# 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.)
- Arbutin (4-hydroxyphenyl-β-D-glucopyranoside) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Butylated hydroxytoluene (BHT) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Calcium chloride ($CaCl_2$) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Carbopol® 980 (Fluka, Buchs, Switzerland)
- Cholesterol (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Cisplatin (Wako Pure Chemical Industries, Ltd., Osaka, Japan)
- Commercial diclofenac emulgel containing 1.16% (w/w) of DCFD salt equivalent to 1% (w/w) of diclofenac sodium (Local pharmacy in Chiang Mai, Thailand)
- Commercial gel containing 6% magnesium ascorbyl phosphate and 1% sodium hyaluronate solution (Local department store in Chiang Mai, Thailand)

- 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.)

- Diaion HP-20 (particle size 200–600 μm) (Mitsubishi Chemical Co., Tokyo, Japan)

- Diclofenac diethyammonium (DCFD) was a gift from Siam Pharmaceutical Co., Ltd., Thailand (90.0–110.0% label amount, USP 25)

- L-α-Dipalmitoyl phosphaditylcholine (DPPC) (Nikko Chemical Company, Tokyo, Japan)

- Dulbecco's modified eagle's medium (DMEM) (GIBCO, Grand Island, NY, U.S.A.)

- Eagle's minimal essential medium (MEM) (GIBCO, Grand Island, NY, U.S.A.)

- EDTA (ethylenediamine tetraacetic acid disodium salt dihydrate) (Fluka Co., Switzerland)

- Ethyl phenylpropiolate (EPP) (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.)

- 5-Fluorouracil (Wako Pure Chemical Industries, Ltd., Osaka, Japan)

- Gallic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Kojic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- α-Melanocyte-stimulating hormone (α-MSH) (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- $N^G$-monomethyl-L-arginine (L-NMMA) (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Octadecyl silica (ODS) gel (100–200 mesh) (Fuji Silysia Chemical, Ltd., Aichi, Japan)

- Phenylbutazone (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Phosphate buffered saline (Gibco BRL; Invitrogen, Burlington, ON, Canada)

- RPMI 1640, non-essential amino acid medium (NEAA) (GIBCO, Grand Island, NY, U.S.A.)

- Sephadex G50 (Fluka Chemicals, Gillingham, Dorset, U.K.)

- 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.)

- Span 60 (Sorbitan monostearate) (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Tris (hydroxymethyl)-methlamine (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Tween 61 (polyoxyethylene sorbitan monostearate) (Sigma Chemical Co., St. Louis, MO, U.S.A.)

- Tyrosine (Sigma Chemical Co., St. Louis, MO, U.S.A.)

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

- Other solvents (analytical grade)

**2.1.2 Cell lines**

- A549 (alveolar basal epithelial cells) (Riken Cell Bank, Tsukuba, Japan)

- AZ521 (stomach cancer cells) (Riken Cell Bank, Tsukuba, Japan)

- B16 (murine melanoma cells) (Riken Cell Bank, Tsukuba, Japan)

- HL60 (human promyelocytic leukemia) (Riken Cell Bank, Tsukuba, Japan)

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

- RAW264.7 (mouse monocyte macrophage) (European Collection of Cell Culture, ECACC; Porton Down, U.K.)

- SK-BR-3 (breast cancer cells) (Riken Cell Bank, Tsukuba, Japan)

**2.1.3 Animals**

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

- Sprague-Dawley rats (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand)

**2.1.4 Human volunteers**

- 31 Thai volunteers (13 men, 18 women, averaged age 33.00 ± 6.76 years, range 27–47 years, averaged BMI 23.11 ± 3.73)

**2.1.5 Equipments**

- Centrivap Console (Labconco, Kansas City, MO, U.S.A.)

- $CO_2$ incubator (Shel Lab, model 2123TC, U.S.A.)

- Corneometer® CM825 (Courage & Khazaka, Cologne, Germany)

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

- Dynamic light scattering (DLS), Zetasizer 300HSA (Malvern Instruments, Malvern, U.K.)

- Fractional collector (Foxy JR, Isco, Inc., Lincoln, U.S.A.)

- Gas Chromatography (GC Shimadzu GC-8A, Japan)

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

- Gradient HPLC (Senshu Scientific Co., Ltd., Tokyo, Japan)

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

- High-resolution (HR)-ESIMS (Agilent 1100 LC/MSD TOF system, Agilent Technologies, U.S.A.)

- Laminar 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.)

- Modified vertical Franz diffusion apparatus (Crown Bio Scientific, Inc., Sommerville, NJ, U.S.A.)

- NMR (JEOL JNM-ECX400, Tokyo, Japan)

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

- Probe sonicator (Vibra Cell™, Sonics & Materials Inc., Newtown, CT, U.S.A.)

