CHAPTER 3

EXPERIMENTAL

3.1 Equipment, materials and chemicals

3.1.1 Equipment

1. Digital balance, Mettler Toledo
2. Hot air oven, MEMMERT
3. 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
4. Herbal slicing machine
5. Mass spectrometer, Fison/VG Autospec-TOF-oa
6. Mass spectrometer, Micromass Qtof 2
7. NMR spectrometers, Varian Mercury 300 and Varian Unity 500
8. Polarimeter DIP-370, JASCO
9. Rotary evaporator, BÜCHI
10. Shaking incubator, Lab Tech
11. Ultrasonic bath super RK510H, Bandelin Sonorex
12. UV Cabinet with long-wave UV light 366 nm and short-wave UV light 254nm
13. UV-Vis Spectrophotometer, Thermo Scientific
14. Vacuum filter machine, BÜCHI

3.1.2 Materials

1. Adjustable air-displacement pipette10 µL, 100 µL, 1,000 µL and 10 mL, Gilson
2. Filter paper No. 1 ∅ 12.5 mm, Whatman
3. Flash silica gel GF_{254} (40-63 µm), Merck
4. TLC aluminium sheet silica gel 60 GF_{254}, Merck
5. TLC Syringe fixed needle 10µl and 25µl, Hamilton
6. 96-Well microplates

3.1.3 Chemicals

1. Acetone, Analytical grade, J.T. Baker
2. Acetylcholinesterase, Sigma Aldrich
3. Albumin bovine serum, Sigma Aldrich
4. Anhydrous sodium sulfate (Na_2SO_4), Laboratory grade
5. Ammonia solution 25-30% (NH_4OH), Laboratory grade, BDH
6. Ammonium molybdate reagent
7. Amphotericin B, Sigma Aldrich
8. Bismuth Subnitrate ($4BiNO_3(OH)_2BiO(OH)$), Analytical grade , Riedel-De Hach
9. BHA (3-tert-Butyl-4-hydroxyanisole), Sigma Aldrich
10. Chloroform ($CHCl_3$), Analytical grade, J.T. Baker
11. Deuterated Chloroform ($CDCl_3$), NMR solvent
12. Deuterated Acetone ($(CD_3)_2CO$), NMR solvent
13. Dichloromethane (CH_2Cl_2), Analytical grade, J.T. Baker
14. Distilled water
15. DPPH (2,2-Diphenyl-1-picrylhydrazyl), Sigma Aldrich

16. Eserine, Sigma Aldrich

17. Ethanol 95% (C_2H_5OH), commercial grade

18. Fast Blue B Salt, Sigma Aldrich

19. Gentamicin, Sigma Aldrich

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

21. Methanol (CH_3OH), Laboratory grade, J.T. Baker

22. Methomyl (*S*-methyl-*N*-(methylcarbamyloxy)thioatimidate 40%), Dupont

23. 1-Naphthyl acetate, Sigma Aldrich

24. Polyethyleneglycol (PEG)

25. Potassium carbonate (K_2CO_3), Laboratory grade

26. Potassium iodide (KI)

27. Tris (hydroxymethyl) amino methane, Sigma Aldrich

28. Tris hydrochloride, Sigma Aldrich

29. α-Tocopherol, Fluka

30. Trolox (6-Hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), Sigma Aldrich

31. Tryptic soy agar (TSA)

32. Tryptic soy broth (TSB)

Reagents

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 (Stahl, 1969).

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 (Gasparic *et al*., 1978).

3.2 Collection of plant materials

Two different *Stemona* spp. i.e. *Stemona curtisii* Hook F. collected from Trang and Petchaboon Province, Thailand and *Stemona aphylla* Craib. collected from Lampang Province, Thailand were identified using Botanical notes on the vascular flora of Chiang Mai (Maxwell, 1991) and deposited in CMU Herbarium, Department of Biology, Faculty of Science, Chiang Mai University. The *Stemona* roots were washed with tap water and sliced into small pieces. The small root chips were under the shade and dried in the hot air oven at 50 °C.

3.3 Extraction and isolation

The general extraction procedure of *S. curtisii* from Trang Province is summarized in Fig. 3.1.

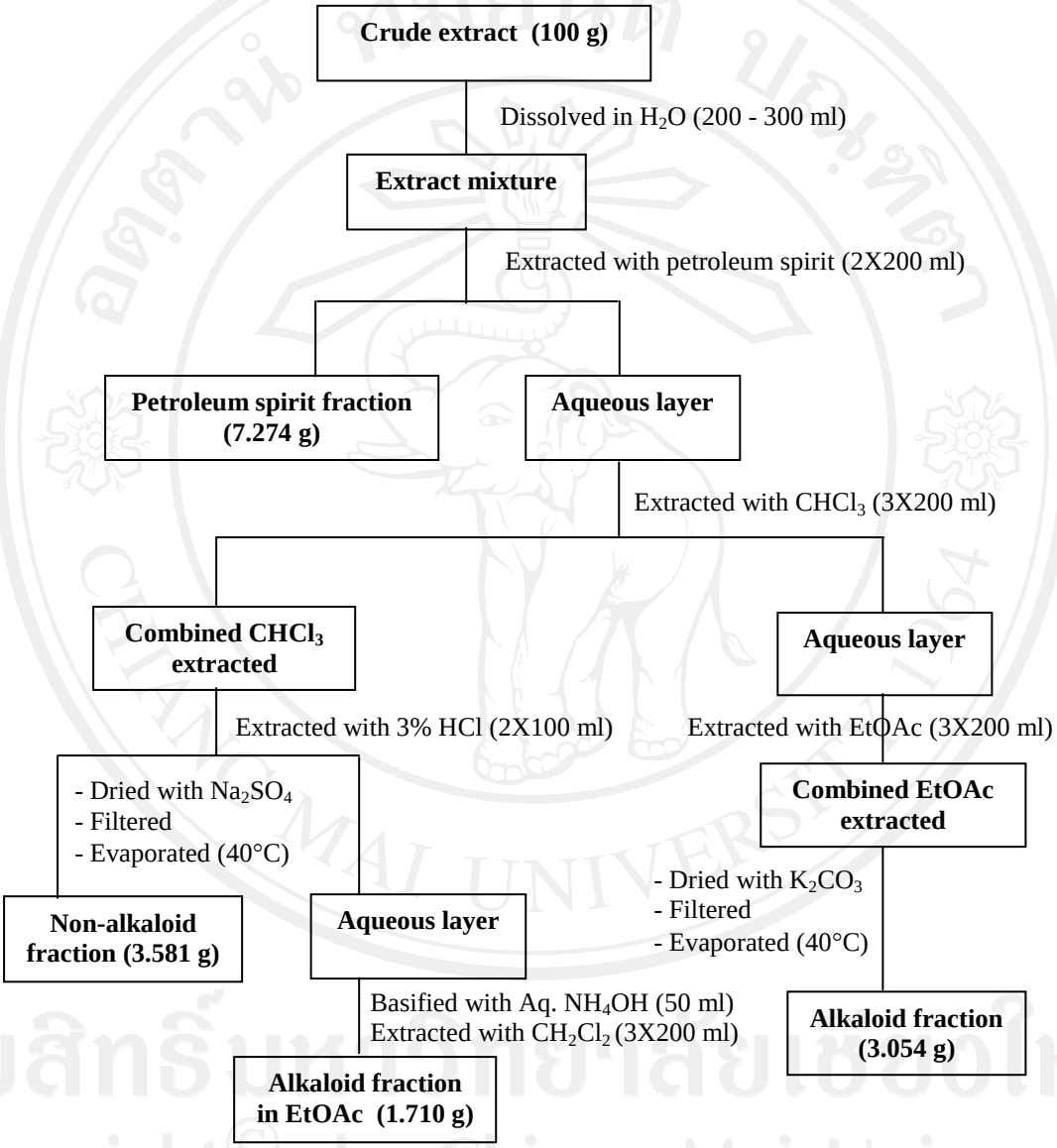


Figure 3.1 General extraction procedure of *S. curtisii* from Trang Province.

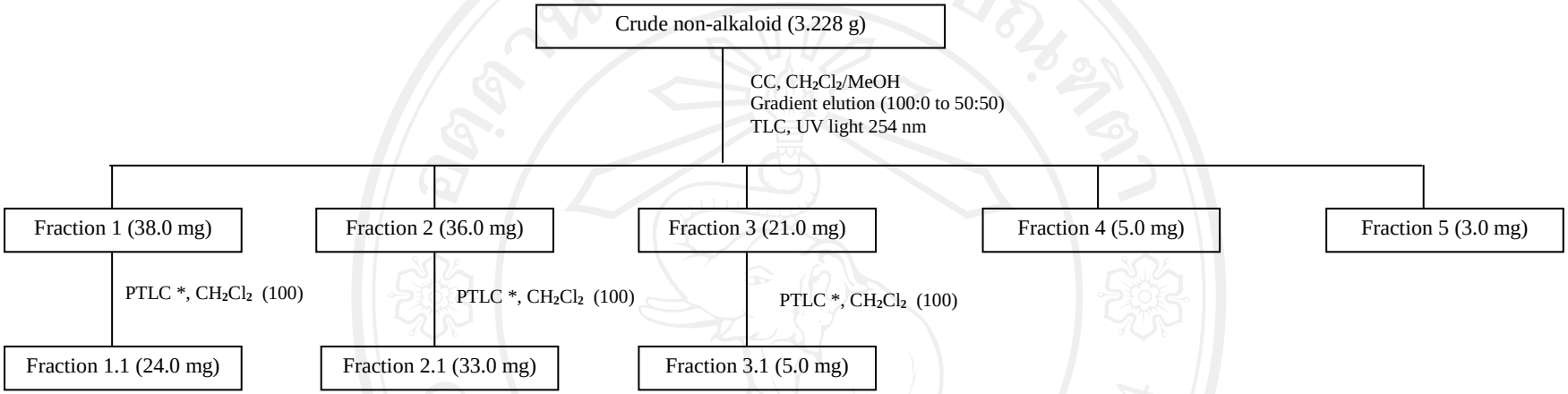
3.3.1 *S. curtisii* extraction and isolation (Trang Province)

The dry ground root of *S. curtisii* (1.0 kg) was extracted with 95% ethanol (3 x 3,000 mL) for 4 days at room temperature. The ethanol extracts were evaporated under reduced pressure to give a dark brown sticky residue (123.1 g). A portion of the crude extract (100.0 g) was dissolved in distilled water (200-300 mL). The resulting aqueous solution was first extracted in petroleum spirit (2 x 200 mL) to yield 7.27 g followed by chloroform and ethyl acetate extraction as shown in Figure 3.1. The combined chloroform extracts were extracted with a solution of 1M hydrochloric acid, water, a solution of saturated NaCl, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure to yield a non-alkaloid crude mixture (3.58 g).

