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Original Article

A STRATEGIC PROCESS DEVELOPMENT AND *IN VITRO* CYTOTOXICITY ANALYSIS OF PACLITAXEL-LOADED LIPOSOMES

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ABSTRACT

Objective: Liposomes are the controlled-release dosage form that improves the therapeutic efficacy of the drugs, prolongs the duration of action, reduces dosage frequency, and improves patient compliance.

Methods: The thin-film hydration method was used to prepare Paclitaxel liposomes. In this process, cholesterol and sodium deoxycholate were used for the formulation, while chloroform and methanol were used as diluents. Percentage (%) drug release study was carried out in phosphate buffer at pH 7.4 in USP apparatus II (Paddle type)Model no VDA-8D, Veego, Mumbai, India.

Results: Paclitaxel liposomes of various batches showed a percentage yield ranging from 38 to 84%. It was observed that (Encapsulation efficiency)EE% of Batches B1 to B10 were 0,62.33,59.51,50.21,44.30,82.25,88.95,72.34,77.37 and 70.63 percentage, respectively. Data fitting to the Peppas, Higuchi, 1st-order, and zero-order models was used to examine the optimized liposome (B7) release kinetic mechanism. Data comparison was done using the correlation coefficient (R^2). Zero-order had an observed correlation coefficient (R^2) of 0.9988, which was greater than that for other models. Therefore, it was clear that the medication was released from the formulation after the zero-order release.

Conclusion: The prepared liposomes were subjected to various evaluation parameters like SEM, zeta potential, particle size analysis, drug release study, etc. Data showed that an increased concentration of cholesterol increases the drug release from liposomes. Microscopic images of the B7 batch revealed that liposomes are spherical and have regular surfaces. Formulation B7 shows good results and can be considered an optimized batch that has been selected for further cell line studies. The statistical analysis was used to support the improved formulation.

Keywords: Paclitaxel, Liposomes, Cancer, Formulation, Efficacy

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INTRODUCTION

Human society places higher importance on natural medicine. The anticancer drug Paclitaxel, which is derived from the Taxus brevifolia or Pacific Yew tree, is widely recognised for working. The way that paclitaxel works is by halting the cancer cells' rapid cell division. By preventing the spindle from forming and preventing the cell from moving from the mitotic phase into the G1 phase, rapid cell division is stopped [1]. On the other hand, paclitaxel's limited water solubility significantly impairs its administration [2]. Cremophore EL [3] and ethanol [4] are often used to increase the solubility of paclitaxel phosphate buffer (disodium hydrogen phosphate, sodium chloride, potassium chloride, and potassium dihydrogen phosphate). But after being administered to a person, all of these solvents exhibit negative responses and undesirable outcomes. Designing a novel drug delivery method offers a chance to prevent these negative side effects. The term "novel dosage forms" refers to formulations created specifically for the safe delivery of active pharmaceutical ingredients into the body, achieving desired therapeutic effects, as well as extending drug half-lives, increasing drug absorption, and administering medications locally, among other things [5]. Thus, it was determined to manufacture liposomes in order to provide an effective and secure delivery system for paclitaxel. Liposomes are spherical vesicles made of one or more phospholipid bilayers. Consequently, we have selected one of the numerous efficient new carriers for the delivery of paclitaxel.

In recent years, innovative medication delivery technologies have replaced conventional dosage forms more and more often. The most popular innovative drug delivery technique in recent years has been controlled release and sustained release formulations. For instance, the liposomal version of Amphotericin B has shown to be much more effective than the conventional form. By releasing the medicine at a predefined pace, sustained-release drug delivery systems maintain constant drug plasma levels throughout time, which reduces adverse drug reactions and improves patient compliance [6].

An anti-neoplastic chemotherapy medication called paclitaxel is used to treat certain kinds of solid tumors. In addition to its amazing promise in the treatment of several malignancies, including lung, breast, and ovarian cancer, it is also sometimes used to treat Kaposi's sarcoma linked to AIDS. It is in BCS Class IV (low solubility and low permeability) [6].

In order to decrease dose frequency and improve patient compliance, the present effort intends to investigate the impact of cholesterol content on the release profile of paclitaxel and to determine the effect of the ratio of lipid matrix and edge activators showing better drug release rates on the formulation of sustained release liposomes of paclitaxel.

