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Preparation and evaluation of liposome formulations for poorly soluble drug itraconazole by complexation

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ABSTRACT

Liposomes are potential carriers for targeting and controlled drug delivery by the intravenous route. Beta cyclodextrin and Hydroxy propyl beta cyclodextrin inclusion complexes with Itraconazole were prepared by kneading method and these complexes were incorporated in the aqueous phase of the liposomes to prepare Itraconazole liposomes. Factors such as ratio of lipids employed, drug:lipid ratio, etc were fine tuned and optimized to achieve maximum entrapment of the Itraconazole in the aqueous phase. In the present work liposomes are prepared by method called Solvent Injection Method. The prepared liposomes are characterized by optical microscopy, Scanning electron microscopy, particle size determination, encapsulation efficiency and also evaluated by using FTIR spectroscopy and in-vitro diffusion studies by using dialysis membrane. The increase in the solubility of Itraconazole with cyclodextrin complexes in comparision with plain drug is an indubitable advantage of this approach.

Key words: Liposomes, Antifungal, Itraconazole, Poorly soluble, Cyclodextrins

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INTRODUCTION

Itraconazole (ITR) is a triazole antifungal agent used in the treatment of superficial and systemic fungal infections. The drug is given orally and highly lipophilic with oral bioavailability of 55% [1]. Itraconazole has a broader spectrum of activity than fluconazole (but not as broad as voriconazole or posaconazole) [2]. In particular Itraconazole is active against *Aspergillus*, where as fluconazole is not active against *Aspergillus*. ITR is licensed for use in blastomycosis, histoplasmosis, onychomycosis and sporotrichosis. Itraconazole is 99% protein bound and has virtually no penetration into cerebrospinal fluid. Because of this it should never be used to treat meningitis or other central nervous system infections [3]. According to the Johns Hopkins Abx Guide, ITR has negligible Cerebro Spinal Fluid penetration, however treatment has been successful for cryptococcal and coccidioidal meningitis [4]. Itraconazole is also prescribed for systemic infections, such as aspergillosis, candidiasis and cryptococcosis, where other antifungal drugs are inappropriate or ineffective.

Fungal infections always remain as a significant cause of morbidity and mortality in spite of advances in medicinal chemistry. Antifungal drugs are identified into three classes of natural products griseofulvin, polyenes and echinocandins [5-7] and four classes of synthetic chemicals namely azoles, allylamines, flucytosine and phenylmorpholines [8-11] according to clinical data against fungal infections. The azoles class of antifungal agent is chemically either an imidazole or a triazole group joined to an asymmetric carbon atom as their functional pharmacophore and work by blocking the active site of an enzyme variously known as lanosterol 14α-demethylase or cytochrome P450DM [12] . The affinity for this P450 and the activity of the azole antifungals is not only determined by the affinity of the nitrogen for the heme iron, but also by that of the N-l substituent for the apoprotein moiety of P450 [13] . This affinity for the apoprotein not only determines the activity of the azole antifungal, but also determines its selectivity. The remaining part of the azole antifungal fits in the similar way like lanosterol in hydrophobic groove by interacting with Met-313 and the P-methyl group of Thr-318 [14] .

Liposomes are the small vesicle of spherical shape that can be produced from cholesterols, non toxic surfactants, glycolipids, long chain fatty acids, sphingolipids and even membrane proteins [15]. Liposomes were first described by British haematologist Dr Alec D Bangham FR Sin 1961 at the Babraham Institute, in Cambridge [16]. Because of unique bilayer-structure properties liposomes are used as carriers for both lipophilic and water-soluble molecules whereas hydrophilic substances are encapsulated in the interior aqueous compartments. Lipophilic drugs are mainly entrapped within lipid bilayers and their sub-cellular size allows relatively higher intracellular uptake than other particulate systems; improving in vivo drug bioavailability [17]. Drug targeting can also be achieved by physical, biological or molecular systems that result in high concentrations of pharmaceutically active agents at the targeted site to prolong localize and have protected drug interaction with diseased tissue [18-21]. Hence, in the present study, it is planned to increase the bioavailability of the Itraconazole by using cyclodextrin complexes. Liposomes are prepared by solvent injection method. The prepared liposomes are characterized by optical microscopy, scanning electron microscopy, particle size determination, encapsulation efficiency, FTIR spectroscopy studies and in vitro diffusion studies using dialysis membrane.

MATERIALS AND METHODS

Materials

Itraconazole was a gift sample from M/s Therdose pharma private limited. Soya lecithin, Cholesterol and dialysis membrane-50 (Molecular Weight. cut off 12000 to 14000) was obtained from M/s. Finar Chemicals Limited, Ahmedabad. Chloroform, methanol, were obtained from M/s. Sisco Research Laboratories Pvt. Ltd. Andheri (E), Mumbai. Beta cyclodextrin and Hydroxy propyl beta cyclodextrin were obtained from Yarrow chemicals, Mumbai. All other materials used in this study were of analytical grade.

Phase solubility studies

Phase solubility studies were carried out according to the method of Higuchi and Connors. Excess amount of Itraconazole (70 mg) was added to 10 mL of deionized water containing increasing amounts of β-CD and HPβ-CD (ranging from 0 to 2.0 mM). The resulting mixture was equilibrated by placing the flasks on the rotary shaker at room temperature for 72 h. To minimize the photochemical degradation the prepared flasks were covered with aluminum foil. Then, suspensions were filtered through using a 0.45 µm millipore filter to remove undissolved solid. An aliquot from each vial was adequately diluted and spectrophotometrically analyzed at 255 nm. Shaking was continued until three consecutive experiments yielded similar results. The apparent stability constant (*K*c) of the complexes were calculated from the phase-solubility diagrams according to the following equation

$$K_c = \frac{\text{Slope}}{S_0(1 - \text{Slope})}$$

Where S_0 is the solubility of Itraconazole at 30°C in the absence of CD and slope means the corresponding slope of the phase-solubility diagrams, i.e. the slope of the drug molar concentration versus CDs molar concentration graph.

Formation and evaluation of Itraconazole cyclodextrin inclusion complexes

The Itraconazole complexes were prepared by kneading method. Kneaded (KN) product was obtained by triturating equimolar quantities of ITR with β-CD and HP β-CD of required molar ratios in a mortar with a small volume of solvent blend of water: methanol: dichloromethane at a volume ratio of 2:5:3. During this kneading process few drops of solvent were introduced to maintain a suitable consistency. The resulting mass was dried in an oven at 55˚C until they get dry and the solid was finally grounded and then sifted through #100 sieve [22].

Dissolution of Itraconazole-Cyclodextrin complexes

20 mg of pure drug and each complex is taken and evaluated for its Dissolution profile to check which complex gives the better solubility by using USP Apparatus 2 (Paddle Type) for 1 hour at the temperature 37±0.5˚C in the buffer 0.1N Hcl solution. 5mL of sample is taken for 10, 20, 30, 45 and 60 min and is replaced with 5 mL of Buffer. Then the absorbance is checked at 255nm by using Elico SL-191 UV-Visible spectrophotometer using 0.1N Hcl as blank.

Preparation of Itraconazole Liposomes

Liposomes were prepared by Solvent Injection Method using different formulations as shown in Table 1 and here drug-CD complexes are used which are prepared by Kneading method. In a beaker 200mg of Lecithin and 100mg of Cholesterol were weighed and dissolved in 10mL of chloroform (Lipid phase). In another beaker 20 mg of drug-CD complex is dissolved in 10mL of methanol and to this 10 mL of buffer is added (Aqueous phase). The beaker with aqueous phase is kept on for stirring at 200 rpm on thermostatically controlled magnetic stirrer (Remi Magnetic Stirrer, Model: LBMS-5886) at a temperature of 45 ºC. The beaker with lipid phase was also kept aside on the

magnetic stirrer to attain 45˚C temperature. To this aqueous phase at 45˚C lipid phase which is also at 45˚C was added by injection at one jet. The mixture was continued for stirring for 1 hour to obtain uniform vesicular dispersion. Finally the liposome dispersion was stored in airtight container at 2-8°C.

Table 1: Composition of Liposomal formulation

Formulation	INGREDIENTS				
	Lecithin (mg)	Cholesterol (mg)	Chloroform (mL)	Buffer (mL)	Complex (mg) (Drug:βCD/ HPβCD)
F1 (βCD)	200	100	10	10	20 (1:0.5)
F2 (βCD)	200	100	10	10	20 (1:1.0)
F3 (βCD)	200	100	10	10	20 (1:1.5)
F4 (βCD)	200	100	10	10	20 (1:2.0)
F5 (HPβCD)	200	100	10	10	20 (1:0.5)
F6 (HPβCD)	200	100	10	10	20 (1:1.0)
F7 (HPβCD)	200	100	10	10	20 (1:1.5)
F8 (HPβCD)	200	100	10	10	20 (1:2.0)

Characterization of Liposomes

Optical microscopy

The prepared Itraconazole liposomes were viewed under for observing the vesicle formation and discreteness of dispersed vesicles. A slide was prepared by placing a drop of liposome dispersion on a glass slide and cover slip was placed over it and this slide was viewed under optical microscope at 40X magnification. Photographs were taken to prepared slides using digital camera.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to characterize the surface morphology of the prepared vesicles. One drop of liposomal dispersion was mounted on a clearglass stub, air-dried, coated with Polaron E 5100 sputter coater (Polaron, Watford, United Kingdom), and visualized under a scanning electron microscope (Leo-435 VP; Leo, Cambridge, United Kingdom

Particle size determination

The particle size determination is done by using Horibu nano particle analyser and the method used here was Dynamic Light Scattering method. Dynamic Light Scattering (DLS also known as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering) is one of the most popular light scattering techniques because it allows particle sizing down to 1 nm diameter. The basic principle is simple: The sample is illuminated by a laser beam and the fluctuations of the scattered light are detected at a known scattering angle θ by a fast photon detector.

Particle size determination of liposomes was done by using Optical microscope. Determination of particle size as mean diameter is based on direct observation under microscope. The procedure includes 2 steps:

Calibration of eye-piece micrometer

The eye piece micrometer contains 100 divisions. The determination of actual length of each division is known as calibration. This is done in comparison with the standard stage micrometer. The number of divisions of eye piece micrometer (x) matching equally with the number of divisions in stage micrometer (y) was noted. Each division of stage micrometer is equal to 0.01 mm or 10 micrometer. Now one division of eye piece micrometer is equal to number of divisions of stage micrometer (y) divided by number of divisions of eye piece micrometer.