- Reversed-phase column, Capcell Pak (C18) (Shiseido Co., Ltd., Tokyo, Japan)

- Rotary evaporator (R-124 Büchi, Switzerland)

- Silicon-based gum material, SILFLO (Flexico Developments Ltd., England)

- Spectrophotometer T80 UV/VIS spectrophotometer, PG instruments Ltd., China)

- Transmission electron microscope (TEM 1200S JEOL, JEOL Ltd., Tokyo, Japan)

- Transmitted light differential interference contrast attachment (Model CH No. 3E0245, Olympus Optical Co. Ltd., Japan)

- 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**

**2.2.1 Development of the modified proper elastic nanovesicular formulations loaded with the model drug (diclofenac diethylammonium; DCFD)**

2.2.1.1 Preparation of DCFD-loaded niosomal system

Eighteen elastic niosomal formulations were prepared by chloroform film method (Bangham, 1965) with sonication. DPPC, Span 60 or Tween 61 mixed with cholesterol (at 1:1, 1:1 and 3:7 molar ratios respectively) of 20 mM, were placed

in a clean, dry round bottom flask. The mixture was dissolved in chloroform. The organic solvent was removed by a rotary evaporator under vacuum. The resulting film was dried overnight under vacuum at room temperature (RT) (27 ± 2°C). The film was hydrated with an aqueous 0–50% ethanolic solution. The resulting dispersion was sonicated by a microtip probe sonicator at pulse on 3.0 and pulse off 1.0, 33% amplitude at 10°C for 15 min. The vesicular dispersions were kept in transparent vials tightly covered with aluminum caps. The most physical stable vesicles at 4 ± 2°C, 27 ± 2°C and 45 ± 2°C for 3 months were selected to load DCFD. To load DCFD in the select vesicles, DCFD was dissolved in chloroform together with other vesicular components and followed the preparation produce as aforementioned.

2.2.1.2 Physicochemical property determination

A. Morphology, vesicle size and zeta potential determination

A drop of niosamal dispersion was applied on a 300-mesh formvar copper grid on paraffin and allow the sample to adhere on the formvar for 10 min. The remaining dispersion was removed and a drop of 2% aqueous solution of ammonium molybdate was applied for 5 min. The remaining solution was then removed. The sample was air dried and examined with a TEM. The morphology and lamellarity of the bilayer vesicles were observed. The diameter of the empty and drug loaded nanovesicles were determined using DLS based on the photon correlation spectroscopy. The analysis ($n$ = 3) was carried out for 100 s at room temperature (27 ± 2°C). The charges on the empty and drug loaded vesicular surface were determined using Zetasizer 300HSA. The analysis time was kept for 60 s. The average zeta potential and charges were determined. The time-dependent correlation function on the

scattered light intensity was measured at a scattering angle of 90°. All samples were diluted 30 times with the freshly filtrated Millipore water for the particle size and zeta potential measurement.

B. Physiochemical stability studies

The selected elastic vesicles loaded with DCFD were stored in transparent vials covered with aluminum cap at 27 ± 2°C (room temperature), 45 ± 2°C and 4 ± 2°C for 3 months. The physical characteristics (color, sedimentation, morphology and particle size by DLS) of the dispersions and the remaining DCFD contents at 1, 2, 3, 4, 8, 12 weeks were determined by HPLC.

C. HPLC assay

The quantitative determination of DCFD were performed by HPLC using a ODS-Hypersil, Hichrom (250 × 4.6 mm i.d., 5 μm) column and a phosphate buffer/methanol (3:7, v/v) as a mobile phase delivered at 1 ml/min. An amount of 20 ml of the injection volume was eluted in column at 27 ± 2°C and monitored at 254 nm UV-detector. Under this condition, the retention time of DCFD was 12.7 min. Every sample was filtered through a 0.45 μm membrane filter, prior to injection onto the HPLC column. The peak areas of the samples were calculated and the concentrations of DCFD in the samples were determined from the standard curve. The experiment was done in triplicate. The standard curve of DCFD demonstrated linear with high correlation ($r^2$=0. 9989). The following regression equation was obtained: $y = 1.84 \times 10^4 x + 9427$, where $y$ was the peak area and $x$ was the quantity of DCFD (μg).

D. Quantitative analysis of ethanol by GC

The gel containing DCFD elastic niosomes at 27 ± 2°C, 45 ± 2°C and 4 ± 2°C were quantitatively analyzed for ethanol contents at initial and after 3 months. Ethanol contents were analyzed by GC equipped with flame-ionization detector and Restex Chromosorb (1.2 m × 5 mm) column; carrier gas: nitrogen gas 1.5 kg/cm$^2$. The temperature of the column was 270°C while the injector and detector were at 300°C. Under this condition, the retention time of ethanol was 3.0 min. The experiment was done in triplicates. The standard curve of ethanol demonstrated linear with high correlation ($r^2$ = 0.9988). The following regression equation was obtained: $y = 13623x - 163.06$, where $y$ was the detector response (mV) and $x$ was the quantity of ethanol (µl).

E. Measurement of elasticity values

Elasticity of the empty and drug loaded of both niosomes and elastic niosomes was carried out by the extrusion method (Cevc et al., 1997 and Jain et al., 2003). Briefly, the vesicles were extruded through a polycarbonate membrane filter with a pore size of 50 nm at constant pressure (2.5 bars). The elasticity of the vesicles was expressed in terms of deformability index according to the following equation:

$$\text{Deformability index} = j \times \left[\frac{r_v}{r_p}\right]^2$$

Where, $j$ was the weight of dispersion, which was extruded in 10 min through a polycarbonate filter of 50 nm pore size, $r_v$ was the size of the vesicle after extrusion and $r_p$ was the pore size of the filter membrane.

