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Lipid shell modified with combination of lipid and phospholipids in solid lipid nanoparticles for engineered specificity of paclitaxel in tumor bearing mice.

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Abstract

Paclitaxel (PTX) is an anticancer drug belonging to the class of Taxan. It is active against various types of carcinomas. The marketed formulation of paclitaxel is associated with deleterious effects with lack of specificity to tumor. Solid lipid nanoparticles (SLN) are colloidal carriers extensively studied and developed for their potential uses especially for controlled release and site specificity. The present study was designed to develop a formulation of PTX in the form of SLN to be administered via IV route with improved tumor specificity, in which the lipid shell was modified by using combination of lipid with phospholipids. Total eight formulations were prepared and were characterized by various in vitro and in vivo parameters. The microemulsification method was used for the preparation of SLN. The production yield of resulting process for all SLN was high. Average particle size was ranged between 209 nm to 385 nm. The developed PTX-SLN showed high percentage entrapment efficiency. The zeta potential values showed the good stable feature of the SLN. The in vitro dissolution study showed that drug release was more retarded and was found to dependent on concentration of lipids employed. *In vitro* cytotoxicity study was performed on MCF-7 cancer cell line, which showed that formulation G2 is having more potentiating effect on cancer cell line. Tissue targeting study and tumor growth inhibition studies were performed on mice where the PTX loaded SLN from batch G2 shown more promising outcome. Results obtained from this study indicated strongly that developed SLN are having potential as an efficient drug delivery system for paclitaxel.

Keywords: PTX, SLN, Modified Shell, Lipid and Phospholipids, Stearic Acid SLN

Introduction

Cancer occurs due to abnormal cell growth, because of cell division without any control, which is an altered functionality of the cells[1]. Chemotherapy is the treatment of cancer with drugs (anticancer drugs) that can destroy cancer cells.[2].

Paclitaxel (PTX) is an taxane, derived from Pacific Yew tree and is active against various cancers.PTX is one of the several cytoskeletal drugs that target tubulin. PTX-treated cells have defects in mitotic spindle assembly, chromosome segregation, and cell division. PTX stabilizes the microtubule polymer and protects it from disassembly. This blocks progression of mitosis, and prolonged activation of the mitotic checkpoint triggers apoptosis or reversion to the G-phase of the cell cycle without cell division. [3,4]

The use of PTX is having its potential advantages as it can cure the cancer but have some drawbacks. The conventional formulation of PTX is in the form of IV solution, formulated with Cremophor EL and Ethanol, marketed as Taxol. This formulation often results in anaphylactic reaction in patients ultimately questioning the therapeutic benefits of PTX. The harmful effects of this drug are seen as they are distributed in cancer cell as well in healthy tissues. The drawbacks of this treatment are due to lack of specificity of cytotoxic agents to cancer cells [5].

An ideal drug delivery system should result maximum therapeutic benefits of drug, which should include the safe and effective management of disease, and it should improve patient compliance [6].

Cancer therapy with chemotherapeutic agents is undergoing a constant improvisation cycle. This is much needed to meet the current demands of disease. The main research is seen with respect to finding new drug delivery systems and constant up gradation of existing ones. In this regard, much focus is given to carriers with site specificity [7].

For the purpose of tissue targeting various moieties were taken on radar namely microparticles [8]. Microballons [9]. bioresponsive systems[10]. colloidal carriers[11]. etc.

The scientists have always given their preference to these carriers as they are having tremendous potential in present drug formulation industry. These carriers are studied extensively and have been produced on large scale because these are the one, which meets the current demand of therapy.

Out of these, colloidal carriers have gained attention of pharmaceutical scientists, as these are associated with an advantage of site specificity and optimal drug release profile. These are the characteristics, which are very important one, as appropriate measure of disease treating is concern. [12].

The nanotech components are very useful findings from the viewpoint of drug delivery system that will hopefully carry therapeutic and diagnostic agents to specific sites in the body allowing for highly targeted treatments that could minimize side effects. [13].

To meet above stated goals of drug delivery system solid lipid nanoparticles have shown great potential. Solid lipid nanoparticles are a novel nano particulate system, which is attracting major attention as an alternative colloidal carrier system to polymeric nanoparticles, liposome's and nanoemulsion [14-17].

These carriers have advantages like improved stability of drugs, encapsulation of agents of diversified physicochemical properties, enhanced drug efficacy, improved pharmacokinetics and less toxicity. They have been reported as useful drug carriers [18-20].

Extensive researches have been done on solid lipid nanoparticles of several chemotherapeutic agents in suitable carriers and their in-vitro and in-vivo efficacy have been evaluated.

However the formulation of SLN by mixed lipid blend and it's in vitro and in vivo biodistribution has not been previously reported. There potential for efficiency of site specificity is not studied. Therefore present study was undertaken to develop PTX loaded SLN designed by lipid shell modified with lipid and phospholipids blend as to study its in vitro characteristics and in vivo biodistribution studies in tumor bearing mice.

