RESEARCH ARTICLE

OPTIMIZATION AND CHARACTERIZATION OF 5-FLUOROURACIL TRANSETHOSOMES FOR SKIN CANCER THERAPY USING RESPONSE SURFACE METHODOLOGY.

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Manuscript Info	*Abstract*

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The purpose of the present study was to develop, optimize and characterize 5-Fluorouracil transethosomes for skin cancer targeting. 5-Fluorouracil transethosomes were prepared by cold method using phospholipon 90G as the lipid and sodium cholate as edge activator. The size reduction was done by probe sonication. Central composite design was used for optimization procedure with different concentration of phospholipon 90G and sodium cholate as independent variables. The response variables selected were particle size and entrapment efficiency. Particle size and entrapment efficiency depends on the quantity of the above independent variables. Mathematical equation and response surface plots were used to relate the dependent and independent variables. The optimized model predicted a particle size of 57nm and entrapment efficiency of 92.06%. Transethosomes proved to be superior in terms of, amount of drug permeated in the skin as compared to conventional 1% flonida cream. The results suggests that transethosomes are efficient carriers for transdermal delivery of 5-Fluorouracil.

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Introduction:-

5- fluorouracil is a small, membrane permeable hydrophilic drug and to retain this molecule in the aqueous compartment of liposomes has been difficult to achieve. Conventional 5- FU is available in several strengths like 1%, 2% and 5% solutions and 1% and 5% creams for topical use. The conventional creams are not able to penetrate into the deeper layers of tumors[1]. Transdermal delivery is an attractive alternative to oral delivery of drugs as it can overcome various shortcomings of oral drug delivery. It avoids first pass metabolism of bioactives, avoids dose dumping, lower fluctuations in plasma drug levels, gastrointestinal side effects and high patient compliance. The skin acts as an excellent barrier for molecular transport, and this is the rationale for the transdermal delivery. Most conventional methods of drug intake namely oral route is not feasible and thus alternative routes must be sought. Intravenous delivery of medicaments avoids many shortcomings such as gastrointestinal and hepatic metabolism but its invasive and apprehensive nature, particularly for chronic administration has encouraged alternative strategies. The transdermal route offers several advantages including large and readily accessible surface area of 1–2 m for drug absorption,ease of application and termination of therapy. Better technologies have evolved for delivering of drug molecules over the past few years. It provides sustained release for drugs with short biological half-lives which requires frequent oral or parenteral administration and controlled release for drugs with narrow therapeutic index[2].
In this investigation, ultradeformable vesicles was developed to deliver 5-FU as an alternative vehicle for topical drug delivery to oral conventional dosage form. 5-FU was the most suitable drug to deliver across the skin for the

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management of skin conditions. Ultra-deformability property of the vesicles makes this system a versatile carrier for systemic and topical delivery of the drug[3]. Different surfactants in different concentration ratio with lipid has been tried to get enhanced drug permeation and drug deposition into the skin.

Materials and methods:-
5-fluorouracil was a generous gift sample from Naprod life science Pvt Ltd, Mumbai. Phospholipon 90G [phosphatidylcholine (PC)] was a gift sample obtained from Lipoid (Ludwigshafen, Germany). Sodium cholate was obtained from S.D. Fine chemicals. All other chemicals used were of analytical grade.

Preparation of transethosomes:-
Phospholipon 90G was dissolved in ethanol in a conical flask with constant stirring at 700 rpm. The temperature of this alcoholic mixture was maintained at 30°C. Drug and sodium cholate was dissolved in water and was maintained at 30°C in a separate vessel. This aqueous phase was then added to the alcoholic phase slowly in a fine stream with constant stirring at 700 rpm in a closed vessel. It was stirred for additional 5 min. The system was kept at 30°C throughout the preparation. Size reduction was done by probe sonication for 5 min at RT.

Experimental design:-
A commercially available software program, Design Expert, Version 10.0.4.0, Stat-Ease Inc, Minneapolis, MN was used. The experimental design chosen was Response Surface, central composite design, 2-factors, 5-level 13 formulations were formulated with five centre points. Run order was kept in a randomize mode to protect against the effects of time related variables and also to satisfy the statistical requirement of independent variables. Analysis of variance (ANOVA) and all statistical analysis were also performed using the same software. Calculation of the effects was performed; half- normal plots and response surface plots were plotted.

