CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

1.1 Acetonitrile, AR grade (Fishers)

1.2 Acetonitrile, HPLC grade (Lab scan)

1.3 Anthrone reagent (Sigma)

1.4 Chloroform, AR grade (Lab scan)

1.5 Dichloromethane (Lab scan)

1.6 Di-potassium hydrogen phosphate (Sigma)

1.7 Ethanol (Lab scan)

1.8 Ethyl acetate (Lab scan)

1.9 Filter paper (Whatman, Number1)

1.10 Isopropyl alcohol (Lab scan)

1.11 Membrane filter (sartolon, diameter 47 and 13 mm)

1.12 Methanol, AR and HPLC grade (Lab scan)

1.13 n-butanol (Lab scan)

1.14 Ortho-phosphoric acid (Lab scan)

1.15 Sodium carbonate (Sigma)

1.16 Solid-phase cartridge, C18 500 mg 3ml (Varians)

1.17 Sulfuric acid (Lab scan)

1.18 TLC plate silica gel 60 F254 (E. Merck)

1.19 Asiaticoside, standard (Quangxi chemical, 90.0%)

1.20 Asiatic acid, standard (Quangxi chemical, 95.0%)

1.21 Madecassoside, standard (Quangxi chemical, 95.0%)

1.22 Madecassic acid, standard (Quangxi chemical, 95.0%)

1.23 Prednisolone, standard (Sigma, 98.4%)

2. Instruments

2.1 Extraction Manifold (Waters)

2.2 Hot air oven (BINDER)

2.3 Humidity/temperature chamber (HOTPACK)

2.4 Electric mill (Retsch Muhle)

2.5 Rotary evaporator (BUCHI)

2.6 pH meter (Metrohm 744)

2.7 Ultrasonic bath (BANDELIN: Sonorex digital 10P)

2.8 Microwave (PROLABO: Microdigest 401)

2.9 Densitometer (Shimadzu: CS9301PC)

2.10 High-Performance Liquid Chromatography (Shimadzu)

2.10.1 Pump	:	System module LC-10ADvp
2.10.2 Autosampler	:	SIL-10ADvp
2.10.3 Degasser	:	DGU-14A
2.10.4 Detector	:	Diode-array detector SPD-M10Avp
2.10.5 Controller	:	System controller SCL-10Avp
2.10.6 Software	:	Class VP

2.10 HPLC column (Alltech Alltima C18: 150 x 4.6 mm, 5μm)

3. Methods

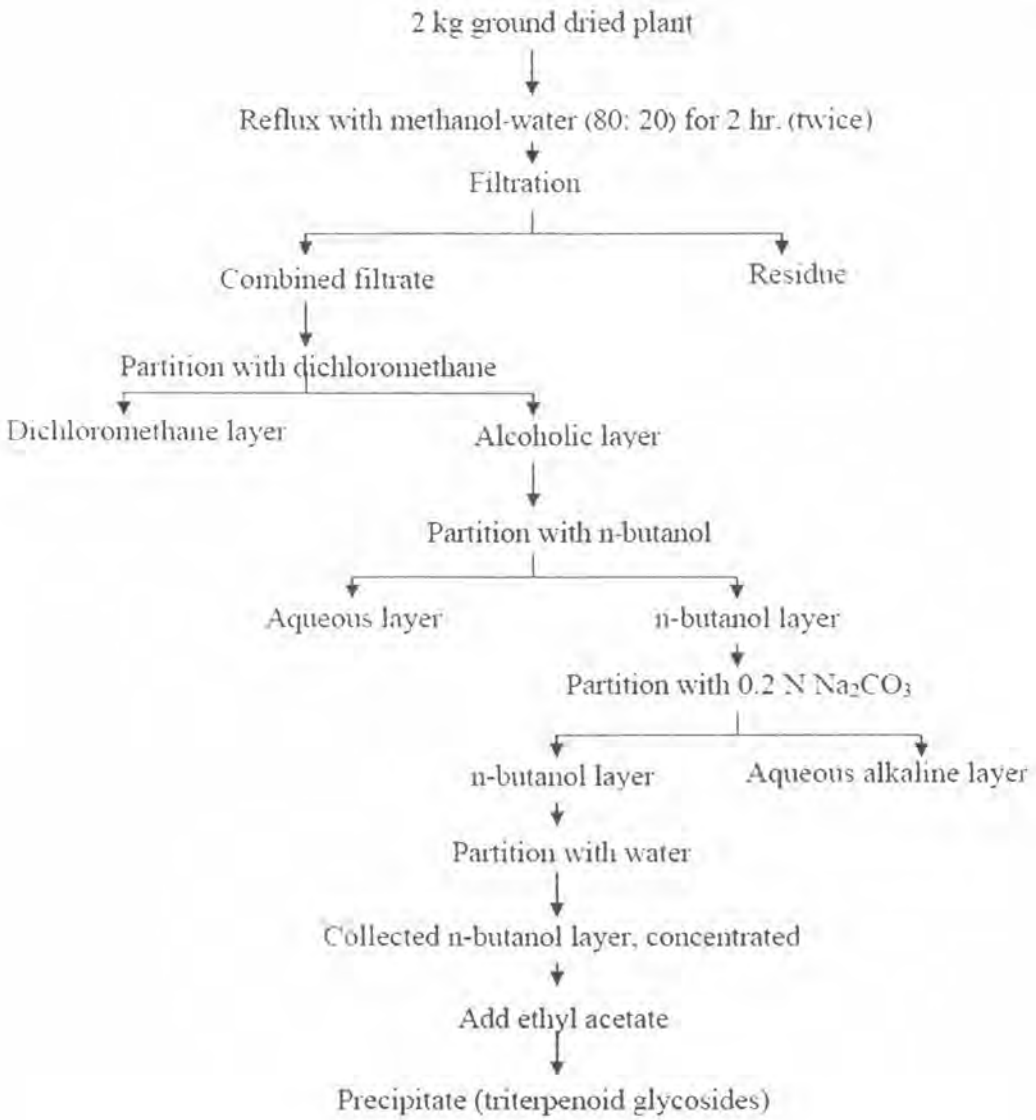
3.1. Extraction and isolation of triterpene glycoside

