CHAPTER III

EXPERIMENTAL

Source of Plant Material

The plant material, *Coleus amboinicus* Lour., investigated in this work was cultivated at the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. It was identified by comparison with the description in Flora of British India Vol.4 (1953).

The voucher specimen of the plant material has been kept at the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

General Techniques

1. Chromatography

1.1 Analytical Thin Layer Chromatography

Technique : One way, ascending

Adsorbent : Mixture (2:1) of silica gel 60G (Number 7731, E. Merck) and silica gel 60 HF254 (Number 7739, E. Merck) 30 g in distilled water.

Plate size : 5 x 20 cm

Layer thickness : 0.25 nm

Activation : Air-dried for 15 minutes and then heated in hot air oven at 110 °C for 1 hour.

Solvent system : Various solvent systems depending on materials.

Distance : 15 cm

Temperature : Room temperature 28-35 °C

Detection : 1) Visual detection under daylight

2) Ultraviolet light at the wavelengths of 254 and 365 nm

3) Spraying with 10% sulphuric acid solution and heating at 100-110 °C for a few minutes

1.2 Column Chromatography

1.2.1 Conventional column chromatography

Column size : The glass columns 1.5-5.0 cm in diameter were used depending on the quantity of sample to be separated.

Adsorbent : Silica gel 60 (No. 9385, E. Merck) particle size 0.040-0.063 mm (230-400 mesh ASTM)

Packing method : Wet packing

Solvent : Various solvent systems depending on materials.

Sample loading : The sample extract was dissolved in a small volume of the eluent and loaded onto the top of the column.

Examination of eluates : Fractions were examined by TLC under ultraviolet light at the wavelengths of 254 and 365 nm and by exposing to 10% sulphuric acid solution and heat, respectively.

2. Spectroscopy

2.1 Ultraviolet (UV) Absorption Spectra

The ultraviolet absorption spectra were obtained on a Milton Roy Sprotronic 3000 Ray Spectrometer. Methanol was employed as the solvent for all compounds.

2.2 Infrared (IR) Absorption Spectra

The spectra were obtained on a Shimadzu IR-440 infrared spectrophotometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.3 Mass Spectra (MS)

The electron impact mass spectra (EIMS) were obtained by operating at 70 eV with a Fisons VG Trio 2000 quadrupole mass spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