F. Entrapment efficiency determination

The loaded DCFD in the vesicles was separated from the not loaded drugs by gel filtration using Sephadex-G-50 as a packing material and purified water as an eluent. Eluates were collected in fractions using a fractional collector at the flow rate of 10 ml/min. The fractions containing the drug loaded vesicles which were detected at 470 nm (Miguel et al. 1997), were pooled, collected and dried with a Centrivap Console. The remaining residues were dissolved in absolute ethanol and assayed for the drug contents by HPLC. The entrapment efficiency was calculated according to the following equation:

$$\% \text{ Entrapment efficiency} = \frac{\textit{DCFD loaded in niosomes}}{\textit{Total DCFD in the niosomal dispersions}} \times 100$$

2.2.1.3 Preparation of gel containing DCFD loaded in elastic niosomes

The elastic and conventional Tween 61 niosomes loaded with DCFD were incorporated into a gel base containing Carbopol® 980. Briefly, gel containing 0.2% Carbopol® 980 was dispersed in the niosomal dispersion with gentle stirring to obtain the total DCFD equivalent to DCFS 1% (w/w).

2.2.1.4 *In vitro* skin permeation study

Rat skin permeation of DCFD from various gel formulations was studied using vertical Franz diffusion cells having the area between the donor and the receiver chamber of 2.46 $cm^2$ and the volume of the receptor compartment of 13 ml. The receptor compartment contained phosphate-buffered saline (PBS, pH 6.5) which was constantly stirred at 100 rpm with a small magnetic bar and in the jacket

controlled temperature at 32 ± 2°C throughout the experiment. The skin was prepared from the abdominal skin of male Sprague-Dawley rats with the body weights in the range of 150 to 200 g. After the abdominal skin of rat was shaved and carefully separated, subcutaneous fat was carefully removed using a scalpel. The freshly shaved skin of the rat was mounted on the receptor compartment with the subcutaneous side facing upwards into the donor compartment. The dermal side was in contact with the receiver medium. One gram of gel was placed into the donor compartment and covered with paraffin film. Experiments were done in triplicate. The cells were stopped at 15, 30 min, 1, 3 and 6 h. Samples in the receiver chamber were analyzed by HPLC. Drug present on the surface of the skin was removed by using the stripping method using 3M Scotch Magic™ tape (1 cm × 1 cm) described by Plessis et al. (1992). After stripping, the skin was cut into small pieces and the drug in the skin was extracted by absolute ethanol under sonication and the DCFD contents were assayed by HPLC.

2.2.1.5 *In vivo* anti-inflammatory assay

The anti-inflammatory activity of various DCFD gel formulations were studied by EPP induced rat ear edema model (Brattsand et al. 1982; Young et al. 1984). The male rats (Sprague-Dawley strain, 30–40 g) were fed with a standard diet ad libitum and housed in a temperature controlled room, in accordance to the European Union regulations (CEC council 86/809). The animals were divided into 6 different groups of three each. The first and the second group served as the positive (phenylbutazone) and negative (gel base) control, respectively. The third group received the gel base containing the unloaded drug. The fourth, fifth and sixth groups received the commercial DCFD emulgel, conventional niosomes loaded with DCFD

in gel and the elastic niosomes loaded with DCFD in gel, respectively. Ear edema was induced by the topical application of EPP at the dose of 1 mg/20 µl per ear to the inner and outer surfaces of both ears by an automatic microliter pipette. An amount of 0.02 g of the samples were applied topically just before EPP application. Before and at 15, 30, 45 min and 1 h after edema induction, the thickness of each ear was measured by a vernier caliper. The percentage of inhibition of the edema formation of the test samples was calculated and statistically evaluated (*t*-test, $p<0.05$) according to the following equation:

$$\text{Percentages of edema inhibition} = \frac{Tc - Tt}{Tc} \times 100$$

Where *Tc* = mean edema thickness (mm) of rat ear in gel base group; *Tt* = mean edema thickness (mm) of rat ear in the test sample.

**2.2.2 *In vitro* anti-aging activities of *Terminalia chebula* gall extract and Thai Lanna medicinal plant extracts**

2.2.2.1 Plant selection

Fifteen medicinal plants including *T. chebula* gall were selected from the Thai Lanna Medicinal Plant Textbook Database version "Manosroi II" developed by NPRDC, Science and Technology Research Institute (STRI), Chiang Mai University in Thailand. The frequent traditional use and scientific evidences for anti-aging and longevity indicated in the recipes were used to select the plants. The selected plants were collected from Chiang Mai Province in Thailand, during January to February in 2008 (**Table 9**). The specimen was authenticated by a botanist at NPRDC, STRI; Chiang Mai University in Thailand.

**Table 9** The 15 selected Thai Lanna plants

| Scientific name (Family) | Thai Lanna name | Part used |
|---|---|---|
| *Acorus gramineus* L. (Araceae) | Wan Nam | Whole plant |
| *Cassia fistula* L. (Fabaceae) | Ratcha Phruek | Fruit |
| *Cyperus rotundus* L. (Cyperaceae) | Ya Haeo Mu | Root |
| *Dregea volubilis* (L.f.) Benth. Ex Hook.f. (Asclepiadaceae) | Kra Thung Ma Ba | Leave |
| *Eclipta prostrate* L. (Asteraceae) | Ka Meng | Whole plant |
| *Myristica fragrans* Houtt. (Myristicaceae) | Chan Thet | Stem |
| *Nigella sativa* L. (Ranunculaceae) | Thian Daeng | Seed |
| *Plumbago indica* L. (Plumbaginaceae) | Chetta Mun Phloeng Daeng | Whole plant |
| *Piper nigrum* L.(Piperaceae) | Phrik Thai | Fruit |
| *Pellacalyx parkinsonii* Fisch. ST (Rhizophoraceae) | Kan Phlu | Flower |
| *Piper sarmentosum* Roxb. (Piperaceae) | Cha Phlu | Leave |
| *Plumbago zeylanica* L. (Plumbaginaceae) | Chetta Mun Phloeng Khao | Whole plant |
| *Terminalia chebula* Retz. (Combretaceae) | Kot Phung Pla | Gall |
| *Tinospora crispa* L. (Menispermaceae) | Boraphet | Stem |
| *Zingiber officinale* Roscoe (Zingiberaceae) | Khing | Rhizome |

