.105 ±1.65	-35.18 ± 4.68	51.24 ± 0.43

Table 3: *In-vivo* effect of PITC-2 on EAC bearing Mice

	PITC-2 5mg/kg b.w	PITC-2 loaded SLN Formulation 5mg/kg b.w	Std drug 5-FU 20mg/kg b.w	Sarcoma 180 Control
Ascites fluid volume (ml)	5.64 ± 0.28*	5.24 ± 0.12*	5.89 ± 0.23*	11.90 ± 0.11*
Packed Cell volume (ml)	1.45 ± 0.08*	1.39 ± 0.04*	1.32 ± 0.02*	4.21 ± 0.08*
Body weight (gm)	22.41 ± 0.43*	22.13± 0.42*	22.68 ± 0.55*	30.10 ± 0.48*
MST (days)	39.67 ± 0.98*	41.83 ± 0.70*	32.00 ± 0.85*	18.40 ± 0.42*
%ILS	215.59	224.89	173.91	0 ± 0
Viable Cell(10^6cells/ml)	3.05± 0.13*	2.92 ± 0.21*	3.45 ± 0.43*	10.65± 0.23*

<0.05 as compared to control group (n = 6 mice per group)

Table 4: Effect of PITC-2 on hematological parameters in EAC bearing Mice

	PITC-2 5mg/kg b.w	PITC-2 loaded SLN Formulation 5mg/kg b.w	Std 5-FU 20mg/kg b.w	EAC control	Normal Mice
Hemoglobin (gm %)	9.4± 0.05*	9.6± 0.08*	9.1± 0.06*	6.3 ± 0.01*	11.8±0.12*
Erythrocyte (RBC) (cells x10^6/mm^3)	5.6± 0.04*	6.53± 0.03*	5.68± 0.08*	3.75 ± 0.08*	7.90±0.02*
Leucocytes (WBC) (cells x10^6/mm^3)	9.1± 0.05*	8.29±0.05*	10.19±0.05*	16.69±0.02*	6.40±0.03*
Neutrophil (%)	31.3± 0.03*	30.46±0.03*	30.1±0.02*	68.5 ± 0.05*	19.4±0.03*
Lymphocyte (%)	53.0± 0.04*	55.4± 0.04*	61.0±0.09*	35.8 ± 0.11*	64.1±0.23*
Monocyte (%)	1.7± 0.03*	2.2± 0.03*	1.6± 0.03*	1.3 ± 0.04*	2.8±0.01*

<0.05 as compared to control group (n = 6 mice per group)

Table 5: Effect of PITC-2 on biochemical parameters in EAC bearing mice

	PITC-2 5mg/kg b.w	PITC-2 loaded SLN formulation 5mg/kg b.w	Std 5-FU 20mg/kg b.w	Sarcoma 180 control	Normal Mice
Bilirubin (total) mg/dl	0.26± 0.02*	0.27± 0.02*	0.24± 0.03*	0.34± 0.01*	0.45± 0.02*
Serum protein (total)g/dl	8.52 ± 0.09*	8.29 ± 0.07*	8.22 ± 0.12*	4.4± 0.08*	8.19±0.08*
AST (SGOT) IU/L	34.5± 0.01*	34.9± 0.01*	35.9± 0.01*	74.9± 0.09*	39.1±0.01*
ALT(SGPT) IU/L	35.6± 0.04*	36.4± 0.01*	39.3± 0.06*	71.4± 0.07*	29.1±0.04*

Serum alkaline phosphatase IU/L	71.1± 0.02*	69.9± 0.02*	72.8± 0.05*	121.1±0.03*	74.4± 0.03*
Creatinine Mg/dl	0.78± 0.01*	0.8± 0.02*	0.81± 0.01*	1.2± 0.02*	0.62± 0.01*

<0.05 as compared to control group (n = 6 mice per group)

