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Formulation of Stable Nanoliposomes of Docetaxel: Design, Optimization, and *in-vitro* Characterization

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Authors' contributions

This work was carried out in collaboration among all authors. Author MSH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KSS and MJC managed the analyses of the study. Authors RBC and RSS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Liposome offers many advantages over conventional dosage forms, like increased bioavailability, possibility of releasing drug at slower and constant rate, accurate drug release. Docetaxel is approved by the FDA for the treatment of locally advanced or metastatic breast cancer, head and neck cancer, gastric cancer. Docetaxel is BCS Class 4 drug; hence efficacy can be improved with liposomal formulation.
Objectives: The Present study prepared Docetaxel loaded liposomal formulation.
Materials and Methods: Formulation batches were designed on the basis of solvent, lipid to cholesterol ratio, lipid to release modifier ratio, hydration temperature and various physicochemical and morphological properties of formulation were examined. The zeta potential, particle size determination, pH, stability, determination of encapsulation efficacy, morphology of formulation and *in-vitro* drug release were investigated.
Results: The zeta potential values of FD5 and FD9 were found to be -12.6 to -12.9 mV and -10.6 to -11.9 mV respectively. The entrapment efficiency of formulations of batch FD5 and FD9 were

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found to be 83.20% and 85.22% respectively. By comparing both FD5 and FD9 it was found that FD9 batch is optimized than FD5 in all aspects. The formulation batch FD9 has particle size of 105 nm, zeta potential in range of -10.6 to -11.9 mV; Drug entrapment efficiency 85.22%, Assay of formulation was 99.56 % and FD9 formulation shows extended release of Drug up to 13 hours.
Conclusion: The study confirmed that the Liposome formulation was successfully prepared and evaluated.

Keywords: Docetaxel-loaded liposomes; pre-formulation; physicochemical and morphological.

1. INTRODUCTION

Liposomes were discovered by Alec D Bangham during the 1960s at the Babraham Institute, University of Cambridge, and comprise of single or different concentric lipid bilayers encapsulating an aqueous compartment [1-2]. The first formulation was made natural lipids; at present they can incorporate regular and additionally manufactured lipids and surfactants. They have the capacity of ensnaring both lipophilic and hydrophilic agent, in the lipid layer and in the aqueous core, respectively. The size of these almost circular lipid vesicles can range from a couple of nanometers to a few micrometers. However, liposomes applied to medical use range between 50 and 450 nm [3]. Liposomes appear to be a practically perfect drug-carrier system, since their morphology is like that of cellular membranes and on account of their capacity to consolidate different substances. Thusly, throughout the previous 50 years liposomes have been broadly examined and they keep on being the subject of extraordinary research. They are esteemed for their natural and technological advantages delivery systems for biologically active substances, both *in vitro* and *in vivo*, and are viewed as the best drug-carrier system known to date [4]. During the last two decades ago, outstanding advancement has been made, and a few biomedical uses of liposomes are either in clinical trials or are going to be put available, while others have just been approved for public use. Docetaxel is approved by the FDA for the treatment of locally advanced or metastatic breast cancer, head and neck cancer, gastric cancer [5]. Docetaxel is BCS Class 4 Drug; hence efficacy can be improved with liposomal formulation [6].

2. MATERIALS AND METHODS

2.1 Pre-Formulation Study

Pre-formulation testing is the first step in rationale development of dosage forms of a drug substance. Pre-formulation study is desired to ensure the development of a stable as well as the therapeutically effective and safe dosage form. Pre-formulation testing is designed to assess the influence of physicochemical properties of drug substances and excipients on formulation properties of dosage form, method of manufacture and pharmacokinetic-biopharmaceutical properties of the resulting product. A thorough understanding of physicochemical properties may ultimately confirm that no significant barriers are present for the formulation development [7].

Basic objectives of Pre-formulation study:

- Characterization of drug.
- Characterization of excipients.
- Compatibility Study.

2.2 Formulation and Development

2.2.1 Characterization of (docetaxel) drug candidate

Characterization of drug is necessary to identify particular drug, and to check the purity of drug, to determine physicochemical properties which help for development of particular dosage form. Docetaxel sample was characterized by identification test, solubility study, melting point, UV analysis, FT-IR analysis [8].

