CHAPTER 3

EXPERIMENT

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. Mass spectrometer, Fison/VG Autospec-TOF-oa
5. Mass spectrometer, Micromass Qtof 2
6. Herbal slicing machine
7. NMR spectrometers, Varian Mercury 300 and Varian Unity 500
8. Polarimeter DIP-370, JASCO
9. Rotary evaporator, Buchi, Switzerland included;
    - Rotavapor R-124
    - Water bath B-480
10. Ultrasonic bath super RK510H, Bandelin Sonorex
11. UV Cabinet with long-wave UV light 366 nm and short-wave UV light 254nm
12. Vacuum filter machine, BÜCHI

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3.1.2 Materials

1. Adjustable air-displacement pipette10μL, 100μL, 1,000μL and 10 mL, Gilson
2. Brine shrimp culturing box and breeding equipments
3. Filter paper No. 1 ∅ 12.5 mm, Whatman
4. Flash silica gel GF_{254} (40-63 μm), Merck
5. Pesticide sprayer machine (carry on type)
6. TLC aluminium sheet silica gel 60 GF_{254}, Merck
7. TLC Syringe fixed needle 10μl and 25μl, Hamilton

3.1.3 Chemicals

1. Acetone (C_3H_6O), Analytical grade, J.T. Baker
2. Ammonia solution 25-30% (NH_4OH), Laboratory grade, BDH
3. Bismuth Subnitrate ($4BiNO_3(OH)_2BiO(OH)$), Analytical grade , Riedel-De Hach
4. Cerium chloride ($CeCl_3.H_2O$), Laboratory grade
5. Dess-Martin periodinane reagent, Analytical grade
6. Deuterated Chloroform ($CDCl_3$), NMR solvent
7. Deuterated Methanol (CD_3OD), NMR solvent
8. Dichloromethane (CH_2Cl_2), Analytical grade, J.T. Baker
9. Distilled water
10. Ethanol 95% (C_2H_5OH), commercial grade
11. Hydrochloric acid fuming 37% (HCl), Analytical grade, Merck
12. Hydrogen peroxide (H_2O_2), Analytical grade
13. Methanol (CH_3OH), Laboratory grade, J.T. Baker

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14. Methomyl (*S*-methyl-*N*-(methylcarbamyloxy)thioatimidate 40%), Dupont
15. Polyethyleneglycol (PEG)
16. Pine oil
17. Potassium iodide 99.5% (KI), Analytical grade, BDH
18. *R*)-(-)- and (*S*)-(+)- Mosher's acid chloride ((*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (MTPACl)), Analytical grade
19. Sodium borohydride ($NaBH_4$), Laboratory grade
20. Sodium sulfate anhydrous (Na_2SO_4), Laboratory grade
21. Tween 80

3.2 Collection of plant materials

The *Stemona* spp. i.e. *Stemona curtisii* Hook F. and 2 unknown species were collected from different locations in Thailand and identified by Botanical notes on the vascular flora of Chiang Mai (Maxwell, 1991). The *Stemona* roots were washed with water. Then, the roots were sliced into small pieces, dried in a shade and incubated in the hot air oven at 50°C to eliminate the water.

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3.3 Extraction and isolation

The general crude alkaloids extraction procedure was summarized in Fig. 3.1.

