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Formulation, and evaluation of transferosomal gel of famciclovir for transdermal use

Short Title: Tranferosomal gel of famciclovir

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Abstract
Famciclovir, the drug of choice for cold sores and recurrent genital herpes, shows poor oral bioavailability and is associated with numerous side effects. The present work emphasizes the possibility of transdermal application of famciclovir through a transferosome-loaded gelling system to localize the drug at the site of application with better penetrability, therapeutic effects, and comfort. Transferosome of famciclovir was prepared using tween 80, phospholipid, and cholesterol. To optimize the entrapment of the drug, and the vesicular size of the transferosomes, central composite design was employed. The optimized formulation was evaluated for physicochemical characteristics, surface morphology study, and degree of deformability. The optimized product was included in Carbopol 940 gelling system. The gel was evaluated for *ex vivo* permeation, skin irritation, drug deposition at various layers of skin, and histopathology study. The design optimization yielded an optimized product (FAMOPT) of nano-sized (339 nm) stable vesicle of transferosome of famciclovir. The surface morphology study revealed the formation of nanovesicles without aggregation. Compatibility between the drug and the excipients was established. The elasticity of the vesicles proved the resistance to leakage. Permeation of the drug was enhanced by 2.8 times. The gel was found to be nonirritating and non-sensitizing to the animal skin. The drug deposition at various layers of skin was remarkably improved indicating effective penetration of the drug. Histopathology study further proved that penetration of nano vesiculate drug through the deeper layers of the skin. Hence, the nano vesicular delivery of famciclovir can be a promising alternative to the conventional delivery of famciclovir with enhanced local and systemic action for the treatment of herpes.
Keywords: Famciclovir, transferosome, deformable vesicle, transdermal penetration, skin deposition

INTRODUCTION

The era of nanotechnology offers a novel therapeutic avenue for antivirals for the successful treatment of viral diseases. Generally, the side effects associated with these small molecules need attention. The novel delivery of drugs in nanocarriers attempts to lower the viral load in a more efficient way than the conventional dosage form of the drugs.

Famciclovir is a guanine analogue used to treat herpes virus infections of the skin expressed through cold sores around the mouth, sores around the anus, and genital herpes.[1] The drug has poor oral bioavailability but suffers from many side effects when administered orally.[2] The dose of the drug is quite high and the treatment lasts for more than 7 days. Hence a new mode of delivery of famciclovir through the transdermal route could be beneficial for the efficacy of the therapy.

Transdermal delivery of drugs through the layers of the stratum corneum is a challenge. One of the most important factors to consider for a successful transdermal formulation is the penetration of the drug through the skin, which is mostly dependent on the physicochemical properties of the drug. Drugs with optimum lipophilicity are best suited for transdermal delivery. Hence for a hydrophilic drug to penetrate through the skin for eliciting systemic effect is a challenge. As a result, in the last decade, lipid-based vesicles or carriers have been routinely studied for topical drug delivery.[3] Niosomes, ethosomes, liposomes, and transferosomes are investigated as promising vesicular carriers for enhancing transdermal drug delivery of drugs.[4,5]

Transferosomes are vesicles of phospholipids with edge activators and are one of the superior drug-delivery systems for topical application.[6] They are elastic in nature which facilitates them to squeeze themselves as intact vesicle through the narrow pores of the skin. The presence of edge activators is responsible for the deformable properties of the transfersomes.[7] The elastic transport is propelled by the trans epidermal osmotic gradient between the surface of the skin and skin interiors. Their flexibility facilitates passing through the pores that are smaller than themselves.[8]

Famciclovir has a log P of 0.6, which does not support transdermal permeation. Hence a novel carrier system is necessary for famciclovir to cross the stratum corneum and localize at the site of action.[9] The composition of transferosomes makes the vesicles self-optimizing to cross the dermal barriers efficiently.[10] Therefore, the present study focuses on the development and characterization of ultra-deformable transferosomes of famciclovir for transdermal delivery.[11] The present study is supported by a well-organized *ex vivo* and histopathological study to establish the enhancement of drug permeation through the layers of skin.

MATERIALS AND METHODS

Materials

Famciclovir was received from Strides Pvt. Ltd., (Bangalore). Acetonitrile, methanol, cholesterol, and carbopol-940 were bought from SD Fine Chemicals Ltd. (Mumbai, India). Tween 80, soya lecithin was supplied by central drug House Pvt, Ltd. (Delhi). All other chemicals used were of analytical grade and distilled water was used throughout the study.

Methods

Design of experiments

Considering the vesicle size, polydispersity index, and entrapment of sparingly soluble famciclovir as dependent variables, the transfersomes of famciclovir were prepared by optimizing three prime independent factors of formulations, namely, phospholipid and cholesterol ratio (SPC:CH), surfactant concentration (Tween 80) (%w/v) and phase volume ratio. A Central composite design was employed using Design Expert software V11. The correlation between the independent and dependent factors was analyzed using response surface methodology (RSM). The design generated 13 experimental runs with three center points. A two-level (low (-1) and high level (+1) testing of each independent variable was done to estimate the effect of composition on the responses as listed in Table 1.[12] Table 2 explains the formulation table as per the design.

Preparation of transferosomes

Thin film hydration technique was employed to formulate the transferosomes.[13,14] To optimize the composition of transfersomes, various formulations were made using the central composite design. Specific amounts of lipids were dissolved in a mixture of organic solvents consisting of methanol and chloroform (1:3) in a dry, round-bottom flask. The evaporation of organic solvents was done under vacuum using a rota evaporator at 100 rpm, at 48-50ºC. The thin lipid film thus obtained was then hydrated using water containing surfactant and drug (1.5% w/v) by rotation for 1 hour at 50ºC. Each formulation was placed in the ultrasonicator bath at 150 W for 20 s. A dialysis bag was used to remove the free drug, the final formulation was stored at 4ºC for further use. Blank transfersomes were prepared using the same method for each formulation.

Evaluation of transfersomes

Vesicle size

The vesicle size of the prepared formulations was measured using Horiba SZ100 (Dynamic light scattering technique) at 25ºC. Samples were diluted with Millipore water as the dispersant. The measurements were performed in triplicate.[15]

Zeta potential

Zeta potential was measured using Horiba SZ100. The formulations were diluted with Millipore water before being subjected to measurements and each measurement was performed in triplicate.[16]

Entrapment efficiency

The prepared dispersion was centrifuged at 3000 rpm at 4ºC for 30 min. The transfersomes were settled as pellets, and the supernatant layer was collected to estimate the unentrapped drug. The pellet was disrupted with an equal volume of methanol in a vortex mixture for 5 min. The sample was diluted and analyzed spectrophotometrically using diluent 0.02 M potassium dihydrogen orthophosphate and acetonitrile (80:20) at 307 nm to estimate the entrapped drug. The unentrapped drug was determined from the analysis of the supernatant layer. Entrapment efficiency was calculated for all formulations in triplicate. The % entrapment efficiency is computed using the formula.[17]

$$\%\ \text{Entrapment efficiency} = \frac{\text{Entrapped Drug}}{\text{Entrapped Drug+Free drug}} * 100$$

Blank transfersomes were handled in a similar way simultaneously and used as blank to terminate the effect of excipients. All studies were carried out in triplicate.

