Chapter 3
Experimentals

3.1 Plant materials

The fresh rhizomes of *Boesenbergia pandurata* (Roxb.) Schltr. were purchased in Hat-Yai Plaza main fresh market, Hat-Yai, Songkhla, in March, 2002. The voucher specimen (accession number: SKP 2060216) was identified by Associate Professor Tanomjit Supavita and kept at the Herbarium center of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai campus, Songkhla 90112, Thailand.

3.2 Chemicals and reagents

3.2.1 Chemicals and reagents for extraction

95% Ethanol, commercial grade (L.B Science®, Thailand) was pre-distilled before used.

n-Hexane, AR grade (Merck®, Germany)

Dichloromethane, AR grade (Merck®, Germany)

Ethyl acetate, AR grade (Merck®, Germany)

Methanol, AR grade (Merck®, Germany)

Acetone, AR grade (Merck®, Germany)

3.2.2 Chemicals and reagents for antioxidant activity assay

Absolute ethanol, AR grade (Merck®, Germany)

Trichloroacetic acid, AR grade (Riedel-deHaen®, Germany)

Thiobarbituric acid, AR grade (Sigma®, USA)

1,1-diphenyl-2-picrylhydrazyl, AR grade (Fluka®, Switzerland)

Folin & Ciocalteu's phenol reagent (Fluka®, Switzerland)

Linoleic acid, AR grade (Sigma®, USA)

Caffeic acid, AR grade (Sigma®, USA)

Butylated hydroxytoluene, AR grade (Sigma®, USA)

3.2.3 Chemicals and reagents for HPLC assay.

Acetic acid, AR grade (Lab Scan®, Thailand)

Acetonitrile, HPLC grade (J.T. Baker®, USA)

Methanol, AR grade (Merck®, Germany)

Ethanol, AR grade (Merck®, Germany)

Distilled water for HPLC

3.3 Instrumentation

3.3.1 Apparatus used for extraction and structure elucidation

Melting points were determined on Buchi® 520 apparatus. CD spectra were recorded in methanol on a Jasco® J-810 spectropolarimeter using sodium D-line wavelength at 589 nm. UV spectra were taken on Shimadzu® UV 2401 PC spectrophotometer. IR spectra were measured as KBr disc on a Jasco® IR-810 spectrophotometer. MS spectra were obtained on a MAT 95 XL mass

spectrometer, Thermofinnigan®. All NMR spectra were recorded on a Varian Inova® 500 NMR spectrometer using TMS as an internal standard with ^{1}H and ^{13}C nuclei observed and were recorded as chemical shift parameter (δ) value in ppm down field from TMS (δ 0.00). Silica gel was used for normal column chromatography and silica gel 60 F_{254} for TLC (Merck®).

3.3.2 Apparatus used for antioxidant activity assay

Spectronic® genesys5 (Miltonroy, USA) spectrophotometer was used for colorimetry. Hot air oven (Memmert, Germany) was set temperature to 45 ºC. Waterbath (Memmert, Germany) was set to 90ºC.

3.3.3 Apparatus used for HPLC assay

HPLC system consists of multisolvents delivery system model 600E (Waters®), autosampler model 717 (Waters®), photodiode array detector model 996 (Waters®), Millenium software and printer (NEC®). Reverse phase HPLC was performed using Apollo®C18 (250 x 4.6 mm I.D., particle size 5 μm) column with a Apollo®C18 (7.5 x 4.6 mm I.D., particle size 5 μm) guard column.

3.4 Methods

3.4.1 Isolation of chemical constituents from the rhizome of *Boesenbergia pandurata*

3.4.1.1 Extraction

Fresh rhizomes (3.0 kg) of *B. pandurata* were homogenized in ethanol. They were then macerated three times with ethanol (3x2L) at room temperature for three days and filtered. The resulting ethanolic extract was concentrated under reduced pressure to give a dark-brown syrupy residue (30.0 g), which was fractionated as shown in Figure 3-1.

