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DEVELOPMENT AND EVALUATION OF DOXORUBICIN HYDROCHLORIDE LOADED LIPOSOMES

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Abstract

Objective: The objective of this study was to formulate and evaluate liposomes loaded with Doxorubicin.

Methods: Liposomal formulation of Doxorubicin was prepared by "thin lipid film method". Mixture of phosphotidyl choline (soya lecithin), Doxorubicin and cholesterol of varying weight ratio was used for the preparation of liposomes. All batches were evaluated by drug entrapment and release kinetic studies. Stability study was carried out with F4 batch.

Results: F2 and F4 formulations showed better drug entrapment efficiency compared to the other batches. The drug entrapment efficiency of all batches was found within the range of 51.42 % to 79.0 %. F4 shows highest release of about 79.0% in 4 h. F4 formulation was found to follow significantly zero-order release kinetics. Short term stability study of formulation F4 showed no significant change in release kinetics.

Conclusion: The F4 batches showed promising results compared to other formulations. No changes were founded during the short-term stability study of F4.

Keywords: Liposomes, Doxorubicin, Phosphatidylcholine, Cholesterol, Release kinetics

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INTRODUCTION:

Liposome is a micro particulate colloidal vesicle, in which aqueous medium is surrounded by single or multiple concentric layers of phospholipids. Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural nontoxic phospholipids. It offers controlled release, targeted drug delivery, thus enhancing therapeutic efficacy, and reduced dosing frequency. Therapeutically, these are used as a carrier for drugs, viruses, bacteria, antigen, peptides, antibiotics, vaccines, genes, and diagnostic agents.

Liposomes are found to be suitable for localization of topically applied drugs at or near the site of application because they may act as slow-releasing vehicles. Topical drug delivery is a pleasing route for local and systemic treatment. The delivery of drug through topical route is the most effective treatment for the skin diseases.

In this study, we prepared Liposomal loaded hydrogel of Doxorubicin hydrochloride for the treatment of wound infection which is caused by mycoses i.e. fungi. After that we evaluate the formulation of liposomal loaded hydrogel by various parameter and then compare with marketed formulation of doxorubicin gel/cream.

The following experimental protocol was therefore designed to allow a systemic approach to study. Doxorubicin drug was selected because it is an anti-cancerous drug and better therapeutic action and this drug is not easily degraded when loaded with hydrogel and are easily available.

Doxorubicin is known to interact with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of the enzyme topoisomerase II, which relaxes super coils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication.

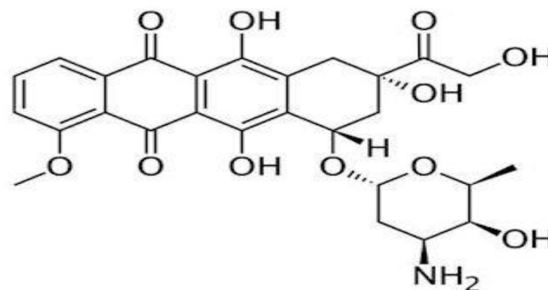
The planar aromatic chromophore portion of the molecule intercalates between two base pairs of the DNA, while the six-membered daunosamine sugar sits in the minor groove and interacts with flanking base pairs immediately adjacent to the intercalation site, as evidenced by several crystal structures.

Liposomes prepared by thin film hydration method (Hand- Shaking Method).

MATERIALS AND METHODS:

Drug profile:

Molecular formula:
$C_{27}H_{29}NO_{11}$



Doxorubicin is a drug used in cancer chemotherapy, it is an anthracycline topoisomerase inhibitor isolated from *streptomyces peucetius var. caesius.*

STANDARD CALIBRATIONCURVE

Standard calibration curve of doxorubicin hydrochloride was developed using phosphate buffer pH 7.4 and estimated by UV-Visible spectrophotometer at 254nm.

Preparation of Liposomes

Method used for the preparation of liposomes is Lipid film hydration by hand shaking method. Initially, cholesterol and soya lecithin were weighed and accurately dissolved in proposed ratio of chloroform and methanol(2:1) stirred for two minutes.100 mg of ketoconazole was added to above solution and continue the stirred for 2 mins. Now, the above solution is subjected for hydration with 10 ml distilled water and added 0.25 ml of Tween 80 and continue the stirring for about one hour for the formation of liposomal vesicles. Finally, the product obtained is collected and stored in a hermetically sealed container for further evaluation studies .

S. No.	INGREDIENTS	F1	F2	F3	F4
1.	Doxorubicin Hydrochloride (mg)	100	100	100	100
2	Soya Lecithin (mg)	100	200	250	100
3.	Cholesterols (mg)	20	30	40	50
4.	Chloroform (ml)	20	20	20	20
5.	Methanol (ml)	10	10	10	10
6.	Distilled water (ml)	10	10	10	10
7.	Tween 80 (ml)	0.25	0.25	0.25	0.25

Evaluations of Liposomes

Determination of percentage drug entrapment efficiency:
Drug entrapment efficiency was calculated by using centrifugation method. The liposomal suspension of 1 ml was taken and centrifuged at 3500 rpm for 15 min. The sediment obtained from the centrifugation was suspended in 100 ml of phosphate buffer pH 7.4, and the absorbance was taken at 294 nm. From that, the amount present in 1 ml of suspension was obtained. The drug entrapment efficiency was calculated from the following formula

Total entrapment efficiency = Amount of drug in supernatent liquid/ Total Amount of drug ×100

Morphology analysis:

Prepared liposomes for all the formulations were viewed under for observing the vesicle formation and discreteness of dispersed vesicles. A slide was prepared by placing over it and this slide was viewed under optical microscope at 40x magnification. Photographs were taken to prepared slides using digital camera .