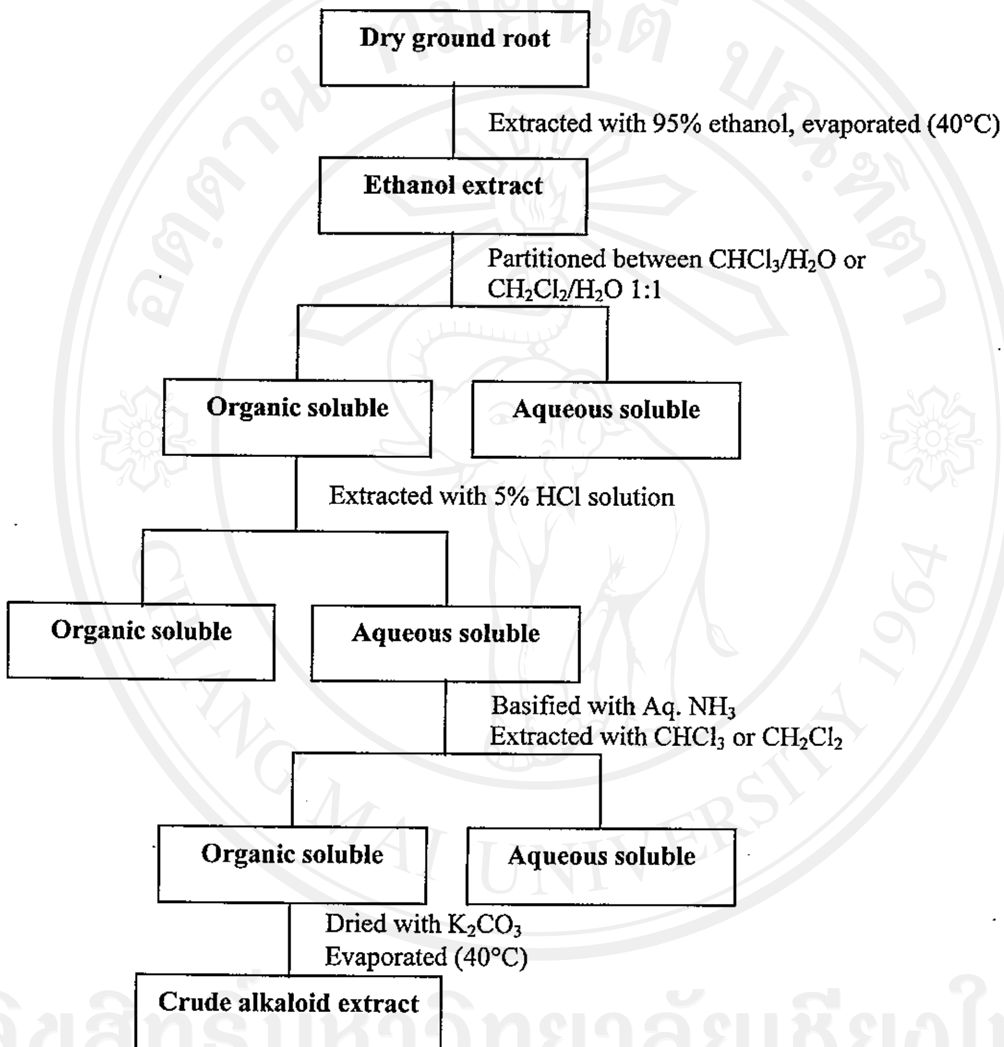

Figure 3.1 Crude alkaloid extraction procedures

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3.3.1 *S. curtisii* extraction and alkaloid isolation

The dry ground root of *S. curtisii* (1.0 kg) was extracted with 95% ethanol (3 x 3,000 mL) over 4 days at room temperature. The ethanolic solution was evaporated to give a dark residue (123.1 g) and a portion of the crude extract (89.7 g) was partitioned between water and dichloromethane. The dichloromethane extract was extracted with 5% hydrochloric acid solution, and the aqueous solution was made basic with aqueous ammonia and extracted with dichloromethane to afford 1.58 g of crude alkaloid material. This material was chromatographed on flash silica gel (150 mL) using gradient elution from 100% dichloromethane to 50% methanol/dichloromethane containing 1% concentrated aqueous ammonia as eluent. A total of 1,500 mL of eluent was collected in test tubes of 20 mL. On the basis of TLC analysis these fractions were pooled to give three alkaloid fractions i.e. fraction 1 (443.4 mg), fraction 2 (328.7 mg), and fraction 3 (760.9 mg). These fractions were further purified by chromatographic techniques.

Separation of fraction 1 by column chromatography gave three alkaloid fractions i.e. fraction 1.1 (178.3 mg), fraction 1.2 (38.7 mg) and fraction 1.3 (165.2 mg). Separation of fraction 1.1 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave stemocurtisinol (**63**, 12.3 mg). Separation of fraction 1.2 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional stemocurtisinol (**63**, 1.8 mg) and oxyprotostemonine (**21**, 1.0 mg). Separation of fraction 1.3 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional oxyprotostemonine (**21**, 8.5 mg).

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Separation of fraction 2 by column chromatography gave two alkaloid fractions i.e. fraction 2.1 (189.6 mg) and fraction 2.2 (139.1 mg). Separation of fraction 2.1 by column chromatography gave two alkaloid fractions i.e. fraction 2.1.1 gave an additional stemocurtisinol (**63**, 7.4 mg), fraction 2.1.2 15.9 mg and fraction 2.1.3 gave an additional oxyprotostemonine (**21**, 8.1 mg). Separation of fraction 2.1.2 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional stemocurtisinol (**63**, 0.8 mg) and oxyprotostemonine (**21**, 0.8 mg). Separation of fraction 2.2 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave stemocurtisine (**58**, 10.5 mg).

Separation of fraction 3 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional stemocurtisine (**58**, 26.0 mg).

This experiment is summarized in Fig. 3.2.