The residue (15.0 g) was preadsorbed on silica gel. It was then eluted with *n*-hexane in a glass column until the *n*-hexane extract gave clear colorless solution. The *n*-hexane extract was combined and evaporated to dryness to give 2.9 g (19.3% yielded) of *n*-hexane fraction. The remaining air dried silica residue was then exhaustively eluted with dichloromethane to give, after evaporating, 1.8 g (12.0% yielded) of dichloromethane fraction. The remaining air dried silica residue was then exhaustively eluted with ethyl acetate to give, after evaporating, 1.7 g (11.6% yielded) of ethyl acetate fraction. Finally, the remaining air dried silica residue was then exhaustively eluted with methanol to give, after evaporating, 1.8 g (12.0% yielded) of methanol fraction. The *n*-hexane, dichloromethane, ethyl acetate and methanol residues were subjected separately to column chromatography for further purification.

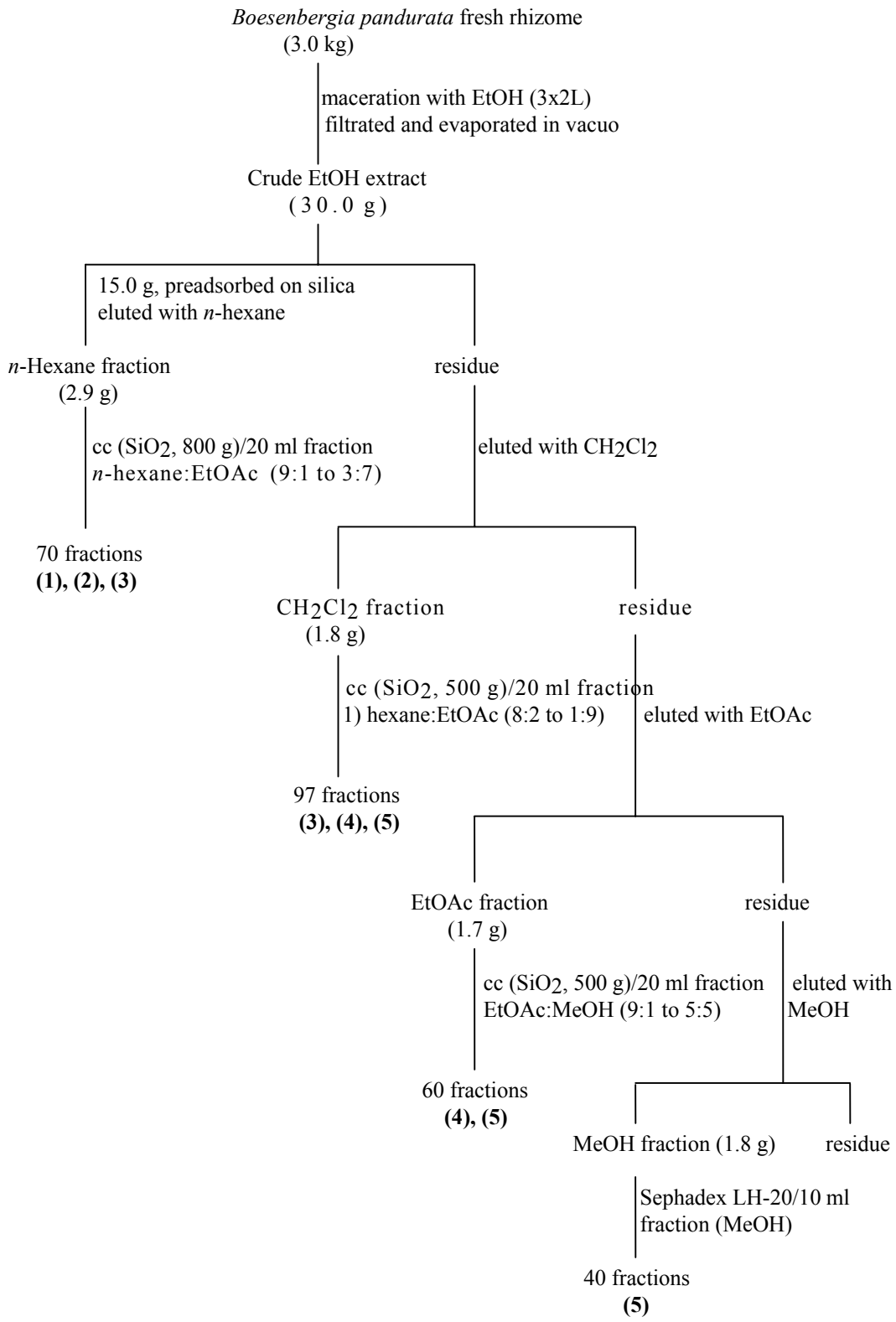

Figure 3-1 Extraction and isolation of *Boesenbergia pandurata* (Rhizome)