The aqueous solution from the chloroform extraction, was basidified with aqueous ammonia to pH 10 and further extracted with ethyl acetate. The combined ethyl acetate extracts were washed with a saturated solution of NaCl, dried over $NaSO_4$, filtered and the solvent was removed to afford the crude alkaloid mixture (1.71 g). The non-alkaloid crude mixture (3.22 g) was separated by flash column chromatography, using silica gel (300 mL) and gradient elution from 100% dichloromethane to 50% methanol/dichloromethane, 15 fractions were obtained from the separation (Fig 3.2). Fraction 6 (23.0 mg) was further separated by preparative TLC using 100% dichloromethane as eluting solvent to give the new benzofuran **1** (stemofuran L, 4.0 mg) and stemofuran K **2** (3.0 mg). Fraction 8 (176.0 mg) was further purified on a silica gel column using gradient elution with dichloromethane-methanol (100:0 to 97:3, v/v) to yield 6 fractions (fractions 8.1-8.6). Further purification of fraction 8.5 (27.0 mg) by preparative TLC (100% CH_2Cl_2) gave stemofuran J **3** (4.0 mg). Fraction 9 (105.0 mg) was also purified by column

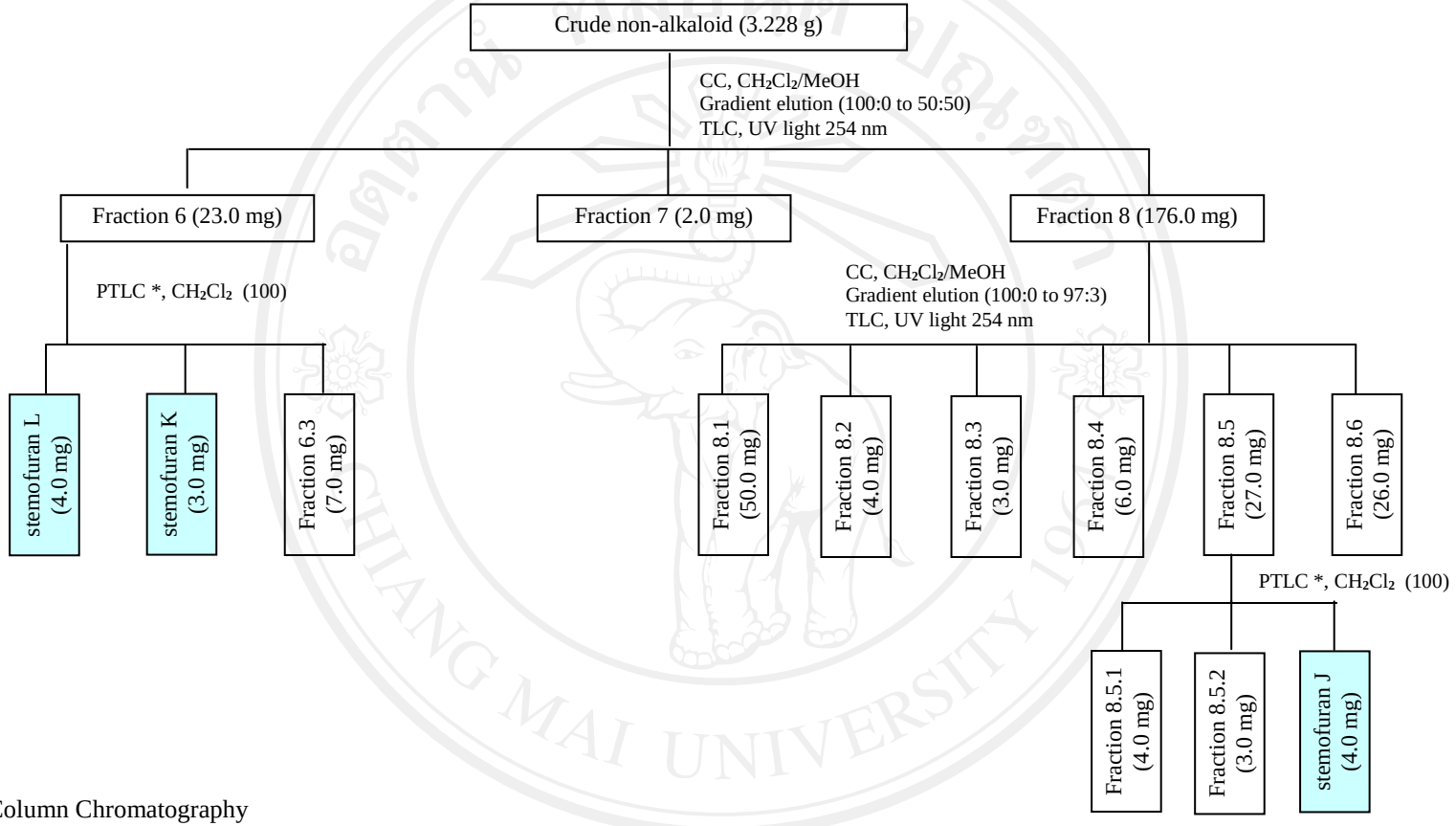
chromatography using gradient elution with dichloromethane-methanol (100:0 to 97:3, v/v) to afford 6.0 mg and 15.0 mg of mixtures of fatty acids (fraction 9.3 and fraction 9.5 respectively). Fraction 12 (273.0 mg) was further purified by column chromatography with gradient elution with dichloromethane-methanol (100:0 to 97:3, v/v) to yield 6 fractions (fractions 12.1-12.6). Fraction 12.1 (45.0 mg) was purified by preparative TLC (100% CH_2Cl_2) to give stemofuran F **4** (4.0 mg) plus a small amount of impurity. Fraction 13 (130.0 mg) was further purified by column chromatography using gradient elution with dichloromethane-methanol (100:0 to 95:5, v/v) to afford stemofuran F **4** (2.0 mg). Fraction 15 (311.0 mg) was further purified by column chromatography using gradient elution with dichloromethane-methanol (100:0 to 95:5, v/v) to yield 4 fractions (fractions 15.1-15.4). Further purification of fraction 15.3 (71.0 mg) by column chromatography with gradient eluent with dichloromethane-methanol (100:0 to 96:4, v/v) afforded stemocurtisinol **5** (4.0 mg, 6.0 mg, 12.0 mg, 8.0 mg and 14.0 mg, fractions 15.3.1-15.3.5, respectively). This experimental process is summarized in Fig. 3.2.

The petroleum spirit crude extract (7.12 g) was separated by flash silica gel column chromatography (600 mL) using gradient elution (100% petroleum spirit to 100% dichloromethane/20% methanol) to give 13 fractions. Fraction 5 (105.8 mg) was purified by column chromatography using gradient elution with petroleum spirit - dichloromethane (100:0 to 0:100, v/v) to yield 10 fractions (fractions 5.1-5.10). Further purification of fraction 5.7 (54.5 mg) by preparative TLC, using 5% ethyl acetate/petroleum spirit as the eluent, gave dehydro-γ-tocopherol **6** (16.3 mg). Fraction 9 (174.0 mg) was purified by recrystallisation from ethanol to give stigmasterol **7** (17 mg). This experimental process is summarized in Fig. 3.3.



