CHAPTER III

EXPERIMENTAL

General techniques

Chromatographic techniques

Thin-layer chromatography (TLC)

Technique : One way ascending

Adsorbent : Silica gel GF_{254} (E.Merck), 30 g/60 ml of distilled water

Plate size : 5 X 20 cm
10 X 20 cm
20 X 20 cm

Layer thickness : 0.25 mm

Activation : Air-dried for 15 minutes/heat at 110°C for 1 hour

Solvent system : a) 30% methanol in chloroform
b) 25% methanol in acetone
c) 30% methanol in benzene
d) 25% methanol in ethylacetate
e) 50% methanol in dichloromethane
f) 20% acetone in chloroform
g) 10% methanol in dichlorometane
h) Chloroform

i) 2% methanol in benzene

j) 2% methanol in chloroform

Distance : 15 cm

Temperature : Laboratory temperature (20-30°C)

Detection on chromatographic plate :

a) Ultraviolet light at wavelength 254 nm

Most compounds which contain unsaturated bonds become visible as quenching spots under UV light at 254 nm

b) Dragendorff's spray reagent

Solution A :

Bismuth subnitrate (850 mg), distilled water (40 ml), and acetic acid (10 ml)

Solution B :

Potassium iodide (8 gm) and distilled water (20 ml)

Solution A and B, each of 5 ml, were mixed then 20 ml of glacial acetic acid and 70 ml distilled water were added and used as a general alkaloidal detecting reagent, the alkaloids give orange spotes as positive result.

Column chromatography (CC)

Column : Flat bottom glass column (various diameter)

Adsorbent : Silica gel 60 (E.Merck)

Packing method : Wet packing

Technique : Short column chromatography

Solvent system : 25% methanol in dichlorometane

Melting point

Melting points were determined on a Gallenkamp, Melting point. Apparatus Model MFB 595 and were uncorrected.

Ultra-violet spectroscopy (UV)

Ultra-violet absorption spectrum were obtained with a Hitachi U 3400 spectrophotometer.

Infrared spectroscopy (IR)

Infrared absorption spectrum were obtained with a Shimadzu model IR 440 spectrophotometer.

Nuclear magnetic resonance spectroscopy (NMR)

The ^{1}H NMR and ^{13}C NMR spectra were obtained on two difference instuments as follows :

a) JEOL FT nuclear magnetic resonance model JNM-A 500 (Alpha Series).

b) Bruker BZH-200 (200 MHz) spectrometer.

Chemical shifts were reported in ppm scale, using Pyridine-d_5 and Acetone-d_6 as operating solvent.

Mass spectroscopy (MS)

Mass spectra were determined on a Jeol FX 3000 double focusing spectrometer for EI. Operating at 70 eV with inlet temperature 150°C-240°C.

Solvent

The solvents used were commercial and analytical grades.

Phytochemical screening

Powder of leaf material (100 g) was macerated with methanol (150 ml) over night. After the mixture was filtered, the filtrate was concentrated to syrupy mass under reduced pressure. It was mixed with kieselguhr and the mixture was eluted by hexane, chloroform and methanol. The extracts were evaporated to dryness under reduced pressure for further screening procedure.

Screening for sterols and triterpenes

Dissolved the small amount of the hexane extract in 3 drops of acetic anhydride, then one drop of conc sulfuric acid was added. The developing of blue to green colors, indicated the present of sterols.

Screening for alkaloids

Dissolved small amount of chloroform extract with 5 ml of dil HCl and filtrated. The filtrate was used for the precipitation test with alkaloidal reagents. The results were shown positive with Dragendorff's and Mayer's reagent.

Screening for flavonoids

Dissolved small amount of the extracts in 2 ml of ethanol, the magnesium ribbons and 2 ml of conc HCl were added. The results showed negative test for flavonoids.

Isolation of Chemical substances from the leaves of *Dysoxylum grande* Hiern.

