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.)
- Benzalkonium chloride (BZKC) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Benzethonium chloride (BZT) (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)
- Cetyl trimethyl ammonium bromide (CTAB) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Cetylpyridinium chloride (CPC) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Cholesterol (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Coomassie® Brilliant Blue G-250 (Bio-Rad Laboratories, Hercules, CA, U.S.A.)
- Commercial cyanoacrylate glue (Altego®, Osaka, Japan)

- Didecyl dimethyl ammonium bromide (DDAB) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- DNA 1 kb ladder (Fermentas, St. Leon-Rot, Germany)- 1,6 diphenyl-1,3,5-hexatriene (DPH) (Wako Pure Chemical Industrial Ltd., Osaka, Japan)
- 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.)
- Dutasteride (Ka-Shing Business Macau Co., Ltd., Macau, China
- Eagle's minimal essential medium (MEM) (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.)
- Finasteride (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Folin–Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Gallic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Kojic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Linoleic acid (Wako Pure Chemical Industrial Ltd., Osaka, Japan)
- Gamma-linolenic acid (99.5%) (Tokyo Chemical Industrial Ltd., Tokyo, Japan)

- Oleic acid (Wako Pure Chemical Industrial Ltd., Osaka, Japan)
- Penicillin-streptomycin for cell culture (PAA, Pasching, Austria)
- Phosphate buffered saline (Gibco BRL; Invitrogen, Burlington, ON, Canada)
- RNA extraction kit, Nucleospin RNA II (Macherey-nagel, Düren, Germany)
- RPMI 1640, non-essential amino acid medium (NEAA) (GIBCO, Grand Island, NY, U.S.A.)
- Siliga gel 60 (Merck, Damstadt, Germany)
- 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.)
- Stearylamine (SA) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Sulphorodamine B (SRB) (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- SuperScript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen, CA, U.S.A.)
- TAE buffer (0.04M Tris, 0.04M Acetate, 0.001M EDTA)
- Tris (hydroxymethyl)-methlamine (Sigma Chemical Co., St. Louis, MO, U.S.A.)
- Trypsin 0.25% (Gibco, Invitrogen Corp., Carlsbad, CA, USA)
- 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.)
- Quant-iT™ dsDNA assay kit (Invitrogen, CA, U.S.A.).

- Quant-iT^{TM} RNA BR assay kit (Invitrogen, CA, U.S.A.).
- Other solvents (analytical grade)

2.1.2 Cell lines

- Normal human skin fibroblasts (Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand)
- Human prostate carcinoma cell line (DU-145) was kindly provided by the College of Science and Technology, Nihon University in Tokyo, Japan

2.1.3 Animals

- New Zealand white rabbits (Faculty of Agriculture, Chiang Mai University, Thailand)
- C57BL/6 mice (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand)

2.1.4 Equipments

- Blender (Twist HR 1701, Philips, Indonesia)
- Centrifuge (Univeral 32 R, Hettich Zentrifugen, Germany)
- CO_2 incubator (Shel Lab, model 2123TC, U.S.A.)
- Differential scanning calorimeter (DSC 8230, Rigaku Co., Tokyo, Japan)
- Dynamic light scattering (DLS), Zetasizer 300HSA (Malvern Instruments, Malvern, U.K.)
- Freeze fracture device (FR-7000A, Hitachi High-tech)
- Gel documentation system and Quantity 1-D analysis software (Bio-Rad Laboratories, U.K.)
- Gel electrophoresis (SUB-CELL® GT, BioRad Laboratories, Milan, Italy)

- High perfomance liquid chromatography (HPLC, AS 1000, Thermo Finigan, U.S.A.)
- iCycler PCR (BioRad Laboratories, USA)
- 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.)
- Microtip probe sonicator (Banderlin Sonoplus GM70, Berlin, Germany)
- Modified vertical Franz diffusion apparatus (Crown Bio Scientific, Inc., Sommerville, NJ, U.S.A.)
- pH meter (Cyberscan pH 510, Euteoh Instruments, Singapore)
- Probe sonicator (Vibra Cell™, Sonics & Materials Inc., Newtown, CT, U.S.A.)
- Qubit fluorometer (Invitrogen, CA, U.S.A.)
- Rotary evaporator (R-124 Büchi, Switzerland)
- SAXSess camera (Anton-Paar, Graz, Austria)
- Spectrofluorophotometer (RF-5300PC, Shimadzu, Japan)
- Spectrophotometer (T80 UV/VIS spectrophotometer, PG instruments Ltd., China)
- Supercritical carbon dioxide fluid apparatus ($scCO_2$) (SFE-500MR-2-C50 System, Thar Instruments, Inc. Pittsburgh, U.S.A.)

- 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)
- Ultracentrifuge (Himac CS100GXII, microultracentrifuge, Hitachi Koki Co. Ltd., Japan)
- 96-Well microplate (Nalge Nunc International, NY, U.S.A.)
- 6 Well plates (Nunc®, USA)
- 96 Well plates (Nunc®, USA)
- X-ray generator (PW3830, PANalytical, operation at 40 kV and 50 mA).
- Zetasizer nano series (Nano-S, Malvern instrument Ltd., Malvern, UK)

