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Research Article

EFFECT OF NATURAL BIOENHANCERS ON SKIN PERMEATION: A JOURNEY FROM ORAL TO TOPICAL

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ABSTRACT

The current focus of the researchers worldwide is to improve the health care therapies by reducing side effects and increasing the specific effects of the drug candidates. The current research work is a dig in this research area as novel concept of bioenhancers has been introduced for the skin permeation of drug candidates. The prime objective of the current research work was to explore the natural bioenhancers to increase the skin permeation. In this research, work the amount of acyclovir permeation increases at the site of herpes virus simplex infection, using the natural compounds. In this work, cream of acyclovir prepared to study the effect of different concentration of three different natural bioenahncers quercetin, silibinin and luteolin. The distribution of the acyclovir in the skin was determined using different experimental protocols including in vitro skin permeation study, which includes skin retention study, *Ex-vivo* skin permeation studies, HaCat cell lines study with MTT assay. The results of the above mentioned experimental studies showed that, the amount of acyclovir with the natural bioenhancers was approximately two folds than the plain acyclovir cream. This type of synthetic and natural therapy can improve acyclovir therapy for the patient.

Keywords: Acyclovir; Cream; Topical; Quercetin; Silibinin; Luteolin

INTRODUCTION

Acyclovir (ACV), is an analogue of 2'-deoxiguanosine and it is highly selective and potent inhibitor of the herpes virus group (Sweetman, 2009). ACV is available in market in different commercial formulations such as oral, intravenous and topical. As the major issue with the molecule is its lower oral bioavailability (~20%) and also having some severe side effects when administered systemically such as nephrotoxicity. Consequently, the most desirable route for such molecule is topical as it can resolve such issues of bioavailability as well as system side effects (Parry, *et al.*, 1992).

Skin plays a vital role as the protective barrier for the vital organs of the body preventing them from the physical, chemical and microbial attacks from the environment. The pathophysiology of the skin has been studied from decades to understand the skin diseases and their effective treatments (Freeman, *et al.*, 1986). It is well known fact that the topical drug delivery has many advantages over oral as it lower the variability in the drug plasma concentration, increase the targeting of the active ingredient for the local effects, it also by-

pass the first pass metabolism hence providing a good patient compliance (Bouwstra, at al., 2002). As described earlier the nature of the skin is protective barrier, so it makes difficult for the major drug classes to penetrate in to the layers and permeate through it (Volpato, *et al.*, 1995). Researchers from the very beginning of the topical drug delivery have been interested in exploring the new protocols or techniques to enhance the drug absorption through this protective barrier and numerous researches has been carried out. However, up to our knowledge till date the natural bioenhancers has not been incorporated in topical drug delivery to increase the permeation of the active ingredient from the protective barrier (Volpato *et al.*, 1995 and Volpato, 1998). In the case of ACV, the topical administration leads to the tenfold higher concentration as compare to the oral administration (Parry, *et al.*, 1992). Although literature suggested that this concentration does not produces desired therapeutic effect (Freeman, *et al.*, 1986). Hence, it is crucial to enhance the penetration of ACV (upto dermis), while maintaining the normal skin barrier function (Shim, *et al.* 2014).

The research envisaged before this proposed work it has been found that efficacy of the ACV topical therapy is low as due to the lack of the penetration of the adequate amount of ACV at the site of action (Volpato, 1998). Researches have tried several approaches for the development of the suitable topical preparation for ACV with an improved efficacy. These different studies carried out by different researcher in the different parts of the world includes several approaches such as a study carried out with different vehicles demonstrates improved therapy (Gide, *et al.*, 2013). The other approaches used by researcher are iontophoresis (Spruance, *et al.*, 1982), percutaneous absorption enhancers (Berthold, *et al.*, 1998) and site specific drug delivery was also another strategy used to improvise the therapy using the particular drug carriers (Chen, *et al.*, 2006).

The prime and major objective of this work was to increase the permeation of the ACV and to explore the natural bioenhancers in the area of topical treatment as literature suggested these molecules has never been used in topical preparations. In this proposed work it has been hypothesized that natural bioenhancers when applied topically with ACV there is the improvement in drug permeation. The hypothesis was studied by using different concentrations of three different natural bioenhancers such as quercetine (QU), silibinin (Sil) and luteoline (LT).

MATERIAL AND METHODS

Material and chemicals

The Model drug ACV was procured from Nestor Pharmaceuticals, New Delhi. The quercetin, silibinin and luteolin were purchased from the sigma Aldrich. All solvents used were of HPLC grade. All other chemicals and reagents were procured from a local supplier and were of AR grade unless mentioned. Deionized double distilled water was used throughout the study.

Preparation of cream

An aqueous cream was prepared using ACV: 250 mg, Cetostearyl alcohol: 337.5 mg, White soft paraffin 625 mg, Liquid paraffin 250 mg, Propylene glycol 2 gm, Purified water (to) 5 gm QU, Sil and LT (Different Concentrations). Different concentrations of bioenhancers have been used in all formulations. In the prepared creams, concentration of QU, Sil and LT was in the range of 1%-5% w/w of ACV. A part of ACV (50mg) and bioenhancer was dissolved in water and propylene glycol at ambient temperature to produce an aqueous solution. The paraffin's and emulsifiers were mixed together and heated to 60°C and emulsified with aqueous solution also at 60°C, using a laboratory mixer. The remaining ACV was added, the mixture dispersed, allowed to cool, and store. Details of different excipients and bioenhancer concentration used have been illustrated in Table 1.

