CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Chemicals

- Acrylamide (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Alpha-tocopherol (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Ascorbic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Calcium chloride ($CaCl_2$) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Carbopol® Ultrez 21 (Lubrizol advance material, Cleveland, OH, U.S.A)
- Coomassie® Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, U.S.A.)
- Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Dulbecco's modified eagle's medium (DMEM) (GIBCO, Grand Island, NY, U.S.A.)
- EDTA (ethylenediamine tetraacetic acid disodium salt dihydrate) (Fluka Co., Switzerland)
- Fetal bovine serum (FBS) (GIBCO, Grand Island, NY, U.S.A.)
- Ferrozine and ferric chloride ($FeCl_2$) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Gallic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Glycerol (Qingdao Baijie International trading Co., Ltd., China)
- Jojoba oil (Guangzhou Mingtai Trading Co.,Ltd, Guangzhou, China)
- Kojic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Methyl paraben (Parchem Fine & Specialty Chemicals, India)
- Mineral oil (Guangzhou Hanglian Chemical Industry Co., Ltd., China)
- Propyl paraben (Parchem Fine & Specialty Chemicals, India)
- Propylene glycol (Qingdao Baijie International trading Co., Ltd., China)
- Sodium azide (NaN_3) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Hercules, CA, U.S.A.)
- Sodium luaryl sulfate (SLS) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Sorbitol 70% solution (Hefei TNJ Chemical Industry Co., Ltd., China)
- TEMED (N,N,N',N'-tetramethyl ethylenediamine) (Fluka Chemicals, Gillingham, Dorset, UK)
- Tris (hydroxymethyl)-methlamine (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Trichloroacetic acid (Merck KGaA, Darmstadt, Germany)
- Triethanolamine (Sigma Chemical Co., St. Louis, MO, USA)
- Trifluoroacetic acid (TFA) (Merck KGaA, Darmstadt, Germany)
- Triton X-100 (BDH Chemicals Ltd., Poole, England)
- Trypsin (Gibco Invitrogen Corp., Carlsbad, CA, USA)
- Tween 20 (polyoxyethylene sorbitan monolaurate) (Jiangyin Huayuan Chemical Co., Ltd., China)
- Tyrosine (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Tyrosinase enzyme, mushroom (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- White petroleum jelly (Dalian Chem Imp. & Exp. Group Co., Ltd., China)

- Other solvents (analytical grade)

2.1.2 Cell lines

- Normal human skin fibroblasts (Department of Tropical Hygine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand)

2.1.3 Animals

- New Zealand white rabbits (Faculty of Agriculture, Chiang Mai University, Thailand)

2.1.4 Human volunteers

- 20 Thai volunteers (10 men, 10 women, averaged age 33.00 ± 6.76 years, range 25–56 years, averaged BMI 23.11 ± 3.73)

2.1.5 Equipments

- CO_2 incubator (Shel Lab, model 2123TC, U.S.A.) Corneometer® CM825 (Courage & Khazaka, Cologne, Germany)

- Cutometer® MPA580 (Courage & Khazaka, Cologne, Germany)6

- Gel documentation system and Quantity 1-D analysis software (Bio-Rad Laboratories, U.K.)

- High Perfomance Liquid Chromatography (HPLC, AS 1000, Thermo Finigan, U.S.A.)

- Larminar air flow cabinet (Cytair 125, Equipments Scientifiques & Industries S.A., France)

- Lyophilizer (Christ FOC-1 Model K-40 equipment, Balzers-Pfeiffer GmbH, Asslar, Germany)

- **Mexameter®** (Courage & Khazaka, Cologne, Germany)

- Microplate Reader (Model 550, BIORAD, U.S.A.)

- Polycarbonate membrane filter with a pore size of 50 nm (Millipore, Billerica, MA, U.S.A.)

- Rotary evaporator (R-124 Büchi, Switzerland)

- 96-Well microplate (Nalge Nunc International, NY, U.S.A.).

- Ultracentrifuge (Univeral 32 R, Hettich Zentrifugen, Germany)

- Visiometer® SV600 FW with CCD camera (Visioscan® VC98, Courage & Khazaka, Cologne, Germany)