Materials

Paclitaxel (IP grade) was supplied by Unan Hande Biotech Co. Ltd. China, Stearic acid was purchased S.D. Fine Chemicals, Mumbai, Di-palmitoyelphosphotidylcholine (DpPC) and 1,2 Dipalmitoyel SN Glycero 3- Phospho Glycerol, Sodium (DPPG,Na) were procured from Genzyme, Switzerland. Sodium glycholate and Soya lecithin were purchased from Across Organics, New Jersey, USA. Potassium dihydrogen phosphate was procured from Ranbaxy Fine Chemicals New Delhi. Ethanol and other solvents were purchased from local suppliers. All the chemicals were used as supplied , without further purification.

Table 1 Formulation Table Design for SLN Prepared by using Combination of 3 Lipids

Form. code	Drug (Parts)	Soy Lecithin (%)	Sodium Taurocholate (%)	Stearic Acid (Parts)	DpPC (Parts)	DPPG.Na (Parts)
	1	15	0.8	10	10	10
	1	15	0.8	5	5	5
	1	15	0.8	10	10	5
	1	15	0.8	10	5	10
	1	15	0.8	5	10	10
	1	15	0.8	5	5	10
	1	15	0.8	5	10	5
	1	15	0.8	10	5	5

Preparation of Paclitaxel Loaded Solid Lipid Nanoparticle

Total 8 batches of PTX loaded SLN were prepared. The formulation table no.1 shows the concentration of various ingredients for preparation of PTX- SLN.

Solid lipid nanoparticles of PTX were prepared by micro emulsification technique using soy lecithin as surfactant, sodium glycholate as co surfactant and 0.1 N Hcl as liquid manufacturing vehicle. Shell material was melted at a temperature of 80^0c to the melted shell constituent PTX drug was added with 5 min. stirring followed by sonication [21].

To this mixture soy lecithin was added and stirred for 2 minutes. Aqueous phase containing co surfactant sodium glycholate was heated at 80o.c and added to melted shell phase mechanical stirring at 800c for 10-15 minutes, formed o/w microemulsion. The micro emulsion was carefully added drop wise into ice cold water in a beaker with continuous stirring a 5 cc glass syringe fitted with 21 gauge needle was used to control the particle size.

The mixture was stirred at 3000rpm and SLN dispersion was stirred for three hrs after complete addition of microemulsion. The SLN dispersion was subjected to ultrasonication for 10 minutes. Collected formulations were lyophilized to get free flowing product.

Particle Size Analysis

The size of particle is an important parameter for SLN to be administered via parentral route. The particle size is crucial for controlled drug release and tissue specificity of drug. Particle size less 100 nm are rapidly taken up in RES and there specificity less So in present study due concern is given to the particle size in the range of 200 nm to 250 nm. The particle size of developed SLN was determined by Dynamic Light Scattering Particle Size Analyzer (Nanotrac Particle Size Analyzer) [22].

Percentage Yield

To check process capability to produce drug loaded SLN percentage practical yield was determined. This assures the output obtained after the completion of production operation. The lyophilized PTX-SLN from each formulation were weighed and the respective percentage yield was calculated using the formula no. [1] [23].

Percentage Yield=(Wt of SLN obtained / Wt of drug +polymers used) X 100 … [1]

Determination of Entrapment Efficiency Percentage

The amount of drug equivalent to one dose from SLN is calculated based on % EE. It is desirable to have high EE as it will allows less quantity of sln/ unit dose of drug, in comparison to low EE which will need more quantity of SLN/unit dose of drug [24].

Entrapment efficiency of PTX loaded SLN was estimated by centrifugation method. The prepared SLN were placed in centrifugation tube and centrifuged at 15000 rpm for 30 min. The supernatant (1ml) was withdrawn and diluted with methanol. The unentrapped PTX was determined by UV spectrophotometer at

227 nm. The samples from the supernatant were diluted suitably before going for absorbance measurement. The free PTX in the supernatant gives the total amount of unentrapped drug. Encapsulation efficiency is expressed as the percent of drug entrapped and was calculated using equation no. [2].

% E.E. = {(Total amount of drug – Free dissolved drug) / Total amount of drug} X 100 .....[2].

SEM Study

Surface topography gives about external feature of prepared formulations. Scanning electron microscopy was done to study the particle surface morphology and shape. SEM was done by using JSM-T330A, JEOL, Hindal, India.

Zeta Potential Determination

Zeta potential was measured by using Zetatrac. Sample was filled into the cell; electrodes are inserted, placed under the microscope, and connected them to the Zeta-Meter 3.0+ unit. All data were taken in triplicate [25].

In Vitro Release Study

The release of PTX from the nanoparticles was measured in triplicate in PBS (PH 7.4). SLN equivalent to 10 mg of PTX were placed in dialysis bag soaked overnight with dissolution fluid. Dialysis bag containing formulation was suspended in 200 ml of PBS (PH 7.4) solution in a capped beaker and placed in an orbital shaker, which was maintained at 37^0 centigrade degree and shaken horizontally at 120 min^{-1}. The temperature of buffer is maintained at 37±1 ^{0}c., release medium was withdrawn at time intervals of 0.5,1, 2, 4, 8, 16, 24, and 48 hrs and replaced by the same volume of PBS [26].
The sample was diluted appropriately with methanol and estimated by UV-Visible spectrophotometer at 227 nm.

