CHAPTER III

MATERIALS AND METHODS

Materials

1. Fresh plant of ***C. asiatica;*** from Richmond, UK which imported from Sri Lanka.
2. Fresh plant of ***C. asiatica***; from Parkkong market, Thailand.
3. Crude extract of ***C. asiatica***; Lot. No.D440002, Ethanol extract, from drug store , Thailand.
4. Authentic **asiaticoside**; from Extrasynthase, France.
5. Snake skin of cobra; from The Thai Red Cross, Thailand.
6. Acetonitrile (HPLC grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
7. Methanol (HPLC grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
8. Methanol (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
9. Ethanol (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
10. Chloroform (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)

11. Hexane (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
12. Isobuthanol (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
13. Buthanol (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
14. Capric/Caprylic triglyceride (Captex 300®) (Abitex. U.S.A.)
15. Soy bean oil (Sigma, U.S.A.)
16. Isopropyl myristate (BP,USP Grade ,Henkel)
17. Polyoxyethylene 10 oleyl ether (Brij 97®) (The East Asiatic Co.,Ltd. , U.S.A.)
18. Polyoxyethylene 20 sobitan monoleate (Tween 80®) (The East Asiatic Co.,Ltd., U.S.A.)
19. Polyoxyethylene 40 stearate (Myrj52 ®) (The East Asiatic Co., Ltd., U.S.A.)
20. Isopropyl alcohol (A.R. grade, Lab-scan Analytical Sciences, Thailand under license and quality assurance by Lab-scan LTD. Ireland.)
21. Propyl paraben (Henkel, Germany)
22. Propylene glycol (Dow,U.S.A.)
23. Methyl Paraben (Henkel, Germany)
24. Sephadex TM LH-20 (Amercham Pharmacia Biotech AB, Sweden)

Glassware

1. Beaker (Pyrex, U.S.A.)
2. Erlenmeyer flasks (Pyrex, U.S.A.)
3. Funnel (Pyrex, U.S.A.)
4. Glass bottle (Pyrex, Germany)
5. Glass slides (Clay Adam, U.S.A.)
6. Measuring cylinders (Pyrex, U.S.A.)
7. Measuring pipettes (HBG, Germany)
8. Pasteur pipettes (John poulten, England)
9. Petri dishes (Pyrex, U.S.A.)
10. Syringes (Nipro, Thailand)
11. Test tubes (Pyrex, U.S.A.)
12. Volumetric flask (Witeg Diffico, Germany)
13. Volumetric pipette (Brand, Germany)
14. Cylinders (Pyrex, U.S.A.)
15. Pipettes (HBG, Germany)
16. Pasteur pipettes (John poulten, England)
17. Petri dishes (Pyrex, U.S.A.)
18. Syringe (Halmilton, Swizerland)

Apparatus

1. High Performance Liquid Chromatography (HPLC) model – LC - 235 Diode Array Detector. Using an octadecylsilylated silica column, Water , USA.
2. High Resolution Liquid Chromatography-Mass Spectrometry (LC-MS) with Electrospray Ionisation, Micromass. USA.
3. Shimadzu C-R 3A Chromatopac-printer, binary LC pump 250 diote array detector, binary gradient
4. Shimadzu LC-10 ADvP, Absorbance 220, 255 nm, binary gradient
5. HPLC (LC-10 ADVP, Shimadzu, Japan)
6. Guard Column (Inertsil ODS-3, 5 um, 4.0 x 10 mm)
7. Column (Inertsil ODS-3, 5 um, 4.6 x 250 mm, 1KI86100)
8. UV-Visible Spectrophotometer(Cary 100 Scan, Compaq de supra Intel inside celeron, U.S.A)
9. Rotary Evaporator (Rotavapor R-114, Buchi)
10. Transmission electron microscope (JEOL, JEM-200CX, Japan)
11. A modified Franz diffusion cell (Bangkok, Thailand)
12. Viscometer (Rheology (International) Shamnon Ltd., Ireland)
13. Capillary tube melting point apparatus (Buchi)
14. pH meter, Orion model 420A, USA.

