# CHAPTER II

# EXPERIMENTAL PROCEDURES

## 2.1 Source of Plant Material

Aerial parts of *Hyptis suaveolens* Poit were collected from Weed Science Sub-division, Botany and Weed Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand in February 2000. This plant was identified by comparison with the herbarium specimen, with voucher number BK 50885 by a botanist of the Princess Sirindhon Plant Herbarium building, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

## 2.2 Instruments and Equipment

Thin Layer Chromatography (TLC) was performed on a aluminum sheets percolated with silica gel (Merch's Kieselgel 60 $PF_{254}$). Column chromatography was conducted on silica gel (No.7731) particle size 0.063-0.200 nm, (No.7734) particle size 0.063-0.200 nm, preparative centrifugal thin layer chromatography was carried out on silica gel 60 (No.7749) and gel filtration chromatography was performed on sephadex LH 20.

The Melting points were determined on a Fisher-Johns melting point apparatus. The IR spectra were recorded on a Fourier Transform-Infrared Spectrophotometer (FT-IR) Nicolet Model impact. Samples were prepared as KBr pellets. The Electron Impact Mass Spectra (EIMS) were obtained on a FISSONS Model TRIO 2000 mass spectrometer. The proton and Carbon-13 Nuclear Magnetic Resonance ($^{1}H$ and $^{13}C$-NMR) experiments were carried out with a JEOL JNM-A 500 FT-NMR spectrometer and a Bruker AC-F 200 FT-NMR spectrometer. The chemical shift in ppm was assigned with reference to the residual proton in deuterated solvent. Solvents for NMR spectra were deuterated chloroform (chloroform-*d*) and deuterated dimethyl sulfoxide (DMSO-*d*).

**2.3 Solvents**

Throughout this work, all organic solvents were commercial grade and were redistilled prior to use. (Hexane, Dichloromethane, Ethanol, Methanol, Ethyl acetate, *n*-Butanol, Chloroform and Acetone).

**2.4 Chemical Test**

**Liberman Burchard's test** (Cook, 1996)

To a solution of the sample to be tested (2-3 mL) in dried chloroform (0.5 mL) was added a few drops of acetic anhydride, followed by one drop of concentrated sulfuric acid. If an unknown was steroid, the color would gradually change from pink to deep green. In case of an unknown was triterpenoid, the color would change to reddish pink.

**2.5 Bioassay on Plant Growth Inhibition** (Camper, 1986)

Plant growth inhibition test was used as a main bioassay to verify the bioactive compound presence. Selected weeds for this investigation were *Mimosa pigra* Linn. and *E. crus-galli* Beauv. Detailed bioassay experiments performed were as follows:

**2.5.1 For crude extract**

Crude extracts of 0.1, 0.5 and 1.0 g dry material were dissolved in an appropriate solvent. Three milliliters were taken and poured into petri dishes (diameter 90 mm) that contained a filter paper disc. Three replicates were performed for each concentration. A control was an equal amount of the solvent used to prepare the tested solution. All petri dishes were dried overnight, followed by the addition of 5.0 mL of distilled water to each plate. Thirty seeds of tested weed were placed in each plate, (3 plates for each concentration). Then, the petri dishes were covered with transparent vinyl film and kept in a growth chamber at 30 °C, 24 h daylight. After seven days, five seedlings were randomly selected to measure the length of root and shoot. Each experiment in was repeated three replications. The inhibitory effect of the substance was calculated with the formula:

$$\% \text{ Inhibition} = [(A - B) / A] \times 100$$

when "A" was the mean root length or height of secondary leaf sheath in the control set.

"B" was the mean root length or height of leaf sheath in the treatment set.

Growth inhibition of 100 % revealed a complete inhibitory effect.

