CHAPTER III

EXPERIMENTAL

1. Source of Plant Materials

The seeds of *Pachyrrhizus erosus* (L.) Urban were collected from Nakornsawan province, Thailand in April 1996. Voucher herbarium specimen (No. 9601) of the plant was identified and deposited at the Forest Herbarium (BKF), Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

The stem bark of *Millettia leucantha* Kurz var. *leucantha* was collected from the World Biosphere Reserve, Sakaeraj Environmental Research Station, Nakorn-Rachasima province, Thailand during April-May, 1999. Authentication was achieved by comparison with the herbarium specimen (BKF No. 18009) at the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. A voucher specimen has been deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. General Techniques

2.1 Analytical Thin –Layer Chromatography (TLC)

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60G F_{254} (E. Merck) precoated plate
Layer thickness	:	0.2 mm
Distance	:	5.0 cm
Temperature	:	Laboratory temperature (28-35 °C)
Detection	:	1. Ultraviolet light at wavelengths of 254 and 365 nm 2. 10% Sulfuric acid in ethanol and heated at 100 °C for a while.

2.2 Column Chromatography

2.2.1 Vacuum Liquid Column Chromatography

Adsorbent	:	Silica gel 60 (No. 7734) particle size 0.063-0.200 nm (70-230 mesh ASTM) (E. Merck)
Packing method	:	Dry packing
Sample loading	:	The sample was dissolved in a small amount of organic

solvent, mixed with a small quantity of adsorbent, triturated, dried and then placed gently on top of the column.

Detection : Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm.

2.2.2 Flash Column Chromatography

Adsorbent : 1. Silica gel 60 (No. 9385) particle size 0.040-0.063 nm (70-230 mesh ASTM) (E. Merck)

2. Silica gel FL100D (Fuji Silysia Chemical Ltd.)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small volume of eluent and then applied gently on top of the column.

Detection : Fractions were examined in the same way as described in section 2.2.1.

2.2.3 Gel Filtration Chromatography

Gel filter : Sephadex LH 20 (Pharmacia)

Packing method : Gel filter was suspended in the eluent and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.

Sample loading : The sample was dissolved in a small volume of eluent and applied on top of the column.

2.2.4 Gas Chromatography

Instrument model : Fisons GC 8000 series

Column : DB-23 GC column [(50% Cyanopropyl) Methylpolysilane, thickness 0.23 μm, inner diameter 0.32 mm, length 25 m]

Detector type : F.I.D. (Flame Ionization Detector)

Column programming: Isotemp. 1 = 80 °C (0 min)

Rate 1 = 10 °C/min

Isotemp. 2 = 180 °C (15 min)

Rate 2 = 4 °C/min

Isotemp. 3 = 220 °C (7 min)

Injector temperature : 80 °C

Helium carrier gas : 1 ml/min

Split ratio : Splitless

Sample size : 1 μl

2.3 Spectroscopy

2.3.1 Ultraviolet (UV) Absorption Spectra

UV (in methanol) spectra were obtained on a Shimadzu UV-160A UV/vis spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3.2 Infrared (IR) Absorption Spectra

IR spectra (film) were recorded on a JASCO FT/IR-300E spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3.3 Mass Spectra

Electrospray ionization mass spectrum (ESIMS) was measured on a mass spectrometer LCT (LCMS) Micromass (National Center for Genetic Engineering and Biotechnology, BIOTECH (NSTDA, Scienec Park, Pathumthani, Thailand). Electron impact mass spectra (EIMS) were measured on a Fison Micromass VG Platform II mass spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University) and JEOL JMS-AM20 mass spectrometer (Chiba University, Chiba, Japan), and high-resolution fast atom bombardment mass spectrometry (HRFABMS) on a JEOL JMS-HX110 spectrometer (Chiba University, Chiba, Japan).

2.3.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H- and ^{13}C-NMR) spectra

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP400 spectrometer, and ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained on a JEOL JNM-GSX500A spectrometer (Chiba University, Chiba, Japan). ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were also obtained on JEOL JNM-A500 (Scientific and Technological Research Equipment Center, Chulalongkorn University).

Solvents for NMR spectra were deuterated chloroform (chloroform-*d*, $CDCl_3$) and deuterated pyridine (pyridine-d_5, C_5D_5N). Chemical shifts were reported in ppm

scale using the chemical shift of the solvent and internal standard (TMS) as the reference signals.

2.4 Physical Properties

2.4.1 Melting Points

Melting points were measured on a micro melting point hot-stage apparatus (Yanagimoto) (Chiba University, Chiba, Japan).

2.4.2 Optical Rotations

Optical rotations were obtained on a Perkin Elmer Polarimeter 341 (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4.3 Elemental analysis data

Elemental analyses were achieved on Perkin Elmer PE-2400 apparatus (Chiba University, Chiba, Japan).

2.5 Solvents

All organic solvents employed throughout this work were of commercial grade and were redistilled prior to use.

3. Extraction and Isolation

3.1 Extraction and Isolation of Compounds from *Pachyrrhizus erosus*

3.1.1 Extraction

The dried powder seeds (2 kg) were successively macerated with hexane (4x3 L), chloroform ($CHCl_3$, 4x3 L) and then 95% ethanol (5x3 L). The filtrates were pooled and evaporated under reduced pressure at temperature not exceeding 40 °C to afford the corresponding hexane (yellow oils, 480 ml), $CHCl_3$ (43 g) and EtOH (127 g) extracts, respectively.

Hexane extract (yellow oils) was analyzed for fatty acid components [**268-278**] by GC. This result was described in **CHAPTER IV**. Furthermore, $CHCl_3$ and EtOH extracts were isolated by chromatographic method to yield purified compounds, subsequently biological activities evaluation were performed.

3.1.2 Isolation of Compounds from $CHCl_3$ Extract

The $CHCl_3$ extract (43 g) was dissolved in a small amount of $CHCl_3$, triturated with silica gel 60 (No. 7734) and dried under room temperature. It was then fractionated by vacuum liquid column chromatography using sintered glass filter column of silica gel 60 (No. 7734). Elution was completed in a polarity gradient manner with mixture of hexane, $CHCl_3$ and methanol (MeOH). The ratio and volumes of solvents used in this column were summarized in **Table 4**.

The eluates were examined by TLC using dichloromethane (CH_2Cl_2) as developing solvent. Fractions (34 fractions) with similar chromatographic pattern were combined to afford eight fractions as shown in **Table 5**.

Table 4 The ratios and volumes of solvents for vacuum liquid column chromatography of $CHCl_3$ extract of *Pachyrrhizus erosus*

Fraction	Ratio (%) of hexane-$CHCl_3$	Ratio (%) of $CHCl_3$-MeOH	Volume of solvents (ml)
1	100 : 0	-	200
2-4	90 : 10	-	600
5-8	80 : 20	-	800
9-12	70 : 30	-	800
13-18	60 : 40	-	1200
19-22	50 : 50	-	800
23-26	40 : 60	-	800
27-30	30 : 70	-	800
31-33	20 : 80	-	600
34-36	10 : 90	-	600
37-39	0 : 100	-	600
40-41	-	95 : 5	600
42-43	-	90 : 10	800
44-46	-	85 : 15	400
47-48	-	80 : 20	800
49-50	-	70 : 30	400
51-52	-	60 : 40	400
53-54	-	50 : 50	400
55-56	-	0 : 100	400

Table 5 Combination of fractions from vacuum liquid column chromatography of $CHCl_3$ extract of *Pachyrrhizus erosus*

Fraction	Combined fractions	Weight (g)	Volume of solvents (ml)
PEC1	1-4	0.1252	800
PEC2	5-10	0.2413	1200
PEC3	11-21	3.5909	2200
PEC4	22-29	3.3234	1600
PEC5	30-36	3.4228	1400
PEC6	37-44	2.0235	1600
PEC7	46-50	0.7852	1200
PEC8	51-56	6.1237	1200

3.1.2.1 Isolation of Compound 4 ((+)-Dolineone)

The residue of PEC3 was fractionated on silica gel column chromatography using $CHCl_3$ as eluent. The eluates were examined by TLC using CH_2Cl_2 as developing solvent. Fractions with similar chromatographic pattern were combined to yield 6 fractions (PEC3-1 to PEC3-6). Fraction PEC3-1 (107 mg) was further fractionated on a silica gel (No. 9385) column and eluted with mixture of hexane and $CHCl_3$ (2:3) to afford white powder of compound **4** (28.1 mg, $1.4x10^{-3}$ % based on dried weight of seeds, R_f 0.68, silica gel, CH_2Cl_2). This compound was later identified as (+)-dolineone [**4**].

3.1.2.2 Isolation of Compound 18 ((+)-Pachyrrhizone)

Fraction PEC3-5 (159.6 mg) was recrystallized in $CHCl_3$-MeOH to yield RPEC3-5 (129.5 mg). Fraction RPEC3-5 combined with fraction PEC3-6-1 (15.5 mg), that fractionated from PEC3-6 (66.7 mg) by column chromatography over silica gel (No. 9385) using $CHCl_3$ as eluent, was recrystallized in $CHCl_3$-MeOH to give 72.4 mg of compound **18**. Furthermore, this compound (161.9 mg) was also obtained from recrystallization of fraction PEC5-3 (873.5 mg) fractionated from fraction PEC5 (2.9 g) by silica gel column chromatography (gradient $CHCl_3$-MeOH system). Thus, compound **18** was separated as white needles (234.3 mg, $11.7x10^{-3}$ % based on dried weight of seeds, R_f 0.57, silica gel, $CHCl_3$). This compound was eventually identified as (+)-pachyrrhizone [**18**].

3.1.2.3 Isolation of Compound 15 (Neotenone)

Fraction PEC4 (1.0 g) was separated on a silica gel (No. 9385) column using isocratic elution with $CHCl_3$. Fractions of 100 ml each were collected. Combination of fractions showing similar chromatographic patterns were completed to give 6 fractions (PEC4-1 to PEC4-6). Fraction PEC4-5 (209.7 mg) was rechromatographed on silica gel (No. 9385) column eluted with $CHCl_3$ to give 4 fractions (PEC4-5-1 to PEC4-5-4). Fraction PEC4-5-2 (96.9 mg) combined with fraction PEC3-4 (94.5 mg) was then fractionated on a column using silica gel (No. 9385) as the adsorbent. Isocratic elution was completed by 30 % hexane in benzene to yield pale yellow powder of compound **15** (82.4 mg, $4.1x10^{-3}$ % based on dried weight of seeds, R_f 0.52, silica gel, CH_2Cl_2). This compound was identified as neotenone [**15**].

