International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 580-592
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Pharmaceutical Sciences | Open Access | MCI Approved
UGC Approved Journal

Formulation and *In Vitro* Evaluation of Efavirenz Loaded Liposomes

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Received: 8 Oct 2018/ Accepted: 6 Nov 2018/ Published online: 01Jan 2019
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Abstract
Efavirenz having antiviral activity and it is a synthetic non-nucleoside reverse transcriptase (RT) Inhibitor Efavirenz is used in combination with other agents in the therapy of Human Immunodeficiency Virus (HIV) infection. The aim of the study is to prepare 10 formulations of liposomes. liposomes acts as carrier for efavirenz for the treatment of HIV-1 that is capable of delivering the drug to the specific target site by using different ratios of phospholipids and cholesterol with a desired amount of drug by thin film hydration technique and to find out the drug release from the liposome's of different ratios, drug release pattern and also to find out the size distribution of liposome's of different ratios and also to increase the bioavailability and efficacy of the drug.

Keywords
Efavirenz, Hydrogenated soybean phosphotidyl choline, Cholesterol, Dichloromethane.

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INTRODUCTION
Liposomes are microscopic sealed vesicles in which the aqueous compartment is enclosed by one or more phospholipid bilayer. liposomes carry the drug and release the drug at the specific site of action. Phospholipid bilayers of liposomes are hydrophilic and lipophilic drugs can be encapsulated in them. Hydrophilic (water-soluble) drugs get encapsulated in aqueous space. Whereas lipophilic drug molecules get entrapped within the lipid bilayer. Liposomes have proved to be an excellent carries for drugs, as these are biodegradable, inert, devoid of antigenic properties and their bilayers are similar in composition as the biological membranes. Efavirenz is a synthetic non-nucleoside reverse transcriptase (RT) inhibitor with antiviral activity. Efavirenz is used in combination with other agents for disease of human immunodeficiency virus (HIV) infection. The present study includes preparation of efavirenz loaded liposomes by thin film hydration technique. Various parameters also performed like entrapment efficiency, particle size analysis, zeta sizing, diffusion studies, accelerated stability studies. The main aim of present study is to formulate drug release and *in-vitro* evaluation of efavirenz.

Table No.1: Materials and Methods

S.No	Name of Chemical	Category	Supplier
1	Efavirenz	Non – nucleoside reverse transcriptase inhibitor(HIV)	Finer chemicals(LR)
2	HSPC (PHOSPHOLIPID)	Phospholipids	Finer chemicals(LR)
3	Cholesterol	Stabiliser	Finer chemicals(LR)
4	Dichloromethane	Solvent	Finer chemicals(LR)
5	Sodium lauryl sulphate	Surfactant	Finer chemicals(LR)
6	Sodium hydroxide	Neutraliser	Finer chemicals(LR)
7	Potassium dihydrogen phosphate	Buffering agent	Finer chemicals(LR)
8	Methanol	Solvent	Finer chemicals(LR)

Table No.2: List of Instruments

S.No	Name of Equipment	Model	Name of Manufacturer
1	Analytical balance	CPA 225 D	Sartorius , India
2	pH meter	pH system 361	Systronics
3	Micro pipette	-	Pfact
4	Water bath	Thermostat	Bio techniques, Mumbai
5	Dialysis membrane 110	-	Purchased from HIMEDIA laboratories, Hyderabad
6	Diffusion apparatus	-	Locally fabricated
7	UV spectrophotometer(UV Probe software)	UV1800	Schimazu, Tokyo , Japan
8	FT-IR(OPUS Software)	-	Alfa-Bruker
9	Zeta sizer	Nano ZS90	Malvern zeta sizer , UK
10	Malvern zeta sizer , UK	-	Millipore (India) Pvt. Ltd
11	Light microscope	Thermosta	Biotechnique; Mumbai

METHODS

1. Preparation of MLVs by physical dispersion:

a) Hand shaken MLVs method

b) Freeze-drying

c) Method involving pro-Liposomes

2. Preparation of SUVs by physical method:

a) Sonicated SUVS method

-bath type sonicator

-probe sonicator

b) French pressure cell

c) pH induced vesiculation

3. Preparation of liposome's by solvent dispersion methods:

a) Ethanol Injection method

b) Ether Injection method

C) Reverse phase evaporation method

Thin film hydration technique:

- Efavirenz multilamellar liposomal vesicles were prepared by using thin film hydration technique.
- This formulation is prepared by hydrogenated soybean phosphatidylcholine as lipid component with cholesterol.
- Accurately weighed quantities of drug, hydrogenated soybean phosphatidylcholine with cholesterol were transferred to 250ml round bottom flask and dissolved in dichloromethane (10ml).
- A layer of lipid film was formed by evaporating the solvent system under reduced pressure using rotary flash evaporator.
- During this process, the conditions of the instrument such as temperature (40ºC) and speed (100rpm) were kept constant.
- After getting thin film reconstitute the thin film with phosphate buffer- 7.4 we can get the milky white dispersion.
- This milky white dispersion is kept it aside for 2hours we can get the liposomes.

Table No.3: Formulation of liposomes by thin film hydration method

Formulation code	Phospholipid (mg)	Cholesterol (mg)	Dichloromethane (ml)
TLF1	150	50	10
TLF2	140	60	10
TLF3	130	70	10
TLF4	120	80	10
TLF5	110	90	10
TLF6	100	100	10
TLF7	160	40	10
TLF8	170	30	10
TLF9	180	20	10
TLF10	190	10	10

Evaluation of liposomes

Drug entrapment efficiency or drug content:

Drug entrapped within the liposomes was estimated after removing the un entrapped drug, which was separated by collecting the supernatant after subjecting the dispersion to centrifugation in a cooling centrifuge at 1000 rpm at a temperature of 4ºC for 30 minutes. Where upon the pellets of Liposomes were washed again with phosphate buffer to remove un entrapped drug. Then it was analyzed at 246 nm.

