CHAPTER III

EXPERIMENTS

3.1 Instruments and equipments

3.1.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared Spectrophotometer. Solid samples were formally examined by incorporating the sample with potassium bromide (KBr) to form a pellete.

3.1.2 Nuclear Magnetic Resonance Spectrometry (NMR)

^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, gHMBC, 1D-NOE, NOESY and TOCSY spectra were recorded on a Mercury Varian 400 Spectrometer operated at 400 MHz for ^{1}H nuclei and at 100 MHz for ^{13}C nuclei. Deuterated solvents, chloroform-*d* and deuterium oxide (D_2O) were used in NMR experiments. Reference signals were the signals of residual protonated solvents at δ_H 7.26(s) ppm and δ_C 77.16 (t) ppm for $CDCl_3$ and at δ_H 4.79 (s) ppm for D_2O.

3.1.3 Mass Spectrometry (MS)

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV. High resolution mass spectroscopy were performed using Electrospray ionization mode on a Micromass LCT (LC/MS) at National Center for Genetic Engineering and Technology (BIOTEC), National Science and Technology Development Agency Building (NSTDA) and Chemistry Department, Mahidol University.

3.1.4 UV-Vis spectrometry

UV-Vis spectra were recorded on a Varian Cary 50 Probe spectrophometer. The samples were diluted by solvent to appreciate concentration.

3.1.5. Polarimetry

Specific optical rotations were recorded on a Perkin Elmer 341 in chloroform, ethanol and H_2O.

3.1.6. Electrothermometer

Melting points were measured on an Electrothermal 9100 melting point apparatus.

3.1.7 X-ray Diffractometer

Crystal data were obtained with a BRUKER SMART CCD Diffractometer at Department of Physics, Factculty of Science and Technology, Thammasart University.

3.2 Chemicals

3.2.1 Solvents

All solvents used in this research such as hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), methanol (MeOH) and acetic acid (AcOH) were commercial grade and purified prior to use by distillation. The reagent grade solvents were used for recrystallization.

3.2.2 Culture media

Culture media used for cultivation of *Emericella variecolor* were Malt extract, Malt Czapek-Dox and Czapek-Dox for liquid and solid media and malt extract of Hi-media were used in this research.

3.2.3 Other chemicals

Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ASTM) was used as adsorbent for column chromatography.

Merck's silica gel 60 Art. 1.09385.1000 (230-400 mesh ASTM) was used as adsorbent for column chromatography.

Merck's silica gel $60GF_{254}$. 1.07731.1000 were applied as adsorbent for preparative TLC.

Merck's TLC aluminium sheet, silica gel $60F_{254}$ procoated 25 sheets, 20x20 cm^2, layer 0.2 mm was used to identical fractions.

3.3 Culture media

Culture media used for culturvation of *Emericella variecolor* were malt extract agar (MEA), Malt Czapek-Dox agar and Czapek-Dox agar.

The medium for growing bacteria was nutrient agar (NA).

3.4 Sample collection

Emericella variecolor was isolated from mature petrioles of *Croton oblongifolius* trees collected in June 22nd, 2002, Amphur Panomsarakam (N 13° 37' 52.9" E 101° 18' 22.7), Chachoengsao Province, Thailand. The fungus was isolated using modified Schulz's surface-sterilization technique (Schulz et al., 1993). The surface-sterilized disk were placed on potato dextrose agar (PDA), incubated at room temperature and purified on PDA. During purification procedures, the strain was identified at genus level by means of light microscopic observation and *E. variecolor* was characterized by slide culture method and ribosomal internal transcribed spacer (ITS) analysis.

