CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

1. 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH, Wako Pure Chemical Industries, Japan)
2. 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma chemical Co., USA)
3. 2-Thiobabituric acid 98% (TBA, Sigma chemical Co., USA)
4. Absolute ethanol(Merck KGaA, Germany)
5. Acetic acid (Lab-scan LTD, Ireland)
6. Butylated hydroxytoluene (BHT, Sigma chemical Co., USA)
7. Cholesterol from lanolin ($C_{27}H_{46}O$, Fluka chemie GmbH., Japan)
8. Chlorofrom(Merck, Germany)
9. Dipotassium hydrogen phosphate(K_2HPO_4, Sigma chemical Co., USA)
10. Gallic acid (Sigma Chemical Co., USA)
11. Octyldodecanol, Eutanol G®
12. Polyoxyethylene glyceryl monococoate PEG-7 glyceryl cocoate, Cetiol HE®
13. Phosphatidylcholine (Epikulon 200, Degussa, Germany)
14. Potassium dihydrogen phosphate (KH_2PO_4, Sigma chemical Co., USA)
15. Quercetin (Sigma chemical Co., USA)
16. t-octylphenoxypolyethoxyethanol (Triton X-100, Sigma chemical Co., USA)
17. Trolox (Sigma chemical Co., USA)

3.2 Plants

The marigold (*T. erecta* L.) flowers were collected from Chiang Mai Province, the northern part of Thailand during winter. Authentication was achieved by Associated Professor Omboon Luanratana, Department of Pharmacognosy, Faculty of Pharmacy, Mahidold University, Bangkok, Thailand. Voucher specimens have been deposited at the Herbarium Section, Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand.

3.3 Instruments

1. Analytical balance (Sartorius®, Model BP610, Germany)
2. Cutometer® (MPA 580, CK Electronic GmbH, Germany)
3. High Pressure homogenizer (Model : AVESTIN C3, Canada)
4. High speed homogenizer (Model : Yellow line DI 25 basic, IKA Werke GmbH & Co, KG Germany)
5. Micropipettes 1-200 µl, 1-1000 µl (Pipetman®, Gilson Co. Ltd., France)
6. Micropipette (Rainin®,Mettler-Toledo Ltd., USA)
7. Microtiter plate reader (Backman coulter®, DTX 880 multimode detector, Austria)
8. Particle size analyzer (Model : Zetasizer ZS, Malvern Instrument Ltd.., Malvern, USA)
9. Refrigerated micro centrifuge (Model : Avanti30, Beckman USA)
10. Rotary evaporator (EYELA®, Japan)
11. Rotary evaporator (Model : Rotavapor R-210, Buchi Labortechnik AG, Switzerland)

12. Skin-visiometer® (SV 600 FireWire, CK Electronic GmbH, Germany)
13. Sonicator
14. Water bath incubator (Model : WNB 14, Memmert GmbH & Co KG, Germany)

3.4 Extraction of marigold flower

All marigold flower extracts used in this study were supported by Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand. The extraction is as followed: the dried and powdered marigold (*T. erecta* L.) flowers (250 g) was extracted continuously by Soxhlet's apparatus with *n*-hexane, ethyl acetate and ethanol, respectively. All extracts were subsequently filtered and evaporated in vacuo. The obtained residues were named as **H**, **EA** and **Et** extracts. From the preliminary study of antioxidant activity, the most active extract, **EA**, was subjected for further isolation by vacuum column chromatography using three solvents (hexane, ethyl acetate and methanol) to obtained 14 fractions (F1-F14). All the marigold extracts and fractions from **EA** were stored in the dark at 4°C until tested.

Figure 3.1 The appearance of three marigold flower extracts (**H**, **EA** and **Et)**

3.5 Determination of antioxidant activity of marigold flower extrats

Antioxidant activities of all marigold flower extracts were determined by two different methods, scavenging effect on 1, 1-diphenyl-2-2picrylhydrazyl (DPPH) method and inhibition on lipid peroxidation (thiobarbituric acid-reactive substance, TBARs method).

