CHAPTER 3

Experimental

3.1 Equipment, materials and chemicals

3.1.1 Equipments

1. Digital balance, Mettler Toledo
2. Direct current from GPS-S Series GPS-303OD power supply, Gwinste
3. High Performance Liquid Chromatography model HP 1100, Agilent Technologies, USA included;
    - High pressure gradient pump
    - UV-Visible absorbance detector
    - ODS hypersil® C-18 column (250 mm x 4 mm, 5.0 μm)
4. High Performance Thin Leyer Chromatography quantitative densitometer, Camag, Switzerland included;
    - Automatic TLC Sampler 4 (ATS 4)
    - Densitometer TLC Scanner 3
    - DigiStore 2 Documentation system with 12 bit CCD camera
5. Infared spectroscopy, FT-IR model TENSER 27, BRUKER, Germany
6. Mass spectrometer, Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer model Q-TOF 2 Micromass, England
7. ^{1}H (400 MHz), ^{13}C (125MHz), and 2D NMR NMR spectrometer, Model DRX 400; 9.3 Tesla NMR, BRUKER, Germany
8. Perkin Elmer Lambda 25 UV/VIS spectrometer, Perkin Elmer
9. Rotary evaporator, Büchi
10. Ultrasonic bath super RK510H, Bandelin Sonorex
11. UV Cabinet with long-wave UV light 366 nm and shot-wave UV light 254 nm

3.1.2 Materials

1. Filter membrane, Nylon, Pore size 0.45 μm, 47 mm, Filtration, USA
2. Filter paper No. 1 Ø 12.5 mm, Whatman, UK
3. Flash silica gel GF_{254} (40-63 µm), Merck, Germany
4. Sephadex LH-20, Phamacia Fine Chemicals, Sweden
5. Silica gel 60 G (5-40 µm), Merck, Germany
6. Syringe Filter membrane, Nylon, Pore size 0.45 μm, 13 mm, Filtration, USA
7. TLC aluminium sheet silica gel 60 GF_{254}, Merck, Germany
8. TLC syring fixed needle 10 µl and 25 µl, Hamilton, New Zealand
9. Aluminum plates, Thailand

3.1.3 Chemicals

1. Acetone (Me_2CO), Analytical grade, RCI Labscan, Thailand
2. Acetonitrile (MeCN), HPLC grade, RCI Labscan, Thailand
3. Acetylcholinesterase, Sigma Aldrich, USA
4. Albumin bovine serum, Sigma Aldrich, USA
5. Anhydrous sodium sulfate (Na_2SO_4), Laboratory grade, BDH, England
6. Ammonia solution 25-30% (NH_4OH), Laboratory grade, BDH, England
7. Ammonium molybdate reagent, Univar, Australia
8. Bismuth Subnitrate ($4BiNO_3(OH)_2BiO(OH)$), Analytical grade,Riedel-De Hach
9. Deuterated Chloroform ($CDCl_3$), NMR solvent, Aldrich, USA

10. Dichloromethane (CH_2Cl_2), Analytical grade, RCI Labscan, Thailand

11. Ethanol 99.9% (C_2H_5OH), absolute, Analytical grade, Merck, Germany

12. Ethanol 95% (C_2H_5OH), Commercial grade, RCI Labscan, Thailand

13. Ethyl acetate ($CH_3COOC_2H_5$), Analytical grade, RCI Labscan, Thailand

14. Fast Blue B Salt, Sigma Aldrich, USA

15. Hydrochloric acid fuming 37% (HCl), Analytical grade, Merck, Germany

16. Methanol (CH_3OH), Laboratory grade, Fisher scientific, UK

17. 1-Naphthyl acetate, Sigma Aldrich, USA

18. Phosphoric acid (H_3PO_4), Sigma Aldrich, USA

19. Potassium iodide (KI), Univar, Australia

20. Sodium chloride (NaCl), Carlo Erba, Italy

21. Sodium dihydrogenphophate dihydrate ($Na_2HPO_4.2H_2O$), Sigma Aldrich, USA

22. Tris (hydroxymethyl) amino methane, Sigma Aldrich, USA

23. Tris hydrochloride, Sigma Aldrich, USA

Reagents

Dragendroff's reagent[36]

Dissolve 600 mg of bismuth subnitrate in 2 mL of 10 M HCl. Add 10 mL of distilled water to obtain solution A. 6 g of KI is dissolved in 10 mL of the solution A. Dilute the mixture by adding distilled water upto 400 mL. Store the reagent in a dark bottle.

Ammonium molybdate reagent[37]

Dissolve 10 g of ammonium molybdate in 360 mL of distilled water and add 4 g of ceric ammonium sulfate. Cool the solution with constant stirring and then mix 40 mL of concentrated sulfuric acid.

