CHAPTER III

EXPERIMENTAL

Source of Plant Material

The leaves of *Aglaia rubiginosa* (Hiern) Pannell was collected from peat swamp forest, Narathiwat, Thailand, in April, 1994. It was identified by Dr. Thawatchai Suntisuk and compared with the voucher specimen BKF. No. 19333 at the Botanical Section, Technical Division, Royal Forest Department, Ministry of Agriculture and Co-operative, Thailand.

The leaves were dried in an oven at low temperature (40-50°C) and ground to powder in the Retsch KG type SK. 1 mill.

General Techniques

2.1 Chromatographic techniques

2.1.1 Thin-layer chromatography (TLC)

Technique	: One way ascending
Stationary phase	: Aluminium oxide (type T, Art. 1090 150 basisch (E. Merck)
	Silica gel GF 254 (E. Merck)
Plate size	: 5x20 cm.
	10x20 cm.
	20x20 cm.
Layer thickness	: 0.25 mm.
Activation	: Air-dried for 15 minutes and then heated in hot air oven at 110°C for one hour.
Solvent systems	: benzene-ethanol (9:1)
	chloroform-ethanol (9:1)

acetone-ethanol (8:2)

benzene-methanol (7:3)

Distance : 15 cm.

Temperature : 28-35°C (room temperature)

Detection : 1) UV light (254 and 366nm)

2) Dragendrorff's reagent

2.1.2 Column Chromatography (CC)

Column : Flat bottom glass column (various diameter)

Stationary phase : Aluminium oxide 90 active, basic (activity stage I), (E.Merck)

Packing method : Wet packing

Technique : Short column chromatography

Long column chromatography

Solvent systems : benzene-ethanol (9.5:0.5)

acetone-benzene (9.5:0.5)

2.2 Spraying reagent

Dragendorff's spraying reagent:

Solution A: bismuth subnitrate (850mg), distilled water (40ml) and acitic acid(10ml)

Solution B: potassium iodide (8 g) and distilled water (20ml)

Solution A and B, each of 5 ml, were mixed, then 20 ml of glacial acetic acid and 70 ml of distilled water were added. The reagent was used as a general alkaloidal reagent giving orange spots as positive result.

2.3 Melting point

Melting point was determined on a Buchi capillary tube melting point apparatus.

2.4 Spectroscopy

2.4.1 Ultraviolet (UV) Absorption Spectrum

The ultraviolet adsorption spectrum was obtained on a Hitachi U 3400 spectrophotometer. (Scientific and Technological Reserch Eqiupment Center,Chulalongkorn University)

2.4.2 Infrared (IR) Absorption Spectrum

The infrared absorption spectrum was obtained on a Shimadzu model IR 440 spectrophotometer. The absorption bands were reported in wave number (cm^{-1}). The materials were examined in KBr cells. (Scientific and Technological Reserch Eqiupment Center, Chulalongkorn University)

2.4.3 Mass Spectrum (MS)

The electron impact mass spectrum (eims) was obtained on a Fisons VG Trio 2000 quadrupole mass spectrometer operating at 70 eV. (Department of Chemistry, Faculty of Science Chulalongkorn University)

2.4.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H-and ^{13}C-NMR) Spcetra

^{1}H-and ^{13}C-NMR spectra were obtained on a JNM-A500 (Alpha series) 500 MHz NMR spectrometer. Chemical shifts were reported in ppm. scale, using deuteromethanol (CD_3OD) as operating solvent and TMS as reference internal standard. (Scientific and Technological Reserch Eqiupment Center, Chulalongkorn University)

2.5 Solvent

The solvents used were redistilled from commercial grade solvent and analytical grade.

Phytochemical screening

Powdered leaves (100 g) were macerated with methanol (150 ml) over night.

After the methanol extract was filtered, it was concentrated to syrupy mass on a water bath for further screening procedure.

3.1 Screening for alkaloids

A small amout of the syrupy mass was dissolved in 5 ml of diluted HCl and filtered. The filtrate gave precipitate with Dragendorff's reagents, indicating the presence of alkaloids.

3.2 Screening for flavonoids

Cyanidin reaction (Shinoda's test):

Small pieces of magnesium ribbon were added to the methanolic extract of the leaves, followed by the dropwise addition of concentrated HCl. The results showed negative test for flavonoids.

3.3 Screening for triterpenoids and sterols

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

Extraction

Dried powdered leaves (7 kg) were macerated for 15 day peroids three times with methanol (22, 18 and 18 L) and filtered. The methanolic extract was concentrated under reduced pressure to give a residue (1.44 kg) which was fractionated according to Figure 1.