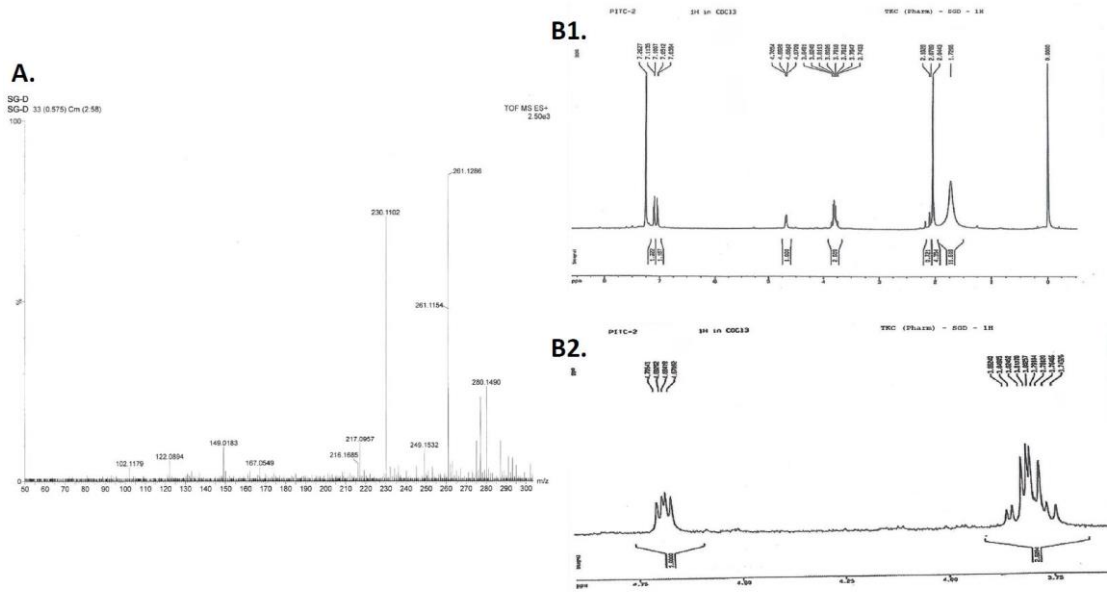

Fig 1: A. Mass spectrum of compound PITC-2, B1 and B2. 1H NMR spectrum of compound PITC-2 in $CDCL_3$ (300MHz)

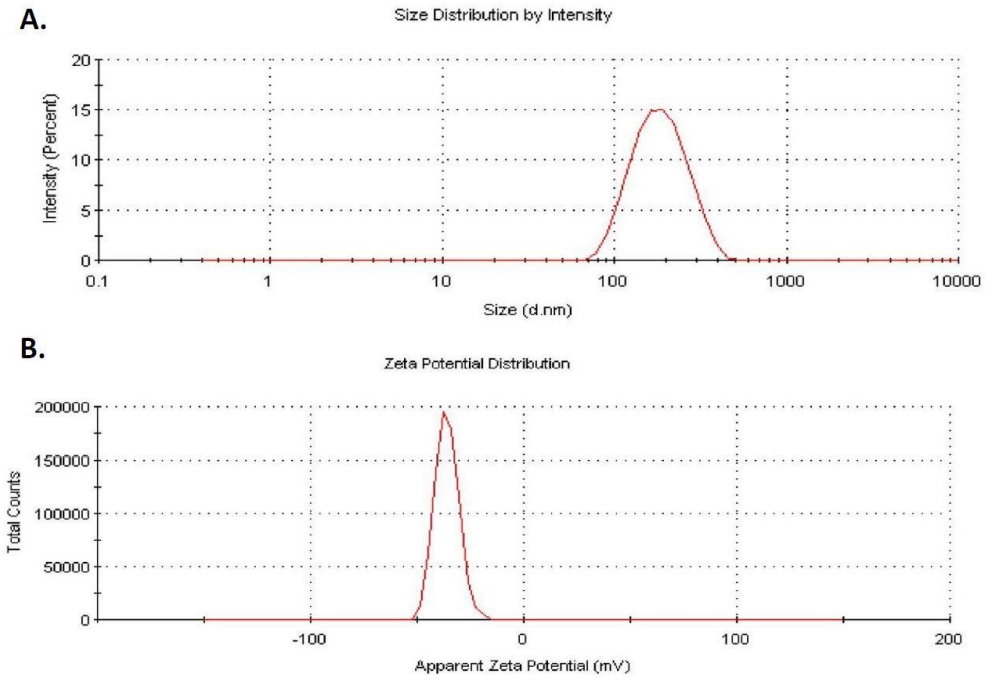

Fig 2: A. Particle size distribution curve of PITC-2 loaded nanoparticles, B. Surface Zeta potential graph showing negative zeta potential value of PITC-2 SLNs.

Entrapment Efficacy:

Total drug used in preparation of formulation was 10 mg. Amount of drug present in the SLNs was found to be (10-4.8) 5.2 mg. Entrapment efficacy of the prepared formulation was 52%.

