CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of the present research including materials, apparatus and methods. The detail of each topic is described below.

Materials

1. Ethyl acetate (LabScan Asia, Co. Ltd., Bangkok, Thailand)
2. Chloroform (LabScan Asia, Co. Ltd., Bangkok, Thailand)
3. Methanol (LabScan Asia, Co. Ltd., Bangkok, Thailand)
4. Formic acid (LabScan Asia, Co. Ltd., Bangkok, Thailand)
5. Sephadex LH20 (Sigma-Aldrich, Inc., Missouri, USA)
6. Folin–Ciocalteu reagent (analytical grade, Sigma-Aldrich Co., St.Loius, Missouri, U.S.A.)
7. Alpha-melanocyte-stimulating hormone (analytical grade, Sigma-Aldrich Co., St.Loius, Missouri, U.S.A.)
8. Catechin (analytical grade, Sigma-Aldrich Co., St.Loius, Missouri, U.S.A.)
9. a,a-diphenyl-b-picrylhydrazyl radical (DPPH, Sigma- Aldrich Chemie GmbH, Steinheim, Germany)
10. Sodium Carbonate (UNIVAR, Aukland, New Zealand)
11. Dulbecco's Modified Eagle's Medium (DMEM, Sigma- Aldrich Chemie GmbH, Steinheim, Germany)
12. 0.25%Trypsin/ 0.01 M EDTA (analytical grade, Sigma-Aldrich Co., St.Loius, Missouri, U.S.A.)
13. Penicillin-Streptomycin (Gibco^{TM}, Invitrogen, New York, U.S.A.)
14. Fetal bovine serum (FBS, cell culture grade, Gibco^{TM}, Invitrogen, New York, U.S.A.)
15. Sodium3,3'-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}-bis(4-metoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay kit

16. Triton x-100 (analytical grade, Sigma-Aldrich Co., St.Loius, Missouri, U.S.A.)

17. L-DOPA (analytical grade, Sigma-Aldrich Co., St.Loius, Missouri, U.S.A.)

18. Ethylene diamine tetraacetic acid(EDTA) (Sigma-Aldrich, Inc., Missouri, USA)

19. Dispase (Gibco™, Invitrogen, New York, U.S.A.)

20. 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich, Inc., Missouri, USA)

21. Sodium chloride (UNIVAR, Aukland, New Zealand)

22. Potassium chloride (UNIVAR, Aukland, New Zealand)

23. Calcium Chloride (UNIVAR, Aukland, New Zealand)

24. Hydrogen Peroxide (UNIVAR, Aukland, New Zealand)

25. Ribonucleic acid, RNase (Gibco™, Invitrogen, New York, U.S.A.)

26. Sulfinpyrazone (Sigma-Aldrich, Inc., Missouri, USA.)

27. Kojic acid (Sigma- Aldrich Chemie GmbH, Steinheim, Germany)

28. Fetal bovine serum (Gibco™, Invitrogen, New York, U.S.A.)

29. serum-free-keratinocyte medium (SFM) with supplements (Gibco™, Invitrogen, New York, U.S.A.)

30. Fluo-3 AM (Molecular Probes Inc., Oregon, USA)

31. Sodium chloride (analytical grade, VWR International Ltd., Poole, England)

32. Potassium dihydrogen orthophosphate (UNIVAR, Aukland, New Zealand)

33. di-Sodium hydrogen orthophosphate anhydrous (Fisher Chemicals, Loughbough, UK)

34. Ethanol (analytical grade, MERCK, New Jersey, U.S.A.)

35. Hydrochloric acid (Analytical Grade, J.T. Baker, New Jersey, U.S.A.)

36. Sodium 3-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonicacidhydrate, XTT) (Roche Diagnostics, Mannheim, Germany)

37. Propidium iodide (Sigma–Aldrich, St. Louis, Missouri, USA)

38. Primary antibodies, rabbit polyclonal to collagen I (Cat no.ab292, Abcam plc, Cambridge, UK)

39. Primary antibodies, rabbit polyclonal to MMP-1 (Cat no. ab292, Abcam plc, Cambridge, UK)

40. Secondary antibodies, Cy5 conjugated goat polyclonal to rabbit IgG (Cat no. ab6564, Abcam plc, Cambridge, UK)

41. Secondary antibodies, FITC conjugated goat polyclonal to mouse IgG (Cat no. ab6785, Abcam plc, Cambridge, UK)

42. 3,3'-Diaminobenzidine (DAPI; Sigma–Aldrich Chemie, Steinheim, Germany)

