CHAPTER III

EXPERIMENTAL

1. Fungal Material

The fungus *Phomopsis* sp. AANN8 was isolated from the twigs of *Artemisia annua* L. grown in Kanchanaburi Province, Thailand (**Figures 4** and **5**). The twigs were washed under running tap water and then air-dried. The cleaned twigs were cut into pieces of 5 cm in length then surface sterilized by 70 % ethanol for 1 minute, 5 % sodium hypochlorite solution for 5 minutes and sterile distilled water for 1 minute three times, respectively. The sterilized samples were cut into small pieces (about 1 cm) using sterile blades and placed on sterile water agar plates. All samples were subsequently incubated at 25 ^{o}C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile pasture pipette and transferred to a sterile potato dextrose agar (PDA) plate. After incubation at 25 ^{o}C for 7 days, the culture purity was determined from colony morphology.

The endophytic fungus was identified by Assistant Professor Dr. Suthep Wiyakrutta and Associate Professor Dr. Nongluksna Sriubolmas based on molecular methods (Phaopongthai *et al.*, 2013). The AANN8 fungus was grown in potato dextrose broth (PDB) at 25 ^{o}C for 4 days and total cellular DNA was extracted from the washed fungal mycelium. The ITS1 region, 5.8S gene and ITS2 region (ITS1-5.8S-ITS2) of the ribosomal RNA gene region were amplified from the fungal genomic DNA by PCR. The PCR products were gel-purified and directly subjected to DNA

sequencing. The DNA sequence of the ITS1-5.8S-ITS2 rRNA gene obtained was used as a query sequence to search for similar sequences in GenBank. DNA sequences of reference and published strains of *Phomopsis* were retrieved for phylogenetic analysis.

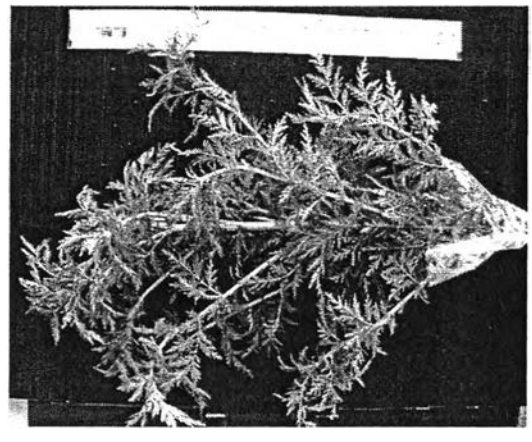


Figure 4. The twigs of *Artemisia annua* L.

Figure 5. The endophytic fungus *Phomopsis* sp. AANN8 on PDA plate.

2. General Experiment Procedures

2.1 Thin Layer Chromatography

Technique : One dimentional, ascending

Adsorbent : Silica gel 60 F_{254} (Merck)

Layer thickness : 0.2 mm

Distance : 5 cm

Temperature : Laboratory temperature (30-35 °C)

Detection : 1. Ultraviolet light at wavelengths 254 and 365 nm

2. Spraying with anisaldehyde-sulfuric acid reagent and heating at 105 °C for 10 minutes

2.2 Column Chromatography

2.2.1 Quick Column Chromatography

Adsorbent : Silica gel 60 (No.7734) particle size 0.063-0.200 nm (70-230 mesh ASTM) (Merck)

Packing method : The adsorbent was packed by the wet method. The adsorbent was mixed with the eluent into a free-flowing suspension and poured into a sintered glass funnel used as the column. The eluent was drained and the adsorbent was evenly settled under reduced pressure using an aspirator or a membrane pump.

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of Kieselgurh, triturated, dried and then placed gently on

top of the column. The elution was generated under reduced pressure using an aspirator or a membrane pump to drain the eluent and elute the compounds from the column for each fraction.

Detection : Fractions were examined by TLC technique in the same manner as described in section 2.1.

2.2.2 Flash Column Chromatography

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

Packing method : The adsorbent was packed by the wet method. The adsorbent was mixed with the eluent into a free-flowing suspension and poured into the column. The eluent was drained and the adsorbent was evenly settled by compressed air or low pressure (about 2 psi). The flow rate was adjusted at 1-2 ml/min by compressed air and the stopcock.

Sample loading : The sample was dissolved in a small amount of the eluent and then applied gently on top of the column.

Detection : Fractions were examined by TLC technique in the same manner as described in section 2.1.

2.2.3 Gel Filtration Chromatography

Adsorbent : Sephadex LH-20 (Pharmacia Biotech AB)

Packing method : Sephadex gel was suspended in methanol and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.

Sample loading : The sample was dissolved in a small amount of eluent and then applied gently on top of the column.

Detection : Fractions were examined by TLC technique in the same manner as described in section 2.1.

2.2.4 High Pressure Liquid Chromatography

Column : LiChrosper RP-18e, 250 x 4 mm, particle size 10 µm (Merck)

Sample : The sample was dissolved in small amount of methanol and filtered through Millipore filter paper before injection.

Pump : LC-20AB (Shimadzu)

Detector : SPD-20A UV Detector (Shimadzu)

Temperature : Room temperature

2.3 Spectroscopy

2.3.1 Ultraviolet Spectra

Ultraviolet (UV) spectra were obtained on a Shimadzu UV-160A UV/VIS spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3.2 Infrared Spectra

Infrared (IR) spectra (KBr disc) were obtained on a Perkin Elmer (Spectrum One) spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.3.3 Mass Spectra

The HR-EIMS, EIMS and FABMS were obtained on a JMS-700 (JEOL) instrument with a direct inlet system operating at 70eV (Meiji Pharmaceutical University, Japan).