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

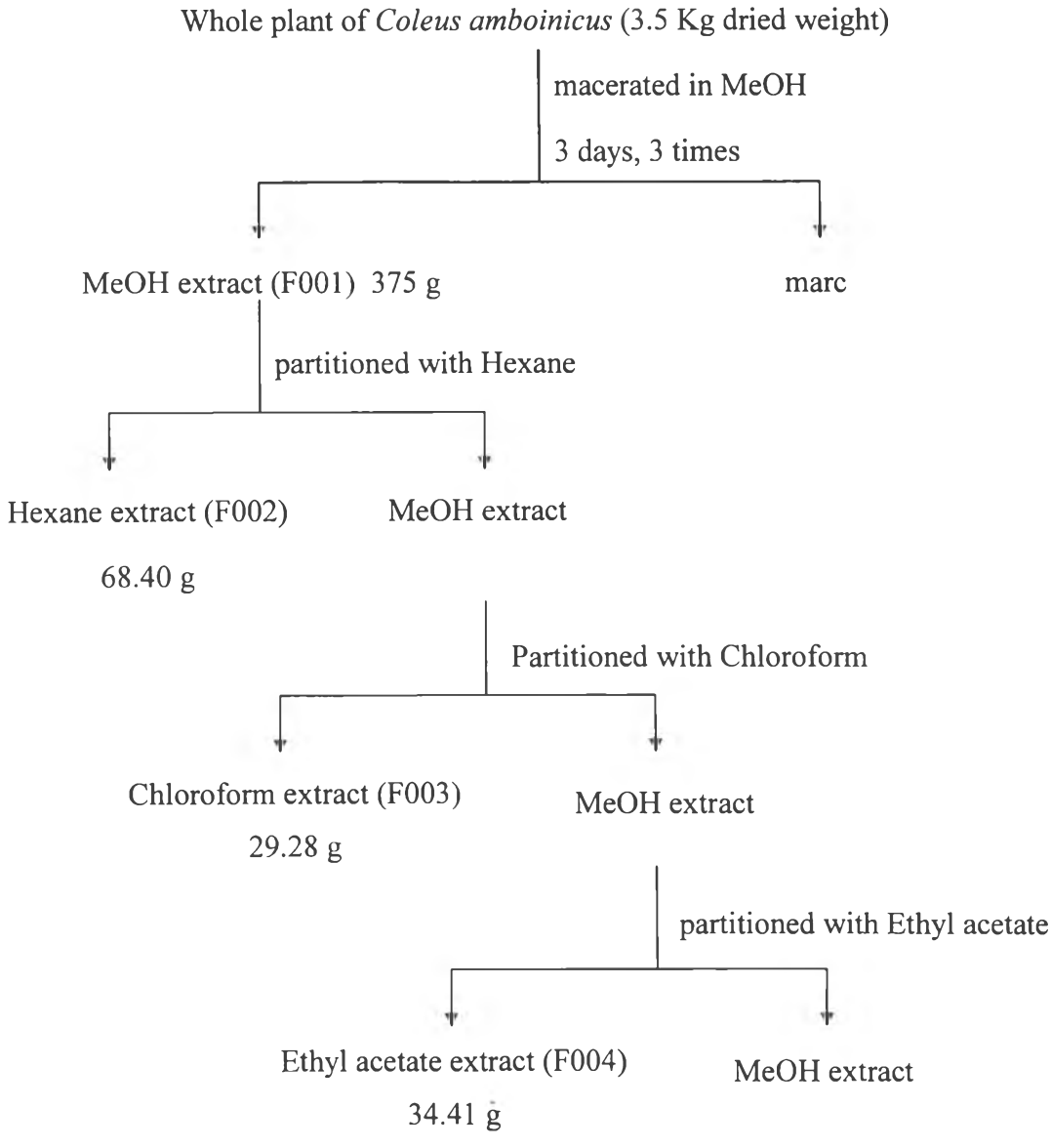
The nmr spectra were obtained on a JEOL JMN-A500 (Alpha series) 500 MHz NMR Spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

3. Solvent

Throughout this work, all organic solvents used, excluding the deuterated solvents for NMR spectra, were commercial grade and had to be redistilled prior to use.

Extraction

The dried whole plant material (3.5 kg dried weight) was chopped into small pieces, macerated three times in methanol (3.5 liters and 3 days each) and then filtered. The filtrate of each batch was combined and concentrated to remove methanol under reduced pressure to yield 375 g of dried crude extract (10.71% of dried weight). A portion of this extract (about 75 g) was reserved as reference sample. The crude extract (300g) was dissolved in methanol to give a final volume (F001) of approximately 1 liter, which was then partitioned with 1 liter of hexane, chloroform and ethyl acetate, respectively. Each fraction was evaporated to dryness under reduced pressure to yield 68.40 g of hexane extract (F002, 1.95% of dried weight), 29.28 g of chloroform extract (F003, 0.84% of dried weight) and 34.41 g of ethyl acetate extract (F004, 0.98% of dried weight). A little amount of each extract was also reserved as reference.



Scheme 3. Extraction scheme of *Coleus amboinicus*

Isolation

The ethyl acetate extract (F004, 20.0 g) was subjected to silica gel column chromatography. The extract was dissolved in a small volume of chloroform and applied to the top of a column (10x20 cm) already packed with a slurry of silica gel (500 g) in chloroform-methanol (7:1). This same solvent mixture was employed as eluting solvent and the collected fractions (30 ml each) were monitored by TLC, with chloroform-methanol (5:1) as the developing solvent system. Two hundred and twenty fractions were collected and combined according to their TLC profiles into seven major fractions (F005-F011) as shown in Table 4.