Scheme 3.1 is the diagram of extraction and isolation of triterpene glycoside. The whole dried ground plant of *Centella asiatica* (L.) Urb. (CA) (2 kg) was refluxed twice with fifteen liters of a mixture of methanol-water (80:20) for two hour and then filtered by vacuum suction. The combined filtrates were partitioned with dichloromethane and n-butanol, respectively. The n-butanol fraction was partition with 0.2 N sodium carbonate solution and finally with water. The collected n-butanol layer was concentrated under reduced pressure at 60 ^{o}C. Ethyl acetate was added to the concentrated n-butanol and the triterpenoid precipitate was obtained.

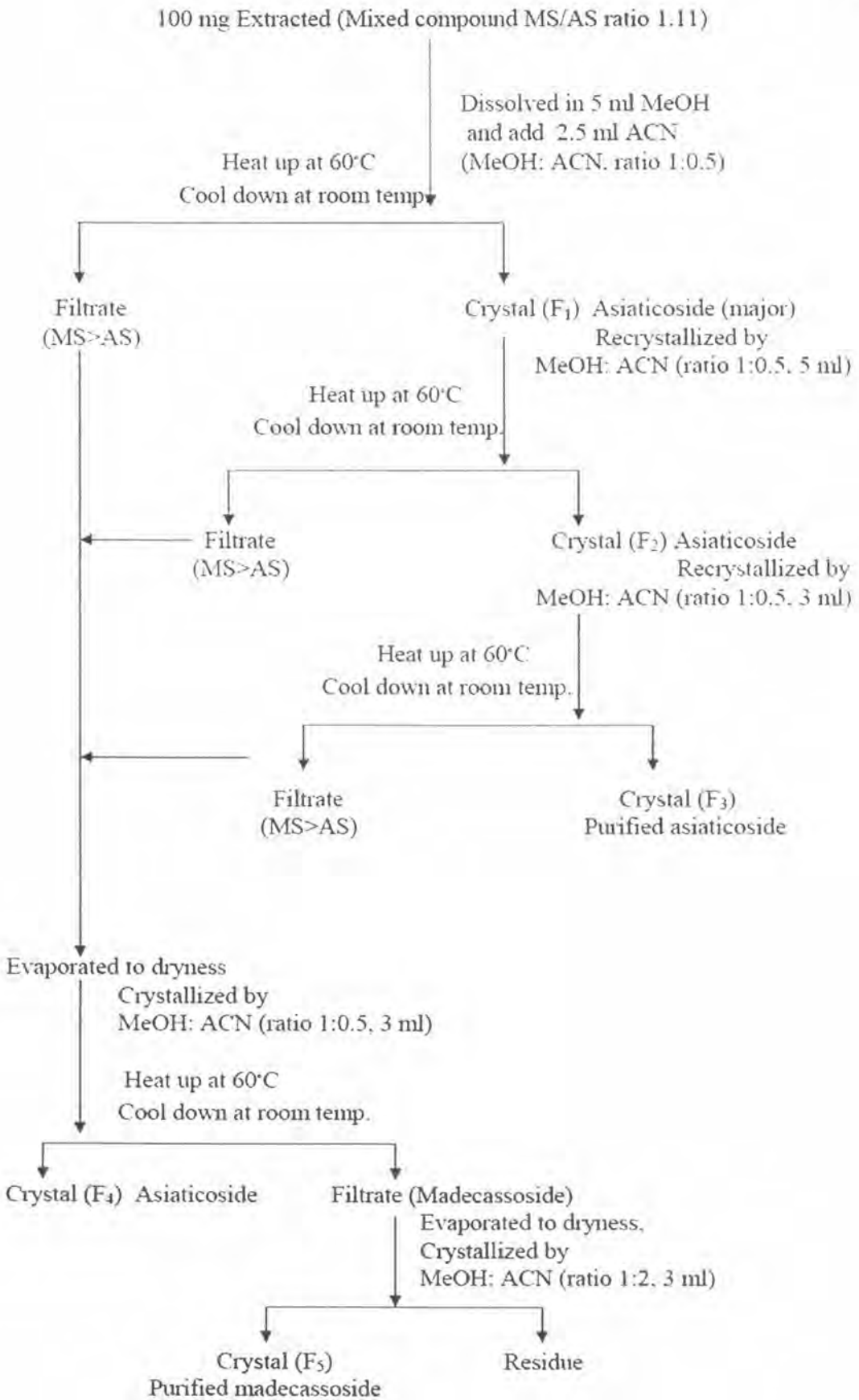
3.2. Preparation of MS, AS, MA and AA as standard

3.2.1 Isolation of AS and MS

The isolation of AS and MS from the triterpenoid precipitate was shown in Scheme 3.2. A 100 mg of triterpenoid glycoside was isolated by fractional crystallization, which used the mixture of methanol: acetonitrile with increasing the ratio of acetonitrile to separate AS and MS. Pure compounds of AS and MS were also finally purified by recrystallization with methanol-acetonitrile.