2.2.2 Melting point determination of docetaxel

Melting point is utilized for assurance of purity and identification of drug. The Melting point of Docetaxel was determined by melting point apparatus utilizing capillary method. Fine powder of Atorvastatin calcium was filled in glass capillary tube which was recently fixed toward one side. The capillary tube was tied with thermometer and afterward immersed into melting point apparatus. The temperature was seen at which drug started to melt by thermometer which was already dip into the liquid paraffin apparatus [9].

2.2.3 IR spectroscopy

FT-IR is used both together information about the structure of a compound and as an analytical tool to assess the purity of a compound. The infrared spectrum sample was recorded and the spectral analysis was done. The dry sample of drug was taken and directly placed and analyzed by IR (Brucker alpha) instrument. IR spectroscopy is one of the important analytical techniques for chemical identification. The drug polymer interaction was studied by FT-IR spectroscopy. The spectra were recorded for pure drug using FTIR. The scanning range was 400-4000 cm^{-1} [10].

2.2.4 Solubility study

The main reason of solubility study is to ensure the solvent drug is dissolve and suitability of solvent for development of dosage form. Solubility study of Docetaxel was done by dissolving 10 mg of drug sample in various solvents like water, ethanol, methanol, acetonitrile [5,11].

2.3 UV Spectrophotometric Method for Docetaxel

2.3.1 Determination of λ_{max}

The standard solution of Docetaxel was scanned between 200-400 nm using UV spectrophotometer in ethanol, Phosphate buffer pH 7.4 [5,12].

2.3.2 Calibration curve of DOC in phosphate buffer pH 7.4

Stock solution was prepared by dissolving 10 mg of accurately weighed in 10 ml of ethanol to get 1000 µg/ml solutions. From this solution 10 ml of sample is diluted to 100 ml with phosphate buffer 7.4 to obtained 100 µg/ml solutions. From this, 0.5, 1.0, 1.5 up to 3.0 ml solutions were pipette into a series of 10 ml volumetric flask and were made-up to 10ml with phosphate buffer pH 7.4 to get 5, 10, 15, 20, 25 and 30 µg/ml solutions of Docetaxel respectively. The absorbance of resulting solutions was measured at 232 nm against the blank. Calibration curves of Docetaxel were performed in triplicate. A graph was plotted by taking concentration on X-axis and absorbance on Y-axis [13].

2.4 Selection of Excipients

After checking the solubility of docetaxel in suitable solvents like ethanol, methanol, chloroform, water; Excipients were selected and solubility study was done with all solvents. These Excipients were selected on the basis of maximum solubility of the excipients in min quantity of different solvents [14].

2.5 Compatibility Study of DOC with Excipients

The compatibility study was done at 55°C for 14 days with Moisture and without Moisture in sealed glass container of individual drug and Drug: Excipient (1:1). Individual IR diagram were taken before putting the ingredient and drug into the glass vials and these vials were kept for 14 days for 55°C in duration of 14 days every one of the vials were watched for any color change, gas formation building up and liquefaction and finally following 14 days its IR was studied [6,15].

2.6 Formulation and Development of Docetaxel Liposomal Injection

2.6.1 Selection of phospholipid and sterol ratio

Firstly ratio of Phospholipid and Sterol was determined by taking in ratio such as 1:0.7, 1:1, 1:0.5, 1:0.9, likewise further given below in table and selected on the basis of phase transition temperature suitable for stable formulation. This method is based on trial and error [5,16].

2.6.2 Trial batch fabrication

Phosphatidylserine & cholesterol were dissolved in Ethanol and Docetaxel added to that under continuous stirring. This mixture was heated at 40-45ºC to evaporate the solvent. The film was hydrated with hydration medium Phosphate buffer pH 6.8 and sonicated for 15 minutes. This mixture was placed in to vacuum oven for complete evaporation of ethanol at 35°C to 45°C for about 1hr at pressure of -400 mmHg. After complete evaporation of ethanol, the final volume was made by using phosphate buffer of pH 6.8. The large unilamellar vesicles reduced to small unilamellar vesicles due to sonication. This mixture was purified by passing through 0.45 µm membrane filter and filtrate was collected (Table 1). Formed liposome was stored in glass vial and kept at 2-8ºC [17].

