# CHAPTER II

# MATERIALS AND METHODS

## 2.1 Chemicals and materials

Name of chemicals and reagents, equipment and instruments are shown in Appendix A and B, respectively. Whereas the details of the reagents or buffer used in this study are shown in Appendix C.

## 2.2 Skin specimens for human keratinocytes culture

Human keratinocytes were cultured from keratome biopsies of juvenile foreskin (circumcision) or abdominal skin (cesarean) which were obtained from Dr. Aram Limtrakul, the obstetrician at Chiang Mai public health promotion center. The passages 4-6 were used in this study.

## 2.3 Antibodies and SDS-PAGE molecular weight standard markers

I. Anti Protein Kinase C-ε rabit polyclonal antibody (cat.no. SC-213) was purchased from Promega Corp, Madison, WI, USA.

II. Anti Protein kinase C-α mouse monoclonal antibody (cat.no. SC-80 ) and anti protein kinase C-δ rabbit polyclonal antibody (cat.no. SC- 213) were purchased from Santa Cruz Biotecnology, USA.

III. Horse raddish peroxidase conjugated anti-rabbit immunoglobulin G was obtained from Amersham, England.

IV. Horse raddish peroxidase conjugated anti-mouse immunoglobulin G was obtained from Amersham, England.

V. SDS-PAGE standard, high range (cat.no.161-0303), broad range (cat.no. 161-0317) and low range (cat.no. 161-0304) were purchased from Bio-Rad, Hercules, CA, USA.

## 2.4 Cell culture : Human epidermal keratinocytes

The keratinocyte medium used in this study was supplied as a basal medium (Keratinocytes -SFM) with a minimum shelf -life of 9 months; BPE (50µg/ml) and rEGF (5 ng/ml) are added at the time of use. The calcium concentration is 0.09 mM, pH value typically fall within the range of 7.0-7.4 and the osmolality values typically fall within the range of 280-310 mOsm/kg (71-72).

### *Cell culture protocol for culture keratinocytes*

Human Keratinocytes were cultured from keratinocyte biopsies of juvenile foreskins (circumcision) or adult skin (cesarean scar) as descirbed by Fisher GJ ,1991 (73).

The skin was placed in transport medium (DMEM free serum) and moved to the laboratory, where it was (proposed) as soon as possible. Using a pair of scissors, all excess dermis and connective tissue were removed, the skin Sections were sterilized by immersing in absolute ethanol for 1 min and soaked in antibiotic solution, 20% penicillin-streptomycin for 30 min and 10% penicillin-streptomycin at 4 °C respectively. The tissue was transferred to dispase solution for 48 h at 4 °C. Then the epidermis layer will be removed and treated with trypsin -EDTA 2 ml and incubated at 37°C in $CO_2$-incubator for 30-45 min, mixed gently every 5 min. The action of trypsin was stopped by adding of soybean trypsin-inhibitor 4 ml. Then cells were centrifuged at 500 rpm, 5 min at room temperature. Supernatant were removed and cell pellet was resuspended in keratinocyte-SFM 5 ml mixed very gently to form a single cell suspension by 5 ml syringe with needle size 22G1/2 .The primary cells were seeded into culture T-75 flask, pretreated with FNC (collagen) solution, at a cell density of approximately $3x10^6$ cells per flask in 10-15 ml complete medium. The flasks were incubated in $CO_2$-incubator, loosely capped, at 37 °C. Fluid was changed with fresh complete medium every 2-3 days. Since primary

keratinocytes show donor to donor variability in their growth characteristics. Flask of primary cells may not reach 70-80% confluently until 10 to 14 days following isolation and set up.

