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. Chitosan 95% deacetylation, viscosity 19 cps, Mw 111,000 (Seafresh chitosan, (LAB) Co., Ltd., Thailand)
5. Centella total triterpenes extract (purchased from Guangxi Chemical Import and Export Corporation, China, batch no. 050703)
6. Colloidal silicon dioxide (Aerosil®) (Degussa, Germany, lot no.713540)
7. Ethyl cellulose aqueous dispersion (Aquacoat®) (FMC Biopolymer Onimax Co., Ltd., USA)
8. Potassium dihydrogen phosphate (Merck, Germany, lot no. A315973 127))
9. Methanol, HPLC grade (Lab Scan Co., Ltd., Thailand)
10. Polyethylene glycol 1000 (Srichand United Dispensary Co., Ltd., Thailand)
11. Polyethylene glycol 4000 (Srichand United Dispensary Co., Ltd., Thailand)
12. Propylene glycol (Srichand United Dispensary Co., Ltd., Thailand)
13. Poloxamer 188, average molecular weight 8270 (BASF, Germany, lot no.BWPWA544C)
14. Poloxamer 407, average molecular weight 12196 (BASF, Germany, lot no.WPHY615B)
15. Sodium chloride (Merck, Germany, lot no.K32104204 324)
16. Sodium dihydrogen phosphate, anhydrous (Asia Pacific Chemicals Limited, batch no. F2F136)
17. Sodium hydrogen phosphate (APS Finechem, batch no.FOJ067)
18. Sodium hydroxide (Mallinckrodt Chemical, Mexico, lot no.7708MVHV)
19. Standard asiaticoside 90% (Guangxi Chemical Import and Export Corporation, China)
20. Standard madecaasoside 95% (Guangxi Chemical Import and Export Corporation, China)

21. Standard prednisolone anhydrous 100.07% (DMSc reference standard, lot no.145052)
22. Suppocire® AM (GATTEFOSSE´ s.a., France, lot no.4226-2)

Apparatus

1. Analytical balance (Model AX105, Mettler Toledo, Switzerland)
2. Cone and plate viscometer (Model LVDV-II+, Brookfield, Scientific Promotion Co., Ltd., USA.)
3. Differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland)
4. Disposable syringe filter nylon 13 mm, 0.45 μm (Chrom Tech, USA)
5. Dissolution apparatus (SOTAX AT7, Sotax AG Basel, Switzerland)
6. Dry cabinet (Model GH-197, Ampore house, Taiwan)
7. 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)
   - Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
   - Precolumn (μBondapack C18, 10 μm, 125 A°, Water Corporation, Ireland)
   - UV-VIS detector (SPD-10A, Shimadzu, Japan)
8. Magnetic stirrer (Model RCT basic, KIKA® Works Guangzhou, China)
9. Mastersizer S (Malvern instrument, UK)
10. Moisture analyzer balance (Model HB43, Mettler Toledo, Switzerland)
11. Peristaltic pump (Model M312, Gilson, France)
12. pH meter (Orion model 420A, Orion Research Inc., USA)
13. Refrigerated incubator (FOC2251, VELP Scientifica, Italy)
14. Scanning electron microscope (Model JSM-T220A, Jeol, Japan)
15. Sonicator (Model TP680DH, Elma, Germany)
16. Spray dryer (Model SD-06, Labplant, Ltd., UK)
17. Stability cabinet (Eurotherm Axyos, Germany)
18. Stopwatch (Heuer, Switzerland)
19. Thermometer digital (AP 105, SILA, Thailand)
20. Water bath (Model WB22, Becthai Co., Ltd., Thailand)
21. X-ray diffraction (Model JDX-3530, Jeol, Japan)

22. Zetasizer (Malvern instrument, UK)
23. Zetasizenano ZS (Malvern instrument, UK)

Methods

A. Determination of Active Constituents from *Centella asiatica*

1. High Performance Liquid Chromatographic (HPLC) Method

The determination of active constituents from *Centella asiatica* was performed by HPLC method because of its specificity and high sensitivity.

1.1 Chromatographic Condition

The chromatographic conditions for the analysis of active constituents 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 7.1, (29:71)
Injection volume	:	20 µl
Flow rate	:	1 ml/min
Detector	:	UV detector at 210 nm
Temperature	:	ambient
Runtime	:	12 min
Internal standard	:	prednisolone

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

1.2 Standard Solution

1.2.1 Preparation of internal standard solution

A stock solution of internal standard was prepared by accurately weighed 12.5 mg of prednisolone into a 25 ml volumetric flask, diluted and adjusted to volume with methanol. The final concentration of prednisolone stock solution was 500 µg/ml.

