CHAPTER III

MATERIALS AND METHODS

Materials

A. Chemicals

Acacia B.P.1998, lot no. PA 84651, distributed by Srichand United Dispensary

Acetic acid (glacial), 100% lot K33266463 422, Merck KGaA, Germany

Benzoic acid. distributed by Srichand United Dispensary

Caprylic/Capric triglyceride (Lexol GT 865), Numsiang Trading, Thailand

Cetyl alcohol, Numsiang Trading, Thailand

Citric acid monohydrate, 99.5-100.5%, lot no. K91334944, Merck, Germany

Cross-linked methylmethacrylate (Micropearl M305), lot no. 50425FS, Adinop, Thailand

Cyclomethicone (Silicone STV-5), Numsiang Trading, Thailand

Cyclopentasiloxane and Dimethicone cross polymer (Dow Corning® 9045), Summit Chemical Company Ltd., USA

DL-Malic acid, ≥ 99%, lot no. S4608137 636, Merck, Germany

Glycerine USP, distributed by Srichand United Dispensary

L(+)-tartaric acid, ≥ 99.5%, lot no. A730304 610, Merck, Germany

Lactic acid, lot no. 3M226124E, Carlo Erba Reagenti

Maltodextrin DE10, lot no. DQ0510015, Nutrition Ltd., Thailand

Mineral oil, distributed by Srichand United Dispensary

Phenolphthalein, lot no. F3E217, Asia Pacific Specific Chemicals Ltd., Australia

Polymethylmethacrylate (MP 2700), lot no. 511275, Adinop, Thailand

Silicon dioxide (Arosil HDK), lot no. ZB56966, Wacker Chemie AG, Germany

Sodium metabisulfite, lot no.F2K105, Asia Pacificific Specialty Chemical

Ltd., Australia

Squalane, Numsiang Trading, Thailand

Steareth 2 (Brij 72), Thai East Asiatic (Thailand) Public Company Ltd.,

Steareth 21(Brij 721), Thai East Asiatic (Thailand) Public Company Ltd.,

Stearic acid NAN-175, Numsiang Trading, Thailand

Tamarind pulp (purchased from Thaladthai, Phatumthanee, Thailand)

B. Accessories

Disposable syringe filter nylon 13mm, 0.45 μm (Chrom Tech, USA)

C. Instruments

Analytical balance (Model Ax105, Mettler Toledo, Switzerland)

Brookfield viscometer (Model LV-II+, Brookfield engineering laboratories, USA)

Centrifuge (Model himac CR 20B3, Hitachi, Japan)

Cone and plate viscometer (Model LVDV-II^{+}, Brookfield, USA)

Corneometer® (Model CM825, Courage + Khazaka electronic GmbH, Germany)

Electronic dry cabinet (Model Dry-60, JWO RUEY Dry-Cabint, Taiwan)

Homogenizer (Model D-7801 Dottingen, Ystral GmbH, Germany)

High Performance Liquid Chromatography

- Auto Injector (SIL-10A, Shimadzu, Japan)
- Comunicator bus module (CBM-10A, Shimadzu, Japan)
- Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
- UV-VIS detector (SPD-10A, Shimadzu, Japan)
- Column (Alltima C18, 5 μm, 150 × 4.6 mm, Lot no. 0611000329.
- Precolumn (μBondapack C18, 10μm, 125 A°, Water Corporation, Ireland)

Magnetic stirrer (Model Mb, Schott, Germany)

Mastersizer S (Malvern, UK)

Mexameter® (Model MX18, Courage + Khazaka electronic GmbH, Germany)

Mini orbital shaker (Model SO5, Stuart Scientific, UK)

Moisture analyzer balance (Model HB43, Mettler Toledo, Switzerland)

pH meter (Model PB20, Sartorius, UK)

Refractometer (Atago 8258, Japan)

Spray dryer (Model SD-06, Labplant, ltd., UK)

Sonicator (Elma, Germany)

