A SYNOPSIS OF THE THESIS ENTITLED

Bioavailability enhancement of poorly bioavailable anti-viral drugs using natural bioenhancers

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Introduction

The prime affliction on the health care system has been imparted by extensive blowout of infectious diseases throughout the world. The appraisal states that a vast range of prescribed drugs suffer from low and variable bioavailability mainly because of metabolism and permeation complications. This issue of bioavailability is the foremost problem which has been carried forward from last many years.

In general practitioner recommend medications on the basis of their characteristics and on the panorama that reliable and reproducible clinical effects will result. However, variability in the drug response among the patient is very common, often leading to challenges in optimizing a dosage regimen for an individual patient. Most drugs are effective in only 25 to 60 percent of patients and million cases of adverse drug reactions occur annually worldwide including thousands of deaths (1). These adverse reactions and effect of drug in a small population is due to variability and low bioavailability of drugs.

There are number of factors responsible for this low and variable bioavailability including release of active ingredient from the formulation, dissolution rate, stability of the drug candidate in the gastrointestinal (GI) atmosphere, permeability through the gut wall and first-pass gut wall and hepatic metabolism. Now as, It is well recognized that biological barriers such as hepatic as well as intestinal drug metabolizing enzymes (DMEs) and efflux drug transporters (DTs), act as concierges of drugs and limit the systemic drug availability (2-4). These systems restrict the appearance of intact drug in the blood circulation either by hampering their passage across intestinal mucosa or by biotransformation carried out by intestinal and hepatic DMEs (5, 6). Although there are various enzymes in the gut wall which may contribute to gut first-pass metabolism in which cytochrome P450 (CYP) 3A has been exposed as to play a most important role and efflux transporter P-glycoprotein (P-gp) is the most extensively studied drug efflux transporter in the gut and have a significant role in the

regulation of GI absorption. Consequently in this a very promising strategy for enhancing drug oral bioavailability entails short-lived inhibition of DMEs and transporters to overcome pre-systemic and first-pass effect (3, 4, 7, 8).

For this approach to be beneficial, a pre-requisite is clear elucidation of the enzyme(s) and/or transporters(s) involved in lowering the systemic availability of the drug candidates. Inhibition of the metabolism of one drug by the addition of another drug causes problems as plasma drug concentrations may quickly increase after one or two doses of the drug that was added. Therefore, there is risk of side effects (which is caused by the interaction of the new agent with the original drug) increases, notwithstanding an earlier stable course on that medication. So to overcome these problem of interactions some natural compounds have demonstrated in past and explored to increase the absorption and bioavailability of co-administered drugs. These natural compounds shown to be have a gifted P-gp and CYP inhibition activity. The intensification of bioavailability through co-administration of drugs with naturally occurring compounds from plants is considered to be very simple and relatively safer than other compounds tried earlier to enhance bioavailability (9, 10).

These herbal origin compounds are called as "Natural bioenhancers" and found to be a divine gift for today's healthcare system. In this research work out of all available techniques new and promising concept of administering natural bioenhancer with poor permeable drugs has been studied.

It has been hypothesized that natural bioenhancers when administered with poor bioavailable drug show improvement in drug bioavailability. In the present work, this new concept of incorporating bioenhancer was used to improve bioavailability pattern of poorly permeable anti-viral drugs such as acyclovir (ACV) and saquinavir (SQU). The hypothesis was studied by using different concentrations of three different natural bioenhancers such as quercetine (QU), silibinin (Sil) and luteoline (LT).

Major Highlights of the Research Work

- **Section A:** Study the effect of quercetin (QU)**,** silibinin **(**Sil) and luteolin (LT) on acyclovir and saquinavir
  - Drug-bioenhancer compatibility study
  - Selection/development/optimization and validation of analytical and bio-analytical method for the determination of drug
  - Computer aided design to predict affinity of drug and bioenhancer
  - Preparation of binary combination of drug and various proportions of bioenhancer using physical mixing.
  - Optimization of proposed binary mixture through determination of permeation coefficient of drugs using In-vitro Caco-2 cell line studies.
  - Pharmacokinetics studies for optimized drug-bioenhancer mixture in rabbits as an animal model
- **Section B:** Study the effect of quercetin (QU)**,** silibinin **(**Sil) and luteolin (LT) on topical delivery of drug: acyclovir
  - 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.
  - MTT Assay

Rationale of Natural Bioenhancers

- This can make the expensive drugs affordable by lowering the dose or dosing frequency.
- Shortening the treatment period also increase the acceptance of patients mainly in case of chemotherapy.
- It comforts the patient in terms of cost also.
- Reduce the required dose ultimately reduce the toxic effects.

PART A: Study the effect of quercetin, silibinin and luteolin on two drugs: acycovir and saquinavir

I. Effect of Quercetin, Silibinin and Luteolin on acyclovir

Inferences form Literature Review and Present Study

In the present research work, this new concept of incorporating bioenhancer was used to improve bioavailability pattern of poor permeable drugs such as acyclovir (ACV) and saquinavir (SQU). Therapeutic effectiveness of a drug depends upon the bioavailability and ultimately upon the solubility of drug molecules. ACV is a potent anti-viral agent useful in the treatment of Herpes Simplex Virus (HSV) infections. The dose ranges between 200 and 800 mg and the oral bioavailability is 10-20%. The therapeutic potential of acyclovir is limited by the low oral bioavailability owing to its limited low permeability.

The present study was a systematic investigation on the effect of QU, Sil and LT for the enhancement of oral bioavailability of ACV. The binary mixture of ACV was prepared by physical mixing method using ACV and QU, Sil and LT. The prepared mixture was characterized by Fourier transform infrared spectroscopy, differential scanning calorimetry. Prior to working on the lab to study these mixture a computer aided study was also performed to confirm the affinity of the ACV, QU, Sil and LT towards P-gp and CYP enzymes. The results from prediction verifies the higher affinity of QU, Sil and LU as compare to the ACV.

In vitro characterization of physical mixture and control was done using Caco-2 cell lines. In vivo data was used to optimize the concentration of the bioenhcancers with drug. In vivo bioavailability of ACV was compared for physical mixture and control in rabbit model. In vivo pharmacokinetic data signify increased rate and extent of ACV absorption from physical mixture, compared to control. Given the promising results in the in vivo studies, it can be concluded that the physical mixture of ACV with QU, Sil and LT could be an

effective and promising approach for successful oral therapy of ACV in the treatment of herpes viruses.

Materials and methods

Firstly, ACV-QU, ACV-Sil, ACV-LT binary systems were prepared using physical mixing method, in this method ACV-QU, ACV-Sil, ACV-LT were prepared by gently blending for 10 minutes in a polythene bag. Binary system were tested for compatibility using FTIR, DSC.

RP-HPLC analytical method was developed and validated to estimate the ACV in presence of QU, Sil and LT. The developed HPLC method was then transferred to the LC-MS. Chromatographic elution was achieved using a Phenomenax C18 5µm (250*4.6) mm column at a flow rate of 0.5 ml/min 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.

Prior to working on the lab to study these mixture a computer aided study was also performed to confirm the affinity of the ACV, QU, Sil and LT towards P-gp and CYP enzymes. The results from prediction verifies the higher affinity of QU, Sil and LT as compare to the ACV.

In vitro Caco-2 cell lines were used to perform the permeation study. Caco-2 cell transport studies were conducted for ACV and ACV-QU, ACV-Sil, ACV-LT binary systems of PM method to determine the extent of improvement in permeability. Apparent permeability coefficient P_{app} values for ACV and ACV-QU, ACV-Sil, ACV-LT binary systems of PM method were calculated. Transepithelial electrical resistance (TEER) and permeation of transcellular marker; phenol red values in representative cell monolayers were assessed to examine the cell morphology and monolayer integrity. Amount of ACV permeated through cell line was estimated using developed and validated method as described above.