Extraction

Dried powdered leaves (8.5 kg) were macerated three times for 7 day periods with methanol (30, 25 and 25 l). The methanolic extract was concentrated under reduced pressure to give residue (2.45 kg) which was fractionated, accordind to Figure 2.

The residue was preadsorbed on kieselguhr. It was then eluted with n-hexane in a large cone percolator until the hexane extract gave negative test to Liebermann-Burchard test. The hexane extract was evaporated to dryness to give 560 g of hexane residue. The residue gave negative test for alkaloids. This residue was not further investigated. The remaining air dried kieselguhr residue was then exhaustively eluted with chloroform to give on evaporation 60 g of chloroform residue containing crude alkaloid. The remaining air dried kieselguhr residue was exhaustively extract with methanol to give on evaporation, 1,178 g of the methanol residue. The chloroform and methanol residue were subjected to column chromatography for further purification.

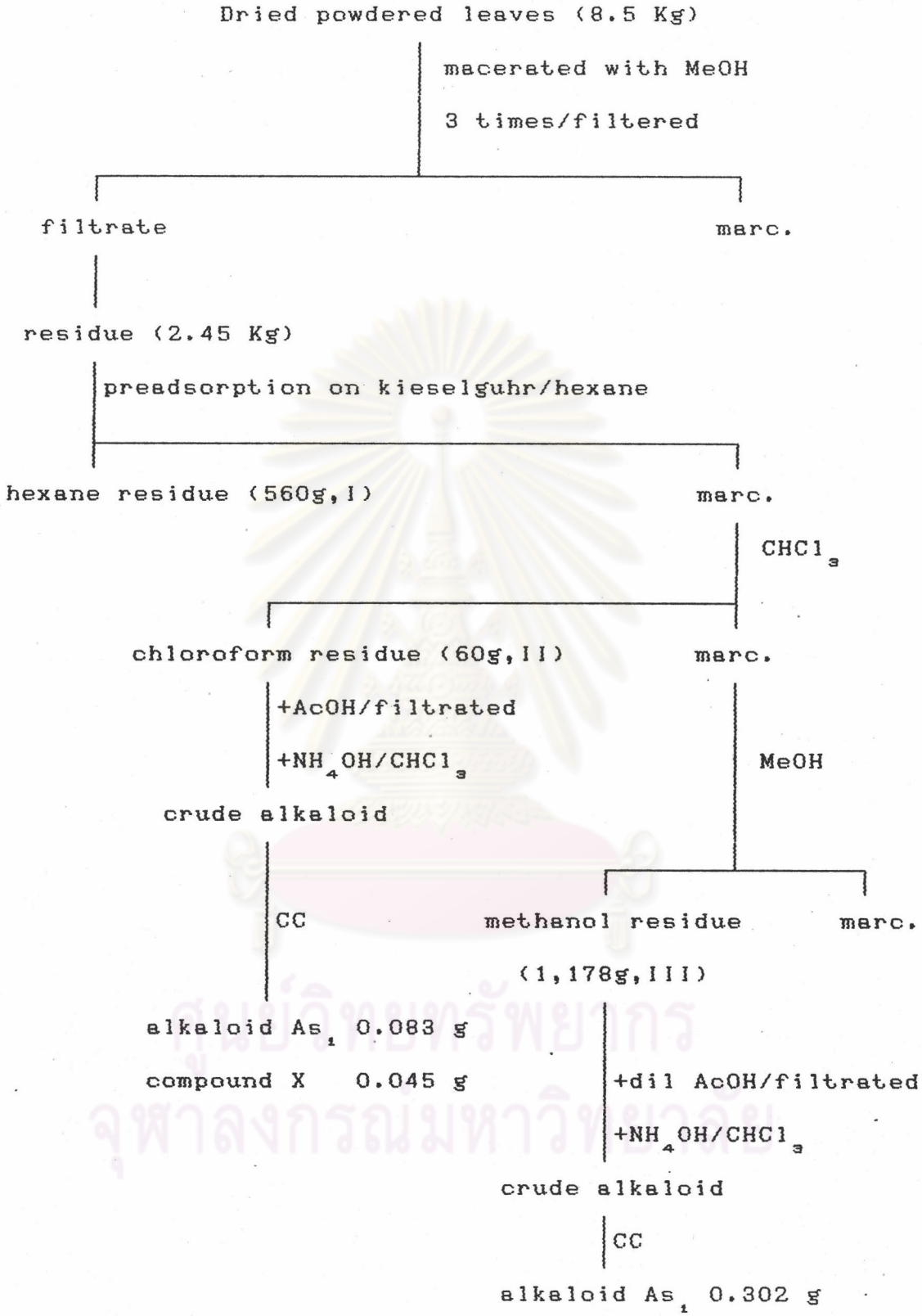

Figure 2 : The extraction and fractionation of *Dysoxylum grande* Hiern Leaves.

Fractionation of chloroform residue.

The chloroform residue (60 g,II) was disolved in glacial acetic acid (250 ml). The acid solution was then diluted with distilled water (200 ml) until complete precipitation of chlorophyll and some other chloroform soluble impurities had occurred. The precipitate was separated by vacuum filtration. The clear acid filtrate was basified with 28 % ammonium hydroxide solution to approximately pH 10, using universal pH paper as an indicator.

The alkaline extract was exhaustively extracted with chloroform to give 3.7 g of crude alkaloid residue. It was then subjected to silica gel column chromatography, using 25 % methanol in dichlorometane as an eluent. Eighty four fractions (40 ml, each) were collected and the column was finally washed with methanol. Those of similar fractions, on TLC plates were combined and evaporated. Fractions 1-2 gave negative tested with modified Dragendorff's reagent and were discarded. Fractions 3-5 gave a pale orange spot on TLC plate when sprayed with modified Dragendofff's reagent. They were combined and evaporated to give residue which was crystallized in methanol/acetone and subsequently recrystallized in methanol to yield 0.045 g (0.0005 %) of colorless needles, designated as compound X.

