ISOLATION AND CHARACTERISATION OF SECONDARY METABOLITES FROM *Dioclea reflexa* (HOOK F.) AND *Cussonia arborea* (HOCHST)

ABDULKABIR OLADELE OLADIMEJI

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BY

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ABSTRACT

The discovery of bioactive natural products is a basis for development of novel pharmaceuticals. Indigenous medicinal plants which are largely unexplored offer chemical diversity with structural complexity and biological potency. Despite their ethnomedicinal usage, there is little information on the bioactive components of *Dioclea reflexa* and *Cussonia arborea*. This study was designed to isolate and characterise the secondary metabolites from the roots of *D. reflexa* and *C. arborea*, and evaluate free radical scavenging activity of the isolates.

One kilogramme each of authenticated *D. reflexa* root and *C. arborea* root-bark were collected from Eruwa, Oyo State. The air-dried samples of *D. reflexa and C. arborea* were purposively extracted with ethanol and methanol respectively. The crude extracts were separately fractionated on silica gel column chromatography with gradient elution using various solvents. The sub-fractions were further subjected to a combination of chromatographic techniques for isolation of pure compounds. Structures of isolated compounds were elucidated using one and two-dimensional Nuclear Magnetic Resonance (NMR), Infrared (IR), Ultra-Violet-Visible and Mass Spectroscopy (MS) techniques, and comparison with literature data for known compounds. The isolated compounds were evaluated for free radical scavenging potential in comparison with butylated hydroxyanisole following standard method.

The ethanol extract (85 g) of *D. reflexa* on column chromatography gave β-sitosterol **1**, lupeol **2**, aurantiamide acetate **3**, mearnsetin **6**, 7,4′-dihydroxyflavone **7** and 3,5-dihydoxy-4-methoxybenzoic acid **8**. The column yielded two new compounds, named lexaflavanone **4** and reflevone **5**. Lexaflavanone showed the presence of sixteen carbons: 8C, $1CH_2$, 6CH and $1CH_3$. The MS gave M^+ peak at m/z 302.0769 (Calcd. 302.0792), with 10 degree of unsaturation, corresponding to $C_{16}H_{14}O_6$. The NMR signals were seen at δ 8.53, 7.70 (1H, d, J= 8.5 Hz), 6.58 (2H, s), 6.55 (1H, dd, J= 2, 8.5 Hz), 6.42 (1H, d, J= 2Hz), 5.36 (1H, dd, J= 2.5, 12.5 Hz), 2.93 (1H, dd, J= 12.5, 17 Hz, H-3_{axial}) and 2.66 (1H, dd, J= 3, 17 Hz, H-3_{equi}). The IR absorptions (υ_{max}, cm^{-1}) for OH (3361) C=O (1652) and C-C in ring (2917) were also detected. Reflevone, molecular formula $C_{16}H_{12}O_6$ suggested δ_H signals: 6.54 (1H, s), 6.97(1H, dd), 7.02(1H, d) and 7.06 (2H, s), and δ_C signal: 177.9 (C=O) typical of flavone. In addition, its infra-red stretching absorptions (υ_{max}, cm^{-1}) revealed C-H sp^2 (1570) and O-H (3377). The

characteristics peaks of retro-Diels-Alder cleavage of ring C were observed in MS spectrum at m/z 137 and 166. Column chromatography of the methanol extract (18 g) of *C. arborea* yielded three known compounds, stigmasterol **9**, oleanolic acid **10** and hederagenin **11** when compared with literature. The compounds were however new to the species of *C. arborea*. Lexaflavanone, reflevone and hederagenin exhibited free radical scavenging activity with IC_{50} of 58.14, 322.0 and 105.0 µM respectively.

The plants have potentials for the development of drugs for the treatment of diseases with oxidative stress due to their free radical scavenging activities. Lexaflavanone and reflevone obtained from *D. reflexa* are new additions to the library of chemical compounds.

Keywords: *Dioclea reflexa*, *Cussonia arborea*, Lexaflavanone, Reflevone, Free radical scavenging activity.

Word count: 489

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……

Abdulkabir Oladele Oladimeji

DEDICATION

This work is dedicated to Almighty Allah, the Creator of all creatures.

CERTIFICATION

I certify that this work was carried out by Mr. Abdulkabir. O. Oladimeji in the Department of Chemistry, University of Ibadan.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

APPENDICES

LIST OF ABBREVIATIONS

MHz = Mega Hertz

Hz = Hertz

J = Coupling constant

d = doublet

s = singlet

t = triplet

m = multiplet

br = broad

MeOD = deuterated methanol

m/z = mass/charge

^{1}H-NMR = Proton Nuclear Magnetic Resonance

^{13}C-NMR =Carbon-13 Nuclear Magnetic Resonance

DEPT = Distortionless Enhancement Polarization Transfer

HSQC = Heteronuclear Single Quatum Coherence

HMBC = Heteronuclear Multiple Bonding Coherence

^{1}H-^{1}H COSY = Proton-Proton Correlation Spectroscopy

NOESY = Nuclear Overhauser Spectroscopy

ESIMS = Electron Spray Ionization Mass Spectrometry

HREISMS = High Resolution Electron Spray Ionization Mass Spectrometry

IR = Infra-Red

UV = Ultra-Violet

MS = Mass Spectrometry

CHAPTER ONE

1.0 INTRODUCTION

Over the years, therapeutic skill in folk medicine and orthodox medical practices depended on pharmacopoeia formulations from plants. Medicinal plants which consist of the flora of wild and cultivated species of trees, shrubs, herbs and lawns are the richest bio-resources of drugs for traditional medicine. It has been estimated that 14-28% of higher plants are used medicinally and that 74 % of pharmacologically active plants-derived components were discovered after follow-up of the ethnomedicinal use of the plants (Gibbons 2003). Medicinal plants are known to attribute their curative abilities to certain biologically active substances which are present in various parts of the plants. These active substances are known as secondary metabolites. They include: terpenes, flavonoids, tannins, saponins, alkaloids, steroids and anthraquinones (Duru *et al*., 2010). Secondary metabolites are created from the building blocks of amino acids, sugars acetyl co-enzyme A, mevalonic acid and nucleotides called primary metabolites. The divergence in the genetic structure of different species has made it possible to hold that arrays of secondary metabolites perform different functions to the benefit of mankind (Nakashi *et al*., 1974). Scientific inquiry has shown the real strength of active principles in many plants, and there are still many species unexplored (Menale *et al*., 2006).

1.1 Plant products as therapeutic agents

The history of drug discovery and even drug chemistry is inexorably linked to plant kingdom and the process of deriving drugs from plant sources is certainly not new (Parfitt, 1978). Natural products chemists, ethnopharmacologists, botanists and microbiologist are searching the earth for phytochemicals, which could be developed for the treatment of infectious diseases (Tanaka, *et al*., 2006) owing to the increasing incidence of side-effects associated with conventional drugs and the emergence of

diseases resistance to antibiotics. Drug discovery from plants involves isolation and identification of secondary metabolites produced by plants and their uses as active principles in medicinal preparations (Taylor *et al*., 2001). A number of plant secondary metabolites have been used as anti-cancer agents. Flavonoid-rich extracts from the mature roots of plants have been shown to exhibit anti-proliferative effects on various cancer lines (Ncube *et al*., 2008). A number of studies have shown saponins to have an inhibitory effect on protozoa (Ncube *et al*., 2008). Quinine, an alkaloid from the bark of *cinchona* tree, is popular for its activity against malaria parasite (Iwu *et al*., 1999). A benzylisoquinoline alkaloid, a papaverine isolated from *Papaver somniferum*, was shown to have an inhibitory effect on several viruses (Ncube *et al*., 2008). Many important compounds that have been found to have antimicrobial activity include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, and coumarins (Okwu 2004).

Another important sub-class of compound under terpenes are the essential oils. Essential oils are volatile, natural mixtures of complex compounds, mainly monoterpenoids and sesquiterpenoids, characterised by strong odour and playing an important role in the protection of the plants against bacteria, virus, fungi, insects, herbivores and as attractant to pollinators (Ogunwande 2010). They are used in agriculture, as food additives and preservatives and as natural remedies, owing to their notable antimicrobial and antioxidant properties (Cinzia *et al*., 2010). Their utilization is influenced by the nature of their constituents which has widespread application in the pharmaceutical, flavour and fragrant industries. Common active components of the essential oils include thymol, carvacol, camphor and terpine-4-ol (Francesca *et al*., 2006).

1.2 Justification for the research

Studies on free radicals and the development of new methods for evaluation of anti-oxidant activity have increased considerably in recent years. The noted deleterious effect of free radicals on cells in relation to certain diseases (such as cardiovascular and neurodegenerative diseases and cancer) has encouraged the search for new substances that can prevent or minimise oxidative damage (Nunes *et al*., 2012).

Free radicals are highly reactive and have the potential to cause damage to cells, including those that may lead to diseases such as cancer, diabetes, etc. Free radicals are formed naturally in the body. In addition, some environmental toxins may contain high levels of free radicals or stimulate the body's cells to produce more free radicals. Anti-oxidants stabilise or deactivate free radicals, often before they attack targets in biological cells. The body makes some of the antioxidants to neutralise free radicals called endogenous anti-oxidants. However, the body relies mainly on exogenous antioxidants are commonly called natural anti-oxidants. Most of these natural anti-oxidants come from fruits, vegetables, spices, grains, and herbs (Velioglu *et al*., 1998; Rababah *et al*., 2005).

Synthetic anti-oxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are reported to be carcinogenic. Thus, the interest in natural anti-oxidants from plants origin has increased considerably (Velioglu *et al*., 1998).

Dioclea and *Cussonia* species were reported to possess these natural anti-oxidants properties (Barreiros *et al*., 2000; Kougan *et al*., 2009). However, few members of these species unlike *Dioclea reflexa* and *Cussonia arborea* have been published in which the promising leads were followed up with an attempt to isolate and identify the active principles. This research is an evidence of continuing interest in anti-oxidant agents from higher plants - *D. reflexa* and *C. arborea.*

1.3 Research objectives

The objectives of the present work are:

(a) To collect and identify the root of *D. reflexa* and *C. arborea.*

(b) To isolate the secondary metabolite constituents of the plant extracts and elucidate the structures of the compounds.

(c) To extract and characterise the volatile oil constituents from the root of *D. reflexa.*

(d) To evaluate free radical scavenging activity of new isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

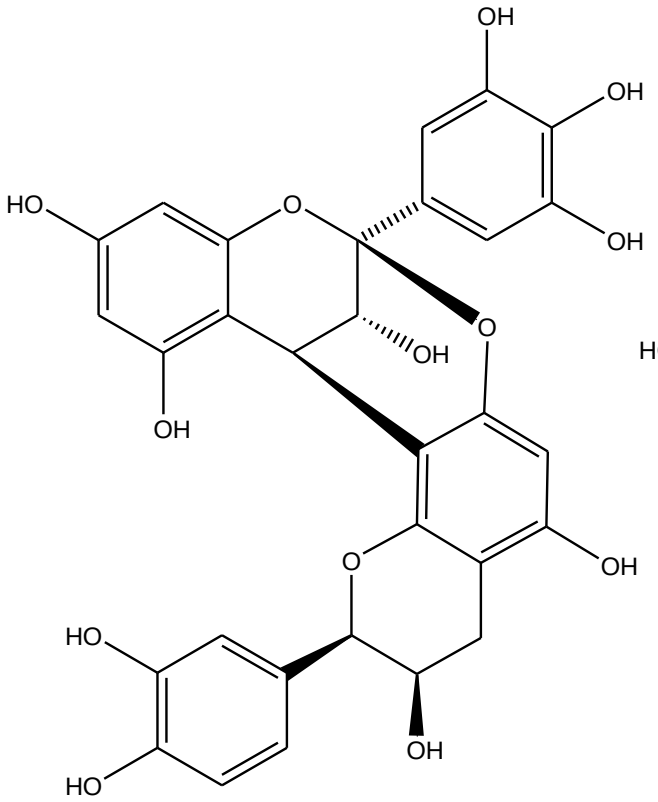
2.1 Plant species used

2.1.1 *Dioclea* species and *Dioclea reflexa* Hook F.

The family *Fabaceae* or *Leguminosae* are commonly referred to as the legume, pea or bean family. *Fabaceae* are usually giant trees, though some are small herbs; and most of them are herbaceous perennials. The plants have indeterminate infloresences and sometimes reduced to a single flower. The family includes three sub-families: *Mimosoideae, Ceasalpinioideae* and *Faboideae. Faboideae* contains 470 genera, which include *Dioclea, Abrus, Collaea, Sophora, Maackia* and *Butea*. Plants of this family are found throughout the world, growing in many different environments and climates (Burkill, 1984).

Dioclea is a small genus containing 40 species, with better distribution in Africa and South America (Burkill, 1984). Examples of *Dioclea* species are *Dioclea albifora* Cowan, *Dioclea aurea* R.H. Maxwell, *Dioclea bicolor* Benth, *Dioclea burkartii* R.H. Maxwell, *Dioclea apurensis* Kunth, *Dioclea cassinoides* Desf., *Dioclea coriacea* Benth., *Dioclea decandra* Amshoff, *Dioclea densiflora* Huber, *Dioclea dichrona* J.F.Macbr., *Dioclea dictyoneura* Diels, *Dioclea grandiflora* Mart, *Dioclea laxiophylla* Mart. *ex* Benth, *Dioclea rostata* Benth, *Dioclea virgata* (Rich.) Amshoff and *Dioclea reflexa* Hook. F. Dioclea species have been of interest in phytochemical and pharmacological research due to their medicinal values. They are well known in folk medicine for the treatment of kidney stones, prostate gland disorders, rheumatism (Barreiros *et al*., 2000) and epilepsy (De Almeida *et al.,* 2010). The pharmacological activities of this genus include anti-convulsant, anxiolytic (De Almeida *et al*., 2010), anti-oxidant, anti-arrhythmogenic (Vianna *et al*., 2006), immunomodulatory (Alves *et al*., 2010), analgesic and a central anti-nociceptive (Almeida *et al*., 2003) effects.

Preliminary studies have shown that A-type proanthocyanidin, epigallocatechin-(2β→7, 4β→8)-epicatechin **2.1** and epicatechin **2.2** isolated from leaves of *D. lasiophylla* possess considerable anti-oxidant activity (Barreiros *et al*., 2000). A new flavone, dioclein **2.3**, isolated as the major constituents of the root-bark of *D. grandiflora* was reported as being a vasodilator, anti-hypertensive (Lemos *et al*., 1999, Trigueiro *et al*., 2000), a potent anti-arrhythmogenic and anti-oxidant (Vianna *et al*., 2006). Also, dioclenol **2.4**, from root-bark of *D. grandiflora* has been reported to have anxiolytic and anti-convulsant effects (De Almeida *et al*., 2010).



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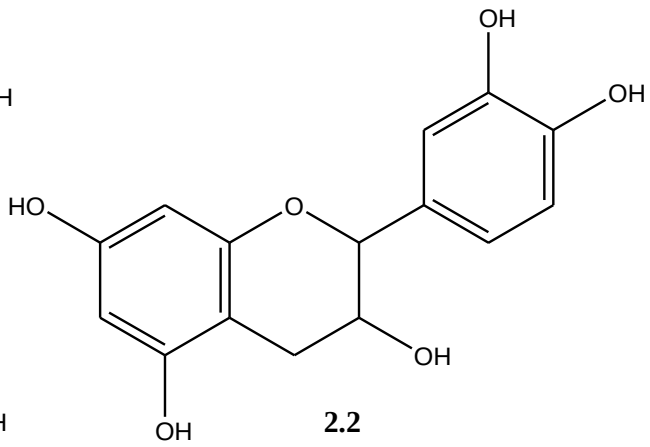
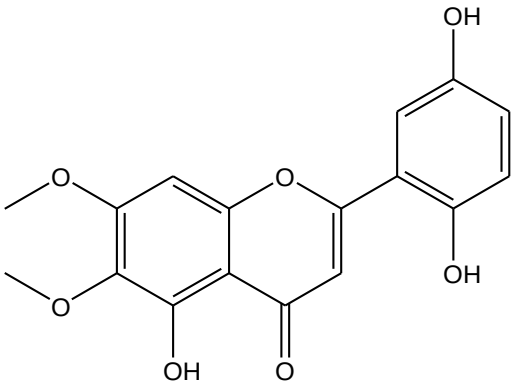


2.2

Agrandol **2.5**, paraibanol **2.6**, diosalol **2.7**, 5,7,2′,5′-tetrahydroxy-6-methoxy-8-prenylflavone **2.8**, floranol **2.9** and β-amyrin **2.10** were among the compounds isolated from *D. grandiflora* (Jenkins *et al*.,1999, and Lemos *et al*., 1999). Barreiros (2000) reported the isolation of luteolin 3′β-D-glucopyranoside **2.11**, chrysoeriol 7β-D-glucoparanoside **2.12** and lipidol, 2-methylpentan-2,4-diol **2.13** from the leaves of *D. lasiophylla*.



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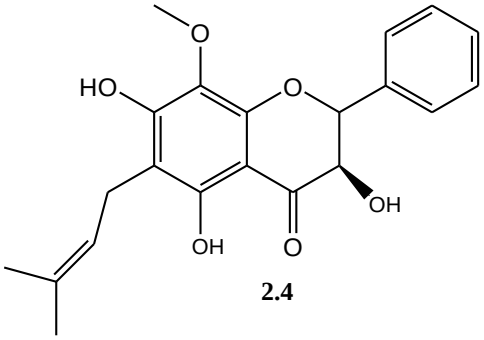

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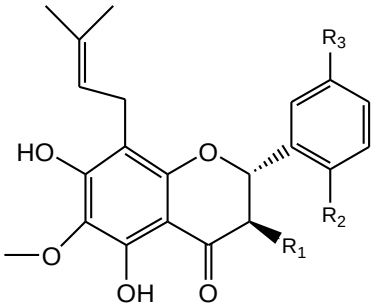
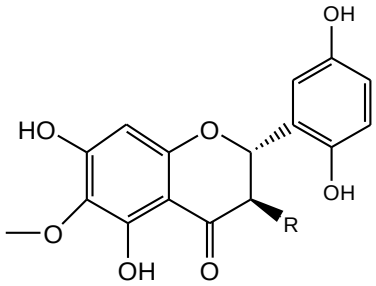


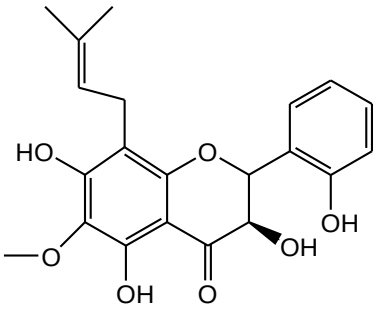
2.5: $R_1 = R_2 = R_3 = H$

2.6: $R_1 = R_2 = R_3 = OH$



2.7 : R = OH

2.8: R = H



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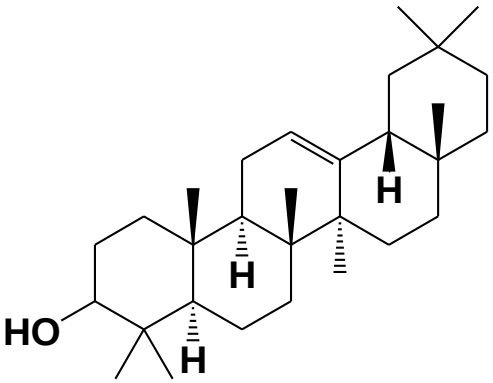
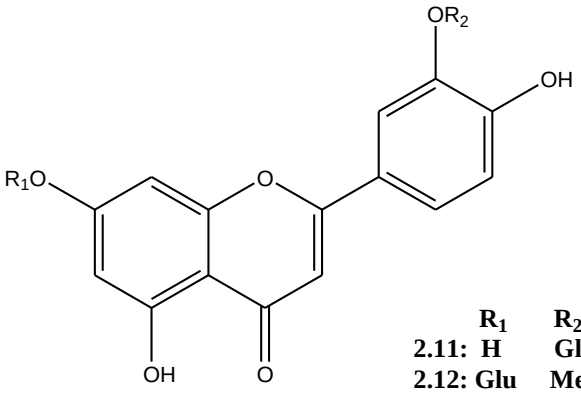
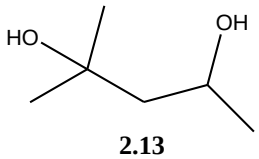


2.10

Other isolated compounds reported in literature from *D. virgata* are 7-hydroxy-6-methoxyflavone **2.14**, 7-hydroxy-6-methoxyflavanone **2.15**, 7-hydroxy-6-methoxyflavonol **2.16** as well as 3-hydroxybenzoic acid **2.17**, 3,4-dihydroxybenzoic acid **2.18**, 4-hydroxy-3-methoxybenzoic acid **2.19**, lupeol **2.20** and glycoside of stigmasterol (Alves *et al*., 2010).



