CHAPTER III

MATERIALS AND METHODS

Materials

Equipment

1. Analytical Balance (Sartorius BA 210S)
2. Brookfield®Digital Viscometer (Model RVT DCP S/N A03969, Brookfield Engineering Laboratories, Inc., USA)
3. Heating Bath (Buchi B-490)
4. Heating magnetic stirrer (Velp Scientifica, model ARE)
5. Hot air oven (Model B40, Memmert, Germany)
6. Modified Franz diffusion cell
7. pH meter (Consort C 832)
8. Reversed – phase High Performance Liquid Chromatography, HPLC (Shimadzu)

   Liquid Chromatography: LC-10ADvp
   System Controller: SCL–10Avp
   UV-VIS Detector: SPD-10Avp
   Auto Injector: Sil-10Advp
   Column Oven: CTO-10Avp
   Degasser: DGU-14A

   Column: C 18 column, Inertsil ODS 5 micron 4.6 mm × 150 mm (GL Science, Japan) with a guard column, Inertsil ODS 5 micron 4.6 mm × 150 mm (GL Science, Japan)
10. Rotary evaporator (Model R-220, Buchi, Switzerland)
11. Stability chamber (Eurotherm, Axyos)
12. Ultrasonic Cleaner (Cavitator, model ME)
13. Universal tensometer (Model H5KS 1509, Tinius Olsen Ltd., UK)
14. UV-Visible spectrophotometer (Model UV-1601, Shimadzu, Japan)

15. Water bath (Gilson, England)

Chemicals

1. Asiaticoside, standard grade (Guanxi Chemical Co., Ltd., China
2. Acetonitrile, HPLC Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
3. Chloroform, A.R. Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
4. Dibutyl phthalate (Carlo Erba.,Ltd Italy, Lot No.403775
5. Disodium hydrogen orthophosphate anhydrous (Fluka Chemi GmbH, Switzerland), Lot No.381225/1
6. Ethanol, A.R. Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
7. Ethyl alcohol absolute anhydrous, A.R. grade (Mallinckrodt Co.,Ltd.,Mexico), Lot No.V569 Y50 D54.
8. Ethyl cellulose, 45 cps (Sigma Co.,Ltd), Lot No. 100K0137.
9. Fresh plant of C.asiatica; from Parkklon market, Thailand
10. Glycerin USP (S. Tong Chemicals Co.,Ltd., Thailand), Lot No.12821203.
11. Hexane, A.R. Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
12. Isobuthanol, A.R. Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
13. Methanol (A.R. grade, Labscan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
14. Methanol, HPLC Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)
15. Propylene glycol (Dow, U.S.A.)
16. Sephadex TM LH-20 (Amercham Pharmacia Biotech AB, Sweden)
17. Sodium sulphide X-hydrate QP (Pancreac Quimica SA., E.U.)
18. Sorbital (70% solution) (Srichan United Dispensary Co.,Ltd, Thailand)
19. Water, HPLC Grade (Lab-Scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD.Ireland.)

Methods

1. Extraction of Asiaticoside from *Centella asiatica*

Asiaticoside was extracted by modified method of Sermboonsang,2001. Three kilogram of *Centella asiatica* dry plant was macerated with 60% ethanol for 3 days, after that, chloroform was used to extract the non-polar part of ethanol macerated extract. The polar part of extracted solution was extracted by isobutanol to separate the intermediate polar component that composed of asiaticoside, madecasoside and asiatic acid. The isobutanol extraction was evaporated by rotavapor (Model R-220, Buchi, Switzerland), the yellow dry powder was collected and was dissolved with 60% methanol. The methanol solution was separated by thin layer chromatography (TLC) method using sephadex LH-20 as stationary phase and the mixture of water, chloroform and ethyl acetate was used as mobile phase in the ratio of 1:2:4. The asiaticoside solution part was collected from the tenth minute of the collected sequence. The HPLC method was used to determine the asiaticoside extract.

1.1 Determination of Asiaticoside Extract using HPLC

Centella asiatica extract was dissolved in methanol–water (60:40) and then was filtered with 0.20 μm membrane(Corning corp., Germany)

The HPLC system composed of the reverse-phase column C18, Inertsil ODS, 5 micron (4.6x150mm) with guard column, Inertsil ODS, 5micron(4.6x10mm). UV-visible detector (SPD-10AV) wavelength at 220 nm. The acetonitrile-water (28:72) were used as mobile phase. A 20 μl volume of sample was injected into the column. The details of assaying were shown in 7.1.

1.2 Determination of Suitable Solubilizing Agent of Asiaticoside

The approximate solubility of asiaticoside was determined in propylene glycol and non-ionic surfactant (Tween20) was used as solubilizing agent. Each

portion of propylene glycol was put in 1 g of asiaticoside, agitation was continued for 24 hr at room temperature.

The various concentration of 0.5, 0.75 and 1.0% of Tween20 was put in 0.5% of asiaticoside in water. The clear solution was observed.

2. Preparation of Polysaccharide Gel (PG) from Durian-Fruit Hulls

The PG dried powder was given by the Department of Biochemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. For the preparation of PG, the 4% w/w concentration of PG was prepared by dispersed 4g of PG dried powder in 96 g of deionized water and continued stirring for 12 h to hydrate and swell the PG.

3. Preparation of PG Facial-Patch Film Base

3.1 Determination of the Amount of Plasticizers

Glycerin and sorbitol at concentration of 5-20% w/w were used as plasticizers.The 4% w/w PG was dispersed in distilled water and stirred continuously at room temperature for 12 h to hydrate and swell the PG. The plasticizers were added to the PG mixtures. The ultrasonic bath was used to remove air bubbles for 1 h. A film was cast by pouring the mixture into 9-cm diameter petridish and drying in hot air oven at 40 °C for 24 h.

The concentration of plasticizers that give a film with satisfactory mechanical properties, maximum detachment force and work of adhesion were selected.

3.2 Determination of a Suitable Concentration of PG

The PG concentration of 4, 5, 6% w/w were dispersed in distilled water respectively. The films were prepared as same as in 3.1. The PG in concentration that

give a film with satisfactory mechanical properties, maximum detachment force and work of adhesion were selected.

3.3 Determination of the Amount of PG Aqueous Solution for Casting

The 4% w/w concentration of PG was prepared as same as in 3.1. A film was cast by pouring 8,10 and 12 g of the mixture into 9-cm diameter petridish and drying in hot air oven at 40 °C 24 h. The amount of PG that give a film with satisfactory mechanical properties, maximum detachment force and work of adhesion were selected.

4. Preparation of PG Facial-Patch Backing Layer

4.1 Determination of a Suitable Film Forming for Backing Layer

The film forming agent such as polyvinyl alcohol (PVA) 72,000 cps, polyvinylpyrrolidone (PVP-K90), polyvinylpyrrolidone (PVP-K30), methylcellulose (MC) 100 cps and ethylcellulose (EC) 45 cps were prepared at concentration of 2% w/w with 20% w/w plasticizer of film forming agent. Films were cast by pouring into the 9-cm diameter petridish and drying in hot air oven at 40 °C 12 h. The satisfactory film was evaluated by physical appearance as thickness, brittle, and compatibility with PG film.

4.2 Determination of Concentration of Film Forming for Backing Layer

The concentration of 2, 5. 10 % w/w of ethylcellulose (EC) 45cps was dispersed in absolute ethanol respectively. Dibutyl phthalate (DBP) was used as plasticizer, the concentration of 10% w/w DBP of EC was added to the solution. The EC solution was cast by pouring into the 9-cm diameter petridish and drying in hot air oven at 40 °C 12 h. The satisfactory film was evaluated by physical appearance as thickness, brittle, and compatibility with PG film.

4.3 Determination of the Amount of Plasticizer for Backing Layer

The suitable concentration of EC was selected and 10, 20,30 % w/w DBP of EC were added respectively. The EC solution was cast as same as in 4.1. The satisfactory film was evaluated by physical appearance as thickness, brittle, and compatibility with PG film.

4.4 Determination of the Amount of EC Solution for Backing Layer

The satisfactory backing layer from 4.3 was cast by pouring 3, 4, 5 g of EC solution into the 9-cm diameter petridish respectively and drying in hot air oven at 40 °C 12 h. The satisfactory film was evaluated by physical appearance as thickness, brittle, and compatibility with PG film.

5. Preparation of PG Facial-Patch Containing Asiaticoside

Ethylcellulose film was cast firstly as the process in 4.4 and drying in hot air oven at 40 °C 12 h. The concentration of 0.5% w/w asiaticoside in propylene glycol was prepared by dissolved 0.5 g of asiaticoside in 4.75 mL of propylene glycol. The solution was added to the mixture of PG aqueous solution in 3.3 with gradually stirred for 30 min. The mixture was cast by the described procedure previously onto the ethylcellulose backing layer and drying in hot air oven at 40 °C 24 h.

6. Physical Evaluation of PG Facial-Patch

6.1 Appearance of PG Facial-Patch

Colors, transparency and integrity of the eye patch was observed. The patch flexibility was roughly determined by hand stretching and bending. The thickness of 4x30 cm^2 patch was measured by micrometer.

6.2 Mechanical Properties of PG Facial-Patch

Mechanical properties of PG facial-patch were evaluated using a tensiometer (Tinius olsen,Model H5KS 1509). The mechanical properties were studied included the tensile strength, percent elongation at break, work of failure and Young's modulus, five replications were presented. The procedure employed was based on the American Society for Testing and Material (1995). A patch specimen was cut into small strips (2x20 mm). Only the strips that were free from air bubbles and physical properties were measured. The mean thickness of each strip was the average value of five measurements taken along the length 2-cm distance using micrometer. Both sides of the test patch were clamped with flat-faced grips and extended by the tensiometer as following conditions:

Rate of grip separation	=	6 mm/min
Gauge length	=	5 mm
Loading weight	=	10 N
Temperature	=	25 ± 2 °C
Relative humidity	=	45 ± 5 %

Five specimens were used for the study of each film formulation. After the specimen was torn off, the breaking force and the change in length at the moment of rupture were recorded by QMAT 4.10 S-series -5K program. The following equations were used to calculate the mechanical of the films:

$$\textit{Tensile strength (MPa)} = \frac{\textit{Maximum load}}{\textit{Original minimum cross-sectional area of the specimen}}$$

$$\textit{\% Elongation} = \frac{\textit{Extension at the moment of rapture of the sample x 100}}{\textit{Initial gage length of the specimen}}$$

$$\textit{Young's modulus} = \frac{\textit{Tensile stress}}{\textit{Elastic strain in tension}}$$

$$\textit{Work of failure (mJ)} = \textit{area of a curve plotting between force and extension}$$

7. Quantitative Analysis of Asiaticoside in PG Facial-Patch

7.1 HPLC Analysis

7.1.1 Preparation of Standard Solution

An accurate weight of 10 mg standard asiaticoside was dissolved in methanol-water (60:40) in 10 mL volumetric flask (1 mg/mL). The 1 mL of solution was transferred to a 10 mL volumetric flask and adjusted to volume with methanol-water (60:40). This stock solution had a final concentration of 100μg/mL. The certain volumes of 20, 40, 60, 80 and 100 μl of asiaticoside solution were transferred into 10 mL volumetric flasks. The methanol-water (60:40) used to adjust the volume and final concentrations of 0.20, 0.40, 0.60, 0.80, 1.0 μg/mL were obtained. The calibration curve of asiaticoside was plotted between the area response and concentration in μg/ml.

7.1.2 HPLC Condition

The HPLC condition used in analysis as following :

Column	:	Inertsil ODS-3 C18, 5 micron, 4.6x150mm
Mobile phase	:	Acetronitrile-ultrapure water (28:72)
Flow rate	:	1 ml /min
Run time	:	15 min
Detector	:	UV detector
Wavelength	:	220 nm
Injection volume	:	20 μl

7.2 Validation of HPLC Method

Analytical parameter validated was specificity, precision, accuracy and linearity.

7.2.1 Precision

7.2.1.1 Within run precision

The within run precision was determined by analyzing five concentrations of standard solution. 0.15, 0.25, 0.30, 0.45 and 0.50 mg/mL asiaticoside in the same day. Three determination of concentration were shown. The percent coefficient of variation (%CV) of the estimated concentration of asiaticoside at each concentration was calculated. The within run precision was accepted if the %CV was within 2% (USP 27, 2004).

7.2.1.2 Between run precision

The between run precision was determined by comparing the same concentrations of standard solutions, 0.15, 0.25, 0.30, 0.45 and 0.50 mg/mL asiaticoside which prepared and injected into HPLC column in different three days. Three determinations of concentration were shown. The percent coefficient of variation (%CV) of the estimated concentration of asiaticoside at each concentration in different three days was calculated. The between run precision was accepted if the %CV was within 2% (USP 27, 2004).

7.2.2 Accuracy

Five concentrations of asiaticoside solution, 0.15, 0.25, 0.30, 0.45 and 0.50 mg/mL, were prepared and injected into HPLC column. The percent recovery of each concentration was calculated as the following equation:

$$\% \text{ recovery} = \text{estimated concentration / known concentration X } 100$$

Three determinations per concentration were shown. The accepted parameter should be within 2% of each nominal concentration (USP 27, 2004)

7.2.3 Linearity

Five standard solutions, 0.15, 0.25, 0.30, 0.45 and 0.50 mg/mL, were prepared and analyzed. The linear regression of the peak area ratio versus their concentration was shown by using least square analytical.

8. *In Vitro* Diffusion Study of Asiaticoside from the PG Facial-Patch

Franz diffusion cell was used for the release study of asiaticoside from the facial-patch. Phosphate buffered saline (PBS; 14mL, pH 7.4) was used as the receptor fluid. The 2x2 cm cellophane membrane was used as diffusion membrane which mounted on the 3.14 cm^2 area receptor cell. The adhesive layer of PG patch was placed on the cellophane membrane and maintained the cell at temperature 37°C±5°C through out the study. The 4 mL of the sample was collected from the receiving chamber at 0, 10, 20, 30, 45, 60, 120, 180, 240, 300, 360, 420 and 480 min via the sampling port of diffusion cell. The receptor fluid was replaced immediately with the same amount of fluid taken.

The HPLC method in 7.1 was used to determine the amount of asiaticoside diffuse through the membrane.

9. *In Vivo* Skin Irritation of PG Facial-Patch

9.1 Draize-FHSA Skin Irritation Test Method in Albino Rabbits

The Draize skin irritation test method involves application of test material to albino rabbit skin in a patch for 24 hr. and subjective grading of the extent of skin erythema and oedema on separate 0 to 4 scales, 24 48 and 72 hr after patch removal (Draize *et al.*,1944). The procedure adopted in the U.S. Federal Hazardous Substance Act (FHSA) was used to assay the skin irritation for the PG facial-patch and described in Table 2. The scoring system was shown in Table 3.

Table 2 Draize-FHSA Model (Alton, 2002)

Number of animals	6 albino rabbit (clipped)
Test sites	2x1 inch^2 sites on dorsum One site intact, the other abraded
Test materials	Applied undiluted to both test sites Solid/semisolid: 0.5 g
Occulsion	1 inch ^{2} surgical gauze over each test site Rubberized cloth over entire trunk
Occlusion period	24 hours
Assessment	24 and 72 hours Visual scoring system

Table 3 Draize-FHSA Scoring System (Aulton, 2002)

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

9.2 Prophetic Patch Testing

The 200 Thai healthy volunteers were selected for *in vivo* skin irritation determined by The National Academy of Sciences (NAS) outline. Occlusive patches may be applied to the intrascapular region of the back or the volar surface of the forearms for 24 hr at 24, 48 and 72 hr. Skin responses were evaluated 30 minutes to 1 hour after removal of the patches, using the animal Draize's scale in table 2. Repeated application was performed after10 days as the described procedure previously.

10.*In Vivo* Skin Efficacy Test of PG Facial-Patch

Determination of Anti-wrinkle Effect

Skin hydration was evaluated by DermaLab® Moisture probe(Barlow and Wirchers, 1999). Transepidermal water loss (TEWL) and elasticity (Young's modulus value) were calculated. The wrinkle was observed using macroscopic photography. Three measurements were performed in each testing. Student's T-Test for paired data was used to determine the differences.

The 20 subjects were selected by the following criteria:

- Thai healthy volunteer
- Aged 30-60 years old
- No cosmetic allergy history
- Healthy skin
- Should not use any anti-wrinkle treatment for 1 week prior to and throughout the experiment

Duration of the application

The volunteers was recommended to use the PG patch after face cleansing 15 min before bedtime at the wrinkle forehead, leave the patch for at least 5 hr for 4 weeks.