CHAPTER 3

RESULTS AND DISCUSSION

3.1 Screening of biological activities of ethanolic extract and each fraction of *Smilax corbularia* Kunth

From the previous research (Tewtrakul *et al*., 2006), it was found that the ethanolic extract of *Smilax corbularia* possessed the most potent inhibitory activity against HIV-1 integrase with an IC_{50} value of 1.9 μg/ml, whereas the water extract showed less effect (IC_{50} = 5.4 μg/ml). The ethanolic extract exhibited the activity approximately two-fold lower than that of a positive control, suramin (IC_{50} = 3.4 μg/ml).

The ethanolic extract of *Smilax corbularia* was prepared as described in section 2.3. Table 3-1 showed the percent yield of each fraction of ethanolic extract (separated by VLC).

Table 3-1 Percent yield of each fraction of ethanolic extract from the rhizome of *Smilax corbularia* (separated by VLC)

Fractions	% Yield (w/w)
Hexane	0.05
Hexane:$CHCl_3$ (1:1)	0.68
$CHCl_3$	0.44
$CHCl_3$: MeOH 1:1(Supernatant)	70.23
$CHCl_3$: MeOH 1:1(Precipitate)	5.04
MeOH	23.31

The results showed that the $CHCl_3$: MeOH (1:1) supernatant fraction showed the highest percentage of yield (70.23 %)

3.1.1 Free radical scavenging activity

The antioxidant activity of the ethanolic extract and each fraction were tested by DPPH radical scavenging assay as described in section 2.4.1 and the results are shown in Table 3-2. The results of antioxidant activity by lipid peroxidation with liposome was described in section 2.4.2 and the results are showe in Table 3-3.

Table 3-2 EC_{50} (μg/ml) of crude ethanolic extract and each fraction tested by DPPH assay

Fractions	**EC_{50} (μg/ml)**
Crude ethanolic extract	4.1 ± 0.2
Hexane	>100
Hexane: $CHCl_3$ (1:1)	>100
$CHCl_3$	>100
$CHCl_3$: MeOH 1:1(Supernatant)	2.1 ± 1.0
$CHCl_3$: MeOH 1:1(Precipitate)	11.1 ± 0.9
MeOH	8.9 ± 0.1
BHT (Positive control)	11.2 ± 2.4

(n=3), n= number of independent experiment which was performed in 3 replicates

The $CHCl_3$: MeOH (1:1) supernate fraction showed the highest antioxidant activity by DPPH assay (EC_{50}=2.1 ± 1.0 μg/ml) followed by the ethanolic extract with the EC_{50} value of 4.1 ± 0.2 μg/ml. The $CHCl_3$: MeOH (1:1) precipitate and MeOH fractions possessed their EC_{50} values of 11.1 ± 0.9 and 8.9 ± 0.1 μg/ml, respectively. Interestingly, crude ethanolic extract and $CHCl_3$: MeOH (1:1) supernate, $CHCl_3$: MeOH (1:1) precipitate and MeOH fractions possessed high antioxidant activites with their value of EC_{50} less than 11.2 μg/ml, which were

lower than that of the standard antioxidant as BHT (EC_{50} = 11.2 ± 2.40 μg/ml). The hexane, hexane: $CHCl_3$ (1:1) and $CHCl_3$ fractions had the lowest antioxidant activity with the EC_{50} values of >100 μg/ml.

3.1.2 Lipid peroxidation of liposome assay

Table 3-3 EC_{50} (μg/ml) of crude ethanolic extract and each fraction of ethanolic extract (separated by VLC) on lipid peroxidation assay

Fractions	EC_{50} (μg/ml)
Crude ethanolic extract	3.4 ± 0.3
Hexane	>100
Hexane: $CHCl_3$ (1:1)	>100
$CHCl_3$	>100
$CHCl_3$: MeOH 1:1(Supernatant)	1.1 ± 0.1
$CHCl_3$: MeOH 1:1(Precipitate)	6.4 ± 0.2
MeOH	5.5 ± 0.0
BHT (Positive control)	6.9 ± 0.5

(n=3), n= number of independent experiment which was performed in 3 replicates

The results showed that the $CHCl_3$: MeOH (1:1) supernate fraction exhibited the highest antioxidant activity by this test with the EC_{50} value of 1.1 ± 0.1 μg/ml, followed by the ethanolic extract with the EC_{50} value of 3.4 ± 0.3 μg/ml. The $CHCl_3$: MeOH (1:1) precipitate and MeOH fractions possessed EC_{50} values of 6.4 ± 0.2 and 5.5 ± 0.0 μg/ml respectively. Interestingly, crude ethanolic extract and each fraction of ethanolic extract possessed high antioxidant activites with their values of EC_{50} less than 6.5 μg/ml which were lower than that of

BHT (EC_{50} = 6.9 ± 0.5 μg/ml), the standard antioxidant. The hexane, hexane: $CHCl_3$ (1:1) and $CHCl_3$ fractions showed the lowest antioxidant activity in this test with the EC_{50} values more than 100 μg/ml.

3.1.3 Anti HIV-1 integrase activity

The anti HIV-1 integrase activity of crude ethanolic extract and each fraction from the rhizome of *Smilax corbularia* (separated by VLC) were evaluated by multiplate integration assay as described in section 2.5. The results are shown in Table 3-4.

Table 3-4 % inhibition of crude ethanolic extract and each fraction on anti HIV-1 IN activity

Fractions	% Inhibition at 100 μg/ml ± S.D.
Crude ethanolic extract	99.4 ± 0.4
Hexane	12.8 ± 1.5
Hexane: $CHCl_3$ (1:1)	-2.0 ± 0.2
$CHCl_3$	-14.1 ± 0.8
$CHCl_3$: MeOH 1:1(Supernatant)	99.8 ± 0.4
$CHCl_3$: MeOH 1:1(Precipitate)	99.4 ± 0.1
MeOH	91.9 ± 1.1

The results showed that the $CHCl_3$: MeOH (1:1) supernate fraction had the highest anti HIV-1 integrase activity in this test with the % inhibition value of 99.8 ± 0.4 μg/ml, followed by the ethanolic extract, $CHCl_3$: MeOH (1:1) precipitate and MeOH fractions with the %

inhibition values of 99.4 ± 0.4, 99.4 ± 0.1 and of 91.9 ± 1.1 μg/ml respectively. Interestingly, crude ethanolic extract and each fraction of ethanolic extract possessed high anti HIV-1 integrase activity with their values of % inhibition more than 90 %. The hexane, hexane: $CHCl_3$ (1:1) and $CHCl_3$ fractions had the lowest anti HIV-1 integrase activity in this test with the % inhibition values of 12.8 ± 1.5, -2.0 ± 0.2 and -14.1 ± 0.8 respectively.

3.2 Analysis of chemical composition and structure determination of the isolated compounds

3.2.1 Structure elucidation of the isolated compounds

Results from the bioassay-guided fractionation for antioxidant and anti HIV-1 integrase activity were shown in section 3.1.1, 3.1.2 and 3.1.3. Thus, the separation of the active extracts was carried out as shown in section 2.7 to give the pure compounds as follows.

3.2.1.1 SC1

SC1 (β-sitosterol): $C_{29}H_{50}O$ (1.5 mg, 0.011 %w/w); white crystal solids. SC1 was the compound isolated from the ethanolic extract of the rhizome of *Smilax corbularia*, obtained as white needle crystal solids. Analysis of chemical shifts, integration and coupling pattern from ^{1}H-NMR data indicate that SC1 was a sterol. The ^{1}H-NMR spectrum are shown in Table 3-6 and Figure 3-3. The TLC analysis of this compound was compared with authentic sample β-sitosterol (Sigma). It was strongly supported that this compound is β-sitosterol. The structure was showed below.

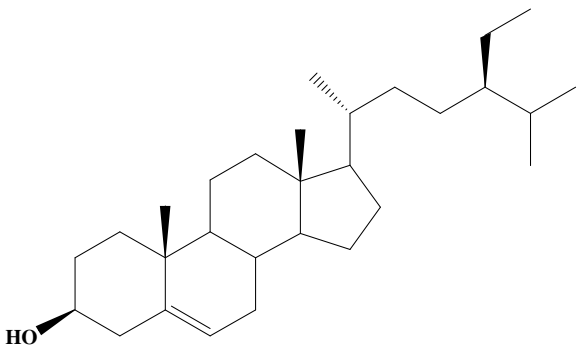


Figure 3-1 Structure of β-sitosterol

3.2.1.2 SC2

SC2 (β-sitosterol-3-*O*-β-D-glucopyranoside): $C_{35}H_{60}O_6$ (3.0 mg, 0.022 %w/w); white amorphous solids. SC2 was compound isolated from the ethanolic extract of the rhizome of *Smilax corbularia* Kunth obtained as white solid and the ^{1}H-NMR spectrum shown in Table 3-5 and Figure 3-4. The ^{1}H-NMR of SC2 was similar to SC1. The difference was ^{1}H-NMR of SC2 showed more signals at δ between 3.24-4.36. This signal could be signal of major moiety and signal δ 4.36 (J= 7.5 Hz) should be signal of anomeric proton. Analysis of sugar part from splitting pattern compared with published paper, this sugar could be glucose (Agrawal, 1985; Shujiro *et al.,* 1978 and Blonquist and Wesserman, 1972). ^{1}H-NMR and TLC analysis of this compound compared with an authentic sample got from Srisopa Ruangnoo (Ruangnoo, 2007). It was strongly supported that this compound is β-sitosterol-3-*O*-β-D-glucopyranoside. The structure was showed below.

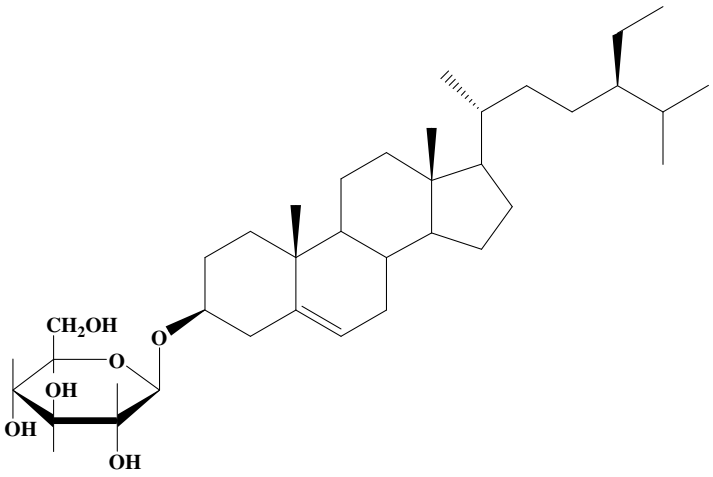


Figure 3-2 Structure of β-sitosterol-3-*O*-β-D-glucopyranoside

Table 3-5 NMR spectral data (500 MHz for ^{1}H) of SC1 (β-sitosterol) in $CDCl_3$ and SC2 (β-sitosterol-3-*O*-β-D-glucopyranoside) in $CDCl_3$:CD_3OD

