CHAPTER III

MATERIALS AND METHODS

Materials

1. Cholesterol (Approx, 99%, Sigma, USA)
2. Cholesterol ester (Lubrizol Company, Indonesia)
3. Citric acid (Ajax Finechem, Australia)
4. Disodium hydrogen orthophosphate (Ajax Finechem, Australia)
5. Ethanol AR grade (Lab-Scan Co., Ltd, Ireland)
6. Folin-Ciocalteu's Reagent (Sigma, USA)
7. Gallic acid (Approx, 99%, Sigma, USA)
8. Methanol AR grade (Lab-Scan Co., Ltd, Ireland
9. Methyl paraben (Acros Organics, USA)
10. Phosphatidylcholine from egg yolk (Lipoid E80, Lipoid German)
11. Potassium hydrogen phosphate (Ajax Finechem, Australia)
12. Propyl paraben (Acros Organics, USA)
13. Sodium carbonate (Ajax Finechem, Australia)
14. Sodium chloride (Ajax Finechem, Australia)

Equipment

1. Analytical balance (Sartorius)
2. Centrifuge (Model K3 system, Centurion, UK)
3. Grinder (Cheng Charoon)
4. Mastersizer (S long bed version 2.11, Malvern, UK)
5. Moisture analyzer (Sartorius MA 30)
6. Optical light microscope (Olympus, Japan)
7. pH meter (832, Consort, Belgium)
8. Rotary evaporator (Model R-200, Buchi, Switzerland)
9. Spray drier (Niro Atomizer, Copenhagen Denmark)
10. Transmission electron Microscope (Model JEM-2100, JOEL, Japan)
11. Ultracentrifuge (L-80, Beckman, USA)
12. Ultrasonic bath (Cavitator, Ultrasonic Mettler Electronic. USA)
13. UV-visible spectrophotometer (UV-160A, Shimadzu, Japan)
14. Vortex mixer (Vortex-genie, model G650E, USA)

Methods

1. Preparation of *P. emblica* extract spray dried powder

Fresh fruits of good quality *P. emblica* were purchased from Mehongson province in northern part of Thailand. The fruits were washed with water and reduced their size by grinder, squeezed out of juice and filtered to obtain the clear solution. Juice was spray dried using Niro Atomizer (Copenhagen, Denmark) to obtain *P. emblica* extract spray dried powder.

2. Preparation of *P. emblica* extract in buffer solution

Accurately weighed 5.0 g of *P. emblica* extract spray dried powder into a 100 mL volumetric flask, adjusted to volume with distilled water and sonicated for 60 min. The concentration of stock solution was 50 mg/mL of *P. emblica* extract.

Aliquots of *P. emblica* extract stock solution of 0.5, 1.0, 1.5, 2.0 and 2.5 mL was each pipetted and transferred to a 25 mL volumetric flask. The solutions were adjusted to volume with buffer solution so that the final concentrations of *P. emblica* extract were 1, 2, 3, 4 and 5 mg/mL, respectively.

Phosphate-citrate buffer solution pH 5.5 was prepared by mixing 56.85 mL of a 2.84 % w/v anhydrous disodium hydrogen orthophosphate solution and 43.15 mL of a 2.1 % w/v citric acid solution.

Phosphate-buffer saline solution (PBS) pH 7.4 was prepared by dissolving 2.38 g of anhydrous disodium hydrogen orthophosphate, 0.19 g of potassium dihydrogen orthophosphate and 8 g of sodium chloride in sufficient water to produce 1000 mL and the pH was adjusted if necessary.

3. Quantitative analysis of total phenolic compounds in *P. emblica* extract by UV-VIS spectrophotometry

3.1 The Folin-Ciocalteau reaction

The total amount of total phenolic compounds in *P. emblica* extract was determined according to the Folin-Ciocalteu procedure, based on complex formation of molybdenum-tungsten blue (Singleton and Rossi, 1965). The sample was allowed to react with Folin-Ciocalteu's reagent and sodium carbonate solution. Absorbance of complex formation was determined at 747 nm and the total phenolic compounds was calculated as gallic acid equivalents (GAE).

3.2 The calibration curve of gallic acid

The standard stock solution of gallic acid in distilled water was prepared in the concentration of 100 μg/mL and six standard solutions of gallic acid were prepared in the concentration of 5, 10, 20, 40, 60 and 80 μg/mL. Distilled water was used as a

blank. The absorbance was determined at 747 nm by UV-VIS spectrophotometer. The absorbances and the concentrations were plotted as the calibration curve.

3.3 The calibration curve of *P. emblica* extract in buffer solution pH 5.5

The sample solution of *P. emblica* extract in buffer was prepared by pipetting *P. emblica* extract stock solution into 25 mL volumetric flask and adjusted to volume with buffer solution pH 5.5 to make the concentrations at 1, 2, 3, 4 and 5 mg/mL of *P. emblica* extract. The total phenolic compounds were determined according to the Folin-Ciocalteu reaction calculated gallic acid equivalent (GAE). The absorbance was determined at 747 nm by UV-VIS spectrophotometer. The calibration curve was plotted between gallic acid equivalent (GAE) and the concentrations of *P. emblica* extract.

