CHAPTER III

EXPERIMENTAL

MATERIALS

Active Ingredients :

- Asiaticoside Extract, Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

- Mangostin Extract, Department of Microbiology, Faculty of Sciences, Prince Songklanakarin University, Songkla, Thailand.

Standard Material :

- Asiaticoside (Batch No. 051209, Jieliang extract Co., Ltd., Shijiazhuang, China)

Formulation Diluents:

- Chitosan M.W. 83,000 Degree of deacetylation 90% (Seafresh Chitosan (lab) Co., Ltd., Bangkok, Thailand)

- Chitosan M.W. 227,000 Degree of deacetylation 85% (Seafresh Chitosan (lab) Co., Ltd., Bangkok, Thailand)

- Pectin (Batch No. 20422602/1, Degussa Texturant System, France)

- Sodium Carboxy Methyl Cellulose (Batch No. 10195289, Maxway Co., Ltd., Bangkok, Thailand)

- Gelatin (Batch No. 3123507, Asia Pacific Africa Co., Ltd., Christchurch, New Zealand)

- Xanthan Gum (Batch No. 3J1992A, Colloides Naturals International Co., Ltd., Rouen, France)
- Polyethylene Low Molecular Weight (Batch No. unknown, Distributed by SVK Pharmaceutical Industry Co., Ltd., Bangkok, Thailand)
- Polyethylene Glycol 6000 (Srichand United Dispensary Co.,Ltd., Bangkok, Thailand)
- Polypropylene (Batch No. unknown, Thai Petrochemical Industry Public Co.,Ltd., Bangkok, Thailand)
- Liquid Paraffin (Batch No. 502235, Srichand United Dispensary Co.,Ltd., Bangkok, Thailand)

Miscellaneous:

- L-Glutamic acid (Batch No. AF411031, Ajax Finechem, New Zealand)
- Dibasic Potassium Phosphate (Batch No. FOB063, APS Finechem, Australia)
- Orthophosphoric acid 85% (Batch No. A1B021, Asia Pacific Specialty Chemicals Limited, Australia)
- Potassium Nitrate (Batch No. unknown, Ajax Finechem, New Zealand)
- Sodium Chloride (Batch No. unknown, APS Finechem, Australia)
- Magnesium Carbonate (Batch No. 63079, Fluka chemical, Germany)
- Potassium Acetrate (Batch No. unknown, Ajax Finechem, New Zealand)
- Hydrochloric acid 37% (Batch No.unknown, Labscan Co., Ltd., Bangkok, Thailand)
- Methanol HPLC Grade (Batch No. 0579328, Fisher Scientific UK Limited, United Kingdom)
- Acetronitrile HPLC Grade (Batch No. 0577413, Fisher Scientific UK Limited, United Kingdom)
- Aluminum Collapsible Tubes (APA Industries Co., Ltd., Bangkok, Thailand)

Equipments

1. Viscosity Machine (Model RV1 Haake®, Karlsruhe, Germany)
2. Differential Scanning Calorimetry (DSC) (Mettler® DSC 822^e, Schwerzenbach, Switzerland)
3. Spray-dryer (Model Hi-tec, Niro atomizer®, Copenhagen, Denmark)
4. Fourier Transform Infrared Spectroscopy (FT-IR) (Model 1760X, Perkin Elmer, UK)
5. Tensile Strength Machine (Lloyd® LR10K, Hampshire, England)
6. Ointment mill (Model AR400S, Erweka®, Heusenstamm, Germany)
7. Analytical Balance (Model A200 S, Sartorius, Germany)
8. Fluorescen 18 watt, 1030 lm (Phillips Electronics, Thailand)
9. Peristaltic Pump (Model 505S, Watson-Marlow Limited, Falmouth, England)
10. pH meter (Model 210A +, Thermo Orion, Germany)
11. High Performance Liquid Chromatography (HPLC) (Model SLC-10AVP, Shimadzu, Japan)
    - Degasser (Model DGU-14A, Shimadzu, Japan)
    - Pump A, B Liquid Chromatography (Model LC-10AD, Shimadzu, Japan)
    - Auto injector (Model SIL-10A, Shimadzu, Japan)
    - Column oven (Model CTD-10AS, Shimadzu, Japan)
    - UV-VIS Detector (Model SPD-10A, Shimadzu, Japan)
    - System Controller (Model SCT-10A, Shimadzu, Japan)