***In-vitro* drug release study**
The *in vitro* release for all formulated ketoconazole liposomes were carried out for 8 hours using in phosphate buffer pH 6.8. The studies were carried in USP dissolution apparatus at $37^0C±0.5^0C$ and 50 rpm speed.900 ml of phosphate buffer pH 6.8 was used as a dissolution medium.1ml of samples were withdrawn at every 30 mins upto 480 mins and make up to 10 ml with pH 6.8 and analyzed for ketoconazole content at 294nm with pH 6.8 as blank using UV- Spectrophotometer.

Percentage Yield of Liposomes:

Percentage(%) Yield= Actual weight of product/ Total weight of drug and excipients×100

The prepared liposomes were collected and weighed. The measured weight was divided by the total amount of drug and excipients which were used for the preparation of liposomes.

Determination of drug content:
Drug entrapped multilamellar liposomes (100 mg) were suspended in 100 ml solution of chloroform: methanol (2:1). The resultant dispersion was kept for 20 min for complete mixing with continuous agitation and filtered through a 0.45 µm membrane filter. The drug content was determined spectrophotometrically at 294 nm using a regression equation derived from the standard graph. Results were based on triplicate determination .

Drug content =Sample absorbance/ Standard absorbance ×100

pH Determination
The pH was determined by using digital pH meter. The glass electrode was calibrated with the solutions determined for the equipment (pH of 4.00 and 7.00). The preparation was left for about 15 min for attaining while measuring. The analysis of formulation were done in triplicate, and average values were calculated.

***In vitro* Drug Release**
The apparatus consists of a glass cylinder open at both ends. A dialysis membrane soaked in distilled water(24 h before use)is fixed to the one end of the cylinder with the aid of an adhesive. Gels equivalent to 10 mg of Ketoconazole is taken inside the cell(donor compartment)and the cell is immersed in a beaker containing 800 ml of phosphate buffer pH 7.4 containing 10% v/v methanol (to maintain sink

condition), act as receptor compartment. The whole assembly is fixed in such a way that the lower end of the cell containing gel is just above the surface of diffusion medium (1-2mm deep) and the medium was agitated using a magnetic stirrer at the temperature 37± 0.5^0 C. Aliquots (5ml) are withdrawn from the receptor compartment periodically and replaced with same volume with fresh buffer. The samples were analyzed by using UV-visible spectrophotometer at 294nm.

Release Kinetics

To analyze the *in vitro* release data various kinetic models were use to describe the release kinetics. The zero order rate Eq. (2) describes the systems where the drug release rate is independent of its concentration. The first order Eq. (3) describes the release from system where release rate is concentration dependent. Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion.

Determination of Particle Size and Polydispersity Index by ZetaSizer

The Ketoconazole loaded liposomes was characterized for their size and Polydispersity index were also analysed by Zetasizer [Beckmen Coulter ™ Delsa Nano Version 3.73/2.30] at 25°C at an angle of 90 °C, after appropriate dilution using double distilled water.

Stability Studies

The drug loaded liposomes were subjected to stability studies for a period of 3 months at room temperature (30±2°C, refrigerator condition i.e. [4±2°C] and at accelerated condition [40±2°C,75%RH]. After 1month,2 months and 3 months of storage ,the formulations was subjected to test for physical stability and pH