3.1.2.4 Isolation of Compounds 17 (Pachyrrhizin) and 8 ((+)-12a-Hydroxydolineone)

Compounds **17** and **8** were obtained from the separation of combined fraction between mother liquor of PEC5-3 (701.6 mg) and PEC5-4 (141.8 mg) on a column using silica gel (No. 9385) as the adsorbent. Elution was performed with benzene to give greenish-yellow needles of compound **17** (107.1 mg, $5.4x10^{-3}$ % based on dried weight of seeds, R_f 0.45, silica gel, CH_2Cl_2) and colourless needles of compound **8** (61.8mg, $3.1x10^{-3}$ % based on dried weight of seeds, R_f 0.38, silica gel, $CHCl_3$). They were later identified as pachyrrhizin [**17**] and (+)-12a-hydroxydolineone [**8**].

3.1.3 Isolation of Compounds from EtOH Extract

The alcoholic extract (27.0g) was separated by vacuum liquid column chromatography (silica gel No. 9385). Elution was performed in a polarity gradient manner of mixtures of hexane-$CHCl_3$ (100:0 to 0:100) and then mixtures of $CHCl_3$-MeOH (100:0 to 0:100) as followed (**Table 6**). Fractions with homogeneous by TLC (silica gel, CH_2Cl_2) were combined to yield 9 fractions (PEA1 to PEA9) as followed (**Table 7**).

Table 6 The ratios and volumes of solvents for vacuum liquid column chromatography of EtOH extract of *Pachyrrhizus erosus*

Fraction	Ratio (%) of hexane-$CHCl_3$	Ratio (%) of $CHCl_3$-MeOH	Volume of solvents (ml)
1	100 : 0	-	200
2	90 : 10	-	200
3	80 : 20	-	200
4-5	70 : 30	-	400
6	60 : 40	-	200
7	50 : 50	-	200
8-9	40 : 60	-	400
10-13	30 : 70	-	800
14-16	20 : 80	-	600
17-20	10 : 90	-	800
21-25	0 : 100	-	1000
26	-	95 : 5	200
27-28	-	90 : 10	400
29-30	-	85 : 15	400
31-32	-	80 : 20	400
33-34	-	70 : 30	400
35-36	-	50 : 50	400
37-38	-	0 : 100	400

Table 7 Combination of fractions from vacuum liquid column chromatography of EtOH extract of *Pachyrrhizus erosus*

Fraction	Combined fractions	Weight (g)	Volume of solvents (ml)
PEA1	1-5	0.2280	1000
PEA2	6-9	0.2987	8000
PEA3	10-13	0.5691	800
PEA4	14-17	1.4832	800
PEA5	18-22	4.4304	1000
PEA6	23-27	3.1927	1000
PEA7	28-30	2.2753	600
PEA8	31-34	2.4635	600
PEA9	35-38	4.1160	800

3.1.3.1 Isolation of Compounds 2 (Dehydroneotenone) and 11 ((+)-12a-Hydroxypachyrrhizone)

Fraction PEA5 (2.0 g) was re-chromatographed on silica gel (FL100D) column. Isocratic elution (20 % hexane in $CHCl_3$) was performed to give 5 fractions (PEA5-1 to PEA5-5). Fraction PEA5-5 (331.0 mg) was further separated by column chromatography (silica gel FL100D, 40-30 % hexane in $CHCl_3$) to yield 4 fractions (PEA5-5-1 to PEA5-5-4). Fraction PEA5-5-2 (162.0 mg) was then purified on a silica gel FL100D column eluting with mixture of hexane-$CHCl_3$-Et_2O (10:40:1) to afford compound **2** as white needles (15.0 mg, $0.8x10^{-3}$ % based on dried weight of seeds, R_f 0.60, silica gel, $CHCl_3$-Et_2O 5:1) and compound **11** (pale yellow crystals, 35.0 mg, $1.8x10^{-3}$ % based on dried weight of seeds, R_f 0.49, silica gel, $CHCl_3$-Et_2O 5:1). They were eventually identified as dehydroneotenone [**2**] and (+)-12a-hydroxypachyrrhizone [**11**].

3.1.3.2 Isolation of Compound 12 ((-)-12a-Hydroxyrotenone)

Fraction PEA5-5-3 (88.0 mg) was repeated column chromatography (silica gel FL100D, 40 % EtOAc in hexane) to afford compound **12** (17.0 mg, $0.9x10^{-3}$ % based on dried weight of seeds, R_f 0.44, silica gel, $CHCl_3$-Et_2O 5:1). It was identified as (-)-12a-hydroxyrotenone [**12**].

3.2 Extraction and Isolation Compounds from *Millettia leucantha* var. *leucantha*

3.2.1 Extraction

The dried pulverized stem bark (2.7 kg) was macerated twice with 95 % EtOH (3 and 2 L) for 3-day period and filtered. The combined filtrates were pooled and evaporated *in vacuo* until dryness to yield a syrupy mass (132.6 g).

3.2.2 Isolation of Compounds from *Millettia leucantha* var. *leucantha*

The alcoholic extract (60.0 g) was fractionated by vacuum liquid column chromatography using a sintered glass filter column of silica gel (No. 7734). Successive gradient elution with mixtures of hexane-$CHCl_3$ and $CHCl_3$-MeOH were employed. The ratios and volumes of solvents used in this column were shown in **Table 8**. Collected fractions with similar chromatographic pattern were combined to afford 12 fractions as shown in **Table 9**.

Table 8 The ratios and volumes of solvents for vacuum liquid column chromatography of EtOH extract of *Millettia leucantha*

Fraction	Ratio (%) of hexane-$CHCl_3$	Ratio (%) of $CHCl_3$-MeOH	Volume of solvents (ml)
1	100 : 0	-	300
2	90 : 10	-	300
3	80 : 20	-	300
4-5	70 : 30	-	600
6-7	60 : 40	-	600
8-9	50 : 50	-	600
10-11	40 : 60	-	600
12-14	30 : 70	-	900
15-17	20 : 80	-	900
18-20	10 : 90	-	900
21-23	0 : 100	-	900
24-28	-	99 : 1	1500
29-30	-	97 : 3	600
31-32	-	95 : 5	600
33-34	-	90 : 10	600
35-36	-	85 : 15	600
37-40	-	80 : 20	1200
41-43	-	70 : 30	900
44-45	-	50 : 50	600
46-47	-	0 : 100	600

Table 9 Combination of fractions from vacuum liquid column chromatography of EtOH extract of *Millettia leucantha*

Fraction	Combined fractions	Weight (g)	Volume of solvents (ml)
ML1	1-5	0.4394	1500
ML2	6-12	1.1801	2100
ML3	13-16	0.6805	1200
ML4	17-20	1.1202	1200
ML5	21-23	1.8339	900
ML6	24-29	8.6343	1800
ML7	30-32	1.0516	900
ML8	33-35	2.4110	900
ML9	36-38	7.3197	900
ML10	39-40	5.2851	600
ML11	41-43	7.5667	900
ML12	44-47	7.1285	1200

3.2.2.1 Isolation of Compound 279 (2',4'-Dimethoxy -3,4-methylenedioxy chalcone)

Fraction ML4 (1.1 g) was further purified by repeated column chromatography using 25 % EtOAc in hexane on silica gel FL100D resulting the fractionation of ML4-1 to ML4-6. Isolation of fraction ML4-5 (289.0 mg) was continued by column chromatography (silica gel FL100D, hexane-EtOAc 8:1) to yield compound **279** (46.0 mg, $5.6x10^{-3}$ % based on dried weight of stem bark, R_f 0.60, silica gel, hexane-Et_2O 2:1). It was identified as (2',4'-dimethoxy-3,4-methylenedioxychalcone) [**279**], which was isolated from natural source for the first time.

3.2.2.2 Isolation of Compound 280 (2'-Hydroxy-3,4,4',6'-tetramethoxy chalcone)

Fraction ML4-6 (88.0 mg) was subjected to column chromatography using silica gel FL100D as adsorbent. Elution with benzene gave 21.0 mg of fraction ML4-6-1 that was rechromatographed on silica gel FL100D, eluted with mixture of hexane-EtOAc (6:1) to afford compound **280** as orange needles (14.0 mg,$1.2x10^{-3}$ % based on dried weight of stem bark, R_f 0.44, silica gel, CH_2Cl_2).

It was identified as 2'-hydroxy-3,4,4',6'-tetramethoxychalcone [**280**].

3.2.2.3 Isolation of Compound 115 (Karanjin)

Fraction ML5 (1.8 g) was separated by column chromatography (silica gel FL100D) using mixture of hexane-EtOAc (4:1) as eluent. After combination of collected fractions according to chromatographic pattern (hexane-EtOAc 2:1), 9 fractions including ML5-1 to ML5-9 were obtained. Fraction ML5-3 (205.1 mg) was further fractionated by repeated column chromatography (silica gel FL100D, hexane-$CHCl_3$ 1:2) to give fractions ML5-3-1 and ML5-3-2. Compound **115,** a colourless plate (4.1 mg, $0.3x10^{-3}$ % based on dried weight of stem bark, R_f 0.50, silica gel, benzene-EtOAc 9:1), was finally obtained from the separation of fraction ML5-3-1 (166.3 mg) by silica gel column chromatography (hexane-EtOAc 7:1). This compound was identified as karanjin [**115**].

3.2.2.4 Isolation of Compounds 281 (2',4',6'-Trimethoxy-3,4-methylenedioxydihydrochalcone), 103 (Lanceolatin B) and 102 (Dihydromilletenone methyl ether)

Fraction ML5-5 (279.1 mg) was subjected to silica gel (FL100D) column chromatography and eluted with equal volumns of hexane and $CHCl_3$, which resulted in the collecting of fractions ML5-5-1 to ML5-5-6. Fraction ML5-5-4 (148.0 mg) was further separated using column chromatography (silica gel FL100D, benzene) to yield compound **281** as pale yellow oil (29.3 mg, $2.4x10^{-3}$ % based on dried weight of stem bark, R_f 0.33, silica gel, hexane-EtOAc 2:1), compound **103** as colourless needles (10.7 mg, $0.9x10^{-3}$ % based on dried weight of stem bark, R_f 0.44, silica gel, benzene-EtOAc 9:1) and compound **102** as colourless oil (21.2 mg, $1.7x10^{-3}$ % based on dried weight of stem bark, R_f 0.60, silica gel, benzene-EtOAc 9:1). They were identified as 2',4',6'-trimethoxy-3,4-methylenedioxydihydrochalcone [**281**], lanceolatin B [**103**] and dihydromilletenone methyl ether [**102**], respectively. Additionally, compound **281** was reported here for the first time.