3.5.1 Determination of antioxidant activity with 1, 1-diphenyl-2-2picrylhydrazyl (DPPH) radical scavenging method

The experimental procedure was adapted from Brem et al. (2004). In this assay, the stable free radical 2, 2-diphenyl-1-picryl hydrazyl (DPPH), which has a strong absorption at 520 nm, reacts with antioxidants and produces colorless 2, 2-diphenyl-1-picryl hydrazine independently of enzymatic activities. Dilution series of test compounds, dissolved in EtOH, were performed in sterile disposable microplates, using freshly prepared 167 µM DPPH $^{\bullet}$/ethanol solutions, 180 µL. Each sample was tested in dilutions with a final volume of 200 µl for all of the assays. The results were determined after 30 min of reaction time in order to analyze antiradical activities. The disappearance of the free radical DPPH$^{\bullet}$ was measured spectrophotometrically at 520 nm with a microplate reader. The percentage inhibition was calculated by the equation: % Inhibition = ($A_{control}$ - A_{sample}/ $A_{control}$)* 100 where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Trolox, quercetin and gallic acid served as reference antioxidants.

3.5.2 Determination of antioxidant activity with thiobarbituric acid-reactive substance (TBARS) method

A modified thiobarbituric acid-reactive substances (TBARS) assay was used to measure the antioxidant activity of the extracts in term of inhibition on lipid peroxidation. Liposome suspension, consisting of Cholesterol, Phosphatidylcholine and 0.2 M potassium phosphate buffer (pH 7.2), was prepare in a sonicator. The marigold flower extracts in ethanol was mixed with a mixture of the sonicated solution and 0.07 M AAPH. The resulting mixture was incubated at 50° C for 24 hours. After incubation, the solution was mixed with 0.2%BHT, 3%Triton-X, 20% acetic acid and 0.6% TBA, respectively. Then the mixture was heated for 30 min at 90° C and cooled the mixture to room temperature. The absorbance of the mixture was measured spectrophotometrically at 540 nm with a microplate reader. The percentage inhibition was calculated same as in DPPH assay. Trolox, quercetin and gallic acid served as reference antioxidants.

3.6 Determination of Total Phenolic content in marigold flower extracts

The total phenolic content of the marigold flower extracts were determined by Folin-Ciocalteu technique using a calibration curve with gallic acid. Each sample was transferred into a tube that contained distilled water and then mixed thoroughly with Folin-Ciocalteu reagent for 30 seconds. After that, 7.5% (w/v) sodium carbonated was added and the mixture was agitated with a vortex mixer. The resulting mixture was incubated at 40°C for 30 minutes in the dark. The absorbances of extracts and blanks were measured at 725 nm using a spectrophotometer (Shimadzu UV-Vis 2450,

Japan). The concentrations of total phenolic compounds in all extracts were expressed as gallic acid equivalent (GAE) in milligram per gram dry sample.

3.7 Skin irritation testing in animal [39-40]

The Draize model and its modification are commonly used to assay skin irritation. In this study, three albino rabbits were used for skin irritation by modified Draize model. Approximately 24 hr before test, three rabbits were removed their fur at dorsal area. Each test substance was applied to a gauze patch (1x1 $inch^2$) then it was covered over the assign test site. After occlusion period (4hr), patches were removed and the test sites were cleaned with water. The erythema and edema formations on rabbits' skin were observed and scored at 1, 24, 48 and 72 hr after removing the patches using Draize scoring system. The primary irritation index (PII) was calculated and classified for their irritation reactions.

Table 3.1 Modified Draize-FHSA Model used in this research.

Topics	Descriptions
1 Number of animals	3 albino rabbits (clipped)
2 Test sites	2 x 1 $inch^2$ sites on dorsum
3 Test material	Applied diluted to the test sites, liquids: 0.5 ml
4 Occlusion	1 $inch^2$ surgical gauze over each test site
5 Occlusion period	4 hr
6 Assessment	1, 24, 48 and 72 hr visual scoring system

Table 3.2 Draize-FHSA Scoring System

Topics	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised > 1 mm)	3
Severe erythema (raised > 1 mm and extending beyond the area of exposure)	4

Table 3.3 Classification of skin irritation

Primary irritation index (PII)	**Classification of skin reaction**
< 2	Non-irritating
2-5	Moderately-irritating
>5	Severe-irritating

3.8 Formulation, characterization, stability test of nanostructured lipid carriers (NLC)

3.8.1 Formulation of NLC (unloaded NLC) [41-43]

In order to reduce the number of experiments, in this first part of the study the unloaded NLC were prepared prior to formulate ME-NLC to investigate the influences of the formulation's composition on characteristic of NLC. The unloaded NLC with optimal particle size and good physical properties were selected for the further study on formulating ME-NLC.

