# CHAPTER III
# MATERIAL AND METHODS

## 3.1 Chemicals

1. Absolute ethanol (Lab-scan LTD, Ireland)
2. Acetic acid (Lab-scan LTD, Ireland)
3. Catechin (Sigma chemical Co., USA)
4. Cholesterol from lanolin ($C_{27}H_{46}O$, Fluka chemie GmbH., Japan)
5. Chlorofrom (Merck, Germany)
6. Dipotassium hydrogen phosphate ($K_2HPO_4$, Sigma chemical Co., USA)
7. Epigallo catechin gallate (EGCG) (Sigma chemical Co., USA)
8. Gallic acid (Sigma chemical Co., USA)
9. Kaempferol (Sigma chemical Co., US)
10. Methanol (Lab-scan LTD, Ireland)
11. Phosphatidylcholine (Epikulon 200, Degussa, Germany)
12. Potassium dihydrogen phosphate ($KH2PO_4$, Sigma chemical Co., USA)
13. Quercetin (Sigma chemical Co., USA)
14. Sodium sulphate anhydrous (Merck, Germany)
15. Trolox (Sigma chemical Co., US)
16. t-octylphenoxypolyethoxyethanol (Triton X-100, Sigma chemical Co., USA)
17. 2,2-diphenyl-1-picryl hydrazyl (DPPH, Sigma chemical Co., USA)
18. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Wako Pure Chemical Industries, Japan)
19. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, Wako Pure Chemical Industries, Japan)
20. 2-Thiobabituric acid 98% (TBA, Sigma chemical Co., USA)

### 3.2 Plant material

Five cultivars (O-Hia, Kwangchao, Chakapad, Hong-Huay and Kim Cheng) of lychee (*Litchi chinensis* Sonn.) seed that cultivated in northern of Thailand were used in this study.

### 3.3 Instruments

1. Analytical balance (Sartorius®, Model BP610, Germany)
2. Clevenger apparatus (Bacthai bankok equipment & chemical Co.Ltd, Thailand)
3. Column HPLC
4. Cutometer® (MPA580, CK Electronic GmbH, Germany)
5. High Pressure Liquid Chromatography (HPLC)
6. pH meter ( Horiba Model EX-20, Korea)
7. Micropipettes 1-200 µl, 1-1000 µl ( Pipetman®, Gilson Co. Ltd., France)
8. Microplate 96 well ( Nunc®, USA)
9. Microtiter plate reader (Backman coulter®, DTX 880 multimode detector, Australia)
10. Rota evaporator ( EYELA®, Japan)
11. Skin-visiometer® (SV 600 FireWire, CK Electronic GmbH, Germany)
12. Water bath ( Memmert®, Germany)

### 3.4 Quality control of raw material

Fresh lychee seeds were dried at 60$^{o}$ C for 24 hours then grounded with the grinder. The lychee seed powder was determined the quality of raw material followed the official method in Thai Herbal Pharmacopoeia (1998, volume 1) [83].



### 3.5 Optimization of lychee seed extraction

The three different extraction methods were compared to find out the highest antioxidant activity of the Lychee seed extract.

**Method 1**

The lychee seed powder was macerated in acetone at room temperature for 4 hours 3 times. All of filtrate was combined and then evaporated to dryness in vacuum. The extracts were stored in the dark at 4°C until tested [76].

**Method 2**

The lychee seed powder was macerated in methanol/1.5 N HCl (85:15 v/v) at 4°C for 20 min 3 times. All lychee seed extract solution was filtered and evaporated to dryness in vacuum. The extracts were stored in the dark at 4°C until tested [75].

**Method 3**

The lychee seed powder was macerated in 85% ethanol at room temperature for 2 hours 3 times. All lychee seed extract solution was filtered and then evaporated to dryness in vacuum. A part of the dried extract was re-dissolved in 85% ethanol and added 20 of water and then partitioned with 30 ml of n-hexane and ethyl acetate sequentially. Each fraction was dried using a rotary evaporator at 40 °C and stored in the dark at 4°C until tested [80, 82].

### 3.6 Determination of antioxidant activities

The in vitro antioxidant assays were done by 3 different methods, DPPH assay, ABTS assay and lipid peroxidation (thiobabituric acid-reactive substance, TBARS method) showed that the lychee seed extracts possessed antioxidant activity.