%Drug entrapped (PDE) = (amount of drug in sediment/ total amount of drug) X100.

pH:

pH of the formulation is determined by the digital pH meter by immersing the electrode in formulation and checking the pH.

Particle size analysis:

The particle size of liposomes was determined by using scanning electron microscope. Optimized batch of liposomes were viewed under microscope to study their size. Size of liposomal vesicles was measured at different locations on slide by taking a small drop of liposomal dispersion on it and average size of liposomal vesicles was determined.

Zeta Sizing:

The redesigned plan got from the above examination is weakened 1:100 with Millipore water and subjected to zeta assessing using Nano ZS 90 Malvern zeta sizer for choosing the typical atom size and zeta potential using laser scattering with a state of 1800.

Diffusion studies:

The Franz Cell device comprises of two essential chambers isolated by a film, a donor compartment and receptor compartment with sampling port. A dialysis film of grade 110 with atomic weight cutoff of 12000 Dalton and test volume limit of 3.63 ml/cm^3 is utilized for this study. 1cm^2 bit of layer is cut and absorbed overnight in buffer of pH 1.2 and are utilized the following day.

The receptor compartment is loaded with buffer and layer is attached onto its surface to such an extent that it covers the opening of compartment and touches the buffer solution. At that point contributor compartment is set above and clasped took after by test expansion, the whole get together is set on a magnetic stirrer, temperature is then set to 37^{0}C with speed of 30 rpm. Intermittently 1ml samples are pulled back and supplanted with measure up to volume of support for consistently. With drawn samples are drawn and afterward analyzed spectrometrically in UV spectrophotometer at a wavelength of 246 nm and computed for Cumulative drug discharge.

RESULTS AND DISCUSSION

Table No.4: Physical characterization of API's

Description	Efavirenz
Color	White
Morphology	Crystalline

Table No.5: Solubility studies of Efavirenz

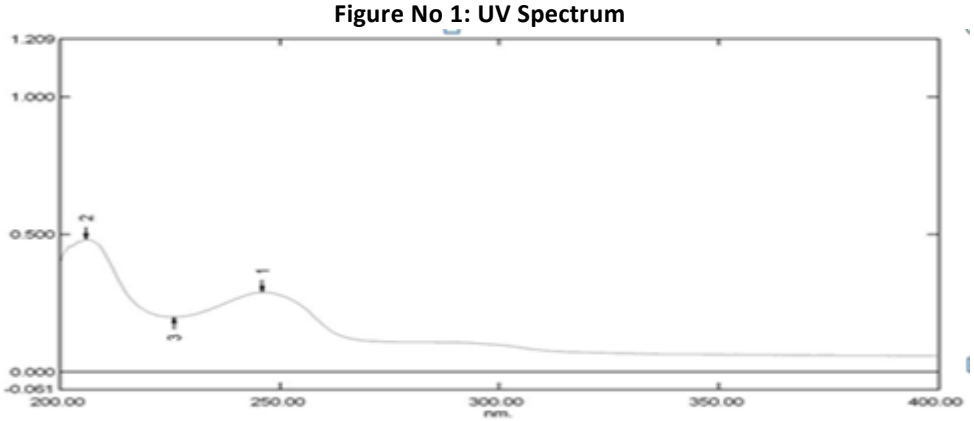
S.No	Solubility	Solvent
1	Water	Insoluble
2	Dichloromethane	Freely soluble
3	Chloroform	Freely soluble
4	Phosphate buffer pH -7.4	Soluble

Efavirenz standard graph:

Determination of standard graph of Efavirenz in pH-7.4:

The spectrum shows maximum absorption at 246 nm.

Figure No 1: UV Spectrum

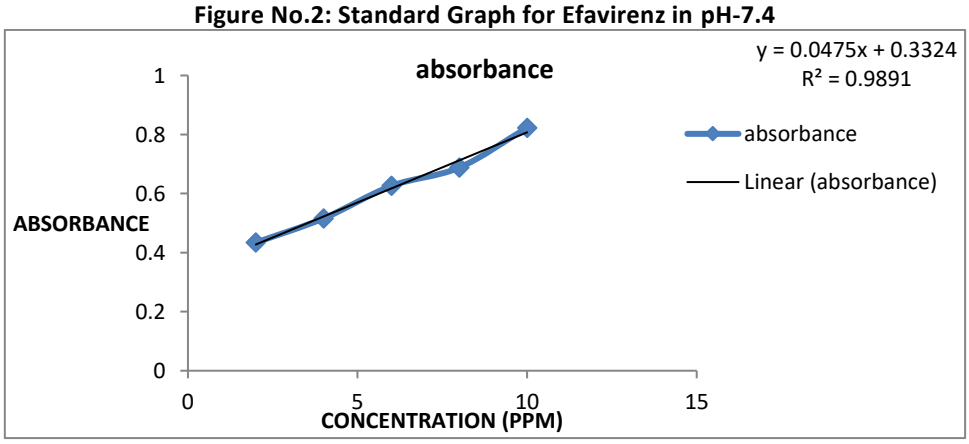


Efavirenz standard graph in pH-7.4 phosphate buffer: Absorption and concentrations are plotted to obtain the standard graph with regression co-efficient 0.989

Table No.6: Efavirenz standard graph in pH-7.4 phosphate buffer:

Concentration (ppm)	Absorbance
2	0.434
4	0.516
6	0.626
8	0.688
10	0.823

Figure No.2: Standard Graph for Efavirenz in pH-7.4



FTIR Studies:

The interaction examines between the medication and Excipients and additionally enhanced detailing was assessed utilizing IR spectrophotometer. Comparable peaks were seen in spectra of various combinations of Excipients and in improved plan (Liposomes), alongside nonappearance of interfering peaks showing there is no undesirable response between Efavirenz and different Excipients utilized as a part of the investigation.

