CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and equipments

2.1.1 Chemicals

- Ammonium thiocyanate (NH_4SCN) (Sigma Co, St. Louis, MO, USA)
- Alpha-tocopherol (Sigma Co, St. Louis, MO, USA)
- Ascorbic acid (Sigma Co, St. Louis, MO, USA)
- Cholesterol (Sigma Chemical Co., St. Louis, MO, USA)
- Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, USA)
- 1, 1 Diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemical Co., St. Louis, MO, USA)
- EDTA (ethylenediamine tetraacetic acid disodium salt dihydrate) (Fluka Co., Switzerland)
- Coomassie® Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, USA)
- Dulbecco's modified eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA)
- Eagle's minimal essential medium (MEM) (GIBCO, Grand Island, NY, USA)
- Fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA)

- Ferrozine and ferric chloride ($FeCl_2$) (Sigma Chemical Co., St. Louis, MO, USA)
- Kojic acid (Sigma Chemical Co., St. Louis, MO, USA)
- Linoleic acid (Sigma Co, St. Louis, MO, USA)
- Other solvents (analytical grade)
- Phosphate buffered saline (Gibco BRL; Invitrogen, Burlington, ON, Canada)
- Tween 61 (polyoxyethylene sorbitan monostearate) (Sigma Chemical Co., St. Louis, MO, USA)
- Tyrosine (Sigma Chemical Co., St. Louis, MO, USA)
- Tyrosinase enzyme, mushroom (Sigma Chemical Co., St. Louis, MO, USA)
- RPMI 1640, non-essential amino acid medium (NEAA) (GIBCO, Grand Island, NY, USA)

2.1.2 Cell lines

- Normal human skin fibroblasts (at the 6^{th} passage from Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand and at the 15^{th} passage from 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 Equipments

- Centrivap Console (Labconco, Kansas City, MO, USA)
- CO_2 incubator (Shel Lab, model 2123TC, USA)

- Dynamic light scattering (DLS), Zetasizer 300HSA (Malvern Instruments, Malvern, U.K.)
- Gas Chromatography-mass spectrometry (GC-MS Thermo, USA)
- Gel documentation system and Quantity 1-D analysis software (Bio-Rad Laboratories, U.K.)
- High Perfomance Liquid Chromatography (HPLC Perkin-Elmer® USA)
- Larminar air flow cabinet (Cytair 125, Equipments Scientifiques & Industries S.A., France)
- Mexameter® (Courage & Khazaka, Cologne, Germany)
- Microplate Reader (Model 550, BIORAD, USA)
- Probe sonicator (Vibra Cell™, Sonics & Materials Inc., Newtown, CT, USA)
- Rotary evaporator (R-124 Buchi, Switzerland)
- Supercritical carbon dioxide fluid ($scCO_2$) apparatus (SFE-500 MR-2- C50 system, Thar Instruments Inc., PA, USA)
- Spectrophotometer T80 UV/VIS spectrophotometer, PG instruments Ltd., China)
- Ultracentrifuge (Univeral 32 R, Hettich Zentrifugen, Germany)
- Ultrasonic bath (Elma® Germany)
- 96 Well plates (Nunc®, USA)

2.2 Methods

2.2.1 Biological activities of the Thai flower extracts

2.2.1.1 Collection of the raw materials

Flowers of *G. jasminoides*, *M. elengi* and *M.hortensis* were collected from Chiang Mai Province in Thailand during April to November in 2009. The specimen were authenticated by a botanist at Faculty of Pharmacy, Chiang Mai University, Thailand and deposited in the Medicinal Plant Herbarium at Faculty of Pharmacy, Chiang Mai University in Thailand for authenticated specimen with the voucher specimen no. 004108 for *M.hortensis* and no. 009595 for *G. jasminoides.* The *M. elengi* 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 and deposited in the NPRDC Medicinal Plant Herbarium, STRI, Chiang Mai University in Thailand for authenticated specimen with the voucher specimen no. Manosroi 0028.

2.2.1.2 Preparation of the flower extracts

A. Flower samples

The whole flowers of *G. jasminoides*, *M. elengi* and *M.hortensis* were used for the extraction.

