CHAPTER III

MATERIAL AND METHOD

3.1 Apparatus and instruments

1. Analytical balance (Sartorius® BP610, Germany)
2. Digital Homoginizer (Polytron® PT3000, USA)
3. High Performance Layer Chromatography equipped with a diode array detector (Agilent® 1200, USA)
4. High Pressure Homogenizer (EmulsiFlex® C3, Canada)
5. HPLC analytical column (Cosmosil® C-18, USA)
6. Micropipettes 1-200 µL, 1-1000 µL (Pipetman®, France)
7. Microplate 96 well (Nunc®, USA)
8. Microtiter plate reader (Backman coulter® DTX 880 multimode detector, Australia)
9. Nylon membrane filter 47 mm, 0.45 µm (Armany®, USA)
10. pH meter (Horiba® EX-20, Korea)
11. Refrigerate Micro Centrifuge (SORVALL® SUPER T21, USA)
12. Rotary vacuum evaporator (Eyela®, Japan)
13. Separatory funnel 500 mL (Witeg®, Germany)
14. Skin Visiometer (SV 600 FireWire, CK Electronic GmbH, Germany)
15. Transmission electron microscopy (TEM) (JEM-2010, Jeol, Japan)
16. Ultrasonic bath (Elma®, Germany)
17. Ultrasonicator (Cole Palmer® 889, USA)
18. UV-Visible Spectrophotometer (Speckol® 1200, Germany)
19. Vortex-Genie 2 (Scientific®, USA)
20. Water bath (Memmert®, Germany)
21. Zetasizer (Nano-ZS 360, Malvern Instruments, England)

3.2 Chemicals

1. (±)-α-Tocopherol (Fluka, Switzerland)
2. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) (Fluka, Switzerland)
3. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma chemical, USA)
4. Absolute ethanol (analytical grade) (Lab-scan, Ireland)
5. Acetone (analytical grade) (Lab-scan, Ireland)
6. Acetronitrile (HPLC grade)(Lab-scan, Ireland)
7. Butanol (analytical grade) (Lab-scan, Ireland)
8. Buthylated hydroxyl aniline (O.V. Chemical and Supply, Thailand)
9. Buthylated hydroxyl toluene (O.V. Chemical and Supply, Thailand)
10. Cetyl alcohol (O.V. Chemical and Supply, Thailand)
11. De-ionized water (Millipore, USA)
12. Dichloromethane (HPLC grade)(Lab-scan, Ireland)
13. Ferric chloride hexahydrate; $FeCl_3.6H_2O$ (Sigma, Germany)
14. Ferrous sulfate heptahydrate; $FeSO_4.7H_2O$ (Sigma, Germany)
15. Glacial acetic acid (analytical grade) (Lab-scan, Ireland)
16. Glycerin (O.V. Chemical and Supply, Thailand)
17. Glycerol monostearate (O.V. Chemical and Supply, Thailand)
18. Hydrochloric acid (analytical grade) (Lab-scan, Ireland)
19. Isopropylmyristate (O.V. Chemical and Supply, Thailand)
20. Jojoba oil (Hong Huat, Thailand)
21. Methanol (HPLC grade)(Lab-scan, Ireland)
22. Petroleum ether (analytical grade) (Lab-scan, Ireland)
23. Propylene glycol (O.V. Chemical and Supply, Thailand)
24. Silicone oil (Hong Huat, Thailand)
25. Sodium acetate (Merck, Germany)
26. Sodium chloride (O.V. Chemical and Supply, Thailand)
27. Span 80 (Numsiang Trading, Thailand)
28. Stearyl alcohol (O.V. Chemical and Supply, Thailand)
29. Tween 80 (Numsiang Trading, Thailand)
30. Vitamin E acetate (Numsiang Trading, Thailand)
31. β-carotene (Sigma, USA)

3.3 Plants material and methods

3.3.1 Medicinal plant materials

Fresh ripen fruits of *Momordica cochinchinensis* (Lour.) Spreng that cultivated in Chiang Mai province, Thailand were used in this study. The fruits were harvested when color became orangish red. After harvested, fruits were stored under refrigerated conditions prior to further processing.