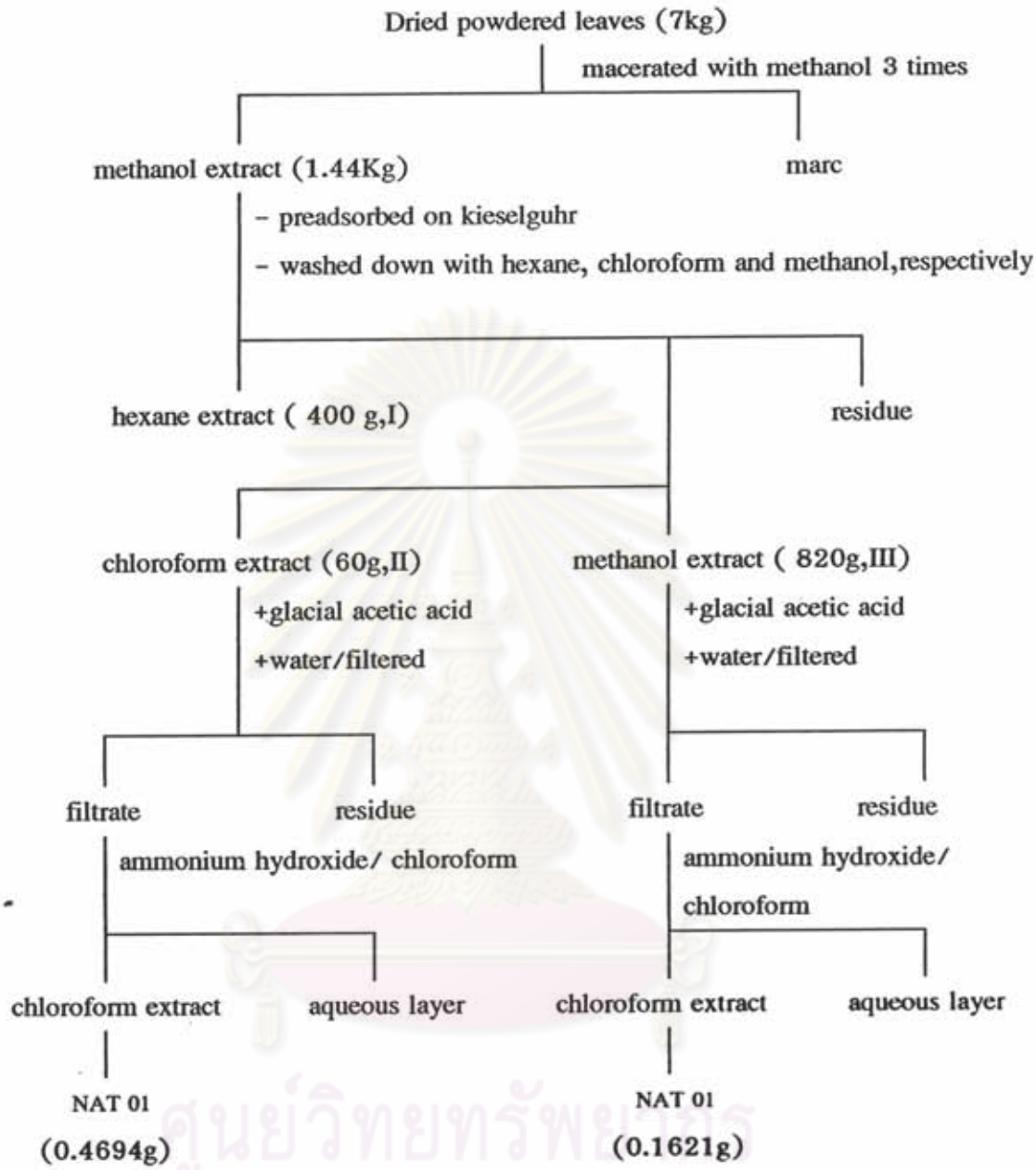

Figure 1 Extraction scheme of *Aglaia rubiginosa* (Hiern) Pannell.

The methanol extract was adsorbed on kieselguhr and put into a large cone percolator. It was then eluted with n-hexane until the eluate stopped giving positive result to Libermann Burchard test. The eluate was evaporated to dryness to give 400g of hexane extract (I). This extract tested negative for alkaloid and was not further investigated. The remaining kieselguhr mixture was air-dried and then exhaustively eluted with chloroform to give, on

evaporation, 60g of chloroform extract (II) containing alkaloids. The air dried kieselguhr mixture was further eluted with methanol to give, on evaporation, 820g of the methanol extract (III).The chloroform and methanol extracts were subjected to column chromatography for further purification.

Isolation Procedure

5.1 Fractionation of chloroform extract

The chloroform extract (60g,II) was dissolved in glacial acetic acid (285ml). The acid solution was diluted with distilled water (1800ml) until complete precipitation of chlorophyll and some other impurities had occured. The precipitate was separated by vacuum filtration. The clear acid filtrate was basified with 25% ammonium hydroxide solution to approximately pH 10 and exhaustively extracted with chloroform to give 6.4830g of crude alkaloid residue. It was then subjected to aluminium oxide basic short column chromatography, using benzene-ethanol (9.5:0.5) as an eluent. Fractions (40ml each) were consequently collected and examined by TLC, using benzene-ethanol (9:1) as developing solvent. Those with similar pattern on TLC plates were combined and evaporated to give 3 major fractions (Table5)

Table 5 Combined fractions from short column chromatography of the chloroform extract

Fraction	Number of eluates	Weight (g)
A-01	1-2	0.0310
A-02	3-15	0.2310
A-03	16-106	0.5194

Fraction A-01 was not further studied and was later discarded

Fraction A-02 showed traces of alkaloids on TLC plates. No pure compound was obtained due to very minute amount of alkaloids presented in this fraction.