3.2.2.5 Isolation of Compounds 282 (2,4,6,β-Tetramethoxy-3',4'-methylenedioxychalcone), 284 (Desmethoxykanugin) and 68 (3',4'-Methylenedioxy-7-methoxyflavone)

Recrystallization of fraction ML6 (8.6 g) with 1% MeOH in $CHCl_3$ acquired pale yellow needles of compound **282** (347.5 mg, $28.5x10^{-3}$ % based on dried weight of stem bark, R_f 0.59, silica gel, hexane-EtOAc 1:1). It was reported for the first time and eventually identified as 2,4,6,β-tetramethoxy-3',4'-methylenedioxychalcone [**282**].

The mother liquor from ML6 was further separated by vacuum liquid column chromatography using hexane-$CHCl_3$-MeOH system with increasing polarity as eluent. Six combined fractions (ML6-1 to ML6-6) were collected from this column. Fraction ML6-1 (1.9 g) was continuously separated on silica gel (FL100D) column, with isocratic elution of hexane-EtOAc (7:1) until giving 17 fractions (ML6-1-1 to ML6-1-17). Fraction ML6-1-11 (333.1 mg) was repeatedly chromatographed on a silica gel (hexane-$CHCl_3$ 1:3) to afford white solid of compound **284** (92.1 mg, $7.5x10^{-3}$ % based on dried weight of stem bark, R_f 0.27, silica gel, hexane-EtOAc 2:1). This compound was successfully identified as desmethoxykanugin [**284**].

Furthermore, the separation of ML6-3 (461.4 mg) was also proceeded by silica gel FL100D column chromatography using $CHCl_3$ as eluent to give 4 combined fractions (ML6-3-1 to ML6-3-4). Fraction ML6-3-3 (304.0 mg) was selected for further purification using column chromatography (silica gel FL100D, hexane-$CHCl_3$ 1:3). From this column, compound **68** was obtained as white needles (6.6 mg, 0.5x 10^{-3} % based on dried weight of stem bark, R_f 0.55, silica gel, hexane-EtOAc 1:1). It was identified as 3',4'-methylenedioxy-7-methoxyflavone [**68**].

3.2.2.6 Isolation of Compound 285 (2',4',6'-Trimethoxy-3,4-methylene dioxychalcone)

Fraction ML7 (1.1 g) was subjected to gel filtration chromatography (sephadex LH 20 , MeOH). The eluates were collected and combined according to their TLC chromatographic patterns (CH_2Cl_2) to give 7 fractions (ML7-1 to ML7-7). Fraction ML7-4 (562.2 mg) was further purified on silica gel (No. 9385, hexane-EtOAc 4:1). After collecting and combinating the eluates with similar chromatographic patterns, eight fractions (ML7-4-1 to ML7-4-8) were obtained. Fraction ML7-4-7 (364.1 mg) was recrystallized in hexane-$CHCl_3$ to give pale yellow needles of compound **285** (142.2 mg, $11.7x10^{-3}$ % based on dried weight of stem bark, R_f 0.66, silica gel, hexane-EtOAc 1:1). This compound was identified as 2',4',6'-trimethoxy-3,4-methylenedioxychalcone [**285**]. Here is the first time to isolate this compound from natural source.

3.2.2.7 Isolation of Compound 287 (3',4'-Methylenedioxy-5,7-dimethoxy flavone)

Fraction ML8 (2.4 g) was fractionated by silica gel (FL100D) column chromatography using gradient elution of $CHCl_3$-MeOH. The eluates were collected and combined according to similarity of chromatographic patterns (hexane-EtOAc

2:1) to obtained 8 fractions (ML8-1 to ML8-8). The purification of fraction ML8-3 (55.4 mg) was further performed by column chromatography (silica gel FL100D, benzene-EtOAc 9:1) to give 4 fractions (ML8-3-1 to ML8-3-4). Colourless needles of compound **287** (14.1 mg, $1.2x10^{-3}$ % based on dried weight of stem bark, R_f 0.29, silica gel, hexane-EtOAc 2:1) were obtained from recrystallization of fraction ML8-3-4 (26.2 mg) from $CHCl_3$-MeOH. This compound was identified as 3',4'-methylenedioxy-5,7-dimethoxyflavone [**287**].

4. Physical and Spectral Data of Isolated Compounds

4.1 Compound 4 ((+)-Dolineone)

Compound **4** was obtained as white crystals, soluble in $CHCl_3$ (28.1 mg, $1.4x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 336 (M^+, 93), 307 (4), 177 (100), 163 (35), 162 (36), 160 (38), 132 (55), 90 (98)

$[\alpha]^{27}_D$: +192° (c 0.35, $CHCl_3$)

UV : λ_{max} nm (log ε), in methanol; 209 (4.72), 236 (4.59), 275 (3.87), 302 (3.73), 341 (3.53)

IR : ν_{max} cm^{-1}, Film; 1681, 1624, 1460, 1154

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 10**, **Figure 16**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 10**, **Figure 17**

4.2 Compound 15 (Neotenone)

Compound **15** was obtained as pale yellow crystals, soluble in $CHCl_3$ (82.4 mg, $4.1x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 338 (M^+, 21), 178 (100), 160 (46), 133 (82), 105 (26), 77 (47), 53 (18)

UV : λ_{max} nm (log ε), in methanol; 207 (4.78), 235 (4.42), 273 (3.62), 299 (3.60), 336 (3.32)

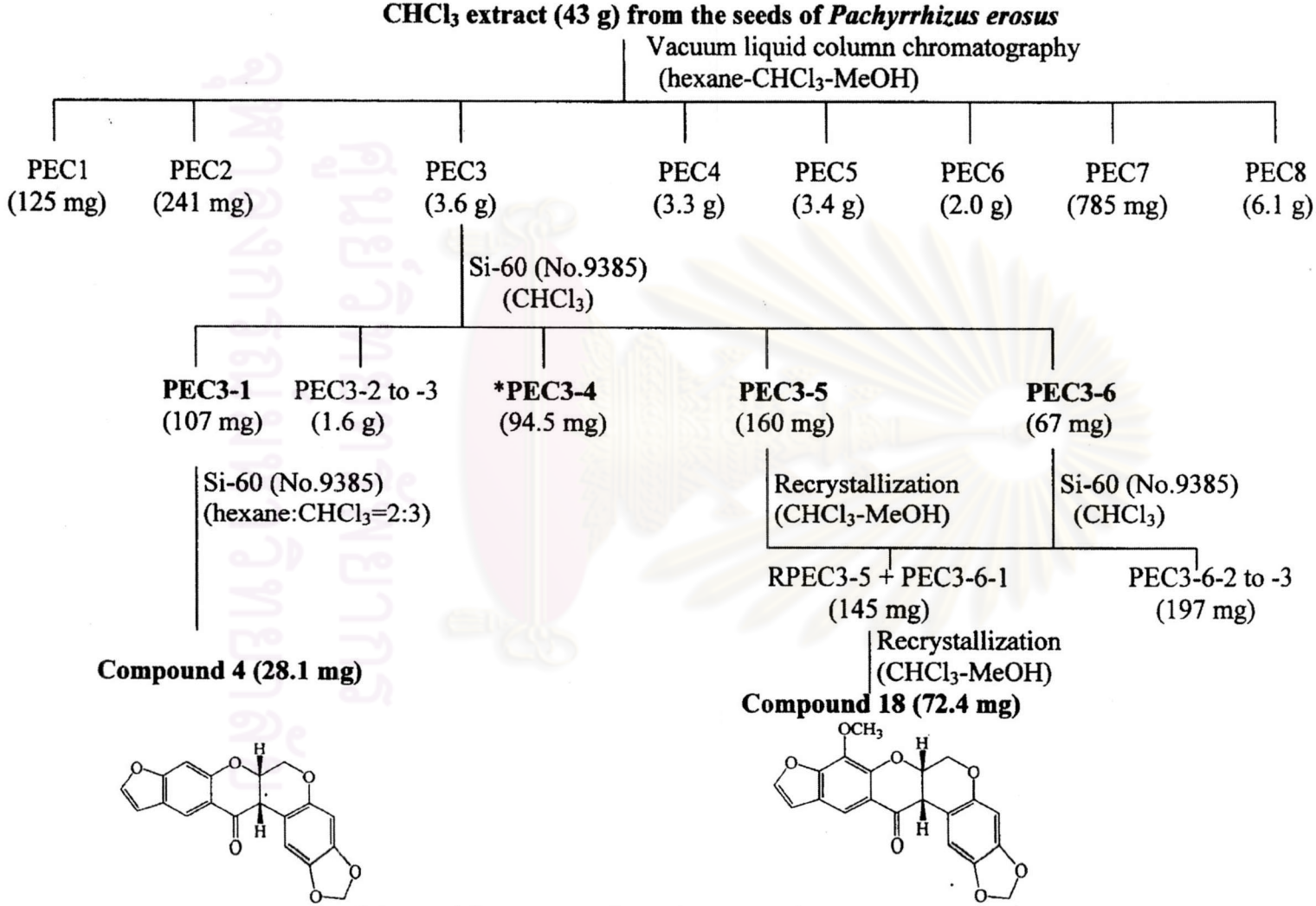
IR : ν_{max} cm^{-1}, Film; 2893, 1687, 1625, 1475

