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Research Article

Development and characterization of paclitaxel and embelin loaded solid lipid nanoparticles for breast cancer

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ABSTRACT

In an effort to develop an alternative formulation of combination of paclitaxel (PTX) and embelin (EMB) suitable for parenteral administration, PTX-EMB loaded sterically stabilized solid lipid nanoparticles (SLNs) were prepared, characterized and examined for *in vitro* cytotoxicity. The SLNs, comprising glycerol mono stearate (GMS) as a solid lipid core, Brij 35 used as surfactant and PEGylated phospholipid used as stabilizer, were prepared using a hot homogenization method. Optimized PTX-EMB loaded formulation, the particle sizes of the prepared SLNs were around 300 nm, suggesting that they would be suitable as a parenteral formulation. Transmission electron microscopy showed that the SLNs were homogeneous and spherical in shape. Entrapment efficiency of paclitaxel and embelin was 92.83 ± 2.2%, 83.25 ± 2.4% respectively. An *in vitro* drug release study were performed in PBS (pH 7.4) for 80 hrs and observed that paclitaxel and embelin released from the PEGylated SLNs was 93.91 ± 4.1 % and 75.63 ± 4.37 % respectively. Furthermore, treatment of the MCF-7 breast cancer cell line with PTX-EMB loaded SLNs yielded cytotoxicities comparable to PTX solution, PTX-EMB mixture solution and PTX loaded PEGylated SLNs. These results collectively suggest that our optimized SLN formulation may have a potential as alternative delivery system for parenteral administration of paclitaxel and embelin.

Keywords: Embelin, Apoptosis, Cancer, Cytotoxicity, Breast Cancer, Solid lipid nanoparticles.

INTRODUCTION

Paclitaxel (PTX) is one of the most commonly used anti-cancer agent. Its unique mode of action was discovered in 1979. PTX has a very complex and unusual chemistry. It is considered as a very expensive drug because it is mainly extracted from the bark of a slow growing Western (Pacific) yew tree, and yields are merely about 0.01% of the dry weight of bark. On an average approximately 3000 trees must be sacrificed in order to obtain 1 kg of PTX and is sufficient to treat about 500 patients; present-day protocol prescribes about 2 gm of total treatment PTX is a mitotic inhibitor used in cancer chemotherapy [1]. The efficacy of Embelin with PTX has already been tested on breast cancer cell line and found to be more efficient than individuals because embelin up-regulate the TNFα induced apoptosis and also potentiate the apoptotic effects of paclitaxel [2].

Embelia ribes Burm F a medicinal woody climber belongs to the Myrsinaceae family. It is also commonly known as false black pepper or vidanga. *E. ribes* is one of the 32 medicinal plant species identified by the Medicinal Board, Govt. of India, New Delhi. Nikolovska-Coleska et al. reported embelin as a fairly potent, non-peptidic, cell-permeable, small-molecule inhibitor of XIAP and represents a promising lead compound for entirely new class of anticancer agents that target the BIR$_3$ domain of XIAP [3]. Paclitaxel is a first-line drug used in the treatment of various cancers like breast, ovarian, lung, head and neck cancers. It works through interfering with normal breakdown of microtubules during cell division. Most common marketed formulation of paclitaxel is available in a vehicle composed of a 50:50 (v/v) mixture of Cremophor EL and dehydrated alcohol.

Cremophor EL has been reported to cause serious hypersensitivity reactions depends upon the carrier system.

This project was aimed to eliminate the problems with existing formulation by encapsulating the combination of drugs i.e. Paclitaxel and Embelin in carrier system like PEGylated solid lipid nanoparticles. The prepared carrier system delivers via i.v. route. PEGylated nanoparticles not only remain in circulation for longer, giving them more time to accumulate in the tumor by EPR effect, but also take longer time to leave the tumor and return to circulation.

MATERIAL AND METHODS

Embelin was purchased from Indofine chemical company, Inc,USA. Paclitaxel was obtained as gift sample from Mac-Chem Products (India) Pvt. Ltd., Mumbai. GMS, Brij-35 were purchased from central drug house, delhi and DSPE-PEG$_{2000}$ were obtained as a gift sample form Lipoid (Germany). MCF-7 was purchased from NCCS, Pune. MTT (3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide), 6-coumarin and Annexin V-FITC Apoptosis Detection Kit were purchased from Sigma Aldrich, USA. All solvents used in the experiment procedure were analytical grade.

Instrumentation and software

All UV-Vis spectrophotometric measurements were carried out with a Shimadzu 1700 double beam UV-Visible spectrophotometer by a fix slit width of 0.1 nm coupled with a computer loaded with Shimadzu UV Probe software of version 2.31. Each and every spectrum was saved in CSV format so as to find out the zero crossing point.

