CHAPTER 3
MATRIALS AND METHODS

3.1 Chemical materials and instruments :

Chemical materials

1. Aminoguanidine hydrocholride (Analytical grade, Sigma-Aldrich, USA)
2. Acetonitrile (HPLC grade, Fisher, Italy)
3. Bovine albumin serum (BSA) (Analytical grade, Fluka analytical, Switzerland)
4. Chloroform (Analytical grade, Labchem, Australia)
5. Corn starch (C.M. Chemical & Lab supplies, Thailand)
6. D-glucose (Analytical grade, Ajax Finechem, Australia)
7. Ethanol 99% (Analytical grade, (ACS) reagent, Merck, Germany)
8. Ethyl acetate (Analytical grade, (ACS) reagent, Ajax Finechem, Australia)
9. Ferric chloride hexahydrate (Analytical grade, Ajax Finechem, Australia)
10. Ferrous sulphate heptahydrate (Analytical grade, RFCI Limited, India)
11. Folin ciocalteu's reagent (Analytical grade, Merck, Germany)
12. Gallic acid (Analytical grade, Sigma-Aldrich, USA)
13. L-Ascorbic acid (Analytical grade, ACS reagent, Sigma-Aldrich, USA)
14. Magnesium stearate (Riedel-de Haen, Germany)
15. Methanol (HPLC grade, Fisher, Italy)
16. Microcrystalline cellulose (Avicel® PH101, JRS Pharma LP., Germany)
17. n-Butanol (Analytical grade, ACS reagent, Merck, Germany)
18. n-Hexane 95% (Analytical grade, Ajax Finechem, Australia)
19. Purified talcum (Vechavit, Thailand)
20. Sodium azide (Analytical grade, Labchem, Australia)
21. Trichloroacetic acid (TCA) (Analytical grade, ACS reagent, Merck, Germany)
22. α-Tocopherol (HPLC grade, Fluka analytical, Switzerland)

23. 2,2-diphenyl-1-picrylhydrazyl (DPPH, Analytical grade, Sigma-Aldrich, USA)
24. 2,4,6-Tris(2-pyridyl)-S-Triazine (TPTZ, Analytical grade, Fluka analytical, Switzerland)

Instruments

1. Analytical balance
   Sartorius AC210 S, Scientific Promotion Co., Ltd., Germany
2. Brookfield viscometer
   Rheometer R/S-CPS (Plate-Plate), Scientific Promotion Co., Ltd., UK
3. Centrifuge
   Avanti 30, Backman, USA
4. Disintegartion test apparatus
   Pharma Test® type PTZ-AUTO 3, Germany
5. Dissolution tester
   Pharma Test® type PTF 20 E, Germany
6. Friabilator, Roche type
   Pharma test® type PTW 600, Germany
7. Hardness tester
   Erweka® type TBH 100, Germany
8. High performance liquid chromatography apparatus (HPLC)
   Hewlett Packard series 100, Agilent Technology, USA
9. Hot air oven
   Binder ED 240/E2, Germany
10. MicroplateUV-Vis Spectrophotometer
    Model 680, Bio-RAD, Japan
11. Milli-Q water filter system
    Milli-Q®, USA
12. Multimode detector
    DTX 880, Beckman Coulter, USA
13. pH meter
    pH Level 2, Inolab, Germany

14. Single stroke tableting machine
    Hanseaten Wilhelm Fette, Germany
15. Sonicator
    Transsonic T460/H, Elma, Germany
16. Vacuum Rotary Evaporator
    N100, Eyela, Japan

3.2 Sources of plant materials:

Fruits of *Phyllanthus emblica* L. were purchased from Muang Mai Market, Chiang Mai province, Thailand. The plant sample was identified as *Phyllanthus emblica* L. by comparison with the herbarium specimens at the herbarium of the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.