METHODS

The experimental methods were performed in 7 parts as following:

1. Polymers Selection for Hydrophobic Base

1.1 Formulation of Hydrophobic Base

Polymers that were used in this experiment are consist of polyethylene, polypropylene and polyethylene glycol (PEG) 6000, respectively. Hydrophobic bases were prepared by melting an appropriate amounts of each polymer with mineral oil at 80-90°C until homogeneous mixture was obtained. Then, the temperature of homogeneous liquids were cool down automatically by using paddle which velocity rate was controlled until the temperature of these hydrophobic bases decrease to approximately 40°C.

After the homogeneous hydrophobic bases were occurred, the physical appearance of each hydrophobic base was observed in order to find the suitable amount of polymers that compatible and give good appeal with mineral oil. The amount of suitable polymer was divided to 4 levels; 1.5%, 3.0%, 4.5% and 6.0%, respectively.

1.2 An Evaluation of Hydrophobic Base

Rheology of hydrophobic bases were studied by using viscosity tester (Haake® RV1). The sample was subjected to shear rates of 0 to 1000 to 0 sec^{-1} in 240 second shear cycle. Mean value of triplicate samples were derived.

The 4 levels of hydrophobic base were evaluated in various parameters such as viscosity at ambient temperature (~ 28° C), relationship between percentage of polymers to viscosity, effect of temperature to viscosity, activation energy of hydrophobic base by using the viscosity modification of the Arrhenius 's equation and rheology of hydrophobic base.

The compatibilities between polymer and mineral oil were investigated by Differential Scanning Calorimetry (DSC) analyzer.

In addition, the hydrophobic bases, packed in close glass containers, were led to physical appearance testing. For one freeze-thaw cycle, samples were kept at 4 ° C (in refrigerator) for 48 hours and then continue at 45 ° C (in hot air oven) for another 48 hours total of 5 cycles and hydrophobic base after treated at -20 ° C were employed to determine the physical stability of these hydrophobic bases.

2. Selection of Gelling Agent

Gelatin, xanthan gum, pectin, sodium carboxy methyl cellulose and chitosan salt at various molecular weights were used in this experiment.

2.1 Preparation and Evaluation of Chitosan Salts

Chitosan M.W. 83,000 and chitosan M.W. 227,000 were used to form chitosan salts. Six grams of chitosan (0.0373 moles of glucosamine) were dissolved in 800 ml water containing different acid (hydrochloric acid or glutamic acid) in molar ratio of 1:1 mole monomers: mole acid. The solutions were stirred constantly for 10 hours and filtered before use. The pH values were check to confirm neutralization and complete reaction between the two. Then solutions were spray-dried by Niro atomizer spray – dryer with an inlet temperature 100 ° C and feed rate of solutions were strickly controlled. The various products of chitosan salt were collected within the collector.

Chitosan salts obtained from spray – dried process were prepared to measure pH and viscosity by adding water to form solutions. The solutions consisted of 0.5%, 1.0%, 2.0% and 3.0% of chitosan salts were brought to evaluated pH and viscosity values.

2.2 Characterization of Chitosan Salts by Fourier Transform Infared Spectrometry (FT-IR)

Infrared (IR) spectra of spray – dry products were recorded with a Perkin Elmer FT-IR spectrometer. The samples were prepared by processing compressed KBr discs.

2.3 Evaluation of Gelling Agent in hydrophobic base

Gelling agents mixed with hydrophobic base were evaluated for their properties in the following three topics.

2.3.1 Moisture Adsorption Study

Hydrophobic bases 80% by weight were mixed with 20% of each gelling agent and divided 1 gram of mixtures placing on glass slide which stored in desiccator. The moisture content was measured gravimetrically after sample had been stored at ambient temperature over saturated salt solutions of Potassium Nitrate (KNO_3), Sodium Chloride (NaCl), Magnesium Carbonate ($Mg(NO_3)_2$) and Potassium Acetate (CH_3COOK) which corresponding to 96, 75, 52 and 25% relative humidity (RH), respectively for at least 240 hours.