	R_1	R_2
2.11:	**H**	**Glu**
2.12:	**Glu**	**Me**



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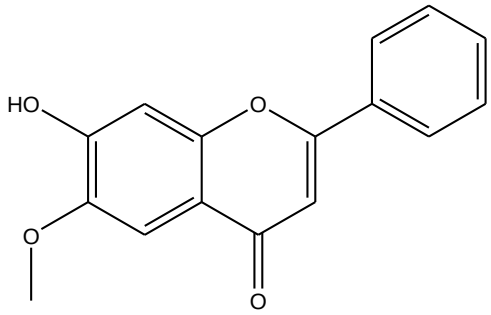

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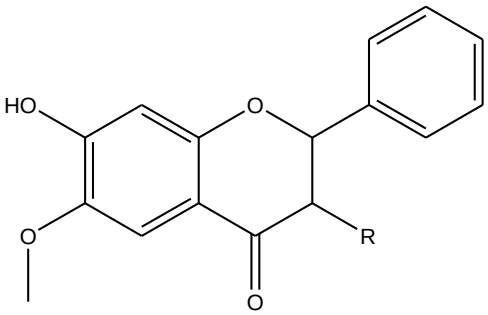
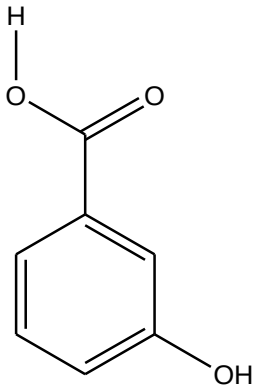


2.15: R = H
2.16: R = OH

Dioclea reflexa Hook. F. (Fig. 2.1) is a woody climber which produces flowers and pods on long, rope-like stems that hang from the forest canopy. The leaf is trifoliate with bluish-white or reddish flowers in long racemes. Pods are glabrescent when mature, with one to two seeds. The seed pods are covered with microscopic velvety hairs. It is known as 'Agbarin' among the Yorubas of the Western Nigeria, where it is used traditionally for the treatment of various ailments such as backache, symptoms of stomach ulcers, asthma, stimulant and tuberculosis (Odugbemi, 2008). The seeds of *D. reflexa* are sometimes used in parts of Africa together with the seeds of *Aframomum spp*. (Grains of Paradise) as a tonic and stimulant. They are also said to be used in hair treatments for killing lice (Pulliah, 2006). In the Caribbean region and Central America, the hairs obtained from the pod, are stirred into honey or syrup as a remedy for intestinal parasites. Alcohol extracts from the leaf of this species has been reported to possess anti-microbial activity (Ogundare and Olorunfemi, 2007).



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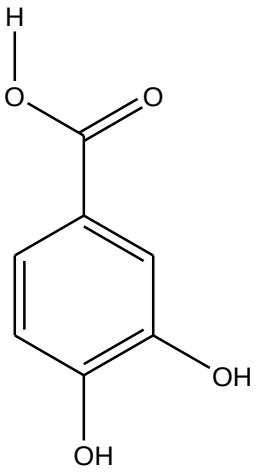

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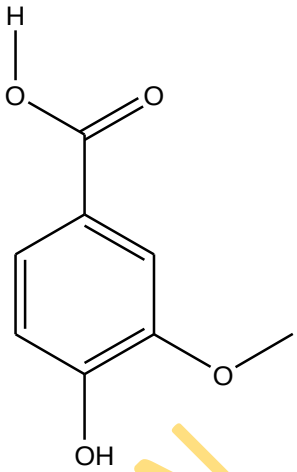

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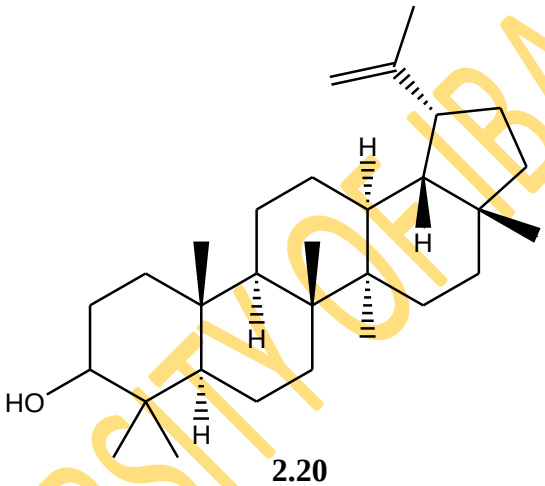
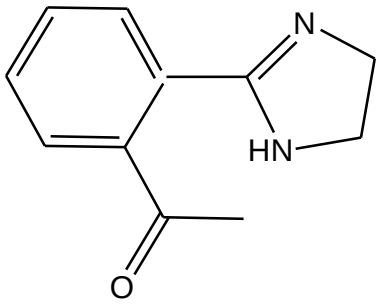


2.20

Chloroform soluble part of the seeds led to the isolation of a new secondary metabolite, dioclimidazole **2.21**, which displayed both anti-cholinesterase and antibacterial activities (Oladosu *et al*., 2010). Faleye (2012) also reported the isolation of taraxasterol **2.22** and stigmasterol from the methanol extract of the seed. A total of 15 volatile compounds have been isolated from the *D. reflexa's* root (Oladosu and Oladimeji, 2012).

However, there is no other report on the secondary metabolites from the roots of *D. reflexa*.



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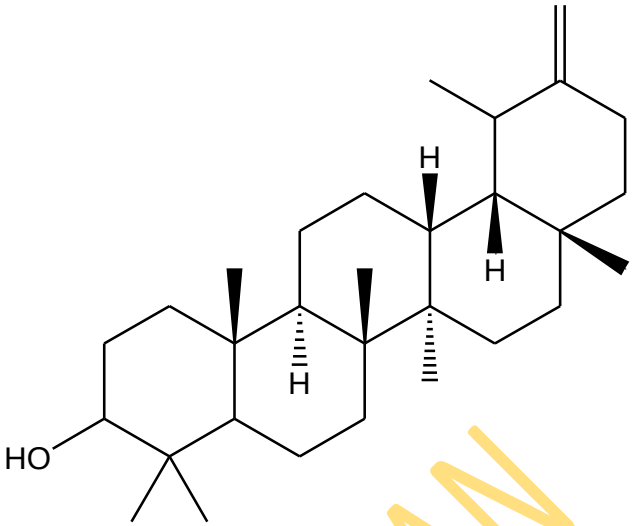


2.22

Figure 2.1. Aerial part of *Dioclea reflexa* (Hook. F.)

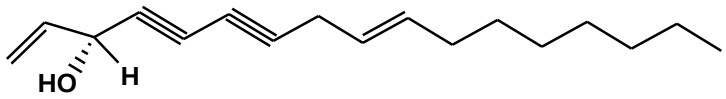
2.1.2 *Cussonia* species and *Cussonia arborea* Hochst *ex* A. Rich

The *Araliaceae* are family of flowering plants, also known as the Aralia or ivy family. They are poorly represented in Africa, with five indigenous genera and one naturalized (Klopper *et al*., 2006); it is only *Cussonia* Thunb. and *Seemannaralia* (Seem.) Vig. that are endemic to Africa (De Villiers *et al*., 2012). *Cussonia* comprises 21 species which include *arborea*, *barteri*, *spicata*, *natalensis*, *racemosa* and *bacoensis*. *Cussonia* are evergreen or deciduous trees or shrubs displaying a considerable range of leaf types: both simple and compound mono- and bi- digitate, as well as ternate. The genus is distributed throughout the grasslands and woodlands of sub-Saharan Africa, Yemen and Comoro Island (De Villiers *et al*., 2010).

Previous phytochemical investigation of the genus revealed the presence of triterpenoid saponins, diterpenoid glycosides, flavonoids (Kougan *et al*., 2009), triterpenoids and C_{18}- polyacetylenes (Papajewski *et al*., 1998). Secondary metabolites present in the plants of *Cussonia* family exhibited various biological activities such as: anti-bacterial, anti-fungal, molluscidal and heamolytic activities (Papajewski *et al*., 1998; Gunzinger *et al*., 1986; Dubois *et al*., 1986). Their anti-ulcer, antimalarial, anticancer (De Villiers *et al*., 2010), antitrichomonas and inhibitory effects on nitric oxide production (Tapondju *et al*., 2003) have also been reported.

Cussonia species are well known in folk medicine in the treatment of mental illness rheumatism and dysmenorrhoea (Dubois *et al*., 1986). They are widely used for the treatment of fever, malaria, eye problems, sexually transmitted diseases, skin problems, cancer and wounds (De Villiers *et al*., 2010).

Falcarinol **2.23**, falcarindol **2.24**, (+)-9(Z),17-octadecadiene-12,14-diyne-1,11,16-triol **2.25** and (+)-9(Z),17-octadecadiene-12,14-diyne-1,11,16-triol triacetate **2.26** isolated from ethyl acetate extract of *C. barteri* had anti-fungal activity against *Cladosporium cucumerinum* and antibacterial activity against both *Bacillus substilis* and *Pseudomonas fluorescens* (Papajewski *et al*., 1998).



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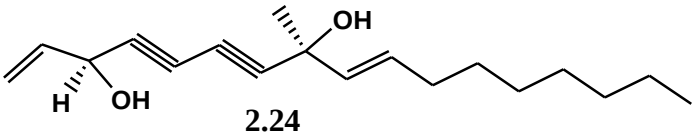

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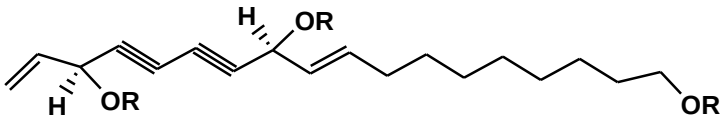
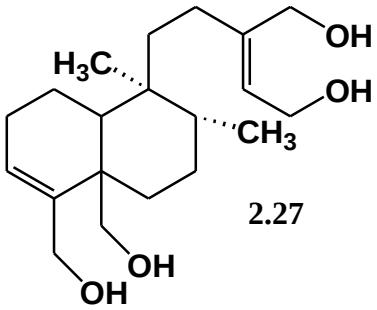


2.25: R = H
2.26: R = Ac

Jewenol A **2.27**, cussosides A **2.28**, cussosides B **2.29**, cussosides C **2.30**, cussosides D **2.31**, cussosides E **2.32**, [α-L-arabinofuranosyl-(1→4)-β-D-glucuronopyranosyl-(1→3)]-3β-hydroxyolean-12-en-28-oic acid **2.33** and [α-L-arabinofuranosyl-(1→4)-β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl-(1→3)]-3β -hydroxyolean-12-en-28-oic acid **2.34**, were among the compounds reported in *Cussonia* (Harinantenaina Liva *et al*., 2002; Gunzinger *et al*., 1986).

The 23-hydroxy-3-oxo-urs-12-en-28-oic acid **2.35** isolated from twigs and leaves of *Cussonia natalensi* has been reported to exhibit anti-ulcer property while triterpenoid saponins, 3-O-(α-L-arabinopyranosyl)-23-hydroxyursolic acid **2.36**, 3-O-(β-D-glucopyranosyl)-23-hydroxyursolic acid **2.37** and 23-hydroxyursolic acid **2.38** isolated from the stem bark of *Cussonia bancoensis* have nitric oxide inhibitory activities (Tapondju *et al*., 2003; Fourie *et al*., 1989). Other compounds reported in literature are cussonosides A and B, β-D-glucopyranosyl-ent-16-β, 17-dihydroxy-kauran-19-oate and paniculoside IV (Harinantenaina Liva *et al*., 2002).



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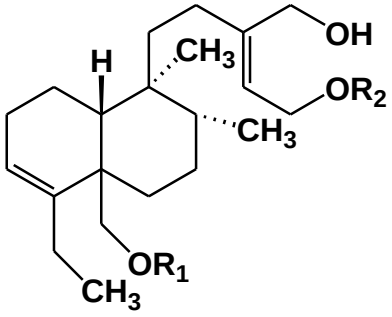
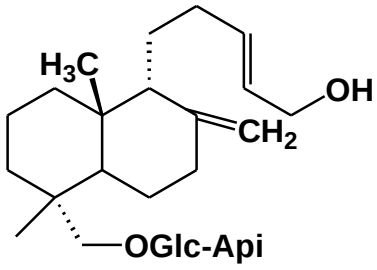


2.28: R_1 = Glc R_2=H
2.29: R_1 = Glc R_2 = Glc
2.30: R_1 = Glc R_2 = Api
2.31: R_1 = Glc-Api R_2=H



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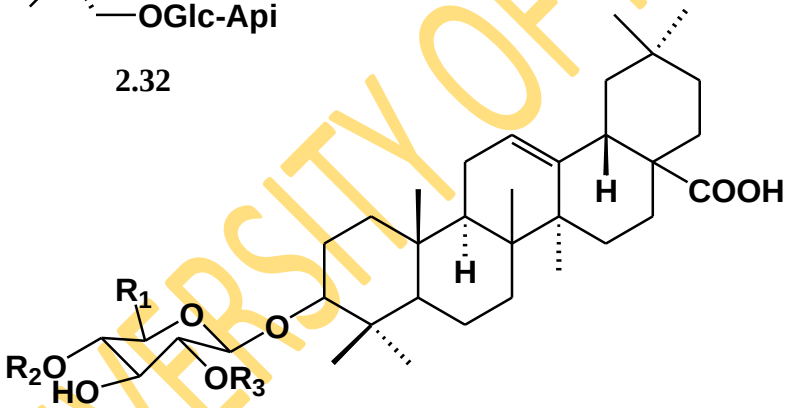
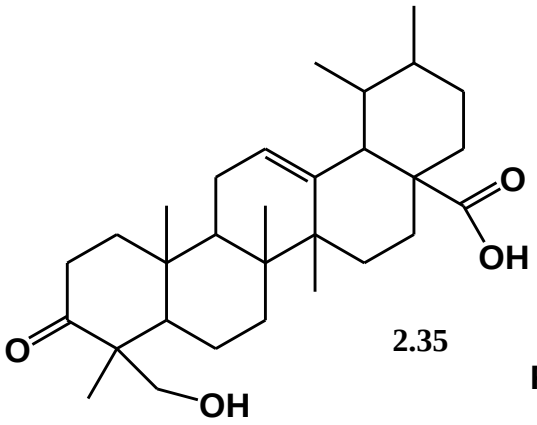


	R_1	R_2	R_3
2.33:	COOH	Ara(f)	H
2.34:	COOH	Ara(f)	Gal

Key:
Glc: b-D-glucopyranosyl
Api: b-D-apiofuranosyl
Ara(f): arabinofuranosyl
Gal: galactopyranosyl



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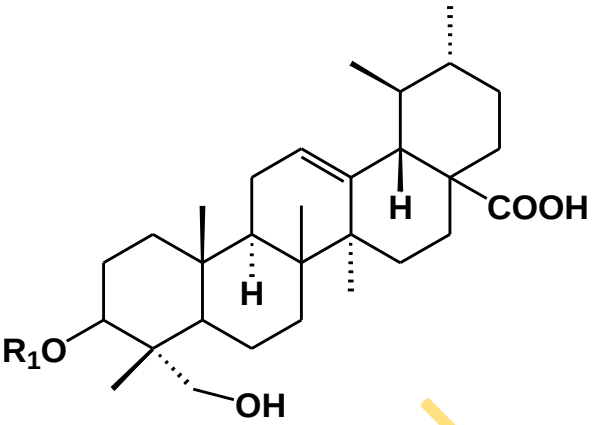
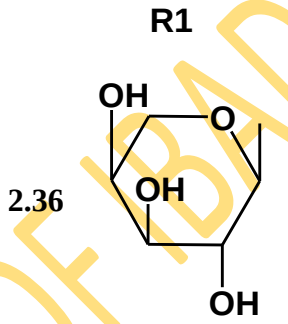
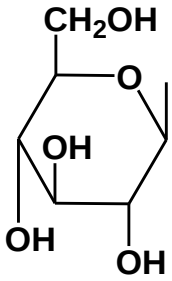




2.36



2.37

2.38 H

Cussonia arborea Hochst. ex A. Rich (Fig. 2.2) is a medium sized deciduous tree with rough and corky bark. It has a wide distribution in Africa, from the Western into the Central and Eastern areas of Africa. The specie is remarkably known for treatment of veneral diseases, women's infertility, paralysis, convulsions and epilepsy among others (Kougan *et al*., 2009). It is known as 'Sigo' among the Yorubas of the western Nigeria, where its leaves are used mainly for the treatment of painful menstruation, biliousness,

constipation and epilepsy (Odugbemi, 2008). Aba and Asuzu (2014), investigated the methanol stem bark extract of *C. arborea* in alloxan-induced diabetic rat models. The result shows that the extract exhibited anti-hyperglycaemic and anti-oxidant properties.

Triterpene glycosides, arboreasides A-E, Ciwujianoside C_3 and 28-O-α-L-rhamnosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside of 23-hydroxyursolic acid only, were reported to have been isolated from the bark of *C. arborea* (Kougan *et al*., 2009). Nvau (2014) reported the isolation of five compounds from ethyl acetate fraction of the stem bark of *C. arborea*. The compounds namely; stigmasterol, 3α,23-dihydroxylursol-12-en-28-oic acid, 3α,23-dihydroxyolean-12-en-28-oic acid, 2,4-dihydoxybenzoic acid and cussomerin were also found to exhibit anti-mycobacterial activity.

A literature survey showed that little or no chemical and biological work has been reported on the root of *C. arborea.*

Figure 2.2. Aerial part of *Cussonia arborea* Hochst *ex* A. Rich

2.2 Spectroscopic Techniques

2.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

This is a technique that explores the magnetic properties of certain atomic nuclei to determine the structure of their organic compounds. Common NMR active nuclei are ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, ^{29}Si etc. The NMR can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules.

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned. The utility of NMR stems from the fact that chemically distinct nuclei differ in resonance frequency in the same magnetic field. This phenomenon is known as the chemical shift. In addition, the resonance frequencies are perturbed by the existence of neighbouring NMR active nuclei, in a manner dependent on the bonding electrons that connect the nuclei. This is known as spin-spin, or *J* coupling. Spin-spin coupling allows one to identify connections between atoms in a molecule, through the bonds that connect them (Pavia *et al.*, 2001; Kalsi, 2004).

2.2.2 Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR)

This is a set of nuclear magnetic resonance spectroscopy (NMR) methods which give data plotted in a space defined by two frequency axes rather than one. The 2D NMR experiments include (i) Homonuclear through-bond correlation methods such as COrrelation SpectroscopY (COSY),) (ii) Heteronuclear through-bond correlation methods, such as Heteronuclear Single-Quantum Correlation spectroscopy (HSQC) and Heteronuclear Multiple-Bond Correlation spectroscopy (HMBC). (iii) Through-space correlation methods, like Nuclear Overhauser Effect Spectroscopy (NOESY) and Rotating frame nuclear Overhauser Effect Spectroscopy (ROESY). The most common types of 2D experiments are homonuclear correlation (COSY) and Heteronuclear Correlation (HETCOR) Spectroscopy (Schram and Bellama, 1988).

^{1}H-^{1}H COSY: This is homonuclear correlation spectroscopy, which shows the correlation between protons that are coupled to each other. There are many modified versions of the basic COSY experiment: DQF-COSY (Double-Quantum Filtered), COSY45, Long Range Correlation Spectroscopy (LRCOSY) and Exclusive Correlation Spectroscopy (ECOSY) (Macomber, 1998).

CH-COSY or **HETCOR**: This 2D NMR indicates heteronuclear correlation, usually between ^{1}H and ^{13}C resonances mediated by $J_{C\text{-}H}$. The experiment can be run using either $^{1}J_{C\text{-}H}$ or longer range couplings. It has poor sensitivity because the observed nucleus is ^{13}C and has been largely replaced by the inverse detection experiments, Heteronuclear Multi-Quantum Coherence (HMQC) and Heteronuclear Single Quantum Coherence **(**HSQC) (Lamber and Mazzola, 2002**)**.

Heteronuclear Multi-Quantum Coherence (HMQC): This experiment is similar to CH-COSY or HETCOR experiment, except that the inverse detection using a Distortionless Ehancement by Polarisation Transfer (DEPT) sequence provides much better sensitivity. It is used to correlate proton and carbon signals using either one bond or longer range couplings (Kalsi, 2004).

Heteronuclear Single Quantum Coherence (HSQC): This is a CH correlation experiment which uses proton detection of the ^{13}C signals using an Insensitive Nuclear Enhancement by Polarisation Transfer (INEPT) sequence. It shows higher resolution in the C-dimension than does the related HMQC experiment (Schram and Bellama, 1988).

Nuclear Overhauser Spectroscopy (NOESY): This experiment shows the correlation between protons that are close in space. The nuclear overhauser effect arises throughout radio frequency saturation of one spin, the effect causes the perturbation via dipolar interactions with further nucleus spins. This enhances the intensity of other spins. This method is a very useful tool to study the conformation of molecules. It is an NMR technique for determining the 3-dimensional structure of molecules (Lamber and Mazzola, 2002**)**.