Thermal analysis by differential scanning calorimetry (DSC):

DSC was a tool which investigate the crystalline behavior and melting point. Fig 3A shows DSC curve of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation. A clear sharp pick observed at 106.26°C for phytochemical PITC-2 which corresponds to its melting temperature. GMS shows its melting pick at 63.4°C. PITC-2 loaded SLN shows pick at 65.14°C whereas drug free SLN shows pick at 64.21°C. This slight shift of pick was due to loading of drug in the lipid matrix. Absence of drug pick at 106°C indicates either solubilization of PITC-2 in lipid matrix upon heating or due to amorphous dispersion of PITC-2 in lipid matrix. A similar type of phenomenon was reported by Das *et.al* for Tretinoin loaded SLNs (15). This reveals a homogeneous dispersion of drug in the lipid.

Powder X-ray diffractometer (PXRD):

Fig 3B shows XRD curve of phytochemical PITC-2, drug free SLN, and PITC-2 loaded SLN formulation. Phytochemical PITC-2 have picks at *2θ* scattered angle shown in the figure which defines its crystalline nature. Those picks are not found in the diffractogram of PITC-2 loaded SLNs which indicates PITC-2 was solubilized and stabilized in lipid matrix of formulation. If PITC-2 was present outside of lipid matrix then due to its poor solubility it would crystallize outside the lipid matrix and effect the diffraction curve. Now the diffractogram of drug free SLNs closely resembles to the diffractogram of PITC-2 loaded SLNs which indicates no change happens in the nature of SLNs due to addition of PITC-2. It shows PITC-2 is no more in a crystalline form in SLN formulations but in an amorphous form. This phenomenon is also observed from the morphological study of the SLNs.

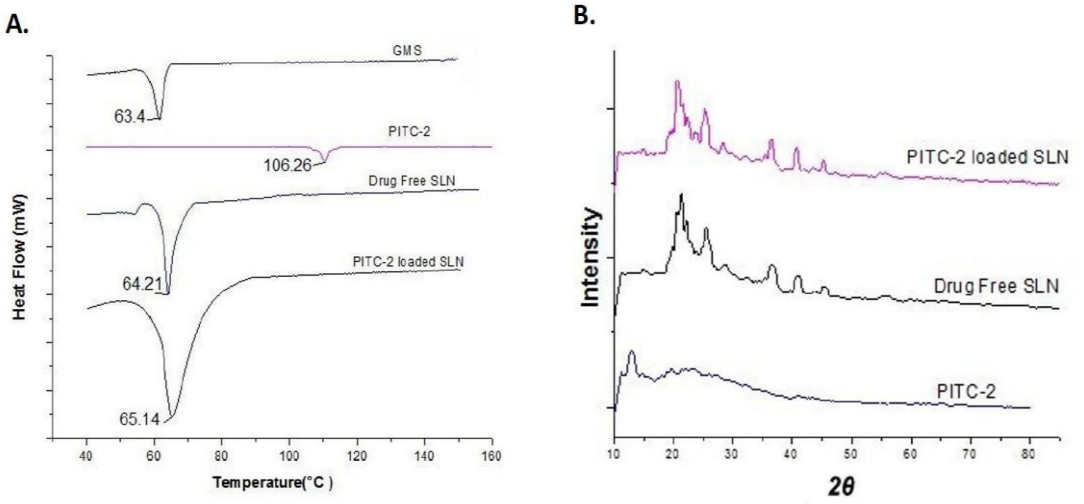

Fig 3: A.DSC Thermogram of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation. B. PXRD profile of phytochemical PITC-2, glycerol monostearate GMS, drug free SLN, and PITC-2 loaded SLN formulation.

Stability of SLNs:

There are minor changes in particle size, PI, ZP and EE after 3 months of storage at 4°C but no changes at all were found in particles stored at 25°C shown in Table 2. However, the changes are not that significant which indicates a good physical stability of PITC-2 SLNs for 3 months at 4°C and 25°C.

Cryogenic field emission scanning electron microscopy (Cryo-FESEM):

To check the surface morphology of nanoparticles Cryo-FESEM was done. Size and shape of the nanoparticles are identified by this method. In our study from Cryo-FESEM of PITC-2 loaded SLNs it was observed that they are almost spherical in shape and have a smooth surface morphology shown in Fig 4A. Agglomeration of nanoparticles are also found which may be due to the lipid as a carrier system. On the other hand, Fig 4B shows crystalline structure of free phytochemical PITC2. Absence of such crystalline structure of PITC-2 in SLNs formulation shows no unentrapped in the formulation (15).