Nanoscale drug delivery systems using liposomes and nanoparticles are emerging technologies for the rational delivery of chemotherapeutic drugs like paclitaxel in the treatment of cancer. Their use offers improved pharmacokinetic properties, controlled and sustained release of drugs, and, more importantly, lower systemic toxicity. A literature survey reveals that liposome-based nano drug delivery has several disadvantages due to various combinations.

Different combinations of phospholipids and polymers used in various research articles are vigorously examined by myself and my research team, including my mentor and guide. The three most commonly used polymers are PEG, egg lecithin, and sodium alginate [7].

It was found that the use of conventional phospholipids and polymers is not highly effective in minimising the limitations of anticancer liposome drug delivery. Due to this problem, commercialization of liposomal drug delivery for cancer on a large scale is still far from reality.

Our research effort aims to formulate the paclitaxel liposome by using cholesterol, sodium deoxycholate, and soy lecithin. The formulation is designed to reduce the various limitations of liposomes associated with the various combinations of polymers

and other stabilisers that are under research right now. Some of them are not good candidates, while others produce toxic effects in patients who are already in a diseased state and are in distress. Our formulation is superior in the selectivity of various combinations of cholesterol, sodium deoxycholate, and soy lecithin with the solvent, which is the methanol-chloroform mixture, which after extensive literature review, was found to be the most suitable combination of solvent used in the preparation of liposomes [7].

This will help bridge the gap between research and commercialization of anticancer drug delivery in the form of liposomes.

MATERIALS AND METHODS

Materials

Paclitaxel was obtained as a gift sample from Fresenius Kabi Oncology in Kolkata; cholesterol, sodium deoxycholate, soy lecithin, chloroform, methanol, and Tween 80 were purchased from Loba Chemie Pvt. Ltd. All other chemicals were of analytical grade.

Characterization of the drug

The gifted sample of Paclitaxel was characterised by physical observations, melting point determination, and recording of ultraviolet, visible, and infrared absorption spectra that were further compared and matched with reference spectra cited in the International Pharmacopoeia.

Physical observation

Paclitaxel is a fine, almost white, crystalline powder that can be seen with the naked eye.

Determining the melting point

The drug sample's melting point was found to be 216–217 °C, which was the same as what was written in the literature. This proved that the sample received was the right one.

Solubility

Almost insoluble in water, soluble in methanol, and easily soluble in methylene chloride, which matched the specification and proved that the sample received was the right one.

Ultraviolet (UV) absorption spectra

In a phosphate buffer with a pH of 7.5, a sample of Paclitaxel was scanned between 200 and 400 nm wavelengths.

Absorbance maximum were found at 230 nm, which was used for quantitative analysis.

Infrared (IR) spectra: The IR spectra of Paclitaxel were matched with those of the reference.

Calibration curve of paclitaxel

A calibration curve of Paclitaxel phosphate buffer pH 7.5 was prepared. An increase in concentration was done in a preset manner. For quantitative estimation of the drug, the regression equation was calculated and utilized. The correlation coefficient was found to be 0.9985.

Preparation of liposomes

Paclitaxel liposomes were prepared by the thin film hydration method (fig. 2) [7]. The compositions of various batches of liposomes are given in table 1.

Liposomes have been prepared by dissolving the drug Paclitaxel (PTX) in methanol, to which cholesterol and soy lecithin (the lipid matrix) were added in varying amounts as per the ratio of different batches of formulations in the round-bottomed flask (RBF). Then chloroform was added to it according to the fixed ratio, and sodium deoxycholate (DCP) and tween 80 were added in varying ratios as per the protocol set forth in composition table 1.

Then the RBF is rotated at an rpm of 80 at a fixed temperature of 60 °C until the solvent is completely evaporated (approximately 1h). After complete evaporation, the flask was sonicated for 30 min, and then hydration was done using distilled water with a drop of acetonitrile added to it. Again, a 30 min sonication cycle was performed before collecting the final liposomes and storing them at negative temperatures to prevent degradation. Fig. 1 depicts the complete procedure in tabulated form.