#### 3.6.1 Determination of antioxidant activity with 2, 2 -diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The experimental procedure was adapted from Brem et al. (2004) [84]. In this assay, the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH), which has a strong absorption at 540 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 •/ethanol solutions, 180 µL. Trolox, Ellagic acid and Gallic acid served as known antioxidants. The samples were tested in dilutions ranging from 0.1-0.5 mg/mL with a final volume of 200 µL for all of the assays. Results were determined after 30 min of reaction time in order to analyse

antiradical activities. The disappearance of the free radical $DPPH^{\bullet}$ was measured spectrophotometrically at 540 nm with a microplatereader. The percentage inhibition was calculated by the following equation**:**

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) * 100$$

**3.6.2 Determination of antioxidant activity with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) cation radical scavenging assay**

ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h. before use. The solution was diluted with ethanol to obtain the absorbance of 0.9±0.1 units at 734 nm. The extracts solution of 70 µl of ethanolic extracts test solution of each sample was added to 630 µl of ABTS free radical cation solution. The absorbance, monitored for 50 min, was measured spectrophotometrically at 734 nm by using a Spectophotometer (Shimadzu UV-Vis 2450, Japan). All measurements were performed in triplicate. The free radical-scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC) [85, 86].

**3.6.3 Determination of antioxidant activity with thiobarbituric acid reaction species (TBARS) assay**.

A modified thiobarbituric acid-reactive substances (TBARS) assay was used to measure the antioxidant activity of the lychee seed extracts in term of inhibition on lipid peroxidation [87, 88]. Liposome suspension, consisting of Cholesterol, Phosphatidylcholine and 0.2 M potassium phosphate buffer (pH 7.2), was prepare in a sonicator for 20 min. The lychee seed extract in ethanol) was mixed with amixture of the sonicated solution ,and AAPH. The resulting mixture was incubated for 24 hr at 50° C. After incubation, the solution was mixed with 0.2% BHT, 3%Triton-X , 20% acetic acid and 0.6% TBA. Then heated at 90° C for 30 min after that cooling the mixture to room temperature. The absorbance of the mixture was measured spectrophotometrically at 540 nm with a microplatereader. The percentage inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) * 100$$

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 was calculated form the graph plotted inhibition percentage against extract concentration. Gallic acid, Quercetin and Ellagic acid served as known antioxidants.

**3.7 Determination of Total Phenolic content in lychee seed extracts**

The total phenolic content of the ethanolic extract was determined using the Folin-Ciocalteu reagent. Each filtrated of sample was transferred into an eppendorf tube that contained distilled water and then mixed thoroughly with of Folin- Ciocalteu reagent. After mixing for 3 min, 7.5% (w/v) sodium carbonate was added. The mixtures were agitated with a vortex mixer, then allowed to stand for a further 30 min in the dark. The absorbance of extracts and blanks were measured at 765 nm using a spectrophotometer (Shimadzu UV-Vis 2450, Japan). The concentration of total phenolic compounds in all extracts were expressed as milligrams of gallic acid equivalents (GAE) per gram of lychee seed extract [89-91].

**3.8 Determination of chromatographic finger print of lychee seed extract by HPLC**

The chromatographic finger print of lychee seed extract was determined by reverse phase high performance liquid chromatography. The separation of phenolic compounds was performed on a HP HPLC series 1100 (Hewlett Packard, Waldbronn, Germany) equipped with CHEM STATION software, a degasser G1322A, a binary gradient pump G1311A, a thermoautosampler G1313A, a column oven G1316A and UV-Visible detection system G1314A. The column was an Alltech Altima 5 mm C (25034.6 mm I.D.) and a C18 guard column (Phenomenex 4 x 3.0 mm.). The column was operated at a temperature of 40°C. The mobile phase consisted of 3% (v/v) acetic acid in water (eluent A) and methanol (eluent B) [92, 93]. The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B

(5 min). The injection volume for all samples was 20 µl. Simultaneous monitoring was performed at 280 nm at a flow-rate of 1 ml /min.

**3.9 Skin irritation testing in animal** [94]

Three albino rabbits were used for skin irritation by modified Draize model. The Draize model and its modification are commonly used to assay skin irritation. Draize used this scoring system to calculate the primary irritation index (PII). This is calculated by averaging the erythema scores and the edema scores of all sites (abraded and nonabraded). These two averages are then added together to give the PII value.

Although the Draize scoring system does not include vesiculation, ulceration, and severe eschar formation, all of the Draize-type tests are used to evaluate corrosion as well as irritation. Therefore, Draize assays continue to be recommened by regulatory bodies for drugs and industrial chemicals.

