Angelica archengelica extract induced perturbation of rat skin and tight junctional protein (ZO-1) of HaCaT cells

[1]Kaushal N., [2]Naz S., [*1]Tiwary AK.

[1]Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala- 147002, Punjab, [2]Dr Paturu Kondaiah's Lab, Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore- 560012,India.

ABSTRACT

Background and purpose of the study: Herbal enhancers compared to the synthetic ones have shown less toxis effects. Coumarins have been shown at concentrations inhibiting phospoliphase C-Y (Phc-Y) are able to enhance tight junction (TJ) permeability due to hyperpoalation of Zonolous Occludense-1 (ZO-1) proteins. The purpose of this study was to evaluate the influence of ethanolic extract of *Angelica archengelica* (AA-E) which contain coumarin on permeation of repaglinide across rat epidermis and on the tight junction plaque protein ZO-1 in HaCaT cells.
Methods: Transepidermal water loss (TEWL) from the rat skin treated with different concentrations of AA-E was assessed by Tewameter. Scanning and Transmission Electron Microscopy (TEM) on were performed on AA-E treated rat skin portions. The possibility of AA-E influence on the architecture of tight junctions by adverse effect on the cytoplasmic ZO-1 in HaCaT cells was investigated. Finally, the systemic delivery of repaglinide from the optimized transdermal formulation was investigated in rats.
Results: The permeation of repaglinide across excised rat epidermis was 7-fold higher in the presence of AA-E (5% w/v) as compared to propylene glycol:ethanol (7:3) mixture. The extract was found to perturb the lipid microconstituents in both excised and viable rat skin, although, the effect was less intense in the later. The enhanced permeation of repaglinide across rat epidermis excised after treatment with AA-E (5% w/v) for different periods was in concordance with the high TEWL values of similarly treated viable rat skin. Further, the observed increase in intercellular space, disordering of lipid structure and corneocyte detachment indicated considerable effect on the ultrastructure of rat epidermis. Treatment of HaCaT cell line with AA-E (0.16% w/v) for 6 hrs influenced ZO-1 as evidenced by reduced immunofluorescence of anti-TJP1 (ZO-1) antibody in Confocal Laser Scanning Microscopy studies (CLSM) studies. The plasma concentration of repaglinide from transdermal formulation was maintained higher and for longer time as compared to oral administration of repaglinide.
Major conclusion: Results suggest the overwhelming influence of *Angelica archengelica* in enhancing the percutaneous permeation of repaglinide to be mediated through perturbation of skin lipids and tight junction protein (ZO-1).

Keywords: Repaglinide, Percutaneous permeation, Transepidermal water loss.

INTRODUCTION

One of the approaches towards broadening the array of drugs that can be successfully delivered transdermally is the use of permeation enhancers. However, the synthetic enhancers (surfactants) that are commonly used in transdermal formulations are reported to be transported across the skin and may exert toxic effect on prolonged uses. Reversibility of the skin perturbation is of prime importance in selecting a permeation enhancer and as a result, there is renewed interest for searching safe and effective permeation enhancers.

Permeation enhancers can act on intercellular lipid domains of the stratum corneum, intercellular keratin, and tight junctional proteins or alter the solvent characteristics of the stratum corneum in order to decrease the barrier resistance of skin (1). The tight junction (TJ) proteins are known to exist in a variety of cells and have been recently reported to be present in the epidermis where they exist in the apical portion of the cells of the stratum granulosum and provide a barrier to both inward and outward movement of molecules (2, 3). The TJ proteins play an important role in cell signaling (4, 5) and

Correspondence: aktiwary2@rediffmail.com

vesicular transport (6). ZO-1 and F- actin have been recognized to be the key elements in the structural organization of TJ complexes (7). Recent reports suggest that disruption of the organization of TJ components, especially ZO-1 and F- actin, can affect the barrier integrity and hence, offer a novel means for enhancement of the percutaneous delivery of drug molecules.

Roots of *Angelica archangelica* contain essential oils, organic acids, steroids, coumarins and flavonoids and have been used for strengthening of the heart, stimulating the circulation and immune system. Ethanolic extract of the roots of *Angelica archangelica* (AA-E) has been reported to contain high concentration of coumarins (8). The in vitro percutaneous absorption and skin metabolism of coumarin (1,2-benzopyrone) has been studied in metabolically viable human, rat (F344), and mouse (CD1 and DBA/2) skin. 3-Nitrocomarin (3-NC), at concentrations inhibiting phospholipase C-y (PLC-y) is able to enhance TJ permeability (1) due to hyperphosphorylation of ZO-1 protein. There is no report relating to the use of AA-E for enhancement of the permeation of drugs across skin. Therefore, it seemed rational to hypothesize the influence of AA-E on the barrier status of skin though its action on ZO-1 protein. Hence, the inclusion of AA-E in transdermal formulations may be anticipated to offer a means for enhancement of the percutaneous permeation of drugs.

This investigation was designed to evaluate the effect of AA-E on the barrier status of rat epidermis. Biochemical constituents, transepidermal water loss (TEWL) and ultrasturactural features were investigated as markers of the barrier integrity of rat epidermis. In addition, the effect of AA-E on tight junction protein (ZO-1) was evaluated in human normal skin keratinocyte cell line (HaCaT). Constant concentration of repaglinide (RGE) an oral antidiabetic drug is required to be maintained in blood for effective control of blood glucose level due to its extremely short half-life of one hour (9). Hence, the possibility of using AA-E for enhancement of the permeation of RGE was evaluated through *in vitro* diffusion studies. Finally, exploratory studies were conducted to assess the systemic delivery of RGE in rats from transdermal formulations containing AA-E as permeation enhancer.

MATERIAL AND METHODS

RGE was obtained from Torrent Pharmaceuticals, (India) as a gift sample. Skin for the in vitro permeation and other studies was obtained from albino Wistar rats (190-210g) of either sex. The protocols for these studies were approved by the Institutional Animal Ethics Committee of Punjabi University, Patiala, India. All chemicals used in this study were of AR grade.

Extraction and standardization of Angelica archangelica

The powdered dried roots of *Angelica archangelica* were extracted using the method described by Ganzera (8).

Preparation of epidermal sheet for in vitro permeation studies

Full thickness skin samples were obtained from Albino Wistar rats of either sex (175–225 g). Epidermal sheets were separated from full thickness sheets utilizing the procedure described by Kligman and Christophers (10). Freshly separated epidermal sheets were used in all the experiments.

