CHAPTER III

MATERIALS AND METHODS

Materials

1. Absolute Ethanol, AR grade (Merck, Germany)
2. Acetonitrile, HPLC grade (Lab Scan Co., Ltd., Thailand)
3. Acetic acid glacial 100% (Lab Scan Co., Ltd., Thailand, lot no.A8401E)
4. Cupper sulfate (Ajax, Finechem, lot no. A171)
5. Bovine serum albumin (BSA) (Sigma-aldrich, Inc., Germany)
6. Dipotassium hydrogen phosphate (Merck, lot no. A687601601)
7. Disodium carbonate (Ajax, Finechem, lot no. A463)
8. Ethylenediaminetetra acetic acid calcium disodium salt (Sigma-aldrich, Inc., USA, lot no. ED2SC)
9. Folin-Ciocalteu reagent (Merck, Germany, lot no. OC683903)
10. Glycerin (Srichand United Dispensary Co., Ltd., Thailand)
11. Hexane (Mallinckrodt Baker, Inc., USA)
12. Petroleum ether (Lab Scan Co., Ltd, Thailand)
13. Phospholic acid (J.T. Baker, USA, lot no. 0260-03A262673045)
14. Potassium dihydrogen phosphate (Merck, Germany, lot no. A315973 127))
15. Methanol, HPLC grade (Lab Scan Co., Ltd., Thailand)
16. Mineral oil (Aketong chemical co., Ltd, Thailand)
17. Propylene glycol (Srichand United Dispensary Co., Ltd., Thailand)
18. Sepicide® HB (ADINOP, Thailand, lot no. T70211)
19. Sodium chloride (Merck, Germany, lot no.K32104204 324)
20. Sodium dihydrogen phosphate, anhydrous (Asia Pacific Chemicals Limited, batch no. F2F136)
21. Sodium hydrogen phosphate (APS Finechem, batch no.FOJ067)
22. Sodium hydroxide (Mallinckrodt Chemical, Mexico, lot no.7708MVHV)
23. Sodium potassium tartrate (Farmitalia Carlo Erba)
24. 70% Sorbitol solution (Srichand United Dispensary Co., Ltd., Thailand)
25. Standard asiaticoside 90% (Guangxi Chemical Corporation, China)

26. Standard triamcinolone acetonide 99.86% (RCV, lot no. 7629/M2)
27. Standard xyloglucan (**Megazyme International Ireland Ltd**, Ireland, lot no. 00401)
28. Tamarind seed
29. Titrated extract of *Centella asiatica* (TECA) (Guangxi Chemical Import and Export Corporation, China, batch no. 050523)
30. Tween 80 (Srichand United Dispensary Co., Ltd., Thailand)

Apparatuses

1. Analytical balance (Model AX105, Mettler Toledo, Switzerland)
2. Centrifuge (Hitachi himac CR20B3, Japan)
3. Differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland)
4. Disposable syringe filter nylon 13 mm, 0.45 μm (Chrom Tech, USA)
5. Dry cabinet (Model GH-197, Ampore House, Taiwan)
6. High performance liquid chromatography system
   - Automatic sample injector (SIL-10A, Shimadzu, Japan)
   - Communications bus module (CBM-10A, Shimadzu, Japan)
   - Column (Alltima HP C18, 5μm, 150mm x 4.6mm, lot no.3461)
   - Column (Sugar Pak I, 6.5mm x 300mm, lot no.002437211A)
   - Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
   - Precolumn (μBondapack C18, 10 μm, 125 A°, Water Corporation, Ireland)
   - Precolumn (Sugar-Pak^{TM} II, Water Corporation, Ireland)
   - RI detector (RID-10A, Shimadzu, Japan)
   - UV-VIS detector (SPD-10A, Shimadzu, Japan)
7. Homogenizer (Model EURO-D, Memmert, Germany)
8. Modified Franz Diffusion cells (Crown Glass Company, USA)
9. Magnetic stirrer (Model RCT basic, KIKA® Works Guangzhou, China)
10. Moisture analyzer balance (Model HB43, Mettler Toledo, Switzerland)
11. pH meter (Orion model 420A, Orion Research Inc., USA)
12. Rotary evaporator (Buchi heating bath B-490, Switzerland)
13. Scanning electron microscope (Model JSM-T220A, Jeol, Japan)
14. Sonicator (Model TP680DH, Elma, Germany)

15. Spray dryer (Model SD-06, Labplant, Ltd., UK)
16. Spray dryer B-290 (Buchi mini, Switzerland)
17. Soxhlet extraction (Chatcharee holding Co., Ltd, Thailand)
18. Stability cabinet (Eurotherm Axyos, Germany)
19. Stopwatch (Heuer, Switzerland)
20. Ultrasonicator (Crest Ultrasonics, Malaysia)
21. Universal tensiometer (Tinius olsen, Model H5KS 1509)
22. UV-Visble Spectophotometer (UV-1601, Shimadzu, Japan)
23. Viscometer (RotoVisco RV1, Germany)
24. Vortex mixer (Vortex Ginies-2, Scientific Industries, USA)
25. Water bath (Model WB22, Becthai Co., Ltd., Thailand)
26. X-ray diffractometer (Model JDX-3530, Jeol, Japan)

Methods

A. Investigation of xyloglucan extraction and preparation of xyloglucan powder from tamarind seeds

1. Preparation of tamarind seed powder

Tamarind seeds were soaked in water for 2-3 days. The seed embryo was manually removed and then they were dried under sunlight. The dried mass were milled and sieved.

