CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Absolute ethanol (Merck)
2. BPE (Gibco)
3. Canada balsam (Fluka)
4. Citric acid (TATE&LYLE GROUP)
5. Chloroform (Lab – Scan)
6. Dimethylsulphoxide (Lab – Scan)
7. DMEM (Gibco)
8. ELISA kit (R&D Systems)
9. r EGF (Gibco)
10. Endothelin (Gibco)
11. Eosin (Sigma)
12. Epigallocatechin (EGC) (Sigma)
13. Epigallocatechin gallate (EGCG) (Sigma)
14. Epicatechin (Sigma)
15. Epicatechin gallate (ECG) (Sigma)
16. Ethyl acetate (Lab – Scan)
17. Ethylenediamine tetra-acetic acid (Merck)
18. L-dopa (Sigma)
19. Fetal bovine serum (Hyclone)
20. basic Fibroblast growth factor (Gibco)
21. Formalin (Sigma)
22. Green tea (Namchai, Doi Mae Salong)
23. Hematoxylin (Sigma)
24. Hydrochloric acid (Sigma)
25. Insulin (Gibco)

26. Isopropyl alcohol (Merck
27. KSFM (Gibco)
28. L-glutamine (Gibco)
29. MCDB153 (Gibco)
30. Melanin (Sigma)
31. MEM (Gibco)
32. α MSH (Gibco)
33. Methanol, HPLC grad (Lab – Scan)
34. Methyl thiazoletetrazolium (Sigma)
35. PBS (Gibco)
36. P/S (Gibco)
37. Phosphoric acid (Lab – Scan)
38. Sodium Hydroxide (Merck)
39. Transferrin (Gibco)
40. Trypsin (Gibco)
41. Xylene (Carlo erba
42. Water (HPLC) (Lab – Scan)

EQUIPMENT

1. Analytical balance (Sartorius)
2. CO_2 water-jacketed incubator (Heraeus)
3. Centrifuge (eppendorf)
4. Freeze dryer (Dura-dry)
5. Hemacytometer (HBG)
6. Inverted microscope (Olympus)
7. 12. Lamina airflow hood (Nuaire)
8. 11. Microplate reader (Molecular Devices)
9. pH meter (Beckman)
10. Nova-Pak® C_{18} 4μm (3.9 X 150 mm) column (Waters)

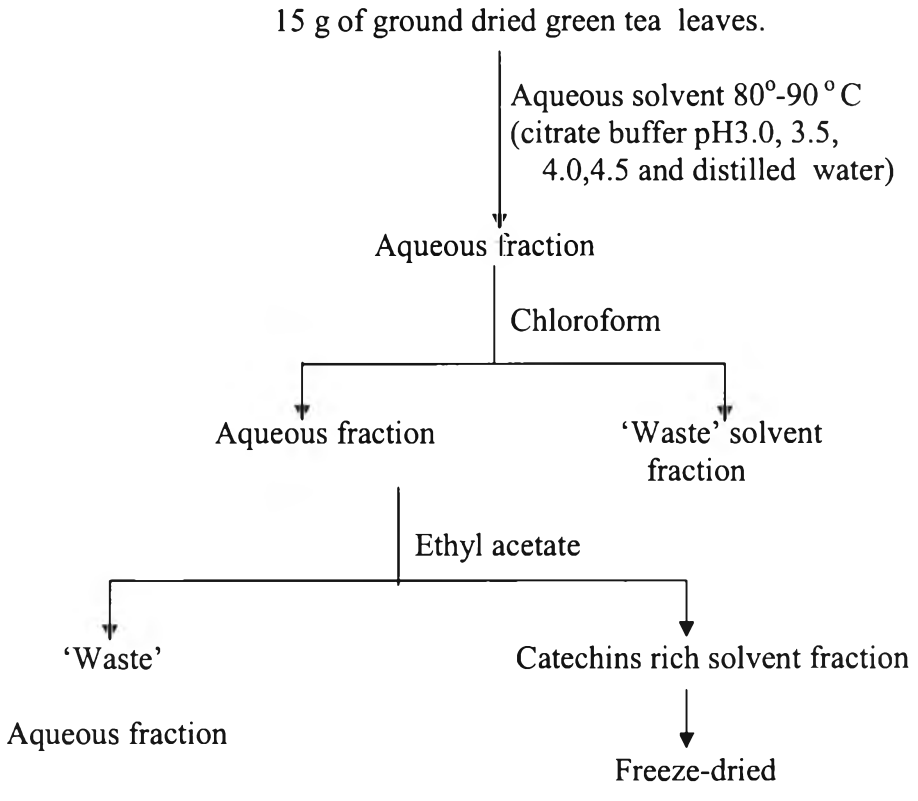
11. Reversed-phase High performance liquid chromatography, HPLC (SHIMADZU)
    - System controller : SCL-10AVP
    - Pump: LC-10ADVP
    - Degasser: DGU-14A
    - UV-Vis detector: SPD-10AVP
    - Auto injector: SIL-10ADVP
    - Column oven: CTO-10AVP
12. Rotary evaporator (EYELA)
13. Sentry^{TM} Guard column and universal holder (Waters)
14. Sonicator (Branson)
15. Vortex mixer (Scientific Industries)
16. Water Bath (Techne)