Simultaneously estimation

UV-Visible spectrophotometry is usually used in analytical laboratories for quantitative and qualitative analyses. The concentration of a model is able to be calculated from the UV-Visible absorbance in sequence by means of Beer's law. Derivative spectrophotometry is extremely developed technique planned for single and mainly multi component analysis by converting the standard spectrum to its first, second and higher derivative spectrum. In derivative spectrophotometry, we include to originate the nil crossing points intended for one drug while other drugs should demonstrate substantial absorbance. Every the spectra of the sample be scanned between 400-200 nm using a 1.0 cm quartz cell. The zero order spectra of the three pure drugs within their concentration range were saved separately. These spectra were derivatized within 1st, 2nd and 3rd order derivative and acceptable results were found by using 1rd derivative spectra in which number of data points used for slope calculation was set as 49. The first derivative spectra were recorded at 300 nm and 249.9 nm for embelin and paclitaxel respectively. Standard laboratory mixture and commercial formulation were scanned, derivatized and analyzed at the same wave length as mentioned above.

Validation: Sample groundwork for validation was given in subsequent manner but dilutions were prepared according to solvent required for derivative method. The methods were validated with respect to linearity, precision, accuracy, limit of detection, limit of quantification [4].

Linearity: Standard stock solutions were prepared by dissolving 10 mg of PTX and 10 mg of EMB in 10 ml volumetric flasks in 10 mL Methanolic PBS (7.4) and the volume was made up with Methanolic PBS (7.4) to get a concentration of 1000 µg/mL of PTX and 1000 µg/mL of EMB. From this, suitable dilutions were made in methanolic PBS (7.4) to get the working standard solutions of 5-21

µg/mL for PTX and 3-11 µg/mL for EMB. The absorbance of the derivatised spectra was measured at 249.9 nm and 300 nm for PTX and EMB, respectively in derivative method. Several aliquots of standard stock solutions were taken in different 10 mL volumetric flask and diluted up to mark with methanolic PBS (7.4), such that the final linearity concentration of PTX and EMB were 5-13 µg/mL and 5-13 µg/mL in Method. Six replicate analyses were carried out. Plot the calibration curve of absorbance Vs respective concentration for PTX and EMB. Find out correlation coefficient and regression line equations for PTX and EMB.

Limit of detection (LOD) and limit of quantization (LOQ): LOD and LOQ were designed from the data obtained from the linearity studies. The slope of the linearity plot was determined. For each of the ten replicate determinations of same conc. standard deviation (SD) of the responses was calculated. From these values, the parameters Limit of Detection and Limit of Quantification were determined on the basis of standard deviation and slope of the regression equation.

$$LOD = (3.3 \times SD) / Slope$$

$$LOQ = (10 \times SD) / Slope$$

Preparation of PTX-EMB loaded PEGylated Solid lipid nanoparticles (SLNs)

PTX-EMB loaded PEGylated SLNs were manufactured by hot homogenization method reported by Reddy et al. slight modification [5]. Lipid and aqueous phase are prepared separately. The lipid was melted (10^0C above the melting point of the lipid used), and both drugs (PTX: EMB) are lipophilic in nature so both are dissolve in lipid phase to obtain a drug-lipid mixture and in aqueous phase surfactant was dissolve in water. Both phases are heated up to 70-80°C and then aqueous phase was poured into lipid phase at same temperature above the melting point of the lipid. This pre-emulsion was homogenize for 5 min at 8000 rpm, and then sonicate immediately subjected to further size reduction by using probe sonication up to 4 min. Then the resultant solution was refrigerated at 2-3°C for 15 min. The concentration of SLNs ingredients and various process variables were optimized on the basis of size, shape, zeta potential and entrapment efficiency. A brief method of preparation of SLNs is depicted by Figure 1.
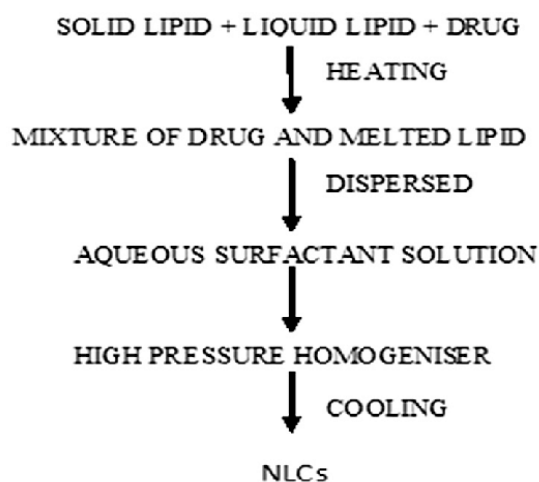

Figure 1: Preparation of SLNs by hot homogenization method

Physical stability of SLNs

The physical stability of the SLNs was evaluated by examining changes of mean particle size of SLNs during storage at 4 and 25°C [6].

In-vitro drug release

The release of PTX and EMB from optimized formulations was determined using the dialysis tubing technique with slight modification as per method reported elsewhere. In brief, an accurately measured PTX-EMB loaded PEGylated SLNs were suspended in 2 mL of PBS (pH 7.4) and transferred into the pre-soaked dialysis membrane (LA393-10MT, HIMEDIA) tied with one end, then other end was tied up and suspended in a conical flask (500 mL) containing 100 mL PBS (pH 7.4). The whole set was placed in a shaking incubator (Daihanlabtech. LSB-1005RE) adjusted to a constant speed (100 rpm) and temperature (37°C). Samples were collected at different intervals for 80 h by maintaining the sink conditions and assayed spectrophotometrically for drug content at 249.9 nm for PTX and at 300 nm for EMB. All experiments were carried out in triplicates.