2.3.1.1. Saturated Salt Solutions of potassium nitrate (KNO_3)

The saturated salt solutions of potassium nitrate were prepared by weighing 260 grams of KNO_3 dissolved in 650 ml water and then the saturated salt solutions were placed in desiccator at ambient temperature. Precipitate of the salt should be appeared in order to maintain constant relative humidity through out the experiment.

2.3.1.2. Saturated Salt Solutions of sodium chloride (NaCl)

The saturated salt solutions of sodium chloride were prepared by weighing 260 grams of NaCl dissolved in 650 ml water and then the saturated salt solutions were placed in desiccator at ambient temperature. Precipitate of the salt should be appeared in order to maintain constant relative humidity through out the experiment.

2.3.1.3. Saturated Salt Solutions of magnesium carbonate ($Mg(NO_3)_2$)

The saturated salt solutions of magnesium carbonate were prepared by weighing 675 grams of $Mg(NO_3)_2$ dissolved in 500 ml water and then the saturated salt solutions were placed in desiccator at ambient temperature. Precipitate of the salt should be appeared in order to maintain constant relative humidity through out the experiment.

2.3.1.4. Saturated Salt Solutions of potassium acetate (CH_3COOK)

The saturated salt solutions of potassium acetate were prepared by weighing 950 grams of CH_3COOK dissolved in 450 ml water and then the saturated salt solutions were placed in desiccator at ambient temperature. Precipitate of the salt should be appeared in order to maintain constant relative humidity through out the experiment.

2.3.2 Tensile Strength Measurement

Gelling agents used in this experiment were divided to four levels; 10%, 20%, 30% and 40%, respectively. Every levels of gelling agent was mixed with hydrophobic base in a mortar until the homogeneity was obtained. The 2 grams of mixtures of gelling agents and hydrophobic base were moisten with 1 ml water and then the mixtures were laid on special apparatus that connected with the stress-strain tester. The tester fitted with 5kN load-detecting transducer. Loads and strain data were collected and converted to tensile strength.

2.3.3 Swelling Index Test

The study of the swelling of gelling agents with respect to time were done by modifying to Ph .Eur. method 2.8.4 in the following way : 0.4 gram of gelling agents were placed in a 10 ml graduated cylinder , adding 16 drops of ethanol 95 % and 10 ml of water and recording the volume at time zero . After this the volume occupied by gelling agents were recorded. Shake vigorously every 10 minutes for 1 hour and then allow to stand for 3 hours. At 1.5 hours after the beginning of the test, release any large volumes of liquid retained in the layer of gelling agents and any particles of the gelling agents floating at the surface of liquid by rotating the cylinder about a vertical axis. Measure the volume occupied by the gelling agents, including any adhering mucilage. Carry out three tests at the same time. Calculate the swelling index from the mean of the three tests.

2.3.4 Compatibility Study by Differential Scanning Calorimetry (DSC)

In the case of using the combination of gelling agents together in the hydrophobic base. Then, the compatibility of gelling agents and hydrophobic base were investigated by differential scanning calorimetry.

3. Determination of Active Ingredients by High Performance Liquid Chromatography Method (HPLC)

Mangostin and asiaticoside were active ingredients used in this study and were analyzed by HPLC method.

3.1 Mangostin

HPLC chromatographic conditions:

Column : Hypersil® gold (C18) column (150 X 4.6 mm), 5 μm (Thermohypersil, UK) equipped with guard column packed with (C18), 5 μm set at an ambient temperature.

Detector	:	UV detector at 244 nm.
Injection volume	:	20 µl
Flow rate	:	1.4 ml/min
Mobile phase	:	methanol: water, 80: 20

Mobile phase was filtrated through a membrane filter with a pore size of 0.45 µm and degassed for at least 30 minute prior to use.

Validation of HPLC Method

The typical analytical parameters to be considered for assay validation are specificity, linearity and precision.

Specificity

The specificity of the active constituent peak was determined by the peak resolution and tailing factor. The well resolving from the other peaks and symmetry of the peaks should be obtained.

The standard solution of mangostin in methanol at the concentration of 20 µg/ml was prepared and evaluated by using chromatographic conditions as described above.

Linearity

Triplicate injection of solutions containing drug in various concentrations ranging from 5 to 60 µg/ml of each reference standard in methanol were prepared and analyzed. The linear equation of the curve obtained by plotting the peak area at each level prepared as a function of the concentrations of each standard was calculated using the linear least square method.