In vivo pharmacokinetic study has been performed using New Zealand white rabbits (2-3 Kg) as animal model. Total 30 rabbits will be randomly divided into 5 groups of 6 animals each (n=6). The animals were housed over two weeks in a temperature (20 – 25 °C) and relative humidity (between 50 and 60 %) controlled room and were given standard rat chow and water which were freely available. The protocol for studies was approved by the Institutional ethical committee at The M S University of Baroda, India. All the animals were fasted overnight with free access to tap water before experiments. ACV (control), ACV along with optimized concentration of QU, Sil and LT from *in-vitro* were administered orally with gavage needle. One group was given ACV by intravenous route to determine the absolute and relative bioavailability. Blood samples were collected from the retro-orbital plexus at 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 24.0 hr into labeled tubes, containing 10 µl of 20 % w/v K_2EDTA solution, as anticoagulant. Plasma was harvested from the blood by centrifugation. Collected plasma samples were further prepared for analysis by adding 500 µL acetonitrile to 100 µL plasma, which were then vortex-mixed briefly. Following centrifugation for 10 min at 10,000×g. Then supernatant was collected and evaporated to dryness under nitrogen and then reconstituted in 10% methanol with 0.1% formic acid to a final volume to 200 µL. Then prepared samples were analysed for the amount of ACV using LC-MS. The results obtained from the LC-MS analysis was then further used for assessment of various pharmacokinetic parameters i.e. C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$, T_{max}, elimination rate constant (Ke) and elimination half-life ($T_{1/2}$) etc.

Results and discussion:

FTIR spectra of ACV, QU, Sil, LT, ACV-QU, ACV-Sil and ACV-LT binary systems were recorded. The absorption band at about 1723 cm^{-1} due to ACV carboxylic acid functional group and 1^o amine of ACV has a strong absorbance at 3309.2 cm-1 is consistent within the all the binary systems. According to the FTIR results, no interaction between ACV

and bioenhancers has been detected. All these peaks of ACV remain unaffected in the IR spectrum of binary systems of each method. The slight change in the intensity is there which is not having any significant difference than the pure ACV. Thus FTIR spectra remains unchanged, explained that there was no interaction between ACV and bioenhancers in binary system. This confirms that QU, Sil, LT is not interacting with ACV at molecular level.

The thermograms of pure ACV, QU, Sil, LT, ACV-QU, ACV-Sil and ACV-LT binary systems were recorded. The thermogram of pure ACV showed a sharp endothermic peak at 254.53 °C, which is due to a melting point as it consumes energy. In the thermogram of binary systems shift in peak to lower temperature which may be explained by weak interaction between ACV and bioenhacncers. Shift in peak to lower temperature is due to conversion of ACV in amorphous form. Thus all thermograms prove that almost negligible interaction between ACV and bioenhancers in all binary systems of each method. The DSC analysis showed a negligible change in melting point of ACV in the presence of bioenhancers.

The Ki values of the ACV for CYP was 76.73 µM and QU, Sil and LT having 1.99 nM, 148.37 nM and 322.88nM respectively. Smaller the value of Ki, the stronger the interaction between the enzyme and inhibitor; thus the greater inhibitory effect. As shown in above results the QU having most inhibitory effect for the CYP enzyme.

The Ki values of the ACV P-gp was 23.56 µM and QU, Sil and LT having 0.33 µM, 12.37 nM and 368.23 nM respectively. Smaller the value of Ki, the stronger the interaction between the enzyme and inhibitor; thus the greater inhibitory effect. As shown in above results the Sil having most inhibitory effect for the P-gp enzyme.

The release profile of mannitol in presence of different concentrations of EDTA is indicates that each EDTA concentration increasing the permeation of mannitol. P_{app} values and enhancement ratio of mannitol is almost twice with 4 mM and higher concentration of

EDTA than control mannitol (without EDTA). The enhancement of permeation with 1 and 2 mM EDTA is almost not seen for mannitol, but when concentration rise to 4 mM almost twice increment in the permeation is observed. Increase in permeation is significantly high than control Mannitol. Presence of EDTA is causing decrease in % TEER values of Caco-2 monolayers during mannitol permeation. This decrease in % TEER suggests that EDTA causing opening of tight junctions and allows more permeation of mannitol which is absorbs by paracellular pathway. EDTA is widely studied bioenhancer, which acts as chelating agent. It has an enhancing effect via a paracellular route by chelating Ca^{+2} to open tight junctions of epithelium. It has low interaction with membrane which leads to lack of high toxicity of membrane. Higher concentration of EDTA can act on transcellular route. All results suggest that after 4 mM of concentration of EDTA all tight junctions are opened completely so after that higher concentrations causes much more similar increase in permeation. An optimum concentration was selected as 8 mM; as such there is no much significance difference in P_{app} values of mannitol with 4 mM and 8 mM EDTA. The reason to optimize 8 mM of EDTA, as decrease in % TEER values suggests there is more opening of tight junctions of monolayers than with 4 mM of EDTA.

The release profile of ACV in presence of optimized concentration of EDTA (8 mM) and with different concentrations of bioenhancers clearly indicates that higher concentrations of QU, Sil and LT are not increasing permeation but with lower concentrations the release i.e. fluxes value of ACV is increasing. While EDTA optimized concentration which was taken as standard bioenhancer shows more release of ACV than bioenhancers concentrations. The presence of QU, Sil and LT is decreasing % TEER values of Caco-2 monolayers. ACV which absorbs by paracellular route has less permeation across the membrane in the absence of QU, Sil and LT. While QU, Sil and LT affects the permeation of ACV, this reflects bioenhancers may have paracellular opening effect that might be related to ready permeation across the

apical membrane. The best concentration of bioenhancers has been further used in the in-vivo pharmacokinetic studies. The results of *in-vitro* studies are shown in **Figure 1, 2**.

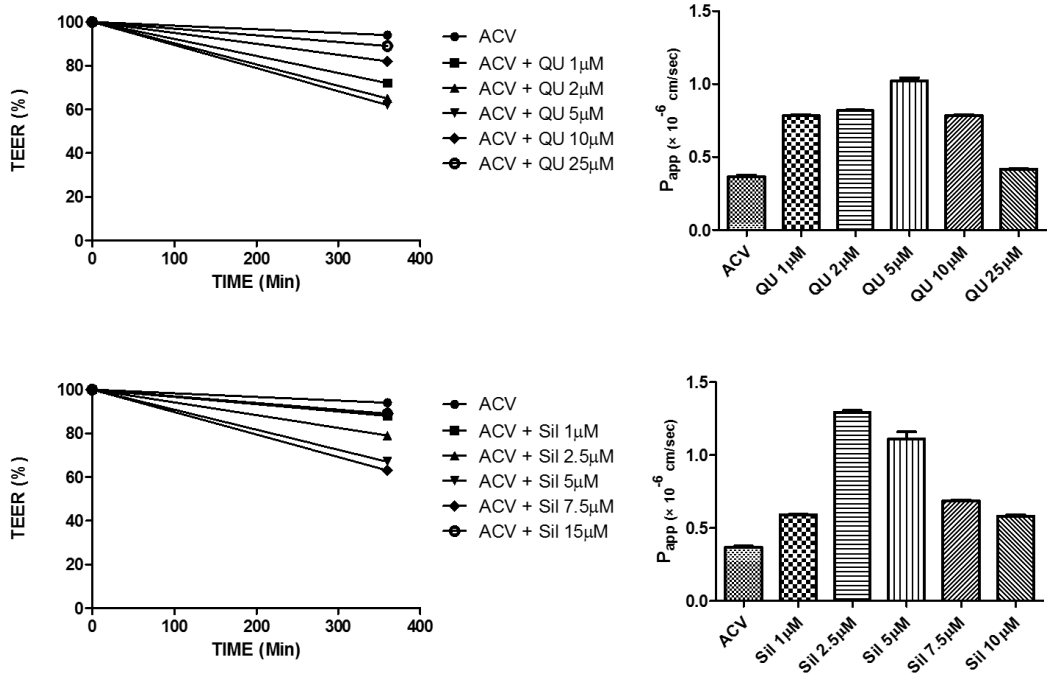

Fig: 1 Papp and effect of various concentrations of Qu, Sil on pre and post % TEER (at 360 min) of Caco-2 monolayers containing ACV