METHODS

1. Preparation of green tea extracts

Catechins or green tea polyphenols are the active ingredients in green tea. To evaluate the activities of green tea, catechins were extracted from dried green tea leaves and used instead of the leaves. Green tea leaves were extracted with five different solvents and compared the yield of each method. Brown precipitate occurred when polyphenol reacted with ferric chloride. The extraction continued until the extracting solvent had no brown precipitate with ferric chloride.

Citrate buffer solutions were prepared by dissolving 1.92 g of anhydrous citric acid in 1000 mL of distilled water and adjusted to pH 3.0, 3.5, 4.0, 4.5 with 0.1 NaOH. About 10 g of ground dried green tea leaves were extracted with 100 mL distilled water or citrate buffer solution at 80 – 90° C for 30 min several times until the extracting solvent had no brown precipitate with 10 % ferric chloride. The green tea solutions were collected and re-extracted with equal volume of Chloroform for about 5 times to remove caffeine, aromatic compounds and pigment. An equal volume of ethyl acetate was used to extract the aqueous fractions several times until the aqueous fraction had no brown precipitate with 10 % ferric chloride. The ethyl acetate fractions were collected and evaporated under vacuum using rotary evaporator. The dried residues were dissolved with 20 mL of distilled water and freeze dried. The freeze dried samples were weighed and analysed for percentage of each catechin.



2. Determination of Polyphenols in green tea extracts

The level of catechins in the extracts were analysed by High Performance Liquid Chromatography (HPLC) technique.

2.1 HPLC condition development

HPLC conditions were developed and used to determine cathechin contents in each green tea extract.

HPLC condition

HPLC column :	Nova-Pack(C18 3.9 x 150 mm Steel column
HPLC guard column :	Waters Sentry TM guard column and Universal guard holder
Mobile phase :	Aqueous solution of 0.15% phosphoric acid : Methanol (85:15)

Detector wave-length: 210 nm
Flow rate: 1.5 mL/ min
Temperature: 30°C
Injection volume: 20 µL
Running time: 35 minutes

2.2 Preparation of sample solutions

Triplicate of stock sample solutions (0.4 mg/mL) were prepared by dissolving each green tea extract in mobile phase. The solutions were subsequently diluted with mobile phase to obtain the sample solution (.04 mg/mL).

2.3 Preparation of standard solutions

Each standard catechin stock solution was prepared by dissolving 5 mg of reference standard (EGC, EGCG, EC and ECG) in 5 ml of mobile phase. Each stock solution was then used to prepare mixed standard stock solution (0.008, 0.02,0.07, and 0.006 mg/mL of EGC, EGCG, EC and ECG, respectively). The mixed standard stock solution was diluted with mobile phase to obtain 5 concentrations of standard solution (3,10,50,70 and 100 % of the mixed standard stock solution) for the calibration curve. Standard caffeine solution was prepared at .01 mg/mL in mobile phase.