Precision

The standard preparation was stepwise diluted with methanol to obtain the final concentration of 20 μg/ml. Six replicated injections of this standard solution was analyzed . Percentages of coefficient of variation (%CV) were calculated for determination of the precision.

3.2 Asiaticoside

HPLC chromatographic conditions:

Column : Hypersil® gold (C18) column (150 X 4.6 mm), 5 μm (Thermohypersil, UK) equipped with guard column packed with (C18), 5 μm set at an ambient temperature.

Detector : UV detector at 210 nm.

Injection volume : 20 μl

Flow rate : 1 ml/min

Mobile phase : Acetronitrile: Phosphate buffer (10Mm K_2HPO_4) pH 7.1 = 29: 71

Mobile phase was filtrated through a membrane filter with a pore size of 0.45 μm and degassed for at least 30 minute prior to use.

Validation of HPLC Method

The typical analytical parameters to be considered for assay validation are specificity, linearity and precision.

Specificity

The specificity of the active constituent peak was determined by the peak resolution and tailing factor. The well resolving from the other peaks and symmetry of the peaks should be obtained.

The standard solution of asiaticoside in methanol at the concentration 200 µg/ml was prepared and evaluated by using chromatographic conditions as described above.

Linearity

Triplicate injection of solutions containing drug in various concentrations ranging from 50 to 600 µg/ml of each reference standard in methanol were prepared and analyzed. The linear equation of the curve obtained by plotting the peak area at each level prepared as a function of the concentrations of each standard was calculated using the least linear square method.

Precision

The standard preparation was stepwise diluted with methanol to obtain the final concentration of 200 µg/ml. Six replicated injections of this standard solution was analyzed . Percentages of coefficient of variation (%CV) were calculated for determination of the precision.

4. Formulation of Herbal Extracts Oral Paste

Hydrophobic base and appropriate gelling agents that were selected in the previous experiment were formulated to oral paste by traditional method in a mortar. The amounts of gelling agents were adjusted before adding active ingredient and other excipients. Physical appearance and taste of mixture of hydrophobic base and gelling agents were evaluated after applying the mixture to mucous area in the mouth. The active ingredients in this study were asiaticoside extract and mangostin, however in each formulation only one active ingredient was employed.

5. An Evaluation of Herbal Extracts Oral Paste.

There are two main active ingredients in this experiment, they are mangostin and asiaticoside extract. In each formulation, mangostin or asiaticoside extract was used as an active ingredient in the oral paste formula.

5.1 Physical Appearances Study

Oral paste containing an appropriate herbal extracts were packed in close aluminum collapsible tubes. The visual appearances of oral paste were observed every month for four months.

5.2 Assay

The percentage of labeled content of active compound was calculated by average of peak area from HPLC method. The parameters to be considered for validation of HPLC method for the assay of pharmaceutical dosage form are specificity and accuracy (recovery).

5.2.1 Mangostin

Validation of HPLC Method for Analyzing the Pharmaceutical Products

Specificity

The specificity of the method was determined by the comparison of standard solutions and test results by analyzing the active components in pharmaceutical dosage forms. Specificity is established by showing that the active components should have no interfere from the other extraneous components and be well resolved from them.

The standard solutions of mangostin in methanol at the concentration of 20 μg/ml and sample blank solutions in methanol were prepared and evaluated by using the chromatographic conditions as described in section 3.1.

Accuracy

The accuracy of the purposed method was performed by analyzing placebos spiked with known quantities of active ingredients and evaluate as percentage of recovery.

Five concentrations (25, 50, 100, 150 and 200% of assay concentration) of the standard solutions spiked into the oral paste placebo were prepared and analyzed. Three sets of the assay were performed.

5.2.2 Asiaticoside

Validation of HPLC Method for Analyzing the Pharmaceutical Products.

Specificity

The specificity of the method was determined by the comparison of standard solutions and test results by analyzing the active components in pharmaceutical dosage forms. Specificity is established by showing that the active components should have no interfere from the other extraneous components and be well resolved from them.

The standard solutions of asiaticoside in methanol at the concentration of 200 µg/ml and sample blank solutions in methanol were prepared and evaluated by using the chromatographic conditions as described in section 3.2.