3.3.2 The oil extraction process from aril of *Momordica cochinchinensis* (Lour.) Spreng

The first step in the oil extraction was scooped out all the aril from the fruit into a large container. Separation of seeds from the wet aril was done manually. The wet aril was spread on a stainless-steel drying tray. The tray was then placed in the hot air oven for about 1 hr under 60°C until the surface of the aril was no longer sticky. The seeds were removed. Aril was dried again in drying oven under 60°C until moisture content was reduced to about 10%. The aril oil was separated by using a mechanical screw press with a boiler under 60°C. The oil was settled in a centrifuge decanter and filtered by Buchner funnel. It was transferred to a sealed amber container and kept in refrigerator.

3.4 Determination of antioxidant activities of aril oil of *Momordica cochinchinensis* (Lour.) Spreng

The antioxidant assays were done by two different methods, namely 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and ferric reducing antioxidant power (FRAP) assay showed that the aril oil possessed antioxidant activity.

3.4.1 1, 1- diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The experimental procedure was adapted from the method of Liu *et al.*(63) and Basuny *et al.*(64). The reaction mixture contained freshly prepared 248 μM ethanol solutions of DPPH$^{\cdot}$ (50 μL) and various concentrations of butanol solution of the test substances, were added with a final volume of 200 μL in sterile disposable 96-well microplate for all of the assays. (±)-α-Tocopherol served as known antioxidants. Results were determined after 30 min of reaction time in order to analyze antiradical activities. The disappearance of the free radical DPPH$^{\cdot}$ was measured spectrophotometrically at 540 nm with a microplate reader (64). The percentage inhibition was calculated by the following equation (1):

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) \times 100 \quad \text{——————} \quad (1)$$

where $A_{control}$ was the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} was the absorbance of test compound.

The antioxidant activity was expressed in term of IC_{50} (50 percent of inhibition concentrations). The IC_{50} values were obtained from linear regression of the percentage (%) inhibition versus various concentrations of the solution of test substances plot. All measurements were taken in triplicate and the mean values were calculated.

3.4.2 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was adapted from the method of Benzie and Strain (65). FRAP reagent contained 5 mL of 10 mM TPTZ in 40 mM HCl plus 5 mLof 20 mM $FeCl_3 \cdot 6H_2O$ and 50 ml of 0.3 M acetate buffer (pH 3.6) and then warmed at 37 °C before using. FRAP reagent (180 µL) was mixed with 20 µL of sample in sterile disposable 96-well microplate. The absorbance at 595 nm was measured at 120 min in the dark condition using a microplate reader. Ethanolic solutions of known concentration of ferrous salt, ranging from 10-100 µM (final concentration) $FeSO_4 \cdot 7H_2O$, was used for the preparation of the calibration curve. The equivalent concentration (EC_1) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4 \cdot 7H_2O$. EC_1 was calculated as the concentration of antioxidant giving an increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mM concentration of ferrous salt solution, determined by using the corresponding regression equation. (±)-α-Tocopherol served as known antioxidants. All measurements were taken in triplicate and the mean values were calculated.

3.5 Determination of total carotenoids content of aril oil of *Momordica cochinchinensis* (Lour.) Spreng

The concentrations of carotenoids used for antioxidant activity were determined spectrophotometrically in appropriate solvent according to Britton (66). The 0.6 g of aril oil was dissolved with hexane in a 50 mL volumetric flask. Hexane was used as the blank solution to monitor the base line. The solution was transferred into a 1 cm quartz cuvette and the absorbance was measured at 446 nm using UV-visible spectrophotometer. The total carotenoids content was expressed as ppm of β-carotene. All measurements were taken in triplicate and the mean values were calculated. The calculation was as follows equation (2):

$$\text{Carotenoids content} = \frac{383 \times \text{Absorbance} \times \text{Volume}}{100 \times \text{Weight}} \quad \text{——————} (2)$$

Where:

383	is the extinction coefficient for carotenoids
Absorbance	is the absorbance of the sample
Volume	is volume used for analysis in mL
Weight	is the weight of the sample in gram