Carbon position	Chemical shift (δ) of ^{1}H (mult, *J* in Hz)			
	SC1	β-sitosterol	SC2	β-sitosterol-3-*O*-β-D-glucopyranoside
3	3.52 (m)	3.43 (m)	3.52 (m)	3.43 (m)
6	5.35 (br d)	5.33 (dt)	5.31 (br d)	5.40 (br d)
18	0.68 (s)	0.66 (s)	0.62 (s)	0.69 (s)
19	1.00 (s)	0.97 (s)	1.19 (s)	1.05 (s)
21	0.92 (d, 6.5)	0.91 (d, 6.5)	0.86 (d, 6)	0.95 (d, 6.6)
26	0.81 (d, 7.5)	0.80 (d, 6.8)	0.75 (d, 7)	0.82 (d, 7.0)
27	0.83 (d, 7)	0.80 (d, 6.8)	0.83 (d, 7)	0.84 (d, 7.5)
29	0.85 (t, 8.5)	0.83 (t, 6.5)	0.85 (t, 7.5)	0.85 (t, 7.5)
Glucose-1	-		4.36 (d, 7.5)	4.40 (d, 7.5)
2	-		3.69-3.72 (m)	3.55-3.62 (m)
3			3.50-3.53 (m)	3.45-3.48 (m)
4	-		3.20-3.24 (m)	3.36-3.40 (m)
5	-		3.77-3.80 (m)	3.75-3.88 (m)
6	-		3.24 (m)	3.28 (m)

Note: β-sitosterol in $CDCl_3$ from Ali *et al.*, 2002; β-sitosterol-3-*O*-β-D-glucopyranoside in $CDCl_3$:CD_3OD from Ruangnoo, 2007

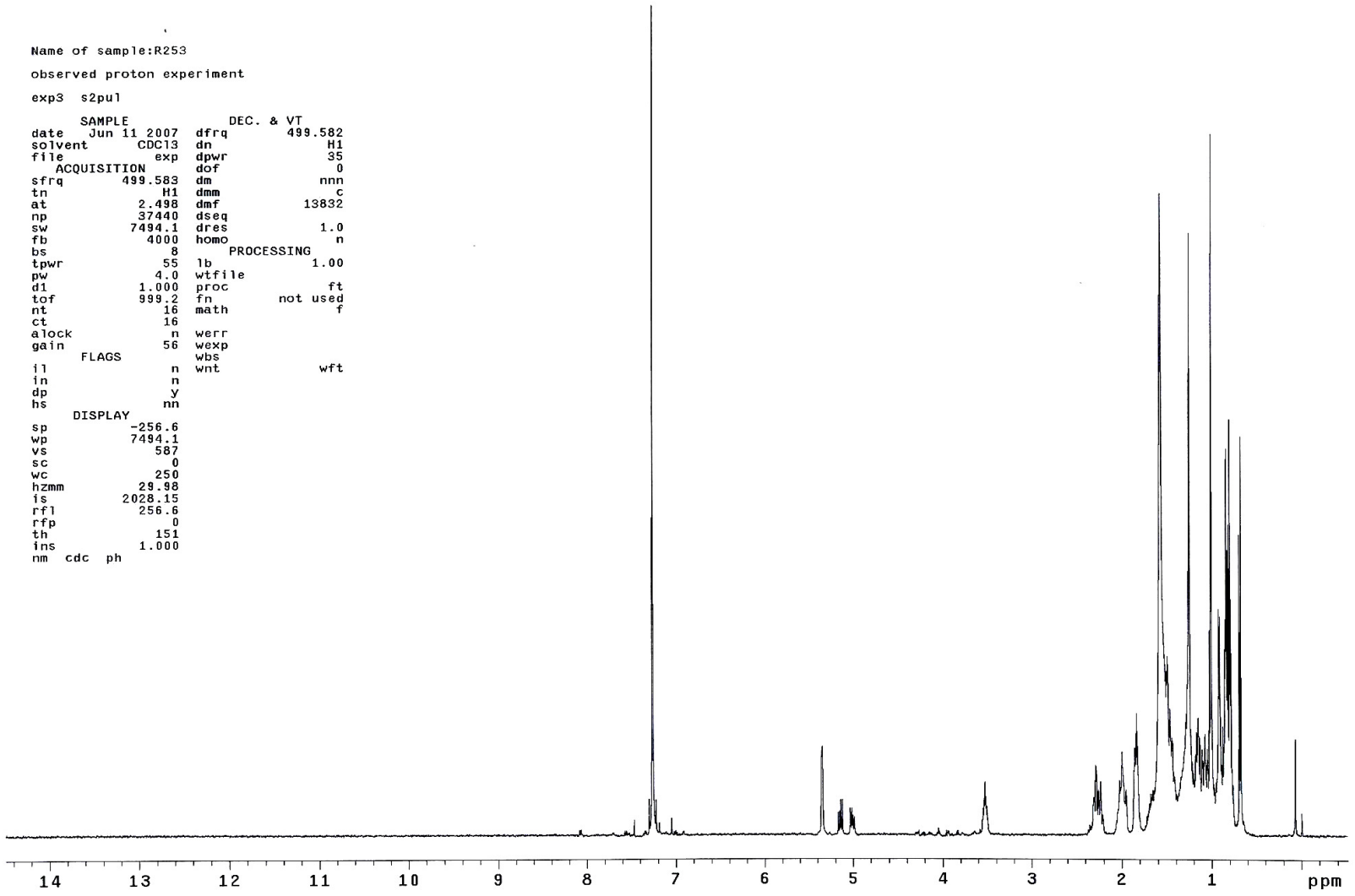


Figure 3-3 ^{1}H-NMR spectrum of β-sitosterol in $CDCl_3$

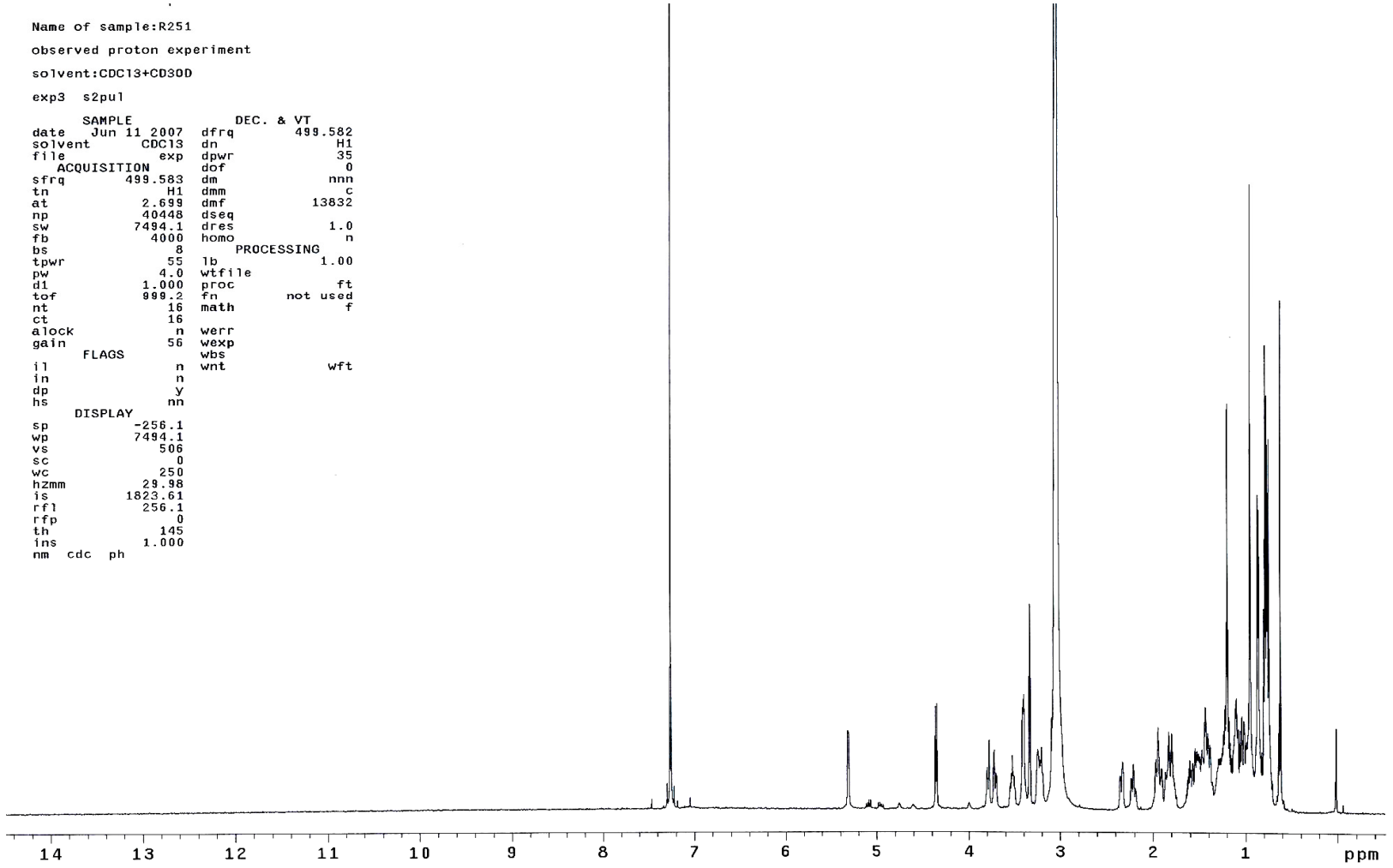


Figure 3-4 ^{1}H-NMR spectrum of β-sitosterol-3-*O*-β-D-glucopyranoside in $CDCl_3$:CD_3OD

3.2.1.3 SC3

SC3 (quercetin): $C_{15}H_{10}O_7$ Yellow crystal solids (12.6 mg, 0.19 %w/w); specific optical rotation $[\alpha]_D$ = +28.07 (c 0.27, MeOH).

SC3 was the major compound isolated from the ethanolic extract of the rhizome of *Smilax corbularia*, obtained as yellow crystal solids. The ^{1}H-NMR spectrum is shown in Table 3-6 and Figure 3-6. Analysis of chemical shifts and integration of these functional groups indicate that SC3 was a quercetin. The ^{1}H-NMR analysis of this compound compared with spectrum in previous journol (Ogundipe *et al*., 2001). It was strongly supported that this compound is quercetin. The structure was showed below.