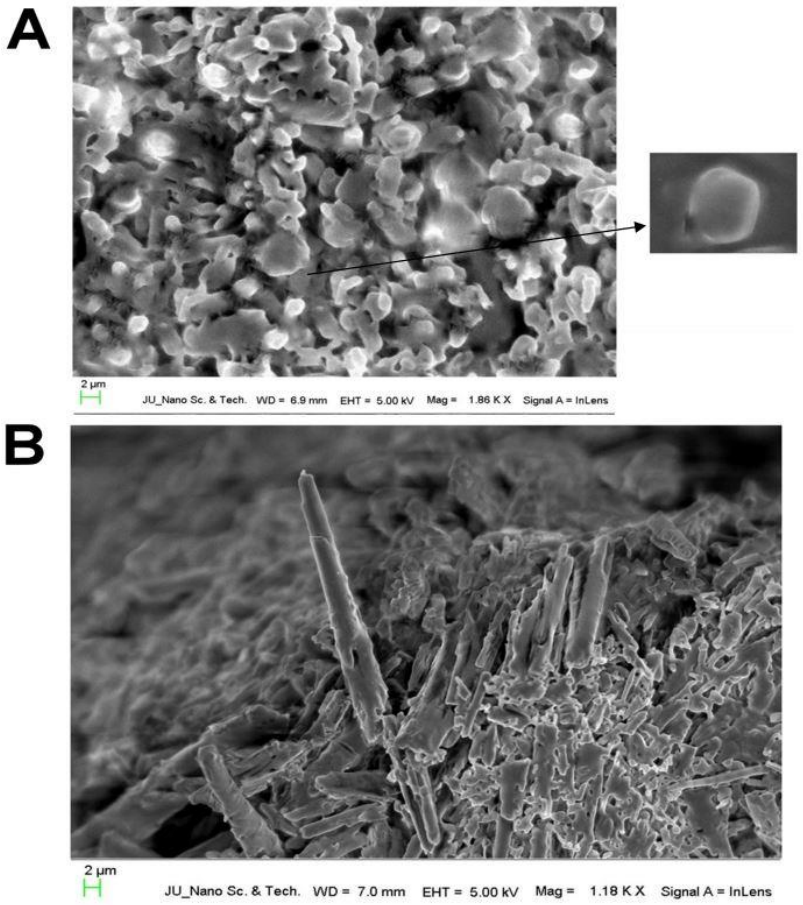

Fig 4: FESEM images of A. PITC-2 loaded SLNs, and B. phytochemical PITC-2.

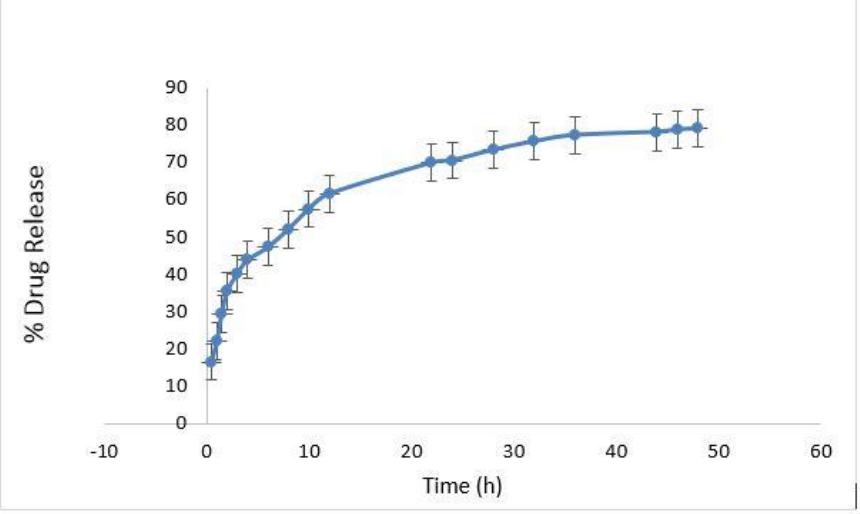

Fig 5: Drug release profile of PITC-2 loaded SLNs (data represents mean ±SD).