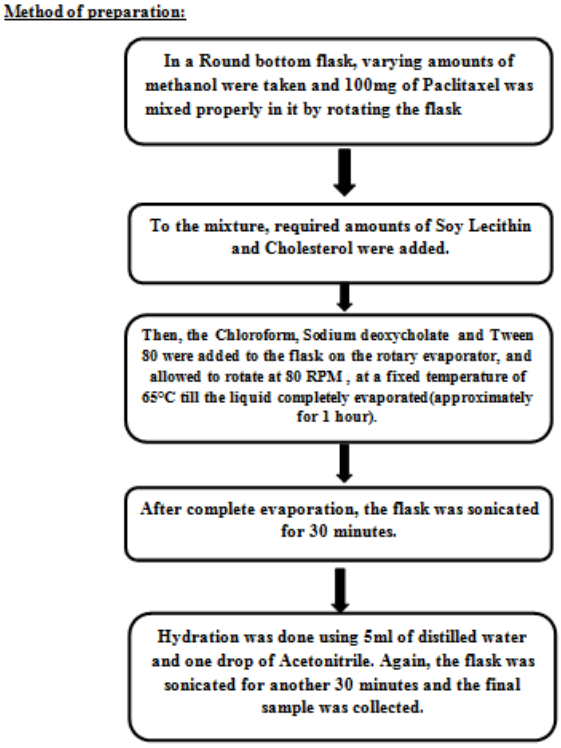

Fig. 1: Procedure of preparation of Paclitaxel liposomes by thin film hydration method

Table 1: Composition of different batches of liposome

Batch	LM ratio (Lipid matrix) cholesterol: Soy lecithin	DCP: Tween 80	PTX in mg	Methanol-chloroform mixture in ml
B1	1:1	Only Tween 80	10	50
B2	1:2	1:1	10	50
B3	1:3	1:1	10	50
B4	2:1	1:1	10	50
B5	3:1	1:1	10	50
B6	1:1	Only DCP	10	50
B7	1:1	1:1	10	50
B8	1:1	1:2	10	50
B9	1:1	2:1	10	50
B10	3:1	1:1	10	50

Evaluation of liposomes

% Yield of liposomes

The formula used to determine the percentage yield of all manufactured Paclitaxel liposome formulations is given below:

$$\textbf{\%}\ \text{yield} = \frac{\text{weight of dried liposomes}}{\text{weight of total drug+polymer}} \times 100\ [6]$$

Swelling index

10 mg of Paclitaxel liposomes were soaked in 100 ml of pH 7.5 phosphate buffer for 24 h at 370 °C. After 24 h, liposomes are removed from phosphate buffer, and the excess buffer is wiped out using filter paper. After that, the final weight of the liposomes was taken.

The swelling index of the different batches of liposomes was calculated using the following formula:

$$\text{Swelling index} = \frac{\text{Wt} - \text{Wo}}{\text{Wo}} \times 100$$

Where Wt is the weight of the liposomes after 24 h and W_0 is the initial weight of the liposomes [7].

Particle size and shape

The test is performed to determine the uniformity of the prepared liposomes [8]. The average particle size of each formulation was assessed using an optical microscope technique, by which the diameters and shapes of 100 particles were recorded.

Percentage drug entrapment efficiency

10 mg of the formulated Paclitaxel liposomes were crushed using a mortar and pestle and suspended in 100 ml of phosphate buffer at pH 7.5 in a 100 ml volumetric flask. Continuous stirring of the medium was done using a magnetic stirrer. On the next pH, the medium was filtered, 1 ml of the filtrate was diluted with 10 ml of the phosphate buffer, and the absorbance was measured by a UV spectrophotometer at a wavelength of 230 nm. [9].

The percentage of drug entrapment efficiency was calculated using the following formula:

$$\text{Drug entrapment efficiency \%} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} * 100$$

Fourier transform infrared (FTIR) spectroscopy

The drug, polymers, and prepared liposomes were subjected to FTIR analysis. Using KBr powder, the samples were scanned on a Bruker Fourier transform infrared (FTIR) spectrometer over a range of 400–500 cm^1.

***In vitro* drug release studies**

An *In vitro* drug release study was performed at 50 rpm using a USP Type II dissolution test apparatus (paddle type). 900 ml of phosphate buffer (pH 7.5) was used as the dissolution medium. The temperature was kept at 37 °C. 5 ml of each sample was taken at predefined intervals and replaced with an equal volume of new medium maintained at the same temperature. Filter paper no. 41, diluted and measured at 230 nm on a Shimadzu UV 1800 spectrophotometer.

Drug release kinetics mechanism

In vitro drug release data of 10 batches (B1–B10) was fitted to the zero-order, first-order, Higuchi, and Korsmeyer–Peppas models to determine the appropriate drug release kinetics model for every batch.