2.2.3 Mass Spectrometry (MS)

Mass Spectrometers use the difference in mass-to-charge ratio (*m*/*z*) of ionized atoms or molecules to separate them. Therefore, mass spectroscopy allows quantitation of atoms or molecules and provides structural information by the identification of distinctive

fragmentation patterns. Detection of compounds can be accomplished with a very minute quantity of samples (Pavia *et al.*, 2001; Kalsi, 2004).

The general operation of a mass spectrometer is in three parts, creation of gas-phase ions, separation of the ions in space or time based on their mass-to-charge ratio and measurement of the quantity of ions of each mass-to-charge ratio. These three phases are carried out by suitable ionisation source, mass analysers and detector respectively.

The Ionisation source: This converts gas phase (sample) molecules into ions (or, in the case of electrospray ionization, move ions that exist in solution into the gas phase) examples include Chemical Ionisation (CI), Atmospheric Pressure CI (APCI), Electron Impact (EI), Electro-Spray Ionization (ESI), Fast Atom Bombardment (FAB), Field Desorption/Field Ionisation (FD/FI), Matrix Assisted Laser Desorption Ionisation (MALDI) and Thermospray Ionisation (TI).

Electron Impact Ionisation (EI): A beam of electrons passes through a gas-phase sample and collides with neutral analyte molcules to produce a positively charged ion or a fragment ion. Generally, electrons with energies of 70 ev are used. This method is applicable to all volatile compounds (>103 Da) and gives reproducible mass spectra with fragmentation to provide structural information (Rose and Johnstone, 2001).

Chemical Ionisation (CI): In this method, a reagent gas is first ionized by electron impact and then subsequently reacts with analyte molecules to produce analyte ions. This method gives molecular weight information and reduced fragmentation in comparison to EI.

Fast Atom Bombardment (FAB): Ions are produced by using a high current of bombarding particles to bombard the analyte which is in low volatile liquid matrix. This is a soft ionisation technique and is suitable for analysis of low volatility species. It produces large peaks for the pseudo-molecular ion species $[M+H]^+$ and $[M-H]^-$ along with other fragment ions and some higher mass cluster ions and dimmers (Kalsi, 2004).

Electro-Spray Ionization (ESI): A solution is nebulized under atmospheric pressure and exposed to a high electrical field which creates a charge on the surface of the droplet. The production of multiple charged ions makes electrospray extremely useful for accurate mass measurement, particularly for thermally labile, high molecular mass

substances (ie. proteins, oligonucleotides, synthetic polymers, etc.) (Hoffman and Stroobank, 2002).

Matrix-Assisted Laser Desorption/Ionization (MALDI): This is a soft ionization technique suitable for the analysis of molecules which tend to be fragile and fragment when ionized by more conventional ionization methods such as biomolecules (biopolymers e.g. DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules). It is similar in character to electrospray ionization both in relative softness and the ions produced (although it causes many fewer multiple charged ions). The MALDI is also more tolerant of salts and complex mixture analysis than ESI (Rose and Johnstone, 2001).

The mass analyzer: This sorts the ions by their masses by applying electromagnetic fields. Examples include quadrupoles, Time-of-Flight (TOF), magnetic sectors, Fourier transform and quadrupole ion traps.

2.2.4 Infrared Spectroscopy (IR)

Infrared spectroscopy measures the vibrations of molecules. The infrared region (10-14000 cm^{-1}) of the electromagnetic spectrum is divided into three regions: the near-, mid-, and far-IR. The mid-IR (400-4000 cm^{-1}) is the most commonly used region for the analysis as all molecules possess characteristic absorbance frequencies and primary molecular vibrations in this range. Mid-infrared spectroscopy methods are based on studying the interaction of infrared radiation with samples. As IR radiation is passed through a sample, specific wavelengths are absorbed causing the chemical bonds in the material to undergo vibrations such as stretching, contracting, and bending. Functional groups present in a molecule tend to absorb IR radiation in the same wave number range, regardless of other structures in the molecule, and spectral peaks are derived from the absorption of bond vibrational energy changes in the IR region (Smith, 1996; Tolstoy *et al.,* 2003). The IR spectroscopic analysis is mainly for determining chemical functional groups in samples.

2.2.5 Ultra violet Spectroscopy

Ultra violet spectroscopy (UV) is a type of absorption spectroscopy in which light of the ultra-violet region (200-400 nm) is absorbed by the molecule. Absorption of the ultra-violet radiations results in the excitation of electrons from the ground state to higher

energy state. The energy of the ultra-violet radiation that are absorbed is equal to the energy difference between the ground state and higher energy states ($\Delta E = hf$). Generally, the most favoured transition is from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) (Pavia *et al.*, 2001; Kalsi, 2004).

The UV spectroscopy employs the Beer-Lambert law, which states that when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with the thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution, or the greater the number of molecules capable of absorbing light of a given wavelength, the greater the extent of light absorption. This is the basic principle of UV spectroscopy. The UV spectroscopy can be used for the detection of chemical functional group and extent of conjugation, determination of purity of a substance and the configuration of geometrical isomers, as well as identification of unknown compounds.

2.2.6 X-ray crystallography

X-ray crystallography is a technique in which the pattern produced by the diffraction of x-rays through the closely spaced lattice of atoms in a crystal is recorded and then analysed to reveal the nature of that lattice. X-ray crystallography allows precise determination of the atomic positions and consequently the bond lengths and angles of molecules within a single crystal. X-rays are electromagnetic radiation with wavelengths between about 0.02 Å and 100 Å (1Å = 10^{-10} meters).

When X-rays are beamed at the crystal, electrons diffract the X-rays, which cause a diffraction pattern. Through the use of the mathematical Fourier transform, these patterns can be converted into electron density maps. These maps show contour lines of electron density. Since electrons more or less surround atoms uniformly, it is possible to determine where atoms are located; only hydrogen is difficult to map because it has one electron thus resulting in very low electron density around it. The crystal is rotated while a computerized detector produces two dimensional electron density maps for each angle of rotation. The third dimension comes from comparing the rotation of the crystal with the series of images. Computer programmes use this method to come up with three dimensional spatial coordinates (Rodes, 1993; Carter *et al*., 1997).

2.3 Anti-oxidant activity of natural products

Oxygen is the most prevalent element in the earth's crust. It exists in air as a diatomic molecule, O_2. Except for a small number of anaerobic bacteria, all living organisms use O_2 for energy production and it is essential for life as we know it. Energy production by organisms from food material requires "oxidation", which implies the loss of electrons. However the potential of O_2 to oxidize also makes it toxic. Oxidation can inactivate important enzymes, and anaerobes that do not have antioxidant mechanisms do not survive in an O_2 environment (Nunes *et al*., 2012). Life under aerobic conditions is characterised by continuous production of free radicals, which is counterbalanced by the activity of antioxidant enzymes and non-enzyme defenses. However, if the production of free radicals exceeds the antioxidant capacity of a living system, these reactive oxygen and nitrogen species can react with lipids, proteins, and DNA causing structural and functional damage to the cell's enzymes and genetic material (Nunes *et al*., 2012). The predominance of oxidants and their consequent damage is called oxidative stress.

The term reactive oxygen species (ROS) includes radicals or chemical species that take part in radical type reactions (i.e. gain or loss of electrons) but are not true radicals in that they do not have unpaired electrons. Examples of non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3). Examples of radical ROS include super oxide anion radicals ($O_2^{\bullet-}$) and hydroxyl radical species ($^{\bullet}OH$). Besides oxygen-based radicals, there are also reactive nitrogen species such as nitric oxide (NO) and nitrogen dioxide (NO_2). An important product of the two radicals $O_2^{\bullet-}$ and NO is peroxynitrite (ONOO-), this reaction occurs at a diffusion limited rate (Nunes *et al*, 2012). Oxidative stress, caused by an imbalance between ROS and the anti-oxidative defense systems is considered to be a major etiological or pathogenic agent of cardiovascular and neurodegenerative diseases, cancers, Alzheimer's, diabetes and aging. Because they inhibit or delay the oxidative process by blocking both the initiation and propagation of oxidizing chain reactions, antioxidants for the treatment of cellular degenerations are beginning to be considered (Jang *et al*., 2010). Oxidative stress and its effects on human health have become a serious issue. Under stress, our bodies end up having more reactive oxygen species than antioxidant species, an imbalance that leads to cell damage (Krishnaiah *et al*., 2011). Cell degradation eventually leads to partial or total functional loss of physiological systems in the body.

Currently, the incidence of free radical imbalance at the onset and during the evolution of more than 100 diseases (cardiovascular, neurological, endocrine, respiratory, immune and self-immune, ischaemia, gastric disorders, tumor progression and carcinogenesis, among others) has been demonstrated (Gupta *et al*, 2010). Interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, replacing synthetic antioxidants which are often restricted due to carcinogenic effects (Djeridane *et al*., 2006; Wannes *et al*., 2010).

2.4 Methods of anti-oxidant activity assessment for natural products

Several techniques have been used to determine the anti-oxidant activity *in vitro* in other to allow rapid screening of substances and/or mixtures of potential interest in the prevention of chronic degenerative diseases.

2.4.1 DPPH assay

This method was first described by Blois (1958), and was later modified slightly by numerous researchers. The DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is considered, from a methodological point of view, one of the easiest, most accurate and productive for evaluation of antioxidant activity in fruit juices, plant extracts and pure substances like flavonoids and terpenoids (Nunes *et al*., 2012). The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourises the DPPH solution. The degree of colour change is proportional to the the concentration and potency of the anti-oxidants. Anti-oxidants activity is then measured by the decrease in absorption at 517 nm. A large decrease in the absorbance of the reaction mixtures indicates significant free radical scavenging activity of the compound under test. The electron donation ability of natural products can be measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The anti-radical activity (three replicates per treatment) is expressed as IC_{50} (µg/ml), the concentration required to cause a 50% DPPH inhibition. The method is influenced by the solvent and the pH of the reactions. The antioxidants BHA, BHT and Trolox® can be used as references in the experiments (Nunes *et al*., 2012).

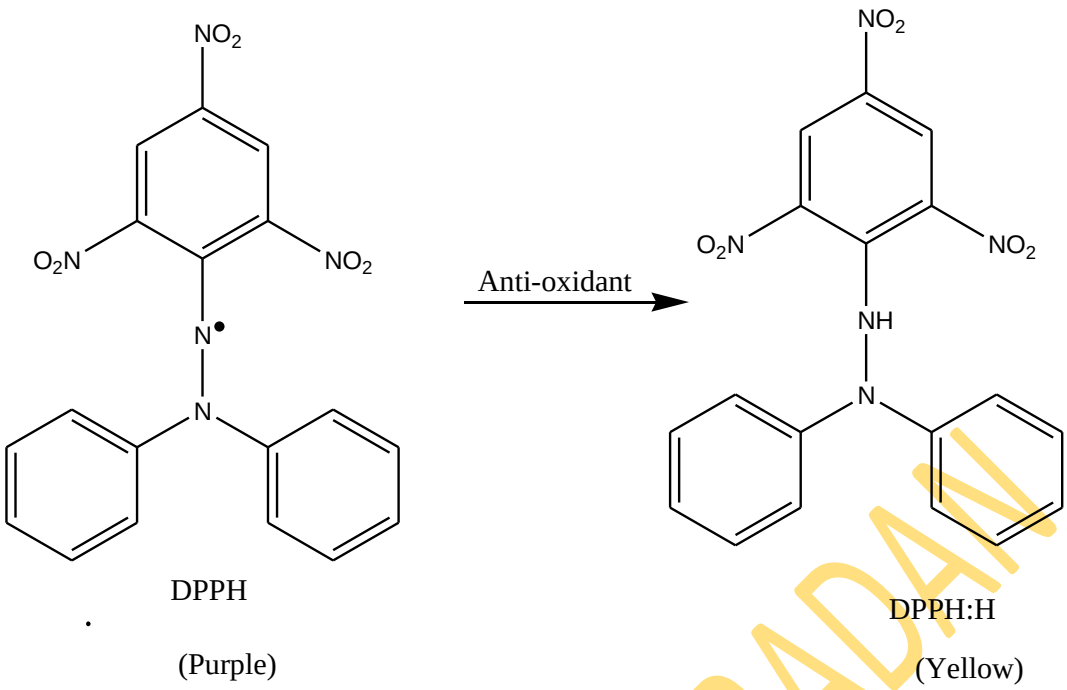


Fig. 3.1 Radicals and non-radical form of DPPH.

2.4.2 β-carotene assay

The β-carotene/linoleic acid oxidation method evaluates the inhibitory activity of free radicals generated during the peroxidation of linoleic acid. The method is based on spectrophotometric discoloration measurements (or oxidation) of β-carotene-induced oxidative degradation products of linoleic acid. This method is suitable for plant samples. The β-carotene bleaching method is based on the loss of β-carotene's yellow colour due to its reaction with radicals formed by linoleic acid oxidation when in an emulsion. The rate of the β-carotene bleaching can be slowed in the presence of antioxidants. The reaction can be monitored by spectrophotometer, β-carotene loss of staining at 470 nm, with intervals of 15 min for a total time of 2 hours. The results are expressed as IC_{50} (μg/ml), the concentration required to cause a 50% β-carotene bleaching inhibition. Tests are realized in triplicate. The results can be compared with synthetic standards such as BHA, BHT and Trolox®, or natural, such as gallic acid and quercetin (Nunes *et al*., 2012).

2.4.3 ABTS method

The 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid), commonly called ABTS, radical scavenging method was developed by Rice-Evans and Miller and was then modified by researchers (Nunes *et al*., 2012). The modification is based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce a radical cation. The improved method generates a blue/green ABTS chromophore via the reaction of ABTS and potassium persulfate. Along with the DPPH method, the ABTS radical scavenging method is one of the most extensively used antioxidant assays for plant samples. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm. Decolourisation assays measure the total antioxidant capacity in both lipophilic and hydrophilic substances. The effects of oxidant concentration and inhibition duration, of the radical cation's absorption are taken into account when the antioxidant activity is determined. Trolox is used as a positive control. The activity is expressed in terms of Trolox-equivalent antioxidant capacity for the extract or substance (TEAC/mg) (Krishnaiah *et al*., 2011).

2.4.4 Reducing power assay

In this assay, the yellow colour of the test solution changes to green depending on the reducing power of the test specimen. The presence of the reductants in the solution causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by absorbance measurement at 700 nm. In the reducing power method, the sample is mixed in 1 mL of methanol with a phosphate buffer (5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5 mL, 1%). The mixture is incubated at 50 ºC for 20 minutes. Next, 5 mL of trichloroacetic acid (10%) are added to the reaction mixture, which is then centrifuged at 3000 RPM for 10 minutes. The upper layer of the solution (5 mL) is mixed with distilled water (5 mL), and ferric chloride (1 mL, 1%), and the absorbance is measured at 700 nm. A stronger absorbance indicates increased reducing power (Krishnaiah *et al*., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 General experimental procedures

Most solvents used were of analytical grade and purchased from Fischer Scientific, Aldrich and chemical distributors in Nigeria; while others were distilled before use. Spray reagents were purchased from Aldrich chemical company.

Melting points were determined on a Buchi 510-K melting point apparatus and were uncorrected. Optical rotations were measured on JASCO P-2000 polarimeter. The IR and UV were recorded using a JASCO 302-A and SHIMADZU UV-240 spectrometers respectively. The ^{1}H-NMR spectra (δ ppm, J in Hz) were recorded on a Bruker AM-300 FT NMR, AM-400 FT NMR, AM-500 FT NMR and AM-600 FT NMR spectrometers. While ^{13}C-NMR spectra were recorded at 75, 100, 125 and 150 MHz on a Bruker AM-300 FT NMR, AM-400 FT NMR, AM-500 FT NMR and AM-600 FT NMR spectrometers respectively. The mass spectra were scanned on a Jeol-JMS 600-H and Finnigan MAT-112 and 312A double focusing mass spectrophotometers connected to DEC PDP 11/34 and IBM-AT compatible PC based system. High Resolution Electron Impact Mass Spectra (HREIMS) were recorded on a MAT 95XP mass spectrometer. X-ray diffraction analysis was carried out on Bruker SMART APEX CCD based diffracometer with Mo Kα radiation, 2000); cell refinement: SAINT (Bruker, 2000); SHELXS97.

Thin Layer Chromatography (TLC) was performed to check the purity of samples on aluminium sheets (0.25 mm thickness) pre-coated with Merck silica gel F_{254}. Column chromatography was performed on silica gel (70-230 mesh) and sephadex LH-20 (Fluka 25-100 mm). Preparative Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) was performed on Shimadzu LC 6A dual pump system with SPD 6AV UV and RI detectors employing RP-18 ODS reverse phase semi-preparative column. The GC-MS analysis of the extracted oil extract was performed using a Shimadzu model QP

2010 chromatograph. An HP-Innowax FSC column (30 m x 0.25 mm, with 0.25 µm film thickness) was used with Helium as the carrier gas at a flow rate of 1 ml/min. The GC oven temperature was kept at 60 °C (hold for 0 min), and programmed to reach 140 °C at a rate of 5 °C/min, then kept constant at 280 °C for 10 min being the final hold time. The split ratio was adjusted to 50:1. The injector temperature was set at 200 °C. Mass spectra were recorded at 70 eV. The acquisition mass range was 30-500 m/z. Data was analysed using MSD ChemStation software.

All glasswares were properly cleaned and the solvents were removed under reduced pressure using a rotary evaporator (Heidolph HB digital, Germany) at a pump pressure of 0.1 mmHg.

3.2 Plant material

The whole plant of *Dioclea reflexa* and *Cussonia arborea* were collected from a farmland at Eruwa in Ibarapa East Local Government area of Oyo state, Nigeria, in May, 2011. The *D. reflexa* was authenticated by Mr Bolu Ajayi of Herbarium Section, Plant Biology Department, University of Ilorin with a voucher specimen (UIH-0027) and *C. arborea* was authenticated the same way with a voucher specimen (UIH-22340). The voucher specimens were deposited at the herbarium.

3.2.1 Extraction of *D. reflexa*

The plant was sorted into different parts (leaves, stems and roots) and air-dried at room temperature for a week. The air-dried, powdered root of *D. reflexa* (1 kg) was soaked in 10 L of ethanol for a week at room temperature. The solution obtained was filtered with whatmann NO 1 filter paper and concentrated under vacuum using rotary evaporator preset at 30 °C. The dried crude extract of the root (85 g) was later stored in refrigerator at 4 °C prior to use.

3.3 Phytochemical Analysis

The following standard procedures (Sofowora, 1984; Harbone, 1993) were used to test for the presence of secondary metabolites in the plant extracts (Table 4.1).

3.3.1 Test for carbohydrates

i. ***Molish test:*** Few drops of Molish reagent were added to 2 mL of test extract in a test-tube. Concentrated H_2SO_4 (1 mL) was then allowed to flow down the side of the inclined test-tube. A reddish brown solution indicated a positive test.

ii. **Fehling's test for reducing sugar:** Five millilitres of equal volumes of Fehling's solutions A and B was added to 2 mL of test extract in a test tube. The resultant mixture was boiled for 2 minutes. A brick red or orange precipitate of copper (I) oxide was an indication of a positive test.

3.3.2 Test for alkaloids

i. **Drangendorff's reagent:** Test sample (0.2 g) was acidified with 1% HCl for 2 minutes and was then treated with few drops of Dragendorff's reagents in a test-tube. The formation of white precipitate indicated the presence of alkaloids.

ii. **Mayer's reagent:** Test sample (0.2 g) was acidified with 1% HCl for two minutes. A cream coloured precipitate formation on addition of Mayer's reagent indicated the presence of alkaloids.

3.3.3 Test for tannins

A dirty-green precipitate, or blue-black, or blue green precipitate, on addition of a few drops of 5% ferric chloride ($FeCl_3$) to the test extract was taken as an indication of the presence of tannins.

3.3.4 Test for glycosides

Five millilitres of H_2SO_4 was added to 0.2 g sample of the test extract, the mixture was heated in boiling water for 15 minutes. Fehling solution was then added and the resulting mixture was heated till it was boiling. A brick-red precipitate indicated the presence of glycosides.

3.3.5 Test for saponins

Test sample (0.2 g) was dissolved in 5 mL of distilled water. Two milliliters of the resulted solution was taken into a test-tube and was shaken vigorously for a few minutes. Frothing which persisted on warming was taken as an evidence of the presence of saponins.

3.3.6 Test for steroids

Test sample (0.2 g) was dissolved in 2 mL of chloroform. Concentrated H_2SO_4 (0.2 ml) was carefully added to form a lower layer. A reddish-brown colour at the interface between the layers indicated the deoxy-sugar characteristics of cadenolides which indicated the presence of steroids.

3.3.7 Test for flavonoids (Shinoda's test)

Test sample (0.2 g) was dissolved in dilute NaOH (50 %). A yellow solution that turns colourless on addition of dilute HCl (50 %) indicated the presence of flavonoids.

3.3.8 Test for resins

Test sample (0.2 g) was shaken with distilled water and filtered. Copper acetate solution (1 mL) was added to 1 mL of the filterates. The resulting solution was shaken vigorously and allowed to separate. A green-colour solution was an evidence of the presence of resins.