1.2.2 Preparation of standard solutions

The 25 mg parts of asiaticoside and madecassoside were accurately weighed and transferred into a 25 ml volumetric flask, diluted and adjusted to volume with methanol. These stock solutions have the final concentrations of asiaticoside and madecassoside of 1000 µg/ml each.

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 or 50% ethanol gave final concentrations of 10, 20 40, 60, 80, 100 µg/ml of asiaticoside, respectively.

The solutions of 50, 200, 400, 600, 800, 1000 µl of madecassoside standard stock solution, and 100 µl of internal standard stock solution were added into 5 ml volumetric flask. The dilution to volume with mobile phase or 50% ethanol gave final concentrations of 10, 40, 80, 120, 160, 200 µg/ml of madecassoside, 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.

1.3 Validation of the HPLC method

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

1.3.1 Specificity

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

1.3.2 Linearity

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

1.3.3 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 asiaticoside and madecassoside at 15, 50, 90 µg/ml and 20, 100, 180 µg/ml were prepared and analyzed, respectively. The percentage of recovery of each concentration was calculated from the ratio of inversely estimated concentration to know concentration multiplied by 100.

1.3.4 Precision

a) Within run precision

The within run precision was determined by analyzed five sets of three concentrations of asiaticoside and madecassoside at 15, 50, 90 µg/ml and 20, 100, 180 µg/ml in the same day. Peak area ratios of asiaticoside and madecassoside to prednisolone 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 analyzed three concentrations of asiaticoside and madecassoside at 15, 50, 90 µg/ml and 20, 100, 180 µg/ml on five different days. Peak area ratios of asiaticoside and madecassoside to prednisolone were 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%.

B. Preparation of Spray Dried Microspheres

1. Preparation of spray drying solution

Chitosan was dissolved in 1% acetic acid solution. The solution was left to stand for 24 hr at room temperature for complete hydration of the polymer. Centella extract was dissolved in the mixed solvent developed in this study. The solvent mixture comprised of 5% propylene glycol, 10% absolute ethanol and distilled water. To prepare the spray drying solution, the centella extract in cosolvent solution was homogeneously mixed with the polymer solution. For formulations containing an additive, silicon dioxide (Aerosil®), it was gradually added into the spray drying solution with aid of a stirrer. The resulting solution was adjusted to the final volume by 1% acetic acid, sonicated 30 min and measured the viscosity (LVDV-II+, Brookfield, USA.)

These feeds were spray-dried using a Spray dryer SD-06 (Labplant, UK) (Figure 8) through a peristaltic pump. The operating parameters were set as follows: dry air rate 200 m^3/hr and nozzle size 0.5mm. The inlet temperature and feed pump

were set according to the experiment design. The resulting dried products were collected and kept away from rehydration until further tests.

As a comparison for test of mucoadhesive, ethyl cellulose (EC) in aqueous dispersion form was used to prepare microspheres. The spray drying conditions, inlet temperature and spray flow rate were set at 120°C and 5 ml/min, respectively.

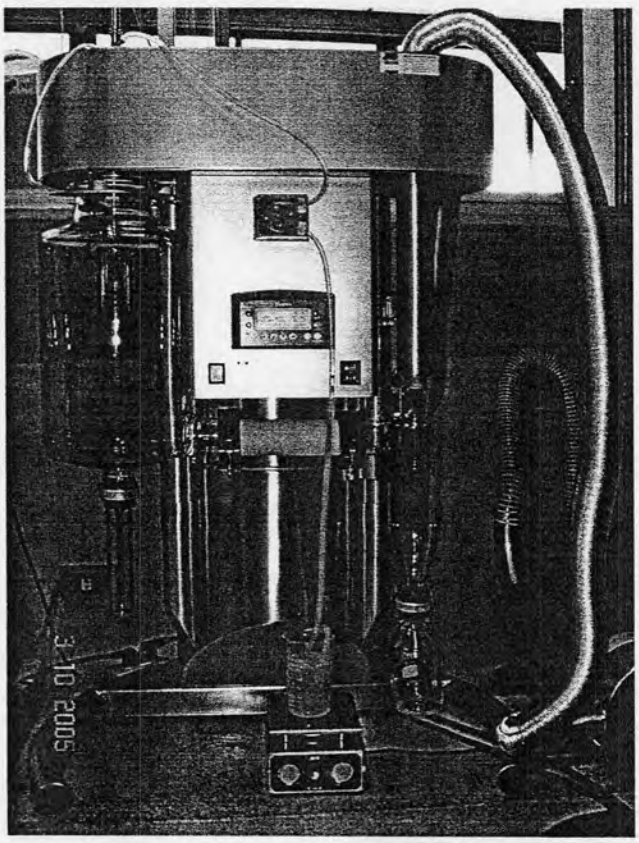


Figure 8 Spray dryer (SD-06, Labplant, Ltd., UK)

