CHAPTER 3

EXPERIMENTAL

3.1 Equipment, materials and chemicals

3.1.1 Equipment

1. HPTLC quantitative densitometer, Camag, Switzerland included;
   - Automatic TLC Sampler 4 (ATS 4)
   - Densitometer TLC Scanner 3
   - DigiStore 2 Documentation system with 12 bit CCD camera
2. Infared spectroscopy, FT-IR model TENSER 27, BRUKER, Germany
3. Mass spectrometer, Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer model Q-TOF 2 Micromass, England
4. ^{1}H (400 MHz), ^{13}C (100MHz), and 2D NMR NMR spectrometer, Model DRX 400; 9.3 Tesla NMR, BRUKER, Germany
5. Ultrasonic bath super RK510H, Bandelin Sonorex
6. UV Cabinet with long-wave UV light 366 nm and shot-wave UV light 254 nm

3.1.2 Materials

1. Filter paper No. 1 Ø 12.5 mm, Whatman
2. Flash silica gel GF_{254} (40-63 µm), Merck, Germany
3. TLC aluminium sheet silica gel 60 GF_{254}, Merck, Germany
4. TLC syring fixed needle 10 µl and 25 µl, Hamilton

3.1.2 Chemicals

1. Acetone, Analytical grade, ACI Labscan, Thailand
2. Acetylcholinesterase, Sigma Aldrich, USA
3. Albumin bovine serum, Sigma Aldrich, USA
4. Anhydrous sodium sulfate (Na_2SO_4), Laboratory grade, BDH, England
5. Ammonia solution 25-30% (NH_4OH), Laboratory grade, BDH, England
6. Ammonium molybdate reagent, Univar, Australia
7. Bismuth Subnitrate ($4BiNO_3(OH)_2BiO(OH)$), Analytical grade,Riedel-De Hach
8. Deuterated Chloroform ($CDCl_3$), NMR solvent, Aldrich, USA
9. Dichloromethane (CH_2Cl_2), Analytical grade, ACI Labscan, Thailand
10. Ethanol % (C_2H_5OH), Analytical grade, Merck, Germany
11. Ethanol 95 % (C_2H_5OH), commercial grade, ACI Labscan, Thailand
12. Fast Blue B Salt, Sigma Aldrich, USA
13. Hydrochloric acid fuming 37% (HCl), Analytical grade, Merck, Germany
14. Methanol (CH_3OH), Laboratory grade, Fisher scientific, UK
15. 1-Naphthyl acetate, Sigma Aldrich, USA
16. Potassium iodide (KI), Univar, Australia
17. Tris (hydroxymethyl) amino methane, Sigma Aldrich, USA
18. Tris hydrochloride, Sigma Aldrich, USA

Reagents

1. Dragendroff's reagent

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 [31].

2. Ammonium molybdate reagent

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 [32].

3.2 Extraction and isolation

3.2.1 *D. glaucum* extraction

The dry leaves of *D. glaucum* (1 kg) were extracted with 95% ethanol (3 x 3000 mL) for 4 days at room temperature. The ethanol extract was evaporated under reduced pressure to give a dark green sticky residue (31.03 g). A portion of the crude extract (30 g) was dissolved in mixture solvent of distilled water and methanol (2:8, v/v, 100 mL). The mixture solution was extracted with dichloromethane (4 x 300 mL) to yield 19.62 g of crude material after evaporation. The summary of extraction as shown in Figure 3.1.