Scanning electron microscopy (SEM)

Drugs containing liposomes were mounted on conducting stubs and vacuum-coated gold palladium film using a Gold Sputter Coater (Model Edwards-S 150 B; Mfg. BOC Edwards UK). To examine the surface morphology, images were taken in SEM at a voltage of 17 kV.

Dark field microscopy

The formed liposomes were seen under Magnus INV manufactured by Olympus Optosystems at magnifications of 10X and 40X with inverse phases for better viewing.

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) assay is one of the most commonly used colorimetric assays to assess cytotoxicity or cell viability. This assay primarily determines cell viability by determining mitochondrial function in cells by measuring the activity of mitochondrial enzymes like succinate dehydrogenase. In this assay, MTT is reduced to purple formazan by NADH. This product can be quantified by light absorbance at a specific wavelength.

The cytotoxicity of a prepared formulation of PTX liposomes was determined via a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. At 37 °C, A375 cell lines ($1*10^4$ cells/well) were cultured in 96-well plates. On confluency, a set of wells were treated with serum-free media only and with standard PTX solution separately, and in other wells, PTX liposomes were added and incubated for a maximum of 90 min. After the treatment, 100 L of 0.5% (w/v) of MTT dissolved in 1X PBS were added to each well, and the cells were incubated. The media were aspirated out, and 150 ml of DMSO was added to each well to dissolve the formazan crystals. The absorbance of each sample was measured at 590 nm on an ELISA reader. Cell viability (%) was calculated by dividing the absorbance of samples obtained from cells incubated with media only and concentrations by the absorbance of samples obtained from cells incubated with culture medium only (Control) and multiplying this ratio by 100. The data were presented as the mean of triplicate readings.

The effect of PTX liposomes at fixed time points on A375 cell lines was examined by MTT assay. As a starting point, the standard PTX solution was used. Cytotoxicity was dependent on the treatment drug formulation and the duration of exposure. The earliest signs of injury in A375 cells were seen after 60 min of exposure to the PTX liposome in the cultured cells, where the percentage of cell survival was approximately 48.34%. MTT assay in A375 cells revealed that after 60 min and 200 g/ml PTX liposome concentration, the cell damage was so severe (survival below 50%) that recovery was difficult. The cell viability (%) of control and PTX liposomes (200 g/ml) was found to be higher for the PTX liposome.

RESULTS

The objective of our work was to determine the effect of the ratio of lipid matrix and edge activators showing better drug release rates on

the formulation of sustained-release liposomes of paclitaxel. Because of their biocompatibility and the ease with which they can be obtained in a high-purity grade, cholesterol and soy lecithin are excellent candidates for the role of vesicle-forming components. As a result of Span 80's biocompatibility and lipophilicity (HLB value of 4.3 and CMC of 0.135 g/l), it was decided to use it as the edge activator.

In addition, sodium deoxycholate was chosen as the edge activator because of its biocompatibility and structural properties. The hydrophilic group of this bile salt molecule is located on the concave face of the molecule, whereas the lipophilic steroidal skeleton is located on the convex face of the molecule. This bile salt molecule displays facial polarity. Because of this structural characteristic, it is simple to incorporate sodium deoxycholate into the lipid bilayers that make up the vesicle membrane [10]. In addition to this, the United State Food and Drug Administration (USFDA) has granted GRAS certification to both of surfactants and recommends their use for systemic administration [11]. We used the film hydration method to create liposomes containing Paclitaxel and investigated the impacts of several parameters, such as cholesterol ratio and methanol-chloroform concentration, on drug release rate.

Particle size analysis of different batches of liposomes

Optical microscopy was used to evaluate the mean particle size of several formulations of Paclitaxel liposomes. Results indicate that the mean particle size of liposomes was decreased by increasing the concentration of cholesterol. The shape of the liposomes was observed through visual observation [12]. The particle size of various batches of liposomes and their shapes are given in table 2.

Percentage yield of liposomes

The percentage yield of different batches of liposomes is shown in table 2.

Liposome swelling index

Swelling studies of all 8 batches of liposomes were performed in a phosphate buffer of pH 7.5. Results are mentioned in table 2. Data from the swelling index indicated that the equilibrium water uptake decreased as the proportion of lipid polymer cholesterol in the matrix increased. This is because increased concentrations of cholesterol do not allow swelling to take place [13, 14].

