Sudan University of Science and Technology

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Isolation and Structure Elucidation of Four Antioxidant Compounds from Sudanese *Sonchus oleraceus* Plant

فصل و تحديد البنيان لاربعة مركبات مضادات للاكسدة من نبتة المولیتا (*Sonchus oleraceus*)

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DEDICATION

FOR MY FATHER'S MEMORY
TO MY FAMILY ESPECIALLY MY
MOTHER MARIAM, BROTHER YOUSIF
SISTER AMNA AND DAUGHTER HANEEN

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Abstract

Sonchus oleraceus (moleita) from the family Asteracae was investigated for its radical scavenging activity. The whole herb was firstly extracted with dicloromethane to remove as much of chlorophyll and other colouring pigments. The plant residue was extracted with methanol. The methanol extract was tested for its antioxidative contents. The methanolic extract showed 88% radical scavenging activity against DPPH(1,1-Diphenyl-2-picyl hydrazyl) a reagent used to test compound for their ability to neutralize free radicals. The methanolic extract of the whole plant was dissolved in 500ml distilled water. The mixture was poured into a separatory funnel and successive addition of 200 milletres of ethyl acetate was added with continuous shaking. Extracts of the ethyl acetate layer were collected. The ethyl acetate was chosen as a solvent that is optimum for extracting antioxidant (polyphenolic) compounds. Primary screening of the ethyl acetate extract showed saponins, coumarins, tannins, alkaloids, flavonoids, anthraquinones, sterols, triterpines and phenols. The highest concentrations were for tannins, flavonoids and phenols.

The ethyl acetate layer was isolated into fractions with column chromatography. Solvent eluent mixtures were used from least to highest polarities (petroleum ether, chloroform, ethyl acetate, and methanol). 14 fractions showed high antioxidant activity. (Fraction 23 –Fraction 36).Fractions were monitored with TLC (Thin Layer Chromatography) and suitable solvent systems.

Fraction 23 gave 69% activity and 1.99 gm yield and solvent system in the ratio (4 : 6) chloroform ; ethyl acetate. Fraction 23 was purified with

preparative thin layer chromatography to give three compounds. Solvent system for purification was (toluene: ethyl acetate:formic acid) 5:4:1.

Fraction 23 purified to give (23_2 and 23_3) which were analysed for structure characterization.Structure chrachterization was done with different spectroscopic techniques:Infra-red, Mass, and Nuclear Magnetic Resonance (NMR).

Fraction 24showed 88% RSA (radical scavenging activity) when analysed with DPPH.Weight of fraction was 0.640 gm.Solvent system for separation was chloroform: ethyl:acetate (3:7). Solvent system for purification was (toluene: ethyl.acetate: formic acid) 5:4:2.Fraction 24 gave one compound for structure characterization .

Sub-column was carried for fractions (30+31) using a smaller column. The solvents used were only ethyl acetate and methanol. Nine sub-fractions were isolated. Sub fraction 7 was chosen for the fourth compound. It gave 40% RSA against DPPH, but its separation was good on TLC .Solvent system for isolation was Ethyl acetate: methanol (4:6).

Four compounds were isolated, compound (1) Lignan glycoside. Brown – green solid, with a solvent system of isolation Toluene: Ethyl Acetate: Formic Acid in the ratio 5: 4: 2. The molecular ion was m/z 869. Weighting 0.466 gm.

Compound (2) Chalcone glycoside. Yellow-Brown solid. Solvent system of isolation (Toluene: Eth. Acet. Form.acid) in the ratio 5:6:3.The molecular ion was m/z 745. Weighting 0.121 gm.

Compound (3) Flavone glycoside. Yellow- brown solid. Solvent system for isolation (Tol: Eth.Acet : Formic Acid). The molecular ion m/z 745.Weighting 0.530 gm.

Compound (4)Tannic acid. Dark brown amorphous solid. Solvent system for isolation was Ethyl Acetate: Methanol (4:6). m/z 789 Weight 0.11 gm.

This investigation proved that *Sonchus oleraceus* plant possesses high activity against RSA, which is prominent for protection of cells against harmful radicals.

المستخلص

اجريت هذة الدراسة البحثية علي نبتة المولينا العشبية (سونكس اوليريشس) من اجل الاستكشاف عن وجود مضادات الاكسدة .

تم استخلاص العشبة باستعمال ثنائي كلورو ميثان لازالة الكلوروفيل والملونات الاخري ثم الاستخلاص بالميثانول الذي أثبت فاعلية استخلاص بنسبة 88% لمضادات الاكسدة بطريقة (1,1 -Diphenyl-2-picyl hydrazyl) وهو محلول لاختبار المركبات التي تحتوي عاي قابلية لاحتواء الشقوق الحرة .

كما ان المسح الاولي لمستخلص النبات كشف عن فعالية عالية للمواد الفينولية .

تم اختيارمستخلص خلات الايثايل الذي استخلص من مستخلص الميثانول بقمع الفصل وتجزئته بواسطة كروماتوغرافيا العمود,14 جزء من الاجزاء التي فصلت تحتوي علي فاعليتة اجريت لها تنقية بواسطة الكروماتوغرافي التحضيري ومن ثم تم فصل اربعة مركبات والتعرف عليها بتقنية ال (NMR) .

المركب الاول (Lignan glycoside) : اللون بني اخضر ,صلب, الوزن 0.44 جرام , M/Z 869, مذيبات الفصل (Toluene : Ethyl acetate : Formic acid) بنسبة 5:4:2

المركب الثاني (Chalcone glycoside) : الوزن 0.121 جرام ,M/Z 745 ,مذيبات الفصل (Toluene : Ethyl acetate : Formic acid)) بنسبة 5:6:3

المركب الثالث (Flavone glycoside) اللون اصفر- بني , الوزن 0.530 جرام ,M/Z 745,مذيبات الفصل (Toluene : Ethyl acetate : Formic acid)

المركب الرابع (Tannic acid) : اللون بني غامق , الوزن 0.11 جرام , M/Z 789 ,مذيبات الفصل (Ethyl acetate : Methanol) بنسبة 4:6 .

اثبتت الدراسات والبحوث العلميه ان مضادات الاكسدة تحمي الخلايا من اضرار الزرات الحرهز وقد اثبت من خلال هذا البحث ان عشبة السونكس اوليريشس (المولينا) يوجد بها مضادات اكسدة عالية الفعالية وموعودة بحماية الخلايا من الذرات الحرة الضارة.

Table of contents

DEDICATION	I
ACKNOWLEDGEMENTS	I I
ABSTRACT (English)	I I I
المستخلص	V
TABLE OF CONTENTS	VII
LIST OF TABLES	XII
LIST OF FIGURES	XII
LIST OF ABBREVIATIONS	I

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1. Introduction …………………………………………………. 1
1.1. Natural products ..…………..………………………….. 2
1. 1.1. Secondary metabolites ……..………..………………. 2
1.1.2 Reactive Oxygen species. …………………………… 4
1.1.3 Antioxidants …..………………………………............... 5
1.1.3.1 Levels of antioxidants action ……………………… 5
1.2 Phenolic compounds ..……………………………………. 6
1.2.1 Phenolic acids . ………..………………………….….... 7
1.2.2 Tannins . …………………………………………… 8
1.2.2.1 Bioactivities of Tannins ………………………… 9
1.2.3 Lignans and Stelbenes ………………………………. 9
1.2.3.1 Bioactivities of lignans………………………….… 10
1.2.3.2Some lignan types with bioactivities …………………… 11
1.2.4 Phenyl propanoids... 12
1.2.5 Flavonoids... 13
1.2.6 Bioactivities of Chalcones... 16
1.2.7 Chemical structures.. 19
1.2.7.1 Antioxidant capacity of phenolic compounds.................. 20
1.3 Methods of spectroscopic separation............................... 21
1.3.1Chromatography... 21
1.3.1.1 Column chromatography.. 22
1.3.2 Methods of identification and structure elucidation............ 22
1.3.2.1 Infra-red spectroscopy.. 22

1.3.2.2 Mass spectroscopy.. 23
1.3.2.2.1 Liquid Chromatography/Mass Spectroscopy……… 25
1.3.3 Nuclear Magnetic Resonance Spectroscopy………….. 26
1.4 Radical Scavenging Assays……………………………….. 30
1.4.1 Radical Scavenging Assays DPPH ……………………… 30
1.5 *Sonchus oleraceus* Species…………………………….. 31
1.5.1 Antioxidant activities of *Sonchus oleraceus*……………. 32
1.6 Taxonomic Hierarchy…………………………………….. 35
Objectives of the Research………………………………… 37

Chapter Two

2. MATERIALS AND METHODS

2.1 Materials... 38
2.1.1 Plant Materials.. 38
2.1.2 chemicals and reagents.. 38
2.1.3 Equipment and instruments...................................... 38
2.1.4 Spray reagents for thin layer chromatography............ 39
2.2 Methods... 39
2.2.1 Sample extraction.. 39
2.2.1.1 Fractionation of methanolic extract: (Solvent-solvent extraction)……………………………………………………….. 40
2.2.2 Preparation of reagents for qualitative phytochemical screening……………………………………………………….. 40
2.2.2.1 Mayer' reagent…………………………………………… 40
2.2.2.2 Valser's reagent………………………………………… 41
2.2.2.3 1 % Aluminum chloride……………………………… 41

2.2.2.4 1 % Potassium hydroxide……………………………… 41
2.2.2.5 Saline solution…………………………………………… 41
2.2.2.6 Gelatin salt reagent……………………………………. 41
2.2.2.7 Ferric chloride…………………………………………… 41
2.2.2.8 0.5 N potassium hydroxides………………………….. 41
2.2.3 Phytochemical screening of the extracts…………………. 41
2.2.3.1 Alkaloids tests………………………………………….. 42
2.2.3.2 Saponins test…………………………………………….. 42
2.2.3.3 Test for Flavonoids and phenols …………………….. 42
2.2.3.4 Test for Tannins……………………………………….. 43
2.2.3.5 Test for Sterols and triterpenes ……………………… 43
2.2.3.6 Test for Coumarins…………………………………… 43
2.2.3.7 Test for Anthraquinone glycosides………………… 43
2.2.4 Thin layer chromatography (TLC)…………………… 44
2.2.4.1 TLC plates……………………………………………. 44
2.2.4.2 Application of sample on TLC plates………………. 44
2.2.4.3 Solvent mobile phases……………………………….. 44
2.2.5 Isolation and purification of compounds………………. 45
2.2.5.1Column chromatography for ethyl acetate extract…… 45
2.2.5.2 Sub- Column chromatography for fractions (30+31)….. 46
2.2.5.3 Preparative TLC of Fractions………………………….. 46
2.2.5.4 Application of sample on preparative T LC plate…… 46
2.2.5.5 Preparative TLC for fraction 23………………………. 47
2.2.5.6 Preparative TLC for fraction 24……………………….. 47
2.2.5.7 Preparative TLC for fraction 25……………………….. 48
2.2.5.8 Preparative TLC for fraction 29……………………….. 48

2.2.6 Antioxidant assay using DPPH for free radical scavenging activity.. 48
2.2.7 Spectroscopic analysis of isolated compounds…………….. 49

Chapter Three

3. Results and Discussion 59
3.1 Methanol solvent extraction………………………………… 60
3.1.1 Results of Radical Scavenging Activity for extracts………….. 61
3.1.2 Results of Radical Scavenging Activity for methanol extracts……………………………………………………… 61
3.2 Results of DPPH Radical scavenging activity for ethyl acetate extract…………………………………………………… 61
3.3 Phytochemical analysis……………………………………….. 62
3.3.1 Qualitative phytochemical analysis………………………….. 62
3.4 Column Chromatography…………………………………. 63
3.4.1 Sub column for fractions (30 +31)………………………. 66
3.5 Purification of fractions with preparative TLC……………. 66
3.5.1 Purification of fraction 23 with preparative TLC……… 66
3.5.2 Purification of fraction 24 with preparative TLC……… 67
3.5.3 Purification of fraction 25 with preparative TLC………… 67
3.5.4 Purification of fraction 29 with preparative TLC…………. 68
3.6 Test of purity with three solvent systems…………………… 68
3.7 Spectroscopic analysis and structure elucidation................ 71
3.7.1 Characterization of first compound...................................... 71
3.7.2 Characterization of compound two....................................... 80
3.7.3 Characterization of compound three...................................... 90

3.7.4 Characterization of compound four....................................... 102
Conclusion and recommendation.. 112
Appendices... 114
Appendix A... 114
Appendix B... 119
Appendix C... 125
References... 129

List of Abbreviations

Eth acet.	Ethyl acetate
Meth.	Methanol
Tol.	Toluene
Form.acid	Formic acid
Chlor.	Chloroform
Acet.acid	Acetic acid
Dichl.meth.	Dichloromethane
Pet.ether	Petroleum ether
RSA	Radical scavenging action
DMSO	Dimethyl sulphoxide
DPPH	1,1-Diphenyl-2-picyl hydrazyl
IR	Infra red
ESI_MS	Electron spray Ionozation-Mass spectroscopy
NMR	Nuclera magnetic resonance
2D NMR(COSY)	Two dimensional nuclear magnetic resonance
HSQC	Heteronuclear single quantum correlation
DEPT	Distortionless enhancement by polarization transferr
TMS	Tetramethyl silane
TLC	Thin layer chromatography
Prep.TLC	Preparative thin layer chromatography

List of figures

Fig.1.1 Origins of secondary metabolites metabolism……….…… 3
Fig.1. 2: p-hydroxy benzoic acid ……………………………….. 8
Fig.1.3 p-hydroxy cinammic acid…………………………………. 8
Fig.1. 4: gallic acid (hydrolysable tannin)…………………… 9
Fig. 1.5: Stelbene monomer………………………………………… 9
Fig. 1.6: Lignan basic structure……………………………………… 10
Fig.1.8: Saururin B………………………………………………… 11
Fig 1.9.: Oferuloylsecoisolariciresinol………………………………. 12
Fig 1.10 Vitecannasides…………………………………………….. 12
Fig1..11: phenylpropanoid unit……………………………………… 13
Fig. 1.12: Flavonoid Skeleton………………………………………. 14
Fig 1.13 Molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone)……………………………………………………………. 14
Fig.1.14: Neoflavonoids structure……………………………… 15
Fig.1.15 Isoflavonoid……………………………………………… 15
Fig1.16 Different flavonoid structures……………………………. 15
Fig 1.17 Anthocyanidins……………………………………………. 16
Fig 1.18 Chalcone…………………………………………………. 17
Fig 1.19 2H-1-benzopyran-2-ones…………………………………. 17
Fig 1.20 Chalcone sulfonamide……………………………………. 17
Fig 1.21 2’, 4’-dihydroxy-3’-methoxychalcone…………………… 18
Fig 1.22 Anti-HIV chalcone……………………………………… 18
Fig1.23 chalconelonchocarpin…………………………….... 18
Fig.1.24 glycolisation positions…………………………………… 19
Fig 1.26 Column chromatography………………………………… 22

Fig.1.27 Mass spectrometer…………………………………… 24
Fig1.28 NMR spectrophotometer2D 1H-1H COSY……………. 27
Fig1.29 *Sonchus oleraceus* picture……………………………… 36
Fig 3.1 IR spectrum of lignan glycoside………………………… 72
Fig 3.2 Proton NMR chemical shifts of lignan glycoside 74
Fig 3.3 2D NMR of lignan compound…………………………… 76
Fig 3.4 HSQC of lignan compound ……………………………. 76
Fig 3.5 Structure of lignan glycoside... 78
Fig 3.6 Mass spectrum of lignan glycoside………………………. 79
Fig 3.7 Structure of lignan fragment……………………………… 79
Fig 3.8Structure of lignan fragment………………………………… 80
Fig. 3.9 IR spectrum of chalcone glycoside……………………….. 82
Fig. 3.8 Proton NMR chemical shifts of chalcone glycoside………… 83
Fig 3.9 2D NMR of chalcone glycoside... 85
Fig. 3.10 HSQC carbon/proton coupling of chalcone glycoside........ 85
Fig.3.11 Strucrure of chalcone glycoside....................................... 88
Fig. 3.12 Mass spectrum for chalcone glycoside……………………... 89
Fig 3.13 Fragment of chalcone of mass *m/e 115*.............................. 89
Fig 3.14 Chalcone mass fragment.. 89
Fig 3.15 Chalcone mass fragment………………………………… 90
Fig. 3.16 IR spectrum of flavone …………………………………… 92
Fig. 3.17 Proton NMR chemical shifts for flavones…………………. 93
Fig. 3.18 2D proton/proton coupling for flavone glycoside......... 95
Fig. 3.19 HSQC carbon/ proton coupling of flavone glycoside....... 95
Fig.3.20 Structure of flavone glycoside.. 98
Fig 3.21 Mass spectrum of flavone glycoside…………………….. 99

Fig 3.22 Flavone mass fragment 103……………………………… 99
Fig 3.23 Flavone mass fragment 171……………………………….. 100
Fig 3.25 Large mass fragment of flavones………………………… 100
Fig. 3.26 Fragment of the sugar molecule linked to the aromatic structures of flavones……………………………………………. 100
Fig. 3.27 IR spectrum of tannic acid……………………………. 102
Fig. 3.28 Proton NMR chemical shifts of tannic acid………………. 105
Fig.3.29 2D proton NMR chemical shifts of tannic acid…………….. 105
Fig. 3.30 HSQC chemical shifts of tannic acid………………………. 106
Fig.3.31 Structure of tannic acid glycoside..................................... 108
Fig. 3.32 Mass spectrum of tannic acid……………………………… 109
Fig 3.33 First mass fragment of tannic acid………………………… 109
Fig 3.34 Mass fragment for sugar of tannin ………………………. 109
Fig 3.35 Calculated fragment for tannic acid………………………. 110
Fig. 3.36 Fragment of tannic acid. *m/e 292*…………………………… 110
Fig. 3.37 Fourth calculated fragment of tannic acid glycoside. *m/e 349*………………………………………………………………. 111
Fig. 3.38 Mass of tannic acid fragment *m/e* 667-3…………………. 111
Fig. A.1 ^{13}C NMR chemical shifts of lignan glycoside…………….. 114
Fig. A.2 DEPT 45 of lignan glycoside………………………….. 116
Fig. A.3 DEPT 90 of lignan glycoside………………………….. 117
Fig. A.4 DEPT 135 for Lignan glycoside………………………. 118
Fig. B.1 ^{13}C NMR chemical shifts of chalcone glycoside 119
Fig. B.2 DEPT 45 chemical shifts of chalcone glycoside…………… 122
Fig. B.3 DEPT 90 chemical shifts of chalcone glycoside……………
Fig. B.4 DEPT 135 chemical shifts of chalcone glycoside…………. 124

Fig. C.1 ^{13}C chemical shifts of flavone glycoside…………………… 125
Fig. C.2 DEPT 45 chemical shifts of flavone glycoside……………… 128
Fig. C.3 DEPT 135 chemical shifts of flavone glycoside…………… 129

List of Tables

Table 2.1 Characteristic IR Absorption Frequencies of Organic groups………………….. 49

Table 2.2 IR Absorption Frequencies of carbonyl compounds…………………………… 50

Table 2.3 NMR chemical shifts chart …………………………………… 53

Table 3.1 Methanol extract of four different plants…………… 60

Table 3.2 RSA against DPPH for methanol extract of four plants 61

Table 3.3: RSA of ethyl acetate extract for two plants…… 61

Table 3.4 Qualitative results of phytochemicals in ethyl acetate extract 62

Table 3.5 Results of column chromatography fractions……………. 63

Table 3.6 Sub column for fractions (30 +31………………………. 66

Table 3.7 Fraction 23 with their weight , RSA and TLC solvent system 67

Table 3.8 s Fraction 24 with their weight , RSA and TLC solvent system 67

Table 3.9 Fraction 25 with their weight , RSA and TLC solvent system 68

Table 3.10 Fraction 29 with their weight , RSA and TLC solvent system 68

Table 3.11 Test of purity with three solvent systems……………. 69

Table 3.12 IR functional group identification of lignan glycoside 72

Table 3.13 Shows NMR spectroscopic data of the lignan glycoside.. 74

Table 3.14 Results of 2D NMR (cosy) and HSQC NMR of lignan glycoside . 80

Table 3.15 IR functional group identification of Chalcone glycoside.. 82

Table 3.16 Spectroscopic identification of NMR for Chalcone glycoside 84

Table 3.17: 2D NMR proton/ proton coupling and HSQC carbon/proton relation for chalcone glycoside... 86

Table 3.18:IR Functional group identification of the flavones glycoside compound……………………………………………………….. 92

Table 3.19: Spectroscopic identification of NMR for Flavone glycoside 94
Table 3. 20: 2D NMR proton/ proton coupling and HSQC chemical shifts for flavone glycoside.. 96
Table 3. 21: 2D NMR proton/ proton coupling and HSQC chemical shifts for flavone glycoside... 96
Table 3.22: IR frequencies of tannic acid glycoside…………………… 103
Table 3.23: Spectroscopic NMR results of tannic acid………………….. 106

Chapter One
Introduction

1. INTRODUCTION

Plants are variable sources of natural products. Other sources of natural products include marine organisms, micro-organisms and arthropods. Other natural products have been used as food flavour ,oils, perfumes and drugs. They are produced through well-established biochemical pathways (Taylor,2000). Lignins, tannins, terpenes,, steroids, alkaloids and flavonoids are the most common examples of natural products. Some of these compounds show the ability to act as antioxidants. (Maymoona,2011). The harmful action of the free radicals can, however, be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers neurodegenerative diseases. Therefore, plant derived antioxidants are now receiving special attention (Maestri *et al*,2006).Oxidative damage due to oxidative stress is involved in the development and progression of many diseases by damaging many components of cells, including lipids, proteins, and nucleic acids. Human cells have various defensive mechanisms in its metabolic activities. Failure of this defense responsible of reversible damage eventually causes cell death. . As oxidative stress is taking part in development of many diseases, the use of antioxidants is intensively studied in medicinal chemistry. Medicinal chemists use the results of the research in the antioxidant drug discovery process, as the industry struggles to find a successful paradigm to fulfill the high expectations for delivering new drugs. In this issue recent development of antioxidant compounds in view of medicinal chemistry is reported. The research related to designing and synthesizing new antioxidant compounds, followed by evaluation of biological testing results and generation of new hypothesis as the basis for further compound design and synthesis is the main focus of the issue (Sibel Susan and Luciano Saso, 2013).

1.1Natural products:

Natural products, well known for their unique chemical diversity and bioactivity, have remarkable developments in the areas of isolation science, spectroscopic techniques, microplate-based ultrasensitive in vitro assays and high-throughput screening (HTS) technologies. The pre-isolation analyses of crude extracts or fractions from different natural matrices, isolation, online detection and duplication of natural products, studies on chemotaxonomy and biosynthesis, chemical finger-printing, quality control of herbal products, and metabolic studies have now become much easier than ever before because of the availability of a number of modern sophisticated hyphenated techniques, e.g., GC-MS, LC-PDA, LC-MS, LC-FTIR, , LC-NMR (Sarker and Nahar ,2012).

1.1.1 Secondary metabolites:

These are produced from universally present precursors (most often acetyl-CoA, amino acids or shikimate) by specific enzymes that probably arose by the duplication and divergence of genes originally coding for primary metabolism. Most secondary metabolites are restricted to single major taxa on the universal phylogenetic tree and so probably originated only once. But different secondary metabolic pathways have originated from different ancestral enzymes at radically different times in evolution. Secondary metabolites are most abundantly produced by microorganisms in crowded habitats and by plants, fungi and sessile animals like sponges, where chemical defense and attack rather than physical escape or fighting are at a premium. The first secondary metabolites were probably antibiotics produced in microbial mats over 3500 million years ago. These first ecosystems probably consisted entirely of eubacteria , archaebacteria and eukaryotes arose much later. (Cavalier Smith,1992).

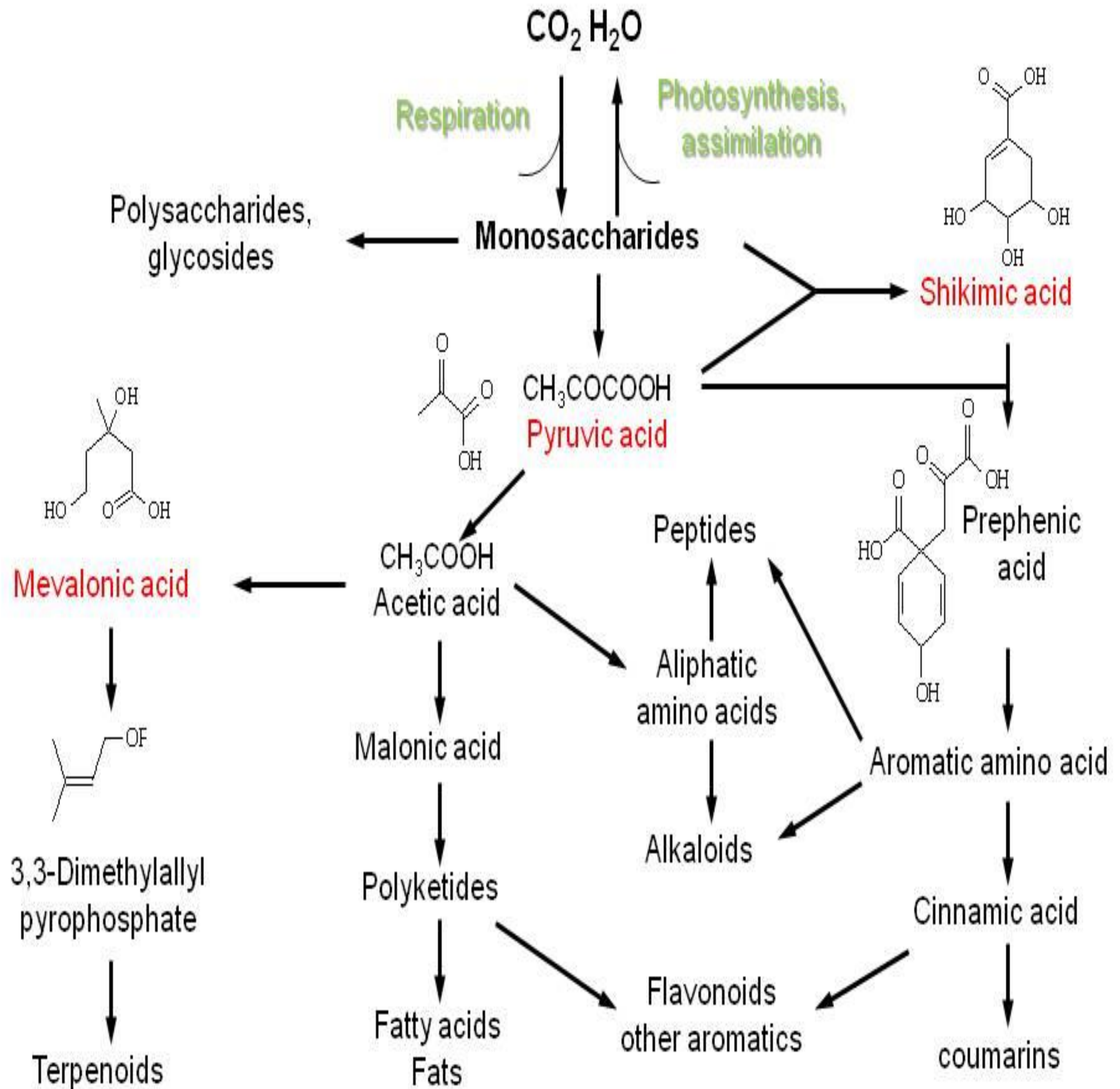


Fig.1.1 (Origins of secondary metabolism.)

1.1.2 Reactive Oxygen Species :(ROS)

A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids. These cytotoxic properties of ROS explain the evolution of complex arrays of nonenzymatic and enzymatic detoxification mechanisms. ROS are continuously produced as byproducts of various metabolic pathways localized in different cellular compartments . Under physiological steady state conditions these molecules are scavenged by different antioxidative defense components that are often confined to particular compartments. Nonenzymatic antioxidants include the major cellular redox buffers ascorbate and glutathione ,as well as tocopherol, flavonoids, alkaloids, and carotenoids.(Klaus and Heribert.2004).

Reactive oxygen species (ROS) is a collective term used for oxygen derived free radicals (superoxide, hydroxyl radical, nitric oxide) and non-radical oxygen derivatives of high reactivity (singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite. (Susinijan ,2015).An imbalance between formation and removal of free radicals can lead to a pathological condition called as oxidative stress . Oxidative stress resulting from an imbalanced ratio between ROS production and detoxification may also disturb physiological signal transduction, lead to chain reactions in lipid layers, and damage DNA repair enzymes (Valko, 2007).

The term ROS is often used to include not only the radicals $OH^{\cdot}$, $RO_2^{\cdot}$, $NO^{\cdot}$ and $O_2^{\cdot-}$ but also the non-radicals HOCl, , $ONOO^-$, O_3 , and H_2O_2. .They are unstable , react with other molecules to achieve stability. ROS can be synthesized by enzyme systems – phagocytic cells, neutrophils and macrophage (NADPH oxidase, myeloperoxidases). Exposure to ionizing radiation, smoking, herbicides, pesticides, fried foods, etc. increases their effect on the body.ROS contribute to cellular aging,mutagenesis,carcinogenesis and coronary heart disease possibly through destabilization of membranes. DNA damage and oxidation of low-density

lipoprotein (LDL). Reactive nitrogen species (RNS) also appear to contribute to the pathology of cardiovascular diseases. Nitrotyrosine, a product of protein nitration by RNS, is present in human atherosclerotic lesions The significance of antioxidants in the diet and their putative value in the intervention and prophylaxis of cardiovascular diseases has been of considerable interest in recent years (Kelly *et a*l. 2002).

1.1.3 Antioxidants:

An antioxidant is a stable molecule and removes potentially damaging oxidizing agents in a living organism and neutralizes it. There are some antioxidants in biological systems 1.Enzymes e.g. catalase, 2. Large molecules as albumin, 3. Small molecules like ascorbic acid, carotenoids and poly-phenols, 4.Hormones e.g. melatonin and estrogen. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property (Halliwell,1995) Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and B-carotene. (Levine and Darwara,1991) .The action of antioxidants depends on two parameters: bond energies and reduction potentials. (Maymoona Salim, 2011).

Polyphenols are considered to be the most effective antioxidants, they can also intensify the activity of other antioxidants soluble in lipids vitamins, and also vitamin C (Elbeita *et al*, 2008) .

1.1.3.1 Levels of antioxidant action

The antioxidants acting in the defense systems act at different levels. The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. Although the precise mechanism and site of radical formation in

vivo are not well elucidated yet, the metal-induced decompositions of hydro-peroxides and hydrogen peroxide must be one of the important sources. To suppress such reactions, some antioxidants reduce hydro-peroxides and hydrogen peroxide beforehand to alcohols and water, respectively, without generation of free radicals and some proteins sequester metal ions.Glutathione peroxidase, glutathione-s-transferase, phospholipid hydro-peroxide glutathione peroxidase (PHGPX), and peroxidase are known to decompose lipid hydro-peroxides to corresponding alcohols. PHGPX (an enzyme acts as oxidoreductases and reducing peroxides into water) is unique in that it can reduce hydro-peroxides of phospholipids integrated into biomembranes. Glutathione peroxidase and catalase reduce hydrogen peroxide to water.

The second line of defense is the antioxidants that scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions. Various endogenous radical-scavenging antioxidants are known: some are hydrophilic and others are lipophilic. Vitamin C, uric acid, bilirubin, albumin, and thiols are hydrophilic, radical-scavenging antioxidants, while vitamin E and ubiquinol are lipophilic radical-scavenging antioxidants. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant.

The third line of defense is the repair and de novo antioxidants. The proteolytic enzymes, proteinases, proteases, and peptidases, present in the cytosol and in the mitochondria of mammalian cells, recognize, degrade, and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins (Hancock, *et al*,2001).

1.2 Phenolic Compounds

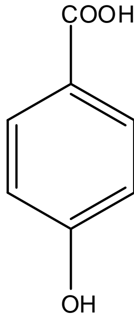
Phenolic compounds comprise a wide variety of molecules that have a polyphenol structure (i.e. several hydroxyl groups on aromatic rings), but also molecules with

one phenol ring, such as phenolic acids and phenolic alcohols. Polyphenols are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main groups of polyphenols are: flavonoids, phenolic acids, tannins (hydrolysable and condensed), stilbenes and lignans (Ioana *et al,* 2011) .

Polyphenols engage in reactions related to their core structure—standard phenolic reactions (e.g., ionization, underlying aromatic chemistry related to the presence of the phenolic hydroxyl,—as well as reactions related to their peripheral structures (e.g., ester linkages of tannic acid being susceptible to alkaline hydrolysis. More critically, per the definition, the polyphenols display behaviors more explicitly limited to the polyphenol class—e.g., formation of particular metal complexes and precipitation of proteins and particular amine-containing organics.(Amprital,2011).

1.2.1 Phenolic acids

Phenolic acids constitute about one-third of the dietary phenols,which may be present in plants in free and bound forms. Bound-phenolics may be linked to various plant components through ester, ether, or acetal bonds .The different forms of phenolic acids result in varying suitability to different extraction conditions and different susceptibilities to degradation .Phenolic acids consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have in common the C6–C1 structure. Hydroxycinnamic acids, on the other hand, are aromatic compounds with a three-carbon side chain (C6–C3), caffeic, ferulic, p-coumaric and sinapic acids being the most common representatives.Derivatives of phenolic acids occur when they bind with cellulose, lignin, sugars and proteins through ester bonds.(Aristidis,2012).



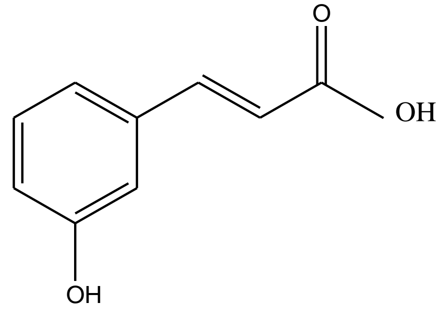


Fig.1. 2: p-hydroxy benzoic acid Fig.1.3 p-hydroxy coumaric acid

1.2.2 Tannins

Tannins, relatively high molecular compounds which constituting the third important group of phenolics, may be subdivided into hydrolysable and condensed tannins.Proanthocyanidins (condensed tannins) are polymeric flavonoids.

The most widely studied condensed tannins are based on flavan-3-ols,epicatechin and (+)-catechin. Hydrolysable tannins are derivatives of gallic acid (3,4,5 trihydroxyl benzoic acid). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively crosslinked to yield more complex hydrolysable tannins .The third subdivision, the phlorotannins consisting entirely of phloroglucinol, has been isolated from several genera of brown algae, but these are not significant in the human diet. Tannins have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents and biological antioxidants.

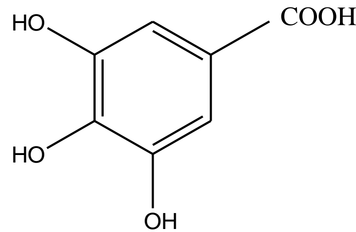


Fig.1. 4: gallic acid (hydrolysable tannin)

1.2.2.1 Bioactivities of Tannins:

Tannins are biological antioxidants, metal chelators and protein precipitators. Metal ion chelating can change the redox potential of a metal or prevent it from participating in redox reactions. Also many low molecular phenolics scavenge radicals as effectively as vitamins E and A .

1.2.3 Stilbenes and lignans

Low quantities of stilbenes are present in the human diet, and the main representative is resveratrol, that exists in both cisand trans isomeric forms, mostly in glycosylated forms. It is produced by plants in response to infection by pathogens or to a variety of stress conditions.It has been detected in more than 70 plant species,including grapes, berries and peanuts.