Figure No.3: Efavirenz FT-IR spectrum

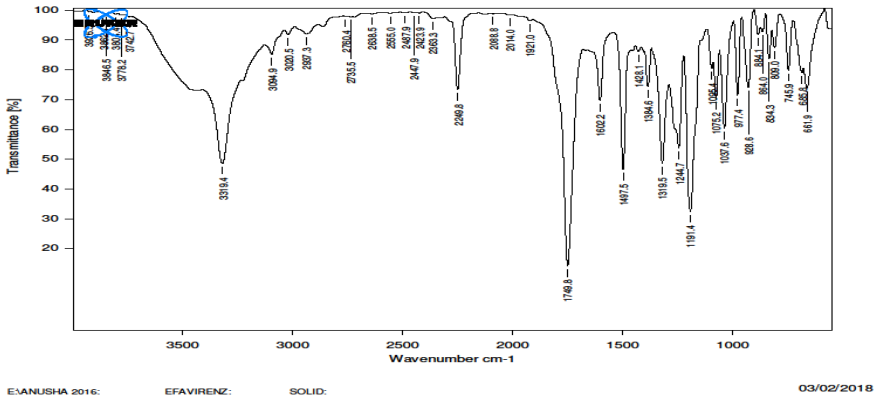


Figure No.4: Cholesterol FTIR spectrum

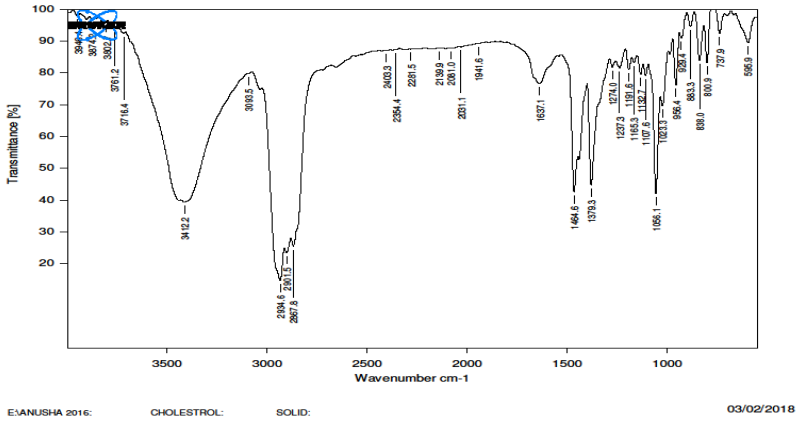


Figure No.5: Hydrogenated soybean phosphotidyl choline FTIR spectrum

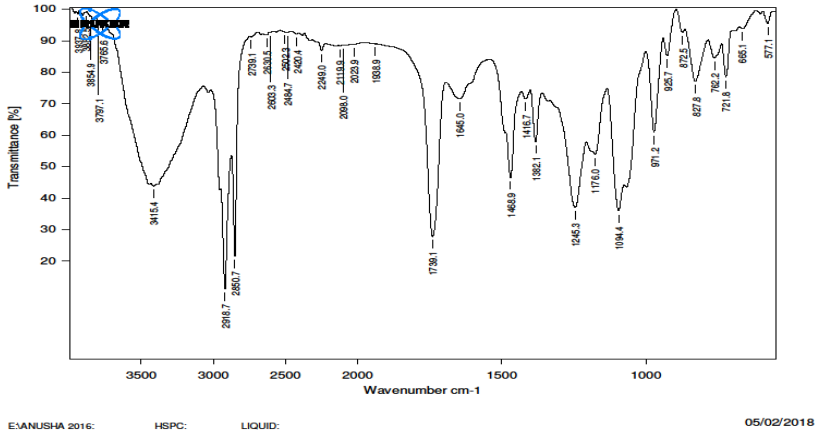


Figure No.6: FT-IR over lay of a) Efavirenz + cholesterol b) Efavirenz

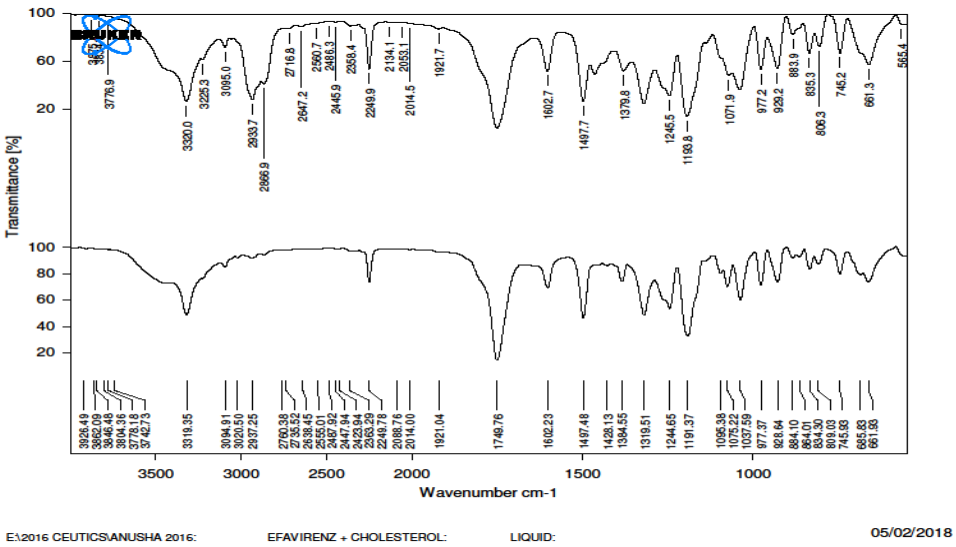


Table No.7: Functional group detection

Efavirenz	Efavirenz + Cholesterol	Functional groups
2249.8	2249.9	C=C stretching bond of alkynes
2014.0	2014.5	Alkynes
1497.5	1497.7	Benzene ring
1191.4	1193.4	Polysaccharides

