CHAPTER III

MATERIALS AND METHODS

Crude Drugs

1. Fresh plant of *Centella asiatica* (purchased from Tavet Market, Bangkok, Thailand in June, 2003.)

Materials

1. Acetonitrile (HPLC grade, Lab Scan Asia Co., Ltd., Thailand)

2. Butan-1-ol (AR grade, Lab Scan Asia Co., Ltd., Thailand)

3. Caprylic/capric triglyceride (Lexol® GT-865) (Namsiang International Co., Ltd., Thailand)

4. Centella selected triterpenes 92-95% (purchased from Guangxi Chemical Import and Export Corporation, China)

5. Chloroform (BDS Laboratory Supplies, England)

6. Disodium hydrogen orthophosphate (AR grade, E. Merck, Germany)

7. Ethanol 95% (Commercial grade, Lab scan Asia Co., Ltd., Thailand)

8. Ethylacetate (AR grade, E. Merck, Germany)

9. Isopropyl myristate (Namsiang International Co., Ltd., Thailand)

10. Methanol (AR grade, Lab Scan Asia Co., Ltd., Thailand)

11. Methanol (HPLC grade, Lab Scan Asia Co., Ltd., Thailand)

12. Polyoxyethylene 10 oleyl ether (Brij® 97) (Donated from East Asiatic Public Company Limited, Thailand)

13. Polyoxyethylene 20 sorbitan monooleate (Tween®80) (Namsiang International Co., Ltd., Thailand)

14. Polyoxyethylene 4 lauryl ether (Brij® 30) (Donated from East Asiatic Public Company Limited, Thailand)

15. Polyoxyethylene 40 sorbitol septaoleate (Arlatone®T) (Donated from East Asiatic Public Company Limited, Thailand)

16. Propan-2-ol (AR grade, Lab Scan Asia Co., Ltd., Thailand)

17. Propylene glycol (Namsiang International Co.,Ltd., Thailand)

18. Sephadex TM LH-20 (Americham Pharmacia Biotech AB, Sweden)

19. Sodium chloride (AR grade, E. Merck, Germany)

20. Sorbitan monooleate (Span® 80) (Namsiang International Co., Ltd., Thailand)

21. Standard asiatic acid 95% (Guangxi Chemical Import and Export Corporation, China)

22. Standard asiaticoside 90% (Guangxi Chemical Import and Export Corporation, China)

23. Standard madecassic acid 93% (Guangxi Chemical Import and Export Corporation, China)

24. Standard madecassoside 95% (Guangxi Chemical Import and Export Corporation, China)

25. Sulfuric acid (AR grade, E. Merck, Germany)

Apparatus

1. Analytical balance (Model AX/MX/UMX, Mettler Toledo, Switzerland)

2. Conductivity meter (Model C535, CONSORT, Belgium)

3. Cone and plate viscometer (Brookfield digital Viscometer, Scientific Industries, USA.)

4. High performance liquid chromatography

- Communications bus module (CBM-10A, Shimadzu, Japan)

- Auto Injector (SIL-10A, Shimadzu, Japan)

- Column (BDS Hypersil C18, 10 µm, 250 x 4.6 mm, Thermo Electron Corporation, England)

- Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)

- UV-VIS detector (SPD-10A, Shimadzu, Japan)

5. Modified Franz difussion cells (Becthai, Thailand)

6. pH meter (Model 420A, Orion,USA)