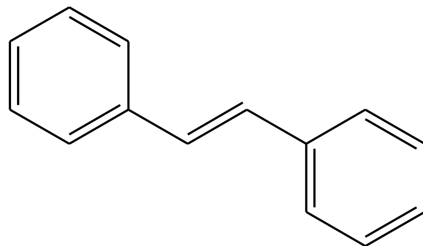


Fig. 1.5: Stelbene monomer

Lignans are produced by oxidative dimerisation of two phenylpropane units; they are mostly present in nature in the free form,while their glycoside derivatives are only a minor form. The interest in lignans and their synthetic derivatives is growing because of potential applications in cancer chemotherapy and various other pharmacological effects(.(Ioana *et al*.2011) .

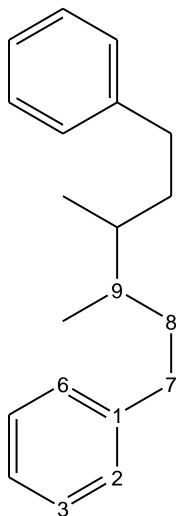


Fig. 1.6: Lignan basic structure

Group of dimeric phenylpropanoid where two C6-C3 are attached by its central carbon. These compounds, having different degrees of oxidation of the side chain or differences in the aromatic substitution, show several biological activities. (Roberta ,et al,2009)

1.2.3.1 Bioactivities of Lignans:

Recent researches work showed that lignans can be **:**

a) Antiviral (Charlton, 1998), (Yousefzadi et al., 2010).

b) Anticancer (Pan et al. 2009) , (Saleem et al.,2005).

c) Anti-inflammatory (Saleem et al., 2005)

d) Antimicrobial (Saleem et al., 2005)
e) Antioxidant (Fauré et al., 1990); (Pan et al., 2009);(Saleem et al., 2005)
f) Immunosuppressive (Saleem et al., 2005)
g) Hepatoprotective (Negi et al., 2008)
h) Osteoporosis prevention .(Habauzit and Horcajada, 2008)

Most of the known natural lignans are oxidized at C9 and C9´ and, based upon the way in which oxygen is incorporated into the skeleton and on the cyclization patterns, a wide range of lignans of very different structural types can be formed. Due to this fact, lignans are classified in eight subgroups. (Wilson et al, 2012)

1.2.3.2 Some lignan types with bioactivities:

Dibenzylbutanes. (Lee et al,2010) isolated a new lignin, saururin B, from the AcOEt extracts of Saururuschinensis rhizomes. Saururin B (1) showed dose-dependent inhibitory activities on HIV-1 protease with an IC50 value of 5.6 micro-mill.

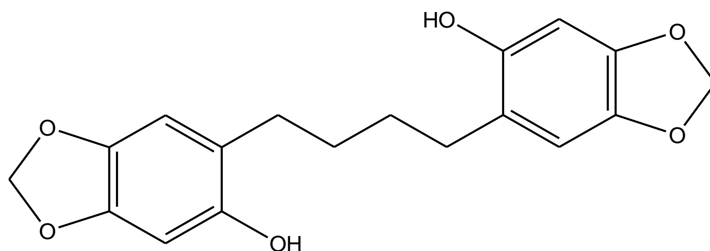


Fig.1.8: Saururin B

Bioactivity-directed fractionation of the extracts from the seeds of Trichosantheskirilowii led to the isolation of a new lignan, Oferuloylsecoisolariciresinol,which exhibited strong cytotoxic effects against human lung carcinoma.(Moon et al, 2008).

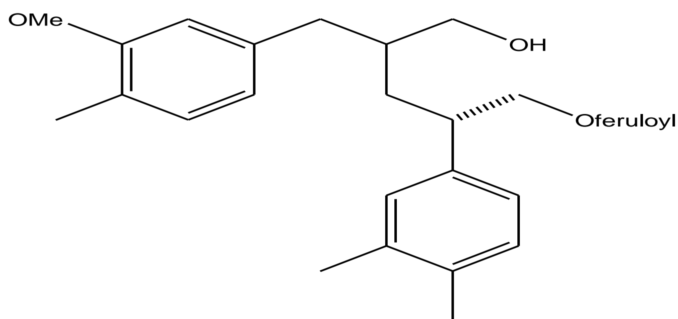
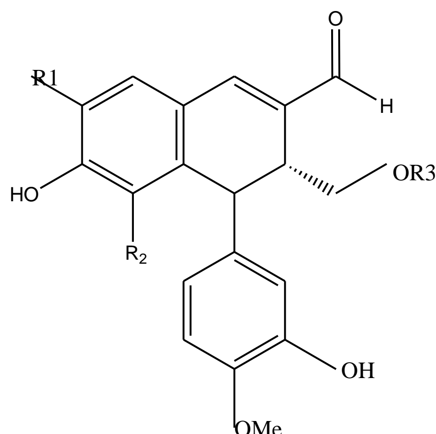


Fig 1.9.: Oferuloylsecoisolariciresinol

Phenyldihydronaphthalene-type lignanglucosides, vitecannasides (Aand B), were isolated from the fruit of Vitexcannabifolia. The scavenging effects of A and B on the stable free radical DPPH was evaluated, and they were found to exhibit stronger activities than that of l-cysteine. [Yamasaki, 2008)



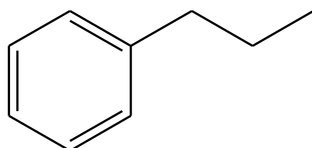
Vitecannasides Fig 1.10

	R1	R2	R3
VitecannasidesA	**H**	**MeO**	**Glc**
VitecannasidesB	**MEO**	**H**	**Glc**

1.2.4 Phenylpropanoids

Phenylpropanoids are naturally occurring phenolic compounds which have an aromatic ring to which a three-carbon side chain is attached. They are derived

biosynthetically from the aromatic protein amino acid phenylalanine and they may contain one or more C6--C3. Among the phenylpropanoids are included hydroxycoumarins, phenylpropenes and lignans. (Harborne,1998).

Fig1.11:  phenylpropanoid unit

1.2.5 Flavonoids

Flavonoids are low-molecular-mass and polyphenolic compounds with a widespread occurrence in many crops, herbs and medicinal plants.(Yongqian, et al.2011).

The term "flavonoid" is generally used to describe a broad collection of natural products that include a C6-C3-C6 carbon framework, or more specifically a phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes: the flavonoids (2-phenylbenzopyrans). Isoflavonoids (3-benzopyrans) and the neoflavonoids (4-benzopyrans). These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related.

Flavonoids and their conjugates form a very large group of natural products. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs. The chemical structures of this class of compounds are based on a C6-C3-C6 skeleton. They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns . The flavonoids may be modified by hydroxylation, methoxylation, or *O*-glycosylation of hydroxyl groups as well as *C*-glycosylation directly to carbon atom of the flavonoid

skeleton. In addition, alkyl groups (often prenyls) may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton of the flavonoid core. The last modification takes place most often in the case of isoflavonoids, where the B ring is condensed to the C-3 carbon atom of the skeleton. Flavonoid glycosides are frequently acylated with aliphatic or aromatic acid molecules. Condensed tannins create a special group of flavonoid compounds formed by polymeric compounds built of flavan-3-ol units (Erich Grotewold.2006).

Flavonoids are generally present in plants bound to sugar as glycosides and any flavonoid aglycone may occur. (Zong-Quan.et al ,2015). They can be further subdivided into six main subclasses, as a function of the type of heterocycle (the C ring) .

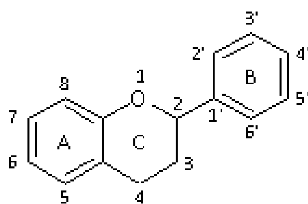


Fig. 1.12: Flavonoid Skeleton

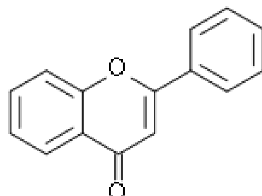
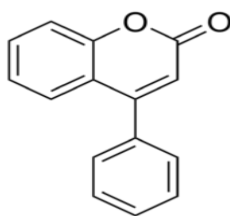


Fig 1.13 Molecular structure of the flavone backbone (2-phenyl-1,4-benzopyrone)

Fig.1.14: Neoflavonoid



Isoflavonoid

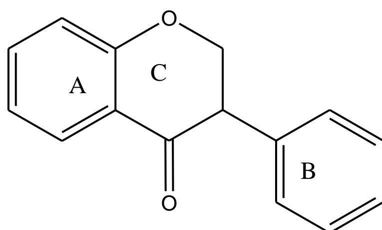


Fig.1.15: Attachment of ring B change to position -3-on C.

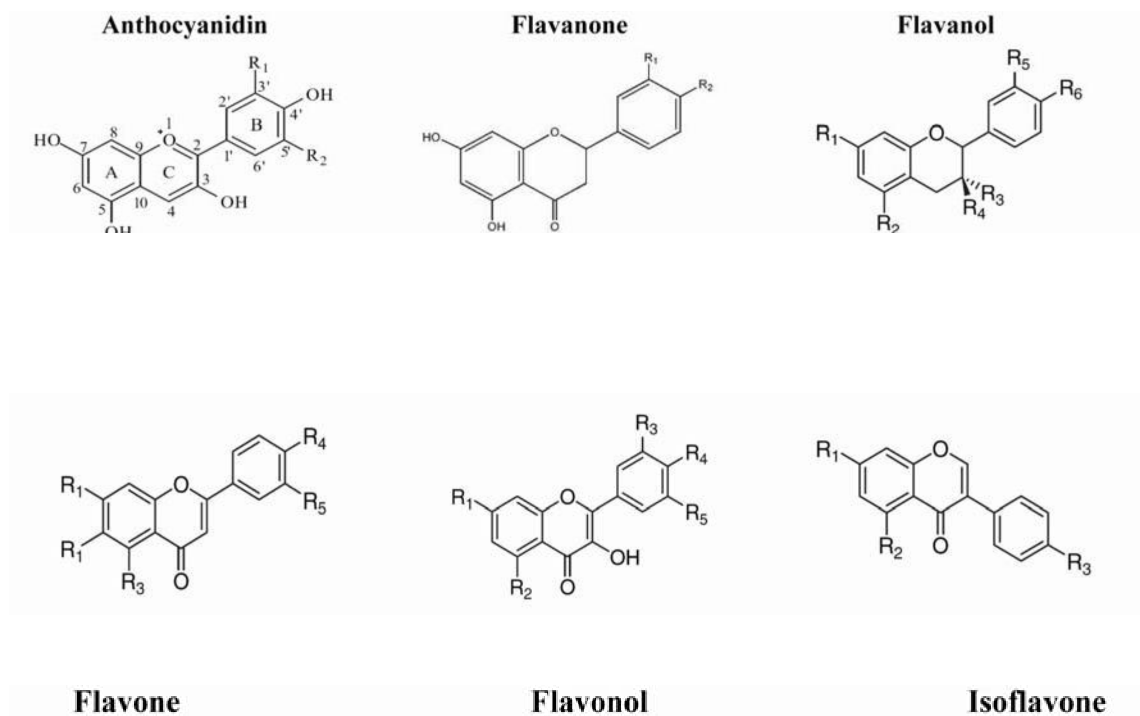
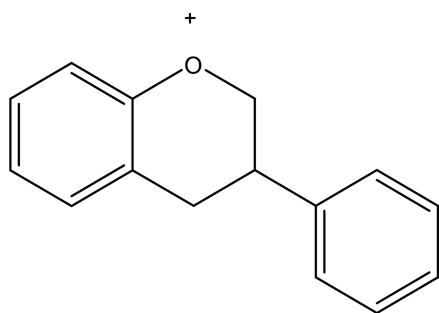


Fig1.16-many structures

These compounds, having different degrees of oxidation of the side chain or differences in the aromatic substitution, show several biological activities (Roberta, *et al* 2009)

Anthocyanins are water-soluble vacuolar pigments that may appear as red, purple, or blue depending on pH. The anthocyanidins are the basic structures of the anthocyanins.The anthocyanidins (or aglycons) consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring B. When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins.

Anthocyanidins/anthocyanins contains a flavylium ion (2-phenyl-1-benzopyranylium or 2-phenylchromenylium) as a core structure.



Anthocyanidins basic structure

Fig 1.17

1.2.6 Bioactivities of chalcones:

Chalcone is a generic term given to compounds bearing the 1, 3-diphenyl-2-propen-1-one framework and belong to the flavonoid family.(-Maayan ,et al. 2005). Chemically they are open-chain flavonoids in which the two aromatic rings are joined by a three carbon carbonyl system. Chalcones are abundantly present in nature starting from ferns to higher plants and a number of them are polyhydroxylated in the aryl rings.(Cushman and Nagarathnam, 1991)

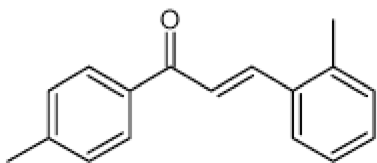


Fig.1.18 Chalcone

The presence of a reactive keto function in chalcones was found to undergo conjugate addition with a nucleophilic group like a thiol group in an essential protein, thus partly contributing for their antimicrobial activity.(Prasad *et al.*2007 Synthesized 3-[1-oxo-3-(2, 4, 5-trimethoxyphenyl)-2-propenyl]-2H-1-benzopyran-2-ones (Fig.1.19) showed significant antimicrobial activity against *B.subtilis, B.pumilis*and and *E.coli.*

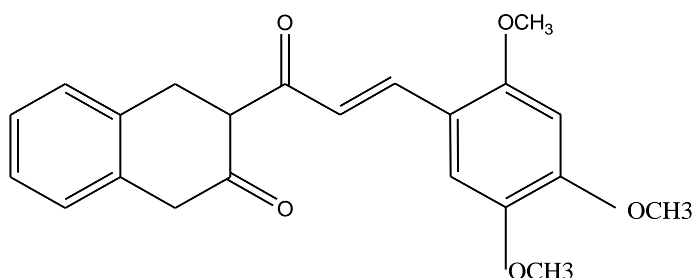


Fig.1.19 3-(1-oxo-3-(2,4, 5-trimethoxyphenyl)-2-prpenyl-2H-1-benzopyran-2-one.

(Dominguez *et al,*2005*)* synthesized chalcones(fig.1.20) with sulfonamide moiety possessing antimalarial activity.

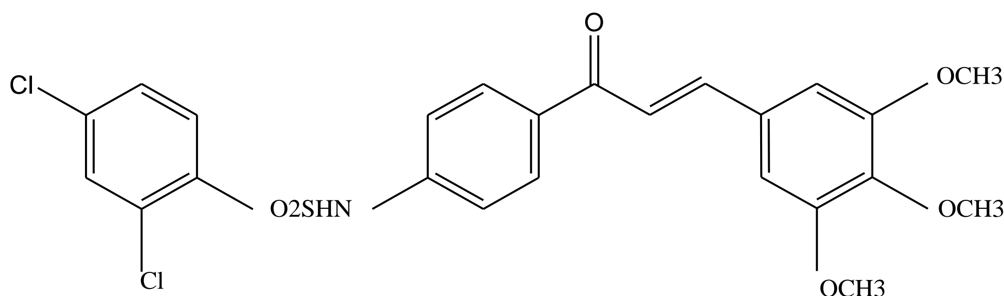


Fig.1.20

(Stevaz, *et al. 2004)* isolated a 2’, 4’-dihydroxy-3’-methoxychalcone(Fig.1.21**)** from the methanolic extract of *Zuccagniapunctata*which exhibited antifungal activity.

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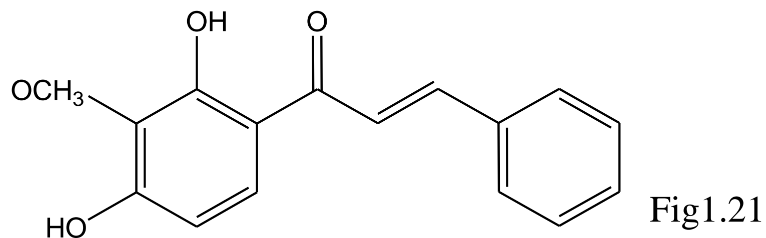
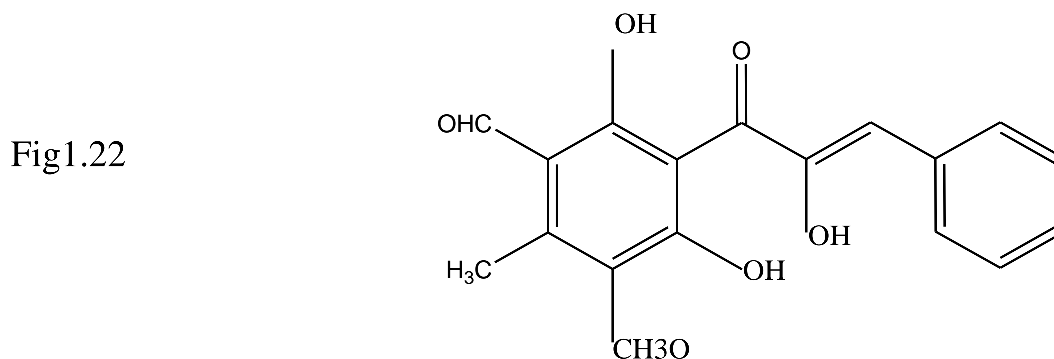


Fig1.21

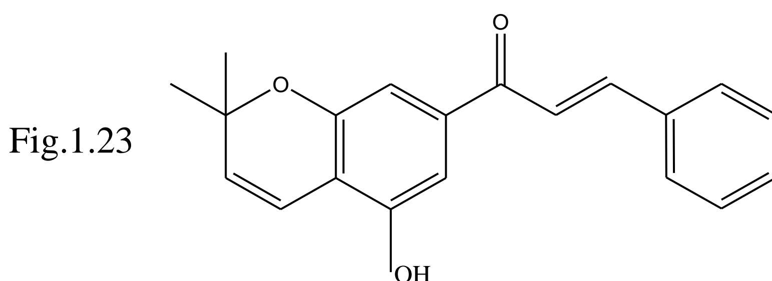
(Nakagawa and lee, 2006.) isolated a unique potent chalcone **(**Fig1.22**)** from genus *Desmos*showing anti-HIV activity.

Fig1.22



(Cunha et al,2003*)* isolated the chalconelonchocarpin **(**Fig1.23**)** from the roots of *Lonchocarpussericeus*which showed cytotoxic activity.

Fig.1.23



1.2.7 Chemical structure:

In plants, flavonoids may occur in various modified forms corresponding to additional hydroxylation, methylation and, most importantly, glycosylation. (Filip and Maya,2003)

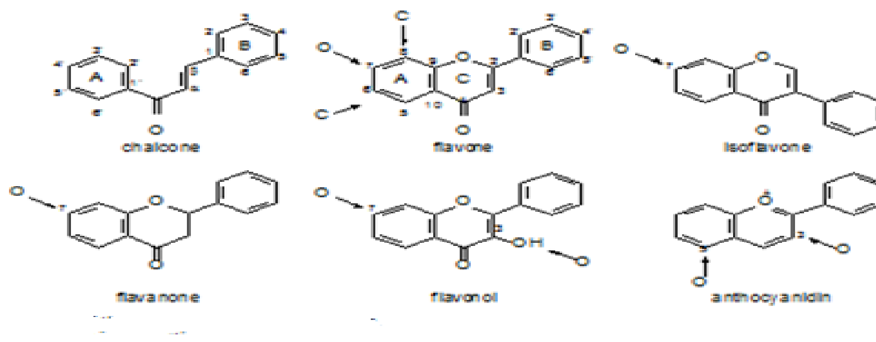


Fig.1.24

Common glycolysation positions are indicated with arrows.

1.2.7.1 Antioxidant capacity of phenolic compounds

The antioxidant capacity of phenolic compounds varies according to the number and position of the hydroxyl groups). Hydroxyl groups on the B-ring donate hydrogen and an electron to ROS radicals, stabilizing them and giving rise to a relatively stable flavonoid radical .Among structurally homologous flavones and flavanones, radical scavenging increases according to the total number of OH groups .The antioxidant activities of phenolic compounds are not only governed by their degree of hydroxylation; but ortho hydroxylation on the benzene ring also influences radical scavenging .

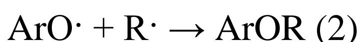
Additionally, the antioxidant activities of flavonoids are influenced by the position and structural properties of the sugar moiety. The antioxidant properties

of flavonoids decreases as the number of glycosidic moieties increases, and thus glycosylated flavonoids are lower in radical scavenging capacity than their corresponding aglycones. (Sundara Mudiyanselage,2014).

Phenolic compounds can modify the lipid packing order of cell membranes, which decreases the fluidity of the membranes sterically hindering the diffusion of ROS thus restricting peroxidation reactions in lipid membranes (Sundara et al, 2014)

Phenolic compounds (ArOH) act as free radical acceptors and chain breakers. They interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals (R): R + ArOH → RH +ArO· (1)

The phenoxy radical intermediates (PO·) are relatively stable due to resonance and therefore a new chain reaction is not easily initiated. Moreover, the phenoxy radical intermediates also act as terminators of propagation route by reacting with other free radicals:


ArO· + R· → ArOR (2)

Phenolic compounds possess ideal structure for chemistry of free radical scavenging activities because they have: (1) phenolic hydroxyl groups that are prone to donate a hydrogen atom or an electron to a free radical; (2) extended conjugated aromatic system to delocalize an unpaired electron. Several relationships between structure and reduction potential have been established as follows. (Jin Dai ,2010). For flavonoids, the major factors that determine the radical-scavenging capability are:(i) *the ortho-dihydroxy structure on the B ring*, which has the best electron-donating properties and confers higher stability to the radical form and participates in electron delocalization.(ii) *the 2,3-double bond with a 4-oxo function in the C ring*, which is responsible for electron delocalization from the B ring.(iii) *the 3- and 5-hydroxyl groups with the 4-oxo function in A and C rings*, which are essential for maximum radical scavenging potential.(iv) *the 3-hydroxyl group is important for antioxidant activity.* The 3-

glycosylation reduces their activity when compared with corresponding aglycones. (Agarwal *et al,*2010.)

1.3 Methods of spectroscopic separation:(Isolation of active compounds)

In earlier times, thin-layer chromatography (TLC), polyamide chromatography, and paper electrophoresis were the major separation techniques for phenolic compounds. Of these methods, TLC is still the workhorse of flavonoid analysis. It is used as a rapid, simple, and versatile method for following polyphenolic compounds in plant extracts and in fractionation work. However, the majority of published work now refers to qualitative and quantitative applications of high performance liquid chromatography (HPLC) for analysis. Flavonoids can be separated, quantified, and identified in one operation by coupling HPLC to ultraviolet (UV), mass, or nuclear magnetic resonance (NMR) detectors.
The bulk of extractions of flavonoid-containing material are performed by simple direct solvent extraction. (Andersen, 2006.)

1.3.1 Chromatography

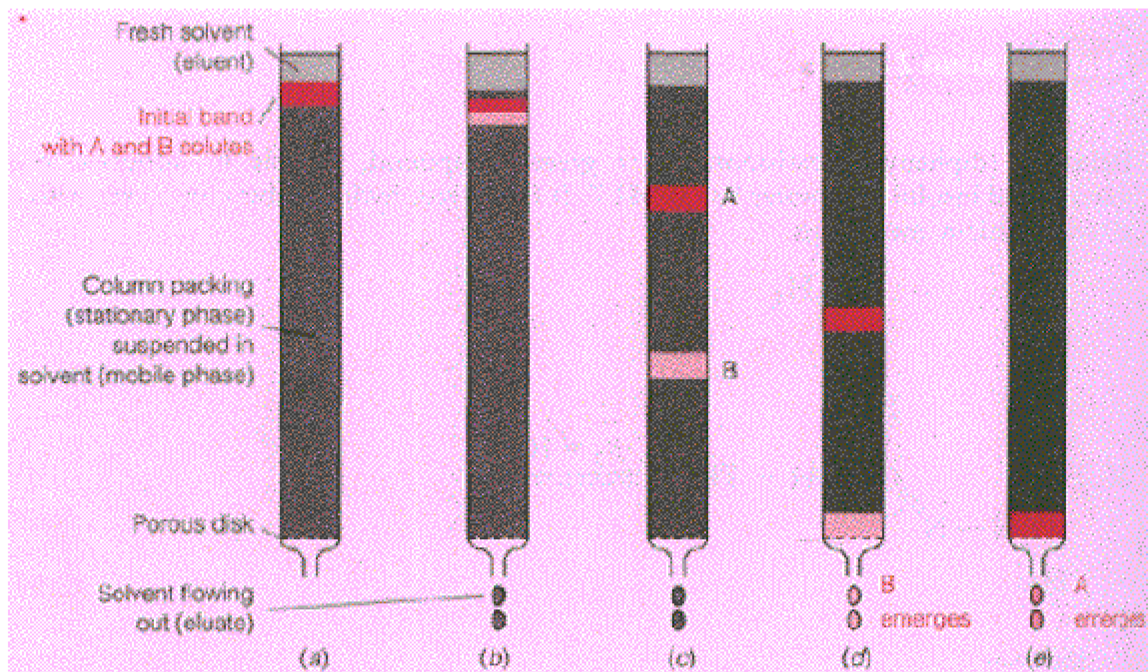
The use of a stationary phase and a mobile phase:

Components of a mixture are carried through the stationary phase by the flow of a gaseous or liquid mobile phase, separations being based on coefficient partition differences in migration rates among the sample components. Chromatographic methods are of two types:In column chromatography, the stationary phase is held in a narrow tube and the mobile phase is forced through the tube under pressure or by gravity.

In *planar chromatography,* the stationary phase *is* supported on a flat plate or in the pores of a paper. Here the mobile phase moves through the stationary phase by capillary action or under the influence of gravity.

1.3.1.1 Column Chromatography

Column chromatography is a separation technique in which the stationary bed is within a tube (Jasper et al,1996). Fig1.26



1.3.2 Methods of identification and structure elucidation

Informative data on plant substances are based on applying spectral characteristics: these include ultraviolet (UV), infra-red (lR), nuclear magnetic resonance (NMR) and mass spectral (MS) measurements and other techniques.

1.3.2.1 Infrared Spectroscopy

IR spectra may be measured on plant substances in an automatic recording IR spectrophotometer either in solution (in chloroform or carbon- tetrachloride (1-5 %, as oil).

The range of measurement is from 4000 to 667 cm-1 (or 2·5 to *15p.)* and the spectrum takes about three minutes to be recorded. The fact that many functional groups can be identified by their characteristic vibration frequencies makes the IR

spectrum the simplest and often the most reliable method of assigning a compound to its class.

Principles of infra red spectroscopy: Spectroscopy is an instrumentally aided interaction between the sample being analyzed and any part of the electromagnetic spectrum. The energy from infra red radiation causes vibrations of electrons in molecules. Energy of a molecule = Electronic energy +Vibrational and Rotational energy.

IR spectroscopy is concerned with vibrations of molecules.Molecules are made of atoms linked by chemical bonds.When IR radiation is applied it causes molecules to vibrate.Different functional groups absorb at charachteristic frequencies of infra-red radiation.There are two types of molecular vibrations.

a) Stretching vibrations which occur 40000-12500cm^{-1}.
b) Bending vibrations occur at lower energy 1400-666 cm^{-1}

1.3.2.2 Mass Spectroscopy

The value of the technique is that it requires only microgram amounts of material, that it can provide an accurate molecular weight and that it may yield a complex fragmentation pattern which is often characteristic of (and may identify) that particular compound. MS, in essence, consists of degrading trace amounts of an organic compound and recording the fragmentation pattern according to mass, at different mass units . The resulting positively charged ions are accelerated in a magnetic field which disperses and permits relative abundance measurements of ions of given mass-to-charge ratio. The resulting record of ion abundance versus mass constitutes the mass spectral graph, which thus consists of a series of lines of varying intensity at different mass. units .

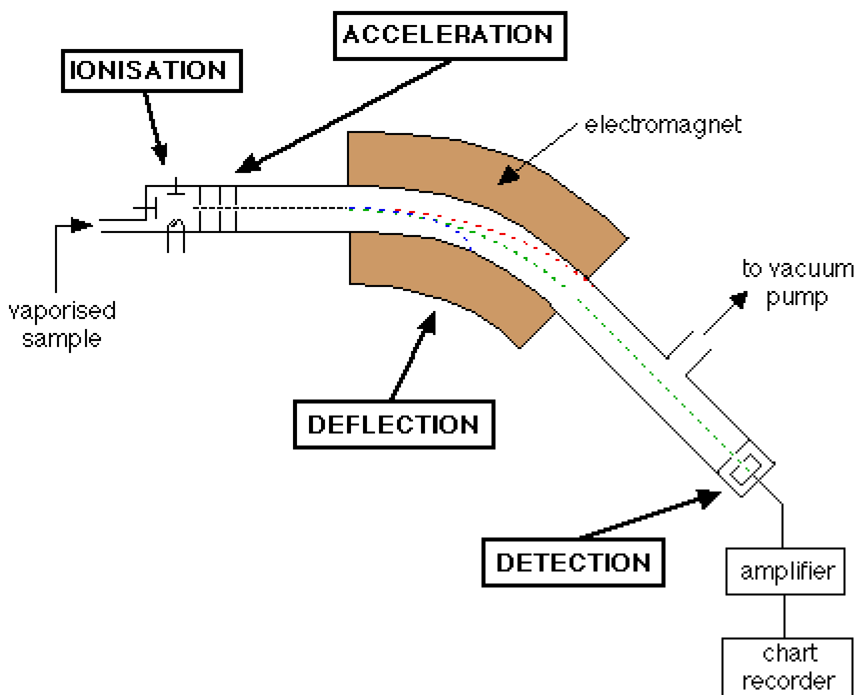


Fig.1.27 Mass spectrometer

Principles of Mass Spectrometry*:*

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample intogaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-

charge ratio (m/z), and then records the relative abundance of each ion type.

The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

1.3.2.2.1 Liquid Chromatography/Mass Spectroscopy

Liquid chromatography coupled to mass spectrometry (LC/MS) represents a very powerful tool for the analysis of natural products. The mass spectrometer is a universal detector that can achieve very high sensitivity and provide information on the molecular mass and on structural features. With regard to structure characterization of flavonoids, information can be obtained on (1) the aglycone moiety, (2) the types of carbohydrates (mono-, di-, tri- or tetrasaccharides and hexoses, deoxyhexoses or pentoses) or other substituents present, (3) the stereochemical assignment of terminal monosaccharide units, (4) the sequence of the glycan part, (5) interglycosidic linkages and (6) attachment points of the substituents to the aglycone). (Philip Cuycken, 2003)

1.3.3 Nuclear Magnetic Resonance Spectroscopy:

NMR spectroscopy essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as,-CH3, -CHO,-NH_2,-CHOH-,etc.) and the NMR spectrum provides a record of the number of hydrogen atoms in these different situations. It cannot, however, give any direct information on the nature of the carbon skeleton of the molecule; this must be obtained in the first instance by application of other spectral techniques. In practice, the sample of the substance is placed in solution, in an inert solvent, between the poles of a powerful magnet and the protons undergo different chemical shifts according to their molecular environments within the molecule. These are measured in the NMR apparatus relative to a standard, usually tetramethylsilane (TMS), which is an inert compound which can be added to the sample solution without the likelihood of chemical reaction occurring. The solvent for NMR measurements has to be inert and without protons.

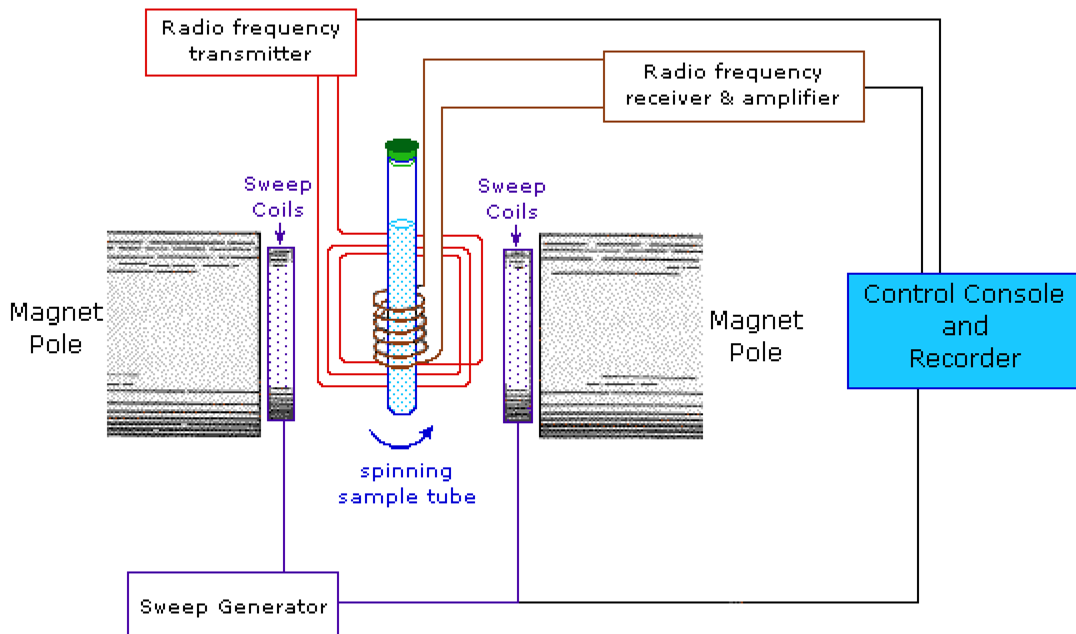


Fig1.28 NMR spectrophotometer2D 1H-1H COSY

The COSY experiment (CorrelationSpectroscopY) provides a means of identifying mutually coupled protons and is the most widely used 2D experiment. It finds use when the homonuclear decoupling experiment is unsuitable, for example in complex spectra, where selective decoupling is not possible because of resonance overlap. The COSY experiment is a very efficient way of establishing connectivities when a large number of coupling networks need to be identified, as it maps all correlations with a single experiment and is now more frequently used than homonuclear decoupling.

^{13}C chemical shifts follow the same principles as those of ^{1}H, although the typical range of chemical shifts is much larger than for ^{1}H (by a factor of about 20). The chemical shift reference standard for ^{13}C is the carbons in tetramethylsilane (TMS), whose chemical shift is considered to be 0.0 ppm.(Silverstein,1991)

1D ^{13}C DEPT .DEPT is "Distortionless Enhancement by Polarisation Transfer" and is used as a means of enhancing signal intensity and for editing spectra. the broadband proton decoupling removes multiplicity in carbon resonances, but the

DEPT sequence allows one to establish the nature of the carbon atom whilst still acquiring broadband decoupled spectra, by making use of changes in signal intensities under differing experimental conditions. Three DEPT spectra are required for a full analysis and are termed DEPT-45, DEPT-90 and DEPT- 135 (the number indicates the flip angle of the editing proton pulse in the sequence) . Non-proton-bearing carbons are not seen in DEPT spectra because the technique relies on polarisation transfer, that is, in this case, the transfer of proton magnetisation onto the *directly bound* carbon .
HSQC (heteronuclear single quantum correlation). Plots proton ^{1}H nmr on x-axis and ^{13}C nmr on y-axis.It utilizes one bond coupling between C and H.

Principles of Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance is defined as a condition when the frequency of the rotating magnetic field becomes equal to the frequency of the processing nucleus. The principle of nuclear magnetic resonance is based on the spins of atomic nuclei. The magnetic measurements depend upon the spin of unpaired electron whereas nuclear magnetic resonance measures magnetic effect caused by the spin of protons and neutrons. Both these nucleons have intrinsic angular moment or spins and hence act as elementary magnet.