2.2 Methods

The scope of the study was divided into 6 parts as the scheme in **Figure 36**

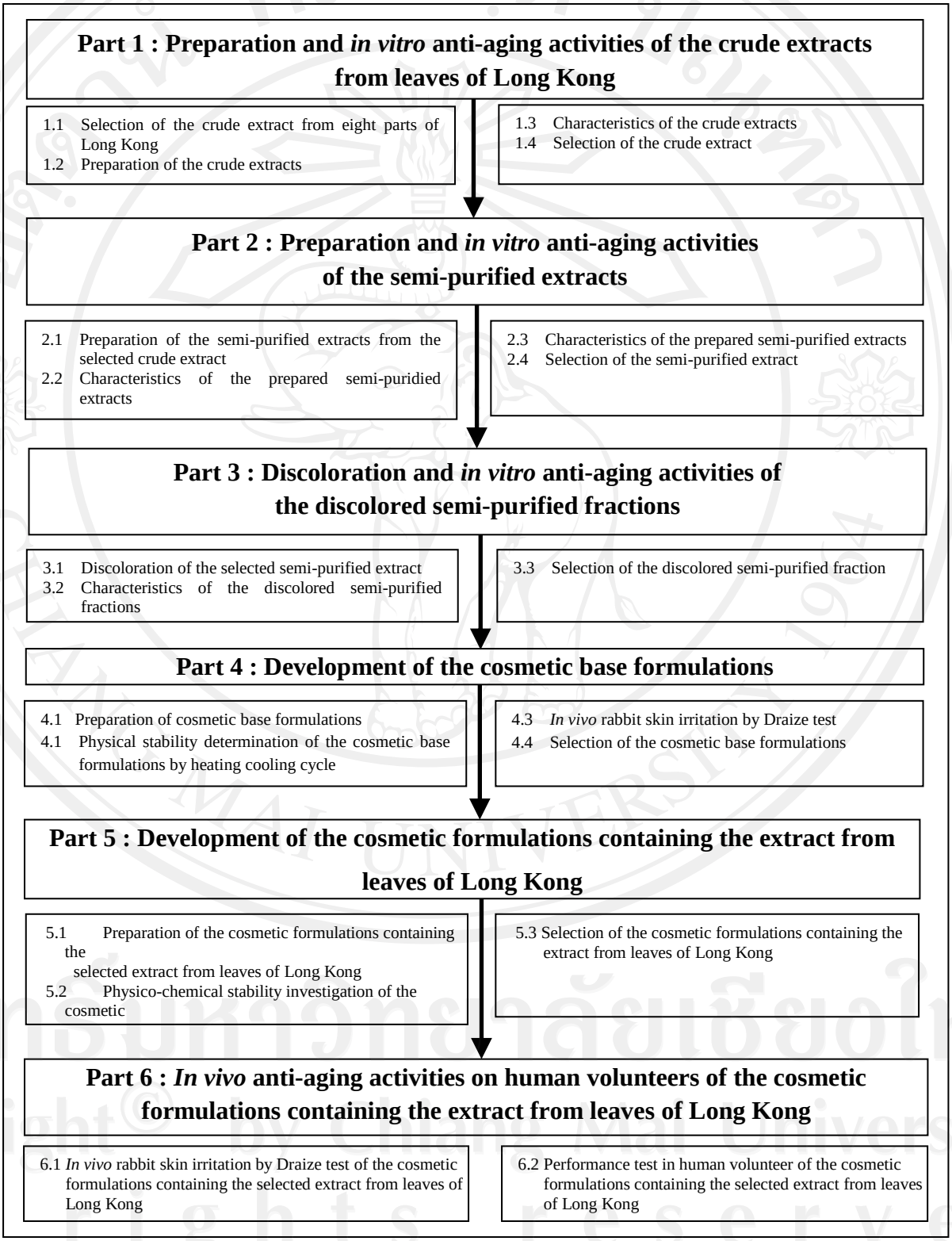

Figure 36 Scheme of the scope of the study

Part 1 : Preparation and *in vitro* anti-aging activities of the crude extracts from leaves of Long Kong

1.1 Selection of the crude extract from eight parts of Long Kong

Eight parts of *L. domesticum* which were ripening fruit, fruit peel, young fruit, stalk, seed, young leaf, old leaf and branch, were extracted with water, methanol and chloroform by the hot and cold processes. After that, 48 extracts were screened for *in vitro* antioxidant activity including DPPH radical scavenging, metal chelating and lipid peroxidation inhibition, tyrosinase inhbibition and cytotoxicity on normal human skin fibroblast under the project of "Development of cosmetic, food supplement and pharmaceutical products from Long Kong" with the financial support from Agricultural Research Development Agency (Public organization) (ARDA), Thailand. The old leaf was selected owning to its interesting antioxidant activities (DPPH radical scavenging, metal chelating and lipid peroxidation inhibition), tyrosinase inhibition activity and gave no cytotoxicity on normal human skin fibroblasts. In addition, the old leaf can be harvested all year. It can be also easily processed due to low moisture which is able to dry in short period of time. The old (3 months or over) leaves of *L. domesticum* were collected from Chantaburi province in the eastern part of Thailand during October to November in 2010. The specimen was authenticated by a botanist at the Natural Products Research and Development Center (NPRDC), Science and Technology Research Institute (STRI), Chiang Mai University in Thailand. The percentage yield of the crude extract was calculated according to the following equation:

$$\text{Percentage Yield (\%)} = \frac{\text{Dry extract weigh (g)}}{\text{Plant powder weigh (g)}} \times 100$$

1.2 Preparation of the crude extracts

The old leaves of *L. domesticum* were washed, dried at 50 ± 2ºC, ground into powder and extracted by the hot and cold processes using 3 different solvents (water, methanol and chloroform). For the hot process, the dried powder was boiled with the solvent at its boiling points [water (100 ± 2ºC), methanol (65 ± 2ºC) and chloroform (60 ± 2ºC)] for 1 h. For the cold process, the dried powder was mixed with the solvents and sonicated in a sonicator bath (Chest ultrasonics corp., NJ, USA) at ambient temperature (30 ± 2ºC) for 1 h. Each extraction was repeated for three times. The mixture from the hot and cold methods was filtered, pooled and dried by a rotary evaporator (Rotavapor R210, Buchi, Flawil, Switzerland) under vacuum at 50ºC and lyophilized. Six extracts were obtained and the percentages yields were calculated on a dry weight basis. The obtained crude extracts were kept at 4 ± 2ºC in a tight container prior to use.

1.3 Characteristics of the crude extracts

1.3.1 Phytochemical test of the crude extracts

Phytochemical constituents in the crude extracts were determined according to the methods previously described (Manosroi et al., 2010). Briefly, the sample (20 mg), dissolved in 20 ml of 80% methanol, was used for detecting the presence of alkaloids, flavonoids, glycosides, saponins, tannins, carotenoids and terpenoids. For alkaloids (Egwaikhide et al., 2007), an amount of 2 ml of the sample solution mixed with 1 ml of 1%HCl was boiled over a water bath and 6 drops of the Dragendorff's reagent were added. The orange precipitate indicated the presence of alkaloids. Quinine sulfate was used as a positive control. For anthraquinone determination (Egwaikhide et al., 2007), 0.5 g of the sample was boiled with 2 ml of

10%HCl for a few minutes in a water bath. It was filtered and allowed to cooled down. Chloroform was added to the filtrated. Few drops of 10% NH_4OH were added to the mixture and heat. Formation of rose-pink color indicated the presence of anthraquinone. The *Senna alata* L. extract (from NPRDC, STRI, Chiang Mai University) was used as positive control for anthraquinones. For the presence of flavonoid (Shinoda test) (Nobakht et al., 2010), 2 ml of the sample solution mixed with 1 ml of the concentrated HCl and magnesium ribbon gave the pink tomato-red color. Luteolin was used as a positive control. The qualitative assay of reducing sugars was performed by TLC method (Sherma, 2000). The extracts dissolving in water were spotted on the silica gel plate compared to standard reducing sugars including glucose, fructose and sucrose. The mobile phase was butanol/acetic acid/diethyl ether/water (9:6:1:3) and 10%H_2SO_4 was used as spraying reagent. After heat, the presence of brown spots which matching position to the positive controls indicated to each reducting sugar existing in the plant extracts. For saponin (Frothing test) (Abba et al., 2009), 0.5 ml of the sample solution was mixed with 5 ml of distilled water. The frothing persistence at 1 cm layer for 15 min indicated the presence of saponins. The *Saponaria officinalis* extract (from NPRDC, STRI, Chiang Mai University) was used as a positive control. For tannins (Egwaikhide et al., 2007), 2 ml of the sample solution were mixed with 2 ml of 15% $FeCl_3$ solution. The blue-black precipitate indicated the presence of tannins. Tannic acid was used as a positive control. For carotenoid (Ajayi et al., 2011), each sample was extracted with chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 0.1 ml of H_2SO_4 was added. A blue color at the interface showed the presence of carotenoids. The carrot extract (from NPRDC, STRI, Chiang Mai University) was

used as a positive control. For triterpenoid and steroid detections (Touchstone et al., 1978), it was performed using a TLC plate. The sample solution was spotted on a silica gel plate. The mixture of hexane/ethyl acetate (9:1) was used as a mobile phase and the plate was sprayed with the 4% vanillin/sulfuric acid reagent. The formation of the blue color spots indicated the presence of triterpenoid, while the violet color spots meant the presence of steroid. Limonene was used as a positive control for triterpenoid and stigmasterol was used as positive control for steroid.