### 2.5.2 For pure compounds

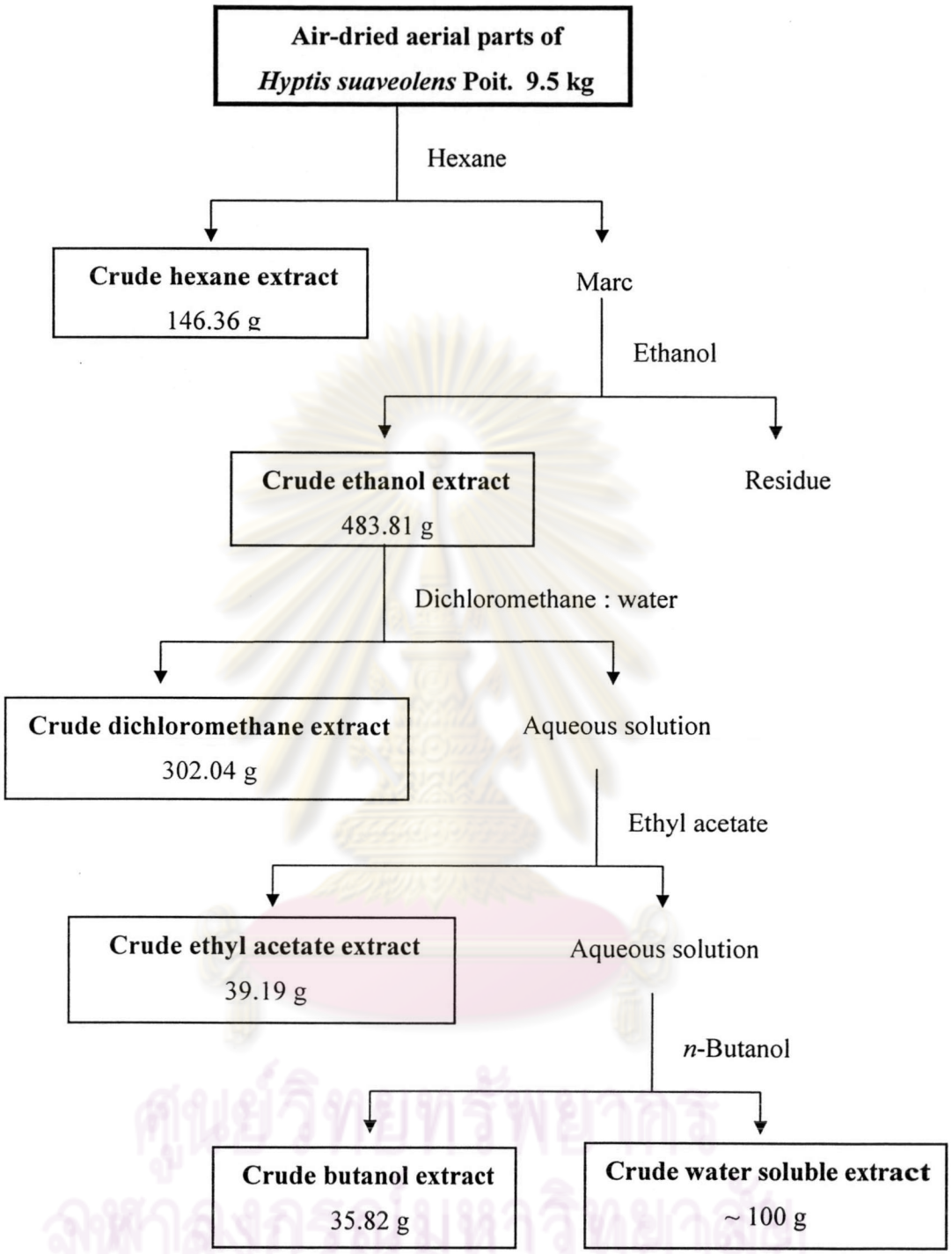
Pure compounds were prepared for the concentrations of 10, 100 and 1000 ppm. Thirty seedlings were transplanted in each petri dish. Each experiment was performed in three replications. Other procedures were conducted in the same manner as 2.5.1.

Most of these tests were conducted at the Weed Science Sub-Division, Botany and Weed Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives.

## 2.6 Extraction and Isolation

### 2.61 Extraction of the aerial parts of *Hyptis suaveolens* Poit.

Plant samples were air-dried (9.5 kg.) and milled. The specimen was soaked in hexane for 7 days at room temperature. The solution was filtered and evaporated by rotary vacuum evaporator to remove the solvent. The hexane soaking procedure was repeated three times to give 146.36 g (yield 1.54% w/w) of hexane extracts. The residue was re-extracted with 95 % ethanol for 5 days at room temperature. The ethanol soaking procedure was repeated three more times or until the solution was colorless. Using rotary vacuum evaporator to almost dryness evaporated the ethanolic extract. The ethanolic crude extract 483.81 g (yield 5.09% w/w) was further partitioned between dichloromethane and water in ratio 1:1 to give a dichloromethane fraction 302.04 g and water-soluble fraction. The water-soluble fraction was extracted with ethyl acetate, then the remaining 39.19 g of ethyl acetate crude extract was further extracted with *n*-butanol to afford the *n*-butanol extract 35.82 g and water extract 100 g. The extraction procedure for preliminary study of plant sample is shown in Scheme 2.1.




**Scheme 2.1** The extraction and fractionation procedure for preliminary study of plant sample.

### 2.6.2 The Separation of Crude Dichloromethane Extract

The dichloromethane extract of the aerial parts (250.23 g) was dissolved in a small amount of dichloromethane, triturated with silica gel 60 and dried under reduced pressure. It was then fractionated by quick column chromatography using a sintered glass filter column of silica gel 60 (No.7734) 400 g. Elution was performed in polarity gradient manner with mixtures of dichloromethane and ethyl acetate as the solvents by increasing ethyl acetate from 5% ethyl acetate in dichloromethane to 100% ethyl acetate (approximately 1000 mL per fraction). Each fraction was examined by TLC using 30% ethyl acetate in dichloromethane as a developing solvent. Fractions with similar chromatographic patterns were combined to yield five fractions, as shown in Table 2.1.