The existence of nuclear magnetism was revealed in the hyper fine structure of spectral lines. If the nucleus with a certain magnetic moment is placed in the magnetic field, we can observe the phenomenon of space quantization and for each allowed direction there will be a slightly different energy level.Atoms like O^{16} and C_{12} which have even number of protons and neutrons have no magnetic moment and hence refuse to give resonance signals. While atoms such as P^{21}, F^{19}, which have odd number of protons and even numbers of neutrons, if any, generate nuclear magnetic moments and “hence give resonance signals.

Experimental Parameter (Chemical shift):

The most important molecular parameter determined by NMR is the chemical shift. The chemical shift is defined as a measure of the resonance frequency of the nuclei in a given chemical environment.

Interpretation of NMR Spectrum:

The number spectrum gives several kinds of information:

(1) The number of signals (peaks) tells us how many kinds of protons (protons with different chemical environments) are present in a molecule.

(2) The position (chemical shift) of the signal informs about the bonding environment of each proton.

(3) The area under each signal tells us how many protons of each kind are in the molecule.

(4) All hydrogens with identical environments in a molecule have same chemical shift, e.g., (a) all the three protons of a methyl CH_3; (b) the protons of a methylene — CH_2; (c) one identical.

(5) Protons on heteroatoms (H—S, H —N, H—O etc.) show highly variable chemical shifts and sometimes broad peaks.

(6) Hydrogen on different carbons yields the same absorptional signal if they are structurally indistinguishable.

(7) Sometimes a proton exhibits an absorption signal which is split into several peaks because of coupling with its neighbouring protons. In such cases a coupling constant J is calculated.

(8) The number of peaks (N) into which a proton signal is split equals one more than the number of vicinal protons (n) (number of equivalent neighbours causing splitting):

$N = n + 1$

N = 2 (one vicinal H) = doublet (d)

N= 3 (two vicinal H's) = triplet (t)

N= 4 (three vicinal H's) = quartet (q).

Principles of 13C NMR spectroscopy:

^{12}C nucleus is not magnetically active, but the ^{13}C nucleus has a spin quantum no. of ½ so ^{13}C exhibit NMR Phenomena where as C^{12} does not Natural Abundance of ^{13}C is only 1.08% & C^{12} – 98.9%. ^{13}C sensitivity is only 1.1% that of ^{1}H. The overall sensitivity of ^{13}C absorption is about 1/5700. Because of this low sensitivity of ^{13}C , gives rise to extremely weak signals and in 13C-NMR require very large sample and large time. ^{13}C chemical shift Range from o to about 250ppm.As In H-NMR, ^{13}C TMS (tetra- methyl silane) is also used as reference standard. Normal ^{13}C spectra are broadband, proton decoupled (technique of cancelling C-H effect) so the peaks show as single lines . Number of peaks indicate number and types of C present.

DEPT-NMR (Distortionless enhancement by polarization transfer):

DEPT-NMR also shows the determination of number of hydrogens attached to each carbon. In this technique three spectra are obtained, one is a normal broadband decoupled spectrum.The second spectrum (DEPT-90) is obtained under special condition in which only carbons bonded to single hydrogen appear. The third spectrum (DEPT-135) is obtained under condition in which CH and CH_3 appear as normal signals, but CH_2 appear as negative signal. There are no peaks for quaternary carbons. (Nikunjavora*, et al*. 2012)

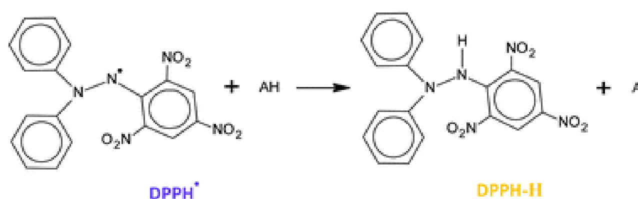
1.4 Radical Scavenging Assays :

1.4.1 DPPH ASSAY

The assay is based on the measurement of the scavenging capacity of antioxidants towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. (Contreras and

Srong ,1982) .DPPH is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule ,so that the molecules do not dimerise, like most other free radicals. The de-localisation also gives rise to the deep violet colour, with an absorption in ethanol solution at around 520 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet colour. Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

Where ZH is the reduced form and A• is free radical produced in the first step. The latter radical will then undergo further reactions which control the overall stoichiometry The reaction (1) is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the auto-oxidation of a lipid or other unsaturated substance; the DPPH molecule Z• is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH. DPPH can accept an electron or hydrogen radical to become a stable, diamagnetic molecule, it can be oxidized only with difficulty, and then irreversibly.(Kedare and Singh,2011).



1.1.10. *Sonchus oleraceus* species:

*Sonchusoleraceus*L. was named by Carolus Linnaeus in 1753 in his "Species Plantarum." "Sonchus" is the Greek name for sow thistle and means "hollow." This is a reference to the hollow stems. The epithet *oleraceus* means "kitchen

vegetable." Sonchus has the questionable distinction of being considered one of the world's worst weeds, a pest in more than 55 countries. (Florence .et al.2011).

1.5.1 Antioxidant activities of *Sonchus oleraceus*

The genus *Sonchus* contains more than 50 species and a study of six wild *Sonchus* species showed that *S.oleraceus* possessed the highest in-vitro antioxidant activity. *S. oleraceus*leaf extracts exhibited four times the antioxidant activity of blueberry extracts, a fruit known for its high antioxidant activity. Key antioxidants identified in *S. oleraceus*leaf extracts were caftaric acid, chlorogenic acid and chicoric acid.(Zong-,et al.2012).

Puha (*Sonchus oleraceus L.*) is a rich source of polyphenols, and exhibits strong antioxidant activity as measured by the 2,2-diphenylpicrylhydrazyl (DPPH) assay. However, the potential of puha to protect against degenerative diseases requires that low molecular weight antioxidants (LMWA) are absorbed and active in human cells. The cellular antioxidant activity (CAA) assay was used to investigate the antioxidant. Methylene blue staining of HepG2 cells indicated that puha extracts were not cytotoxic at concentrations below 100mg DW/mL. The data indicate the potential of puha as a nutraceutical supplement for human health.(Arlene,et al.2011).

Several bioactive compounds have been identified in the leaves of *Sonchus oleraceus*, including alkaloids, flavonoids, tannins, terpenes, steroids and phenols *S. oleraceus* presents toxicity against *Artemia salina* ,findings suggest that *S. oleraceus* whole plant possess both antidiabetic and antioxidant properties, and therefore could be used as starting point for the development of herbal medicines and/or source of new drug molecules against diabetes.

S. oleraceus extracts protect cells against H_2O_2-induced senescence by mediating. Cells were treated with *S. oleraceus* extracts before or after H_2O_2 stress . *S. oleraceus* extracts showed higher cellular antioxidant activity than chlorogenic

acid in WI-38 cells. *S. oleraceus* extracts suppressed H_2O_2 stress-induced premature senescence in a concentration-dependent manner.

The antioxidant activity of *S. oleraceus* extracts was concentration dependent and its IC_{50} values ranged from 47.1 to 210.5 μg/ml and IC_{50} of 70% MeOH, boiling water and 70% EtOH extracts were 47.1, 52.7 and 56.5 μg/ml, respectively. 70% MeOH extract of *S. oleraceus* contained the greatest amount of both phenolic and flavonoid contents. (JieYin,et al,2007)

Eight compounds, from ethyl acetate layer including 2 flavones, luteolin (1) and apigenin (2), 2 flavonols, kaempferol (3) and quercetin (4), and 4 flavonoid glucosides, luteolin-7-O-β-D-glucoside (5), apigetrin (6), astragalin (7), and isoquercitrin (8), isolated from the whole herb of Sonchus oleraceus L. This was the first time to report compounds 3, 4, 6, 7 and 8 from the *Sonchus oleraceus L.* The antioxidant activities of the isolated flavonoids and their glucoside derivatives were evaluated by DPPH free radical-scavenging assay, showing that compounds 1, 3, 4 and 8 exhibited stronger antioxidant activities compared with α, tocopherol and curcumin. (Clautilde, et al. 2008).

Ten compounds were isolated and elucidated as luteolin(Ⅰ),luteolin-7-O-β-D-glucoside(Ⅱ),apigenin(Ⅲ),apigenin-7-O-β-D-glucuronide, methyl ester(Ⅳ),apigenin-7-O-β-D-glucuronide ethyl ester(Ⅴ),apigenin-7-O-β-D-glucopyranuronide(Ⅵ),germanicyl acetate(Ⅶ),3β-hydroxy-6β,7α,11β-H-eudesm-4-en-6,12-olide(Ⅷ),oleanolic acid(Ⅸ) and 1-cerotol(Ⅹ) from *Sonchus oleraceus*.

(Xu and Liang,2005).

Silica gel column chromatograply (CC) and thin-layer chromatography were adopted to isolate six chemical compounds from the acetone-extracted product from *Sonchus oleraceus* and they were identified by such modern spectrum techniques as 1H NMR, 13 CNMR and 1R to be

apigenim(1),tuteolin(2),quercetin-3-O-glucose(3),3β-hydroxy-12-en-ursolic acid(4),(p-methoxyphenyl) acetic acid (5) and (p-hydroxyphenyl) acetic acid(6),which were first found in the plant. (**-**HU Pei, et al.2006**)**

1.6 Taxonomic Hierarchy

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridaeplantae – green plants
Infra-kingdom	Streptophyta – land plants
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – spermatophytes, seed plants, phanérogames
Infradivision	Angiospermae – flowering plants, angiosperms, plantas com flor, angiosperma, plantes à fleurs, angiospermes, plantes à fruits
Class	Magnoliopsida
Superorder	Asteranae
Order	Asterales
Family	Asteraceae – sunflowers, tournesols
Genus	Sonchus L. – sowthistle, sow thistle
Species	Sonchusoleraceus L. – common sowthistle, sow-thistle, common sow-thistle, annual sowthistle, pualele, sow thistle

Sonchus oleraceus **herb** **Fig.1.29**

Objectives of the Research:

The main aim of this work is to carry out antioxidants investigation for some plants. *Adensonia digitata*, *Sonchus oleraceus*, *Tamarindus indicum*, *Balanites aegyptiaca* .To isolate and identify compounds using spectroscopic techniques.

Antioxidant compounds are proved to be a) Antiviral (Charlton, 1998), (Yousefzadi et al., 2010).

b) Anticancer (Pan et al. 2009) , (Saleem et al.,2005).

i) Anti-inflammatory (Saleem et al., 2005)

j) Antimicrobial (Saleem et al., 2005)

k) Immunosuppressive (Saleem et al., 2005)

l) Hepatoprotective (Negi et al., 2008)

m) Osteoporosis prevention .(Habauzit and Horcajada, 2008)

General objective:

The aim of this study is to evaluate and isolate antioxidant compounds from some Sudanese plants.

Specific objectives:

1-To screen for antioxidant activity from selected medicinal plants used traditionally in alleviating clinical signs and symptoms.

2-To identify the bioactive compounds by biologically directed fractionation of the most active extracts from selected Sudanese plants.

3-To carry the process of isolation,purification and structure elucidation of the most active fractions from selected plant extracts.

Chapter Two
Materials and Methods

2 Materials and Methods

2.1. Materials

2.1.1 Plant Materials

Four different plants were collected .1-Fruit part of *Adensonia digitata*.2-Fruit part of *Balanites aegyptica*.3-Fruit part of *Tamarindus indicum* and 4-Whole plant of *Sonchus oleraceus* herb.The Sonchus oleraceus herb was collected from River Nile bank, Shambat area. The other three plants were purchased from Omdurman local market. Plant material of Sonchus oleraceus was left to dry under shade with continuous rotation.

2.1.2 Chemicals and Reagents

General reagents

Acetone, Chloroform, Dichloromethane, Ethyl acetate, Formic acid,Methanol, Petroleum ether, toluene.All the mentioned chemicals were purchased from Loba chemicals in India. The DPPH was purchased from Sigma Aldrich company in Germany.

Silica gel for TLC was 254GF.Silica gel for column chromatography was 60 mesh in size.

2.1.3 Equipments and instruments

Equipment

Beakers, capillary tubes, conical flasks, glass funnels, filter papers, glass plates 20×20, glass column, droppers, graduated measuring cylinder, 50 ml graduated cylinder for column chromatography,oven,sample bottles , separatory funnel, sprayer, rotary evaporator, round flasks(500 ml), silica gel spreador and solvent tanks.

Instruments

Cromatic ultra violet (UV) cabinets with two wavelenghths.Short wave length (254) and long (366) nm. Ultra spectrophotometer with micro plates (96 plates).

Infra- red (IR) spectra were recorded on Shimadzu spectrometer.

For MS analysis a Shimazduliquid chromatographic system (LCMS-8040(Shimazdu) linked with bench top triple quadra-pole tandem mass spectrometer equipped with an electrospray ionization (ESI) source was used for identification of compounds. The mobile phase was composed of solvents A (0.1% formic acid in water) and B (70% acetonitrile) for isocratic use. A Q3 scan was used of total rum of 2 minutes, the solvent flow rate was $0.6mlmin^{-1}$, and the injection volume was 5μl. Desolvation line temperature, heat block temperature and the cell temperature were 250°C and 400°C and 40°C respectively. The capillary voltage was 4.5 kV, the collision-induced dissociation (CID) gas pressure was 230 kPa and the dell time was 10 msec. for optimal analytical conditions. The nebulizing gas flow and drying gas flow were $3Lmin^{-1}$ and $15Lmin^{-1}$, respectively.
H^1 and 2D proton NMR . ^{13}C and ^{13}C (DEPT) NMR spectra were recorded with 700 MHz Brucker Advance by using tetramethylsilane (TMS) as an internal standard for samples dissolved in CD_3OD (deuterated methanol).The chemical shifts (δ) were reported in ppm relative to TMS = 0.

2.1.4 Spray reagents for thin layer chromatography

1-Anisaldehyde

2- glacial acetic acid

3- concentrated sulphuric acid and vanillin.

2.2 Methods

2.2.1 Sample extraction

3 kgs. of the coarse plant powder *(Sonchus oleraceus*) was extracted by soaking in dichloro- methane for seventy two hours with daily filtration and evaporation. Solvent was evaporated under reduced pressure to dryness using rotary evaporator apparatus .The plant residue was left to dry from the dicloromethane. When dried was re-extracted with methanol with continuous filtration and evaporation of solvent. The collected extract was left to dry in glass sample bottles. (Hilmi et al,2014). The yield percentage was calculated as follows:(Weight of extract obtained / weight of plant sample) X100.

The above process was repeated for *Adensonia digitata*,*Balanites aegyptica* and *Tamarindus indicum*.

2.2.1.1 Fractionation of methanolic extract: (Solvent-solvent extraction)

Ethyl acetate extract

250 gms methanolic extract were dissolved in 500 ml distilled water and shaked three times successively with 500 ml ethyl acetate solvent using a separatory funnel. Ethyl acetate layers were combined together and evaporated under reduced pressure using rotary evaporator apparatus. Successive extraction was done for three days. The percentage yield was calculated as follows:

Weight of extract obtained / weight of plant sample ×100

2.2.2 Preparation of reagents for qualitative phytochemical screening

2.2.2.1 Mayer' reagent:

1- 1.36 g of mercuric chloride dissolved in 60 ml of distilled water.
2- 5 g of potassium iodide was dissolved in 10 ml of distilled water.

Solution (1) and (2) were mixed and diluted to 100 ml with distilled water.

2.2.2.2 Valser's reagent:

10 g of potassium iodide dissolved in 100 ml of distilled water. Mercuric iodide was added till the solution became saturated. Excess of mercuric iodide was removed out.

2.2.2.3 1 % Methanolic Aluminum chloride:

1 g of aluminum chloride dissolved in 100 ml methanol.

2.2.2.4 1 % Methanolic Potassium hydroxide:

1 g of potassium hydroxide dissolved in 100 ml methanol.

2.2.2.5 Saline solution:

0.9 g of sodium chloride dissolved in 100 of distilled water.

2.2.2.6 Gelatin salt reagent:

10 g of gelatin powder dissolved in 100 ml hot distilled water.

2.2.2.7 Methanolic Ferric chloride:

3 g of ferric chloride dissolved in 100 ml methanol.

2.2.2.8 0.5 N potassium hydroxides:

0.89 g of potassium hydroxide dissolved in 100 ml distilled water.

2.2.3 Phytochemical screening of the extracts:

The extracts of the plants were subjected to phytochemical activity screening for the qualitative identification of the various classes of phyto-constituents (Harborne, 1984) The screening covered alkaloids, saponins, flavonoids, tannins, sterols, triterpenes, cumarins and anthraquinones .

2.2.3.1 Alkaloids tests:

0.5 g of each extract was dissolved in 2 ml of 2N Hcl, stirred while heating in water bath for 10 minutes, cooled, filtered and divided into two test tubes. To one test tube a few drops of Mayer's reagent were added, while to the other tube a few drops of Valser's reagent were added. A slight turbidity or heavy precipitate in either one or the two test tubes was taken as presumptive evidence for the presence of alkaloids.

2.2.3.2 Saponins test:

0.5 g of each extract was placed in a clean test tube and 10 mls of distilled water were added. The tube was stoppered and vigorously shaken for 30 seconds, then allowed to stand for 15-20 minutes, and classified for saponins content as follows: no froth = negative ; froth less than 1 cm = weakly positive; froth 1cm = medium; froth 1-2 cm = highly positive; and froth greater than 2 cm = strong.

2.2.3.3 Test for Flavonoids and phenols

0.5 g of each extract was dissolved in 15 ml of 96% ethanol and filtered. The filtrate was used for the following tests:

a) To 3 ml of the filtrate in a test tube, 1 ml of 1% aluminium chloride solution in methanol was added .Formation of a yellow colour indicated the presence of flavonoids.

b) To 3 ml of the filtrate in a test tube, 1 ml of potassium hydroxide solution was added. A dark yellow colour indicated the presence of flavonoids compounds.

c) To 3 ml of filtrate, 0.5 ml of concentrated HCL and a few magnesium turnings (0.5 g) were added .Production of definite color change to pink or red was taken as presumptive evidence that flavonoids compounds were present in the plants sample.

d) Dissolve a small amount of extract in ethanol, add $FecL_3$ reagent. The formation a red, blue or purple colour indicates presence of phenols

2.2.3.4 Test for Tannins

0.5 g of each extract was stirred with 10 ml of hot saline solution. The mixture was cooled and filtered. About 5 ml of this solution was treated with few drops of the Gelatin-salt reagents. Formation of an immediate precipitate was taken as evidence for the presence of tannins. Positive test confirmed by the addition of few drops of 1% $FecL_3$, test reagent to another portion of the solution and should result in a characteristic blue, blue-black, green or blue-green color and precipitate.

2.2.3.5 Test for Sterols and triterpenes

0.5 g of each extract were dissolved in 10 ml chloroform, 0.5 ml acetic anhydride (0.5 ml) was added and the solution was transferred into a dry test tube. Few drops of concentrated sulphuric acid were poured carefully down the walls of the test tube so as to form a lower layer. Brownish-red or violet ring at the zone of the contact with supernatant and green or violet coloration denoted the presence of sterols and /or triterpenes pink to purple.

2.2.3.6 Test for Coumarins:

0.5 g of each extract were dissolved in 10 ml distilled water in a test tube and filter paper was attached to the test tube to be saturated with the vapor after a spotted with 0.5 KOH . Then the filter paper was inspected under ultra violet (UV) light, the presence of coumrins was indicated if the spot was found to absorb the ultra violet (UV) light.

2.2.3.7 Test for Anthraquinone glycosides:

0.5 g of each extract were boiled with 10 ml of 0.5N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. A 5ml of the benzene solution was shaked with 3ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraquinones was indicated if the alkaline layer aquires pink or red color.

2.2.4 Thin layer chromatography (TLC)

2.2.4.1 TLC plates

The plates were prepared in the laboratory according to the method of Stashl (1969). 30 g of silica gel were shaken with 60 ml distilled water for two minutes using 250 ml stopper conical flask. The slurry was spread using spreader making 0.25 mm thickness on each glass plate.. The coated plates were then allowed to dry at room temperature and activated at 105°C for one hour. The hot plates were stored and allowed to cool down and stored till use.

2.2.4.2 Preparation of sample on TLC plates

A micro ml of sample was applied to the plates using capillary tube. Samples were spotted at about 2 cm from the bottom of the plate and 2 cm from each edge. The plate was kept to dry by air and then inserted in tank containing the selected solvent system. After reaching the height of 15 to 17 cm, the plate was taken out of the tank and subjected to air till solvent was completely evaporated. The plate was inspected in day light, then examined under UV lamp and finally sprayed with the specific spraying reagent. Rf values of separated spots which appeared in day light or under UV or after sprayed and heated were calculated as followed:

Distance crossed by spot / distance crossed by solvent front

2.2.4.3 Solvent mobile phases

1- Chloroform : Methanol (8: 2)

2- Ethyl acetate :Acetic acid : formic acid (10 :1.5 : 1)

3- Toluene : Methanol : formic acid (6 : 4 : 2 ml)

4- Toluene : Ethyl acetate : formic acid (4:5:1) ,(6 : 3: 3)

5- Ethyl acetate : Methanol : Acetic acid (40 : 10 : 2.5)

6- Chloroform: Acetone : Formic acid (70 : 17 : 9)

2.2.5 Isolation and purification of compounds

2.2.5.1 Preparation Column chromatography for ethyl acetate extract

The active phyto-constituents were separated from the extract by the column Chromatography (Jasper,1996).The glass column of size 90 cm length and 10 cm diameter was thoroughly washed with detergent and water to make grease free and kept for drying .A round cotton wool was introduced into the bottom of column with help of a rod .The cotton is to prevent silica out flow. 500 gms of packing material silica gel –of column (60 mesh size) was slurred with petroleum ether and poured in column. A homogeneous packing of the silica gel was done by maintaining gentle agitation while there was a solvent flow through the column. The column set up was left to stand for 24 hours.30 gms of ethyl acetate extract was mixed with 60 gms of silica in a beaker. There was a continous stirring with a glass rod over a water bath till ethyl. acetate solvent dries. The extract- silica mixture was introduced into top of column .Cotton wool was placed on top of extract to prevent it from bubbling upwards. When all the extract has been adsorbed on the top of the column the vacant space above it was filled with petroleum ether solvent and the column allowed to run, the supply of the solvent and combinations of solvent system was replenished from a beaker .The eluents were allowed to run into 50 ml graduated glass tubes. The various fractions thus obtained were concentrated by evaporation on rotary evaporatorThey were collected in small glass vials.Each collected fraction was developed on TLC plates and a suitable solvent system. The different solvents(starting with 100% pet.ether as shown on results of column chromatography table) used were according to the procedure given by (Herborne ,1984).

The collection of fractions was done when RF (retention factors) values are similar. The run- rate was 50 ml/5 minutes.

2.2.5.2 Sub- Column chromatography for fractions (30+31)

Sub column chromatography was used for 4 gms of the two fractions(30+31) for further isolation. These two fractions had the same separation profiles on TLC plates. A smaller column was used size 50 cm length and 5cm diameter. The eluent solvent started from ethyl acetate ending with methanol(starting with 100% ethyl acetate and adding 10% methanol every 400 mls of solvent mixtures). In between, different ratios of the two solvents were used. Seven sub fractions were collected when monitored with TLC.Solvent systems on TLC plates were toluene: ethyl acetate: formic acid (4:6:3).The purified compound designated sub-7.

2.2.5.3 Preparative TLC for Fractions

60 g of Silica gel GF_{254} silica gel were shaken with 120 ml distilled water for two minutes using 250 ml Stoppard conical flask. The slurry was spread using spreader making 0.5 mm thickness on five glass 20×20 cm plates. The coated plates were then allowed to dry at room temperature and activated at 105°C for one hour. The hot plates were stored and allowed to cool down and stored till use.

2.2.5.4 Application of sample on preparative T LC plate

Column fraction was applied to the plate as line using capillary tube. Sample was spotted at about 2 cm from the bottom of the plate and 2 cm from each edge. Plate was kept to dry by air and then inserted in tank containing solvent system .After reached the height of 15 to 20 cm, the plate was taken out of the tank and allowed to air till solvent was completely evaporated. The plate was inspected in day light, and then examined under UV lamp. Bands that are florescent were scratched and the scratches of all plates were collected together. Collected scratches were then dissolved in methanol and filtered using filter paper. The filtrate was allowed to

air-dry to concentrate. Concentrated solution was then tested by thin layer chromatography using the following solvent systems:

- Ethyl acetate :Acetic acid : formic acid (10 :1.5 : 1)

-Toluene : Methanol : formic acid (6 : 4 : 2 ml)

- Toluene : Ethyl acetate : formic acid (4:5:1) ,(6 : 3: 3)

- Chloroform: Acetone : Formic acid (70 : 17 : 9)

-Ethyl acetat e : Acetic acid : Formic acid (10 :1 : 1)

Plates were inspected in day light, under UV lamp and sprayed with 5%, Vanillin-sulphuric acid and P-Anisaldehyde.

2.2.5.5 Preparative TLC for fraction 23

Fraction 23 (1.200 grams) was dissolved in 2: 3 (chloroform: ethyl acetate).The sample was introduced as a band on 15 preparative TLC plates. Three layers were isolated. The silica containing the compound was washed several times with methanol. The solvent was evaporated and the compound was stored in glass vials.The purity was tested with three different solvent systems. The purified compounds were designated 23_2 and 23_3

1-Chloroform:acetone:formic acid.

2-Ethyl acetate acetic acid:formic acid(10:1:1)

3-Toluene eth.acet:formic acid (50:40:3).

2.2.5.6 Preparative TLC for fraction 24

Fraction 24 (0.640) gms sample was introduced as a band on 10 preparative TLC plates. One layer was isolated. .The silica containing the compound was washed several times with methanol .The solvent was evaporated and the compound was

stored in glass vials .The purity was tested with three different solvent systems.The purified compound designated 24_1.

1-Chloroform:acetone:formic acid.

2-Ethyl acetate acetic acid:formic acid (10:1:1)

3-Toluene eth.acet:formic acid (50:40:3).

2.2.5.7 Preparative TLC for fraction 25

Fraction 25 (0.880) gms were dissolved in 6:4 (methanol : ethyl acetate). Sample was applied as a band on 10 preparative TLC plates. Two florescent bands were isolated .Solvent system was Tol. :Ethyl ac: formic acid (50 :40 : 3ml). Purity was tested with three different solvent systems.

1-Chloroform:acetone:formic acid.

2-Ethyl acetate acetic acid:formic acid (10:1:1)

3-Toluene eth.acet:formic acid (50:40:3).

2.2.5.8 Preparative TLC for fraction 29

Fraction 29 (0.40) gms dissolved in (1 :4) methanol : Ethyl acetate..Sample was applied as a band on 10 preparative TLC plates.Three florescent bands were isolated. Solvent system for isolation was - ethyl.acet :methanol (10 : 1). Three layers were isolated.

2.2.6 Antioxidant assay using DPPH for free radical scavenging activity

The DPPH radical scavenging was determined according to the modified method of (Shimada et al.] in 96-wells plate, the test samples were allowed to react with 1,1-Diphenyl-2-picryl-hydrazyl stable free radical (DPPH) for half an hour at 37°C. The concentration of DPPH was kept as 300 μM. The test samples were

dissolved in DMSO (dimethyl sulfoxide reagent) while DPPH was prepared in ethanol. After 30 min. incubation, decrease in absorbance was measured at 517 nm using multiple reader spectrophotometer. Percentage radical scavenging activity of samples was determined in comparison with a DMSO treated control group .All the tests and analysis were run in triplicate .(Shimada,1992)

2.2.7 Spectroscopic analysis of isolated compounds

Pure fractions were characterized by: a) IR b) MS c) Proton NMR. d) ^{13}C NMR. e) DEPT NMR. f) 2D NMR (cosy) and g) HSQC NMR. These compounds were identified by data analysis and literature previously reported.

To elucidate a structure from spectroscopic data is not an easy job. Principles and theories about the different spectroscopic techniques were studied from relevant references. (Silverstrein et al. 2005). First studied about the infra red spectroscopy to know the main functional groups in the compound.The analysisof the IR spectra was done by consulting many frequency tables and compare the findings to get a final result. An example of IR frequency table:

Table 2.1

Characteristic IR Absorption Frequencies of Organic Functional Groups

Functional Group	Type of Vibration	Characteristic Absorptions (cm-1)	Intensity
Alcohol			
O-H	(stretch, H-bonded)	3200-3600	strong, broad
O-H	(stretch, free)	3500-3700	strong, sharp
C-O	(stretch)	1050-1150	Strong
Alkane			
C-H	stretch	2850-3000	Strong
-C-H	bending	1350-1480	Variable
Alkene			
=C-H	stretch	3010-3100	Medium
=C-H	bending	675-1000	Strong
C=C	stretch	1620-1680	Variable

Alkyl Halide			
C-F	stretch	1000-1400	Strong
C-Cl	stretch	600-800	Strong
C-Br	stretch	500-600	Strong
C-I	stretch	500	Strong
Alkyne			
C-H	stretch	3300	strong,sharp
$-C\equiv C-$	stretch	2100-2260	variable, not present in symmetrical alkynes
Amine			
N-H	stretch	3300-3500	medium (primary amines have two bands; secondary have one band, often very weak)
C-N	stretch	1080-1360	medium-weak
N-H	bending	1600	Medium
Aromatic			
C-H	stretch	3000-3100	Medium
C=C	stretch	1400-1600	medium-weak, multiple bands
Analysis of C-H out-of-plane bending can often distinguish substitution patterns			
Carbonyl	Detailed Information on Carbonyl IR		
C=O	stretch	1670-1820	Strong
(conjugation moves absorptions to lower wave numbers)			
Ether			
C-O	stretch	1000-1300 (1070-1150)	Strong
Nitrile			
CN	stretch	2210-2260	Medium
Nitro			
N-O	stretch	1515-1560 & 1345-1385	strong, two bands

Table 2.2

IR Absorption Frequencies of Functional Groups Containing a Carbonyl (C=O)

Functional Group	Type of Vibration	Characteristic Absorptions (cm-1)	Intensity
Carbonyl			
C=O	stretch	1670-1820	Strong
(conjugation moves absorptions to lower wave numbers)			

Acid			
C=O	stretch	1700-1725	Strong
O-H	stretch	2500-3300	strong, very broad
C-O	stretch	1210-1320	Strong
Aldehyde			
C=O	stretch	1740-1720	Strong
=C-H	stretch	2820-2850 & 2720-2750	medium, two peaks
Amide			
C=O	stretch	1640-1690	Strong
N-H	stretch	3100-3500	unsubstituted have two bands
N-H	bending	1550-1640	
Anhydride			
C=O	stretch	1800-1830 & 1740-1775	two bands
Ester			
C=O	stretch	1735-1750	Strong
C-O	stretch	1000-1300	two bands or more
Ketone			
Acyclic	stretch	1705-1725	Strong
Cyclic	stretch	3-membered - 1850 4-membered - 1780 5-membered - 1745 6-membered - 1715 7-membered - 1705	Strong
α,β-unsaturated	stretch	1665-1685	Strong
aryl ketone	stretch	1680-1700	Strong

Also much literature was cited for IR ranges of flavonoids, (Oyvind,2006).

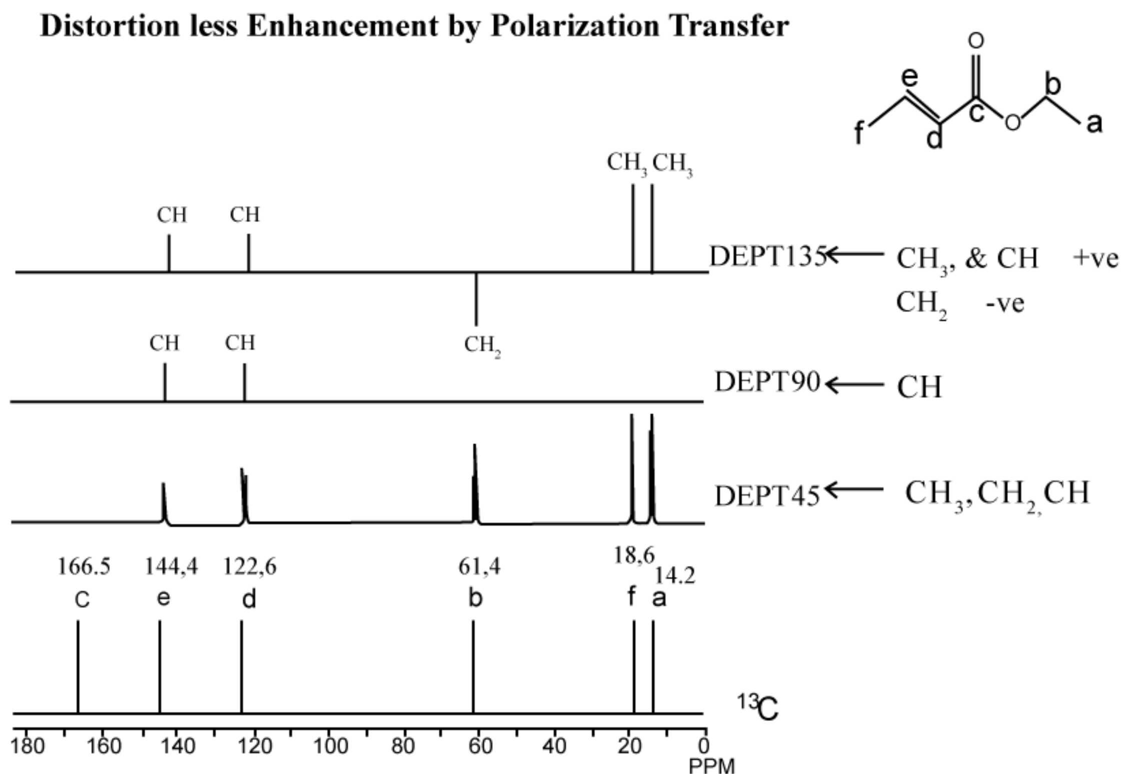
Secondly, as much information about NMR spectroscopy was collected. To characterize a compound with NMR data as much analysis should be done. Many NMR techniques were used in this research. Starting with ^{13}C analysis for each compound.

Three ^{13}C DEPT experiments were done to determine CH_3,CH_2 and CH carbons and locate their positions. DEPT 45,DEPT 90 and DEPT 135DEPT.

DEPT-NMR — Through a technique known as DEPT-NMR it is possible to determine which signals in a carbon-13 NMR spectrum come from carbons with no hydrogens attached, which come from carbons with one hydrogen attached,

which come from carbons with two hydrogens attached, and which come from carbons with 3 hydrogens attached. Step 1 Run an ordinary (broadband-decoupled) C-13 NMR spectrum of the compound. A signal will appear for each non-equivalent carbon. (Equivalent carbons will allcontribute to the same signal.) Step 2 Run a DEPT-90 C-13 NMR of the compound. A signal will appear for each non-equivalent carbon that has one hydrogen attached (CH). No peaks will appear for the C, CH2 , or CH3 carbons. Step 3 Run a DEPT-135 C-13 NMR of the compound. A positive signal (peak) will appear for CH3 and CH carbons. A negative peak will appear for CH2 carbons.

An example of DEPT analysis:



Analyses of carbon- 13 spectra was done from ^{13}C chart for each of the isolated compounds. With the aid of 13C charts ,the different types of carbons were located according to their chemical shifts. An example of 13C chemical chart together with proton NMR chemical shifts chart is as follows:

Table 2.3 NMR chemical shifts chart

1H and ^{13}C Chemical Shifts

Where ranges are not given, a wise assumption would be ± 0.4 ppm for H, and ± 3 ppm for C.