2.4 HPLC Assay

To quantify each catechin in green tea extracts using the developed HPLC conditions, the sample and standard solutions were injected through the system. Standard curve of EGC, EGCG, EC and ECG were constructed by plotting peak area of chromatogram versus concentration. The percentage of each catechin was calculated by comparing the peak area of sample with the standard.

3. Analytical Method Validation

The developed analytical method was validated, according to USP 26. Specificity of the method was proved by injection of each reference standard solution and the retention time of chromatograms were observed. Accuracy was performed by duplicate injection of a triplicate of 5 concentrations of sample solutions. The calibration curves were prepared as described. The actual concentration, the observed concentration, and % recovery of EGC, EGCG, EC and ECG were determined. Peak area was used to calculate the measured concentration. Linearity was obtained by plotting the observed concentration versus the actual concentration, and the least squares regression equation was calculated. Precision was measured by duplicate injections of six replicated sample solutions, and the % RSD (Relative Standard Deviation) of EGC, EGCG, EC and ECG was determined.

4. Preparation of monolayer cell cultures.

To study the effects of green tea extracts in cell cultures; monolayer cell cultures of melanocyte/keratinocyte co-culture and melanocytes were prepared from dark normal human foreskin, keratinocytes were prepared from white normal human foreskin and fibroblasts were prepared from any normal human foreskin.

4.1 Isolation of Normal Human Epidermal cells

Newborn foreskins (already approved by ethical committee from Facullty of Pharmaceutical Science, Chulalongkorn University) were transfeered from hospital in Dulbecco' Modified Eagle's Medium (containing 200 U/ml Penicillin and 100 µg/mL Streptomycin). They were transferred to a sterile tube with 0.5 % dispase in phosphate buffer saline (PBS). The skins were left overnight at 4°C until the very thin sheet of opaque epithelium could be easily removed from dermis with forceps. All of the epidermal layers were removed and soaked in 0.25% trypsin for 5 minutes (minimum) at 37°C. The reaction of trypsin was stopped by adding 10% fetal bovine serum. To isolate the cells, the cell suspension was gently pipetted to break up

clumps, centrifuged, the supernatant discarded and the cells resuspended in medium according to the requirement of the cells for further studies.

4.2 Preparation of melanocyte / keratinocyte co-cultures

The previously isolated epidermal cells were suspended in the melanocyte/ keratinocyte co-culture medium consisting of 1:1 MCDB 153 and KSFM medium with 25 µg/mL Bovine Pituitary Extract (BPE), and 0.20 ng/mL Recombinant Epidermal Growth Factor (rEGF), 5% heat- inactivated serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 5 µg/mL insulin, 5 µg/ml transferrin, 0.6 ng/mL basic Fibroblast Growth Factor (bFGF),10 nM/mL endothelin-1, and 10 nM/mL Melanocyt Stimulating Hormone (MSH). The cells were cultured in tissue culture (TC) dish (35mm) at 37°C in humidified CO_2 incubator. The medium was changed every two or three days until the co-cultures of melanocyte / keratinocyte were nearly confluent then they were subcultured (see below) to use for further studies.

Subculture procedure

The cultures were washed with PBS to remove serum and free-floating cells, and then incubated with 0.06 % trypsin / EDTA at 37 °C for about 5 min. When cells could be seen to be rounded up and detached, 10% FBS was added to inhibit trypsin activity. The cells were centrifuged, the supernatant discarded, resuspended in fresh medium, and cultured on a new TC dish.

4.3 Preparation of Normal Human Foreskin Keratinocyte (NHFK)

Normal Human Epidermal cells were isolated from newborn foreskin as described previously. The cells were suspended in keratinocyte growth medium containing Keratinocyte-SFM with 100 U/mL Penicillin, 100 µg/mL Streptomycin, 25 µg/mL Bovine Pituitary Extract (BPE), and 0.2 ng/mL Recombinant Epidermal Growth Factor (rEGF), plated on TC dishes and incubated at 37 °C in a 5% CO_2 incubator. The medium was changed two - three times weekly until the cells were

confluent, then they were subcultured to separate melanocytes from keratinocytes. Melanocytes react more quickly to trypsin and this can be used to separate them from the keratinocyte/melanocyte coculture. When melanocytes could be seen to be rounded up and detached, the trypsin solution with melanocytes was removed. and used to prepared melanocyte cell culture. Another aliquot of 0.06 % trypsin / EDTA was added to the remaining attached cells, left at 37 °C until the cells were rounded up and detached then 10% FBS was added to inhibit trypsin activity. The cells were centrifuged, the pellet resuspended in fresh medium and cultured on a new TC dish. The medium was changed every 2-3 days until a confluent culture of keratinocytes was established.