1.3.2 Determination of the total phenolic and flavonoid contents of the crude extracts (Ardestani et al., 2007; Tepe et al., 2007)

The total phenolic contents of the crude extracts were determined by the modified Folin-Ciocalteu assay (Tepe et al., 2007). Briefly, 5 µl of each sample dissolved in DMSO at the concentraction of 10 mg/ml were mixed with 50 µl of Folin-Ciocalteu reagent, and adjusted to the total volume of 200 µl with distilled water. After vigorous shaking in dark at room temperature for 2 h, 25 µl of 2% (v/v) Na_2CO_3 were added and mixed. The absorbance was measured at 760 nm by a well reader. The total phenolic contents were quantified by the calibration standard curve obtained from various known concentrations of gallic acid [absorbance = 9.7836 gallic acid (Kumguan et al.) + 0.3394; R^2=0.9859]. The concentrations were expressed as mg of Gallic Acid Equivalents (GAE)/g of the dry crude extracts or semi-purified fractions. All measurements were done in triplicate.

The total flavonoid contents of each crude extract and semi-purified fraction were determined by a colorimetric method as described previously with some modifications (Ardestani et al., 2007). Briefly, 20 µl of the appropriate diluted sample (20 mg/ml dissolved in MeOH) were mixed with 80 µl of distilled water and

followed by the immediate addition of 6 µl of 15% (w/v) $NaNO_2$ and 6 µl of 10% (w/v) $AlCl_3$. After 5 min, 80 µl of 4% (w/v) NaOH were added. After 1 min, 8 µl of water were immediately added to adjust the final volume to 200 µl. Then, the mixture was thoroughly mixed with shaking for 15 min at room temperature (27ºC). The absorbance of the mixture was determined at 510 nm by a well reader. The total flavonoid content of the samples was expressed as mg of Quercetin Equivalents (QE)/g of the dry crude extract or semi-purified fraction and calculated by the linear equation based on the calibration curve [absorbance = 0.1968 quercetin (Kumguan et al.) + 0.405; R^2=0.9464]. All measurements were done in triplicate.

1.3.*3* *In vitro* anti-aging activities of the crude extracts determination

1.3.3.1 DPPH free radical scavenging activity assay (Tachibana et al., 2001)

Free radical scavenging activity of the crude extracts were determined by the modified DPPH assay (Tachibana et al., 2001). Briefly, 50 µl of five serial concentrations of the samples (0.001–10 mg/ml) dissolved in DMSO, and 50 µl of DPPH in ethanol solution were put into each well of a 96-well microplate (Nalge Nunc International, NY). The reaction mixtures were allowed to stand for 30 min at room temperature (30 ± 2ºC), and the absorbance was measured at 515 nm by a well reader (Model 680 microplate reader, Bio-Rad Laboratories Ltd., Corston, UK) against the negative control (DMSO). Ascorbic acid (0.001–10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of free radical scavenging activity were calculated according to the following equation:

$$\text{Scavenging (\%)} = [(A-B)/A] \times 100$$

Where *A* was the absorbance of the negative control and *B* was the absorbance of the sample. The sample concentration providing 50% of scavenging (SC_{50}) activity was calculated from the graph plotted between the percentages of the scavenging activity and the sample concentrations.

1.3.3.2 Lipid peroxidation inhibition assay (Osawa et al., 1981)

The lipid peroxidation inhibition of the crude extracts and were assayed by the modified ferric-thiocyanate method (Osawa et al., 1981). An amount of 50 µl of five serial concentrations of the samples (0.001–10 mg/ml) dissolved in DMSO was added to 50 µl of linoleic acid in 50% (v/v) DMSO. The reaction was initiated by the addition of 50 µl of NH_4SCN (5mM) and 50 µl of $FeCl_2$ (2mM). The mixture was incubated at 37 ± 2ºC in a 96-well microplate for 1 h and the absorbance was measured at 490 nm. The solution without the sample was used as a negative control. The α-tocopherol (0.001–10 mg/ml) was used as a positive control. All determinations were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = [(A-B)/A] \times 100$$

Where *A* was the absorbance of the negative control and *B* was the absorbance of the sample. The sample concentration providing 50% inhibition of lipid peroxidation (IPC_{50}) was calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

1.3.3.3 Metal ion chelating assay (Decker et al., 1990)

The metal ion chelating activity of the crude extracts were assayed by the modified ferrous ion chelating method (Decker et al., 1990). Briefly, 100 µl of five serial concentrations of the samples (0.001–10 mg/ml) dissolved in

DMSO were added to the solution of 2 mM $FeCl_2$ (50 µl) in distilled water. The reaction was initiated by the addition of 5 mM ferrozine (50 µl) and the total volume was adjusted to 300 µl by distilled water. Then, the mixture was left at room temperature (30 ± 2ºC) for 15 min. Absorbance of the resulting solution was then measured at 570 nm by a microplate reader. EDTA (0.001–10 mg/ml) was used as a positive control. The negative control containing $FeCl_2$ and ferrozine, was the complex formation molecules. All experiments were performed in triplicate. The percentages inhibition of ferrozine–Fe^{2+} complex formation were calculated by the following equation:

$$\text{Metal chelating activity (\%)} = [(A-B)/A] \times 100$$

Where *A* was the absorbance of the negative control and *B* was the absorbance of the sample. The sample concentration providing 50% metal chelating activity (CC_{50}) was calculated from the graph plotted between the percentages of metal chelating activity and the sample concentrations.

1.3.4 Tyrosinase inhibition assay (Shimizu et al., 1998)

The tyrosinase inhibition activity of the crude extracts were assayed by the modified dopachrome method using tyrosine as a substrate (Shimizu et al., 1998). Briefly, 50 µl of five serial concentrations of the samples (0.001–10 mg/ml) dissolved in DMSO, and 50 µl of 100 units of the mushroom tyrosinase solution in 0.1 M phosphate buffer, and 50 µl of tyrosine solution (1 mg/ml) in 0.1 M phosphate buffer, and 50 µl of 0.1 M phosphate buffer were added into each well of a 96-well plate. The mixture was incubated at 37 ± 2ºC for 60 min and the absorbance at 450 nm was measured. Kojic acid (0.001–10 mg/ml) was used as a positive control. The solution without the sample was used as a negative control. All experiments were performed

in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation:

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

Where *A* was the absorbance of the negative control after incubation, *B* was the absorbance of the negative control before incubation, *C* was the absorbance of the sample after incubation, and *D* was the absorbance of the sample before incubation. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted between the percentages of the tyrosinase inhibition and the sample concentrations.