Table 4. Combined fractions from the ethyl acetate extract, F004

Code	Number of eluates	Weight of combined fraction (g)
F005	1 - 27	1.24
F006	28-36	0.37
F007	37-50	0.30
F008	51-100	1.13
F009	101-140	5.30
F010	141-200	1.92
F011	201-220	1.33

1. Isolation of chemical constituents of fraction F006

Fraction F006 was separated by column chromatography using a column of silica gel (50 g, 3.0x10 cm) with chloroform-methanol (50:1) as the packing solvent. The sample (0.37 g) was dissolved in a small volume of chloroform-methanol (50:1) and loaded on the top of the column. This same solvent mixture was employed as eluting solvent. The fractional volumn was about 15 ml. All eluates were collected and combined by using TLC examination, with chloroform-methanol (5:1) as the

developing solvent system. Fractionation of F006 gave four fractions (F012-F015) as summarized in Table 5.

Table 5. Combined fractions from F006

Code	Number of eluates	Weight of combined fraction (mg)
F012	1-31	45
F013	32-41	220
F014	41-45	51
F015	46-50	33

1.1 Isolation of compound RAT 1

Fraction F013 was subjected to further column chromatography. The fraction (220 mg) was dissolved in a small volume of chloroform-methanol (50:1) and loaded on the top of a column (3.0x10 cm) already packed with a slurry of silica gel (20 g) in chloroform-methanol-H_2O (50:1:1). The same solvent mixture was employed as eluting solvent and the collected fractions were examined by TLC, with chloroform-methanol (5:1) as the developing solvent system. The fractional volume was approximately 15 ml. Twenty fractions were collected and combined according to their TLC profiles into four fractions (F016-F019) as shown in Table 6.

Table 6. Combined fractions from F013

Code	Number of eluates	Weight of combined fraction (mg)
F016	1-4	12.3
F017	5-9	48.9
F018	10-15	34.5
F019	15-20	16.5

Fraction F017 appeared as a brown spot on TLC plate after being sprayed with 10% sulfuric acid solution and heated for 5 minutes. Compound RAT 1 was thus characterized as a dark-brown oil (48.9 mg, 0.0014 % of dried weight). NMR techniques, both 1D and 2D, were applied to identify the chemical structure of RAT 1.

1.2 Isolation of compound RAT 2

F009, another fraction obtained from F004, displayed several purple spots on TLC plate upon detection with 10% sulfuric acid solution and, thus, was further investigated. The sample (5.30 g) was dissolved in a small volume of chloroform-methanol (10:3) and loaded on the top of a silica gel column (50 g, 3.0x10 cm). Elution was performed utilizing the same solvent system. Each 30 ml fraction was collected and compared by TLC, using chloroform-methanol (10:3) as the developing solvent system. Twenty-five fractions were combined into four major fractions (F020-F023) as shown in Table 7.

Table 7. Combined fractions from F009

Code	Number of eluates	Weight of combined fraction (g)
F020	1-4	0.52
F021	5-13	2.33
F022	14-20	1.52
F023	20-25	0.87

Fraction F021 (2.33 g) was selected for further isolation by using a silica gel column (50 g, 3.0x10 cm) with chloroform-methanol (10:1) as the eluent. The volume of each collected fraction was approximately 25 ml. Thirty-five fractions were

collected and combined according to their TLC patterns to give three major fractions (F024-F026) as shown in Table 8.

Table 8. Combined fractions from F021

Code	Number of eluates	Weight of combined fraction (g)
F024	1-16	1.32
F025	17-27	0.94
F026	28-35	0.26

Fraction F025 (0.94 g) was furthur purified by using a silica gel column (30 g, 2.0x10 cm) with acetone-chloroform (2:1) as the eluent. The volume of each collected fraction was approximately 15 ml. Twenty-five fractions were collected and combined according to their TLC patterns to give three major fractions (F027-F029) as shown in Table 9.

