University of Benghazi

Faculty of Science

Department of Chemistry

ISOLATION AND STRUCTURAL IDENTIFICATION OF SOME CHEMICAL CONSTITUENTS FROM THE LEAVES OF *ARBUTUS PAVARII*

M.Sc. Thesis

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University of Benghazi

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This thesis entitled

ISOLATION AND STRUCTURAL IDENTIFICATION OF SOME CHEMICAL CONSTITUENTS FORM THE LEAVES OF *ARBUTUS PAVARII*

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Submitted in Partial Fulfillment for the Requirements for the Master Science degree in Chemistry

Final Approval

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(Head, Chemistry Department)

Dr. Ahmed M.Mami
(Dean, Faculty of Science)

Dedication

I Dedicate This Work To

My mother ,my Mather Fatma

my waif , and my children

Acknowledgement

I thank the almighty Allah for giving me courage and the determination, as well as guidance in conducting this research study, despite all difficulties. First I would like to thank my supervisor Ast.Prof .Fakhri Elabar. For guidance,advice, courteous supports and helpful comments throughout all stages of this work.

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. . . .Mohamed Elerybi

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ABSTRACT

A.pavarii (Ericaceae Family) is one of endemic plants growing in Libya (AL-Jabel AL-Akhdar region), which known locally as SHMARY.

A. pavarii is a member of the genus Arbutus used for honey production.

It's used in folk medicine in treatment of kidney diseases. The chemical screening showed the presence of Flavonoids, phenolics, triterpenoids, and sterols compounds. Through chromatographic techniques of acetone extract two compounds were obtained and identified as Lupeol and Epicatechin in addition to one compound was obtained from hydrolyzed methanol extract, which is identified as hydroquinone. The structure of these compounds had been elucidated using spectroscopic methods (^{1}H- NMR, ^{13}C- NMR and Mass). Two dimensions nuclear magnetic resonance technique (2D-NMR) and DEPT had been used to confirm the structure of these compounds.

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 Ericaceae Family

Arbutus is a genus of at least 14 species of flowering plants in the family Ericaceae, native to warm temperate regions of the Mediterranean, Western Europe, and North America. The North American members of the genus are called strawberry tree. ***Arbutus*** species are used as food plants; several species are widely cultivated as ornamental plants. ***Arbutus*** bark and leaves are used as medicines for colds, stomach problems, and tuberculosis.[1]

Most of the pharmacological applications of the Arbutus species are shown in table (1).

Table (1): Pharmacology Activeties of Arbutus Species

Species	Part Use	Pharmacology Activeti	Ref.
A.unedo.	Leaves	Against Trichomonas vaginalis	[2]
		Trophozoites	[3]
		Antihyperglycemic activity	
		Antioxidants	[4],[5]
		Hypertension and anti flammatory	[6]
		Antiaggregant action	[7]
		Gastrointestinal disorders	
		Urological problems;	
		Dermatologic problems	
		Cardio-vascular application	[8],[9], [10],[11]
		Kidney diseases	
		Cardiac diseases	
		Diabetes	
		Antihaemorrhoidal	
		Diuretic	
		Anti-inflammatory Anti-diarrheal	

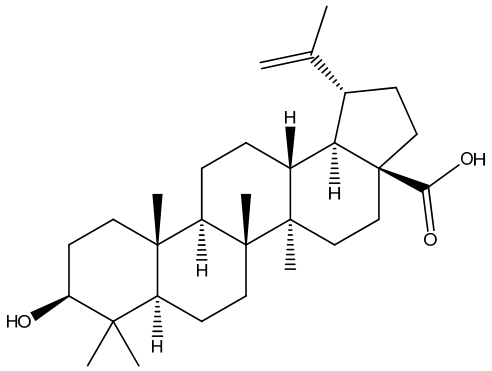
A.unedo	Fruits	Gastrointestinal disorders Urological problems Dermatologic problems Kidney diseases Cardio-vascular application	[8],[11], [12]
A.unedo	Bark	Gastrointestinal disorders Urological problems Dermatologic problems Cardio-vascular application Hypertension	[12]
A.unedo	Roots	Cardiac diseases Diabetes Diuretic Anti-inflammatory Anti-diarrheal.	[12],[9], [13],[10]
***A.*menziesii**	Leaves	Antibacterial	[14]
***A.*archorids**	Leaves	Antioxidants	[15],[16]

1.2 Phytochemistry

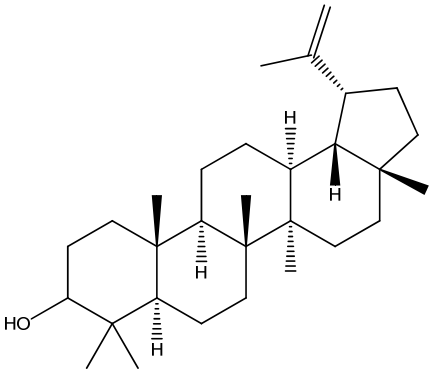
Most available literatures revealed the presence of different phyto-constituents in the leaves and fruits of ***A. unedo*** and the most of the compounds isolated from this species are triterpenes, irridoid glycosides, organic acids, tannins, flavonoids, sterols, and phenolic compounds [1]. The isolated compounds from this species are shown below:

Table (2): Some Terpenoids And Steroids Isolated From Arbutus Species.

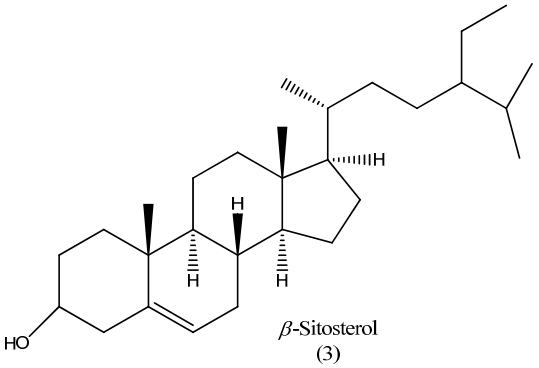
Species	Part Used	Compounds	Structure	Ref.
		***Triterpenoids* & steroids**		
A.menziesii	Leaves	Betulinic acid Lupeol β-Sitosterol	1 2 3	[17] [18] [18]
A.unedo	Leaves&Steem	Betulinic acid	1	[19],[20]
	Leaves&Steem	Lupeol	2	[19],[20]
	Leaves	α-amyrin acetate Pomolic acid 3-acetate Betulin	4 5 6	[19] [19] [19]
	Steem	*β*-Sitosterol 7*β*-hydroxystigmast-4-en-3-one. Ursolic acid	3 7 8	[19] [19] [20]



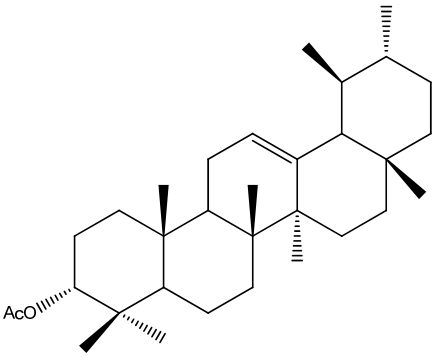
betulinic acid
(1)



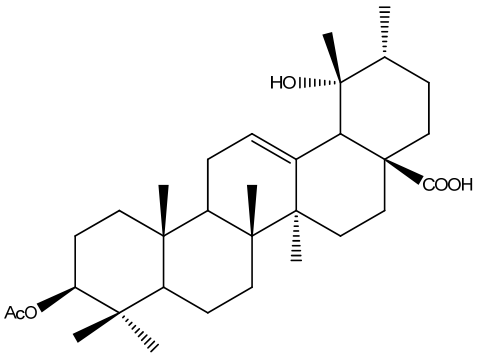
lupeol
(2)



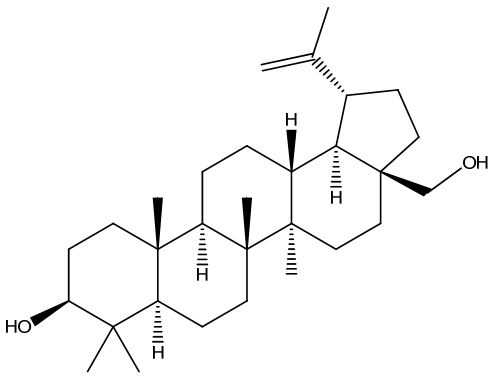
β-Sitosterol
(3)



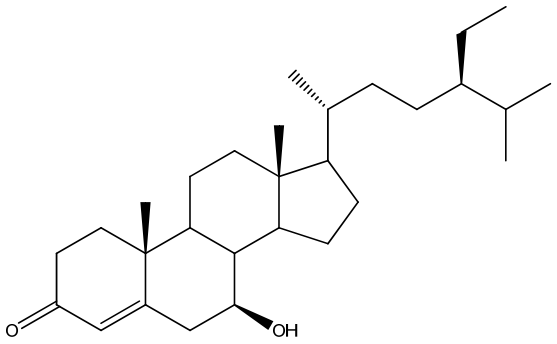
α-amyrin acetate
(4)



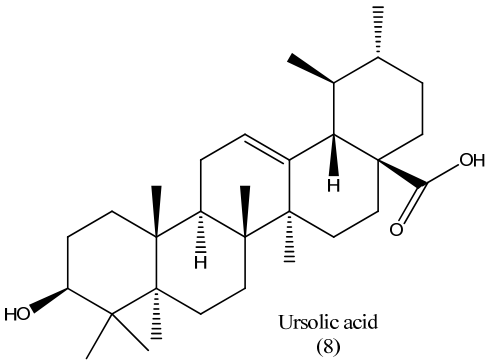
pomolic acid 3-acetate
(5)



Betulin
(6)



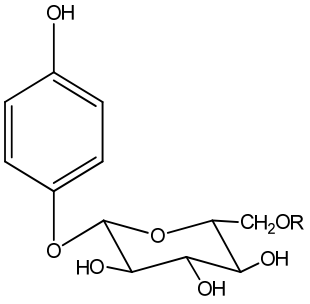
7β-hydroxystigmast-4-en-3-one.
(7)



Ursolic acid
(8)

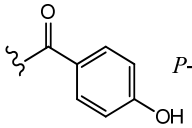
Table (3): Some Phenolic Compounds Isolated From *Arbutus* Species.

Species	Part Used	Compounds	Structure	Ref.
Phenolic acids &phenylpropanoids				
A.andrachne	Leaves	Arbutin	9	[21]
A.unedo	Leaves	*P*-hydroxybenzoyl arbutin	10	[22]
		galloylarbutin	11	[22]
Flavenoid&Flavenoid glycoside				
A.unedo	Leaves	Kaempferol 3-O-rhamnoside	13-a	[22],[23]
		Kaempferol 3-O-arabinoside	13-b	[23] ,[22]
		Quercetin 3-O-arabofuranoside	13-c	[23],[22]
		Quercetin 3-O- rhamnoside	13-d	[23],[22]
		Quercetin 3-O- galactoside	13-e	[22]
		Myricetin 3-O- arabofuranoside	13-f	[19]
		(-)-catechin	26	[22]
		Gallocatechin	26	
	Root	Gallocatechin-4,8-catechin	14	[24]
		(+)-catechin	22	[25]
		(+) catechin gallate	23	[25]
		(-)epicatechin	20	[25]
		gentisic acid	16	[25]
		caffeic acid	19	[25]
		benzoic acid, 4-(acetyloxy)-3-methoxy methyl ester	17	[25]
		4-hydroxy phenyl acetic acid		[25]
Cyanidin &Anthocyanidin				
A.unedo.	fruits	Cyanidin-3-O-golactoglucoside	21	[26],[24]
		Cyanidin-3-O-golactoside	24	
		delphinidin-3-O-golactoside	25	
A.unedo.	Fruit	***Ellagic acid derivatives***		[24]
		Ellagic acid diglucoside	27-a	
		Ellagic acid glucoside	27-b	
		Methylellagic acid rhamnoside	27-c	
		Ellgic acid arabinoside	27-d	



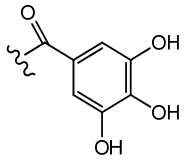
R= H Arbutin
(9)

R=

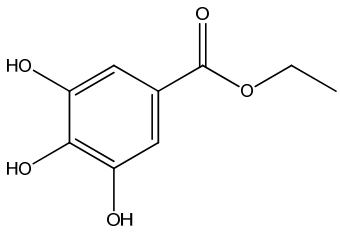


P-hydroxybenzoyl arbutin
(10)

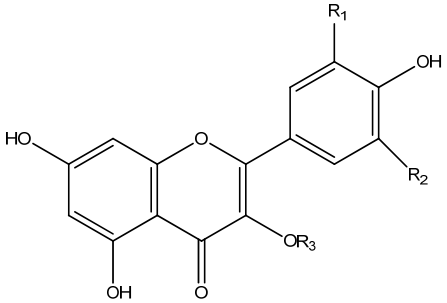
R =



galloylarbutin
(11)

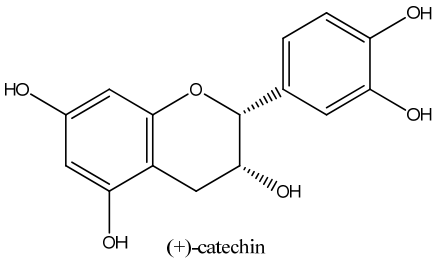


ethyl gallate
(12)

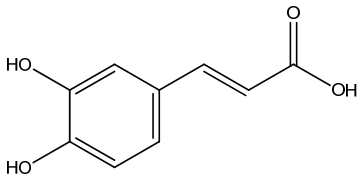


(13-a) R_1=H, R_2= OH,R_3=arabinofuranosyl= [quercetin 3-O-arabofuranoside]
(13-b) R_1=H, R_2= OH,R_3=rahmnopyranosyl = [Quercetin 3-O-rhamnoside]
(13-c) R_1=H, R_2= OH,R_3=galactopyranosyl =[Quercetin 3-O- galactoside]
(13-d) R_1=H, R_2= H, R_3=rahmnopyranosyl =[kaempferol 3-O-rhamnoside]
(13-e) R_1=H, R_2= H ,R_3=arabinofuranosyl=[kaempferol 3-O-arabinoside]
(13-f) R_1=OH,R_2=OH,R_3=arabinofuranosyl= [Myricetin 3-O - arabofuranoside]

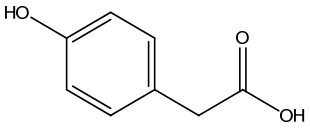
(13)



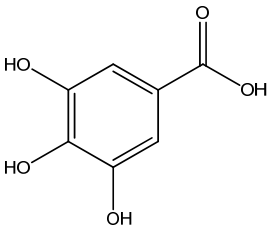
(+)-catechin
(14)



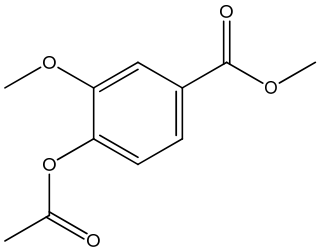
caffeic acid
(16)



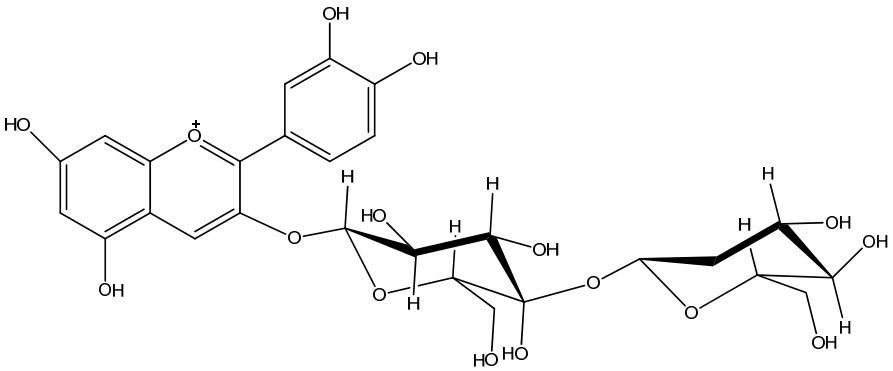
4-hydroxy phenyl acetic acid
(17)



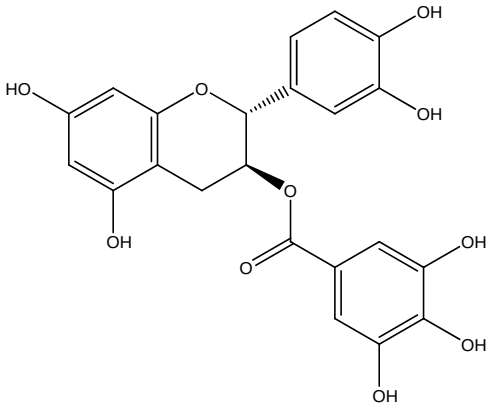
gentisic acid
(20)



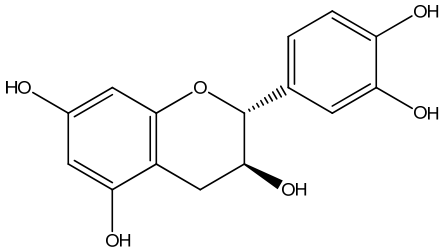
benzoic acid, 4-(acetyloxy)-3-methoxy-, methyl ester
(19)



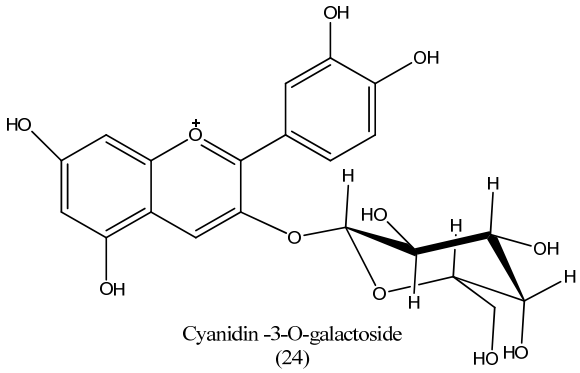
Cyanidin-3-O-galactoglycoside
(21)



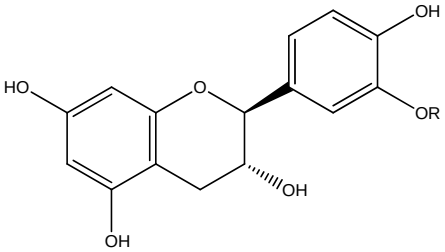
(+) catechin gallate
(22)



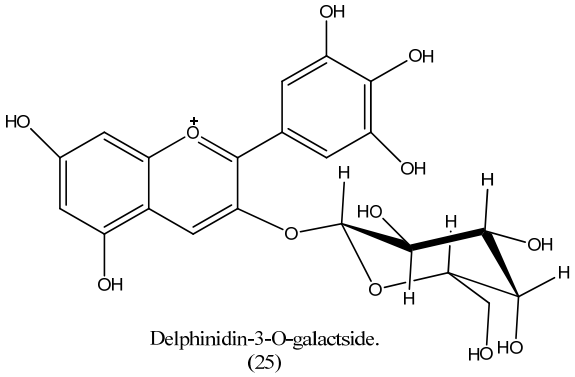
(-)Epicatechin
(23)



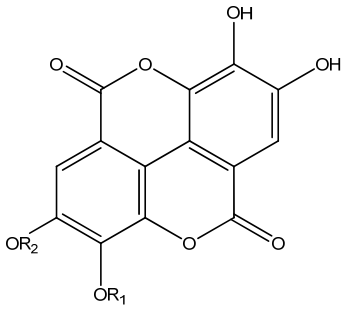
Cyanidin -3-O-galactoside
(24)



(26-a) R=H (-)-catechin
(26-b) R=OH gallocatechin
(26)



Delphinidin-3-O-galactside.
(25)

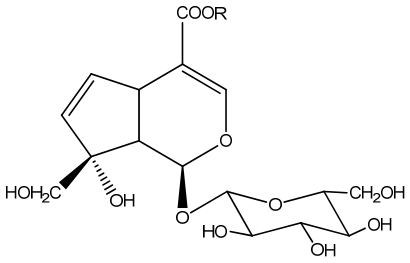


(27-a) R_1,R_2=glucopyrnosyl ellagic acid diglucoside
(27-b) R_1=glucopyrnosyl ,R_2=H ellagic acid glucoside
(27-c) R_1=rhamnopyrnsyl ,R_2= CH_3 methylellagic acid rhamnoside
(27-d) R_1=arabinopyrnsyl , R_2=H ellagic acid arabinoside

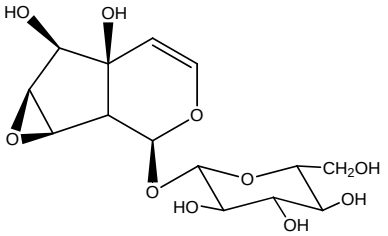
(27)

Table (4): Some Iridoids And Iridoids Glycoside Isolated From *Arbutus* Species.