Figure No.7: FT-IR over lay of a) Efavirenz + phospholipid b) Efavirenz

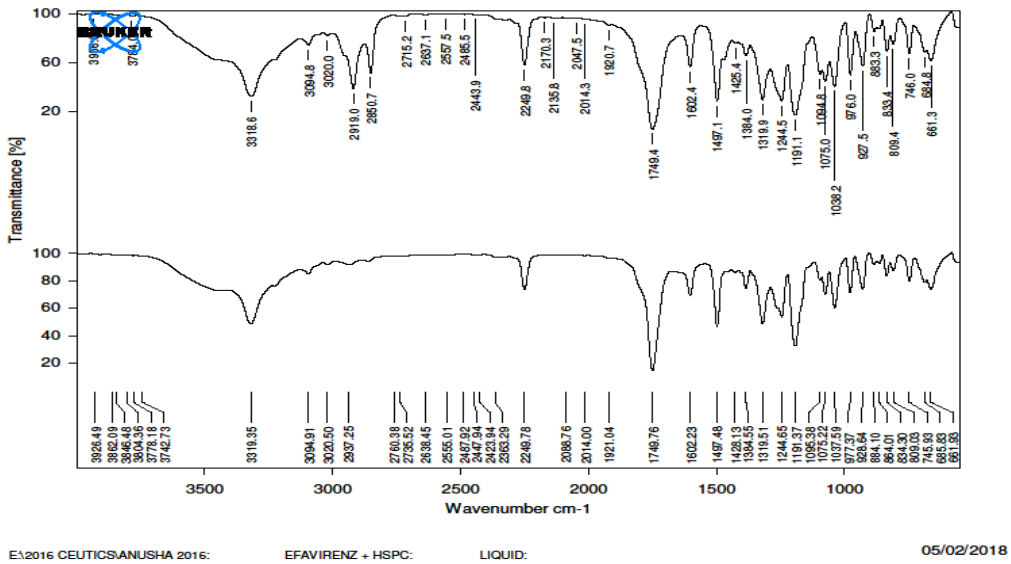


Table No.8: Functional group detection:

Efavirenz	Efavirenz + HSPC	Functional groups
3778.2	3784.6	Alkynes
3319.4	3318.6	Alkyne
3094.9	3094.8	Carboxylic acid O-H
3020.5	3020.0	Alkene

Figure No.8: FT-IR over lay of a) Efavirenz + Dichloromethane b) Efavirenz

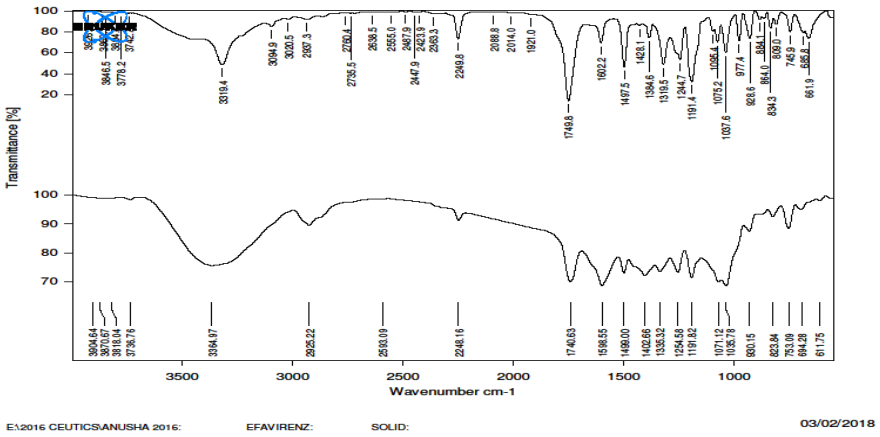
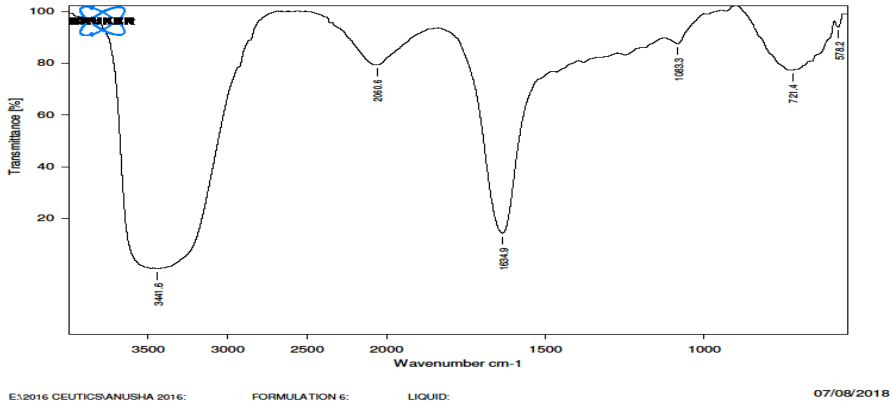


Table No.9: Functional group detection

Efavirenz	Efavirenz + Dichloromethane	Functional groups
1191.4	1191.8	Phosphine oxide
2249.8	2248.7	C=CStretching bond of alkynes molecules
1497.5	1499.0	Benzene ring
1749.8	1740.6	Ester

Figure No.9: FT-IR of optimized liposome formulation (TLF-6)



Drug entrapment and % entrapment efficiency of the formulations:

Drug entrapped within the liposome's was estimated after removing the un entrapped drug, which is separated by collecting the supernatant solution after subjecting the dispersion to centrifugation in a cooling centrifuge at 1000 rpm at a temperature of 4°C for 30 minutes. Where upon the pellets of Liposomes were washed again with phosphate buffer to remove un entrapped drug. Then it was analyzed at 246 nm.