Measurement of globule size

A droplet of liposome formulation was mounted on glass slide and placed on mechanical stage of microscope then the globule diameter was measured and recorded for 100 globules and average particle size was determined.

Drug entrapment efficiency

The drug entrapment efficiency was calculated using the total drug content of liposome dispersion and unentrapped drug content of the dispersion. The total dug content of the dispersion is determined estimating total drug entrapped and unentrapped. 5 ml of liposome dispersion was taken in a volumetric flask. The dispersion was subjected to sonication in bath sonicator (M/s. Remi) for 30 minutes. Then the mixture was filtered and estimated after suitable dilution at 280 nm wavelengths by using UV Visible Spectrophotometer (Shimadzu, UV1800). For the free unentrapped drug, 5 ml of the liposome dispersion subjected to centrifugation at 18000 rpm using Remi centrifuge for 40 min at 50 C. The supernatant clear solution was collected separately and the free drug present in the supernatant was estimated after suitable dilution at 255 nm wavelength by using UV Visible Spectrophotometer. The entrapment efficiency of all the formulation was calculated by using following formula.

$$\% \text{ Entrapment Efficiency} = 1 - \frac{\text{Unentrapped drug content}}{\text{Total drug content}} \times 100$$

In vitro diffusion studies

In vitro diffusion studies were carried by using Franz diffusion cell appratus. The capacity of the receptor compartment was 20 ml and the area of the donor compartment exposed to receptor compartment was 1.41cm^2. Dialysis membrane-50 with molecular weight cut off 12000 to 14000 Da from Hi-Media Laboratories Pvt. Ltd having flat width of 24.26 mm and diameter of 14.3 mm with approximate capacity of 1.61 mL/cm was used for the study. The membrane was soaked overnight in phosphate buffer pH 7.4. 10 ml of prepared liposomal dispersion which contains 10 mg of drug was taken and placed in the donor cell. Dialysis membrane was placed in between donor cell and receptor cell. 20 ml of phosphate buffer (pH 7.4) was taken in receptor cell to touch the bottom surface of dialysis membrane. The temperature of the receptor phase was maintained at 37 ± 0.5° C and the receptor compartment was stirred with magnetic stirrer to maintain homogeneous condition. The aliquots of 3 ml were withdrawn at different time intervals. Fresh medium was used to replace with equal volume of the sample withdrawn. The samples were analyzed at 255 nm in a UV-Visible spectrophotometer and amount of drug released at different time intervals was calculated.

Fourier Transform Infrared Spectroscopy (FTIR)

To investigate any possible interaction between the drug and the excipients utilized under investigation FTIR spectrophotometry was used. The IR Spectra of pure drug (Itraconazole) and the combination of drug with excipients were carried out by using Alfa brooker FTIR with KBr background. Sample preparation includes grinding a small quantity of the sample with a purified salt usually potassium bromide finely to remove scattering effects from large crystals. The powder mixture was crushed in a mechanical die press to form a translucent pellet through which the beam of the spectrometer can pass. The pressed sample was carefully removed from the die and was placed in the FTIR sample holder. The IR spectrum was recorded from 4000 cm^{-1} to 400 cm$^{-1.}$

Microbiological assay

The microbiological assay of Itraconazole was carried out by cup plate method. The potato dextrose agar medium was prepared, sterilized and inoculated with candida albicans micro-organism at a temperature 27˚ C and immediately pored the inoculated medium into petri plates to give a depth of (4 to 5) mm uniformly and kept aside for solidification. Small cavities of 10 mm diameter were made on solidified agar petri plates by using sterilized cylinder shaped borer. 500 µl of the prepared standard solutions and sample solutions (i.e equivalent to 1 µg/ml and 5 µg/ml drug concentration) were added into each cavity. These petri plates are left for 1 to 4 hours at room temperature as a pre-incubation diffusion to minimize the effects of variation in time between different solutions. Prepared petri plates were incubated for 48 hours at 27˚ C and measured the diameter of circular inhibited zones. T-test is also conducted for microbiological studies to test the significant difference between standard and Itraconazole liposomal formulations.

RESULTS AND DISCUSSION

Phase Solubility Studies

The phase-solubility studies were conducted for Itraconazole by using different concentrations of both beta cyclodextrin and hydroxyl propyl beta cyclodextrin. The solubility of Itraconazole in water is 2.15mM and the solubility of ITR with β-CD was 19.7mM and with HP-β-CD it was 27.3mM. The results indicated that the solubility of the drug was increased positively with increasing CD concentration (as shown in Fig 1 and 2). These phase solubility diagrams can be classified as AP type according to the model proposed by Higuchi and Connors and the stability constant for ITR- β-CD was found to be 0.046 and for ITR-HPβ-CD it was 0.068.

Table 2: Phase solubility data of Itraconazole with β-CD

Conc of β-CD (mM)*10^{-3}	Conc of ITR with β-CD (mM) *10^{-3}
0	2.15409
9.956122575	2.25008
19.91224515	3.16332
39.91259758	3.31186
59.91295001	4.07656
79.91330244	5.74349
99.91365487	10.54623
149.8704823	14.43574
199.8273097	19.70611
Stability constant	0.04600

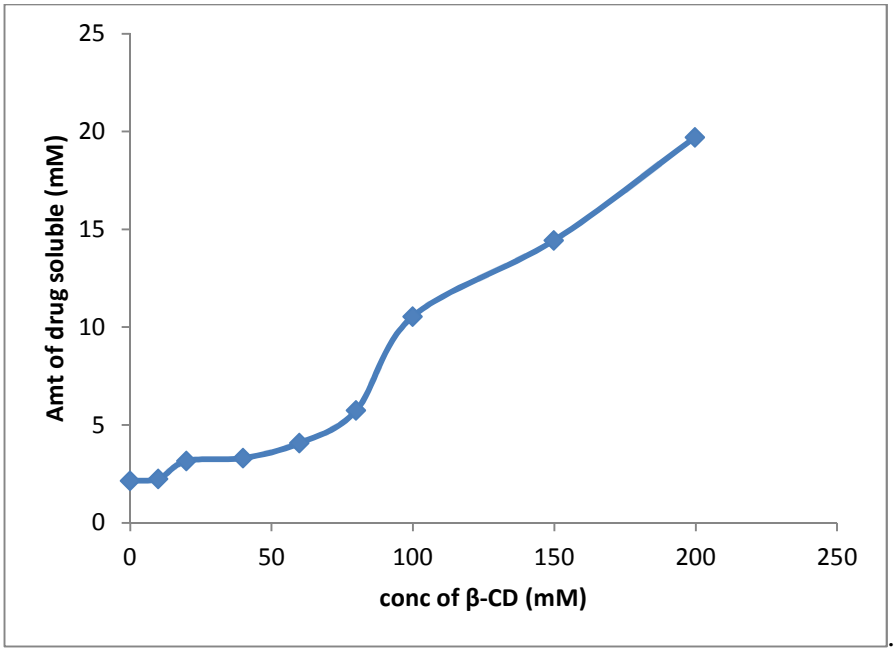

Figure 1: Phase solubility graph of Itraconazole with beta cyclodextrin

Table 3: Phase solubility data of Itraconazole with HPβ-CD

Conc of HPβ-CD (mM)*10^{-3}	Conc of ITR with HPβ-CD (mM) *10^{-3}
0	2.15409
9.993510707	3.63094
19.98702141	4.00504
39.97404283	4.79724
59.96106424	5.06131
79.94808566	7.72950
100.0000000	13.68755
149.9675535	20.27275
200.0000000	27.36959
Stability constant	0.068000

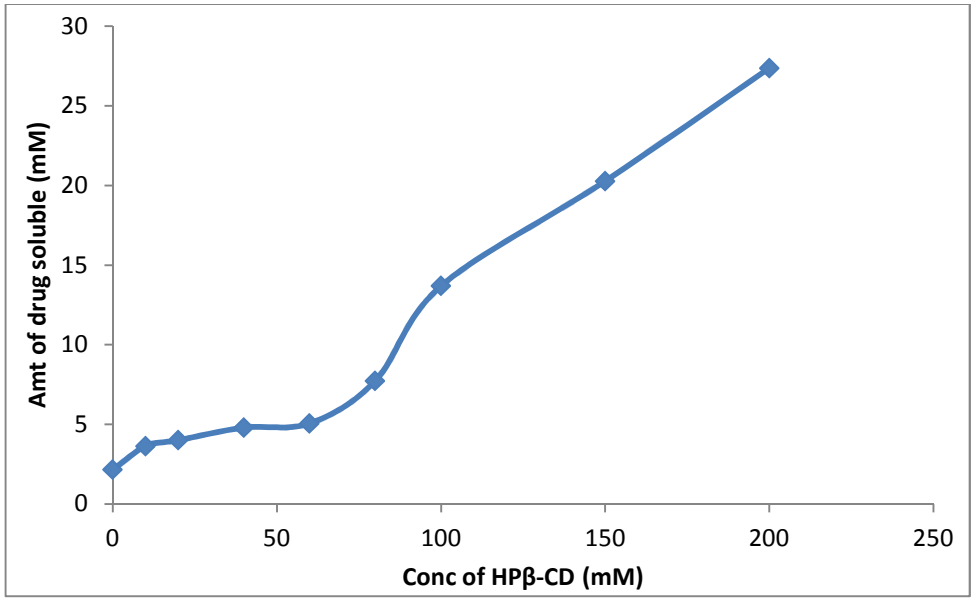

Figure 2: Phase solubility graph of Itraconazole with Hydroxy propyl beta cyclodextrin

Dissolution Profile of Drug-Cyclodextrin Complexes

The inclusion complexes of ITR were prepared with both β-CD and HP-β-CD by kneading technique at drug to cyclodextrin weight ratio of 1:0.5, 1:1, 1:1.5 and 1:2. All the inclusion complexes are amorphous free flowing

powders. The dissolution rate of itraconazole of pure drug was found to be 12.56 %. The complexes showed dissolution rate of 49.32 % & 56.72 % for -CD and HP-β-CD respectively (Shown in Fig 3 & 4). Further the complexes were used for the formulation of liposomes.

