Chapter 4
Results and Discussions

4.1 Characterization of compounds

The fresh rhizomes of *Boesenbergia pandurata* were extracted with ethanol. The crude extract was further fractionated using solvents with increasing polarity, as described in experimental (Chapter 3). Compounds **1**, **2**, **3** were found in *n*-hexane fraction, whereas compounds **3**, **4**, **5** were obtained from dichloromethane part. Compounds **4**, **5** were found in ethyl acetate fraction and the methanol part contained compounds **5**. The structure determination of these compounds (**1 – 5**) were decribed below.

4.1.1 Identification of compound 1

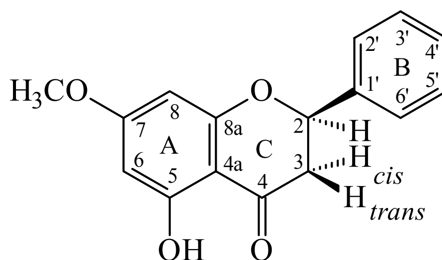


Figure 4-1 Structure of compound **1**

Compound **1** was obtained and re-crystallized as needles from acetone, with m.p. 99-100˚C. The UV spectrum (Figure A-1) shows the maximum absorption at λ_{max} 288 nm (ε 1.9 x 10^3, c 0.001 in MeOH). The molecular formula was suggested as $C_{16}H_{14}O_4$, the molecular ion peak at m/z 271.0970 (calc. 271.2927)

in the HR-FAB mass spectrum and confirmed by the NMR spectrum data (Table 4-1 and 4-2), exhibited IR absorption band at 3400 (OH-stretching), 1640 (C=O-stretching) and 1620(C=C- stretching) cm^{-1}, respectively (Figure A-3).

In the ^1H NMR spectrum (Table 4-1), a methoxy group was seen as a singlet at δ 3.80. A one-proton singlet at δ 12.00, interchangeable with D_2O, sited the free hydroxyl at position 5 with a H-bridge to O at the carbonyl at position 4. Two doublets at δ 6.08 (J = 2 Hz) and 6.06 (J = 2 Hz) attributable to *meta* H-6 and H-8, together with a fragment at *m/z* 193 in MS (generated by the loss of the B-ring), placed the methoxyl group on C-7. A five-proton multiplet at δ 7.37-7.47 confirmed the non-substitution of the B-ring. The characterized of the ^1H NMR was the absence of the typical flavanone H-3 proton and the presence of an ABX system. The X part appeared as a doublet-doublet for a proton center at δ 5.40 (J = 13, 3 Hz) attributable to H-2 and the AB portion, as two doublet-doublet of one proton each at δ 2.80 (J = 17, 3 Hz) and 3.10 (J = 17, 13 Hz) attributable to H-3 (*cis*) and H-3 (*trans*), respectively.

Table 4-1 ^{1}H NMR data and ^{1}H-^{1}H COSY of compound **1** (500 MHz, $CDCl_3$)

H	δ (ppm)	M	J (Hz)	H-H cosy	Literature* δ (ppm)	Δ δ (ppm)
2	5.40	dd	13,3	3	5.40	0.00
3-*cis*	2.80	dd	17,3	-	2.85	-0.05
3-*trans*	3.10	dd	17,13	2	3.10	0.00
6	6.08	d	2	-	6.03	0.05
8	6.06	d	2	-	6.03	0.03
2',3',4',5',6'	7.37-7.47	m	-	-	7.40	-
5-OH	12.00	s	-	-	12.00	0.00
7-OMe	3.80	s	-	-	3.90	-0.10

* Burke, B. and Nair, M. 1986. Phytochemistry. 25. 1427-1430.

The ^{13}C NMR spectrum showed 16 carbons signals including one methyl, one methylene, 8 methine and 6 quarternary carbon atoms. Among these signals the carbon signal of methoxy group is clearly assigned due to the presence of chemical shift 55.61. Assigned and confirmed by using HMQC and HMBC techniques (Table 4-2), 6 carbon atoms of mono-substituted aromatic ring (ring B) are located at δ 138.31 (C-1'), 126.07 (C-2', C-6') and 128.80 (C-3', C-4' and C-5'). The spectrum showed 6 signals of carbon in Ring A, located at δ 103.05 (C-4a), 162.70 (C-5), 95.05 (C-6), 164.06 (C-7), 94.18 (C-8) and 167.89 (C-8a). At ring C, the chemical shift of a carbonyl carbon was assigned at δ 195.70 (C-4) and two carbon signal (C-2 and C-3) were located at δ 79.13 and 43.28, respectively. The CD spectrum (Figure A-4) of compound **1** exhibited the negative Cotton effect ([θ]

$_{288}$ = -8250). This agreed with a *S* configuration at C-2. This assignment is supported by the comparison with that previously reported by Burke and Nair (1986). All of data were in agreement with the structure of (-) (*S*)-5-hydroxy-7-methoxyflavanone or pinostrobin (Figure 4-1).

Table 4-2 ^{13}C NMR data HMQC and HMBC of compound **1** (500 MHz, $CDCl_3$)

C	**δ (ppm)**	**HMQC**	**HMBC**	**Literature* δ (ppm)**	**Δ δ (ppm)**
2	79.13	2	-	78.89	0.24
3	43.28	3	-	43.05	0.23
4	195.70	-	-	197.25	-1.55
4a	103.05	-	6,8,5-OH	102.80	0.25
5	162.70	-	5-OH	163.80	-1.10
6	95.05	6	5-OH	94.80	0.25
7	164.06	-	-	167.62	-3.56
8	94.18	8	-	93.93	0.25
8a	167.89	-	-	167.62	0.27
1'	138.31	-	1'	138.02	0.29
2',6'	126.07	-	-	125.81	0.26
3',4',5'	128.80	-	-	128.54	0.26
7-OMe	55.61	7-OMe	-	55.36	0.25

* Burke, B. and Nair, M. 1986. Phytochemistry. 25. 1427-1430.

4.1.2 Identification of compound 2

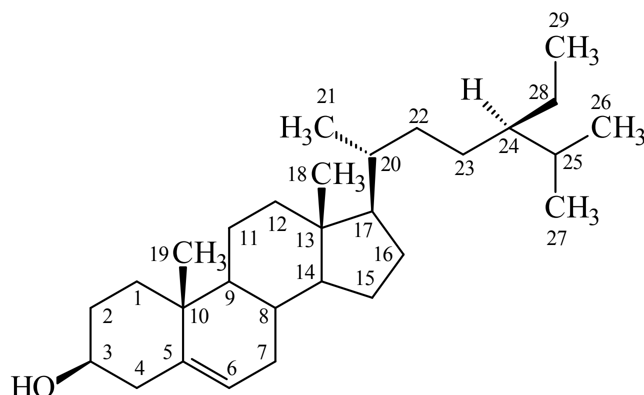


Figure 4-2 Structure of compound **2**

Compound **2** was obtained and re-crystallized as needles from acetone, with m.p. 266-270˚C. Mass spectrum of this compound show the molecular ion peak at m/z 576.