2.2 Methods

The scope of this study was divided into 6 parts as the scheme in **Figure 12**

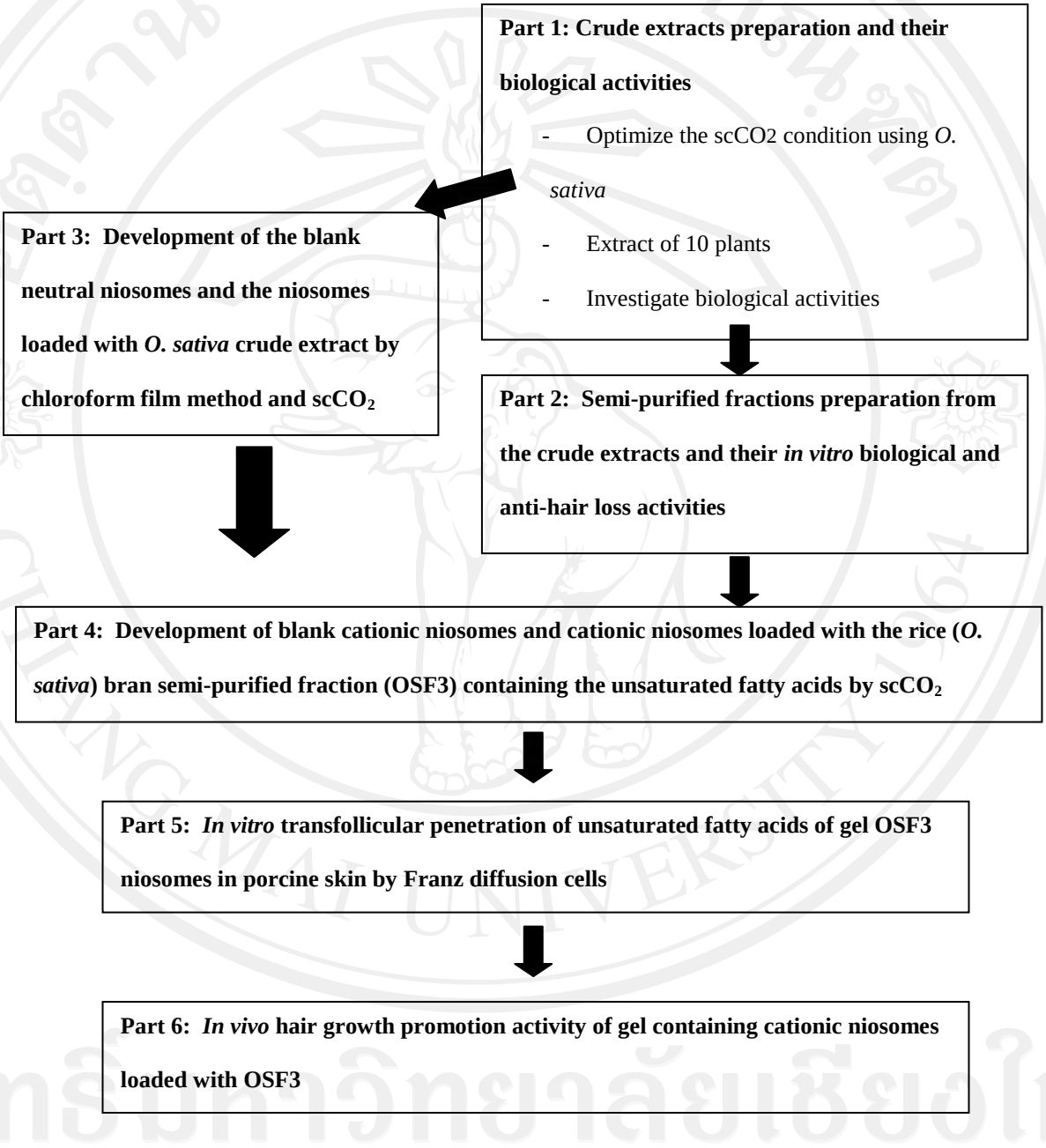

Figure 12 Scope of the study

Part 1: Crude extracts preparation and their biological activities

1.1 Plant crude extract

1.1.1 Plant samples

Parts of the ten edible plants which have been searched from the literature reviews to contain high amount of unsaturated fatty acids (gamma-linolenic acid, linoleic acid and oleic acid) were collected from Chiang Mai Province in Thailand during October to November in 2008. The plant seeds used in this study were packed in vacuum plastic bags and purchased from Thai Cereals World Co., Ltd., Bangkok, Thailand. The % moisture contents in the seeds were in the range of 6-10%. The specimen samples were authenticated by a botanist at the Natural Products Research Development Center (NPRDC), Science and Technology Research Institute (STRI) at Chiang Mai University, Chiang Mai in Thailand.

1.1.2 Plant preparation

All plant parts were seeds, except *O. sativa* and *C. tinctorius* were bran and flower, respectively. The bran of *O. sativa* was passed through sieve No. 25 (0.707 mm). Other plant parts were ground into small pieces by a blender and passed through sieve No. 20 (0.841 mm). The plant powder was kept in a tight container at 4^{o}C until use.

1.1.3 Maceration method

Briefly, 200 grams of the plant powder were macerated with 1 L of 95% v/v ethanol at room temperature (27 ± 2^{o}C) for 8 hours and stirred every 2 hours. The extract was filtered through the paper filter Whatman No. 1, connected with a vacuum pump. The residues were re-extracted more by the same process twice. All

filtrates were collected, pooled and dried by a rotary evaporator at 40°C. The crude extracts were kept at -80°C until use.

1.1.4 Supercritical carbon dioxide fluid technique

1.1.4.1 Optimization of the extraction condition

The bran of *O. sativa* (rice bran) was selected to be a representation for the optimization of the $scCO_2$extraction conditions. Briefly, 200 grams of the rice bran were extracted by $scCO_2$ with 10-35% (w/v) of 95% (v/v) ethanol as a co-solvent. The temperature in the collecting vessel was raised to 40°C. Carbon dioxide gas was introduced into the collecting vessel and the pressure was maintained at 200 bar. After 2 hours, the pressure was released and the extract was collected. The rice bran residues were re-extracted by the same procedure for 4 times. The total exposure time of the rice bran to $scCO_2$ was 8 hours. All extract were collected, pooled and dried by a rotary evaporator at 40°C. The dried extracts were kept at -80°C until use.