In vitro permeation of RGE using excised rat epidermis

Freshly obtained epidermal sheets were mounted between the donor and receptor compartments of vertical Franz glass diffusion cells. The epidermal sheet was equilibrated for 4 hrs using phosphate buffer of pH 7.4 (PB) in receptor compartment. The equilibration was judged to be completed when the fluid content in receptor compartment (phosphate buffer of pH 7.4) did not exhibit any fluorescence on being analyzed spectrofluorimetrically at excitation and emission spectra of 282 nm and 379 nm, respectively. The receptor compartment was filled with fresh PB (pH of 7.4) containing sodium azide (0.05% w/v) as preservative and PEG 400 (10% v/v) as solubilizing agent. The temperature of the receptor compartment was maintained at 35±2°C. The donor compartment was loaded with dispersion of RGE (2 mg) in PG: EtOH (7:3) mixture or dispersion of RGE (2 mg) and AA-E (1-6% w/v) in PG: EtOH (7:3) mixture and sealed with parafilm. Samples (1.0 ml) from the receptor fluid were withdrawn at different time intervals during 48 hrs and immediately analyzed spectrofluorimetrically using excitation and emission wavelengths of 282 nm and 379 nm, respectively. Standard plot of RGE solution was prepared in PB containing sodium azide (0.05% w/v) and PEG 400 (10% v/v) spectrofluorimetrically using excitation and emission wavelengths of 282 nm and 379 nm, respectively.

In vitro permeation of RGE across epidermis obtained after treatment of viable rat skin

One patch (7cm^2) was prepared on dorsal side of rat by shaving with mechanical clipper. The patch was treated with selected dose of AA-E (5% w/v). Surface area exposed to treatment was kept constant. The animals were sacrificed after 4, 8, 12, 24, 36 or 48 hrs. The treated skin patches were excised, epidermis separated and used for different in vitro permeation studies of RGE. All experiments were carried out in triplicate.

Biochemical estimation of skin microconstituents

Transdermal patches containing AA-E (1-6%

w/v) dispersed in PG: ETOH (7:3) mixture were prepared using adhesive tape, polyethylene backing membrane, and a rubber ring and applied to shaved skin of the dorsal portion of rats. The animals were sacrificed after 4, 8, 12, 24, 36 or 48 hrs and the epidermis was separated from treated skin patches. All epidermal sheets were dried to constant weight at 50ºC and total lipids were extracted by the Folch method (11). Cholesterol and triglycerides contents in these extracts were determined by using respective kits. Sphingosine content was determined spectroflourometrically (SL 174, Elico, India) using excitation and emission wavelengths of 340 and 455 nm, respectively, according to the method outlined by Sharma et al. (12). All determinations were carried out in triplicate.

Transmission electron microscopic (TEM) studies
Excised epidermal samples treated with AA-E (1-6% w/v) for 48 hrs or epidermis obtained from viable skin excised after similar treatments for different time intervals (4, 12, 24, 36 and 48 hrs) were fixed in Karnovosky's fixative [(2% *para*formaldehyde and 1% (w/v) glutaraldehyde in 0.1M phosphate buffer (pH of 7.4)] and processed. Ultra-thin (60-90°A) sections were cut and double stained with uranyl acetate and lead citrate for TEM observation (Morgagni 268 D, Sei, Netherlands).

Scanning electron microscopic (SEM) studies
The methods used were similar to those reported earlier (13). Photographs were taken under scanning electron microscope (LEO 435 SVP, Cambridge, UK).

Cells, media and culture condition
Human normal skin keratinocyte cell line (HaCaT), provided by Professor Sudhir Krishna, (National Centre for Biological Sciences, Bangalore, India) was maintained in Dulbecco modified eagle medium (DMEM) (Sigma-Aldrich USA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen life sciences, USA). The FBS for culturing HaCaT cells was heat inactivated for 30 min at 55°C. HaCaT cells were cultivated according to the method described by Brandner et al. (4). The cells were incubated until they were approximately 50-60% confluent. Cells were made serum free at 16-24 hrs prior fixation and treated with solutions containing different concentrations of AA-E for 6 hrs. AA-E (50 mg) was dissolved in PG: EtOH (7:3) mixture. The solutions were sterilized by filteration (Sartolab. vl15, pore size 0.2 lam) and serially diluted in Dulbecco modified eagle medium to obtain solutions of 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.8 or 1 µg/µl of AA-E. Confluent cells were treated with different concentrations of AA-E. Untreated cells served as control. The cells were cultured in the conditions described above for 6 hrs. The growth of cells was monitored under a light microscope. All experiments were repeated three times.

Immunoflourescence
Fixation was performed by immersing the cells into freshly prepared 4% v/v paraformaldehyde for 10 min. Immediately after fixation, cells were permeabilized with 0.1% v/v TritonX-100 (Sigma) for 5 min. Cells were then rinsed with phosphate buffer saline (PBS) for 3 min. and blocked in 10% FBS in PBS for one hour. This was followed by incubation with primary anti-TJP1 (ZO-1) antibody (1:100 dilution) for 2 hrs at room temperature (or overnight at 4°C). After rinsing of each of them three times in PBS for 10 min, cover slips were incubated with FITC-conjugated anti-rabbit IgG (10 µg/ml) in PBS containing 2% w/v FBS for 2 hrs. Slides were then rinsed three times in PBS and cover slips were mounted in antifade mounting medium (Vector laboratories, USA). Nucleus was stained using propidium iodide (5 µg/ml). Control for antibody specificity was prepared by omitting the primary antibody. The control demonstrated minimal or no staining. For microscopic examination, a Zeiss Axioplan fluorescence microscope (Zeiss) equipped with a confocal scanning unit MRC-600 (Bio-rad) and an argon-Krypton laser were used.

RNA Isolation and Semi-quantitative RT-PCR
Total RNA was extracted from HaCaT cells after treatment with different concentrations of AA-E using TRI-reagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed using high capacity cDNA archive kit (Applied Biosystems, USA). cDNA equivalent to 20 ng of total RNA was used for the PCR reactions. The PCR reactions were performed using 2X DyNAzymeTM Master Mix (FINNZYMES, Finland) with ZO-1 and occludin specific primers in a final reaction volume of 20µl. The primer sequence used for ZO-1 and occludin was 5'CTTCAAAGGGAAAGCCTC3'(f orward)5'TACCTTCACCATGCTCC 3'(reverse),5 'CTGGATCAGGGAATATCCAC3'(forward)and5' TCAGCAGCAGCCATGTAC 3' (reverse).