1.3.5 Cytotoxicity of the crude extracts on human skin fibroblasts by sulforhodamine B (SRB) assay (Papazisis et al., 1997)

The normal human skin fibroblasts at the 8th passage were provided from the Faculty of Dentistry, Chiang Mai University in Chiang Mai, Thailand. Cells were cultured under the standard conditions in the complete culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 mg/ml). Cells were incubated in a temperature-controlled and humidified incubator (Shel Lab, model 2123TC, Cornelius, USA) with 5% CO_2 at 37ºC. The crude extracts were tested for cytotoxicity on the normal human skin fibroblasts by the SRB assay as previous described (Papazisis et al., 1997). The cells were plated at the density of 1.0×10^4 cells/well in 96-well plates and left overnight for cell attachment on the plate in 5% CO_2 at 37ºC. Cells were then exposed to five serial concentrations of the samples (0.001–10 mg/ml) for 24 h. After incubation, the adherent cells were fixed *in situ*, washed and dyed with SRB. The bound dye was solubilized and the absorbance

was measured at 540 nm by a well reader. Ascorbic acid (0.001–10 mg/ml) was used as a positive control. The experiments were done in triplicate. The percentages of cell viability were calculated according to the following equation:

$$\text{Cell viability (\%)} = \left(\frac{A_{sample}}{A_{control}}\right) \times 100$$

Where A_{sample} was the absorbance of the cells treated with the samples and $A_{control}$ was the absorbance of the non-treated cells.

1.3.6 Gelatinolytic activity on MMP-2 inhibition zymography of the crude extracts on human skin fibroblasts (Kim et al., 2008)

The six crude extracts (water, methanol and chloroform by hot and cold processes) were tested for gelatinolytic activity of MMP-2 inhibition in comparing to ascorbic acid (Manosroi et al., 2010). A monolayer of 5×10^5 cells of normal human skin fibroblasts was maintained in the culture medium without FBS for 24 h, treated with the samples and ascorbic acid with three serial dilution concentrations (0.001- 0.1 mg/ml) dissolved in 5% (v/v) DMSO and incubated for 48 h. The culture supernatants were collected to assess the gelatinolytic activities of MMP-2. The SDS-PAGE zymography using gelatin as a substrate was performed. Briefly, 20 μL of the cell culture supernatant were suspended in the loading buffer [0.125 M Tris (pH 6.8), 4% (w/w) SDS and 0.04% bromophenol blue] and run on 10% SDS polyacrylamide gel containing 0.1% (w/v) gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 20 min in the renaturing buffer [50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3 and 2.5% Triton X-100]. The gels were then incubated for 24 h at 37ºC in the developing buffer [50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100]. Gels were subsequently stained with 0.5% Coomassie brilliant blue G-250 and de-stained in 30% methanol and 10% acetic

acid (v/v) to detect the gelatinolytic activity (Kim et al., 2007). The gel was documented by a gel documentation system (Bio-Rad Laboratories, UK) and analyzed by the Quantity 1-D analysis software. The area multiplied by intensity (mm^2) of the bands on the gel was determined as the relative MMP-2 content (Arican et al., 1999; Carmeliet et al., 1997). The percentages of MMP-2 inhibition in comparing to the control (the untreated systems) were calculated by the following equation:

$$\text{MMP-2 inhibition (\%)} = 100 - \left(\left(\frac{\textit{MMP-2 content of sample}}{\textit{MMP-2 content of control}} \right) \times 100 \right)$$

The assays were done in three independent separate experiments. The MMP-2 inhibition potency of the samples was compared with the positive control (ascorbic acid).

1.4 Selection of the crude extract

The crude extract which gave the best *in vitro* anti-aging activities including DPPH radical scavenging, lipid peroxidation inhibition and metal ion chelating activity, tyrosinase inhibition activity, cytotoxiciy and gelatinolytic activity on MMP-2 inhibition zymography on human skin fibroblasts were selected for further development to semi-purified extracts.

Part 2 : Preparation and *in vitro* anti-aging activities of the semi-purified extracts

2.1 Preparation of the semi-purified extracts from the selected crude extract

The hot water crude extract which showed the highest biological activities was selected to prepare the semi-purified fractions by the solvent-solvent partition as described previously (Hideaki, 2005). Briefly, the crude extract (2.0 g) was suspended in 100 ml of H_2O and partitioned with 100 ml of ethyl acetate, leading

to the ethyl acetate and water fractions. The water fraction was partitioned between 100 ml of H_2O and butanol giving the water and butanol fractions as shown in the **Figure 37**. The resulting solutions were evaporated to dryness by a rotary evaporator. The dried residues were stored at 4ºC in a tight container until use. The percentage yield of each fraction was determined as the following equation:

$$\text{Percentage yield (\%)} = \frac{\text{Dry semi-purified extract weigh (g)}}{\text{Dry crude extract weigh (g)}} \times 100$$

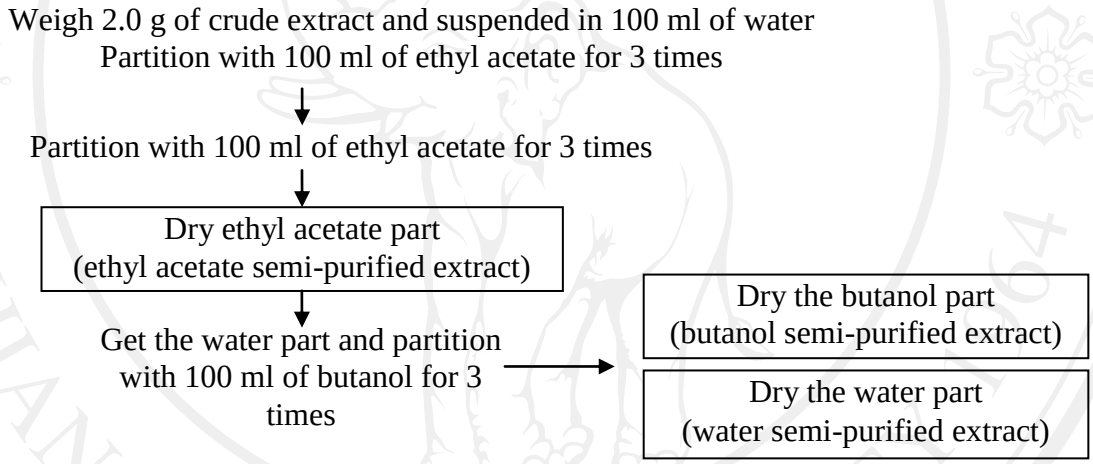

Figure 37 Scheme of purification of the water crude extract from old leaves of Long Kong which gave the three semi-purified extracts including ethyl acetate, butanol and water soluble fractions (in frames)

2.2 Characteristics of the prepared semi-purified extracts

2.2.1 Phytochemical tests of the semi-purified extracts

The prepared semi-purified extracts were carried following the phytochemical tests as in topic **1.3.1**.