3.6 Reverse phase - High Performance Liquid Chromatography (RP-HPLC) analysis

3.6.1 Chromatographic condition

The analysis was determined by reverse phase high performance liquid chromatography (HPLC). The HPLC was performed on a HP HPLC series 1200 (Hewlett Packard, Waldbronn, Germany) equipped with CHEM STATION software, a degasser G1322A, a quaternary gradient pump G1311A, a thermoautosampler G1313A, a column oven G1316A and diode array detection system G1314A. The column used was a symmetry C18 column (250 x 4.6 mm i.d., 5 µm.; Cosmosil®). The column was operated at a temperature of 40°C. The mobile phase consisted of (A) acetronitrile: dichloromethane: methanol (v/v 7:2:1). The column was eluted by using isocratic 100% of solution A for 20 min. The flow rate was 1 mL/min, detection was at 450 nm, and the injection volume for all samples was 5 µL. Prior to measuring by HPLC analysis, the sample solutions were filtered through a 0.45 µm nylon syringe filter.

3.6.2 Preparation of β-carotene standard solution

Five individual working β-carotene standard solutions of around 5–100 µg/mL were freshly prepared everyday from stock standard solution which dissolved petroleum ether and mixing until complete dissolution; then the solutions were transferred to a volumetric flask and making to volume with mobile phase. The different concentrations of the standards measured in triplicate. Retention time and peak area

of each preparation were recorded. The peak area was plotted against the concentration of β-carotene standard for calibration curve.

3.6.3 Analytical validation of HPLC equipment

Precision

The precision of the analytical method was determined in terms of repeatability and reproducibility. Repeatability or intraday precision was evaluated by 5 replicate injections of 3 different concentrations of β-carotene standard solutions on the same day. Reproducibility or interday precision was performed the same way of repeatability test but over 5 different days. Each sample was injected twice times onto HPLC column.

The percentage of coefficient of variation (%CV) of peak areas determined by the following equation (3):

$$\%CV = \frac{SD}{X} \times 100 \qquad \text{——————} \quad (3)$$

Where:

SD is the standard deviation

X is the mean value

Accuracy

The accuracy of the analytical method was determined in terms of repeatability and reproducibility. The accuracy of the analytical method was determined by 5 replicate injections of 3 different concentrations of β-carotene standard solutions. Reproducibility or interday accuracy was performed the same way of repeatability test but over 5 different days. Each sample was injected twice times onto HPLC column.

The percentage of inaccuracy was calculated by the following equation (4):

$$\%\ \text{Inaccuracy} = \frac{(\text{Measured concentration} - \text{Target concentration})}{\text{Target concentration}} \times 100 \qquad \text{——————} \quad (4)$$

3.6.4 Determination of β-carotene in aril oil of *Momordica cochinchinensis* (Lour.) Spreng

0.4 grams of oil was first extracted with 15 mL solution of dichloromethane (DCM) and acetone (1:4) in 100-mL separatory funnel, shaking for 5 minutes each time until the sample was colorless. About three extractions were generally sufficient. The mixture was filtered and washed 3 times with a saturated sodium chloride solution. The organic layer was taken and dehydrated with anhydrous sodium sulphate and evaporated under reduced pressure at 25°C. The residue was dissolved in 10 mL solution of dichloromethane (DCM) and diluted at appropriate with 10 ml mobile phase for HPLC analysis. The concentration of β-carotene was obtained by referring to a standard curve of β-carotene prepared under the same conditions, then calculated yield of β-carotene of aril oil (%w/w).

3.7 Preparation of nanostructured lipid carriers (NLC)

3.7.1 Optimal condition for NLC preparation

NLC was produced by hot high pressure homogenizer (HPH). The melted lipid phase containing cetyl alcohol and jojoba oil were dispersed in a hot surfactant (tween 80) solution (75°C) obtaining a pre-emulsion using a high speed homoginizer at 8000 rpm for 10 min. The combination of antioxidants (BHA, BHT and (±)-α-tocopherol) were added in the melted lipid phase during the production. This hot pre-emulsion was further processed by HPH at 60°C. Several batches were prepared by varying the homogenization pressure and number of homogenization cycle to study their effects on the characteristics of the NLC. The number of homogenization cycles was varied from 3 to 8 at a fixed homogenization pressure at 1,000 bars and homogenization pressures were varied from 600 to 1,000 bars at a fixed number of homogenization cycles at 5 cycles. The lipid dispersion was cooled at to room temperature leading to the lipid phase recrystallization and finally the NLC was formed. The compositions of formulation were shown Table 7.