Table 9. Combined fractions from F025

Code	Number of eluates	Weight of combined fraction (g)
F027	1-8	0.61
F028	9-18	0.54
F029	19-25	0.27

Fraction F028 appeared as a purple spot on TLC plate after being sprayed with 10% sulfuric acid solution and heated for 5 minutes. Compound RAT 2 was thus characterized as a light yellow solid (540 mg, 0.015 % of dried weight). NMR techniques were applied to identify the chemical structure of RAT 2.

1.3 Isolation of compound RAT 3

F008 (1.13 g), another fraction obtained from F004, was selected for further isolation by using a silica gel column (50 g, 3.0x10 cm) chloroform-methanol (5:1) as the eluent. The volume of each collected fraction was approximately 30 ml. Twenty fractions were collected and combined according to their TLC patterns to give four major fractions (F030-F033) as shown in Table 10.

Table 10. Combined fractions from F008

Code	Number of eluates	Weight of combined fraction (g)
F030	1-2	0.08
F031	3-8	0.64
F032	9-14	0.19
F033	15-20	0.12

Fraction F031 (0.64 g) was chosen for further isolation by using a silica gel column (50 g, 3.0x10 cm) with chloroform-methanol (5:1) as the eluent. The volume of each collected fraction was approximately 25 ml. Twenty fractions were collected and combined according to their TLC patterns to give three major fractions (F034-F036) as shown in Table 11.

Table 11. Combined fractions from F031

Code	Number of eluates	Weight of combined fraction (g)
F034	1-3	0.25
F035	4-11	0.34
F036	12-20	0.16

