CHAPTER 3

MATERIAL AND METHODS

3.1 Apparatus and Instruments

1. Rotary evaporator, Eyela, Tokyo
2. Hot air oven, Binder, Germany
3. Spectrophotometer, Shimadzu model UV-2540, Japan
4. Quartz cuvette
5. Centrifuge, Sorvall model Super T21 Refrigerated centrifuge, UK
6. Centrifuge tube 15 and 50 ml
7. High performance liquid chromatograph, HP 1100, Hewlett Packard, Germany.
8. Hypersil® ODS C-18 column (250 x 4.6 mm, 5.0 μm) Analytical balance, Precisa model 303A, Switzerland
9. Digital cosmetic camera, Coscam® model USB-225, South Korea
10. Nylon syringe membrane filter
11. High pressure homogenizer, Avestin model Emilsiflex C-3, Canada
12. Particle size analyzer, Malvern model Zetasizer nano ZS, UK
13. Differential scanning calorimeter, Perkin Elmer, USA
14. Water purifier, Millipore model Mili-Q series synthesis, USA

3.2 Reagents and Standards

1. 95 % Ethyl alcohol, Liquor Distillery Organization Excise Department, Thailand
2. Absolute ethyl alcohol, Liquor Distillery Organization Excise Department, Thailand
3. Dichloromethane, Fisher Scientific, UK
4. Methyl alcohol, HPLC grade, Fisher Scientific, UK
5. Propane-1,2-diol, Fisher Scientific, UK
6. Gallic acid, Sigma chemicals, USA
7. Testosterone, Sigma chemicals, USA
8. Finasteride, Sigma chemicals, USA
9. Dithiotreitol, Sigma chemicals, USA
10. NADPH, Sigma chemicals, USA
11. Sucrose, Sigma chemicals, USA
12. Dibasic sodium phosphate
13. Monobasic sodium phosphate
14. Propyl *p*-hydroxybenzoate, Sigma chemicals, USA
15. Minoxidil, Sigma chemicals, USA
16. Monostearin, TCI chemicals, Tokyo
17. Pluronic® F-68, Sigma Chemicals, USA
18. Polyoxyethylene sorbitan monostearate, Tween™ 80, Croda, UK
19. Polyoxyethylene sorbitan monooleate, Tween™ 60, Amresco, USA
20. Sorbitan monooleate, Span™ 80, Croda, UK
21. Sorbitan monostearate, Span™ 60, Croda, UK
22. Capric/caprylic triglyceride, Miglyol® 812, Sasol, Germany

23. Laureth-4, Brij^{TM}-L4, Croda, UK
24. 1,3-Dimethylol-5,5-dimethylhydantoin (DMDM hydantoin), Glydant^{TM}, Lonza, Switzerland
25. Safflower yellow, TCI chemicals, Tokyo
26. De-ionized water (DI water)

3.3 Animals

1. Male Sprague-Dawley rats, National Laboratory Animal Center, Thailand
2. Male C57BL/6Mlac mice, National Laboratory Animal Center, Thailand

All animals were housed under the 12 h light/dark cycle with freely accessed to food and water. All animals were allowed to adapt to environment one week prior to the study.

3.4 Plant Materials

1. *Centella asiatica* (L.) Urb.
2. *Carthamus tinctorius* L.
3. *Terminalia chebula* Retz.
4. *Terminalia bellirica* (Geartn.) Roxb.
5. *Oryza sativa* L.
6. *Garcinia mangostana* L.
7. *Ocimum basilicum* L.
8. *Piper nigrum* Wall.
9. *Citrus reticulata* Blanco
10. *Curcuma longa* L.