## *Secondary passage of epidermal keratinocytes*

Upon reaching 80% confluent (Figure 12). The medium was removed and the flask washed once with 10-15 ml of PBS. Trypsin –EDTA solution (2 ml) was then added to the flask and it was incubated for 5-10 minutes. When approximately 70% of the cells were round up, trypsin inhibitor was added to stop reaction then centrifuged at 500 rpm for 3 minutes at room temperature. The cell pellet was gently resuspended in 5-10 ml of incomplete-DMEM to wash and then centrifuged. The pellet was resuspended in 10-15 ml of complete medium and seeded into T-75 flask at a density of $3x10^6$ cells per flask. The flasks were then incubated at 37°C and fluid were changed every 2-3 days until the cells reached 80% confluent, the cells could then be repassaged .

## *Cryopreservation of human epidermal keratinocytes*

Flasks of human keratinocytes were washed and harvested as above. The cells were resuspended in freezing media containing fetal calf serum (92%) and DMSO (8%). Then the cells suspension were slowly added to cryovials (i.e. $2x10^6$ cells per vial in 1 ml) and kept to freezing temperature (- 80 °C). After approximately 24 hours at -80 °C, the vials were transferred to a liqiud nitrogen cell freezer.

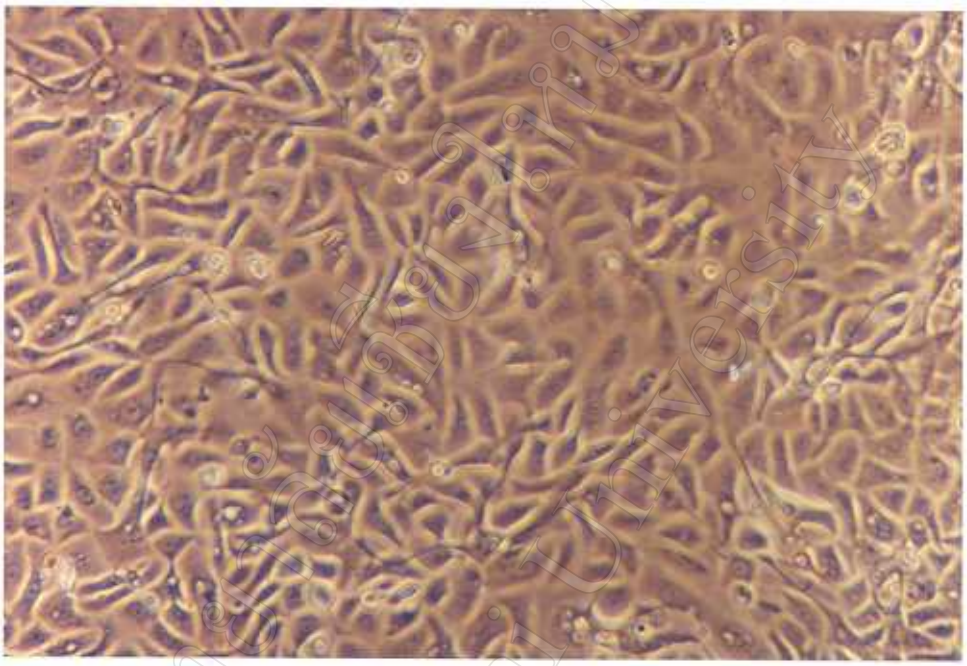


Figure 12. Phase contrast micrograph of human keratinocytes growing in presence of feeder layer of collagen-FNC at different magnification (magnification 100x)

## 2.5 Preparation of cytosolic and membrane fractions of PKC

After removal of the medium, the monolayer of confluent cells was washed twice with ice cold $Ca^{2+}$ and $Mg^{2+}$-free phosphate-buffer saline (PBS) and detatched by adding 0.05% of trypsin-EDTA. The reaction was stopped with trypsin-inhibitor. Cells were collected by low speed (500 rpm) centrifugation and then washed in PBS. Cell cytosolic and membrane fractions were prepared as follows (70). The cell pellets were resuspended in buffer A and homoginized using a glass dounce homoginizer. Cells homogenates were centrifuged at 35000 rpm for 1 h. The supernatant were collected. These supernatants constituted the cytosolic PKC fraction. The pellet were resuspended in Buffer B and centrifuged at 6000 g ,15 min. The resulting pellets was treated with 0.2%Triton X-100 in buffer A for 1 h on ice, homoginized, then centrifuged at 35000 rpm for 1 h. The resulting supernatant were concentrated using Centricon 30 concentrators (Amicon Corp.). The concentrated supernatant represented the solubilized membrane PKC fraction.