The optimization was carried out after the evaluation of the thirteen formulations. The optimized formulation (FAMOPT) was taken for further evaluation

Fourier transform infrared spectroscopy (FTIR)

FTIR spectrophotometric analysis was carried out to investigate the compatibility between the drug and the excipients. Bruker ATR alpha technique was used to do the analysis. The spectra of pure drug, physical mixture of optimized blank, and FAMOPT were recorded at a temperature of 25.0 ± 0.5ºC by placing the samples on a zinc solenoid crystal plate over the wave number 4000 to 400 cm^{-1}. [18]

Transmission electron microscopy (TEM)

The morphology of FAMOPT was examined using a transmission electron microscope FEI Technai T20 (North America). Transferosome dispersion was placed on a paraffin sheet on which a carbon-coated grid was put to allow the sample to adhere to it. The excess sample was removed by adsorption on a small piece of filter paper. A drop of phosphotungstate (1%) was added to the grid. The samples were air-dried and imaged.[19]

Degree of deformability

The elasticity of FAMOPT vesicles was determined using extrusion method. The transfersomes were extruded at a pressure of 2.5 bar through a polycarbonate membrane (pore diameter, 0.2 microns), (Merck, India). Vesicle size was noted before and after passing through the membrane using Horiba SZ100. The degree of deformability was calculated by estimating the ratio of vesicle size before and after the extrusion process[20]. The entrapment efficiency was also determined after the extrusion method to estimate the leakage if any.

Preparation of transferosomal gel of famciclovir

The transferosomal gel of famciclovir was formulated by incorporating FAMOPT (5 %v/v) in the aqueous dispersion of carbopol 940. Carbopol 940 (0.5 %w/w) was accurately weighed and dispersed into distilled water in a beaker. Propylene glycol (7% w/w) was added to this solution. Stirring was continued at 500 rpm for 2 h and then the final pH of the gel base was adjusted to 5.5 using sodium hydroxide.[21] A gel of pure drug gel of equivalent strength to the optimized product was prepared using the same composition and was used for comparative evaluation for the drug release study.

Evaluation of gel

Physicochemical characterization of the transferosomal gel of FAMOPT

The transferosomal gel of FAMOPT was tested for appearance, feel, odor, clarity, and homogeneity.[22]

The pH of the selected gel was determined using a digital pH meter.

Viscosity measurements of prepared transferosomal gel were measured by Brookfield viscometer using spindle T-D with the optimum speed of 10 rpm at 25ºC. The measurements were performed in triplicate.[23]

Ex vivo diffusion studies of transfersomes loaded gel and skin deposition studies

The *ex vivo* diffusion studies of transferosomal gel of FAMOPT were performed using Wistar albino rat skin. The animals were euthanized with excess carbon dioxide, the abdominal skin was surgically removed. The excised skin was cleaned with saline water and placed on the receptor compartment. The receptor compartment was filled with a freshly prepared buffer solution of pH 5.5. The diffusion medium was stirred at 100 rpm, at 37.0 ± 0.5ºC. The transfersomes loaded gel (500 µl) was placed in the donor compartment and samples of diffusion medium (1 ml) were collected at different time intervals for 24 h. The samples were withdrawn at an interval of a specific time, donor cell was replenished with an equal volume of fresh medium. The samples were analyzed spectrophotometrically. A gel of pure drug of equivalent strength and the optimized drug-loaded transferosomal formulation (FAMOPT) were subjected to a comparative drug diffusion study using the same procedure.

The drug release kinetics was studied by plotting the diffusion into different rate kinetics models.

For all selected formulations the amount of drug permeated (mg/cm^2) with time was also calculated. Flux and permeation constant is calculated using the following formula. [24]

$$Jss=dq/dt$$

$$P=dq/dt\ .1/AC_D$$

Where, A- diffusion area (4.512)

dq/dt-slope of the linear region of the *ex vivo* diffusion curve

C_D- is the donor concentration

At the end of the *ex vivo* permeation study, the skin was removed from the diffusion flask, the remaining gel was swabbed, and the skin was washed repeatedly with 0.02M potassium dihydrogen orthophosphate and acetonitrile (80:20) solution to remove the surplus drug.[25]

The skin was carefully sectioned into the dermis and epidermis layer using a tweezer .[20] The separated layers were homogenized with phosphate buffer and centrifuged at 10000 rpm for 5 min, and the supernatant was analyzed by UV spectrophotometrically by spiking (known concentration 10 µg/ml) experiments to estimate the drug deposition in various layers of skin.

Skin irritation study

The skin irritation study was performed on albino Wistar rats. The back of the animal was shaved carefully. The animals were divided into three groups each containing 6 animals- control, test, and placebo. The optimized formulation was applied on the back side area of the animal for 7 days to perform an irritation study. The change in the skin colour, morphology, and the development of erythema and oedema were observed daily for 7 days of the study.[26]

Histopathological studies
The skin from the animals of Group I and Group II was taken for histopathological studies. The excised skin was dipped in 10% formalin solution, and subjected to histopathological study. The sections were observed with a light microscope equipped with digital camera using hematoxylin and eosin stains.[27]

RESULTS

Evaluation of experimental design
The experimental runs of the thirteen trials are listed in table 3. The response surface graphs showed the most statistically significant variables on the evaluated responses as shown in figure 1. The model was established for all dependent variables at a significance level of $P<0.05$ as shown in table 4. The main factors' effects on the formulation responses are listed here. It was found that lipid ratio (SPC: CH) had a significant effect on drug entrapment, while % nonionic surfactant had a remarkable effect on particle size. PDI was found to be greatly affected by both phase volume ratio and surfactant concentration as indicated in table 4.
The model optimization was carried out at a desirability of 0.77 and an optimized condition was predicted at a SPC:CH ratio of 1.44:1, surfactant concentration of 6.65 %w/v, and phase volume of 0.42, to produce drug-loaded transfersomes with high entrapment efficiency and low vesicle size and polydispersity. The optimized formulation (FAMOPT) was prepared and exhibited the properties as predicted by the design with a %bias within 10% as shown in table 5. The zeta potential of the optimized formulation was found to be -9.7mV. The zeta potential and vesicle size of the optimized formulations are shown in figure 2.