2. Experimental design

2.1 Screening design: fractional factorial design (FFD)

Fractional factorial design experiments 2^{5-1} with three center points (16+3) were initially built. They allowed evaluation, by 19 experiments. The chosen design, resolution V designs, considers only two-factor interactions. The factors were classified as two processing parameters and three formulation parameters. All parameters at low and high levels of spray-dried conditions and formulations for preparing microspheres and the matrix of the experiments are shown in Table 1 and Table 2, respectively.

Table 1 Parameters of spray drying process and formulation variables

Parameters	Level	
	low (-)	high (+)
Processing parameters		
inlet temperature (°C)	140	160
feed rate (ml/min)	5	7
Formulation parameters		
%solid content (%)	2	4
%additive (%w/v)	0	0.5
polymer/extract ratio	1/1	1.5/1

Table 2 Matrix of experiments of the fractional factorial design

Code	Inlet temp (°C)	Feed rate (ml/min)	% Solid content	%Additive (%w/v)	Polymer/extract ratio
F1	(-) 140	(-) 5	(-) 2%	(-) 0%	(+) 1.5/1
F2	(+) 160	(-) 5	(-) 2%	(-) 0%	(-) 1/1
F3	(-) 140	(+) 7	(-) 2%	(-) 0%	(-) 1/1
F4	(+) 160	(+) 7	(-) 2%	(-) 0%	(+) 1.5/1
F5	(-) 140	(-) 5	(+) 4%	(-) 0%	(-) 1/1
F6	(+) 160	(-) 5	(+) 4%	(-) 0%	(+) 1.5/1
F7	(-) 140	(+) 7	(+) 4%	(-) 0%	(+) 1.5/1
F8	(+) 160	(+) 7	(+) 4%	(-) 0%	(-) 1/1
F9	(-) 140	(-) 5	(-) 2%	(+) 0.5%	(-) 1/1
F10	(+) 160	(-) 5	(-) 2%	(+) 0.5%	(+) 1.5/1
F11	(-) 140	(+) 7	(-) 2%	(+) 0.5%	(+) 1.5/1
F12	(+) 160	(+) 7	(-) 2%	(+) 0.5%	(-) 1/1
F13	(-) 140	(-) 5	(+) 4%	(+) 0.5%	(+) 1.5/1
F14	(+) 160	(-) 5	(+) 4%	(+) 0.5%	(-) 1/1
F15	(-) 140	(+) 7	(+) 4%	(+) 0.5%	(-) 1/1
F16	(+) 160	(+) 7	(+) 4%	(+) 0.5%	(+) 1.5/1
F17	(0) 150	(0) 6	(0) 3%	(0) 0.25%	(0) 1.25/1
F18	(0) 150	(0) 6	(0) 3%	(0) 0.25%	(0) 1.25/1
F19	(0) 150	(0) 6	(0) 3%	(0) 0.25%	(0) 1.25/1

2.2 Optimization design: central composite design (CCD)

In a second step, the optimal operating condition was estimated by response surface methodology. A central composite design was performed to obtain the response surface and to determine which combination of factor values would produce maximal yield and minimal moisture content (optimal formulation).

The response surface plots indicate the effects of the factors on the %yield and %moisture content responses at each level of the factors. Statistical analyses were performed using Design-Expert version 7.0.3 statistical software. Since the results from fractional factorial design (Section 2.1), inlet temperature and %solid content showed significant effects on %yield and %moisture content. Then, a full central composite design for two variables was built. It consisted of the following parts (Figure 9) (Lundstedt et al., 1998):

- A full factorial design (2^2)
- Experiment at the axial points (2×2). Axial points are situated on the axis in co-ordinate system and with distance $\pm\alpha$ from the origin. The value of α was varying with the number of variables ($\alpha = \pm 2^{1/2}$). So, value of α for two variables = 1.414 (coded value)
- Replicating of experiments at the center point. In this study, five replicating were used (5).

Thus, the central composite design in this study was $2^2 + (2\times2) + 5 = 13$ runs. The design matrix with responses was given in Table 3.