2.2.2.2 Preparation of the extracts

The plants were washed, cut into pieces, dried at 40 ± 2°C in a hot air oven, ground to powder and kept in an airtight plastic bag at 4 ± 2°C until use. For the extraction process, 100 g of the dried plant powder were extracted using four different conditions. For HM (hot-methanol), the powder was extracted by continuous Soxhlet extraction for 1 h in 400 ml methanol until exhaust (65 ± 2°C). For HW (hot-water), the powder was heated for 1 h with 400 ml distilled water at 100 ± 2°C and then cooled to room temperature (27 ± 2°C). For CM (cold-methanol) and CW (cold-water), 100 g of the powder were macerated in 400 ml of methanol or distilled water

and sonicated in a bath sonicator for 1 h at room temperature (27 ± 2°C). The mixtures were filtered through Whatman No. 1 filter paper and the plant residues were re-extracted twice under the same conditions. The filtrates were pooled and concentrated under vacuum by a rotary evaporator, and lyophilized. The dried extracts were stored at 4 ± 2°C prior to use. Sixty extracts were obtained and the percentage yields were calculated on a dry weight basis.

2.2.2.3 Phytochemical test of the extracts

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 and 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.

2.2.2.4 Biological assays to evaluate the *in vitro* anti-aging activities of the extracts

A. DPPH free radical scavenging activity assay

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 assay

The $Fe^{2+}$ chelating ability of the extract was measured by the ferrous iron–ferrozine complex method (Decker and 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 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 from the three plants showing the highest activity of DPPH scavenging, chelating and tyrosinase inhibition activities were selected to test 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 $1.0 \times 10^5$ 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 on MMP-2 inhibition (Zymography)

The CM, CW, HM and HW extract of the three plants which showed the highest activity of human fibroblasts proliferation activity were selected to assay for gelatinolytic activity of MMP-2 inhibition in comparing to ascorbic acid. Monolayer of $5.0 \times 10^5$ 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 and 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).

**2.2.3 Biological activities of phenolic compounds isolated from galls of *Terminalia chebula* Retz. (Combretaceae)**

2.2.3.1 Fractionation and isolation of phenolic compounds from the gall extracts

The galls were washed, cut into small pieces, dried at 40 ± 2 °C in a hot air oven and ground to powder. One hundred grams of the dried powder was macerated and extracted with 400 ml of distilled water and sonicated in a bath sonicator for 1 h at room temperature (27 ± 2 °C). The extract was filtered and concentrated under vacuum by a rotary evaporator, and lyophilized. The yields of the extracts were 49.85% w/w of the dried plant material. Approximately 50 g of the extract was applied on a Diaion HP-20 column (8 × 90 cm) eluted with 50% methanol

(MeOH) in water to give 17.38 g. A portion of this fraction (8 g) was subjected to an ODS column (4 × 85 cm) eluted with MeOH/$H_2O$/acetic acid (AcOH) gradient to give 3 fractions. The eluted solvent of each fraction were as followed: fr.1: MeOH/$H_2O$/AcOH (20:80:1), fr.2: MeOH/$H_2O$/AcOH (40:60:1) and fr.3: MeOH/$H_2O$/AcOH (60:40:1). All fractions from Diaion and ODS columns were tested for their DPPH radical scavenging activity before further purification. Each fraction was purified by a preparative gradient HPLC. The HPLC was performed using the gradient HPLC system with a binary pump and UV-VIS detector. A 250 mm × 10 mm i.d., 5 µm reversed-phase column, Capcell Pak (C18), was used. The injection volume of the sample was 500 µl and eluted with a gradient system of acetonitrile (solvent-A): 0.1% formic acid (solvent-B) at 2 ml/min flow rate and the UV detection at 270 nm. The gradient system started with 35% solvent-A at 0 min and was linearly changed to 84% solvent-A in 35 min, with the total run time of 45 min. All fractions were further purified by an isocratic HPLC system with constant 20% solvent-A for 40 min.

2.2.3.2 Structure elucidation of the phenolic compounds isolated from the gall extracts

NMR spectra were recorded with a JEOL JNM-ECX400 ($^1$H, 400 MHz; $^{13}$C, 100 MHz) spectrometer in $CD_3COCD_3$ with or without $D_2O$, with tetramethylsilane (TMS) as an internal standard. ESIMS and high-resolution (HR)-ESIMS were recorded on an Agilent 1100 LC/MSD TOF system. The isolated compounds were identified by comparison with the chemical/physical data reported in the literature. Identification and NMR signal assignment were supported by the analysis of the $^{13}$C DEPT, $^1$H–$^1$H COSY, HMQC and HMBC data.