2.2.2 Determination of the total phenolic and flavonoid contents of the semi-purified extracts

The prepared semi-purified extracts were carried following the determination of the total phenolic and flavonoid contents of the semi-purified extracts as in topic **1.3.2**.

2.2.3 *In vitro* anti-aging activities of the semi-purified extracts determination

The prepared semi-purified extracts were carried following the *in vitro* anti-aging activities which are DPPH free radical scavenging activity assay, lipid peroxidation inhibition assay, metal chelating assay, tyrosinase inhibition assay, cytotoxicity and gelatinolytic activity on MMP-2 inhibition on human skin fibroblasts as in topic **1.3.3** − **1.3.6**.

2.2.4 Specification of the selected semi-purified extracts

2.2.4.1 Solubility test (European Pharmacopoeia 5th ed., 2004)

The solubility tests of the semi-purified extracts were performed by a modified method of European Pharmacopoeia. Seven kinds of solvent were examined which were hot water (80±2°C), cold water (25±2°C), ethanol, methanol, propylene glycol, glycerol and mineral oil. The word "soluble" means substance dissolve in the solvent with no cloudy, precipitate and clear solution.

Dissolving procedure was used as the supporting technique to dissolve the sample. First, the sample after adding an amount of solvent was shaken vigorously for 1 min and placed in a room temperature (25±2°C) for 15 min. If the substance was not completely dissolved, repeated the shaking for 1 min and placed the tube at room temperature (25±2°C) for 15 min.

Briefly, 10 mg of each sample was put into stoppered tubes, added 1 µl of solvent and proceeded as described under *dissolving procedure*. If the substance was completely dissolved, it is very soluble. If the sample was not completely dissolved, add 9 µl of the solvent and proceed as described in the *dissolving procedure*. If the sample is completely dissolved, it is freely soluble. If the sample is not completely dissolved, adjust volume of the solvent to 30 µl and proceed as described under *dissolving procedure*. If the sample is completely dissolved, it is soluble. If the sample is not completely dissolved, adjust volume of the solvent to 100 µl and proceed as described under *dissolving procedure*. If the sample is completely dissolved, it is sparingly soluble. If the sample is not completely dissolved, adjust volume of the solvent to 1,000 µl and proceed as described under *dissolving procedure*. If the sample is completely dissolved, it is slightly soluble. If the sample is not completely dissolved, adjust volume of the solvent to 10,000 µl and proceed as described under *dissolving procedure*. If the substance is completely dissolved, it is very slightly soluble. The solubility definitions were defined based on the volume of the used solvent as described in **Table 24**.

Table 24 The descriptive term used for explaining solubility of the substance (European Pharmacopoeia 5th ed., 2004)

Descriptive term	**Approximate volume of solvent in microliters per gram of solute**
Very soluble	Less than 1
Freely soluble	1 – 10
Soluble	10 – 30
Sparingly soluble	30 – 100
Slightly soluble	100 – 1,000
Very slightly soluble	1,000 – 10,000
Practically insoluble	More than 10,000

2.2.4.2 Chemical stability test (European Pharmacopoeia 5th ed., 2004)

The chemical stability test was examined in order to observe the durable of the samples under suffering condition such as reducing agent, oxidizing agent, acidic and basic conditions. Seven types of the reagents which were 10% v/v hydrochloric acid, 10% w/v sodium hydroxide, 10% v/v acetic acid, 10% w/v ammonium hydroxide, 10% v/v hydrogen peroxide, 10% w/v ferric chloride and 10% w/v sodium acetate were prepared and the type of each reagent was listed in **Table 25**.

Table 25 Reagents used for the chemical resistant test (European Pharmacopoeia 5th ed., 2004)

Reagents	Types of reagent
10% v/v hydrochloric acid	Strong acid agent
10% w/v sodium hydroxide	Strong base agent
10% v/v acetic acid	Weak acid agent
10% w/v ammonium hydroxide	Weak base agent
10% v/v hydrogen peroxide	Oxidizing agent
10% w/v ferric chloride	Reducing agent
10% w/v sodium acetate	Sodium salt of weak acid

Briefly, weigh 10 mg of each sample into the stoppered tube and add the proper solvent (water or ethanol), shake vigorously until clear solution. Universal indicator was used to identify the pH value of each extract. After that, add reagent drop by drop until reach to 50 drops. Dropping is finished when color changing, cloudy or precipitate appears, count the numbers of the reagent drop and defined as listed in **Table 26**.

Table 26 The descriptive definition of the chemical stability test (European Pharmacopoeia 5th ed., 2004)

Descriptive term	Numbers of dropping reagent when changing occur
Stable	50
Medium stable	40 – 49
Slightly stable	20 – 39
Unstable	1 – 19

2.2.4.3 HPLC fingerprint profile (Lazarowych et al., 1998)

The HPLC fingerprint profile of the prepared semi-purified extracts were investigated by using high performance liquid chromatography (HPLC) technique. The 0.1% acetic acid in 50% acetonitrile in distilled water was used as a mobile phase delivered at 1 ml/min. HPLC was performed at ambient temperature (25±2ºC) with the isocratic of for 20 min. An amount of 10 µl of the injection volume was eluted in the column and monitored at 254 nm UV-detector. Each sample was extracted with 95% acetonitrile and hexane (1:1). Sample residue from 95% acetronitrile part was obtained, evaporated and dissolved in mobile phase. Sample solution was filtered through a 0.45-µm membrane filter, prior to injection onto the HPLC column. The peak areas were calculated and the concentrations of gallic acid were determined from the standard curve.

2.3 Selection of the semi-purified extract

The criteria for selection of the semi-purified extract were considered following the selection of the crude extract as described in topic **1.4**.

Part 3 : Discoloration and *in vitro* anti-aging activities of the selected semi-purified extracts

3.1 Discoloration of the selected semi-purified extract

The color of the ethyl acetate semi-purified extract was eliminated by using the partition technique. Briefly, 1.0 g of the extract was dissolved in 50% methanol in water and chloroform (1:1). Each part of the solution was collected and evaporated to dryness by a rotary evaporator at 50±2ºC. The dried residues were stored at 4ºC in a tight container until use.

3.2 Characteristics of the discolored semi-purified fractions

The prepared chloroform and methanol-water discolored semi-purified fracitons were characterized as in topic **2.2**.

3.3 Selection of the discolored semi-purified fractions

The criteria for selection of the discolored semi-purified fractions were considered following the selection of the crude extract as described in topic **2.2**.