Compound Class	Structure	^{1}H, ppm	^{13}C, ppm
Alkanes	CH_3	0.6 - 1.2	15 – 30
	CH_2	1.2 - 1.5	22 – 45
	CH	1.4 - 1.8	30 – 58
Cycloalkanes	3-ring CH_2	-0.2 - 0.2	-2.9
	4-ring CH_2	1.95	22.3
	5-ring CH_2	1.50	26.5
	6-ring CH_2	1.44	27.3
Various CH_3	CH_3-C-C-G (G = X, OH, OR, N ..)	0.8 - 1.4	27 – 29
	CH_3-C-G (G = C=C, Ar)	1.05 - 1.20	15 – 30
	CH_3-C-G (G = X, OH, OR, C=O)	1.0 - 2.0	25 – 30
	CH_3 -C=C	1.5 - 2.0	12 – 25
	CH_3-COR, CH_3-Ar	2.1 - 2.4	20 – 30
	CH_3-C≡ C	1.7	5 – 30
	CH_3-G (G = N, X)	2.2 - 3.5	25 – 35

	CH_3-G (G = OR, OAr)	3.2 - 3.8	56 – 60
Various CH_2	R-CH_2-G, G = C=O	2.3 - 2.6	32 – 45
	G = C=C	1.9 - 2.3	32 – 35
	G = Ar	2.4 - 2.7	38 – 40
	G = F	4.3	88
	G = Cl	3.4	51
	G = Br	3.3	40
	G = I	3.1	13
	G = OH, OR	3.5	67 – 69
	G = NH_2	2.5	47 – 49
	G = NR_2	2.5	60 – 62
	R = CO_2H	2.4	39 – 41
	G = CN	2.5	25 – 27
Various CH	R_2CH-G, G = C=O	2.5	40
	G = C=C	2.2	?
	G = Ar	2.8	32
	G = F	4.6	83
	G = Cl	4.0	52
	G = Br	4.1	45
	G = I	4.2	20
	G = OH, OR	3.9	57 – 58
	G = NH_2	2.8	43

	G = NR_2	2.8	56
	G = CO_2H	2.6	?
	G = CN	2.7	23
Alkenes	=CH_2	4.5 - 5.0	115
	=CH_2 (conjugated)	5.3 - 5.8	117
	=CHR	5.1 - 5.8	120 – 140
	=CHR (conjugated)	5.8 - 6.6	130 -140
	C=C=CH_2	4.4	75 – 90
	C=C=C	N.A..	210 – 220
Alkynes	RC≡CH	2.4 - 2.7	65 – 70
	RC≡CH	N.A.	85 – 90
Benzenes	General Ranges	6.5 - 8.5	115 – 160
Specific examples:	$PhNO_2$, ipso- ortho- meta- para-	N.A. 8.2 7.4 7.6	148.5 123.5 129.4 134.3
	$PhOCH_3$, ipso- ortho- meta- para-	N.A. 6.8 7.2 6.7	159.9 114.1 129.5 120.8
	PhBr, ipso- ortho-	N.A. 7.5	123.0 131.9

	meta- para-	7.1 6.7	130.2 126.9
	$PhCH_3$, ipso- ortho- meta- para-	N.A. 7.4 7.2 7.1	137.8 129.3 128.5 125.6
Carbonyl Groups (aldehydes)	RCHO	9.4 - 9.7	200
	ArCHO	9.7 - 10.0	190
(ketones)	R_2CO	N.A.	205 – 215
	5-ring C=O	N.A.	214
	6-ring C=O	N.A.	209
	ArCOR	N.A.	190 – 200
(carboxyls)	RCO_2H, $ArCO_2H$	N.A.	170 – 180
(esters)	RCO_2R, $ArCO_2R$	N.A.	165 – 172
(acid chlorides)	RCOCl, ArCOCl	N.A.	168 – 170
(amides)	$RCONH_2$, $ArCONH_2$	N.A.	170
Nitriles	RC≡N	N.A.	115 – 125
Exchangeable (Acidic) Hydrogens	ROH (free)	0.5 - 1.0	N.A.
	ROH (H-bonded)	4.0 - 6.0	N.A.
	ArOH (free)	4.5	N.A.
	ArOH (H-bonded)	9.0 - 12.0	N.A.
	CO_2H (H-bonded)	9.6 - 13.3	N.A.

	NH, NH_2 (free)	0.5 - 1.5	N.A.
	ArNHR, $ArNH_2$ (free)	2.5 - 4.0	N.A.
	R_3NH^+, $R_2NH_2^+$, RNH_3^+ (in CF_3CO_2H)	7.0 - 8.0	N.A.
	Ar_3NH^+ , etc. (in CF_3CO_2H)	8.5 - 9.5	N.A.
	RSH	1.0 - 1.6	N.A.
	ArSH	3.0 - 4.0	N.A.

(Silvetrein,2005) , (Wehrli, 1976)

Proton NMR results interpretation:

The most important part in structure elucidation of the compounds were the proton NMR results. Proton chemical shifts tell a lot of information A)For aromatic protons,place of substitution was indicated by calculating the J values.J values were calculated by finding interaction between pair of proton shifts and multiply result by NMR machine in hertz. B) Number of signals were found,they reflect types of protons. C) Areas ratio of peaks intensities were calculated from the NMR spectrum. They showed the numbers of protons present. D) Chemical shifts values were read from NMR reference tables. They showed positions of protons.These tables tell a lot of information about the proton shift. E) Signal spin-spin splitting indicated that equivalent protons give similar signals. N+1 rule was followed; a single signal tells that there is no neighboring proton.

Mass fragmentation**:**

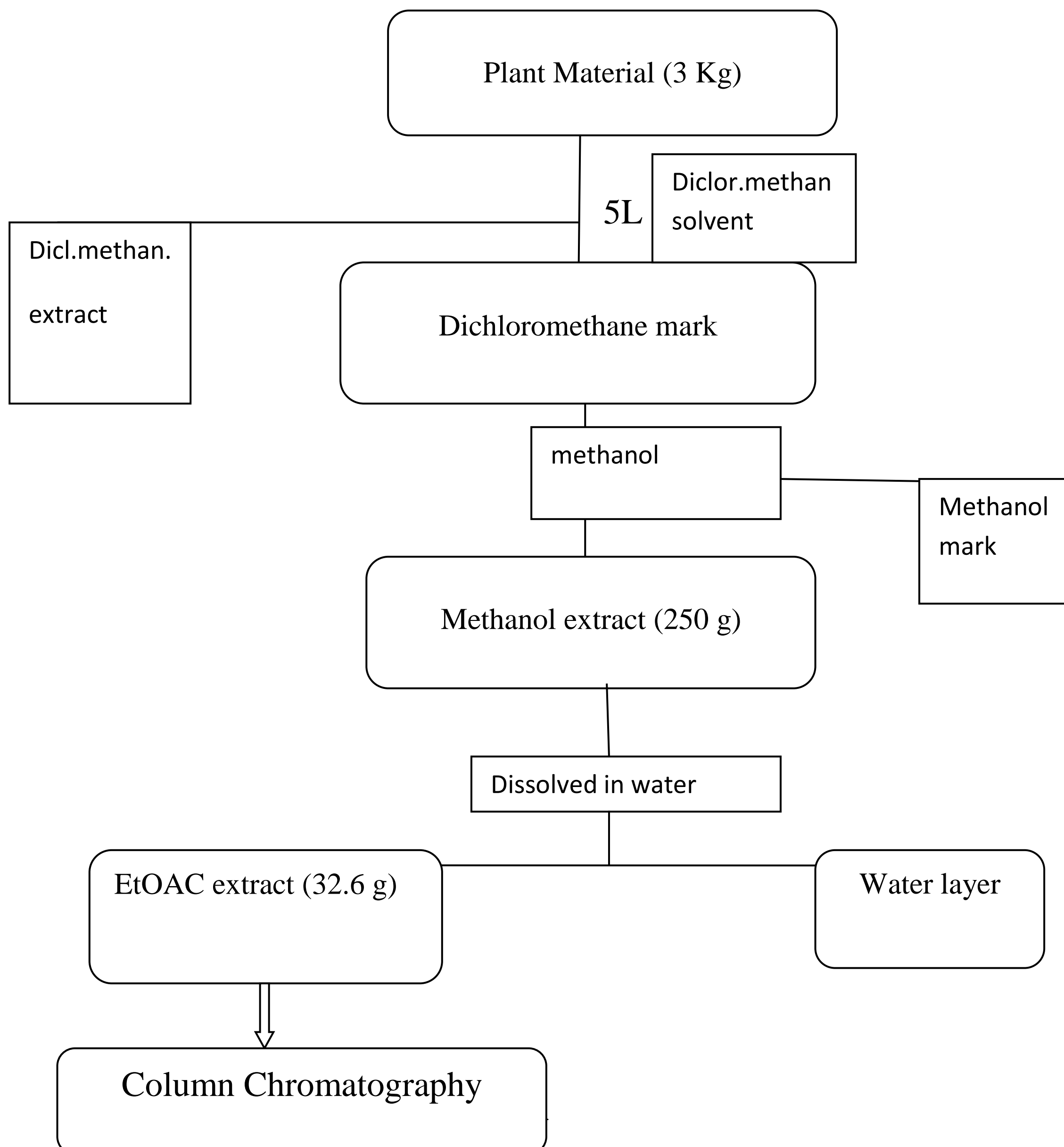
The molecular ions are energetically unstable, and some of them will break up into smaller pieces. All sorts of fragmentations of the original molecular ion are possible- and that means there will be many lines in the mass spectrum. By calculations and many trials, the structure of fragments were found. There is a rule when molecular ion mass is odd, the structure might contain nitrogen atom.(Plazonic, 2009), (Pedro, 2012).

All through this research a lot of literature citation was adopted. Comparison of result from published papers from many reliable journals. The search was focused on characterization and structure elucidation of poly-phenolic compounds.When the final structure was elucidated, the molecular ion mass was calculated. The mass fragments were calculated and their structures were drawn accordingly from the mass spectra of each compound.

Chapter Three
Results and Discussion

3. Results and Discussion

S. oleraceus was selected for antioxidants benefits .In order to establish these benefits the plant was extracted and screened to discover and elucidate structures of antioxidants in the plant.



3.1 Methanol extract of four different plants.

Table 3.1: methanol extract of four different plants.

Plant	Wt. of extract	%yield
Adensonia digitata	24.5 gm	12.25
Sonchus oleraceus	32.6 gm	16.3
Tamrindus indica	20.3 gm	10.15
Balanites aegyptiaca	27.5 gm	13.75

Sonchus oleraceus whole herb extract gave the highest yield with methanol solvent extraction.

3.1.1 Results of Radical Scavenging Activity for extracts

* **Antioxidant activity** of plant methanol and ethyl acetate extract was detected using DPPH solution.DPPH is a stable free radical freshly prepared solution of DPPH has a deep purple color with absorption at 517 nm.This purple color fades when antioxidant molecules from plant extract attack the free radicals of DPPH reagent. This converts them into substituted hydrazine resulting in decrease of absorption wave length (Amarowicz *et al*,2003).The activity was expressed as inhibition percentage value.(µg/ml).The values of antioxidants of plant extracts were compared with a standard. The standard is propyl gallate.The ethyl acetate extract has a high DPPH RSA compared to other solvents extracts (Mohammed *et al*, 2013).

3.1.2 Results of Radical Scavenging Activity for methanol extracts

Table: 3.2 RSA against DPPH for methanol extract of four plants.

Num.	Sample	%RSA+SD (DPPH)
1	*Adensonia digitata*	80+_0.05
2	*Tamarindus indica*	29+-0.70
3	*Balanites aegyptiaca*	41+-0.02
4	*Sonchus oleraceous*	88+_0.31
5	PG/EDTA	90+_0.01

Methanolic extract of Adensonia digitata and Sonchus oleraceus gave the highest RSA against DPPH reagent.

3.2 Results of DPPH Radical scavenging activity for ethyl acetate extract

Table 3.3: RSA of ethyl acetate extract for two plants

Sample.	Ethyl acetate extract
Adensoniadigitata	52+_0.01
Sonchus oleraceus	89+_0.00
PG	92+-0.01

It is shown that sonchus oleraceus gave 88% RSA in comparison with *Adensonia digitata*.Therefor Sonchus oleraceus plant was chosen to carry the tests for this research.

In conclusion methanolic extract of *Sonchus* gave a yield of 16.3% and the weight was 250 gms. On screening the extract with DPPH for radical scavenging activity (RSA) it showed 88% in comparison to 90% of a reference The ethyl acetate extract showed RSA of 89%.. The ethyl acetate extract showed the highest activitv for DPPH radical scavenging activity.

3.3 Phytochemical analysis

3. 3.1 Qualitative phytochemical analysis

Primary screening for sonchus oleraceus herb was performed with different chemical reagents to detect phyto constituents in the chosen plant (sonchus oleraceus). Results are presented below

Table 3.4 Qualitative results of phytochemicals in ethyl acetate extract

Class of compound	Test reagent	Sonchus eth.acet. extract
Alkaloid	Mayers and Valser's	+
Anthraquinone glycoside	KOH, 3% H_2O_2,benzene,NH_4OH	-
Coumarins	KOH	-
Flavonoids	$ALcL_3$,KOH	+++
Saponins		+
Sterols and triterpines	Acetic anhydride and conc.H_2SO_4	+
Tannins		
Phenols	$FecL_3$	+++

(-) Absent (+) Low concentration

(++) Moderate concentration (+++) High concentration

The results indicated a high concentration of Flavonoids ,tannins and phenols.

3.4 Column Chromatography

Column chromatography technique:

Column chromatography is an isolation technique in which phyto- constituents are eluted by adsorption separation of constituents at interphase between solid and liquid. The components have different affinity towards adsorption to achieve separation. (Maysa, et al.2016). The different polarities of solvent mixture determine the type of components that will separate.

Column chromatography fractionation:

The best mobile phase for TLC was chosen by trials. The best solvent separation is the one that gave best separation on TLC plates.

The ethyl acetate extract of S.oleraceus (30 gms) was mixed with silica gel and placed on top of the settled column. A cotton wool was placed on top of silca+ extract.The first solvent used was petroleum ether .Increasing the polarity of the solvents, petroleum ether, chloroform, ethyl acetate and methanol and their mixtures. Fractions obtained from the column were analyzed with TLC in suitable solvent systems. Elements with similar r.f *(*retention factor) value were collected together. High activity results from fraction 23 (chloroform : ethyl acetate) 4:6. Fraction 36 was the last with a high activity (ethyl acetate : methanol) 1 : 9.

Table 3.5 Results of column chromatography fractions

Fraction num.	Eluent (mobile phase)	Weight of fraction	%RSA±SD (DPPH)	TlC solvent system
F1	100% pet.ether	0.460 gm	26+_0.06	Pet.Ether:chloroform 9:1
F2	95%:5%	0.538 gm	38+_0.06	

	Pet.Ether: chloroform			
F3	90% :10% Pet ether :chloroform	0.200	22+_0.09	
F4	85 : 15	0.355	19+_0.07	
F5	80 :20	0.177	20+_0.04	
F6	75:25	0.300	14+_0.04	
F7	70:30		2+_0.04	
F8	60:40		8+_0.02	
F9	50:50	0.638	3+_0.03	
F10	40:60	1.142	9+_0.04	
F11	30:70	0.383	16+_0.07	

Continued:

Fraction num.	Eluent (mobile phase)	Weight of fraction	%RSA±SD (DPPH)	TlC solvent system
F12	20:80	0.358	16+-0.02	
F13	10:90	0.538	10+-0.10	Pet. Ether : chloroform 8 : 2
F14	5 pet.ether:95 chloroform	0.510	9+_0.06	
F15	100% chloroform	0.258	7+_0.01	6 chloroform : 4 pet.ether
F16	95chlor:5 eth.acet	0.870	8+-0.06	
F17	90:10		8+-0.03	
F18	85:15		7+_ 0.04	
F19	80:20	1.767	7+-0.04	

F20	70:30	0.423	8+-0.05	Toluene:ethyl acetate: formic acid 9: 1 : 1
F21	60:40	0.335	24+_0.04	
F22	50lclor:50ethacet	0.136	37+-0.01	
F23	40:60	1.199	69+-0.08	
F24	30:70	0.640	88+_0.04	Tol:ethac:f.a (8;2:1)
F25	20:80	0.880	90+-0.01	
F26	10:90	0.444	89+-0.00	
F27	100%eth acet	0.152	77+-0.03	
F28	10 meth : 90eth acet	0.076	69+-0.08	

Fraction num.	Eluent (mobile phase)	Weight of fraction	%RSA±SD (DPPH)	TlC solvent system
F29	20:80	0.463	82+-0.10	Tol. Eth.Ac. Meth 80 . 20 .20
F30	30:70.	2.931	86+-0.02	
F31	40:60	2.401	74+-0.08	60:40:10 ml
F32	50:50	2.100	57+-0.04	
F33	60meth:40eth.acet	0.838	70+-0.03	50tol:40meth:10formic
F34	70:30	0.776	90+-0.05	
F35	20:80	2.188	84+-0.31	
F36	10:90	0.732	81+-0.01	
F37	100% methanol	0.742	23+-0.11	
38	25% water	0.820	60±0.09	

39	50% water	0.901	61±0.01	
40	75% water	0.220	52±0.04	
41	100% water	0.087	26±0.05	
	PG		91±0.01	

3.4.1 Sub column for fractions (30 +31).

Table 3.6

Sub.fractions	Solv.eluent	DPPH	TLC solv.sys	Weight(gm)
1	100%eth.ac	75±0.06	Tol:ethacet.:Form 40 : 60 :3 ml	0.210
2	Eth .ac:meth 90: 10	41±0.04		2.473
3	80: 20	47±0.00		0.64
4	70: 30	66±0.01		0.16
5	60: 40	39±0.03		0.11
6	50:50	14±0.08		0.038
7	40: 60	40±0.020		0.05
8	20meth:80eth.ac	17±0.07		0.317
9	100% methanol	44±0.01		0.042

Fractions 30 and 31 were combined because they have similar separation profiles. The solvents for the run were ethyl acetate and methanol at different successive ratios.

3.5 Purification of fractions with preparative TLC

3.5.1 Purification of fraction 23 with preparative TLC

Table 3.7 fraction 23 with their weight , RSA and TLC solvent system

Sub fraction	DPPH	Solvent syst.	R_f	Wt .gm
23_1	29%	Toluen :Eth.Acet.: Form. Acid (5:4:2)	10/15	0.525
23_2	52%		7.7/15	0.466
23_3	55%		13/15	0.121

Fraction 23 gave three sub fractions, solvent system for separation was toluene ethyl acetate formic acid (5:4:1).Their weight and RF values were different.23_2 and 23_3 were chosen for structure identification.

3.5.2 Purification of fraction 24 with preparative TLC

Table 3.8 s fraction 24 with their weight , RSA and TLC solvent system

Fraction	DPPH	Solvent syst.	R_f	Wt .gm
24	68%	Toluen :eth.acet.: form. Acid (5:4:2l)	6/15	0,530

Fraction 24 gave one component. Solvent system was toluene:ethyl acetate:formic acid (5:4:2). RSA was 68%.It was chosen for structure elucidation.

3.5.3 Purification of fraction 25 with preparative TLC

Table 3.9 fraction 25 with their weight , RSA and TLC solvent system

Number	DPPH	Solvent syst.	R_f wt
25_1	12±0.02	Tol: EthAcet: Formic acid 80:20:2ml	7/16 0.80
25_2	69±0.04		4/16 0.151
PG	91%		

3.6.4 Purification of fraction 29 with preparative TLC

Table 3.10 fraction 29 with their weight , RSA and TLC solvent system

Number Fraction 29	Sub fraction	DPPH ASS.	Solv.system	R_f value	Weight
1	29_1	20± 0.01	Eth.ac.meth.formic 100 : 14 : 3 ml	11/15	0.1 gm
2	29_2	29± 0.06		11.5/15	0.05
3	29_3	40±0.03		10/15	0.327
	PG	91±0.01			

Fraction 29 gave three sub fractions 29_1, 29_2 and 29_3.Their RSA were small percentage. Solvent system for separation was ethyl.acet:methanol:formic acid (100:14:3)

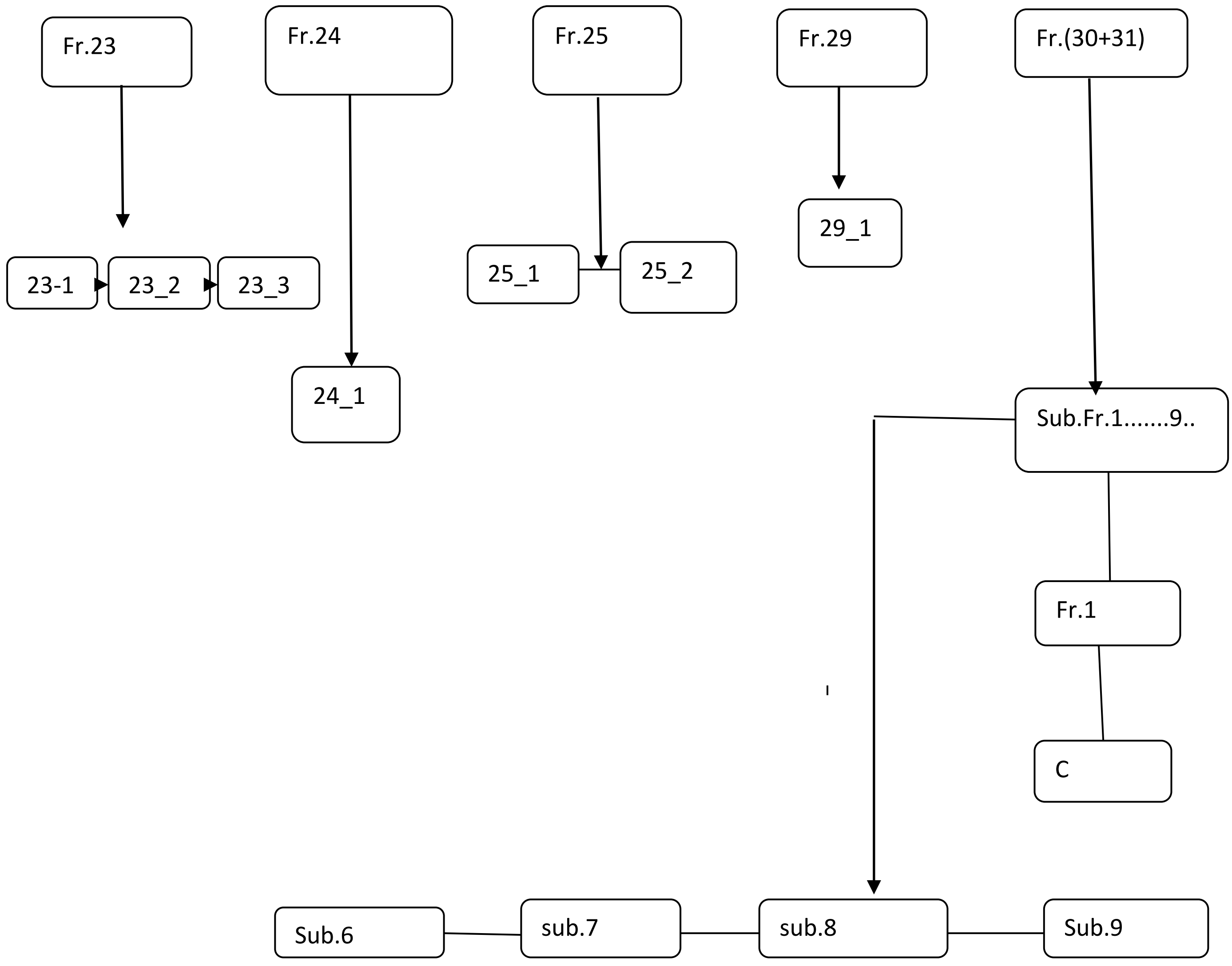
3.6 Test of purity with three solvent systems:

Table 3.11

Name of sample	Solvent syst. 1	Solvent syst. 2	Solvent syst. 3
	Chloroform:acetone: Formic (70:17:9)	Ethyl.acet: aceticacid:formic (10:1:1)	Toluene:eth.acet: Formic Acid (50 :40: 3)
23_1	12/15	-	10/15
23_2	-	-	7/15
23_3	13/14	-	14/15
24	6/14	12/13	5/16
25_1	7/14	7.5/15	4.5/15
25_2	-	12/15	5/15
29_1	4/14	5/13	15/16
29_2	2/14 , 6/14	5/13	5/16 , 6/16
29_3	2/14 , 4/14 , 7/14	5/13, 9/13	4/16 , 8/16
(30+31)sub4	-	5/13 ,6/13	2/16 ,5/16, 14/16
(30+31)sub6	-	-	14/16
(30+31)sub7	2/14	-	15/16
(30+31)sub8	4/14	7.5/13	15.5/16

Sub fraction 23 gave single spots with the three solvent systems. Fraction 24 gave a single spot with each of the three solvent systems. Sub-fractions of 29 gave multiple spots. Subs 6, 7 and 8 gave single spots.

Fig. 3



3.7 Spectroscopic analysis and structure elucidation:

3.7.1 Characterization of first compound (23_2)

compound 1: Lignan glycoside

Brown-green amorphous solid.This compound was isolated from ethyl acetate extract. Column chromatography mobile phase was chloroform : ethyl acetate (3:7). RSA was 88 % and weight of compound was 0.64 gm.Molecular mass ion m/z (M+H) at m/z 869.

IR frequencies : Broad stretch at 3411 cm^{-1} (-OH- stretch). C-H –stretch at 2935 cm^{-1}.Strong aromatic bending at 1598 $cm-^{1}$. Ar-O bending at 1388 $cm-^{1}$.

Proton NMR: 7.7 ,7.6 two symmetrical aromatic chemical shifts , substitution in meta position ,coupling is(J 0.1 x700 Hz).

7.4 is a proton chemical shift of -OH on ring B which is disubstituted . 7-6.9 roton chemical shifts (singlet no neighbouring protons) is characteristic for Ar--O. 6.5 chemical shift is singlet and characteristic for a phenol. 4.5 chemical shift is for Ar-O- R a singlet (no neighbouring protons). 3.6- 3.5 is characteristic of sugars –CH_2OH units. 2.8-2.5 isa a proton chemical shift for Ar-R. 1.5 is a chemical shift of sugar cyclohexane.

^{13}C NMR : 136 carbon shift at C1 in etherified units. 132 .129 .128 are aromatic carbons. 125 is C5 of the aromatic ring., 76, 74, -OH- on sugar. 64 ,33 ,30 –CH_2 on sugar ,20 ,14 aliphatic carbons.

Fig.3.1 IR spectrum of lignan glycoside

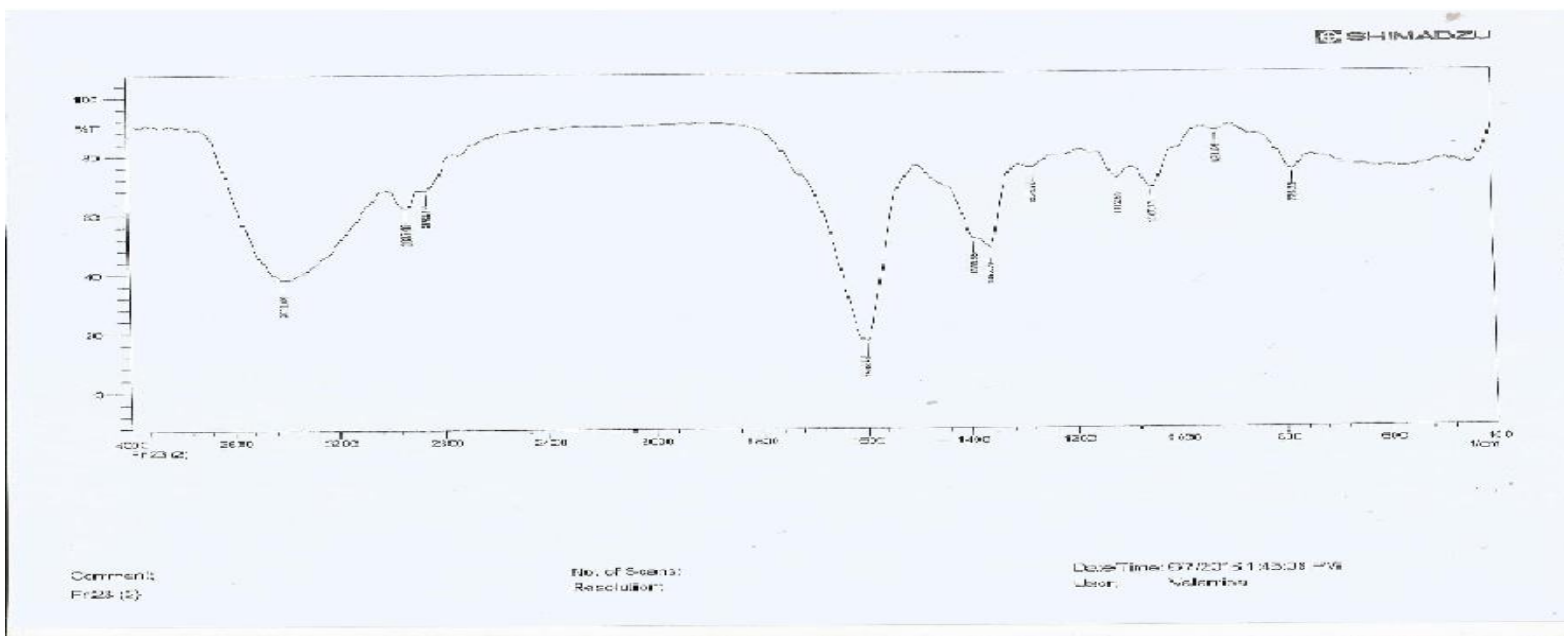
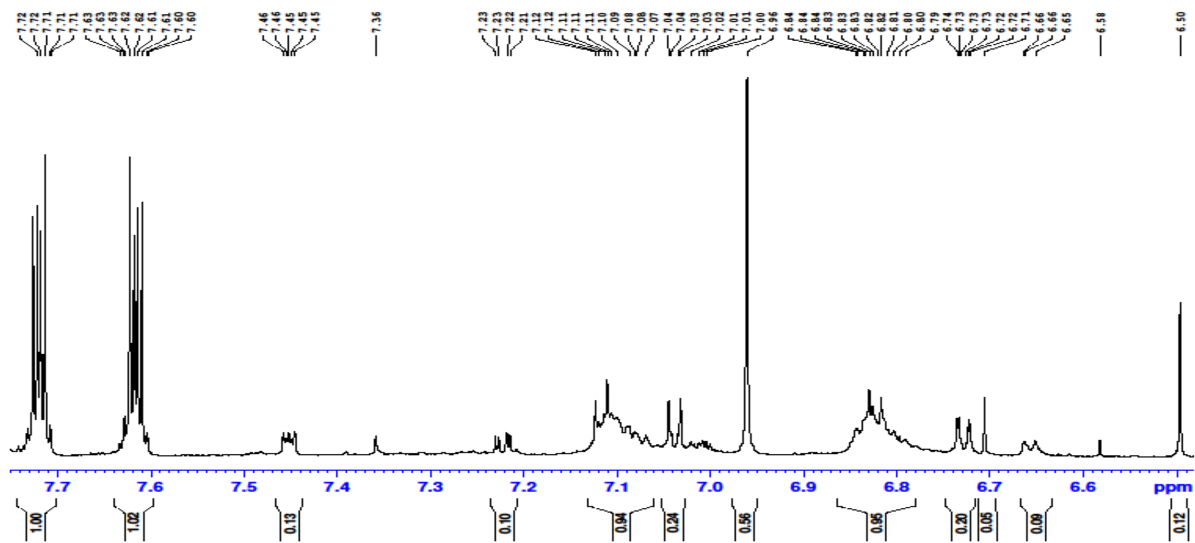
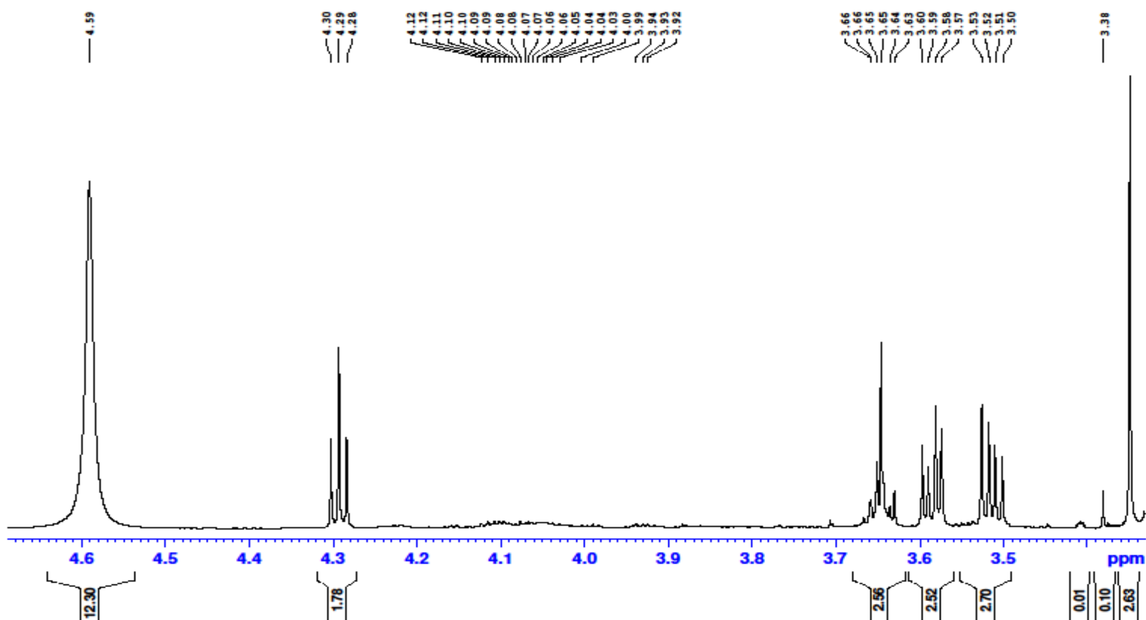


Table 3.12 IR functional group identification of lignan glycoside

IR frequencies (cm^{-1})	Indication
3411	Broad stretch of –OH- group
2935	-CH- stretch
1598	Strong aromatic bending
1388	Aryl ether bending


23-2 proton
ppm


23-2 proton
ppm

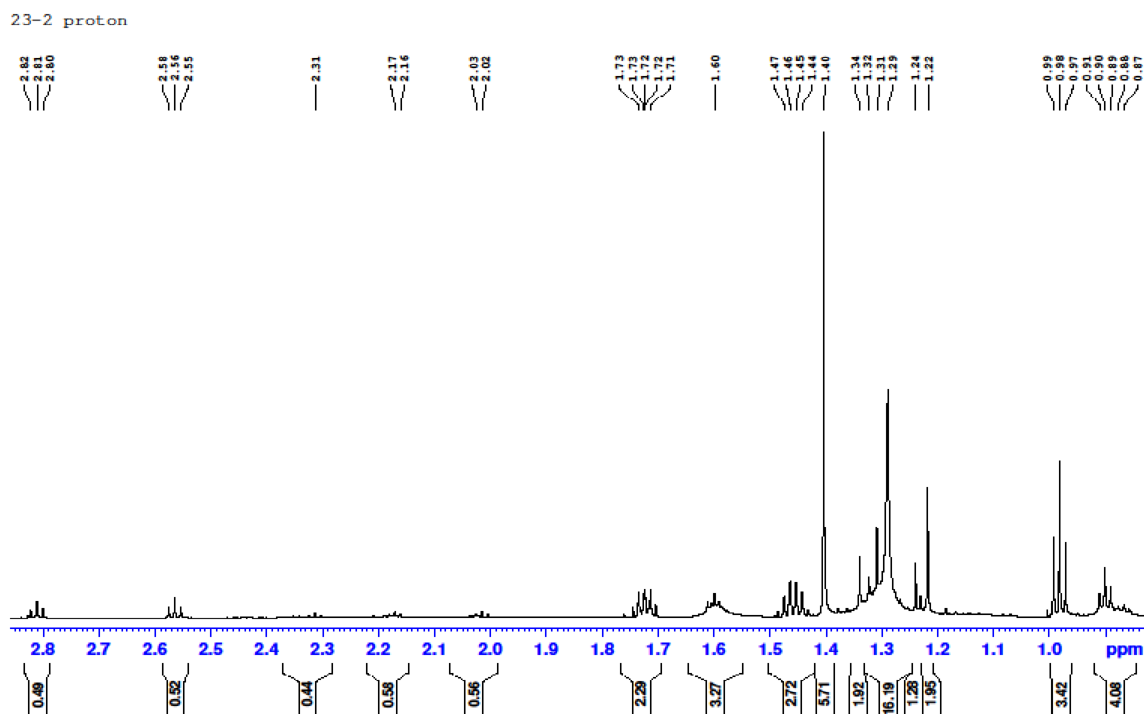


Fig. 3.2 Proton NMR chemical shifts of lignan glycoside .