Evaluation of pharmacokinetic parameters of RGE in animals
Wistar albino rats of either sex (230-250g) were used for the bioavailability studies. The rats were housed under controlled environmental conditions (23±2°C; 55±5% RH) and fed with a commercial diet with free access to water. About 10 cm² of skin was shaved with electric razor on the dorsal side. Rats were observed for 24 hrs for any untoward effect of shaving. The rats were divided into 4 groups (n=6). Group I received oral administration of RGE (0.5 mg) after overnight fasting. Group II received

Table 1. *In vitro* permeation of RGE from different formulations across excised rat epidermis.

Treatment	Flux of RGE ($\mu g/cm^2/h$)[*]	Enhancement ratio
PG:EtOH (7:3)	12.470 ± 1.12	-
AA-E (1% w/v)	32.671 ± 0.97	[#a] 2.620
AA-E (3% w/v)	54.232 ± 1.23	[#a] 4.349
AA-E (5% w/v)	87.439 ± 0.94	[#a] 7.012
AA-E (6% w/v)	85.420 ± 1.19	[#a] 6.850

[*]Mean ± S.D of three experiments; # With respect to PG: EtOH (7:3); 'a' indicates significant difference ($p < 0.05$) with respect to PG:EtOH (7:3).

Enhamcement ratio is the ratio of flux of RGE across rat epidermis in the presence of respective concentration of ethanolic extract of *Angelica archengelica* (AA-E) dispersed in propylene glycol: ethanol mixture to that of the control in 7:3 mixture of PG: EtOH.

transdermal patch A containing RGE and AA-E (1% w/v) dispersed in PG: EtOH (7:3) mixture, group III and IV received transdermal patch (B) containing RGE and AA-E (5% w/v) or (6% w/v) dispersed in PG:EtOH (7:3) mixture, respectively. Blood sample (0.3 ml) via the post-orbital venous plexus vein was collected in heparinized tubes at 1, 2, 4, 6, 8, 12, 24, 28, 36, 42 and 48 hrs. The blood samples were transferred to heparinized eppendroff tubes mixed gently and centrifuged (4000 rpm, 5 min) to obtain 200 μl plasma and stored at −20ºC until analysis. The concentration of RGE in the plasma was determined by HPLC analysis. The plasma samples (1 ml) were extracted using the procedure reported by Ruzilawati et al. (14).

Transepidermal water loss (TEWL) determination
Wistar rats of either sex (approximately 250 g) were anesthetized. Patches were prepared as reported in pharmacokinetic studies. The patch received treatment with selected dose of AA (5% w/v) dispersed in PG:ETOH (7:3) mixture for different studies. The laboratory temperature and humidity were maintained at 23±2°C; 55±5% RH, respectively. Surface area exposed to treatment was kept constant. TEWL was recorded on each site after removal of the patch at 4, 8, 12, 24, 36 or 48 hrs by using Tewameter TM 210 (Courage + Khazaka Electronic GmbH, Koln, Germany). Data was stored and analyzed by the TEWAMETERÒ computer software.

Draize Test
The study employed albino rabbits (1.2-2.5 kg) of either sex (n=5) for testing skin irritation. Two patches each of two square inch area were prepared by shaving the dorsal surface of one rabbit. Patch made from two layers of light gauze was dipped in solutions containing different concentrations of extracts prepared in PG:EtOH (7:3). The animals were immobilized in the special holder during the 24 hrs patch exposure. Upon removal of the patches the animals were observed for any sign of erythema or oedema for a period of 72 hrs. The observations were repeated after 72 hrs.

Statistical analysis
Studentized t test was employed for comparing the data between two groups. The level of significance was fixed at 0.05.

RESULTS AND DISCUSSION

Characterization of Coumarin present in AA-E
AA-E subjected to HPTLC analysis using toluene: ethylacetate (9:1) as mobile phase gave single band characteristic of bergapten at 322 nm with Rf value of 0.30 (8). The AA-E was found to contain 5.234% w/w of bergapten. Fig 1 (a) depicts the characteristic bands exhibited by pure bergapten (A1) as well as those exhibited by AA-E (A2-A4). Fig 1(b) shows the spectrograph corresponding to these samples.

In vitro permeation of RGE across rat epidermis
Table 1 summarizes the flux of RGE across excised rat epidermis treated with different concentrations of AA-E (1-6% w/v). The permeation of RGE across excised epidermis in the presence of 1% w/v AA-E was 2.62 fold greater than that in the presence of donor vehicle (PG:EtOH, 7:3). The permeation increased to 4.35-fold and 7.01-fold in the presence of AA-E 3% w/v or 5% w/v respectively as compared to that in the presence of vehicle. The permeation was ~7-fold higher in the presence of AA-E 6% w/v concentration as compared to that of vehicle which is almost comparable to that in the presence of AA-E 5% w/v.
Previous studies have reported coumarins are rapidly and extensively absorbed across skin into the receptor fluid (15). This high topical bioavailability of coumarins to some extent has been attributed to their physicochemical properties. An optimal lipophilicity (log Po/w =1.2) enables it to be readily taken up into the lipid regions of the stratum corneum. At the same time, the sufficient (albeit low) aqueous solubility allows its partitioning from the stratum corneum into the relatively aqueous viable epidermis, dermis, and receptor fluid (15). During the initial stages, absorption of coumarins across mouse skin into the receptor fluid was observed to be considerably fast

Table 2. Effect of treatment with *Angelica archangelica* extract (AA-E) on microconstituents of epidermis in excised and viable rat skin.

Formulation	Skin microconstituent extracted (%) [#]		
	Cholesterol	Triglyceride	Sphingosine
Skin Type: Excised after 48 hrs of treatment			
AA-E (1%w/v)	22.934 ± 3.23	12.345 ± 1.96	7.121 ± 2.34
AA-E (3%w/v)	25.305 ± 0.99	14.087± 2.99	8.293 ± 3.26
AA-E (5%w/v)	57.987 ± 1.76	25.087 ± 0.66	14.234 ± 2.17
AA-E (6%w/v)	31.237 ± 2.09	17.456 ± 2.97	9.345 ± 1.07
Skin Type: Viable treated with AA-E (5% w/v)			
4hrs	17.118 ± 2.87	6.027 ± 0.99	3.093 ± 1.34
8hrs	21.235 ± 3.98	13.924 ± 2.07	6.125 ± 0.91
12 hrs	34.143 ± 4.02	27.176 ±1.35	23.012 ± 2.09
24 hrs	27.567 ± 1.29	16.623 ± 2.87	15.637 ± 2.56
36 hrs	24.109 ± 1.35	13.154 ± 1.97	11.734 ± 0.91
48 hrs	16.345 ± 1.75	5.034 ± 3.02	4.467 ± 2.09

