CHAPTER II

EXPERIMENTAL

2.1 Plant Materials

The fresh stems of *Piper aurantiacum* Miq. were collected from the forest at Chieng-rai province in February, 1995. The voucher specimen (BKF 5937) has been lodged with the Herbarium of the Royal Forest Department. The fresh stems were sun-dried and crushed to coarse pieces.

2.2 Equipments

2.2.1 Rotary Evaporator

Eyela rotary evaporator model N-1 and/or Buchi rotary evaporator model R114 were used for the rapid removal of large amounts of volatile solvents under reduced pressure.

2.2.2 Chromatotron Equipment

Chromatotron equipment on Harrison Research model 7924T was operated for certain separation.

2.2.3 Melting Point Apparatus

The melting points were recorded on a Fisher-John melting point apparatus.

2.2.4 Infrared Spectrophotometer (IR)

The IR spectra were recorded on a Perkin-Elmer model 781, USA spectrophotometer. Solid samples which were examined by this equipment were incorperated into potassium bromide to form a pellet.

2.2.5 Fourie Transform-Infrared Spectrophotometer (FT-IR)

The FT-IR were recorded on a Perkin-Elmer model 1760x Fourie Transform Infrared Spectrophotometer. Solid samples were examined by incorperating the

sample into a pellet of potassium bromide. Liquid samples were dropped on a sodium chloride cell.

2.2.6 Gas Chromatography-Mass Spectrometer (GC-MS)

The GC-MS analysis was performed on a Fison Gas-Liquid Chromatography model GC 8000 coupled with a Fison Mass Spectrometer model Trio 2000.

2.2.7 ^{1}H and ^{13}C-Nuclear Magnetic Resonance Spectrometer

Routine ^{1}H-NMR spectra were recorded on Bruker spectrometer Model ACF 200 operated at 200.13 MHz for ^{1}H and 50.26 MHz for ^{13}C-nuclei. The chemical shift (δ) in ppm were referenced to the signal from the residaul proton in deuterated solvents. Assignments of carbon spectra were assisted by a Distortionless Enhancement by Polarization Transfer (DEPT) experiments. Specialized NMR experiments (COSY, NOESY, HMBC, HMQC, etc.) was performed on JEOL 500 MHz NMR spectrometer model JNM-A500 by Dr.Sathorn Suwan.

2.6.8 Gas Chromatography (GC)

The GC analysis was performed on a Shimadzu Gas Chromatograph model GC-7AG

2.6.8 Elemental Analyser

Elemental analysis was determined by a Perkin-Elmer CHN/O Analyser model 2400 Series II

2.3 Solvents and Chromatographic Media

2.3.1 All solvents used in this research were purified prior to use by simple distillation except for those which were reagent grade.

2.3.2 Merck's silica gel 60 Art. 7729.1000 was used as adsorbent for Quick Column Chromatography.

2.3.3 Merck's silica gel 60 Art.7734.1000 (70-230 mesh ASTM) was used as adsorbent for column chromatography.

2.3.4 Merck's silica gel 60 Art.7749 PF_{254} containing gypsum was used for chromatotron technique.

2.3.5 Merck's TLC aluminium sheets silica gel 60 F_{254} pre-coated 25 sheets, 20 x 20 cm^2, layer thickness 0.2 mm were used for TLC analysis.

2.4 Physical Separation Techniques

2.4.1 Quick Column Chromatography

Quick Column Chromatography was performed as described in reference [53].

2.4.2 Column Chromatography (CC)

Column Chromatography was performed as described in reference [54,55].

2.4.3 Chromatotron Technique

Chromatotron technique was operated for separation which was described in reference [56].