The unloaded NLC formulations with 10% of lipid phase and 5% of surfactant prepared in this study can classify into 3 groups. First, the group of formulation A (A1-A9) consists of glycerylmonostearate (GMS) and stearic acid (SA) as solid lipid, PEG-7 glyceryl cocoate (GC) and cyclomethicone (CCM) as liquid lipid and stabilized by the combination of Tween® and Span®. Second, the group of formulation B (B1-B6), the composition was similar with formulation A but liquid lipid was changed and consists of only octyldodecanol (OD). Third, the group of formulation C (C1-C6) consists of glyceryl behenate (GB) and behenyl alcohol (BA) as solid lipid, GC as liquid lipid and Poloxamer 188® as surfactant. The effect of solid: liquid lipid ratio on characteristic of NLCs was also observed in each group of formulations by varying it into 3 ratios of solid lipid: liquid lipid; 2:1, 1:1 and 1:2.

A lipid phase (10%w/w) which contains the blend of solid lipids, liquid lipids and lipophillic surfactant was melted at about 70°C. Then the hot aqueous phase containing hydrophilic surfactant was gradually dispersed into hot lipid phase using high speed homogenizer (Yellow line DI 25 basic, IKA Werke GmbH & Co.KG

Germany) at 8,000 rpm for 5 minutes. The total surfactants content used in each formulation is 5%w/w. Then the pre-emulsion was processed at 800 bar, 70°C for 5 cycles using a high pressure homogenizer (AVESTIN C3, Malvern, Canada). After homogenization, the obtained hot lipid dispersion was cooled down to room temperature while stirring with magnetic stirrer. The lipids recrystallized and formed the nanostructured lipid carriers (unloaded-NLC).

3.8.2 Characterization method for unloaded NLC [42-43]

3.8.2.1 Particle size measurement

The average particle size (Z-average size) and size distribution evaluated from polydispersity index (PDI) were measured by photon correlation spectroscopy (PCS) on a Zetasizer ZS (Malvern Instruments Ltd., Malvern, UK). Zeta potential was evaluated on the same equipment using folded capillary cells. The experimental measurements were repeated 3 times for each sample. Before the measurement, the NLC dispersions were diluted with deionized water (initial NLC dispersion: water = 1:1,000) to obtain the suitable concentration for the measurement. The characterization was performed at 24 hours (1 day) after preparations for initial value.

3.8.2.2 Zeta potential measurement

Zeta potential was evaluated with the same equipment and method as particle size measurement using folded capillary cells.

3.8.3 Stability test of unloaded NLC

The unloaded NLCs dispersions were stored at room temperature in a well-tight container and protected from light for three months. An aliquot of sample was taken at predetermined time intervals of 30, 60 and 90 days after preparations to investigate the particle size and zeta potential. The physical properties: color, odor, pH and aggregation or any unstable state (creaming or cracking), of NLC dispersions were also observed at the same time. The unloaded NLCs dispersions with proper particle size, zeta potential and stable after stability testing will be consider for preparing the marigold flower extract NLC (ME-NLC).

3.9 Formulation, characterization, stability test and antioxidant activity of marigold flower extract loaded nanostructured lipid carriers (ME-NLC)

3.9.1 Formulation of marigold flower extract loaded NLC (ME-NLC)

The marigold flower extract loaded NLC were prepared and developed based on the study of unloaded NLC by varying the amount of surfactant from 5 -12 % in formulation. ME-NLC was prepared using the same method as unloaded NLC by adding a certain amount of marigold flower extract selected from the result of antioxidant activity test and mixing thoroughly with the blend of melted lipids phase. After homogenization, the obtained hot lipid dispersion was cooled down to room temperature while stirring with magnetic stirrer. The lipids recrystallized and formed the ME-NLC. We kept it as NLC dispersion at room temperature for further processes.

3.9.2 Characterization method for marigold flower extract loaded nanostructured lipid carriers (ME-NLC)

3.9.2.1 Particle size measurement

The average particle size (Z-average size) and size distribution evaluated from polydispersity index (PDI) were measured by photon correlation spectroscopy (PCS) on a Zetasizer ZS (Malvern Instruments Ltd., Malvern, UK). Zeta potential was evaluated on the same equipment using folded capillary cells. The experimental measurements were repeated 3 times for each sample. Before the measurement, the NLC dispersions were diluted with deionized water (initial NLC dispersion: water = 1:1,000) to obtain the suitable concentration for the measurement. The characterization was performed at 1, 30, 60 and 90 days after preparation.

3.9.2.2 Zeta potential measurement

Zeta potential was evaluated with the same equipment and method as particle size measurement using folded capillary cells.

3.9.3 Stability test of marigold flower extract loaded nanostructured lipid carriers (ME-NLC)

Stability test of ME-NLC was investigated in four conditions, i.e. room temperature, 4°C as cool place and 45 °C as hot place for 3 months and 6 cycles of heating-cooling cycling method which defined as alternation of storage conditions from 45° for 48 hours to 4°C for another 48 hours. At the predetermine times; 30, 60

and 90 days after test, each sample was investigated for the particles size, PDI, zeta potential, antioxidant activity and physical properties.