43. ELISA kit for type I procollagen (Takara Bio, Inc., Shiga, Japan)

44. ELISA kit for MMP-1 (Ray Biotech, Inc., Norcross, Germany

45. Total glutathione assay kit (Dojindo Molecular Technology, Inc., Kumamoto, Japan)

46. Calcium carbonate (analytical grade, Riedel-dehaen, Seelze, Germany)

47. Calcium chloride (analytical grade, Ajax Finechem, Aukland, New Zealand)

48. Bovine serum albumin (Sigma–Aldrich, St. Louis, Missouri, U.S.A.)

49. 10X DMEM (Gibco^{TM}, Invitrogen, New York, U.S.A.)

50. Rat tail type I collagen (Gibco^{TM}, Invitrogen, New York, U.S.A.)

51. Acetic acid (LabScan Asia, Co. Ltd., Bangkok, Thailand)

52. Sodium Hydroxide (UNIVAR, Aukland, New Zealand)

Instruments

1. Magnetic stirrer (HL instrument, MS 115, Harikul Science, Bangkok, Thailand)

2. Microplate spectrophotometer (Spectra Count, Perkin Elmer, Massachusetts, U.S.A.)

3. Inverted microscope (model TS100, Nikon Eclipse, Tokyo, Japan)

4. Vertical laminar air flow cabinet (model BHG2004S, Faster SRL, Ferrara, Italy)

5. Water bath (model LWB-211A, Daihen LabTech Co. Ltd., Seoul, Korea)

6. UV-spectrophotometer (Biochrom Ltd, Cambridge, England)
7. Inverted light microscope (TS100, Nikon Corporation, Tokyo, Japan)
8. Fluorescence microscope (Observer. A1, Carl Zeiss, Aalen, Germany)
9. CO_2 incubator (model 311, ThermoForma, Marietta, U.S.A.)
10. Multimode detector (Beckman Coulter Inc., California, USA)
11. UVA Source, F-lamps equipped with a H-1 filter (Honle, Gräfelfing, Germany)
12. UV meter with UVA probe (Honle, Gräfelfing, Germany)
13. Multimode detector (Beckman Coulter Inc., California, USA)
14. Flow cytometer (model FACScalibur, Becton Dickinson, New Jersey, USA)

Methodology

1. Preparation of tamarind seed coat extract

1.1 Plant collection

The seeds of tamarind were purchased from local market in Petchaboon Province, Thailand. The studies were performed between the time periods of June 2009 to October 2011. Moreover, the plant was collected to make the herbarium specimen (Appendix A).

1.2 Solvent extraction

The seeds were heated in a hot air oven at 140°C for 45 min, cooled and cracked to separate the outside brown layer. Only brown-red seed coats were collected and then ground into fine powder. Liquid extraction with 70% ethanol was used for seed coat extraction. After drying under vacuum, the solids were extracted with chloroform in separated funnel and the chloroform part was discards. The collected part was then extracted with ethyl acetate and the ethyl acetate part was discards. The collected part was then dried under vacuum (Pumthong, 1999). After removing of tannin by Sephadex LH20 column, the extract was dried under vacuum and stored in a tight-amber glass at 4°C for further studies.

2. Qualification of tamarind seed coat extract

2.1 Quantification of total phenol in the extract

Since polyphenolic flavonoid is the active compound in tamarind seed coat extract (Sudjaroen, 2005), the amount of total phenol of the extract was determined by Folin-Ciocalteu assay. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate using for the colorimetric assay of phenolic antioxidants and polyphenol antioxidants. The reagent will react with phenols and nonphenolic reducing substances to form chromogens that can be detected spectrophotometrically (Singleton, 1999).

An aliquot of the samples (40 µl) was mixed with 1.8 ml of Folin–Ciocalteu reagent which was previously diluted with distilled water (1:10). The solution was incubated at 25°C for 5 min before adding 1.2 ml of 15% sodium carbonate solution in distilled water. After incubation at room temperature for 30 min, the absorbance at 765 nm was detected. Catechin, one of the phenolic compounds founded in tamarind seed coat extract was used as a standard. The total amount of phenolic compounds were calculated and expressed as catechin (mg/g).