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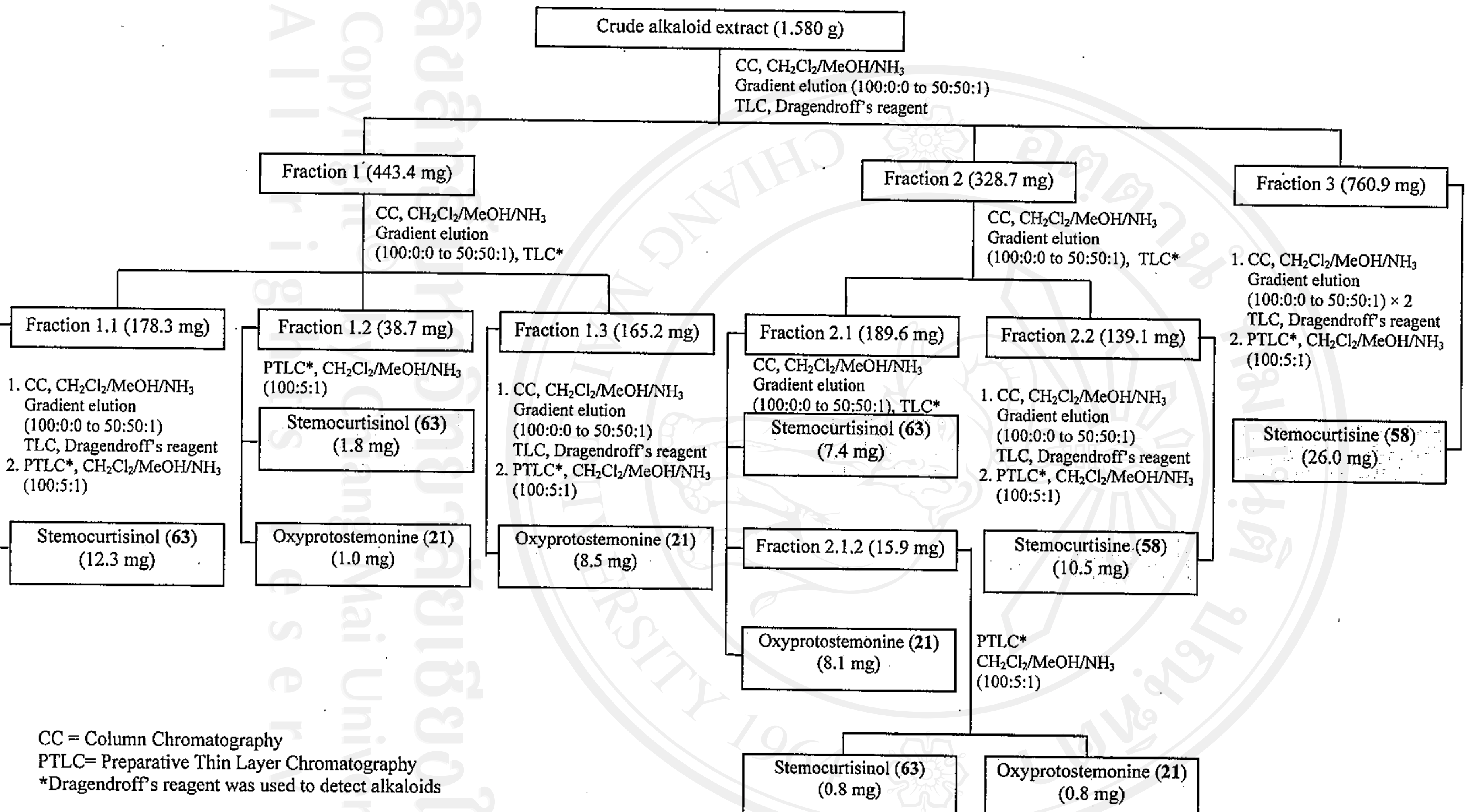

Figure 3.2 Isolation of alkaloids from *S. curtisii*

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3.3.2 Unknown *S.* sp. 1 extraction and alkaloid isolation

The dry ground root of *Stemona* sp. (unknown 1) (1.44 kg) was extracted with 95% ethanol (4 x 4,500 mL) over 4 days at room temperature. The ethanolic solution was evaporated to give a dark residue (99.7 g) and a portion of the extract (37.2 g) was partitioned between water and dichloromethane. The dichloromethane extract was extracted with 5% hydrochloric acid solution, and the aqueous solution was made basic with aqueous ammonia and extracted with dichloromethane to afford 0.952 g of crude alkaloid material. This material was chromatographed on flash silica gel (100 mL) using gradient elution from 100% dichloromethane to 50% methanol/dichloromethane containing 1% concentrated aqueous ammonia as eluent. A total of 900 mL of eluent was collected in test tubes of 20 mL. On the basis of TLC analysis these fractions were pooled to give six alkaloid fractions i.e. fraction 1 (171.0 mg), fraction 2 (100.3 mg), fraction 3 (255.6 mg), fraction 4 (47.1 mg), fraction 5 (29.3 mg), and fraction 6 (63.7 mg). These fractions were further purified by chromatographic techniques.

Separation of fraction 1 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave neotuberostemonine (**13**, 59.6 mg) and (2′*S*)-hydroxystemofoline (**51**, 69.6 mg).

Separation of fraction 2 by column chromatography gave two alkaloid fractions i.e. fraction 2.1 (76.0 mg) and fraction 2.2 (15.9 mg). Separation of fraction 2.1 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional neotuberostemonine (**13**, 11.7 mg) and (2′*S*)-hydroxystemofoline (**51**, 36.8 mg). Separation of fraction 2.2 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3= 100: 5: 1) gave tuberostemonine L (**69**, 8.7 mg).

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Separation of fraction 3 by column chromatography gave two alkaloid fractions i.e. fraction 3.1 (109.9 mg) and fraction 3.2 (148.7 mg). Separation of fraction 3.1 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional neotuberostemonine (**13**, 29.9 mg) and (2′*S*)-hydroxystemofoline (**51**, 29.7 mg). Separation of fraction 3.2 by column chromatography gave three alkaloid fractions i.e. fraction 3.2.1, which was additional (2′*S*)-hydroxystemofoline (**51**, 9.6 mg), fraction 3.2.2 60.5 mg and fraction 3.2.3, which was additional tuberostemonine L (**69**, 46.7 mg). Separation of fraction 3.2.2 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave an additional (2′*S*)-hydroxystemofoline (**51**, 25.9 mg) and tuberostemonine L (**69**, 20.0 mg).