3.9.4 Antioxidant activity test of marigold flower extract loaded nanostructured lipid carriers (ME-NLC)

ME-NLC dispersions were centrifuge at 20 °C, 10,000 rpm for 60 minutes using refrigerated microcentrifuge (Model: Avanti 30, Beckman, USA). The sediment was collected and kept in well-tight protected from light container for antioxidant activity test.

One gram of sediment was accurately weighed and dissolved in 5 ml of absolute ethanol before sonicate for 60 minutes. Then this solution was refrigerated at 4°C, the lipid recrystallized and further centrifuged at 20 °C, 10,000 rpm for 30 minutes. The supernatant was decanted for antioxidant activity test. The antioxidant activity test of ME-NLCs were done by two different methods, scavenging effect on DPPH assay and the percent inhibition on TBARS assay as mentioned in antioxidant activity test of marigold flower extracts.

3.10 Formulation and stability test of cream base

3.10.1 Formulation of cream base

Six formulas of cream bases (formula I, II, III, IV, V and VI) containing various oily materials such as stearyl alcohol, stearic acid, glycerylmonostearate, mineral oil as lipid phase in concentration of 19-26%, cetereth 25, Tween®, Span® or triethanolamine stearate as surfactants in concentration of 1-6% and propylene glycol

as humectants, preservatives and water, were prepared using conventional hot process. The ingredients of aqueous phase (part A) and oil phase (part B) were weighed and placed into two separated containers. The ingredients in part A were mixed and melted at about 70°C, while the ingredients in part B was mixed and heated to 70°C. Then part A was gradually added to part B with constant agitation to form emulsion and mixed until the homogenous emulsion was obtained. The emulsion was cooled down to room temperature.

3.10.2 Stability test of cream base

The stability of each cream base was tested by accelerated test freeze-thaw cycling method which defined as alternation of storage conditions at 45°C for 48 hours and 4°C for another 48 hours for 6 cycles. The changing of physical properties was observed such as color, odor, pH, smoothness and unstable states. In addition, spreadability and feeling on the skin were also observed and compared before and after accelerated test.

3.10.3 Selection of good cream base

The physical property, spreadability and feeling on skin of all formulations were compared and selected the most suitable cream base for the incorporation of marigold flower extract loaded nanostructured lipid carriers as ME-NLC cream.

3.11 Formulation, characterization, stability test and antioxidant activity of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream) [44-46]

3.11.1 Formulation of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

After cream base formulation had been chosen, the selected ME-NLC formulation was directly combined to cream base. The concentrations of ME-NLC in studied creams are 15 and 30%.

3.11.2 Characterization of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

The method for investigating the particle size and zeta potential of ME-NLC cream is the same method as ME-NLC. The physical properties were also observed.

3.11.3 Stability test of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

Stability test of ME-NLC cream was investigated in 5 conditions, i.e. 6 cycles of freeze-thaw cycling, 4°C as cool place, 45°C as hot place, room temperature with and without light for 3 months. At the predetermine times; 30, 60 and 90 days after test, each sample was investigated for the particles size, PDI, zeta potential, antioxidant activity and physical properties.

3.11.4 Antioxidant activity of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream) [47]

One gram of sample was accurately weighed and dissolved in 5 ml of absolute ethanol before sonicate for 60 min. Then this solution was refrigerated at 4°C, the lipid recrystallized and further centrifuged at 20°C, 10,000 rpm for 30 minutes. The supernatant was determined for antioxidant activity. The antioxidant activity of ME-NLC cream was done by DPPH and TBARS assay as mentioned in antioxidant activity of marigold flower extracts.

3.12 Skin irritation testing in human volunteers [39, 48]

The skin irritation test protocol of this study was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. Before participating in the clinical study, all of volunteers received the information of this study and signed a written informed consent that contained all the basic elements outlined.

3.12.1 Subjects of the study

Twenty-five Thai volunteers aged 30-55 years were selected by using inclusion and exclusion criteria. (Two test sites per volunteer; n = 50)

Inclusion criteria

1. Healthy skin, no skin diseases such as dermatitis
2. Unnecessary using, receiving or taking any preparation such as antihistamine drug or any other drugs.