2.2 Free radical scavenging activity on DPPH

The antioxidant activity of tamarind seed coat extract was measured in terms of hydrogen donating or radical scavenging ability, using the a,a-diphenyl-b-picrylhydrazyl radical (DPPH) method (Brand-Williams, Cuvelier and Berset, 1995) modified by Sanchez-Moreno, Larrauri, and Saura-Calixto (1998). This assay is based on the measurement of scavenging ability of antioxidant test substances towards the stable radical. Hydrogen atom or electron-donating ability of the extracts was measured from the bleaching of the purple-colored methanol solution of DPPH.

In 96-well plate, 75 µl of various concentrations of the extract (0.5-1000 µl/ml in methanol) were added followed by 150 µl of 0.2 mM methanolic solution of DPPH. After incubation for 30 min at room temperature, the absorbance was evaluated with a spectrophotometer at 515 nm against a blank (which contained the same amount of methanol and DPPH without the test compound). L-ascorbic acid (vitamins C) and tocopherol (vitamin E) were used as the antioxidant standard. The percentage of remaining DPPH ($DPPH_R$) was calculated as follows:

$$\%DPPH_R = [1 - [A_{(sample)}/A_{(blank)}]] \times 100$$

where

A(sample) was absorbance intensity of sample solution

A(blank) was absorbance intensity of blank solution

The percentage of remaining DPPH against the sample/standard concentration was plotted to obtain the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50% (EC_{50}). The study was performed in triplicates.

2.3 Preliminary study of the extract on cytotoxicity of skin cells

Cytotoxicity of the extract was study based on the viability of HaCaT (normal human keratinocyte cell line, Cell Line Service, DKFZ, Heidelberg, Germany) after 24 h of treating with 50 – 200 µg/ml of the extract. HaCaT cells (passage 18) were initially cultured in a 25-cm^2 flask with DMEM supplemented with 10% FBS and incubated at 37°C with a humid atmosphere containing 5% CO_2. The medium was changed every two days.

To determine the effect of the extract on cytotoxicity, the cell suspension was transferred from a 25- cm^2 flask into a 96-well plate (1 x 10^4 cells per well). The old medium was then replaced with 250 µl DMEM. After 24 h incubation, 50 – 200 µg/ml extract was exposed for 24 h. The control cells were cultured in DMEM medium without the extract. The viability of cells was determined by XTT assay

2.3.1 XTT assay

Sodium 3,3'-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}-bis (4-metoxy-6-nitro) benzene sulfonic acid hydrate (XTT) was used for cell proliferation assay. The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT to orange compounds of formazan. The dye formed is water-soluble and the dye intensity can be read at a given wavelength with a spectrophotometer. The greater number of active cells in the well means the greater activity of mitochondria enzymes and leads to the higher concentration of the dye formed, which can then be measured and quantitated.

Following incubation, 50 ul XTT labeling mixture was added to each well. The 96-well plate was further incubated for 4 h. The intensity of dye was measured with a spectrophotometer. The fraction of viable cells was calculated by subtracting the optical density fraction of treated cells from the untreated cells. Each study had a minimum of three measurements.

3. The study of lightening properties

3.1 Effects of extract on melanogenesis in B16-F1, mouse melanoma cell

3.1.1 Cells and treatments

This study was designed to primarily screen inhibitory action on melanin production of the extract using B16-F1 mouse melanoma cells (ATCC No. CRL-6323, American Type Culture Collection, Virginia, USA), a well-known model for determination of melanogenesis inhibitory activity (Donsing, et al., 2008; Siegrist and Eberle, 1986). B16-F1 cells were initially cultured in a 25-cm^2 flask with DMEM supplemented with 10% FBS incubated at 37°C with a humid atmosphere containing 5% CO_2. The medium was changed every two days and the number of passages was not exceed 6.