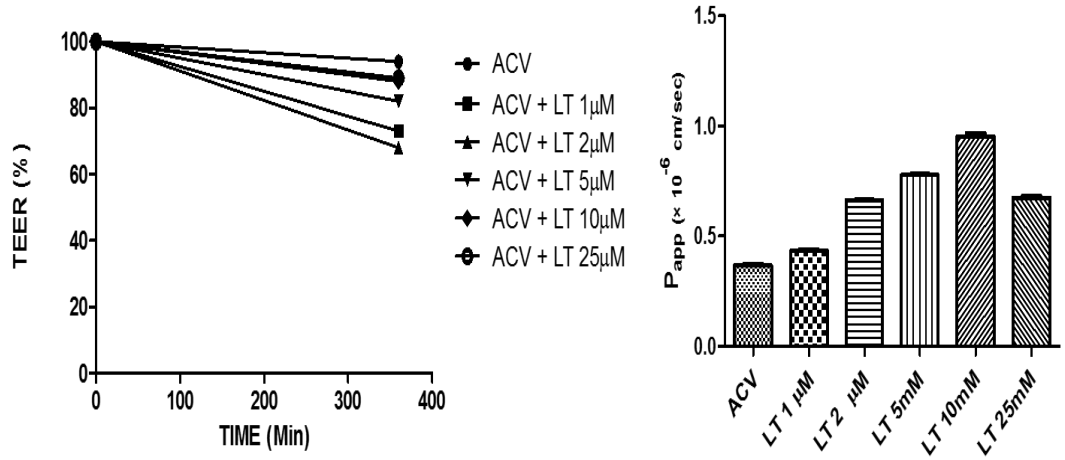

Fig: 2 Papp and effect of various concentrations of LT on pre and post % TEER (at 360 min) of Caco-2 monolayers containing ACV

In-vivo pharmacokinetic studies initiates with the development of bio-analytical method. The chromatograph of rabbit blank plasma and plasma spiked with ACV standard is shown in **Figure 3**. It is clearly observed from the chromatogram that there was no interference from blank plasma peaks to peaks of drug; as well as all the bioenhancers QU, Sil and LT are not showing any peak at the retention time of ACV. Calibration curve was plotted between area of ACV versus ACV concentration in plasma was constructed by spiking seven different concentrations of ACV. Chromatographic responses were found to be linear over an analytical range of 80–2500 ng/ml and found to be quite satisfactory and reproducible with time. Correlation coefficient equals 0.9975, indicating a strong linear relationship between the variables. Extraction efficiency was greater than 89 %. Accuracy data in the present study ranged from 98.38 to 99.87 % indicates that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of ACV, gave %RSD below 5.0 (should be less than 15 according to CDER guidance for Bio-analytical Method Validation), revealed that proposed method is highly precise.

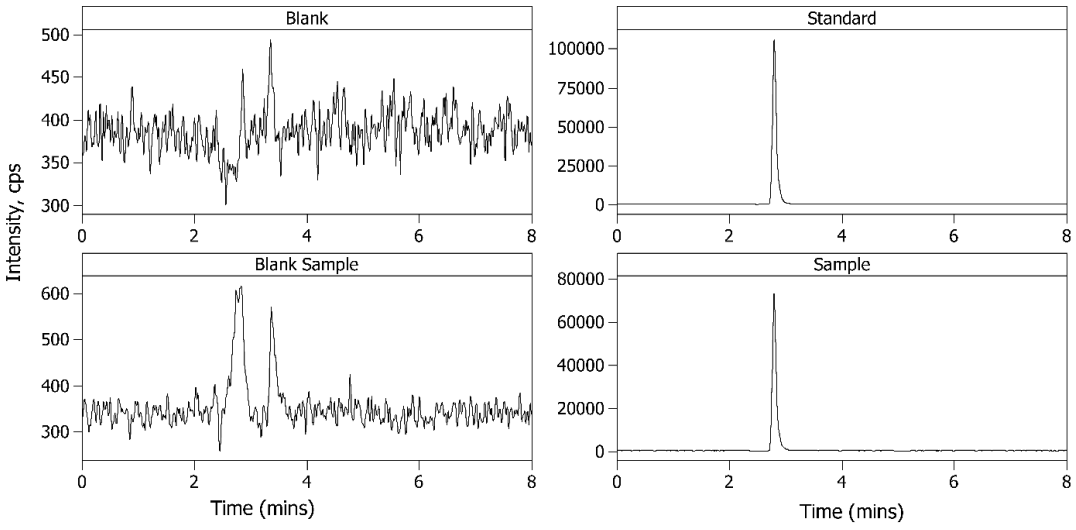

Fig 3: LC-MS chromatogram of sample, standard and blank of ACV

Plasma samples collected at different time points from the rabbits were analyzed using LC-MS and drug (ACV) plasma concentration values were determined from calibration

curve. Mean plasma concentrations of ACV in each of rabbit i.e. control rabbit and rabbit treated with each of three concentrations of ACV and bioenhancers. The average plasma drug concentrations versus time profiles in presence of each concentrations of QU, Sil and LT are represented. It is clearly observed that plasma drug concentrations with each concentrations of bioenhancers are highly significant ($p < 0.001$) and increased than control ACV.

Different concentrations of QU, Sil and LT were administered to rabbits with ACV, it significantly increase the mean plasma concentration of ACV. It is found that administration of lowest concentration i.e 5 mg/kg of QU, 2.5 mg/kg Sil and 0.25 mg/kg LT cause maximum bioenhancement of ACV. It cause 2.85, 2.65 and 1.97 fold increment in permeation of ACV.

The enhanced absorption could be due to an interaction between QU, Sil, LT and ACV in gastrointestinal tract. Possible mechanisms proposed are,

- The results obtained from the Caco-2 cell line studies postulates that bioenhancers can modulate paracellular openings and cause inhibition of P-glycoprotein (plays minor role in ACV permeation). This synergistic action (paracellular opening and P-glycoprotein inhibition) increases permeation of ACV significantly in presence of QU, Sil and LT.
- Results suggest that the QU induced increase in bioavailability of oral ACV can be attributed to enhanced ACV absorption in the gastrointestinal tract via QU induced inhibition of P-gp and reduced first-pass metabolism due to QU induced inhibition of CYP3A in the small intestine and/or in the liver rather than reduced renal and/or hepatic elimination of ACV. Sil could mainly be due to the increased intestinal absorption of ACV via P-gp inhibition. While LT could increase bioavailability by inhibition of P-gp and reduced first-pass metabolism of ACV by LT inhibition of CYP3A in the small intestine and/or in the liver rather than reduced renal and/or hepatic elimination of ACV.

Conclusion:

The results of DSC and IR of binary mixture suggests that there is no physical interaction between ACV and bioenhancers.

The results of mannitol permeation studies showed that 8 mM concentration of EDTA is sufficient enough to open paracellular pathway and leads to more permeation of mannitol. In permeation study of ACV it is found that QU, Sil and LT significantly improve permeation of ACV in lower concentrations than the higher concentrations of bioenhancers. QU with 5 µM concentration cause 1.9 fold increases in permeation, 2.5 µM of Sil is sufficient to cause 2.4 fold enhancement, while 10 mM LT cause 1.65 fold enhancement permeation of ACV. Bioenhancers seems to cause opening of paracellular pathway which can lead to more permeation of ACV across cell monolayers. These all studies indicates all bioenhancers are most effective as bioenhancers in lowest concentrations only. Higher concentrations may cause reduction in permeation of ACV. Bioenhancers can modulate membrane dynamics, and cause increase in intestinal surface and its brush border effect allows more permeation of ACV.

The results of pharmacokinetic data suggests that all the three bioenhancers QU, Sil and LT effectively cause increment in mean ACV plasma concentration. All the bioenhancers QU, Sil and LT administration with ACV cause's increment in area under the curve and absorption rate. They don't affect elimination rate but prolong T_{max}. The results of *in vivo* studies of ACV with Sil and LT are almost similar but more enhancement of ACV found with QU. The *in vivo* results of enhancement in permeation are same as the results obtained in previous studies of *in vitro* cell line studies.

In the *in vivo* studies of ACV with QU, lowest concentration of QU (8:1) 5 mg/kg is sufficient enough to act as bioenhancer and cause almost 3 fold increase in bioavailability. While in *in vitro* studies It has been found that QU (ACV: QU 10:2) cause 2 fold increase in permeation of ACV.