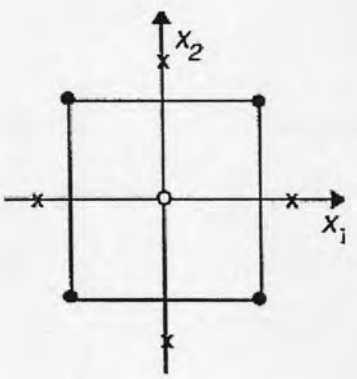


Figure 9 Central composite designs for two variables. The different markings mean (•) factorial design, (o) center point and (×) axial points.

Table 3 Central composite design matrix of two parameters

Code	Parameters	
	Inlet temperature (°C)	**Solid content (%)**
C1	(-) 140	(-) 2
C2	(+) 160	(-) 2
C3	(-) 140	(+) 4
C4	(+) 160	(+) 4
C5	(-α) 136	(0) 3
C6	(α) 164	(0) 3
C7	(0) 150	(-α) 1.6
C8	(0) 150	(α) 4.4
C9	(0) 150	(0) 3
C10	(0) 150	(0) 3
C11	(0) 150	(0) 3
C12	(0) 150	(0) 3
C13	(0) 150	(0) 3

A graphical optimization was also conducted using the Design-Expert version 7.0.3 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 140-160 °C and 2-4 % for inlet temperature and solid content, respectively. The main objective was to obtained %yield as high as possible and %moisture content as low as possible.

C. Evaluation of Physicochemical Properties of Spray Dried Microspheres

1. Percentage yield

The percentage yield (w/w) was calculated as the weight of the dried microspheres recovered from each batch divided by the sum of the initial dry weight of the starting materials and multiplied with 100.

2. Determination of moisture content

A sample of microspheres 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 percent moisture content was calculated automatically. The mean and standard deviation of three determinations were calculated.

3. Determination of scanning electron microscopy

The microspheres were viewed using a scanning electron microscope (SEM), (JSM-T220A, Jeol, Japan) for morphological examination. 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 spray dried microspheres were ×750 and ×2000.

4. Determination of size and size distribution

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

5. Determination of zeta potential

The zeta potential is a representative of particle charge. Zeta potential was determined by electrophoresis, which was performed with Zetąsizenano ZS (Malvern instrument, UK). The microspheres were suspended in phosphate buffer solution pH 7.4 (0.0001 M) by sonication for 15 min. The cell was filled with a measured amount of sample and inserted with its integral gold electrode close to the lid. The mean and standard deviation of three determinations were calculated.

6. In vitro evaluation of mucoadhesive properties of microspheres

The mucoadhesive properties of microspheres were determined in vitro by Harikarnpakdee (2003). The principle of this test based on simulating a biological flow by washing off a mucous membrane covered with the product to be tested. The

mucoadhesive properties were carried out on centella microspheres, optimal microspheres from optimization, ethyl cellulose microspheres (control) and optimal microspheres dispersed in liquid suppository (centella liquid suppository).

Fresh pig rectum was obtained from the local abattoir within 1 hr of killing the animal. A freshly cut 5 cm long piece of rectum of pig was obtained and cleaned by washing with isotonic saline solution. Accurately weighed microspheres of 125 mg were placed on mucosal surface, which was fixed over polyethylene support. The plate with the tissue was then fixed in an angle of 40° relative to the horizontal plane. Phosphate buffer saline (pH 7.4) controlled at 37°C (WaterBath22, Becthai, Thailand) was pumped at rate of 5 ml/min over the tissue using a peristaltic pump (M312, Gilson, France). The apparatus was assembled in the laboratory (Figure 10). The duration time for complete washing off microspheres from the rectal mucosa was recorded. The change of microsphere adhesion on the rectal mucosa was also observed.