3.3.9 Test for cardiac glycosides

Test sample (0.2 g) was dissolved in 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was then reacted with 1 mL concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxy sugar characteristic of cardiac glycosides.

3.3.10 Test for phenols

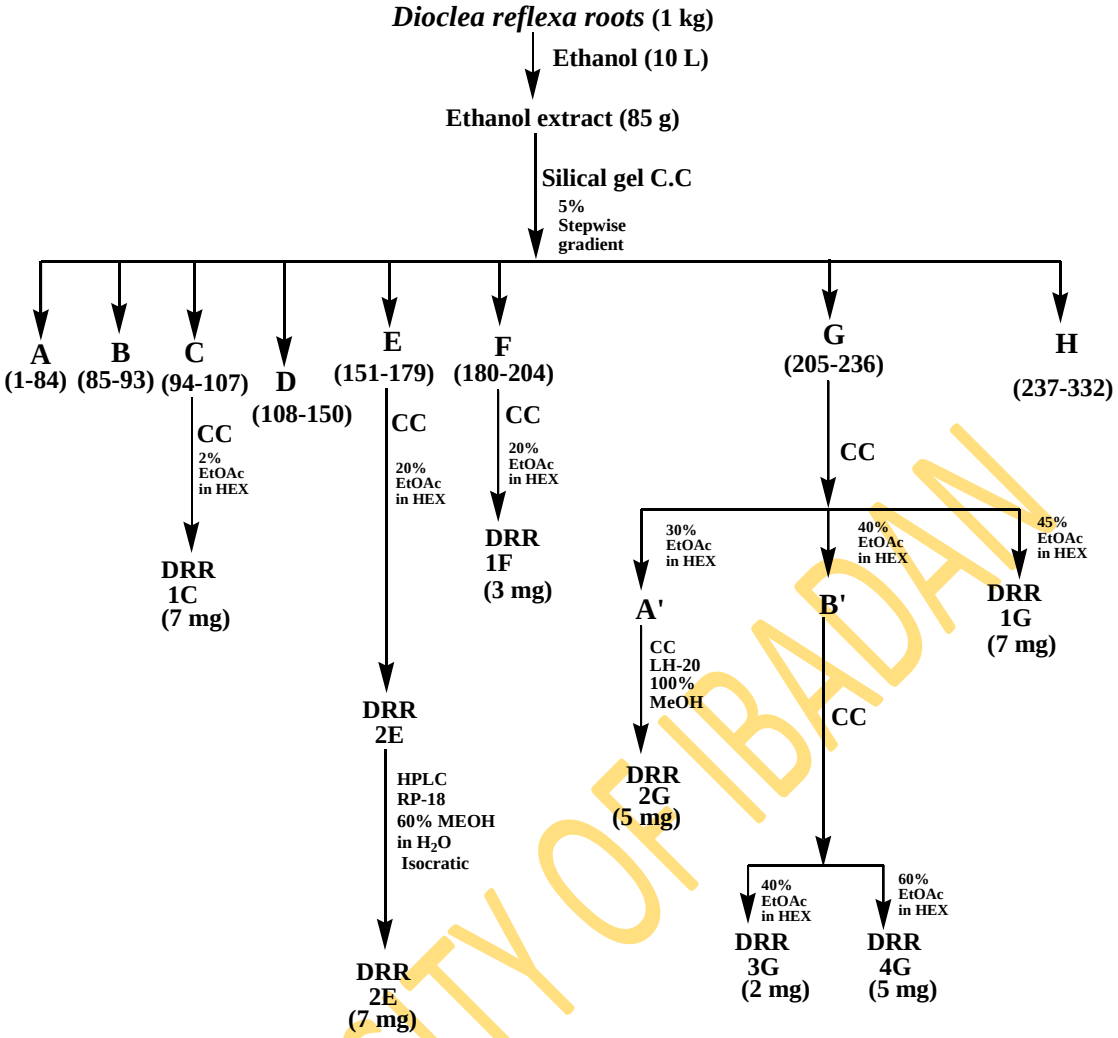
Test sample (0.2 g) of test extract was dissolved in 2 mL ferric chloride solution. Blue-black or brown colouration indicated the presence of phenol.

3.3.11 Test for anthraquinones (Born-Trager's test)

Test sample (0.2 g) was shaken with 4 mL of benzene. The mixture was filtered and 2 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of pink red or violet colour in ammoniacal solution (lower phase) indicated the presence of free anthraquinones.

3.4 Column chromatography of crude ethanol extracts of *D. reflexa* roots

The dried crude ethanol extract (85 g) of the root was re-dissolved in MeOH and then pre-adsorbed on silica gel (42 g) to form a homogenous solid. The adsorbed extract was subjected to column (length = 82 cm, internal diameter = 5.0 cm) chromatography using silica gel (2.55 kg) and successively eluted with a gradient of organic solvents with increasing polarities of n-hexane (HEX), HEX/dichloromethane (DCM), DCM, DCM/ethyl acetate (EtOAc), EtOAc, EtOAc/methanol (MeOH). The 332 (100 ml / vial) fractions collected were pooled together to 8 sub-fractions (A-H) according to TLC profile (Table 4.2; Scheme 3.1).



Scheme 3.1. Scheme of fractionation and isolation of *D. reflexa* roots extract

Keys:

A-H = Combined fractions

DRR = ***D****ioclea* ***r****eflexa* **r**oots

3.4.1 Purification of sub-fraction C

Sub-fraction C (40 mg) was loaded on silica-gel column chromatography (length = 22 cm, internal diameter = 1.0 cm). Elution was carried out using gradient of n-hexane and ethyl acetate [n-hexane (100%, 60 mL); n-hexane: ethyl acetate (98:2, 75 mL); (95:5, 75 mL); and (92:8, 30 mL)]. The eluents were collected in the fractions of 15 mL/vial. A total of 16 fractions were collected. Combined fractions 8-9 eluted with hexane-ethyl acetate (49:1) gave white crystalline substance tagged DRR 1C, which was further purified by washing with n-hexane repeatedly (Table 4.3).

Characterisation of DRR-1C

White crystals, yield = 7 mg.

M.pt 214-215˚C.

UV λ_{max} nm (log ε): 243(0.25).

IR V_{max} cm^{-1}: 3364 (OH), 2941, 2859 (C-H aliphatic), 1455, 1381 cm^{-1}.

EI-MS *m/z* (rel. int): 426 (M^+, 70), 411 (28), 393 (10), 315 (21), 257 (16), 234 (23), 218 (69), 207 (100), 189 (95), 135 (84).

HREIMS= 426.3714 (Calcd. 426.3864 for $C_{30}H_{50}O$).

^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.74 (3H, s, H-24), 0.77 (3H, s, H-28), 0.81 (3H, s, H-25), 0.92 (3H, s, H-27), 0.95 (3H, s, H-23), 1.01 (3H, s, H-26), 1.66 (3H, s, H-30), 2.33 (1H, m, H-19), 3.15 (1H, dd, J =4.8, 12.0 Hz, H-3β), 4.54 (1H, d, J= 2.0 Hz, H-29α), 4.67 (1H, d, J= 2.0 Hz, H-29β). (Table 4.4)

^{13}C-NMR ($CDCl_3$, 125MHz): δ 15.4 (C-24), 18.0 (C-28), 16.2 (C-25), 14.6 (C-27), 28.0 (C-23), 16.0 (C-26), 19.3 (C-30), 48.0 (C-19), 79.0 (C-3), 109.3 (C-29), 151.0 (C-20), 27.5 (C-2), 35.6 (C-16), 43.0 (C-17), 38.9 (C-4), 55.3 (C-5), 18.3 (C-6), 40.9 (C-8), 37.2 (C-10), 20.9 (C-11), 25.2 (C-12), 38.1 (C-13), 42.8 (C-14), 27.4 (C-15), 38.7 (C-1), 34.3 (C-7), 50.5 (C-9), 48.3 (C-18). (Table 4.4)

HSQC, HMBC and COSY experiments are presented in Appendix 1-3.

3.4.2 Purification of sub-fraction E

Sub-fraction E (170 mg) was rechromatographed on silica gel column (length = 22 cm, internal diameter = 1.5 cm) chromatography using n-hexane/EtOAc as eluents. The solvent systems used in eluting the column are: n- hexane/EtOAc (90:10, 89:11, 87:13, 85:15, 83:17, 80:20, 75:25, 65:35, 80:20, 50:50, and 40:60). A total of 370 fractions were collected which were pooled to 7 sub-fractions (E1-7) using TLC analysis (Table 4.5). The E4 (55 mg) eluted with 20 % EtOAc in hexane afforded DRR 2E as semi-pure sample and was further purified via HPLC on RP-18, ODS column, eluted with MeOH/H_2O (60:40) at flow rate of 1 mL/min.

Characterisation of DRR-2E

Yellow powder, yield = 7 mg

M.pt. 222 °C

IR (KBr): 3361 (OH), 1652 (C=O), 1459 (ring C-C) cm^{-1}

UV λ_{max} nm (log ε): 311 (0.55), 276 (1.03), 230 (1.44), 214 (1.56).

EIMS *m/z* (relative intensity %): 43 (23), 83 (46), 136 (9), 137 (65), 151 (43), 153 (29), 163 (19), 166 (29), 259 (9), 271 (2), 287 (8), 302 (M^+, 100).

HREI MS: *m/z*: 302.0769 [calculated 302.0792 for $C_{16}H_{14}O_6$]

^{1}H-NMR (CD_3COCD_3, 500MHz): δ 8.54 (2H, br s, OH-3′/5′), 7.71 (1H, d, J= 8.5 Hz, H-5), 6.59 (2H, s, H-2′/6′), 6.55 (1H, dd, J= 2, 8.5 Hz, H-6), 6.42 (1H, d, J= 2Hz, H-8), 5.37 (1H, dd, J= 2.5, 12.5 Hz, H-2), 2.93 (1H, dd, J= 12.5, 17 Hz, H-3a), 2.66 (1H, dd, J= 3, 17 Hz, H-3b), 3.80 (3H, s, OCH_3). (Table 4.6)

^{13}C-NMR (CD_3COCD_3, 125MHz): δ 80.3 (C-2), 44.7 (C-3), 190.2 (C-4), 129.4 (C-5), 111.3 (C-6), 165.4 (C-7), 103.7 (C-8), 164.3 (C-9), 115.2 (C-10), 136.2 (C-1′), 106.6 (C-2′/6′), 151.4 (C-3′/5′), 136.2 (C-4′), 60.7 (OCH_3). (Table 4.6)

3.4.3 Purification of sub-fraction F

The sub-fraction F (668 mg) was pre-adsorbed on silica gel and chromatographed on silica gel column (length = 23 cm, internal diameter = 2.0 cm). Fractionation was carried out using n-hexane: EtOAc (92:8, 200 mL); (90:10, 200 mL); (85:15, 200 mL);

(4:1, 200 mL); (3:1, 200 mL); (7:3, 100 mL); (65:35, 200 mL); (60:40, 200 mL); (1:1, 200 mL) and (3:7, 200 mL) separately and EtOAc (100%, 200 mL) followed by EtOAc: methanol (1:1, 200 mL and methanol (100%, 200 mL) individually to give 305 fractions which were pooled to 14 sub-fractions (F1-14) using TLC analysis. Fraction F5 eluted with n-hexane/EtOAc (4:1) gave white needle-like crystals of DRR-1F (Table 4.7).

Characterisation of DRR-1F

White needle-like crystal, yield = 3 mg.

M.pt. 186-188 ˚C.

IR Vmax (cm^{-1}) : 3300, 1729, 1658, 1632.

UV λ_{max} nm (log ε) = 244 (1.18).

[α]^{30} = -45 ($CHCl_3$, c 0.07).

EIMS: *m/z* (rel int %):444.1 (M^+, 2), 384 (3), 353 (4), 323 (4), 311 (11), 293 (5), 269 (11), 252 (89), 224 (78), 176 (9), 176 (7), 172 (15), 134 (9), 131 (14), 120 (10), 105 (100), 91 (10), 77 (25), 51(2) 43 (6).

HREI MS: *m/z*: 444.1000 [calculated 444.5200 for $C_{27}H_{28}O_4N_2$]

^{1}H-NMR ($CDCl_3$, 400 MHz) : δ 2.08 (3H, s,H-1), 2.74 (2H, m,H-11), 3.00 (1H, dd, J=13.6, 8.0, H-10a), 3.18 (1H, dd, J=13.6, 5.2, H-10b), 3.81 (1H, dd, J=11.2, 6.4, H-3a), 3.93 (1H, dd, J=11.2, 7.2, H-3b) , 4.33 (1H, m, H-4), 4.72 (1H, q, H-7), 5.87 (1H, d, J= 8.0, H-5), 6.69 (1H, d, J= 6.8, H-8), 7.04-7.21 (5H, m), 7.21-7.30 (5H, m), 7.42(2H, t) 7.51 (1H, d), 7.70 (2H, dd). (Table 4.8)

^{13}C-NMR (CD_3Cl_3, 100MHz): See Table 4.8

3.4.4 Purification of sub-fraction G

Sub-fraction G (1.2 g) was purified over silica gel column (length = 18 cm, internal diameter = 2.5 cm) and gradient elution was carried out with n-hexane/ EtOAc (19:1, 500 mL); (9:1, 500 mL); (85:15, 500 mL) and (4:1, 500 mL); (3:1, 500 mL mL); (7:3, 500 mL); (65:35, 500 mL); (6:4, 500 mL); (55:45; 250 mL); (1:1, 500 mL mL); (40:60, 250 mL); (35:65, 250 mL); (25:75, 250 mL) and (1:9, 250 mL) individually to give 205

fractions. The TLC analysis of the fractions resulted in 15 pooled sub-fractions (G1-15). The G-9 (12 mg) eluted with 40% ethyl acetate in hexane gave a pure yellow solid which was coded DRR 1G (7.0 mg). The G-6 (262 mg) and G-8 (176 mg) eluted with 30% and 40% ethyl acetate in hexane respectively were further purified.

Characterisation of DRR-1G

Yellow crystals, yield = 7 mg.

M.pt.: 225 ˚C

UV λ_{max} nm (log ε) = 212 (1.45), 311 (0.9)

IR (KBr): 3377 (OH), 1627 (C=O), 1570 (C=C) cm^{-1}

EIMS *m/z* (relative intensity %): 53(48), 77 (22), 108 (20), 121 (40), 137 (40), 149 (29), 164 (8), 200 (8), 229 (100), 257 (96), 285(22), 300 (89).

HREIMS = 300.0621 [calculated 300.2700 for $C_{16}H_{12}O_6$].

^{1}H-NMR (CD_3COCD_3, 400 MHz): δ 3.87 (3H, s, OCH_3), 6.54 (1H, s, H-3), 6.97 (1H, dd, J= 2.4, 8.8 Hz, H-6), 7.01 (1H, d, J= 2.4Hz, H-8), 7.07 (2H, s, H-2′/6′), 7.95 (1H, d, J=8.8Hz, H-5), 9.64 (1H, br s, OH-7). (Table 4.10).

^{13}C-NMR (CD_3COCD_3, 100 MHz): δ 163.3 (C-2), 107.0 (C-3), 177.2 (C=O), 127.7 (C-5), 115.4 (C-6), 163.3 (C-7), 103.5 (C-8), 158.7 (C-9), 118.0 (C-10), 128.3 (C-1′), 106.6 (C-2′/6′), 151.8 (C-3′/5′), 139.0 (C-4′), 60.7 (OCH_3). (Table 4.10).

NOESY experiment is presented in Appendix 4.

3.4.5 Purification of chromatographic fraction G-6

The G-6 (262 mg) was chromatographed on silica gel column (length = 19 cm, internal diameter = 2 cm) using hexane: EtOAc (9:1, 25 mL), (4:1, 25 mL), (7:3, 25 mL), (3:2, 20 mL), (1:1, 20 mL), (2:3, 20 mL), (3:7, 20 mL), (1:4, 20 mL) and (1:9, 20 mL) separately. Thirty-nine fractions were collected. The fractions were pooled to six sub-fractions using TLC analysis (GA 1-6). Rechromatography of GA-5 (50 mg) on a sephadex LH-20 column using isocratic system with 100 % MeOH yielded DRR 2G (5 mg), a yellow powder (Table 4.11).

Characterisation of DRR-2G

Yellow powder, yield = 5 mg.

M.pt.: 208-210 ˚C.

UV λ_{max} nm (log ε) = 212 (1.268), 260 (0.749), 365 (0.845).

IR (KBr): 3380 (OH), 1659 (C=O ketone), 1597, 1507 cm^{-1};

EIMS *m/z* (relative intensity %): 332.1 (91), 317 (M^+, 100), 289 (20), 261 (48), 233 (11), 205 (10), 177(6), 153(20), 108(12), 77(32).

HREIMS = 332.0574 (Calcd. 332.25287 for $C_{16}H_{12}O_8$).

^{1}H-NMR (CD_3COCD_3, 400 MHz): δ 3.88 (3H, s, OCH_3), 6.26 (1H, d, J= 2.0 Hz, H-6), 6.52 (1H, d, J= 2.0 Hz, H-8), 7.38 (2H, s, H-2′/6′), 8.36 (2H, s, OH-3′/5′), 12.10 (1H, s, OH-5). (Table 4.12)

^{13}C-NMR (CD_3COCD_3, 100 MHz): δ 146.2 (C-2), 137.5 (C-3), 176.7 (C=O), 162.3 (C-5), 99.2 (C-6), 165.1 (C-7), 94.4 (C-8), 157.8 (C-9), 104.2 (C-10), 127.3 (C-1′), 108.3 (C-2′/6′), 151.3 (C-3′/5′), 138.0 (C-4′), 60.7 (OCH_3). (Table 4.12)

HMBC, HSQC, COSY and NOESY experiments are presented in Appendix 5-8

3.4.6 Purification of chromatographic fraction G-8

The G-8 (176 mg) was chromatographed on a finger column chromatography using silica gel and eluted with gradient of hexane: EtOAc (9:1, 20 mL), (4:2, 20 mL), (7:3, 20 mL) (6:4, 20 mL), (1:1, 20 mL), (2:3, 20 mL), (3:7, 20 mL) and (1:4, 20 mL) separately. Fifty-one fractions were collected and pooled to 9 sub-fractions (GB 1-9) (Table 4.13). The GB-2 (2 mg) and GB-9 (5 mg) from the elution of 10% and 40% ethyl acetate in hexane afforded yellow powders coded DRR-3G (2 mg, n-hexane/EtOAc, R_f 60:40) and DRR-4G (5 mg, n-hexane/EtOAc, R_f 40:60) respectively.

Characterisation of DRR-3G

Yellow powder, yield = 2 mg

M.pt.: 101 ˚C

UV λ_{max} nm (log ε) = 213(1.13), 255(0.65).

EIMS: *m/z* (relative intensity %): 51 (6), 67 (9), 113 (30), 141 (22), 151 (4), 169 (95), 184 (100).

HREIMS: *m/z*: 184.0379 [calculated 184.1272 for $C_8H_8O_5$]

^{1}H-NMR (CD_3OD, 400MHz): δ 3.84 (3H, s), 7.02 (2H, s). (Table 4.14)

^{13}C-NMR (CD_3OD, 100MHz): δ 170 (C=O), 151.6 (C-3), 140.8 (C-4), 127.9 (C-1), 110.3 (C-2), 60.7 (OCH_3). (Table 4.14)

HMBC, HSQC, COSY and NOESY experiments are presented in Appendix 9-12.

Characterisation of DRR-4G

Yellow powder, yield = 5 mg.

M.pt.: 115 ˚C

UV λ_{max} nm (log ε) = 213 (1.627), 230 (1.362), 328 (1.677)

IR (KBr) = 3619 (Ar-OH), 1697 (ketones C=O), 1649 (ring C=C) cm^{-1}

EIMS *m/z* (relative intensity %): 254.2 (M^+, 100), 237 (5.5), 226 (41), 136 (9), 137 (40), 118 (24), 108 (12), 86 (39),

HREIMS= 254.0567 (Calcd. 254.24425 for $C_{15}H_{10}O_4$).

^{1}H-NMR (CD_3OD, 400 MHz): δ 7.96 (1H, d, J= 8.8 Hz, H-5), 7.86 (2H, dt, H-2'/6'), 6.97 (1H, d, J= 2.0 Hz H-8), 6.91 (3H, m, H-3'/5'/6), 6.68 (1H, s, H-3). (Table 4.15)

^{13}C-NMR (CD_3OD, 100 MHz): δ 166.0 (C-2), 105.1 (C-3), 180.3 (C=O), 127.8 (C-5), 116.3 (C-6), 165.0 (C-7), 103.5 (C-8), 159.5 (C-9), 114.3 (C-10), 123.5 (C-1′), 129.4 (C-2′/6′), 117.0 (C-3′/5′), 162.5 (C-4′). (Table 4.15)

HMBC, HSQC, COSY and NOESY experiments are presented in Appendix 13-16.