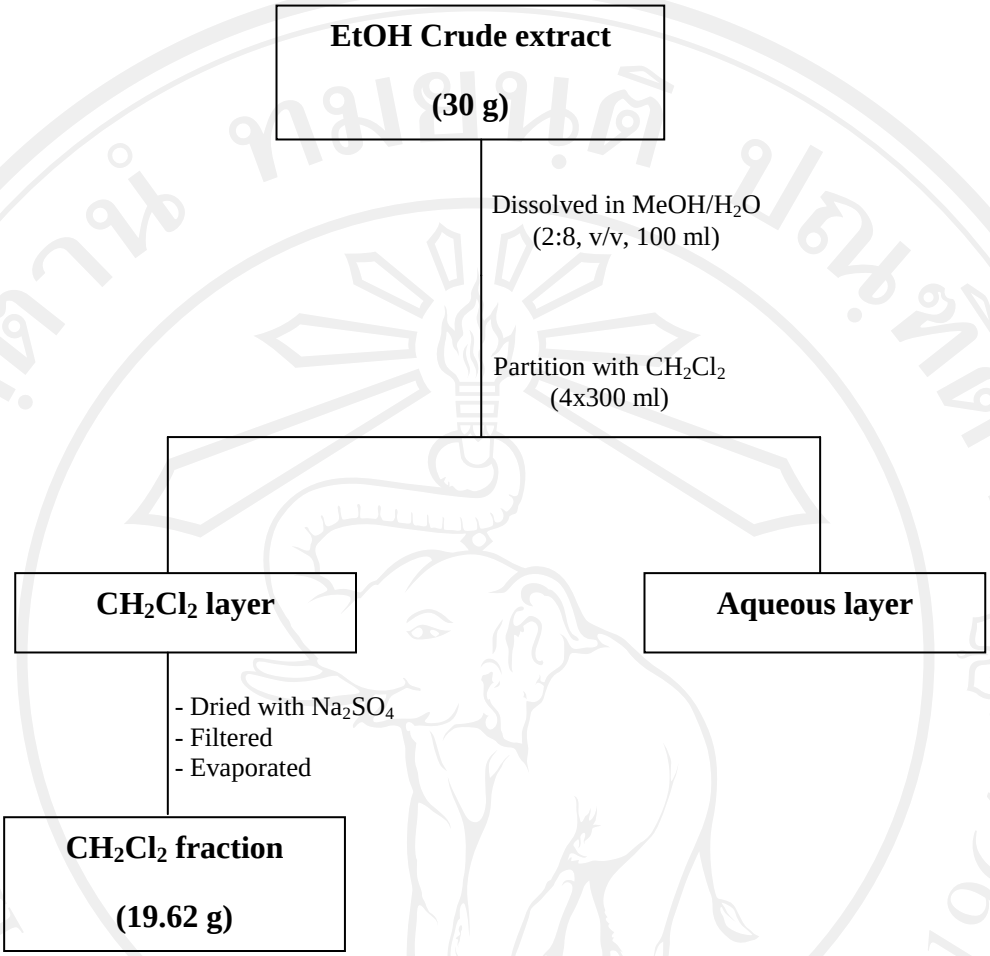

Figure 3.1 General extraction procedure of *D. glaucum*

3.2.2 *D. glaucum* isolation

A portion of the dichloromethane crude extract (3.06 g) was separated by flash column chromatography, using silica gel (300 ml) and gradient elution (100% dichloromethane to 100% methanol) to gave 7 fractions (Figure 3.2).

Fraction 5 (263 mg, eluted with 5% MeOH- CH_2Cl_2) was further purified by silica gel column chromatography using a gradient elution with dichloromethane-methanol (100:0 to 50:50, v/v) to yield 4 fractions (Fraction 5.1-5.4). Fraction 5.2 (174 mg, eluted with 2%-5% MeOH- CH_2Cl_2) was further purified by gradient column chromatography with dichloromethane-methanol (100:0 to 50:50, v/v) to yield 5 fractions (Fraction 5.2.1-5.2.5). Further purification of fraction 5.2.2 (130 mg, eluted with 1%-3% MeOH- CH_2Cl_2) by gradient column chromatography with dichloromethane-methanol (100:0 to 50:50, v/v) gave F5.2.2.5 (**DG1**, 3 mg, eluted with 10% MeOH- CH_2Cl_2). Fraction 5.3 (17 mg, eluted with 10% MeOH- CH_2Cl_2) was further purified by preparative TLC (methanol: dichloromethane: ammonia, 7:92:1, v/v, 2 elutions) gave F5.3.2 (**DG2**, 5 mg, R_f = 0.3). The summary of fraction 5 as shown in Figure 3.2.

Fraction 4 (210 mg, eluted with 3% MeOH- CH_2Cl_2) was rechromatograph gradient elution with dichloromethane-methanol (100:0 to 50:50, v/v) to gave seven fractions (fraction 4.1-4.7). Fraction 4.4 (9 mg, eluted with 2% MeOH- CH_2Cl_2) was further purified by preparative TLC (methanol: dichloromethane: ammonia, 3:96:1, v/v, 3 elutions) to yield F4.4.1 (**DG3**, 2 mg, R_f = 0.6). Further purification of fraction 4.3 (99 mg, eluted with 2% MeOH- CH_2Cl_2) by gradient column chromatography with dichloromethane-methanol (100:0 to 10:90, v/v) gave 7 fractions (fraction 4.3.1-4.3.7). Fraction 4.3.6 (15 mg, eluted with 2% MeOH- CH_2Cl_2) was further purified by preparative TLC (methanol:

dichloromethane: ammonia, 3:96:1, v/v, 2 elutions) to yield F4.3.6.1 (**DG4**, 6 mg, R_f = 0.7). The summary of fraction 4 as shown in Figure 3.3.