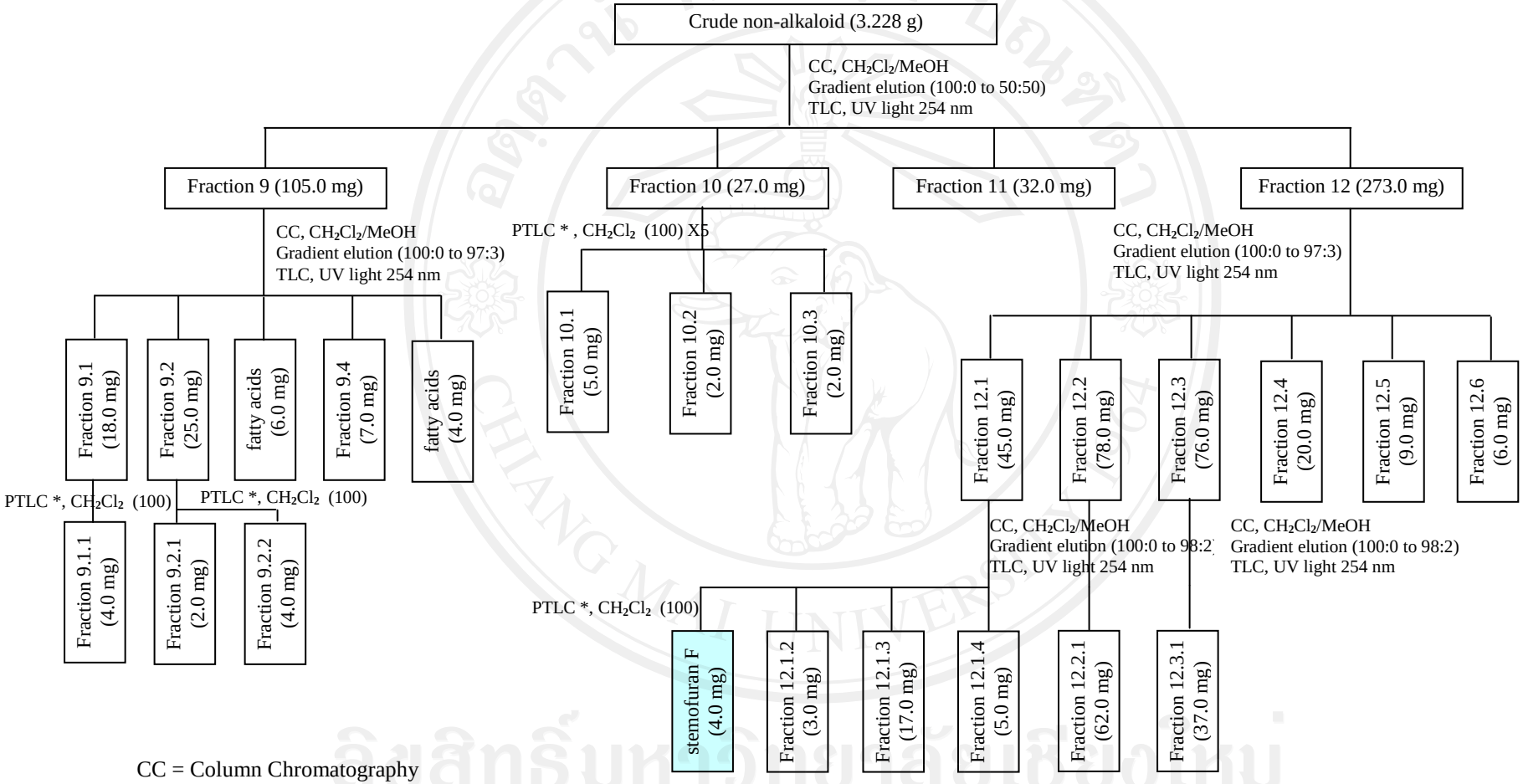
CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.2 Isolation of the chloroform crude extract of *S. curtisii* from Trang Province.



CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.2 (Continued).



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PTLC= Preparative Thin Layer Chromatography

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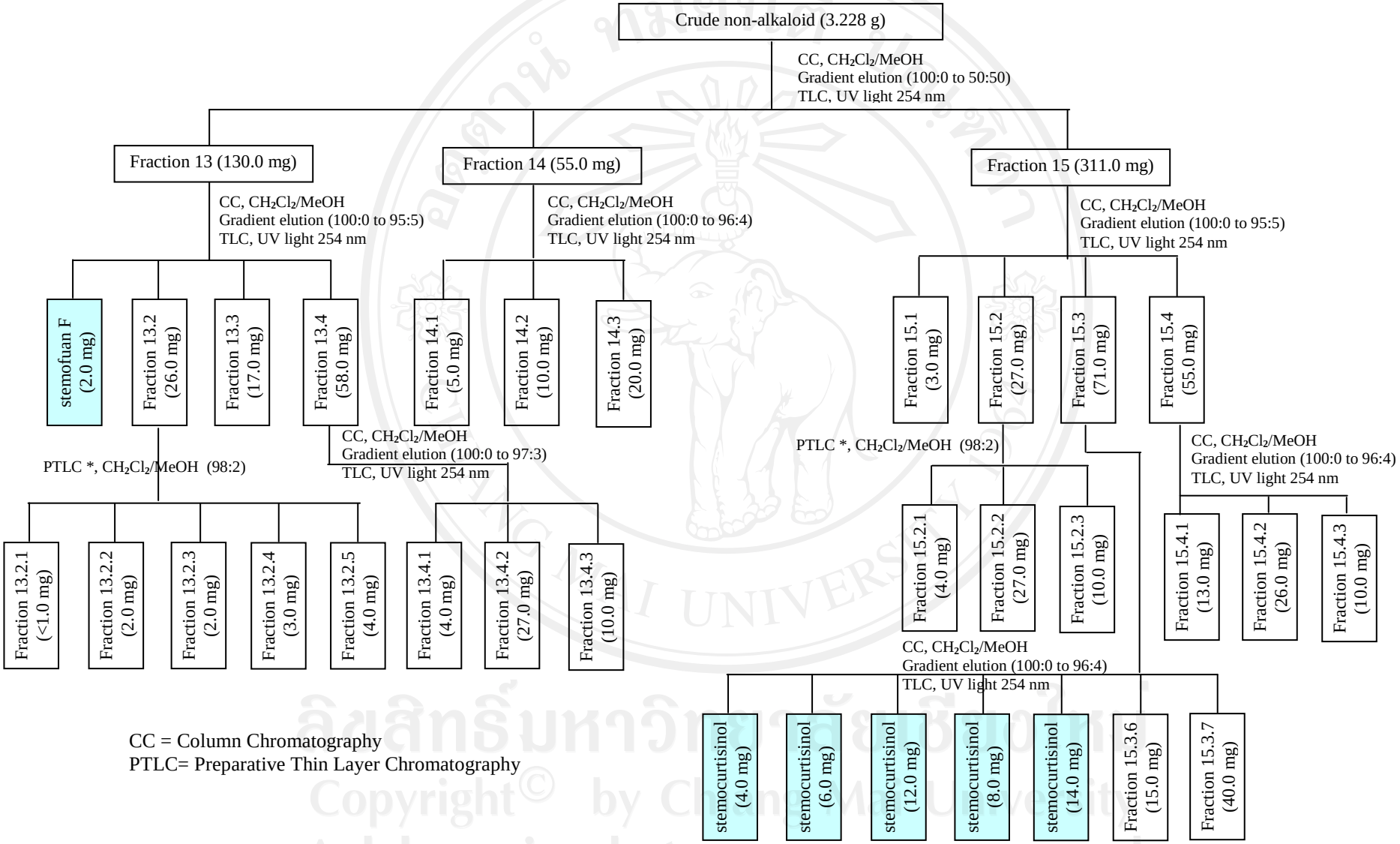

CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

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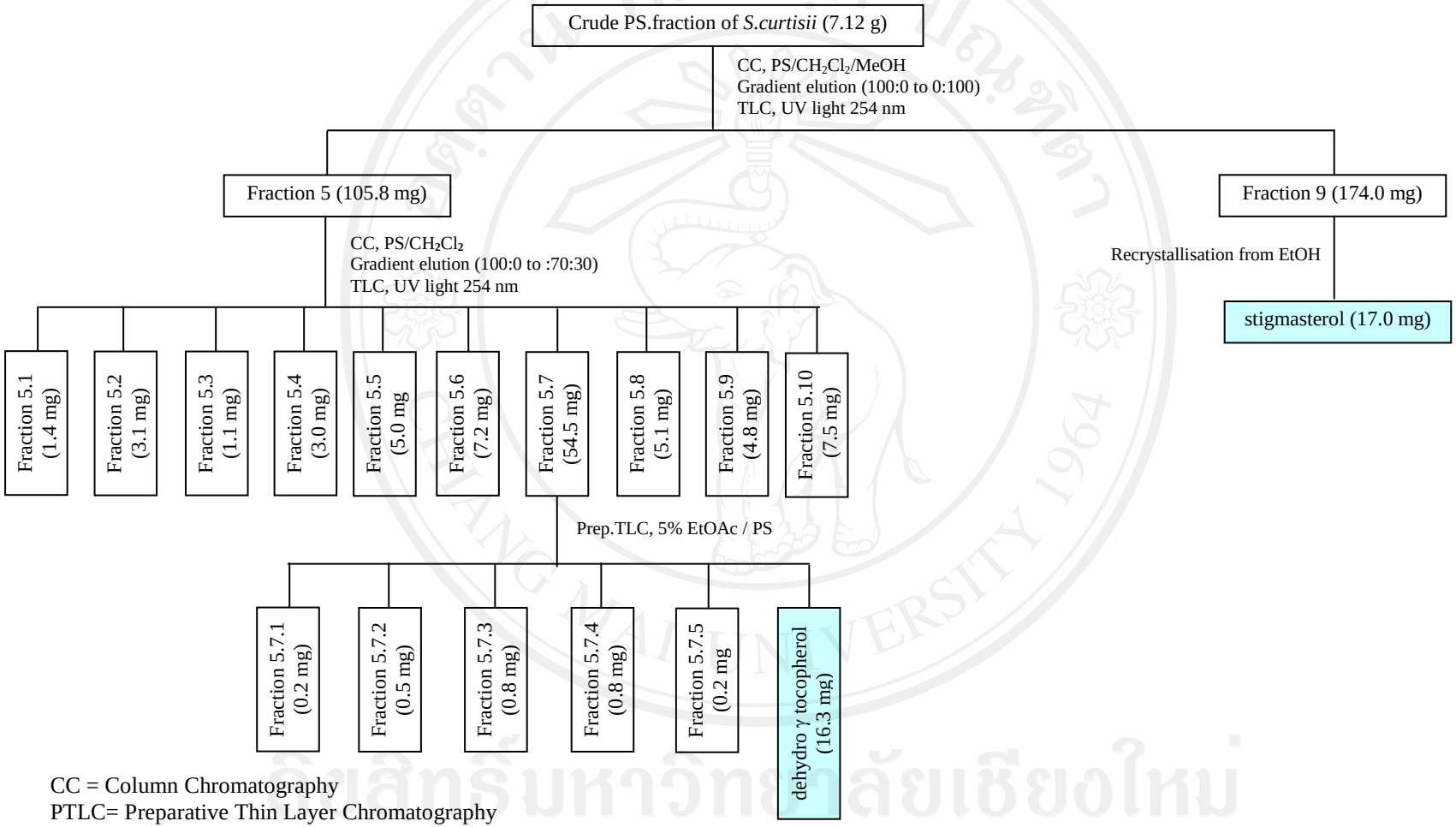


CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.3 Isolation of the petroleum spirit crude extract of *S. curtisii* from Trang Province.