11. *Acacia concinna* Wall.
12. *Alpinia galanga* Willd.
13. *Andrographis paniculata* Nees
14. *Averrhoa carambola* L.
15. *Cassia siamea* Lam.
16. *Citrus hystrix* DC.
17. *Clitoria ternatea* L.
18. *Cymbopogon citratus* Stapf
19. *Ipomoea aquatica* Forssk.
20. *Lawsonia inermis* L.
21. *Phyllanthus emblica* L.
22. *Rhinacanthus nasutus* Kuntze
23. *Sapindus rarak* DC.
24. *Tinospora rumphii* Boerl.
25. *Trichosanthes cucumerina* L.
26. *Zingiber officinale* Roscoe

3.5 Methodology

3.5.1 Extraction of plants

Plants were purchased from local markets in Chiang Mai, Thailand. After the comparison with herbarium specimens at Faculty of Pharmacy, Chiang Mai University, they were dried at 45 °C in a hot-air oven. Next, they were ground by using an electric grinder and extracted by maceration with 95 % ethyl alcohol. The

solvents were evaporated to dryness under controlled pressure and temperature using a rotary evaporator to obtain crude extracts.

3.5.2 Determination of total phenolic contents (TPC)

TPC was determined from the method of Kumar et al. (2011a), as described. Diluted plant extracts (0.2 ml) was added to 1.0 ml of 0.2 N Folin-Ciocalteu phenol reagent in a test tube and kept for 5 min. Then sodium carbonate solution (7.5 % w/w, 0.3 ml) was added. After that, the reactions were kept in a dark place for 2 h, and then read for UV absorbance at 750 nm.

Gallic acid was used as a standard. TPC of each sample was expressed as mg gallic acid equivalent (GAE) per 1 g extract.

3.5.3 Determination 5α-reductase inhibitory activity

3.5.3.1 Preparation of 5α-reductase suspension

5α-reductase suspension was prepared from the liver of male SD rats according to the method reported by Kumar et al. (2011a). Three rats were sacrificed, the livers were then removed, cut with scissors and homogenized in a solution containing 0.32 M sucrose and 1 mM dithiothreitol in the 0.02 M phosphate buffer at pH 6.5. The homogenate was then centrifuged at 4500 × *g* at 0 °C for 30 min, twice. The supernatants were pooled and assessed for the soluble protein using the Lowry method (Lowry et al., 1951).

3.5.3.2 Determination of 5α-reductase inhibitory activity

5α-reductase inhibitory activity was assessed according to the method report by Kumar et al. (2011a). Each test compound (0.2 ml in 50 % ethanol) was added in the test tube that contained 1.0 ml of 0.02 mM phosphate buffer (pH 6.5), 0.3 ml of freshly prepared 500 ppm testosterone solution in 50% ethanol solution, and 1.0 ml of microsomal suspension. Reaction was then initiated by the addition of 0.5 ml of 0.77 mg/ml NADPH in phosphate buffer. After that, the test tube was incubated at 37 °C for 30 min. Then the reaction was stopped by adding 5.0 ml dichloromethane, followed by adding 0.5 ml of 100 ppm propyl *p*-hydroxybenzoate in 50 % ethanol (as an internal standard for HPLC). After addition of internal standard, it was shaken for 60 s and then centrifuged at 400 × *g* for 10 min. Four ml of organic phase was decanted and evaporated to dryness. The residue was re-dissolved in 5.0 ml methanol. An aliquot of 10.0 µl was analyzed for remaining testosterone content using high pressure liquid chromatography (HPLC). Using analytical Hypersil®-ODS column (Thermo Scientific, USA) 250 x 4.6 mm i.d. with 5µm internal particle size, 40 °C. The mobile phase used was 65 % methanol with a flow rate of 1 ml/min and detected by UV absorbance at 242 nm.

In order to determine inhibitory activity, two extra reactions were conducted, first: reaction control (rxn) contains 0.2 ml of 50 % ethanol as a sample. Second: an enzyme blank (ctrl) receives 5.0 ml dichloromethane to denature the enzyme, so that testosterone cannot be metabolized into DHT. The % inhibition was calculated using peak area ratio (*r*) of testosterone/internal standard following the equation:

$$\% \text{ inhibition} = [(r_{\text{sample}} - r_{\text{rxn}})/(r_{\text{ctrl}} - r_{\text{rxn}})] \times 100$$

Where r_{sample} is r of the tested sample

r_{rxn} is r of the reaction control

r_{ctrl} is r of the enzyme blank

Finasteride was used as a standard enzyme inhibitor. Ten concentrations of finasteride ranging from 0.1 to 1.0 μM were studied. The standard inhibition curve was constructed and the IC_{50} of finasteride was calculated from the obtained standard equation. From standard equation of the finasteride, enzyme inhibitory activity of each plant extract was calculated and reported in terms of finasteride equivalent anti-5α-reductase activity (FEA value) as a unit of mg finasteride equivalent per 1 g extract.