Fractions 6-21 gave negative test with modified Dragendorff's reagent and were discarded. Fractions 22-26 gave an identical alkaloid positive spot on TLC plate. They were combined and evaporated to give residue which was crystallized in methanol/acetone and subsequently recrystallized in methanol to yield 0.083 g (0.001 %) of pale yellow crystals, designated as alkaloid As_1.

Fractionation of methanol residue.

The methanol residue (1,178 g,III) was dissolved in glacial acetic acid (950 ml). The acid solution was then diluted with distilled water (1,050 ml) until complete precipitation of chlorophyll and some other methanol soluble impurities had occurred. The precipitate was separated by vacuum filtration. The clear acid filtrate was basified with 28% ammonium hydroxide solution to approximately pH 10, using universal pH paper as an indicator.

The alkaline extract was exhaustively extracted with chloroform to give 45.5 g of crude alkaloid residue. It was divided into four portions, each portion (11 g) was subjected to silica gel column chromatography, using 25% methanol in dichlorometane as an eluent. One hundred and ten fractions (40 ml, each) were collected and the column was eluted with methanol. Those similar fractions on TLC plates were combined and evaporated.

Fractions 1-64 gave negative tested with modified Dragendorff's reagent and were discarded. Fraction 65-110 gave an identical alkaloid positive spot on TLC plate. They were combined and evaporated to give residue which was crystallized in methanol to yeild 0.302 g (0.0036%) of pale yellow crystals, was designated as alkaloid As_1.

Characterization of alkaloid As_1

Alkaloid As_1 was obtained as pale yellow crystals, recrystallized from methanol. It gave positive test with modified Dragendorff's reagent. It is soluble in water, methanol and insoluble in other non polar organic solvents.

hRf Value

a) 17.3 (25% methanol in acetone)
(Figure 9)

b) 14.7 (30% methanol in Chloroform)
(Figure 10)

c) 10.7 (30% methanol in benzene)
(Figure 11)

d) 4 (25% methanol in ethyl acetate)
(Figure 12)

e) 30.7 (50% methanol in dichloromethane)
(Figure 13)

Melting Point

218-219°C

Molecular Weight

305

Molecular formula

$C_{16}H_{19}NO_5$

Ultra-violet absorption spectrum (Figure 14)

λmax (MeOH) 220, 260, 325 nm.