2.2.3.3 Biological assays

A. Free radical scavenging assay

Free radical scavenging activity of the isolated compounds was determined by DPPH with a slight modification of the method previously described (Akazawa et al., 2006). An amount of 10 µl of the samples in a DMSO, 200 µl of ethanol (EtOH), 190 µl of 0.1 M acetate buffer (pH 5.5), and 100 µl of 500 µM DPPH in EtOH were mixed in a test tube. For the negative control, DMSO was used instead of the sample solution. The reaction mixtures were mixed at 30°C for 30 min. The absorbance at 517 nm of the mixture was measured by a microplate reader. The experiments were done in triplicate. Ascorbic acid, α-tocopherol and BHT were used as reference compounds. The free radical scavenging activity was calculated according to the following equation as described in the determination of % scavenging.

B. Tyrosinase inhibition assay

The samples were assayed by the tyrosinase inhibition method previously described with slightly modification (Nerya et al., 2003). Briefly, 138.5 µl of 700 µM of tyrosine solution in 0.1 M McIlvaine phosphate buffer (pH 6.8) and 1.5 µl of five serial concentrations of the samples (at 1–500 µM dissolved in DMSO) were mixed in a 96-well microplate for 2 min. Then, 10 µl of tyrosinase enzyme solution (150 Unit) in McIlvaine phosphate buffer were added and incubated at room temperature (25 ± 1°C) for 5 min. After that, 50 µl of isopropanol was added and the absorbance was measured by a Sunrise-Basic microplate reader at 475 nm. Arbutin and kojic acid were used as reference compounds. The experiments were done in triplicate. The

inhibition percentages of tyrosinase were calculated according to the following equation as described in the determination of % Tyrosinase inhibition.

C. Inhibition of α-MSH induced melanin production on B16 murine melanoma

The B16 cells, plated at $5 \times 10^3$ cells/ well in a 24-well plate, were pre-incubated for 24 h. The samples dissolved in DMSO at the final concentration of 10–100 μM, and α-MSH (100 nM) were added to the medium and cultured for 96 h. The medium was removed and the cells were dissolved in 200 μl of 2 M NaOH in 10% DMSO. The amount of melanin was determined spectrophotometrically by a Sunrise-Basic microplate reader at the wavelength of 405 nm. The experiments were performed in triplicate. Arbutin and kojic acid were used as reference compounds.

D. Cytotoxic activity on human tumor cell lines

Cytotoxic activities of the 6 isolated phenolic compounds from *T.chebula* galls on human tumor cell lines (HL60, A549, AZ521 and SK-BR-3) were determined by MTT based colorimetric assay by the method previously described (Tabata et al., 2005) with slight modification. Briefly, cell lines ($3 \times 10^3$ cells/well) were seeded onto a 96-well culture plate with medium and maintained for 24 h. Then, the test samples dissolved in DMSO were added to the cell culture to give the final concentrations (1–100 μM). After 48 h incubation, MTT (10 μl, 5 mg/ml in phosphate-buffered saline) solution was added to the wells. After 3 h of incubation, isopropanol containing 0.04 M HCl was added to dissolve the produced formazan. The

absorbance of each well was then read at 570 nm (reference at 630 nm) using the Sunrise-Basic microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan). The optical density of formazan formed by the control cells was used as a reference. Cisplatin and 5-fluorouracil were used as positive controls. The percentages of cell proliferation in comparing to the control were calculated according to the following equation:

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

Where $A_{control}$ was the absorbance of the control, $A_{sample}$ was the absorbance of the sample and $A_{blank}$ was the absorbance of medium. The $EC_{50}$ value which was the effective concentration required for 50% of cell viability was determined.

E. Nitric oxide (NO) production from macrophages stimulated by LPS

Inhibitory effects of the samples on the NO production in LPS-activated mouse macrophages were evaluated by the method previously described (Tao et al., 2002) with slight modification. Briefly, RAW264.7 cells ($1 \times 10^5$ cells/well) were seeded in 100 µl of D-MEM and pre-cultured in the 96-well microplates at 37 °C in 5% $CO_2$ for 2 h. After addition of the fresh medium containing 2 µg/ml LPS and the samples dissolved in DMSO (1–100 µM) in each well, the mixture was further incubated for 16 h at 37 °C in 5% $CO_2$. The NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent. L-NMMA

was used as a reference. Experiments were performed in triplicate. Cytotoxicity was determined by MTT colorimetric assay after 16 h incubation with the test compounds. Percentages of inhibition (%) were calculated according to the following equation:

$$\% \text{ NO Inhibition} = \frac{A - B}{A - C} \times 100$$

Where, *A* was the absorbance of the system treated with LPS, *B* was the absorbance of the system treated with LPS and the sample and *C* was the absorbance of the system treated without LPS or the sample. $IC_{50}$ was determined graphically.

**2.2.4 The entrapment of the semi-purified fraction and gallic acid in the selected niosomal formulation**

2.2.4.1 Preparation of the semi-purified fraction containing gallic acid from *T. chebula* galls crude extract

The semi-purified fractions containing gallic acid were prepared as previously described (Manosroi et al., 2010b). Briefly, 100 g of the dried powder was macerated and extracted with 400 ml of distilled water and sonicated in a bath sonicator for 1 h at room temperature (27 ± 2 °C). The extract was filtered and concentrated under vacuum by a rotary evaporator, and lyophilized. The yields of the crude extracts were 49.85% w/w of the dried plant material. Approximately 50 g of the crude extract was applied on a Diaion HP-20 column (8 × 90 cm) eluted with 50% methanol (MeOH) in water to give 17.38 g. A portion of this fraction (8 g) was subjected to an ODS column (4 × 85 cm) eluted with MeOH/$H_2O$/acetic acid gradient

to give 3 fractions (250 ml of each fraction). The eluted solvent and the yield of each fraction were as followed: fr.1: MeOH/$H_2O$/AcOH (2:8:0.1), fr.2: MeOH/$H_2O$/AcOH (4:6:0.1) and fr.3: MeOH/$H_2O$/AcOH (6:4:0.1).The fraction which gave the highest total phenolic contents was selected in further study.