2.3.4 Proton and Carbon-13 Nuclear Magnetic Resonance Spectra

^{1}H- and ^{13}C-NMR, DEPT135, ^{1}H,^{1}H-COSY, HSQC, and HMBC spectra were obtained on a Bruker Fourier 300 spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University) operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C.

Deuterated solvents for NMR spectra were used, including deuterated chloroform ($CDCl_3$), deuterated acetone (acetone-d_6) and deuterated methanol (CD_3OD). Chemical shifts were reported in ppm scale and coupling constants were reported in Hz using the chemical shift of the residue in NMR solvent as the reference signal.

Proton-detected heteronuclear correlations were measured using HSQC (optimized for $^{1}J_{HC}$ = 145 Hz) and HMBC (optimized for $^{n}J_{HC}$ = 4 and 8 Hz) pulse sequences.

2.4 Solvents

All organic solvents employed throughout this work were of commercial grade and were redistilled prior to use.

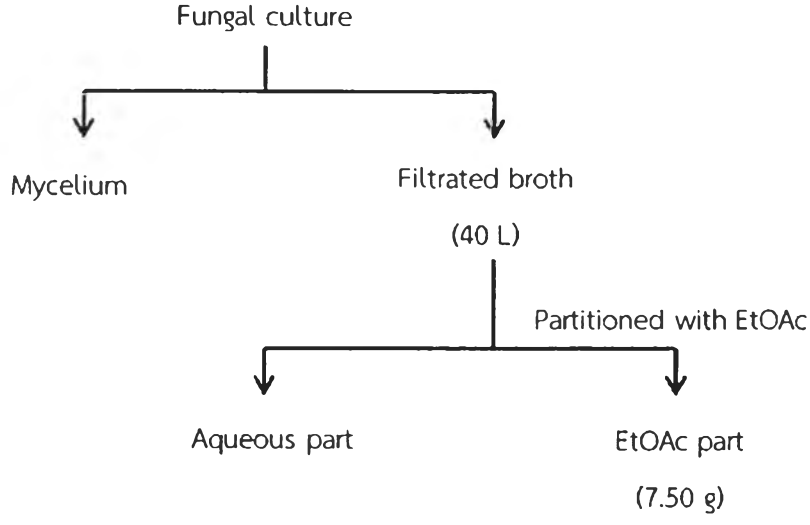
3. Fermentation, Extraction and Isolation

3.1 Fermentation

The endophytic fungus *Phomopsis* sp. AANN8 was grown on PDA plates at 25 ^{o}C approximately for 7 days depending on growth rate. Six pieces (1 x 1 cm^2) of the grown culture were inoculated into a 1000-ml Erlenmeyer flask containing 200 ml of yeast extract sucrose broth (YSB) and incubated at 25 ^{o}C for 21 days under a stationary condition.

3.2 Extraction

The fungal culture was separated into the mycelium and filtrate parts. The filtrated broth (40 l) was partitioned with ethyl acetate three times (20 l each). The ethyl acetate phase was evaporated to dryness under reduced pressure using a rotary evaporator to yield the ethyl acetate extract (7.50 g), as presented in **Scheme 1**.



Scheme 1. Extraction of the *Phomopsis* sp. AANN8 fungal culture.

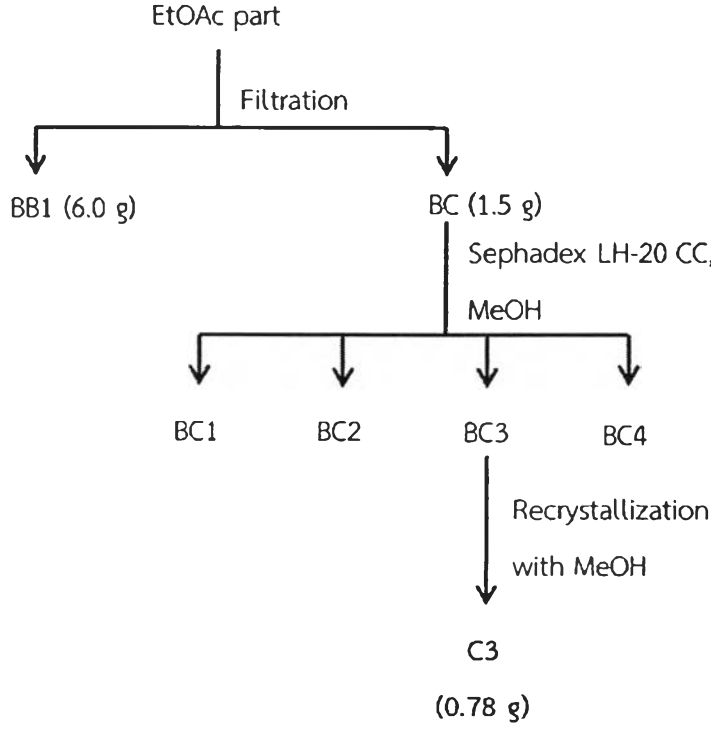
3.3 Fractionation of the Ethyl Acetate Extract

The ethyl acetate extract (7.50 g) was re-dissolved in ethyl acetate and separated by filter paper (Whatman™ No.1) to afford brown precipitates or BC (1.50 g), and the filtrate part. The filtrate was evaporated to dryness under reduced pressure using a rotary evaporator to yield BB1 (6.00 g).