2.4.4 Thin-Layer Chromatography (TLC)

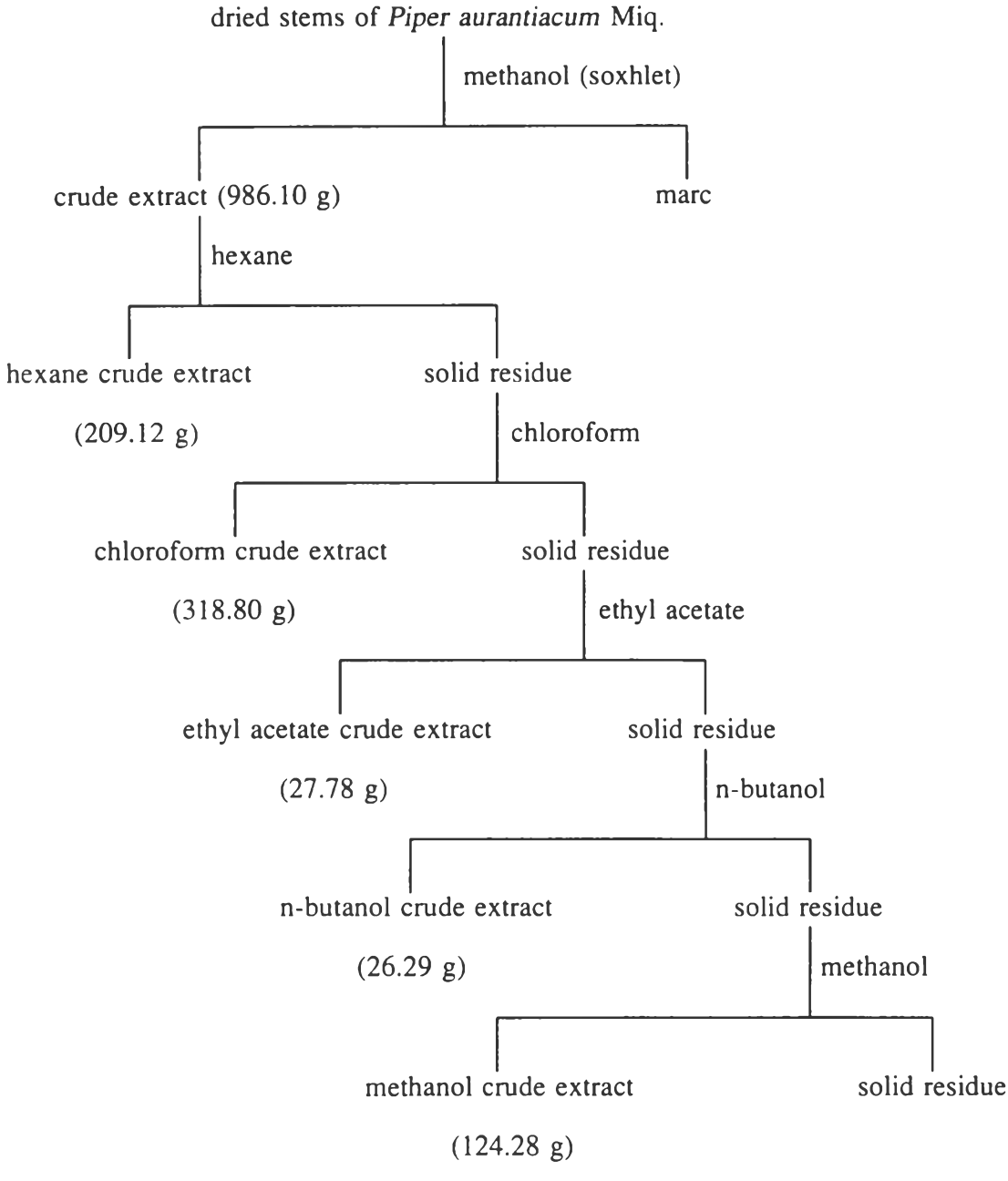
TLC analysis was performed as described in reference [57,58].

2.5 Extraction

The dried and crushed stems of *Piper aurantiacum* Miq. (19 kg) were extracted with methanol by using the soxhlet appararus until the solution was colourless. It was filtered and methanol was evaporated, then 986.10 g (5.19% wt. by wt. of the dried stems) of the black-brown and sticky crude extract was obtained.