1.1.4.2 Supercritical carbon dioxide fluid extraction

Briefly, 200 grams of the plant powder were put in the supercritical carbon dioxide fluid apparatus ($scCO_2$) together with 25% (w/v) of 95% (v/v) ethanol as a co-solvent, in the chamber at 40°C and 200 bar (Manosroi et al., 2010). After 2 hours, the pressure was released and the extract was collected. The plant extract residues were re-extracted more by the same procedure 3 times. All extracts were collected, pooled, mixed and dried by a rotary evaporator at 40°C. The crude extracts were kept at -80°C until use.

1.2 Determination of bioactive compounds and biological activities of the crude extract

1.2.1 Determination of unsaturated fatty acid contents in the extracts by HPLC

Gamma-linolenic acid, linoleic acid and oleic acid contents in the extracts were determined by HPLC (Luna® C18 10 micron 250 x 4.60 mm Phenomenex USA Column, LC1200UV/VIS Detector and LC1100HPLC pump) using the mobile phase of 95% v/v acetonitrile mixed with 5% v/v of 0.1% v/v glacial acetic acid, injection volume at 20 µl, flow rate of 1 ml/min and the UV detector at 205 nm. The gamma-linolenic acid, linoleic acid and oleic acid contents in the extracts were determined from the HPLC chromatogram in comparing to the standard gamma-linolenic acid, linoleic acid and oleic acid, respectively.

1.2.2 Determination of total phenolic contents (TPC)

Total phenolic contents (TPC) in the form of gallic acid in the extracts were determined using the Folin–Ciocalteu reagent (Zhou et al., 2004). Briefly, 100 mg/ml of the extracts were mixed with Folin–Ciocalteu reagent and 20% w/v of sodium carbonate (Na_2CO_3) at ambient temperature (27±2ºC). After incubation for 30 minutes, the absorbance of blue color developed in each assay mixture was recorded at 760 nm by a well reader. The TPC of the extracts were expressed in mg of gallic acid equivalents (GAE) per gram of extract.

1.2.3 Phytochemical test of the extracts

The extract (20 mg), dissolved in 20 ml of 80% methanol, was used for detecting the presence of alkaloids, anthraquinones, carotenoids, flavonoids, glycosides, 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 carotenoids, 2 ml of the extract solution were mixed with 2 ml of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85 % sulphuric acid was added. A blue colour at the interface showed the presence of carotenoids (Ajayi et al., 2011). *Daucus carota* Linn extract 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 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 (Onwukaeme et al., 2007;

VanMiddlesworth and Cannell, 1998). 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.

1.2.4 Antioxidative activities of the extracts

1.2.4.1 DPPH radical scavenging assay

Free radical scavenging activities of the extracts, standard antioxidants (vitamin C and E) and standard unsaturated fatty acids were determined by a modified DPPH assay (Tachibana et al., 2001). Briefly, 50 µl of five serial concentrations of the extracts or the standard unsaturated fatty acids including gamma-linolenic acid, linoleic acid and oleic acid (at 0.01-100 mg/ml) dissolved in 95% v/v ethanol and 50 µl of ethanol solution of DPPH were put into each well of a 96-well microplate. The reaction mixtures were allowed to stand for 30 minutes at 27 ± 2^{o}C, and the absorbance was measured at 515 nm by a well reader against a blank (95% v/v ethanol). Vitamin C and E (0.001-10 mg/ml) were used as positive controls. The experiments were done in triplicate. The percentages of radical scavenging activity were calculated as follows:

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

, where A was the absorbance of the control and B was the absorbance of the sample. The sample concentrations providing 50% of scavenging (SC_{50}) were calculated from the graph plotted between the percentages of scavenging and the sample concentrations.

1.2.4.2 Lipid peroxidation inhibition activity

The antioxidant activity of the extracts was assayed by the modified Ferric-thiocyanate method (Osawa and Namiki, 1981). An amount of 50 µl of five serial concentrations of the extracts and the standard unsaturated fatty acids at 0.01-100 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 (5 mM) and 50 µl of $FeCl_2$ (2 mM). The mixture was incubated at 37 ± 2°C in a 96-well microplate for 1 hour. During the oxidation of linoleic acid, peroxides are formed leading to the oxidation of Fe^{+2} to Fe^{+3}. The latter ions forming a complex with thiocyanate can be detected at 490 nm. The solution without the sample was used as a negative control. Vitamin C and E at 0.001-10 mg/ml were used as positive controls. All determinations were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation:

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

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

1.2.4.3 Metal ion chelating assay

The metal ion chelating activity of the extracts was assayed by the modified ferrous ion chelating method (Decker and Welch, 1990). Briefly, 100 µl of five serial concentrations of the extracts or the standard unsaturated fatty acids at 0.01-100 mg/ml dissolved in 95% v/v ethanol 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 27 ± 2°C for 15 minutes. 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 contains $FeCl_2$ and ferrozine, which were complex formation molecules. 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] x 100

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

1.2.5 Tyrosinase inhibition assay

The tyrosinase inhibition activity of the extracts was assayed by the modified dopachrome method using tyrosine as a substrate (Shimizu et al., 1998). Briefly, 50 µl of five serial concentrations of the extracts or the standard unsaturated fatty acids at 0.01-100 mg/ml dissolved in DMSO, 50 µl of 200 units tyrosinase solution in 0.1M phosphate buffer, and 50 µl of 1 mg/ml tyrosine solution in 0.1M phosphate buffer, and 50 µl of 0.1M phosphate buffer were added into a 96-well plate. The mixture was incubated at 37 ± 2°C for 60 minutes and the absorbance at 450 nm was measured. Vitamin C and kojic acid (0.001-10 mg/ml) were used as positive controls. All experiments were performed in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation:

Tyrosinase inhibition activity (%) = [(A-B)-(C-D)/(A-B)] x 100

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

1.2.6 Cytotoxicity on aged human skin fibroblasts of the extracts

1.2.6.1 Cell culture

The normal human skin fibroblasts were provided by Dr. Natthanej Luplertlop at the Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Cells were cultured under standard conditions in the complete culture medium containing α-modified Eagles culture medium (MEM-Alpha) supplemented with 10% v/v fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated in a temperature-controlled and humidified incubator with 5% CO_2 at 37°C. Cells were used at the 30^{th} passage.