The general extraction procedure of *S. curtisii* from Petchaboon Province is summarized in Fig. 3.4.

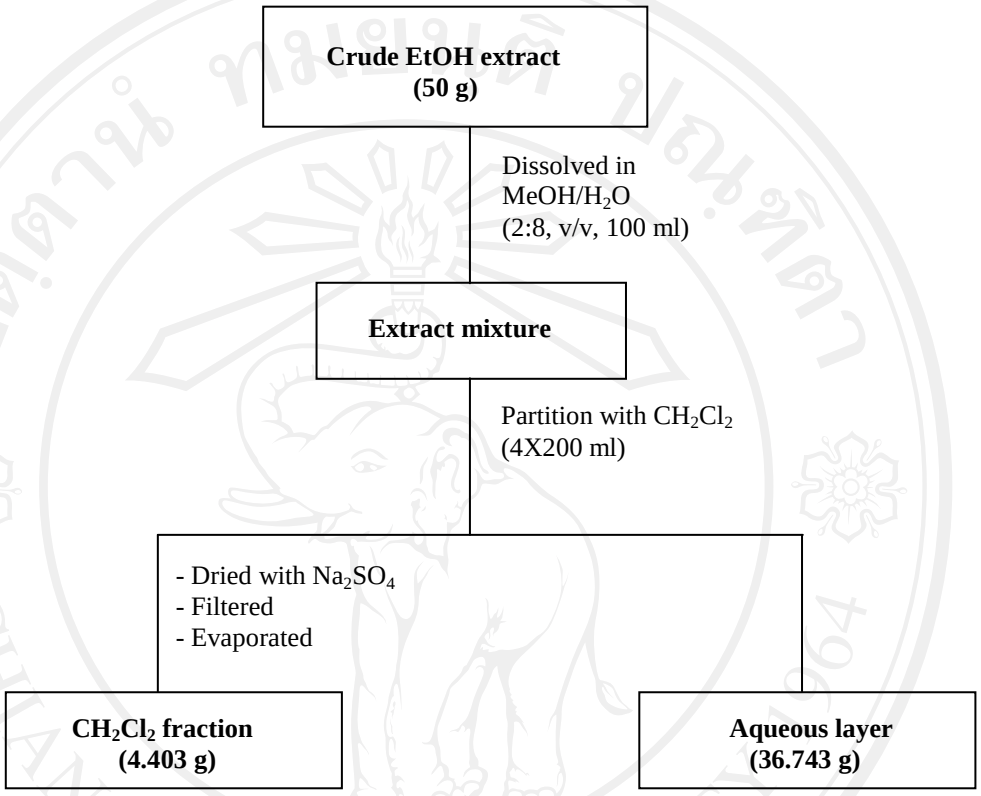


Figure 3.4 General extraction procedure of *S. curtisii* from Petchaboon Province.

3.3.2 *S. curtisii* extraction and isolation (Petchaboon Province)

The dry ground root of *S. curtisii* (2.5 kg) was extracted with 95% ethanol (3 x 3,000 mL) for 4 days at room temperature. The ethanol extracts were evaporated under reduced pressure to give a dark brown sticky residue (259.89 g). A portion of the crude extract (50.0 g) was dissolved in distilled water and methanol (8:2, v/v, 100 mL). The resulting mixture was extracted in dichloromethane (4 x 200 mL) to yield, 4.403 g of crude material after drying (Na_2SO_4) and evarporation as shown in Figure 3.4. A portion of the dichloromethane crude extract (2.776 g) was separated by flash column chromatography, using silica gel (200 ml) and gradient elution (100% dichloromethane to 100% methanol), 6 fractions were obtained from the separation (Figure 3.5).

Fraction 3 (361.0 mg) was further purified by silica gel column chromatography using a gradient elution with dichloromethane-methanol (100:0 to 90:10, v/v) to yield 4 fractions (fractions 3.1-3.4). Fraction 3.3 (169.0 mg) was further purified by column chromatography with gradient elution (100% dichloromethane to 100% methanol) to yield 2 fractions. Further purification of fraction 3.3.1 (66.0 mg) by preparative TLC (hexane: ethyl acetate, 30:70, v/v) gave a new compound, stemofuran S **8** (30 mg). Further purification of fraction 3.3.2 (48.0 mg) by preparative TLC was carried out, using ethyl acetate/hexane (1:9) as eluent to give oxystemokerrin **9** (9.0 mg).

Fraction 4 (1,774.0 mg) was also separated by flash column chromatography, using silica gel (200 ml) and gradient elution (100% dichloromethane to 100% methanol). Five fractions were obtained from the separation (fractions 4.1-4.5). Fraction 4.1 (1,514.0 mg) was further purified on silica gel column using gradient

elution with dichloromethane-methanol (100:0 to 80:20, v/v) to yield 9 fractions (fractions 4.1.1-4.1.9). Fraction 4.1.6 (125.0 mg) was further purified by column chromatography using gradient elution (hexane-ethyl acetate 50:0 to 0:50, v/v) to yield 2 fractions (fractions 4.1.6.1 and 4.1.6.2). Further purification of fraction 4.1.6.2 (26.0 mg) by preparative TLC (methanol: ethyl acetate: NH_4OH, 10:89:1, v/v) gave oxystemokerrin-*N*-oxide **10** (4.0 mg) and oxyprotostemonine **11** (6.0 mg). Fraction 4.1.7 (357.0 mg) was further purified on a silica gel column, using gradient elution with ethyl acetate-methanol (50:0 to 0:50, v/v) to yield 3 fractions (fractions 4.1.7.1-4.1.7.3). Further purification of fraction 4.1.7.3 (40.0 mg) by preparative TLC was carried out, using methanol/ethyl acetate (1:4) as eluent to give oxystemokerrin-*N*-oxide **10** (7.0 mg). Fraction 4.1.7.2 (252.0 mg) was purified by column chromatography using gradient elution with ethyl acetate-methanol (100:0 to 0:100, v/v) to yield 2 fractions (fractions 4.1.7.2.1 and 4.1.7.2.2). Fraction 4.1.7.2.2 (85 mg) was further separated by preparative TLC using methanol/ethyl acetate (1:9) as the eluting solvent to give oxystemokerrin-*N*-oxide **10** (15.0 mg). This experimental procedure is summarized in Fig. 3.5.

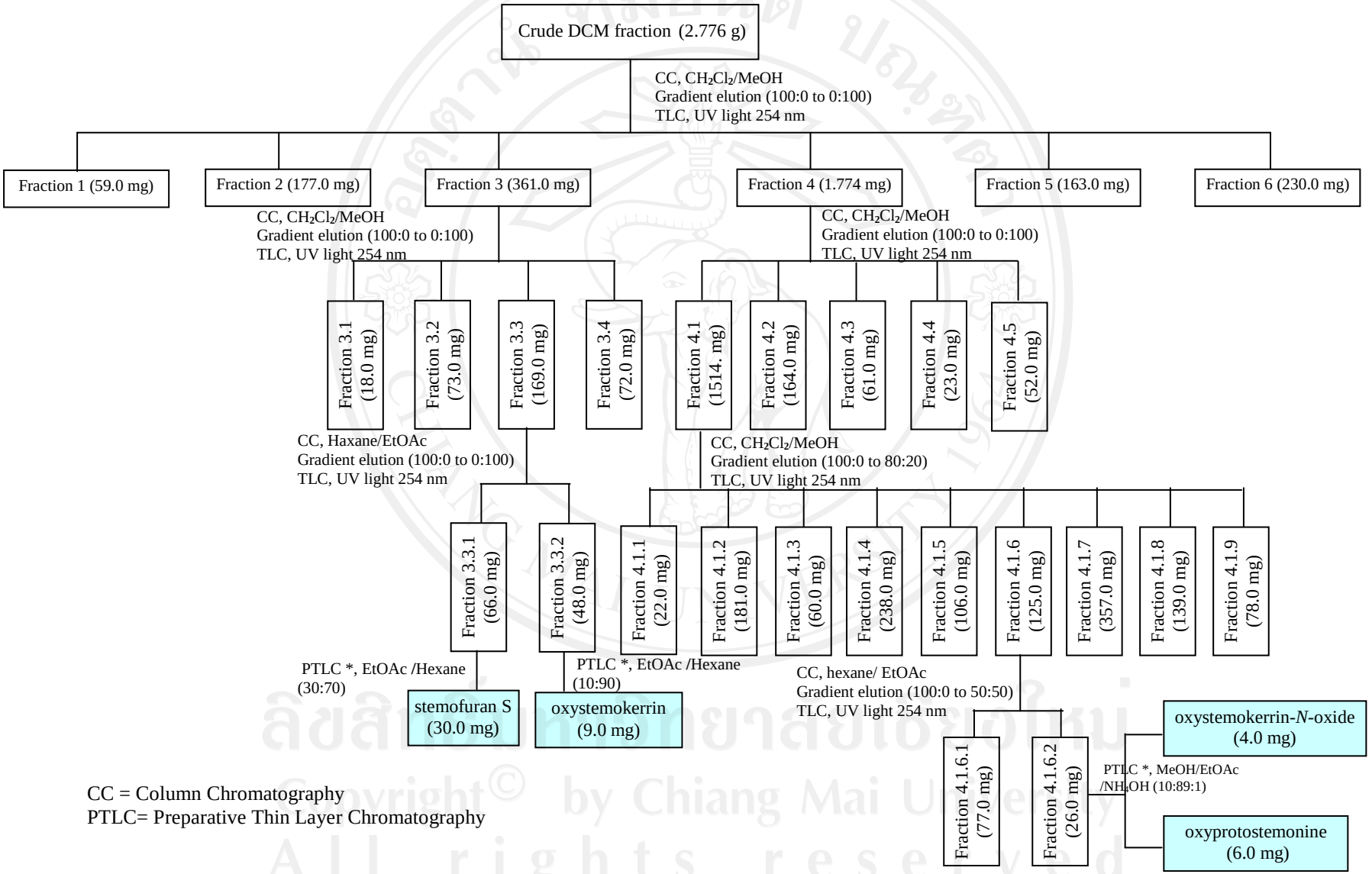
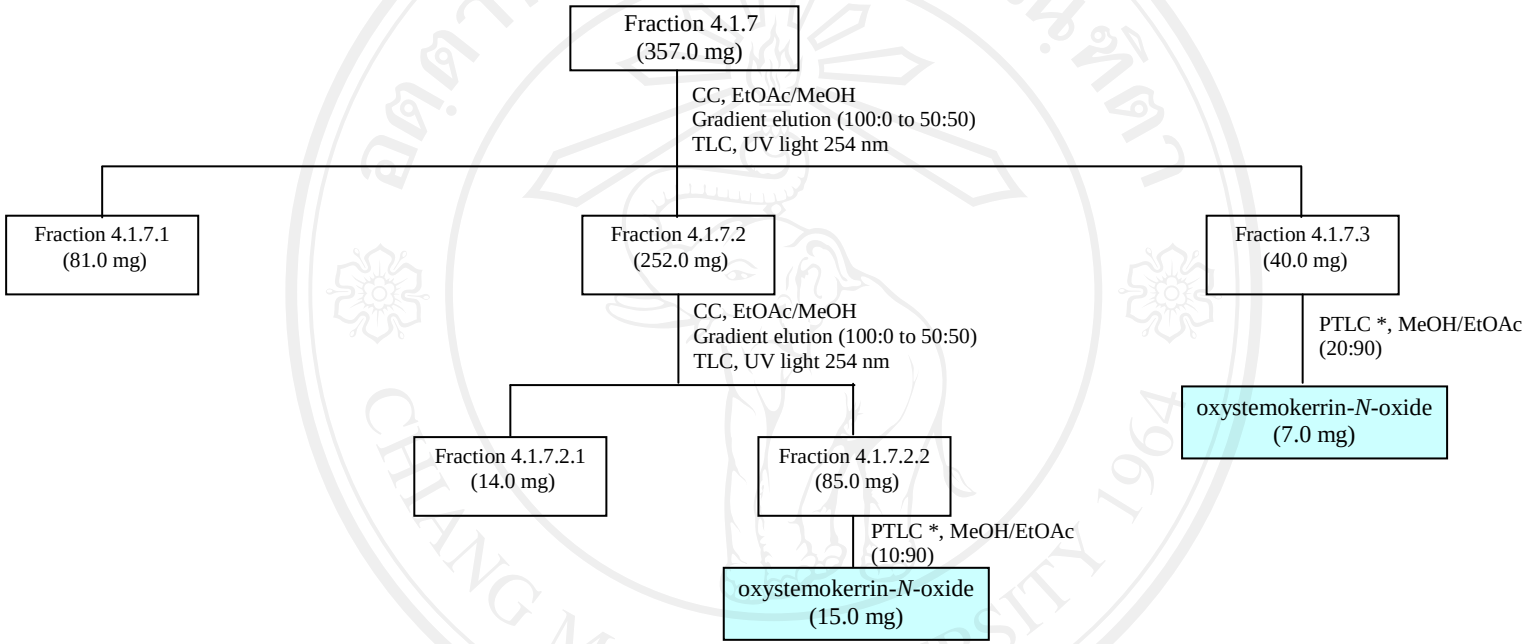