Table 1: Preparation of different creams with natural bioenhancers.

Formulation code	ACV (mg)	bioenhancer	CA[#] (mg)	SLS* (mg)	WSP[$] (mg)	LP[@] (mg)	PG[&] (gm)	Water (to gm)
Acv	250	NA	338	37.5	625	250	2	5
ACSI-1	250	Silibinin 1 %	338	37.5	625	250	2	5
ACSI-2	250	Silibinin 2 %	338	37.5	625	250	2	5
ACSI-3	250	Silibinin 3 %	338	37.5	625	250	2	5
ACSI-4	250	Silibinin 4 %	338	37.5	625	250	2	5
ACSI-5	250	Silibinin 5 %	338	37.5	625	250	2	5
ACQU-1	250	Quercetin 1 %	338	37.5	625	250	2	5
ACQU-2	250	Quercetin 2 %	338	37.5	625	250	2	5
ACQU-3	250	Quercetin 3 %	338	37.5	625	250	2	5
ACQU-4	250	Quercetin 4 %	338	37.5	625	250	2	5
ACQU-5	250	Quercetin 5 %	338	37.5	625	250	2	5
ACLU-1	250	Luteolin 1 %	338	37.5	625	250	2	5
ACLU-2	250	Luteolin 2 %	338	37.5	625	250	2	5
ACLU-3	250	Luteolin 3 %	338	37.5	625	250	2	5
ACLU-4	250	Luteolin 4 %	338	37.5	625	250	2	5
ACLU-5	250	Luteolin 5 %	338	37.5	625	250	2	5

Drug content studies

Dummy cream formulations prepared in lab was analyzed for drug content using validated LC-MS method as per ICH guidelines. Chromatographic separation of acyclovir was effected on C18 column (Milford, MA) using a isocratic mobile phases (60:40): A, 2 mM aqueous

ammonium acetate with 0.1% formic acid and B, 100% methanol with 0.1% formic acid having a flow rate of 1.0 ml/min.

Spreadibility

Spreadibility of the formulated cream was determined, by measuring diameter of 1gm cream between horizontal plates after 1min. standardized weigh on the upper plate was 125 gm. The spreadibility was calculated using formula:

$$S = \frac{m.l}{t}$$

In vitro skin permeation studies

In vitro permeation study has been carried out to explore the effect of the bioenhancers on the permeability of the ACV cream. The abdominal hair of wistar rats was removed using hair remover cream 24 h before use in the experimentation. After anaesthetizing the rat with chloroform the abdominal skin was surgically removed from the animal and adhering subcutaneous fat was carefully cleaned with hot water cotton swab and kept in freeze. Finally the skin was taken and examined carefully using microscope to ensure that is free from surface irregularity. Skin permeation is the diffusion of the drug across the skin layer into the receptor phase which represents blood vessels. Skin permeation of ACV, ACV-QU, ACV-Sil and ACV-LT at different concentration level was studied using locally fabricated Franz diffusion cell with an effective permeation area and receptor cell volume of 1.0 cm^2 and 10 ml, respectively. The temperature was maintained at 37 ± 0.5°C. The receptor compartment contained 10 ml PBS (Phosphate Buffer Solution) (pH 6.4) containing sodium azide (0.05% w/v) as preservative and was constantly stirred by a magnetic stirrer at 100 rpm. Sample of the receptor phase were collected up to 24 hrs. An aliquot of 1 ml sample was withdrawn at suitable time intervals and replaced immediately with fresh volumes of diffusion medium. The samples were analyzed using above mentioned LC-MS method after suitable dilutions.

Skin Retention Study

At the end of the permeation experiments (after 24 h), the remaining formulation in the donor phase was scrapped off the skin, and the exposed skin surface was rinsed with water/DMSO (1:3) to remove excess drug from the surface. The receptor media was then replaced with fresh water/DMSO (1:3). Receptor contents were allowed to stir for the next 24 h. After 24 h, the media was analyzed for the amount of drug retained in skin.

Ex vivo skin permeation studies

Ex vivo studies has been carried out on the wistar rats. The hairs of the abdominal part of the

rats has been removed 24 h before the experimentation. After anaesthetizing the rat with chloroform the abdominal part was divided in to two-four different section each having area 1 cm^2. Then the different formulations were applied on the abdominal part in each section carefully and equally force. One section in each rabbit is kept as blank for the analysis. After 60 mins the skin samples were collected from the rat. The skin sample collected from the rats were analysed for the ACV using the LC-MS method. The amount remain unabsorbed has been calculated and reported.