The cytosolic and membrane protein concentrations were determined by Lowry's method (74).

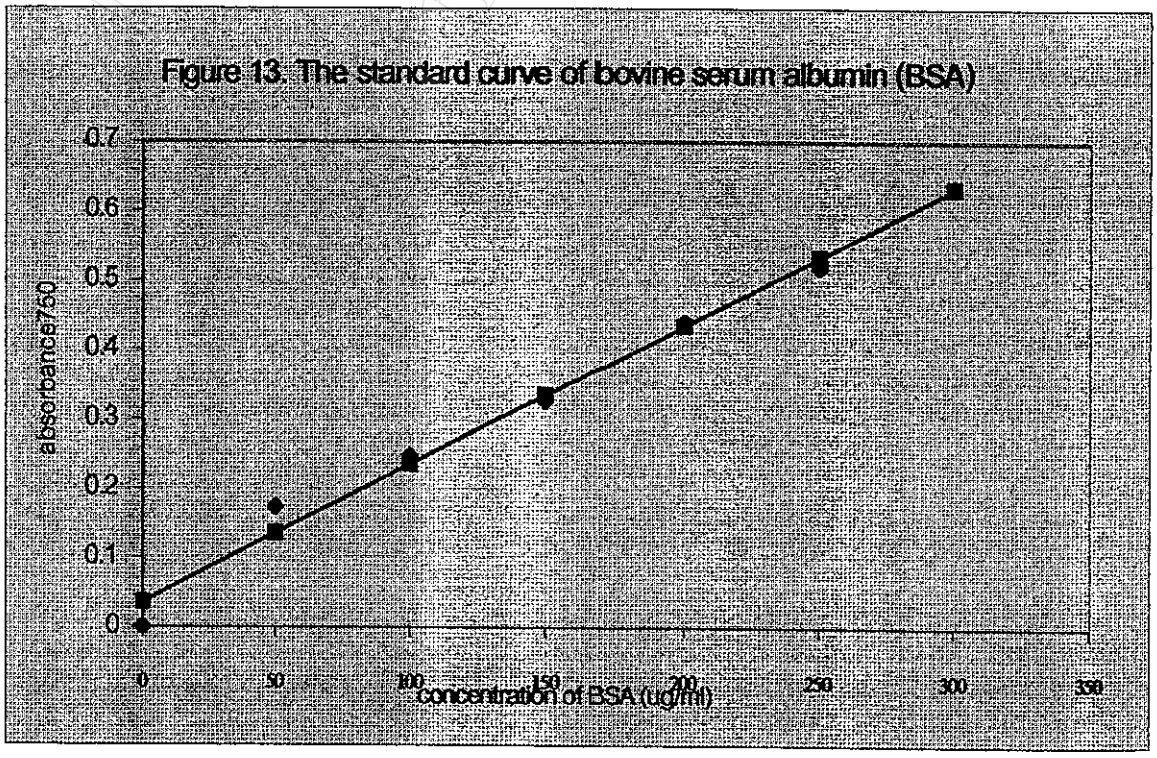
## 2.6 Protein determination

To construct a standard protein curve; 0, 0.5, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35 μg/ml of BSA solutions were used and treated exactly the same as the protein samples (Table 5). An aliquot of solubilized protein samples were diluted with distilled water to 1 ml. Five ml of alkaline copper solution (solution C) were added and resulting solution was mixed. After standing at room temperature for 10 mins, 0.5 ml of Folin-phenol reagent (solution D) was measured into the test tube, mixed gently and allowed to stand for another 30 min at room temperature. The absorbance at 750 nm of standard BSA and tested samples were determined using the spectrophotometer. Another tube containing distilled water replacing

the protein samples was treated as above and served as a blank. A standard curve of total protein content determination is presented in Figure 13, from which the protein content in the sample was determined.