7. Refrigerated incubator (FOC 225I, VELP Scientifica, Italy)

8. Rotary evaporator (Rotavapor RE-120, Buchi, Switzerland)

9. Sonicator (Elma, Germany)

10. Transmission electron microscopy (JEM-1210, JEOL , Japan)

11. UV- visible spectrophotometer (UV-1601, Shimadzu, Japan)

12. Vacuum Pump (CB 169 Vacuum System, Buchi, Switzerland)

13. Vortex mixer (Vortex Genie-2, Scientific Industries,Inc., USA)

Others

1. Parafilm (American National Can TM, USA)

2. Polyamide membrane filter 47 mm, 0.45 µm (Sartorius AG, Germany)

3. Syringe filter cellulose acetate 13 mm, 0.45 μm (Chrom Tech Inc., USA)

4. TLC Alumina sheet siliga gel 60F 254 20 X 20 cm (E. Merck, Germany)

5. Whatmann filter paper No. 1, 150 mm (Whatman International Ltd., England)

Methods

A. Extraction and Isolation of Active Constituents from *Centella asiatica*

1. Extraction of Chemical Components from Fresh Leaves

Fresh leaves of *Centella asiatica* (5.99 kg) were macerated with 60 liters of ethanol at ambient temperature for 3 days. The filtration of the extracted liquid was made through filter paper (Whatman no.1) under vacuum. The marc was successively extracted until exhausted. Then, the filtrate was concentrated under reduced pressure. Next, the water was added to the ethanol extract and used to further extract with chloroform in separating flask. The extraction was separated into chloroform phase and water phase. The chloroform phase was determined by TLC method. The water phase was further extracted with n-butanol in another separating flask. The butanol phase was evaporated by a rotary evaporator to dryness to obtain the brown extract. The dried butanol extract was dissolved in methanol and passed through Sephadex™-LH20 and eluted with methanol. The 20 ml fraction which were primarily identified by TLC method for asiaticoside, madecassoside, madecassic acid and asiatic acid were collected (Appendix A).

2. Extraction of Chemical Components from Dried Leaves

The dried leaves of *Centella asiatica* (2 kg) were refluxed at 80 °C with the mixture of methanol and water in ratio of 7:3 for 3 hours. The extract was filtered through Whatman filter paper no.1 under vacuum. The marc was successively refluxed until exhausted. Next, the filtrate was evaporated under reduced pressure. After being concentrated, the extract was partitioned with dichloromethane. The extraction was separated into dichloromethane phase and water phase. The water phase was further partition with n-butanol in separating flask. Subsequently, the butanol phase was washed several times with 0.2 N sodium carbonate until receiving clear water layer and then with water. The butanol layer was separated from water layer and the butanol extraction was precipitated with ethylacetate to obtain yellow precipitates (Appendix A).

B. Determination of Active Constituents from *Centella asiatica*

1. Thin Layer Chromatographic (TLC) Method

TLC is the most versatile and flexible chromatographic method. It is rapid and available for a number of samples to beidentified since up to 30 individual samples and standards can be applied to a single plate and separated at the same time.

The fractionated extractions were dissolved with methanol and spotted on TLC Alumina sheet compared standard solution, asiaticoside, madecassoside, madecassic acid and asiatic acid and dried. Afterthat, the plates were placed into equilibrated mobile phase, ethylacetate: methanol: water = 7.5: 2.5: 1 inside a closed chamber. When the mobile phase has moved to an appropriate distance, the plates

have been removed and dried. The plates were visualized by spraying with 10% sulfuric acid in methanol, followed by heating air for 15 minutes. The basic parameter used to describe migration is the Rf value, where

$$\text{Rf} \quad = \quad \frac{\text{distance moved by the solute}}{\text{distance moved by mobile phase front}}$$

2. High Performance Liquid Chromatrographic (HPLC) Method

The determination of active constituents from *Centella asiatica* was performed by HPLC (gradient system) method because of its specificity and high sensitivity. The condition for HPLC analysis was developed and validated.

2.1 Chromatographic Condition

The chromatrographic conditions for the analysis of active constituents from *Centella asiatica* were as follows:

Column   : BDS Hypersil C18 (5 µm, 250 x 4.6 mm)

Mobile phase   : Solvent A : mixtures of acetonitrile and water (30:70 v/v)

                        Solvent B : acetonitrile

Gradient system :

Time (min)	Solvent A	Solvent B
0 - 20	100 - 45 %	0 – 55 %
20-30	45-100 %	55-0 %

Flow rate   : 1 ml/min

Detector   : UV detector 210 nm

Temperature   : ambient

Internal standard : prednisolone 100 μg/ml

Solvent A and B were prepared, mixed well and filtered through a filtering membrane with a 0.45 μm pore size and then degassed by sonication for 30 min prior to use.