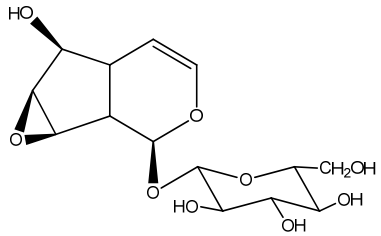
Species	Part Used	Compounds	Structure	Ref.
A.andrachne	Leaves &Bark	Monotropein Monotropein methyl ester Stilbericoside Unedoside	29-a 29-b 28 30	[21]
A.unedo	Leaves Leaves	Arbutoside Unedide (6,7-dihydro-6β-hyroxymonotropein)	32 31	[27] [27]
	Steem Steem Steem Steem Steem	Monotropein Monotropein methyl ester Stilbericoside Unedoside Iridoids geniposide	29-a 29-b 28 30 33	[20] [20] [20] [20] [20]



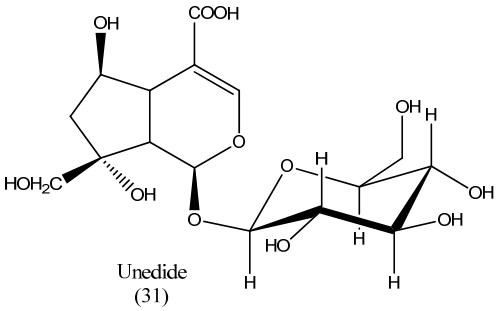
(29-a) R = H = Monotropein
(29-b) R= CH_3 =Monotropein methyl ester
(29)



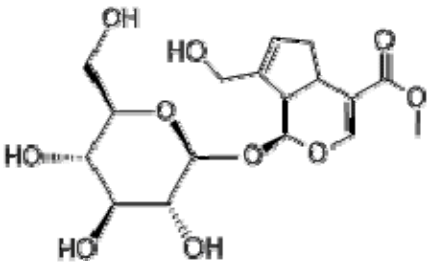
Stilbericoside
(28)



Unedoside
(30)



Unedide
(31)



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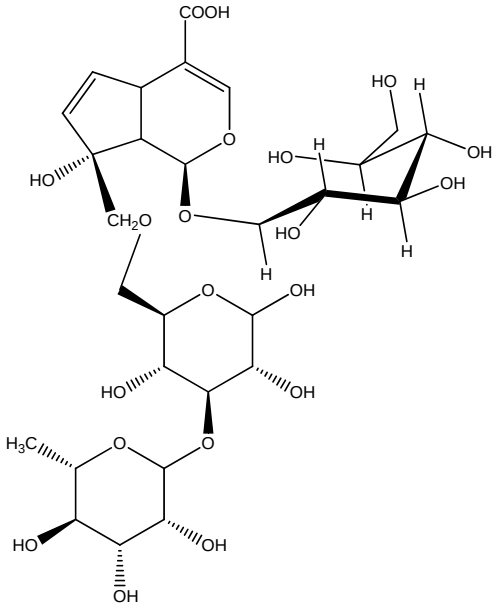


Arbtoside
(32)

1.3. *Arbutus pavarii* Pamp.

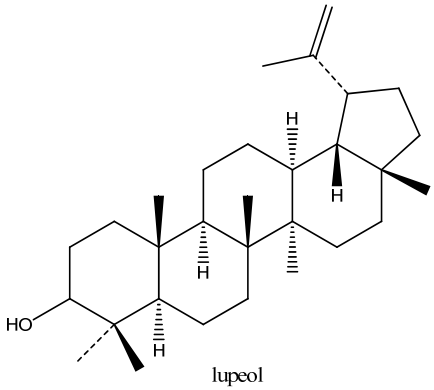
***A.pavarii* Pamp**. is one of the endemic species in Al-Jabel Al-Akhdar .As it's described in the Libyan flora; it is an evergreen shrub or small tree, 1.5 to 3 m tall. The bark is reddish brown fissured and peeling in small flakes. The flowers have an attractive scent, like honey, which is locally called "ALHANNON", is very expansive and widely used for medicinal purposes. It is used for relief and as protection against a number of diseases in folk medicine.[28]

A.pavarii is a member of the genus ***Arbutus***. It's used for honey production, as food due to its berries, as ornament trees. It also has a medicinal use in the treatment of kidney diseases. It is recorded as an endemic medicinal species with a high relative importance value.[28]

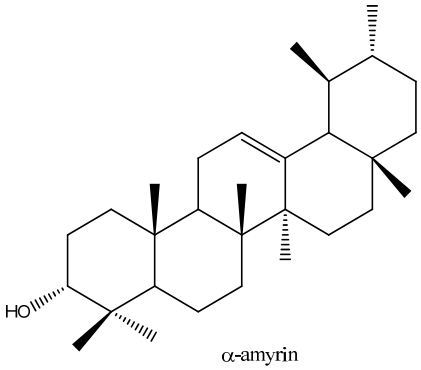
Picture (1): *A.pavarii* .Pmp Tree

1.3.1. Phytochemistry of *A. Pavarii*

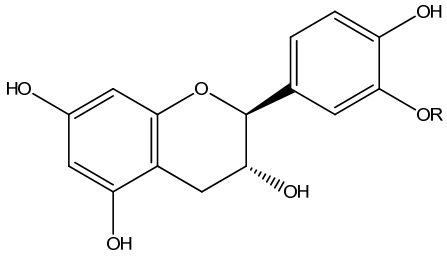
The first chemical study was performed by F.Elabbar**(1999)** which isolated the following compounds[29]



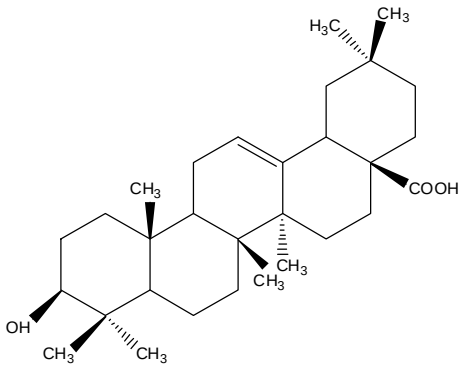
lupeol



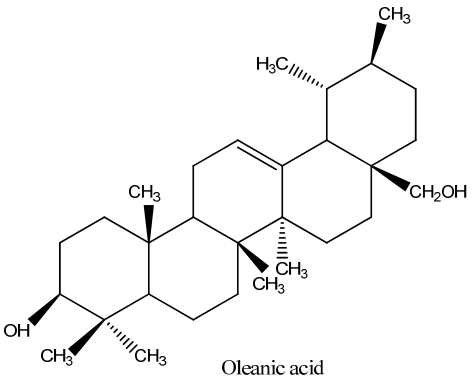
α-amyrin



R=H (-)-catechin
R=OH gallocatechin



Uvaol



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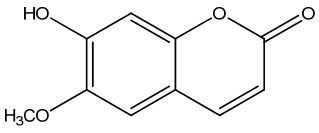
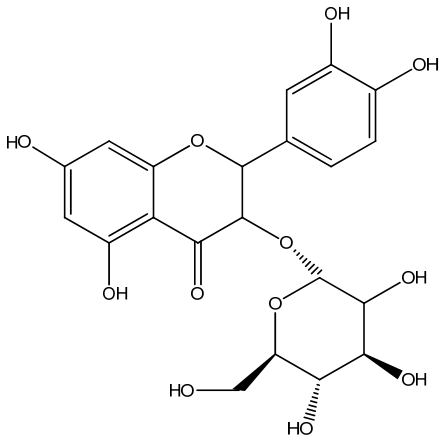


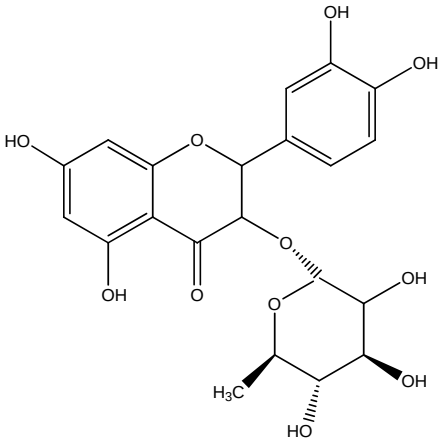
Scopoletin

CHAPTER TWO

EXPRIMENTAL



Iso quercitin



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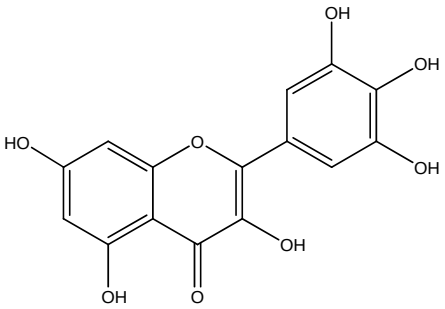
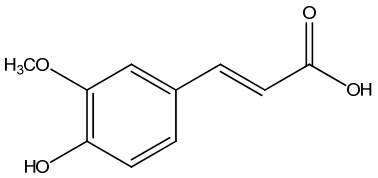


Myricetin



Ferulic Acid

2. EXPERIMNTAL

2.1General Technique

The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer and Bruker DPX. ^{1}H-NMR spectra were run at 300 and 400 MHz and ^{13}C-NMR spectra were run at 100 MHz in CD_3OD, $CDCl_3$ and DMSO-d_6. Chemical shift are quoted in δ and were related to that of the solvent signals.MS Finnigan mat SSQ7000 Ionization mode EIeV 70. Analytical thin layer chromatography (TLC) was carried out on precoated 0.25mm silica gel plates with fluorescent indicator (Macherey-Nagel - GF_{254}). Preparative TLC was conducted on glass plates (20cm × 20cm) coated with silica gel 60 and the spots were visualized either by UV light (254-366) or I_2 vapor. Wet column chromatography was carried out using RDH silica gel S (230-400 mesh ASTM) and silica gel (70-230mesh).

2.2 Plant Collection and Identification

The plant material of ***A.pavarii*** confined in the wild in AL-Jabel AL-Akhdar and adjoining area between Alhemda and Darna collected from AL-Abiar during August 2010, the plant was identified by Botany department of Benghazi University .The Leaves were allowed to dry in air and then grounded into a fine powder and used for the preparation of extract.

2.3 Chemical Screening of *A.pavarii*

2.3.1 Preparation of the Plant Extract

Samples of dry leaves 100g were placed in Erlenmeyer flasks with 300ml of methanol covered with a funnel and refluxed for an hour followed by concentration of the extract to about 20ml in vacuum[30].

2.3.2 Screening for alkaloids

An equivalent of 20g of leaves extract was evaporated to a syrupy consistency by evaporating over a steam bath. 0.5ml hydrochloric acid (2*N*) were added to the concentrated extract, and then heated for about five minutes. After cooling the mixture, about 0.5g of powdered sodium chloride were added to the mixture then filtered. The filtrate was divided into two equal portions; one portion was treated with a few drops of Mayer's reagent and the other with similar amount of Wagner's reagent. The leaves sample showed no sign of turbidity, which clearly indicate the absence of alkaloids in the sample[30],[31].

2.3.3 Screening for Saponins

Volume of the alcohol extract equivalent to 2g of sample was shook with 10ml of distilled water in stoppered test tube for 30 seconds. Then, it was allowed to stand and observed for a period of over 30 minutes. If the forth persisted up to30 minutes above the liquid surface in about 3cm height, then the sample is presumed to contain saponin in a high concentration. The sample showed no forth and that was firm evidence that Saponins were absent in the sample[30],[31].

2.3.4 Screening for Phenolic Compounds

2.3.4.1 Preparation of the Defatted Plant Extract

The equivalent of 10 g methanol plant extract was evaporated over water bath then cooled to room temperature. The residue was treated with 10 ml of *n*-hexane. The treatment of the residue was repeated with fresh volume of the solvent until the solvent was almost colorless. The defatted residue was dissolved in 20 ml of methanol and the insoluble residue was filtered away, then divided into 2 test tubes.

2.3.4.2 Test for Leucoanthocyanins

0.5 ml of concentrated hydrochloric acid was added to test tube 1 then it was warmed on water bath for 5 minutes. The sample's color changed to red indicating the presence of Leucoanthocyanins[30],[31].

2.3.4.3 Test for Cyanidin

0.5 ml of concentrated hydrochloric acid and three to four pieces of magnesium turnings were added to test tube 2. The sample's color changed from brown to red indicating the presence of Cyanidin in sample[30],[31].

2.3.4.4 Two Dimensional Paper Chromatography Detection of Flavonoids

A spot of the defatted extract of sample was chromatographed on paper chromatography. The paper was developed by solvent mixture of t-butanol : acetic acid : water (3:1:1) (TAW), when the solvent reached the desired range the paper was removed from the developing chamber, and dried by warm air using a hair dryer. Then, the paper turned a whole (90^{o}) and developed in the second direction using acetic acid (15%).

The examination of the paper chromatography was done in three stages

- Stage one marking all spots under visible light
- Stage two marking all spots under UV light
- Stage three exposures all spots to ammonia vapor

Conclusion: upon using the ammonia vapor, other spots and/or colors appeared indicating the presence of flavonoids in the sample[30],[31].

2.3.5 Screening for Tannins

The equivalent of 10 g of methanol extract sample was evaporated to dryness on a hot water bath and cooled. Then 20 ml of hot distilled water was added and cooled, then 5 drops of sodium chloride (10%) solution was added to the mixture, then it was filtered off. To the clear solution three drops of gelatin-salt regent was added. The sample extract shows a precipitate that indicated the presence of tannins[30],[31].

2.3.6 Screening for Triterpenoids and Steroids

The equivalent of 10 g of methanol extract was evaporated to dryness on a hot water bath; about 10 ml *n*-hexane was added to the cooled extract to remove most of the color of the residue. About 10 ml of chloroform were added, stirred and decanted into a test tube containing about 100 mg of anhydrous sodium sulphate. The mixture was filtered and it was treated with three drops of acetic anhydride then one drop of concentrated sulphuric acid was added. The appearing of deep red color indicates the presence of triterpenoids and /or steroids in the sample [30],[31].

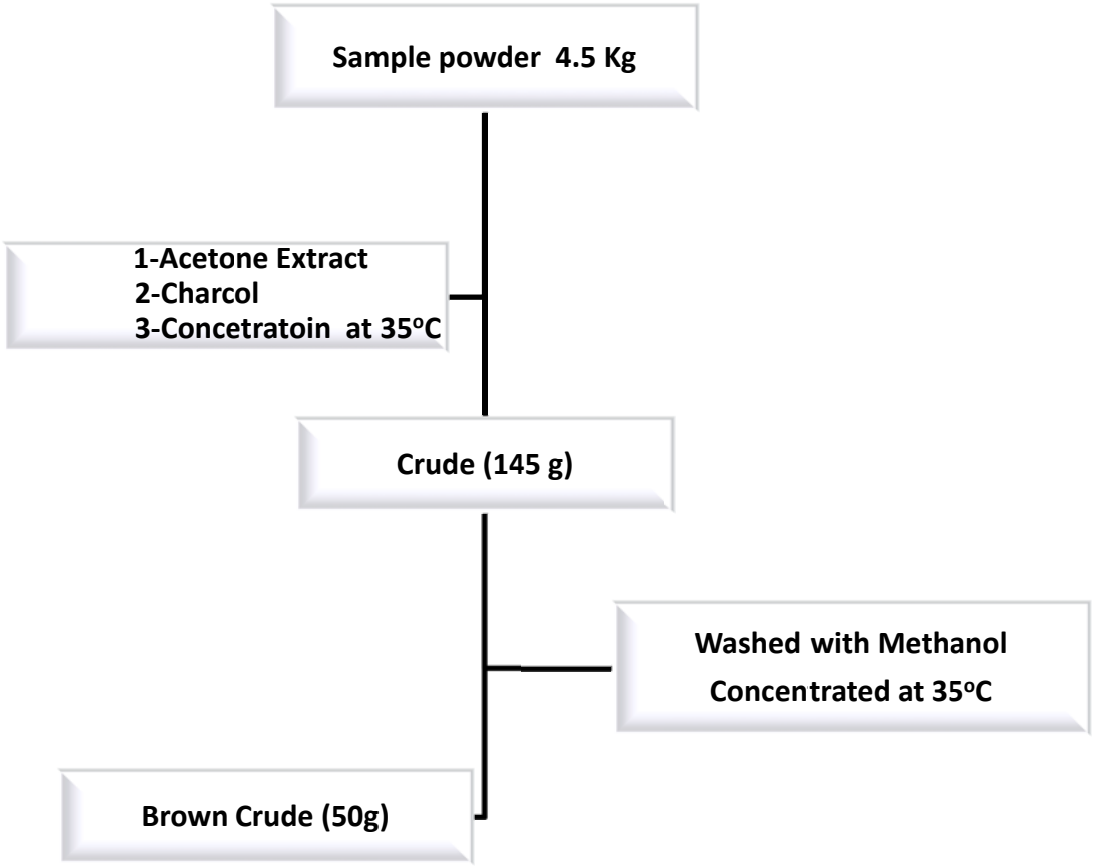
2.4 Extraction the Leaves of *A.Pavarii*

The plant leaves has been divided into two parts; part (A) extracted with acetone and part (B) extracted with petroleum ether, chloroform and methanol.

2.4.1 Acetone Extract (Part A):

The powdered leaves of ***A.pavarii*** 4.5 Kg were extracted with acetone (10L× 3) over 12 days at room temperature. The combined acetone extract passed through charcoal to remove the colored pigments, then filtered and evaporated under reduced pressure using rotary evaporator at 35^{o}C to produce a brown crude extract 145 g. The crude extract was washed with methanol (250 ml × 2) to produce brown crude 50 g. as shown in Scheme (1).