**Table 2.1** The separation of dichloromethane extract by quick column chromatography.

| Fraction code | Number of fraction | Weight (g) |
|---|---|---|
| A | 1-2 | 1.32 |
| B | 3-10 | 12.64 |
| C | 11-26 | 55.43 |
| D | 27-42 | 126.95 |
| E | 43-50 | 11.66 |

#### 2.6.2.1 Isolation of Mixture HS-1

Fraction C of the dichloromethane extract (44.76 g) was further chromatographed by column chromatography using silica gel 60 (No.7734) as adsorbent. Gradient elution from 4 % ethyl acetate in dichloromethane to 100% ethyl acetate was performed (approximately 100 mL per fraction). The elution with 30% methanol in ethyl acetate was carried out to receive the final fraction. Each portion was examined by TLC using 20% ethyl acetate in dichloromethane as a developing solvent. Fractions with similar chromatographic patterns were combined to yield eight fractions, as shown in Table 2.2.

**Table 2.2** The separation of dichloromethane extract by long column chromatography.

| Fraction code | Number of fraction | Weight (g) |
|---|---|---|
| C1 | 1-28 | 0.20 |
| C2 | 29-47 | 0.45 |
| C3 | 48-62 | 0.54 |
| C4 | 63-91 | 0.56 |
| C5 | 92-108 | 0.63 |
| C6 | 109-165 | 5.92 |
| C7 | 166-397 | 21.35 |
| C8 | 398-415 | 0.86 |

Fraction C3 (1.54 g) was further chromatographed by column chromatography using silica gel 60 (No.7734) as adsorbent. Gradient elution from 10% ethyl acetate in dichloromethane to 100% ethyl acetate was performed (approximately 30 mL per fraction). Three fractions (C31-C33) were obtained, as presented in Table 2.3.

**Table 2.3** Fractionation of the fraction C3 by column chromatography.

| Fraction code | Number of fraction | Weight (g) |
|---|---|---|
| C31 | 1-3 | 0.09 |
| C32 | 4-6 | 0.21 |
| C33 | 7-28 | 0.65 |

Fraction C32 (0.21 g) was further chromatographed by gel filtration chromatography using sephadex LH-20 as adsorbent. The elution from 50% methanol in chloroform was performed (approximately 10 mL per fraction). Two fractions (C321-C322) were obtained, as shown in Scheme 2.2.

Fraction C322 was recrystallized by methanol to furnish Mixture **HS-1** as colorless needle crystals (102.1 mg).

**2.6.2.2 Isolation of Compound HS-2**

Fraction C7 (21.35 g) was further chromatographed by column chromatography using silica gel (No. 7749) as an adsorbent. The elution from 20% ethyl acetate in hexane was performed (approximately 30 mL per fraction). Six fractions (C71-C76) were obtained, as shown in Scheme 2.2.

Fraction C75 (7.53 g) was recrystallized by methanol to furnish Compound **HS-2** as powder (3.12 g).

**2.6.2.3 Isolation of Compound HS-3**

After separation of Compound **HS-2**, the remained residue of fraction C75 (4.42 g), was further chromatographed by column chromatography using silica gel (No.7745) as an adsorbent. The elution from 3% methanol in dichloromethane was performed (approximately 30 mL per fraction). Four fractions (C751-C754) were achieved.

Fraction C752 (2.54 g) was then purified by gel filtration chromatography using a column of sephadex LH-20 with 50% methanol in chloroform as the solvent. Each portion was examined by TLC using 10% methanol in chloroform as developing solvent. Isolation fractions C7521-C7523 were accomplished, as revealed in Scheme 2.2.