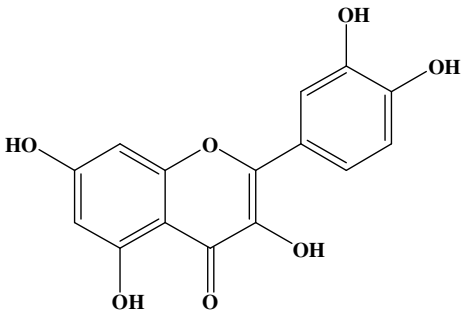


Figure 3-5 Structure of quercetin

Table 3-6 NMR spectral data (500 MHz for ^{1}H) of SC3 quercetin in CD_3OD

Carbon position	**δ_H (mult., *J* in Hz) (SC3)**	**δ_H (mult., *J* in Hz) quercetin**
6	6.38 (d, 2.0)	6.36 (d, 2.11)
8	6.17 (d, 2.0)	6.16 (d, 2.11)
2′	7.72 (d, 2.5)	7.73 (d, 2.11)
5′	6.87 (d, 8.0)	6.89 (d, 8.54)
6′	7.62 (dd, 8.5, 2.5)	7.63 (dd, 8.56, 2.08)

Note: Quercetin in CD_3OD from Ogundipe *et al*., 2001

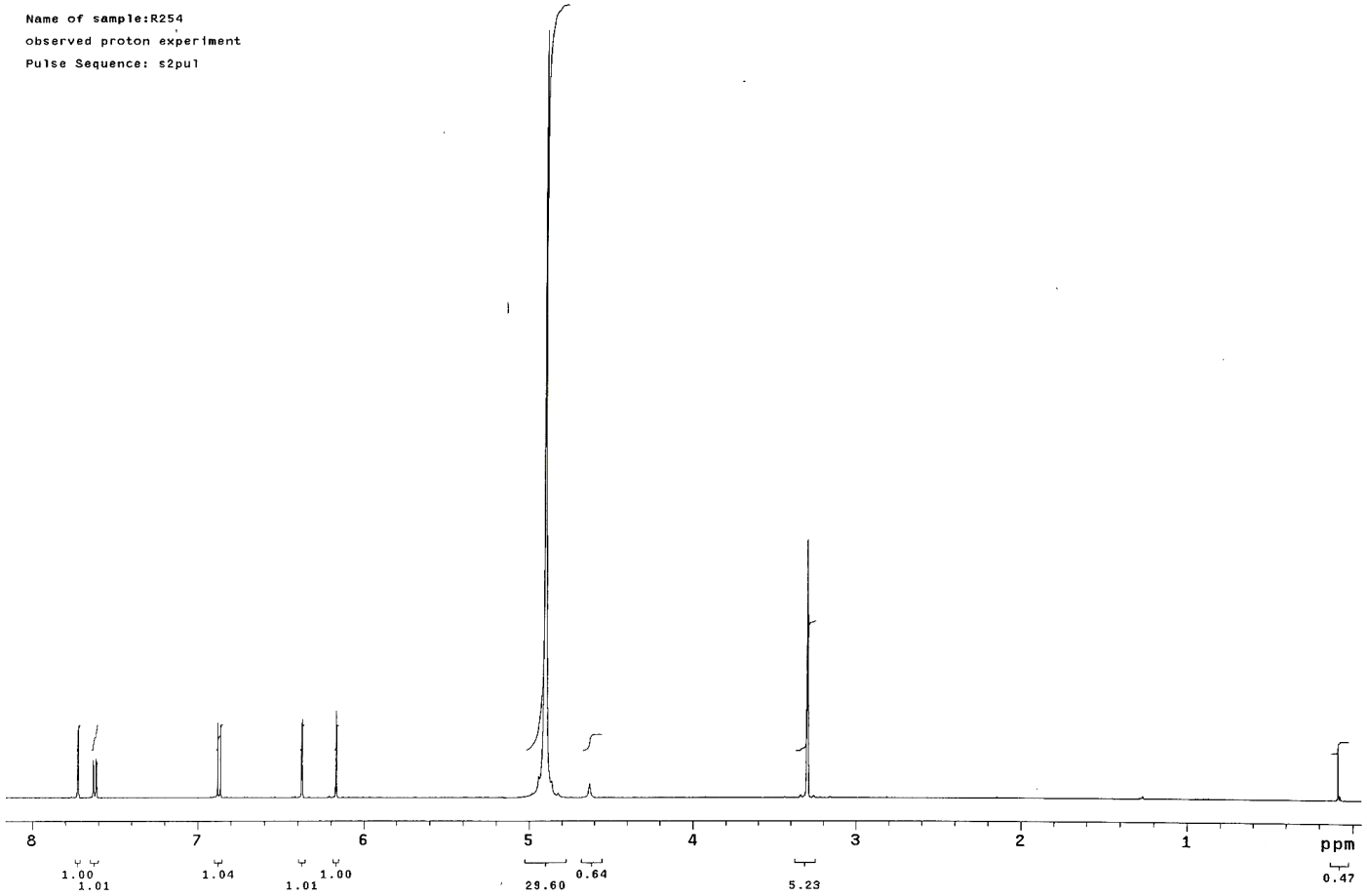

Name of sample:R254
observed proton experiment
Pulse Sequence: s2pul
8
7
6
5
4
3
2
1
ppm
1.00
1.01
1.04
1.01
1.00
29.60
0.64
5.23
0.47

Figure 3-6 ^{1}H-NMR spectrum of quercetin in CD_3OD

3.2.1.4 SC4

SC4 (astilbin): $C_{18}H_{22}O_{11}$ white crystal (49.00 mg, 0.27 %w/w); optical rotation [α]D = -2.92 (c 0.25, MeOH), UV (MeOH) λmax (logε) 330.44 (4.41), 290.73 (4.99), 226.60 (5.02) and 214.94 (5.11) nm (Figure 3-12). IR (KBr disc) λmax 3400.53, 2922.50, 1638.07, 1601.84, 820.57 cm^{-1} (Figure 3-11). The EI-MS m/z 450.1167 (Calc. for $C_{21}H_{22}O_{11}$ 450.1167).

SC4 was the major compound isolated from the ethanolic extract of the rhizome of *Smilax corbularia*, obtained as white crystals and showed protonated molecular ion peak in EI mass spectrum at *m/z* 450.1167 (Figure 3-10), corresponding with a molecular formula of $C_{21}H_{22}O_{11}$ (MW= 450).

The ^{1}H-NMR and ^{13}C-NMR spectra of SC4 showed in Figure 3-8 and Figure 3-9. Analysis of chemical shifts and integration of these functional groups indicate that SC4 was an astilbin (Table 3-7). The ^{1}H-NMR and ^{13}C-NMR analysis of this compound compared with an authentic sample from the previous publication (Du *et al*., 2005). It was strongly supported that this compound is astilbin. The structure was showed below.

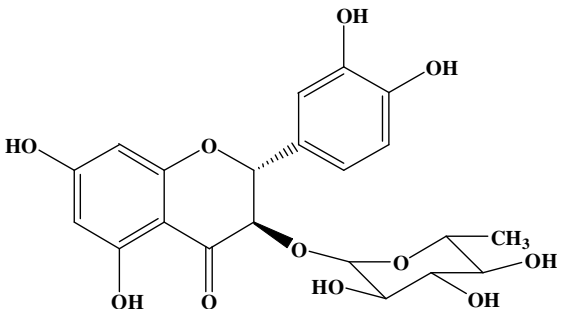


Figure 3-7 Structure of astilbin

Table 3-7 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of SC4 astilbin in CD_3OD

Carbon position	δ_C (SC4)	δ_C (astibin)	δ_H **(mult., *J* in Hz)** (SC4)	δ_H **(mult., *J* in Hz)** (astilbin)
2	83.9	81.5	5.07 (d, 10.5)	5.24 (d, 9.8)
3	78.6	75.6	4.57 (d, 10.5)	4.63 (d, 9.8)
4	196.0	194.3		
5	165.4	163.3		
6	96.2	96.0	5.90 (d, 2.0)	5.90 (d, 2.1)
7	168.0	166.9		
8	97.4	95.0	5.92 (d, 2.0)	5.88 (d, 2.1)
9	164.1	162.1		
10	102.5	101.0		
1′	129.1	126.8		
2′	115.5	114.7	6.95 (s)	6.88 (s)
3′	147.3	145.8		
4′	146.5	145.1		
5′	116.4	115.3	6.81 (d, 8.0)	6.74 (s)
6′	120.5	118.7	6.84 (dd, 8.0, 2.0)	6.74 (s)
1″	102.1	100.0	4.03 (s)	4.07 (s)
2″	71.7	70.1	3.50 (br,s)	3.36 (br,s)
3″	72.1	70.4	3.65 (dd, 9.5, 3.0)	3.42 (dd, 9.4, 2.8)
4″	73.7	71.6	3.30 (dd, 9.4, 9.4)	3.15 (dd, 9.4, 9.4)
5″	70.5	68.9	4.23 (dq, 9.5, 6.5)	3.88 (dq, 9.4, 6.2)
6″	17.8	17.6	1.17 (d, 5.5)	1.05 (d, 6.2)