The crude extract was re-extracted by hexane until the solution was colourless and the filtered hexane solution was concentrated under reduced pressure to afford the hexane crude extract as dark-brown oil of 209.12 g (1.10% wt. by wt. of the dried stems). Then, the residue was futher processed by chloroform, ethyl acetate, n-butanol and methanol in the same manner. The chloroform and ethyl acetate crude extracts were dark-brown oil weighed 318.80 g and 27.28 g, respectively (1.68% and 0.15% wt. by wt. of the dried stems) while the butanol crude extract was dark-brown sticky liquid weighed 26.29 g (0.14% wt. by wt. of the dried stems) and the methanol crude extract was dark-brown sticky substance weighed 124.28 g (0.65% wt. by wt. of the dried stems).

The procedure of the extractions is shown in Scheme 2.1 and the results of crude extracts were shown in Table 2.1.



Scheme 2.1 : Extraction of the dried stems of *Piper aurantiacum* Miq.

Table 2.1 : The results of dried stems extraction of *Piper aurantiacum* Miq.

Crude extract	Remark	Weight (g)	% wt. by wt. of dried stems
MeOH (soxhlet)	sticky dark-brown gum	986.10	5.19
Hexane	dark-brown oil	209.12	1.10
$CHCl_3$	dark-brown oil	318.80	1.68
EtOAc	dark-brown oil	27.78	0.15
n-BuOH	sticky dark-brown liquid	26.29	0.14
MeOH	sticky dark-brown substance	124.28	0.65

2.6 Isolation of the Chemical Constituents of the *Piper aurantiacum* Miq.

2.6.1 Separation of Hexane Extract

80 g of Hexane extract was chromatographed on silica gel 60 Art.7729 using a quick column chromatography. The column was eluted with hexane, hexane-chloroform, chloroform-methanol and methanol, respectively and approximately 200 ml of eluent was collected for each fraction. The solution in each fraction was removed by a rotary evaporator to a volume of about 30 ml. After each fraction was concentrated on a water bath to 10 ml, and then they were monitored by TLC and the identical fractions were combined. The results of the separation of hexane extract are presented in Table 2.2.

Table 2.2 : The results of the separation of the hexane extract by quick column chromatography

Eluent	Fraction No.	Remark	Weight(g)
100%Hexane	1-3	yellow oil	6.48
	4-9	trace yellow solid in yellow oil	8.03
	10-12	yellow solid in yellow oil (Compound I and II)	0.65
10%$CHCl_3$-Hexane	13-18	yellow solid in yellow oil (Compound I and II)	1.05
	19-24	yellow needle crystal in yellow oil (Compound I and II)	2.31
	25-34	deep yellow oil	3.64
	35-54	deep yellow oil	5.23
30%$CHCl_3$-Hexane	55-58	deep yellow oil	0.80
	59-62	dark yellow oil	1.29
	63-74	dark yellow oil	3.54
50%$CHCl_3$-Hexane	75-80	dark yellow oil	2.04
	81-113	white needle crystal in dark yellow oil (Compound III)	10.39
	114-117	brown oil	1.38
	118-120	brown oil	1.58
70%$CHCl_3$-Hexane	121-124	deep yellow oil	0.55
	125-130	deep yellow oil	0.73
	131-135	deep yellow oil	0.47
100%$CHCl_3$	136-143	deep yellow oil	2.97
1,3%$CHCl_3$-MeOH	144-152	brown oil (Compound IV)	5.16
5%$CHCl_3$-MeOH	153-157	brown oil	0.40

Table 2.2 (continue) : The results of the separation of the hexane extract by quick column chromatography

Eluent	Fraction No.	Remark	Weight(g)
5%$CHCl_3$-MeOH	158-159	brown oil	3.37
10,20%$CHCl_3$-MeOH	158-159	brown oil	2.39
50%$CHCl_3$-MeOH	166-172	dark brown tar	3.44
100%MeOH	173-180	dark brown tar	2.21

2.6.1.1 The Separation of the Eluted Fraction No.10-24

TLC indicated that fraction No.10-12, 13-18 and 19-24 should be combined. The combined fractions No.10-24 appeared as 4.01 g of yellow solid in deep yellow oil and was chromatographed on silica gel 60 Art.7734. The column was eluted with 20% ethyl acetate in hexane and eluted solution from this column was collected approximately 50 ml for each fraction. It was evaporated by a rotary evaporator to a volume of about 10 ml and was monitored by TLC. The fractions with similar composition were combined together. Table 2.3 shows the results of the separation.