In-Vitro Cytotoxicity Study

In vitro cytotoxicity of prepared SLN was done by using Breast cancer cell line (MCF-7 cell line).The drug, formulated in the SLN with the drug concentration ranging from 0.25 to 25μg/ml was used [27,28].
To perform *in-vitro* cytotoxicity study trypan blue dye test was used. Trypan Blue dye is not permeable in live cells due to the presence of plasma membrane. When the cells are, dead cells take up the dye and appear as blue colour. The method is an index of the dead cells in a cell population.
Cell concentration was diluted to 1x 10^6 cells/ml using phosphate buffered saline (pH 7.4). Trypan blue dye (0.4%) was prepared in PBS pH 7.4 and 0.1ml of breast cancer cell line was mixed. To this formulation with highest particle size, and with lowest particle size were added. Marketed formulation of PTX (Taxol) was also subjected for study.
All the samples were diluted with phosphate buffered saline (pH 7.4) to make various concentrations of PTX in the range of 0.25 to 25 mcg/ml, were added and incubated for 24 hours in incubator at 37^0C. After this, the mixture was added with 0.1ml of trypan blue dye and incubated for 5 minutes at 37^0C. Blue colored dead cells and unstained viable cells were counted by using a Neubauer chamber using low power objective. Percentage of dead cells were calculated and recorded. The percentage viability and % dead cells were calculated by the using formula [3] and [4],

$$\%\text{viability} = \frac{\text{Viable cell}}{(\text{Viable cell} + \text{Dead cell})} \times 100 \quad .....[3].$$

$$\%\text{Dead Cells} = 100 - \%\text{of viable cells} \quad .....[4].$$

In-Vivo Studies

These studies were carried out after obtaining the due permission for conducting experiments from institutional ethics committee which is registered for "Teaching and Research on Animals".

Tissue Targeting Study

To perform tissue targeting studies, female Swiss Albino mice (weighing 25-30 Gms) were divided in 3 groups six in each group. Each mouse was inoculated with Ehlrich Ascites Carcinoma cell line. To perform inoculation cancer cells at a number of 1 to 2 10^6 were suspended in 0.2mL of culture medium (DMEM) and subcutaneously inoculated at the right thigh of each mouse using a 1.0mL syringe. Animals were kept in a SPF facility and had free access to food and water [29,30].
After the inoculation, tumor allowed to attain volume of 100-200 mm^3.Selected formulations (G1 and G2) were administered to mice in two groups and third group was administered with Taxol(marketed formulation of PTX) at a dose of 7.5 mg per kg after redispersing into phosphate saline buffer pH 7.4. At predetermined time points (1, 4, 8, 16 and 24h), six animals from each group were given anesthesia and blood was collected via cardiac puncture. Then animals were euthanized by cervical dislocation and dissected. Tissues of interest (liver, kidney, hear, lungs, etc) were collected at different time points. Plasma and tissue samples were frozen at -20^{o}C until analysis.
Tissue samples of mice were homogenized in tissue homogenizer by adding 10 time volumes of distilled water containing 4% bovine serum albumin for 5 minute. The supernant was collected and was spiked with 50microlitre of internal standard methanolic solution of carbamazepine (5mcg/ml).The sample was then extracted twice with 2ml ethyl acetate. The ethyl acetate fractions were combined and were dried. Dried residue was dissolved in 100 microlitre of zinc sulphate solution, centrifuged and supernant was collected. Collected supernant was injected in HPLC using water: acetonitrille (60:40) as mobile phase.

Tumor Growth Inhibition Studies

To study *in vivo* anti tumor activity, female Swiss Albino mice were selected weighing 25-30 gms. Mice were divided in 4 groups, six in each group [31].

The, Ehlrich Ascites Carcinoma cell line in its exponential growth was inoculated into each mouse. Cancer cells at a number of 2 to 4 10^6 were suspended in 0.2mL of culture medium and subcutaneously inoculated at the right thigh of each mouse using a 1.0mL syringe. Animals were kept in a SPF facility and had free access to food and water.

When the tumor volume became about 25-50 mm^3, selected formulations (G1 and G2) were administered to mice in respective two groups and third group was administered with Taxol. The dose of total 7.5 mg/kg, three times at days 0, 4 and 8 (total 22.5 mg/kg), after redespersing into phosphate saline buffer pH 7.4 (1.5 mg/mL of PTX).

Group 4 was maintained as untreated control group with normal saline.

The tumor volumes of mice were monitored twice a week for up to 30 days. The tumor volume calculation was performed using the formula,

$$\text{Tumor Volume} = 0.4\,(a\,b^2) \quad \text{.....[5].}$$

Where, a is the largest and b is the smallest diameter.

Result and Discussion

Percentage Yield

Total percentage yield of all formulations were nearly uniform. Percentage yield was high in formulation G1.The results of percent practical yield are shown in Table 2. It increases with increase in concentration of lipid added to the formulation.