3.2 Collection and extraction of plant materials

The leaves of *Dasymaschalon obtusipetalum* collected from Doi Tung, Chiang Rai Province, Thailand. The dried leaves were extracyed with 95% ethanol (3 days, 3 times) at the room temperature. The ethanol extract was evaporated under reduced pressure to give a dark brown gum (430 g). Aportion of ethanolic crude extract was investigated by conventional and EC technique.

3.3 Alkaloid extraction and isolation

The general extraction procedure of *D. obtusipetalum* summarized in Fig. 3.1.

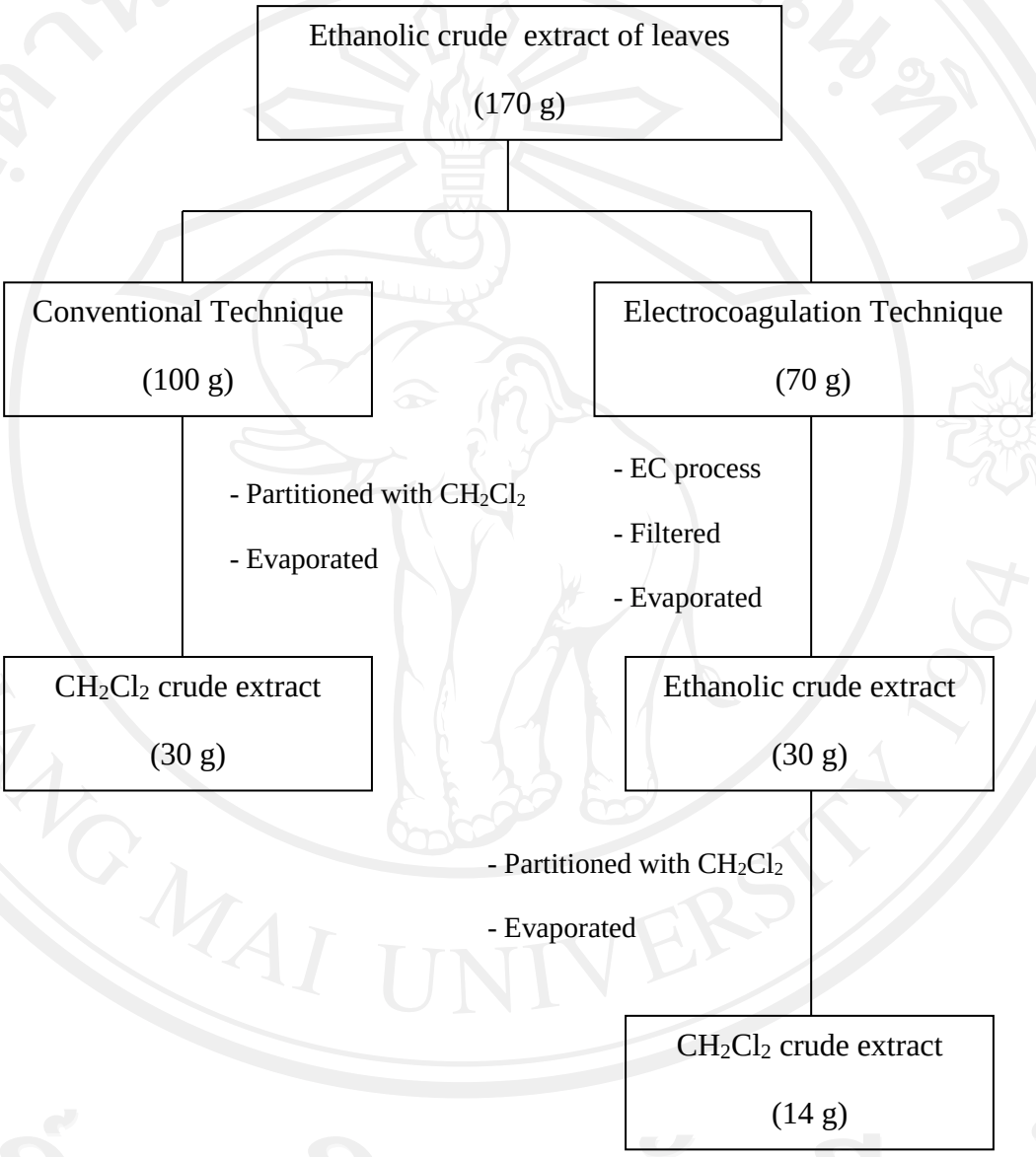


Figure 3.1 General extraction procedure of *D. obtusipetalum*

3.3.1 Electrocoagulation technique (EC)

A portion of ethanolic crude extract (0.5040 g) was dissolved in 85% ethanol and adjusted 0.2% sodium chloride for supporting electrolyte. Two aluminum plates (4 x 15 cm) were used as electrodes and depth into a magnetically-stirred solution. Direct current (3 A, 31 V) from DC power supply was passed through the solution. At every 15 min interval during 2 hours, collected 3 mL of the sample. Sample solution was diluted 100 times and taken for UV absorption measurement at the maximum absorption wavelength (242 nm). To determine the optimum time for applying the current to the sample solution, absorbance and time were plot and gave the optimum time at 30 min. A simple electrocoagulation set-up was shown in Fig. 3.2.