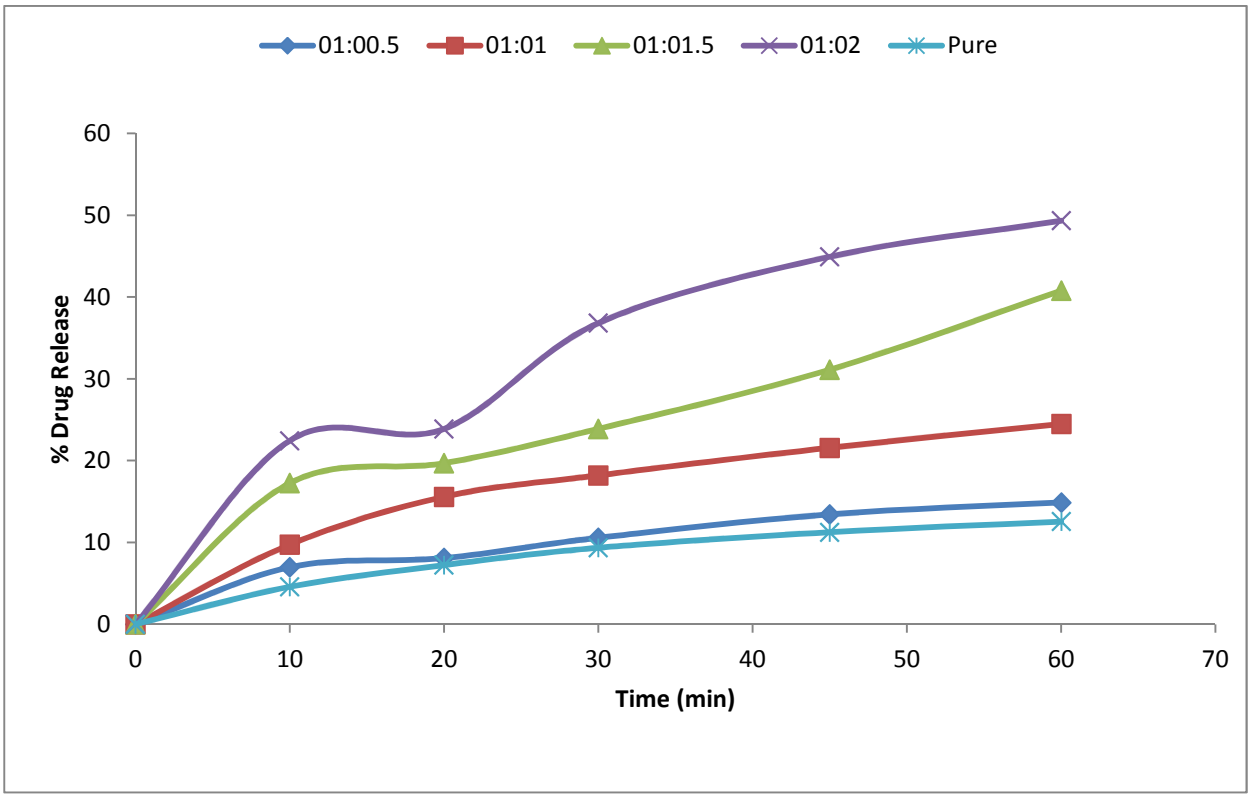

Figure 3: Dissolution profile of Itraconazole-β-CD complexes and pure drug

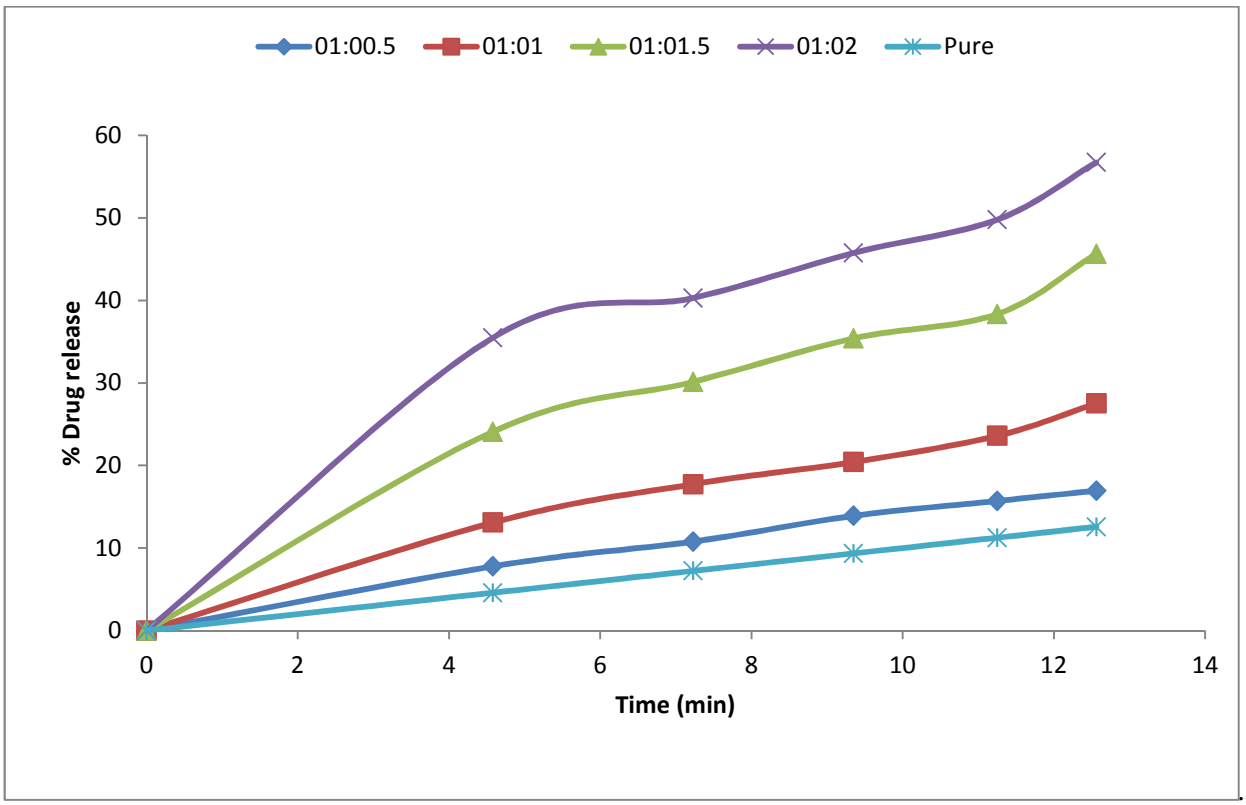

Figure 4: Dissolution profile of Itraconazole-HPβ-CD complexes

Optical Microscopy

The vesicles were observed under optical microscope was found to be discrete and spherical in shape. The images (shown in Fig 5 and 6) clearly indicated the discrete structures of liposomes vesicles.