[#] Mean ± S.D of three experiments
This table shows the percentage of extracted skin microconstituents across excised rat epidermis using Folch method by applying transdermal patches containing *Angelica archengelica* extract (AA-E) (1-6% w/v) and across viable rat epidermis using AA-E (5% w/v) dispersed in PG: ETOH (7:3) mixture.

and more extensive, probably due to significant role of the hair follicles as suggested previously for other compounds (16). Further, greater flux of coumarin through excised cadaver scalp skin compared to that through abdominal skin has been reported. This was again attributed to the higher hair follicle density in the former (169/cm²) than in the latter (24/cm²) (17). Therefore, the ability of coumarins to enhance the permeation seems to be a combination of its physicochemical properties and the number of functional permeation pathway.

The effect of coumarins on membrane fluidity is attributed to its easy partitioning in the membrane (due to its apolar nature) and the configuration it assumes 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 (18). Coumarins contain benzopyrene ring due to which they affect the protein portion of the skin constituents. The reduction of high protein oedema has been detected 4 hrs after administration and the maximum effect was evident at 12 hrs (19). In view of these reports and observed enhancement of RGE permeation across excised rat epidermis, it seemed essential to investigate the influence of AA-E on skin micro constituents and ultrastructure for gaining an insight into the exact mechanism of percutaneous permeation enhancement.

Biochemical estimation
The quantity of cholesterol, triglyceride, or sphingosine extracted from excised epidermis increased in the presence of increasing concentration of AA-E up to 5% w/v. Treatment with AA-E (6% w/v) for 48 hrs extracted the maximum amount of cholesterol (57.987%), followed by triglyceride (25.087%), and least amount of sphingosine 14.234% (Table 2). It is interesting to note that all these treatments were less effective when applied to viable skin as shown in table 2. Further, the maximum effect in viable skin was observed when epidermis was excised after 12 hrs treatment with 5% w/v AA-E (Fig 2). Therefore, AA-E may be suggested to influence the permeation of RGE through modulation of epidermal barrier status by affecting the skin micro constituents.

Transepidermal water loss (TEWL) studies
The most important function of the skin is the control of the transepidermal water loss (TEWL). The TEWL of viable rat skin portions increased rapidly in the AA-E treated skin portion and was maximum (~ 6-fold) higher after 12 hrs as compared to the untreated portion (Fig 2). Further, the permeation of RGE across the skin portions excised after 12 hrs of treatment with AA-E was significantly (P<0.05) higher as compared to that across skin portions excised at other time periods (Fig 2). The flux of RGE and TEWL at later time intervals decreased continuously. The lack of significant difference (P<0.05) in the permeation of RGE across AA-E treated epidermal sheets excised after 48 hr of treatment suggested normalization of skin microconstituents that would have accompanied the restoration of barrier properties of skin. It is evident from figure 2 that the TEWL of skin treated with AA (5% w/v) was only ~2-fold higher in the AA-E
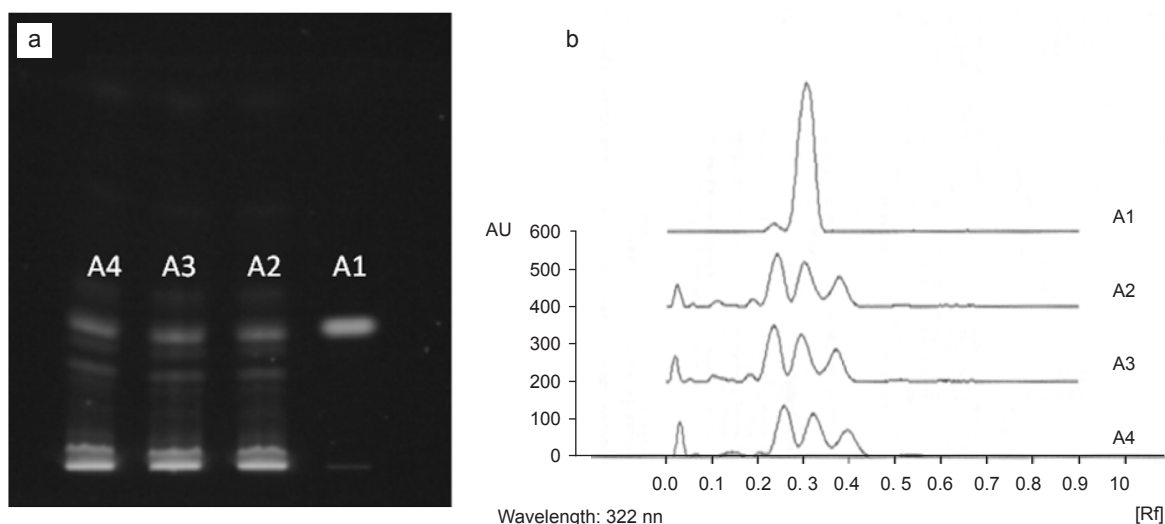
Figure 1. Bands charaterstic of bergapten (a) and corresponding retention time (b) obtained by HPTLC analysis of pure bergapten (A1) or replicate samples of AA-E (A2-A3).
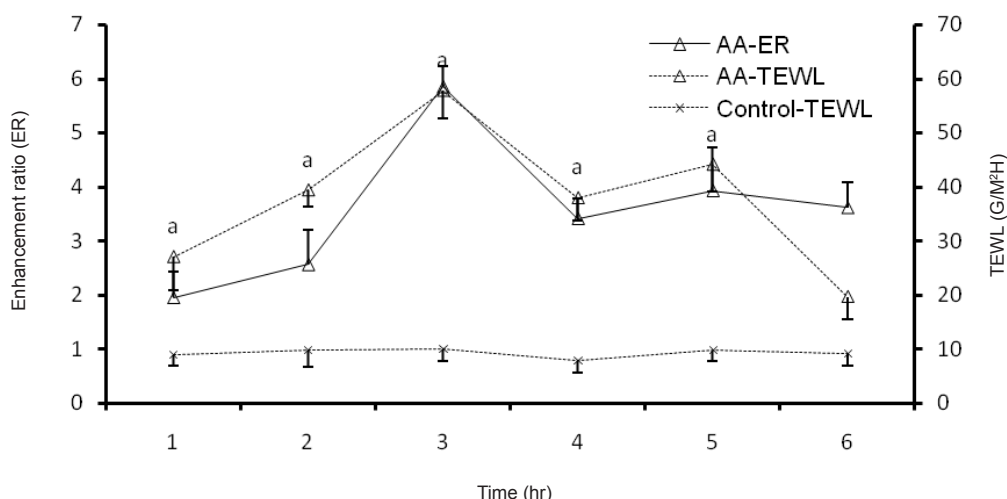

Figure 2. Enhancement ratio (ER) of RGE and TEWL of viable rat epidermis after treatment with AA-E (5% w/v) . The horizontal bars for each observation represent standard deviation of three experiments (n=3). "a" indicates significant difference ($p<0.05$) with respect to TEWL of control group.

treated skin portion after 48 hrs as compared to untreated skin portion. Hence, weaning off the effect of AA-E after 48 hrs 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.