2. Xyloglucan extraction process

Generally, the extraction of xyloglucan was composed of two processes as follows:

Defatting process with hexane

The extraction of fat by hexane might be done with either mixing hexane directly with dried tamarind seed powder or mixing hexane with slurry of tamarind seed powder in water.

The sedimentation by centrifugation

The slurry of defatted tamarind seed powder in water might be centrifuged to sediment undissolved fraction of the powder either immediately or left standing overnight.

Thus, four methods of xyloglucan extraction were designed and investigated as depicted in Figure 10.

Method I

Tamarind seed powder was firstly soaked with hexane overnight by packing in a percolator. The percolate was slowly collected on the next day. Then, the powder was removed from the percolator and dried in a hot air oven under 60°C. Thirty grams of dried powder were mixed with 60 volumes of distilled water to make slurry using a homogenizer for 90 min at 90°C (Somsiri, 1997). The slurry was then centrifuged at 12,000 rpm for 30 min at 25°C. The supernatant liquid was removed for the spray drying.

Method II

Tamarind seed powder was firstly soaked with hexane overnight by packing in a percolator. The percolate was slowly collected on the next day. Then, the powder was removed from the percolator and dried in a hot air oven under 60°C. Thirty grams of dried powder were mixed with 60 volumes of distilled water to make slurry using a homogenizer for 90 min at 90°C. The slurry was left standing overnight and then centrifuged at 12,000 rpm for 30 min at 25°C. The supernatant liquid was removed for the spray drying.

Method III

Thirty grams of tamarind seed powder were mixed with 60 volumes of distilled water to make slurry using a homogenizer for 90 min at 90°C. The extraction of fat was performed by the addition of hexane in triplicate. The slurry was then centrifuged at 12,000 rpm for 30 min at 25°C. The supernatant liquid was removed for the spray drying.

Method IV

Thirty grams of tamarind seed powder were mixed with 60 volumes of distilled water to make slurry using a homogenizer for 90 min at 90°C. The extraction of fat was performed by the addition of hexane in triplicate. The slurry was left standing overnight and then centrifuged at 12,000 rpm for 30 min at 25°C. The supernatant liquid was removed for the spray drying.

3. Spray drying condition

The supernatant solution was dried in spray dryer (SD-06, Labplant, Ltd., UK) (Figure 11) at a condition of inlet temperature of 160±2°C, feed rate 167.17 ml per hour (pump = 2), spray air flow 300 cubic meter per hour (fan = 50) and slow deblocker.

The collected xyloglucan spray dried powders prepared under different methods of xyloglucan extraction (Method I-IV) were characterized and compared.