Table 1. Design of trial batches

Parameters	FD5	FD9
Drug	Docetaxel 10 mg	Docetaxel 10 mg
Solvent	Ethanol 4 ml	Ethanol 4 ml
Carrier ratio:	1:6	1:8.5
Cholesterol: PS		
Hydration medium (phosphate buffer of pH 6.8)	20 mL	20 mL
Operating Temperature	35-45°C	35-45°C

2.7 Optimization of Liposome Formulation

2.7.1 Droplet size determination

Liposome formulation (1 ml) was diluted with 10 ml deionized water in a beaker with constant stirring using a glass rod. The resultant solution was then subjected to particle size analysis. The droplet size so formed determined by Dynamic light scattering (DLS) technique using a zetasizer (Nano ZS, Malvern Instruments, UK) [5,18].

2.7.2 Zeta potential determination

The Zeta potential of the selected formulation was determined by laser diffraction analysis using particle size analyzer (Malvern Zetasizer Nano Series ZS 90). The samples were diluted with a ratio of 1:100 (v/v) with distilled water and mixed for 1 min using a magnetic stirrer. All studies were repeated in triplicate [5,19].

2.7.3 Determination of encapsulation efficiency

Encapsulation efficiency of nanoliposomes was determined by separating non-encapsulated DOC from DOCNL suspension by centrifugation at 5000 rpm for 15 min at room temperature. The sediment nanoliposomes were disrupted with ethanol to release the entrapped drug; suitably diluted with phosphate buffer pH 6.8 and the absorbance measured at 230 nm to calculate the encapsulation efficiency using the calibration curve equation y = 0.04x + 0.0032. Here, 'y' is the measured absorbance and 'x' is the concentration of DOC in mg/mL. The percent encapsulation efficiency was calculated using following equation [6,20].

Encapsulation efficiency %= (Total drug – free drug)/ (Total Drug) ×100 (1)

2.7.4 pH of formulation

pH governs the ionization and tonicity in blood of the formulation. The pH of the formulation was measured with Digital pH meter [21].

2.7.5 Morphology

Scanning electron microscopy was employed to determine the shape and surface morphology of the produced liposomes [22].

2.7.6 *In vitro* drug release study

Liposome dispersion was placed on one side of the cellophane membrane in a vertical Franz diffusion cell. Other side of the membrane was in contact with the dissolution medium. Entire dissolution assembly was placed on a magnetic stirrer at temperature of 37°C. Dissolution medium was 40 ml of PBS pH 6.8 containing. Different aliquots of dissolution medium was withdrawn at different time intervals- 60 min, 2 ,3, up to 13 h, Whenever sample was withdrawn equal volume of fresh dissolution medium was added to the cell to maintain a constant volume. Drug concentrations in the dissolution medium were determined by UV spectrophotometric method [5].

2.7.7 Stability study

The stability of the docetaxel liposome was evaluated after storage at 2-8°C and 25°C for 1 month and 3 month, respectively. The particle size distribution and drug encapsulation efficiency of the samples were determined as a function of the storage time [6].

3. RESULTS AND DISCUSSION

3.1 Melting Point Determination

The melting point of the Docetaxel was found 234-236°C that is matched with standard values that indicating the purity of sample.

3.2 Solubility

DOC was found to be insoluble in water, soluble in ethanol, chloroform, acetonitrile and methanol. Solubility of DOC in to water was found to be 0.0127 mg/mL.

3.3 UV Spectrophotometric Method for DOC

3.3.1 Determination of λ_{max}

The standard solutions of DOC in ethanol and phosphate buffer pH 7.4 solvent media were scanned between 200-400 nm in UV spectrophotometer. The maximum absorbance was observed at 232 nm in case of ethanol and 234 nm in case of phosphate buffer 7.4. The working λ_{max} was chosen as 232 nm as shown in Fig. 1.

3.3.2 Calibration curve of DOC in ethanol

The calibration curves of DOC in ethanol was found to be linear in the concentration range of 5-30 µg/ml and having coefficient of regression (R^2) value 0.997 as shown in Fig. 2.