1.2.6.2 Cytotoxicity by the SRB assay

The extracts were tested for cell proliferation of the aged normal human skin fibroblasts (30^{th} passage) by the SRB assay as previous described (Papazisis et al., 1997). Vitamin C (0.00001 - 1 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 overnight for cell attachment on the plate in 5% CO_2 at 37°C. Cells were then exposed to five serial concentrations of the extracts (0.00001- 1 mg/ml) for 24 hours. 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 experiments were done in triplicate. The percentages of cell proliferation were calculated according to the following equation:

Cell proliferation activity (%) = (A-B / C-B) × 100

, where A was the absorbance of the sample, B was the absorbance of the blank and C was the absorbance of the control. Stimulation index (SI), the ratio between the percentages of cell proliferation treated with the extracts (at 0.1 mg/ml) and the control (no treatment), was presented.

Part 2: Semi-purified fractions preparation from the crude extracts and their *in vitro* biological and anti-hair loss activities

2.1 Preparation of the semi-purified fraction containing unsaturated fatty acids from *O. sativa, C. tinctorius* and *S. bicolor* crude extracts

The semi-purified fractions of the three selected crude extracts, including *O. sativa*, *C. tinctorius* and *S. bicolor* from the $scCO_2$ process which gave the highest unsaturated fatty acid contents and antioxidative activities were prepared as previously described with modification (Chatterjee et al., 2007). Briefly, 25 grams of the crude extracts were loaded on the 750 grams of silica gel 60 column (4 cmϕ x100 cm) and eluted with petroleum ether/ethyl acetate (8:1) at the flow rate of 1 ml/min. Each fraction of 100 ml was collected and the solvent was evaporated by a rotary evaporator. The total of 30 fractions were obtained. Each dried fraction was analyzed for unsaturated fatty acid contents by HPLC with the previously described method (Manosroi et al., 2010). The consecutive fractions which contained the same unsaturated fatty acids were pooled and evaporated. There were four dried fractions from each crude plant extracts. The percentages yields, the unsaturated fatty acids and the total phenolic contents of the dried fractions were determined.

2.2 Biological activities of the semi-purified fractions

The semi-purified fractions were tested for the DPPH radical scavenging, lipid peroxidation inhibition, metal ion chelating, tyrosinase inhibition and cell proliferation activity on the aged normal human skin fibroblasts with the previously described methods (section 1.2.1, 1.2.2, 1.2.4, 1.2.5 and 1.2.6).

2.3 Cytotoxicity of the semi-purified fractions on DU-145 cell line

2.3.1 Cell culture

The human prostate carcinoma cell line (DU-145) was provided by Prof. Dr. Toshihiro Akihisa at the College of Science and Technology, Nihon University in Tokyo, Japan. Cells were cultured under the standard conditions in the complete culture medium containing RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were incubated in a temperature-controlled and humidified incubator (Shel Lab, model 2123TC, U.S.A.) with 5% CO_2 at 37°C.

2.3.2 Cytotoxicity by the SRB assay

The semi-purified fractions were tested for cytotoxicity on DU-145 cells by the SRB assay as previous described (Papazisis et al., 1997). The standard unsaturated fatty acids (gamma-linolenic acid, linoleic acid and oleic acid), the standard finasteride and dutasteride at 0.0001 - 1 mg/ml were used as positive controls. 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 37°C. Cells were then exposed to five serial concentrations of the crude extracts and their semi-purified fractions (0.00001- 1 mg/ml) for 24 hours. 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 microplate reader. The experiments were done in triplicate. The percentages of cell proliferation were calculated according to the following equation:

$$\text{Cell viability (\%)} = (\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}}) \times 100$$

The concentrations of the samples which gave % cell viability of more than 90% were used in the 5α-reductase inhibition experiment.

2.3.3 Inhibition of 5α-reductase activity

2.3.3.1 Cultivation of the cells

The pellets of human DU-145 cells were plated onto the 6-well plates separately at the density of 2.5×10^5 cells/well, incubated with 10% (v/v) FBS-DMEM medium containing penicillin (100 U/ml) and streptomycin (100 mg/ml) in a 5% CO_2 incubator at 37°C. Cells were then exposed to the semi-purified fractions, the standard finasteride and dutasteride at the final concentration of 0.1 mg/ml and the standard unsaturated fatty acids at 0.1-0.001 mg/ml for 24 hours. The medium were removed, and the cells were washed with PBS, trypsinized with 0.25% trypsin solution for 2 min and suspended in PBS.

2.3.3.2 Total RNA extraction

The total RNA from the cell pellets was extracted by the RNA extraction kit according to the instructions of the manufacturer. The concentration of the total RNA was quantified by Qubit Fluorometer and Quant-iT™ RNA BR assay kit. The total RNA solution was kept at -20°C until used.

2.3.3.3 Reverse transcription-polymerase chain reaction (RT-PCR)

The 5α-reductase type 1 and 2 genes were amplified from the extracted RNA by SuperScrip™ One-Step RT-PCR with Platinum® Taq kit according

to the manufacturer's protocol. Briefly, five micrograms of the total RNAs were reverse transcribed with RT/ Platinum Taq® mix and subjected to PCR cycles with the primers for human 5α-reductase type 1 and 2 (SRD5A1 and 2) as follows: 94ºC for 15 s, 55ºC for 30 s, 72ºC for 45 sec for 35 cycles. The human 5α-reductase type 1 primers were designed based on GenBank accession no. NM_001047.2 and NM_000348, respectively, with a forward (5'- CCA TGT TCC TCG TCC ACT AC-3') and reverse (5'- TTC AAC CTC CAT TTC AGC GT -3'), produced 707 bp amplicon and human 5α-reductase type 2 (SRD5A2) forward (5'- GGG TGG TAC ACA GAC ATA CG-3') and reverse (5'- TCA CGA CTA TGA GGA GAG GG-3'), produced 938 bp amplicon (Torres and Ortega, 2004). The RT-PCR products were loaded on 1% agarose gel in the 1X tris-acetate-EDTA (TAE) buffer chamber at 100 V for 30 min. The human 5α-reductase type 1 and 2 dsDNA samples were quantified by the Qubit fluorometer and Quant-iT™ dsDNA assay kit.