In the *in vivo* studies of ACV with Sil, (16:1) 2.5 mg/kg of is causing almost 4 fold increase in bioavailability of ACV. ACV: Sil (10:2) cause almost 2.5 fold increase in permeation of ACV in *in vitro* permeation studies.

In the *in vivo* studies of ACV with LT, (ACV: LT 0.25 mg/kg of LT) is causing 2 fold increase in bioavailability of ACV. ACV: LT cause 1.7 fold increase in permeation of ACV in *in vitro* permeation studies.

These herbal origin bioenhancers are not causing any toxicity on the intestinal membrane like synthetic bioenhancers. Thus QU, Sil and LT can be successfully utilized as bioenhancer for ACV.

Effect of quercetin, silibinin and luteolin on Saquinavir

Inferences form Literature Review and Present State of Knowledge

The oral bioavailability of the human immunodeficiency virus (HIV) protease inhibitor saquinavir (SQU) is also low and variable in patients. The reasons for low oral bioavailability are unclear (11). One explanation is that absorption is decreased by an active efflux pump in the lever and intestine such as P-glycoprotein (P-gp). P-gp is a 170 kDa transmembrane protein (12). It is localized at the apical secretory surface of various tissues where it mediates the active transmembrane transport of a variety of lipophilic substrates. It appears to act as a general detoxification system protecting tissues from a broad spectrum of lipophilic endogenous or exogenous toxic compounds, most of which tend to be large, aromatic and amphiphilic (13, 14). Several studies have demonstrated the possible use of P-gp inhibitors that reverse P-gp-mediated efflux in an attempt to improve the efficiency of drug transport across the epithelia, thus resulting in enhanced oral bioavailability. P-gp inhibitors may also influence absorption, distribution, metabolism and elimination of P-gp substrates in the process of modulating pharmacokinetics (15).

Materials and methods:

SQU-QU, SQU-Sil, SQU-LT binary systems were prepared at three different weight ratios. In physical mixtures in this method SQU-QU, SQU-Sil, SQU-LT were prepared by gently blending for 10 minutes in a polythene bag. Binary system were tested for compatibility using FTIR, DSC.

RP-HPLC analytical method was developed and validated to estimate the SQU in presence of QU, Sil and LT. The developed HPLC method was then transferred to the LC-MS. Chromatographic elution was achieved using a Phenomenax C18 5µm (250*4.6) mm column at a flow rate of 0.5 ml/min having run time 10 mins. The isocratic composition of eluent a (water with 0.1% formic acid) and eluent b (acetonitrile) was in 35:65 % v/v.

Prior to working on the lab study these mixture a computer aided study was also performed to confirm the affinity of the SQU, QU, Sil and LT towards P-gp and CYP enzymes. The results from prediction verifies the higher affinity of QU, Sil and LT as compare to the SQU.

In vitro Caco-2 cell lines were used to perform the permeation study. Caco-2 cell transport studies were conducted for SQU and SQU-QU, SQU-Sil, SQU-LT binary systems to determine the extent of improvement in permeability. Apparent permeability coefficient P_{app} values for SQU, SQU-QU, SQU-Sil, SQU-LT binary systems were calculated. Transepithelial electrical resistance and permeation of transcellular marker; phenol red values in representative cell monolayers were assessed to examine the cell morphology and monolayer integrity. Amount of SQU permeated through cell line was estimated using developed and validated method as described above. The best concentration of bioenhancers has been further used in the in-vivo pharmacokinetic studies.

In vivo pharmacokinetic study has been performed using New Zealand white rabbits (2-3 Kg) as animal model. Total 30 rabbits will be randomly divided into 5 groups of 6

animals each (n=6). The animals were housed over two weeks in a temperature (20 – 25 °C) and relative humidity (between 50 and 60 %) controlled room and were given standard rat chow and water which were freely available. The protocol for studies was approved by the Institutional ethical committee at The M S University of Baroda, India. All the animals were fasted overnight with free access to tap water before experiments. SQU (control), SQU along with optimized concentration of QU, Sil and LT from *in-vitro* were administered orally with gavage needle. One group was given SQU by intravenous route to determine the absolute bioavailability. Blood samples were collected from the retro-orbital plexus at 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 24.0 and 48 hr into labelled tubes, containing 10 µl of 20 % w/v K_2EDTA solution, as anticoagulant. Plasma was harvested from the blood by centrifugation. Collected plasma samples were further prepared for analysis by adding 500 µL acetonitrile to 100 µL plasma, which were then vortex-mixed briefly. Following centrifugation for 10 min at 10,000×g. Then supernatant was make up to 200 µL using mobile phase. Then prepared samples were analysed for the amount of SQU using LC-MS. The results obtained from the LC-MS analysis was then further used for assessment of various pharmacokinetic parameters i.e. C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$, T_{max}, elimination rate constant (Ke) and elimination half-life ($T_{1/2}$) etc.

Results and discussion:

FTIR spectra of SQU, QU, Sil, LT, SQU-QU, SQU-Sil and SQU-LT binary systems were recorded. According to the FTIR results, no interaction between SQU and bioenhancers has been detected. All these peaks of SQU remain unaffected in the IR spectrum of binary systems of each method. The slight change in the intensity is there which is not having any significant difference than the pure SQU. Thus FTIR spectra remains unchanged, explained that there was no interaction between SQU and bioenhancers in the binary system. This confirms that QU, SIL, LU is not interacting with SQU at molecular level.

The thermograms of pure SQU, QU, Sil, LT, SQU-QU, SQU-Sil and SQU-LT binary systems were recorded. The thermogram of pure SQU showed a sharp endothermic peak at 250.58 °C, which is due to a melting point as it consumes energy. In the thermogram of binary systems shift in peak to lower temperature which may be explained by weak interaction between SQU and bioenhacncers. All thermograms prove that almost negligible interaction between SQU and bioenhancers in binary systems. The DSC analysis showed a negligible change in melting point of QU in the presence of bioenhancers.

Bi-directional Caco-2 permeability of SQU alone and in presence of bioenhancers was determined. SQU has shown an efflux ratio (B-A/A-B) of > 25. This efflux ratio reduced to 2.6 when SQU co-administered with verapamil, a positive control P-gp inhibitor. This result confirms that SQU is a P-gp substrate. Similarly experiments with SQU in presence of QU, Sil and LT reduced the efflux ratio to ~2 suggesting potential inhibition of P-gp. Caco-2 mediated SQU transport were completely blocked by QU, Sil and LT which is reflected by a decrease of efflux ratios down to 3.6, 3.2 and 2.7 respectively. At the 10 µM, 5 µM and 10 µM concentration, these compounds almost completely inhibited the activity of P-gp. These results suggested that QU, Sil and LT are appeared to be an inhibitor of P-gp. The results of in-vitro studies has been illustrate in **Figure 4, 5**.

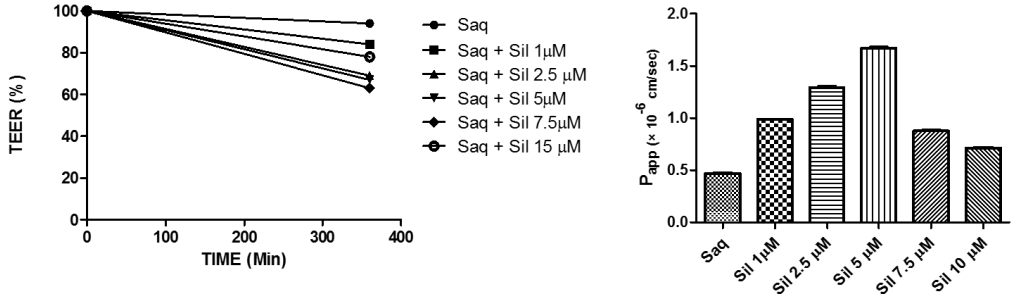

Fig: 4 Papp and effect of various concentrations of Sil on pre and post % TEER (at 360 min) of Caco-2 monolayers containing SQU