Scheme 3.1 Extraction and isolation scheme for triterpenoid glycosides from CA

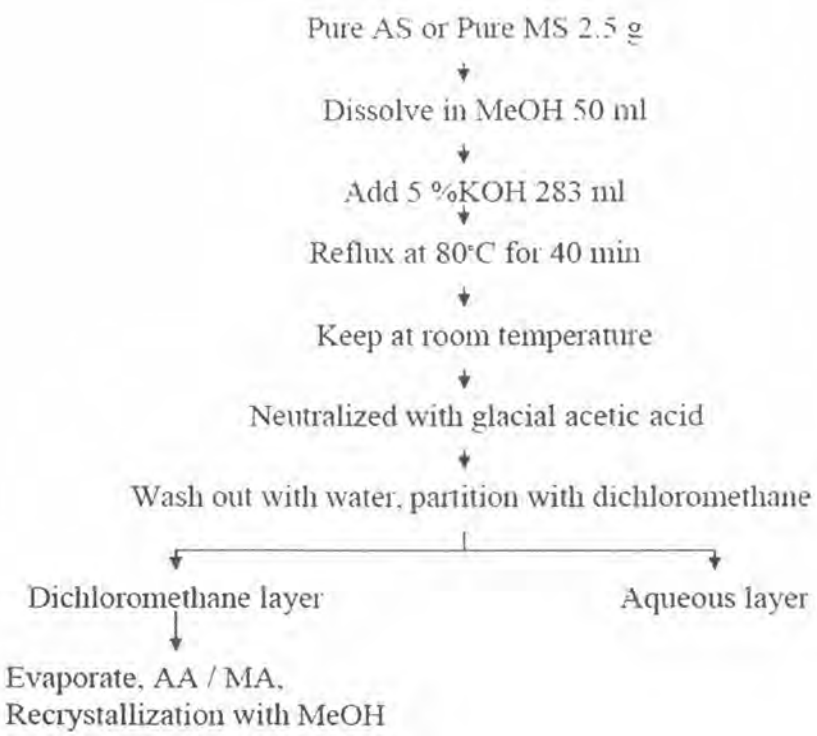


Scheme 3.2 Isolation of AS and MS from triterpenoid glycosides

3.2.2 Preparation of AA and MA

Scheme 3.3 is the diagram of alkali hydrolysis of glycosides to obtain the corresponding aglycones. A 2.5 g of each pure compounds (AS and MS) was separately dissolved in methanol 50 ml and then added 283 ml of 5% potassium hydroxide. The mixture was refluxed for 40 minute at 80 °C, the mixture was neutralized with glacial acetic acid and partitioned twice with 100 ml of dichloromethane. Collected dichloromethane layer was evaporated under reduced pressured to dryness to obtain AA and MA, respectively.

Pure AA and MA were obtained by recrystallization with methanol.



Scheme 3.3 Diagram of alkali hydrolysis of glycosides

3.2.3 Identification of triterpene glycosides and its aglycone

3.2.3.1 TLC method

A 10 mg of each isolated compounds and standard: AS, MS, AA and MA were dissolved in methanol. The solution was applied on a silica gel plate, which was then developed in the mobile phase. Finally, the R_f value of each compounds was detected with anthrone spraying reagent and compared to the standard AS, MS, MA and AA.

Spraying reagent

A 100 mg of anthrone was dissolved in 5 ml of concentrated sulfuric acid and diluted with 50 ml of ethanol.

TLC condition

Stationary phase:	silica gel plate GF_{254} 10 x 20 cm
Developing solvent:	chloroform: methanol: water (30: 15: 2)
Applied:	2 µl

Densitometer parameter

Photo mode:	Reflection
Scan mode:	Linear
Set zero mode:	At start
Beam size:	0.4 x 1.0 mm.
Wavelength:	525 nm.

The developed TLC-plate was sprayed with anthrone reagent and heated at 110 °C for 10 min. The TLC-plate was kept at room temperature for 30 min before determining the absorbance of AS, MS, AA and MA with densitometer at the wavelength of 525 nm.

3.2.3.2 HPLC method