The cell suspension was transferred to a 24-well plate (1×10^5 cells per well). To determine the effect of the extract on melanin production in α-MSH-stimulated melanogenesis. The cells were tested with two different two protocol: (i) 1 nM α-MSH (Morandini, et al., 1998) was present for 48 h and at 24 h, 50 – 200 µg/ml of the extract was also added. (ii) 50 – 200 µg/ml extract was present throughout the 48 h and 1 nM α-MSH added after the first 24 h. In addition, 50 µg/ml kojic acid, a well-known lightening compound was also tested using the same protocols. Each experiment was performed in triplicate. Control cells were incubated with 1 nM α-MSH without extract.

3.1.2 Melanin content assay

The melanin content assay was performed as previously described with slight modifications (Donsing, et al., 2008; Mun, et al., 2004). The assay based on destroying the retractile cells leaving behind the melanin granules, which could then be quantified spectrophotometrically. The cells within the wells were trypsinized and washed twice with phosphate buffer saline (PBS), air-dried, dissolved

in 200 µl of 1 N NaOH, heated at 80°C for 1 h and then cooled. The melanin content was measured at 490 nm using a microplate reader (Spectra Count®; Perkin Elmer Inc., Boston, Massachusetts, USA). The content of melanin was calculated by comparing the averaged absorbance of the 3 wells with that of the control cells and expressed as percentage.

3.2 Melanogenesic activity of the extract in human primary cells

3.2.1 Isolation of cells

Co-culture of isolated keratinocytes and melanocytes were used as an in vitro model in this study. Keratinocytes and melanocytes were isolated from foreskin tissues coming from humans aged ≤3 years. Tissues were incubated in 10 mL of 5% dispase solution at 4°C for 24 h and the epidermal layer was then separated from the tissue using forceps. This epidermal layer was cut into small pieces (2x2mm) by using surgical blade and then incubated in 4 ml of 0.25% trypsin solution at 37°C and under 5% CO_2 for 20 min. 4 ml of DMEM medium with 10% FBS were used to stop trypsin reaction. The cell suspension containing both keratinocytes and melanocytes was centrifuged at 1,500 rpm for 5 min and cells maintained in serum-free medium with supplements under air/5% CO_2 and at 37°C.

3.2.2 Determination of tyrosinase inhibitory activity from keratinocyte/melanocyte co-cultures

To determine inhibition of tyrosinase, the modified method of Huang, et al., 2005 was used. A suspension of cells from the primary cultures was transferred from a 25- cm^2 flask into a 24-well plate (1 x 10^5 cells per well) and then incubated with the extract (50–500 µg/ml) or kojic acid (0.5-500 µg/ml) at 37°C under 5% CO_2 for 72 h. The cells were washed with ice-cold PBS and then lysed by adding 0.5 ml PBS pH 6.8 containing 1% triton X-100 with sonication. A cell-free supernatant was collected by centrifugation and tested for the tyrosinase activity. Ninety micro litters of each sample solution adjusted to equal protein value were added in a 96-well plate. After incubation at room temperature for 5 min, 10 µl substrate (10 mM L-DOPA) was added to each well. After a further 30 min of incubation, the optical densities of the L-DOPA oxidation product, dopachrome, produced by tyrosinase were measured at 475 nm with the Spectra Count microplate reader. The concentration of the extract giving 50% inhibition (IC_{50}) was determined

from plot of percent inhibition against log concentration of the extract or kojic acid using Prism (GraphPad, California, USA.). Percent inhibition was calculated by using the following equation:

$$\text{Percent inhibition} = [1 - [A_{(\text{treatment})}/A_{(\text{control})}]] \times 100$$

where

A(treatment) is absorbance intensity of extract-treated group

A(control) is absorbance intensity of untreated group

The study was performed in three batches of keratinocyte-melanocyte co-cultures isolated from one-skin tissues.

3.2.3 Intracellular calcium and PAR-2 activity

PAR-2 activity was assessed by the rise in intracellular calcium (Ca^{2+}) when released from the endoplasmic reticulum by the action of PAR-2 (Bohm, et al., 1996; Kaufmann, et al., 2011). Briefly, the co-cultured cells were collected by using EDTA-containing calcium-free isotonic PBS. Keratinocyte-melanocyte coculture cells were suspended in DMEM containing 10%FBS and 200 µl of this was added to black 96 well-plates. 0.25 mM sulfinpyrazone and 1 µM fluo-3 AM were then added. The mixture was incubated at room temperature for 20-25 min and the cells were then washed twice. Two hundred microliters of assay buffer (150 mM NaCl, 3 mM KCl, 1.5 mM CaCl2, 20 mM HEPES, 10 mM glucose and 0.25 mM sulfinpyrazone) (untreated group), assay buffer plus 10 µM trypsin (as a PAR-2 activator) or assay buffer plus 200 µg/ml extract were added and incubated for a further 60 min.

