# CHAPTER III

## EXPERIMENTS

### 1. Source of Plant Materials

The leaves of *Andrographis paniculata* Nees, was collected from the botanical garden in the Faculty of Pharmaceutical sciences at Chulalongkorn University. This plant was identified at the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

The leaves of *Andrographis paniculata* Nees, was dried in hot air oven at 50 $^{o}$C for 24 hours and then ground into powder by an electric mill and passed through sive no. 60.

### 2. General Techniques

#### 2.1 Thin Layer Chromatography (TLC)

The experimental details are summarised as follows :-

Analytical

Technique : one way, ascending

Adsorbents : silica gel G (E.merk), silica gel 60 $F_{254}$, calcium sulphate binder 13%, 30 g/60 ml of distilled water.

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

Layer thichness : 250 microns

Activation : air dried for 15 miniutes and then at 105 $^{o}$C for 1 hour

Distance : 15 cm.

Laboratory temperature : 25-30 $^{o}$C

Solvent system

| System | Component | Ratio |
|---|---|---|
| 1 | Chloroform | - |
| 2 | Chloroform : Methanol | 9 : 1 |
| 3 | Chloroform : Methanol | 8 : 1 |
| 4 | Chloroform : Acetone | 9 : 1 |
| 5 | Chloroform : Absolute ethanol | 85 : 15 |
| 6 | Chloroform : Benzene | 1 : 1 |

Detection of Compounds on TLC Plate

Ultraviolet Detection :- The spot of Andrographolide, Dehydroandrographolide, and Deoxyandrographolide-19β-D-glucoside gave violet colour on TLC plate under ultraviolet light at 254 nm.

Spraying Reagent for TLC :-

1. Kedde's Reagent For unsaturated-γ-lactone compound

2. Iodine Vapour For unsaturated organic compounds.

Reagent : Iodine crystal

Note : The chromatographic plate was placed a closed vessel containing some crystal of Iodine. Iodine vapour generated bind to the spot of organic compound presented as brown spots.

2.2 Column Chromatography

Column size : 10 inch x 5 inch, 1 inch x 14 inch,

Adsorbent : silica gel (silica gel G 60 230-400 mesh ASTM), silica gel G 0.040-0.063 mm. (E.merk)

Packing : Adsorbent was packed as wet method into the column.

Sampling loading : The portion of crude extract was dissolved in a small amount of solvent which was used in packing the column, and transfered directly to the top of the column by a pipet.

Solvent : Hexane (E.merk)
Chloroform (E.merk)
Methanol (E.merk)

## 2.3 Crystallization of The Compound

The technique involved dissolving the material in hot solvent ( or solvent mixture ) and cooled the solution slowly. Crystallization was started upon cooling.

## 2.4 Identification

### 2.4.1 Physical Constant

Melting Point :- Melting point of the compounds were determined by Electrothermal Melting Point Apparatus.

A few mg of sample was filled into a 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 miniute at the melting point range.

### 2.4.2 Spectroscopy

Infrared Spectra :- A11 Infrared absorption spectra were obtained by Shimaszu IR-400. Infrared Spectrophotometer of the Science and Technological Research Equipment Centre, 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 Ib/Sq inch pressure was enough to make a good pellet which can be used to obtain a good IR spectrum.

MS Spectra :- EIMS spectra were determined by Jeol Fx 300 double focusing Mass spectrometer, of the Scientific and Technological Research Equipment Centre, 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 scaned. The number of scan was selected and recorded as a mass spectrum.

3. Isolation of Chemical Substance from the Leaves of *Andrographis paniculata*

3.1 Extraction

A 2 Kg dried leaves powder of *Andrographis paniculata* Nees, was extracted by percolation with ethanol. The ethanolic filtrate was concentrated under reduced pressure to give a gummy residue ( 200 g )

3.2 Isolation of Chemical Substance

30 g of gummy residue (from 3.1) was separated by silica gel quick column chromatography (the diameter of column was 10 inchs and 5 inchs height). The column was eluted with chloroform and increase with

methanol to 20% methanol in chloroform. Each fraction from quick column was monitored by TLC (solvent system no 2). Each eluted fraction which contain major spot from TLC will be combined.

The combined chloroform eluted fractions were evaporated under reduced pressure to give a dark brown syrupy mass. It was dissolved in methanol, chilled and scratched until crystalline precipitate formed. The precipitated was collected and further purify by recrystallization from methanol. Two gram of dehydroandrographolide (C-2) (1% yield) was obtained.