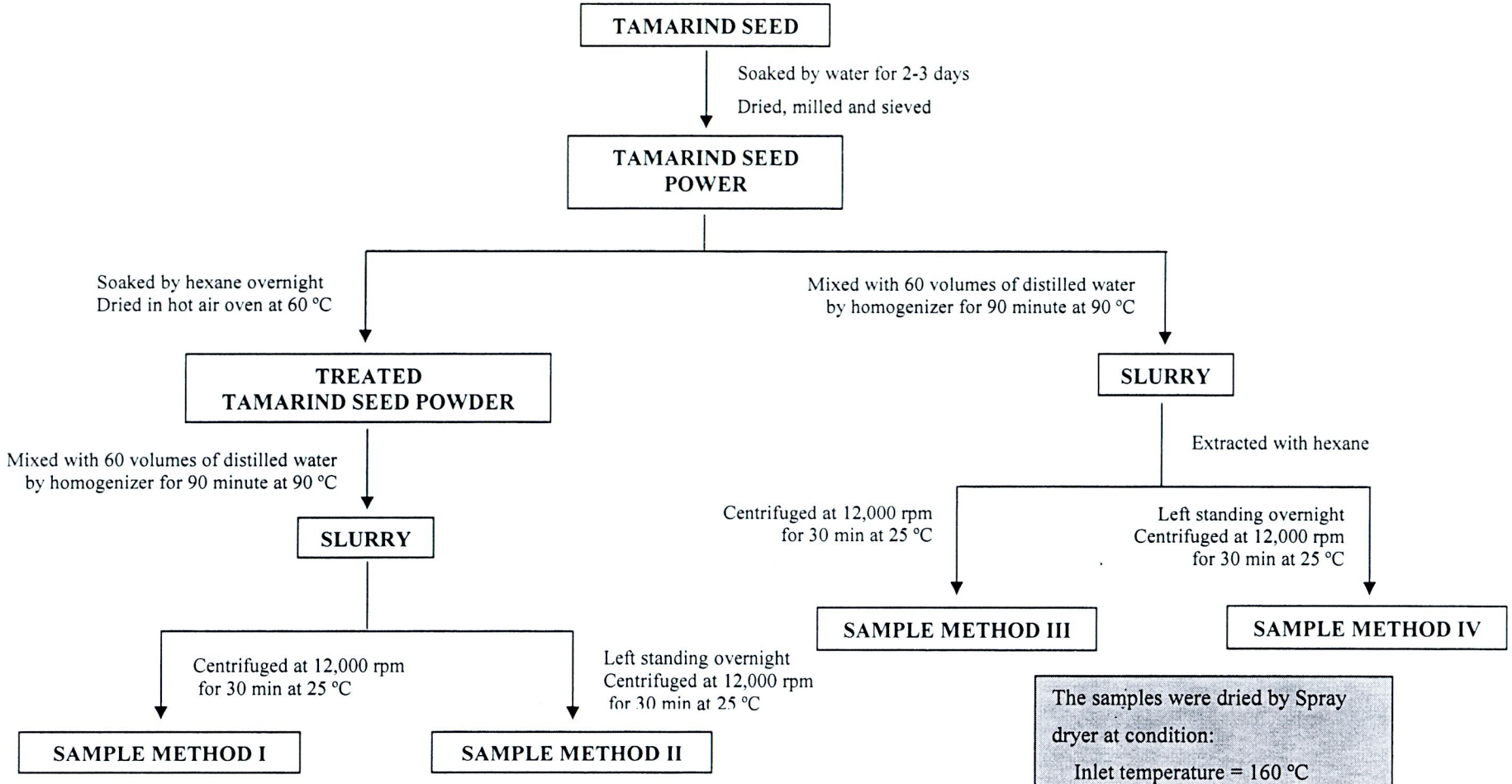

Figure 10 Diagram of xyloglucan extraction

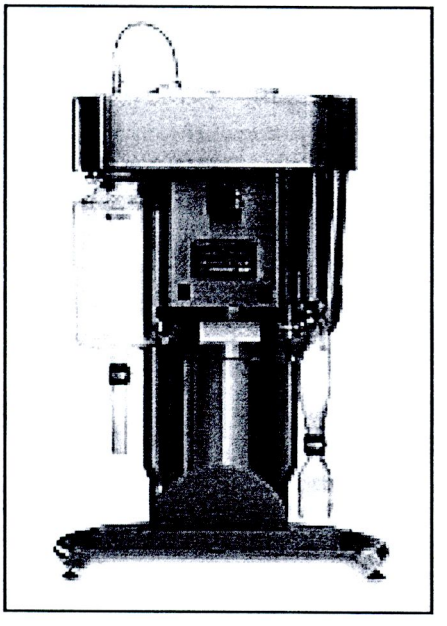

Figure 11 Spray dryer (Model SD-06, Labplant, Ltd., UK)

B. Characterization of xyloglucan spray dried powder from Tamarind seeds.

To determine the appropriate method for the xyloglucan extraction, the xyloglucan spray dried powders prepared by four different methods of defatting and sedimentation were studied.

1. Percentage yield

The calculation of percentage yield (w/w) was the weight of the dried powder recovered from collecting chamber divided by the sum of the initial weight of tamarind seed powder and multiplied with 100.

2. Xyloglucan content

The determination of xyloglucan content of xyloglucan spray dried powder was performed by high performance liquid chromatography (HPLC), which was developed in this investigation.

2.1 Chromatographic Condition

The chromatographic conditions for the analysis of active constituents from xyloglucan were as follows (modified from **Megazyme International Ireland Ltd**):

Column	:	Sugar Pak I® 6.5 mm x 300 mm
Precolumn	:	μBondapack C18, 10 μm, 125A°
Mobile phase	:	Ultra pure water
Injection volume	:	100 μl
Flow rate	:	0.6 ml/min
Detector	:	RI detector
Temperature	:	80 °C
Runtime	:	15 min

The mobile phase was freshly prepared, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min before using.

2.2 Standard Solution

- Preparation of standard stock solutions

The 125 mg of xyloglucan were accurately weighed and transferred into a 25 ml volumetric flask, ultrapure water was used to dilute and adjust to volume. The stock solution had the final concentration of xyloglucan of 5000 μg/ml.

- Preparation of standard solutions

The solutions of 400, 500, 600, 700, 800, 900 μl of xyloglucan standard stock solution were pipetted and added into 5 ml volumetric flasks. The dilution to volume with ultra pure water gave the final concentrations of 400, 500, 600, 700, 800, 900 μg/ml of xyloglucan, respectively.

The standard solutions were freshly prepared and used for the HPLC determination. As a result, the standard curve between concentration and peak area was plotted.

2.3 Validation of the HPLC method

The analytical parameters used in the assay validation of the HPLC assay method were specificity, linearity, accuracy and precision.

2.3.1 Specificity

The specificity of the method was determined by comparing the test results from analyses of xyloglucan in ethanol with standard solutions. Under the chromatographic conditions used, the peak of xyloglucan must be completely separated from and not be interfered by the peaks of ethanol.

2.3.2 Linearity

The linearity was determined from the coefficient of determination (R^2). Six concentrations of standard solutions and three replicates of each concentration were prepared and analyzed. The relation between the peak area and concentrations were plotted and the least square linear regressions were calculated.

2.3.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Five sets of three concentrations (low, medium, high) of xyloglucan at 450, 650, 850 μg/ml were prepared and analyzed, respectively. The accuracy of the method was determined from the percentage of recovery. The percentage of recovery of each concentration was calculated from the estimated concentration to know concentration multiplied by 100.

2.3.4 Precision

a) Within run precision

The within run precision was determined by analyzed five sets of three concentrations (low, medium, high) of xyloglucan at 450, 650, 850 μg/ml in the same run. Peak area of xyloglucan was calculated and the percent of coefficient of variation (%CV) of each concentration was determined.

b) Between run precision

The precision during the operation run was determined by analyzing three concentrations (low, medium, high) of xyloglucan at 450, 650, 850 μg/ml on five different runs. Peak area of xyloglucan was calculated and the percent of coefficient of variation of each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 98-102% of each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2%.