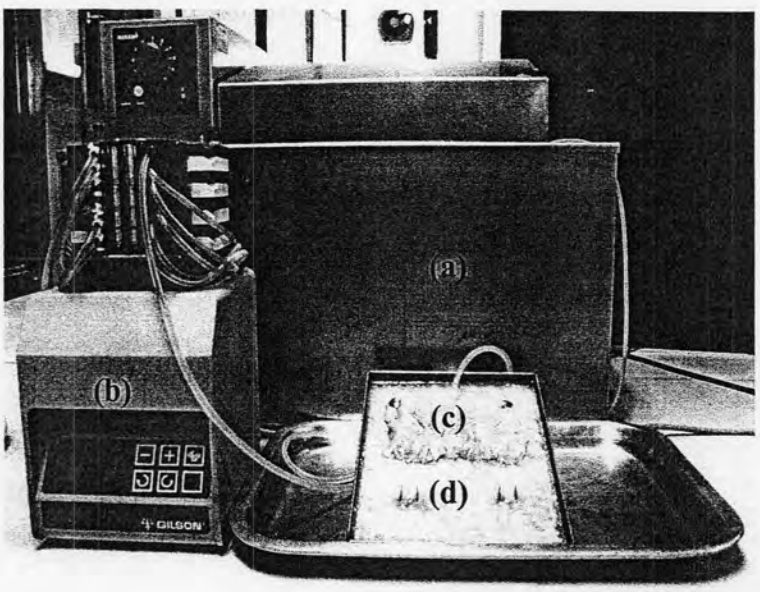


Figure 10 Mucoadhesive property testing apparatus (assembled in the laboratory);
(a) water bath, (b) peristaltic pump, (c) fresh pig rectum tissue,
(d) polyethylene support.

7. Determination of differential scanning calorimetric thermograms

Differentials scanning calorimetry (DSC) is an effective thermal method to study the equilibrium phase of either a pure compound or a mixture. Different effects, associated with physical or chemical changes, are registered as a function of temperature as the substance is heated at a uniform rate. The diffractograms of spray dried microspheres of different formulations were investigated using differential scanning calorimeter (DSC822^e, Mettler Toledo, Switzerland).

The diffractograms of centella total triterpenes extract standard substance, physical mixture and blank microspheres were also carried out in comparison. The samples (10-15 mg) were accurately weighed into aluminum pans (40 µl) and then sealed. The DSC runs were conducted over a temperature range 0-300°C at rate of 10°C/min. Inert atmosphere was maintained by purging nitrogen gas as a flow rate of 200 ml/min.

8. Determination of powder x-ray diffraction

The crystallity of centella extract in the spray dried microspheres was examined by powder x-ray diffractometry (JDX -3530, Jeol, Japan). The data were investigated over an angular range form 5° to 45° in continuous mode using a step angle of 0.004° and step time of 1 second.

9. Determination of %content

Samples from each batch of microspheres were weighed accurately (10 mg) into test tubes to which 10 ml of 50% ethanol solutions were added. The samples were sonicated for 30 min and shaked over night at 25°C to extract the entrapped centella extract. After the tube was centrifuged at 6000 rpm for 10 min, the supernatant was separated and put into a 25 ml volumetric flask. Then, the remaining solid was further extracted using 10 ml of 50% ethanol solution by sonication for 30 min, centrifuged and separated. Total supernatants were collected and adjusted to volume with the medium. They were assayed by HPLC method.

The amount of centella total triterpenes extract in the microspheres were calculated form the ratio of actual to theoretical content and expressed as a percentage. The mean and standard deviation of three determinations were calculated.

10. Determination of microspheres dissolution

Dissolution testing for microsphere samples were carried out in triplicate using a dissolution apparatus (SOTAX AT7, Switzerland) following the USP apparatus II (paddle) method. Accurately weighed of microspheres equivalent to 60 mg of centella extract were dispersed into 500 ml of phosphate buffer (pH 7.4). Each sample was stirred at 100 rpm and the temperature was maintained at 37±5°C throughout the experiment.

Samples in the amount of 5 ml were withdrawn at 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min interval, and fresh dissolution mediums were simultaneously replaced into the apparatus to maintain a constant volume and to ensure a sink condition. Five ml of sample solution were pipetted into a 5 ml volumetric flask. Then, 100 µl of prednisolone stock solution was added, mixed well and filtered using a 0.45 µm filter (Chrom Tech, USA) to ensure that no precipitation occurred during HPLC analysis. The filtrates were assayed by HPLC to determine the amounts dissolved of asiaticoside and madecassoside in the medium.

D. Preparation of Suppositories

1. Preparation of the rectal liquid suppositories

1.1 Preparation of the plain rectal liquid suppository bases

The plain liquid suppository bases were prepared using cold method (Schmolka, 1972). Using different concentrations of poloxamer 407 (P 407) and/or poloxamer 188 (P188), as shown in Table 4, the poloxamers were slowly added into the calculated amount of cold distilled water with continuous agitation using magnetic stirrer bar. The formed dispersions were left at 4°C until clear transparent solutions were obtained. The physical characterizations of these liquid suppository bases were evaluated.

Table 4 Compositions of the liquid suppository bases

Code	P 407 (%w/w)	P 188 (%w/w)
1	10	-
2	15	-
3	17	-
4	20	-
5	23	-
6	25	-
7	27	-
8	30	-
9	-	20
10	-	25
11	-	30
12	15	10
13	15	15
14	16	4
15	16	8
16	17	5
17	17	10
18	18	4
19	20	5
20	20	10
21	25	5
22	25	10

1.2 Preparation of centella liquid suppositories

The liquid suppository formulations which showed appropriate physical properties were selected from the above formulations.