3.5.4 Determination of hair growth promoting activity of the plant extracts

Three plants with highest 5α-reductase inhibitory activity were selected to evaluate the hair growth promoting activity by the method of Kumar et al. (2012). Seven-week-old C57BL/6Mlac mice were randomly divided into five groups for three treatments, as follows: vehicle control group, positive control group, and three of each extracts group. Dorsal hair of the mice was removed by using a depilatory cream. On the next day, 100 μl of the test solutions were gently applied once a day, for 28 consecutive days. Minoxidil (2 %) was used as a positive control. A concentration of 1 % w/w of each plant extract in vehicle composed of propylene glycol:ethanol:water = 5:3:2 was used. Same vehicle was used in the negative control group.

The hair growth promoting activity of the substances was checked by the darkening of the dorsal skin. Hair growth was measured at days 1, 7, 14, 21 and 28 by assigning a hair growth score (Figure 7), as follows:

Score 0 = no growth observed

Score 1 = up to 20 % growth observed

Score 2 = 20 – 40 % growth observed

Score 3 = 40 – 60 % growth observed

Score 4 = 60 – 80 % growth observed

Score 5 = 80% to full growth observed

HGS = 0 HGS = 1 HGS = 2

HGS = 3 HGS = 4 HGS = 5

Figure 7 Assigning of hair growth score (HGS) in C57BL/6 mice

Digital images of total hair growth on day 28 were obtained using a Coscam® USB-225 (Seoul, South Korea) with a 40 × magnification lens.

On Day 28, the mice were sacrificed; their dorsal skins were then removed and sectioned by two different pathways, longitudinal section for hair follicle histology determination and cross section for hair follicle count. For assessment of total hair follicle, hair follicles of each mouse were count manually under the light microscope with 100 × magnification lens.

3.5.5 Preparation of NLC base and NLC containing plant extract

Plant extract with the highest 5α-reductase inhibitory activity and hair growth promoting activity was selected for incorporation in the wax matrix of NLC. The NLC were prepared by using hot high pressure homogenization technique, as followed. The oil compartment and plant extract (if needed) were melted at 70 °C and the water compartment was also heated at 70 °C. Then, the oil compartment was slowly incorporated into the water compartment and continuously stirred by using glass stirrer to obtain the coarse emulsion. After that, the hot coarse emulsion was forced through the high pressure homogenizer with controlled pressure at 750 bar, 85 °C for 7 continuous cycles to form the nanoemulsion. Finally, the nanoemulsion was allowed to cool down to room temperature to obtain the NLC.

3.5.6 Assessment of NLC base

3.5.6.1 Physical appearance

Prepared NLCs were assessed for physical appearances such as color, odor, texture, phase separation, and crystal growth by using organoleptic techniques.

3.5.6.2 Physical stability of prepared NLC

Prepared NLC formulations were assessed for physical stability by using heating-cooling cycle and room temperature keeping. Physical assessment for changes in their physical appearance, such as color, odor, texture and phase separation, particle size, size distribution and zeta potential were evaluated to decide the appropriate formulation. Heating-cooling cycle was determined by keeping the tested formula at 4 ± 2 °C for 24h, then keeping at 45 ± 2 °C for another 24 h, for 6 consecutive cycles. Heating-cooling cycle was used as the accelerated stability study for the selection of the appropriate base, if the formulations have passed the cycle and remains unchanged. For long term stability study, only the formulation that passed the heating-cooling cycle testing was kept at room temperature for 12 months, respectively.

3.5.6.3 Particle size and size distribution

Prepared NLC formulations were assessed for particle size and size distribution by photon correlation spectroscopy using the Zetasizer nano ZS, Malvern ,UK. Prior to the experiment, samples were diluted with DI water to obtain appropriate scattering intensity. Hydrodynamic radius of particles were computed from Stokes-Einstein equation, as followed:

$$D = \frac{kT}{3\pi\eta d}$$

Where D is the diffusion coefficient, k is the boltzmann constant, T is absolute temperature, η is viscosity of the dispersing lipid and d is the particle size diameter.