4.4 Preparation of Normal Human Foreskin Melanocyte (NHFM)

The isolated melanocytes were cultured in melanocyte growth medium containing MCDB 153 medium with 5% heat- inactivated serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 5µg/mL insulin, 5µg/mL transferrin, 0.6ng/mL basic Fibroblast Growth Factor (bFGF), 10nM/mL endothelin-1, and 10nM/mL Melanocyte Stimulating Hormone (MSH). The cells were incubated at 37°C in a humidified CO_2 incubator. The medium was changed every two or three days.

4.5 Preparation of Normal Human Foreskin Fibroblast (NHFF)

The dermis was cut into very tiny pieces with a scalpel and transferred to TC dish, allowed to settle and attach for 15 minutes. The tissues were then cultured in fibroblast growth medium containing Dulbecco' Modified Eagle's Medium (DMEM) with 10% FBS ,1% L glutamine and 100 U/mL Penicillin and 100 µg/mL Streptomycin and incubated at 37° C in a 5 % CO_2 incubator. The medium was changed every three or four day. Within 1- 3 weeks the fibroblasts started growing from the edges of the attached tissue. The confluent fibroblasts from explants could be subcultured and harvested using trypsin / EDTA, resuspended in fresh medium and replated on to a new TC dish to expand the population.

5. Preparation of Reconstituted 3-dimentional human skin (3D skin)

To investigate photo-protective activity of green tea extracts, 3D skin was prepared by culturing of human epidermal cells on collagen raft containing fibroblasts.

5.1. Preparation of rat tail tendon collagen

Tendons were pulled from rat tails using sterile forceps. They were weighed to make an extraction of 1 % w/v tendons in 0.5 M acetic acid (14.30 mL glacial acetic acid in 500 ml sterile water) and left for 48 hours (hr) at 4 °C. The extraction was filtered through 4 layers of sterile gauze in a sterile funnel. Dialysis tubing was rinsed with distilled water and 0.1X DMEM and clamped one end with double knot. The collagen solution was loaded into the tubing using a sterile funnel, and the end was closed with clamp. Dialysis, with one change, was carried out against 0.1x DMEM for 24 hr at 4° C. The collagen was loaded into sterile centrifuge bottles (250 ml volume) and sterilized by centrifugation at $1x10^4$ rpm for 2 hr at 6 °C. The supernatant containing collagen was transferred to sterile bottles and used to prepare collagen rafts. The concentration of collagen was calculated by pipetting 3 mL of the collagen solution into a preweighed Petri dish, left to dry at 37 °C, then reweighed.

Concentration of the collagen (mg/mL) = (final weight-preweighed Petri dish)/3

5.2 Preparation of collagen rafts with human fibroblast cells

Collagen raft solution containing 1.33 mL of Mini Essential Medium (MEM 10X) / 0.67mL of 0.34 M NaOH, 6 mL of the rat tail tendon collagen (2 mg/mL in 1mM acetic acid), 1 mL of DMEM and 1 mL of $1x10^6$ human fibroblast cells in FBS was prepared for two rafts. The solution in 5 mL aliquots was dispensed into 35 mm TC dishes and allowed to set at room temperature for 30 minutes. The raft gel was loosen from dish surface by running around the edge with a micro pipette tip and incubated with DMEM at 37° C in a 5 % CO_2 incubator. The medium was changed every 3-4 days until no more contraction of raft.

