Chapter III

Experiment

1. Source of plant material

The aerial part of Andrographis paniculata Nees were collected in August 1988, from the campus of the Faculty of Pharmaceutical Science, Chulalongkorn University, Bangkok Thailand. This plant was identified by comparison with the voucher at the Royal Florest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

2. Extraction

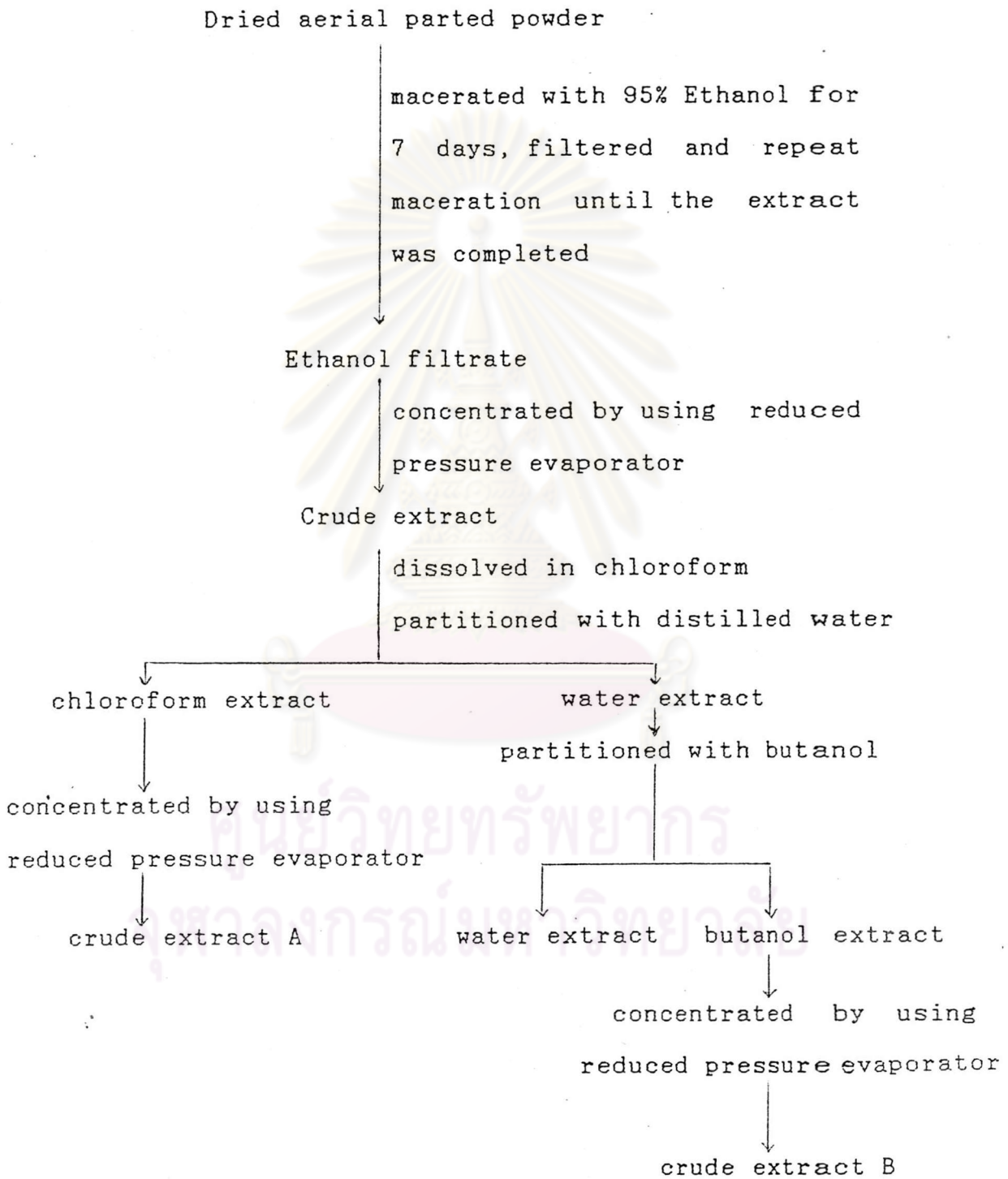
The dried aerial part powder of Andrographis paniculata Nees. (100 g) was macerated with 95% ethanol. The ethanol filtrate was concentrated under reduced pressure evaporator to give a crude extract (22.21 g). The extract was dissolved in chloroform and partitioned with distilled water.

The chloroform extract was concentrated under reduced pressure evaporator to give a crude extract A 11.5 g).

The water extract was partitioned by n-butanol to give butanol extract. The butanol extract was concentrated

under reduced pressure evaporator to give a crude extract B (3.75 g).

