CHAPTER II

EXPERIMENT

2.1 Plant Material

Roots of *Tylophora indica* Merr. were purchased from Vetchapong-osot, a Thai medicinal plant shop in Bangkok, Thailand, on March, 2001. This plant was identified by Associate Professor Dr. Obchan Thaithong, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. A voucher specimen has been deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

2.2 Chemicals

2.2.1 Solvent

Most of solvents in this research were a commercial grade and distilled before using. HPLC grade solvents were used for high performance liquid chromatography (HPLC) and reagent grade solvents were used for crystallization.

2.2.2 Adsorbent

Adsorbents such as silica gel 60 Merck, cat. No. 7731, 7734, 7749 and 9815 were used for quick column chromatography, column chromatography, chromatotron and flash column chromatography, respectively. TLC were performed on precoated Merk silica gel 60 F254 (0.25 mm-thick layer). Gel filtration chromatography was performed on sephadex LH 20.

2.2.3 Dipping Reagent

The 10% of sulfuric acid in ethanol was routinely used for detecting spots of some compounds, the following reagents were used to detect certain functional groups or class of compounds.

2.2.3.1 Liebermann-Burchard Test

A small sample was dissolved in chloroform and treat with acetic anhydride and a few drops of concentrated sulfuric acid. A steroid sample, the color would gradually change from pink to deep green. If the sample was triterpenoid, the color would change to reddish pink.

2.2.3.2 Dragendorff's reagent

2.2.3.2.1 Dragendorff' s spray reagent

This reagent was used for detect alkaloids on TLC plate. The positive test showed the orange spot. The preparation of this reagent was described as below:

Stock solution A : 0.6 g of bismuth nitrate was dissolved in 2 mL of conc. HCl and added 10 mL distilled water.

Stock solution B : 6 g of KI dissolved in 10 mL distilled water.

Stock solution A and B were mixed and 7 mL conc. HCl and 373 mL distilled water were added.

2.2.3.2.2 Dragendorff's reagent

A solution of bismuth nitrate (8g) in HNO_3 (12 mL) was mixed with a solution of potassium iodide (27.2 mL) in distilled water (50 mL). Distilled water were added to make 100 mL of this solution.

2.3 Equipments

2.3.1 Rotary evaporation

The large amounts of all volatile organic solvents were evaporated by the Buchi rotary evaporator.

2.3.2 Chromatotron equipment

Chromatotron (model 7924 T, Harrison Research) on a silica gel plate of 1 and 2 mm thickness was performed on centrifugal thin layer chromatography.

2.3.3 High Performance Liquid Chromatography (HPLC)

HPLC was performed on Gilson model 303 with a Alltech RP-C_{18} (250×22 mm) column and a UV detector.

2.3.4 Melting point apparatus (m.p.)

Melting Points were determined with Fisher-John Melting Point apparatus and the value was uncorrected.

2.3.5 Fourier Transform-Infrared Spectrophotometer (FT-IR)

Nicolet Impact 410 FT/IR spectrophotometer was used for record infrared spectra.

2.3.6 Mass Spectrometer

The Electron Impact Mass Spectra (EIMS) were obtained on Mass Spectrometer Model VG TRIO 2000.

2.3.7 ^{1}H and ^{13}C-Nuclear Magnetic Resonance Spectrometer

^{1}H and ^{13}C spectra were carried out with a JEOL JNM-A 500 FT-NMR spectrometer and a Bruker AC-F 200 FT-NMR spectrometer. The chemical shift in ppm was assigned with reference to the residual proton in deuterated solvent. Solvent for NMR spectra were deuterated chloroform ($CDCl_3$), deuterated acetone (acetone- d_6) and deuterated dimethyl sulfoxide (DMSO-d_6).

2.3.8 Gas Chromatography (GC)

Steroid analysis was performed on Shimadzu GC-7AG at Scientific and Technology Research Equipment Center, Chulalongkorn University.

2.3.9 GC-MS

Methylated carboxylic acid analysis was performed by CP-Wax57CB column (25 m × 0.25 mm i.d., 0.25 μm film thickness), GC Model Star 3400 Cx and Ms Model Saturn 4D from Varian. The temperature program was set 60 °C for 1 min, 60-270 °C at 25 °C/min and 5 min hold at 270 °C.