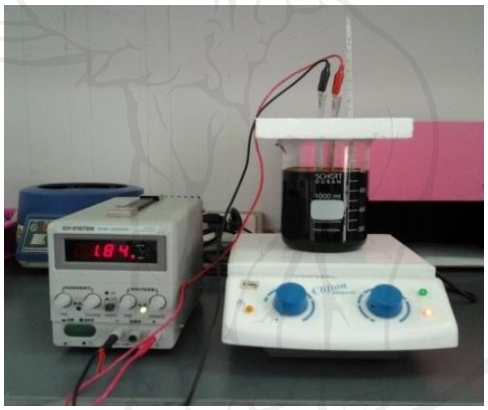


Figure 3.2 A simple electrocoagulation set-up

At the optimum time, 70 g of ethanolic crude extract was dissolved in 85% ethanol and adjusted 0.2% sodium chloride solution. Two aluminum plates (9 x 18 cm) were dipped into a magnetically-stirred solution. Direct current (3 A, 31 V) from DC power supply was passed through the solution 30 minutes followed by filtration. The filtrates were evaporated by evaporator to gave 30 g of ethanolic crude extract. Then, ethanolic crude extract was patitioned with with dichloromethane and evaporated by evaporator to gave 14 g of dichloromethane extract. This extract was separated by Quick Column Chromatography (QCC) using polarity gradient elution with Hexane-CH_2Cl_2-MeOH (100:0:0 to 0:80:20) to give 30 fractions (E1-E30). Fraction E22 (1.1 g)

was subjected to silica gel column chromatography (CC) by using polarity gradient system with Me_2CO-CH_2Cl_2 (20:80 to 100:0) to give 8 fractions (E22.1–E22.8) and fraction E22.5 was compound 21 (61.5 mg). This experimental process is summarized in Fig. 3.3.

Fraction E22.9 (1.1613 g) was combined from fraction E22.2 (308.0 mg), E22.3 (181.5 mg), E22.4 (579.0 mg), E22.6 (82.7 mg) and E22.7 (7.1 mg). This fraction was separated by Sephadex LH-20 with MeOH to give 3 fractions (E22.9.1–E22.9.3). Fraction E22.9.1 (37.5 mg), E22.9.2 (287.5 mg) and E22.9.3 (205.1 mg) were combined to fraction E22.9.4 (530.4 mg) and determinated by RP-HPLC [MeCN/Buffer pH 2.6 (30:70), 1.0 $mL.min^{-1}$] to obtain compound 21 (164.4 mg). This experimental process is summarized in Fig. 3.4.

Fraction E21* (3.0739 g) was combined from fraction E21 (2.4207 g) and E23 (3.0739 g). And then, this fraction was separated by using QCC with Hexane-EtOAc-MeOH (100:0:0 to 0:90:10) to give 6 fractions (E21*1 – E21*6). Fraction E21*7 (1.214 g) was combined from fraction E21*2 (907.8 mg), E21*3 (124.3 mg), E21*4 (77.3 mg), E21*5 (103.7 mg) and E21*6 (143.0 mg) and determinated by RP-HPLC [MeCN/Buffer pH 2.6 (30:70), 1.0 $mL.min^{-1}$] to obtain compound 21 (407.9 mg). This experimental process is summarized in Fig. 3.5.