5.3. Preparation of 3D skin

Epidermal cells and a collagen raft were prepared as described. Medium was removed from the raft prior to use. The cells were resuspended after trypsinization and pelleted by low speed centrifugation (1000 rpm) in 50 µL of FBS and then placed on top of raft. The dish with cells on the raft was placed in the incubator to allow cells to attach on raft. One day later, 2 ml of keratinocyte growth medium was gently added to the dish and incubated at 37° C in a 5 % CO_2 incubator. Medium was changed every 2-3 days. After 10 days, the DMEM was replaced with 1:1 keratinocyte/fibroblast medium to the air-liquid interface of the raft and changed every 2-3 days. After about 3 - 4 weeks, 3D skin was formed and ready to use.

6. Cytotoxicity testing for green tea extract and EGCG

6.1 Cell culture

L929 cells (mouse C3H/An connective tissue; ECACC NO. 85011425) are epithelial like cells which have morphology similar to keratinocytes. They are widely used in the standard test method for cytotoxicity (follow BSEN 30993-5 and ISO 10933-5: Biological evaluation of medical devices part 5; Test for cytotoxicity, *in vitro* methods). In these studies, L 929 and NHFF (Normal Human Foreskin Fibroblasts), were separately cultured in DMEM, 10% foetal bovine serum (FBS), 1% L glutamine and 100 U/mL Penicillin and 100 µg/mL Streptomycin ; NHFK (Normal Human Foreskin Keratinocytes) were cultured in the keratinocyte growth medium; NHFM (Normal Human Foreskin Melanocytes) were cultured in melanocyte growth medium. Cells were plated in 96-well plates at an optimum concentration (cells/well) in 200 µl / well of medium. Optimum cell seeding density for each cell was determined: 3x 10^3 cells/well for NHFF, 1x 10^3 cells/well for L929, 1x 10^3 cells/well for 3T3, 1x 10^3 cells/well for IEG6,1.2x10^4 cells/well for NHFK and 1.4 x10^4 cells/well for NHFM. All cells were incubated at 37 °C in a 5 % CO_2 incubator for 48 hr to allow attachment and initiation of growth before applying the test samples.

6.2 Cytotoxicity assays

The cytotoxicity of GTE was compared to pure EGCG. For test samples; eight concentrations of EGCG or green tea extract (GTE) in growth medium were prepared for each cell as shown in Table 2 and Table 3.

Table 2. The concentrations of EGCG in test sample

Cell	EGCG (μg/mL)								
L929	0	10	20	30	40	50	70	90	_
NHFM	0	10	20	30	40	50	60	70	80
NHFK	0	_	20	30	40	50	60	70	80
NHFF	0	10	20	25	30	40	50	70	90

Table 3. The concentrations of GTE in test sample

Cell	GTE (μg/mL)								
L929	0	10	20	30	40	50	70	90	200
NHFM	0	30	60	90	120	150	200	_	_
NHFK	0	30	60	90	120	150	200	_	_
NHFF	0	20	30	60	70	90	120	150	_

After 48 hr cultivation, the growth medium was aspirated from the wells and replaced with the test samples. The cells were exposed to the samples for 24 h. Medium with toxin was then aspirated, replaced with fresh medium, and the cultures were incubated for a further 24 h. The cytotoxicity of the samples was quantified with a modified 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Plumb, Milroy & Kaye 1989), which measures the metabolic conversion in the mitochondria of tetrazolium (yellow) to formazan (blue). 50 μl of MTT in Phosphate buffered saline (PBS) (5 mg/ mL) was added to the medium in

each well. The cells were incubated for 4 h and then the medium with MTT was aspirated from the wells, the formazan was solubilized with dimethyl sulfoxide (DMSO) and stabilized with 25 µL of Sorensen's glycine buffer (0.1 M glycine in 0.1 M NaCl , pH 10.5). The optical density was read by spectrophotometry with a micro-plate reader at 570 nm. The average of four wells was used to determine the mean of each point. The first and the last columns contained medium only and were used as the blank. The data were analysed with the SoftMax program (Molecular Devices) to determine the dose response curve of each sample.

7. Inhibition of melanin synthesis studies in melanocyte cell cultures

Kojic acid has been shown to reduce melanin production and tyrosinase activity in the cell cultures (Hata *et al.*, 1986). In this study, kojic acid was used to compare this activity with green tea extract (GTE).