3.3.1 Isolation of C3

The precipitate BC (1.50 g) was subjected to a Sephadex LH-20 column (2.5 cm x 90 cm) and eluted with methanol. Fifteen fractions (30 ml each) were collected and examined by TLC using 5 % methanol in dichloromethane as the mobile phase, and then combined into 4 fractions: BC1 (2.1 mg), BC2 (39.1 mg), BC3 (1.42 g), and BC4 (32.2 mg).

Fraction BC3 (1.42 g) was crystallized in methanol to afford compound C3 as colorless needles (0.78 g). The isolation of compound C3 is presented in **Scheme 2.**



Scheme 2. Isolation of C3.

3.4 Fractionation of Fraction BB1

Fraction BB1 (6.00 g) was separated by quick column chromatography using a sintered glass filter column of silica gel (70 g, 7.5 cm x 3.5 cm). BB1 was dissolved in a small amount of ethyl acetate and methanol, triturated with Kieselgurh and dried under vacuum. Elution was performed in a polarity gradient manner with mixtures of hexane-ethyl acetate (10:0 to 0:10) and methanol. Forty two fractions (50 ml each) were collected and combined according to their TLC patterns by using a mixture of

hexane-ethyl acetate (3:7) as the mobile phase into 5 fractions: BB2 (0.30 g), BB3 (0.56 g), BB4 (2.10 g), BB5 (0.89 g), and BB6 (1.30 g). Their TLC patterns are shown in **Figure 6**.

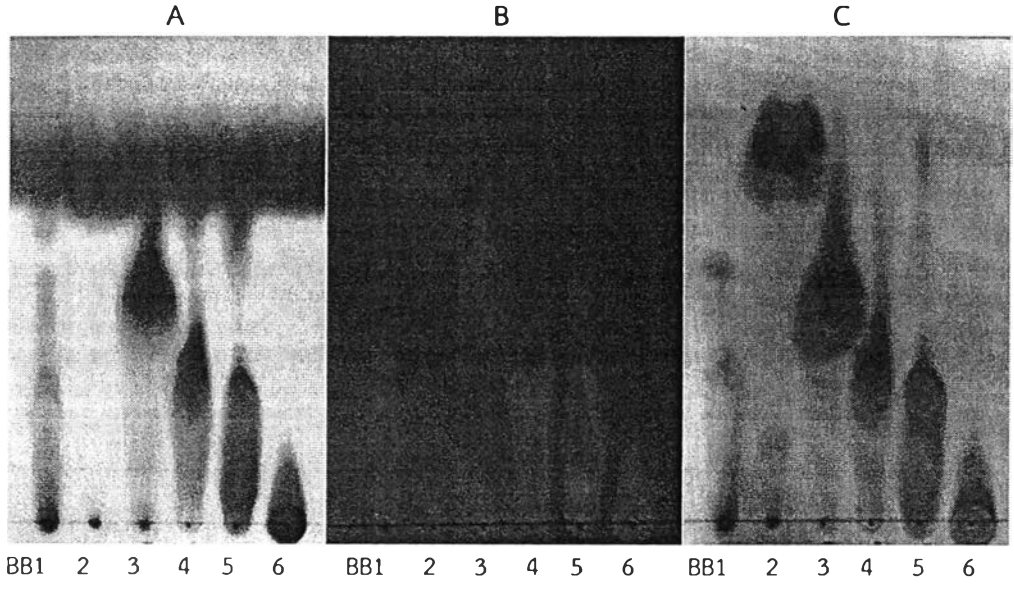


Figure 6. TLC patterns of fractions BB1-BB6 (Silica gel, hexane : EtOAc (3:7), A = under UV 254 nm, B = under UV 365 nm and C = spraying anisaldehyde-sulfuric acid reagent).

3.4.1 Isolation of BB21-C

Fraction BB4 (2.10 g) was subjected to gel filtration chromatography using a Sephadex LH-20 column (2.5 cm x 90 cm) eluting with methanol. Thirty fractions (20 ml each) were collected and examined by TLC using hexane-ethyl acetate (3:7) as the mobile phase, and then combined into 7 fractions: BB7 (11.5 mg), BB8 (110.4 mg), BB9 (931.5 mg), BB10 (723.0 mg), BB11 (20.3 mg), BB12 (30.9 mg), and BB13 (20.7 mg). Their TLC patterns are shown in **Figure 7**.

