CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemical	Company
2-aminoanthracene	Sigma-Aldrich, USA
Ascorbic acid	Mreck, Germany
Dipotassium hydrogen phosphate	Fluka, Switzerland
Bacto agar	Difco, USA
Biotin	Sigma-Aldrich, USA
Citric acid monohydrate	Sigma-Aldrich, USA
3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
Disodium hydrogen phosphate	Carlo Erbo, Italy
Ethanol 95%	BDH, England
Ethylene diaminetetraacetic acid	Sigma-Aldrich, USA
Fetal bovine serum	Gibco, England
2-(2-furyl)-3-5-nitro-2-furyl) acrylamide	Sigma-Aldrich, USA
Glucose	Sigma-Aldrich, USA
D-glucose-6-phosphate	Sigma-Aldrich, USA

Chemical	**Company**
Histidine	Sigma-Aldrich, USA
5-hydroxy-2-(hydroxymethyl)-4-pyrone	Sigma-Aldrich, USA
L-3,4-dihydroxyphenylalanine	Sigma-Aldrich, USA
Magnesium sulphate heptahydrate	Sigma-Aldrich, USA
Nicotinamide adenine dinucleotide (NADH)	Sigma-Aldrich, USA
Nicotinamide adenine dinucleotide phosphate (NAPDH)	Sigma-Aldrich, USA
Oxoid nutrient broth	Criterion, USA
Penicillin	Gibco, England
Potassium chloride	Carlo Erbo, Italy
Potassium dihydrogen phosphate	BDH, UK
RPMI1640 medium	Gibco, England
Sodium chloride	Carlo Erbo, Italy
Sodium dihydrogen phosphate	Carlo Erbo, Italy
Sodium hydroxide	Carlo Erbo, Italy
Streptomycin	Gibco, England
Trypsin	Gibco, England
Tryphan blue	Sigma-Aldrich, USA
Tyrosinase	Sigma-Aldrich, USA

2.1.2 Instruments

Instruments	Company
Analytically balance Model PIONEER PA64	Ohaus corporation, USA
Autoclave Model MLS-3780	Sanyo, Japan
Centrifuge Model1720	Kubota, Japan
Centrifuge tube	Vivantis, USA
Counting chamber	Hausser scientific, USA
Electronically balance Model PB 1502-S	Mettler Toledo, Switzerland
Freezer	Whirlpool, USA
Incubator shaker	New brunswick scientific, USA
Infrared spectroscopy Model Spectum Gx Series	PerkinElmer, USA
Laminar flow Model Telstar Bio-II-A	Telstar, Spain
Microcentrifuge	Eppendrof, Canada
Microcentrifuge tube	Molecular bio product, USA
Microfilter sterile (0.45 μm)	Whatman, USA
Microplate reader	Biotek, USA
Micropipette	Gilson, France
Microscope	Olympus optical, Japan
Nuclear magnetic resonance Model AV400	Bruker, Germany
Oven Model ULE 400	Memmert, Germany

Instruments	Company
pH meter Model 744 pH meter	Metrohm, Switzerland
Pipette tips	Molecular Bio product, USA
Spectrophotometer Model 20 Genesys	Spectronic instrument, USA
Ultraviolet–visible spectroscopy Lamda 25	Perkin Elmer, USA

2.1.3 Microorganisms

Salmonella typhimurium strains TA98 and TA100 were used to test mutagenicity and antimutagenicity of the plant extracts. Both bacterial strains were kindly provided by Prof. Dr. Usanee Viniketkummuen, Department of Biochemistry, Faculty of Medicine, Chiang Mai University. The genotypes of bacterial strains were shown in Table 2.1. Both *Salmonella* strains which were used for mutagenic screening had different mutations in various genes of histidine operon (Mortelmans and Zeiger *et. al.*, 2000). Bacterial strain TA98 was mutated in *hisD3052* that caused base-pair substitution mutations in a nitrogen base of triplet codon in histidine gene. For example, a leucine (GAG/CTC) was substituted by proline (GGG/CCC) in histidine gene. The deletion or insertion of *hisG46* in bacterial strain TA100 has caused frameshifts mutations of nucleotide sequences. In addition, the bacterial tester strains (gram-nagative bacteria) were developed the genetic to sensitive for chemical mutagens. For example, *uvrB* deletion caused the defect of DNA repair pathway. *Rfa* mutation leaded to defective lipolysaccahaide (LPS) layer that coated of bacterial cell