3.3 Preparation of *P. emblica* fruit extracts

3.3.1 *P. emblica* crude extraction

Fresh fruits of *P. emblica* were washed and dried at room temperature. The washed fruits were incubated at 50°C in tray dryer for 72 hr. The dried fruits of *P. emblica* were ground to powder and then extracted with 95% ethanol by maceration method with dried plant and solvent ratio of 1:4 for 3 days, 3 cycles.

The macerated solvent was collected by filtration and evaporated using a vacuum rotary evaporator (EYELA N100, Tokyo) operated at 45°C until dryness. The crude extract of *P.emblica* fruits was collected and calculated for the %yield value by the following formula.

$$\% \text{ yield value} = \frac{\text{weight of the extract}}{\text{weight of the dried plant}} \times 100 \qquad \text{-------- (1)}$$

3.3.2 Fractional extraction of *P. emblica* extract

The crude extract of *P. emblica* was dissolved in de-ionized water and then fractional extracted with different solvents namely n-hexane, chloroform, ethyl acetate and n-butanol, respectively according to solvent polarity from non-polar to polar. The fractional extraction was performed in a separating funnel using the crude

extract:organic solvent ratio of 1:1. The mixture was gently mixed for 15 min and then was left for another 30 min to achieve the complete separation. The fractional extraction for each solvent was carried out 3 times. The aqueous layer was separated for further extraction with the next organic solvent in respective order of the polarity under the same condition. The organic layers were collected and evaporated to dryness using a vacuum rotary evaporator (EYELA N100, Tokyo) operated at 45°C for n-hexane, chloroform and ethyl acetate fractions and at 60°C for n-butanol fraction.

3.3.3 Rheological property of *P. emblica* crude and fractional extracts

The rheological property of *P. emblica* crude and fractional extracts was investigated using Brookfield viscometer (Rheometer R/S-CPS (Plate-Plate), UK).

3.4 Biological activity of *P. emblica* crude and fractional extracts

3.4.1 Antioxidant activity test

3.4.1.1 DPPH radical scavenging activity

The free radical scavenging activity of the crude extract and its fractions was determined by DPPH method (53) with some modifications. The samples were dissolved in ethanol to prepare solutions of various concentrations. Twenty microliters of each sample solution was added with 180 µl of ethanolic solution containing DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals at a concentration of 100 mM. The mixtures were kept in the dark for 30 min at room temperature. The absorbance was measured at 540 nm using Microplate reader (Bio-Rad Model 680, USA). The % inhibition of each sample was calculated according to the formula below. The IC_{50} values, the effective concentration of sample to obtain 50% antioxidant activity, were determined from the % inhibition and concentration curve.

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad \text{------ (2)}$$

3.4.1.2 Ferric reducing antioxidant power (FRAP) assay

The reducing power of the crude extract and its fractions was determined by FRAP assay (50) with some modifications. The FRAP reagent, containing 1 ml of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl plus 1 ml of 20 mM $FeCl_3$ and 10 ml of acetate buffer pH 3.6, was freshly prepared. The crude or the fractional extracts were dissolved in ethanol to a concentration of 150 µg/ml. An aliquot (20 µl) of the extract solution was mixed with 180 µl of FRAP reagent, and the absorbance of the mixture was measured at 595 nm using Microplate reader (Bio-Rad Model 680, USA). The $FeSO_4$ solutions were used to obtain a calibration curve. The reducing power was expressed as an equivalent concentration (EC); the concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

3.4.2 Total phenolic content (TPC)

The total phenolic contents of *P. emblica* crude and fractional extracts were determined using Folin-Ciocalteu's reagent (FCR). An aliquot of 20 µl of the crude and fractional extract solution (400 µg/ml) was mixed with 45 µl of FCR followed by 135 µl of 2% w/v Na_2CO_3 solution. The absorbance was then measured at 790 nm using Microplate reader (Bio-Rad Model 680, USA) after incubation at room temperature for 2 hr. Gallic acid was used as a standard phenolic compound. Results were expressed in terms of gallic acid equivalent (GAE) mg/g dry extract.