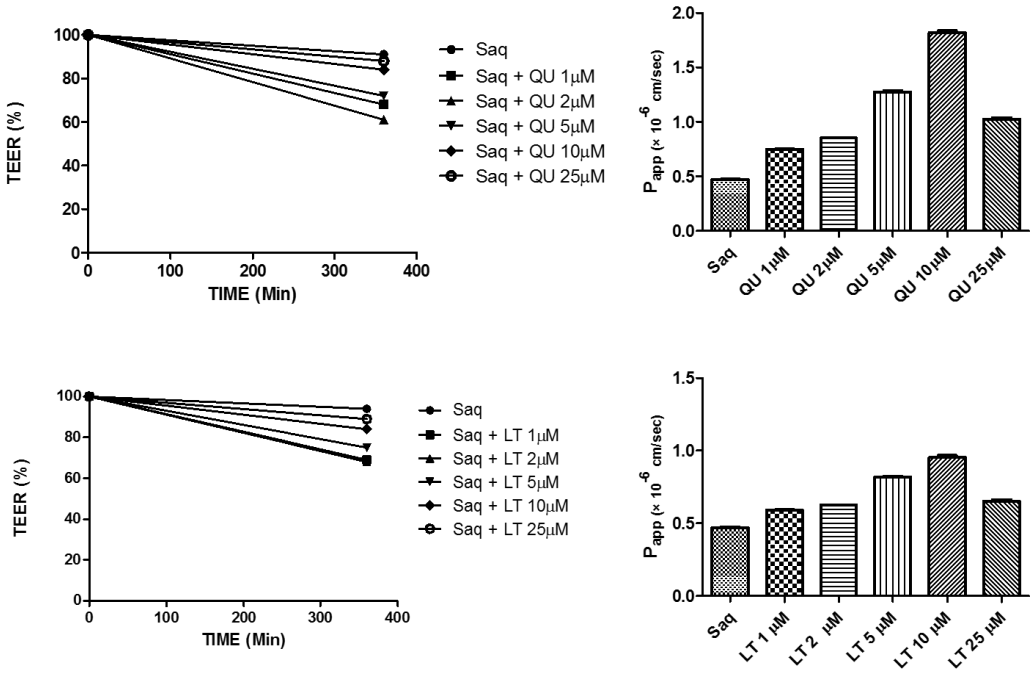

Fig: 5 Papp and effect of various concentrations of LT and QU on pre and post % TEER (at 360 min) of Caco-2 monolayers containing SQU

To further investigate the interactions of QU, Sil and LT with SQU. *In-vivo*, pharmacokinetic studies of SQU after i.v. and p.o. administration were performed in rabbits with and without the co-administration of QU, Sil and LT. *In-vivo* pharmacokinetic studies initiates with the development of bio-analytical method. The chromatograph of rabbit blank plasma and plasma spiked with SQU standard is shown in **Figure 6**. It is clearly observed

from the chromatogram that there was no interference from blank plasma peaks to peaks of drug; as well as all the bioenhancers QU, Sil and LT are not showing any peak at the retention time of SQU. Calibration curve was plotted between areas of SQU versus SQU concentration in plasma was constructed by spiking seven different concentrations of SQU. Chromatographic responses were found to be linear over an analytical range of 25–2000 ng/ml and found to be quite satisfactory and reproducible with time. Correlation coefficient equals 0.9915, indicating a strong linear relationship between the variables. Extraction efficiency was greater than 85 %. Accuracy data in the present study ranged from 98.42 to 100.07 % indicates that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of SQU, gave R.S.D. below 5.0 (should be less than 15 according to CDER guidance for Bio-analytical Method Validation), revealed that proposed method is highly precise.

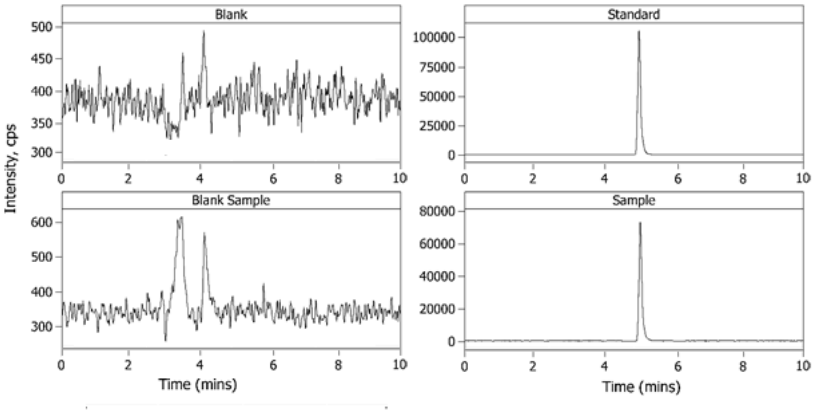

Fig 6: LC-MS chromatogram of sample, standard and blank of SQU

Plasma samples collected at different time points from the rabbits were analyzed using LC-MS and drug (SQU) plasma concentration values were determined from calibration curve. Mean plasma concentrations of SQU in each of rabbit i.e. control rabbit and rabbit treated with each of three concentrations of SQU and bioenhancers. The average plasma drug concentrations versus time profiles in presence of each concentrations of QU, Sil and LT are

represented. It is clearly observed that plasma drug concentrations with each concentrations of bioenhancers are highly significant ($p < 0.001$) and increased than control SQU.

Different concentrations of QU, Sil and LT were administered to rabbits with SQU, it significantly increase the mean plasma concentration of SQU. It is found that administration of lowest concentration i.e 10 mg/kg of QU, 5 mg/kg Sil and 2 mg/kg LT cause maximum bioenhancement of QU. It cause 3.91, 5.32 and 2.19 fold increment in permeation of SQU.

The enhanced absorption could be due to an interaction between QU, Sil, LT and SQU in gastrointestinal tract. The results obtained from the Caco-2 cell line studies postulates that bioenhancers can modulate paracellular openings and cause inhibition of P-glycoprotein (plays minor role in SQU permeation). This synergistic action (paracellular opening and P-glycoprotein inhibition) increases permeation of SQU significantly in presence of QU, Sil and LT. Since QU, Sil and LT are well absorbed through intestine, non-toxic and has many health-benificial activities; these might be good candidates of efflux transporter(s) modulator in improving the bioavailability of low bioavalable compound SQU. P-gp inhibition by QU, Sil and LT, possibly attributed to the enhanced intestinal absorption and/or decreased\ systemic elimination of SQU After p.o. administration of QU, Sil and LT separately, the altered p.o. pharmacokinetics of SQU is more likely because of the intestine and/or systemic interactions of QU, Sil and LT with SQU.

Conclusion:

The results of DSC and IR of binary systems suggests that there is no physical interaction between SQU and QU, Sil and LT.

The results of mannitol permeation studies showed that 8 mM concentration of EDTA is sufficient enough to open paracellular pathway and leads to more permeation of mannitol. In permeation study of SQU it is found that QU, Sil and LT significantly improve permeation of SQU in lower concentrations than the higher concentrations of bioenhancers. QU with 10

µM concentration cause 3.6 fold increases in permeation, 5 µM of Sil is sufficient to cause 3.2 fold enhancement, while 10 µM of LT causes 2.7 fold enhancement in permeation of SQU. Bioenhancers seems to cause opening of paracellular pathway which can lead to more permeation of SQU across cell monolayers. These all studies indicates all bioenhancers are most effective as bioenhancers in lowest concentrations only. Higher concentrations may cause reduction in permeation of SQU. Bioenhancers can modulate membrane dynamics, and cause increase in intestinal surface and its brush border effect allows more permeation of SQU.

The results pharmacokinetic data suggests that all three bioenhancers QU, Sil and LT effectively cause increment in mean SQU plasma concentration. All the bioenhancers QU, Sil and LT administration with SQU cause's increment in area under the curve and absorption rate. They don't affect elimination rate but prolong T_{max}. The results of *in vivo* studies of SQU with QU, Sil and LT are almost similar. The *in vivo* results of enhancement in permeation are same as the results obtained in previous studies of *in vitro* cell line studies.

In the *in vivo* studies of SQU with QU, lowest concentration of QU (SQU: QU 5:1 i.e. 10 mg/kg of QU) is sufficient enough to act as bioenhancer and cause almost 4 fold increase in bioavailability. While in *in vitro* studies It has been found that SQU (SQU: QU 5:2) cause 3.7 fold increase in permeation of SQU.

In the *in vivo* studies of SQU with Sil, (SQU: Sil 5 mg/kg of Sil) is causing 5.3 fold increase in bioavailability of SQU. While SQU: Sil (5:1) cause 3.2 fold increase in permeation of SQU in *in vitro* permeation studies.