2.2.4.2 Determination of the phenolic components in the selected semi-purified fractions

The total phenolic contents of the 3 fractions from the ODS column were determined by a modified Folin-Ciocalteu method (Yeh and Yen, 2005), calibrating against gallic acid as the reference standard. Briefly, the fraction (500 µl) at the concentration of 10 mg/ml was mixed with an equal volume of 1N Folin-Ciocalteu reagent and 1 ml of 20% sodium carbonate ($Na_2CO_3$) and incubated for 25 min at room temperature (27 ± 2°C). The reaction mixture was then centrifuged at 5000 rpm for 10 min. The absorbance of the supernatant was measured at 730 nm using a spectrophotometer. The experiments were done in triplicate. The total phenolic contents were calculated on the basis of the calibration curve of the standard gallic acid. The semi-purified fraction which gave the highest total phenolic content was selected to load in niosomes. The phenolic compounds in the selected semi-purified fraction were analyzed using the gradient HPLC system with the evaporative light scattering detector (Sedex 55 ELSD system, Alfortville, France). The equipment consisted of a binary pump with a gradient controller and a degasser. The column used was a Capcell Pak AQ (5 µm, 250 × 4.6 mm i.d.). The injection volume of the sample was 20 µl and eluted by acetonitrile (solvent-A)/0.1% formic acid (solvent-B) at 0.5 ml/min. The gradient system started with 10% solvent-A at 5 min and then linearly changed to the 60% solvent-A in 75 min.

2.2.4.3 Entrapment of the semi-purified fraction and gallic acid in the selected elastic niosomes

The blank elastic and non-elastic niosomal formulations were selected from the best formulations in topic **2.2.1** and prepared by the modified chloroform film method previously described (Manosroi et al., 2008). Tween 61 mixed with cholesterol (at 1:1 molar ratios) of 20 mM were placed in a clean and dry round bottom flask. The mixture was dissolved in chloroform. The organic solvent was removed by a rotary evaporator under vacuum. The resulting film was dried overnight under vacuum at room temperature (27 ± 2°C). The film was rehydrated with 5 mM phosphate buffer (PB), pH 7.0 for non-elastic niosomes or 25% ethanol in PB for elastic niosomes. The niosomal dispersion was put in an ice bath (4°C) while sonicating by a microtip probe sonicator at pulse on 3.0 and pulse off 1.0, 33% amplitude for 10 min. The dispersions were kept in vials tightly covered with aluminum caps. For the loading of gallic acid or the semi-purified fraction, gallic acid or the semi-purified fraction dissolved in PB were used to hydrate the film to obtain the niosomal dispersion.

2.2.4.4 Physicochemical characteristics of the loaded niosomes

A. Morphology, vesicle size and zeta potential determination

The morphology, vesicle size and zeta potential were determined following the preparation produce as aforementioned above.

B. Measurement of elasticity value

Elasticity of the loaded or unloaded elastic and non-elastic niosomes was carried following the preparation produce as aforementioned above.

C. Entrapment efficiency determination

The entrapment efficiencies of the pure gallic acid or gallic acid containing in the semi-purified fraction loaded in elastic and non-elastic niosomes were determined by gel-filtration using Sephadex® G-50 as a packing material and phosphate buffer (pH 7.0) as an eluent. Eluates were collected in tubes using a fractional collector at the flow rate of 6 ml/min. The fractions containing niosomes detected at 470 nm (Castanho et al., 1997), were pooled, collected and dried with a freeze-dryer. The residues were dissolved in absolute ethanol and assayed for gallic acid contents by HPLC. The percentages of the entrapment efficiencies in the term of gallic acid were calculated according to the following equation:

$$\% \text{ Entrapment efficiency} = \frac{\textit{gallic acid amounts loaded in niosomes}}{\textit{total gallic acid in the niosomal dispersion}} \times 100.$$

2.2.4.5 Stability of the semi-purified fraction and gallic acid loaded in the selected elastic niosomes

A. Physical stability determination

The elastic and non-elastic niosomes loaded with the pure gallic acid or gallic acid in the semi-purified fraction were put in vials covered with aluminum cap and stored at room temperature (27 ± 2°C), 4 ± 2°C and 45 ± 2°C for 3 months. The physical characteristics (color, sedimentation, morphology and particle size by DLS) were determined at initial, 1, 2 and 3 months.