Invitro drug release:-
Invitro drug release was evaluated using franz diffusion cell. A cellophane dialysis membrane with molecular weight cut-off of 8000 daltons (Hi-media) was hydrated with phosphate buffer saline Ph 7.4 (PBS 7.4) overnight. Vesicular formulation of 1ml or 1g of gel of 5- fluorouracil was placed in the donor compartment. The receptor compartment was filled with 13ml of PBS 7.4 and stirred with a magnetic bead at 300-400rpm and the temperature of the system was maintained at 32 ± 1^OC to mimic human skin. The available diffusion area was $2.61cm^2$. 1ml aliquot was withdrawn at predetermined time intervals and was immediately replaced with an equal volume of fresh buffer. All samples were analyzed for 5- fluorouracil content by U.V spectrophotometry at 266nm.

Exvivo skin permeation study:-
Porcine abdomine skin obtained from slaughter house was used as a model membrane for the skin permeation study because of its similarity with human skin in lipid content and permeability. The skin sample was mounted between the donor and receptor compartments of the diffusion cell. The receptor compartment was filled with 1ml of TEL or 1g of TEL gel of 5-fluorouracil. The receptor compartment was filled with 13ml of PBS 7.4 and stirred with a magnetic bead at 300-400rpm and the temperature of the system was maintained at 35 ± 1^OC to mimic human skin. The available diffusion area was $2.61cm^2$. 1ml aliquot was withdrawn at predetermined time intervals and was immediately replaced with an equal volume of fresh buffer. All samples were analyzed for 5- fluorouracil content by U.V spectrophotometry at 266nm.

Permeation data analysis:-
the flux (J,ug cm^{-2} hr^{-1}) was calculated from the slope of linear portion of the plot divided by the skin surface area. The steady state permeability coefficient (Kp) of the drug through porcine skin was calculated by using the following equation:

Kp= J/Co

Where, J is the flux and Co is the concentration of 5-fluorouracil in the gel. The penetration enhancing activity of the enhancer may be calculated in terms of enhancement ratio (ER), using the following equation:

ER= Drug permeability coefficient (treated)
$\quad$ Drug permeability coefficient (control)

Skin deposition studies:-
At the end of 24hrs of permeation experiment, the surface of the skin was washed five times with 5ml of 30% methanol to remove excess 5- fluorouracil from the surface of the skin. The skin was cut into small pieces which were further homogenized with 5ml of 30% methanol. The resulting solution was then centrifuged for 10mins at 10,000 rpm. The supernatant was then separated to determine the 5- fluorouracil content by U.V spectrophotometer at 266nm.

In-vitro cell lines study:-
The cell lines used were SK-MEL-2. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37° C, 5 % CO2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs. Experimental drugs were initially solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml.

Results and discussion:-
Experimental design:-
The technique of central composite design with 2 factors at 5 different levels affecting the particle size and entrapment efficiency was considered. All experiments were carried out in random order to nullify the effects of extraneous or nuisance variables. The results of the experimental design were analyzed using Design Expert software that provided considerable useful information and reaffirmed the utility of statistical design for conducting experiments. The selected independent variables like the concentration of edge activator and concentration of phospholipid significantly influenced the particle size and drug entrapment efficiency that is very much evident from the results shown in Table 2.

Factor A- Phospholipid concentration
Phospholipid concentration was varied to study the effect of phospholipid concentration on particle size and %EE. Levels of factor A are shown in Table 1
Factor B- Surfactant concentration
Surfactant concentration was varied to study the effect of surfactant concentration on particle size and %EE. Levels of factor B are shown in Table 1.

