CHAPTER 3
RESULT AND DISCUSSION

3.1 The Result of Extraction for Preliminary Screening

The ethanolic crude extract was further extracted with various solvents according to the procedure described in chapter 2, in order to seek for bioactive fractions The results of extraction are shown in Table 3.1

Table 3.1 The results of extraction

Fraction	Solvent	Weight(g), Percentage % (wt. by wt. of EtOH crude extract)
2.1A	$CHCl_3$	18.5, (37.0)
2.1B	hexane	5.8, (11.6)
2.1C	90% MeOH	9.8, (19.6)
2.1D	EtOAc	12.3, (24.7)
2.1E	H_2O	19.5, (39)
2.2A	EtOAc	17.2, (34.4)
2.2B	H_2O	6.9, (13.8)
2.2C	BuOH	15.3, (30.6)

3.2 The Result of Biological Activity Screening Tests

3.2.1 The Result of Biological Activity Screening of the Ethanolic Crude Extract

The preliminary screening test of the ethanolic crude extracts of the whole plants of *S. africanus*, revealed high cytotoxic activity against brine shrimp *Artemia salina* (LC_{50} = 0.0001 μg/ml) and six carcinoma cell lines was presented in Table 3.2

Table 3.2 Percentage inhibition of six carcinoma cell lines with ethanolic crude extract

Method	Concentration (µg/ml)			Estimation
	1	10	100	
HL-60	3.43	40.10	79.37	+
KB	1.08	3.23	48.79	-
BGC-823	-0.83	-4.98	-	-
HCT-8	24.74	59.10	100	++
B	5.41	52.53	74.52	++
T	-55.08	-16.10	83.05	+

HL-60 Human Luekemia Carcinoma
KB Human Naspharyngal Carcinoma
BGC-823 Human Gastric Carcinoma
HCT-8 Human Colon Carcinoma
B Proliferation of Mouse (B) Lymphocyte
T Proliferation of Mouse (T) Lymphocyte

3.2.2 The Result of Biological Screening of Various Crude Extract

The ethanolic crude showed significant activity against brine shrimp and carcinoma cell lines, especially HCT-8, B, T and HL-60. The ethanolic crude extract was further extracted with various solvents. The Result of biological activity screening of various are presented in Tables 3.3-3.6.

Table 3.3 The Result of Brine Shrimp Cytotoxic Lethality Test

Fraction	Solvent	LC_{50} µg/ml	Bioactivity
2.1A	$CHCl_3$	0.00001	high activity
2.1B	Hexane	0.04491	high activity
2.1C	90% MeOH	0.00005	high activity
2.1D	EtOAc	6.44667	high activity
2.1E	H_2O	16.26521	medium activity
2.2A	EtOAc	2.20039	high activity
2.2B	H_2O	6.40240	high activity
2.2C	BuOH	17.2678	medium activity

Table 3.4 Percentage inhibition of Human Colon Carcinoma Cell with various crude extracts

Fraction	Solvent	Concentration (µg/ml)			% Estimation
		1	10	100	
2.1A	$CHCl_3$	-8.31	78.03	97.13	++
2.1B	hexane	0.63	88.18	96.50	++
2.1C	90% MeOH	-1.37	89.62	96.50	++
2.1D	EtOAc	-3.84	85.88	98.87	++
2.1E	H_2O	-15.74	-6.06	-6.91	-
2.2A	EtOAc	-15.36	-14.39	43.23	-
2.2B	H_2O	-15.74	-4.98	9.61	-
2.2C	BuOH	-0.28	-7.62	28.32	-

Table 3.5 Percentage inhibition of Human Leukemia Carcinoma Cell with various crude extract

Fraction	Solvent	Concentration (µg/ml)			% Estimation
		1	10	100	
2.1A	$CHCl_3$	14.97	73.75	75.03	++
2.1B	hexane	-44.50	62.91	79.17	++
2.1C	90% MeOH	-16.11	72.32	78.60	++
2.1D	EtOAc	20.90	89.85	97.72	++
2.1E	H_2O	-0.43	-6.99	12.59	-
2.2A	EtOAc	7.56	4.57	60.07	+
2.2B	H_2O	1.39	8.04	66.40	+
2.2C	BuOH	6.68	1.84	10.11	-

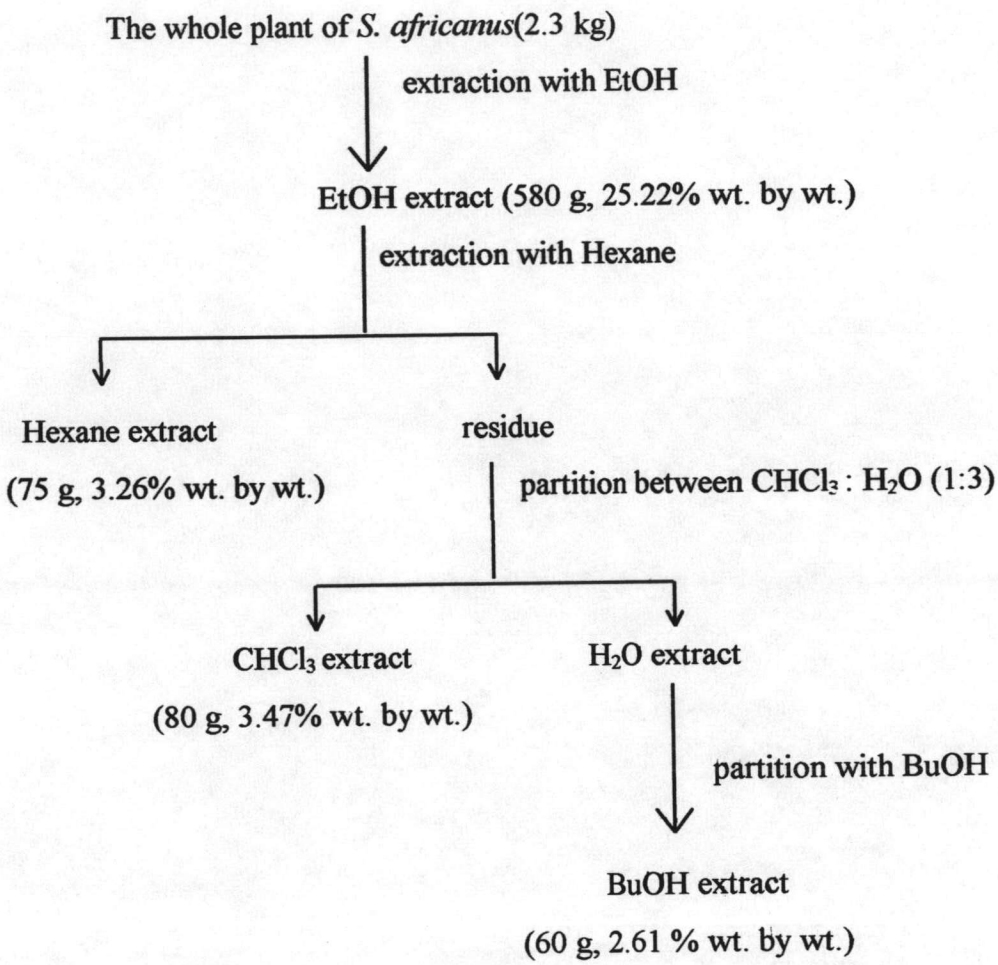
Table 3.6 Percentage inhibition of Human Hepatocellular Carcinoma Cell with various crude extract

Fraction	Solvent	Concentration (µg/ml)			% Estimation
		1	10	100	
2.1A	$CHCl_3$	-37.39	97.22	98.06	++
2.1B	hexane	-25.34	97.36	99.16	++
2.1C	90% MeOH	-30.33	97.36	98.61	++
2.1D	EtOAc	-25.54	-12.39	96.05	+
2.1E	H_2O	-12.33	12.31	29.37	-
2.2A	EtOAc	-35.31	-32.54	15.37	-
2.2B	H_2O	1.70	23.12	26.21	-
2.2C	BuOH	-23.54	15.23	-7.89	-

3.3 The Extraction for Separation

Noticeably, the chloroform and ethyl acetate crude extract revealed significant cytotoxicity against brine shrimp and various carcinoma cell lines, this fraction was further investigated. Thus, the ethanolic crude was defatted by extraction with hexane. The residue was extracted by partition with chloroform and butanol, respectively. The extraction is showed in Scheme 3.1

Scheme 3.1 The result of extraction for the whole plant of *S. africanus*



3.3 Separation

3.3.1 The Separation of Chloroform Crude Extract

The chloroform extract, 70 g was separated by open column chromatography. After the column was packed with silica gel as adsorbent. The crude mixture, which was mixed with some silica gel and to dried before the addition to the top of column. The column was eluted by increasing polarity of solvent. Each fraction (about 500 ml) was collected and concentrated to a small volume and then checked by TLC. Fractions containing the same components were combined. The results of separation and combination are indicated in Table 3.7.

3.3.2 The Separation of Butanol Crude Extract

The butanol crude extract, 60g was separated by open column. The separation and the elution procedure were the same as the separation of chloroform crude extract. The result of separation and combination indicated in Table 3.8.

Table 3.7 The result of separation of chloroform crude extract

Eluents	Fraction No.	Remark	Weight (g)
50% $CHCl_3$: hexane	1-14	white ppt in yellow oil (compound **1**)	3.48
100% $CHCl_3$	15-20	white needle crystal in yellow oil (compound **2**)	4.48
5% MeOH in $CHCl_3$	21-24	brown oil	6.12
10%MeOH in $CHCl_3$	25-29	yellow solid amorphous (compound **3**)	11.01
15%MeOH in $CHCl_3$	30-34	brown oil	8.03
20%MeOH in $CHCl_3$	35-39	dark brown oil (compound **4,5**)	4.05
30%MeOH in $CHCl_3$	40-44	dark brown oil	3.29
40%MeOH in $CHCl_3$	45-49	yellowish crystal in brown oil (compound 6)	5.26
50%MeOH in $CHCl_3$	50-53	dark brown oil	4.72
60%MeOH in $CHCl_3$	54-60	black tar	8.24
80%MeOH in $CHCl_3$	61-65	black tar	6.24
100%MeOH	66-69	black tar	10.80

Table 3.8 The result of separation of buthanol crude extract

Eluents	Fraction No.	Remark	weight (g)
15% MeOH in $CHCl_3$	1-4	yellow ptt in brown oil (compound 7)	4.75
$CHCl_3$: MeOH: H_2O (85: 15: 5)	5-11	pale brown oil	8.29
$CHCl_3$: MeOH: H_2O (80:20:5)	12-18	colorless crystal in yellow oil (compound 8,9)	3.46
$CHCl_3$: MeOH: H_2O (80:20:10)	19-30	black tar	12.04
100% MeOH	31-35	black tar	10.26

3.4 Purification, Properties and Structural Elucidation of Substances from *S. africanus*

3.4.1 The structural elucidation of compound 1

Compound **1** (50 mg) was isolated from $CHCl_3$ crude extract by eluting with 50% $CHCl_3$ in hexane. After recrystalization several times in 1:1 $CHCl_3$: hexane, a colorless crystal of melting point 278-280 °C was formed. This compound was tested with Liebermann-Burchard reagent and showed a red color, which is the characteristic of the presence of a triterpenoid structure. However, compound **1** shown negative results with 1% $FeCl_3$ and 2,4-DNP and Br_2/CCl_4. Thus, compound **1** should be saturated triterpenoid.

The IR spectrum of this compound (Fig 3.1) gave some major absorption bands at 3550-3400 cm^{-1} of hydroxy group and 1173 $cm^{-1}$of C-O streching.

The mass spectrum of compound **1** (Fig 3.2) showed a molecular ion peak at m/z 428. The other significant fragmentation peaks and fragmentation pattern of this compound was demonstrated in Scheme 3.2

The ^1H NMR spectrum ($CDCl_3$) of compound **1** (Fig 3.3) displayed signal at 3.84 (1H,s), which should be CH-OH and the signals at δ 0.85, 0.99 and 1.20 (24H, m) indicated the presence of methyl group. The signals of methylene protons and methine protons of triterpene part displayed at δ1.26-1.54 (27H, m)

The ^{13}C NMR spectrum (Fig 3.4) showed 30 signals of carbon which looked closely resemble to those of certain triterpenoid structure, friedelan-3β-ol. The comparison of the ^{13}C NMR of compound **1** and friedelan-3β-ol is shown in Table 3.9.

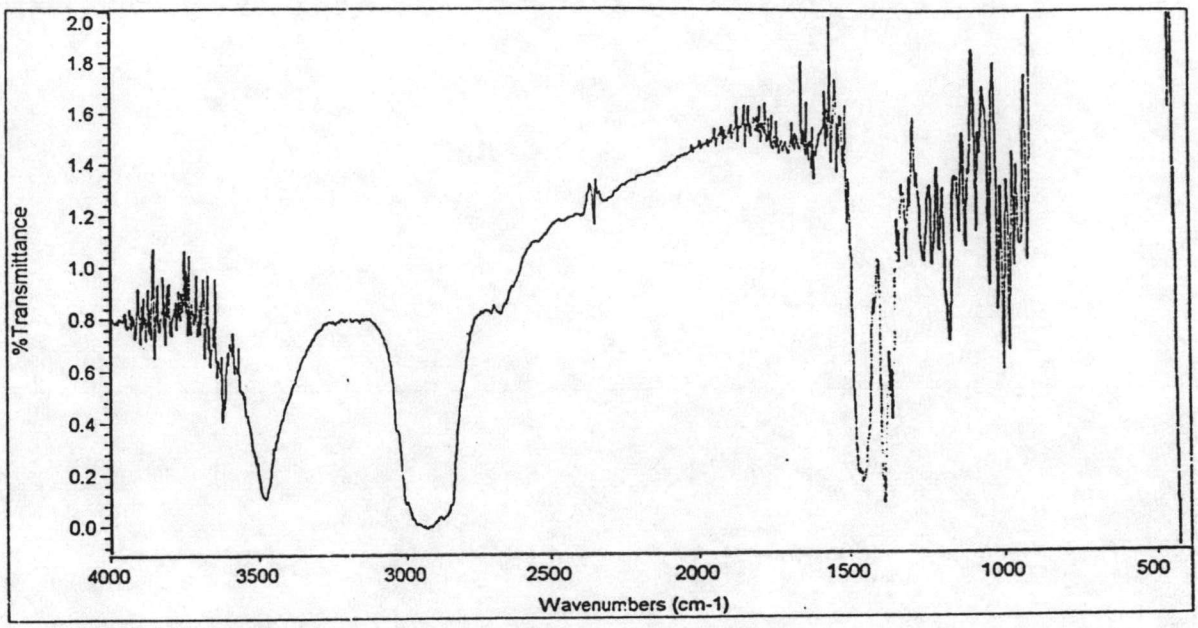


Figure 3.1 The IR spectrum of compound **1**

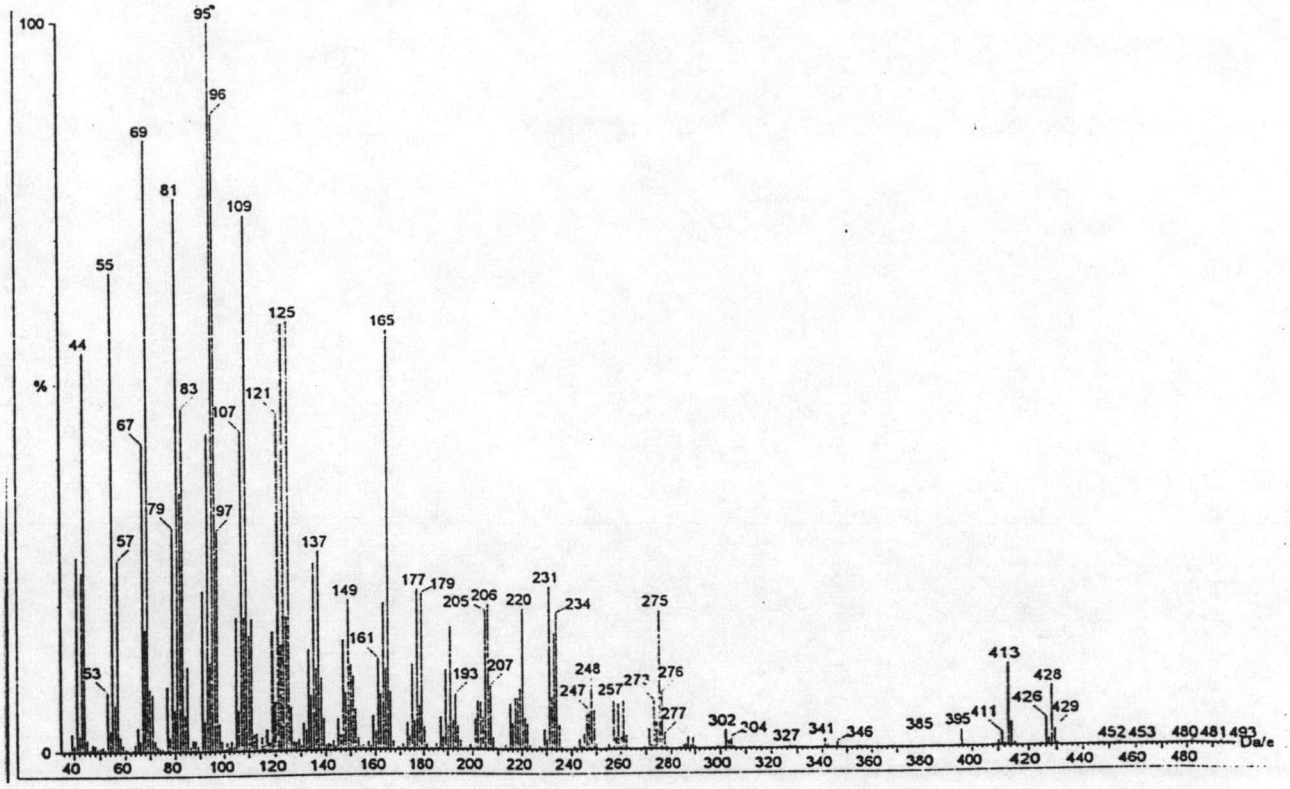
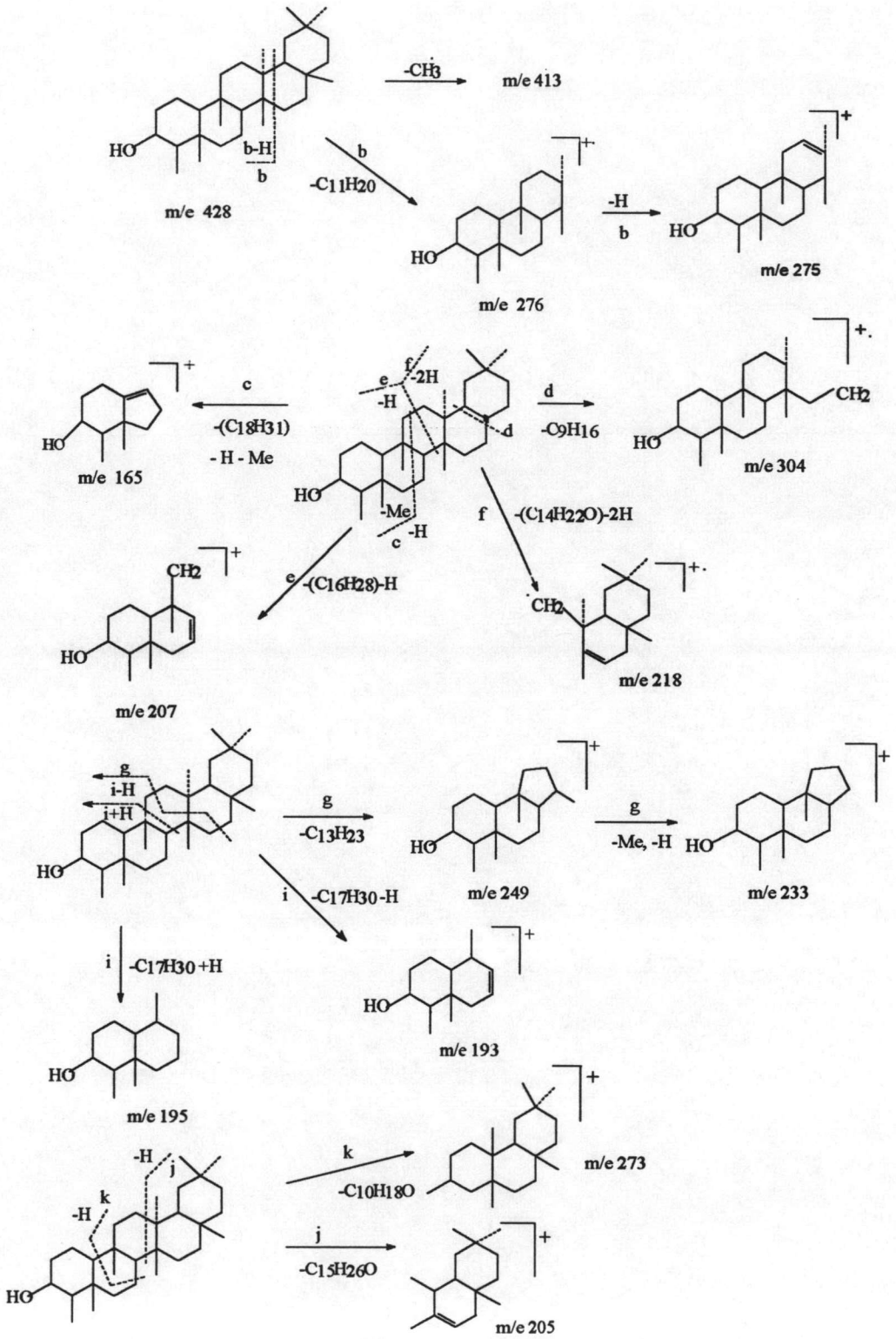


Figure 3.2 The Mass spectrum of compound **1**



Scheme 3.2 Mass fragmentation Pattern of Compound 1

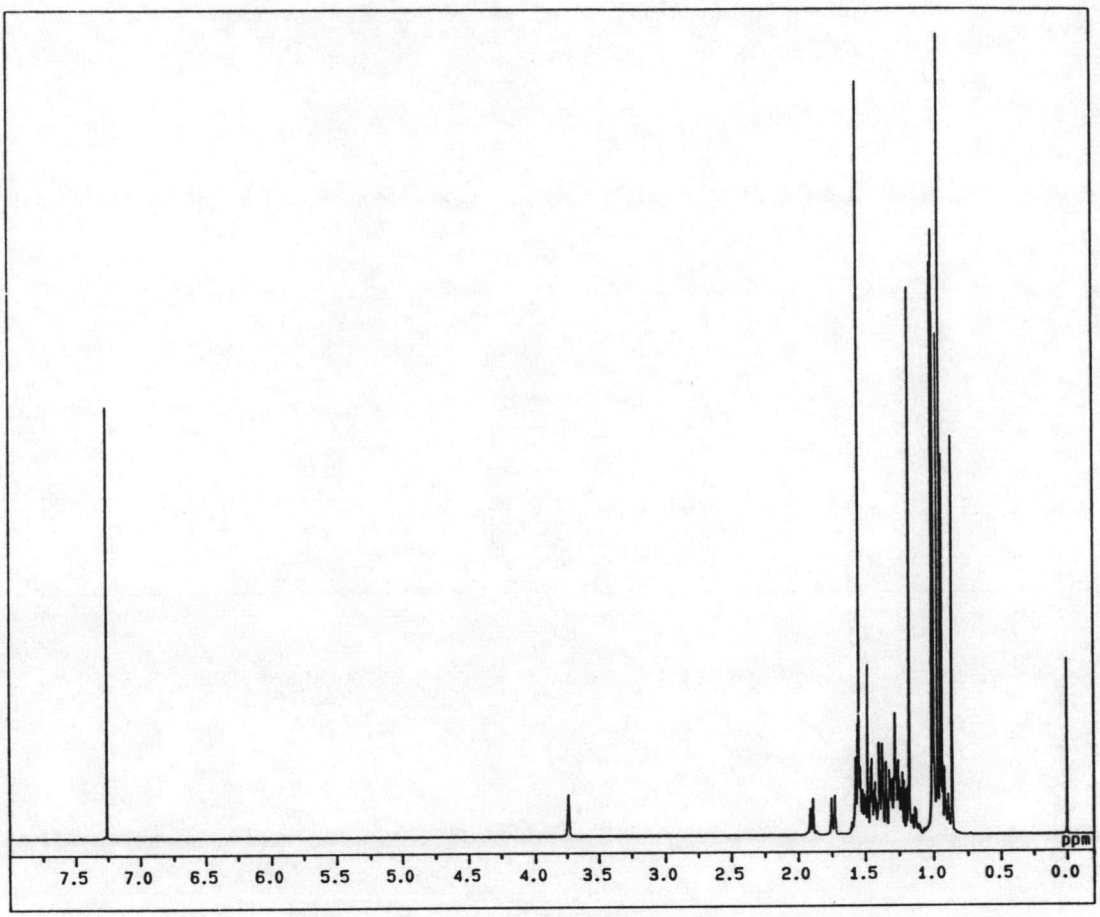


Figure 3.3 The ^{1}H NMR spectrum of compound **1**

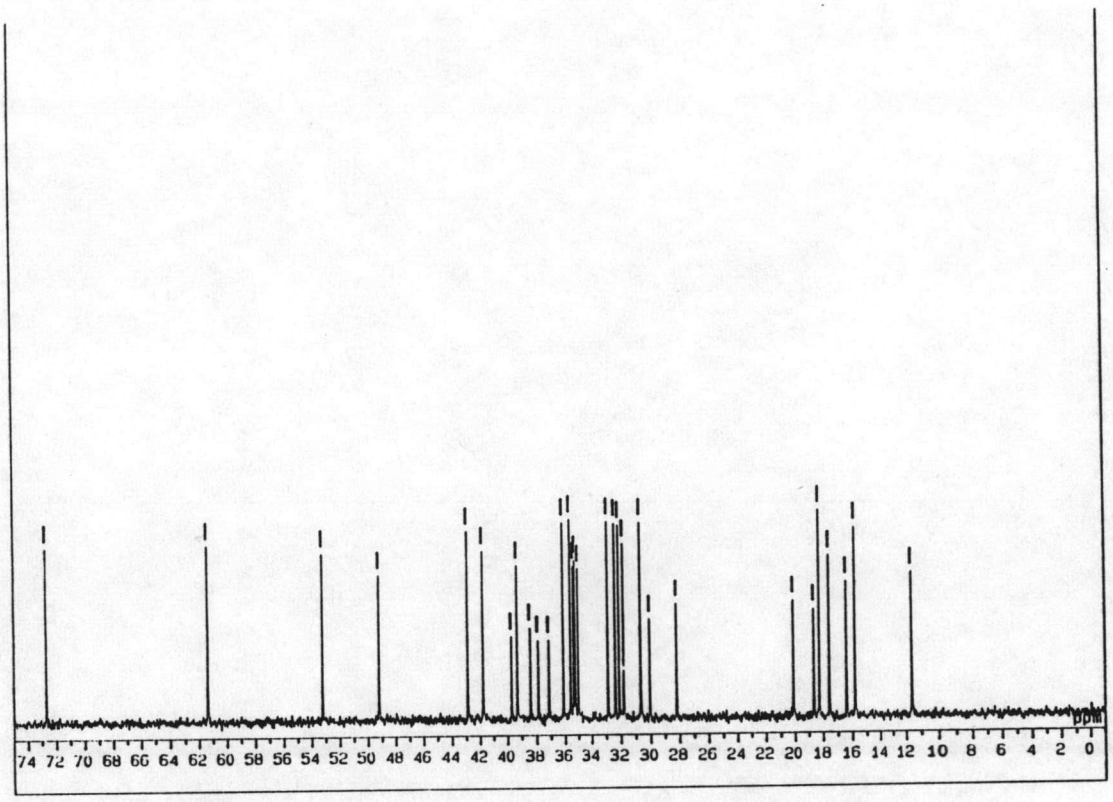


Figure 3.4 The ^{13}C NMR spectrum of compound **1**

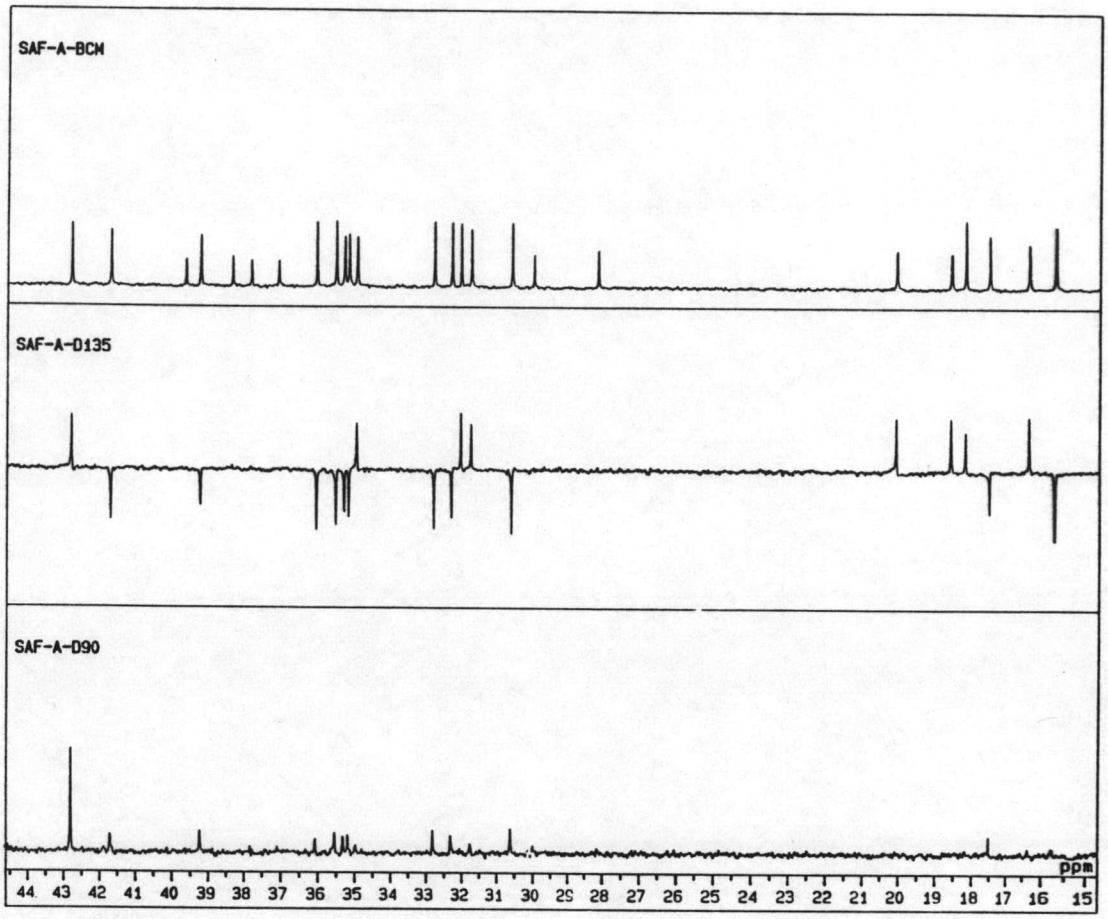
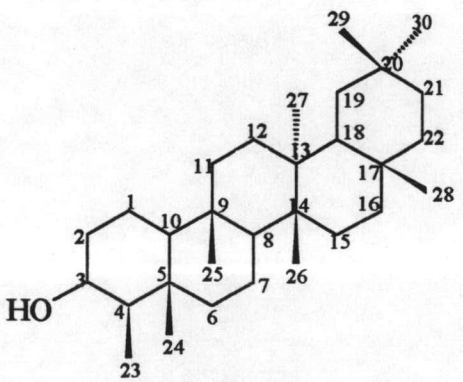


Figure 3.5 The DEPT 90, 135 spectrum of compound **1**

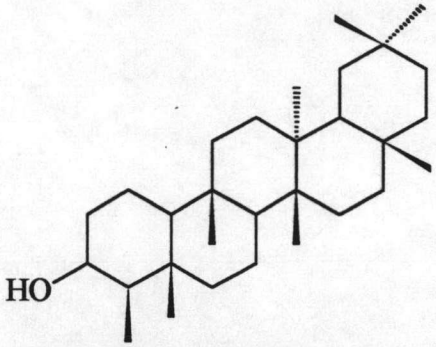
Table3.9 The ^{13}C NMR assignment of friedelan-3β-ol and compound **1**

Position of carbon	Chemical shift(ppm)	
	Friedelan-3β-ol	Compound **1**
1	16.4	16.3
2	35.3	35.3
3	72.8	72.7
4	49.3	49.1
5	37.9	37.1
6	41.8	41.7
7	17.6	17.5
8	53.3	53.2
9	35.2	35.2
10	61.4	61.3
11	35.7	36.0
12	30.7	30.6
13	38.4	37.8
14	39.7	39.6
15	32.4	32.3
16	36.2	36.1
17	30.1	30.0
18	42.9	42.8
19	35.4	35.5
20	28.2	28.1
21	32.9	32.8
22	39.3	39.2
23	11.6	11.5
24	15.8	15.8
25	18.3	18.2
26	18.7	18.6
27	20.1	20.0
28	32.1	32.0
29	35.0	35.0
30	31.8	31.7



Friedelan-3β-ol

All the above data indicated that compound **1** should be friedelan-3β-ol. The TLC employing various solvent systems found that this compound gave the same R_f value as that of friedelan-3β-ol. Therefore, compound **1** was friedelan-3β-ol.

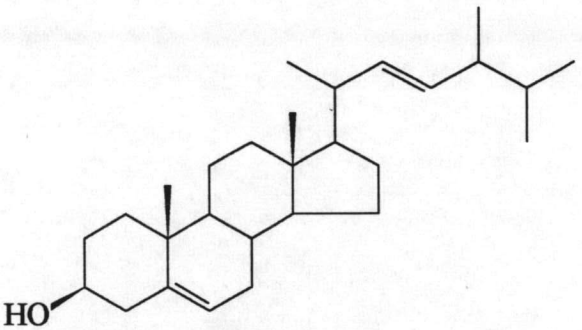


Friedelan-3β-ol

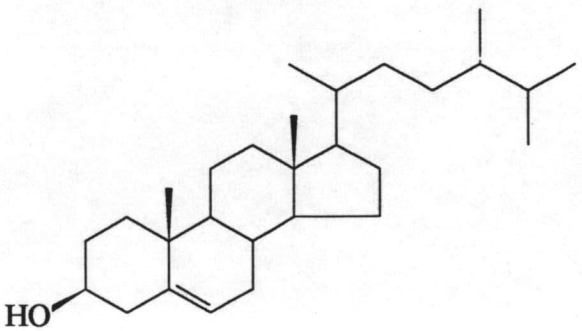
3.4.2 The structural elucidation of compound 2

Compound **2** was white needle crystal in a yellow oil, which was obtained from chloroform extract. This compound was crystallized from hexane and yielded 1.23 g of the white needles. R_f value was 0.65 ((3:2) hexane: $CHCl_3$), mp. 143-146 °C. This compound was tested with Liebermann-Burchard reagent and showed a blue color, which is the characteristic of a steroidal structure.

The IR (Fig 3.6) absorption band at 3600-3300 cm^{-1} suggested the presence of hydroxy group, an absorption band due to a disubstituted alkene at 969 cm^{-1} was also observed. The physical properties and chemical test suggested that compound **2** was the mixture of steroid. As a previously report, the mixture of steroids were stigmastrol and β-sitosterol. However this mixture were confirm by GLC technique.



stigmasterol



β-sitosterol

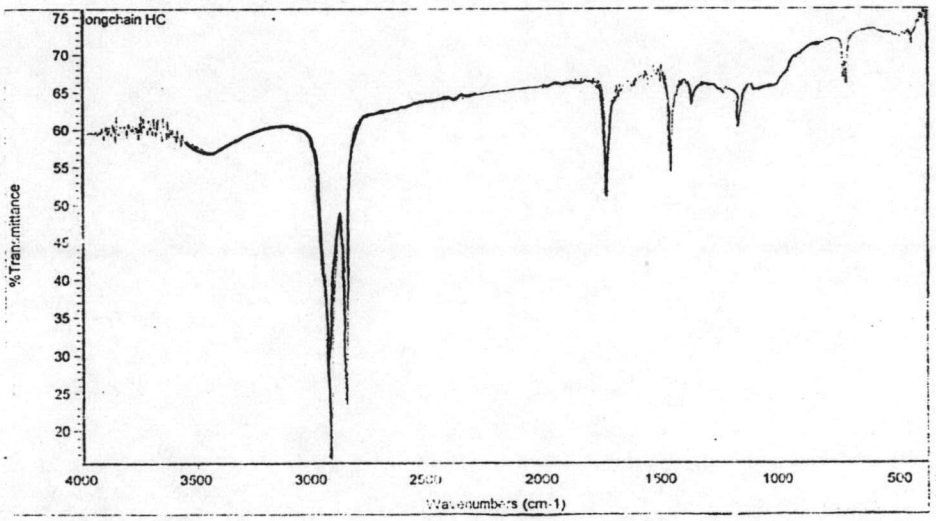


Figure 3.6 The IR spectrum of compound **2**

3.4.3 The structural elucidation of compound 3

The yellow amorphous solid of compound **3** (138 mg) was isolated from chloroform crude extract. After several recrystallizations in 1:1 MeOH/ CH_2Cl_2, a yellow amorphous solid was obtained. The melting point was 240-242 °C and R_f value was 0.55 (1:1 $CHCl_2$ /MeOH). Compound **3** was tested with a cyanidin reagent and showed red color, which is the characteristic of the presence of the flavonol compound.

The IR spectrum (Fig 3.7) showed a broad band of hydroxy group at 3500-3000 cm^{-1} and the characteristic band of C=O stretching of conjugated ketone at 1653 cm^{-1}.

The mass spectrum (Fig 3.8) showed a molecular ion peak at m/z 360 ($C_{18}H_{16}O_8$) and other fragments at m/z 317(M^+-CH_3CO), 345(M^+-CH_3), 331(M^+-C_2H_5), 302 (M^+-C_3H_6O), 181(M^+-$C_{10}H_{11}O_3$) and 153(M^+-$C_{11}H_{11}O_4$).

The ^1H NMR spectrum ($CDCl_3$) (Fig 3.9) with signal integration revealed the presence of aromatic protons at δ (ppm): 7.05(1H, d, J=5.5 Hz), 6.52(1H, s), 6.60(1H, s) and 5.90 (1H, s). Methoxy groups at δ 4.00, 3.96, 3.92(each 3H, s) and featured protons of hydroxy protons at δ 11.63(1H, s), 7.90(1H, d, J = 2.14 Hz) and 7.75(1H, dd, J = 2.8, 1.8 Hz) were also observed.

The ^{13}C NMR spectrum of compound **3** (Fig 3.10) exhibited resonance for all carbons. The multiplicity of each carbon was established the presence of four tertiary carbons at δ 121.9, 114.8, 110.7 and 90.9 ppm. The quaternary carbons at δ 175.4, 159.4, 152.5, 151.6(x2), 140.0, 146.6, 145.8, 135.6, 123.1 and 104.5, respectively and the presence of methoxy carbons at 60.8, 56.4 and 56.3.

All the above data suggested compound **3** to be quercetagentin-3,6,7-trimethyl ether. Since both compound **3** and quercetagentin-3,6,7-trimethyl ether displayed the same spot on TLC plate in various solvents by TLC technique. Thus, compound **3** was actually quercetagentin-3,6,7-trimethyl ether. The proton and carbon assignment of compound **3** shown in figure were achieved on the basis of comparison with previous reports[15].

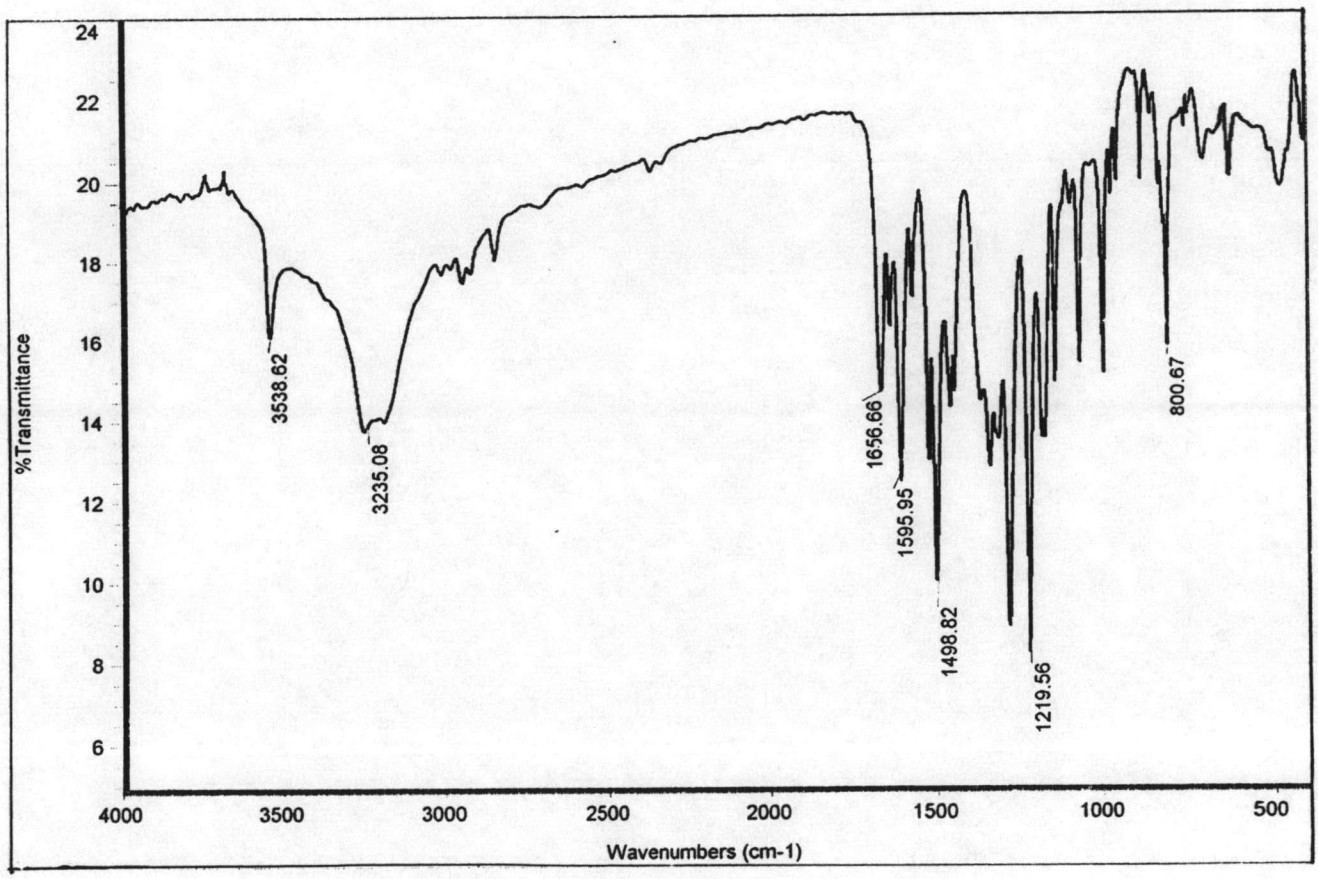


Figure 3.7 The IR spectrum of compound **3**

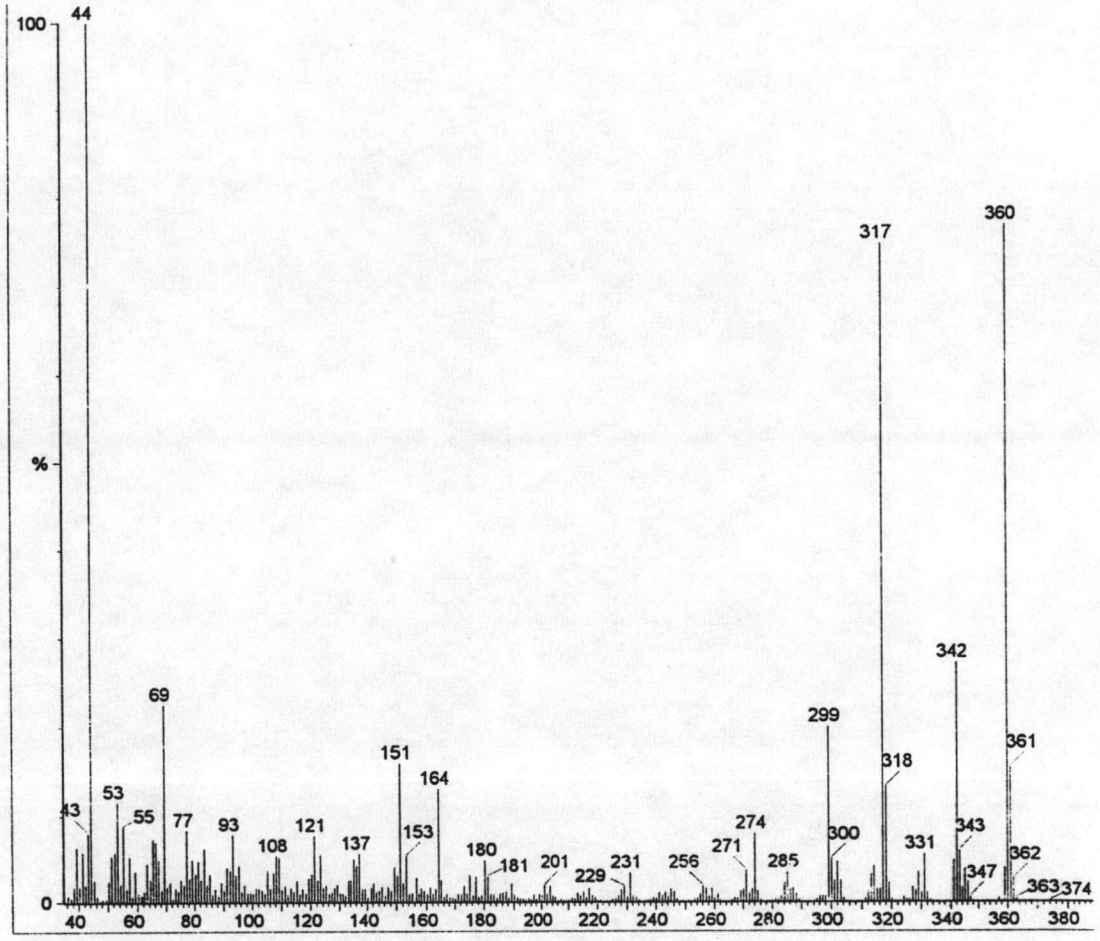
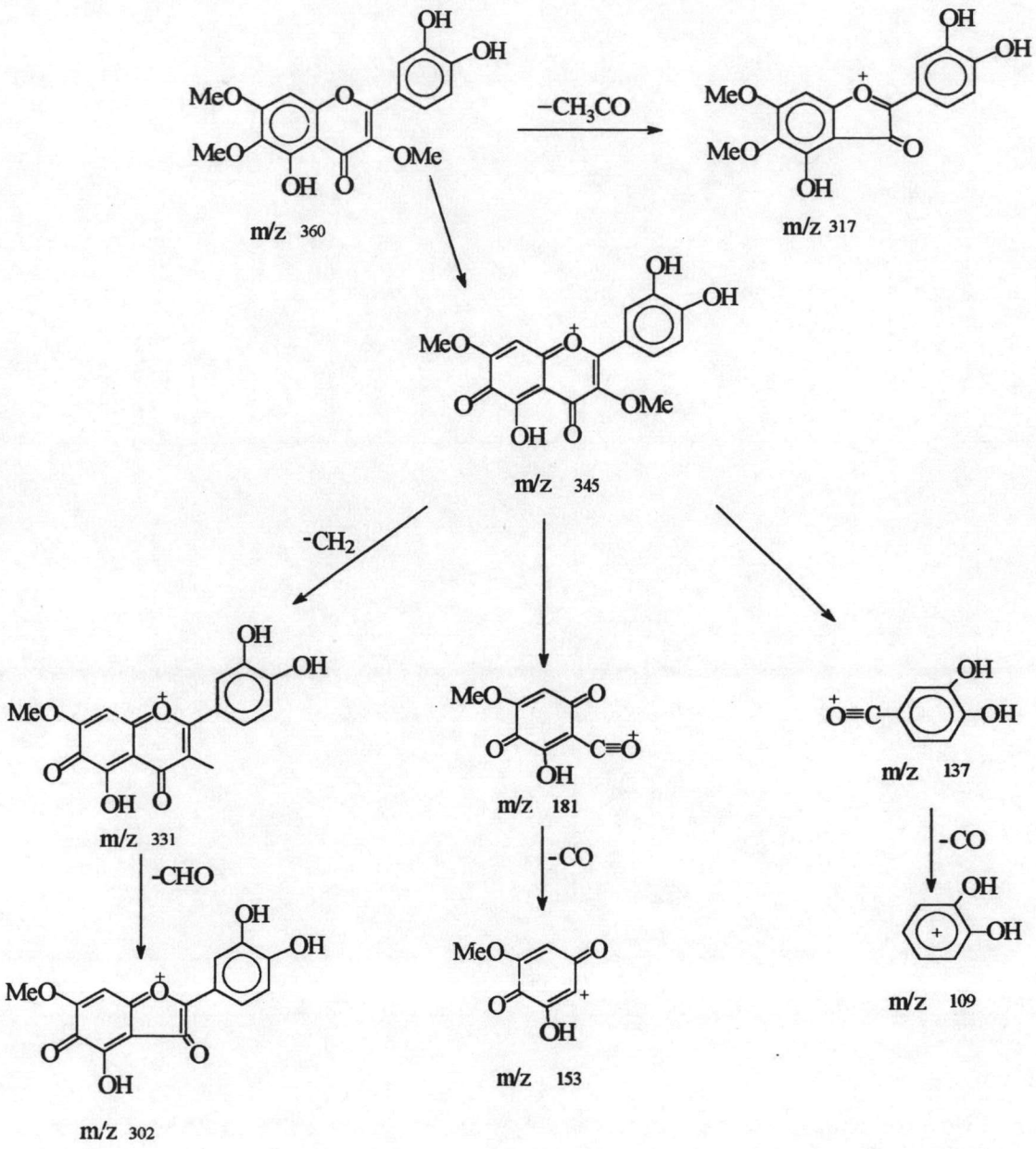


Figure 3.8 The mass spectrum of compound **3**



Scheme 3.3 The Mass fragmentation of compound **3**

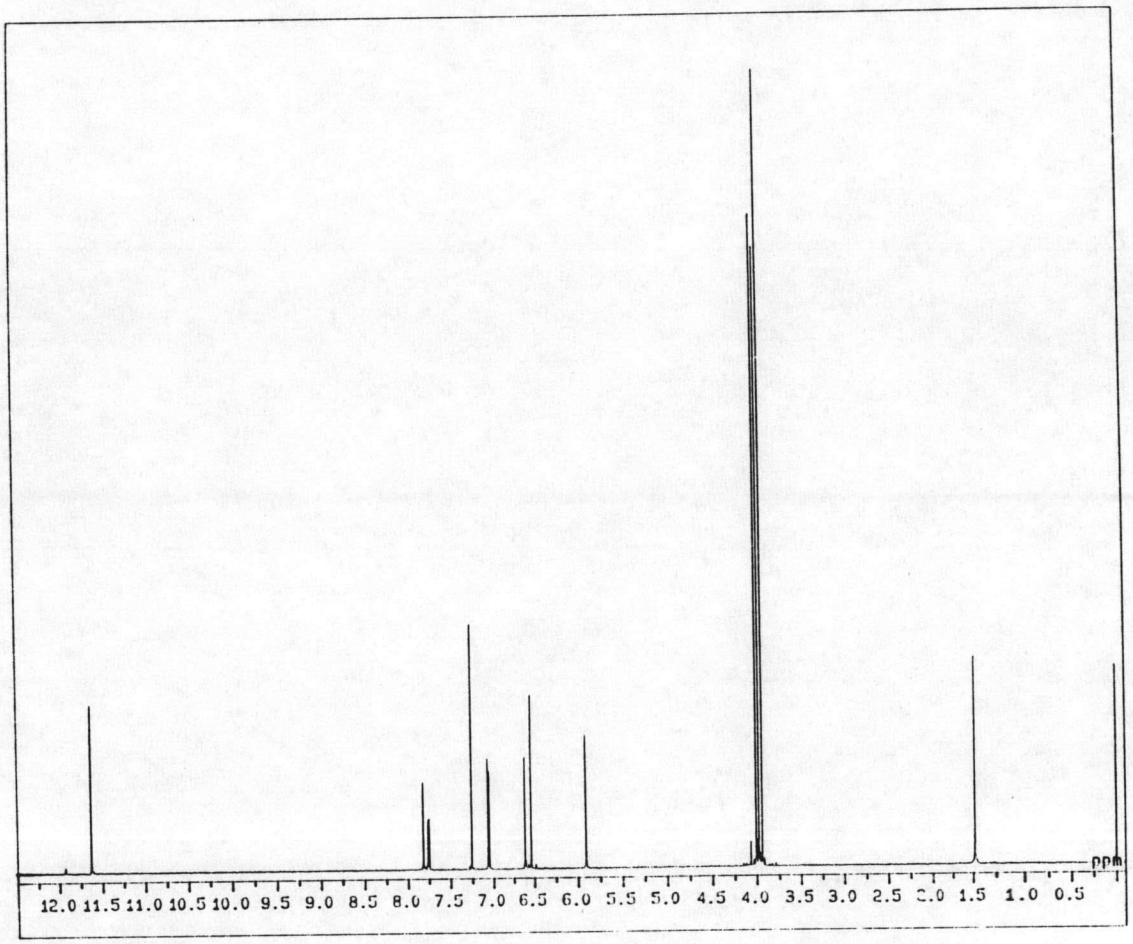


Figure 3.9 The ^{1}H NMR spectrum of compound **3**

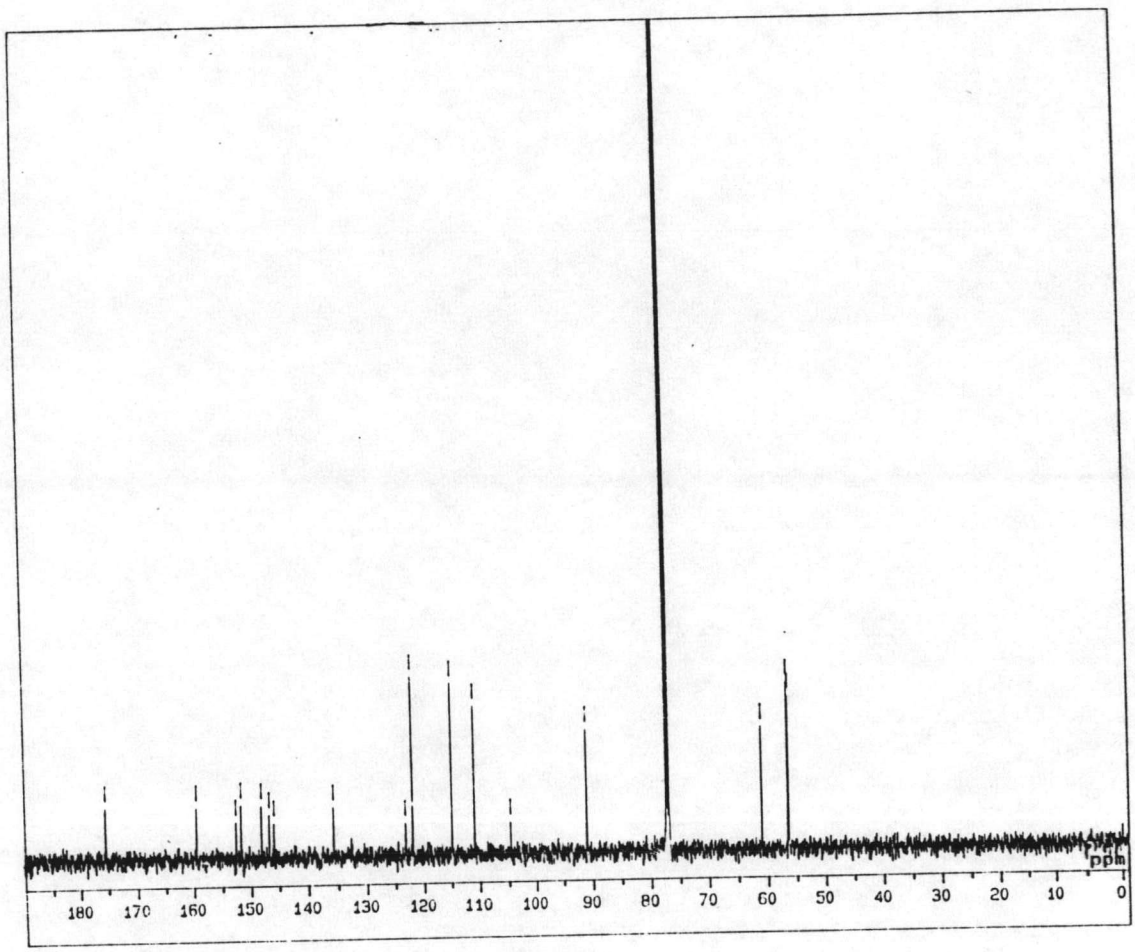


Figure 3.10 The ^{13}C NMR spectrum of compound **3**

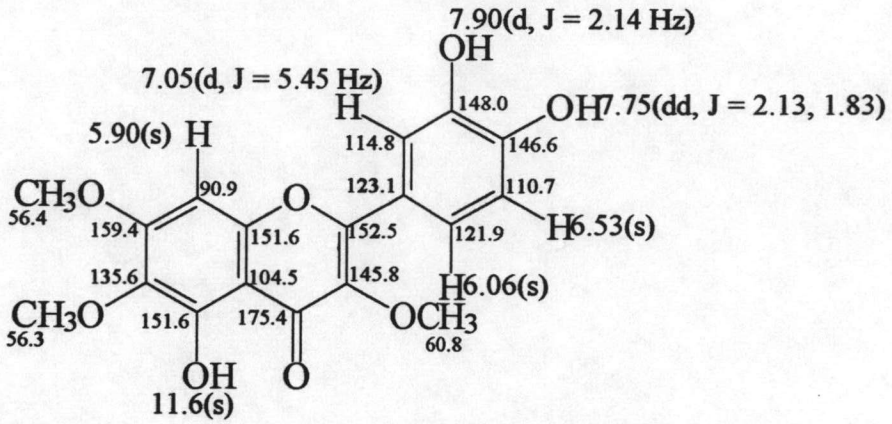


Figure 3.11 Proton and carbon assignment of (compound **3**) quercetagentin-3,6,7-trimethyl ether

3.3.4 The structural elucidation of compound 4

Compound **4** (168 mg) was obtained from chloroform crude extract by eluting with 20 % MeOH in $CHCl_3$. This fraction was further purified by chromatotron technique, starting from 10% EtOAc in hexane. The second fraction eluted with 20% EtOAc in hexane was evaporated to afford colorless oil.

The IR spectrum (Fig 3.20) showed an intense band of OH stretching at 3532 cm^{-1}, the characteristic band of α, β unsaturated ester ($C{=}CCO_2R$) at 1711 cm^{-1} and α, β unsaturated ketone (C=CC=O) at 1655 cm^{-1}.

The molecular formula of compound **4** was established by high resolution mass spectroscopy which revealed a molecular ion peak at m/z 364.6, calculated for $C_{20}H_{28}O_6$. The loss of one molecule of angelate and two molecules of angelate gave peak at m/z 264 and m/z 164, respectively. Other fragmentation peaks were found at m/z 83 $[C_4H_7CO]^+$, the characteristic of cyclic ketone and m/z 55 $[83\text{-}CO]^+$. The possible mass fragmentation pattern of this compound was shown in scheme 3.4.

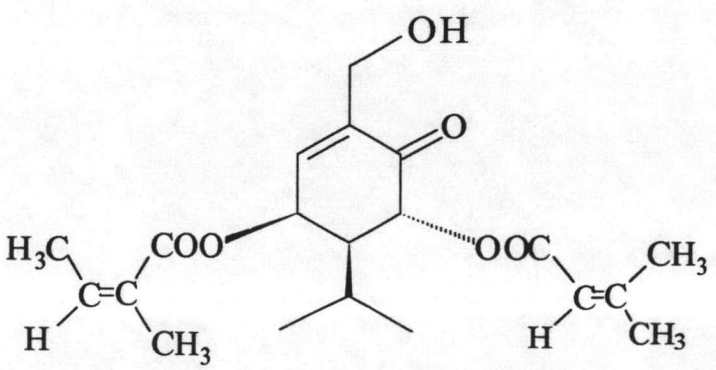
The ^1H NMR spectrum (Fig 3.17) and signal integration (Fig 3.18) indicated important proton signals at δ (ppm) : 7.03 (1H, dt, J = 5.5, 2.7 Hz), 6.16 (2H, dq, J = 7.3, 1.5 Hz), 5.84 (1H, d, J = 12.5 Hz), 5.76 (1H, m), 4.32 (2H, br dd, J = 7.9, 5.5 Hz), 2.51 (1H, ddd J = 5.2, 4.3, 4.9 Hz), 2.03(1H, m), 1.94 ($4CH_3$, m), 1.05(CH_3, d, J = 7.01 Hz), 0.99 (CH_3, d, J = 7.0 Hz).

The ^{13}C NMR spectrum (Fig 3.20) and The DEPT 90, 135 spectra (Fig 3.21) showed ketonic carbon (δ 195.2), ester carbons (δ 167.0 and 166.7), methine carbons (δ 73.1, 66.9, 46.2 and 27.8), metylene carbon (δ 60.6), methyl carbons (δ 19.9, 19.6 and 15.9), tertiary carbon (δ 138.8) and quarternary carbons (δ 139.3, 138.4 and 127.2).

From literature survey[6] , the ^1H NMR spectral data of compound **4** was close to those of 3∝-angeloxoyloxy-5β-tigloyloxy-7-hydroxycarvotacetone isolated previously from *S. bullatus.*

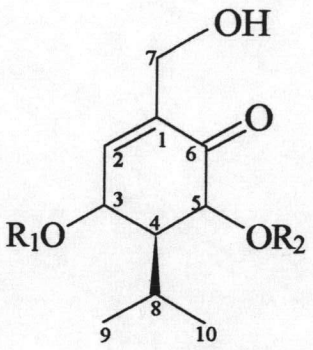
Table 3.10 The comparison of proton NMR of 3∝-angeloxoyloxy-5β-tigloyloxy-7-hydroxycarvotacetone and compound **4**

Proton position	3∝-angeloxoyloxy-5β-tigloyloxy-7-hydroxycarvotacetone	Compound **4**
2	7.02	7.03
3	5.74	5.76
4	2.49	2.51
5	5.82	5.84
7	4.35	4.32
8	2.04	2.03
9	1.03	1.03
10	0.98	0.99
$OCOR^1$	6.97	6.16
	1.83	1.94
	1.90	1.94
$OCOR^2$	6.15	6.16
	2.01	1.94
	1.91	1.94

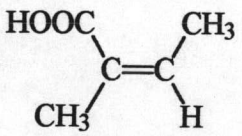


3α-angeloxoyloxy -5β-tigeloyloxy-7-hydroxycarvoacetone

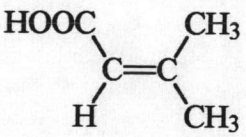
Generally, most cavotacetones had hydroxy groups at C-3, C-5 and C-7 which were always esterified by most common acids such as tiglic acid, angelic acid and acetic acid.



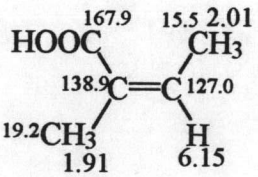
Basic skeleton of carvotacetone



Angelic acid



Tiglic acid



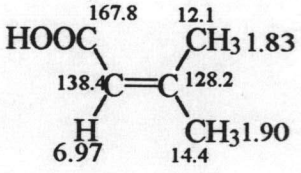


Figure 3.12 Proton and carbon assignment of angelic acid and tiglic Acid

Nonetheless, the ^{13}C NMR spectrum exhibited signals of 2-methylbut-2-enolate that might be angelate (*Z*-isomer) or tiglate (*E*-isomer)[28]. The conjugated ester group caused an upfield shift (δ = 6.16) of neighboring proton more pronounce for angelate than for tiglate. Through-space coupling (see NOESY spectrum) of methine proton (δ 6.16) and two groups of methyl (δ2.03, 1.94) certainly indicated that only two angelates existed as substituents.

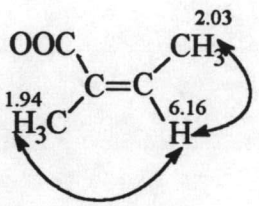


Figure 3.13 NOESY correlation of proton and methyl groups of angelate

Relative stereochemistry of three methine protons at δ 5.76 (H-3), 2.51(H-4), and 5.74 (H-5) was deduced from their coupling constant. Owing to the greater coupling constant of $^{3}J_{4,5}$ (12.5 Hz), H-4 and H-5 were arranged in the opposite direction, while H-3 and H-4 were oriented in the same plane. Additionally, NOESY spectrum also supported the relative stereochemistry of H-3, H-4 and H-5. Only contour plots due to the correlation of H-3 and H-4 were observed. The ^{1}H-^{1}H COSY spectrum allowed the assignment of all proton signals while the ^{13}C NMR signals were assigned by HMQC and HMBC experiments.

Table 3.11 The carbon and attached proton determined by one bond correlation in HMQC spectrum

Carbon position	Chemical shift (ppm)	Attached proton
C-1	139.3	-
C-2	138.8	7.03(1H, dt, J = 5.5 Hz)
C-3	67.5	5.76(1H, m)
C-4	46.2	2.51(1H, ddd, J = 5.2, 4.3, 4.5 Hz)
C-5	73.1	5.84(1H, d, J = 12.5)
C-6	195.2	-
C-7	60.6	4.32(2H, br dd, J =7.9, 5.5 Hz)
C-8	27.7	2.03(1H, m)
C-9	19.9	1.05(3H, d, J =7.0 Hz)
C-10	19.6-	0.99(3H, d, J = 7.0 Hz
OR^1	Oang	
C-1'	166.9	-
C-2'	127.2	-
C-3'	139.1	6.16(2H, dq, J = 7.3, 1.5 Hz)
C-4'	15.9	1.94 (m)
C-5'	19.1	1.94 (m)
OR^2	Oang	
C-1"	166.7	-
C-2"	127.0	-
C-3"	140.0	6.16(m)
C-4"	15.8	1.94 (m)
C-5"	19.5	1.94 (m)

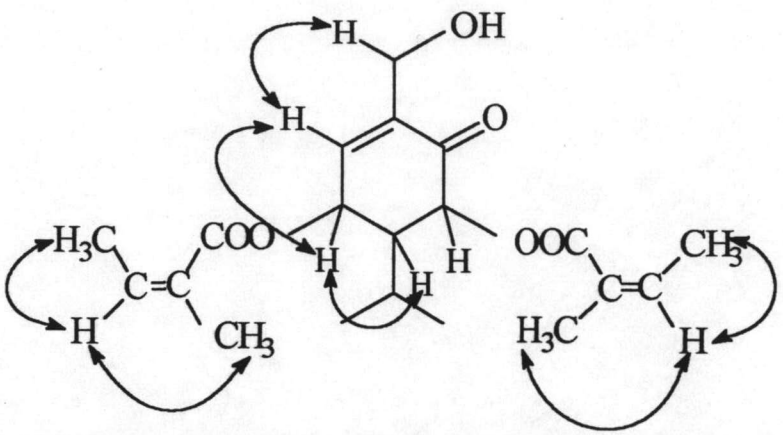


Figure 3.14 Through space coupling of proton as deduce from NOESY

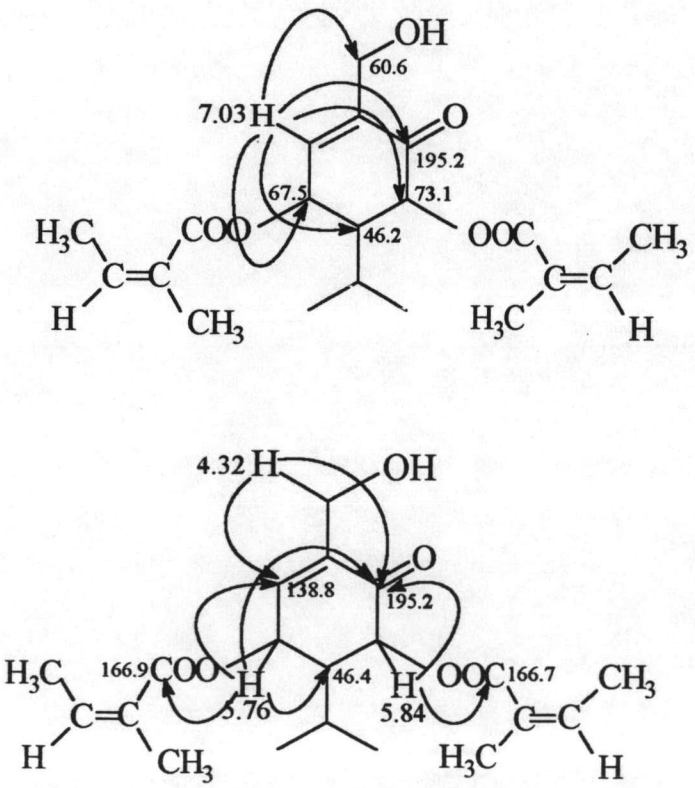


Figure 3.15 Long range ^{1}H-^{13}C coupling as detected in HMBC

All above data allowed us to assign compound **4** as 3α, 5β-diangeloxoyloxy-7-hydroxycarvotacetone. Computational structure searching suggested that there was no literature reported the isolation of this compound; consequently, it was a new carvotacetone.

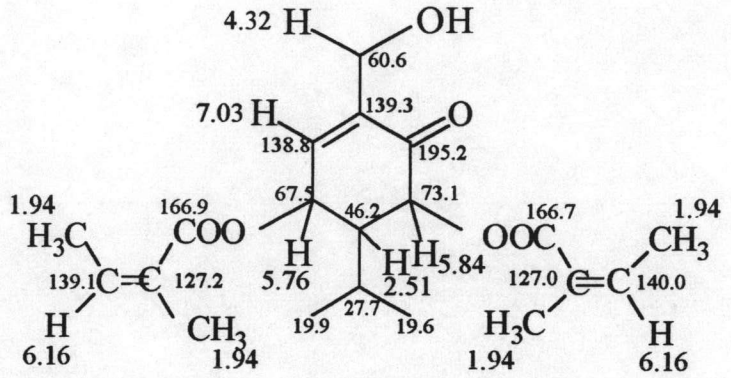
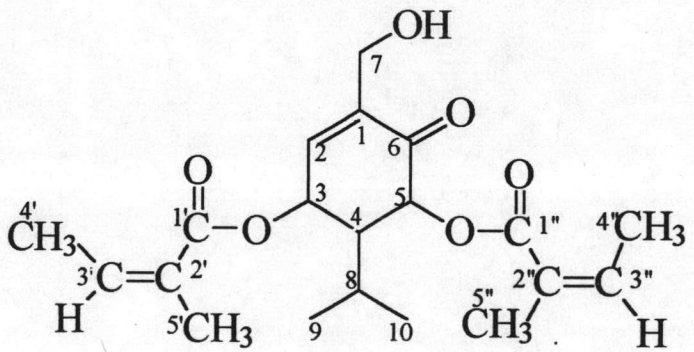
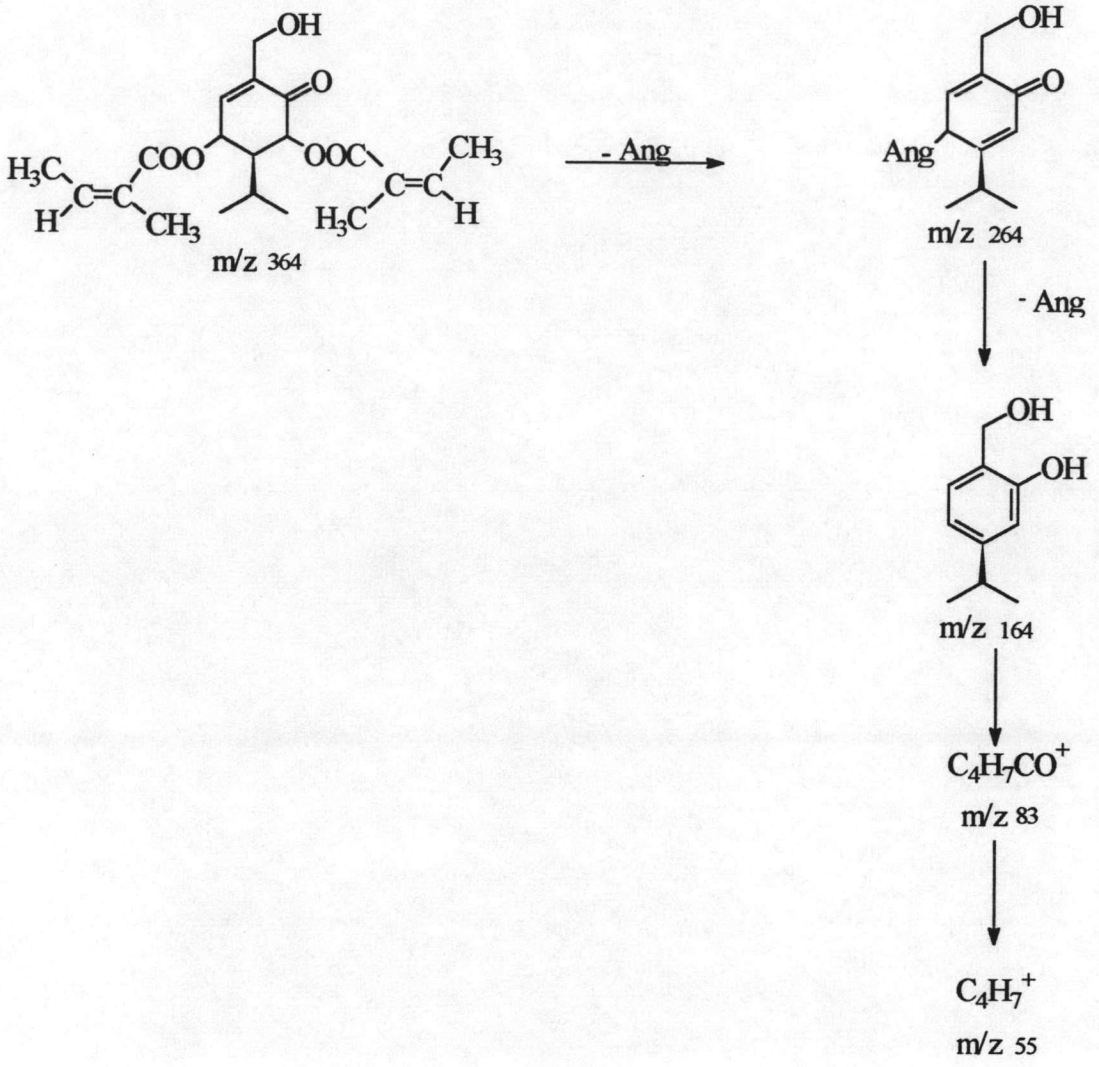


Figure 3.16 Proton and carbon assignment of compound **4**



3α,5β -diangeloxoyloxy-7-hydroxycarvotacetone



Scheme 3.4 The mass fragmentation pattern of compound **4**

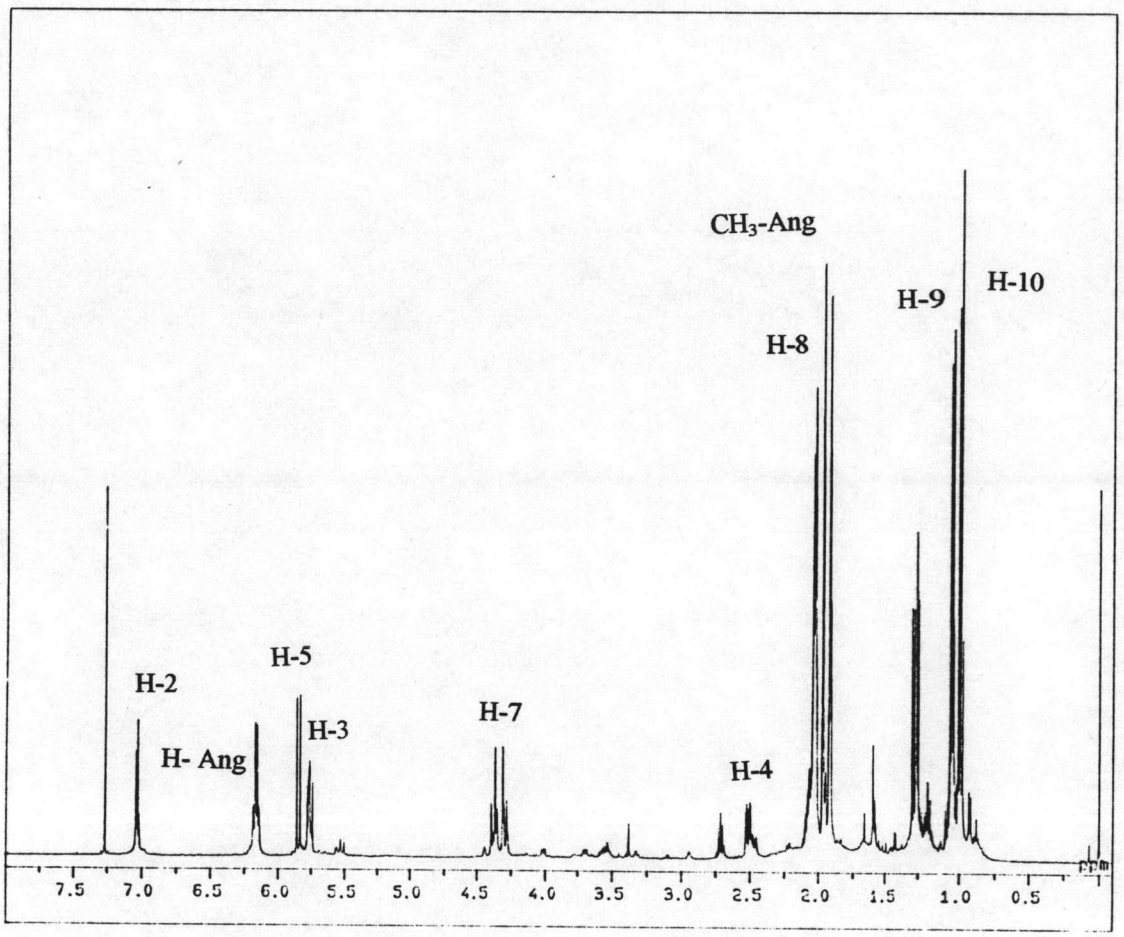


Figure 3.17 The ^{1}H NMR spectrum of compound **4**

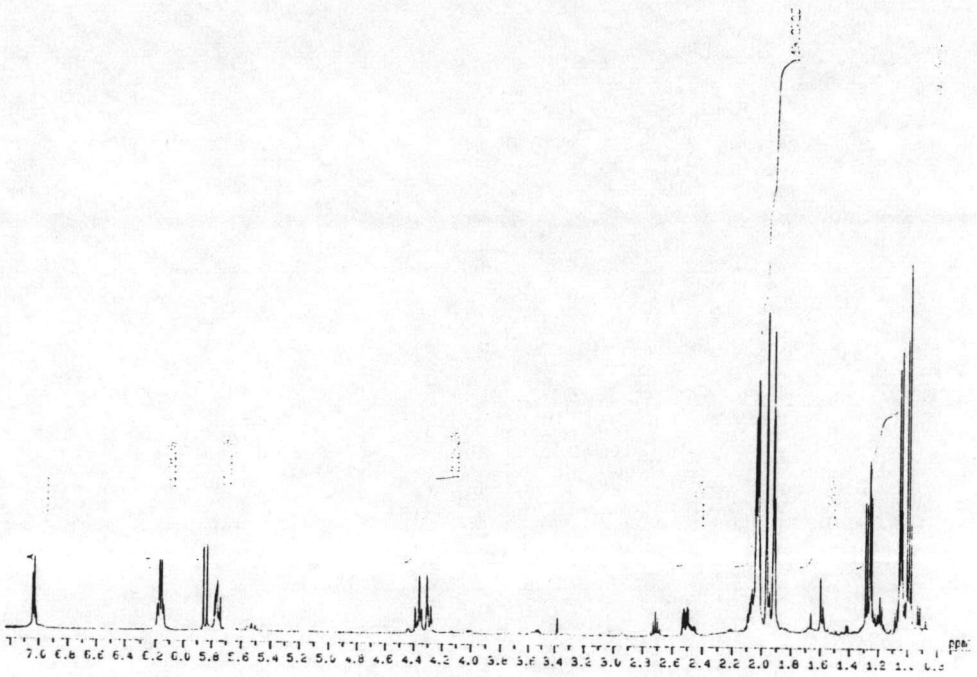


Figure 3.18 The proton integration signals of compound 4

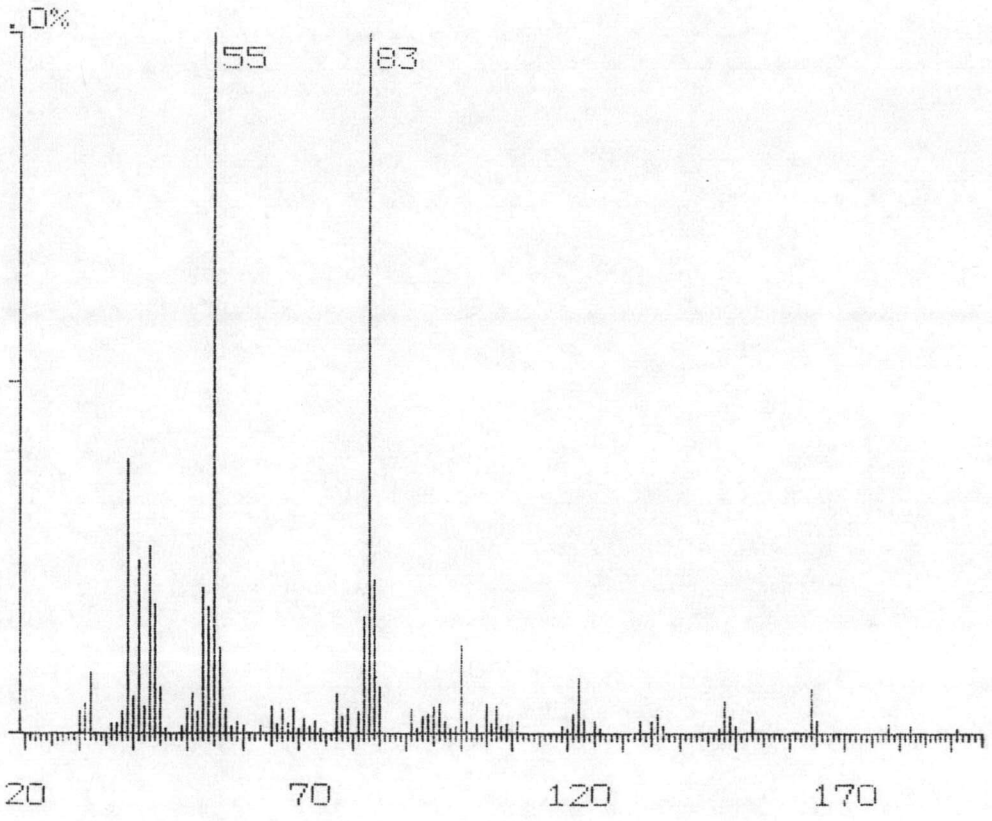


Figure 3.19 The mass spectrum of compound **4**

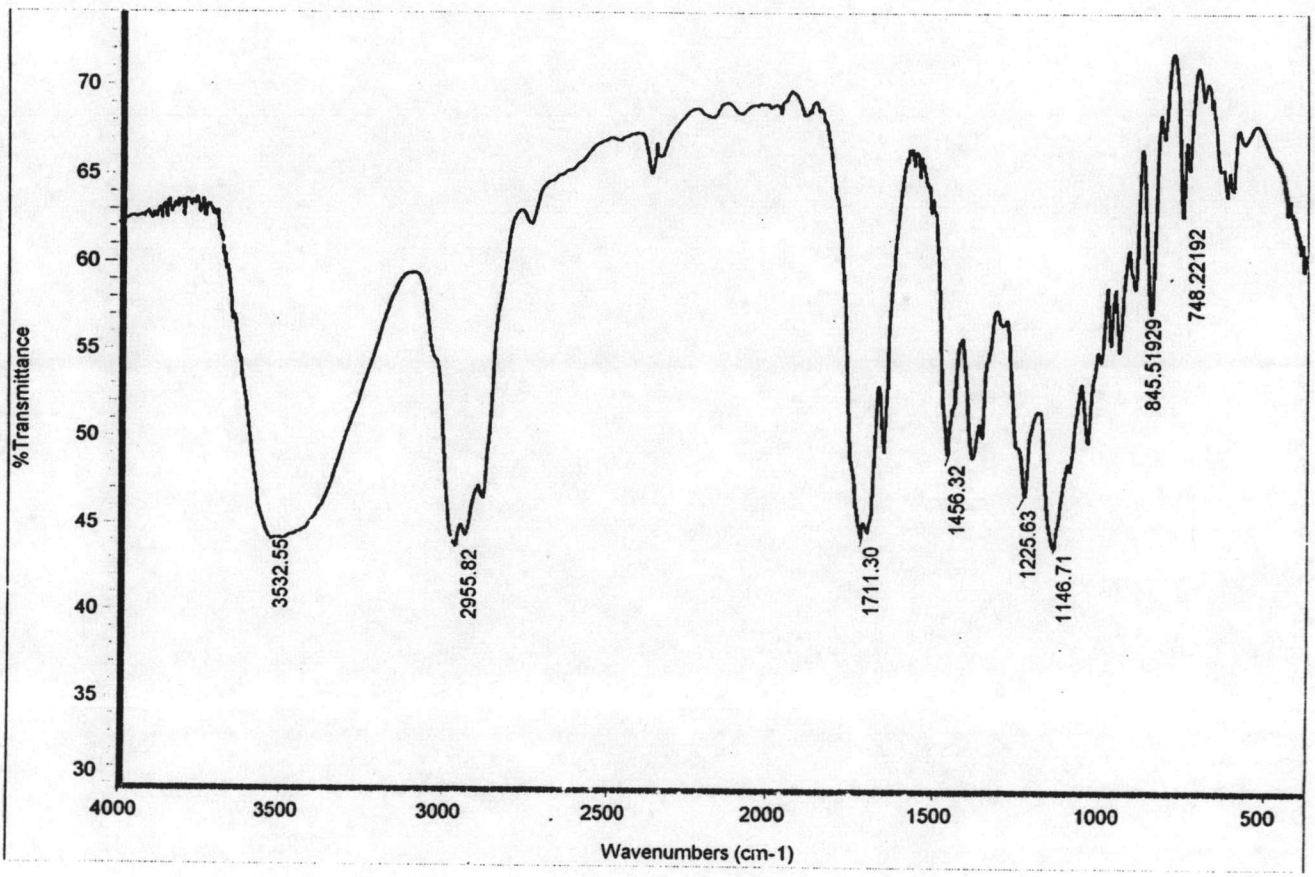


Figure 3.20 The IR spectrum of compound 4

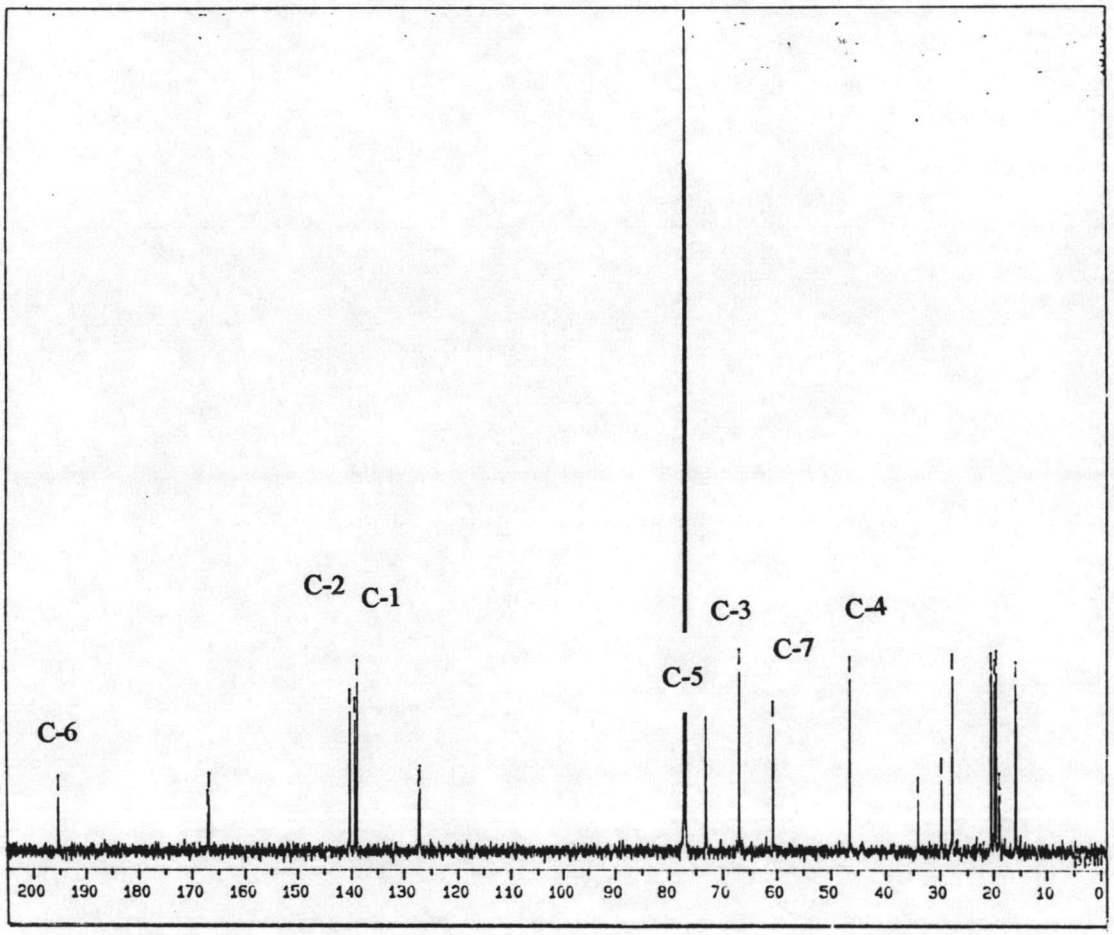


Figure 3.21 The ^{13}C NMR spectrum of compound 4

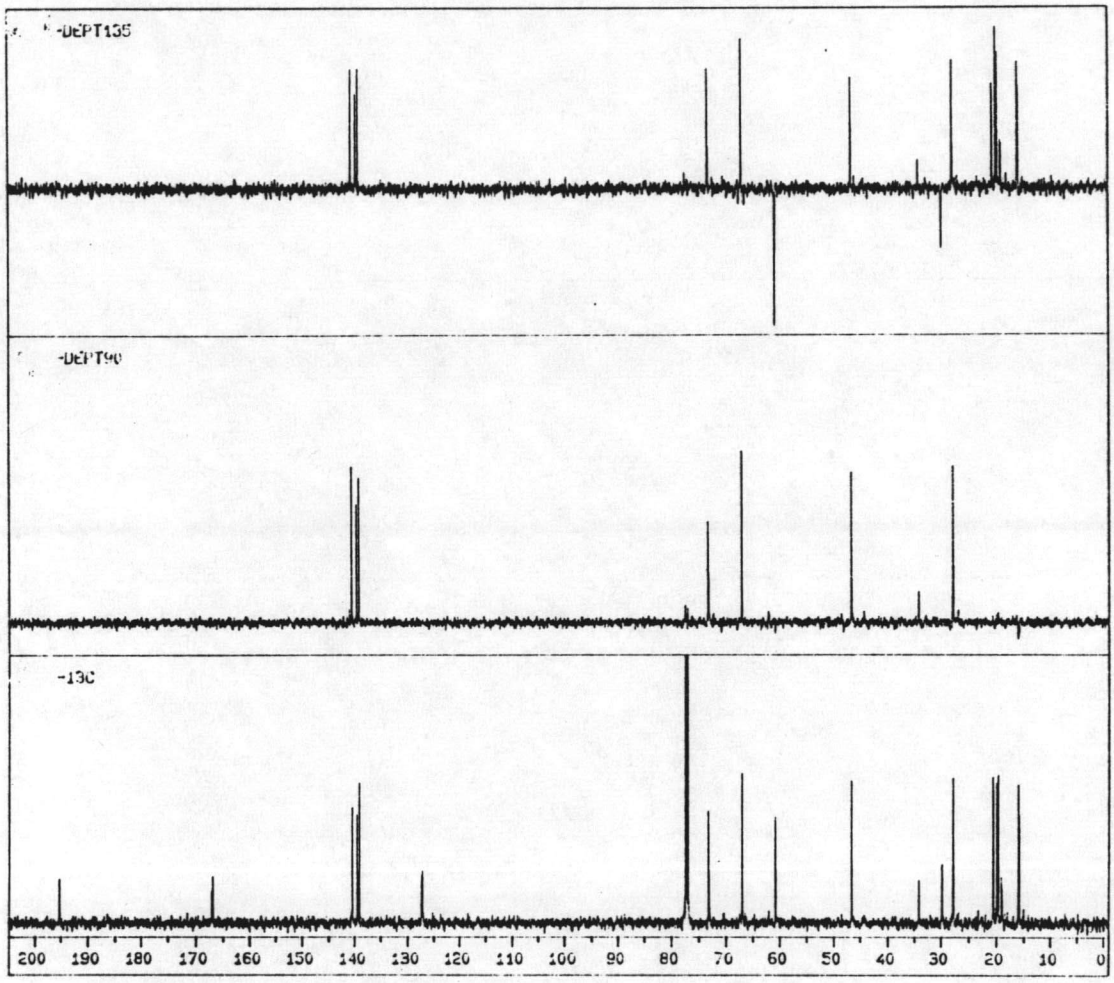


Figure 3.22 The 90, 135 DEPT spectra of compound **4**

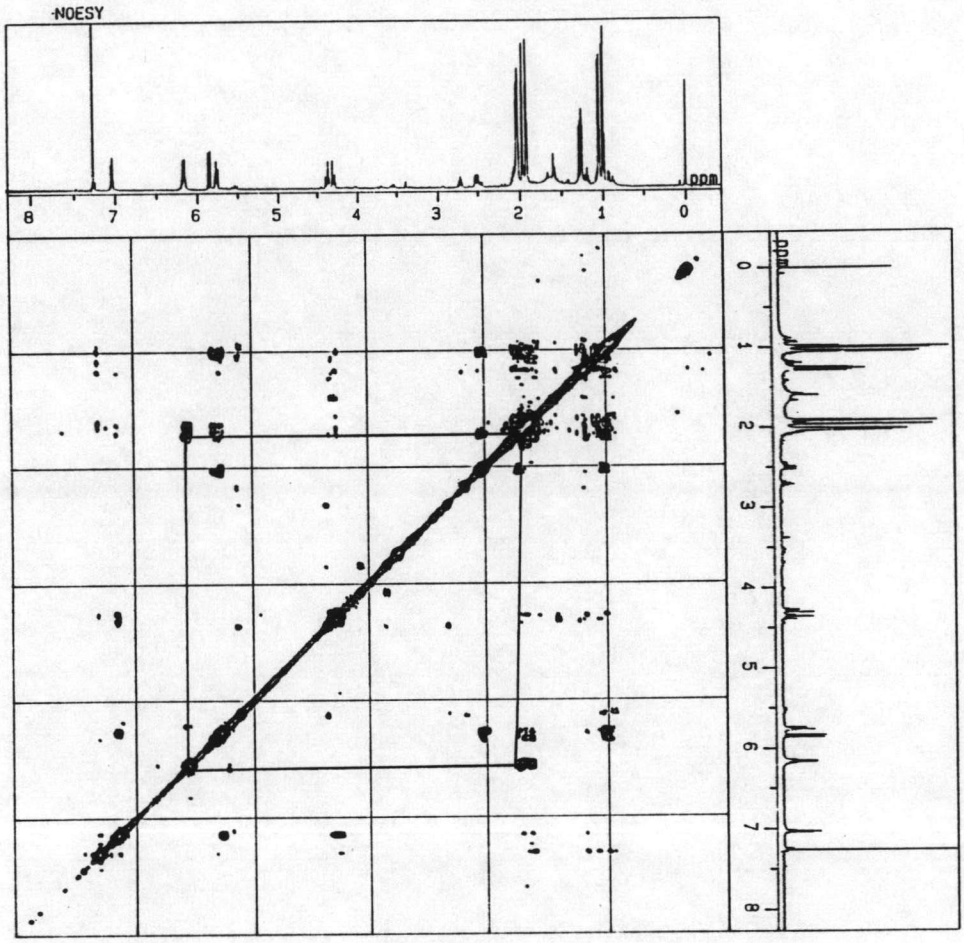


Figure 3.23 The NOESY spectrum of compound 4

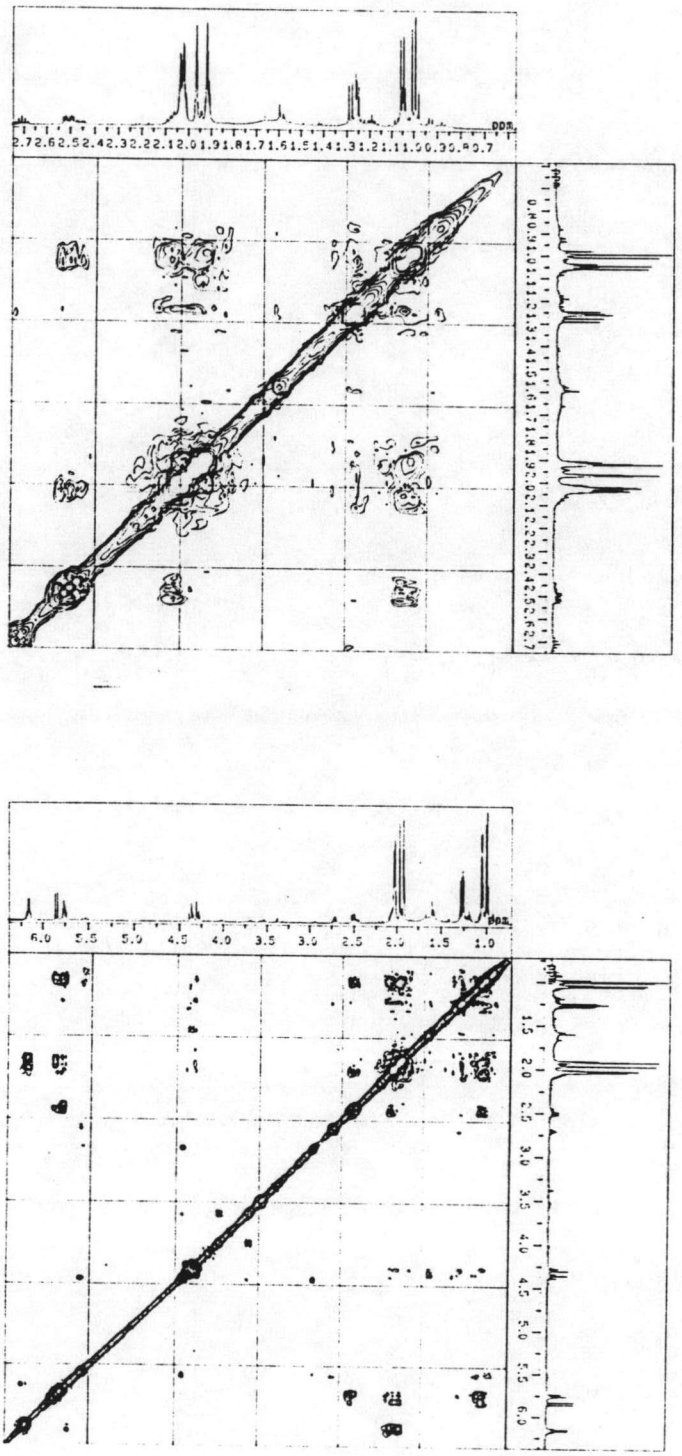


Figure 3.24 The expanded NOESY spectrum of compound **4**

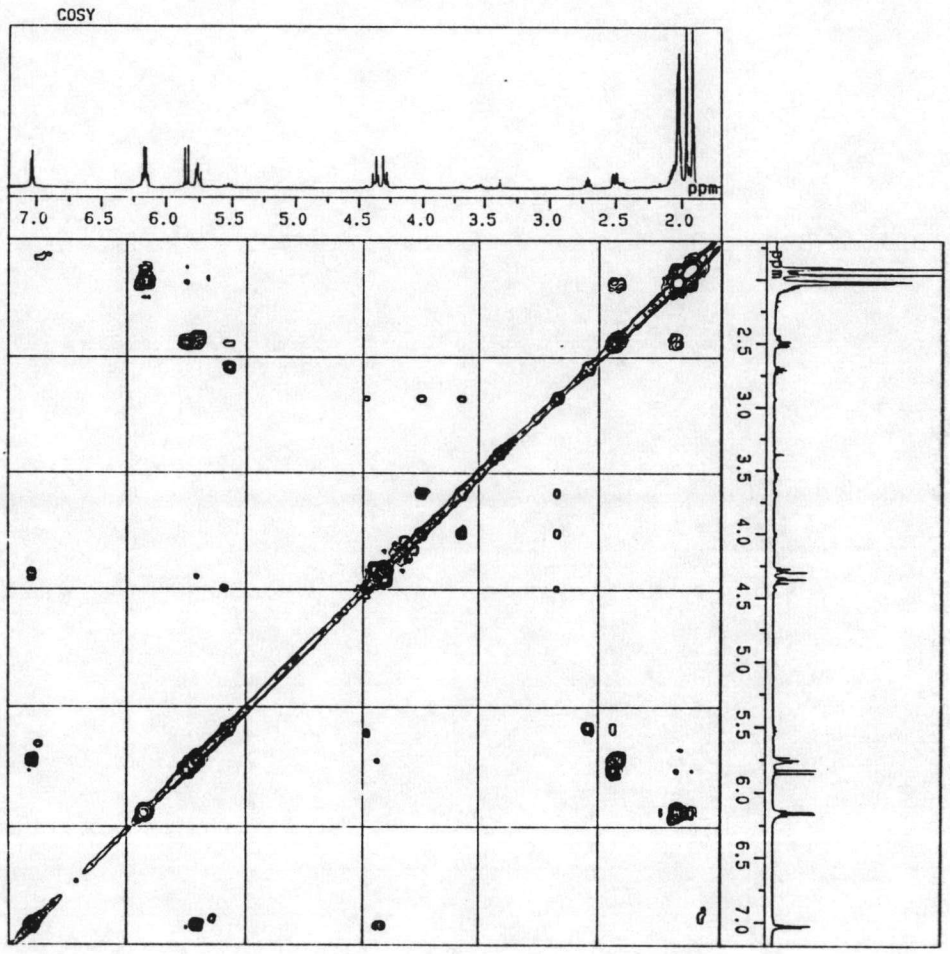


Figure 3.25 The ^{1}H-^{1}H COSY spectrum of compound 4

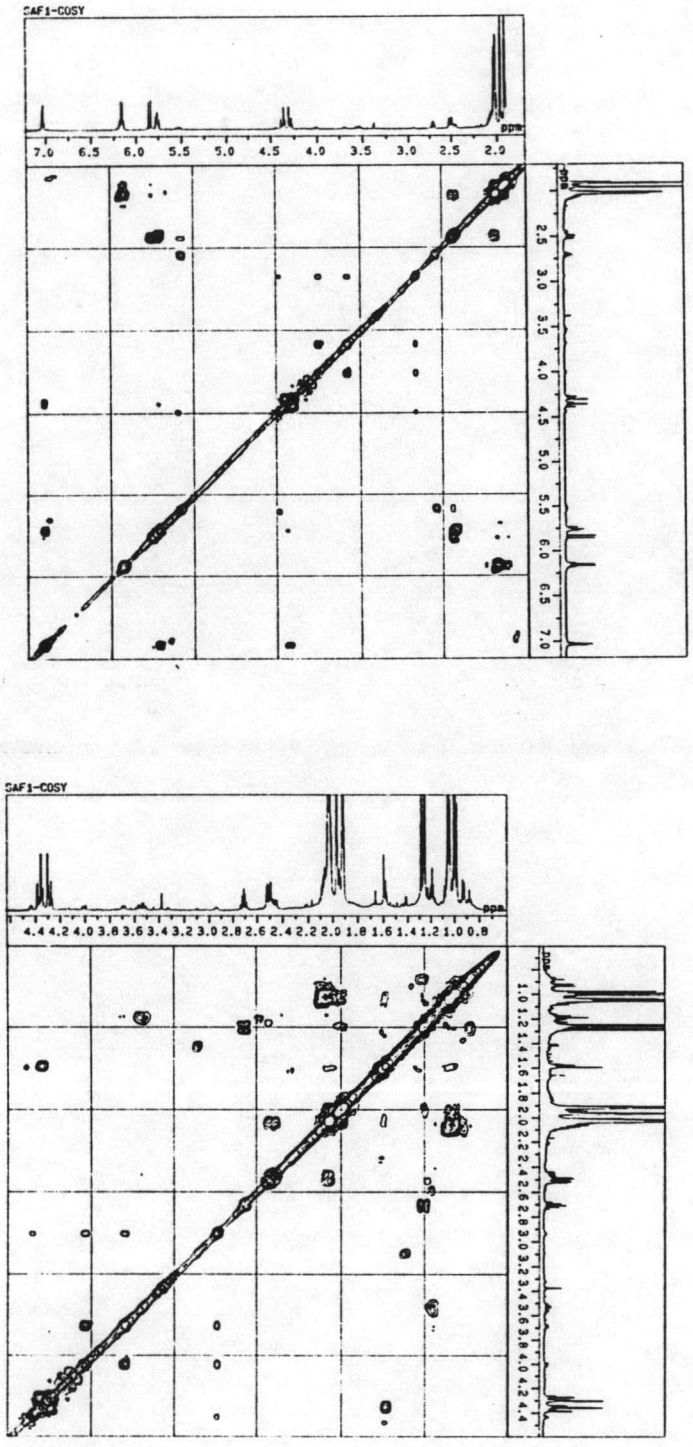


Figure 3.26 The expanded ^{1}H-^{1}H COSY spectrum of compound **4**

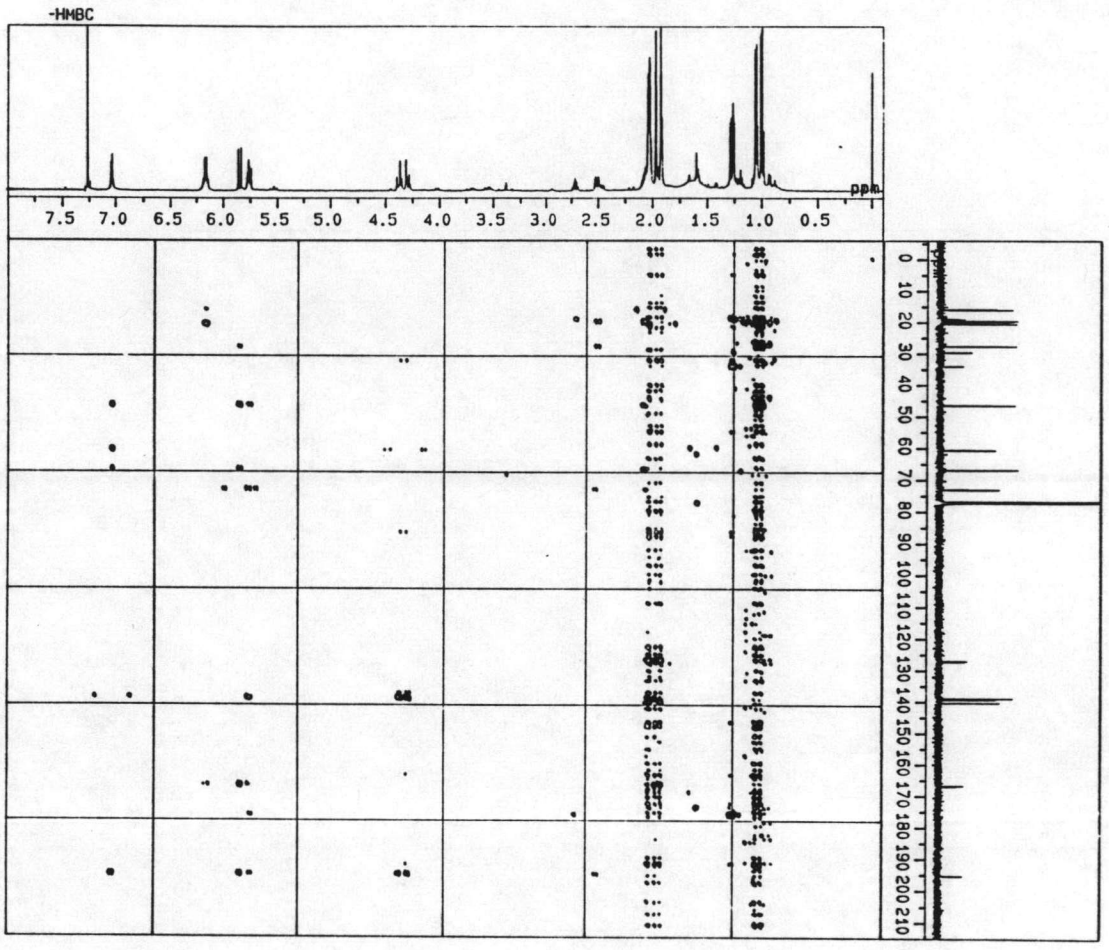


Figure 3.27 The HMBC spectrum of compound **4**

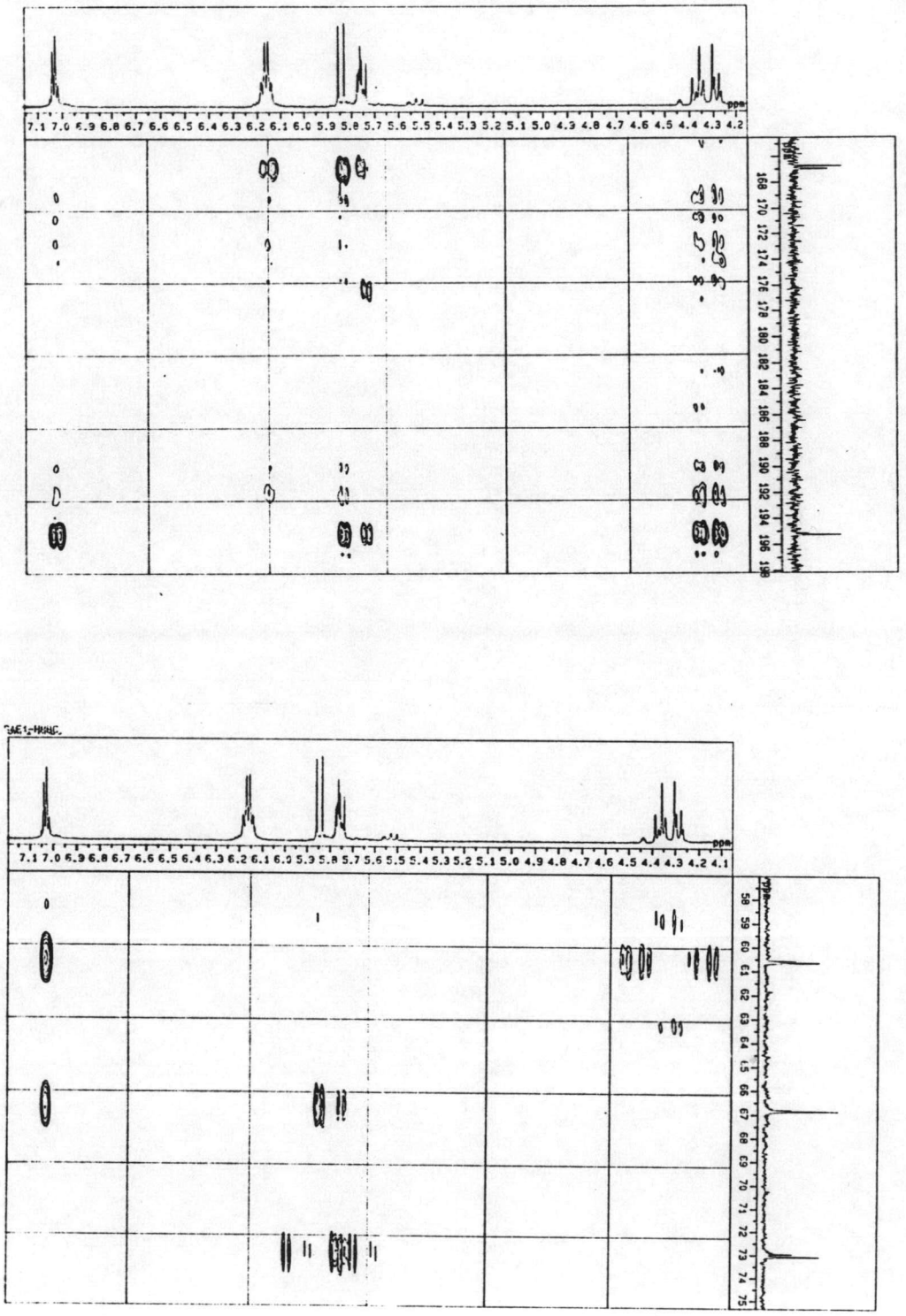


Figure 3.28 The expanded HMBC spectrum of compound **4**

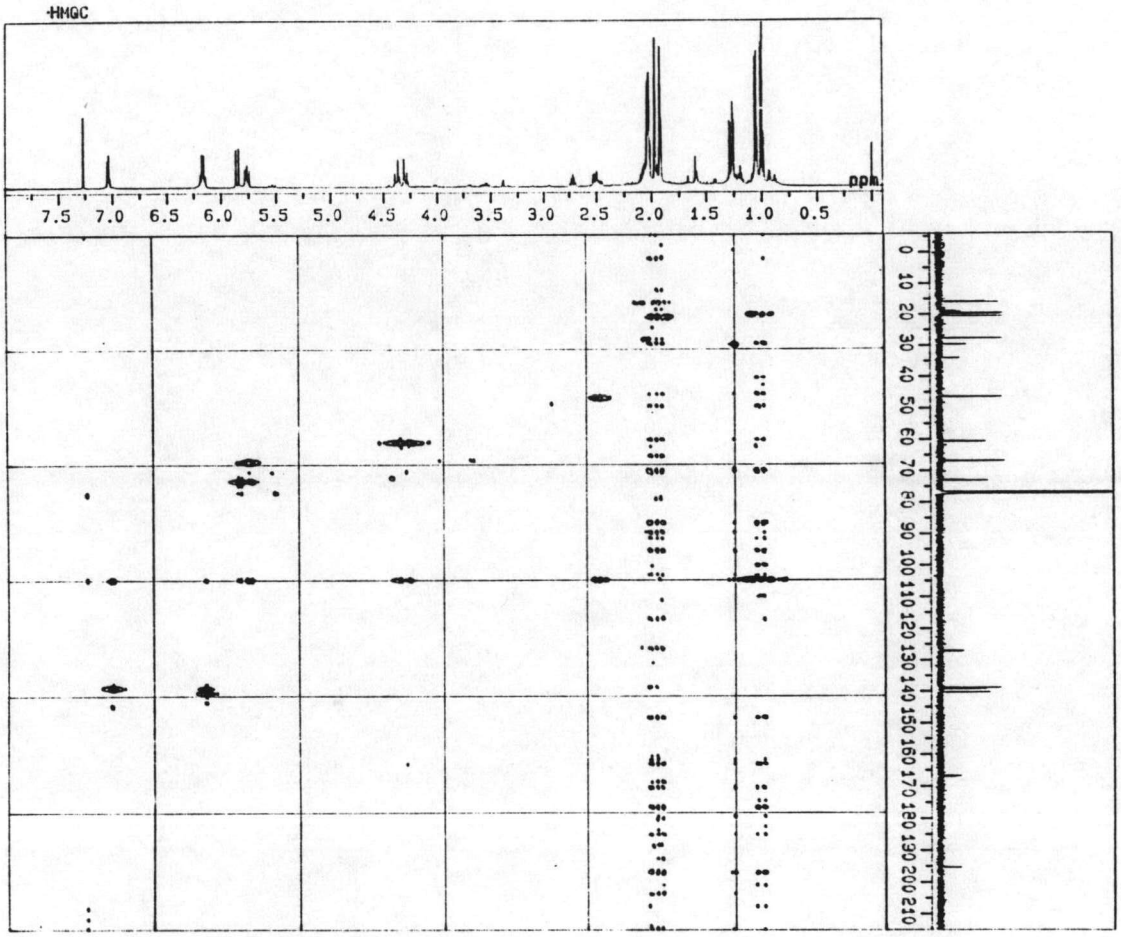


Figure 3.29 The HMQC spectrum of compound **4**

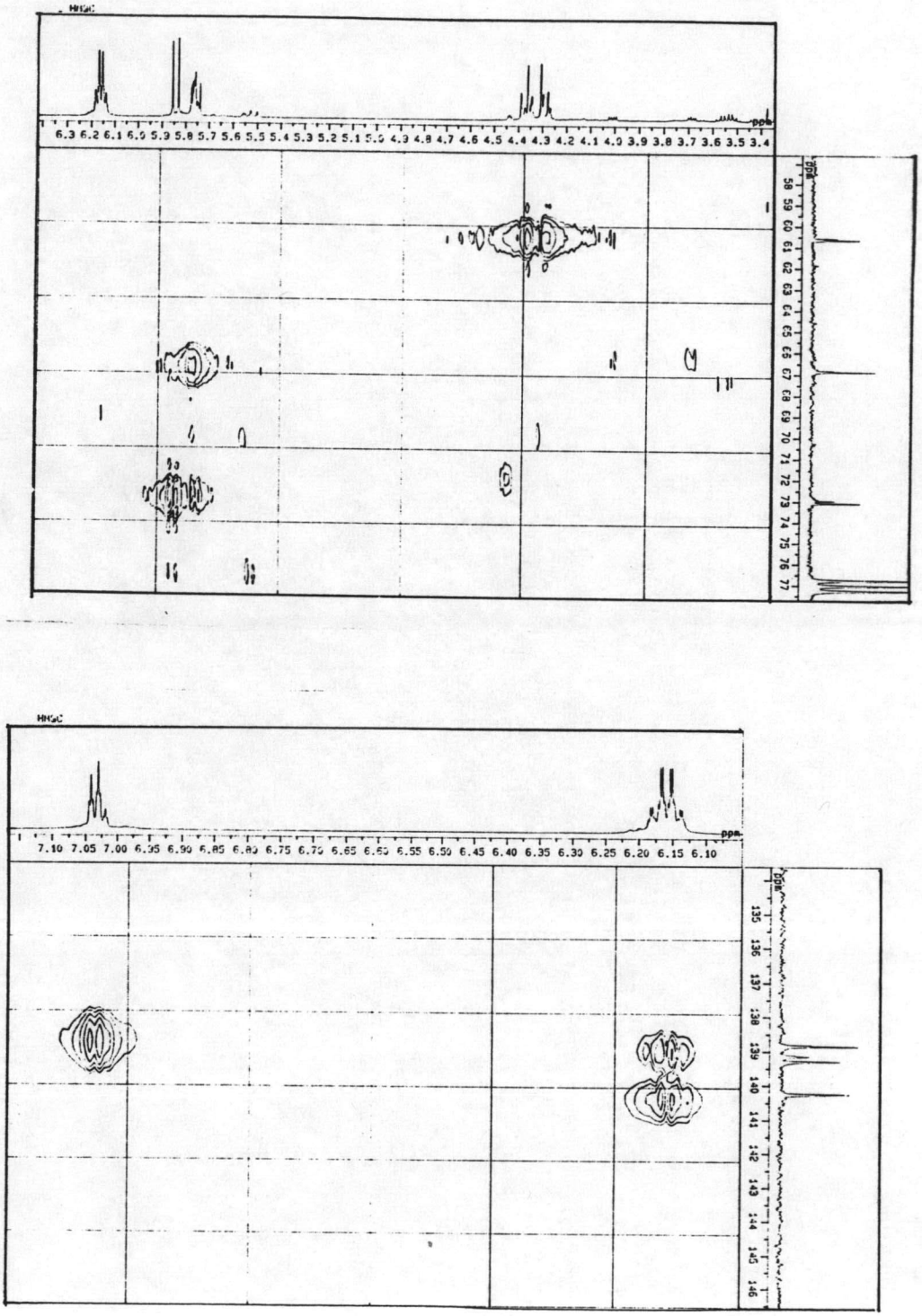


Figure 3.30 The expanded HMQC spectrum of compound **4**

3.3.5 The structural elucidation of compound 5

Compound **5** obtained in the same fraction as compound **4**. This elute was further purified with chromototron technique, the third fraction eluted with 20% ethyl acetate in hexane gave colorless oil after the solvent was evaporated.

The IR spectrum of compound **5** (Fig 3.35) showed an intense band at 3550 cm^{-1} the presence of hydroxy group and the presence of the α, β unsaturated ketone at 1735 cm^{-1}

The molecular formula of compound **5** (Fig 3.33) was determined as $C_{24}H_{32}O_7$ by high-resolution mass spectrometry(M^+) m/z 432. The loss of angelate fragments led to the presence of the ion peak at m/z 332 [$(432\text{-}C_5H_8O_2)^+$] and 232 [$332\text{-}C_5H_8O_2)^+$].

The ^1H NMR spectrum (Fig 3.36) and signal integration (Fig 3.37) indicated the important proton signal at δ (ppm): 6.84 (1H, dd, J = 1.5, 5.8 Hz), 6.14 (3H, dq, J = 7.3, 1.5 Hz), 5.83 (1H, d, J = 12.8 Hz), 5.67 (1H, dd, J = 3.4, 2.5), 2.47 (1H, ddd, J = 12.5, 4.88, 3.35 Hz), 2.03 (1H, m), 1.94 ($6CH_3$, m), 1.04 (CH_3, d, J = 7.02 Hz) and 0.99 (CH_3, d, J = 7.01 Hz)

The ^{13}C NMR spectrum of compound **5** (Fig 3.38) showed 24 carbon signals. Multiplicity of the carbon was determined by employing DEPT 90 and 135. This established that there were eight methines (δ 138.9, 138.7, 138.1, 139.2, 127.2 and 127.4), one methylene (δ 27.7), five quaternary carbons (δ 194.8, 166.8, 166.9 and 167.1) and eight methyl groups (δ 19.8, 19.5, 15.9, 15.8, 15.5, 14.2, and 14.1)

The ^1H and ^{13}C NMR spectral data of compound **5** were similar to those of compound **4**. However, unlike compound **4**, compound **5** showed the absence of the methylene proton signal, H-7 at δ 4.32. The integration of angelate proton at δ 6.14 suggested the presence of three angelate groups. Three angelate groups composed of two groups located at C-3 and C-5 like compound **4** while the another replaced hydroxy methyl(CH_2-OH) group.

Table 3.12 The carbon and attached proton s determined by one bond correlation in HMQC spectrum

Carbon position	Chemical shift (ppm)	Attached proton
C-1	140.1	-
C-2	139.8	6.84(1H, dd, J = 1.5, 5.8 Hz)
C-3	67.4	5.67(1H, dd, J = 3.4, 2.5 Hz)
C-4	46.7	2.47(1H, ddd, J = 12.5, 4.88, 3.55 Hz)
C-5	73.1	5.83(1H, d, J = 12.8 Hz)
C-6	194.8	-
C-7	27.7	2.04(1H, m)
C-8	19.9	1.04(CH_3, d, J= 7.02 Hz)
C-9	19.6	0.99(CH_3, d, J= 7.02 Hz)
OR^1	Ang	
C-1	166.8	-
C-2	127.5	-
C-3	138.7	6.14(1H, dq, J = 7.3, 1.5 Hz)
C-4	15.9	1.94(CH_3, m)
C-5	19.9	1.94(CH_3, m)
OR^2	Ang	
C-1′	166.9	-
C-2′	127.4	
C-3′	138.9	6.14(1H, dq, J = 7.3, 1.5 Hz)
C-4′	15.7	1.94(CH_3, m
C-5′	19.6	1.94(CH_3, m
OR^3	Ang	
C-1″	167.1	-
C-2″	127.1	-
C-3″	138.1	6.14(1H, dq, J = 7.3, 1.5 Hz)
C-4″	14.5	1.94(CH_3, m
C-5″	20.5	1.94(CH_3, m

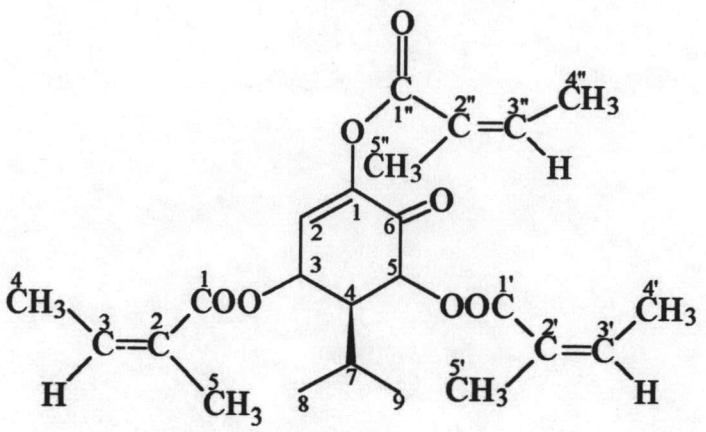


Figure 3.31 Carbon assignment of compound **5**

The ^{1}H-^{1}H COSY spectrum allowed the assignment of all proton signals while the ^{13}C NMR signals were assigned by HMQC and DEPT experiments. The relative position of the ester group was finally proved by HMBC experiment, which showed connection between C-1 of 3-O-Ang at δ 166.8, C-1 of 5-O-Ang at 166.9 with H-3 and H-5 of the terpene part, respectively. The relative stereochemistry of H-3, H-4, H-5 were determined by means of NOESY, which revealed the correlation between H-3 and H-4 but through space coupling between H-4 and H-5 disappeared. It could be implied that H-3 and H-4 were placed in the same plane. In addition the greater coupling constant $^{3}J_{4,5}$ = 12.8 Hz. Accordingly, H-3 and H-4 were oriented in the same plane as well as compound **4**.

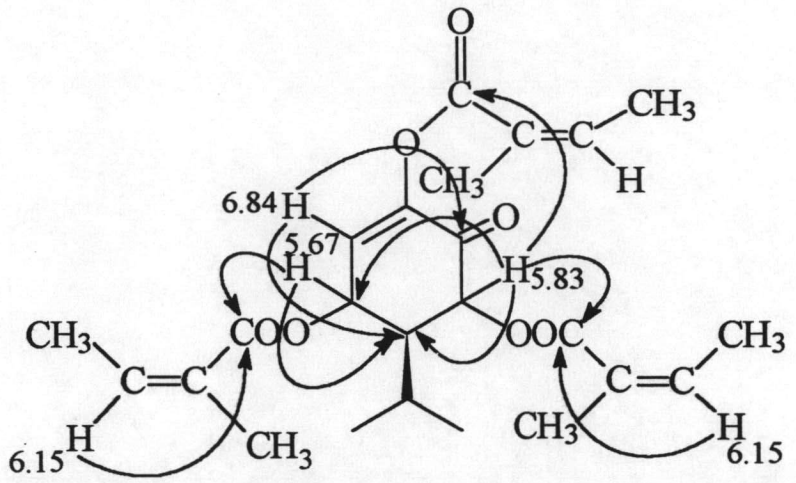


figure 3.32 Long range ^{1}H-^{13}C coupling as detected in HMBC

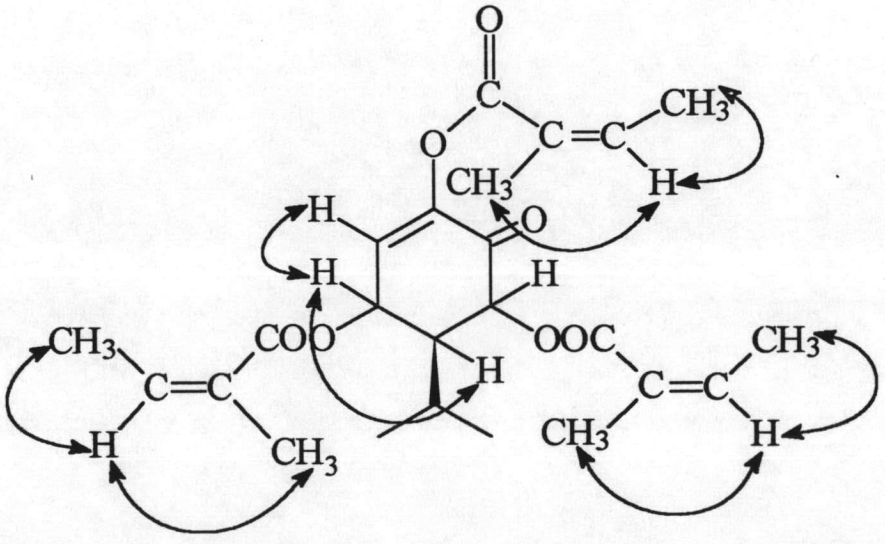
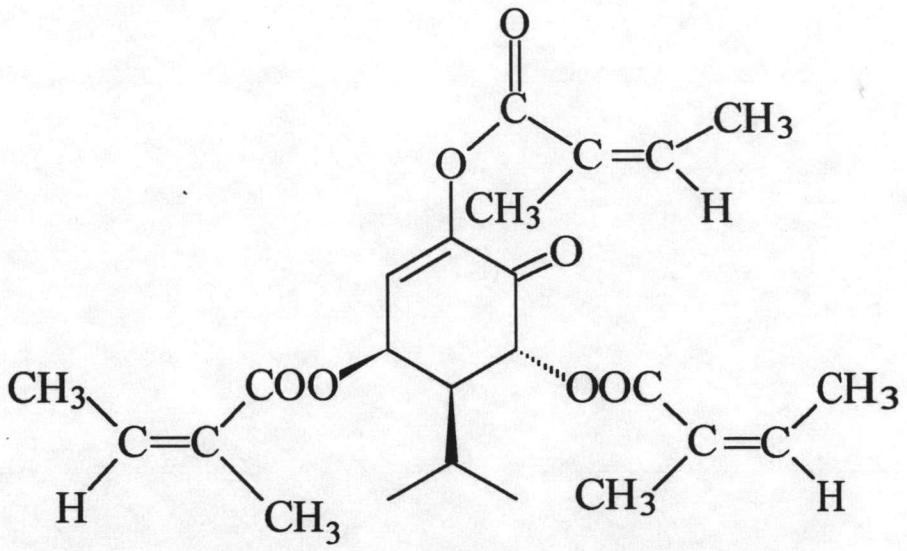
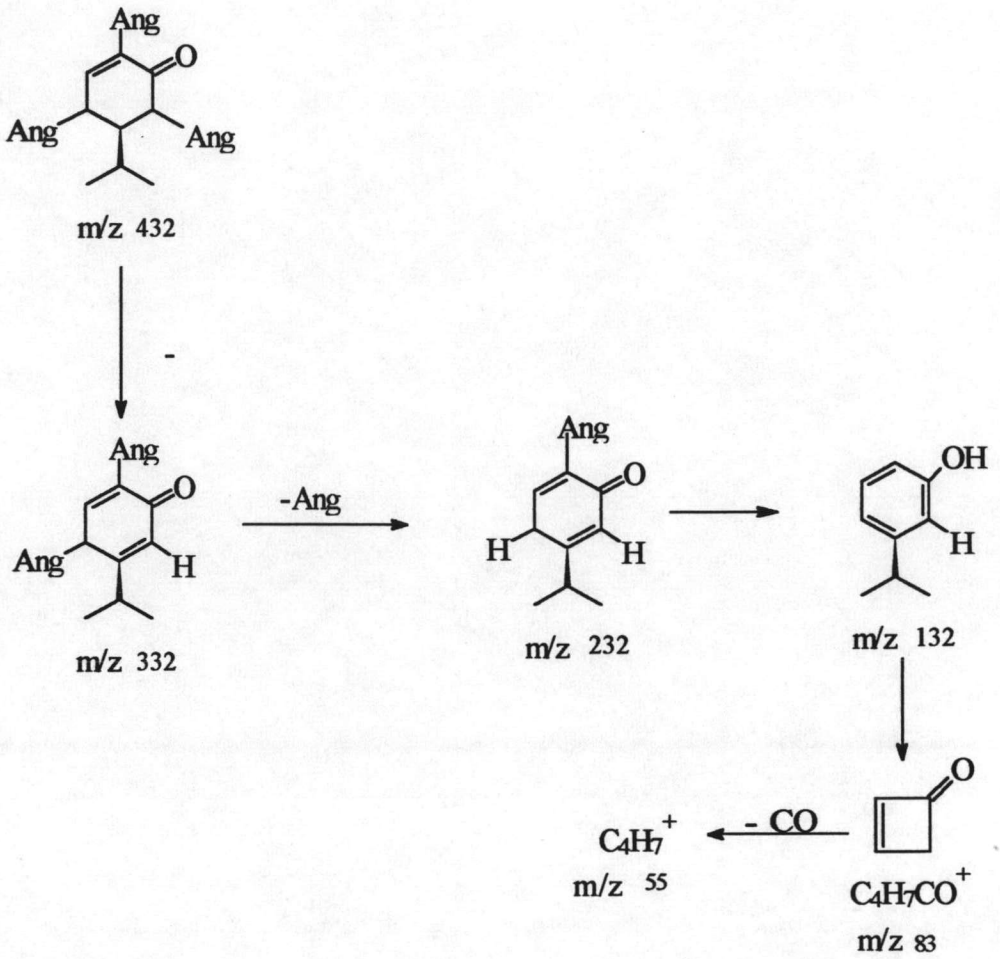


Figure 3.33 Through space coupling of proton as deduce from NOESY



carvotacetone analogue

Generally, carvotacetone derivatives must have carbon at 7-position, but C-7 of compound **5** disappeared. Thus, compound **5** was named 2,4α,6β-triangeloxoyloxy-5-(sec-propyl)-2-cyclohexenone (carvotacetone analogue) However, this compound was analogue of carvotacetone, Since other carbons were similar to 3α,5β-diangeloxy-7-hydroxycarvotaceone. Computational searching suggested that there was no reported the isolation of this compound; consequenly, it was a new compound.



Scheme 3.5 The mass fragmentation pattern of compound **5**

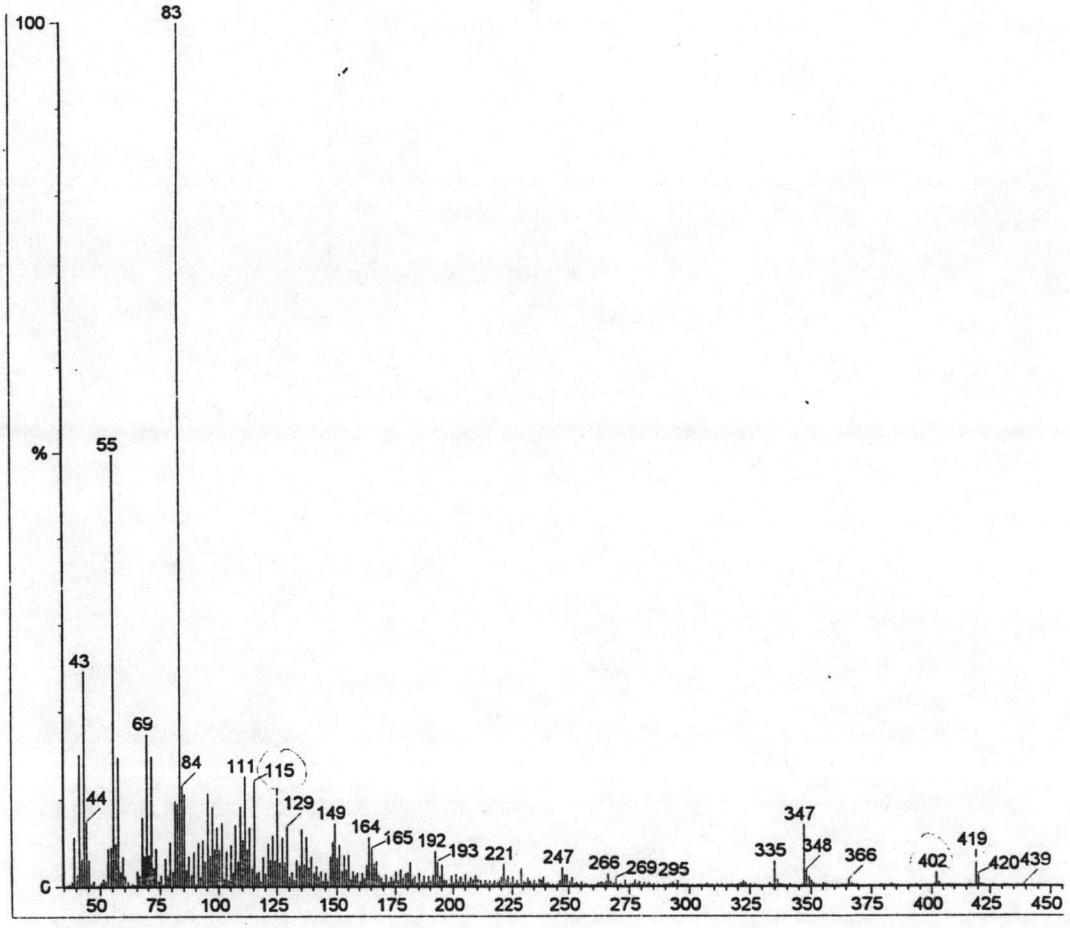


Figure 3.34 The mass spectrum of compound **5**

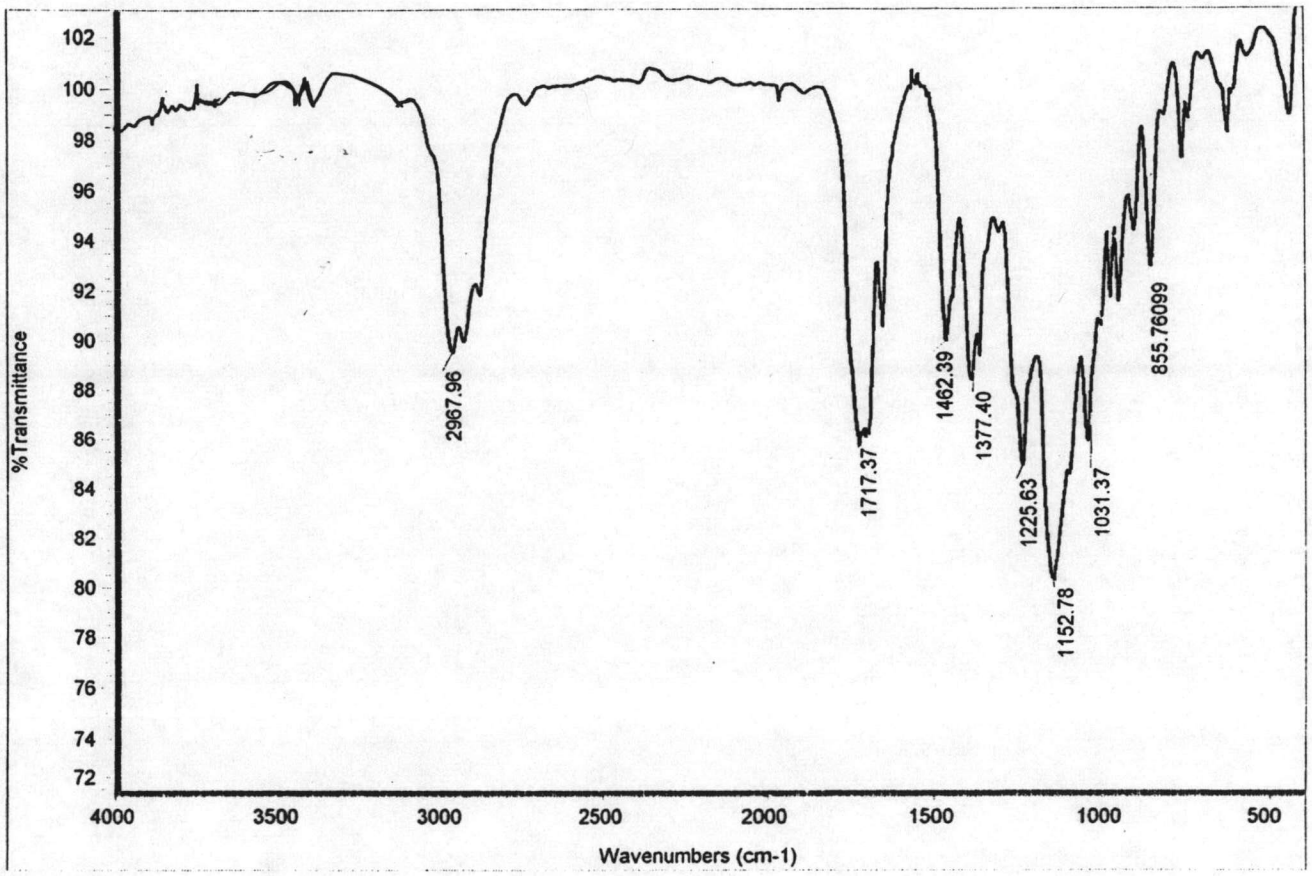


Figure 3.35 The IR spectrum of compound **5**

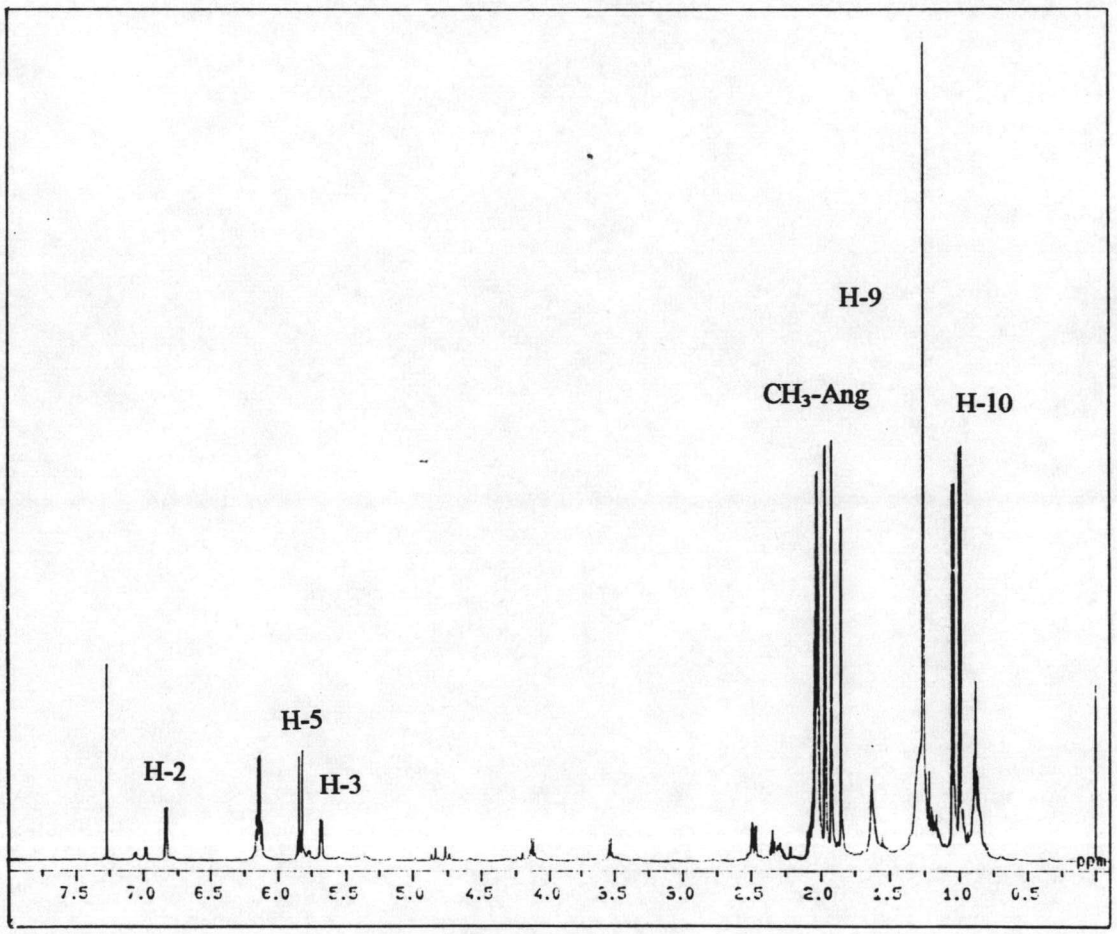


Figure 3.36 The ^{1}H NMR spectrum of compound **5**

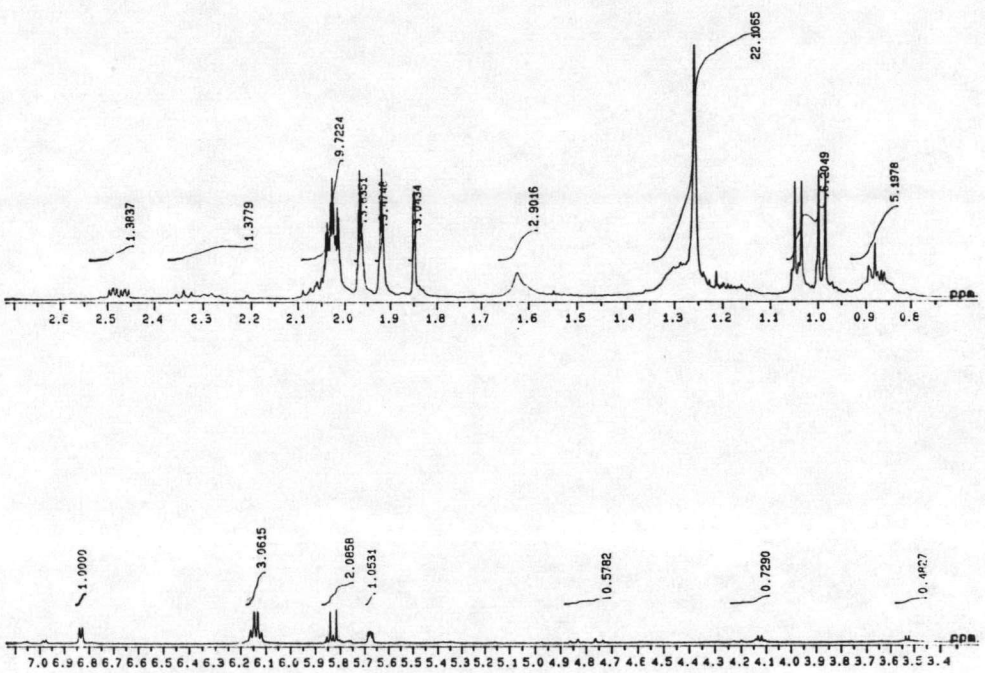


Figure 3.37 Proton integration signal of compound **5**

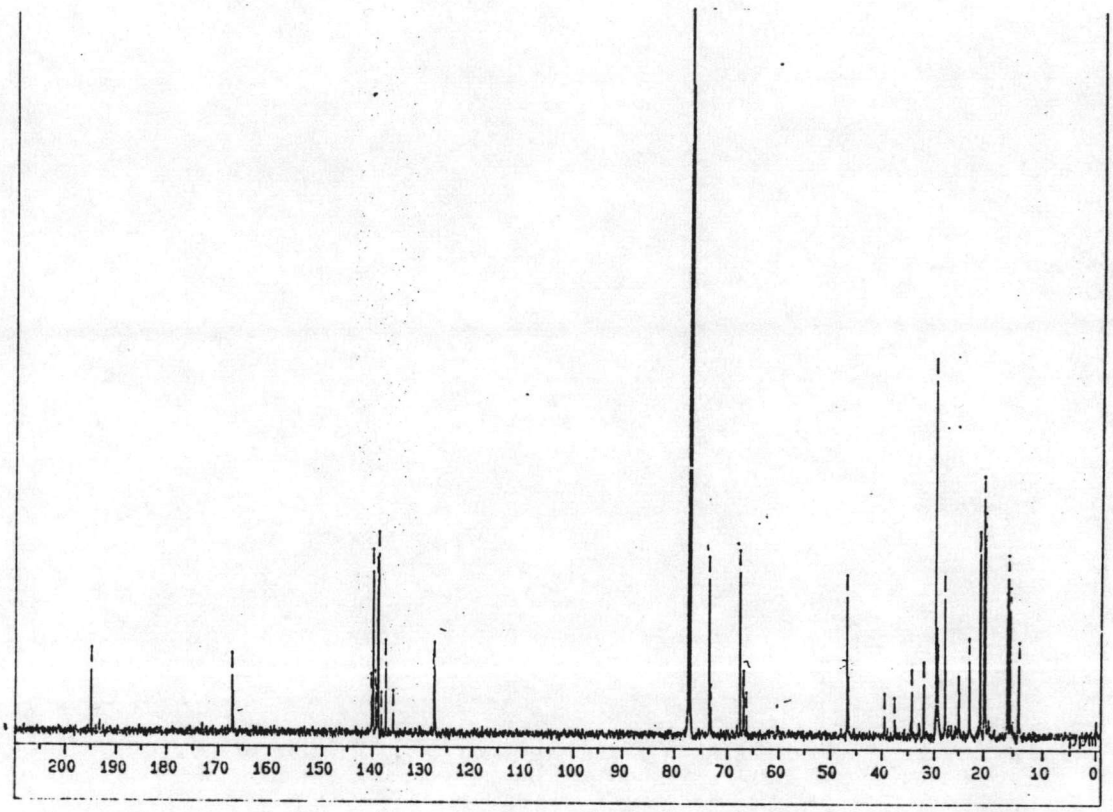


Figure 3.38 The ^{13}C NMR spectrum of compound **5**

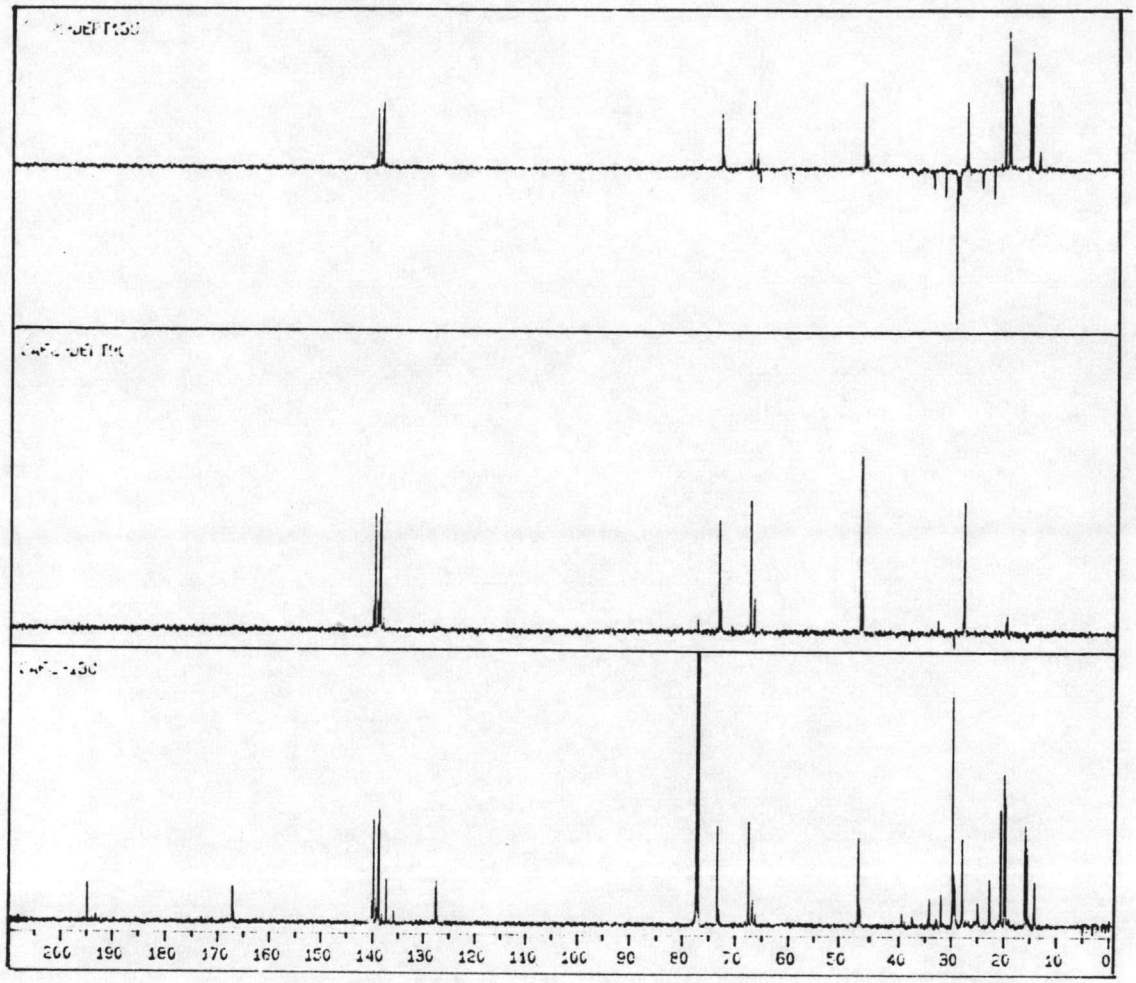


Figure 3.39 The DEPT 90, 135 spectra of compound **5**

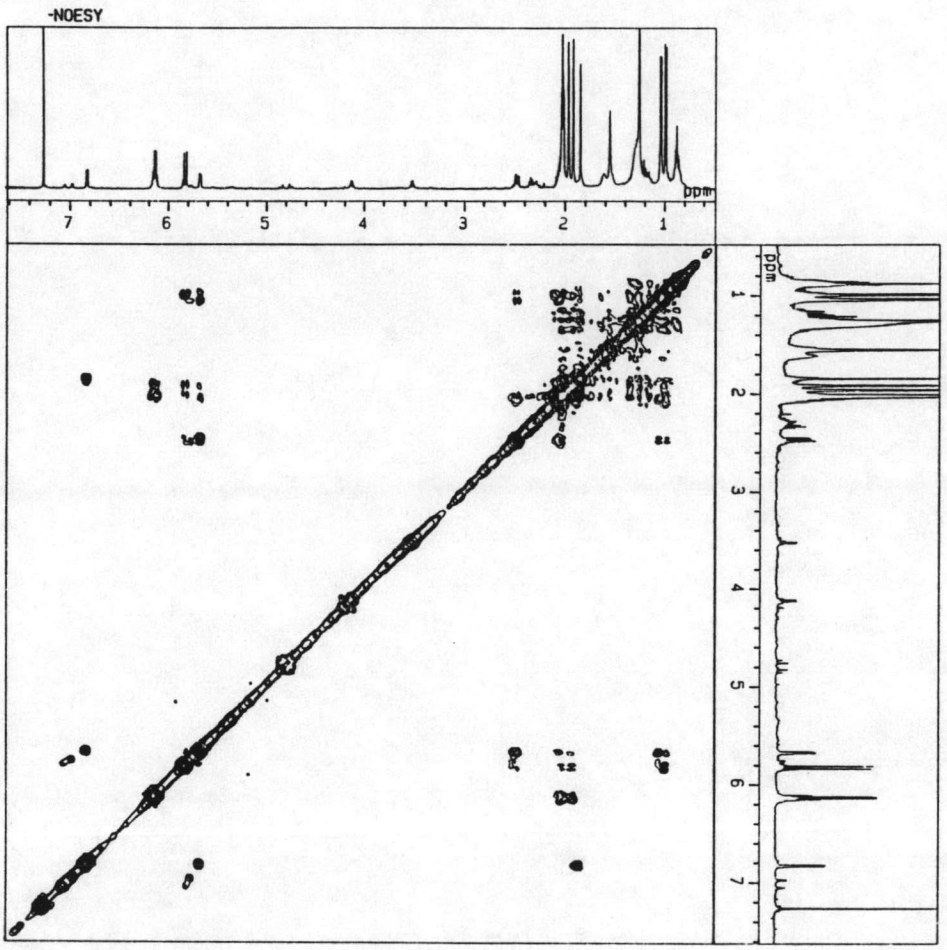


Figure 3.40 The NOESY spectrum of compound **5**

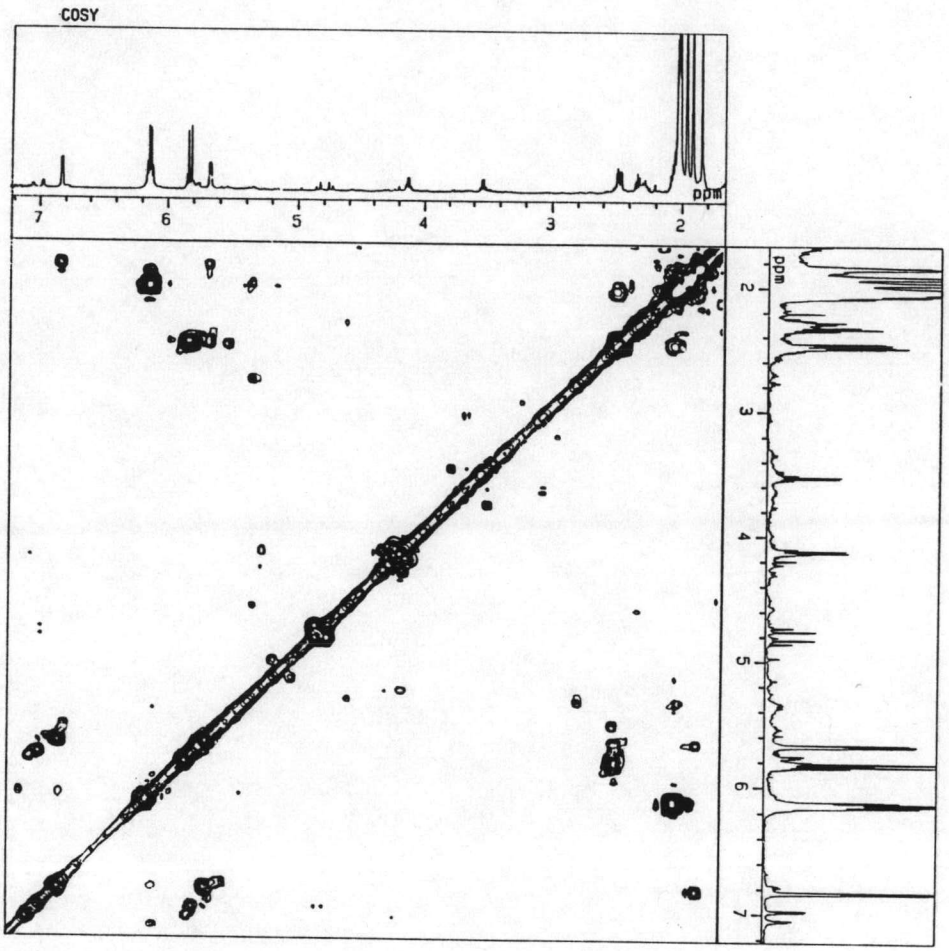


Figure 3.41 The COSY spectrum of compound **5**

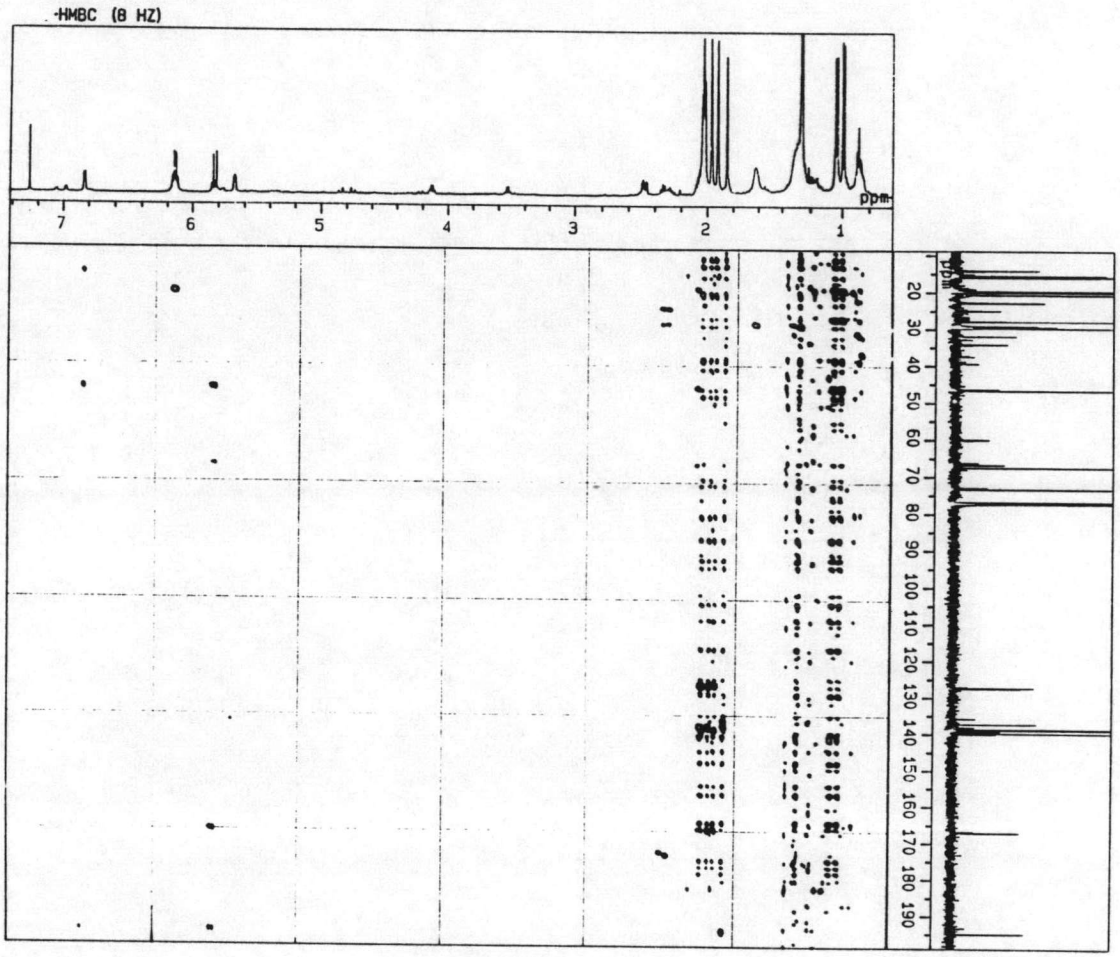


Figure 3.42 The HMBC spectrum of compound **5**

3.3.6 The structural elucidation of Compound 6

Compound **6** (150 mg), the major constituent of this plant which was isolated as a yellow amorphous solid. Its R_f value was 0.55 (50% $CHCl_2$ in MeOH), m.p. 240-242 °C. The physical properties (melting point, R_f value and solubility) of this compound was almost identical to those of Chrysosphenol-C (quercetagetin-3,3′,7-trimethoxy ether) which was isolated from *S.africanus*.

The IR spectrum of compound **6** (Fig 3.44) showed the existence of hydroxy group and carbonyl group at 3200-3500 cm^{-1} and 1653 cm^{-1}, respectively.

The mass spectrum (Fig 3.45) showed the molecular peak at m/z 360 which is consistent with a molecular weight of $C_{18}H_{16}O_8$. Other fragmentation and fragmentation pattern displayed in Scheme 3.6.

The ^1H NMR (Fig 3.46)showed signals for methyl groups at δ 3.80, 3.84 and 3.96 (each 3H, s), aromatic protons at δ 6.73 (1H, s), 6.91 (1H, d, J = 13.0 Hz), 7.55 (1H, dd, J = 5.0 Hz) and 7.65 (1H, d, J = 4.0 Hz).

The ^{13}C NMR (Fig 3.47) also exhibited 18 signals. The signals at δ 180.1 indicated the presence of carbonyl group. In addition four methine carbons (δ 92.1, 116.0, 122.5 and 133.3), quaternary carbons (δ 107.3, 122.5, 133.3, 133.6, 139.7, 146.5, 150.1, 153.4, 153.9 and 160.0) as well as methoxy carbons (δ 57.6, 60.5 and 61.1) were observed.

The spectral data of compound **6** were similar to those of Chrysosphenol-C (quercetagetin-3,3′,7-trimethoxy ether). Especially, ^{13}C NMR data was nearly identical. All the above data were in good agreed with Chrysosphenol-C[15].

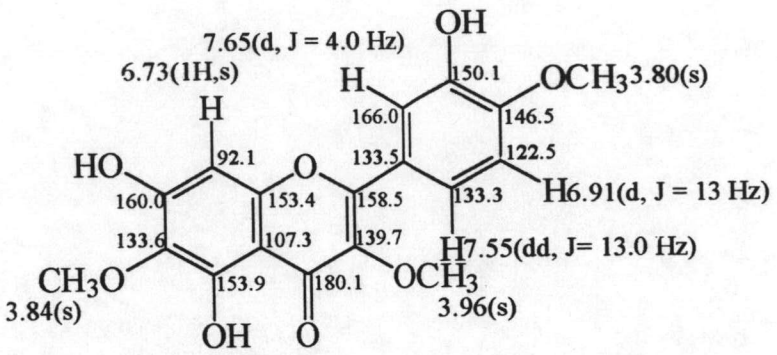
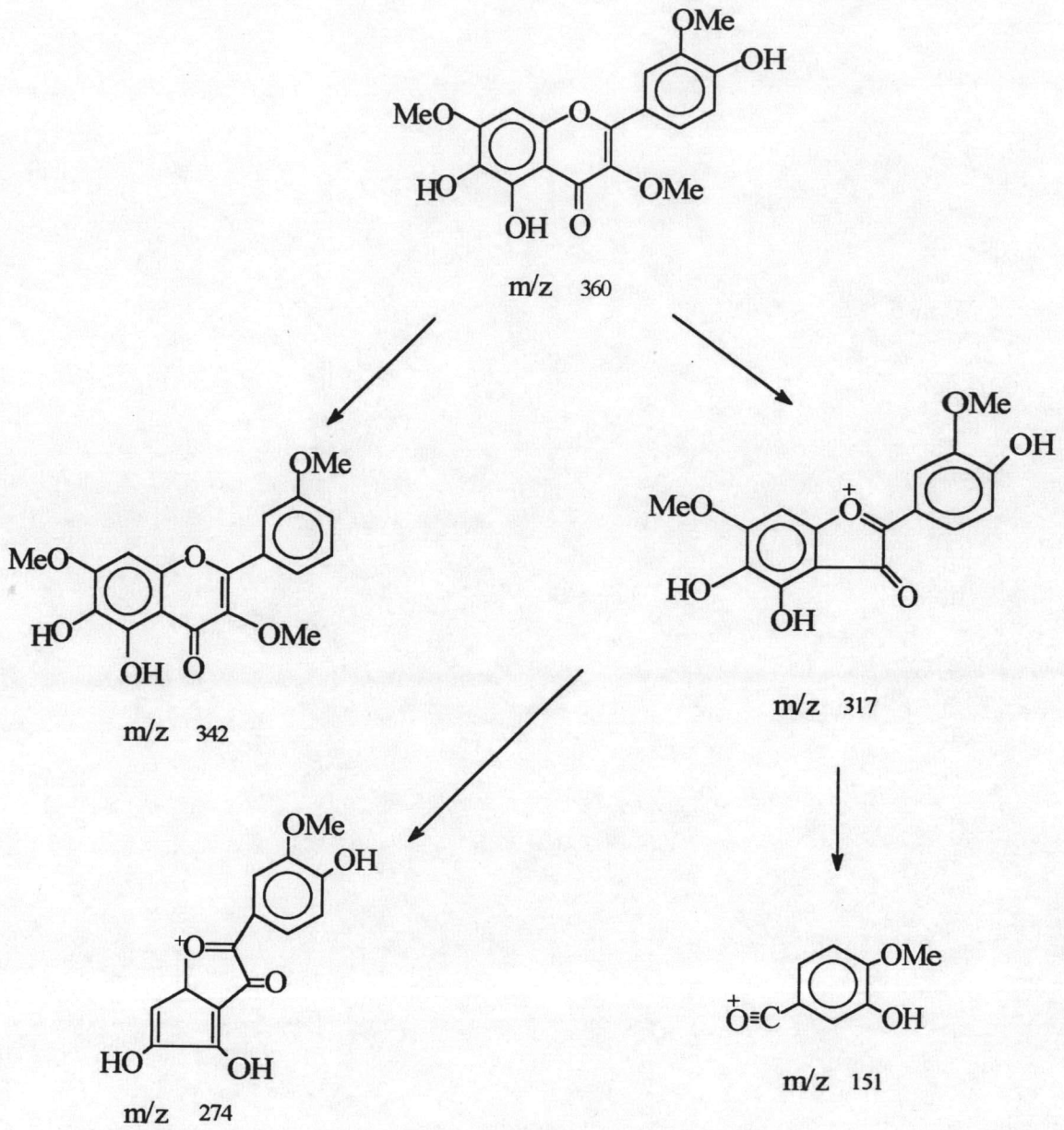


Figure 3.43 Proton and carbon assignment of compound **6**, Chrysosphenol-C



Scheme 3.6 The mass fragmentation pattern of compound **6**

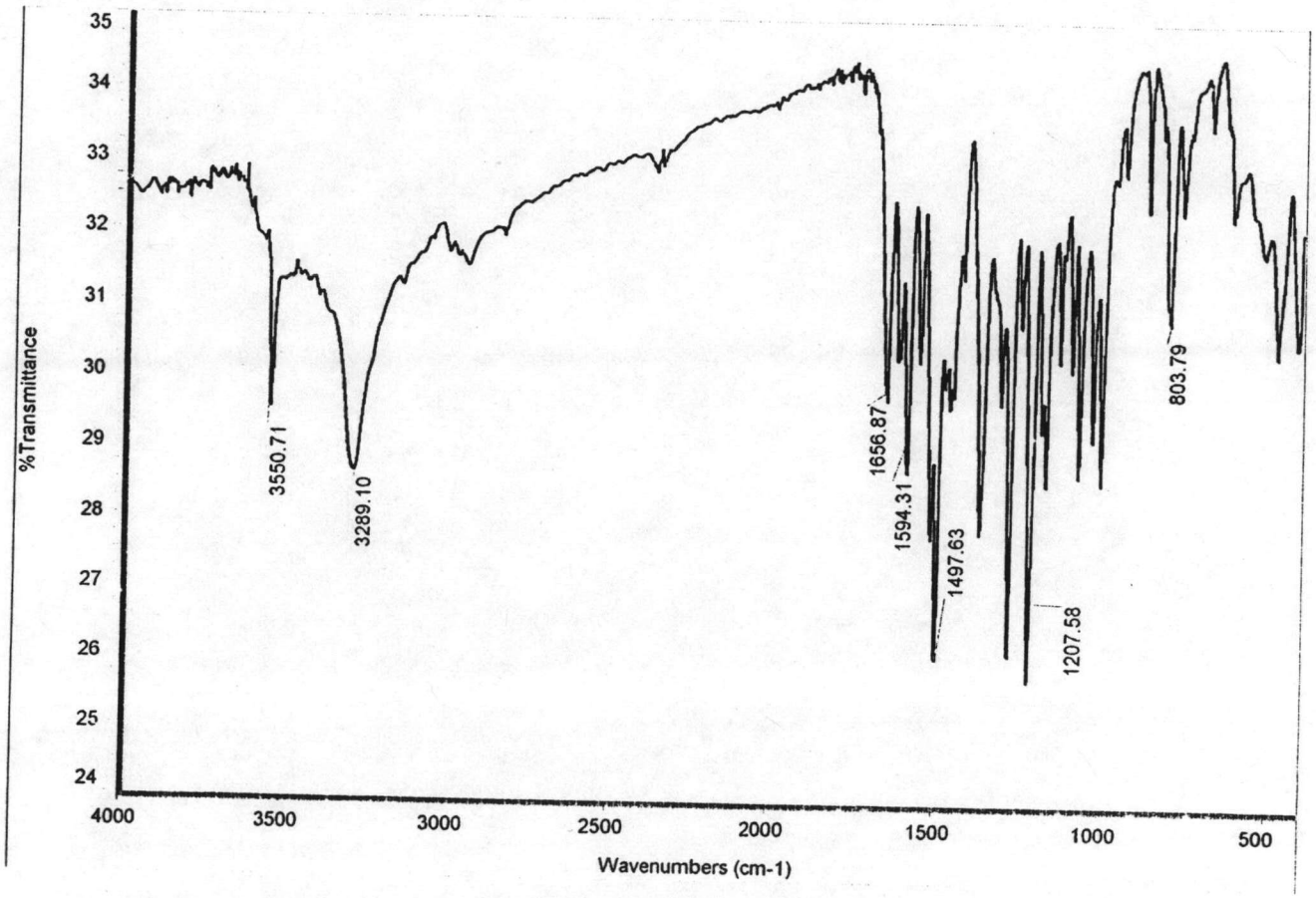


Figure 3.44 The IR spectrum of compound 6

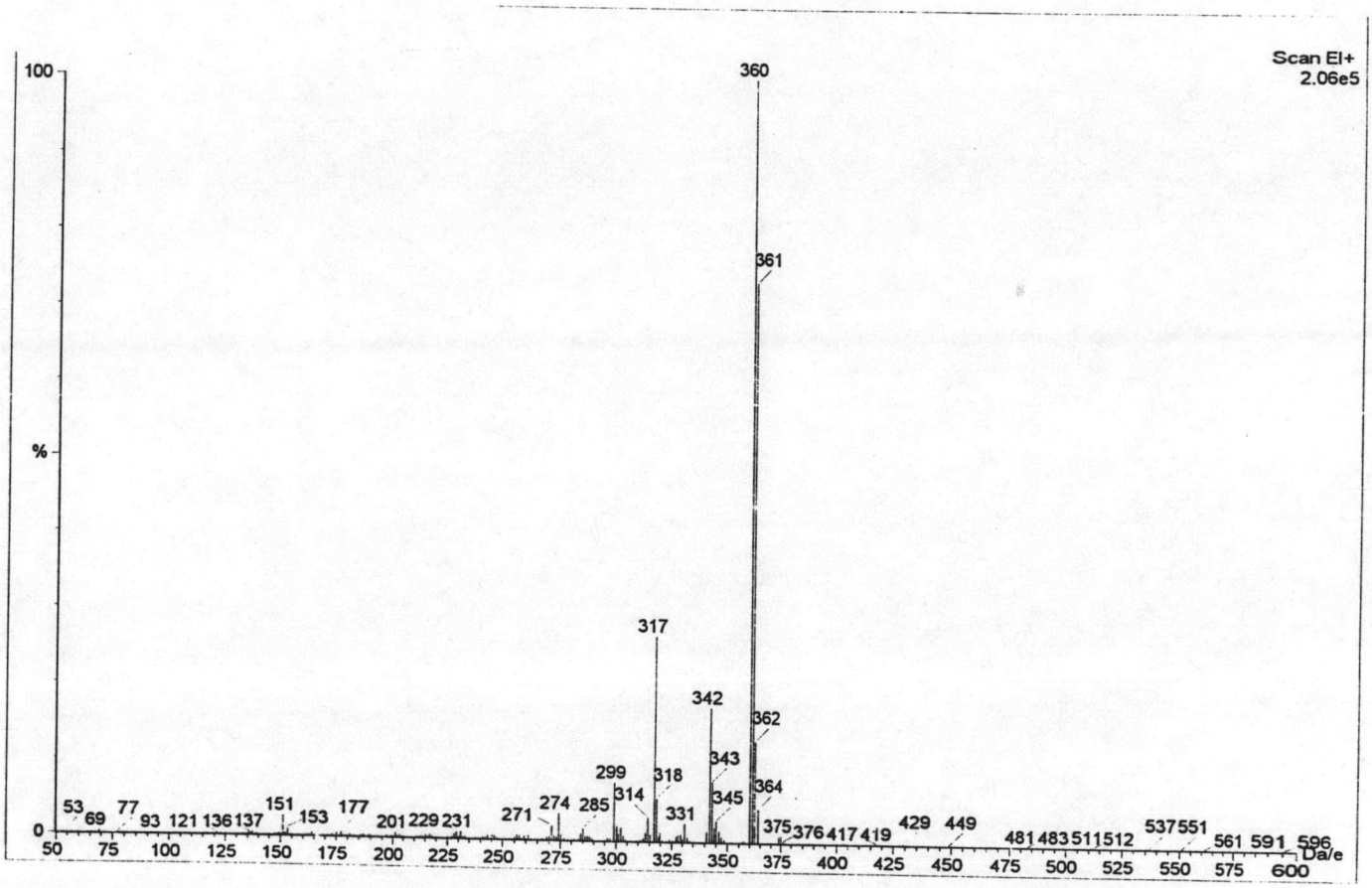


Figure 3.45 The mass spectrum of compound **6**

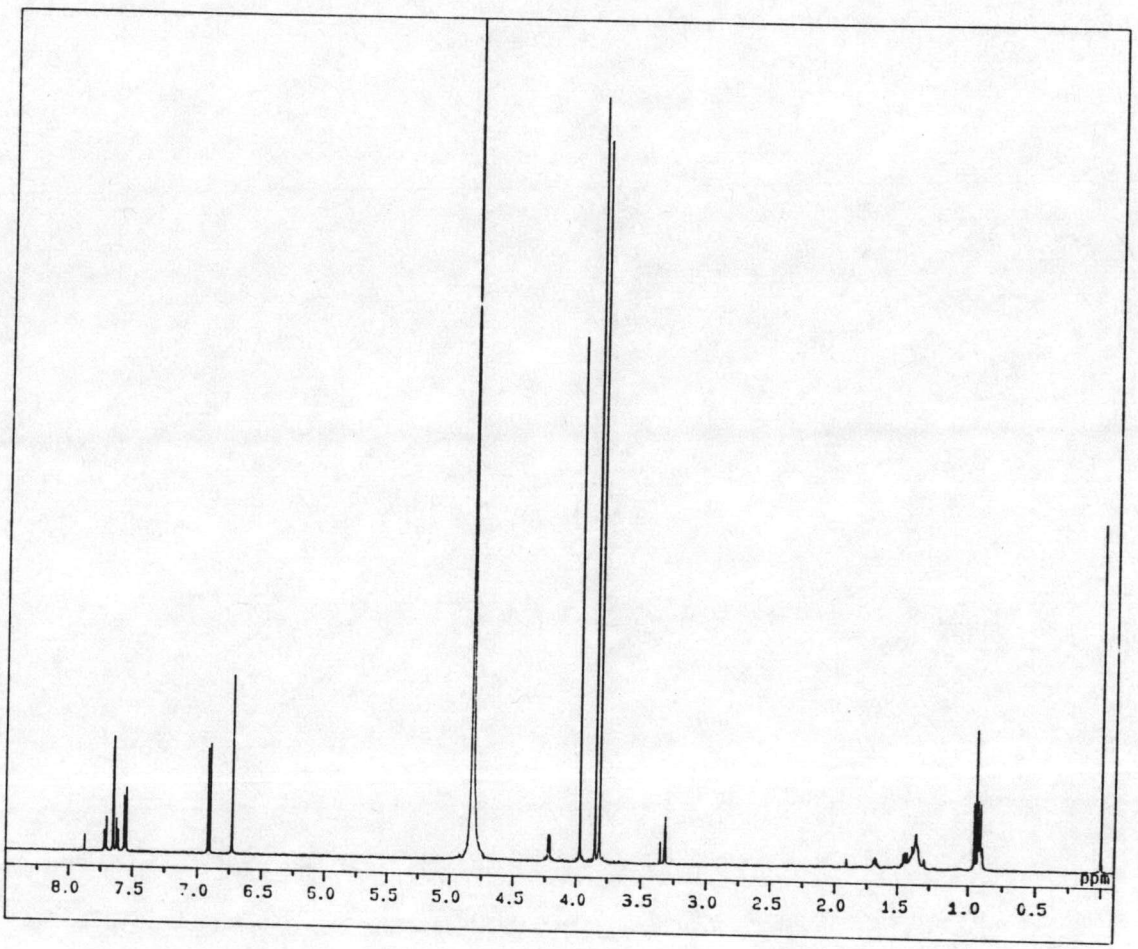


Figure 3.46 The ^{1}H NMR spectrum of compound 6

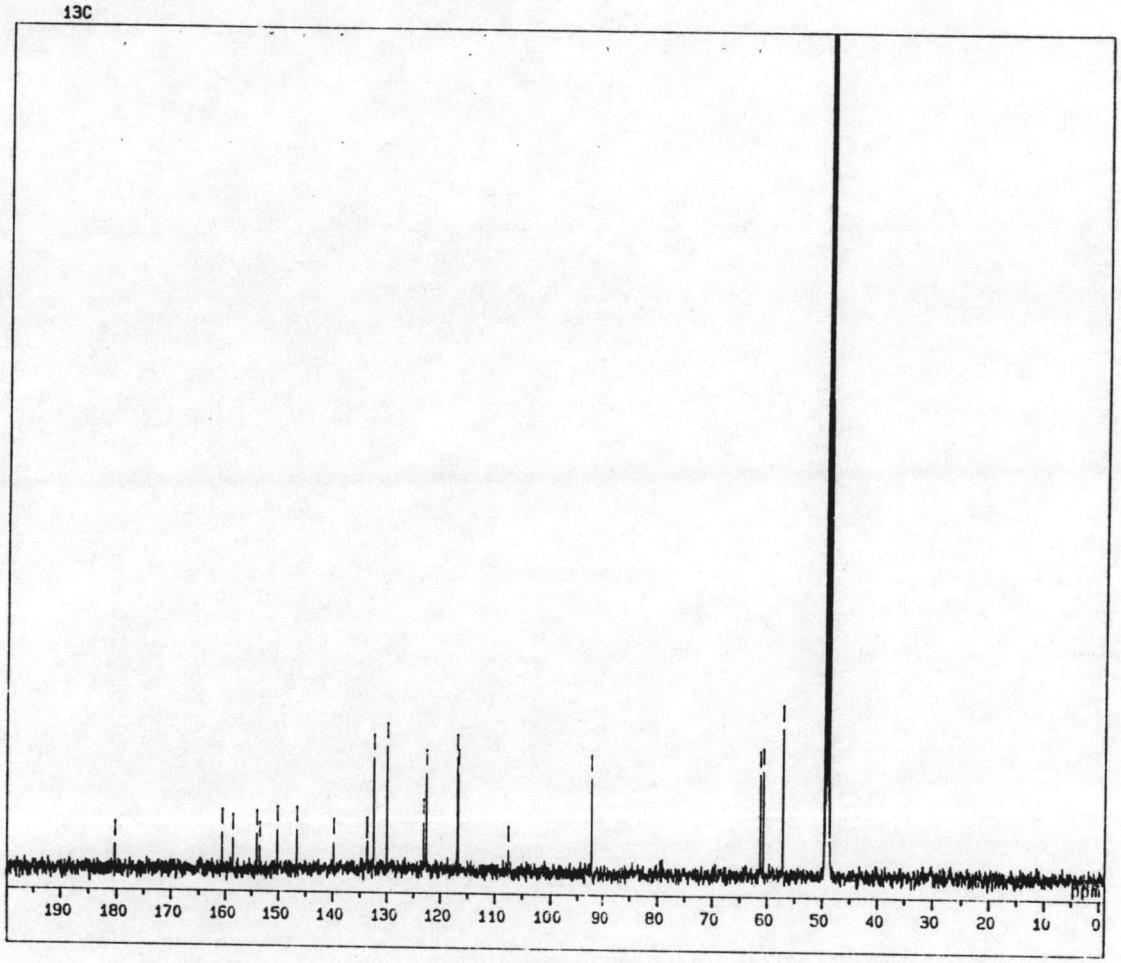


Figure 3.47 The ^{13}C NMR spectrum of compound 6

3.3.7 The structural elucidation of compound 7

Compound 7 (35 mg) was obtained as yellow amorphous substance in relatively low yield, from the isolation of BuOH crude extract. It meltd at 280-282 °C.

The IR spectrum (Fig 3.49)showed the absorption band of stretching at 3550 cm^{-1} of OH group including the absorption peak of C=O and C-O at 1653, 1277 cm^{-1}, respectively.

The molecular formula of compound 7 was confirmed by the molecular peak at m/z 346, displayed in molecular mass spectrum (Fig 3.50) and other fragmentation at m/z 317, 303, and 328.

The ^{1}H NMR spectrum (Fig 3.51) indicated the aromatic proton at δ 6.89 (1H, d, J = 8.55 Hz), 7.77 (1H, d, J = 2.1 Hz), 7.65 (1H, dd, J= 1.83, 2.13 Hz) and 6.7 (1H, s), the methoxy proton δ 3.84 and 3.70 (each 3H, s). The hydroxy proton at δ 7.90 (each 1H, s), 8.10 and 8.5 (each 1H, s).

In the ^{13}C NMR spectrum (Fig 3.52) showed 18 signals of carbon. The quaternary carbons at 177.5, 160.3, three methine carbons at 116.3, 116.4, 121.8 and 91.8 and the methoxy carbons at 61.1, 56.9.

All above data, compound 7 were suggested indicated to be a methoxygenated flavonol compound, that was isolated from *S. africanus*[15]. Compound 7 was postulated as quercetagentin-3,7-dimethyl ether. To confirm compound 7 whether it was quercetagentin-3,7-dimethyl ether, it was compared with an authentic sample on TLC plate. Both compound 7 and quercetagentin-3,7-dimethyl ether also gave the same spot in various solvents. Thus, compound 7 was certainly quercetagentin-3,7-dimethyl ether.

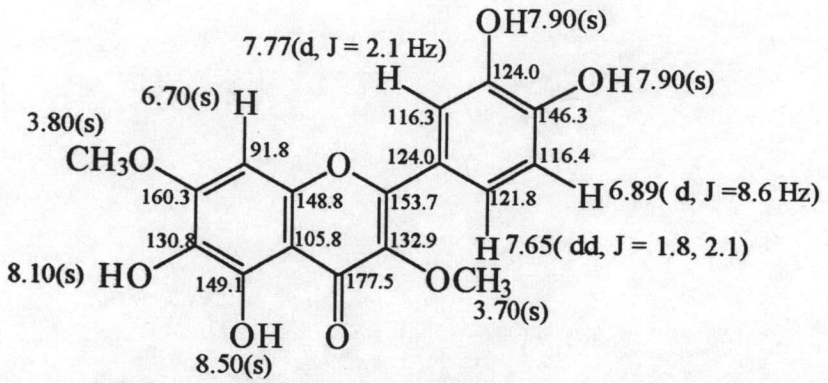
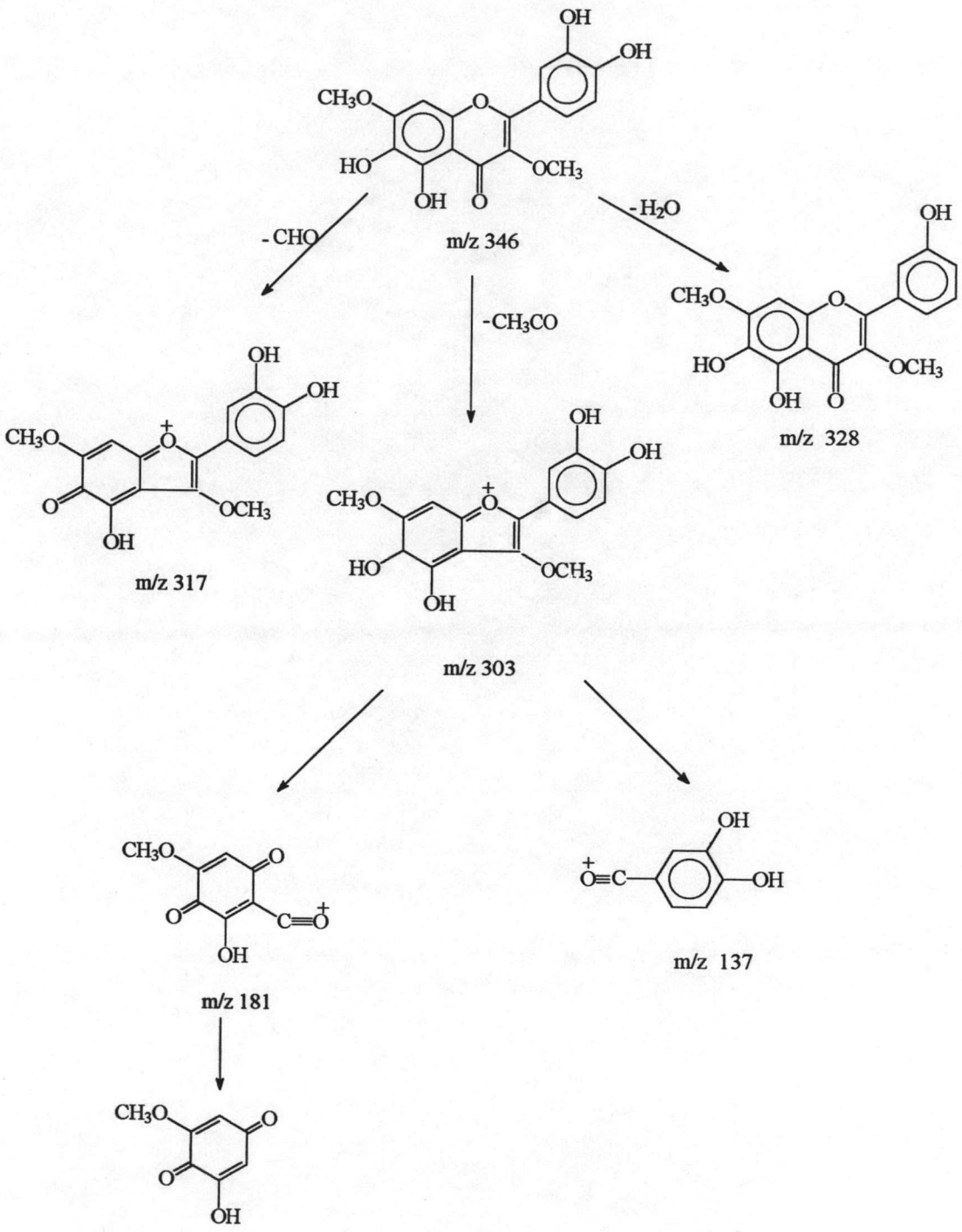


Figure 3.48 Proton and carbon assignment of compound 7, quercetagentin-3,7-dimethyl ether



Scheme 3.7 The mass fragmentation of compound 7

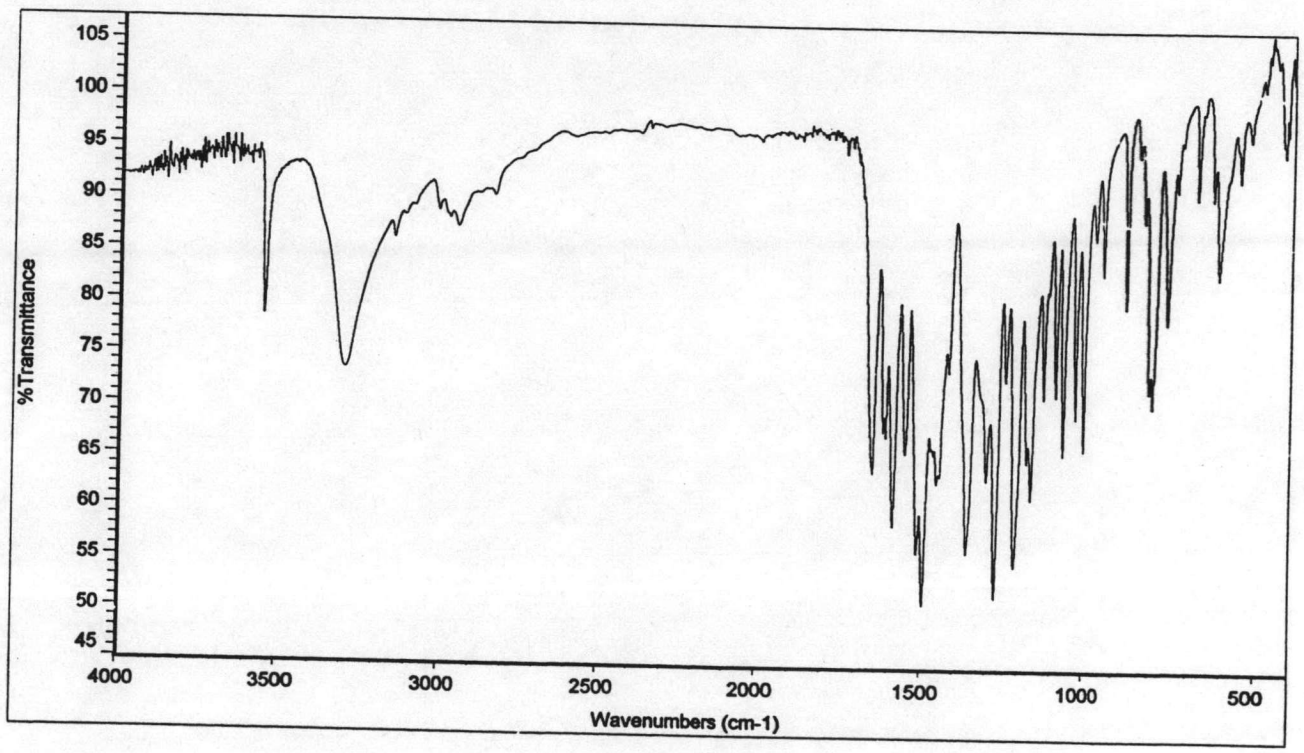


Figure 3.49 The IR spectrum of compound 7

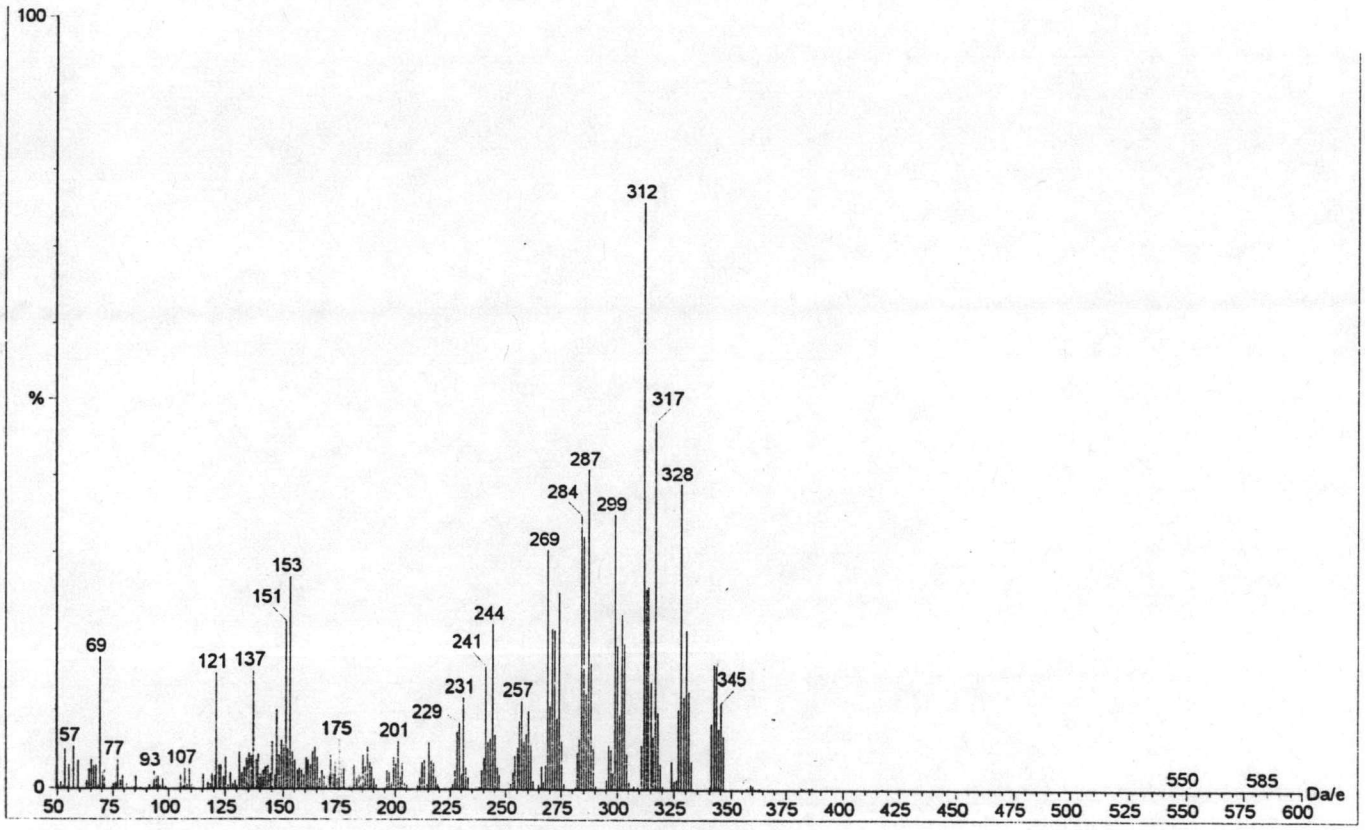


Figure 3.50 The mass spectrum of compound 7

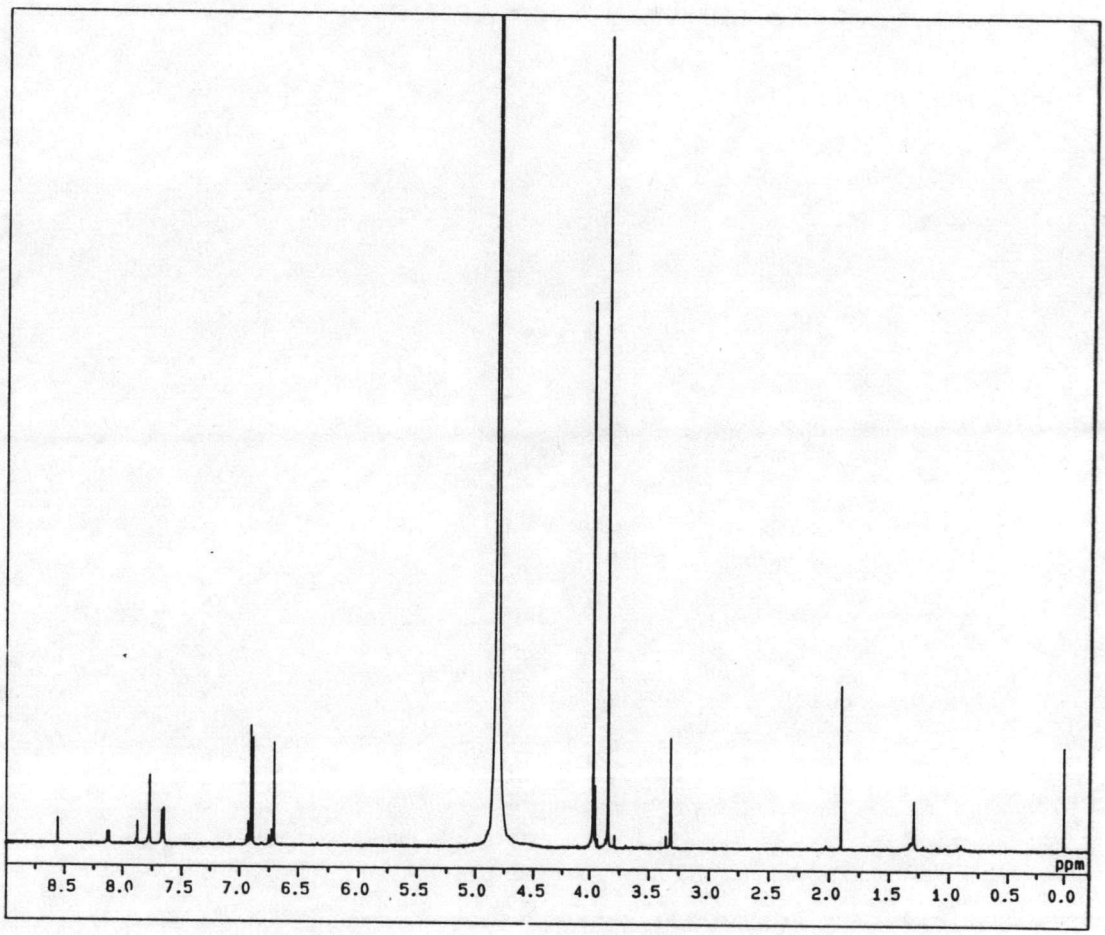


Figure 3.51 The ^{1}H NMR spectrum of compound 7

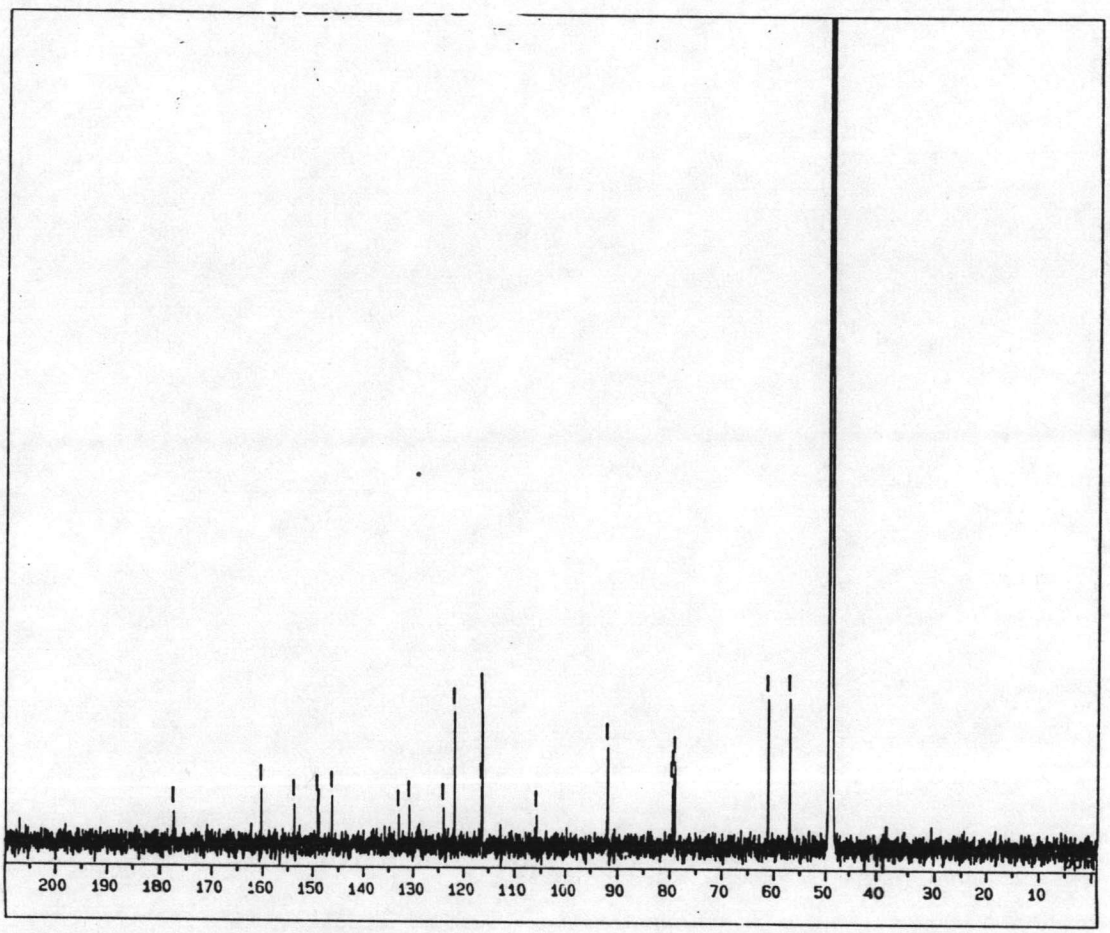


Figure 3.52 The ^{13}C NMR spectrum of compound 7

3.3.8 The structural elucidation of compound 8

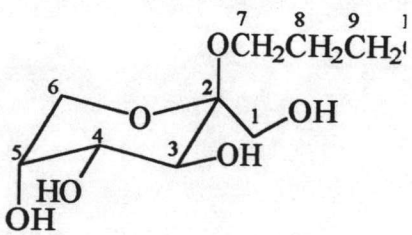
Compound **8**, the second compound from polar part, butanol crude extract. The isolation of compound **8** was accomplished by recolumn chromotography and recrystallization to afford compound **8** as colorless crystal (70 mg) mp. 150-152 °C

The ^{1}H NMR spectrum (Fig 3.53) of compound **8** contained resonance for a methyl group at δ 0.93 (3H, t), methylene protons at δ 1.38, 1.55 and 3.51 (each 2H, m).

The ^{13}C NMR spectrum (Fig 3.54) indicated the presence of methylene carbons at δ 65.1, 70.5, 61.6, 33.3 and 19.7, three methines carbon at δ 71.5, 69.8 and 62.5, quaternary carbon at 101.5 and methyl carbon at 14.3. All multiplicity assignment was supported by DEPT experiment. Both the ^{1}H- and ^{13}C-NMR spectral data of compound **8** was closely resembled to those of 2-*O*-*n*-butyl-*β*-fructupyranose[29].

Table 3.13 The carbon assignment of compound **4** and 2-*O*-*n* buthy *β*-fructupyranose

Carbon position	Chemical shift (ppm)	
	2-*O*-*n* buthy *β*-fructupyranose	Compound 8
C-1	63.6	65.1
C-2	99.9	101.5
C-3	69.4	70.5
C-4	70.2	71.5
C-5	69.2	69.8
C-6	62.2	62.5
C-7	60.5	61.6
C-8	31.8	33.3
C-9	19.1	19.7
C-10	13.6	14.3



The ^{13}C NMR data of compound **8** also supported the structural assigned for 2-*O*-*n*-buthyl-*β*-fructupyranose. Thus, compound **8** was 2-O-*n*-butyl-β-Fructupyranose.

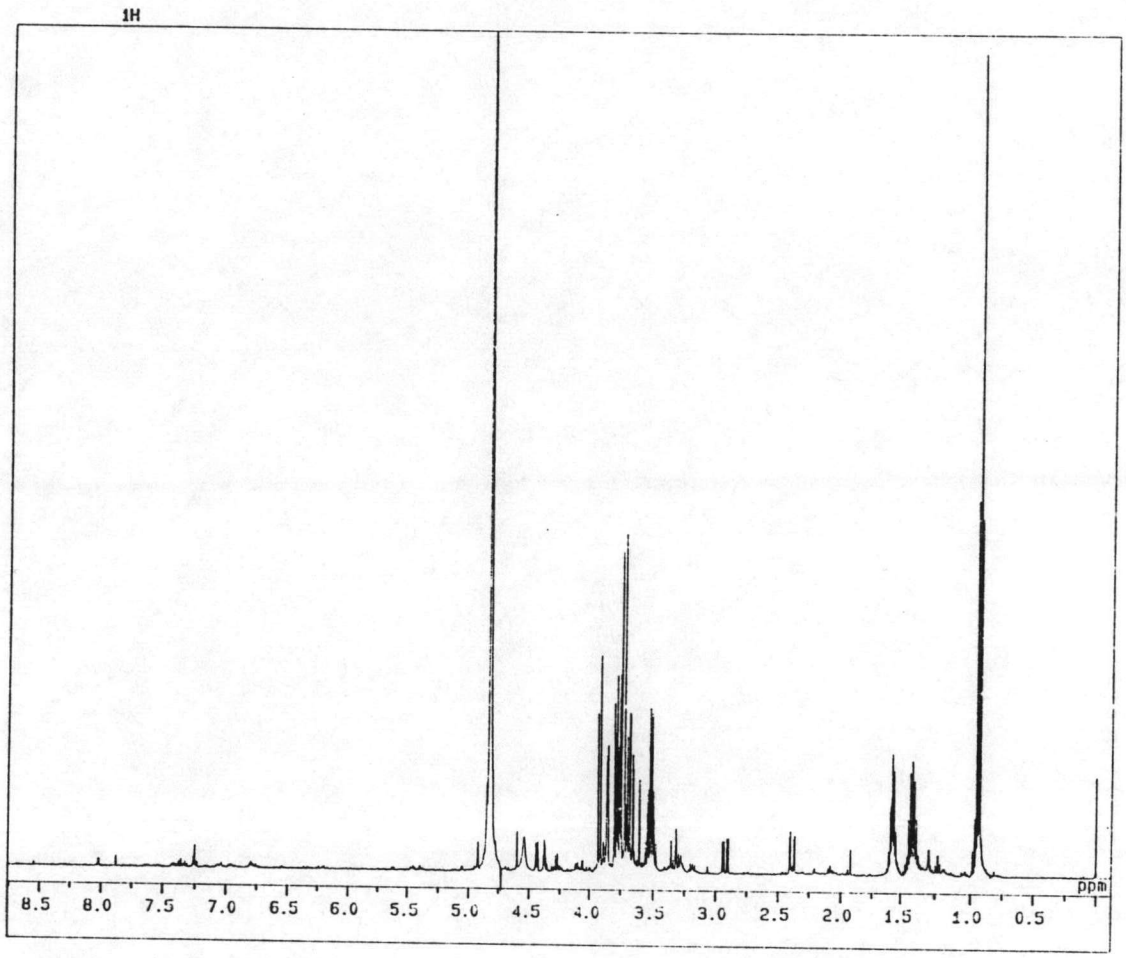


Figure 3.53 ^{1}H NMR spectrum of compound **8**

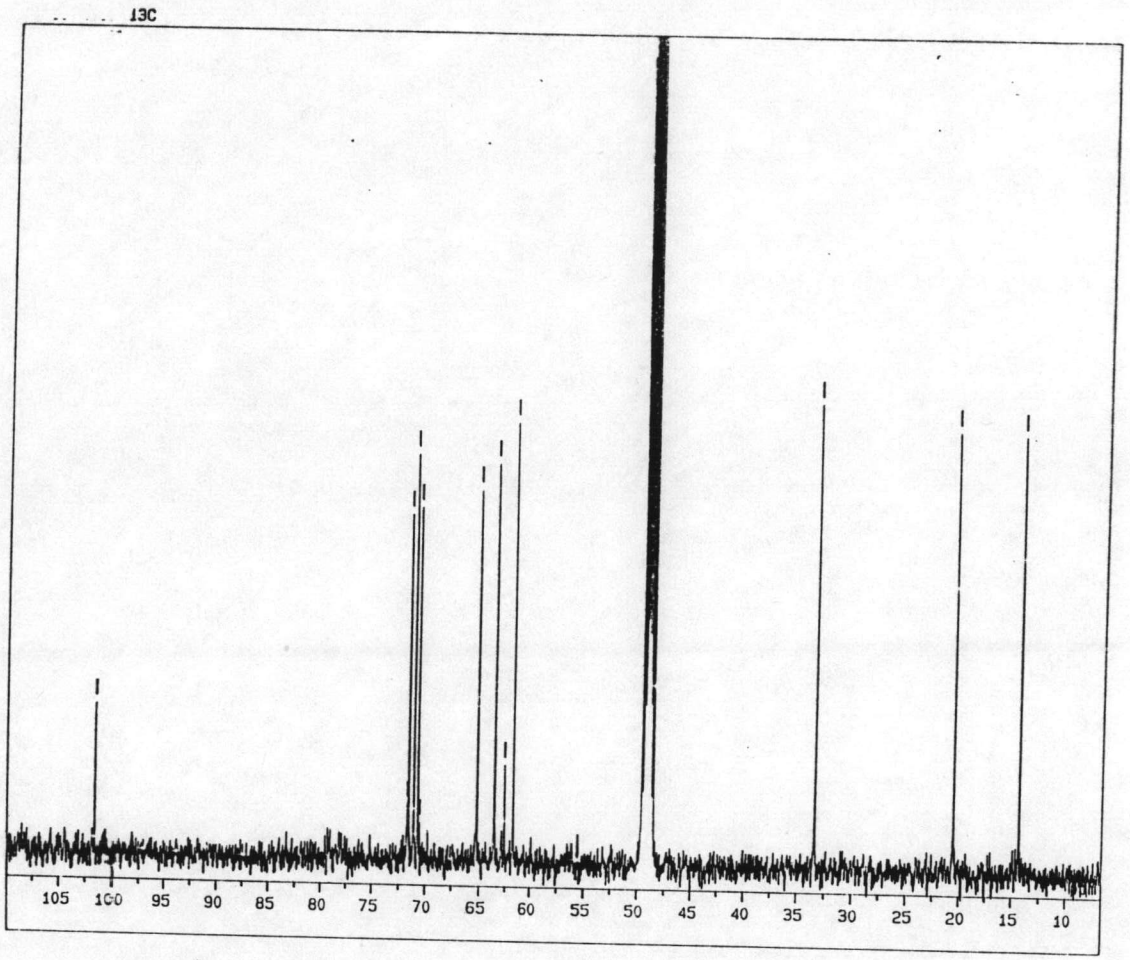
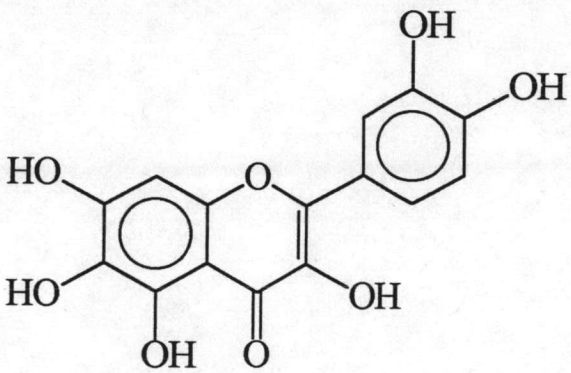


Figure 3.54 ^{13}C NMR spectrum of compound **8**

3.3.9 The structural elucidation of compound 9

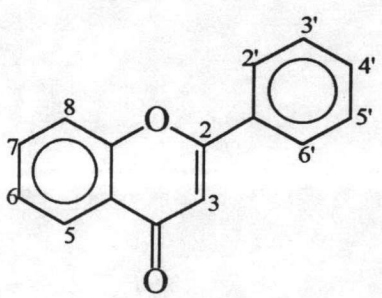
Compound **9** (45 mg) was obtained from butanol crude extract by recrystallization in methanol. Its melting was 240-242 °C and R_f value was 0.4 (100% EtOAc). Compound **9** and authentic quercetin (Sigma) showed the same spot on TLC plate in various solvents. The ^{13}C NMR of compound **9** is close to those of quercetin[30]. Thus, compound **9** should be quercetin. The ^{13}C NMR data of compound **9** and quercetin is showed in Table 3.14



Quercetin

Table 3.14 The ^{13}C NMR data of quercetin and Compound **9**

carbon position	quercetin	compound 9
C-2	146.9	146.2
C-3	135.8	137.2
C-4	175.9	177.3
C-5	156.2	158.3
C-6	98.3	99.3
C-7	164.0	164.9
C-8	93.5	94.4
C-9	160.8	160.6
C-10	103.1	104.5
C-1′	122.1	121.8
C-2′	115.2	115.2
C-3′	145.1	145.6
C-4′	147.7	148.1
C-5′	155.7	158.3
C-6′	120.1	121.0



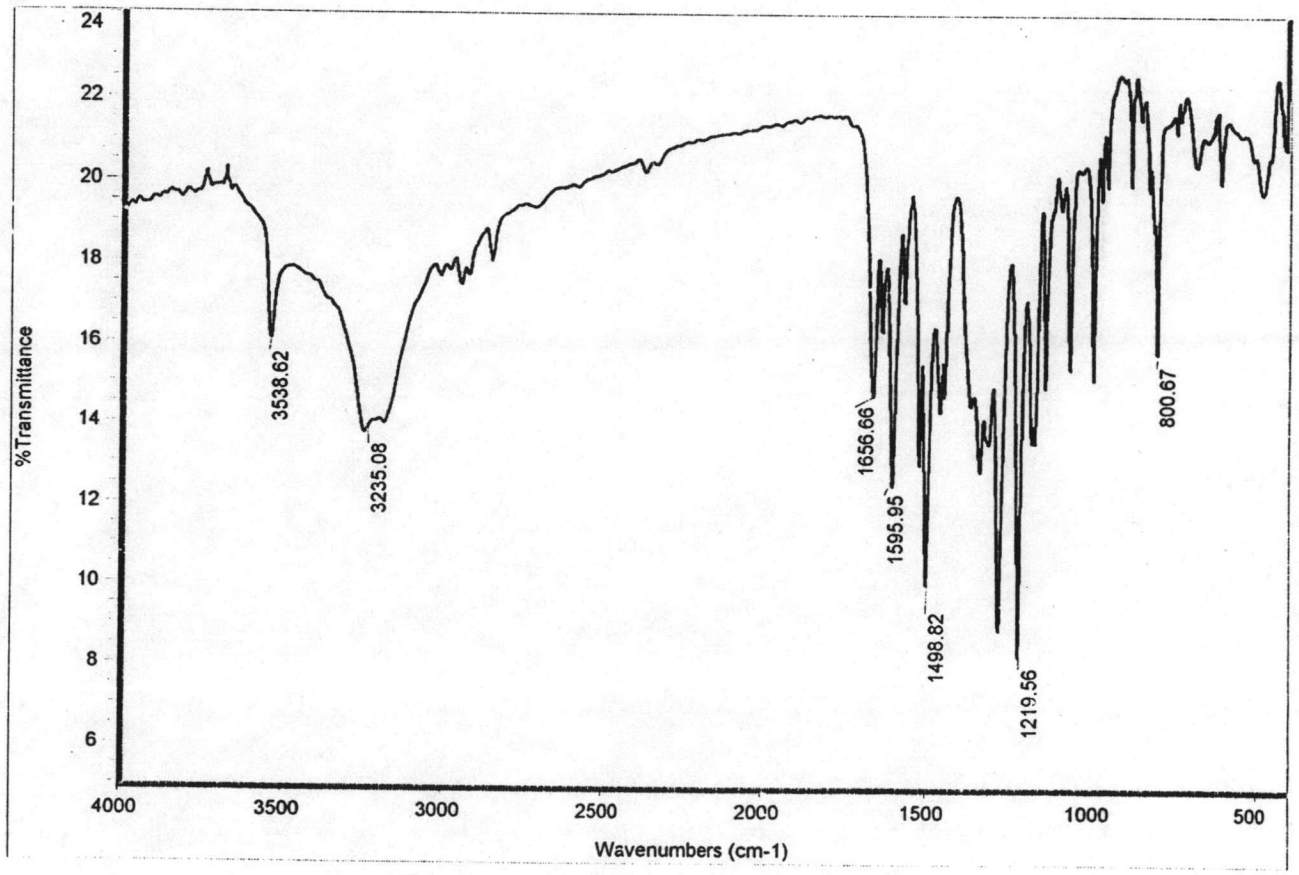


Figure 3.55 The IR spectrum of compound **9**

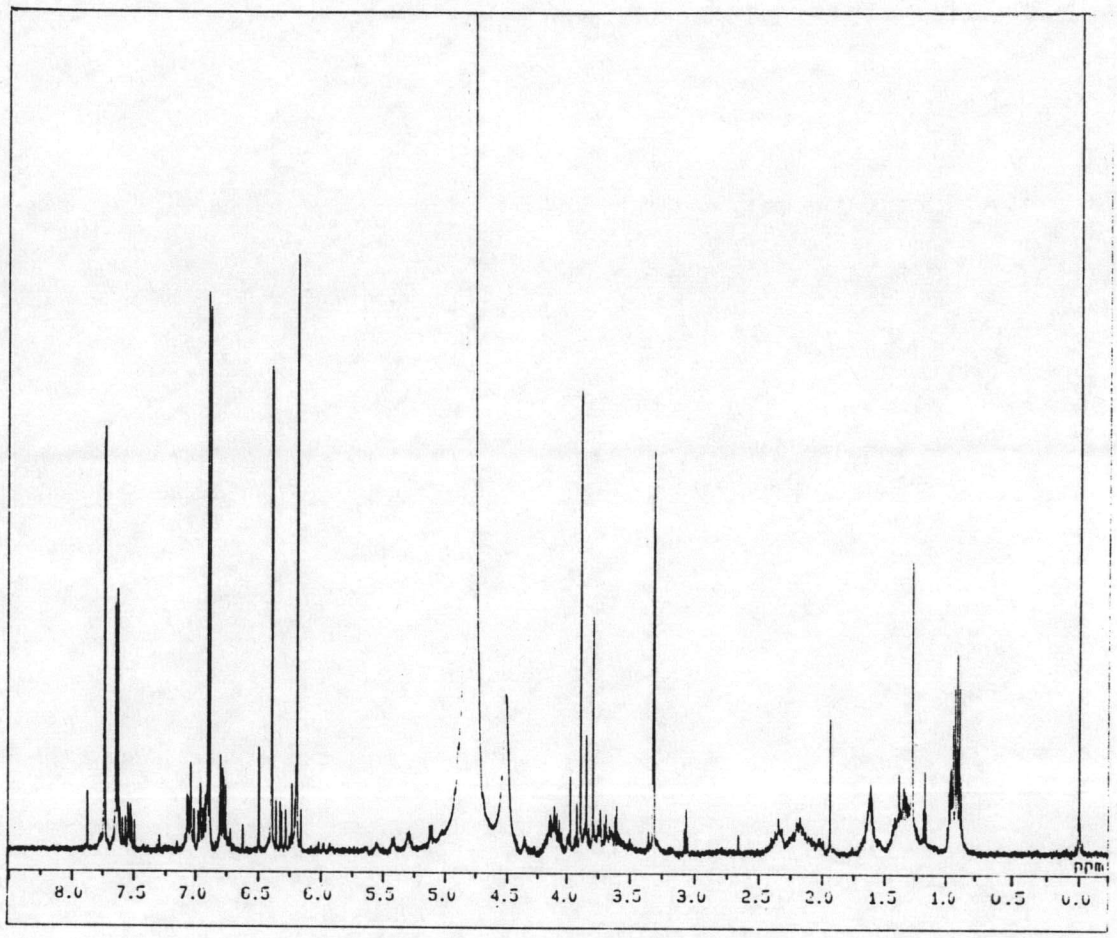


Figure 3.56 The ^{1}H NMR spectrum of compound **9**

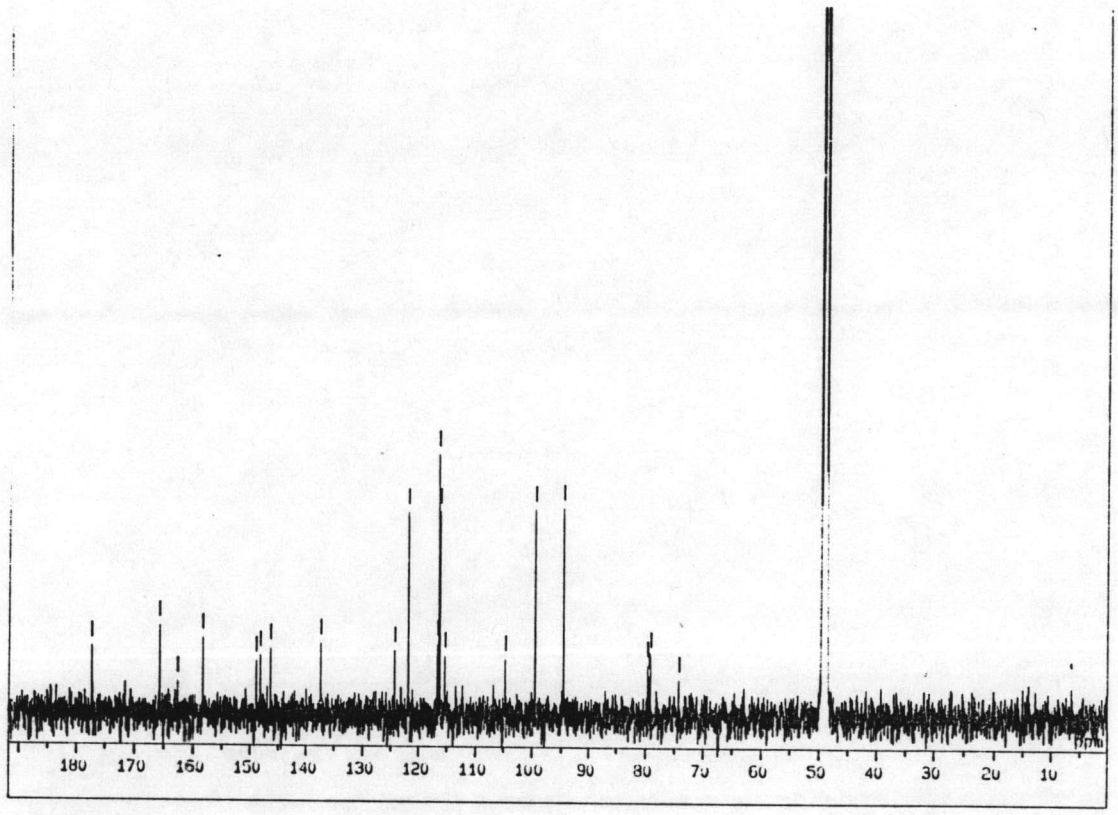


Figure 3.57 The ^{13}C NMR spectrum of compound **9**

3.5 The Result of Biological Activities of Isolated Compound

3.5.1 The BSCLT of Isolated Compounds

According to the preliminary cytotoxicity screening test, chloroform and butanol crude extract revealed high cytotoxic against brine shrimp (*Artemia salina*). Both of them were selected for further investigation of bioactive compounds. The isolated compounds were tested the cytotoxic against brine shrimp. The results of BSCLT displayed in Table 3.15.

Table 3.15 The Result of Brine Shrimp Lethality Cytotoxicity Test of Isolated Compound

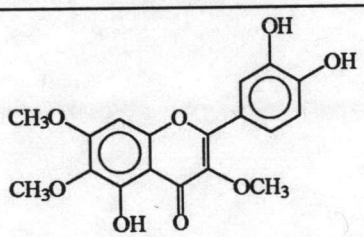
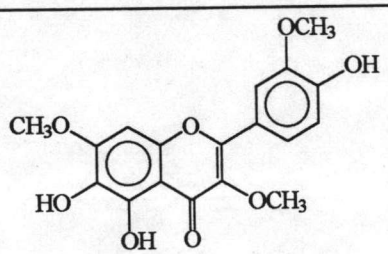
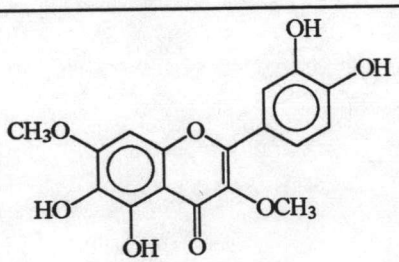
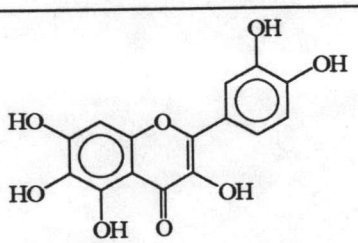
Sample	LC_{50} (μg/ml)	Activity
compound 1	180.62	Low activity
compound 2	>1000	No activity
compound 3	241.82	Low activity
compound 4	12.34	Medium activity
compound 5	11.45	Medium activity
compound 6	145.61	Low activity
compound 7	72.32	Medium activity
compound 8	>1000	No activity

Carvotacetones were reported since 1990, but there was no reportd about their biological activities. From Table 3.15, both compounds **4** and **5** revealed significant cytotoxicity against brine shrimp (*Artemia salina.*)

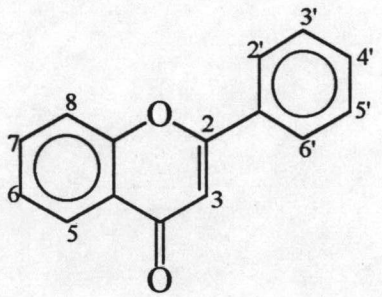
3.5.2 The result of cAMP inhibition

As a previous report, the flavonol compound showed cAMP inhibiting effect. Thus, four isolated flavonol compounds from *S. africanus* were tested by method described in Chapter 2. Four flavonol compounds revealed significant cAMP inhibiting effect at dose 10 mg/ml. The percentage inhibition of cAMP with compound **3**, **6**, **7** and **9** shown in table 3.16.

Table 3.16 The percentage inhibition of cAMP with compound **3**, **6**, **7** and **9**

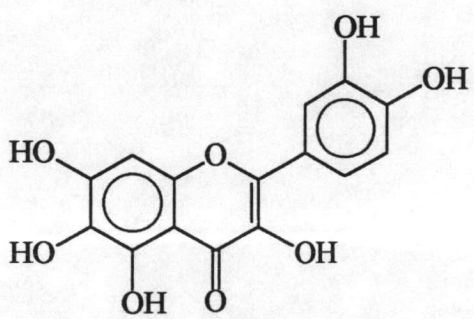
compound	chemical structure	% inhibition
3		75.0
6		50.0
7		42.0
9		87.5

During the last few years[31], the inhibition of enzyme by flavonoids was studied using MLC (miosin light chain) peptide as CDPK (Ca^{+}-depent protein kinase) substrate. A major feature of the inhibition by flavonoids of wheat embryo CDPK. It appears that the minimal structural requirements for inhibition of CDPK are:



a) 2,3 unsaturation
b) 3′-and 4′ hydroxy
c) an additional 3-hydroxyl in ring C or hydroxyls in ring A

Among flavonoids which was isolated from *S. africanus*. Compound **9** showed strong inhibition cAMP. The same reason as previously mentioned, compound **9** has 3′, 4′ hydroxyl and 3-hydroxyl. Thus, compound **9** are strong inhibitor, which comparing with compound **3, 6,** and **7**



Compound **9**