2.4 Bioassay on Plant Growth Regulators

Plant growth regulator test was used as a main bioassay to verify the presence of bioactive compounds. Selected plants for this investigation were *Brassica pekinensis* Rupr. (lettuce) and *Echinochloa crus-galli* (L.) Beauv. The bioassay were performed as follows:

2.4.1 Brassica pekinensis Rupr.[28]

Test solutions were prepared at concentrations of 1000, 100 and 10 ppm. One milliliter of test solutions was added into petri-dish (25 mm diameter) that contained a filter paper disc. A control was an equal amount of the solvent used to prepare the test solution. All petri-dishes were dried overnight. Ten seedlings of *B. pekinensis* Rupr. were placed in a Petri-dish wetted with 1 mL of distilled water. The petri-dishes were covered with the lid and sealed with parafilm to

prevent the evaporation of water. These petri-dishes kept under daylight for seven days. Growth was quantified by measuring the length of roots and shoots. All tests and controls were run in three replicate and averaged. Percent of growth inhibition could be calculated from the following formula.

$$\% \text{ Growth Inhibition} = [1-(T/C)] \times 100\ \%$$

When "T" was the mean roots or shoots length of treated seedling.

"C" was the mean roots or shoots length of controlled seedling.

Growth inhibition of 100% revealed a complete inhibitory effect.

2.4.2 Echinochloa crus-galli (L.) Beauv.[29]

Seed of *E. crus-galli* Beauv. were soaked in distilled water for overnight. Five germinant seeds were taken into a glass tube (10 mm diameter) containing test solution concentrations which were prepared in the same manner as 2.4.1. The final volume was 500 µl by adding water and then the tube is tightly covered with parafilm. The tube was left under daylight for seven days, and then the lengths of the second leaf sheaths were compared with those of seedlings grown in only water. Percent of growth inhibition could be calculated from the same formula as 2.4.1.