Particle Size Analysis

Dynamic Light Scattering Particle Size Analyzer measured the size distributions along with the volume mean diameter of the nanoparticles. As the total lipid concentration was raised they showed is an increase in average particle size (formulation G1). The average particle sizes of all six formulations were listed in Table 2.

Determination of Entrapment Efficiency Percentage

The results of entrapment efficiency were found to be excellent and were high for the formulations, which were prepared with high concentration combination of lipid with phospholipid. High lipid concentration resulted in maximum drug entrapment in formulation G1. The data is given in Table 2.

Zeta Potential

The stability study of the nanoparticle was evaluated by measuring the zeta potential of the SLN by the zeta meter. The G1shown high zeta potential value. The high zeta potential value indicate better stability of product as particles continue to repel each other and remain non aggregated. The results are tabulated in Table no. 2.

Table 2 Characterization of Paclitaxel SLN Percentage Practical Yield, Particle Size, % Entrapment Efficiency, Zeta Potential and CDR after 48 Hrs of Formulations

Form. Code	% Percentage yield	Particle size (nm)	% Entrapment Efficiency	Zeta Potential(mV)	CDR After 48 hrs
G1	93.16±1.15	385.0±4.0	93±2.0	-28.58±0.044	5.844±2.12
G2	93.26±3.25	209.0±2.4	86±2.0	-24.65±0.040	7.993±2.10
G3	92.93±2.48	320.0±1.0	90±3.0	-26.88±0.031	6.950±2.56
G4	93.26±3.60	334.0±2.0	91±2.0	-25.84±0.028	6.170±2.68
G5	92.77±2.60	342.0±1.0	92±3.0	-26.48±0.024	5.976±2.32
G6	92.19±2.80	272.0±1.0	86±1.0	-23.64±0.041	7.468±3.20
G7	91.37±3.20	276.0±2.0	86±4.0	-23.72±0.048	7.225±3.33
G8	91.37±1.60	284.0±3.0	87±1.0	-24.08±0.016	7.423±1.20

Each value represents mean ± S.D. (n=3)

Surface Morphology

Scanning Electron Microscopy (JSM-T330A, JEOL) did shape and surface morphology of nanoparticles. SEM photograph of selected formulation G2 is shown in Figure 1. The PTX SLN has shown smooth surface and spherical shape.

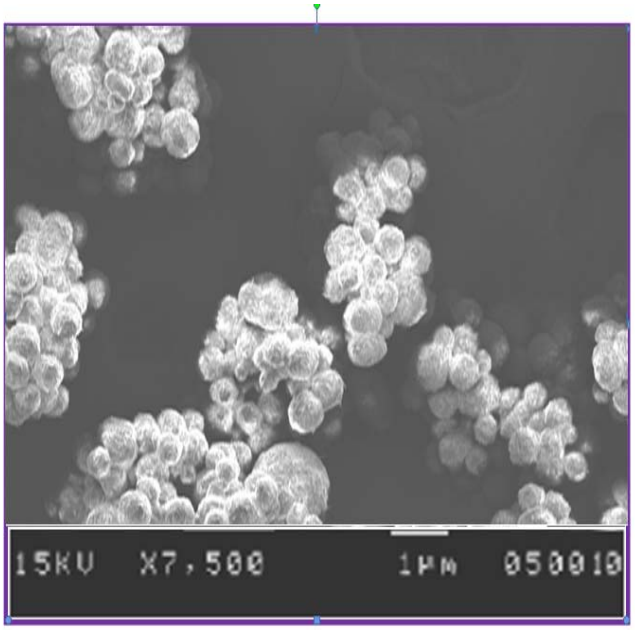

Figure 1 Scanning Electron Microscopy study SEM Formulation G2

In Vitro Drug Release Studies

In vitro drug release from the SLN in phosphate buffer pH 7.4 was performed using dialysis bag diffusion technique. The in vitro drug release profile of SLN formulations obtained from dialysis experiment is shown in graph no. 1. It was observed that the drug release from the formulations slightly increases as the particle size of the formulation decreases and all the eight formulations showed a biphasic release with initial fast release. The mechanism for the burst release may be attributed to the drug adsorbed on SLN or due to leakage of drug from SLN.

The drug release found to be dependent on the concentration of shell material employed. Nanoparticles prepared with high concentration of stearic acid, DpPC and DPPG,na showed 58.44% (G1) release. When lipid concentration was at low values, release was reported as 79.93% (G2 after 48 hours).

The results indicated that at high concentration of shell material there is retardation of drug release in comparison to low concentration of shell material.