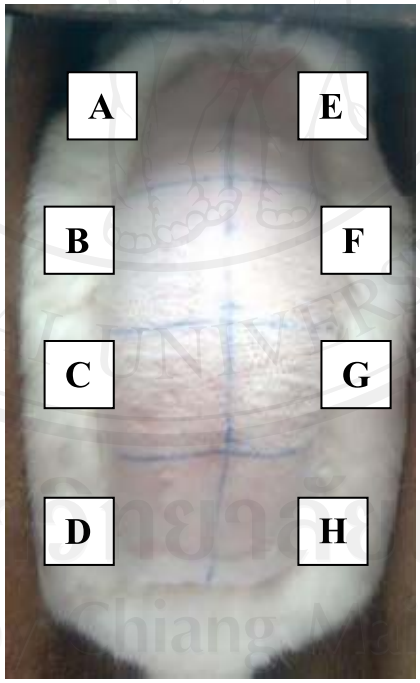


**Figure 3.1** Profile of test site on rabbit skin (A = untreated site, B = crude extract, C = ethyl acetate part extract, D = cream base, E = cream contain ethanolic part extract, F = ethanol, G = ethyl acetate, H = cream contain ethyl acetate part extract)

**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 2 $inch^2$ 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<br>Rubberized cloth over entire trunk |
| 5 Occlusion period | 4 hours |
| 6 Assessment | 1, 24, 48 and 72 hours<br>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** Type of Skin Irritation score

| Primary Irritation Index | Type of Skin Irritation |
|---|---|
| < 0.5 | Non-irritating |
| 0.5 – 2.0 | Slightly irritating |
| 2.1 – 5.0 | Moderately irritation |
| > 5.0 | Severe irritation |

### 3.10 Formulation and stability test of cream base

#### 3.9.1 Formulation of cream base

The composition of the o/w cream base were cetyl alcohol, stearyl alcohol, stearic acid, glyceryl monostearate, cyclomethicone, jojoba oil, carbopol ETD 2020, cremophore, tween 80, propylene glycol, triethanolamine, sodium metabisulfite, conc. paraben and purified water. They were varied in different concentration for three formulas.

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 and mix until homogeneous. The emulsion was then cool down to the room temperature

#### 3.10.2 Stability test of cream base

The stability of cream base was tested by accelerated test: freeze-thaw cycling 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

#### 3.10.3 pH of cream base

The pH of all cream bases were measured and compared before and after accelerated test.

**3.10.4 Visualization of physical changing**

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

**3.10.5 Spreadability and feel on skin**

Spreadability and feel on skin of all cream bases were tested and compared before and after accelerated test.

**3.10.6 Selection of good cream base**

The pH, visualization of physical changing, spreadability and feel on skin of all cream bases formulas were compared and selected as cream base for lychee seed extract cream

**3.11 Formulation and stability test of lychee extract cream**

The lychee extract with good antioxidant activity and good fragrance property were chosen to add in the selected cream base for anti-wrinkle cream. Lychee seed extract cream as well as cream base were determinated pH, stability, physical property, spreadability and feel on skin.

**3.12 Skin irritation testing in human volunteer**

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 and signed a written informed consent that contained all the basic elements outlined.

**3.12.1 Subjects of study**

Forty-eight Thai volunteers (aged 25– 60; n = 48) 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. 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 back area of volunteer was divided into two parts, left and right. Each part was treated by Finn chamber that contain the sample (cream base, cream of ethanolic part, cream of ethyl acetate part, and 1% sodium lauryl sulfate solution). After 48 hours, the irritate value was evaluated at 0 and 7 days by using Draize scoring system.