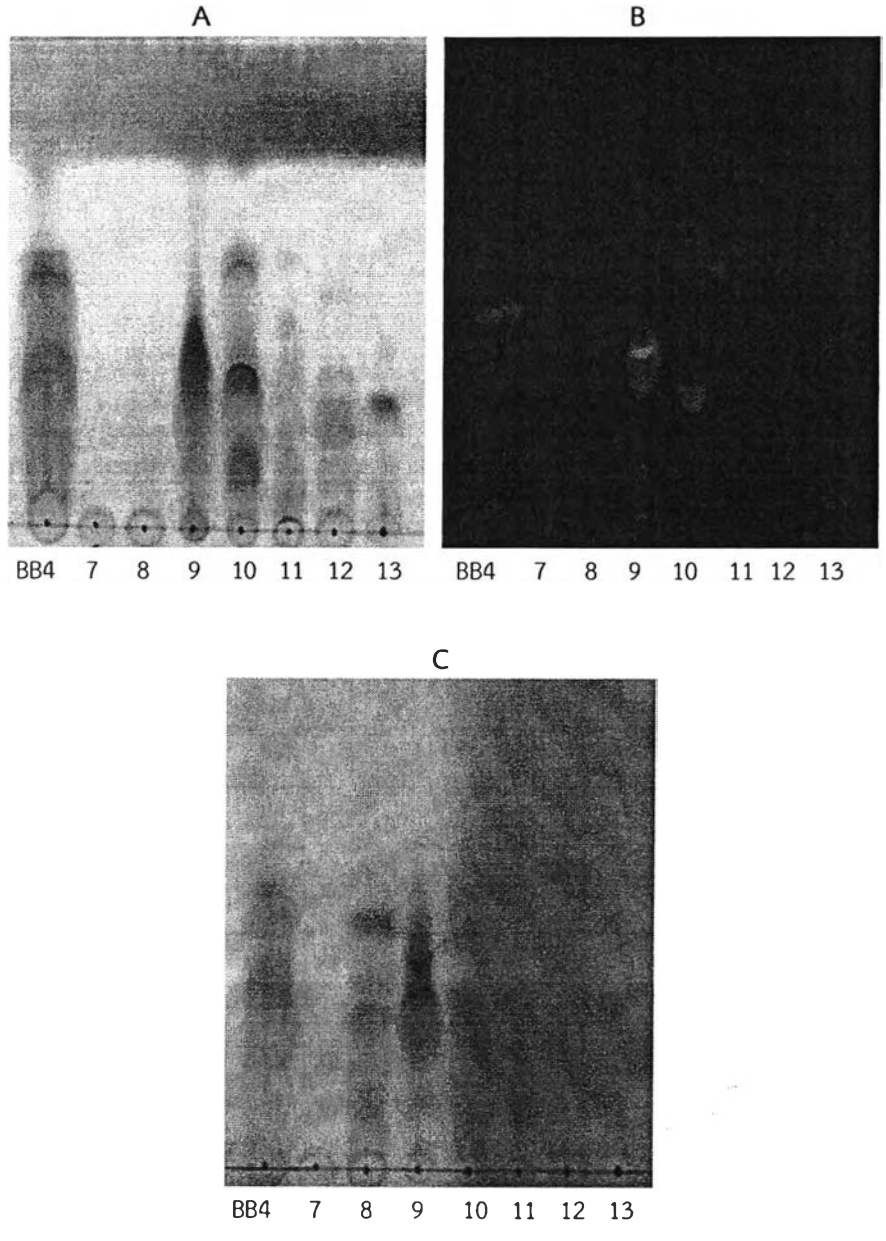


Figure 7. TLC patterns of fractions BB4 and BB7-BB13 (Silica gel, hexane : EtOAc (3:7), A = under UV 254 nm, B = under UV 365 nm and C = spraying anisaldehyde-sulfuric acid reagent).

Fraction BB10 (723.0 mg) was further separated on a silica gel column (40 g, 2.5 cm x 24 cm) using hexane-ethyl acetate (4:6) as the mobile phase. Fifty two

collected fractions (10 ml each) were combined into 8 fractions: BB19 (15.4 mg), BB20 (74.5 mg), BB21 (90.9 mg), BB22 (75.7 mg), BB23 (94.7 mg), BB24 (57.2 mg), BB25 (49.1 mg), and BB26 (83.2 mg). Their TLC patterns are shown in **Figure 8**.

