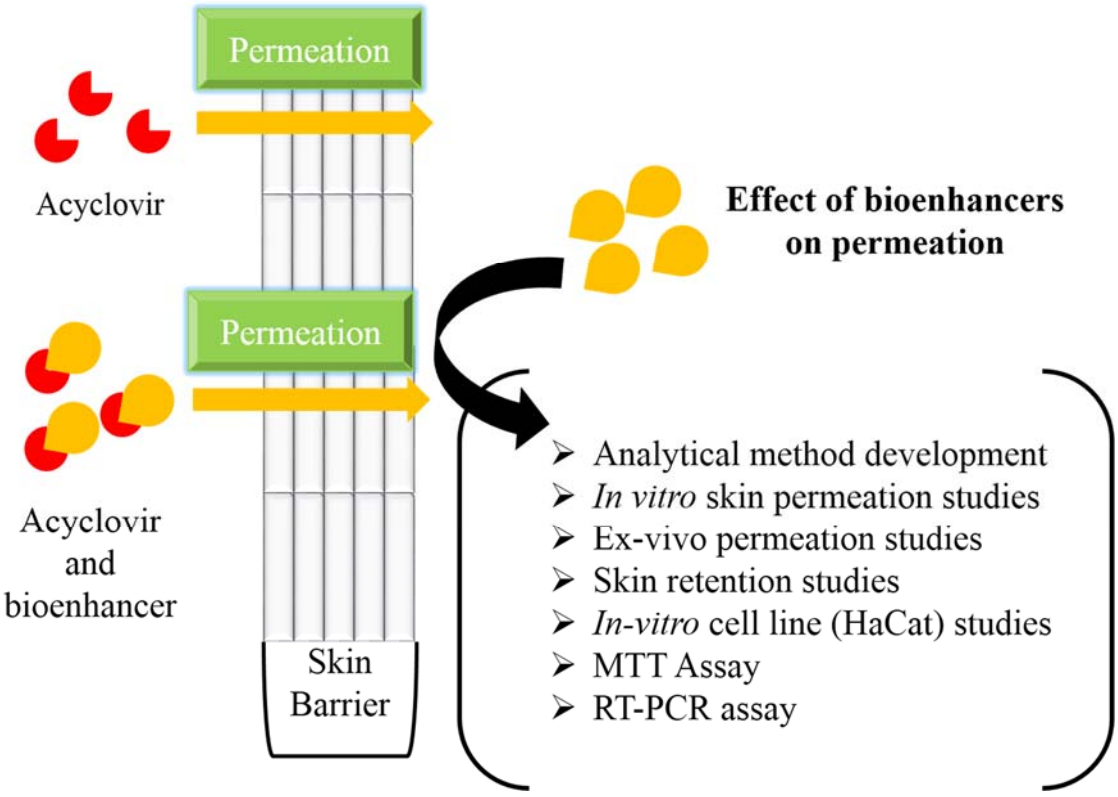
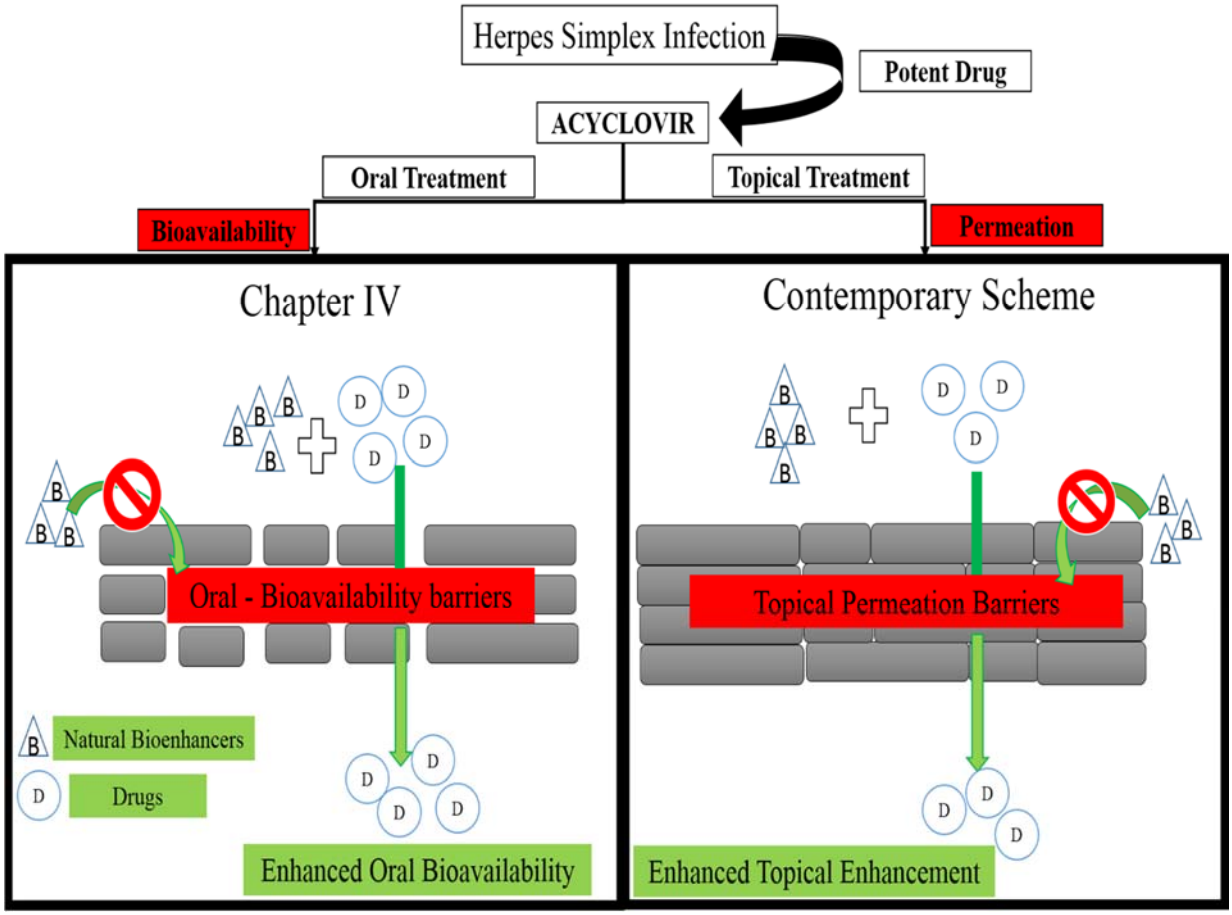
Graphical Presentation of Chapter VI



Natural bioenhancers journey from oral to topical

In the dissertation research work primarily bioenhancers were included to increase the bioavailability of the ACV and SQU. During the research work it was observed that bioenhancers shows a good results in the oral treatment. The acyclovir has also been available in market as topical preparation (skin cream) for the treatment of herpes simplex. To explore the effect of natural bioenhancers on the topical permeation few studies were added up in the research work. To carry out this work a cream containing different concentrations of QU, Sil and LT has been prepared and evaluated for different parameters. Then these prepared creams were used in different experiments for estimation of ACV permeation in the presence of QU, Sil and LT.



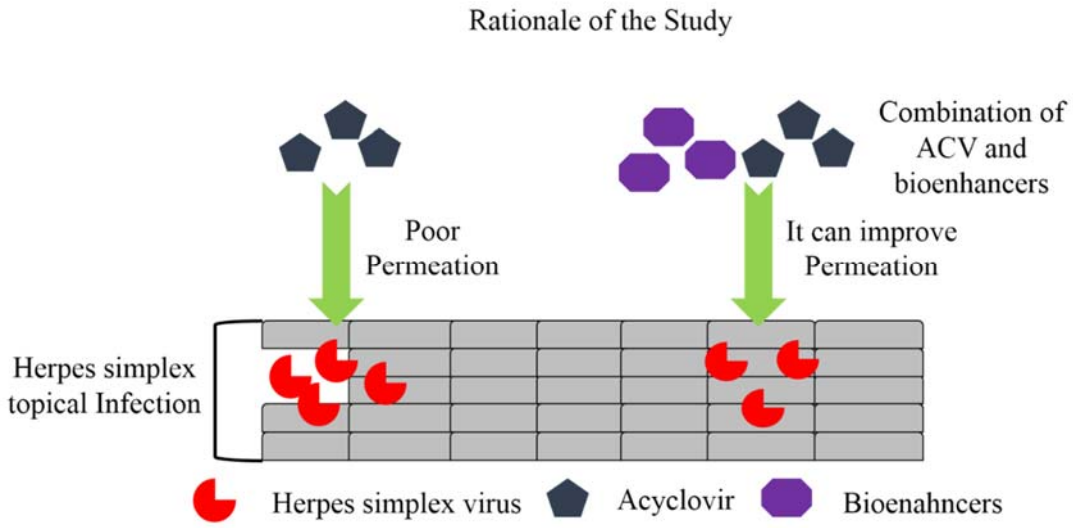
6.0. Introduction

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[3]. 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[4]. 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[5]. Researchers from the very beginning of the topical drug delivery has 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. But 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[6-7]. In the case of ACV, the topical administration leads to the tenfold higher concentration as compare to the oral administration[2]. Although literature suggested that this concentration does not produces desired therapeutic effect[3]. Hence, it is crucial to enhance the penetration of ACV (upto dermis), while maintaining the normal skin barrier function[8].