The percentage xyloglucan content (w/w) was calculated as the amount of xyloglucan divided by the sum of the initial weight of tamarind seed powder and multiplied with 100

3. Total protein content

The determination of protein content in xyloglucan spray dried powder prepared from four extraction methods was performed by Lowry's method. Lowry's method for total protein is one of the most common colorimetric assays. This procedure is particularly sensitive because it employs two color forming reactions. It uses the Biuret reaction in which Cu^{2+} (in the presence of base) reacts with the peptide bond to give a deep blue color. In addition, Folin-Ciocalteu reagent, in which a complex mixture of inorganic salts reacts with tyrosine and tryptophan residues bovine serum albumin (BSA), was used as standard protein.

3.1 Standard Solution

Preparation of standard BSA stock solutions

The 20 mg parts of BSA were accurately weighed and transferred into a 10 ml volumetric flask, diluted and adjusted to volume with ultrapure water. The stock solutions had the final concentrations of BSA of 2000 µg/ml.

The solutions of 25, 50, 75, 100, 125, 150 µl of BSA standard stock solution were added into glass tubes. The dilution to volume with ultrapure water gave final concentrations of 50, 100, 150, 200, 250, 300 µg/ml of BSA, respectively.

The standard solutions were analyzed spectrophotometrically at 550 nm. The standard curve was plotted between concentration and absorbance.

3.2 Validation of the protein assay method

The analytical parameters were applied in the assay validation for linearity, accuracy and precision of spectrophotometric assay.

3.2.1 Linearity

Three sets of six concentrations of standard solutions ranging from 50 to 300 μg/ml. Linear regression analysis of the absorbance versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

3.2.2 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations (low, medium, high) of BSA at 80, 160, 240 μg/ml were prepared and analyzed, respectively. The percentage of recovery of each concentration was calculated from the estimated concentration to know concentration multiplied by 100.

3.2.3 Precision

a) Within run precision

The within run precision was determined by analyzing five sets of three concentrations of BSA at 80, 160, 240 μg/ml in the same run. Absorbances of BSA were calculated and the percent of coefficient of variation (%CV) of each concentration was determined.

b) Between run precision

The precision during the operation run was determined by analyzing three concentrations of BSA at 80, 160, 240 μg/ml on five different runs. Absorbance of BSA was calculated and the percent of coefficient of variation of each concentration was determined.

Acceptance criteria:

For accuracy, the percentage of recovery should be within 85-115% of each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 15%.

4. Fat content

The assay of fat content in xyloglucan spray dried powder was performed by AOAC.920.39. Amount of fat was calculated by the increased weight of flask multiplied with 100.

One gram of xyloglucan spray dried powder was packed in a filter paper and extracted with 300 ml of petroleum ether for 3 hour by soxhlet extraction (Figure 12). The petroleum ether extract was collected and evaporated under vacuum by rotary evaporator. The percentage fat content was calculated as the amount of fat, divided by the initial weight of tamarind seed powder and multiplied with 100.

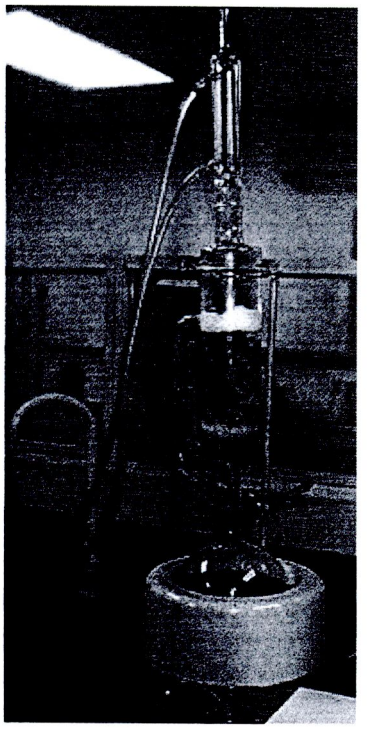

Figure 12 Soxhlet extraction

5. Moisture content

A sample of xyloglucan spray dried powder was accurately weighed on the pan of moisture analyzer (Model HB43, Mettler Toledo, Switzerland). The temperature was set at 105°C and the maintainable constant weight for 20 s was detected. The sample was exposed to a halogen lamp until a constant weight was

obtained. The percentage moisture content was calculated automatically. The mean and standard deviation of three determinations were calculated.

6. Powder topography by scanning electron microscopy

The morphological feature of xyloglucan spray dried powder was displayed using scanning electron microscope (SEM), (JSM-T220A, Jeol, Japan). Powder samples were mounted onto aluminum stubs using double-sides adhesive tape and then sputter coated with a thin layer of gold before examination. The samples were imaged using a 15 kv electron beam. The magnifications of the photomicrographs of powder were ×500 and ×2000.

C. Optimization of spray drying condition

From the investigation of xyloglucan extraction method in topic B, the method I was selected. The spray drying solution was prepared from 600 grams of tamarind seed powder, the method was shown under method I. Sepicide® HB was added to the solution with the concentration of 0.5%w/v for preservation action. The solution was divided into 20 equal parts and kept frozen. The frozen was thawed at room temperature before spray drying process.