Table 2.3 : The results of the separation of fraction No.10-24 by chromatotron technique

Fraction No.10-24	Remark	Weight (g)
1	pale yellow oil	0.01
2-4 (Fraction A)	yellow needle crystal (Compound I)	1.85
5-9 (Fraction B)	yellow needle crystal (Compound II)	0.92
10-49	trace light yellow oil	0.03
50-75	yellow oil	0.69

2.6.1.2 The Separation of the Eluted Fraction No.144-152

5.16 g of fraciton No.144-152 was chromatographed on silica gel 60 Art.7734 and this column was eluted with 20% ethyl acetate in hexane. Approximately 200 ml of the eluate was collected for each fraction and was evaporated to 10 ml. Each fraction was checked by TLC and the identical fractions were combined together. The results of the separation are presented in Table 2.4.

Table 2.4 : The results of the separation of fraction No.144-152 by column chromatography

Fraction No.	Remark	Weight (g)
1-3	yellow oil	0.06
4-17	yellow oil	0.14
18-19	yellow oil	0.01
20-35	deep yellow oil	0.50
36-50	white solidin deep yellow oil	0.42
(Fraction C)	(Compound IV)	
51-55	trace solid in yellow oil	0.17
56-66	yellow oil	0.51
67-75	deep yellow oil	0.39
76-80	yellow oil	0.02
81-83	brownish-yellow oil	0.08
84-87	brownish-yellow oil	0.01
88-89	brownish-yellow oil	0.08
90-92	brownish-yellow oil	0.05
93-98	brownish-yellow oil	0.04
99-105	brownish-yellow oil	0.30
106-110	brown oil	0.09

2.6.2 Separation of Chloroform Extract

80 g of chloroform extract was separated into various fraction by quick column chromatography. Silica gel 60 Art.7729 was used as an adsorbent. Eluting solvent used for each fraction was 200 ml. The solution in each fraction was evaporated to 30 ml, then it was concentrated to 10 ml on a water bath and checked their similarities by TLC before they were combined together. The results of the separation are shown in Table 2.5.

Table 2.5 : The results of the separation of the chloroform extract by quick column chromatography

Eluent	Fraction No.	Remark	Weight(g)
100%Hexane	1-11	trace white semi solid	0.42
10%$CHCl_3$-Hexane	12-21	trace white semi solid	0.46
30%$CHCl_3$-Hexane	22-30	white semi solid	0.87
50%$CHCl_3$-Hexane	31-38	white semi solid	0.74
	39-47	yellow solid in yellow oil (Compound II)	1.53
	48-54	yellow solid in brown oil (Compound II)	0.96
	55-60	brown oil	2.98
70%$CHCl_3$-Hexane	61-75	white needle crystal in dark brown oil (Compound III)	8.22
	76-85	white solid in dark brown oil (Compound III)	4.39
	86-99	yellow brown oil	1.27
	100-118	yellow brown oil	2.30
100%$CHCl_3$	119-144	yellow brown oil	4.16

Table 2.5 (continue) : The results of the separation of the chloroform extract by quick column chromatography

Eluent	Fraction No.	Remark	Weight(g)
1,3%MeOH-$CHCl_3$	145-155	yellow brown oil	2.83
5%MeOH-$CHCl_3$	156-163	dark brown oil	1.49
	164-180	dark brown oil	4.26
5,10%MeOH-$CHCl_3$	181-187	dark brown oil	2.93
10%MeOH-$CHCl_3$	188-194	dark brown oil	2.18
20%MeOH-$CHCl_3$	195-210	dark brown oil	3.12
50%MeOH-$CHCl_3$	211-216	dark brown tar	1.01
100%MeOH	217-220	dark brown tar	0.99

2.6.3 Separation of Ethyl Acetate Extract

27.78 g of Ethyl acetate extract was applied on the top of the quick column chromatography and it was initially eluted by 50% chloroform in hexane. The polarity of eluent was gradually increased from chloroform in hexane to pure chloroform, methanol in chloroform and then to 100% methanol. Approximately 200 ml of eluted solution was collected for each fraction and was evaporated to a volume of about 30 ml; next, the solvents in each fraction were removed by using a water bath until about 10 ml of the solution was obtained. The similar fractions were combined after they were monitored by TLC and the Table 2.6 shows the results of the separation.