2



Scheme (1): Acetone Extraction of Leaves *A. pavarii*

2.4 .2 Separation And Purification of Part (A)

15 g of the brown crude was subjected to flash column chromatography using silica gel 500g (70 – 230 mesh ASTM) eluted with 100 % *n*-hexane followed by gradient elution with *n*-hexane : EtOAc (9:1 to 1:9) and EtOAc 100 % and elution with EtOAc : Methanol(1:1) and100 % Methanol to give 17 fractions (500 ml each), all fractions volume were reduced using rotator evaporator to 50 ml followed by examination with TLC using 100% hexane, hexane / EtOAc (1:1).Resulting of combination of similar fractions are shown in table (5).

Table (5): Combined Fractions of Column Chromatagraphy of the Leaves *A.Pavarii*

Fractions	Solvent System of Elution
A(1-3)	Two 100% n-hexane then (9:1) n-hexane /EtOAc
B(4)	8:2 n-hexane /EtOAc
C(5-6)	7:3 n-hexane /EtOAc- 6:4 n-hexane /EtOAc
D(7-9)	3:7 n-hexane /EtOAc - 1:9 n-hexane /EtOAc
E(10-11)	100% EtOAc
12-17	Discounted

Fractions **B** and **D** were further investigated by other chromatographic techniques.

2.4.2.1 Separation and Purification of Compound *M1*

Fraction **B** was pale yellow crude, 0.215g of which was subjected to wet column chromatography using 60g of silica gel (70 -230 mesh ASTM) eluted with 100% hexane followed by gradient elution with Hexane / chloroform (9.9:0.1 to 0.1:9.9) and 100% chloroform to produce 22 fractions (25 ml each) as shown in table (6). Each fraction was examined by TLC using 100% hexane, hexane / chloroform 1:1 and chloroform 100% as solvent system.

Table (6): Fractions of Column Chromatography of Fraction B

Fraction Number	Solvent System of Elution
1	100% n-hexane
2	9.9:0.1 n-hexane / chloroform
3	9.8:0.2 n-hexane / chloroform
4	9.7:0.3 n-hexane / chloroform
5	9.6:0.4 n-hexane / chloroform
6	9.5:0.5 n-hexane / chloroform
7	9.4:0.6 n-hexane / chloroform
8	9.3:0.7 n-hexane / chloroform
9	9.2:0.8 n-hexane / chloroform
10	9.1:0.9 n-hexane / chloroform
11	9:1 n-hexane / chloroform
12	9:1 n-hexane / chloroform
13	8:2 n-hexane / chloroform
14	7:3 n-hexane / chloroform
15	6:4 n-hexane / chloroform
16	5:5 n-hexane / chloroform
17	4:6 n-hexane / chloroform
18	3:7 n-hexane / chloroform
19	2:8 n-hexane / chloroform
20	1:9 n-hexane / chloroform
21	100% chloroform
22	**100% chloroform**

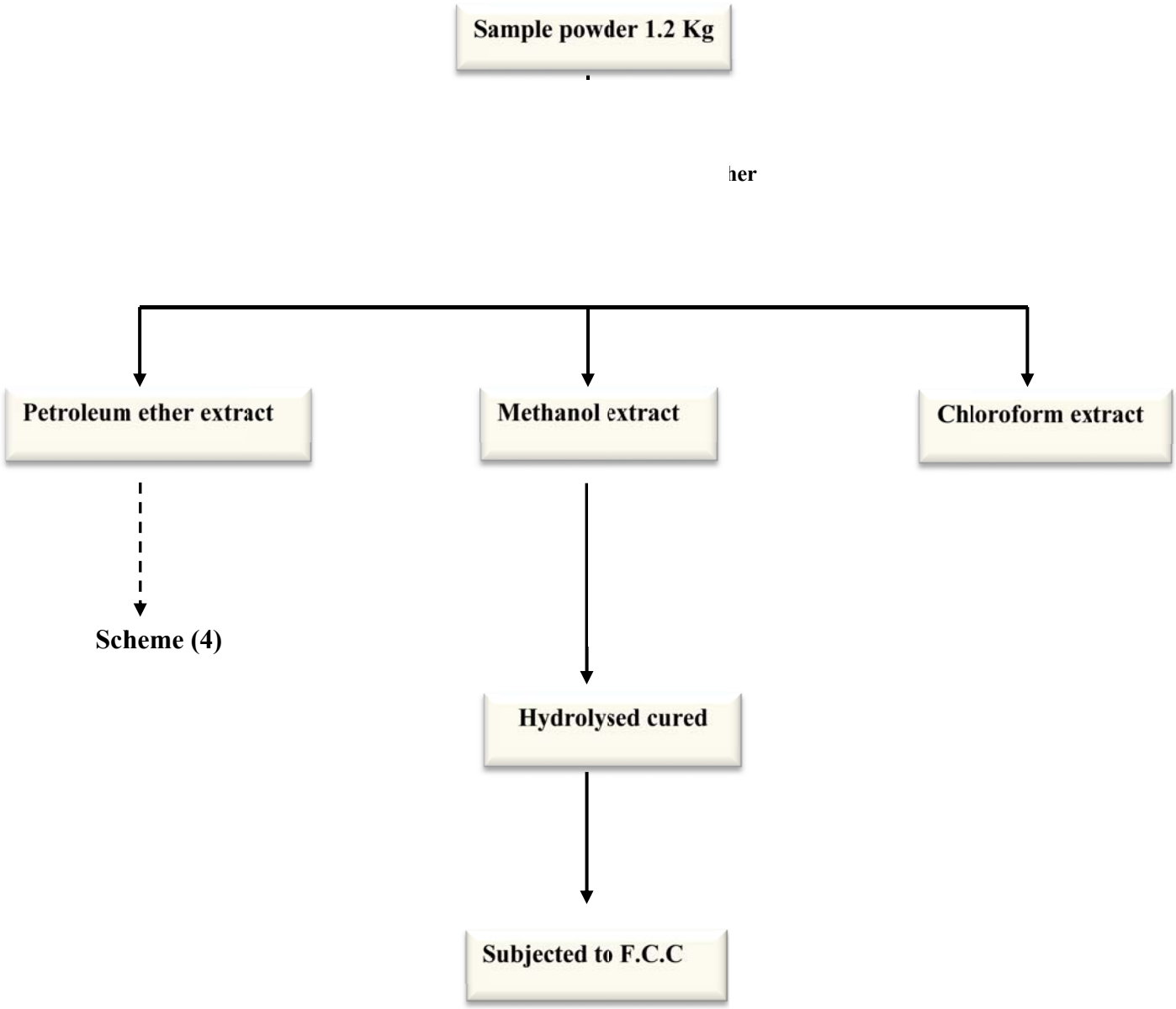
Fraction **22** produced pure compound ***M1*** (4mg) of which has spectra data as follows: EI mass spectrum: m/z = 426 (19%), 411(5%), 384 (0.3%), 207 (50%), 189(48.5%). ^{1}H NMR : 0.77(s,3H), 0.81(s,3H), 0.84(s,3H) , 0.95(s,3H), 0.96(s, 3H), 1.02 (s, 3H),1.69(s,3H), 3.18(dd,1H), 4.68(brs,1H), 4.57 (brs,1H). ^{13}C NMR : 38.35 (C-1),26.58 (C-2), 78.99(C-3), 38.78(C-4), 55.35(C-5), 18.35(C-6), 34.84(C-7), 40.58(C-8) ,50.48 (C-9), 37.19(C-10), 21.69(C-11), 25.02(C-12), 38.10(C-13), 42.38 (C-14), 27.44(C-15) , 35.9(C-16) , 42.78(C-17) , 48.35(C-18), 47.48(C-19) , 150.88 (C-20), 29.71(C-21) , 39.68(C-22) , 28.12 (C-23) ,15.60(C-24),16.13(C-25),16.01(C-26) , 14.58(C-27) ,18.02 (C-28) ,109.34(C-29),20.025(C-30) .

2.4.2.2 Separation and Purification of Compound *M2*

Fraction **D** was subjected to preparative TLC eluted with hexane / EtOA (5.5:4.5). Three bands with different R_f values were separated , the band at R_f= 0.45 was recovered and evaporated till dryness to give yellow crude 0.2 g this crude was subjected to flash chromatography using 30g of dry silica gel and eluted with hexane / EtOAc (6:4) to produce three fractions (100 ml each). fraction (1) contains pure compound M2(5 mg) which has spectra data as follows: EI mass spectrum : m/z = 291 [M^{+*}] , 152(32.5%), 110(10.73%), 139(100%), 123(49.10%).^{1}H NMR : 2.74 (dd , 1H), 2.83 (dd ,1 H),4.18(brs ,1H),4.81(brs ,1H),5.93(d ,1H),5.96 (d ,1H) ,6.76(d,1H),6.81 (dd, 1H),6.97(d,1H). ^{13}C NMR:79.78(C-2),67.39(C-3), 29.15(C-4),157.48(C-5) ,96.5 (C-6),157.82(C-7), 95.96(C-8),157.25(C-9), 100.17(C-10), 132.20(C-1'), 115.33(C-2'), 145.82(C-3'), 145.67(C-4'), 116.0(C-5'), 119.45(C-6'). #

2.4.3 Petroleum Ether, Chloroform and Methanol Extract (Part B)

About 1.2 Kg of dry leaves of ***A.pavarii*** was subjected and extracted with petroleum ether, chloroform and methanol as in scheme (2)



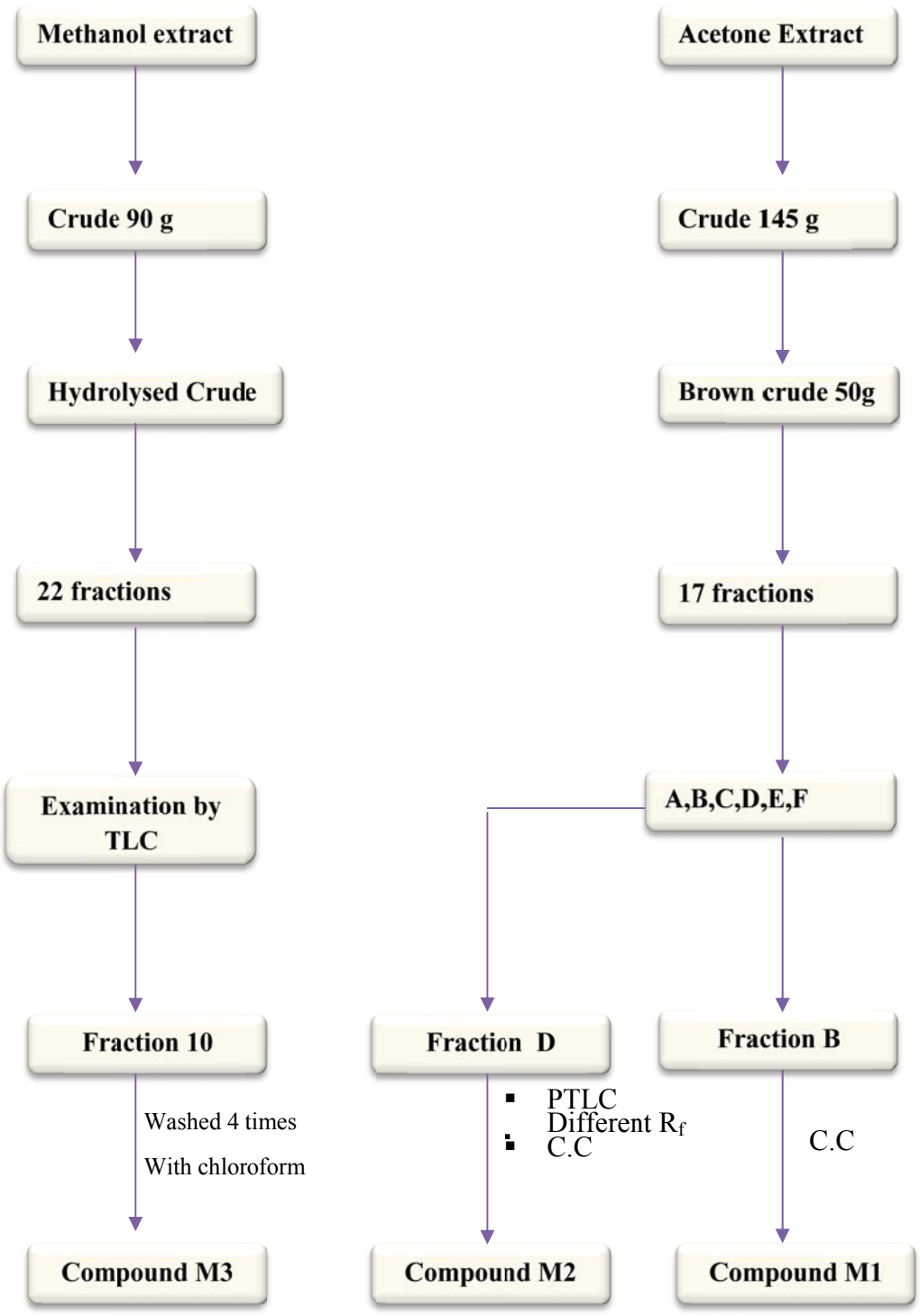
Scheme (2): Extraction of leaves *A.pavarii* (Part B)

2.4.3.1 Separation and Purification of Part (B)

Methanol extract was evaporated at 40 C^{o} under vacuum till dryness to produce 80g of crude. About 40 g of this crude were subjected to hydrolysis with HCl (2N) /methanol (1:1) under reflux for 5 hours to cleavage the glycoside unit, and yield 15g of hydrolysed crude. The hydrolysed crude was subjected to flash chromatography and eluted with hexane / chloroform (1:1) followed by gradient elution with n-hexane / chloroform (4:6 to 1:9) and 100% chloroform, followed by gradient elution with chloroform / EtOAc (9:1- 1:9), and 100% EtOAc to produce 16 fractions (400 ml each).

2.4.3.1.1 Separation and Purification of Compound*M3*

Fraction **10 (**chloroform / EtOAc) (6:4) containes compound M3 which was purified by washing with chloroform (4 Times) to produce pure white crystals (8mg), which has spectra data as follows: EI mass spectrum : m/z =110(100%) [M^{+*}] , 109(4%), 82(7%) , 81(1%) ,53(27%), 39(23%).^{1}H- NMR: 6.55(s, 4H Ar) ,8.58 (s, 2H) . ^{13}C -NMR : 117.20 , 151.65.



Scheme (3): Separation of Leaves *A.pavarii*

2.5 Extraction of Lipids Fraction:

The combined petroleum ether extract from previous section (2.4.3) was extracted with diethyl ether, and passed through charcoal to remove the colored pigments, then filtered, dried over anhydrous sodium sulphate and evaporated under vacuum at 30 C^0 till dryness to give a pale yellow residue (8g). The Ether residue was dissolved in boiling acetone (250 ml) and left overnight at room temperature.
An amorphous precipitate was filtered, washed with cold acetone and recrystallized from chloroform / methanol to give bright white crystals (2g) of acetone insoluble fraction (hydrocarbons and fatty alcohols mixture). The filtrate (acetone soluble fraction) was evaporated till dryness[32] (4. 5g).

2.5.1 GC/MS Analysis of Fatty Alcohols Fraction (Acetone Insoluble):

The fatty alcohols and hydrocarbons mixture was dissolved in ether and subjected to GC/MS analysis using the conditions as shown in table (7) below. The results of GC/ MS were shown in Table (8).

Table (7): GC/MS Instrument Parameters

Instrument	SHIMADZU GC/MS-QP550A
Column	DB1, 30m ; .53mm ID; 1.5um film (J&W scientific)
Carrier gas	Helium
Ionization method	EI
Ionization voltage	70ev
Detector temperature	230 C^0
Injector temperature	280C^0

2.5.2. Saponification of Acetone Soluble Fraction:

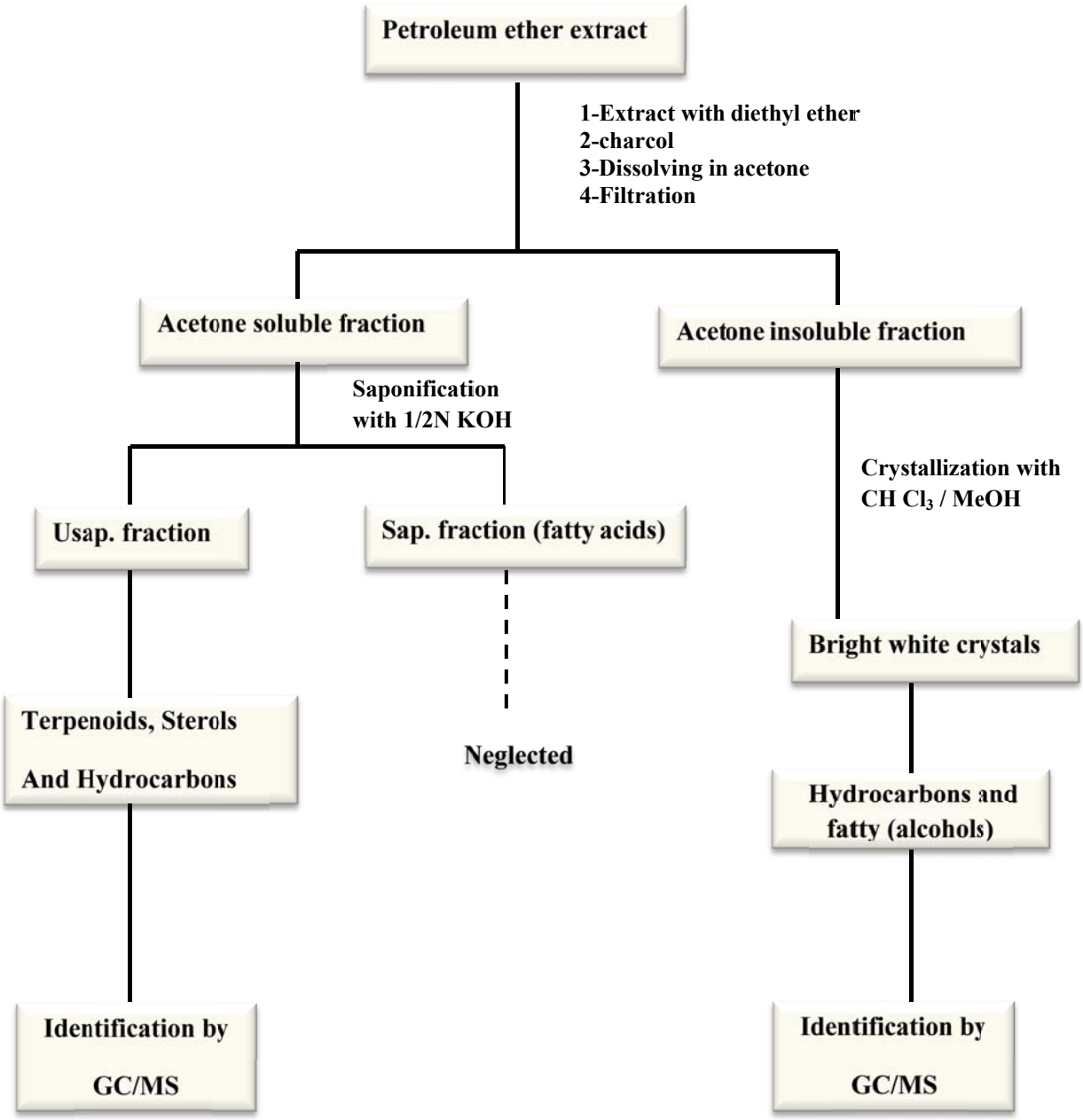
The acetone soluble fraction (4. 5 g) was saponified by refluxing with 100ml (0.5 N) alcoholic KOH. The alcoholic solution was concentrated to about 25 ml and diluted with cold distilled water. The unsaponifiable constituents were extracted by shaking with successive portions of diethyl ether (3 x 100 ml). The combined ether extracts were washed with distilled water, dried over anhydrous sodium sulphate and evaporated under vacuum till dryness to give a yellowish brown semi-solid residue of unsaponifiable matters (2.5g).[32]

CHAPTER THREE

RESULTS & DISCUSSION

2.5.2.1. Gas Chromatographic Analysis of Unsaponifiable Compounds:

The unsaponifiable compounds are subjected to GC/MS analysis under the same conditions in table (7) and the obtained results are shown in Table (9).