3.5 Isolation of metabolites of *Emericella variecolor*

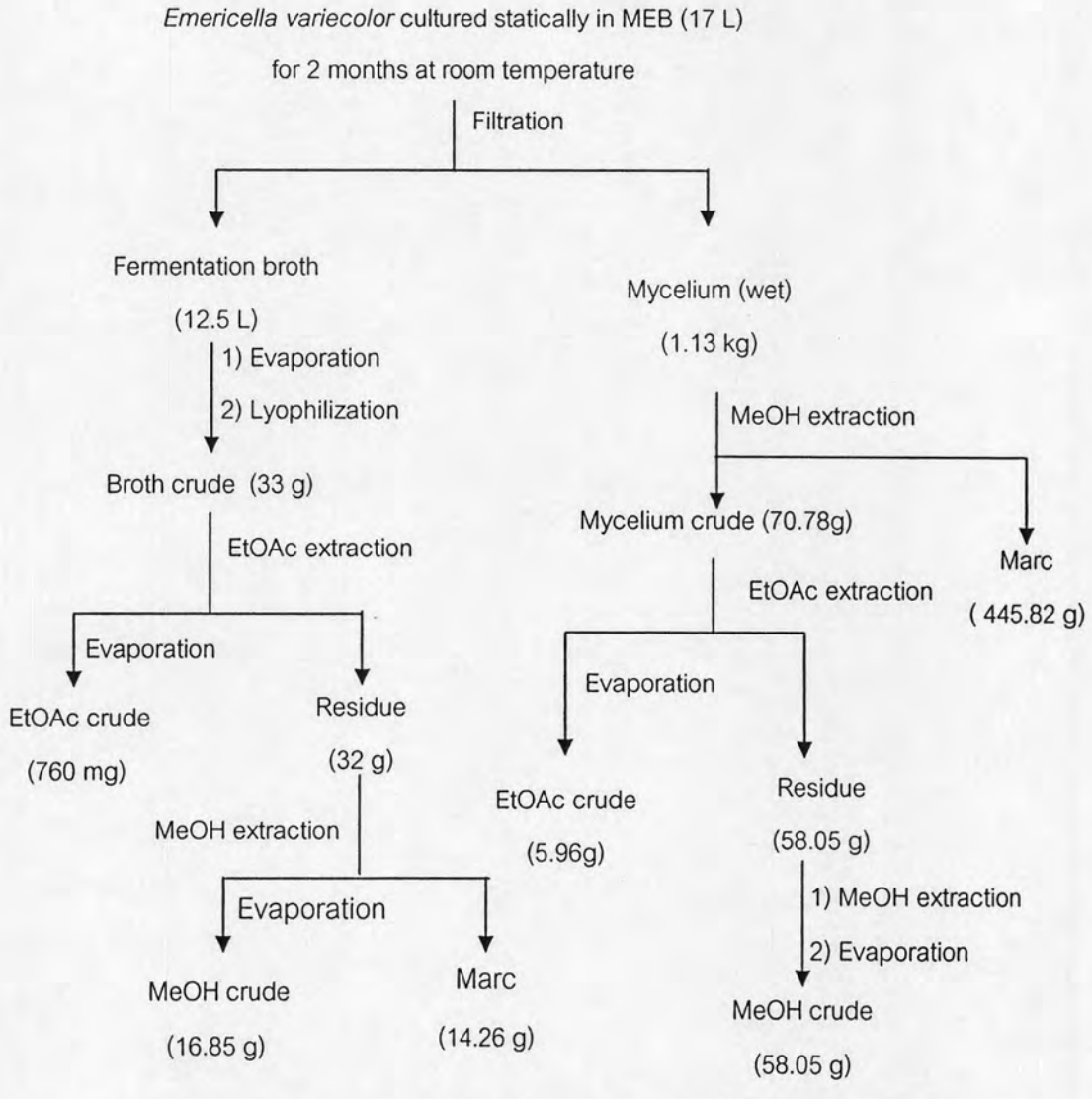
3.5.1 Metabolite of *Emericella variecolor* in Malt extract broth (MEB)

Stock cultures of *Emericella variecolor* cultured on the malt extract agar plates at room temperature for 7 days were cut into 8 mm diameter by cork hole borrer and then inoculated five disks into 250 ml flask containing 100 ml of MEB (x 170). *Emericella variecolor* were cultured statically at room temperature for 2 months. Fermentation broth and mycelium were filtered through a filter paper Whatman no.1.

Emericella variecolor cultured in Malt extract broth (MEB) (17 L) at room temperature for 2 months was filtered through filter paper Whatman No. 1 to obtain the fermentation broth (12.5 L) and mycelium (1.13 kg of wet weight). Methanol (500 ml) was added into the culture medium for preserved and evaporated by rotary evaporator *in vacuo* to remove methanol and partial water. Then the fermentation broth was lyophilized to give a residue as a dark brown solid (33.0 g). The residue was extracted with ethyl acetate (500 ml x 5) in ultrasonic bath. The ethyl acetate extract was evaporated *in vacuo* to obtain a dark brown solid (760 mg). The remaining residue (32.0 g) was further extracted with methanol (500 ml x 5) in ultrasonic bath. After evaporating

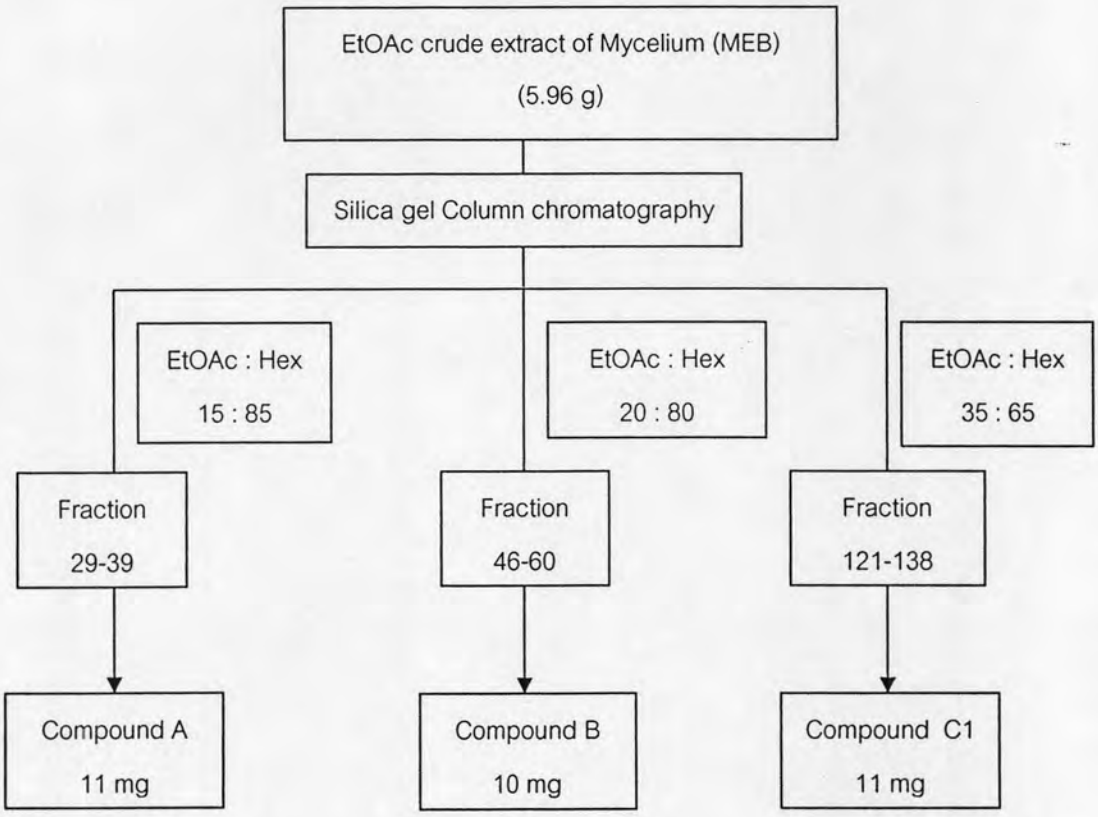
methanol extract (16.85 g) was obtained as a brown solid of residue (14.26 g). The extraction procedure and the results are shown in **Scheme 3.1**.