3.4 The calibration curve of *P. emblica* extract in buffer solution pH 7.4

The sample solution of *P. emblica* extract at the concentrations of 1, 2, 3, 4 and 5 mg/mL in buffer solution pH 7.4 were determined using Folin-Ciocalteu's reaction. The absorbances were determined at 747 nm by UV-VIS spectrophotometer. The amount of total phenolic compounds was calculated as GAE from calibration curve. The calibration curve was plotted between gallic acid equivalent (GAE) and concentration of *P. emblica* extract.

4. Preparation of *P. emblica* extract in liposomes

The basic compositions of the lipid fraction of liposomal suspensions are 72% w/w egg phophatidylcholine (EPC) (Lipoid E80, Ludwigshafen, Germany) and 28% w/w cholesterol (extra pure, Sigma). At the mole ratio of 2:1 was known as the suitable composition to produce stable membranes (Brisaert, 2001). *P. emblica* extract spray dried powder in buffer solution pH 5.5 and 7.4 was individually in the

concentration ranged of 1-5 mg/mL and cholesterol ester was used as a stabilizer.. Liposomes were prepared using thin film methods.

The amount of 540 mg phosphatidylcholine (LipoidE80) and 168 mg of cholesterol were dissolved in 25 mL ethanol to obtain clear solution. Ethanol was evaporated out using rotary evaporator at 40°C in water bath under reduced pressure of 100 mbar until the thin film was obtained. *P. emblica* extract at each concentration varied from 1-5 mg/mL in phosphate-citrate buffer solution pH 5.5 and PBS pH 7.4 was used in the formulation (Table 1). Then cholesterol ester was added and shaking for 30 min. Then the liposome suspensions were allowed to swell for 2 h at room temperature.

Table 1 Formulation of *P. emblica* extract in buffer solution for encapsulated in liposomes

Formulation	**Concentration of *P. emblica* extract**
1	1 %w/v of *P. emblica* extract in buffer pH 5.5
2	2 %w/v of *P. emblica* extract in buffer pH 5.5
3	3 %w/v of *P. emblica* extract in buffer pH 5.5
4	4 %w/v of *P. emblica* extract in buffer pH 5.5
5	5 %w/v of *P. emblica* extract in buffer pH 5.5
6	1 %w/v of *P. emblica* extract in buffer pH 7.4
7	2 %w/v of *P. emblica* extract in buffer pH 7.4
8	3 %w/v of *P. emblica* extract in buffer pH 7.4
9	4 %w/v of *P. emblica* extract in buffer pH 7.4
10	5 %w/v of *P. emblica* extract in buffer pH 7.4

P. emblica extract in liposomes were stored in the refrigerator (4 $\pm$ 1°C) and in room temperature (30 $\pm$ 1°C) to determine the stability at 0, 1, 2, 4, 8 and 12 weeks for analysis of total phenolic compounds by UV-VIS spectrophotometry using gallic acid to evaluated (GAE) as a marker.

5. Determination of entrapment efficiency (EE)

5.1 Separation method

One mL of different amount of *P. emblica* in liposomes were pipetted into ultracentrifuge tubes and adjusted with distilled water to the same weight of each tubes and then centrifuged at 65,000 rpm at 4°C for 1 h.

5.2 UV-VIS spectrophotometry determination of total phenolic compounds of *P. emblica* extract in liposomes

Total phenolic compounds in *P. emblica* extract in liposomes were investigated using UV-VIS spectrophotometry. *P. emblica* extract in liposomes was put into 25 mL volumetric flask and liposomes were broken down by adding methanol and sonicating for 30 min, then adjusted to volume with distilled water. The sample was allowed to react with Folin-Ciocalteu's reagent and sodium carbonate solution. Absorbance at 747 nm was measured and the total phenolic compounds were calculated as gallic acid equivalents (GAE).

5.3 Encapsulation efficiency

5.3.1 Determination of unencapsulated *P. emblica* extract from liposome suspensions

The supernatant was separated by ultracentrifuge at 65,000 rpm at 4°C for 1 h. Then it was put into a 25 mL volumetric flask and adjusted with water to determine unenencapsulated *P. emblica* extract in liposomes.

5.3.2 Determination of *P. emblica* extract in liposomes

The precipitant was broken down by 1 mL methanol and sonicated for 30 min then the solution was pipetted into 25 ml volumetric flask and adjusted to volume with water to determine encapsulation efficiency of *P. emblica* extract in liposomes.