In-vitro anticancer activity of SLNs by cell line study

MCF-7 cell line (breast carcinoma) was obtained from National Center for Cell Sciences, Pune, India. Cell lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum. Cells were maintained in 5% CO_2 humidified incubator at 37°C [7]. During subculture, cells were detached by trypsinization when they reached 80% confluency and split (1:4). Growth medium was changed every 3 days.

Cell uptake study: Cell uptake study of the formulation was performed for qualitative estimation of the uptake of liposome by the cancer cells. Cell uptake study of drug loaded SLNs was performed on MCF 7 cells (breast cancer cell lines) according to the procedure reported earlier by Mauro Gigli [8]. The cell culture was freshly prepared by sub culturing the cells using Dulbecco's Minimum Essential Medium (DMEM). Formulation was incubated with the cells using 6 well culture plates for 2 hours [9]. Plain cells in media served as positive control. Images of control and test sample were taken using Fluoroscent Microscope (Olympus, CK X 41**).**

Apoptosis Assay: Human breast cancer cell line (MCF-7) was treated Annexin V-FITC Apoptosis detection kit. The cells were plated in 6 well plate and treated with different concentration of PTX: EMB loaded PEGylated SLNs and incubated it. Media was removed and washed with PBS pH 7.4 and re-suspended in annexin binding buffer. 1 μL annexin V dye and PI was added and kept in dark for 30 min. Finally cells were washed and observed under fluorescent microscope using appropriate filters.

The cells should separate into three groups: live, apoptotic and dead. Live cells show only weak annexin V staining of the cellular membrane, while apoptotic cells show asignificantly higher degree of surface labelling. Dead cells show both membrane staining by Annexin V and strong nuclear staining from the propidium iodide [10].

Cell viability by MTT assay: The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye reduction assay was performed to determine the cytotoxic effect of the different ratio of drugs encapsulated SLNs at various concentrations. The assay depends on the reduction of MTT by mitochondrial dehydrogenase, an enzyme present in the mitochondria of viable cells, to a blue formazan product [11]. Briefly, the MCF-7 cells were at a concentration 3×10^4 cells/mL plated onto 96-well flat bottom culture plates with various concentrations of formulation. All cultures were incubated for 24 hours at 37°C in a humidified incubator. After 24 hours of incubation (37°C, 5% CO_2 in a humid atmosphere), 5μl of MTT (5 mg/mL in DMSO) was added to each well, and the plate was incubated for a further four hours at 37°C. The resulting formazan was dissolved in 200 μL dimethyl sulphoxide and absorbance of the solution was read at 595 nm using ELISA plate reader (Biorad, Model 680, Japan). All determinations were carried out in triplicate. Concentrations of SLNs showing 50% reduction in cell viability (i.e. IC_{50} values) were then calculated [12].

Statistical analysis

All the data were run in triplicate for each sample. All data were expressed as mean ± standard deviation (SD)for $n = 3$. Student's t-test analysis was done to assess the statistical significance of the data sets. A p value less than 0.05 was considered to indicate statistical significance for all comparisons.

RESULTS AND DISCUSSION

Selection of Wavelength for Simultaneous Estimation of x and y:

Derivative Method: First order derivative spectrum for PTX showed zero crossing points: 249.9 nm. The wavelength selected for estimation of PTX was 249.9 nm because it showed adequate absorbance at this wavelength in mixture. Similarly, first order derivative spectrum for EMB was taken and it showed zero crossing point: 300 nm. The wavelength selected for estimation of EMB was 300 nm because it showed adequate absorbance at this wavelength in mixture.

Table 1: Analytical data from the calibration graphs for the determination of PTX and EMB by spectrophotometry

Parameters	Paclitaxel (PTX)	Embelin (EMB)
ZCP (nm)	249.9	300
Linearity range	5-21 μg/mL	3-11 μg/mL
Regression equation [a]	0.0371x + 0.0066	0.07x - 0.034
r^2	0.998	0.999
Absorbance	0.158	0.234
LOD	0.03522	0.0187
LOQ	0.10567	0.0566

[a] absorbance value (A) versus concentration (C) of each drug in μg/mL

Linerity: The assay showed a good linear response over the range 5-21 μg/mL for PTX and 3-11 μg/mL for EMB. The regression equations (n = 5) were y = 0.0371x + 0.0066 (r^2 = 0.998) for PTX and y = 0.0844x + 0.043 (r^2 = 0.999) for EMB, where y is the peak area ratio of analyte to drug (PTX or EMB) and x is the methanolic PBS (7.4) concentration. At calibration curve concentrations, the RSD value ranged from 0.19% to 0.93% for PTX and 0.83% to 1.10% for EMB. Analytical data from the calibration graphs for the determination of PTX-EMB by spectrophotometry is mentioned in table 1.