Release mechanism model was chosen, based on goodness of fit test. Based on the highest regression values (r), the best–fit model for all six formulations was Higuchi Model. Peppa's model with 'n ~ 0.5'value indicating non-fickian diffusion release. Thus showing release of PTX was found to follow Higuchi's classical diffusion model and diffusion was non-Fickian

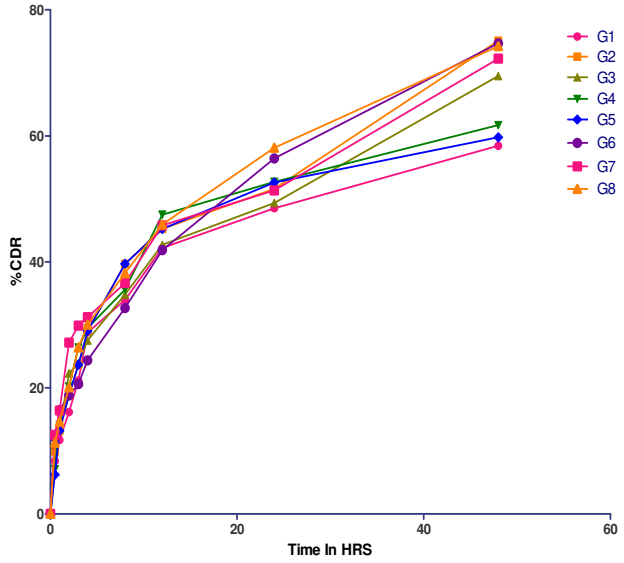

Figure 2: %CDR Vs Time Plots for formulation G1-G8

In vitro Cytotoxicity Study

Two formulations (G1) and (G2) were selected for this test. (Formulations with high particle size and low particle size) and were compared with Taxol, marketed formulation of PTX. The results are depicted in figure. no.3

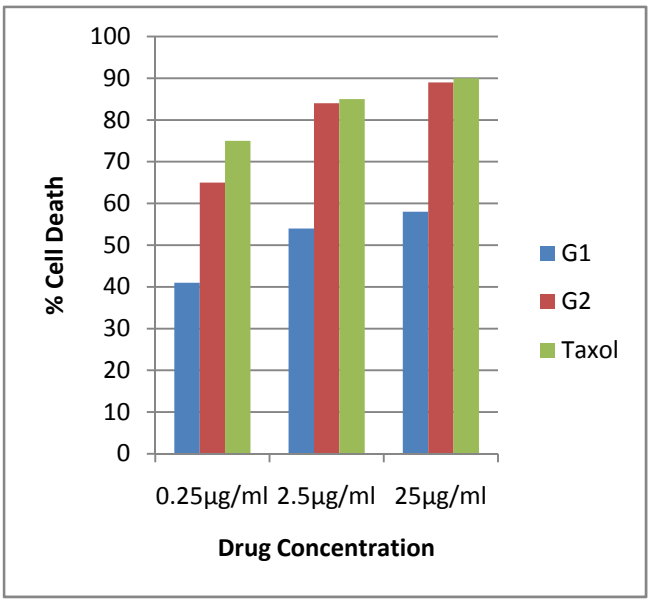

Figure 3: *In Vitro* Cytotoxicity study.Graph of % Cell death Vs Paclitaxel Concentration from Formulations G1, G2 and Taxol

Data indicates a significant reduction in MCF-7 cell viability observed when the cells were exposed to the Formulation G2, in comparison with SLN formulation G1. The cytotoxicity of marketed product, Taxol was also recorded. It was found that formulations G2 have shown nearly same cell cytotoxicity in comparison to

Taxol. The enhancement of cytotoxicy in G2 was is in accordance with its in vitro release profile while formulation G1 released fewer drugs for cell toxicity. Activity of PTX formulated in the nanoparticles prepared with low concentration of shell material can be justified by that the tumor cells can internalize the drug-loaded nanoparticles, allowing the drug to be released inside the cells. When lipid shell concentration was high resulted in high particle size with more difficulty to internalize into cells.

In vivo Tissue Targeting Studies

Formulation G1 and G2 were selected for this study along with Taxol. Female Swiss albino mice weighing 25-30 gm were selected for this study. Mice were inoculated with EAC and after growth of tumor to size of 100-200 mm^3, mice were injected with selected SLN formulations of PTX and marketed formulation Taxol (7.5 mg/kg as PTX) intravenously via tail vein of all mice. Tissues were collected by sacrificing the mice at 1, 4,8,16 and 24 hours of drug administration. Biodistribution of drug from formulation G2 were found to be excellent. This has shown more accumulation of drug in tumor tissue as compared with formulation G1 and Taxol. The particle size criteria was found to be crucial the SLN with smallest particle size shown more inhibition of tumor growth. The release of drug from formulation G1 was not significant in comparison to formulation G2, which may be due to less amount of drug released from the formulation. The results are indicated in figure.no.,4,5,6 ,7 and 8.

Analysis of plasma samples at different time points showed that drug is rapidly untaken and eliminated for marketed product Taxol. In case prepared SLN they showed presence of drug in plasma up to 24 hours. The biodistribution of drug from formulation G2 was found to be more promising, as they showed maximum drug concentration in tumor tissue when compared with G1 formulation and Taxol.