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 11**, **Figure 23**

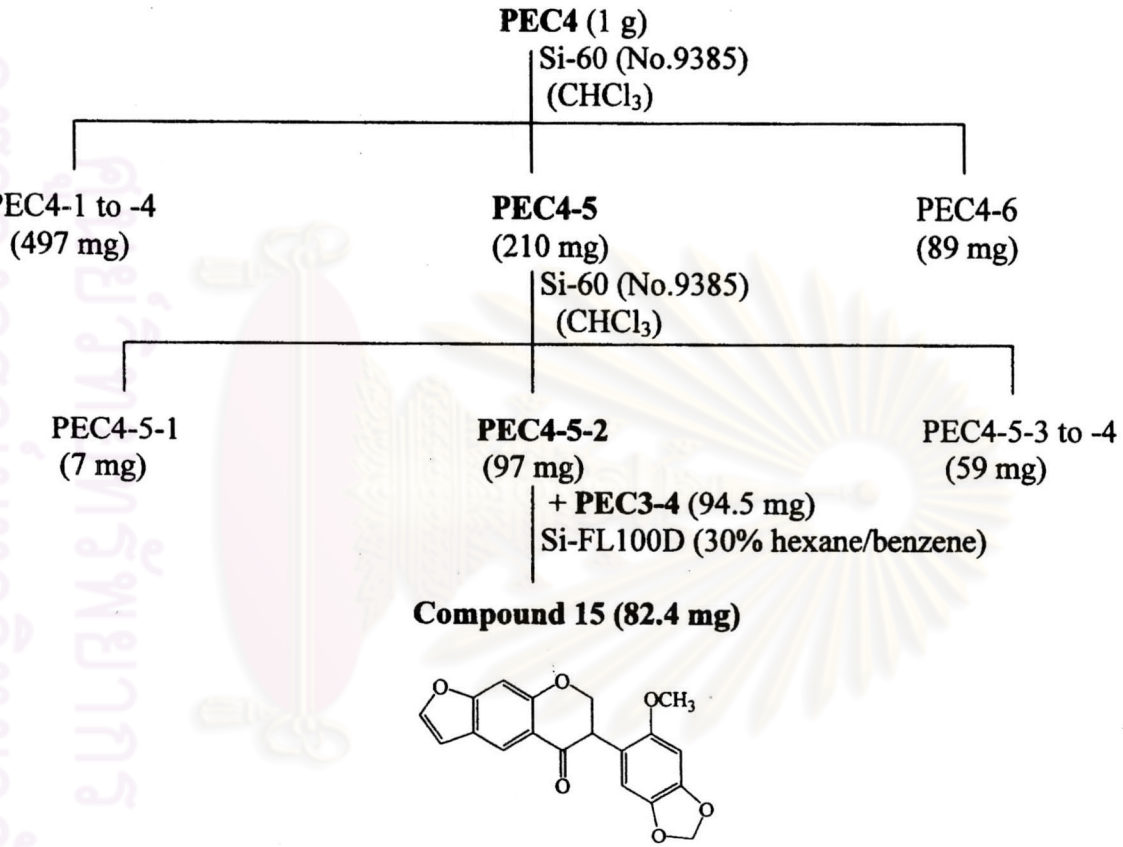
^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 11**, **Figure 24**

4.3 Compound 18 ((+)-Pachyrrhizone)

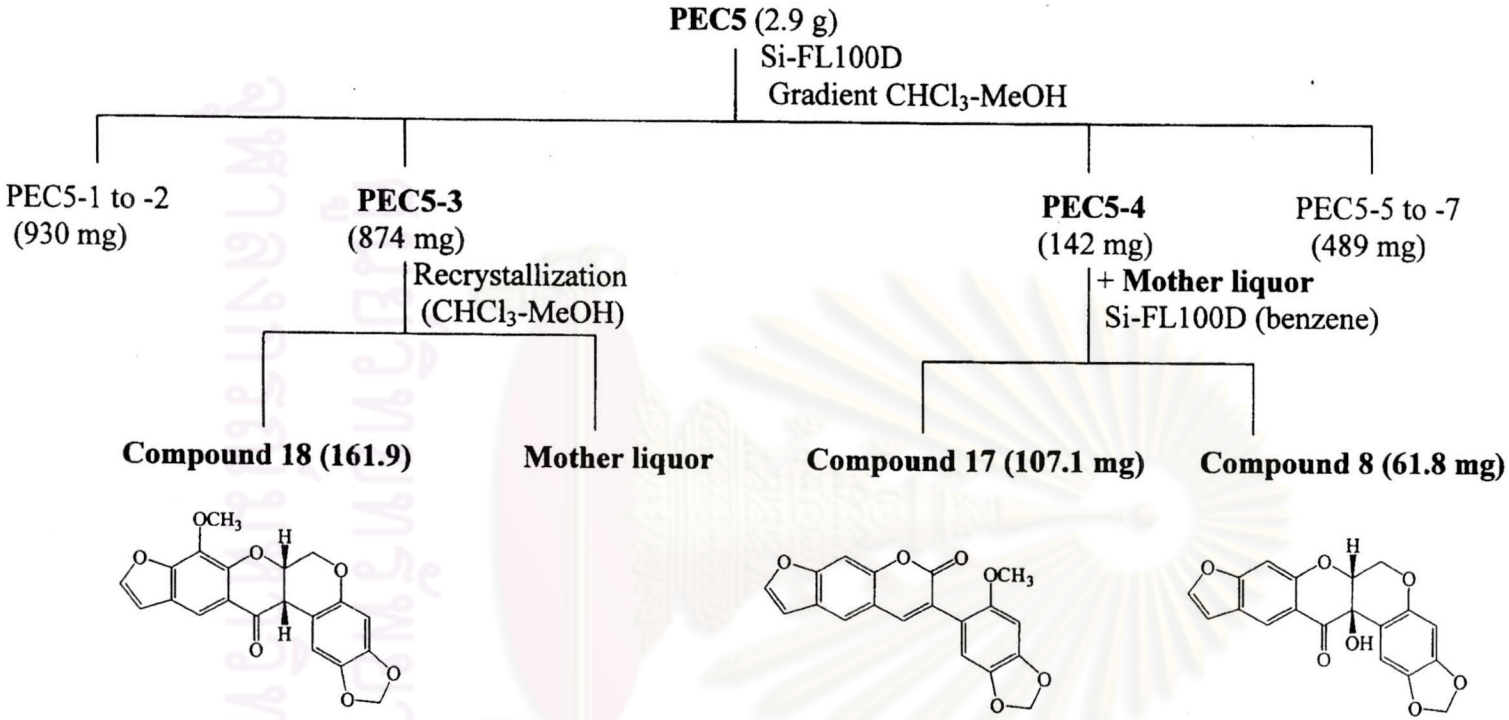
Compound **18** was obtained as white needles, soluble in $CHCl_3$ (234.3 mg, $11.7x10^{-3}$ % based on dried weight of the seeds).



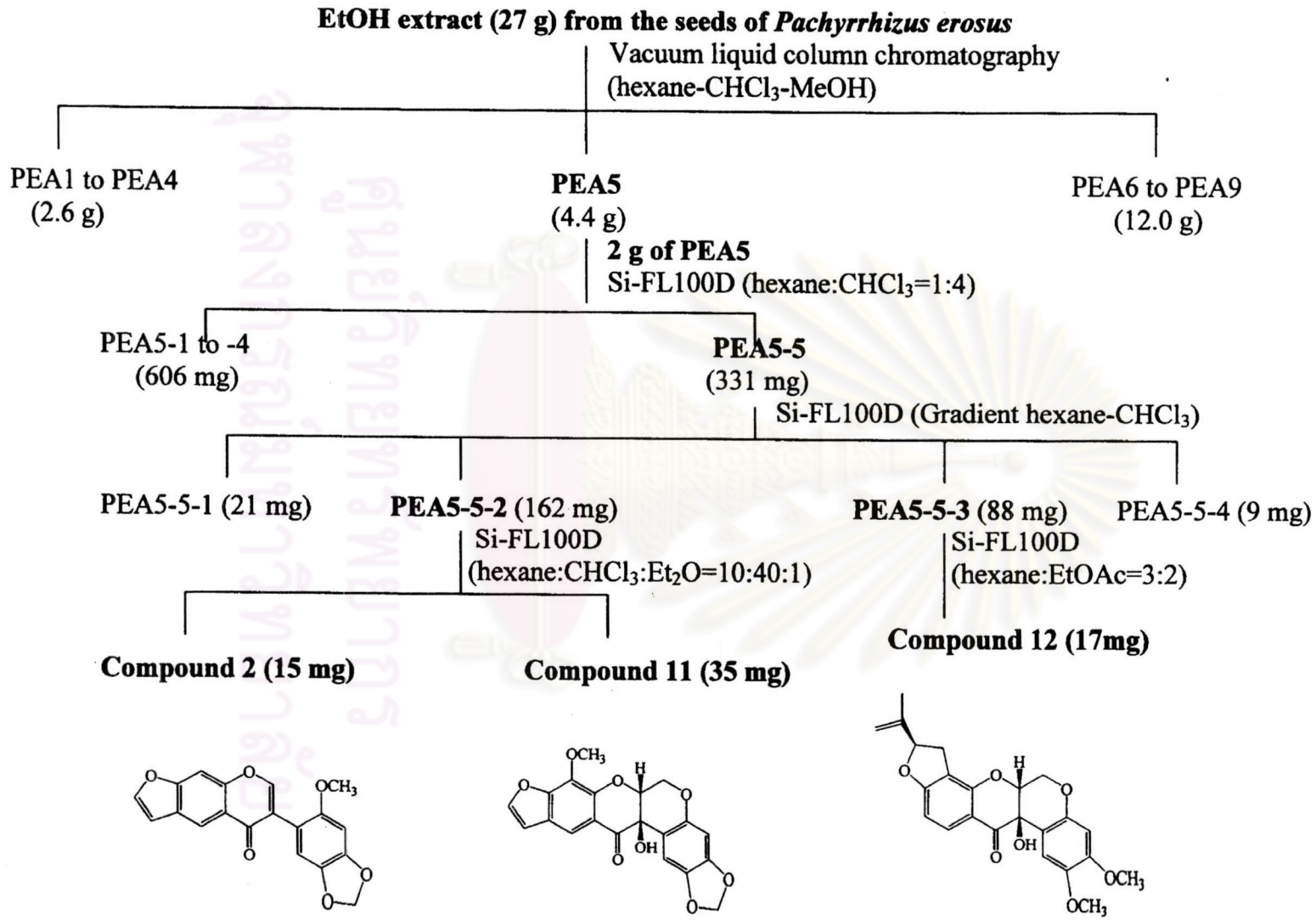
Scheme 4 Separation of $CHCl_3$ extract of *Pachyrrhizus erosus* seeds