Other

1. Filter membrane 0.45 µm 47, 13 mm (Sartolon polyamide, Lada Manufacturing Corp. Germany)
2. Parafilm "M" (Laboratory Film, U.S.A.)
3. Centrifuge tube plastic 50,500 ml (Nalgene, U.S.A.)
4. Clip (Medi-clip, England)
5. Whatman filter paper No.1 (hatman, England)

Methodology

1. Extraction and Isolation of Chemical Components from *C. siatica*

1.1 Extraction of Chemical Components from ***C. asiatica*** by using Ethanol and Hexane.

C. asiatica preparations use only fresh leaves, which were washed properly using distilled water. Three different batches of 22.8 g. each were weighed out and macerated in 200-ml. ethanol at ambient temperature for 3 days. These batches were labeled for analysis, which were green in color.

Weighing 3 batches of dried finely powdered of ***C. asiatica*** extraction from drug store 10 g. from each batches and ethanol 200-ml.into each and

macerated 3 days as same as above method. The colors of macerated extraction were yellow.

All of samples were filtrated by whatman paper No.1 under vacuum, a 5 ml. portion of each was reserved for analysis with HPLC model – LC - 235 Diode Array Detector and high resolution LC-MS with electrospray ionization.

Samples F1 and CA1 were chosen for further partitioned extraction. To either F1 or CA1 was added 200 ml. hexane for 3 days at ambient temperature. After filtration the solvent was removed on a rotary evaporator at 50 °C. The extracted sample of 0.1 g. was weighted and dissolved in 9 : 1 methanol : water and stirred for complete dissolution at ambient temperature and filtered, for further analysis.

Chloroform, isobuthanol and water were used to extract further components from the residue as the Figure 10. Each extract was dried and dissolved in methanol for analysis.

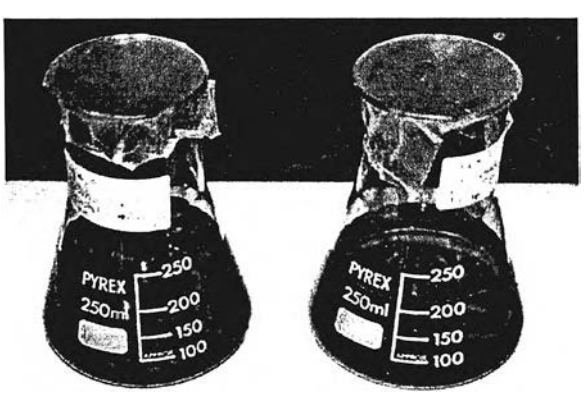

Figure 10 Fresh leaves and of ***C. asiatica*** macerated in chloroform and isobuthanol