Table 1:- Variables selected for optimization

Variables	Low (-1)	Medium (0)	High (1)
Amount of Phospholipid	400mg	600mg	800mg
Amount of Surfactant	40mg	60mg	80mg

Table 2:- Design matrix and responses

Std	Run	Factor 1 A: Amount of phospholipid	Factor 2 B: Amount of surfactant	Response 1 Particle size nm	Response 2 Entrapment efficiency %
1	1	-1	-1	104	85.44
10	2	0	0	57	89.28
8	3	0	1	69	76.88
12	4	0	0	58	89.89
7	5	0	-1	66	92.06
5	6	-1	0	97	79.35
4	7	1	1	78	81.03
9	8	0	0	57	89.45
2	9	1	-1	49	73.55

13	10	0	0	57	89.28
3	11	-1	1	109	67.01
6	12	1	0	99	75.08
11	13	0	0	59	89.15

Table 3:- Formula for optimized batch of TEL

| Ingredients	Quantity
Drug	100mg
Sodium cholate	40mg
Phospholipon 90G	600mg
Ethanol	30%
Water upto	20ml

Effect of amount of surfactant on TEL:-
It is seen in fig 1 and 4 that an optimum amount of 40mg of surfactant generates particles of optimum size. Increasing or decreasing this amount causes changes in the particle size. The entrapment efficiency is also highest for 40mg of surfactant concentration.

In fig 2 and 3 it is seen that the initial increment in drug entrapment in the presence of low concentrations of EAs may be credited to the growth in vesicle size owing to the incorporation of more amount of drug. The decreased entrapment efficiency beyond a certain optimum concentration of EA can be ascribed to the pore formation of phospholipid bilayers in EA. A particle size in the range of 49 to 100 was desired as these transethosomes would be further loaded into gel.

Effect of amount of lipid on TEL:-
As shown in response plot Fig 1 & 4 initially particle size decreases with lower lipid concentration and then increases with increase in concentration. In Fig 2 & 3 it is seen that increase in lipid concentration increases entrapment efficiency upto an optimum level. Increase in drug encapsulation may be due to the presence of sodium deoxycholate in the bilayer, which "solubilise" and "hold" the drug in the lipid bilayer and hence enhances the encapsulation efficiency for the TELs.
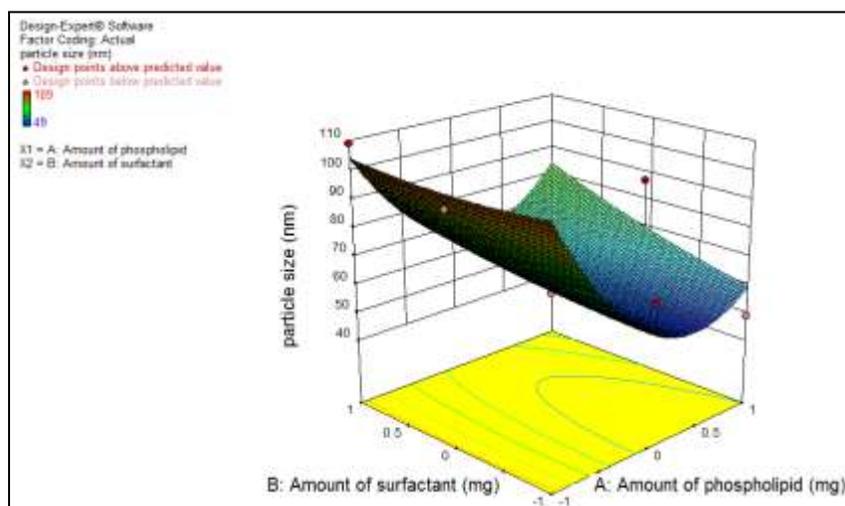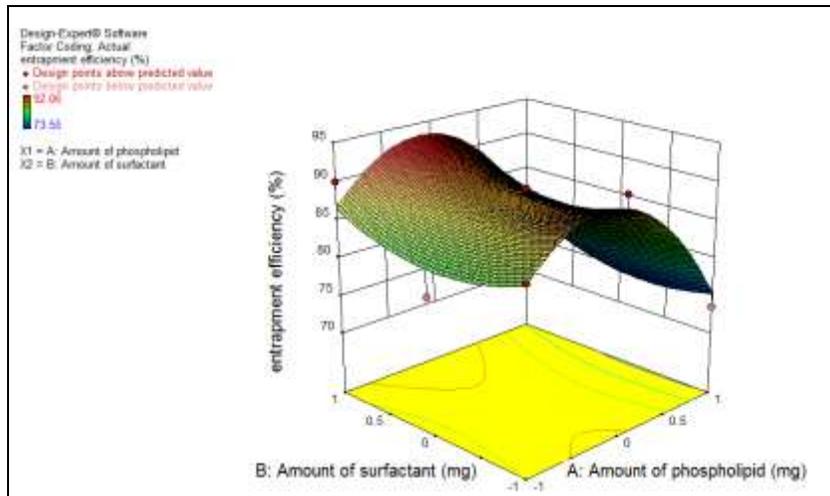