The mycelium (1.13 kg of wet weight) was extracted with methanol (2 L x 5) and then evaporated the solvent to give a dark brown solid (70.78 g). The dark brown solid was extracted with ethyl acetate (2 L x 5) and with methanol (2 L x 5), respectively. After removal of the solvent, ethyl acetate crude extract (5.96 g) and the methanol crude residue (58.05 g) was obtained. The extraction procedure was summarized in **Scheme 3.1**.



Scheme 3.1 Extraction of fermentation broth and mycelium of *Emericella variecolor* cultured in Malt Extract broth

The ethyl acetate crude from mycelium (1.53 g) was subjected to column chromatography (silica gel, 300 g) using eluents of increasing polarity from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH, MeOH and AcOH 1% in MeOH, respectively.

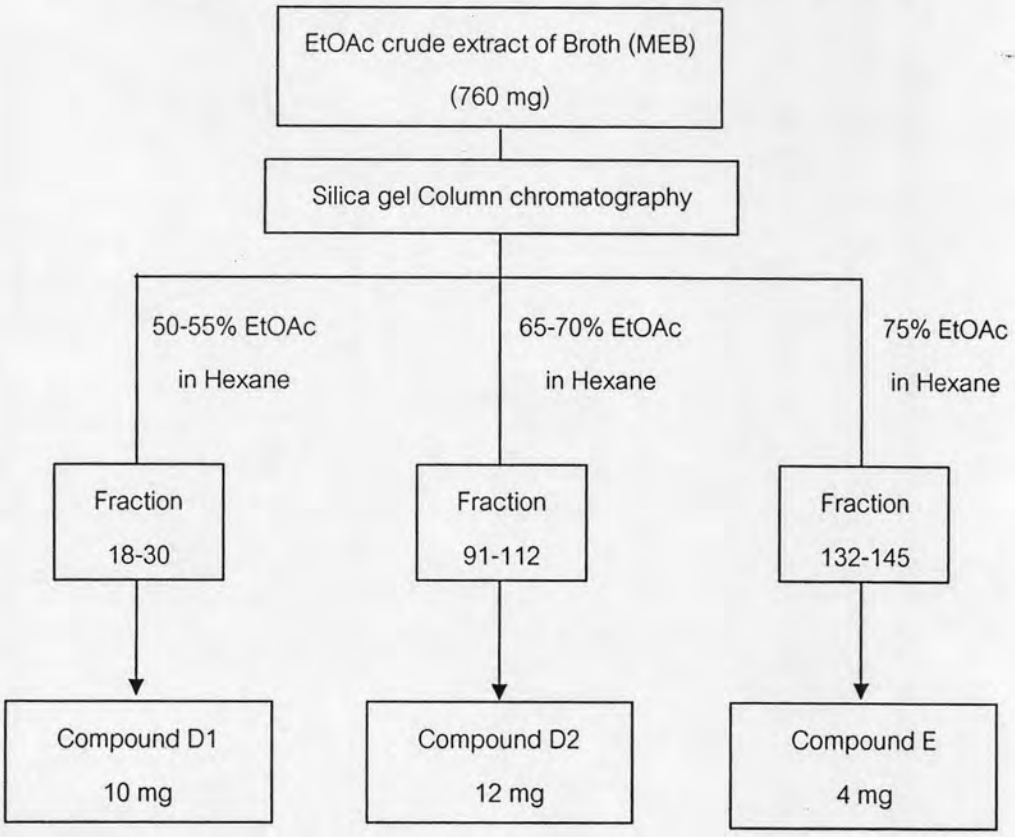


Scheme 3.2 Isolation procedure of ethyl acetate crude from mycelium culture in MEB of *Emericella variecolor*

Isolation procedure of EtOAc crude extract of *Emericella variecolor* mycelium cultured in MEB by silica gel column chromatography was shown in **Scheme 3.2**. Total 385 fractions were obtained from isolation of EtOAc crude extract of mycelium (MEB). Compound A was crystallized from combined fraction 29-39, eluted with 15 % EtOAc in hexane. After filtration and washing with hexane and ethyl acetate, respectively, compound A (11 mg) was obtained as a white solid. Compound B obtained in fraction 46-60, eluted with 20 % EtOAc in hexane was crystallized during evaporation. The precipitate was filtered and then washed with hexane and ethyl acetate, respectively, to give compound B (10 mg) as a white crystal. Fraction 121-138 eluted with 35 % EtOAc

in hexane was crystallized from chloroform to obtain compound C1 (11 mg) as a colorless crystal.

The ethyl acetate crude extract of the fermentation broth (MEB) (760 mg) was subjected to column chromatography (silica gel, 20 g) using eluents of increasing polarity from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH, respectively.