3.5. Isolation of volatile oil from *D. reflexa* root

The *D. reflexa* root (300 g) was hydrodistilled for 4 hours in an all-glass Clevenger-type apparatus in accordance with the British pharmacopoeia method (1980). The oil collected in well-capped bottles was dried over anhydrous sodium sulphate and stored in refrigerator at 4 °C in the dark prior to analysis.

3.5.1 Identification of oil constituents

Identification of constituents was done by comparing the retention times, the retention indices and the mass spectra of the chromatographic peaks with those of standards analysed under the same conditions. The peak assignments of the other volatile components were based on computer matching of the mass spectra obtained with the WILEY 275, NIST 08 and ADAMS libraries, taking into account the coherence of the retention indices of the analysed compounds with those reported by Adams (2007) and NIST08 libraries (2008).

3.6 Extraction of *C. arborea* root

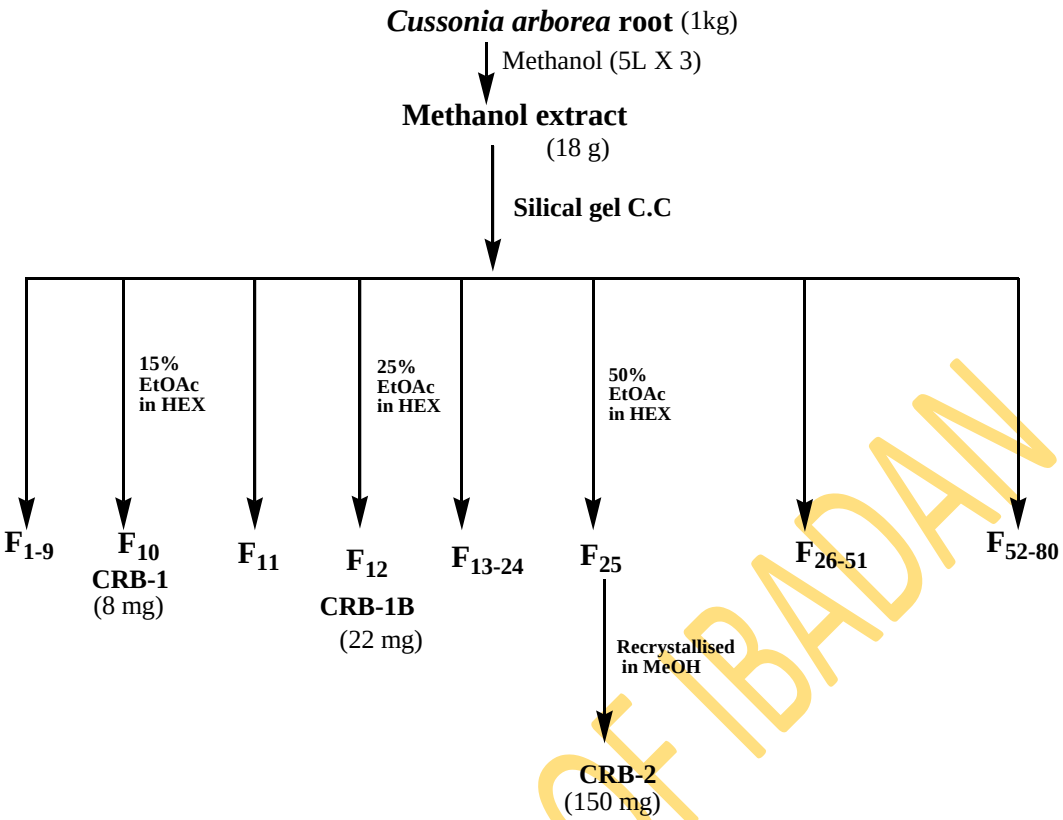
The pulverised air-dried roots' bark of *Cussonia arborea* (1000 kg) were extracted by percolation with distilled methanol (3 × 2.5 L) for two weeks at room temperature. The combined extracts were concentrated under reduced pressure using rotary evaporator, preset at 37 °C yielding a light greenish powder (18 g).

3.6.1 Column chromatography of crude methanol extract of *C. arborea* root bark

The methanol extract (18 g) was pre-adsorbed on equal weight of silica gel and introduced into a column (length = 82 cm, internal diameter = 5.0 cm) packed with silica gel (25-40 µm) as stationary phase. The solvent system used in eluting the column were: hexane (100%, 1000 mL), hexane/EtOAc (19:1, 9:1, 17:3, 4:1, 3:1,7:3, 13:7, 3:2, 11:9, 1:1, 2:3, 3:7, 1:4, 1:9, 1000 mL each), EtOAc (100%, 1000 ml), EtOAc/MeOH (19:1, 9:1, 1000 mL each) successively (Scheme 3.2). The volume of eluent collected was 1000 mL/vial.

A total of 80 fractions were collected (F_1- F_{80}). Fraction F_{10} and F_{12} eluted with hexane: EtOAc, 17:3 and 3:1 afforded two white crystalline solids, coded as CRB-1 (8.0 mg) and CRB-1B (22.0 mg) respectively. Fraction F_{25} eluted with hexane: EtOAc, 1:1 was

concentrated under reduced pressure to yield a white solid. The solid was then collected by filtration and purified by recrystallisation from mixtures MeOH and n-hexane (1:19). The pure colourless crystal obtained was tagged CRB-2 (150.0 mg).



Scheme 3.2. Scheme of isolation of *C. arborea* roots

Keys:

F = Fraction(s)

CRB = ***C****ussonia* ***a****rborea* **r**oot

3.6.2 Spectroscopic data of CRB-1

M.pt.: 168-169˚C

White crystalline solid, Yield = 8.0 mg.

UV λ_{max} nm (log ε) = 243 (0.25).

IR (KBr): 3419 (OH), 2934, 2866 (C-H aliphatic), 1457, 1376, 1056 cm^{-1}.

EI-MS: *m/z* (rel. int): 412 (M^+, 100), 396 (47), 381 (34), 369 (17), 329 (40), 303 (46), 255 (66), 233 (42), 213 (48), 159 (43), 107 (52), 83 (44).

HREIMS= 412.3683 (Calcd. 412.3707 for $C_{29}H_{48}O$).

^{1}H NMR: ($CDCl_3$, 400 MHz,): δ 0.67 (3H,s, H-18), 0.78 (3H,t, H-29), 0.82 (3H,d, J=7.5 Hz, H-26), 1.01 (3H, d, J=6.5 Hz, H-21), 0.99 (3H, s, H-19), 4.96 (1H,dd, J=15.2, 8.8 Hz, H-23), 5.10 (1H, dd, J=15.2, 8.4 Hz, H-22), 5.32 (1H, m, H-6). (Table 4.18)

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 51.2 (C-24), 25.4 (C-28), 31.9 (C-25), 19.0 (C-27), 129.3 (C-23), 21.2 (C-26), 19.4 (C-19), 71.8 (C-3), 12.2 (C-29), 40.5 (C-20), 31.7 (C-2), 28.9 (C-16), 56.0 (C-17), 42.3 (C-4), 140.8 (C-5), 121.7 (C-6), 32.0 (C-8), 36.5 (C-10), 21.1 (C-11), 39.7 (C-12), 42.2 (C-13), 56.9 (C-14), 24.4 (C-15), 37.3 (C-1), 31.7 (C-7), 50.2 (C-9), 12.1 (C-18). (Table 4.18)

HMBC, HSQC, COSY and NOESY experiments are presented in Appendix 17-20.

3.6.3 Spectroscopic data of CRB-1B

M.pt.: 271-273° C

White crystalline solid, Yield = 22.0 mg.

EI-MS *m/z* (rel. int.): 456 (M^+, 2), 410 (2), 300 (2), 248 (100), 233 (10), 203 (77), 189 (12), 133 (12), 133 (13), 119 (9).

HREIMS= 456.3566 (Calcd. 456.3605 for $C_{30}H_{48}O_3$).

^{1}H- NMR (400 MHz, $CDCl_3$): δ 0.75 (3H, s, H-24), 0.88 (3H, s, H-29), 0.88 (3H, s, H-25), 1.11 (3H, s, H-27), 0.96 (3H, s, H-23), 0.73 (3H, s, H-26), 0.89 (3H, s, H-30), 1.57 (2H, m, H-19), 2.78 (1H, m, H-18), 3.18 (1H, t, H-3), 5.26 (1H, m, H-12). (Table 4.19)

^{13}C-NMR ($CDCl_3$, 100 MHz): δ 15.5 (C-24), 183.1 (C-28), 15.3 (C-25), 25.9 (C-27), 28.1 (C-23), 17.1 (C-26), 23.6 (C-30), 45.8 (C-19), 79.0 (C-3), 33.1 (C-29), 30.7 (C-20), 27.1 (C-2), 23.4 (C-16), 46.5 (C-17), 38.7 (C-4), 55.2 (C-5), 18.3 (C-6), 39.2 (C-8), 37.1 (C-10), 22.9 (C-11), 122.6 (C-12), 143.6 (C-13), 41.6 (C-14), 27.6 (C-15), 38.4 (C-1), 32.6 (C-7), 47.6 (C-9), 41.0 (C-18). (Table 4.19)

HMBC, HSQC, COSY and NOESY experiments are presented in Appendix 21-24.

3.6.4 Spectroscopic data of CRB-2

M.pt.: 292 ˚C.

Colourless crystals, Yield = 150.0 mg.

UV λ_{max} nm (log ε) = 211 (0.66).

IR(KBr) : 3453 (OH), 2944, 1699 (C=O), 1463, 1384, 1038 cm^{-1} .

EI-MS: *m/z* (rel. int): 472 (M^+,6), 454 (7), 396(7), 248(100), 223 (23), 203(86), 175 (69), 161 (9), (45), 119(25).

HREIMS= 472.3573 (Calcd. 472.3554 for $C_{30}H_{48}O_4$).

^{1}H NMR: (400 MHz, CD_3OD): δ 0.69 (3H, *s*, H-24), 0.81 (3H, *s*, H-26), 0.90 (3H, *s*, H-29), 0.93 (3H, *s*, H-30), 0.97 (3H, *s*, H-25), 1.12 (3H, *s*, H-27), 2.83 (1H, *dd, J =4.0*, 14 Hz H-18), 3.50 (1H, *d*, *J* = 10.8 Hz, H-23), 3.57 (1H, *m*,H-3), 5.21 (1H, *m*,H-12). (Table 4.20).

^{13}C-NMR (100 MHz, CD_3OD): δ 12.7 (C-24), 181.9 (C-28), 16.3 (C-25), 24.0 (C-27), 67.2 (C-23), 17.7 (C-26), 21.6 (C-30), 47.2 (C-19), 73.8 (C-3), 33.6 (C-29), 34.9 (C-20), 25.3 (C-2), 24.5 (C-16), 47.6 (C-17), 43.0 (C-4), 19.1 (C-6), 37.9 (C-8), 24.4 (C-11), 123.6 (C-12), 143.3 (C-13), 43.3 (C-14), 28.8 (C-15), 39.4 (C-1), 33.5 (C-7), 42.7 (C-18). (Table 4.20).

X-ray Crystallography of CRB-2

The CRB-2 crystals (150 mg) was recrystallised in hexane-methanol at room temperature to obtain a single crystal for x-ray crystallography.

3.7 Antioxidant assay

The free radical scavenging effect was measured through 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the standard method employed by Gulcin *et al*., (2005). A 0.3 µM solution of DPPH was prepared in ethanol. Five µL of each sample of different concentrations (10–1000 µg) were mixed with 95 µL of a DPPH solution in ethanol separately. The mixture was then dispensed in a 96 well-plate and incubated at 37 ºC for 30 min. The absorbance at 515 nm was measured by a microtitre plate reader (Spectramax plus 384 Molecular Device, USA), and the percentage of radical scavenging activity was determined in comparison with the DMSO-treated control. Butylated hydroxyanisole (BHA) was used as standard.

The DPPH scavenging effect (%) was then calculated thus:

$$\frac{Ac - As}{Ac} \times 100$$

,

Where, Ac = Absorbance of control (DMSO treated),

As = Absorbance of sample.

The IC_{50} of the compounds were determined by means of EZ fit enzyme software, designed by Pellera Scientific Inc. Amherst, U.S.A.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Extraction and Phytochemical analysis of *Dioclea reflexa* (Hook F.)

The air-dried and powdered root of *D. reflexa* (1 kg) yielded ethanol extract (85 g) which gave a percentage yield of 8.5 % w/w. Preliminary phytochemical screening of the ethanol root extracts revealed the presence of alkaloid, tannins, glycoside, saponins, steroid, flavonoids, resins, phenols and carbohydrate (Table 4.1). The crude ethanol extracts (85 g) of *D. reflexa* (roots) was chromatographed on silica gel and eluted with gradient of hexane, dichloromethane, ethylacetate and methanol to give 332 chromatographic fractions, which were pooled to 8 sub-fractions labelled A-H (Table 4.2). Further purification of sub-fractions using various column chromatography techniques afforded seven compounds which were tagged DRR-1C, DRR-2E, DRR-1F, DRR-1G, DRR-2G, DRR-3G and DRR-4G.

4.2 Characterisation of isolated compounds

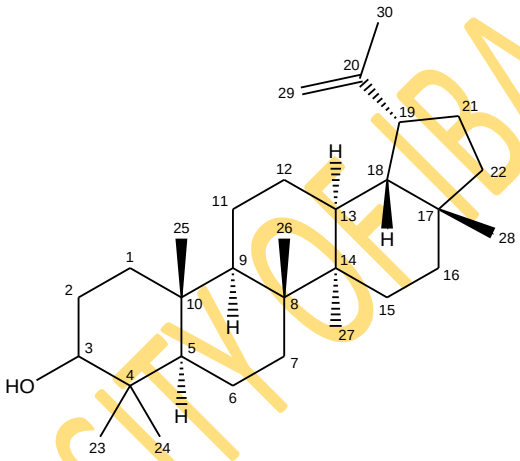
4.2.1 Isolation and characterisation of DRR-1C

Sub-fraction C (40 mg) was purified using column chromatography. Elution was carried out using gradient of n-hexane and ethyl acetate [n-hexane (100%, 60 mL); n-hexane: ethyl acetate (98:2, 75 mL); (95:5, 75 mL); and (92:8, 30 mL)]. A total of 16 fractions were collected (Table 4.3). Combined fractions 8-9 eluted with 2% ethyl acetate in hexane led to the isolation of DRR-1C (7 mg), which was washed with hexane to obtain white crystals.

The EIMS of DRR-1C (Fig. 4.1) established the molecular ion peak at *m/z* 426.3714 (calcd. 426.3864), corresponding to the molecular formula $C_{30}H_{50}O$. The mass spectrum showed other fragment ion peaks at *m/z* 411 [M^+ - CH_3], 218 [M^+- $C_{14}H_{28}$], 207 [M^+- $C_{18}H_{27}$], besides the molecular ion at *m/z* 426 which are diagnostic features for pentacyclic triterpene with an isopropenyl group (Waller, 1980).

The ^{1}H-NMR (Fig. 4.2) of DRR-1C exhibited the presence of seven tertiary methyl groups at 0.74, 0.77, 0.81, 0.92, 0.95, 1.01 and 1.66. The δ_H at 4.54 (1H, d, J= 1.8 Hz) and 4.67 (1H, d, J = 1.8 Hz) was assigned to position 29, a characteristic feature of a terminal isopropenyl group. One proton doublet of doublet at δ_H 3.15 (J= 4.8, 12.0 Hz) was due to proton attached to the carbon bearing hydroxyl group at C-3. A proton sextet at δ_H 2.33 could be attributed to H-19 which was characteristics for lupeol.

The ^{13}C-NMR (Table 4.4) assignments were substantiated by DEPT experiments, which showed the presence of seven methyl, eleven methylene, six methine and six quartenary carbons. Comparison of the spectra data with existing literatures identified DRR-1C to be lupeol **4.1** (Mahato and Kundu, 1994; Jager *et al*., 2009).



4.1

Table 4.1. Phytochemical screening of crude ethanol extract of *D. reflexa* root.

Test	Remarks
Alkaloid	+
Tannins	+
Glycoside	+
Saponins	+
Steroid	+
Flavonoid	+
Resins	+
Phenols	+
Carbohydrate	+
Anthraquinones	–

Keys:

+ = present

– = absent

Table 4.2 Column chromatography of crude ethanol extract of *D. reflexa* roots

No.	Column eluents	Pooled fraction	Pool code
1.	Hexane (100%)	1-30	
2.	HEX: DCM (19:1)	31-44	
3.	HEX: DCM (17:3)	45-50	
4.	HEX: DCM (4:1)	51-56	
5.	HEX: DCM (3:1)	57-62	
6.	HEX: DCM (7:3)	63-68	A
7.	HEX: DCM (13:7)	69-74	
8.	HEX: DCM (3:2)	75-80	
9.	HEX: DCM (11:9)	81-84	
10	HEX: DCM (1:1)	85-93	B
11	HEX: DCM (9:11)	94-97	
12	HEX: DCM (2:3)	98-107	C
13	HEX: DCM (7:13)	108-110	
14	HEX: DCM (3:7)	111-119	
15	HEX: DCM (1:3)	120-128	
16	HEX: DCM (1:4)	129-132	D
17	HEX: DCM (3:17)	133-135	
18	HEX: DCM (1:9)	136-148	
19	DCM (100%)	149-150	
20	DCM:EtOAc (19:1)	151-155	
21	DCM:EtOAc (9:1)	156-158	
22	DCM:EtOAc (17:3)	159-163	
23	DCM:EtOAc ((4:1)	164-165	E
24	DCM:EtOAc (3:1)	166-170	
25	DCM:EtOAc (7:3)	171-174	
26	DCM:EtOAc (13:7)	175-177	
27	DCM:EtOAc (3:2)	178-179	
28	DCM:EtOAc (11:9)	180-189	
29	DCM:EtOAc (1:1)	190-196	F
30	DCM:EtOAc (9:11)	197-204	
31	DCM:EtOAc (2:3)	205-207	
32	DCM:EtOAc (7:13)	208-212	
33	DCM:EtOAc (3:7)	213-214	
34	DCM:EtOAc (1:3)	215-219	
35	DCM:EtOAc (1:4)	220-226	G
36	DCM:EtOAc (3:17)	227-230	
37	DCM:EtOAc (1:9)	231-233	
38	DCM:EtOAc (1:19)	234-236	
39	EtOAc (100%)	237-250	
40	EtOAc:MeOH (19:1)	251-260	
41	EtOAc:MeOH (9:1)	261-266	H
42	EtOAc:MeOH (17:3)	267-285	
43	EtOAc:MeOH (4:1)	286-332	

Table 4.3. Column chromatography of sub-fraction C

Fraction nos.	Column eluents	Pool code
1.	Hexane (100%)	
2.	Hexane (100%)	
3.	Hexane (100%)	
4.	Hexane (100%)	
5.	HEX : EtOAc (98:2)	
6.	HEX : EtOAc (98:2)	
7.	HEX : EtOAc (98:2)	
8.	HEX : EtOAc (98:2)	DRR-1C
9.	HEX : EtOAc (98:2)	
10.	HEX : EtOAc (95:5)	
11.	HEX : EtOAc (95:5)	
12.	HEX : EtOAc (95:5)	
13.	HEX : EtOAc (95:5)	
14.	HEX : EtOAc (95:5)	
15.	HEX : EtOAc (92:8)	
16.	HEX : EtOAc (92:8)	

Table 4.4. The ^{1}H and ^{13}C NMR spectra data of DRR-1C (500 and 125 MHz, ppm in $CDCl_3$ respectively) compared with ^{13}C NMR data of literature.

Position	^{1}H δ (multiplicity,J Hz)	^{13}C	^{13}C*	DEPT
1.	0.87 (m)	38.7	38.7	CH_2
2.	1.61 (m)	27.5	27.4	CH_2
3.	3.15 (m)	79.0	78.9	CH
4.	3.15(dd,4.8,12.0)	38.9	38.8	C
5.	0.65 (m)	55.3	55.3	CH
6.	1.36 (m)	18.3	18.3	CH_2
7.	1.36 (m)	34.3	34.2	CH_2
8.	-	40.9	40.8	C
9.	1.23 (m)	50.5	50.4	CH
10.	-	37.2	37.1	C
11.	1.23 (m)	20.9	20.9	CH_2
12.	1.66 (m)	25.2	25.1	CH_2
13.	1.6 (m)	38.1	38.0	CH
14.	-	42.8	42.8	C
15.	1.54 (m)	27.4	27.4	CH_2
16.	1.36 (m)	35.6	35.5	CH_2
17.	-	43.0	43.0	C
18.	1.33 (m)	48.3	48.2	CH
19.	2.33 (m)	48.0	47.9	CH
20.	-	151.0	150.9	C
21.	1.90 (m)	29.9	29.8	CH_2
22.	1.16 (m),1.36 (m)	40.0	40.0	CH_2
23.	0.95 (s)	28.0	28.0	CH_3
24.	0.74 (s)	15.4	15.4	CH_3
25.	0.81 (s)	16.2	16.1	CH_3
26.	1.01 (s)	16.0	15.9	CH_3
27.	0.92 (s)	14.6	14.5	CH_3
28.	0.77 (s)	18.0	18.0	CH_3
29.	4.54(d, 1.8), 4.67(d, 1.8)	109.3	109.3	CH_2
30.	1.66 (s)	19.3	19.1	CH_3

* Mahato and Kundu, 1994.