Table 3.13 Shows NMR spectroscopic data of the lignan glycoside

Position	Proton NMR	J(Hz)	^{13}C NMR	DEPT
C1	4.8		120	CH
C2	5.5		114	CH
C1’			129	CH
C3’	7.4	1.2	132	CH
C4’	2.8	3.6	125	CH

C2''			128	CH
C3'''	6.9	2.3	136	CH

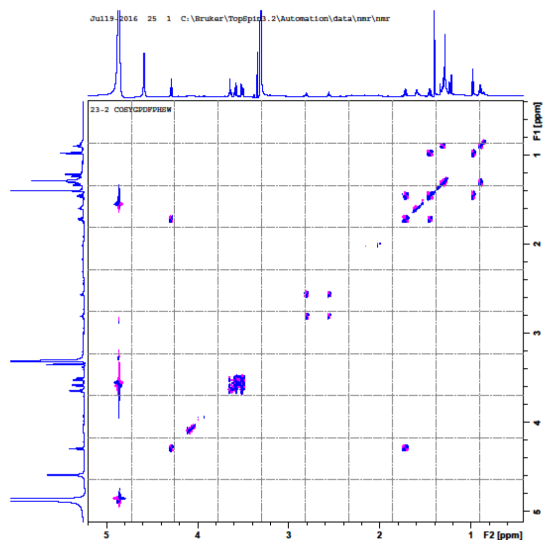
Sugar 1 of lignan glycoside

Position	Proton NMR	13CNMR	DEPT
C1		81 (c—o)	
C2	3.5	75 (C—OH)	CH_2
C3	1.6	13 (R)	CH
C4	1.7	35	CH
C5		39	CH_2
C6	4.5	65 (CH_2-O)	

Sugar 2 of lignan glycoside

	1.4	**30**	**CH_3**
	1.6	14	CH_3
	1.3	29	CH_3

Fig. 3.3 2D NMR of lignan compound



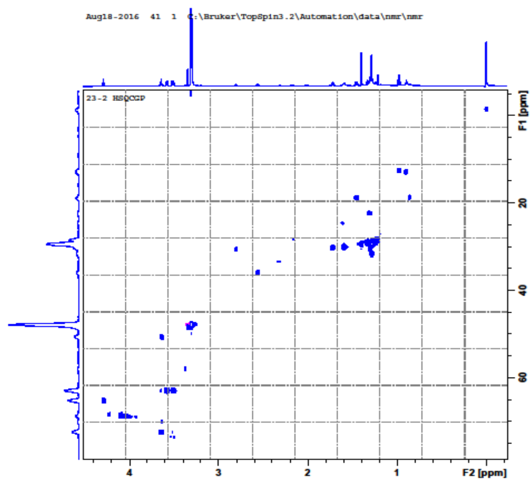


Fig. 3.4 HSQC of lignan compound

Table 3.14

Results of 2D NMR (cosy) and HSQC NMR of lignan glycoside

2D NMR coupling chemical shifts of protons	**Indication**	**HSQC correlations**	**Indication**
5↔2	Ar—CH-	0.9↔10	$(R)_n$
5↔2.4	CH_2=	1.9↔19	$(R)_n$-CH
5↔4.2	C=OH	1.3↔35	CH_2 O—R
5.5↔2.2	CH=	3.5↔70	CH_2-OH

The IR spectrum indicated the presence of hydroxyl group (3411 cm^{-1}), the presence of aromatic group (1598 cm^{-1}), and the presence of ether aryl bonds (1388 cm-^{1}).

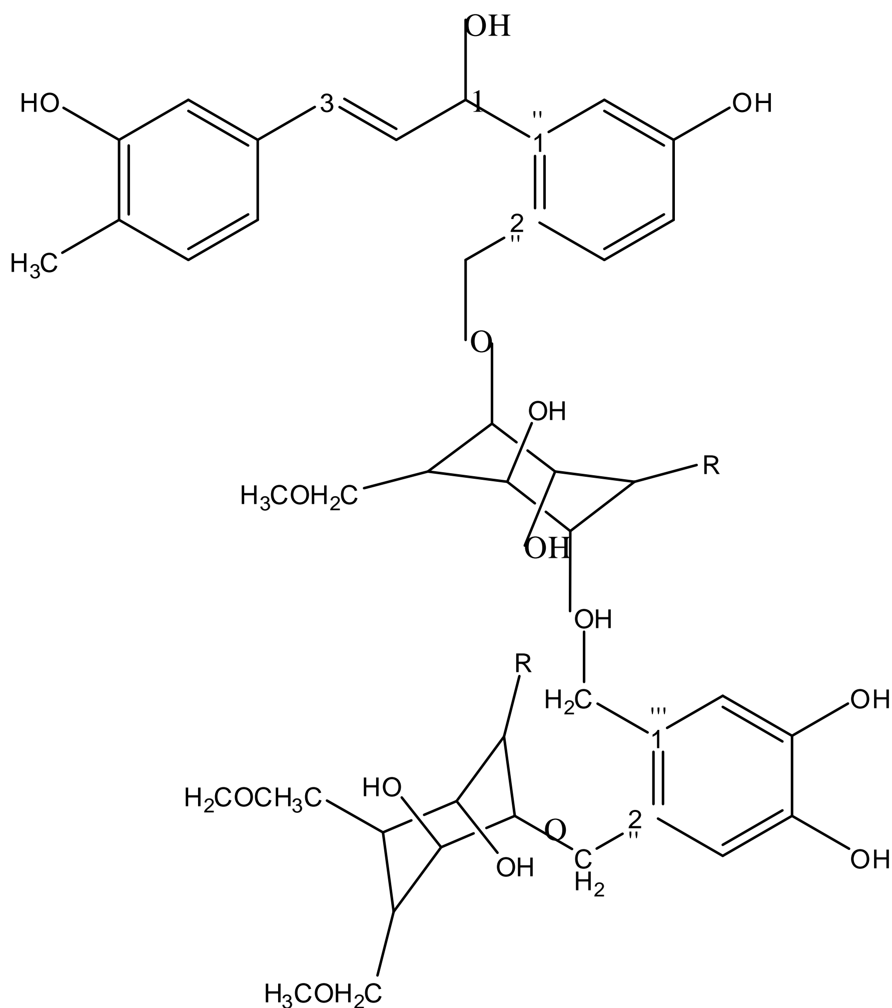
The proton NMR analysis for the same compound indicated two symmetrical aromatic chemical shifts at δ 7.7, 6.5 singlet indicating a phenol with no neighboring protons chemical shifts. 4.3 triplet for Ar-CH_3-O.ortho to –OH (J=15). 4.5 sharp signal for Ar-O (J=11,orth substituted).

^{13}C NMR indicated chemical shifts at 136 for aromatic –ether correlation.δ132 presence of a phenol. 129 shift indicates Ar-OCH_2.

Compared with research results,two equivalent aromatic protons at 7.7-7.6.Sharp OH singlet at 6.5 under symmetrical environment.4.3 triplet for Ar-CH3-O.ortho to –OH(J=15).4.5 sharp signal for Ar-O (J=11,orth substituted).

Compound 1:Lignan glycoside. Calculated mass $C_{47}O_{15}H_{63}$.

Fig.3.5 structure of lignan glycoside



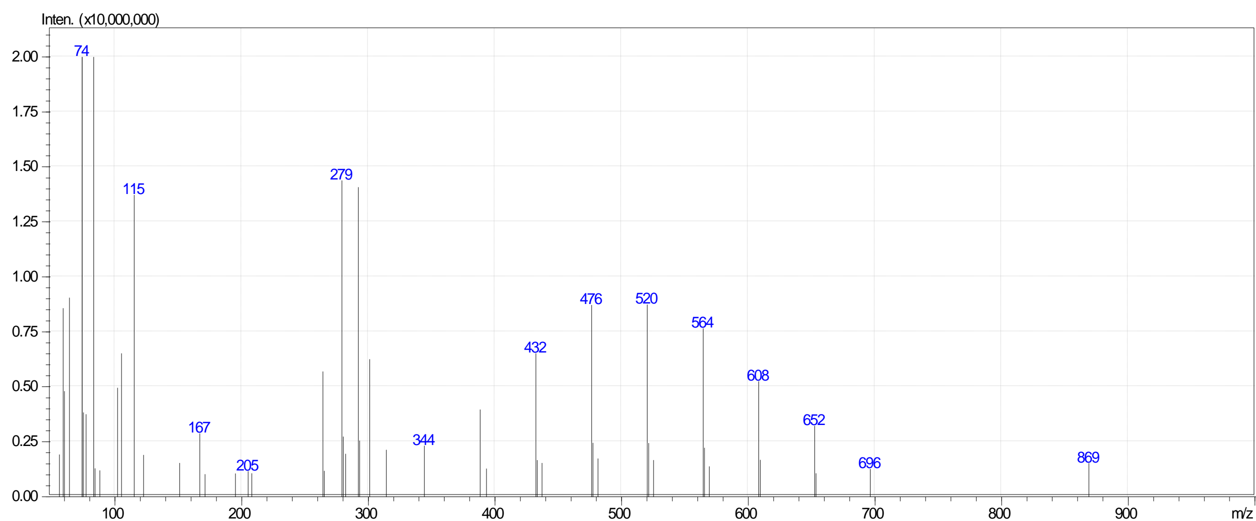


Fig. 3.6 Mass spectrum of lignan glycoside

Fragmentation pattern of lignan glycoside:

Mass of fragments from spectrum were:205, 279, 432, 520, 608 *m/e*

Fig 3.7 Structure of lignan fragment. *m/e* 279

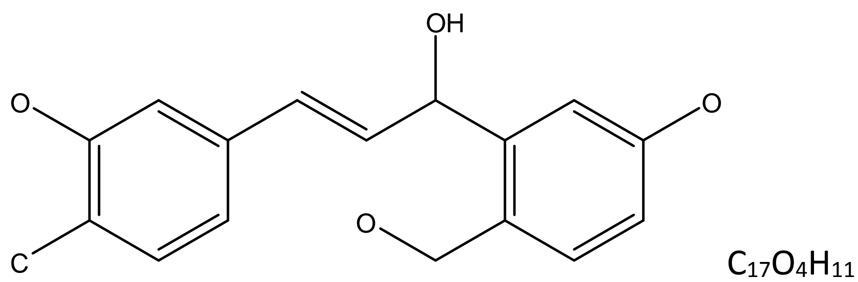
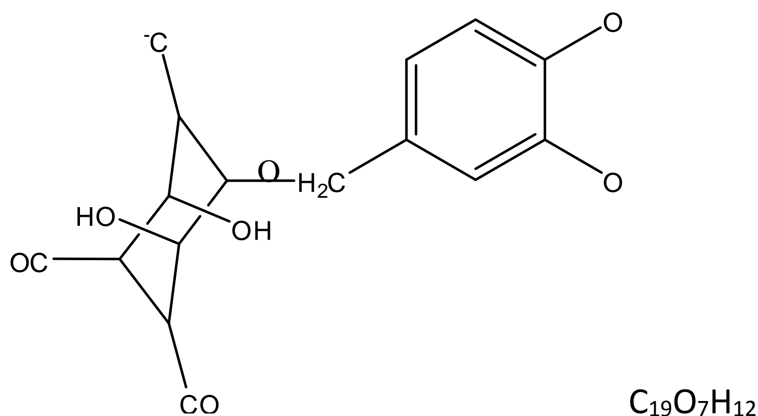


Fig 3.8 Structure of lignan fragment. *m/e* 432



3.8.2 Characterization of compound two (23_3)

Dark brown amorphous solid. Column chromatography mobile phase was chloroform : ethyl acetate (2:8). RSA was 90 % and weight of compound was 0.88 gm .Molecular mass ion at m/z is 745.

IR frequencies :3388 cm-^{1} is OH stretch. 2831 cm^{-1} is C-H –stretching frequency. 1728 cm^{-1} is a carbonyl stretch –C=O- ,but highly shielded . 1596 cm^{-1} aromatic –C=C- stretch .1112.8 cm^{-1} is aryl-O-CH_2 bending.

Proton NMR chemical shifts : 8.5 singlet (1 H) of an amide meta substituted on an aromatic ring (J 2.8). 7.7-6.7 protons, with respect to carbonyl group appear as two doublets with large J values (5.9). 6.8 ,6.7, 6.5 ,sharp singlet, is a chemical shift of -OH- on aromatic ring. 5.5-5.3 is the double bonds shifts region. 4.5 triplet considered for –CH_2-O-glucose.3.5-3.2 CH_2-OH of glucose unit. 1.5 is chemical shift of cyclohexane of glucose units.1-0.9 aliphatic group (R) shifts.

^{13}C NMR chemical shifts : 139 ,129, 128,. aromatic carbon. 114-125 is chemical shift of carbonyl carbon of chalcone. 70 - 60 is aliphatic CH_2OH shift. 35-30 are carbon shifts of-CH_2- sugar moieties .

Cosy 2D NMR : 1.5......4.5 glucose –O-CH_2-.3.5......3.6 glycerol units connected.

2.5.......2.8 CH_2 in cyclo hexane. Also CH_3-N gives a a proton chemical split around this value. 1.6......1.5 sugar multiples.

1.5.........1 aliphatic group attached to sugar.

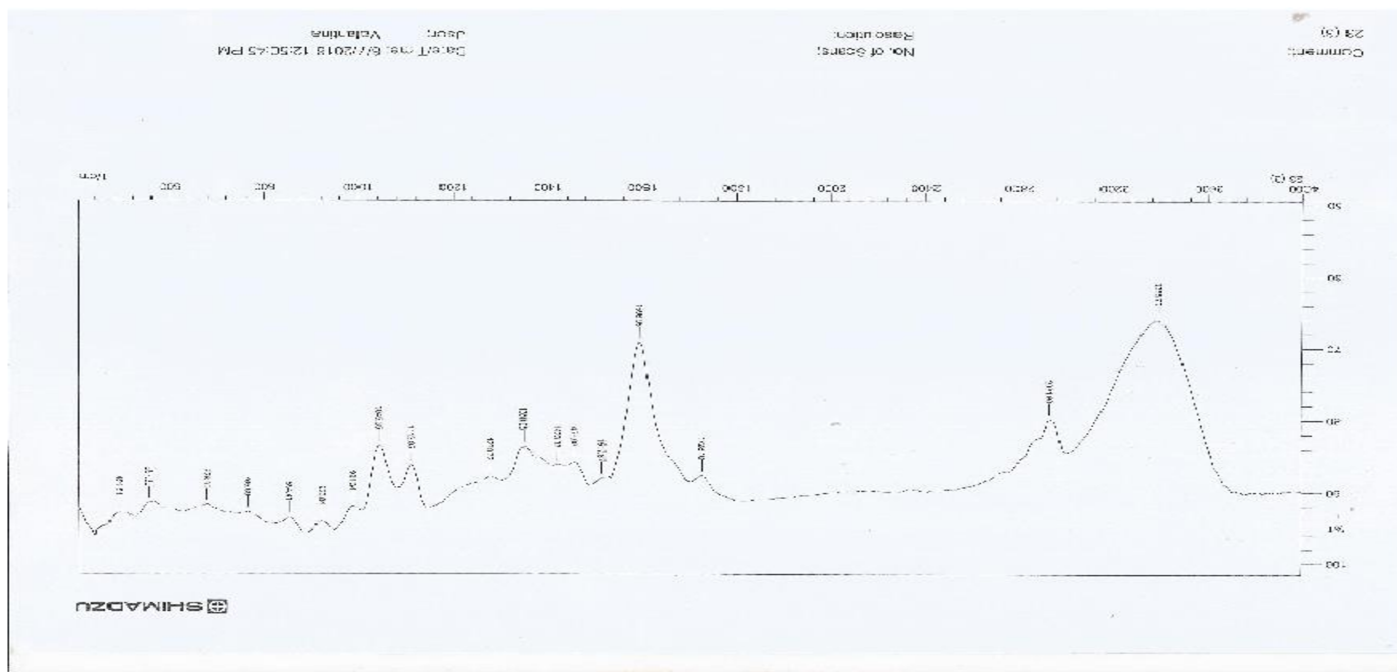


Fig. 3.9 IR spectrum of chalcone glycoside

Table 3.15 IR functional group identification of Chalcone glycoside

IR frequencies cm^{-1}	Indication
3388	-OH- stretch
2831	-CH- stretch
1728	-C=O- carbonyl stretch
1596	Aromatic –C=C- stretch
1112.8	Aryl –O-CH_2 bending

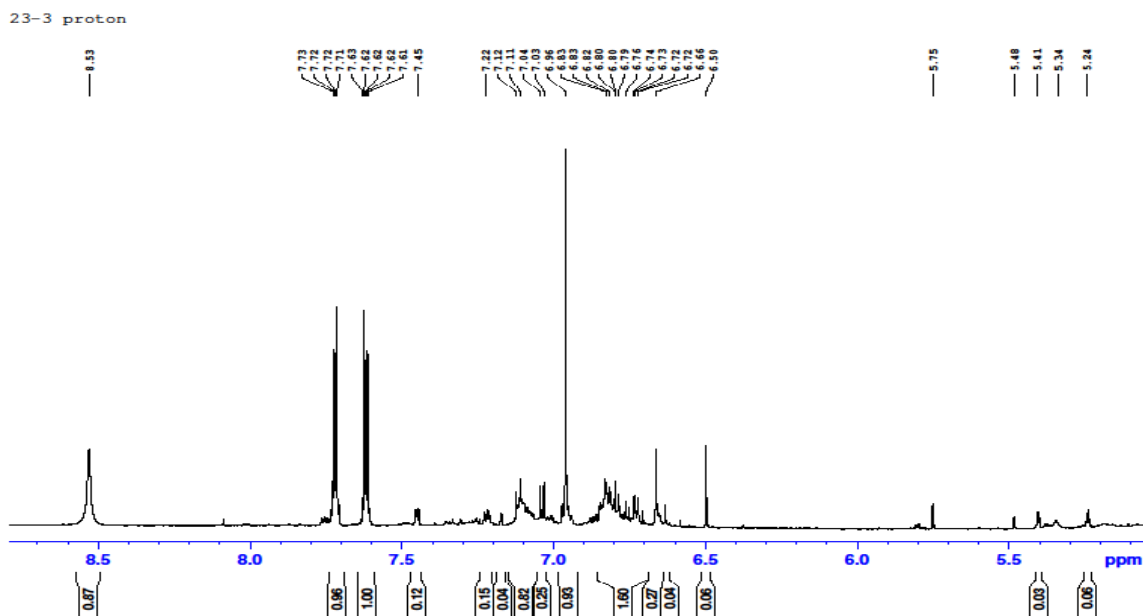
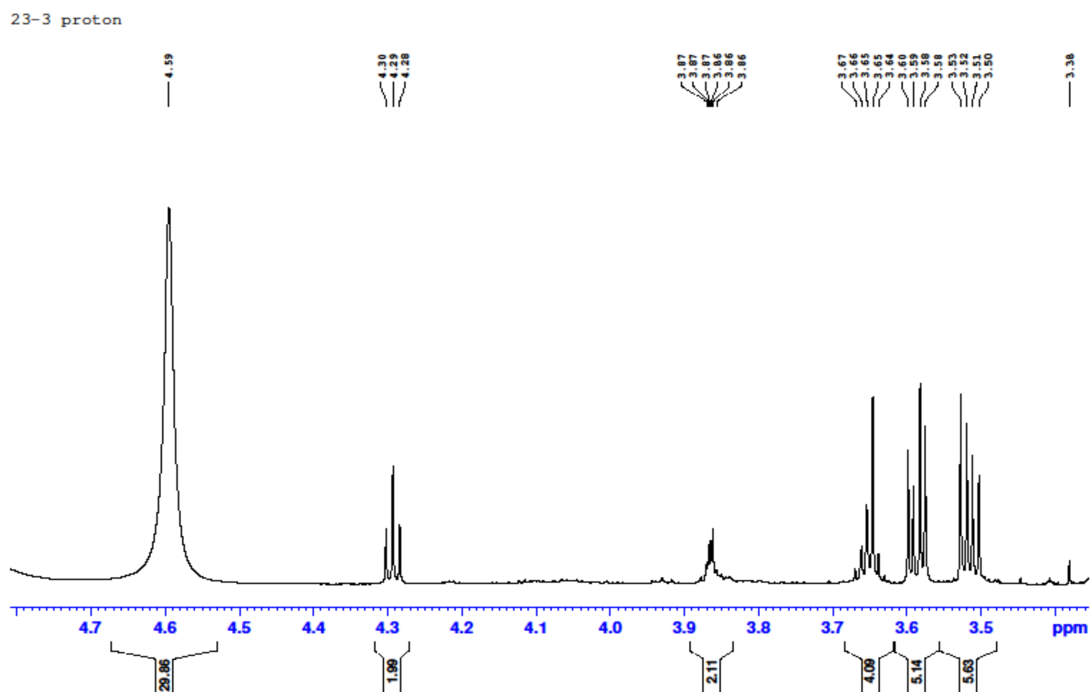


Fig. 3.8 Proton NMR chemical shifts of chalcone glycoside



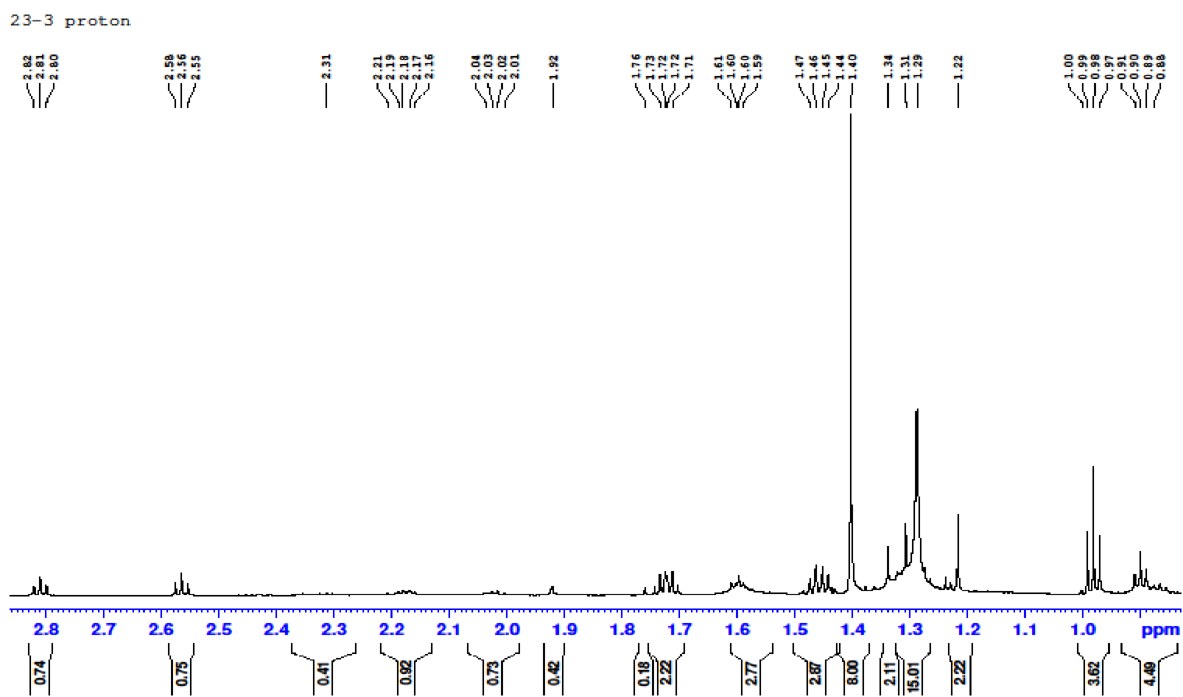
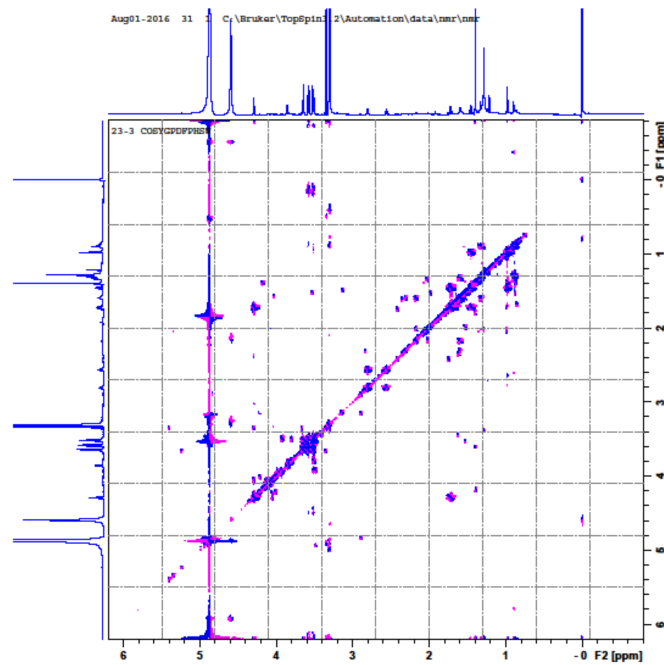


Fig. 3.8 Proton NMR chemical shifts of chalcone glycoside

Table 3.16: Spectroscopic identification for NMRof Chalcone glycoside.

Position	**^{1}H NMR**	**J (Hz)**	**^{13}C NMR**	**DEPT**
C1	4.3		120	CH
C2	4.3			
C3	5.5		114	CH
C4	8.5	2.8	139	CH
C5	7.7	5	132	CH
C1'	5.5		130	CH
C3'	6.8	2.5	128	CH
C4'	6.5			
C1''	3.5		73	CH_2
C2''	3		60	CH_2
C3''	3.2		30	CH
C4''	2.8		31	CH

Fig 3.9 2D NMR of chalcone glycoside



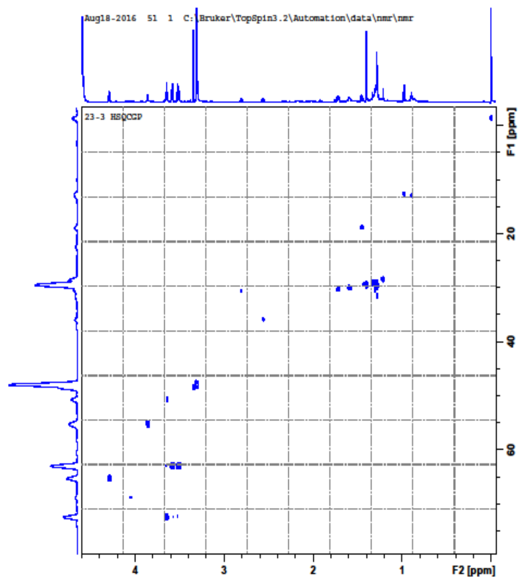


Fig. 3.10 HSQC carbon/proton coupling of chalcone glycoside

Table 3.17: 2D NMR proton/ proton coupling and HSQC carbon/proton relation for chalcone glycoside

2D NMR coupling chemical shifts	Indication	Carbon/proton NMR chemical shifts (HSQC)	Indication
0.9↔1.2	(CH3)n	3.5↔65	CH_2-OH
0.6↔4.5	N—CH_3	1.5↔30	Cyclohexane-CH_2
0.9↔3.5	CH-O-CH_3		
4.5↔5.5	Ar-C=O		
2.2↔5.5	=CH-CH_2O-		
2.2↔3.4	CH_2-O		
1.5↔2.5	CH_2-CH_3		

The IR spectrum for the chalcone indicated the presence of hydroxyl group (3388 cm^- the presence of –CH- stretch (2831cm^{-1}), the presence of a carbonyl group (1427 cm^{-1}), the presence of aromatic electrons (1596 cm^{-1}) and the presence of aryl-O-C-bending (112.8 cm^{-1})

^{1}H NMR, 8.5δ singlet meta substituted (J 2.8). 7.7-6.7 protons, with respect to carbonyl group appear as two doublets with large J value.6.8,6.7. A chemical shift

6.5 ,sharp singlets (no neighbouring protons), is a chemical shift of -OH- on aromatic ring.(meta substituted J=3 Hz and para 6.7).

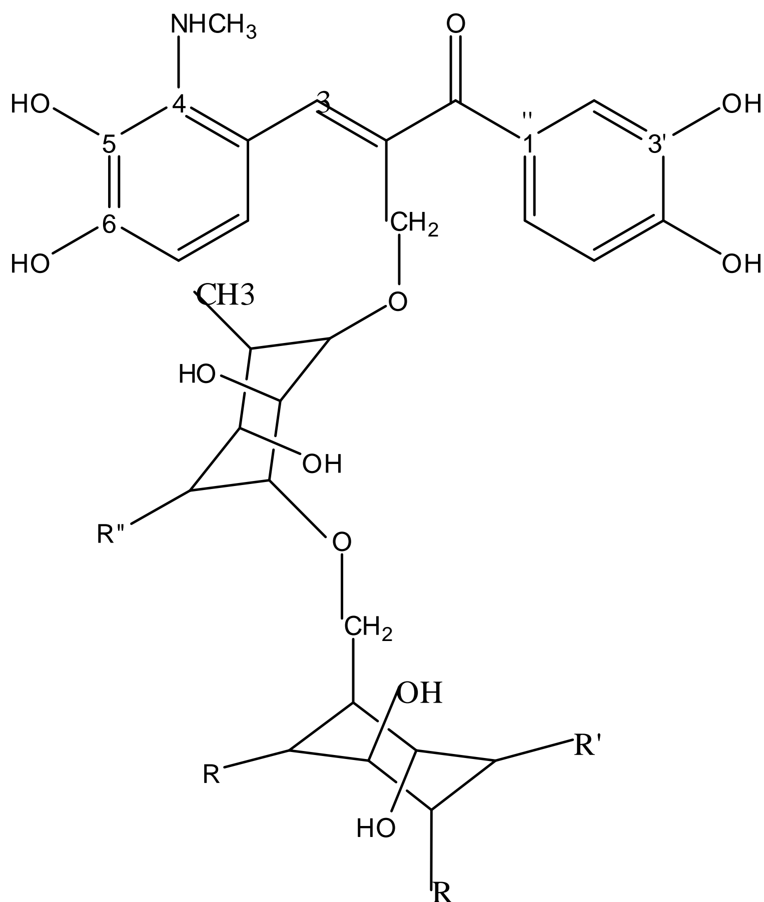
^{13}C NMR, 139 chemical shift at C4" of Ar-NH-.Chemical shift at 129 at C4 for ArOH. δ115 at C2" for double bonds. α and β protons of chalcones appear as two douplets with large J value ranging 6.4 -7.5 ppm. 13C values 116- 128 for α carbon and 139-145 for β carbons with respect to carbonyl. .(Sheik Khadar,et al.2015).

^{13}C NMR chemical shifts : 139 ,129, 128,. aromatic carbons.114-125 is chemical shift of carbonyl carbon of chalcone. 125αcarbon with respect to carbonyl group.

^{1}H NMR ,7.7-6.7 protons, with respect to carbonyl group appear as two doublets with large J values. 6.8 ,6.7, 6.5 ,sharp singlets (no neighbouring protons), is a chemical shift of -OH- on aromatic ring.(meta substituted J=3 Hz and para 6.7 Hz).

Compound 2: Chalcone glycoside.

Fig.3.11 Strucrure of chalcone glycoside. Calculated mass $C_{39}O_{13}H_{55}$



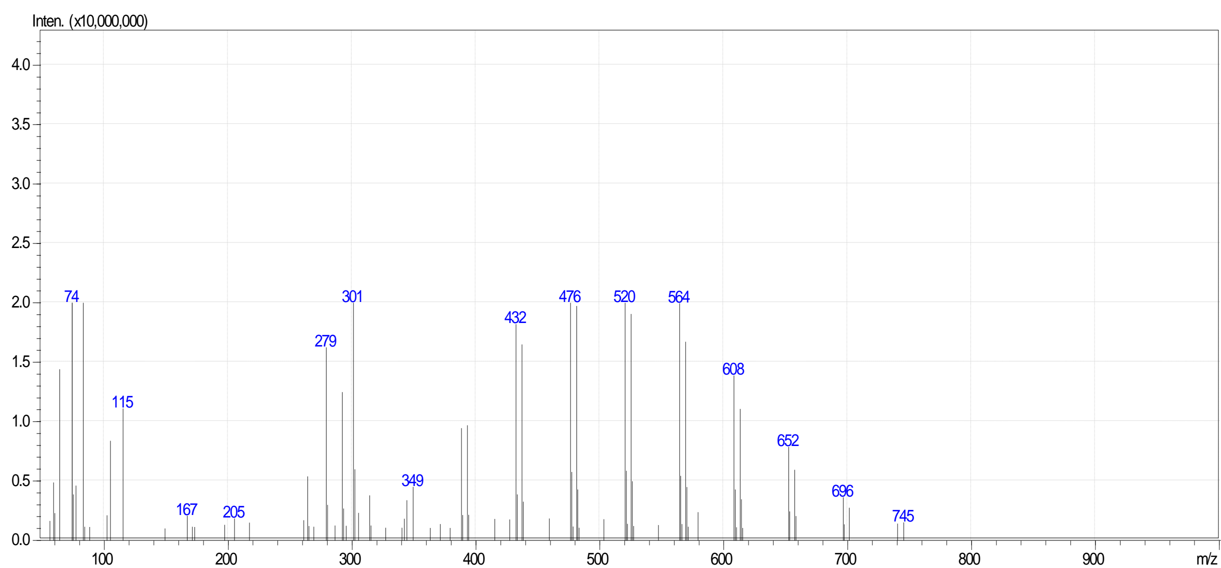


Fig. 3.12 Mass spectrum for chalcone glycoside

Fragmentation pattern for chalcone glycoside:

Mass of fragments for chalcone glycoside were 74, 115, 205, 432, 520, 652 *m/e*.