3.4.1.2 Isolation and purification

The *n*-hexane fraction (2.8 g) was chromatographed by CC (silica gel, 800 g). Elution was accomplished with a gradient of *n*-hexane:ethyl acetate, 9:1 (200 ml); 7:3 (400 ml); 5:5 (600 ml); 3:7 (200 ml). A total of 70 fractions (fraction size 20 ml) were collected and combined on the basis of the TLC profiles after detection with UV lamp and 50% sulfuric acid in ethanol as spraying reagent. Fractions 4-7, 22-23 and 55-59 resulted in the isolation of compounds **1** , **2** and **3** , respectively.

The dichloromethane fraction (1.5 g) was chromatographed on silica gel (500 g) column with step gradient of *n*-hexane:ethyl acetate, 8:2 (200 ml); 6:4 (740 ml); 3:7 (660 ml) and 1:9 (200 ml). A total of 90 fractions (fraction size 20 ml) were collected and combined on the basis of the TLC profiles after detection with UV lamp and 50% sulfuric acid in ethanol as spraying reagent. Fractions 13-15, 34-36 and 56-57 resulted in the isolation of compounds **3**, **4** and **5,** respectively.

The ethyl acetate fraction (1.5 g) was chromatographed on silica gel (500 g) column with step gradient of ethyl acetate:methanol, 9:1 (200 ml); 7:3 (800 ml) and 5:5 (200 ml). A total of 92 fractions (fraction size 20 ml) were collected and combined on the methanol of the TLC profiles after detection with UV lamp and 50% sulfuric acid in ethanol as spraying reagent. Fractions 6-7 and 44-45 resulted in the isolation of compounds **4** and **5**, respectively**.**

The methanol fraction (1.0 g) was fractionated by chromatographic technique using a column of Sephadex LH-20 with methanol as

eluant. The eluates were collected approximately 10 ml per fraction. A total of 40 fractions combined on the basis of the TLC profiles after detection with UV lamp and 50% sulfuric acid in ethanol as spraying reagent. Fractions 12-13 resulted in the isolation of compound **5**.

3.4.1.3 Characterization of the isolated compounds

Compound 1

Character	needle recrystallized with acetone
m.p.	99-100 °C
UV	λ_{max} 288 (ε 1.9x10^3) (c 0.001, MeOH)
IR	ν_{max} 3400, 1640, 1620 cm^{-1}
MS	HRFAB: [M+H$^+$] m/z 271.0970
	($C_{16}H_{14}O_4$+H$^+$; cal. 271.2927)
CD	$[\theta]_{253}$= -289, $[\theta]_{288}$= -8250,
^{1}H NMR	see table 4-1
^{13}C NMR	see table 4-2

Compound 2

Character	colorless crytal recrystallized with acetone
m.p.	266-270°C
^{1}H NMR	see table 4-3

Compound 3

Character	colorless crytal recrystallized with acetone
m.p.	185-186°C
UV	λ_{max} 289 (ε 2.14x10^4) (c 0.001, MeOH)

IR	ν_{max} 3100, 1625, 1600 cm^{-1}
^{1}H NMR	see table 4-4
^{13}C NMR	see table 4-5

Compound 4

Character	yellow needle recrystallized with acetone
Mp.	199-200°C
UV	λ_{max} 343 (ε 3.82x10^{4}) (c 0.0005, MeOH)
IR	ν_{max} 3170, 1625, 970 cm^{-1}
MS	HRFAB: [M+H^{+}] m/z 271.0970
	($C_{16}H_{14}O_4$+H^{+}; cal. 271.2927)
^{1}H NMR	see table 4-6
^{13}C NMR	see table 4-7

Compound 5

Character	colorless needle recrystallized with acetone
m.p.	219-220°C
UV	λ_{max} 285 (ε 1.74x10^{4}) (c 0.001, MeOH)
IR	ν_{max}3500,3350,3100,1600,1580,1100 cm^{-1}
MS	HRFAB: [M+H^{+}] m/z 271.0970
	($C_{16}H_{14}O_4$+H^{+}; cal. 271.2927)
^{1}H NMR	see table 4-8
^{13}C NMR	see table 4-9