RESULT AND DISCUSSION

Table: Standard readings of Doxorubicin hydrochloride in UV

S. No.	Concentration (μg/ml)	Absorbance at 294nm
1.	0	0
2.	10	0.184
3.	20	0.348
4.	30	0.526
5.	40	0.721
6.	50	0.901

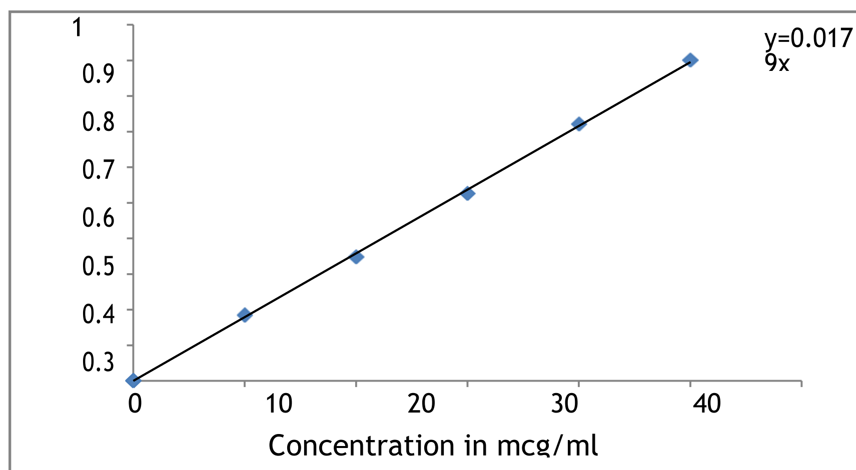
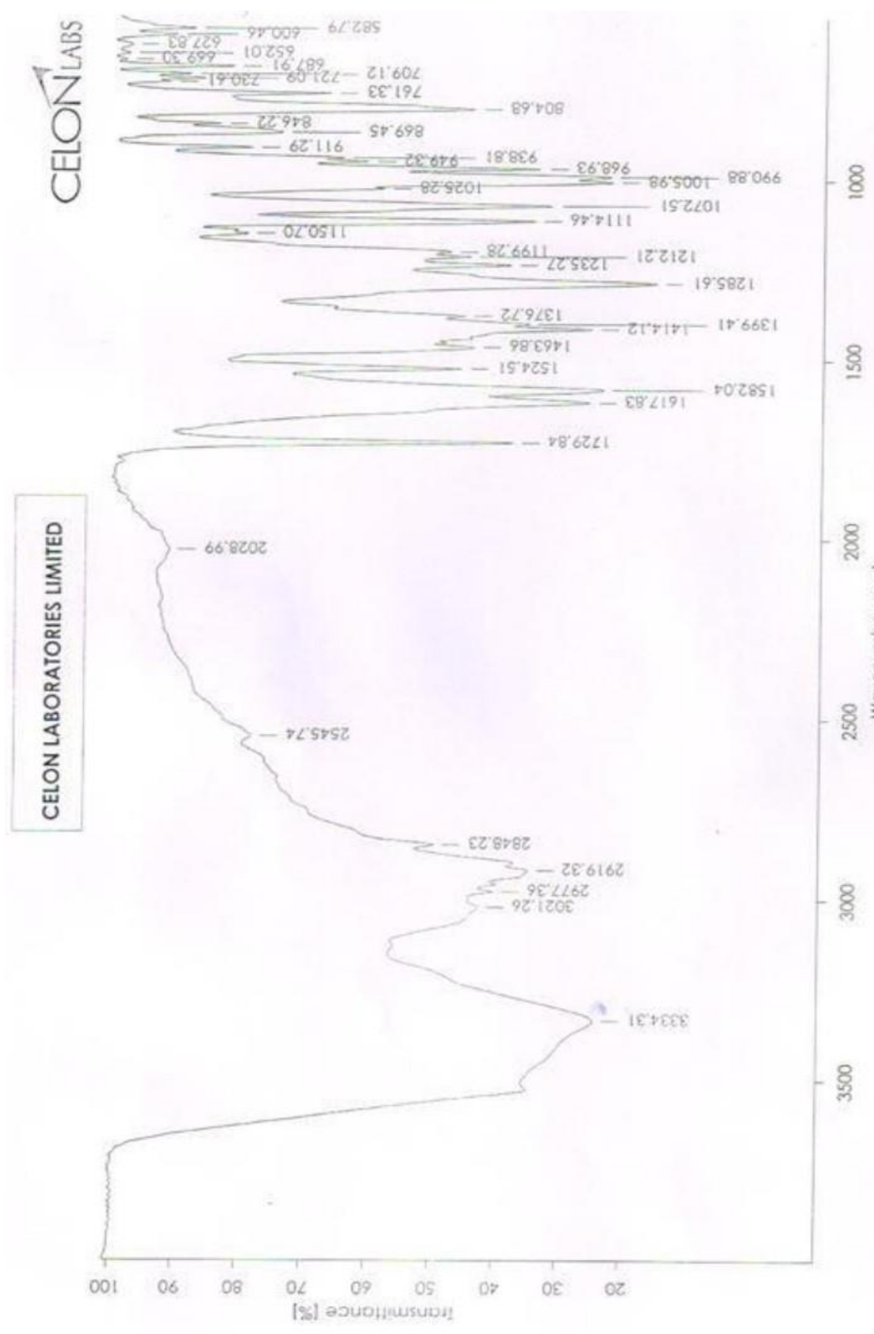
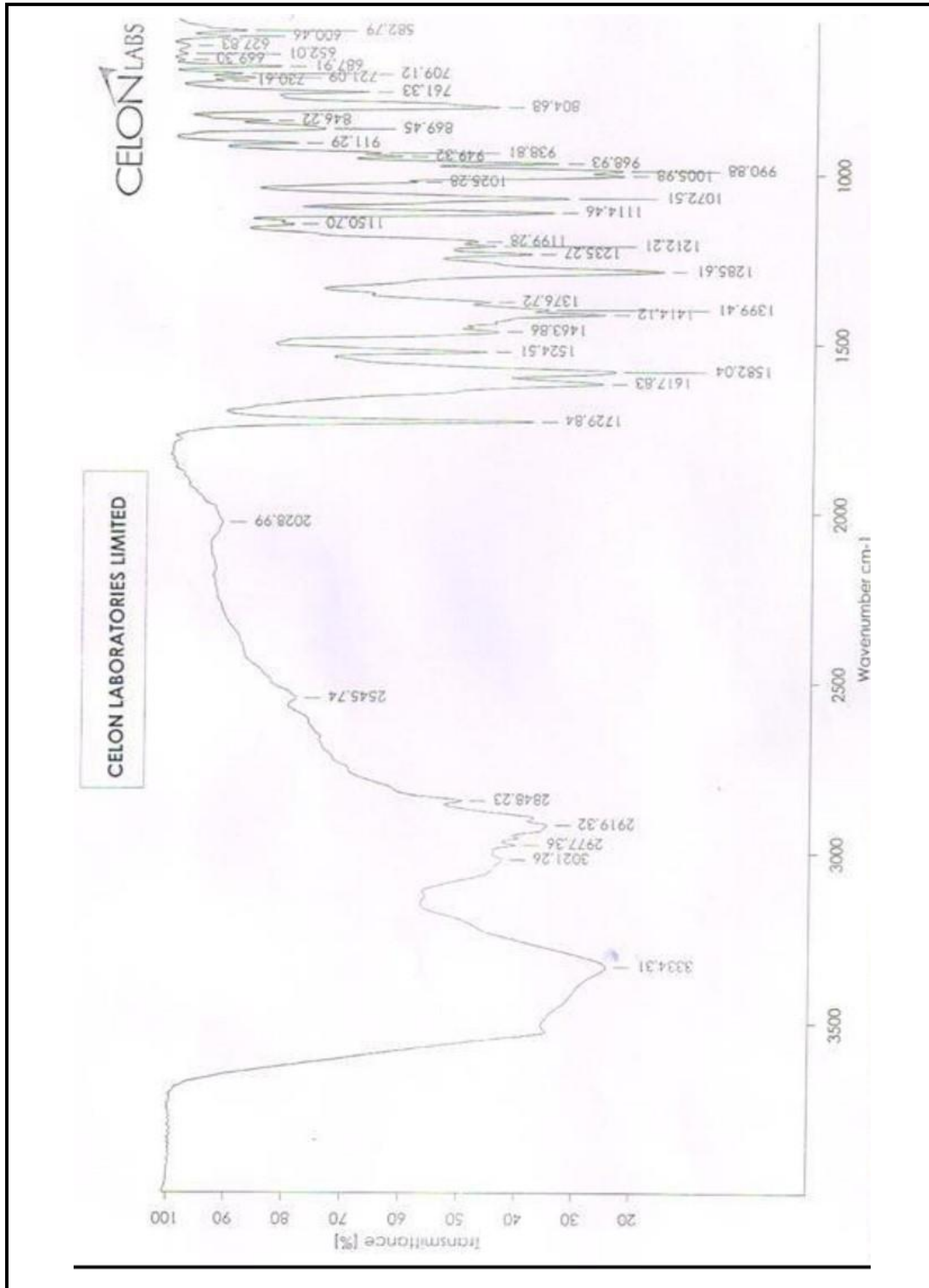