Stability carbinet (Eurotherm Axyos, Germany)

Viscometer (Model R1 : 2 : H2, Shannon, Ireland)

Methods

1. PREPARATION OF TAMARIND PULP EXTRACT

1.1 Tamarind pulp extraction

The method for preparing tamarind pulp extract was modified from สุรัญญา เกียรติกำพล, 2522. One hundred gram of non-seed tamarind pulp was weighed and then 500 mL deionized water was added. The mixture was mixed until homogenous. Tamarind pulp mixture was shaked by orbital shaker at speed of 100 rpm for 60 min. Tamarind extract was separated from the mixture by using filter cloth and collected. Residual from first extraction was extracted again with 250 mL deionized water. The second and the third tamarind pulp extracts were pooled together with the first extract. The final tamarind pulp residuals that soaked with water were separated by centrifugation at 10,000 rpm for 15 min. Whole tamarind pulp extract was centrifuged at the same condition for sedimentation of the fine particles that could not be separated by filter cloth. Finally, the supernatant of tamarind pulp extract was collected and kept in refrigerator before use in further study.

1.2 Physical and chemical properties evaluation of tamarind pulp extract.

1.2.1 Density measurement of tamarind pulp extract

Density was measured by using pycnometer at ambient temperature. Empty flask was weighed. Tamarind fruit extract was placed in pycnometer and weighed again. Different weight of empty flask and flask that fulfill with sample were calculated. Density of tamarind pulp extract was compared with water as reference at the same volume. Measurements were triplicates and mean of density were calculated. Density of samples were calculated from equation as following

$$\text{Density} = \frac{\text{mass of substance}}{\text{volume of substance}} \quad \text{(1)}$$

$$\text{Density of tamarind pulp extract} = \frac{\text{mass of tamarind pulp extract} \times \text{density of water}}{\text{mass of water with same volume}} \quad \text{(2)}$$

1.2.2 Viscosity of tamarind pulp extract

Viscosity of tamarind pulp extract was determined by using viscometer (Brookfield viscometer DVII+, Scientific Industries, USA) The determination of viscosity was performed by using spindle number 1. with speed 100 rpm at ambient temperature. Viscosity measurements of sample were performed in triplicate.

1.2.3 pH of tamarind extract

The pH of tamarind pulp extract were determined by using pH meter. pH measurements of sample were performed in triplicate.

1.2.4 Titratable acidity of tamarind pulp extract

Titratable acidity was studied as follow by AOAC official method of analysis (Horwitz, 2000; James, 1995). Ten grams of tamarind pulp extract were accurately weighed and diluted with carbon dioxide-free water. Added 0.3 mL

phenolphthalein TS into each 100 mL solution being titrated. Titrated to just before end point with 0.1 N Sodium hydroxide, transfer 2 mL of solution into 20 mL carbon dioxide-free water in a small beaker. In this extra dilution, the colors of tamarind pulp extract was so pale that phenolphthalein color was easy to be observed. If the test showed that end point is not reached, pour extra diluted portion back into the original solution, more alkali was added, and titration was continued into faint pink end point. Titratable acidity of tamarind pulp extract could be expressed as milliliters of 0.1 N sodium hydroxide per 100 mL of tamarind pulp extract.

1.2.5 Determination of soluble solid in tamarind pulp extract

The soluble solid of tamarind pulp extract was determined as followed by AOAC official method of analysis using refractometry method (Horwitz, 2000; Bayram et al., 2005). Two drops of sample were place over the measuring prism of refractometer (Atago 8258, Japan). The scale of refractometer was read. Line of shadow indicated value of degree brix as show in fugure 6. Degree brix was the percentage of solid matter; or the total solid, which was dissolved in a liquid. Degree brix could be described as the weight of solid sample dissolved in 100 grams solution.