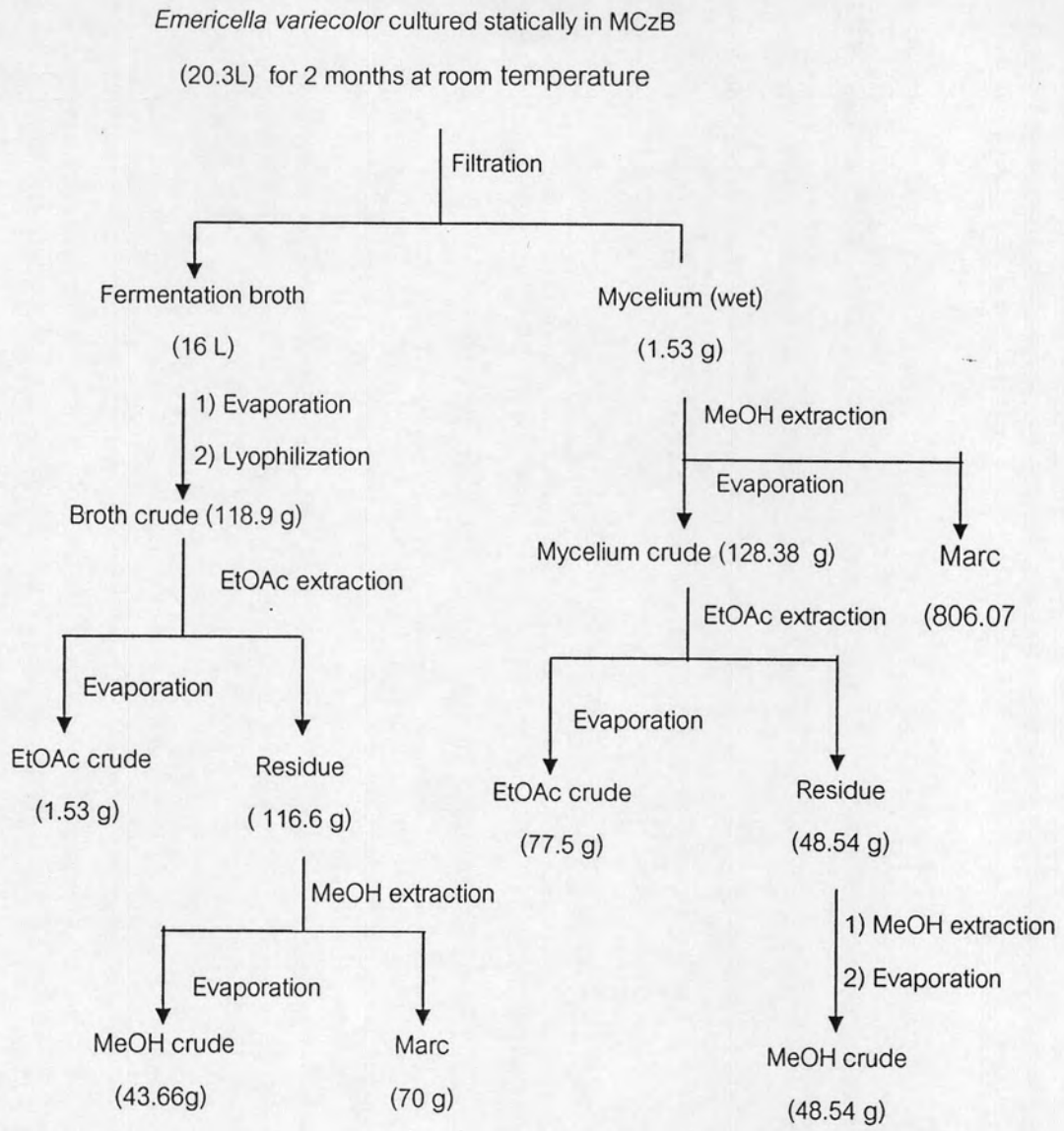
Scheme 3.3 Isolation procedure of ethyl acetate crude from MEB broth of *Emericella variecolor*

Isolation procedure of EtOAc crude extract of fermentation MEB by silica gel column chromatography was shown in **Scheme 3.3**. Total 325 fractions isolated from EtOAc crude extract of broth (MEB). Fraction 18-30 eluted with 50-55% EtOAc in hexane was crystallized with chloroform to obtain compound D1 (10 mg) as a colorless crystal. Fraction 91-112 eluted with 65-70 % EtOAc in hexane was washed by hexane and ethyl acetate, respectively to give compound D2 (12 mg) as a white solid. Fraction132-145 eluted with 75 % EtOAc in hexane was purified by PTLC (silica gel) using 75 % EtOAc in hexane as mobile phase to obtain compound E (4 mg) as brown oil.