Fraction 6 (353 mg, eluted with 10% MeOH- CH_2Cl_2) was rechromatograph gradient elution with dichloromethane-methanol (100:0 to 50:50, v/v) gave 9 fractions (fraction 6.1-6.9). Further purification of fraction 6.3 (11 mg, eluted with 5% MeOH- CH_2Cl_2) and fraction 6.7 (13 mg, eluted with 10% MeOH- CH_2Cl_2) by preparative TLC (methanol: dichloromethane: ammonia, 5:94:1and 10:89:1, v/v, respectively) to yield F6.3.1 (**DG5**, 6 mg, R_f = 0.5) and F6.7.1 (**DG6**, 4 mg, R_f = 0.4), respectively. The summary of fraction 6 as shown in Figure 3.4.

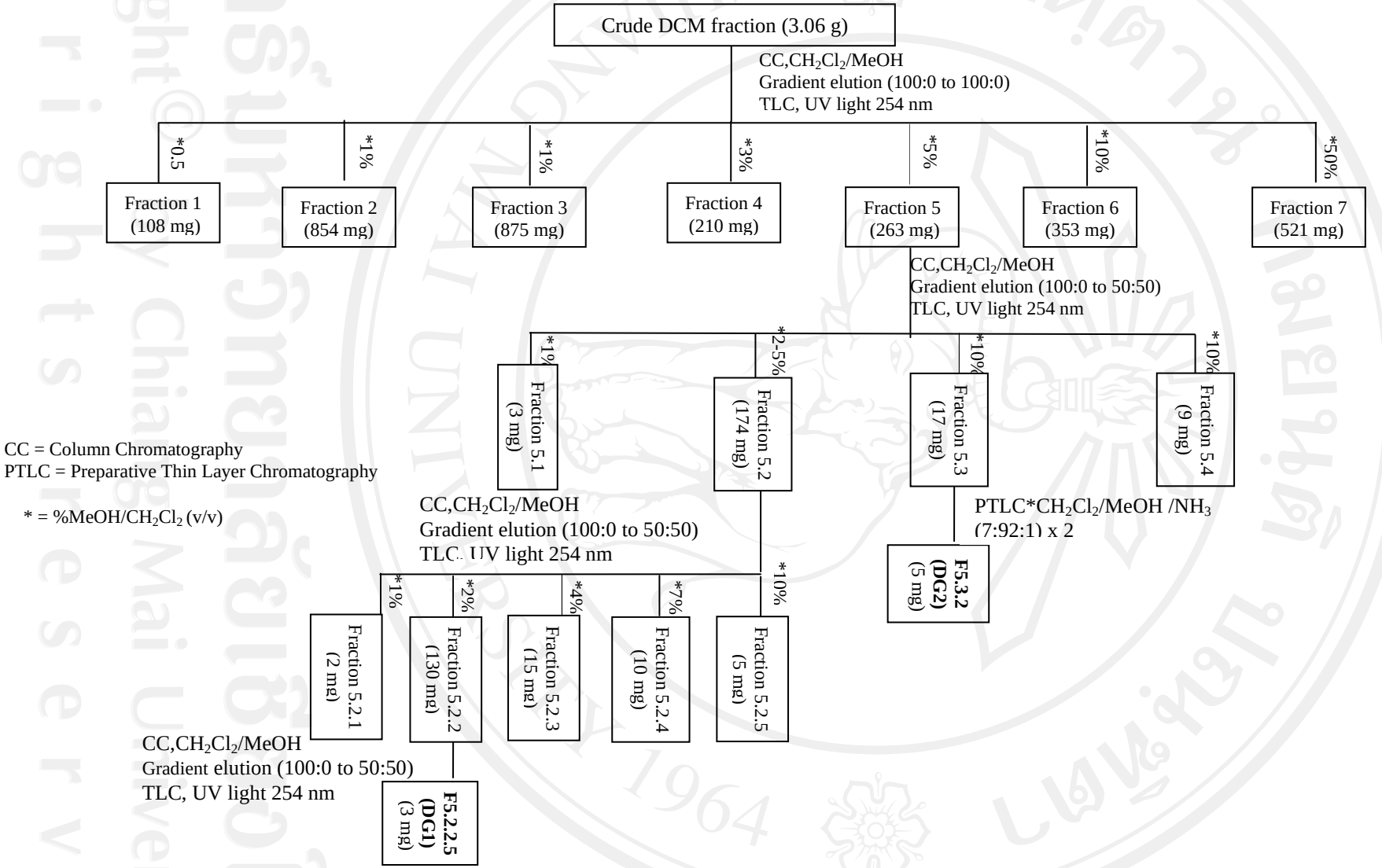

Figure 3.2 Isolation of the dichloromethane crude extract of *D. glaucum* (fraction 5)

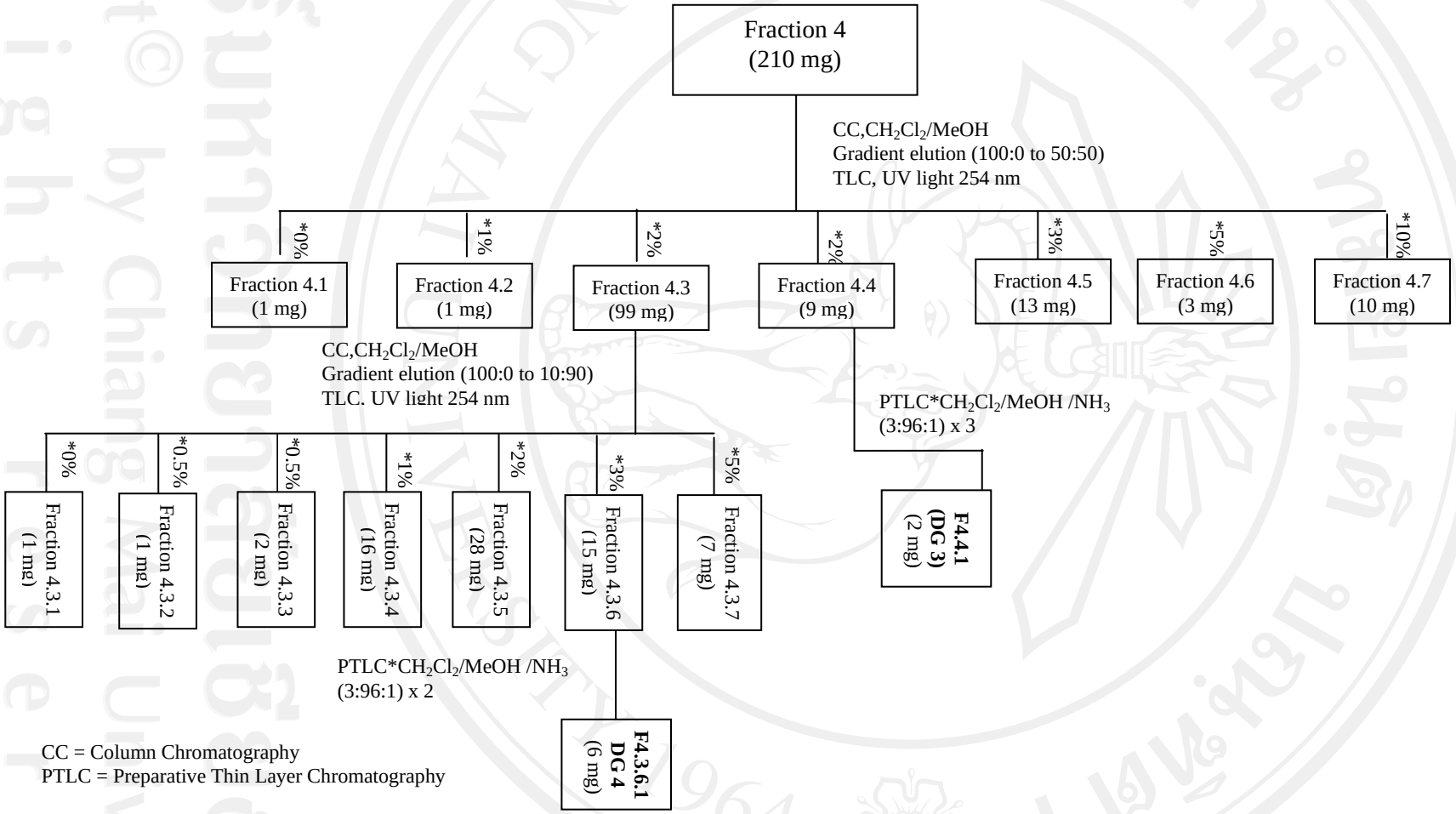

Figure 3.3 Isolation of the dichloromethane crude extract of *D. glaucum* (fraction 4)