The ^{1}H NMR spectrum shown 49 proton signals, separated to 2 area, upfield (δ 0.6-2.3) and downfield (δ 3.0-5.4). Signals of six methyl groups were relatively upfield at between δ 0.6-2.3 including two singlet, three doublet and one triplet. A hydroxylated proton (H-3) was relatively located at downfield at δ 3.53, multiplet and a olefin methine proton (H-6) at δ 5.35, multiplet. The ^{1}H NMR chemical shift assignments of the methyl protons were mostly in accordance with those of reference

In comparison with authentic β-sitosterol; m.p., TLC, ^{1}H NMR spectrum, the characteristic of compound **2** was identified.

Table 4-3 ^{1}H NMR data of compound **2** (500 MHz, $CDCl_3$)

H	***δ* (ppm)**	**M**	**J (Hz)**	**Literature[*] *δ* (ppm)**	**Δ *δ* (ppm)**
3	3.53	m	-	3.52	0.01
6	5.35	dd	2.0, 5.1	5.35	0.00
18	0.68	s	-	0.68	0.00
19	1.01	s	-	1.01	0.00
21	0.92	d	4.0	0.92	0.00
26	0.83	d	8.3	0.83	0.00
27	0.79	d	8.5	0.81	-0.02
29	0.82	t	6.6	0.84	-0.02

* Kojima *et al.*,1990. phytochemistry. 2351-2355.

4.1.3 Identification of compound 3

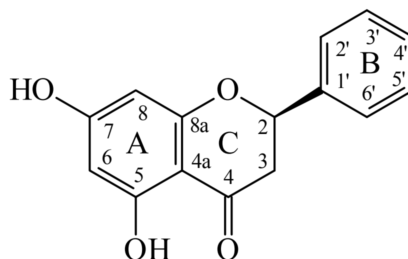


Figure 4-3 Structure of compound **3**

Compound **3** was isolated and re-crystallized as colorless crystalline from acetone, m.p. 185-186˚C. The UV spectrum (Figure A-11) shows the maximum absorption at λ 289 nm (ε 2.14 x 10^4, c 0.001 in MeOH). Its IR spectraum (Figure A-12) showed absorption band at 3400 (OH streching), 1640 (C=O stretching) and 1600 (C=C stretching) cm^{-1}, repectively. The MS spectrum (FAB) showed the molecular ion peak at m/z 256, indicated the formal formula $C_{15}H_{14}O_4$.

In the ^{1}H NMR spectrum (Table 4-4), one-proton singlet at δ 12.00, interchangeable with D_2O, sited the free hydroxyl group (position 5) with a H-bridge to O at the carbonyl (position 4). Two doublets at δ 6.00 (J = 2 Hz) and 6.01 (J = 2 Hz) attributable to *meta* H-6 and H-8. A five-proton multiplet at δ 7.40-7.47 confirmed the non-substitution of the B-ring. The characterized of the ^{1}NMR was the absence of the typical flavanone H-3 proton and the presence of an ABX system. The X part appeared as a doublet-doublet for a proton center at δ 5.43 (J = 13, 3 Hz) attributable to H-2 and the AB portion, as two doublet-doublet of one proton each at δ 2.83 (J = 17, 3 Hz) and 3.10 (J = 17, 13 Hz) attributable to H-3 (*cis*) and H-3 (*trans*), respectively.

Table 4-4 ^{1}H NMR data of compound **3** (500 MHz, $CDCl_3$)

H	δ (ppm)	M	J (Hz)	Literature*,** δ (ppm)	Δ δ (ppm)
2	5.43	dd	13,3	5.58	-0.15
3-*cis*	2.83	dd	17,3	2.72	0.11
3-*trans*	3.10	dd	17,13	3.23	-0.13
6	6.00	d	2	5.90	0.10
8	6.01	d	2	5.93	0.08
2'-6'	7.40-7.47	m	-	7.55-7.41	-
5-OH	12.00	s	-	12.13	-0.13

* Liu *et al*. 1992. J. Nat .Prod. 55. 357-363.

**The 1H NMR was recorded in DMSO-d_6 with TMS as standard.

The ^{13}C NMR spectrum showed 15 carbons signals including one methylene, 8 methine and 6 quarternary carbon atoms. Assigned and confirmed by using HMQC and HMBC techniques (Table 4-5), 6 carbon atoms of mono-substituted aromatic ring (ring B) are located at δ 138 .22 (C-1'), 126.13 (C-2', C-6'), 128.88 (C-3', C-4') and 128.91 (C-5'). The spectrum showed 6 signals of carbon in Ring A, located at δ 103.18 (C-4a), 164.30 (C-5), 96.73 (C-6), 164.59 (C-7), 95.48 (C-8) and 163.50 (C-8a). At ring C, the chemical shift of a carbonyl carbon was assigned at δ 195.83 (C-4) and two carbon signals (C-2 and C-3) were located at δ 79.21 and 43.29, respectively.

Table 4-5 ^{13}C NMR data of compound **3** (500 MHz, $CDCl_3$)

C	**δ (ppm)**	**Literature*, ** δ (ppm)**	**Δ δ (ppm)**
2	79.21	78.20	1.01
3	43.29	42.00	1.29
4	195.83	195.50	0.33
4a	103.18	101.6	1.58
5	164.30	163.50	0.80
6	96.73	95.80	0.93
7	164.59	166.60	-2.01
8	95.48	94.90	0.58
8a	163.50	162.60	0.90
1'	138.22	138.60	-0.38
2', 6'	126.13	126.40	-0.27
3', 5'	128.88	128.70	0.18
4'	128.91	128.70	0.21

* Liu *et al.* 1992. J. Nat .Prod. 55. 357-363.

** The 13C NMR was recorded in DMSO-d_6 with TMS as standard.

In comparison with ^1H and ^{13}C NMR spectra and data of compound **1** (pinostrobin), compound **3** show the similar signals. The differences of ^1H NMR between compound **1** and **3** were the absence of 7-OMe (δ 3.80) in compound **3** and the integration of the area under peak of proton at δ 7.3 - 7.4 from 5 in compound **1** to 6 in compound **3.** Moreover, the comparison of the ^{13}C NMR spectrum of compound **3** with that of compound **1** showed that the chemical shifts of the carbon atoms of compound **3**, except 7-OMe, are similar to those of the relevant carbon

atoms of compound **1**. This indicates that compound **3** has a hydroxyl group at C-7 not a methoxyl group as occurs in compound **1**. Finally, the compound **3** can be clearly established as 5,7-dihydroxyflavanone or pinocembrin (Figure 4-3) from the aforementioned spectroscopic data.

4.1.4 Identification of compound 4

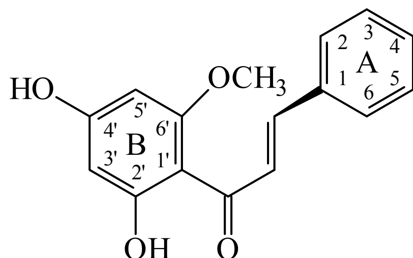


Figure 4-4 Structure of compound **4**

Compound **4** was isolated and re-crystallized as yellow crystalline from acetone, with m.p. 199-200˚C. The UV spectrum (Figure A-15) shows the maximum absorption at λ 343 nm (ε 3.82 x 10^4, c 0.0005 in MeOH). The IR spectraum (Figure A-16) had bands at 3170 (OH-stretching) and 1625 (C=O-stretching) cm^{-1}, respectively. The MS spectrum (FAB) showed the molecular ion peak at m/z 270, indicated the formal formula $C_{16}H_{14}O_4$.