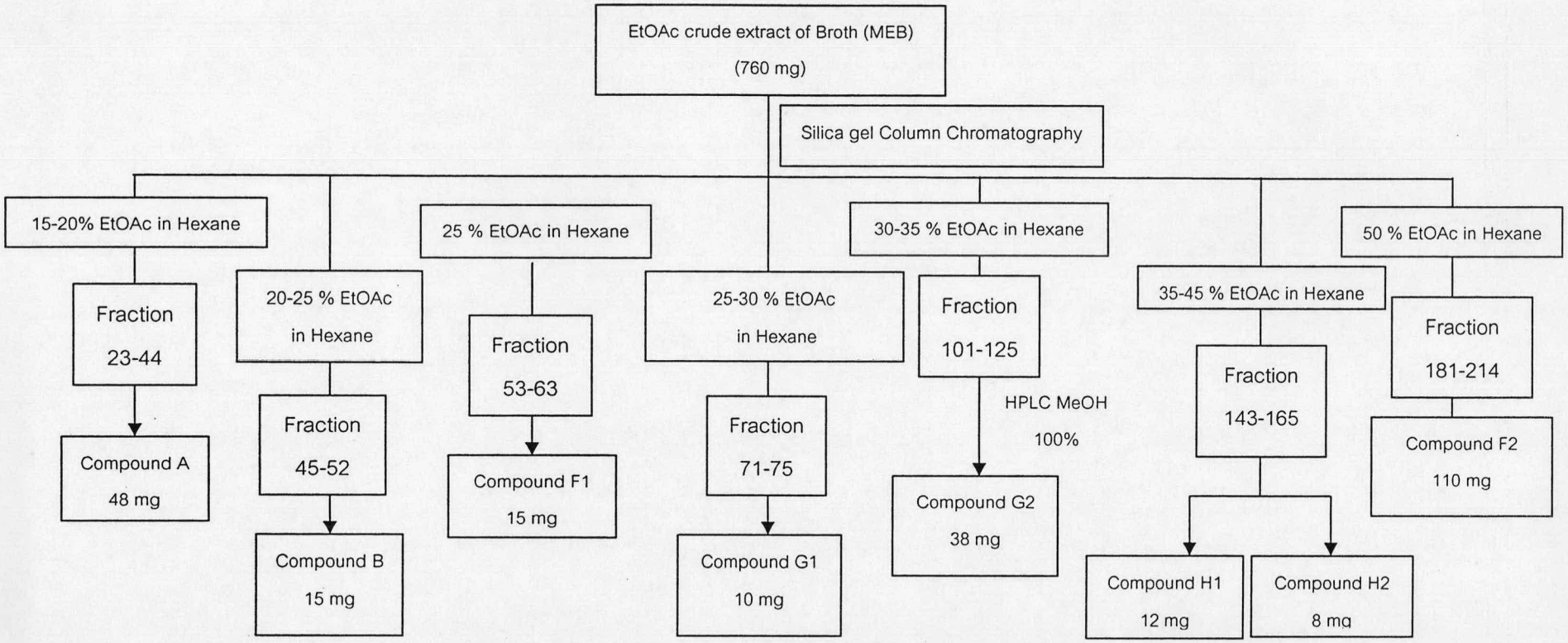
3.5.2 Metabolites of *Emericella variecolor* in Malt Czapek-Dox broth (MCzB)

Stock cultures of *Emericella variecolor* cultured on the Malt Czapek-Dox agar at room temperature for 7 days were cut into 8 mm diameter by cork hole borrer and then inoculated five disks into 250 ml flask containing 100 ml of MCzB (x 203). *Emericella variecolor* were cultured statically at room temperature for 2 months at room temperature. Fermentation broth and mycelium were filtered through a filter paper Whatman no.1 to obtain the fermentation broth (16 L) and mycelium (1.53 kg of wet weight). Methanol (500 ml) was added into the fermentation broth for preservation and evaporated by rotary evaporator *in vacuo* to remove methanol and partial water. Then the fermentation broth was lyophilized to give a residue as a dark brown solid (118.9 g). The residue was extracted with ethyl acetate (500 ml x 5) in ultrasonic bath. The ethyl acetate extract was evaporated *in vacuo* to obtain a dark brown solid (1.53 g). The remaining residue (116.6 g) was further extracted with methanol (500 ml x 5) in ultrasonic bath. After evaporation of methanol) a residue (70 g) was obtained.

The mycelium (1.53 kg of wet weight) was extracted with methanol (2 L x 5) and the solvent was evaporated to give a dark brown solid (128.38 g). The dark brown solid was extracted with ethyl acetate (2 L x 5) and methanol (2 L x 5), respectively. After removal of the solvents, ethyl acetate crude extract (77.5 g) and the methanol crude residue (48.54 g) was obtained. The extraction procedure was summarized in **Scheme 3.4.**