HaCat cell line

Human normal skin keratinocyte cell line (HaCaT), was maintained in Dulbecco modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. The FBS for culturing HaCaT cells was heat inactivated for 30 mins at 55°C. The cells were maintained at 37°C in a humified atmosphere with 5% CO_2. Second or third passage HaCaT cells were plated on coverslips in a 12 well plate at a density of about 5,000 cells/cm^2. After overnight incubation at 37°C in a humified 5% CO_2 incubator, cells were supplemented with fresh DMEM medium containing 10% FBS. Solution of ACV (10 µM) and QU, Sil and LT (range 2 µM–10 µM) has been prepared in PBS buffer, followed by serial dilution in DMEM to obtain solutions of different concentrations. The 100% confluent cells in 12 well plate were treated with solutions containing concentrations of ACV and QU, Sil and LT. A control without addition of any solution was also kept. The cells were cultured under the conditions described above for 24 hr. The growth of cells was monitored on an inverted-phase microscope. All concentrations were used in triplicate.

MTT assay

The effect of ACV, QU, Sil and LT on the HaCat cell lines toxicity was performed using MTT assay. The HaCaT cell proliferation on treated and untreated cell was determined after 4 days of culturing by MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is yellow, to a purple formazan product]. A volume of 10 µL of 12 mM MTT was taken for cell incubation performed at 37 °C for 4 h in the darkness. The media were then decanted and washed with phosphate-buffered saline solution (PBS). The produced formazan salts were dissolved with dimethylsulphoxide (DMSO, Sigma-Aldrich, USA), and the absorbance was measured at 570 nm to estimate the formazan concentration.

RESULTS AND DISCUSSION

Drug content studies

Dummy cream formulations prepared in lab was analyzed for drug content using validated LC-MS method as per ICH guidelines. The Mass spectra of ACV have been illustrated in Figure 1. The amount of drug found in the different creams has been in the range of 98-101%. The amount of drug founded shows that cream passes the assay test and can be used

for the further studies. The result of assay for different formulations has been compiled in the Table 2.

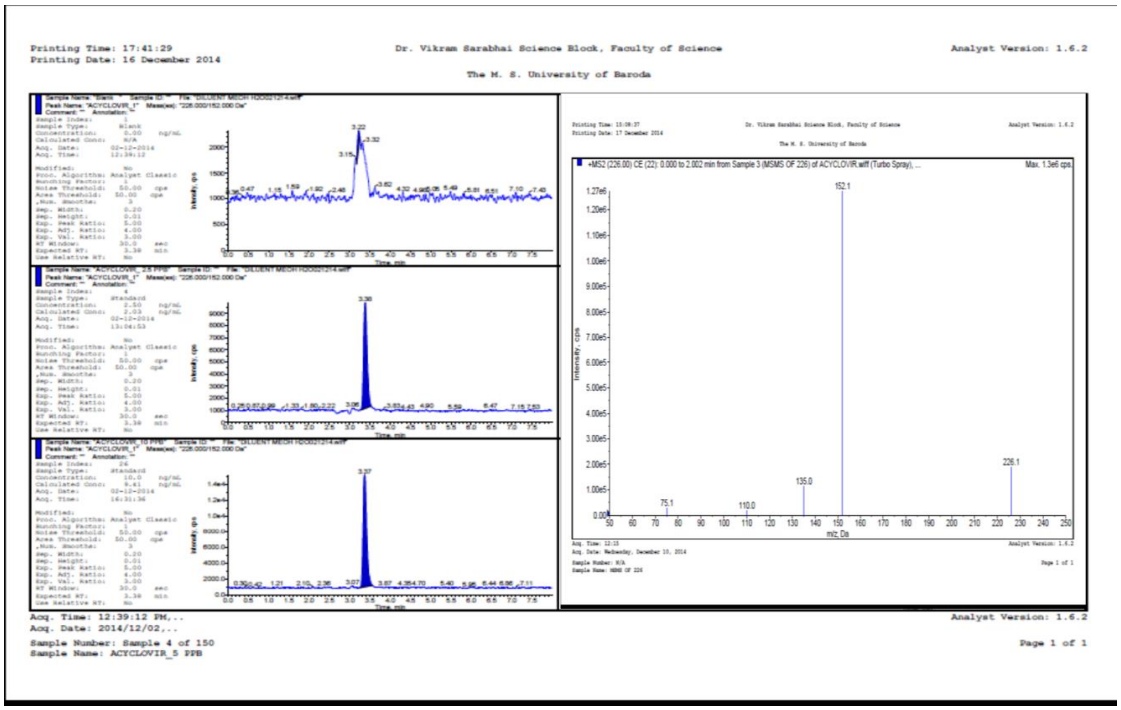

Figure 1: LC-MS Chromatogram of ACV.

Table 2: Results of drug content and spreadability studies of different creams.

Formulation code	Drug Content (%)	Spreadability
Acv	99.42	17.7
ACSI-1	99.62	17.8
ACSI-2	99.45	17.8
ACSI-3	99.62	17.8
ACSI-4	98.96	17.9
ACSI-5	99.12	17.7
ACQU-1	99.36	18.2
ACQU-2	100.02	18.1
ACQU-3	99.48	18.2
ACQU-4	98.89	18.3
ACQU-5	99.23	18.1
ACLU-1	100.07	18.2
ACLU-2	99.56	17.9
ACLU-3	98.72	17.9
ACLU-4	99.34	18.1
ACLU-5	99.15	17.8

Spreadibility

Spreadibility of the formulated cream was determined and found to be satisfactory in the range of 17.6 -18.4 for the different formulations. The results of the spreadability have been summed up in Table 2.