In the ^1H NMR spectrum (Table 4-6) exhibited the resonance of two hydroxyl groups at δ 13.68 (s), interchangeable with D_2O, sited the free hydroxyl group (position 5) with a H-bridge to O at the carbonyl (position 4) and at δ 10.64 (s, br). A sharp singlet signal at δ 3.87 with 3 protons integration was assigned methoxy group at 6'-OMe. The two doublets at δ 5.92 (J = 2 Hz) and 6.02 (J = 2 Hz) attributable to *meta* H-3' and H-5', respectively. A five-proton multiplet at δ 7.40-7.71 confirmed the non-substitution of the aromatic ring (H-1 – H-6). Furthermore, there have 2 olefinic *trans* protons signal at δ 7.63 (J = 16 Hz) and 7.88 (J = 16 Hz) attributable to H_α and H_β, respectively.

Table 4-6 ^{1}H NMR data and ^{1}H -^{1}H cosy of compound **4** (500 MHz, DMSO-d_6)

H	**δ (ppm)**	**M**	**J (Hz)**	**H-H cosy**	**Literature[*, **] δ (ppm)**	**Δ δ (ppm)**
2 , 6	7.71		-	4	-	-
3 , 5	7.45	m	-	2	-	-
4	7.71		-	-	-	-
α-H	7.63	d	16	β-H	7.80	-0.17
β-H	7.88	d	16	α-H	7.94	-0.06
3'	5.92	d	2	5'	6.02	-0.10
5'	6.02	d	2	3'	6.08	-0.06
2'-OH	13.68	s	-	-	14.14	0.46
4'-OH	10.64	s	-	-	-	-
6'-OMe	3.87	s	-	-	3.98	-0.11

* Itokawa *et al*. 1981. Phytochemistry. 20. 2503-2506.

** The 1H NMR was recorded in $(D_3C)_2CO$-d_6 with TMS as standard.

The ^{13}C NMR spectrum showed 16 carbon signals including one methyl group, 9 methin and 6 quarternary carbon atoms. Assigned and confirmed by using HMQC and HMBC techniques (Table 4-7), 6 carbon atoms of mono-substituted aromatic ring are located at δ 135.1 (C-1), 128.8 (C-2), 129.7 (C-3), 127.5 (C4), 129.5 (C-5) and 128.4 (C-6). The spectrum showed 6 signals of carbon in tetra-substituted aromatic ring indicated at δ 104.3 (C-1'), 164.5 (C-2'), 95.6 (C-3'), 165.2 (C-4)', 94.3 (C-5') and 164.5 (C-6'). However, there have two olefinic carbon atoms at δ 128.3 (C_α) and 141.9 (C_β), respectively.

The assignment of compound **4** is supported by the comparison with that previously reported by Itokawa *et al*. (1981). All of data were in agreement with the structure of 2',4'-dihydroxy-6'-methoxy chalcone or cardamonin (Figure 4-4).

Table 4-7 ^{13}C NMR data HMQC and HMBC of compound **4** (500MHz,DMSO-d_6)

C	δ (ppm)	HMQC	HMBC	Literature*,** δ (ppm)	$\Delta\ \delta$ (ppm)
1	135.08	-	α-H	136.5	-1.42
2,6	128.51	2,6	β-H	129.0	-0.49
3,5	129.19	3,5	2	129.7	-0.51
4	130.45	4	-	130.7	-0.25
α-C	127.67	α-H	β-H	128.6	-0.93
β-C	141.92	β-H	α-H, 2, 6	142.4	-0.48
C=O	191.92	-	-	193.0	-1.08
1'	105.31	-	3',5',2'-OH	106.4	-1.09
2'	166.37	-	3'	168.3	-1.93
3'	95.99	3'	5'	97.0	-1.01
4'	165.17	-	3', 5'	165.8	-0.63
5'	91.86	5'	3'	92.3	-0.44
6'	162.84	-	5', 6'-OMe	164.3	-1.46
6'-OMe	56.18	6'-OMe	-	56.3	-0.12

* Itokawa *et al*. 1981. Phytochemistry. 20. 2503-2506.

** The 1H NMR was recorded in $(D_3C)_2CO$-d_6 with TMS as standard.

4.1.5 Identification of compound 5

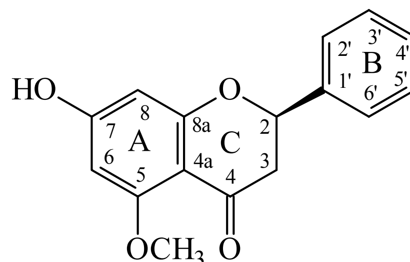


Figure 4-5 Structure of compound **5**

Compound **5** was obtained and re-crystallized as colorless crystalline from acetone, with m.p. 219-220°C. The UV spectrum (Figure A-24) shows the maximum absorption at λ 285 nm (ε 1.74 x 10^4, c 0.001 in MeOH). The molecular formula was suggests as $C_{16}H_{14}O_4$, the molecular ion peak at m/z 271.0970 (calc. 271.2927) in the HR-FAB mass spectrum and confirmed by the NMR spectrum data (Table 4-8), exhibited IR absorption band at 3500, 3350, 3100 (OH-streching), 1600 (C=O-stretching) and 1580 (C=C-stretching) cm^{-1}, respectively (Figure A-25).

In the ^1H NMR spectrum (Table 4-8), a methyl group was seen as a sharp singlet at δ 3.73 (5-OMe). A one-proton singlet (broad) at δ 10.54 (7-OH), interchangeable with D_2O. Two doublets at δ 6.00 (J = 2 Hz) and 6.07 (J = 2 Hz) attributable to *meta* H-6 and H-8, placed the methoxyl group on C-7. A five-proton multiplet at δ 7.37-7.47 (C-2' – C-6') confirmed the non-substitution of the B-ring. The characterized of the ^1H NMR was the absence of the typical flavanone H-3 proton and the presence of an ABX system. The X part appeared as a doublet-

doublet for a proton center at δ 5.48 (J = 16, 3 Hz) attributable to H-2 and the AB portion, as two doublet-doublet of one proton each at δ 2.62 (J = 16, 12 Hz) and 2.97 (J = 12, 3 Hz) attributable to H-3 (*cis*) and H-3 (*trans*), respectively.

Table 4-8 ^{1}H NMR data and ^{1}H -^{1}H cosy of compound **5** (500MHz, DMSO-d_6)

H	**δ (ppm)**	**M**	**J (Hz)**	**H-H cosy**	**Literature*, ** δ (ppm)**	**Δ δ (ppm)**
2	5.48	dd	16,3	3	5.44	0.04
3-*cis*	2.62	dd	16,12	-	2.59	0.03
3-*trans*	2.97	dd	12,3	3-*cis*	2.98	-0.01
6	6.00	d	2	6	5.98	0.02
8	6.07	d	2	8	6.06	0.01
2',3',4',5',6'	7.36-7.49	m	-	-	7.40	-
5-OMe	3.73	s	-	-	3.72	0.01
7-OH	10.54	s (br.)	-	-	-	-

* Itokawa *et al*. 1981. Phytochemistry. 20. 2503-2506.