Table 2.6 : The results of the separation of ethyl acetate extract by quick column chromatography

Elutent	Fraction No.	Remark	Weight(g)
50%$CHCl_3$-Hexane	1-2	trace pale orange oil	0.09
	3-4	trace yellow oil	0.09
	5-12	yellow brown oil	0.48
60%$CHCl_3$-Hexane	13-32	white needle crystal in brown oil (Compound III)	2.36
60%$CHCl_3$-Hexane	33-38	solid in brown oil	0.15
80%$CHCl_3$-Hexane	39-50	solid in brownish-yellow oil	0.53
	51-54	solid in yellow oil	0.31
100%$CHCl_3$	55-66	solid in yellow oil	0.74
1%MeOH-$CHCl_3$	67-78	solid in brown oil	0.13
	79-90	dark brown oil	0.97
3,5%MeOH-$CHCl_3$	91-107	brown oil	1.86
10,20%MeOH-$CHCl_3$	108-132	reddish-brown oil	2.53
50%MeOH-$CHCl_3$	133-140	reddish brown oil	2.09
100%MeOH	141-145	brown tar	0.88

2.6.4 Separation of Butanol Extract

Quick column chromatography was used for separating the n-butanol extract (26.29 g) into various fractions. Eluting solvent system was the same as described in the separation of ethyl acetate extract. Each fraction was collected and concentrated in the same manner and was checked by TLC, then the identical fractions were combined. The results of the separation of n-butanol extract are presented in Table 2.7.

Table 2.7 : The results of the separation of n-butanol extract by quick column chromatography

Elutent	Fraction No.	Remark	Weight(g)
50%$CHCl_3$-Hexane	1-6	light yellow oil	0.12
	7-20	yellow oil	2.65
60%$CHCl_3$-Hexane	21-46	yellow oil	1.48
80%$CHCl_3$-Hexane	47-62	yellow oil	1.36
100%$CHCl_3$	63-90	brown oil	3.27
1%MeOH-$CHCl_3$	91-99	brown oil	0.66
3%MeOH-$CHCl_3$	100-106	brown oil	0.49
5,10%MeOH-$CHCl_3$	107-125	dark brown oil	1.15
10%MeOH-$CHCl_3$	126-134	dark brown oil	0.31
20%MeOH-$CHCl_3$	135-142	dark brown oil	0.44
50%MeOH-$CHCl_3$	143-156	dark brown oil	0.87
100%MeOH	157-160	dark brown tar	0.31

2.6.5 Separation of Methanol Extract

About 2 g of methanol extract was chromatographed on sephadex LH20 column. The column was eluted with 50% methanol in chloroform and each eluted fractions were collected approximately 20 ml. They were concentrated on a water bath and were checked by TLC, then the identical fractions were combined together. The results of the separation of methanol extract are shown in Table 2.8.

Table 2.8 : The results of the separation of methanol extract

Fraction No.	Remark
1-2	light brown oil
3-4	brown oil
5-7	dark brown oil
8-9	solid in dark brown oil (Compound V)
10-13	dark brown oil
14-16	brown oil
17-20	light brown oil

2.7 Purifications And Properties of the Eluted Compounds

2.7.1 Purification and Properties of Compound I

Compound I was obtained as yellow needle-like solid from Fraction A (Table 2.3) which was eluted from silica gel column with 20% ethyl acetate in hexane. It was purified by recrystallization from hot hexane for several times to afford Compound I as colourless needles, 0.99 g (1.36×10^{-2} % wt. by wt. of the dried stems), m.p. 137-138°C and R_f value 0.63 (SiO_2 : 40% ethyl acetate in hexane). This compound was soluble in chloroform, ethyl acetate and acetone, slightly soluble in hexane and insoluble in methanol. After monitoring by colour test, this substance gave a green colour with Liebermann-Burchard's reagent which suggested the appearance of a steroidal moiety.