2.2 Standard Solution

From the prelimination study, prednisolone was chosen to be the internal standard. A stock solution of internal standard was prepared by accurately weighing of 25 mg of prednisolone into a 25 ml volumetric flask. Methanol was added to dissolve the internal standard and adjust to the final volume to obtain the final concentration of 100 μg/ml.

The 50 mg parts of asiaticoside, madecassic acid and asiatic acid were accurately weighed and transferred into a 50 ml volumetric flask. Methanol was used to dissolve and adjusted to volume. This stock solution had the final concentrations of asiaticoside, madecassic acid and asiatic acid of 1000 μg/ml each. Afterthat, the stock solution was further diluted to obtain the standard solutions with the final concentrations of 10, 20, 30, 40, 50 and 60μg/ml, respectively.

Two ml of each standard solutions were pipetted into test tubes. Then, 120 μl of prednisolone stock solution was added and mixed well for the HPLC run.

2.3 Sample Solutions of HPLC Method

One ml of each of microemulsion preparations was weighed and transferred into a 10 ml volumetric flask. Methanol was added to volume and vortexed for 5 min. One ml of solutions was further pipetted into 10 ml volumetric flask and adjusted to volume with methanol. The clear solutions were obtained and used for the HPLC assay.

In case of sample solutions from the permeation study, two ml were pipetted from Franz diffusion cell into test tubes. Then, 120 µl of prednisolone stock solution was added and mixed well for HPLC run.

2.4 Validation of HPLC Method

Typical parameters to be considered in validation study of the HPLC analysis are specificity, linearity, accuracy and precision (USP 25, 2002).

2.4.1 Specificity

The specificity of the method was determined by comparing the test results from analyses of active components in microemulsion preparations with standard solutions. The test was aimed to ensure that the peak of the interested components was free from interference by other components in the sample.

Solutions of blank methanol and 40% ethanol in isotonic phosphate buffer pH 7.4 were prepared. The chromatograms of these solutions were evaluated

by comparing with that of 100 µg/ml standard mixture solution of asiaticoside, madecassic acid and asiatic acid.

2.4.2 Linearity

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. The linearity was determined from the coefficient of determination (R^2).

2.4.3 Accuracy

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Three concentrations (low, medium, high) of standard mixture solutions, covering linearity range and five replicates of each concentration were prepared and analyzed. The accuracy of the method was expressed as the percentage of recovery.

2.4.4 Precision

a) Within Run Precision

Within run precision was determined by analyzed three concentrations (low, medium, high) of standard mixture solutions and five replicates of each concentration in the same day. Peak area ratios were compared and the percent of coefficient of variation (%CV) of each concentration was determined.

b) Between Run Precision

Between run precision was determined by analyzed three concentrations (low, medium, high) of standard mixture solutions for five different days. Peak area ratios were compared and the percent of coefficient of variation (%CV) of each concentration was determined.

C. Preparation of Microemulsion

1. Microemulsion formulation

Microemulsion preparations were formulated by using various oils, surfactants and cosurfactants as follows (Appendix F):

Oil : caprylic/capric triglyceride , isopropyl myristate

Surfactant : Brij 30, Brij 97, Arlatone T, mixture of Tween 80 and Span 80

at the ratio of 1:1 and 2:1

Cosurfactant : propan-2-ol, propylene glycol

The microemulsions were prepared by weighing the ingredients into Taflon-sealed screw-cap tubes and vigorously mixed with a vortex mixer for 5 min. The systems were stored for 1 week at ambient temperature before further studied.