** The 1H NMR was recorded in DMSO-d_6 with TMS as standard.

The ^{13}C NMR spectrum showed 16 carbons signals including one methyl, one methylene, 8 methin and 6 quarternary carbon atoms. Among these signals the carbon signal of methoxy group is clearly assigned due to the presence of chemical shift 55.77 (5-OMe). Assigned and confirmed by using HMQC and HMBC techniques (Table 4-9), 6 carbon atoms of mono-substituted aromatic ring (ring B) are located at δ 139.31 (C-1'), 126.55 (C-2', C-6'), 128.63 (C-3', C-5') and 128.44 (C-4'). The spectrum showed 6 signals of carbon in Ring A, located at δ 104.66 (C-4a), 164.16 (C-5), 95.79 (C-6), 164.49 (C-7), 93.52 (C-8) and 162.35 (C-

8a). At ring C, the chemical shift of a carbonyl carbon was assigned at δ 187.43 (C-4) and two carbon signal (C-2 and C-3) were located at δ 78.17 and 44.99, respectively.

The ^{1}H and ^{13}C NMR spectra of compound **5** show similar signals to that compound **1**. The only difference between compound **1** and compound **5** were the resonance of proton at the lowest field. The compound **1** has a characteristic pattern of a sharp singlet signal at δ 12.00, interchangeable with D_2O, sited the free hydroxyl (position 5) with a H-bridge to O at the carbonyl (position 4), but compound **5** has only a broad singlet signal at δ 10.54 ($\Delta\ \delta$ = 1.46), that mean in compound **5** the H-bonding was disappeared. This assignment is supported by the comparison with that previously reported by Itokawa *et al*., (1973). All of data were in agreement with the structure 5-Methoxy-7-hydroxyflavanone or alpinetin (Figure 4-5). This compound was isolated from *Alpinia speciosa* (Itokawa *et al*., 1973).

Table 4-9 ^{13}C NMR data HMQC and HMBC of compound **5** (500 MHz, DMSO-d_6)

C	***δ* (ppm)**	**HMQC**	**HMBC**	**Literature[*, **] *δ* (ppm)**	**Δ *δ* (ppm)**
2	78.17	2	3-*trans*	78.10	0.07
3	44.99	3	-	45.00	-0.01
4	187.43	-	3	187.40	0.03
4a	104.66	-	6,8	104.60	0.06
5	164.16	-	6	164.10	0.06
6	95.78	6	8	95.80	-0.02
7	164.49	-	8	164.40	0.09
8	93.52	8	6	93.50	0.02
8a	162.35	-	8,5-OMe	162.20	0.15
1'	139.31	-	-	139.20	0.11
2',6'	126.55	-	-	126.40	0.15
3',5'	128.63	-	-	128.50	0.13
4'	128.44	-	-	128.30	0.14
5-OMe	55.77	5-OMe	-	55.70	0.07

* Itokawa *et al*. 1981. Phytochemistry. 20. 2503-2506.

** The 1H NMR was recorded in DMSO-d_6 with TMS as standard.

4.2 Total phenolic content

Source of antioxidant compounds have been searched from several types of plant materials, and using a number of different methods. Flavonoids and other plant phenolics are especially common in plant. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Rice-Evans *et al*., 1998). The purpose of this study is to screen the total phenolic content and antioxidative activity of extracts from the rhizome of *Boesenbergia pandurata*.

The total phenolic content by Folin-Ciocalteu colorimetric assay applied in this study is a simple method and requires a few reagents, thus being suitable for crude estimation of the content of total phenols, even though it is limited by the low specificity toward polyphenols, and relies on the use of a standard compound (Mosca *et al*., 2000).

The results of total phenolic contents of four fractions from crude ethanolic extract are given in Table 4-10. The total phenolic content of crude ethanolic extract measured by the Folin-ciocalteu method was found to be 10.6 $\pm$ 0.2 %w/w (determinated as caffeic acid). The highest total phenolic content occurred in the dichloromethane fraction of crude extract of *Boesenbergia pandurata*. The lowest was represented by the methanol fraction.

Table 4-10 Total phenolic content of fractions from ethanolic extract of *B. pandurata*

Sample	**Total phenolics (%w/w)**
Crude ethanol extract	10.6±0.2 c
n-Hexane	12.4±0.1 b
Dichloromethane	13.5±0.2 a
Ethyl acetate	7.6±0.1 d
Methanol	6.8±0.1 e
F-test	**
C.V. (%)	1.27

Mean ± S.D. (n = 3) followed by the different letters in the same column denote the significant differences, according to Duncan's multiple range test.

** significant at $p<0.01$

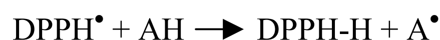
4.3 Antioxidant activity

The fractions prepared by using a variety of solvents, including n-hexane, dichloromethane, ethyl acetate and methanol were tested for antioxidant effect. The tests can be categorized into two groups: assays for radical scavenging ability and assays that test the ability to inhibit lipid oxidation under accelerated conditions.

4.3.1 Free radical scavenging activity by DPPH assay

Various phytochemical components, such as flavonoids, phenylpropanoids and phenolic acids, are known to be responsible for the antioxidant capacity of fruits, vegetables and spices. Free radical scavenging is generally accepted to be the means by which antioxidant compounds inhibit lipid peroxidation.

The method employed is based on the reduction of an alcoholic DPPH solution at 520 nm in the presence of a hydrogen donating antioxidant (AH) due to the formation of the non-radical form (DPPH-H), according to the following reaction:



$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$

The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the sample. Through radical-radical interactions, the radical $A^{\bullet}$ can contribute to the formation of stable molecules. This method is simple, rapid and sensitive. No expensive reagents or sophisticated instrumentation are required.

Different extraction media have been tried to ensure the maximum extraction of the available antioxidants from the samples (Kahkonen *et al*., 1999). In the present study, two different solvent extractions viz. water and ethanol were used. Ethanol was found to be more efficient than water in extracting the antioxidants present in the *B. pandurata* extracts. The antioxidant activities of aqueous and ethanolic extracts of the *B. pandurata* are given in Figure 4-6. The results indicated that antioxidant activity of *B. pandurata* ethanolic extract increased with increasing concentrations. Thus, crude ethanolic extract was able to scavenge the DPPH radical scavenging significantly.

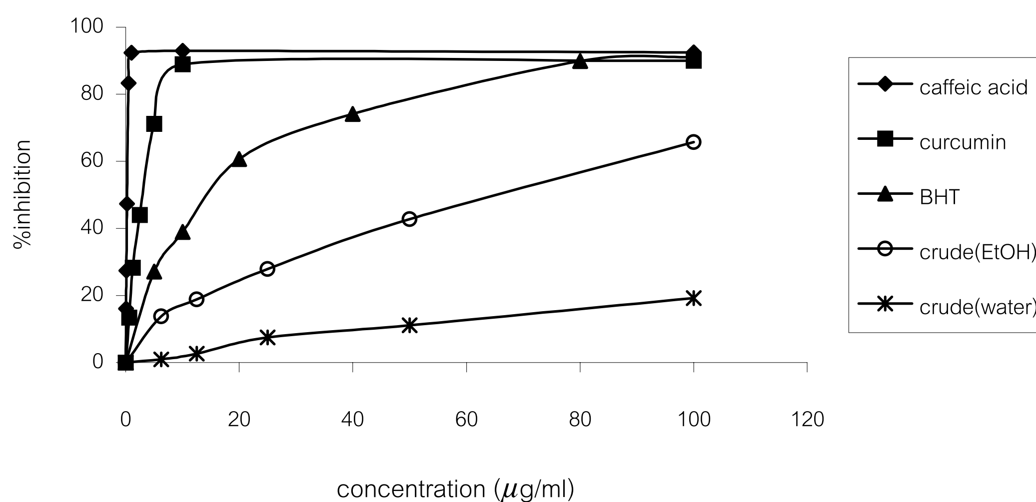


Figure 4-6 Radical scavenging effect of ethanolic extract of *B. pandurata* on the DPPH radical concentration (μg/m1)

The ethanolic extract of fresh rhizome of *B. pandurata* showed free radical scavenging activity (EC_{50}) against DPPH less than dichloromethane and and methanol fractions but higher than *n*-hexane and ethyl acetate fraction (Table 4-11).

Table 4-11 DPPH radical scavenging activity of *B. pandurata* rhizome extract.

Sample	**DPPH radical scavenging, EC_{50} (μg/ml)**
Crude ethanol extract	67.7$\pm$2.6 bc
n-Hexane fraction	75.7$\pm$1.9 a
CH_2Cl_2 fraction	67.5$\pm$1.3 bc
EtOAc fraction	71.7$\pm$0.6 ab
MeOH fraction	66.8$\pm$0.6 c
Curcumin (reference)	3.1$\pm$0.6
Caffeic acid (reference)	0.6$\pm$0.9
BHT (reference)	15.5$\pm$1.4
F-test	**
C.V. (%)	2.26

Mean $\pm$ S.D. (n = 3) followed by the different letters in the same column denote the significant differences, according to Duncan's multiple range test.

** significant at $p<0.01$

4.3.2 Free radical scavenging activity of pure compounds

In order to determine the active substances in the ethanolic extract, it was subjected to phytochemical investigation. Pinostrobin , β-sitosterol , pinocembrin, cardamonin and alpinetin, were isolated and evaluated for the antioxidant activity by DPPH assay. The result showed that pinocembrin exhibited the strong DPPH radical scavenging activity (Table 4-12). However, it indicated the ability of that pinocembrin as radical scavenger was lower than crude extract and fractions of *B. pandurata*.

Table 4-12 DPPH radical scavenging activity of compounds isolated from *B .pandurata*

Compounds	**DPPH radical scavenging, EC_{50} (μg/ml)**
pinostrobin	> 500
β -sitosterol	> 500
pinocembrin	294.7
cardamonin	> 500
alpinetin	> 500

The antioxidant activity of the individual compounds are lower than the crude extract. The results reported by Lu and Foo (2001) indicated that the structure in the B-ring is important for enhancement of radical-scavenging activity. These isolated compounds lack of an ortho-dihydroxyl moiety in their B-ring, thus possess rather weak antioxidant activity.

4.3.3 Inhibition of autoxidation on linoleic acid system

Various phytochemical components, such as flavonoids, phenylpropanoids and phenolic acids, are known to be responsible for the antioxidant capacity of fruits, vegetables and spices. Free radical scavenging is generally accepted to be the means by which antioxidant compounds inhibit lipid peroxidation.

Table 4-13 Inhibition of autoxidation of ethanolic extract from *B. pandurata* rhizome on the linoleic acid system

Sample	**Linoleic acid autoxidation, %Inhibition (at 100 μg/ml)**
Crude ethanol extract	64.4 a
n-Hexane fraction	42.1 d
CH_2Cl_2 fraction	54.3 b
EtOAc fraction	45.5 c
MeOH fraction	42.2 d
Caffeic acid (reference)	74.9
BHT (reference)	89.0
F-test	**
C.V. (%)	0.79

Mean $\pm$ S.D. (n = 3) followed by the different letters in the same column denote the significant differences, according to Duncan's multiple range test.

** significant at p<0.01

4.4 HPLC analysis

4.4.1 Selection of the mobile phase

The phenolic compounds of natural origin have the positive property of being soluble in polar solvents. This leads to the possibility of using reversed phase HPLC (RP-HPLC) in their analysis, sufficient retention being achieved by using acidic conditions in order to avoid the presence of ionized forms of the analyzes. Octadodecyl Silane (ODS, C18, RP-18) is by far the most popular of the stationary phases, used for general compounds or phenolics separation. However, polymeric condensed tannins cannot be analysed using RP-HPLC because of too strong sorption to the stationary phases (Schofield *et.al*. 1998). Condensed tannins therefore have to be analysed using the normal phase HPLC technique, which is not so widely used since it has much poorer resolution compared to RP-HPLC (Waksmundzka-hajnos 1998).

The eluents used in the RP-HPLC analysis of phenolics are mixtures of pH modifiers with a polar, water-soluble organic solvent: methanol (MeOH) or acetonitrile (ACN). The pH is a major factor especially in the separation of ionisable compounds such as phenolic acids. (Waksmundzka-hajnos 1998).

Another positive property of phenolic compounds is their conjugated C-C double bonds which act as chromophores. A combination of RP-HPLC and an ultraviolet/visible light (UV/Vis) detector is commonly used in both qualitative and quantitative analysis of nature-derived samples containing phenolics (Markham and Bloor 1998). The UV wavelength of 280 nm has proved to be suitable for the

universal detection of all phenolics, ranging from simple hydroxybenzoic acids to larger tannins.

The ultimate goal of good HPLC chromatogram is the attainment of sharp well-resolved peaks in the minimum analysis time.

A total of four mobile phase from recent registered cases were applied to separate pure compounds; pinostrobin (**1**), pinocembrin (**3**), cardamonin (**4**) and alpinetin (**5**); and retention time (t_R), tailing factor (T_f), selectivity factor (α), capacity factor (k') and resolution (Rs) obtained from each mobile phase calculated (M1-M4, see Table 3-1). Based on the literature, the criteria for a good separation are: tailing factor (T_f) less than 2, number of theoretical plates (N) not more than 2000, resolution (Rs) more than 2 and retention time (t_R) not more than 30 mins. The results showed that the resolution was less than 2 for mobile phase (M1). The retention times were too long for mobile phase (M3 and M4). Therefore, M2 was chosen as the mobile phase for later study. The compound with a retention time of 26.0 min has high content in the crude extract. As the result showed, with the exception of mobile phase containing 0.5% aqueous acetic acid and acetonitrile with ratio of 45:55. The chromatographic parameters of interested peaks are listed in Table 4-14.

Table 4-14 Chromatographic parameters from HPLC chromatogram of ethanolic extract from *B. pandurata* by using 0.5% aqueous acetic acid:acetonitrile (45:55) as mobile phase (M2)

Compound	**Parameter**					
	$\mathbf{t_R}$	**k**	**α**	**Rs**	**N**	$\mathbf{T_f}$
Pinostrobin	26.29	12.20	1.68	9.41	4255	0.83
Pinocembrin	10.01	4.02	1.18	2.22	2844	1.00
Cardomonin	16.44	7.25	1.38	7.11	17424	1.13
Alpinetin	5.03	1.52	1.32	3.20	2844	1.25

The chromatogram obtained from the extract of *B. pandurata* was shown in Figure 4-7. Eleven well-resolved peaks identified by relative retention time and characterized of spectra (Figure 4-8) as:

Peak at t_R = 5.0 min corresponded to alpinetin

Peak at t_R = 10.0 min corresponded to pinocembrin

Peak at t_R = 16.0 min corresponded to cardamonin

Peak at t_R = 26.0 min corresponded to pinostrobin

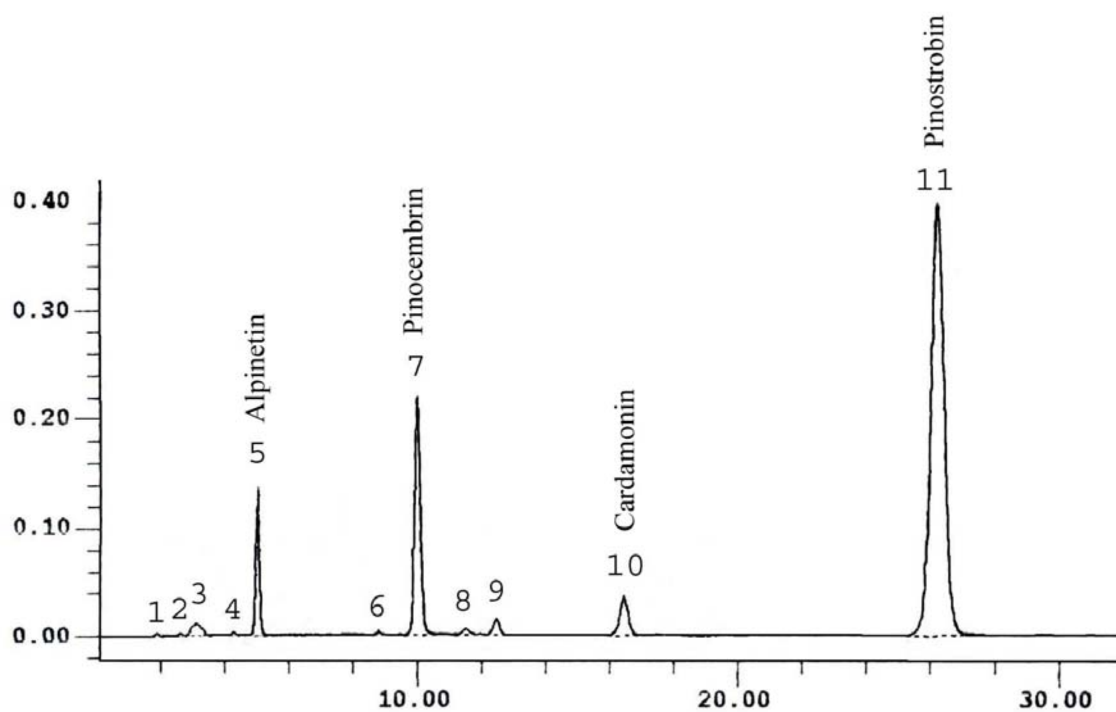


Figure 4-7 Chromatogram of the ethanolic extract from *Boesenbergia pandurata.*

Chromatographic conditions: as described in experimental section

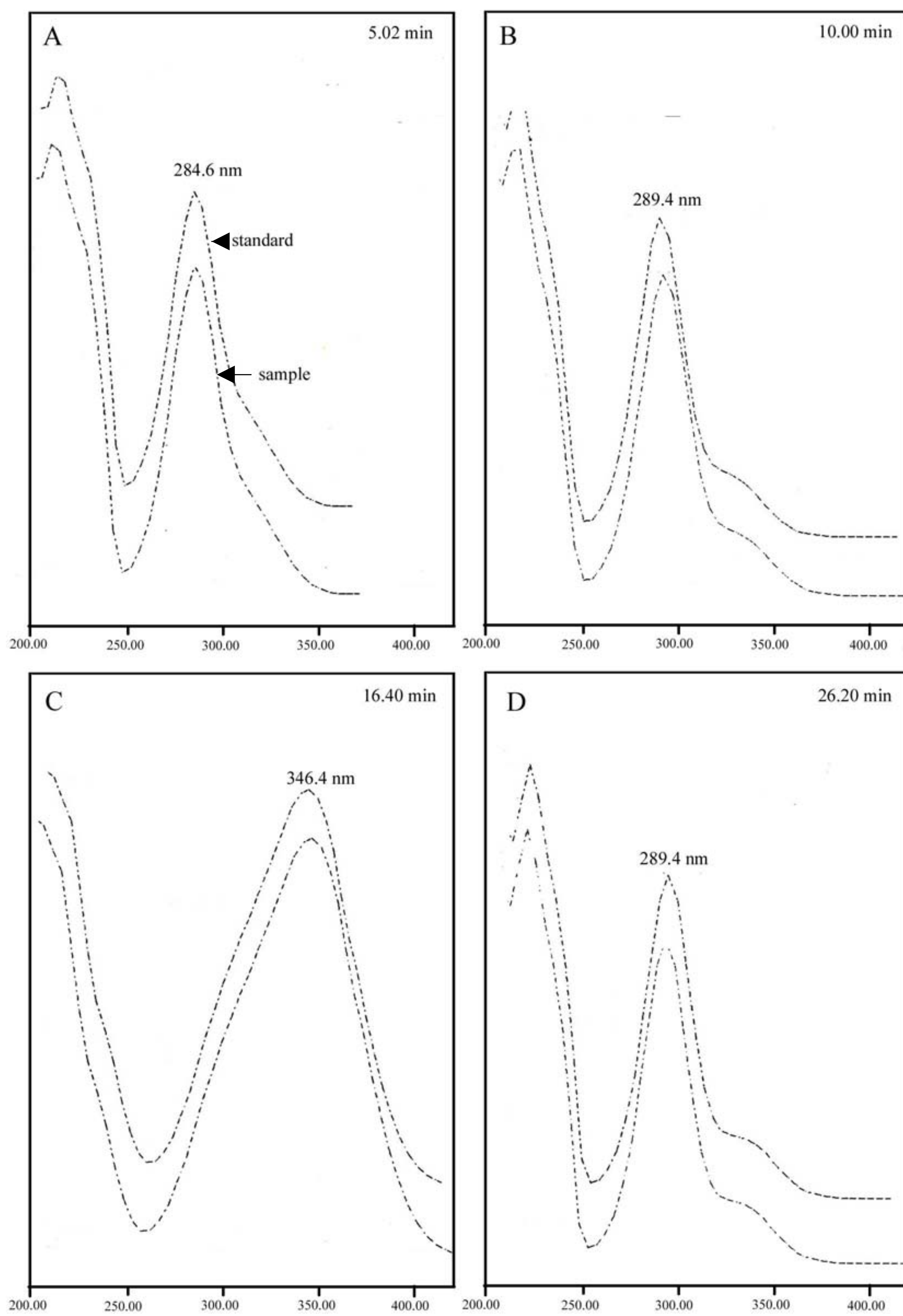

A
5.02 min
284.6 nm
standard
sample
B
10.00 min
289.4 nm
C
16.40 min
346.4 nm
D
26.20 min
289.4 nm
200.00
250.00
300.00
350.00
400.00

Figure 4-8 UV spectra of alpinetin (A); pinocembrin (B); cardamonin (C) and pinostrobin (D) over the wavelength range 200-400 nm

4.4.2 Calibration curve of pinostrobin

A linear relationship between the relative peak areas versus pinostrobin concentrations (12.5-200 μg/ml) was observed with coefficient of determination (r^2) = 0.9999 and regression equation of Y = 57349X – 187012 (Figure 4-9). This calibration curve was used for quantitative determination of pinostrobin contents in *Boesenbergia pandurata* in the next section.

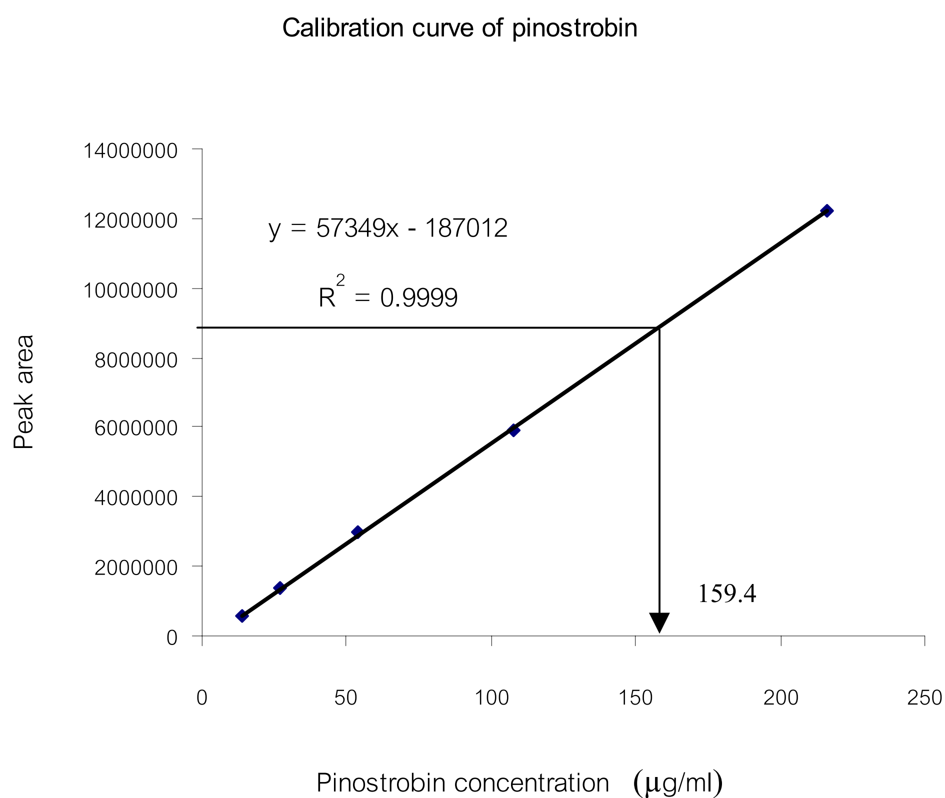


Figure 4-9 A calibration curve of pinostrobin at λ 280 nm

The repeatability test for precision demonstrated that the analyzed peaks presented as %RSD in Table 4-15. Rather high repeatability in the peak areas, intra-day and inter-day relative standard deviations (%RSD) were always better than 2 and 3 %, respectively. According to the USP25/NF20 criteria should not more than 2%.

Table 4-15 Intra-day and inter-day precisions of pinostrobin; n=3

Concentration	Mean area * (%RSD)	
(μg/ml)	Intra-day	Inter-day
12.5	610316 (1.7)	600738 (1.6)
25	1331279 (1.8)	1356469 (1.8)
50	2911305 (1.4)	2956565 (2.8)
100	5906185 (1.1)	5919958 (1.3)
200	11880328 (1.2)	12176391 (2.1)

Pinostrobin contents extracted from fresh rhizome of *B. pandurata* was found to be 0.5 % w/w.

4.5 Application for commercial dry crudes of *B. pandurata* rhizome

Five crude of *B. pandurata* rhizome were randomly selected from local Thai herb drug stores in Hat-Yai and analyzed using the current methods. Chromatographic profiles of their ethanolic extract exhibited similarly as shown in Figure 4-10.

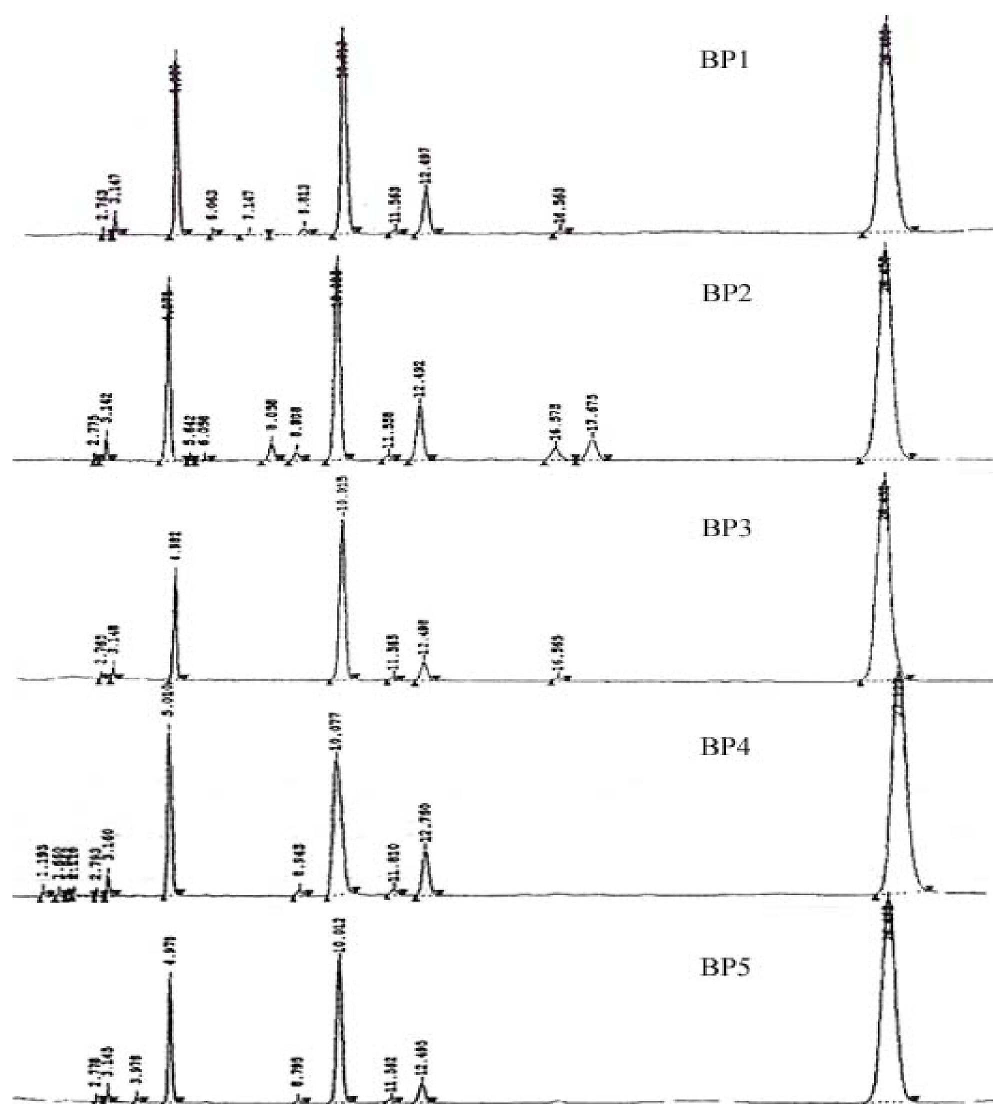


Figure 4-10 Chromatographic profile of ethanolic extract from five commercial *B. pandurata* rhizome

Data showed the existence of a correlation between the pinostrobin content in crude extract and the antioxidant activity. Of the five samples tested, *B. pandurata* was the richest in pinostrobin content (Figure 4-11) and its extracts proved the most effective on quenching free radicals. BP2 had the highest content of pinostrobin and its extracts displayed the highest activity againtst DPPH radicals (Table 4-16).

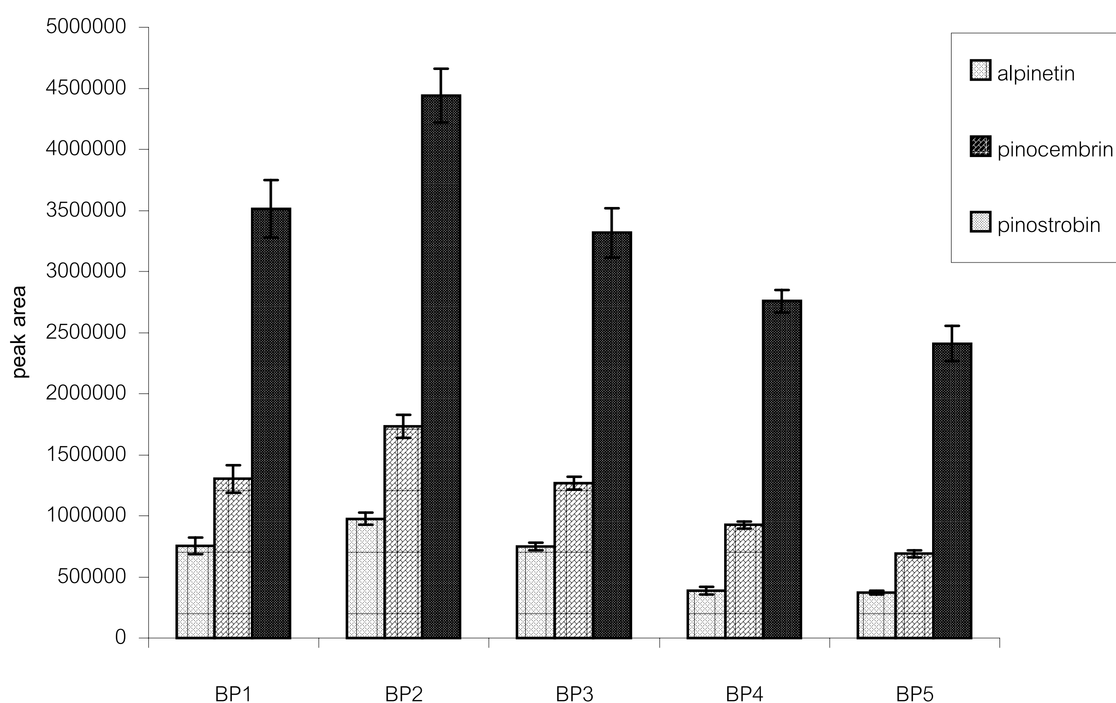


Figure 4-11 Average peak areas of alpinetin (**5**), pinocembrin (**3**) and pinostrobin (**1**) in crude drug of *B. pandurata* from five sources; n=3

Table 4-16 Peak area and ratio of alpinetin, pinocembrin and pinostrobin from five *B. pandurata* samples (BP1-BP5)

samples	Mean area* (SD) x 10^5			Ratio
	Alpinetin (**5**)	Pinocembrin (**3**)	Pinostrobin (**1**)	Al:Pc:Ps**
BP1	7.54 (0.70)	13.02 (1.11)	35.12 (2.34)	1:2:5
BP2	9.76 (0.48)	17.32 (0.94)	44.37 (2.01)	1:2:5
BP3	7.49 (0.30)	12.65 (0.52)	33.17 (1.19)	1:2:5
BP4	3.88 (0.30)	9.24 (0.30)	27.57 (0.35)	1:2:5
BP5	3.70 (0.14)	6.90 (0.29)	24.10 (1.01)	1:2:5

* Mean from triplicate determinations

** Al = Alpinetin, Pc = Pinocembrin, Ps = Pinostrobin

Table 4-17 Pinostrobin contents (%w/w) and DPPH radical scavenging activity of five *B. pandurata* samples (BP1-BP5)

Sample	Pinostrobin content (%w/w) ± SD*	DPPH radical scavenging EC_{50} (mg/ml) ± SD*
BP1	3.2 ± 0.2	0.97 ± 0.04
BP2	4.0 ± 0.2	0.62 ± 0.06
BP3	3.1 ± 0.1	1.07 ± 0.02
BP4	2.6 ± 0.0	1.51 ± 0.04
BP5	3.1 ± 0.0	1.22 ± 0.01

* Mean ± SD from triplicate determinations.

The correlation coefficient between the DPPH radical scavenging activity and pinostrobin content was determined (Figure 4-12). The values of the DPPH radical scavenging activity showed negative correlation coefficient (r) was 0.9668.

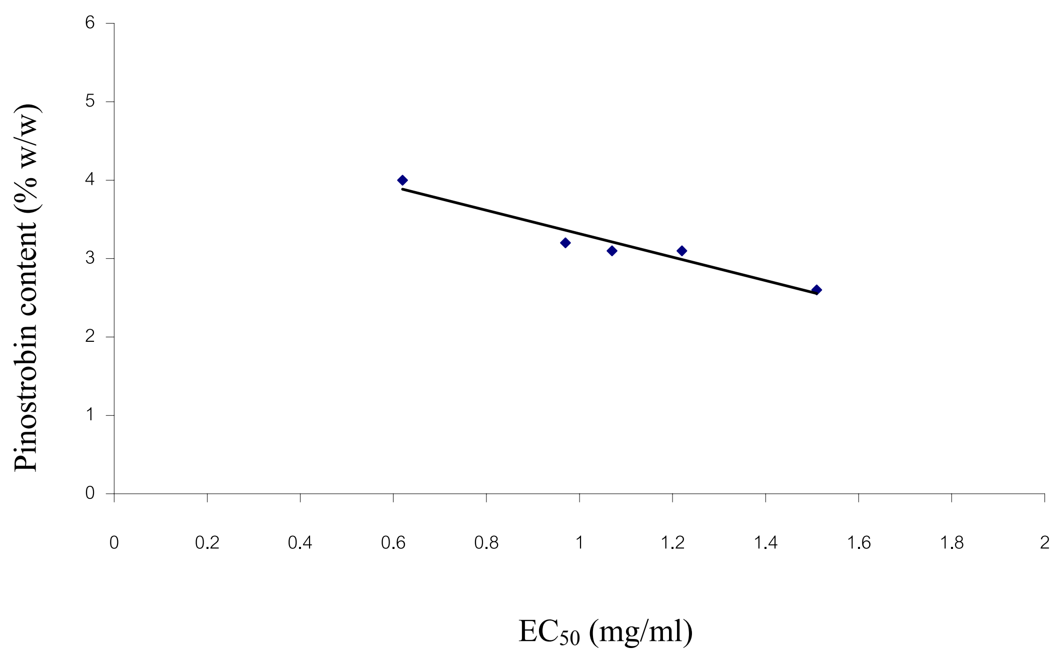


Figure 4-12 Free radical scavenging activity of crude drug of *B. pandurata* by DPPH assay and pinostrobin contents