Figure 3.5 Isolation of the dichloromethane crude extract of *S. curtisii* from Petchaboon Province.



CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.5 (Continued).

The general extraction procedure of *S. aphylla* from Lampang Province is summarized in Fig. 3.6.

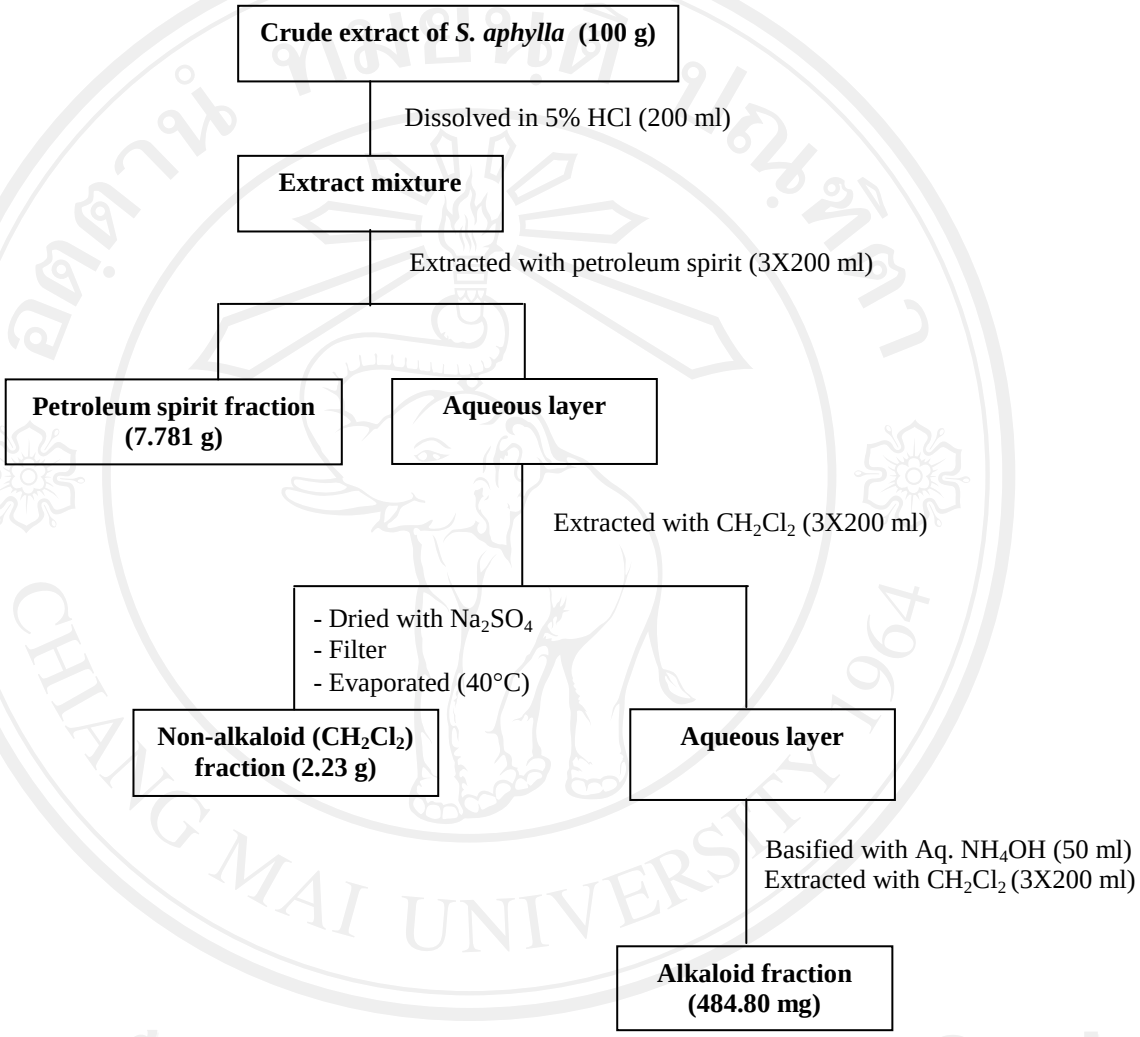


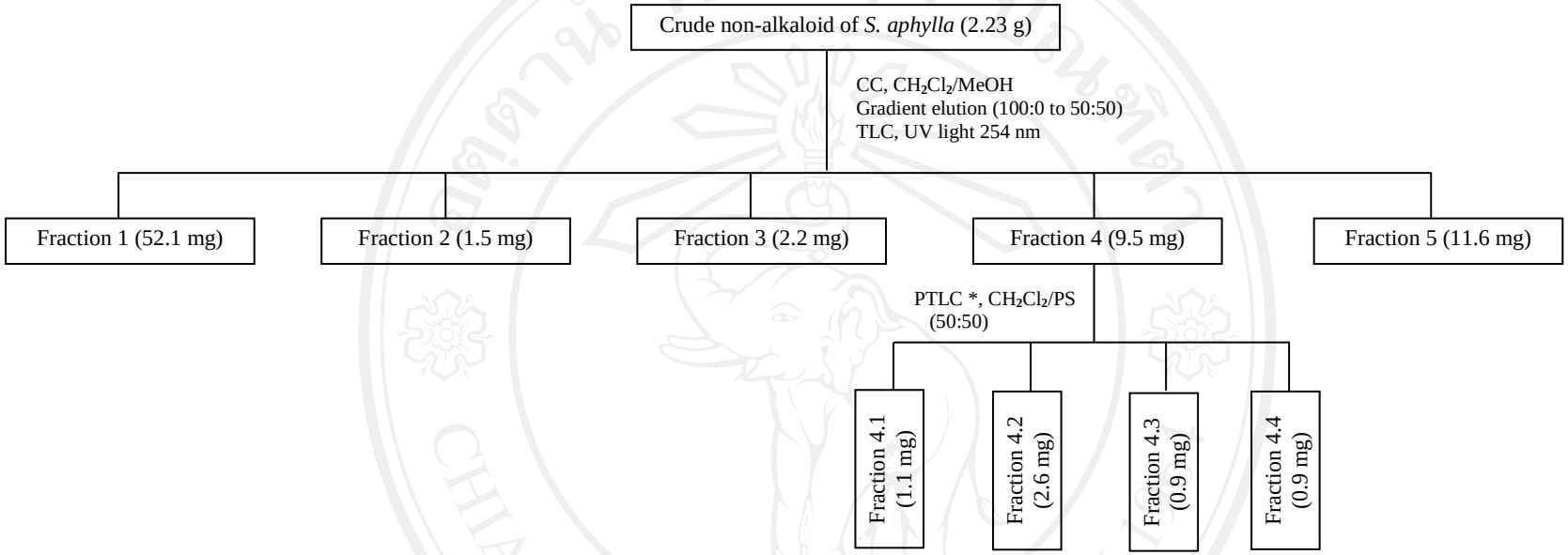
Figure 3.6 General extraction procedure of *S. aphylla* from Lampang Province**.**