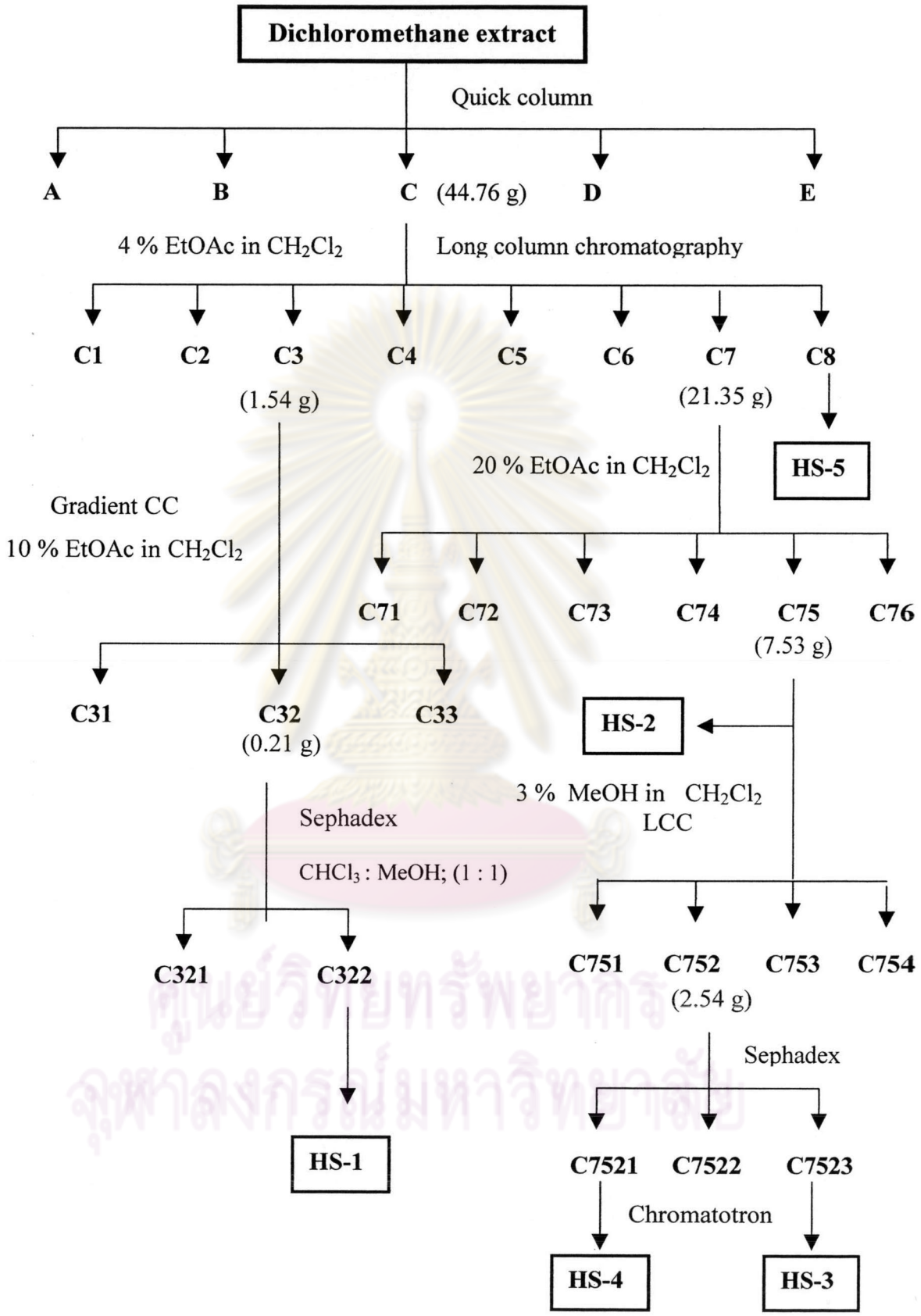
Fraction C7523 exhibited only one red-violet colored fluorescent spot under UV light at 254 nm. Fraction C7523 was further recrystallized in methanol to furnish Compound **HS-3** (25.2 mg) of a yellow crystal.

**2.6.2.4 Isolation of Compound HS-4**

Compound **HS-4** could be isolated from fraction C7521 by preparative centrifugal thin layer chromatography (Chromatotron). Isocratic elution by hexane:ethyl acetate (3 : 1) was performed. The eluents was collected by employing UV light at wavelength of 254 nm to detect bands of UV absorbing compounds on the adsorbent layer, and also analyzed by TLC technique. The fourth of five fractions (C75214) gave compound **HS-4** (14.5 mg).

**2.6.2.5 Isolation of Mixture HS-5**

Mixture **HS-5** (17.1 mg) was obtained as white amorphous from fraction C8 through recrystallization from methanol and dichloromethane.