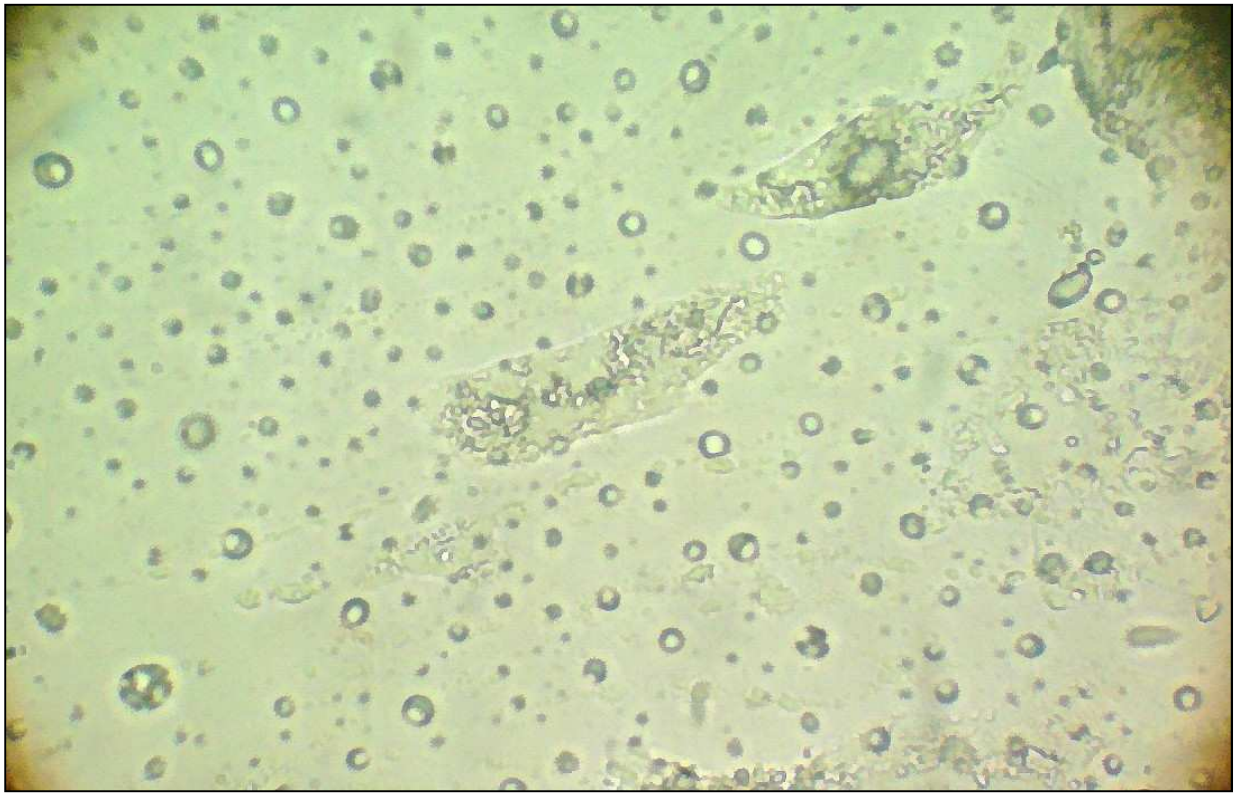

Figure 5: Optical microscope image for Formulation 4

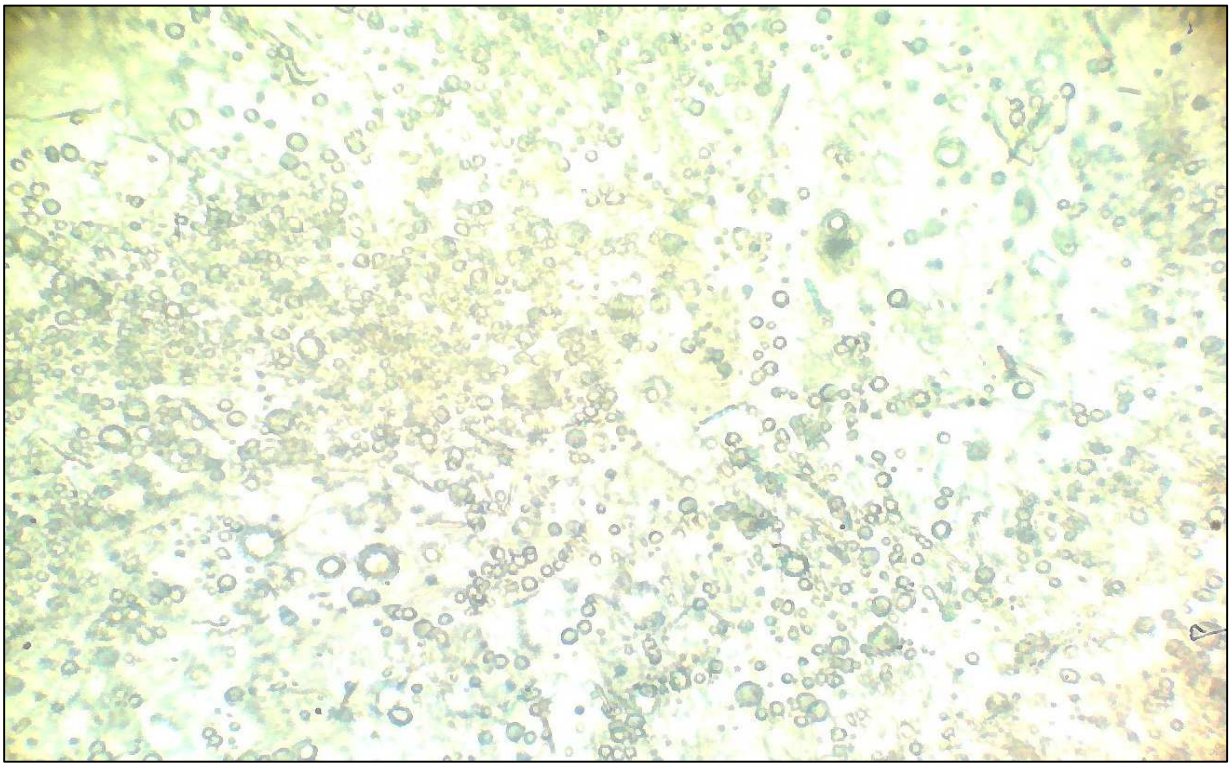

Figure 6: Optical microscope image for Formulation 8

Particle Size Determination by Nano Particle Analyser

The particle size distribution analysis was performed by using particle size analyzer and the results showed that the mean particle size Z-Average of the liposome vesicle in F8 was 3679.4nm and PI of 5.215 (as shown in Fig 24). The particle size distribution analysis was performed by using optical microscope and the mean particle size of F8 was 4.47µm (as shown in Table 7). This indicated vesicles are good and unilamellar.

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Measurement Results

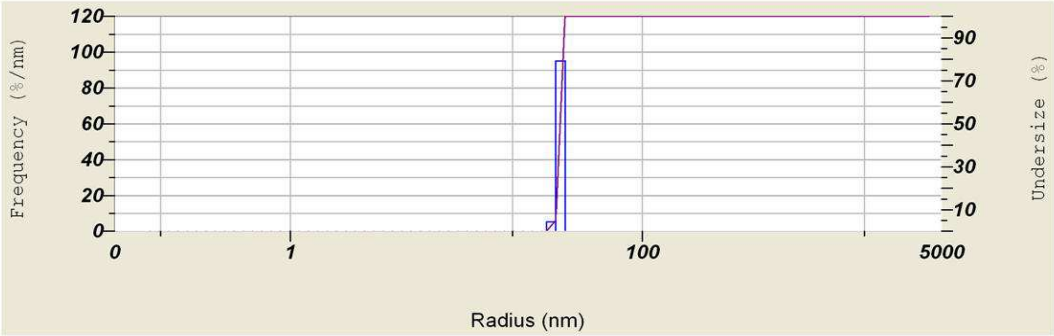
Date	:	Monday, February 02, 2015 3:24:37 PM
Measurement Type	:	Particle Size
Sample Name	:	git pharmy modi 1
Scattering Angle	:	173
Temperature of the holder	:	25.1 °C
T% before meas.	:	5
Viscosity of the dispersion medium	:	0.893 mPa·s
Form Of Distribution	:	Standard
Representation of result	:	Volume
Count rate	:	2419 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	34.1 nm	0.9 nm	34.2 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	34.1 nm	0.9 nm	34.2 nm

Cumulant Operations

Z-Average	:	3679.4 nm
PI	:	5.215



No.	Radius	Frequency	Cumulation	No.	Radius	Frequency	Cumulation	No.	Radius	Frequency	Cumulation	No.	Radius	Frequency	Cumulation
1	0.17	0.000	0.000	27	4.05	0.000	0.000	53	96.74	0.000	100.000	79	2311.41	0.000	100.000
2	0.19	0.000	0.000	28	4.57	0.000	0.000	54	109.30	0.000	100.000	80	2611.48	0.000	100.000
3	0.22	0.000	0.000	29	5.17	0.000	0.000	55	123.49	0.000	100.000	81	2950.51	0.000	100.000
4	0.24	0.000	0.000	30	5.84	0.000	0.000	56	139.52	0.000	100.000	82	3333.55	0.000	100.000
5	0.28	0.000	0.000	31	6.60	0.000	0.000	57	157.63	0.000	100.000	83	3766.32	0.000	100.000
6	0.31	0.000	0.000	32	7.45	0.000	0.000	58	178.10	0.000	100.000	84	4255.28	0.000	100.000
7	0.35	0.000	0.000	33	8.42	0.000	0.000	59	201.22	0.000	100.000				
8	0.40	0.000	0.000	34	9.52	0.000	0.000	60	227.34	0.000	100.000				
9	0.45	0.000	0.000	35	10.75	0.000	0.000	61	256.86	0.000	100.000				
10	0.51	0.000	0.000	36	12.15	0.000	0.000	62	290.20	0.000	100.000				
11	0.57	0.000	0.000	37	13.72	0.000	0.000	63	327.88	0.000	100.000				
12	0.65	0.000	0.000	38	15.50	0.000	0.000	64	370.44	0.000	100.000				
13	0.73	0.000	0.000	39	17.52	0.000	0.000	65	418.54	0.000	100.000				
14	0.83	0.000	0.000	40	19.79	0.000	0.000	66	472.87	0.000	100.000				
15	0.94	0.000	0.000	41	22.36	0.000	0.000	67	534.26	0.000	100.000				
16	1.06	0.000	0.000	42	25.26	0.000	0.000	68	603.62	0.000	100.000				
17	1.19	0.000	0.000	43	28.54	0.000	0.000	69	681.98	0.000	100.000				
18	1.35	0.000	0.000	44	32.25	5.096	5.096	70	770.52	0.000	100.000				
19	1.52	0.000	0.000	45	36.44	94.904	100.000	71	870.55	0.000	100.000				
20	1.72	0.000	0.000	46	41.17	0.000	100.000	72	983.57	0.000	100.000				
21	1.95	0.000	0.000	47	46.51	0.000	100.000	73	1111.26	0.000	100.000				
22	2.20	0.000	0.000	48	52.55	0.000	100.000	74	1255.52	0.000	100.000				
23	2.48	0.000	0.000	49	59.37	0.000	100.000	75	1418.52	0.000	100.000				
24	2.81	0.000	0.000	50	67.08	0.000	100.000	76	1602.68	0.000	100.000				
25	3.17	0.000	0.000	51	75.79	0.000	100.000	77	1810.74	0.000	100.000				
26	3.58	0.000	0.000	52	85.62	0.000	100.000	78	2045.81	0.000	100.000				

1 / 1

Figure 7: Particle size determination by nanoparticle analyser

Table 4: Mean particle size determination of Itraconazole Liposome formulations

Formulation	Mean size of each liposome (µm)
F1	6.09
F2	5.79
F3	6.30
F4	5.49
F5	4.92
F6	5.34
F7	4.59
F8	4.47

Scanning Electron Microscope (SEM)

The scanning electron microscopy describes the surface morphology of Liposomes and SEM of formulation F8 and pure drug (as shown in Fig 8 & 9) showed the liposomes were spherical and smooth vesicular structures.