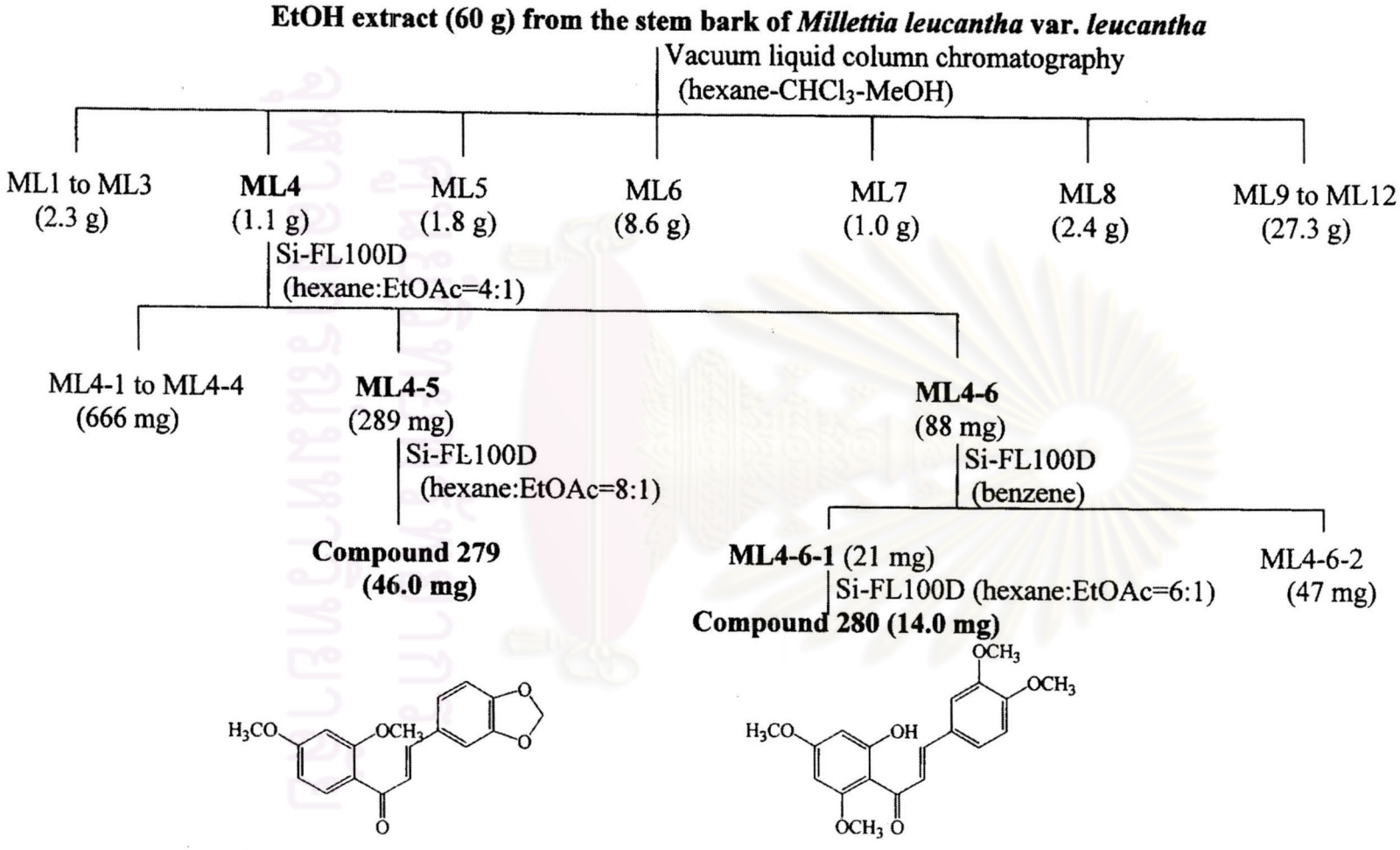
Scheme 5 Separation of fraction PEC4 from the $CHCl_3$ extract of *Pachyrrhizus erosus* seeds



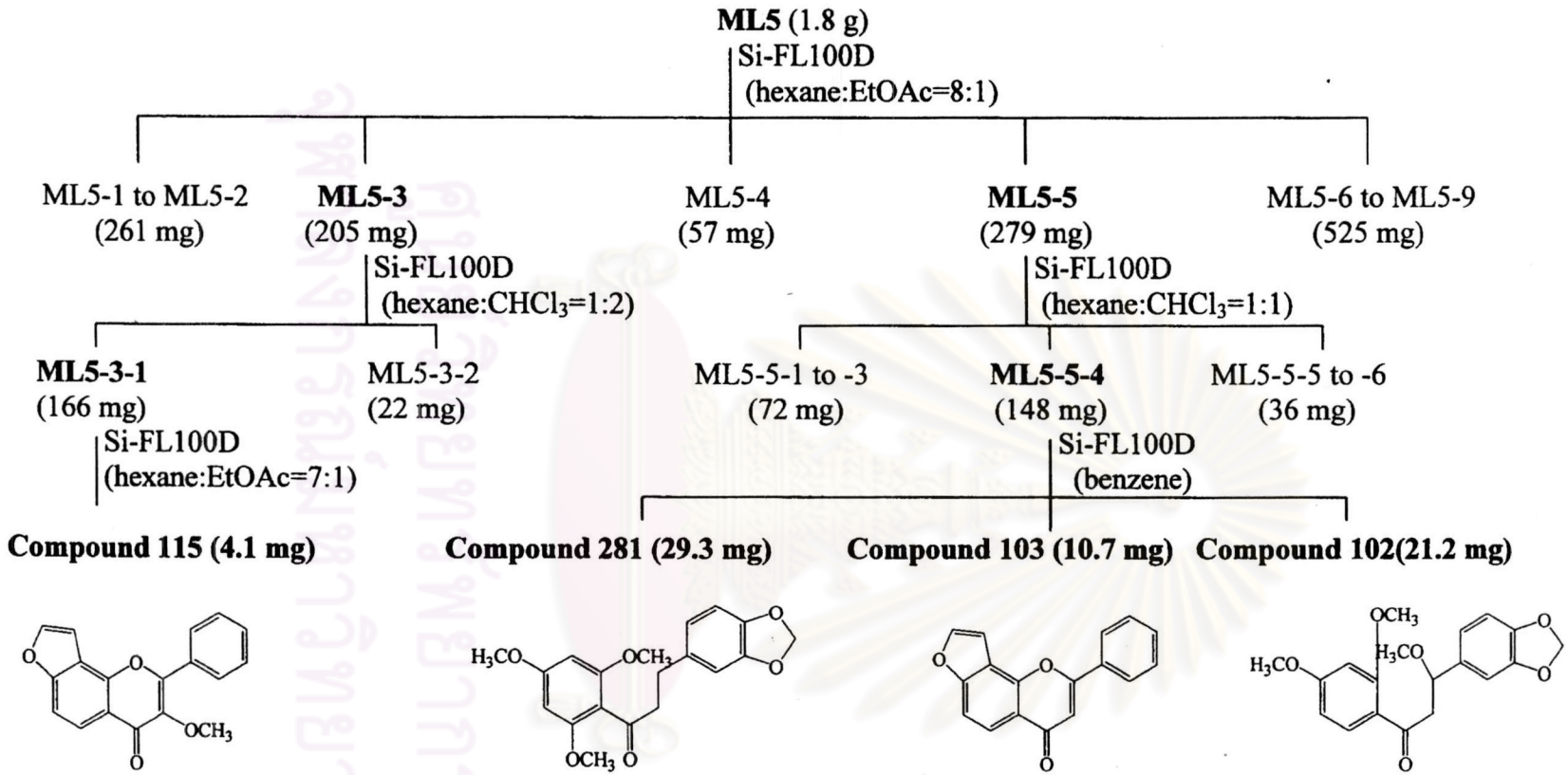
Scheme 6 Separation of fraction PEC5 from the $CHCl_3$ extract of *Pachyrrhizus erosus* seeds



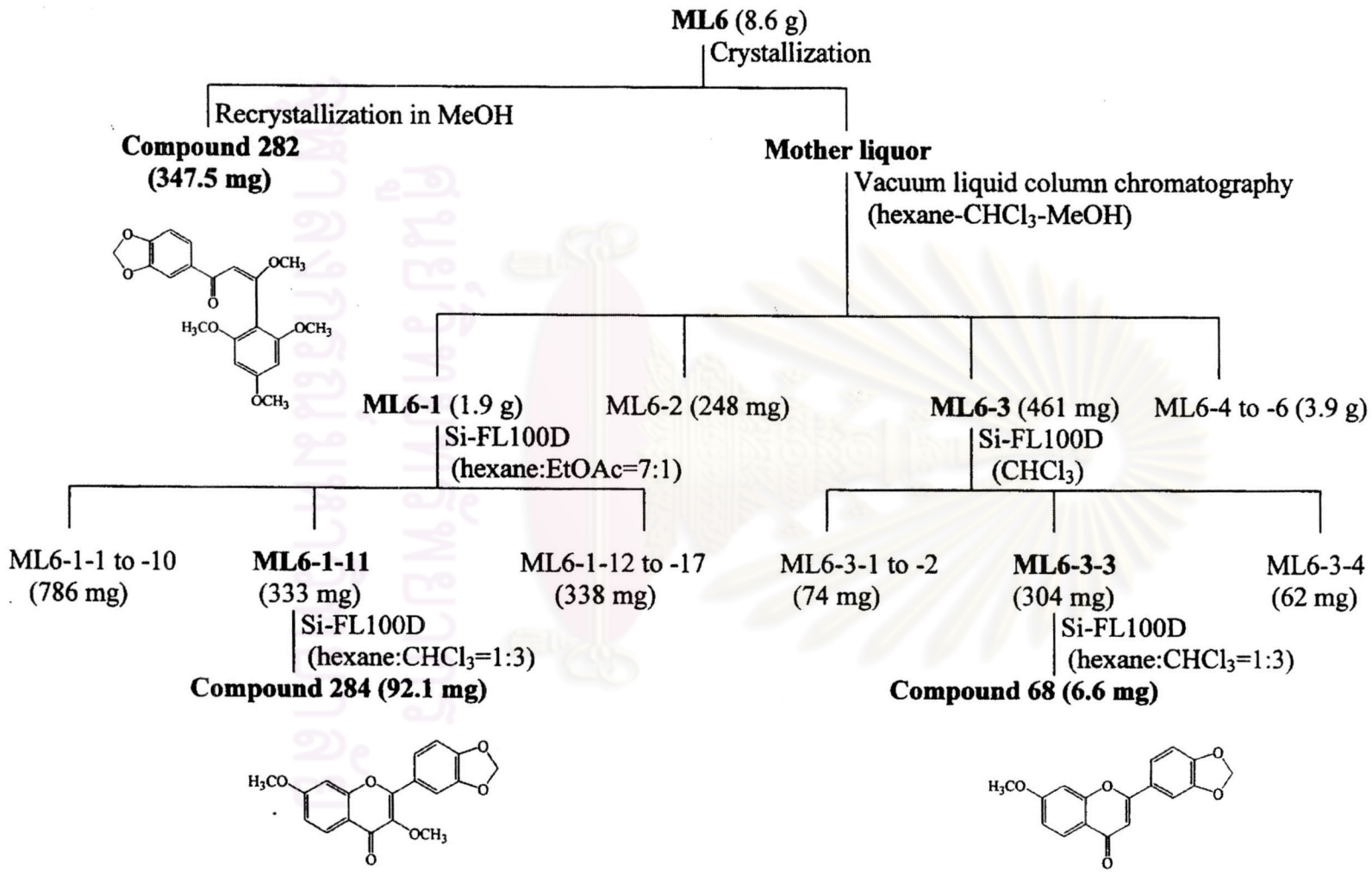
Scheme 7 Separation of the EtOH extract of *Pachyrrhizus erosus* seeds