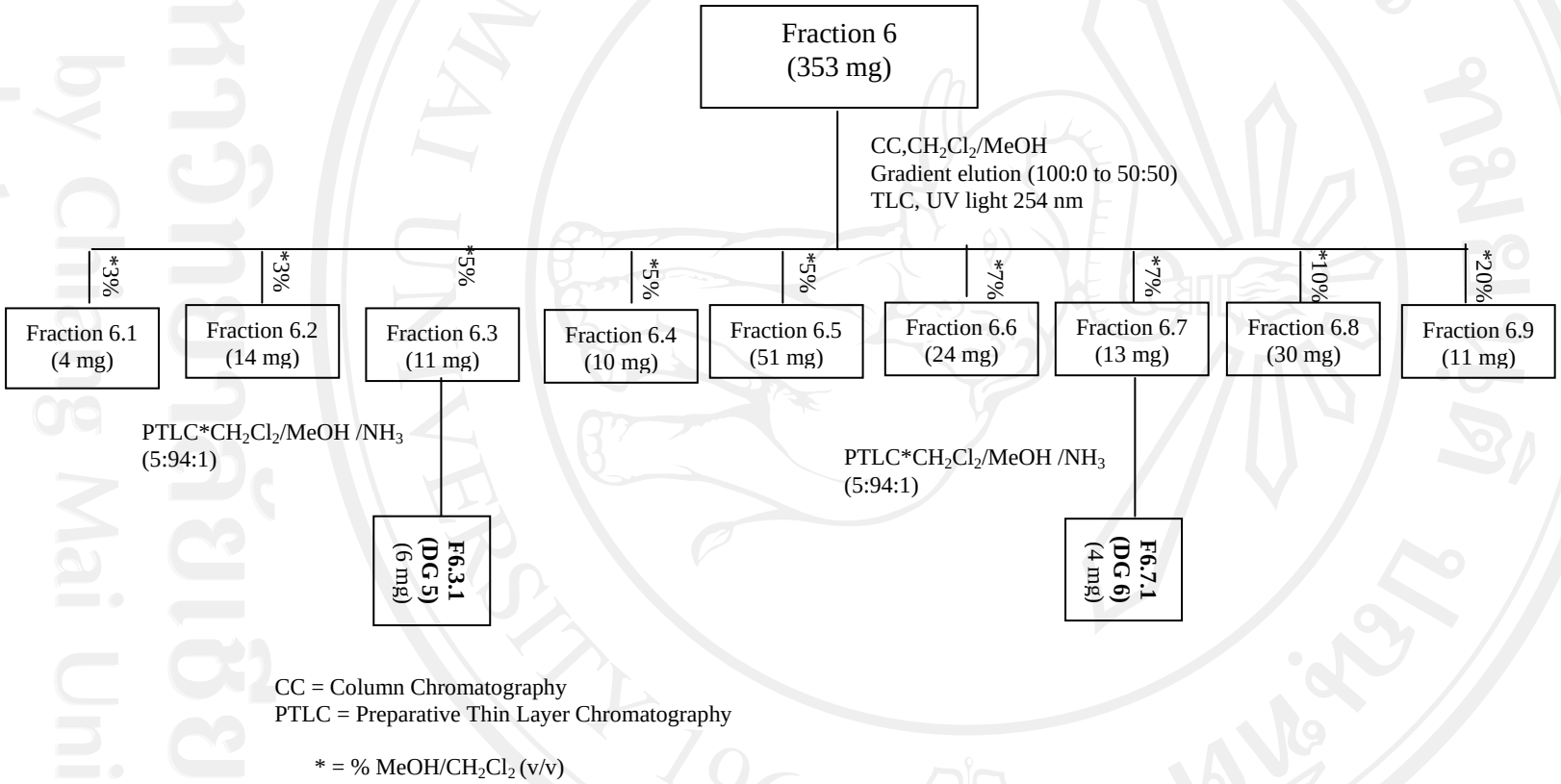

Figure 3.4 Isolation of the dichloromethane crude extract of *D. glaucum* (fraction 6)