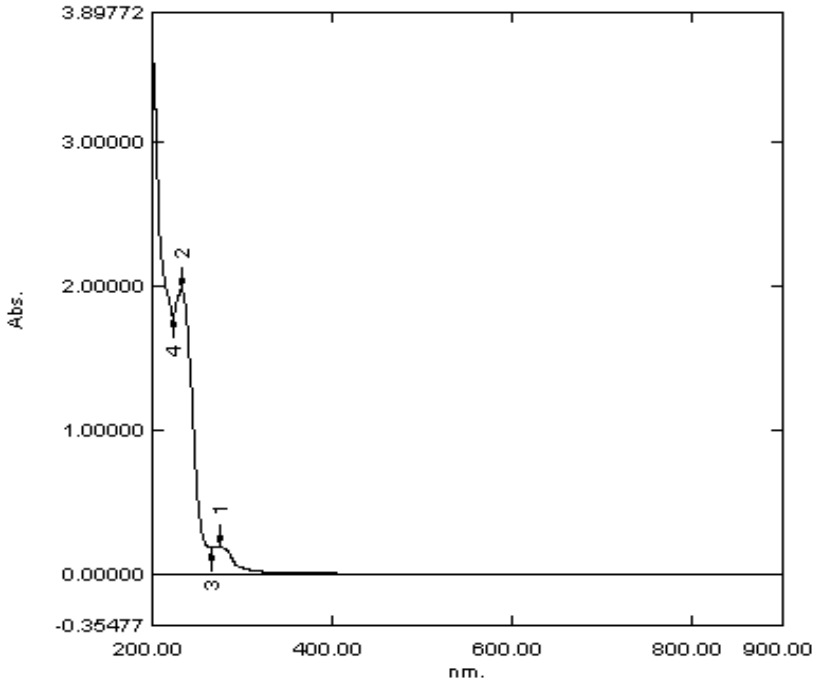


Fig. 1. λmax of docetaxel (DOC)

Conc.	Absorbance
5	0.263
10	0.358
15	0.569
20	0.686
25	0.901
30	1.031

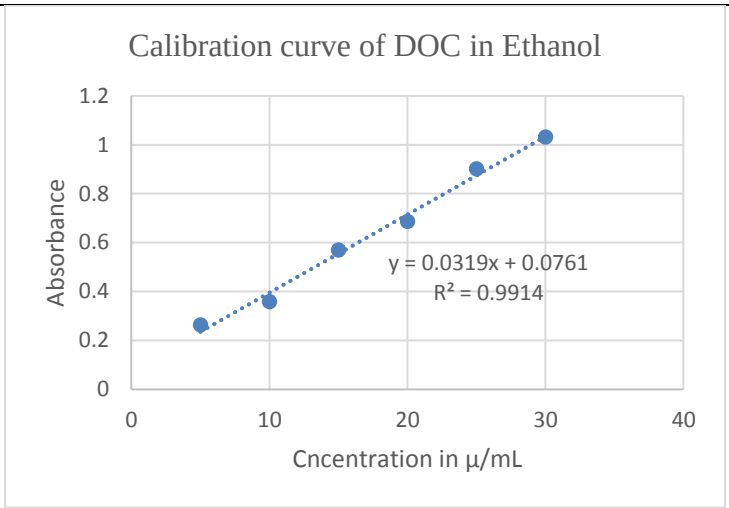


Fig. 2. Calibration curve of DOC in ethanol

3.3.3 Calibration curve of DOC in buffer pH 7.4

The calibration curves of DOC in pH 7.4 was found to be linear in the concentration range of 5-30 µg/ml and having coefficient of regression (R^2) value 0.997 (Fig. 3).

3.4 Compatibility by FT-IR Study

IR spectroscopy has been employed as a useful tool to identify the drug excipients interaction. IR spectra of pure DOC (Fig. 4) and excipients were taken before starting & after completion of compatibility study.

3.4.1 FT-IR of cholesterol

The cholesterol shows functional frequency peak at 3744.34, 2818.32, 1888.88, 1104.62, 1082.19 cm^{-1} (Table 2) and confirmed the functional group like (-OH), (-CH2 & -CH3 (S)), C=O, (-CH in plane and (-CH deformation) respectively as shown in Fig. 5.