B. Preparation of the extracts by the maceration process

Briefly, 200 g of the fresh flowers were macerated with 1 L of hexane at room temperature (27±2 ◦C) for 6 hours with continuous stirring. The extract was filtered through the paper filter (Whatman No. 1) connected with a vacuum pump. All filtrates were collected, pooled and dried by a rotary evaporator (Rotavapor R210, Buchi, Switzerland) at 40 ◦C. The percentage yields were determined. The dried extracts were kept at −80 ◦C until use.

C. Preparation of the extracts by supercritical carbon dioxide fluid ($ScCO_2$)

Fresh flowers of *G. jasminoides*, *M. elengi* and *M.elengi* were extracted by the $ScCO_2$ technique. Briefly, 100 g of each plant were separately extracted by $ScCO_2$ with 4-50% (w/v) of 95% (v/v) ethanol as a co-solvent. The temperature in the collecting vessel was raised to 40^{o}C. Carbon dioxide gas was introduced into the collecting vessel and the pressure was maintained at 200 Bar. After 6 h, the pressure was released and the extract was collected. The percentage yields were determined. All extracts were dried by a rotary evaporator (R-124 Buchi, Flawil, Switzerland) at 40^{o}C and kept at -80^{o}C until use.

2.2.1.3 Determination of the physico-chemical properties and phytochemicals of the extracts

The physical appearances as well as the physico-chemical stabitity of the extracts in various reagents were examined. The extract (20 mg), dissolved in 20 ml of 80% methanol, was used for detecting the presence of alkaloids, flavonoids, glycosides, saponins, tannins and xanthones according to the methods previously described. For alkaloid, 2 ml of the extract solution mixed with 1 ml of 1% HCl were boiled on a water bath and 6 drops of Dragendroff's reagent were added. Creamish or brownish-red or orange precipitate indicated the presence of alkaloids (Brimer et al., 1989). Quinine sulphate was used as a positive control. For anthraquinones (Borntrager's test) determination, 0.1 g of the powder extract was boiled with 4 ml of alcoholic KOH for 2–3 minutes and diluted with 4 ml of water and filtered. The filtrate was acidified with dilute HCl, filtered, cooled and shaken with benzene. The benzene layer was separate and put into a clean test tube and shaken with 2 ml of the

dilute ammonia solution. Extracts consisting of anthraquinones gave an orange-red to deep orange-red color in the aqueous layer (Allen, 1974; Harbone, 1976). Anthraquinone was used as a positive control. For the presence of flavonoid (Shinoda test), 2 ml of the extract solution mixed with 1 ml of conc. HCl and magnesium ribbon gave the pink-tomato red color (Allen, 1974). Luteolin was used as a positive control. For the qualitative assay of glycoside (Fehling's test for reducing sugars), 2 ml of the extract solution mixed with 1 ml of Fehling's solution was heated in a water bath for 10 min. The brick-red precipitate indicated the presence of reducing sugar contained in glycosides (Harbone, 1976; Onwukaeme et al., 2007). Glucose, fructose and galactose from Sigma Chemical (USA) were used as positive controls. For saponin (Frothing test), 0.5 ml of the extract solution was mixed with 5 ml of distilled water. The frothing persistence indicated the presence of saponins (Allen, 1974; Harbone, 1976). *Saponaria officinalis* extract was used as a positive control. For tannins, 2 ml of the extract solution were mixed with 2 ml of 15% $FeCl_3$ solution. The blue-black precipitate indicated the presence of tannins (VanMiddlesworth & Cannell, 1998; Onwukaeme et al., 2007). Tannic acid was used as a positive control. For xanthones, 2 ml of the extract solution were mixed with 1 ml of 5% KOH reagent. The formation of yellow precipitate indicated the presence of xanthones (Allen, 1974; Harbone, 1976). *Garcinia mangostana* Linn extract was used as a positive control. For carotenoid , the extract of 0.05 mg were mixed with 2 ml of chloroform and 10 µl sulfuric acid were added. Blue or bluegreen indicated the presence of carotenoid (Farmsworth , 1966).