Table 7 Composition of the NLC formulation (%, w/w)

Composition	(%w/w)
Cetyl alcohol	8.0
Jojoba oil	5.0
Aril oil	-
Tween 80	1.0
BHA	3.0
BHT	3.5
(±)-α-tocopherol	1.5
Deionized water q.s.	100.0

The NLC formulations were transferred to airtight clear glass containers. All formulations were then measured immediately for their particle size, size distribution and zeta potential value.

3.7.2 Preparation of aril oil of *Momordica cochinchinensis* (Lour.) Spreng - loaded NLC

Aril oil-loaded NLC was produced by hot high pressure homogenizer (HPH). The melted lipid phase containing cetyl alcohol and jojoba oil were dispersed in a hot surfactant (tween 80) solution (75°C) obtaining a pre-emulsion using a high speed homoginizer at 8000 rpm for 10 min. The combination of antioxidants (BHA, BHT and (±)-α-tocopherol) and aril oil were added in the melted lipid phase during the production at 60°C. This hot pre-emulsion was further processed by HPH at 60°C applying the homogenization pressure and cycle was selected from 3.7.1. The lipid dispersion was cooled at room temperature leading to the lipid phase recrystallization and finally the aril oil-loaded NLC was formed. The compositions of formulation ingredients were shown Table 8.

Table 8 Composition of the aril oil loaded-NLC formulation (%, w/w)

Composition	A1	A3	A5
Cetyl alcohol	8.0	8.0	8.0
Jojoba oil	5.0	5.0	5.0
Aril oil	1.0	3.0	5.0
Tween 80	1.0	1.0	1.0
BHA	3.0	3.0	3.0
BHT	3.5	3.5	3.5
α-tocopherol	1.5	1.5	1.5
Deionized water q.s.	100.0	100.0	100.0

The aril oil loaded-NLC formulations were transferred to airtight clear glass containers. All formulations were then measured immediately for their particle size, size distribution and zeta potential value.

3.7.3 Characterization of aril oil of *Momordica cochinchinnensis* (Lour.) Spreng-loaded NLC

The morphology of the NLC and aril oil-loaded NLC were determined using a transmission electron micrometer (TEM). One drop of sample was placed on to copper grid and waited until dry. After being stained by 2% (w/v) solution of uranyl acetate, it was dried at room temperature for about half an hour, and the sample was gained for the TEM investigation.

3.7.4 Stability test of the NLC and aril oil of *Momordica cochinchinnensis* - loaded NLC formulation

Each sample was divided into three groups which were stored at 4, 25 and 45°C for 90 days and kept in the airtight clear glass containers. At the end of periods, the samples were determined for particle size, size distribution and zeta potential value using a zetasizer. Before measurement, each sample was diluted with de-ionized water at room temperature.

3.8 Formulation cream containing aril oil and cream containing aril oil-loaded NLC

3.8.1 Preparation cream base and cream containing aril oil

The oil in water type cream base was prepared according to the formula shown in Table 10. The method for preparing cream base was conventional hot process. The ingredients of oil phase (part A) and aqueous phase (part B) were weighed and placed into two separated containers. The ingredients in part A were combined and melted to about 70°C. At the same time, ingredients in part B were combined and heated to about 75°C. Then, part A was added slowly to part B with constant agitation to from emulsion using mixing stirrer at 1,000 rpm for 10 min. For preparation of cream containing aril oil; aril oil (1% of formulation) was added in cream at 60°C and stirred until an emulsion homogeneous. The emulsion was cool down to the room temperature and filled into the airtight clear glass container.

Table 9 The formula preparation of oil in water cream base

Oil phase (part A)	Water phase (part B)
Cetyl alcohol Stearyl alcohol Glyceryl monostearate Silicone oil Isopropyl meristate (IPM) *Vitamin E acetate* Span 80	Tween 80 Propylene glycol Glycerin Paraben conc. Deionized water

3.8.2 Preparation cream containing NLC and cream containing aril oil-loaded NLC

The oil in water type cream base was prepared according to the formula shown in Table 10. The method for preparing cream base was conventional hot process. The ingredients of oil phase (part A) and aqueous phase (part B) were weighed and placed into two separated containers. The ingredients in part A were combined and melted to about 70°C. At the same time, ingredients in part B were combined and heated to about 75°C. Then, part A was added slowly to part B with constant agitation to from emulsion using mixing stirrer at 1,000 rpm for 10 min. For preparation of cream

containing NLC and cream containing aril oil-loaded NLC; NLC and aril oil-loaded NLC (50% of formulation) was added in cream at 40°C, respectively and stirred until an emulsion homogeneous. The emulsion was cool down to the room temperature and filled into the airtight clear glass container.