**Table 5.**Preparation of bovine serum albumin standard solution.

| Concentration(μg/ml) | Stock standard(ml) | Distilled water(ml) |
|---|---|---|
| 0 | 0 | 1.00 |
| 50 | 0.05 | 0.95 |
| 100 | 0.10 | 0.90 |
| 150 | 0.15 | 0.85 |
| 200 | 0.20 | 0.80 |
| 250 | 0.25 | 0.75 |
| 300 | 0.30 | 0.70 |
| 350 | 0.35 | 0.65 |



## 2.7 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The samples were taken from Section 2.5, protein contents were determined according to Lowry's method (see Section 2.6) then SDS-PAGE was performed according to Laemmli's method (75).

Electrophoresis was run as described; the glass was first cleaned in detergent, washed with water and ethanol and allowed drying. The longer rectangular glass plate was lay down first, the two spacers of equal thickness were placed along the short edges of the rectangular glass plate was lay down first. Next, the shorter glass plates were aligned and taken to the clamp. The separating gel monomer solution was prepared by combining all reagents except ammoniumpersulfate (APS) and N, N, N' N'-tetramethyl ethylene diamine (TEMED) .The solution was deaerated under vacuum for at least 15 min. A comb was completing placed into the assembled gel sandwich. A marker pen was placed a mark on the glass plate 1 cm below the teeth of comb. To which the level of separating gel was poured. Then, The APS and TEMED were added to the deaerated monomer solution and the solution was smoothly poured to the mark using a glass pipette and bulb to prevent it from mixing with air. The monomer solution was immediately overlaid with isobutanol or distilled water. The gel was allowed to polymerize for 45 min to 1 hr. The overlay solution was completely rinsed off with distilled water. The stacking gel monomer solution was prepared by combining all reagents except APS and TEMED .The solution was deaerated under vacuum for at least 15 min. The area above the separating gel was dried with filter paper before pouring the stacking gel. The comb was placed in the gel sandwich and tilted it so that the teeth are at slight angle to prevent air from being trapped under the comb teeth while the monomer solution were poured. The APS and TEMED were added to the degassed monomer solution and the solution was poured down the spacer nearest the upturned side of the comb, until all the teeth have been covered by solution. Then the comb

was properly aligned in the sandwich and the monomer was added to fill completely. The comb was properly seated when the T portions of the comb rest on top of the spacers. The gel was allowed to polymerize 30-45 min. After polymerization was completed, the comb was removed by pulling at straight up slowly and gently. The wells were completely rinse with distilled water. The gel was ready to be attached to the inner cooling core, the samples loaded and the gel run .The clamps assembly gel sandwiches were released from the casting stand. The inner cooling core was layered down flat on a lab bench. With the glass plates of the gel sandwich butts up against the notch in the U-shape gasket. The inner cooling core was turned over and attached clamp assembly to the other side of the core in the same manner. The electrode buffer was prepared by mixing one volume of protein samples with five volumes of samples solubilization buffer and heated to 95 °C for 5 min, were loaded into wells under the electrode buffer with a Hamilton syringe. After sample application (30 µg of protein samples) the electrophoresis was carried out using 100 Volt and 30-19 mA until the front of tracking dye had migrated approximately 0.5 cm from bottom (60-90 min). The gel was removed from between the plates and electrotransferred onto Hybond ECL membrane (Amersham Co.,Arlington Heights IL) in a transblot electrophoretic transfer cell (Bio-Rad laboratories) (76) .

## 2.8 Protein Western blot analysis

Following electrophoresis, the gels were subjected to immunoblotting (76).