The combined 5% methanol in chloroform eluted fractions were evaporated under reduced pressure to give a dark brown syrupy mass. It was redissolved in methanol, chilled and scratched until crystalline precipitate formed. The precipitated was collected and further purify by recrystallization from methanol. Four gram of andrographolide (C-3) (2% yield) was obtained.

The combined 10% methanol in chloroform eluted fractions were evaporated under reduced pressure to give a dark brown syrupy mass. It was dissolved in methanol, chilled and scratched until crystalline precipitate formed. The precipitated was collected and further purify by recrystallization from methanol. One gram of neoandrographolide (C-4) (0.5% yield) was obtained.

The combined after 10 % methanol in chloroform until 20% methanol in chloroform eluted fractions were concentrated under reduced pressure on rotary evaporator and rechromatograph on fractional column chromatography that packed with silica gel (column size 1" x 14"). Eluted with 10% methanol in chloroform. Deoxyandrographolide-19β-D-glucoside (C-5) was separated from column and recrystallization in methanol. 200 mg of crystal (0.1 % yield) was obtained.

The Authentic samples for andrographolide, neoandrographolide, deoxyandrographolide-19β-D-glucoside were the gift from Professor Lian Xiaotian, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, People's Republic of China.

4. Acetylation

4.1 Acetylation of Andrographolide

Andrographolide (120 mg) was refluxed in pyridine (4 ml) and acetic anhydride (2 ml) for 2.30 hours and the mixture was left at room temperature overnight. The mixture was poured into 30 ml of water, then the solution was extracted three time with 30 ml chiroform. The chloroform extracted was partitioned with water to remove acid. After the evaporation of solvent, pale semisolid product was obtained. The product was further purifyed by column chromatography using silica gel as adsorbent and mixture of hexane : chloroform (3:1) as

elutent. The major product was collected from the column elution. Crystallization from chloroform : ether gave 100 mg needle crystal with mp 134-135 $^{o}$C.

4.2 Acetylation of C-2 Compound

The C-2 compound (100 mg) was stired with acetic anhydride ( 2.5 ml) and pyridine (2.5 ml) at room temperature over night. The isolation of the acetylated product was performed as in 4.1. Crytallization from chloroform-ether the diacetate (105 mg) as needles, mp 134-135 $^{o}$C was obtained.

5. Assay of Four Diterpene Lactones Contents in the leaves of *Andrographis paniculata* by HPLC

5.1 Instruments

1. Pump : PERKIN ELMER series 410
2. Integrator : PERKIN ELMER LCI-100
3. Detector : PERKIN ELMER LC-235 Diode Array

5.2 Chemical

1. Dehydroandrographolide (C-2),
   Andrographolide (C-3),
   Neoandrographolide (C-4),
   Deoxyandrographolide-19β-D-glucoside (C-5)
   was used as standard substance for the analysis. Purity of each compound was checked by TLC (see Table 3).

2. Methanol (E.merk)

5.3 Chromatographic Conditions

| | |
|---|---|
| Column | : μ-Bondapak $C_{18}$ (30 cm x o.39 mm i.d.) |
| Pre Column | : LiChrosorb ® RP-18 5 μm |
| Mobile Phase | : methanol : water (42:58,v/v) |
| Flow Rate | : 2 ml/min |
| Detector Wavelength | : 255 nm, 220 nm |
| Sensitivity | : 0.1 AUFS |
| Chart Speed | : 0.1 cm/min |
| Injection Volume | : 20 mcl |

5.4 Determination of Adherence to Beer's Law

Calibration curve for dehydroandrographolide (C-2) : Five solution of dehydroandrographolide (C-2) were prepared to contain 0.10, 0.20, 0.30, 0.57 and 0.97 mg/ml in methanol. A 20 mcl of each solution were chromatographed twice. Retention time, peak heights and peak areas for each chromatogram were recorded. The resulted obtained were shown in Table 4. The calibration curve was plotted between peak heights or peak areas against the concentrations of dehydroandrographolide (C-2) (see Figure 17).

Calibration curve for andrographolide (C-3) : Four solution of andrographolide (C-3) were prepared to

contain 0.10, 0.21, 0.31 and 0.42 mg/ml in methanol. A 20 mcl of each solution were chromatographed twice. Retention time, peak heights and peak areas for each chromatogram were recorded. The resulted obtained were shown in Table 5. The calibration curve was plotted between peak heights or peak areas against the concentrations of andrographolide (C-3) (see Figure 18).