Drug entrapment efficiency as a percentage

The determination of entrapment efficiency was used to determine the amount of Paclitaxel present in the various batches of liposome formulations. The efficiency of drug entrapment as a percentage was found to be dependent on the balanced ratios of all ingredients. As the concentration of the ingredients was not varied in the formulation, the entrapment efficiency also increased [15, 16]. As shown in table 2, the B7 batch with LM Ratio and edge activator ratio both 1:1 demonstrated the highest entrapment efficiency.

Fourier transform infrared spectroscopy (FTIR)

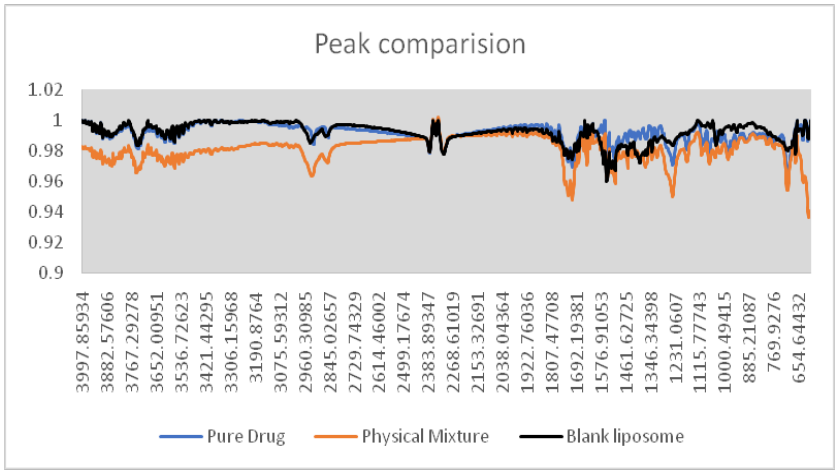

Fig. 2: IR spectra of Pure drug, Physical mixture and blank liposome

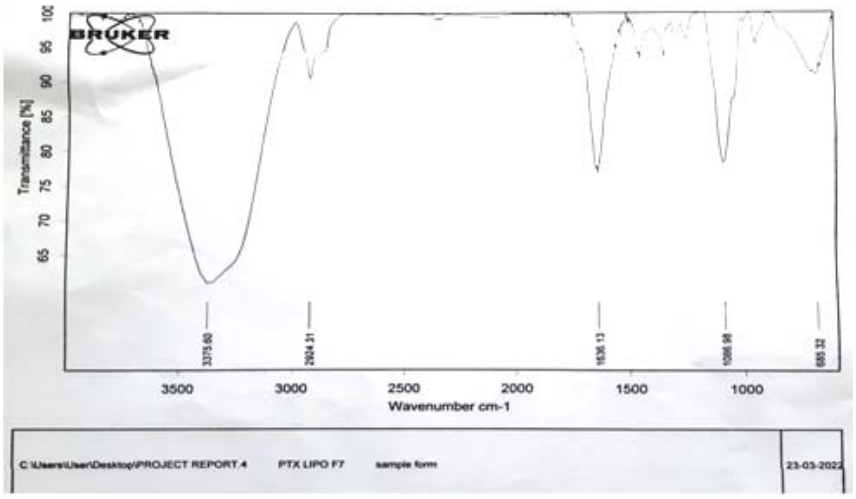

Fig. 3: IR spectra of paclitaxel liposome

Table 2: Percentage yield, swelling index, drug entrapment efficiency of 10 batches of liposome

Batch code	% Yield	Swelling index	Encapsulation efficiency
B1	47.33±1.3	78.12±0.1	0
B2	53.1±1.5	60.78±0.4	62.33±0.51
B3	60.24±0.9	82.06±0.29	59.51±0.22
B4	62.6±0.5	47.017±0.1	50.21±0.3
B5	75.0±0.9	49.08±0.03	44.30±0.07
B6	77.5±0.7	79.46±0.03	82.25±0.32
B7	95.4±0.5	78.11±0.09	88.95±0.03
B8	75.26±0.5	52.19±0.5	72.34±0.03
B9	85.0±0.4	56.11±0.3	77.37±0.21
B10	72.9±1.3	47.14±0.2	70.63±0.18