Separation of fraction 4 preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave stemocurtisine (**58**, 3.1 mg).

Separation of fraction 5 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave (3′*S*)-hydroxystemofoline (**70**, 4.3 mg).

Separation of fraction 6 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave tuberostemonine M (**71**, 5.6 mg).

This experiment is summarized in Fig. 3.3.

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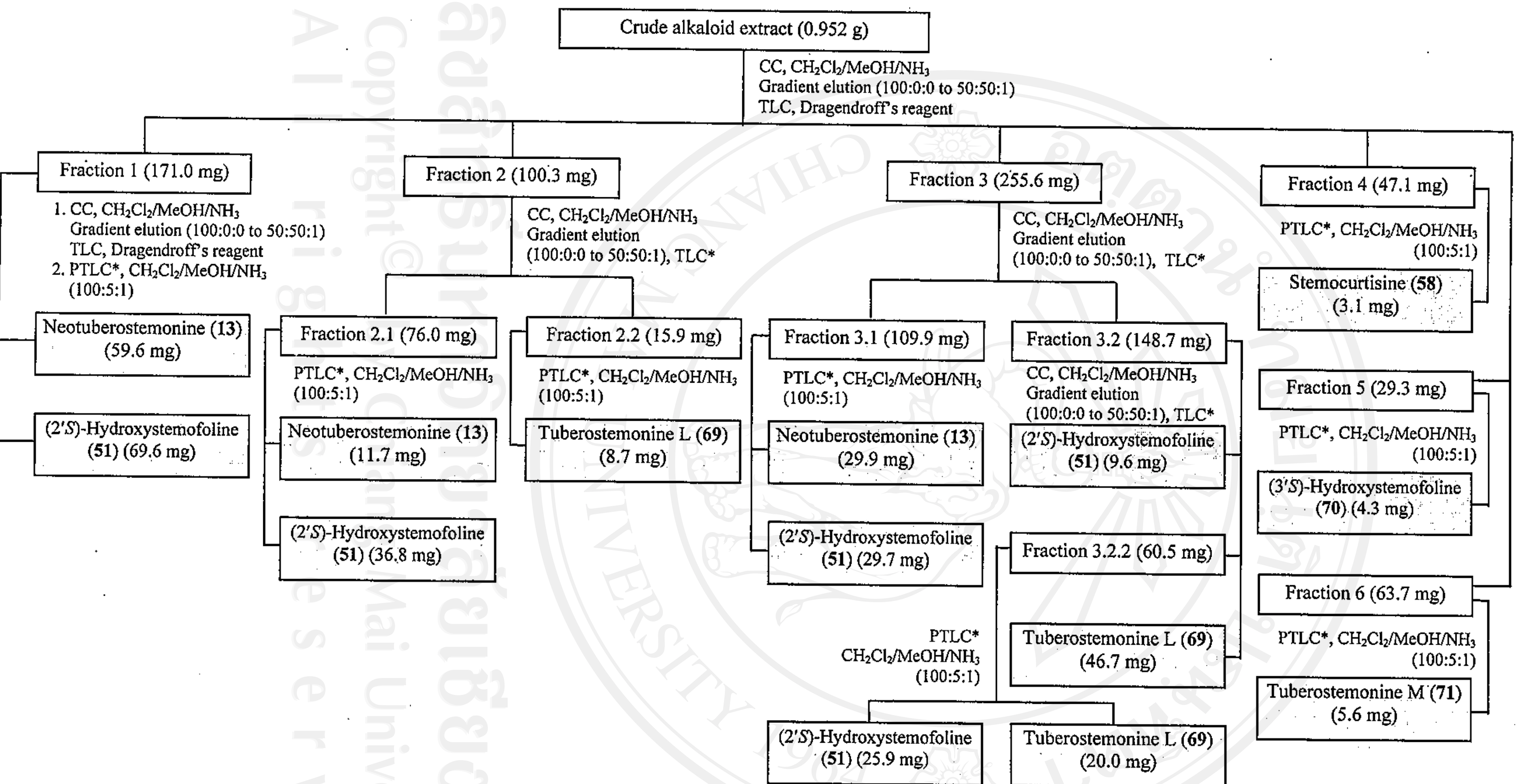

Figure 3.3 Isolation of alkaloids from unknown 1

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3.3.3 Unknown *S.* sp. 2 extraction and alkaloid isolation

The dry ground root of *Stemona* sp. (unknown 2) (1.1 kg) was extracted with 95% ethanol (4 x 3,000 mL) over 4 days at room temperature. The ethanolic solution was evaporated to give a dark residue (223 g) and a portion of the extract (58.8 g) was partitioned between water and dichloromethane. The dichloromethane extract was extracted with 5% hydrochloric acid solution, and the aqueous solution was made basic with aqueous ammonia and extracted with dichloromethane to afford 0.732 g of crude alkaloid material. This material was chromatographed on flash silica gel (100 mL) using gradient elution from 100% dichloromethane to 50% methanol/dichloromethane containing 1% concentrated aqueous ammonia as eluent. A total of 900 mL of eluent was collected in test tubes of 20 mL. On the basis of TLC analysis these fractions were pooled to give five alkaloid fractions i.e. fraction 1 (490.6 mg), fraction 2 (28.0 mg), fraction 3 (32.3 mg), fraction 4 (84.3 mg) and fraction 5 (18.0 mg). These fractions were further purified by chromatographic techniques.