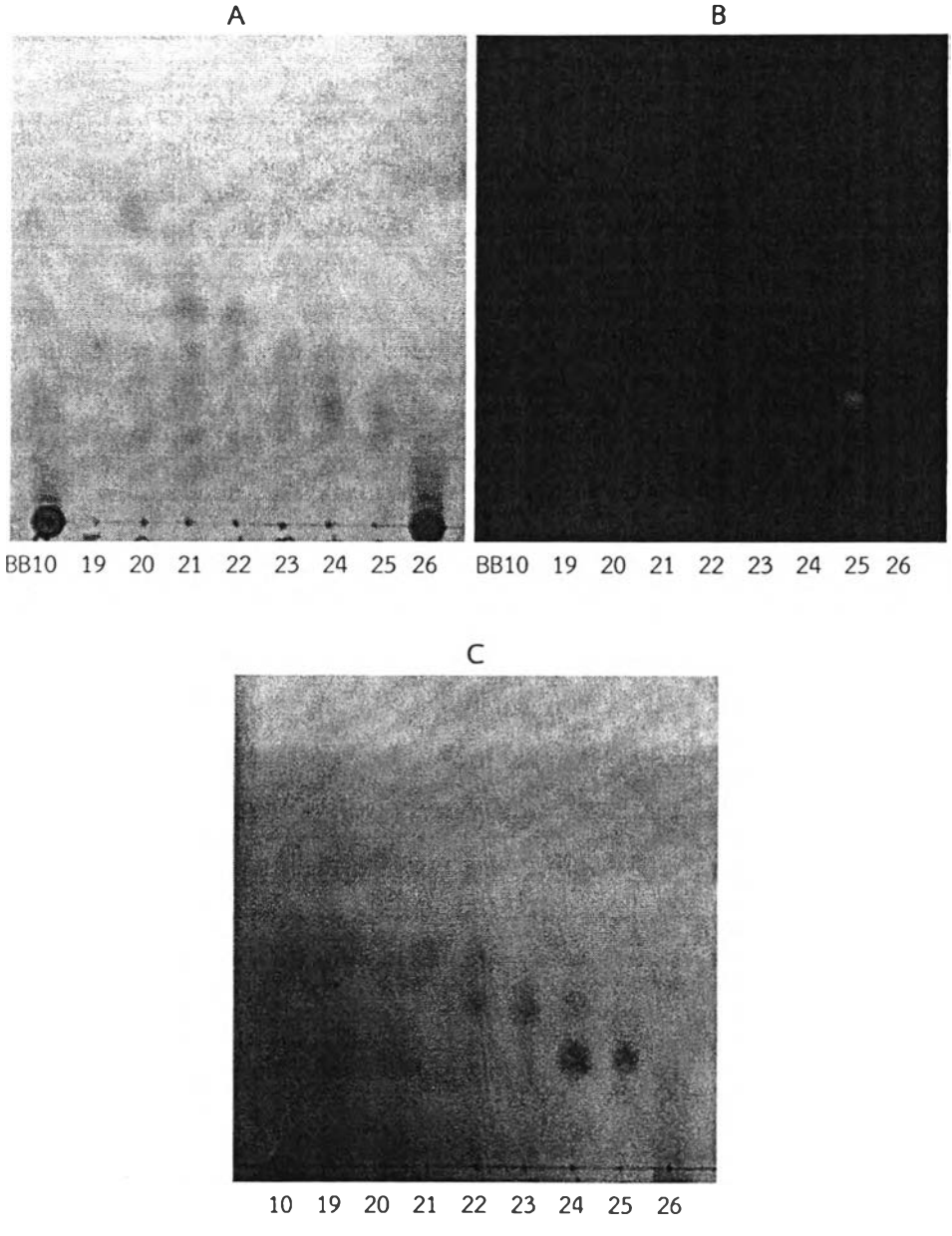


Figure 8. TLC patterns of fractions BB10 and BB19-BB26 (Silica gel, hexane : EtOAc (4:6), A = under UV 254 nm, B = under UV 365 nm and C = spraying anisaldehyde-sulfuric acid reagent).

Fraction BB21 (90.9 mg) at the concentration 90.9 mg/ml was purified by HPLC using 30 % methanol in water as the