Results are given in mean±SD

Table 3: Probable peaks on performing IR spectroscopy of drug

Phosphatidylcholine	3424.9	Water band
	2922	$V_s[(CH_3)_4]$
	2852.2	$V_s[(CH_3)_4]$
	1736.6	$V_s[C=O]$
	700-1500	δ[C−H]
	1467.4	scissoring $\delta[(CH_2)_n]$
	1378.1	$\delta[CH_2]$
	1242.6	$v_{as}[PO_2^-]$
	1091.5	$v[PO_2^-]$
	970.4	Asymmetric $[^+N-(CH_3)_3]$ stretching
	927.0	v[C−C−N]
	826.9	$v[P(-O-C)_n]$
	721.8	rocking $v[(CH_2)_n]$
Taxol	3063	−C−H
	1735	Ester
	1709	Ketone
	1647	Amide
	1072	Finger print
	1244	Finger print
	708	Finger print
Phosphatidylcholinetaxol complex	709	Finger print
	969	Asymmetric$[^+N-(CH_3)_3]$ stretching
	1095	Symmetric $v_s[PO_2^-]$
	1245	Antisymmetric $v_{as}[PO_2^-]$
	1467	Scissoring CH_{28}(hexagonal)
	1647	C=O; symmetric alyl C=C
	1737	C=O stretching
	2853	Symmetric $v_s[(CH_3)_4]$
	2923	Symmetric $v_s[(CH_3)_4]$
	3441	H−bound OH groups

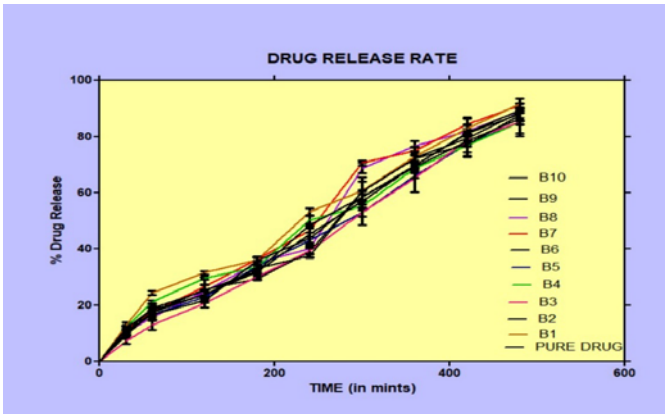

Fig. 4: Drug release rate of 10 batches of liposomes. Error bars indicate SD values

***In vitro* drug release studies**

In vitro drug release studies of all the batches of the liposomes (B1-B10) revealed that the drug release range was 76%-98% shown in fig. 4 [17, 18]. *In vitro* drug release study of 10 batches of liposomes can be presented in the following order: B3<pure drug<B10 <B4<B2<B5<B9<B6<B1<B8<B7.

Drug release kinetics mechanism

In vitro drug release data of 10 batches of liposomes (B1-B10) were fitted to zero order, first order, Higuchi, Korsmeyer-peppas model to determine the appropriate drug release kinetics model [19-21] (fig. 5).

Selection of the optimized batch

Selection of the optimum batch from 10 batches of prepared Paclitaxel liposomes was based on results from the size and shape of the liposomes, % entrapment efficiency, drug release rates and cumulative % drug release values. From the results, it can be concluded that the B7 batch is the optimized batch [22, 23].

DSC measurements

DSC measurements were performed using a DSC4000 device (Perkin Elmer). Before the calorimetric experiments, the solutions were degassed and then filled into the sample cell (4 mg) [24]. A heating rate of 10 °C min^{-1} in the 30–300 °C range was applied. Three up and down scans were performed for each sample to prove the reproducibility. All curves shown in the fig. originate from the first heating scan [25].