Fluorescence was excited at 485 nm and emission measured at 530 nm using a multimode detector (Beckman Coulter Inc., California, USA). The emission signal was adjusted to 100% using the untreated cells and intracellular free calcium expressed as a percentage assuming that this is linearly proportional to calcium at low concentrations (<500 nM). The study was performed in three batches of keratinocyte-melanocyte co-culture cells isolated from one-skin tissues.

3.3 Cell viability assay

Viability following treatment was determined by counting the number of trypan blue positive cells in each well under x100 microscope. The study was performed in triplicate to obtain average number of viable cells.

4. The study of anti-aging properties

4.1 Fibroblast isolation

Fibroblast is the major cell type of the dermis, the second layer of the skin, which functions in producing the viscoelastic components of that skin such as collagen and elastin. The explant technique was used in fibroblast isolation. The dermis layer of human excess surgery skin tissue (eyelid, breast, abdominal from woman age 29-65) was cut into small pieces by surgical blade. Four to five skin disks were then placed in a culture dish and subsequently incubated at 37°C with a humid atmosphere containing 5% CO_2 for 30 min. After incubation, the tissue disks could be well attached on the wall of the culture flask. The culture medium consisted of DMEM, 10% FBS and 1% of a stock penicillin/streptomycin solution were added to each flask. After incubation, the fibroblast cells were migrated from the original site. The obtained fibroblast cell was cultured in DMEM with supplement at air containing 5% CO_2 and temperature of 37°C.

4.2 Prevention of H_2O_2 induced fibroblast damage.

In skin aging, the reduction of viscoelastic components mostly cause by oxidative stress. Reactive oxygen species (ROS) cause peroxidation of the lipid, activation of the protease that destroys cells. Determination on H_2O_2 induced cell damage refers to the anti-oxidation activities of the extract on skin simulation.

A suspension of fibroblast cells from the primary cultures (passage 6) was transferred from a 175- cm^2 flask into a 24-well plate (1×10^5 cells per well) and then incubated at 37°C under 5% CO_2 for 24 h. After cultivation of cell, various concentrations (100-1000 µM/ml) of H_2O_2 were added to activate the oxidative stress in fibroblast (Tanaka, 2001). Then, cells were exposed to 200 µg/ml extract for 24 h. The control cells were cultured in DMEM medium without the extract. The viability of cells was determined by XTT assay. The study was performed in triplicates.

4.3 Study of cell response after UVA irradiation

UVA radiation has long been known to generate an oxidative stress in cells irradiated in culture (Tyrrell, 1991) and this has been linked to many studies that show that both endogenous antioxidant pathways and added antioxidants can protect against the damage that arises from exposure to high UVA doses. Endogenous antioxidant defense molecules such as glutathione are depleted in skin and skin cells by UVA radiation and this efflux may lead not only to apoptosis in human keratinocytes (He, et al., 2003) but also to the induction of the interstitial collagenase, metalloproteinase-1 (MMP-1) in cultured skin fibroblasts (Yin, 2001). This study was investigated the effects of tamarind seed coat extract on skin fibroblast after UVA irradiation in various factors.

4.3.1 Cell treatment after UVA-irradiation

Honle F-lamps equipped with a H-1 filter (Honle, Germany) to eliminated emission spectrum between 320 and 400 nm were used as a UVA source. UVA strength was measured using a Honle UV meter with UVA probe. A suspension of fibroblast cells (passage not exceed 8) from the primary cultures was transferred from a 175- cm^2 flask into a 12-well plate (2×10^5 cells per well) and then incubated at 37°C under 5% CO_2. Before irradiation, culture medium of fibroblast at subconfluence (80-90% confluence) was replaced with PBS. Cells were divided into 3 groups: control group, UVA irradiation (5-40 J/cm^2) untreated group and UVA irradiation treated group. Six hours after irradiation, PBS was replaced with DMEM medium with/without extract. At the time of study period, cells and supernatant were harvest for the assay as follow:

4.3.2 Cell viability

Cell viability of 3 test groups as mention in 4.3.1 was assessed 24, 48 and 72 h after exposure UVA radiation using XTT assay.