A 10 mg of each isolated compounds and standard was dissolved in methanol and adjusted volume to 100.0 ml (0.1 mg/ml, concentration). The solution was injected on to the HPLC system and then compared the retention time of each compound to standard AS, MS, MA and AA.

HPLC system

Column:	Alltech (Alltima C18, 4.6 x 150 mm, 5 μm.)
Mobile phase:	Acetonitrile-phosphate buffer (10 mM), pH 7.1 (29:71)
Flow rate:	1 ml/min
Detector:	Photodiode array at 210 nm
Injection volume:	20 μl

3.2.3.3 Spectroscopic methods

3.2.3.3.1 Infrared Spectroscopic method (IR)

Approximately 2 or 3 mg of each isolated compounds was mixed and ground with about fifteen mg of previously dried potassium bromide (KBr). The solid mixture was compressed to thin film KBr disc and was scanned with IR spectrophotometer.

3.2.3.3.2 Nuclear-Magnetic Resonance (NMR)

Each isolated compounds was weighed about 10 mg and dissolved with deuterated DMSO in a NMR tube. The mixture was measured in the NMR spectrospin.

3.2.3.4 Physical properties

Approximately 1 mg of isolated compounds was packed and trapped in a capillary melting point tube. The melting points were measured by a melting point apparatus.

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3.2.4 Determination of purity of isolated AS, MS, AA and MA (as working standard)

3.2.4.1 Preparation of reference standard solution

Accurately weighed about 10 mg of each reference standards and dissolved in a mixture of 50% methanol in water. The mixture was adjusted volume to 100.0 ml to obtain the 0.1 mg/ml of standard solution.

3.2.4.2 Preparation of working standard solution

Accurately weighed about 10 mg of each working standards and dissolved in a mixture of 50% methanol in water. The mixture was adjusted volume to 100.0 ml to obtain the 0.1 mg/ml of working standard solution

3.2.4.3 Analysis of standard and working standard solutions

The solutions from 3.2.4.1 and 3.2.4.2 were injected onto a HPLC system, the retention time of each working standard substances was compared to their reference standards and the purity of each working standards was determined and calculated from the peak area as chromatographic purity.