In vitro skin permeation studies

The results of the *in-vitro* skin permeation studies show there is a significant increase in the amount of the ACV permeated in the skin in the presence of QU, Sil and LT. The results show that there was maximum increase in the permeation of ACV in the formulation having 4% of QU. There is 1.98 fold increase in the permeation of ACV using 4% of QU, while in the combination of ACV-Sil and ACV-LT maximum enhancement was achieved at 2% and 1% having 2.14 and 1.57 fold increase in the flux of the ACV respectively. The Comparison of mean cumulative amount of drug permeation and flux has been illustrated in Figures 2 and 3, respectively.

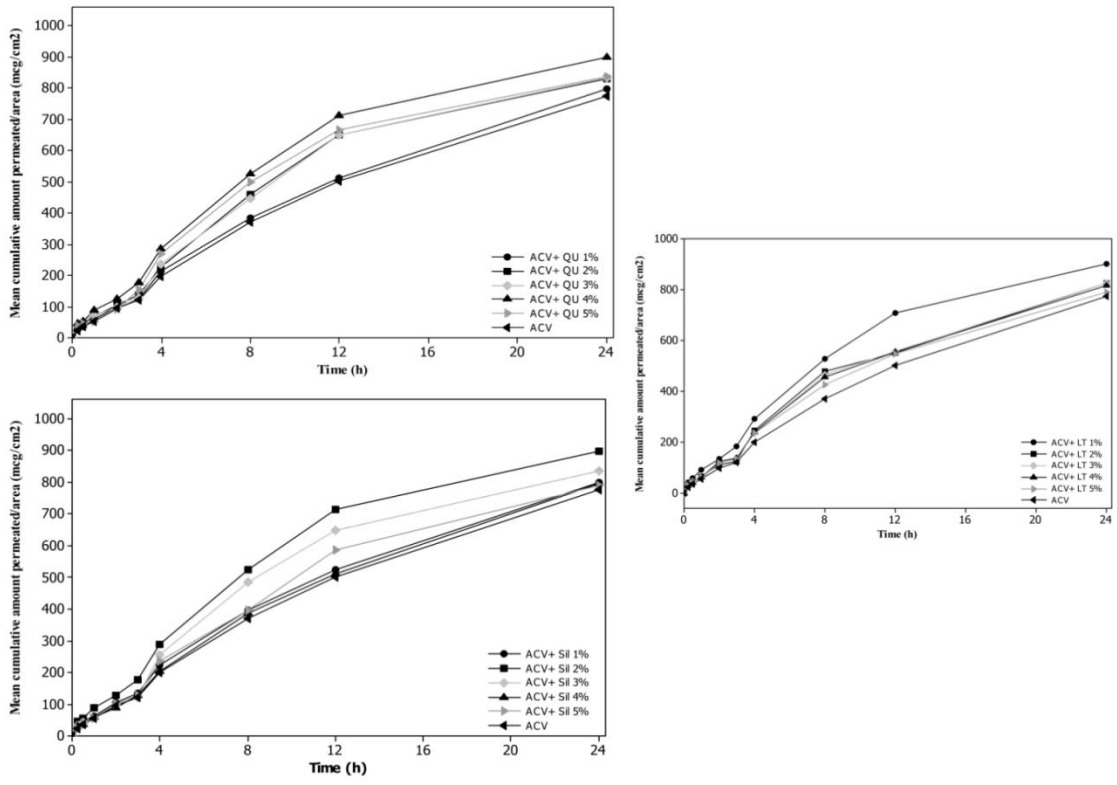

Figure 2: Comparison of mean cumulative amount of ACV released per unit area of skin.