3.4.2 Assay for total phenolic contents

3.4.2.1 Sample preparation and testing procedure

The total phenolic content for each fraction was measured according to the method described by Lee, *et al*. (2000). After the solvent was evaporated from a 0.2 ml aliquot of each extract, the dried residue was mixed with 2 ml of 10% Na_2CO_3 for 2 min at room temperature and treated with 0.2 ml of Folin & Ciocalteu's phenol reagent and adjusted to 5 ml with water. After a 30 min incubation period at room temperature, absorbance at 752 nm was measured.

3.4.2.2 Standard curve of caffeic acid

The caffeic acid was dissolved in 10% Na_2CO_3 (stock solution) to procedure concentrations of 10, 8, 6, 4, and 2 μg/ml, determinated by the described method. The calibration curve fitted to linear regression with the coefficient of determination (r^2) of 0.9991 was demonstrated in Figure 3-2. This calibration curve was used for quantitative determination of total phenolic content.

3.4.2.3 Data analysis

The total phenolic content of each fraction was estimated by comparison with a standard curve generated from analysis of caffeic acid and the percentage of total phenolic content was calculated as total phenolic content in the samples in the unit of w/w.

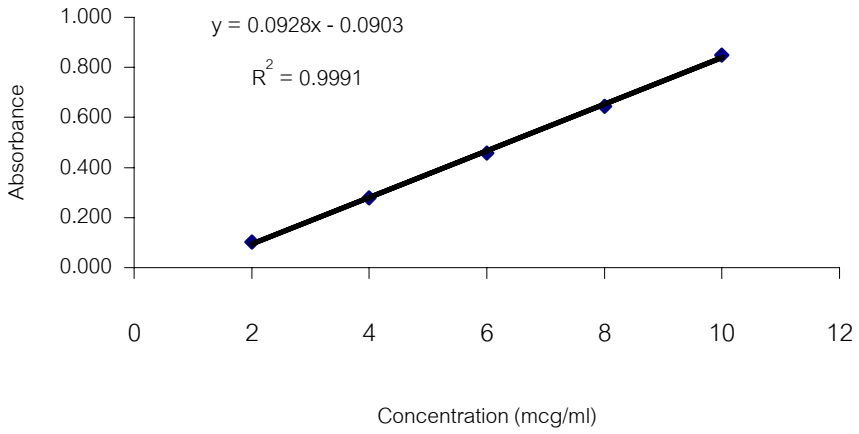

Figure 3-2 A calibration curve of caffeic acid standard solutions. This data was used to calculate total phenolic content in crude extraction

3.4.3 Assays for DPPH radical scavenger

3.4.3.1 Preparation of sample

Each extract was dissolved in absolute ethanol and prepared to several dilutions (two-fold dilution) for free radical scavenging activity test by DPPH assay (Yamaguchi *et al*., 1998).

3.4.3.2 Testing procedure

Each concentration was tested in triplicate. A portion of sample solution (500 μl) was mixed to an equal volume of $6x10^{-5}$ M DPPH solution (in absolute ethanol). After 20 minutes at room temperature, the mixture were measured the UV absorbance at 520 nm. BHT, caffeic acid and curcumin were tested in the same system as positive standards.

3.4.3.3 Data analysis

The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The result was expressed as the percentage inhibition calculating as shown below:

$$\%\text{inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where $A_{control}$ is the absorbance of DPPH solution without sample solution and A_{sample} is the absorbance of DPPH solution with sample solution.

EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations.

3.4.4 Assay for linoleic acid inhibition

3.4.4.1 Preparation of reaction

The antioxidative activity with linoleic acid was measured according to the method of Sekiwa *et al*. (2000). A sample (2.0 ml) of each extract was added to a 2.5% w/v linoleic acid solution consisting of ethanol (8.0 ml), 10 mM phosphate buffer (pH 7.0, 8.0 ml), and purified water (2.0 ml) in a screw-top vial. A solution without the antioxidant sample was used as a control. BHT and caffeic acid were taken as positive controls. Duplicate vials were prepared for each sample. Each vial was incubated at 45 °C for 7 days in the dark and measured antioxidant activity. The malonaldehyde generated from linoleic acid in each sample solution was determined by thiobarbituric acid (TBA) method.