The developed method was novel, simple, accurate, and precise, economical, which would be used to estimate PTX and EMB in their combined dosage form in routine analysis.

Hot homogenization method was used to prepare PTX-EMB loaded PEGylated SLNs. Optimization of process and formulation parameters results in the successfully production of PTX-EMB loaded PEGylated SLNs with particle size 316.3 ± 15.7 with PDI 0.075 ± 0.08 while that of SLNs without drug incorporation have particle size 137.3 ± 6.7 nm. However, the PTX-EMB loaded PEGylated SLNs remained within the injectable range for intravenous administration

(~200–400 nm) [13]. The PEGylated phospholipid (DSPE-PEG2000) is usual to provide additional steric stabilization of SLNs. Consequently, the prepared PEGylated SLNs in this study should theoretically stable for long periods and having long circulation time. Ultimately, TEM (transmission electron microscope) was used to inspect the morphology and submicron particle size of PEGylated SLNs (**Figure 2**). TEM studies show that particle size of PTX-EMB loaded PEGylated SLNs was found to be 300nm.

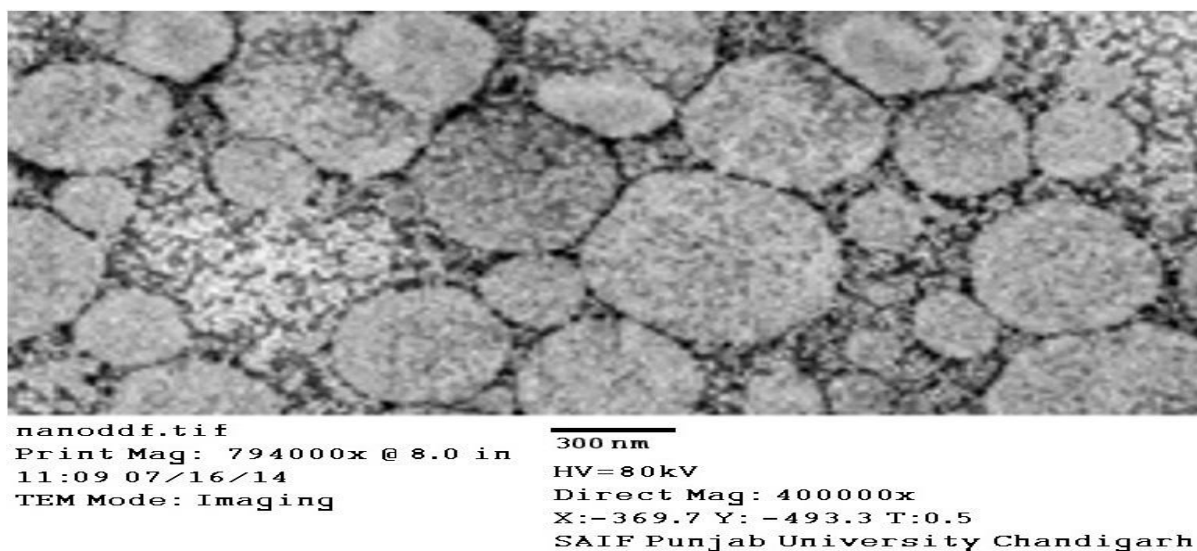Entrapment efficiency of drugs in the SLNs is 92.83 and 83.25 for PTX and EMB respectively.

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SAIF Punjab University Chandigarh

Figure 2 TEM photograph of PTX and EMB loaded PEGylated SLNs

Physical stability

Physical stability of PTX-EMB loaded PEGylated SLNs was measured by investigating the changes in mean particle size for the duration of 30 days at 4°C and 25°C. Particle size values obtained at different days at both temperatures are shown in Figure 3.
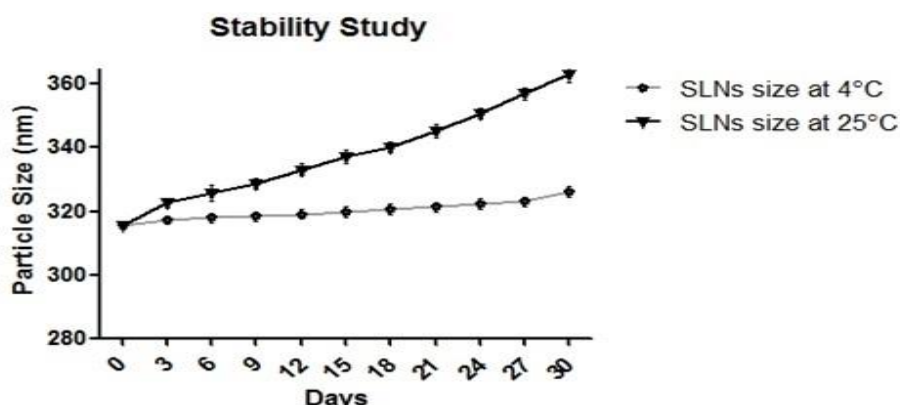

Figure 3: Stability study of PTX-EMB loaded PEGylated SLNs at 4°C and 25°C. Data presented as Mean ±SEM, n =3