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[7]. Researches has 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[9]. The other approaches used by researcher are iontophoresis[10], percutaneous absorption enhancers[11] and site specific drug delivery was also another strategy used to improvise the therapy using the particular drug carriers[12].

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).



6.1. Plan of Work (Aim & Objectives)

The specific aim of the research work was to study the effect of quercetin (QU)**,** silibinin **(**Sil) and luteolin (LT) on topical delivery of ACV. It includes following studies

- ❖ Analytical method development for the estimation of ACV in different sample matrixes.
- ❖ Optimization of bioenhancer concentration on basis of permeation coefficient of drug using skin permeation studies.
- ❖ Optimization of bioenhancer concentration on basis of permeation coefficient of drug using *In-vitro* cell line (HaCat) studies incuding MTT Assay
- ❖ RT-PCR assay

6.2. Materials and Methods

6.2.1. Materials

The Model drug ACV was procured from Nestor Pharmaceuticals, New Delhi. The quercetin, silibinin and luteolin was purchased from the sigma Aldrich. Cell line was procured from NCCS Pune. 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.

6.2.2 LC-MS Method for estimation of ACV in Cell lines and plasma samples

6.2.2.1. Instrumentation & LC-MS Conditions

Chromatographic study was performed using ekspertTM ultraLC with ekspertTM ultraLC 100 pump system (eksigent-AB Sciex, USA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, USA), located at Dr. Vikram Sarabhai Science Center, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat, INDIA. 20 µL of each sample was injected. The autosampler system (ekspertTM ultraLC 100 XL, eksigent-AB Sciex, USA) was tempered to 8°C equipped with column oven (ekspertTM ultraLC 100, eksigent-AB Sciex, USA) fixed at 40°C. Chromatographic elution of analyte was achieved using a Phenomenax C18 5µm (250*4.6) mm column at a flow rate of 0.5 ml/min for having run time 8 mins. The isocratic composition of eluent a (water with 0.1% formic acid) and eluent b (methanol) was in 60:40 % v/v. Analysis was conducted using 3200 QTRAP mass spectrometer (AB Sciex, USA) equipped with electro spray ionization (ESI) source. The mass spectrometer was operated in the positive ion mode with a potential of 5.5 kV applied on the electro spray ionization needle. The ionization source temperature was 600 °C. Acv was identified and quantified using Multiple

Reaction Monitoring (MRM) mode. The curtain gas (CUR) was at 25.0 psi, the nebulizer source gas 1 at 50.0 psi and the turbo ion source gas 2 at 50.0 psi was utilized. The optimized Declustering potential and entrance potential were 60.0 V and 5.6 V respectively. Acv fragmentation was achieved by collisionally activated dissociation (CAD) with nitrogen gas. The collision gas pressure was fixed at 2.0 psi for MRM quantitation. The collision energy 22.0 V and collision cell exit potential 3.0 V were optimized. Dwell time 200 ms was used. The product ion at m/z 226.00 was selected. Detailed validation has been discussed in Chapter IV.

6.2.2.2. Cell line and permeation studies sample preparation

The samples for the acv permeation studies were collected at different time points were filtered and diluted with the mobile phase. The prepared samples with unknown concentrations has been injected.

6.2.2. 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 has 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^{o}c and emulsified with aqueous solution also at 60^{o}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 has been illustrated in Table 6.1.

Table 6.1 Preparation of different creams with bioenhancers

Formulation code	ACV (mg)	bioenhancer	CA[#] (mg)	SLS* (mg)	WSP[$] (mg)	LP[@] (mg)	PG[&] (gm)	Water (to gm)
Acv	250	NA	337.5	37.5	625	250	2	5
ACSI-1	250	Silibinin 1 %	337.5	37.5	625	250	2	5
ACSI-2	250	Silibinin 2 %	337.5	37.5	625	250	2	5
ACSI-3	250	Silibinin 3 %	337.5	37.5	625	250	2	5
ACSI-4	250	Silibinin 4 %	337.5	37.5	625	250	2	5
ACSI-5	250	Silibinin 5 %	337.5	37.5	625	250	2	5
ACQU-1	250	Quercetin 1 %	337.5	37.5	625	250	2	5
ACQU-2	250	Quercetin 2 %	337.5	37.5	625	250	2	5
ACQU-3	250	Quercetin 3 %	337.5	37.5	625	250	2	5
ACQU-4	250	Quercetin 4 %	337.5	37.5	625	250	2	5
ACQU-5	250	Quercetin 5 %	337.5	37.5	625	250	2	5
ACLU-1	250	Luteolin 1 %	337.5	37.5	625	250	2	5
ACLU-2	250	Luteolin 2 %	337.5	37.5	625	250	2	5
ACLU-3	250	Luteolin 3 %	337.5	37.5	625	250	2	5
ACLU-4	250	Luteolin 4 %	337.5	37.5	625	250	2	5
ACLU-5	250	Luteolin 5 %	337.5	37.5	625	250	2	5

6.2.2.1. ACV content estimation

Dummy cream formulations prepared in lab was analyzed for drug content using validated (Discussed in Chapter IV) UV spectrometry method. Spectrophotometric measurements were made on a Shimadzu 1700 double beam UV–VIS spectrophotometer with a fix slit width of 1 nm coupled with Shimadzu UV PC software (UV probe) version 2.31. Weighing balance of Shimadzu AX120, bath sonicator (Electroquip) and borosil glass apparatus were used for experimental purpose.

6.2.2.1.1. Preparation of standard solutions

An accurately weighed amount of ACV was transferred into a 10 mL calibrated flask and dissolved in approximately 4 mL of 0.1M HCl. The resulting solutions were completed to the mark with 0.1M HCl obtaining stock standard solution containing 1000 µg/mL. Different volumes of this stock solution were then further diluted with 0.1M HCl to obtain the working standard solutions.

6.2.2.1.2. Cream samples

An accurately weighed amount of the cream equivalent to 10 mg of ACV was shaken with 5 mL of 0.1M HCl and sonicated for 15 min and then the volume was made up to the mark with 0.1M HCl. The resulting solution was filtered and first portion of the filtrate was discarded. The working solutions were prepared by further diluting with 0.1M HCl for analysis.

6.2.2.2. 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 125gm. The spreadibility was calculated using formula:

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

Value S is spreadibility, m is the weight tied to the upper slides, l is the length of glass slide and t is time taken.

6.2.3. *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 ketamine 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 1ml sample was withdrawn at suitable time intervals and replaced immediately with fresh volumes of diffusion medium. The samples were analyzed using above mentioned UV method after suitable dilutions.

6.2.4. 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.

6.2.5. 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 hrs before the experimentation. After anaesthetizing the rat with ketamine the abdominal part was divided in to two-four different section each having area 1cm^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.

6.2.6. 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.

The cells were cultured under the conditions described above for 24 hr. The growth of cells was monitored on an inverted-phase microscope. Solution of ACV (10 μM) and QU, Sil and LT (range 2 μM–10 μM) has been prepared in HBBS 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. All concentrations were used in triplicate. Samples from the receptor well were collected at different time points and ACV concentration was estimated using LC-MS.

6.2.7. 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 hours 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.

6.2.8. RT-PCR

RT-PCR studies were carried out using ZO-1 primer with sequence 5′CTTCAAAGGGAAAGCCTC3′ (forward) 5′TACCTTCACCATGCTCC3′ (reverse) (13), for tight junction. In this study the HaCat cells were treated with the different concentration of the bioenhancers and after the treatment RNA was extracted from the cell

lines using the TRI-reagent from sigma Aldrich. Then RNA was reverse transcribed using cDNA kit obtained commercially. Then cDNA was used for the PCR reaction having quantity equivalent to 20 ng of RNA. The PCR reactions was carried out with ZO-1 using 2X DyNAzymeTM Master Mix (FINNZYMES, Finland).

6.2.9. Draize Test

In the skin irritation test, New Zeland rabbits were used (2– 3kg) of either sex (n=3). The dorsal part of the rabbits were shaved and different cream samples were applied on the skin. It was covered with the transparent tapes. After applying the sample animals were kept in chamber for 24 hrs. After the completion of 24 hrs animals were observed for erythema or oedema for a period of 72 hrs.

6.3. Result and Discussion

6.3.1. LC-MS Method

The chromatogram and mass spectra of ACV has been illustrated in Figure 6.1 and 6.2. Chromatogram of blank, blank sample, standard and cell line sample has been illustrated in Figure 6.3. The method has been validated as discussed earlier in the Chapter IV.