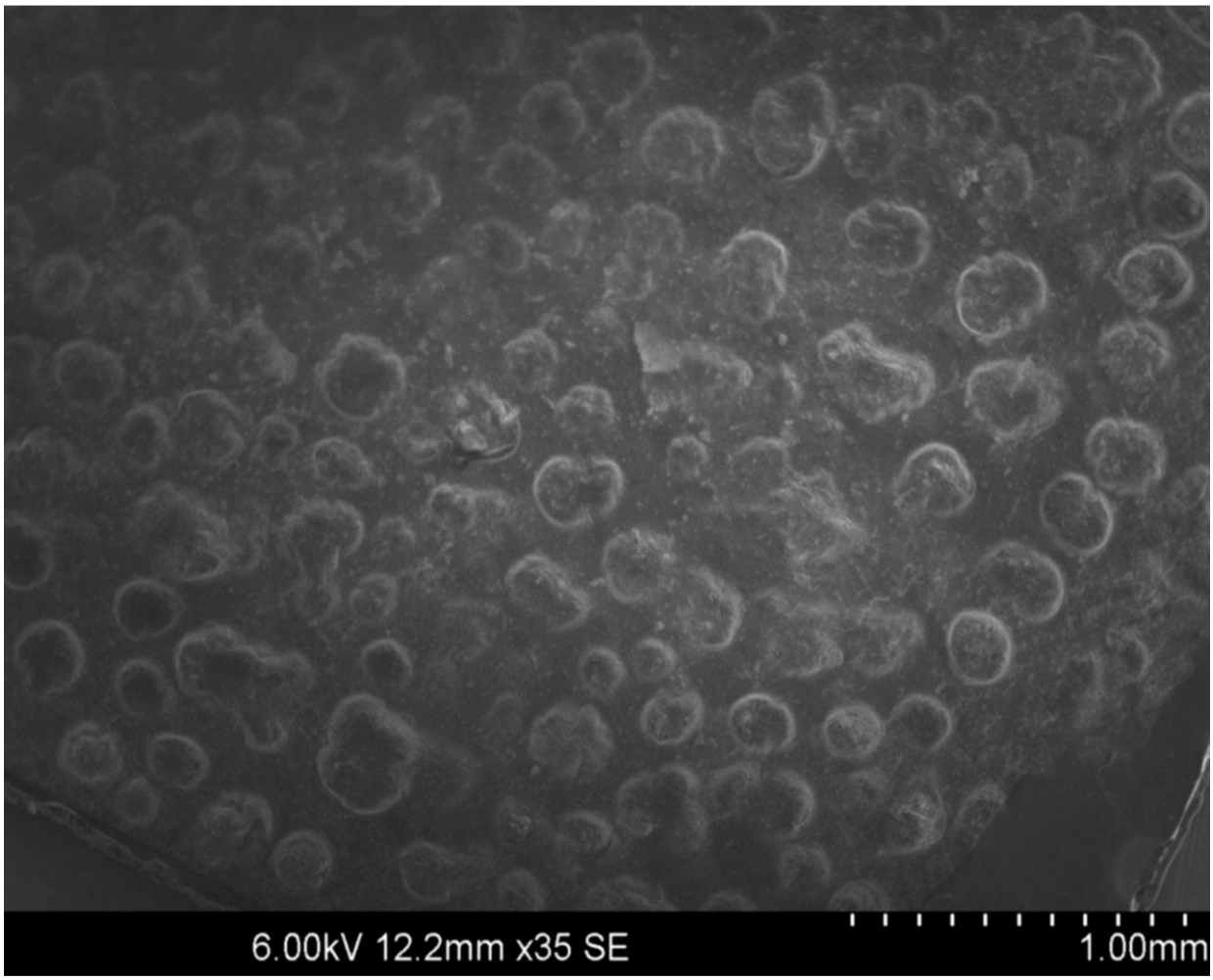


Figure 8: SEM result of Formulation F8

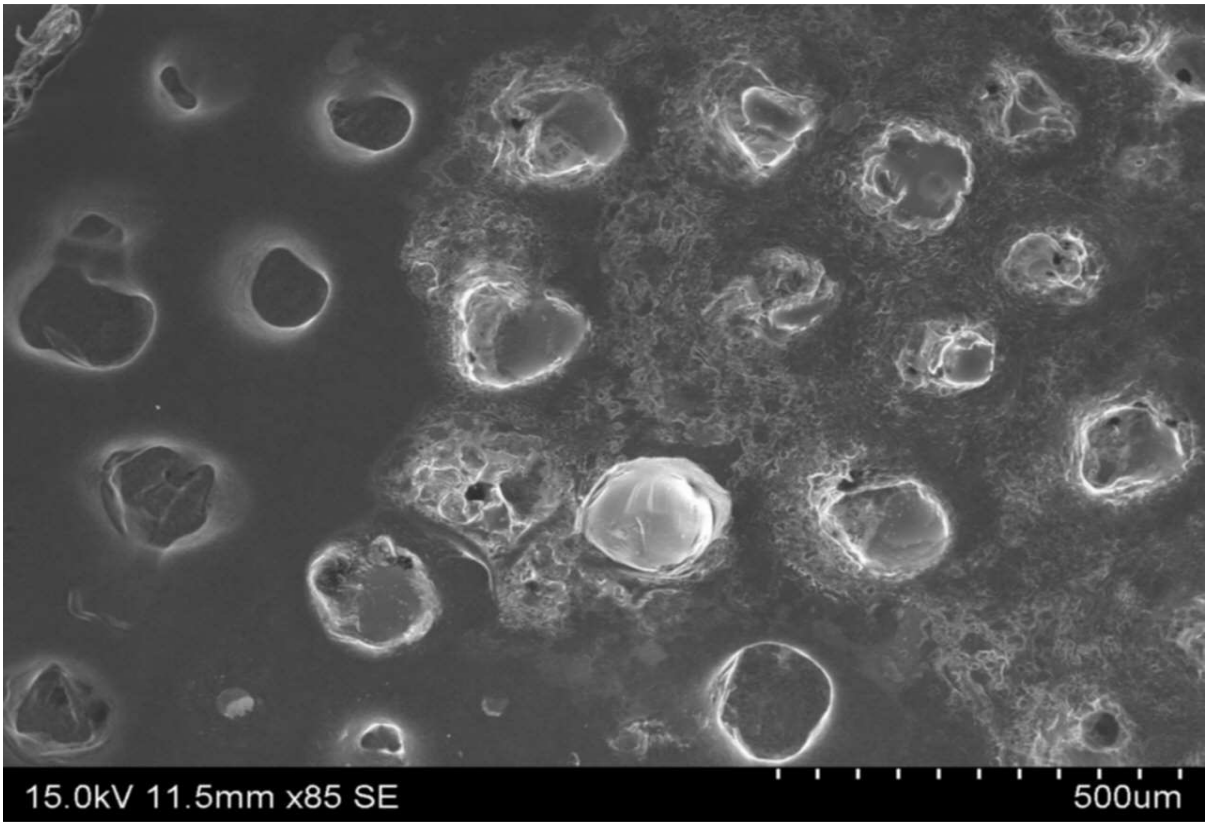


Figure 9: SEM result of Itraconazole pure drug

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of itraconazole were shown in fig. Itraconazole FTIR (shown in Table 10) showed that the characteristics peaks at 508.17, 532.23, 1698.30, 671.82, 1551.91, 1381.38, 1510.36, 2964, 2823, 824.37 Itraconazole liposomes were shown in fig. Itraconazole Liposomes FTIR (shown in Table 11) showed that the characteristics peaks at 563, 582, 1643.33, 667, 1451.71, 1112.20, 1406.16, 2952, 2842, 633.05cm-1. These results indicated that there is no interaction between drug and excipients used in the formulation.

Table 5: FTIR bands of Itraconazole liposomes

Functional group	IR band of Itraconazole (cm^{-1})	IR band of Itraconazole liposomes (cm^{-1})
C–Cl stretch	508.17, 532.23	563, 582
C=O stretch	1698.30	1643.33
(Aromatic) R-C-H bonding	671.82	667
(Aromatic) R-C-C bonding	1551.91	1451.71
(Ether linkage) R-O-R	1381.38	1112.20
(Aromatic) R-C=C bonding	1510.36	1406.16
(Alkyl) R-C-H stretch	2964, 2823	2952, 2842
Mono substituted benzene	824.37	633.05