3.4 *In vivo* rabbit skin irritation by Draize test of the ethyl acetate semi-purified extract and the discolored water fraction

Three male rabbits (1.5–2.5 kg) were kept carefully following an acclimation period of 7 days to ensure their suitability for the study within a limited-access rodent facility with environmental conditions set at 25 ± 2°C, 60–90% RH and 12-h light/12-h dark cycle. Animals were provided ad labium access to a commercial rabbit-diet and drinking water was supplied to each cage. Back of the animals was shaved to be free of fur with an electric clipper 24 h before topical application of the samples. The shaved areas were divided into 10 sites of 2.5 × 2.5 cm each. An amount of 0.5 g of each sample and 20% SLS solution (positive control) was placed

on each site. The untreated site was used as a negative control. The treated sites were covered with gauze and wrapped with a non-occlusive bandage. After 24 h, the bandage and the test materials were removed and the treated sites were washed 2 times by distilled water and air dried. One h later, the sites were examined for skin edema and erythema by optical visualization and measured by a **Mexameter®**, respectively. Scoring of erythema and edema was performed at 24, 48 and 72 h according to Draize et al., in 1944 and adopted by OECD Test Guideline 404. The Primary Irritation Index (PII) was calculated according to the following equation:

$$PII = \frac{\sum \textit{erythema grade at 24/48/72 h} + \sum \textit{edema grade at 24/48/72 h}}{\textit{Number of animals}}$$

Where $\sum$ *erythema grade at 24/48/72 h* was the summation of the erythema grade by optical observation at 24 or 48 or 72 h and $\sum$ *edema grade at 24/48/72 h* was the summation of the edema grade by optical observation at 24 or 48 or 72 h. The irritation degree was categorized as negligible, slight, moderate or severe irritation based on the PII values. This study protocol has been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (protocol number 27/2554) (Please see appendix A).

Part 4 : Development of the cosmetic base formulations

4.1 Preparation of the cosmetic base formulations

Cosmetic base formulations which were gel, serum and cream were prepared with the compositions shown in **Tables 27** and **28**. Each formulation was prepared in 3 different formulas.

Table 27 Compositions of the gel and serum base formulations

Types of cosmetic base formulations	Gelling agent	Compositions
Gel No.1	3% HPMC	Distilled water Propylene glycol Glycerol Polysorbate 20 Sodium metabisulfite
Gel No.2	1.5% SCMC	Distilled water Propylene glycol Glycerol Polysorbate 20 Conc. paraben
Gel No.3	1.5% SCMC	Distilled water Propylene glycol Glycerol Polysorbate 20 Conc. paraben
Serum No.1	1% HPMC	Distilled water Propylene glycol Polysorbate 20 Sodium metabisulfite
Serum No.2	1% SCMC	Distilled water Propylene glycol Polysorbate 20 Conc. paraben
Serum No.3	0.8% Carbopol Ultrez®	Distilled water Propylene glycol Polysorbate 20 Conc. paraben

Note : Conc. paraben prepared by dissolve 18 g methyl paraben and 2 g propyl paraben in 80 ml propylene glycol

Each formula of gel and serum had the same compositions except the type of gel forming agents. Gel formulation was prepared by dispersing the gelling powder (HPMC, SCMC and Carbopol Ultrez® 21) in the distilled water, and then gently stirred by a magnetic stirrer until homogeneity. The mixture of propylene glycol, glycerol, polysorbate 20 and preservative were added into the dispersion with continuous mixing until homogenous and an amount of triethanolamine was added with vigorously mixing until the homogeneous gel was obtained. Serum was prepared

Table 28 Compositions of the cream base formulations

Cosmetic formulations	Compositions	
	Aqueous phase	**Oil phase**
Cream No.1	Distilled water Glycerol Sorbitol 70% solution Propylene glycol 0.8% Carbopol Ultrez® 21	Glyceryl monostearate Mineral oil Jojoba oil White petrolatum jelly Stearic acid Polysorbate 20 Conc. paraben
Cream No.2	Distilled water Glycerol Propylene glycol 0.6% Carbopol 934	Glyceryl monostearate Mineral oil Cyclomethicone White petrolatum jelly Myristic acid Polysorbate 20 Sorbitan monolaurate Conc. paraben
Cream No.3	Distilled water Sorbitol 70% solution Propylene glycol 0.8% Carbopol 980	PEG-400 monostearate Beeswax White petrolatum jelly Cetyl alcohol Polysorbate 20 Conc. paraben

Note : Conc. paraben prepared by dissolving 18 g of methyl paraben and 2 g of propyl paraben in 80 ml propylene glycol

by dispersing gelling powder in the distilled water, prior to stir vigorously by a magnetic stirrer until homogeneity. Triethanolamine was added with gently mixing until homogeneous gel was obtained. Amount of gel solution was added into the mixture of propylene glycol, polysorbate 20 and preservatives, and slightly stirred continuously until homogeneous.

For cream preparation, oil phase was melted at 65 ± 2 ^{o}C. The mixture of an aqueous phase except conc. paraben was heated at 65 ± 2 ^{o}C. The temperature of the oil and aqueous phase was maintained at 65 ± 2 ^{o}C. After that, the oil phase was

poured into the aqueous phase and stirred until the mixture cooled to 40^{o}C. The conc. paraben solution was added into the mixture and stirred continuously.

4.2 Physical stability determination of the cosmetic base formulations by heating cooling cycles

The physical stability of the cosmetic base formulations was evaluated by heating-cooling method as described previouly (Garrett, 1962). Gel, serum and cream base formulations were stored in transparent glass bottles and kept at 45 ± 2^{o}C and 4 ± 2^{o}C for 6 cycles with 24 h for each heating and 24 h for each cooling. The physical appearances (viscosity, uniformity, color, odor and pH) of the bases were examined initial and after 6 cycles (12 days). Gel, serum and cream base formulations which gave the best physical stability were selected for the further studies.

4.3 *In vivo* rabbit skin irritation by Draize test

In vivo irritation on rabbit skin by Draize test of the cosmetic base formulaions were performed as in **Topic 3.4**.

4.4 Selection of the cosmetic base formulations

The cosmetic base formulations were considered selected according to the physical stability after heating cooling cycles. The physical appearance of each formulation including uniformity, pH, color, sedimentation and viscosity was collected. Then, the stable formulations were subjected to test for rabbit skin test. The best formula of each type of cosmetic formulations which are gel, serum and cream was selected for further development.

Part 5 : Development of the cosmetic formulations containing the extract from leaves of Long Kong

5.1 Preparation of the cosmetic formulations containing the selected extract from leaves of Long Kong

The selected semi-purified extract was incorporated into each selected each cosmetic base formulation (gel, serum and cream). The concentrations of the extract were 0.1, 0.3 and 0.5% (w/w). Gel formulation was prepared by dispersing the gelling powder in the distilled water, and an amount of triethanolamine was added with vigorously mixing until the homogeneous gel was obtained. The mixture of the extract, propylene glycol, glycerol, polysorbate 20 and preservative were added into the gel with continuous mixing until homogenous. Serum was prepared by dispersing the gelling powder in the distilled water, prior to stir vigorously by a magnetic stirrer until homogeneity. Triethanolamine was added with gently mixing until homogeneous gel was obtained. An aliquot of the gel solution was added into the mixture of propylene glycol, polysorbate 20 and preservatives, and slightly stir continuously until homogeneous.

