# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Chemicals

1. Acetic acid (Lab-Scan Ltd., Ireland)
2. Chlorogenic acid (Sigma, USA)
3. De-ionized water (Millipore, USA)
4. Dimethyl sulfoxide (DMSO) (RCI LABSCAN, Thailand)
5. Dipotassium hydrogen phosphate (Sigma, USA)
6. Ellagic acid (Sigma chemical Co., USA)
7. Eudragit® E100 (Jebsen & Jessen Nutrilife Ltd., Germany)
8. Eudragit® NE30D (Jebsen & Jessen Nutrilife Ltd., Germany)
9. Formic acid (Fisher Scientific UK Ltd., UK)
10. Gallic acid (Sigma, USA)
11. Glycerin (VIDHYASOM Co., LTD., Thailand)
12. Hydroxyethylcellulose (VIDHYASOM Co., LTD., Thailand)
13. Hydroxypropyl methylcellulose (VIDHYASOM Co., LTD., Thailand)
14. Lemon oil (United Chemical & Trading, Thailand)

15. Methanol (HPLC grade, Labscan., Thailand)
16. Methyl paraben (VIDHYASOM Co., LTD., Thailand)
17. Oleic acid (VIDHYASOM Co., LTD., Thailand)
18. PEG 400 (Srichand United Dispensary Co., Ltd. Thailand)
19. Potassium dihydrogen phosphate (Sigma-Aldrich Inc., USA)
20. Propylene glycol (VIDHYASOM Co., LTD., Thailand)
21. Propyl paraben (VIDHYASOM Co., LTD., Thailand)
22. PVP K90 (United Chemical & Trading, Thailand)
23. Shrimp chitosan (Taming Co., LTD., Thailand)
24. Sodium metabisulfite (RCI LABSCAN, Thailand)
25. Squid chitosan (Taming Co., LTD., Thailand)
26. Triethyl citrate (Merck, Germany)
27. Tween 80 (VIDHYASOM Co., LTD., Thailand)

### 3.1.2 Equipments

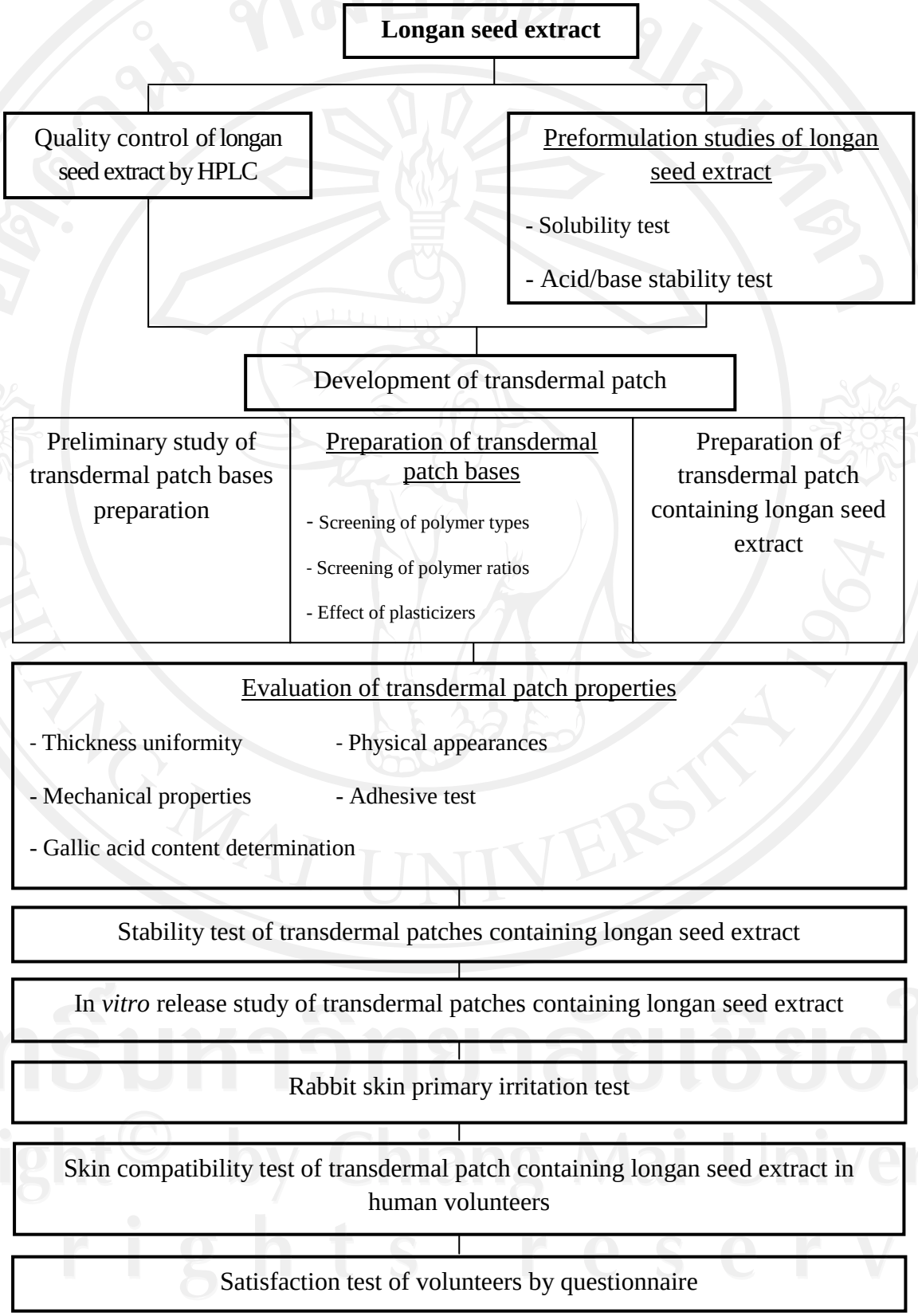
1. Analytical balance 2 position (A&D. Co., LTD. Japan)
2. Analytical balance 4 position (Precisa XT220A, Switzerland)
3. Auto pipett 1-200 µl, 1-1000 µl (Pipetman®, Gilson Co. Ltd., France)
4. Glass plates
5. Homogenizer (KIKA® Yellow line, Germany)
6. Hot air oven (Memmert®, CMbH Co., Ltd., Germany)
7. HPLC (hp 1100 series, Hewlett Packard, USA)
8. Magnetic stirrer (KIKA®, Yellow line, Germany)

9. Franz-diffusion cell
10. pH Meter (Horiba Model EX-20, Korea)
11. Rolling Ball Tack Tester (ASTM D 3121-06)
12. Tensile (HOUNSFIELD, S1KS).
13. Ultrasonic sonicator (Elma®, Elma GmbH & Co KG, Germany)
14. Vortex mixer (Scientific industry, USA)

### 3.1.3 Other Materials

1. Backing membrane
2. Cellulose membrane (Sigma, USA)
3. Release liner (3M company, USA)

**Research designs**



## 3.2 Methods

### 3.2.1 Quality control of longan seed extract by HPLC

The longan seed extract was dissolved in DMSO and filtered through 13 mm, 0.45 µm nylon membrane and gallic acid was identified and also quantified by using HPLC. The HPLC system consisted of HPLC series hp 1100 (Hewlett Packard, USA) equipped with CHEM STATION software, degasser G1322A, binary gradient pump G1311A and thermoautosampler G1313A. The column was an Zorbax Eclipse XDB - C18 4.6 x 250 mm 5 microns and a C18 guard column (Eclipse XDB 4.6 x 12.5 mm, 5 micron). Solvent gradients were formed by varying the proportion of solvent A (0.4% formic acid) to solvent B (80% methanol). Solvent A was increased to 100% in 5 min and subsequently decreased to 0% in 40 min at a flow rate of 1 ml/min. The injection volume for all samples was 50 µl and the gallic acid was detected at 270 nm.

### 3.2.2 Preformulation studies of longan seed extract

#### Physicochemical characterization

#### Solubility test

The solubility of the longan seed extract in various vehicles were tested as DI water, glycerin, propylene glycol, 10% tween 80, DMSO, PEG 400 and acetic acid. In this study, the extract was dissolved in each solvent in the ratios of 1:1, 1:10, 1:20 and 1:30 (w/v). The descriptive terms of solubility were showed in Table 3.1. Then the solutions were mixed with vortex mixer at room temperature and observed for their solubility and compatibility.

**Table 3.1** The descriptive terms of solubility (European Pharmacopoeia 5th ed., 2004)

| Descriptive Term | Parts of Solvent Required for 1 Part of Solute |
|---|---|
| Very soluble | Less than 1 |
| Freely soluble | From 1 to 10 |
| Soluble | From 10 to 30 |
| Sparingly soluble | From 30 to100 |
| Slightly soluble | From 100 to 1,000 |
| Very slightly soluble | From 1,000 to 10,000 |
| Practically insoluble, or Insoluble | Greater than or equal to 10,000 |

**Acid/base stability test of longan seed extract**

The solution of HCl (1N) or NaOH (10% w/v) was added into the longan seed extract solution for adjusting the pH to 3, 4, 5, 6, 7 and 8 (the longan seed extract solution was used as a control). The changing of physical characteristics such as color and precipitation were observed immediately and after storage at various conditions [room temperature, room temperature no light, 4°C and 45°C for 1 month and at accelerated test: heating/cooling cycling method (4°C, 48 hrs alternated 48°C, 48 hrs as 1 cycle) for 6 cycles].

### 3.2.3 Preliminary study of transdermal patch bases preparation

The preliminary study purpose to screen the types of polymer, plasticizer and penetration enhancer for suitable transdermal patch base. Several types of polymer (synthetic polymer: PVP K90, semi-synthetic polymer: HPMC and natural polymer: squid chitosan) were used to combine in different ratios. Additive such as plasticizers (PEG 400, propylene glycol and glycerin) and penetration enhancers (DMSO, oleic acid and lemon oil) were selected and added in the transdermal patch bases. The transdermal patch formulations prepared with different polymers ratio, plasticizers and penetration enhancers were shown in **Table 3.2**. The mechanical property and stability test were determined (data not shown). Their adhesive properties were expressed in term of good or poor or no adhesion by the perception of the tester (data not shown).

**Table 3.2** Composition of the transdermal patch base

| Code | Polymer ratio | Plasticizer (%) | | | Enhancer (%) | | |
|---|---|---|---|---|---|---|---|
| | HPMC: Sq: PVP | PEG | PG | GC | D | L | O |
| F1 | 3:12:1 | - | - | - | - | - | - |
| F2 | 6:9:1 | - | - | - | - | - | - |
| F3 | 9:6:1 | - | - | - | - | - | - |
| F4 | 12:3:1 | - | - | - | - | - | - |
| F5 | 15:15:1 | - | - | - | - | - | - |
| F4-PEG | 12:3:1 | 15 | - | - | - | - | - |
| F4-PG | 12:3:1 | - | 15 | - | - | - | - |
| F4-GC | 12:3:1 | - | - | 15 | - | - | - |
| F4-PEG-D | 12:3:1 | 15 | - | - | 1 | - | - |
| F4-PEG-L | 12:3:1 | 15 | - | - | - | 1 | - |
| F4-PEG-O | 12:3:1 | 15 | - | - | - | - | 1 |

Sq = squid chitosan, HPMC = hydroxypropyl methylcellulose, PVP = polyvinylpyrrolidone, GC = glycerin, PG = propylene glycol, D = dimethyl sulfoxide, L = lemon oil and O = oleic acid

### 3.2.4 Preparation of transdermal patch bases

#### 3.2.4.1 Screening of polymer types

The primary screening, aimed to screen the new suitable types of polymer base on the preliminary study data. Several types of synthetic polymer; Eudragit® NE 30 D, Eudragit® E100, and semi-synthetic polymer; HEC, HPMC were used in the combination with natural polymers: squid chitosan and shrimp chitosan in the base of formulation of transdermal patches. Each type of polymer was combined in the same ratio as shown in **Table 3.3**. Plasticizers such as propylene glycol and

triethyl citrate were added in the formulation. The suitable transdermal patches formulations were obtained after screening with visually physical appearances.

**Table 3.3** Types of combined polymer in the transdermal patch bases

| **Types of polymer Syn: Nat: Semi-syn*** | **Ratio of polymers** |
|---|---|
| Eu NE: Sq: HPMC | 1:1:1 |
| Eu NE: Sq: HEC | 1:1:1 |
| Eu NE: Sh: HPMC | 1:1:1 |
| Eu NE: Sh: HEC | 1:1:1 |
| Eu E100: Sq: HPMC | 1:1:1 |
| Eu E100: Sq: HEC | 1:1:1 |
| Eu E100: Sh: HPMC | 1:1:1 |
| Eu E100: Sh: HEC | 1:1:1 |

* Syn = Synthetic polymer, Nat = Natural polymer, Semi-syn = Semi-synthetic polymer, Eu NE = Eudragit® NE 30 D, Sq = Squid chitosan, HEC = Hydroxyethylcellulose and HPMC = Hydroxypropyl methylcellulose

### 3.2.4.2 Screening of polymer ratios

For the secondary screening, the selected polymers which obtained from primary screening; Eudragit® NE 30 D, squid chitosan and HEC were combined in various ratios as shown in **Table 3.4**. Propylene glycol and triethyl citrate were used as plasticizers. The suitable formulation was then screened based on physical appearances, mechanical property and adhesive property.

**Table 3.4** Combination of the polymers in various ratios, formulation P1-P5

| Formulation No. | Ratio of polymers Syn: Nat: Semi-syn* |
|---|---|
| P1 | 4: 1: 5 |
| P2 | 4: 2: 4 |
| P3 | 4: 3: 3 |
| P4 | 4: 4: 2 |
| P5 | 4: 5: 1 |

* Syn = Synthetic polymer, Nat = Natural polymer and Semi-syn = Semi-synthetic polymer

### 3.2.4.3 Effect of plasticizers

The most optimal transdermal patch formulation (P2) was selected for adding plasticizers; propylene glycol and triethyl citrate. Propylene glycol was varied from 15% to 25% and 5% - 15% of triethyl citrate that were used in the formulations as shown in **Table 3.5**. The appropriate transdermal patch formulation was carried out on evaluation of the physical appearances, mechanical properties and adhesive properties.

**Table 3.5** Compositions of plasticizers in transdermal patch bases

| Formulation No. | Plasticizers | |
|---|---|---|
| | Propylene glycol | Triethyl citrate |
| 1A | 15 | 5 |
| 2A | 15 | 10 |
| 3A | 15 | 15 |
| 1B | 20 | 5 |
| 2B | 20 | 10 |
| 3B | 20 | 15 |
| 1C | 25 | 5 |
| 2C | 25 | 10 |
| 3C | 25 | 15 |

### 3.2.5 Preparation of transdermal patch containing longan seed extract

The transdermal patches were prepared by film casting techniques as follow. Firstly, the polymers were dissolved in suitable solvents. Longan seed extract (0.5% w/w) was dissolved in a suitable solvent before added in the polymer solution. Propylene glycol or triethyl citrate served as plasticizer where DMSO (3C-D-E), oleic acid (3C-O-E) or lemon oil (3C-L-E) as penetration enhancer. The final solution was then poured into a glass plate and dried in a hot air oven at 45°C. The dried films were packed in aluminum foil and stored in a desiccator until further use.

### 3.2.6 Evaluation of transdermal patch properties

#### 3.2.6.1 Physical appearances

The appearances of all transdermal patches were determined visually. These characteristics included color, flexibility and residue after removal.

#### 3.2.6.2 Thickness uniformity [38]

The thickness of each film was measured using micrometer (Digital caliper). The thickness was measured at six different places of each film and the averages were recorded.

#### 3.2.6.3 Mechanical property [39]

The apparatus used for measuring this property was a tensile tester (HOUNSFIELD, S1KS) as shown in **Figure 3.1**. The test film of size (10 mm × 50 mm) and free from air bubbles was fixed between two clamps positioned at a distance of 3 cm. The rate of clamps separation was 0.5 mm/sec, to a distance of 5 cm. Each formulation was also evaluated in triplicate. The tensile strength and elongation at break were calculated.

*Tensile strength* = Breaking force (N)/ Cross-sectional area of sample ($mm^2$)

*Elongation (%)* = Increase in length at breaking point (mm)/

Original length (mm) × 100


**Figure 3.1** Tensile testing machine (HOUNSFIELD, S1KS)

#### 3.2.6.4 Adhesive test (Modified Rolling Ball Tack Tester)

Rolling ball tack tester (ASTM D 3121-06) was used for the adhesion test, as shown in **Figure 3.2** The transdermal patches were tested for their adhesion related with distance running of a stainless steel ball on aluminum tray. When the stainless steel ball was released at the top of an incline, allowed to accelerate down the incline and roll on to a horizontal surface covered with the transdermal patches. Tack was determined by measuring the distance that the ball travels across the adhesive before stopping. The tack value represents adhesiveness. Each formulation was evaluated in triplicate.

**Figure 3.2** Modified Rolling Ball Tack Tester

**3.2.6.5 Gallic acid content determination**

The content of gallic acid from the transdermal patches containing longan seed extract was determined by dissolving a portion of the transdermal patch in a solvent. The patch was cut in rectangular 1 $cm^2$ and accurately weighed. The sample was dissolved in 10 ml of methanol. Then, it was shaken on magnetic stirrer for 24 hrs and sonicated in Ultrasonic sonicator for 2 hrs. The obtained solution was filtered and the amount of released active compound was determined by HPLC.

**3.2.7 Stability test of transdermal patches containing longan seed extract**

The stability studies of the transdermal patches containing longan seed extract were carried out at different temperatures: 4°C, 45°C, room temperature (27 ± 2°C) with 75% relative humidity for 3 months and accelerated test: heating-cooling cycling method (6 cycles). Thereafter, the tested transdermal patches were observed for physical and chemical properties.

**3.2.8 In *vitro* release study of transdermal patches containing longan seed extract** [38]

The gallic acid released from the transdermal patches was determined by using Franz-diffusion cell **(Figure 3.3).** The certain surface area of transdermal patch containing longan seed extract, 2.25 $cm^2$, was cut into the circular shape. The cellulose membrane with molecular weight cut off 12,000 (Sigma, USA) was for 24 hrs soaked in 40% v/v PEG 400 in PBS of pH 7.4. The condition of these studies was performed at 32± 0.5°C. The samples of 3 ml were collected at 1, 2, 3, 6, 12 and 24 hr and replaced with fresh medium. The gallic acid content in the samples was evaluated

by using HPLC method. Cumulative percentage of the released gallic acid was calculated and plotted against time.

**Figure 3.3** Modified Franz diffusion cell

**3.2.9 Rabbit skin primary irritation test** [41-42]

The safety of transdermal patches was determined by skin primary irritation. Three healthy young adult male albino rabbits – *Oryctolagus cuniculus* (strain: New Zealand white; NZW) were used as tested animals, and kept separately in the 20 ± 3 °C experimental animal room. Approximately 24 hrs before the test, fur had been removed from the test area by clipping from the dorsal area. Then, 2 x 2 $mm^2$ of tested transdermal patches, including transdermal patch base, transdermal patches containing longan seed extract, 1 % w/v sodium lauryl sulfate (SLS) as positive control and deionized water as negative control, were applied to 25 x 25 $mm^2$. After 4 hr of exposure, gauze patches were removed and the areas were cleaned with water. The tested areas were observed at 1, 24, 48 and 72 hr after removal for erythematous and edematic reactions. The reactions were scored based on Draize scoring system. The primary irritation index (PII) was calculated and categorized for type of irritation.

**Table 3.6** Draize scoring system

| Reactions | Value |
|---|---|
| Erythema and Eschar Formation: | |
| No erythema | 0 |
| Very slight erythema (barely perceptible) | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema (beef 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 approximately 1 mm) | 3 |
| Severe edema (raised > 1 mm and extending beyond area of exposure) | 4 |

**Table 3.7** Classification of skin irritation

| Primary Irritation Index (PII) | Classification of skin reaction |
|---|---|
| 0 – 0.4 | No irritation |
| 0.5 – 1.9 | Slightly irritation |
| 2.0 – 4.9 | Moderately irritation |
| 5.0 – 8.0 | Severe irritation |

**3.2.10 Skin compatibility test of transdermal patch containing longan seed extract in human volunteers**

Skin compatibility, as defined in Walker AP et al (1996) [43], was evaluated with single application closed patch epicutaneous test under occlusion. Initially, the test protocol was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. The volunteers received the information regarding the nature of study, timetable, limitation and possible risks, and gave their written informed consent prior participation.

**3.2.10.1 Study population**

Thirty healthy Thai volunteers (aged 20-50) were selected on the basis of inclusion and non-inclusion criteria.

**Inclusion criteria**

- Informed volunteers
- Subjects agreeing to follow the conditions in the study information sheet

**Non-inclusion criteria**

- Any active skin disease that may interfere with the study
- Blemishes or marks (e.g. tattoos, scars, sunburn) on the test sites
- Irritated skin on test sites
- Pregnancy or nursing condition
- Medication that may affect skin response (e.g. using antiallergic drugs), or past medical history
- Participation in another clinical study

**Withdrawal criteria**

- Having any severe adverse effects
- No longer wish to participate in the study

**3.2.10.2 Test materials**

Tested samples were transdermal patch base, selected transdermal patch containing longan seed extract, while 1 % w/v sodium lauryl sulfate (SLS) as positive control and deionized water as negative control. Test sites were both left and right of upper back (duplication).

**3.2.10.3 Test method**

The test sites were firstly cleaned by gently swabbing with water. Then, 1×1 $cm^2$ of test substances, with the sequence according to **Figure 3.4**, was adhered and covered with waterproof hypoallergic film for 48 hrs. The test sites were also cleaned suddenly with water after patch removal, and skin compatibility was then assessed at 1, 24, 48 and 72 hr for the irritation responses based on Draize scoring system.

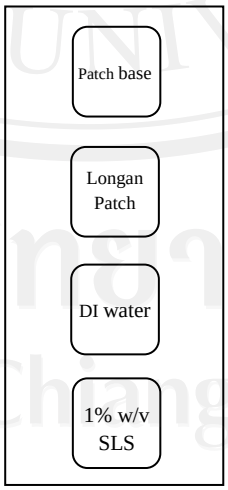



**Figure 3.4** Sequence of application on skin compatibility test

### 3.2.11 Satisfaction test of volunteers by questionnaire

The protocol was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. Thirty Thai volunteers (male and female) were informed and agreed with the consent before participation in the study. Subjects were instructed to hold a test patch on one side of the knee for 6 hours. After finishing the test, volunteers were questioned on the product satisfaction. The questionnaire was regarding product appearances, comfortable in use, feeling on used, feeling after used and overall satisfaction.