Summary and Conclusion
Historically, developments in transdermal drug delivery have been incremental, focusing on overcoming problems associated with the barrier properties of the skin, reducing skin irritation rates and improving the aesthetics associated with patch systems. Recently, the transdermal route has vied with oral treatment as the most successful innovative research area in drug delivery. Drug delivery through the complex structure of human skin provides a fascinating scientific challenge in an important therapeutic area.

Transdermal drug delivery is gaining more and more interest in the pharmaceutical industry. It also represents the most successful and most innovative area of research in drug delivery. Transdermal drug delivery systems encompass a wide array of non-invasive or minimally invasive technologies for delivering drugs and vaccines across the skin without needles.

A useful approach for increasing percutaneous absorption of drugs is to employ a permeation enhancer(s). Typically, these agents should be able to partition into and interact with skin constituents to induce a temporary and reversible increase in skin permeability. Most notably, the mechanism by which drug molecules penetrate the dermal barrier and the physicochemical properties of the permeant are inviting greater attention. These issues are now more amenable to discussion because of the improved understanding of the stratum corneum (SC) structure.

The use of synthetic chemical enhancers for enhancing the permeation of drugs is decreasing due to their absorption into the systemic circulation often associated with long-term effects and potential systemic toxicity. Therefore, herbal enhancers are being widely investigated for percutaneous enhancement. Hence, the main objectives of this study are:-

a) To screen glycosides and alkaloids from natural sources for enhancing the \textit{in vitro} percutaneous permeation of repaglinide across excised rat epidermis.
b) To study the influence of above-mentioned agents on biochemical constituents, biophysical status and ultra structural manifestations of rat epidermis.
c) To study the pharmacokinetics of repaglinide after application of the optimized transdermal formulation.
d) To test the performance of optimized transdermal formulation by assessing its hypoglycemic effect in rats.

Due to their apolar nature the effect of, alkaloids and vannilloids on membrane fluidity may be attributed to their easy partitioning in the membrane and resulting in altered lipid dynamics. Coumarins contain benzopyrene ring due to which they have a potential to interact with protein portion and influence the lipid fluidity of the skin. They are characterized by their membrane partitioning properties and hence, possess great potential for use as percutaneous permeation enhancement of drugs. The present investigation was aimed at studying the perse effect of alkaloids and vanniloids present in *Piper nigrum* and *Zingiber officinale* (ZO-E) as well as of coumarins and furanocoumarin present in *Angelica archangelica* (AA-E) and *Melilotus officinalis* (MO-E) containing extracts as well as their combination with chitosan and unveiling their influence on microstructure, microconstituents and biophysical manifestations of rat epidermis.

The solubility of RGE in PB was observed to increase to 118 µg/ml in the presence of 0.008% w/v piperine, 43.96 µg/ml in 0.05% ZO-E, 22.47 µg/ml in 5% w/v AA-E or 20.43 µg/ml in 3% w/v MO-E, respectively. The enhancement of aqueous solubility of RGE resulted in a decrease in Ko/w of RGE. The enhanced solubility of RGE in the presence of these extracts may be due to the effect of the physicochemical properties of alkaloids and coumarins present in theses extracts.

The permeation of RGE using any extract, CTN or extract-CTN mixture as donor formulation across excised as well as epidermis excised from viable rat epidermis was observed to be significantly higher ($p < 0.05$) as compared to that
using propylene glycol-ethanol (7:3) mixture. Maximum flux of RGE was observed when mixture of the respective extract was used in combination with CTN as donor vehicle. Amongst alkaloids and vanniloids (piperine and ZO-E extract), piperine was more effective in increasing the permeation of RGE. Amongst coumarins and furanocoumarins (AA-E and ZO-E extract), AA-E was found to be more effective in increasing the permeation of RGE. The permeation of RGE was maximum across epidermis excised at 36 h treatment of viable skin with piperine or piperine-CTN mixture whereas for the other extracts permeation was maximum at 12 h. The flux of RGE across epidermis excised at later time periods decreased continuously. The decreased permeation of RGE at later time intervals may be due to normalization of skin microconstituents that would accompany the restoration of barrier properties of skin. The maximum enhancement of RGE permeation was observed in the presence of AA-E-CTN combination.