The HPLC system used was the same as in 3.2.3.2

3.2.5 Accelerated stability study of isolated AS, MS, AA and MA

A 10 mg of each standards was accurately weighed and packed in well-close container for four replicates and then kept its into the humidity/temperature chamber at 45 °C, 75 %RH throughout four months. Each standards was sampling from the humidity chamber monthly for determined a content by the HPLC system as in 3.2.3.2

3.3 Development of sample preparation for the HPLC quantitative determination of MS, AS, MA and AA in CA

3.3.1 Comparative study of extraction methods

3.3.1.1 Heat reflux

A 5 g of dried ground plant of CA was refluxing at 70°C into 90 ml of the mixture of 80% methanol-water for 1, 5, 10, 30, 60, 90, 120 and 180 minute. The extracts were filtered, cooled and cleaned up by solid phase extraction. Finally eluted samples were injected onto the HPLC systems. The percentage contents of each triterpenoid compounds were calculated.

3.3.1.2 Ultrasonic-assisted extraction (UAE)

For the UAE experiments, an ultrasonic bath was used as an ultrasound sources. The bath, Sonorex Digital 10P (BANDELIN, GERMANY), was a rectangular container (300 x 240 x 200, mm), which produced HF-frequency at 35 kHz. The bath power rating was 205 W on the scale 0-100 %. The extraction of CA was performed by adding a five gram of dried ground plant into 90 ml of the mixture of 80% methanol-water in the flask. The flask was then partially immersed into the ultrasonic bath, which contains 6 L of water. The solvent surface in the flask was kept at the level of the water in the ultrasonic bath and regulated at constant temperature (70°C) to avoid the water temperature rise, caused by ultrasonic exposure. The extracts were sampling during the extraction time, which carried out for 1, 2, 3, 5, 10, 20, 30 and 60 minute and the ultrasonic bath power was carried out with the three series of 10%, 50% and 100% of power. The extracts were filtered, cooled and cleaned up by solid phase extraction. Finally eluted samples were injected onto the HPLC systems. The percentage contents of each triterpenoid compounds were calculated.

The stability of active compounds during ultrasound exposure has also been investigated by using asiaticoside and madecassoside standards. The solutions were sampling during the extraction time, which carried out for 1, 3, 5, 10, 20, 30 and 60 minute with 50% of power. The mixtures were cooled, adjusted to volume and then injected onto the

HPLC systems. The remained percentage contents of asiaticoside and madecassoside were also calculated.

3.3.1.3 Microwave-assisted extraction (MAE)

The MAE experiment was carried out using an opened-vessel system at atmospheric pressure as a microwave apparatus (Microwave digestion 401, Prolabo). A 2.5 g of dried ground plant of CA was extracted with 45 ml of the mixture of 80% methanol in water. The opened MAE system can be operated at the temperature, which results to refluxing solution. The extracts were sampling during the extraction time, which subjected to the different time of irradiation for 0.5, 1, 1.5, 2, 3, 4, 6, and 8 minute with the same power. After the extraction time had elapsed, the vessels were allowed to cool at room temperature and then filtered, and cleaned up the sample by solid phase extraction (SPE). Finally, eluted samples were injected onto the HPLC systems. The percentage contents of each triterpenoid compounds were calculated.

The stability of active compounds during microwave irradiation has also been investigated by using asiaticoside and madecassoside standards instead of the dried ground plant to perform the microwave-assisted extraction and the remained percentage contents of each standard were also calculated.

3.3.2 Preparation of sample solutions

3.3.2.1 Extracted solution

A 5 g of dried ground plant of CA was refluxed with 90 ml of the mixture of 80% methanol in water for 1.5 hour. The extract solution was cooled down to room temperature, filtered and adjusted volume to 100 ml with deionized water.

3.3.2.2 Internal standard solution

A 10 mg of Prednisolone (PL) was accurately weighed into the volumetric flask and then dissolved in methanol and adjusted volume to 25.0 ml to obtain the 0.4 mg/ml of working internal standard solution.

3.3.2.3 Sample solutions

For each sample solution, which prepared by pipette 5.0 ml of extracted solution, then added 1.0 ml of working internal standard solution (Prednisolone) and adjusted volume to 10.0 ml with mobile phase (the mixture of acetonitrile: phosphate buffer, 10 mM pH 7.1 (29: 71)).

3.3.3 Sample preparation clean up by solid phase extraction

3.3.3.1 Impurities eluting solvent

A 2 ml of sample solution was loaded onto a 500 mg C18 SPE cartridge. A 3 ml of the mixture of 10% of acetonitrile in water was used to wash the impurities from the SPE. The washed eluent was injected onto the HPLC system to detect the absence of the triterpenoid compounds.