***In vitro* release kinetics of SLNs:**

Fig 5 shows a sustained drug release of PITC-2 loaded SLNs at 7.4 pH phosphate buffer. Percentage drug release verses time is plotted to determine the pattern of drug release. The release study shows biphasic pattern. It was observed that 35 % of the drug was released in first 2 hr and then a sustained release of SLNs occurs. These burst releases occur may be due to presence of absorbed drug in the surface of the nanoparticles. After 48 hrs of percentage drug release was 79.02%. This is important for the physiological requirements of human system. A similar type of pharmacokinetic study was observed by Shengpeng Wang et.al (28).

Inhibition of EAC cells growth by PITC-2:

A significant reduction of ascetic fluid volume, packed cell volume and viable tumor cell count was found with PITC-2 SLNs formulation at dose 5mg/kg and phytochemical PITC-2 at concentration 5mg/kg b.w compared to that of control group mice. A same type of finding was observed by de Costa *et al.* on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives (29). Moreover, a good increase in lifespan and median survival time (MST) was also observed in EAC cell bearing mice when treated with PITC-2 loaded SLNs, shown in Table 3.

Effect of PITC-2 on hematological parameters

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte and lymphocyte count was found in phytochemical PITC 2 treated groups as well as in PITC-2 loaded SLNs with compared to that of EAC control group (Table 4). Even SLNs shows comparative good result than free PITC-2. This reveals protective activity of PITC-2 SLNs on the hematopoietic system.

Effect of PITC-2 on biochemical parameters

One major problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver (30). Toxicity is a major problem of drug delivery system too. One can have a neat delivery system, but it is more important to be a safe delivery system with low toxicity for its introduction in pharmaceutical and clinical market. Toxicity is due to the excipients in carrier system. Use of glycerides composed of fatty acid, contained in oils of parenteral fat emulsion may reduce toxicity (4). In our study we had used glyceryl monostearate which may not induce any toxicity for the SLNs. Furthermore, surfactant used in our study was poloxomer which is recommended as an acceptable surfactant for use in parenteral formulations. (4). Here liver toxicity was analyzed in this experiment by biochemical studies and histopathology of liver tissue. Phytochemical PITC-2 treated group and PITC-2 loaded SLNs group shows significant decrease in SGPT, SGOT and serum alkaline phosphatase parameters in comparison with EAC control group shown in Table 5. It was found that groups treated with PITC-2 loaded SLNs had less toxic liver as the level of AST, ALT and ALP are close to normal value. An more increase in total protein was also found in PITC-2 formulation treated groups.

Histopathology of liver and kidney tissue

Lesion in liver was caused by many drugs as well as carrier systems which are analyzed by biopsy. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver has a unique regenerative ability and great adaptive efficiency. For example, an adaptive phenomenon of liver is if an anticonvulsant drug was used for a long time then it causes increase in endoplasmic reticulum. Again, regeneration of hepatic tissue occurs by the conjugative tissues after a hepatocellular necrosis due to treatment with alginates (31). In our study H&E stained section of liver of a healthy mouse was shown in Fig. 6A, bearing all the normal features which includes circular hepatic portal vein and of hepatic artery, as marked by arrow. The tissue section comprises of hepatic sinusoid and nuclei which are usual. But none of the above-mentioned regular features are observed in case of Sarcoma-180 control group showed in Fig. 6B, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Although little changes are found in group receiving standard 5-FU like deformed hepatic artery and irregular bile duct yet lesser amount of hepatocellular lesions observed which are close to normal as shown in Fig. 6C. Groups receiving phytochemical PITC 2 and PITC-2 loaded SLNs also shows a little hepatic deformation and altered hepatocyte population shown in Fig. 6D and 6E. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed found. This all features show SLNs are less toxic to liver.

Histopathological analysis of kidney was also done for control, 5-FU, phytochemical PITC-2 and PITC-2 loaded SLNs. H&E stained section of kidney of a healthy mice was shown in Fig. 7A, bearing all the normal features, while Sarcoma-180 control group shows severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders showed in Fig. 7B. Group treated with 5-FU and PITC-2 shows light glomerular and tubular hemorrhage and cellular features are less deformation which are close to

normal shown in Fig. 7C, 7D and 7E respectively. The characterization of necrosis is done by no nuclear staining and deeply eosinophilic cytoplasm (31).This show PITC-2 loaded SLNs are less toxic to kidney than standard drug and quite resembles to the action of phytochemical PITC-2.