In the *in vivo* studies of SQU with LT, (SQU: LT 2 mg/kg of LT) is causing 2.19 fold increase in bioavailability of SQU. While SQU: LT (5:2) cause almost 2 fold increase in permeation of SQU in *in vitro* permeation studies.

These herbal origin bioenhancers are not causing any toxicity on the intestinal membrane like synthetic bioenhancers. Thus QU, Sil and LT can be successfully utilized as bioenhancer for SQU.

Section B: Study the effect of quercetin, silibinin and luteolin on topical delivery of ACV

Introduction

In the dissertation research work primarily boienhancers 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). The physic chemical properties of ACV are: molecular weight (225.21), partition coefficient (log Ko/w-1.56), melting point (256.5^{O}C), and dissociation constant (2.94 and 9.23) (15, 16). Therefore this compound may serve as a good model drug for the transdermal drug delivery. So to explore the effect of natural bioenhacers 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.

Effect of bioenhancers on topical permeation:

➢ Cream preparation:

An aqueous cream was prepared using following ingredients:

1. ACV: 250 mg
2. Cetostearyl alcohol: 337.5 mg
3. White soft paraffin 625 mg
4. Liquid paraffin 250 mg
5. Propylene glycol 2 gm
6. Purified water (to) 5 gm
7. 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 1**.

Drug content studies:

Dummy cream formulations prepared in lab was analyzed for drug content using validated HPLC method. The amount of drug in formulation was found to be in between 98.12 % - 99.76%.

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. Spreadibility was found to be satisfactory in the range of 17.6 -18.4.

Table 1: Different formulations with excipients

Formulation code	ACV (mg)	bioenhancer	CA[#] (mg)	SLS* (mg)	WSP[$] (mg)	LP[@] (mg)	P G[&] (gm)	Water (to gm)
Acv	250	NA	337.5	37.5	625	250	2	5
ACSI-1	250	Silibinin 0.4 mg	337.5	37.5	625	250	2	5
ACSI-2	250	Silibinin 0.5 mg	337.5	37.5	625	250	2	5
ACSI-3	250	Silibinin 1 mg	337.5	37.5	625	250	2	5
ACSI-4	250	Silibinin 2 mg	337.5	37.5	625	250	2	5
ACSI-5	250	Silibinin 2.5 mg	337.5	37.5	625	250	2	5
ACQU-1	250	Quercetin 0.4 mg	337.5	37.5	625	250	2	5
ACQU-2	250	Quercetin 0.5 mg	337.5	37.5	625	250	2	5
ACQU-3	250	Quercetin 1 mg	337.5	37.5	625	250	2	5
ACQU-4	250	Quercetin 2 mg	337.5	37.5	625	250	2	5
ACQU-5	250	Quercetin 2.5 mg	337.5	37.5	625	250	2	5
ACLU-1	250	Luteolin 0.4 mg	337.5	37.5	625	250	2	5
ACLU-2	250	Luteolin 0.5 mg	337.5	37.5	625	250	2	5
ACLU-3	250	Luteolin 1 mg	337.5	37.5	625	250	2	5
ACLU-4	250	Luteolin 2 mg	337.5	37.5	625	250	2	5
ACLU-5	250	Luteolin 2.5 mg	337.5	37.5	625	250	2	5

***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 24h 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 1ml sample was withdrawn at suitable time intervals and replaced immediately with fresh volumes of diffusion medium. The samples were analyzed using above mentioned HPLC method after suitable dilutions. The *in-vitro* studies show there is a significant increase in the permeation of the ACV from the skin in the presence of QU, Sil and LT. In ACV-QU the maximum enhancement was found at 4% having 1.98 fold increase in the flux of the drug. While in the ACV-Sil and ACV-LT maximum enhancement was at 2% and 1% having 2.14 and 1.57 fold increase in the flux of the drug respectively. Comparison of mean cumulative amount and flux has been illustrated in **Figure 7 and 8** respectively.

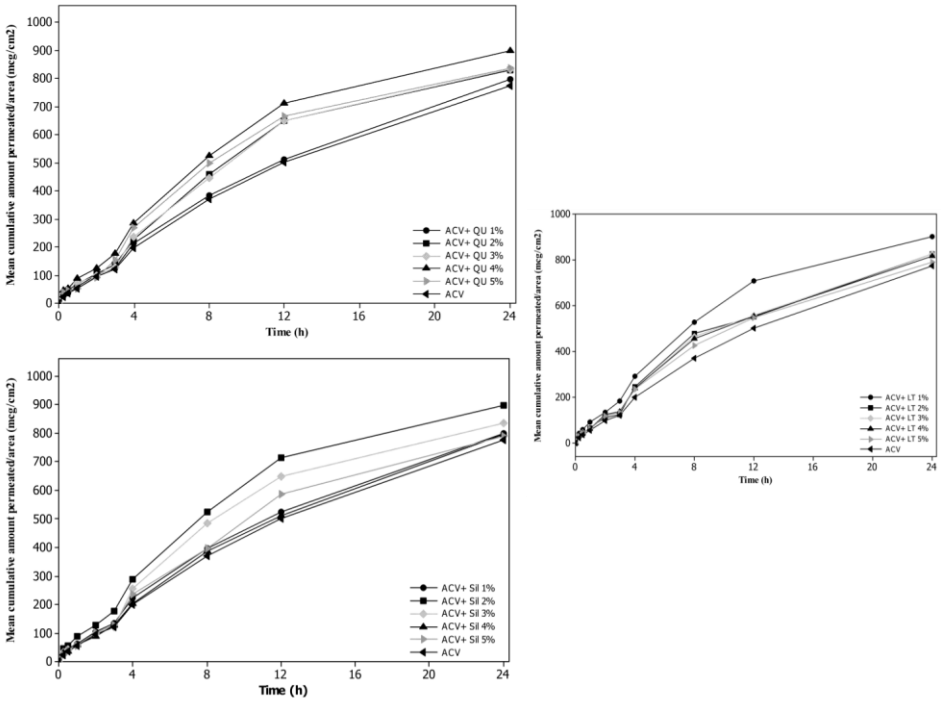

Fig: 7 Comparison of mean cumulative amount of ACV released per unit area of skin

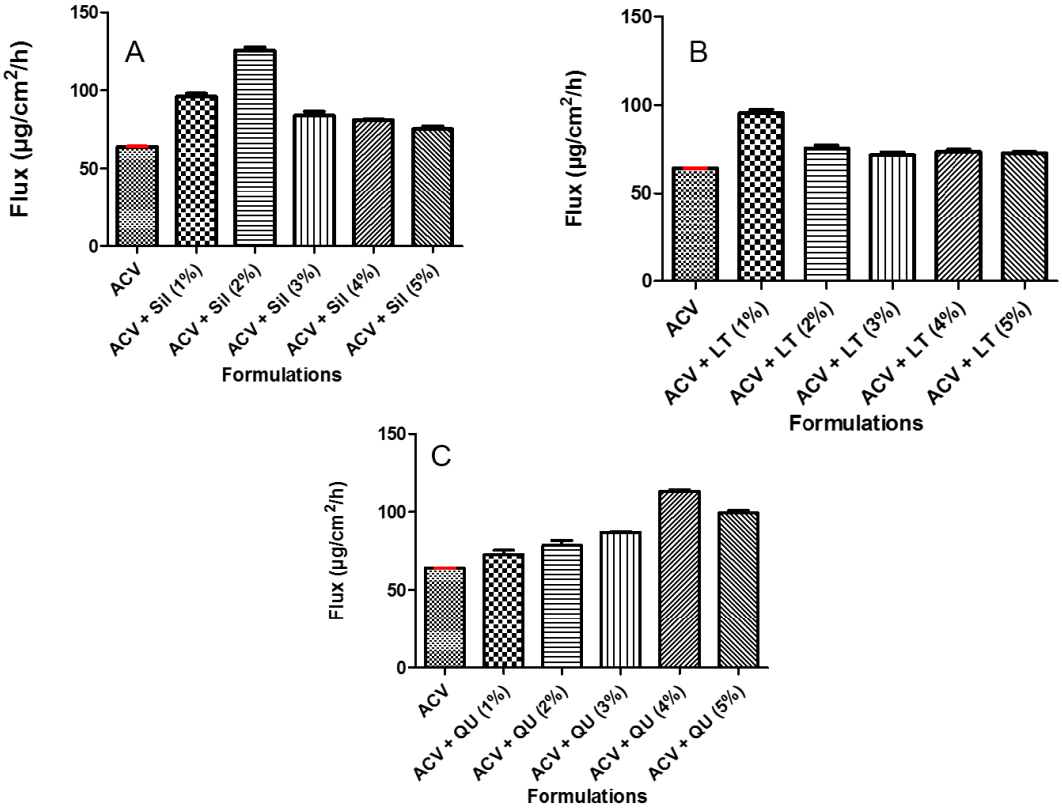

Fig: 8 Comparison of Flux of ACV in different creams

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. The amount of drug retained in skin was significantly higher in all the prepared creams. It indicates that concentration of drug penetrate in to the skin is higher in the presence of QU, Sil and LT. Comparison of amount retain in skin has been illustrated in **Figure 9**.