Part 3: Development of blank neutral niosomes and the niosomes loaded with *O. sativa* crude extract by chloroform film method and $scCO_2$

3.1 Niosomes preparation

3.1.1 The chloroform film method with sonication (cm)

The total amounts of 20 mM of Tween 61 mixed with cholesterol (at 1:1 molar ratio) together with the *O. sativa* crude extract at various concentrations (0.06, 0.12 and 0.25 % w/w) were dissolved with chloroform in a round bottom flask. The solvent was removed by a rotary evaporator to get a thin film and the residual solvent was dried overnight in a vacuum desiccator. An amount of 20 ml of distilled water was added to the film and mixed at 50 ± 1°C for 15 minutes by a rotary

evaporator. The dispersion put in the ice bath was sonicated for 2 minutes by an ultrasonic generator.

3.1.2 The supercritical carbon dioxide fluid technique with sonication ($scCO_2$)

The conditions for niosome preparation by the $scCO_2$ method were performed as previously described (Imura et al., 2003; Manosroi et al., 2003; Otake et al., 2001). The total amounts of 20 mM of Tween 61 mixed with cholesterol (at 1:1 molar ratio) together with the extract (0.06, 0.12 and 0.25 % w/w) were added into the view cell. The volume of the view cell was about 20 cm^3. The temperature in the cell was raised to 50°C and the CO_2 gas was introduced into the view cell. The pressure and temperature in the view cell were maintained at 200 bar and 50 ± 1°C, respectively. The pressure was released after 30 minutes. The niosomal dispersion was obtained and further put in an ice bath to sonicate for 2 minutes by an ultrasonic generator.

3.2 Physical characteristics of the niosomes

3.2.1 The maximum loading of the extracts in niosomes

The concentrations of the extract entrapped in niosomes were increased from 0.06 to 4.00 % w/w. The maximum loading of the extracts in niosomes was determined from the maximum concentration of the extracts which gave no precipitation.

3.2.2 Particle size

The particle size of niosomes was measured by the dynamic light scattering (DLS) apparatus. The niosomal dispersions were diluted to 10 times with

distilled water. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90° and wavelength at 535 nm.

3.2.3 Morphology

The morphology of niosomes was investigated by the freezed fracture (FF) TEM. The niosomal dispersion was immediately frozen in liquid ethane and then the frozen sample was fractured in a freeze-replica apparatus at −150°C. The sample replica was prepared with platinum–carbon shadowing, followed by initial platinum shadowing. After acetone-water cleaning, the replica was set on a TEM copper grid coated with a holey carbon film. FF-TEM measurements were made using a Hitachi H-7650 at an accelerating voltage of 120 kV under a low electron dose.

3.2.4 Transition temperature of niosomes

Thermal analysis of the niosomal dispersion was determined by differential scanning calorimeter. Distilled water was used as a reference. Twenty microliters of the niosomal dispersion were placed in the aluminium sample pan, which was then carefully sealed with the cap. The measurement condition was at 1K/min for scanning rate, 20-90°C for the scanning range and 0.1 mcal/s for the sensitivity.

3.2.5 Microviscosity of the niosomal membrane

DPH (1,6 diphenyl-1,3,5-hexatriene) in tetrahydrofuran (THF) was used as a fluorescent probe. The molar ratio of niosomes to DPH was 300:1. One millimolar of DPH in THF was added to 10 ml of the niosomal dispersion. The mixture was incubated for 24 h at 25 ± 1°C. The microviscosity of niosomal

membrane was determined by fluorescence polarization (*P*) which was calculated according to the following equation:

$$\text{Fluorescence polarization} = (I_p - GI_v) / (I_p + GI_v)$$

, where, I_p and I_v were the fluorescence intensity of the emitted light polarized parallel and vertical to the exciting light, respectively, and *G* was the grating correction factor (Prendergast et al., 2002; Roy et al., 2005). The fluorescence intensities, I_p and I_v, were measured at various temperatures (30-90°C) by a spectrofluorophotometer. The excitation and emission wavelength were 350 and 450 nm, respectively.

Part 4: Development of blank cationic niosomes and cationic niosomes loaded with the rice (*Oryza sativa*) bran semi-purified fraction (OSF3) containing the unsaturated fatty acids by $scCO_2$ technique

4.1 Selection of the niosomal formulation

4.1.1 Niosome preparation

4.1.1.1 Loading of OSF3 in niosomes by supercritical carbon dioxide fluid technique with sonication ($scCO_2$)

Niosomes were prepared by the $scCO_2$ method as previously described with some modifications (Manosroi et al., 2010). For blank niosomes, the composition at 20 mM of Tween 61 mixed with cholesterol and cationic surfactants (CTAB, CPC, SA, BZKC, BZT and DDAB) at 1:1:0.05, 1:1:0.25 and 1:1:0.5 molar ratios, respectively was added into the view cell of the $scCO_2$ apparatus. The volume of the view cell was about 100 cm^3. The temperature in the cell was raised to 50°C and the CO_2 gas was introduced into the view cell. The pressure and temperature in the view cell were maintained at 250 bar and 50 ± 1°C, respectively. The pressure

was released after 30 minutes. The niosomal dispersion was collected in a test tube placing in an ice bath (4°C) and sonicated by a microtip probe sonicator at 40% amplitude for 5 min.