Fourier transform infrared spectroscopy (FTIR)
The characteristic peaks of $COOCH_3$ stretching, C=C, C=N, and C-0 stretching, and C-N bending of the pure drug were observed at 1745,1653,1614,1211 and 1249 cm^{-1} respectively (figure 3A). The spectra of blank formulation (Figure 3B) and FAMOPT (Figure 3C) were analyzed for compatibility.

Transmission electron microscopy (TEM)
The nanovesicles of FAMOPT were observed under transmission electron microscopy studies as shown in figure 4.

Degree of deformability
The ratio of the change in the size of the vesicles of FAMOPT was found to be less than 1 as listed in table 6, The % of leakage was calculated to be 3.46%.

Evaluation of transferosomal gel of famciclovir
The optimized formulation (FAMOPT) was dispersed in the Carbopol 940 gel matrix. The gel was found to be translucent; pH was found to be 5.5 with a viscosity of 88.6 cp. The drug content in the gel was estimated to be 69.5%.

Ex vivo diffusion studies of transfersomes loaded gel
The *ex vivo* drug release study was conducted for FAMOPT, transferosomal gel of FAMOPT, and pure drug gel of famciclovir. The formulation FAMOPT and gel of FAMOPT showed sustained release of the drug. The pure drug gel release reached a steady state at 6 h as shown in figure 5.
The permeation of the drug from the transferosomal gel and FAMOPT was found to be approximately 2.8 and 3 times respectively higher compared to the pure drug gel as listed in table 7. The drug deposition in the various layers of skin is presented in Figure 6 and it was seen that permeation of the drug from the transferosomal formulations was remarkably higher compared to the pure drug.

Skin irritation study
The skin irritation test was done for the animals such that the first group received control, the second group with the application of placebo, and the third group with the application of test formulation (transferosomal gel of FAMOPT) all animals in the test groups showed no signs of the erythema and edema as shown in figure 7.

Histopathology study
The histopathology study of the rat skin is presented in figure 8.

DISCUSSION

The central composite design was employed in the study to investigate the experimental variables at five different levels on the responses with a minimum number of experiments. The results were subjected to regression analysis using Design Expert software V13 to develop a relationship between factors and responses. The use of center points in the design increased the confidence level and helped to minimize the errors in experimentation. The response surface diagrams showed the significant effect of lipid composition, surfactant concentration, and phase volume ratio on entrapment efficiency. The vesicle size of the transfersomes was greatly affected by surfactant concentration and phase volume ratio as indicated by the extent of curvature in the response surface diagram. The effect of the main factors on the responses was evaluated with model validation statistics. The drug has good water solubility, hence entrapment of the drug in the core of the bilayer vesicle was a challenge. The bilayer should be stable enough to prevent the leakage of the drug. The nonionic surfactant was

included to contribute elasticity of the vesicle. The outcome of the main factors effect on the properties of the transferosomes signified the same.
The model equation revealed a fit summary of the factors with the responses. The positive sign in the equation is the indication of the critical parameters' significant contribution to the responses. The ANOVA test showed the model was significant for the approximation of the effects of the variables on entrapment efficiency, vesicle size, and PDI. The optimization of the model was carried out considering the high entrapment efficiency of the drug, minimizing the particle size and PDI at high desirability. The vesicle size of the FAMOPT after experimentation was found to be within the predicted range, and the surface charge indicated the stability of the vesicles with lesser aggregation and flocculation.
The compatibility study of the pure drug with the excipients was confirmed with the retention of specific peaks in the fingerprint region of the formulation. The optimized formulation could retain the major peaks of the pure drug and the excipients. Hence it establishes the compatibility between the drug and excipients.
TEM study revealed that the vesicles were spherical and without aggregation. The size range of the vesicles of the optimized product was found to be within the predicted range as per the design.
The degree of deformability of the optimized formulation was calculated by estimating the vesicle size of the formulation before and after the extrusion process through the polymeric membrane of lower aperture size. The obtained deformability index (<1) of the optimized formulation is an indication of the retention of vesicle size and also proved the minimum leakage of the vesicles. The elasticity of the prepared nanovesicles was manifested.
The physicochemical properties of the hydrogel of the optimized formulation in Carbopol 940 were found to be suitable for spreading and suitable for application over the skin.
The *ex vivo* diffusion study revealed that the drug release was very high from the transferosomal gel of the optimized formulation compared to the gel of pure drug. The diffusion of the drug from the nanocarriers was found to be much higher and more sustained compared to the gel of the pure drug. The transferosomal gel of FAMOPT was almost comparable to FAMOPT, a slight reduction in the drug release from the gel is attributed to its viscosity. The release study of famciclovir from the transferosomal gel followed first-order kinetics. The exponent of the Korsmeyer Peppas model (n) is 0.96 which indicates non-fickian anomalous diffusion of the drug from the gel.
Skin permeation studies showed significantly higher permeation of the drug from the nanocarriers, hence the penetrability of famciclovir was improved significantly.
The drug deposition in various layers represents a high deposition of the drug in the layers of skin from the gel of nanovesicles compared to the gel of pure drug and the result was in conformation with the *ex vivo* permeation study. This further established the efficient permeation of the drug into different layers of the skin from the nanovesicles.
The gel was found to be non-sensitive to the skin. Histopathology study revealed that there were no sensitization and irritations on the different layers of skin on the application of the nano gel, a near normal morphology was observed as similar to the control group. A focal inflammation on the dermal layers on the application of transferosomal gel gives evidence of penetration of nano vesiculate drug.

CONCLUSION

In this study, novel deformable vesicles of famciclovir were developed and evaluated for a nanocarrier for transdermal delivery. These nano-sized vesicles exhibited deformable properties. These properties of the vesicle could deliver the drug into the deeper layers of the skin. Moreover, the incorporation of the drug in the Carbopol 940 gel could also enhance the penetration and deposition of the drug in the dermis layer. The permeation of famciclovir was enhanced significantly from the transferosomal gel compared to a gel of pure drug. The transferosomal gel was non-sensitive, and non-irritating to the animal skin. Hence it can be concluded that transdermal application of transferosomal gel of famciclovir could be an alternative for conventional delivery in the reduction of virus load and could be a noninvasively promising technique for the treatment of cold sores and genital herpes.