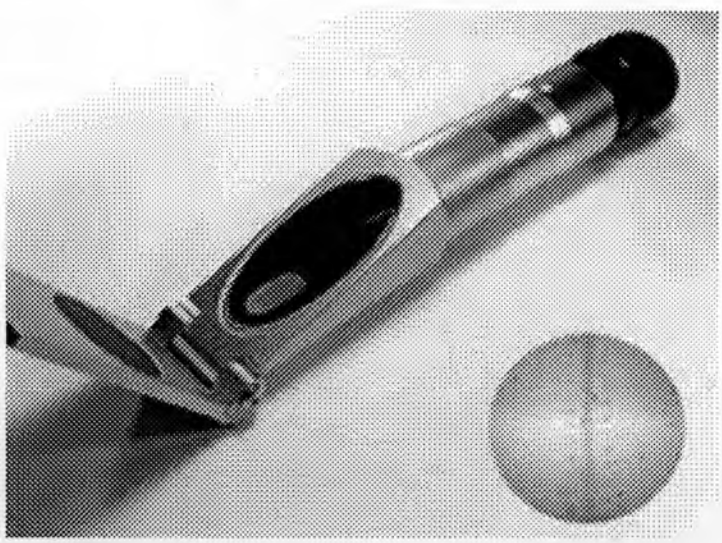

Figure 6 Refractometer

1.2.6 Determination of tartaric acid in tamarind pulp extract

Tartaric acid was determined by high performance liquid chromatography method. One gram of tamarind pulp extract was accurately weighed and put into 10 mL volumetric flask, diluted and adjusted to volume with mobile phase. Acetic acid was used as internal standard. One mL sample solution was transferred into 10 mL volumetric flask, 1 mL of internal standard stock solution, was added and mixed well, diluted and adjusted to volume with mobile phase. Solutions were filterred through 0.45 μm membrane filter before analysis, and then injected into HPLC column. The peak area ratio of tartaric acid and acetic acid was calculated and concentration of tartaric acid was determined from standard curve.

2. DETERMINATION OF TARTARIC ACID BY HPLC METHOD

In this study, HPLC method was used for determining tartaric acid in tamarind pulp extract, spray-dried tamarind pulp extract and oil-in-water emulsion containing spray-dried tamarind pulp extract. The condition for quantitative analysis of tartaric acid was modified from Nicoletti (1999).

Chromatographic condition

The HPLC condition for analysis of tartaric acid was as follows:

Column	: Alltima C18 5μm, 150 mm
Mobile Phase	: 0.05 mM KH_2PO_4 pH 2.5
Injection volume	: 20 μl
Flow rate	: 0.5 mL/min
Detector	: UV detector 210 nm
Temperature	: ambient
Run time	: 11 min
Internal standard	: Acetic acid

The mobile phase was prepared by using phosphate buffer, 0.05 mM potassium dihydrogen phosphate adjust to pH 2.50 with phosphoric acid. The

solutions was filtered through a 0.45 μm nylon membrane filter and degassed by sonicator for 30 min prior to use.

2.1 Standard solutions for HPLC method

From preliminary study, acetic acid was chosen to be internal standard. A stock solution of internal standard was prepared by accurately weighing of 240 mg of acetic acid into a 100 mL volumetric flask. Mobile phase was added to dilute the internal standard and the solution was adjusted to final volume by mobile phase. The stock solution of acetic acid had final concentration 2.4 mg/mL

A stock solution of tartaric acid was prepared by accurately weighing 100 mg of tartaric acid into 100 mL volumetric flask. Mobile phase was used to dilute tartaric acid and the solution was adjusted to the final volume by mobile phase. This stock solution of tartaric acid had final concentration 1 mg/mL.

Standard solutions of tartaric acid were prepared by pipetting 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL of tartaric acid stock solution into 10 mL volumetric flasks, respectively. Then 1 mL of acetic acid stock solution was added in to each of these volumetric flasks. The solutions were adjusted to volume with mobile phase. The concentration of tartaric acid in standard solutions were 50, 100, 150, 200, 250 and 300 μg/mL, respectively, and acetic acid was 240 μg/mL.