***In-vitro* drug release**

The in-vitro drug release study of the PTX-EMB loaded PEGylated SLNs was done in Methanolic PBS pH 7.4 using dialysis bag. The release study of the formulation was done thrice to check the reproducibility. Figure 4 shows the release of PTX and EMB from SLNs.
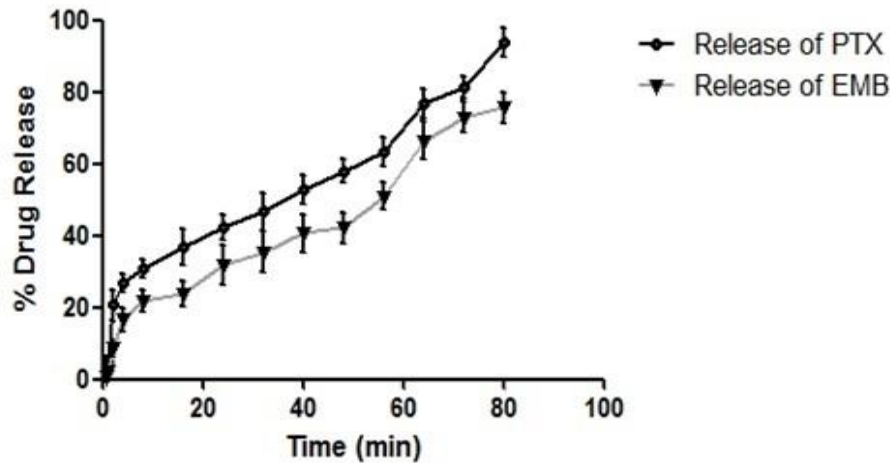
Figure 4: Percentage cumulative release of PTX and EMB vs. Time curve. Data presented as Mean ±SEM, n =3

It is shown in the results that more than 50% of the total PTX was released in 40 hours while EMB in 56 hrs. This data further proves that there is sustain release of drug from the SLNs. Kinetics of drug release from controlled release formulation was evaluated by model dependent methods. PTX and EMB both drugs followed zero order kinetics. It

shows that release of both was not depends upon time and concentration, release of both drugs follows the diffusion. Various kinetic models for PTX and EMB release from PTX-EMB loaded PEGylated SLNs are given in Figure 5 and Figure 6 respectively and in Table 2, regression value of PTX and EMB in various kinetic models of release study are given.
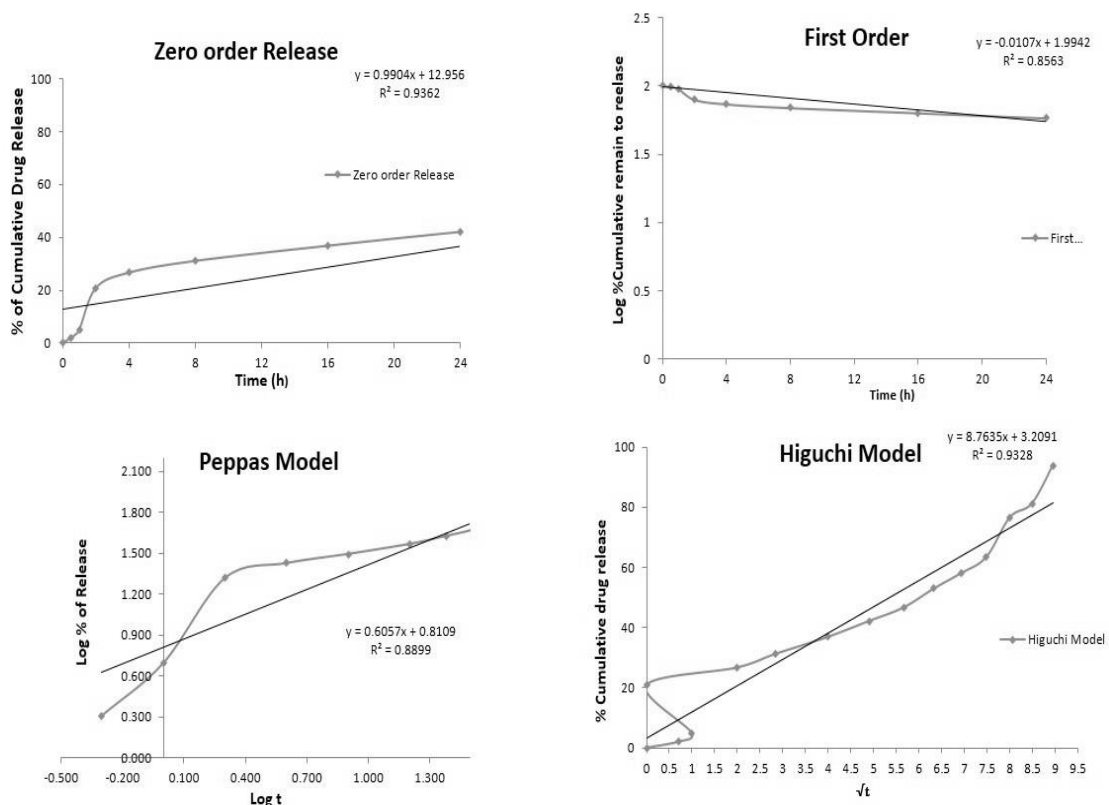