1. Preparation of spray drying solution

A Spray dryer B-290 (Buchi Mini, Switzerland) was used for the preparation of spray dried powder (Figure 13). The operating parameters were set with the feed rate of 10% and nozzle size of 0.7 mm. The spray drying solution was controlled at 37 °C through spray drying process. The inlet temperature and aspirator rate were set according to the experiment design. The resulting spray dried powders were collected and kept away from moisture until further investigations.

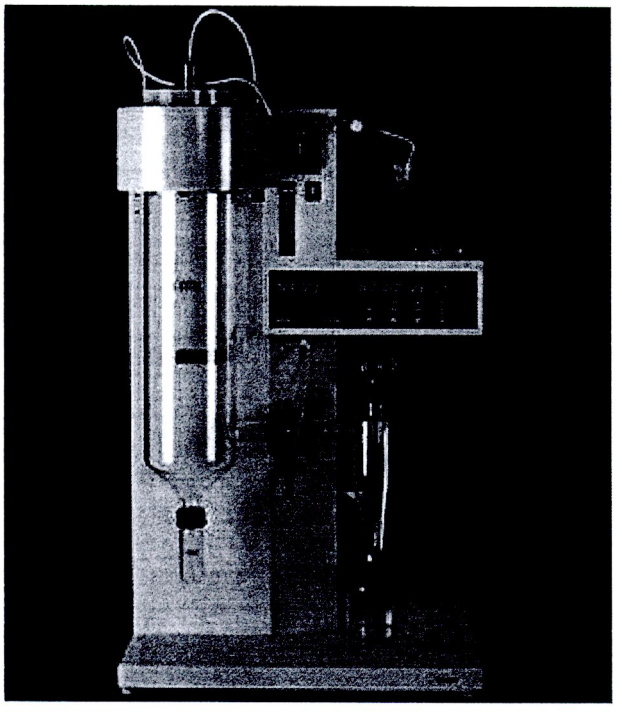

Figure 13 Spray dryer B-290 (Buchi Mini, Switzerland)

2. Optimization design: face centered design (FCD)

A face centered design is a central composite design with alpha equals to 1. For this purpose, a face centered design with two factors at two levels was used to study the response surface and to determine the combination of variables would produce the maximal yield and the minimal moisture content. The combination effect of inlet temperature and aspirator rate was studied by response surface methodology. The response surface plots indicated the effects of the factors on the percentage yield and percentage moisture content responses at each level of the factors. The face centered design in this study was 13 runs. The design matrix with responses was given in Table 1.

Table 1 A face central design of two parameters

Code	Parameters	
	Inlet temperature (°C)	**Aspirator rate (%)**
C1	(-) 120	(-) 80
C2	(+) 200	(-) 80
C3	(-) 120	(+) 100
C4	(+) 200	(+) 100
C5	(-) 120	(0) 90
C6	(+) 200	(0) 90
C7	(0) 160	(-) 80
C8	(0) 160	(+) 100
C9	(0) 160	(0) 90
C10	(0) 160	(0) 90
C11	(0) 160	(0) 90
C12	(0) 160	(0) 90
C13	(0) 160	(0) 90

Statistical analysis and a graphical optimization were performed using Design-Expert version 7.1 statistical software. The method basically consisted of overlaying the curves of the models accroding to the criteria imposed. The selection of conditions to overlay plot were range from 120-200 °C of inlet temperature and 80-100 % of aspirator rate. The main objective was to maximize %yield and minimize %moisture content.

3. Characterization of xyloglucan spray dried powder

The xyloglucan spray dried powders prepared from the 13 designed conditions in Table 1 was evaluated for percentage yield and moisture content (mention in topic B1 and B5). Additionally, size and size distribution were also studied.

D. Determination of physicochemical properties of xyloglucan power from tamarind seeds.

1. pH

The pH of 1%w/v xyloglucan powder from tamarind seeds was measured by using pH meter. The measurements were done in triplicate.

2. Solubility in water

The excessed amount of weighed xyloglucan powder was added into 10 ml of distilled water and left shaking at room temperature for 24 hrs. The mixture was centrifuged and filtered through a 0.45 μm membrane filter. The determination of amount of xyloglucan which dissolved in distilled water was performed by HPLC method. The examination was performed in triplicate.

3. Viscosity and rheology property

Viscosity and rheology property of xyloglucan solution was measured by using viscometer (RotoVisco RV1, Germany) (Figure 14). The cone C35/1° Ti was selected in the experiment. The viscosity and rheology measurements were made on solutions of tamarind seed xyloglucan powder dissolved in distilled water at 1, 1.5 and 2% w/v. The measurement was performed in triplicate.

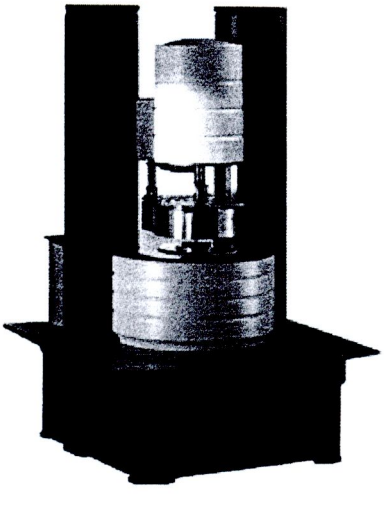

Figure 14 Viscometer (RotoVisco RV1, Germany)

4. Incompatibility of xyloglucan and ethanol

The 1%w/v of tamarind seed xyloglucan solution was prepared and studied for the incompatibility of xyloglucan and ethanol. Tamarind seed xyloglucan solution was added with ethanol in different mixing ratios, mixed thoroughly and left at room temperature for 24 hrs (Table 2). These liquid were centrifuged and filtered through 0.45 μm membrane filter. The determination of amount of xyloglucan was performed by HPLC method. The examination was performed in triplicate.