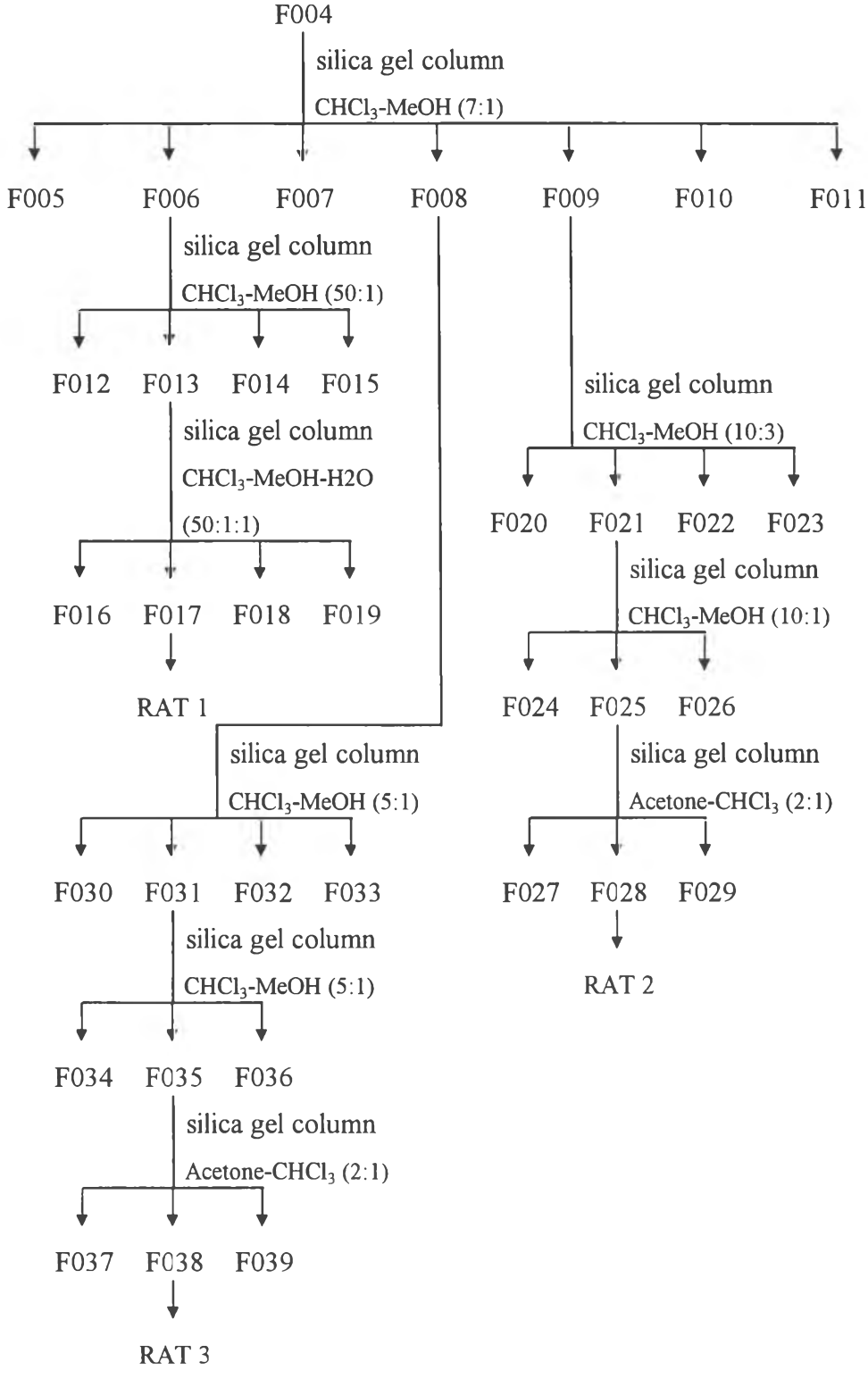
Fraction F035 (0.34 g) was further purified by using a silica gel column (30 g, 2.0x10 cm) with acetone-chloroform (2:1) as the eluent. The volume of each collected fraction was approximately 15 ml. Fifteen fractions were collected and combined according to their TLC patterns to give three major fractions (F034-F036) as shown in Table 12.

Table 12. Combined fractions from F035

Code	Number of eluates	Weight of combined fraction (g)
F037	1-3	0.24
F038	4-8	0.19
F039	9-15	0.08

When developed with chloroform-methanol (5:1), fraction F038 displayed single purple spot on TLC plate after being sprayed with 10% sulfuric acid solution and heated for 5 minutes. Compound RAT 3 was thus characterized as a light yellow solid (190 mg, 0.0054 % of dried weight). NMR techniques were applied to identify the chemical structure of RAT 3.

The isolation of these compounds can be summarized as shown in Scheme 4.



Scheme 4. Isolation scheme of compounds from the ethyl acetate extract of *Coleus amboinicus*

Characterization of isolated compounds

1. Compound RAT 1

Dark brown oil.

MS *m/z* (% rel. int.) : 126 (6), 125 (9), 124 (100), 123 (68), 109 (6), 105 (10), 97 (9), 96 (10), 95 (44), 84 (18) and 68 (3) (Figure 7, page 64)

IR ν_{max}, KBr disc, cm^{-1} : 3380, 2930, 2850, 1710, 1670, 1520, 1190 and 1020 (Figure 8, page 65)

UV λ_{max}, (MeOH), nm (log ε) : 224 (2.05) and 279 (2.34) (Figure 9, page 66)

^1H NMR (δ ppm, 500 MHz, acetone-d_6) : 9.59 (1H, *s*), 7.37 (1H, *d*, *J* = 3.6 Hz), 6.58 (1H, *d*, *J* = 3.6 Hz) and 4.65 (2H, *s*) (Figure 13, page 70)

^{13}C NMR (δ ppm, 125 MHz, acetone-d_6) : 178.2 (*d*), 163.0 (*s*), 153.5 (*s*), 123.9 (*d*), 110.3 (*d*) and 57.5 (*t*) (Figure 10, page 67)

2. Compound RAT 2

Light yellow solid. Soluble in ethyl acetate, acetone and methanol.