Figure 5: Kinetic models for PTX release from PTX-EMB loaded PEGylated SLNs
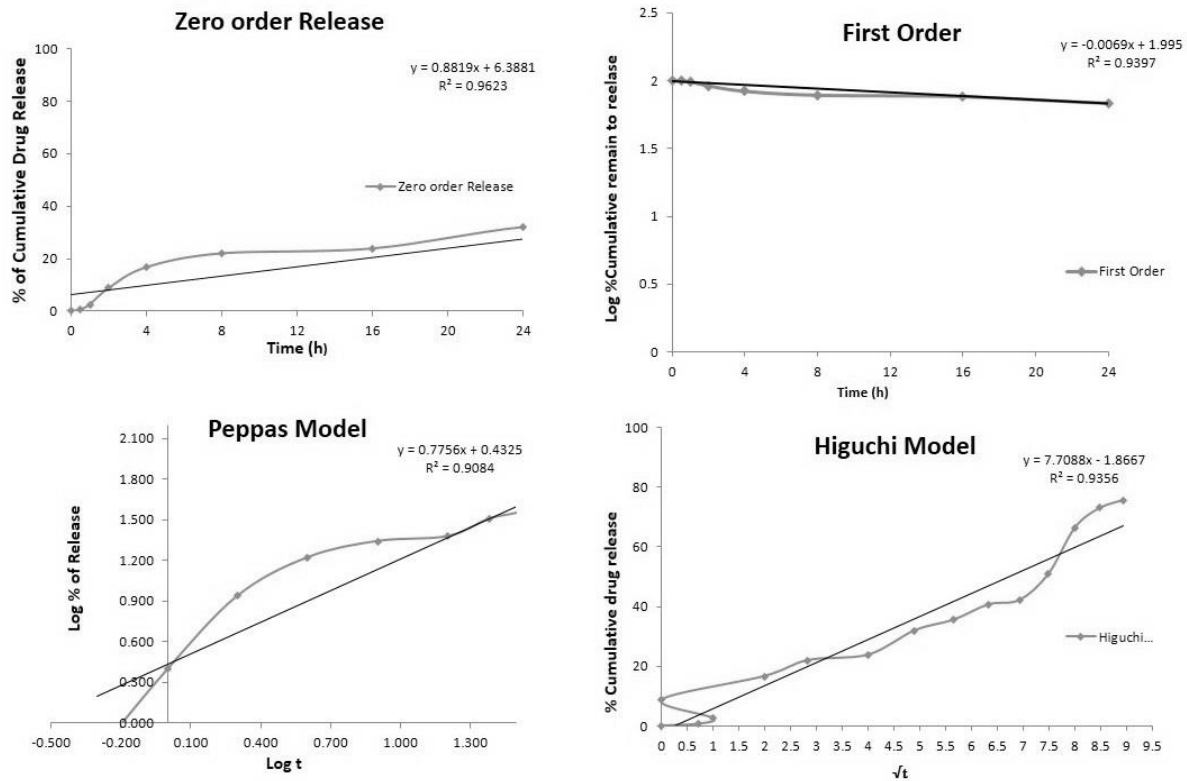
Figure 6: Kinetic models for EMB release from PTX-EMB loaded PEGylated SLNs

The release kinetics of drug from SLNs can be described using four different kinetic models, which are Zero order, First order, Peppas model and Higuchi model. All of these models explain the release pattern to be dependent on some individual property. The mechanism of drug release was studied using zero order equation.

$$Q_t = Q_0 + K_0 t$$

Where Q_t is cumulative amount of drug release at time t, Q_0 initial amount of drug, K_0 is a zero-order release constant and t is the time.

Table 2: Regression value of PTX and EMB in various kinetic models of release study

Kinetic Models	Regression(R^2) Value of PTX	Regression(R^2) Value of EMB
Zero Order Release	**0.9362**	**0.9523**
First Order	0.8563	0.9397
Peppas Model	0.8899	0.9084
Higuchi Model	0.9328	0.9356

***In-vitro* cell line study**

Cell Uptake Study: Cell uptake study of the formulation was performed for qualitative estimation of the uptake of SLNs by the cancer cells. Images of the cells were taken after 2 hours of incubation with the suspension of plain Coumarin-6, Coumarin-6 loaded SLNs and Coumarin-6 loaded PEGylated SLNs on Olympus CKX41 fluorescence microscope. We were able to see the fluorescence on B excitation filter of the microscope and the images were taken at 20 X magnification.