Separation of fraction 1 by column chromatography gave three alkaloid fractions i.e. fraction 1.1, which was pure (11*Z*)-1′, 2′-didehydrostemofoline (**52**, 191.3 mg), fraction 1.2 187.9 mg and fraction 1.3 15.8 mg. Separation of fraction 1.2 by column chromatography gave three alkaloid fractions i.e. fraction 1.2.1, which was additional (11*Z*)-1′,2′-didehydrostemofoline (**52**, 44.6 mg), fraction 1.2.2 85.7 mg and fraction 1.2.3 16.9 mg. Separation of fraction 1.2.2 by column chromatography gave pure sample of (2′*S*)-hydroxystemofoline (**51**, 45.1 mg). Separation of fraction 1.2.3 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 2.5: 1) gave an additional of (2′*S*)-hydroxystemofoline (**51**, 13.3 mg). Separation of fraction 1.3 by preparative

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TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 2.5: 1) gave (11*E*)-1′,2′-didehydrostemofoline (**53**, 2.2 mg) that contained 10% of (11*Z*)-1′,2′-didehydrostemofoline (**52**).

Separation of fraction 2 by two successive preparative TLC purifications (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1 and then CH_2Cl_2: MeOH: aqueous NH_3, 100: 2.5:1) gave a pure sample of methylstemofoline (**72**, 1.9 mg).

Separation of fraction 3 by column chromatography and then preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 2.5: 1) gave a pure sample of (2′*R*)-hydroxystemofoline (**73**, 5.7 mg).

Separation of fraction 4 by column chromatography gave two alkaloid fractions i.e. fraction 4.1 39.5 mg and fraction 4.2 19.9 mg. Separation of fraction 4.1 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 4: 1) gave a mixture (50: 50) of (3′R)-stemofolenol (**74**) and (3′S)-stemofolenol (**75**) (**74** and **75**, 26.8 mg). Separation of fraction 4.2 by preparative TLC (CH_2Cl_2-MeOH-aqueous NH_3 = 100: 4 : 1) gave a pure sample of stemofolinoside (**76**, 3.6 mg).

Separation of fraction 5 by preparative TLC (CH_2Cl_2: MeOH: aqueous NH_3 = 100: 5: 1) gave a pure sample of 1′, 2′-didehydrostemofoline-*N*-oxide (**77**) 2.3 mg.

This experiment is summarized in Fig. 3.4.

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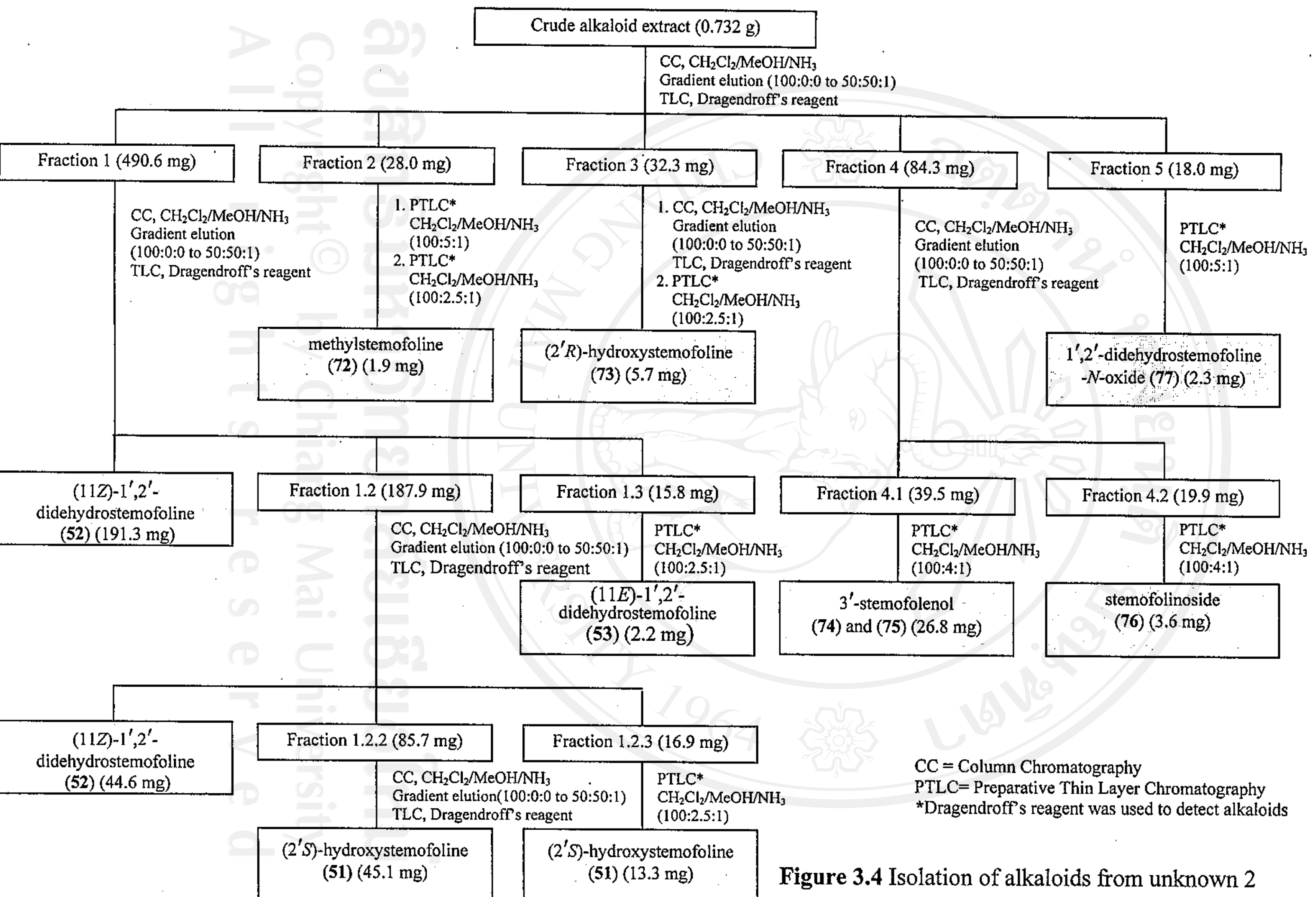