%Drug entrapped (PDE) = (amount of drug in sediment/ total amount of drug X 100)

Table No.10: Drug entrapment and % entrapment efficiency of the formulations

S.No	Formulation Code	Drug entrapment	% Entrapment efficiency
1	TLF1	9.7±0.04	97%
2	TLF2	9.6±0.05	96%
3	TLF3	9.8±0.04	98%
4	TLF4	9.7±0.03	97%
5	TLF5	9.5±0.01	95%
6	TLF6	9.9±0.03	99%
7	TLF7	9.5±0.01	95%
8	TLF8	9.4±0.05	94%
9	TLF9	9.3±0.04	93%
10	TLF10	9.5±0.02	95%

Liposomes diffusion studies:

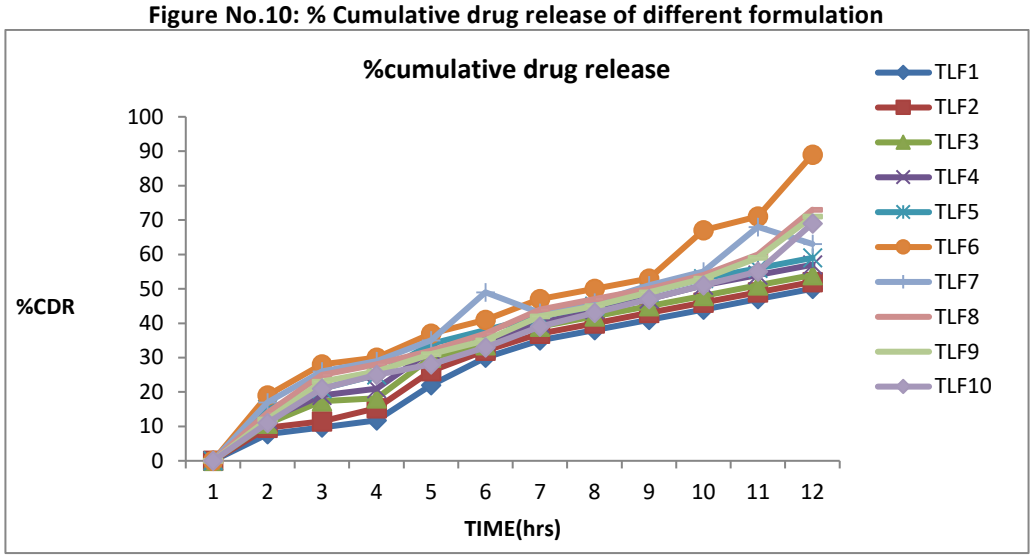
Diffusion studies are conducted for various formulations containing varying concentrations of Phospholipids and cholesterol in pH -7.4 phosphate buffer. In case of Liposomes dispersions release action within 12hours. The various formulation codes along with cumulative drug release values are given in table and fig below.

Table No.11: % Cumulative drug release values

S.No	% Cumulative Drug Release									
	TLF1	TLF2	TLF3	TLF4	TLF5	TLF6	TLF7	TLF8	TLF9	TLF10
0	0	0	0	0	0	0	0	0	0	0
1	7.8±0.91	9.6±0.17	10.8±0.23	12±0.87	15±0.35	19±0.23	17±0.56	14±0.12	12±0.12	11±0,33
2	9.7±0.53	11.5±0.47	17.3±0.39	19±0.45	21±0.24	28±0.54	26±0.76	25±0.32	23±0.14	21±0.43
3	11.8±0.22	15.3±0.82	18.2±0.54	21±0.34	25±0.56	30±0.76	29±0.45	28±0.23	26±0.23	25±0.12
4	22±0.86	26.1±0.47	30±0.68	32±0.56	34±0.34	37±0.89	35±0.24	32±0.56	31±0.45	28±0.75
5	30±0.45	32±o.17	33.6±0.33	35±0.74	38±0.95	41±0.56	49±0.54	37±0.53	35±0.76	33±0.87
6	35±0.73	37±0.47	39±0.37	40±0.53	42±0.4	47±0.45	43±0.43	44±0.56	42±0.56	39±0.96
7	38±0.66	40±0.33	42±0.65	44±0.12	45±0.76	50±0.34	46±0.32	47±0.7	45±0.34	43±0.85
8	41±0.46	43±0.5	45±0.98	47±0.16	49±0.86	53±0.65	51±0.12	50±0.34	49±0.13	47±0.45
9	44±0.82	46±0.23	48±0.67	51±0.25	53±0.45	67±0.74	55±0.11	54±0.74	53±0.23	51±0.47
10	47±0.33	49±0.34	51±0.45	54±0.56	56±0.46	71±0.78	68±0.34	60±0.76	59±0.43	55±0.7
11	50±0.66	52±0.33	54±0.76	57±0.43	59±0.34	89±0.12	63±0.45	73±0.87	71±0.14	69±0.96
12	55±0.33	58±0.25	61±0.56	67±0.45	73±0.54	96.08±0.24	85±0.56	80±0.89	79±0.15	75±0.45