Accuracy

The accuracy of the purposed method was performed by analyzing placebos spiked with known quantities of active ingredients and evaluate as percentage of recovery.

Five concentrations (25, 50, 100, 150 and 200% of assay concentration) of the standard solutions spiked into the oral paste placebo were prepared and analyzed. Three sets of the assay were performed.

5.3 Assay for The Pharmaceutical Products

5.3.1 Mangostin

Standard Preparation

The amount of 12.5 mg of standard mangostin was accurately weighed into 50-ml volumetric flask, dissolved in methanol and diluted to volume. Then, 2 ml of standard solution was pipetted into 25-ml of volumetric flask and diluted to volume. The solution was diluted with methanol to obtain the concentration of 20 μg/ml. The solution was filtered through 0.45 μm membrane filter and injected into HPLC column.

Sample Preparation

The amount of 0.5 gram of oral paste containing 0.2% by weight of active ingredient was accurately weighted into 50-ml volumetric flask and diluted with methanol to volume. The volumetric flask was brought to sonicate for 30 minutes. Solution was filtered through 0.45 μm membrane filter and injected into HPLC column.

5.3.2 Asiaticoside

Standard Preparation

The amount of 62.5 mg of standard mangostin was accurately weighed into 25-ml volumetric flask , dissolved in methanol and diluted to volume. Then, 2 ml of standard solution was pipetted into 25-ml of volumetric flask and diluted to volume. The solution was diluted with methanol obtaining the concentration of 200 μg/ml. The solution was filtered through 0.45 μm membrane filter and injected into HPLC column.

Sample Preparation

The amount of 2.5 gram of oral paste containing 0.2% of active ingredient was accurately weighted into 25-ml volumetric flask and diluted with methanol to volume. The volumetric flask was brought to sonicate for 30 minutes. Solution was filtered through 0.45 μm membrane filter and injected into HPLC column.

6. Photooxidation Study

From the functional moiety of mangostin and asiaticoside showed that, these two compounds might be sensitive to light. In this case, the formulas containing these two compounds were subjected for photooxidation studies.

In this experiment the oral paste formulations containing active ingredient were added with antioxidant before study. Butylated hydroxytoluene (BHT) used in these experiments as antioxidant were divided into 3 levels (0.1%, 0.2% and 0.3%). Two different containers were used to pack the oral paste formulation, one of them was the container that protected from light but for the other container could allow light to pass through it. For experimental design both containers were placed under daylight fluorescent lamp (18 watt, 1030 lm) within the closed cabinet at ambient temperature and humidity at suitable storage duration. The oral paste formulation contained in containers that light could pass through were analyzed at day 0, day 2, day 5, day 8, day 11 and day 14 but the formulation contained in aluminum collapsible tubes were analyzed at an initial condition, day 14 and day 28, respectively by HPLC method including observation for the physical appearance. The details of these experiments are showed in Table 2.

Table 2 Photooxidation studies of mangostin and asiaticoside oral paste at various conditions, with and without butylated hydroxytoluene under daylight fluorescent

	Mangostin			Asiaticoside		
	Concentration of BHT (%)			Concentration of BHT (%)		
Well close tight light resistant containers (blank)	-	-	-	-	-	-
Well close tight light resistant containers	0.1	0.2	0.3	0.1	0.2	0.3
Well close but light could pass through containers	0.1	0.2	0.3	0.1	0.2	0.3

7. Stability Study

Oral paste formulas, packed in well closed, protected from light containers, were stored under both accelerated (45 ± 2° C, 75 ±5 %RH) and at ambient conditions for 4 months. The storage assay values for the percentage remaining for either mangostin or asiaticoside at 2 weeks, 1, 2, 3 and 4 months, respectively were obtained by using HPLC method. The sample preparations were prepared as described in section 5.3.1 and 5.3.2. In addition, the physical appearance of the oral pastes were observed every time of sampling along with assay by HPLC.

In the case of using chitosan as one of gelling agents , the physical appearance of chitosan were also observed by placing the formulation in the desiccators which stored in hot air oven at controlled temperature.

After stability study, the formulations that changed in physical appearance were brought to compatibility study by Differential Scanning Calorimetry (DSC).