Fig 1:- Response surface plot for particle size
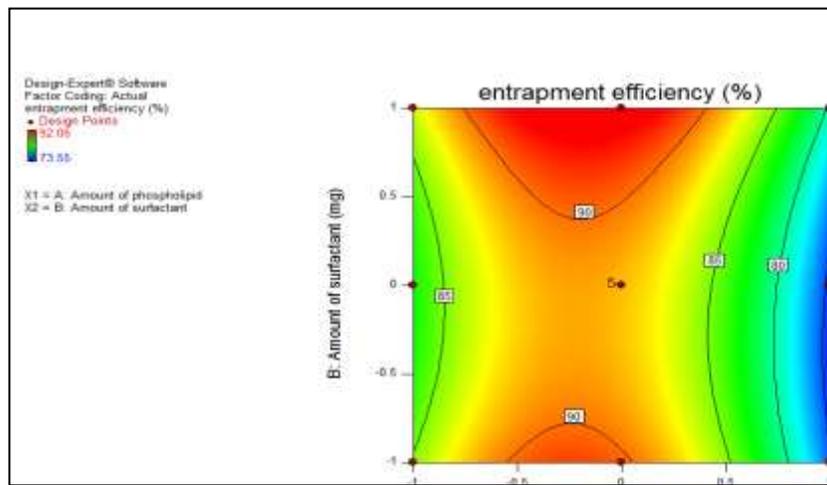
Fig 2:- response surface plot for entrapment efficiency

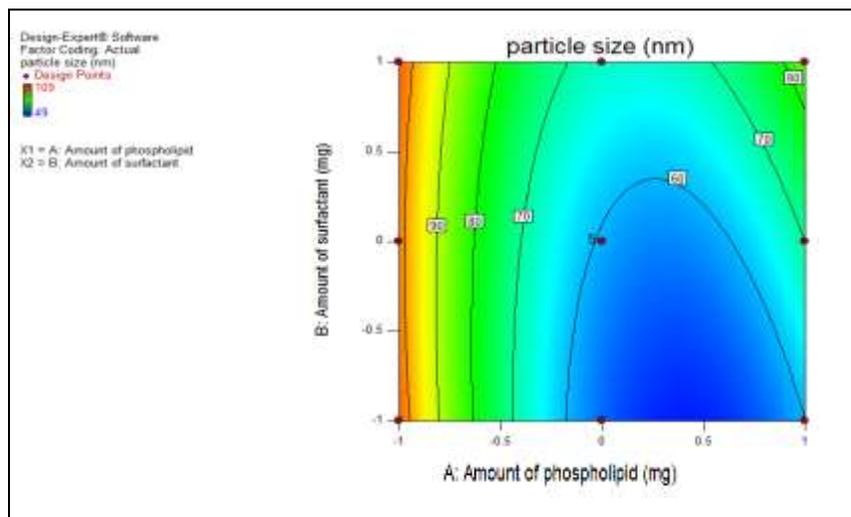**Fig 3:-** Contour plot for entrapment efficiency