For cream preparation, the oil phase was melted at 65 ± 2 ^{o}C. The mixture of aqueous phase (except the extract and conc. paraben) was heated at 65 ± 2 ^{o}C. The temperature of the oil and aqueous phase was maintained at 65 ± 2 ^{o}C. After that, the oil phase was poured into the aqueous phase and stirred until the mixture cooled to 40 ^{o}C. The extract and conc. paraben solution were added into the mixture and stirred continuously.

5.2 Physico-chemical stability investigation of the cosmetic formulations containing the selected extract from leaves of Long Kong

5.2.1 Physical stability determination

The prepared cosmetic formulations with and without various semi-purified extract concentrations and the semi-purified extract at various concentrations at 0.1, 0.3 and 0.5% in propylene glycol were put in tightly containers and stored at room temperature (27 ± 2°C), 4 ± 2°C and 45 ± 2°C for 3 months. The physical characteristics including viscosity, color, uniformity and pH, were evaluated at initial, 1, 2 and 3 months.

5.2.2 Chemical stability determination

The percentages remaining of gallic acid contents determined by HPLC at 0, 1, 2, 3 months of various gel formulations were investigated. The HPLC conditions of the analysis of gallic acid were LC1200 UV/VIS detector and LC1100 HPLC pump using Luna® C18 (10 µm, 250 mm × 4.0 mm i.d.) Shiseido Japan column. The mobile phase containing MeCN/H_2O/AcOH (10:90:1, v/v) and the flow rate of 1 ml/min were used. The samples were extracted with 95% acetonitrile and hexane (1:1). Then, 95% acetonitrile was collected, evaporated to dryness, and the dry residue was dissolved in the mobile phase before filtering through the 0.45 µm membrane filter to obtain the clear solution, prior to the injection onto the HPLC column. An amount of 10 µl of the samples was injected into the column and monitored at 254 nm UV detector. The retention time of gallic acid was 3.7 min. The gallic acid contents were determined from the standard curve of the standard gallic acid, which demonstrated linear with high correlation (r^2 = 1). The following regression equation was obtained: $y = 6928x + 12549$, where y was the peak area and x was the quantity of gallic acid (µg). The experiment was done in triplicate.

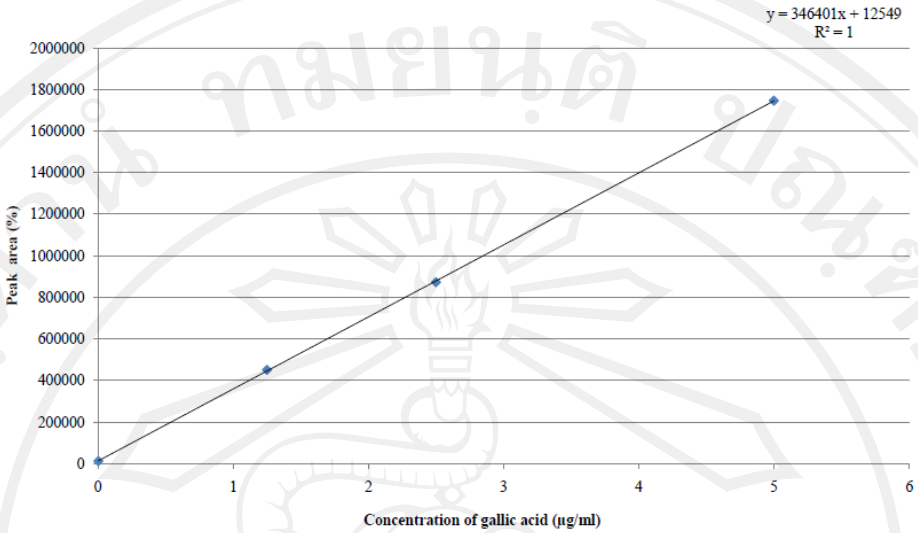

Figure 39 The standard curve of gallic acid

The degradation rate constants (k) of gallic acid were estimated from the slope of the plot between the bioactive concentrations versus times, by the least-squares fitting of various kinetic equations including zero order, first order and second order calculated according to the following equations:

Zero order equation : $[A]_t = -k_0 t + [A]_0$

First order equation : $\ln[A]_t = -k_1 t + \ln[A]_0$

Second order equation : $1/[A]_t = 1/[A]_0 + k_2 t$

Where $[A]_t$ represents the concentration of gallic acid at a particular time, and $[A]_0$ represents the initial concentration of gallic acid and kt represents the slope of the resulting linear of the order rate constants.

The predicted shelf life (t_{90}) and half life (t_{50}), which were the time required when gallic acid in the formulations remained at 90 and 50%, respectively were estimated by substituting k into the following shelf life and half life equations

(Florence, A. T. and Attwood, D., 2006; Manosroi, A., Podjanasoonthon, K. and Manosroi, J., 2002):

$$\text{Zero order : Shelf life } (t_{90}) = 0.2/t_{50}$$

$$\text{Half life } (t_{50}) = [A]_0/2k_0$$

Where $[A_0]$ was the amounts of gallic acid (µg/ml) at initial and k_0 was the degradation rate constants of the zero order kinetic equations.

$$\text{First order : Shelf life } (t_{90}) = 0.152t_{50}$$

$$\text{Half life } (t_{50}) = 0.693/k_1$$

Where k_1 was the degradation rate constants of the first order kinetic equations.

$$\text{Second order : Shelf life } (t_{90}) = 0.11t_{50}$$

$$\text{Half life } (t_{50}) = 1/[A]_0k_2$$

Where $[A_0]$ was the amounts of gallic acid (µg/ml) at initial and k_2 was the degradation rate constants of the second order kinetic equations.

$$\text{Shelf life } (t_{90}) = 0.11/[A_0]k_2$$

$$\text{Half life } (t_{50}) = 1/[A_0]k_2$$

Where $[A_0]$ was the amounts of gallic acid (µg/ml) at initial and k_2 was the degradation rate constants of the second order kinetic equation.

5.3 Selection of the cosmetic formulations containing the extract from leaves of Long Kong

Each type of cosmetic formulations containing the extract from leaves of *L. domesticum* were considered selected according to the physical and chemical stability after kept at various temperatures (27 ± 2°C, 4 ± 2°C and 45 ± 2°C) for 3 months. The physical appearance of each formulation including uniformity, pH, color, sedimentation and viscosity was collected. The chemical stability of each of

formulations was analyzed. Then, the stable formulations containing the extract at various concentrations were selected for performance test in human volunteers.