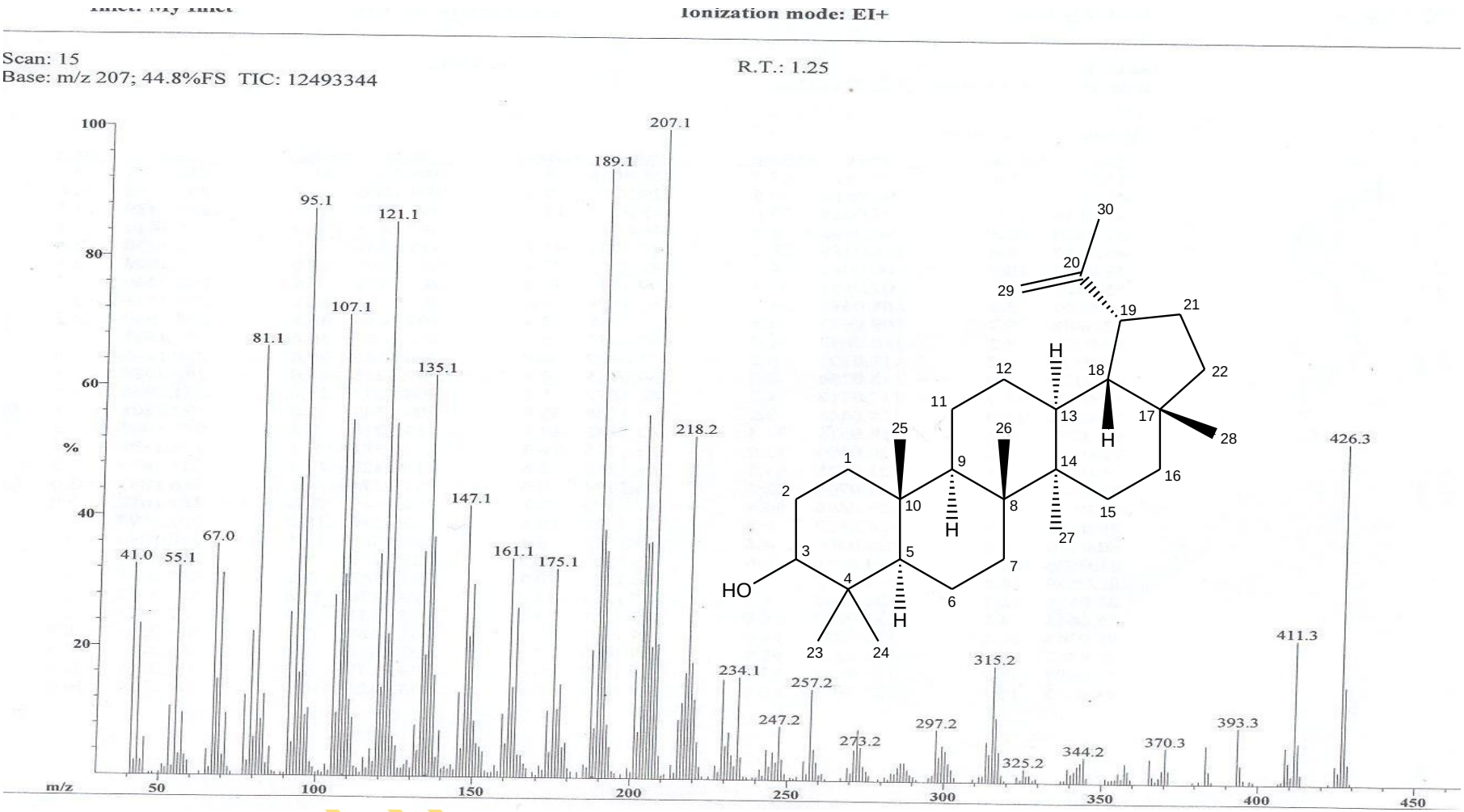


Fig. 4.1. EIMS of DRR-1C

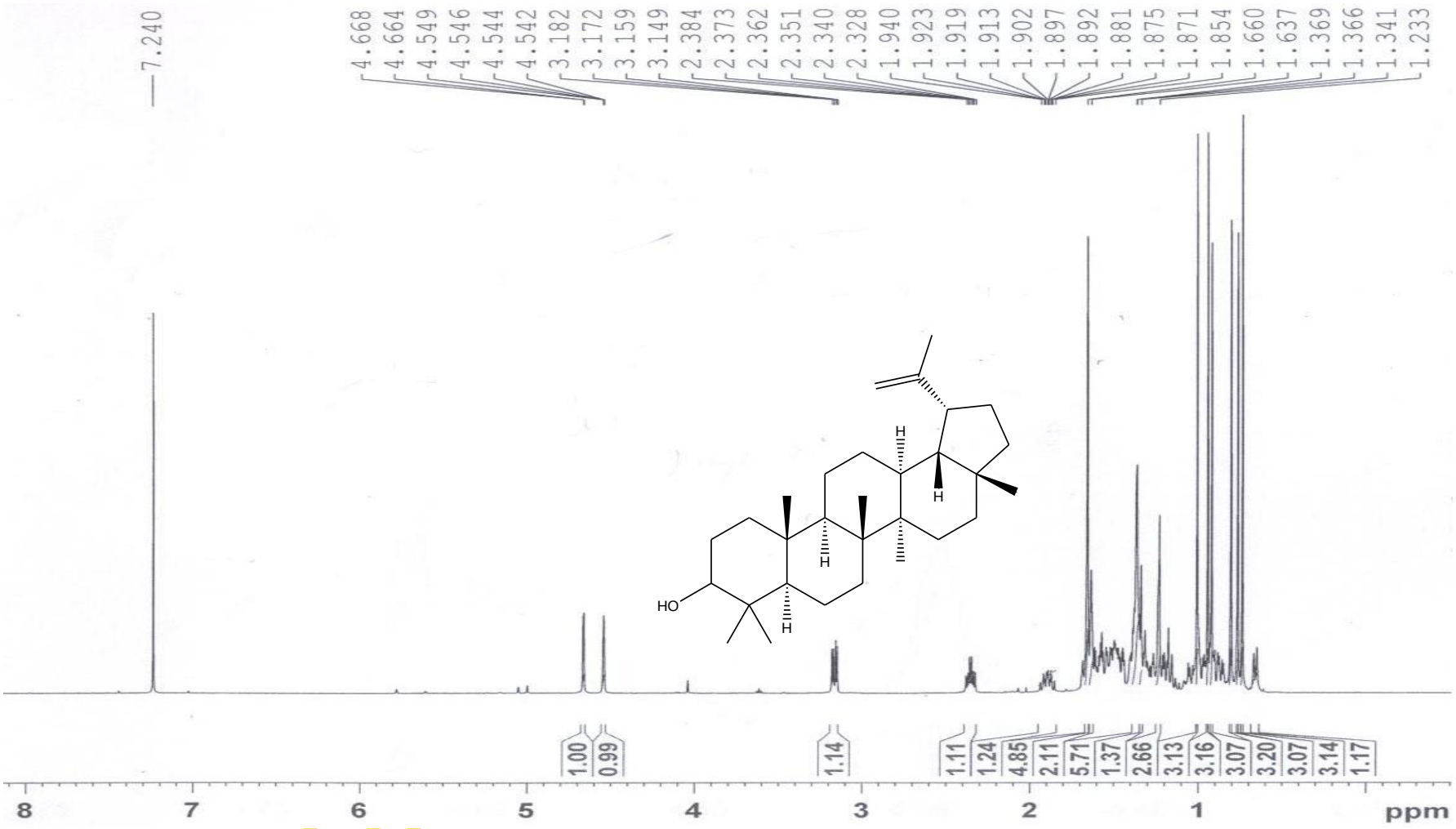


Fig. 4.2. ^{1}H NMR spectrum of DRR-1C ($CDCl_3$, 500 MHz)

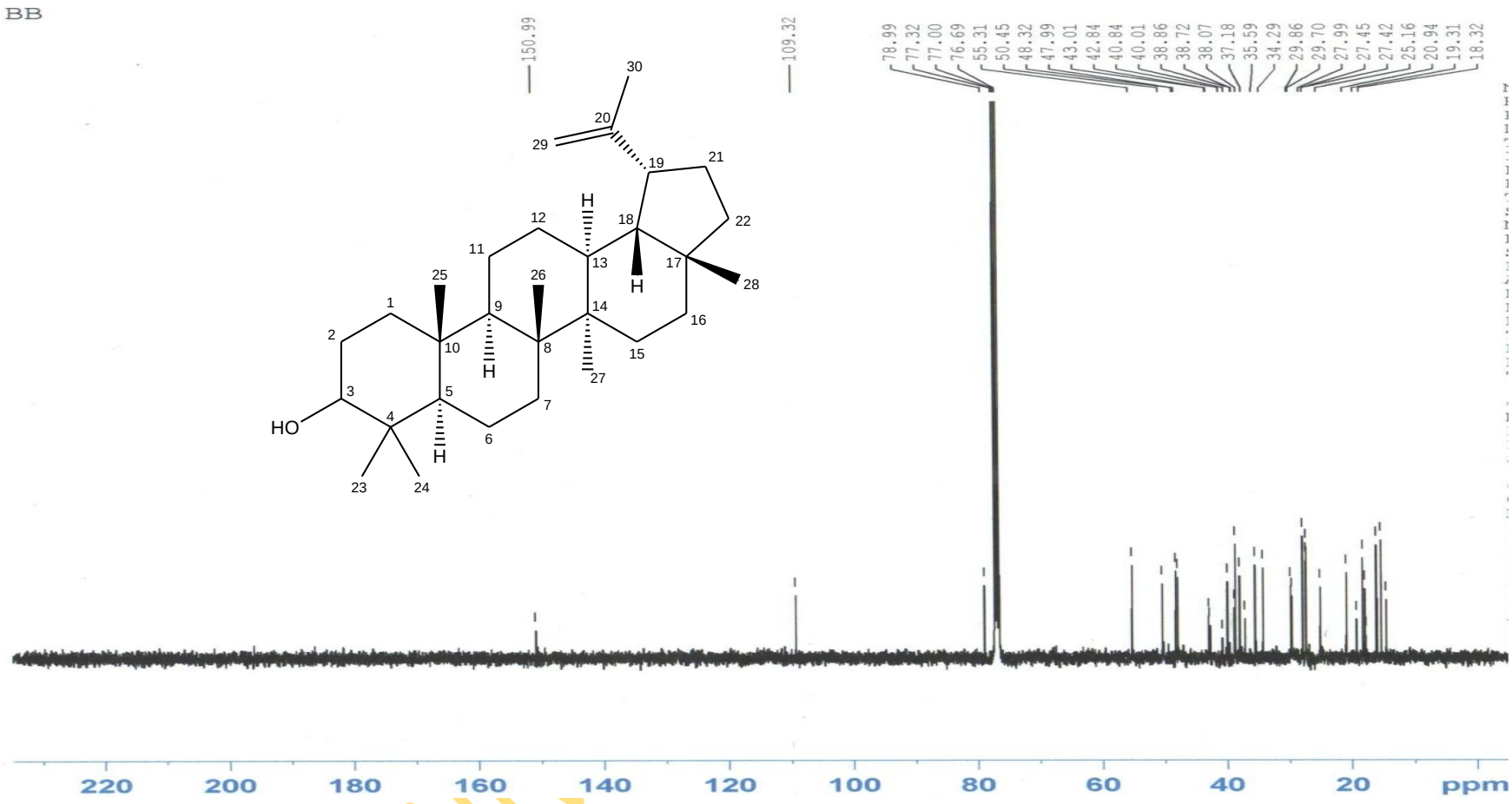


Fig. 4.3. ^{13}C-NMR spectrum of DRR-1C ($CDCl_3$, 125 MHz)

4.2.2 Isolation and characterisation of DRR-2E

Sub-fraction E was also rechromatographed on silica gel by column chromatography using gradient mixtures of n-hexane and EtOAc as eluents (Table 4.5). The fraction E4 eluted with 20 % EtOAc in hexane afforded DRR-2E as semi-pure compound and was further purified via preparative HPLC on RP-18, ODS column, eluted with MeOH/H_2O (60:40) in isocratic system (Flow rate: 1ml/min; Detector: RI; Retention time: 10.5 min).

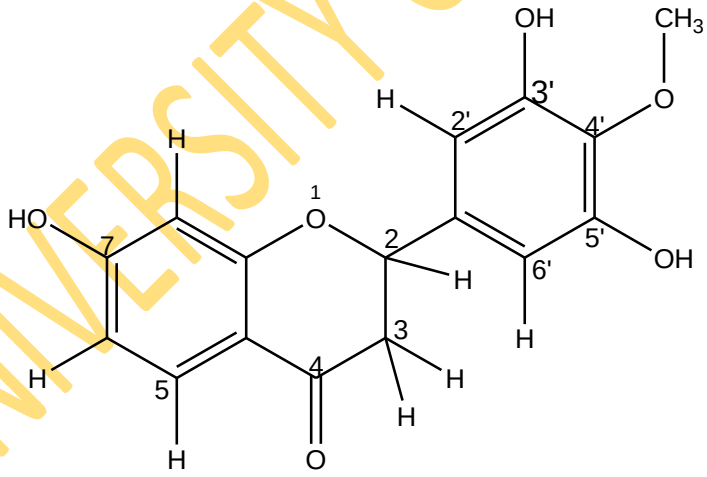
Compound DRR-2E (7 mg) was obtained as yellow powder. The EIMS (Fig. 4.13) gave molecular ion peak at *m/z* 302, corresponding to the molecular formula $C_{16}H_{14}O_6$. This was supported by its HR-EI MS, which also showed the M^+ at *m/z* 302.0769 (calc. 302.0792) with 10 degree of unsaturation in the molecule, eight of which were accounted for by the aromatic rings A and B, one by the carbonyl group and other one by heterocyclic ring C. The retro-Diels-Alder cleavage of the heterocyclic ring produced ions at m/z 137$[A+1]^+$ and 166 $[B]^+$ (Scheme 4.1). The prominent ions were observed at m/z 287 [M^+-CH_3], 259 [M^+-CH_3-CO] and 151[B-CH_3].

The ^{1}H-NMR spectrum (Fig. 4.5- 4.5a) of DRR-2E showed the 3H singlet of methoxyl at δ 3.80 (C-4′), 2H singlet at δ 8.54 (C-3′/5′) and another 2H singlet appeared at δ 6.59 assigned to C-2′ and C-6′: typical for a symmetrical myricetin-type, B-ring (Abbas *et al.*, 2007). Two 1H doublets appeared at δ 6.42 (J= 2 Hz) and δ 7.71(J= 8.5 Hz) along with another 1H doublet of doublets appeared at δ 6.55 (J= 2, 8.5 Hz), correlated with the carbons at 103.7, 129.4 and 111.3 ppm respectively in HSQC spectrum characterized the C-8, C-5 and C-6 of a flavonoids 7-hydroxy A-ring (Park *et al*., 2007). An examination of its NMR data (Table 4.6) and its comparison with literature (Hammami *et al*., 2004; Jenkins *et al*., 1999; Ye *et al*., 2004; Bhattacharya *et al*., 1998) revealed that compound DRR-2E was a flavanone. One proton doublet of doublets observed at δ 5.37 (J= 2.5, 12.5 Hz), assigned to C-2 is a distinctive feature for a flavanone (Jenkins *et al*., 1999; Mabry *et al*., 1975), as the splitting pattern observed for the methylene at C-3 produced two 1H doublet of doublets at δ 2.93 for the C-3a axial proton (J= 17, 12.5 Hz) and another at δ 2.66 for the C-3b equatorial proton (J= 3, 17 Hz).

The ^{13}C and DEPT spectra (Fig. 4.6 – 4.8) of DRR-2E confirmed the presence of sixteen carbons: eight quaternary, one methylene, six methines and one methyl carbons.

Three ^{13}C peaks at 106.6, 136.2 and 151.4 ppm showed double intensities, they were assigned to C-2′/C-6′, C-1′/4′ and C-3′/5′ at the B ring respectively. The position of the methoxyl was further confirmed by a 3-bond HMBC (Fig. 4.4) connectivity between the proton and C-4′ (δ 136.22). Two proton singlet at 6.59 attached directly to 106.58 showed long range coupling with ^{13}C NMR peak at 44.67 (C-2) were assigned C-2′/C-6′. The other 2H singlet at 8.54 had no carbon attached in HSQC (Fig. 4.9) were assigned C-3′/5′.

The chemical shift of C-7 in unsubstituted flavones usually appears at 134.0 (± 1.5 ppm), in the ^{13}C NMR spectrum (Park *et al.*, 2007). The C-7 signal appeared at 165.4 ppm, which is downfield, indicated the presence of a substituent at C-7 of the ring A. The substituent is a hydroxyl group as supported by the presence of the ion at m/z 137 in the EIMS. The most downfield shifted ^{13}C peak at 190.2 was assigned to C-4. The COSY spectrum (Fig. 4.11) showed correlations between two methines at C-5 (δ 7.7) and C-6 (δ 6.55). From the spectra data (Table 4.6), the novel structure of DRR-2E was determined as 7, 3′, 5′-trihydoxy-4′-methoxyflavanone which has been given trivial name lexaflavanone **4.2**.



4.2

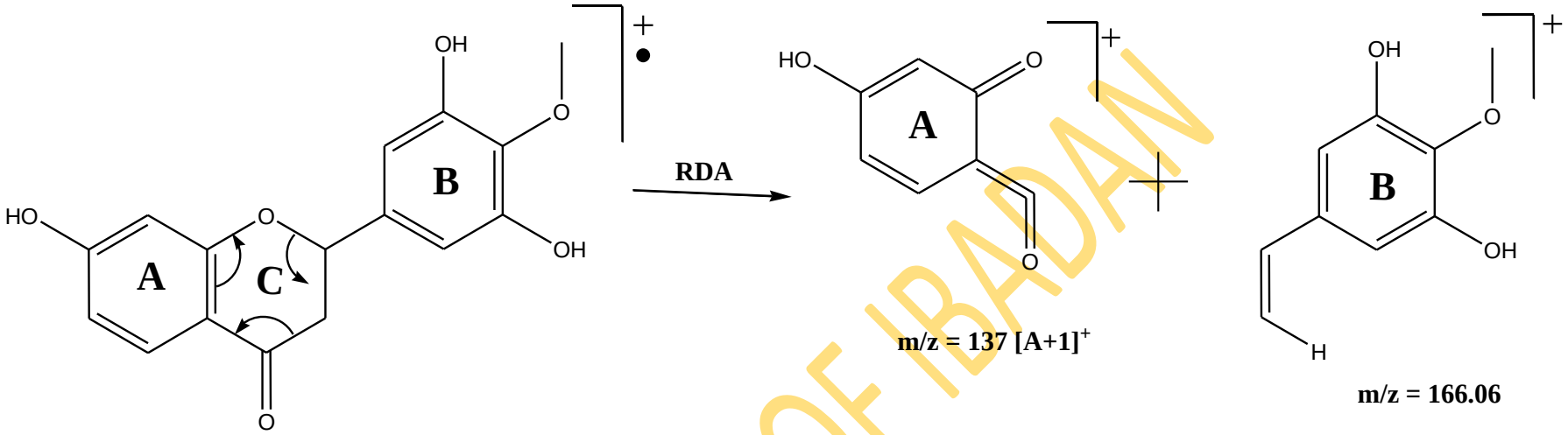
Table 4.5. Column chromatography of sub-fraction E

Column eluents	Pooled fraction	Pool code
HEX : EtOAc (90:10)	1-46	E1
HEX : EtOAc (90:10)	47-69	
HEX : EtOAc (90:10)	71-120	E2
HEX : EtOAc (89:11)	121-164	
HEX : EtOAc (87:13)	165-188	E3
HEX : EtOAc (85:15)	189-196	
HEX : EtOAc (83:17)	197-218	
HEX : EtOAc (80:20)	220-267	E4
HEX : EtOAc (75:25)	268-281	E5
HEX : EtOAc (65:35)	282-297	
HEX : EtOAc (80:20)	298-318	E6
HEX : EtOAc (50:50)	319-338	
HEX : EtOAc (40:60)	339-370	E7

Table 4.6. ^{1}H and ^{13}C-NMR spectra data of DRR-2E (500 and 125 MHz, Acetone)

Position	^{1}H δ (integration, multiplicity, J Hz)	^{13}C	DEPT	HMBC
2	5.37(1H, dd, J= 2.5, 12.5)	80.3	CH	C-4, C-1′, C-2′
3a	2.93(1H, dd, J= 12.5, 17)	44.7	CH_2	C-2, C-4, C-1′
b	2.66(1H, dd, J= 3, 17)	-		C-4
4	-	190.2	C	
5	7.71 (1H, d, J= 8.5)	129.4	CH	C-4, C-7
6	6.55 (1H, dd, J= 2, 8.5)	111.3	CH	C-8, C-10
7	-	165.4	C	
8	6.42 (1H, d, J= 2)	103.7	CH	C-6, C-7, C-10
9	-	164.3	C	
10	-	115.2	C	
1′	-	136.2	C	
2′/6′	6.59 (2H, s)	106.6	CH	C-2, C-1′, C-3/5′
3′/5′	-	151.4	C	
3′/5′-OH	8.54			
4′	-	136.2	C	
OCH_3	3.80 (3H, s)	60.7	CH_3	C-4′