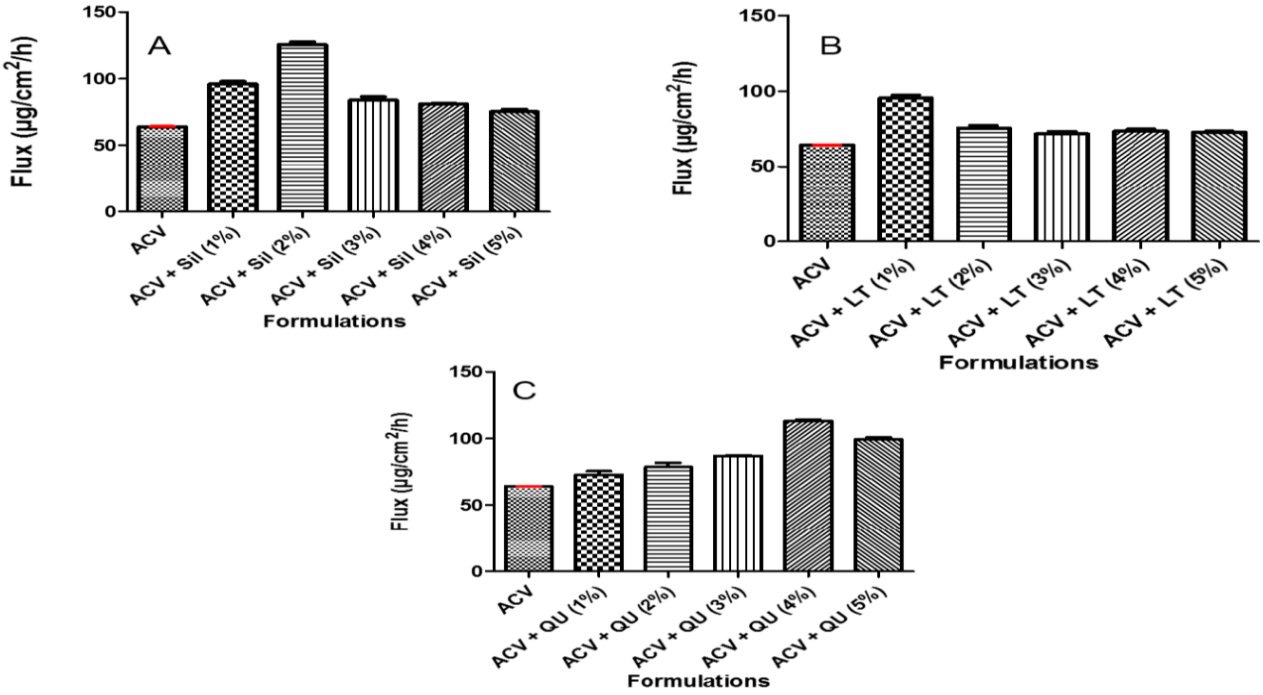

Figure 3: Comparison of Flux of ACV in different dummy formulations.

Skin Retention Study

The amount of drug retained in skin was significantly higher in all the prepared creams using QU, Sil and LT as compared to the cream without bioenhancers. The comparison of the amount retained in the skin has been illustrated in Figure 4.

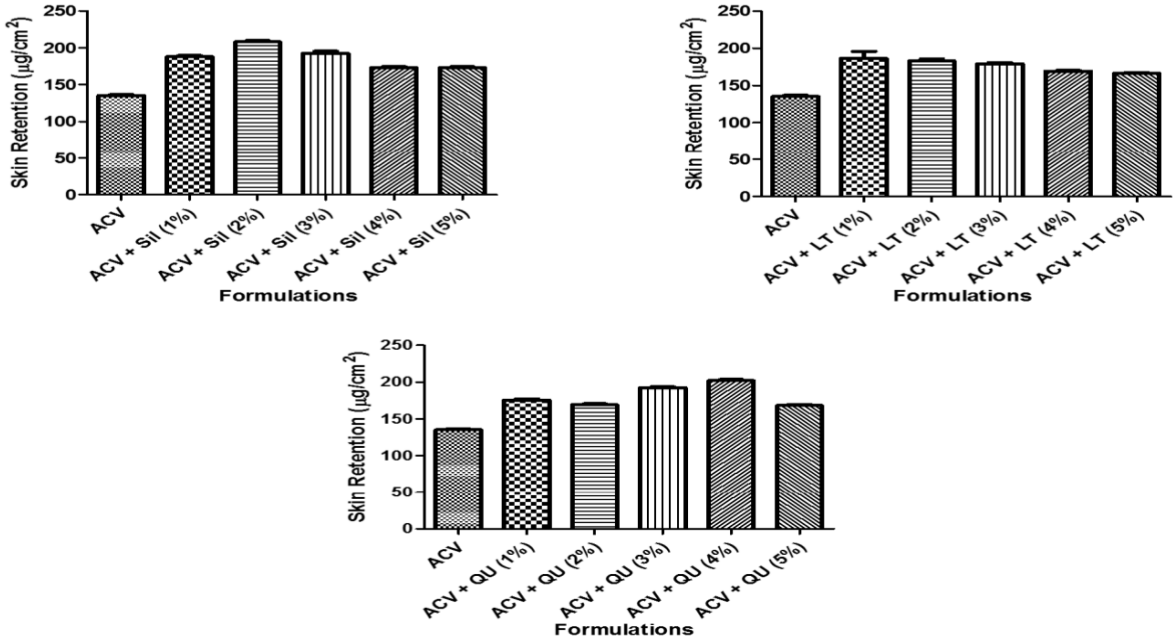

Figure 4: Comparison of amount of drug retain in the skin.

Ex vivo skin permeation studies

In the *Ex vivo* skin permeation studies the unabsorbed ACV has been estimated using LC-MS method. The unabsorbed drug in the plain ACV cream was found to be at the higher side as compared to the other creams. The Figure 5 shows the application of the cream on the abdominal part of the rat. The upper layer of the skin was washed out using 0.1 N Hcl and the ACV drug was estimated. Figure 6 shows the comparison of the unabsorbed drug in the plain and the cream containing different concentration of bioenhancers. The unabsorbed ACV in the cream containing bioenhnacers was approx. 40% of the drug as compared to the ACV plain cream.