**Scheme 2.2** Isolation scheme of dichloromethane extract.

**2.6.2.6 Isolation of Mixture HS-6**

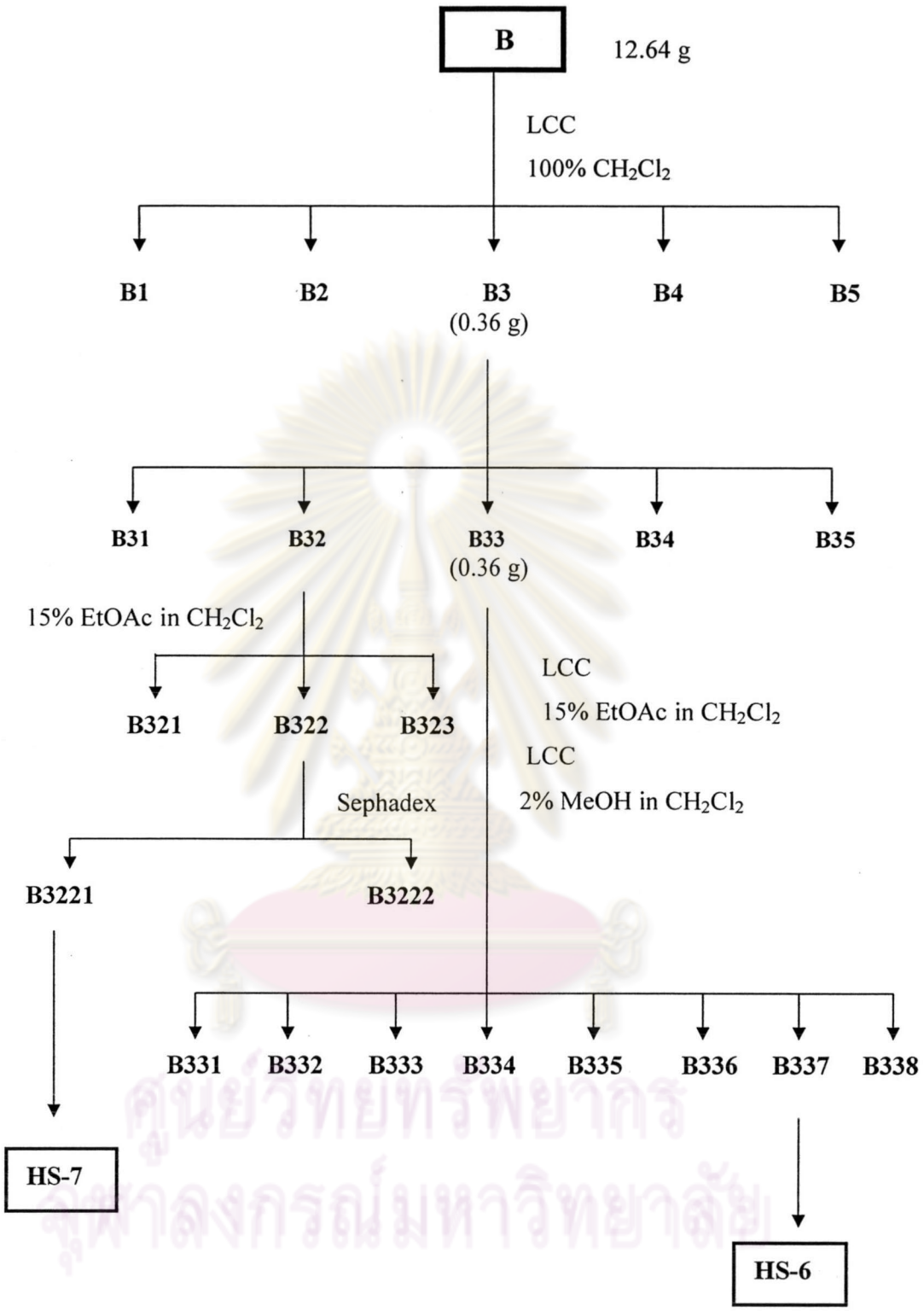
Fraction B (12.64 g) was further chromatographed by column chromatography using silica gel 60 (No.7344) as an adsorbent. The elution from 100% dichloromethane was performed (approximately 30 mL per fraction). Five fractions (B1-B5) were obtained. Fraction B3 (0.36 g) was further fractionated using a column chromatography (see Scheme 2.3). Fraction B33 was then purified by column chromatography using a silica gel 60 column with 2% methanol in dichloromethane as an eluting solvent. Eight fractions (B331-B338) were obtained, as shown in Scheme 2.3. Fraction B337 was recrystallized in methanol to furnish Mixture **HS-6** as crystals, 31.6 mg.

**2.6.2.7 Isolation of Mixture HS-7**

Fraction B32 was further separated by column chromatography using silica gel (No.7734) as an adsorbent. The elution from 5% ethyl acetate in dichloromethane was performed (approximately 20 mL per fraction). Three fractions (B321-B323) were obtained.

Fraction B322 was then purified by gel filtration chromatography using a column of sephadex LH-20 with 50% methanol in chloroform as an eluting solvent. Two fractions (B3221-B3222) were combined according to the TLC examination using 10% ethyl acetate in chloroform as a developing solvent. Isolation fractions B3221-B3222 were obtained, as shown in Scheme 2.3.

Fraction B3221 was recrystallized in methanol to furnish mixture **HS-7** of a white amorphous, 17.5 mg.