Table 2 Mixing ratios of absolute ethanol and tamarind seed xyloglucan solution

%ethanol (v/v)	1% tamarind seed xyloglucan solution (ml)	Amounts of added ethanol (μl)
0	5.00	-
1	4.95	50
2	4.90	100
3	4.85	150
4	4.80	200
5	4.75	250

5. Thermal analysis by differential scanning calorimetric method

The differential scanning calorimetric (DSC) thermogram was determined by using differential scanning calorimeter (DSC822e, Mettler Toledo, Switzerland). A highly sensitive ceramic sensor in the DSC instrument was used to measure the difference between the heat flows to the sample and reference crucibles. The samples (3-5 mg) were accurately weighed into standard aluminum pans (40 μl) and then sealed. The DSC runs were conducted over a temperature range 30-330 °C at rate of 10°C/min. All tests were performed under a nitrogen atmosphere of 2 ml/min.

6. Powder X-ray Diffractogram

Powder X-ray diffractograms were carried out by using powder X-ray diffractometer (Bruker AXS model D8 Discover, Germany) with Cu-K radiation as the source of X-rays. The measurement conditions were set as voltage of 40 kV, current of 40 mA, scanning speed of 0.3 °/min in the 0.02° angle range of 0.8-30°.

7. Morphology

Morphology of xyloglucan powder was examined by scanning electron microscope (SEM), (JSM-T220A, Jeol, Japan).

8. Determination of size and size distribution

Particle size analysis was performed on a sample of powder suspended in light mineral oil as a non-dissolving dispersion medium. The samples were analyzed by using a laser light scattering (Mastersizer S, Malvern, UK). The mean and standard deviation of three determinations were calculated.

E. Formulation of films prepared from tamarind seed xyloglucan

1. Development of films formulation prepared from tamarind seed xyloglucan

The effect of concentration of combined plasticizers and concentration of tamarind seed xyloglucan in film formulations was determined using a small composite design (Table 3). From the results of preliminary study, the concentration ranges of glycerin and 70%sorbitol solution were 0-4% and 2-6% w/w, respectively, whereas the concentrations range of tamarind seed xyloglucan was 1-2%w/w. The formulations were shown in Table 4. The tamarind seed xyloglucan was dispersed in mixed solvent. The solvent mixture comprised of propylene glycol, Tween 80, glycerin, 70%sorbitol, Sepicide® HB, ethanol and distilled water. The mixture was poured into a plate. The casting mixture was dried by a hot air oven at 50°C for 24 hrs.

2. Evaluation of films prepared from tamarind seed

2.1 Physical Appearances

Color, transparency, flexibility and integrity of all films were visually observed. The thickness of film was measured by using a micrometer having a sensitivity of 0.01 mm.

Table 3 Small central composite design of film formulations

Film No.	tamarind seed xyloglucan	70% sorbitol solution	glycerin
1	(+) 2%	(+) 6%	(-) 0%
2	(+) 2%	(-) 2%	(+) 4%
3	(-) 1%	(+) 6%	(+) 4%
4	(-) 1%	(-) 2%	(-) 0%
5	(-) 1%	(0) 4%	(0) 2%
6	(+) 2%	(0) 4%	(0) 2%
7	(0) 1.5%	(-) 2%	(0) 2%
8	(0) 1.5%	(+) 6%	(0) 2%
9	(0) 1.5%	(0) 4%	(-) 0%
10	(0) 1.5%	(0) 4%	(+) 4%
11	(0) 1.5%	(0) 4%	(0) 2%
12	(0) 1.5%	(0) 4%	(0) 2%
13	(0) 1.5%	(0) 4%	(0) 2%
14	(0) 1.5%	(0) 4%	(0) 2%
15	(0) 1.5%	(0) 4%	(0) 2%

2.2 Film weight

The weight of films (1×1 cm^2) was investigated using an analytical balance (Mettler Toedo, Switzerland), that has a sensitivity of 0.00001 g. The measurement was preformed in 5 samples in each formulation.

2.3 Adhesive force

Tensile mucoadhesive experiments was examined by tensiometer (Tinius Olsen, Model H5KS 1509) with a 10 N load cell and a software-controlled program, QMat 4.10 S-Series-5K). The withdrawal and retune speeds were set at 15 mm/min. A probe was an aluminium cylinder having a diameter of 2.5 cm. A film product, whose the radius of a circle was 2.5 cm, was taped to the base of aluminium probe fixed to the mobile arm of the tensiometer (Tinmanee, 2004). A piece of porcine skin was securely set in place on a platform. Before testing, the piece of porcine skin was dropped with 100 µl distilled water. The film was brought into contact with the

porcine skin with a constant force of 0.1 N for 10 s. The value of the resistance to withdraw of the probe indicating the adhesive force of the film to porcine skin was displayed by adhesive force (N/cm^2). Five specimens were examined for one film formulation. A new porcine skin was replaced for each run.