mobile phase, injection volume 40 µl, flow rate 3 ml/min to yield compound BB21-C as colorless needles (9.5 mg) at retention time 11.0-12.5 minute. The HPLC chromatogram is shown in **Figure 9** and the isolation of compound BB21-C is presented in **Scheme 3**.

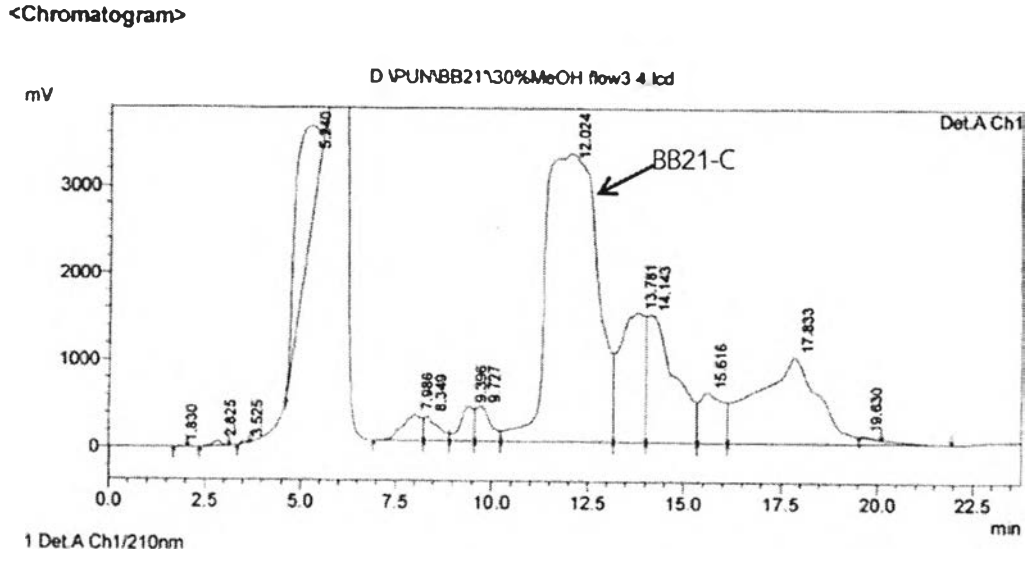
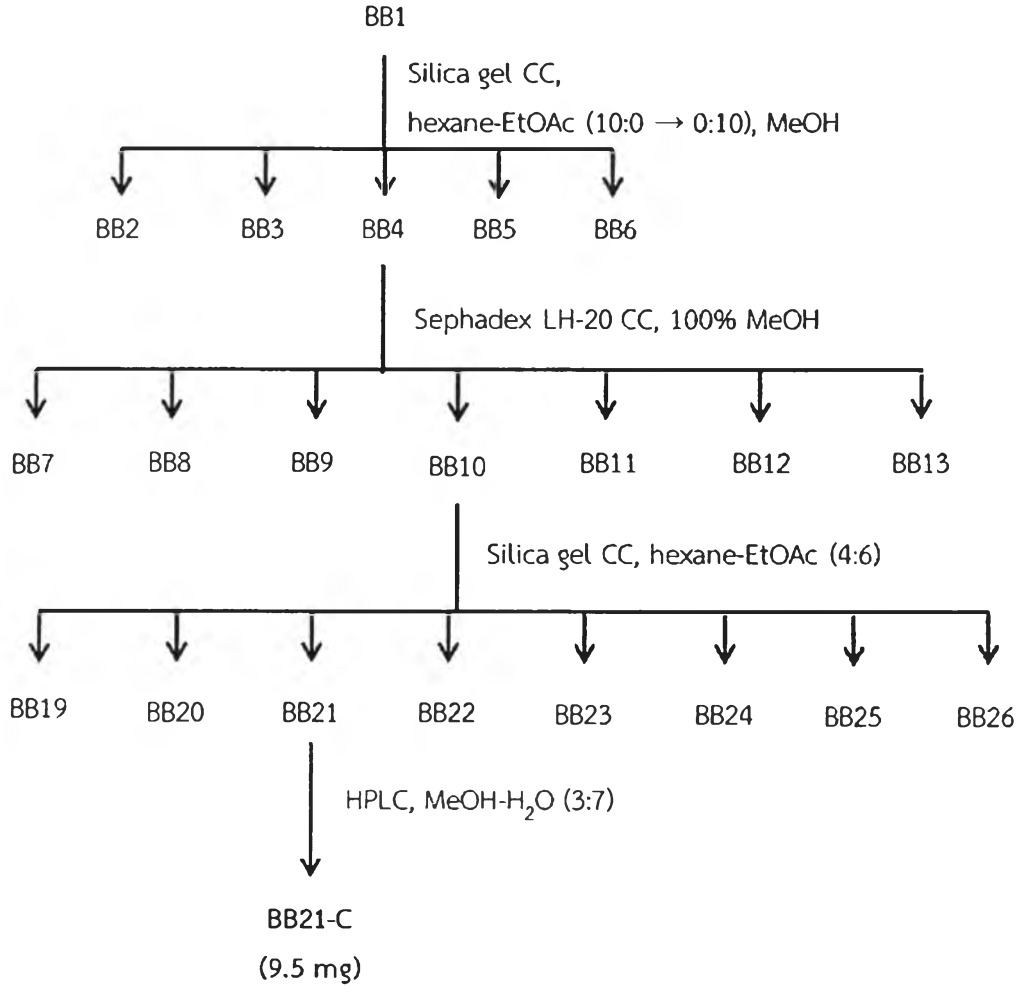