Calibration curve for neoandrographolide (C-4) : Four solution of neoandrographolide (C-4) were prepared to contain 0.10, 0.15, 0.20, and 0.30 mg/ml in methanol. A 20 mcl of each solution were chromatographed twice. Retention time, peak heights and peak areas for each chromatogram were recorded. The resulted obtained were shown in Table 6. The calibration curve was plotted between peak heights or peak areas against the concentrations of neoandrographolide (C-4) (see Figure 19).

Calibration curve for deoxyandrographolide--19β-D-glucoside (C-5) : Five solution of deoxyandrographolide-19β-D-glucoside (C-5) were prerared to contain 0.02, 0.04, 0.08, 0.10, and 0.20 mg/ml in methanol. A 20 mcl of each solution were chromatographed twice. Retention time, peak heights and peak areas for each chromatogram were recorded. The resulted obtained were shown in Table 7. The calibration curve was plotted between peak heights or peak areas against the concentrations of deoxyandrographolide-19β-D-glucoside

(C-5) (see Figure 20).

5.5 Determination of the Reproducibility of Peak Height and Peak Area

20 mcl of each four diterpene lactones solution (dehydroandrographolide (C-2) 0.30 mg/ml in methanol, andrographolide (C-3) 0.31 mg/ml in methanol, neoandrographolide (C-4) 0.20 mg/ml in methanol and deoxyandrographolide-19β-D-glucoside (C-5) 0.08 mg/ml in methanol), were chromatographed five times. The retention time peak height, peak area were recorded in the chromatogram. The results obtained were shown in Table 8--11.

5.6 Determination of Four Diterpene Lactones Contents in the leaves of *Andrographis paniculata*

Standard Solution :

Standard solution of four diterpene lactones used as external standard solution were prepared as follow.

Four solution of dehydroandrographolide (C-2) were prepared to contain 0.10, 0.30, 0.50, 0.70 mg/ml in the methanol.

Four solution of andrographolide (C-3) were prepared to contain 0.10, 0.30, 0.50, 0.60 mg/ml in methanol.

Four solution of neoandrographolide (C-4) were

prepared to contain 0.05, 0.10, 0.15, 0.20 mg/ml in methanol.

And Four solution of deoxyandrographolide-19β-D-glucoside (C-5) were prepared to contain 0.02, 0.09, 0.20, 0.30 mg/ml in methanol.

Sample Collection :

The one year old leaves of *Andrographis paniculata* was collected from the botanical garden in the Faculty of Pharmaceutical Sciences at Chulalongkorn University. The tenth pair leaves counted from the top of the plant were collected at three o'clock in the afternoon, on the 20$^{th}$ of each month started from January 1988 until December 1988. The leaves were dried in the vacuum desicator which contains silica gel as the drying agent for 24 hours. The dried sample was kept in tight container containing silica gel. Prior to the assay, the dried leaves was ground into fine powder with electric mill and passed through sieve no. 60.

Assay Preparation

A accurately weighed 250 mg of the crude powder was placed in a Soxhlet's extraction apparatus, 100 ml of methanol, was used to extract the diterpene by refluxing for 1.30 hours. The methanolic extract was evaporated under reduced pressure until 20 ml of solution remained. The concentrated methanolic was transfered quantitative,

to a 25 ml volumetric flask, then the volume was adjust with methanol.

Chromatographic Procedure

20 mcl of the assay preparations and the standard preparation were injected into a high performance liquid chromatographic system. The UV detecter was set at 255 nm from the start of injection to 22 minutes, then the wavelength was changed to at 220 nm until the end of chromatogram. External standard solution was injected after every two injection of the sample solution injection. The peak height for each injection were recorded in the chromatogram and the results was shown in Table 12-15.

Calculations

The percent w/w of each diterpene lactone in *Andrographis paniculata* powder was calculated as follow :

$$\% \text{ w/w of each diterpene lactone in the crude drug} = \frac{Hp}{Hs} \times \frac{Cs}{Wt} \times \frac{V}{1000} \times 100$$

where,

Hp = peak height of diterpene lactone in assay preparation

Hs = peak height of diterpene lactone standard solution

Cs = concentration of diterpene lactone standard solution in mg/ml

Wt = weight of the crude powder used (mg)

V = final adjust volume in ml ( V = 25 )

Quantity (% w/w) of each diterpenoid contents in the leaves of *Andrographis paniculata* Nees collected monthly are shown in Table 16 and Figure 25.