For niosomes loaded with OSF3, the same condition was used as the blank niosomes (250 bar, 50°C and 30 minutes), except the 2% (w/v) of OSF3 was adding in the $scCO_2$ view cell together with Tween 61 mixed with cholesterol and the cationic surfactant.

4.1.1.2 The maximum loading of OSF3 in niosomes

The concentrations of the OSF3 loaded in niosomes were increased from 1.00 to 4.00 % (w/v). The maximum loading of the OSF3 in niosomes was determined from the maximum concentration of the OSF3 which gave no precipitation or oil separation layer.

4.1.2 Physicochemical characteristics of the blank and loaded niosomes

The niosomes loaded and not loaded with OSF3 were stored in transparent vials covered with aluminum cap at 4±2, 25±2 and 45±2 °C for 3 months. At initial and 1, 2, 3 months, the samples were withdrawn and assayed for physical characteristics (appearance, particle size and zeta potential) and the unsaturated fatty acid contents by HPLC.

4.1.2.1 Physical characteristics of niosomes

A. Appearances

The blank and OSF3 loaded niosomes were investigated for the sedimentation, separation layer and color optically at initial and 1, 2, 3 months.

B. Particle sizes

The particle sizes of blank and OSF3 loaded niosomes were measured by dynamic light scattering (DLS) by the Zetasizer 300HSA based on photon correlation spectroscopy. The niosomal dispersions were diluted to 15 times with distilled water. The diameters of blank and loaded niosomes were carried out for 100 s at room temperature (25±2 ◦C). The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90° and wavelength at 535 nm.

C. Zeta potential

The charges of blank and OSF3 loaded niosomes were determined using the 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 15 times with freshly filtrated Millipore water for the particle size and zeta potential measurement.

4.1.2.2 Chemical stability of the unsaturated fatty acids in OSF3 loaded in niosomes

The remaining unsaturated fatty acid contents in OSF3 in the loaded niosomes were determined at 0, 1, 2 and 3 months in comparing to the OSF3 solution which dispersed in 70% (v/v) propylene glycol. The 500 µl of the OSF3 loaded niosomes or OSF3 solution were mixed with 1000 µl of the extraction mixture [50% (v/v) triton X-100 and 50% (v/v) of 95% v/v of acetonitrile with 0.1 % glacial acetic acid]. Then, the unsaturated fatty acid contents were determined by HPLC using the same condition as previously described (Manosroi et al., 2010).

4.1.3 Cytotoxicity on aged human skin fibroblasts of niosomes

The blank and OSF3 loaded cationic niosomes were investigated for cytotoxicity on normal human skin fibroblast as previously described (Manosroi et al., 2010). The blank niosomes which composed of various cationic surfactants were determined for cytotoxicity. The least cytotoxic blank niosomes were selected to load with OSF3 and tested again for cytotoxicity. The final concentration of 0.0002 – 2 mM of the blank and the loaded niosomes were incubated with cells for 24 hours.

4.2 Physico-chemical characteristics of CTAB cationic niosomes loaded with OSF3 prepared by supercritical carbon dioxide fluid ($scCO_2$)

4.2.1 Preparation of CTAB cationic niosomes loaded with OSF3 by supercritical carbon dioxide fluid technique with sonication ($scCO_2$)

The niosomes were prepared by the $scCO_2$ method as previously described (Manosroi et al., 2012a; Manosroi et al., 2010). The composition at 20 mM of Tween 61 mixed with cholesterol and CTAB at 1:1:0.5 molar ratio together with 0.1, 0.5, 1.0 and 2.0 % w/v of OSF3 was added into the view cell of the $scCO_2$ apparatus. The volume of the view cell was about 100 cm^3. The temperature in the cell was raised to 50°C and the CO_2 gas was introduced into the view cell. The pressure and temperature in the view cell were maintained at 250 bar and 50 ± 1°C, respectively. The pressure was released after 30 minutes. The niosomal dispersion was collected in a test tube placing in an ice bath (4°C) and sonicated by a microtip probe sonicator at 40% amplitude for 5 min.

4.2.2 Entrapment efficiency of OSF3 loaded in CTAB cationic niosomes

In order to separate the non-loaded OSF3, the niosomal suspensions loaded with 0.5% OSF3 were centrifuged at 47,000 rpm for 90 min at 4◦C and washed

with distilled water. The amounts of OSF3 in the supernatant and in the pellet sediment were analyzed by HPLC, after disrupting the niosomes by adding 500 µl of 10% (v/v) Triton X-100. Amount of the 500 µl of the supernatant or the pellet were mixed with 1000 µl of the mobile phase (95% v/v of acetonitrile mixed with 0.1 % glacial acetic acid). Then, the unsaturated fatty acid (gamma-linolenic acid, linoleic acid and oleic acid) contents containing in OSF3 were determined by HPLC using the same condition as previously described (Manosroi et al., 2010).

4.2.3 Physical characteristics of the blank and OSF3 loaded CTAB cationic niosomes

Appearances, vesicular sizes and zeta potential values of the blank and OSF3 loaded niosomes before and after centrifuged were investigated as previously described as in section 4.1.2.1. The evaluation of transition temperature and microviscosity of the niosomal membrane were determined as previously described in section 3.2.4 and 3.2.5, respectively.

4.2.3.1 Morphology

A. Transmission Electron Microscopic observation

The niosomal dispersion was frozen quickly in liquid propane using a cryopreparation chamber. The frozen sample was transferred to a freeze fracture device and fractured at -150 °C. Platinum was deposited onto the fractured surface at an angle of 45° and the carbon was evaporated at an angle of 90°. The replica was recovered onto the copper 400 meshes after washing with acetone and water, and observed by a transmission microscope.