Fraction A-03 showed one alkaloid positive spot at hRf value of 3.5 on Alumina TLC plates, using benzene-ethanol (9.5:0.5) as developing solvent. It was crystallized / recrystallized in benzene/methanol (9.5:0.5) to yield NAT 01 as colorless needle. (0.4694 g, 0.0067%)

5.1 Fractionation of methanol extract

The methanol extract (820g,III) was dissolved in glacial acetic acid (1575 ml). The acid solution was diluted with distilled water (2000 ml) until complete precipitation of chlorophyll and some other impurities had occured. The precipitate was separated by vacuum filtration. The clear acid filtrate was basified with 25% ammonium hydroxide solution to approximately pH 10 and exhaustively extracted with chloroform to give 5.0112g of crude alkaloid residue. It was then subjected to aluminium oxide basic short column chromatography, using acetone:benzene (9.5:0.5) as an eluent. Fractions (30 ml each) were consequently collected and examined by TLC, using acetone:benzene (9.5:0.5) as developing solvent. Those with similar pattern on TLC plates were combined and evaporated to give 3 major fractions (Table 6)

Table 6 Combined fractions from short column chromatography of the methanol extract

Fraction	Number of eluates	Weight (g)
A-01	1-2	0.0315
A-02	3-69	2.5636
A-03	70-109	0.2310

Fraction A-01 was not further studied and was later discarded

Fraction A-02 showed traces of alkaloids on TLC plates. It was dissolved in glacial acetic acid (5 ml.). The acid solution was diluted with distilled water (10 ml.) until complete precipitation of impuritied had occured the precipitation was separated. The clear acid filtrate was basified with 25% ammonium hydroxide solution to approximately pH10 and exhaustively extracted with chloroform to give 1.076 g of crude alkaloid residue. No pure

Fraction A-03 showed one alkaloid positive spot on TLC plates . It was crystallized/ recrystallized in benzene/methanol (9.5:0.5) to yield compound NAT 01 as colorless needle. (0.1621g, 0.0023%)

Characterization of isolated compound

6.1 Compound NAT01

Apperance : colorless needles

Solubility : very soluble in methanol, slightly soluble in pyridine

hRf value : a) 85.00 [Aluminium oxide /chloroform-ethanol (9:1)]

b) 20.00 [Aluminium oxide /benzene-methanol (7:3))]

c) 75.00 [Silica gel GF 254/acetone-ethanol (8:2)]

Melting Point : 116-117°C

Spectral Data : a) EIMS [*m/z* (% relative intensity, Fig.3 page 122]

55(3.85%), 56(2.31%),69(6.15%),70(35.38%),77(17.69%), 82(12.31%),84(3.08%),98(6.15%),99(10.77%),103 (40.00%),104(4.62%),123(4.23%),131(100%),132(11.52%), 146(5.00%),151(19.62%),160(5.38%),168(8.46%),169 (3.08%),188(6.15%),189(1.15%),200(21.54%),201 (10.77%),202(0.35%),219((0.35%),298(0.20%),317(0.30%)

b) UV (λmax nm (ε) in MeOH: Fig.4 page 123)

275(0.6706),274(0.6719),273(0.6729),272(0.6727),270 (0.6646),275(0.6701),271(0.6692)

c) IR (ν,cm^{-1},KBr disc; Fig.5 page 124)

3981,3942,3884,3842,3816,3769,3744,3700,3610,3539, 3504,3436,3285,3059,2950,2875,2334,1655,1617,1532, 1483,1446,1378,1330,1292,1221,1166,1092,1072,1016, 972,878,765,728,693,666,573,542,489,419

d) ^{1}H-NMR (δ ppm, 500MHz, in CD_3OD; Fig.6 page125)

1.60 (4H,*m*),1.83(3H,*dt*,*J*=1.83,1.22Hz),3.28(2H,*m*),3.32(2H,*m*),4.23 (2H,*dq*,*J*=6.10,1.02Hz),6.33(1H,*tq*,*J*=6.10,1.53Hz),6.59 (1H,*d*,*J*=15Hz),7.36(1H,*m*),7.38(2H,*m*),7.52(1H,*d*,*J*=15Hz),7.54 (2H,*dd*,*J*=7.63,1.53Hz)

e) ^{1}H-NMR (δ ppm, 500MHz, in C_5D_5N; Fig.9 page 128)

8.40(1H),8.03(1H),7.56(2H),7.26(3H),7.01(1H),6.92(1H), 4.55(2H),3.56(4H),2.00(3H),1.72(4H)

f) ^{13}C-NMR (δ ppm,125MHz,in CD_3OD; Fig.12 page 131)

129.4(2-C),130.1(2-C),130.7,132.5,140.8,121.8,172.0, 40.3,40.2,27.8(2-C),168.6,59.5,136.2,135.9,13.00