Electron microscopic studies
SEM photomicrographs of rat skin treated with different concentrations of AA-E (1-6% w/v) revealed loosening of stratum corneum (SC) surface layers in comparison to control (Figs 3a-3f). Treatment with AA-E (5% w/v) resulted in formation of largest pores

coupled with loosening of SC surface (Fig 3d). The effect was apparently of less intensity when AA-E (5% w/v) was applied to viable skin (Fig 3f). TEM micrographs of untreated rat epidermis revealed closely packed SC cell layers and tightly packed keratin pattern (Fig 4a). Greatest extent of lipid area disordering and increase in intercellular space along with corneocyte detachment was observed after treatment of excised skin with 5% w/v of AA-E (Fig 4d). These effects were apparently less severe when the viable skin was treated with 5% w/v of AA-E (Fig 4f). The less severe influence of these treatments on ultrastructural features of viable skin
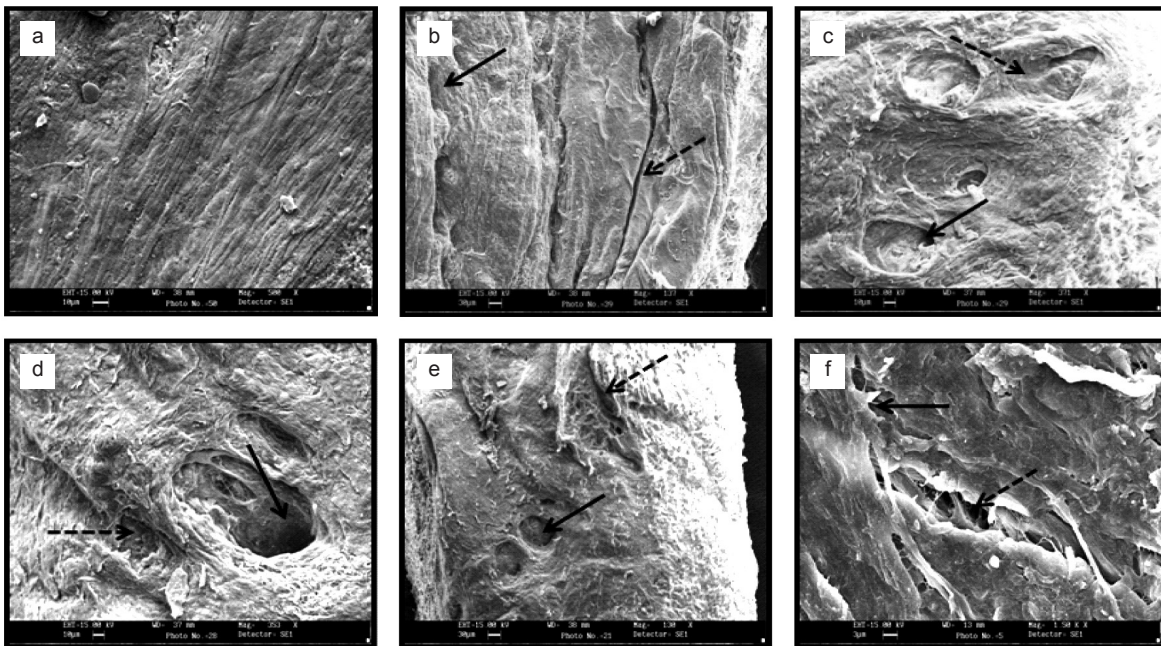
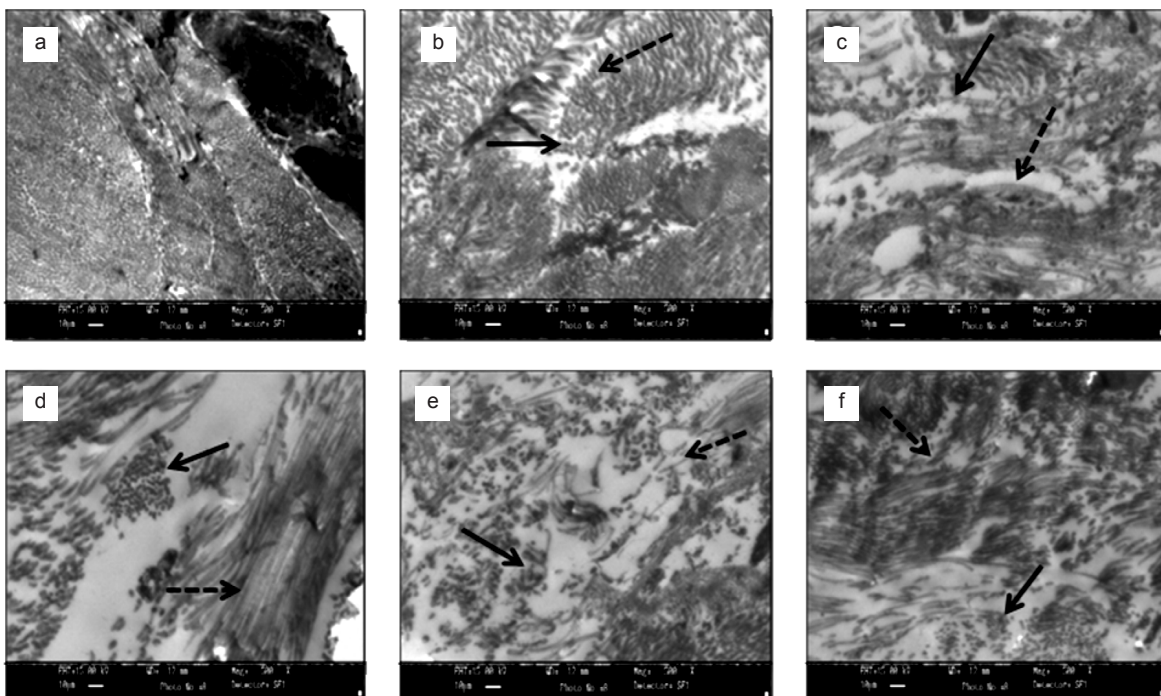**Figure 3.** Scanning electron photomicrographs of rat skin: untreated epidermis (a); excised skin treated for: 48 hrs with AA-E (1-6% w/v) (b-e); as well as viable skin treated for 12 hrs with 5% w/v AA-E (f).
[Broken arrows indicate loosening of SC surface and solid arrows indicate formation of surface pores, magnification is ×500, shorter scale bar is 10 μm, and longer scale bar is 20 μm