3. Non-atopic, with no past or present history of skin diseases
4. No any scar, wound, blemish, and any skin diseases
5. No irregular skin color at test site
6. Subjects agree to sign an informed consent form
7. Comfortable involve in this study

Exclusion criteria

1. Subjects who did not or could not sign an informed consent form, unable to comply with the requirements of the protocol
2. Subjects who were participating in any other clinical study

Discontinuation criteria

1. Have skin irritation
2. Subjects who want to quit from the experiment for any reason
3. Subjects who couldn't practice following instruction criteria of study

3.12.2 Test substance application protocol

The upper back area of each volunteer was divided into two parts, left and right (duplication). Each side of the upper back was treated by Finn chamber that contain the samples. After 48 hr of application, the patches were removed and the test sites were cleaned suddenly with purified water. The skin irritation was evaluated at 1, 24 and 48 hr, after patch removal, based on Draize scoring system.

3.13 Wrinkle reducing capacity test of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

The protocol of clinical study was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. All of volunteers also received the information of this study (Appendix A) and signed a written informed consent that contained all the basic elements outlined before participating in the study (Appendix B).

3.13.1 Volunteers of the study

Twenty-five Thai volunteers aged 30-55 years were selected by using inclusion and exclusion criteria same as the skin irritation testing in human.

3.13.2 Test substance application protocol

The purpose of this study was to evaluate the wrinkle reducing capacity of ME-NLC cream, compared with cream base and cream containing marigold flower extract (ME cream). Application of any cosmetic products was prohibited 3 days before starting the test. The volunteers had to apply the test creams twice daily, once in the morning and once in the evening, for 8 weeks. The test sites were both left and right forearms. Each volunteer was assigned to apply the test creams in difference orders.

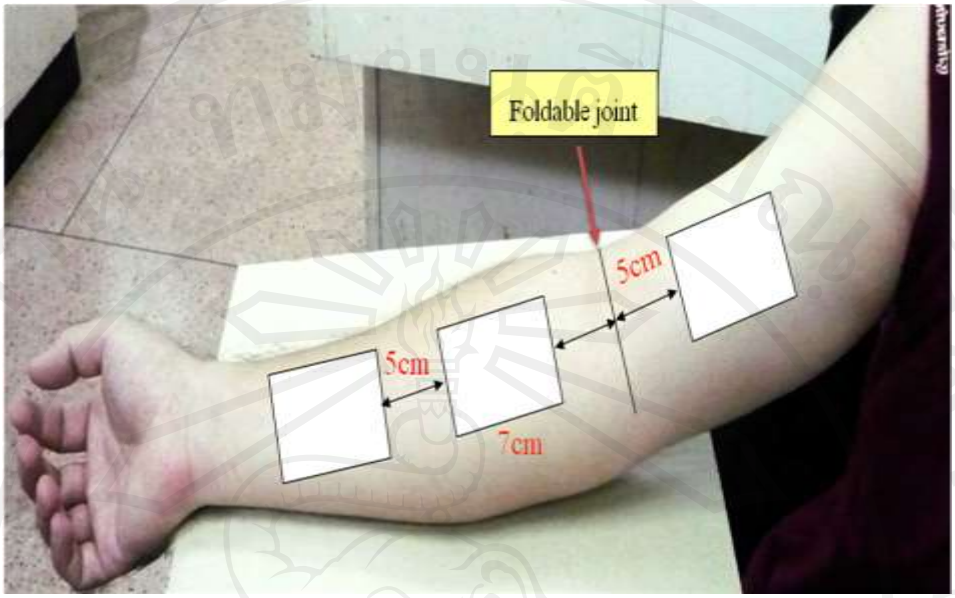

Figure 3.2 Application sites of wrinkle reducing capacity test

3.13.3 Assessment period and methodologies

The subjects were enrolled in a randomized, double-blinded, placebo controlled trial. The study protocol included the evaluation at day 0 for initial value, 4 and 8 weeks after the treatment. Skin-visiometer SV600 FW was used as device measuring four parameters which are R*a*, Rz, Surface and Volume (Appendix C) that referring to the difference of skin condition by the test creams.

3.13.4 Statistical analyze

Paired t-test was used to examine changes in R*a*, Rz, surface and volume values, before and after of each treatment. The percentage efficiency values were evaluated by the following equation:

% Efficiency value = [(Vm-Vo)/Vo] x 100

Where Vo is the value at initial point (day 0) and Vm is the value at measuring point (4 and 8 weeks). The data were subjected to two way analysis of variance and the significance of the difference between means was determined by Duncan's multiple range test ($P<0.05$) using SPSS for Windows.

3.13.5 Satisfaction on the test creams

After finishing the test, all volunteers were questioned on the product satisfaction (Appendix D).