DECLARATION OF CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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Table 1: List of independent factors

Factors	Low level (-1)	High level (+1)
SPC: Cholesterol	1:1	2:1
Surfactant concentration(%w/v)	5	20
Phase volume ratio	0.4	0.6

Table 2: Central Composite design for the formulation of transferosomes

Runs	Factor 1 SPC:Cholesterol	Factor 2 % Non ionic surfactant	Factor 3 Phase volume ratio
1	1	1	-1
2	0	0	0
3	-1	1	1
4	0	1.41421	0
5	0	0	0
6	0	-1.41421	0
7	-1	-1	-1
8	1	-1	1
9	-1.41421	0	0
10	0	0	0
11	0	0	-1.41421
12	1.41421	0	0
13	0	0	1.41421

Table 3: Experimental evaluation of transferosomes as per central composite design

Formulation code	%Entrapment efficiency	Particle size(nm)	PDI
F1	70.9±0.001	452.9±20.1	0.553±2.32
F2	71.6±0.096	483.0±31.2	0.73±1.45
F3	94.3±0.007	440.7±33.4	0.604±0.95
F4	49.2±0.014	508.5±11.5	0.951±1.02
F5	76.0±0.011	519.0±26.8	0.77±2.01
F6	71.0±0.172	314.2±30.4	0.196±0.95
F7	55.2±0.036	213.7±16.9	0.434±1.48
F8	62.9±0.037	415.8±25.9	0.337±1.21
F9	53.7±0.006	493.5±39.7	0.317±2.13
F10	68.9±0.007	456.0±24.4	0.750±1.06
F11	66.3±0.060	416.8±36.1	0.361±0.78
F12	86.3±0.023	344.9±19.2	0.381±1.71
F13	61.3±0.012	278.6±28.5	0.769±1.82

All the values are mean (n=3)±SD

Table 4: Model validation statistics

Response	Suggested fit summary	P value	R^2	Model equation
Entrapment efficiency	2FI	0.0243*	0.855	Entrapment efficiency = +68.33+11.51A-7.69B-1.78C-9.54AB-19.43AC+15.43BC
	A-SPC:CH	0.0147*		
	B-% Non ionic surfactant	0.0643		
	C-Phase volume ratio	0.6194		
Particle size	Quadratic	0.0456*	0.966	Particle size = +477.85-52.54A+68.70B -48.86C-96.34AB+2.67AC-106.11BC-23.21A^2-27.13B^2-58.96C^2
	A-SPC:CH	0.0579		
	B-% Non ionic surfactant	0.0296*		
	C-Phase volume ratio	0.0687		
PDI	Quadratic	0.0374*	0.970	PDI=+0.723353+0.022627A+0.0266933B+0.14425C+0.15575AB+0.170433AC+0.059627BC-0.16719A^2-0.05494B^2-0.051919C^2
	A-SPC:CH	0.6085		
	B-% Non ionic surfactant	0.0067*		
	C-Phase volume ratio	0.0359*		

*denotes significant.

Table 5: Model prediction VS. observed responses for the optimized product (FAMOPT)

Responses	Predicted	Observed	%Bias
Entrapment efficiency (%)	84.89	81.78 ±0.02	3.65
Particle size (nm)	335	340.5±23.7	1.64
PDI	0.383	0.419±1.16	9.39

All the values are mean (n=3)±SD

Table 6: Degree of deformability of the optimized product (FAMOPT)

	Size of vesicles (nm)	% Entrapment of drug
Before extrusion	340.5±0.02	84.15±0.02
After extrusion	320.33±5.05	81.23±0.05
Deformability	0.94	-
%Leakage	-	3.46

All the values are mean (n=3)±SD

Table 7: Permeation data of famciclovir after 24h

Formulation	Permeated amount of drug at 24h ($\mu g/cm^2$)	Drug flux (($\mu g/cm^2/h$)	Permeability coefficient (Kp) (cm/h)	Permeation enhanced over gel of pure drug
FAMOPT	1977	46.32	579	3
Gel of FAMOPT	1751	42.7	534	2.77
Gel of pure drug	767	19.26	192	-

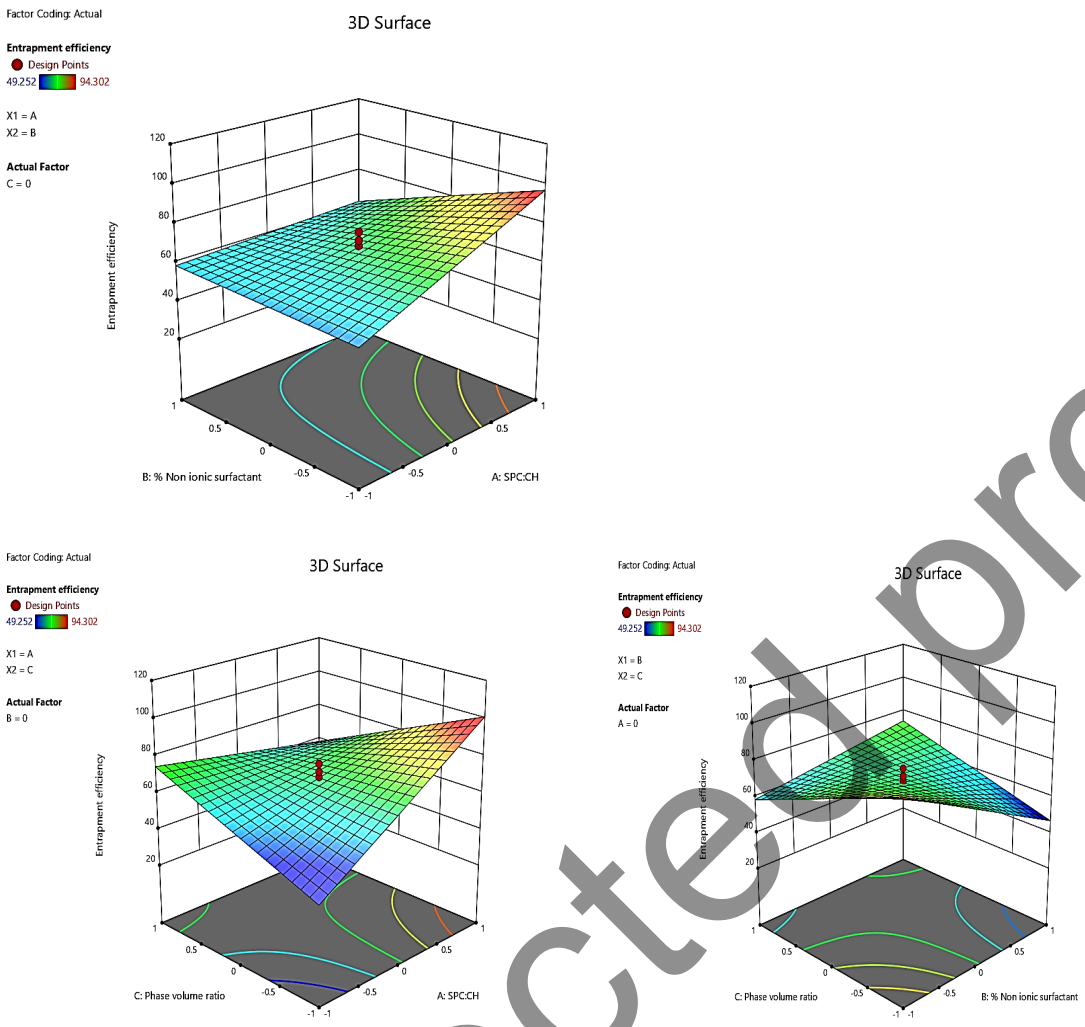


Figure 1A: Response surface diagram for entrapment efficiency(A)