3.3 Determination of efficiency on acetylcholinesterase inhibitory activity by TLC bioautographic assay [33**]**

Acetylcholinesterase (906 U/mg) was dissolved in 150 ml of 0.05 M tris-hydrochloric acid buffer at pH 7.8 and bovine serum albumin (150 mg) was added to the solution in order to stabilize the enzyme during the bioassay. The stock solution was kept at 4°C. TLC plates were eluted with acetone to wash them, and were thoroughly dried just before use. After spotting with pure sample from *D. glaucum* at different amounts, galanthamine was also used as positive controls. The TLC plate was dried with a hair dryer for complete removal of the solvent. The plate was then sprayed with enzyme stock solution and thoroughly dried again. For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing a little water; by this mean, water did not come directly into contact with the plate but the atmosphere was kept humid. The cover was placed on the tank and incubation was performed at 37°C for 20 min. The enzyme had satisfactory stability under these conditions. For detection of the enzyme activity, solution of 1-naphthyl acetate (250 mg) in ethanol (100 ml) and Fast Blue B salt (400 mg) in distilled water (160 ml) were prepared immediately before use (in order to prevent decomposition). After incubation of the TLC plate, 10 ml of the naphthyl acetate solution and 40 ml of the Fast Blue B salt solution were mix and sprayed onto the plate to give a purple coloration after 1-2 min.

The separated constituents can be directly detected on the TLC plate. The test relies on the cleavage by acetylcholinesterase of 1-naphthyl acetate to form 1-naphthol, which in turn reacts with Fast Blue B salt to give a purple coloured diazonium dye as shown in

Figure 3.5. Regions of the TLC plate which contain acetylcholinesterase inhibitors show up as white spots against the purple background.

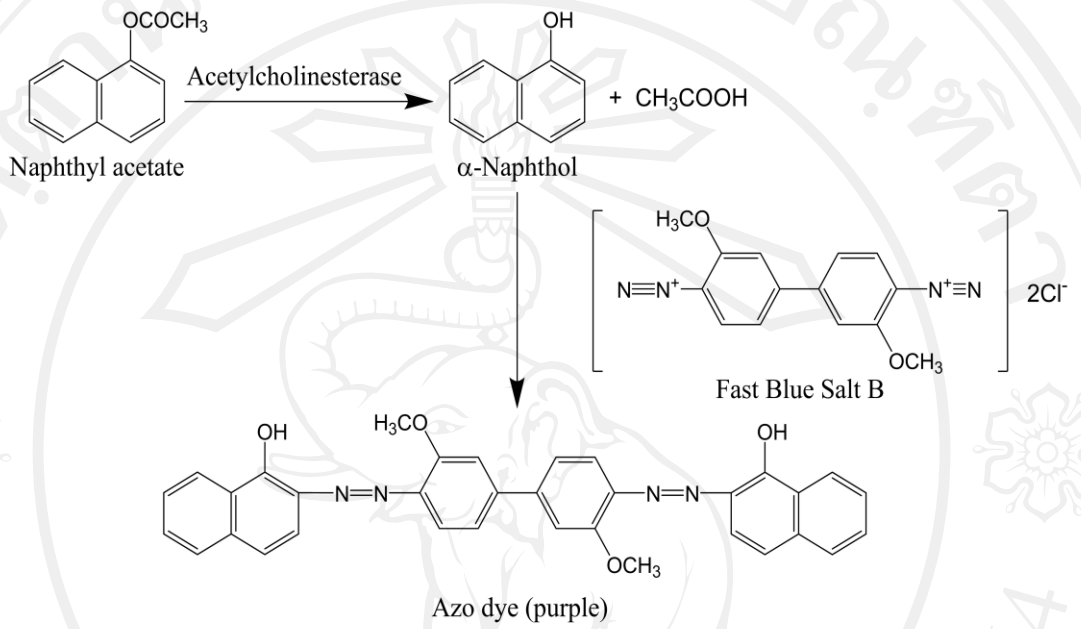

Figure 3.5 Reaction of acetylcholinesterase with naphthyl acetate and the subsequent formation of the purple dye in the TLC bioassay.