MS *m/z* (% rel. int.) : 328 (1), 167 (10), 166 (100), 151 (53) and 91 (6) (Figure 21, page 82)

IR ν_{max}, KBr disc, cm^{-1} : 3390, 2960, 1700, 1510, 1420, 1360, 1200 and 1080 (Figure 22, page 83)

UV λ_{max}, (MeOH), nm (log ε) : 216 (3.23) and 286 (2.84) (Figure 23, page 84)

^{1}H NMR (δ ppm, 500 MHz, acetone-d_6) : 6.92 (1H, *s*), 6.68 (1H, *s*), 4.75 (1H, *d*, *J* = 7.3 Hz), 3.88 (1H, *dd*, *J* = 10.9, 1.9 Hz), 3.73 (1H, *dd*, *J* = 10.9, 4.9 Hz), 3.53 (1H, *m*), 3.50 (1H, *m*), 3.49 (1H, *m*), 3.47 (1H, *septet*, *J* = 7.0 Hz), 3.42 (1H, *m*), 2.13 (3H, *s*) and 1.13 (6H, *d*, *J* = 7.0 Hz) (Figure 29, page 90)

^{13}C NMR (δ ppm, 125 MHz, acetone-d_6) : 151.5 (*s*), 148.9 (*s*), 137.6 (*s*), 122.5 (*s*), 120.2 (*d*), 112.9 (*d*), 104.0 (*d*), 78.1 (*d*), 77.4 (*d*), 74.9 (*d*), 71.4 (*d*), 62.7 (*t*), 26.5 (*d*), 23.5 (*q*), 23.4 (*q*) and 16.0 (*q*) (Figure 24, page 85)

3. Compound RAT 3

Viscous light yellow solid. Soluble in ethyl acetate, acetone and methanol.

MS *m/z* (% rel. int.) : 167 (1), 166 (9), 151 (15), 150 (100), 135 (65), 133 (5), 105 (7) and 91 (15) (Figure 41, page 104)

IR ν_{max}, KBr disc, cm^{-1} : 3390, 2960, 1710, 1510, 1420, 1250 and 1080 (Figure 42, page 105)

UV λ_{max}, (MeOH), nm (log ε) : 216 (3.30), 269 (2.86) and 276 (2.82) (Figure 43, page 106)

^{1}H NMR (δ ppm, 500 MHz, acetone-d_6) : 7.02 (1H, *d*, *J* = 7.5 Hz), 7.02 (1H, *d*, *J* = 1.8 Hz), 6.78 (1H, *dd*, *J* = 7.5, 1.8 Hz), 4.93 (1H, *d*, *J* = 7.6 Hz), 3.89 (1H, *dd*, *J* = 11.5, 1.8 Hz), 3.72 (1H, *dd*, *J* =11.5, 4.9 Hz), 3.54 (1H, *dd*, *J* = 7.6, 1.8 Hz), 3.54 (1H, *m*), 3.50 (1H, *m*), 3.49 (1H, *m*), 2.84 (1H, *septet*, *J* = 6.7 Hz), 2.20 (3H, *s*) and 1.20 (6H, *d*, *J* = 6.7 Hz) (Figure 49, page 112)

^{13}C NMR (δ ppm, 125 MHz, acetone-d_6) : 156.7 (*s*), 148.5 (*s*), 131.0 (*d*), 125.2 (*s*), 120.5 (*d*), 114.0 (*d*), 102.1 (*d*), 78.1 (*d*), 77.5 (*d*), 74.7 (*d*), 71.4 (*d*), 62.6 (*t*), 34.5 (*d*), 24.3 (*q*), 24.2 (*q*) and 16.0 (*q*) (Figure 44, page 107)