3.4.3 HPLC analysis

The crude and fractions of *P. emblica* extract were characterized by HPLC (Hewlett packard/hp1100, USA) analysis on a reverse phase C_{18} column (5 µm, 4.0x250 mm, Hypersil ODS, Agilent, USA) using UV-detector operating at 220 nm. A solvent system was acetonitrile: 0.05% phosphoric acid (isocratic; 10:90) at a flow rate of 0.5 ml/min. All extracts were dissolved in methanol (1 mg/ml) and filtered through a membrane filter 0.45 µm before injection (10 µl) into HPLC system. Gallic acid and ascorbic acid were used as standards.

3.4.4 Antiglycation assay

The antiglycation activity of *P. emblica* crude and fractional extracts was investigated with some modifications(52). One milliliter of the reaction mixture containing 1mg/ml bovine serum albumin (BSA), 200 mM glucose with either aminoguanidine (positive control) or the plant extract as an inhibitor in phosphate buffer saline (PBS) pH 7.4 with sodium azide 0.02% w/v as a preservative was prepared. The negative control was unreacting solution that used PBS instead of an inhibitor. The mixtures were incubated at 60°C for 30 hr. After cooling, the reaction mixtures were added with 100 µl of 100% w/v trichloroacetic acid (TCA) and then centrifuged at 10000 rpm, 4 °C, for 15 min to obtain AGEs precipitates. The obtained precipitates were dissolved with 500 µl alkaline PBS and the fluorescence intensity (ex. 360 nm, em. 465 nm) was monitored by using Multimode detector (Backman Coulter, DTX 880, USA). The % inhibitory activities of *P. emblica* crude and fractional extract on AGEs formation were determined and the IC_{50} values of sample were also calculated.

3.5 Tablet formulation

The suitable fractional extract showing the highest antioxidant and antiglycation activity was brought to the subject of tablet formulation. The dose of *P. emblica* extract tablet was calculated equivalent to the antioxidant activity of the usual daily dose of vitamin E (α-tocopherol) via DPPH method. The vitamin E dose that is not harmful and used for dietary supplement is 400-1000 IU (international unit) or 266.7-666.7 mg per day. The details of the calculation were shown in the appendix. The tablet formulation development procedure was as follows.

3.5.1 Evaluation of excipients

Tablets containing five hundred milligrams of *P. emblica* extract were prepared by wet granulation method. Types and amounts of suitable diluent was evaluated to gain the tablets which had suitable pharmaceutical properties on hardness, disintegration time and low moisture adsorption.

3.5.1.1 Evaluation of diluents

The diluents commonly used in tablet formulation, i.e. lactose, corn starch, rice starch, dibasic calcium phosphate (Emcompress®) and microcrystalline cellulose (Avicel® PH 101) were evaluated in this study. Purified talcum and (2%) and magnesium stearate (0.5%) were added as glidant and lubricant in the formulation, respectively. The ratio of diluents and the extract was investigated and the value should not more than 1:1 to find out the diluents which can absorb the extract and provide the tablet which not double of weight from the extract used.

3.5.1.2 Evaluation of binders

At the beginning of the study, *P. emblica* fractional extract itself was used as a binder in the formula. In case the tablets have insufficient hardness (below 40 N), the addition of binder such as polyvinyl pyrrolidone will be considered.

3.5.1.3 Evaluation of the concentrations of superdisintegrant

If the tablets prepared from the selected diluent from 3.5.1.1 does not disintegrate within the acceptance limit of 15 min, a superdisintegrant (Ac-Di-Sol®) at various concentrations, i.e. 1%, 3%, and 5% will be added into a formulation as a superdisintegrant by intragranular and extragranular methods.