Conc.	Absorbance
5	0.249
10	0.373
15	0.525
20	0.669
25	0.820
30	1.003

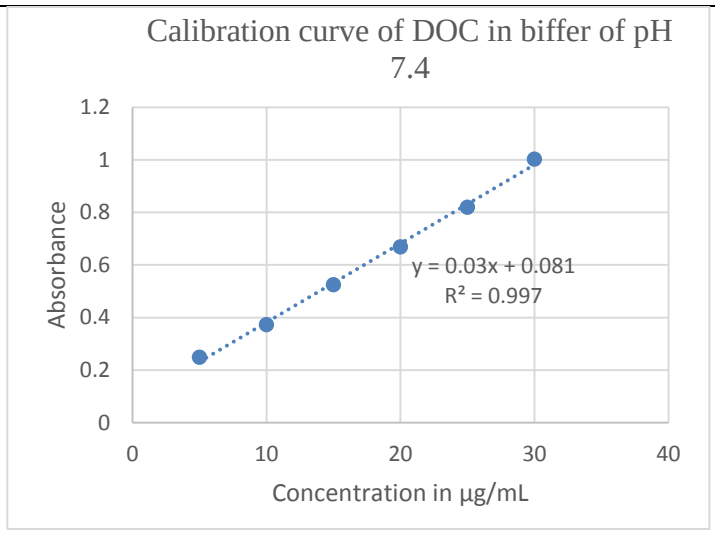


Fig. 3. Calibration curve of DOC in buffer pH 7.4

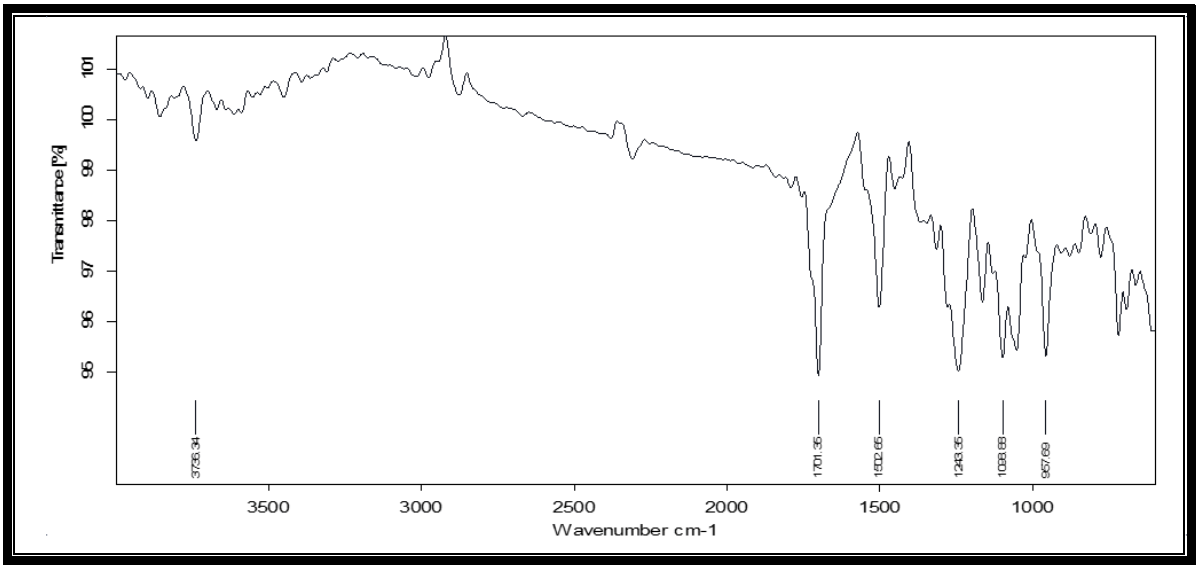


Fig. 4. FT-IR of docetaxel

Table 2. Reported IR frequencies of cholesterol

Sr. No.	FT-IR frequency ranges (cm^{-1})	Interpretation
1	3744.34 cm^{-1}	-OH (Stretching)
2	2818.32 cm^{-1}	$-CH_2$ & $-CH_3$ (Stretching)
3	1888.88 cm^{-1}	-C=O
4	1104.62 cm^{-1}	-CH in Plane
5	1082.19 cm^{-1}	-CH deformation out of the plane