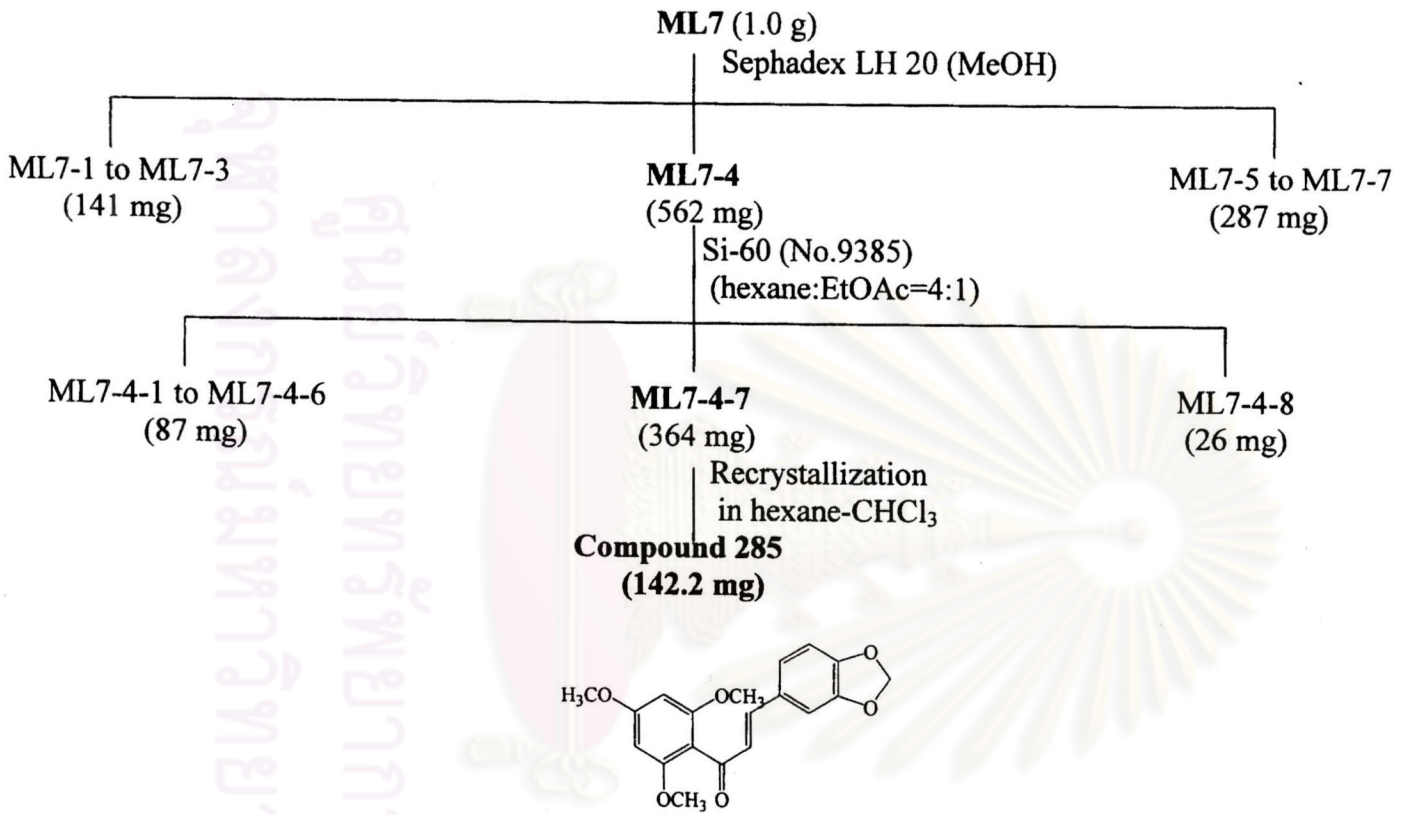
Scheme 8 Separation of the EtOH extract of *Millettia leucantha* var. *leucantha* stem bark



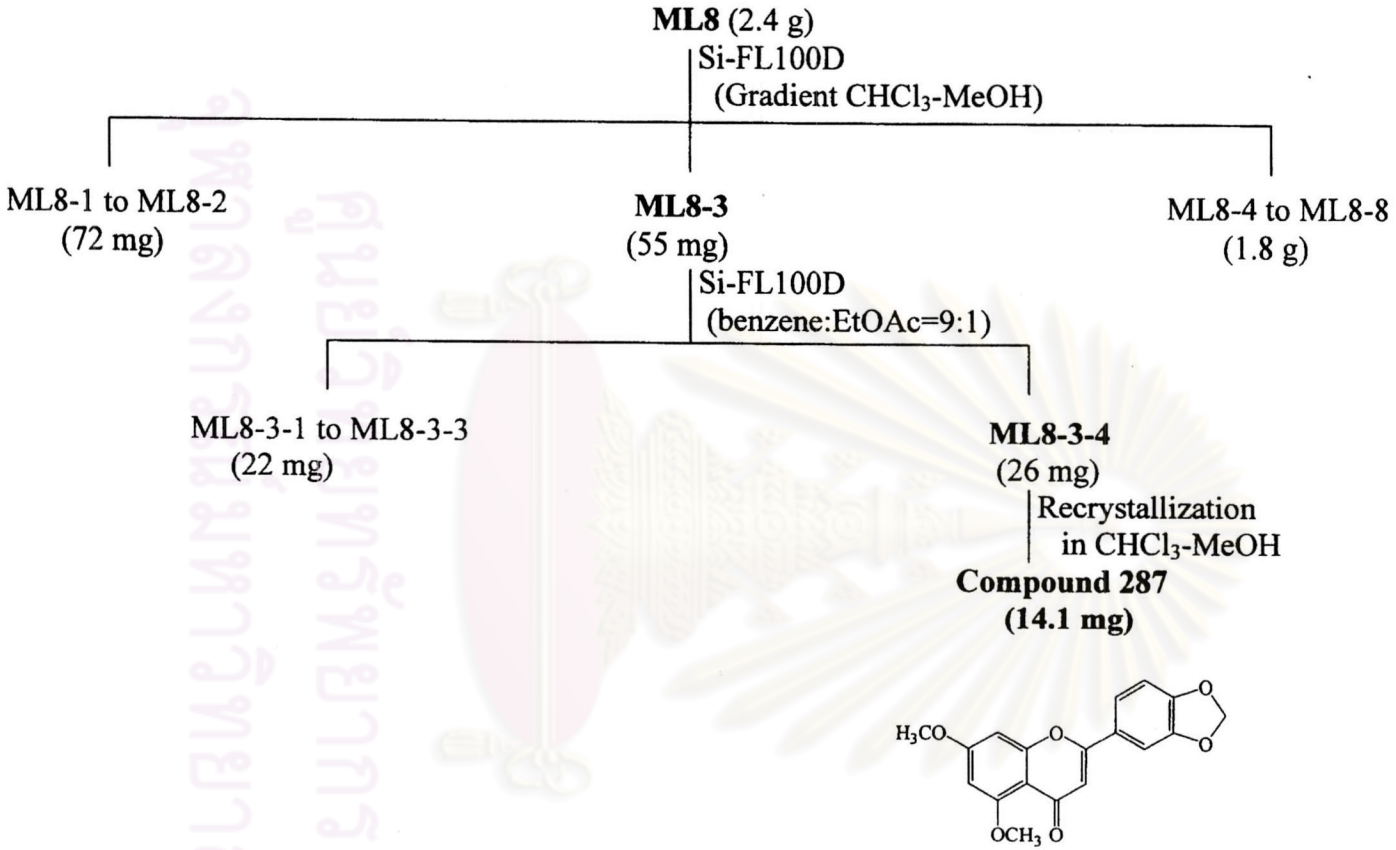
Scheme 9 Separation of fraction ML5 from the EtOH extract of *Millettia leucantha* var. *leucantha* stem bark



Scheme 10 Separation of fraction ML6 from the EtOH extract of *Millettia leucantha* var. *leucantha* stem bark



Scheme 11 Separation of fraction ML7 from the EtOH extract of *Millettia leucantha* var. *leucantha* stem bark



Scheme 12 Separation of fraction ML8 from the EtOH extract of *Millettia leucantha* var. *leucantha* stem bark

EIMS : *m/z* (% relative intensity); 366 (M^+, 73), 191 (27), 177 (59), 175 (100), 147 (80), 89(72)

$[\alpha]^{27}_D$: +116° (c 0.45, $CHCl_3$)

UV : λ_{max} nm (log ε), in methanol; 208 (5.09), 243 (4.75), 283 (4.05), 344 (3.56)

IR : ν_{max} cm^{-1}, Film; 1676, 1619, 1481, 1156

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 12, Figure 30**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 12, Figure 31**