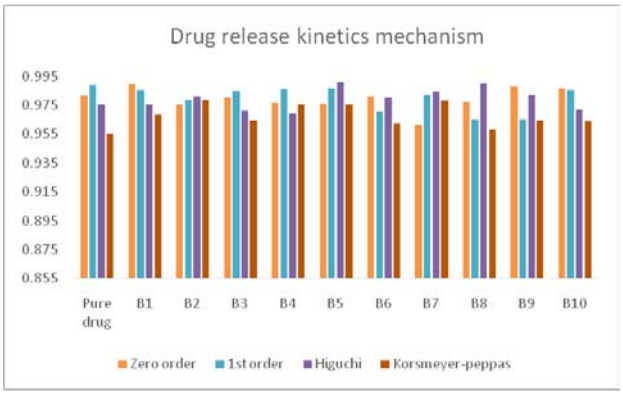

Fig. 5: Drug release kinetics mechanism

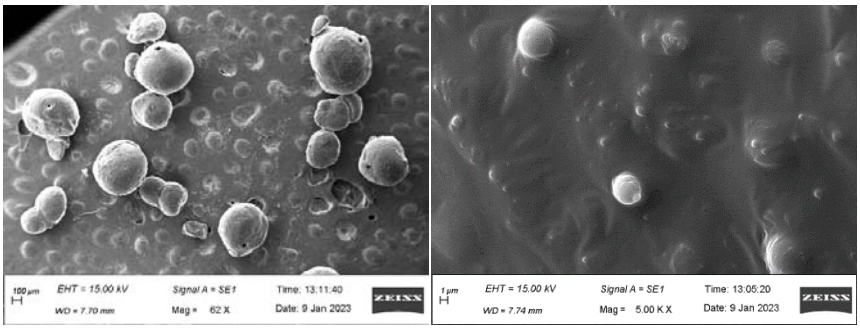

Fig. 6: SEM image of optimised liposome batch in different size ranges (100 µm and 1 µm)

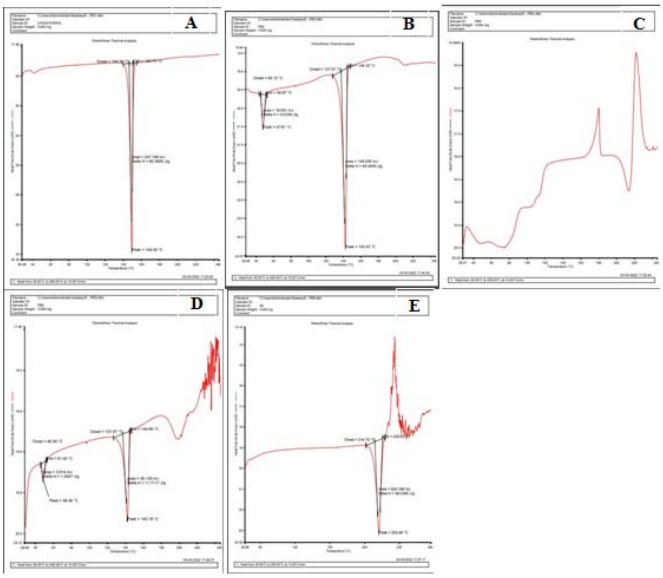

Fig. 7: Differential scanning calorimetry (DSC) A. Cholesterol, B. Sodium deoxycholate, C. Cholesterol, and Sodium deoxycholate, D. Cholesterol, Sodium deoxycholate, and Paclitaxel, E. Paclitaxel)

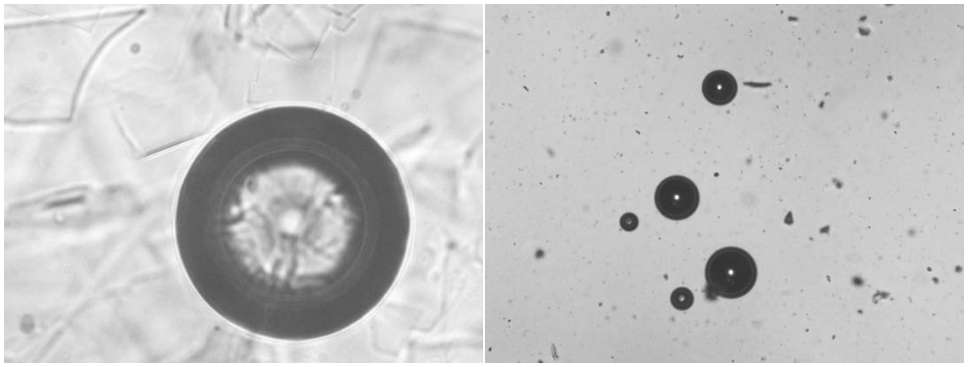

Fig. 8: Image of liposome under darkfield microscope 40X and 10X, respectively

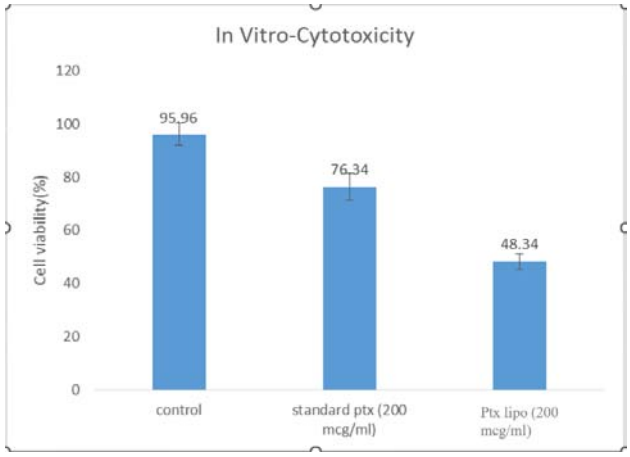

Fig. 9: Comparison between cell viability% of control, standard drug and prepared liposomes. Error bars indicate SD values