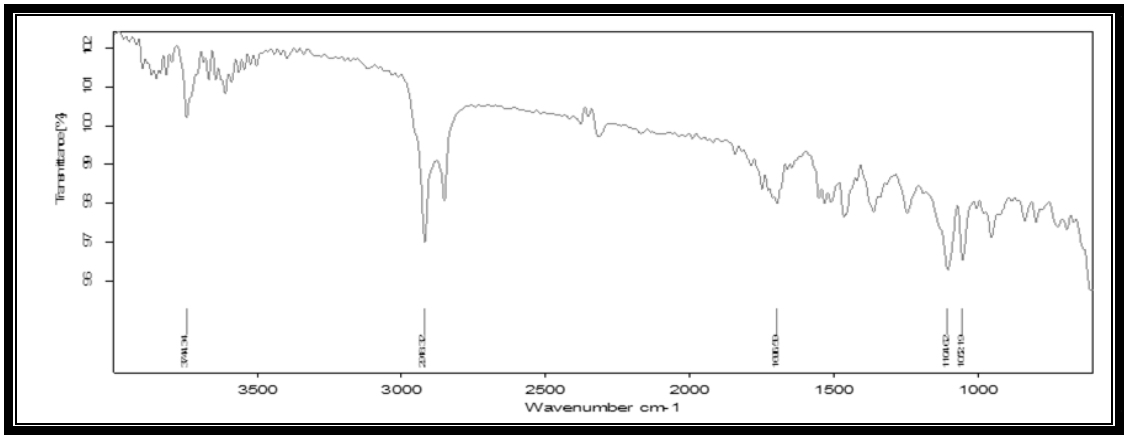


Fig. 5. FT-IR of cholesterol

3.4.2 FT-IR of phosphatidylserine (PS)

The data of analysis presence of the (-NH), (-CH2 & CH3 group), (-CH3 (S), (-C=O) and (-PO2) group has been confirmed at frequency range of 3400, 2916.32, 2849.86, 1728.50, 1096.56-1467.32 cm^{-1} (Table 3) respectively and all identification data together confirmed the structure of phosphatidylserine (Fig. 6).

3.4.3 FT-IR of mixture of API + PS + cholesterol

According to the IR spectra of drug and excipients after and before compatibility, there was no any significant change in the spectra that shows all functional group of individual agent are stable (Fig. 7). Thus drug and excipients were compatible with each other.

3.5 Optimization of Liposome Formulation

For the good and stable liposome formulation the particle size should be below 400nm, zeta potential should be in the range of 40 to -40 mV and entrapment efficiency must be higher. Hence by considering these critical parameters, batch FD5 and FD9 was found to be good.

3.5.1 Particle size determination

Mean globule size of optimized formulation where showed globule size of between 105 to 113 nm.

3.5.2 Zeta potential measurement

ZP governs the degree of repulsion between adjacent or similarly charged and dispersed droplet, it shows the practical application in the stability. ZP-values of FD5 and FD9 were found to be -12.6 to -12.9 mV and -10.6 to -11.9 mV respectively. ZP values in range of 40 to -40 mV of either charge characterize a stable formulation.

3.5.3 Entrapment efficiency

The entrapment efficiency of formulations of batch FD5 and FD9 were found to be 83.20% and 85.22% respectively.

3.5.4 *In-vitro* drug release

By comparing both FD5 and FD9, it was found that FD9 (series 3) batch is optimized than FD5 (series 2). The formulation batch FD9 has particle size of 105 nm, zeta potential in range of -10.6 to -11.9 mV; drug entrapment efficiency of 85.22%, Assay of formulation was 99.56% and FD9 formulation shows extended release of drug up to 13 hours (Fig. 8). Hence, optimized Batch

FD9 was subjected to the stability study for 6 months at 2-8°C and 3 months at 25°C.

3.5.5 Stability study

The stability of the docetaxel liposome was evaluated after storage at 2-8 and 25°C. The particle size distribution and drug encapsulation efficiency of the samples were determined as a function of the storage time. Before the stability study, particle size and entrapment efficiency value where 105 nm and 85.22%, respectively. After the stability study, particle size and entrapment efficiency value was found to be increased.

3.5.6 Morphology

Scanning electron microscopy images of batch number FD5 & FD9 (Fig. 9).

Table 3. Reported IR frequencies of phosphatidylserine (PS)

Sr. No.	FT-IR frequency ranges (cm^{-1})	Interpretation
1	3400.00 cm-^{1}	-NH_3 group
2	2916.32 cm $^{-1}$	-CH_2 & CH_3 group
3	2849.86 cm $^{-1}$	-CH_3 (Stretching)
4	1728.50 cm $^{-1}$	-C=O group
5	1096.56 cm -1 to 1467.32 cm -1	(-PO2) group