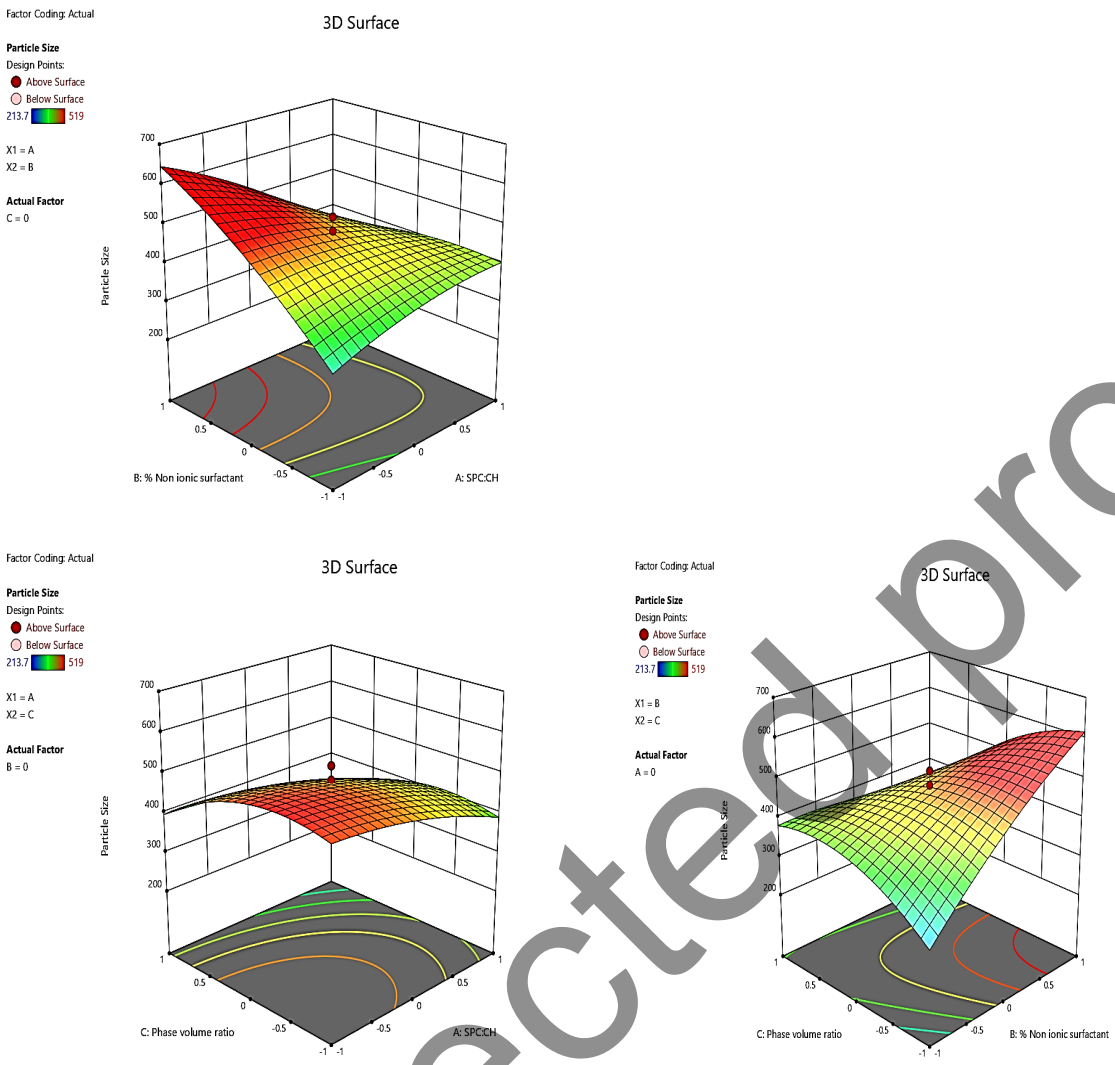


Figure 1B: Response surface diagram for particle size(B)

