CHAPTER III
RESULTS AND DISCUSSION

The goal of this research is the isolation and structural elucidation of bioactive compounds from the roots of *C. orientalis*. This plant was selected for examination due to the activity of crude extracts against brine shrimp and anticell lines.

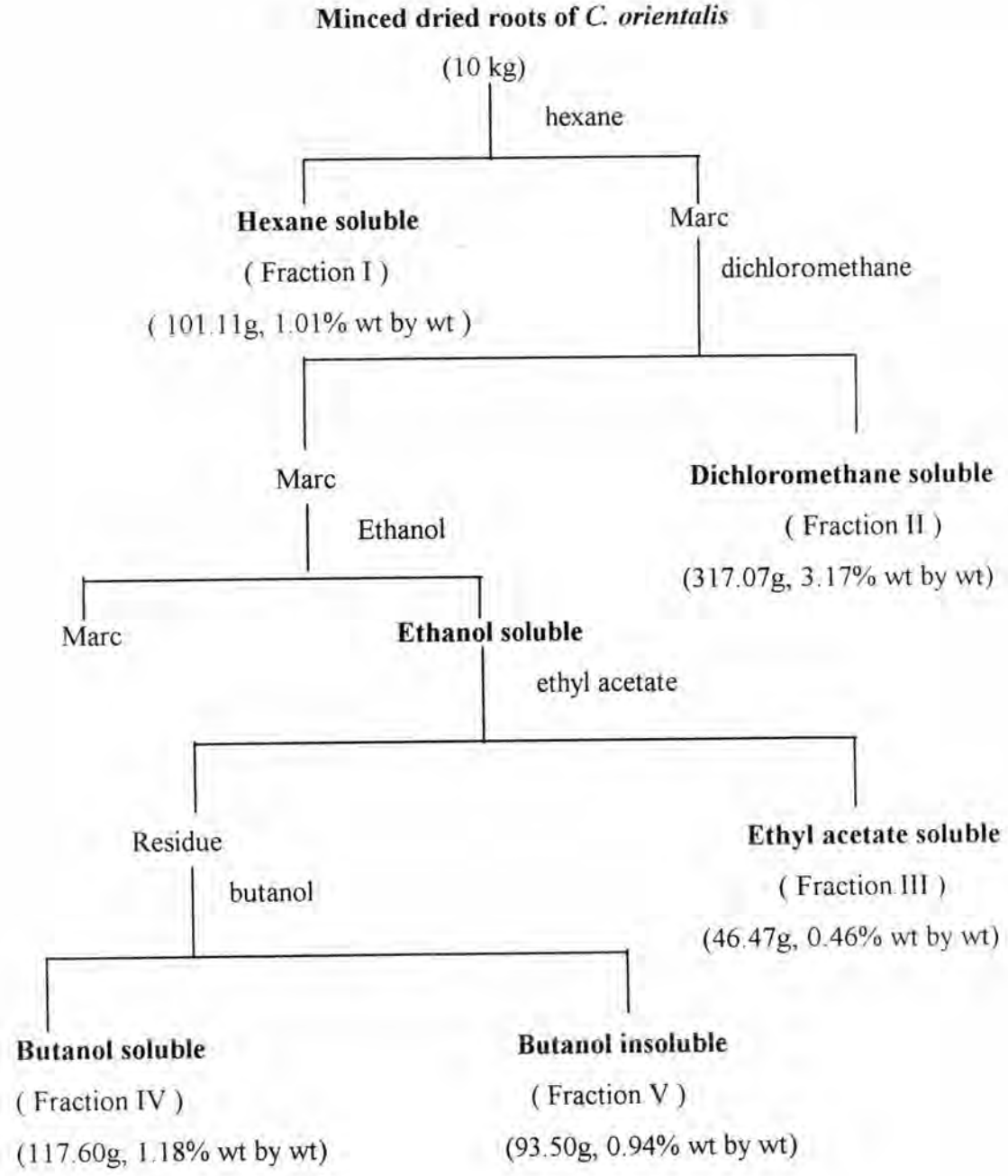
The extracts of the roots of *C. orientalis* were obtained according to the extraction procedure shown in scheme 2. The roots of *C. orientalis* were extracted with hexane, dichloromethane and ethanol. The ethanol residue was then partition with ethyl acetate and butanol.

Each crude extract of *C .orientalis* was bioassayed for cytotoxicity with brine shrimp. According to the procedure described in Chapter II. The results of brine shrimp cytotoxicity are reported in Table 1.

Brine Shrimp Cytotoxicity Test

Table 1. The brine shrimp cytotoxicity test of extracts from *C. orientalis*

Fraction/Solvent extract	LC_{50}	Activity
I (Hexane)	0.04	High activity
II (Dichloromethane)	0.07	High activity
III (Ethyl acetate)	5.35	High activity
IV (Butanol)	18.78	Medium activity
V (Ethanol)	100.00	Low activity



Scheme. 2 The procedure and results of extraction of the roots of *C. orientalis*

Anticell Lines Cytotoxicity Test

Hexane extract, ethyl acetate extract, butanol extract and ethanol residue were inactive against anticell lines cytotoxicity test. Only dichloromethane extract showed medium activity against *Erythroleukemia carcinoma* (K-562) cell lines. This result is presented in Table 2.

Table 2. The cytotoxicity test against *Erythroleukemia carcinoma* (K-562) of crude dichloromethane extract

Sample	Concentration (μg/ml)	Inhibition (%)	Estimation
Dichloromethane crude extract	1	26.00	++
	10	51.58	
	100	54.54	

The results of biological activity screening test revealed that nonpolar crude extracts showed high cytotoxicity test against brine shrimp. Only dichloromethane crude extract showed promising result against *E. carcinoma* (K-562).

Separation

Separation of Hexane Crude Extract (Fraction I)

Hexane crude extract (Fraction I, 68 g) was separated on silica gel Art. 7734(900 g) by open column chromatography using hexane, hexane-dichloromethane mixture and dichloromethane-ethyl acetate mixture of increasing polarity. The combined portions (TLC guided using organic solvents as solvent and 10% Conc.H_2SO_4 in EtOH as a detection reagent) were concentrated to obtain 20 main fractions. Separation of hexane crude extract are summarized in Table 3.

Table 3 Column chromatography of Hexane Crude Extract (Fraction I)

Eluents	Fraction no.	Remarks	Weight (g)
Hexane	1	Yellow wax	0.7758
5% CH_2Cl_2 in Hexane	2	Green oil	1.2358
10% CH_2Cl_2 in Hexane	3-4	Yellow oil	2.3364
15% CH_2Cl_2 in Hexane	5-9	Yellow oil	1.9084
20% CH_2Cl_2 in Hexane	10-17	Yellow oil	1.0356
25% CH_2Cl_2 in Hexane	18-21	Yellow oil	1.0036
	22-25	Yellow oil	1.5863
30% CH_2Cl_2 in Hexane	26-32	Pale Yellow oil	1.9860
40% CH_2Cl_2 in Hexane	33-35	Orange oil	2.0451
45% CH_2Cl_2 in Hexane	36-41	White solid in brown oil (Compound **6**)	2.5639
50% CH_2Cl_2 in Hexane	42-45	White solid in brown oil (Compound **9**)	1.0692
60% CH_2Cl_2 in Hexane	46-50	Brown oil	0.9941
70% CH_2Cl_2 in Hexane	51-56	Brown oil	1.6981
80% CH_2Cl_2 in Hexane	57-67	Brown oil	2.6980
90% CH_2Cl_2 in Hexane	68-69	Brown oil	3.5964
95% CH_2Cl_2 in Hexane	70-76	Brown oil	0.9852
CH_2Cl_2	77-87	Brown oil	1.4736
5% EtOAc in CH_2Cl_2	88-91	Dark brown oil	1.0586
10% EtOAc in CH_2Cl_2	92-107	Dark brown oil	1.5338

Separation of Dichloromethane Crude Extract (Fraction II)

The dichloromethane crude extract (40g) was dissolved in 200ml of dichloromethane and added to 40 grams of silica gel. Dichloromethane was removed in vacuo, and the dried sample placed on a glass chromatography column (diameter 12 cm with 480 g of silica gel as the absorbent). The column was eluted with hexane and then the solvent polarity was increased by addition of dichloromethane, ethyl acetate and methanol.

The eluates were collected in 500 ml portions. The solvent were removed in vacuo. All fractions were monitorted by thin layer chromatography. Components were located by ultraviolet light or iodine absorption. Fractions containing the same component were combined, and the results are presented in Table 4.

Chromatography of dichloromethane extract yield mixture **1** which was isolated from fraction, 5-19 (Table 4).

Table 4 Column Chromatography of Dichloromethane Crude Extract (Fraction II)

Eluents	Fraction no.	Remarks	Weight (g)
20 %CH_2Cl_2 in hexane	1-4	Yellow wax	1.5232
	5-19	White solid in yellow oil (Mixture **1**)	1.0031
	20-22	Yellow wax	0.5563
30 %CH_2Cl_2 in hexane	23-37	Yellow wax	1.2530
	38-43	White solid in yellow oil (Compound **2**)	2.0150
	44-54	Orange oil	0.88915
50 %CH_2Cl_2 in hexane	55-67	Brown oil	2.1358
60 %CH_2Cl_2 in hexane	68-77	White solid in brown oil (Mixture **3**)	2.4639

Table 4 (cont.)

Eluents	Fraction no.	Remarks	Weight (g)
90 %CH_2Cl_2 in hexane	78-81	Brown oil	2.0377
CH_2Cl_2	82-85	Brown oil	0.5579
	86-93	Brown oil	1.6513
	94-105	Brown oil	3.7321
10% EtOAc in CH_2Cl_2	106-110	yellow oil	1.1130
10% EtOAc in CH_2Cl_2	111-117	Brown oil	1.9320
	118-131	Brown oil	0.7729
20% EtOAc in CH_2Cl_2	132-153	White solid in brown oil (Compound **6**)	4.3890
40% EtOAc in CH_2Cl_2	154-169	Dark brown oil	3.7815
50% EtOAc in CH_2Cl_2	170-184	Dark brown oil	1.1173
60% EtOAc in CH_2Cl_2	185-204	Dark brown oil	1.3941
80% EtOAc in CH_2Cl_2	205-212	Brown tar	0.7921
90% EtOAc in CH_2Cl_2	213-221	Brown tar	1.3377
EtOAc	222-227	Brown tar	0.6532
5% MeOH in EtOAc	228-234	Brown tar	0.7128
10% MeOH in EtOAc	235-247	Brown tar	0.6448
20% MeOH in EtOAc	248-259	Brown tar	0.4615

The dichloromethane crude extract (40g) was subjected to chromatography on a silica gel (230-400 mesh, 480 g) column. The column was gradient eluted with an increasing concentration of dichloromethane in hexane, ethyl acetate in dichloromethane, then methanol in ethyl acetate to give 25 fractions.

The separation of the eluted fraction no. 78-81

Fraction no. 78-81 was further separated by chromatotron using stepwise elution with hexane, 5% EtOAc in hexane and 10% EtOAc in hexane to give fraction a, b, c. The results are shown in Table 5.

Table 5 Separation of fraction no. 78-81 by chromatotron

Eluents	Fraction no.	Remarks	Weight (g)
Hexane	a	Trace	-
5% EtOAC in hexane	b	Yellow oil	1.3521
10% EtOAC in hexane	c	Yellow oil	0.3973

The separation of the eluted fraction no. b

TLC showed the compounds which were visible under U.V. light. Similarly, fraction no. b was separated into 2 fractions, fraction b1 (compound **4**) and fraction b2 by preparative TLC, developed in dichloromethane, afforded 27.9 mg of compound **4**.

Table 6 Separation of fraction no. b by preparative TLC

Eluents	Fraction no.	Remarks	Weights (g)
CH_2Cl_2	b1	Yellow solid (Compound **4**)	0.0279
	b2	Yellow oil	0.6531

The separation of the eluted fraction No. 94-105

The combined fractions was also separated by chromatotron using the same procedure as for the separation of the eluted fraction no. 78-81 but using hexane, 10% diethyl ether in hexane, and 20% diethyl ether in hexane as solvent to give 3 major fractions.

Table 7 Separation of fraction no. 94-105 by chromatotron

Eluents	Fraction no.	Remarks	Weight (g)
Hexane	d	Trace	-
10% diethyl ether in Hexane	e	Yellow oil	1.2311
20% diethyl ether in Hexane	f	Brown oil	1.9356

The separation of the eluted fraction no. e

The appropriate fraction (e) was purified by preparative TLC which precoated with silica gel GF254 using 70% diethyl ether in hexane as solvent to yield compound 5.

Table 8 Separation of fraction no. e by preparative TLC

Eluents	Fraction no.	Remarks	Weight (g)
70% diethyl ether in hexane	e1	Pale yellow oil (compound 5)	0.0402
	e2	Yellow oil	0.0124

The separation of the eluted fraction no. 111-117

Fraction no. 111-117 was separated by chromatotron with hexane and gradient between ethyl acetate and hexane to give 4 fractions.

Table 9 Separation of fraction no. 111-117 by chromatotron

Eluents	Fraction no.	Remarks	Weight (g)
Hexane	g	Trace	-
10% EtOAc in hexane	h	White solid (Compound **6**)	0.0484
20% EtOAc in hexane	i	Yellow oil	1.4133
30% EtOAc in hexane	j	Yellow solid (Compound **8**)	0.2118

The separation of the eluted fraction no. i

TLC was shown a major component which was obtained from the separation of the eluted fraction no. 111-117 (Table 10). It was eluted with 20% EtOAc in hexane by using chromatotron. Then this fraction (i) was separated by chromatotron with 20% EtOAc in hexane again to afford 0.1946 g of compound **7**.

Table 10 Separation of fraction no.i by chromatotron

Eluents	Fraction no.	Remarks	Weight (g)
10% EtOAc in hexane	i-1	Trace	-
20% EtOAc in hexane	i-2	Pale yellow oil (Compound 7)	0.1946

Separation of Ethyl Acetate Crude Extract (Fraction III)

Fraction III, the ethyl acetate crude extract (45 g) was separated into 12 fractions by column chromatography on silica gel (450 g) and eluting with gradient between dichloromethane and ethyl acetate, ethyl acetate and methanol. Each fraction was collected in 5 ml and checked by TLC. The fractions having the same components were combined. The separation of ethyl acetate crude extract was reported in Table 5.

Table 11 Column Chromatography of Ethyl Acetate Crude Extract (Fraction III)

Eluents	Fraction no.	Remarks	Weight (g)
20% EtOAc in CH_2Cl_2	1-2	White solid in yellow oil (Mixture **3**)	1.3720
	3-4	Red oil	4.5011
	5-10	White solid in yellow oil (Compound **10**)	6.3009
	11-17	Yellow oil	1.2446
	18-24	Brown oil	2.4190
50% EtOAc in CH_2Cl_2	25-34	Brown oil	2.6992
100% EtOAc	35-45	Dark brown oil	2.3913
	46-55	Dark brown oil	1.0552
10% MeOH in % EtOAc	56-65	Dark brown oil	2.0459
	66-69	Dark brown oil	1.0438
40% MeOH in % EtOAc	70-75	Dark brown oil	1.7350
	76-85	Dark brown oil	0.9967

Purification, properties and structure elucidation of substances from *C. orientalis*

Structure elucidation of Mixture 1

This fraction after washing yellow oil with hexane and recrystallization with dichloromethane and hexane for several time, mixture **1** was obtained as pale yellow solid, 7 mg ($0.07x10^{-3}$% wt by wt of the roots), m.p. 81-82 ^{0}C, R_f values 0.53 in 50 % CH_2Cl_2 in hexane. This mixture was soluble in dichloromethane, chloroform but not in hexane.

The IR spectrum (Fig. 4) exhibited a strong absorption band at 1737 cm^{-1}, which is characteristic of an ester carbonyl group. The absorption band at 1189 cm^{-1} ought to be C-O stretching vibration mode of ester group. The IR absorption band is shown in Table 12.

Table. 12 The IR absorption band assignments of Mixture **1**

Vibration	Wave number (cm^{-1})	Intensity
C-H stretching of -CH_2, CH_3	2925,2848	Strong
C=O stretching of ester	1737	Strong
C-H bending of -CH_2, CH_3	1465,1375	Moderate
C-O stretching	1189	Moderate
-CH_2 rocking in C-$(CH_2)_n$-C	690	Weak

The IR spectrum results indicated that mixture **1** should be a mixture of long chain aliphatic esters.

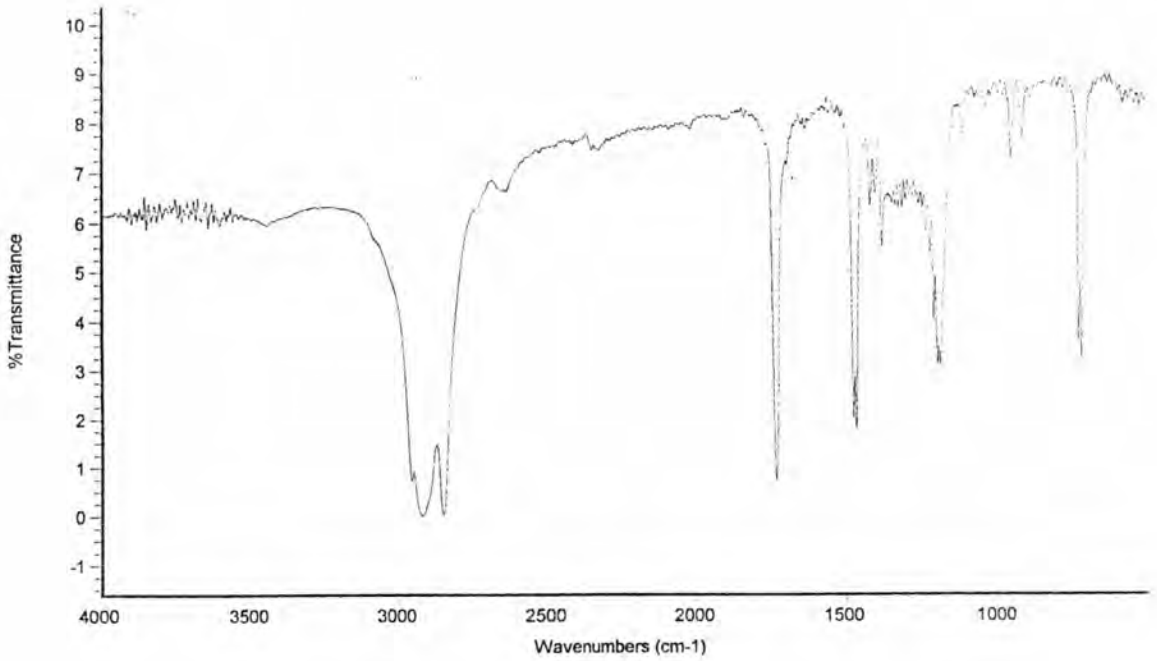


Fig. 4 The IR spectrum of Mixture **1**

Structure elucidation of Compound 2

Compound **2** was white amorphous in a yellow oil in fraction no. 38-43, which was obtained from the column chromatography of dichloromethane crude extract (Table 4). It was eluted with 30% CH_2Cl_2 in hexane from the silica gel column. After yellow oil was removed by washing with hexane and recrystallization with dichloromethane for several times. It gave white amorphous 17 mg ($0.17x10^{-3}$% wt by wt of the roots); m.p. 138-140 ^{0}C. TLC revealed only one spot at R_f 0.24 (solvent system as 20% EtOAc in hexane). This compound was soluble in dichloromethane, chloroform but not in hexane. Compound **2** gave positive test (purple color) with Liebermann Burchard's reagent.

The IR spectrum (Fig. 5) displayed the band of hydroxy group at 3200-3600 cm^{-1}, long chain hydrocarbon at 2929 and 2843 $cm.^{-1}$

Table 13 The IR absorption band assignments of Compound **2**

Vibration	Wave number	Intensity
O-H stretching of R-OH	3200-3600	Moderate
C-H stretching of vinyl	3100	Weak
C-H stretching of -CH_2, -CH_3	2929, 2842	Strong
C-O bending of -CH_2, CH_3	1475, 1383	Moderate
C-O stretching	1034	Weak

Compound **2**, was analyzed by gas chromatography. The result was compared with a standard mixture of steroids (Campesterol, stigmasterol and β-sitosterol). The retention time of standard steroids were 19.59, 20.90 and 23.76 and retention time of compound **2** was 23.29, which indicated that this compound was β-sitosterol (Fig. 6) .

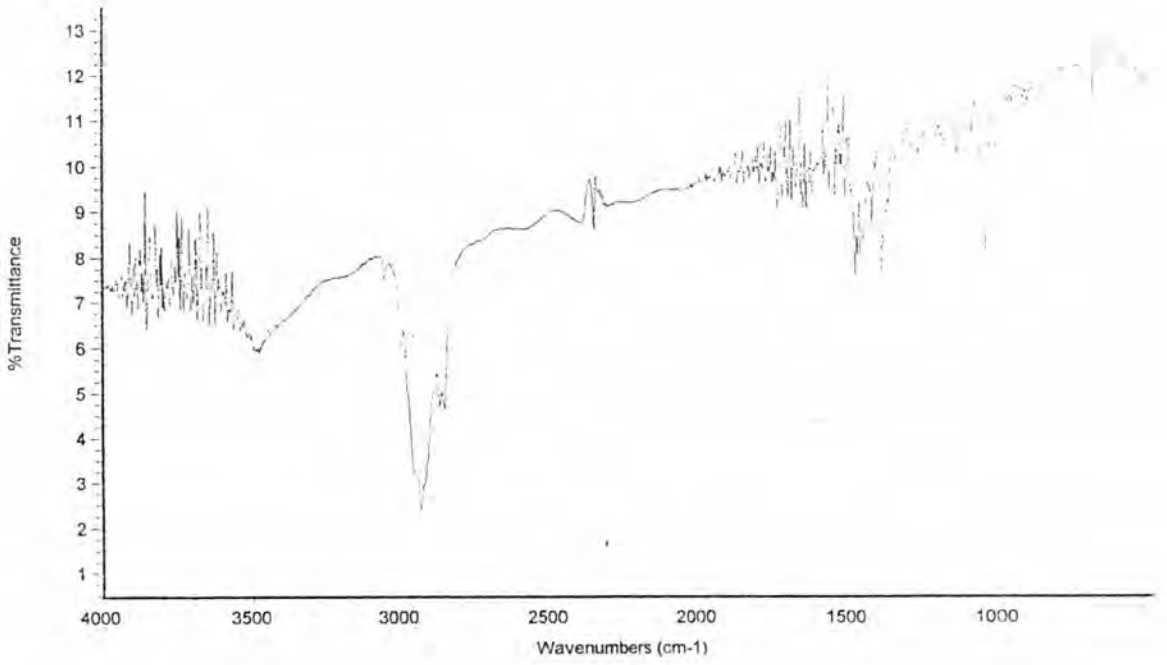


Fig. 5 The IR spectrum of Compound **2**

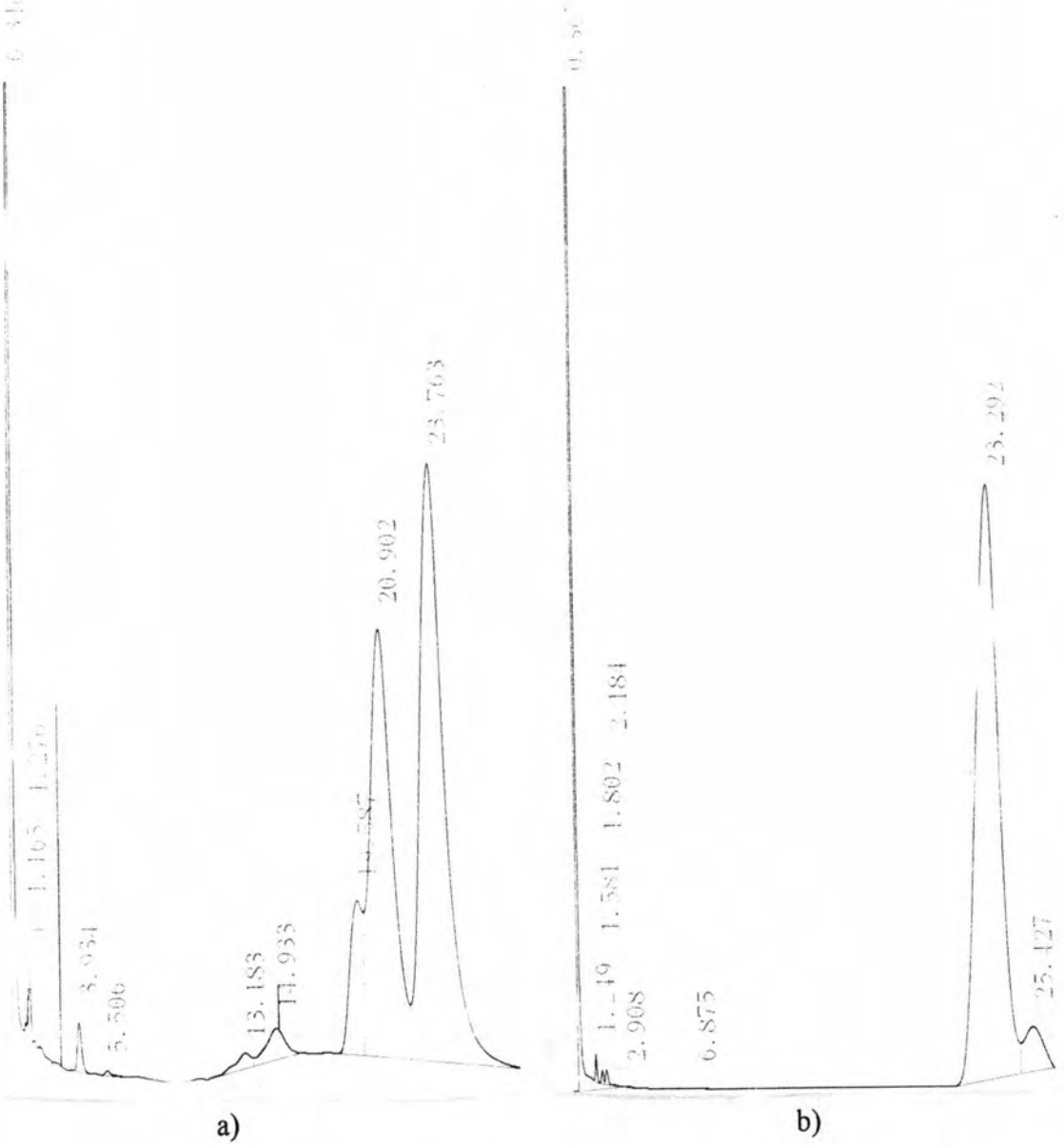


Fig. 6 The GC analysis results of

a) Standard steroids ; campesterol, stigmasterol, β-sitosterol

b) Compound **2**

Structure elucidation of Mixture 3

The dichloromethane crude extract was subjected to a silica gel column eluted with various solvent systems(Table 4). Fraction no. 68-77 eluted with 60% CH_2Cl_2 in hexane, afforded 23 mg of mixture **3**, ($0.23x10^{-3}$% wt by wt of the roots), white amorphous, which repeated purification by recrystallization in dichloromethane; m.p. 138-140 ^{0}C, (R_f value 0.16 in 20% EtOAc-hexane). It was soluble in dichloromethane, chloroform but slightly soluble in hexane and gave positive test with Liebermann-Burchard's reagent.

The IR spectrum of mixture **3** was shown in figure 7 which exhibited the absorption band of hydroxy group (OH) at 3150-3600 cm^{-1}, absorption band of disubstituted and trisubstituted vinyl were presenced at 898 and 845 cm^{-1}, respectively.

Table 14 The IR absorption band assignments of the Mixture **3**

Vibration	Wave number (cm^{-1})	Intensity
O-H stretching of R-OH	3150-3600	Moderate
C-H stretching of -CH_2, CH_3	2925, 2858	Strong
C-H bending of -CH_2, CH_3	1455, 1383	Weak
C-O stretching	1050	Moderate
C-H out of plane blending	979	Moderate

The structure of mixture **3** was confirmed with gas chromatography by comparision the retention time of mixture **3** with mixture of standard of steroids: stigmasterol and β-sitosterol (Fig.8). The retention time of mixture **3** were 18.06 and 20.65 and the retention time of the standard were 18.56 and 21.02.

From GC analysis and IR spectrum indicated that mixture **3** was a mixture of stigmasterol and β-sitosterol.

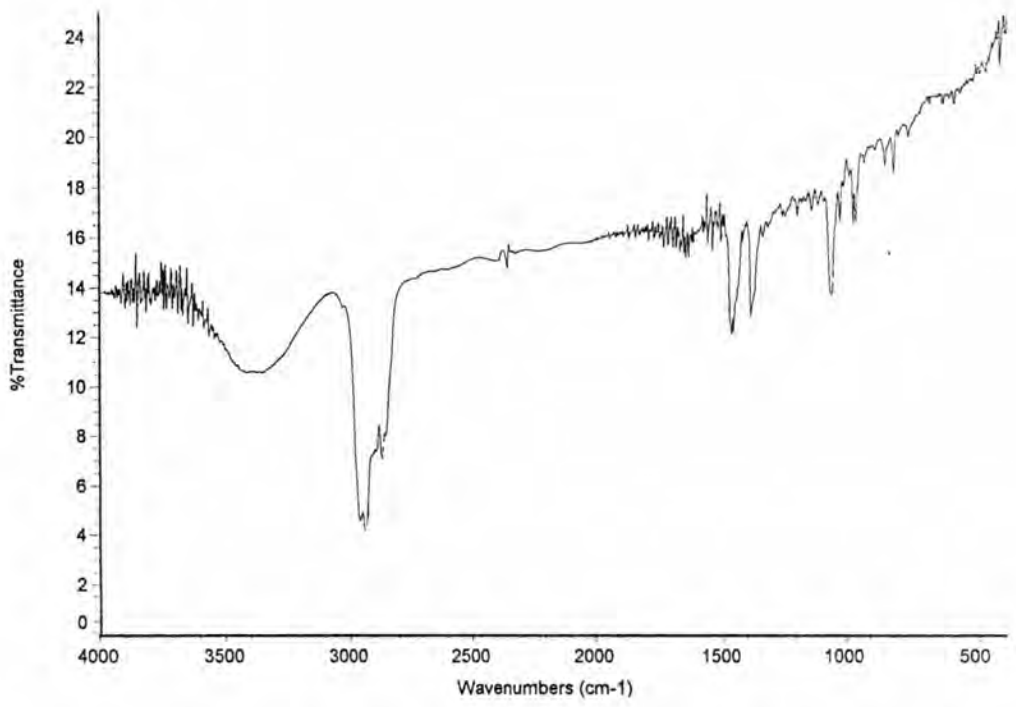
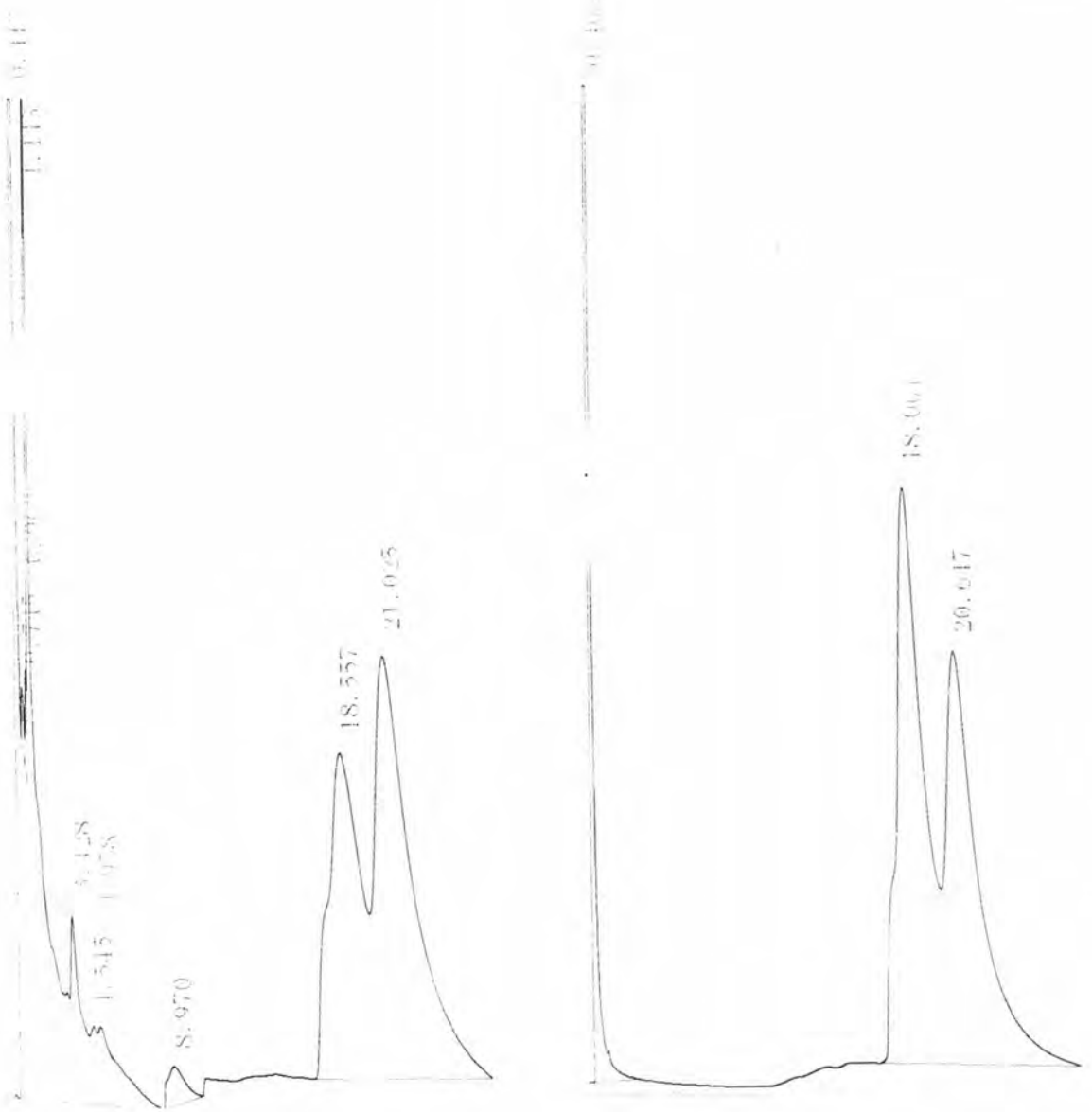


Fig. 7 The IR spectrum of Mixture **3**



a) b)

Fig. 8 The GC analysis results of

a) Standard steroids ; campesterol, stigmasterol, β-sitosterol

b) Mixture **3**

Structure elucidation of Compound 4

Compound **4**, yellow solid was obtained from dichloromethane crude extract which was separated by column chromatography eluted with 90% CH_2Cl_2 in hexane and reseparated by using chromatotron, finally purified with preparative TLC (CH_2Cl_2 as solvent); m.p. 89-90 ^{0}C(R_f value 0.28 in CH_2Cl_2). Compound **4**, 27.9 mg ($0.28x10^{-3}$ % wt by wt of the roots) was soluble in dichloromethane, chloroform and methanol but not in hexane.

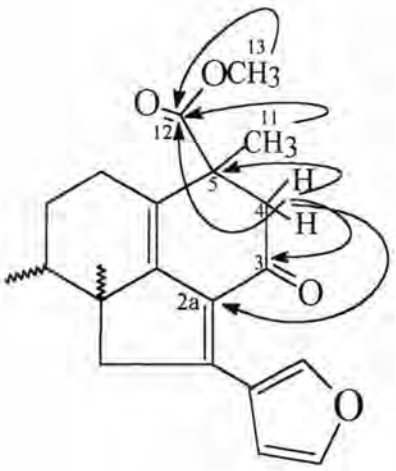
The IR spectrum of compound **4** (Fig. 9) indicated that this compound contained a carbonyl ester group at 1721 cm^{-1}. The olefinic and an α,β-unsaturated carbonyl group absorption showed at 1690 cm^{-1} and 1654 cm^{-1} respectively. There was evidenced for a furan ring at 3110, 1460 and 830 cm^{-1}. However, type of double bond and the substitution of the furan ring were accounted by NMR and MS.

The molecular ion was observed at m/z 340 and other fragments were at m/z 281, 266, 284, 95 and 81 (Fig.10).

The ^{1}H-NMR spectrum of compound **4** (Fig. 11-13) showed signals for β-substituted of furan ring proton at δ 8.56 [1H, s], 7.43 [1H, t, J=1.50-2.00 Hz] and 7.00 [1H, dd, J=0.61, 2.14 Hz]. [2,5,31,32] The ester methoxy group gave rise to a broad singlet at δ 3.57 [3H, s]. Singlet at δ 0.99, 1.38 and doublet at δ 0.97 demonstrated the presence of three methyl group. The ^{13}C-NMR spectrum exhibited 21 signals (Fig. 14). DEPT 90 and DEPT 135 experiments (Fig. 15) showed three methyl carbons at δ 16.4, 20.3 and 23.8, four methylene carbons at δ 23.8, 27.1, 50.3 and 52.1, one methoxy carbon at δ 52.2, four tertiary carbons at δ 37.1, 111.0, 142.7 and 146.3 and nine quaternary carbons at δ 42.5, 48.5, 121.9, 125.1, 128.0, 139.7, 150.4, 174.6 and 195.1 [It showed that this molecule must contained a keto group, which was corresponded with HMQC (Fig. 16-19)]. Compound **4** had the molecular formula $C_{21}H_{24}O_4$, as determined by mass spectra, ^{1}H- and ^{13}C-NMR (Fig. 11-14). Further structure identification of this compound involved other two-dimentional NMR analysis.

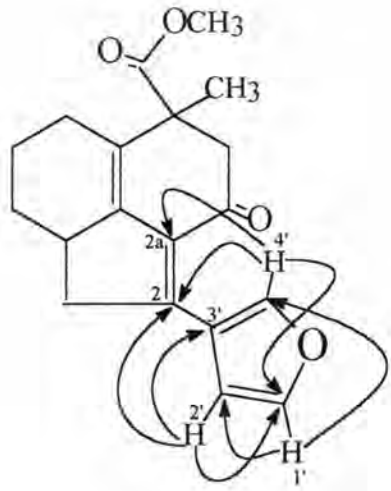
However, the existence of a singlet signal at δ 195.1, attributable to an keto group (C-3), was observed. The position of (C-3) keto group was established on the

basis of HMBC correlation (Fig. 20-28) between H-4 and C-3. A ester carbonyl (C-12) showed significant correlation between the signal corresponding to methoxy proton ($-OCH_3$) and C-12 between methyl proton (H-11) and between H-4 and C-5, were observed.