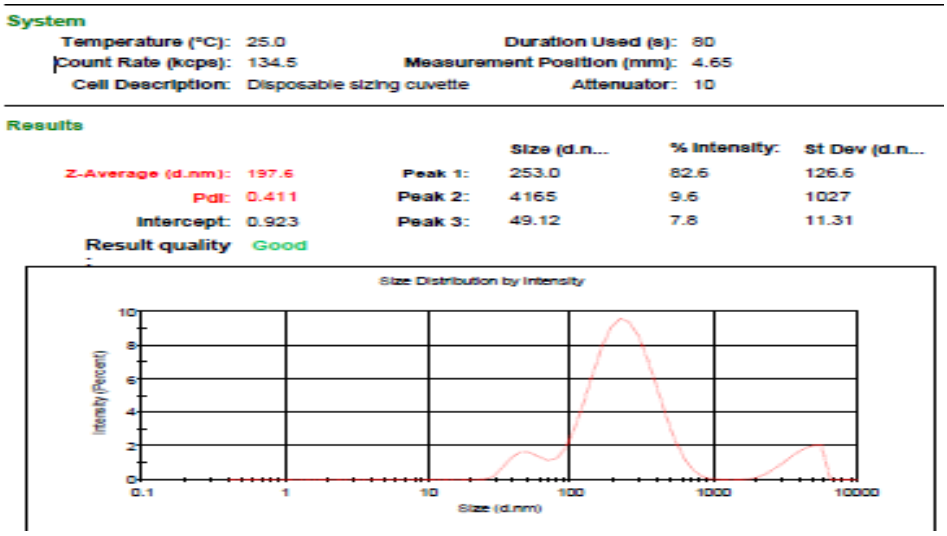
Values in mean of cumulative % drug release ± Standard deviation (n=3)

Figure No.10: % Cumulative drug release of different formulation



Droplet size determination:

From the diffusion study it was concluded that the formulation TLF6 showed more drug release than other formulation. So it is selected as the best formulation. Size and potential values are determined for the formulation. Size of the particles was found to be197 nm and PDI value was found to be 0.411 and its zeta potential was found to be -23.1mv.



Zeta potential determination:

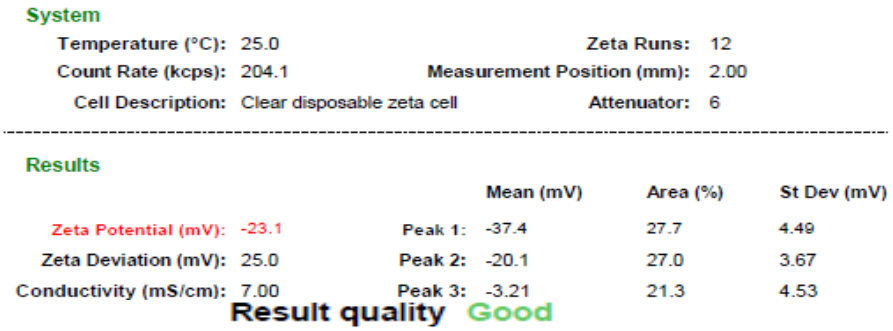
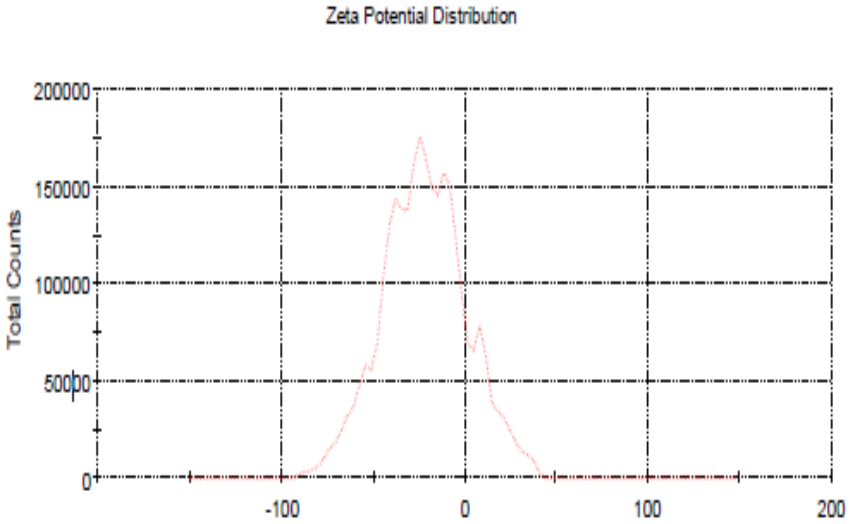
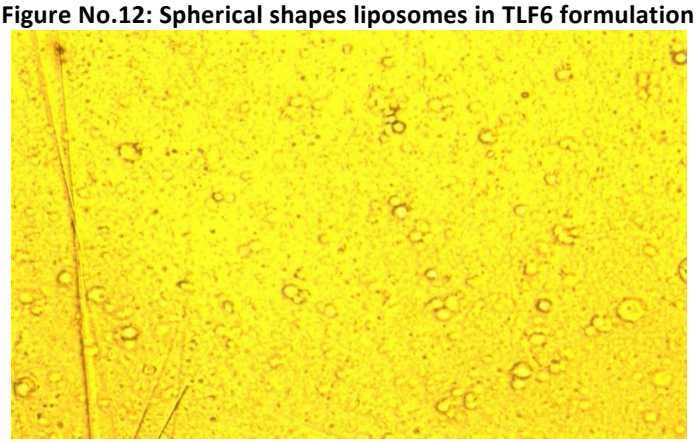


Figure No.11: Zeta potential of TLF6 formulation



Microscopy:
At a magnification of up to 100x, liposomes are clearly visible. Spherical like structures are evident in optimized formulation. (Multilamellar liposomes size range from 100nm-10 µm)

Figure No.12: Spherical shapes liposomes in TLF6 formulation



Kinetics of drug release:
Mathematical modeling of % drug release in-vitro diffusion for optimized formulation

Table No.12: Kinetics of drug release:

Time (hrs)	% CDR	%cumulative drug remaining	square root time	log %cumulative drug remaining	log time	log cumulative % drug released	cube root of % drug remaining (wt)	wo-wt
0	0	100	0	2	0	0	4.64	0
1	19	81	1	1.90	0	1.27	4.32	0.32
2	28	72	1.41	1.85	0.30	1.44	4.16	0.48
3	30	70	1.73	1.84	0.47	1.47	4.12	0.52
4	37	63	2	1.79	0.60	1.56	3.97	0.67
5	41	59	2.23	1.77	0.69	1.61	3.89	0.75
6	47	53	2.44	1.72	0.77	1.67	3.75	0.89
7	50	50	2.64	1.69	0.84	1.69	3.68	0.96

Time (hrs)	% CDR	%cumulative drug remaining	square root time	log %cumulative drug remaining	log time	log cumulative % drug released	cube root of % drug remaining (wt)	wo-wt
8	53	47	2.82	1.67	0.90	1.72	3.60	1.04
9	67	33	3	1.51	0.95	1.82	3.20	1.44
10	71	29	3.16	1.46	1	1.85	3.07	1.57
11	89	11	3.31	1.04	1.04	1.94	2.22	2.42
12	93	7	3.46	0.84	1.07	1.96	1.91	2.73