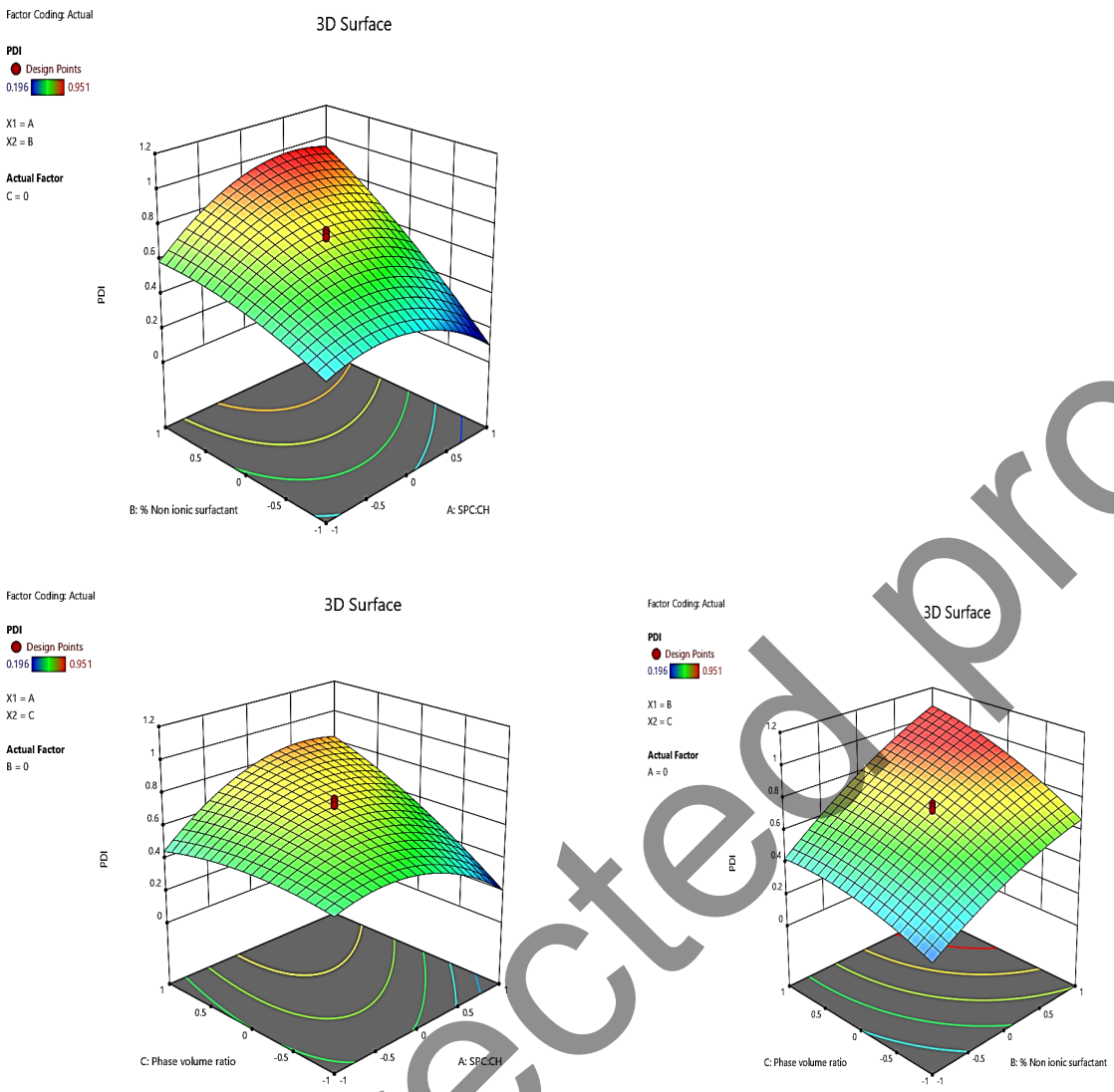
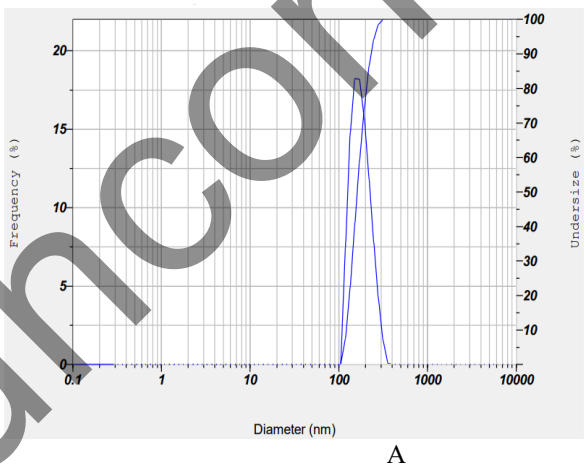
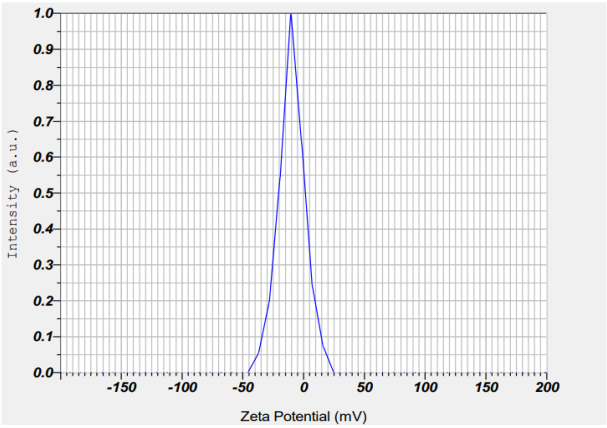


Figure 1C: Response surface diagram for PDI (C)