Fig 3.13 Fragment of chalcone of mass *m/e* 115

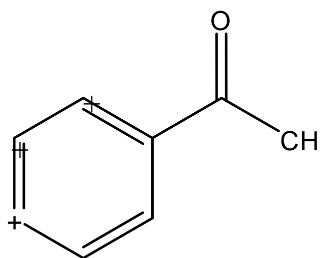
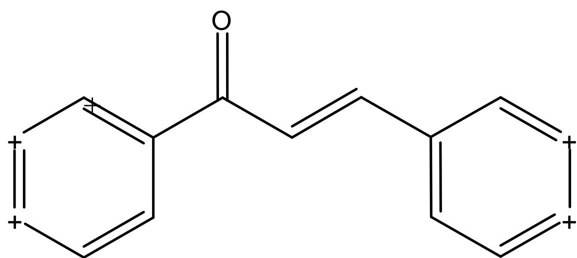


Fig 3.14 Fragment of mass *m/e* 203



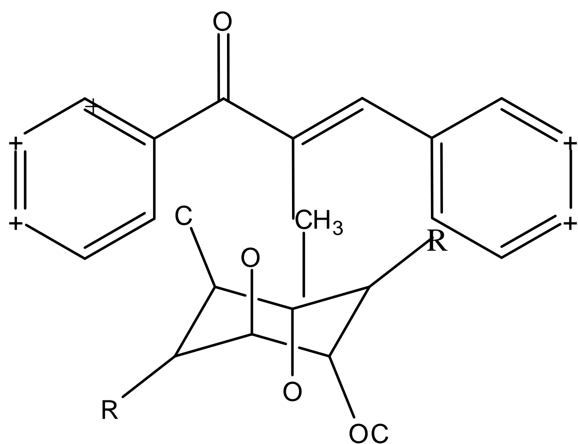


Fig 3.15 Fragment *m/e* 436

3.8.3 Characterization of compound three (24_1)

Compound 3:Flavone glycoside

Dark brown solid. . Column chromatography mobile phase was chloroform : ethyl acetate (1:9). RSA was 89 % and weight of compound was 0.4 gm .Molecular ion mass at mass m/z is 745.

Infra-red readings : 3360 cm^{-1} broad –OH- stretch. 2929cm^{-1} C-H stretch. 1602 cm^{-1} aromatic resonance stretch. 1714 cm^{-1} carbonyl stretch but structurally attached to an aromatic ring.

^{1}H NMR chemical shifts :8.1 is phenol single shift next to electron rich centre.

7.7 is proton shift in C5 adjacent to the fused ring. 6.9 proton shift in C8 singlet (no neighbouring protons). 6.5 proton shift of a phenol. 6.2 is a chemical shift of ph-OAr. 5.3 is a proton in conjugated double bond (singlet). 2.8 is methylene proton between phenyl group and a sugar molecule.

^{13}C NMR : chemical shifts 145,130, 129, 112, 103 .145 , 130 are aromatic ring carbons. 129 is the chemical shift of carbon C7 . 112 is chemical shift of C3 (double bond conjugation). 103 is a characteristic of C-O anomeric carbon of ring C. Chemical shifts 30,29,27,25 are _CH_2_ of the sugar units.

2D proton NMR (cosy): 7.8↔6 , -OH- in the fused ring. 8↔.6 , _OH- in pyridine ring. 2.8↔5.5 (2.8 is chemical shift of –CH_2- attached to resonating aromatic ring.). 1.9↔1.5 (methyl group proton in a hexyl glucose molecule)

HSQC: Chemical shifts.130↔5.5 double bonds of aromatic ring.

110↔6.1 singlet (-OH in a conjugating system and no neighbouring protons).

100↔6.8 singlet , –OH in a conjugating system. 50↔30 splitting signal

30↔1.9 is CH_3 proton next to OH group of a sugar.

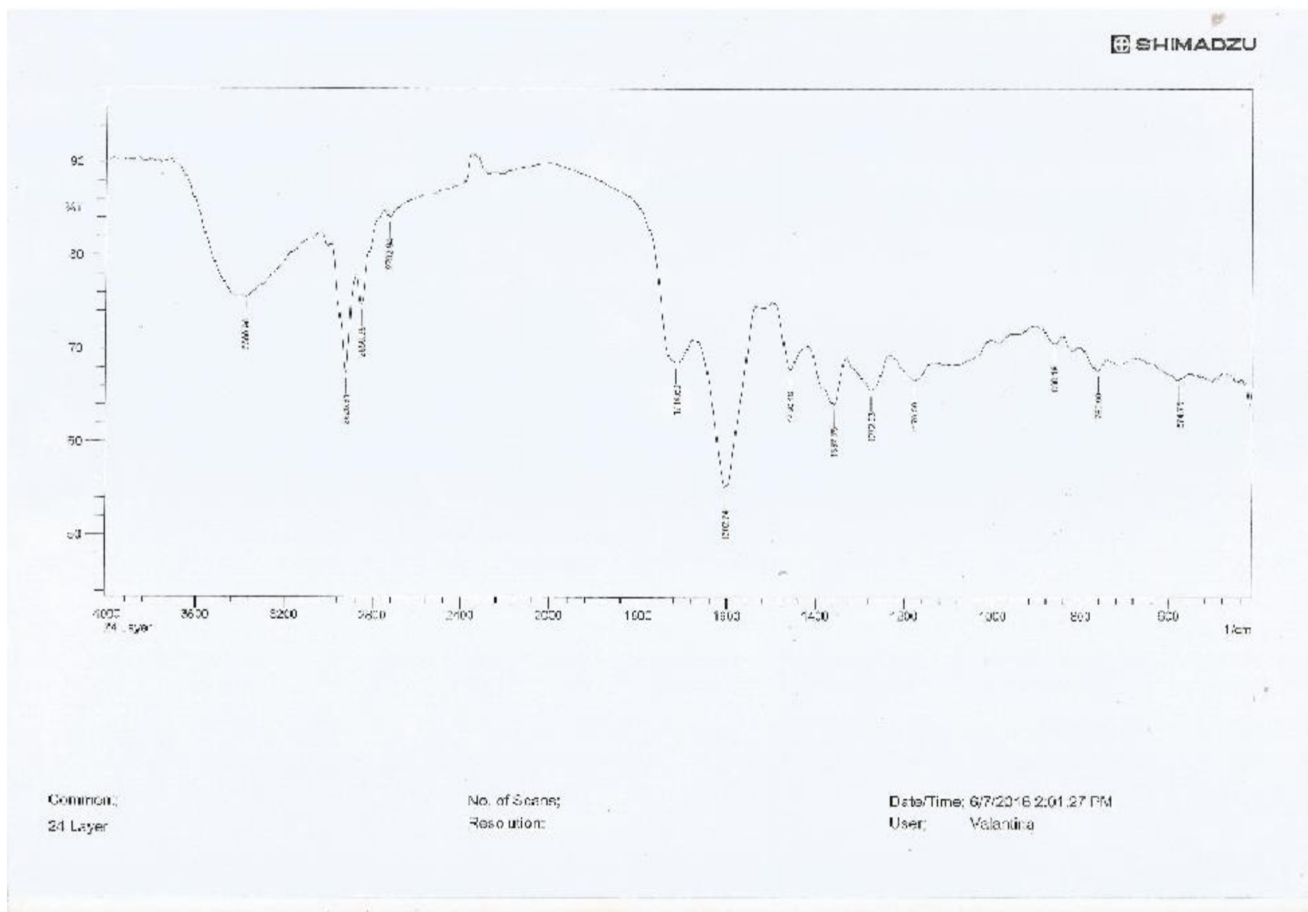
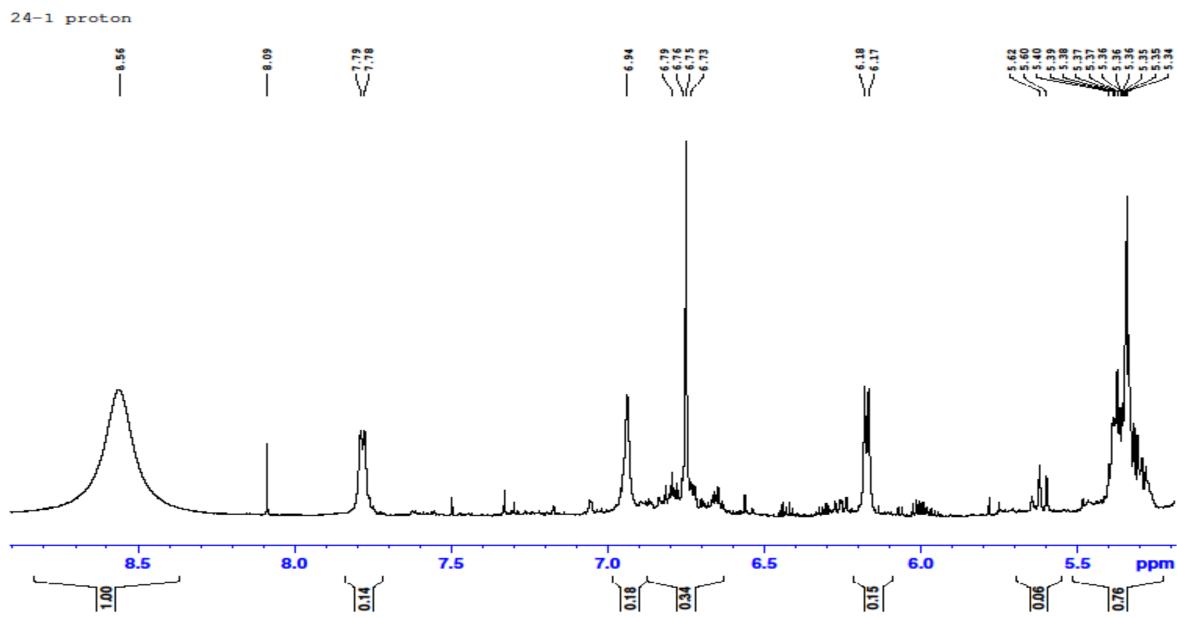


Fig. 3.16 IR spectrum of flavone glycoside

Table 3.18: IR functional group identification of the flavones glycoside compound.

IR frequency(cm^{-1})	Indication
3360	Phenolic stretch
2929	CH- stretch
1714	Carbonyl stretch
1602	Double bond and aromatic resonance stretch.



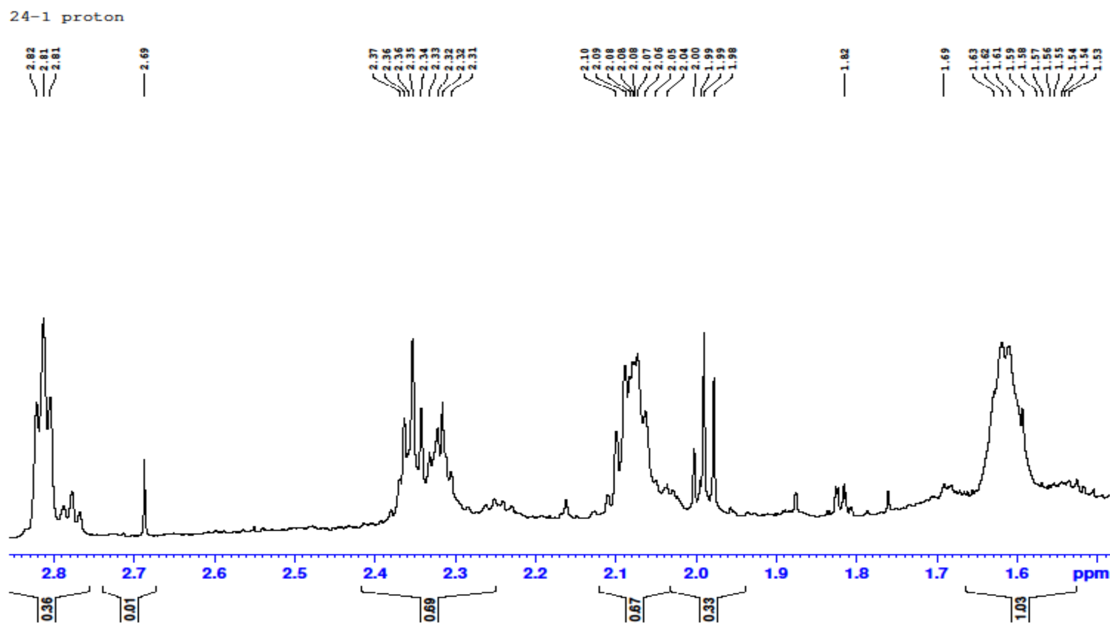
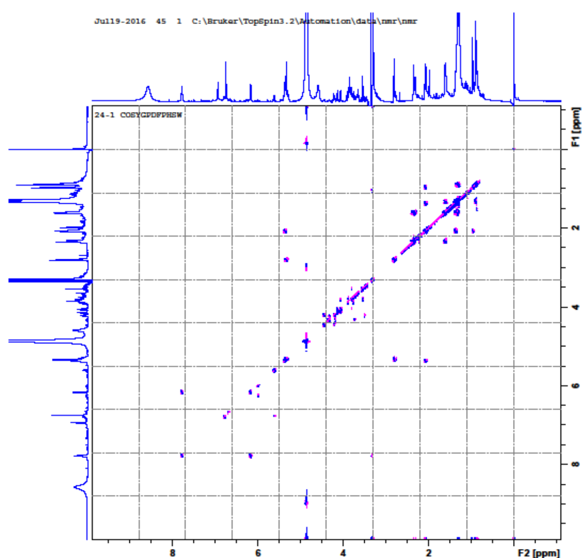


Fig. 3.17 Proton NMR chemical shifts for flavones glycoside

Table 3.19: Spectroscopic identification of NMR for Flavone glycoside

Position	^{1}HNMR	J(Hz)	^{13}CNMR	DEPT
C2	8		103	CH
C4	8.5		112	CH
C7	7.9		135	CH
C2’	6.1	1.2	129	CH
C4’	6.7	8	145	CH
C1’’	2.8			
C4’’	5.3		126	
C5’’	2.3			
Sugar1 C6	2.3		38	CH2
C2	1.6		25	CH3
C3	1.5		16	CH3
C4	3.8		35	CH2
Sugar2 C1	2.8		38	CH2
C2	3.5		30	CH2
C3	3.5		60	CH2
C5	1.3		27	CH3

Fig. 3.18 2D proton/proton coupling for flavone glycoside



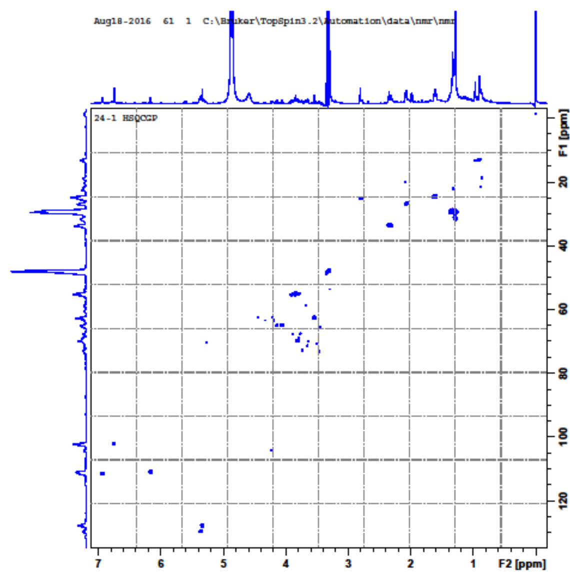


Fig. 3.19 HSQC carbon/ proton coupling of flavone glycoside

Table 3.20 : 2D NMR proton/ proton coupling and HSQC chemical shifts for flavone glycoside

2D NMR proton coupling chemical shifts	Indication	HSQC SHIFTS	Indication
2↔5	CH2-Ar	1↔19	CH3
2.5↔5	CH=CH	1↔14	R-CH3
6↔8	Aromatic and phenolic	1.5↔20	R-cyclohexane
6↔7.8	Phenolic protons and fused ring	1.6↔30	CH2-cyclohexane
		2.5↔38	CH2-O

The infra red frequencies of the isolated flavone indicated the presence of medium N-H stretch(3360 cm^{-1}) ,-CH-stretch(2929), carbonyl group stretch band (1714) and bouble bonds and aromatic resonance (1602).

Proton NMR for the flavones were wide signal at 8.5 ppm for a proton meta to N atom (J=2) .Sharp single signal at 8.1 next to an electron rich centre.7.7ppm signal for fused rings.6.5 and 6.7 are signals of meta substituted phenols.6.2 ,6.0 singlets are protons ortho to OH (J=9).5.3 signal at double bond area.5.8 doublet of doublet Ar-O-CH- proton in a conjugating double bond area.

2D NMR chemical shifts for the isolated flavones indicated proton correlation between H and N at 8 and 6 chemical shifts. Sharp splits when CH_2 correlated to

C=C and CH-Ar bonding at (5.5and 2.8) ppmThe correlation between 1.9 and 1.5 shifts indicates methyl proton in hexyl sugar molecule.

^{13}C NMR for the isolated flavones is 145 of phenol carbon. 130 ppm is a shift for aromatic carbon.112 ppm double bond carbon shift and 103 is –C-O- on ring C.

Lastly for HSQC 130↔5.5 double bonds correlated with aromatic ring.

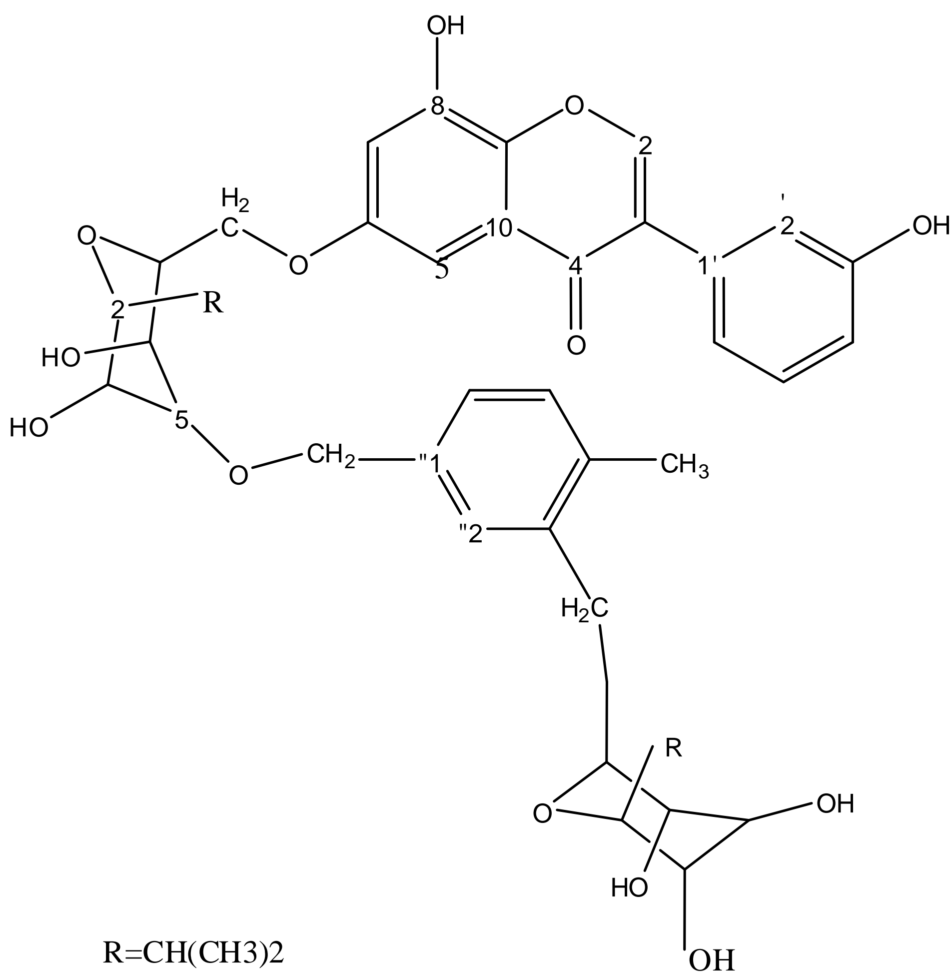
110↔6.1 is a correlation of C-OH in a conjugating system .

100---6.8 carbon in resonating area and a proton downfield due to N atom.

The ^{1}H-NMR spectrum of the isolated compound showed signals at chemical shifts of aromatic region δ 6-8 ppm. The 1H-NMR spectrum of the isolated compound displayed two meta-coupled doublets at δ 6.4ppm (d, J = 2.0 Hz, 1H) and δ 6.21 ppm (d, J = 2.0 Hz, 1H) respectively for aromatic protons H-6 and H-8 on the ring A. The position of H-6 and H-8 are meta to each other. This is evidenced by the coupling constant values (J) 2.0 Hz, which is Jmeta 1-3 Hz This also proves the ring A contain two substituents at position C-5 and C-7. Two doublet signals at δ 6.92 ppm (d, J = 9.1 Hz, 1H) and δ 7.86 ppm (d, J = 9.1 Hz, 1H) showed the orientation of the ortho proton signal for the proton H-3', 5' and H-2', 6' ring B with the substituents at position C-4'. A singlet signal at δ 6.61 ppm (1H, s) showed proton H-3 in ring C has isolated proton. The 1H-NMR spectrum exhibited proton signals characteristic of flavone nucleus. (Sovia Lenny,et al.2013).

ProtonNMR values 8.1- 6.5 which are aromatic.6.5-6.2 coupled aromatic protons (J= 3Hz) meta substituted.7.7 shift is aromatic proton of fused rings with very small coupling constant.Protons H*2 and H*4 show singlet signals on ring B.J=9 Hz ortho substituted.6.9 singlet is shift for proton α to a carbonyl .5.5 is double bond shift and 6.4 is an isolated proton of ring C.

Fig.3.20 Structure of flavone glycoside



Flavone glycoside structure . Calculated mass $C_{41}O_{13}H_{47}$

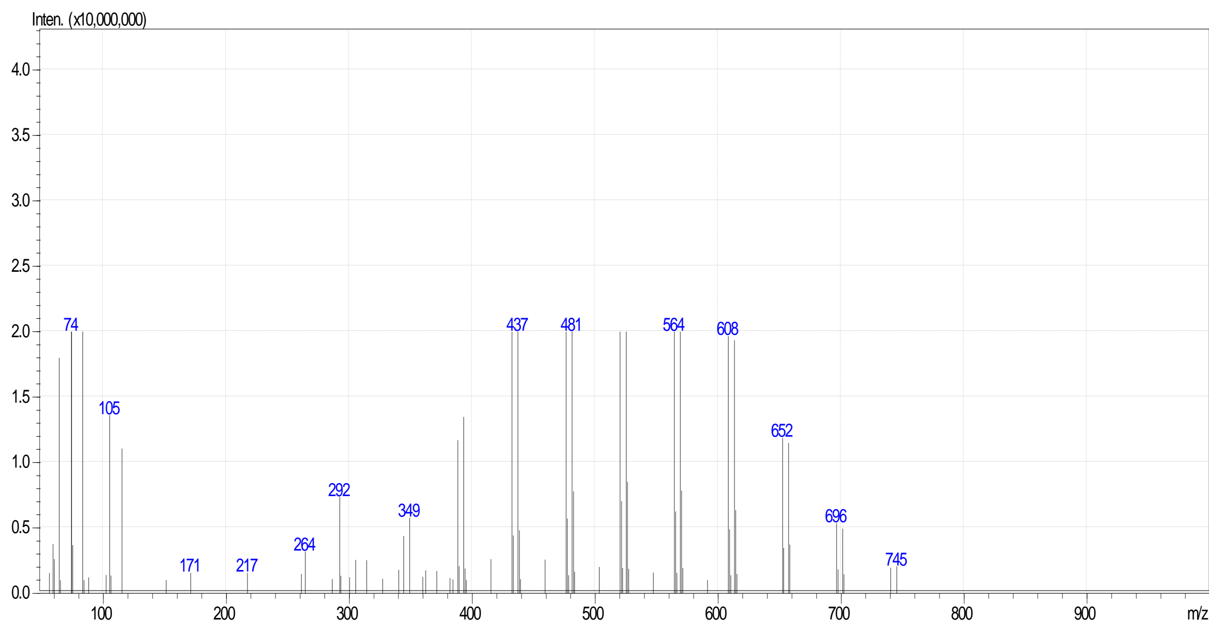


Fig 3.21 Mass spectrum of flavone glycoside

Fragmentation pattern of flavone glycoside :

74,105,171,217,254,437,554,696 *m/e* .The fragments below indicate some of the possible mass fragments.

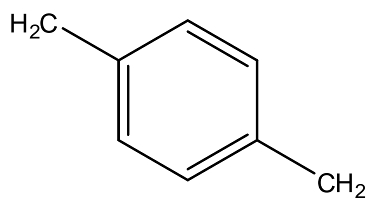
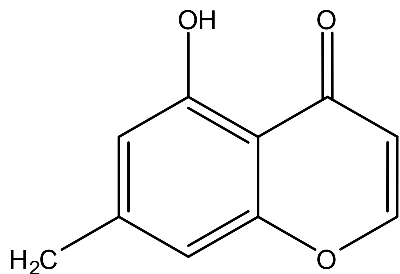


Fig 3.22 *m/e* 103. of flavone fragment



m/e 171 $C_{10}O_3H_3$ Fig 3.23 mass fragment of flavone

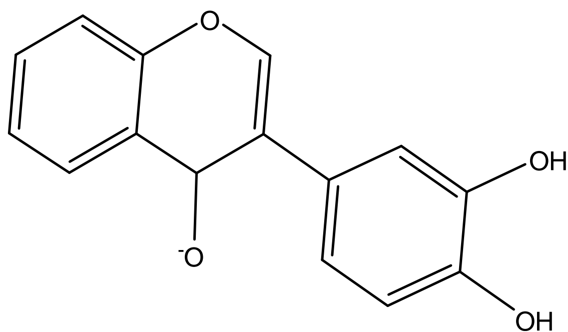
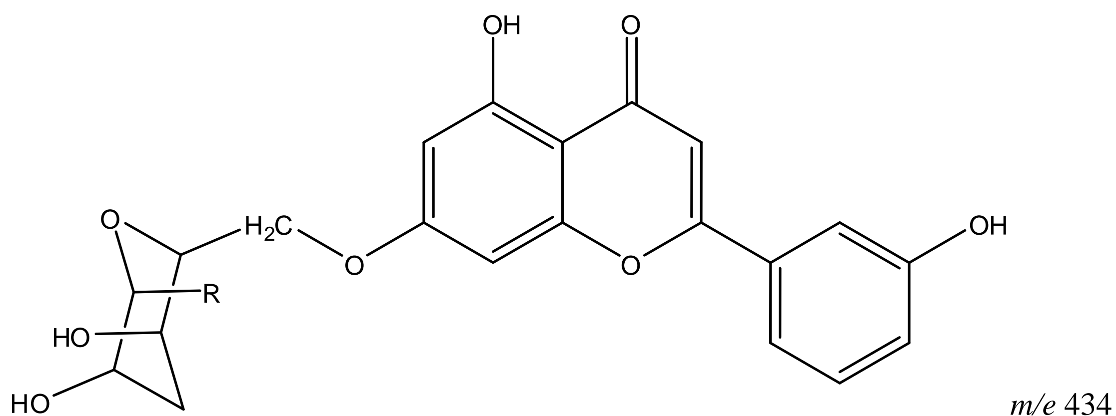
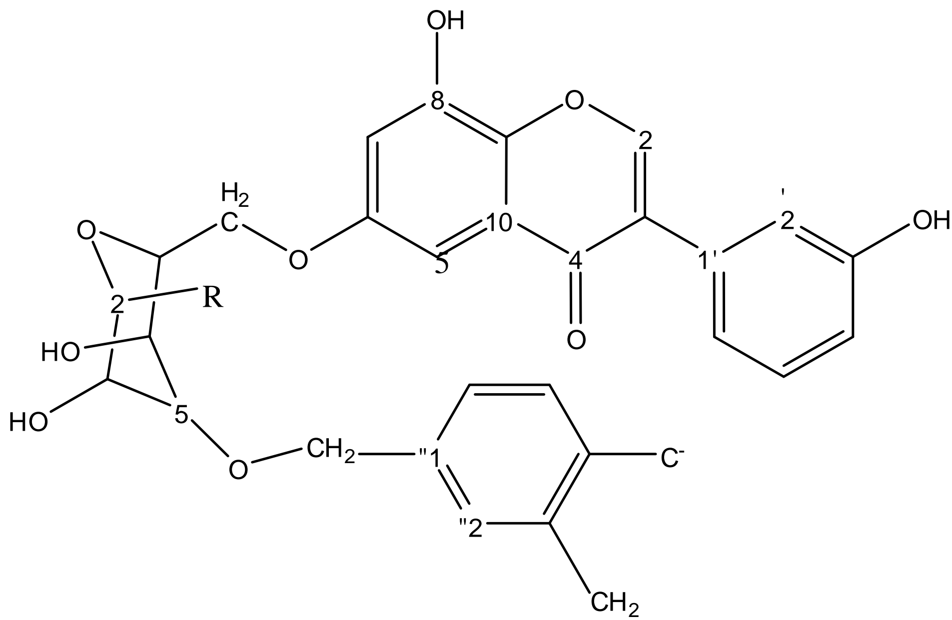


Fig 3.24 *m/e* 254 mass of a flavone unit



R=$CH(CH_3)_2$

Fig.3.25 Larger fragment of flavone glycoside showing glycoside linkage.



m/e 554

Fig. 3.26 Fragment of the sugar molecule linked to the aromatic structures of flavone

3.8.4 Characterization of compound four (sub-7)

Compound 4: Tannic acid glycoside

Brown amorphous solid. Mobile phase for sub-column chromatography was (ethyl acetate : methanol) 4 : 6.weight of fraction 0.16 gm. M+H at m/z 789

Infra –red readings: 1415 cm^{-1} is a bending of carboxylate ion dimer.

^{1}HNMR: 7.8 chemical shifts of resonating aromatic rings. (1 H adjacent to a ring) 7.2.There are sharp characteristic signals at 7.4, 7.2, 7.0, 6.8, 6.6, 6.5 representing phenolic structures .There is identical duplet at 6.2 indicating –COOH-CH_2, attachment of carboxylate to sugar structure.3.9 is proton chemical shifts of –CH2-O .

^{13} C DEPT 90 -CH- chemical shifts 49.5 , 48.7 ,48.5. of hydroxyl groups .

180 i s –C- of Ar-COOH. 67,79 carbon shifts of CH_2OH

2D NMR (cosy): combination of proton chemical shifts: 1.5↔3.5(hydroxyl in cyclohexane i.e sugar molecule). 3.6↔3.8(proton shifts of two adjacent protons with –OH- groups). 2↔3.4 (protons adjacent to –OH- and coo- ion).

HSQC: is a relation between carbon and protons. 4.9↔45 (-OH – attached to aromatic ring. 5.2 proton shift with 105 carbon shift (5.2↔105) shifts of conjugated double bonds.

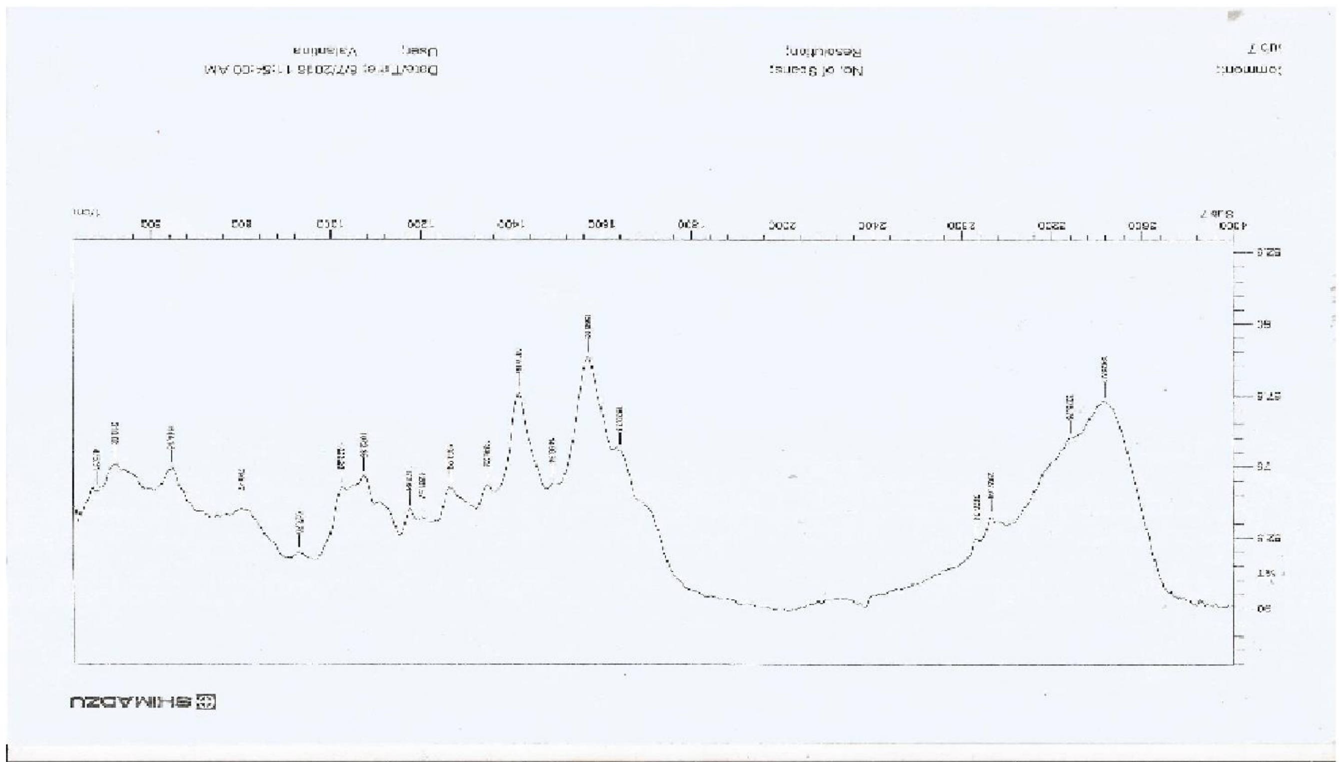
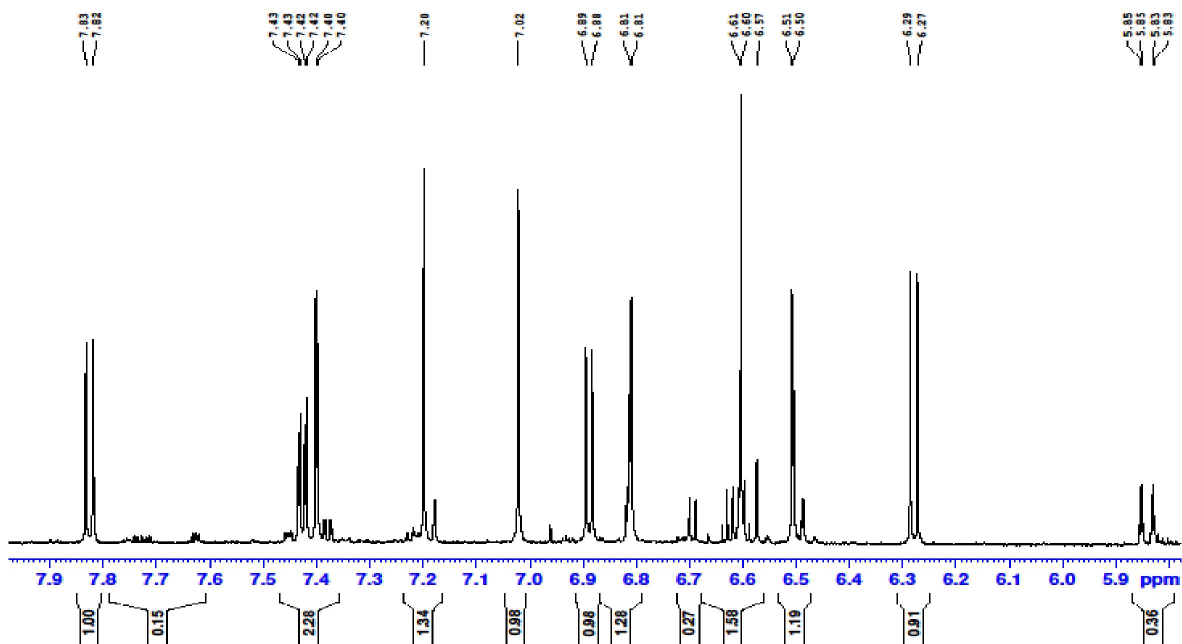
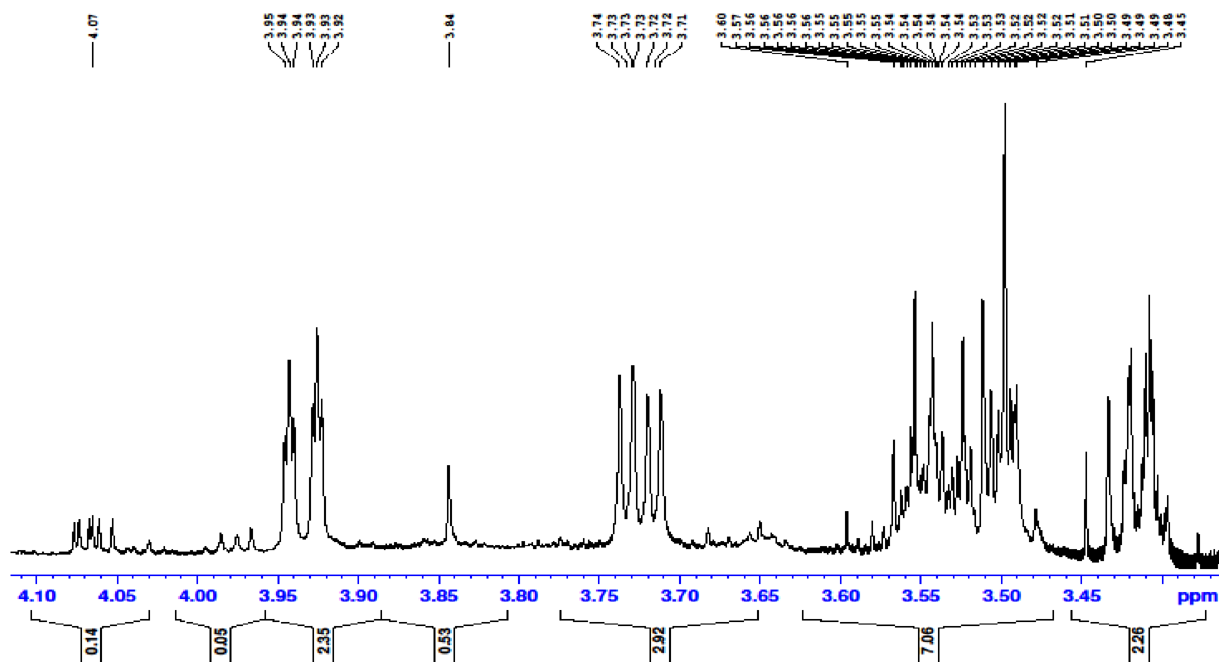