4.4 Compound 17 (Pachyrrhizin)

Compound **17** was obtained as greenish-yellow needles, soluble in $CHCl_3$ (107.1 mg, $5.4x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 336 (M^+, 100), 293 (42), 265 (21), 179 (9)

UV : λ_{max} nm (log ε), in methanol; 210 (4.81), 242 (4.49), 292(4.19), 348 (4.09)

IR : ν_{max} cm^{-1}, Film; 1716, 1625, 1429, 1193, 1012

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 13, Figure 37**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 13, Figure 38**

4.5 Compound 8 ((+)-12a-Hydroxydolineone)

Compound **8** was obtained as pale yellow crystals, soluble in $CHCl_3$ (61.8 mg, $3.1x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 352 (M^+, 47), 192 (73), 191 (100), 161 (10), 76 (52)

$[\alpha]^{27}_D$: +140° (c 0.36, $CHCl_3$)

UV : λ_{max} nm (log ε), in methanol; 208 (4.42), 237 (4.67), 276 (3.98), 304 (3.82), 339 (3.66)

IR : ν_{max} cm^{-1}, Film; 3461, 2907, 1682, 1625, 1480

^{1}H NMR : δ ppm, 600 MHz, in $CDCl_3$; see **Table 14, Figure 44**

^{13}C NMR : δ ppm, 150 MHz, in $CDCl_3$; see **Table 14, Figure 45**

4.6 Compound 2 (Dehydroneotenone)

Compound **2** was obtained as white crystals, soluble in $CHCl_3$ (15.0 mg, $0.8x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 336 (M^+, 100), 306 (13), 305 (46), 161 (23), 132 (20), 104 (15), 76 (23)

UV : λ_{max} nm (log ε), in methanol; 208 (4.82), 237 (4.38), 303 (3.94)

IR : ν_{max} cm^{-1}, Film; 1645, 1622, 1474, 1196

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 15**, **Figure 51**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 15**, **Figure 52**

4.7 Compound 11 ((+)-12a-Hydroxypachyrrhizone)

Compound **11** was obtained as pale yellow crystals, soluble in $CHCl_3$ (35.0 mg, $1.8x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 382 (M^+, 8), 192 (47), 191 (100), 165 (19) 147 (24), 107 (10)

$[\alpha]^{27}_D$: +91.6° (c 0.43, $CHCl_3$)

UV : λ_{max} nm (log ε), in methanol; 206 (4.64), 243 (4.47), 284 (3.79), 349 (3.16)

IR : ν_{max} cm^{-1}, Film; 3461, 2937, 1682, 1621, 1482

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 16**, **Figure 58**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 16**, **Figure 59**

4.8 Compound 12 ((-)-12a-Hydroxyrotenone)

Compound **12** was obtained as colourless oil (17.0 mg, $0.9x10^{-3}$ % based on dried weight of the seeds).

EIMS : *m/z* (% relative intensity); 410 (M^+, 8), 209 (35), 208 (100), 297 (60) 187 (11), 165 (14), 109 (13), 78 (19)

$[\alpha]^{27}_D$: -145.0° (c 0.32, $CHCl_3$)

UV : λ_{max} nm (log ε), in methanol; 205 (4.70), 244 (3.78), 293 (3.89)

IR : ν_{max} cm^{-1}, Film; 3446, 2962, 1673, 1614, 1507

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 17**, **Figure 65**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 17**, **Figure 66**

4.9 Compound 279 (2',4'-Dimethoxy-3,4-methylenedioxychalcone)

Compound **279** was obtained as yellow needles, soluble in $CHCl_3$ (46.0 mg, $5.6x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 312 (M^+, 70), 297 (23), 284 (23), 165 (43) 147 (7), 135 (100), 107 (33), 89 (72)

***Anal.* Calcd** : For $C_{18}H_{16}O_5$•1/6 H_2O; C, 68.60; H, 5.21 Found; C, 68.66; H, 5.00

UV : λ_{max} nm (log ε), in methanol; 206 (4.51), 245 (4.10), 304 (4.08), 348 (4.43)

IR : ν_{max} cm^{-1}, Film; 1651, 1601, 1489

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 18**, **Figure72**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 18**, **Figure 73**

4.10 Compound 280 (2'-Hydroxy-3,4,4',6'-tetramethoxychalcone)

Compound **280** was obtained as orange needles, soluble in $CHCl_3$ (14.0 mg, $1.2x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 344 (M^+,48), 207 (33), 181 (15), 164 (44), 151 (100)

UV : λ_{max} nm (log ε), in methanol; 220 (4.18), 257 (3.63), 370 (4.17)

IR : ν_{max} cm^{-1}, Film; 3446, 1622, 1510, 1219

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 19**, **Figure 79**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 19**, **Figure 80**

4.11 Compound 115 (Karanjin)

Compound **115** was obtained as colourless plates, soluble in $CHCl_3$ (4.1 mg, $0.3x10^{-3}$ % based on dried weight of the stem bark).

ESIMS : $[M+Na]^+$ *m/z* 315.1 (positive ion mode)
$[M+H]^+$ *m/z* 293.1 (positive ion mode)

UV : λ_{max} nm (log ε), in methanol; 219 (4.19), 260 (3.98), 304 (3.80)

IR : ν_{max} cm^{-1}, Film; 1633, 1625, 1458, 1165

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 20**, **Figure 84**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 20**, **Figure 85**

4.12 Compound 281 (2',4',6'-Trimethoxy-3,4-methylenedioxy dihydrochalcone)

Compound **281** was obtained as pale yellow oil (4.1 mg, $0.3x10^{-3}$ % based on dried weight of the stem bark).

HRFABMS : *m/z* 345.1333 $[M+H]^+$; calcd for $C_{19}H_{21}O_6$ 345.1347

EIMS : *m/z* (% relative intensity); 344 (M^+, 26), 313 (10), 195 (82), 168 (48), 148 (100), 109 (8), 91 (29)

UV : λ_{max} nm (log ε), in methanol; 207 (4.84), 233(4.51), 285 (4.31)

IR : ν_{max} cm^{-1}, Film; 2940, 1698, 1606, 1455, 1206, 1155, 1126

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 21**, **Figure 89**

^{13}C NMR : δ ppm, 125MHz, in $CDCl_3$; see **Table 21**, **Figure 90**

4.13 Compound 103 (Lanceolatin B)

Compound **103** was obtained as colourless needless, soluble in $CHCl_3$ (10.7 mg, $0.9x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 262 (M^+, 73), 234 (9), 160(81), 132 (40), 104 (55), 102 (47), 83 (87), 76 (100)

UV : λ_{max} nm (log ε), in methanol; 219 (4.28), 263 (4.08), 297 (3.95)

IR : ν_{max} cm^{-1}, Film; 1645, 1405, 1362, 1067

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 22**, **Figure 97**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 22**, **Figure 98**

4.14 Compound 102 (Dihydromilletenone methyl ether)

Compound **102** was obtained as colourless oil (21.2 mg, $1.7x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 344 (M^+, 2), 329 (5), 165(100), 149 (25), 148 (6), 107 (14), 83 (19)

UV : λ_{max} nm (log ε), in methanol; 220(4.70), 269 (4.39), 295 (4.29)

IR : ν_{max} cm^{-1}, Film; 2935, 1666, 1600, 1488, 1244

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 23**, **Figure 102**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 23**, **Figure 103**

4.15 Compound 282 (2,4,6,β-Tetramethoxy-3',4'-methylenedioxychalcone)

Compound **282** was obtained as pale yellow needless, soluble in $CHCl_3$ (347.5mg, $28.5x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 372 (M^+, 3), 342 (99), 341(100), 149(27), 121(20)

***Anal.* Calcd** : For $C_{20}H_{20}O_7 \cdot 1/4\ H_2O$; C, 63.74; H, 5.48 Found; C, 64.04; H, 5.48

UV : λ_{max} nm (log ε), in methanol; 211 (5.05), 277(4.08), 320 (4.17)

IR : ν_{max} cm^{-1}, Film; 2940, 1659, 1587, 1487, 1439, 1234, 1127

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 24**, **Figure 107**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 24**, **Figure 108**

4.16 Compound 284 (Desmethoxykanugin)

Compound **284** was obtained as white solid, soluble in $CHCl_3$ (92.1 mg, $7.5x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 326 (M^+, 100), 308 (12), 283(10), 253 (7), 146 (15), 119 (57), 83 (55), 63 (40)

UV : λ_{max} nm (log ε), in methanol; 208(5.08), 242 (4.56), 314 (4.47), 339 (4.52)

IR : ν_{max} cm^{-1}, Film; 1619, 1503, 1445, 1383, 1251

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; see **Table 25**, **Figure 115**

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; see **Table 25**, **Figure 116**

4.17 Compound 68 (3',4'-Methylenedioxy-7-methoxyflavone)

Compound **68** was obtained as colourless needles (6.6 mg, $0.5x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 296 (M^+, 100), 269 (11), 268 (18) 253 (24), 146 (67), 107(30), 88 (26)

UV : λ_{max} nm (log ε), in methanol; 220(4.13), 237 (3.96), 311 (3.81), 334 (3.89)

IR : ν_{max} cm^{-1}, Film; 1638, 1502, 1433, 1236

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 26**, **Figure 120**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 26**, **Figure 121**

4.18 Compound 285 (2',4',6'-Trimethoxy-3,4-methylenedioxychalcone)

Compound **285** was obtained as pale yellow needles (142.2 mg, $11.7x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 342 (M^+, 46), 327 (11), 315(81), 314 (100), 283 (10)

***Anal.* Calcd** : For $C_{19}H_{18}O_6$; C, 66.66; H, 5.30 Found; C, 66.49; H, 5.24

UV : λ_{max} nm (log ε), in methanol; 208(4.94), 254 (4.08), 296 (4.09), 344 (4.40)

IR : ν_{max} cm^{-1}, Film; 2941, 1646, 1603, 1489, 1255, 1127

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; see **Table 27**, **Figure 125**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; see **Table 27**, **Figure 126**

4.19 Compound 287 (3',4'-Methylenedioxy-5,7-dimethoxyflavone)

Compound **287** was obtained as colourless needles (14.1 mg, $1.2x10^{-3}$ % based on dried weight of the stem bark).