2. Physical Characterization

2.1 Visual inspection

Visual observation was used to characterize and identify the systems prepared from Topic C1. The physical appearance were classified as isotropic

systems, emulsions or milky suspensions. Only clear isotropic one phase system was elucidated as microemulsions or liquid crystalline systems in Topic 2.2 whereas emulsions were nontransparent.

2.2 Polarized light microscopy

A drop of sample from Topic 2.1 was placed on a glass slide and then examined under polarized light microscopy. Liquid crystals showed birefringence with typical oily streaks, maltese crosses, or fan-shaped textures whereas microemulsions weere nonbirefringent and generate a dark background when investigated under polarizing microscope (DePolo, 1988; Alany et al., 2001).

3. Ternary and Pseudo-ternary Phase Diagram Determination

3.1 Construction of ternary and pseudo-ternary phase diagrams

The physical state of the system obtained from topics 2.1 and 2.2 was marked on a ternary and pseudo-ternary phase diagram with the top apex representing surfactant or mixture of surfactant and cosurfactant at the ratio of 1:1, 2:1 or 4:1 and the other two apices representing oil and water.

3.2 Determination of ternary and pseudo-ternary phase diagrams

3.2.1 Effects of oil and surfactant on ternary and pseudo-ternary phase diagrams

The systems of microemulsion are shown in Table 1.

Table 1 Compositions of oil and surfactant in microemulsion.

System	Oil	Surfactant
1	caprylic/capric triglyceride	Brij 30
2	caprylic/capric triglyceride	Brij 97
3	caprylic/capric triglyceride	Arlatone T
4	caprylic/capric triglyceride	Tween 80 and Span 80 (1:1)
5	caprylic/capric triglyceride	Tween 80 and Span 80 (2:1)
6	IPM	Brij 30
7	IPM	Brij 97
8	IPM	Arlatone T
9	IPM	Tween 80 and Span 80 (1:1)
10	IPM	Tween 80 and Span 80 (2:1)

3.2.2 Effects of cosurfactant on pseudo-ternary phase diagrams

The systems of microemulsion of various ratios of mixture of surfactant and cosurfactant using caprylic/capric triglyceride as oil and propan-2-ol, propylene glycol as cosurfactant are shown in Tables 2 and 3, respectively. Similarly, the systems with isopropyl myristate as oil component are shown in Tables 4 and 5, respectively.

Table 2 Compositions of surfactant and cosurfactant in microemulsion with caprylic/capric triglyceride as oil and propan-2-ol as cosurfactant.

System	Surfactant	ratio of surfactant and cosurfactant
11	Brij 30	1:1
12	Brij 30	2:1
13	Brij 30	4:1
14	Brij 97	1:1
15	Brij 97	2:1
16	Brij 97	4:1
17	Arlatone T	1:1
18	Arlatone T	2:1
19	Arlatone T	4:1
20	Tween 80 and Span 80 (1:1)	1:1
21	Tween 80 and Span 80 (1:1)	2:1
22	Tween 80 and Span 80 (1:1)	4:1
23	Tween 80 and Span 80 (2:1)	1:1
24	Tween 80 and Span 80 (2:1)	2:1
25	Tween 80 and Span 80 (2:1)	4:1

Table 3 Compositions of surfactant and cosurfactant in microemulsion with caprylic/capric triglyceride as oil and propylene glycol as cosurfactant.

System	Surfactant	ratio of surfactant and cosurfactant
26	Brij 30	1:1
27	Brij 30	2:1
28	Brij 30	4:1
29	Brij 97	1:1
30	Brij 97	2:1
31	Brij 97	4:1
32	Arlatone T	1:1
33	Arlatone T	2:1
34	Arlatone T	4:1
35	Tween 80 and Span 80 (1:1)	1:1
36	Tween 80 and Span 80 (1:1)	2:1
37	Tween 80 and Span 80 (1:1)	4:1
38	Tween 80 and Span 80 (2:1)	1:1
39	Tween 80 and Span 80 (2:1)	2:1
40	Tween 80 and Span 80 (2:1)	4:1

Table 4 Compositions of surfactant and cosurfactant in microemulsion with isopropyl myristate as oil and propan-2-ol as cosurfactant.