Fig. 3.27 IR spectrum of tannic acid

Table 3.22 IR frequencies of tannic acid glycoside

IR frequency cm^{-1}	Indication
3436	Phenol stretch band (broad)
1588	Aromatic stretch
1415	Carboxylate ion bending


SUB-7 proton
ppm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ppm

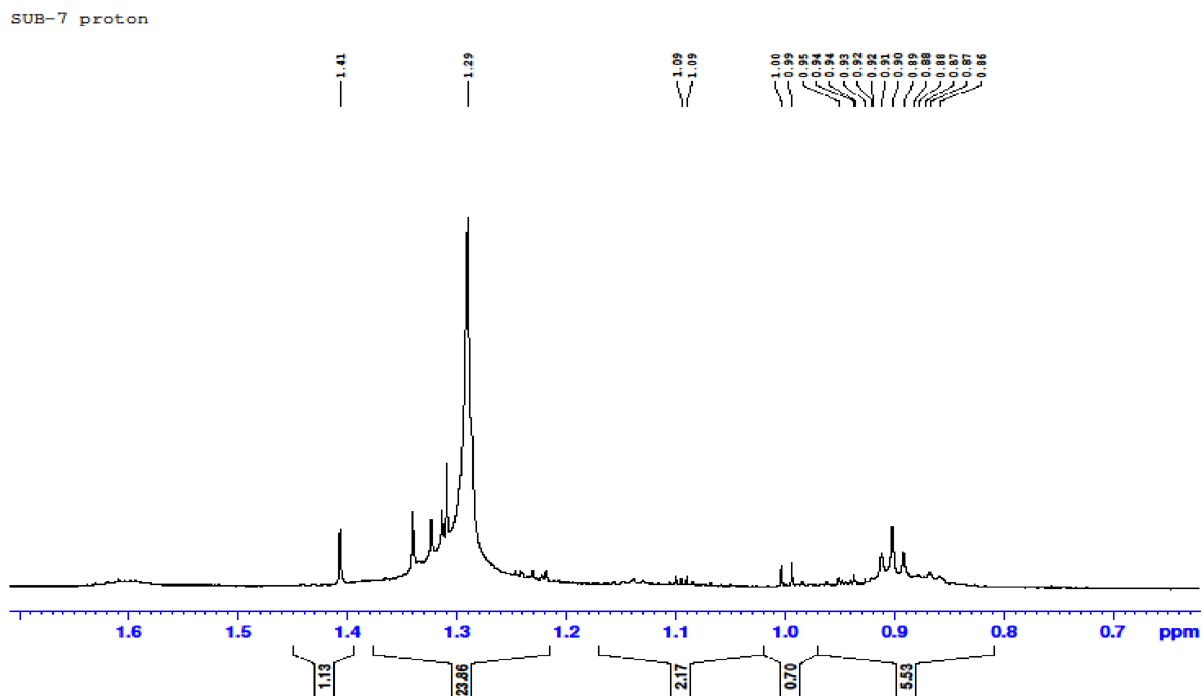


Fig. 3.28 Proton NMR chemical shifts of tannic acid

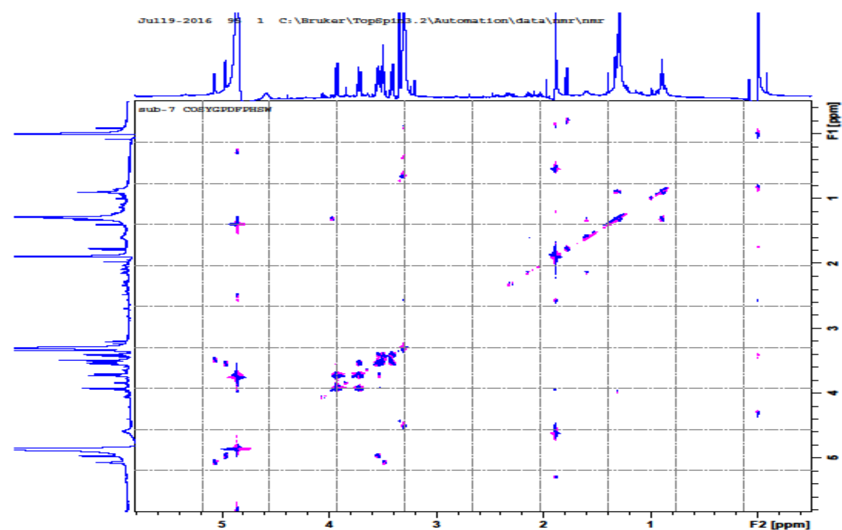


Fig.3.29 2D proton NMR chemical shifts of tannic acid

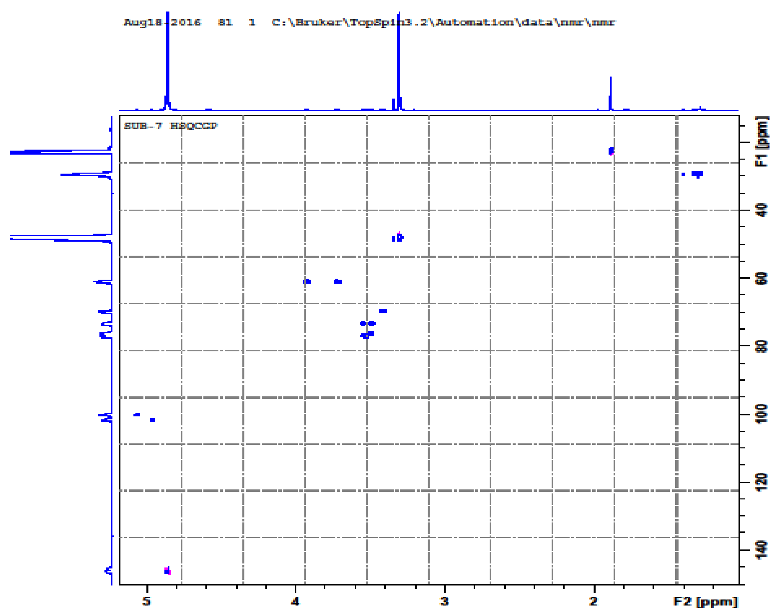


Fig. 3.30 HSQC chemical shifts of tannic acid

Table 3.23: Spectroscopic NMR results of tannic acid

ProtonNMR chemical shifts	J(Hz)	2D NMR	Indication	HSQC NMR	Indication
7.8	7 ortho	5↔4.6	C=O—O	45↔0.9	O—CH3
7.4	2	5↔4	C=O—O	141↔5	Aromatic
7.3	9	5↔3.5	C=CH	110↔5	Double bonds
7.1	11	5↔1	Ar—CH_3	70↔3.5	R—OH
6.8	1 para	4↔1.5	O—CH_3	70↔4	R—O-

3.5↔0.9	CH—CH_3
2—0.9	CH_2—R

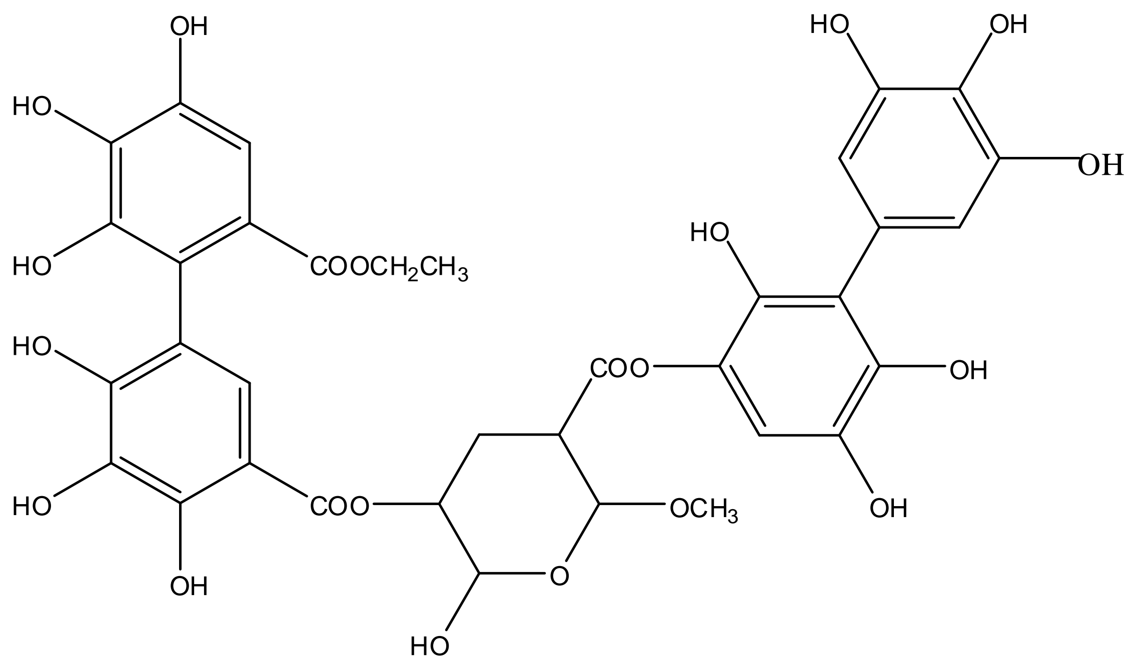
Infra red frequencies of the isolated tannic acid dimer indicated a broad phenolic OH stretch at 3434 cm^{-1}.An aromatic stretch at 1588cm^{-1}.A carboxylate bending at 1415 cm^{-1}

Proton NMR showed a chemical shift at 7.8 of resonating aromatic rings. 7.4 (2H j is small substitution is in meta position).There are sharp symmetrical signals at, 7.2, 7.0, 6.8, 6.6 and 6.5 representing phenolic structures .There is identical duplet at 6.2 indicating –COOH-CH_2,attachment of carboxylate to sugar structure .Also there is a proton shift at.3.9 indicating –CH_2-O .

Concerning 2D NMR combination of proton chemical shifts: 1.5↔3.5 is (hydroxyl in cyclohexane i.e. sugar molecule).Correlation of 3.6 with 3.8(proton shifts of two adjacent protons with –OH- groups).

For HSQC correlation indication of 5.2 proton shift with 105 carbon shift (5.2↔105) of conjugated double bonds.

Fig.3.31 Structure of tannic acid glycoside



Tannin glycoside

Mass of compound 789. $C_{35}O_{21}H_{33}$

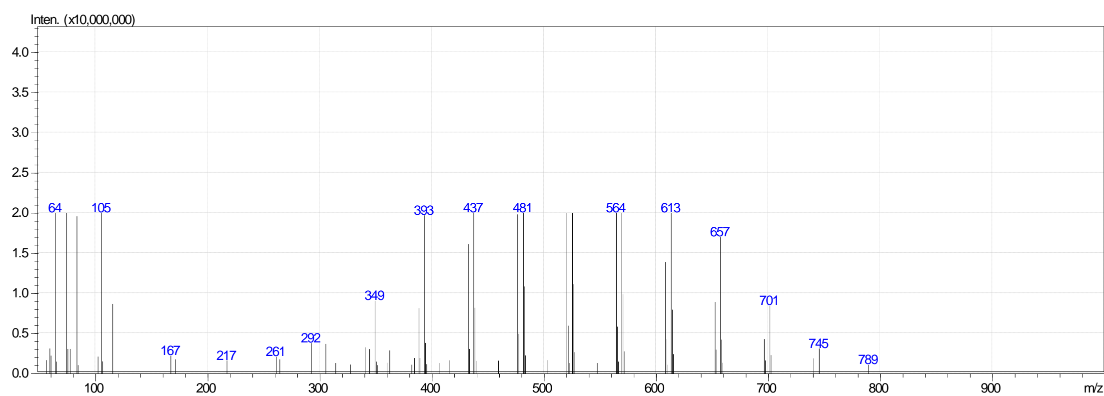


Fig. 3.32 Mass spectrum of tannic acid

Fragmentation pattern of tannic acid glycoside :

Mass of fragments were *m/e:* 64, 105, 167, 217, 261, 292, 349, 481, 564, 613, 667, 701.

The first fragment calculated for tannin

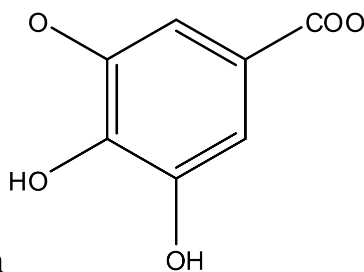


Fig.3.33

$C_7O_5H_3$ *m/e*167

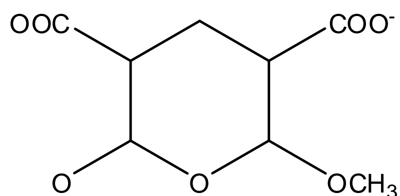


Fig3. 34 Mass fragment for sugar of tannin *m/e* 217

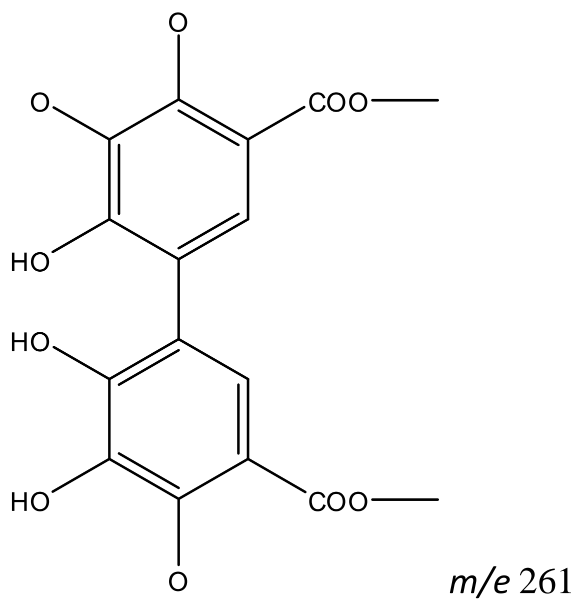


Fig 3.35 Calculated fragment for tannic acid

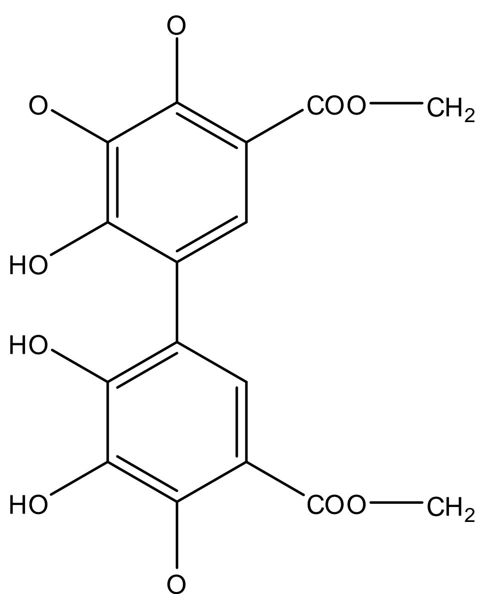


Fig. 3.36 fragment of tannic acid. *m/e* 292

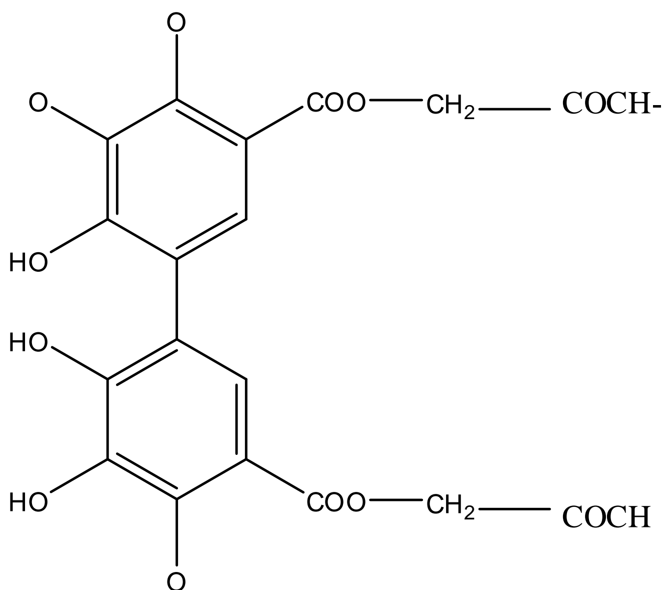


Fig. 3.37 Fourth calculated fragment of tannic acid glycoside. *m/e* 349

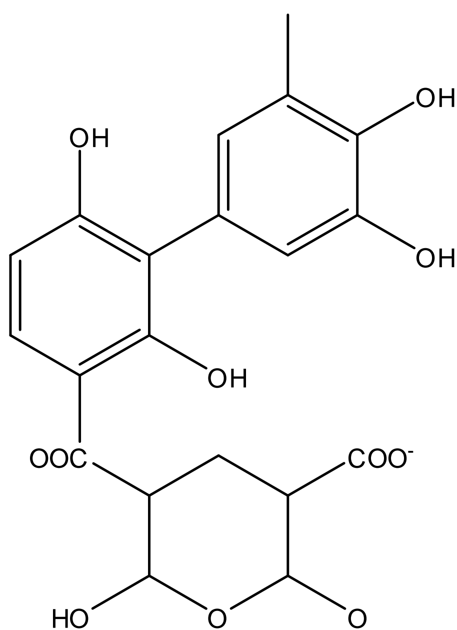


Fig. 3.38 Mass of tannic acid fragment *m/e* 667-3

Conclusion:

The dried crushed aerial parts of *sonchus oleraceus* plant was subjected to successive solvent extraction. The solvents were dichloromethane, methanol and ethyl acetate.Phytochemical screening was carried on the ethyl acetate extract and considerable amounts of flavonoids and poly-phenols were found. Chromatographic methods were used to isolate and purify compounds from ethyl acetate extract. Column fractions with less polar mobile solvents mixtures showed very low antioxidant activities. The activity is concentration dependent. A sufficient amount of extract must be introduced into the column . Spectroscopic analysis was done to characterize structures of four compounds.

I. Lignan glycoside

II. Chalcone glycoside

III. Flavone glycoside

IV. Tannic acid glycoside

The results obtained in this work are noteworthy, because of the strong activities and isolation of important compounds of non-nutritional health benefits.

Recommendations:

In this study bioactivity-guided isolation of ethyl acetate extract, showed that it has the highest antioxidant activities.

I. It is recommend that *Sonchu soleraceu*s plant should be investigated with wider range of solvents and under different conditions. There is no much literature about this plant it needs a lot of study, since it proved to contain compounds of importance.

II. Different bioactivities to be tested for this plant.

III. Antifungal , antiviral ,anti-tumour, anti-malarial ,anti-parasitic, immunoregulator etc. Need to be closely investigated.

IV. D-Test for bioactivities in non-polar solvents or solvents of low polarity, and isolate structures of compounds,should be subjected to more studies.

V. E-Soxhlet extraction of the plant with different solvents grading from low to high, then test for the aim of study,should be stated.

VI. F-More pharmacological test of the different solvents extracts in mice and comparison of the results of the plant with that of a drug on a target disease, will be appreciated.

VII. E-To study the markers on the plant ,and to point to differences with its family, is recommended.

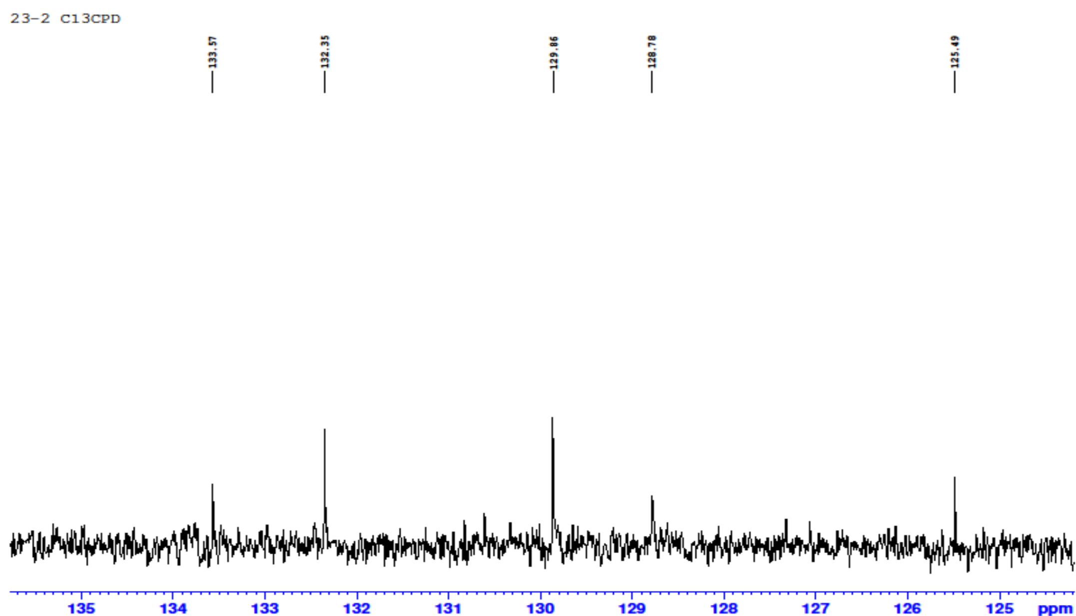
VIII. F-To test whether the isolated compounds can serve as ligands for enzymes activities.

IX. G-Assessment of cytotoxicity, for the isolated active fractions,is recommended.

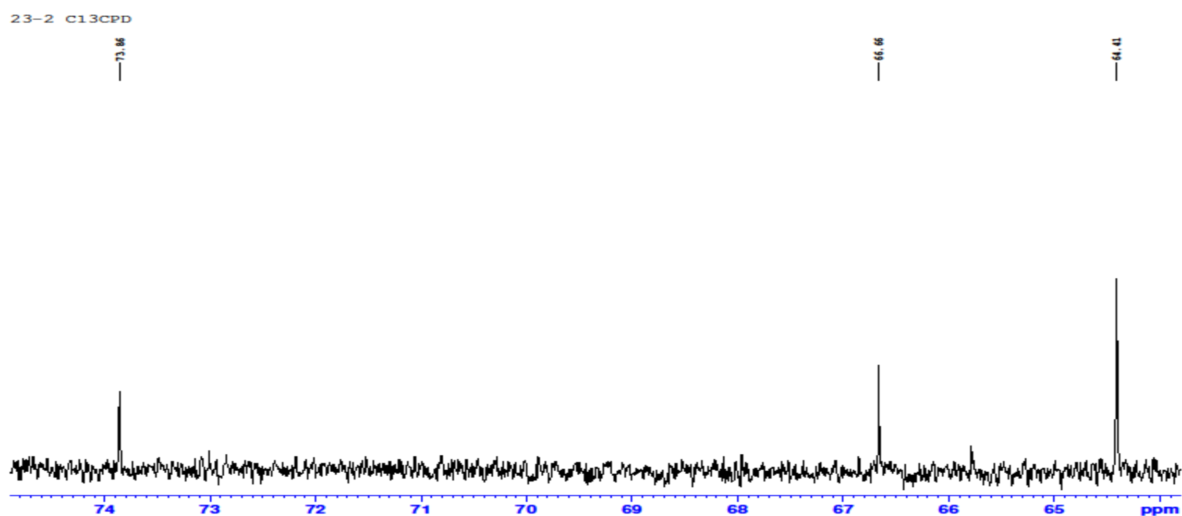
X. H-Chemical synthesis of the isolated compounds and do tests as drugs precursors, will be of highly medical value.

Appendix A

Fig. A.1 ^{13}C NMR chemical shifts of lignan glycoside



^{13}C chemical shifts for lignan glycoside (continued)….



^{13}C chemical shifts of lignan glycoside (continued)….

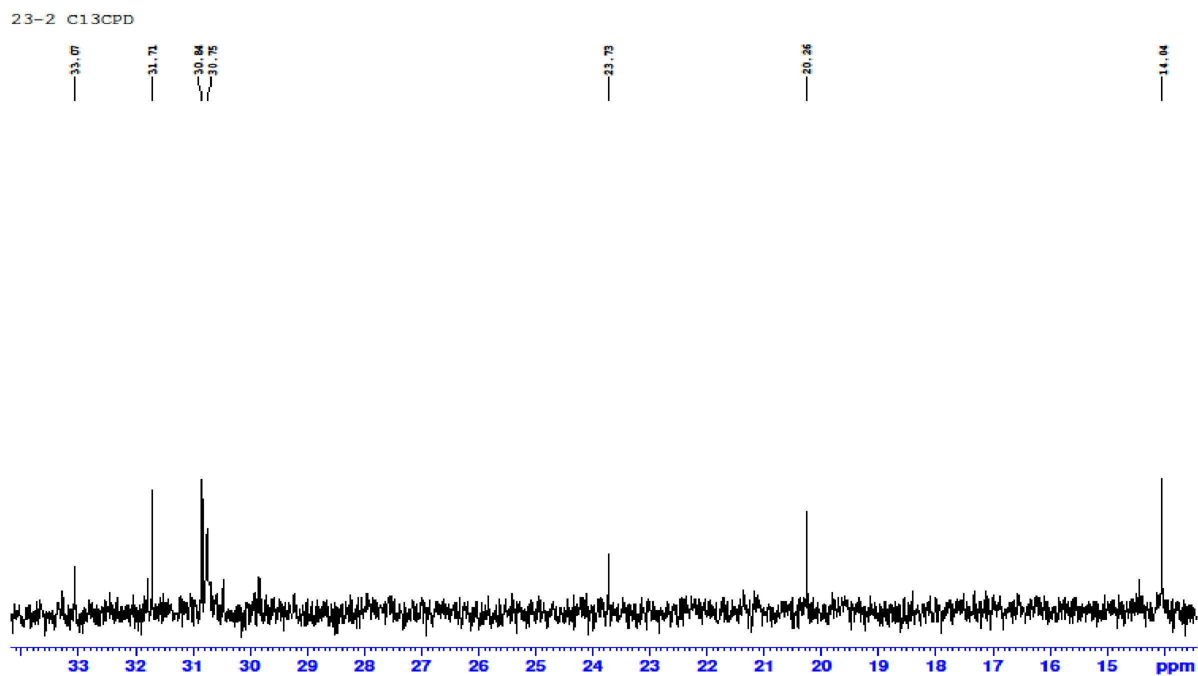
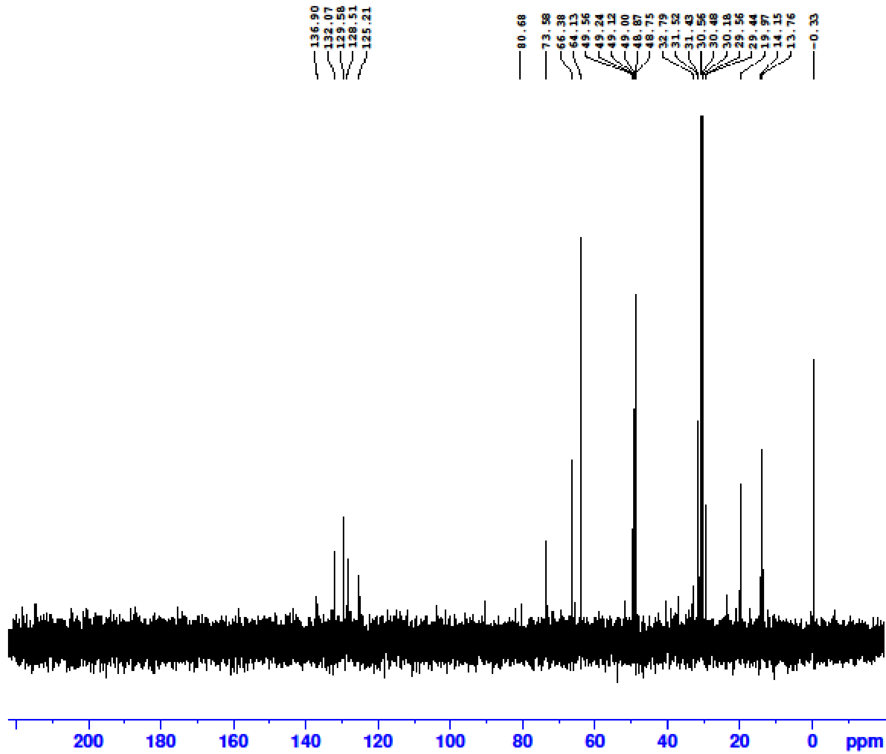


Fig. A.2 DEPT 45 of lignan glycoside



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RG                    185.67
DW                    11.733 usec
DE                     18.00 usec
TE                     298.1 K
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D2                0.00344828 sec
D12               0.00002000 sec
TD0                        1

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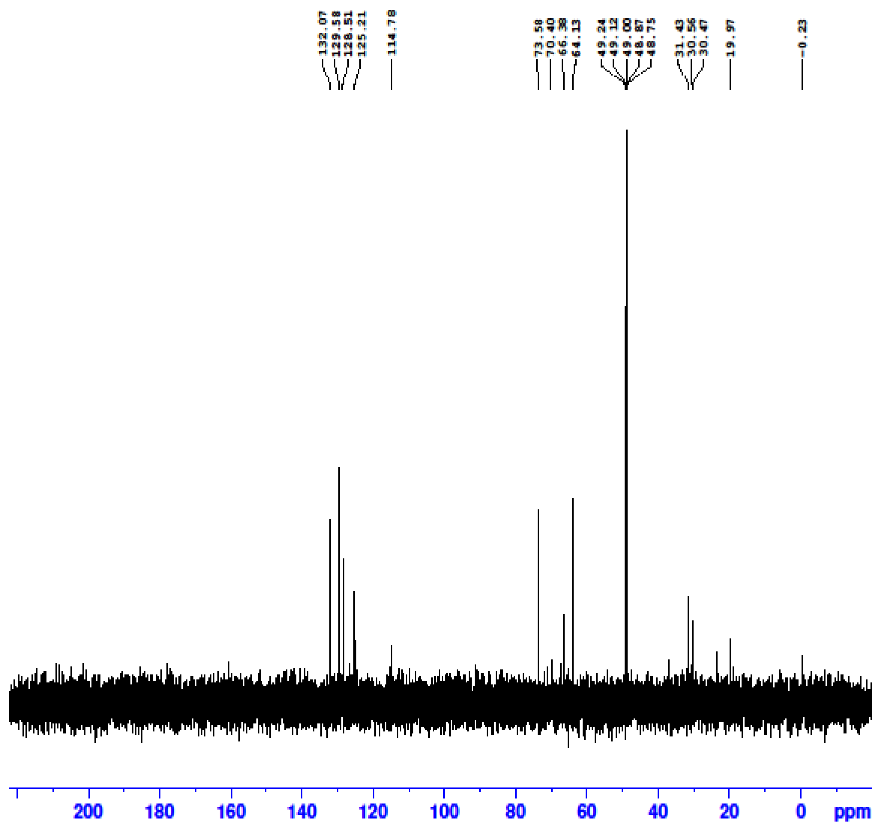
Fig. A.3 DEPT 90 of lignan glycoside



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RG                    185.67
DW                    11.733 usec
DE                     18.00 usec
TE                     298.1 K
CNST2            145.0000000
D1                2.00000000 sec
D2                0.00344828 sec
D12               0.00002000 sec
TD0                        1

======== CHANNEL f1 ========
SFO1             176.0780071 MHz
NUC1                     13C
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P13                  2000.00 usec
PLW0         0 W
PLW1             90.00000000 W
SPNAM[5]         Crp60comp.4
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SPOFFS5      0 Hz
SPW5             19.80100060 W

======== CHANNEL f2 ========
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NUC2                      1H
CPDPRG[2             waltz16
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P4                     16.00 usec
PCPD2                  65.00 usec
PLW2              6.69999981 W
PLW12             0.10149000 W