Samples were analyzed five times; the values were reported in the term of mean ± SD.

3.5.6.4 Zeta potential

Prepared NLC formulations were assessed for zeta potential by photon correlation spectroscopy using the Zetasizer nano ZS, Malvern ,UK. Zeta potential (ZP) of the formulations were assessed in the water; where measurement of ZP in water yield the stern potential which is directly related to the surface charge (Nernst potential). Zeta potential was calculated from Helmholtz-Smoluchowski equation, as followed.

$$ZP = \frac{4\nu\pi\eta}{E\varepsilon}$$

Where ZP is the zeta potential, ν is the particle velocity, η is the viscosity of the dispersing medium, *E* is the field strength, ε is the dielectric constant.

Prior to the experiment, 0.5 g of samples were diluted with 20 ml DI water.

3.5.6.5 Transmission electron microscopy (TEM)

TEM was performed by using negative staining method with saturated uranyl acetate in 30 % ethanol. Prior to the experiment, appropriate NLC base was diluted with particle-free DI water at the ratio of 1:10, the diluted NLC was dropped on copper grid and left dried at room temperature. After that, the uranyl acetate solution was dropped on the copper grid, left dried and then observed with TEM.

3.5.6.6 Lipid crystallinity

Selected appropriate NLC formulation was assessed for lipid crystallinity by differential scanning calorimetry and X-ray diffractometry.

3.5.6.6.1 Differential scanning calorimetry

NLC Samples were accurately weighed and put into the aluminium pan with holes, then it was hermetically sealed. Sealed sample pan were put in the DSC, scanning was done over the temperature range from 25 to 85 °C. Empty aluminium pan was used as the reference pan. Monostearin was used as a standard. Crystallinity of the NLC was calculated as the crystallinity index (CI) by the following equation (Teeranachaideekul et al., 2007):

$$CI\ (\%) = \frac{\Delta H_{NLC}}{\Delta H_{monostearin} \times Concentration_{lipid\ phase}} \times 100$$

Where ΔH_{NLC} is the melting enthalpy of NLC dispersion

$\Delta H_{monostearin}$ is the melting enthalpy of monostearin

3.5.6.6.2 X-ray diffraction (XRD)

XRD was performed by using wide-angle X-ray scattering (WAXS) to investigate lipid crystallinity and polymorphism of the selected formulation and to confirm the results obtain from DSC. Analysis was performed by a D8 X-ray Diffractometer (Brüker AXS GmbH, Germany) with an X-ray tube equipped with copper anode (Cu-Kα radiation, 40 kV, 40 mA, λ = 1.54184 nm) using the Si(Li) semiconductor and scintillation counter as a detector. Analyses were done at 2 Theta

from 2.0° to 40.0°, data collection was done with a step width of 0.04° and step time of 1 s. All WAXS experiments were conducted at 25 °C.

3.5.7 Assessment of plant extract-loaded NLC

Selected appropriate NLC formulation was used as the carrier for selected plant extract. Plant extract-loaded NLC was evaluated for physical appearance, stability, particle size and size distribution, zeta potential, TEM, lipid crystallinity by the same method as NLC base. In addition, entrapment efficacy of plant extracted-loaded NLC were evaluated by the HPLC-UV method. Chemical stability of plant extract-loaded NLC was determined by HPLC-UV method.

3.5.7.1 Development of extraction method

Cold methanol precipitation method was used to extract the unentrapped safflower from NLC matrix (Kumar, 2011b). Formulations (0.5 g) were mixed with methanol (4.5 g) and centrifuged at 6000 rpm, 0 °C for 30 min. Confirmation of extraction method was done by UV spectrum scanning and HPLC analyses.

3.5.7.2 Development of HPLC-UV method for determination of safflower yellow

Simple isocratic HPLC-UV chromatographic method was developed and validated under the criteria from Tripartite International Conference on Harmonization (ICH) text on validation of analytical procedure (ICH, 1995a, 1995b, and 1996), as followed.