4.3.3 Cell cycle analysis

After the 72 h of cultivation, the proportions of cells in each of the cell cycle (G1, S and G2) were determined by staining the cell DNA with propidium iodide (PI). Briefly, cells were detached by trypsinization and wash twice with phosphate buffer saline containing 2 mM disodium ethylenediamine tetracetic acid (Na EDTA). The cells were fixed overnight with cold absolute ethanol and then

stained with solution containing 5 µl of 0.1 mg/ml PI, 1 µl of 1 mg/ml RNAse and 49 µl of 2 mM Na EDTA in PBS. After 20 min incubation at room temperature in the dark, fluorescent cells were sorted in a Flow Cytometry System equipped with a 488-nm argon laser. The data were analyzed on the CellQuestPro software.

4.3.4 Total glutathione assay

Determination of total glutathione refers to the presence of free radical scavenging enzyme in fibroblast after UVA irradiated and whether tamarind seed coat extract could induce the activity of glutathione. This assay was performed using commercial total glutathione quantification kit. The assay base on the principle that shown in Figure 17. DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)), known as Ellman's Reagent, was developed for the detection of thiol compounds. The glutathione recycling system by DTNB and glutathione reductase created a highly sensitive glutathione detection method. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5- thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the measurement at 412 nm absorbance. GSH is generated from GSSG by glutathione reductase, and reacts with DTNB again to produce 2-nitro-5-thiobenzoic acid. Therefore, this recycling reaction improves the sensitivity of total glutathione detection.

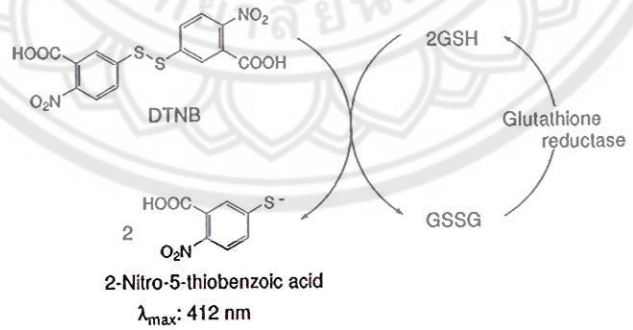

Figure 17 Principle of total glutathione quantification

After 6, 24, 48 and 72 h of cultivation, cells were collected and lysis with T-PER buffer for 20 min. Supernatant after centrifugation at 5000 RPM/ 5 min were collected. For total glutathione assay, each selected well in 96-well plate was added 20 µl of enzyme working solution, 140 µl of coenzyme working solution and 20

µl of either of one of the GSH standard solutions or the sample solution. Then incubate the plate at 37°C for 10 min. Add 20 µl of DTNB solution, and incubate the plate at 37°C for 20-40 min. Detected absorbance at 405 nm or 415 nm using a microplate reader and determined concentrations of GSH in sample solutions using a calibration curve.

4.3.5 The study of MMP-1 inhibitors

Matrix metalloproteinase 1 (MMP-1) or collagenases, belong to a large family of proteases, which function in collagen degradation resulting in skin aging. The less MMP-1 content in fibroblast after treated with the extract than control cells could indicate the potential of the extract in inhibiting dermal matrix breakdown.

1) Quantitative by enzyme immunoassay

Enzyme-linked immunosorbent assay was study for the quantitative measurement of human MMP-1 pro and active forms in fibroblast cell culture supernatants. Retinoic acid (50 ng/ml), well-known MMP-1 inhibitor was used as reference. After UVA irradiation and incubation with the extract for 18, 48 and 72 h, the cell-free supernatant were collected, stored at -80 °C and then assayed later. The amount of MMP-1 was measure by using a commercial human MMP-1 EIA kit. This assay employs an antibody specific for human MMP-1 coated on a 96-well plate. Briefly, standards and samples were pipetted into the wells and MMP-1 presented in a sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human MMP-1 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. The wells were again washed, a TMB substrate solution was added to the wells and color develops in proportion to the amount of MMP-1 bound. The stop solution changes the color from blue to yellow, and the intensity of the color was measured at 450 nm using microplate reader.