6.3.2. ACV content studies in cream

Dummy cream formulations prepared in lab was analyzed for drug content using validated UV methos as per ICH guidelines. 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 Table 6.2.

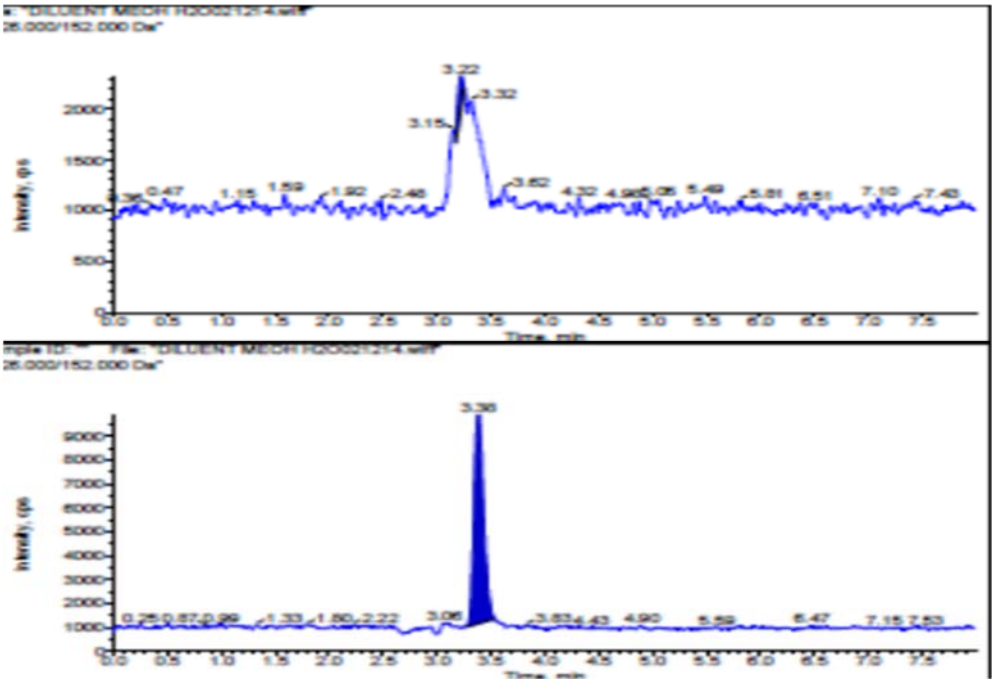


Figure 6.1 Chromatogram of ACV and Blank

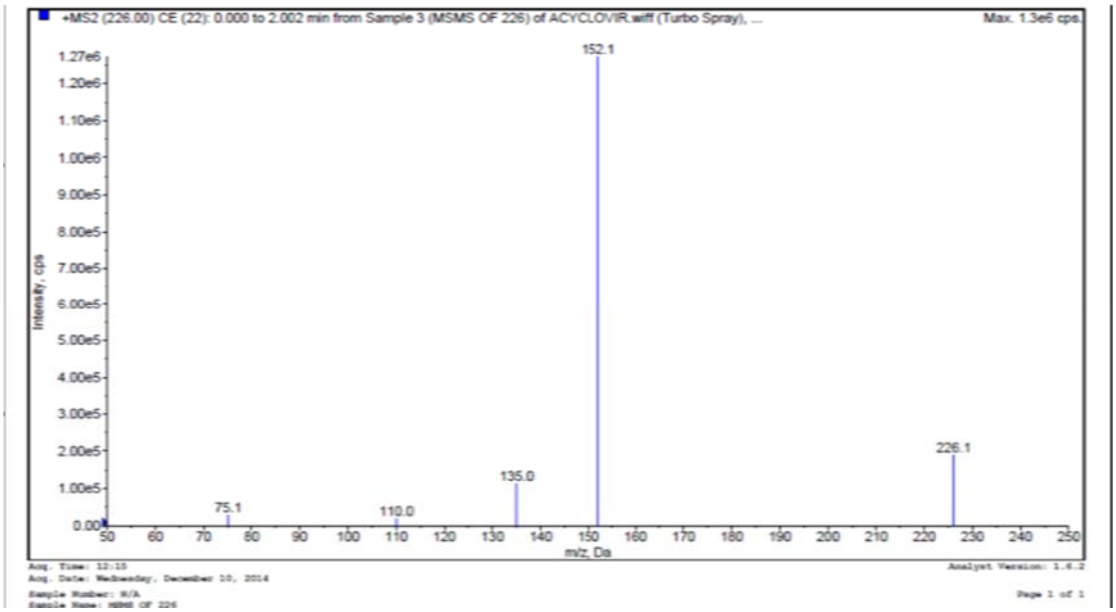


Figure 6.2 Mass spectra of ACV

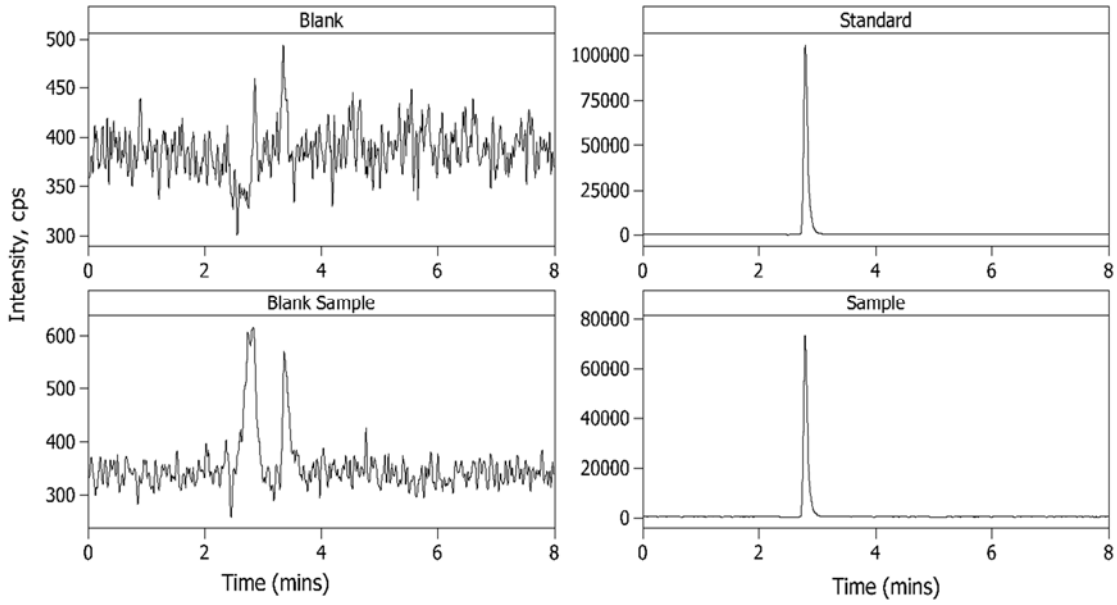


Figure 6.3 Chromatogram of Blank, Blank sample, Standard and cell line sample

6.3.3. 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 has been summed up in Table 6.2.

Table 6.2 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

6.3.4. *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 time profile of mean cumulative amount of drug permeation in the presence and absence of

different ratio of QU has been shown in Figure 6.4 which clearly indicates increase in the permeation with the time in the presence of the QU. Figure 6.5 and 6.6 shows the time and cumulative amount profile in the presence of Sil and LT. The flux was calculated for all the different ratios of three bioenhancers and it was observed that Sil shows the maximum at 2% level while QU at 4% and LT at 1% as shown in Figure 6.7. 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 maximum enhancement was observed with the Sil followed by QU and LT. The maximum permeation due to Sil can be due to its skin junction pore opening capacity.