System	Surfactant	ratio of surfactant and cosurfactant
41	Brij 30	1:1
42	Brij 30	2:1
43	Brij 30	4:1
44	Brij 97	1:1
45	Brij 97	2:1
46	Brij 97	4:1
47	Arlatone T	1:1
48	Arlatone T	2:1
49	Arlatone T	4:1
50	Tween 80 and Span 80 (1:1)	1:1
51	Tween 80 and Span 80 (1:1)	2:1
52	Tween 80 and Span 80 (1:1)	4:1
53	Tween 80 and Span 80 (2:1)	1:1
54	Tween 80 and Span 80 (2:1)	2:1
55	Tween 80 and Span 80 (2:1)	4:1

Table 5 Compositions of surfactant and cosurfactant in microemulsion with IPM

as oil and propylene glycol as cosurfactant.

System	Surfactant	ratio of surfactant and cosurfactant
56	Brij 30	1:1
57	Brij 30	2:1
58	Brij 30	4:1
59	Brij 97	1:1
60	Brij 97	2:1
61	Brij 97	4:1
62	Arlatone T	1:1
63	Arlatone T	2:1
64	Arlatone T	4:1
65	Tween 80 and Span 80 (1:1)	1:1
66	Tween 80 and Span 80 (1:1)	2:1
67	Tween 80 and Span 80 (1:1)	4:1
68	Tween 80 and Span 80 (2:1)	1:1
69	Tween 80 and Span 80 (2:1)	2:1
70	Tween 80 and Span 80 (2:1)	4:1

4. Physical Stability

Heating-cooling cycle was used as procedure of the stability studies. Selected microemulsions from isotropic zone in the pseudo-ternary phase diagram were stored in the refrigerator at 4 ± 0.5 °C for 48 hours and in the hot air oven at 45 ± 0.5°C for 48 hours. This was called as one cycle, and six cycles were carried out. The clarity and phase separation of the systems were observed at the end of storage period. The system without phase separation was recorded as physically stable.

D. Centella Extract Microemulsion

1. Formulation of Centella Triterpene Extract Microemulsion

Stable formulations of microemulsions from the study in topic C 4 were selected. The selection criteria were various surfactants at the ratio of oil:

surfactant: water as 30: 60: 10. The ratio represented the possible lowest proportion of surfactant that could be identically used to form stable microemulsions (Table 6). Microemulsions were prepared with the same method as those described in C 1. Centella triterpenes extract was weighed in an amount equivalent to 2% of formula and directly incorporated into micromulsions. The mixture was mixed by a magnetic stirrer for 30 minutes.

Table 6 Microemulsion formulas for permeation study of effect of oil and surfactant

Formula	Oil	Surfactant	Water	Ratio (%by weight)
1	IPM	tween80/span80(1:1)	water	30:60:10
2	caprylic/capric triglyceride	tween80/span80(1:1)	water	30:60:10
3	IPM	tween80/span80(2:1)	water	30:60:10
4	caprylic/capric triglyceride	tween80/span80(2:1)	water	30:60:10
5	IPM	Brij30	water	30:60:10
6	caprylic/capric triglyceride	Brij30	water	30:60:10
7	IPM	Brij97	water	30:60:10
8	caprylic/capric triglyceride	Brij97	water	30:60:10
9	IPM	Arlatone T	water	30:60:10
10	caprylic/capric triglyceride	Arlatone T	water	30:60:10

2. Determination of Physicochemical Properties of Centella Triterpene Extract Containing Microemulsion

The physicochemical properties of microemulsions before and after incorporating Centella triterpenes extract were determined as followings:

2.1 Physical Appearance

The physical appearances of prepared Centella triterpenes extract microemulsions such as color, clarity, phase separation were observed.