F2 - Processing parameters
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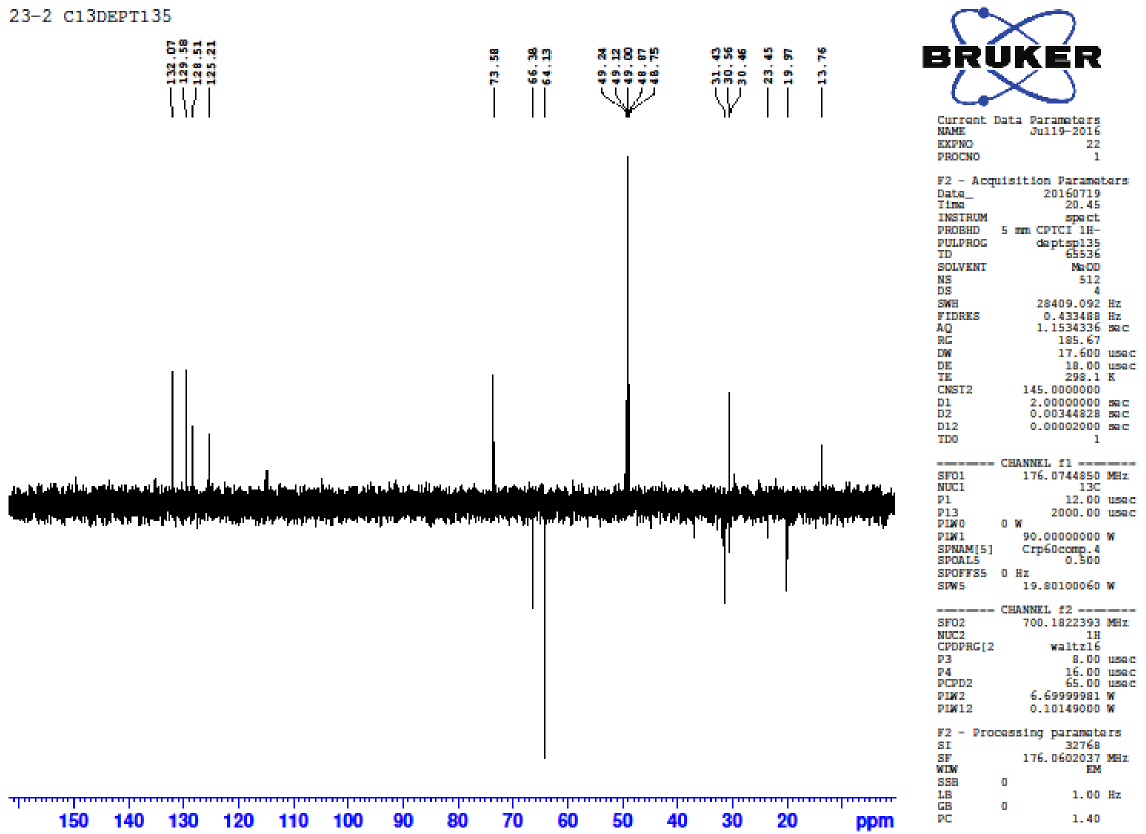
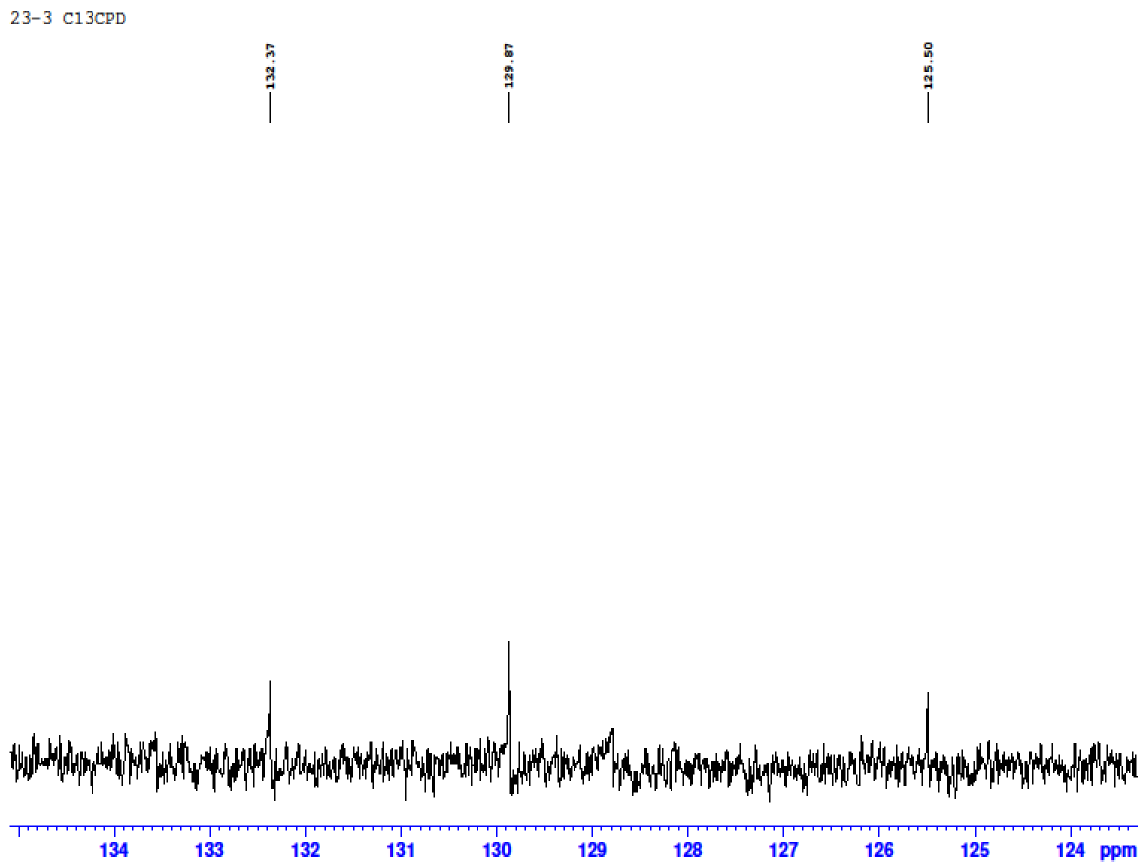
Fig. A.4 DEPT 135 for Lignan glycoside

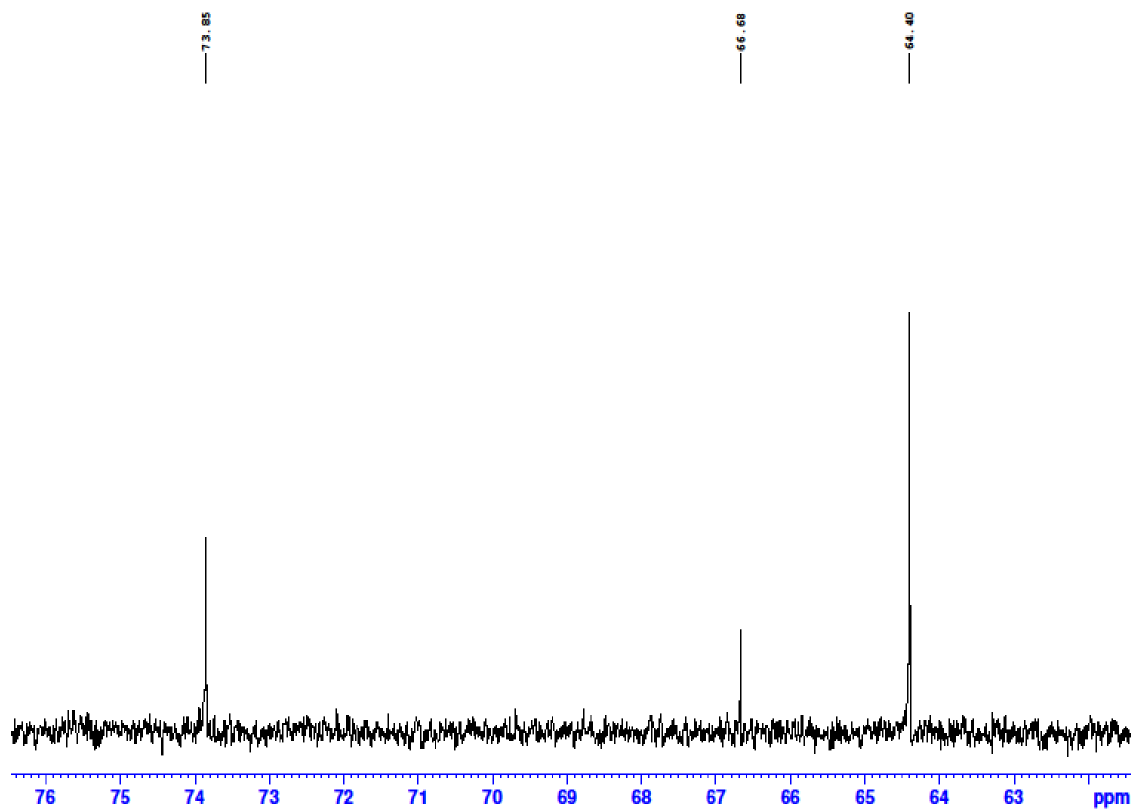


Appendix B

Fig. B.1 ^{13}C NMR chemical shifts of chalcone glycoside



^{13}C NMR chemical shifts for chalcone glycoside (cont…)



^{13}C NMR chemical shifts for chalcone glycoside (cont…)

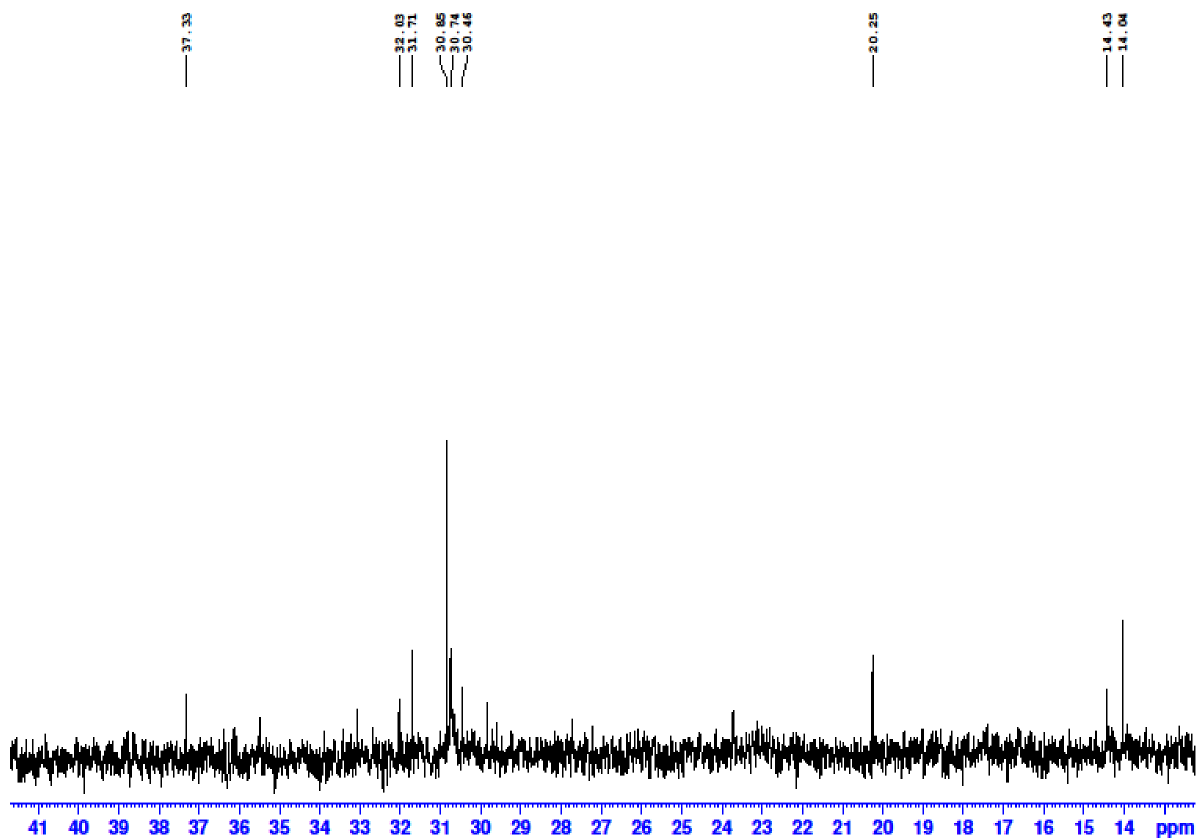


Fig. B.2 DEPT 45 chemical shifts of chalcone glycoside

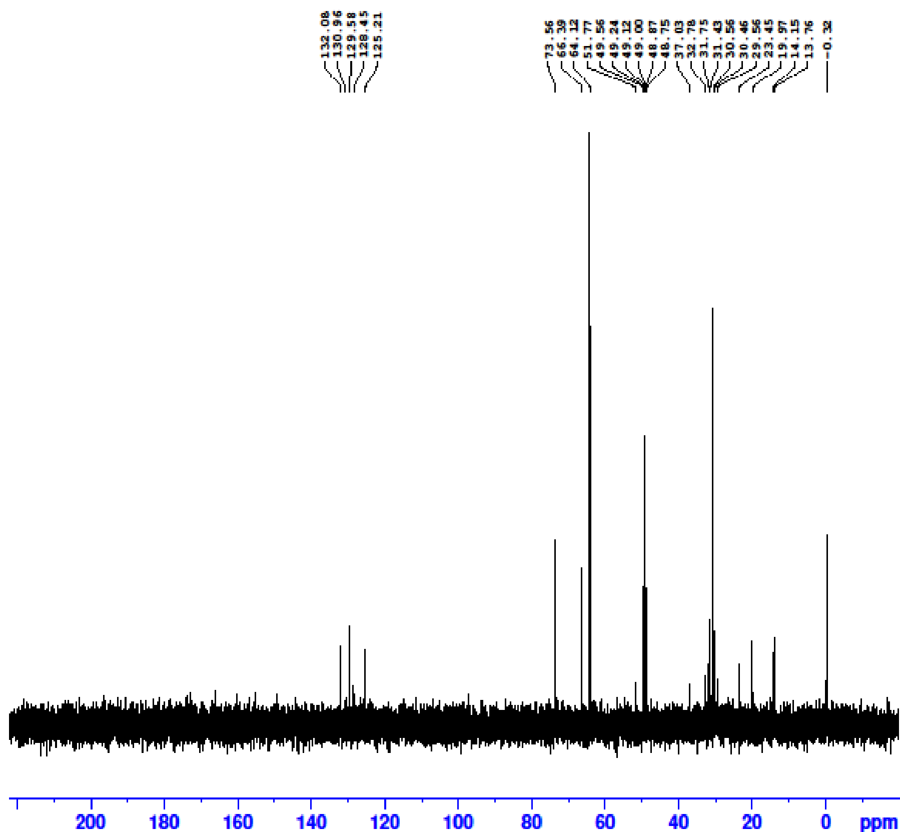
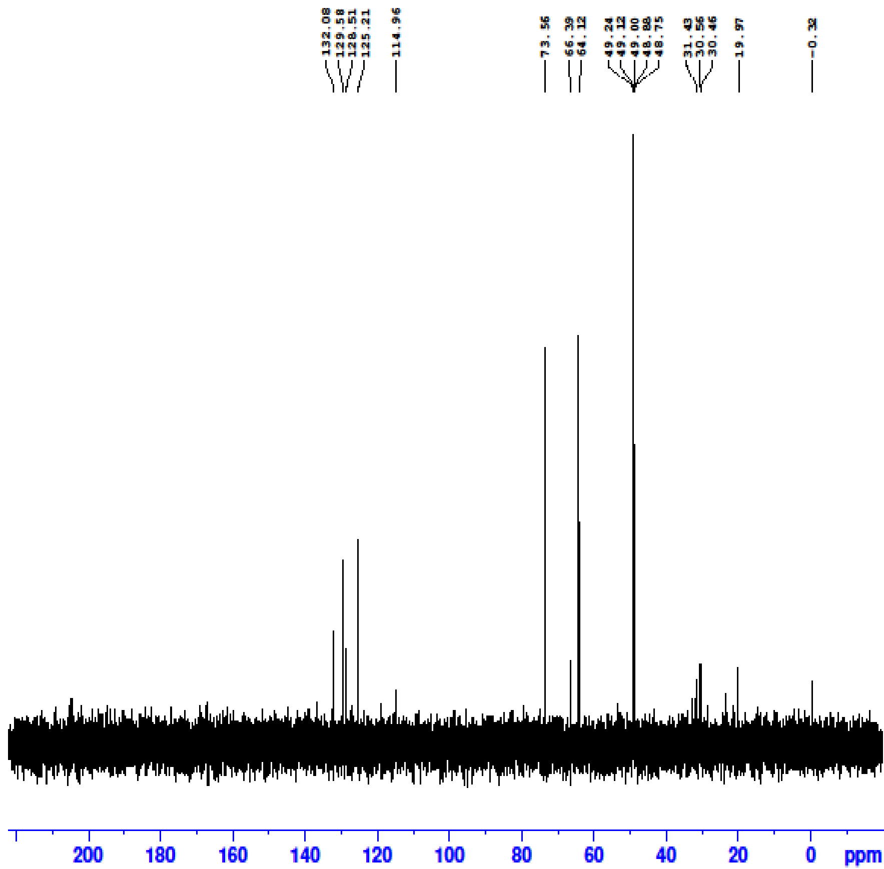


Fig. B.3 DEPT 90 chemical shifts ofchalcone glycoside



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DW 11.733 usec
DE 18.00 usec
TE 298.2 K
CNST2 145.0000000
D1 2.00000000 sec
D2 0.00344828 sec
D12 0.00002000 sec
TD0 1

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P1 12.00 usec
P13 2000.00 usec
PLW0 0 W
PLW1 90.00000000 W
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SPOFFS5 0 Hz
SPW5 19.80100060 W

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CPDPRG[2 waltz16
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PCPD2 65.00 usec
PLW2 6.69999981 W
PLW12 0.10149000 W

F2 - Processing parameters
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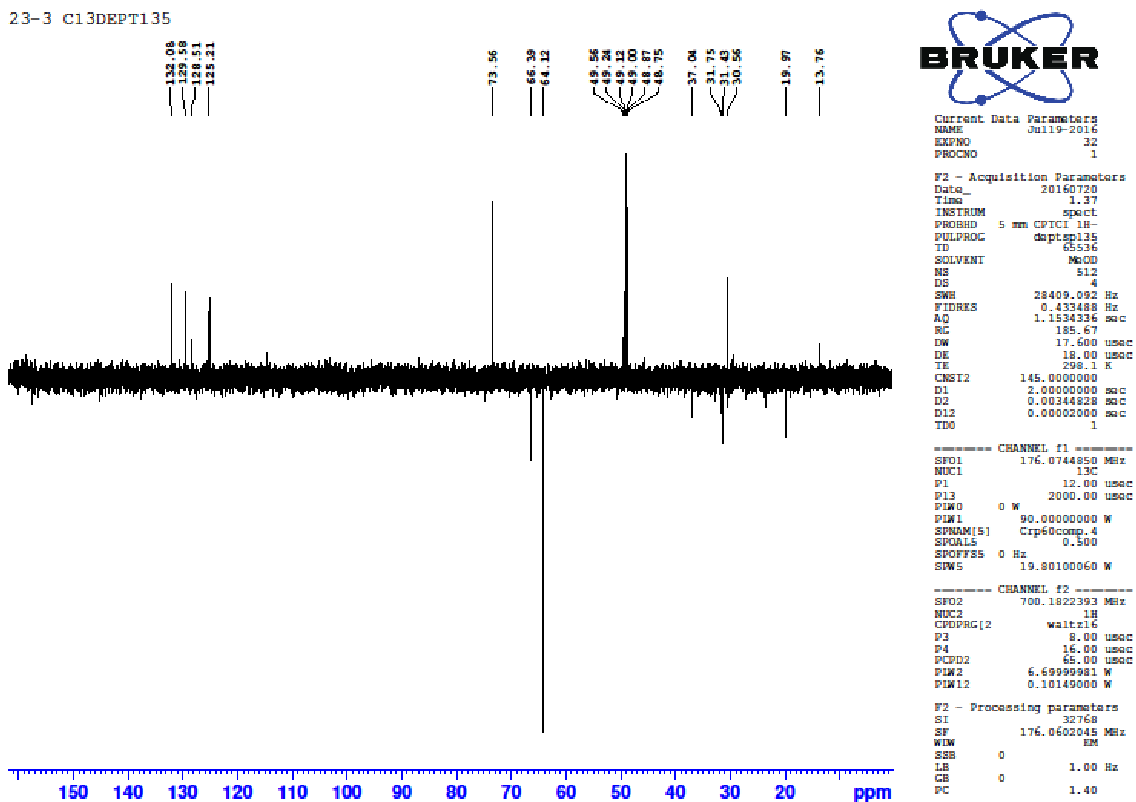
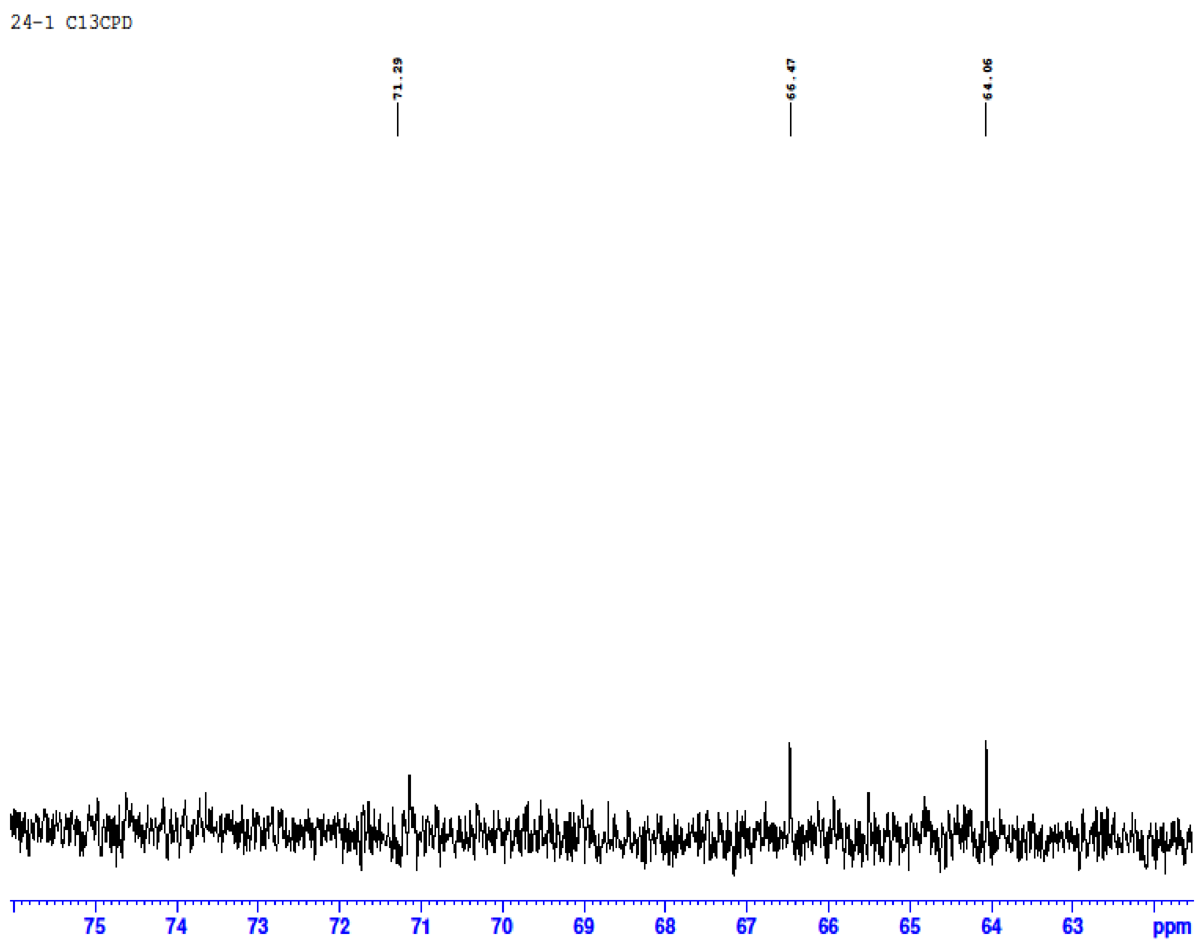
PC 1.40

Fig. B.4 DEPT 135 chemical shifts of chalcone glycoside

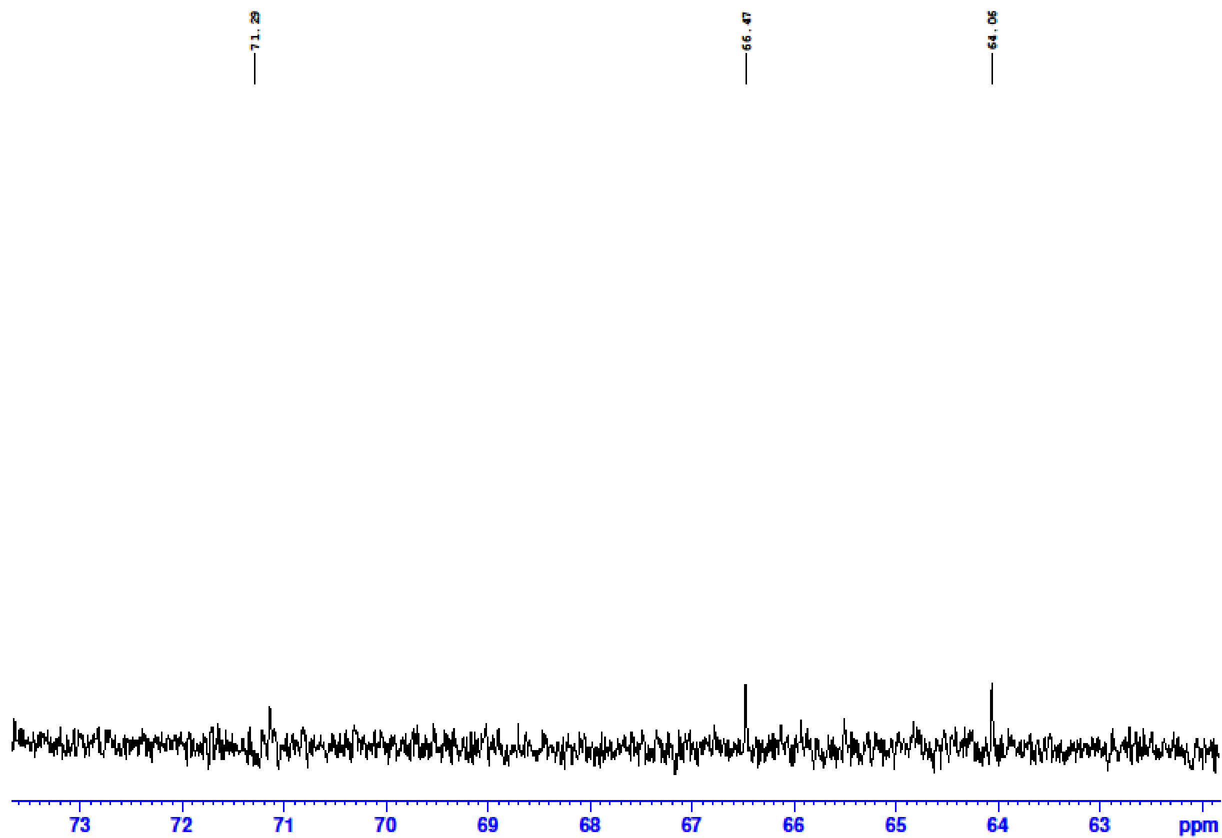


Appendix C

Fig. C.1 ^{13}C chemical shifts of flavone glycoside



^{13}Cchemical shifts for flavones glycoside (cont….)



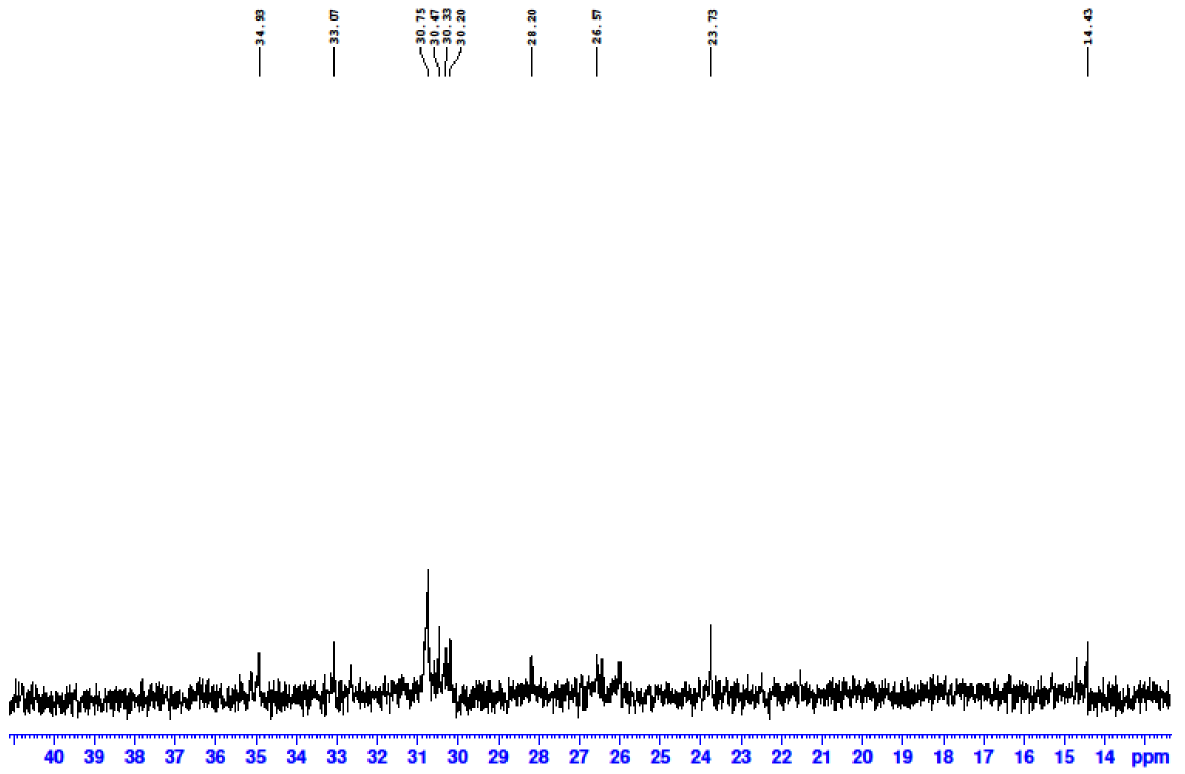

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Fig. C.2 DEPT 45 chemical shifts of flavone glycoside

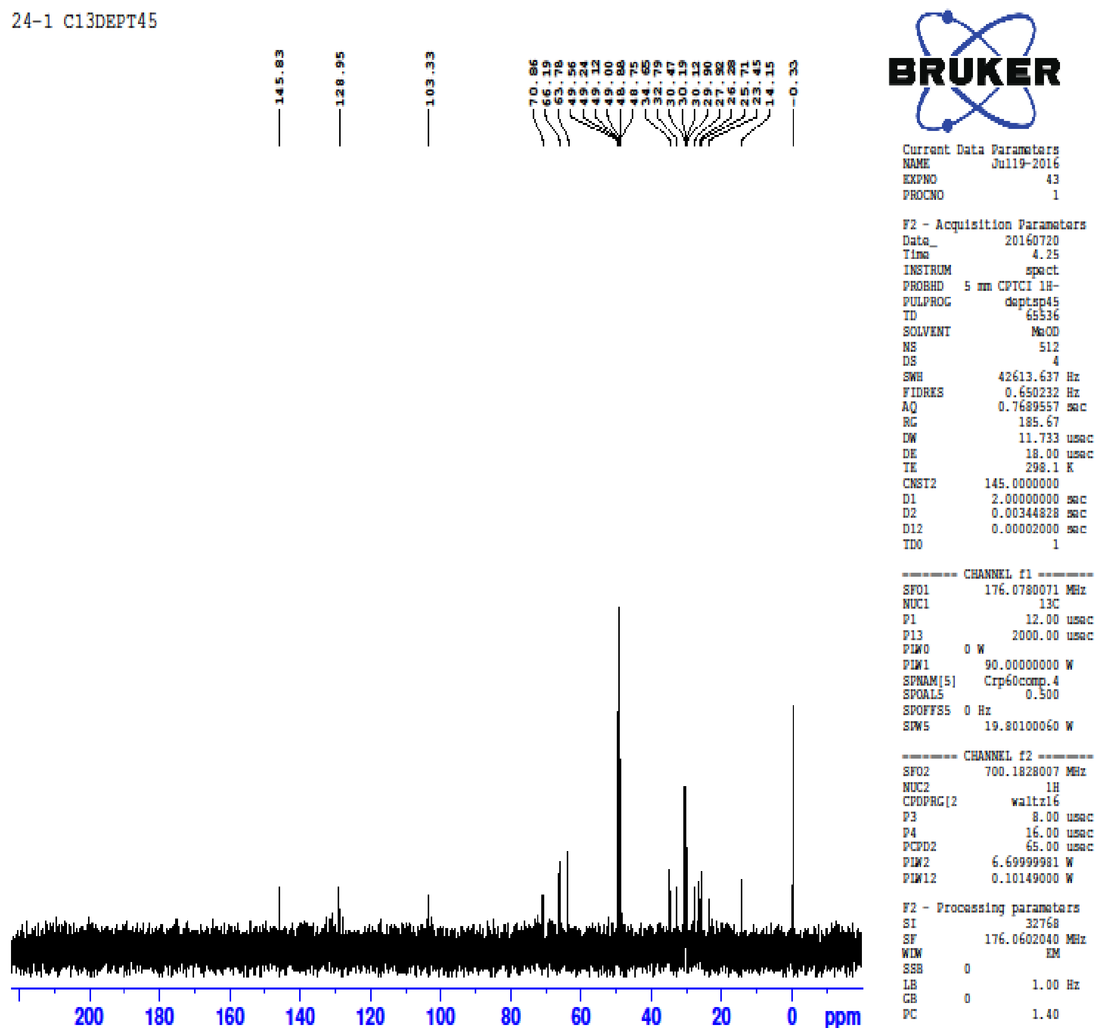
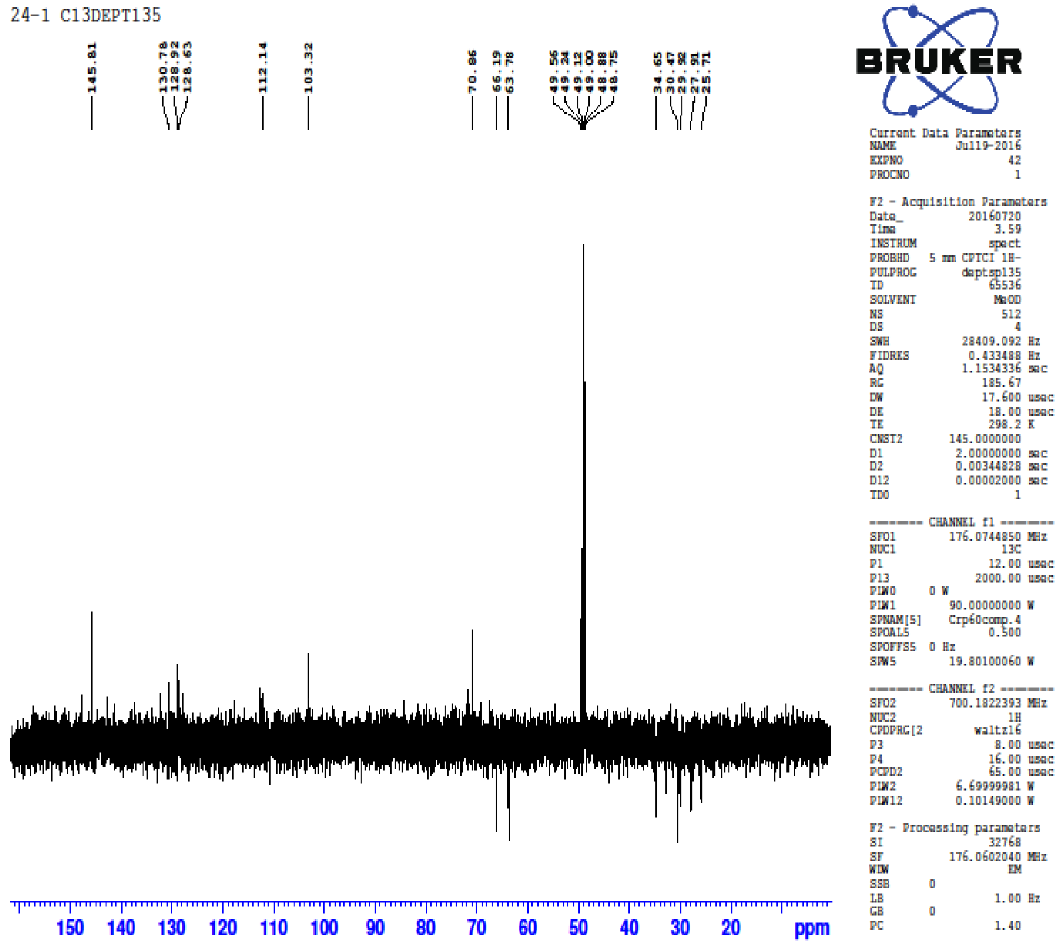


Fig. C.3 DEPT 135 chemical shifts of flavone glycoside



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