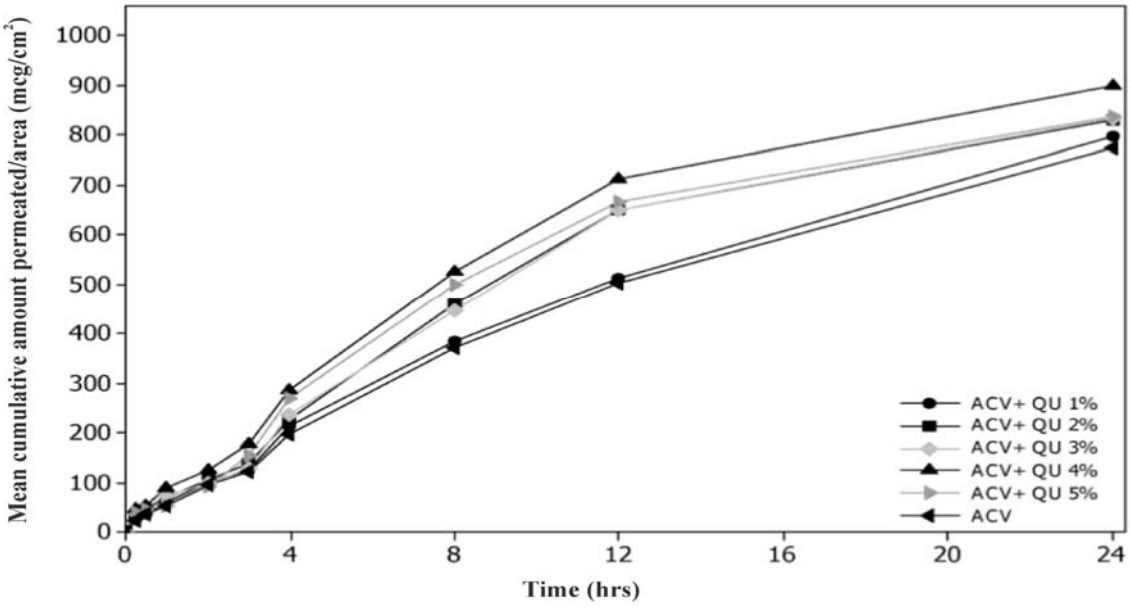


Figure 6.4 Comparison of mean cumulative amount of ACV released in presence and absence of QU per unit area of skin

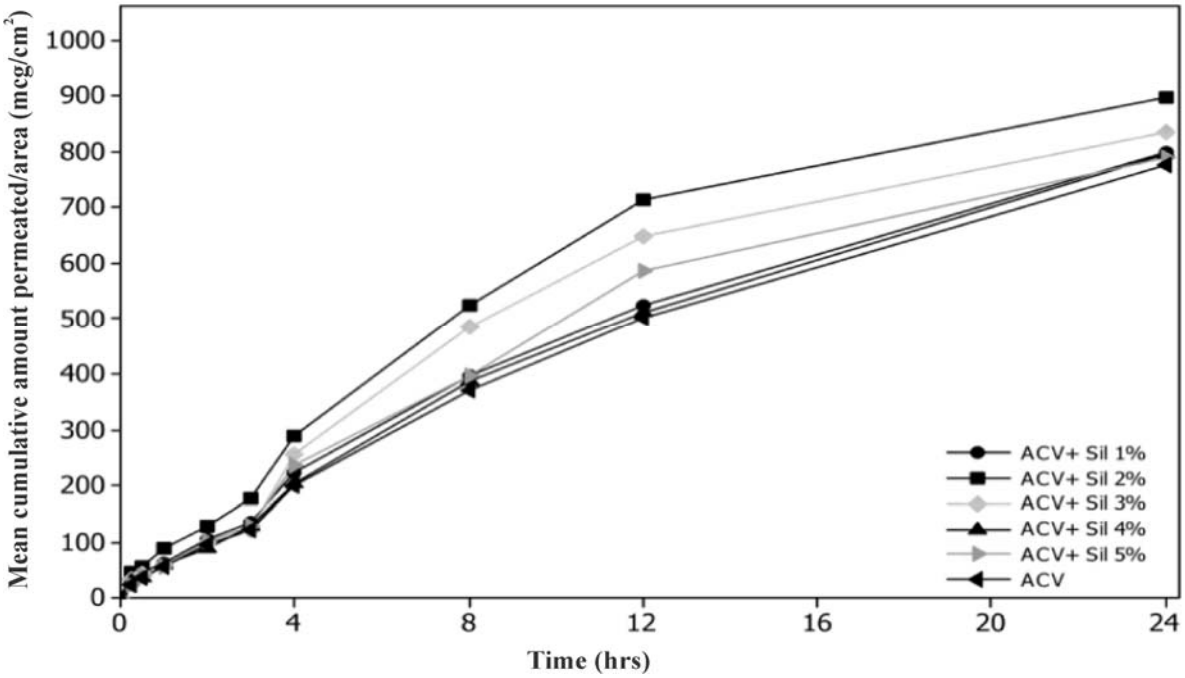


Figure 6.5 Comparison of mean cumulative amount of ACV released in presence and absence of QU per unit area of skin

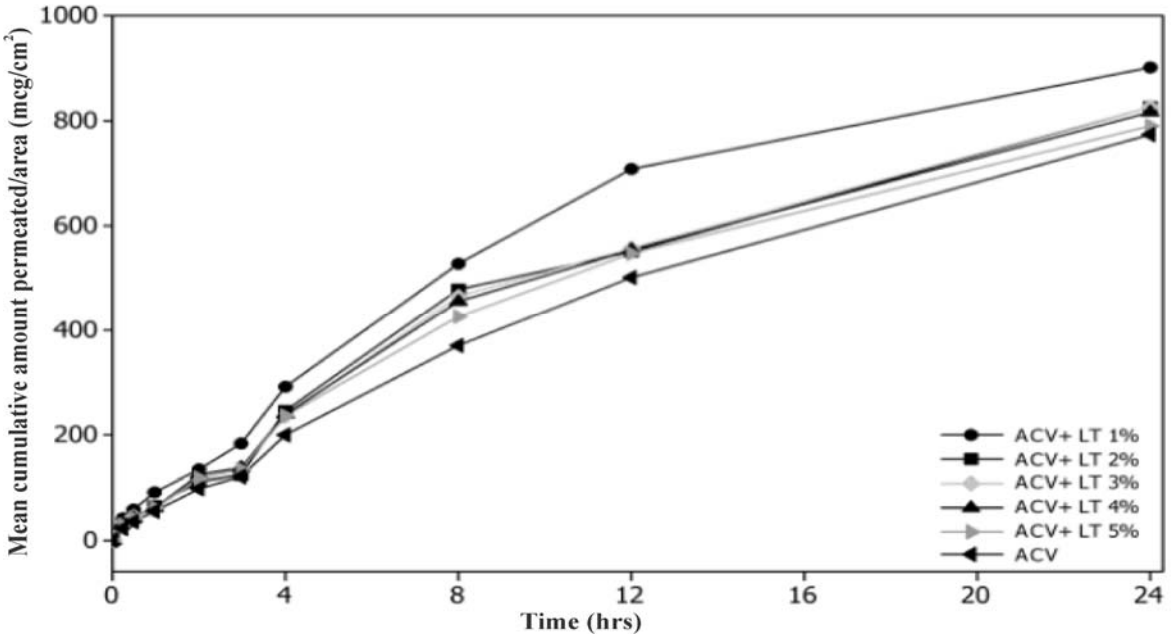


Figure 6.6 Comparison of mean cumulative amount of ACV released in presence and absence of QU per unit area of skin

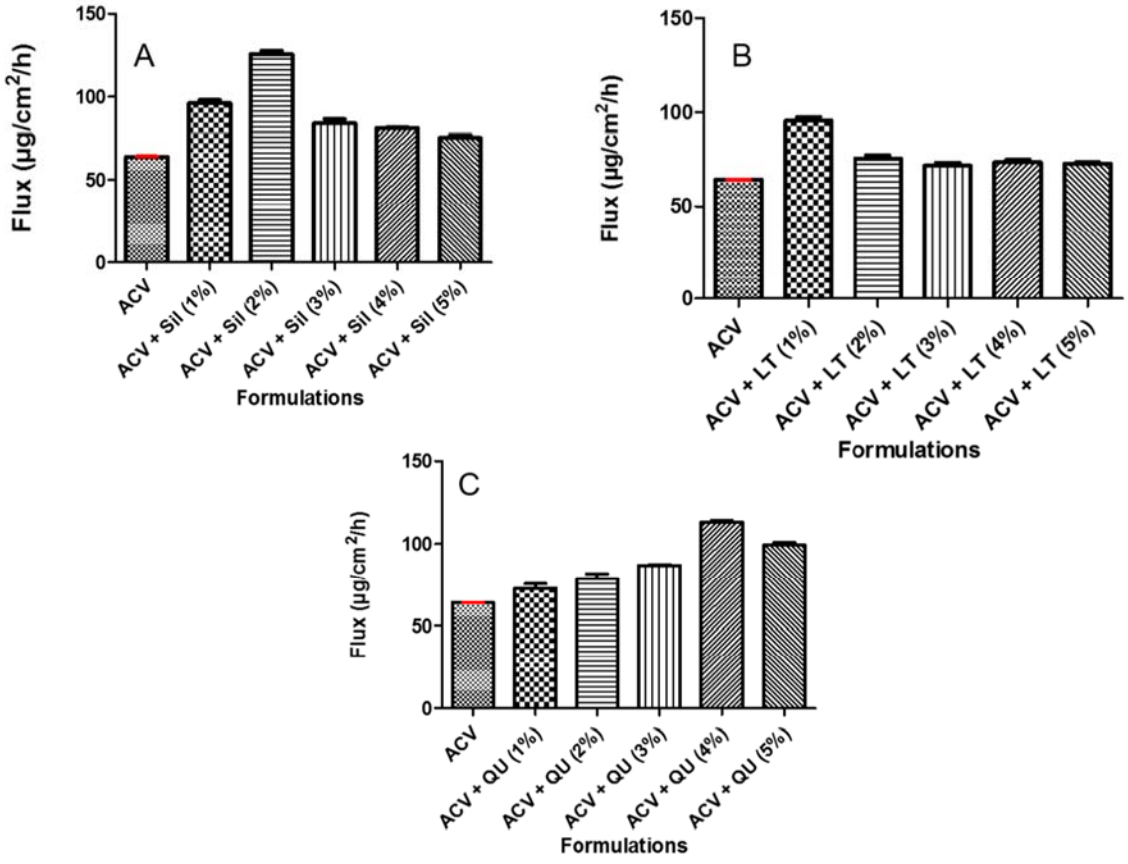


Figure 6.7 Comparison of Flux of ACV in the presence and absence of different ratios of bioenhancers.