Extraction Scheme of *C. asiatica*

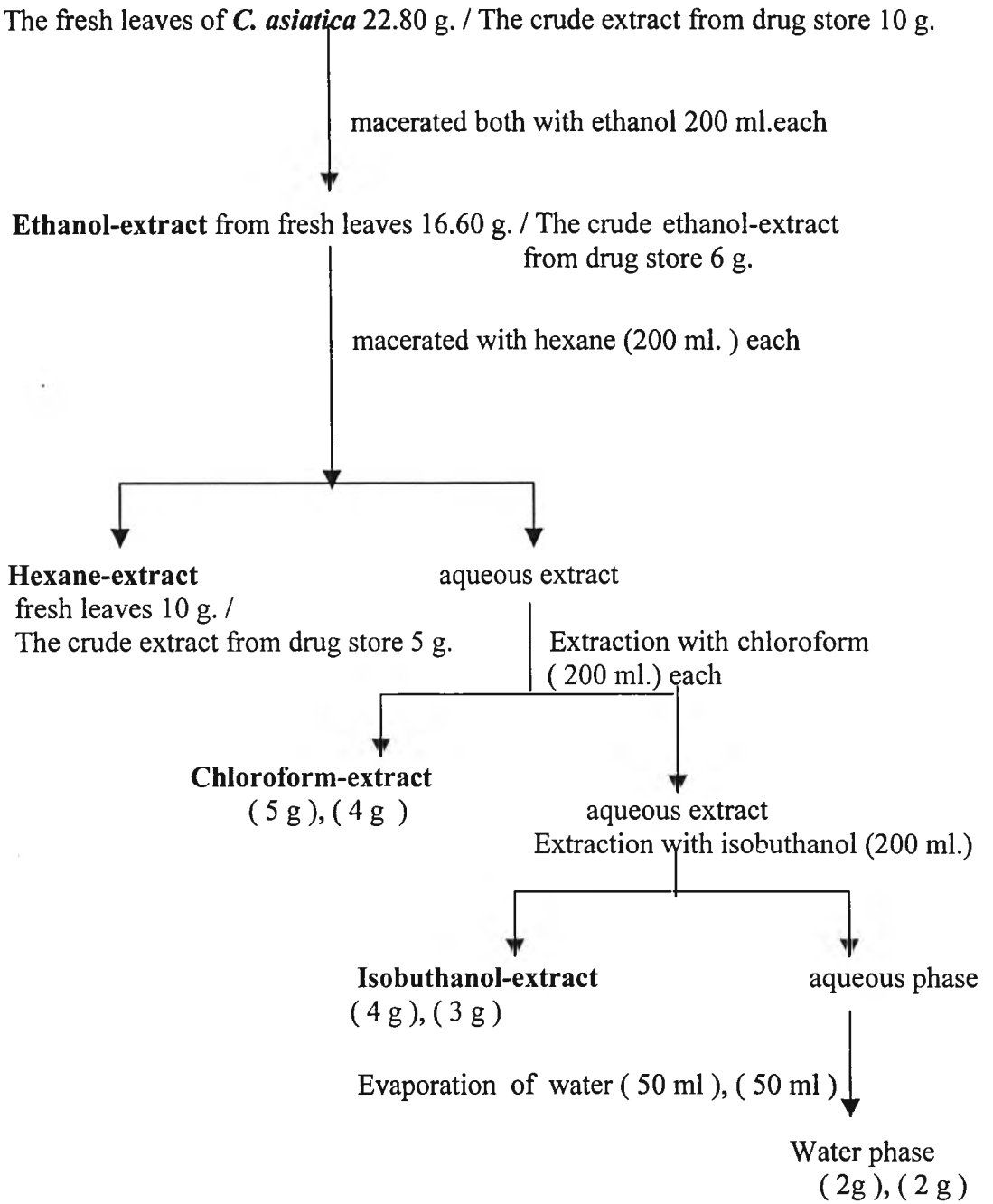

Figure 11 Extraction the components from the fresh leaves of ***C. asiatica*** using ethanol, hexane, chloroform, and isobuthanol. (Rao, et al.1996)

1.2 Extraction of Chemical Components from ***C. asiatica*** using Ethanol, Chloroform and Buthanol and Purification using Sephadex LH-20.

Approximately weight of 18.5 Kg cleaned fresh leaves of *C.asiatica* was macerated with 20 liter ethanol at ambient temperature for 3 days. The maceration was filter by whatman filter paper number 1 under vacuum. The marc was successively extracted until exhausted. The filtrate was evaporated until dry. The ethanol extract was 8 Kg. The 1500 ml of water was added to ethanol extract. Chloroform was used for further extract. The extraction was separate into 2 phase. Phase 1 was chloroform extract which evaporated until dry with rotar evaporator. The aqueous phase was further extract with buthanol 500 ml with 3 successive extraction. The buthanol phase was evaporated using rotar evaporator. The dried extract of buthanol extraction was diluted with methanol and then passed through Sephadex^{TM} LH-20 two times. The receiver solution was recrystallization in methanol. The purified **asiaticoside** was kept for further study. The white crystalline needles of **asiaticoside** were obtained.