3. RESULTS AND DISCUSSION

3.1 GC/MS Data Results

3.1.1 GC/MS Data of Fatty Alcohols Fraction (the Acetone Insoluble)

The results from the mass spectra of the separated peaks from the chromatograph were compared with the ***Wiley mass spectral data base*** and results are shown in table (8)

Table (8): GC/MS Data Result of Fatty Alcohols Fraction

Number	R.Time	Compound Name	%	Structure No.
1	4.931	Tetradecane	4.71	3
2	5.838	Hexadecane	7.62	2
3	6.818	Octadecanoic acid	12.17	9
4	10.432	Hexadecanoic acid	13.84	4
5	12.11	Apiol	1.97	13
6	15.942	Butyl phthalate	21.89	17
7	16.770	Palmitol	9.52	19
8	20.670	Linalool	3.73	1
9	24.742	Dioctyl phthalate	20.04	8
10	29.411	Tricosane	3.51	10

3.1.2 GC/MS Data Results of Unsaponifiable compounds

The results from the mass spectra of the separated peaks from the chromatograph were compared with the ***Wiley mass spectral data base*** and results are shown in table (9).

Table (9): GC/MS Data Results of Unsaponifiable Compounds

Number	R.Time	Compound Name	%	Structure No.
1	5.480	Tetradecanoic acid	17.59	7
2	6.992	Terpineol	26.28	6
3	7.527	1,8-Cineol	5.02	15
4	8.055	Ocimene	2.36	16
5	10.092	Beta-Elemene	35.46	12
6	12.6	Butyl phthalate	14.73	17
7	13.795	Trans-Caryophllene	2.64	14
8	15.709	p-methoxystyrene	0.86	18
9	20.942	Octadecanoic acid	1.06	9
10	21.81	Eugenol	7.22	15
11	22.842	Caryophllene Oxide	1.34	11

3.2. Identification of Compound M1

The ^{1}H-NMR spectrums in table (10) and fig.(7) showed two olefinic protons as broad singlets at δ_H 4.57 ppm and δ_H 4.68 ppm due to the 2H at C-29; this effect is due to Geminal proton-proton coupling which ranges from 0-1 Hz resulting of broad unequivalent signals. Other signal that appeared as singlet at δ_H 1.69 ppm due to 3H on C- 30 (allylic proton), which is attached directly with methen carbon C-20. Another signal appeared as doublet of doublet at (δ_H 3.18 ppm *J*=10.8, 5.4 Hz) due to H -3 which directly attached to C-3 that connected to a hydroxyl group. An additional signal appeared as a multiple at δ_H 2.38 ppm due to proton at C-19 this is a characteristic signal for proton on ring (**E**) holding the side chain . The spectrum showed six signals as singlets at δ 0.96,0.77, 0.84, 1.02, 0.95 and 0.80 ppm (3H each) assignable to methyl group protons at C-23, C-24, C-25,C-26 , C-27 and C-28. All assignments are in good agreement with published data of Lupeol in Table (10).

Table (10):^{1}H-NMR Spectral Data of Compound M1 and Literature of Lupeol

No.of H	δ_H *M1*ppm, *J* (Hz)	δ_H ppm , *J* (Hz)[33]	δ_H ppm, *J* (Hz)[34]	δ_H ppm, *J* (Hz)[35]	δ_H ppm, *J* (Hz)[36]
29	4.57 br s	4.55	4.56	4.57	4.55
29	4.68 br s	4.67	4.68	4.69	4.67
3	3.18 dd , 10.8,5.4	3.17dd	3.21, 11.5 ,5.03	3.20 dd	3.17 10.2 ,5.1
30	1.69 s	1.67	1.68	1.64	1.66
26	1.02 s	1.02	1.02	1.04	1.01
23	0.96 s	0.95	0.95	0.97	0.94
27	0.95 s	0.94	0.93	0.94	0.92
25	0.84 s	0.84	0.83	0.84	0.81
28	0.80 s	0.82	0.8	0.79	0.76
24	0.77 s	0.78	0.79	0.76	0.74

M1-^{1}H-NMR spectral data measured at 300 MHz in $CDCl_3$
[33]-^{1}H-NMR spectral data measured at 400 MHz in $CDCl_3$
[34]-^{1}H-NMR spectral data measured at 400 MHz in $CDCl_3$
[35]-^{1}H-NMR spectral data measured at 400 MHz in $CDCl_3$
[36]-^{1}H-NMR spectral data measured at 300 MHz in $CDCl_3$

The ^{13}C-NMR spectrum in fig.(8) was indicative of 30 carbon resonances the DEPT technique suggested the presence of seven methyl, eleven methylene, six methine and six quaternary carbon atoms as revealed in Fig.(9) .Two signals at δ_C 109.34 and 150.88 ppm were due to two olefinic carbons of C-29 and C-20, respectively, of which the deshielded signal at δ_C 150.88 ppm was assigned for quaternary olefinic carbon C-20. The other deshielded signal at δ_C 78.99 was due to C-3 with a hydroxyl group attached. A comparison of carbon resonances of compound M1 and Lupeol published data as shown in Table (11) revealed a complete agreement in all data.

However from the HMQC of this compound showed the presence of oxymethine at δ_H 3.18 ppm and olefinic proton δ_H 4.68 and 4.57 ppm were assigned to C-3 and C-29 respectively . And another protons at δ_H 1.69 ppm were assigned to C-30 . and six methyl proton signals δ_H 0.96, 0.95,0.77, 0.84 , 1.02 , 0.80 ppm were correlated to C-23 C-27 , C-24 , C-25 ,C-26 , C-28 respectively and as shown in Table (11) and Fig. (10).

Table (11):^{13}C-NMR Data Spectral of Compound M1and Literature of Lupeol

No. of C	δ_C M1ppm	$\delta_C$ppm[37]	$\delta_C$ppm[38]	$\delta_C$ppm[39]
1	38.35	38.0	38.9	38.7
2	26.58	27.4	27.6	27.5
3	78.99	79.00	78.2	79.3
4	38.78	38.7	39.0	39.8
5	55.35	55.3	55.5	55.5
6	18.35	18.3	18.5	19.0
7	34.84	34.2	34.4	34.2
8	40.58	41.1	41.0	41.1
9	50.48	50.4	50.6	50.9
10	37.19	37.7	37.3	37.2
11	21.69	20.9	21.1	21.2
12	25.02	25.1	25.3	25.3
13	38.10	38.10	38.2	38.5
14	42.38	42.8	43.0	42.8
15	27.44	27.4	27.7	27.2
16	35.92	35.6	35.8	35.9
17	42.78	43.27	43.2	43.2
18	48.35	48.2	48.5	48.5
19	47.48	48.0	48.2	47.8
20	150.88	150.9	151.2	151.2
21	29.71	28.5	30.0	30.1
22	39.68	40.0	40.2	40.3
23	28.12	28.1	28.2	28.4
24	15.60	15.4	15.6	15.6
25	16.13	16.1	16.2	16.2
26	16.01	15.9	16.3	16.1
27	14.580	14.6	14.7	14.8
28	18.02	18.0	18.1	18.1
29	109.34	109.5	109.5	109.5
30	20.025	19.4	19.5	19.8

M1-^{13}C-NMR spectral data measured at 100 MHz in CD Cl_3
[37]-^{13}C-NMR spectral data measured at 100 MHz in CD Cl_3
[38]-^{13}C-NMR spectral data measured at 125MHz in CD Cl_3
[39]-^{13}C-NMR spectral data measured at 125 MHz in CD Cl_3

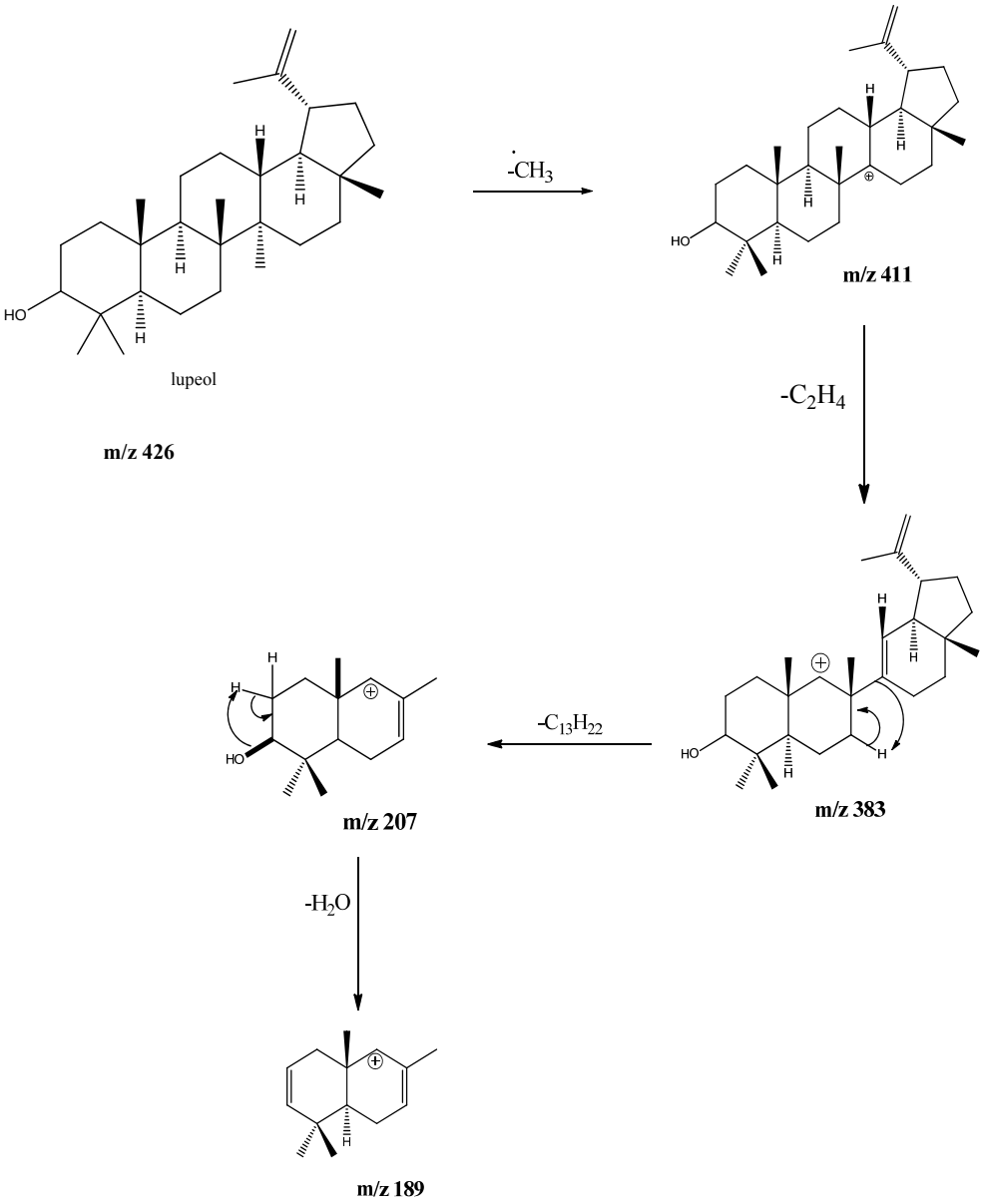
Table (12)**:HMQC Correlation of Compound M1**

^{1}H-NMR ppm	^{13}C-NMR ppm
3.18 dd	(78.99) C-3
0.96 s	(28.12) C-23
0.77 s	(15.6) C-24
0.84 s	(16.13) C-25
1.02 s	(16.01) C-26
0.80 s	(18.02) C-28
4.68 br s ,4.57 br s	(109.34) C-29
1.69 s	(20.02) C-30

The mass spectrum of compound M1 displayed a molecular ion peak at m/z = 426 in agreement with molecular formula $C_{30}H_{50}O$. And peak at *m/z* = 411 resulting from bonds cleavage at C-14 - C-27 and consequent $^{\bullet}CH_3$ elimination , the fragment ion at m/z = 411 convert to fragment ion at m/z = 384 by losing ($-C_2H_4$) further it converts to fragment ion at m/z 207 by lose water molecule to give fragment ion at m/z = 189. This fragmentation pattern was seen in more than one reference[40].

Table (13): Mass Fragments of Compound M1

Mass m/z	Abundance %
426	19
411	5
384	0.3
207	50
189	48.5



Scheme (5): Mass Fragmentation of Compound M1

On the basis of spectral data discussed above, and by comparative literature, the compound M1 was identified as **20 (29) lupen-3β-ol** (Lupeol).

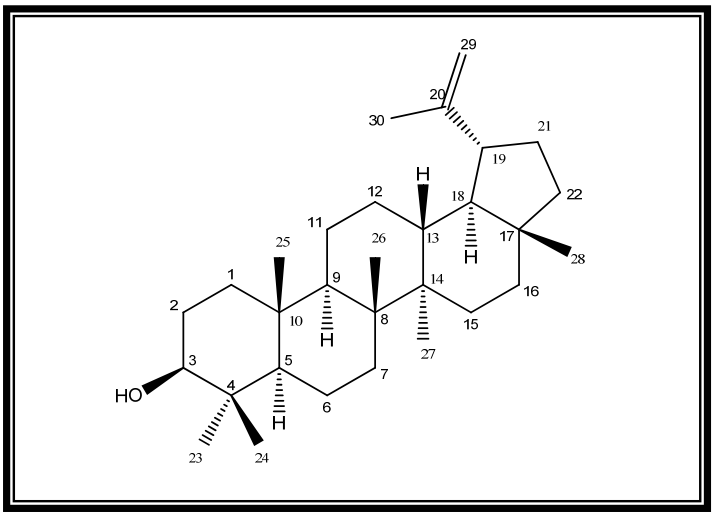


Figure (1): Structure of Compound M1(Lupeol)

3.3 Identification of Compound M2

The ^{1}H NMR spectrum of the compound explained *AB* system ring **A** with meta coupling , two doublet protons at δ_A 5.96 ppm (H-6, ^{4}J= 2.18 Hz) and δ_B 5.93 ppm (H-8, ^{4}J= 2.16 Hz) ,were due to two phenyl protons situated at 1,3 position to each other of ring **A** which contain 5,7-dihydroxy substitution pattern which give rise to doublet in range 5.9 to 5.96 ppm , the H-6 doublet occurs consistently at higher filed than the signal for the H-8 .The spectrum also showed two signals at δ_H 6.76 (d, *J*= 8.12 Hz) and 6.97 (d, *J*= 2.14 Hz) assigned to two phenyl protons situated at C-5' and C-2' in ring B respectively, and signal was recorded as doublet of doublet at 6.81ppm (dd , *J*= 8.35 , 1.82 Hz) which occurs due to 6'-H, 2'-H as *meta* coupling which has coupling constant 1.82 Hz and 6'-H ,5'-H *ortho* coupling which has constant 8.5 Hz . A singlet at δ_H 4.81ppm (brs) was due to methine proton (C-3) having an adjacent –OH group and situated between methylene and a methine carbon. A singlet at δ_H 4.18(brs) for a methine proton(C-2) attached with an oxygen atom and CHOH group .Two doublet of doublets resonating at δ_H 2.74 ppm(dd, *J*=16.90 , 3.0 Hz) and 2.83ppm (dd, *J*=16.77 ,4.3 Hz) were due to two methylene protons (C-4 eq & C-4ax) adjacent with a methine carbon as showed in figure (12).

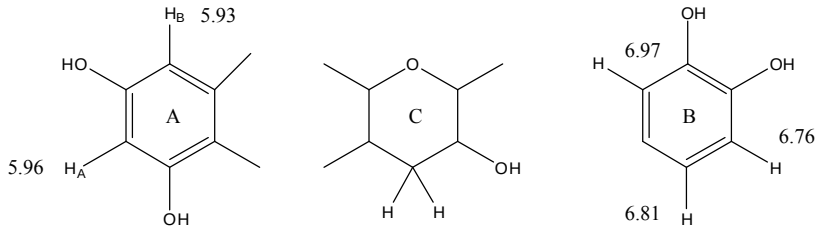


Figure (2): Data Volume of Ring System by H-NMR Compound M2

Table (14):^{1}H-NMR Spectral Data of Compound M2 and Literature Data of Epicatechin

No.of H	δ_HM2ppm, J (Hz)^{M2}	$\delta_H$ppm , J(Hz) [41]	$\delta_H$ppm, J(Hz) [42]	$\delta_H$ppm, J(Hz) [43]
2	4.81 br s	4.80	4.80	4.81
3	4.18 br s	4.19	4.16	4.18
4eq	2.74 dd ,16.77 , 3.0	2.74 , 16.8 . 2.9	2.72 , 16.8 , 2.7	2.74 ,16.5 , 3.0
4ax	2.83dd , 16.77 ,4.30	2.86 16.8 , 4.3	2.86 , 16.8 , 4.5	2.86 ,16.5 , 4.5
6	5.96 d ,2.18	5.95 , 2.3	5.93 , 2.4	5.96 , 2.0
8	5.93 d , 2.16	5.92. 2.3	5.90 , 2.4	5.90 , 2.0
2'	6.97 d , 2.14	6.98 , 1.8	6.97 , 1.8	6.98 , 2.0
5'	6.76 d , 8.12	6.76 , 8.2	6.75 , 8.1	6.76 , 8.0
6'	6.81dd , 8.35 ,1.82	6.8 , 8.2 , 1.8	6.79 , 8.1 , 1.8	6.80 , 8.0 , 2.0

M2-^{1}H-NMR spectral data measured at 400MHz in CD_3DO
[41]-^{1}H-NMR spectral data measured at 400 MHz in CD3DO
[42]-^{1}H-NMR spectral data measured at 300 MHz in CD3DO
[43]-^{1}H-NMR spectral data measured at 300 MHz in CD_3DO

The ^{13}C-NMR of this compound had exhibited fifteen signals representing fifteen carbon skeleton , including one secondary carbon δ_C 29.15 ppm assigned to C-4 as in DEPT technique ,showed in figure (14) where there were five non-substituted aromatic carbon signal at δ_C 96.5 representing carbon C-6 and signal at δ_C 95.96 assigned to carbon C-8 and signal at δ_C 115.3 related to carbon C-2' and signal at 116.0 assigned to C-5'and signal at δ_C 119.45 assigned to C-6', the flavonoid ring junction appeared at δ_C 157.25 and 100.17 ppm for carbon C-9 and C-10 respectively, other aromatic carbon attached to hydroxyl group appeared at C-5, C-7, C-3' , and C-4' at δ_C 157.48, 157.82 ,145.82 and 145.67 ppm respectively the quaternary aromatic carbon appeared as C-1' at δ_C 132.209 ppm and the tertiary carbon at δ_C 79.78 ppm assigned to C-2 and another tertiary carbon attached to hydroxyl group appeared at δ_C 67.39 ppm assigned to C-3 , the table (15) also determine a comparison of carbon resonances of isolated flavonol with the literature data of Epicatechin [44],[45],[46] ,figure (4) illustrated ring system by ^{13}C-NMR.