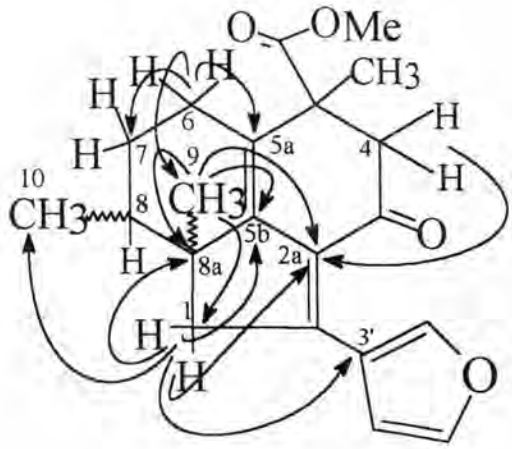


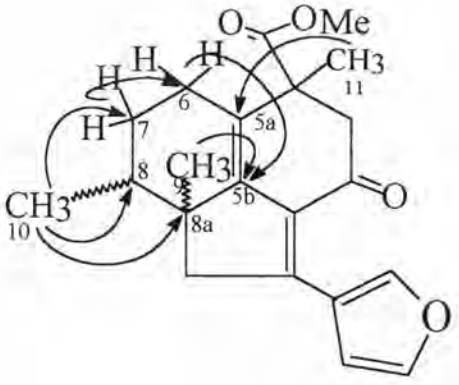
These observations indicated that the furan ring occupied the C-2 position, on the basis of HMBC experiments:

1. H-4′(δ 8.56) showed significant correlation between C-2a (δ 150.4) and C-1′(δ 142.7)
2. H-2′(δ 7.00) correlated with C-2 (δ 139.7), C-1′(δ142.7) and C-3′(δ 121.9)
3. H-1′(δ 7.43) correlated with C-4′(δ146.3)
4. H-4′(δ8.56) correlated with C-2′(δ 111.0).

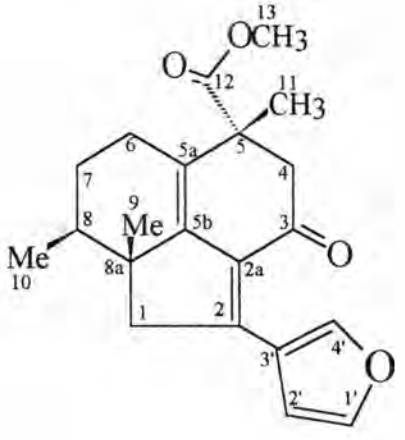


This allowed the assignment of the signals of H-6 and H-7, as δ 2.30 [2H,m] signal (H-6) was a long range coupling with H-7 [δ ca 1.64] and correlated with C-2a, C-7, C-8a and C-9. At δ 1.58 was obviously that of H-8 which coupled with H-7 and correlated with C-10. The H-1 correlated with C-2a, C-5, C-5a, C-5b, C-8a, C-10 and C-3. Furthermore, H-4 correlated with C-2a.





On the basis of these data, this compound was assigned as 5-Acenaphthylene-carboxylic acid, 2-(3′-furanyl)-1,3,4,5,6,7,8,8a-octahydro-5,8,8a-trimethyl-3-oxo, methyl ester, chettaphanin II.[5,33]



Chettaphanin II

Table 15 The ^{1}H-NMR, ^{13}C-NMR and multiple bond correlation of Compound **4**

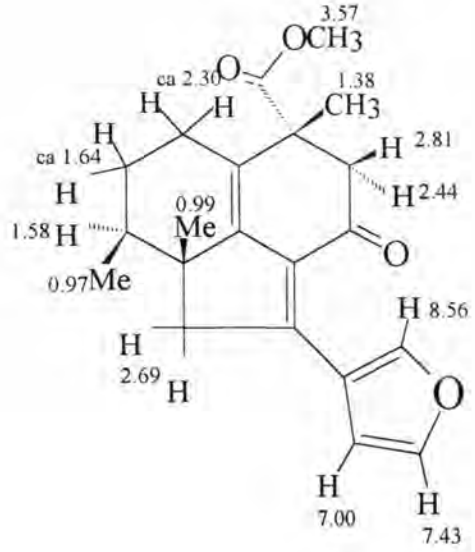
Position	Attached proton	^{13}C	multiple bond correlations attached carbond
C-1	2.69(dd) J=32.95, 16.79 Hz	50.3	C-2a, C-5, C-5a, C-5b
	-		C-8a, C-10
C-2	-	139.7	-
C-2a	-	150.4	-
C-3	-	195.1	-
C-4	2.44(d) J=15.81 Hz		C-2a, C-3, C-5, C-5a,
	2.81(d) J=15.81 Hz	52.1	C-11, C-12
C-5	-	48.5	-
C-5a	-	125.1	-
C-5b	-	128.0	-
C-6	ca 2.30 (m)	23.8	C-2a, C-7, C-9
C-7	ca 1.64 (m)	27.1	C-5a
C-8	1.58 (m)	37.1	C-5b
C-8a	-	42.5	-
C-9	0.99 (s)	16.4	C-1, C-2a, C-8, C-8a
C-10	0.97 (d) J=6.41 Hz	20.3	C-7, C-8, C-8a
C-11	1.38 (s)	22.3	C-4, C-5, C-5a, C-12
C-12	-	174.6	-
C-1′	7.43 (t) J=1.50-2.00 Hz	142.7	C-2′, C-4′
C-2′	7.00 (dd) J=0.61, 2.41 Hz	111.0	C-2, C-1′, C-3′, C-4′
C-3′	-	121.9	-
C-4′	8.56 (s)	146.3	C-2a, C-1′, C-2′, C-3′
OMe	3.57 (s)	52.2	C-12

From these data and a comparison with the reported data of closely related compounds, the structure of compound **4** was identified as 5-Acenaphthylene carboxylic acid, 2-(3′-furanyl)-1,3,4,5,6,7,8,8a-octahydro-5α,8α,8aα-trimethyl-3-oxo, methyl ester, chettaphaninII (Fig. 2). On literature review in 1971, chettaphanin II was obtained from *A. siamensis*.[5] It presented only IR and ^{1}H-NMR data but did not show any ^{1}H-NMR spectrum. The comparison of ^{1}H-NMR data of chettaphanin II with compound **4** is shown in Table 16.

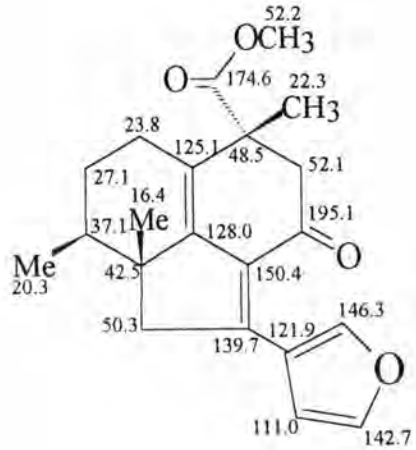
Table 16 The comparison of ^{1}H-NMR of Chettaphanin II with Compound **4**

Position	Chettaphanin II[5]	Compound **4**
methylene proton adjacent	2.44 [1H, J=16 Hz]	2.44 [1H, d, J=15.87 Hz]
to a carbonyl group	2.86 [1H, J=16 Hz]	2.81 [1H, d, J=15.87 Hz]
secondary methyl group	0.98 [3H, J=5.1 Hz]	0.97 [3H, d, J=6.41 Hz]
tertiary methyl group	1.00 [3H, s]	0.99 [3H, s]
	1.39 [3H, s]	1.38 [3H, s]
β-monosubstituted of	7.00 [1H, q]	7.00 [1H, dd, J=0.61, 2.14 Hz]
furan ring	7.44 [1H, q]	7.43 [1H, t, J=1.50-2.00 Hz]
	8.68 [1H, q]	8.56 [1H, s]

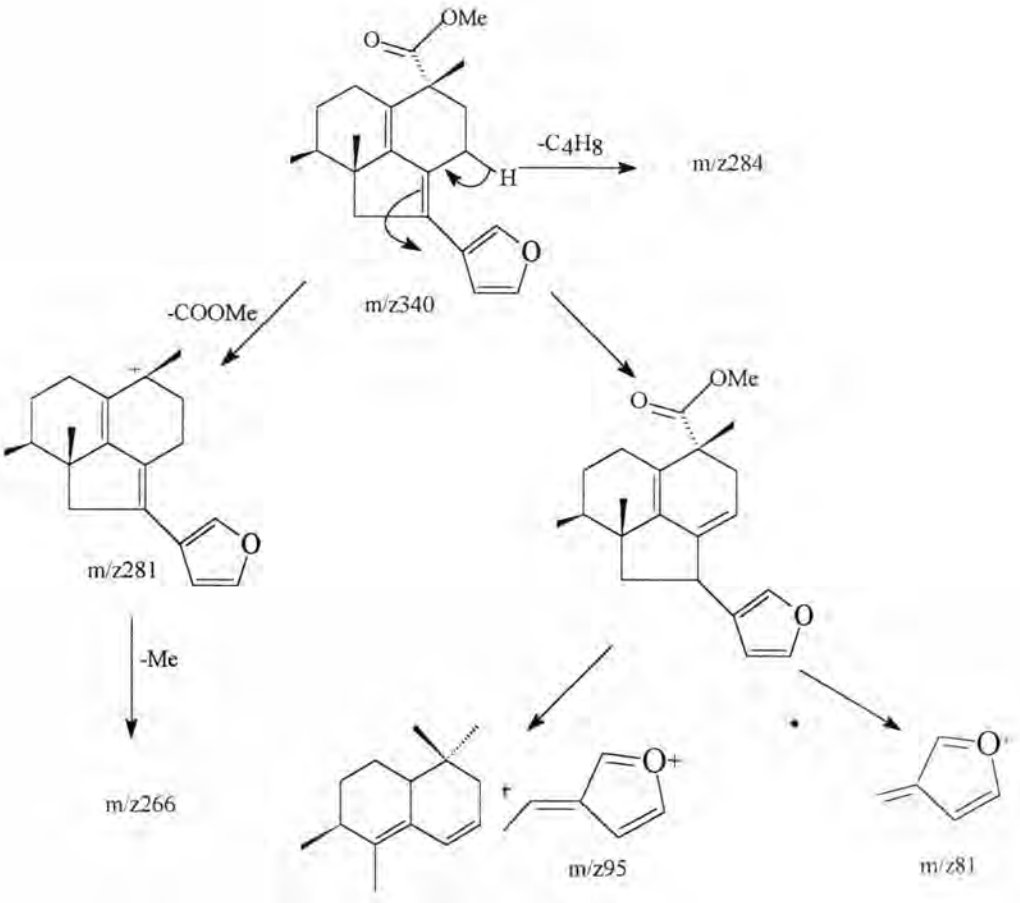
Compound **4** was assigned as chettaphanin II. This compound showed cytotoxicity to brine shrimp at LD_{50} =19.95μg/ml. This is the first report giving a complete structural assignment for this compound.