2.2.1.4 Chemical identification of the extracts by HPLC and GC-MS

HPLC

A: Instruments

Vacuum degasser series (Perkin-Elmer®)

Pump series 200LC (Perkin-Elmer®)

Diode array detector series 200 Diode Array (Perkin-Elmer®)

Spheri-5 RP18 column, 220 mm x 4.6 mm i.d. (Perkin-Elmer®)

20 µl ryringe loading sample injector model 7725i (Rheodyne®)

B. Chromatographic conditions

Analytical column : BDS Hypersil C18 (250×4.6 mm, 5 µm)

Flow rate : 1 ml/min

Temperature : 25 °C

Detector : UV 205 nm

Injection volume : 20 µl

Mobile phase : as the following table

	Time	**Flow (ml/min)**	**CH_3CN** (ml)	**0.1% CH_3COOH**
Step 0	0.0	1	95	5
Step 1	20	1	95	5

GC-MS

A: Instruments

Column : DB-WAX

122-7062

60m × 0.25mm, 0.25µm

B. Gas chromatographic conditions

Carrier : Helium at 25cm/sec

(1ml/min)

Oven : as the following table

	RATE **ºC/MIN**	**VALUE** **ºC**	**HOLD TIME** **MIN**	**RUN TIME** **MIN**
∇ (Initial)		60	4	4
Ramp1	4	250	15	66.5
*	Split 200:1/Solvent Delay 2.00 min			

Injection : Split ratio 200:1

Detector : MS

Sample : 1µL neat

2.2.1.5 Biological activity determination of the extracts

A. DPPH radical scavenging activity

The DPPH radical scavenging activity of all extracts was determined by a modified method previously described (Tachibana et al., 2001).

Briefly, 50 µl of the five serial concentration extracts [0.001–10 mg/ml dissolved in methanol and 20% v/v DMSO (1:1)] and 50 µl of ethanolic solution of DPPH were put into each well of a 96-well microplate. The reaction mixture was allowed to stand for 30 min at 27 ± 2°C, and the absorbance was measured at 515 nm by a well reader against a blank [methanol mixed with 20% v/v DMSO (1:1)]. Ascorbic acid, BHT and α-tocopherol (0.001–10 mg/ml) were used as positive controls. The experiments were done in triplicate. The IC_{50} value which was the concentration of the sample that scavenged 50% of the DPPH radical was determined. The percentages of DPPH radical scavenging activity were calculated according to the following equation:

$$\% \text{ Scavenging} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the sample. The histogram of the percentages of DPPH radical scavenging activity of the extract at 0.1 mg/ml was presented.

B. Metal chelating activity

The Fe^{2+} chelating ability of the extracts was measured by the ferrous iron–ferrozine complex method (Decker & Welch, 1990). Briefly, the reaction mixture containing 2 mM $FeCl_2$ (10 µl) and 5 mM ferrozine (10 µl) and 100 µl of the five serial concentration extracts [0.001–10 mg/ml dissolved in methanol and 20% v/v DMSO (1:1) solution] were mixed in a 96-well plate and incubated for 10 min at 27 ± 2°C. The absorbance was recorded by a well reader at 570 nm. The absorbance of the control was determined by replacing the extract with methanol. EDTA (0.001–10 mg/ml) was used as a positive control. The experiments were done in triplicate. The IC_{50} value which was the concentration of the sample that chelated

50% of the ferrous iron was determined. The ability of the sample to chelate ferrous ion was calculated according to the following equation

$$\% \text{ Chelating effect} = 1 - \left(\frac{Abs_{sample}}{Abs_{control}} \times 100 \right)$$

Where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the sample. The histogram of the percentages of the chelating effect of the extract at 0.1 mg/ml was presented.

C. Tyrosinase inhibition assay

The extracts were assayed by the modified tyrosinase inhibition method previously described (Shimizu et al., 1998). Briefly, 120 µl of 1.66 mM of tyrosine solution in 0.1 M phosphate buffer (pH 6.8), 60 µl of five serial concentrations of the extracts [0.001–10 mg/ml dissolved in methanol and 20% v/v DMSO (1:1)] and 60 µl of phosphate buffer were mixed in a 96-well plate and incubated at 37 ± 2°C for 60 min. Then, 60 µl of tyrosinase enzyme solution (0.6 mg/ml) in phosphate buffer were added. The enzyme activity at 37 ± 2°C was measured by a well reader at 450 nm. Ascorbic acid and kojic acid (0.001–10 mg/ml) were used as positive controls. The experiments were done in triplicate. The IC_{50} value which was the concentration of the sample that inhibited 50% of the enzyme activity was determined. The inhibition percentage of tyrosinase was calculated according to the following equation :

$$\% \text{ Tyrosinase inhibition} = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \right) \times 100$$

Where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the sample. The histogram of the percentages of the inhibition effect of the extract at 0.1 mg/ml was presented.