3.3.3.2 Analytes eluting solvent

A 2 ml of sample solution was loaded onto a 500 mg C18 SPE cartridge. A 3 ml of the mixture of 10% of acetonitrile in water was used to wash the impurities from the SPE. Six individual cycles of 0.5 ml of the mixture of 45% of acetonitrile in 10 mM (pH 7.10) phosphate buffer was used to elute the analytes out of the SPE cartridge. The eluent was injected onto the HPLC system as in 3.2.3.2 to detect the completeness of the eluting of the triterpenoid compounds.

3.4 Quantitative determination of MS, AS, MA and AA in CA by the HPLC method

3.4.1 Collection of plant sample and storage

3.4.1.1 Sources of plant sample

12 Accessions of CA sample, cultivated by Thailand Institute of Scientific and Technological Research have been labeled as CA1, CA2 ...and CA12. The plant samples were collected from various locations in Thailand as shown in Table 3.4. Another CA sample (labeled as CA13) was collected from the garden in Nongdindang district, Ampur Muang of Nakhon Pathom province as shown in Table 3.5.

3.4.1.2 Sampling time

CA sample from Thailand Institute of Scientific and Technological Research was collected during the middle of the month on July and November, 2005. Another CA sample from Nakhon Pathom province was collected every 2 month from November, 2005 to September, 2006 for annual study.

CA samples in Thailand Institute of Scientific and Technological Research that collected from Ubon Ratchathani province (CA3) and Nakhon Si Thammarat province (CA5) have also been annually studied by

collected that plant from original sources every 2 month during the year 2006 as shown in Table 3.5.

Furthermore, the leaf of CA sample with 1 week, 2 week, 3 week and 4 week old was collected to determine the content of triterpenes that produced in the life cycle of plants.

Each CA samples was refrained from insecticide around 1 week before harvesting and cut about 2 inch above the ground. At least 5 kg of fresh plant were collected each month.

3.4.1.3 Storage of plant sample

Fresh collecting plant was cleaned with water to remove soil and other solid particle, and then air dried at room temperature for 3 days and subsequently dried by hot air oven at 50 °C for 24 hour. The dried plant samples were ground with milling machine. The dried ground plant samples were kept in 3 layers' polyethylene bag and the bag was kept in dry place.

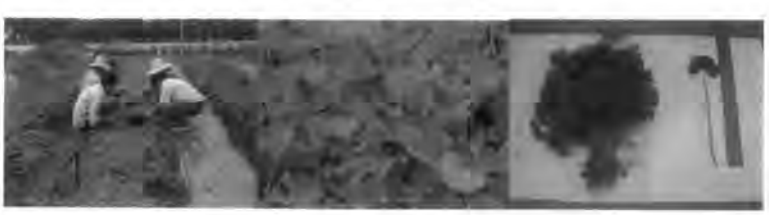

Figure 3.1 Picture of CA planted at Thailand Institute of Scientific and Technological Research and Nakhon Pathom province