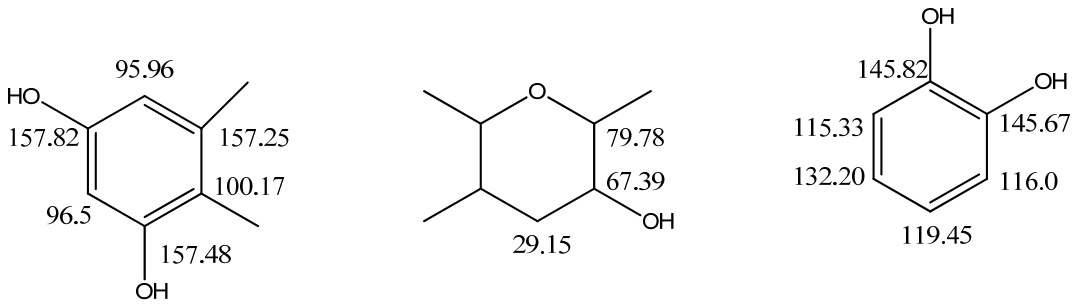


Figure (3): Data Volume of Rin System by 13C-NMR Compound M2

Table (15): ^{13} C-NMR Spectra of Compound M2 and Epicatechin

No. of C	δ_CM2ppm	$\delta_C$ppm[44]	$\delta_C$ppm[45]	$\delta_C$ppm[46]
2	79.78	79.8	79.8	78.31
3	67.39	67.4	67.4	65.19
4	29.151	29.2	29.2	28.47
5	157.48	157.6	157.6	156.79
6	96.508	96.4	96.4	95.36
7	157.82	157.9	157.9	156.47
8	95.96	95.9	95.8	94.38
9	157.25	157.3	157.3	156.03
10	100.17	100.1	100.1	98.79
1'	132.209	132.3	132.2	130.9
2'	115.33	115.3	115.3	115.12
3'	145.82	145.9	145.9	144.66
4'	145.67	145.7	145.7	144.73
5'	116.00	115.9	115.9	115.06
6'	119.45	119.4	119.7	118.27

M1-^{13}C-NMR spectral data measured at 100 MHz in CD_3DO
[44]-^{13}C-NMR spectral data measured at 100 MHz in CD_3DO
[45]-^{13}C-NMR spectral data measured at 100 MHz in CD_3DO
[46]-^{13}C-NMR spectral data measured at 125MHz in DMSO-d6

However the carbon signals at δ_C 96.5 ppm and δ_C 95.96 ppm corroborated a direct connectivity with proton signals δ_H 5.93 ppm and δ_H 5.96 ppm in the HMQC spectrum respectively and δ_C 115.33 ppm and δ_C 116.0 ppm corroborated a direct connectivity with proton signals δ_H 6.97 ppm and δ_H 6.76 ppm respectively.

Table (16) and fig. (15) Showing correlation HMQC.

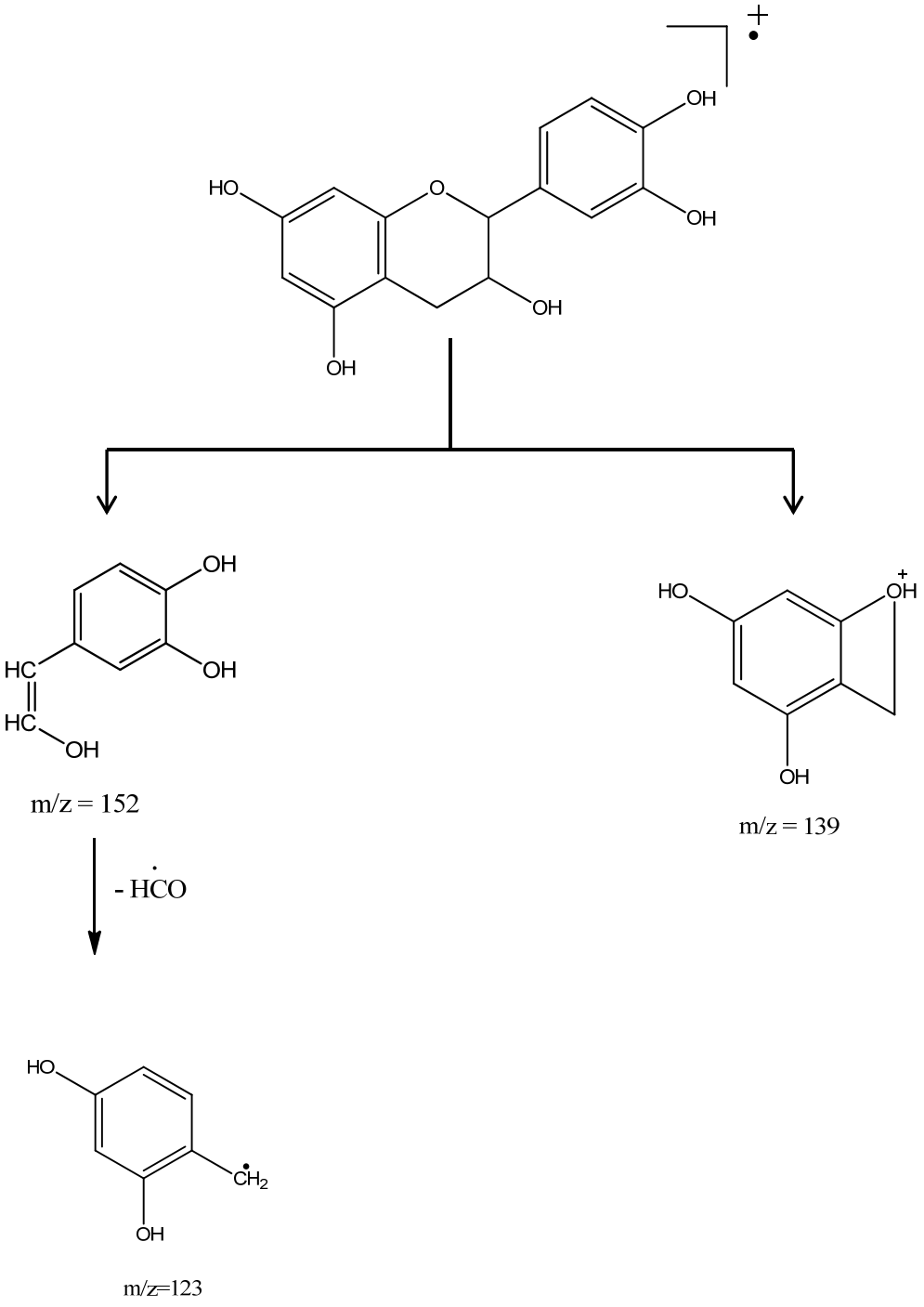
Table (16): Correlation HMQC of Compound M2

^{1}H-NMR ppm	^{13}C-NMR ppm
2.74dd, 2.83dd	(29.15) C-4
4.18 brs	(67.39) C-3
4.81 brs	(79.78) C-2
5.93d	(95.96) C-8
5.96d	(96.50) C-6
6.76d	(116.00) C-5'
6.81dd	(119.45) C-6'
6.97d	(115.33) C-2'

The mass spectrum of compound M2 displayed a molecular ion peak at m/z = 290 in agreement with molecular formula $\mathbf{C_{15}H_{14}O_6}$ cleavage of bonds 1,2 and 3,4 produce fragment ion m/z = 139 and m/z =152 the fragment ion m/z =152 by losing HCO^{*} to give fragment ion m/z =123 as showed in scheme (6).

Table (17): Mass Fragmentation of Compound M1

Mass m/z	Abundance %
139	100
123	49.10
152	32.5



Scheme (6): Mass Fagmentation of Compound M2

The ^{1}H-NMR and ^{13}C-NMR spectrums confirmed that isolated compound M2 was (Epicatechin).

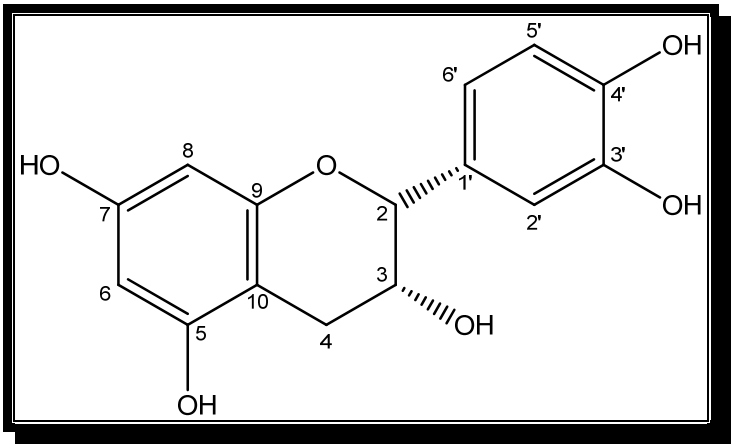


Figure (4): Structure of Compound M2 (Epicatechin)

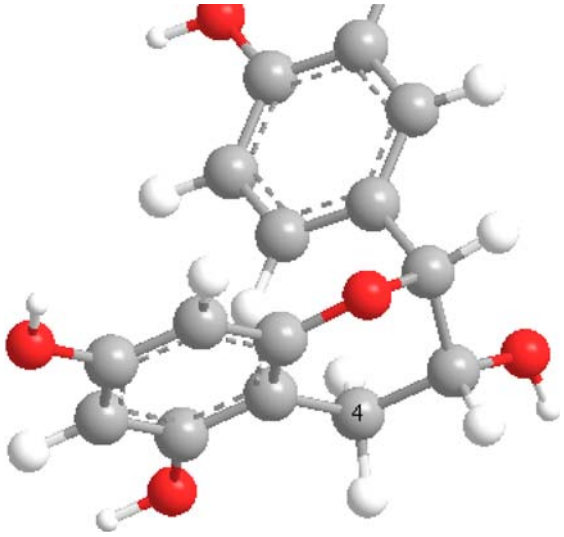


Figure (5): 3D Structure of Compound M2

3.4 Identification of Compound M3

The ^{1}H-NMR spectrum of compound M3 Fig (17) showed single aromatic peak at δ_H 6.55 (4H,s) as singlet consistent with an A_4 spin system for a symmetrically para-disubstituted benzene and proton as singlet at δ_H 8.58 duo to (2H , s) , characteristic of H-bonded hydroxyl . The ^{13}C-NMR spectrum Fig (18) showed two aromatic carbon resonances at δ_C 117.20 for aromatic methine and at δ_C 151.65 for aromatic quaternary carrying hydroxyl group. This supported the symmetrical structure of this compound.

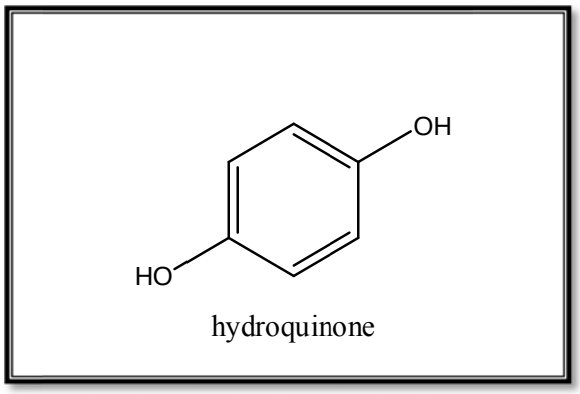
The mass spectrum Fig (20) and scheme (7) is in complete agreement with this assignment showing the molecular ion at m/z 110 (100 %), as base peak and two peaks at m/z 82(21 %) and 81 (2 %) due to the loss of CO and CHO respectively. The finding of this compound was expected, because of the high probability of separating the marker compound of arbutus which is arbutin (9), but as a result of the hydrolysis, the deglycosidic compound hydroquinone was found.

The agreement of isolated compound M3 was good with published data as shown in Table (18)

Table (18): Spectra Data of Compound M3 and Published Hydroquinone

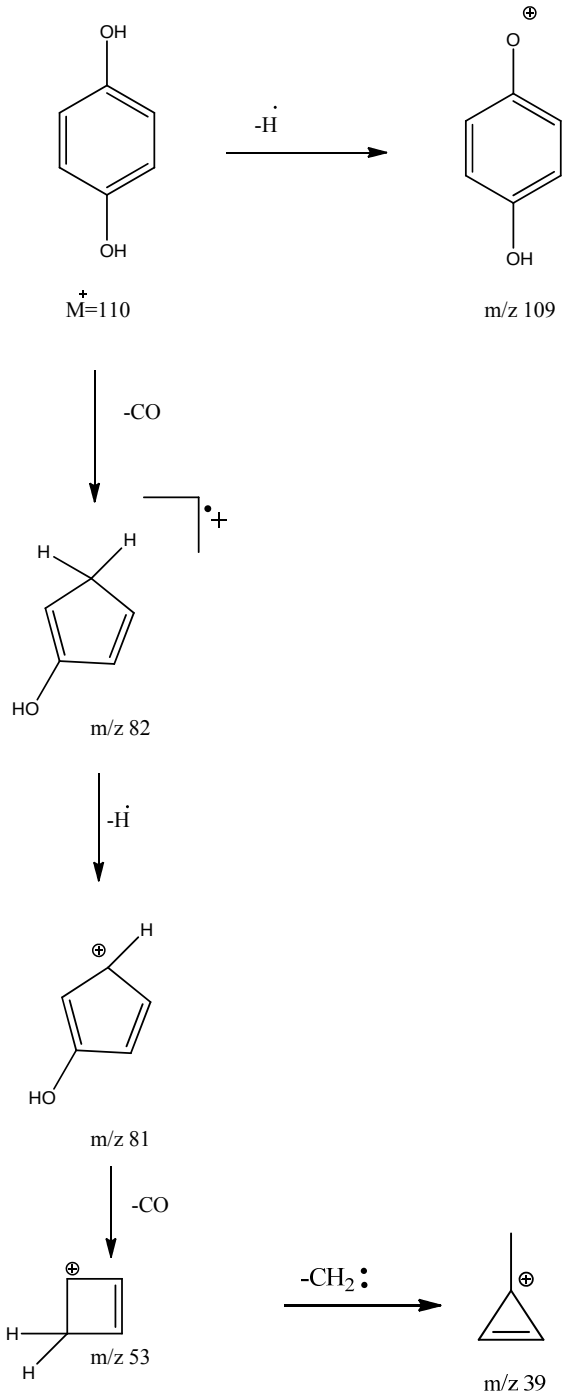
Position	Compound M3		Hydroquinone[47]	
	δ_H ppm	δ_C ppm	δ_H ppm	δ_C ppm
2,3,5,6	6.55 s	117.20	6.81 s	119.08
1,4	8.58 s	151.65	8.19 s	151.65

The above results are consistent with the hydroquinone structure Fig (6).



hydroquinone

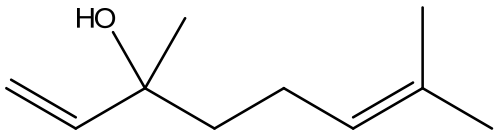
oquinone)



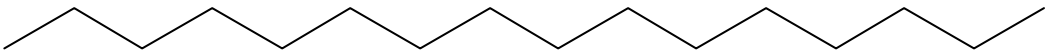
Scheme (7): Mass Fragmentation of Compound M3

Appendix

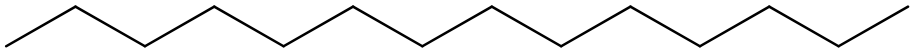
Appendix



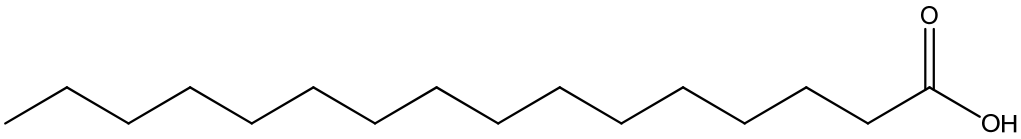
Linalool
(1)



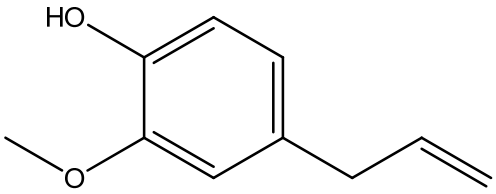
hexadecane
(2)



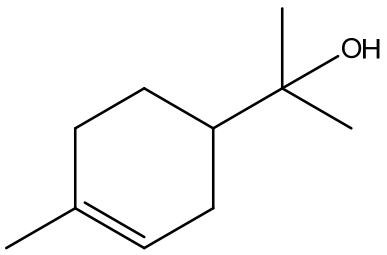
Tetradecane
(3)



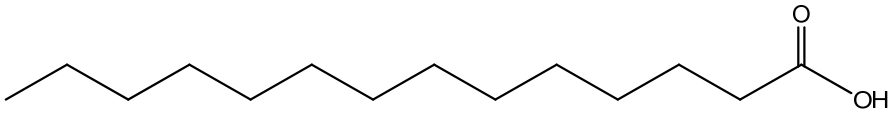
Hexadecanoic acid
(4)



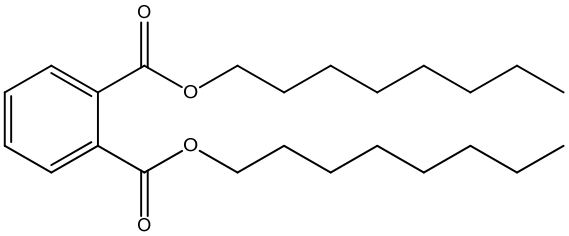
Eugenol
(5)



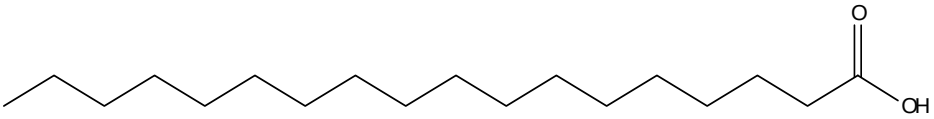
Terpineol
(6)



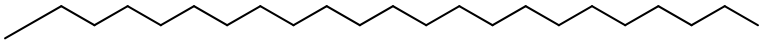
Tetradecanoic acid
(7)