2.5 Extraction

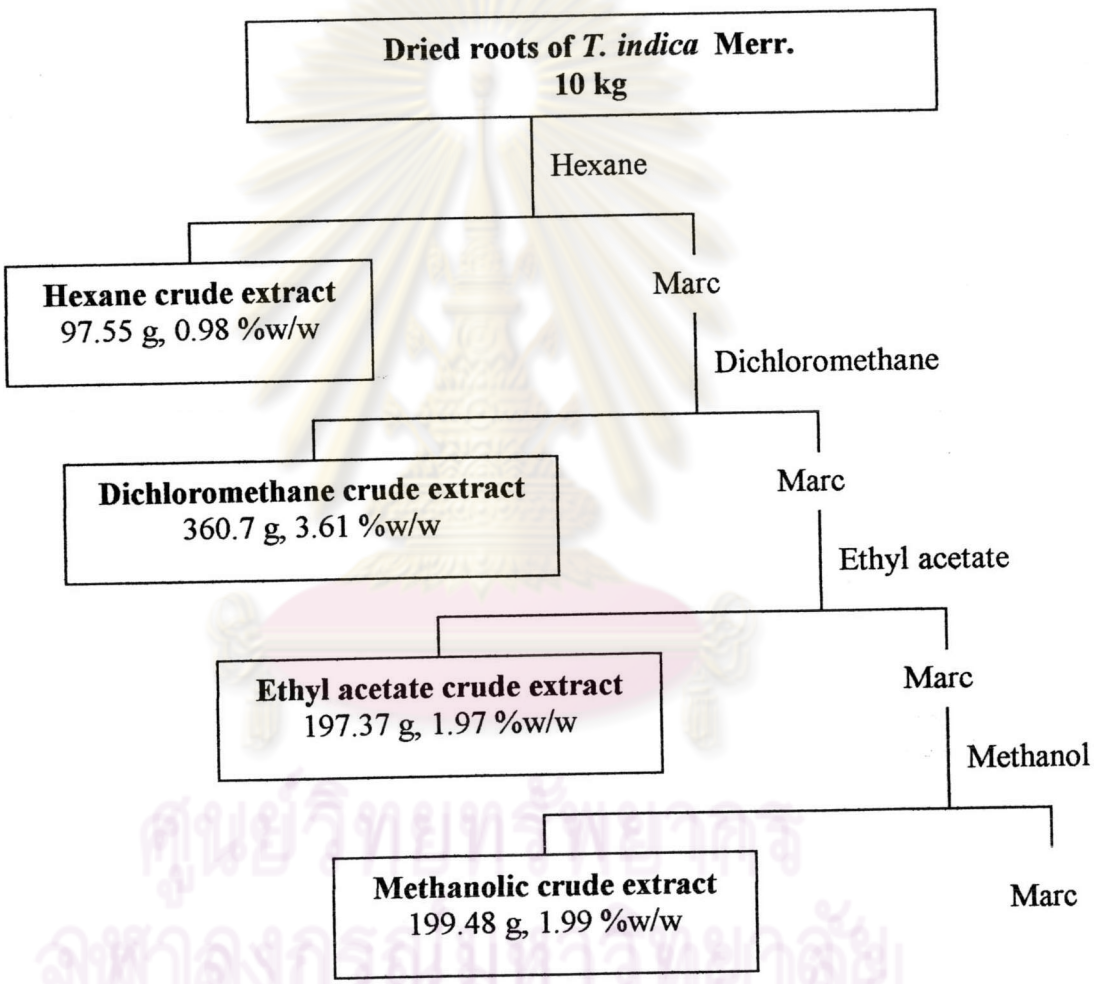
2.5.1 Extraction of 12 plants

Twelve plants were air-dried and milled. Each of specimens was soaked in dichloromethane and methanol, respectively. The two extracts of each plant were filtered and evaporated by rotary evaporator to dryness, yielding dichloromethane and methanolic crude extract.

2.5.2 Extraction of root of T. indica Merr.

Dried and coarsely roots of *T. indica* Merr. (10 kg) were soaked in hexane for 7 days at room temperature. After filtered, the solution was evaporated under reduced pressure to dryness. This procedure was repeated three times to give hexane crude extract 97.55 g (yield 0.98 %w/w). The residue was re-extracted with

organic solvents, dichloromethane, ethyl acetate and methanol, respectively in the same manner as crude hexane, yielding dichloromethane crude extract 360.7 g (yield 3.61 %w/w), ethyl acetate crude extract 197.37 g (yield 1.97 %w/w) and methanolic crude extract 199.48 g (yield 1.99 %w/w), respectively. The extraction procedure and results for preliminary study are epitomized in **Scheme 2.1**



Scheme2.1 The extraction procedure for preliminary study of the roots of *T. indica* Merr.

2.6 Separation and purification

First, dichloromethane and ethyl acetate crude extracts were separated by quick column chromatography techniques. The crude extract was dissolved in a little amount of a suitable solvent and mixed with silica gel (1:1) to afford the extract paste. The paste was evaporated to dryness by rotary evaporator before to subject on the top of sintered glass filter column of silica gel 60 (No. 7731). The column was eluted by increasing polarity of solvent. Each subfraction was monitored by TLC and subfractions which had the same components were combined. Subfractions from dichloromethane and ethyl acetate crude extracts of the roots of *T. indica* Merr. are shown in Tables 2.1 and 2.2.

Table 2.1 The separation of dichloromethane crude extract by quick column chromatography.

Subfraction code	Solvent system	Weight (g)
A	hexane – 1:1 CH_2Cl_2/hexane	1.89
B	3:2 – 7:3 CH_2Cl_2/hexane	5.63
C	9:1 CH_2Cl_2/hexane - CH_2Cl_2	5.05
D	1:9 - 3:7 EtOAc/CH_2Cl_2	11.94
E	1:1 - 7:3 EtOAc/CH_2Cl_2	26.51
F	EtOAc	35.61

Table 2.2 The separation of ethyl acetate crude extract by quick column chromatography.