6.3.5. Skin Retention Study

At the end of the in vitro experimentation the amount of drug retained in skin was calculated and it was observed that it was significantly higher in all the creams contains QU, Sil and LT. The comparison of the amount retained in the skin has been illustrated in Figure 6.8. Although the drug retained in the skin is very slightly different in the presence of different ratios of the bioenhancers but it is significantly differ from the plain ACV, hence these results evidently shows that there is increase in the permeation of the ACV in the skin.

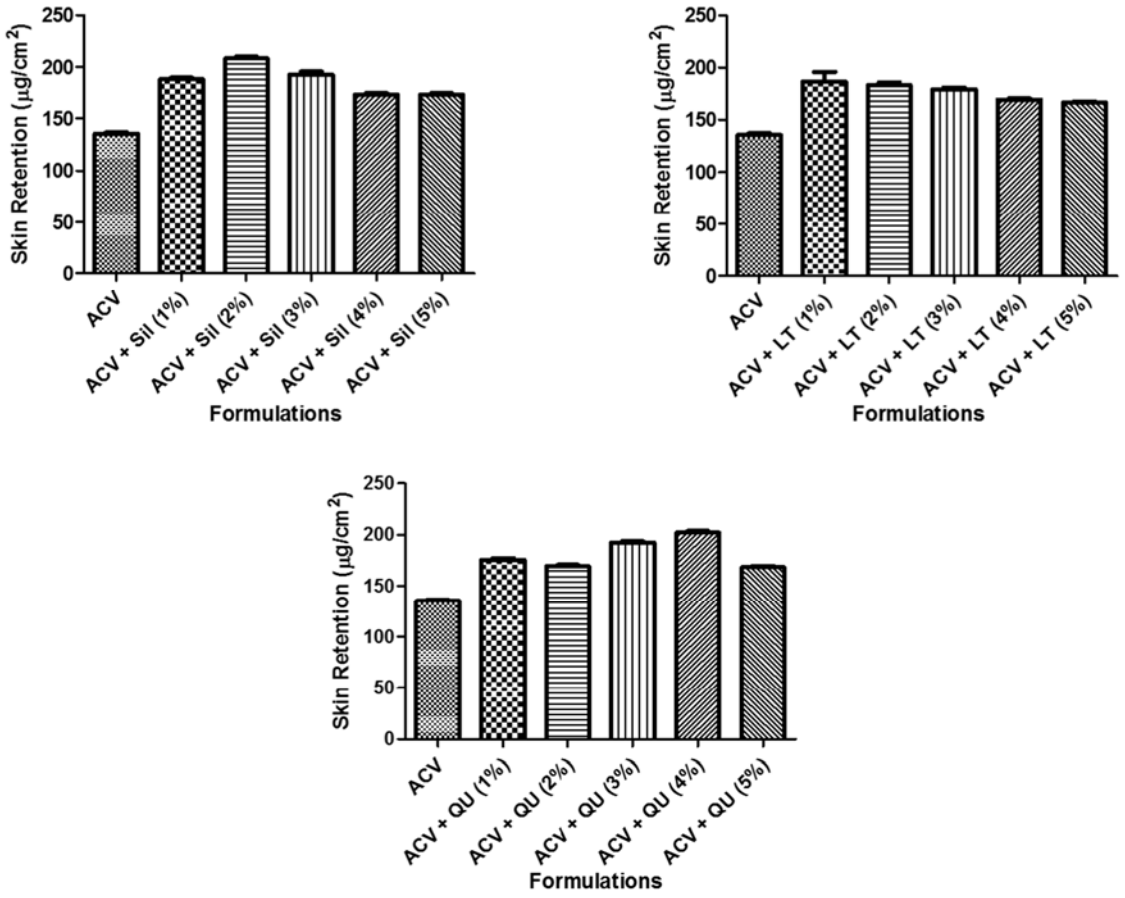


Figure 6.8 Comparison of amount of drug retain in the skin

6.3.6. *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 6.9 shows the application of the cream on the abdominal part of the rat. The upper layer of the skin was washed out using 0.1N Hcl and the ACV drug was estimated. Figure 6.10 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 which quite less as compared to the ACV plain cream which is around 67.42%. Different

bioenhancers ratio effect on unabsorbed drug has been shown in Figure 6.10, 6.11 and 6.12 for QU, Sil and LT respectively.