3.3.3 *S. aphylla* extraction and isolation (Lampang Province)

The dry ground root of *Stemona aphylla* (15.14 kg) was extracted with 95% ethanol (4 x 10 L) for 4 days at room temperature. The ethanolic solution was evaporated to give a dark residue (1.45 kg). A portion of the extract (100.0 g) was partitioned between water (200 ml) and 5% hydrochloric acid solution (50 ml). The resulting aqueous solution was extracteded with petroleum spirit. The petroleum spirit was removed to yield (7.78 g) of crude mixture. The aqueous solution was further extract with dichloromethane. After removing the dichloromethane a crude mixture (2.23 g) was obtained. The aqueous solution was made basic with aqueous ammonia to pH 10. The basic aqueous solution was extracted with dichloromethane and the solvent was removed to afford the crude alkaloid mixture (484.4 mg). The chloroform crude extract (2.23 g) was separated by flash column chromatography on silica gel (200 ml) using gradient elution (100% dichloromethane to 50% methanol/dichloromethane) to give 12 fractions as shown in fig. 3.7. Fraction 6 (55.3 mg) was applied to a silica gel column with gradient elution using dichloromethane-methanol (100:0 to 97:3, v/v) to afford a new benzofuran **1** (stemofuran L, 1.0 mg), corresponding to that isolated from *S. curtisii* in Trang province. This experimental procedure is summarized in Fig. 3.6 and 3.7.

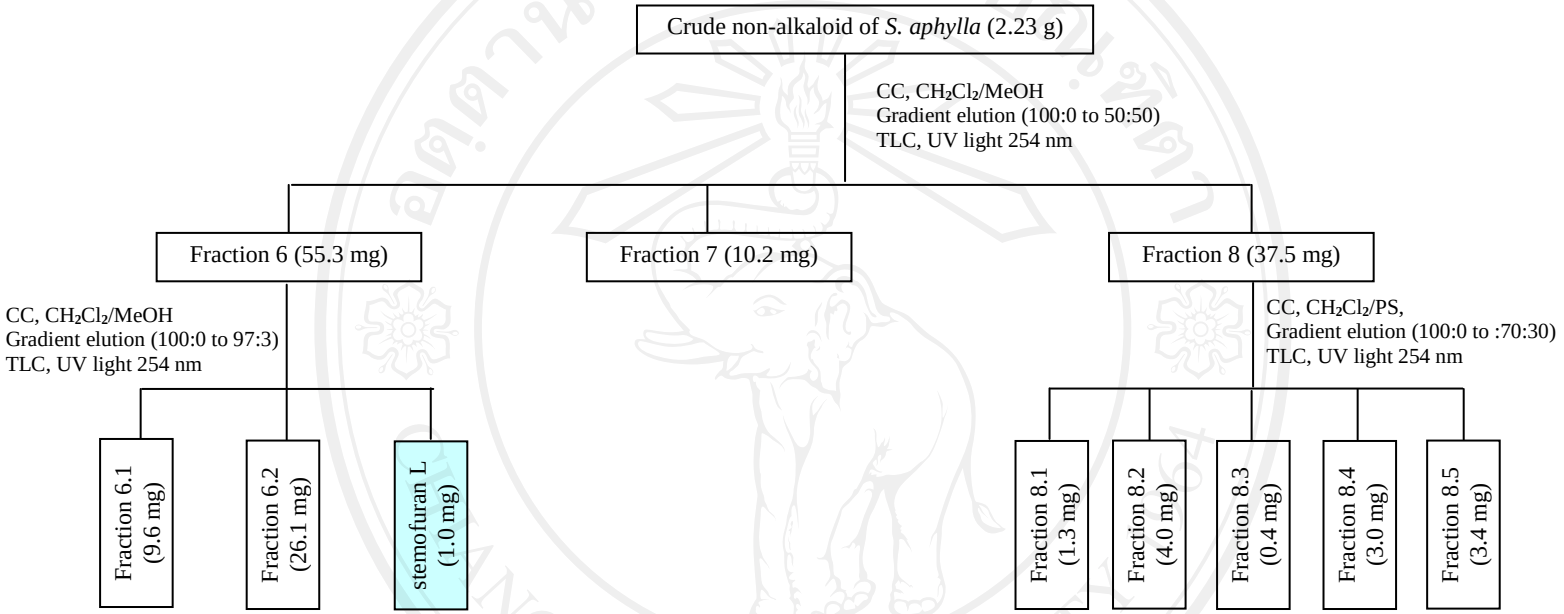
A portion of the petroleum spirit crude extract (5.98 g) was separated by flash column chromatography on silica gel (600 ml) using gradient elution (100% petroleum spirit to 100% ethyl acetate) to give 8 fractions. Fraction 3 (65.0 mg) was purified by column chromatography with gradient elution (toluene:ethyl acetate (100:0 to 1:99, v/v) to yield 7 fractions (fractions 3.1-3.7). Further purification of fraction 3.5 (45.1 mg) by preparative TLC using 1% toluene/ethyl acetate as the

eluting solvent gave dehydro-δ-tocopherol **12** (20.9 mg). Fraction 6 (121.9 mg) was further purified by column chromatography using gradient elution (100% petroleum spirit to 20% methanol) to afford stigmasterol **7** (34.0 mg) and stemofuran J **3** (11.4 mg). Fraction 7 (80.4 mg) was purified by column chromatography with gradient elution from 50% petroleum spirit to 50% methanol/dichloromethane to give an additional amount of stemofuran J **3** (7.0 mg). This experimental procedure is summarized in Fig. 3.8.



CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.7 Isolation of the chloroform crude extract of *S. aphylla* from Lampang Province.



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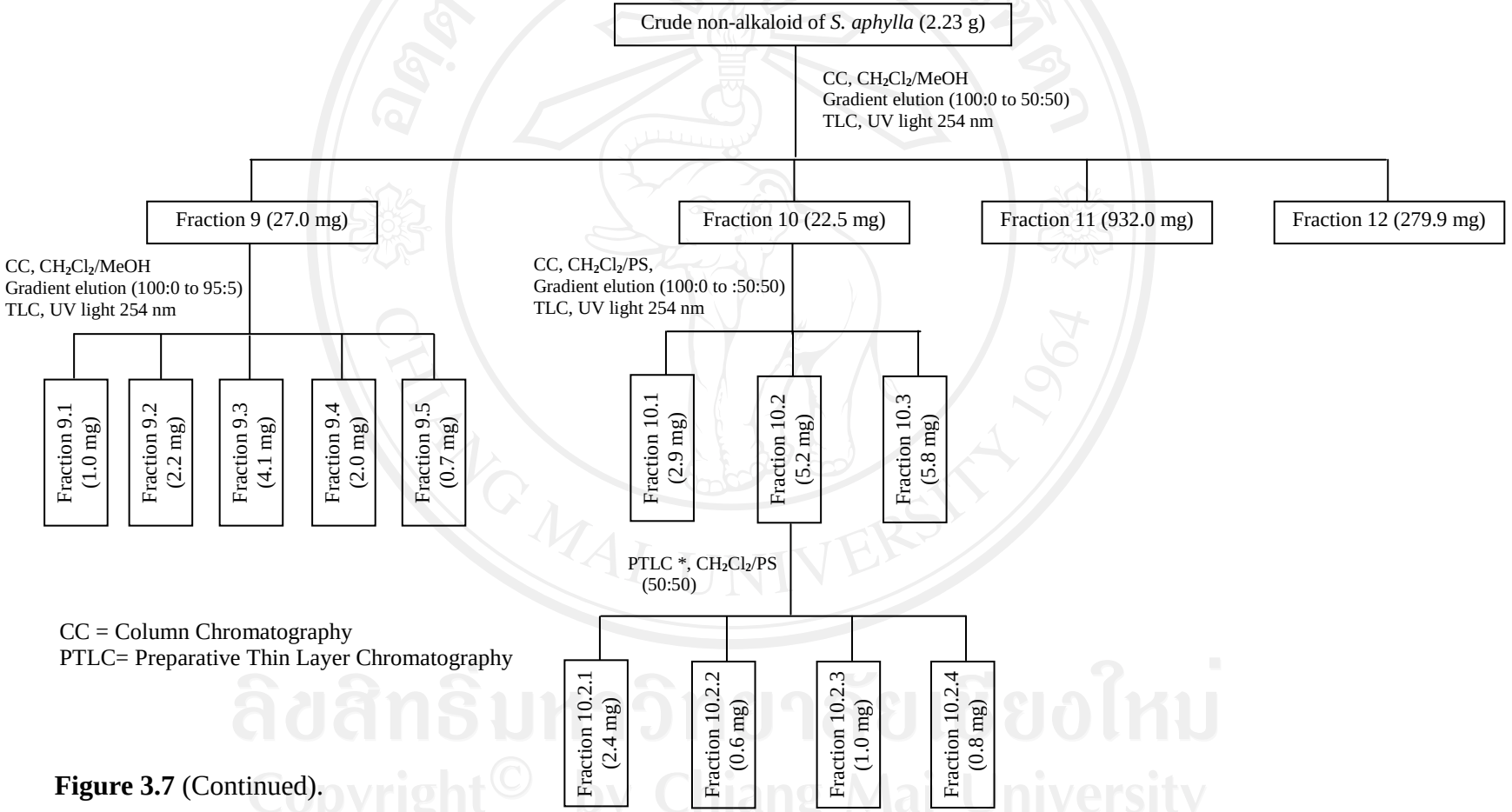

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Figure 3.7 (Continued).