Figure 3.4 Isolation of alkaloids from unknown 2

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3.4 Determination of bioactive compounds

Brine shrimp assays

The toxic activities of the pure alkaloids from *Stemona* spp. on *Artemia salina* Leach (brine shrimp) (Fig. 3.5) were determined. Brine shrimp eggs were hatched in artificial seawater (35 g of salt per litre) and the larvae were available for the experiment 48 hrs after sowing. The brine shrimp were treated with the crude extracts from *S. curtisii* and the unknown *S.* spp., unknown 1 and unknown 2. They were also treated with pure samples of stemocurtisinol **(63)**, oxyprotostemonine **(21)**, stemocurtisine **(58)**, (11*Z*)-1′,2′-didehydrostemofoline **(52)**, (2′*S*)-hydroxystemofoline **(51)** and the mixture of 3′-stemofolenol **(74+75)**. The LC_{50} of these extracts and pure compounds was determined within 24 hrs using the Reed Muench Method (Teng, 1993). All samples were dissolved in 95% ethanol and made into different concentrations at 25, 50, 100, 200 and 400 ppm, with 3 mL in each test tube. Each test solution was prepared in triplicate. Ten brine shrimps were dropped into the test solutions in test tubes. A parallel series of tests were conducted with control solvent and control seawater.

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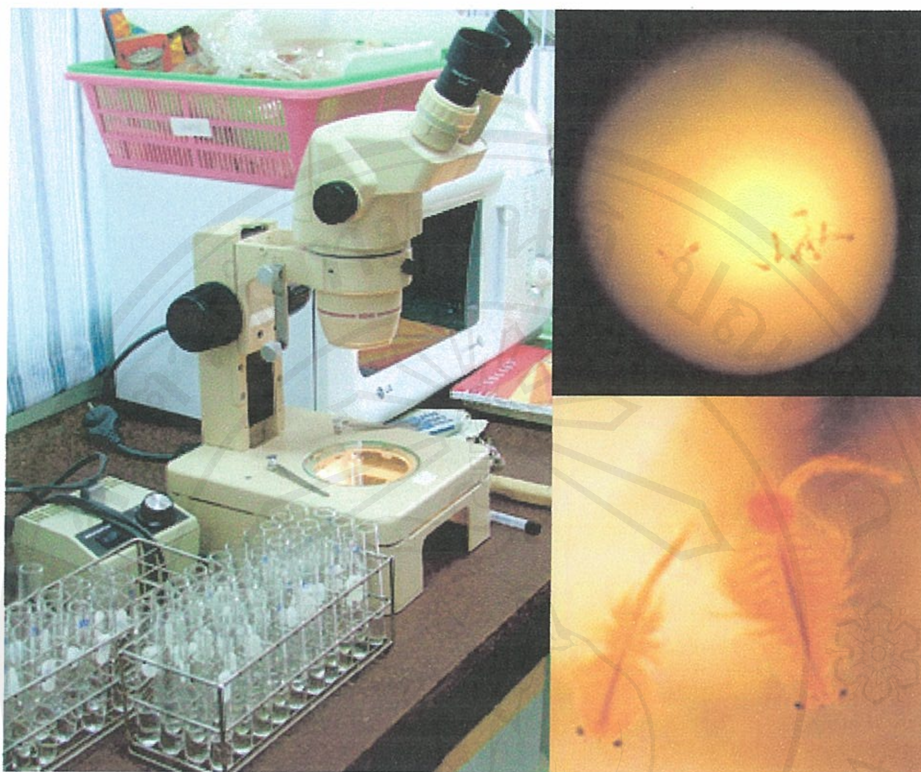

Figure 3.5 Brine shrimp assays

To determine LC_{50}, the number of dead nauplii from each test tube was observed after application for 24 hrs by stereomicroscopy. The ratio of dead/alive nauplii was analysed to determine the LC_{50} by probit analysis.