2.4 Mechanical properties

Mechanical properties of films from tamarind seed xyloglucan were determined by using tensiometer (Tinius Olsen, Model H5KS 1509) with a 10 N load cell. The mechanical properties studied included the tensile strength, percent elongation at break, work of failure, and Young's modulus were evaluated. The measurements were done in five replicates. The procedure employed was based on the guideline of the American Society for Testing and Material ASTM (1995).

The film specimens were cut into small strips of 3 × 40 mm. The thickness of each strip was the averaged value of five separate measurements by using micrometer. The test machine was set according to conditions as follows:

Rate of grip separation	=	12 mm/min
Gauge length	=	5 mm
Loading weight	=	2 Newton
Temperature	=	37±2 °C
Relative humidity	=	24±3%

Five specimens were examined for one film formulation. After the specimen was ruptured, the breaking force and the change in length at the moment of rupture, were analyzed by the software-controlled program, QMat 4.10 S-Series-5K). The calculation of the mechanical properties of the films was as:

$$\text{Tensile strength (MPa)} = \frac{\text{maximum load}}{\text{original minimum cross – sectional area of the specimen}}.$$

$$\text{\% elongation} = \frac{\text{extension at the moment of rupture of the sample}}{\text{initial gauge length of the specimen}} \times 100$$

$$\text{Young's modulus} = \frac{\text{tensile stress}}{\text{elastic strain in tension}}.$$

Work of failure (mJ) = area of a curve plotting between force and extension

F. Formulation of films prepared from tamarind seed xyloglucan containing *Centella asiatica* extract

1. Prepared film from tamarind seed containing *Centella asiatica* extract

According to the evaluation of all films in topic E2.3, the formulation 6 with the highest adhesive force (N/cm^2) value, was selected. *Centella asiatica* extract was weighed in an amount equivalent to 1% asiaticoside (w/w). *Centella asiatica* extract was dissolved in the solvent mixture comprised of 1.5% propylene glycol, 1% Tween 80, 2% absolute ethanol and distilled water.

2. The analysis of asiaticoside in *Centella asiatica* extract

The determination of asiaticoside content was performed by high performance liquid chromatography, HPLC).

2.1 Chromatographic Condition

The chromatographic conditions for the analysis of asiaticoside from *Centella asiatica* were as follows (Kongthong, 2004):

Column	:	Alltima® HP C18, 5 µm, 150 mm x 4.6 mm
Precolumn	:	µBondapack C18, 10 µm, 125A°
Mobile phase	:	Acetonitrile: 10mM phosphate buffer pH 6.8, (35:65)
Injection volume	:	20 µl
Flow rate	:	0.8 ml/min
Detector	:	UV detector at 210 nm
Temperature	:	ambient
Run time	:	9 min
Internal standard	:	triamcinolone acetonide

The mobile phase was freshly prepared, filtered through 0.45 µm membrane filter and then degassed by sonication for 30 min before using.

Table 4 Composition of tamarind seed xyloglucan film formulations

Ingredient	Rx (w/w)														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Xyloglucan powder	2	2	1	1	1	2	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Propylene glycol	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tween 80	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Ethanol	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
70% Sorbitol solution	6	2	6	2	4	4	2	6	4	4	4	4	4	4	4
Glycerin	0	4	4	0	2	2	2	2	0	4	2	2	2	2	2
Sepicide HB	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water qs to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

2.2 Standard Solutions

2.2.1 Preparation of internal standard solution

A stock solution of internal standard was prepared by accurately weighed 5 mg of triamcinolone acetonide into a 10 ml volumetric flask, diluted and adjusted to volume with methanol. The final concentration of triamcinolone acetonide stock solution was 500 μg/ml.

2.2.2 Preparation of standard asiaticoside solutions

The 25 mg part of asiaticoside was accurately weighed and transferred into a 25 ml volumetric flask, diluted and adjusted to volume with methanol. This stock solution had the final concentration of asiaticoside of 1000 μg/ml.

The solutions of 50, 100, 200, 300, 400, 500 μl of asiaticoside standard stock solution, and 100 μl of internal standard stock solution were added into 5 ml volumetric flasks. The dilution to volume with mobile phase gave final concentrations of 10, 20 40, 60, 80 and 100 μg/ml of asiaticoside, respectively.

The standard solutions were freshly prepared and used for the HPLC run. As a result, the standard curve between concentration and peak area ratio was plotted.

2.3 Validation of the HPLC method

The analysis of parameters in the assay validation for the HPLC method was specificity, linearity, precision and accuracy.

2.3.1 Specificity

The specificity of the method was determined by comparing the test results from analyses of asiaticoside in each film preparation with standard solutions. Under the chromatographic conditions used, the peak of asiaticoside must be completely separated from and not be interfered by the peaks of other components in the preparation.

2.3.2 Linearity

The linearity was determined from the coefficient of determination (R^2). Six concentrations of standard asiaticoside solutions and three replicates of each

concentration were prepared and analyzed. The relation between the peak height ratios and concentrations were plotted and the least square linear regressions were calculated.

2.3.3 Accuracy

a) Accuracy of analysis of solution

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations (low, medium, high) of asiaticoside at 30, 50, 90 µg/ml were prepared and analyzed, respectively. The percentage of recovery for each concentration was calculated from the ratio of inversely estimated actual concentration multiplied by 100.

b) Accuracy of analysis of film formulation

The accuracy formulation of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations (low, medium, high) of asiaticoside at 30, 50, 90 µg/ml that spiked into selected formulation in topic F1 were prepared and analyzed, respectively. The percentage of recovery for each concentration was calculated from the ratio of inversely estimated actual concentration multiplied by 100.