Note: Astilbin in DMSO from Du *et al*., 2005

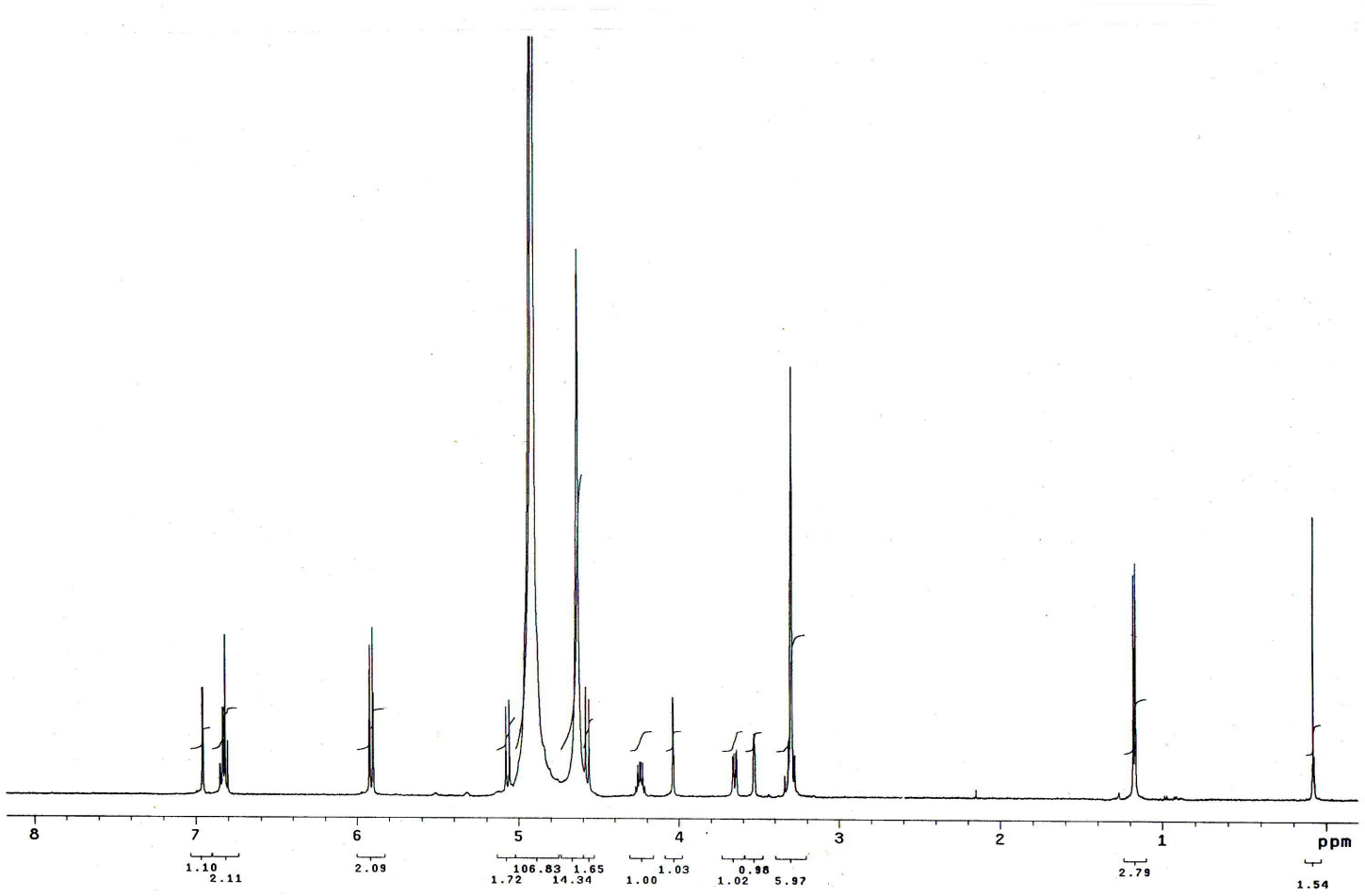
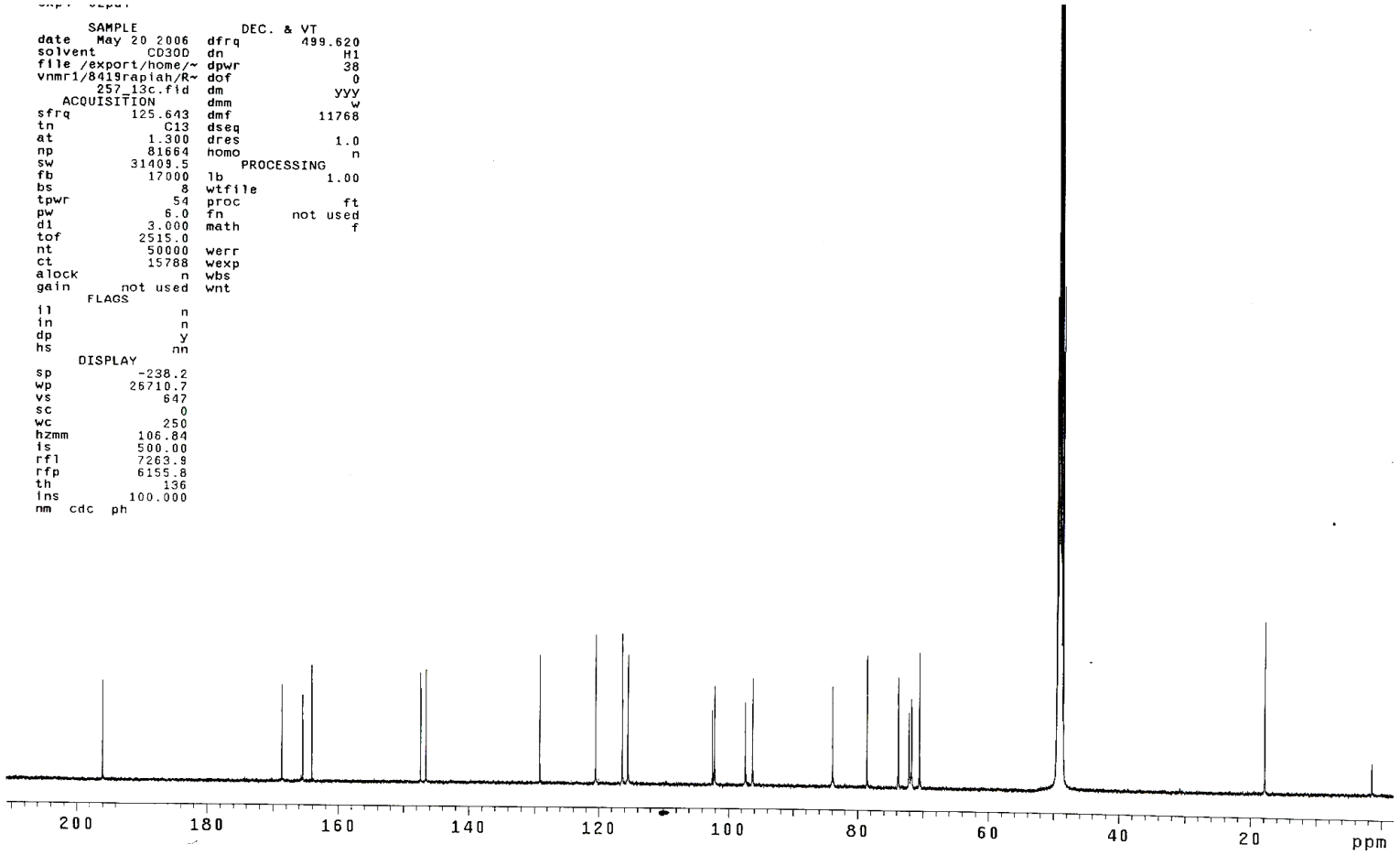


Figure 3-8 ^{1}H-NMR spectrum of astilbin in CD_3OD


SAMPLE
date May 20 2006
solvent CD3OD
file /export/home/~
vnmr1/8419rapiah/R~
257_13c.fid
ACQUISITION
sfrq 125.643
tn C13
at 1.300
np 81664
sw 31409.5
fb 17000
bs 8
tpwr 54
pw 6.0
d1 3.000
tof 2515.0
nt 50000
ct 15788
alock n
gain not used
FLAGS
il n
in n
dp y
hs nn
DISPLAY
sp -238.2
wp 26710.7
vs 647
sc 0
wc 250
hzmm 106.84
is 500.00
rfl 7263.8
rfp 6155.8
th 136
ins 100.000
nm cdc ph
DEC. & VT
dfrq 499.620
dn H1
dpwr 38
dof 0
dm yyy
dmm w
dmf 11768
dseq
dres 1.0
homo n
PROCESSING
lb 1.00
wtfile
proc ft
fn not used
math f
werr
wexp
wbs
wnt
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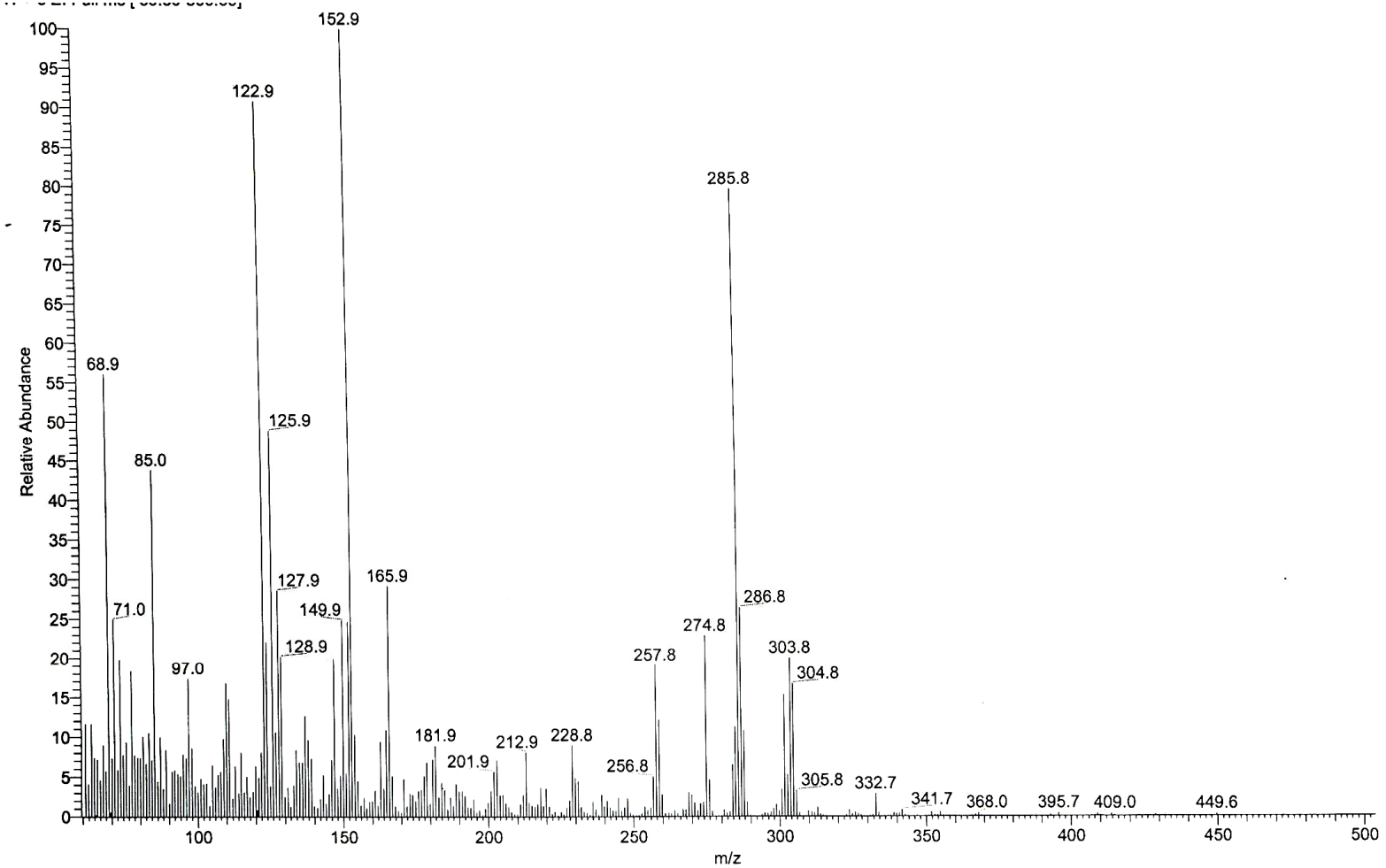
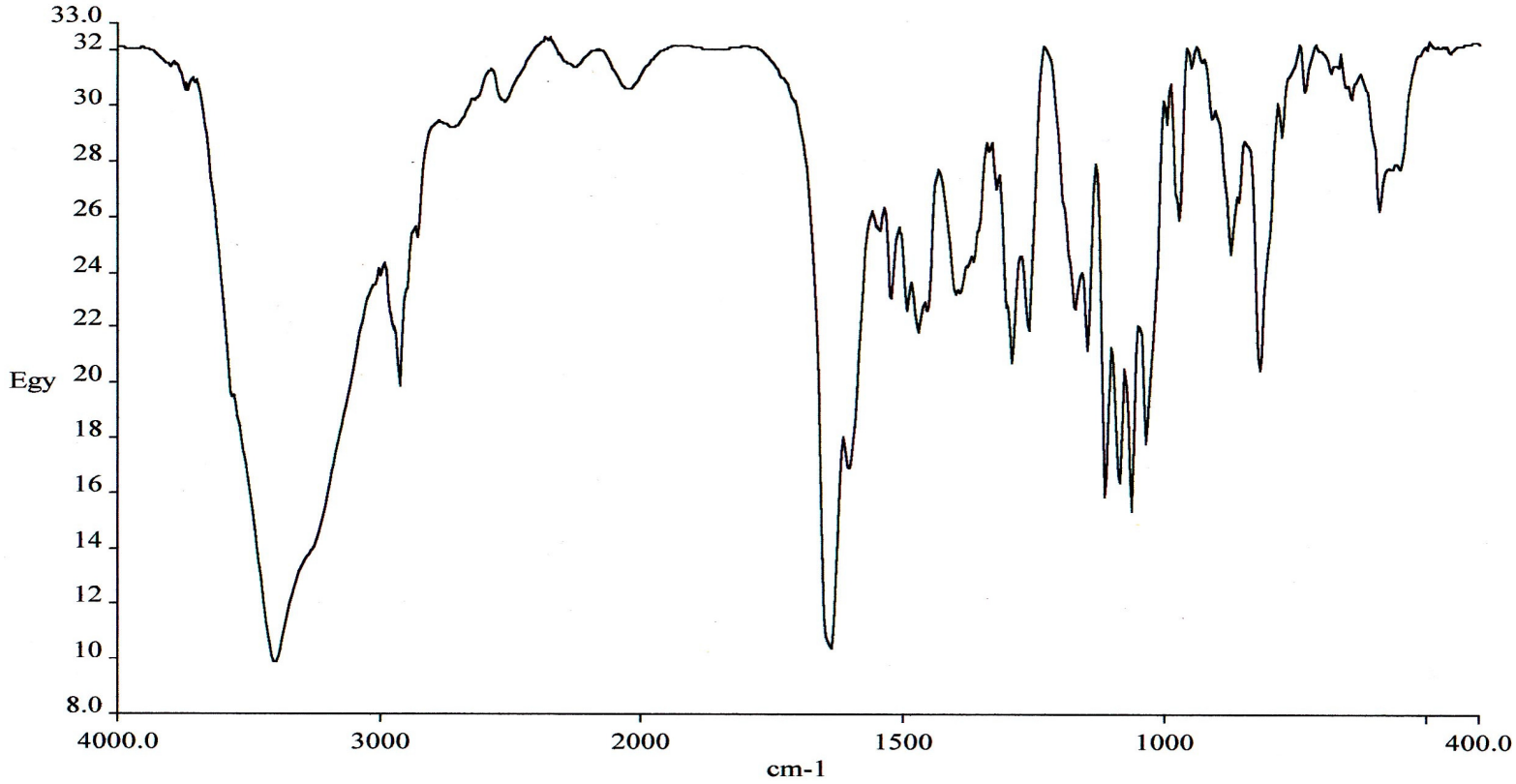
180
160
140
120
100
80
60
40
20
ppm

Figure 3-9 ^{13}C-NMR spectrum of astilbin in CD_3OD




33.0
32
30
28
26
24
22
20
18
16
14
12
10
8.0
Egy
4000.0
3000
2000
1500
1000
400.0
cm-1

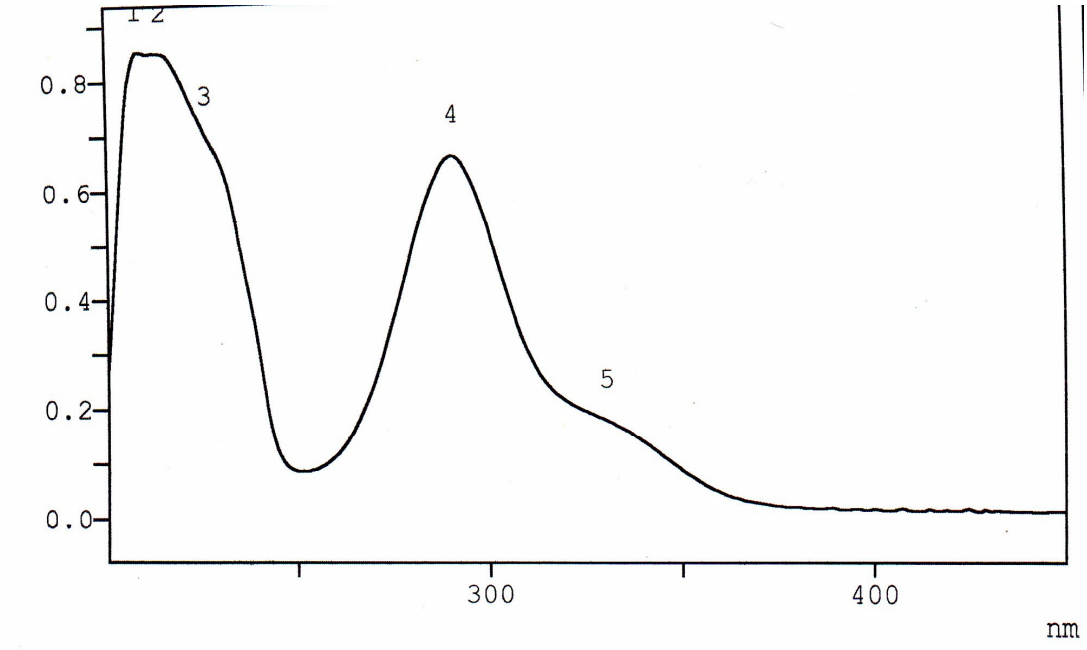


Figure 3-12 UV spectrum of astilbin

3.2.1.5 SC5

SC5 (engeletin): $C_{21}H_{22}O_{10}$, Brown powder; (26.30 mg, 0.15 %w/w); specific optical rotation [α]D = +11.50 (c 0.20, MeOH), UV (MeOH) λmax (logε) 329.80 (4.76), 292.50 (5.30), 217.92 (5.46) nm (Figure 3-18). IR (KBr disc) λmax 3369.91, 2922.50, 1638.73, 1587.09, 826.56 cm^{-1} (Figure 3-17). EI-MS m/z 434.1204 (Calc. for $C_{21}H_{22}O_{10}$ 434.1204).

SC5 was the major compound isolated from the ethanolic extract of the rhizome of *Smilax corbularia*, obtained as brown powder and showed protonated molecular ion peak in EI mass spectrum at *m/z* 434.1204 (Figure 3-16), corresponding with a molecular formula of $C_{21}H_{22}O_{10}$ (MW= 434).

The ^{1}H-NMR and ^{13}C-NMR spectra of SC5 was showed in Figure 3-14 and Figure 3-15. Analysis of chemical shifts, integration and spin coupling patterns of these functional groups indicate that SC5 was an engeletin (Table 3-8). The ^{1}H-NMR and ^{13}C-NMR analysis of this compound compared with spectrum of the previous publication (Lu and Foo, 1999). It was strongly supported that this compound is engeletin. The structure was showed below.

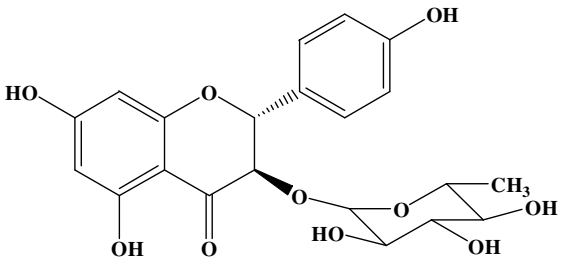


Figure 3-13 Structure of engeletin

Table 3-8 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of SC5 engeletin in CD_3OD

Carbon position	δ_C (SC5)	δ_C (engeletin)	δ_H (mult., *J* in Hz) (SC5)	δ_H (mult., *J* in Hz) (engeletin)
2	83.9	81.28	5.13 (d, 11)	5.28 (d, 10.3)
3	78.7	75.75	4.60 (d, 11)	4.73 (d, 10.3)
4	196.0	194.32		
5	165.0	163.21		
6	96.2	95.97	5.89 (d, 2.5)	5.87 (d, 1.9)
7	168.6	167.28		
8	97.4	94.97	5.92 (d, 2.0)	5.90 (d, 1.9)
9	164.1	161.99		
10	102.5	100.61		
1′	128.6	126.32		
2′	130.1	128.82	7.35 (d, 8.5)	7.33 (d, 8.5)
3′	116.4	114.96	6.84 (d, 8.5)	6.79 (d, 8.5)
4′	159.4	157.63		
5′	116.4	114.96	6.84 (d, 8.5)	6.79 (d, 8.5)
6′	130.1	128.82	7.35 (d, 8.5)	7.33 (d, 8.5)
1″	102.2	100.10	3.90 (s)	3.98 (s)
2″	71.7	70.20	3.49 (br,s)	-
3″	72.1	69.95	3.64 (dd, 9.5, 3.0)	-
4″	73.7	71.42	3.29 (dd, 9.5, 9.5)	-
5″	70.5	68.77	4.24 (dq, 9.5, 6.5)	-
6″	17.9	17.52	1.17 (d, 6.0)	1.05 (d, 6.2)

Note: Engeletin in DMSO from Lu and Foo, 1999

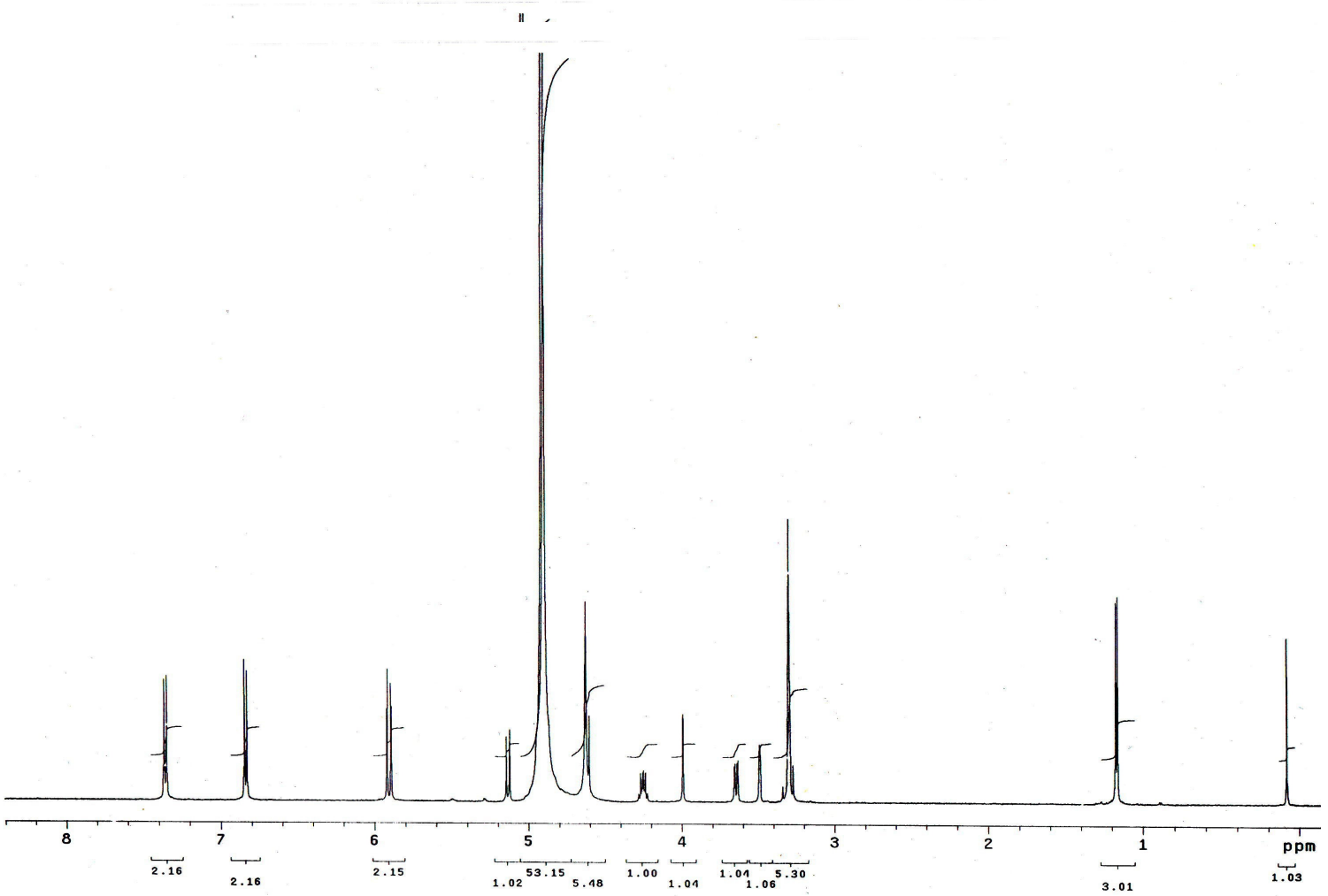


Figure 3-14 ^{1}H-NMR spectrum of engeletin in CD_3OD

Name of sample:R 258

observed carbon experiment

exp3 s2pul

SAMPLE		DEC. & VT	
date	May 21 2006	dfrq	499.620
solvent	CD3OD	dn	H1
file	exp	dpwr	38
ACQUISITION		dof	0
sfrq	125.643	dm	yyy
tn	C13	dmm	w
at	1.300	dmf	11768
np	81664	dseq	
sw	31409.5	dres	1.0
fb	17000	homo	n
bs	8	PROCESSING	
tpwr	54	lb	1.00
pw	6.0	wtfile	
d1	3.000	proc	ft
tof	2515.0	fn	not used
nt	50000	math	f
ct	5128		
alock	n	werr	
gain	not used	wexp	
FLAGS		wbs	
il	n	wnt	
in	n		
dp	y		
hs	nn		
DISPLAY			
sp	-498.0		
wp	27349.1		
vs	959		
sc	0		
wc	250		
hzmm	109.40		
is	500.00		
rfl	7262.5		
rfp	6155.8		
th	116		
ins	100.000		

nm cdc ph

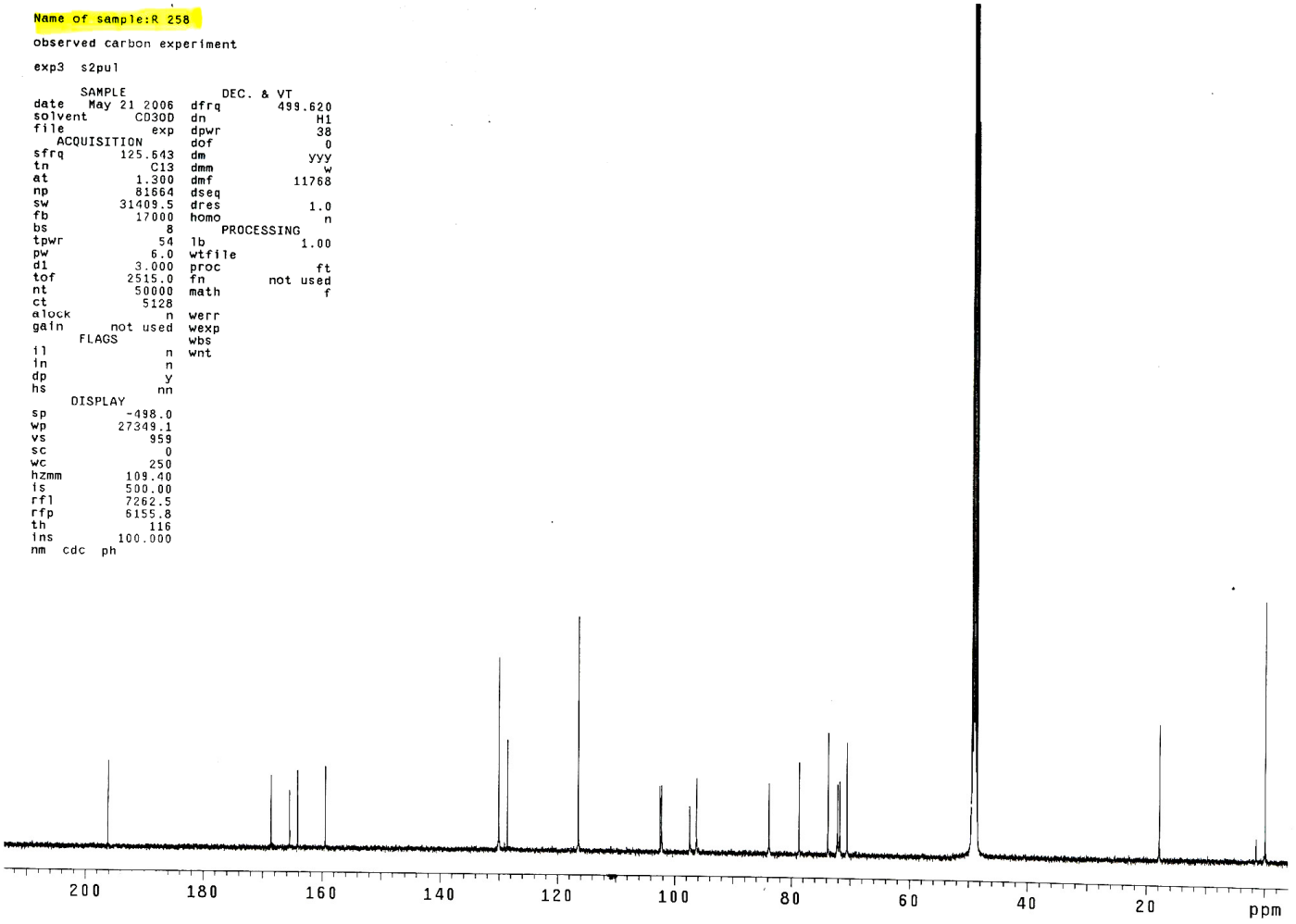
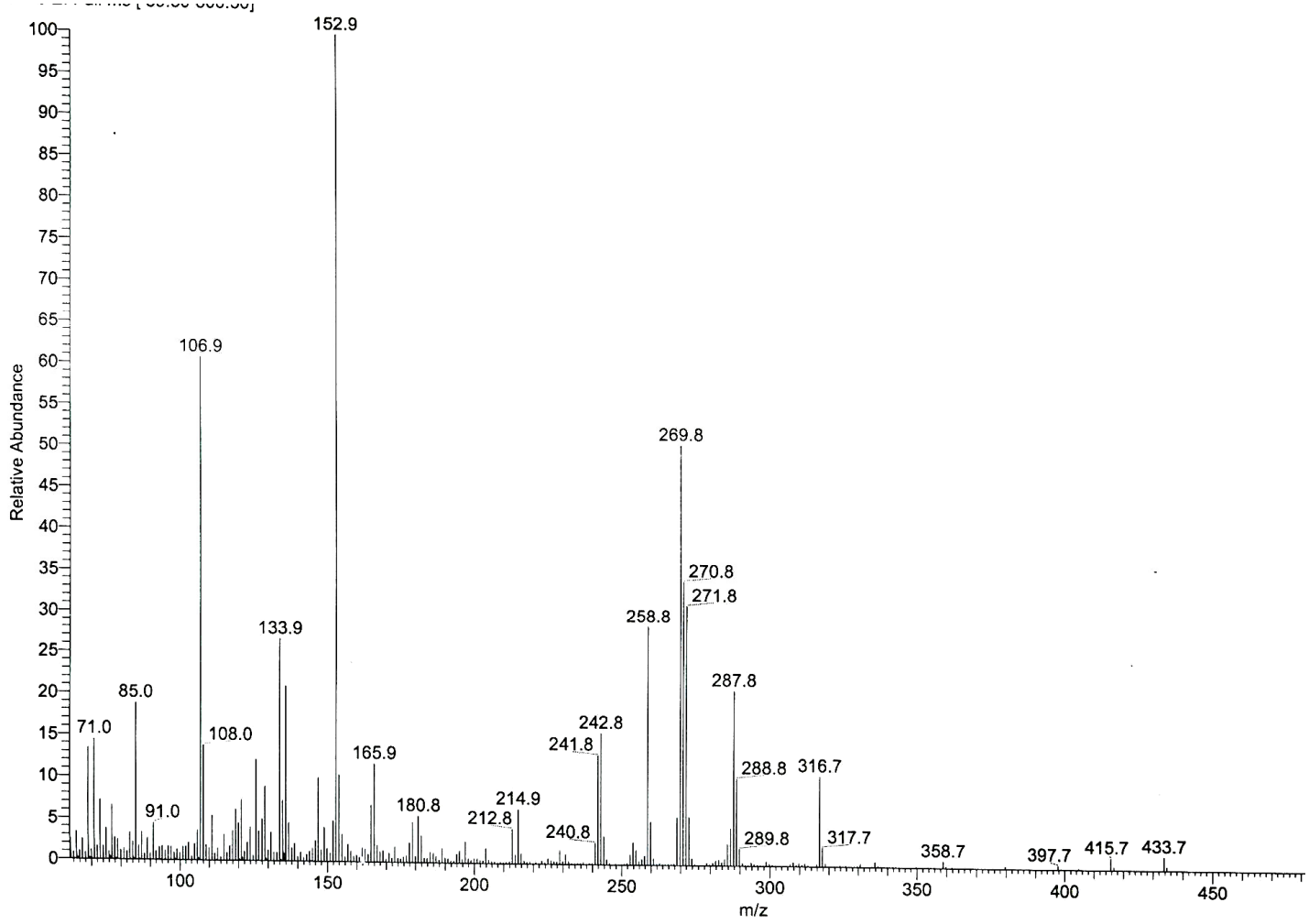


Figure 3-15 ^{13}C-NMR spectrum of engeletin in CD_3OD



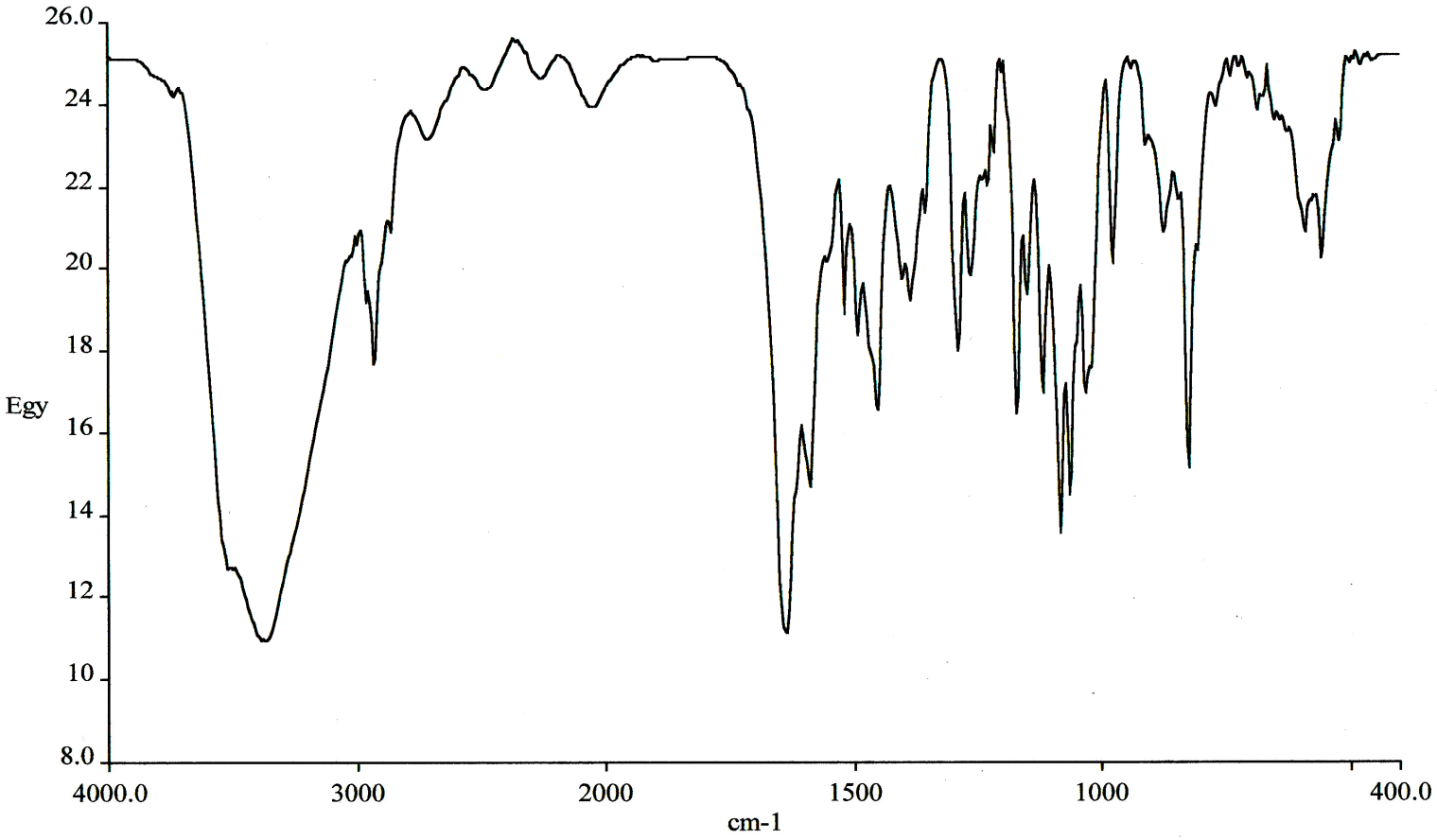


Figure 3-16 EIMS spectrum of engeletin

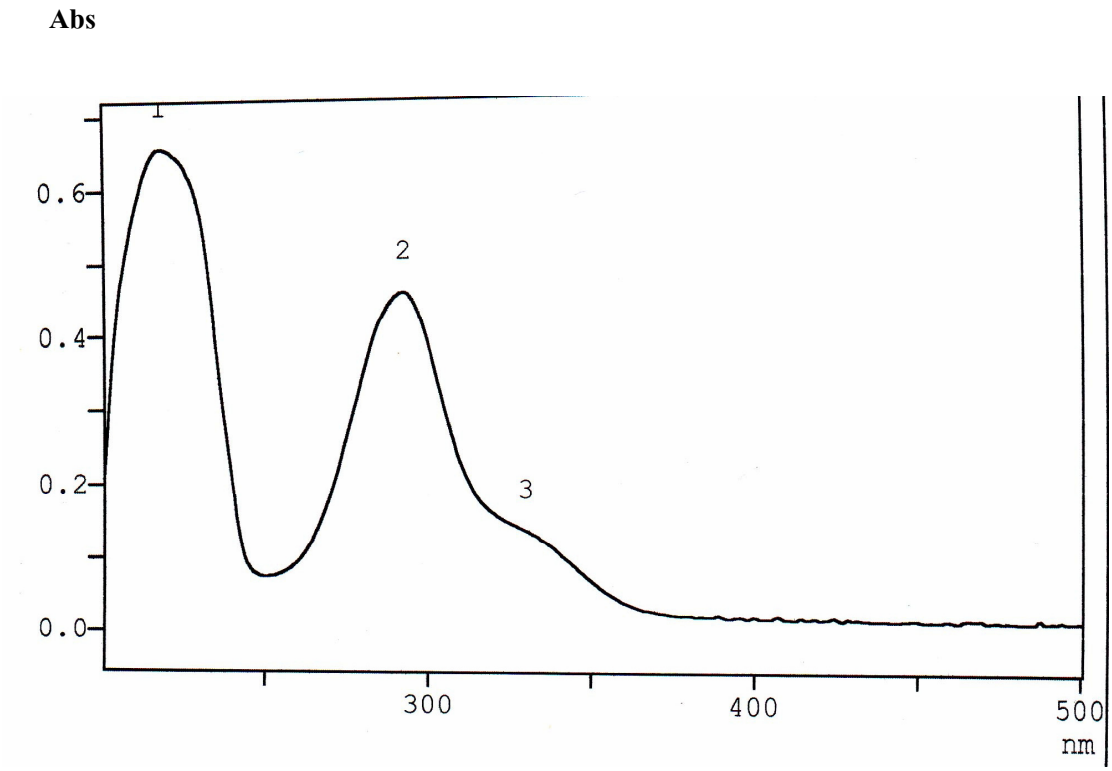
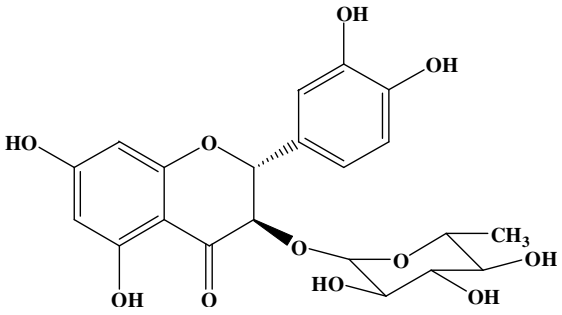


Figure 3-18 UV spectrum of engeletin

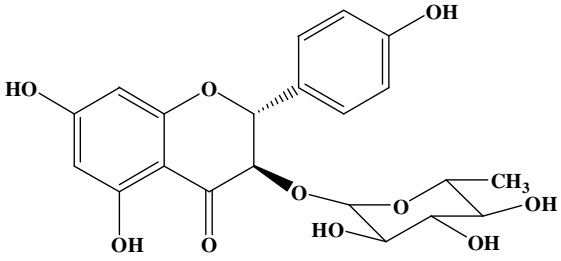
3.3 Discussion on phytochemical investigation

The ethanolic extract of the rhizome of *Smilax corbularia* Kunth was separated by column chromatography using an isocratic solvents: chloroform and methanol. Five compounds were isolated. All pure compounds were detected by application of the general spraying reagent anisaldehyde in sulphuric acid, giving different colours after heating. SC1 and SC2 were violet colour. SC3, SC4 and SC5 were yellow colour. Only three compounds (SC3, SC4 and SC5) could be detected by UV 254 nm.

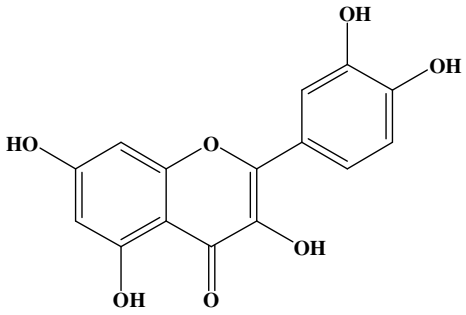
The five pure compounds could be divided into two chemical groups. They were two sterols (β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside) and three flavonoids (quercetin, astilbin and engeletin). The structures are shown in Figure 3-19. The investigation on chemical constituents of the rhizome of *Smilax corbularia* Kunth was found that quercetin, astibin and engeletin, the major antioxidant compound, are the main compounds and normally were also found in *Smilax glabra* (Chien and Adam, 1979, Cao *et al.,* 1993, Ng *et al.,* 2001, Du *et al.,* 2005). β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside were found in small amount in ethanolic extract and these compounds are commonly sterols and found in higher plants and have also been found in many plant species.



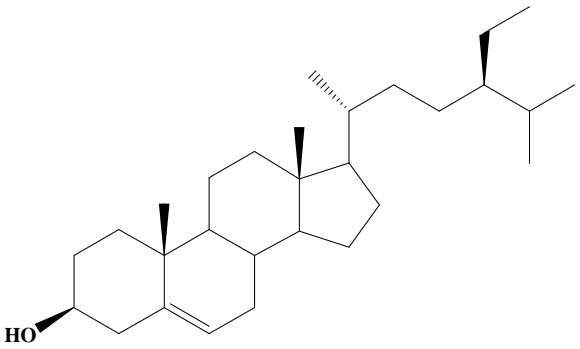
Astilbin



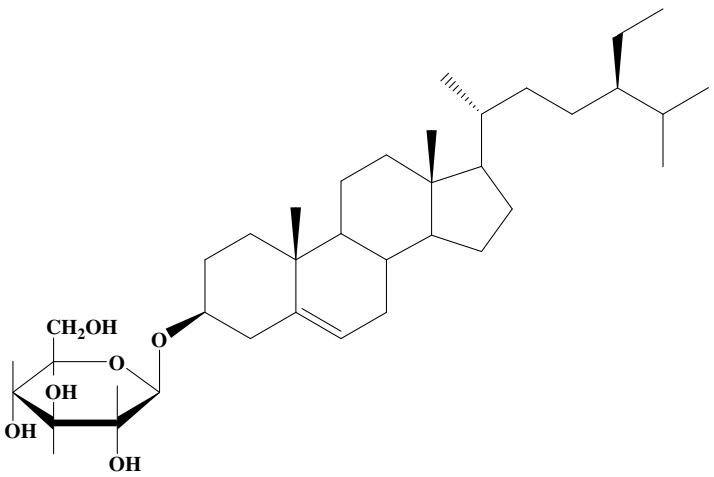
Engeletin



Quercetin



β-sitosterol



β-sitosterol-3-*O*-β-D-glucopyranoside

Figure 3-19 The chemical structures of five compounds isolated from the ethanolic extract of the rhizome of *Smilax corbularia* Kunth

3.4 Activities of the isolated compounds

The β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside, quercetin, astilbin and engeletin were isolated from the ethanolic extract of *Smilax corbularia.* They were assessed for testing antioxidant and anti-HIV-1 integrase activities.

3.4.1 Antioxidant activity

Quercetin exhibited high antioxidant activity on both antioxidant assay (DPPH and lipid peroxidation assay) with EC_{50} of 0.6 and 0.3 μg/ml, respectively. These results related with the previous study which showed that quercetin possessed antioxidant effect by the DPPH radical scavenging assay (EC_{50} = 4.9 ± 0.6 μM) (Rao *et al.,* 2007), followed by astilbin (EC_{50}= 2.5 μg/ml) and engeletin (EC_{50}= 3.9 μg/ml), whereas β-sitosterol-3-*O*-β-D-glucopyranoside and β-sitosterol were >100 μg/ml. Moreover antioxidant test was determined by the lipid peroxidation of liposome assay. The EC_{50} of astilbin and engeletin were found to be 0.8 and 1.2 μg/ml, respectively. The resuls also related with the previous study which found that quercetin, astilbin and engeletin showed high antioxidant activity (Closa *et al.,* 1997; Sanches *et al.,* 2005). β-sitosterol-3-*O*-β-D-glucopyranoside and β-sitosterol (EC_{50} >100 μg/ml) showed less antioxidant activity (Table 3-9). These results indicated that quercetin, astilbin and engeletin are

markers for antioxidant assay of the ethanolic extract and they will be useful for quality control of this extract in terms of chemical finger print.

Table 3-9 Antioxidant activity of compounds isolated from *Smilax corbularia* by DPPH assay and lipid peroxidation assay

Compounds	EC_{50} (μg/ml)	
	DPPH assay	**Lipid peroxidation**
β-sitosterol	>100	>100
β-sitosterol-3-*O*-β-D-glucopyranoside	>100	>100
Quercetin	0.6 ± 0.1	0.3 ± 0.1
Astilbin	2.5 ± 0.3	0.8 ± 9.1
Engeletin	3.9 ± 0.2	1.2 ± 0.1

(n=3), n= number of independent experiment which was performed in 3 replicates and NT= not test

3.4.2 Anti HIV-1 integrase activity

The results indicated that quercetin possessed the most potent inhibitory activity against HIV-1 integrase with an IC_{50} value of 8.9 ± 1.2 μM, followed by astilbin (IC_{50}= 50.3 ± 0.9 μM), β-sitosterol-3-*O*-β-D-glucopyranoside (IC_{50}= 80.5 ± 1.0 μM), β-sitosterol (IC_{50}= 80.8 ± 0.9 μM) and engeletin (IC_{50}= 174.3 ± 0.8 μM), respectively (Table 3-10). These results related with the previous study which showed that quercetin exhibited HIV-1 integrase inhibitory activity with an IC_{50} value of 15 μM (Tewtrakul *et al.,* 2001). The structure-activity findings for flavones in the current study of the inhibition of HIV-1 integrase can be summarized as follows (1) activity required the presence of at least three hydroxyl groups and (2) activity was reduced or eliminated by the presence of glycosyl or methoxy substituents. The planarity, aromaticity and polarity may allow quercetin to bind by stacking with adenine or guanine, or to compete with purine moieties for binding to enzyme sites. Many of the molecules also have oxidation-reduction and metal chelation capacities (Fesen *et al.,* 1994).

Table 3-10 Inhibitory effect against HIV-1 integrase of compounds isolated from *Smilax corbularia*

Compounds	IC_{50} (μM)± S.D.
β-sitosterol-3-*O*-β-D-glucopyranoside	80.5 ± 1.0
β-sitosterol	80.8 ± 0.9
Quercetin	8.9 ± 1.2
Astilbin	50.3 ± 0.9
Engeletin	174.3 ± 0.8

The results are the mean ± S.D (n=4)

3.5 Determination of astilbin and engeletin

3.5.1 Standardization of astilbin and engeletin

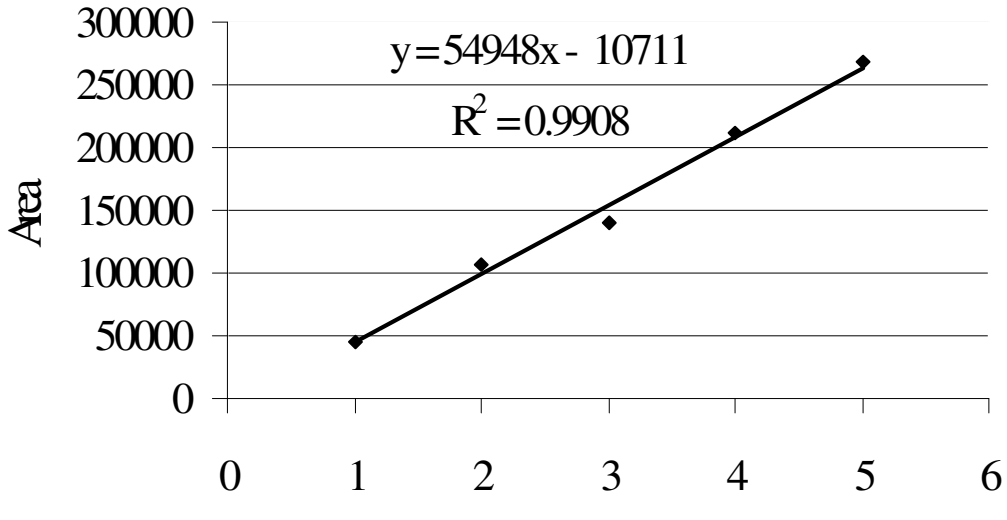
For quantitative determination, astilbin and engeletin were used as the marker substances evaluate the quantity of an active compound from the crude extract. The content of astilbin and engeletin were determined in chapter 2.8. The retention time and the peak area were calculated as percentage for astilbin and engeletin. To determine the linearity equations and linear scope for the analysis, a series of mixed standard solutions ranged from 1.0-5.0 μg/ml were tested for astilbin whereas those of engeletin ranged from 0.81-4.05. The results were summarized in Table 3-11 for astilbin and engeletin. The chromatogram of crude extract and $CHCl_3$:MeOH (1:1) supernate fraction showed in Figure 3-22. Astilbin and engeletin were also indicated in its chromatogram.

3.5.2 Analysis of astilbin and engeletin content by HPLC

The contents of astilbin and engeletin in crude ethanolic extract of the rhizome of *Smilax corbularia* and $CHCl_3$:MeOH (1:1) supernate fraction were determined as described in the chapter 2.8. The results were obtained within 9 min and 13 min respectively, for the HPLC separation. During sample analysis, the UV absorbance of the targeted peak was compared with standard for confirmation. The contents of astilbin in crude ethanolic extract and $CHCl_3$:MeOH (1:1) supernate were 0.218 and 0.216 %w/w, respectively and the contents of engeletin in crude ethanolic extract and $CHCl_3$:MeOH (1:1) supernate were 0.021 and 0.016 %w/w, respectively. The results are showed in Table 3-13.

Table 3-11 The regression equation for astilbin and engeletin

Compound	**Regression equation**	**Correlation coefficient**	**Linear range (μg/ml)**
Astilbin	y= 54948x-10711	0.9908	1.0-5.0
Engeletin	y= 46388x-5268.9	0.9946	0.81-4.05



Concentration (μg/ml)

Figure 3-20 Standard curve of astilbin, the "y" value is the peak area of analysis and the "x" value is the concentration of the analysis (μg/ml)

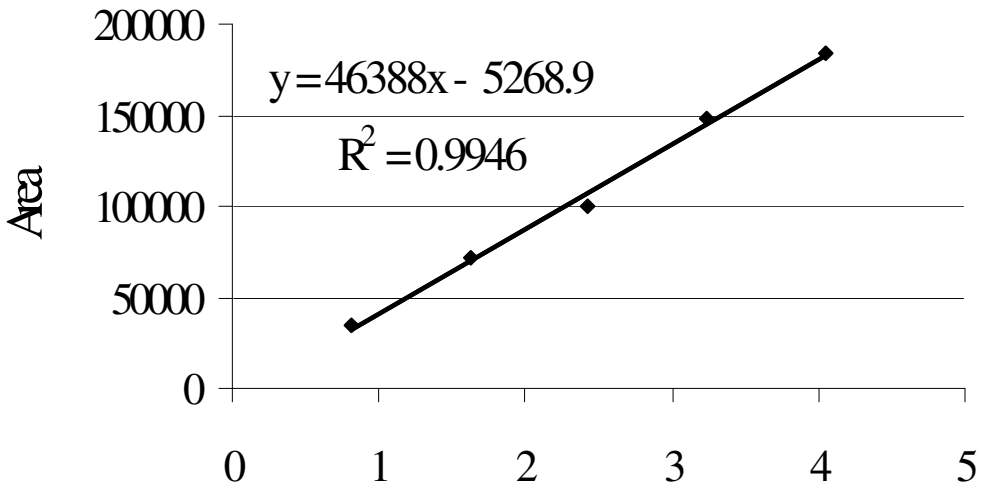
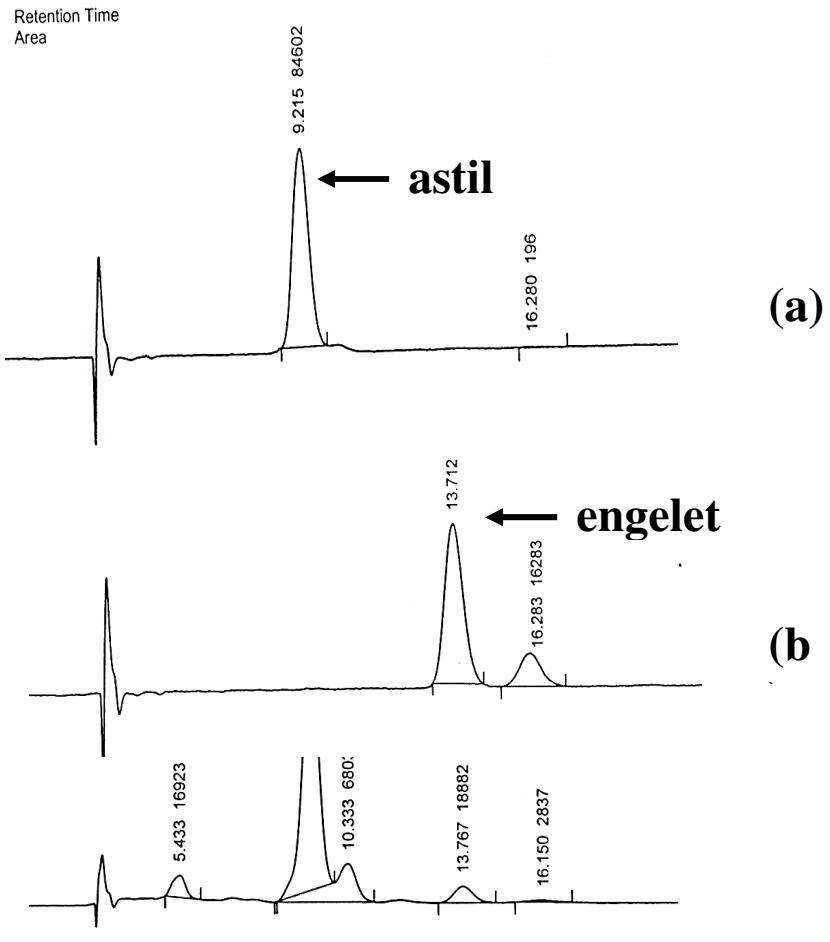


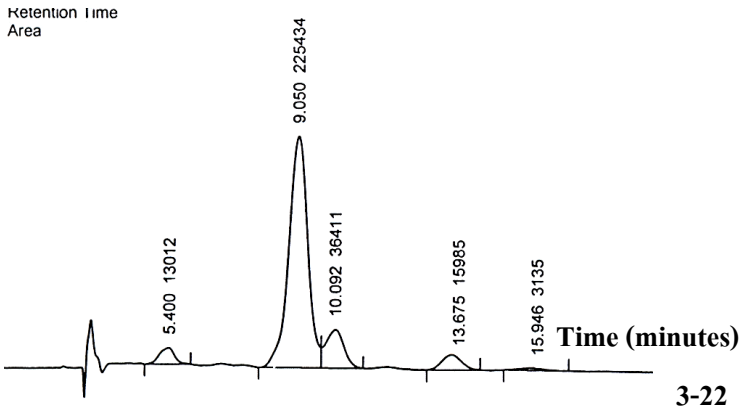
Figure 3-21 Standard curve of engeletin, the "y" value is the peak area of analysis and the "x" value is the concentration of the analysis (μg/ml)

Table 3-12 Astilbin and engeletin contents of crude extract and $CHCl_3$:MeOH 1:1 S by HPLC analysis

	Astilbin	**Engeletin**
	% content (w/w)	**% content (w/w)**
crude ethanolic extract	0.218	0.021
$CHCl_3$:MeOH 1:1) S	0.216	0.016



(c)



(d)

Figure 3-22 Typical HPLC chromatogra

ethanolic extract (c) and $CHCl_3$:MeOH 1:1 S (d) recorded at 291 nm