3.5 Formulation of the bioinsecticide

Different solvents and other supplement substances such as some surfactants and emulsifiers were considered to be used for producing the bioinsecticidal formulation. The percentage of crude extract, solvents and supplement substances for bioinsecticidal formulation were investigated.

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3.6 Efficiency of bioinsecticidal formulation

3.6.1 Leaf disk choice test

To determine the mode of action of bioinsecticidal formulations, leaf disk assays (Fig. 3.6) were carried out with third instar larvae of *Spodoptera littoralis* Boisduval (*S. littoralis*). Leaf disks of a standard size (1.33 cm^2) were cut from *Brassica oleracea* L. CV. (Chinese kale), and then were spreaded on their upper surface with 20 µL of the bioinsecticidal formulation dissolved in acetone at the concentrations of 0.015, 0.030, 0.15 and 0.30%. Control leaf disks were spreaded with the same volume of acetone. After the solvent was evaporated, two treated and two control leaf disks were placed on moist filter paper in a Petri dish. Two third 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.

3.6.2 Topical application method (Fig. 3.7)

Different levels of concentration of the bioinsecticidal formulation i.e. 0.15, 1.5, 3.0 and 15% were topically applied to the dorsal thorax of the instar 3 of *S. littoralis* and compared with a 0.15% formulation of the chemical pesticide (methomyl). All treatments were replicated 3 times, and 10 insects were used for each replication. Control solvent (acetone) was also conducted. The percentage of the mortality was determined 24 hrs after application (Busvine, 1980).

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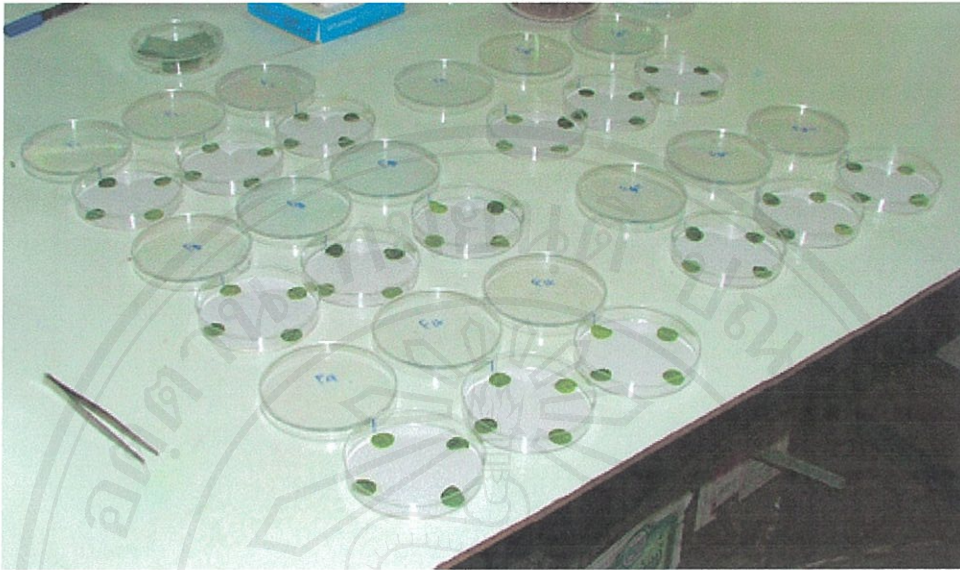

Figure 3.6 Leaf disk choice test

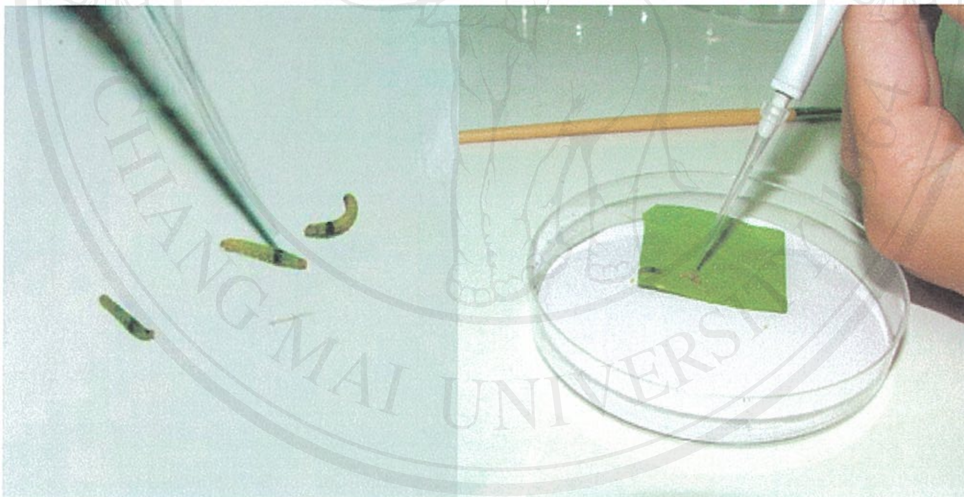

Figure 3.7 Topical application method

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3.7 Formulation of bioinsecticide on a pilot scale

The bioinsecticide production process on a pilot scale was studied and summarized in Fig. 3.8. The chromatographic pattern (fingerprint) of the formulation produced was established for quality control by using HPTLC quantitative analysis densitometry.