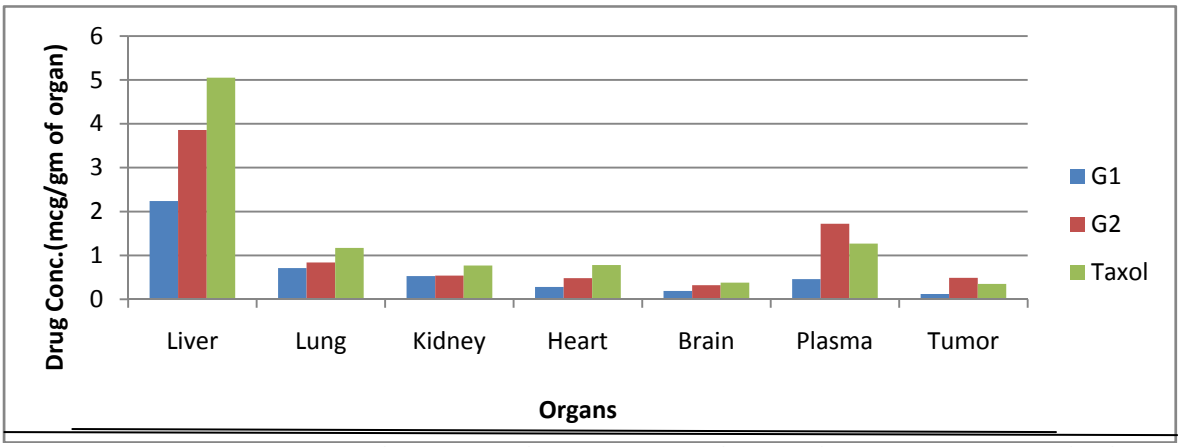

Figure 4: In Vivo Tissue Targeting study Graph of Drug Conc. in mcg/gm of Organ Vs Organ from Formulations G1, G2 and Taxol after 1 hour

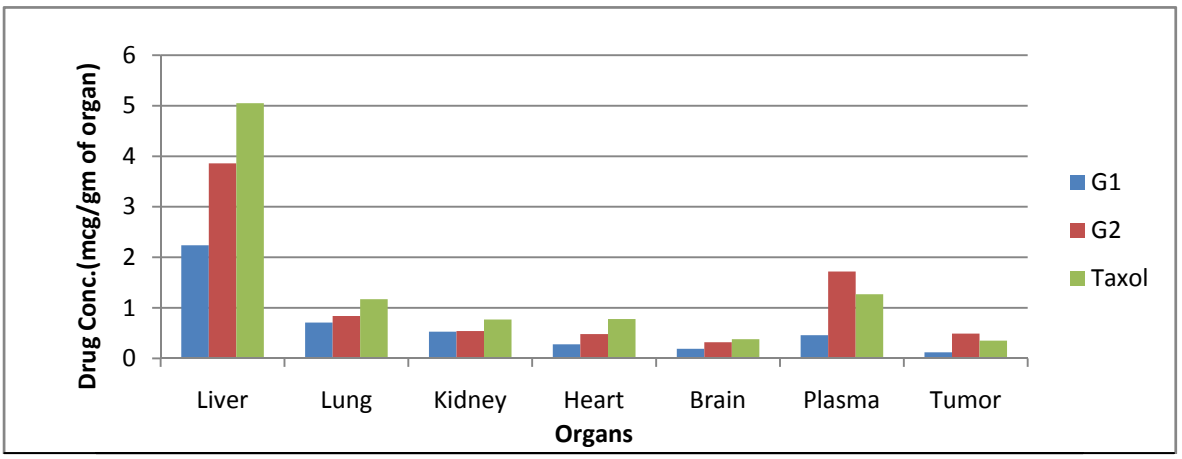

Figure 5: In Vivo Tissue Targeting studyGraph of Drug Conc. in mcg/gm of Organ Vs Organ from Formulations G1, G2 and Taxol after 4 hour