Part 6 : *In vivo* anti-aging evaluation of the cosmetic formulations containing extract from leaves of Long Kong

6.1 *In vivo* irritation on rabbit skin by Draize test of the cosmetic formulations containing the semi-purified extract from leaves of Long Kong

The selected cosmetic base formulations and cosmetic formulation containing the ethyl acetate semi-purified extract at various concentrations (0.1, 0.3 and 0.5%) and the semi-purified extract solution dissolving in propylene glycol at various concentrations (0.1, 0.3 and 0.5%) were evaluated for skin irritation by Draize test as in topic ***4.3***.

6.2 *In vivo* anti-aging activities in human volunteers of the cosmetic formulations containing the selected extract from leaves of Long Kong

6.2.1 Subjects and study protocol

The total of 20 Thai volunteers (10 men, 10 women, averaged age 37.35 ± 9.71 years, range 24–56 years, averaged BMI 23.53 ± 5.12) was recruited from Faculty of Pharmacy, Chiang Mai University in Chiang Mai, Thailand.

All volunteers were free of pathological systems on their arms and were not using any topical agents on the test areas during the study. The samples were applied on the 4 × 4 cm^2 area on the volar forearm. Each forearm was virtually divided into six areas. Nine areas were treated with 0.2 ml of the 9 formulations listed in **Table 29**. The area without the treatment was used as a negative control. The formulations were applied on the areas once daily for 4 weeks. The areas were measured for anti-

aging activity before sample application, after application at 1, 2, 3 and 4 weeks and the washout period at 1 and 2 weeks. Before the measurements, the volunteers were accommodated in a controlled room at 25 ± 2°C and 75 ± 2% RH for 20 min. All volunteers finished the study and without any drop-outs. This study protocol was reviewed and approved by the ethical committee of Faculty of Pharmacy, Chiang Mai University in Thailand (protocol number 20/2012) (please see appendix A).

6.2.2 Skin elasticity measurement

Skin elasticity of the treated areas was determined using a non-invasive *in vivo* suction skin elasticity meter, Cutometer® MPA580 which was equipped with a 2-mm measuring probe. The time/strain was used with a 5-s application of a constant negative pressure of 450 mbar, followed by a 3-s relaxation period and repeat for 3 times for each area. The immediate distention (Ue), the final distention (Uf), and the immediate retraction (Ur) were measured. The delayed distention (Uv) was obtained from the difference between Uf and Ue. The elasticity ratio Uv/Ue or skin elastic extension, as well as the ability of the skin to return to its initial position after deformation (Ur/Uf) or skin elastic recovery were calculated from the above parameters by Win-Cutometer MPA Version 2.12.15.9 software which have been reported to be independent of skin thickness (Elsner et al., 1990; Takema et al., 1994). Therefore, the Ur/Uf and Uv/Ue values were used as parameters of skin elasticity. For relative inspection of the data, the significant difference results were calculated as parameter changes (%) by the following equation:

$$\% \text{ Parameter changes} = \left(\frac{\sum Q_{ti}}{\sum Q_{0i}} - 1 \right) \times 100$$

Where Q_{ti} was the quotient after application time t of 4 weeks for each volunteer i, and Q_{0i} was the quotient before application time for each volunteer i.

Table 29 Descriptions of the samples in skin anti-aging evaluation in human volunteers

Samples	Descriptions
cream base	cream without semi-purified extract
0.1% cream	cream incorporated with 0.1% of semi-purified extract
0.3% cream	cream incorporated with 0.3% of semi-purified extract
0.5% cream	cream incorporated with 0.5% of semi-purified extracts
propylene glycol	propylene glycol without semi-purified extract
0.1% propylene glycol	0.1% of semi-purified extract dissolved in propylene glycol
0.3% propylene glycol	0.3% of semi-purified extract dissolved in propylene glycol
0.5% propylene glycol	0.5% of semi-purified extract dissolved in propylene glycol
Commercial product	Cream containing extract from heartwood of *Artocarpus lakoocha* Roxb. which has anti-aging and tyrosinase inhibition activities (Singhatong et al., 2010; Suwannalert et al., 2012).

6.2.3 Skin roughness measurement

The skin roughness was assessed using the image analysis software. Briefly, light was directed at a 208 angle and the images were observed from the tested skin area using a CCD camera (Visioscan® VC98). The parameters of the image to evaluate average skin roughness were analyzed by CK Visiometer® SV600 FW; image analyzing program Version 1.6.6.1 software. The parameter changes (%) were calculated the following equation as described in the determination of % parameter changes in topic ***6.2.2.***

6.2.4 Skin hydration measurement

Skin hydration was investigated using a Corneometer® CM825 which was mounted on a Multi Probe Adapter® MPA5. Capacitance changes depending almost solely upon the water content in the stratum corneum were detected and evaluated by CK MPA Multiprobe Adaptor Version 1.4.2.2. Five measurements were performed on each tested skin area.

6.2.5 Skin erythema and melanin measurements

The degree of skin erythema (redness) and pigmentation (melanin) was measured using a **Mexameter® MX18**. Five measurements were performed on each tested skin area.

6.2.6 Satisfactory questionnaires of the cosmetic formulations

The cream formulations containing the semi-purified extract at various concentrations (0.1, 0.3 and 0.5%) were selected to explore the satisfaction according to the further development of the topical cosmetic product to the market. Therefore, the cream formulations containing the semi-purified extract at 0.1, 0.3 and 0.5% were estimated for physical appearance and sensation based on individual satisfaction of each volunteer by a questionnaire (please see the questionnaires in Appendix B). The satisfaction on the physical appearance including absorption, consistency, color and odor, and the organoleptic evaluations including sticky, moisture, smoothness and whiteness after application of the topical cream formulations on the skin were assessed after 4 weeks application. The data were collected and interpreted by the five levels of Likert's rating scale as described in **Table 30**. The percentages of the satisfactory scale were calculated as the following equation:

$$(\%)\ \text{Satisfaction} = \frac{\text{Total obtained score}}{\text{Total score}} \times 100$$

Table 30 The description of five levels of Likert's rating scale of the satisfactory score estimation of volunteers

Level of the Likert's rating scale	Description
1.00 – 1.49	Not satisfied
1.50 – 2.49	Less satisfied
2.50 – 3.49	Fairly satisfied
3.50 – 4.49	Satisfied / Good
4.50 – 5.00	Very satisfied / Excellent

6.2.7 Statistical analysis

All data were presented as mean±standard deviation (SD) and standard error of mean (SEM). Student's paired T-test and the One-way ANOVA test were used to evaluate the significance of differences at the significant level of $p<0.05$. Statistical analysis was performed using SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA).