CHAPTER III

MATERIALS AND METHODS

Materials

1. Citric acid (Ajax Finechem, Australia)
2. Disodium hydrogen orthophosphate anhydrous (Fluka Chemi GmbH, Switzerland)
3. Ethanol HPLC grade (Lab-Scan Analytical Sciences, Thailand under licence and quality assurance by Lab-Scan Ltd, Ireland.)
4. Gallic acid (Approx, 99%, Sigma, USA)
5. Glycerin (Sigma-Aldrich Chemical, Germany)
6. Methanol HPLC grade (Lab-Scan Analytical Sciences, Thailand under licence and quality assurance by Lab-Scan Ltd, Ireland.)
7. Methyl paraben (Acros Organics, USA)
8. Phosphatidylcholine from soybean lecithin Epikuron 200 ® 92% (lot no. 159089) Lucas Meyer, Germany
9. Polyvinyl Alcohol (Average Molecular weight ~67,000) (Fluka, Sigma-Aldrich Chemical, Germany)
10. Trifluoroacetic Acid (Fluka, Sigma-Aldrich Chemical, Germany)
11. Polyoxyethylene Sorbitan Mono-oleate (Tween®80) (Srichand United Dispensary Co., LTD, Thaialnd)

Equipment

1. Analytical balance (Sartorius)
2. DermaLab® Elasticity probe
3. Heating bath (Buchi B-490)
4. Heating magnetic stirrer (VELP Scientifica, Europe)
5. High performance liquid chromatography (HPLC, Shimadzu, Japan) instrument equipped with the following:
   - System controller (SCL-10AVP)
   - Pump (LC 10ADVP)
   - Diode array detector (SPD-M10AVP)
   - Autoinjector (SIL-10ADVP)
6. Hot air oven (Model B40, Memmert, Germany)
7. Mastersizer (S long bed version 2.11, Malvern, UK)
8. Modified Franz diffusion cell
9. Moisture analyzer (Sartorius MA 30)
10. Optical light microscope (Olympus, Japan)
11. pH meter (832, Consort, Belgium)
12. Rotary evaporator (Model R-200, Buchi, Switzerland)
13. Skin Diagnostic SD 27, Germany
14. Tensiometer (Tinius Oslen, Model H5KS 1509)
15. Transmission electron Microscope (Model JEM-2100, JOEL, Japan)
16. Ultracentrifuge (L-80, Beckman, USA)
17. Ultrasonic bath (Cavitator, Ultrasonic Mettler Electronic, USA)
18. Vortex mixer (Vortex-genie, model G650E, USA)
19. Water bath (Gilson, England)

Methods

1. Preparation of *P. emblica* extract nanoliposomes

Nanoliposomes were prepared by modified ethanol injection method (New, 1987; Sirirat et al., 2007). The amount of 30 mg soybean phosphatidylcholine (SPC) was dissolved in 1 mL of ethanol. Tween®80 30 mg was dissolved in aqueous medium (citrate-phosphate buffer pH 5.5) and was adjusted to 10 ml. Also, *P. emblica* extract 100 mg was dissolved in aqueous medium. The mixture was introduced into a 25 ml conical flask, and stirred rapidly with a magnetic stirrer. Then, fit a find gauge needle and the lipid solution was drawn up. The tip of the needle was positioned below the surface of the stirred aqueous, and the organic solution was injected as rapidly as possible into the medium. Liposomes were formed immediately and ethanol was evaporated out using rotary evaporator at 40°C in water bath under reduced pressure of 100 mbar. In addition, liposomes obtained from SPC: Tween®80 in the ratio of 1:1, 3:1, and 5:1 by weight were varied to get high entrapment efficiency. Thus, different ratio of liposomal membrane and Tween®80 were prepared by repeating following above method. *P. emblica* extract at each concentration varied from 1-2% w/v was used in the formulation as shown in Table 4.

Table 4 Formulation of *P. emblica* extract in different ratio of liposomal membrane and Tween®80

Formulation	Compositions	Ratio	Concentration of *P. emblica* extract
1	SPC: Tween®80	1:1	1 %w/v of *P. emblica* extract
2	SPC: Tween®80	1:1	2 %w/v of *P. emblica* extract
3	SPC: Tween®80	3:1	1 %w/v of *P. emblica* extract
4	SPC: Tween®80	3:1	2 %w/v of *P. emblica* extract
5	SPC: Tween®80	5:1	1 %w/v of *P. emblica* extract
6	SPC: Tween®80	5:1	2 %w/v of *P. emblica* extract

Sizing of liposomes was performed by Lipex Extruder using 100 nm polycarbonate membrane filtered to nanoliposomes.

A citrate-phosphate buffer (1.0 mM), pH 5.5 was prepared from citric acid and disodium hydrogen orthophosphate anhydrous. All the reagents were of analytical grade. The final pH was adjusted using a pH meter.

2. Physical characterization of *P. emblica* extract nanoliposomes

2.1 Particle size determination

The particle sizes of *P. emblica* extract nanoliposomes were measured by means of the laser light scattering (Mastersizer, Malvern, UK). The measurements were repeated three times for each sample.

The unscattered light was brought and passed through the detector and out of the optical system. The total laser power passing out of the system in this way was monitored allowing the sample volume concentration to be determined and shown on computer (Scientific and Technology Research Equipment Center [STREC]).

The process for particle size determination was involved with three basic steps. Firstly, the sample was prepared and dispersed to the corrected concentration and then delivered to the optical unit. This is the purpose of the sample dispersion accessories. Sample preparation was the most important stage of making a measurement. It should be remembered that, if the product is poorly prepared (i.e. being unrepresentative or badly dispersed) then the basic data will be incorrect no amount of analysis of these data will give a correct answer. Secondly, there was the capturing of the scattering pattern from the prepared sample. This is known as the measurement. This is the function of the optical unit. Then, the sample was dropped into the chamber that use de-mineralized water as the dispersing agent. Finally, once the measurement was complete, the raw data contained in the measurement was analyzed.

2.2 Morphology

The shape and the surface morphology of *P. emblica* extract nanoliposomes were investigated by means of the transmission electron microscope (TEM). They were normally characterized under optical microscopes and scanning electron microscopes. However, prepared nanoliposomes had noticeably small sizes that could not be characterized under optical microscopes and scanning electron microscopes. Thus, these nanoliposomes were characterized under transmission electron microscopy.

The procedure for negative staining of a nanoliposomes preparation sample was as follows. A drop of *P. emblica* extract nanoliposomes suspensions was applied onto carbon coated grids. After leaving for 1-3 min to allow adsorption of *P. emblica* extract nanoliposomes to a grid, the excess was removed by filter paper. A drop of 2% phosphotungstic acid was applied onto the grid, leaving for 1 min, drawn off by filter paper. Then the grid was air-dried and examined under a transmission electron microscope.

2.3 Determination of encapsulation efficiency

2.3.1 Separation method

To separate nanoliposomes from the suspensions ultracentrifuge method was used. The ultracentrifuge tube containing 8 mL of *P. emblica* extract nanoliposomes suspensions was put in the rotor. The maximum speed of the ultracentrifuge was 65,000 rpm at which the ultracentrifuge tube can tolerate. The temperature was set at 4°C. The ultracentrifugation process was set for 1.30 h to separate them efficiently.

2.3.2 Determination of unencapsulated *P. emblica* extract from nanoliposomes suspensions

The supernatant was separated by ultracentrifuge at 65,000 rpm at 4°C for 1.30 h. Then, it was put into a 25 mL volumetric flask and adjusted with citrate-phosphate buffer pH 5.5 to determine unencapsulated *P. emblica* extract in nanoliposomes.

2.3.3 Determination of *P. emblica* extract in nanoliposomes

The precipitant was broken down by 1 mL methanol and sonicated for 30 min and then the solution was pipetted into 25 mL volumetric flask and adjusted to volume with citrate-phosphate buffer pH 5.5 to determine encapsulation efficiency of *P. emblica* extract in nanoliposomes.

2.3.4 Calculation of the percentage of encapsulation efficiency and the percentage of recovery

The encapsulation efficiency was calculated as the ratio between the amount of *P. emblica* extract in the nanoliposomes and initial amount of *P. emblica* extract used to formulate. The percentage of encapsulation efficiency and the percentage of recovery of *P. emblica* extract of each preparation were determined from the following equation:

$$\% \text{ Encapsulation efficiency} = \frac{\text{Amount of } P.\ emblica \text{ extract in the nanoliposomes} \times 100}{\text{Initial amount of } P.\ emblica \text{ extract used to formulate}}$$

$$\% \text{ Recovery} = \frac{\text{Sum of amount of } P.\ emblica \text{ extract in nanoliposomes and unencapsulated } P.\ emblica \text{ extract} \times 100}{\text{Initial amount of } P.\ emblica \text{ extract used to formulate}}$$

3. Preparation of facial patch containing *P. emblica* extract nanoliposomes

3.1 Determination of the amount of plasticizers

Glycerin at concentration of 1-5% w/v was used as plasticizer. The 2.5% w/v polyvinyl alcohol (PVA) was dispersed in distilled water and stirred continuously at room temperature until dissolved. The plasticizers were added to the mixture. 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 24h.

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

3.2 Preparation of backing layer of facial patch containing *P. emblica* extract nanoliposomes

PVA 2.5% w/v and glycerin 1% w/v were selected from 3.1 and were dispersed in distilled water and continuously stirred until dissolved. The ultrasonic bath was used to remove air bubbles for 1 h. Films were cast by pouring the mixture into 9 cm diameter petridish and drying in hot air oven at 40°C for 24 h.

3.3 Determination of the amount of aqueous solution for casting

The film containing 2.5% w/v concentration of PVA and 1% w/v glycerin were prepared as same as in 3.2. A film was cast by pouring 10, 15, and 20 g of the mixture into 9 cm diameter petridish and drying in hot air oven at 40 °C for 24h. The amount of aqueous solution that gives a film with satisfactory mechanical properties, maximum detachment force, and work of adhesion were selected.

3.4 Preparation of facial patch containing *P. emblica* extract nanoliposomes

Backing layer is cast firstly as the process in 3.2 and drying in hot air oven at 40°C 24 h. The concentrations of 1.0% w/v *P. emblica* extract nanoliposomes were used in active layer together with 2.5% w/v PVA and 1% w/v glycerin in preparation. The mixture was dispersed in distilled water and continuously stirred until dissolved. Then, the mixture was cast by the procedure described previously onto the well dry backing layer and drying in hot air oven at 40°C for 24 h.

4. Physical evaluation of facial patch containing *P. emblica* extract nanoliposomes

4.1 Appearance of facial patch containing *P. emblica* extract nanoliposomes

Color, transparency and integrity of patch were observed by the eyes. The patch flexibility was roughly determined by hand stretching and bending.

4.2 Mechanical properties of facial patch containing *P. emblica* extract nanoliposomes

Mechanical properties of facial patch containing *P. emblica* extract nanoliposomes were evaluated using a tensiometer (Tinius Oslen, 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. A patch specimen was cut into small strips (2*2 cm). 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 of 2 cm distance using micrometer. Both sides of the test patch were clamped with flat-faced grips and extended by the tensiometer as following:

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

Five specimens were used for the study of each film formulation. After the specimen was torn off, the breaking force and the change in the length at the moment of rupture were recorded by QMAT 4.10 S-series -5K program (digital system).

The following equations were used to calculate the mechanical of the films:

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

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

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

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

5. Determination of *P. emblica* extract

Gallic acid was used as the marker in *P. emblica* extract.

5.1 HPLC Analysis

The high performance liquid chromatography technique was used for analysis of gallic acid. The system was composed of two pumps able to generate the variable flow of mobile phase, an autoinjector, an adjustable wavelength UV detector, system controller and degasser. All of these were operated by the data station software.

HPLC condition

Column:	C18 (Alltech 4.6 x 150 mm, 5μ)
Mobile Phase:	0.3%v/v Trifluoroacetic Acid : Methanol (92:8)
Flow Rate:	1 mL/min
Detection:	270 nm
Injection volume:	20 μL
Temperature:	Ambient
Run Time:	15 minutes

5.2 The calibration curve of gallic acid using HPLC

The standard stock solution of gallic acid was prepared in the concentration of 100 μg/mL and seven standard solutions of gallic acid were prepared in the concentration of 0.01, 0.2, 0.4, 0.6, 0.8, 1.5, and 2 μg/mL. The concentrations and peak areas were plotted as the calibration curve.

5.3 Validation of HPLC method

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

5.3.1 Accuracy

Accuracy in term of recovery was determined by calculating the ratio of observed concentration and actual concentration. Observed concentration was obtained from linear regression equation of a calibration curve.

The preparations were processed in triplicate at three different concentrations. The actual concentration, the observed concentrations, and % recovery of gallic acid were determined.

% recovery = Observed concentration / actual concentration x 100

Three determinations per concentration were shown. The accepted parameter should be within 2% of each nominal concentration

5.3.2 Precision

5.3.2.1 Intra-day precision

Six replicates of three different concentrations of aqueous solutions of gallic acid were prepared and analyzed by HPLC. The percentage of coefficient of variation (% CV) was calculated.

$$\% CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

5.3.2.2 Inter-day precision

The same range of concentrations of gallic acid in the inter-day precision was used and analyzed in other three different days. The percentage of coefficient of variation (% CV) was calculated.

5.3.3 Linearity

Five standard solutions were prepared and analyzed. The linearity graphs of gallic acid were constructed from the actual concentrations and the observed concentrations of gallic acid. The graph was fitted with a straight line using the least square linear regression analysis.

5.3.4 Limit of quantitation (LOQ)

The concentration of gallic acid was reduced until the signal-to-noise ratios from HPLC chromatogram was 10:1.

5.3.5 Limit of detection (LOD)

The concentration of gallic acid was reduced until the signal-to-noise ratios from HPLC chromatogram was 3:1.

5.3.6 Specificity

The citrate-phosphate buffer pH 5.5 with and without gallic acid was analyzed by HPLC to ensure that the peaks of buffers were not overlapped with peaks of analytes.

5.4 Quantitative analysis of gallic acid using High Performance Liquid Chromatography (HPLC)

P. emblica extract was dissolved in citrate-phosphate buffer pH 5.5 and then was filtered with 0.45 μm membrane (Whatman NYL w/GMF). The HPLC system composed of column C18, alltech, 5 micron (4.6 x 150 mm) with guard column, UV-Visible detector (SPD-10A VP Shimadzu) wavelength 270 nm. The 0.3%v/v Trifluoroacetic Acid-Methanol (92:8) was used as mobile phase. A 20 μL volume of sample was injected into the column.

Concentration of gallic acid in *P. emblica* extract and nanoliposomes were determined using HPLC and calculated back to *P. emblica* extract.

6. *In vitro* diffusion study of *P. emblica* extract from nanoliposomes from the facial patch

Franz diffusion cell was used for the released study of *P. embliça* extract from nanoliposomes from the facial patch. Citrate-phosphate buffer (14mL, pH 5.5) was used as the receptor fluid. The round shaped cellophane membrane (r = 0.75 cm) was used as diffusion membrane which mounted on the 1.77 cm^2 area receptor cell. The adhesive layer of facial patch was placed on the cellophane membrane and maintained the cell at temperature 37°C ± 5°C through out the study. The 1 mL of the sample was collected from the receiving chamber at 0, 5, 15, 30, 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 the fluid taken.

The HPLC method in 5.4 was used to determine the amount of *P. emblica* extract diffuse through the membrane.

7. *In vivo* skin moisturizing and elasticity efficacy test of facial patch containing *P. emblica* extract nanoliposomes

Determination of moisture and elasticity of the skin

Moisture content was evaluated by Skin Diagnostic SD 27 and skin elasticity was evaluated by DermaLab® Elasticity probe (Barlow and Wircher, 1999). Moisture content and elasticity (Young' modulus value) were calculated. Repeated measurement for paired data was used to determine the differences among four groups:

- Control group (Backing layer only)
- Pure nanoliposomes group,
- Pure *P. emblica* group,
- Nanoliposomes and *P. emblica* group

Then, the 21 subjects are selected by the following criteria:

- Thai healthy volunteer
- Aged 25-60 years old
- No cosmetic allergy history
- Healthy skin
- Should not use any facial treatment for 1 week prior to and throughout the experiment

Duration of the application

The volunteers were recommended to use the facial patch containing *P. emblica* extract nanoliposomes after 15 min face cleaning before bedtime at the forehead, leaving the patch for at least 5 h. everyday for 4 weeks.