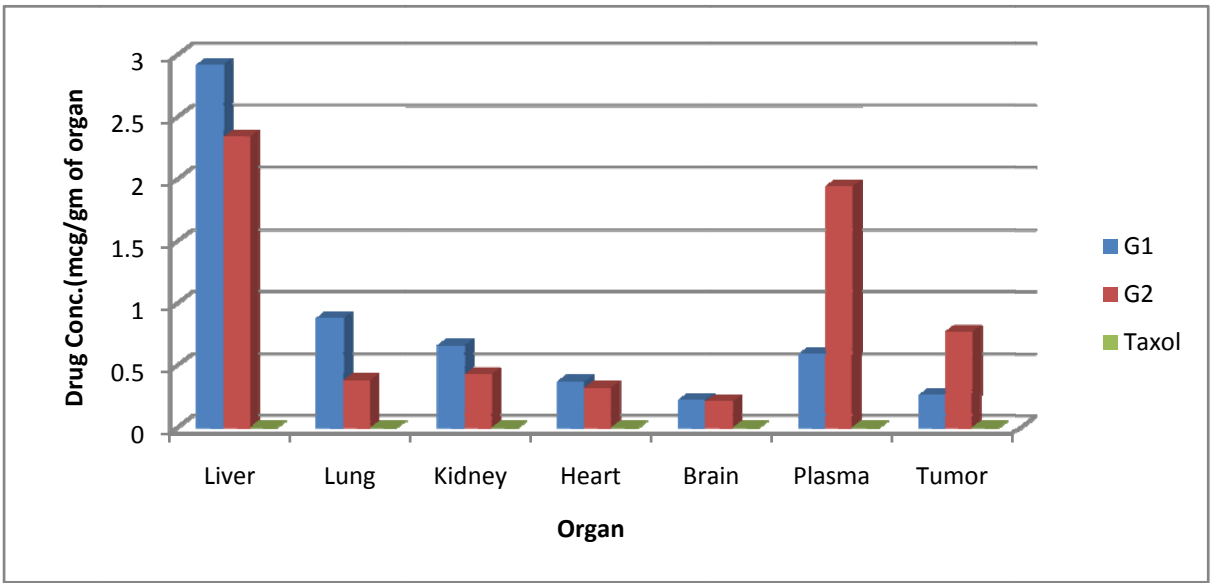

Figure 6: In Vivo Tissue Targeting study Graph of Drug Conc. in mcg/gm of Organ Vs Organ from Formulations G1, G2 and Taxol after 8 hour

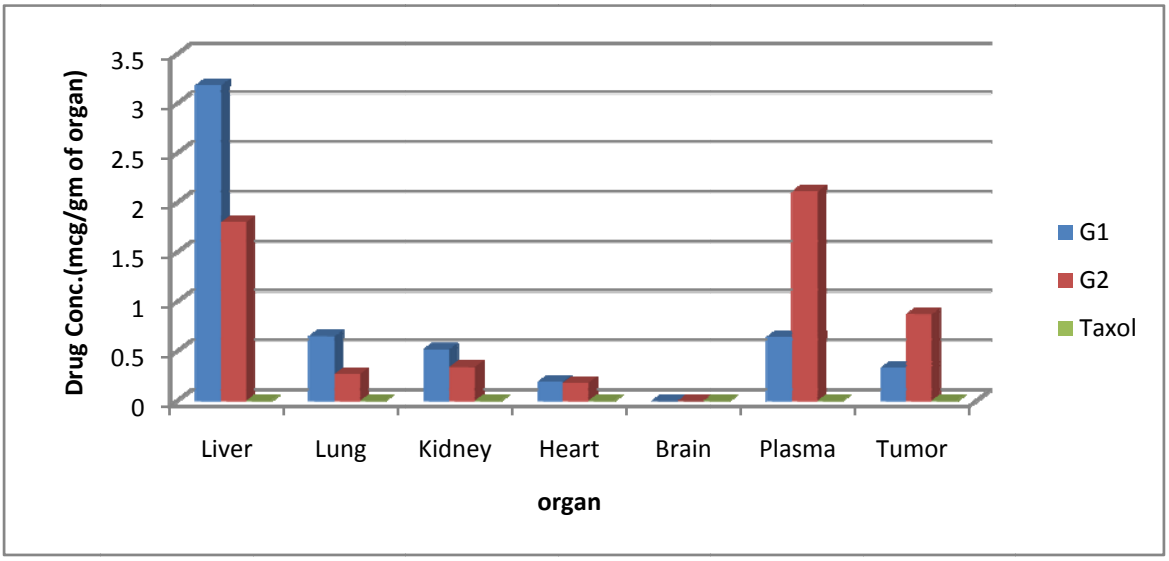

Figure 7: In Vivo Tissue Targeting study Graph of Drug Conc. in mcg/gm of Organ Vs Organ from Formulations G1, G2 and Taxol after 16 hour

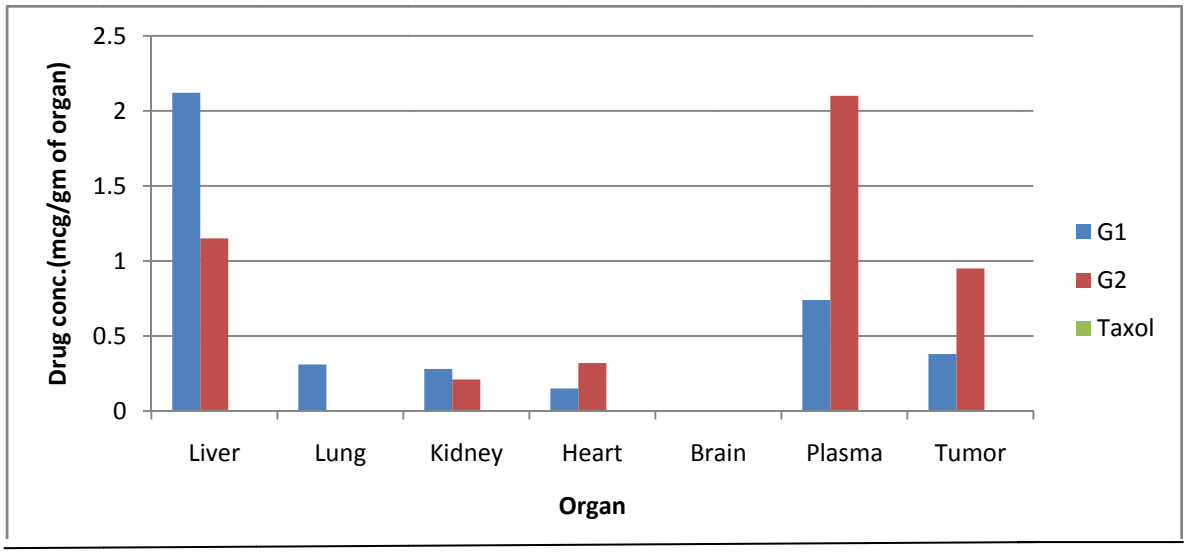

Figure 8: *In Vivo* Tissue Targeting study Graph of Drug Conc. in mcg/gm of Organ Vs Organ from Formulations G1, G2 and Taxol after 24 hour