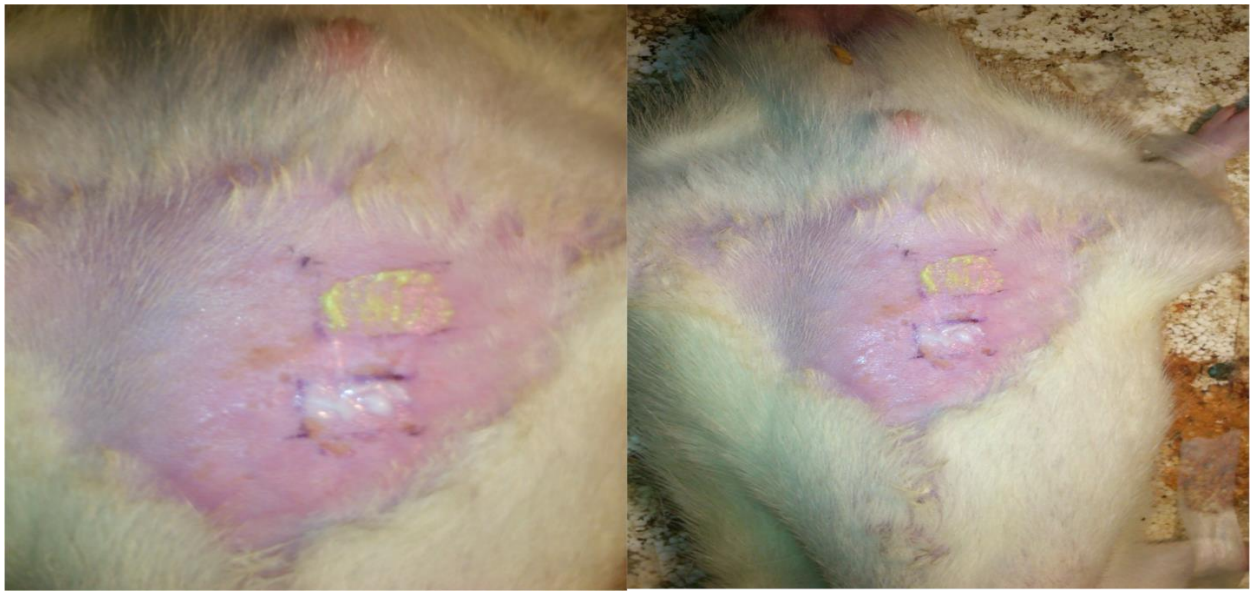

Figure 5: Application of the cream on the abdominal part of the rat.

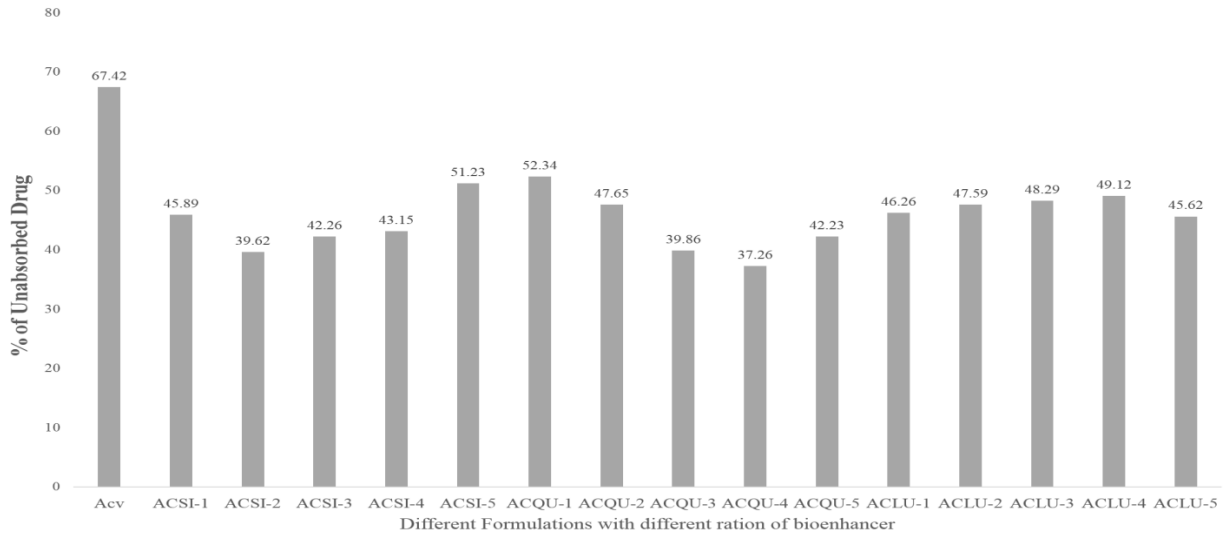

Figure 6: Comparison of the % unabsorbed drug in the ex-vivo studies.

HaCat cell line

The *in-vitro* cell line studies show there is a significant increase in the permeation of the ACV in the presence of QU, Sil and LT. In ACV-QU the maximum enhancement was found at 6 µM having 1.36 fold enhancement ratio of the ACV. While in the ACV-Sil and ACV-LT maximum enhancement was at 4 µM and 2 µM having 1.41 and 1.23 fold increase in the concentration of the drug respectively. Comparison of amount permeated of ACV using different concentrations of LT, QU and Sil has been shown in Figures 7-9, respectively.