The scheme of extraction was performed as the following



3. General techniques for isolation

3.1 Thin-layer chromatography (TLC)

The experimental details were summarised as the following

Analytical TLC

Technique : one way ascending

Adsorbents : Silica gel G (E. merk), Silica gel 60 GF 254 30 g/60 ml of distilled water

Plate size : 20 cm x 20 cm, 20 cm x 5 cm

Layer thickness : 250 μ

Activation : Air dried for 15 minutes and then at 110°C for 1 hour

Distance : 15 cm

Laboratory temperature : 25-30°C

Solvent system

System	Component	Ratio
1	Chloroform	
2	Chloroform : Methanol	8 : 1
3	Chloroform : Methanol	9 : 1
4	Chloroform : Absolute ethanol	85 : 15
5	Chloroform : Acetone	1 : 1
6	Ethyl acetate : Methanol	8 : 2
7	Chloroform : Ethyl acetate : Methanol	5 : 3 : 2

8	Acetone : Methanol	8 : 2
9	Ether : Methanol	7 : 3
10	Benzene : Methanol	1 : 1

Detection of Compound on TLC plate

1. Ultraviolet light at wavelength 254 nm

Unsaturated organic compounds could fluoresce under UV light. Diterpenoid lactone compounds presented violet spots.

2. Iodine vapour

Iodine vapour generated from iodine crystal in a closed vessel could bind to unsaturated organic compounds and presented as brown spot.

3.2 Column chromatography

3.2.1 Open column chromatography

Column size : 3 inch x 10 inch, 2 inch x 14 inch, 1 cm x 50 cm

Adsorbent : Silica gel 60 particle size 0.040-0.063 mm. (E. merck)

Packing : Adsorbent was mixed with solvent system to be slurry then pour into the column.

Sample loading : The portion of crude extract was dissolved in small volume of solvent used for packing

the column and gently applied on top of the adsorbent. If the crude extract was insoluble in solvent used for packing column, dry loading would be operated. The crude extract was dissolved in small volume of volatile solvent then mixed with small amount of silica gel 60 for column chromatography. The mixture should be dried by keeping in the vacuum desiccator. The dried mixture was gently apply on top of the adsorbent.

Solvent : n-Hexane (E. Merck)

Chloroform (E. merck)

Methanol

Ethyl acetate

3.2.2 Quick column chromatography

Column size : 5 inch x 8 inch, 3.5 inch x 4.5 inch, 3 inch x 2 inch

Adsorbent : Silica gel 60 for column chromatography particle size 0.015-0.040 mm

Packing : Adsorbent was packed dried into the column in a single portion to give a depth of 2-3 cm. The column was clamped and connected to the suctioned flask. A low viscosity solvent (e.g. petroleum ether, hexane) in appropriate amount was poured carefully over the adsorbent. Then started to suction by suction pump. The solvent would rapidly flow pass the column. The adsorbent would be packed. The top of the adsorbent bed should be flattened

Sample loading : The portion of crude extract was mixed with silica gel 60 for column chromatography in ratio 1 : 2. The dried mixture was gently applied on top of the adsorbent and flattened.

Solvent : n-Hexane
Chloroform
Ethyl acetate
Methanol

3.3 Crystallisation technique

The purification of impure crystalline compounds was usually effected by crystallisation from a suitable solvent . The crystallisation process consisted of

1. Dissolving the impure substance in some suitable solvent at or near the boiling point.
2. Filtering the hot solution .
3. Allowing the hot solution to cool.
4. Adding the second solvent incase of the crystal was not formed
5. Separating the crystal from the supernatant solution.

The resulting solid after drying was tested for purity by a melting point determination and thin layer chromatography and if found impure was again recrystallised

The process was repeated until the pure compound was obtained.

4. Isolation of chemical substance

4.1 Isolation from crude extract A (Chloroform extract) Crude extract A (11.5 g) was separated by quick column chromatography (The column had 5 inches diameter and 3.5 inches high) with silica gel G 60 100 g. The column was eluted with hexane : chloroform (1:1) , chloroform , and chloroform : methanol (95:5). The volume of eluting solvent were 2500,600, and 4000 ml respectively. Eighty ml fraction was collected from quick column and monitored on TLC. The liked fractions were combined to give the following portions.