Tumor Growth Inhibition Study

Tumor growth inhibition studies were performed to confirm the localization of PTX in tumor tissue when administered with PTX loaded SLN. Formulations G1, G2, and marketed formulation Taxol were used for this study, after the growth of tumor to volume of 25-50 mm^3.Formulation G2 was shown more inhibition in tumor growth in comparison with G1 formulation loaded with PTX and marketed product Taxol. The results are shown in fig no.9.

The results were found to be in good agreement with the results of in vitro cytotoxicity and in vivo tissue targeting study. The formulation G2 which indicated more accumulation of encapsulated drug in tumor tissues and resulted in more inhibitory effect as compared to other formulation. The study was discontinued after 10 days for mice of group maintained on normal saline as tumor volume was enlarged excessively.

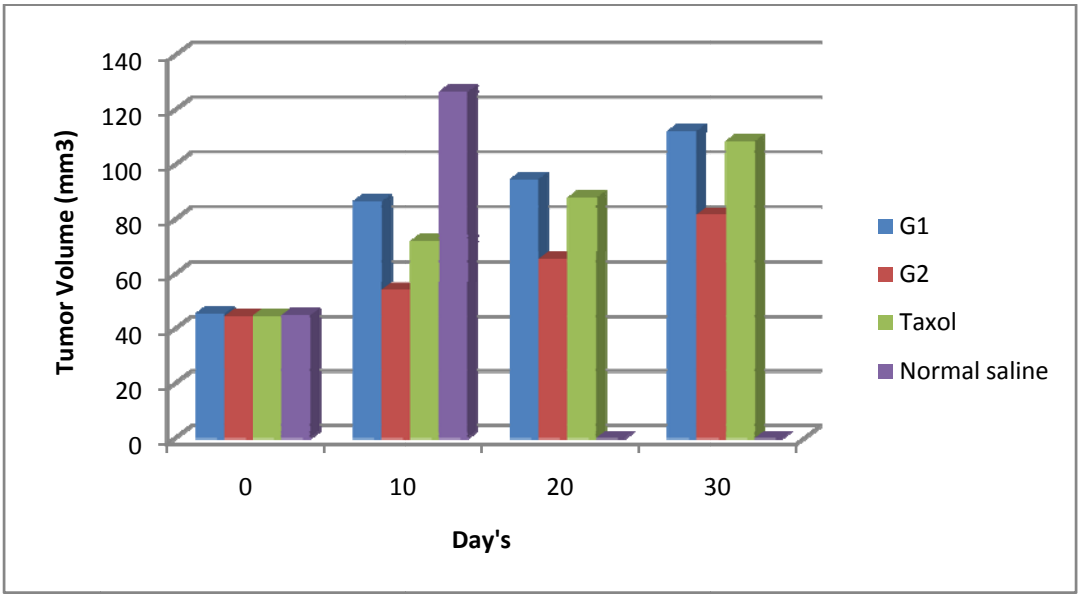

Figure 9: Tumor Growth Inhibition Study. Graph of Tumor Volume (mm^3) Vs Time (Day's) in Mice Maintained on Formulations G1, G2, Taxol and Normal Saline after 24 hour

Statistical Analysis

The results were subjected to Dunnettes Multiple Comparison test. The statistical results for formulation in comparison to Taxol was found to very significant for all the in vivo studies ($p<0.05$).

Conclusion

The present study shown high promise as per the set objectives. The formulation were prepared by combination of stearic acid and phospholipids DpPC and DPPG,Na..The fabricated SLN were subjected to characterization by various *in vitro* and *in vivo* methods. The SLN fabricated in present study were shown to perform in better way in comparison to marketed formulation of PTX,

Developed SLN was shown sustained drug release that further advantageous in chemotherapy. The combination of lipid with phospholipids shown better performance in terms of there *in vitro* and *in vivo* characteristics. Formulation prepared with combination of lipid with phospholipids showed excellent in-vitro cytotoxicity study ,tissue targeting efficiency and in vivo tumor growth inhibition studies suggesting there suitability for tumor targeting.

Our preliminary research findings strengthened the need of developing colloidal carrier in the form of SLN for PTX. This will be very useful in improving the therapeutic performance of PTX. Results obtained from this study indicated strongly that developed SLN are having potential as an efficient drug delivery system. However it is recommended and convincing, to carry out more *in-vivo* experimentation to support present evidence of feasibility prior to their clinical application

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