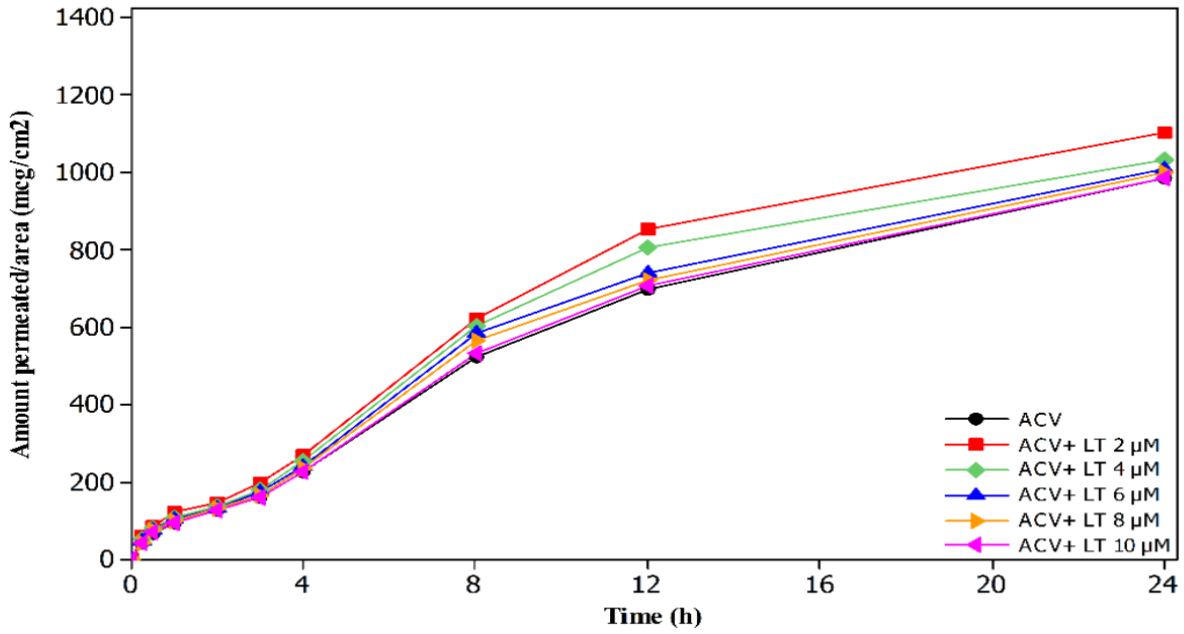

Figure 7: Amount of drug permeated in HaCat cell lines at different concentrations of LT.

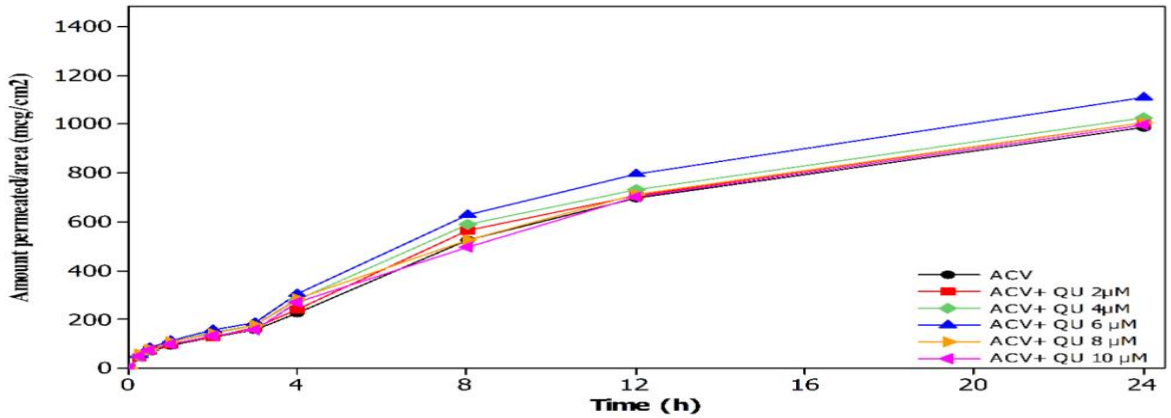

Figure 8: Amount of drug permeated in HaCat cell lines at different concentrations of QU