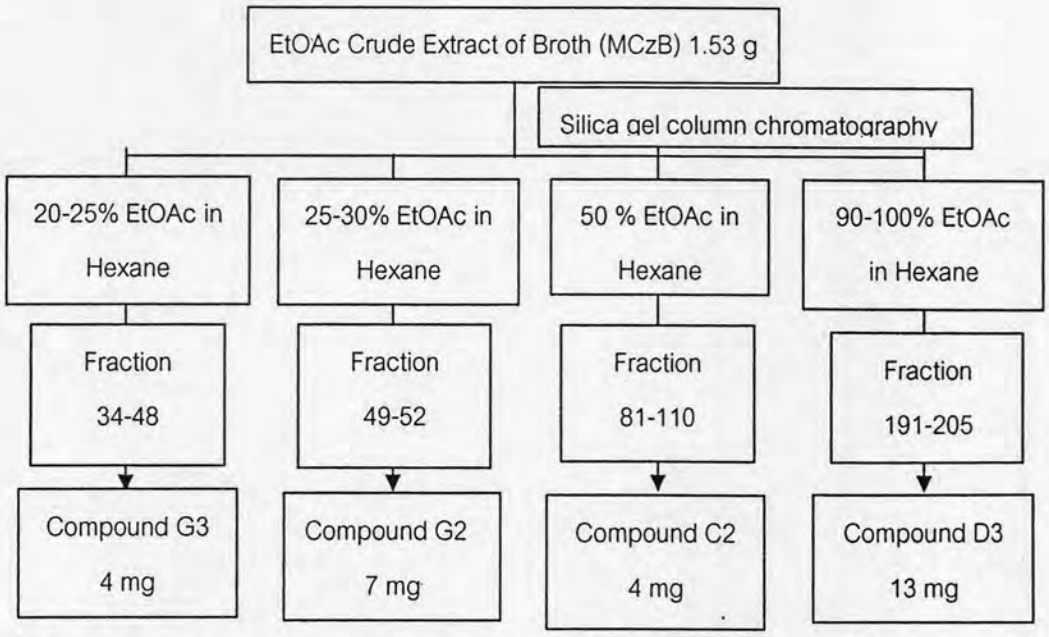


Scheme 3.4 Extraction of fermentation broth and mycelium of *Emericella variecolor* cultured in Malt Czapek Dox broth



Scheme 3.5 Isolation procedure of ethyl acetate crude from mycelium cultured in MEB of *Emericella variecolor*

Isolation procedure of EtOAc crude extract of *E. variecolor* mycelium cultured in MCzB was shown in **Scheme 3.5**. Total 312 fractions were obtained by silica gel column chromatography. Fraction 23-44 eluted with 15-20 %EtOAc in hexane was washed with hexane and ethyl acetate, respectively to give a white solid of compound A (48 mg). Fraction 45-52 eluted with 20-25% EtOAc in hexane to give compound B (15mg) as white crystal. Compound F1 was precipitate from combined fraction 53-63, eluted with 25 % EtOAc in hexane. After filtration and washing with hexane and ethyl acetate respectively, compound F1 (15 mg) was obtained as a yellow solid. Fraction 71-75 eluted with 25-30 % EtOAc in hexane was washed with hexane and ethyl acetate respectively to obtain compound G1 (10 mg) as orange solid. Compound G2 was precipitated from combined fraction 101-125 eluted with 30-35 % EtOAc in hexane, was precipitated during evaporation. The precipitate was filtered and then washed with hexane and ethyl acetate respectively, to obtain compound G2 (38 mg) as orange solid. Fraction 143-165 eluted with 35-45 % EtOAc in hexane was purified by reverse phase HPLC using methanol as mobile phase to give a orange amorphous of compound H1 (12 mg) from retention time at 17 minutes and a orange solid of compound H2 (8 mg) from retention time at 26 minutes. In addition, Fraction 181-214 eluted with 50 % EtOAc in hexane was washed by ethyl acetate, compound F2 (110 mg) to afford as a yellow solid.



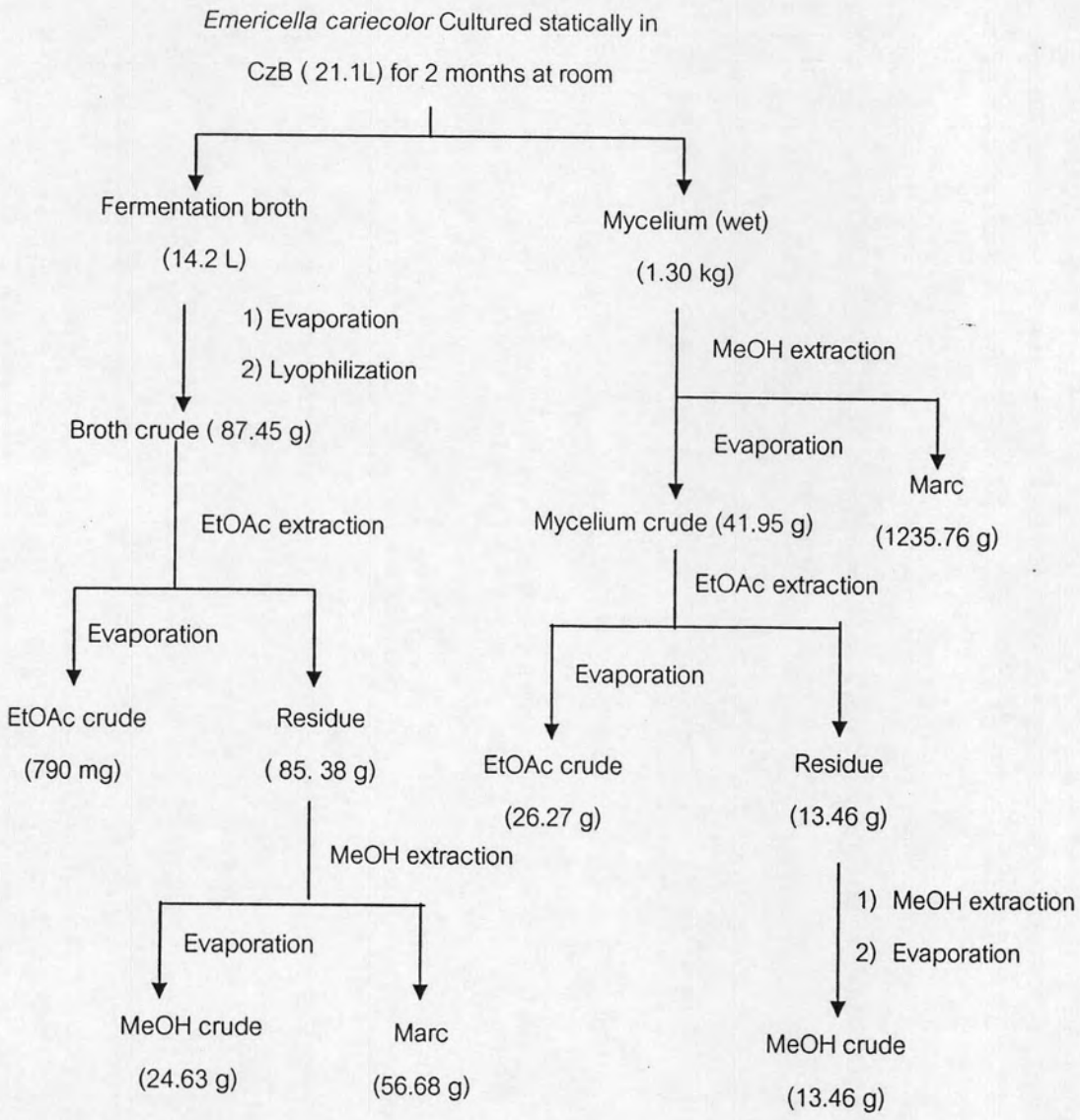
Scheme 3.6 Isolation procedure of ethyl acetate crude in MCzB of *Emericella variecolor.*