B. Small Angle X-ray Scattering (SAXS) measurements

SAXS experiment was carried out using a SAXSess camera and a PW3830 X-ray generator. All samples were filled into a thin quartz capillary and set in a sample holder unit for 1 hour before the measurement. An imaging plate was used to record the scattering data and read out by a cyclone storage phosphor system to get a scattering pattern.

4.2.3.2 Micropolarity environments of the niosomal membrane

In order to estimate the micropolarity environments of the vesicular membrane, the steady-state fluorescence measurements were performed using a fluorescence spectrophotometer. Briefly, an amount of 5 ml of the sample was incubated with 10 µl of pyrene solution (1 µmol/ml) for 72 hours at 25±2 °C (Kalyanasundaram, 1987; Takamatsu et al., 2009). The spectrum was recorded between 360 and 400 nm with the excitation wavelength at 335 nm. The fluorescence intensity ratio (I1/I3) of the first (I1, 373 nm) to the third (I3, 384 nm) vibrational peaks indicated the micro-environmental polarity around the pyrene molecules. The decrease in the I1/I3 value was the indication of the more hydrophobic environments formation.

Part 5: *In vitro* transfollicular penetration of unsaturated fatty acids of gel OSF3 niosomes in porcine skin by Franz diffusion cells

5.1 Preparation of gel containing the cationic niosomes loaded with OSF3

The cationic niosomes loaded with OSF3 were incorporated into the gel base containing Carbopol® 980. Briefly, 0.3% (w/w) Carbopol® 980 was dispersed in the niosomal dispersion with gentle stirring, resulting in the gel containing niosomes loaded with 2.00 % (w/v) of OSF3 (gel OSF3 niosomes). For gel OSF3, the 2.00 %

(w/v) OSF3 was dissolved in hot water and then dispersed with 0.3% Carbopol® 980 with gentle stirring.

5.2 Physicochemical characteristics of the gel containing OSF3 loaded niosomes

The gel OSF3 niosomes and gel OSF3 were stored in transparent vials covered with aluminum cap and kept at 4±2, 25±2 and 45±2 °C for 3 months. At initial and 1, 2, 3 months, the samples were withdrawn and assayed for physical characteristics and chemical characteristics with the same procedures as in **section 4.2 and 4.3**.

5.3 *In vitro* transfollicular penetration of the unsaturated fatty acid containing in various samples through porcine skin by Franz diffusion cells

5.3.1 Skin sample

The porcine skin was taken freshly from the male newborn large white-landrace hybrid pigs (1.0 - 1.5 kg) which died of natural causes few days after birth and obtained from the farm of Betagro Northern Agro-Industry Co., Ltd. in Lamphun, Thailand during April- May in 2011. The *in vitro* transfollicular delivery in porcine skin protocol has already been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (30 September 2010, Protocol Number: 20/2553).

5.3.2 Preparation of the porcine skin

The newborn porcine back skin was shaved and carefully separated. Then, the subcutaneous fat was carefully removed using a scalpel. For cyanoacrylate skin surface biopsy, a drop of the power glue (99.0 %, (w/w) cyanoacrylate, Altego®, Osaka, Japan) was placed on the skin and covered with a glass slide under slight pressure as previously described (Otberg et al., 2004). After 5 minutes, the

cyanoacrylate polymerized and the glass slide was removed with one quick movement to open all hair follicles.

5.3.3 Follicular closing technique

The number of hair follicles on the porcine skin was counted. A pair of skin sample with an equal number of hair follicles was used as opened and blocked hair follicle skin sample. All hair follicles of the blocked hair follicle skin were closed using the follicular closing technique (Otberg et al., 2008) with modification. Briefly, each follicular orifice of the block hair follicle skin sample was closed by placing a small drop of nail varnish and dried for 5 minutes to completely close the follicular shunt.

5.3.4 Transfollicular delivery

Transfollicular delivery by vertical Franz diffusion cells of niosomes loaded with OSF3 (OSF3 niosomes), gel incorporated with OSF3 (gel OSF3), gel incorporated with niosomes loaded OSF3 (gel OSF3 niosomes), OSF3 solution, gel base and blank niosomes using the three unsaturated fatty acids (gamma-linolenic acid, linoleic acid and oleic acid contents) as the markers was performed by the follicular closing technique. The blocked and opened hair follicle skins were mounted with the subcutaneous side facing upwards to the donor compartment, while the dermal side contacting with the receiver. The contact area between the donor and the receiver chamber was 2.46 cm^2. The volume of the receptor compartment [phosphate-buffered saline (PBS), pH 7.4] was 13 ml, which was constantly stirred at 100 rpm with a magnetic bar and the temperature was controlled at 37 ± 2°C throughout the experiment.

An amount of 500 µl of the samples (the OSF3 niosomes, gel OSF3 niosomes, gel OSF3, OSF3 solution, gel base and blank niosomes) was placed into the donor compartment and covered with a paraffin film. Cells were stopped at 1, 2, 4 and 6 hours. The treated porcine skin samples were removed and swung twice in 100 ml of distilled water. The skin was stripped with an adhesive tape using 3M Scotch MagicTM tape (1 cm × 1 cm) (Plessis et al., 1992). Each tape was charged with a weight of 300 g for 10s and then removed rapidly (Foldvari et al., 2006; Van der Molen et al., 1997). The stripping tapes were discarded to remove excess samples (Padula et al., 2010). The total unsaturated fatty acid contents were extracted from the skin. The stripped skin was cut into small pieces, extracted with 1 ml of the extraction mixture, sonicated for 10 min in an ice bath and filtered. For the receiver compartment, the receiving solution was freeze-dried and reconstituted with 1 ml of the extraction mixture. The experiments were done in triplicate.