5.3.3 Quantitative analysis of encapsulated *P. emblica* extract in liposomes

The encapsulation efficiency was calculated as the ratio between the amount of *P. emblica* extract in the liposomes and the initial amount of *P. emblica* extract used in the formulation. The percentage of entrapment efficiency of *P. emblica* extract of each preparation was determined using the following equation

%Encapsulation efficiency =

$$\frac{\text{Amount of } P.\ emblica \text{ extract in liposomes} \times 100}{\text{Sum of amount of } P.\ emblica \text{ extract in liposomes and unencapsulated of them}}$$

%Recovery =

$$\frac{\text{Sum of amount of } P.\ emblica \text{ extract in liposomes and unencapsulated of them} \times 100}{\text{Initial amount of } P.\ emblica \text{ extract}}$$

6. Physical characterization of *P. emblica* in liposomes

6.1 Particle size and size distribution determination

The unscattered light was brought and passes 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 particle sizes of *P. emblica* extract in liposomes were measured by mean of the laser light scattering. Triplicate measurements were done for each sample (n=3)

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.

6.2 Morphology determination

The shape and the surface morphology of liposomes containing *P. emblica* extract were investigated by mean of the transmission electron microscope (TEM). The procedure for negative staining of liposomes preparation sample was as follows. A drop of liposomes suspension was applied onto carbon coated grids. After leaving for 1-3 min to allow adsorption of liposomes 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. Then it was drawn off by filter paper. Then the grid was air-dried and examined under a transmission electron microscope.

7. Chemical stability of *P. emblica* extract in buffer solution

The stability of *P. emblica* extract in buffer solutions were studied to compare with *P. emblica* extract in liposomes. Stability of *P. emblica* extract in buffer solutions pH 5.5 and 7.4 at $4 \pm 1^{\circ}C$ and $25 \pm 1^{\circ}C$ were determined at 0, 1, 2, 4, 8 and 12 weeks. The amount of *P. emblica* extract at each time intervals was determined as total phenolic compounds using UV-VIS spectrophotometer. The percent remaining of *P. emblica* extract was calculated at 12 weeks of storage to show the stability in buffer solutions at pH 5.5 and 7.4.

The concentrations of *P. emblica* extract in buffer solution varied at 1, 2, 3, 4 and 5 mg/mL in buffer pH 5.5 and pH 7.4 were protected from light and stored in

different condition at room temperature (30 ± 1°C) and in refrigerator (4±1°C). The absorbance of total phenolic compounds was measured by UV-VIS spectrophotometry. The concentration of phenolic compounds was calculated from calibration curve of gallic acid as GAE.

8. Stability of *P. emblica* extract in liposomes

8.1 Chemical stability of *P. emblica* extract in liposomes

The chemical stability of the preparations was measured the total phenolic compounds in different time. The samples were stored in a well closed bottles protected from light in the refrigerator (4 ± 1°C) and room temperature (30 ± 1°C) The samples were taken immediately after preparation as 0 and then 1, 2, 4, 8 and 12 weeks. The amounts of total phenolic compounds of undegraded *P. emblica* extract in sample were quantitative determined by UV-VIS spectrophotometry by pipetting *P. emblica* extract in liposomes into a 25 mL volumetric flask and liposomes membrane was broken down by adding methanol and sonicating, then adjusted to volume with water. The sample was allowed to react with Folin-Ciocalteu's reagent and sodium carbonate solution. Absorbance at 747 nm was measured and the total phenolic content was calculated as gallic acid equivalents (GAE). The results were presented as the remaining of GAE in different time and % remaining at 12 weeks of storage of *P. emblica* extract in liposome suspensions.

% remaining of *P. emblica* extract in liposomes suspensions was calculated from the following equation:

$$\% \text{ remaining} = \frac{\text{The amount of } P.\ emblica \text{ in suspensions at 12 weeks} \times 100}{\text{The amount of } P.\ emblica \text{ in suspensions at time 0}}$$

8.2 Physical stability of *P. emblica* extract in liposomes

The physical stability (size and morphology) of *P. emblica* extract in liposomes were examined in the preparations that exhibited the best encapsulation efficiency.

Particle size distribution and morphology were analyzed at time 0 and after storage 12 weeks in refrigerator ($4 \pm 1^{\circ}C$) and room temperature ($30 \pm 1^{\circ}C$) using Mastersizer.

Morphology was determined by Transmission electron microscope (TEM) at time 0 and after storage 12 weeks in refrigerator ($4 \pm 1\ ^{\circ}C$) and at room temperature ($30 \pm 1^{\circ}C$).

9. Comparative of % remaining of *P. emblica* extract in buffer solution and in liposomes at 12 weeks of storage

Percent remaining of total phenolic compounds calculated as gallic acid equivalent (GAE) in *P. emblica* extract was compared the % remaining of *P. emblica* extract in buffer solution pH 5.5, pH 7.4 and in liposomes pH 5.5 and 7.4 stored in a refrigerator ($4 \pm 1\ ^{\circ}C$) and room temperature ($30 \pm 1^{\circ}C$) at 12 weeks of storage.