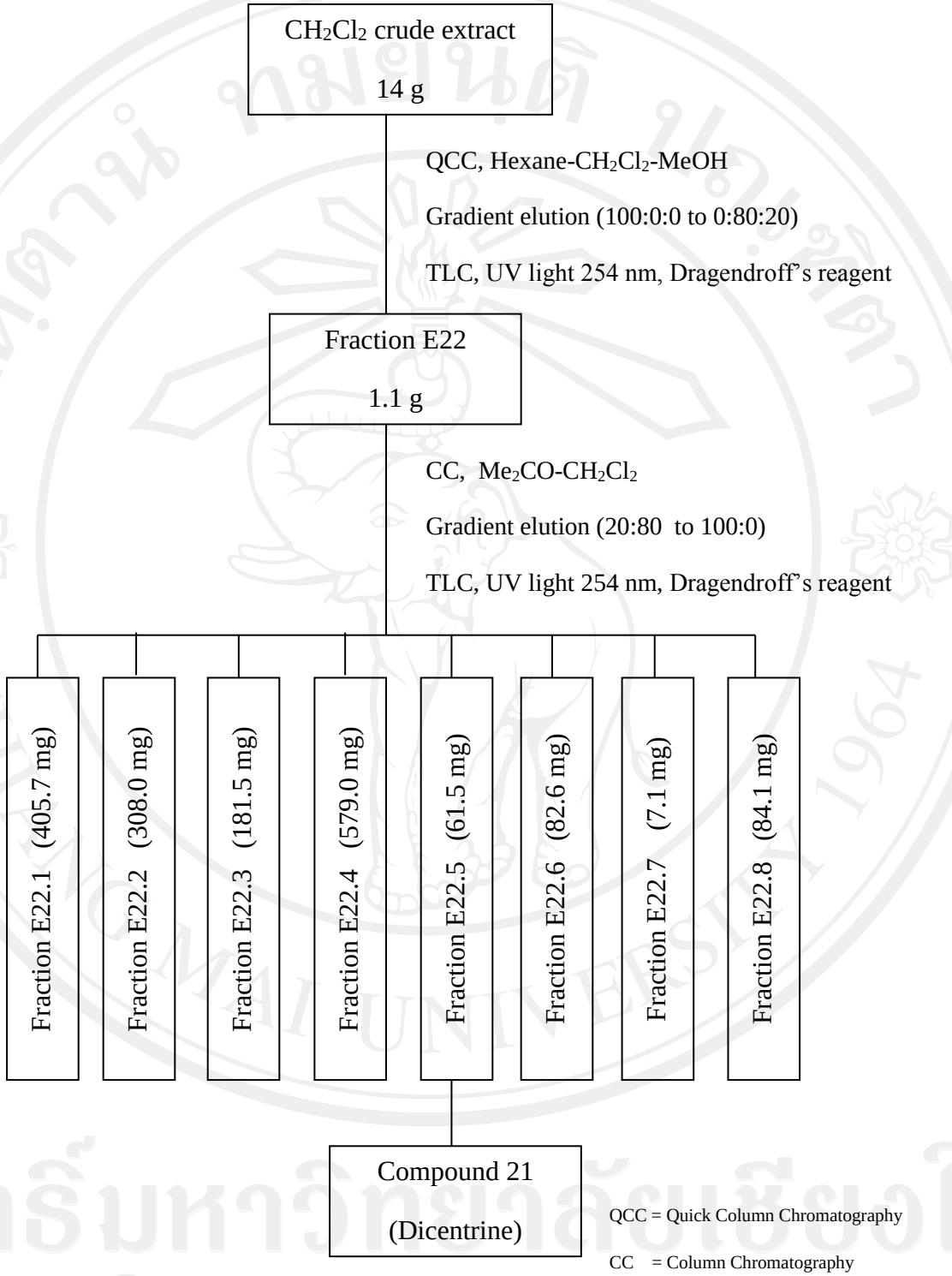
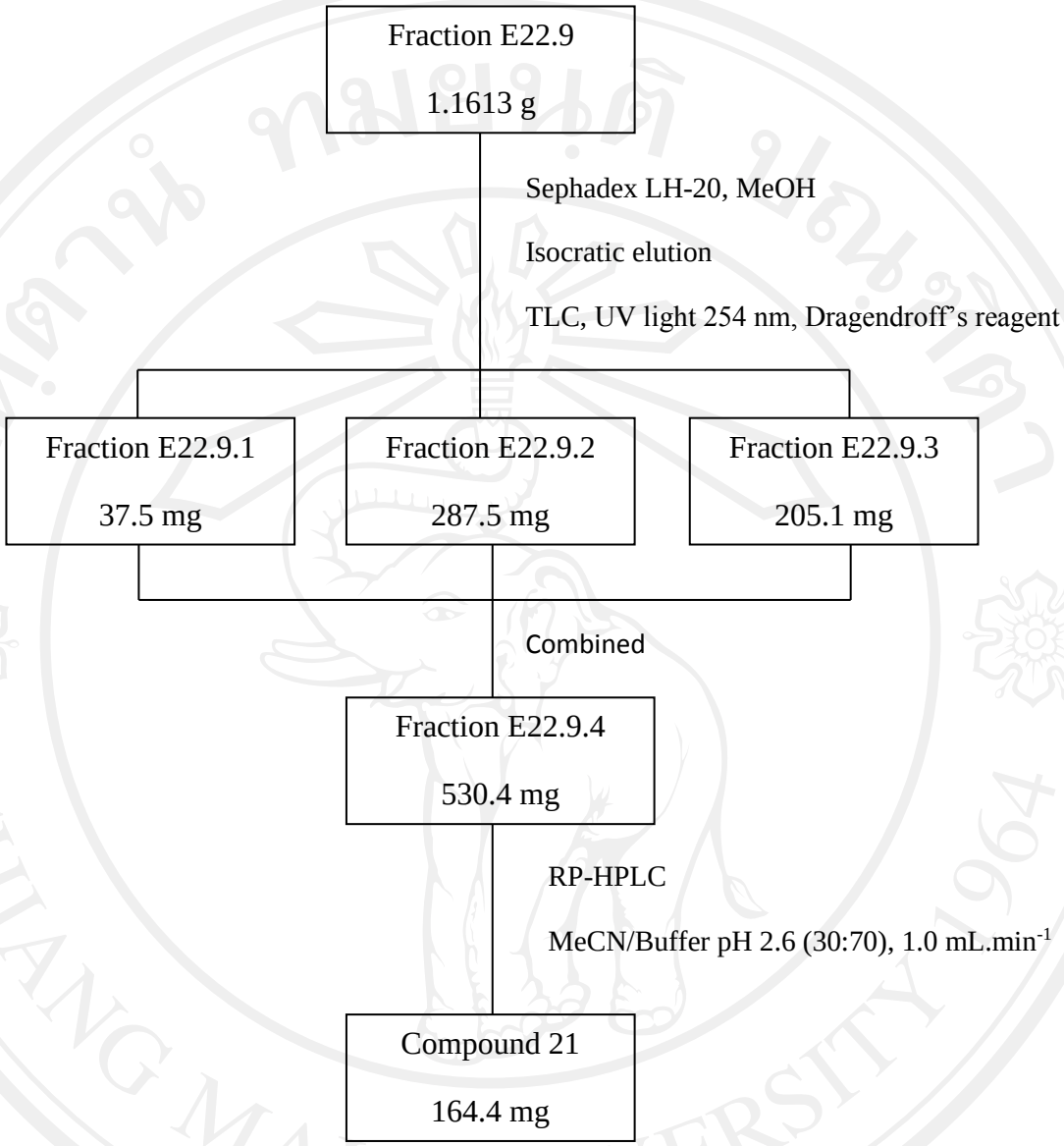