Isolation procedure of EtOAc crude extract of MCzB fermentation broth was shown in **Scheme 3.6**. Total 330 fractions were obtained by silica gel column chromatography. Fraction 34-48 eluted with 20-25 % EtOAc in hexane was washed by hexane and ethyl acetate, respectively to give a orange solid as compound G3 (4 mg). Fraction 49-52 eluted with 25-30 % EtOAc in hexane was washed by ethyl acetate to obtain a orange solid as compound C2 (4 mg). Fraction 81-110 eluted with 50 % EtOAc in hexane was washed by hexane and ethyl acetate, respectively to give compound G2 (7 mg) as orange solid. Fraction 191-205 eluted with 90-100 % EtOAc in hexane was washed by hexane and ethyl acetate, respectively to afford compound D3 (13 mg) as white solid.

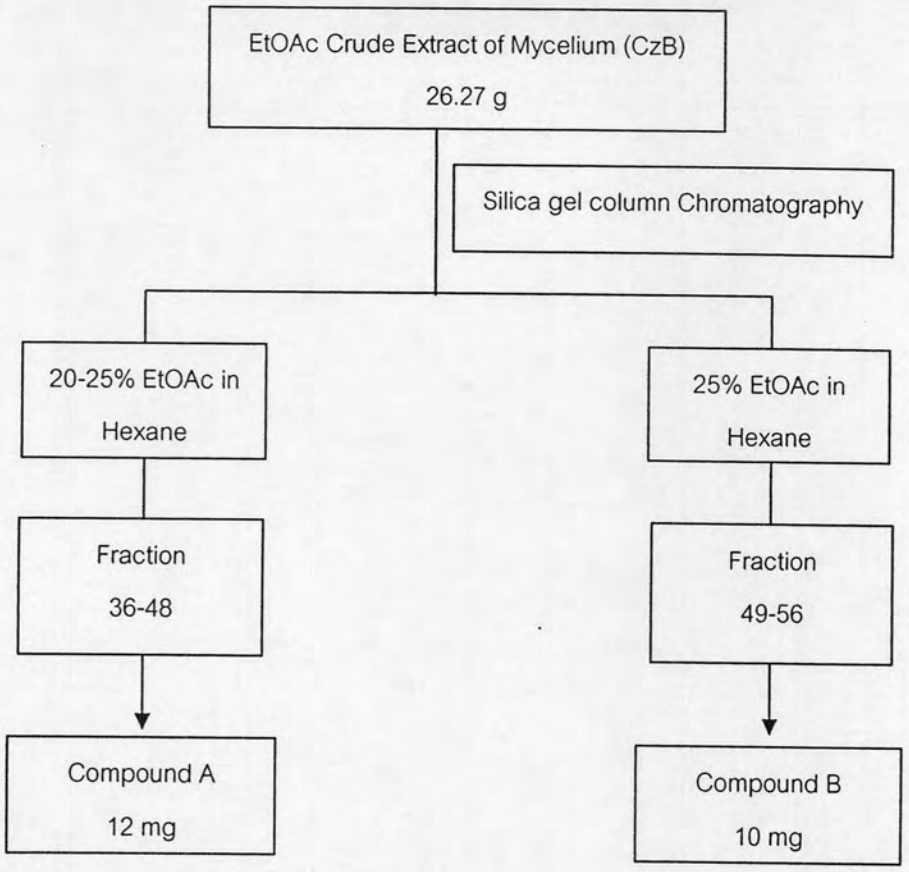
3.5.3 Metabolites of *Emericella variecolor* in Czapek-Dox broth (CzB)

Stock cultures of *Emericella variecolor* cultured on the Czapek-dox agar plates at room temperature for 7 days were cut into 8 mm diameter by cork hole borrer and then inoculated five disks into 250 ml flask containing 100 ml of CzB (x 211). *Emericella variecolor* were cultured statically at room temperature for 2 months. Fermentation broth and mycelium were filtered through a filter paper Whatman no.1 to obtain the fermentation broth (14.2 L) and mycelium (1.30 kg of wet weight). Methanol (2 L) was added into the fermentation broth for preserved and evaporated by rotary evaporator *in vacuo* to remove methanol and partial water. Then the fermentation broth was lyophilized to give a residue as a dark brown solid (87.45 g). The residue was extracted with ethyl acetate (2L x 5) in ultrasonic bath. The ethyl acetate extract was evaporated *in vacuo* to obtain a dark brown solid (790 mg). The remaining residue (85.38 g) was further extracted with methanol (2 L x 5) in ultrasonic bath. After evaporating methanol extract (24.63 g) was obtained as brown solid (56.68 g).