The effect of alkaloids and vanniloids on membrane fluidity may be attributed to their easy partitioning in the membrane (due to their apolar nature) and the configuration they assume with neighboring molecules in the membrane, resulting in altered lipid dynamics. Modulation of membrane protein function by its lipid environment is a well known phenomenon. Coumarins contain benzopyrene ring due to which they possess a potential to interact with protein components of the skin. Coumarins can easily partition in the SC and diffusion across a epidermis. However, the observed decrease in Ko/w of RGE by the enhancers and enhanced permeation across excised rat epidermis indicated that RGE permeation could not be a simple function of concentrations of extracts employed in the donor vehicle. Therefore, the influence of these extracts on skin micro constituents and ultrastructural features was critically evaluated for gaining an insight into exact mechanism of permeation enhancement by these extracts. Treatment of excised rat epidermis with different extracts for 48 h extracted cholesterol, triglycerides and sphingosine. It is interesting to note that all these treatments were significantly less effective (p < 0.05) for viable skin as compared
to that for excised skin. This could be attributed to the fact that the skin recognizes the impairment in its barrier function and rapidly restores itself by synthesizing lipids in order to replace those that have been extracted. The data indicates that lipid extraction followed the same trend as that of permeation studies where flux decreased when skin was excised after 36 h of treatment with piperine or piperine-CTN mixture and 24 h after treatment with ZO-E, AA-E or MO-E or their combination with CTN.

DSC analysis of untreated epidermal samples revealed four sharp endothermic transitions at 36°C (T₁), 70°C (T₂), 81°C (T₃), and 96°C (T₄). The first endotherm (T₁) has been attributed to sebaceous secretions, surface contamination, or minor structural rearrangements within the bilayer and has not been detected by many investigators. Due to the very small enthalpy and absence from thermograms of many samples, the importance of this endotherm (T₁) while investigating the mechanism of action of penetration enhancers remains obscure. It is generally accepted that the T₂ endotherm at 70°C arises from the melting of lipid bilayer. The T₃ endotherm at 81°C might be associated with the disruption of polar head groups of lipids or the melting of lipids associated with keratin. The occurrence of these lipid-associated endotherms has further been suggested to be due to the heterogeneous distribution of lipids in the intercellular bilayers, leading to the melting of SC lipids in two stages: T₂ and T₃. The reheated sample showed only one broad transition at approximately 70°C. This broad transition has been suggested to be a combination of T₂ and T₃ transition. These studies together suggest that T₂ and T₃ transitions arise from thermal events within the epidermal lipids, while the T₄ transition owes its origin to a protein component that was thermally irreversible.

Propylene glycol:ethanol (7:3) mixture (PG: EtOH) was used as a donor vehicle in our experiments. The thermogram of excised rat epidermis treated with PG:EtOH for 48 h showed the presence of only T₂ transition. Both enthalpy and peak transition temperature (Tm) of this endotherm were insignificantly different than that observed in normal epidermis (P< 0.05). The earlier reports of an
insignificant effect on the enthalpies of lipid transition with a minor shift in Tm of this transition after treatment with PG suggests no major contribution of PG alone towards permeation enhancement of drugs. In addition, ethanol at low concentration (30% w/w) has been reported to increase lipid fluidity especially at the polar interface of the lipid bilayer without causing any overall alteration in the degree of SC lipid-alkyl chain interaction. The absence of lipidprotein transition (T_3) and protein transition (T_4) in PG:EtOH-treated rat epidermis seems to be due to their dehydrating and protein denaturation effects that are expected to replace water molecules bound to polar protein side chains. The broad nature of endotherm and shifting of Tm towards higher temperature could be attributed to the merger of T_2 and T_3 transitions. This suggested the effect of extracts on epidermal lipids as well protein. CTN treatment of excised rat epidermis was also observed to result in merger of T_2 and T_3 endotherms. Similarly, treatment with mixture of any extract with CTN resulted in merger of T_2 and T_3 endotherms as well as obliteration of T_4 endotherm. Therefore, the observed findings of merger of T_2 and T_3 endotherms coupled with obliteration of T_4 endotherm after treatment with extracts, CTN or their mixture suggested their influence on both epidermal lipids and proteins of excised rat epidermis.