D. Cell proliferation activity on aged human skin fibroblasts by SRB assay

The normal human skin fibroblasts were cultured under standard conditions in the complete culture medium containing α-modified Eagles culture medium (MEM-Alpha) supplemented with 10% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated in a temperature-controlled, humidified incubator with 5% CO_2 at 37°C. Cells were used at the 15^{th} passage. The extracts were tested for cell proliferation activity of the 15^{th} passage normal human skin fibroblasts by the SRB assay according to the method of Papazisis et al., 1997. Ascorbic acid (0.001–10 mg/ml) was used as a positive control. The cells were plated at a density of 1.0×10^5 cells/well in 96-well plates and left for cell attachment on the plate overnight in 5% CO_2 at 37°C. Cells were then exposed to five serial concentrations of the extracts (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. The assays were done in three independent and separate experiments. The percentage of cell growth was calculated according to the following equation:

$$\%G = \frac{A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance of the sample. Stimulation index (SI) which was the ratio between the

percentages of %G treated with the extracts at 0.1 mg/ml and the control (no treatment) was presented.

E. Gelatinolytic activity

The extracts were assayed for gelatinolytic activity of MMP-2 inhibition in comparing to ascorbic acid. Monolayer of 5.0×10^5 cells at the 15^{th} passage normal human skin fibroblasts were maintained in the culture medium without FBS for 24 h, treated with the extracts and ascorbic acid at the concentration at 0.1 mg/ml and incubated for 48 h. The culture supernatants were collected. To assess the gelatinolytic activities of MMP-2 in the culture media, SDS-PAGE zymography using gelatin as a substrate was performed. Briefly, 20 µl of the cell culture supernatant were suspended in loading buffer [0.125 M Tris (pH 6.8), 4% SDS and 0.04% bromophenol blue] and, without prior denaturation, run on 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 20 min in the renaturing buffer (50 mM Tris, 5mM $CaCl_2$, 0.02% NaN_3, 2.5% Triton X-100). The gels were then incubated for 24 h at 37°C in 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 destained in 30% methanol and 10% acetic acid (v/v) to detect gelatinolytic activity (Kim et al., 2007). The gel was documented by a gel documentation system and analyzed by 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 (Carmeliet et al., 1997; Arican & Ceylan, 1999). The percentages of MMP-2 inhibition in comparing to the control (the untreated systems) were calculated according to the following equation:

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

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

2.2.1.6 Selection of the extracts

The extract which gave the best activity was selected to load in niosomes.

2.2.2 Characteristics and biological activities of Thai flower extracts loaded in niosomes

2.2.2.1 Preparation of niosomal formulations loaded with the extracts / maximum loading determination

An amount of 20 mM niosomal dispersion was prepared by chloroform film method with sonication. Tween 61 and cholesterol were mixed at 1:1 molar ratio dissolved in chloroform. The methanolic solution of each flower extract was added to the flask (1 g of the extract per 100 ml of niosomes) and the solvents were then removed by a rotary evaporator under vacuum. The resulting film was dried and hydrated with 100 ml distilled water at 50±2^{o}C for 30 min followed by sonication using a microtip probe sonicator (Vibra Cell™, Sonics & Materials, Inc., Newton, CT, USA) at pulse on 3.0 and pulse off 1.0, 35% amplitude for 10 min. The temperature was controlled by placing the flask containing the loaded niosomes in an ice bath. Then, the niosomal dispersion was centrifuged at 5,000 rpm, 4^{o}C for 1 min. The concentrations of the flower extracts loaded in niosomes were increased from 1 to

3% (w/v). The maximum loading of the flower extracts in niosomes was determined from the maximum concentration of the flower extracts which gave no precipitation.

2.2.2.2 Morphology, vesicular size and zeta potential determination

The vesicular size and zeta potential of the non-loaded and loaded niosomes were investigated by dynamic light scattering technique using Zetasizer Nano ZS (Malvern Instrument, Malvern, UK), with DTSv5.0 software. All samples were diluted to 30 folds with freshly distilled water prior to the measurement. The mean diameter was calculated from the Brownian motion of the vesicles of 5 individual runs. For the zeta potential measurement, all samples were also diluted with distilled water to 30 folds. The measurement was performed at 25^{o}C for 5 individual runs.