membrane and regulated permeable chemicals. The insertion of plasmid pKM101 leaded to increasing of sensitive chemical and mutagenesis.

Table 2.1 Genotypes of the mutant bacterial *S. typhimurium* strains TA98 and TA100 (Mortelmans and Zeiger *et. al.*, 2000).

Strains	**Mutation marker**	***bio chlD uvrB gal***	**LPS Defect**	**Plasmid**	**DNA target**	**Reversion event**
TA98	*hisD3052*	Deletion mutation	*Rfa*	pKM101	-C-G-C-G-C-G-C-G-	Base-pair substitution
TA100	*hisG46*	Deletion mutation	*Rfa*	pKM101	-G-G-G-G-	Frameshifts

2.1.4 Cell cultures

In this study, normal mouse fibroblast L929 and mouse melanoma B16F10 cell lines were purchased from American Type Culture Collection (ATCC), USA which were partially supported by Assoc. Prof. Somboon Anuntalabhochai, Department of Biology, Faculty of Science, Chiang Mai University. Both cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml).

2.1.5 Plant Extracts

In this study, five kinds of plants were obtained from S&J International Enterprise Pubic Company Limited. There were *Terminalia chebula* Retzius, *Terminalia bellerica*, *Etlingera elatior* (Jack) R.M. Smith, *Rosa damascena* and *Rafflesia kerrii* Meijer.

2.2 Methods

2.2.1 Preparation of the plant extracts

The plant extracts were prepared and supplied by S&J International Enterprise Pubic Company Limited. Briefly, the crude extracts of *E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer were extracted from dry flowers by maceration in 50% (v/v) hydroglycol. Whereas, *T. chebula* Retz. and *T. bellerica* extracts were prepared from the dry fruits by maceration in 70% (v/v) hydroglycol as shown in the Table 2.2. After three days of maceration, the extracts were filtered though Whatman filter paper No.1. The filtrates were kept at 4 °C until used.

Table 2.2 List of plants, parts and solvent used for the study.

Species	Code	Extracted Part	Extracted solvent
Terminalia chebula Retzius	MB	fruit	70% hydroglycol
Terminalia bellerica	BM	fruit	70% hydroglycol
Etlingera elatior (Jack) R.M. Smith	EE	flower	50% hydroglycol
Rosa damascena	DR	flower	50% hydroglycol
Rafflesia kerrii Meijer	RM	flower	50% hydroglycol

2.2.2 Characterization of plant extracts

The physical character of the extracts were noted and recorded including color and precipitation of the plant extracts. Then, the plant extracts were characterized using Infrared spectroscopy (IR), Nuclear magnetic resonance (NMR) and Ultraviolet-visible spectrophotometry (UV-visible).

2.2.3 Determination of total phenolic content by using Folin-Ciocalteu method

The total phenolic contents (TPCs) of the five plant extracts were determined by Folin-Ciocalteu method (Chan *et al.,* 2008). In this experiment, the total phenolic contents were estimated as gallic acid equivalents. The mixtures contained 0.1 ml of the plant extracts at different concentrations (between 0.1 and 5.0 mg/ml) were added 4 ml of water and 0.5 ml of 2 N Folin-Ciocalteu reagent. The mixtures were pre-incubated at room temperature for 5 min before adding 0.4 ml of 20% (w/v) sodium carbonate. Then, the mixtures were incubated at room temperature for 30 min. The absorbance at 765 nm was measured with UV–visible spectrophotometer model Lamda 25. The experiments were done in triplicates. Gallic acid was used as standard for the calibration curve and the total phenolic contents were expressed as mg of gallic acid equivalents per mg of the extract.

2.2.4 Cytotoxicity assay

2.2.4.1 Cytotoxicity of the plant extracts in mouse fibroblast cell lines by MTT assay

In this study, MTT assay was performed to investigate the cytotoxic effect of the plant extracts by using normal mouse fibroblast L929 and mouse melanoma

B16F10 cell lines. MTT (3–(4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was used as a standard colorimetric assay for measuring cellular proliferation or cell growth (Mosmann *et al.,* 1983). Cell culture facility and MTT assay were kindly provided by Dr. Nisa Chawapun, Department of Radiology, Faculty of Medicine and Dr. Angkana Saovapakhiran, Department of Chemistry, Faculty of Science, Chiang Mai University. Both cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium in a T-25 cm^2 flask until cells were grown about 60-70% confluent. The cells were trypsinized and evaluated in a hemocytometer chamber under a phase contrast optical microscope with tryphan blue staining. Next, the fibroblast cell lines were seeded in 96-well plate at a concentration of 1 x 10^5 cells/ml and cultured at 37 °C in an atmosphere of 5% CO_2 for 48 h. Then, the medium was removed from the plate and washed with 100 µl of 0.1 mM phosphate buffer saline (PBS), pH 7.4. Next, RPMI 1640 medium containing the plant extracts at different concentrations between 0.008 - 87.340 mg/ml were added to the cells and incubated at 37 °C in an atmosphere of 5% CO_2 for 48 h. Twenty microlites of MTT solution was added and the cells were incubated for 4 h at 37 °C in an atmosphere of 5% CO_2. Then, the mixture was removed from the plate and the formazan crystal was solubilized with 100 µl of dimethyl sulfoxide (DMSO). The absorbance at 550 nm and 620 nm were measured by a microplate reader. The 50% cytotoxic dose (CD_{50}) or the concentrations of the extracts at 50% of the viable cells after incubated with *T. chebula* Retz., *T. bellerica*, *E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer were calculated and compared with the untreated control group. The experiments were done in triplicates.

2.2.4.2 Cytotoxicity of the plant extracts in mouse fibroblast cell lines by Dye exclusion method

Dye exclusion was used to determine cell viability by measuring the number of viable cells which presented in a cell suspension and binded to chemical dye. The cytotoxicity of plant extracts were performed in normal mouse fibroblast L929 and mouse melanoma B16F10 cell lines by dye exclusion method. Firstly, the cell lines were seeded at 1 x 10^5 cells/well in 24-well plates and incubated at 37 °C under an atmosphere of 5% CO_2 for 48 h. Next, the medium was removed from the plate and washed by adding 500 µl of 0.1 mM phosphate buffer saline, pH 7.4. Then, RPMI 1640 medium containing the extracts at different concentrations were added to the cell lines and incubated for 24 h. After that, the medium was removed and cell lines were trypsinized. Finally, the cell lines were stained with trypan blue and the number of cells viability were counted under the phase contrast microscope. The percentage of cells viability was calculated. The experiments were done in duplicates.

2.2.5 Genotoxicity assay

The plant extracts were also investigated for mutagenic properties by Ames test (Moron and Ames *et al.*, 1983). Ames test was performed using histidine-dependent strains of *S. typhimurium* TA98 for detection of base-pair substitution mutations of *hisD3052* and *S. typhimurium* TA100 for detection of frameshift mutations of *hisG46*. Both bacterial strains were modified histidine genes which were unable to synthesize histidine for growth and forming colonies. The mutagenicity test was referred to a “back mutation” which could occur by adding mutagens to resotore the synthesis of histidine in these bacteria. (Mortelmans and Zeiger *et al.*, 2000).