3.4.4.2 Thiobarbituric acid reactive substance (TBARS) assay

Sample solutions after the last day of incubation were subjected to the TBA assay. Two milliliters of the reaction mixture was mixed with 20% trichloroacetic acid (1.0 ml) and 1% thiobarbituric acid in 0.05 N NaOH (1.0 ml) and the mixture was heated in a boiling-water bath for 20 min. After cooling and centrifugation at 4000 rmp for 10 min, the generated TBA reactive substances were measured the absorbance at 532 nm.

3.4.4.3 Data analysis

The %inhibition of lipid peroxidation was assessed by comparing the absorbance of the full reaction mixture with that of extract test reaction mixtures where the substance to be assessed was included. Calculation of %inhibition was as follows:

$$\%\text{inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where $A_{control}$ is the absorbance of TBARS solution without sample solution and A_{sample} is the absorbance of TBARS solution with sample solution.

3.4.5 HPLC Analysis

3.4.5.1 Study of the HPLC operating conditions

3.4.5.1.1 Selection of the mobile phase

Four different mobile phases (Table 3-1) were evaluated to use for separating the pure compounds; pinostrobin, pinocembrin, cardamonin and alpinetin; and the crude extract. The chromatographic separation was carried out using the mixture of 0.5% aqueous acetic acid and acetonitrile as mobile phase at a flow rate of 1.0 ml/min. The 0.5% aqueous acetic acid was freshly prepared for daily use and filtered through 0.45 μm nylon membrane and degassed before using. The injection volume was 20 μl. The peaks were detected at 280 nm. All chromatographic analyses were conducted isocratically at ambient temperature.

Table 3-1 Different ratios of solvent for HPLC mobile phase

Mobile phase	Ratio of 0.5% aq.acetic acid:acetonitrile
M1	40:60
M2	45:55
M3	50:50
M4	55:45

The chromatographic parameters; retention time ($\mathbf{t_R}$), tailing factor ($\mathbf{T_f}$), resolution (**Rs**), capacity factor (**k´**) and relative retention ($\boldsymbol{\alpha}$); of each system were calculated. The most appropriate mobile phase could be selected. Chromatographic peaks were identified by comparison of the retention time with standards of pinostrobin, pinocembrin, cardamonin and alpinetin and by photodiode array UV spectra.

3.4.5.2 Standard curves of Pinostrobin

3.4.5.2.1 Operating condition

Pinostrobin stock solution in ethanol was diluted to produce final working solution 200, 100, 50, 25 and 12.5 μg/ml in triplicate. The solutions were filtered through 0.45 μm Nylon filter and then analyzed by HPLC. The standard curves were obtained by plotting the peak areas versus the concentrations. According to these standard curves, the linear regessions and coefficients of determination were calculated.

3.4.5.2.2 Repeatability (intra-day and Inter-day assays)

The pinostrobin solutions within the concentration ranges of standard curves were analyzed by HPLC for three times/day for three consecutive days. The resulting peak areas were used to calculate standard deviations and relative standard deviations (%RSD).

3.4.6 Application for commercial crudes of *B. pandurata* rhizome

3.4.6.1 Preparation of sample

Ten grams of fine powder *B. pandurata* rhizome were macerated with 90 ml of 95% ethanol for 24 hours. The extract was filtered and collected. The filtrate of samples were examined according to the method described below.

3.4.6.2 DPPH radical scavenging assay

Each filtrate was diluted with absolute ethanol in two-fold dilutions for free radical scavenging activity test by DPPH assay (3.4.3)

3.4.6.3 HPLC assay

1 ml of the filtrate was transferred to a 50 ml volumetric flask and adjusted to volume with 0.5% aqueous acetic acid and acetonitrile (45:55). Prior to use, all samples were filtered through a 0.45 μm Nylon filter. Each sample was extracted in triplicate.

3.4.7 Statistical analysis

Data for total phenolic content, EC_{50} values from DPPH assay and %inhibition values from linoleic acid assay of ethanolic extracts of *B. pandurata* rhizome and data for pinostrobin content (%w/w) from different sources of *B. pandurata* at different local were subjected to analysis of variance and Duncan's multiple range test was used to determine significant difference between means.