Co-culture of melanocytes and keratonocytes were subcultured and plated in 35 mm TC dish at $5x10^5$ cells/ dish. The cells were cultured in the melanocyte / keratonocyte co-culture medium for 48 hr to allow attachment and initiation of growth before applying the test samples. For test samples; the growth medium (as control), 60 µg/mL of GTE in growth medium, and 500 µg/mL of kojic acid in the growth medium were prepared. The growth medium consists of DMEM, 1% L- glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 10 % FBS. The cells were cultured with the test samples, after 2 days the medium was replaced with fresh test sample and cultured for another 2 days. Then the cells were evaluated for melanin content by Dopa reaction and optical density measurement.

7.1 Dopa reaction (Tomohisa Hirobe, *et al.*1988)

To stop metabolic activities, the medium was replaced with 5% formalin in phosphate buffered saline (PBS) and the cultures were incubated at 4°C for 30 min, rinsed with distilled water. They were then incubated with 0.1% L-Dopa solution in PBS, pH 7 at 37°C for 4 hrs. The cells were fixed with 10% formalin in PBS at 4°C for 1hr. A drop

of fixed cells was air-dried on a slide and sealed with glycerin. Photos were taken to compare results visually.

7.2 Melanin measurements (Susan *et al.*,1998)

The cells were trypsinized, and counted, After centrifugation, the cell pellets were dissolved in 100 µL 1 N NaOH and transferred to 96 well plates. Melanin concentrations were determined by measurement of the optical density at 405 nm, using a standard curve generated from synthetic melanin (Sigma). Melanin values were expressed as melanin per cell in picograms.

8. UV protection studies in keratinocyte cell cultures

Ectoin, a biopolymer from bacteria has been shown to protect the harmful effect of UV irradiation on skin (Joachim Bünger, *et al.* 2000). In this study, Ectoin was used to compare the activity with GTE.

Keratinocytes were seeded in 96 well plates (at $1.2x10^4$ cells/well) in 200 µl / well of medium and incubated at 37 ^{o}C in a 5 % CO_2 incubator for 48 hr to allow attachment and initiation of growth before applying the test samples. For test samples; the growth medium (as control), 60 µg/mL of green tea extract (GTE), 30 µg/mL of EGCG and 4 mM of Ectoin in the growth medium were prepared. The growth medium consisted of DMEM, 1% L- glutamine, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 10 % FBS.The cells were exposed to the samples for 24 hrs. After that the plate was irradiated with 400 or 600 mj of UVB. Then medium with toxin was aspirated, replaced with fresh medium, and the cultures were incubated for a further 24 hr. Cell viability was quantified with a modified 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described previously.

9. UV protection studies on 3D skin model

Test samples (the growth medium, 60 µg/mL of green tea extract and 4 mM of Ectoin in the growth medium) were prepared and applied to the 3D skin model. The skin models were incubated at 37° C in a 5 % CO_2 incubator for 24 hr. After that they were irradiated with 400 mj of UVB. Then the test samples were replaced with fresh medium, and the skin models were incubated for a further 24 hr. The culture medium was assayed for released cytokine: Interleukin 1alpha (IL-1α). The skins were evaluated for sunburn cells (SBC) and cell viability.

9.1 Evaluation of sunburn cells

The skin models were fixed in 10 % formalin. Vertical sections of paraffin embedded cultures were stained with Haematoxylin /Eosin and examined skin histology for sunburn cells. A photo was taken and number of Sunburn cell per millimeter of skin model were counted using a scale in eyepiece of microscope

Preparation Histological Sections

Dehydration: After fixing in 10% formalin for 48-72 h, the skin models were rinsed overnight with tap water and stored dehydrated sequentially in 30%, 50%, 70%, 95%, and 100% ethanol for 30 minutes (min) of each.

De-alcoholization: After dehydration, the skin models were stored in 1:1 xylene/ethanol for 2x30 min and xylene for 2x30 min.

Infiltration after De-alcoholization: The skin models were stored in 1:1 xylene/paraffin for 2x30 min and 45° C paraffin for 2x30 min.