Apparatus

HPLC analyses were carried out by Hewlett Packard Model 1100 liquid chromatograph. The column used was C-18 (Hypersil®-ODS, Thermo Scientific, USA) 250 × 4.6 mm i.d. with 5μm internal particle size. Methanol (60% v/v in DI water) was used as a mobile phase using flow rate at 1 ml/min. Column temperature was set at 40 °C. An injection volume was 10 μL. The detection wavelength was fixed at 401 nm. Total run time was 10 min per sample.

Accuracy

Accuracy of conducted method was expressed as the % recovery of safflower yellow, which was defined by analysis of known amount of standard safflower yellow added into extracted NLC base. Each concentration was analyzed in triplicate to calculate the accuracy.

Precision

Relative standard deviation or RSD was used as a parameter for determining repeatability and intermediate precision. Three replications of each concentration were analyzed in same day and on the different day for repeatability and intermediate precision, respectively.

Specificity

Peak purity of safflower yellow in safflower extract and extracted safflower extract-loaded NLC (CT-NLC) was firstly checked by standard addition technique and then they were confirmed by UV scan over the wavelength between 200 and 600

nm. Mass spectrometric determination was also used to confirm the peak purity, by using Agilent LC/MSD SL. The nebulising gas was nitrogen, which set at a pressure of 50 psi and temperature was 320 °C with the flow rate of 13 l/min. The voltage was set at 4 kV. The full scan covered the mass range from m/z 300 – 900. Atmospheric pressure ionization electrospray (API-ES) technique was used.

Linearity and range

Six concentrations, from 5 to 100 ppm, of standard safflower yellow were analyzed. Concentration response curve was constructed from concentration of safflower yellow (as *X* scale) and peak area (as *Y* scale). The range was obtained from the linear response.

Lower limit of detection (LOD) and lower limit of quantitation (LOQ)

LOD and LOQ were determined from the concentrations of safflower yellow that gave signal intensity three times and ten times greater than that of the noise, respectively.

3.5.7. Drug entrapment efficacy determination

The entrapment efficacy of safflower extract into NLC lipid core was analyzed by indirect method, which is the determination of unentrapped safflower extract in the medium. For this purpose, cold methanol precipitation technique was used to aggregate the wax matrix and to dissolve the solubilized safflower extract into the methanol phase. After that, the methanol phase was injected to HPLC system by using previously described condition. Entrapment efficacy (EE) and drug loading (L)

were calculated by following equations (Hu et al., 2006, Teeranachaideekul et al., 2007):

$$EE\ (\%) = \frac{W_a - W_s}{W_a} \times 100$$

$$L\ (\%) = \frac{W_a - W_s}{W_a - W_s + W_L} \times 100$$

Where W_a, W_s, and W_L representing the weight of safflower extract added in the system, analyzed weight in supernatant and weight of lipid added in the system, respectively (Hu et al., 2006).

For confirmation of cold methanol precipitation technique, the precipitated wax were dissolved in hexane, then partitioned with methanol and analyzed by UV and HPLC as a direct method.

3.5.8 Formulation and assessment of hair lotion containing NLC

3.5.8.1 Formulation of hair lotion

NLC were incorporated into three different dosage forms: nanosuspensions, hydrogels, and O/W emulsion. Physical properties of these hair lotions were assessed by the same direction explained before. Stability of these hair lotions was assessed by heating-cooling cycle and real time room temperature keeping. Particle size was analyzed by PCS.

3.5.8.2 Hair growth promoting efficacy and irritation assessment of hair lotion

The most appropriate hair lotion was tested for hair growth promoting activity in C57BL/6Mlac mice with the same direction explained before, using NLC base and

purify water as a control. Irritation of hair lotion was studied by observing the irritation signs such as redness, erythema, or skin lesions after application in mice. Digital images of total hair growth at day 28 of the mice were also obtained from Coscam®.

3.6 Statistics

All samples were analyzed in triplicate. All values were expressed as mean ± SD. Student t-test was used to determine the significant differences between the treatment and standard. Analysis of variance (ANOVA) was used to compare results of the several treatments. Significant differences between means were determined by Duncan's multiple range tests. Pearson's correlation coefficient was used to predict the relationship between two parameters. A probability value of $p < 0.05$ was adopted as the criteria for the significant differences.