The gels were rinsed in transfer buffer prior to blotting to facilitate the removal of electrophoresis buffer salts and detergents. Then the membrane were cut to the dimension of the gel and wet it by slowly sliding at 45 ° angle into transfer buffer (soaked for 15-30 minutes). Two pieces of filter papers per gel were needed for each gel/membrane sandwich. The filter paper and filter pads were completely saturated by soaking in transfer buffer. The buffer tank was filled half-full with transfer buffer and opened the gel holder by

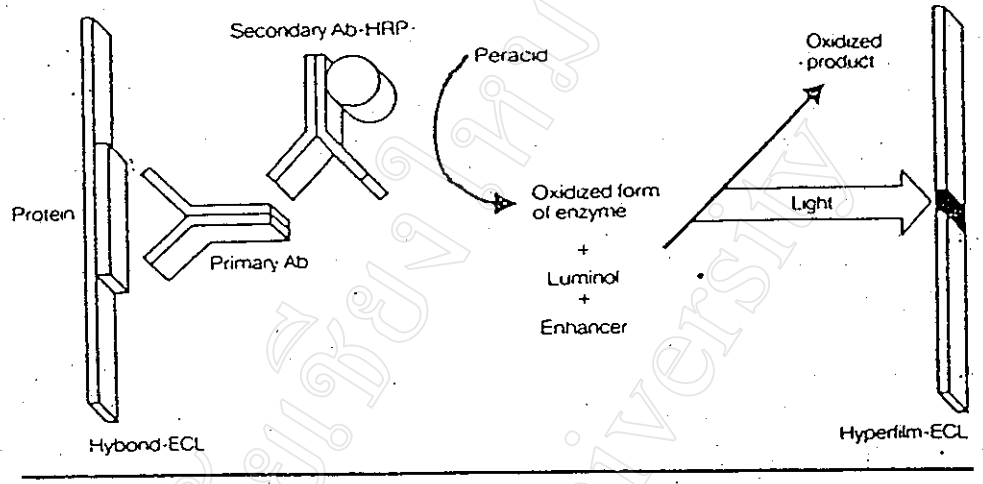
sliding and lifting the latch. Noted that one panel of the holder was clear and the other panel was tined smoky gray. The clear panel was the anode (+) side, and the gray panel was the cathode ( - ) side. A black disc identified the cathode itself. A pre-wetted fiber pad was placed on the gray panel of the holder and placed 2 pieces of saturated filter paper on top of the pad. The pre-equilibrated gel was placed on the top of the filter paper. Then 2 pieces of pre-wetted transfer membranes were placed on it. Completed the sandwich by placing a piece of saturated filter paper on the top of the membrane, the air was removed by rolling a glass pipette over the filter paper. Before closing, a saturated fiber pad was placed on top of filter paper. Then closed the gel holder, held it firmly and placed it in the Transfer-Blot tank . Filled the buffer tank to the bottom of the red anode disc with transfer buffer and a stir bar was added to the cell before putting the lid in place, plugged into the power supply and turned on the power supply for transfer condition (30 volts, overnight). Following transfer, the power supply was turned off and disconnected the unit from the power supply. The lid was removed and the gel cassettes from the unit. The transfer buffer and filter paper were discarded. The transfer efficiency can be monitored with prestained molecular weight standard, or by staining the gel with amido black. Alternatively, the gel can be stained with Coomassie Blue-250. In order to examine the PKC-isoenzmyes the blots were further detected by ECL detection system.

## 2.9 ECL detection

ECL Western blotting is a light emitting nonradioactive method for detection of immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase -labeled antibodies. The principle are outline in Figure 14 (76).