Figure :3.1 Standard graph of Doxorubicin hydrochloride in phosphate buffer of pH 7.4.

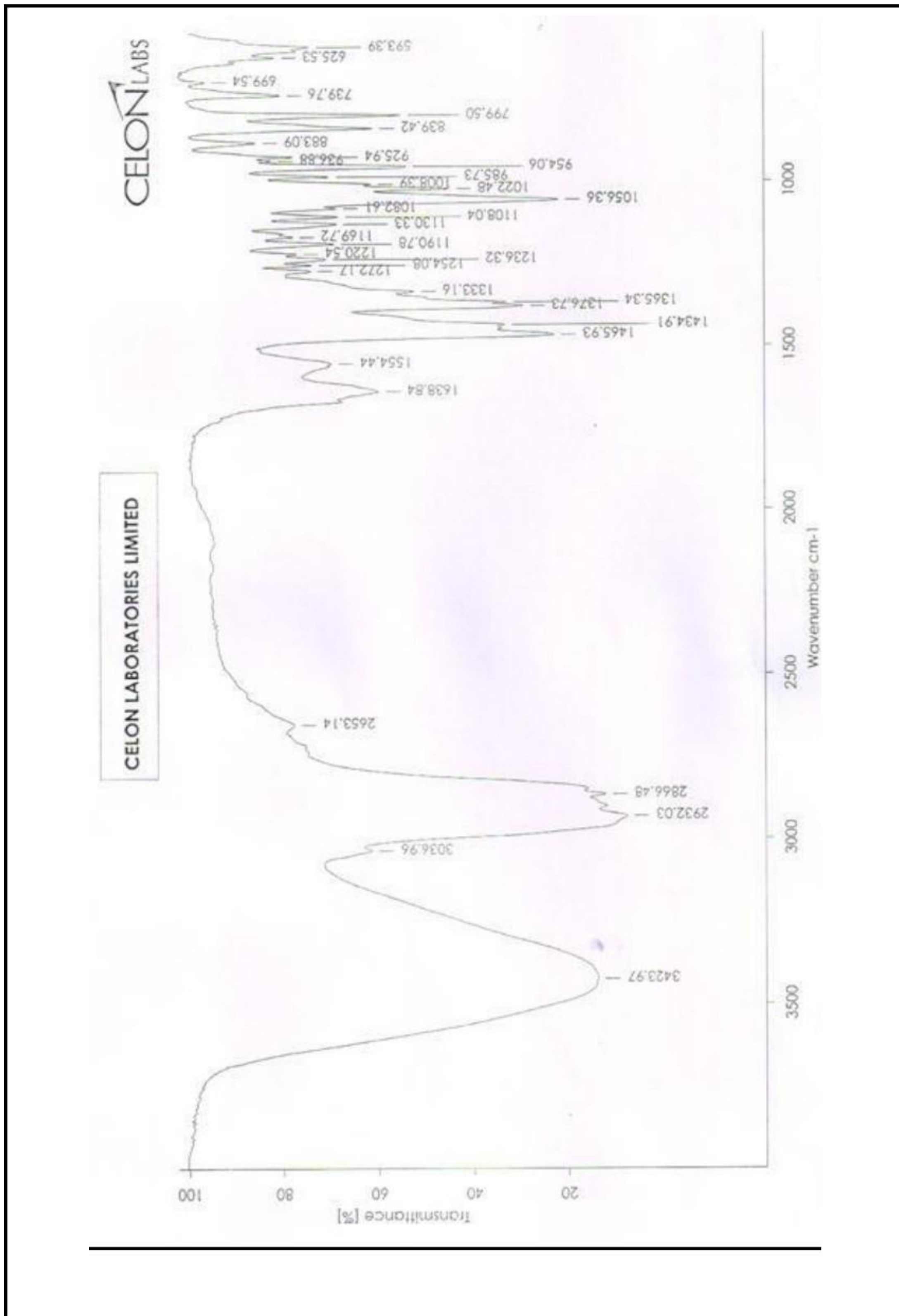
COMPATIBILITY STUDIES: -



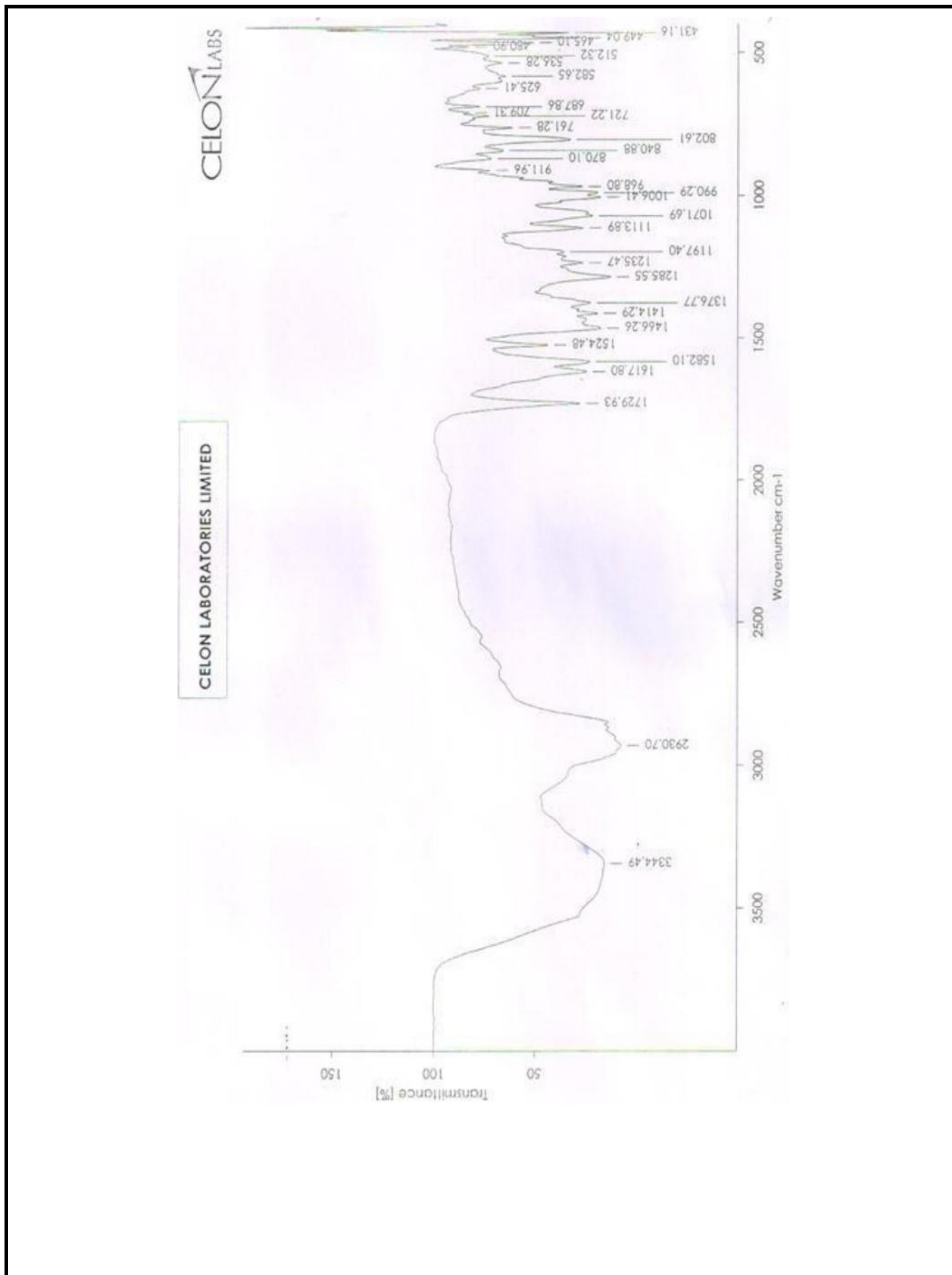
Spectra No: 1 FTIR of Doxorubicin Hcl



Spectra No: 3. 2 FTIR of Soy lecithin



Spectra No: 3 FTIR of Cholesterol



Spectra No3. 4: FTIR of Doxorubicin Hcl, Soy lecithin, Cholesterol, Dicetyl phosphate.

Table :3.2 Interpretations of FTIR Spectra for pure drug.

S. No.	Functional Groups	Assesment Peak of Pure Drug Cm^{-1}	Range of Groups Cm^{-1}
1.	C=C Streching (Aromatic)	1463.86 1524.51	1450 – 1600
2.	O-H Bending (Alcohol)	1072.51	1050 – 1150
3.	C=O Streching	1729.84	1705 – 1735
4.	N-H Bending	1617.83	1500 – 1650
5.	C-O Streching (6-Membered cyclic)	1114.46	1100 – 1120

Table : 3.3 Interpretations of FTIR Spectra for pure drug and Spectra-5

S. No.	Functional Groups	Assesment Peak of Pure Drug Cm^{-1}	Assesment Peak of Spectra-1 Cm^{-1}
1.	C=C Streching (Aromatic)	1463.86 1524.51	1466.26 1524.48
2.	O-H Bending (Alcohol)	1072.51	1071.69
3.	C=O Streching	1729.84	1729.93
4.	N-H Bending	1617.83	1617.80
5.	C-O Streching (6-Membe red cyclic)	1114.46	1113.89

3.1.1 Evaluation of Liposomes

Following parameters were used for the evaluation of liposomes which were listed below:

3.1.2 Physical examination

Liposomes formulation has smooth texture and appeared to be translucent and whitish in colour and results are listed in table no.3.4 .

Table : 3.4 Results of physical appearance of liposomes

S. No	Formulation Code	Appearance	Feel on Application
1.	F-1	Whitish and Translucent	Smooth texture
2.	F-2	Whitish and Translucent	Smooth texture
3.	F-3	Whitish and Translucent	Smooth texture
4.	F-4	Whitish and Translucent	Smooth texture

3.1.3 pH Determination

The pH of all liposomes formulations was found to be in the range of 3.83 to3.90. These pH values showed that formulated gel probably would not produce skin irritation. The conductivities values gel remained stable. Hence, prepared gel is suitable for topical applications. Results were listed in table no.3.5 .