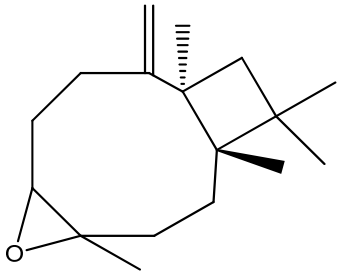
Dioctyl phthalate
(8)



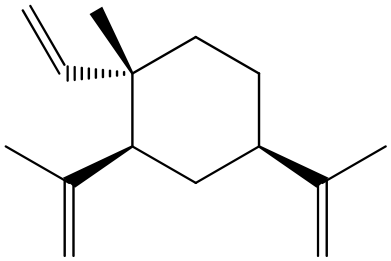
Octadecanoic acid
(9)



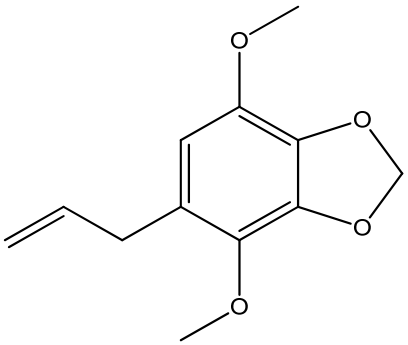
Tricosane
(10)



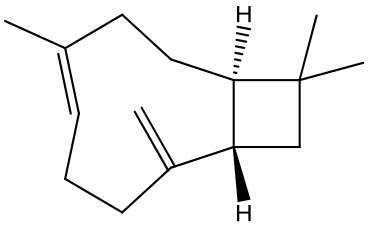
Caryophllene Oxide
(11)



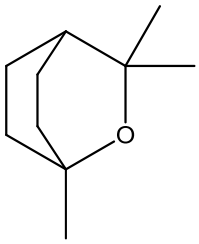
Beta-Elemene
(12)



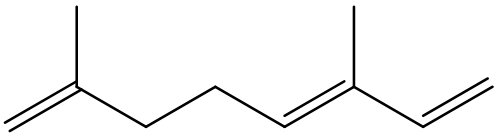
4,7-dimethoxy-5-prop-2-enyl-1,3-benzodioxole (Apiol)
(13)



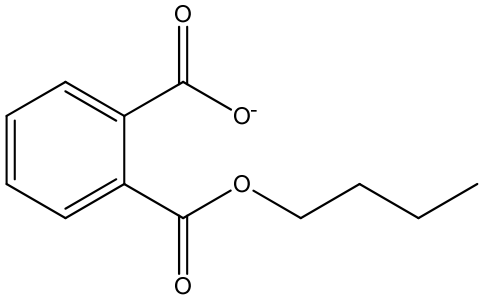
Trans-Caryophllene
(14)



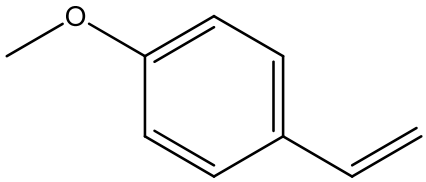
1,8-Epoxy-p-menthane
1,8-Cineole
(15)



3,7-dimethyl-1,3,7-octatriene
(Ocimene)
(16)



Butyl phthalate
(17)



p-methoxystyrene
(18)