Figure 3.3 Isolation of the dichloromethane crude extract from EC technique



CC = Column Chromatography

TLC = Thin Layer Chromatography

RP-HPLC = Reverse Phase – High Performance Liquid Chromatography

Figure 3.4 Isolation of the acetone crude extract from EC technique

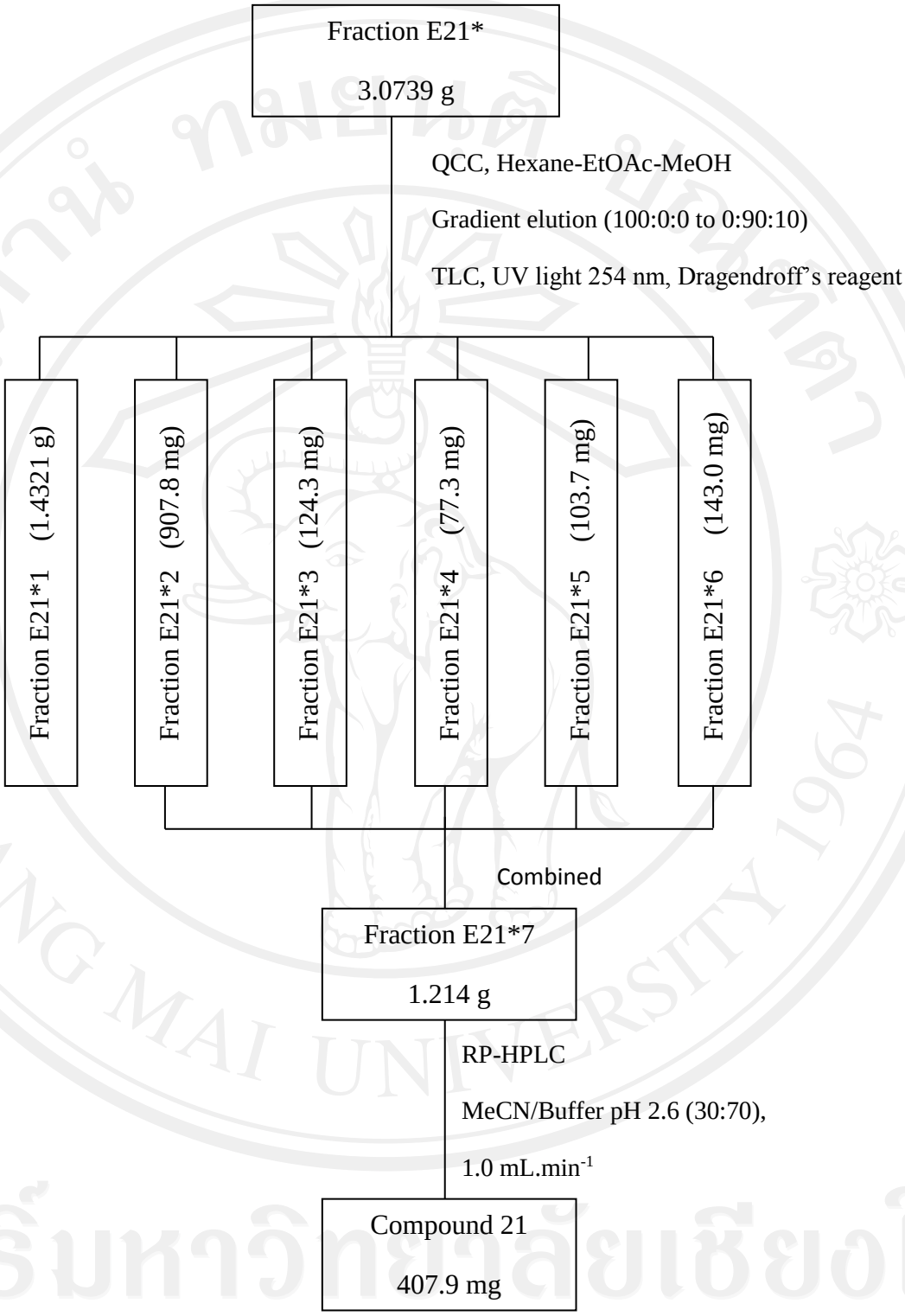
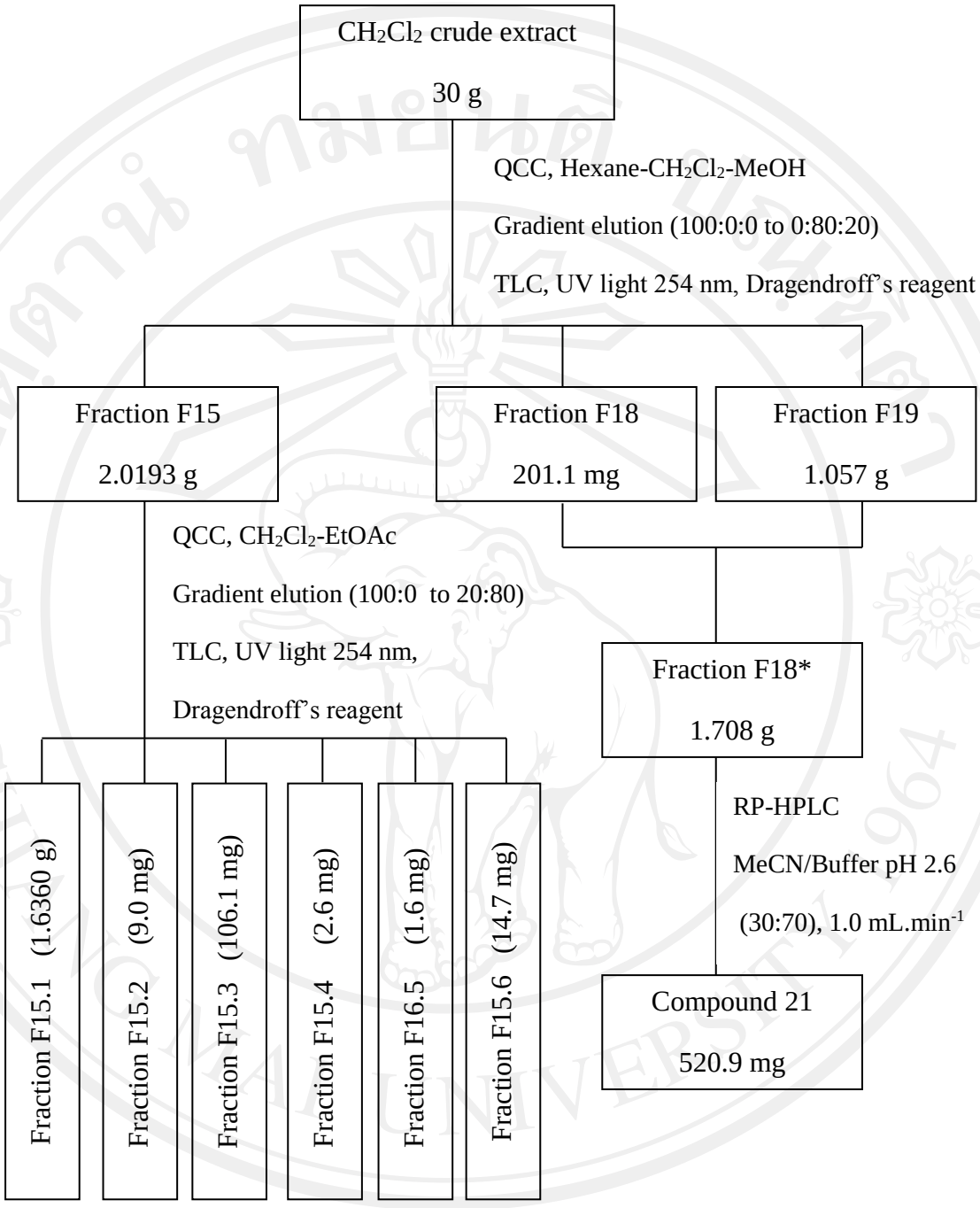


Figure 3.5 Isolation of the dichloromethane crude extract from EC technique

3.3.2 Conventional technique (Solvent extraction)

A portion of ethanolic extract 100 g was dissolved in 30% ethanol and patitioned with dichloromethane to give dichloromethane extract 30 g and separated by QCC using polarity gradient elution with Hexane-CH_2Cl_2-MeOH (100:0:0 to 0:80:20) to give 22 fractions (F1-F22). Fraction F15 (2.0193 g) was separated by QCC with CH_2Cl_2–EtOAc (100:0 to 20:80) to give 6 fractions (F15.1–F15.6). Fraction F18 (201.1 mg) and F19 (1.5072 g) were combined and determinated by RP-HPLC [MeCN/Buffer pH 2.6 (30:70)], 1.0 $mL.min^{-1}$] to obtain compound 21 (520.9 mg). This experimental process is summarized in Fig 3.6.