2.3.4 Precision

a) Within run precision

The within run precision was determined by analyzed five sets of three concentrations of asiaticoside at 30, 50, 90 µg/ml in the same run. Peak height ratios of asiaticoside to triamcinolone acetonide were calculated and the percent of coefficient of variation (%CV) of each concentration was determined.

b) Between run precision

The between run precision was determined by analyzing three concentrations of asiaticoside at 30, 50, 90 µg/ml on five different runs. Peak height ratios of

asiaticoside to triamcinolone acetonide were calculated and the percentage of coefficient of variation for each nominal concentration was determined.

Acceptance criteria:

In order to get high accuracy, the percentage of recovery should be within 98-102% for each nominal concentration, whereas the different coefficient percentage for both within run precision and between run precision should be less than 2%.

3. Evaluation of film prepared from tamarind seed xyloglucan containing *Centella asiatica* extract

Physical appearances

Physical appearances, thickness, weight, mechanical properties and adhesive force of the film containing *Centella asiatica* extract were investigated as the same procedures mentioned in topic E2.

Thermal analysis by differential scanning calorimetry

The differential scanning calorimetric (DSC) thermogram was determined by using differential scanning calorimeter (DSC822e, Mettler Toledo, Switzerland). The samples (3-5 mg) were accurately weighed into standard aluminum pans (40 µl) and then sealed. The DSC runs were conducted over a temperature range 25-360°C at rate of 10°C/min. All tests were performed under a nitrogen atmosphere of 2 ml/min.

Powder X-ray diffractometry

Powder X-ray diffractograms were carried out by using powder X-ray diffractometer (Bruker AXS model D8 Discover, Germany) with Cu-K radiation as the source of X-rays. The measurement conditions were as follows: voltage of 40 kV, current of 40 mA, scanning speed of 0.2°/min increment the 0.02° angle range of 3-40°.

In vitro release study of films prepared from tamarind seed xyloglucan containing *Centella asiatica* extract

The *in vitro* release study was performed by using Franz diffusion cell, which consisted of donor and receiver compartments. The dialysis membrane was placed between two compartments of Franz diffusion cell. The membrane was soaked in distilled water for 24 hours, then washed by hot distilled water and soaked in isotonic phosphate buffer saline buffer pH 7.4 for an hour before use. The receiving compartment contained 14 ml of 40% ethanol in isotonic phosphate buffer saline buffer pH 7.4 which was maintained at 37±0.5°C by a circulating water jacket (Kabbovloi, 2004). The receptor fluid and membranes were equilibrated to the desired temperature for 1 hr before this study. After equilibration, the sample of film (diameter 1.8 cm) was carefully placed into the donor compartment and 200 µl of isotonic phosphate buffer saline buffer pH 7.4 was also dropped into donor compartment then covered with paraffin to prevent evaporation. The receptor fluid was continuously mixed by magnetic stirring bar throughout the time of study. A volume of 1 ml were taken from receiver medium at certain time intervals of 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 28, 32, 36, 40, 44 and 48 hours. The receptor compartment was replaced with receptor solution to keep the constant volume during the experiment. The examination was performed in triplicate. The samples were analyzed for amount of asiaticoside released from the films by HPLC method as mentioned in topic F2.

Skin permeation of films prepared from tamarind seed xyloglucan containing *Centella Asiatica* extract

Permeation experiments were performed by using Franz diffusion cell, which consisted of donor and receiver compartments. The porcine skin was placed between two compartments of Franz diffusion cell. The porcine skin was soaked in isotonic phosphate buffer saline buffer pH 7.4 for an hour before use. The receiving compartment contained 14 ml of 40% ethanol in isotonic phosphate buffer saline buffer pH 7.4 which was maintained at 37±0.5°C by a circulating water jacket. The receptor fluid and porcine skin were equilibrated to the desired temperature for 1 hr before the permeation study. After equilibration, the sample of film (diameter 1.8 cm)

was carefully placed into the donor compartment and 200 µl of isotonic phosphate buffer saline buffer pH 7.4 was also dropped into donor compartment then covered with paraffin to prevent evaporation. The receptor fluid was continuously mixed by magnetic stirring bar throughout the time of study. A volume of 5 ml were taken from receiver medium at certain time intervals of 1, 2, 4, 6, 8, 10, 12, 15, 18, 21 and 24 hours. The receptor compartment was replaced with receptor solution to keep the constant volume during the experiment. The samples were analyzed for amount of asiaticoside permeated from the film into the receptor compartment by HPLC method as mentioned in topic F2. Additionally, at the end of study, the remaining portion of the film in the donor compartment and the porcine skin were removed and analyzed for asiaticoside by the same method. The examination was performed in triplicate.

Stability of films prepared from tamarind seed xyloglucan containing *Centella asiatica* extract

The films were stored in glass vials, which were tightly sealed with rubber closure and aluminum caps at 40±2°C and 75±5 %RH for three months (Cartensen, 1990). Samples were withdrawn at 0, 1, 2, and 3 months and were analyzed for the amounts remaining of asiaticoside. The tensiometer study, differential scanning calorimetry study and powder X-ray diffraction study were also performed. The analysis of asiaticoside in film samples followed the previously described HPLC method. The examination was performed in triplicate.