As a result, the standard curve of tartaric acid between concentration and peak area ratio was plotted

2.2 Validation of HPLC method

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

2.2.1 Specificity

Under the chromatographic conditions used, the peak of tartaric acid must be completely separated from and was not interfered by the peak of other components in the sample.

2.2.2 Linearity

Three sets of six standard solutions were prepared and analyzed. Linear regression analysis of the peak area ratios versus their concentrations was performed. The linearity was determined from the coefficient of determination (R^2).

2.2.3 Accuracy

The accuracy of an analytical method is the closeness of test result obtained by that method to the true value. The accuracy of method was determined from the percentage of recovery. Five sets of three concentrations at 75, 175 and 275 µg/mL were prepared and analyzed. The percentage of recovery of each concentration was calculated from the ratio of estimated concentration to known concentration multiply by 100. The first one was diluted in mobile phase without any diluents. The other one was also in mobile phase with all diluents in formation of oil-in-water emulsion. Both accuracy test fix the concentration of tartaric acid at 75, 175 and 275 µg/mL, and all of processing accuracy test were operated similarly for both conditions.

2.2.4 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

a. Within run precision

The within run precision was determined by analyzing five sets of three concentrations at 75, 175 and 275 µg/mL in the same day. Peak area ratios of tartaric acid to acetic acid were calculated and the percent coefficient of variation (%CV) of each concentration was determined.

b. Between run precision

The between run precision was determined by analyzing five sets of three concentrations at 75, 175 and 275 μg/mL on five different days. The percent coefficient of variation (% CV) of each concentration was determined.

Acceptance criteria:

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

2.3 Determination of tartaric acid in tamarind pulp extract, spray-dried tamarind pulp extract and oil-in-water emulsions containing spray-dried tamarind pulp extract.

2.3.1 Determination of tartaric acid in tamarind pulp extract

Tartaric acid was determined by high performance liquid chromatography method. One gram of tamarind pulp extract was accurately weighed and put into 10 mL volumetric flask, diluted and adjusted to volume with mobile phase. Acetic acid was used as internal standard. One mL sample solution was transferred into 10 mL volumetric flask, 1 mL of internal standard stock solution, was added and mixed well, diluted and adjusted to volume with mobile phase. Solutions were filterred through 0.45 μm membrane filter before analysis, and then injected into HPLC column. The peak area ratio of tartaric acid and acetic acid was calculated and concentration of tartaric acid was determined from standard curve.

2.3.2 Determination of tartaric acid in spray-dried tamarind pulp extract

Fifty milligrams of spray-dried tamarind pulp extract was accurately weighed into 10 mL volumetric flask; and diluted with mobile phase. The mixture was shaking and sonicated for 30 min for dissolving spray-dried pulp extract until clear solution was received, solution was adjusted to volume with mobile phase. Two milliliters of sample solution was transferred into 10 mL volumetric flask. One milliliter of internal standard stock solution was mixed well then diluted and adjusted

to volume with mobile phase. Solutions were filtered through 0.45 μm membrane filter before analysis, and then injected into HPLC column. The peak area ratio of tartaric acid and acetic acid was calculated and concentration of tartaric acid was determined from the standard curve.

2.3.3 Determination of tartaric acid in oil-in-water emulsion containing tamarind pulp extract

One thousand and six hundred milligrams of an oil-in-water emulsion containing spray-dried tamarind pulp extract was accurately weighed into 10 mL volumetric flask, diluted and adjusted to volume with mobile phase. The mixture was shaken and then sonicated for 30 min. One milliliter of sample solution was transfered into 10 mL volumetric flask, 1 mL of internal standard stock solution was added, mix well and then diluted and adjusted to volume with mobile phase. The mixture was filtered through 0.45 μm membrane filter before analysis, and then injected into HPLC column. The peak area ratio of tartaric acid and acetic acid was calculated and concentration of tartaric acid was determined from the standard curve.