Accurately weighed amounts of the optimized formulation of microspheres which contained asiaticoside equivalent to 8 mg were blended with 2 g of the liquid suppository bases. The centella liquid suppositories were freshly prepared before used in the further studies.

2. Preparation of the conventional suppositories

2.1 Selection of the hydrophilic suppository base

The conventional hydrophilic (water-soluble) suppository base formulation was selected (Lawrence, 1990), since it gave rapid dissolution and did not interfere the quantitative analysis of the centella extract. The selected formulation was as follows:

Rx

Polyethylene glycol 1000	96%
Polyethylene glycol 4000	4%

2.2 Selection of the lipophilic suppository base

The lipophilic semisynthetic glyceride base, Suppocire® AM, was used, since it was easily melted, dislodged from the mold and suitable melting point with 35-36.5 °C.

2.3 Preparation of the conventional suppositories

The conventional suppositories of PEG mixtures and Suppocire® AM were prepared by fusion method in water bath at 48°C. The optimal microspheres or pure extract (asiaticoside equivalent to 8 mg) were added into the molten suppository bases and stirred until nearly congealed. The mixtures were poured into a steel suppository mold and allowed to solidify at room temperature.

All conventional suppositories were wrapped individually in aluminum foil and stored in the refrigerator at 4°C until used.

E. Evaluation of Physicochemical Properties of Liquid Suppositories

1. Measurement of the gelation temperature

Gelation temperature is the temperature at which liquid phase makes transition to gel. An accepted liquid suppository must have a gelation temperature in the range of 30 - 36°C so as to be a liquid at room temperature and a gel phase instantly in the rectum.

The gelation temperature was determined by an adapted method described by Choi et al. (1998). The apparatus was designed and assembled in the laboratory by the investigator which was calibrated compare with Choi et al. (1998). The observed

values were different from reference method which could be several causes such as average molecular weight of poloxamers. However, the measurement obtained from this apparatus demonstrated a high precision (not more than 0.6).

The apparatus consisted of a heating unit equipped with a thermostat controlling the temperature, stainless steel tank, magnetic stirrer with a magnetic bar (1 x 5/16 inch) rotated at 100±5 rpm and temperature sensors for detecting temperature of gels. The apparatus which was equipped and employed in this study is displayed in Figures 11 – 12.

A 10 g sample of the poloxamer solution at 4 °C was transferred to a 25 ml of beaker and put in the modified water bath at 4°C. The temperature of the water bath was increased with the increments of 3°C (or 1°C in the region of sol-gel transition temperature). The temperature at which the rotation of the bar stopped was taken as the gelation temperature. The maximum temperature tested was 50°C. The results were recorded as the mean of five measurements.

The gelation temperature of the prepared centella liquid suppositories was also measured. This study was aimed to investigate the effect of the added microspheres on the gelation temperature. This experiment was conducted in triplicates.

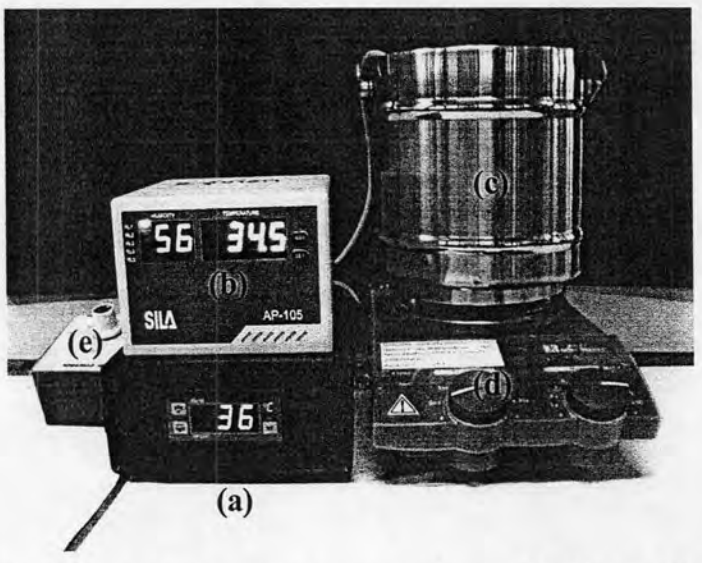


Figure 11 Gelation temperature testing apparatus (assembled in the laboratory);