2) Qualitative by immunofluorescence assay

To confirm the results of enzyme immunoassay, the immunofluorescence assay was performed to detect the location and relative abundance of MMP-1 in fibroblast cells. All procedure in this assay was performed at room temperature. Briefly, after collected supernatant for enzyme immunoassay, fix cells by added 300-400 µl of 4% formaldehyde fixative solution to each well, and

incubated for 20 min. Wash the wells twice with PBS and cover with 400 µL of wash buffer (Tris buffer saline (TBS) with 0.025% Triton X-100). Block non-specific staining by added 400 µL of blocking buffer (TBS with 10% bovine serum albunine (BSA)) and incubated for 30 min. After removed blocking buffer, the unconjugated primary MMP-1 antibody in ratio 1:200 dilution buffer (TBS with 1%BSA) was added and incubated for 2 h, then wash two times with wash buffer. Stained with the secondary antibody, fluorescein (FITC) in ratio 1:250 dilution buffer and incubated for 1 h in the dark. Then, counterstained by added 1:500 4',6-diamidino-2-phenylindole (DAPI) solution to each well, and incubated 5 min. Rinsed twice with PBS.

Visualized using a fluorescence microscope and filter sets appropriate for the label used. FITC is excited by a blue (488nm) light, it will emits green color (520 nm). DAPI counterstain can obscure visualization of targets localized in cell nuclei. It has an absorption maximum at 358 nm and emits blue color (461 nm).

4.3.6 The study of type-I procollagen stimulators

Collagen is the major viscoelasticity component of the skin. To improve the anti-aging properties of the extract, the determination of collagen content were performed.

1) Quantitative by enzyme immunoassay

In this study, the procollagen assay was used an antibody against the C-terminal propeptide region that is part of the transcribed collagen protein, which is then proteolytically cleaved after secretion. Thus, this assay is a measure of newly synthesized collagen. L-ascobic acid (100 µg/ml), a well-known collagen promotor was used as positive control. After UVA irradiation and incubation with the extract for 18, 48 and 72 h, the cell-free supernatant were collected, stored at -80 °C and then assayed later. The amount of type I procollagen was measure by using a commercial human procollagen type-I C-peptide EIA kit. The levels of type-I procollagen were normalized against a standard dose-response curve based on the absorption at the wavelength of 450 nm using a microplate reader. The determinations were performed in triplicate.

2) Qualitative by immunofluorescence assay

The same procedure as MMP-1 was performed for type-1 procollagen immunofluorescence assay while using the unconjugated primary type-1

procollagen antibody and the secondary antibody was Cyanine Dyes (Cy5), which excited by a red (650 nm) light and emits red color (670 nm).

4.4 Effect of the extract on the contraction of fibroblasts-embedded collagen lattice

4.4.1 Fibroblast-embedded collagen lattice preparation

Three-dimensional collagen lattice was prepared according to the previous studies (Jouandeaud, et al., 2004, Vinnet, et al., 2004) with modification. Fibroblasts from nonwrinkled and old (wrinkled) skin collected from the same explants as mentioned in 4.1 were used to prepare the fibroblast-embedded collagen lattice. Briefly, the disk-shaped matrix was prepared in 60 mm Petri dishes consisted of 0.45 ml of 10X DMEM, 1.5 ml of 0.6% rat tail type I collagen, 2 ml of dH_2O, 0.25 ml of 0.1 N NaOH, 0.3 ml of 5% $NaHCO_3$ and 0.5 ml of cell suspension ($8x10^5$ cells/ml). Matrices were maintained in DMEM medium with 200 µg/ml of the extract (treated group) or without the extract (control group) and then placed in a 37°C incubator in a humid atmosphere containing 5% CO_2, 20 µg/ml catechin was used as standerd. Matrix disks were prepared in triplicate for each group and experiments were performed in duplicate.

4.4.2 Contraction capacity determination

The contraction capacity of fibroblasts-embedded lattice was visually determined from lattice diameter. To measure matrix diameters, they were placed on a scanner with a scale paper on background. The matrix diameters were measured every day within 7 days culture period.

5. Statistical analysis

All quantitative data were expressed as means of samples for each treatment. Student's unpaired t-test was used for comparison between two groups. $p<0.05$ was considered significant.