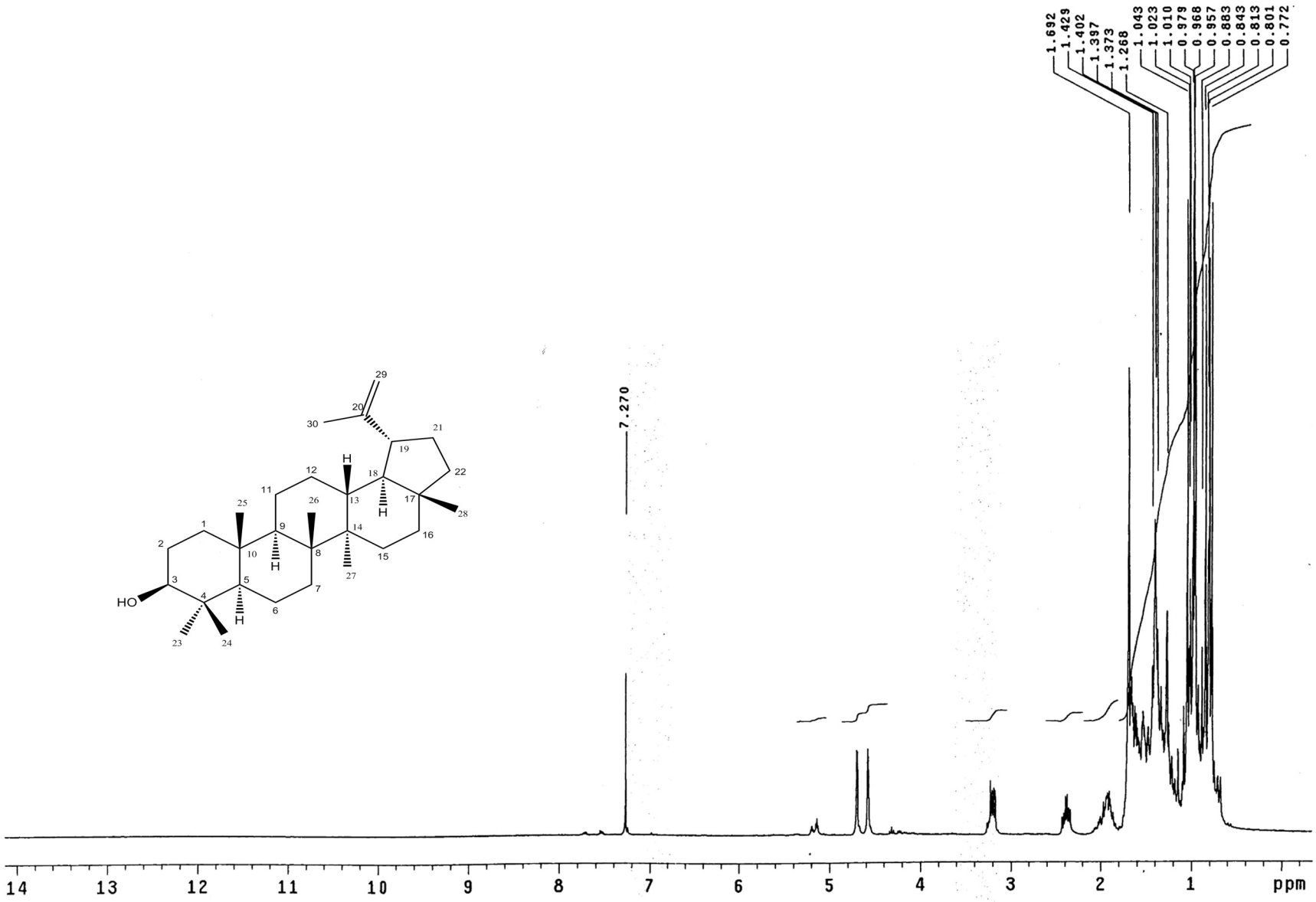


Figure (7): H-NMR Spectrum of Compound M1

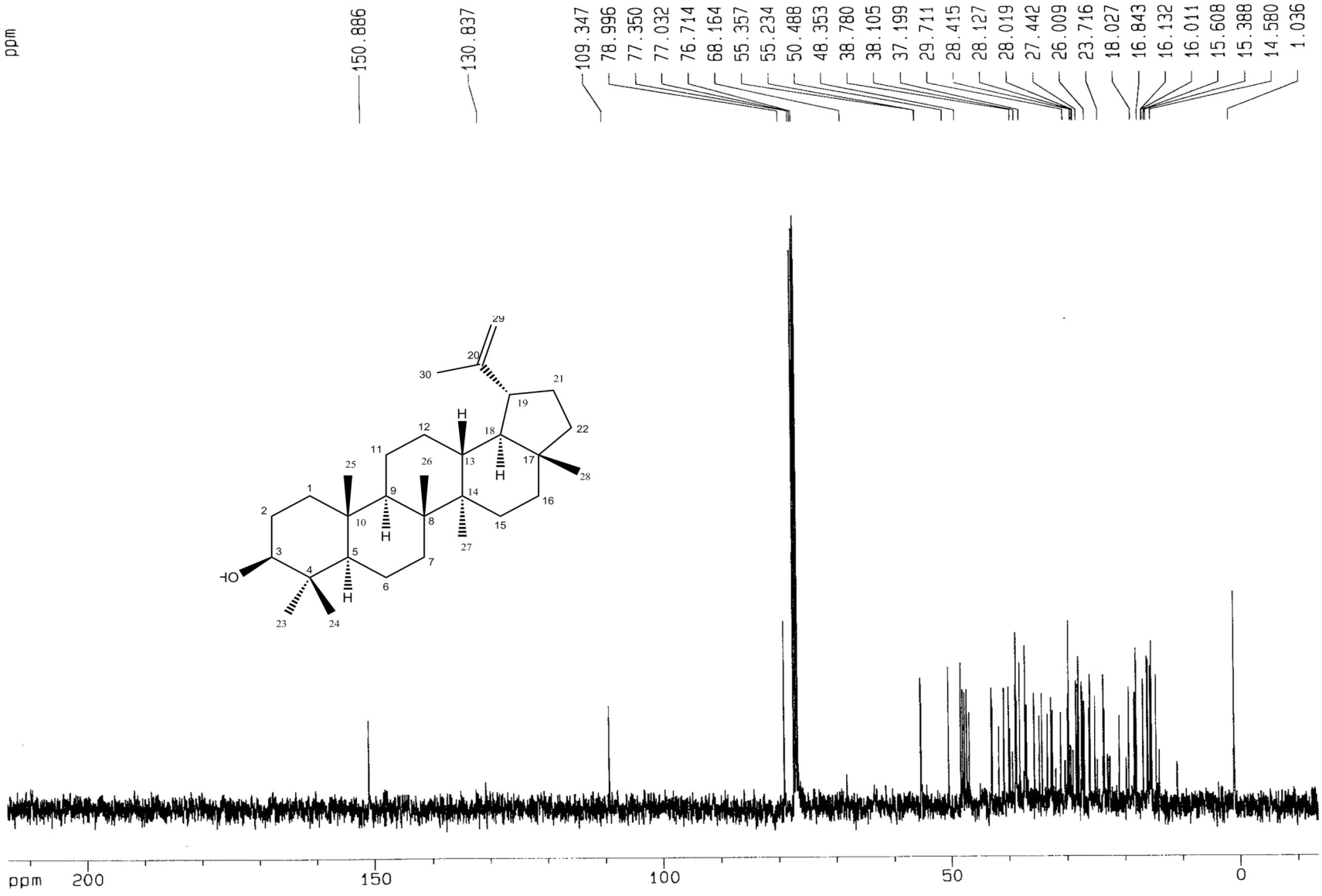


Figure (8) : 13C-NMR Spctrum of Compound M1

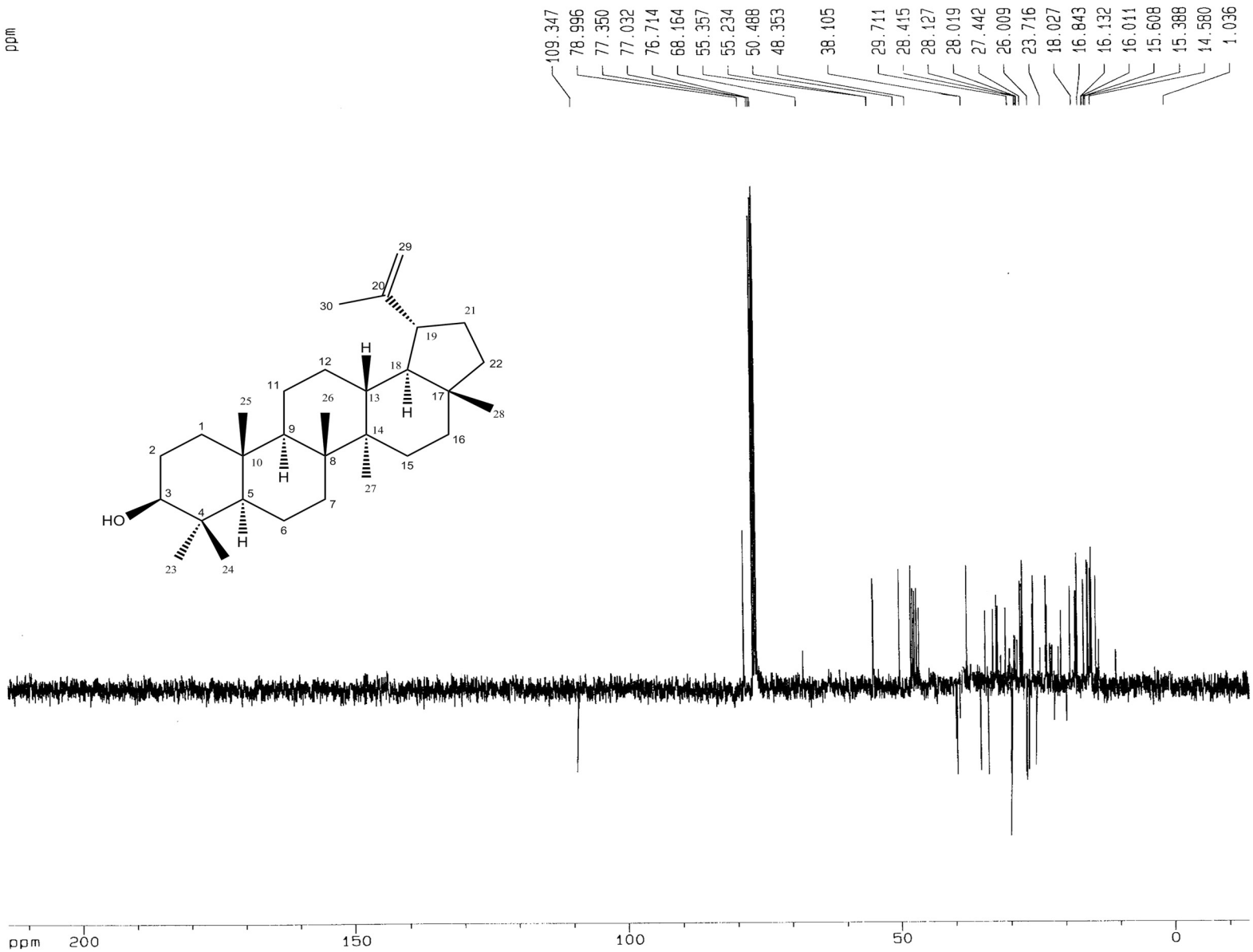


Figure (9) : DEPT Spectrum of Compound M1

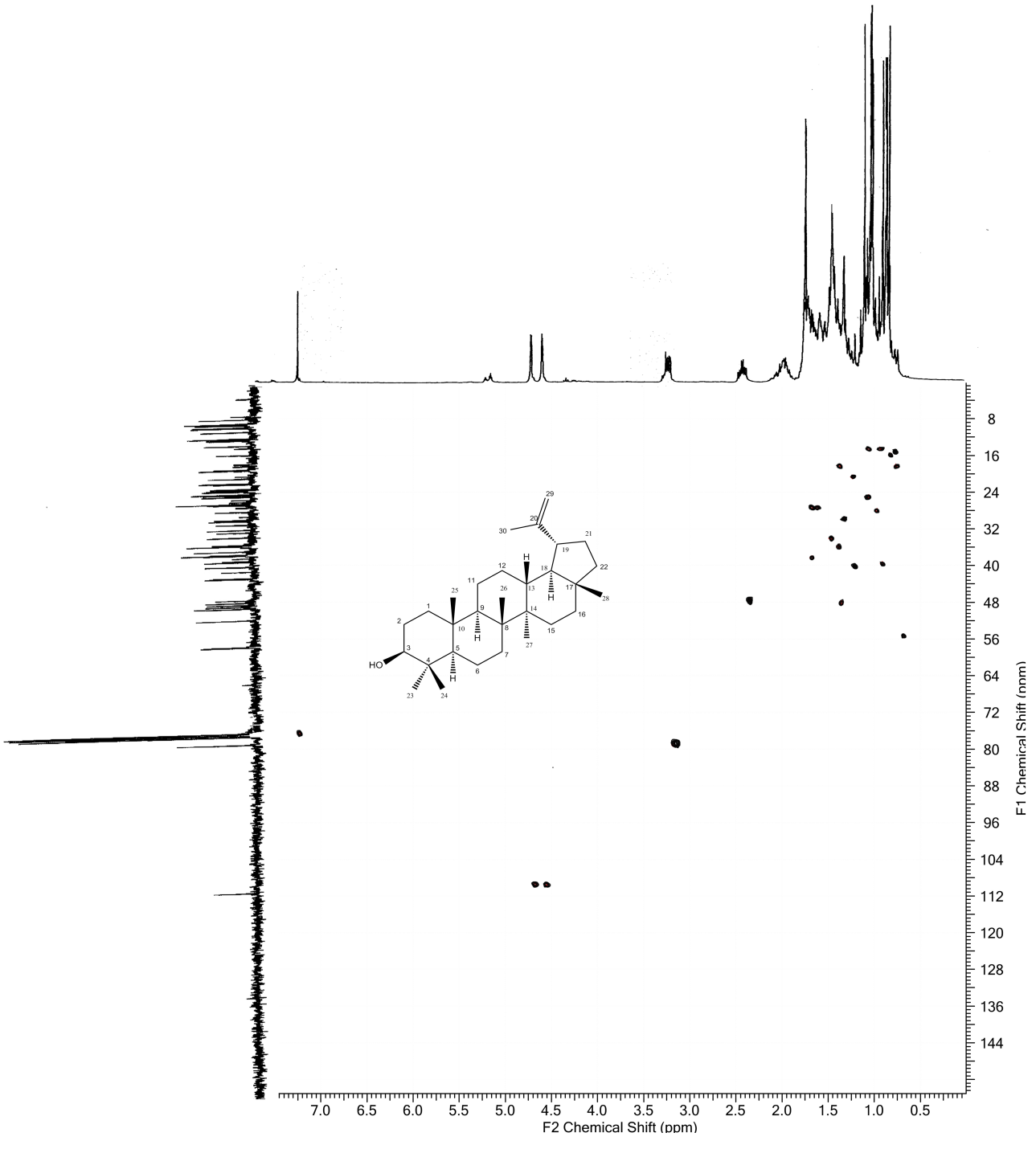
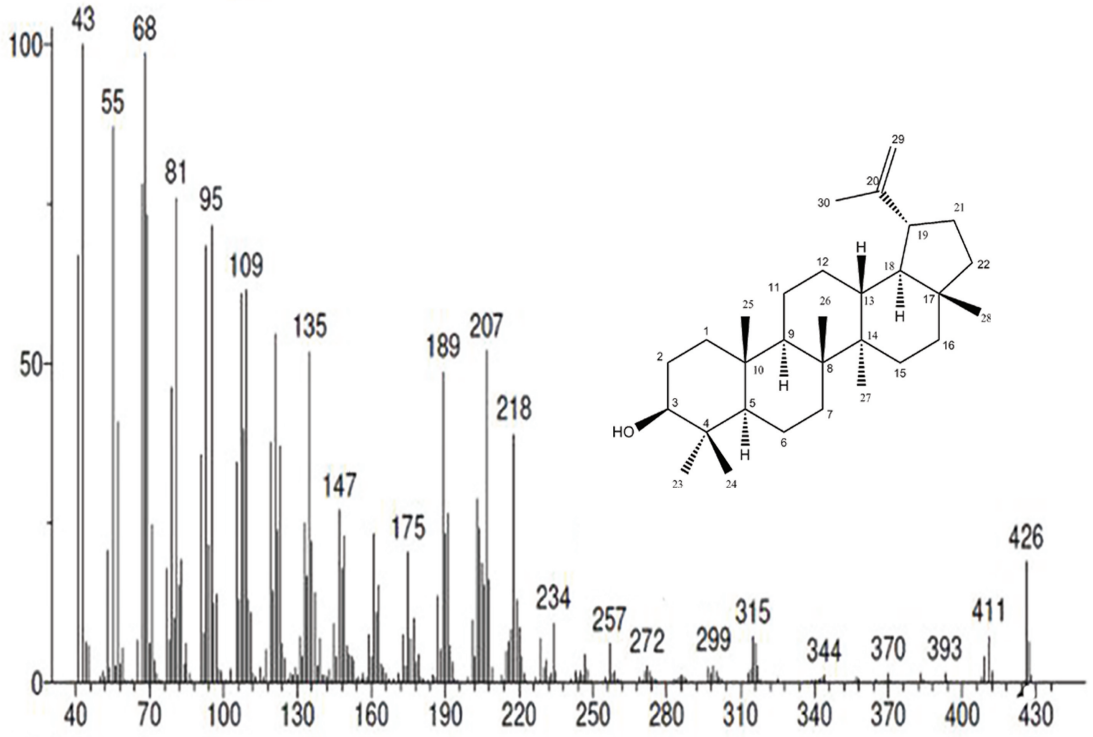
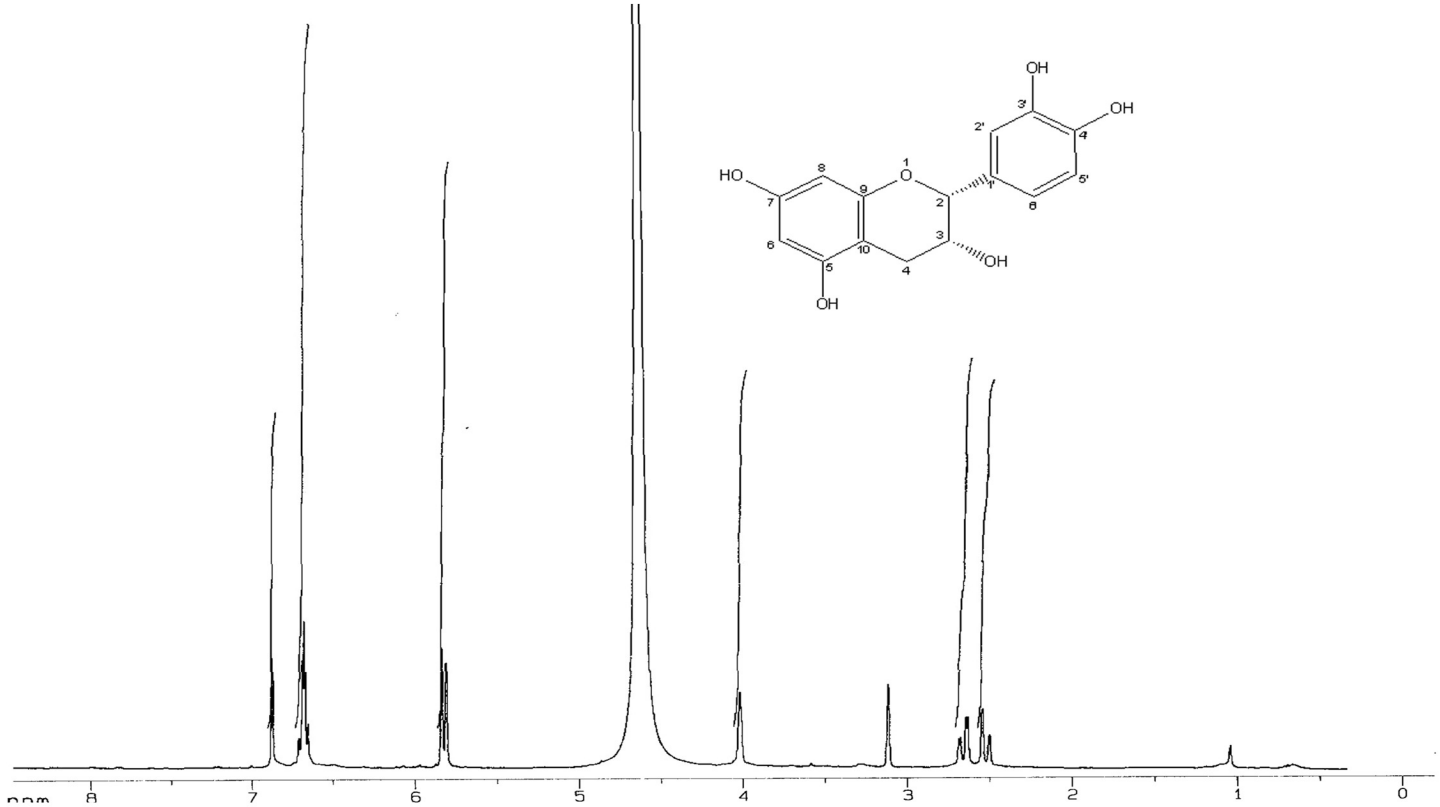


Figure 11 : HMQC Correlation of Compound M1



Figure(11): Mass Spectrum of Compound M1



Figure(12) : H-NMR Spectrum of Compound M2

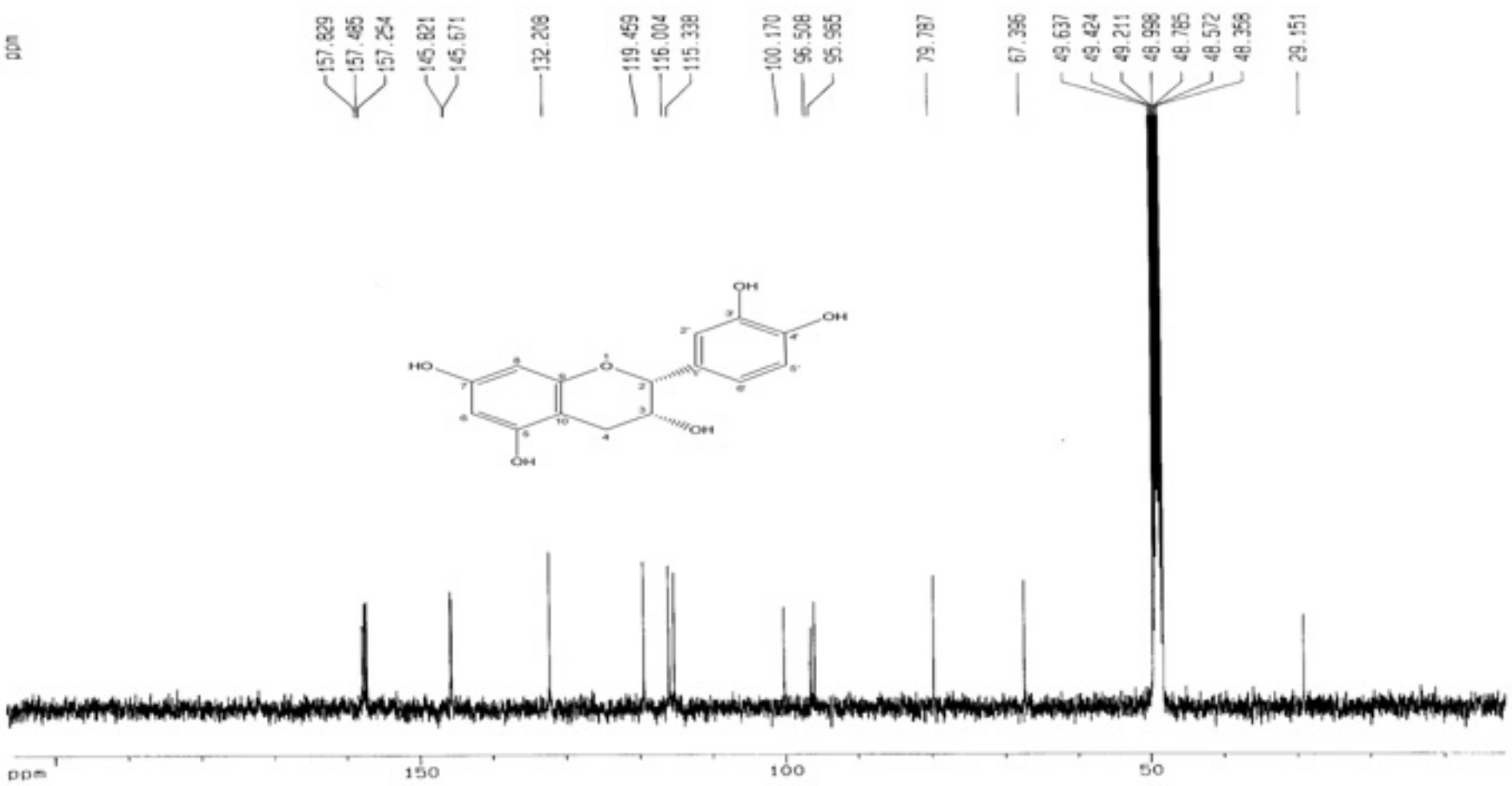


Figure (13) : C NMR Spectraum of compound M2

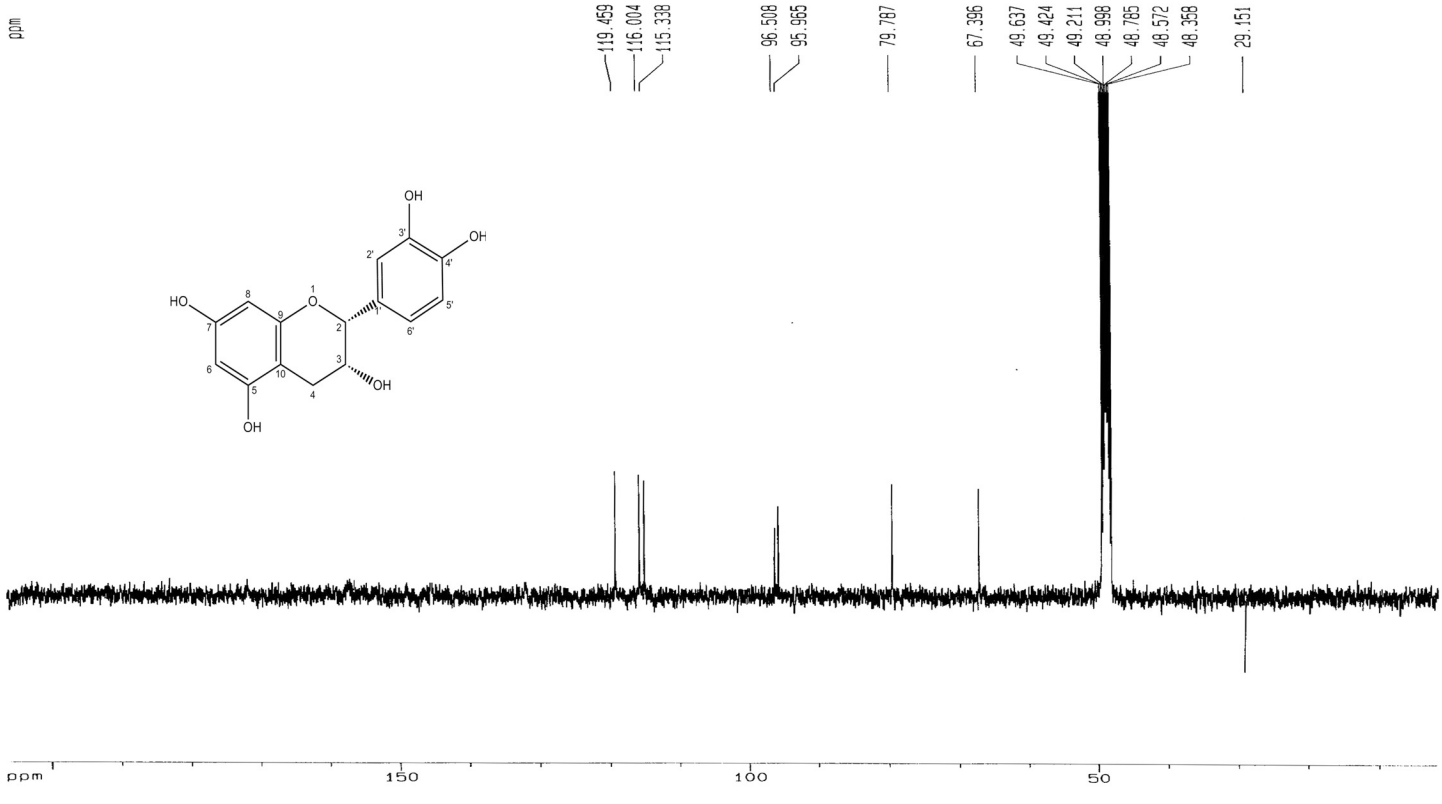


Figure (14) : DEPT Spectrum of Compound M2

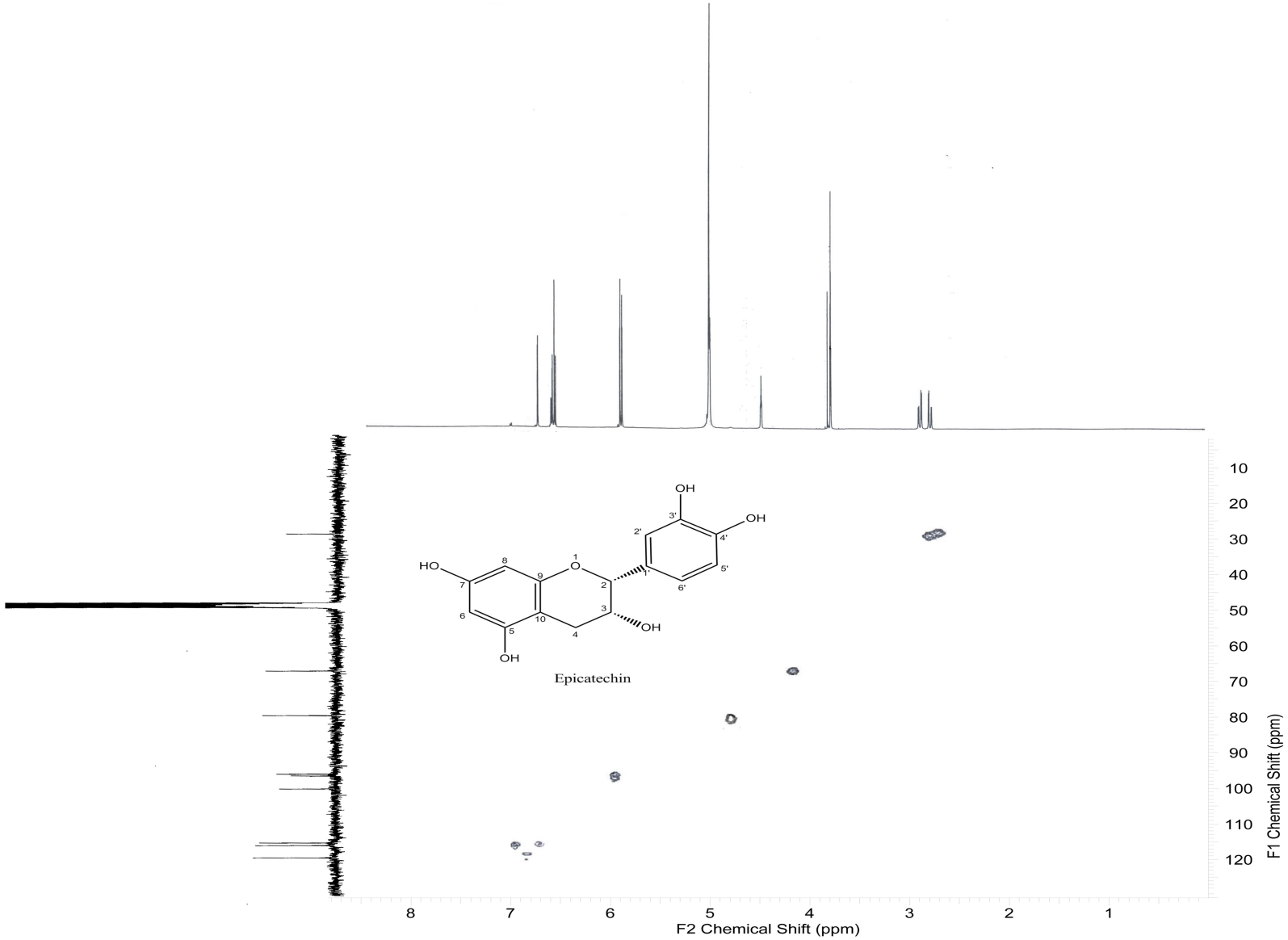
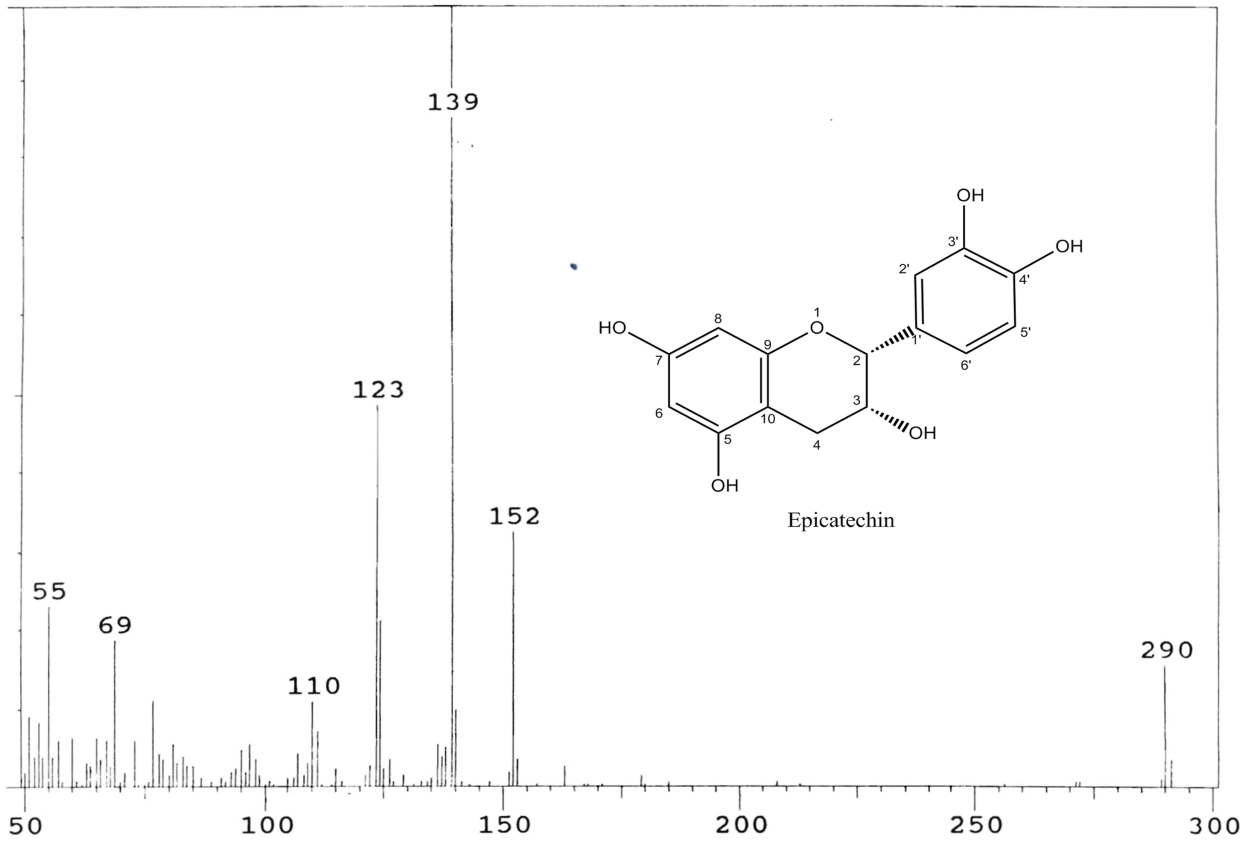