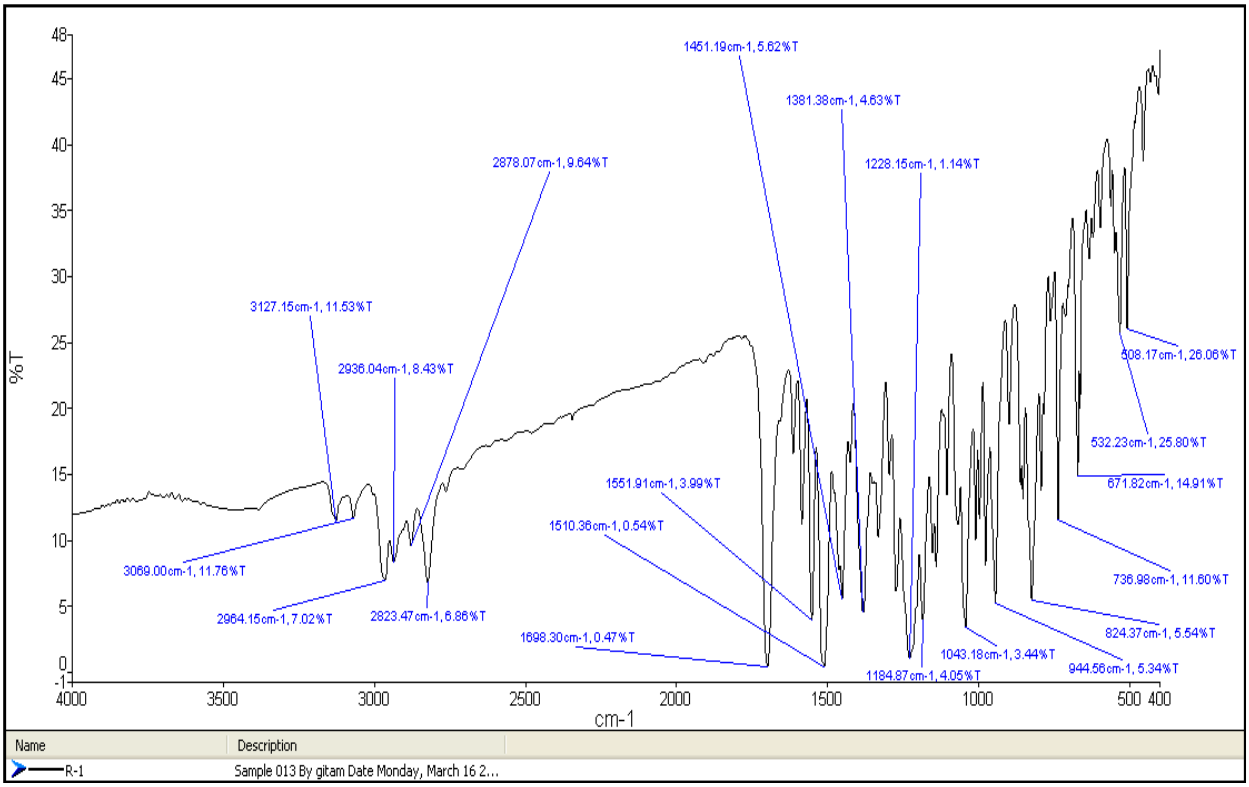


Figure 10: FTIR spectra of Itraconazole pure drug

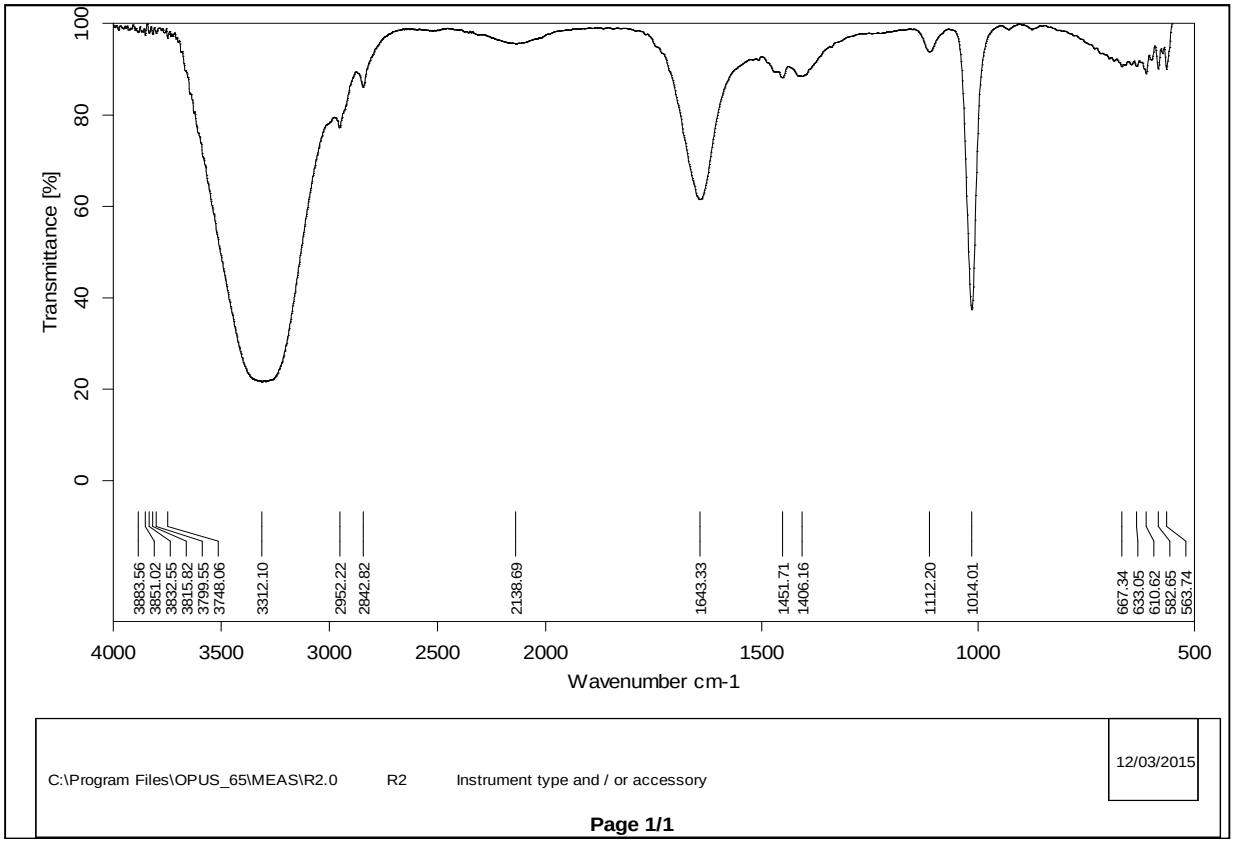


Figure 11: FTIR spectra of Itraconazole Liposomes

Drug Content, Entrapment Efficacy and Percentage Drug Release of Liposomes

The Itraconazole liposomal formulations were subjected to the estimation of drug content and encapsulation efficiency. The drug content was in the range of 94.78 % w/w to 101.81 % w/w for the liposome formulations. The encapsulation efficiency was found to be 37.99 % to 55.01 %. The results indicated that the formulation F-8 i.e. liposomes prepared by using high concentration of HPβ-CD showed highest drug content (% w/w) of 101.81 and highest encapsulation efficiency (% w/w) of 55.01. Liposomes prepared by lowest concentration of β-CD i.e.., F1 showed lowest drug content (% w/w) of 94.78 and lowest encapsulation efficiency (% w/w) of 37.99 (as shown in Table 10)

Table 6: %Drug content, % Drug entrapped and % Drug release (25 hrs) of Itraconazole liposomal formulations

Formulation	%Drug content	% Drug entrapped	% Drug release
F1	94.78	37.99	17.25
F2	95.24	43.91	19.93
F3	98.54	47.01	21.8
F4	100.71	52.01	24.78
F5	97.09	41.00	19.38
F6	97.34	45.98	24.95
F7	99.59	48.99	32.58
F8	101.81	55.01	39.62

***Invitro* Drug Release Studies**

The Itraconazole Liposomes were subjected to in vitro diffusion studies and the results indicated that the formulation F-8 showed highest *in vitro* drug release (% w/w) of 39.62 (as shown in Fig 13). The formulation F1 showed lowest *In vitro* drug release of 17.25 (as shown in Fig 12). The diffusion constants of formulations F8 and F1 were 0.029 and 0.009 respectively (as shown in Table 7). The formulation F8 showed greater diffusion constant and F1 showed lesser diffusion constant. The drug diffusion increased as the concentration HP-B-CD concentration increased.

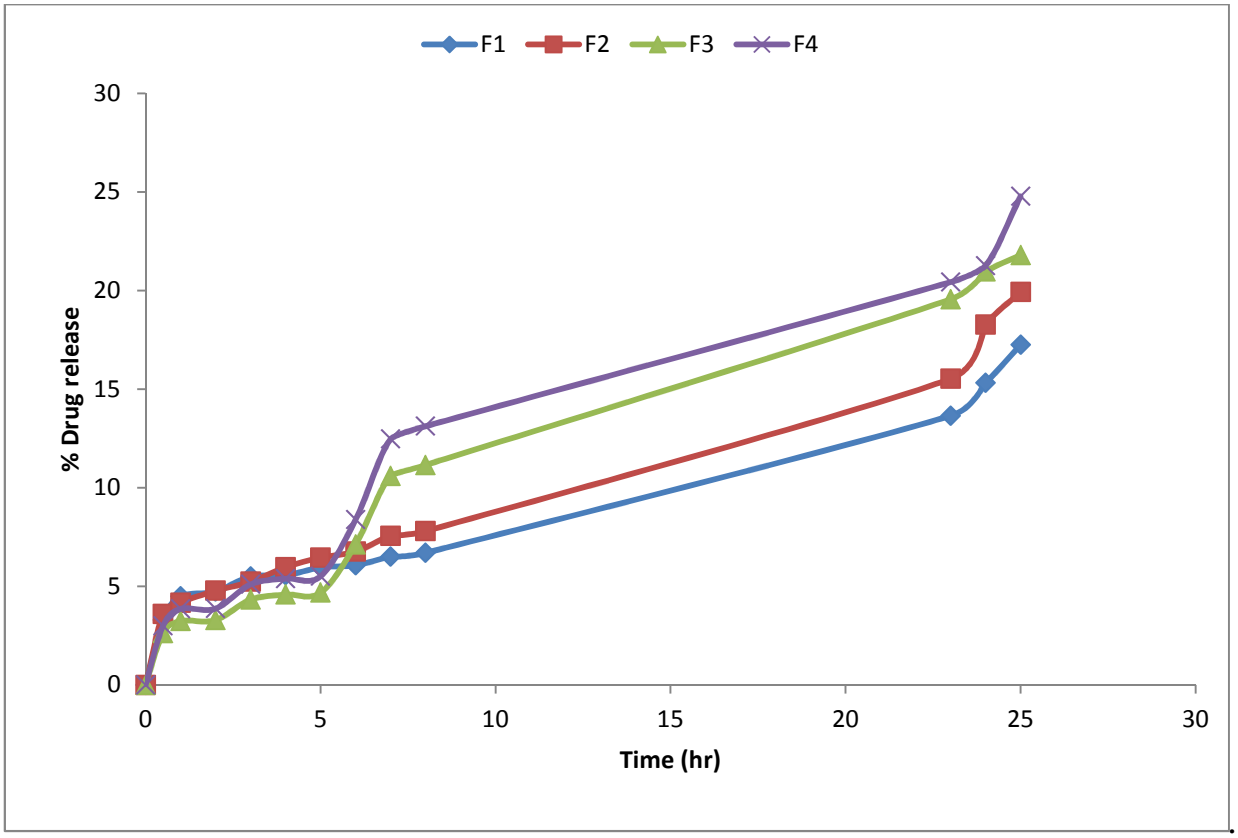


Figure 12: Percentage Drug release of ITR Liposomal formulations F1, F2, F3, F4

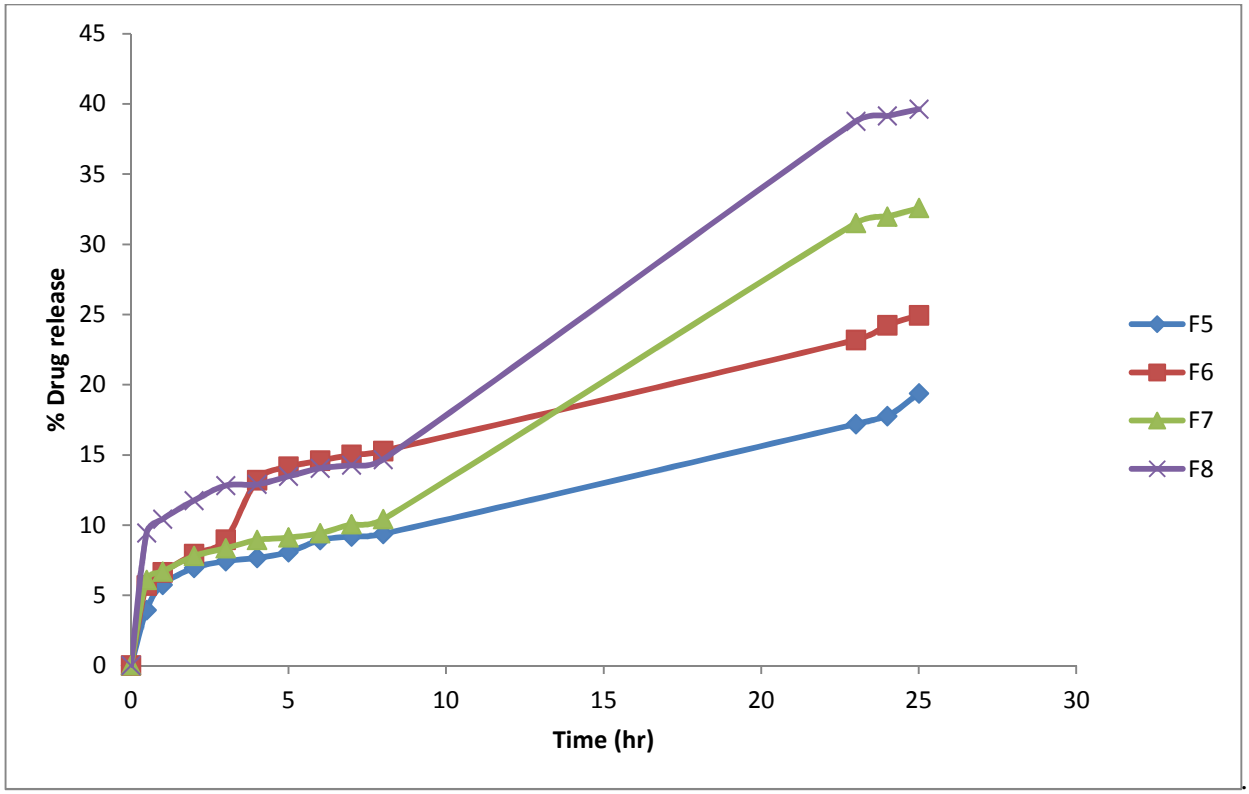


Figure 13: Percentage Drug release of ITR Liposomal formulations F5, F6, F7, F8

Table 7: Diffusion pattern and diffusion constants of Itraconazole Liposomal formulations