(a) temperature controller for water bath,

(b) digital thermometer for poloxamer gel

(c) applied water bath

(d) magnetic stirrer

(e) dimmer

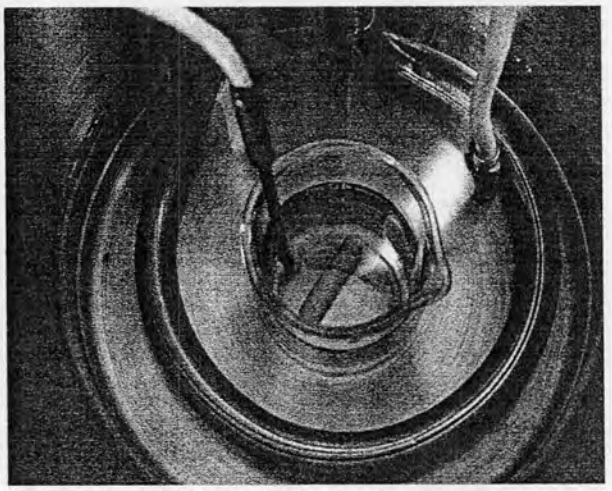


Figure 12 Gelation temperature testing apparatus; top view (assembled in the laboratory)

2. Measurement of the gel strength

The gel strength is important in finding the condition, which allows the easy insertion of the suppository and no leakage from the anus. The previous study has been investigated for suitable gel strength. The proper range was 10-50 seconds (Choi et al., 1998). For this range, above the upper threshold of gel strength, it was difficult to insert the poloxamer gel. Under the lower limit, the poloxamer gel leaked out from the anus.

The gel strength was determined by an adapted method described by Choi et al. (1998). Poloxamer solution (50 g) was put in a 100 ml-graduated cylinder and gelled in thermostatically controlled water bath at 37°C. The apparatus for measuring gel strength equipped with a 35 g weight was then placed above the poloxamer gel (Figures 13 - 15). The gel strength was determined by the time in seconds that the apparatus sank 5 cm down through the poloxamer gel. A range of 10-50 seconds was accepted and the maximum time was tested 50 seconds. The results were recorded as the mean of five measurements.

The gel strength of centella liquid suppositories was also determined according to the procedure described above. This experiment was conducted in triplicates

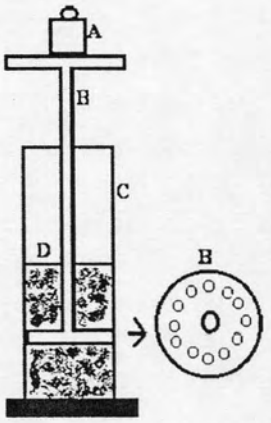


Figure 13 Schematic illustration of gel strength-measuring device (assembled in the laboratory);
(a) weights, (b) device, (c) graduated cylinder, (d) poloxamer gel

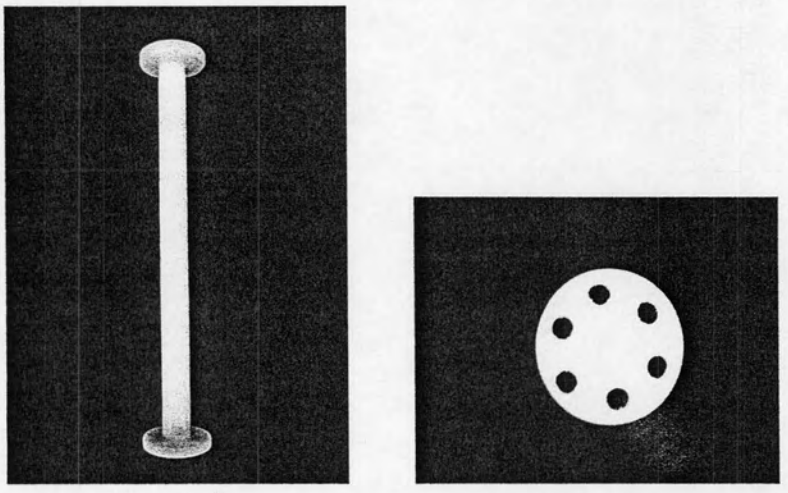


Figure 14 Gel strength device; (a) side view, (b) top view

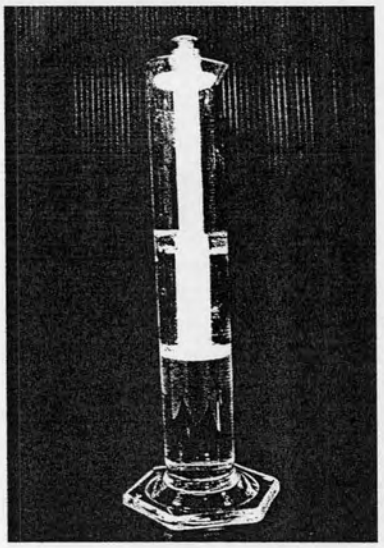


Figure 15 Gel strength testing apparatus (assembled in the laboratory)

3. Measurement of setting time of gel

Setting time of gel is time which liquid phase makes transition to gel. If setting time is short, the liquid suppository will not leak from the anus.