The most important function of the skin is the control of the transepidermal water loss (TEWL). The effect of treatment with extracts on barrier status of rat skin was assessed by measurement of TEWL. The TEWL of viable rat skin portions increased rapidly in the skin portion treated with extracts and was highest after 36 h for piperine or 12 h for ZO-E, AA-E or MO-E or their combination with CTN as compared to the untreated portion. The in vitro permeation of RGE across epidermal sheets excised after these treatments also followed the same trend as TEWL. Further, the TEWL was observed to decrease continuously after 36 h (piperine) or 12 h (ZO-E, AA-E or MO-E) of treatment. Hence, weaning off the effect after 48 h of treatment could be attributed to the recovery of barrier status of skin as a natural response phenomenon in a bid to restore its original condition.
Different theoretical and experimental results have suggested that the drug penetration occurs through cavitation-induced keratinocytes or intercellular lipid bilayer disordering. SEM studies revealed loosening and creation of pores in SC surface layers by AA-E-CTN mixture treatment in excised epidermis. The effect was of apparently less intensity when these formulations were applied to viable skin. The purpose of the TEM study was to observe the perturbation induced by enhancers in the intercellular/intracellular path of the transdermal permeation. The TEM studies also revealed disordering of lipid areas and increase in intercellular space along with corneocyte detachment in excised epidermis. These effects were apparently less severe when the treatments were given to viable skin. It is important to note that these microscopic observations correlated very well with the findings of SC microconstituent estimations after similar treatments.

SEM studies revealed loosening and creation of larger pores with selected concentration of extracts in SC surface layers, creation of smaller pores by CTN and greatest loosening and pore formation by extracts-CTN mixture treatment in excised epidermis. The effect was of apparently less intensity when these formulations were applied to viable skin.

The plasma profile of RGE after oral administration and transdermal application of patches containing respective extract as well as its combination with CTN was investigated in Wistar rats. The maximum drug concentration (Cmax) after administration of tablets containing 0.5 mg of RGE was 17.96 ng/ml while the time taken to produce this concentration (Tmax) was 1 h. Application of transdermal patch containing AA-E-CTN mixture or 5% w/v AA-E to rats was observed to produce the highest Cmax of RGE of respectively, 52.89 ng/ml and 42.19 ng/ml as compared to that after oral administration. The time taken to achieve Cmax after application of transdermal patches was 12 h as compared to 1 h after oral administration. However, RGE concentration in rat plasma increased slowly and remained higher than Cmax for longer duration in the former case.
The transdermal patch containing AA-E (5% w/v)-CTN (1% w/v) mixture was observed to start exerting its effect after 4 h of application to streptozotocin induced hyperglycemic rats. The patch was able to maintain normal blood glucose level for 24 h.

Confocal laser scanning microscopy was utilized to determine the alterations in structural proteins. Staining of the HaCaT cells with propidium iodide (PI) without treatment with antibodies resulted in red color staining of the nucleus. Treatment with primary anti-TJP1 (ZO-1) antibody labeled with FITC-conjugated anti rabbit IgG was observed to exhibit continuous punctuate localization of ZO-1 along the cytoplasmic surface of the cell membrane, staining specifically the HaCaT cell borders. TJ-associated protein, ZO-1, was found localized to a sharp, continuous band around the cell periphery in untreated cells. Treatment with different concentrations of extracts for 6 h resulted in loss of ZO-1 from membrane regions indicating the loss of functional tight junctions from such areas indicative of diminished TJ integrity. Increasing the concentration of extracts decreased the immunofluorescence intensity of HaCaT cells. The best effect was observed at concentration of piperine (0.008% w/v), ZO-E (0.16% w/v), AA-E (0.08% w/v) or MO-E (0.04% w/v) on HaCaT cells where the immunofluorescence intensity of anti-TJP1 (ZO-1) antibody was observed to decrease around the cell boundaries. Less severe effect of CTN as compared to any extract on ZO-1 protein of HaCaT cells was observed. The mixture of AA-E (0.08 % w/v)-CTN (1% w/v) was observed to remarkably decrease the intensity of immunofluorescence of anti-TJP1 (ZO-1) antibody with rare cell-to-cell connection. Overall, these studies indicated most severe effect of AA-E (0.08 % w/v)-CTN (1% w/v) mixtures followed by 0.08 % w/v AA-E and 1% w/v CTN.

The result of the present investigation revealed that overwhelming influence of *Piper nigrum, Zingiber officinale, Angelica archangelica* and *Melilotus officinalis* extract on rat epidermis as well as on HaCaT cells. The data obtained from lipid perturbation studies, TEWL studies, differential scanning calorimetry studies and microscopic studies coorelated with the observed permeation.
enhancement of repaglinide (RGE). On the basis of data obtained from these investigations it could be concluded that the maximum enhancement of in vitro percutaneous permeation of RGE was exhibited by ethanolic extract of Angelica archangelica (7.01-fold enhancement). It would be worthy to investigate the possibility of using AAIE extract for enhancing the percutaneous permeation of other drugs (especially hydrophilic). Further, it is essential to evaluate compatibility of AAIE extract with excipients and ingredients likely to be present in a transdermal formulation before optimizing a formulation for clinical use.