In this study, the mutagenicity assay were performed by using Ames test to confirm the safety for cosmetic application of the plant extracts. The Ames test without or with metabolic pathway activation by adding mouse liver microsome S9 (S9 microsomal fraction) were performed. The *S. typhimurium* strains TA98 and TA100 were cultured in 10 ml of oxoid nutrient broth No. 2. The culture was incubated at 37 °C and shaken at 120 rpm for 14 h until the bacteria were contained cells about $2x10^9$ cells/ml. The assay was performed using pre-incubation technique. The mixture consisted of 50 µl of the plant extracts at different concentrations (between 0.088 and 87.43 mg/ml), 100 µl of bacterial culture (approximately 1-2 $x10^9$cells/tube), was added either 500 µl of S9 mix for metabolic pathway activation or 0.2 M sodium phosphate buffer, pH 7.4 for non metabolic pathway activation. Then, the mixture was pre-incubated at 30 °C in a shaker water bath for 30 min. After pre-incubation, the mixture was added to 2 ml of molten top agar which contained 0.6% (w/v) agar, 0.5% (w/v) NaCl and 0.5 mM histidine-biotin and then poured onto the surface of minimal glucose agar plate. The plates were inverted and incubated at 37 °C for 48 h. The numbers of revertant colonies were counted and compared with 50% hydroglycol, 70% hydroglycol and DMSO which were used as negative controls. 2-aminoanthracene (2-AA) and 2-(2-furyl)-3-5-nitro-2-furyl) acrylamide (AF-2) were used as positive controls for metabolic pathway and non metabolic pathway activation, respectively. The experiments were performed in duplicates and two plates were used for the calculation of the average of revertant colonies.

2.2.6 Antimutagenicity assay

The plant extracts were also investigated for their antimutagenicity at different concentrations. The antimutagenicity was done by modified Ames test. *S. typhimurium* strains TA98 and TA100 were cultured in 10 ml of oxoid nutrient broth No. 2. The culture was incubated at 37 °C and shaken at 120 rpm for 14 h until the bacteria was grown about $2x10^9$ cells/ml. For metabolic pathway activation, the reaction mixture consisted of 50 µl of 2-AA mutagen, 50 µl of the plant extracts at different concentrations (between 10 and 80 mg/ml), 500 µl of S9 mix and 100 µl of bacterial culture. For non metabolic pathway activation, the reaction mixture consisted of 50 µl of AF-2, 50 µl of the plant extracts at different concentrations, 500 µl of 0.2 M sodium phosphate buffer, pH 7.4 and 100 µl of bacterial culture. The mixture was pre-incubated at 30 °C in a shaker water bath for 30 min. Then, the mixture was added into 2 ml of molten top agar and poured onto the surface of minimal glucose agar plate. The plates were inverted and incubated at 37 °C for 48 h. The numbers of revertant colonies were counted and compared with 50% hydroglycol, 70% hydroglycol and DMSO which were used as negative controls. 2-AA and AF-2 were used as positive controls for metabolic pathway and non metabolic pathway activation, respectively. The experiments were performed in duplicates. The number of revertants were used to calculate the % inhibition of mutagenesis as shown in this equation.

$$\% \text{ inhibition of mutagenesis} = 100 - \left[\frac{\text{No. of } his+ \text{ revertants from extract - spontaneous reversion (solvent)}}{\text{No. of } his+ \text{ revertants from mutagen - spontaneous reversion (DMSO)}} \times 100 \right]$$