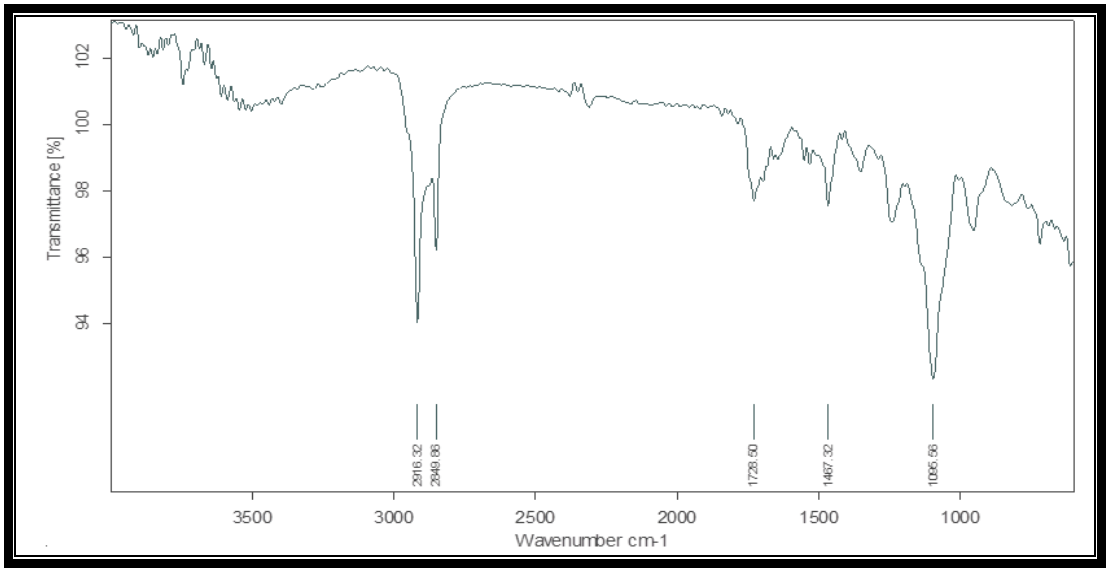
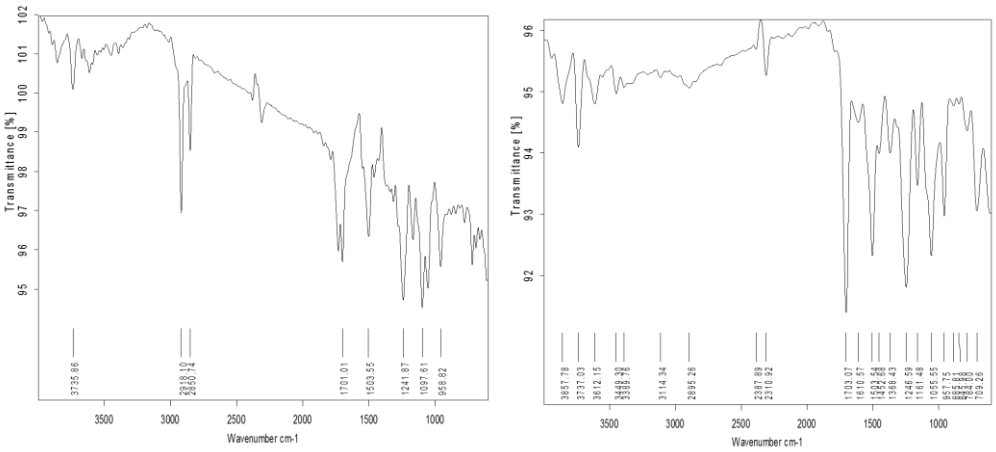


Fig. 6. FT-IR of phosphatidylserine (PS)