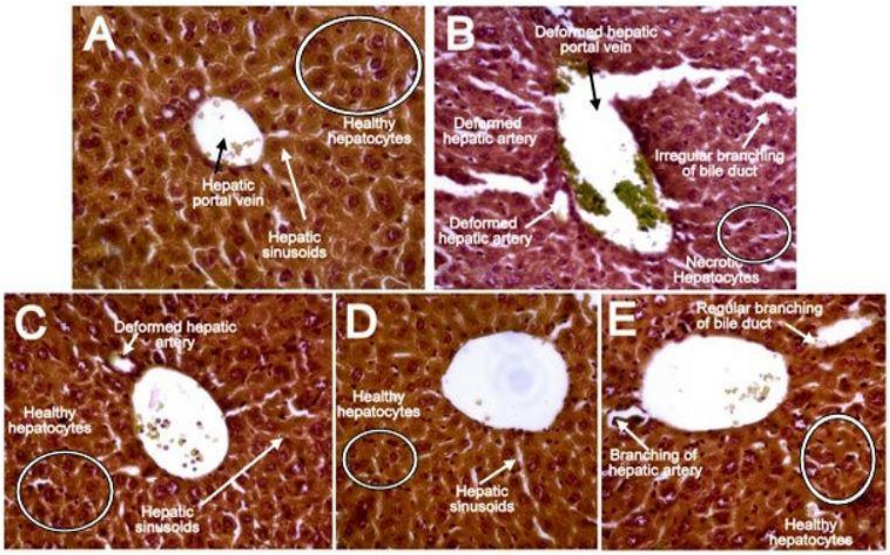

Fig 6: H & E stained section of liver of mice showing cell morphology of groups A. Normal Mice, B. EAC control mice, C. 5 FU standard drug, D. 5 mg/kg PITC-2 and E. 5mg/kg PITC-2 SLNs.

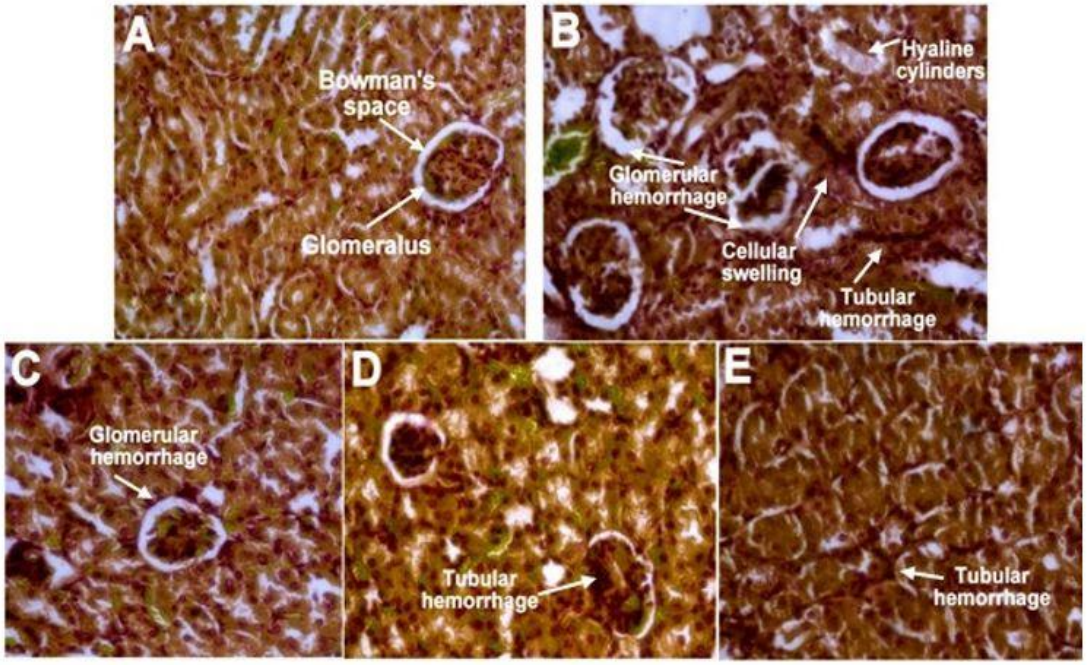

Fig 7: H & E stained section of kidney of mice showing cell morphology of groups A. Normal Mice, B. EAC control mice, C. 5 FU standard drug, D. 5 mg/kg PITC-2 and E. 5mg/kg PITC-2 SLNs.