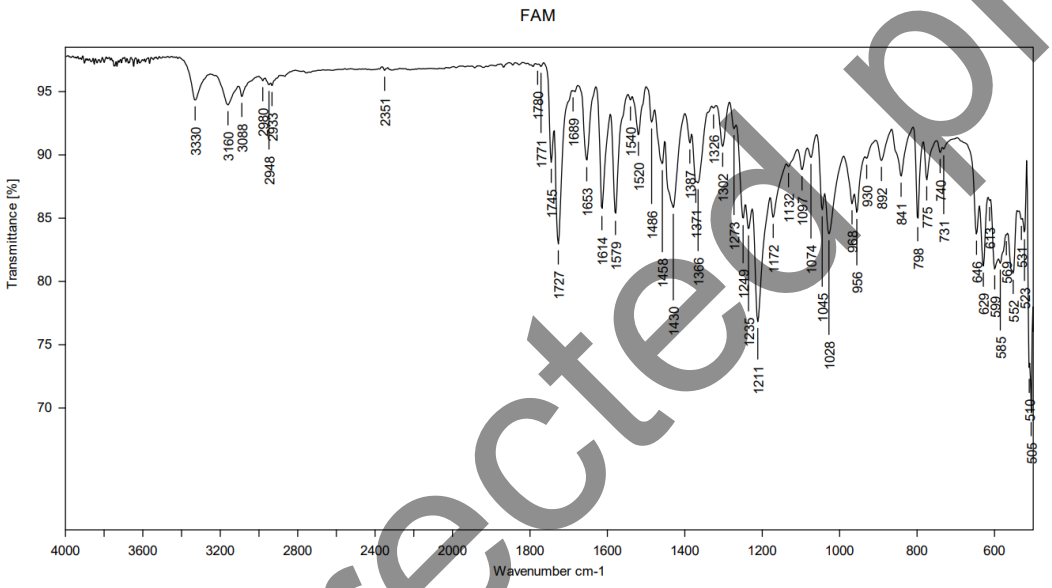


A

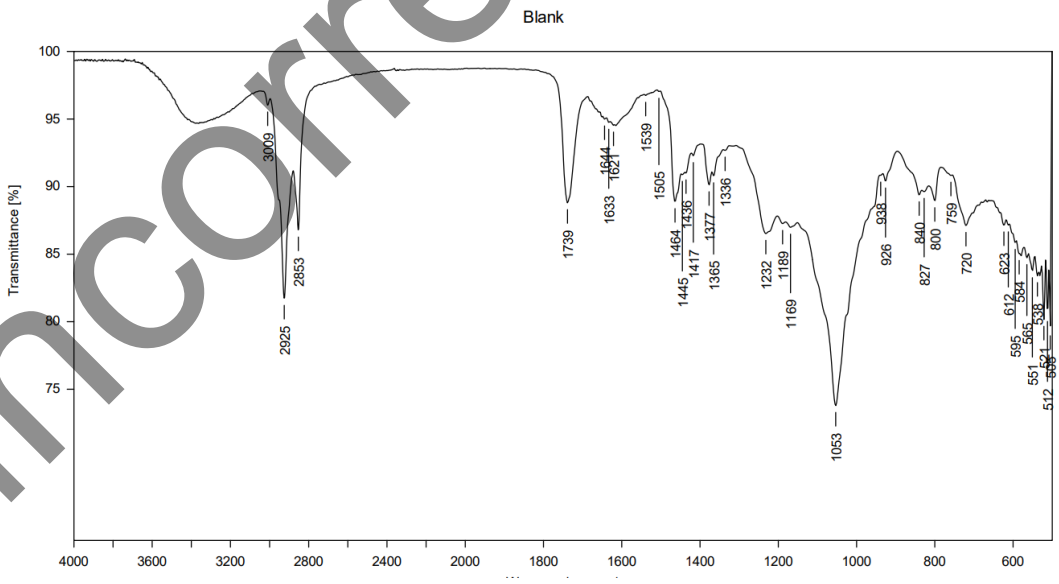


B

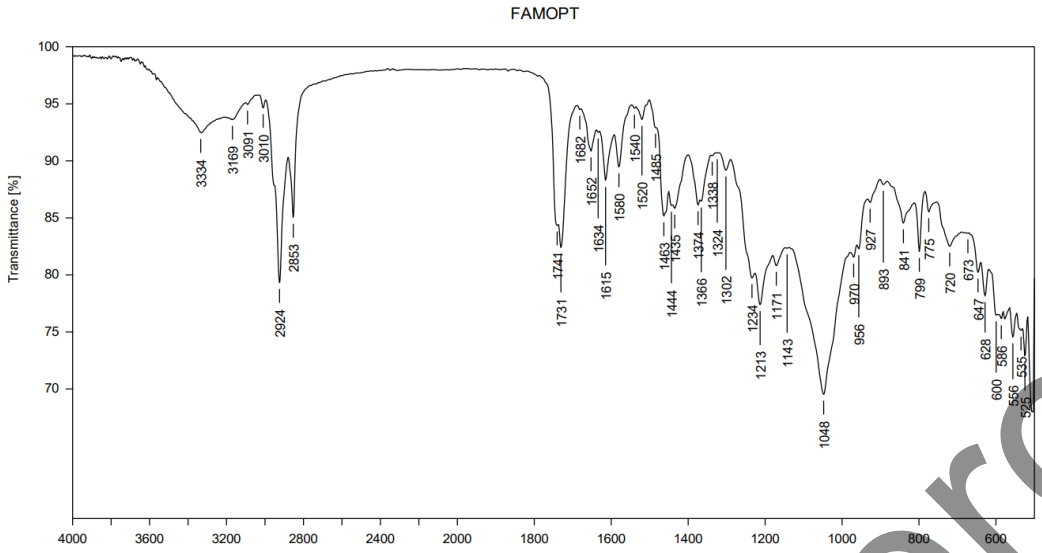
Figure 2: Particle size (A) and Zeta Potential(B) of FAMOPT



A



B



C

Figure 3: FTIR Spectra of Pure Drug(A), Blank FAMOPT(B), and FAMOPT(C)