Figure 4. Transmission electron photomicrographs of rat skin: untreated epidermis (a); excised skin treated for: 48 hrs with AA-E (1-6% w/v) (b-e); as well as viable skin treated for 12 hrs with 5% w/v AA-E (f).
[Broken arrows indicate disruption of lipid areas and solid arrows indicate corneocyte detachment, magnification is ×20,000, scale bar is 100 nm]
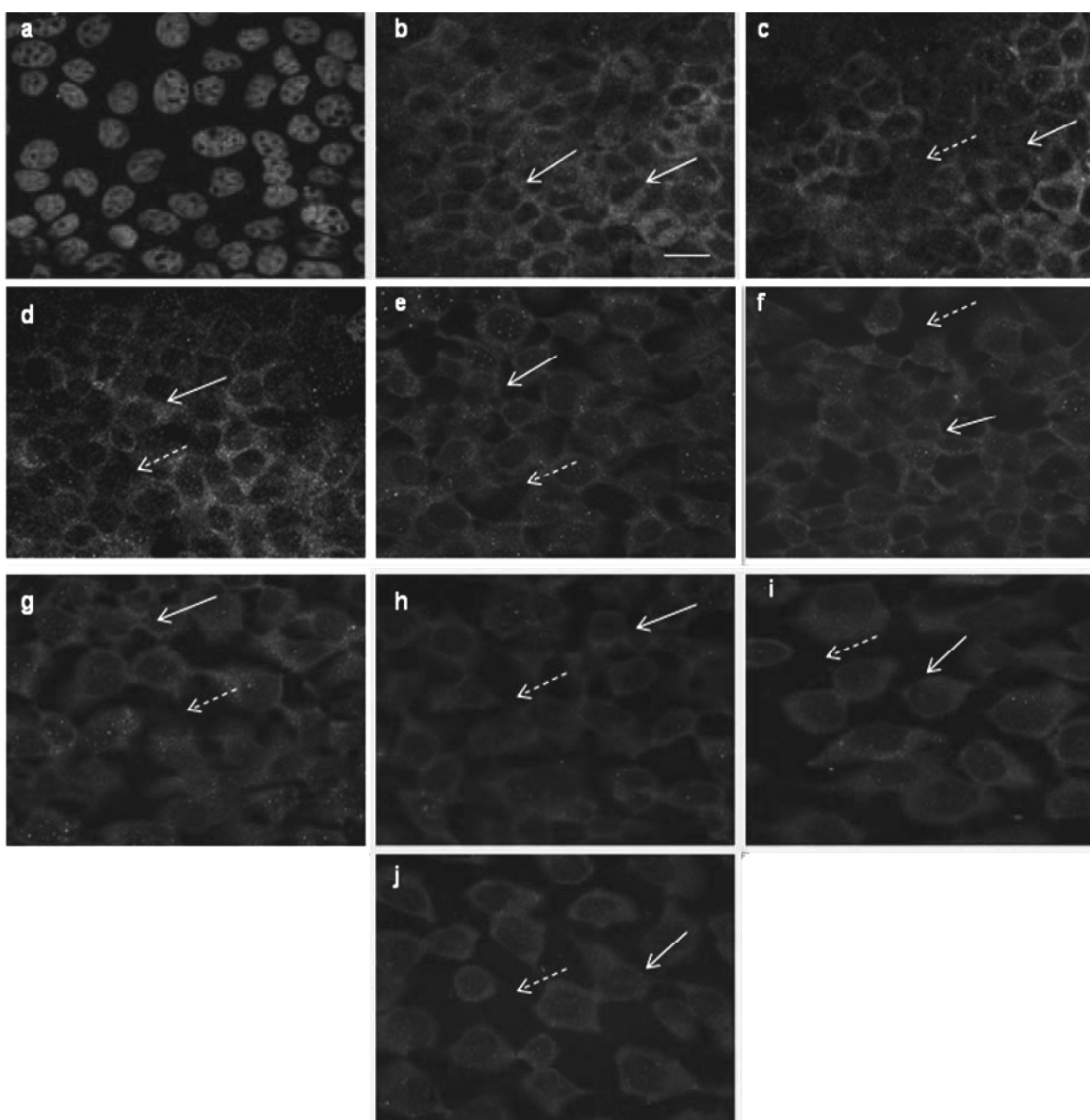
Figure 5. Confocal laser scanning photomicrographs of HaCaT cells: without treatment with antibodies (a); after treatment with primary anti-TJP1 (ZO-1) antibody labeled with FITC-conjugated anti rabbit IgG antibodies only (b); after treatment with 0.0025% w/v AA-E (c); 0.01% w/v AA-E (d); 0.02% w/v AA-E (e) ; 0.04% w/v AA-E (f); 0.08% w/v AA-E (g); 0.16% AA-E w/v (h), 0.8% w/v AA-E (i) and 1% w/v AA-E (j).
[Propidium iodide (PI) stained red colored nucleus (a); Fluorescence due to attachment of primary anti-TJP1 (ZO-1) antibody labeled with FITC-conjugated anti rabbit IgG antibody indicates presence of ZO-1 protein in cell boundaries (b-j). The solid arrows indicate presence of demarcating cell boundaries and broken arrows indicate disruption of demarcating cell boundaries, Scale mode plane, scale bar: 0.7μm]

could be ascribed to the continuous attempt of the skin to normalize its barrier status through enhanced lipid synthesis.