### Principles of ECL detection

Luminescence is defined as the emission of light resulting from the dissipation of energy from a substance in an excited state. In chemiluminescence the excitation is effected by a chemical reaction. The chemical reaction of cyclic diacylhydrazides such as luminol has been widely used in chemical analysis and extensively studies. One of the most clearly understood system is the HRP/ hydrogen peroxide catalyzed oxidation of luminol in alkaline conditions. Immediately following oxidation, the luminol is in an excited state which then decays to ground state *via* a light emitted pathway. Enhanced HPR in the light output approximately 1000 folds and extending the time of light emission. The light proceed by this enhance chemiluminescent reaction peak after 5-20 minutes and decays slowly thereafter with a half-life approximately 60 minutes. The maximum light emission is at a wavelength of 428 nm can be detected by a short exposure to blue-light sensitive autoradiography film ( Hyperfilm ECL).



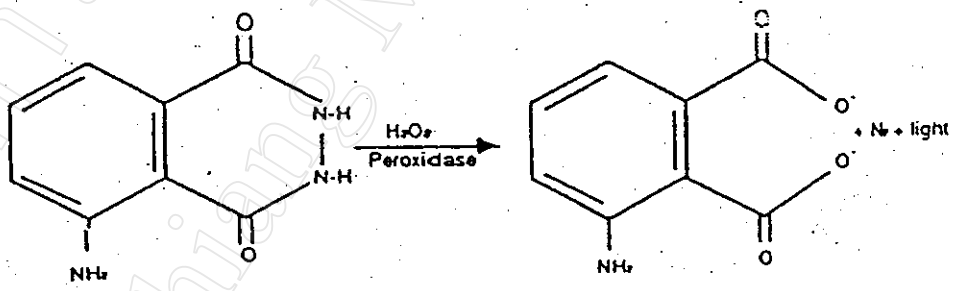




Figure 14. Principle of ECL detection

After transblotting the electophoretically resolves protein, the blots were blocked at non-specific binding by immersing the membrane in 5 % blocking reagent (5% skim milk dissolve in PBS) for 2 hour at room temperature on an orbital shaker. After that the blots were briefly rinsed with two changes of washing buffer (PBS containing 0.05% tween 20) then washed once for 15 minutes and twice for 5 minutes with fresh changes of the washing buffer. Blots were then incubated with PKC-ε polyclonal antibody diluted 1:2,500 in blocking reagent for 2 hours at room temperature. The membrane was washed extensively in washing buffer and reincubated with peroxidase-linked secondary antibody (Antirabbit IgG, Amersham) diluted 1: 10,000 in blocking reagent for one hour at room temperature, the membrane was further washed for 15 minutes and 4x for 5 minutes in fresh change of washing buffer. For detection step, equal volume of detection solution 1 with detection solution 2 were mixed to give sufficient to cover the membrane. The excess buffer was drained and placed on a piece of saranwrap, protein side up. The detection reagent was added to the protein side of the membrane. Do not allow the surface of the membrane to become uncovered and incubated for precisely 3-5 minute at room temperature without agitation. The excess detection reagent was drained and wrapped in the saranwrap. The membrane was placed protein side up, in the film cassette. Work as quickly as possible; minimized the delay between incubating the membrane in the detection reagent and exposing them to the film (next step). The lights were switch off and the hyperfilm ECL was placed on the top of the membranes for one min. The film was removed and developed by incubated in developing buffer for one min and fixed in fixing solution for 50 second.

## 2.10 Effect of TPA on level and distribution of PKC-alpha (α), -delta (δ), and -epsilon (ε) isoenzymes in human keratinocytes

PKC isoenzymes activation is associated with translocation from cytosol to membrane which is occurred within few hours of agonist stimulation and subsequent proteolytic degradation leading to down regulation. It was found that human keratinocytes expressed PKC-α, -δ, -ε, -ζ and η. This study were examined the effect of TPA on PKC -α, -δ, and -ε isoenzymes because they are TPA- dependent isotypes.