Figure 9. HPLC chromatogram of fraction BB21.



Scheme 3. Isolation of BB21-C.

3.4.2 Isolation of BB28-C

Fraction BB9 (931.5 mg) was chromatographed on a silica gel column (60 g, 2.5 cm x 34 cm), eluted with dichloromethane-acetone (9:1). Ninety five fractions (20 ml each) were collected and combined into 7 fractions: BB27 (14.2 mg), BB28 (42.1 mg), BB29 (90.0 mg), BB30 (339.4 mg), BB31 (6.2 mg), BB32 (77.5 mg), and BB33 (109.7 mg). Their TLC patterns are shown in **Figure 10**.

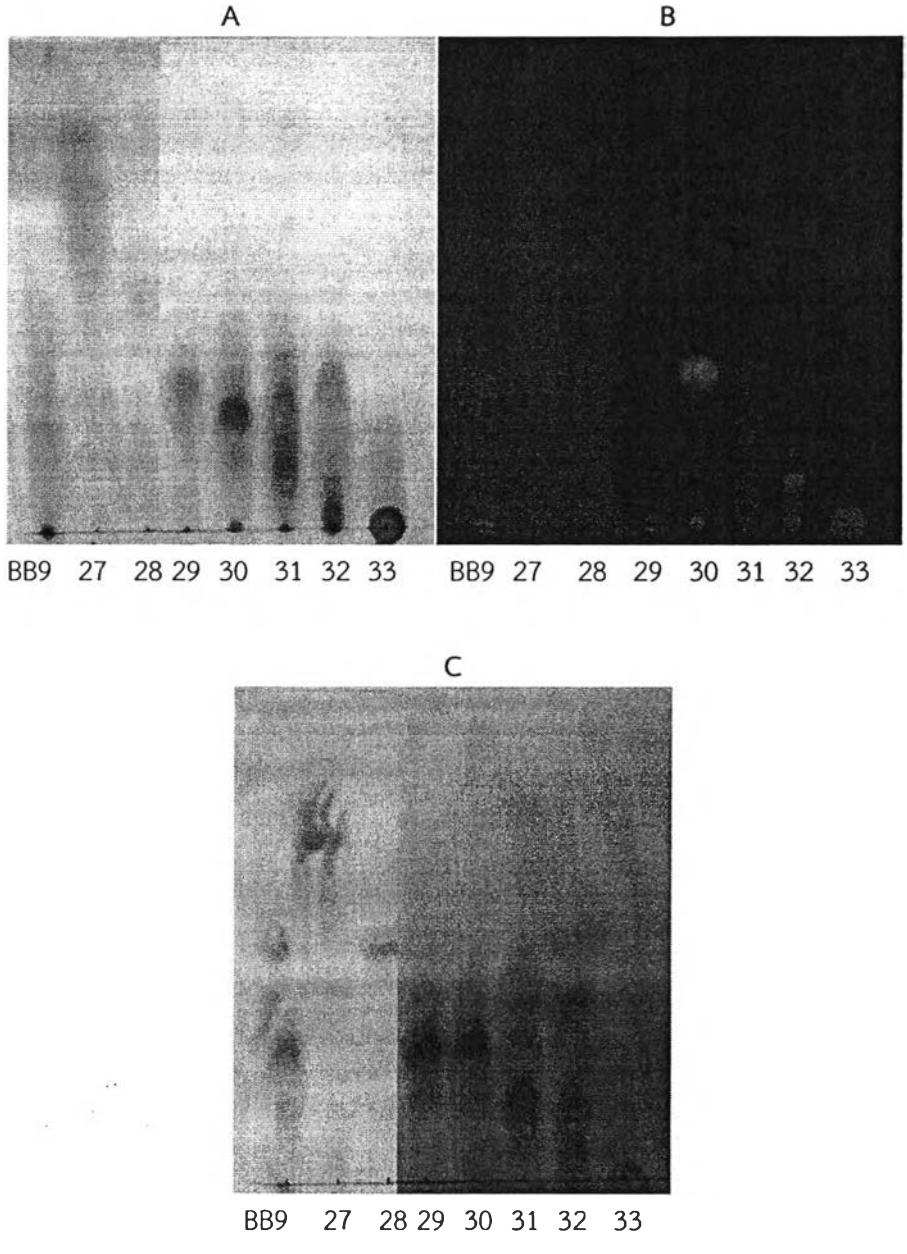


Figure 10. TLC patterns of fractions BB9 and BB27-BB33 (Silica gel, hexane : EtOAc (3:7), A = under UV 254 nm, B = under UV 365 nm and C = spraying anisaldehyde-sulfuric acid reagent).

Fraction BB28 (42.1 mg) at the concentration 42.1 mg/ml was purified by HPLC using 30 % methanol in water as the mobile phase, injection volume 20 µl, flow rate 2 ml/min to provide BB28-C as a colorless amorphous powder (6.9 mg) at retention time 20.0-23.0 minute. The HPLC chromatogram is shown in **Figure 11** and the isolation of BB28-C is presented in **Scheme 4**.

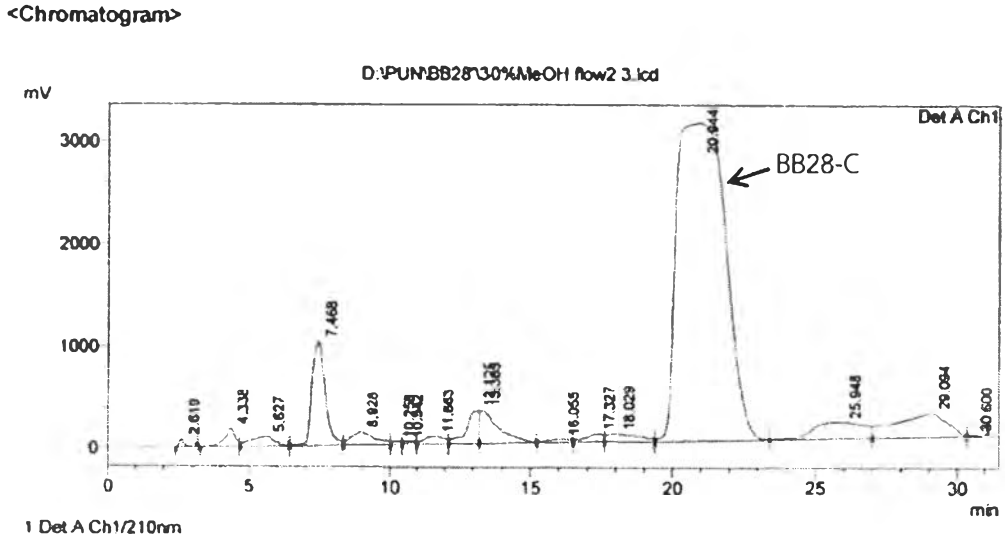
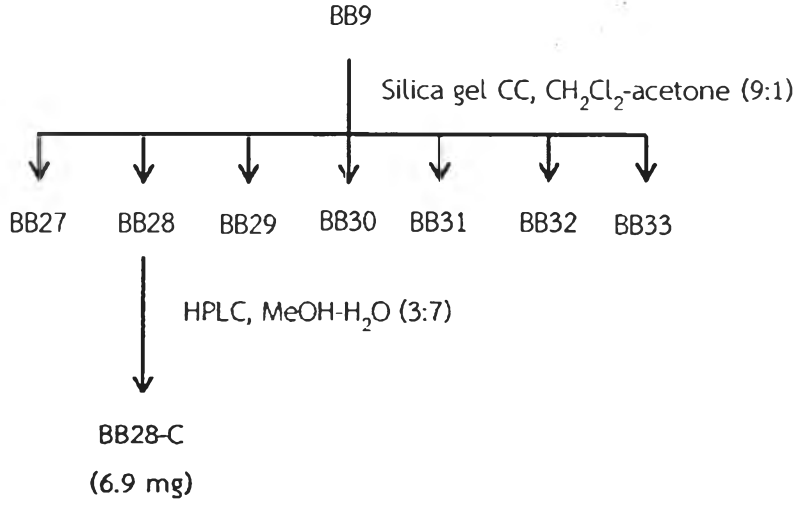


Figure 11. HPLC chromatogram of fraction BB28.



Scheme 4. Isolation of BB28-C.

4. Physical and Spectral Data of the Isolated Compounds

4.1 C3

Compound C3 was obtained as colorless needles (783.7 mg, 1.96 %w/v of the *Phomopsis* fermentation broth). The compound is soluble in methanol.

UV : λ_{max} (MeOH) nm (log ε): 209 (3.99); **Figure 13**.

IR : ν_{max} cm^{-1} (KBr): 3041, 1694, 1418, 1201, 924, 639; **Figure 14**.

FABMS : *m/z* 117 $[M-H]^-$ (Glycerol); **Figure 15**.

^{1}H NMR : δ ppm, 300 MHz, in CD_3OD; 2.58 (4H, s); **Table 4, Figure 16**.

^{13}C NMR : δ ppm, 75 MHz, in CD_3OD; 174.8 and 28.4; **Table 4, Figure 17**.