3.5.1.4 Evaluation of the moisture content of the tablets

The tablets of each preformulation study formulas were weighed individually and the kept in the desiccator which saturated with sodium chloride solution, refer to the percent relative humidity of 75%. After 24 hour, the tablets were individually weighed again and the change in weight was calculated to moisture content (%).

3.5.2 Tableting process

The wet granulation process was used for the preparation of *P. emblica* extract tablets. The wet mass was prepared by mixing *P. emblica* extract, diluent and a superdisintegrant (in case of necessary) and passed through a 14 mesh sieve. After drying in hot air oven, granules were screened through a sieve no. 16 mesh and then mixed with the remaining superdisintegrant followed by a glidant and a lubricant.

The granules were compressed on single stoke tableting machine (HANSEATEN Wilhelm Fette, Germany). The suitable formulation of *P. emblica* extract tablets was scaled up and evaluated for the pharmaceutical properties and stability study.

3.5.3 Evaluation of the pharmaceutical properties of *P. emblica* extract tablets

3.5.3.1 Hardness

Ten tablets of *P. emblica* extract formulation were randomly drawn and measured for their hardness using a hardness tester (Erweka® type TBH 100, Germany). The mean and standard deviation of crushing strength values in newton (N) were shown.

3.5.3.2 Thickness and diameter

The thickness and diameter of *P. emblica* extract tablets were measured by a digital micrometer. The mean and standard deviation of the tablets were demonstrated.

3.5.3.3 Weight variation

Weight variation test was determined by weighting 20 tablets individually, calculating the average weight and comparing the individual tablet weights to the average. The requirements are met if the weights of not more than 2 tablets differ from the average weight by more than the percentage listed of 5 percents for the uncoated tablet which has average than 324 mg and no tablet differ in weight by more double than percentage (54).

3.5.3.4 Friability test

The percent tablet friability was determined by the Roche-type friabilator. Twenty tablets were accurately weighed and then placed in the friabilitor and rotated for 4 min (100 revolutions). The tablets were weighed again and % friability was calculated from the equation 3.

$$\%\text{Friability} = \frac{\text{weight before test} - \text{weight after test}}{\text{weight before test}} \times 100 \qquad \text{----(3)}$$

3.5.3.5 Disintegration test

The disintegration time of the *P. emblica* extract tablets were determined individually in distilled water using USP method with discs. The results were reported as the mean of twelve tablets and standard deviation.

3.5.3.6 Dissolution test

The dissolution profiles of *P. emblica* extract tablets was determined by a dissolution tester (Pharma test, PTW 600, Germany) apparatus 2 (paddle method). Nine hundred ml of phosphate buffer saline pH 6.8 was used as a dissolution medium. The paddle speed was maintained at 100 rpm. Five milliliters of the medium was sampling from the vessel at 5, 10, 15, 30, 45, and 60 min. The amount of gallic acid in the *P. emblica* extract tablet was measured by HPLC at wavelength of 220 nm and calculated form a standard curve.

3.6 Stability of *P. emblica* extract tablets

The *P. emblica* fractional extract tablets were investigated for the stability of the active ingredients for 4 months under the storage conditions of 30°C, 65% relative humidity (RH) and 45°C, 75% RH. The tablets were sampling 7 times after storage for 0, 7, 15, 30, 60, 90 and 120 days and were evaluated for the following items.

- Scavenging activity by DPPH method
- Ferric reducing antioxidant power
- Total phenolic content
- Antiglycation activity
- HPLC analysis (only on the first and the last day of stability study)

3.7 Statistical analysis

The data from the experiments were expressed as mean ± standard deviation (S.D.). Statistical analysis of the experimental data was performed using the software package SPSS version 15 by applying one-way ANOVA tests. Least-significant difference (LSD) was used as a post hoc test to assess the significance of differences. To compare the significance of the difference between the means of two groups, the *t*-test was performed; in all cases, a value of $p < 0.05$ was accepted as significant.