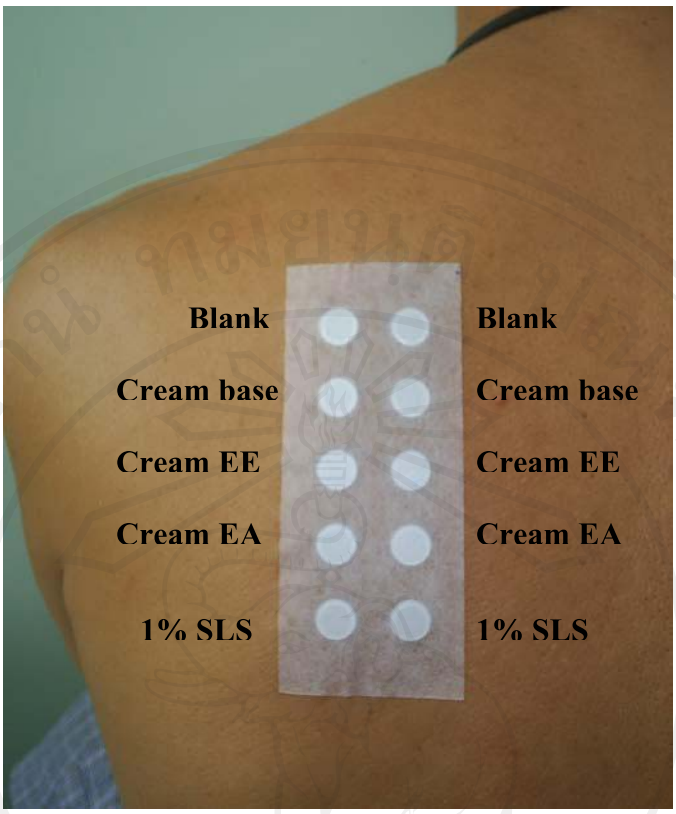


**Figure 3.2** Profile of test site on human skin (Cream EE = cream contain ethanolic part extract, Cream EA cream contain ethyl acetate part extract, 1% SLS = positive control)

### 3.13 Antioxidant test of lychee seed extract cream

The ethanolic part of lychee seed extract cream and ethyl acetate part of lychee seed extract cream were prepared in 4 concentrations, 0.1%, 0.2%, 0.5% and 1.0%, then extracted by absolute ethanol and centrifuge at 4500 rpm for 30 min. The antioxidant activity of supernatant was determined the by DPPH radical scavenging before and after stability test.



### 3.14 Wrinkle reducing capacity tests of lychee seed extract cream

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 [95, 96].

### 3.14.1 Location and duration time of study

The study was started in May 2009 at the Faculty of Pharmacy, Chiang Mai University.

### 3.14.2 Subjects of study

Twenty Thai volunteers (aged 25– 60; n = 24) 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. 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

### 3.14.3 Test substance application protocol

Application of any cosmetic products was prohibited 3 days prior to the start of the study. Subjects were instructed to apply the test creams twice daily (once in the morning, once in the evening) for 8 weeks. Test creams (B and C) were applied at the test sites (Figure 3.3). Subjects used the left-hand fore and middle fingers to apply test cream to right forearm by gentle circular massaging motion. To each site of test was applied a approximately amount (0.2g) of each assigned test formulation.

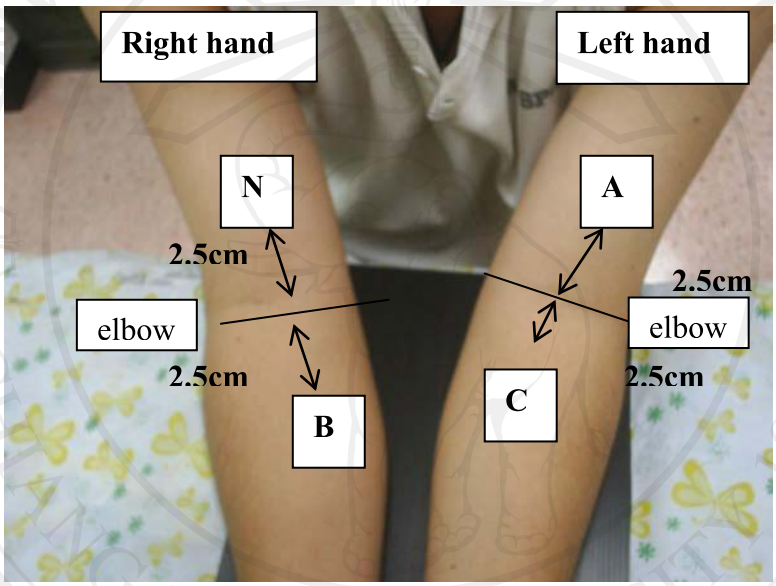


**Figure 3.3** Profile of test site (**N** = untreated site; **A** = placebo-cream site; **B** = Active cream I; C = Active cream II)

### 3.14.4 Assessment calendar and methodologies

The subjects were enrolled in a single-blind, placebo-controlled. The study protocol included evaluation at day 0 and after 8 weeks of treatment. The improvements of skin condition were evaluated by wrinkle. The instrument used is Skin Visiometer® Sv 600 for analysis of the skin profile with the 'classical' DIN parameters (Ra and Rz) method, surface and volume (Appendix C) at four test site (N = untreated site; A = placebo-cream site; B = Active cream I; C = Active cream II)

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### 3.14.5 Statistical analyzes

Paired t-test were 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 :

(value at measuring point – value at initial point)*100 / value at initial point

The data were subjected to a 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. of the three test site ( untreated site, active-cream site, placebo-cream site) was evaluated using test.

### 3.14.6 Questionnaire

After testing was finished, all volunteers were asked some question (see Appendix D)