Fraction F16* (6.5542 g) was combined from fraction F16 (2.4698 g), F17 (3.9783 g) and F15.3 (106.1 mg) and separated by QCC with CH_2Cl_2–EtOAc (100:0 to 20:80) to give 6 fractions (F16*1 – F16*6). Fraction F16*3 (26.0 mg), F16*4 (33.6 mg) and F16*5 (19.1 mg) were combined to fraction F16*7 (81.3 mg) and determinated by RP-HPLC [MeCN/Buffer pH 2.6 (30:70), 1.0 $mL.min^{-1}$] to obtain compound 21 (25.6 mg). This experimental process is summarized in Fig 3.7.

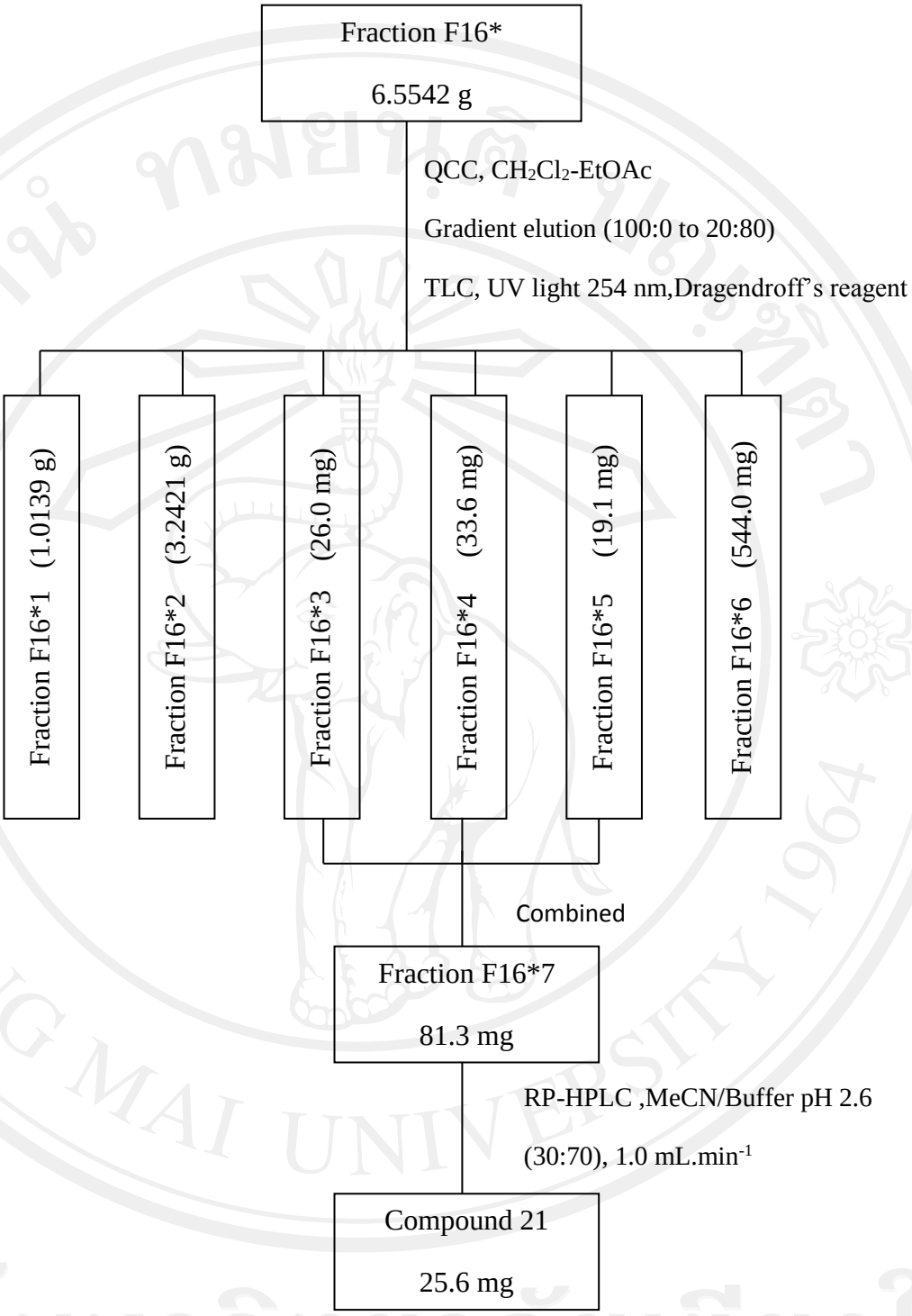


QCC = Quick Column Chromatography

TLC = Thin Layer Chromatography

RP-HPLC = Reverse Phase – High Performance

Figure 3.6 Isolation of the dichloromethane crude extract from conventional technique