The 80 % confluent human keratinocytes ($3X10^6$ cells/ml) passages 4-6 were placed in growth factor-free medium (keratinocyte - SFM without hydrocortisone, insulin, BPE, EGE and rEGF; cat.no. 10726-016) for 48 h. The cells were treated with 160 nM TPA at different time 1, 2 and 18 h. Control cells were received DMSO with the final concentration of 4%.

| components | cocktail TPA (4000 ng/ml) | Control (DMSO) |
|---|---|---|
| 1 mg/ml TPA | 20 µl | - |
| Incompleted -keratinocyte SFM | 4800 µl | 4800 µl |
| DMSO | 180 µl | 200 µl |
| Total volume | 5000 µl | 5000 µl |

Note: 250 µl of TPA cocktail solution was added to each flask containing 10ml medium. Therefore, the final concentration of TPA was 160 nM and final concentration of DMSO both TPA and the control were 4%.

The 30 μg protein/ 35 μl of cytosolic and membrane fractions were separated on 7.5% polyacrylamide gels (Section 2.7) and transferred to nitrocellulose membrane (Section 2.8). Then the blots were blocked with 5% skim milk in PBS for 2 h on shaker bath at room temperature. After that, to detect PKC-epsilon, the blot was incubated with anti-PKC epsilon which was diluted 1:2500 in the blocking solution for 2 h on the shaker bath. Whereas for PKC-alpha and delta detection, the blots were incubated with the anti-PKC-alpha or -delta for 1 h on shaker bath. Following washing, immunoreactive proteins were visualized using the enhance chemiluminnescence Western blotting detection system (Amersham). The intensities of PKC band were quantified by laser densitometer.

## 2.11 Effect of TPA on the enzyme content of the PKC - ε isoenzyme in human keratinocytes

To determine whether TPA increased the level or induced newly synthesis of PKC - ε isoenzyme in human keratinocytes. The cytosolic and membrane fractions (30 μg protein/ 35 μl) of TPA - treated cells (1, 2 and 18 h) were pooled and then assayed for PKC - ε isoenzyme by Western blot analysis compared with the control (DMSO-treated cells).

## 2.12 Effect of curcumin on level and distribution of PKC- epsilon isoenzyme in human keratinocytes

Curcumin itself may causes the translocation or downregulation of PKC - ε isoenzyme or affects the PKC in some others way. To examine this possibility the study was evaluated as follows.

The 80% confluent human keratinocytes ($3X10^6$ cells/ml) at early cell passage (passage 4-6) were placed in serum free keratinocyte medium for 47 h as described in the Section 2.4 and then treated for 1 h with different concentration of curcumin (0-50 μM).The cytosolic and membrane fractions (30 μg protein/ 35 μl) were tested for PKC-epsilon by SDS-PAGE and Western blot as described in the Section 2.7- 2.9.

## 2.13 Effect of curcumin on level and distribution of TPA-induced PKC-epsilon in human keratinocytes

Since it has been reported that curcumin inhibited many biological effect of TPA in mouse skin fibroblast and mouse skin model (6-11).This study was further examined whether curcumin inhibit the effect of TPA-induced PKC-epsilon in human keratinocytes.

The cells ($3X10^6$ cells/ml) were preincubated for 1 hr with different concentration of curcumin 20, 40, 50 μM followed by treatment with TPA 160 nM for 1 h at 37 °C. The control cells were received DMSO at the final concentration of 4%. Cytosolic and membrane fractions were prepared as describe in Section 2.5 and then analyzed the level of PKC-epsilon by SDS-PAGE (Section 2.7) and Western blot (Section 2.8-2.9).

## 2.14 Effect of time on curcumin addition on TPA-induced PKC-epsilon in human keratinocyes

To explore the kinetic inhibition of curcumin on TPA-induced PKC-epsilon isoenzyme, human keratinocytes were incubated at 37 °C with curcumin for 1 h before, at the same time and after the addition of 160 nM TPA for 2 h. The cytosolic and membrane extracts were prepared as described in Section 2.5 and then assayed for PKC-epsilon as described in experimental procedure Section 2.7-2.9.