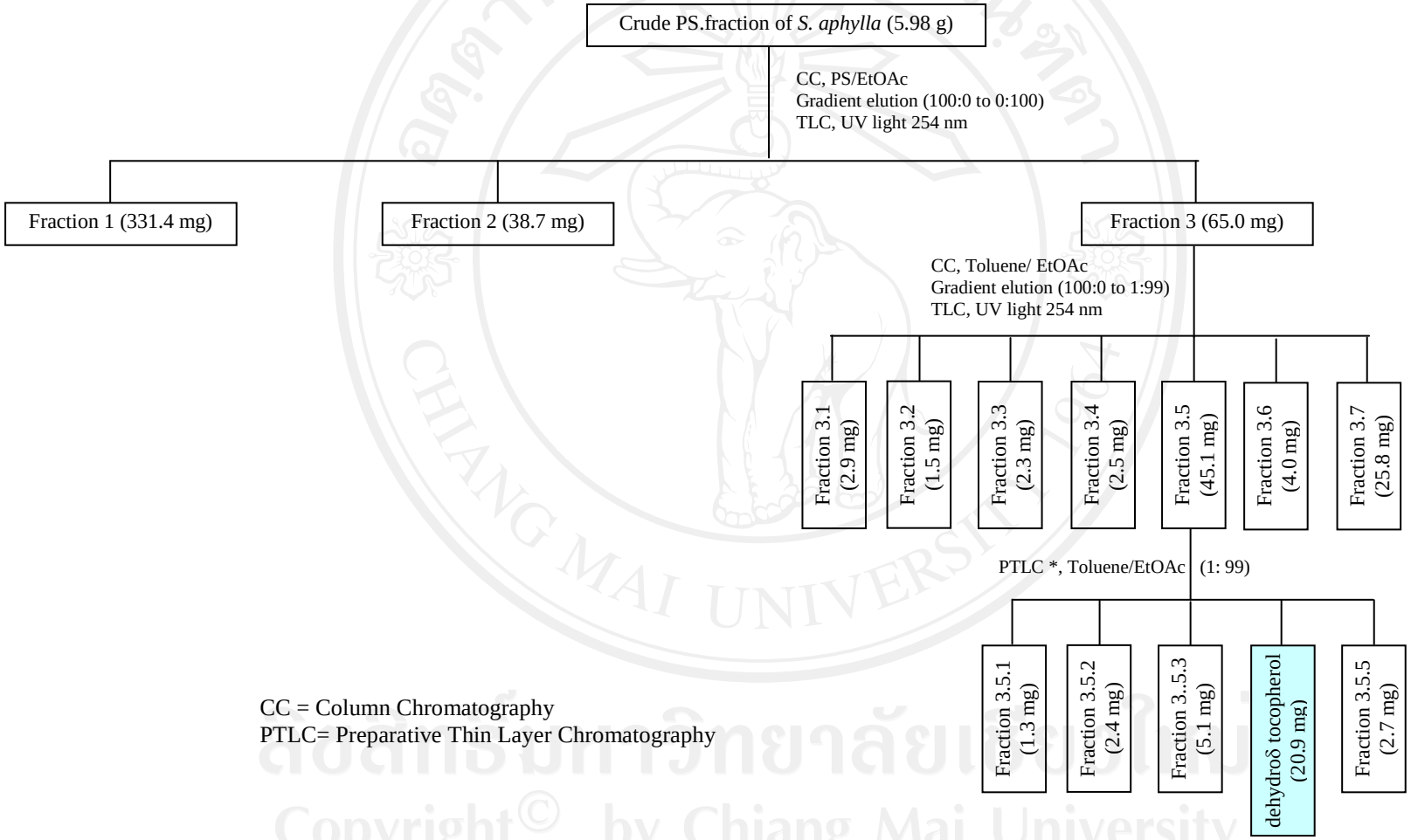
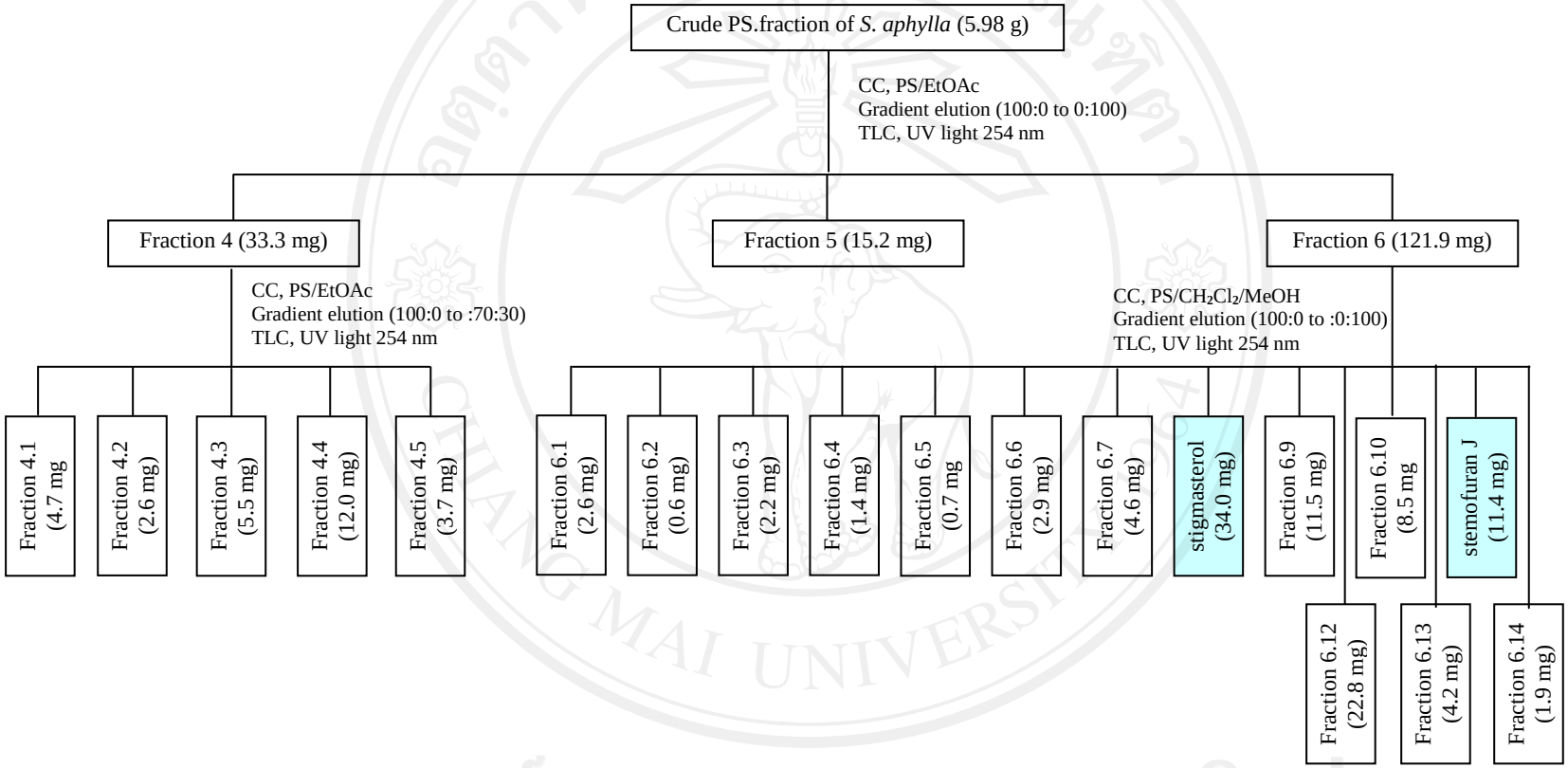
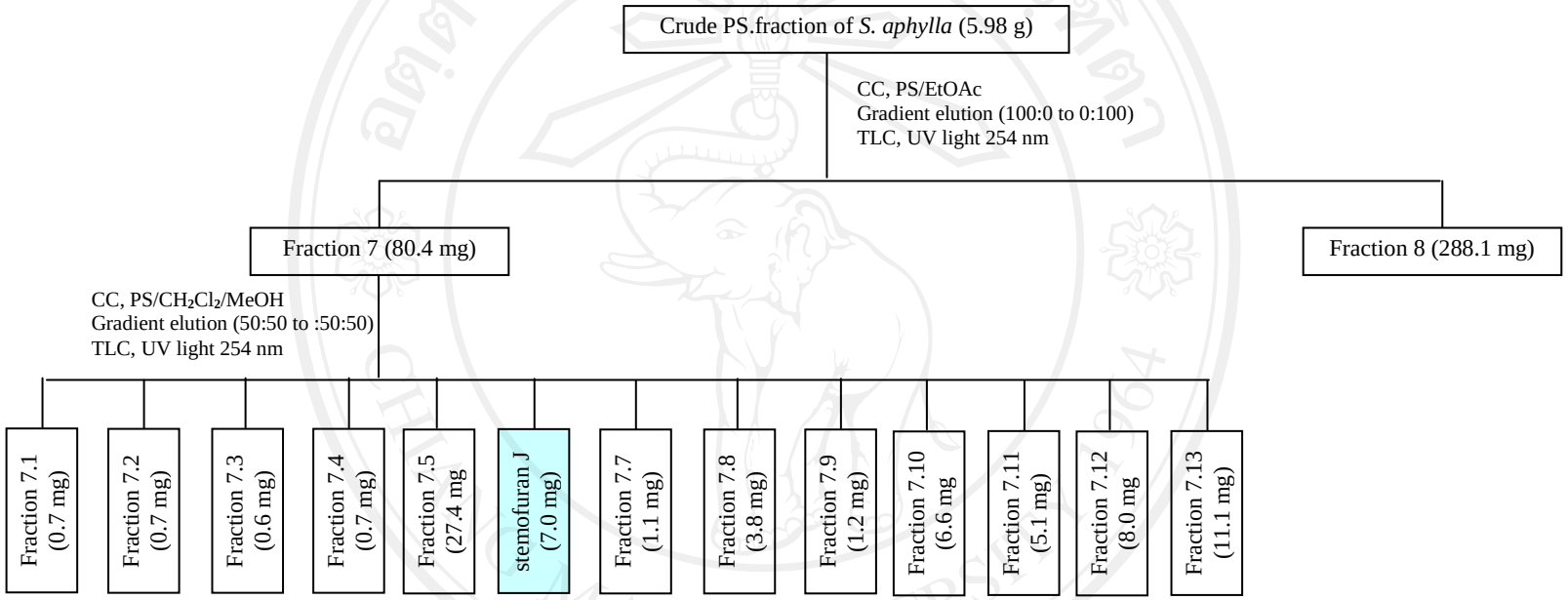


Figure 3.8 Isolation of the petroleum spirit crude extract of *S. aphylla* from Lampang Province.



CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.8 (Continued).



CC = Column Chromatography
PTLC= Preparative Thin Layer Chromatography

Figure 3.8 (Continued).

3.4 Structure elucidation

The isolated compounds were identified by their proton-nuclear magnetic resonance (^{1}H-NMR), carbon-nuclear magnetic resonance (^{13}C-NMR), two dimensional NMR, UV spectroscopy and mass spectrometry (MS). ^{1}H (500 MHz), ^{13}C (125 MHz), and 2D NMR spectra were recorded on a Varian Unity INOVA 500 MHz spectrometer. High-resolution EIMS were recorded on a Fison/VG Autospec-TOF-oa mass spectrometer (70 eV). High-resolution ESIMS were obtained with a Micromass QTOF 2 mass spectrometer using a cone voltage of 30 V and polyethylene glycol (PEG) as an internal reference.

3.5 Screening bioactive compounds

The study of bioactive compounds from plant sources and extracts in the chemical laboratory is often hampered by the lack of a suitable, simple and rapid screening procedures. The most general screening bioassays are the brine shrimp lethality test (BST) (Teng, 1993) and the crown-gall tumor inhibition test (Ghisalberti, 1993; McLaughlin, 1991). The first technique is an *in vivo* lethality test on a tiny crustacean, the brine shrimp (*Artemia salina*). Since its introduction in 1982 (Mayer *et al*., 1982), this test has been used for the detection *in vivo* of active antitumour agents and pesticides produced by plants. However, it can also be used to evaluate plants for different pharmacological activities (McLaughin, 1991). Herbicidal, insect-antifeedant, larvicidal and molluscidal activities can be determined by simple bioassays, which can function as surrogate assays to isolate bioactive compounds from plant extracts. The general toxic activities of the pure compounds from *Stemona* spp. on *Artemia salina* Leach (brine shrimp) (Fig. 3.9) were determined. Brine shrimp

eggs were hatched in artificial seawater (37.5 g of salt per litre) and the larvae were available for the experiment 48 hrs after sowing. The brine shrimp were treated with pure samples of stemofuran L (**1**), stemofuran K (**2**), stemofuran J (**3**), stemofuran F (**4**), stemocurtisinol (**5**), dehydro-γ-tocopherol (**6**), stigmasterol (**7**), stemofuran S (**8**), oxystemokerrin (**9**), oxystemokerrin-*N*-oxide (**10**), oxyprotostemonine (**11**) and dehydro-δ-tocopherol (**12**). The LC_{50} of pure compounds was determined within 24 hrs. All samples of compounds were dissolved in 95% ethanol and made into different concentrations ranging from 10 to 500 ppm, with 3 mL in each test tube. Each test solution was prepared in triplicate. Ten brine shrimps were dropped into the test solutions. A parallel series of tests were conducted with control solvent and control seawater.

The mortality of brine shrimp was observed under a stereomicroscope at 24 hours after application. The dead nauplii were used to determine the LC_{50}. The data were analyzed by probit analysis, SPSS for Windows.

(a) (b) (c)

Figure 3.9 Brine shrimp assays.

(a) hatching brine shrimp in artificial seawater.

(b) live brine shrimp.

(c) determination of lethal brine shrimp under stereomicroscopy.

3.6 Determination of efficiency on acetylcholinesterase inhibitory activity by TLC bioautographic assay (Marston *et al*., 2002)

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 stabilise 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 samples from *Stemona* spp. at different amounts, galanthamine and eserine were 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 means, 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 enzyme activity, solutions 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 mixed and sprayed onto the plate to give a purple coloration after 1–2 min.

A TLC bioautographic assay was chosen because it gives quick access to information concerning the activity. 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 (Fig. 3.10). Regions of the TLC plate which contain acetylcholinesterase inhibitors show up as white spots against the purple background.

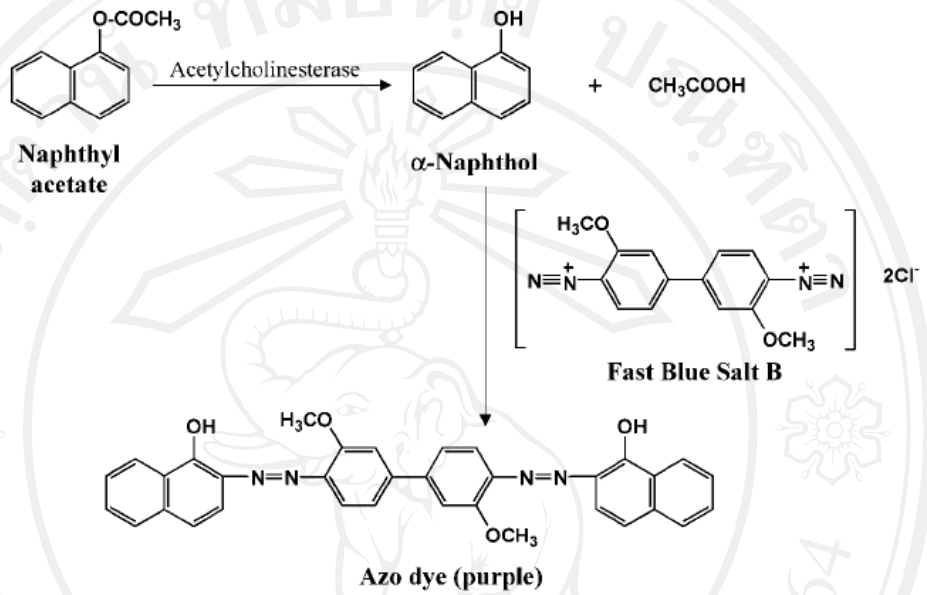


Figure 3.10 Reaction of acetylcholinesterase with naphthyl acetate and the subsequent formation of the purple dye in the TLC bioassay.

3.7 Determination of antimicrobial activities

Studies of antimicrobial activities of isolated compounds from *Stemona* species against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, *Candida albicans* and *Cryptococcus neoformans* were carried out by the broth dilution method (Richard, *et al*., 2003). Sterile 96-well microplates were used for the assay (0.5 mL volume, Fisher Scientific). Test samples were dissolved in a minimal amount of ethanol for stock solutions (1,000 μg/mL). Samples were diluted to twice

the desired initial test concentration with TSB. All wells, except the first, were filled with TSB (50 μL). Test samples (100 μL) were added to the first well and serial two-fold dilutions were made down to the desired minimum concentration. Day-old cultures of bacteria grown were suspended in TSB until turbidity was equal to a 0.5 McFarland Standard (Koneman *et al*., 1997). The plates were inoculated with the bacterial suspension (50 μL per well) and incubated at 37 °C overnight. Antibiotics, gentamicin and amphotericin B were used as positive controls. The MIC was determined as the lowest sample concentration (signifying live growth) that gave rise to a clear solution. Minimum bactericidal concentrations (MBC) were considered by transferring approximately 0.01 mL of test samples from each well showing no growth of the test organism to a Petri dish. Spread over the surface of a tryptic soy agar plate. The plates were incubated at 37°C for 18-48 h., triplicates of each samples were made. MBC was defined as the lowest concentration of sample concentration which prevented growth and reduced the inoculum by ≥ 99.99% killing within 24 hr.

3.8 Determination of insecticidal properties

The insecticidal property of *Stemona* spp. crude extracts were investigated against third instar larvae of *Spodoptera littoralis* Boisduval (*S. littoralis*) by leaf disk assays (Fig. 3.11), in comparison with the commercial insecticide methomyl and control sample. Leaf disks of a standard size (1.33 cm^2) were cut from *Brassica oleracea* L. CV. (Chinese kale), and then were spread on their upper surfaces with 20 μL of crude extract solution at the concentrations of 0.01, 0.05, 0.1, 0.5 and 1.0%. Control leaf disks were spread with the same volume of 95% ethanol. After the solvent was evaporated, two treated and two control leaf disks were placed on moist

filter paper in a Petri dish. Two 3rd instar larvae were randomly selected and fed on the leaf disks in the Petri dishes. (Brem *et al*., 2002)

To avoid a no choice situation, results were taken when approximately 50% of controls were eaten within 24 hrs after application. Each experiment was conducted in triplicate.

(a) (b)

Figure 3.11 Leaf disk choice test.

(a) Chinese kale leaf infested with 3rd instar larvae of *S. littoralis*.

(b) Leaf disks with test solution.

3.9 Determination of antioxidant activities

The radical scavenging activities of *Stemona* spp. crude extracts and isolated compounds were assessed by the DPPH assay (Yen *et al*., 1997). An aliquot 100 mL of the DPPH radical solution (0.2 mM, in ethanol) was added to the samples at different concentrations (0–1000 mg/L). The mixture was vigorously shaken and left to stand for 30 min at room temperature in the dark. The absorbance was measured by a UV-Vis spectrophotometer at 517 nm. The DPPH radical scavenging activities of the samples were compared with that of standard α-tocopherol, butylated

hydroxyanisole (BHA) and trolox. The radical scavenging effect was calculated by the following equation:

radical scavenging (%) = [1- (a_{sample} / $a_{control}$)] x 100

where $a_{control}$ is the absorbance of the control at 517 nm, and a_{sample} is the absorbance of the extract/standard at 517 nm and the IC_{50} was determined.