Fluorescence in the cells can be clearly seen in the Figure 7, which shows that coumarin-6 loaded SLNs were taken up by the cells. Coumarin-6 loaded PEGylated SLNs (D) shows better uptake as compared to plain Coumarin-6 (B) and Coumarin-6 loaded SLNs (C). SLNs formulation travels inside the cells via mechanism of passive transport and so increase bioavailability of drug. PEGylation SLNs Inhibit the RES uptake of cells and make more effective the formulation.
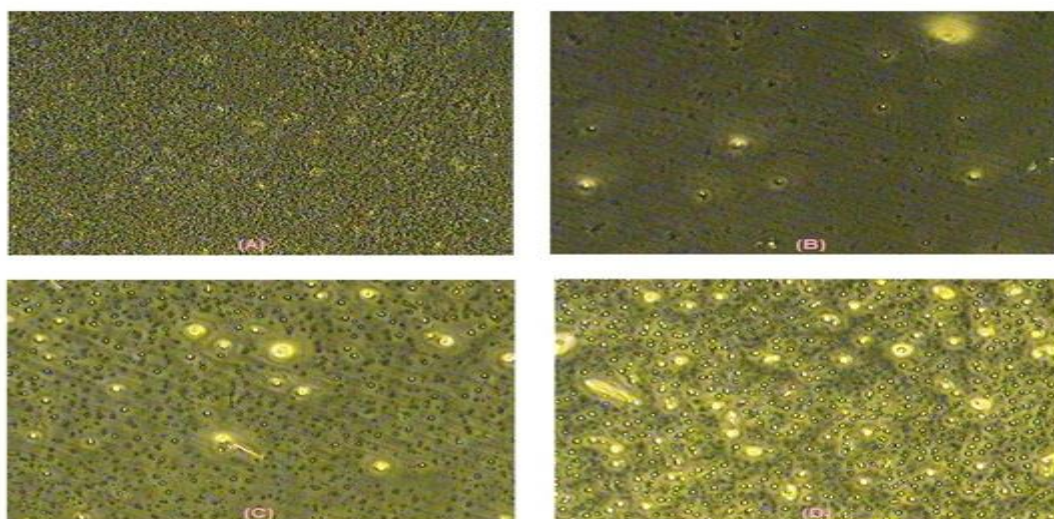
Figure 7: Images of cell uptake (A) Plain MCF-7 cells (B) Plain Coumarin-6 treated cells (C) Coumarin-6 loaded SLNs treated cells (D) Coumarin-6 loaded PEGylated SLNs treated cells

Apoptosis Assay: Apoptosis study of optimized formulation PTX-EMB loaded PEGylated SLNs was conducted using Annexin V-FITC detection kit in MCF-7 cell lines, shown in Figure 8. In MCF-7 cells, control group (A) shows negligible presence of apoptosis and necrotic cells (less than 5%). The percentage of apoptotic cells was higher in case of cells treated with PTX-EMB loaded PEGylated SLNs (D) than PTX loaded PEGylated SLNs (C). With plain PTX treatment (B), we observed very few cells at pro-apoptotic phase as compared to PTX-EMB loaded PEGylated SLNs (D) and PTX loaded PEGylated SLNs (C). All formulations showed negligible amount of necrotic cells, while control group showed negligible apoptotic activity.
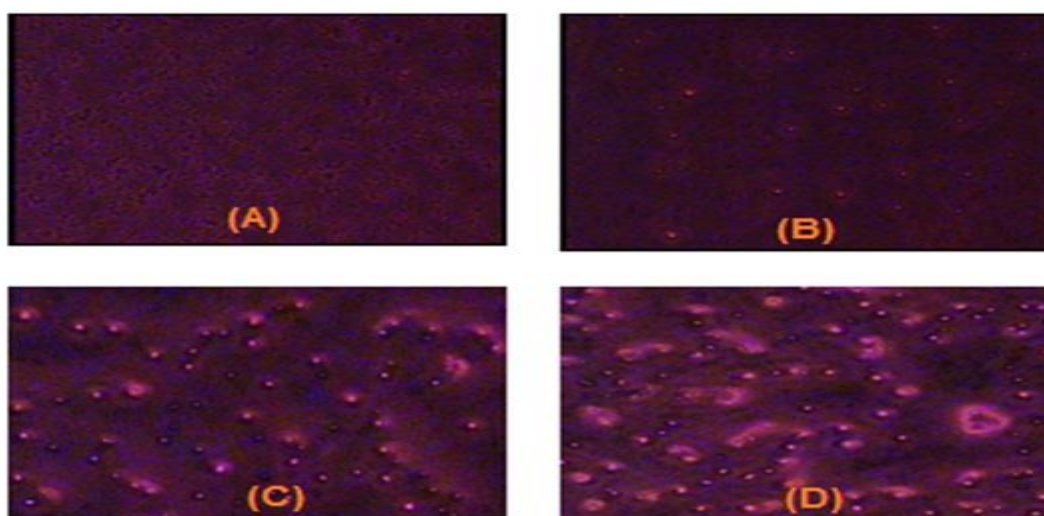

Figure 8: Images of Apoptosis Assay (A) Plain MCF-7 cells (B) Plain PTX treated cells (C) PTX loaded PEGylated SLNs treated cells (D) PTX-EMB loaded PEGylated SLNs treated cells

Optimized PTX-EMB loaded PEGylated SLNs (D) was able to cause a significant increase in programmed cell death. PTX loaded PEGylated SLNs (C) also cause a significant increase in programmed cell death as compare to plain PTX (B). But in combination with EMB i.e. PTX-EMB loaded PEGylated SLNs (D) showed better results because EMB serves to be apoptotic inducer and hence in synergism with PTX shows enhanced effect and better apoptosis.