3. PREPARATION OF SPRAY-DRIED TAMARIND PULP EXTRACT

3.1 Preparation of tamarind pulp extract-carrier solution

3.1.1 Preparation of tamarind pulp extract and maltodextrin

Maltodextrin was dispersed in tamarind pulp extract until homogeneous with clear mixture for spray-dried in Mini Spray-drier (model SD06, Labplant Ltd., UK). Formulation that used silicon dioxide was prepared by adding silicon dioxide in mixture and completely dispersed before spray-drying and stirring with magnetic stirrer along the process of spray-drying.

3.1.2 Preparation of tamarind pulp extract and acacia

Acacia was dispersed in tamarind pulp extract until homogeneous with clear mixture for spray-dried in Mini Spray-drier. Formulation that used silicon dioxide was prepared by adding silicon dioxide in mixture and completely dispersed before spray-drying and stirring with magnetic stirrer along the process of spray-drying.

3.2 Preparation of spray-dried tamarind pulp extract

Factors influenced on properties of spray-dried tamarind pulp extract were studied. Experimental design was 2^5 factorial designs with five factors and each factor has 2 levels. The factors of this experimental were as following.

Factor 1: Type of spray-dried carrier has 2 levels, acacia and maltodextrin.

Factor 2: Concentration of spray-dried carrier has 2 levels, 5% and 10% by weight.

Factor 3: Concentration of silicon dioxide has 2 levels, 0% and 1% by weight.

Factor 4: Inlet temperature has 2 levels, 110 °C and 130 °C.

Factor 5: Fan setting has 2 levels, 30 and 50

Factors effect on properties of spray-dried tamarind pulp extract result on percent yield and percent moisture content of spray-dried pulp extract. The 2^5 factorial designs are shown in table 4.

Method of preparation of tamarind pulp extract using spray-drying technique was studied by varying factors that affected on properties of spray-dried powders i.e. type and concentration of carrier and condition of spray- drier. Carriers used in this experiment were acacia and maltodextrin with 2 variable concentration, 5 and 10 percent by weight, with or without silicon dioxide concentration of 1 percent by weight. Mixture of tamarind pulp extract and carrier were spray dried in condition of inlet air temperature 110 °C or 130 °C and flow rates were adjusted with fan setting 30 and 50, feed rate was adjusted to level 3 (3 mL/min). The particles were collected

from the collecting chamber and cyclone of spray-drier. These spray-dried tamarind pulp extract were filled in air-tight amber glass bottle, and then sealed with parafilm and stored in desicator to prevent moisture absorption prior to further studies.

Criteria to selected spray-dried tamarind pulp extract for formulation of oil in water emultions containing spray-dried tamarind pulp extract are as follows.

1. High percent yield and most are in collecting chamber.
2. Low percent moisture content and loosely aggregation when keeping spray-dried tamarind pulp extract in desicator for 1 week.
3. Low percent carrier added in formulation.

Table 4. The 2^5 factorial designs for studying factors affected on properties of spray-dried tamarind pulp extract.

Experiment	Type of carrier	Concentration of carrier (% w/w)	Concentration of silicon dioxide (% w/w)	Inlet temperature (°C)	Fan setting
1	Maltodextrin	10 %	1 %	130	50
2	Maltodextrin	10 %	1 %	130	30
3	Maltodextrin	10 %	1 %	110	50
4	Maltodextrin	10 %	1 %	110	30
5	Maltodextrin	10 %	0 %	130	50
6	Maltodextrin	10 %	0 %	130	30
7	Maltodextrin	10 %	0 %	110	50
8	Maltodextrin	10 %	0 %	110	30
9	Maltodextrin	5 %	1 %	130	50
10	Maltodextrin	5 %	1 %	130	30
11	Maltodextrin	5 %	1 %	110	50
12	Maltodextrin	5 %	1 %	110	30
13	Maltodextrin	5 %	0 %	130	50
14	Maltodextrin	5 %	0 %	130	30
15	Maltodextrin	5 %	0 %	110	50
16	Maltodextrin	5 %	0 %	110	30
17	Acacia	10 %	1 %	130	50
18	Acacia	10 %	1 %	130	30
19	Acacia	10 %	1 %	110	50
20	Acacia	10 %	1 %	110	30
21	Acacia	10 %	0 %	130	50
22	Acacia	10 %	0 %	130	30
23	Acacia	10 %	0 %	110	50
24	Acacia	10 %	0 %	110	30
25	Acacia	5 %	1 %	130	50
26	Acacia	5 %	1 %	130	30
27	Acacia	5 %	1 %	110	50
28	Acacia	5 %	1 %	110	30
29	Acacia	5 %	0 %	130	50
30	Acacia	5 %	0 %	130	30
31	Acacia	5 %	0 %	110	50
32	Acacia	5 %	0 %	110	30