A 2 ml sample of poloxamer solution was transferred to a test tube which put in the water bath at 37°C. The sample was then carefully examined for gelation every 5 seconds which was said to have occurred when the meniscus would no longer move upon tilting through 90° (Figure 16). The results were recorded as the mean of five measurements. For centella liquid suppositories, the measurements were conducted in triplicates

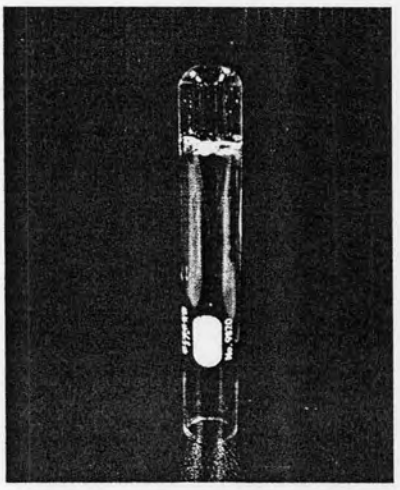


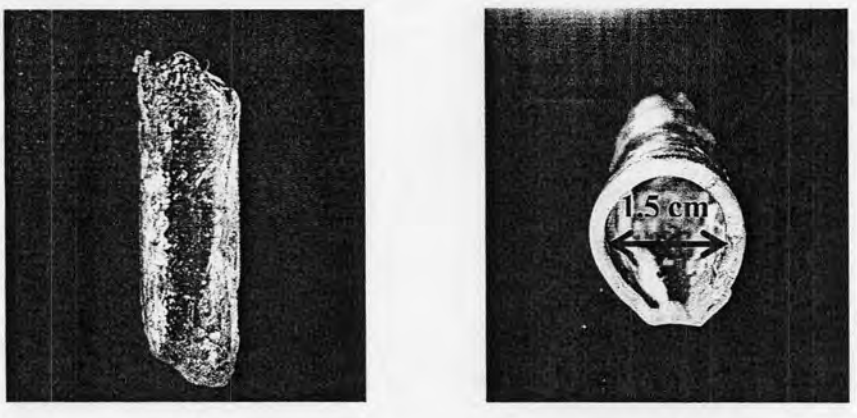
Figure 16 The measuring of setting time of poloxamer solution

4. In vitro release from *in situ* suppositories

In vitro release was carried out on *in situ* gel suppositories and conventional suppositories (hydrophilic and lipophilic bases). Each type of suppositories was contained optimal centella-chitosan microspheres or pure centella extract for comparison.

Each of centella liquid suppositories was filled into sugar tubes for holding the shape of *in situ* gel suppositories and controlled surface area near to conventional suppositories. The sugar tubes were made from sucrose which were melted and formed cylinder shape. The weight of each sugar tubes was about 5 g with 1.5 cm diameter (Figure 17). Since this sugar tube dissolved in medium at 37°C within 5 minutes while liquid suppository could be also transformed to gel with cylinder shape at this range time.

Release studies for centella suppositories were carried out in triplicate using a dissolution apparatus (SOTAX AT7, Switzerland) following the USP apparatus II (paddle) method. The sugar tube of centella liquid suppositories or conventional suppositories was put into 500 ml of phosphate buffer (pH 7.4) at 37±5°C and the speed of the paddle was set at 100 rpm. Aliquot samples of 5 ml were withdrawn at 10, 20, 30, 45, 60, 120, 180 and 240 min intervals (lag time 5 minutes for liquid suppository formulations). The contents of asiaticoside and madecassoside in the samples were analyzed with HPLC using the same method as described for the determination of dissolution of microspheres (Section C.10).



(a) **(b)**

Figure 17 The sugar tube prepared for filling liquid suppositories;
(a) side view, (b) top view with 1.5 cm diameter of tube

F. Stability Study of Chitosan Microspheres Containing Centella Extract

The microspheres 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 1, 2, and 3 months and were analyzed for the amounts remaining of asiaticoside and madecassoside. The differential scanning calorimetry study and powder x-ray diffraction study were also performed. For the analysis of asiaticoside and madecassoside in microspheres samples followed the previously described HPLC method.