Fig 4:- Contour plot for particle size

In vitro drug release:-

Encapsulation of 5- fluorouracil into TEL led to significant prolongation of 5- FU release across the artificial membrane. The results indicate that TEL dispersion and gel release more drug than the conventional 1% flonida cream. High permeation of TEL might be due to combination of both ethanol and edge activator. The release profile of gel indicated slow release as compared to TEL dispersion. This can be explained by the fact that drug diffusion from the transethosomal carrier followed by diffusion from the gel matrix resulted in sustained release effects.
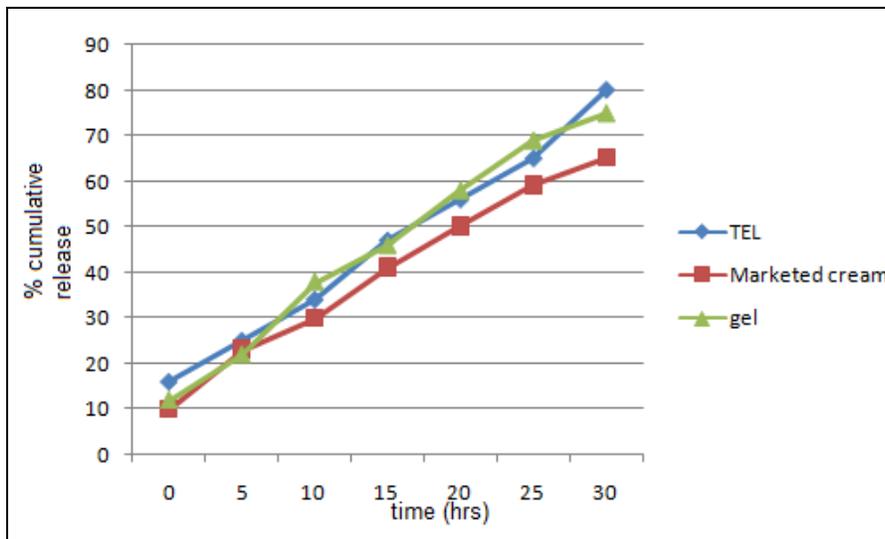

Fig 5:- Invitro drug release of 5-FU from [A] TEL dispersion [B] TEL gel [C] Marketed cream

Exvivo skin permeation studies:-

The exvivo skin permeation studies provided valuable information about the product behavior in vivo since they indicate the amount of drug available for absorption.
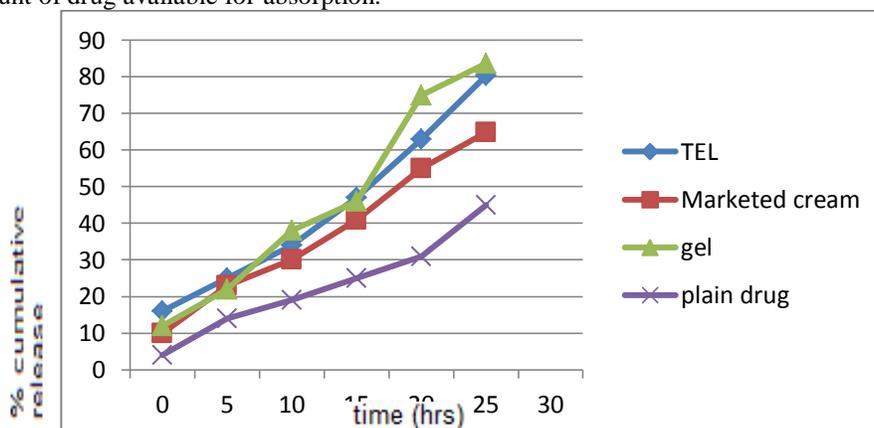

Fig 6:- Exvivo skin permeation from [A] Plain drug [B] TEL Dispersion [C] TEL gel [D] Marketed cream

Permeation of TEL gel and TEL dispersion are much enhanced as compared to plain drug solution. It has been suggested that ethanol may provide the vesicles with soft flexible characteristics, which allow them to easily penetrate into deeper layers of the skin. Synergistic mechanism is observed between phospholipid vesicles, ethanol and skin lipids. Ethanol fluidizes the SC lipids resulting in enhanced drug penetration. On the other hand ethanol interacts with lipid vesicles increases the lipid fluidity and makes them flexible. These flexible vesicles squeeze themselves intact through the disturbed SC to the deeper layers of skin and the consequent release of the drug occurs by the fusion of transethosomal vesicles with skin lipids.