Table : 3.5 Results of pH of all liposomes

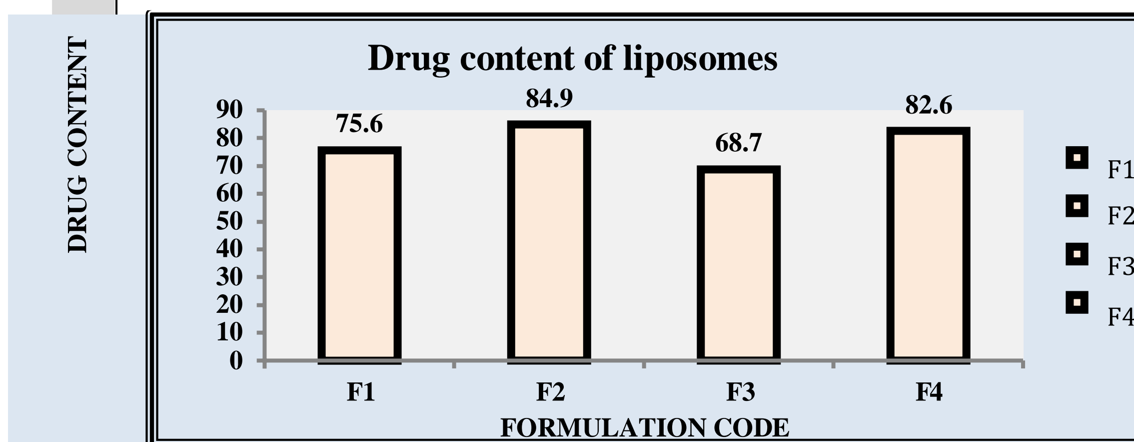
Sr. No.	Formulation Code	pH
1.	F-1	5.83
2.	F-2	5.89
3.	F-3	5.87
4.	F-4	5.90
Mean± S.D		5.87±0.030

Drug Content

Drug content estimation was determined by using UV Spectrophotometer at 294 nm. Drug content of all the formulation was found between 68.7 to 84.9%, which represent uniformity in drug content. Results of drug content estimation depicted in table no.3.6 and figureno.3.5.

Table : Drug content of liposomes.

S.No	Formulations	Drug Content%
1.	**F1**	75.6
2.	**F2**	84.9
3.	**F3**	68.7
4.	**F4**	82.6
Mean±S.D		77.95±6.344



3.1.4 *In vitro* drug release

The percentage in vitro release of liposomes was carried out after 1 hour of interval up to 8 hours. The results were shown graphically represented in figure no. 3.6 and 3.7.

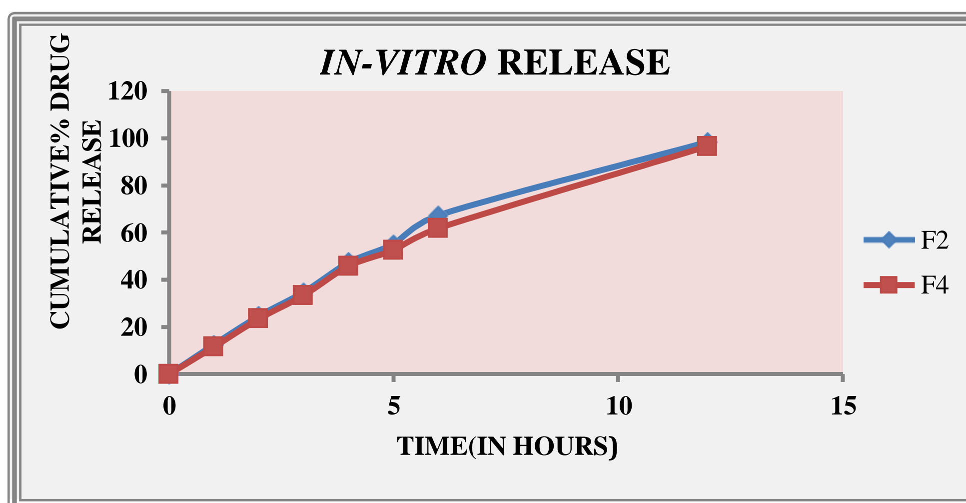


Figure : 3.6 Grahically represenred *in- vitro* release of F-2 and F-4 formulatio**n**

From the above graph, it can be concluded that the drug released form liposomes formulation was characterized by initial burst release in first few hours and later on providing a sustained release drug profile.

Determination of Particle Size and Poly Dispersity Index by the use of Zeta Sizer

The liposomes formulation was characterized for their size Polydispersity index by using Zeta Sizer [Beckman Coulter]. Results of average particle size and Polydispersity index were obtained from instrumental based calculation system. Average particle size of liposomes and polydispersity index was shown in figure no.3.8 ,3.9 and 3.10 and table no.3.7 .

Table: 3.7 Particle Size and Poly Dispersity Index by the use of Zeta Sizer

Sr. No.	Formulation Code	Particle Size	Polydispersity index
1.	F-2	795.3nm	0.303
2.	F-4	1220.1nm	0.281