Confocal laser scanning microscopic (CLSM) studies
Confocal laser scanning microscopy was utilized to determine 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 (Fig 5a). 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 (Fig 5b). TJ-associated protein, ZO-1, was localized as a sharp, continuous band around the cell periphery in untreated cells. Treatment with different concentrations of AA-E for 6 hrs resulted in loss of ZO-1 from membrane regions indicating the loss of functional tight junctions from such
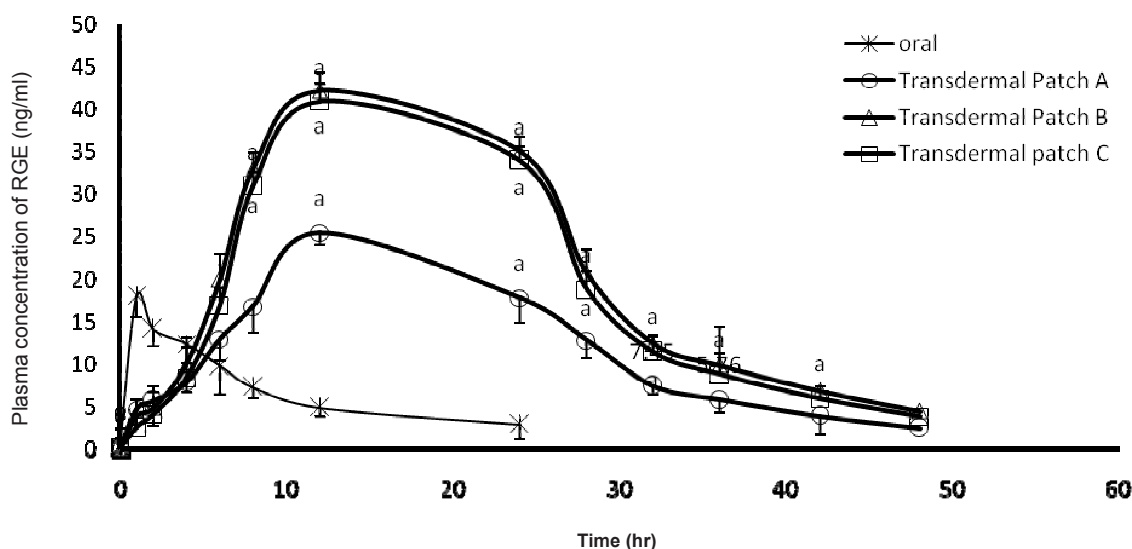
Figure 6. Pharmacokinetic profile of RGE after oral administration of tablets (0.5 mg) and transdermal patches (A: 1%w/v AA-E; B: 5%w/v AA-E; C: 6%w/v AA-E) formulations. The horizontal bars for each observation represent standard deviation of three experiments (n=3). "a" indicates significant difference (p<0.05) with respect to oral administration.

areas indicative of diminished TJ integrity (Figs 5c-j). Treatment of HaCaT cells with 0.0025% w/v of AA-E resulted in a decrease in the intensity of immunofluorescence around the cell boundaries (Fig 5c) as compared to the untreated cells (Fig 5b). Increasing the concentration of AA-E to 0.01% w/v further decreased the immunofluorescence of HaCaT cells (Fig 5d). The cells started separating from each other on treatment with 0.02% or 0.04% w/v AA-E (Fig 5e and 5f). At higher concentration of 0.08% w/v of AA-E (Fig 5g) HaCaT cells were observed as pentagonal shaped cells with rare cell-to-cell connection. The cell boundaries appeared highly diffused after treatment with 0.16% w/v AA-E (Fig 5h). The immunofluorescence was significantly diffused along the cell borders as well as in the cytoplasmic region with no demarcation of cell–cell connection between adjacent cells when 0.8% w/v (Fig 5i) or 1% w/v (Fig 5j) concentrations of AA-E were employed.

Evaluation of pharmacokinetic parameters of RGE in animals
Transdermal patch (A) containing 1% w/v of AA-E yielded C_{max} of 26.2 ng/ml as compared to17.96 ng/ml observed after oral administration. The C_{max} was further enhanced by ~2-fold (34.3 ng/ml) and ~2.5-fold (42.3 ng/ml) as compared to oral administration after application of transdermal patch containing, 5% w/v or 6% w/v of AA-E respectively. However, the time taken to achieve C_{max} was significantly delayed (P> 0.05) from one hour (oral administration) to 12 hrs (transdermal administration). It is important to note that the minimum effective concentration reported for human after an oral dose of 2 mg of RGE is 27.74 ng/ml (20). This concentration was not achieved in rats in the present investigation after oral administration of tablets containing 0.5 mg RGE. Further, the plasma RGE concentration declined sharply after one hour and was negligible after 2 hrs of oral administration of tablets. On the other hand, the plasma RGE concentration attributed to maintenance of minimum effective concentration of 27.7 ng/ml for 18.5 or 19.1 hrs after application of transdermal patch containing, 5% and 6% w/v of AA-E respectively. However, transdermal patch containing AA-E (1% w/v) was unable to maintain minimum effective concentration of 27.7 ng/ml.

Effect of AA-E on ZO-1 and occludin expression in HaCaT cells
The densitometric data obtained indicated that the m-RNA levels related to both ZO-1 as well as occludin remained unaffected when the HaCaT cells were incubated with 0.02, 0.08, 0.16, 0.8 and 1% w/v concentrations of AA-E for 12 hrs (Fig 7). However, the gene expression for these tight junctional proteins decreased slightly by 1% w/v concentration of AA-E. Although, it is difficult to extrapolate these findings to the exact safe concentration range of AA-E for use on rat skin, the data does suggest a wide concentration range of AA-E that seems safe for use on HaCaT cells and possibly on rat skin.

Draize Test:
The results of draize test did not suggest any irritation potential of AA-E on rabbit skin.

CONCLUSION
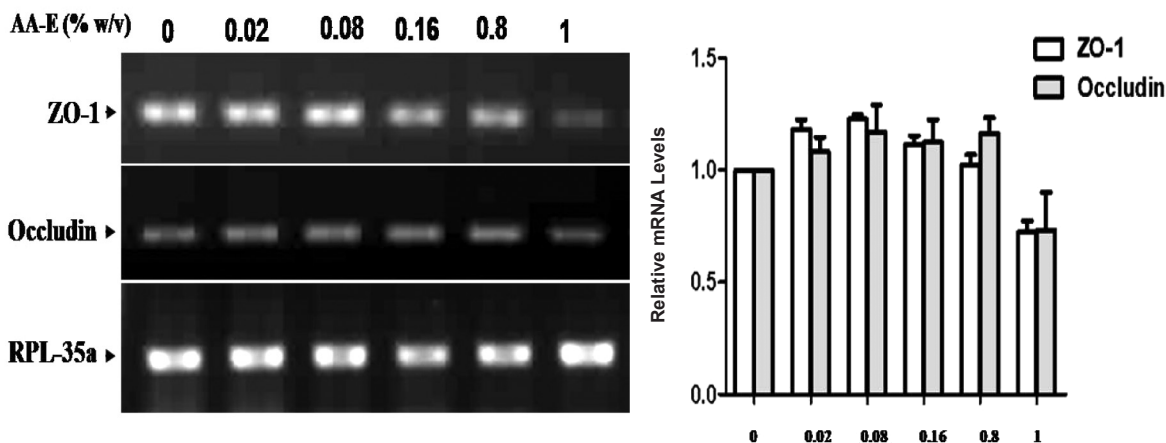Result of the present investigation revealed that