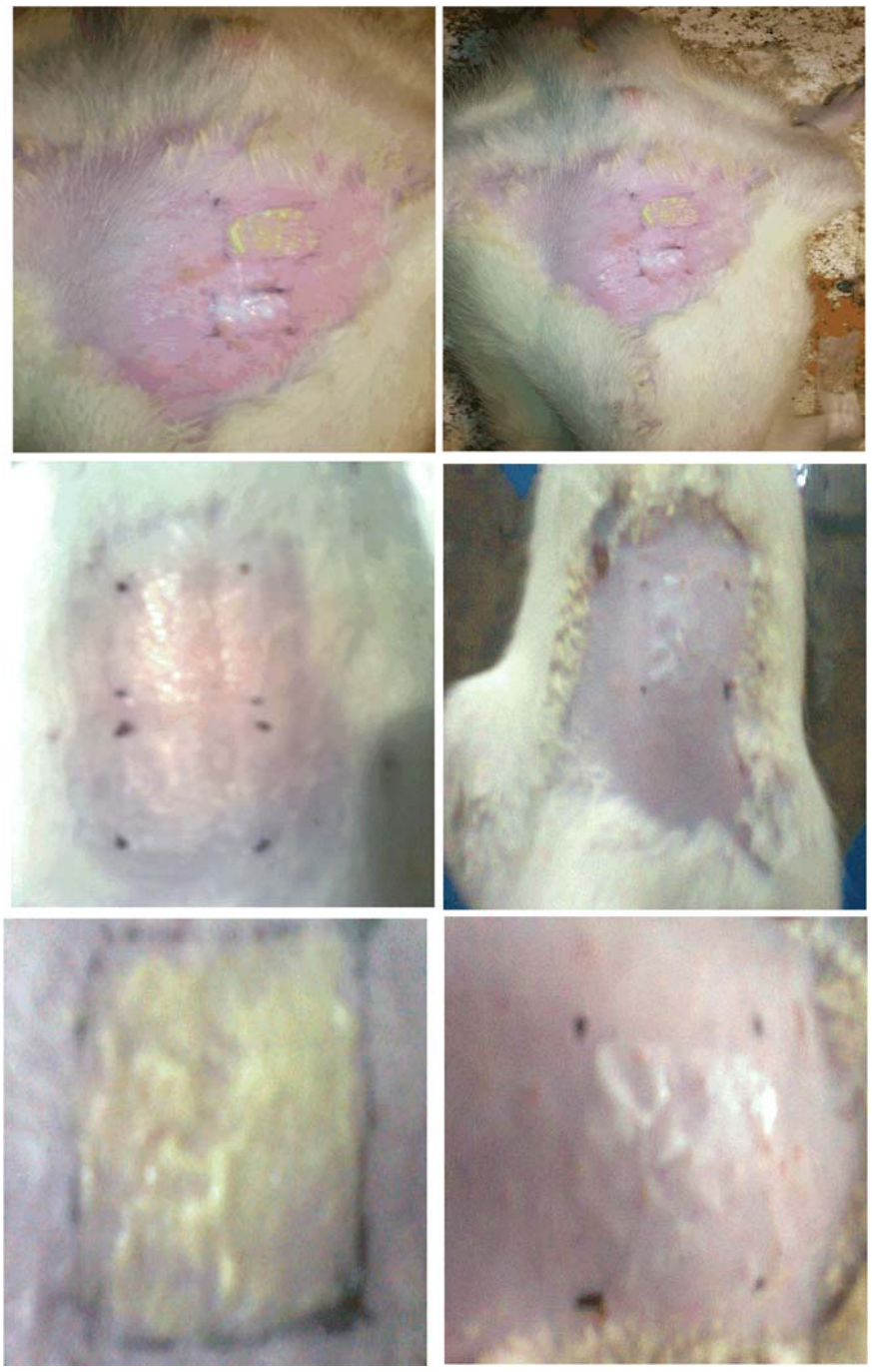


Figure 6.9 Application of the cream on the abdominal part of the rat

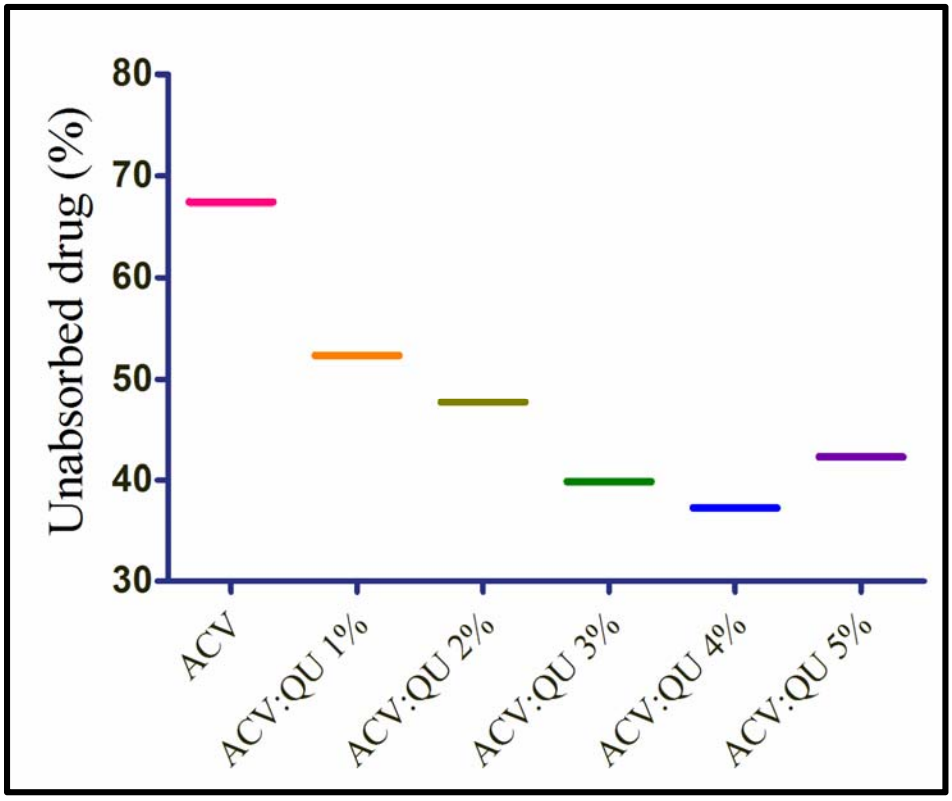


Figure 6.10 Comparison of the % unabsorbed drug in the presence and absence of QU.

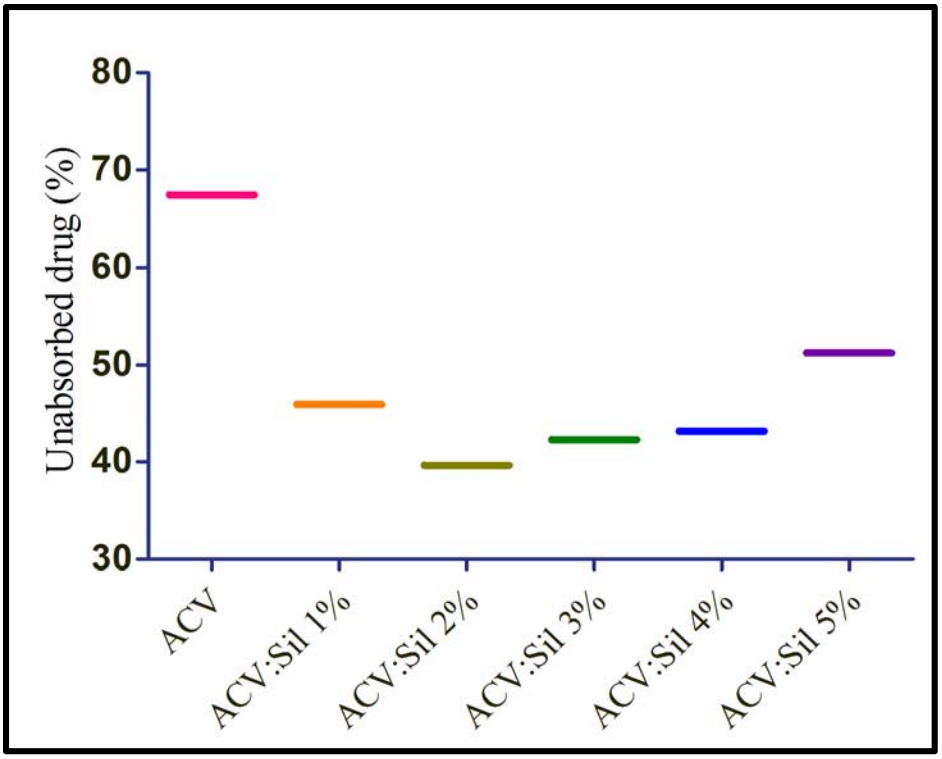


Figure 6.11 Comparison of the % unabsorbed drug in the presence and absence of Sil.

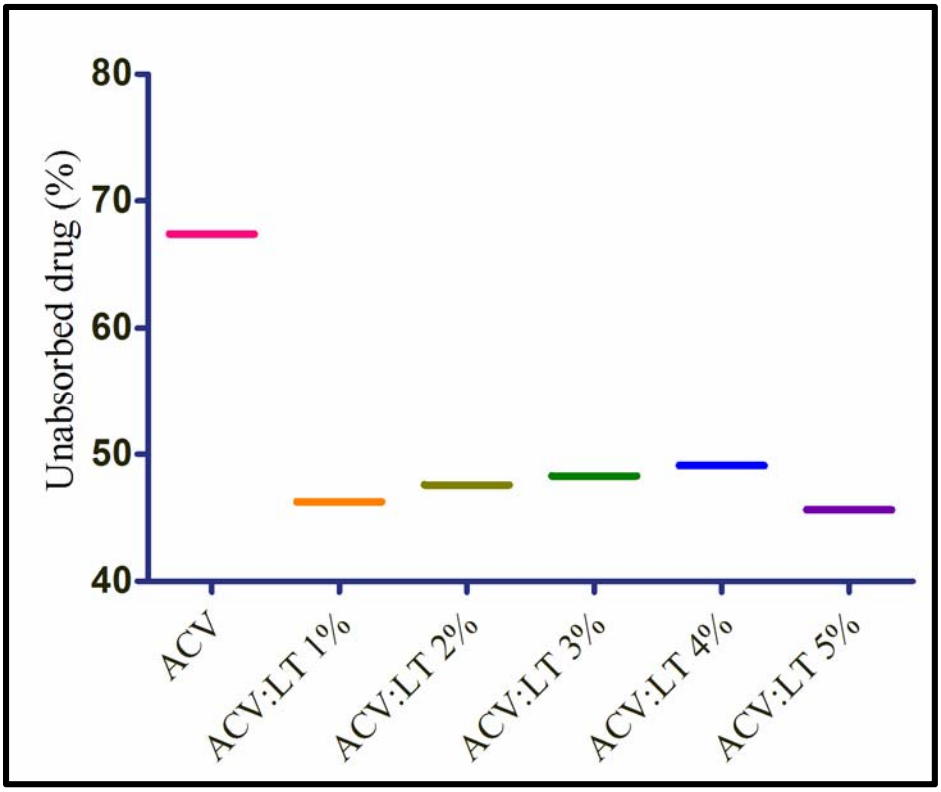


Figure 6.12 Comparison of the % unabsorbed drug in the presence and absence of LT.

6.3.7. 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 the cell lines the permeation of the ACV through the cell layer has been studied and it was found that there is increase in the permeated amount with the time. Time profile of amount permeated of ACV using different concentrations of LT, QU and Sil has been shown in Figure 6.13, 6.14 and 6.15 respectively. This may be due to mechanism of bioenhancers ability to open the pores of the skin membrane. In ACV:QU the maximum enhancement was found at 6 µM having 1.36 fold enhancement ratio. 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. The comparison of the ER has been shown in the Figure 6.16 which clearly

indicates the maximum enhancement was achieved with the Sil followed by QU and LT. The LT and QU have very similar structure while Sil is bulky molecule than the QU and LT so the structure of the compound may be playing a role in the permeation of the compound.