EIMS : *m/z* (% relative intensity); 326 (M^+, 100), 325 (52), 297 (38), 296 (28), 280 (31), 253 (13), 146 (42), 134 (10), 88 (22)

UV : λ_{max} nm (log ε), in methanol; 220(4.82), 240 (4.35), 265 (4.18), 334 (4.33)

IR : ν_{max} cm^{-1}, Film; 1653, 1616, 1456, 1325, 1114

^{1}H NMR : δ ppm, 500 MHz, in C_5D_5N; see **Table 28**, **Figure 134**

^{13}C NMR : δ ppm, 125 MHz, in C_5D_5N; see **Table 28**, **Figure 135**

5. Correlation of Compound 285 to Compound 281 by Reduction with Et_3SiH-CF_3CO_2H

To a solution of compound **285** (19.7 mg, 0.058 mmol) in CH_2Cl_2 (0.5 ml), Et_3SiH (0.028 ml, 0.18 mmol) and CF_3CO_2H (0.018 ml, 0.23 mmol) were added. The whole mixture was stirred at room temperature for 18 h. Additional reagents [Et_3SiH (0.028 ml, 0.18 mmol) and CF_3CO_2H (0.015 ml, 0.20 mmol)] were increased and the reaction mixture was stirred at room temperature for further 12 h. Excess amount of $NaHCO_3$ (200 mg) was added to the reaction mixture for neutralization, followed by filtration of insoluble material and evaporation to afford orange oil (21.8 mg). The crude product was purified by preparative TLC (Merck, Art. 5715, hexane-EtOAc 3:1) to give three fractions. Fraction 1 (less polar fraction), colourless oil was purified to give perhydro-compound **286** as by-product (7.9 mg, 40.1 % based on 19.7 mg of compound **285**), whereas fraction 2 as colourless oil was purified to afford compound **281** (4.4 mg, 22.3% based on 19.7 mg of compound **285**). The more polar, fraction 3, was purified to obtain starting compound **285** (2.7 mg, 13.7% based on 19.7 mg of compound **285**). The reaction of this chemical correlation was demonstrated in **Scheme 5** of **CHAPTER IV**.

6. Evaluation of Biological Activties

6.1 Antimicrobial Activity

Antimicrobial activity of the crude extracts and pure compounds were tested by using the agar cup/disc diffusion methods (Lorian, 1980). Activity was performed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538P, *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763. All tested bacteria were cultivated on Mueller Hinton agar (MHA), while all tested yeast were cultivated on Sabouraud dextrose agar (SDA) at 37 °C for 24-48 h. The cell culture were washed from the agar surface and suspended in sterile normal saline solution (NSS), and standardized to match turbidity standard of McFarland No. 0.5, provided approximately $1x10^8$ CFU/ml (colony forming unit/ml). Each of molten (20

ml) media (MHA and SDA) was separated and poured into 9 cm diameter petri dish and allowed to solidify to form base layer. Tested microorganism (0.2 ml) was mixed with 5 ml of molten media and immediately added into base layer media in petri dish. All tested samples were diluted in various concentrations in 10% DMSO in NSS (the maximum concentration 5 mg/ml) and then applied on steriled cup (300 µl in each) or paper disc (20 µl in each) for cup/disc diffusion assay. In the case of disc diffusion assay, these discs were left in sterile petri dish until the solvent was completely dried. Cups or dried paper discs were placed on the surface of inoculated petri dishes and incubated at 37 °C for 24 h for antibacterial assay and 48 h for antifungal assay. The diameters of inhibition zones were measured.

6.2 Evaluation of Anti-Herpes Simplex Virus (HSV) Activity

6.2.1 Anti-HSV Activity

Evaluation of antiviral activity by the plaque reduction assay (Abou-karam and Shier, 1990) was performed against HSV-1 (KOS) and HSV-2 (186). Virus (30 PFU/25 µl) was mixed with 25 µl of complete medium containing various concentrations of tested compound and incubated at 37 °C for an hour. After incubation, the mixtures were added into Vero cells ($6x10^5$ cells/ml, 50 µl/well) in 96-well microtiter plates and incubated at 37 °C for 2 hour. The overlay medium containing various concentration of test compound (100 µl/well) was added to the Vero cell and incubated at 37 °C in humidified CO_2 incubator for 2 days. The plaques were counted under an inverted microscope. The cells were fixed in 10% formalin and stained with 1 % crystal violet for an hour. The percent plaque inhibition was determined. Acyclovir was employed to be positive control. The graph plotted between values of various concentrations and its percent inhibition was used for IC_{50} (inhibition concentration at 50 % of virus growth) determination.

6.2.2 Cytotoxicity Test

Cytotoxicity was determined by incubating Vero cell monolayer with complete medium containing various dilutions of sample at 37 °C for 3 days. The cytotoxicity was then examined by trypan blue exclusion method. The maximum non-cytotoxic concentration of the sample was used for the anti-HSV evaluation.

6.3 *In Vitro* Cytotoxic Assay

The cytotoxicity of sample against human lung cancer cell line, NCI-H460, was assessed by methyleneblue staining method. Briefly, $1.5x10^3$ cells in RPMI1640 medium supplemented with 10 % fetal bovine serum and 1 mM sodium pyruvate were incubated in 96-well plates in the presence of serially diluted samples. After a 3-day culture, cell were stained with 0.05 % methyleneblue dye for 30 minutes, and then thoroughly washed with distilled water. The stained dye was extracted with 3 % HCl and OD660 was measured with microplate reader (Dynatech MR600) to determine cell growth inhibition.

6.4 Determination of COX Inhibitory Activity

6.4.1 Cell Culture and Treatment

Stock DMEM

All tissue culture medium and supplements were purchased from Gibco BRL. The powder of Dulbecco's modified medium (DMEM, 10 g) and 49.3 ml of 7.5 % $NaHCO_3$ were added to distilled water. Medium was adjusted to 1 l with distilled water after pH of 7-7.4 was reached by adding 1 N NaOH. Medium was then filter sterilized with 0.2 μM nylon membrane. Ten ml of filter sterilized 200 mM L-glutamine was then added to this stock solution.

Cell Culture Medium

For growing and maintaining cells, stocked DMEM was supplemented with fetal bovine serum (10 % final concentration), 50 mg/ml ascorbic acid, 10 ml/l non-essential amino acid and 800 μg/l hygromycin B. For drug treatment experiment, cells were grown in the same media without hygromycin B.

Treatment of Cells

Immortalized murine COX-$1^{-/-}$ and COX-$2^{-/-}$ cells (Kirtikara, Swangkul and Ballou, 2001) were seeded at $1x10^5$ cells/ml in DMEM in 96-well flat bottomed tissue culture plates, 83 μl/well, and incubated in a humidified incubator with 5 % CO_2 for 72 h. Subsequently, cells were washed gently with DMEM without fetal bovine serum and incubated with serum-free DMEM containing vehicle and tested samples for 30 min. Medium was replaced with fresh serum-free DMEM containing vehicle, tested samples and 2 μM A23187, a calcium ionophore. After additional 30 min of

incubation, medium from each well was collected and placed in -80°C freezer until further use.

6.4.2 Preparation of Tested Samples

Pure dimethyl sulfoxide (DMSO) was used to initially solubilize the tested samples at $1x10^{-1}$ g/ml. Sample ($1x10^{-2}$ g/ml in 10 % DMSO) was subsequently prepared and later diluted before adding to culture medium to yield various concentrations of samples. Final DMSO concentration in the medium was 0.1%.

6.4.3 Radioimmunoassay (RIA) of Prostaglandin E_2 (PGE_2)

Materials

Anti-PGE_2 antibody was purchased from Sigma Chemicals and ^{3}H-PGE_2 was from Amersham.

Preparation of Solutions

RIA buffer was prepared by adding 22.8 g K_2HPO_4, 13.6 g KH_2PO_4, 9 g NaCl and 1 g sodium azide in total volumn of 1 l of distilled water. Subsequently, 1 g of gelatin was dissolved in 1 l of this solution at 37 °C until completely solubilized . The solution was kept at 4 °C.

Charcoal dextran solution was prepared by adding 2 g dextrans (T-70) in 1 l of RIA buffer at 37°C. Twenty grams of charcoal was later added and mixed thoroughly.

Stock anti-PGE_2 was prepared by adding 5 ml of 0.01 M sodium phosphate buffer saline, pH 7.4, containing 0.1 % BSA and 0.1 % sodium azide into one vial of lyophilized powder. The vial was rotated gently until the powder was dissolved. In a RIA, anti-PGE_2 working solution was prepared by diluting antiserum stock solution 10 fold with the buffer used to prepare the stock solution.

RIA

Media (50 µl) from each well were placed in the 1.5 ml microfuge tube containing 50 µl ^{3}H-PGE_2. Anti-PGE_2 working solution (50 µl) was then added to the tube. Two blank tubes containing all solutions similar to above sample tubes except replacing anti-PGE_2 with RIA buffer were prepared. Similarly, two zero tubes containing DMEM instead of medium from well were prepared. All tubes were kept on ice and subsequently incubated at 4 °C overnight. Then, 100 µl of charcoal/dextran were added to tubes that were being kept on ice. After 15 min, tubes

were centrifuged at 1500 g for 10 min at 4 °C. The amount of radioactive material in the supernatant were measured using a Packard scintillation counter.

6.4.4 Calculation of PGE_2 Level

PGE_2 levels were calculated from the following equation for percent binding and compared with the standard curve of known PGE_2.

$$\% \text{ binding} = \frac{(\text{average CPMA-average blank}) \, 100}{(\text{average zero-average blank})}$$

The levels of PGE2 produced in these COX-1 and COX-2 null cell lines correspond to the activity of COX-2 and COX-1 enzymes, respectively. DMSO (0.1%) was used as a control for 100 % COX activity. Aspirin, indomethacin and N-(2-[cyclohexyloxy]-4-nitrophenyl)methanesulfonamide (NS-398) were used as positive controls.

6.4.5 Determination of IC_{50}

Samples were diluted to 8 concentrations, including 10^{-9}, 10^{-8}, $3.3x10^{-8}$, 10^{-7}, $3.3x10^{-7}$, 10^{-6}, $3.3x10^{-6}$and 10^{-5} g/ml. Each of these concentrations was tested for the COX inhibitory activity as described above. The % COX inhibition of each concentration was calculated and used to plot graph. The IC_{50} value of each pure compound was then obtained from the graph.