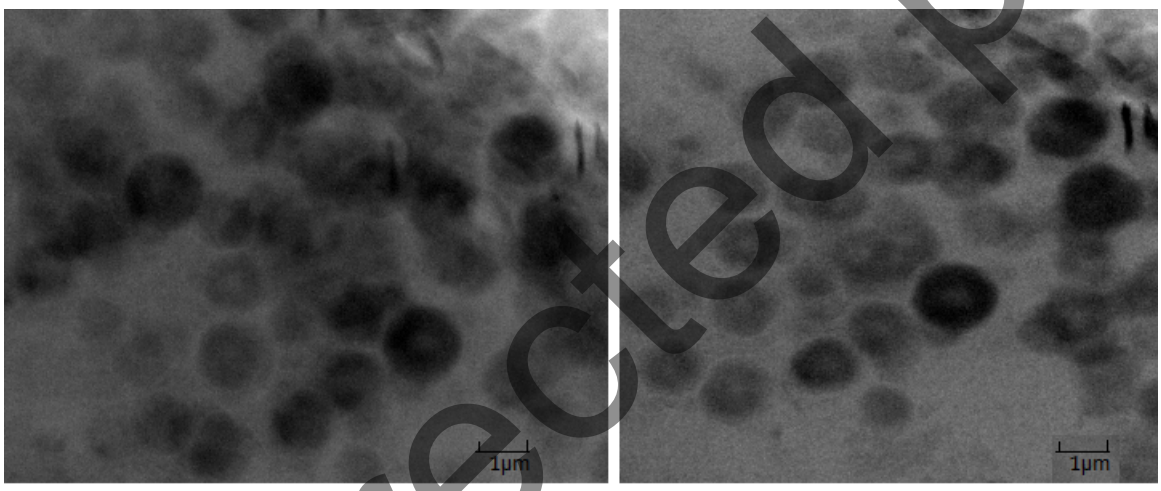


Figure 4: TEM images of transferosomes of famciclovir

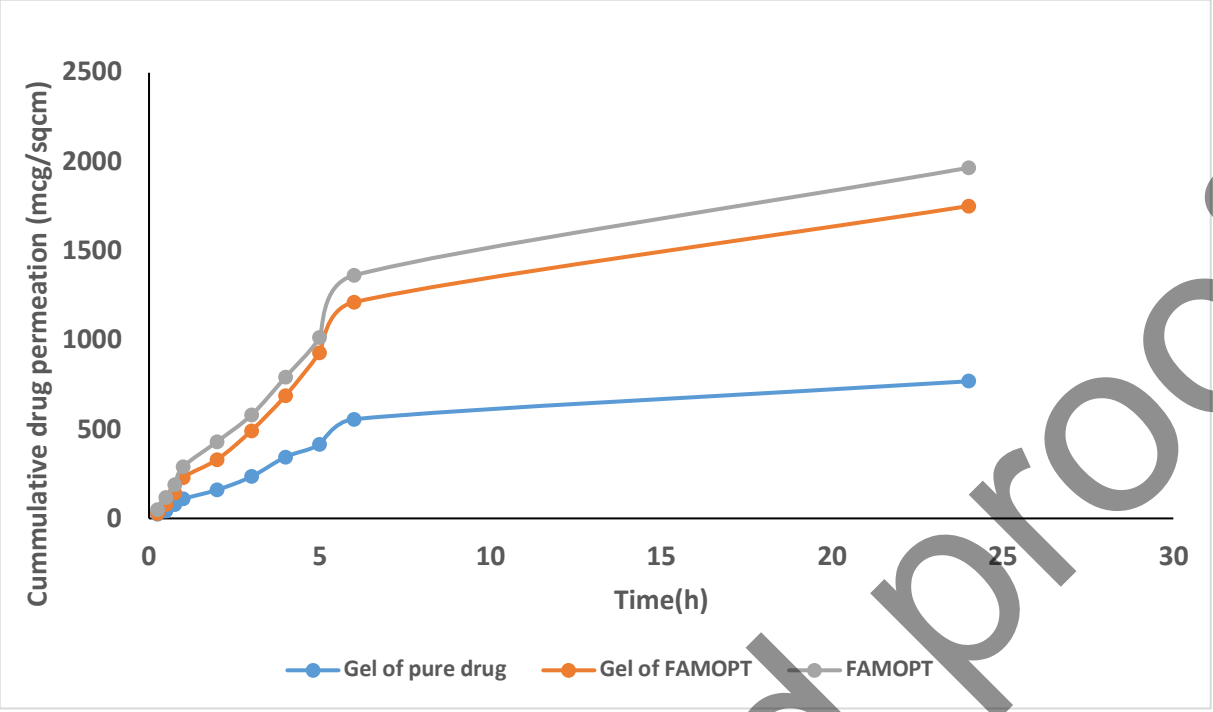


Figure 5: *Ex vivo* diffusion curve of famciclovir

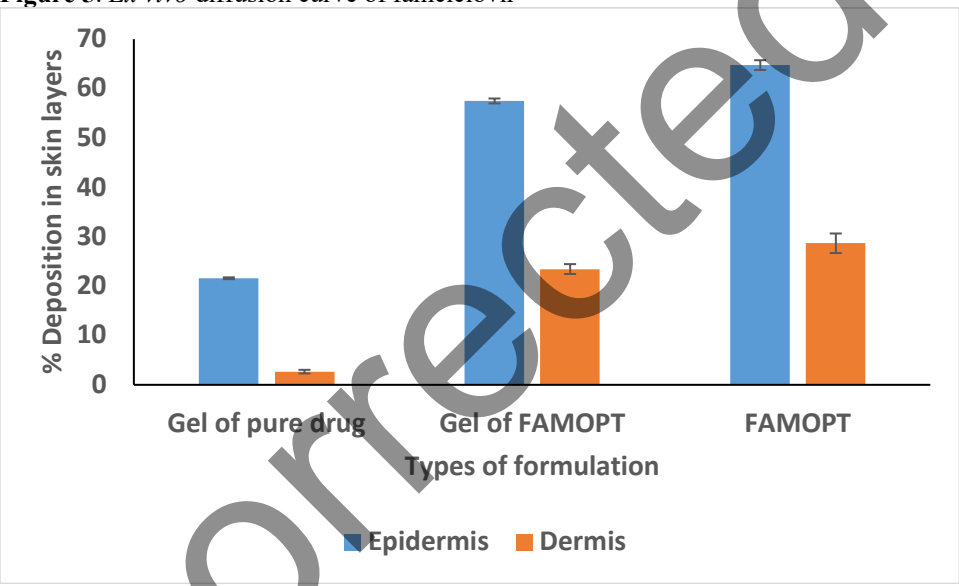
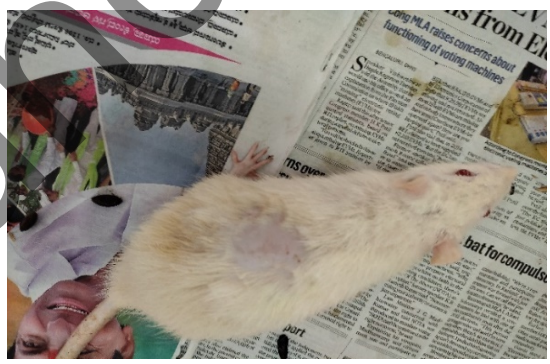
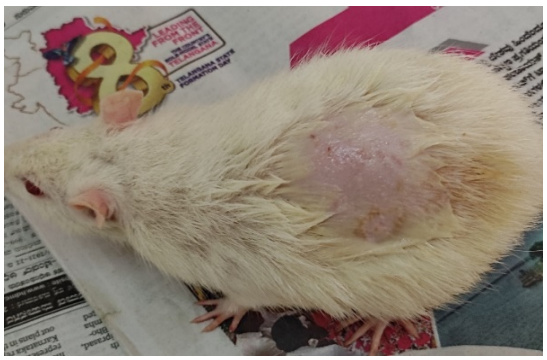


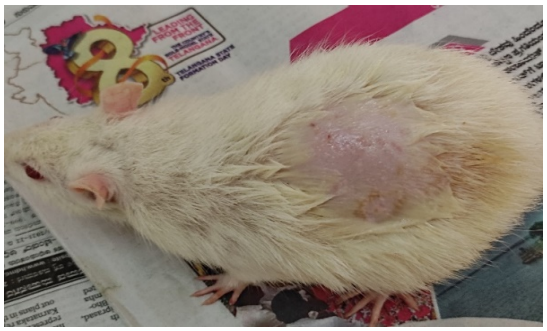
Figure 6: Estimation of deposition of famciclovir in skin layers



A

B

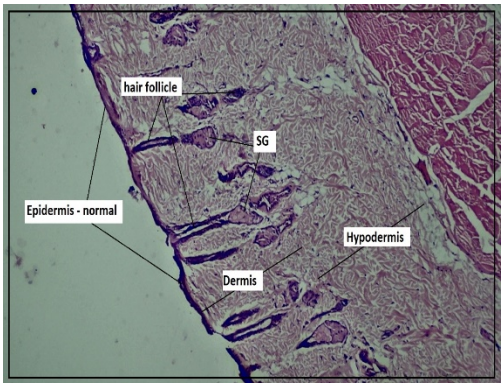




C

Figure 7: Skin irritation study for 7 days: Group 1-control(A), Group 2- placebo(B), and Group III (C) test formulation (gel of FAMOPT)

A



B

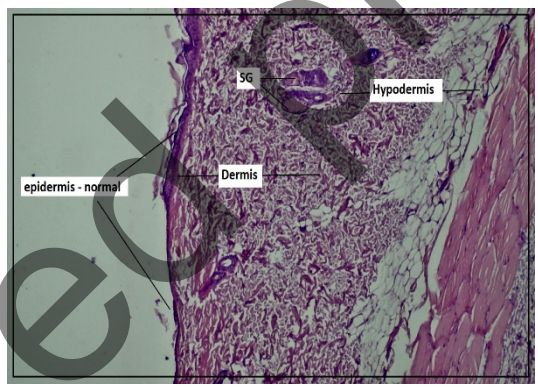


Figure 8: Histopathological Examination of rat skin (A): Control and (B): After application of transferosomal gel