2.2 pH

The pH of microemulsions was measured by using pH meter at 23±2 °C.

2.3 Viscosity

Viscosity of microemulsions was measured using a Brookfield digital viscometer fitted with CP-40 cone spindle at 23±2 °C.

2.4 Droplet Size

The structure and droplet sizes of microemulsions were characterized by transmission electron microscopy (TEM). Samples were prepared by negative staining with alkaline phosphotungstic acid.

2.5 Electrical Conductivity

Conductivity was measured using conductivity electrode at 23±2 °C. Reading were taken after the value had remained constant for at least 5 min.

All pH, viscosity and conductivity measurements were carried out in triplicate.

3. Permeation Study

3.1 Permeation Procedure

Permeation experiments were performed using modified Franz

diffusion apparatus. The modified Franz diffusion cell consists of a donor compartment and a receptor compartment. These cells were made of glass with a contact area of 2.40 cm^2. The membranes were mounted between the cell compartments and an o-ring was used to position the membrane. The upper surface of membrane was exposed to Centella triterpenes extract microemulsions. The receptor compartment was filled with 14 ml of 40% ethanol in isotonic phosphate buffer saline pH 7.4. It was kept at 37 ± 0.5 °C by circulating water through an external water jacket. After equilibration of membrane with the receptor solution, 1 ml of Centella selected triterpenes extract microemulsions was pipetted into the donor compartment. The donor compartment was covered with parafin film to prevent evaporation of the solvent. The receptor fluid was continuously stirred by magnetic stirring bar. A volume of 3 ml was withdrawn from the receptor cell for analysis at 1, 6, 9, 12, 24, 36, 48, 60 and 72 hours. The receptor compartment was refilled with receptor solution to keep the volume constant during the experiment.

3.2 Preparation of Membrane

The shed snake skin of *Elaphe obsoleta* donated from Pata Private Zoo, bangkok was used. The shed snake skin was storaged in the freezer -20 ± 0.5 °C before use. The dorsal skin was cut to size and hydrated by allowing them to soak overnight in water in covered beaker at 37 ± 0.5 °C. Before experiments, hydrated skin was soaked in receptor fluid for 1 hour at 37 ± 0.5 °C.

3.3 Receptor Fluid

3.3.1 Solubility Studies of Receptor Fluid

Selection of appropriate receptor fluid is important to maintain sink conditions during the permeation study. Ethanol was used to enhance drug solubility in receptor fluid. Excess amount of Centella triterpenes extract was weighed into screw-cap tubes. Varying ratios of ethanol in isotonic PBS were prepared ranging 0-40 % and added in tubes. The tubes were continuously rotated at 37 ± 0.5 °C for 72 hours to achieve equilibration. At equilibrium, the suspensions were filtered through 0.45 µm cellulose acetate membrane filters and then diluted with methanol to make up appropriate concentrations. The concentrations of asiaticoside, madecassic acid and asiatic acid were determined by HPLC. The experiment was carried out in triplicate. Among the isotonic PBS with different concentrations of ethanol evaluated, 40 % ethanol in isotonic PBS was considered to give an optimal increase of the solubility of asiaticoside, madecassic acid and Asiatic acid. Thus, it was used as the receptor fluid in this study.

3.3.2 Receptor Fluid Preparation

Isotonic PBS was prepared by dissolved 2.38 g of disodium hydrogen orthophosphate, 0.19 g of potassium dihydrogen orthophosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 ml and adjust pH if necessary (BP,1999).

After that a mixture of ethanol and isotonic PBS at the ratio of 40:60 v/v was prepared and kept in a refrigerated incubator at 37 ± 0.5 °C.