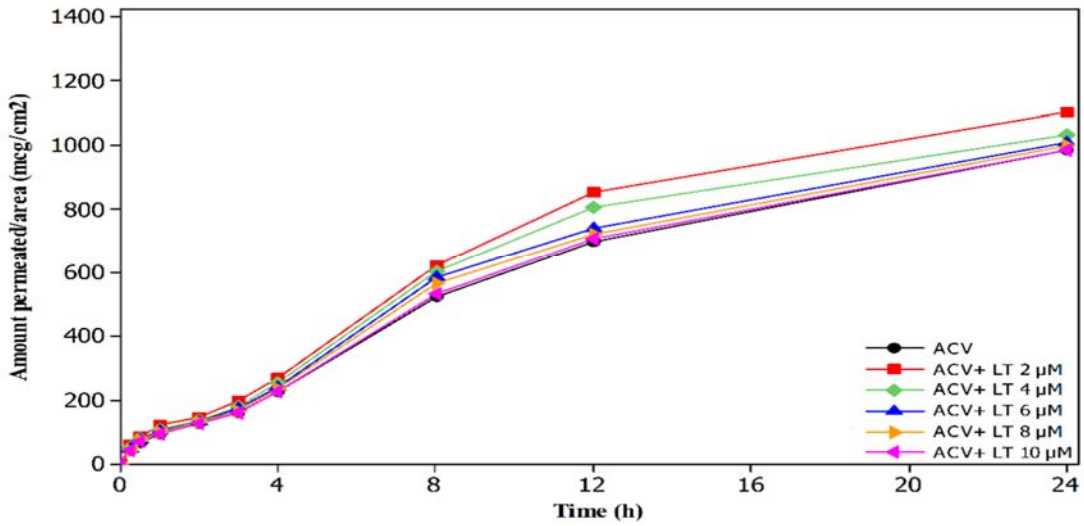


Figure 6.13 Amount of drug permeated with time in HaCat cell lines at different concentrations of LT

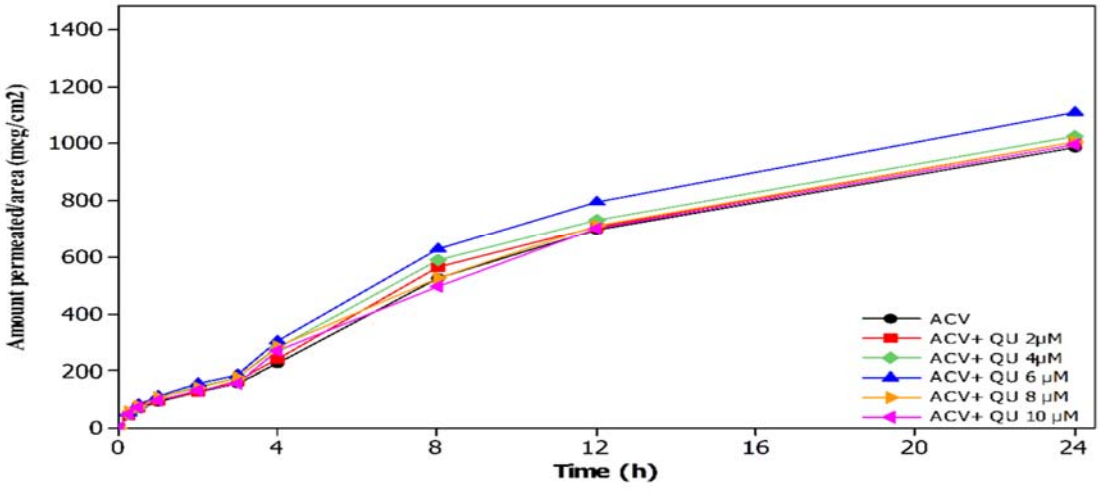


Figure 6.14 Amount of drug permeated with time in HaCat cell lines at different concentrations of QU

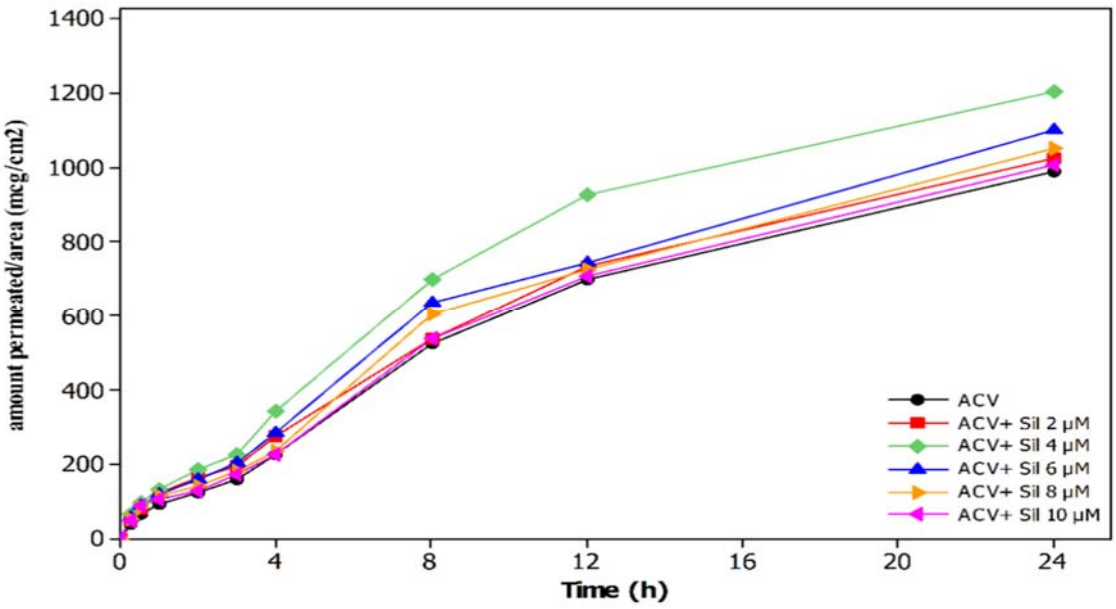


Figure 6.15 Amount of drug permeated with time in HaCat cell lines at different concentrations of Sil

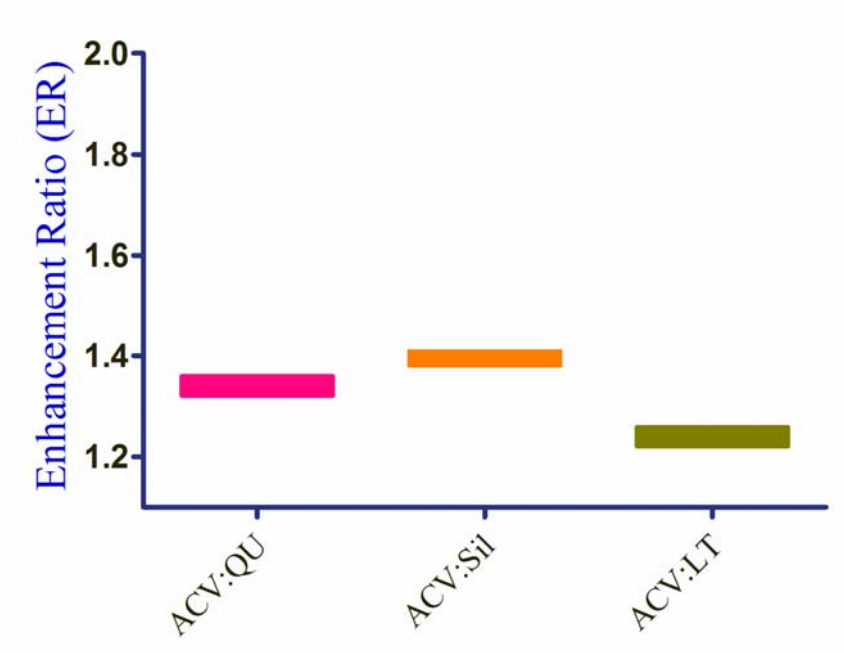


Figure 6.16 Comparison of ER of three different bioenhancers

6.3.8. 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 6.17, 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.

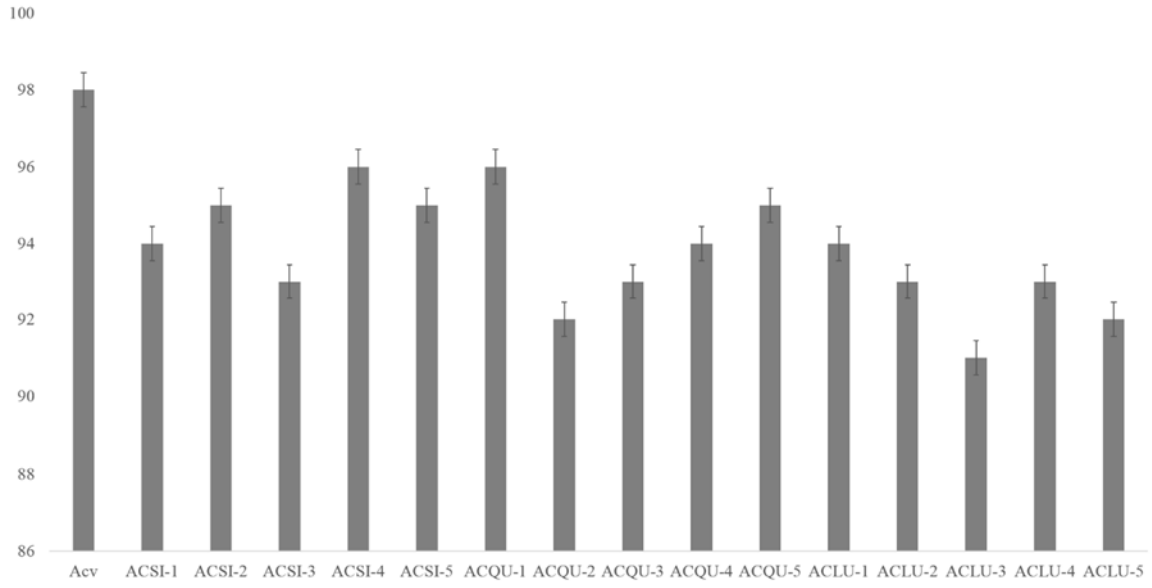


Figure 6.17 Cell viability in MTT Assay

6.3.9. RT-PCR

In the RT-PCR studies it was found that there is a very slight effect on the gene expression of the ZO-1 tight junctions in the case of QU and LT while it was slightly extra in the case of Sil. The slight decrease in expression in ZO-1 is observed at from the 2-5% of Sil and 3-5% of QU and 2-5% of LT as shown in Figure 6.18, 6.19 and 6.20 respectively. From this data it can be concluded that there is slight effect of the bioenhancers on the tight junction proteins but exact and cell level data needs a depth study to confirm that.