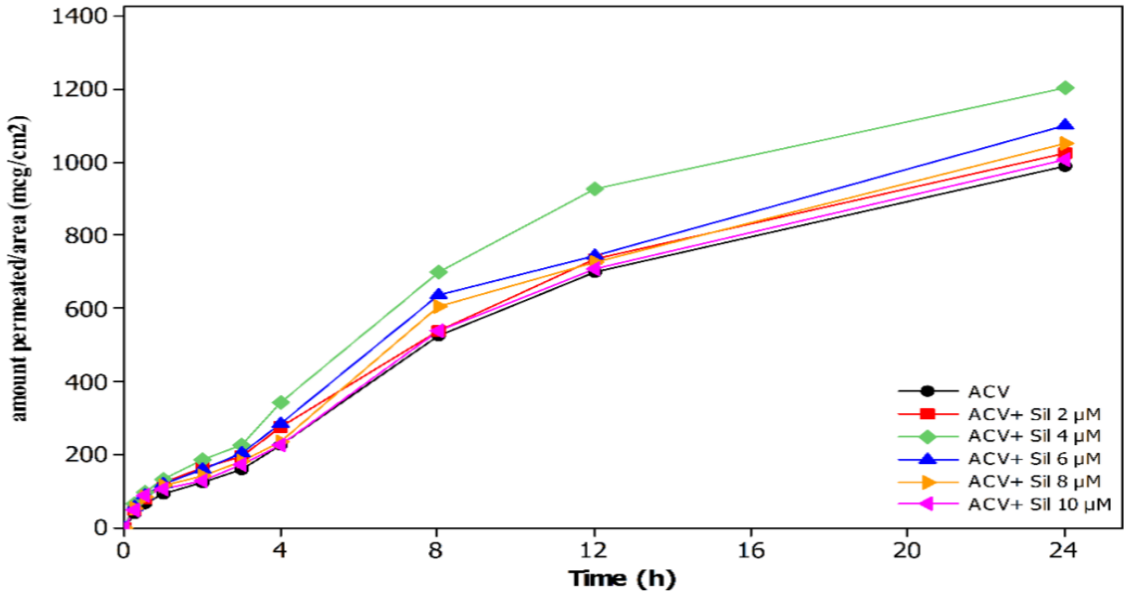

Figure 9: Amount of drug permeated in HaCat cell lines at different concentrations of Sil.

MTT Assay

In the MTT assay, there was no significant catalytical activity shown due to dead or damaged cells. The duration of MTT assay was similar as permeation studies 24 h. As shown in Figure 10, neither individual bioenhancers nor ACV showed significant cell death or cytotoxicity. All the excipients tested showed cell viability >90% revealing no cytotoxic effect of bioenhancers and excipients on cell monolayer during the 24 h incubation period.

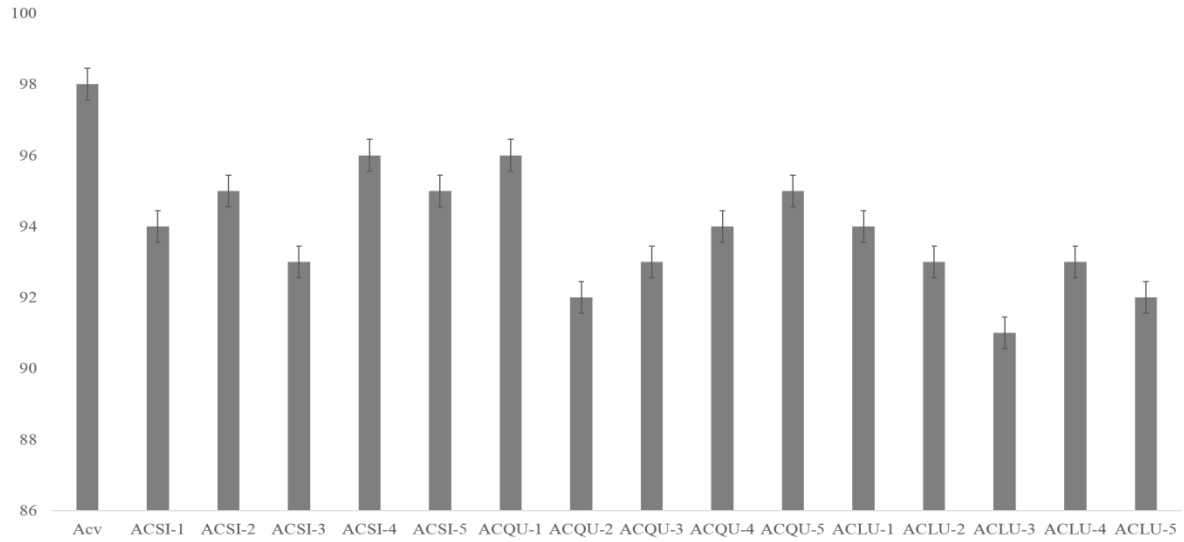

Figure 10: MTT studies showing % cell viability of HaCat cells after exposure to different samples (n=6).

DISCUSSION AND CONCLUSION

Cream formulations containing QU, Sil and LT shows an increase in the permeation of ACV as compare to the control in rat skin as well as in cell lines. The observations shows more increase in the flux of ACV at low concentration Sil (2%) and LT (1%) as compared to the high concentration (3, 4 and 5%). While in the QU more increment was observed at the higher concentrations (4%) as compare to lower. These promising observations can excited the researchers to further explore the exact mechanism of the bioenhancers effect on the skin permeation. The incorporation of these bioenhancers in the topical therapy can improve the patient compliance and therapy for Herpes simplex virus. The MTT assay observations also supports the previous observations as from MTT assay it has been observed that there is no toxicity due to QU, Sil and LT. The irritation studies revealed that no irritation caused by the bioenhancers on the skin, hence they are safer for the topical delivery. These promising observations from the all studies carried out excited the researchers to further explore the exact mechanism of the bioenhancers effect on the skin permeation as these molecules can dig up a new era for the topical delivery of the drugs with poor permeation and improve the therapy. The incorporation of these bioenhancers in the topical therapy of herpes simplex virus can improve the patient compliance and therapy for Herpes simplex virus. These studies open several paths for the researcher to expand and improve the topical therapy.

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CONFLICT OF INTEREST

The Author declares no conflict of interest

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