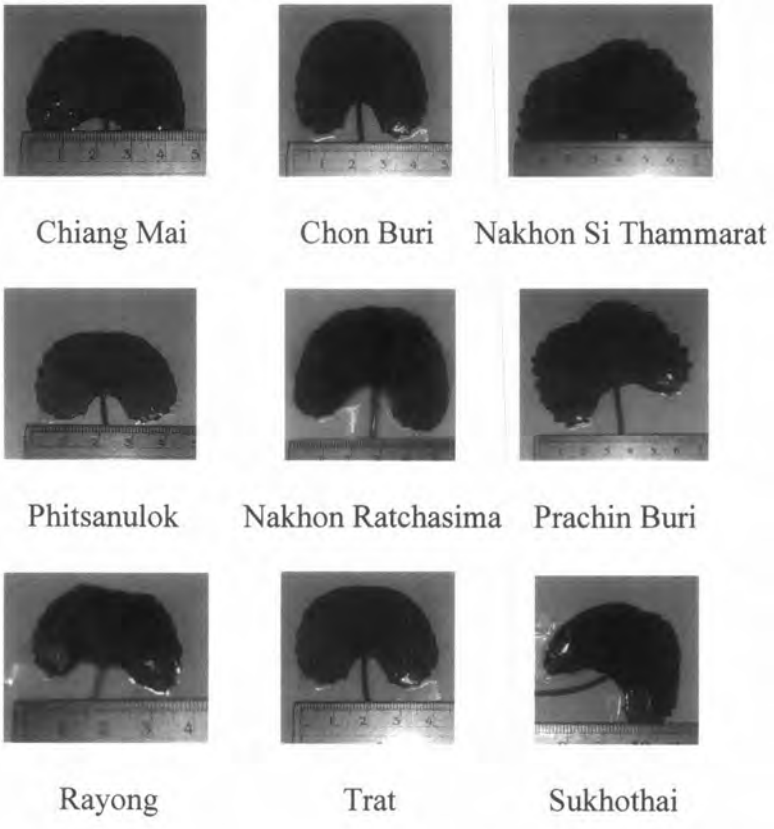

Figure 3.2 Picture of various CA samples at Thailand Institute of Scientific and Technological Research

Figure 3.3 Picture of CA sample at Ubon Ratchathani province

Figure 3.4 Picture of CA sample at Nakhon Si Thammarat province

3.4.2 Determination of MS, AS, MA and AA

3.4.2.1 Preparation of standard solutions

3.4.2.1.1 Stock standard mixture solutions

1) Acid standard solutions

2 aglycone stock standard solutions (A_1 and A_2) were prepared according to table 3.1.

Solution	MA (mg)	AA (mg)	Volume (ml)
A_1	20.0	10.0	25.0
A_2	30.0	15.0	25.0

Table 3.1 Preparation of acid standard solutions for sample analysis

2) Stock standard mixture solutions

3 stock standard mixture solutions (S_1, S_2 and S_3) were prepared according to table 3.2.

Items	Solutions		
	S_1	S_2	S_3
MS (mg)	7.0	27.0	40.0
AS (mg)	5.0	20.0	30.0
A_1 (ml)	1.0	4.0	-
A_2(ml)	-	-	4.0
Volume (ml)	10.0	10.0	10.0

Table 3.2 Preparation of stock standard mixture solutions for sample analysis

3) Internal standard solution

Prednisolone 10.0 mg was dissolved in methanol and adjusted volume to 25.0 ml.

3.4.2.1.2 Working standard solutions

A 1.0 ml of each stock standard mixture solutions (S_1, S_2 and S_3) and internal standard solution were mixed together and adjusted volume to 10.0 with mobile phase as working standard solutions WS_1, WS_2 and WS_3, as sequentially.

Items	Concentration of working standard solutions (mg/ml)		
	WS_1	WS_3	WS_5
MS	0.070	0.270	0.400
AS	0.050	0.200	0.300
MA	0.008	0.032	0.048
AA	0.004	0.016	0.024

Table 3.3 Concentration of working standard solutions

3.4.2.2 Preparation of extract sample solutions

3.4.2.2.1 Extracted solution

A 5 g of dried ground plant was refluxed with 90 ml of the mixture of 80% methanol in water for 1.5 hour. After the extract solution was cooled down to room temperature, the extract solution was filtered and was adjusted to volume 100.0 ml with deionized water.

3.4.2.2.2 Sample solutions

A 5.0 ml of extracted solution and 1.0 ml of internal standard solution were mixed together and adjusted volume to 10.0 ml with the mobile phase of HPLC condition.

3.4.2.2.3 Clean up of sample solution

Each sample was loaded 2.0 ml onto a SPE cartridge (C18, 500 mg). The impurities were washed out with acetonitrile: water (1:9) 3.0 ml. The analytes were eluted with six cycle of 0.5 ml of the mixture of acetonitrile: phosphate buffer, 10 mM pH 7.1 (45: 55).

The analytes were injected onto the HPLC system as in 3.2.3.2 and the peak area of triterpenes was also calculated.

3.4.2.3 Stability study of sample solution

A 10 mg of triterpenoid glycoside was dissolved in methanol and adjusted volume to 100 ml for prepared as stock standard solution.

A 5.0 ml of stock standard solution was spiked into the mixture of acetonitrile and phosphate buffer solution as ratio 29: 71 (the mobile phase of HPLC condition) instead of extracted sample solution as in 3.4.2.2.2.

The sample solution that prepared by standard spiked was filtered through 0.45 μm membrane and injected onto the HPLC system as in 3.2.3.2 for evaluated the stability of sample solution under the room temperature, cooling condition at 4°C during 24 hours and the autosampler of HPLC instrument during 8 hours.