3.3 Characterization of spray-dried tamarind pulp extract

3.3.1 Percent yield of spray-dried tamarind pulp extract

Spray-dried tamarind pulp extracts from collecting chamber and cyclone of spray-drier were collected and weighed. Percent yield of spray-dried tamarind pulp extract was determined from this equation

$$\% \text{ Yield} = \frac{\text{weight of spray-dried tamarind pulp extract} \times 100}{\text{weight of total solid in mixture before spray-drying}} \quad (3)$$

Where,

Total solid in mixture = weight of soluble solid in tamarind pulp extract + weight of carrier

3.3.2 Aggregation test of spray-dried tamarind pulp extract

Aggregation test of spray-dried tamarind pulp extract was observed suddenly after spray-drying of each sample. Then all of spray-dried tamarind pulp extract were kept in desicator for 1 week, after that aggregation test was established for each sample. The glass bottles were tapped for 5 times, turned the bottle up to down. The sticky particles were observed and compared between each condition of spray-drying.

3.3.3 Morphology

Morphology of spray-dried tamarind pulp extract was observed by scanning electron microscope (SEM). The samples were coated with gold by ion sputtering under a high vacuum and high voltage. The coated samples were examined under SEM.

3.3.4 Particle sizes and size distribution

The particle sizes and size distribution were measured using a laser light scattering. The spray-dried tamarind pulp extract were uniformly dispersed in liquid paraffin prior to measurement.

3.3.5 Percent moisture content

Percent moisture content of spray-dried tamarind pulp extracts were evaluated using moisture analyzer balance (Mettler Toledo, model HB 43) with thermogravimetric method. Moisture determinations were carried out on 0.5 gram of spray-dried tamarind pulp extract in pan, which was dried at 105 °C until it reached the constant weight. Percent moisture content of sample was performed in triplicate.

3.3.5 Content of tartaric acid in spray-dried tamarind pulp extract

Tartaric acid was determined by high performance liquid chromatography method. Fifty milligrams of spray-dried tamarind pulp extract were accurately weighed into 10 mL volumetric flask, diluted and adjusted to volume with mobile phase. The mixture was shaken and sonicated for 30 min for dissolving spray-dried tamarind pulp extract until clear solution was received. Solution was adjusted to volumn with mobile phase. One milliliter of sample solution was transferred into 10 mL volumetric flask, added 1 mL internal standard stock solution, mixed well and then diluted and adjusted to volume with mobile phase. Solutions were filtered through a 0.45 μm membrane filter before analysis, and then injected into HPLC column. The peak area ratio of tartaric acid and acetic acid was calculated and concentration of tartaric acid was determined from the calibration curve.

3.3.6 Stability of spray-dried tamarind pulp extract

Stability of spray-dried tamarind pulp extract were studied in accelerated condition. They were kept in tight, clear and light-resistant glass bottles at temperature of 40 ± 2 °C and relative humidity 75 ± 5 % for 6 months. Samples were sampling at time 0, 1, 2, 3 and 6 month. Percent remaining tartaric acid in samples were determined by HPLC and percent moisture contents were measured with moisture analyzer balance (European medicines agency, 2007).