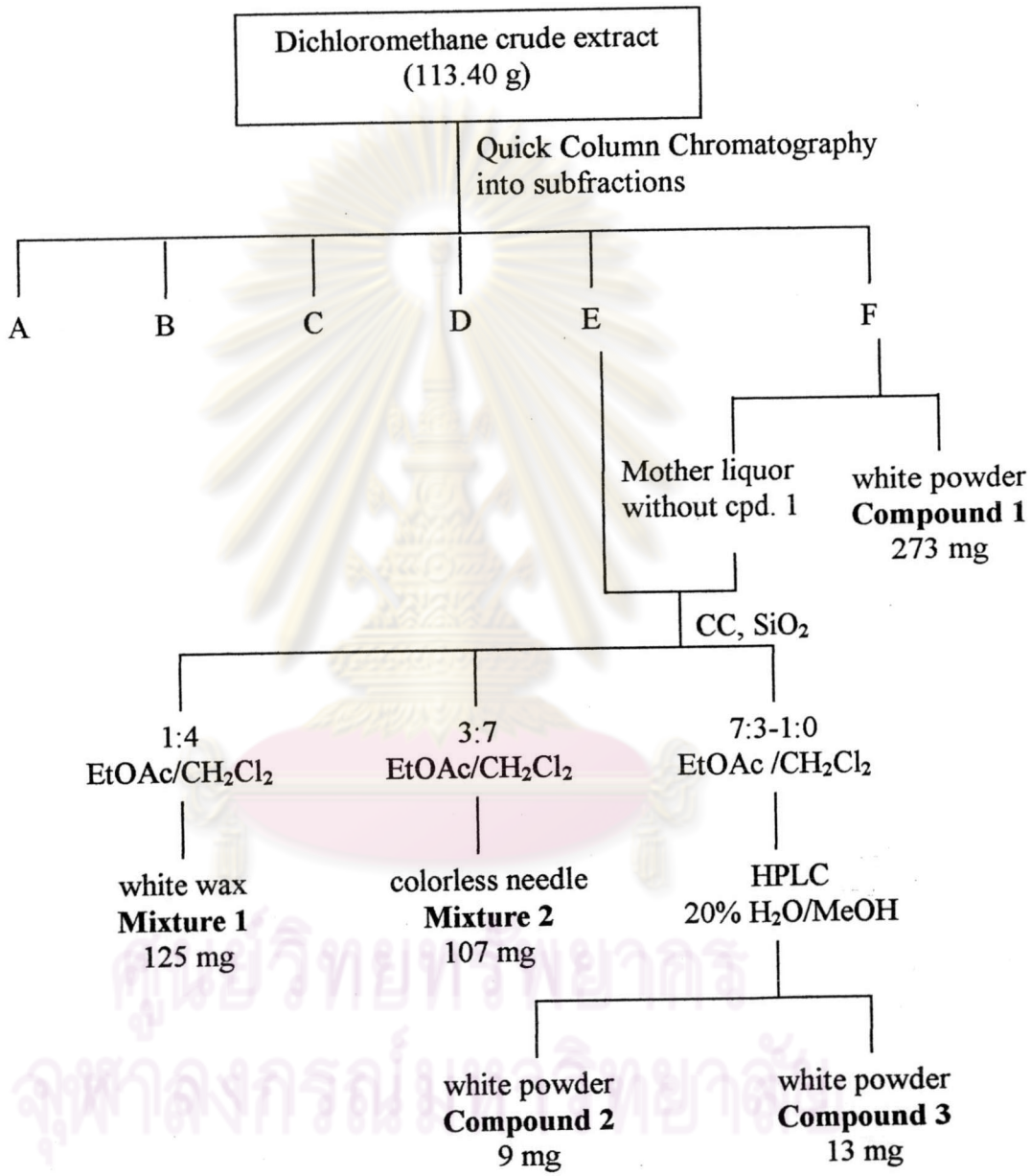
Subfraction code	Solvent system	Weight (g)
1	CH_2Cl_2-2:3 EtOAc/CH_2Cl_2	7.14
2	3:2 – 4:1 EtOAc/CH_2Cl_2	19.77
3	EtOAc	10.80
4	1:4 – 2:3 MeOH/EtOAc	42.44
5	3:2 – 4:1 MeOH/EtOAc	11.61
6	MeOH	0.29

After the concentrated subfraction F was left overnight, the white powder was deposited. It was recrystallized in hot MeOH for several time to yield **compound 1** (273 mg, 0.24 %w/w). Based on their TLC pattern, the remained subfraction F was combined with subfraction E. The combined fraction was subjected to silica gel column chromatography which packed in CH_2Cl_2. Elution was performed with CH_2Cl_2 gradiant of increasing amounts of EtOAc. Each fraction was monitored by TLC. The fraction eluting with CH_2Cl_2-EtOAc (4:1) gave a **mixture 1** (125 mg, 0.11 %w/w). The fraction eluting with CH_2Cl_2-EtOAc (7:3) gave a **mixture 2** (107 mg, 0.09 %w/w). The mixture from the fraction eluting with 70-100% EtOAc in CH_2Cl_2 was purified by HPLC (C_{18} reversed-phase, 20% H_2O-MeOH) to yield **compound 2** (9 mg, 0.008 %w/w) and **compound 3** (13 mg, 0.01 %w/w).