Figure 4.4. HMBC connectivities of DRR-2E. Key: COSY(—) and HMBC (→)



Scheme 4.1. Fragmentation pattern of compound DRR-2E

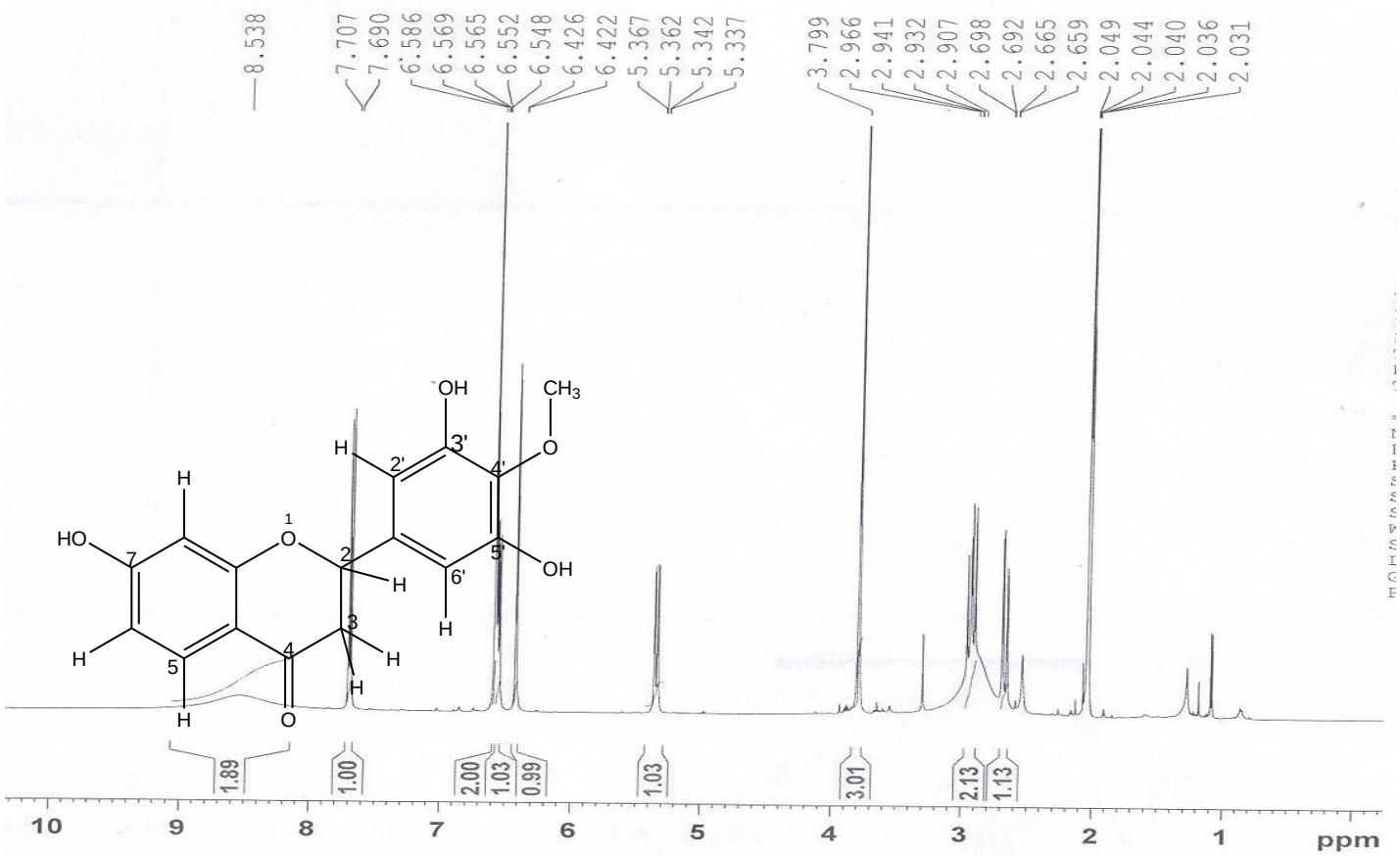


Fig. 4.5. ^{1}H-NMR spectrum of DRR-2E (CD_3COCD_3, 500 MHz)

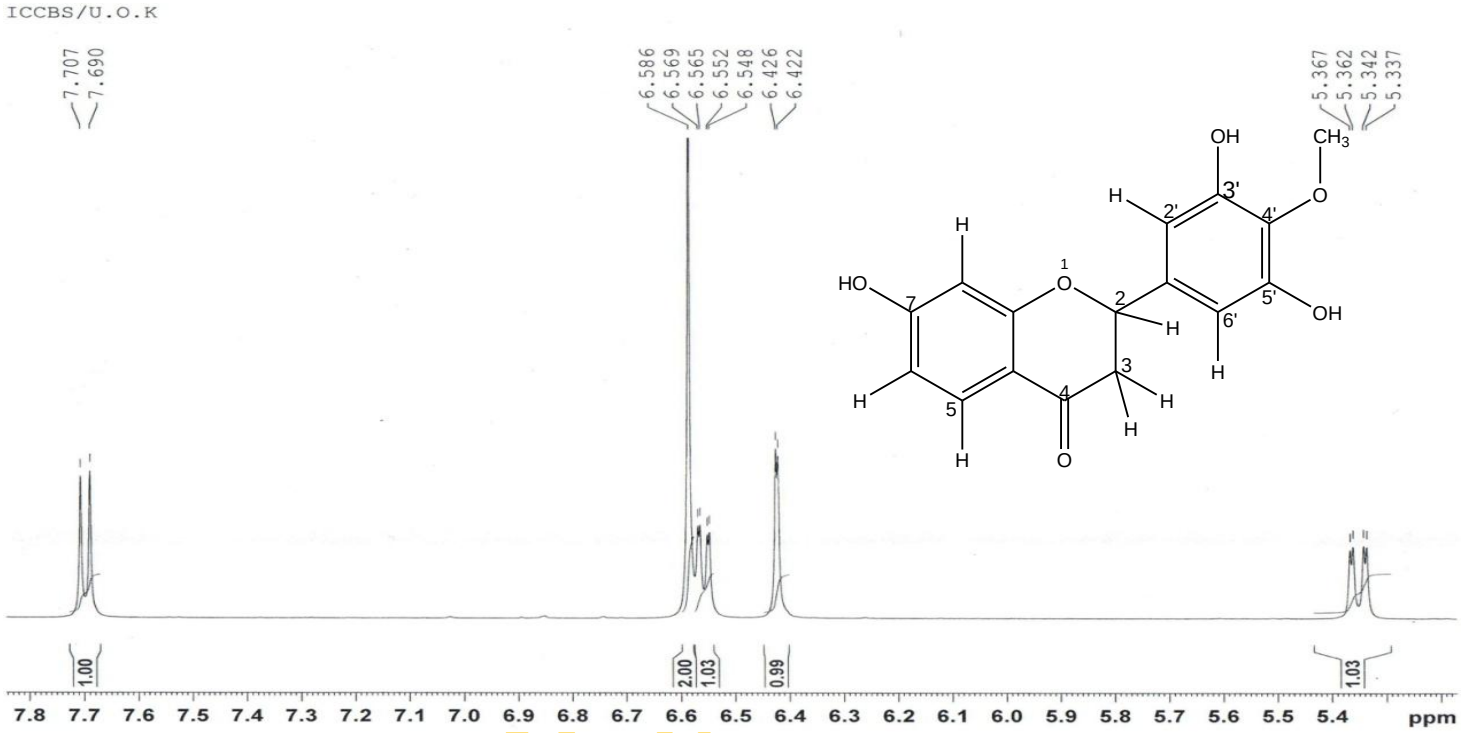


Fig. 4.5a. Expanded ^{1}H-NMR spectrum of DRR-2E (CD_3COCD_3, 500 MHz)

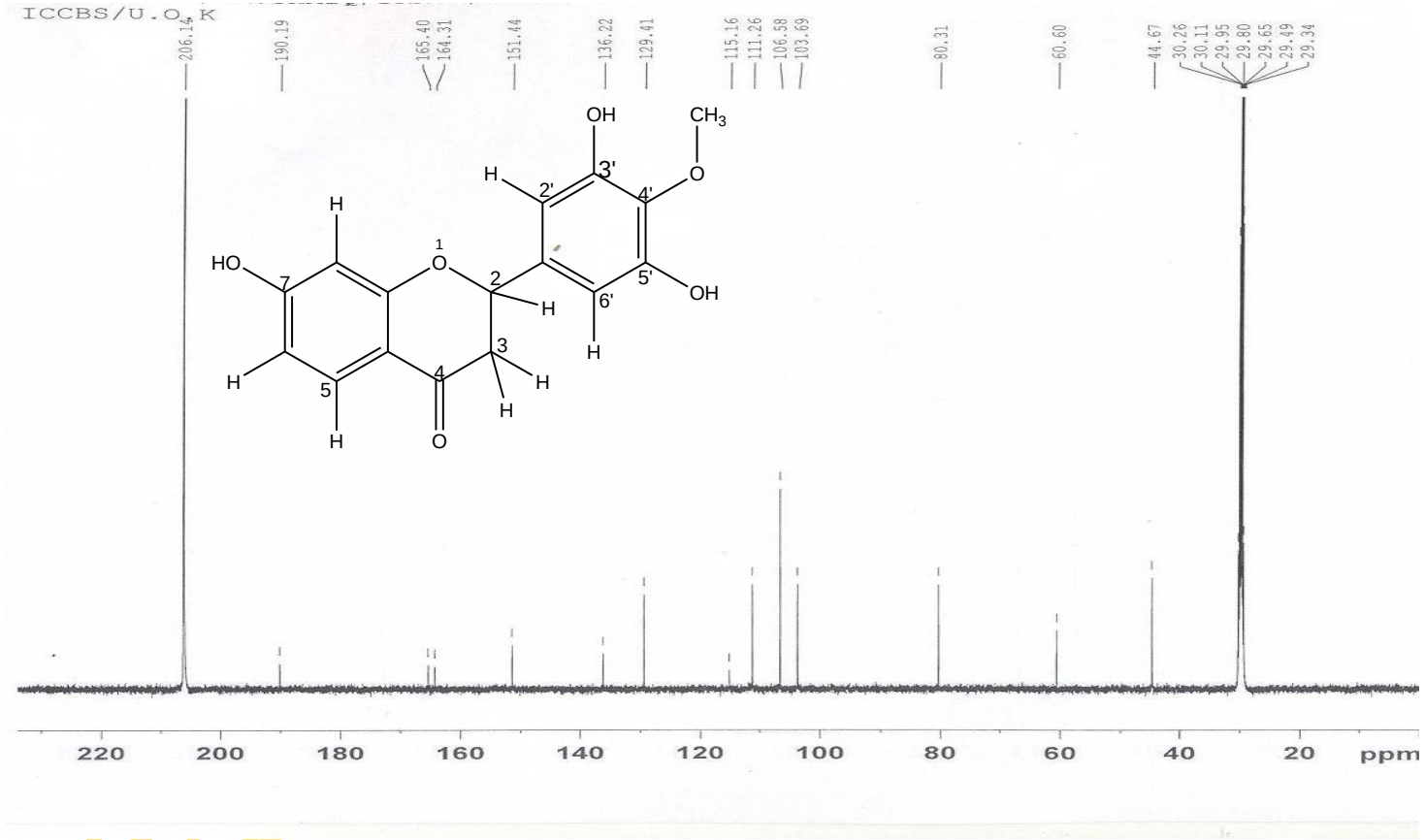


Fig. 4.6. ^{13}C-NMR spectrum of DRR-2E (CD_3COCD_3, 125 MHz)

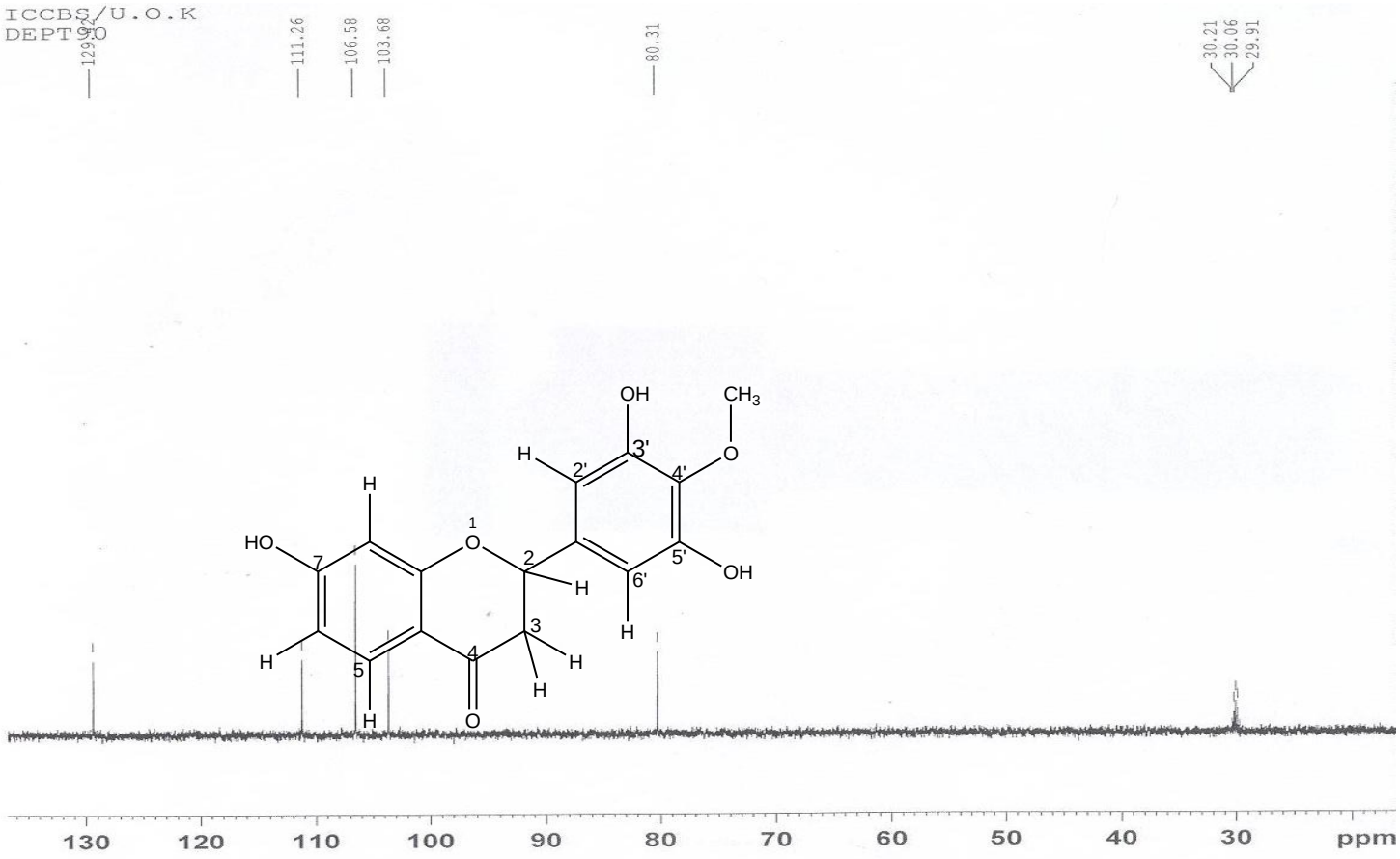


Fig. 4.8. DEPT 90 spectrum of DRR-2E

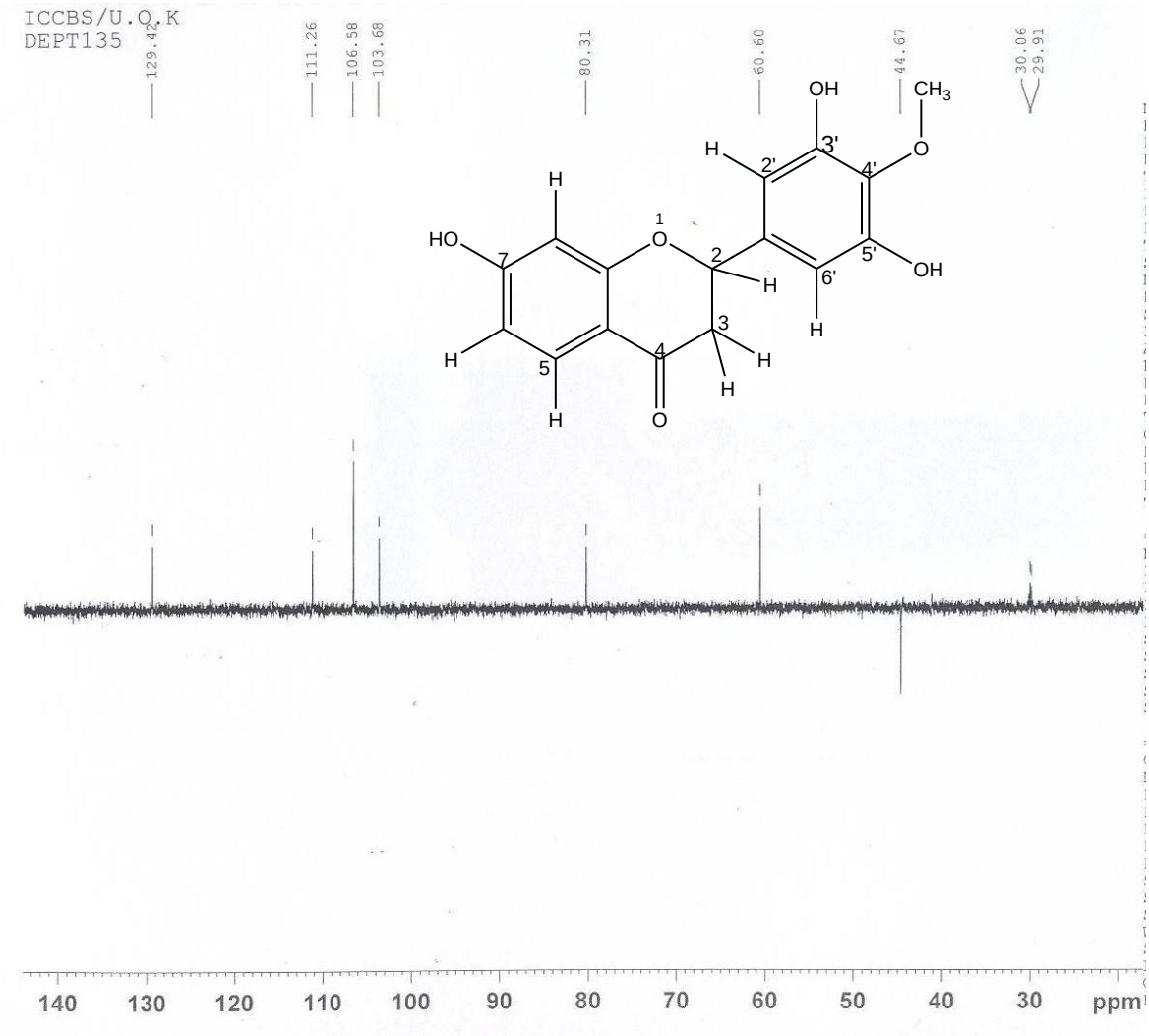


Fig. 4.8. DEPT 135 spectrum of DRR-2E (CD_3COCD_3)