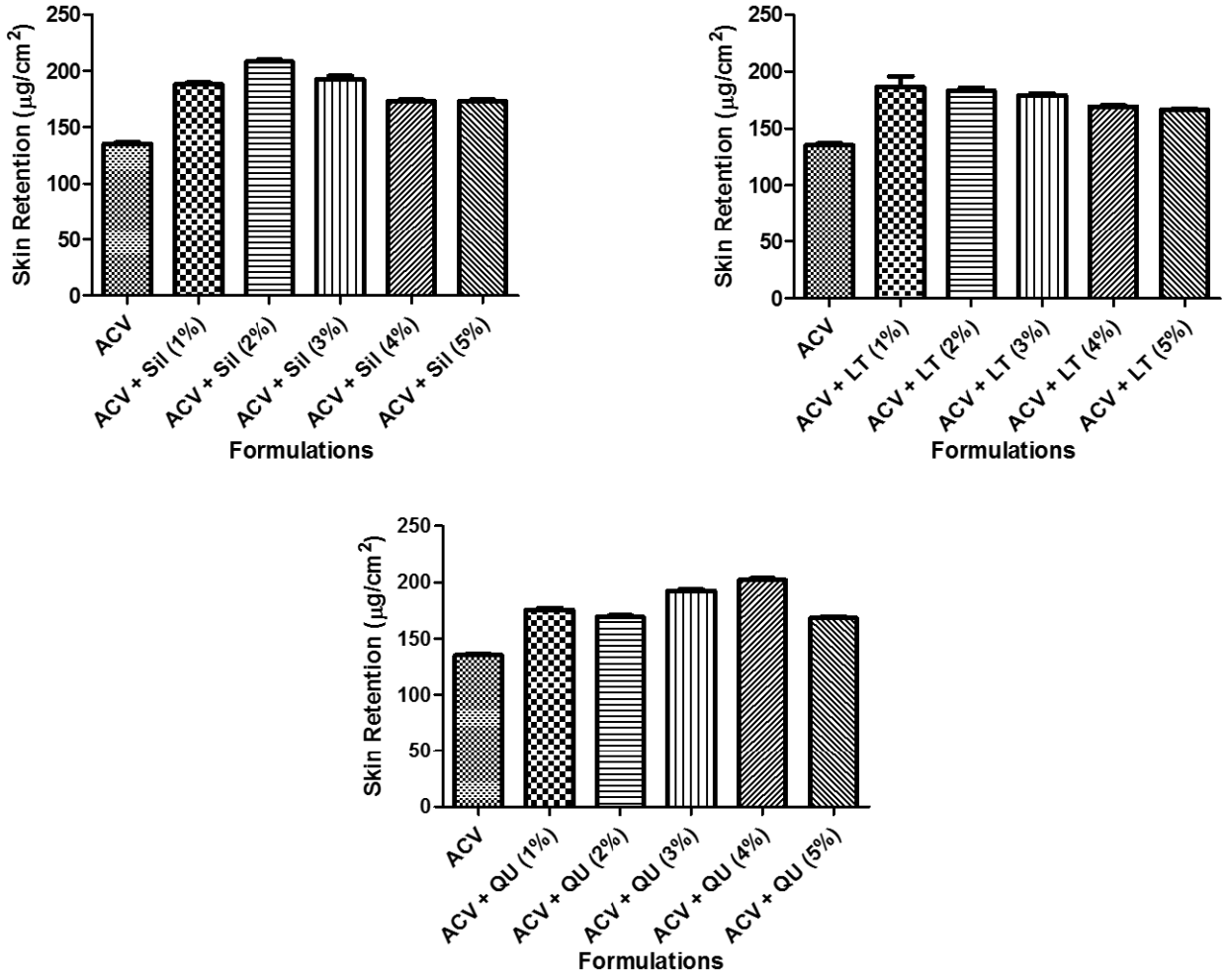

Fig: 9 Comparison of Skin retention of ACV in different creams

ACV permeation studies across 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. The *in-vitro* cell line studies show there is a significant increase in the permeation of the ACV from the skin 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 drug. 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 **Figure 10, 11 and 12** respectively