Figure No.13: Zero Order graph

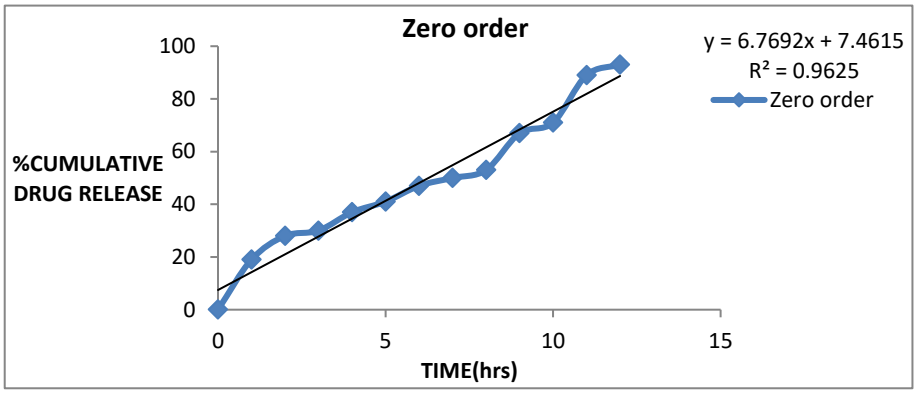


Figure No.14: First Order graph

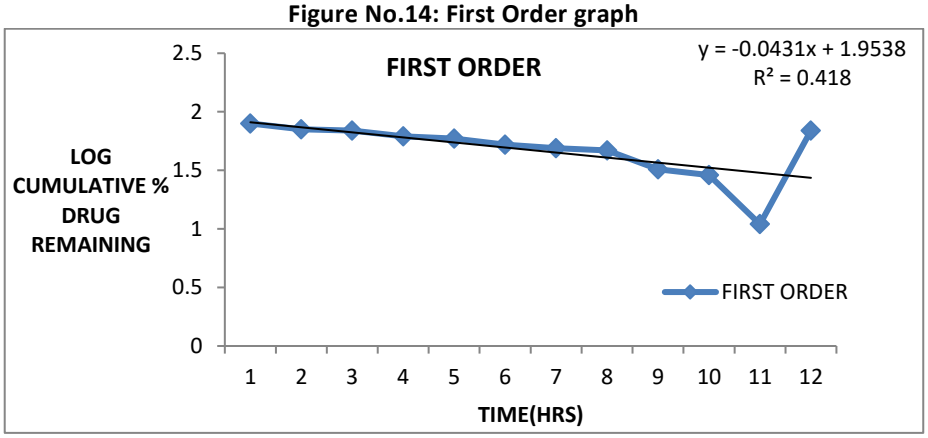


Figure No.15: Kors Peppas Graph

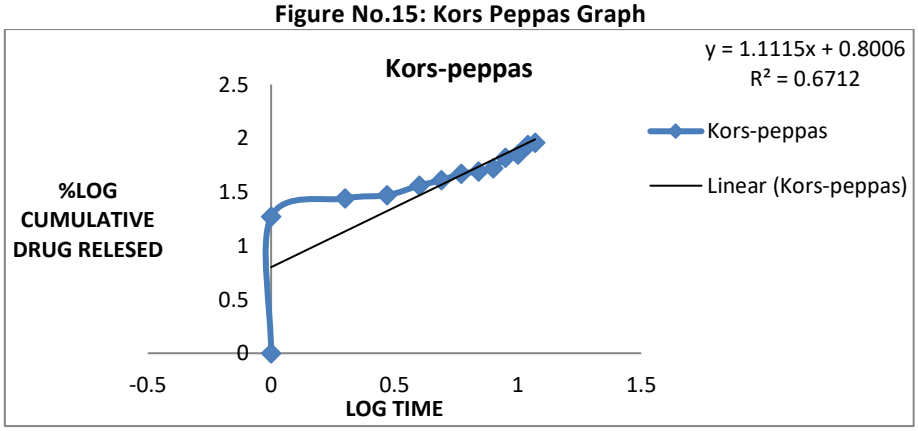


Figure No.16: Hixson Graph

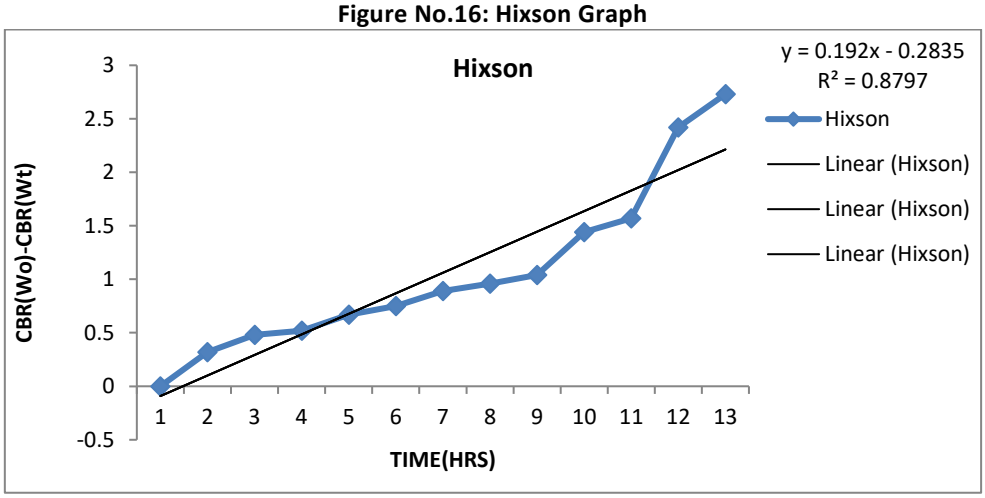
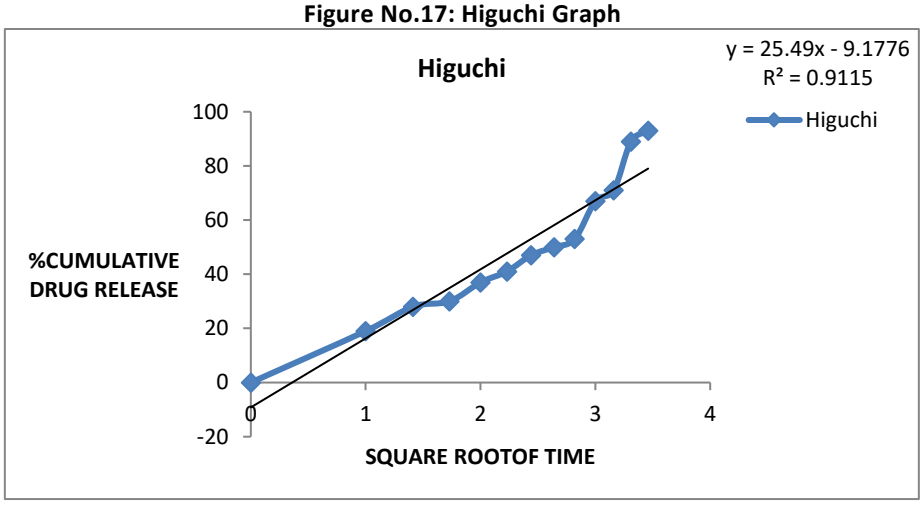


Figure No.17: Higuchi Graph



a, b, c and d: Drug release from optimized liposomes formulation (TLF6).

Table No.13: Results of standard deviation kinetic models:

Kinetic model	r^2 values
ZERO ORDER	0.962
FIRST ORDER	0.418
KORSMAEYER-PEPPAS	0.6712
HICKSON CROWEL	0.8797
HIGUCHI	0.9115

Based on the above results it is inferred that liposomes formulation (TLF6) follows zero order kinetics with anomalous/non -fickian diffusion (swell able and cylindrical matrix).

Table No.14: pH of optimized formulation THF6:

Time (days)	pH of the THF6
0	7.4
30	7.2
60	7.0
90	6.8
120	6.8

Figure No.18: pH of the optimized formulation

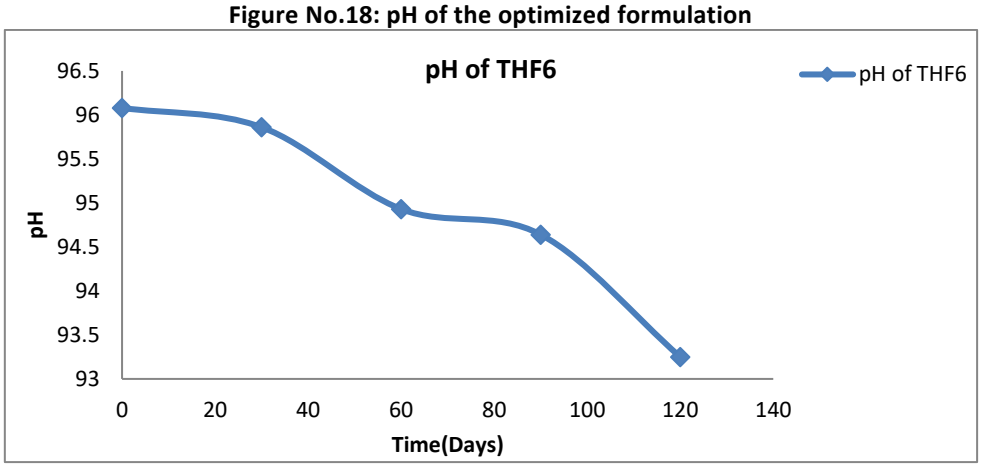


Table No.15: Stability studies of the optimized formulation

% DRUG RELEASE	TIME (days)				
	0	30	60	90	120
THF6	96.08±0.24	95.86±0.25	94.93±0.04	94.64±0.57	93.25±0.65

Values in mean of cumulative drug release ± standard deviation (n=3) Minor changes in parameters are observed confirming the stability of selected optimized formulations.

SUMMARY AND CONCLUSION

The different formulations of liposome's containing Efavirenz were prepared by using thin film hydration method. The percentage entrapment efficiency was determined and maximum entrapment efficiency was found to be 99%.

From FTIR spectra of the drug and physical mixture it was found that there is no significant interaction. Zeta potential analysis was done for optimized formulation TLF6. Average zeta potential and charge on the liposome was determined. The value was -23 Mv which indicates that the surface of Liposomes is dominated by the anions and proved that prepared liposome have sufficient charge to avoid aggregation of vesicles.

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