B. Chemical stability determination

The remaining gallic acid contents in the niosomal dispersion determined by HPLC at 0, 0.5, 1, 1.5, 2, 3 months, were investigated. Analysis of gallic acid was by HPLC, LC1200 UV/VIS detector and LC1100HPLC pump using Luna® C18 (10 µm i.d., 250 mm × 4.0 mm i.d.) Phenomenex USA column and a mobile phase containing MeCN/$H_2O$/AcOH (10:90:1, v/v) at the flow rate of 1 ml/min. The samples were filtered through a 0.45 µm membrane filter, prior to the injection onto the HPLC column. An amount of 20 µl of the samples was injected into the column and monitored at 254 nm UV detector. The retention time of gallic acid was 3.8 min. The gallic acid contents were determined from the standard curve of the standard gallic acid, which demonstrated linear with high correlation ($r^2$ = 0.9993). The following regression equation was obtained: $y = (9 \times 10^7)x + 510586$, where $y$ was the peak area and $x$ was the quantity of gallic acid (µg). The experiment was done in triplicate.

**2.2.5 Preparation of gel formulations containing the semi-purified fraction from *T. chebula* galls**

2.2.5.1 Selection of the gel base formulation

The gel was prepared by dispersing Carbopol® in the distilled water then gently stirred by a magnetic stirrer until homogeneity and stored in transparent glass bottles, then kept at 4 ± 2°C, 27 ± 2°C and 45 ± 2°C for 3 months. The physical appearances (uniformity, color, odor and pH) of the gel bases were examined every month. Gel base formulation which gave the best physical stability was selected for further studies.

2.2.5.2 Preparation of gel containing the semi-purified fraction loaded in niosomes

The elastic and non-elastic niosomes loaded with the pure gallic acid or gallic acid containing in the semi-purified fraction were incorporated into the selected gel base containing Carbopol® 980. Briefly, the 0.6% Carbopol® 980 gel was dispersed in the niosomal dispersion with gentle stirring, resulting in the gel containing 0.5% w/w of pure gallic acid, and the gel containing 0.08% w/w of gallic acid in the semi-purified fraction.

2.2.5.3 Physical stability determination

The gel containing non-elastic and elastic niosomes loaded with the pure gallic acid or the semi-purified fraction containing gallic acid 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 color, phase separation and particle size of niosomes determined by dynamic light scattering (DLS) and transmission electron microscope (TEM), respectively were evaluated at initial, 1, 2 and 3 months.

2.2.5.4 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.) Phenomenex USA column and a mobile phase containing MeCN/$H_2O$/AcOH (10:90:1, v/v) at the flow rate of 1 ml/min. The gel samples were diluted to 30 times with distilled water before filtered through a 0.45 µm membrane filter to obtain clear solution, prior to the injection onto the HPLC column. An amount of 20 µl of the samples was injected into

the column and monitored at 254 nm UV detector. The retention time of gallic acid was 3.8 min. The gallic acid contents were determined from the standard curve of the standard gallic acid, which demonstrated linear with high correlation ($r^2$ = 0.9993). The following regression equation was obtained: $y = (9 \times 10^7)x + 510586$, where $y$ was the peak area and $x$ was the quantity of gallic acid (µg). The experiment was done in triplicate.

**2.2.6 Transdermal absorption of gel containing niosomes loaded with gallic acid from *Terminalia chebula* Retz. (Combretaceae) galls**

2.2.6.1 Preparation of the rat skin

Full-thickness abdominal skin was obtained from the male Sprague-Dawley rats (body weights of 150 to 200 g). Hair on abdominal area was shaved off and left overnight. The rats were sacrificed and the abdominal skin was removed. The subcutaneous fat was carefully removed using a scalpel.

2.2.6.2 Formulations for transdermal absorption study

All formulations used in this study were listed in **Table 10**.

2.2.6.3 Transdermal absorption study

Rat skin permeation of phenolic compounds using gallic acid as a marker from various gel formulations was performed using vertical Franz diffusion cells having the contact area between the donor and the receiver chamber of 2.46 $cm^2$ and the volume of the receptor compartment of 13 ml. The freshly skin was mounted on the receptor

**Table 10** Descriptions and compositions of the samples in the transdermal absorption study

| Sample code | Gel sample descriptions | Compositions |
|---|---|---|
| GS | gel incorporated with free gallic acid | 0.5% gallic acid in gel |
| GN | gel incorporated with non-elastic niosomes loaded with gallic acid | 87% non-elastic niosomes loaded with 0.5% gallic acid- |
| GE | gel incorporated with elastic niosomes loaded with gallic acid | 87% elastic niosomes loaded with 0.5% gallic acid |
| SS | gel incorporated with the semi-purified extracts containing gallic acid | 0.5% semi-purified extracts containing 0.08% gallic acid in gel |
| SN | gel incorporated with non-elastic niosomes loaded the semi-purified extracts containing gallic acid | 87% non-elastic niosomes loaded with 0.5% semi-purified extracts containing 0.08% gallic acid - |
| SE | gel incorporated with elastic niosomes loaded the semi-purified extracts containing gallic acid | 87% elastic niosomes loaded with 0.5% semi-purified extracts containing 0.08% gallic acid |

compartment with the subcutaneous side facing upwards into the donor compartment, while the dermal side was in contact with the receiver medium. The receptor compartment contained phosphate-buffered saline (PBS, pH 6.5) which was constantly stirred at 100 rpm with a magnetic bar and in the jacket with the controlled temperature at 32 ± 2°C throughout the experiment. The gel (0.5 g) was placed into the donor compartment and covered with paraffin film. Cells were stopped at 1, 3, 6, 9 and 12 h. Samples in the receiver chamber were analyzed for gallic acid contents by HPLC.