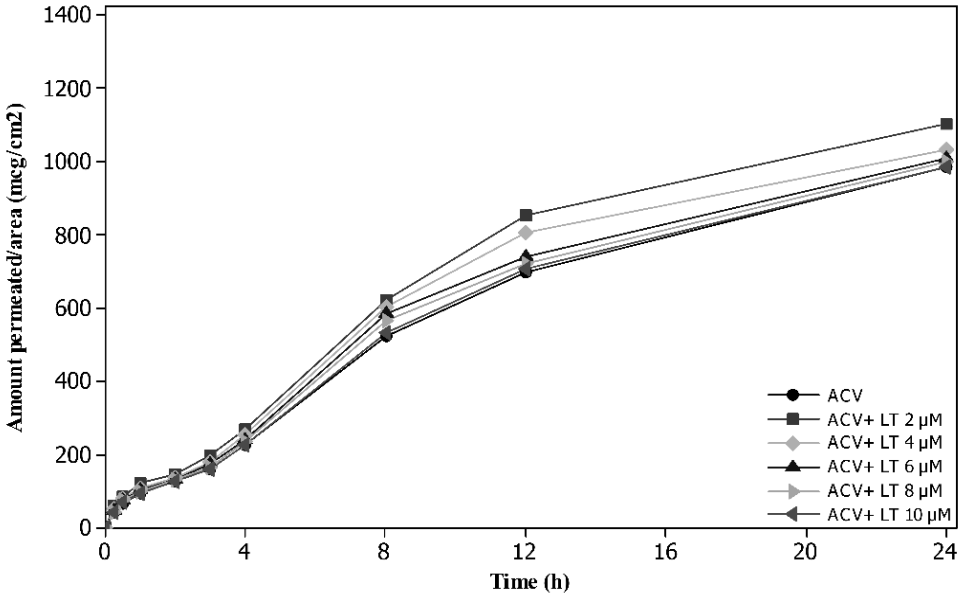

Fig: 10 Comparison of amount permeated of ACV and different concentrations of LT

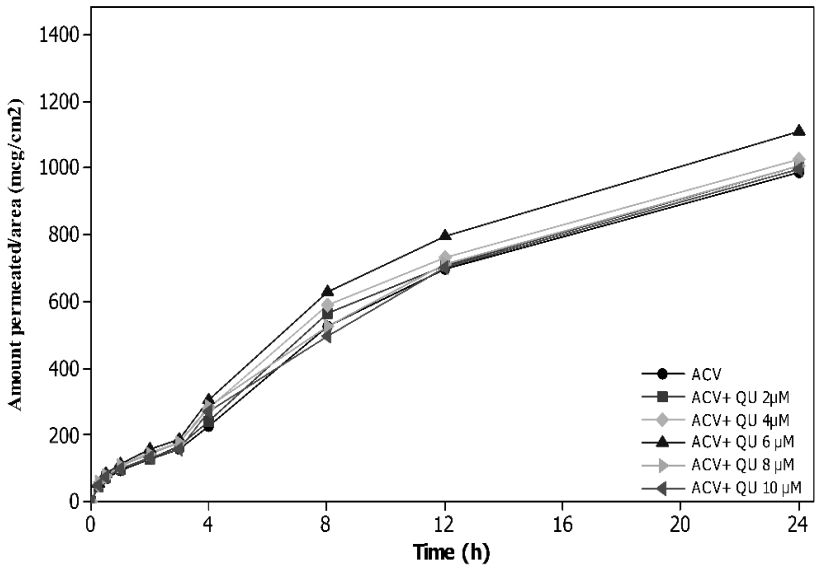

Fig: 11 Comparison of amount permeated of ACV and different concentrations of QU

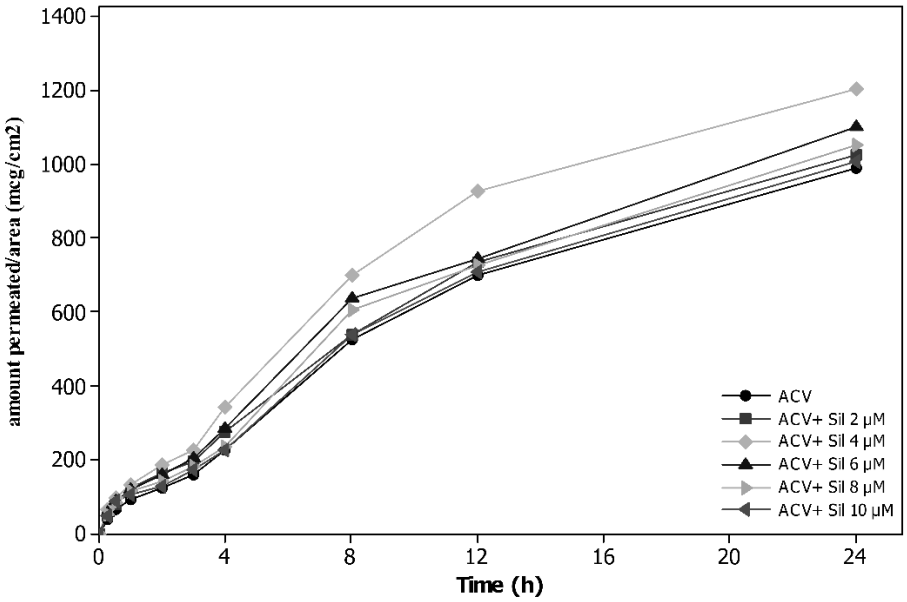

Fig: 12 Comparison of amount permeated of ACV and different concentrations of Sil

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 (17). MTT assay revealed that the tested QU, Sil and LT concentrations were found to be non-toxic to the cell monolayer.

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. But 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.

Summary:

The results of *In-vitro* and In-vivo studies for acyclovir and saquinavir has been illustrated in the **Table 2 and 3 respectively**

Table 2: Results of increase in the bioavailability of acyclovir in the presence of different bioenhancers observed in In-vitro and In-vivo study.

Bioenhancer	***In-vitro*** ***(fold increase than control)***	***In-vivo*** ***(fold increase than control)***
Quercetin	**1.9**	**2.85**
Silibinin	**2.4**	**2.65**
Luteolin	**1.65**	**1.97**

Table 3 Results of increase in the bioavailability of saquinavir in the presence of different bioenhancers observed in In-vitro and In-vivo study.

Bioenhancer	*In-vitro* *(fold increase than control)*	*In-vivo* *(fold increase than control)*
Quercetin	**3.7**	**3.91**
Silibinin	**3.2**	**5.32**
Luteolin	**2.09**	**2.19**

The results of *In-vitro* skin permeation study and in-vitro cell line studies for acyclovir topical delivery has been illustrated in the **Table 4**

Table 4: Results of increase in the topical permeation of acyclovir in the presence of different bioenhancers observed in In-vitro and In-vivo study.

Bioenhancer	*Skin permeation study (In-vitro)* *(fold increase than control)*	*In-vitro cell line study* *(fold increase than control)*
Quercetin	**1.98**	**1.36**
Silibinin	**2.14**	**1.41**
Luteolin	**1.57**	**1.23**

References:

1. Spear BB, Heath-Chiozzi M, Huff J. Clinical application of pharmacogenetics. Trends Mol Med. 2002;7 201-4.
2. Chan LMS, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. Eur J Pharm Sci. 2004;21(1):25-51.
3. Breedveld P, Beijnen JH, Schellens JHM. Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. Trends Pharmacol Sci. 2006;27(1):17-24.
4. Kuppens IELM, Breedveld P, Beijnen JH, Schellens JHM. Modulation of oral drug bioavailability: from preclinical mechanism to therapeutic application. Cancer Invest. 2005;23(5):443-64.
5. Zhang Y, Benet LZ. The gut as a barrier to drug absorption: combined role of cytochrome P450 3A and P-glycoprotein. Clin Pharmacokinet. 2001;40(3):159-68.
6. Benet LZ, Cummins CL, Wu CY. Unmasking the dynamic interplay between efflux transporters and metabolic enzymes. Int J Pharm. 2004;277(1-2):3-9.
7. Kwan KC. Oral bioavailability and first-pass effects. Drug Metab Dispos. 1997;25(12):1329-36.
8. Kruijtzer CMF, Beijnen JH, Schellens JHM. Improvement of oral drug treatment by temporary inhibition of drug transporters and/or cytochrome P450 in the gastrointestinal tract and liver: an overview. Oncologist. 2002;7(6):516-30.
9. Benet LZ. The role of BCS (biopharmaceutics classification system) and BDDCS (biopharmaceutics drug disposition classification system) in drug development. J Pharm Sci. 2013;102(1):34-42.

10. Kerb R, Fux R, Mörike K, Kremsner PG, Gil JP, Gleiter CH, et al. Pharmacogenetics of antimalarial drugs: effect on metabolism and transport. Lancet Infect Dis. 2009;9(12):760-74.

11. Noble S, Faulds D. Saquinavir; A review of its pharmacology and clinical potential in management of HIV infection. Drugs. 1996;52: 93-112.

12. Germann U.A. P-gycloprotein – a mediator for multidrug resistance in tumor cells. Eur J. Cancer. 1996;32A: 927-944.

13. Pajeva I.K., Wiese M., Cordes H.P. and Seydel J.K. Membrane interactions of some cataphiphilic drugs and relation to their multi-drug resistance reversing ability. J. Cancer. Res. Clin. Oncol. 1996;122: 27-40.

14. Ecker G., Chiba P. Structure activity relationship studies on modulators of multidrug transporter P-glycoprotein-an overview. Wien. Klin. Wochenschr. 1997;107(22): 681-686.

15. Varma, M.V., Ashokraj, Y., Dey, C.S., Panchagnula, R.. (2003). P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. Pharmacol. Res. 2003;48: 347–359.

16. Franklin KA, Gray PM, Andy GM, Mare BB. Effect of abrasion induced by rotating brush on the skin penetration of routes with varying physicochemical. Eur. J. Pharm. Biopharm. 1007; 10; 1016-1027.

17. B.P. Pharmaceutical Codex, 1994, 711-713.

18. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Meth. 1983;65:55–63.

Paper published:

- A newfangled study using risk silhouette and uncertainty approximation for quantification of acyclovir in diverse formulation, Journal of Pharmaceutical Analysis, 5(1), 2015, 58-69.
- Challenges in Oral Delivery: Role of P-gp Efflux Pump, Current drug therapy, 9(1), 47-55.
- Myriad molecules to overcome efflux drug transporters and drug metabolizing enzymes: a journey from synthetic to natural, CRT-12019, ***Critical Reviews™ in Therapeutic Drug Carrier Systems.*** **(Manuscript Accepted)**
- Risk outlining and uncertainty contour for quantification of acyclovir using LC-MS: solicitation to *In Vitro* cell line studies, **EJAC (Manuscript Accepted)**

Book Chapter:

- Karan Mittal, Rajashree Mashru, Arti R Thakkar, Novel and Nanostructured Drug Delivery Strategies for Enhancing Bioavailability of BCS Class III Drugs, Nanostructured Drug Delivery, 4, 2014

Paper in communication:

- Effect of quercetin, silibinin and luteolin on the topical permeation of acyclovir, ***Acta pharmacologica silica.***