5.3.5 HPLC analysis

The unsaturated fatty acids in skin and receiver compartments were assayed by HPLC using the same conditions as previously described (Manosroi et al., 2010). Then, the cumulative amounts, fluxes and transfollicular penetration per one hair follicle both in skin and the receiving solution were calculated by the following equations:

$$\text{Cumulative amounts } (ng/cm^2) = \frac{(FA_{open} - FA_{block})}{2.46}$$

$$\text{Fluxes } (ng/cm^2/hour) = \frac{(FA_{open} - FA_{block})}{2.46 \times 6}$$

$$\text{Transfollicular penetration per one HF } (ng/\text{one HF}) = \frac{(FA_{open} - FA_{block})}{\text{number of hair follicles}}$$

,where FA_{open} was the unsaturated fatty acid amounts in the open system, FA_{block} was the unsaturated fatty acid amounts in the block system and HF was the hair follicle.

Part 6: *In vivo* hair growth promotion activity of gel containing cationic niosomes loaded with OSF3

6.1 *In vitro* and *in vivo* toxicity evaluation of the formulations containing OSF3

6.1.1 *In vitro* cytotoxicity on aged human skin fibroblasts

The formulations containing 2.0% (w/v) of OSF3 (OSF3 solution, OSF3 niosomes, gel OSF3 and gel OSF3 niosomes) were investigated for cytotoxicity on normal human skin fibroblasts (passage 14^{th}) provided by Dr. Natthanej Luplertlop at the Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand using the same condition as previously described (Manosroi et al., 2010). The final concentration with the serial of 10-fold dilutions (0.0002 – 2 mM) of OSF3 niosomes containing in each formulation were prepared and incubated with the cells for 24 hours.

6.1.2 *In vivo* rabbit skin irritation test by the closed patch test

Three male rabbits (New Zealand White, 1.5–2.5 kg) purchased from Department of Animal And Aquatic Science, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand were kept carefully following an acclimation period of 7 days to ensure the limited-access rodent facility with the environmental conditions set at 25±2°C, 60–90% RH and 12 h light/12 h dark cycle. The animals were provided *ad labium* access to a commercial rabbit-diet and the 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 sample application. The shaved back of the animals was divided into 10 sites with the area of 2.5 × 2.5 cm each. An amount of 500 µl of each

sample (OSF3 solution, OSF3 niosomes, gel OSF3 and gel OSF3 niosomes) 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 samples were removed and the treated sites were washed 2 times with distilled water and air dried. One hour later, the sites were examined optically and measured by Mexameter® for skin edema and erythema. Scoring of erythema and edema was performed at 24, 48 and 72 h (Draize et al., 1944). Dermal irritation was scored and recorded according to the grades described in the OECD Guideline method 404, 2002 (OECD, 2002). The Primary Irritation Index (PII) was calculated using the following equation:

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

The irritation degree was categorized based on the PII values as negligible (PII = 0–0.4), or slight (PII = 0.5–1.9), moderate (PII = 2–4.9) or severe (PII = 5–8) irritation. This study protocol has been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Protocol Number: 20/2553).

6.2 *In vivo* hair growth promotion activity

6.2.1 Animals

The *in vivo* hair growth promotion protocol has already been reviewed and approved by the ethical committee of Faculty of Medicine, Chiang Mai University in Thailand (Protocol Number: 20/2553). Six-week-old C57BL/6 male mice (n = 32) purchased from the National Laboratory Animal Center, Mahidol University in Nakhon Pathom, Thailand were housed individually in stainless steel cages in a room with controlled temperature (25 ± 2°C), a 12 h cycle of light and dark

and maintained on a standard laboratory diet and water *ad libitum* at least 7 days prior to the experiment.

6.2.2 The sample treatment

The 4 cm^2 areas of the dorsal skins of all mice were shaved 1 day before topical application of the samples. The 7-week-old male mice were randomly allocated into eight experimental groups of four animals each and provided with one of the following treatments: distilled water, 95% (v/v) ethanol, gel base, 2.0% (w/v) of OSF3 in ethanol, niosomes loaded with OSF3 (OSF3 niosomes), gel incorporated with niosomes loaded with OSF3 (gel OSF3 niosomes), dutasteride 2% (w/v) as a positive control, no treatment as a negative control. All mice were topically applied to the test area with 200 µl of the samples every day for 21 days. The animals were kept in isolation for 30 minutes and housed back to the respective cages. At initial and every week before the daily sample application, the hair growth promotion activity was investigated. Hair growth promotion in the wash-out period for 21 days more was further determined. Thus, the total investigation period was 42 days.

6.2.3 Determination of hair growth promotion activity

6.2.3.1 Observation and photography

The dorsal skin of the mice was observed visually, recorded and photographed. The hair regrowth was evaluated at initial and every week before daily sample application (at day 0, 7, 14, 21, 28, 35 and 42) using the following hair growth scores: score 0 (no hair growth), score 1 (less than 20% growth) , score 2 (20% to less than 40% growth), score 3 (40% to less than 60% growth), score 4 (60% to less than 80% growth) and score 5 (80% to 100% growth) (Matsuda *et al.*, 2002).

6.2.3.2 Hair length determination

The determination of hair length was evaluated according to the method previously described (Yoon et al., 2010). Briefly, hairs were plucked randomly from the shaved area of all mice on day 7, 14, 21, 28, 35 and 42 of the experiment. The length of 10 hairs was measured and the average length was determined. The results were expressed as the mean length ± S.D. of 10 hairs.

6.2.3.3 Histological studies

Skin specimens were taken from the dorsal of the mice from each group at day 0 and 21 after sample application. Individual skin samples were fixed in 4% paraformaldehyde and the tissues were then dehydrated and embedded in the paraffin using an automatic tissue processor, sectioned to 4 μm thickness with a rotary microtome, stained with hematoxylin and eosin, and then the histological morphology was examined using light microscopy. Hair follicles were counted manually in dermis and subcutis layer by a blinded observer at a fixed size.