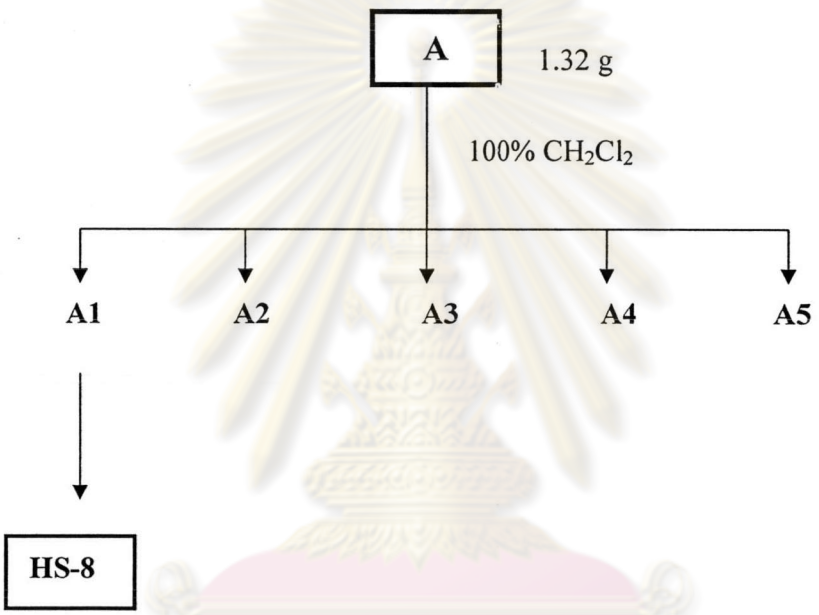



**Scheme 2.3** The isolation procedure of fraction B of dichloromethane extract.

**2.6.2.8 Isolation of Mixture HS-8**

Fraction A (1.32 g) was further chromatographed by column chromatography using silica gel (No.7734) as an adsorbent. The elution from 100% dichloromethane was performed (approximately 30 mL per fraction). Five fractions (A1-A5) were obtained, as shown in Scheme 2.4.

Fraction A1 was recrystallized with methanol to furnish Mixture **HS-8** as white amorphous, (22.7 mg).




**Scheme 2.4** The isolation procedure of fraction A of dichloromethane extract.

### 2.6.3 Separation of Crude Hexane Extract

The hexane extract of the aerial parts (121.48 g) was dissolved in a small amount of hexane, triturated with silica gel 60 and dried under reduced pressure. It was then fractionated by quick column chromatography using a sintered glass filter column of silica gel 60 (No.7734) 400 g. Elution was performed in polarity gradient manner with mixtures of hexane and dichloromethane as the solvents by increasing dichloromethane from 5% dichloromethane in hexane to 100% dichloromethane (approximately 1000 mL per fraction). Each fraction was examined by TLC using 20% dichloromethane in hexane as a developing solvent. Fractions with similar chromatographic pattern were combined to yield six fractions, as shown in Table 2.4.

**Table 2.4** The separation of hexane extract by quick column chromatography.

| Fraction code | Number of fraction | Weight (g) |
|---|---|---|
| F | 1-7 | 51.85 |
| G | 8-14 | 10.61 |
| H | 15-21 | 6.74 |
| I | 22-27 | 5.27 |
| J | 28-40 | 11.67 |
| K | 41-55 | 28.50 |