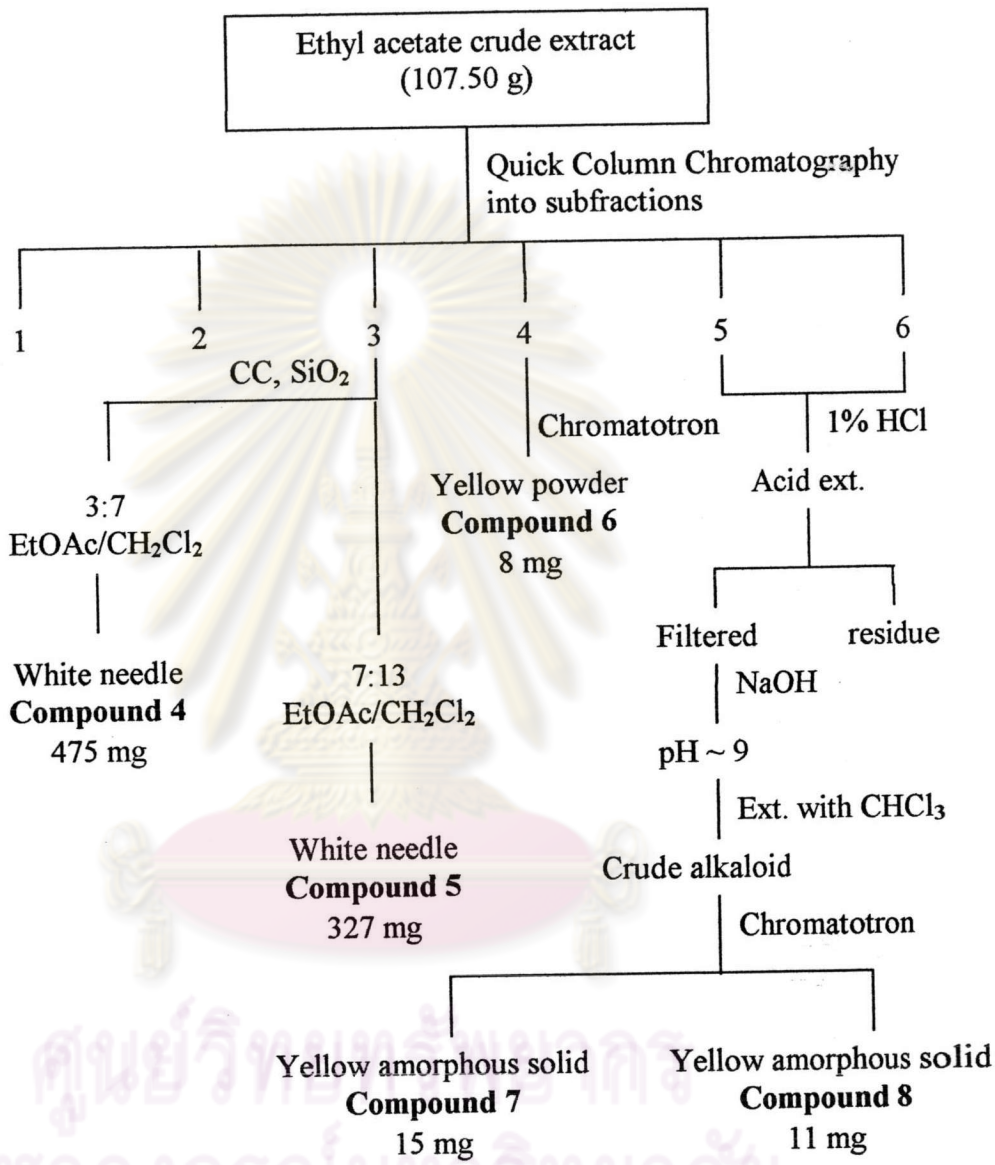
Subfraction 3 from ethyl acetate crude extract was chromatographed on silica gel column by elution with mixtures of CH_2Cl_2-EtOAc (from 9:1 to 1:4, then pure EtOAc). Fractions were combined based on their TLC patterns. Fraction eluting with CH_2Cl_2-EtOAc (7:3, 7:13 and 2:3) gave **compound 4** (475 mg, 0.44 %w/w) and **compound 5** (327 mg, 0.30 %w/w). From TLC patterns, subfraction 4 showed fluorescence spot under UV light (long-wavelength, 365 nm), then chromatotron was used advantageously for the reseparation of this fraction. Elution with EtOAc-hexane (1:1) yielded **compound 6** (8 mg, 0.007 %w/w).

Subfraction 5 and 6 gave positive test to Dragendorff reagent. The separation of crude alkaloid, subfraction 5 and 6 was extracted with 1% HCl. The acid filtered extract was adjust with NaOH until to be alkali (pH 9) and then successively extracted with $CHCl_3$. The alkaloid content of the $CHCl_3$ extract was monitored by TLC using Dragendorff's reagent to visualize spots. The $CHCl_3$ extract was purified by chromatotron using mixtures of EtOAc-MeOH (19:1 to 4:1) to give **compound 7** (15 mg, 0.01 %w/w) and **compound 8** (11 mg, 0.01 %w/w).

The isolation of the mixtures and compounds from dichloromethane and ethyl acetate crude extracts of the roots of *T. indica* Merr. is briefly summarized in **Schemes 2.2** and **2.3**, respectively.



Scheme 2.2 Isolation procedure of the dichloromethane crude extract



Scheme 2.3 Isolation procedure of the ethyl acetate crude extract