2.2.2.3 Biological activities of niosomes loaded with the extracts

A. Cytotoxicity assay on human skin fibroblast by SRB assay

The human skin fibroblast at the 6th passage from the Faculty of Dentistry, Chiang Mai University, Chiang Mai in Thailand were cultured in DMEM medium. The medium were supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cultivation was performed at 37^{o}C, 5% CO_2.

Cytotoxicity of the extracts, and the extracts loaded in niosomes were determined by sulforhodamine B (SRB) assay. All samples were separately diluted in DMEM medium to the final total concentration of the extracts at 10 mg/ml. The cells were cultured and placed into 96-well plate for 10,000 cells / well. The cultures were incubated at 37±2^{o}C, 5% CO_2 for 24 h. Each sample was added into each well, incubated at 37±2^{o}C, 5% CO_2 for 24 h. Then, fixed the cells

with 50% trichloroacetic acid and incubated at 4^{o}C for 1 h. The cells were rinsed with distilled water, air-dried and stained with 0.4% SRB solution for 30 min at room temperature. The unbound SRB was removed by washing with 1% glacial acetic acid solution for four times. The remaining SRB was dissolved by 10 mM Tris HCl with shaking for 30 min. Vitamin C was used as a standard. The untreated cells were used as a negative control. The absorbance was measured at 540 nm and the percentages of cell viability were calculated by the following equation;

$$\%\text{Cell viability} = (T_{test}/T_{ctrl})\times 100$$

where T_{test} indicated the A_{540} of the cells treated with the sample and T_{ctrl} represented the A_{540} of the cells without any treatment.

B. Lipid peroxidation activity

The lipid peroxidation inhibition activity of the extracts and the extracts loaded in niosomes was assayed by the modified Ferric-thiocyanate method (Sun et al., 2011;Osawa et al., 1981). An amount of 50 µl of five serial concentrations of the samples or vitamin E at 0.001-10 mg/ml in DMSO was added to 50 µl of linoleic acid in 50% (v/v) DMSO in each 96-well microplate. The reaction was initiated by the addition of 50 µl of NH_4SCN (5 mM) and 50 µl of $FeCl_2$ (2 mM). The mixture was incubated at 37±2^{o}C for 1 h. During the oxidation of linoleic acid, peroxides are formed leading to the oxidation of Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate which can be detected at 490 nm. The dissolving solution without the sample was used as a negative control. Vitamin E at 0.001-10 mg/ml was used as a positive control. All experiments were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated as the following;

Inhibition of lipid peroxidation (%) = [(A-B)/A]×100

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

C. Metal ion chelating activity

The metal ion chelating activity of the extracts and the extracts loaded in niosomes was assayed by the modified ferrous ion chelating method [14]. Briefly, 100 µl of five serial concentrations of the samples or EDTA at 0.001-10 mg/ml were added to 50 µl of 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 50 µl of 5 mM ferrozine and the total volume was adjusted to 300 µl by distilled water. The mixture was left at 27±2^{o}C 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 contained $FeCl_2$ and ferrozine. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe^{2+} complex formation were calculated by the following equation;

Metal chelating activity (%) = [(A-B)/A]×100

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

D. Gelatinolytic activity (zymography) on MMP-2 inhibition

Various concentrations (0.01, 0.1 and 1 mg/ml) of the samples were tested for gelatinolytic activity of MMP-2 inhibition in comparing to vitamin C. The human skin fibroblasts at 6^{th} passage were seeded in 6-well plates at an amount of 5×10^5 cells/well. The monolayer of the cells was maintained in the culture medium without FBS for 24 h, treated with the samples and incubated for 72 h. The culture supernatants were collected to assess the gelanolytic activities of MMP-2 in the culture media.