Table 2 Fractions eluted from crude extract A

Eluent	Number of fraction	Crude appearance	weight (g)
Hexane:chloroform 1 : 1	1-24	White Crystal in green-yellow oil	0.36
	25-27	Black-green mass	1.08
Chloroform	28-34	Black-green mass	1.91
Chloroform:Methanol 95 : 5	35-43	White Crystal in black-green oil	4.65
	44-47	Clear-square Crystal in black oil	2.300
	48-57	White Crystal in black green mass	3.70
	58-64	Black-brown mass	1.15
	65-75	Black-brown oil	3.07

Isolation of compound C-2 from fraction 28-43

Fraction 28-43 was chromatographed over column chromatography (size 3" x 20")with silica gel 60 100 g and eluted with chloroform : methanol (95 : 5). Thirty ml fraction being collected. Compound C-2 (4.53 g) was separated from fraction 1-10 and was recrystallised in chloroform.

Isolation of compound C-3 and compound C-4 from fraction 44-47 and 48-57

Fraction 44-47 and 48-57 contained white crystal in black-green mass. The white crystal was separated and recrystallised in chloroform and methanol. The black-green mass was chromatographed over column chromatography (size 3" x 20") with silica gel 60 .The column was eluted with chloroform : methanol(95 : 5). Thirty ml fraction being collected compound C-3 (3.52 g) was separated from the combined fraction 1-30 and was recrystallised in chloroform and methanol.

Fraction 31-55 was crystallised in chloroform and methanol. Compound C-4 (1.13 g) was separated and recrystallised.

Isolation of compound C-4 and compound C-5 from fraction 58-64 .

Fraction 58-64 was chromatograph over column chromatography (size 2" x 24") with silica gel 60 70 g. The column was eluted with chloroform : methanol (95 : 5). Thirty ml fraction being collected. Compound C-4(1.22 g) was separated from fraction 17-28 and recrystallised in chloroform and methanol. Compound C-5(0.37 g) was separated from fraction 31-52 and recrystallised in ethyl acetate and methanol.

4.2 Isolation from crude extract B (Butanol extract)

Crude extract B (3.745 g) was mixed with silica gel 60 for column chromatography 7 g and packed to column chromatography (size 3" x 24") by dried loading method. The column was eluted with chloroform : Methanol (95 : 5). Thirty ml fraction being collected. Compound C-5 was separated from fraction 27-40 and recrystallised in ethyl acetate and methanol (0.165 g).

Compound C-6 was separate from fraction 51-61 and recrystallised in ethyl acetate and methanol (0.26 g).

5. Physicochemical property determination

5.1 Physical constant

Melting point : The melting point was determinated by Electrothermal Melting Point Apparatus in Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Science, Chulalongkorn University.

A few mg of sample was ground in agate mortar and a finely powder was filled into capillary tube which was sealed at one end. The sample tube was put into the instrument and the temperature was raised at a rate of 4-5^{o}C per minute and 1^{o}C per minute at the melting point range.

5.2 Spectroscopy

Ultraviolet spectra

Ultraviolet spectra were obtained with a Hitachi 220 A spectrophotometer in the Scientific and Technological Research Equipment Center, Chulalongkorn University.

Accurately weighed an amount of sample in 10 ml volumetric flask, then dissolve with suitable solvent, adjusted to volume with the same solvent. Dilution can be made in order to obtain a suitable concentration. This solution was used to obtain the UV spectrum.

Infrared Spectra

Infrared adsorption spectra were obtained with a Shimaszu IR-440-Infrared spectrophotometer of the Scientific and Technological Research Equipment Center, Chulalongkorn University.

A few mg of sample was ground with small amount of anhydrous potassium bromide in agate mortar. The homogeneous mixture was transfered to a pellet maker. Applying 18,000-20,000 lb/sq inch was enough to make a good pellet which can be used to obtain a good IR spectrum.

Mass Spectra

EIMS spectra were obtained with Jeol Fx 300 double focusing Mass spectrometer of the Scientific and Technological Research Equipment Center, Chulalongkorn University.

A few mcg of sample was introduced directly into the ionization chamber using sample probe. The sample was heated and the mass was scanned. The number of scan was selected and recorded as a mass spectrum.

Nuclear Magnetic Resonance Spectra

NMR spectra were obtained with Bruker NR/200 AF FT-NMR spectrometer in Department of Chemistry, Faculty of Science, Chulalongkorn University.

30-50 mg of sample was dissolved in 1-2 ml of deuterated solvent, filtered, and transfered to a 5 mm NMR tube. Push the sample tube into a spinner then insert the sample tube and spinner into the sample depth gauge. The tube was adjusted until the round bottom was just below the bottom mark appropriate to the sample tube diameter. Insert the sample tube into the magnet, the sample tube would start spinning and signals was adjusted and locked be presented in a color display. Adjusted the "FIELD" in order to position the up and down sweep signals symmetrically about the center of the screen. After the

field was locked shimming was operated to produce maximum amplitude and decay time (ringing) of the observe signal. The parameters were adjusted for required experiment such as proton NMR carbon-13 NMR, DEPT 90, DEPT 135, H,H COSY and C, H correlation.