The percent cumulative permeation in 24hrs was found to be 80.35% and 83.67% from TEL dispersion and gel respectively which was higher than the plain drug 45% and marketed 1% flonida cream 64.78%.

Skin permeation analysis:-

Formulation	Permeability coefficient (x 10^{-3}) [cm hr^{-1}]	Flux [ug hr^{-1} cm^{-2}]	ER
TEL gel	9.457	47.432	3.150
TEL dispersion	8.538	40.383	2.682
Marketed cream	3.156	16.674	1.234
Plain drug solution	2.004	11.256	0.453

Table 4:- Permeation coefficient, flux and enhancement ratio[ER] for the formulations assayed

The ER of TEL gel was found to be 3 fold and TEL dispersion was found to be 2 fold as compared to marketed cream and plain drug solution.

Skin deposition studies:-
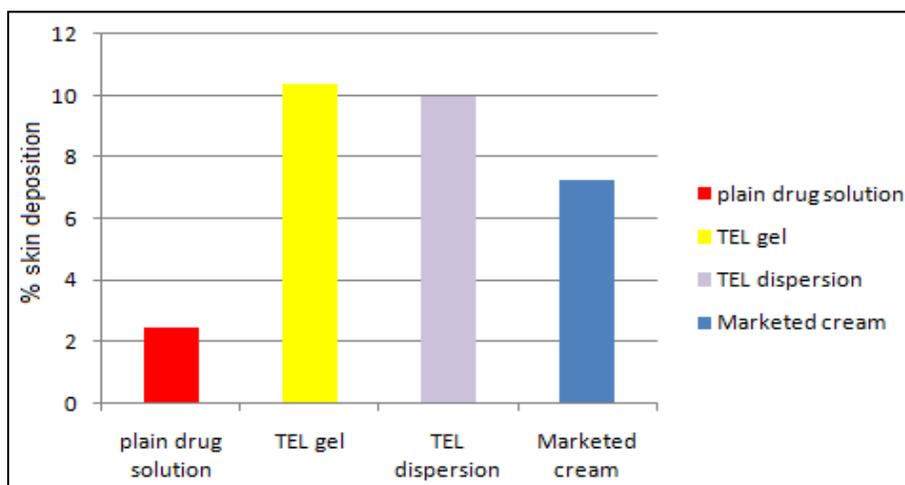
Fig 7:- Comparative study of skin deposition of 5-FU for various formulations

The amount of the drug retained on the skin from plain drug solution, TEL, TEL gel and marketed cream at the end of 24hrs are depicted in fig 7. 5-FU deposition on skin from TEL dispersion (9.985% and TEL gel 10.344% was much higher than plain drug 2.456% and conventional marketed cream 7.224%. Higher skin deposition of TEL gel and dispersion is due to the combined effect of phospholipid, edge activator and ethanol on skin, thus providing a mode for sustained drug delivery for greater period of time.

***In-vitro* cell lines study:-**
Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.
Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:
[Ti/C] x 100 %

The optimized formulation showed the inhibition of less than 10 which is considered as good % control growth value.

Conclusion:-
Water soluble drug 5- Fluorouracil was entrapped into the aqueous phase of stable unilamellar transethosomes by cold method of preparation followed by sonication. By the experimental design the crucial contributing factors for controlling the particle size and drug entrapment efficiency were determined.

Release profile of 5- fluorouracil loaded transethosomes was 80.35% and transethosomal gel was 83.67% as compared to ~65% for conventional 1% 5-FU loaded flonida cream . Thus, the 5- FU loaded transethosomes released more drug than marketed cream, the drug release being related to entrapment efficiency.

The results obtained from this study indicates that new phospholipid carrier transethosomes which consists of high concentration of ethanol and edge activator enhances the permeation of 5- Fluorouracil. Thus the developed transethosomal formulation could be the potential carrier for 5-Fluorouracil and other similar drugs especially due to their simple production and ease of scale up.

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