4. FORMULATION OF OIL-IN-WATER EMULSIONS CONTAINING SPRAY-DRIED TAMARIND PULP EXTRACT

4.1 Formulation of oil-in-water emulsions

In this study, non-ionic emulsifier was selected. Compositions of oil-in-water emulsion are shown in table 5. These emulsions were used as creams base for spray-dried tamarind pulp extract.

Beaker method was used to prepare oil-in-water emulsion. The ingredients were divided into 2 separated phase, oil and water phase. Steric acid, cetyl alcohol, steareth 2, steareth 21, squalane, caprylic/capric triglyceride, rice bran oil, cyclomethicone and cyclopentasiloxane and dimethicone crosspolymer were melted, respectively in oil phase. Oil phase was heated individually to 75 °C. Xantan Gum, polymethylmethacrylate and cross-linked methylmethacrylate were dispersed in water before mix in water phase. Water phase was heated and controlled at 78 °C in water phase. The oil phase was poured into water phase. Homogenizer was used with speed level 5 for few minutes and stirred until congeal.

Criteria for oil-in-water emulsion selection

1. Smooth texture, non greasy when applied
2. Stable, no separation in room temperature
3. Good spreadability
4. Pass heating-cooling cycle from accelerated stability test temperature

Table 5 Formulation of oil-in-water emulsion (cream base)

Formulation	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Oil phase (percentage by weight)																				
Cyclomethicone	7	7	7	6	6	6	6	6	6	4	4	4	4	4	4	4	4	4	4	4
Steric acid	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Squalane	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Caprylic/capric triglyceride	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1
Rice bran oil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Cetyl alcohol	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Steareth 2	4	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2.8	2.6	3
Steareth 21	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Cyclopentasiloxane and Dimethicone crosspolymer	-	-	-	-	-	-	-	-	-	2	2	2	2	2	2	2	3	2.2	2.4	2
Water phase (percentage by weight)																				
Xantan Gum	0.1	0.1	0.1	0.15	0.2	0.1	-	-	-	0.2	0.1	0.1	0.1	-	-	-	-	-	-	-
Polymethylmethacrylate	-	-	-	-	-	-	1	-	0.5	-	1	-	0.5	1	-	0.5	-	1	1	1
Cross-linked methylmethacrylate	-	-	-	-	-	-	-	1	0.5	-	-	1	0.5	-	1	0.5	-	-	-	-
Glycerol	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Paraben concentrate	0.5	0.5	0.5	0.5	0.5	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzoic acid	-	-	-	-	-	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sodium metabisulfite	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	0.5	0.5	0.5	0.5	0.5
Water q.s. to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

4.2 Formulations of oil-in-water emulsions containing spray-dried tamarind pulp extract

The oil-in-water emulsion containing spray-dried tamarind pulp extract was formulated by using emulsion base selected from 4.1. Spray-dried tamarind pulp extract was used as active ingredient in this study with concentration of 8% by weight. Spray-dried tamarind pulp extract and emulsion base was mixed using geometric dilution on cleaned slab and spatula.

4.3 Accelerated stability testing

Emulsions was kept at temperature 4 °C for 24 h and temperature 45 °C for 24 h this was 1 cycle then repeated for 6 cycles (Carstensen, 1995). The characteristics of emulsion were examined as following.

A. Appearance and phase separation of emulsion.

Appearance and phase separation of emulsion were observed by eye inspection.

B. Skin feeling

Skin feeling, smooth texture, non-greasy and good spreadability when applied on skin, were evaluated by sense of touch.

C. Odor of emulsion

Odor of emulsion was investigated by smelling.

D. Color of emulsion

Color of emulsion was evaluated by eye inspection.

E. pH of emulsion

pH of emulsion was determined by pH meter (Satorius, UK).

F. Viscosity of emulsion

Viscosity of emulsion was determined by cone and plate viscometer (Model LVDV-II^{+}, Brookfield, USA).