√T	Q/A							
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
0	0	0	0	0	0	0	0	0
0.71	0.0116	0.0110	0.0112	0.0109	0.0165	0.0213	0.0265	0.0357
1.00	0.0158	0.0127	0.0138	0.0140	0.0240	0.0248	0.0291	0.0394
1.41	0.0166	0.0146	0.0140	0.0140	0.0290	0.0296	0.0339	0.0444
1.73	0.0193	0.0160	0.0185	0.0185	0.0310	0.0335	0.0363	0.0484
2.00	0.0195	0.0182	0.0195	0.0195	0.0320	0.0493	0.0388	0.0487
2.24	0.0209	0.0197	0.0200	0.0200	0.0337	0.0528	0.0396	0.0509
2.45	0.0213	0.0206	0.0303	0.0303	0.0373	0.0545	0.0409	0.0531
2.65	0.0228	0.0231	0.0451	0.0451	0.0383	0.0560	0.0437	0.0539
2.83	0.0235	0.0238	0.0475	0.0475	0.0391	0.0571	0.0452	0.0554
4.80	0.0479	0.0474	0.0833	0.0740	0.0718	0.0866	0.1369	0.1464
4.90	0.0538	0.0558	0.0893	0.0770	0.0741	0.0905	0.1389	0.1479
5.00	0.0606	0.0609	0.0929	0.0897	0.0809	0.0932	0.1416	0.1497
5.10	0.0509	0.0581	0.0669	0.0669	0.0790	0.0857	0.1243	0.1461
5.20	0.0478	0.0573	0.0600	0.0600	0.0478	0.0831	0.1175	0.1445
Diffusion constants	0.009	0.011	0.017	0.015	0.012	0.015	0.027	0.029

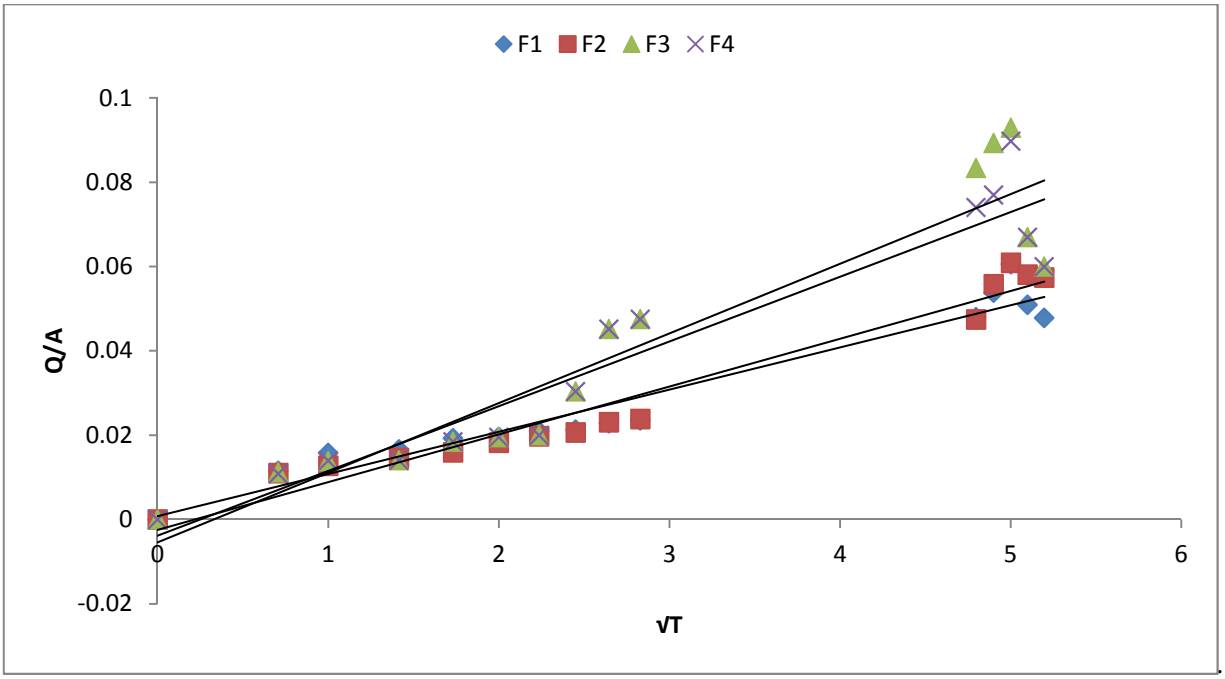


Figure 14: Diffusion pattern of Itraconazole Liposomal formulations F1, F2, F3, F4

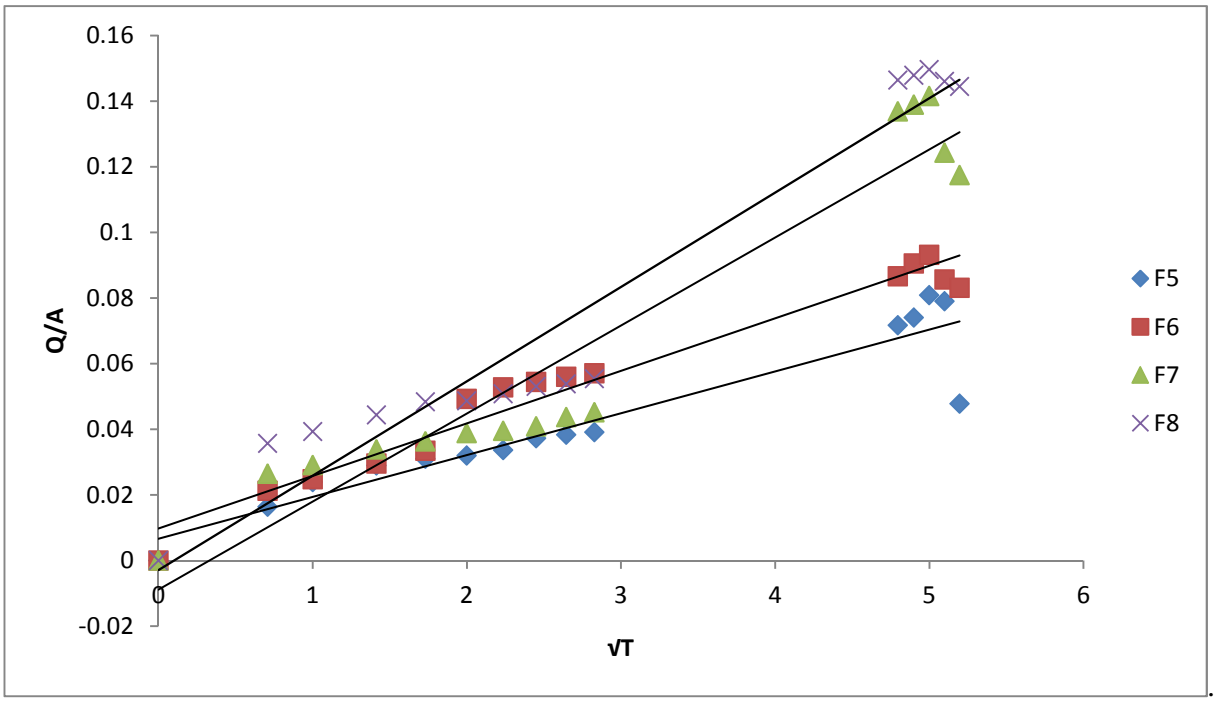


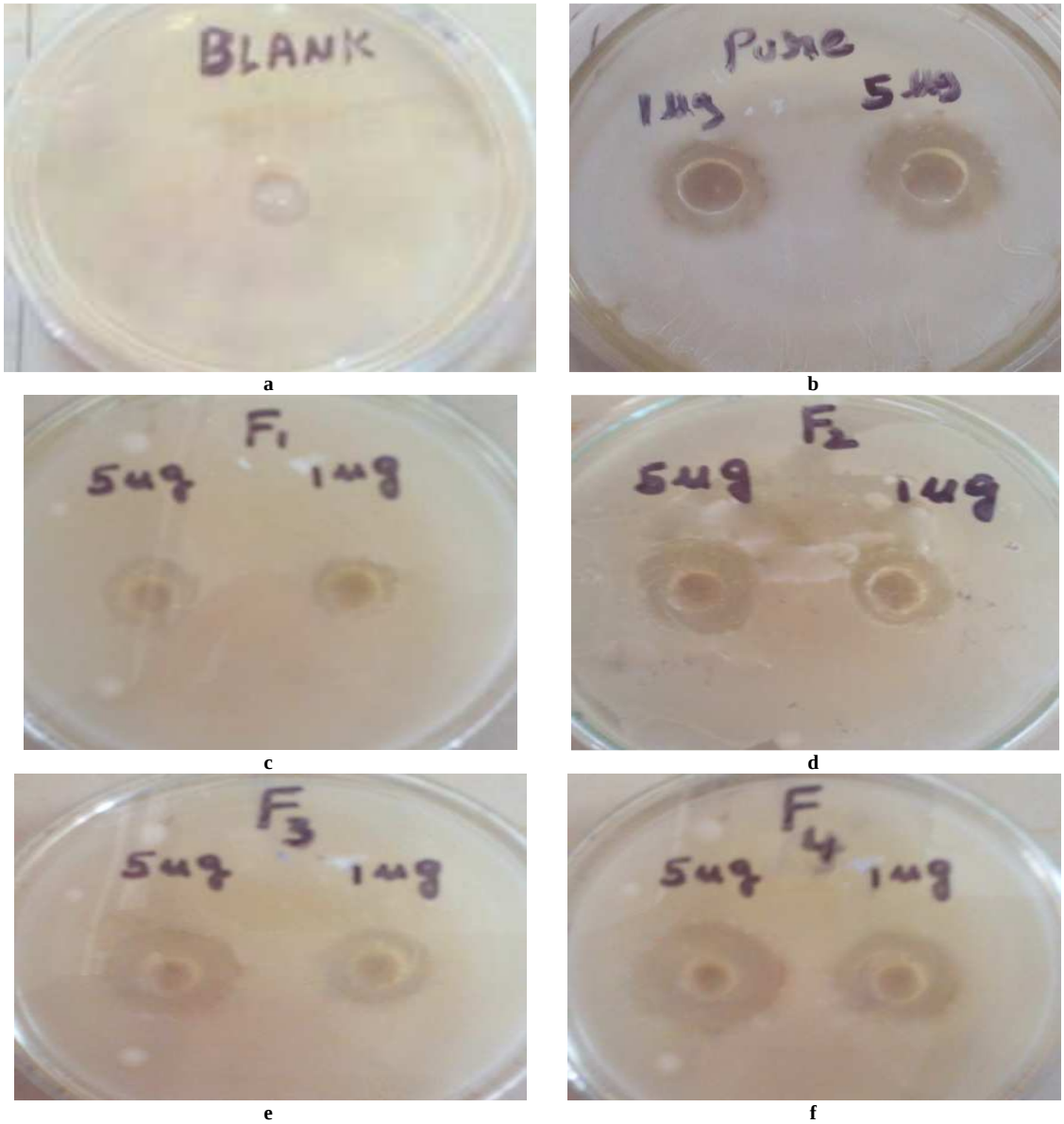
Figure 15: Diffusion pattern of Itraconazole Liposomal formulations F5, F6, F7, F8