3.8.3 Stability test

All formulations were kept in the airtight clear glass containers. They were stored at 4, 25 and 45°C for 90 days and tested by accelerated test: heating-cooling cycle method which defined as alternation of storage conditions from 45°C for 48 hr to 4° C for 48 hr (1 cycle) for 6 cycles.

1. Viscosity and pH

The viscosity and pH of all formulations were measured and compared before and after accelerated test. Each sample was triplicately measured.

2. Physical properties

The changing of physical properties was also observed such as odor, color, smoothness and unstable condition (creaming and cracking).

3.9 Chemical stability studies β-carotene of cream containing aril oil and cream containing aril oil-loaded NLC

3.9.1 Temperature stability testing

All samples were placed in the airtight clear glass containers and stored at 4, 25 and 45°C for 90 days. The samples were analyzed for β-carotene remaining at the end of the storage period by RP-HPLC as described in the section 2.5.

3.9.2 Photo-stability testing

All samples were placed the airtight clear glass containers and exposed to light from a fluorescent bulb (600 lux) in the black box at room temperature for 8 hours. The samples were analyzed for β-carotene remaining at the end of the storage period by RP-HPLC following in 2.5.

3.9.3 Determination of β-carotene of cream containing aril oil and cream containing aril oil-loaded NLC for RP-HPLC analysis

All samples were determined modifying from the method described by

Ritter and Purcell (27). Samples (50 mg) were first extracted with 20 mL of acetone in 100-mL beaker, stirring for 5 min each time until the sample was colorless. About three extractions were generally sufficient. The samples were filtered and transferred into a 250-mL separatory funnel. 60 mL of petroleum ether was added. After the mixture was well shaken, the petroleum ether phase was collected. Added approximately 5 gm of a saturated sodium chloride solution to the separator and washed three times. The petroleum ether phase was taken and dehydrated with anhydrous sodium sulphate and evaporated under low pressure at 25°C. The residue was dissolved in petroleum ether and diluted with mobile phase for RP-HPLC analysis. The samples were then quantitatively evaluated by RP-HPLC. The concentration of β-carotene was obtained by referring to a standard curve of β-carotene prepared under the same conditions. Stability of β-carotene from aril oil in cream and aril oil loaded NLC in a cream were expressed as the percentage of β-carotene concentration remaining in relation to the concentration in freshly prepared aril oil in cream and aril oil loaded NLC in a cream, were analyzed for beta-carotene concentration at 0 day equivalent to be 100%.

3.10 Primary skin irritation test

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, each subject received the information of this study (Appendix A) and signed a written informed consent that contained all the basic elements outlined. (Appendix B).

Subjects of study

Twenty Thai volunteers (aged 30– 60 years) were selected by using inclusion and exclusion criteria.

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. Subjects who 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.

Test substance application protocol (67, 68)

The patch test procedure involved application of 0.2 g on a webril pad to the skin of the upper outer arm of 20 human volunteers for up to 4 hr. Test materials were applied progressively from 15 and 30 min through 1, 2, 3 and 4 h. Each progressive application was at a new skin site. The shorter exposure periods were omitted if the study directors were satisfied that excessive reactions would not occurred following longer exposure. Treatment sites were assessed for the presence of irritation using a 5 point visual scoring scale at 24, 48, and 72 hr after patch removal. A volunteer with a positive or greater reaction at any of the assessments was considered to have demonstrated a positive irritant reaction, and treatment with the causative substance did not proceed on that person. For volunteers with a positive or greater response at application times of less than 4 hr to a particular test substance, it was assumed that they would present a stronger irritant reaction if exposed for 4 hr. However, once a positive or greater response was obtained, there was no need to subject these volunteer to further treatment with that substance. In these studies, 1% sodium lauryl sulfate (SLS) was used as positive control. The primary skin irritation was evaluated by visual scoring according to International Contact Dermatitis Research Group

(ICDRG) scale from 0 to 5 (0, no erythema; 1, very slight erythema, well-defined uniform erythema; 3, moderate to severe erythema; 4, severe erythema to slight eschar formation; 5, severe erythema with oedema) then calculated the primary irritation index (PII).