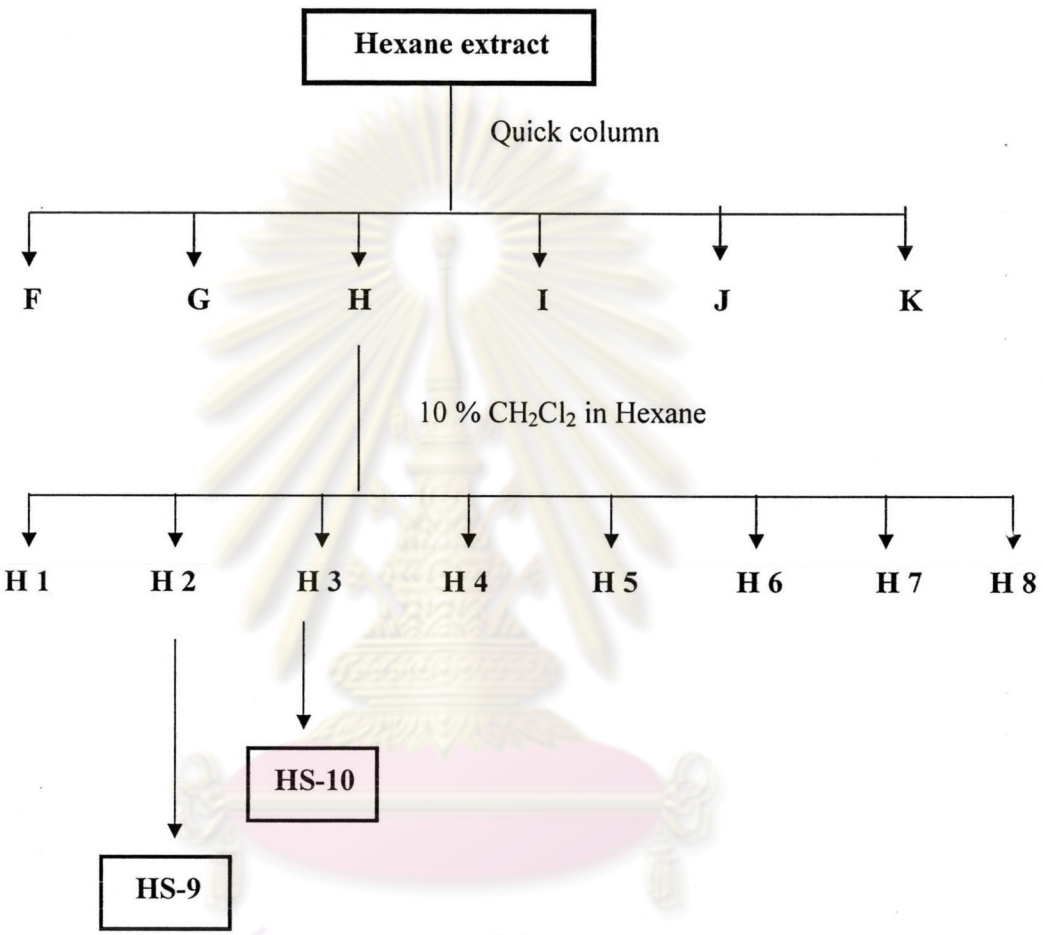
#### 2.6.3.1 Isolation of Compound HS-9

The mother liquor from the crude hexane of the aerial parts of *H. suaveolens*, fraction H (6.74 g) was further chromatographed by column chromatography using silica gel (No.7734) as an adsorbent. The elution from 10% dichloromethane in hexane was performed (approximately 30 mL per fraction). Each portion was examined by TLC using 15% dichloromethane in hexane as a developing solvent. Fractions with similar chromatographic pattern were combined to yield eight fractions, as shown in Scheme 2.5.

Fraction H2 was recrystallized with a mixture of acetone and methanol to furnish Compound **HS-9** (184.9 mg) as white crystals.

**2.6.3.2 Isolation of Compound HS-10**

Fraction H3 was recrystallized with a mixture of acetone and methanol to furnish Compound **HS-10** (21.4 mg) as white crystals, as shown in Scheme 2.5.




**Scheme 2.5** Isolation scheme of hexane extract.

**2.6.3.3 Isolation of Compound HS-11**

Fraction J (11.67 g) was further chromatographed by column chromatography using silica gel 60 (No.7734) as an adsorbent. Gradient elution from 100% dichloromethane to 100% ethyl acetate was performed (approximately 100 mL per fraction). Each fraction was examined by TLC using 30% ethyl acetate in dichloromethane as a developing solvent. Fractions with similar chromatographic pattern were combined to yield eight fractions, as shown in Table 2.5.

**Table 2.5** The separation of fraction J of hexane extract by long column chromatography.

| Fraction code | Number of fraction | Weight (g) |
|---|---|---|
| J1 | 1-10 | 1.95 |
| J2 | 11-20 | 0.67 |
| J3 | 21-28 | 3.69 |
| J4 | 29-55 | 1.77 |
| J5 | 56-87 | 0.65 |
| J6 | 88-95 | 0.46 |
| J7 | 96-130 | 1.20 |

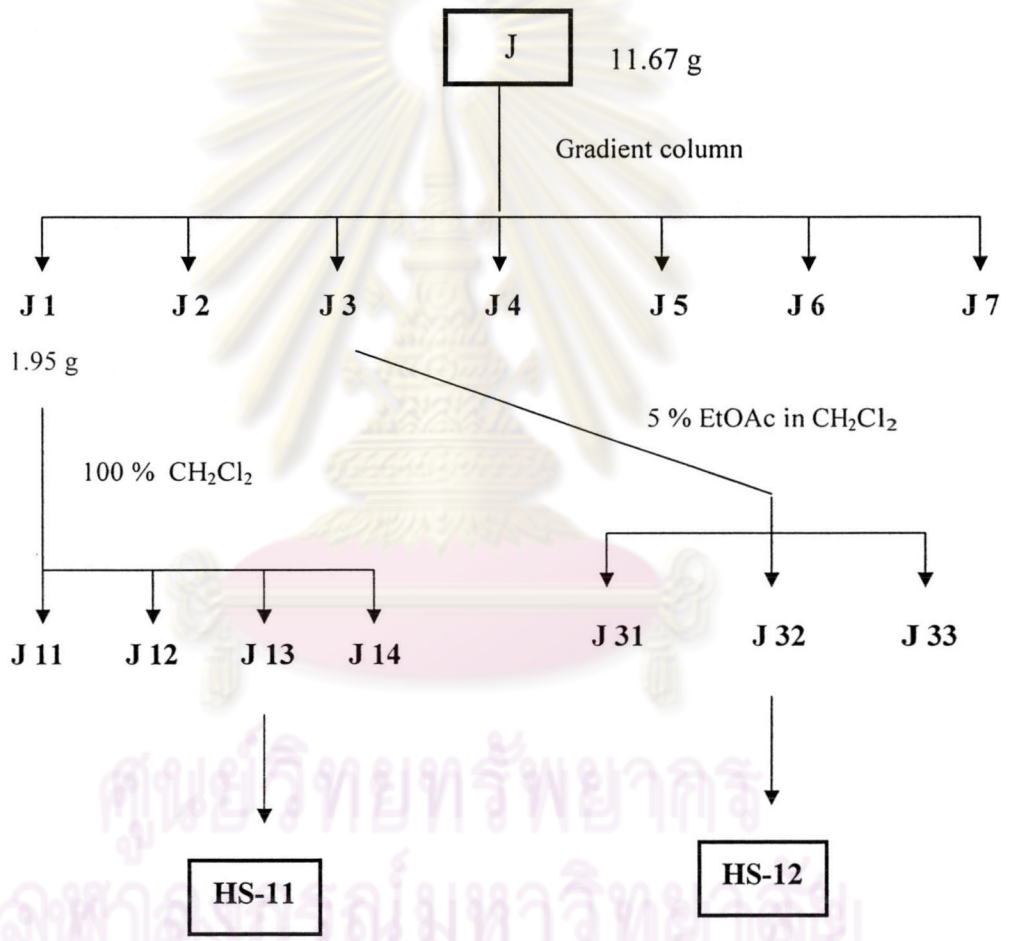
Fraction J1 (1.95 g) was further chromatographed by column chromatography using silica gel 60 (No.7734) as an adsorbent. The elution from 100% dichloromethane was performed (approximately 30 mL per fraction). Four fractions (J11-J14) were accomplished, as shown in Scheme 2.7.