Morphological changes of EAC cells

Now for analyzing the cytotoxic effect of the formulation histopathology of ascetic fluid was done. Cell apoptosis takes place and a significant morphological change was observed in EAC cell with both Haematoxylin & Eosin (H&E) and Papanicolaou (Pap) staining shown in Fig 8. Apoptosis is a cell regulatory mechanism which is distinguished by diverse alteration in cell morphology including asymmetry in plasma membrane, cytoplasm shrinkage, chromatin condensation, formation of apoptotic bodies, etc (32) (30). In our study, H&E

staining cells of control group have good circular morphology, intact plasma membrane and nucleus. But cell of groups treated with standard drug shows apoptotic bodies and nuclear contraction. Irregularity in cell morphology, blebbing of plasma membrane, chromatin condensation was also found in groups treated with phytochemical PTIC-2. In case of groups treated with PITC-2 loaded SLNs evident change in cell morphology was found which includes cell shrinkage, formation of apoptotic bodies, nuclear contraction and blebbing of plasma membrane. Similarly, in case of cells stained with Pap stain numerous features of cellular apoptosis was observed in standard and test drug groups which are shown in the figure by arrow. Degree of change in cell morphology was more in SLN PITC-2 than phytochemical PITC-2.

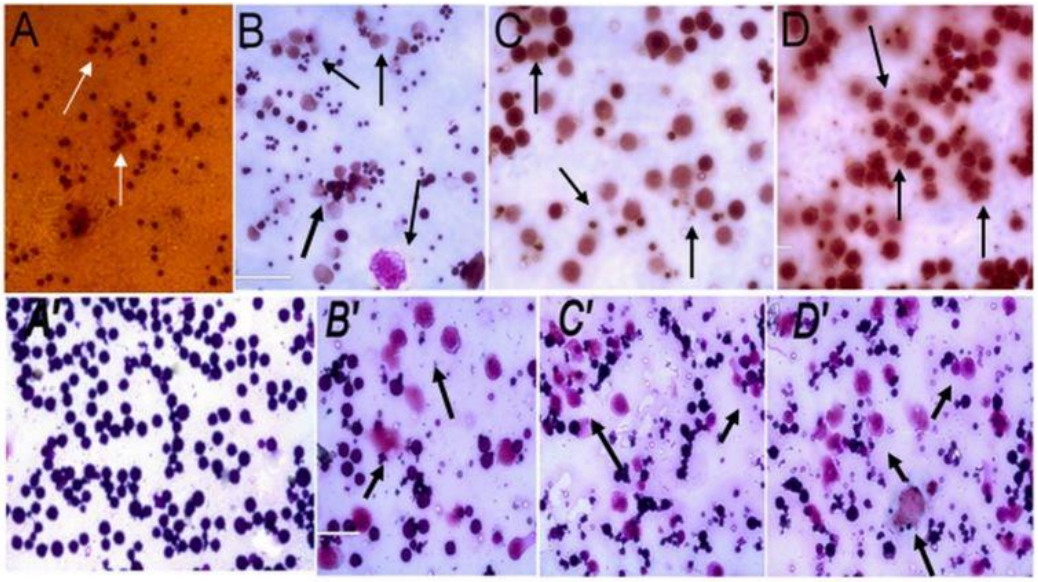

Fig 8: Change in cell morphology of EAC cells by H&E stain of groups A. EAC control mice, B. 5 FU standard drug, C. 5 mg/kg PITC-2 and D. 5mg/kg PITC-2 SLNs and Papanicolaou stain of groups A'. EAC control mice, B'. 5 FU standard drug, C'. 5 mg/kg PITC-2 and D'. 5mg/kg PITC-2 SLNs

CONCLUSION:

In this study PITC-2 loaded SLNs were successfully prepared by emulsion evaporation method. Poorly water soluble phytochemical PITC-2 was effectively loaded in the nanoparticles. The method resulted in producing consistent smaller size nanoparticles in the range of 170- 180 nm with narrow size distribution. Most of the process variables shows significant effect on the formulation properties. SLNs prepared for systematic drug delivery obtains better therapeutic efficiency by sustained drug release, thus by improving bioavailability and will decrease dosing with lower side effects. Finally, this planning methodology has clearly shown its usefulness in this optimization process, and this research produces a framework for the understanding of SLN formation.

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