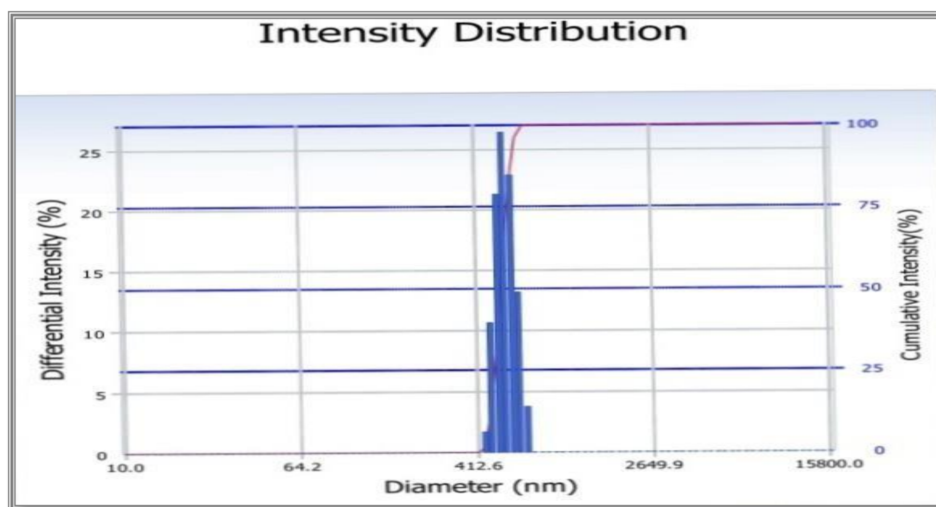


Figure : 3.8 Particle size of liposomes F2) Formulation by Zeta Sizer

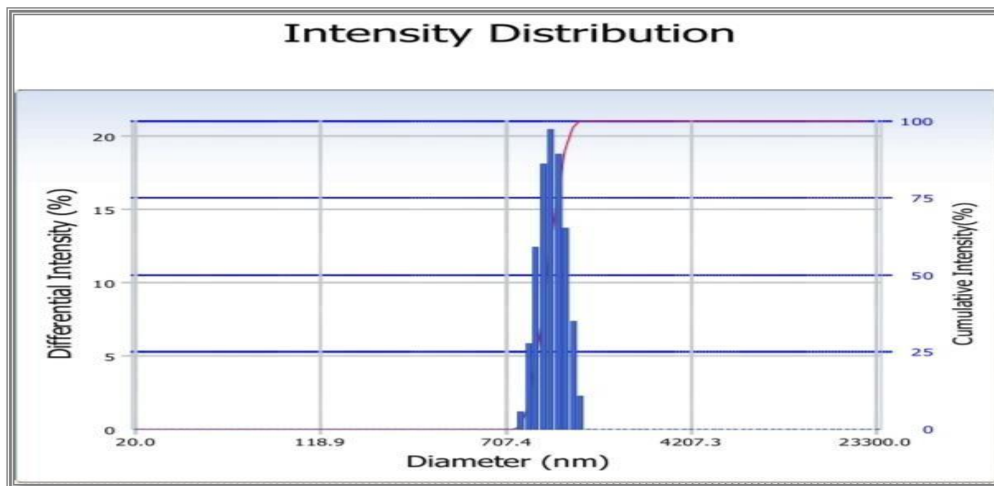


Figure : 3.9 Particle size of liposomes (F-4) Formulation by Zeta Sizer

Morphological Characterization Using Tem Study

The morphology of the liposomes was analysed by the help of the transmission electron microscope [FEI Technai G^2 F20 Netherlands]. The results are shown in figure no.3.11. Most of the particles were spherical with only few irregular shapedparticles.

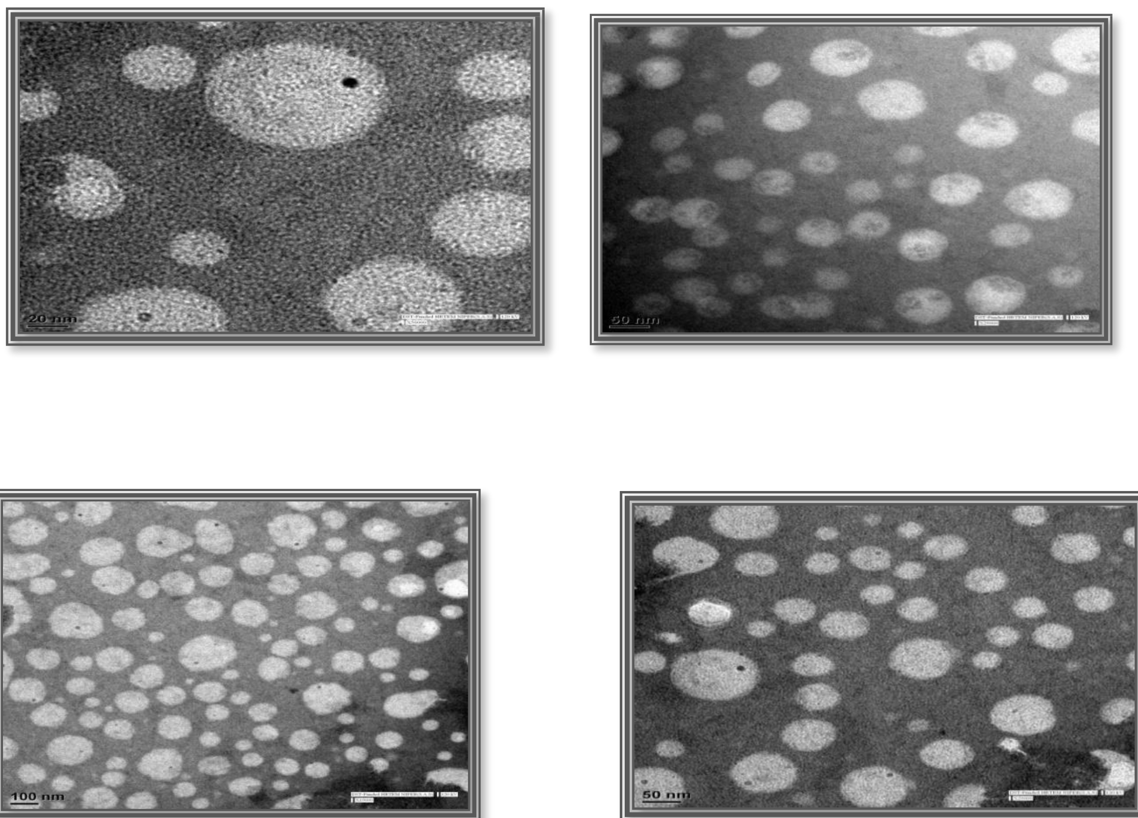


Figure : 3.11 TEM images of (F-2) liposomes Stability Study

3.Stability studies:

The stability studies are very important evaluation parameter and they were performed in order to study whether the formulation can bear changes in temperature, humidity etc. So., stability studies were done for 3 months. The formulation was stable up to 1 months in terms of appearance and pH. But after 2 months, the formulation kept under room temperature and accelerated started to change in physical apperance and pH. The changes in appearance of the formulation at different temperature are shown in figure no.10,11,12 and table no.,11,12,13.The change in pH of the formulation at different temperature are shown in figure no.13,14,15. and table no.14,15,16.