Figure 7. Effect of AA-E on the expression of tight junction proteins in human keratinocyte
Exponentially growing HaCaT cells were serum starved for 24 hrs and treated with different concentrations of AA-E for 12hrs. mRNA levels of ZO-1 and occludin upon AA-E treatment and relative mRNA expression from the densitometric data after normalization to RPL-35a [Data is representative of three independent experiments].

AA-E (5% w/v) enhanced the permeation of RGE across excised rat epidermis by 7-fold. AA-E was found to perturb the lipid microconstituents in both excised and viable rat skin, although, the effect was less intense in the later. The perturbation was manifested in the higher TEWL values of the viable rat skin treated with AA-E (5% w/v). Further, intercellular loosening and pores were evident in SEM photomicrographs of AA-E treated rat epidermis. The TEM photomicrographs also revealed distorted lipid area along with increase in intercellular space and corneocytes detachment after similar treatment of rat epidermis. The CLSM investigations on HaCaT cells revealed loss of functional tight junction after treatment with AA-E 0.16% w/v for 6 hrs. Draize test revealed that AA-E did not irritate rabbit skin. The plasma concentration was maintained higher and for longer time by transdermal formulation as compared to oral administration of RGE. Overall, the findings suggested that the overwhelming influence of AA-E in enhancing the percutaneous permeation of RGE to be mediated by its influence on skin lipids and tight junction protein (ZO-1).

REFERENCES
1. Ward PD, Klein RR, Troutman MD, Desai S, Thakker DR. Phospholipase C-γ modulates epithelial tight junction permeability through hyperphosphorylation of tight junction proteins. J Biol Chem, 2002; 277:35760-35765.
2. Brandner JM. Tight junctions and tight junction proteins in mammalian epidermis. Eur J Pharm Biopharm, 2009; 72:289-294.
3. Brandner JM, Kief S, Grund C, Rendl M, Houdek P, Kuhn C. Organization and formation of the tight junction system in human epidermis and cultured keratinocytes. Eur J Cell Biol, 2002; 81: 253-263.
4. Ebnet K, Schulz CU, Brickwedde MM, Pendl GG, Vestweber D. Junctional Adhesion Molecule Interacts with the PDZ Domain-containing Proteins AF-6 and ZO-1. J Biol Chem, 2000; 275: 27979-27988.
5. Balda MS, Matter K. The tight junction protein ZO-1 and an interacting transcripiton factor regulate ErbB-2 expression. EMBOJ, 2000; 19:2024 -2033.
6. Lapierre LA. The molecular structure of the tight junction. Adv Drug Deliv Rev, 2000; 41:255-264.
7. Ju Y, Wang T, Li Y, Xin W, Wang S, Li J. Coxsackievirus B3 affects endothelial tight junctions: Possible relationship to ZO-1 and F-actin, as well as p38 MAPK activity. Cell Biol Int, 2007; 31:1207-1213.
8. Ganzera M. Recent advancements and applications in the analysis of traditional Chinese medicines. Planta Med, 2009; 75:776-783.

9. Culy CR, Jaruis B. Repaglinide: A review of its thrapeutic use in type 2 diabetes mellitus. Drugs, 2001; 61: 1625-1660.

10. Kligman AM, Christophers E. Preparation of isolated sheets of human stratum corneum. Arch Dermatol, 1963; 88:702-705.

11. Babita K, Tiwary AK. Transcutaneous delivery of levodopa: enhancement by fatty acid synthesis inhibition. Mol Pharm, 2005; 2:57-63.

12. Sharma C, Smith T, Li S, Schroepfer GJ, Jr., Needleman DH. Inhibition of Ca2+ release channel (ryanodine receptor) activity by sphingolipid bases: mechanism of action. Chem Phys Lipids, 2000; 104: 1-11.

13. Fatouros DG, Groenink HW, de Graaff AM, van Aelst AC, Koerten HK, Bouwstra JA. Visualization studies of human skin in vitro/in vivo under the influence of an electrical field. Eur J Pharm Sci, 2006; 29:160-170.

14. Ruzilawati AB, Wahab MSA, Imran A, Ismail Z, Gana SH. Method development and validation of repaglinide in human plasma by HPLC and its application in pharmacokinetic studies. J Pharma Biomed Anal, 2007; 43:1831-1835.

15. Beckley-Kartey SAJ, Hotchkiss SAM, Capel M. Comparative in Vitro skin absorption and metabolism of coumarin (1,2-Benzopyrone) in human, rat, and mouse. Toxicol Appl Pharmacol, 1997; 145:34-42.

16. Ford RA, Hawkins DR, Mayo BC, Api AM. The in vivo dermal absorption and metabolism of [4-14C] coumarin by rats and by human volunteers under simulated conditions of use in fragrances. Food Chem Toxicol, 2001; 39:153-162.

17. Kao J, Hall J, Helman G. In vitro percutaneous absorption in mouse skin: Influence of skin appendages. Toxicol Appl Pharmacol, 1988; 94: 93-103.

18. Wang W, Camenisch G, Sane DC. A coumarin-based prodrug strategy to improve the oral absorption of RGD peptidomimetics. J Control Release, 2000; 65:245-251.

19. Camenisch G, Wang W, Wang B, Borchardt R. A comparison of the bioconversion rates and the Caco-2 cell permeation characteristics of Coumarin-based cyclic prodrugs and methylester-based linear prodrugs of RGD peptidomimetics. Pharm Res, 1998; 15:1174-1181.

20. Van Heiningen PN, Hatorp V, Nielsen K, Hansen KT, Van Lier JJ, De Merbel NC. Absorption, metabolism and excretion of a single oral dose of (14)C-repaglinide during repaglinide multiple dosing. Eur J Clin Pharmacol, 1999; 55: 521-525.