(a) Before exposure **(b) After exposure**

Fig. 7. FT-IR Spectra of API + PS + cholesterol mixture

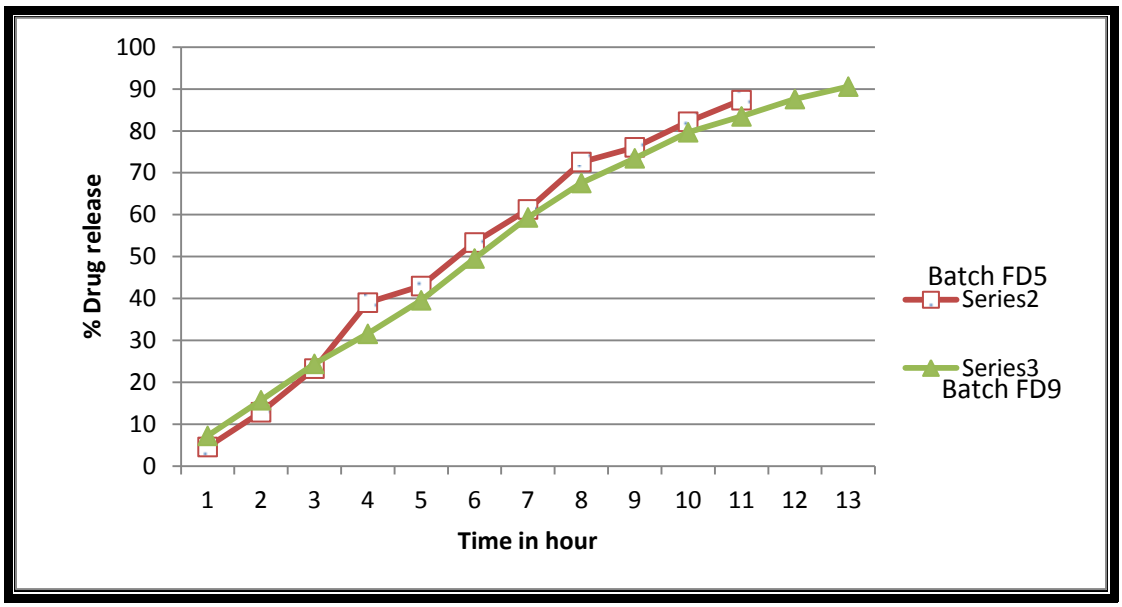


Fig. 8. *In-vitro* drug release study

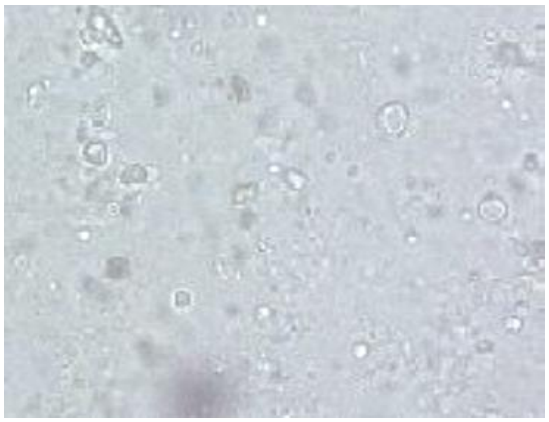
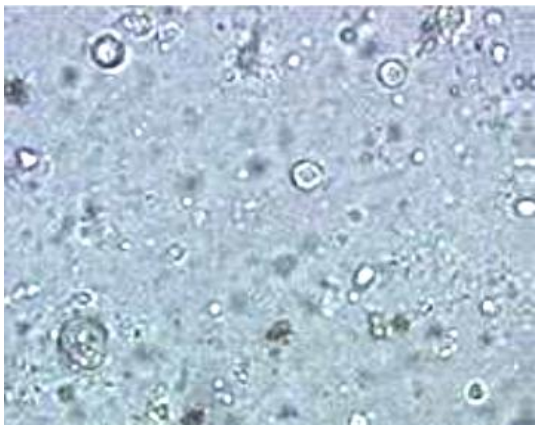


Fig. 9. A. SEM image of FD5



B. SEM image of FD9

4. CONCLUSION

Liposome offers many advantages over conventional dosage forms, like increased bioavailability, possibility of releasing drug at slower and constant rate, accurate drug release. Docetaxel is approved by the FDA for treatment of locally advanced or metastatic breast cancer, head and neck cancer, gastric cancer. Docetaxel is BCS Class 4 drug; hence efficacy can be improved with liposomal formulation. Docetaxel anhydrous is insoluble in water with Partition coefficient (log *P* = 2.4). Considering all above parameter, Docetaxel was selected as model drug candidate for increasing its water permeability and bioavailability through liposome formulation. As part of Pre-formulation studies, solubility of drug in various organic solvent and water, melting point was determined to enable selection of excipients with good drug loading ability. Selected lipid (PS) and sterol (cholesterol) were tried in different proportions for liposome. Formulation batches were designed on the basis of solvent, lipid to cholesterol ratio, hydration temperature etc. Formulations were optimised on three test *i.e.,* particle size, zeta potential, entrapment efficiency. Optimized formulations were further characterized for droplet size measurement, Zeta potential, entrapment efficiency. Batch FD5 and FD9 have shown good results. Hence, these batches were further subjected to in vitro drug release and assay of the formulation. The formulation batch FD9 has particle size of 105 nm, zeta potential in range of -10.6 to -11.9 mV; drug entrapment efficiency 85.22%, Assay of formulation was 99.56% and FD9 formulation shows extended release of drug up to 13 hours. The morphology images of

Liposomes by SEM showed formation of proper liposomes with some free drug. From the above study, it was concluded that Docetaxel liposome was successfully developed.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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