3.3.3 Permeation Data Analysis

The cumulative amount penetrated through shed snake skin was calculated as follows:

Receptor compartment volume = V

Sample volume withdrawn = 3 ml

Sample #1 (1hr), #2 (2hr), #3 (4hr), #4 (6hr), #5 (9hr), #6 (12hr), #7 (24hr), # 8 (36hr), #9(48hr)

Concentration C1 (1hr), C2 (2hr), C3 (4hr), C4 (6hr), C5 (9hr), C6 (12hr), C7 (24hr), C 8 (36hr), C9(48hr)

Cumulative amount of sample #1 (1hr) = V x C1

Cumulative amount of sample #2 (2hr) = V x C2 + 3ml x (C1)

Cumulative amount of sample #3 (4hr) = V x C3 + 3ml x (C1+C2)

Cumulative amount of sample #4 (6hr) = V x C4 + 3ml x (C1+C2+C3)

Cumulative amount of sample #5 (9hr) = V x C5+ 3ml x (C1+C2+C3+C4)

Cumulative amount of sample #6 (12hr) = V x C6+ 3ml x

(C1+C2+C3+C4+C5)

Cumulative amount of sample #7 (24hr) =V x C7+ 3ml x

(C1+C2+C3+C4+C5+C6)

Cumulative amount of sample # 8 (36hr)=V x C8+ 3ml x

(C1+C2+C3+C4+C5+C6+C7)

Cumulative amount of sample # 9 (78hr)=V x C9+ 3ml x

(C1+C2+C3+C4+C5+C6+C7+C8)

Linear regression analysis of the data was performed by plotting the cumulative amount of asiaticoside, madecassic acid and asiatic acid penetrated through a unit area versus time. From these plots, the flux was calculated corresponding to the slope. The permeability was calculated by dividing the flux by the initial concentration of asiaticoside, madecassic acid and asiatic acid. The effect of oil, surfactant and cosurfactant on permeation profile were studied and selected as following:

a) Effects of Oils

Ten microemulsion formulas were prepared by using either isopropyl myristate or caprylic/capric triglyceride as oil with various surfactants at the ratio of oil: surfactant: water 30: 60:10. Microemulsion formulas are shown in table 6. The selection criteria of the best formula were based on the permeation profiles.

b) Effects of Surfactant

The effects of surfactant in selected oil from section D 3.3.3a on permeation through shed snake skin were determined. The appropriated surfactant resulting in the highest permeation was selected for further study.

c) Effects of Cosurfactant

The effects of type and concentration of cosurfactant on permeation through shed snake skin were studied. Microemulsion formulas are shown in Table 7. The component in formulas 11 to 14, used IPM as oil and Brij 97 as surfactant. In formula 15, tween 80 and span 80 in the ratio of 2:1 were used instead of Brij 97. As

the same criteria as in D 3.3.3a, the appropriate formula was selected based on the permeation study.

Table 7 Microemulsion formulas for permeation study of effect of cosurfactant

Formula	Surfactant	Cosurfactant	Ratio (%by weight) (surfactant: cosurfactant)
11	Brij 97	propan-2-ol	1:01
12	Brij 97	propylene glycol	1:01
13	Brij 97	propan-2-ol	2:01
14	Brij 97	propan-2-ol	4:01
15	Tween 80: Span 80 (2:1)	propan-2-ol	1:01

4. Formulation of *Centella asiatica* Extract Microemulsion

4.1 Preparation of *Centella asiatica* Extract Microemulsion

The microemulsion was prepared by using the selected formula from section D 3.3.3c (Formula 15). Then, *Centella asiatica* extract was incorporated to obtain 2% w/w preparation. The complete formulation was as follows:

Centella asiatica Extract	2%
IPM	30%
Tween 80	20%
Span 80	10%
Propan-2-ol	30%
Water	10%

4.2 Permeation Study of *Centella asiatica* Extract Microemulsion

The procedure of permeation was performed as in section D 3. The permeation profile of Centella selected triterpenes extract microemulsion and *Centella asiatica* extract microemulsion were compared.

4.3 Stability study

The procedure of stability study was performed in the same way as in section C 4. The percent remaining of active ingredients of *Centella asiatica* extract Microemulsion before and after passing the heating-cooling cycles were evaluated.