Table: 3.8 Result of liposomes for stability study at room temperature (Physical appearance)

Temperature	Formulation code	Physical appearance		
		(After 1month)	(After 2months)	(After3months)
Room temperature (25°C±2°C)	F-1	No change	Light brown	Light brown
	F-2	No change	No change	No change
	F-3	No change	Light brown	Whitish brown
	F-4	No change	No change	No change

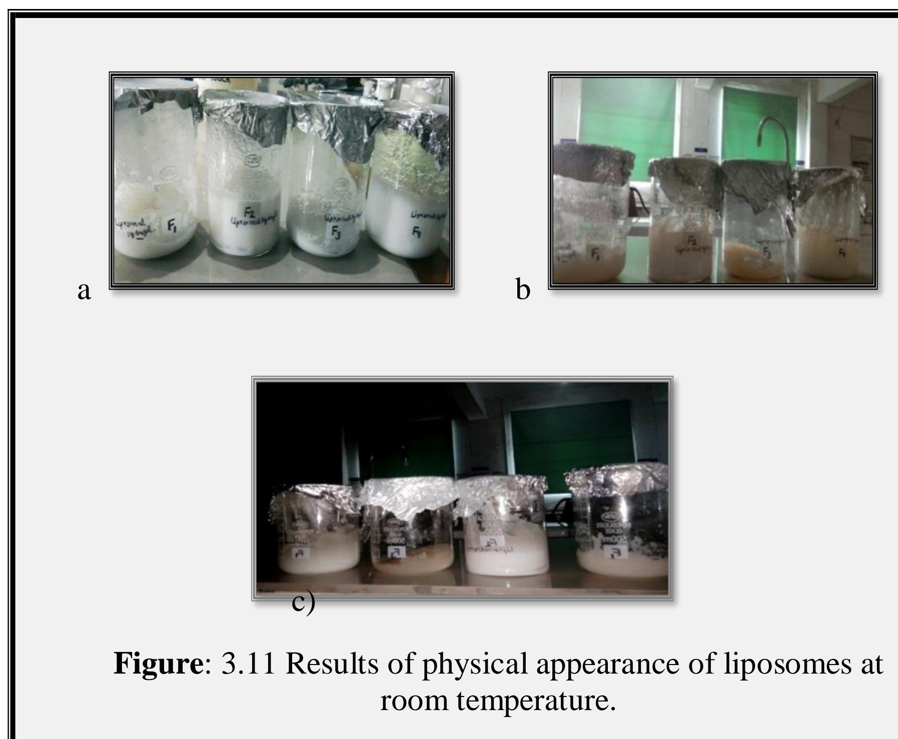


Figure: 3.11 Results of physical appearance of liposomes at room temperature.

Table: Result of liposomes for stability study at refrigerator condition (Physical appearance)

Temperature	**Formulation code**	**Physical appearance**		
		(After 1month)	**(After 2months)**	**(After 3months)**
Refrigerated condition (4°C ±2°C)	F-1	No change	No change	No change
	F-2	No change	No change	No change
	F-3	No change	No change	No change
	F-4	No change	No change	No change

Temperature	Formulation code	Physical appearance		
		(After 1month)	(After 2months)	(After 3months)
Accelerated temperature (40° C±2°C),	F-1	No change	Light yellow	Whitish brown
	F-2	No change	No change	Whitish brown Translucent
	F-3	Light brown	Yellowish	Dark brownish
	F-4	No change	No change	Whitish brown

Temperature	Formulation code	pH		
		(After 1month)	(After 2months)	(After3months)
Room temperature(25°C±2°C)	F-1	3.83	3.63	3.71
	F-2	3.89	3.78	3.75
	F-3	3.87	3.69	4.32
	F-4	3.90	3.79	3.78

Table : 3.12 Result of liposomes for stability study pH at refrigerator condition

Temperature	Formulation code	pH		
		(After 1month)	(After 2months)	(After 3months)
Refrigerated condition(4°C ± 2°C)	F-1	5.43	5.54	5.55
	F-2	5.90	5.91	5.92
	F-3	6.12	6.11	6.13
	F-4	5.90	5.92	5.93

Table : 3.13 Result of liposomes for stability study pH at accelerated temperature

Temperature	Formulation code	pH		
		(After 1month)	(After 2months)	(After 3months)
Accelerated temperature (40° C±2°C),	F-1	5.91	5.90	5.91
	F-2	5.90	5.91	5.93
	F-3	6.11	6.12	6.00
	F-4	5.90	5.91	5.93

According to the above observations it can be concluded that the formulation was most stable at refrigerated conditions as compared to room or accelerated condition. So, refrigerated conditions are optimum for storage of formulations.

CONCLUSION:

The Method used for the preparation of liposomes is Lipid film hydration by hand shaking method. Initially, cholestrol and soya lecithin were weighed and accurately dissolved in proposed ratio of chloroform and methanol(2:1) stirred for two minutes.100 mg of doxorubicin was added to above solution and continue the stirred for 2 minutes [14]. Now, the above solution is subjected for hydration with 10 ml distilled water and added 0.25 ml of Tween 80 and continue the stirring for about one hour for the formation of liposomal vesicles. Finally, the product obtained is collected and stored in a hermetically sealed container for further evaluation studies.

It was concluded that F2 gel formulation was having best having best anti-microbial activity when compared to other prepared formulations. (F1, F3. F4). For future prospective these gel formulations can be good for hydrogel formulation to treat the mouth ulcer and the effect of dosage form can be further studied by carried out *in-vivo* studies[15].

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Author's contributions All the authors have contribute equally.

Conflicts of interests Declared none

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