4.4 Stability study of oil-in-water emulsions containing spray-dried tamarind pulp extract

For the reason of this experiment, to confirm the stability of oil-in-water emulsion base and oil-in water emulsion containing 8% w/w spray-dried pulp extract that used in volunteers. The period of this study was 6 weeks and all of volunteer were suggested to keep both emulsions in ambient temperature, so this is the reason for established stabilities of both emulsion for 6 weeks at ambient temperature.

In this study, stability of oil-in-water emulsions containing spray-dried tamarind pulp extract and emulsion base were evaluated in short term. Emulsions was kept in the same type of container as that used in volunteers. They were kept at ambient temperature for 6 weeks. Appearance, physical properties and chemical properties of oil-in-water emulsion were investigated every 2 weeks.

4.4.1 Physical stability of emulsions

The characteristics of emulsions were examined.

A. Appearance and phase separation of emulsions.

Appearance and phase separation of emulsions were observed by eye inspection.

B. Skin feeling

Skin feeling, smooth texture, non-greasy, and good spreadability when applied on skin, were evaluated by sense of touch.

C. Odor of emulsions

Odor of emulsion was investigated by smelling.

D. Color of emulsions

Color of emulsions was evaluated by eye inspection.

E. pH of emulsions

pH of emulsions was determined by pH meter (Satorius, UK).

F. Viscosity of emulsions

Viscosity of emulsions was determined by viscometer (Model R1 : 2 : H2, Shannon, Ireland).

4.4.2 Chemical stability of emulsion

Oil-in-water emulsions containing spray-dried tamarind pulp extract was studied at ambient temperature for 6 weeks. Percent remaining of tartaric acid in emulsion was determined by HPLC technique.

5. STUDY OF WHITENING OF EFFICACY TEST IN VOLUNTEERS

Oil-in-water emulsions containing spray-dried tamarind pulp extract and emulsion base were used in this study. Whitening efficacy test was evaluated in 35 volunteers.

5.1 Subject selection

Inclusion criteria

- Healthy female volunteers
- Ages in range 20 – 45 years
- All of them given signature in informed consents before participate in this study
- No history to allergy of chemical or cosmetic substances
- No scar or blemish in studied area.

Exclusion criteria

- Allergy to chemical or cosmetic sunstances
- History of skin disease at studied region
- Use medicine or cosmetic that might effected the results in studied area
- Do not perform to follow condition between participated

5.2 Study design

The protocol of efficacy test was approved by The Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Thirty five healthy volunteers participated with single blind study. Participants were half test in

forehead area. Each sited of forehead was assigned to apply oil-in-water emulsions containing spray-dried tamarind pulp extract or emulsions base. In this study, they were allowed to leave from this experiment anytime.

5.3 Product use

Prior to study, the participants was stopped to use any medicine or cosmetic such as lightening product or moisturizer for one week. They had to visit for evaluation moisture and skin color as base line for two times. This studied results were compared with their base line. Periods of this study were 8 weeks. In first 2 weeks, base line of each region was measured, left and right of forehead. Afterthat they were assigned to use emulsions for 6 weeks. Five hundred milligrams of emulsions were applied everyday in each area, two times per day in morning and evening after shower. All participants were recommended to avoid from sunlight and stop to use medication or cosmetics that might interfere skin color and skin moisture.

5.4 Skin measurement

Skins were measured after application of emulsion every weeks for 3 weeks. In this study, skin moisture and skin color were measured.

5.4.1 Skin color measurement

Skin color was measured at each site of forehead with Mexameter® MX18. Plastic template was used to remark the area of measurement at same area every week. Forehead area had diameter 1 inch. Probe of Mexameter® MX18 was placed on skin in three times at fix area. The value was calculated and statistic was use for analysis.

5.4.2 Skin moisture measurement

Skin miosture was measured with Corneometer® at the same area of skin color at forehead. Probe of Corneometer® was placed on skin in triplicate at fix area. The value was calculated and statistic was used for analysis.