Images under dark field microscope

Optimized batch B3 under a dark field microscope had been viewed in inversed phase to view the interior of the liposomes in 40X and 10X, respectively. Instrument used Magnus INVS (220-240V-0.3A, 50-60Hz). The liposomes were regular and spherical.

MTT assay

The measure of cell viability shows a stark difference between the three formulations, that is control, standard PTX and PTX liposomes (fig. 9) where cell viability was found to be lowest in liposomes (48.34%), proving it to be indicative of positive response to move this formulation for further studies.

DISCUSSION

The pharmaceutical sector is facing a growing challenge with chemicals that are difficult to solubilize. Formulation scientists are facing a rising issue as they try to deliver an ever-increasing number of poorly soluble chemicals through various routes for its assessment. Bose *et al.* did a study on improving the bioavailability of paclitaxel using various novel drug delivery approaches out of which the about method was selected after doing numerous trial and error [26]. In order to boost the dissolution rate and improve the bioavailability of poorly soluble compounds, novel ingredient combination have been used, which has never been used before. The chemotherapy drug paclitaxel has widespread use despite its very low solubility. So, sodium deoxycholate has been used as an edge activator.

Therefore, a novel delivery form, liposome, has been used as a formulation technique to enhance the administration of poorly soluble drugs. Liposome formulations have the benefit of decreasing the organic solvent concentration often necessary for poorly soluble drugs, making them suitable for administration. Similar work of other authors has highlighted the use of Cremophor EL and Zein, which are highly expensive additional materials so our product is economically advantageous than the currently available marketed preparations [27, 28]. In this particular investigation, cholesterol and soy lecithin were used as vesicle-forming components, and various quantities of span 80 and sodium deoxycholate were utilized as edge activators. The fluidity of the lipid membranes of the vesicles is provided by the surfactant when it is present at sublytic concentrations, and the membrane of the vesicle becomes more flexible. Cholesterol and soy lecithin are chosen as vesicle-forming components primarily due to the biocompatibility of both substances and the readily available high-purity grades of both. As a result of Span 80's biocompatibility and lipophilicity (HLB value of 4.3 and CMC of 0.135 g/l), it was decided to use it as the edge activator. In addition, sodium deoxycholate was chosen as the edge activator because of the biocompatibility of its nature and its structural property it has. The hydrophilic group of this bile salt molecule is located on the concave face of the molecule, whereas the lipophilic steroidal skeleton is located on the convex face of the molecule. This bile salt molecule displays facial polarity. As a result of this structural characteristics, it is not difficult to incorporate sodium deoxycholate into the lipid bilayers that make up the vesicle membrane [29-32]. Ongoing research is the pharmacokinetic study which will improve the claim further. In the future BA/BE studies will be done of the optimized liposome of paclitaxel.

CONCLUSION

Liposomal drug delivery methods are the most well-established, with multiple formulations having received regulatory clearance. Liposomes are lipid vesicles with a phospholipid bilayer membrane capable of encapsulating both hydrophilic and lipophilic medicines. For cancer therapy, there are many FDA-approved liposomal formulations. Because of its biocompatibility, biodegradability, low toxicity, and immunogenicity, liposomal formulations have the greatest influence in the field of cancer. So, to solve such problems,

preparation, characterization, and evaluation of paclitaxel liposomes have been made for enhanced bioavailability and cost effectivity in comparison to current available anticancer novel delivery forms in the market. The investigation of the anticancer properties of Paclitaxel-loaded-liposome on other cell line studies will be evaluated to demonstrate the real efficacy of this nano-medicine in preclinical and clinical applications.

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AUTHORS CONTRIBUTIONS

All authors contributed equally.

CONFLICT OF INTERESTS

The authors report no financial or other conflicts of interest in this work.

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