ν_{max}(KBr, cm^{-1}) 3500-3430(s,br) , 2960(s), 2840(m), 1650(w), 1460(w), 1380(w), 1050(m), 950(w), 840(w), 800(w) ; m/z(EI) 414 (M^+,67%), 396(30), 381 (21), 329(38), 303(49), 273(24), 255(33), 231(24), 213(42), 161(47), 159(48), 145 (66), 133(40), 119(42), 107(69), 95(65), 81(79), 69(64), 55(100) ; δ_H (200.13

MHz, $CDCl_3$) 0.62-2.26(m), 3.50(m), 5.33(d, J=5.1Hz) ; δ_C (50.26 MHz, $CDCl_3$) 11.85, 11.97, 18.76, 19.02, 19.39, 19.81, 21.07, 23.05, 24.29, 26.05, 28.23, 29.13, 31.61, 31.89, 33.93, 36.13, 36.49, 37.23, 39.76, 42.24, 45.82, 50.11, 56.04, 56.75, 71.81, 121.72, 140.73.

GLC analysis of standard steroids (Fig.A.7a) namely cholesterol, campesterol, stigmasterol and β-sitosterol showed retention times at 13.06, 17.61, 18.76 and 21.00 min, respectively. (Condition of GLC analysis : column OV-1, column temperature 260°C, injection temperature 290°C, N_2 flow rate 50 ml/min). The GLC chromatogram of Compound I showed one peak at retention time 20.84 min (Fig.A.7b).

2.7.2 Purification and Properties of Compound II

0.92 g of Compound II yellow needle solid, from fraction B (Table 2.3) was washed by a mixture of hexane and chloroform. The remaining solid was purified by recrystallization with a mixture of hexane and ethyl acetate for several times to afford yellow needle crystal (0.44 g). The TLC (SiO_2 : 50% ethyl acetate in hexane) exhibited two spots that the higher one was bigger than the other. They were separated by chromatotron technique and the results are presented in Table 2.9 and the TLC of fraction B-1 (0.13 g) showed only one spot.

Table 2.9 : The results of the separation of fraction B by chromatotron technique

Elutent	Fraction No.	Remark
100%Hexane	1-5	colourless oil
5%EtOAc-Hexane	6-9	colourless oil
	10-16	pale yellow oil
	17-19	pale yellow semi solid
	20-27 (Fraction B-1)	yellow solid

Table 2.9 (continue) : The results of the separation of fraction B by chromatotron technique

Elutent	Fraction No.	Remark
5%EtOAc-Hexane	28-29	pale yellow semi solid
10%EtOAc-Hexane	30-37	light yellow oil
25%EtOAc-Hexane	38-44	light yellow oil
50,70%EtOAc-Hexane	45-60	yellow oil
100%EtOAc	61-65	yellow oil
100%MeOH	66-70	pale yellow oil

Besides, Compound II was obtained from fraction No. 39-54 of the chloform extract (Table 2.5) and it was purified by removing yellow and brown oil with a mixture of hexane and chloroform, recrystallization from a mixture of hexane and ethyl acetate and purification by using chromatotron equipment with the same method as described above. Finally, 0.96 g of yellow solid was obtained.

Total weight of Compound II was 1.09 g (0.02% wt. by wt. of the dried stems), the melting point was 142-143°C and the R_f value revealed one spot at 0.71 (SiO_2 : 50% ethyl acetate in hexane). This compound was soluble in hexane and chloroform but slightly soluble in ethyl acetate and insoluble in acetate and methanol.

ν_{max} (KBr, cm^{-1}) 3010(w), 2960(w), 2910(w), 1720(s), 1630(s), 1620(s), 1520(s), 1510(s), 1470(s), 1445(s), 1380(m), 1340(m), 1320(m), 1285(s), 1260(s), 1160(s), 1055(s), 1020(s) ; m/z(EI) 232 (M^+,71%), 201(23), 173(95), 143(38), 115 (100), 100(20), 89(13), 63(17) ; δ_H (200.13 MHz, $CDCl_3$) 3.74(3H, s), 5.95(2H, s), 6.46(1H, dd, J=10.0,15.2Hz), 6.73(1H, d, J=15.1Hz), 6.75(1H, d, J=8.0Hz), 6.82 (1H, d, J=15.2Hz), 6.88(1H, dd, J=1.7, 8.0Hz), 6.96(1H, d, J=1.6Hz), 7.39(1H, dd,

J=10.0, 15.2Hz) ; δ_C (50.26 MHz, $CDCl_3$) 51.50, 101.47, 106.01, 108.62, 120.44, 123.10, 124.98, 130.68, 140.73, 145.02, 148.89, 149.00, 167.94.

2.7.3 Purification and Properties of Compound III

Compound III was obtained as a white needle crystal in brown oil from fraction No.81-113 of hexane extract (Table 2.2), fraction No.61-85 of chloroform extract (Table 2.5) and fraction No.13-32 of ethyl acetate extract (Table 2.6).

After removing brown oil by washing with ethyl acetate and recrystallizing the solid with a mixture of hexane and ethyl acetate for several times to afford colourless rectangular-like crystal, 8.74 g (0.42% wt. by wt. of the dried stems). The melting point was 158-159°C and the R_f value was 0.61 (SiO_2 : 40% ethyl acetate in hexane).

ν_{max} (KBr, cm^{-1}) 3600-3200(s,br), 3075(w), 2960(s), 2880(m), 1680(s), 1630 (m), 1600(s), 1515(s), 1450(m), 1370(m), 1330(m), 1280(m), 1190(s), 1170(s), 1032 (m), 980(m), 830(m), 760(w), 555(w) ; m/z(EI) 300 (M^+,10%), 164(6), 148(14), 147 (100), 119(8), 109(6), 91(9) ; δ_H (200.13 MHz, $CDCl_3$) 0.86(3H, s), 0.88(3H, s), 0.91(3H, s), 1.04(1H, dd, J=13.7, 4.0Hz), 1.25(1H, m), 1.34(1H, m), 1.69(1H, t, J=4.0Hz), 1.75(1H, m), 2.00(1H, m), 2.40(1H, m), 5.00(1H, dt, J=10.0, 2.8Hz), 6.31 (1H, br s), 6.32(1H, d, J=16.0Hz), 6.85(2H, br d, J=8.6Hz), 7.42(2H, br d, J=8.6Hz), 7.61(1H, d, J=16.0Hz) ; δ_C (50.26 MHz, $CDCl_3$) 13.58, 18.95, 19.72, 27.23, 28.06, 36.85, 44.93, 47.86, 48.97, 80.41, 115.51, 115.98, 126.75, 130.06, 144.78, 158.41, 168.72.

2.7.4 Purification and Properties of Compound IV

Trace of Compound IV was obtained from fraction C (Table 2.4) of hexane extract. The yellow oil was removed by washing with a mixture of ethyl acetate and

hexane and the remaining solid was also recrystallized with ethyl acetate-hexane to afford white precipitate (trace) as a Compound IV.

ν_{max} (KBr, cm^{-1}) 3300-2500(br), 2920(w), 1675(s), 1621(m), 1616(m), 1509 (w), 1455(s), 1416(m), 1367(w), 1304(s), 1265(s), 1240(m), 918(w), 800(w) ; m/z (EI) 166 (M^+,83%), 165(100), 149(33), 121(14), 119(17), 91(10) ; δ_H (200.13 MHz, $CDCl_3$) 6.05(1H, s), 6.85(1H, br, d, J=8.2Hz), 7.50(1H, d), 7.71(1H, dd, J=8.2Hz)

2.7.5 Purification and Properties of Compound V

Compound V was obtained as a solid in dark brown oil from fraction No.8-9 of methanol extract which was eluted from Sephadex LH20 with 50% methanol in chloroform (Table 2.8). Dark brown oil was removed by washing with acetone and the remaining solid was purified by recrystallization from water and methanol for several times to afford Compound V as a colourless cubic-like crystal, 42 mg ($1.37x10^{-3}$% wt. by wt. of the dried stems) and the melting point was over 270°C. This compound was soluble in water and slightly soluble in methanol, but insoluble in organic solvents. It gave yellow precipitate with sodiom cobaltinitrite ($Na_3Co(NO_2)_6$) and gave white precipitate with $AgNO_3$. After monitoring by flame test, Compound V gave purple colour.