Extraction and Purification of Asiaticoside

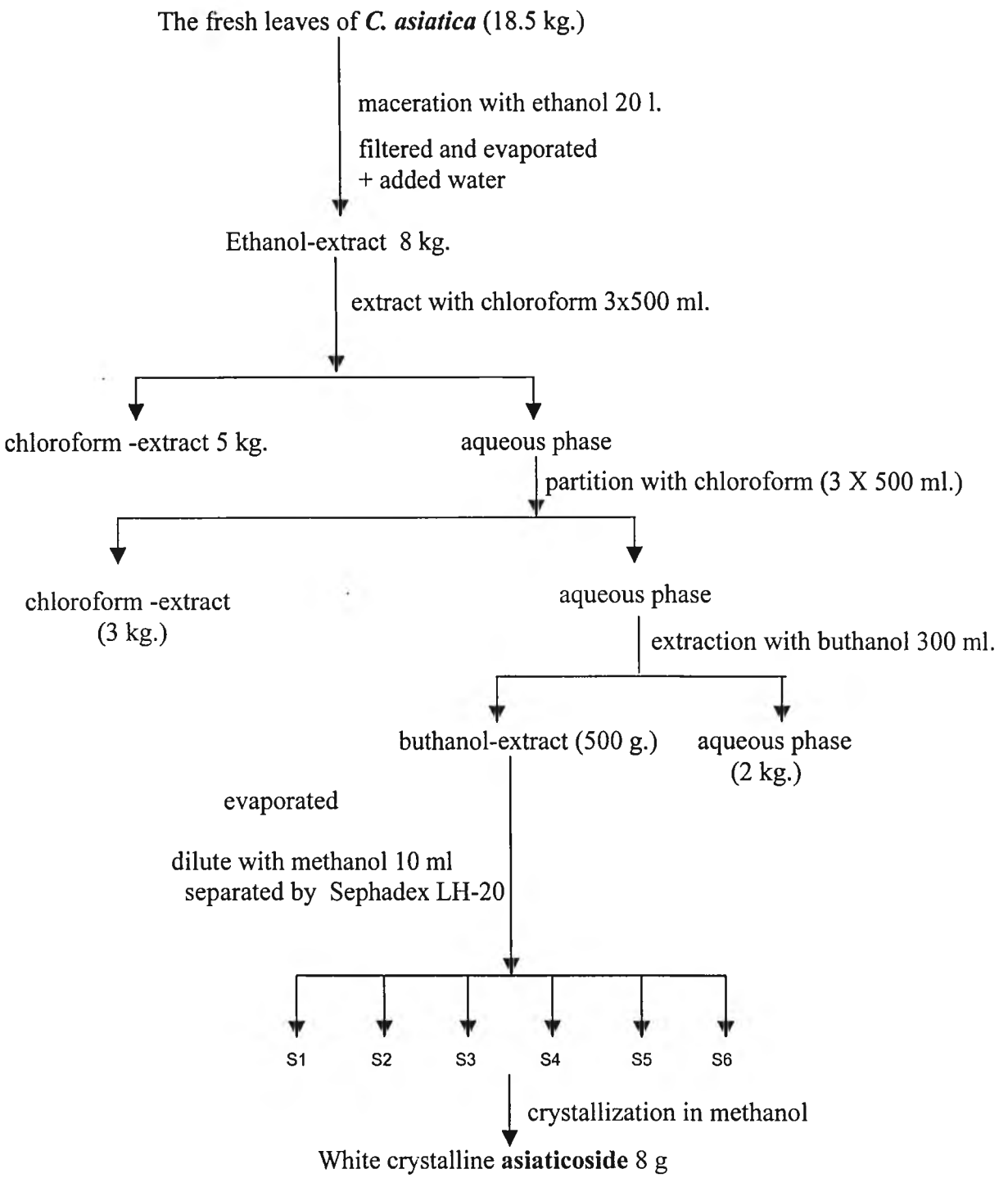

Figure 12 Schematic diagram present the method of extraction and purification of **asiaticeside** from fresh *C. asiatica* leaves

2. Characterization of Purified Asiaticoside

2.1 Melting Point Determination.

The meltimg point of purified **asiaticoside** received by recrystallization in methanol as a white crystalline needle was determined by using Buchi capillary tube melting point apparatus.

2.2 UV-Visible Spectrophotometer

Distilled water was used as a reference for analysis. Each of the samples extracted by the different solvents in 1.1 were analyzed using UV absorption spectroscopy. An accurate weight of purified **asiaticoside** was dissolved in water the maximum absorption curve of **asiaticoside** was determined.

2.3 High Resolution LC-MS with Electrospray Ionization

2.3.1 Preparation of Standard Solution of Dry Extraction of *C. asiatica*

The dry powers of trained from 1.1 and 1.2 were investigated an accurately weighted to be 100 mg. A 10 ml of 9 : 1 methanol : water was added. The solution was filtered and injected into LC-MS instrument. The chromatographic condition used are as following .

LC condition Column Inertsil ODS_3 250 mm X 4.6 X 5 um.

Mobile phase : acetonitrile : Distilled water

Gradient

Flowrate 1 µl/min

Injection 10 µl

UV 220 and 255 nm.

Source parameter

Ionmode : Electrospray positive

Desolvertion Temperature 60°C

Source Temperature 80°C

The chromatogram of fresh ***C.asiatica*** extract and ***C.asiatica*** obtained from drug store were also investigated.

2.4 HPLC Analysis

2.4.1 Preparation of Standard Solution.

An accurate weight of purified **asiaticoside** was dissolved in 9 : 1 Methanol : Water. Four appropriate dilution of **asiaticoside** were prepared to contain 0.2, 0.4, 0.6, 0.8 mg/ml. The calibration curve was plotted between the area response and concentration in mg/ml . The condition used in analysis are as following.