Cell viability by MTT assay: Cytotoxic curve and IC$_{50}$ value were determined for different SLNs formulations (PTX loaded PEGylated SLNs and PTX-EMB loaded PEGylated SLNs) and further compared with PTX solution and PTX-EMB mixture solution in dose dependent manner in MCF-7 cell line using MTT assay. IC$_{50}$ value, concentration of drug required for 50% cell death listed in table 3.

Table 3: IC$_{50}$ values of PTX solution, PTX-EMB mixture solution, PTX loaded PEGylated SLNs and PTX-EMB loaded PEGylated SLNs.

| Formulation	IC$_{50}$ Value
PTX solution	12 μg/ml
PTX-EMB mixture solution	10 μg/ml
PTX loaded PEGylated SLNs	9.4 μg/ml
PTX-EMB loaded PEGylated SLNs	6 μg/ml

From result it is observed that PTX-EMB loaded PEGylated SLNs exhibited significantly higher cytotoxicity as compared to PTX solution, PTX-EMB mixture solution and PTX loaded PEGylated SLNs at all drug concentration. IC$_{50}$ value for MCF-7 cells treated with PTX-EMB loaded PEGylated SLNs was found to be 6 μg/ml that was less than from PTX solution (12 μg/ml), PTX-EMB mixture solution (10 μg/ml) and PTX loaded PEGylated SLNs (9.4 μg/ml).

Therapeutic efficacy of drug loaded SLNs depends on cellular uptake, intracellular distribution and more importantly the amount of drug available from the internalized SLNs inside the cell [14]. The enhanced cytotoxicity effect of PTX-EMB loaded PEGylated SLNs could be attributed by higher uptake due to PEGylation of SLNs (Table 4, Figure 9).

Table 4: Cytotoxicity study of PTX solution, PTX-EMB mixture solution, PTX loaded PEGylated SLNs and PTX-EMB loaded PEGylated SLNs in MCF-7 using MTT assay.

Conc. (μg/ml)	PTX solution	PTX-EMB mixture solution	PTX loaded PEGylated SLNs	PTX-EMB loaded PEGylated SLNs
	% Cell Viability			
0	99.3	97.86	99.03	97.8
2	97.61	91.25	89.09	74.5
4	88.28	83.43	76.71	65.17
6	75.42	71.5	64.33	53.51
8	64.66	60.27	55.64	45.54
10	56.46	49.76	47.54	39.22
12	48.56	36.68	34.25	30.96
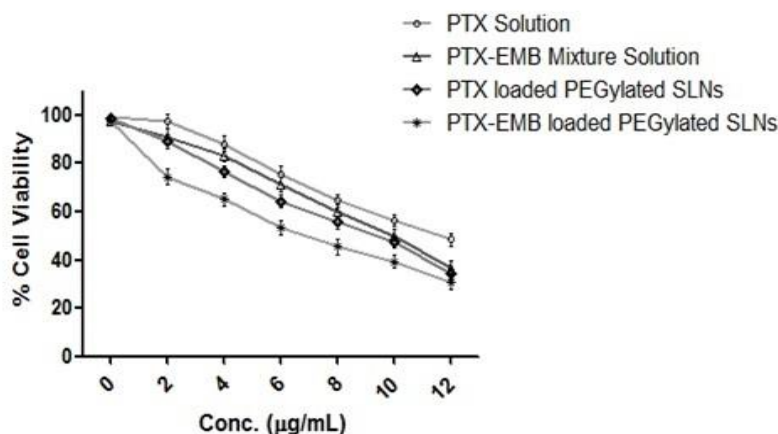
Figure 9: Cytotoxicity study of PTX solution, PTX-EMB mixture solution, PTX loaded PEGylated SLNs and PTX-EMB loaded PEGylated SLNs in MCF-7 cell line using MTT assay. Data presented as Mean ± SEM, n =3

This is because both the cytotoxic agents synergistically produced enhanced cytotoxicity. But interestingly, cytotoxicity of PTX- EMB loaded SLNs was found to be much higher than the physical mixture of the PTX-EMB. This proves that through drug loaded SLNs, drug is better uptake to and retained in the cancerous cells. These results indicate that PTX and EMB loading PEGylated SLNs is a novel and effective approach towards breast cancer management of the highly toxic chemotherapeutic drugs and thus producing fewer side effects.

CONCLUSION

From above studies, it has been concluded that the aim of the project has been achieved by developing the Paclitaxel and Embelin loaded PEGylated SLNs. The formulation showed good potential ability towards breast cancer. Also it was concluded that embelin played a significant role in up-regulation of the TNFα induced apoptosis of paclitaxel. The optimized formulation (Paclitaxel and Embelin loaded PEGylated SLNs) exerts synergistic anti cancer activity

because of anti cancer properties of both paclitaxel and embelin.

From the study we conclude that the formulation is effective and can be recommended for pre-clinical studies.

Declaration of interest

The authors confirm that this article content has no conflict of interest.

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