4.2 BB21-C

Compound BB21-C was obtained as colorless needles (9.5 mg, 0.02 %w/v of the *Phomopsis* fermentation broth). The compound is soluble in acetone and methanol.

UV : λ_{max} (MeOH) nm (log ε): 210 (4.16), 282 (3.01); **Figure 18**.

IR : ν_{max} cm^{-1} (KBr): 3392, 3151, 2063, 1598, 1512, 1232, 818; **Figure 19**.

HR-EIMS : *m/z* 138.0680 $[M]^{+\cdot}$, calc for $C_8H_{10}O_2$, 138.0681; **Figure 20**.

^{1}H NMR : δ ppm, 300 MHz, in acetone-d_6; 8.15 (1H, s), 7.03 (2H, d, *J* = 8.5 Hz), 6.72 (2H, d, *J* = 8.5 Hz), 3.69 (1H, s), 3.66 (2H, t, *J* = 7.2 Hz) and 2.69 (2H, t, *J* = 7.2 Hz); **Table 5, Figure 21**.

^{13}C NMR : δ ppm, 75 MHz, in acetone-d_6; 156.5, 131.6, 130.6, 115.8, 64.2 and 39.4; **Table 5, Figure 22**.

4.3 BB28-C

BB28-C was obtained as colorless amorphous powder (6.9 mg, 0.02 %w/v of the *Phomopsis* fermentation broth). BB28-C is soluble in dichloromethane.

IR : ν_{max} cm^{-1} (KBr): 3435, 1733, 1630, 1557; **Figure 24.**

EIMS : *m/z* 242 $[M]^{+\cdot}$; **Figure 25.**

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; 4.96 (1H, dq, *J* = 6.3, 3.3 Hz), 4.86 (1H, p, *J* = 6.3 Hz), 4.69 (2H, t, *J* = 6.0 Hz), 4.68 (2H, t, *J* = 6.0 Hz), 3.93 (1H, dq, *J* = 6.3, 3.3 Hz), 3.79 (1H, p, *J* = 6.3 Hz), 3.02 (2H, dt, *J* = 6.0 Hz), 3.01 (2H, t, *J* = 6.0 Hz), 1.27 (3H, d, *J* = 6.3 Hz), 1.26 (3H, d, *J* = 6.3 Hz), 1.21 (3H, d, *J* = 6.3 Hz) and 1.19 (3H, d, *J* = 6.3 Hz); **Table 6, Figure 26.**

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; 169.10, 169.01, 76.20, 75.77, 69.87, 69.82, 69.25, 31.41, 19.10, 17.91, 16.18 and 13.94; **Table 6, Figure 28.**

5. Determination of Antileukemic Activity

The antileukemic activity evaluation in this study was performed by the Department of Microbiology, Faculty of Science, Mahidol University.

The crude extract, fractions and isolated compounds from *Phomopsis* fermentation broth were evaluated for their antileukemic activity by sulforhodamine B (SRB) colorimetric method (Skehan *et al.*, 1990) using the human acute monocytic leukemia (THP-1) cell line. Ellipticine was used as the positive control and 10 % DMSO was used as the negative control. The samples were diluted to a final concentration 20 µg/ml. The protocols are as follows:

1. The THP-1 cell line was grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 % foetal bovine serum (FBS) and 2 mM glutamine,

incubated at 37 °C in 100 % humidity with a 5 % CO_2 atmosphere in air. Cells at exponential growth phase were harvested and diluted to 1 x 10^5 cells/ml with fresh medium.

2. Successively, the cell suspension was mixed gently before aliquots of 190 µl were plated in a 96-well microplate and incubated at 37 °C in 5 % CO_2 incubator for 72 hours.

3. After cell growth, 10 µl of test sample solution in 10 % DMSO was dispensed into each well and incubated at 37 °C in 5 % CO_2 incubator for 24 hours.

4. At the end of the incubation period, 50 µl of cold 50 % trichloroacetic acid (TCA) solution was added to each well, and the plate was further incubated at 4 °C for 30 minutes.

5. After 30 minutes, the supernatant of culture was removed, cells were washed with tap water 4 times and plate was air dried.

6. 100 µl of 0.4 % SRB solution in 1 % acetic acid was added to each well, and the plate was then incubated at room temperature for 30 minutes.

7. After 30 minutes, 100 µl of 1 % acetic acid was added to each well for 4 times to rinse off unbound dye and the plate was air dried.

8. 200 µl of 10 mM Tris base (pH 10) was added to each well. The plate was shaken for 5 minutes and the optical density (OD) was measured using an ELISA plate reader at 515 nm. The cytotoxicity percentage was calculated using the following equation:

$$\% \text{ Cytotoxicity} = [1 - (OD_{test} / OD_{control})] \times 100$$

Whereas OD_{test} and $OD_{control}$ are the absorbance from treated and untreated conditions, respectively.

The crude extract, fractions and isolated compounds that exhibited antileukemic activity against THP-1 cell line over 55 % cytotoxicity at the final concentration 20 µg/ml were further tested for dose response curves.

Dose response curves were plotted from 4 concentrations (µg/ml) of 5-fold serially diluted test samples and the effective concentrations of the samples that exhibited cytotoxicity against THP-1 cell line by 50 % (EC_{50} value). The antileukemic activity of the samples tested was compared with ellipticine, which its EC_{50} was at 19.5 µg/ml.

The criteria of interpretation are shown below

Crude extract	active; EC_{50} < 20 µg/ml
Pure compound	active; EC_{50} < 5 µg/ml