HPLC condition:

Column Inertsil ODS3 5µm (250 X 4.6 mm i.d)

Mobile phase : acetronitrile : distilled water = 20 : 80

Flow rate 1 µl/min

Injection volume 10 µl

UV Detector diode array in the UV range of 220

Formulation of Microemulsion Gel Containing 1% Asiaticoside

Microemulsion gel was formulated using different oil and surfactant concentrations (Ubonthip N., et al.2001) as in Table 2

The amount used in the system was prepared by weight, so the ingredients were weighing in grams and mix together warmed to temperature 60 °C-70 °C using waterbath and stirred together with vortex mixer. In this system microemulsion gels were received with clear appearance.

Brij 97 ®, Myrj 52®, and Tween 80 ® were used as a surfactant. The oil components were capric/caprylic triglyceride, Soy bean oil, and IPM.

Table 2 The composition of ingredients in microemulsion gel

Ingredient	Formula					
	1	2	3	4	5	6
Capric/caprylic triglyceride	15	-	-	-	-	-
Soy bean oil	-	10	10	-	-	-
Isopropylmyristate	-	-	-	10	60	5
Polyoxyethylene 10 oleyl ether (Brij 97®)	69	40	80	-	-	-
Polyoxyethylene 40 stearate (Myrj 52®)	-	-	-	60	-	-
Polyoxyethylene 20 sorbitanmonoleate (Tween 80®)	-	-	-	-	30	40
Water	13	49	9	29	9	54
Asiaticoside	1	1	1	1	1	1

3.2 Preparation of **Asiaticoside** in Microemulsion Gel

The composition and method were carried out as same as described in 3.1. An accurate weight of **asiaticoside** was weighed and added into each of microemulsion gel. The obtained **asiaticoside** microemulsion gels contained 1% **asiaticoside** were kept for further studied.

3.3 Stability Studied

All of six formulae of **asiaticoside** microemulsion gels were tested for there stability by incubating in oven at temperature of 45 °C for 48 hours then transfer to refrigerator at temperature –4 °C for 48 hours. This freeze-thaw cycle was continued for 3 cycles.

3.4 Viscosity Measurement

The viscosities of six formulae were measured by using viscometer at room temperature.

3.5 The pH Measurement

The pH of **asiaticoside** microemulsion gel of 6 formulae was measured by using pH meter, Orion model 420A.

3.6 Transmission Electron Microscopy

The morphologies and particle sizes of **asiaticoside** were investigated by transmission electron microscopy. The samples were freezing before mounted on the stub and coated with gold for four to five minutes with ion sputter. Finally it was observed under transmission electron microscopy.

4. Permeation Study

Three formulae of **asiaticoside** microemulsion gel were selected from physical properties such as viscosity of preparations. After **asiaticoside** was incorporated into microemulsion gel the viscosity was not change. The selected formulae were formula No. 1, 3 and 6.

4.1 Franz Diffusion Cell

The apparatus for permeation studies was modified Franz diffusion cell, which consisted of 2 compartments that are donor compartment and receptor compartment.

The donor and receptor compartments were separated by the cobra shed snake skin which was used as model membrane for human skin. The dorsal region of the shed snake skin was face to the donor chamber containing 1% **asiaticoside** microemulsion gel. The solution of 9: 1 methanol: water was added to the receptor chamber as a receptor fluid the temperature was controlled at 37°C. The receptor chamber was able to contain the fluid with the volume of 13.6 g by weight the diameter of Franz cell was 1.6 cm. corresponding to an effectively permeable area of 2.01 cm^2. The receptor compartment was equipped with a magnetic stirring bar and thermostat at 37°C by circulating water through a jacket surrounding the cell body through out the experiment.

4.2 Preparation and Treatment of Membrane

Cobra shed snake skin was used as a skin membrane model. A whole shed snake skin was cut into square then each piece of shed snake skin was hydrated over night prior to conducting the experiment. Then the piece of skin was clamped in place between the donor chamber and the receptor chamber of the cell.

4.3 Determination of Permeation

Three sets of each formula 1, 3 and 6 were studied. The standard **asiaticoside**, which obtained from France and from drug store were also prepared by using formula1. The **asiaticoside** microemulsion gel was placed in the donor compartment. The receptor fluid was collected at time 1, 24, 48 and 72 hours. The **asiaticoside** was analyzed using HPLC method as same as condition in 2.4.1