Infared absorption spectrum (Figure 15)

ν max (KBr) (cm^{-1}) 3400, 3070, 1660, 1610, 1555, 1413, 1155, 1370, 1110, 1080, 836, 555.

Mass spectrum (Figure 16)

(EIMS)

m/z (% ret int) 305 (15.20), 277 (5.00), 276 (29.35), 219 (7.58), 205 (16.55), 71 (15.83),58 (100.00), 44 (35.24), 43 (49.28), 42 (29.27).

Proton nuclear magnetic resonance (^{1}H NMR)

(500 MHz, Pyridine-d_5) (Figure 17)

δ 1.53 (bd,J=11 Hz,1H), δ 2.16 (bd,J=11 Hz,1H), δ 2.18 (s,3H), δ 2.23 (s,3H), δ 2.34 (bd,J=11 Hz,1H), δ 2.96 (m, 2H), δ 3.12 (bd, J=11 Hz, 1H), δ3.61 (bd, J = 13.4 Hz, 1H), δ 4.39 (bs, H), δ 6.12 (s, 1H), δ 6.73 (s, 1H).

Carbon nuclear magnetic resonance (^{13}C NMR)

(125 MHz, Pyridine-d_5) (Figure 19)

(δ 19.90, CH_3), (δ 25.29, CH_2), (δ 38.12, CH), (δ 46.09, CH_3), (δ 56.76, CH_2), (δ 62.42, CH_2), (δ 69.83, CH), (δ 101.50, CH), (δ 104.66, C), (δ 108.42, CH), (δ 108.49, C), (δ 156.23, C), (δ 161.40, C),(δ 165.08,C), (δ 166.80, C), (δ 183.14, C).

Characterization of Compound X

Compound X was obtained as colorless needles recrystallized from methanol. It was soluble in methanol, acetone.

hRf Value

a) 46.7 (20% acetone in chloroform)

(Figure 23)

b) 78.7 (10% methanol in dichloromethane)

(Figure 24)

c) 10 (Chloroform)

(Figure 25)

d) 64.7 (2% methanol in chloroform)

(Figure 26)

e) 30.7 (2% methanol in benzene)

(Figure 27)

Melting point

275-278° C (decomp.)

Molecular weight

192

Molecular formula

$C_{10}H_8O_4$

Ultra-violet absorption spectrum (Figure 28)

λ max (MeOH) 227, 248, 255, 294 nm.

Infrared absorption spectrum (Figure 29)

ν max (KBr) (cm^{-1}) 3400, 1650, 1620, 1560, 1505,

1470, 1420, 1020, 850.

Mass spectrum (Figure 30)

(EIMS)

m/z (% rel int) 192 (100.00), 164 (40.99), 163 (30.03), 152 (18.27), 136 (7.58) 124 (28.72), 96 (14.08), 69 (33.20) 39 (20.31).

Proton nuclear magnetic resonance (^{1}H NMR)

(200 MHz, Acetone-d_6) (Figure 31)

δ 2.36 (s,3H), δ 6.06 (s,1H), δ6.21 (d, J= 2 Hz,1H) δ 6.36 (d, J=2 Hz,1H), δ9.57 (s,1H), δ12.88 (s,1H).

Carbon nuclear magnetic resonance (^{13}C NMR)

(50 MHz, Acetone-d_6) (Figure 32)

(δ 19.72, CH_3), (δ 93.94, CH), (δ 99.06, CH), (δ 104.33, C), (δ 108.33,CH), (δ 158.66,C), (δ 162.74,C), (δ 164.48, C), (δ 167.81, C),(δ 182.58, C).