Microbiological Assay

Itraconazole liposomes were subjected to Microbiological studies and the results showed the formulation F8 showed greater percentage zone of inhibition of 116.67 when compared with the pure drug and F1 showed lowest percentage zone of inhibition of 62.50 (as shown in Table 8).

Table 8: Microbiological data of itraconazole pure drug and liposomes

Formulation	Conc	Log C	Inhibition diameter (mm)	% Inhibition
F1	1	0.00	11	45.83
	5	0.70	15	62.50
F2	1	0.00	13	54.17
	5	0.70	18	75.00
F3	1	0.00	15	62.50
	5	0.70	20	83.33
F4	1	0.00	17	70.83
	5	0.70	22	91.67
F5	1	0.00	16	66.67
	5	0.70	20	83.33
F6	1	0.00	18	75.00
	5	0.70	22	91.67
F7	1	0.00	19	79.17
	5	0.70	24	100.00
F8	1	0.00	22	91.67
	5	0.70	28	116.67
Pure	1	0.00	20	83.33
	5	0.70	24	100.00

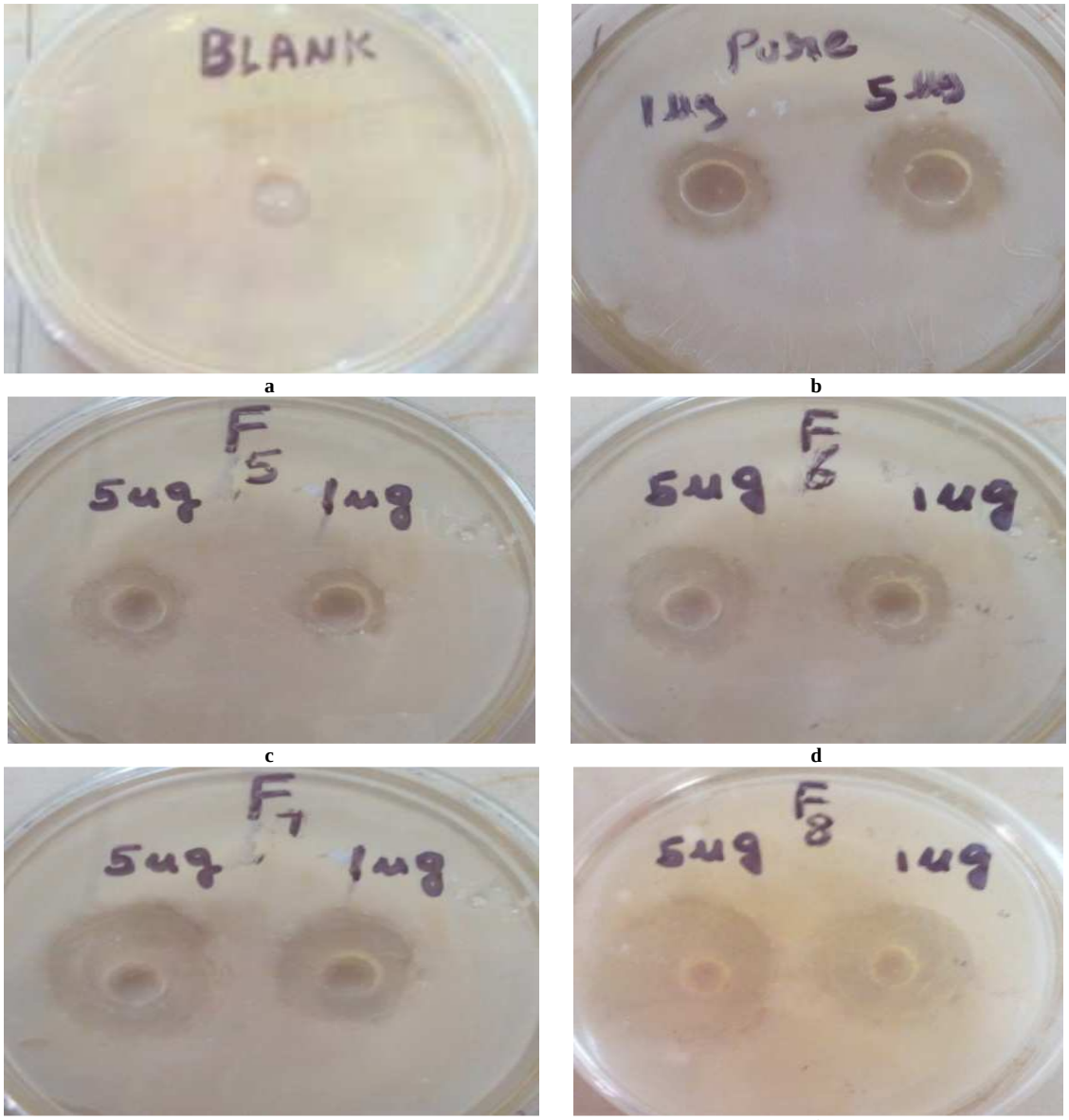


a **b**

c **d**

e **f**

Fig 16: Zone of inhibition of a. Blank, b. Pure, c. Formulation 1, d. Formulation 2, e. Formulation 3, f. Formulation 4



a **b**

c **d**

e **f**

Fig 17: Zone of inhibition of a. Blank, b. Pure, c. Formulation 5, d. Formulation 6, e. Formulation 7, f. Formulation 8

Microbiological studies of Itraconazole liposomes were subjected to t-test. It is conducted between pure drug with F8 and pure drug with F4 and F8 with F4. The results (Table 9) showed that calculated $|t|$ for pure drug with F8 is 2.596 and F8 with F4 was 3.875 and pure drug with F4 was 2.001. The critical value or tabulated value of t at 0.05 level of significance 4 degrees of freedom $|0.05, 4|$ is. 2.132. Calculated t values of pure drug with F8 and F8 with F4 were found to be greater than critical value or tabulated value which indicates there is significant difference between them and calculated t-value of pure drug with F4 is lower than the tabulated value indicates there is no significant difference them. These results clearly indicated that the liposomes are more efficient when compared with pure drug.

Table 9: t-test results for Microbiological assay of Itraconazole Liposomes

S. No.	Comparison	Calculated t-Value	Tabulated t-value	Inference
1.	Pure drug with F8	2.596	2.132	Significantly different
2.	Pure drug with F4	2.001	2.132	Significantly indifferent
3.	F8 with F4	3.875	2.132	Significantly different

Microbiological studies of Itraconazole liposomes were subjected to ANOVA test (Table 10). The table 16 shows that the calculated value for pure drug Vs F8 was 311.38 and for pure drug Vs F4 was 265.36 which are greater than the tabular value of 5.14 at 5% level of significance with degrees of freedom being $v_1 = 2$ (between the groups) and

v_2 = 6 (within the group). Therefore it is concluded that the difference in the microbiological study is significant. Hence, this analysis does not support the null hypothesis of no difference in sample means.

Table 10: ANOVA results for Microbiological assay of Itraconazole Liposomes

S. No	Comparision	Source of variation	Sum of squares	Degrees of freedom	Mean squares	Calculated F value	Tabulated F value	Inference
1	Pure drug Vs F8	Between	759.78	k-1= 2	379.89	311.38	5.14	Significantly different
		Within	7.34	n-k = 6	1.22			
2	Pure drug Vs F4	Between	575.56	k-1= 2	287.88	265.36	5.14	Significantly different
		Within	13.34	n-k = 6	2.22			

CONCLUSION

Hereby, it is concluded that the solubility of Itraconazole can be increased by using Cyclodextrin complexes and Itraconazole can be formulated in Liposomal formulation by using drug-cyclodextrin complexes which are prepared by kneading method. The solubility and the bioavailability of the drug is increased by formulating into liposomal formulations when compared to the pure drug. Itraconazole liposomes prepared with Hydroxy propyl beta cyclodextrin complexes ie, F8 showed greater solubility and bio-availability when compared with beta cyclodextrin complexes. The formulation with highest proportion of hydroxy propyl beta cyclodextrin ie.., F8 showed maximum bio-availability with less particle size and maximum drug content, encapsulation efficacy and invitro diffusion. This formulation also showed greater effect on Candida albicans ie.., it showed greater zone of inhibition than pure drug in microbiological assay. Hence, it is concluded that the Itraconazole can be formulated into Liposomes by using cyclodextrin complexes and the HPβ-CD showed greater increase in the solubility of Itraconazole than β-CD complexes. Further studies can be carried for these formulations to optimize for the stability and *in vivo* bio availability to make promising drug delivery system.

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