Figure (15): HMQC of Compound M2



Figure(16): Mass Spectrum of Compound M2

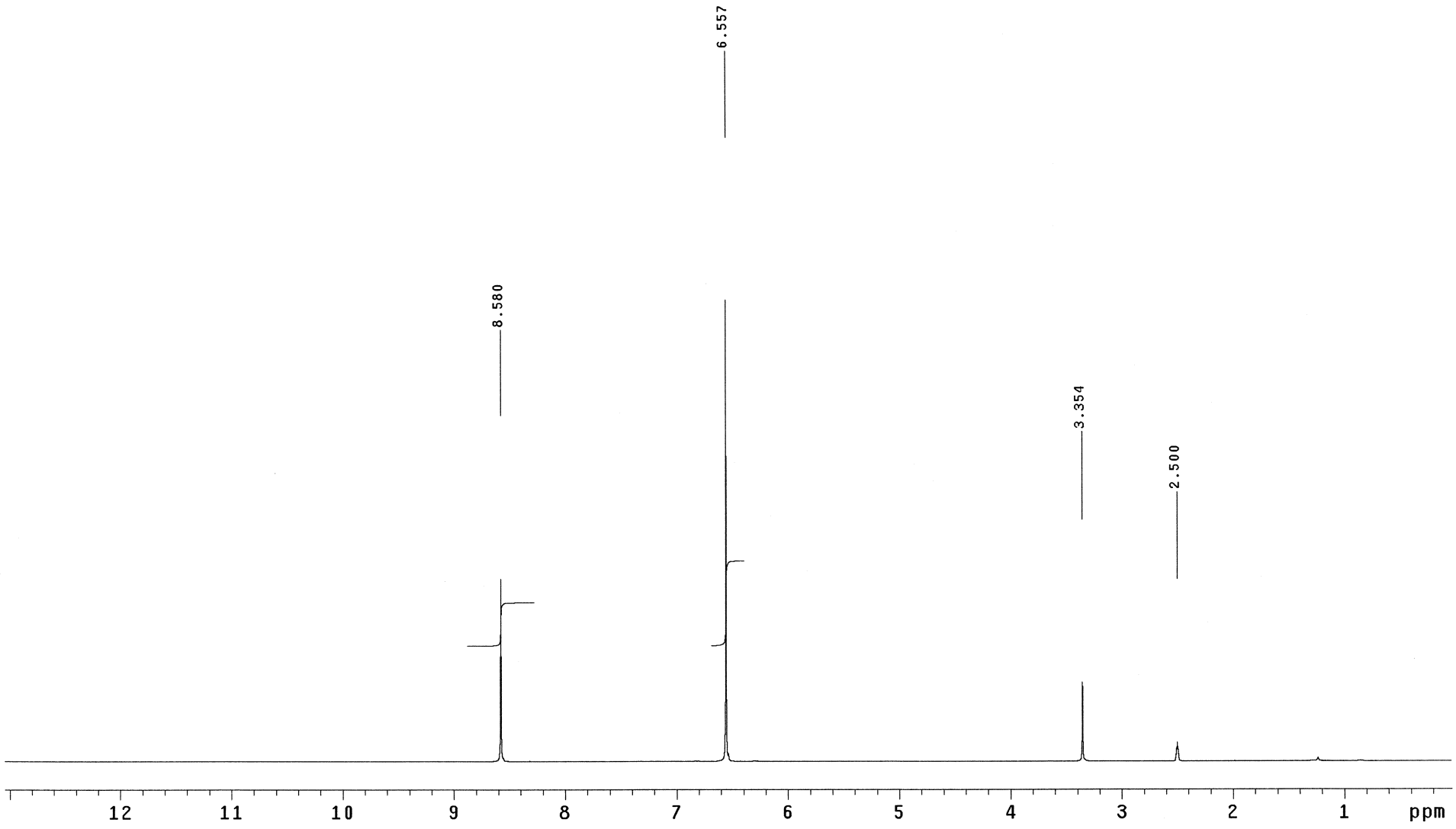


Figure (17) : H NMR Spectrum of Compound M3

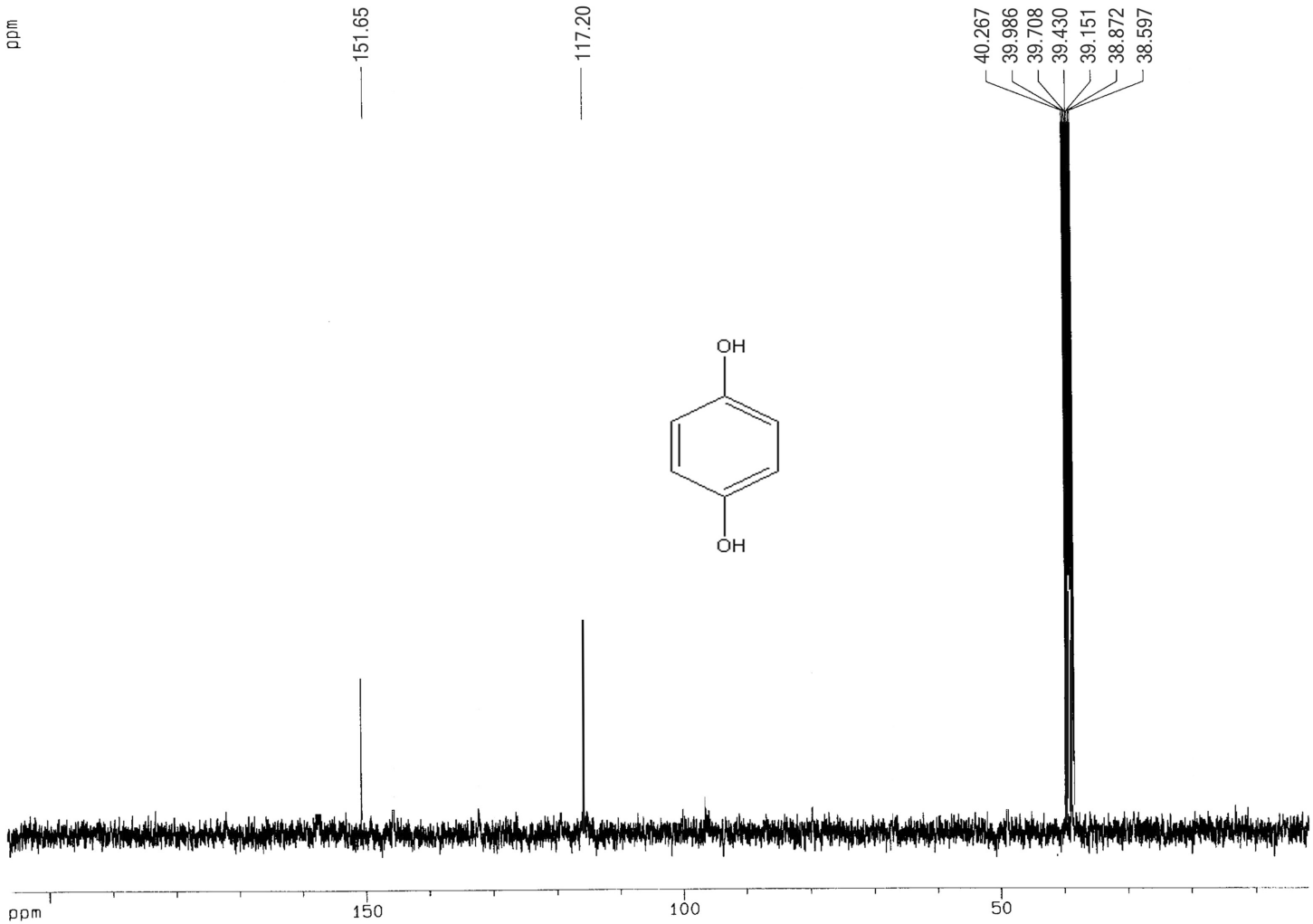
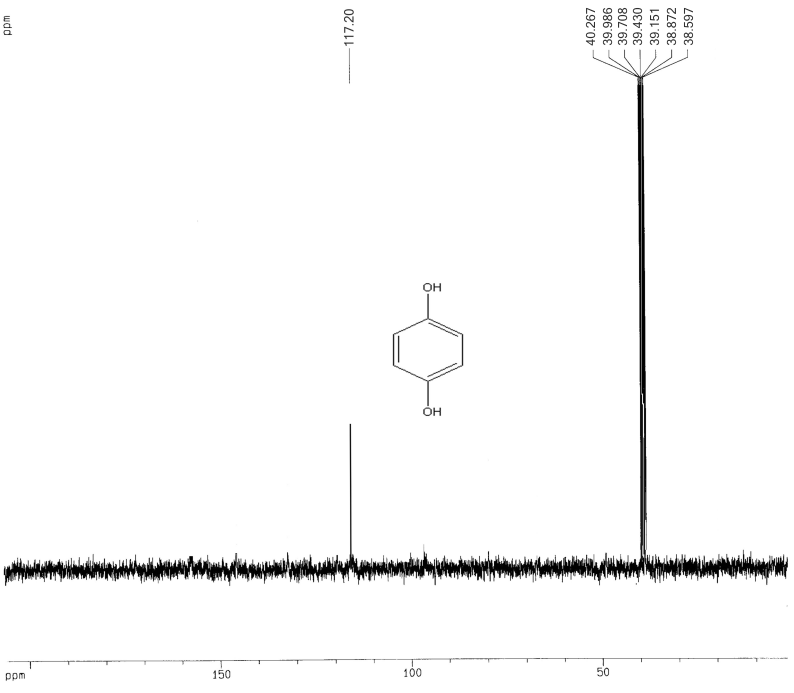


Figure (18): C-NMR Spectrum of Compound M3



Figure(19): DEPT Spectrum of Compound M3

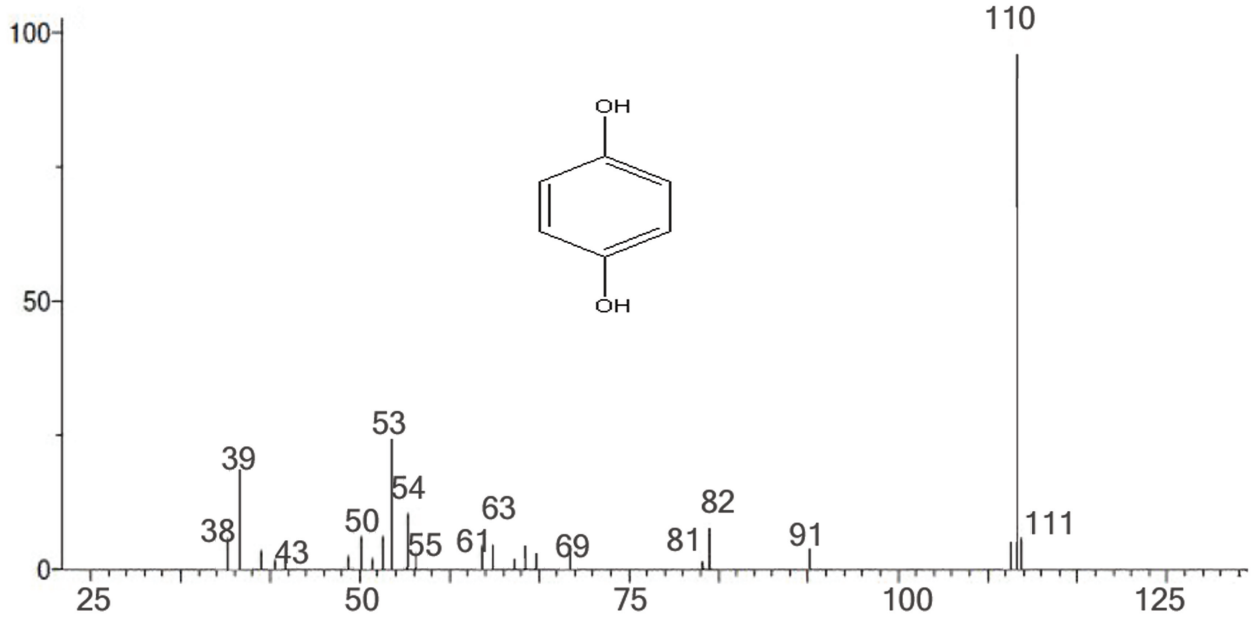
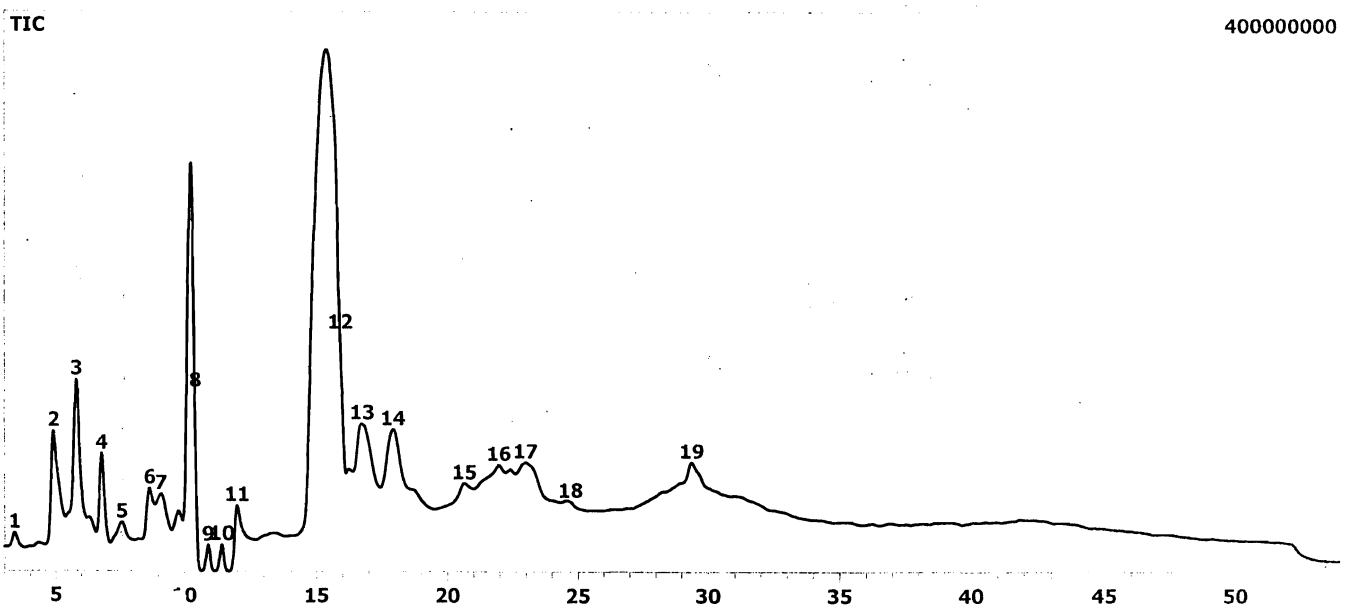
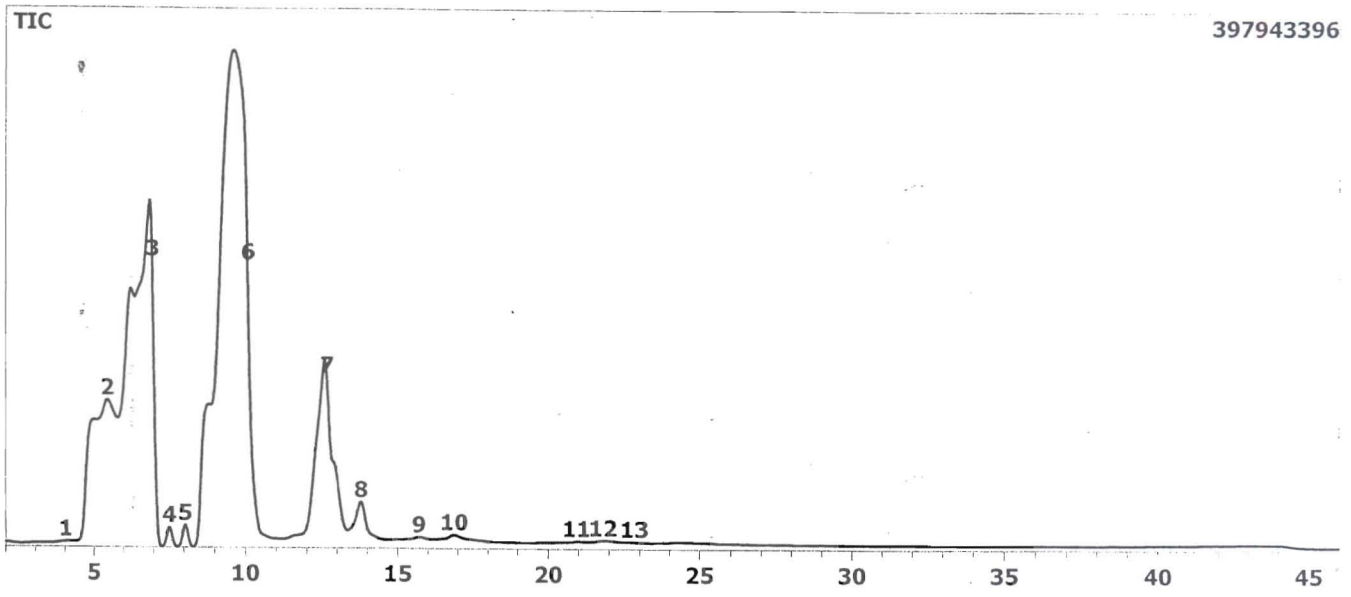


Figure (20): Mass Spectrum Of Compound M3

Data : EP'BY1.D01
Sample : Dr/ M. Eriby
ID : 1
Sample Amount : 0
Dilution Factor : 0
Type : Unknown
Operator :
Vial No. : 0
Barcode :



Data : ERIBY2.D02
Sample : Dr/ M. Eriby
ID : Sample 2
Sample Amount : 0
Dilution Factor : 0
Type : Unknown
Operator :
Vial No. : 0
Barcode :



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