3.11 Wrinkle reducing capacity test

The protocol of clinical study was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. Before participating in the clinical study, each subject received the information of this study (Appendix A) and signed a written informed consent that contained all the basic elements outlined. (Appendix B). Wrinkle development in aged skin has been associated with free radicals. This clinical study was modified from two studies (69, 70)

3.11.1 Location and duration time of study

The study was started in between 1 August – 30 September 2010 at the Faculty of Pharmacy, Chiang Mai University.

3.11.2 Subjects of study

Twenty Thai volunteers (aged 30– 60 years) were selected by using inclusion and exclusion criteria.

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. Subjects were 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. Subjects who 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.

3.11.3 Test substance application protocol

The subjects were enrolled in a single-blind, placebo-controlled. Application of any cosmetic products was prohibited a week prior to the start of the study. Test creams (A, B, C and D) were applied at the test sites (Figure 9). Subjects applied test cream to forearm by gentle circular massaging motion. To each site of test was applied an approximately amount (0.2 g) of each assigned test formulation. The study protocol included evaluation at day 0 and after 8 weeks of treatment. Subjects were instructed to apply the test creams twice daily (once in the morning, once in the evening) continuously for 8 weeks. The improvements of skin condition were evaluated by reducing of wrinkle. The instrument used is Skin Visiometer® SV 600 for analysis of the skin profile with the 'classical' DIN parameters (R*a* and R*z*) method, surface and volume (Appendix C) at five test sites (untreated area, N; cream containing NLC, A; cream containing aril oil loaded NLC area, B; cream base area, C; cream containing aril oil area, D).

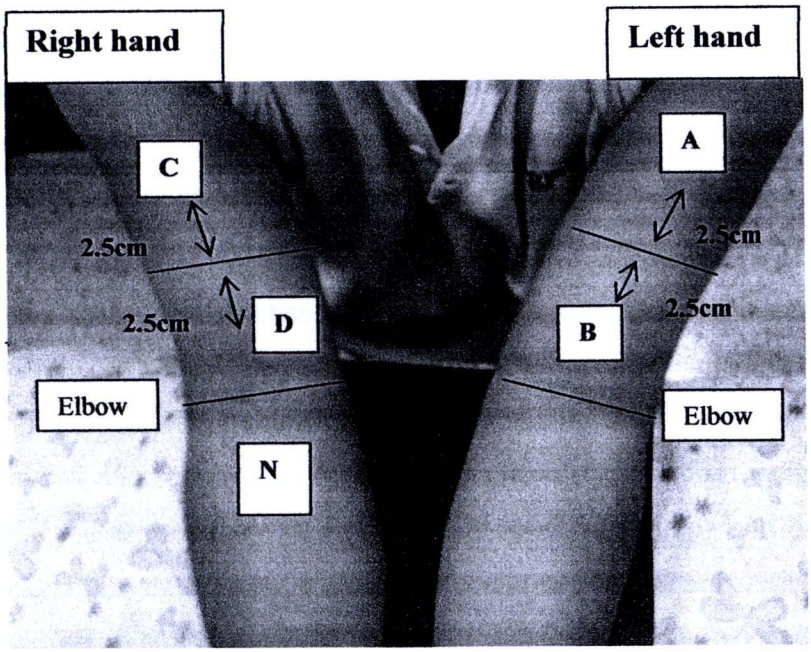

Figure 9 Profile of test sites (N = untreated area; A = cream containing NLC area; B = cream containing aril oil loaded NLC area; C = cream base area; D = cream containing aril oil area)

3.11.4 Statistical analyzes

Paired t-test ($P<0.05$) was used to examine changes in R*a*, R*z*, volume and surface values, before and after of each treatment (untreated cream, treated active-cream, treated placebo cream). The percentage efficiency values were calculated by the following equation (5):

$$\% \text{ Efficiency} = \frac{\text{value at measuring point-value at initial point}}{\text{value at initial point}} \times 100 \quad \text{———(5)}$$