QCC = Quick Column Chromatography

TLC = Thin Layer Chromatography

RP-HPLC = Reverse Phase – High Performance Liquid Chromatography

Figure 3.7 Isolation of the dichloromethane crude extract from conventional technique

3.4 Determination of compound 21 by RP-HPLC

3.4.1 HPLC conditions

The chromatographic column was ODS hypersil® C-18 (250 mm x 4 mm, 5.0 μm) for analytical column. Mobile phase consisted of Acetonitrile (MeCN) : Buffer pH 2.6 (10 mM Phosphoric acid (sodium) buffer solution) for 30 : 70. The flow rate was 1.0 ml.min^{-1} and detecting wavelength was set at 210 nm. The volume for each injection was 20 μL.

3.4.2 Preparation of standard solutions

Compound 21 was used as a standard. Compound 21 100 μg.mL^{-1} was prepared as the standard stock solutions. The stock solution was diluted by adding mobile phase [MeCN/Buffer pH 2.6 (30:70)] as needed to prepare a series of standard solutions. A series of each standard solution containing 10, 20, 30, 40 and 50 μg.mL^{-1} were prepared from stock standard 100 μg.mL^{-1} by dilution and filtered with syringe fiter membrane (Nylon).

3.4.3 Preparation of sample solutions

Each samples; fraction E22.9.4, E21*7, F18* and F16*7 1,000 μg.mL^{-1} were prepared as the stock solutions. The stock solution was diluted into 100 μg.mL^{-1} by adding mobile phase [MeCN/Buffer pH 2.6 (30:70)] and filtered with syringe fiter membrane (Nylon).

3.4.4 Calibration Curve

A series of standard solution containing 10, 20, 30, 40 and 50 μg.mL^{-1} were injected 20 μL into the RP-HPLC. Peak area and concentration of standard solutions were ploted to give calibration curve. The amounts of compound 21 contained in each sample were calculated by reference to the calibration curve.

3.5 Structure elucidation

Compound 21 was identified by proton-nuclear magnatic resonance (^{1}H-NMR), carbon-nuclear magnatic resonance (^{13}C-NMR), two dimensional-nuclear magnatic resonance (2D NMR), Infrarad spectroscopy (IR), UV spectroscopy and mass spectrometry (MS). ^{1}H (400 MHz), ^{13}C (125 MHz) and 2D-NMR spectra were recorded on a Model DRX 400 MHz spectrometer. Molecular weight were taken with Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer model Q-TOF 2 Micromass. IR-spectra were obtained by FT-IR model TENSER 27. Absorbance were taken with Perkin Elmer Lambda 25 UV/VIS spectrometer.

3.6 Determination of efficiency on acetylcholinesterase inhibitory activity by TLC bioautographic assay[38]

Acetylcholinesterase (906 U/mg) was dissolved in 0.05 M tris-hydrochloric acid buffer at pH 7.8 (150 mL) and added bovine serum albumin (150 mg) for stabilize the enzyme during test. The stock solution was kept at 4℃. TLC plates were washed with acetone and thoroughly dried before use. Each sample; conventional extract, EC extract and compound 21 was applied on TLC plate in varying quantities (10, 50, 100, 250 and 500 ng) and Galantamine was used as a standard. The plate was sprayed with enzyme stock solution. It were incubated at 37℃ for 20 min and sprayed freshly indicator solution (1-naphthyl acetate (250 mg) in ethanol (100 mL)and Fast Blue B salt(400 mg) in distilled water (160 mL) were mix) to give purple coloration after 1-2 min on plate. A white spot showed the AChE inhibition by the samples.

3.7 Brine Shrimp Lethality Activity test (BST)

Brine shrimp eggs were hatched in sea water (39 g of salt in 1 liter of distilled water) for 48 hours. Ten nauplii were collected into test tube by using Pasteur pipette. Each sample; conventional extract, EC extract and compound 21 was prepared in varying quantities (10, 20, 40, 80 and 160 μg.mL^{-1}), $K_2Cr_2O_7$ and DMSO were used for positive and negative control ,respectively. Sample solutions were added into test tube and adjusted with sea water for 3 mL. Each sample solutions were prepared in triplicate. After 24 hours, the dead nauplii were observed and used to determine the LC_{50} by using statistic analysis. The data were analyzed by probit analysis, SPSS for Windows.

(a) (b) (c)

Figure 3.8 Brine shrimp assays; (a) brine shrimp eggs

(b) hatching brine shrimp in seawater

(c) live brine shrimp[39]