The assignment chemical shift for protons of Compound 4



The assignment chemical shift for carbons of Compound 4



Scheme 3 The possible mass fragmentation pattern of Compound **4**

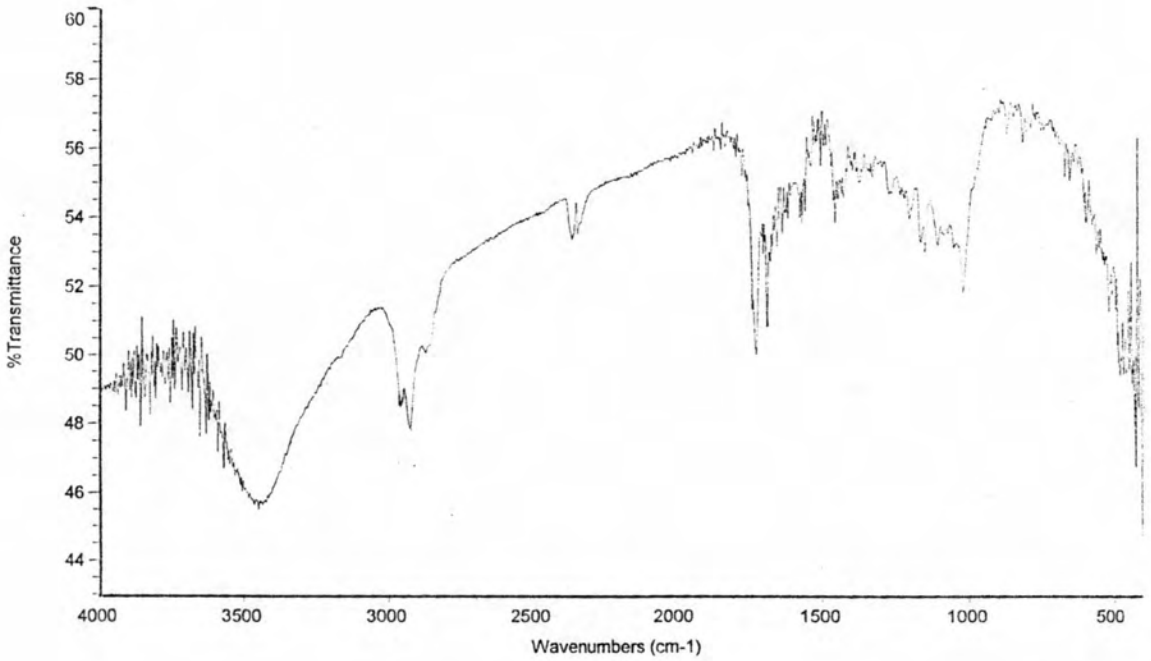


Fig. 9 The IR spectrum of Compound **4**

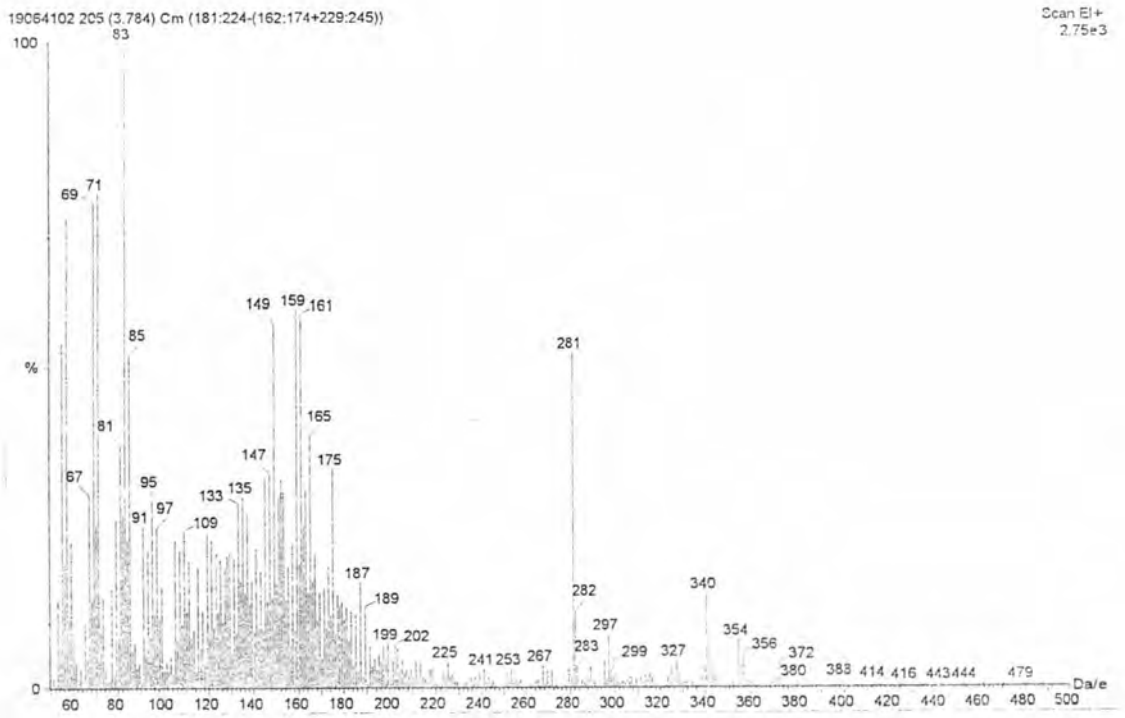


Fig. 10 The mass spectrum of Compound **4**

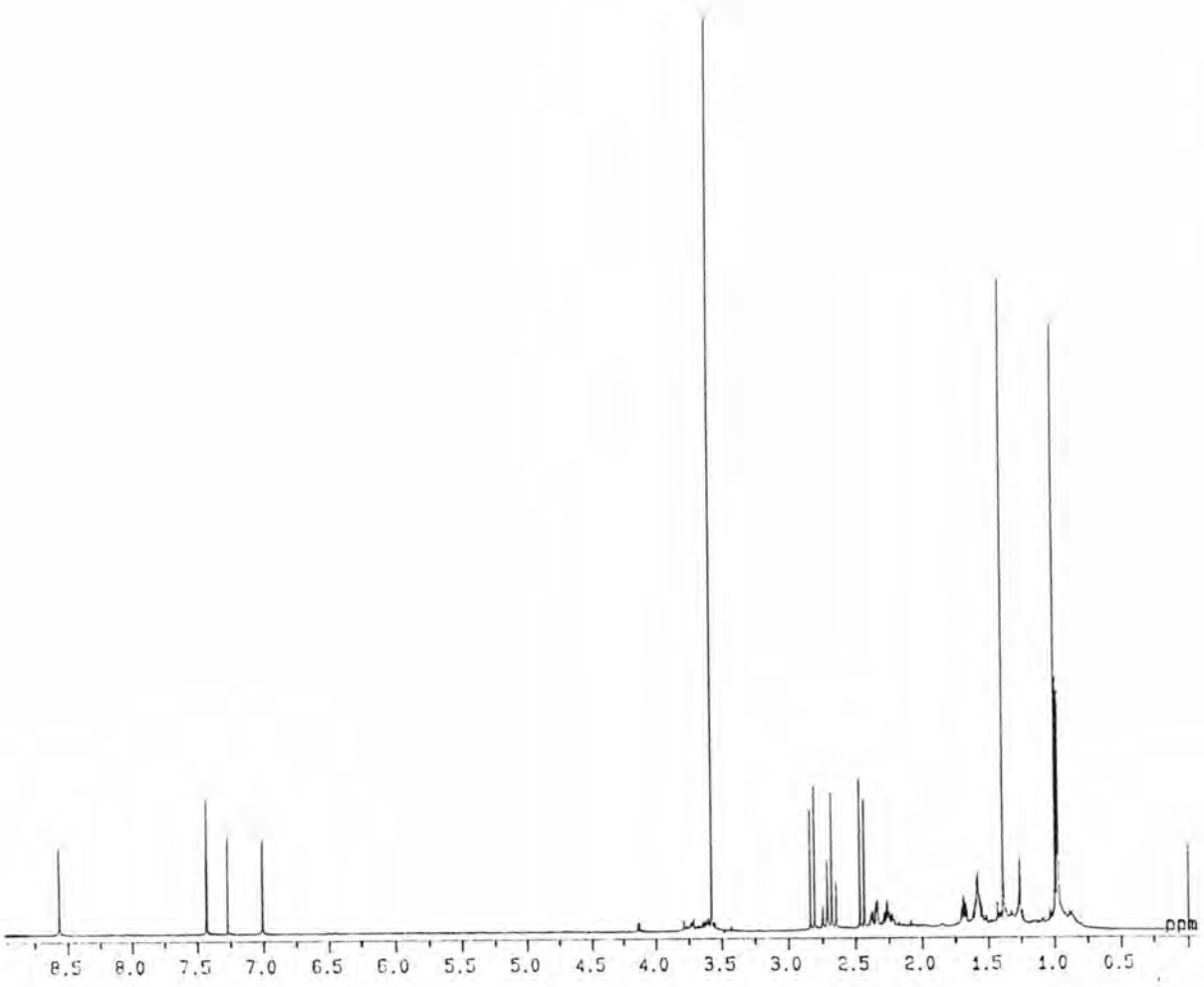


Fig. 11 The ^{1}H-NMR spectrum of Compound **4**

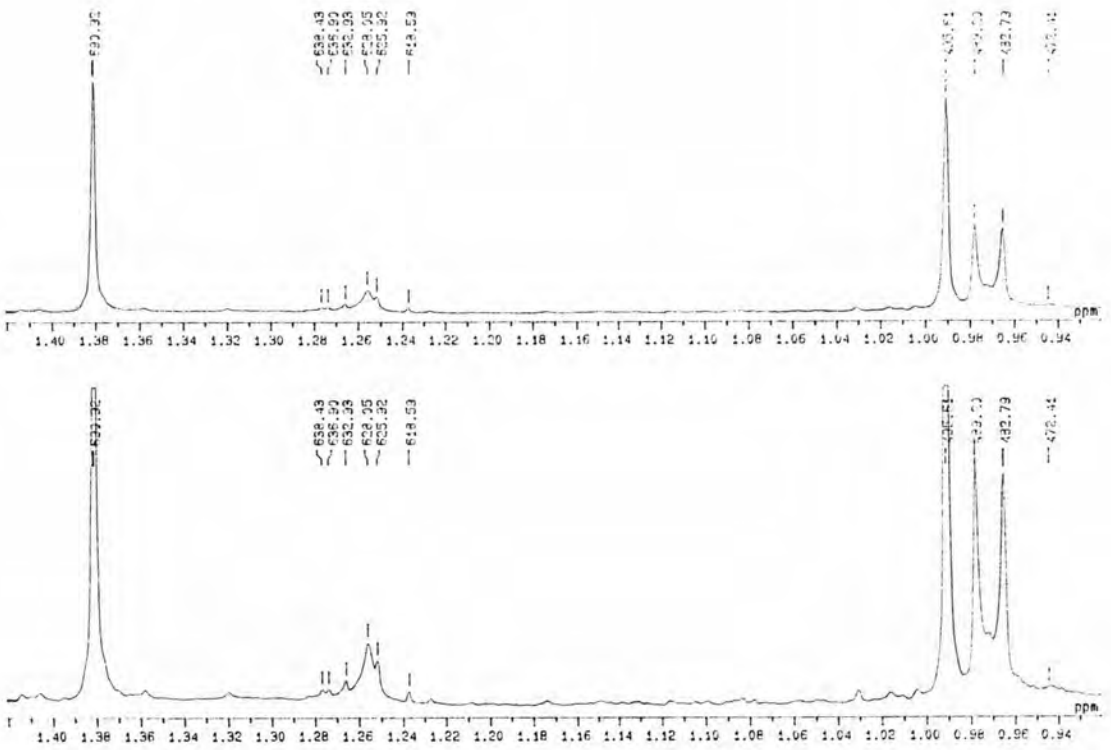


Fig. 12 The expansion of ^{1}H-NMR spectrum of Compound **4**

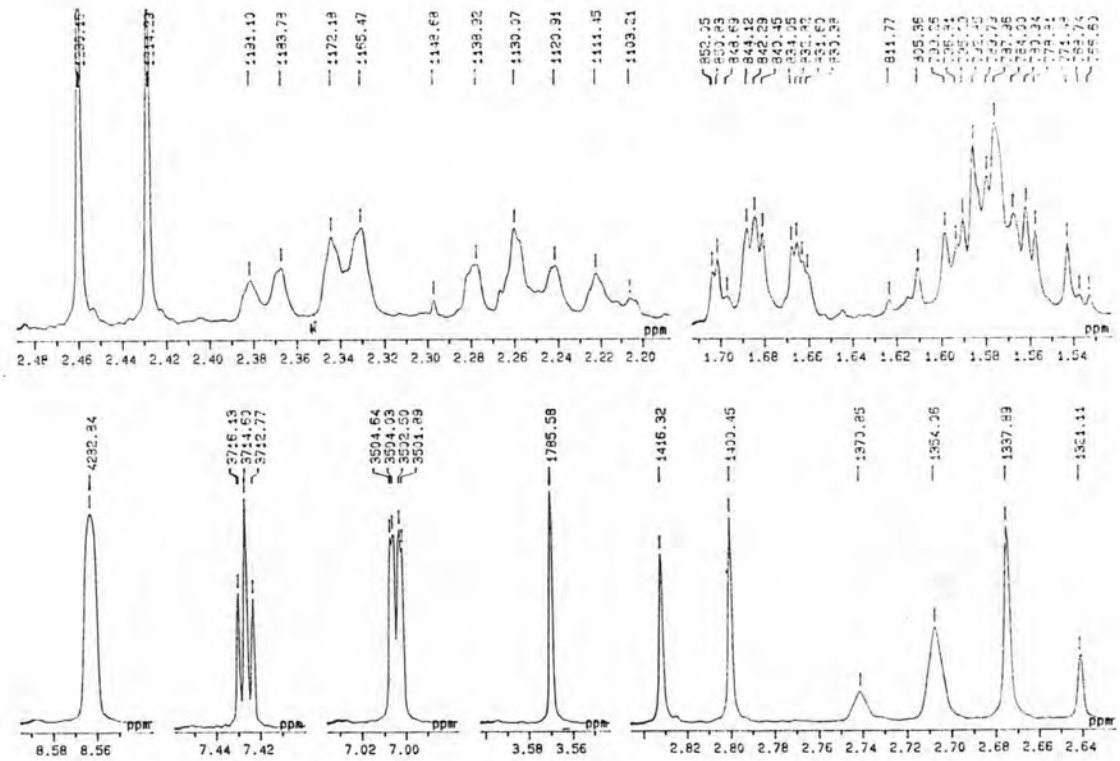


Fig. 13 The expansion of ^{1}H-NMR spectrum of Compound **4**

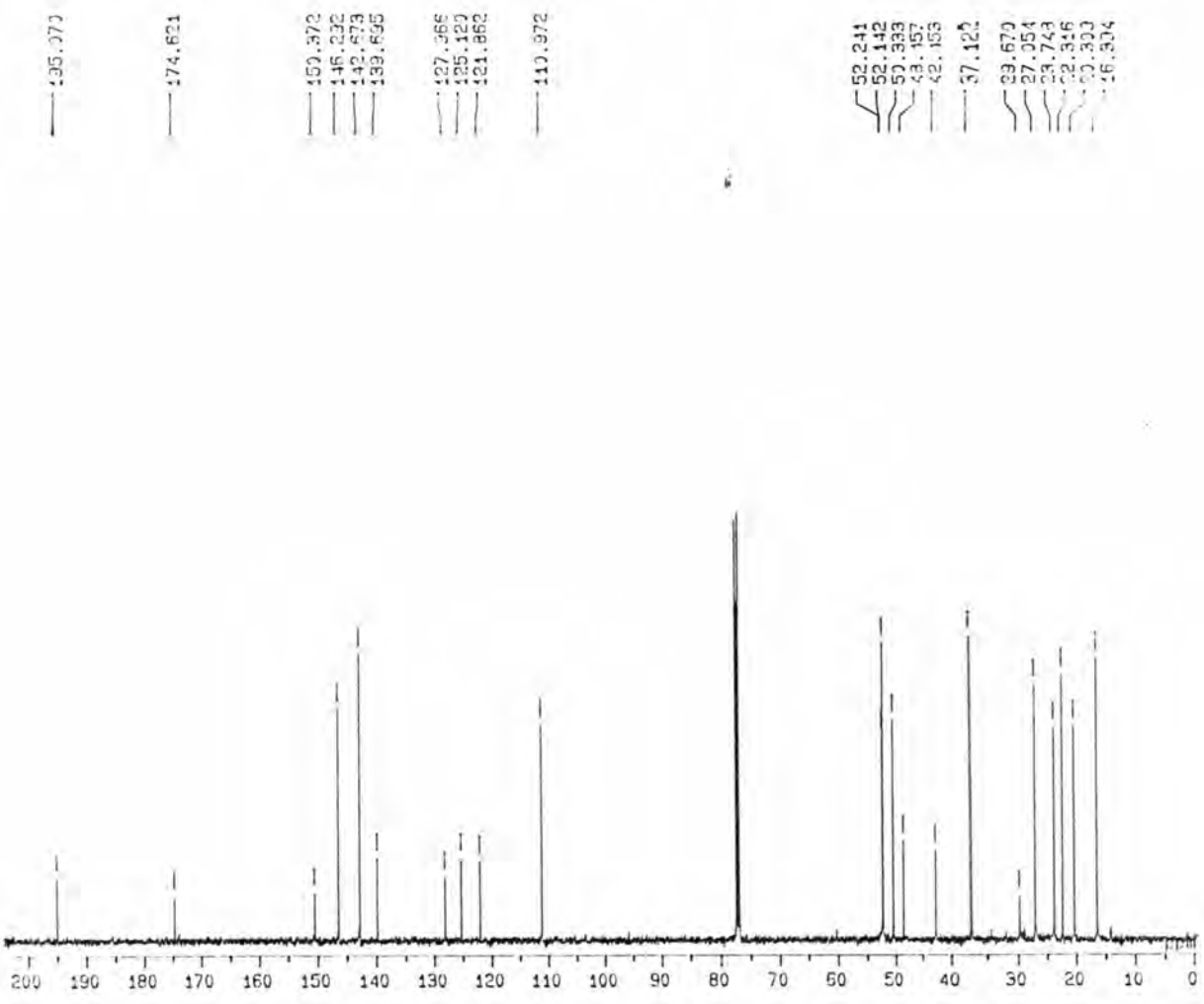


Fig. 14 The ^{13}C-NMR spectrum of Compound **4**

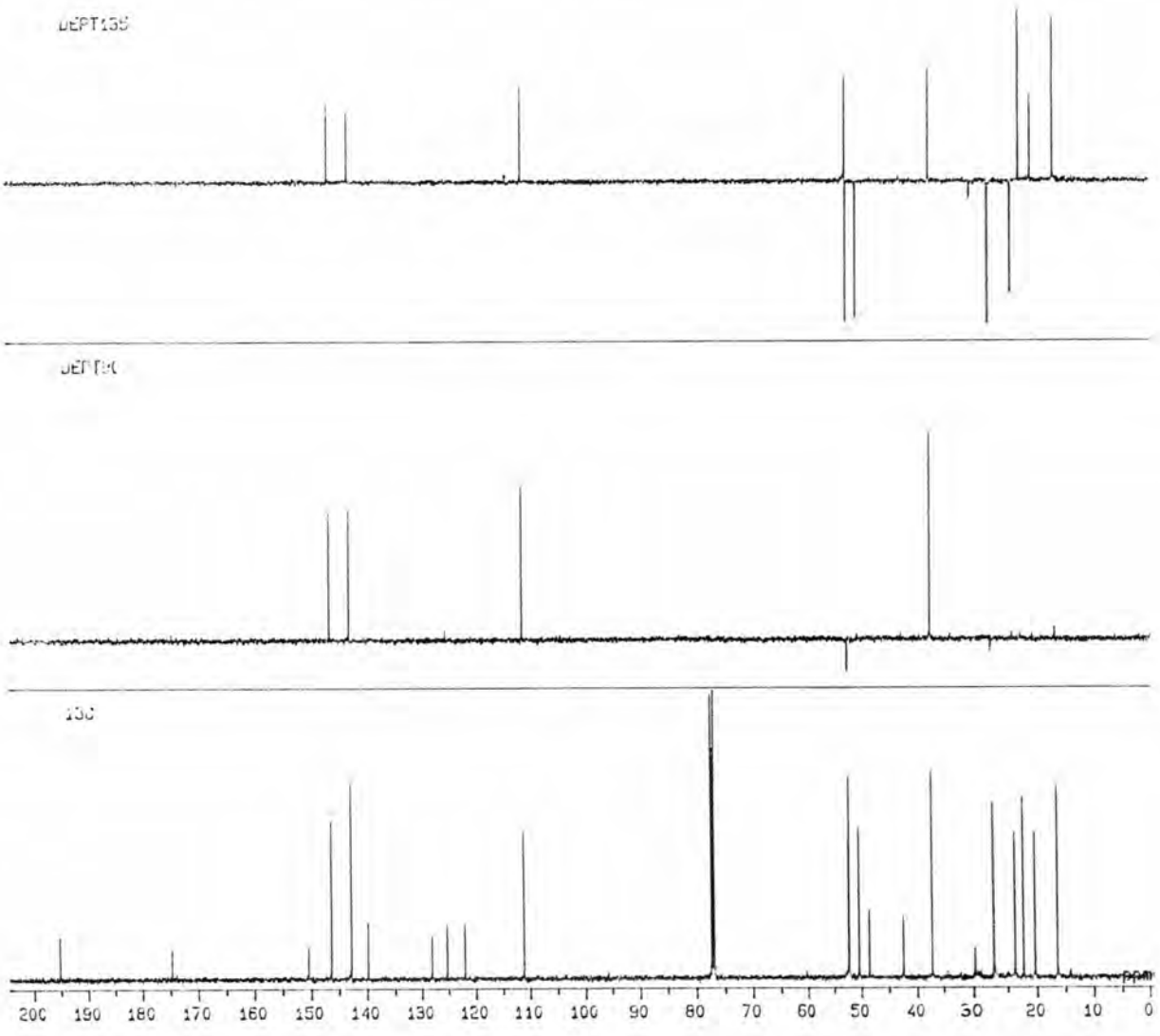


Fig. 15 The DEPT 90, 135, ^{13}C-NMR spectrum of Compound **4**

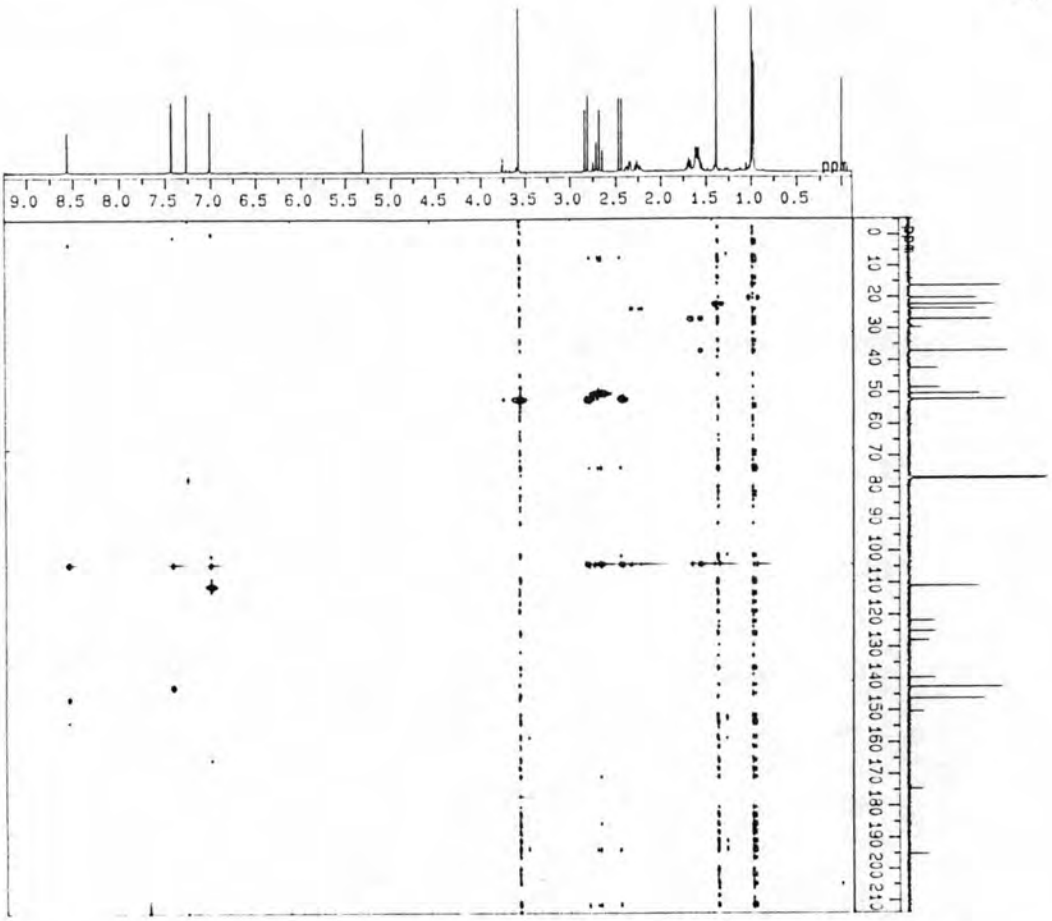


Fig. 16 The HMQC spectrum of Compound **4**

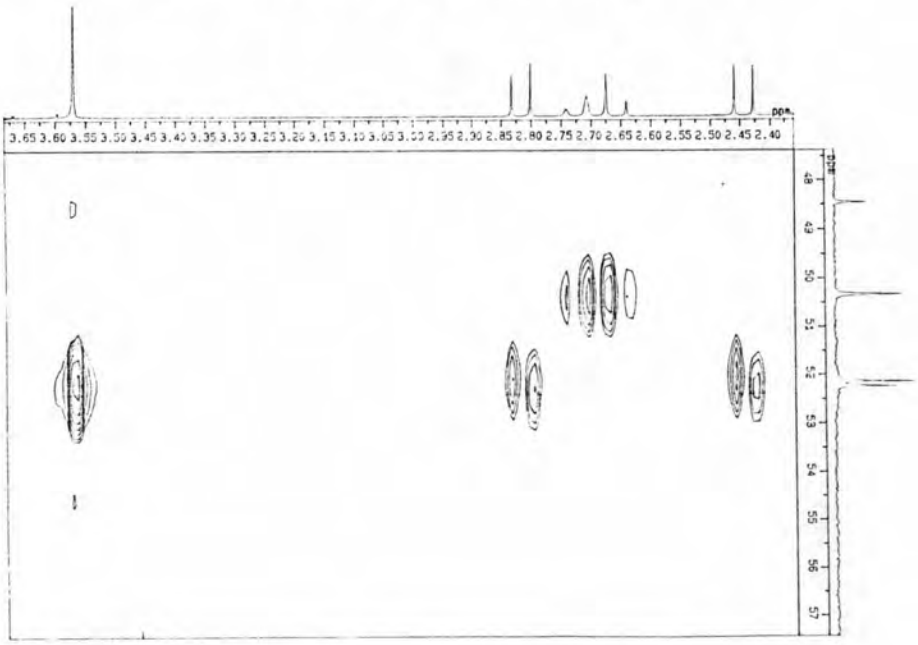


Fig. 17 The expansion of HMQC spectrum of Compound **4**

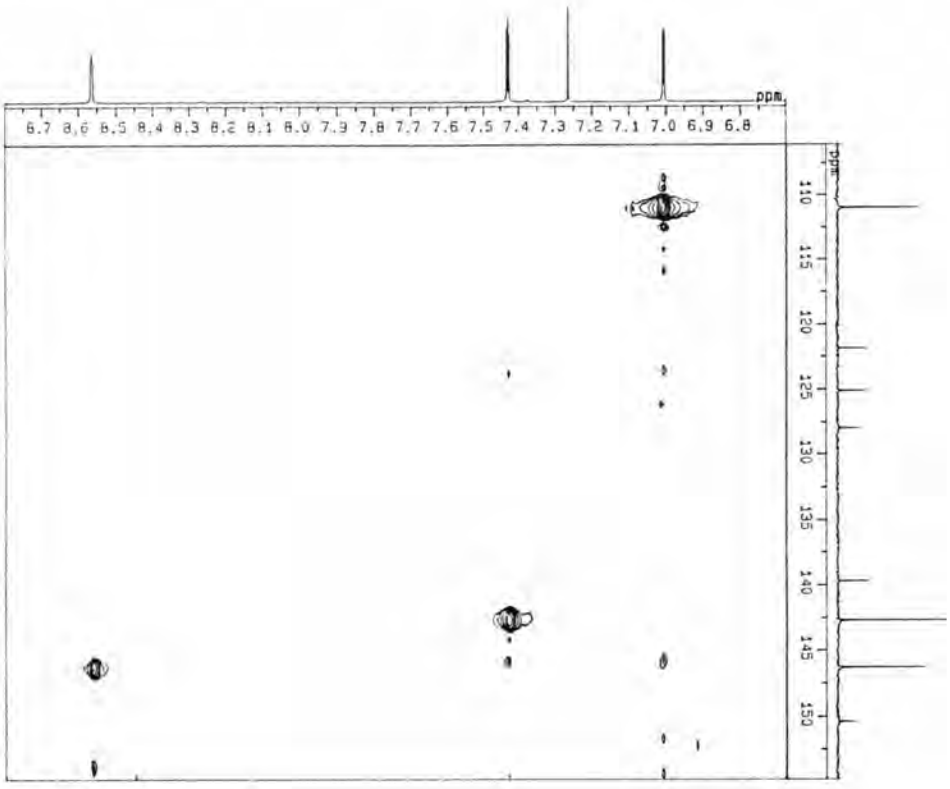


Fig. 18 The expansion of HMQC spectrum of Compound **4**

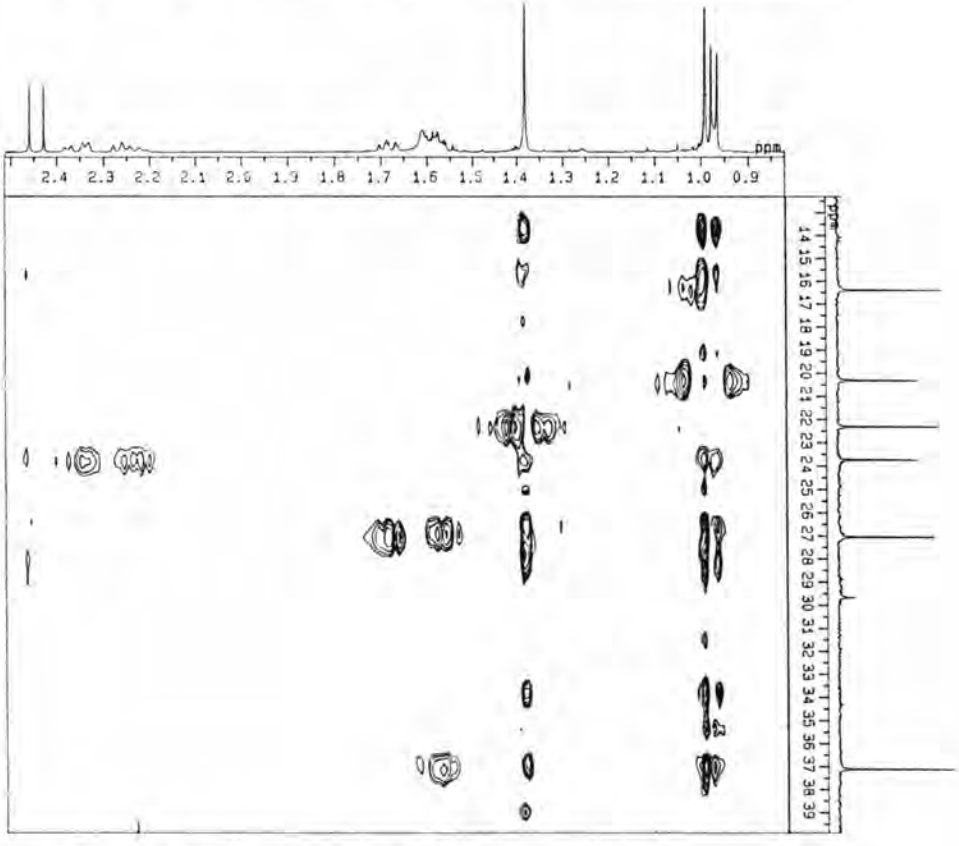


Fig. 19 The expansion of HMQC spectrum of Compound **4**

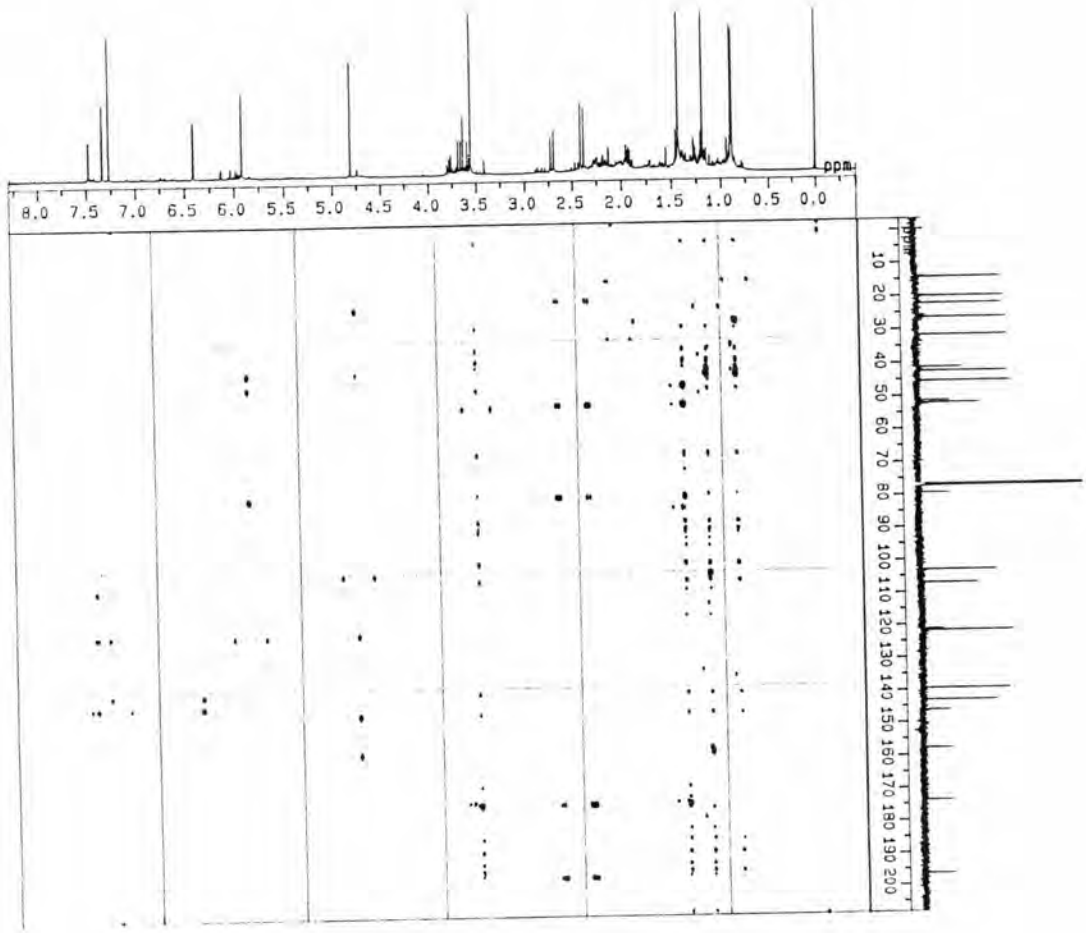


Fig. 20 The HMBC spectrum of Compound **4**

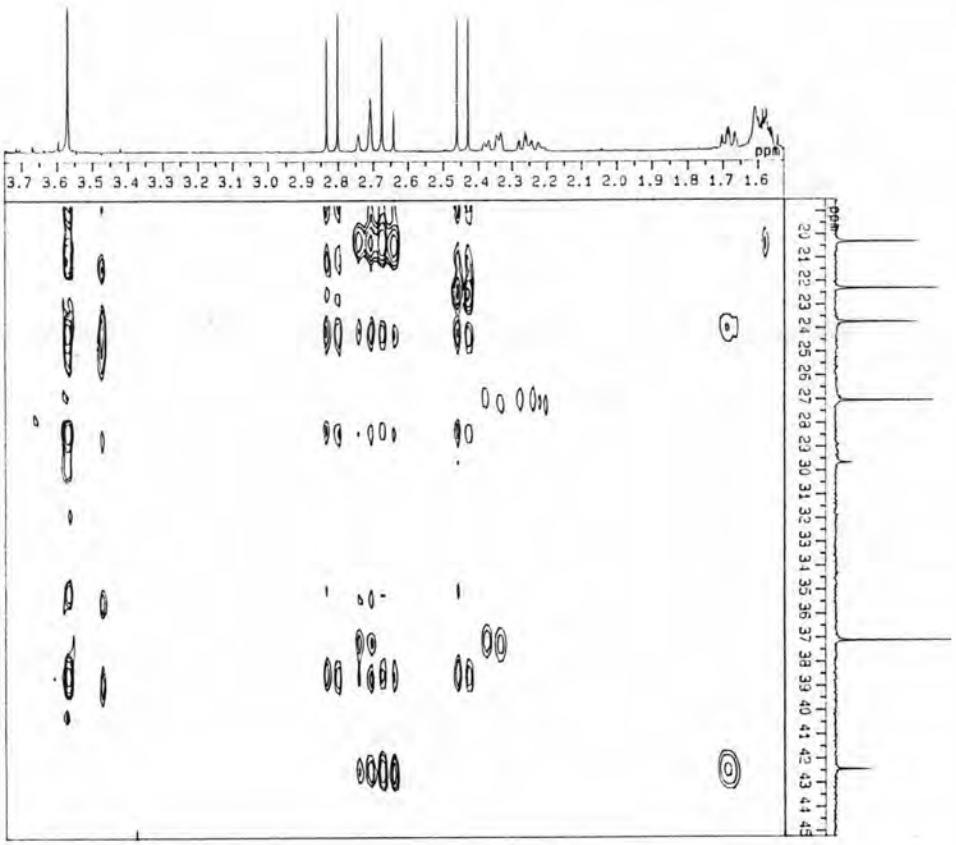


Fig. 21 The expansion of HMBC spectrum of Compound **4**

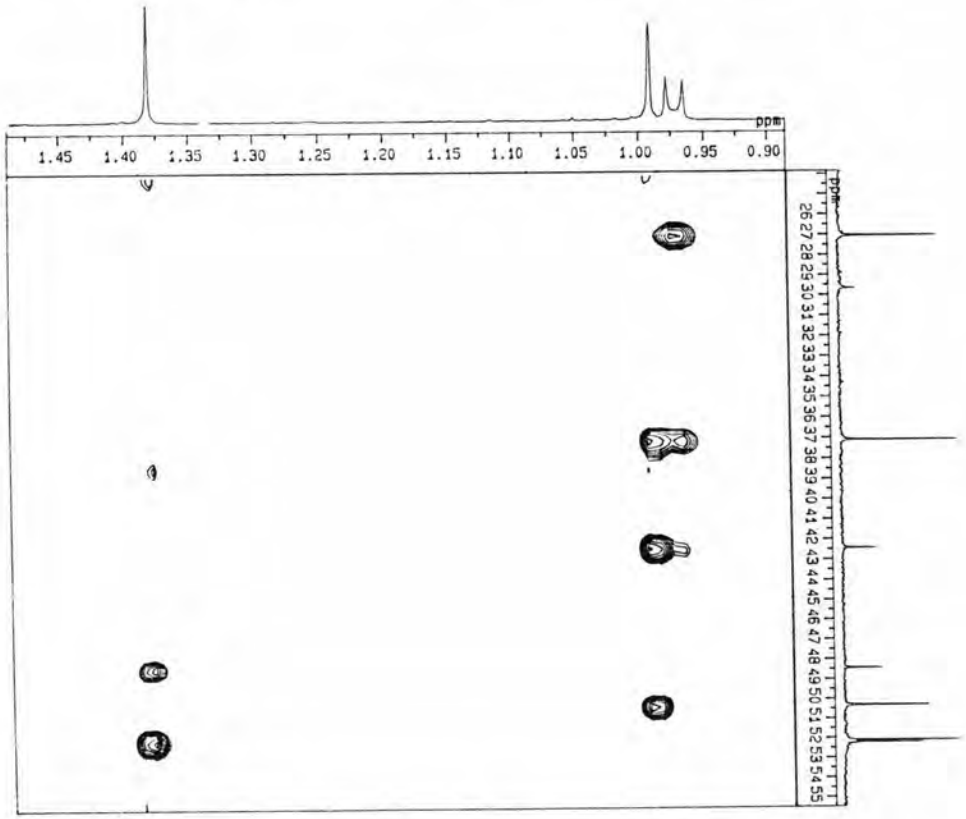


Fig. 22 The expansion of HMBC spectrum of Compound **4**

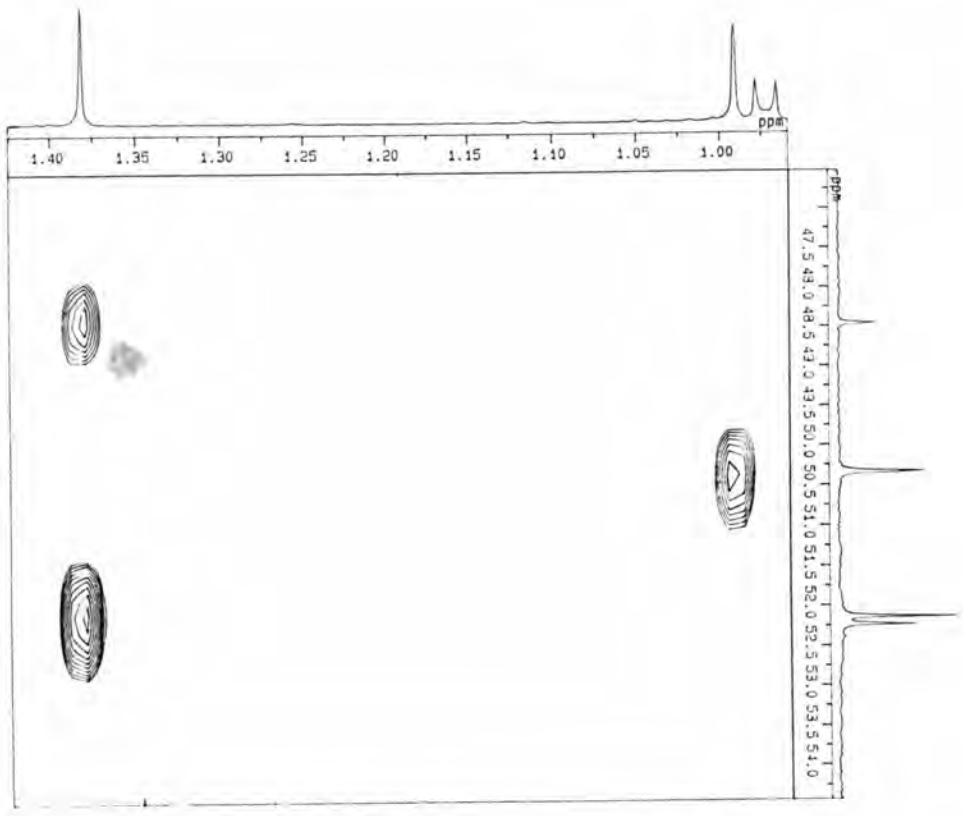


Fig. 23 The expansion of HMBC spectrum of Compound **4**

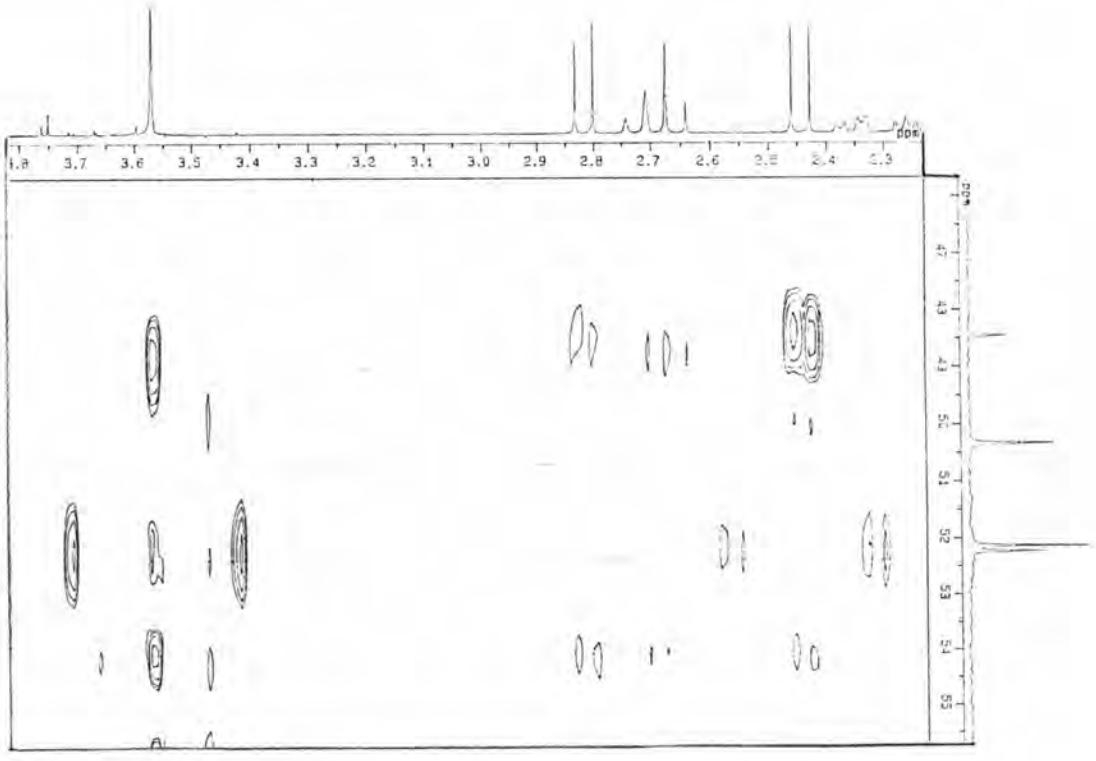


Fig. 24 The expansion of HMBC spectrum of Compound **4**

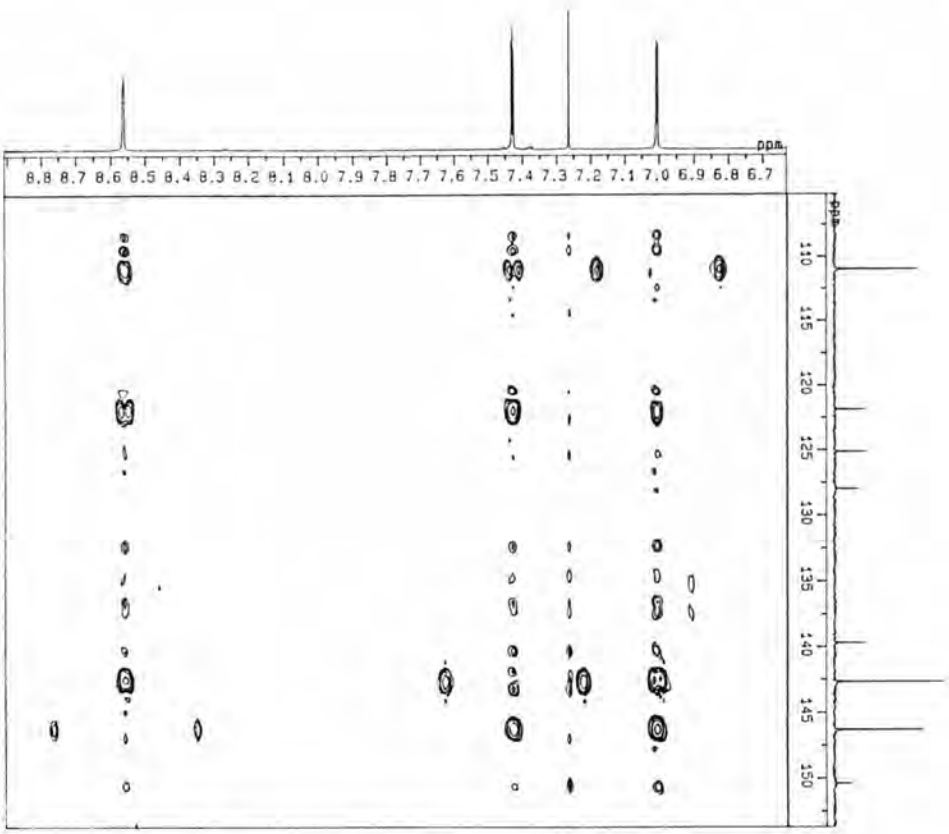


Fig. 25 The expansion of HMBC spectrum of Compound **4**

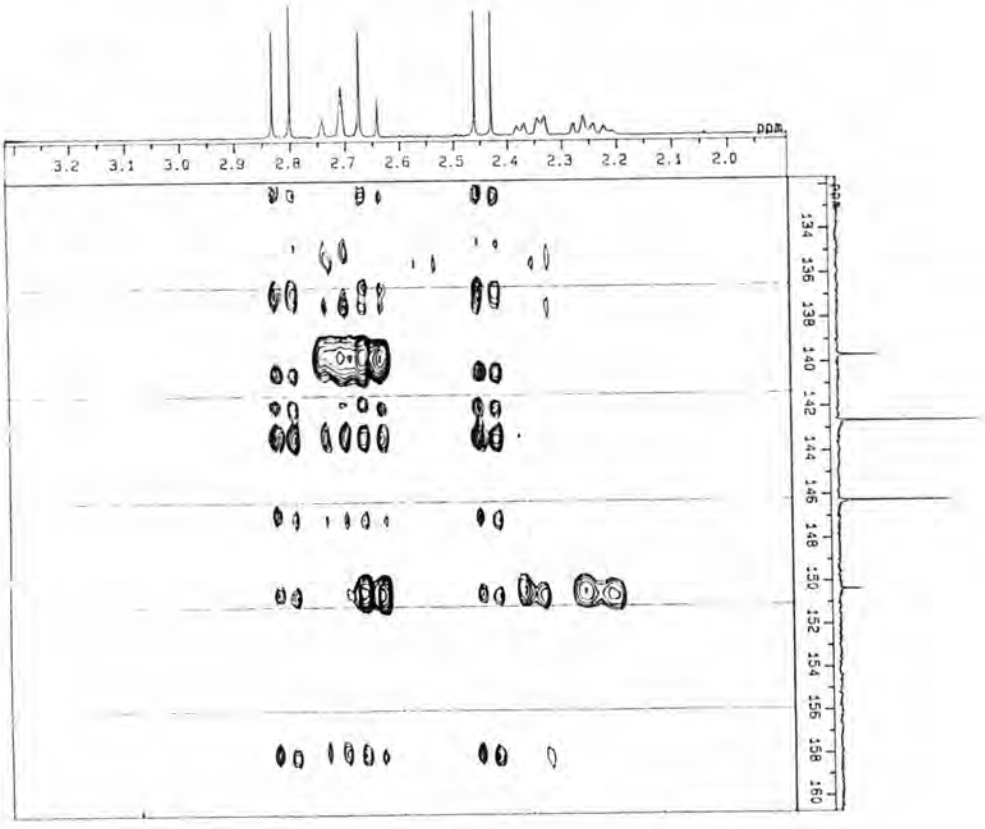


Fig. 26 The expansion of HMBC spectrum of Compound **4**

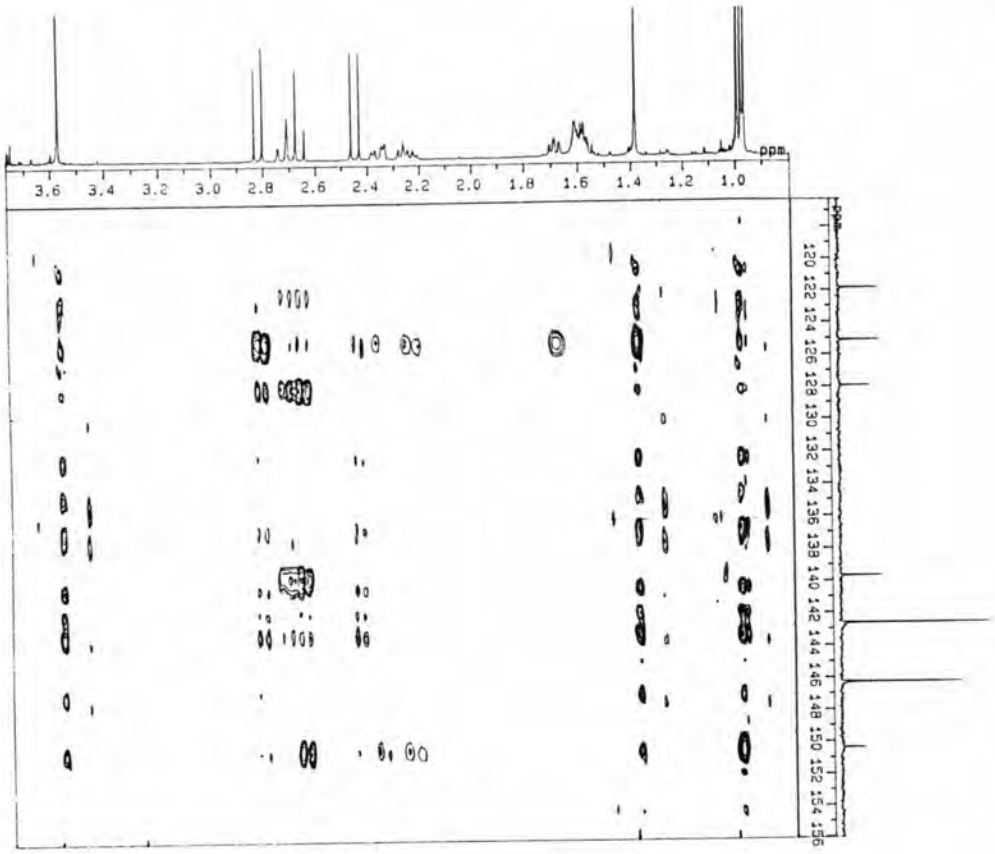


Fig. 27 The expansion of HMBC spectrum of Compound **4**

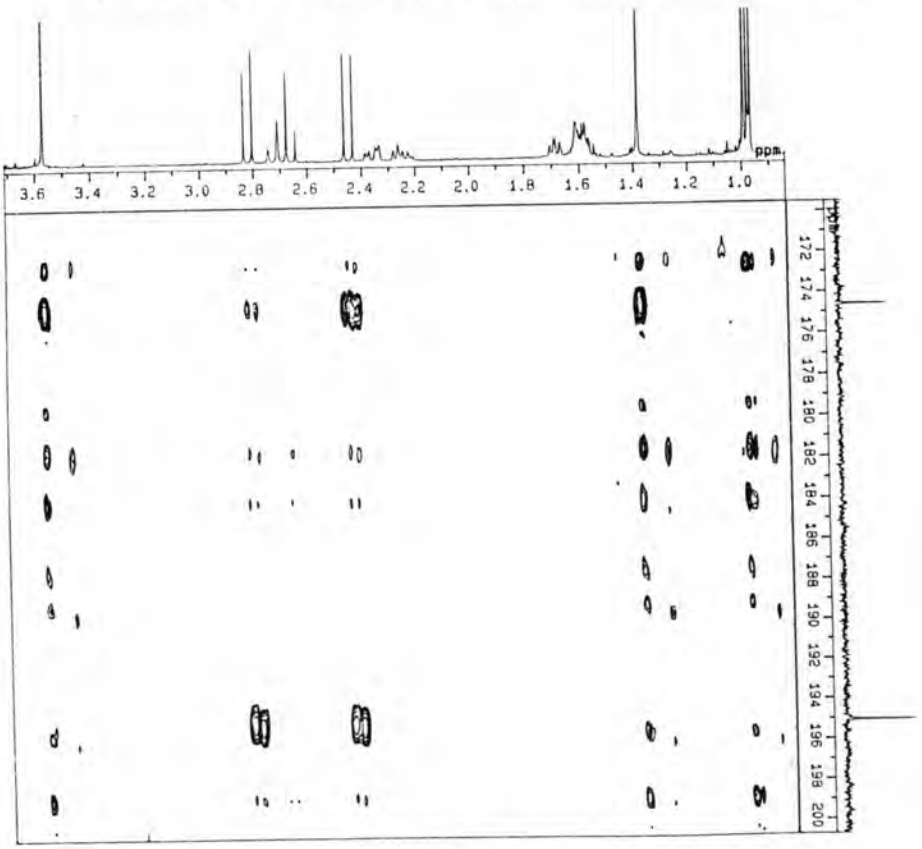


Fig. 28 The expansion of HMBC spectrum of Compound **4**

Structure elucidation of Compound 5

The fraction no. 94-105 was reseparated with hexane and increasing polarity with EtOEt (0-30%) by chromatotron. Accordingly fraction no. e was subjected by using preparative TLC and 70% ether in hexane was used as developing solvent to give 2 fractions, fraction e1 and e2 respectively. Fraction e1, 40.2 mg ($0.40x10^{-3}$ wt by wt of the roots), although showing a single spot on TLC, was compound **5**, as yellow oil. The R_f value was 0.36 in dichloromethane. It gave positive test with 2,4-DNP and Br_2 in CCl_4 reagents which indicated that it had a carbonyl functional groups of ketone and unsaturated part in its structure.

The IR confirmed the presence of α,β-unsaturated of ketone at 1680 cm^{-1} and C=O stretching of carbonyl ester at 1735 cm^{-1}. A furan ring would accommodate these requirements. Furan bands appeared at 1457, 1050 and 870 cm^{-1} (sharp and characteristic) and 720 cm^{-1} (Fig. 29). Mass spectrometry indicated M^+=356. The mass spectrum showed mass fragments at 325, 297, 281, 228, 227, 95 and 83(Fig. 30).

The ^{1}H-NMR spectrum (Fig. 31-33) showed β-substituted furan ring[32] at δ 7.48 [1H, t, J=1.22 Hz], δ 7.34 [1H, t, J=1.83 Hz] and δ 6.41 [1H, dd, J=0.91,1.83 Hz], two vinylic protons at δ 5.91 [1H, s] and δ 4.80 [1H, s], one methoxy proton at δ 3.55 [3H, s], three methylene protons at δ 2.38 [1H, d, J=16.48 Hz], 2.70 [1H, dd, J=0.61,16.48 Hz], δ 2.25 [1H, dd, J=3.70,12.96 Hz] and δ 1.92 [1H, dd, J=5.17, 13.44 Hz], one methine proton at δ 2.16 [1H, m] and δ 1.40 [1H, m] and three methyl protons at δ 1.42 [3H, s], δ 1.18 [3H, s] and δ 0.89 [3H, s].

The ^{13}C-NMR spectra (Fig. 34)and DEPT experiments (Fig. 35), also showed signals attributable to three methyl carbons (14.3, 20.1 and 22.3), three methylene carbons (26.5, 31.8 and 45.5), five methine carbons (42.6,103.2, 107.2, 121.8, 139.5 and 143.2), eight quaternary carbons (41.4, 51.4, 79.5, 120.8, 146.2, 157.8, 173.9 and 196.5) and one methoxy carbon (52.2).

From these data indicated that compound **5** had 21 carbons and 16 protons, the presence of one methoxy, two vinylic protons, one furan ring and two carbonyl groups suggested this compound had at least 5 oxygen atoms in the molecule.

The molecular ion at m/z 356 indicated that molecular formular was $C_{21}H_{24}O_5$, corresponding to a degree of unsaturation of ten. Two dimensional NMR techniques were used to provide further information, One Bond Correlation (CHSHF) data revealed the protons were attached to the carbons (Fig. 36-38). The results are given in Table 17.

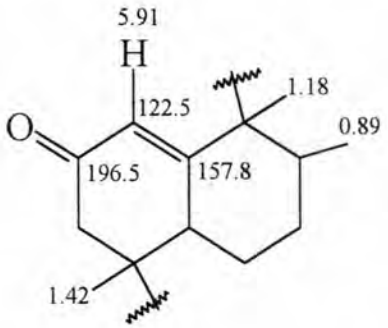
Table 17 The ^{1}H-and ^{13}C-NMR spectral data of Compound **5** (500/125 MHz, $CDCl_3$)

Position	ppm	Attached protons
C-18	14.3	0.89 (d) J=7.02 Hz
C-19	20.1	1.42 (s)
C-17	22.3	1.18 (s)
C-7	26.5	1.40 (m)
		2.16 (m)
C-6	31.8	1.92 (dd) J=5.17, 13.44 Hz
		2.25 (dd) J=3.70, 12.96 Hz
C-9	41.4	-
C-8	42.6	1.90 (m)
C-3	45.5	2.38 (d) J=16.48 Hz
		2.70 (d) J=16.48 Hz
C-4	51.4	-
C-21	52.1	3.55 (s)
C-5	79.5	-
C-11	103.2	4.80 (s)
C-14	107.2	6.41 (dd) J=0.91, 1.83 Hz
C-13	121.8	-
C-1	122.5	5.91 (s)
C-15	139.5	7.48 (t) J=1.22 Hz
C-16	143.2	7.34 (t) J=1.83 Hz
C-12	146.2	-

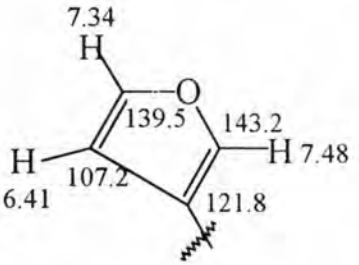
Table 17 (cont.)

Position	ppm	Attached protons
C-10	157.8	-
C-20	173.9	-
C-2	196.5	-

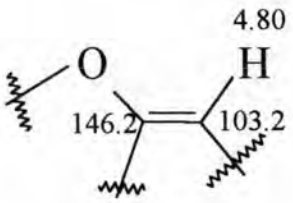
From ^{1}H-NMR spectral data, compound **5** was presumed to be a clerodane diterpene.[34] Thus, it showed the signals for a secondary and a tertiary methyl group at δ 0.89, 1.18 and 1.42. The ^{1}H- and ^{13}C-NMR spectra (Table 17) also showed presence of β-substituted furan moiety [2,5,35], which was confirmed by C-H COSY. In addition compound **5** possessed a α, β-unsaturated ketone grouping with α-olefinic protons (δ 196.5, 122.5 and 157.8)[14,15,36] and carbonyl ester function (δ 173.9). The location of enol form, C-O-C=C<u>H</u>- signal showed a singlet at δ 4.80 by comparision with literature data. [37]



Clerodane



β-substituted furan

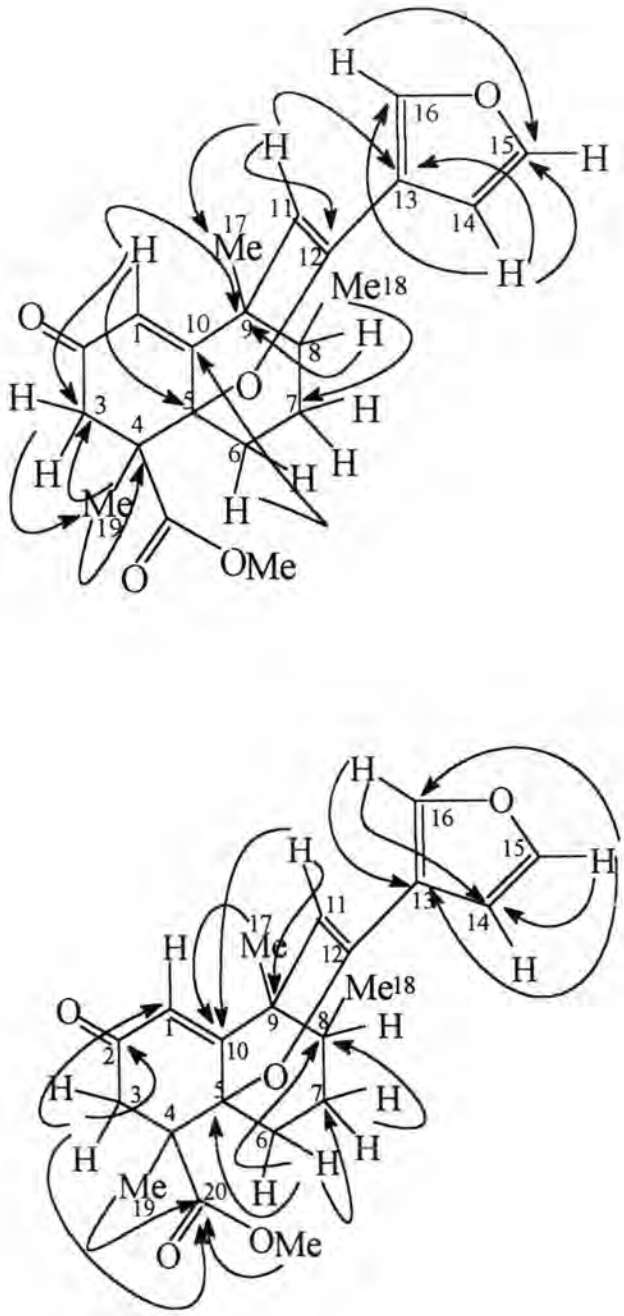


Enol form

In the H-C long range coupling spectrum was obtained by HMBC (Heteronuclear multiple bond correlation), furan proton at δ 6.41 showed cross peaks with the carbons at δ121.8 (C-13) and 139.5 (C-16) and 143.2 (C-15), the proton of enol showed cross peaks with the carbons at δ 22.3 (C-17), 41.4 (C-9), 121.8 (C-13), 146.2 (C-12) and 157.8 (C-10), the vinyl proton at δ 5.91 showed cross peaks with the carbons at δ 41.4 (C-9), 45.5 (C-3) and 79.5 (C-5). The methylene protons at δ 2.38 and 2.70 showed cross peaks with the carbons at δ 20.1 (C-19), 51.4 (C-4), 79.5 (C-5), 173.9 (C-20) and 196.5 (C-2)(Fig. 39-44). The couplings clearly observed that enol function connected with furan ring at β-substituted furan and the oxygen of enol form occupied at the C-5 of clerodane. By analysis of the ^{1}H-^{1}H COSY (Fig. 45-47) and HMBC spectra, the possible structure of compound are shown below.

Table 18 One bond and multiple bond correlation of Compound **5**

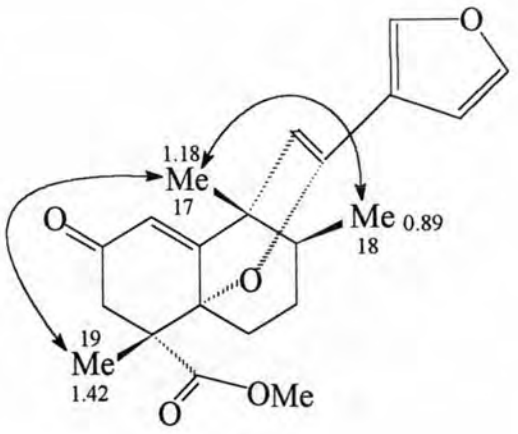
Proton (δ ppm)	One bond correlations attached carbon	Multiple bond correlations attached carbon
7.48	143.2	139.5,121.8,107.2
7.34	139.5	143.2,121.8,107.2
6.41	107.2	143.2,139.5,121.8
5.91	122.5	79.5,45.5,41.4
4.80	103.2	157.8,146.2,121.8,41.4,22.3
3.50	52.1	173.9
2.70,2.38	45.5	196.5,173.9,122.5,20.1
2.25,1.92	31.8	157.8,79.5,42.5,26.5
2.16,1.40	26.5	42.5
1.90	42.5	41.4
1.42	20.1	173.9,51.4,45.5
1.18	22.3	157.8
0.89	14.3	26.5



Most significant correlations observed in HMBC of Compound 5

A degree of unsaturation of this compound was ten, which corresponding to molecular formular ($C_{21}H_{24}O_5$).

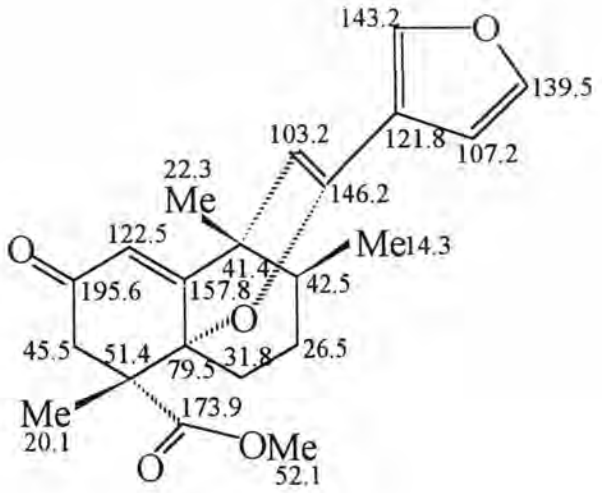
The relative configuration was supported by NOESY experiments(Fig. 48-50).



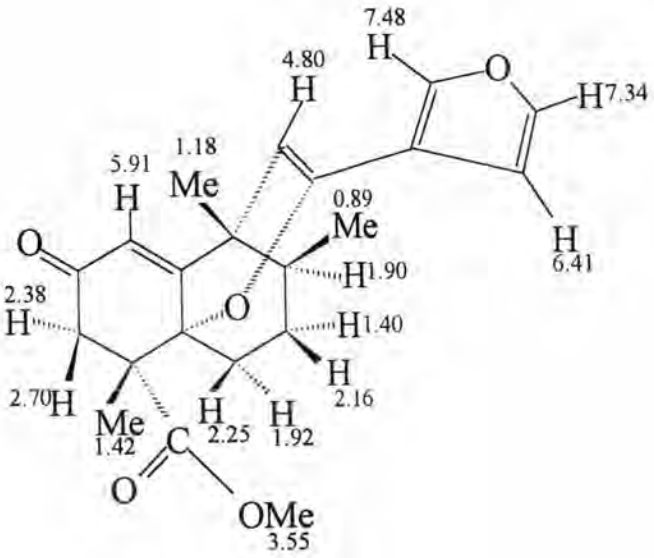
Selected NOESY correlation for Compound 5

The spectrum data is consistent with the identification as 4α, 8α ,9α, trimethyl 12(13-furanyl)-5,12-epoxy-2-oxo-cleroda-1(10),11(12),-diene-4-methyl ester. To our knowlenge, this compound has not been previously reported. We named this new compound as chettaphanin III.

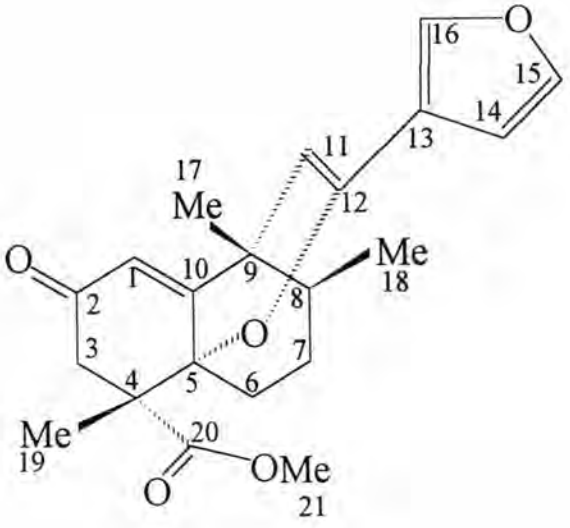
Compound **5** showed medium activity to brine shrimp cytotoxicity test at LD_{50}= 79.54 μg/ml.



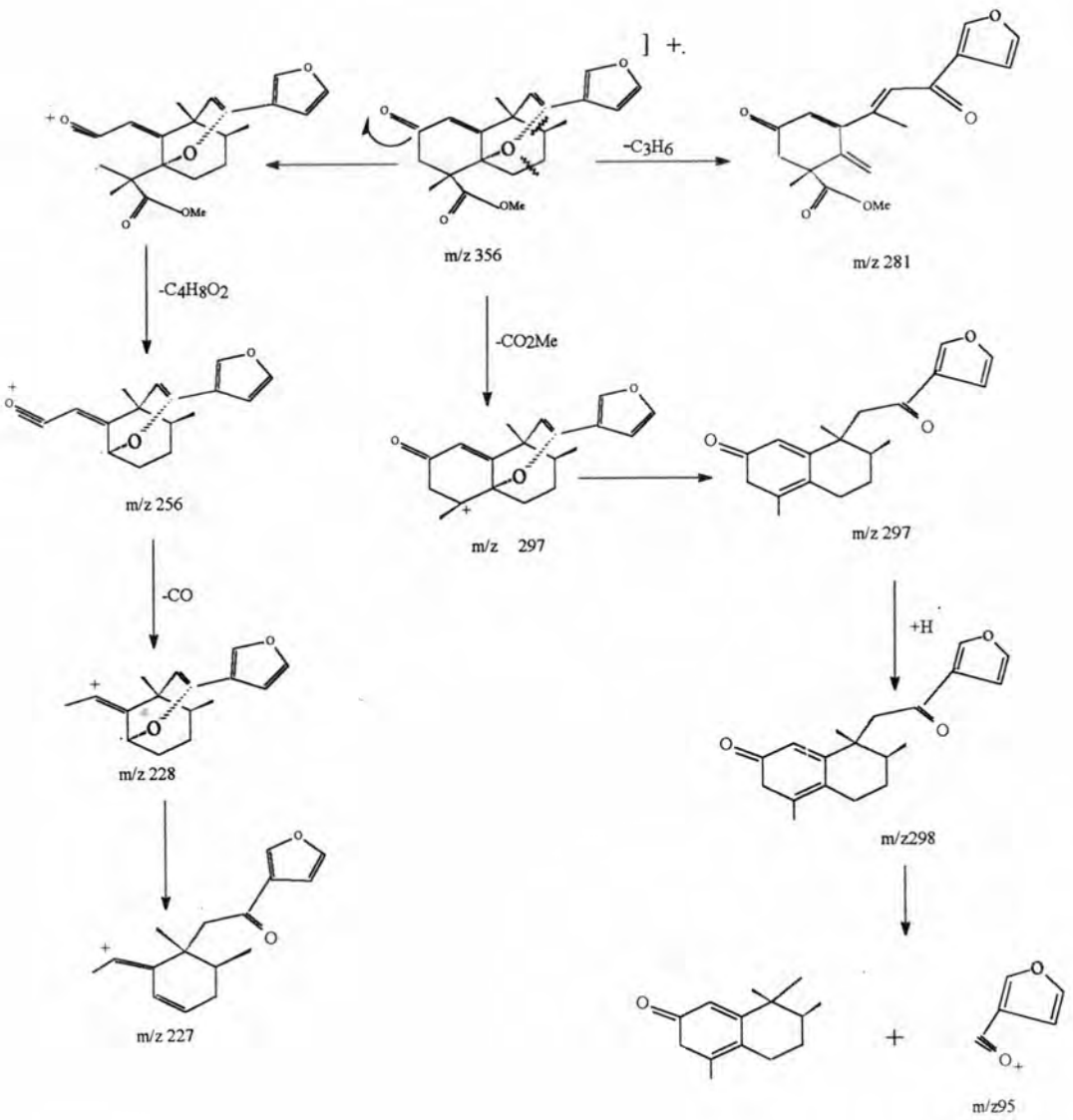
The assignment chemical shift for carbons of compound 5



The assignments chemical shift for protons of compound 5



Chettaphanin III (new furanoditerpene)



Scheme 4 The possible mass fragmentation pattern of Compound **5**

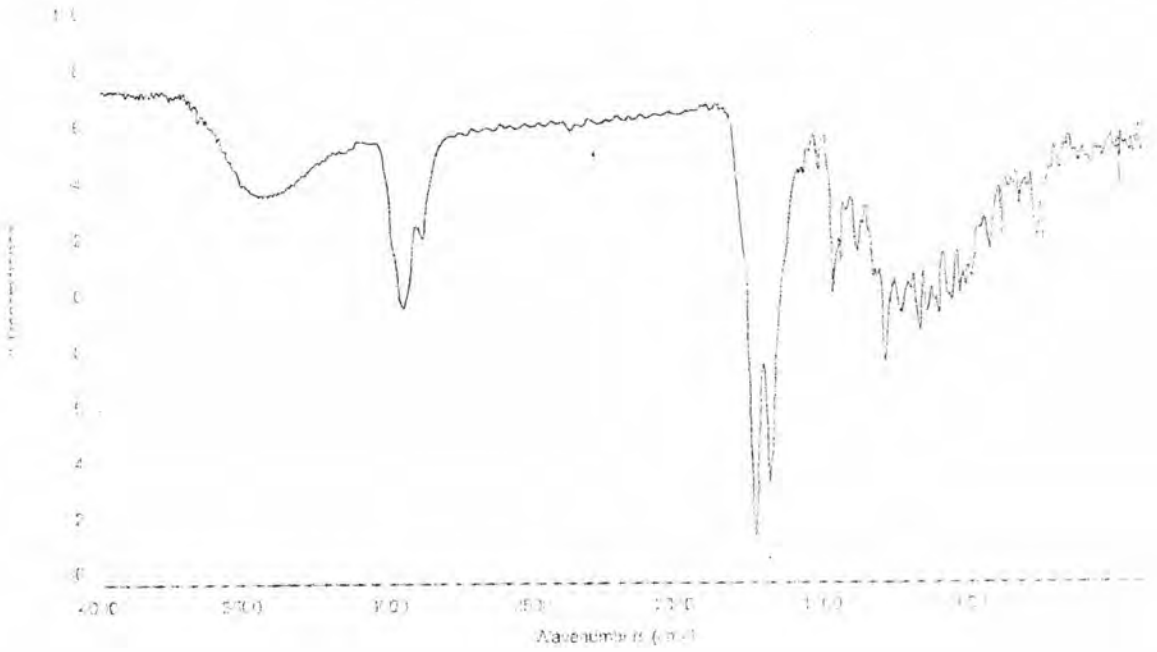


Fig. 29 The IR spectrum of Compound **5**

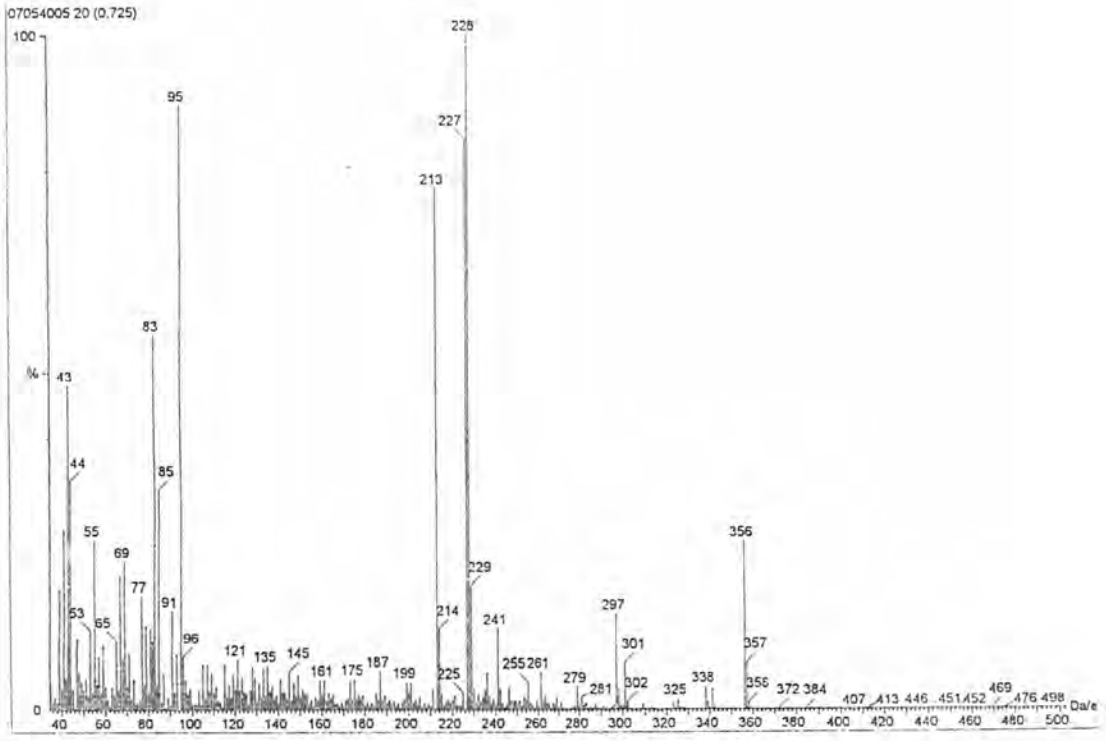


Fig. 30 The mass spectrum of Compound **5**

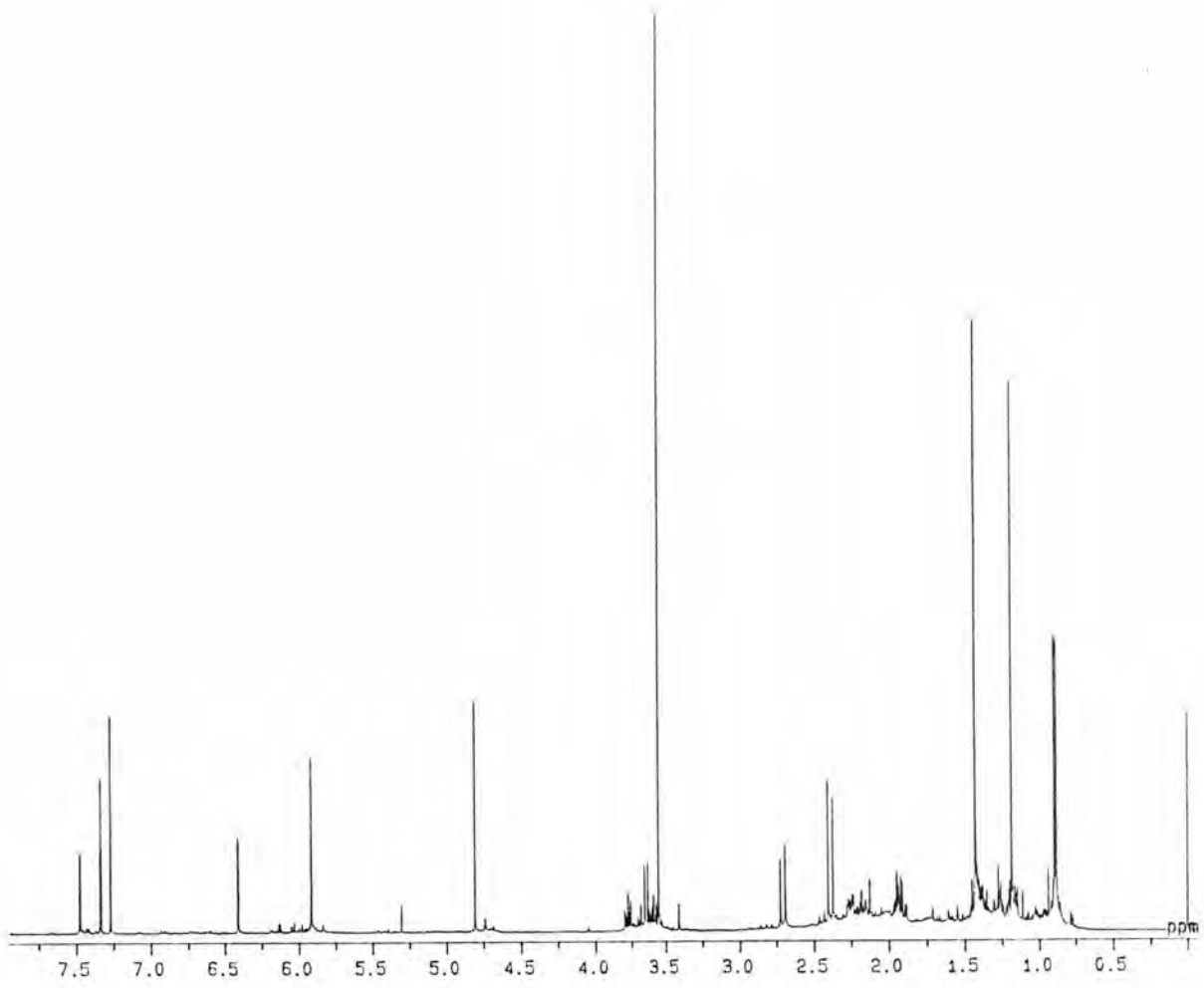


Fig. 31 The ^{1}H-NMR spectrum of Compound **5**

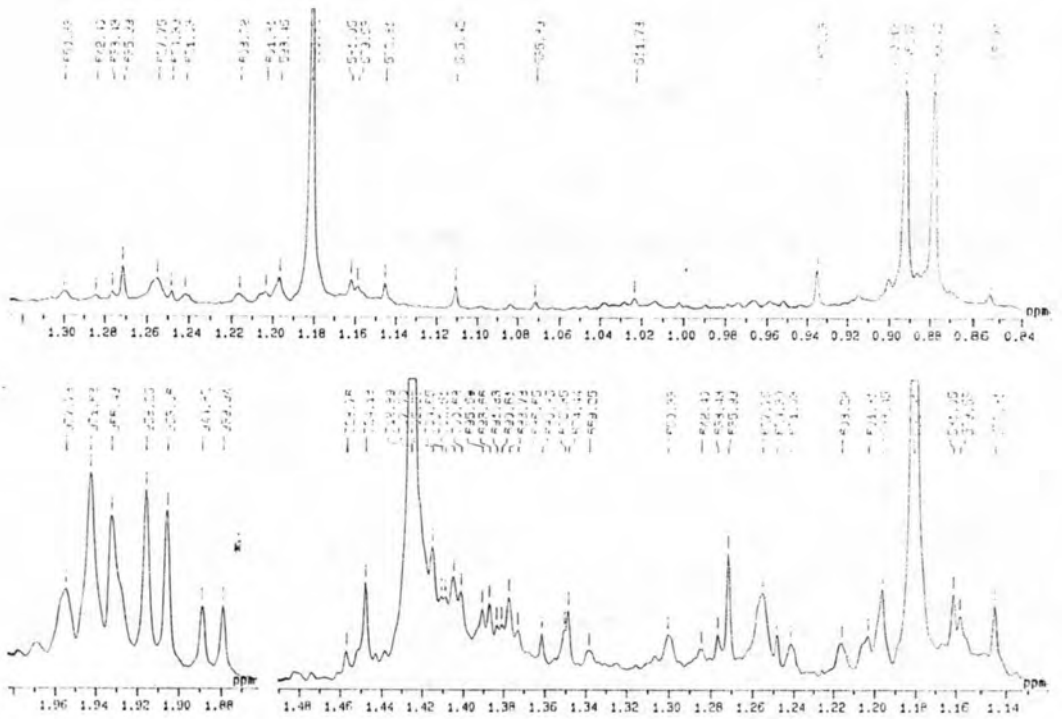


Fig. 32 The expansion of ^{1}H-NMR spectrum of Compound **5**

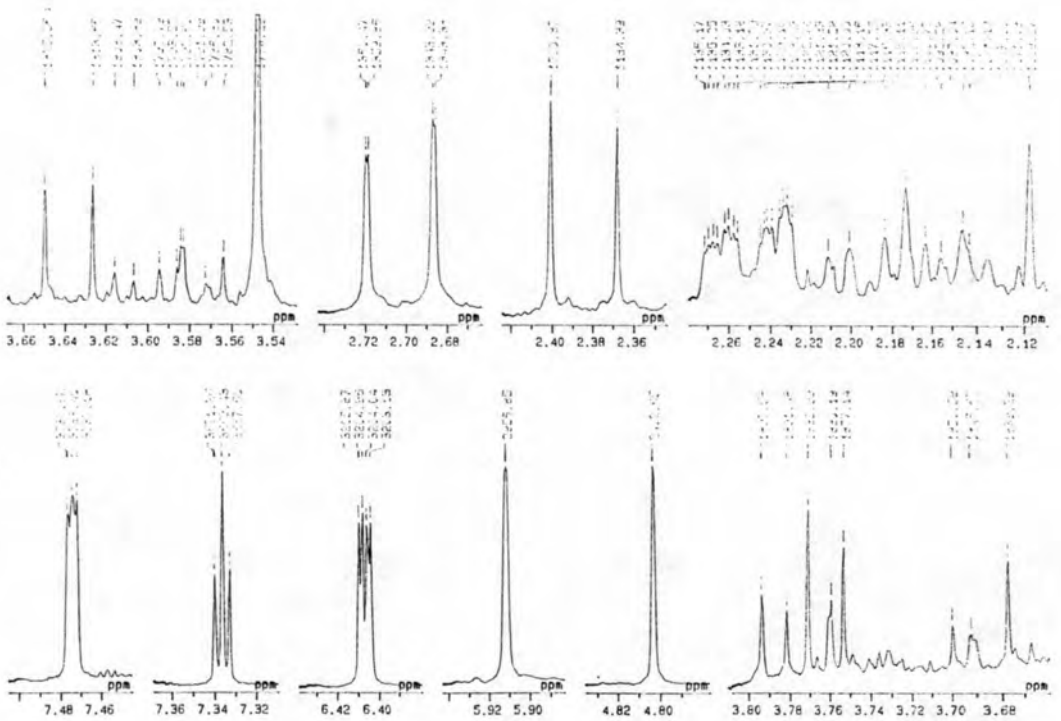


Fig. 33 The expansion of ^{1}H-NMR of Compound **5**

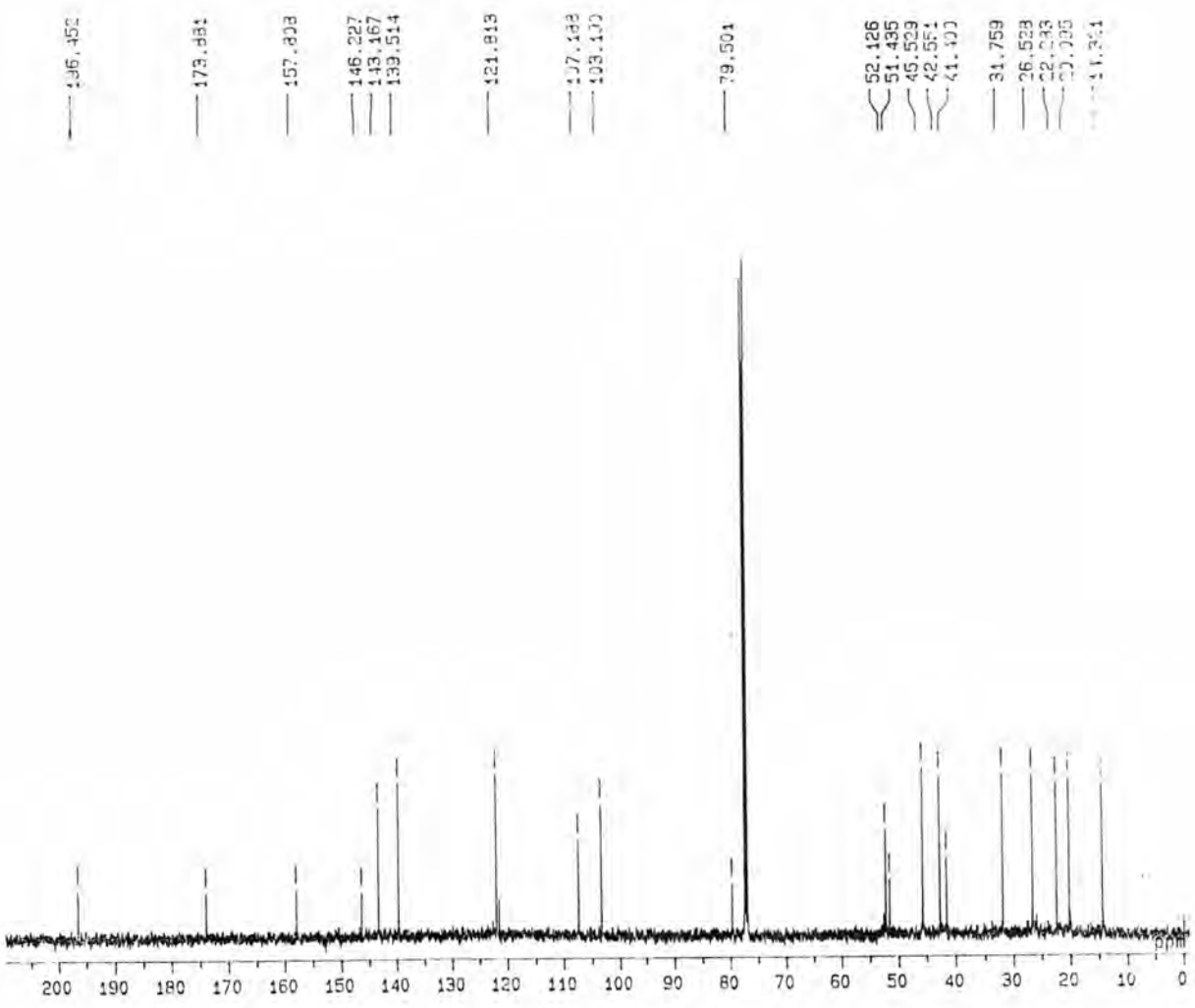


Fig. 34 The ^{13}C-NMR spectrum of Compound **5**

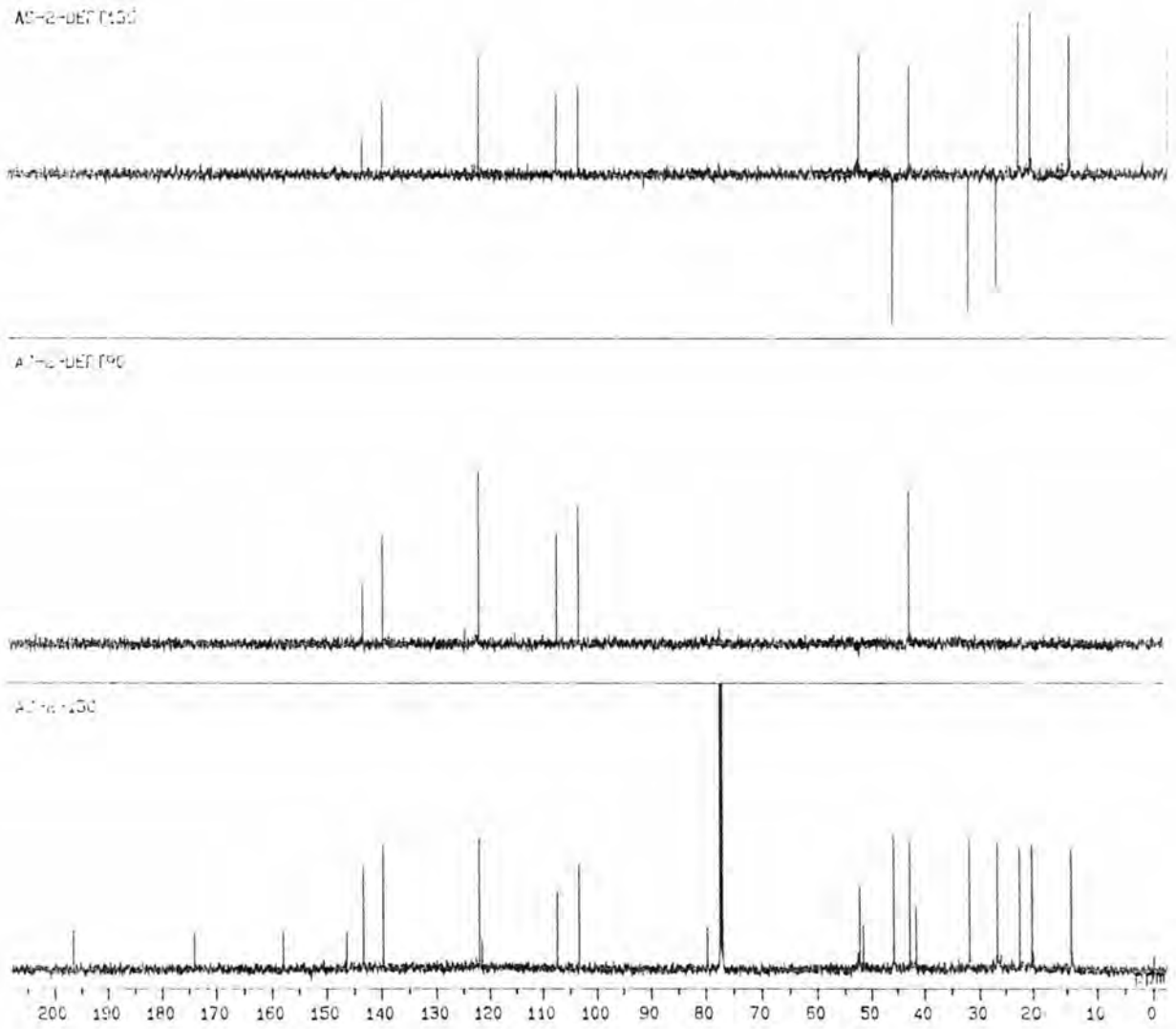


Fig. 35 The DEPT 90 and 135 ^{13}C-NMR spectrum of Compound **5**

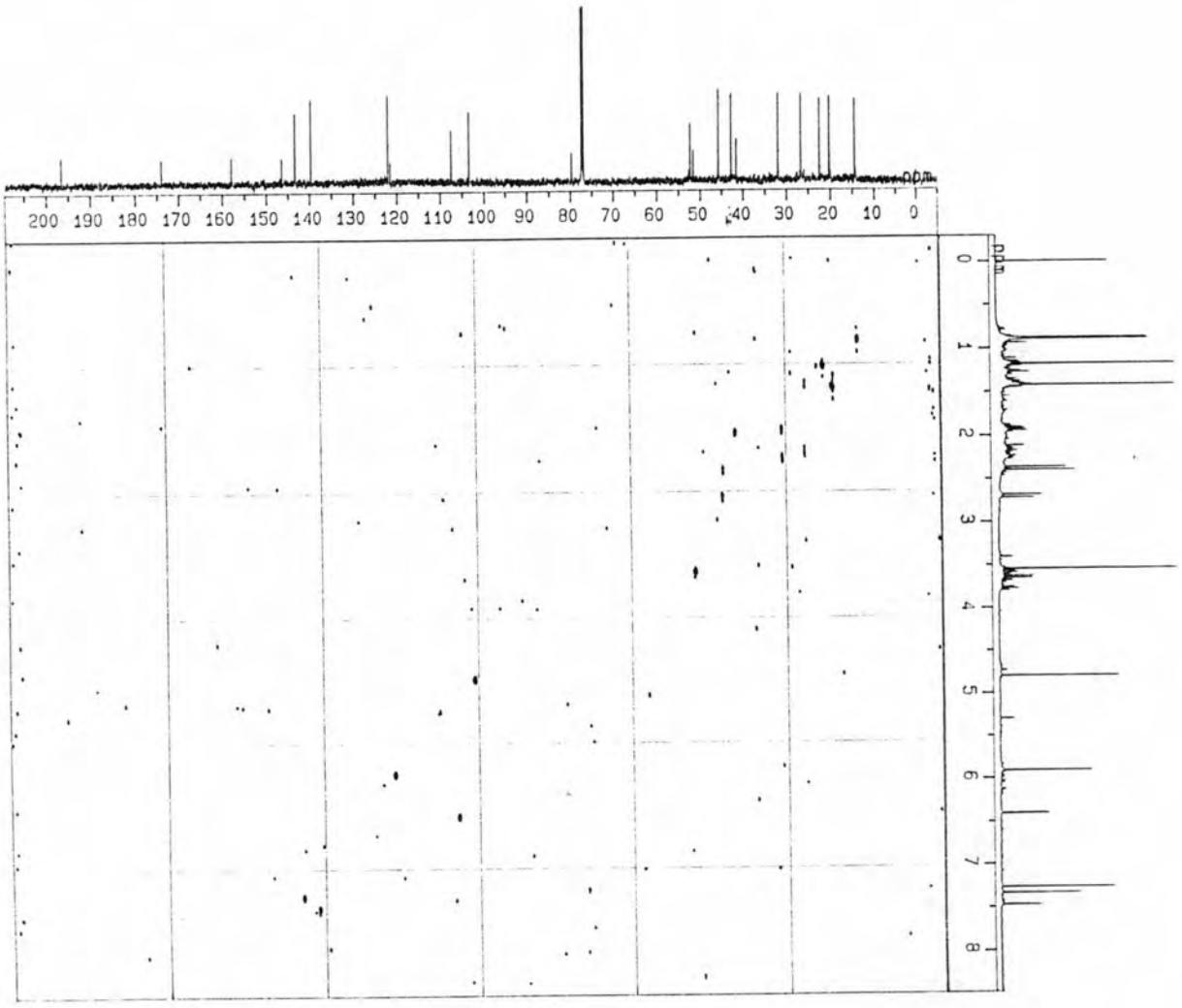


Fig. 36 The CHSHF spectrum of Compound **5**

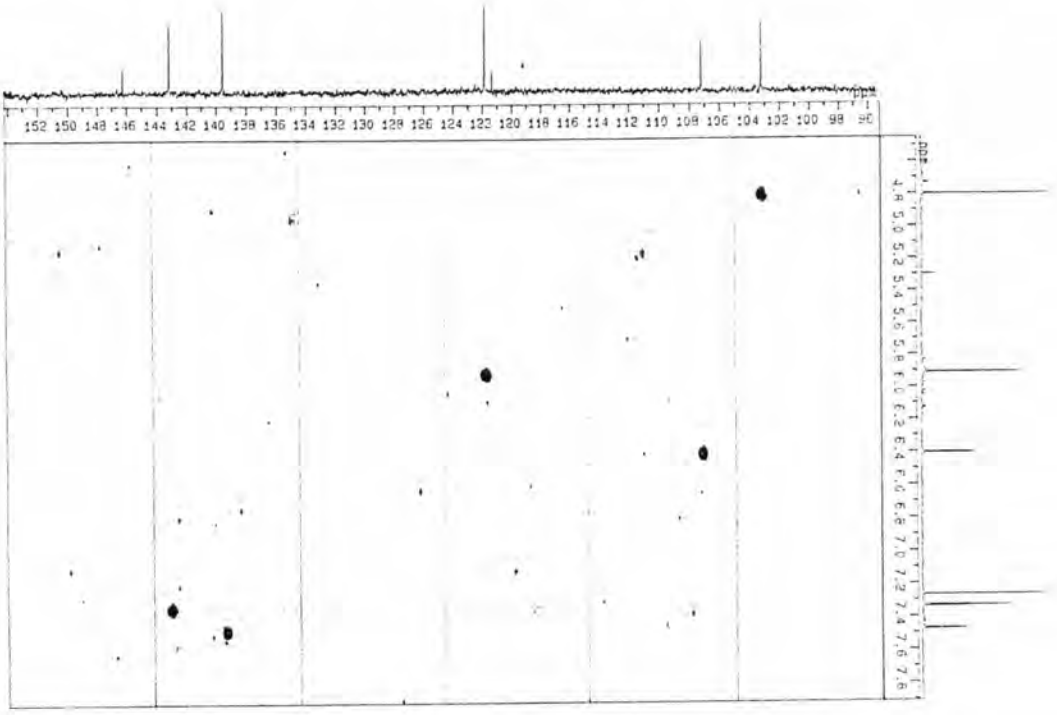


Fig. 37 The expansion of CHSHF spectrum of Compound 5

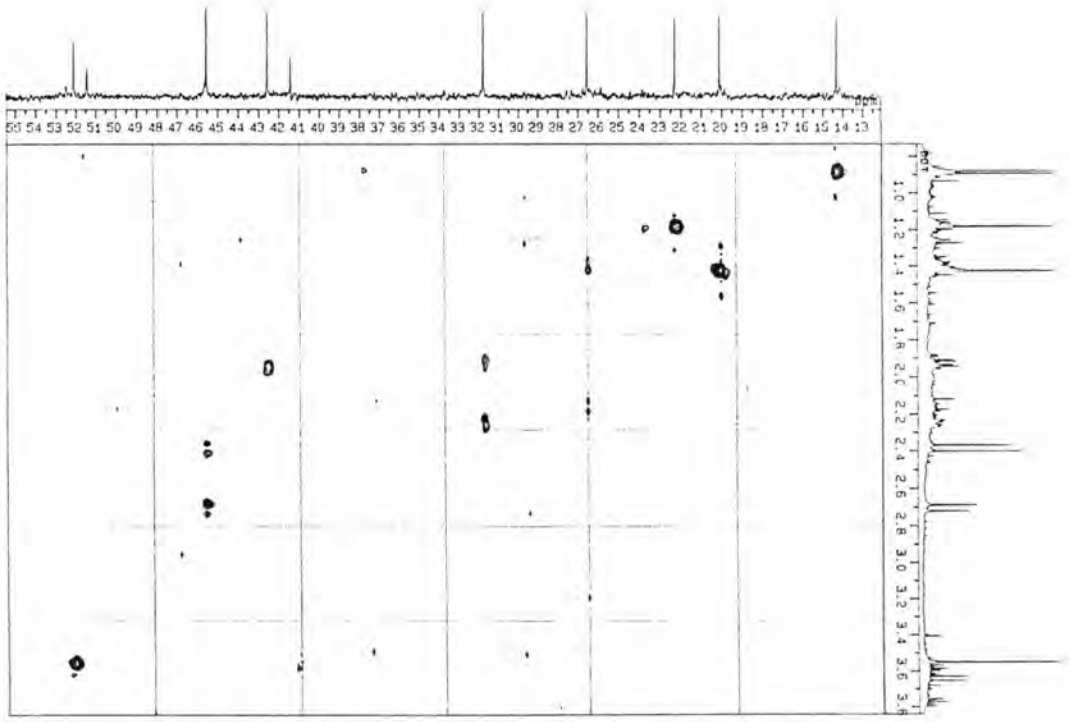


Fig. 38 The expansion of CHSHF spectrum of Compound 5

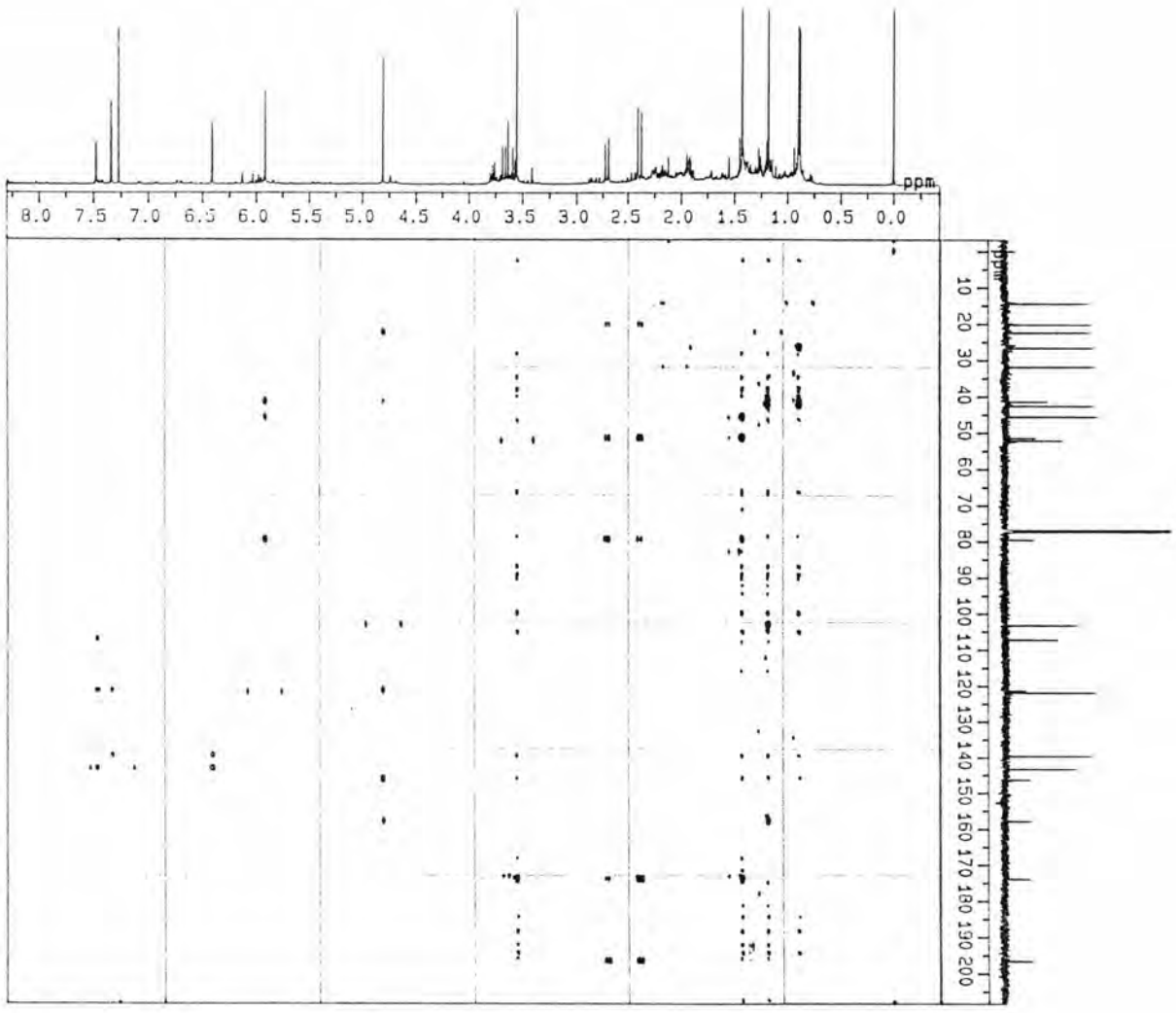


Fig. 39 The HMBC spectrum of Compound **5**

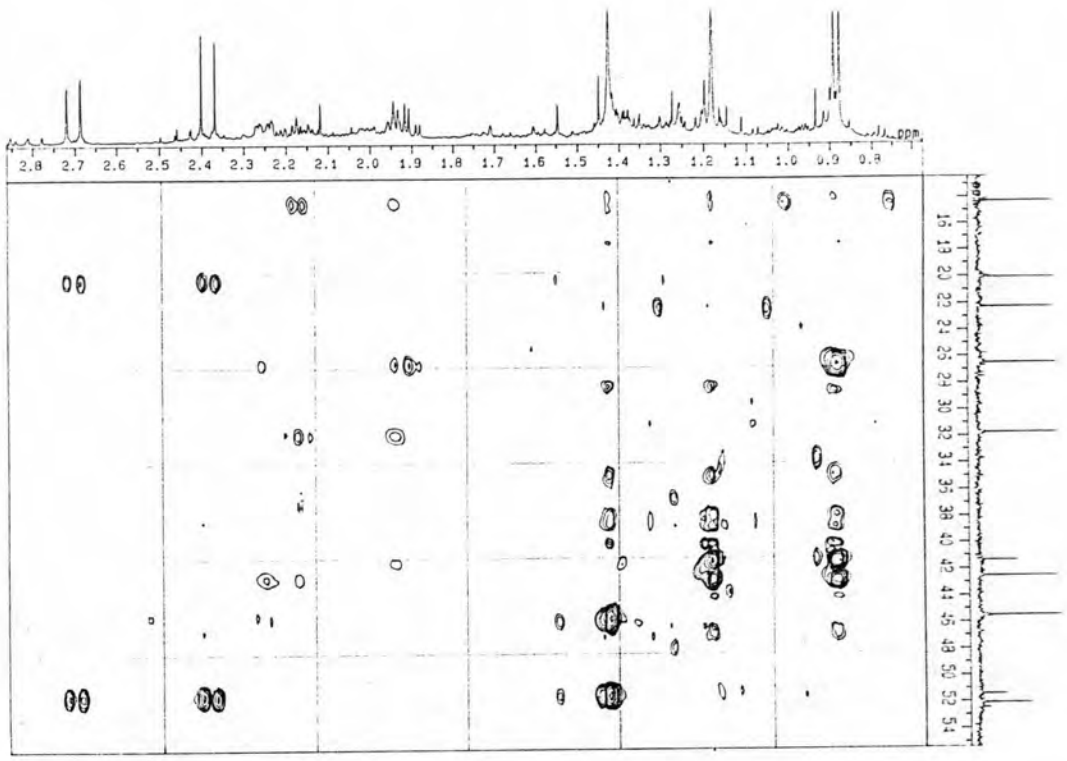


Fig. 40 The expansion of HMBC spectrum of Compound **5**

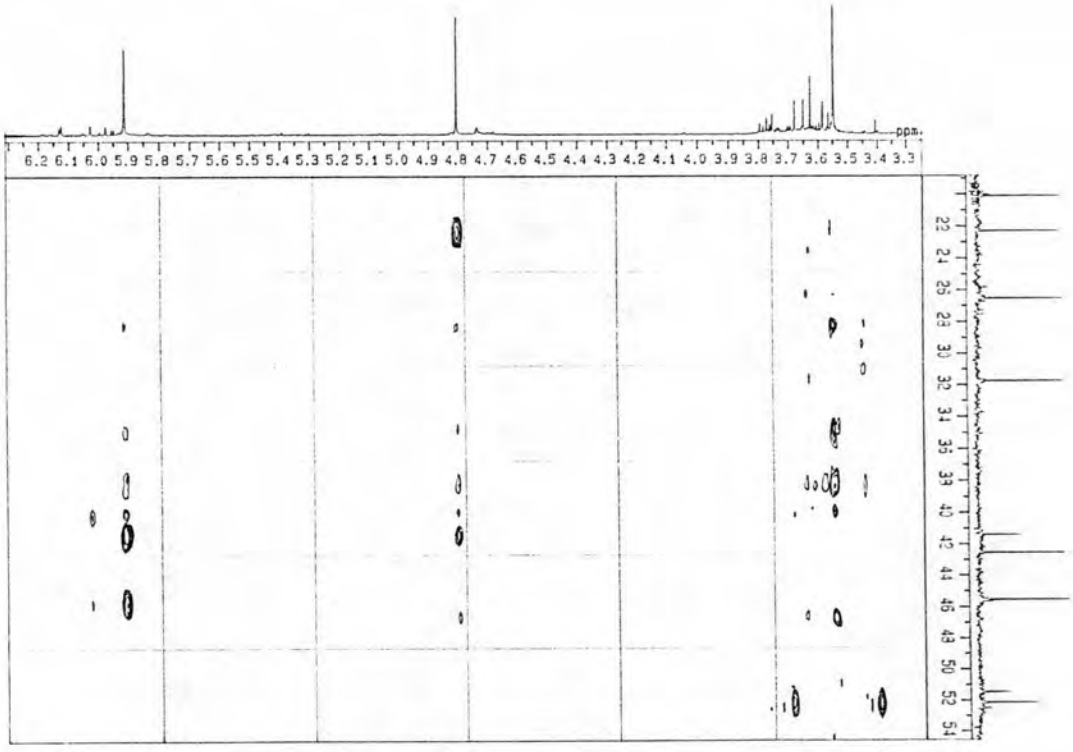


Fig. 41 The expansion HMBC spectrum of Compound **5**

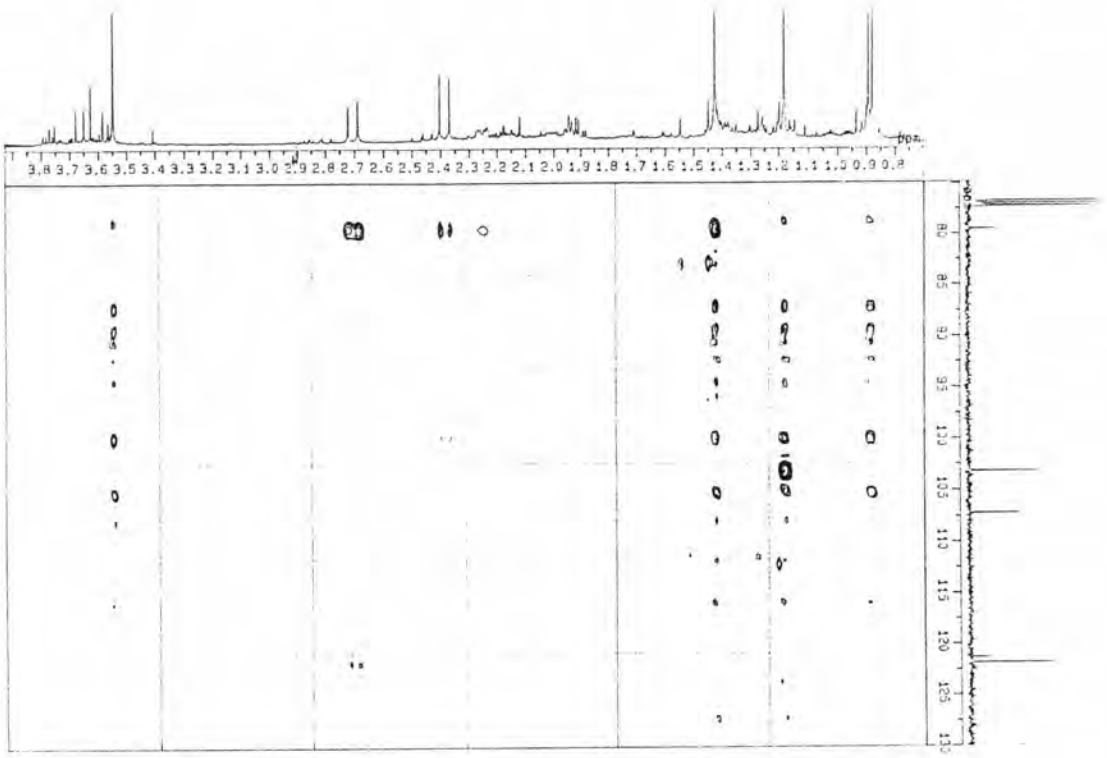


Fig. 42 The expansion HMBC spectrum of Compound **5**

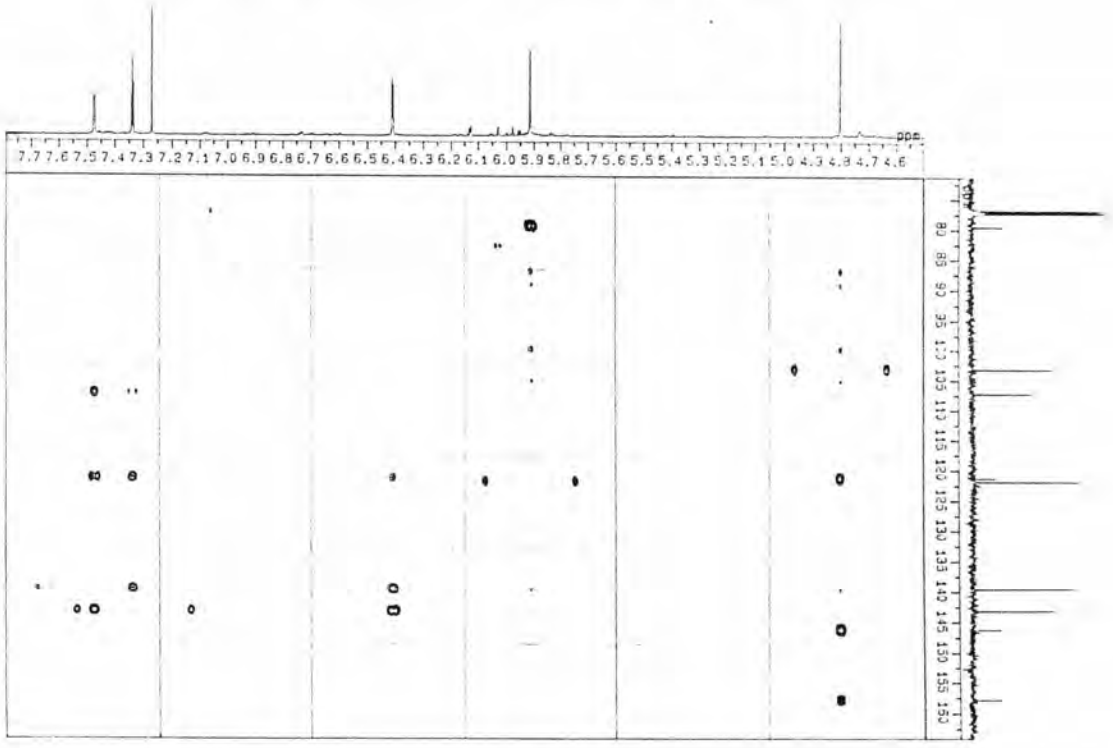


Fig. 43 The expansion HMBC spectrum of Compound **5**

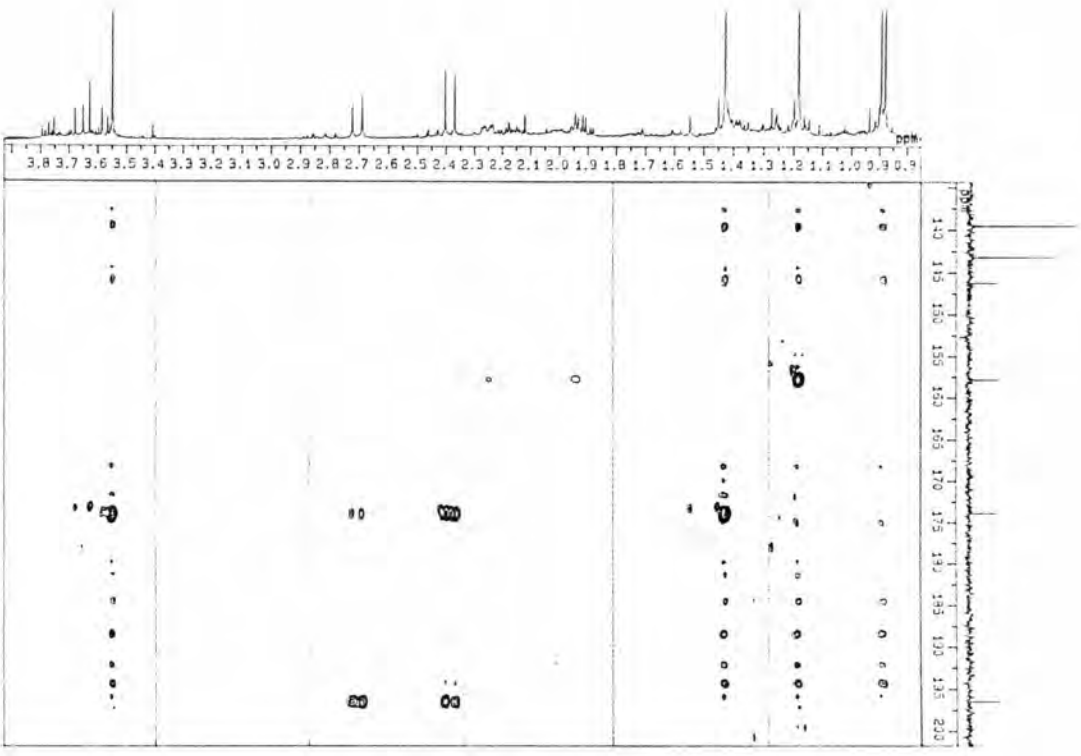


Fig. 44 The expansion HMBC spectrum of Compound 5

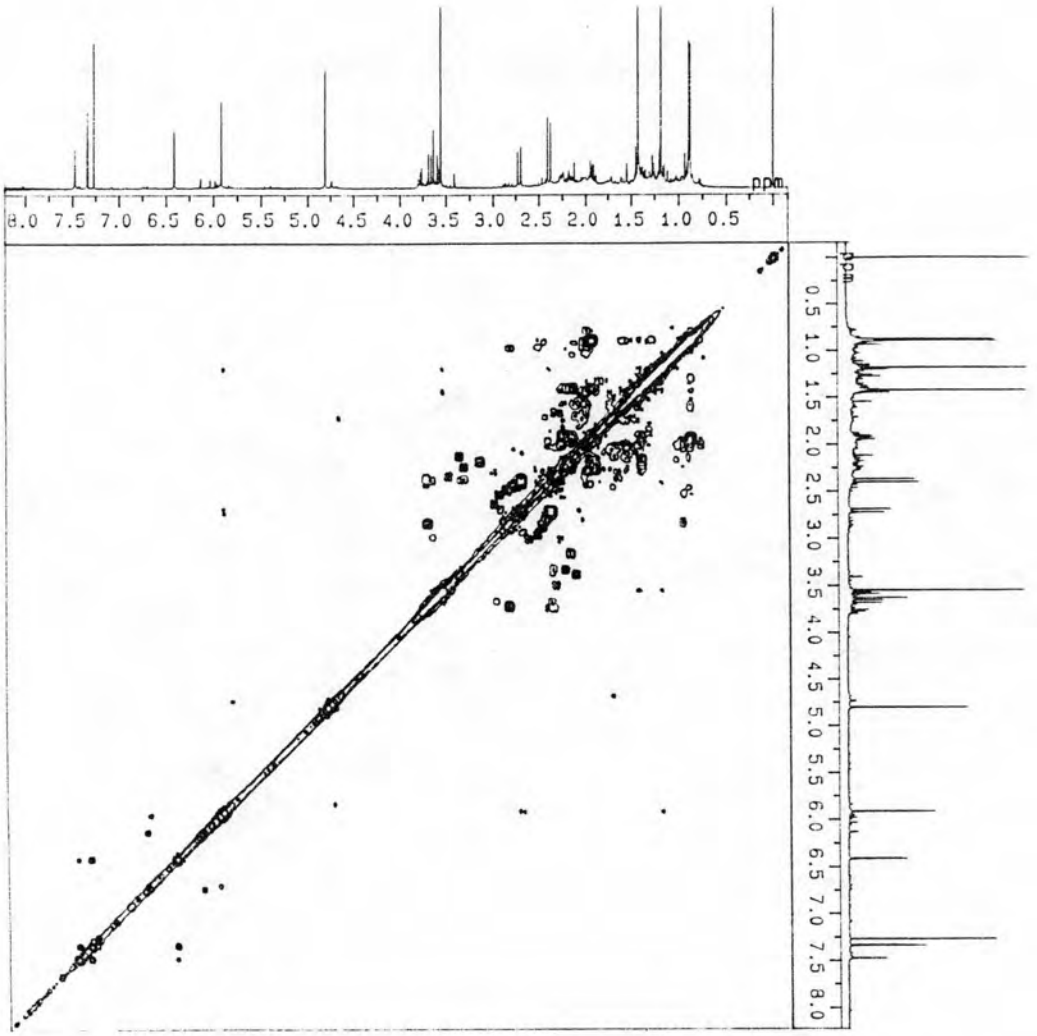


Fig. 45 The ^{1}H-^{1}H COSY spectrum of compound **5**

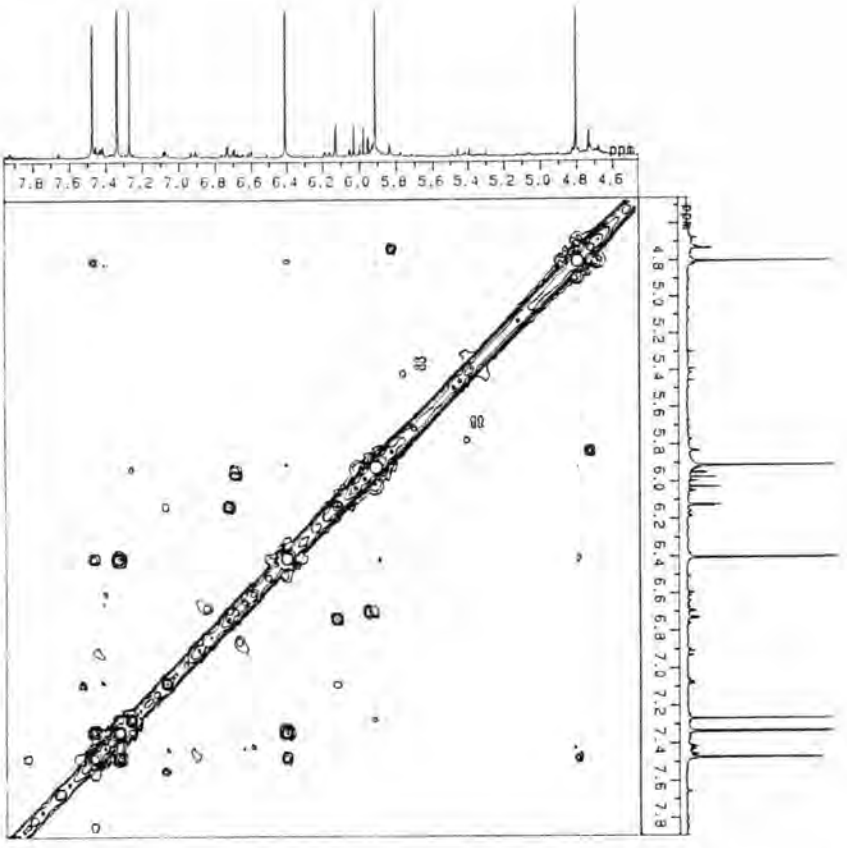


Fig. 46 The expansion of ^{1}H-^{1}H COSY spectrum of compound **5**

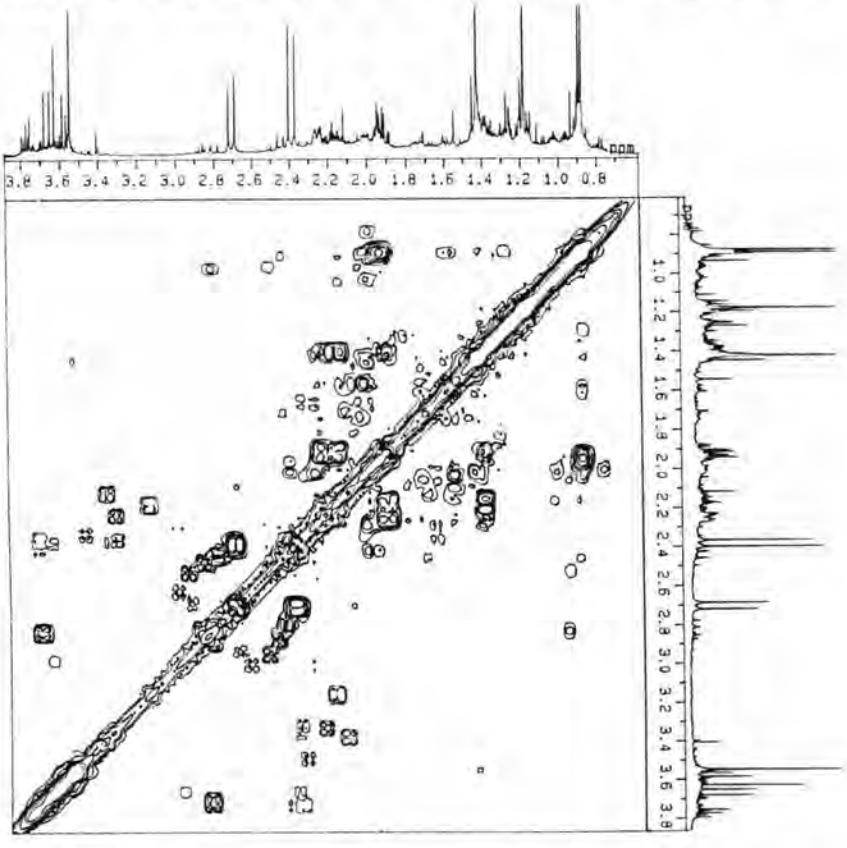


Fig. 47 The expansion of ^{1}H-^{1}H COSY spectrum of compound **5**

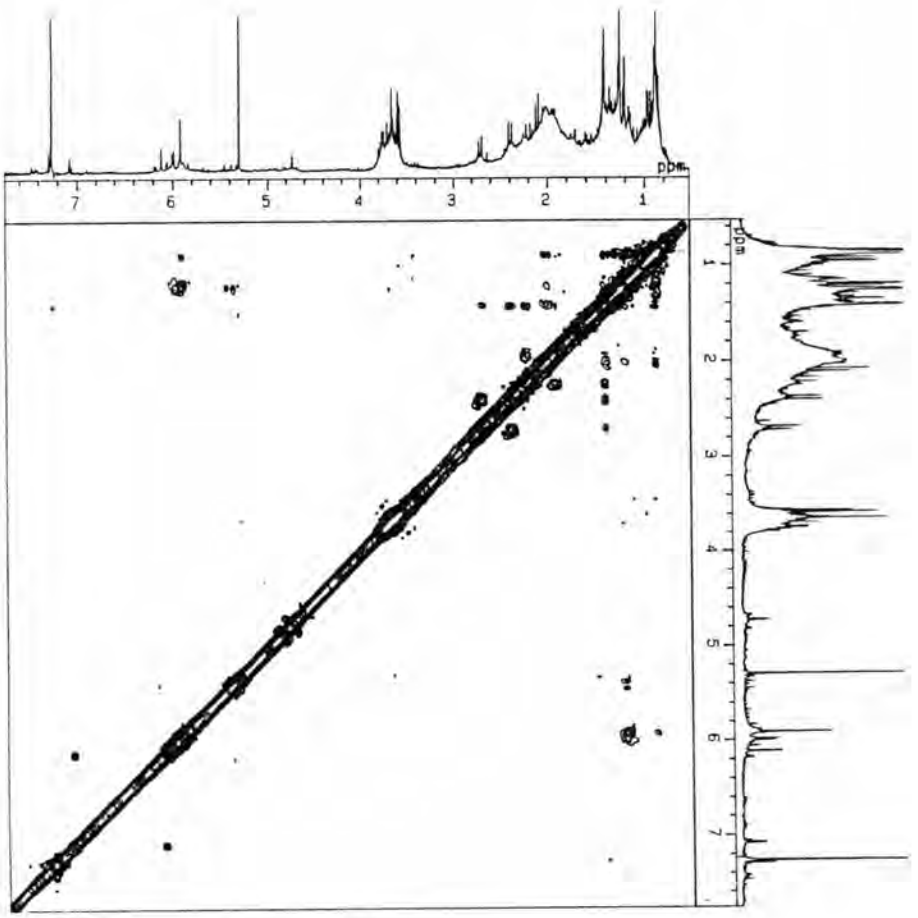


Fig. 48 The NOESY spectrum of compound **5**

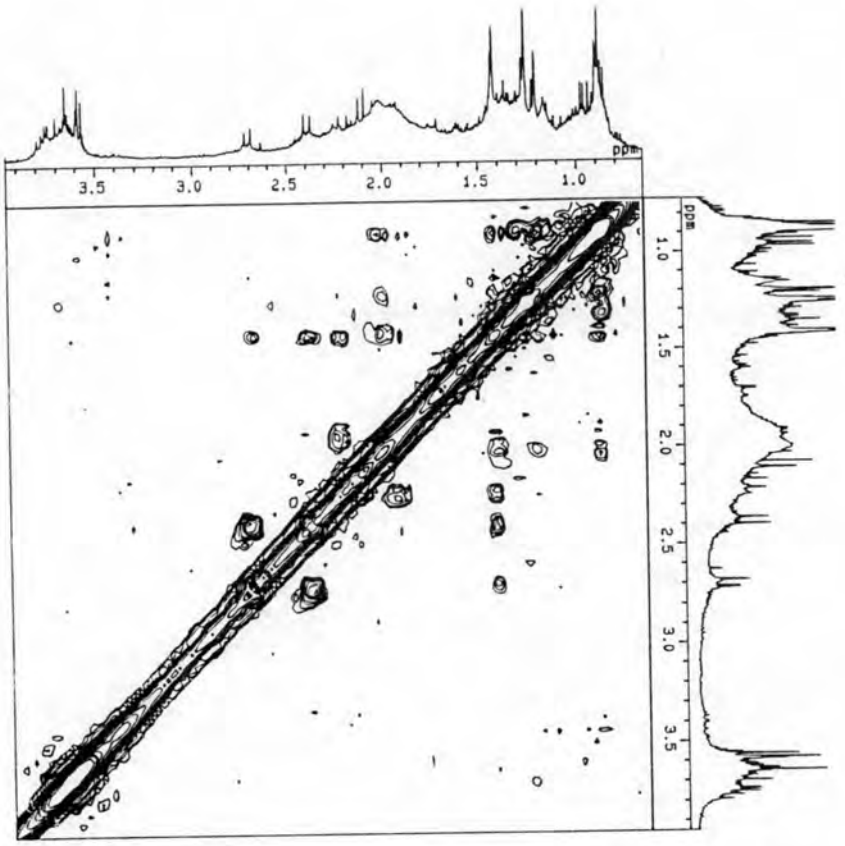


Fig. 49 The expansion of NOESY spectrum of compound **5**

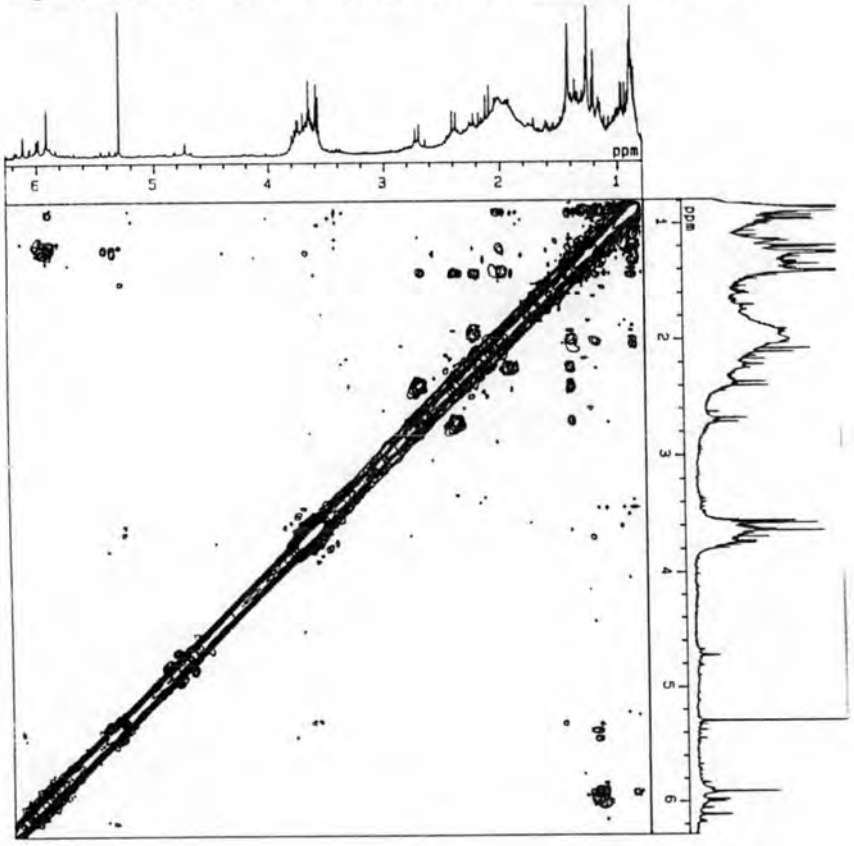


Fig. 50 The expansion of NOESY spectrum of compound **5**

Structure elucidation of Compound 6

Compound **6** was isolated from Fraction I and II, 0.0752 g ($0.75x10^{-3}$ wt by wt of the roots). This compound was white solid; m.p. 159-160 ^{0}C.(R_f value was 0.46, 70% EtOAc in hexane). This compound was soluble in dichloromethane, and chloroform and slightly soluble in hexane.

The IR spectrum (Fig. 51) indicated absorption band of hydroxy group at 2500-3300 cm^{-1} and absorption of C=O stretching vibration of conjucated carboxylic acid at 1675 cm^{-1}. The C=C stretching vibration of vinyl at 1644 and 1434 cm^{-1} and other IR spectrum bands were shown in Table 19.

Table 19 The IR absorption band assignments of Compound **6**

Vibration	Wave number (cm^{-1})	Intensity
O-H stretching of carboxylic acid	2500-3300	Strong (broad)
C-H stretching of -CH_2, -CH_3	2919,2858	Strong, Moderate
C=O stretching of carboxylic acid	1675	Strong
C=C stretching of vinyl	1644	Moderate
C-O stretching	1286	Moderate
CH_2 rocking in C-$(CH_2)_n$-C	718	Moderate

The ^{1}H-NMR spectrum of compound **6** showed signals for -CH_3, -CH_2- and -CH- of alicyclic at δ 0.75-2.80 (Fig. 52).

The ^{13}C-NMR spectrum and DEPT experiments (Fig. 53-54) displayed 15 signals, that contained three methyl carbons (δ 18.0, 19.3 and 26.2), five methylene carbons (δ 25.7, 26.9, 27.9, 31.3 and 36.3), two methine carbons (δ 36.0 and 48.2) and five quaternary carbons (δ 41.7, 68.2, 123.1, 171.0 and 173.1).

This compound displayed a molecular ion peak at m/z 234 (Fig. 55), consisted with a molecular formula of $C_{15}H_{22}O_2$. The presence of fifteen carbons in ^{13}C-NMR spectrum suggested a sesquiterpene skeleton, and IR absorption peaks at 2500-3300 and 1675 cm^{-1} revealed a carboxylic acid functional group. From ^{13}C-NMR, olefinic

carbon peaks appeared at δ 123.1 and 173.1 ppm. However, an olefinic proton was absent in this compound; instead, an α,β-unsaturated acid moiety constituted as a part of the structure.

From literature search, for patchoulane type has been found in Euphorbiaeceae family. This information suggested that this compound might be cyperenoic acid.

The ^{13}C-NMR spectrum of compound **6** was compared with cyperenoic acid to confirm this structure (Table 20).

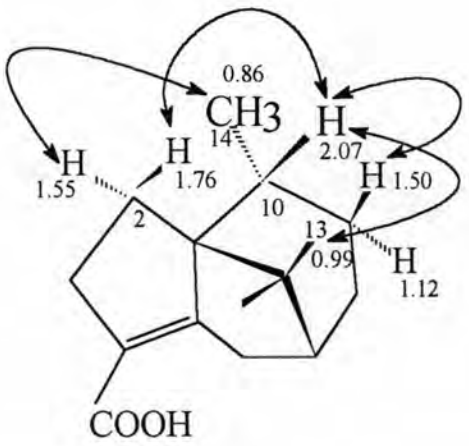
Table 20 The ^{13}C-NMR spectral data of Compound **6** (125 MHz) compared with Cyperenoic acid [38]

Position	Chemical shioft (δ ppm)	
	Cyperenoic acid[38]	Compound **6**
1	68.4	68.2
2	25.9	25.7
3	36.3	36.3
4	122.9	123.1
5	169.3	171.0
6	31.5	31.3
7	48.3	48.2
8	27.1	26.9
9	28.0	27.9
10	36.1	36.0
11	41.9	41.7
12	26.4	26.2
13	19.4	19.3
14	173.2	173.1
15	18.1	18.0

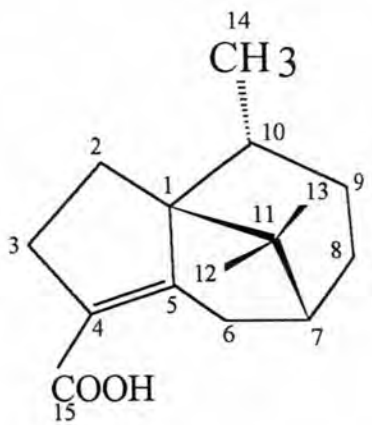
Relative stereochemistry at C-10 was proved by NOE difference technique (Fig. 56-57). Cross peaks were observed between the signal corresponding to

(I) H-10 (δ 2.07), H-13 (δ 0.99), H-2(δ 1.76) and H-9 (δ 1.50).

(II) H-14 (δ 0.86) and H-2 (δ 1.55).



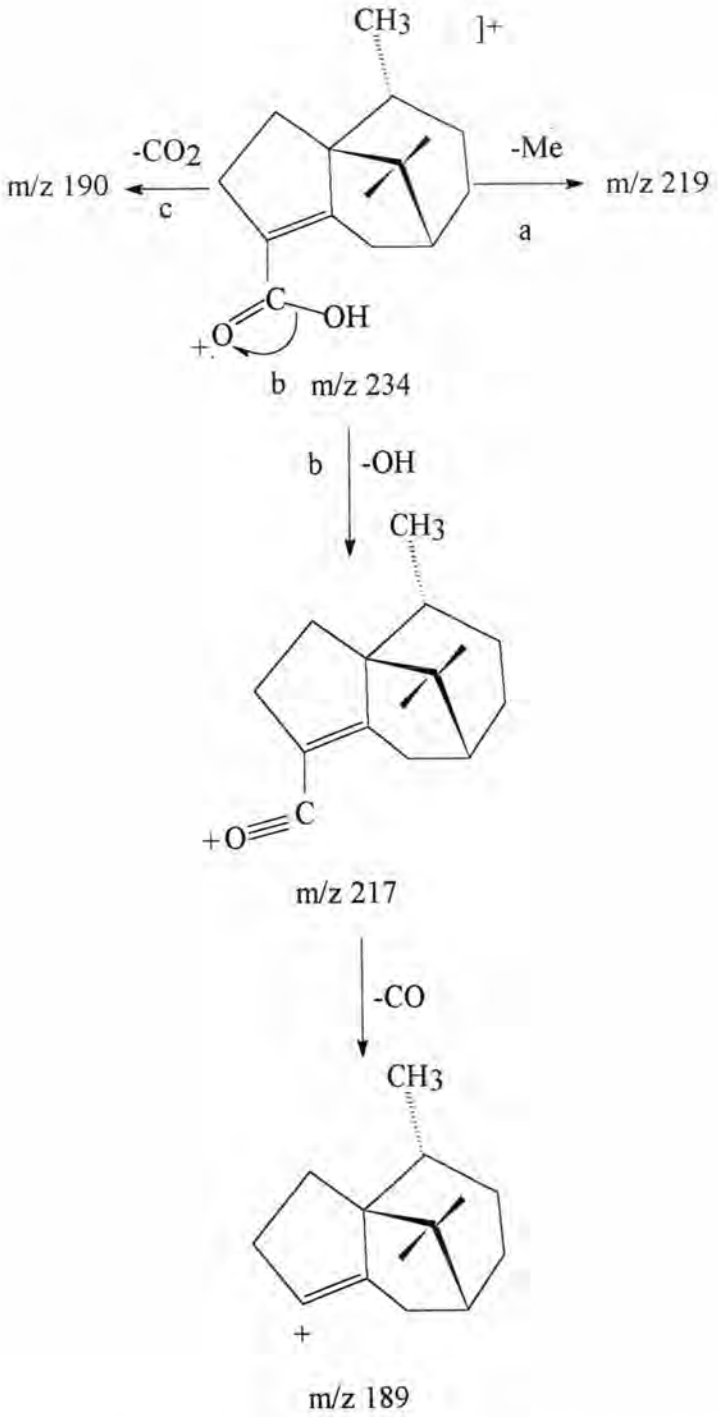
From these results, it was deduced that CH_3-14 was β-oriented, while H-10 was α- oriented.



Cyperenoic acid

Base on these results, the structure of compound **6** is established to be cyperenoic acid, which has been found for the first time in this plant.

In 1987, cyperenoic acid was isolated from *Sandwitbia guyanensis* Lanj., an endemic plant of Guyana, which was confirmed structure and stereochemistry by 2D-NMR spectroscopy. [39] In the same year, Cyperenoic acid were isolated from *C. crassifolius*, inactive (ED_{50} > 50 μg/ml) in the KB and *P*-388 in *vitro* cytotoxicity test system.[12] One year latter, Boonyaratavej, S. and Roengsumran, S. reported the isolation of cyperenoic acid from the roots of *C. crassifolius* and its characterized by X-ray diffraction.[40] However, compound **6** showed moderately cytotoxic to brine shrimp at LC_{50} = 32.58 μg/ml, weakly inhibition against bacterial; *E. coli*, *B. cereus*, *S. aureus* and *S. derby* and also showed activity against antifungal *C. albicans*.



Scheme 5 The possible mass fragmentation pattern of Compound **6**

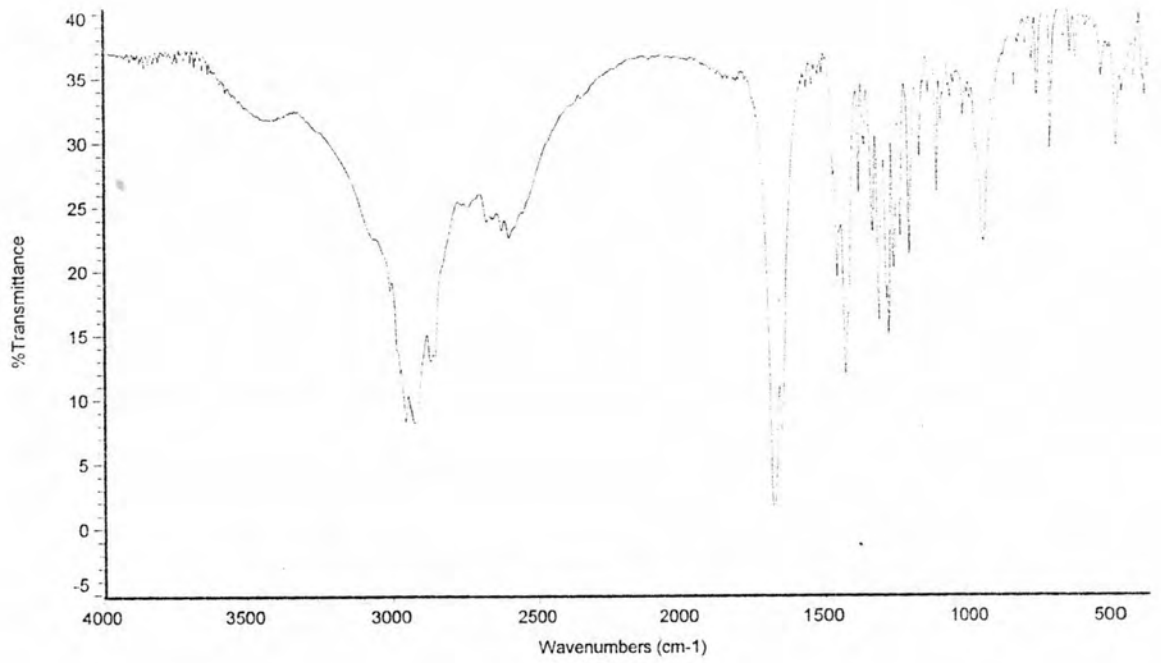


Fig. 51 The IR spectrum of Compound **6**

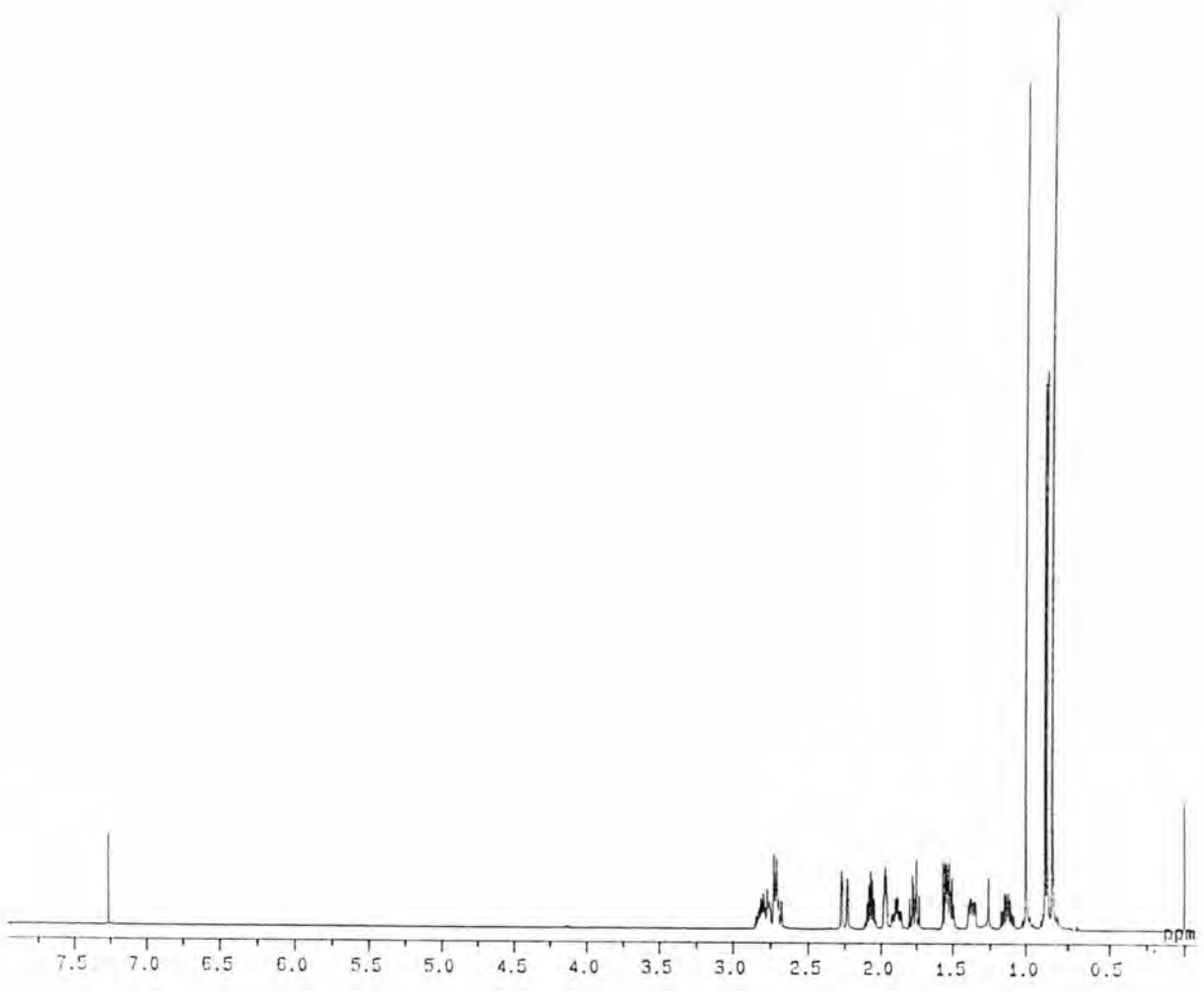


Fig. 52 The ^{1}H-NMR spectrum of Compound **6**

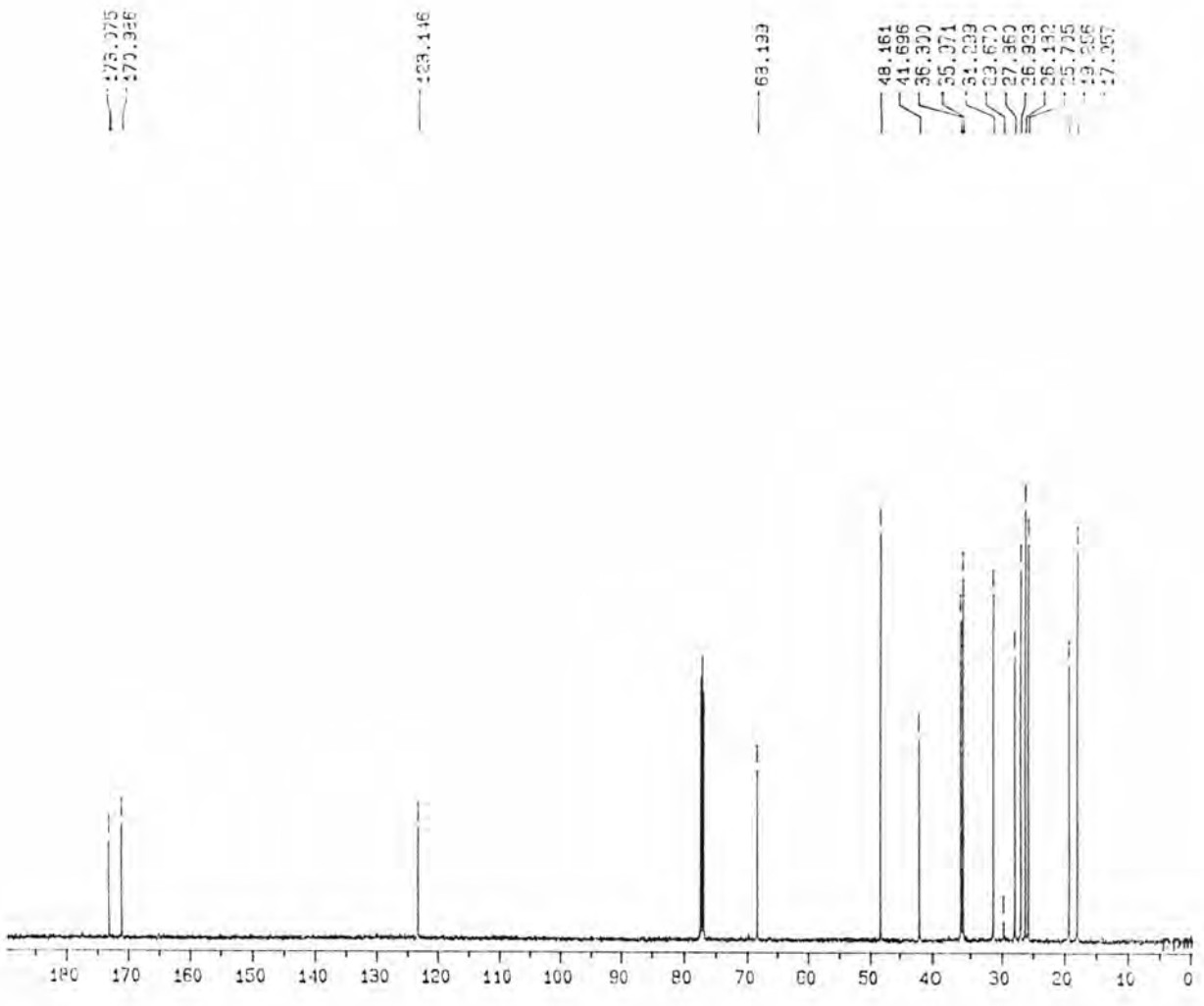


Fig. 53 The ^{13}C-NMR spectrum of Compound **6**

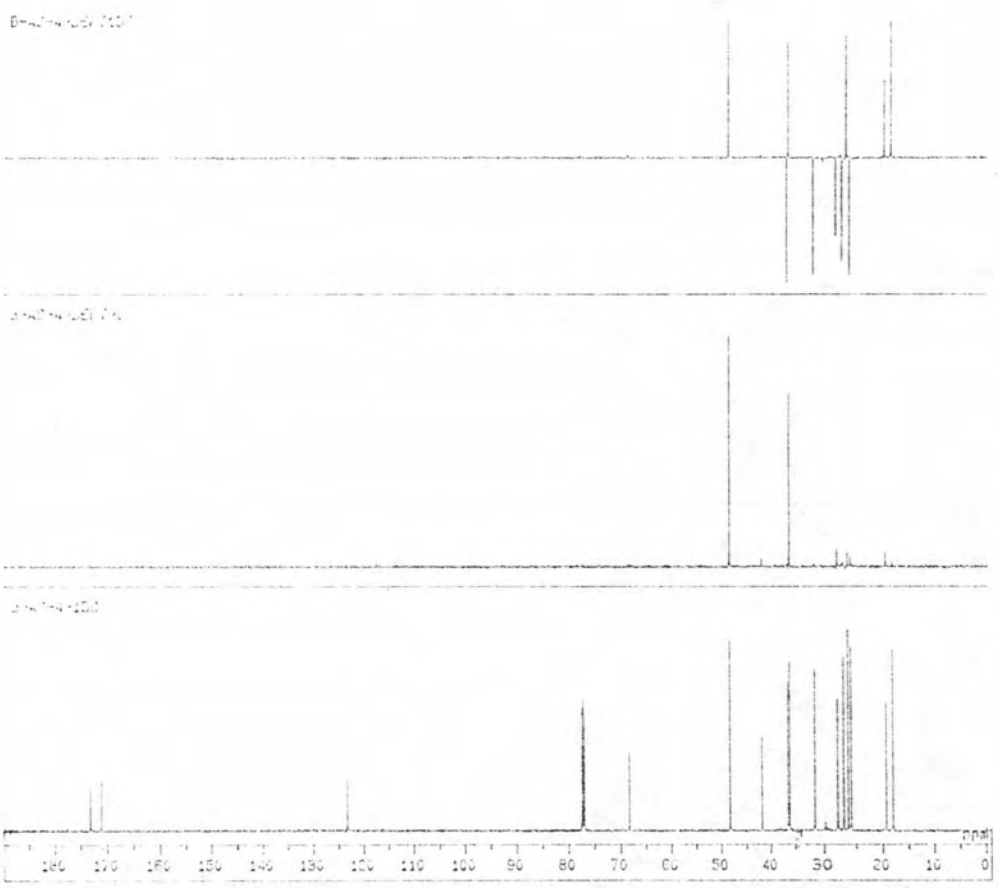


Fig. 54 The DEPT 90^0, 135^0 ^{13}C-NMR spectrum of Compound **6**

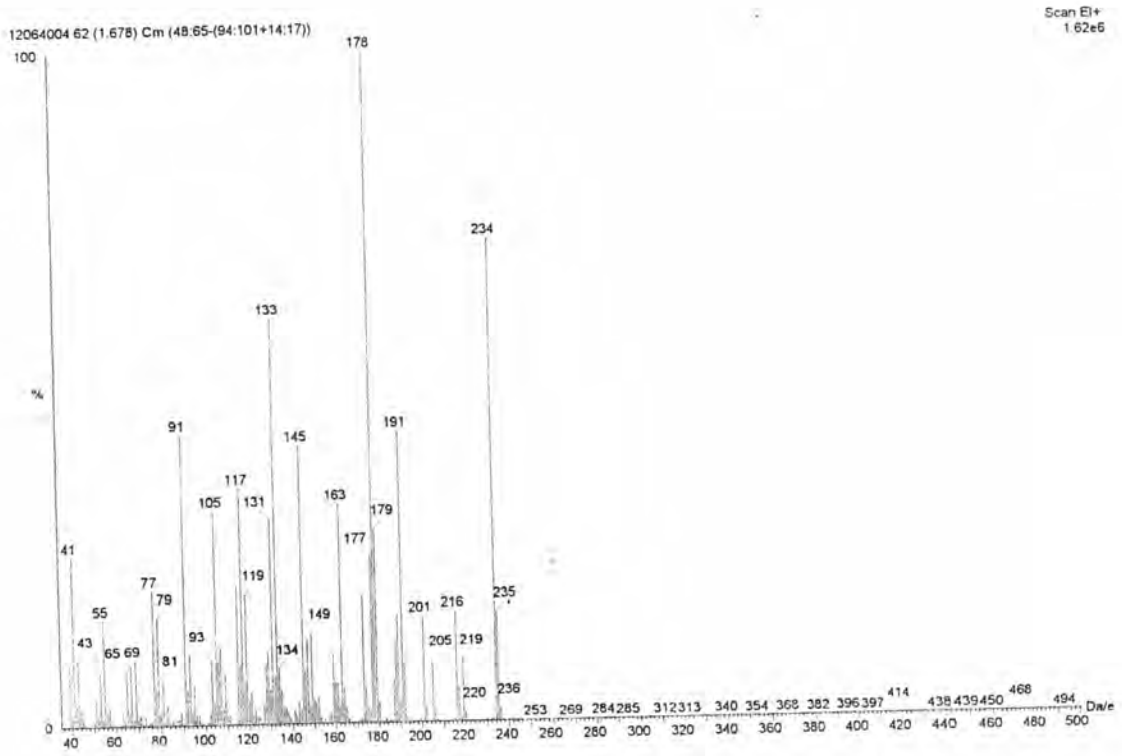


Fig. 55 The mass spectrum of Compound **6**

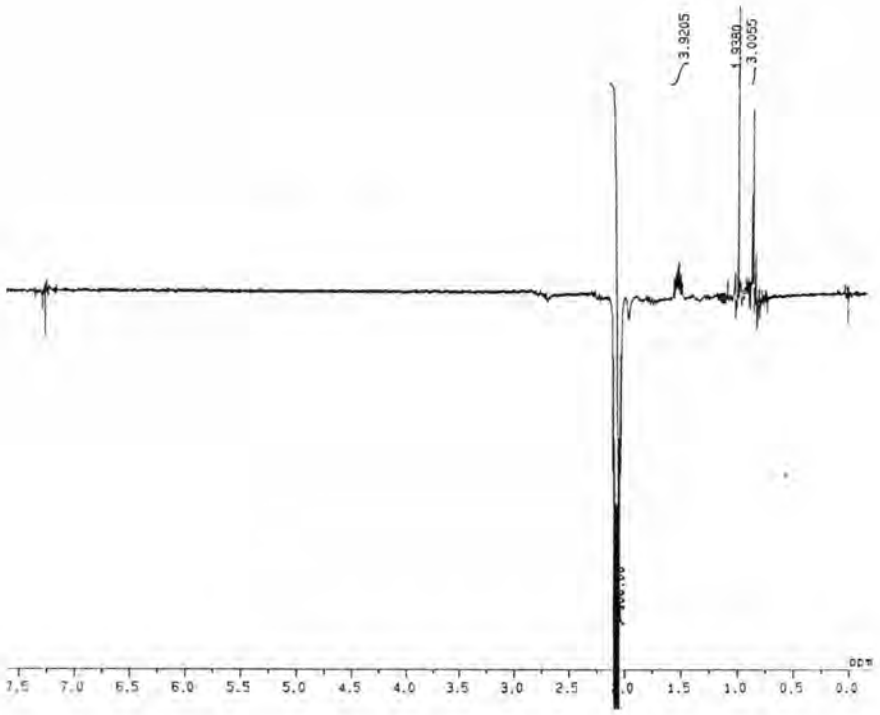


Fig. 56 The NOE difference spectrum of Compound **6**
(irradiated at δ 2.07 ppm)

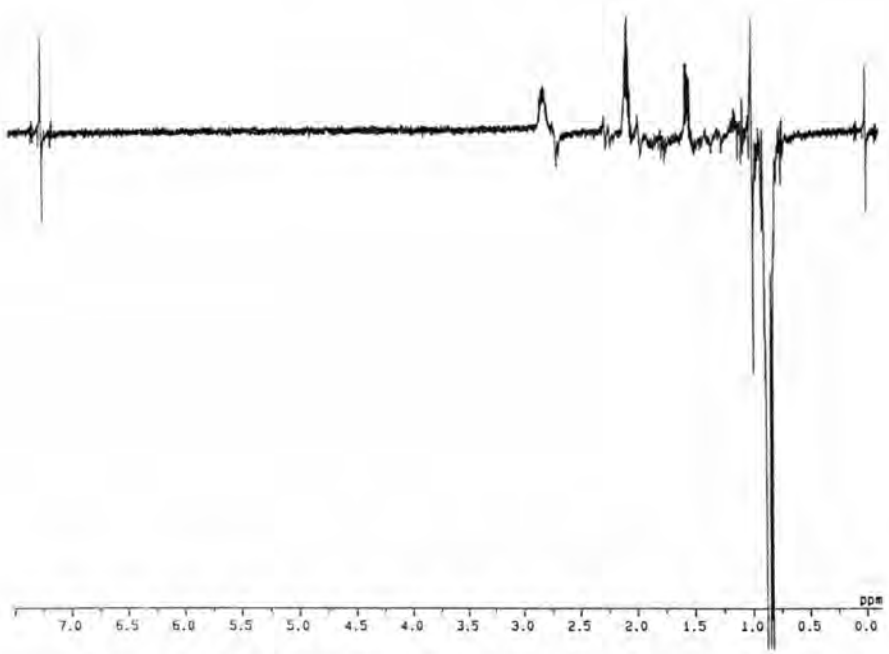


Fig. 57 The NOE difference spectrum of Compound **6**
(irradiated at δ 0.86 ppm)

Structure elucidation of Compound 7

Compound 7 was isolated by silica gel column chromatography of the dichloromethane crude extract and further purified by chromatotron. This compound was obtained as pale yellow oil, 194.6 mg ($1.95x10^{-3}$% wt by wt) The R_f value was 0.43 (50% EtOAc in hexane as developing solvent).

The IR spectrum presented of hydroxy group which indicated by -OH stretching at 3200-3600 cm^{-1}, C-O stretching at 1396 cm^{-1}. The olefinic absorption was also apparent C-H stretching of vinyl at 3125 cm^{-1} and C=C band at 1640 cm^{-1}(Fig. 58).

The ^{1}H-NMR spectrum (Fig. 59) of compound 8 also showed resonances at δ 1.04 (s), 1.06 (s) and 1.28 (s) for methyl group and at δ 4.68 ppm [2H, dd, J=1.25,12.98 Hz] for vinyl protons.

The ^{13}C-NMR and DEPT experiments (Table 21) at δ 106.3 and 153.4 were distributed to a vinyl moiety CH_2=C< and at δ 80.9 for carbinyl carbon -C(-OH)- (Fig. 60-61).

This compound was assigned the molecular formula $C_{15}H_{24}O$ [(M^+)=m/z 220], the mass spectrum of each peak was compared with authentic spectra through the library search (NIST data base) (Fig. 62).

On the basis, the structure of compound 7 was established as (-) spathulenol.

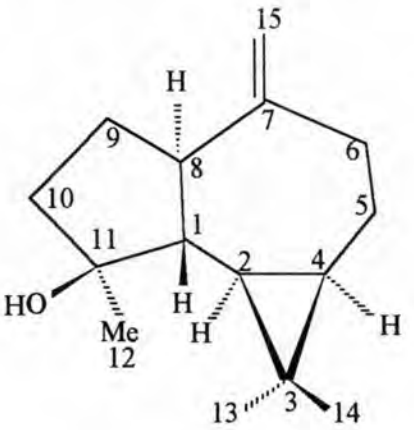
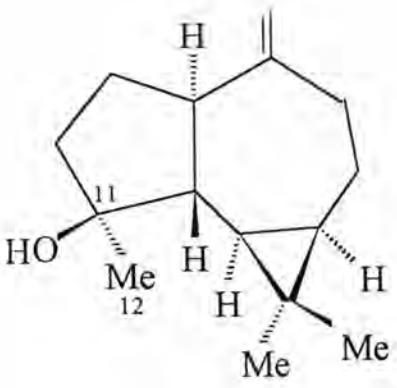
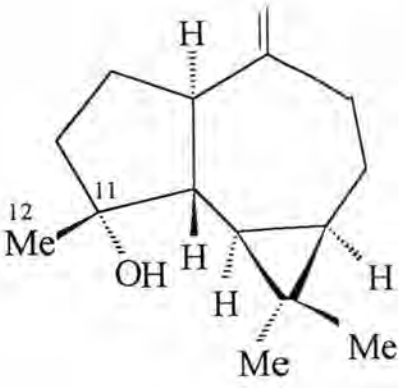


Table 21 The ^{13}C-NMR spectral data of Compound 7 (125 MHz) compared with (-)- spathulenol[41]

Position	Chemical shift (δ ppm)	
	(-)-Spathulenol [41]	Compound 7
1	54.3	54.3
2	29.8	29.9
3	20.3	20.2
4	27.5	27.5
5	24.8	24.8
6	38.9	38.8
7	153.4	153.4
8	53.4	53.4
9	26.7	26.7
10	41.8	41.7
11	80.9	81.0
12	26.1	26.0
13	28.7	28.6
14	16.3	16.3
15	106.3	106.2

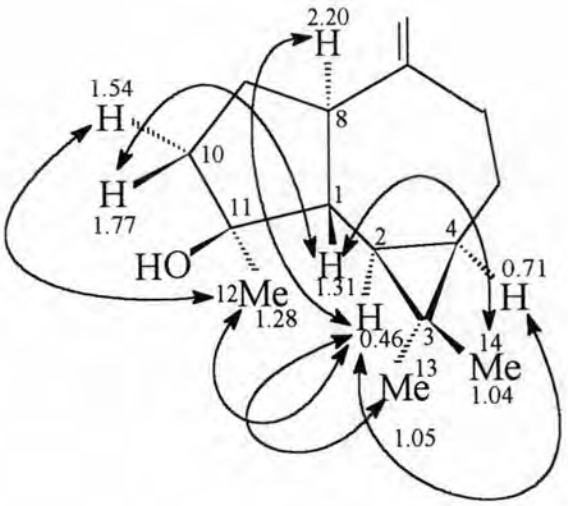


(A) (-)-spathulenol



(B) (+)-11-epispathulenol

The isomeric (+)-11-epispathulenol, (B) was having ^{1}H- and ^{13}C-NMR spectra very similar to (-)-spathulenol.[42] Thus, the structural differences between (A) and (B) were restricted to the stereochemistry of the chiral centres at C-1(δ 54.3), C-4 (δ 27.5)and C-8(δ 53.4). The stereochemistry of compound 7 was detected by NOE difference technique (Fig. 63-65). On irradiation at the methine proton resonance H-1 (δ 1.31). NOE was observed on the methyl proton resonance H-14(δ 1.04) (Fig. 63). When the methyl proton resonance H-2 (δ 0.46) was irradiated (Fig. 64), NOE was observed on the methyl proton resonance H-12 (δ 1.28), H-8(δ 2.20) and H-4 (δ 0.71). Moreover, the methyl proton resonance H-12(δ 1.28) was irradiated (Fig. 65), NOE was observed on the methine resonance H-2(δ 0.46) and H-10 (δ 1.54).

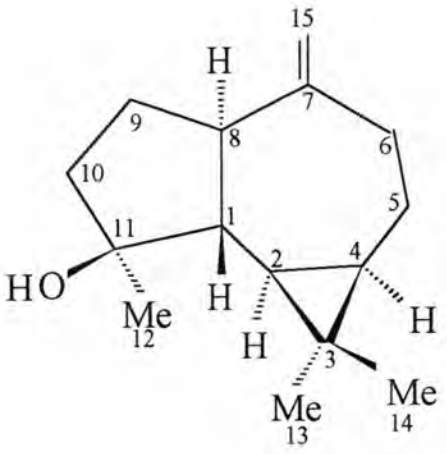


The NOE difference experiments:

irradiation at δ 0.46 (H-2), 1.28 (H-12) and 1.31 (H-1) ppm

So, the configuration at the junction between the five- and seven-member ring was found to be *trans*. From the NOE meansurements, the configuration and conformation of compound **7** was determined as (-)-spathulenol, (A).

Furthermore , (-)-spathulenol had been isolated from *Simularia mayi* [43], *Brasilia sickii* [44], *Phebalium tuberculosum* ssp., *Drummondita bassellii*, *Eriostemon brucei* ssp.[45] and Liverworts.[46] (-)-Spathulenol has been showed moderate antifungal activity against *C. cucumerinum*.[47] However, compound **7** was weakly inhibited in antibacterial (*S. derby*) and moderate activity against brine shrimp (LC_{50}= 32.64μg/ml).



(-)-spathulenol

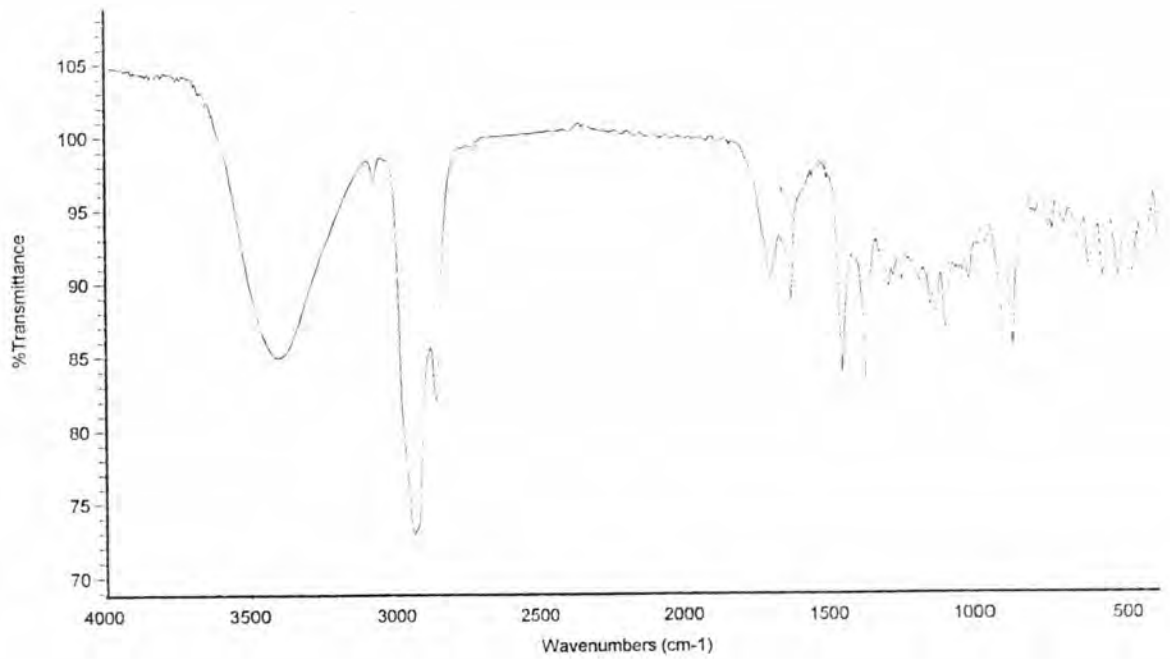


Fig. 58 The IR spectrum of Compound 7

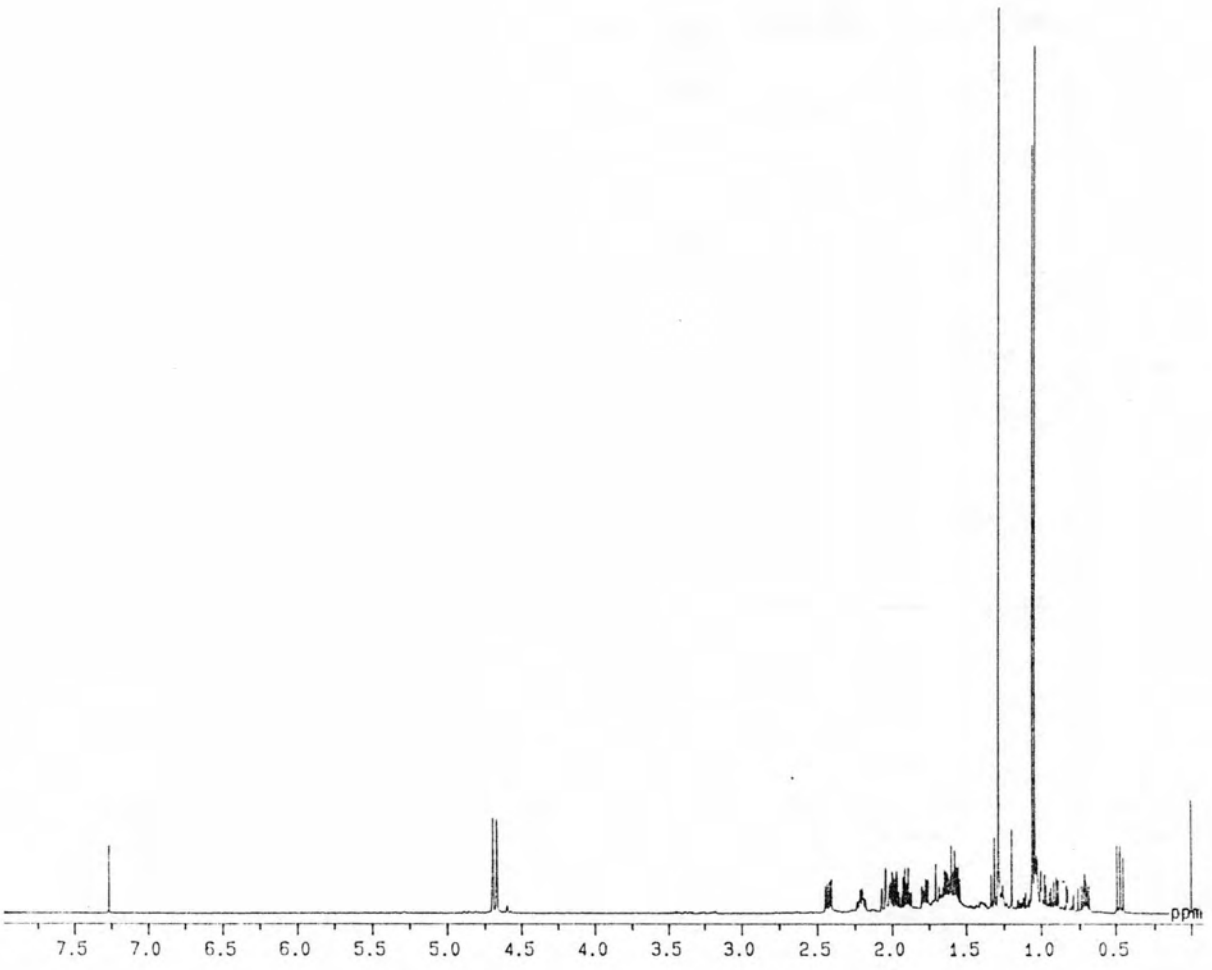


Fig. 59 The ^{1}H-NMR spectrum of compound 7

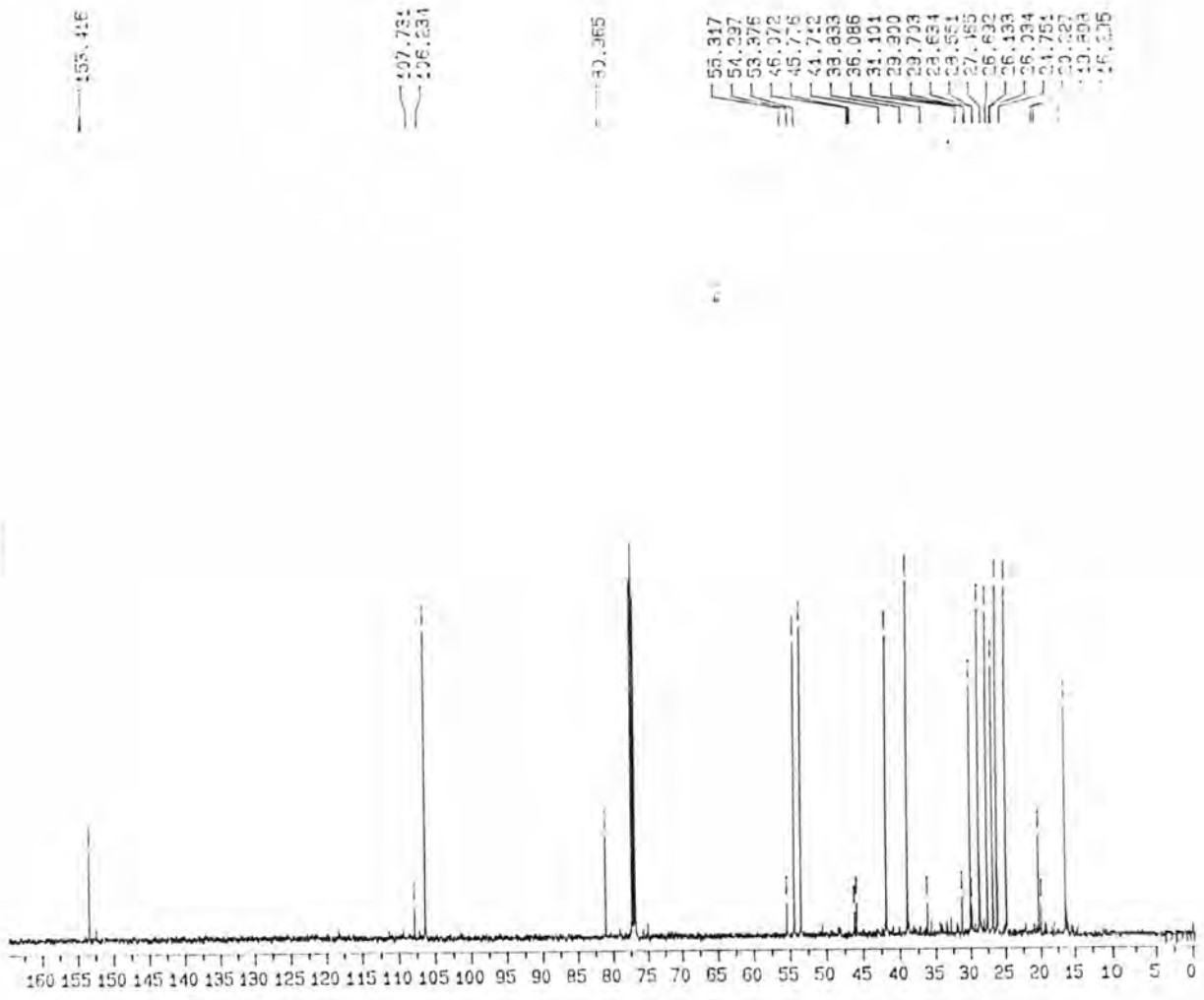


Fig. 60 The ^{13}C-NMR spectrum of compound **7**

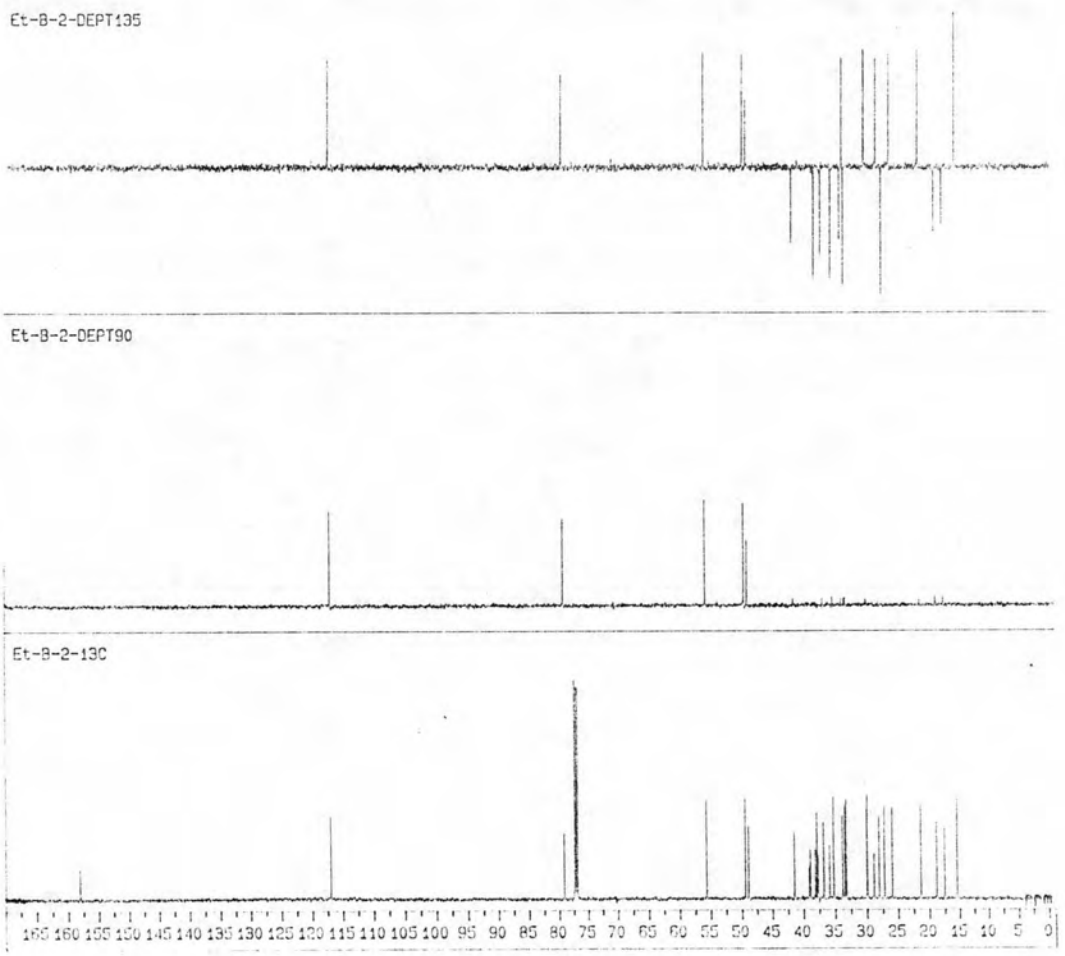
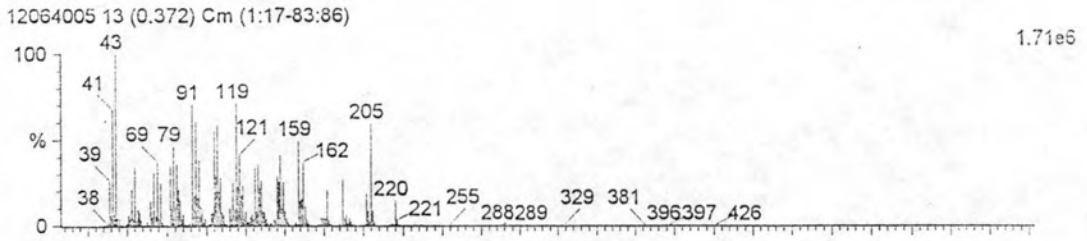
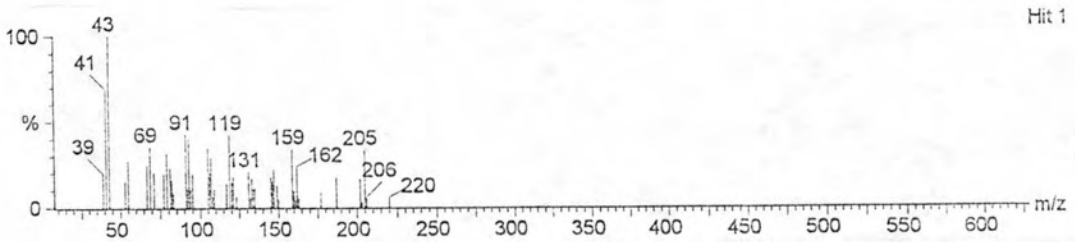


Fig. 61 The DEPT 90 and 135 ^{13}C-NMR spectrum of compound 7



a)



b)

Fig. 62 The mass spectrum of Compound **7** and (-)-Spathulenol (standard)

a) (-)-Spathulenol (standard)

b) Compound **7**

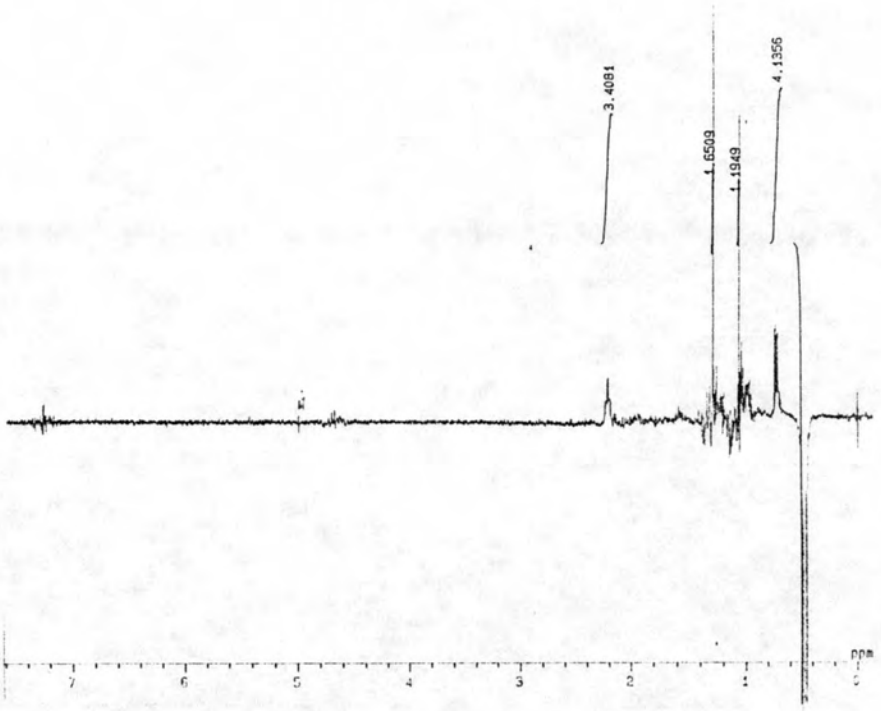


Fig. 63 The NOE difference spectrum of Compound 7
(irradiated at δ 0.46 ppm)

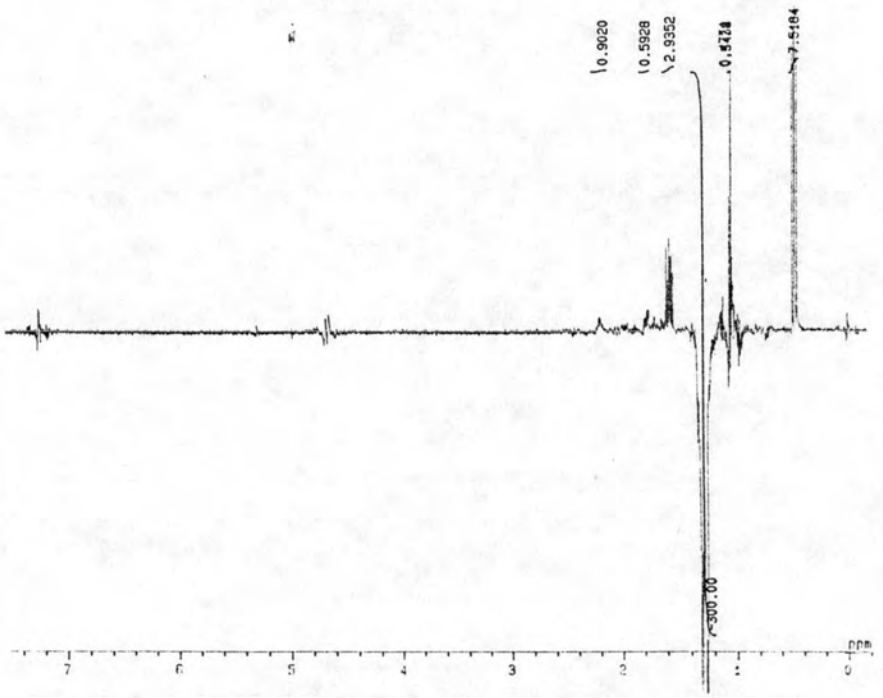


Fig. 64 The NOE difference spectrum of Compound 7
(irradiated at δ 1.28 ppm)

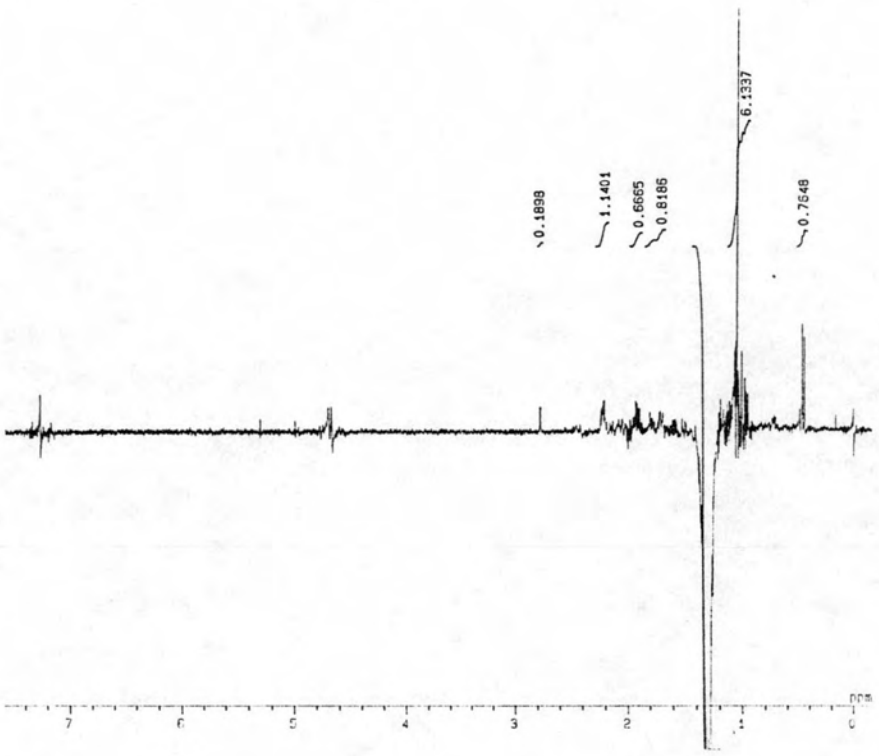


Fig. 65 The NOE difference spectrum of Compound 7

(irradiated at δ 1.31 ppm)

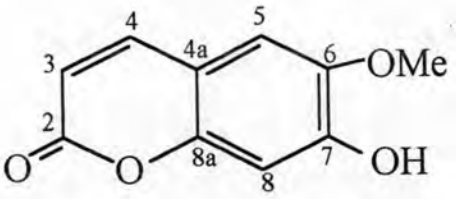
Structure elucidation of Compound 8

Compound **8**, yellow needle solid, was obtained from fraction no. 111-117 (small fraction of dichloromethane crude extract) by using chromatotron and eluted with 30% EtOAc in hexane (Table 22), 7.1 mg ($0.07x10^{-3}$% wt by wt); m.p. 182-184 ^{0}C. The R_f value was 0.46 (70% ethyl acetate in hexane as solvent). It was soluble in methanol. The IR spectrum showed the presence of the hydroxy group at 3000-3150 cm^{-1}, C=O stretching at 1715 cm^{-1} and C=C stretching aromatic at 1562 cm^{-1}(Fig. 66). Mass spectra (Fig. 67) indicated M^+ = 192 and showed mass fragments at 177 (M^+-Me), 149 (M^+-COMe) and 121 (M^+-$C_3H_3O_2$).

The ^{1}H-NMR spectrum displayed a typical pair of doublets (J =9.46 Hz) for H-3 (δ 6.16) and H-4(δ 7.84) and two uncoupled aromatic protons at δ 6.74 and δ 7.07 ppm, which were characteristic of a 6,7-disubstituted coumarin [48,49] and methoxy proton at δ 3.90 (Fig. 68).

The ^{13}C-NMR, DEPT 90 and 135 spectrum (Fig. 69-70) showed 10 signals as follow: one methoxy group at δ 56.8, four methine carbons at δ 104.1, 111.7, 111.9 and 147.2 and five quaternary carbons at δ 109.7, 146.2, 151.8, 154.6 and 164.3.

The information obtained from the IR spectrum suggested that compound **8** should be 6,7-disubstituted coumarin and the fragmentation ion pattern of mass spectra of this compound compared with those of reference mass spectra (NIST data base). The mass fragmentation pattern of compound **8** matched 7-hydroxy-6-methoxy coumarin.



7-hydroxy-6-methoxy coumarin

Further characterization of compound **8** was carried out by ^{13}C-NMR spectroscopy (Table 22). The assignments of the signals were made on the basis of a DEPT experiment. On data base indicated that compound **8** was 7-hydroxy-6-methoxy coumarin. The position of a methoxy group was also confirmed by the NOE difference technique. When the methoxy group (δ 3.90) was irradiated (Fig. 71), NOE was observed in the proton at δ 7.07 (H-5)which showed a NOE on irradiation of the proton at C-5 (Fig. 72). A H-5 was irradiated, NOE was observed in the methoxy group at δ 3.90 and proton at δ 7.84 (H-4) (Fig. 73).

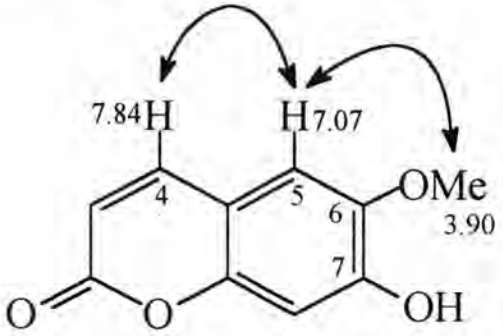


Table 22 The ^{1}H- and ^{13}C-NMR spectral data of Compound **8**

Position	^{1}H-NMR	^{13}C-NMR
2	-	164.3
3	6.16 [1H, d, J=9.46 Hz]	111.7
4	7.84 [1H, d, J=9.46 Hz]	147.6
5	-	109.7
5a	-	111.9
6	7.07 (s)	154.6
7	-	146.2
8	6.74 (s)	104.1
8a	-	151.8
OMe	3.90 (s)	56.8

From the above data, compound **8** could be assigned as 7-hydroxy-6-methoxy-coumarin, scopoletin.

A recently, scopoletin was isolated from *Santolina oblogifolia* Boiss. (Compositae), had not inhibition against Enzyme Immunoassay (EIA) on LTC_4 and PGE_2-release, but scopoletin and 6-methoxy-7-glycosidylcoumarin at the hightest dosage showed an inhibition rate of 55% on LTC_4 release.[50] Compound **8** showed cytotoxicity to brine shrimp at LC_{50} = 125.00 μg/ml.

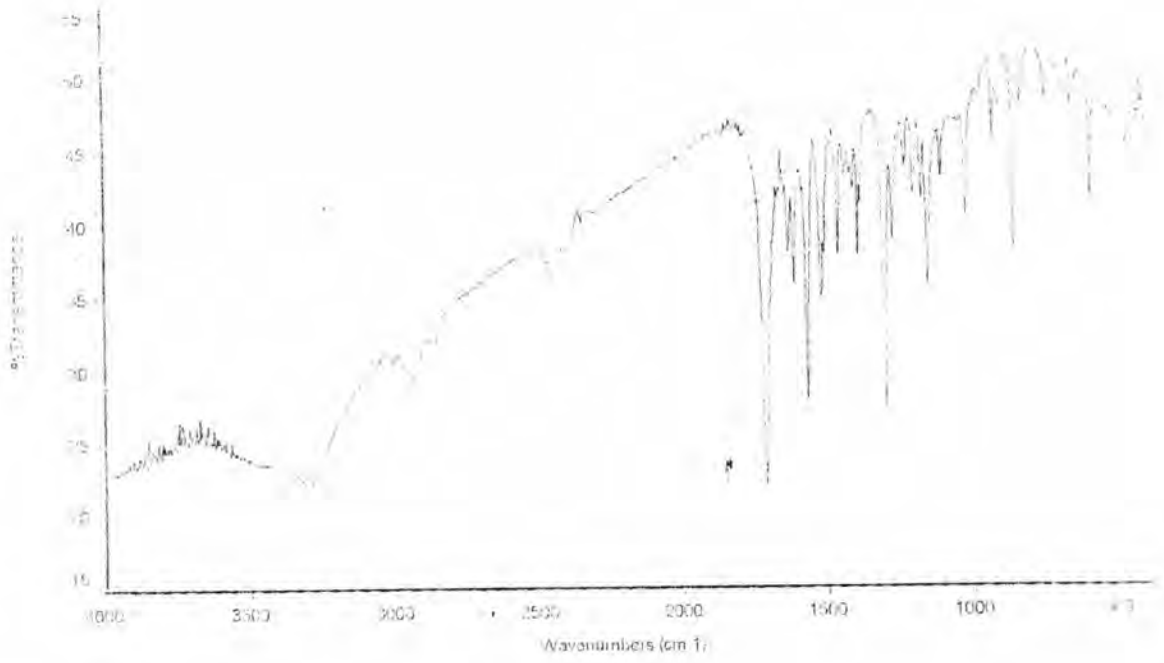
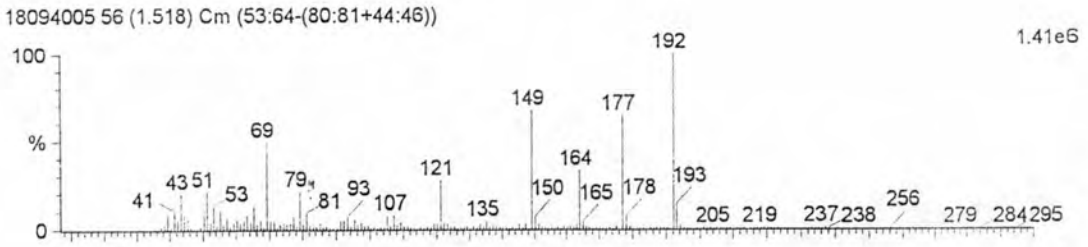
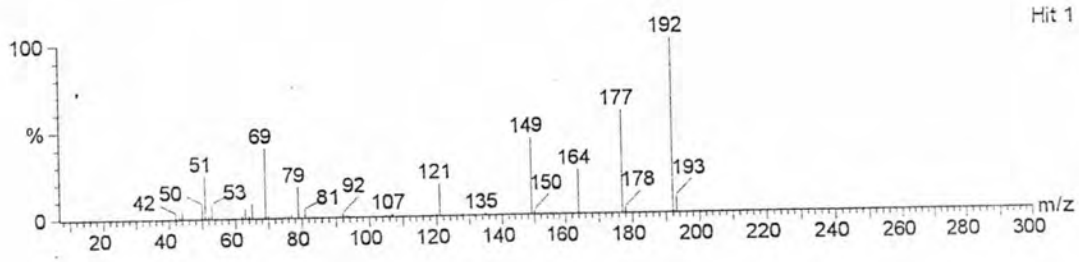


Fig. 66 The IR spectrum of Compound **8**



a)



b)

Fig. 67 The mass spectrum of Compound **8** and Scopoletin (Standard)

a) Scopoletin (Standard)

b) Compound **8**

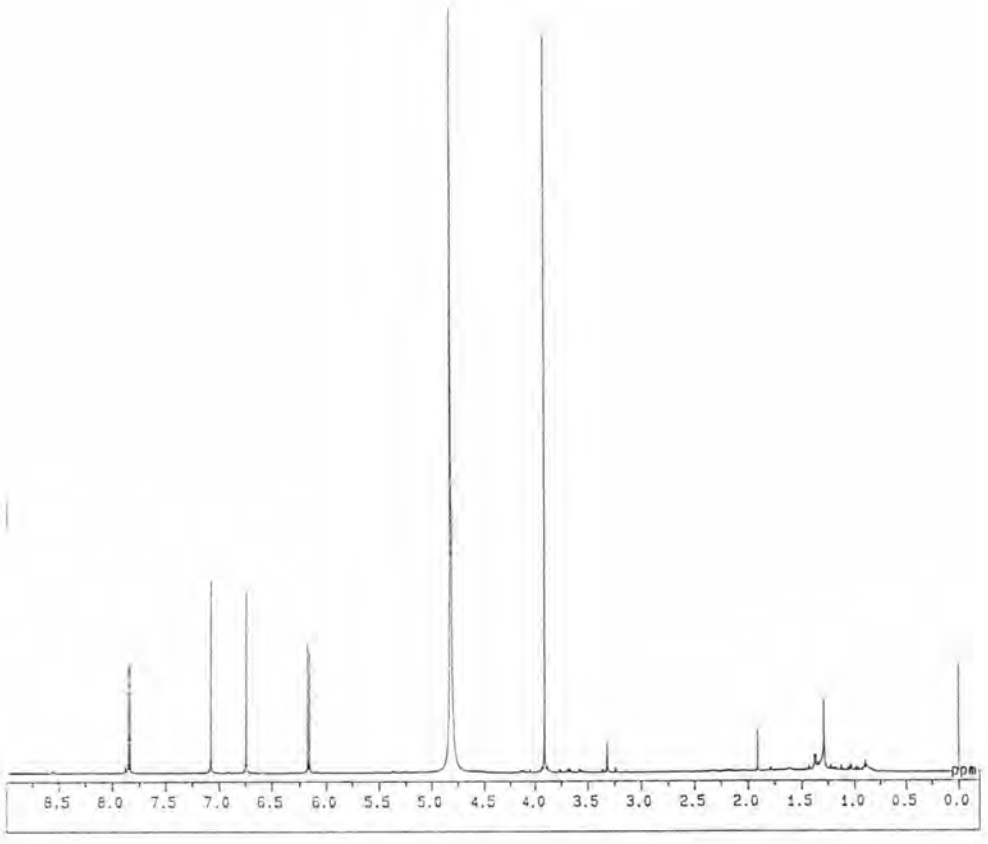


Fig. 68 The ^{1}H-NMR spectrum of Compound **8**

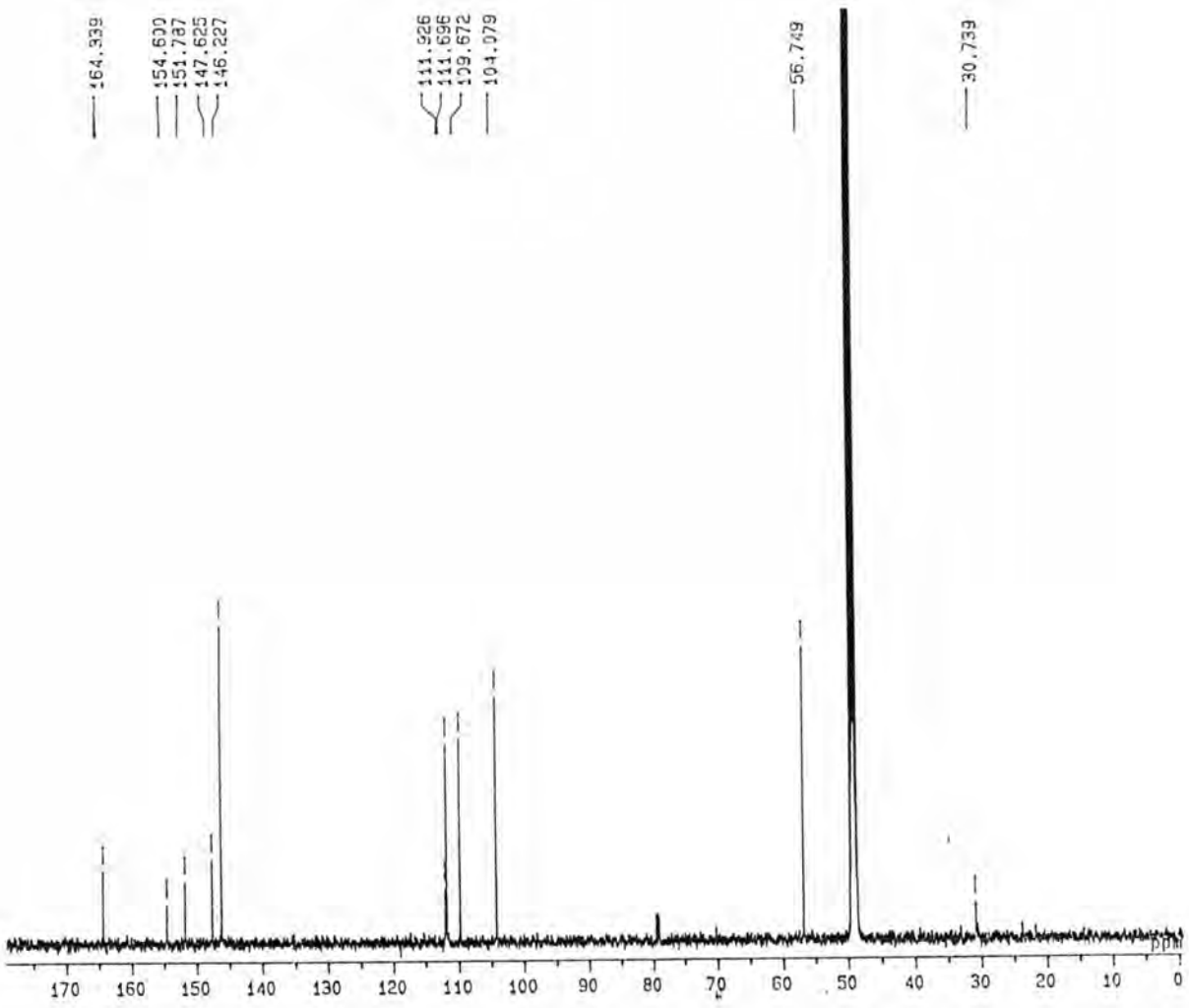


Fig. 69 The ^{13}C-NMR spectrum of Compound **8**

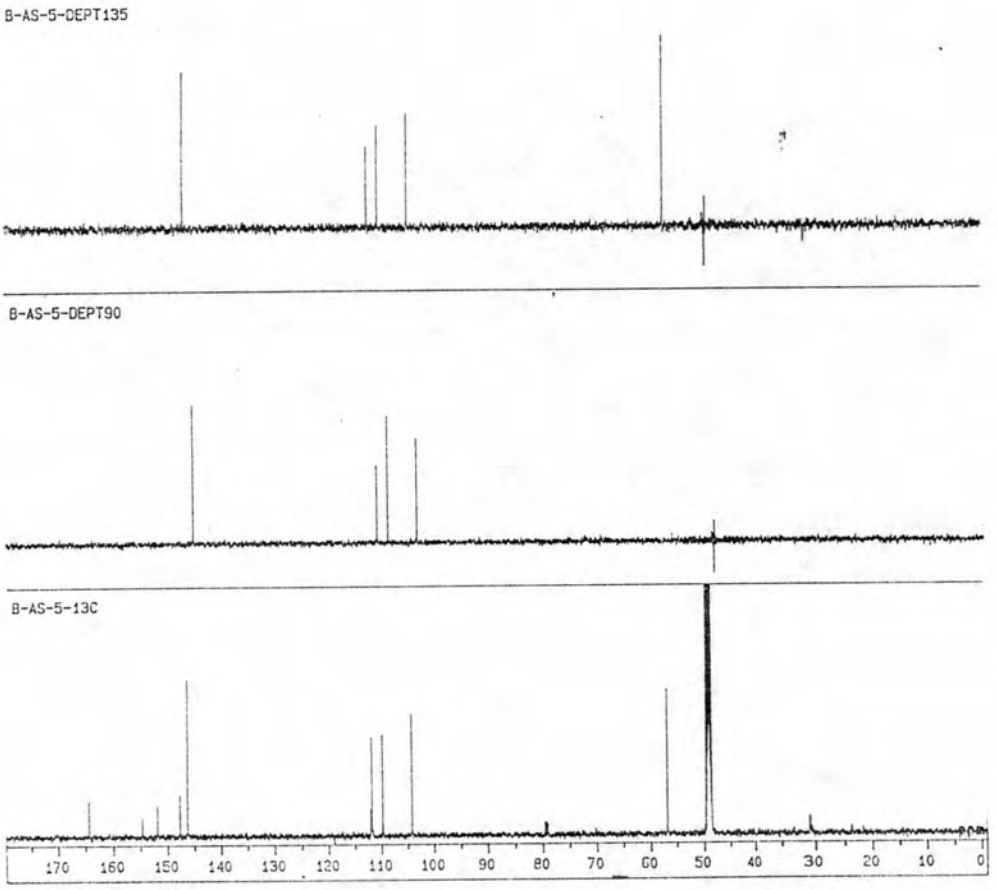


Fig. 70 The DEPT 90^0 and 135^0 ^{13}C-NMR spectrum of Compound **8**

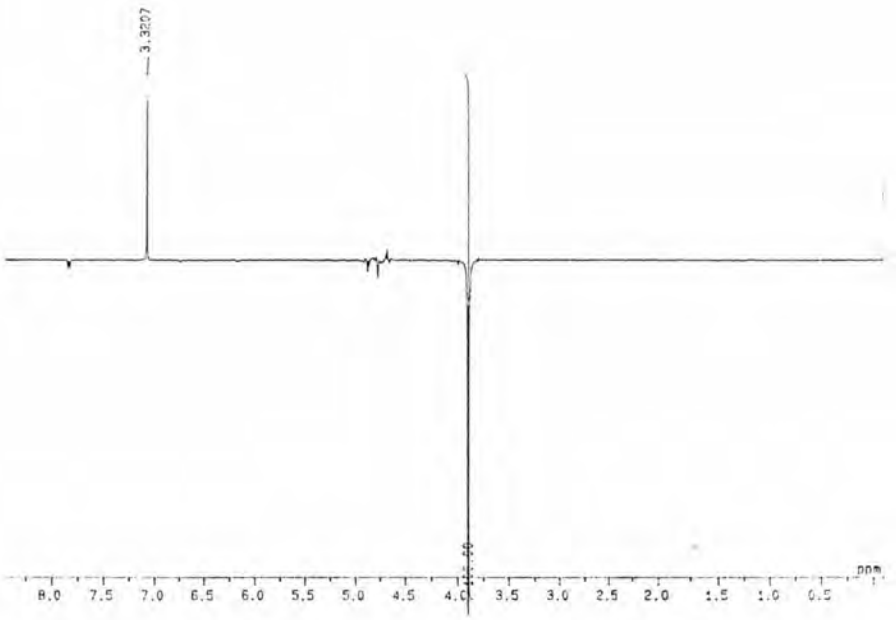


Fig. 71 The NOE difference spectrum of Compound **8**

(irradiated at δ 3.90 ppm)

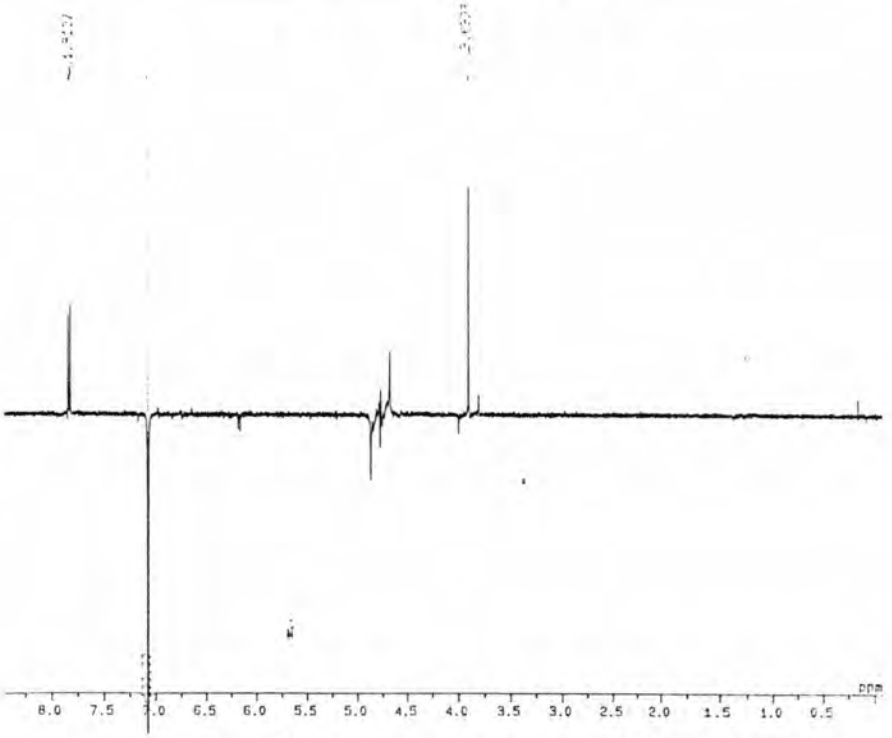


Fig. 72 The NOE difference spectrum of Compound **8**

(irradiated at δ 7.08 ppm)

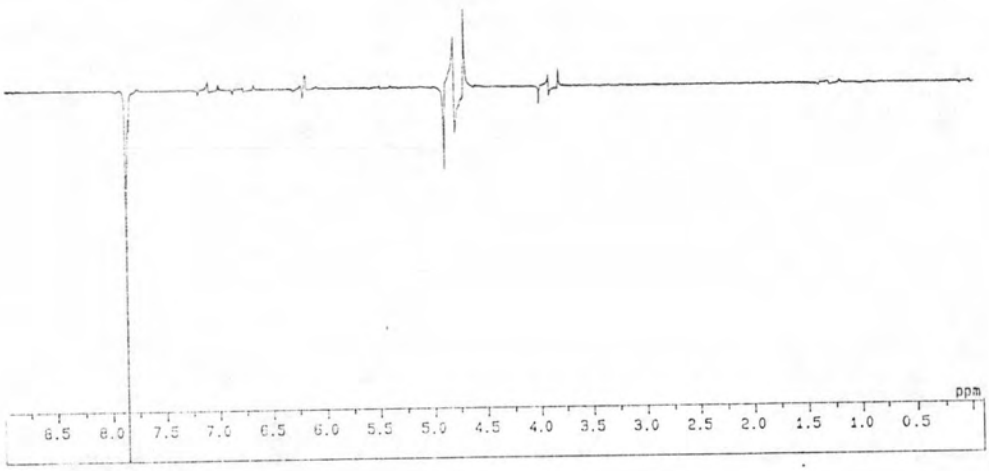


Fig. 73 The NOE difference spectrum of Compound **8**

(irradiated at δ 7.84 ppm)

Structure elucidation of Compound 9

Compound **9** was white solid in a brown oils, which was obtained from both the hexane and dichloromethane crude extracts. This compound was crystallized from chloroform and yielded 2.40 g (0.02% wt by wt of the roots). The R_f value was 0.36 [ethyl acetate : hexane (3:7)]; m.p. 157-158^{0}C. This compound was soluble in ethyl acetate, chloroform and dichloromethane but not in hexane.

The IR absorption band at 3420 cm^{-1} suggested the presented of hydroxy group (-OH). Unsaturation was also presented, C=C 1690 cm^{-1} and C-H stretching at 3010 cm^{-1}. The carbonyl absorption at 1730 cm^{-1} indicated to carbonyl ester group. Aliphatic absorption, C-H stretching region was also presented. Furan ring displayed at 1450, 1005 and 875 cm^{-1} (Fig. 74).

Table 23 The IR absorption band assignments of Compound **9**

Vibration	Wave number (cm^{-1})	Intensity
O-H stretching of R-OH	3420	Strong
C-H stretching of vinyl	3045	Moderate
C-H stretching of -CH_2, -CH_3	2961	Strong
C=O stretching of carbonyl ester	1730	Strong
C=C stretching of α,β-unsaturated carbonyl ester group	1690	Moderate
C-H bending of -CH2, -CH3	1450, 1325	Moderate
C-O stretching	1005	Moderate

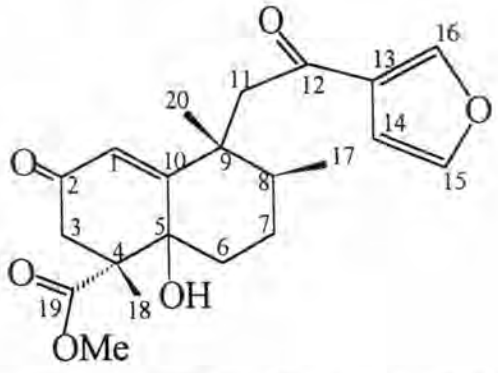
The molecular ion was observed at m/z 374 (Fig. 75) and other fragments were at m/z 265, 264, 189, 164 and 95 (indicated that it was carbonyl furanoditerpene). The ^{1}H-NMR (Fig. 76) showed three protons corresponding to β-substituted furan ring at δ 7.99, 7.41 and 6.64 (1H each, H-15, H-16 and H-14) [2,14], α,β-unsaturated keton

proton at δ 5.82 [15], methoxy group at δ 3.70 and clerodane protons at δ 0.89 to 3.26 ppm [34,36].

The ^{13}C-NMR spectrum and DEPT 90, 135 experiments (Fig 77-78) displayed 21 signals as follows: three methyl carbons (16.8, 19.5 and 25.9), one methoxy group (52.4), four methylene carbons (25.1, 31.8, 43.2 and 47.8), five methine carbons (35.2, 108.4, 125.6, 144.1 and 146.3) and eight quaternary carbons (41.2, 53.0, 72.7, 128.0, 167.6, 174.6, 190.9 and 197.8).

From these data indicated that compound **9** had 21 carbons and 26 protons, the presence of one methoxy group, one hydroxy group, three carbonyl groups and one furan ring suggested this compound had at least six oxygen atoms in the molecule. The molecular ion at m/z 374 indicated the molecular formular was $C_{21}H_{26}O_6$, corresponding to ^{1}H- and ^{13}C-NMR.

On literature search, in 1970 Aiyar Sato and coworker have been studied chemical constituents of *A. siamensis*, they isolated major substance, Chettaphanin I [5]. Its molecular formula was $C_{21}H_{26}O_6$; m.p. 157-158 ^{0}C. This data revealed that compound **9** was Chettaphanin I, which that recently isolated from the roots of *C. cassifolius*. [12] The ^{1}H- and ^{13}C- NMR data closely matched those reported.



Chettaphanin I was inactive (ED_{50}> 50μg/ml) in the KB and P-388 *in vitro* cytotoxicity test system [12] and inactive with antifungal (*C. cucumerinum* and *C. albicans*), antioxidant (DPPH and β-carotene), lavicidal (*Aedes aegypti*), brine shrimp (LC_{50}>1000) but weakly inhibition against antifeedant (*G. mellonella*).

Table 24 The ^{13}C-NMR spectral of Compound **9** (125 MHz) compared with Chettaphanin I[12]

Position	Chemical shift (δ ppm)	
	Chettaphanin I[12]	Compound **9**
1	125.7	125.6
2	190.9	190.9
3	53.1	53.0
4	43.2	43.2
5	72.8	72.7
6	32.1	31.8
7	25.3	25.1
8	35.4	35.2
9	41.7	41.2
10	167.2	167.6
11	48.0	47.8
12	197.4	197.8
13	128.2	128.0
14	108.4	108.4
15	144.0	144.1
16	149.6	146.3
17	16.7	16.7
18	25.8	25.9
19	174.5	174.6
20	19.5	19.5
OMe	52.3	52.4

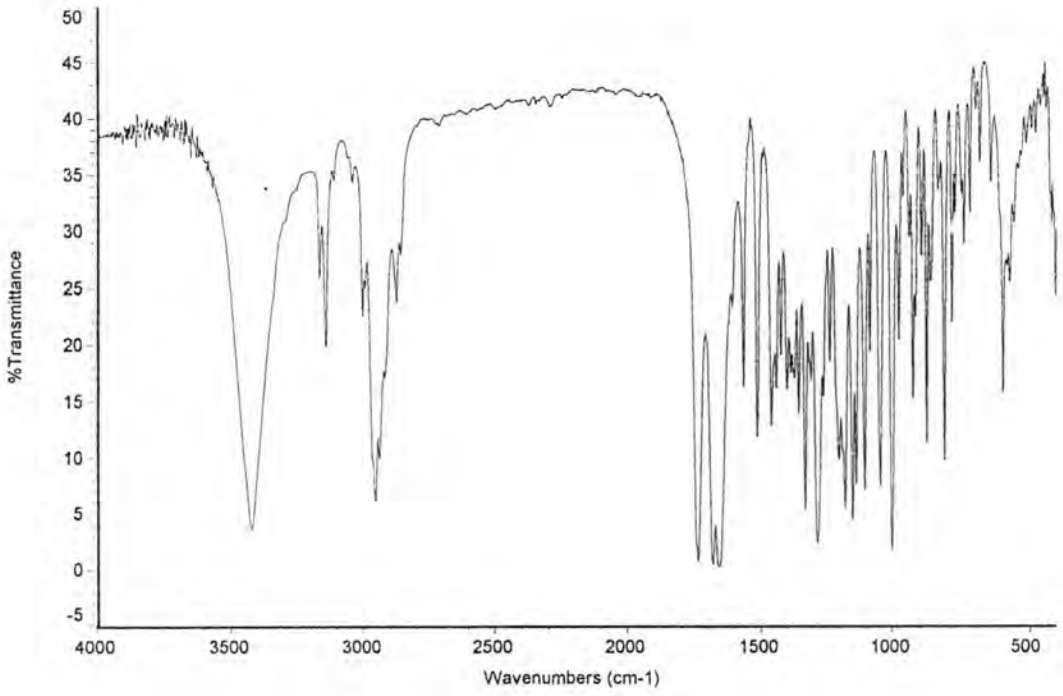


Fig. 74 The IR spectrum of Compound **9**

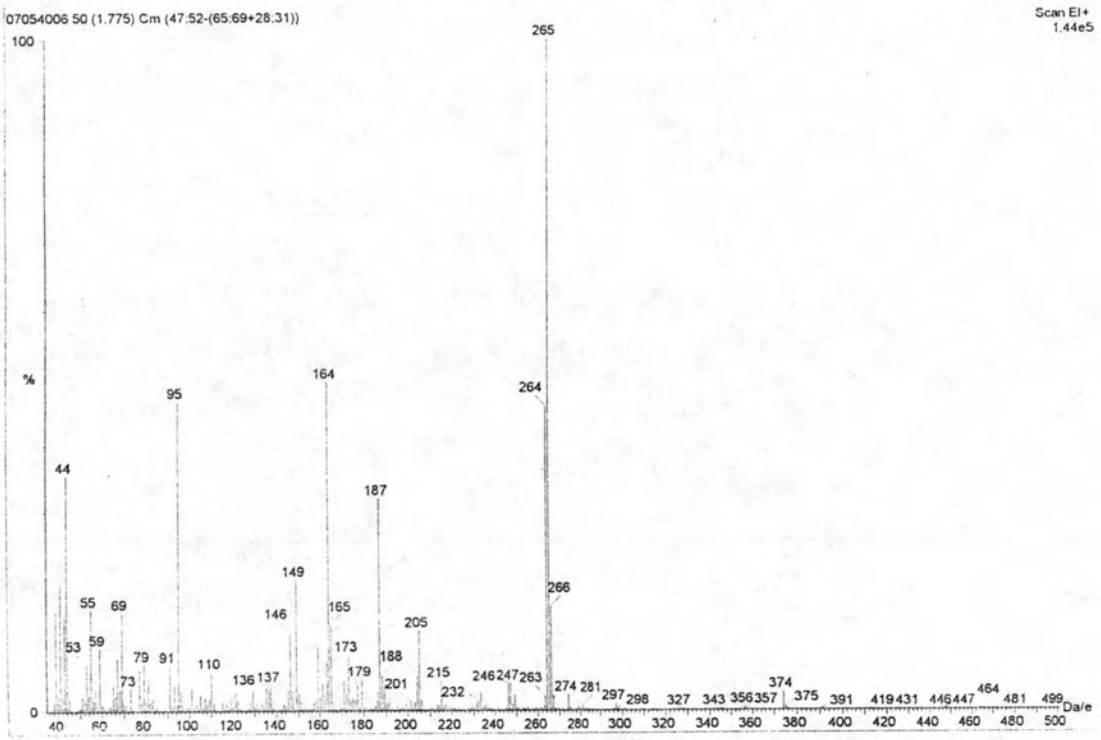


Fig. 75 The mass spectrum of Compound **9**

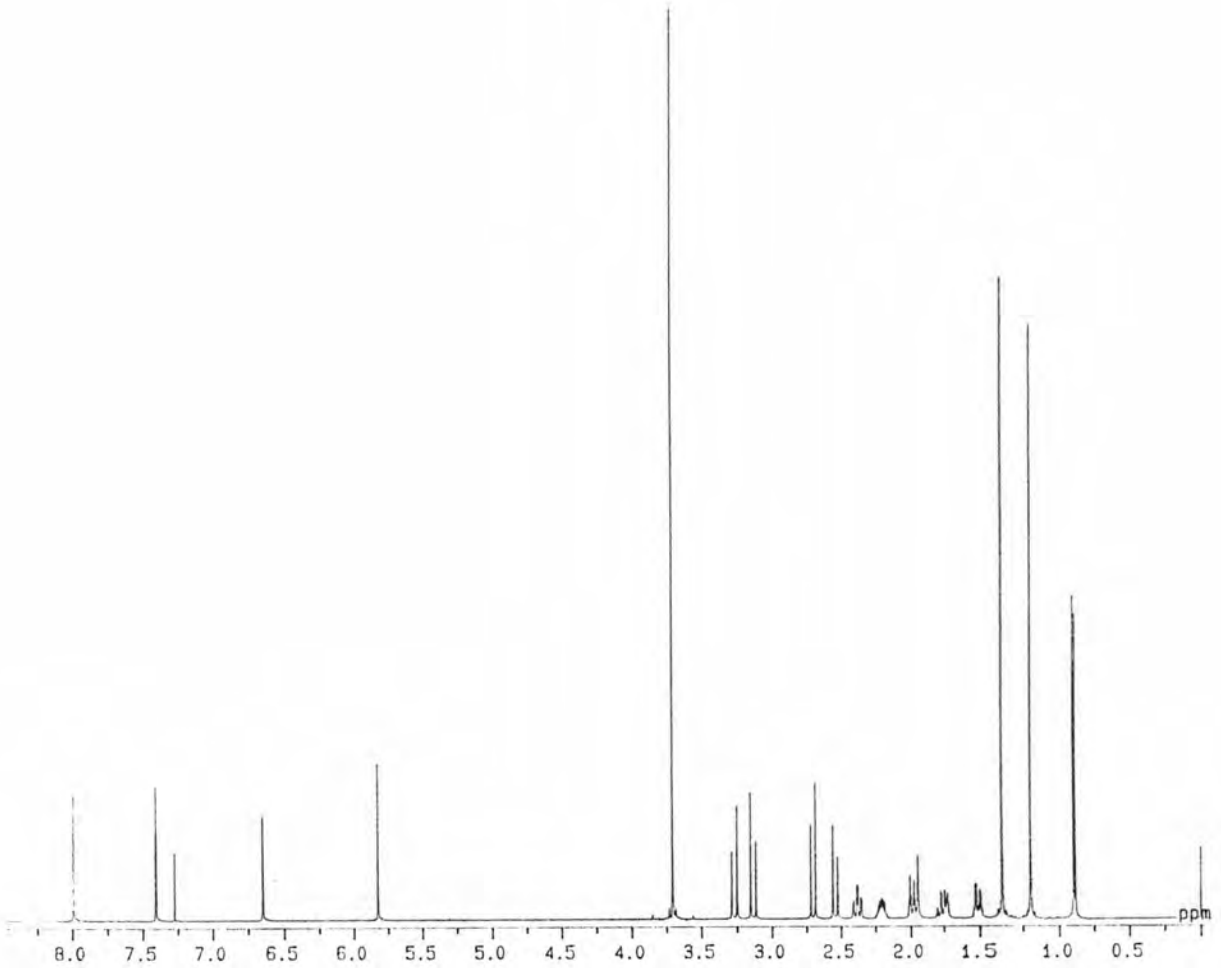


Fig. 76 The ^{1}H-NMR spectrum of Compound **9**

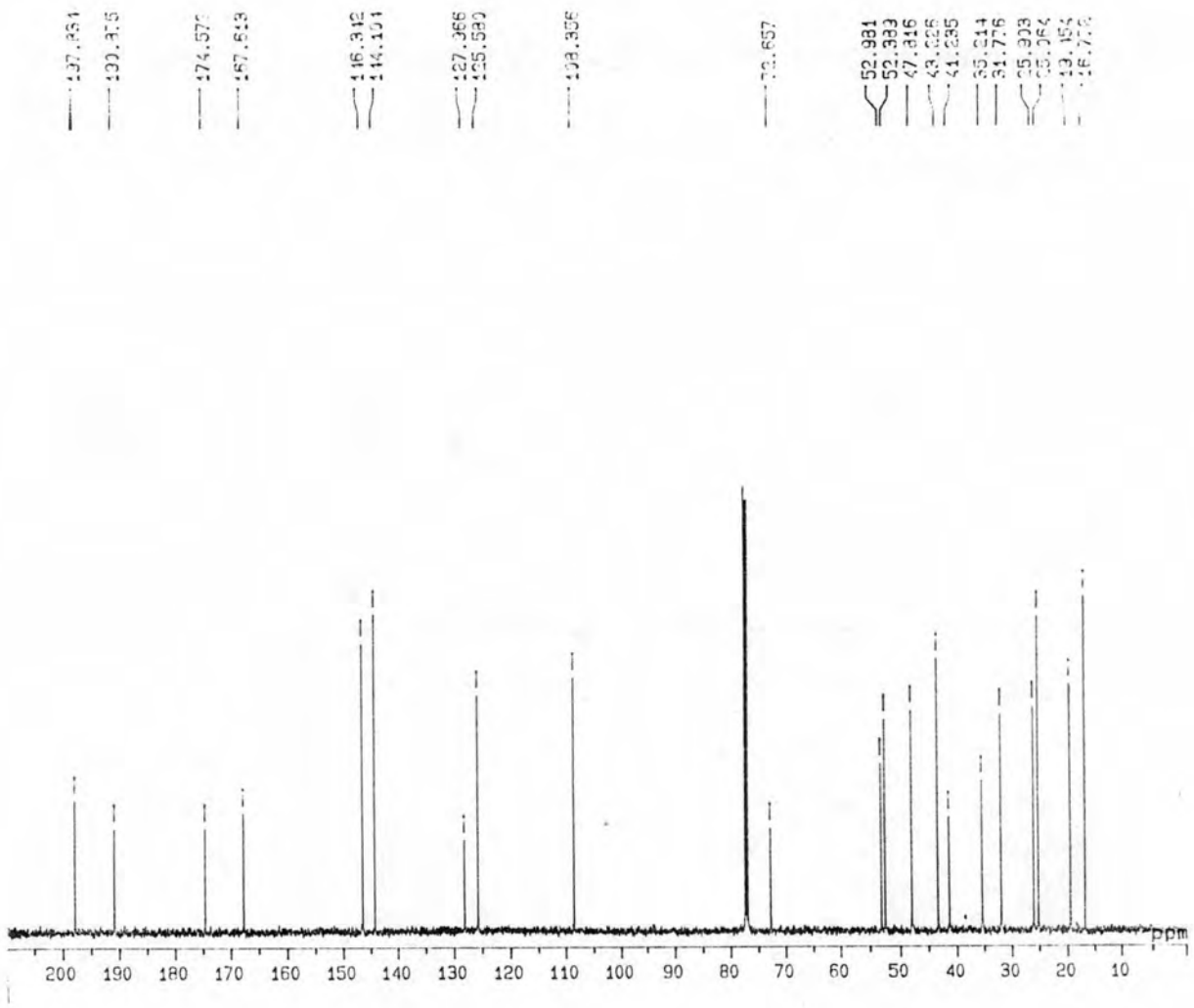


Fig. 77 The ^{13}C-NMR spectrum of Compound **9**

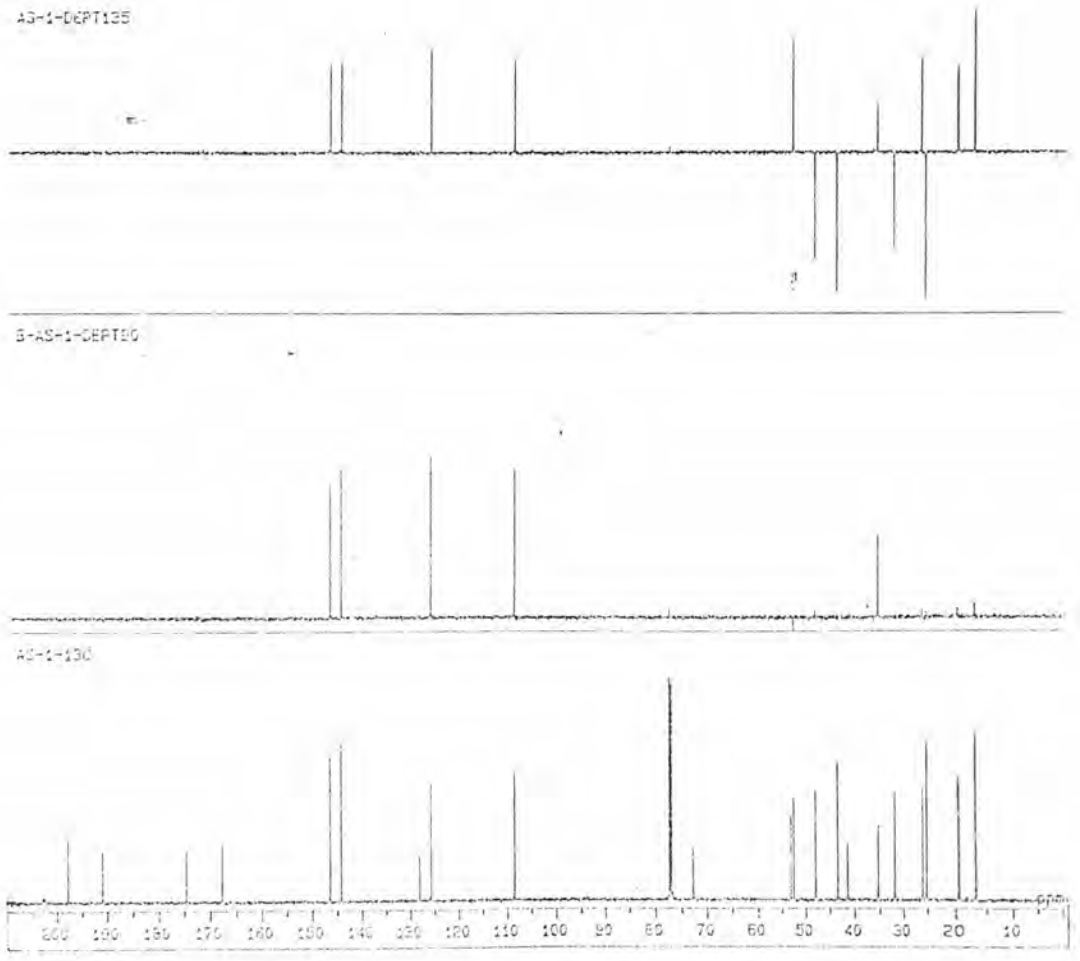


Fig. 78 The DEPT 90^0 and 135^0 ^{13}C-NMR spectrum of Compound **9**

Structure elucidation of Compound 10

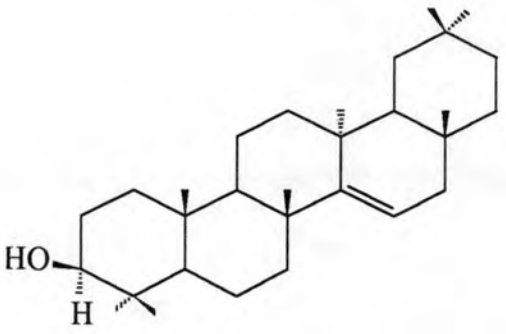
Compound **10** was white needle crystal, 52 mg ($0.52x10^{-3}$ % wt by wt of the roots),isolated from both hexane and ethyl acetate crude extracts (Table 3-5). This compound was purified by recrystalization with hot chloroform. The melting point was 278-280 ^{0}C and R_f value was 0.70 in 50% ethyl acetate in hexane. It was soluble in hot chloroform but not in hexane and dichloromethane and gave positive test with Liebermann-berchard reagent.

The IR spectrum of compound **10** was shown hydroxyl absorption at 3493cm^{-1}. Peaks at 3052 and 1644 cm^{-1} suggested the presence of vinyl group and at 2935, 2863, 1481 and 1383 cm^{-1} revealed an hydrocarbon system. This spectrum contained additional peak at 1040 cm^{-1} for C-O stretching (Fig. 79).

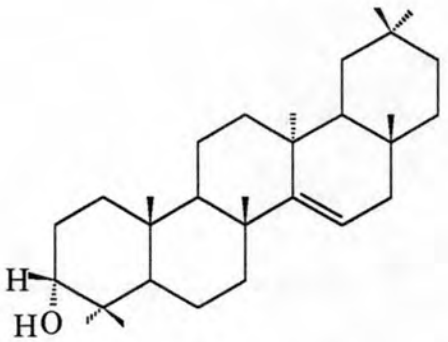
The mass spectra presented a molecular ion at m/z 426 showed fragments at m/z 411, 393, 302, 204, 189 and 133 (Fig. 80).

The ^{1}H-NMR spectrum of compound **11** showed signals at δ 5.53 due to a vinyl protons quartet at δ 3.19 demonstrated the presence of carbinal proton and eight tertiary methyl signals between δ 0.81 to 1.10 and methylene proton at δ 1.22 to 2.03 (Fig. 81).

The ^{13}C-NMR spectrum (Table 25) showed 20 lines. DEPT analysis using a nutation angle of 90^0, indicated five methine at δ116.4, 79.1, 55.6, 49.3 and 48.7. Besides the methine carbons, the DEPT 135^0 spectrum showed ten methylene carbons and eight methyl carbons indicating that the carbons at δ 153.1, 39.0, 38.8, 37.7, 37.6, 35.8 and 28.8 were non-hydrogenated, after comparison with the decoupled spectrum (Fig. 82-83). From ^{13}C-NMR , this compound was taraxerol or isotaraxerol.

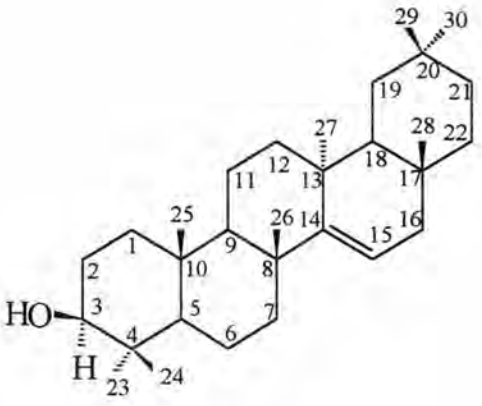


Taraxerol



Isotaraxerol

The ^{1}H-NMR spectra of taraxerol and isotaraxerol showed the expected differences in the carbinol proton region. The C-3 proton in isotaraxerol appeared as a well defined triplet centred at δ 3.38 ppm (J=3Hz), typical of an equatorial proton associated with a 3α-hydroxy group in ring A of triterpenoid, whereas the C-3 proton in taraxerol appeared as ill-defined quartet (δ 3.22 ppm), typical of the axial proton associated with a 3β-hydroxy group. Melting point of taraxerol was 282-283 ^{0}C but melting point of isotaraxerol was 267-269 ^{0}C [51], which indicated that compound **10** was taraxerol. By confirm, this compound was direct compared with an authentic sample.



Taraxerol

This compound was inactive to brine shrimp cytotoxicity test, fungal (*C. cucumerinum* and *C. albicans*), bacterial *E. coli*, *B. cereus* and *S. derby* and insect antifeedant (*G. mellonella*).

Table 25 The ^{13}C-NMR spectral data of Compound **10** (125 MHz) compared with taraxerol [52]

Position	Chemical shift (δ ppm)	
	Taraxerol [52]	Compound **10**
1	38.7	38.0
2	27.3	27.2
3	79.1	79.1
4	38.8	38.8
5	55.6	55.5
6	18.9	18.8
7	33.2	33.1
8	39.1	39.0
9	49.4	49.3
10	37.6	37.6
11	17.6	17.5
12	36.8	36.7
13	37.7	37.7
14	158.2	158.1
15	117.0	116.9
16	33.8	33.4
17	35.8	35.8
18	48.9	48.8
19	41.4	41.3
20	28.8	28.8
21	35.2	35.1
22	37.8	37.7
23	28.0	28.0
24	15.5	15.5
25	15.5	15.4
26	26.0	25.9

Table 25 (cont.)

Position	Chemical shift (δ ppm)	
	Taraxerol [52]	Compound **10**
27	29.7	29.8
28	29.9	29.9
29	33.4	33.7
30	21.4	21.3

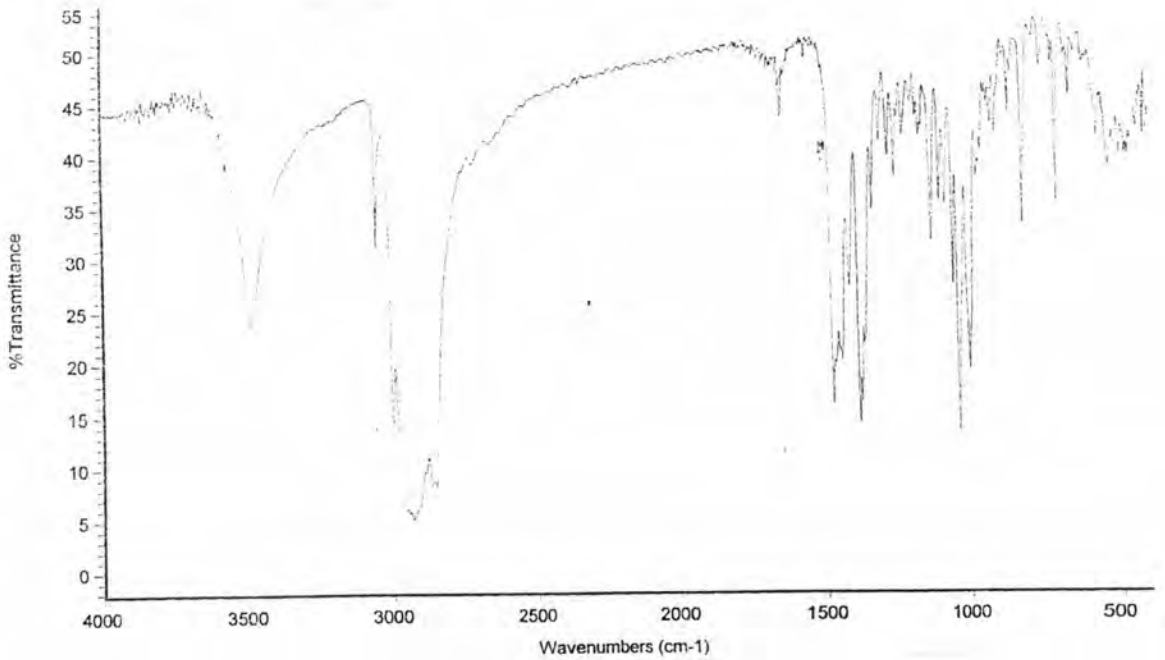


Fig. 79 The IR spectrum of Compound **10**

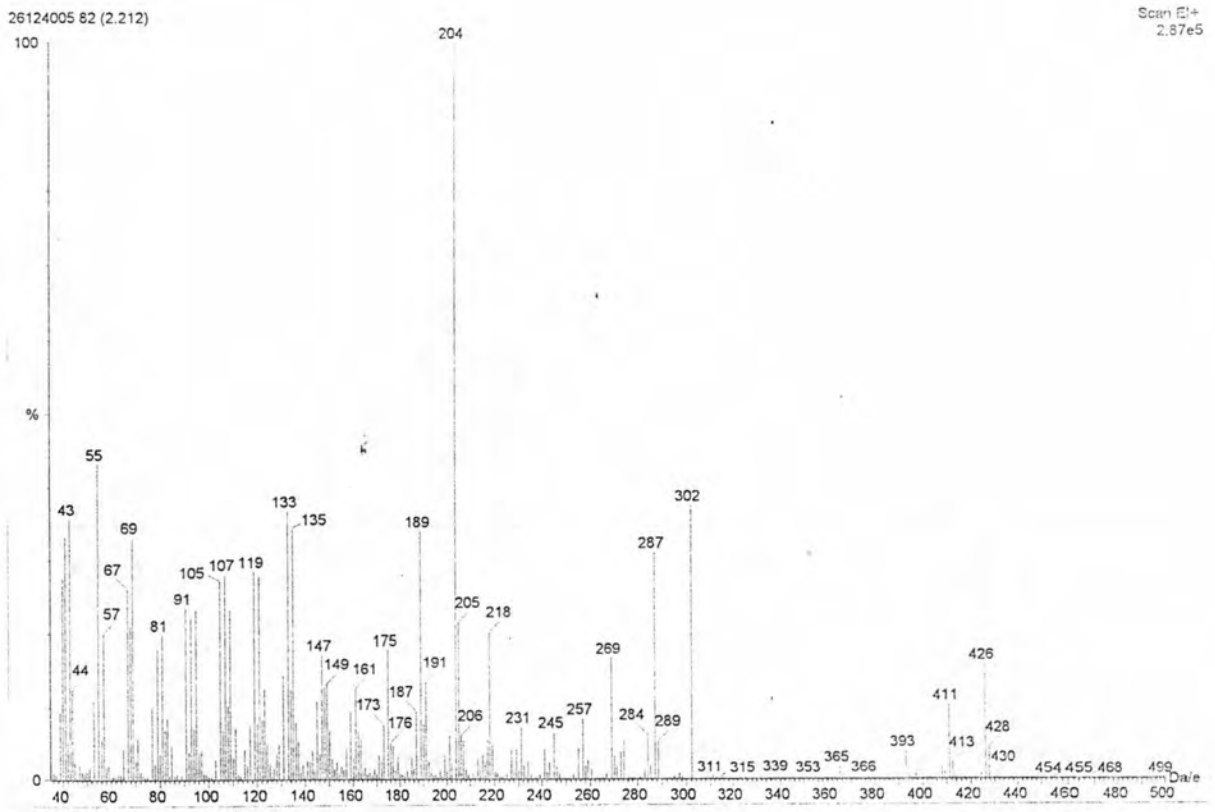


Fig. 80 The mass spectrum of Compound **10**

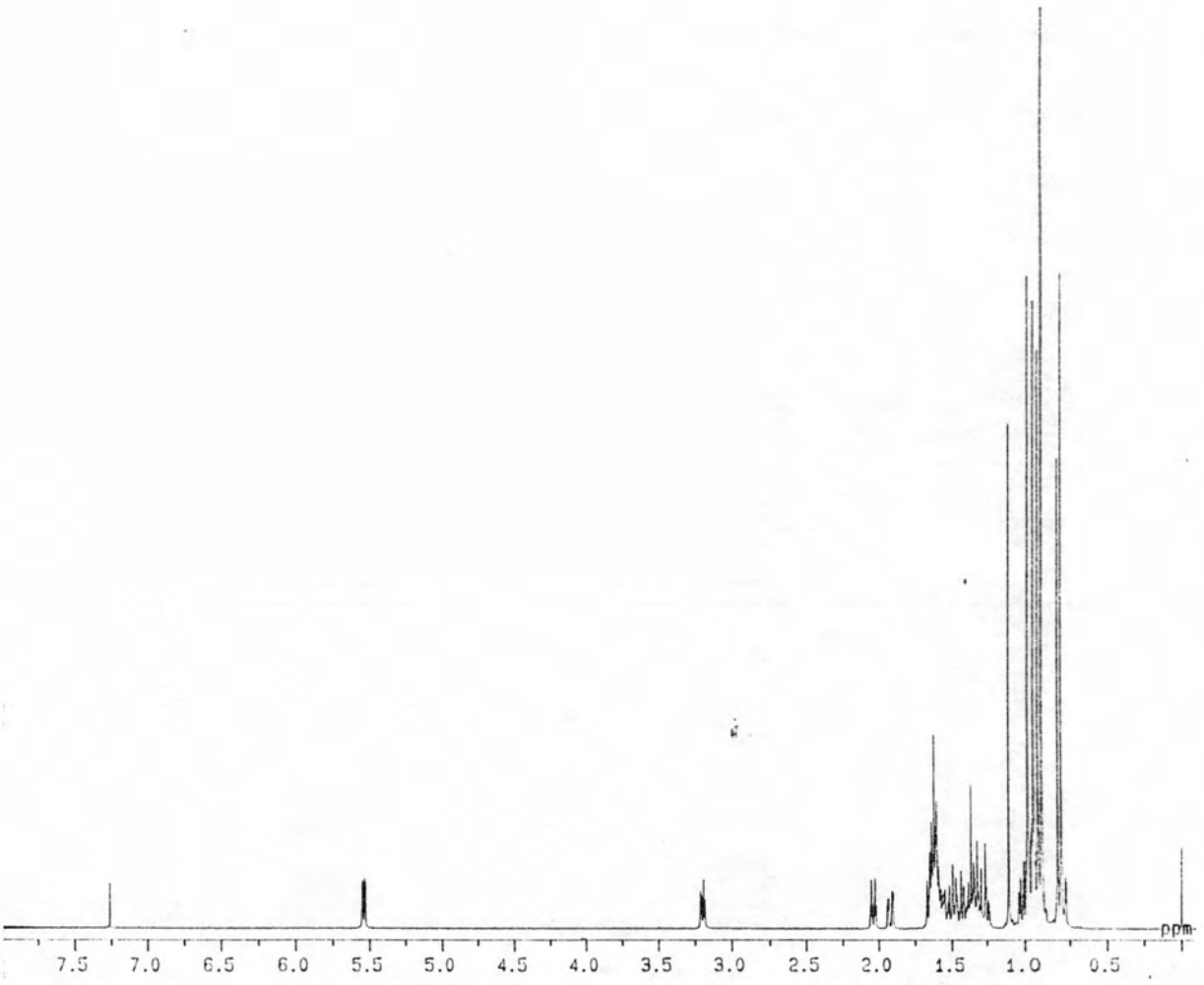


Fig. 81 The ^{1}H-NMR spectrum of Compound **10**

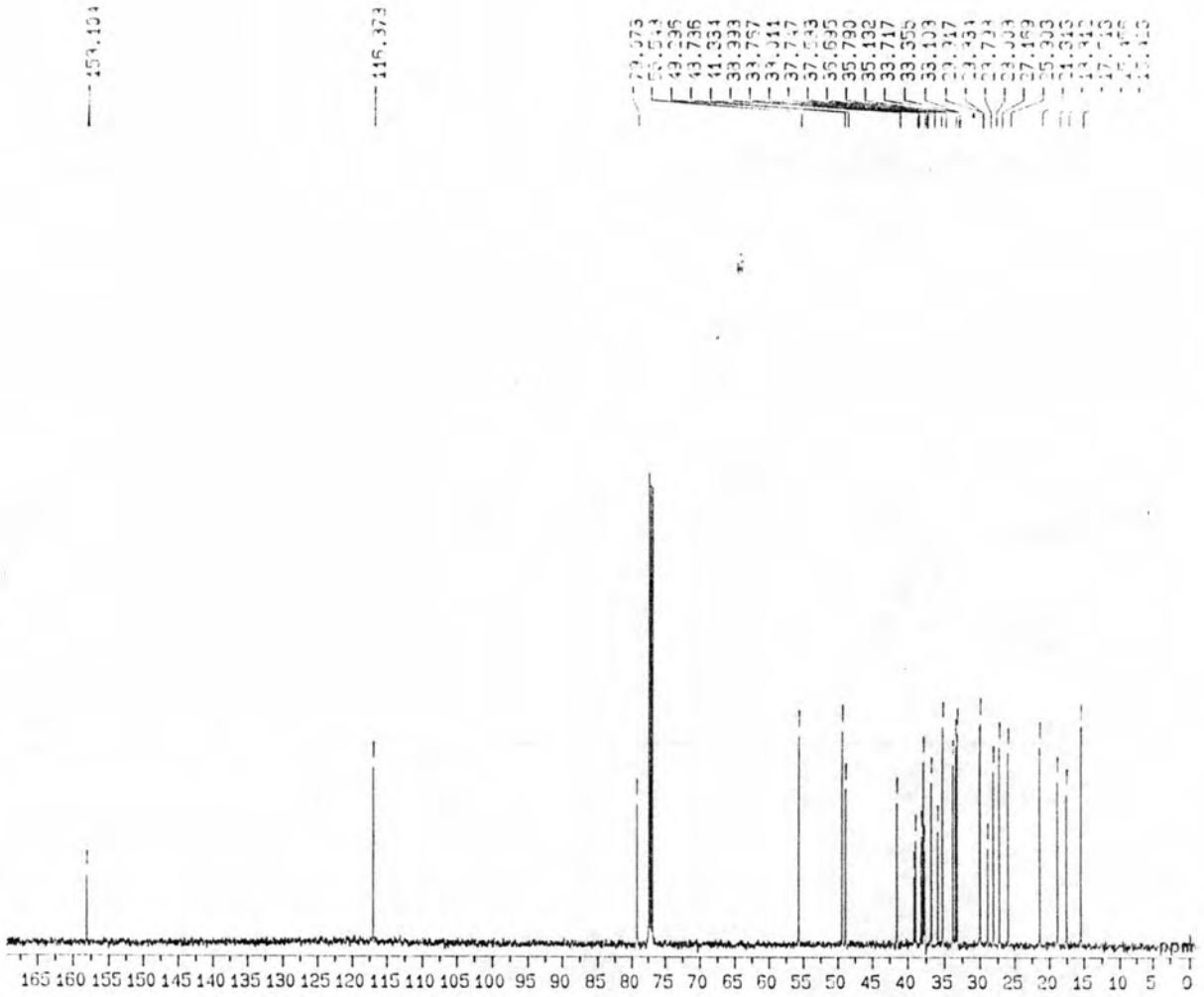


Fig. 82 The ^{13}C-NMR spectrum of Compound **10**

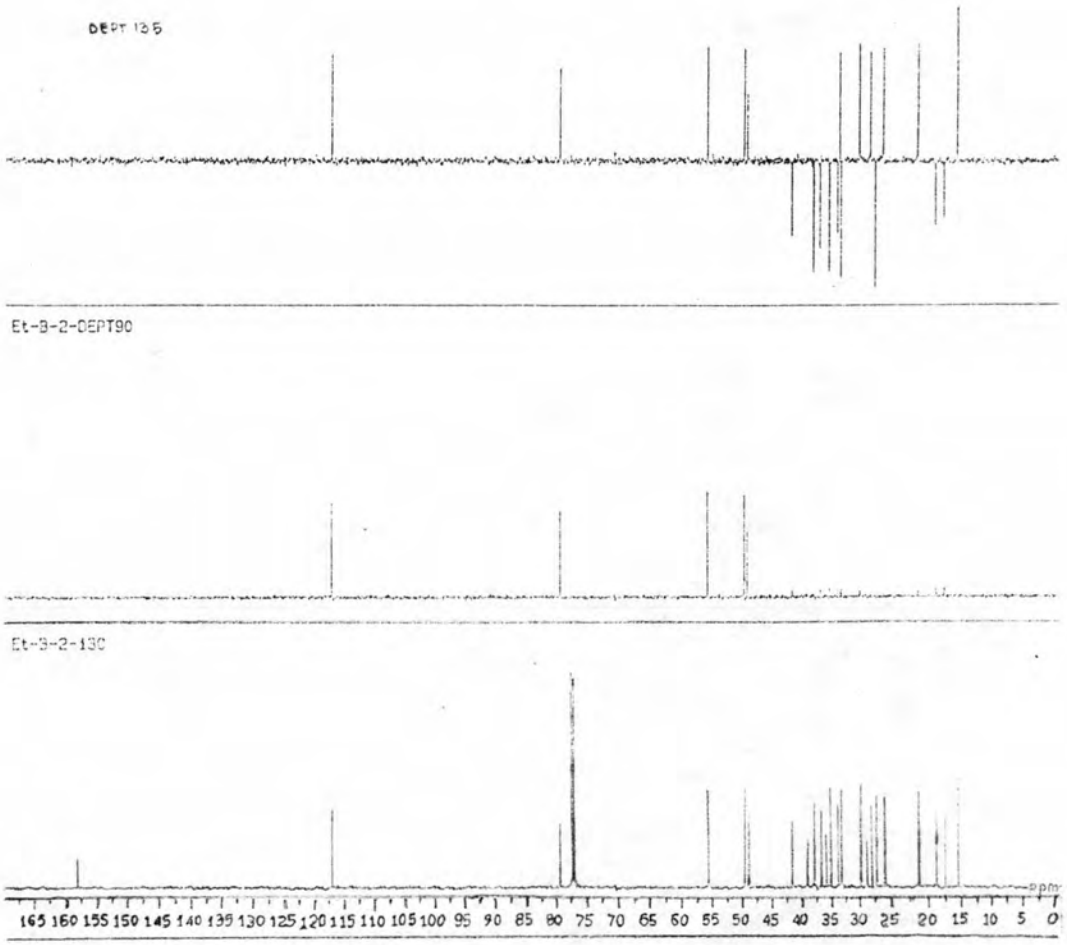


Fig. 83 The DEPT 90^0 and 135^0 ^{13}C-NMR spectrum of Compound **10**