SDS-PAGE zymography using gelatin as a substrate was performed according to the method previously described with some modifications (Kobayashi et al., 2003; Balitaan et al.,2010). Briefly, the cell culture supernatant was suspended in the loading buffer [0.125 M Tris (pH 6.8), 4% SDS and 0.04% bromophenol blue, without prior denaturation] and run on the 10% SDS polyacrylamide gel with the presence of 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, 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) at room temperature (27±2 °C) to visualize the bands (Kim et al., 2008). The two bands of MMP-2 were observed on the gelatin-zymography gel. The positions of the pro MMP-2 (upper band) and the active MMP-2 (lower band) were indicated. Electrophoretic data was determined by gel documentation system (Bio-Rad Laboratories,UK) and analysed by 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 (Manosroi et al., 2010). The MMP-2 inhibition in comparing to the control (the untreated systems) was calculated by the following equation:

MMP-2 inhibition (%) = 100 - [(MMP-2content$_{sample}$/MMP-2 content$_{control}$)×100]

The assays were done in three independent separate experiments. The potency of MMP-2 inhibition of the samples was compared with vitamin C.

2.2.2.4 Physical stability of the flower extracts loaded and not loaded in niosomes

A. Physical stability

The niosomal dispersions loaded and not loaded with the extract were put in vials covered with aluminium cap and stored at room temperature (27±2 ^{O}C), 4 ±2 ^{O}C and 45±2 ^{O}C for 3 months. The physical characteristics (color, sedimentation, morphology and particle size by DLS) as well as the pH values were determined at initial, 1, 2 and 3 months

B. Chemical stability

At initial, 1, 2 and 3 months, the samples were withdrawn and the remaining γ-linoleic acid and oleic and contents in the niosomal dispersion were determined by HPLC. The linoleic and oleic acid contents were determined from the standard curve. The experiment was done in triplicate. The marker used for the stability study was γ-linoleic acid.

2.2.3 Irritation study on rabbits' skin of the extracts and niosomes loaded with the extracts

2.2.3.1 Preparation of the animals

This study employed six male rabbits (New Zealand

White, 2.5-3.0 kg) from The Royal Project Foundation in Chiang Mai, Thailand to test for skin irritation.

2.2.3.2 Method

Animal care and handling throughout the experimental were performed in accordance to the CPCSEA guidelines. The fur on the back of the rabbits was shaved with an electrical clipper 24 hrs prior sample application. The area on the back of each rabbits was shaved prior to the experiment. The back was divided into 8 marked areas (6 cm^2) for topical application of the samples. Distilled water was used as a negative control and 15% sodium lauryl sulfate solution (SLS) was used as a positive control. An amount of 0.2 g of the samples was applied on the skin and covered with a gauze patch for 24 hrs. Then, the patches were removed and the tested site was rinsed with distilled water. The skin pigment (melanin and erythema) was measured by a Mexameter (Courage & Khazaka electronic GmbH, Germany) at 1, 24, 48 and 72 hrs after patch removal (Draize et al., 1944). Grading of the severity of erythema and edema formation was also investigated (Kirwin, 1984). The erythemal scores were recorded visually in ranging from 0 to 4 depending on the degree of erythema [no erythema = 0, slight erythema (barely perceptible-light pink) = 1, moderate erythema (dark pink) = 2, moderate to severe erythema (light red) = 3, severe erythema (extreme redness) = 4]. The degree of edema was recorded in ranging from 0 to 4 as the followings: no edema = 0, very slight edema (barely perceptible) = 1, slight edema (edges of area well defined by definite raising) = 2, moderate edema (raising approximately 1 millimeter) = 3, severe edema (raised more than 1 mm and extending beyond the area of exposure) = 4.

2.2.4 Cost estimation

2.2.4.1 The selected extract

The extract which gave the best and highest biological activity was selected to load in niosomes. The estimated cost of the selected extract was calculated by the same as the following topic.2.2.4.2

2.2.4.2 The selected niosomes loaded with the flower extract

Calculation of the production cost consisted of the following;

- Raw material cost
- Power supply cost
- Depreciation cost of the machines and equipments
- Labour cost

Cost per unit calculated by the total number of the consumption multiply by the unit cost and divided by the output as the following:

$$\text{Cost per unit} = \frac{(\text{QTT RM x Price})+(\text{QTT Power supply x Price})+ (\text{QTT work hour x Depreciation cost/Unit})+((\text{Work hours x labour Cost/hour})}{\text{Production yield}}$$

QTT : Quantity

RM : Raw materials