Paraffin Embedding: Liquid paraffin (59° C) was filled in a plastic dish (2.5x4.0 cm). When the paraffin in the bottom of the dish had slightly hardened, the

skin model was placed upright the dish and hold upright with a pair of tweezers until it could no longer topple over. Secured the gird on the dish with paraffin and allowed the paraffin dish to cool for approximately 1 hr.

Sectioning: The cool paraffin block was carefully removed from the plastic dish. Slice 30 μm sections from the cooled paraffin block until the optimum cross – section was found. Cooled the blocks for approximately 30 min and then prepared 5 μm sections. Placed the sections in a water bath (45° C) and attached them to slides in a drying cabinet at 35 – 40° C; this also removed the paraffin.

Staining: The skin model sections were placed in a xylene bath for 2x5 min, then rehydrated in 100% ethanol for 2x5 min and 95%, 70%, 50%, 30% ethanol for 5 min each. They were rinsed with tap water, stained with Haematoxylin for approximately 15 min and rinse in tap water for 5-10 min. (slide will turn blue),and rinsed again in distilled water. They were then incubated in Eosin solution (cytoplasm stain) for approximately 10 min, rinsed in tap water, incubated in 70% ethanol (to wash out stain) for several minutes depending on staining intensity and incubated in 95% to 100% ethanol for 5 minutes each. Finally, they were placed in a xylene bath for approximately 10 min. The coverslips were mounted with Canada balsam (Bunger *et al.*, 2001).

9.2 Analysis of tissue viability

MTT assay for tissue viability

Tissue viability was measured with the MTT assay method (Plum et al., 1989). After the removal of the culture media, the skin models were washed twice with PBS and transferred to 24 well plates. Then 500 μl/well MTT solution (5.0 mg/mL) was added and left in the CO_2 incubator for 4 hours (until purple crystals appeared). The medium with MTT was removed from the wells and replaced with 2 mL/well of MTT solubilizing medium (0.04 N HCl in Isopropanol). The skin models were left

overnight. After the extraction period was complete, the extractant solutions were pipetted up and down at least 3 times to insure they were well mixed, and then transferred to 96 well plates (200 µL/well). The optical density was measured at 570 nm using 200 µL of extractant as a blank.(Zhu *et al.*, 2001). The percent viability was determined using the following formula.

% viability = 100x [OD (sample)/OD (negative control)]

9.3 Analysis of the released cytokines (IL-1α) after UV irradiation

The amount of the cytokine interleukin 1α (IL-1α), in these culture medium samples was measured by enzyme-linked immunosorbent assay (ELISA). The standard cytokine and the culture medium samples were added to each well of a 96-well microplate in duplicate. The amount of IL-1α was measured using commercial kits (R&D System Inc., Minneapolis, MN, U.S.A.) according to the package instructions. The absorbance of cytokine in wells was measured with a microplate reader at 450 nm. Amount of IL-1α was determined by plotting on a calibration curve.

10. UV protection studies on excised human breast skin

Test samples (the medium, 60 µg/mL of green tea extract (GTE) and 4mM of Ectoin in the medium) were prepared and applied on human skin biopsies (excised breast skin from plastic surgery). The excised skin was incubated at 37 °C in a 5 % CO_2 incubator for 24 hours. After that they were irradiated with 400 mj of UVB. Then the medium with test samples was replaced with fresh medium, and the skins were incubated for further 24 hrs. The culture medium was removed and assayed for released IL 1α cytokine. The excised skins were evaluated for sunburn cells and cell viability.

10.1 Evaluation of sunburn cells

The excised skins were fixed in 10 % formalin. Vertical sections of paraffin embedded cultures were stained with Haematoxylin /Eosin and histologically examined for sunburn cells. as described previously.

10.2 Analysis of tissue viability

Tissue viability of the excised skin was measured by MTT assay method as the same method as 3D skin model.

10.3 Analysis of the released cytokines after UV irradiation

The amount of cytokine (IL-1α) in these culture medium samples was measured by enzyme-linked immunosorbent assay (ELISA) with the same method as 3D skin model.