Figure 3.8 Bioinsecticide production process on a pilot scale

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3.8 Efficiency of bioinsecticide produced on a pilot scale

The efficiency of the bioinsecticide produced on a pilot scale was tested on an agricultural crop. The insect pest, the quality and quantity of crop production were compared not only with those produced by a commercial chemical pesticide but also by the control treatment.

The effect of the bioinsecticidal formulation on *Brassica oleracea* L. CV. (Chinese kale) in the field

These experiments were done at the field trial of the Department of Agronomy, Chiang Mai University, in the period from 6 June 2006 to 13 July 2006.

1. Randomized complete block design with 3 treatments and 4 replications (75 experimental units/ treatment/ replication) were performed in a 10 x 12 m^2 field which was divided into different plots shown in Fig. 3.9.

2. Bioinsecticidal formulation of plant extract, chemical pesticide (methomyl) and control (water) were applied to Chinese kale from the first week after transplantation until harvesting.

3. To compare the effect of bioinsecticidal formulations with a synthetic pesticide and control treatment, the 5 pest insects, the quality and quantity of 30 sampling plants from each replication were observed and analysed by using ANOVA and LSD for parametric tests and Friedman and Wilcoxon for nonparametric tests. For plant quality data, the ordinal scale of 1, 3, 5, 7 and 9 was used to indicate the quality of very poor, poor, moderate, good and excellent, respectively.

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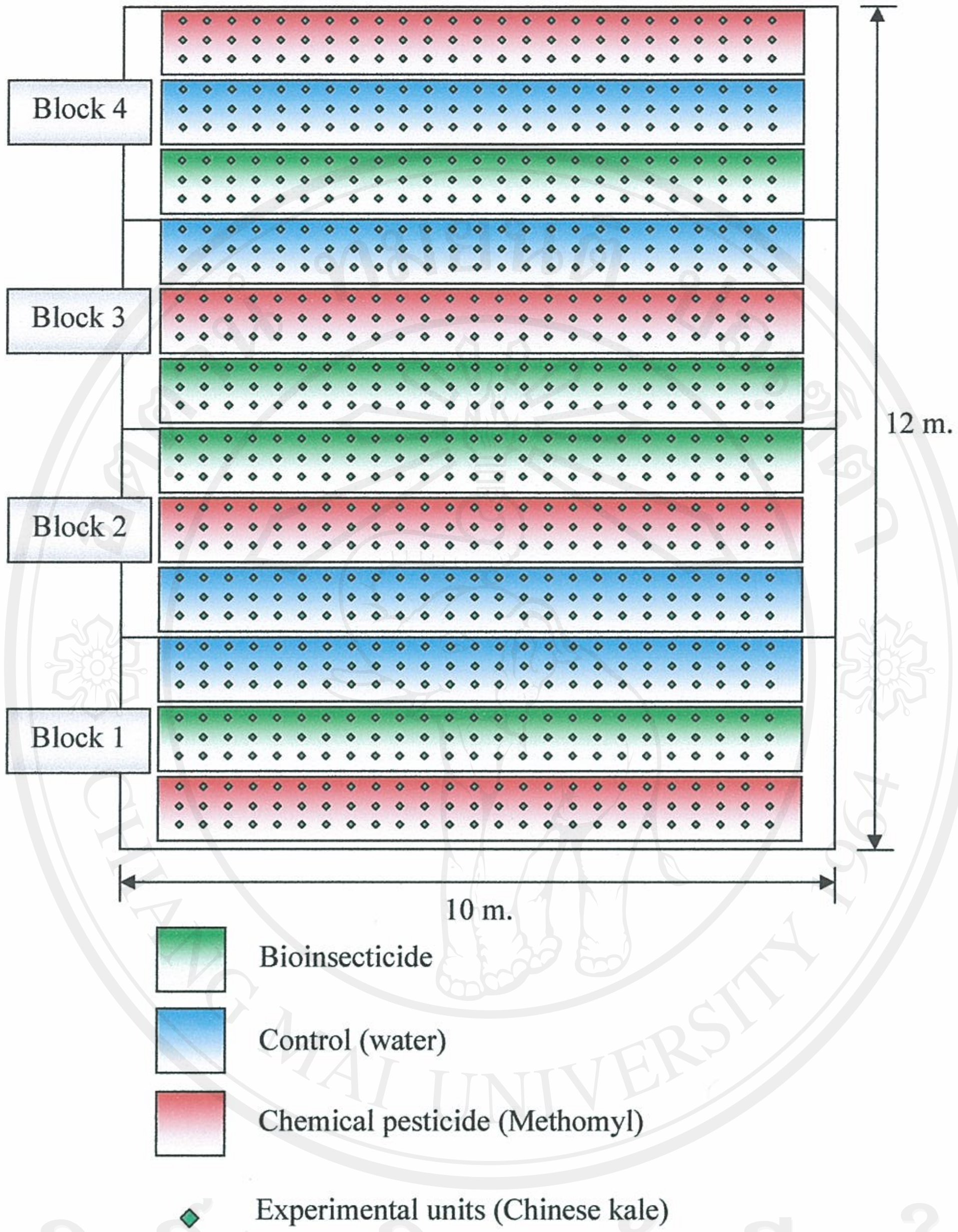

Figure 3.9 Experimental plots

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