2.2.7 Investigation of antityrosinase activities in plant extracts (Chan *et al.*, 2008)

The plant extracts were determined for antityrosinase activity by the dopachrome method. In this experiment, L-3,4-dihydroxyphenylalanine (L-Dopa) was used as a substrate. The mixtures were contained the plant extracts at different concentrations (between 0.1 and 80.0 mg/ml), 0.1 mM potassium phosphate buffer, pH 6.8 and 0.06 mg/ml of mushroom tyrosinase solution. The mixture was pre-incubated at 37 °C for 5 min before adding 2.5 mM L-Dopa and then incubated at 37 °C for another 5 min. The absorbance at 475 nm was measured for tyrosinase activity by UV–visible spectrophotometer. Ascorbic acid and Kojic acid were used as positive controls for tyrosinase inhibitors. 50% and 70% hydroglycol were used as negative controls. The experiments were done in triplicates. From these results, % inhibition of tyrosinase were calculated and reported with the 50% inhibitory concentration (IC_{50}) or the concentration of the extract inhibited tyrosinase activity at 50% after incubated with *T. chebula* Retz., *T. bellerica*, *E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer extract. The percentage inhibitions of tyrosinase were calculated with the following formula:

$$\% \text{ Tyrosinase inhibition} = \left[\frac{(A\text{-}B) - (C\text{-}D)}{(A\text{-}B)}\right] \times 100$$

A = the absorbance of the control (L-Dopa mixed with tyrosinase in buffer)

B = the absorbance of the blank (L-Dopa in buffer)

C = the absorbance of the reaction mixture

D = the absorbance of the blank of C (L-Dopa mixed with test sample without adding tyrosinase in buffer)

2.2.8 Determination of UVA induced morphological changes (Heo *et al.*, 2010)

The morphological changes of cell lines after induced by UVA radiation were determined under a microscope. The normal mouse fibroblast L929 and mouse melanoma fibroblast B16F10 cell lines were used in this experiment. The cell lines were cultured at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 medium in T-25 cm^2 flask until cells were grown about 60-70% confluent. The cells were trypsinized and evaluated in a hemocytometer chamber under a phase contrast optical microscope with tryphan blue staining. Then, the cell lines were seeded at a 1 x 10^5 cells/well in 24-well plates and incubated at 37 ◦C in an atmosphere of 5% CO_2 for 48 h. Then, the medium was removed from plates and washed with 500 µl of 0.1 mM phosphate buffer saline, pH 7.4. The cell lines were exposed to UVA radiation with 0.3 J/cm^2 for 22 min 56 sec. Exposure time was calculated with the formula:

$$H_\lambda = t \cdot E_\lambda$$

H_λ = the energy level indicated as exposure per unit area (J/cm^2)

t = the exposure time (second)

E_λ = the irradiance (W/cm^2)

Then, RPMI 1640 medium containing the extracts at different concentrations were added to the UVA treated cells. The extracts concentrations in the range of $CD_{3.75}$, $CD_{6.25}$ and $CD_{12.5}$ which were calculated from the corresponding cytotoxicity assay were applied. The cells were incubated at 37 ◦C in an atmosphere of 5% CO_2 for 24 h. The morphological changes of cells after exposured to UVA were investigated under a light microscope with 10x magnification and photographed. The

solvent of each extract either 50% hydroglycol or 70% hydroglycol were used as negative controls for each experiment. Addition of extracts to the cell lines without exposure to UVA radiation were done as positive controls. The experiments were performed in triplicates.

2.2.9 Determination of UVB induced morphological changes

The morphological changes of normal mouse fibroblast L929 and mouse melanoma fibroblast B16F10 cell lines induced by UVB radiation were also investigated. The changes of cell morphology after induced by UVB radiation were done the same as procedure as described in Section 2.2.8. Both cell lines were exposed to UVB radiation with 30 mJ/cm^2 for 6 min 45 sec. The morphological changes of cells after exposured to UVB were investigated under a light microscope with 10x magnification and photographed. The solvent of each extract either 50% hydroglycol or 70% hydroglycol were used as negative controls for each experiment. The positive control experiment was carried out under the same conditions excepted that no radiation was used. The experiments were done in triplicates.