The mycelium (1.30 kg of wet weight) was extracted with methanol (2 L x 5) and then evaporated the solvent to give a dark brown solid (41.95 g). The dark brown solid was extracted with ethyl acetate (2 L x 5) and followed by extraction with methanol (2 L x 5). After removal of the solvent, ethyl acetate crude extract (26.27 g) was obtained and the methanol crude residue (13.46 g) was remained. The extraction procedure was summarized in **Scheme 3.7**.

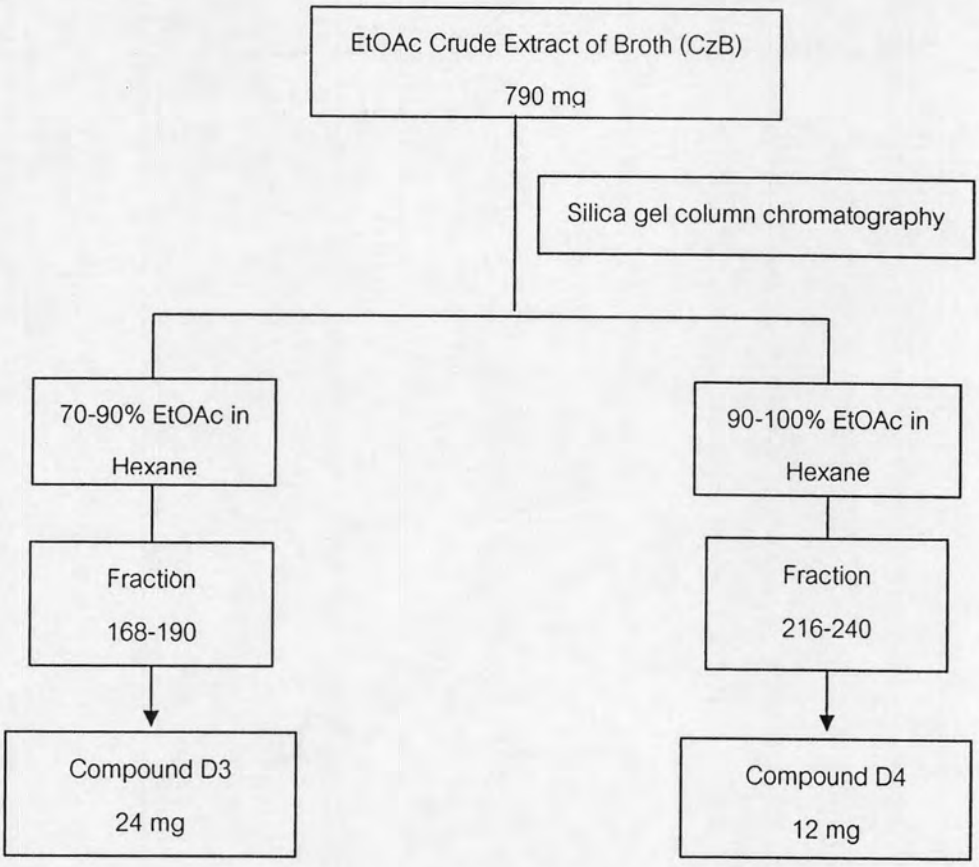


Scheme 3.7 Extraction of fermentation broth and mycelium of *Emericella variecolor* cultured in Czapek Dox broth



Scheme 3.8 Isolation procedure of ethyl acetate crude from mycelium cultured in CzB of *Emericella variecolor*.

Isolation procedure of EtOAc crude extract of mycelium culture in CzB of *Emericella variecolor* was shown in **Scheme 3.8**. Total 280 fractions were obtained by silica gel column chromatography. Compound A was precipitated from combined fraction 36-48, eluted with 20-25% EtOAc in hexane. After filtration and washing with hexane and ethyl acetate respectively, compound A (12 mg) obtained as white solid. Fraction 49-56 eluted with 25 % EtOAc in hexane was washed with hexane and ethyl acetate respectively, compound B (10 mg) was obtained as a colorless crystal.



Scheme 3.9 Isolation procedure of ethyl acetate crude from CzB broth of *Emericella variecolor*

Isolation procedure of EtOAc crude extract of CzB broth of *Emericella variecolor* by silica gel column chromatography was shown in **Scheme 3.9**. Total 340 fractions were obtained from isolation of EtOAc crude extract of broth (CzB). Fraction 168-190 eluted with 70-90 EtOAc in hexane and washed with hexane and ethyl acetate, respectively to give compound D3 (24 mg) as white solid. Fraction 216-240 eluted with 90-100 % EtOAc in hexane and washed with hexane and ethyl acetate, respectively to give compound D4 (12 mg) as white solid.

3.6 Bioassay of the isolated metabolites

3.6.1 Cytotoxicity test

Cytotoxicity activity against 5 human tumor cell lines was carried out at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. The bioassay for in vitro cytotoxic activity toward five cell lines comprising of BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), KATO-3 (gastric) and SW620 (colon) were performed by the MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] colorimetric method (Carmichael et. al., 1987).

3.6.3 Antimicrobial test.

The metabolites were tested against microorganisms, including *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10231, by microdilution tests in 96 well sterile microtiter plates.

3.6.2 Antioxidant test.

Antioxidant activity was tested by using DPPH solution at 200 μM in absolute ethanol in 96 well-plates and was also measured at 517 nm and vitamin E as positive control. The procedure work in dark by covered with aluminum foil.