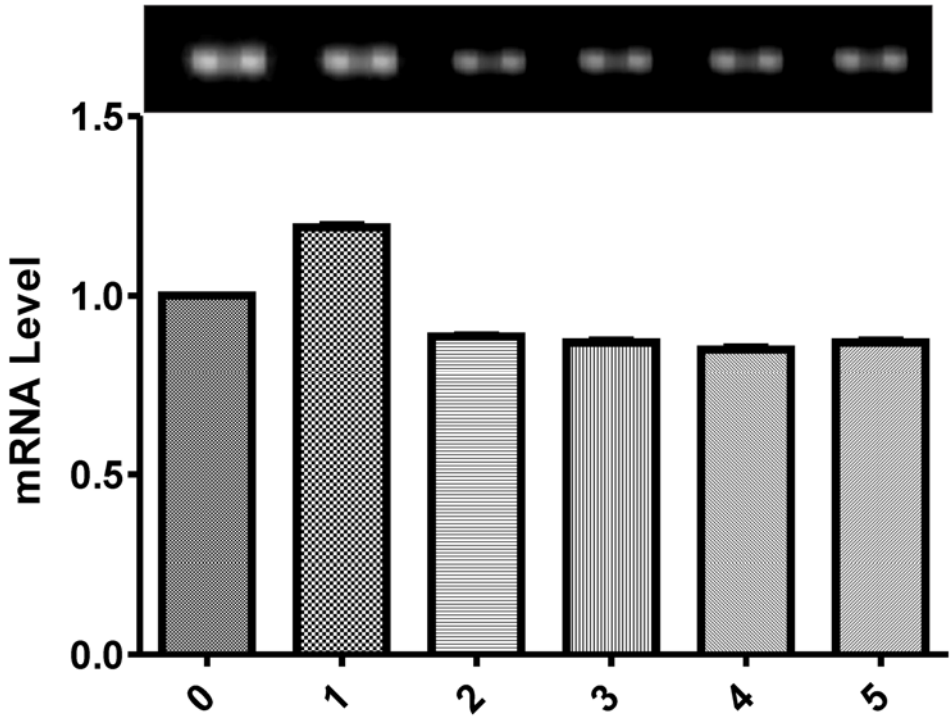


Figure 6.18 mRNA level at different concentration of Sil from (0-5%)

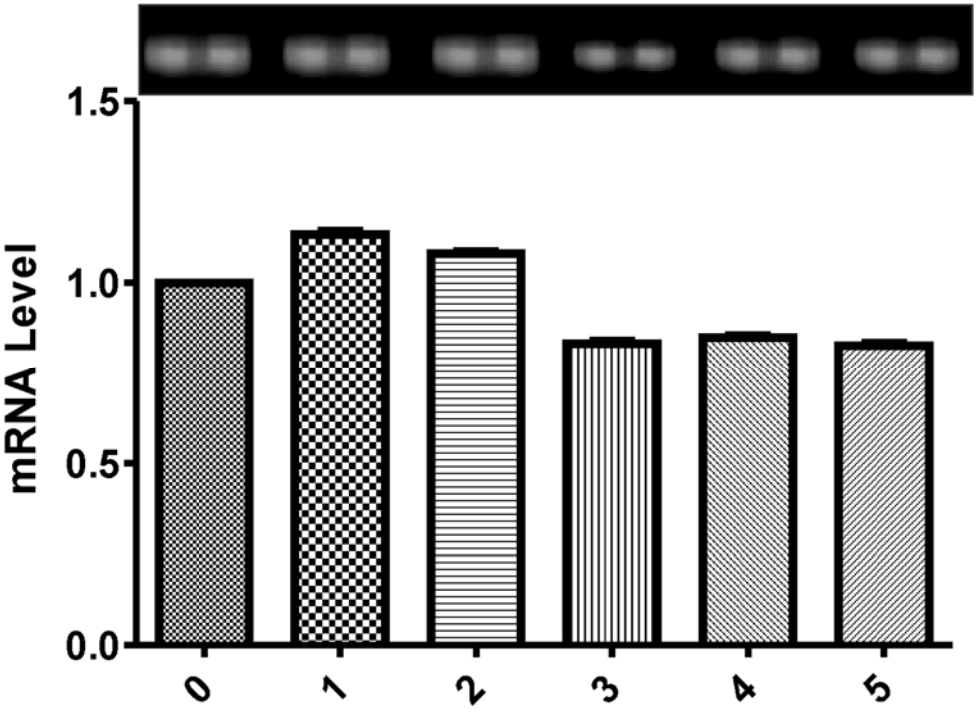


Figure 6.19 mRNA level at different concentration of QU from (0-5%)

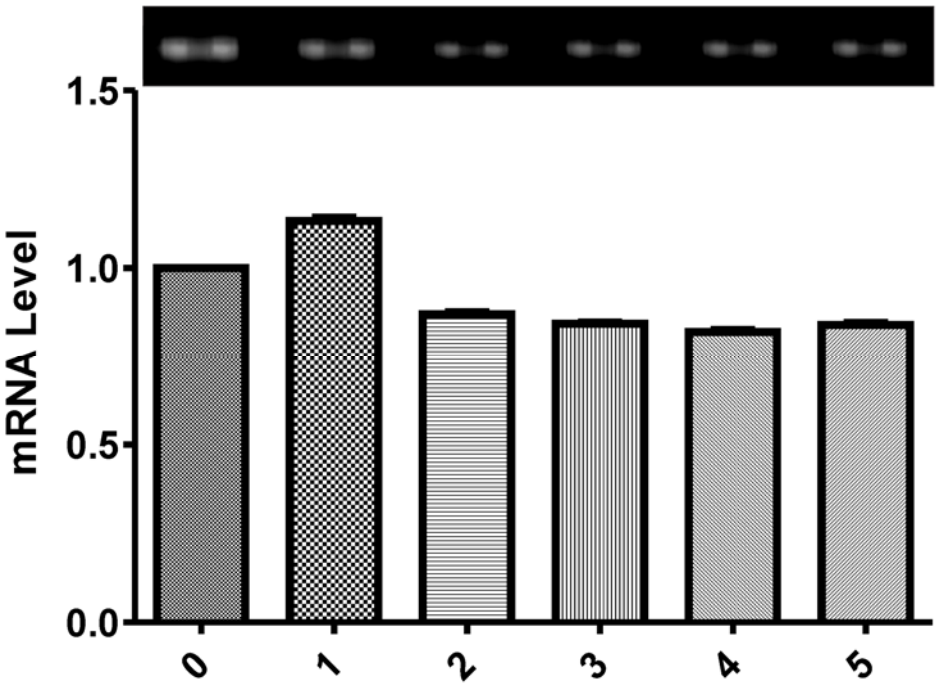


Figure 6.20 mRNA level at different concentration of LT from (0-5%)

6.3.10. Draize Test

In skin irritation test, no redness, swelling or other type of irritation has been observed in the animal, which concludes the safety of the compounds when applied to the skin.

6.4. Discussion

The bioenhancers QU, Sil and LT shows an increase in the permeation of ACV in the in-vitro permeation study, ex-vivo permeation and cell lines study. 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. MTT assay results shows the damage to the cell layer was minimum with the bioenhancers. 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. RT-PCR assay although not clearly represents the activity but a slight change in the expression of tight junctions revealed that there is need of cellular level study is further needed up to reveal the exact mechanism of the bioenhancers. In the comparison of the ER of the three bioenhancers it has been observed that the all of them shows almost same although keeps Sil on the higher edge. Sil is found to be more active in the studies as it shows activity in very less concentration as compare to the QU and LT. Sil can be concluded as the first choice of bioenhancers for topical permeation enhancement.

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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