Fraction J13 was recrystallized in methanol to furnish Compound **HS-11**, (42.7 mg) as colorless needle crystals.

**2.6.3.4 Isolation of Compound HS-12**

Fraction J3 (3.69 g) was further chromatographed by column chromatography using silica gel 60 (No.7734) as an adsorbent. The elution from 25% ethyl acetate in dichloromethane was performed (approximately 30 mL per fraction). Three fractions (J31-J33) were obtained, as displayed in Scheme 2.7.

Compound **HS-12** (39.7 mg) was obtained as colorless needles from fraction J32 through recrystallized from methanol.




**Scheme 2.6** The isolation procedure of fraction J of hexane extract.

**2.6.3.5 Isolation of Compound HS-13**

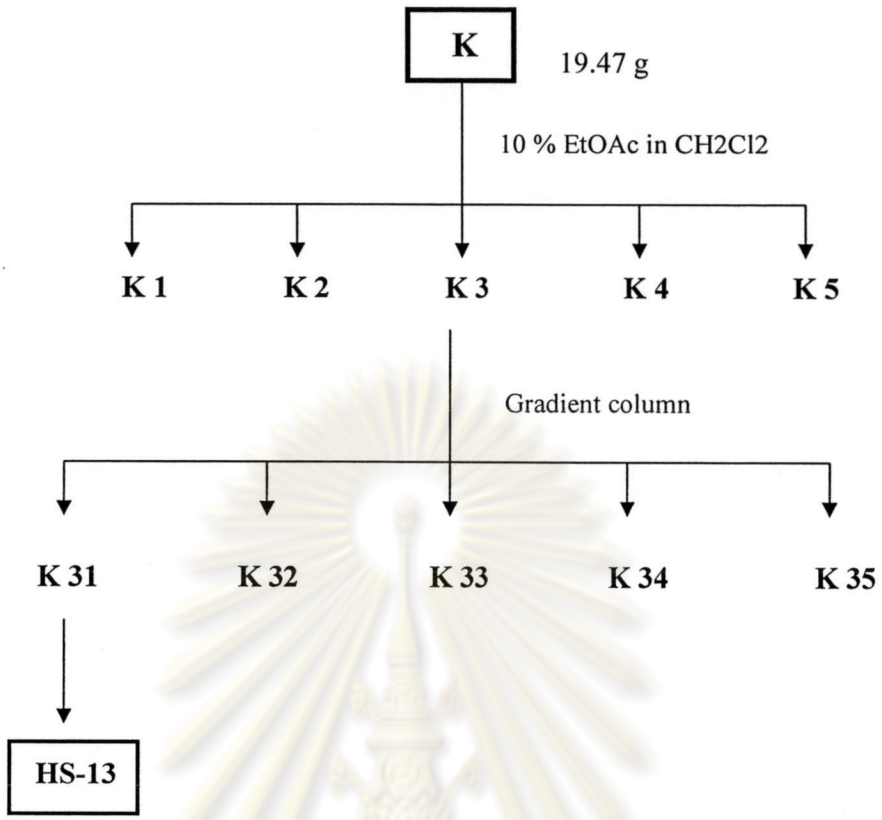
Fraction K (19.47 g) was further chromatographed by column chromatography using silica gel 60 (No. 7734) as an adsorbent. The elution from 10% ethyl acetate in dichloromethane was conducted (approximately 30 mL per fraction). Each portion was examined by TLC using 15% ethyl acetate in dichloromethane as a developing solvent. Fractions with similar chromatographic pattern were combined to yield five fractions.

Fraction K3 (4.31 g) was further chromatographed by column chromatography using silica gel 60 (No.7734) as an adsorbent. Gradient elution from 10% ethyl acetate in dichloromethane to 100% ethyl acetate was performed (approximately 30 mL per fraction). Five fractions (K31-K33) were obtained, as shown in Table 2.6.

**Table 2.6** The separation of fraction K of hexane extract by long column chromatography.

| Fraction code | Number of fraction | Weight (g) |
|---|---|---|
| K31 | 1-15 | 0.31 |
| K32 | 16-26 | 0.92 |
| K33 | 27-41 | 0.74 |
| K34 | 42-64 | 0.19 |
| K35 | 65-80 | 0.87 |

Fraction K31 was recrystallized in methanol to furnish Compound **HS-13** as powder (1.42 g).




**Scheme 2.7** The isolation procedure of fraction K of hexane extract.