2.2.6.4 Extraction of gallic acid from the skin strips and whole skin

The skin was removed from the cell and swung twice in 100 ml of distilled water. The rinsed water was collected and combined with the above remaining donor solution. The amount of gallic acid in the stratum corneum (SC) was

collected by stripping the skin with a 3M Scotch Magic™ tape (1 cm × 1 cm) (Plessis et al., 1992). Nine tapes were used for each skin sample and pooled in a 5-ml vial, containing 5 ml of absolute ethanol under sonication and assayed by HPLC. The amounts of gallic acid in the viable epidermis and dermis (VED) were determined by cutting the skin into small pieces, and pooled in a 5-ml vial, containing 5 ml of ethanol. Then, the subsequent steps followed the similar procedures as described in the determination of gallic acid contents in SC in topic **2.2.5.3**.

**2.2.7 *In vivo* anti-aging evaluation of gel containing niosomes loaded with phenolic compounds extracted from *Terminalia chebula* galls**

2.2.7.1 Rabbit skin irritation test by the closed patch test

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 to 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 5% 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 preformed at 24, 48 and 72 h according to Draize *et al.*

(1944) and adopted by OECD Test Guideline 404 (**Table 11**). 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 (**Table 12**). This study protocol has been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Please see appendix C).

**Table 11** Classification system for skin reaction by optical observation

| Reaction | Grade |
|---|---|
| ***Erythema*** | |
| No erythema | 0 |
| Very slightly erythema | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema to eschar formation | 4 |
| ***Edema*** | |
| No edema | 0 |
| Very slightly edema | 1 |
| Well-defined edema | 2 |
| Moderate edema (raising approximately 1 mm) | 3 |
| Severe edema (raising more than 1 mm) | 4 |

2.2.7.2 Skin anti-aging evaluation in human volunteers

A. Subjects and study protocol

A total of 31 Thai volunteers (13 men, 18 women, averaged age 33.00 ± 6.76 years, range 27–47 years, averaged BMI 23.11 ± 3.73) recruited from Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand were enrolled in the study.

**Table 12** Response categories of irritation in rabbit skin irritation test

| Category | Primary Irritation Index (PII) |
|---|---|
| Negligible | 0-0.4 |
| Slight irritation | 0.5-1.9 |
| Moderate irritation | 2.0-4.9 |
| Severe irritation | 5.0-8.0 |

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 four areas. Seven areas were treated with 0.2 ml of the 7 gel formulations listed in **Table 13**. The area without the treatment was used as a negative control. The gel formulations were applied on the areas twice daily for 8 weeks. The areas were measured for anti-aging activity before sample application and after application at 2, 4, 6 and 8 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 with out any drop-outs. This study protocol was reviewed and approved by the ethical committee of Faculty of Pharmacy, Chiang Mai University in Thailand.

**Table 13** Descriptions and compositions of the samples in skin anti-aging evaluation in human volunteers

| Sample code | Sample descriptions | Compositions |
|---|---|---|
| Gel base | gel without niosomes and the active compounds | gel base composition (Carbopol® 980, propylene glycol, triethanolamine, propyl paraben, methyl paraben) |
| GN | gel incorporated with non-elastic niosomes loaded with gallic acid | 87% non-elastic niosomes loaded with 0.5% gallic acid |
| GE | gel incorporated with elastic niosomes loaded with gallic acid | 87% elastic niosomes loaded with 0.5% gallic acid |
| SS | gel incorporated with the semi-purified extracts containing gallic acid | 0.5% semi-purified extracts containing 0.08% gallic acid in gel |
| SN | gel incorporated with non-elastic niosomes loaded the semi-purified extracts containing gallic acid | 87% non-elastic niosomes loaded with 0.5% semi-purified extracts containing 0.08% gallic acid |
| SE | gel incorporated with elastic niosomes loaded the semi-purified extracts containing gallic acid | 87% elastic niosomes loaded with 0.5% semi-purified extracts containing 0.08% gallic acid |
| Commercial product | gel incorporated with magnesium ascorbyl phosphate | 6% magnesium ascorbyl phosphate, panthenol, potassium hydroxide, propyl paraben, methyl paraben |

B. 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 low 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 8 weeks for each volunteer $i$, and $Q_{0i}$ was the quotient before application time for each volunteer $i$.

C. Skin surface microstructure measurement

Skin negative replicas were taken from the volar forearm surface area by applying a silicon-based gum material (SILFLO) according to the manufacturer's instructions. In order to obtain the replicas from the same skin area, the skin was marked using an oil-based marking pen. The skin surface microstructure was assessed using the image analysis software. Briefly, light was directed at a 208 angle and the images were observed from the replica using a CCD camera (Visioscan® VC98). The parameters of the replica image to evaluate skin surface microstructure including the level of wrinkle, the maximum roughness value (Rm) and the average roughness (Ra)

were analyzed by CK Visiometer® SV600 FW; image analyzing program Version 1.6.6.1 software, which can scan the microrelief from replicas using a light transmission method. The parameter changes (%) were calculated the following equation as described in the determination of % parameter changes in topic skin elasticity measurement.

D. 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.

E. Skin erythema and pigmentation 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.

**2.2.8 Statistical analysis**

All data of this study results were presented as mean ± SD. Student's paired *t*-test, the Kruskall-Wallis test, and the Wilcoxon Signed-Rank test were used to evaluate the significance of differences at the significant level of $p$-value < 0.05. Statistical analysis was performed using SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA).