CHAPTER 3
RESULT AND DISCUSSION

3.1 Primary Bioassay Screening Results of Crude Extracts

3.1.1 Brine Shrimp Cytotoxic Lethality Test (BSCLT) Result

This primary screening was carried out by the staffs at the Department of Biology, Faculty of Science, Chulalongkorn University. Firstly, ethanolic extract of the stems exhibited medium activity with LC_{50} 67.17. The stems were extracted again with various solvents to afford 5 crude extracts. BSCLT result of these extracts was displayed in table 3.1.

Table 3.1 Brine shrimp cytotoxic lethality test of various crude extracts

Crude extracts	LC_{50}'s (μg/ml)	Bioactivity*
Hexane extract	1.38	high activity
Dichlomethane extract	0.04	high activity
Ethyl acetate extract	47.18	medium activity
Butanol extract	16.05	medium activity
Ethanolic residue	2.29	high activity

3.1.2 Anticell Lines Screening Result

The screening result of various crude extracts inhibited against seven cell lines was presented in Figure 3.1-3.7

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* High activity (LC_{50}< 10 μg/ml)
Medium activity (LC_{50}< 100 μg/ml)
Low activity (LC_{50}< 1000 μg/ml)
No activity (LC_{50}>1000 μg/ml)

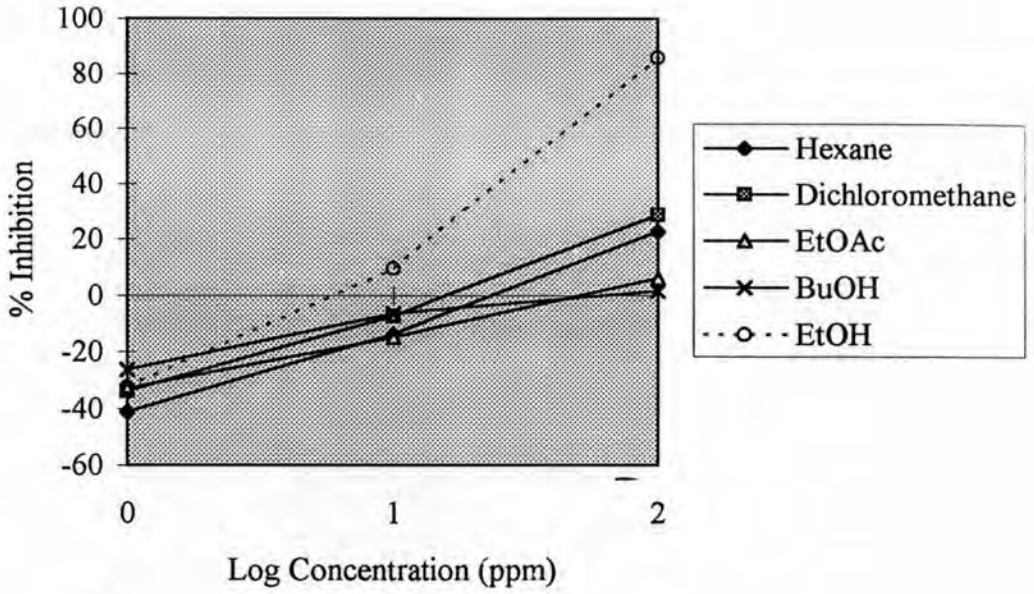


Figure 3.1 Primary screening of various crude extracts inhibited against Human Gastric Carcinoma (BGC-823)

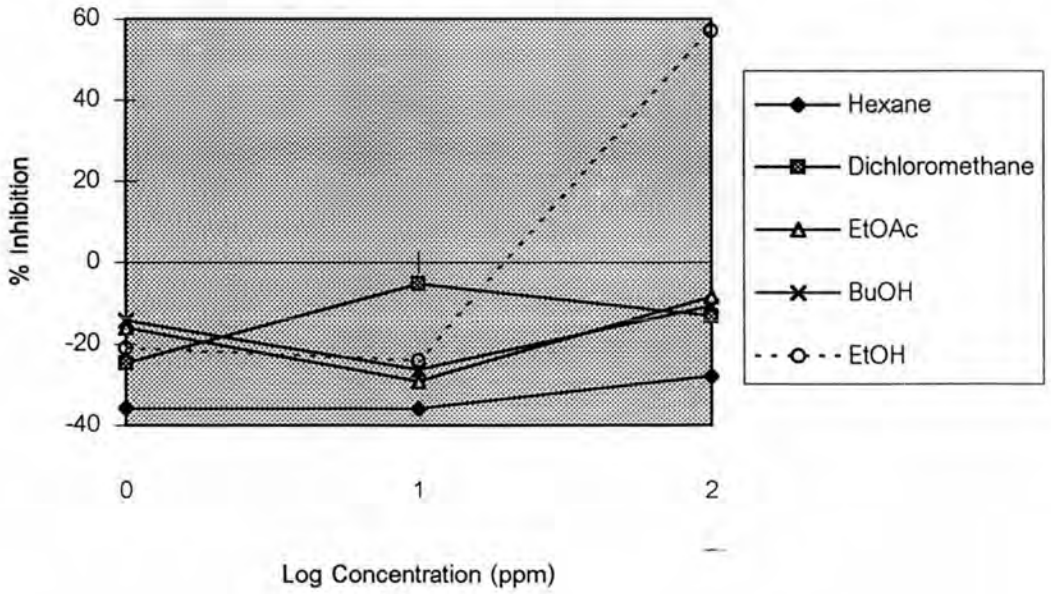


Figure 3.2 Primary screening of various crude extracts inhibited against Human Hepatocellular Carcinoma (Bel-7402)

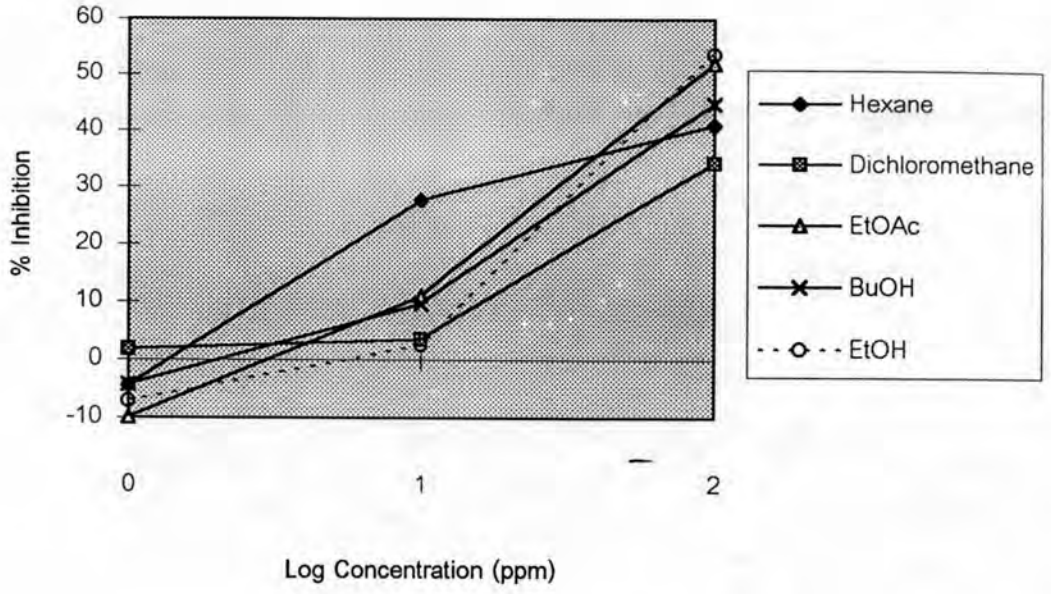


Figure 3.3 Primary screening of various crude extracts inhibited against Human Erythroleukemia Carcinoma (K562)

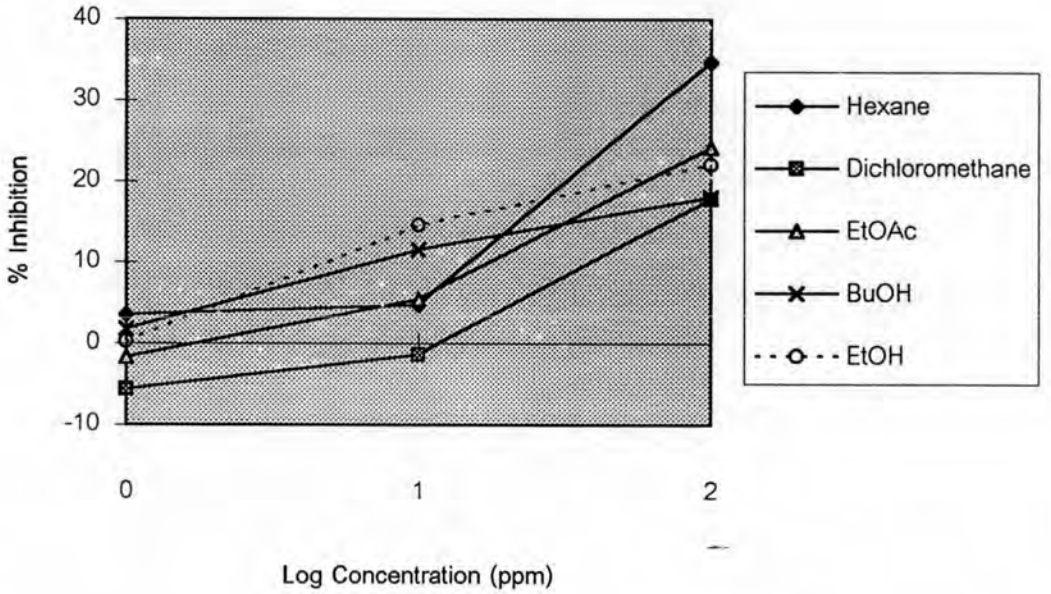


Figure 3.4 Primary screening of various crude extracts inhibited against Human Bladder Carcinoma (BIU)

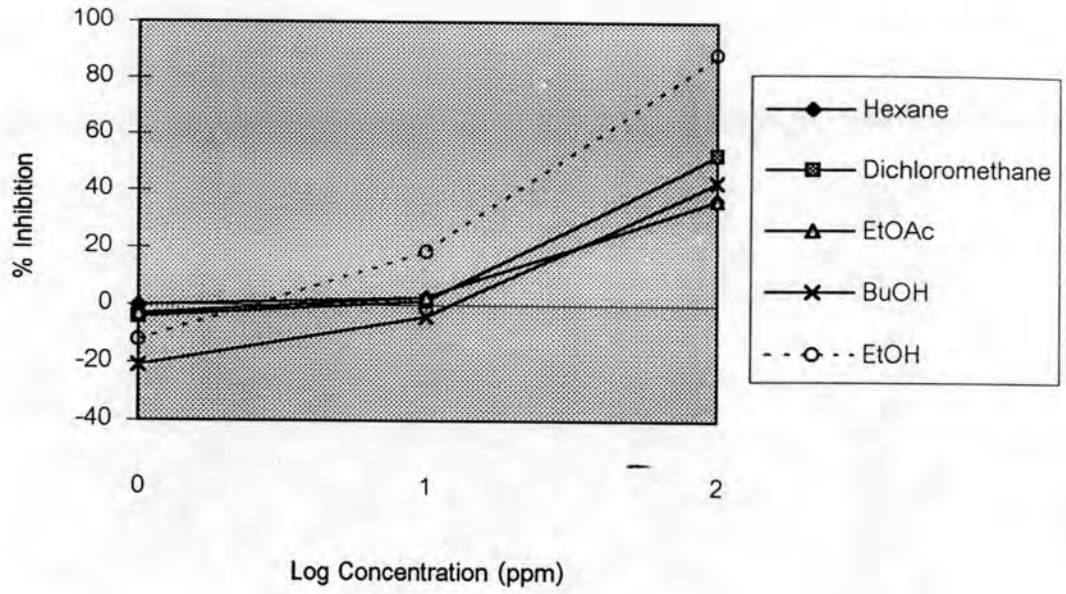


Figure 3.5 Primary screening of various crude extracts inhibited against Human Nasopharyngeal Carcinoma (KB)

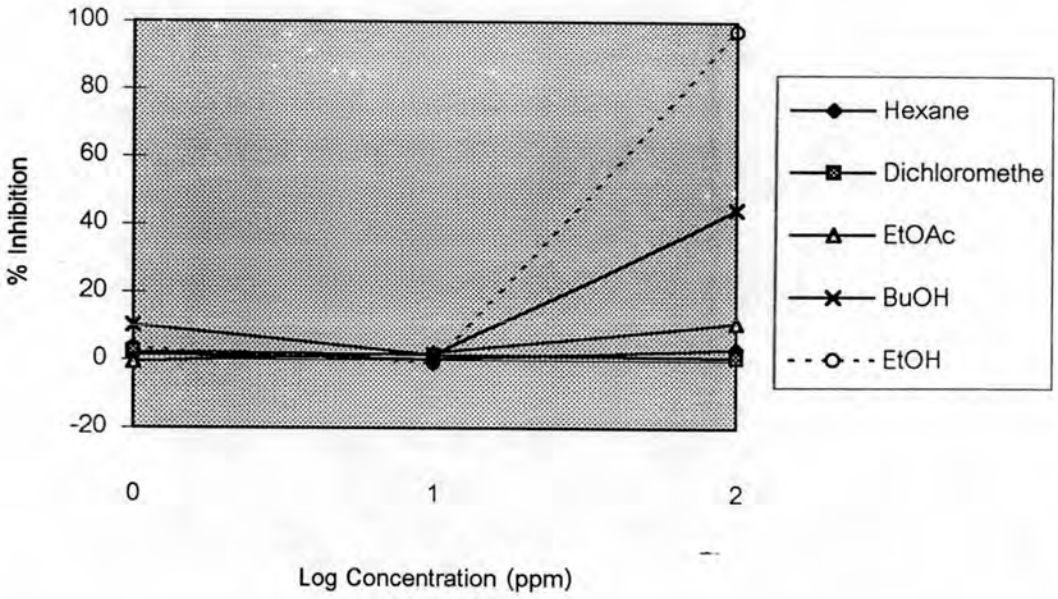


Figure 3.6 Primary screening of various crude extracts inhibited against Human Leukemia Carcinoma (HL-60)

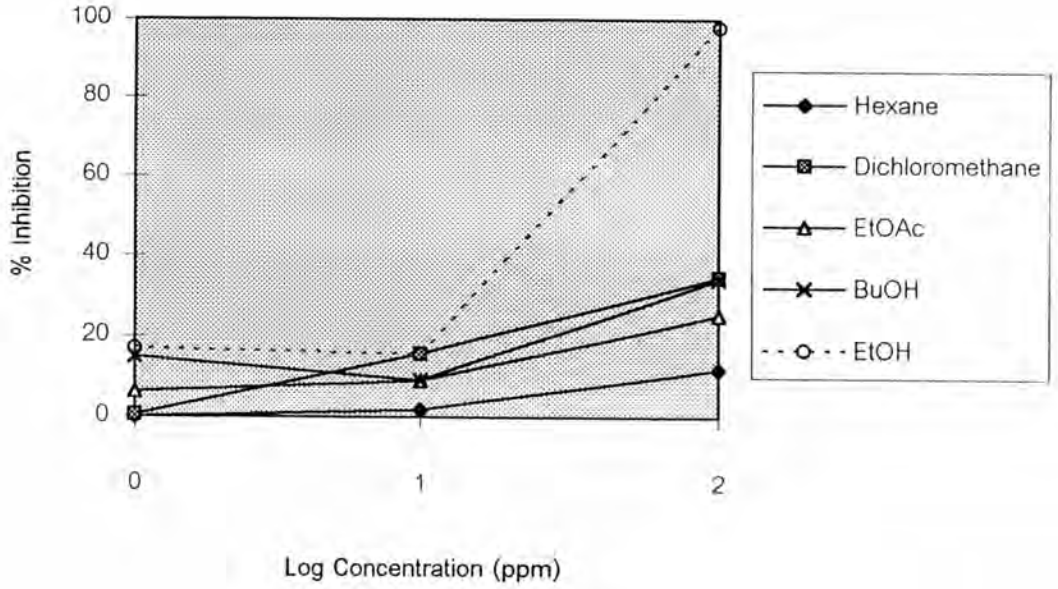


Figure 3.7 Primary screening of various crude extracts inhibited against Human Colon Carcinoma (HCT-8)

Among crude extracts of *A. arborescens*, ethanolic residue apparently inhibited most cell lines with 53.76 to 97.23% inhibition interval. Noticeably, a dramatically increasing change of inhibition from negative (at 1 and 10 ppm) to strong inhibition (at 100 ppm) was commonly observed. This result suggested that the ethanolic residue was likely to be cytotoxic agent.

3.2 Properties and Structure Elucidation of Isolated Mixtures and Compounds

3.2.1 Mixture 1

The mixture **1**, a white wax of melting point 68-72°C (470 mg), was obtained from the hexane extract which was fractionated by column chromatography over siliga gel eluting with 1:4 CH_2Cl_2/hexane. It was purified by several crystallizations from the same eluting solvent. The IR spectrum indicated the absence of any functional groups, and only a strong C-H stretching of long chain aliphatic hydrocarbons at 2920 cm^{-1}. The GC (HP-1 column, initial temperature 230 °C, carrier gas N_2, FID detector) revealed 8 peaks which corresponded to standard long chain aliphatic hydrocarbons (C_{25}-C_{30} and C_{32}-C_{33}) as well as 3 peaks of C_{18}, C_{21} and C_{34} obtained by extrapolation. Hence, it could be concluded that the mixture **1** composed of 11 components: octadecosane ($C_{18}H_{38}$), ecosane ($C_{21}H_{44}$), pentacosane ($C_{25}H_{52}$), hexacosane ($C_{26}H_{54}$), heptacosane ($C_{27}H_{56}$), octacosane ($C_{28}H_{58}$), nonacosane ($C_{29}H_{60}$), triacotane ($C_{30}H_{62}$), dotriacontane ($C_{32}H_{66}$), tritriacontane ($C_{33}H_{68}$) and tetratriacontane ($C_{34}H_{70}$). The composition of each compound was presented in Figure 3.11

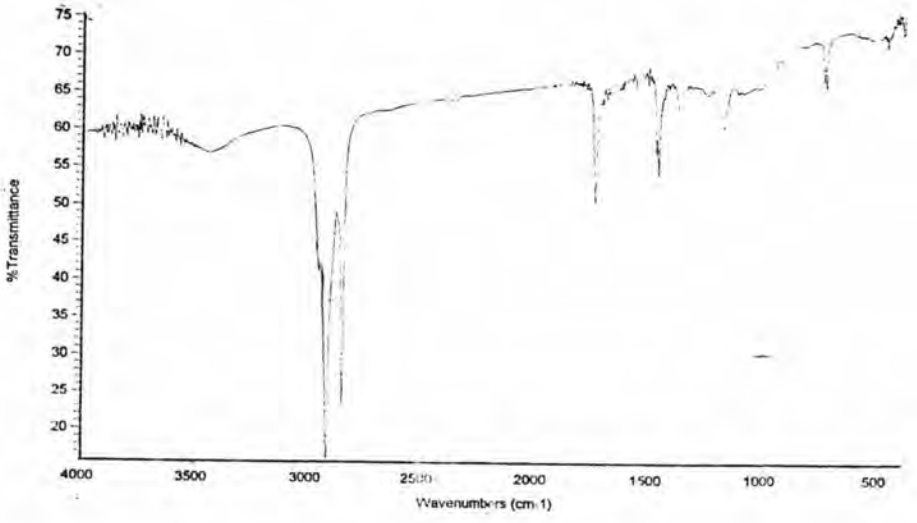


Figure 3.8 IR spectrum of mixture **1**

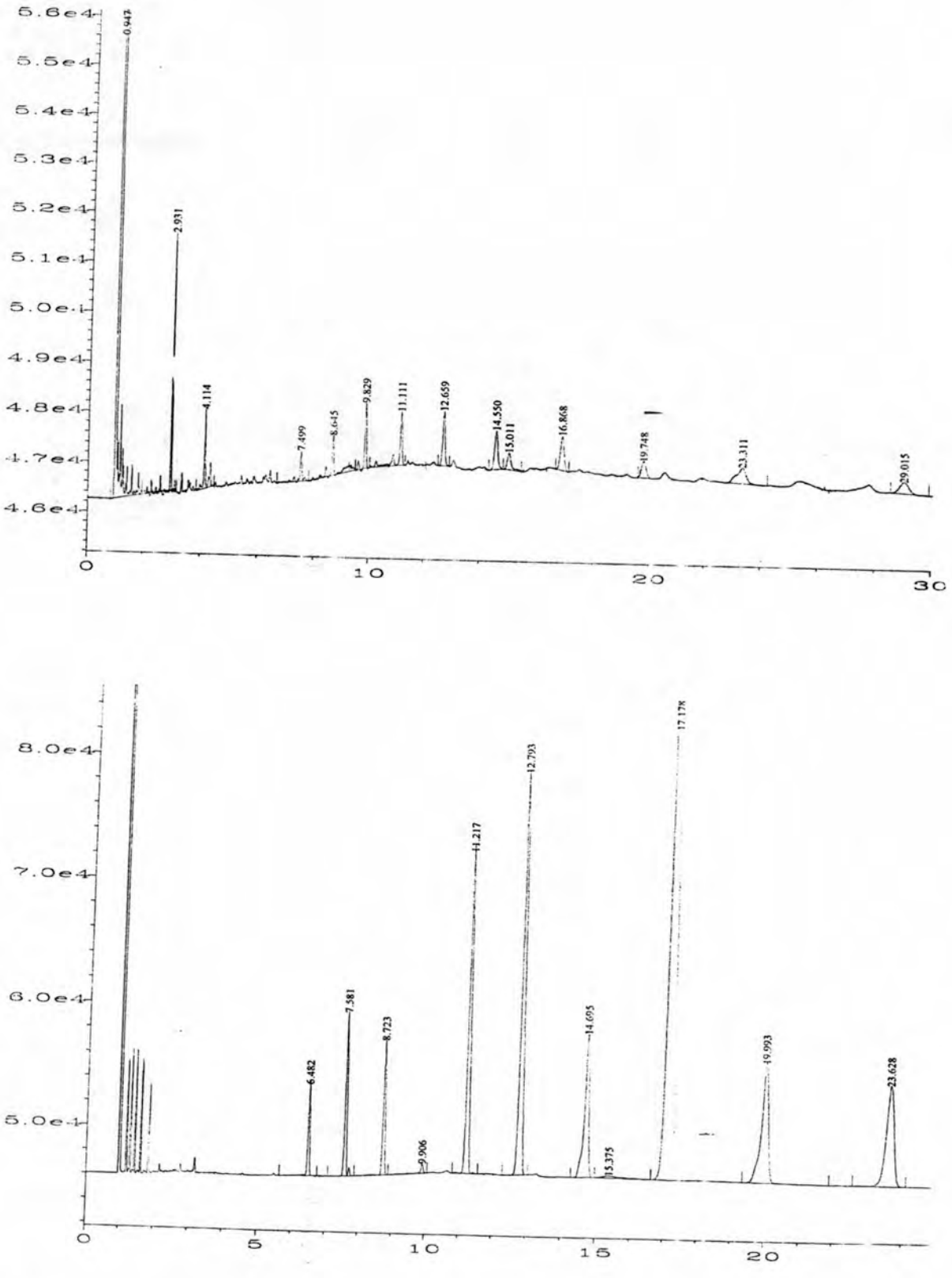


Figure 3.9 The GC chromatograms of mixture **1** (a) and standard long chain hydrocarbons (b)

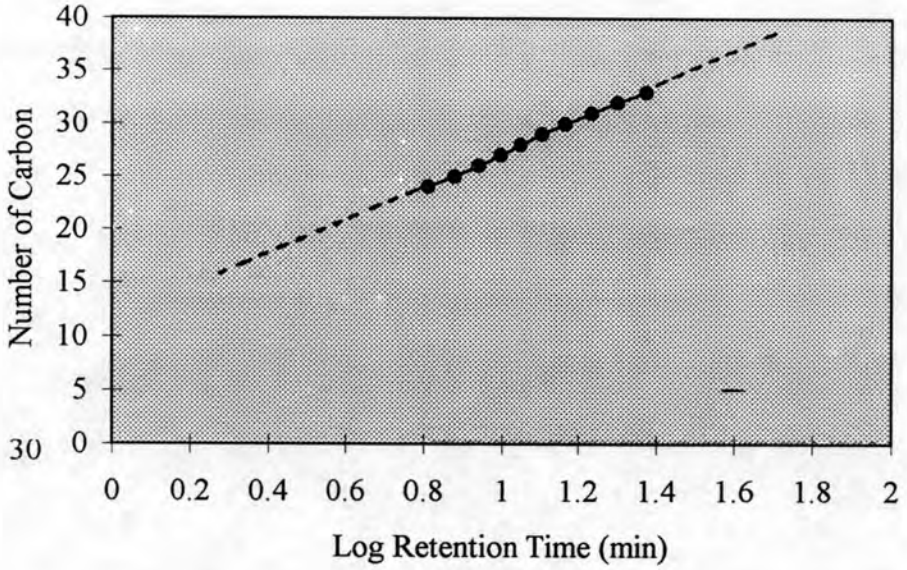
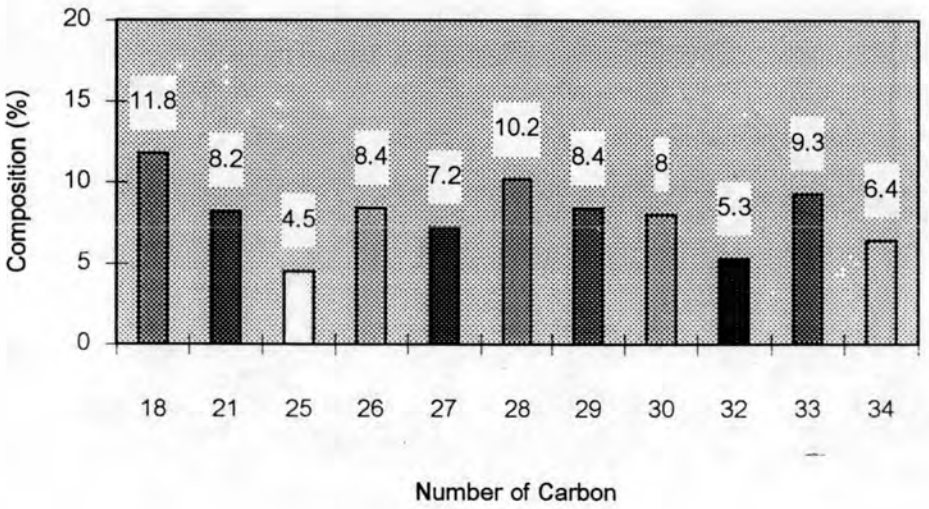


Figure 3.10 The standard calibration curve of long chain hydrocarbon

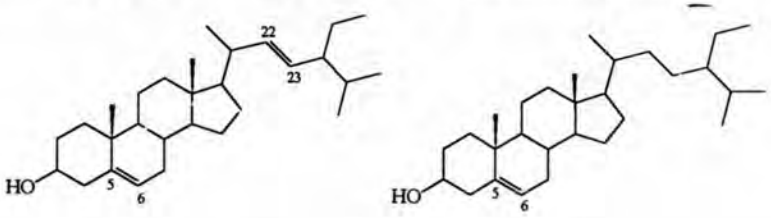


CH_3-$(CH_2)_n$-CH_3 n = 16, 19, 23-28, 30-32

Figure 3.11 The composition of mixture **1**

Mixture 2

About 152 mg of colorless needle of melting point 148-150°C was obtained by a similar treatment of the hexane extract using 1:1 CH_2Cl_2/hexane as an eluting solvent. A deep green solution was shown after reacting with Liebermann-Burchard's reagent indicating of a steroid. The GC (OV-1 column, initial column temperature 255 °C, carrier gas N_2, FID detector) gave two peaks with retention time of 17.62 and 20.84 which corresponded to those of the authentic stigmasterol (**2.1**) and β-sitosterol (**2.2**), respectively. Then, it could be decided that the mixture **2** mostly consisted of stigmasterol (98.1%) and a trace amount of β-sitosterol (1.9%).



(2.1) **(2.2)**

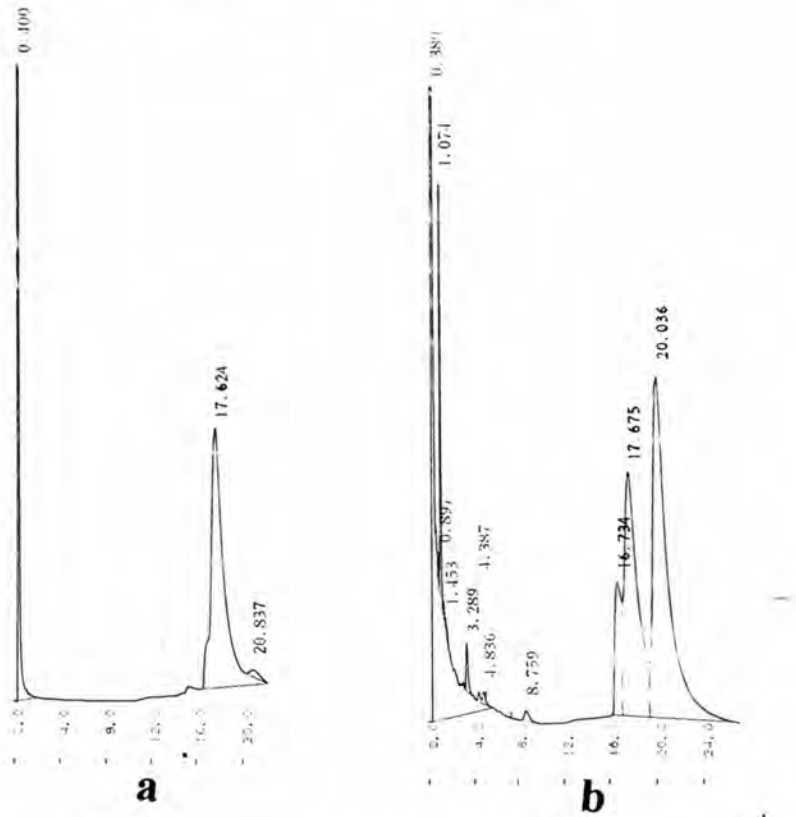


Figure 3.12 Chromatograms of (a) mixture **2** and (b) standard steroids

3.2.3 Mixture 3

The pale yellow wax, obtained from dichloromethane crude extract by eluting with 1:4 to 3:7 CH_2Cl_2/hexane, was recrystalized from 1:4 CH_2Cl_2/hexane to afford 110 mg of the white wax. Its melting point was 78-85 °C, a broad range of melting point, could indicate that the wax was a mixture. The IR spectrum showed strong absorption bands at ν_{max} 2915 (C-H, aliphatic), 1735 (C=O, ester) and 1195 (C-O) cm^{-1}. It could be concluded that the white wax should be a mixture of long chain aliphatic esters.

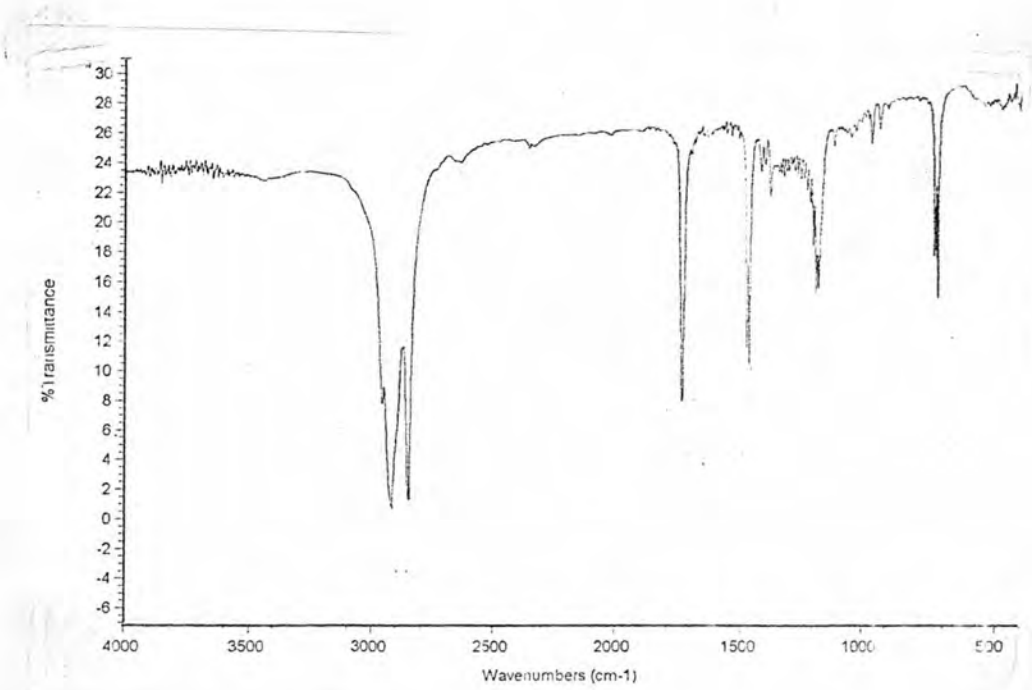
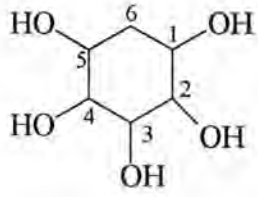


Figure 3.13 IR spectrum of mixture 3

3.2.4 Compound 1

Compound **1** (*ca* 87.0 g) was a colorless sugarlike crystal of melting point 235-236°C and surprisingly had a sweet taste, obtained as a major component from this plant. After the concentrated ethanolic extract was left overnight, the colorless crystal was deposited. It was recrystalized in hot MeOH/H_2O for several times and was readily soluble in hot water. The IR spectrum contained a very strong and broad absorption due to hydroxy groups at ν_{max} 3308 cm^{-1}. The molecular formula could be also calculated as $C_6H_{12}O_5$ (M.W. 164) from ^1H- and DEPT-NMR spectra. Although the molecular peak at m/z 164 was absent but the hydroxy ion loss $(M-OH)^+$ at m/z 147 could be observed Five most downfield signals from δ 4.26 to 4.66 were unambiguously assigned as hydroxy protons since they did not have any correlations with carbons in HMQC spectrum, an alternative mean to decide which signals were hydroxy protons. Additionally, there were one methylene carbon (CH_2) and five methine ones (CH) at δ 34.9, 68.4, 68.9, 71.4, 72.9 and 75.0 ppm. The only one double bond equivalent (dbe) suggested that this compound possibly had one cyclic. NMR spectra of this compound were similar to those of cyclitols, the polyhydroxycyclohexanes. From all above data, compound **1** was established as pentahydroxycyclohexane or quercitol, a generic name for the ten diastreomeric deoxyinositol: four meso isomers and six dl-pairs.



Ten prefixes: *cis* (a), *muco* (b), *neo* (c), *scyllo* (d), *allo* (e), *epi* (f), *talo* (g), *vibo* (h), *proto* (i) and *galo* (j), were introduced to facilely specify the configuration of each diastereoisomers.

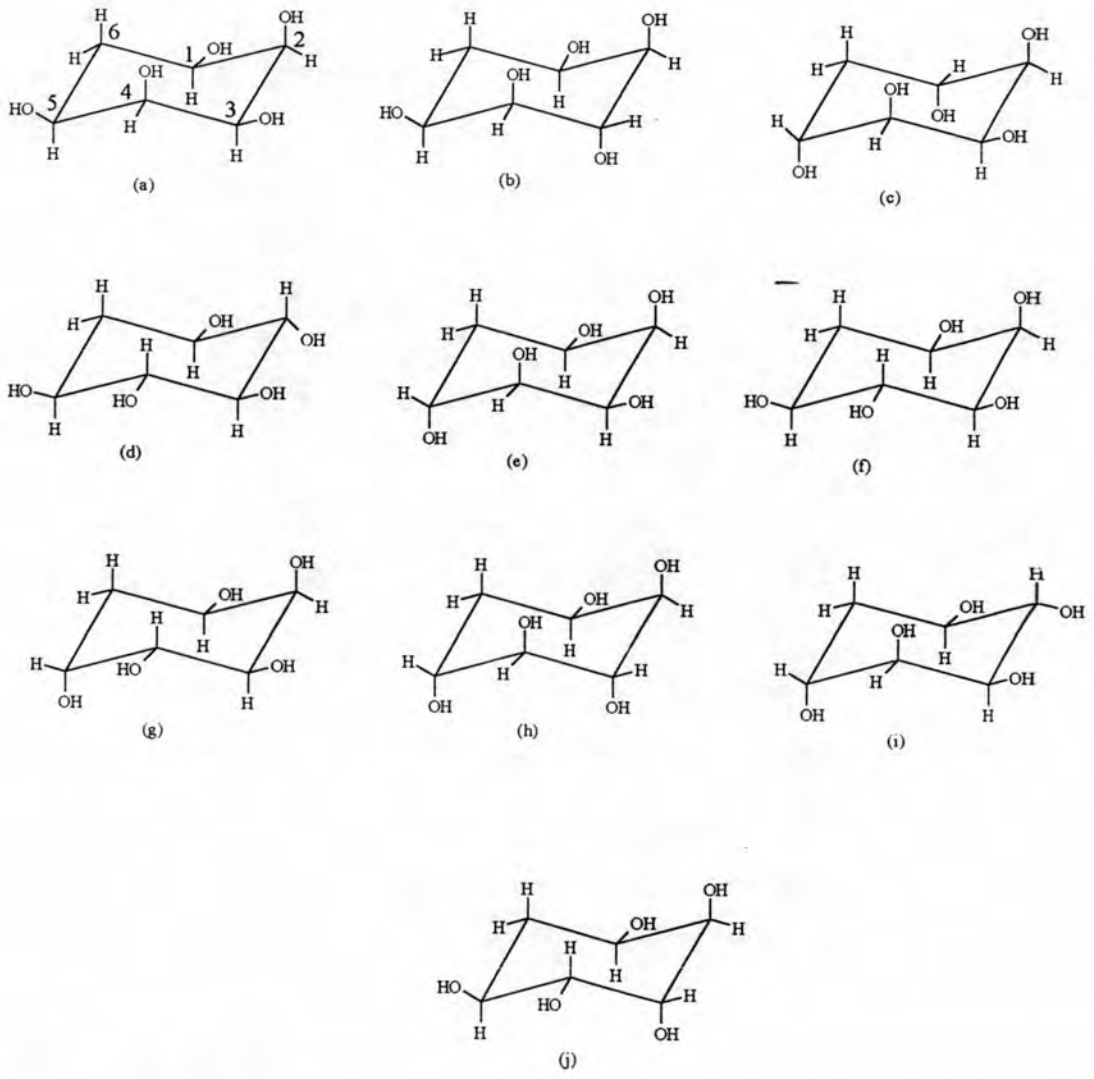
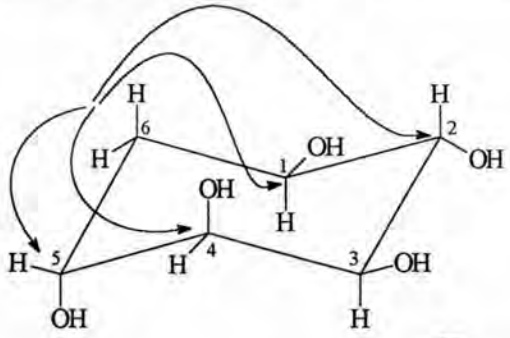


Figure 3.14 Configurations of four mesoisomers (a-d) and six dl-pairs (e-j, each structure had both d- and l-form determined by optical rotation)

Before determining the configuration of this compound, it needed to assign chemical shifts of each signal. To begin with methylene proton (H-6) at δ 1.60 (2H, m) in HMBC spectrum, there were cross peaks due to the correlation between H-6 and four methine carbons (δ 68.4, 68.9, 72.9 and 75.0) via $^{2}J_{H,C}$ or $^{3}J_{H,C}$.

However, the correspondent correlation between H-6 and the another methine carbon at δ 71.4 was not observed. This information implied that the uncorrelated methine carbon was C-3 because it was too far from H-6 to correlate via $^{2}J_{H,C}$ or $^{3}J_{H,C}$.



As a result of certain assignment of C-3, the signal of H-3 was determined to resonate at δ *ca* 3.42 via one bond correlation in HMQC spectrum. In ^{1}H-^{1}H COSY spectrum, the H-3 also showed $^{3}J_{H,H}$ correlations to H-2 and H-4. Therefore, signals of C-2 (δ 75.0) and C-4 (δ 72.9) could be further assigned on the basis of one bond correlation. Although chemical shifts of nearby protons and carbon of C-3 could be interchangeable, but the sequence of connection was fixed. Signal assignment of H-1, H-5, C-1 and C-5 was the same as H-2, H-4, C-2 and C-4. In addition, contour plots due to $^{3}J_{H,OH}$ correlation between methine and their geminal hydroxy protons were also useful to assign chemical shifts of hydroxy ones. For example, H-3 methine signal showed a correlation to geminal 3-OH (δ 4.27, 1H, d, J = 6.1 Hz). The rest of hydroxy protons could be assigned by this correlation. The complete assignments of compound **1** were listed in table 3.2

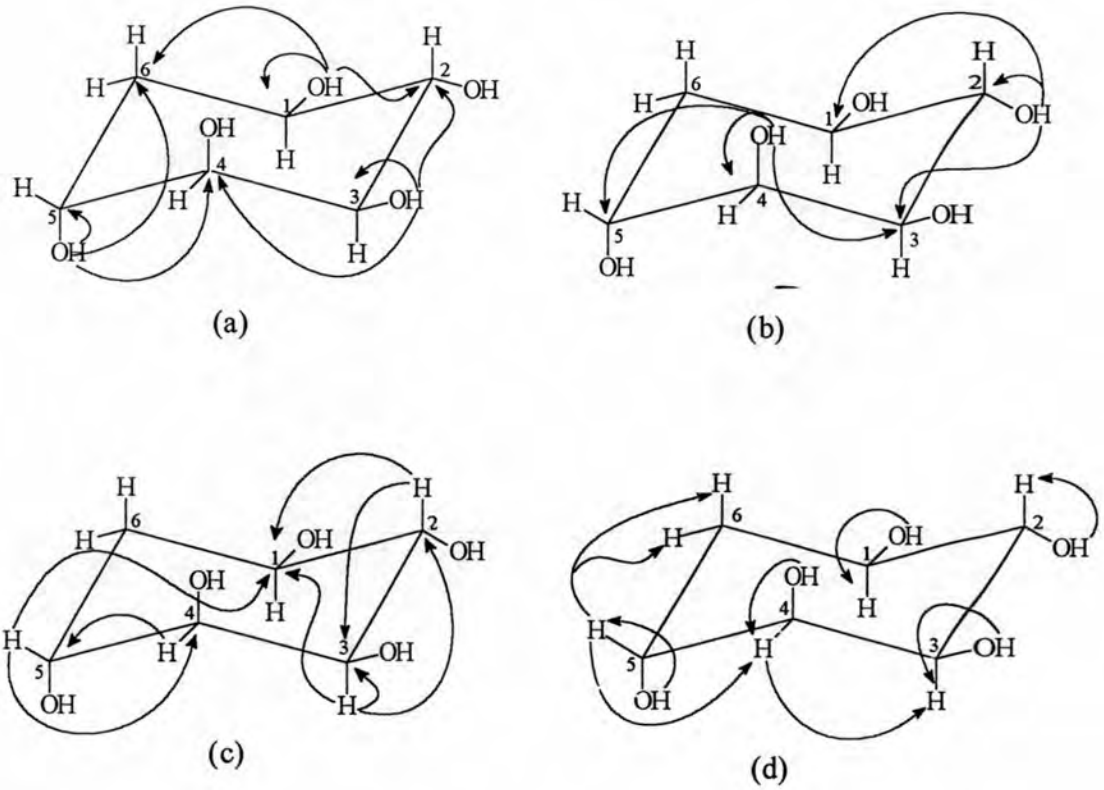


Figure 3.15 HMBC correlation in alcoholic (a-b) and methine (c) regions including ^{1}H-^{1}H COSY correlation (d)

Table 3.2 ^{1}H- and ^{13}C-NMR shift values and long-range H-C connectivity in compound **1** was established by HMBC (DMSO-d_6)

Position	δ_H(mult., J)[a]	δ_C(mult.)[b,c]	$^{2}J_{H,C}$	$^{3}J_{H,C}$
1	3.48 (m)	68.9 (CH)	-	-
1-OH	4.37 (d, 4.9)	-	C-1	C-2, C-6
2	3.29 (dt, 4.0, 8.9)	75.0 (CH)	C-1, C-3	-
2-OH	4.42 (d, 4.3)	-	C-2	C-1, C-3
3	3.42 (m)	71.4 (CH)	C-2	-
3-OH	4.27 (d, 6.1)	-	C-3	C-2, C-4
4	3.59 (q, *ca* 3.4-3.7)	72.9 (CH)	C-5	-
4-OH	4.49 (d, 3.7)	-	C-4	C-5, C-3
5	3.67 (quint, *ca* 3.4-3.7)	68.4 (CH)	C-4	C-1
5-OH	4.66 (d, 3.4)	-	C-5	C-4, C-6
6	ca 1.59-1.62 (2H, m) 1.80[d](dt, 3.1, 13.1) 1.98[d](dtd, 1.2, 4.0, 14.0)	34.9 (CH_2)	C-1, C-5	C-2, C-4

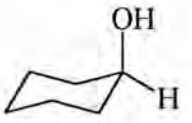
[a] 1H, unless noted.

[b] Multiplicities were determined by DEPT

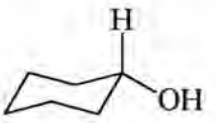
[c] Assignments were based on HMQC correlation

[d] Methylene protons could be resolved into H_{6ax} (δ 1.80) and H_{6eq} (δ 1.98) where they were recorded in D_2O

In dimethyl sulfoxide or in acid free solution, the coupling constant of hydroxy protons could indicate stereochemistry itself, axial or equatorial[20]. It significantly showed different values:



J_{HOCH} = 3.0 ... 4.2



J_{HOCH} = 4.2 ... 5.7

Among five hydroxy protons, 4-OH and 5-OH had coupling constants less than 4.2 Hz and were assigned as axial while the other were equatorial. The complete assignment with stereochemistry of compound **1** was shown in Figure 3.16

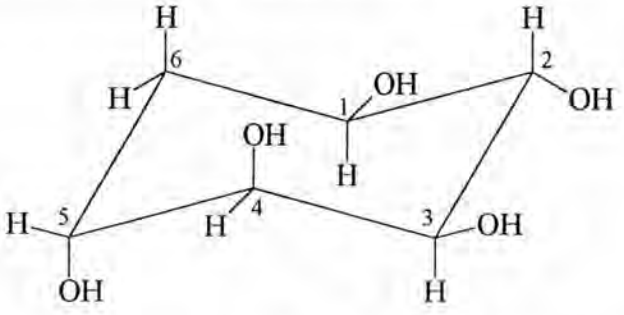
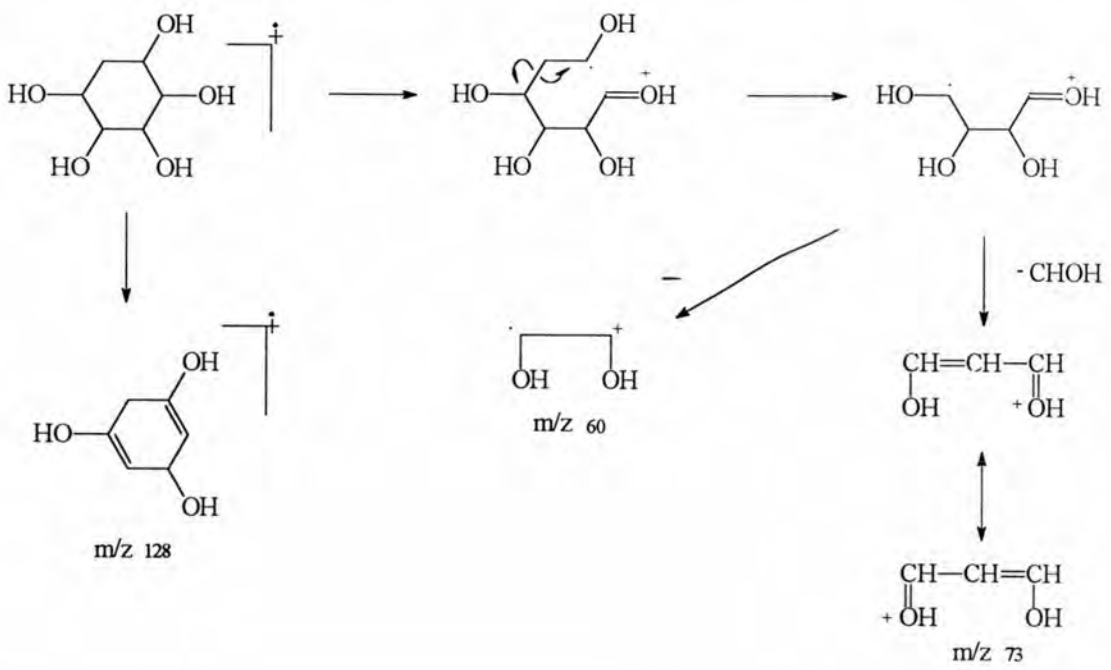


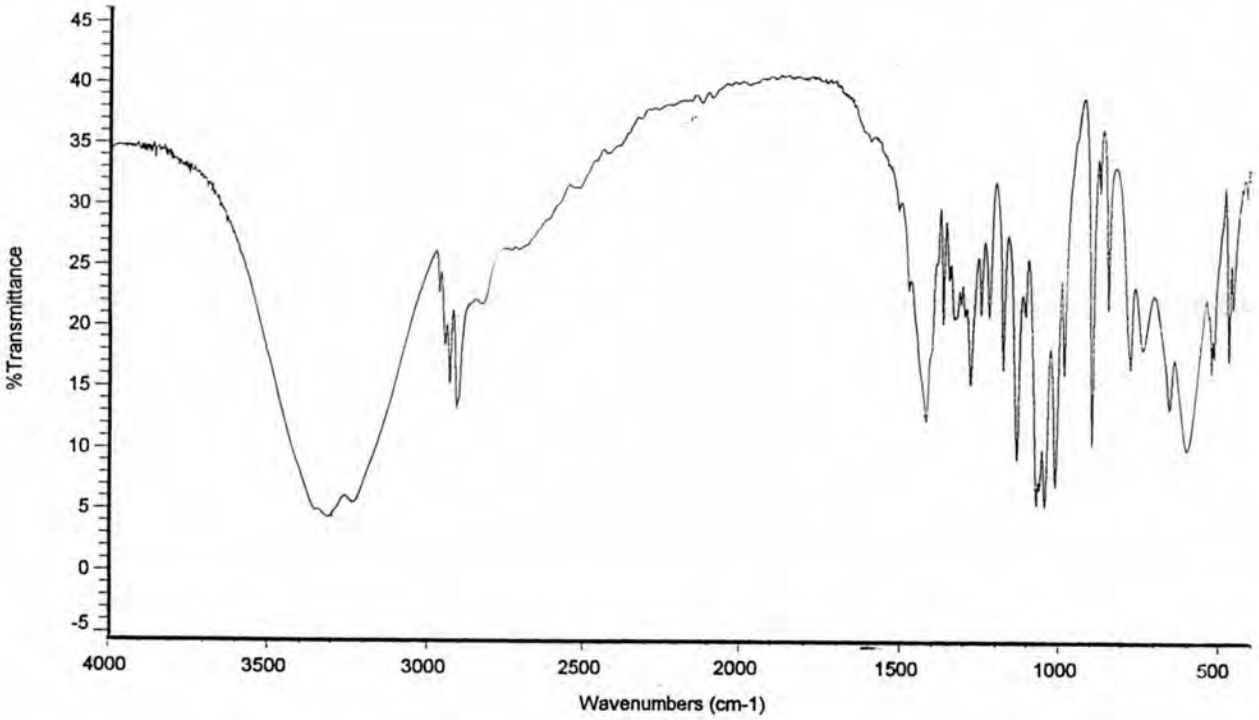
Figure 3.16 Complete assignment with stereochemistry of compound **1**

The stereochemistry of compound **1** was similar to that of quercitol (Figure 3.11(i)). Natural occurring *proto*-quercitol has been found in two forms, (+)-*proto*-quercitol and (-)-*proto*-quercitol. Optical rotation of compound **1**, $[\alpha]^{27}_{D}$ +27.9 (*c* 0.02, H_2O), [lit. $[\alpha]_D^{20}$ +24, H_2O] could indicate itself as (+)-*proto*-quercitol.

Proto-quercitol mostly exists in (+)-form, especially, in acorns of *Quercus spp.* On the contrary, (-)-form occurs in only *Eucalyptus populnea*. In addition, this is the first report for finding (+)-*proto*-quercitol in Sapindaceae.



Scheme 3.1 Mass fragmentation pattern of compound **1**



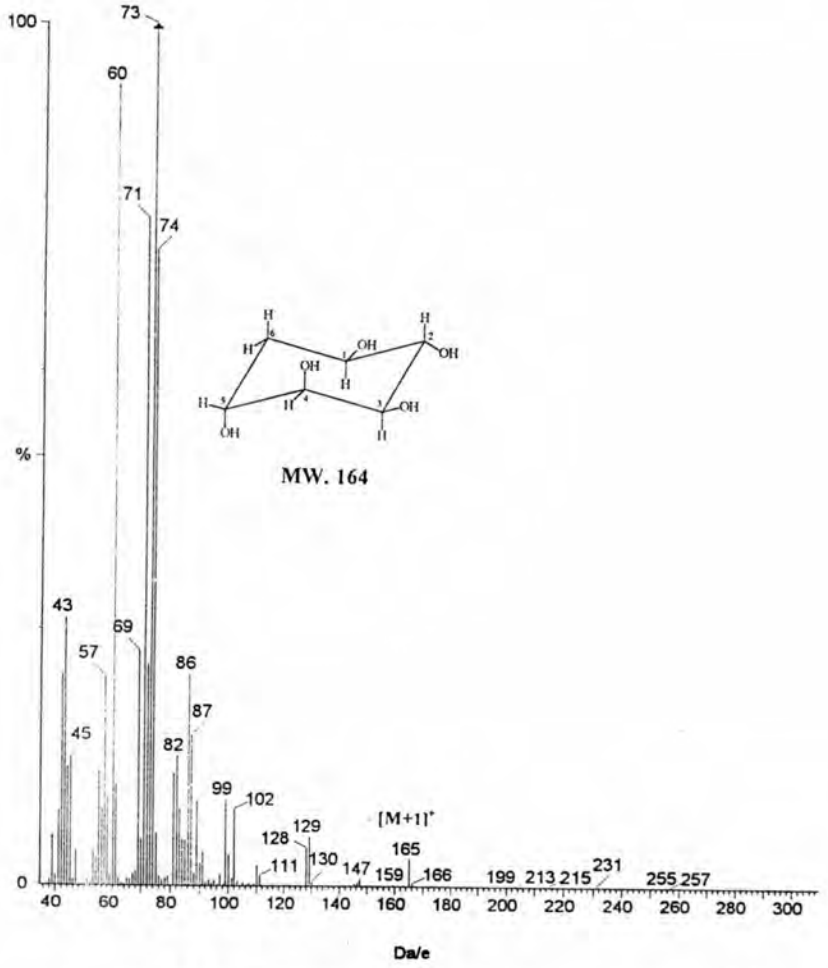


Figure 3.17 Infrared and mass spectra of compound **1**

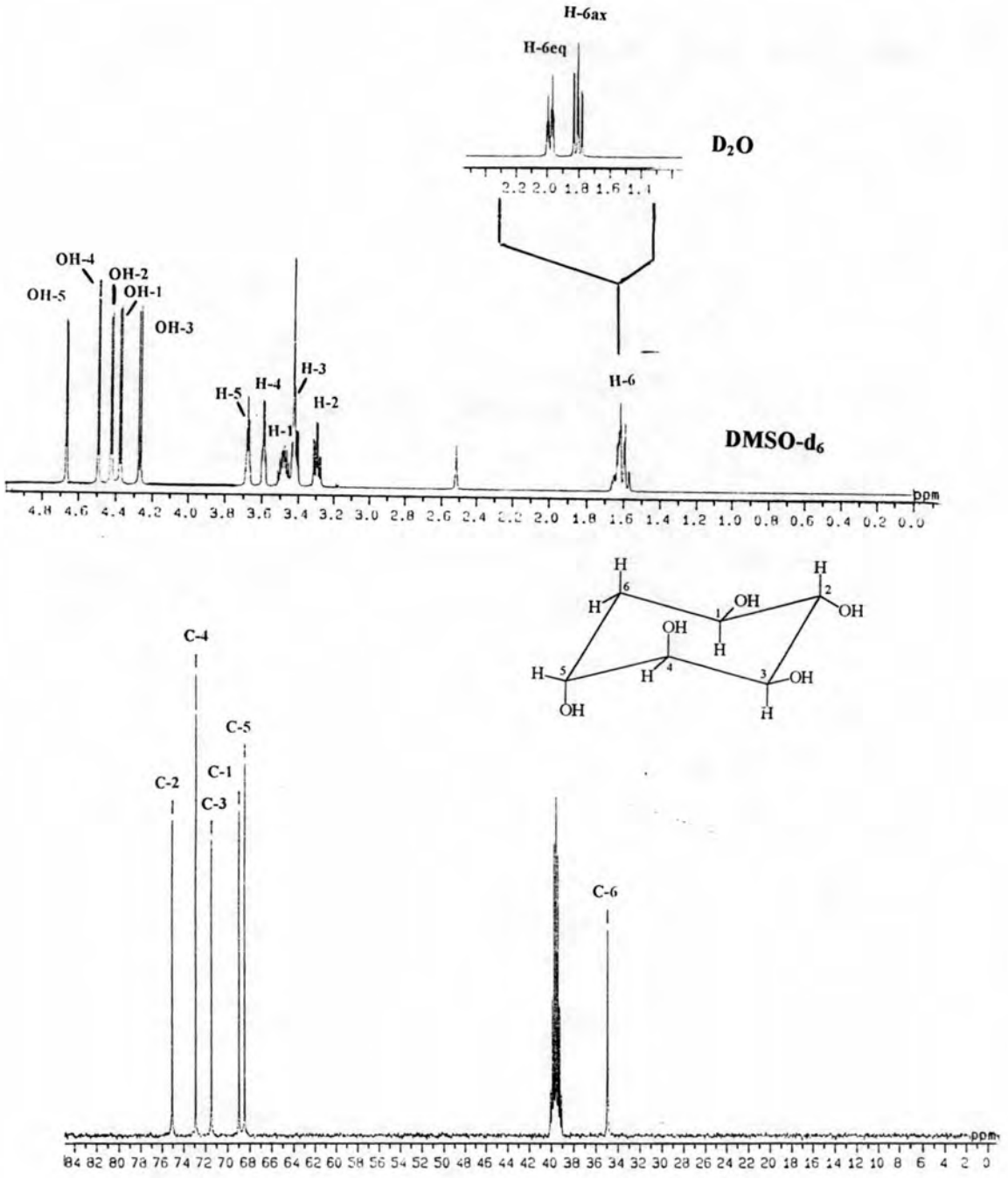


Figure 3.18 ^{1}H- and ^{13}C-NMR spectra (DMSO-d_6) of compound **1**. A complex multiplet signal of methylene proton was resolved into doublet and triplet while hydroxy signals were disappear (recorded in D_2O)

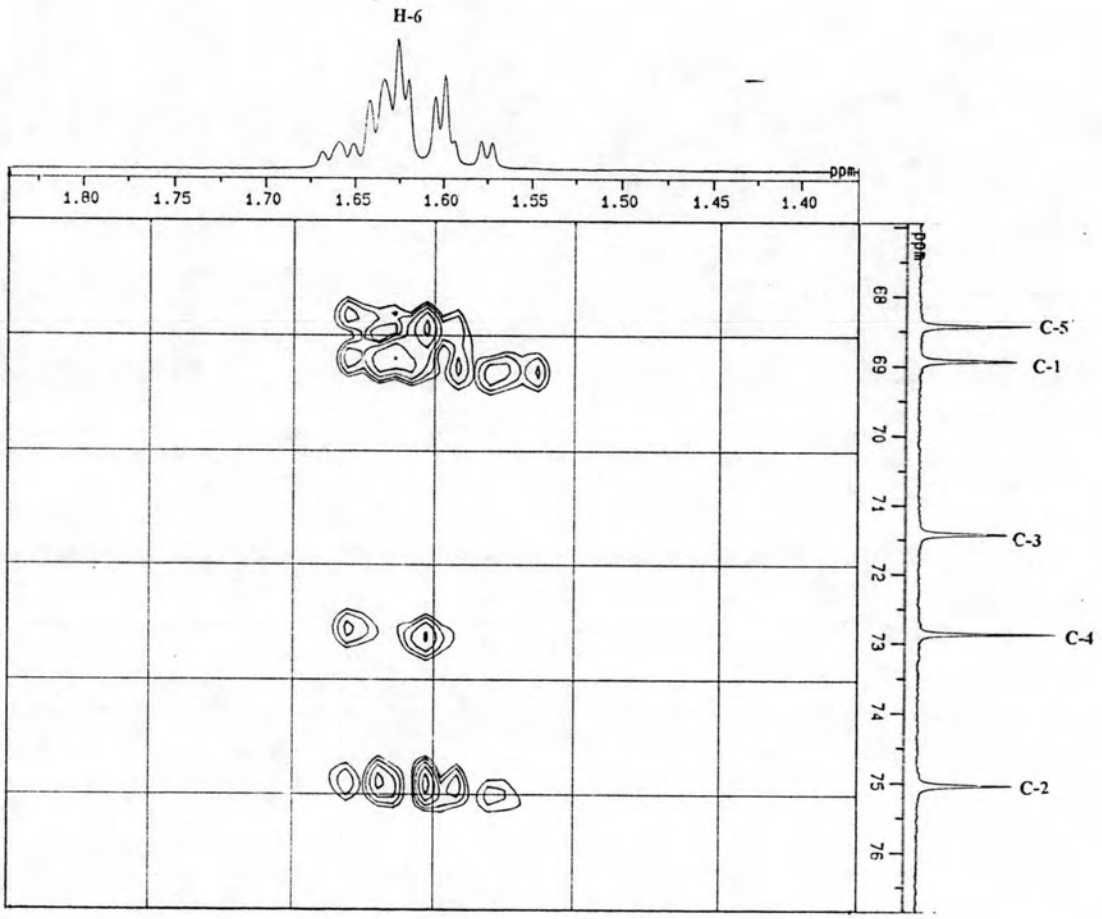


Figure 3.19 HMBC spectrum of compound **1** focused on methylene region

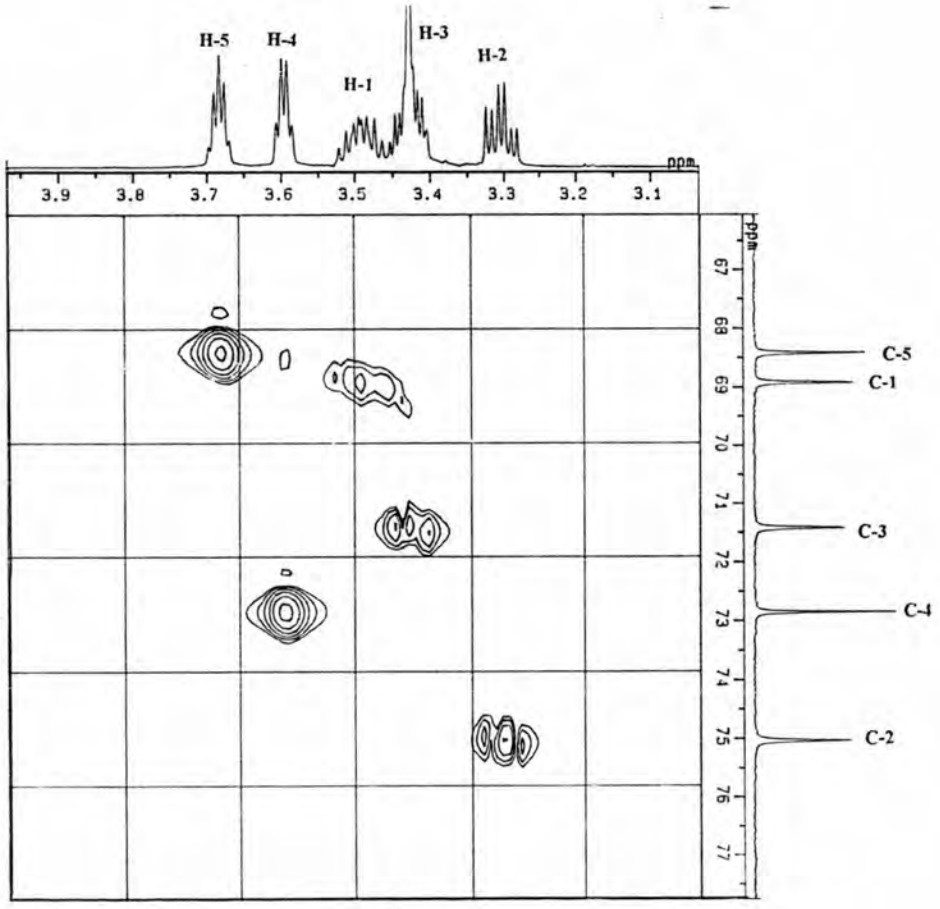


Figure 3.20 HMQC spectrum of compound **1**

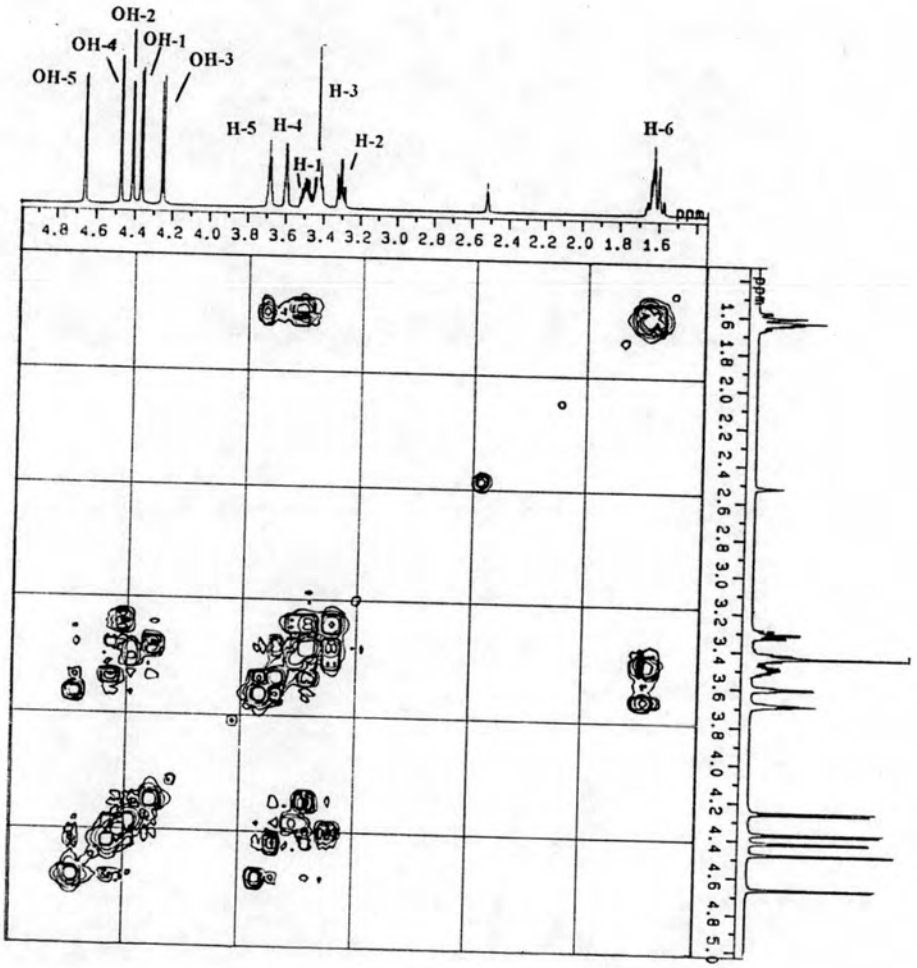
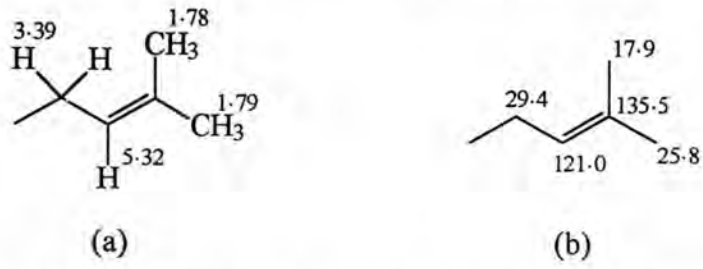


Figure 3.21 ^{1}H-^{1}H COSY spectrum of compound **1**

3.2.5 Compound 2

Compound **2** (37 mg) was obtained from dichloromethane crude extract by eluting with 2:3 CH_2Cl_2/hexane to afford the yellow viscous liquid. This fraction was further separated by chromatotron, starting from 5% ethyl acetate in hexane, the fourth fraction eluted with 20% ethyl acetate in hexane gave a colorless crystal after the solvent was evaporated. It had R_f 0.27 (SiO_2, 3:2 CH_2Cl_2/hexane) and melting point of 101-103 °C. The compound immediately gave a deep red spot after dipping in DNPH reagent, suggesting the presence of a carbonyl group. The IR spectrum showed an intense band of O-H stretching at ν_{max} 3190 cm^{-1} and the characteristic band of C-H stretching of aldehyde at ν_{max} 2965 and 2915 cm^{-1} as well as an unusual C=O stretching at 1659 cm^{-1}. The molecular peak at m/z 258 in mass spectrum corresponded to the molecular formula $C_{17}H_{22}O_4$ of compound **2**. Other fragment peaks at m/z 241, 229, 203 and 77 suggested $[M\text{-}OH]^+$, $[M\text{-}CHO]^+$, $[M\text{-}C_4H_7]^+$ and a tropylium ion of a benzene ring, respectively.

^{1}H-NMR spectrum and signal integration indicated the presence of two prenyl groups: δ 1.78 ($2CH_3$, s), 1.80 ($2CH_3$, br s), 3.39 (4H, d, J = 7.0 Hz) and 5.32 (2H, qtquin., J = 7.0, 1.0 Hz). ^{13}C-NMR spectrum also corresponded to the proton assignment. Methyl groups of the prenyl resonated at δ 17.9 and 25.8 while the secondary, tertiary and quaternary carbon were found at δ 29.4, 121.0 and 135.5, respectively. The proton and carbon assignment were shown in figure 3.21(a) and (b).

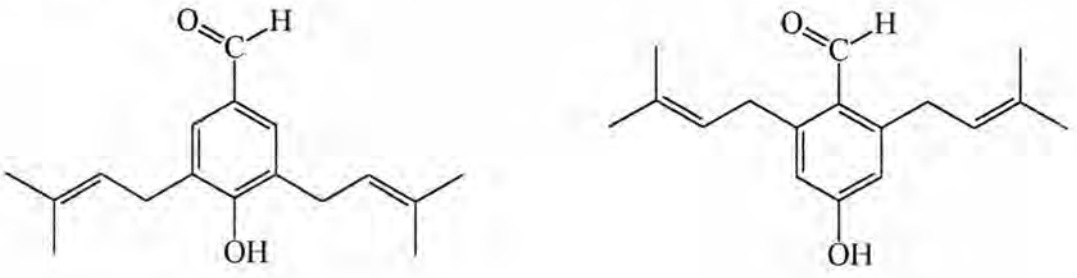


(a) (b)

Figure 3.22 (a) proton and (b) carbon assignments of prenyl group

The most downfield signal at δ 9.82 was assigned to be an aldehyde proton. The rest of protons at δ 7.63 (2H, s) and 6.07 (1H, s) were the symmetry aromatic protons and the phenolic one.

The combination of these data suggested that compound **2** was hyroxybenzaldehyde with two prenyl groups. Additionally, it could be inferred from the symmetry aromatic protons that there were only two possible structures of compound **2** shown below:

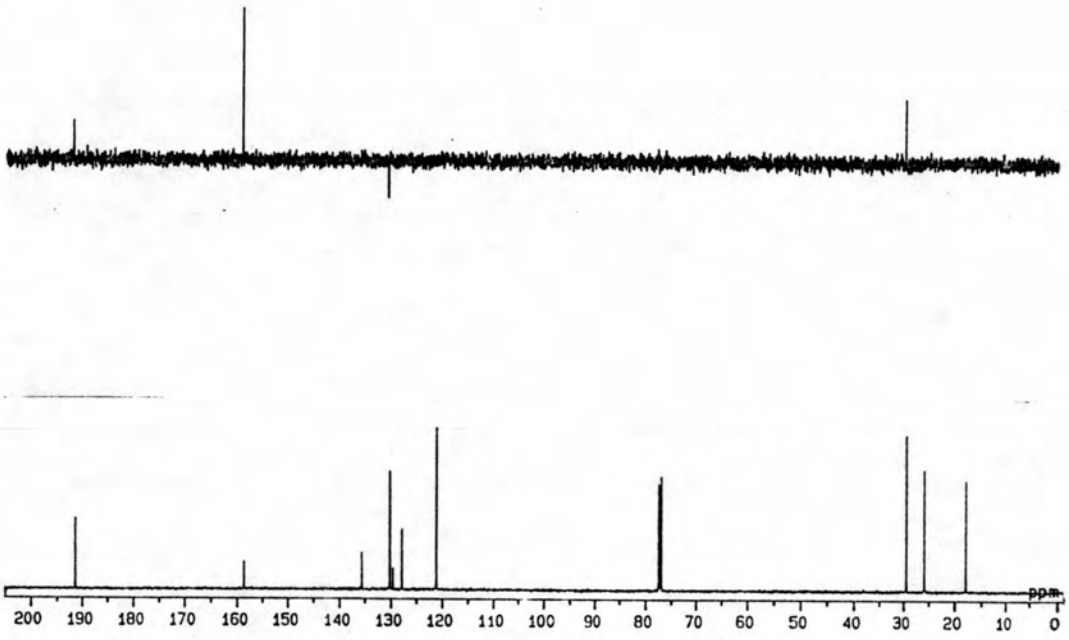


(a) (b)

Figure 3.23 Two possible structures of compound **2**

Biosynthesis point of view[22], indicated a prenyl group meta to a hydroxy group was seldom found. Calculation for chemical shift of the aromatic proton ortho to a hydroxy group gave 6.70 ppm while the same calculation for the proton ortho to an aldehyde group provided the chemical shift (7.82 ppm) which was as close as the observe value. Although, the two previous papers[23, 24] have been reported the isolation of 3,5-*Bis*-[3,3-dimethylallyl]-*p*-hydroxybenzaldehyde as well as ^{1}H-NMR spectral data which were rather similar to those of compound **2**, but melting point of compound **2** (101-103°C) was quite different from the reports (118°C).

To settle conclusively the structure of compound **2** was either as (a) or (b), selective INEPT (a 1D-NMR technique involved irradiation of the proton with an appropriate $^3J_{CH}$ value prior to observe signal of enhancement carbon three bonds away from the irradiated proton) was suitable for this purpose. Irradiation of H-2/H-6 (δ 7.53) with 10 Hz of coupling constant (*J*) resulted in enhancements of δ 29.4, 158.6 and 191.4, which could be assigned as CH_2 of prenyl group, C-4 and CHO. The irradiation result indicated that structure (a) fit for compound **2**. This is the first report giving a complete assignment of compound **2**.



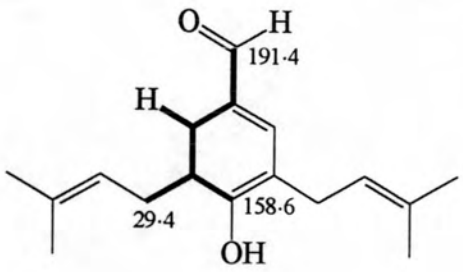


Figure 3.24 Selective INEPT spectra of compound **2**

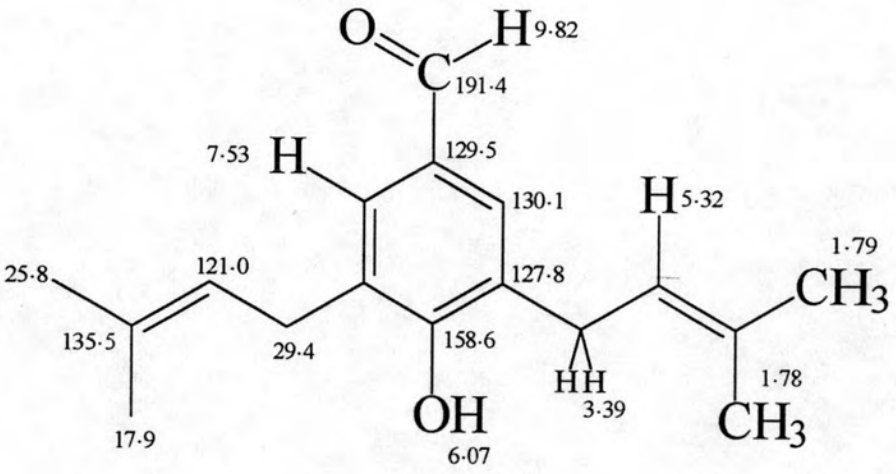
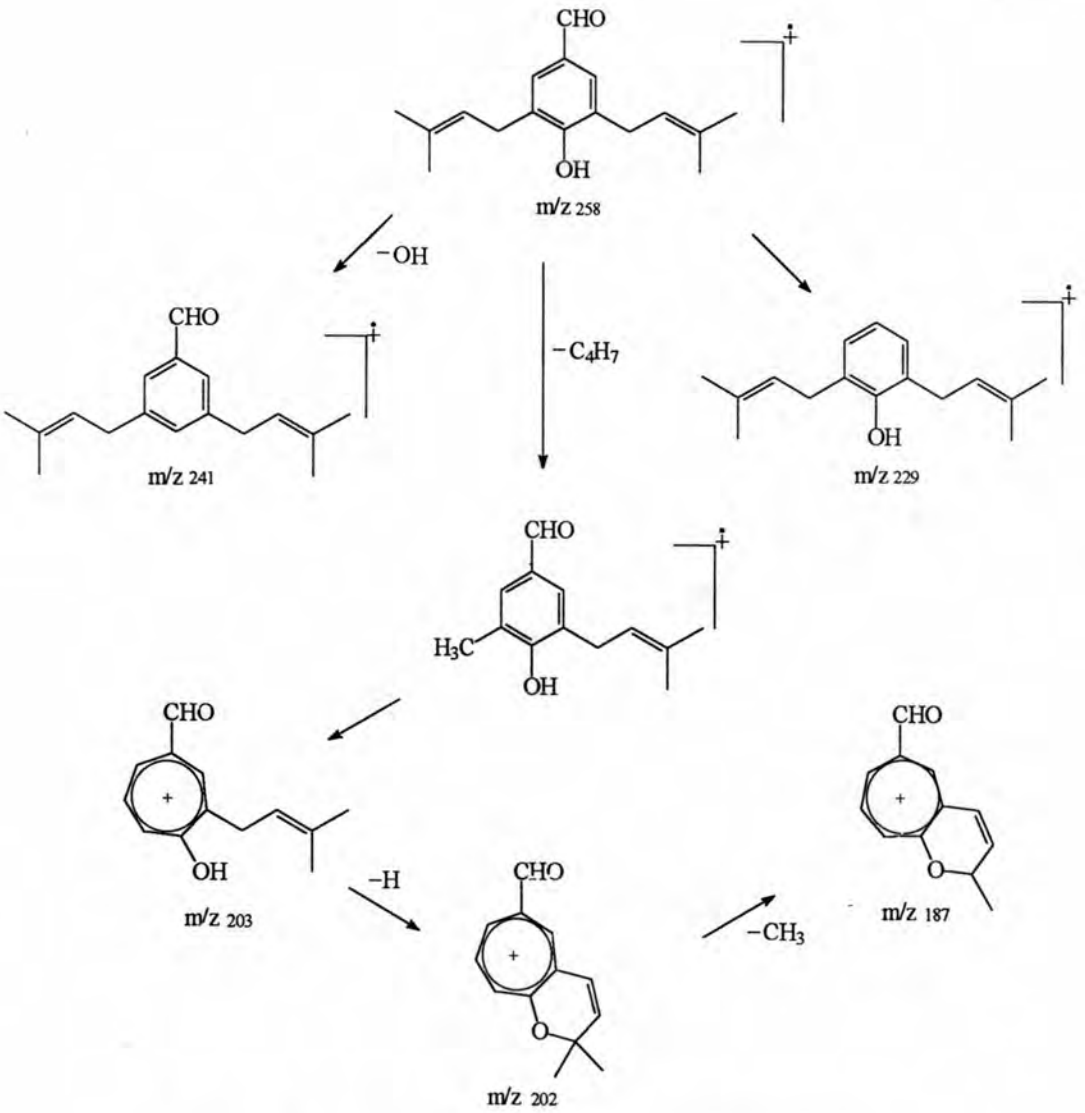
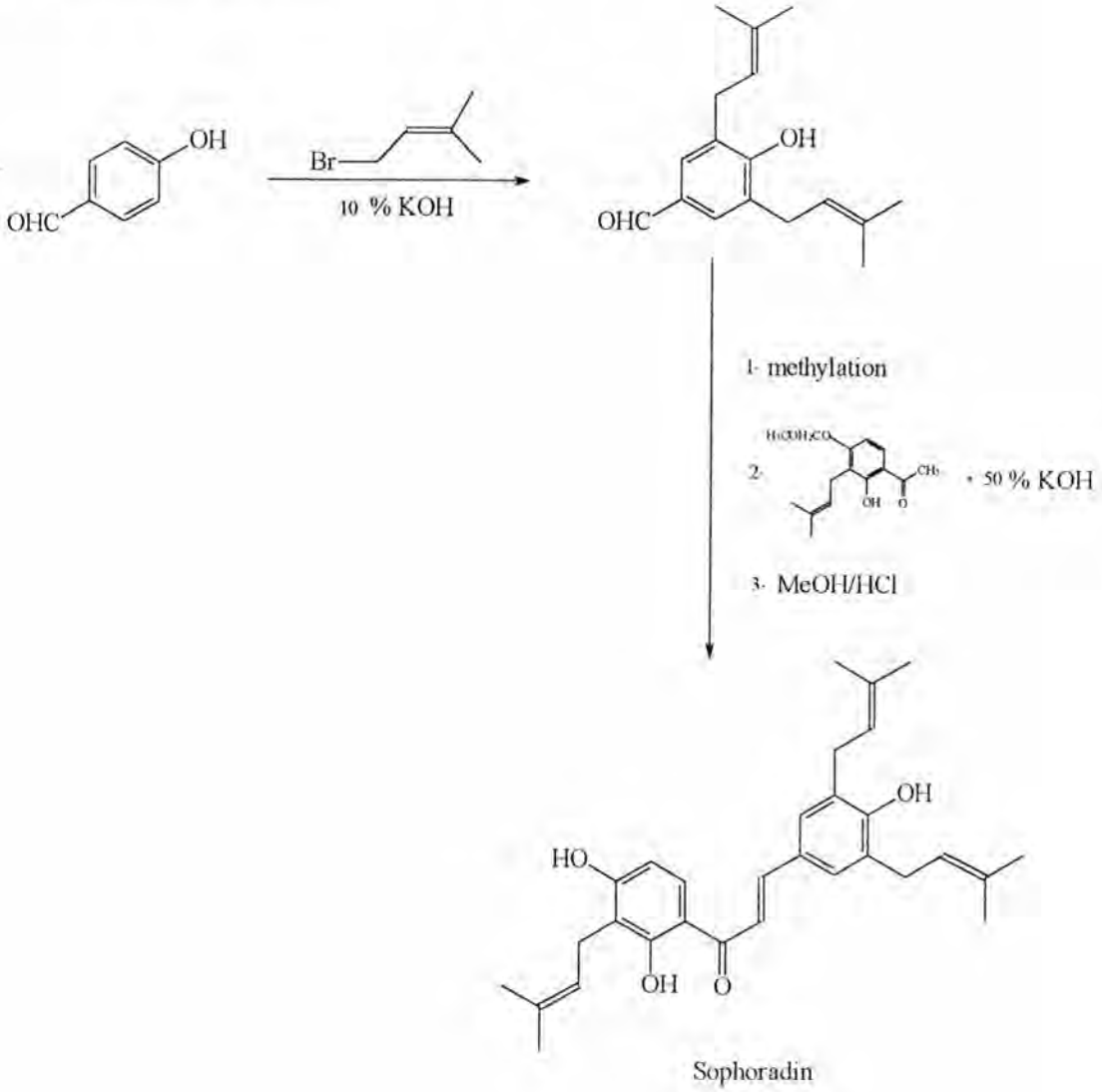


Figure 3.25 Proton and carbon assignments of compound **2**



Scheme 3.2 Mass fragmentation pattern of compound **2**

Compound **2** was first naturally isolated from aerial parts of *Senecio erubescens* Ait. var. *crepidifolius* DC. by Bohlmann and coworkers in 1985.[23] Nine years later, Hemlata and Kalidhar.[24] reported the isolation of this compound from the stems of *Dodonaea viscosa*, a plant in the same family of *A. arborescens*. However, compound **2** was synthesized as an intermediate for Sophoradin synthesis[25] in 1975. It was prepared from the reaction of *p*-hydroxybenzaldehyde with 3,3-dimethylallylbromide in 10% KOH. Preparation of Sophoradin, anti-ulcer agent in rat, was summarized as follow:



Sophoradin

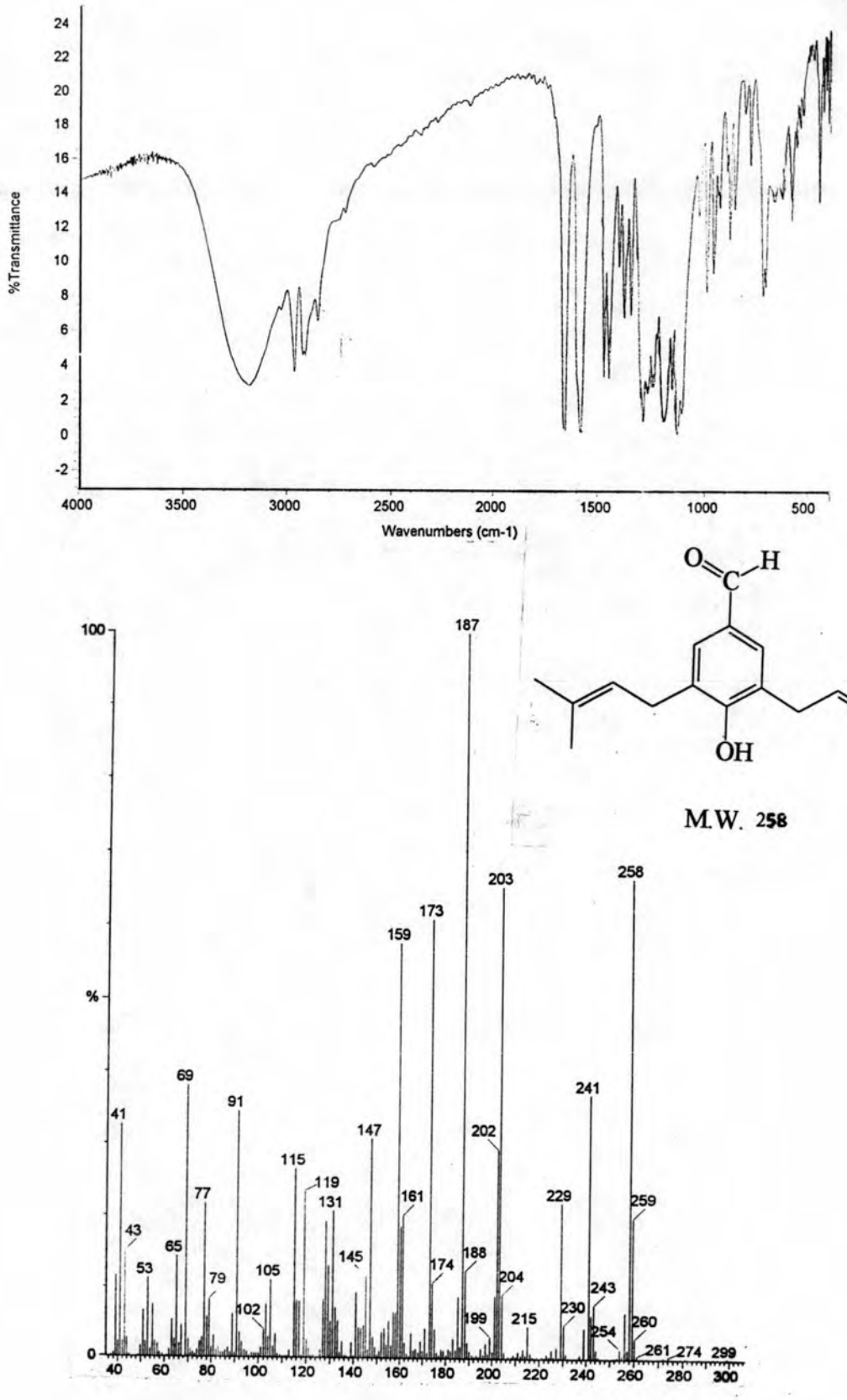


Figure 3.26 Infrared and mass spectra of compound **2**

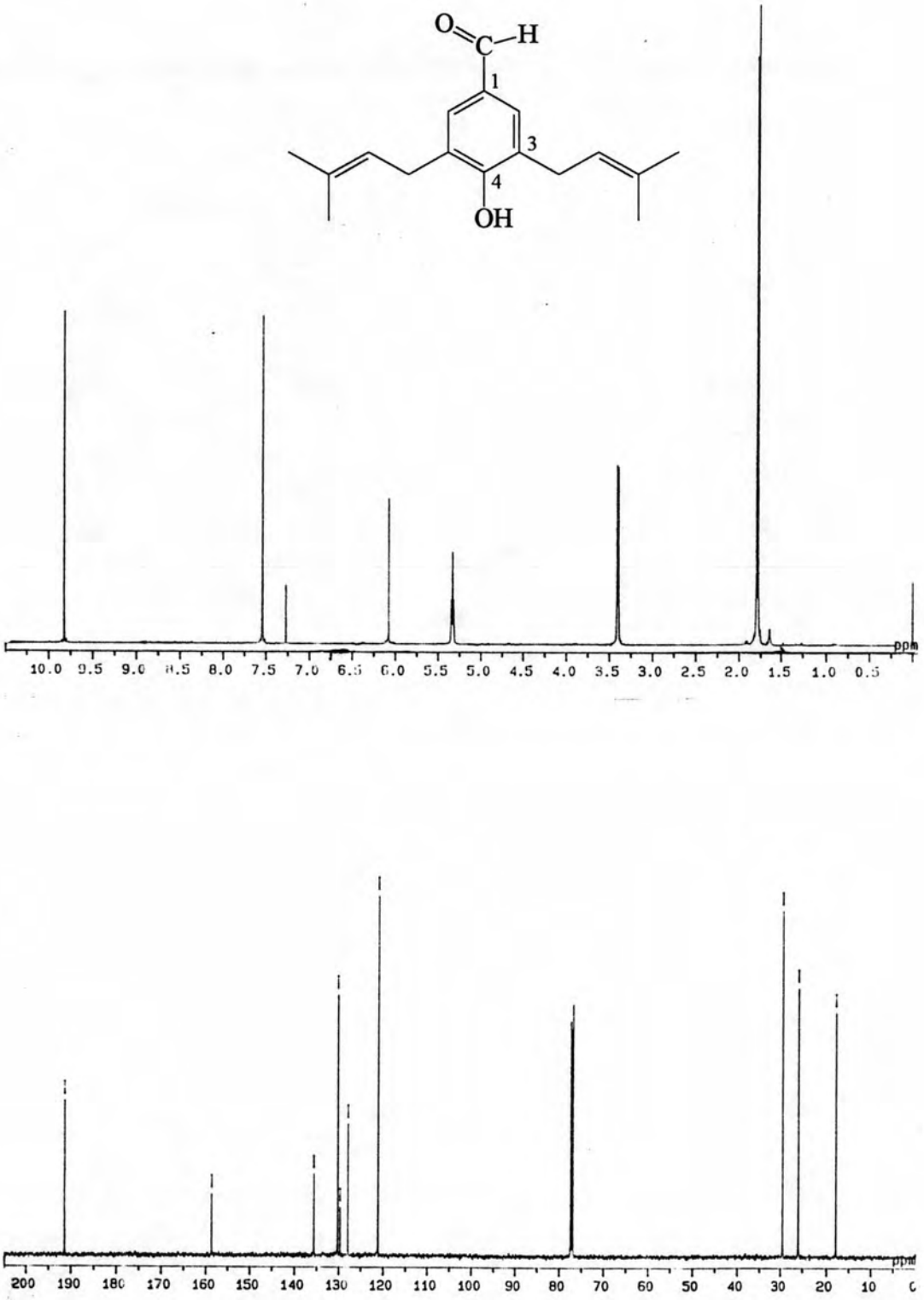


Figure 3.27 ^{1}H- and ^{13}C-NMR spectra($CDCl_3$) of compound **2**

3.2.6 Compound 3

Compound 3 (185 mg) was a colorless needle after recrystallizing from CH_2Cl_2. It melted at 294-295 °C, and had R_f 0.33 (SiO_2, 1:4 EtOAc/CH_2Cl_2). IR spectrum exhibited strong C=O stretching of ester at 1721 cm^{-1}. Base peak at m/z 533 in mass spectrum suggested this component might be one nitrogen containing compound. On the other hand, elemental analysis result confirmed the absence of any nitrogen atoms, and provided empirical formula $C_5H_4O_2$. A relative low intensity at m/z 576 was possibly to be a molecular peak which corresponded to molecular formula $C_{30}H_{24}O_{12}$ (Anal. C 62.6%, H 4.5%; Cal. for $C_{30}H_{24}O_{12}$ C 62.5%, H 4.2%), thus indicating nineteen degrees of unsaturation in the molecule. Singlet of aromatic protons at δ 8.10 could indicate equivalent para-substituent groups.

Owing to 1:1 signal integration of aromatic and methylene protons (δ 4.70, s), twelve protons belonged to aromatic ones, it could be implied that this compound composed of three aromatic rings. Consequently, the rest twelve protons were six of methylene ones. Among them, methylene protons should be divided into three pairs oriented in a symmetrical way.

^{13}C-NMR also supported the presence of ester and methylene carbons which were directly attached to oxygen of ester at δ165.3 and 62.7, respectively. Other signals were tertiary (129.7 ppm) and quaternary (133.8 ppm) carbons. Partial structure of compound **3** was deduced as diethylenyl terephthalate. The above spectral information led to the establishment of the structure of this compound as cyclic tris(ethylene terephthalate). Complete structure of compound **3** was proved by single-crystal X-ray analysis.

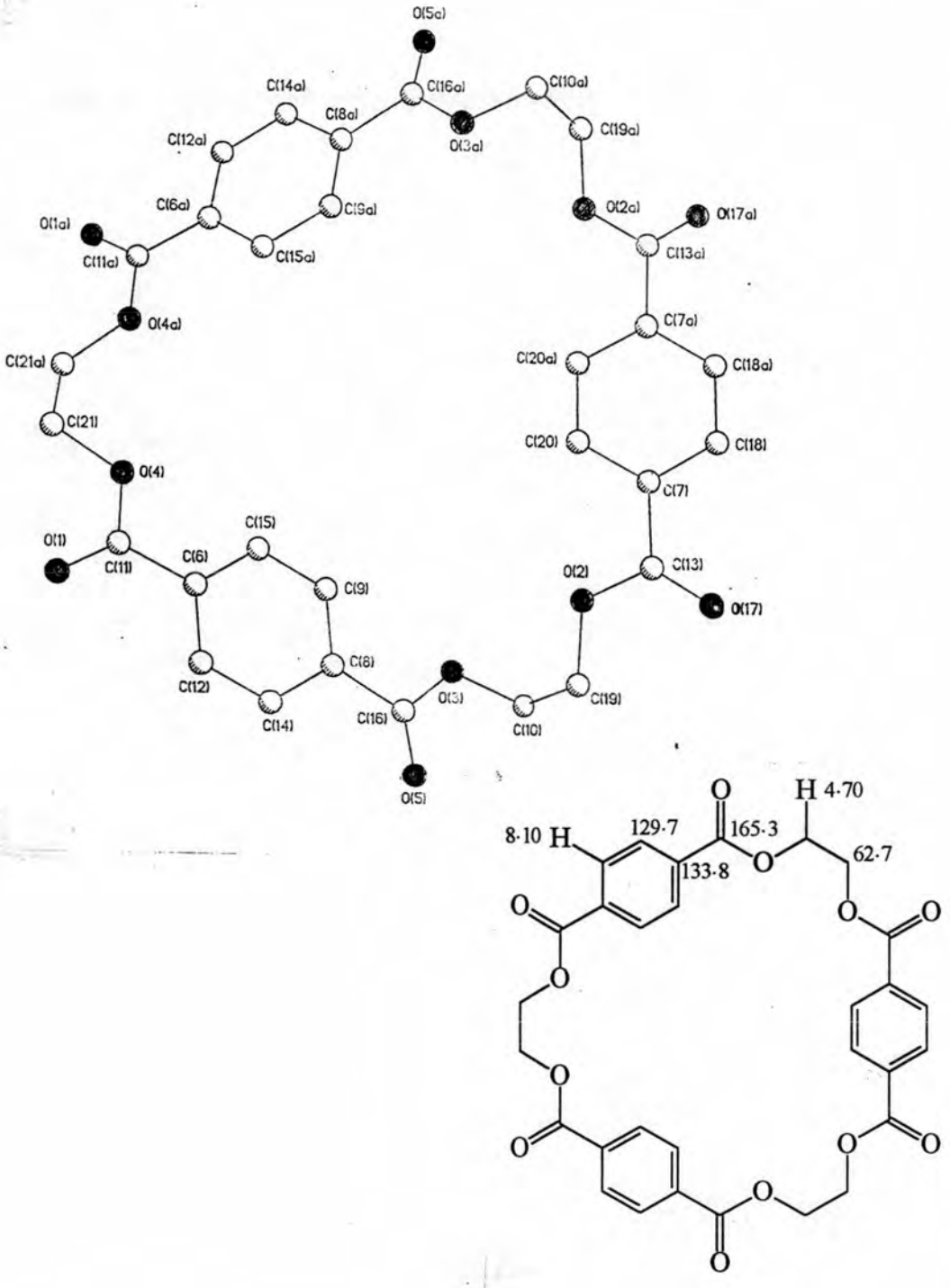


Figure 3.28 Perspective ORTEP drawing and NMR assignments of compound **3**

Cyclic tris(ethylene terephthalate) has been found in plastic films and nylons, not discovered as a natural component. In order to ascertain that compound **3** was whether an impurity or a constituent of *A. arborescens*. It might be contaminated during extraction and isolation procedures from two sources, distilled commercial solvent or plastic containers. To proof the suspecting, all solvents used for extraction and isolation were analytical grade (J.T. Beaker) including avoid contacting plastic ware throughout the experiment.

About 2.1 Kg of the stems bought from the same shop (April, 1997, see source of plant material) were carefully minced and soaked in EtOH. The ethanolic extract was concentrated to dryness under reduced pressure and further partitioned with hexane and CH_2Cl_2 yielded 3.4 and 6.9 g of two extracts, respectively. Dichloromethane extract was selected to separate since the target compound had been mainly disclosed in this extract. It was subjected to column chromatography using silica gel 60 (70-230 mesh), and CH_2Cl_2 as first eluent to afford fraction 1-7 which did not contain compound **3** (TLC guide). The ninth and tenth fraction obtained by eluting with 1:4 EtOAc/ CH_2Cl_2 showed the upper UV-active spot the same as compound **3** did, even in several solvent systems. The result allowed us to conclude that compound **3** was actually presence in the stems of *A. arborescens*. However, it was suspected that cyclic tris(ethylene terephthalate) might be adsorbed by the plant.

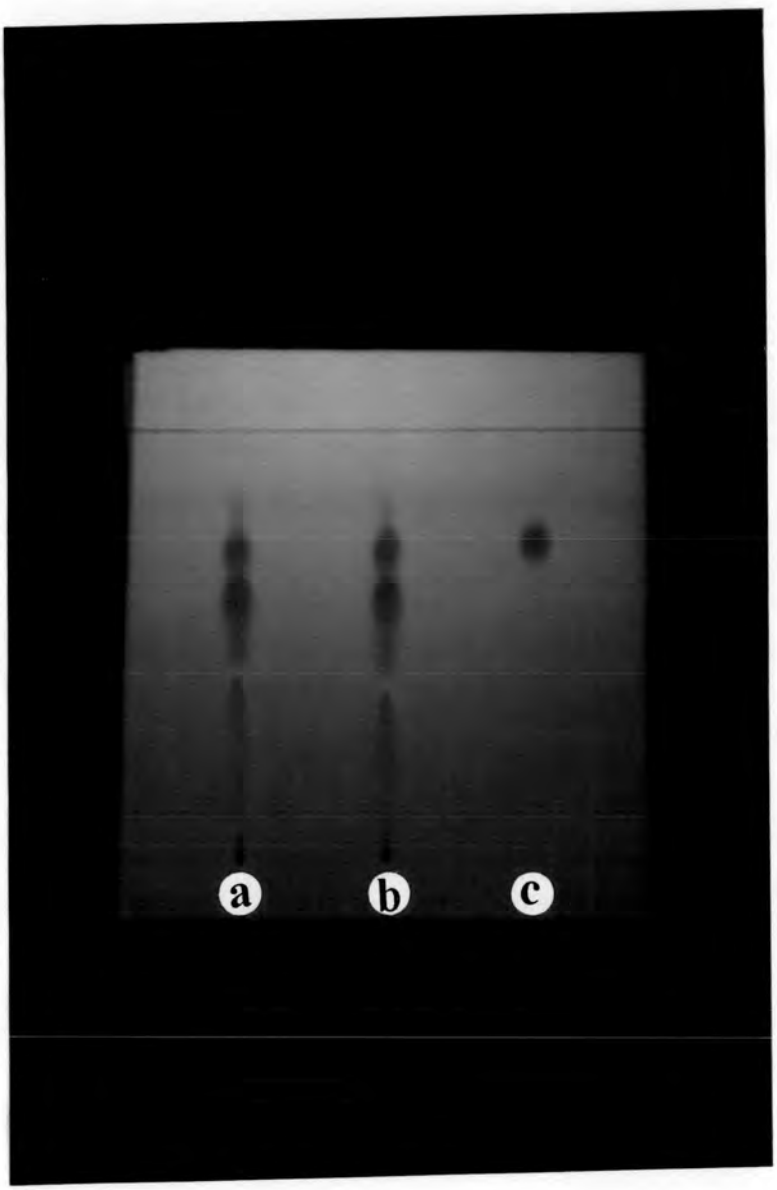
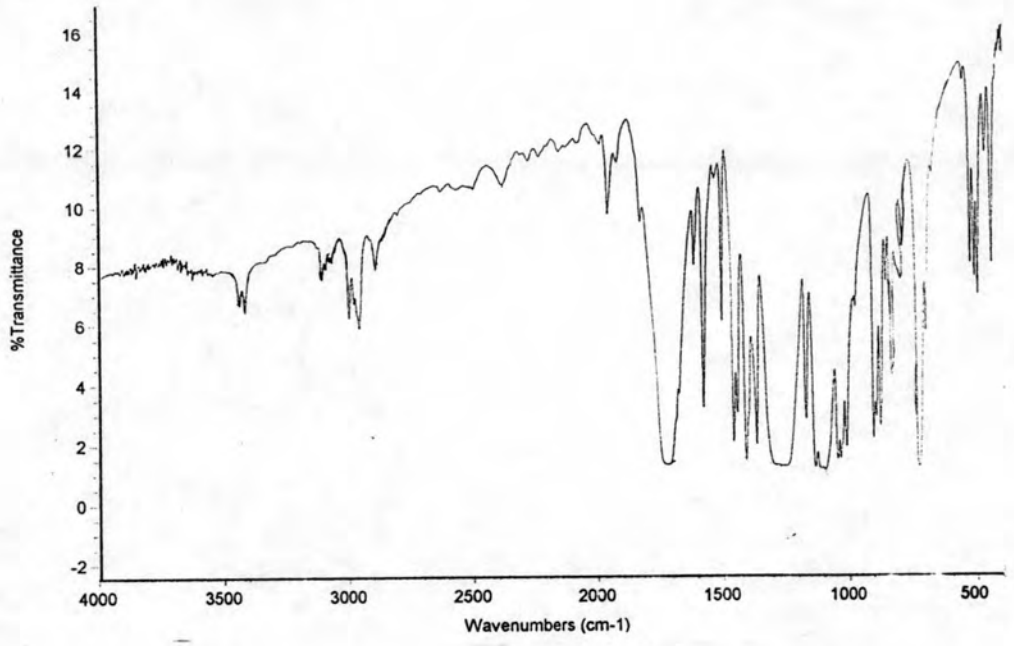


Figure 3.29 TLC (20x20 cm, 0.5 mm thickness, SiO_2-1:4 EtOAc/CH_2Cl_2) of (a) fraction 9-10, (b) fraction 9-10 plus cyclic tris(ethylene terephthalate) and (c) cyclic tris(ethylene terephthalate) illuminated under UV 254



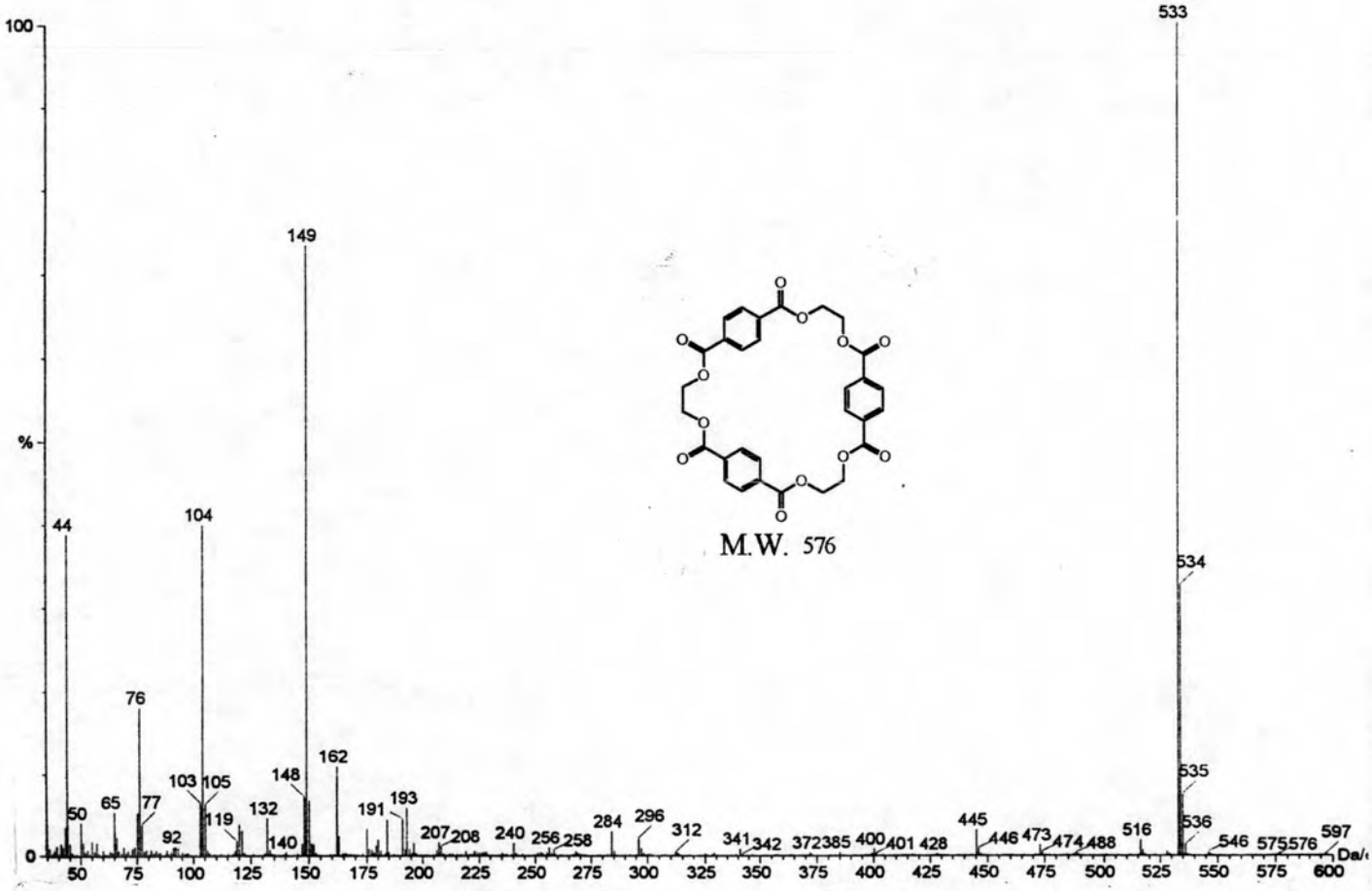


Figure 3.30 Infrared and mass spectra of compound **3**

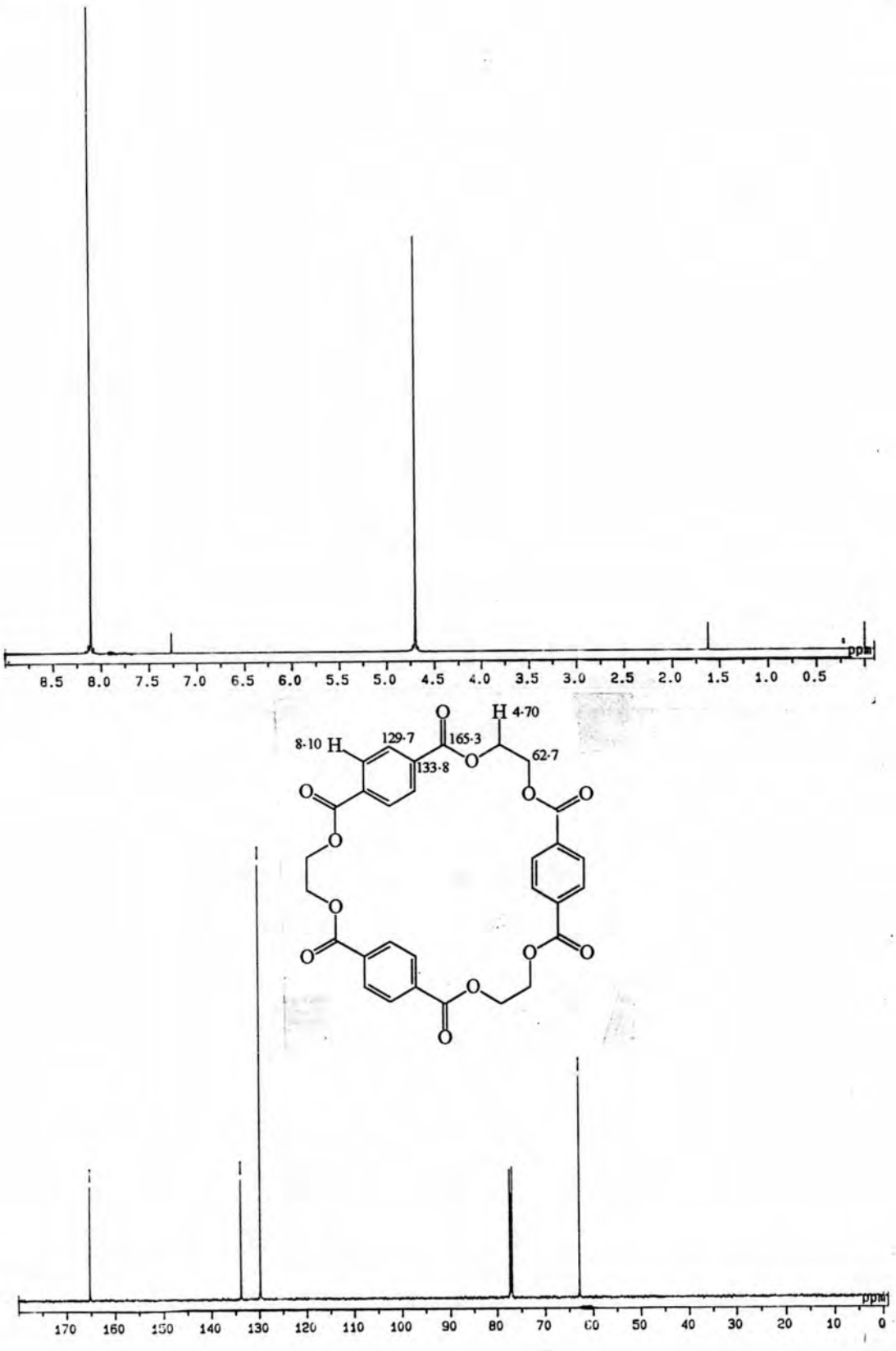


Figure 3.31 ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) of compound **3**

3.2.7 Compound 4

White powder (842 mg) of compound **4** was obtained from both dichloromethane and ethyl acetate crude extracts. After several recrystalization in hot 1:1 MeOH/CH_2Cl_2, a white powder was formed, m.p. 264-266 °C (dec.), R_f 0.56 (SiO_2, 3:7 MeOH/CH_2Cl_2) From the less solubility in various solvents and UV-inactive character, compound **4** seemed to be a steroid or a triterpenoid glycoside. Therefore, it was tested with Liebermann-Burchard reagent, and a deep green solution was gradually visible. This provided clear evidence that compound 4 was a steroid glycoside. IR spectrum also showed characteristic absorption bands of steroid glycosides: O-H stretching due to hydroxyl groups of sugar, C-H deformation of β-sugar and C-O stretching vibrations of glycosidic linkage at ν_{max} 3395, 890 and 1025, respectively.

A group of six carbon signals resonated at δ 100.8, 73.4, 76.9, 70.1, 76.7 and 61.1 was exactly resemble to a distinctive manner of D-glucose[26]. An anomeric proton at δ 4.22 (1H, d, J = 7.6 Hz) could point out the β-anomer of this sugar while a group of multiplet between δ 4.39-5.00 was the signals of other sugar moiety.

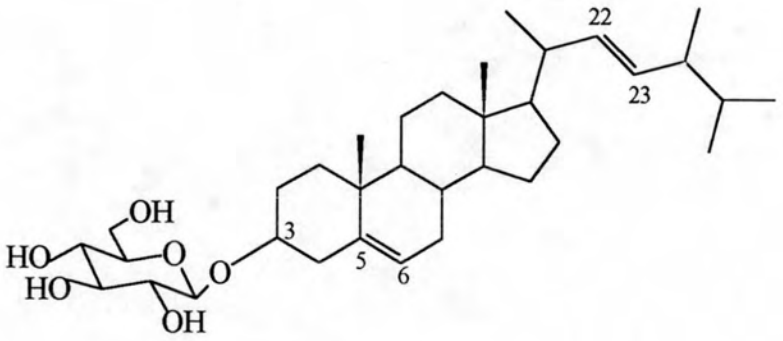
The mass spectrum did not show the molecular peak of this compound, only a molecular peak of a steroid aglycone presented relatively trace at m/z 412. Other fragments at m/z 394, 255 and 213 were similar to the fragmentation pattern of stigmasterol. Additionally, the lack of peaks at m/z 414 and 400 also provided a useful data that β-sitosteryl-3-O-β-D-glucopyranoside and campesteryl-3-O-β-D-glucopyranoside were not actually contaminated in this compound. All of the above results and literature comparison indicated that compound 4 was stigmasteryl-3-O-β-D- glucopyranoside.

Table 3.3 ^{13}C-NMR assignment of aglycones of compound **4** and stigmasteryl-3-O-β-D-glucopyranoside[27]

Carbon	Chemical shift (ppm)	
	Compound **3**	Stigmasteryl-3-O--β-D-glucopyranoside
C-1	36.2	37.4
C-2	31.4	31.7
C-3	70.1	71.8
C-4	41.8	42.4
C-5	140.4	140.0
C-6	121.1	121.7
C-7	31.3	31.9
C-8	31.4	31.9
C-9	49.6	50.3
C-10	36.8	36.6
C-11	21.0	21.1
C-12	38.3	39.8
C-13	41.8	42.4
C-14	56.1	57.0
C-15	23.8	24.4
C-16	28.4	28.9
C-17	56.2	56.0
C-18	12.0	12.2
C-19	19.6	19.4
C-20	41.7	40.5
C-21	21.0	21.1
C-22	138.0	138.4
C-23	128.8	129.4
C-24	50.6	51.3
C-25	31.3	31.9

Table 3.3(continue) ^{13}C-NMR assignment of aglycones of compound **4** and stigmasteryl-3-O-β-D-glucopyranoside

	Chemical shift (ppm)	
Carbon	Compound **3**	Stigmasteryl-3-O--β-D-glucopyranoside
C-26	19.0	19.0
C-27	21.0	21.1
C-28	24.8	25.4
C-29	12.0	12.0



stigmasteryl-3-O-β-D-glucopyranoside

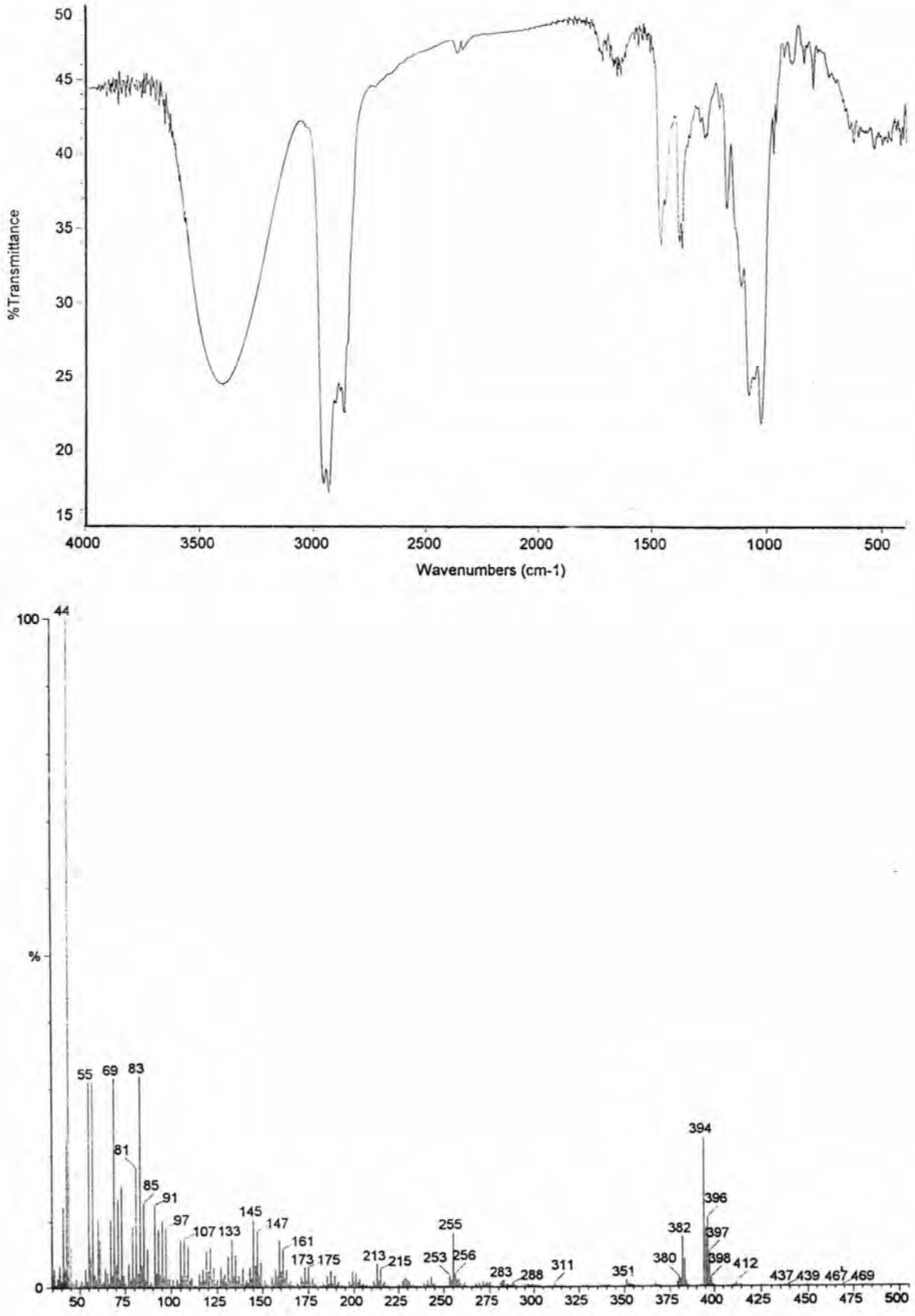


Figure 3.32 Infrared and mass spectra of compound **4**

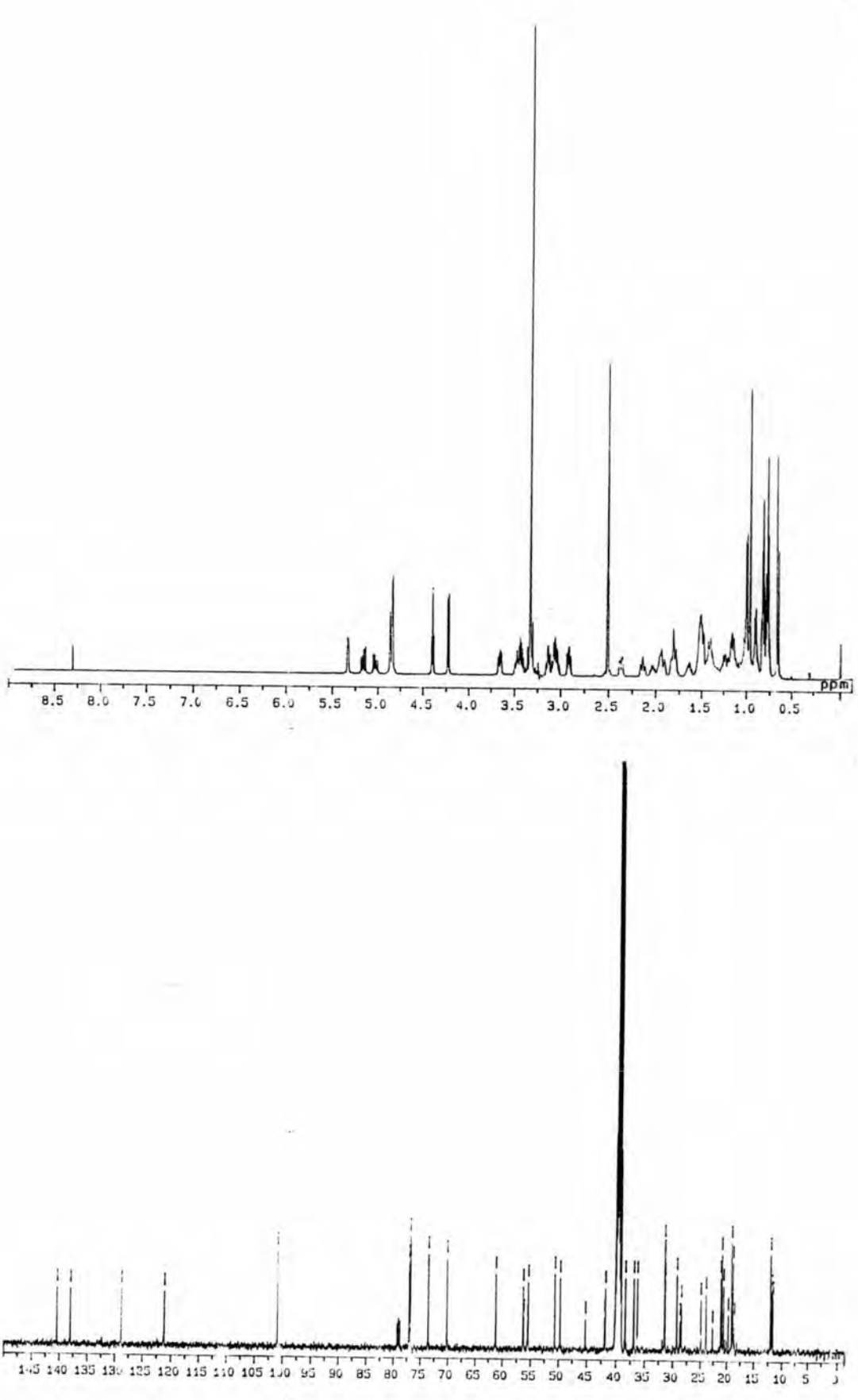
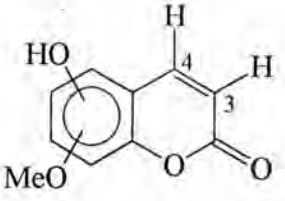


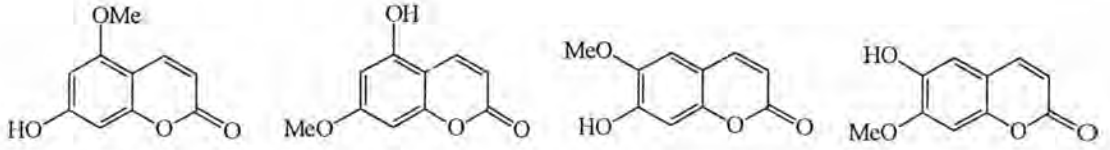
Figure 3.33 ^{1}H- and ^{13}C-NMR spectra (DMSO-d_6) of compound **4**

3.2.8 Compound 5

Eight milligrams of compound **5** was obtained as a yellow powder of melting point 169-170 °C and R_f 0.47 (SiO_2, 1:4 EtOAc/CH_2Cl_2). Compound **5** displayed bright blue fluorescence under UV lamp (365 nm) which suggested that it might be 7-hydroxycoumarins or 6,7-dialkoxycoumarins[28]. IR spectrum also clearly confirmed the presence of a hydroxy group at ν_{max} 3330 cm^{-1} and also showed the characteristic absorption band of unsaturated lactone of coumarin at 1705 (C=O) and 1290 (C-O) cm^{-1}. Molecular peak at m/z 192 corresponded to a molecular formula $C_{10}H_8O_4$. The ion peak at m/z 177 suggested the presence of methyl group that might be a part of methoxy one. Chemical shifts of carbon at δ 56.7 and proton at δ 3.91 were unambiguously confirmed the presence of one methoxy group. Other chemical shifts in NMR spectra and all above data corresponded to the structure of coumarin that contained one hydroxy and one methoxy as substituents. Typical chemical shifts of H-3 (δ 6.17, 1H, d, J = 9.4 Hz) and H-4 (δ 7.83, 1H, d, J = 9.4 Hz) implied that the substituents did not locate at an unsaturated lactone ring but at an aromatic one.



Two singlet peaks of aromatic proton at δ 6.79 (1H, s) and 7.19 (1H, s) guided that they were not adjacent to each other. Four possible structures of compound **5** could be established as follow.



(a) (b) (c) (d)

NOE Difference was an useful technique to determine which one was compound **5**. Irradiation of an aromatic proton at δ 7.19 resulted in enhancement of H-4 at δ 7.83. This provided clear evidence that the irradiated proton belonged to

H-5; therefore, structure (a) and (b) was canceled. Methoxy proton at δ 3.91 which was also enhanced by the irradiation of H-5 could be assigned as 6-OCH_3. Thus, the structure of compound **5** should be a structure (c). Once again, irradiation of 6-OCH_3 led to only one enhancement of H-5, indicated C-7 was then replaced by a hydroxy group. Similarly, irradiation of an aromatic proton at δ 6.79 caused no signal to rise. This unambiguously confirmed the presence of H-8 (δ 6.79). The above data led to the establishment of the structure of this compound as 7-hydroxy-6-methoxycoumarin or scopoletin. Complete proton and carbon assignments were achieved on the basis of comparison to NMR spectra of scopoletin[28] and shown in figure 3.34.

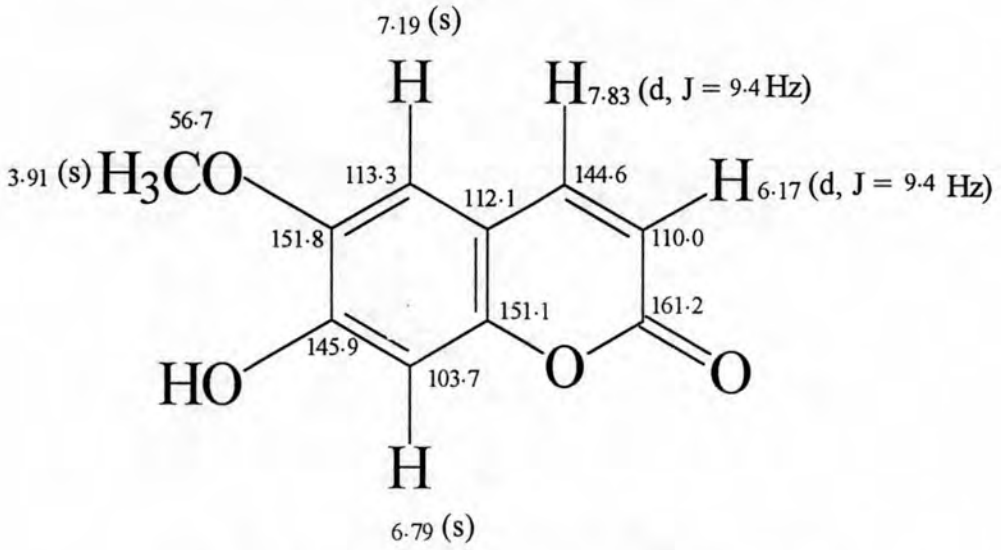
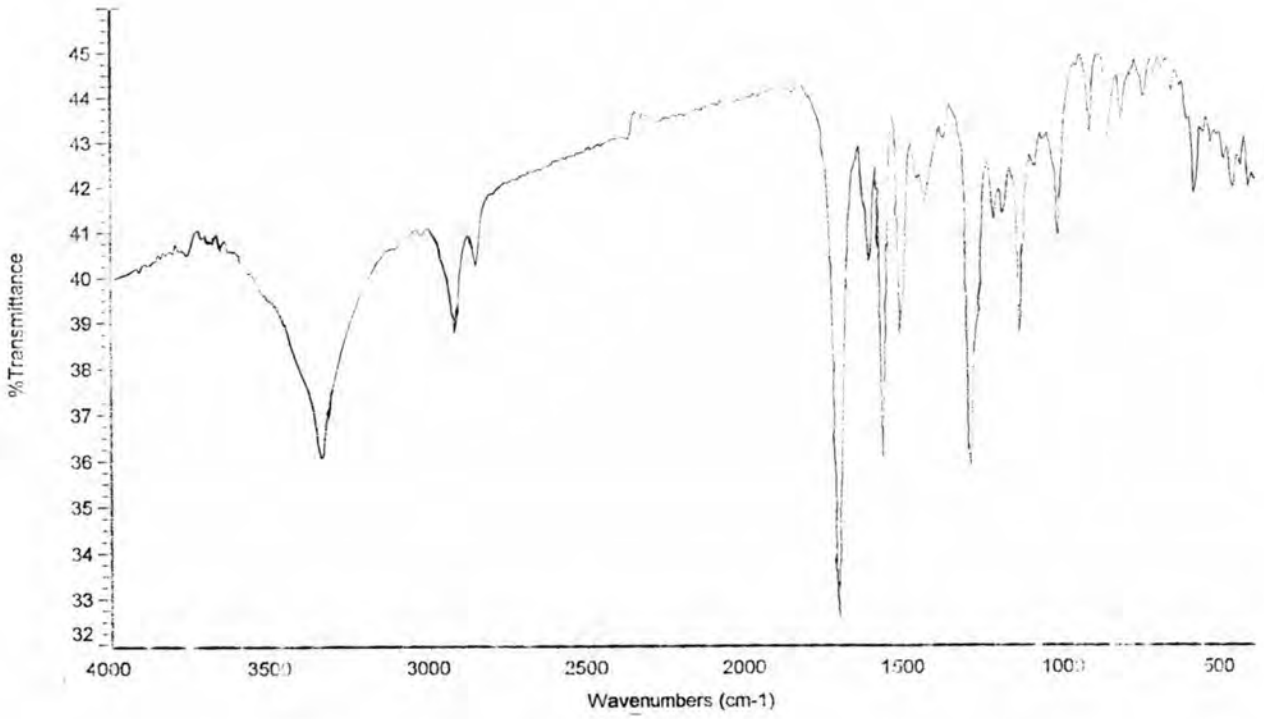
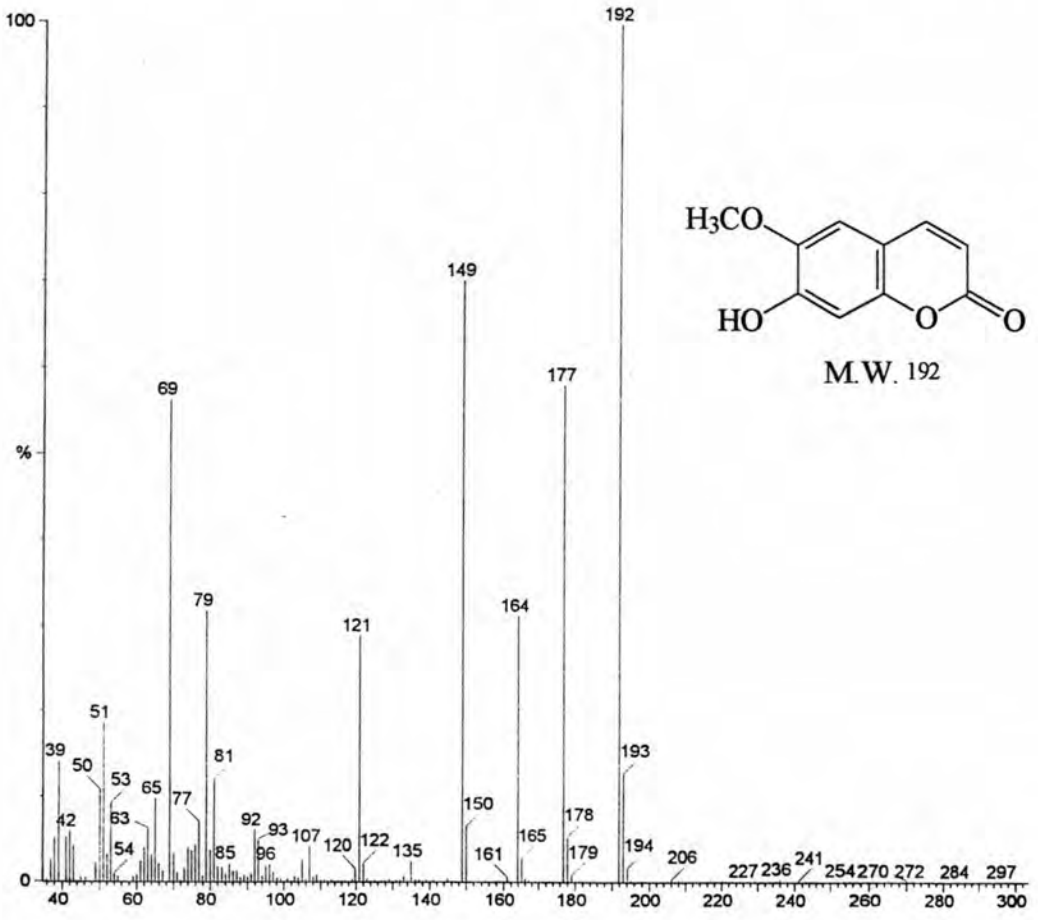


Figure 3.34 NMR assignment of compound **5**





M.W. 192

Figure 3.35 Infrared and mass spectra of compound **5**

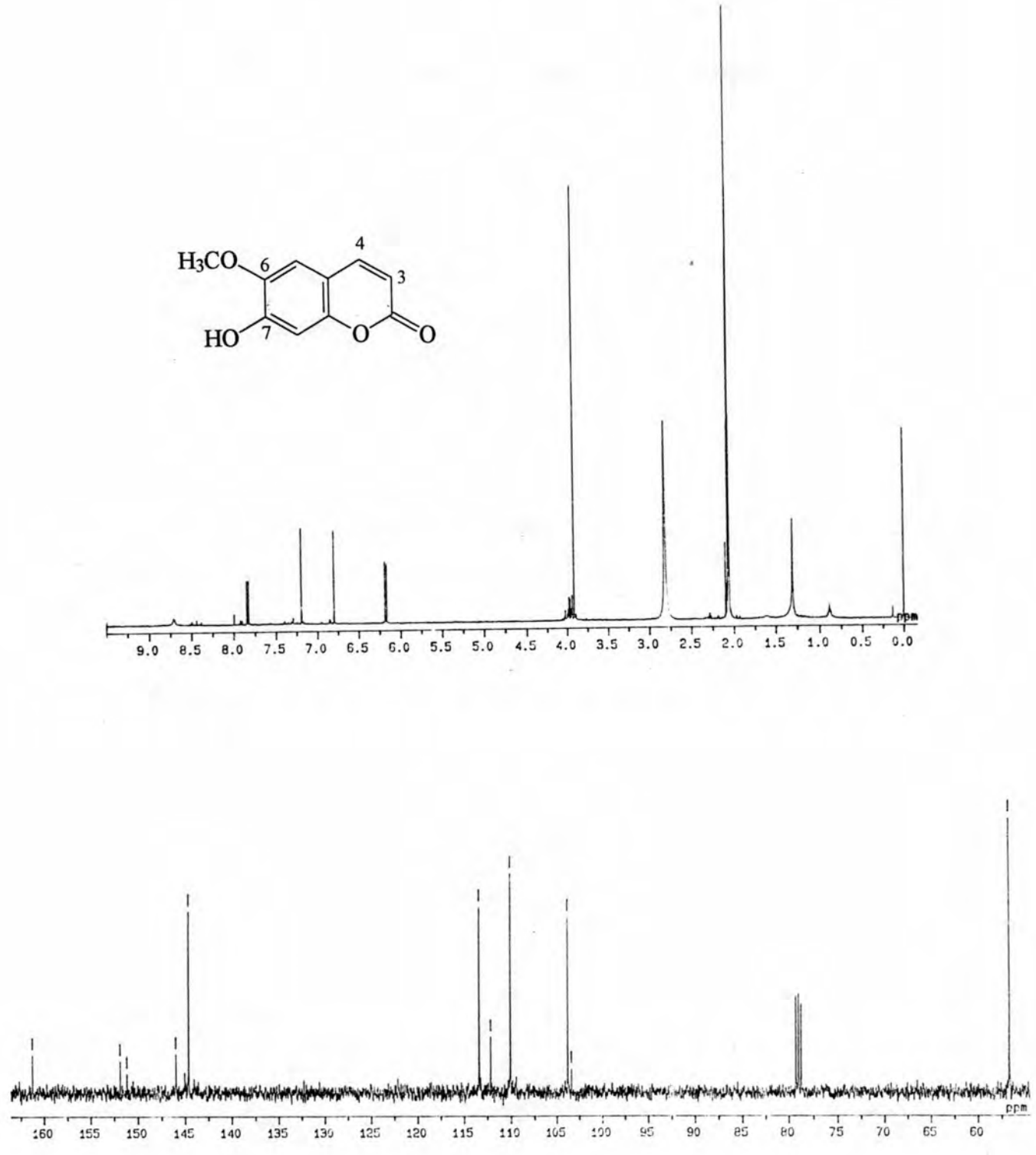
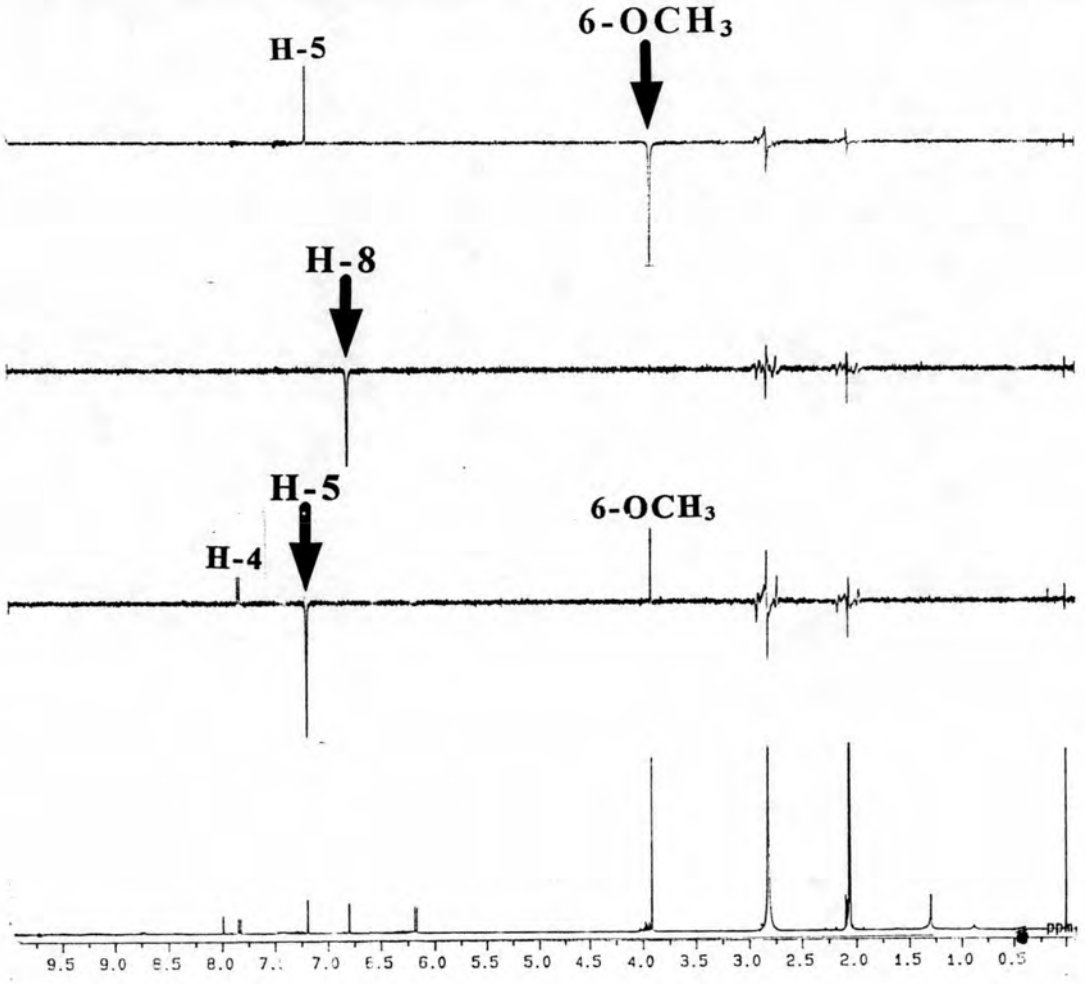


Figure 3.36 ^{1}H- and ^{13}C-NMR spectra (Acetone-d_6) of compound **5**



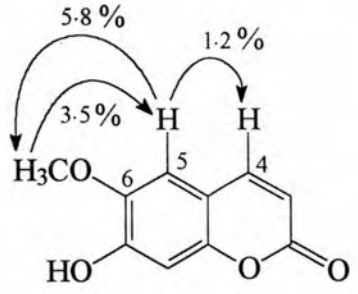


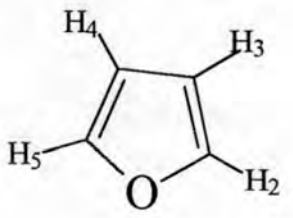
Figure 3.37 NOE Difference spectra with percent enhancement of compound **5**

3.2.9 Compound 6

Orange viscous liquid of compound **6** (285 mg) was obtained from ethyl acetate extract. It showed a deep red spot with DNPH dipping reagent, R_f 0.67 (SiO_2, 1:4 hexane/EtOAc). This suggested the presence of a carbonyl group, and the IR spectrum further specified this carbonyl would be an aldehyde owing to the characteristic C-H stretching at 2930 and 2845 cm^{-1}. Other functional group was a hydroxy one (ν_{max} 3375 cm^{-1}) that also implied from IR spectrum. Moreover, there was the absorption band at 1028 cm^{-1} due to ring breathing of furan. Mass spectrum also idicated the presence of aldehyde and hydroxy group: m/z 125 $[M-H]^+$ for an aldehyde and m/z 109 $[M-OH]^+$ for a hydroxy group. From the NMR spectrum data and molecular peak at m/z 126 could establish the molecular formula, $C_6H_6O_3$.

^{13}C-NMR spectrum showed the most downfield tertiary carbon of an aldehyde at δ 177.7 and methylene carbon which directly attacked to oxygen atom at δ 57.6. Then, there were two substituent groups attached to furan ring, one group was certainly aldehyde and the another should be hydroxymethyl group ($HOCH_2$-). The four carbon signal at δ 110.0, 122.8, 152.4 and 160.7 belong to furan skeleton.

^{1}H-NMR spectrum also exhibited signals corresponded to those of already assigned carbons such aldehyde and methylene protons resonated at δ 9.58 (1H, s) and 4.72 (2H, s), respectively. Furano protons resonated at δ 6.52 (1H, d, J = 3.7 Hz) and 7.22 (1H, d, J = 3.4 Hz). Magnitude of coupling constants resulted from the coupling of H-3 and H-4. Then, the disubstituted furan as mentioned above was 2,5-disubstituted one.



J_{nm}	J_{23}	J_{34}	J_{24}	J_{25}
Coupling constant (Hz)	1.6-2.0	3.2-3.8	0.6-1.0	1.3-1.8

All spectroscopy results allowed to assign compound **6** as 5-hydroxymethyl-furfuraldehyde or 5-HMF, and complete assignment was carried out on the basis of comparison to the previous reports[29,30].

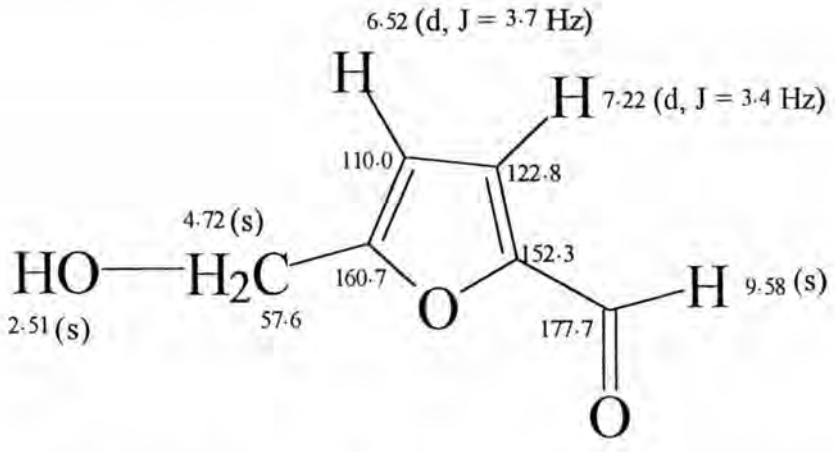
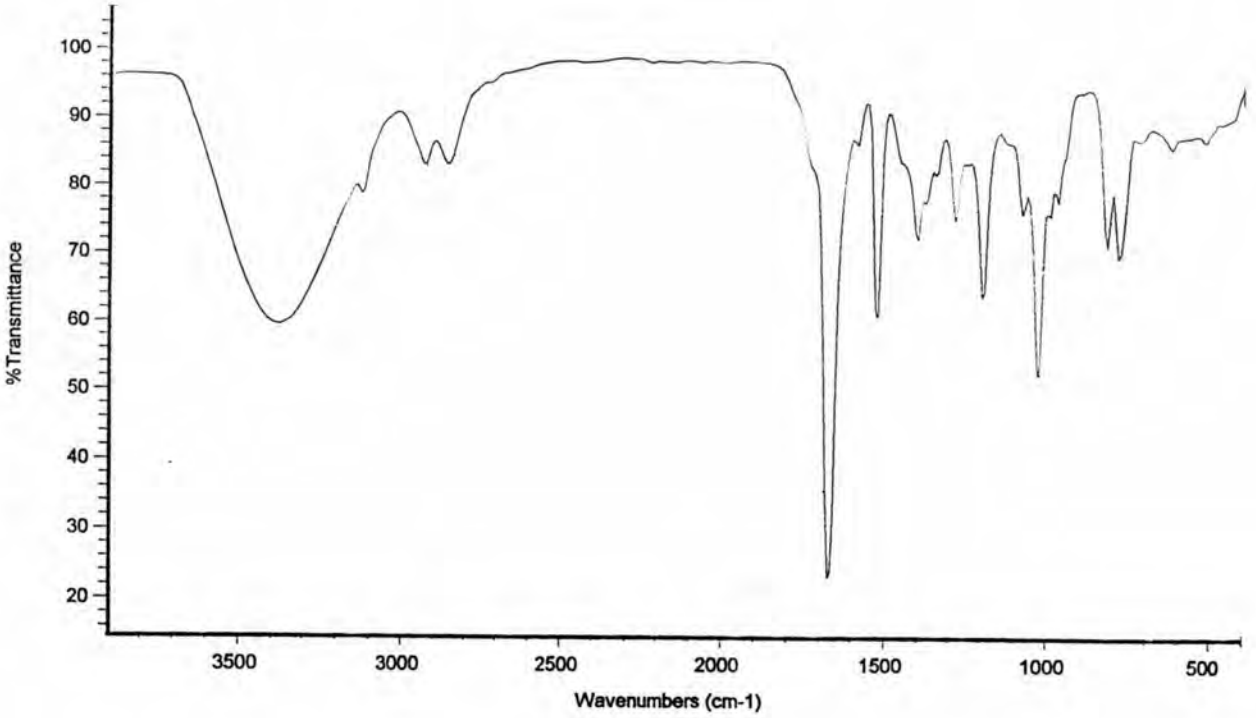


Figure 3.38 Proton and carbon assignments of compound **6**

5-Hydroxymethylfurfuraldehyde had been isolated from several plants in various families such *Pteris inaequalis* (Pteridaceae)[31], *Trachelospermum asiaticum* (Apocynaceae)[30], *Azadirachta indica* (Meliaceae)[32], and recently found in bulks of *Gladiolus spp*[33]. It could be commercially prepared by acid dehydration from several kinds of sugar, especially, from hexose.

5-HMF was of interest owing to a lot of their pharmacological activities. Reaction products between 5-HMF and monohydroxy-1,4-naphthoquinones were used as sunburn-prevention cosmetics containing[34]. More attractively, it was active as anthelmintic against *Clonorchia sinensis* (Chinese liver fluke)[35]. This evidence assuredly supported the anthelmintic activity of *A. arborescens*. In 1997, Wubert *et al.* found that 5-HMF isolated from *Gladiolus spp.* could inhibit chlorophyll synthesis in seedling of *Lepidium sativum* Linn., this activity could lead to a potential herbicide.



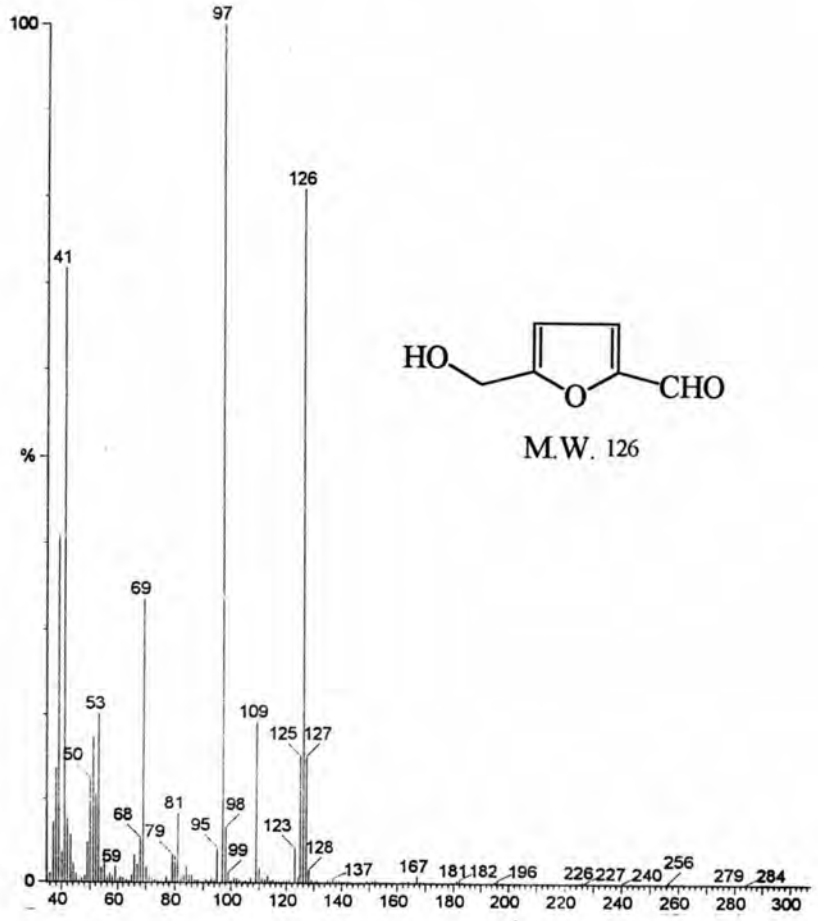


Figure 3.39 Infrared and mass spectra of compound **6**

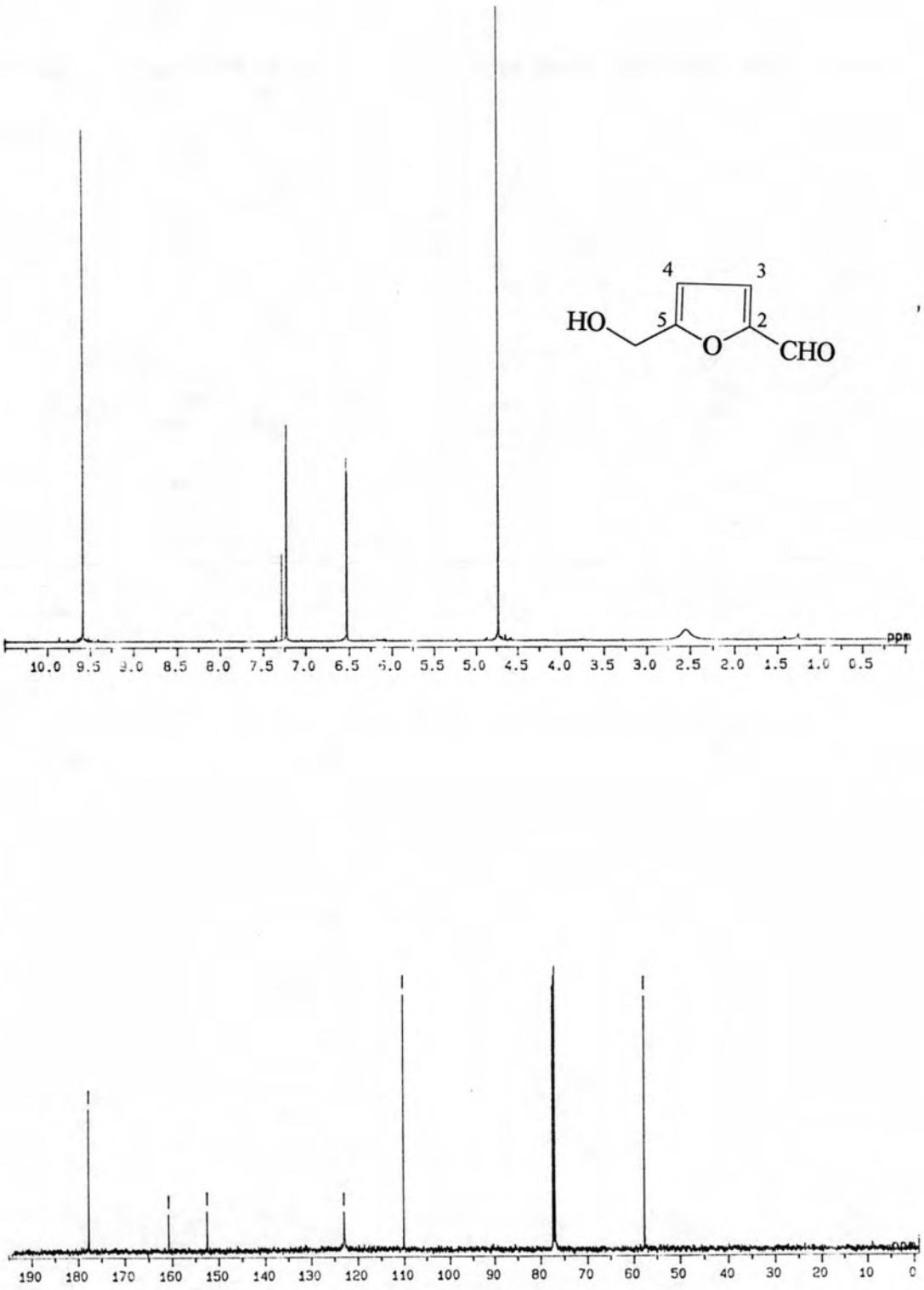


Figure 3.40 ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) of compound **6**

3.2.10 Compound 7

compound 7 (5 mg) was obtained as a white powder of melting point 214-216 °C, and had R_f 0.64 (SiO_2, 1:3 hexane/EtOAc). ^{1}H-NMR spectrum showed aromatic protons at δ 6.91 (d, 2H, J = 8.9 Hz) and δ 7.91 (d, 2H, J = 9.2 Hz). The AA'XX' splitting pattern (distorted doublet) suggested the presence of unequivalent *para*-substituted groups. One group that could be clearly implied from IR spectrum was carboxylic one since there were very broad absorption bands of O-H stretching at 3090-2535 cm^{-1} including C=O and C-O stretching at 1680 and 1245 cm^{-1}, respectively. Another one might be a phenolic group because there was a quaternary carbon signal resonated at δ 162.5, a typical range of aromatic carbon attached to a hydroxy group.

All spectral data suggested that compound 7 might be *p*-hydroxybenzoic acid. This compound was confirmed with authentic *p*-hydroxybenzoic acid (Hopkin & Williams Ltd.). NMR assignment of compound 7 shown in figure 3.41 were achieved base on previous literature[36].

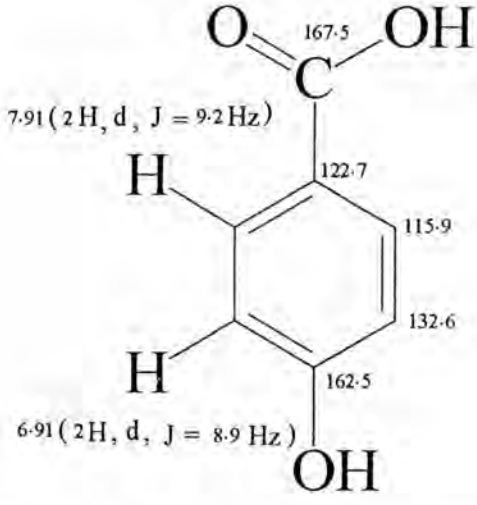


Figure 3.41 Proton and carbon assignments of compound 7 . Carboxylic and phenolic protons were absence because of their exchangeability with H_2O from deuterated solvent.

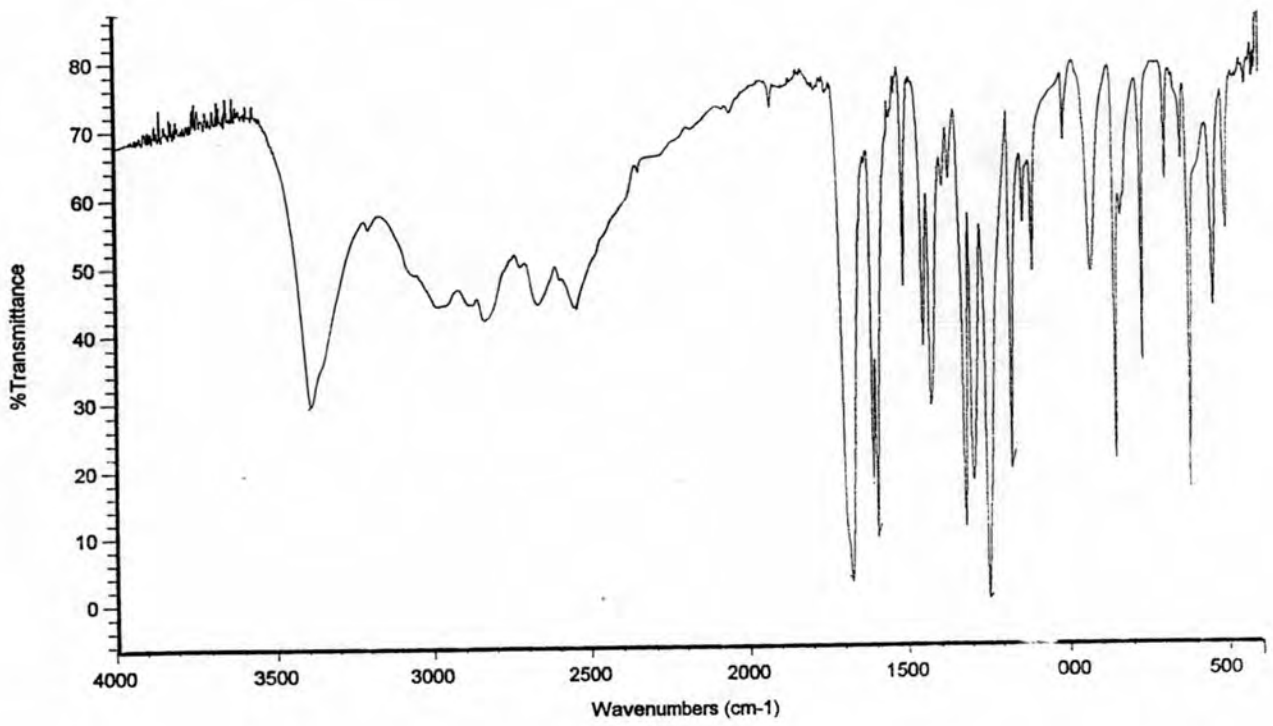


Figure 3.42 Infrared spectrum of compound 7

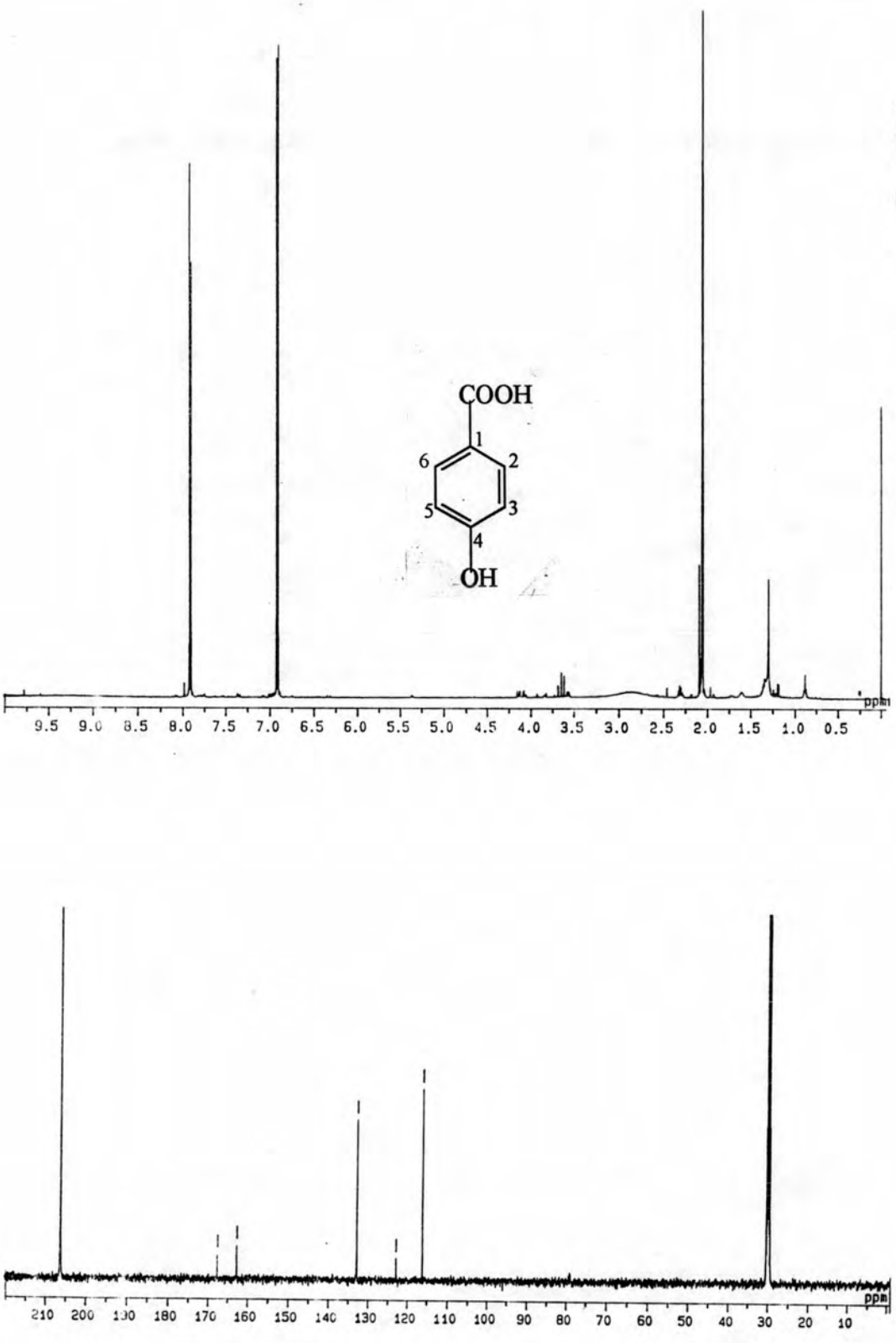
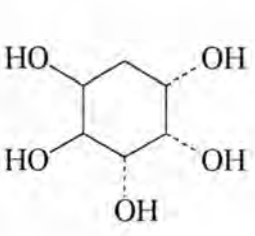


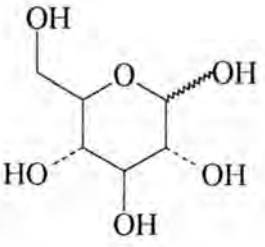
Figure 3.43 ^{1}H- and ^{13}C-NMR spectra (Acetone-d_6) of compound **7**

Most isolated compounds seemed to be primary and secondary metabolites in the early stage of the plant biosynthetic pathway. For support our assumption, (+)-*proto*-quercitol, the most abundant component isolated from this plant, was a good example to illustrate the structure similarity relation between it and glucose. Experiment on the incorporation of labeled possible precursors into (+)-quercitol confirmed that only D-glucose was transformed[37].



(+)-*proto*-quercitol

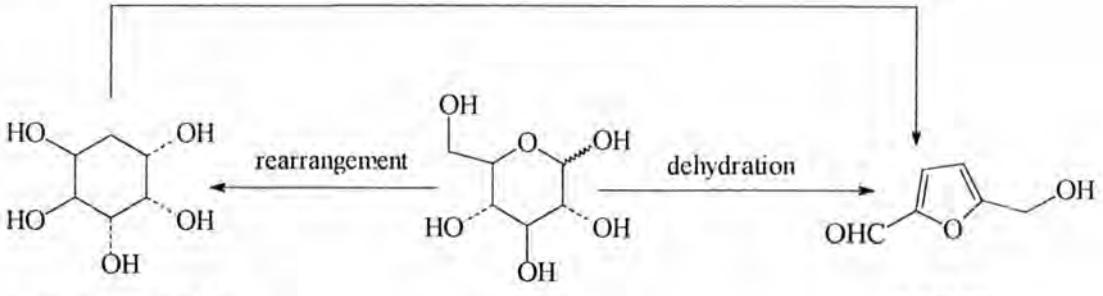
($C_6H_{12}O_5$)



glucose

($C_6H_{12}O_6$)

Furthermore, glucose and other hexoses could be transformed to 5-hydroxy-methylfurfural which was also separated from this plant. 5-Hydroxymethylfurfural might be derived from (+)-*proto*-quercitol, a possible pool of precursor.



(+)-*proto*-quercitol glucose 5-HMF

Other compounds like 3,5-Bis-[3,3-dimethylallyl]-*p*-hydroxybenzaldehyde, scopoletin and *p*-hydroxybenzoic acid were regarded as phenolic C_6-C_n type compounds.

3.3 Brine Shrimp Cytotoxic Lethality Test Result of Isolated Compounds

$LC_{50's}$ and 95% confidence intervals were determined by a probit analysis program. In case where data were not sufficient, and amount of compounds was available enough, motalities in each concentration were considered to determine the proper set of new concentrations for further test to obtain $LC_{50's}$ and 95% confidence intervals. If the tested compounds were not available enough, and data were insufficient, $LC_{50's}$ were estimated using a logit transformation.

Table 3.4 BSCLT result of some isolated compounds.

Tested Compounds	Motality at 24 hours			$LC_{50's}$ (μg/ml)	95% confidence intervals
	1000	100	10		
compound **1**	33.3	20.0	13.3	>1000	-
compound **2**	73.3	26.7	16.7	460	215-1028
compound **3**	29.3	20.3	13.3	>1000	-
compound **5**	66.7	60.0	16.7	123	-
compound **6**	93.3	73.3	23.3	71.2	40.7-89.2
compound **7**[*]	100.0	96.7	30.0	33.1	20.8-47.3

Among the tested compounds, compound **6** and **7** showed an attractive result against brine shrimp, however, an unextended study of biological activities of compound **6** was an interesting point for further study of other activities including BSCLT of this compound and its analogues. Consequently, furfural (**6a**, Merck, redistilled prior used), a furanocarboxaldehyde which was more available and resemble to **6**, was used as a starting material in order to synthesized other furan derivatives. Furfuryl alcohol (**6b**) and 2-furoic acid (**6c**) were prepared from Canizzaro reaction while semicarbazone (**6d**) and 2,4-dinitrophenylhydrazone (**6e**) of furfural were

[*] compound 7 or *p*-hydroxybenzoic acid used in BSCLT and antibacterial assay was purchased from Hopkin & Williams Ltd.

from conventional means[38]. All structures of them were characterized by comparison to spectral data and physical property of previous reports[38-40].

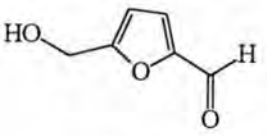
Compound ***6b***: IR(neat): 3360 (OH), 782 (CH oop,furan); ^{1}H-NMR (DMSO-d_6): δ 3.32 (s, 1H, OH), 4.50 (s, 2H, CH_2), 6.29 (m, 2H), 7.38 (d, 1H, J=1.6 Hz)

Compound ***6c***: M.p. 132-133 °C (ref. 133-134 °C); IR (KBr): 3145-2577 (OH), 1685 (C=O), 770 (CH oop, furan); ^{1}H-NMR (DMSO-d_6): δ 6.71 (dd, 1H, J=1.8, 3.7 Hz), 7.32 (d, 1H, J = 3.7 Hz), 7.89 (d, 1H, J = 1.7 Hz), 10.51 (s, 1H,COOH)

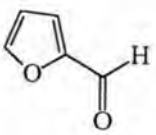
Compound ***6d***: M.p. 191-192 °C (ref. 190-192 °C); IR (KBr): 3465 (-NH-CO-), 1662 (-NH-CO-NH_2), 1598 (-CH=N), 753 (CH oop, furan)

Compound ***6e***: M.p. 201 °C (ref. 202 °C); IR (KBr): 3280 (-NH-aro), 1510 (-CH=N-), 1615 ($-NO_2$ asym.), 1325 ($-NO_2$ sym.), 770 (CH oop, furan)

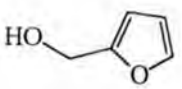
Furfural (**6a**) and its derivatives (**6b-6e**) were tested with BSCLT as well as antibacterial assay and plant growth inhibition.



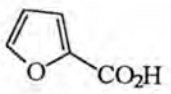
5-HMF (6)



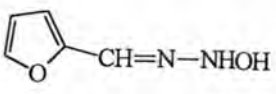
Furfural (**6a**)



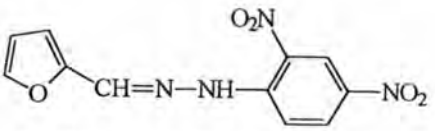
Furfuryl alcohol (**6b**)



2-Furoic acid (**6c**)



Semicarbazone (**6d**)



2,4-Dinitrophenylhydrazone (**6e**)

Table 3.5 BSCLT result of compound **6** and its analogues.

Tested Compounds	Motality at 24 hours			LC_{50}'s (μg/ml)	95% confidence intervals
	1000	100	10		
compound **6**	93.3	73.3	23.3	71.2	40.7-89.2
compound **6a**	83.3	30.0	13.3	278	141-512
compound **6b**	90.0	13.3	10.0	204	-
compound **6c**	100.0	100.0	100.0	0.91	0.33-1.47
compound **6d**	100.0	26.3	12.0	293	153-386
compound **6e**	100.0	96.8	39.0	32.6	11.8-52.2

3.4 Antibacterial result

After 24 hours of incubation, diameter of clear zone was measured. More than 10 mm diameter of inhibition zone was estimated as high inhibition (++). Weak inhibition (+) was established in case of 7-10 mm diameter of clear zone while the tested compounds were inactive (-) where diameter of inhibition zone was less than 7 mm.

Table 3.6 Antibacterial result of isolated compounds against *E. coli*, *B. cereus*, *S. aureus* and *S. derby* at dose 300 μg/disc.

Tested compounds	Antibacterial estimation of tested compounds against			
	E. coli	*B. cereus*	*S. aureus*	*S. derby*
compound **1**	-	-	-	-
compound **2**	-	-	-	-
compound **3**	-	-	-	-
compound **6**	++	++	++	++
compound **7**	-	-	-	-

As a result of strong activity of compound **6** against four susceptible, it was further tested with these four bacteria including three more resistance ones: *Escherichia coli* 0157: H7* , *Listeria monocytogenes* and flat sour spoilage. Herein, antibacterial assay was carried out using spot-on-lawn technique[41], and %T/C values were determined as follow:

$$\%T/C = \frac{\text{inhibition zone radius in mm caused by sample}}{\text{inhibition zone radius in mm caused by control}} \times 100$$

Table 3.7 Antibacterial result of isolated compounds against *E. coli*, *B. cereus*, *S. aureus*, *S. derby*, *E. coli* 0157: H7, *L. monocytogenes* and flat sour spoilage at dose 10 μg/well.

Microorganisms	Inhibition zone radius (mm) caused by		%T/C
	Compound **6** (T)	Streptomycin (C)	
E. coli	2.5	4.0	63
B. cereus	3.0	5.5	55
S. aureus	1.5	5.0	30
S. derby	2.5	4.0	63
E. coli 0157: H7	2.5	4.0	63
L. monocytogenes	1.5	3.0	50
flat sour spoilage	3.5	5.0	70

Nevertheless antibacterial activity of compound **6** against 7 microorganisms was approximately half fold of streptomycin, but compound **6** had some advantages such cost and material availability over streptomycin. To our best knowledge, compound **6** was not toxic to human and animals, this implied that it would be an alternatively potent antibacterial agent.

* This strain caused serious diarrhea in Japanese children, and some lethally injured cases were died.

Compound **6a-6e**, analogues of compound **6**, were also tested with four susceptible bacteria to disclose which analogues exhibited strong activity like compound **6**. However, none of them gave antibacterial activity.

Table 3.8 Antibacterial result of compound **6** and its analogues against *E. coli*, *B. cereus*, *S. aureus* and *S. derby*, at dose 300 μg/discl.

Tested compounds	Antibacterial estimation of tested compounds against			
	E. coli	*B. cereus*	*S. aureus*	*S. derby*
compound **6**	++	++	++	++
compound **6a**	-	-	-	-
compound **6b**	-	-	-	-
compound **6c**	-	-	-	-
compound **6d**	-	-	-	-
compound **6e**	-	-	-	-

3.5 Plant Growth Inhibition Result

In general, herbicidal effect of any chemicals on growth of root or shoot usually showed parallel result. In spite of some cases where data was not compatible, effect on growth of root was mainly considered because root contacted directly to the chemical. Three plants used in this experiment were *Mimosa pigra* Linn., *Echinochloa crus-galli* Beauv. and *Oryza sativa* var. RD.23. The two former plants were weeds that cause serious problems in almost every parts of Thailand while the latter was selected as a model of monocotyledon weeds.

Table 3. 9 Effect of some synthetic furan analogues and certain isolated compounds on growth of *Mimosa pigra* Linn. at various concentration (ppm)

Tested compounds	% Inhibition* on					
	Root			Shoot		
	10	100	1000	10	100	1000
compound **1**	3.1	14.8	-14.0	19.5	13.0	15.6
compound **6**	-15.5	-23.8	31.9	-10.3	-2.1	6.1
compound **6a**	-26.0	27.5	19.3	-4.2	-20.5	8.1
compound **6b**	-23.0	7.3	75.0	-12.3	-0.1	10.1
compound **6c**	-20.0	-23.0	100.0	-10.3	-28.7	100.0
compound **6d**	7.5	-14.0	86.0	-13.7	9.1	28.8
compound **6e**	-20.0	-23.0	10.5	-26.4	-7.5	-18.8

* %Inhibition less than zero referred to % promotion.

Table 3.10 Effect of some synthetic furan analogues and certain isolated compounds on growth of *Echinochloa crus-galli* Beauv. at various concentration (ppm)

Tested compounds	% Inhibition on					
	Root			Shoot		
	10	100	1000	10	100	1000
compound **1**	-34.6	-30.7	-33.2	10.9	15.2	5.7
compound **6**	-18.8	-56.7	-34.5	-2.7	5.4	24.3
compound **6a**	-52.6	-10.2	79.2	30.6	8.7	39.6
compound **6b**	-19.5	-17.1	90.8	27.9	31.5	56.8
compound **6c**	-5.7	16.8	98.9	99.1	99.3	99.8
compound **6d**	0.1	24.9	72.9	-3.8	-5.7	19.5
compound **6e**	31.1	8.2	13.7	20.0	21.7	8.3

Table 3.11 Effect of some synthetic furan analogues and certain isolated compounds on growth of *Oryza sativa* var. RD. 23 at various concentration (ppm)

Tested compounds	% Inhibition on					
	Root			Shoot		
	10	100	1000	10	100	1000
compound **1**	-12.1	-3.9	-16.9	7.7	0.3	2.8
compound **6**	15.8	24.0	80.8	13.1	-0.4	7.1
compound **6a**	13.9	30.6	66.9	44.0	24.3	50.0
compound **6b**	30.6	32.6	71.7	2.6	9.7	28.7
compound **6c**	-24.8	-7.9	26.5	2.5	9.2	11.1
compound **6d**	13.5	-5.3	10.5	0.9	-3.4	7.1
compound **6e**	3.2	-8.5	-3.5	1.4	0.3	1.7

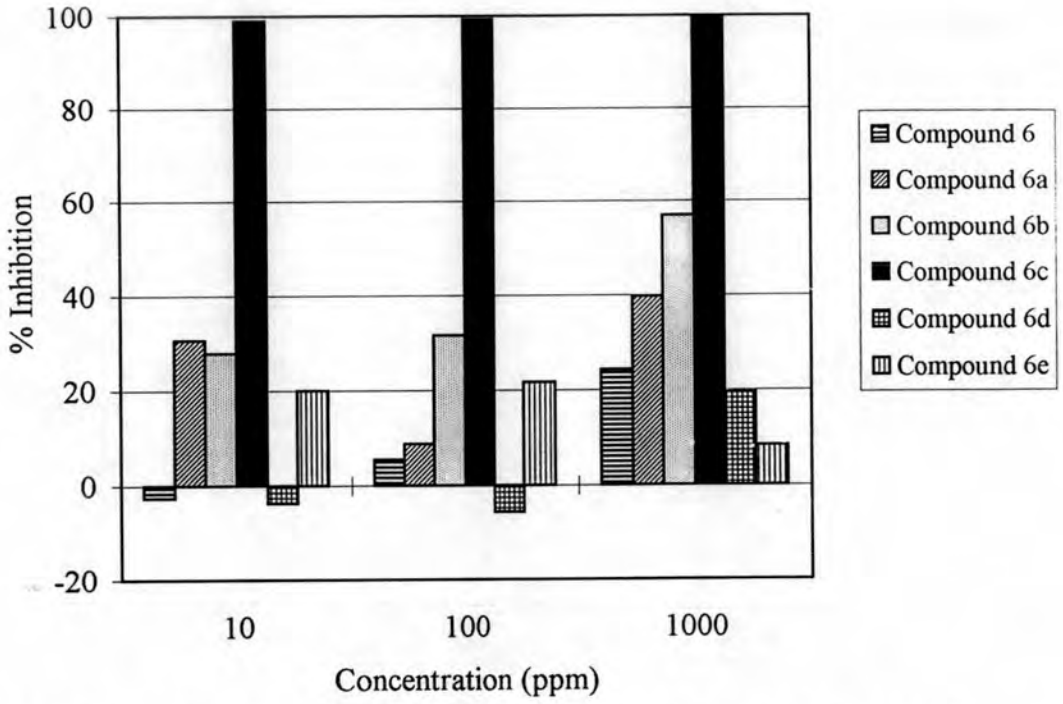


Figure 3.44 Effect of 2-furoic acid (**6c**) on shoot growth of *Echinochloa crus-galli* Beauv. compared with 5-HMF (**6**) and other furan derivatives (**6a-b, 6d-e**)

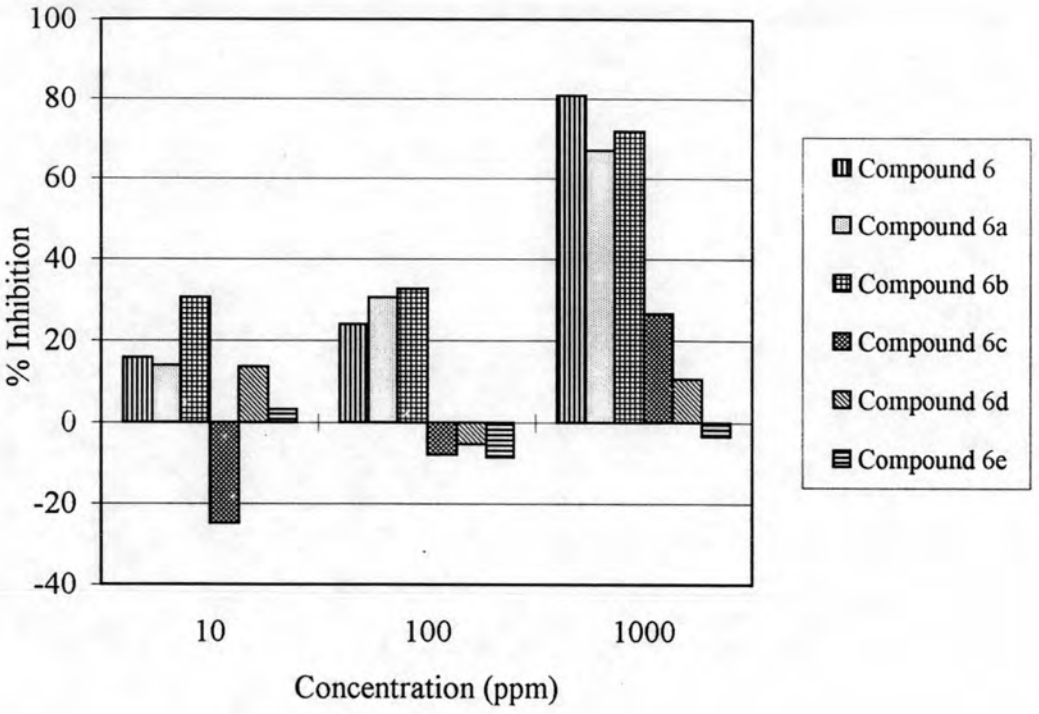


Figure 3.45 Effect of 5-HMF (**6**) and furfuryl alcohol(**6b**) on root growth of *Oryza sativa* var. RD. 23 compared other furan derivatives (**6a**, **6c-e**)

All synthetic furan analogues except **6e** showed more satisfactory inhibition efect (more than 70% of inhibition) on three tested plants than naturally isolated compounds (**1** and **6**). 2-Furoic acid (**6c**) significantly exhibited so strong herbicidal effect (more than 90% inhibition) on *E. crus-galli* Beauv. even at low concentration like 10 ppm. As for *O. sativa* var. RD.23, its growth was rather retarded by only compound **6** and furfuryl alcohol (**6b**). This result also supported the herbicidal effect of 5-HMF (**6**) on green cress or *Lepidium sativum*.

Other than cytotoxicity, antibacterial activity and plant growth inhibition of compound **6**, antifungal activity against *Cladosporium cucumerinum* and antioxidant* (DPPH** and β-carotene as detecting agents) were also observed.

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* These TLC autographic assays were performed by Dr. Worawan Bhundumnavin during her short course training (April, 1998) on rapid screening bioassays kindly provided by Professor Kurt Hostettmann of the Institute of Pharmacognosy and Phytochemistry, University of Lausane, Switzerland.

** 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl radical