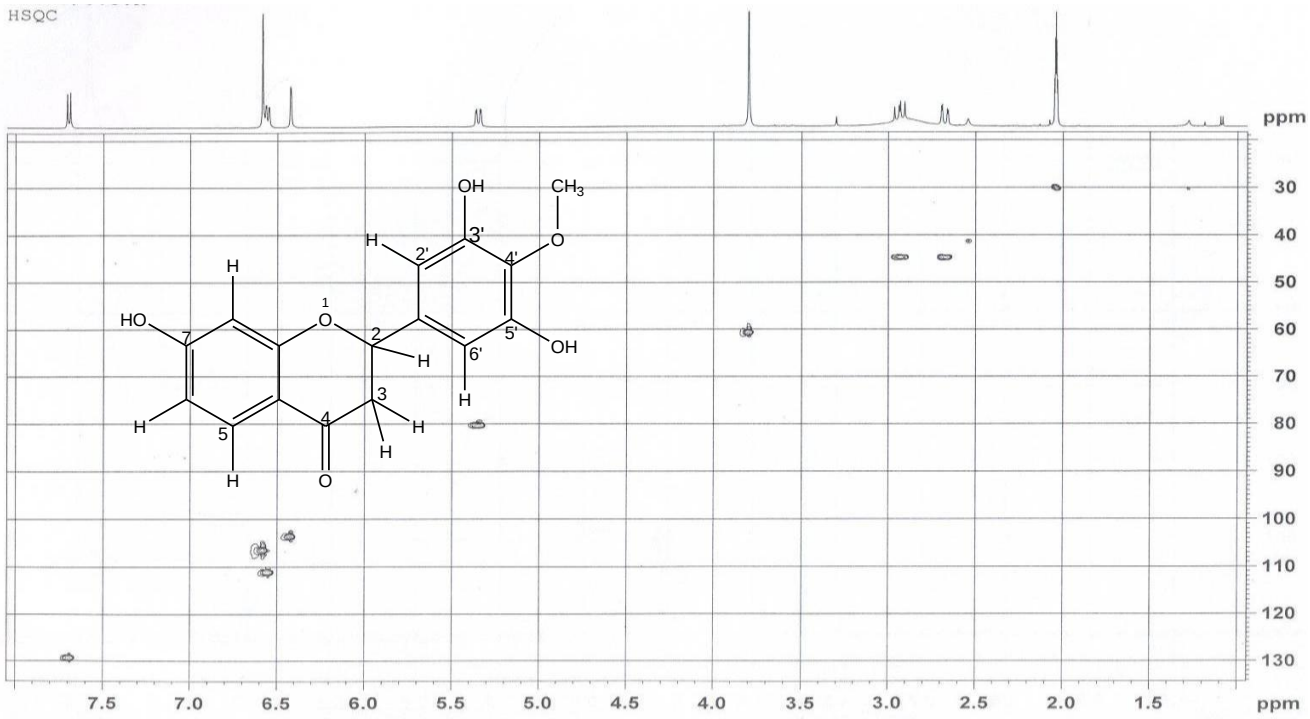


Fig. 4.9. HSQC spectrum of DRR-2E (CD_3COCD_3)

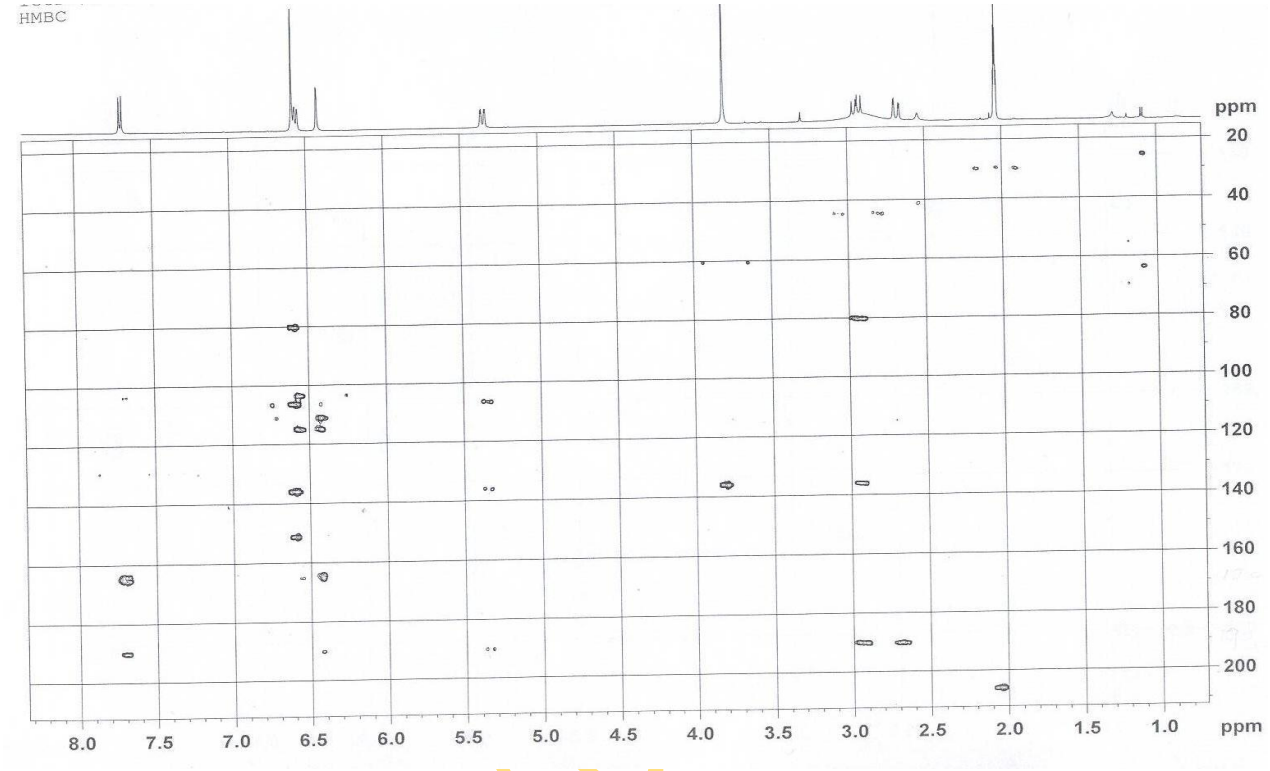


Fig. 4.10. HMBC spectrum of DRR-2E (CD_3COCD_3)

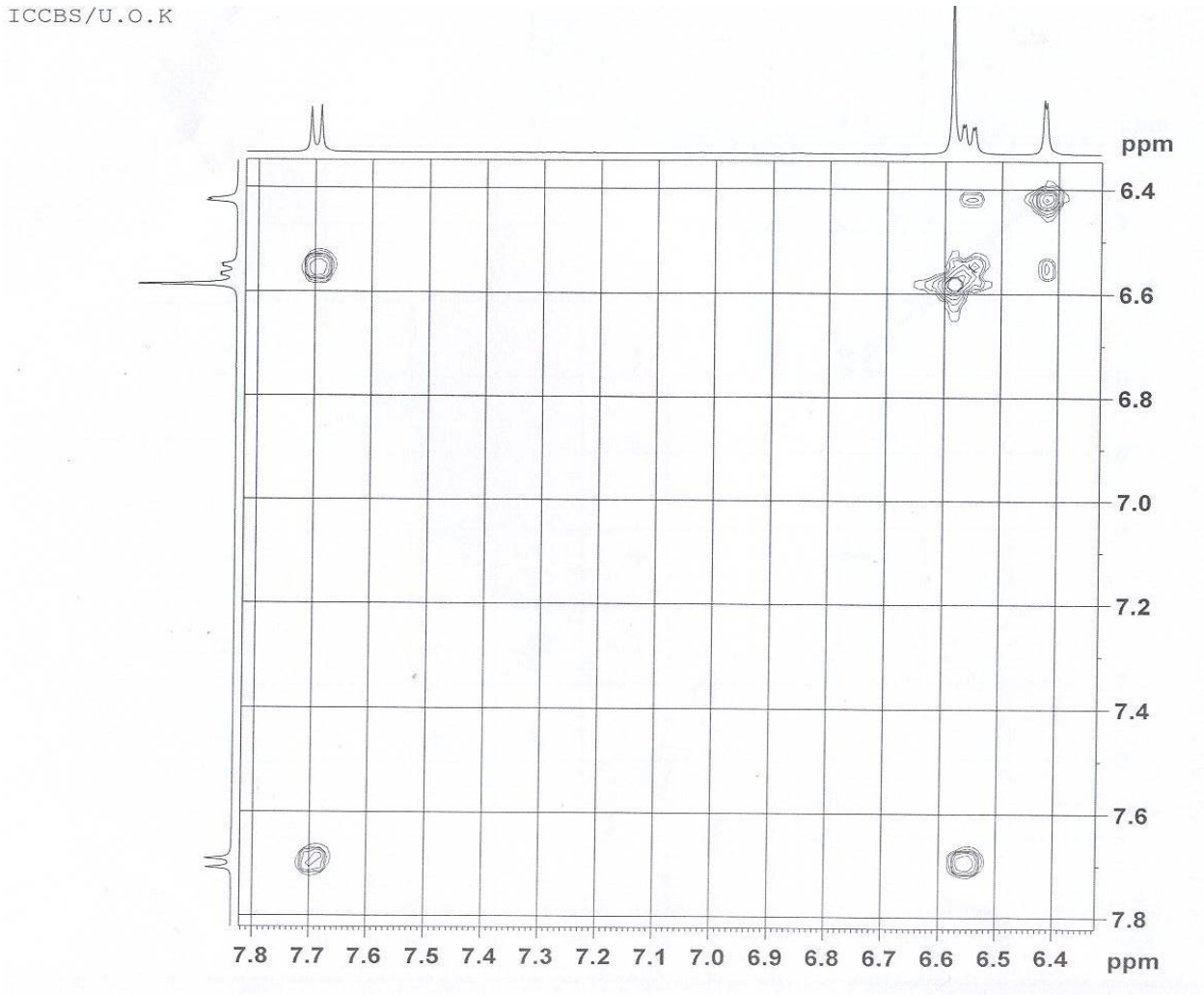


Fig. 4.11. COSY spectrum of DRR-2E (CD_3COCD_3)

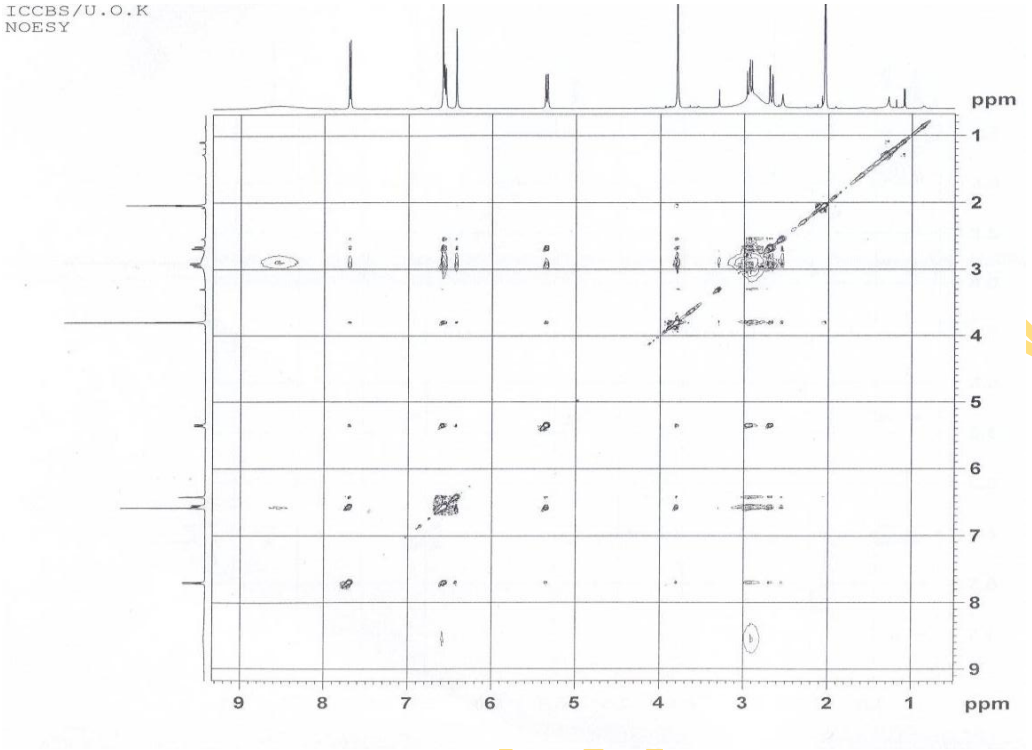


Fig. 4.12. NOESY spectrum of DRR-2E (CD_3COCD_3)

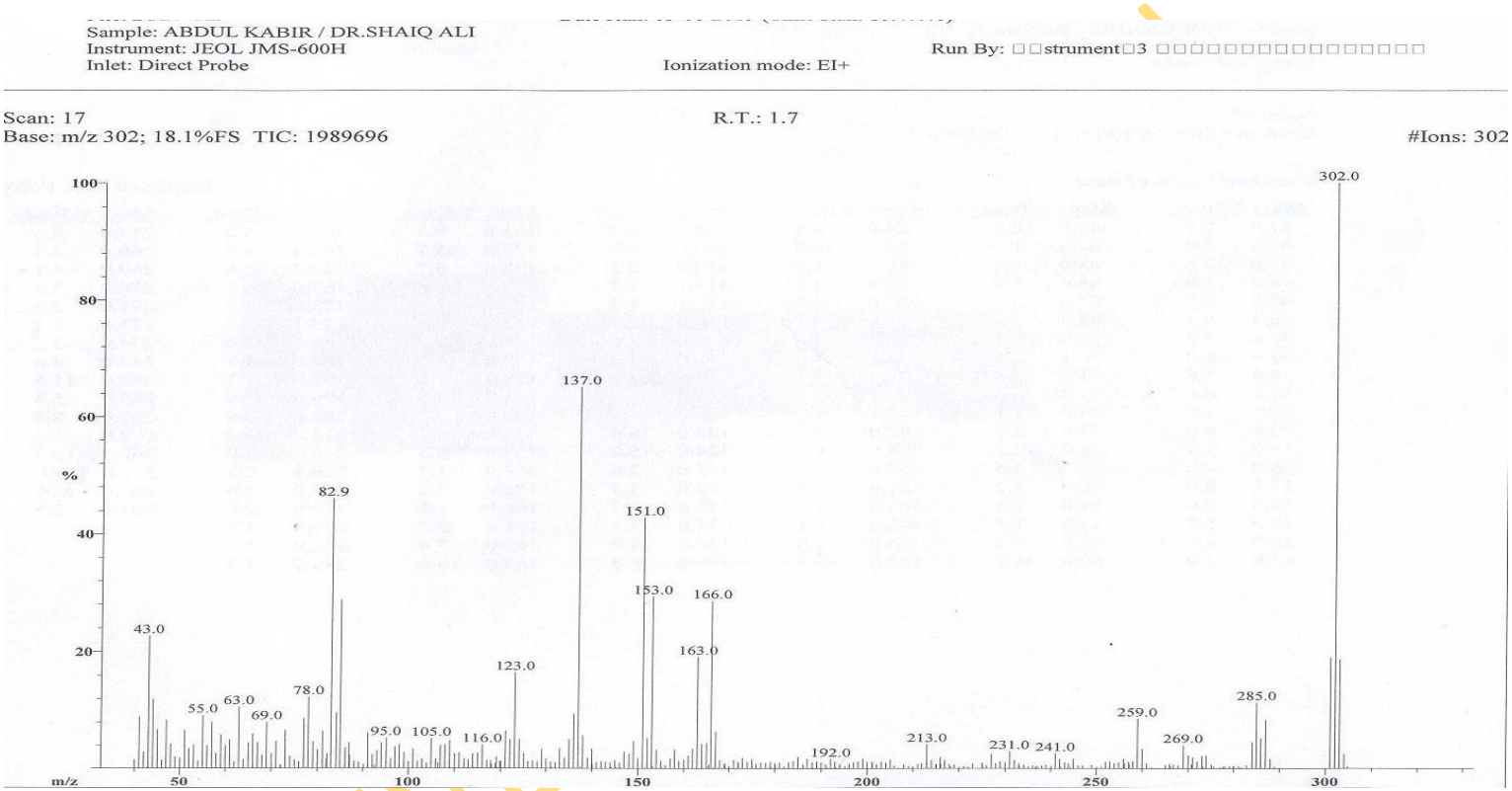


Fig. 4.13. EIMS spectrum of DRR-2E

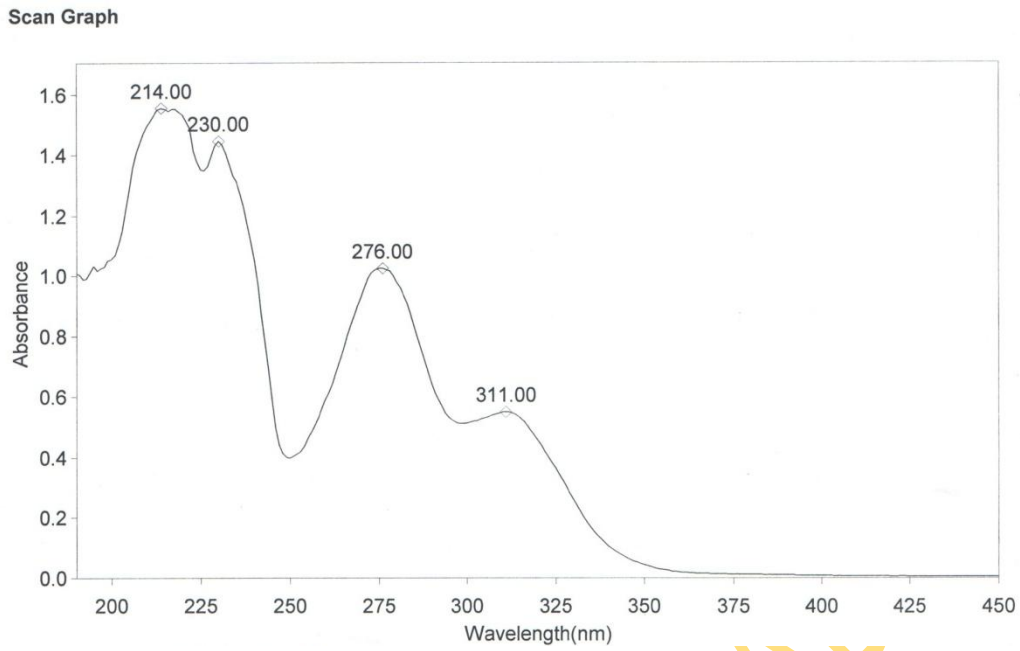


Fig. 4.14. UV spectrum of DRR-2E

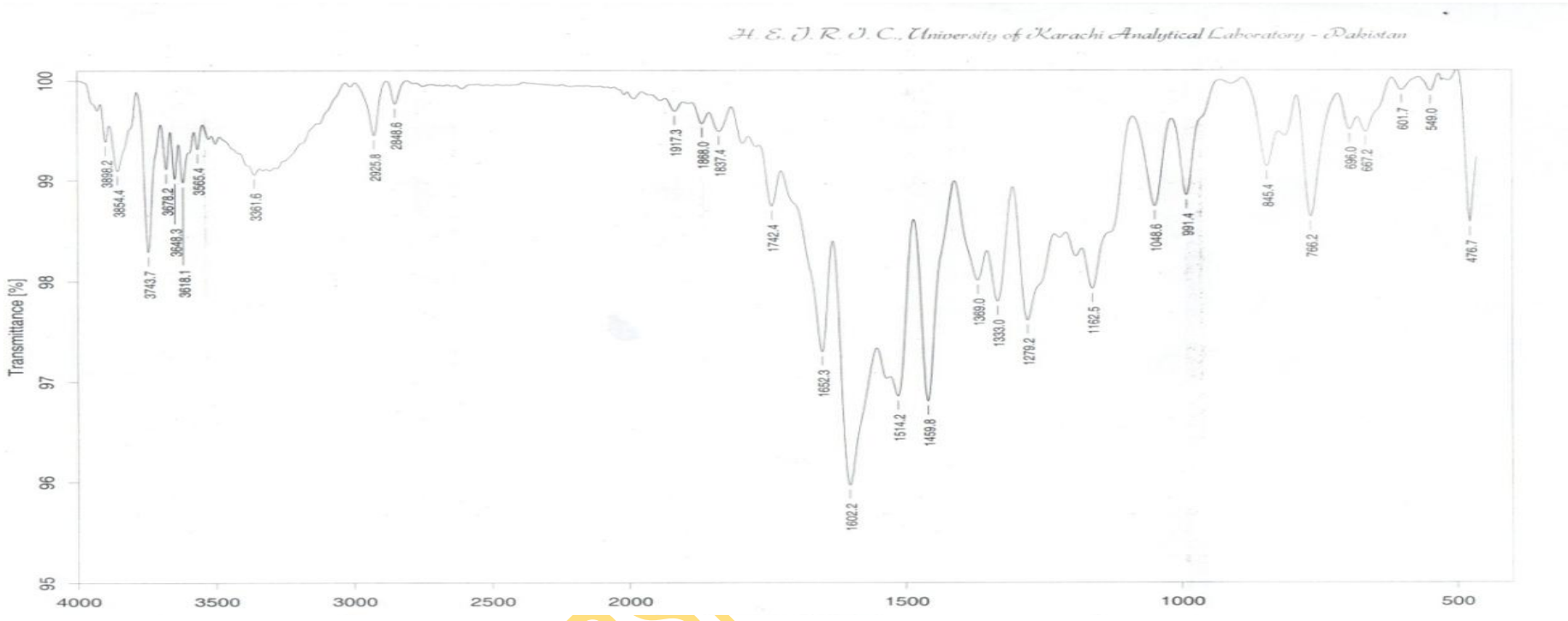


Fig. 4.15. IR spectrum of DRR-2E

4.2.3 Isolation and Characterisation of DRR-1F

The sub-fraction F (668 mg) was purified over a silica gel column and eluted with mixtures of increasing polarity of n-hexane/EtOAc/MeOH. Three hundred and five fractions (10 ml/vial) were collected (Table 4.7). Fraction F5 eluted with n-hexane/EtOAc (4:1) gave white needle-like crystals labelled DRR-IF (3 mg).

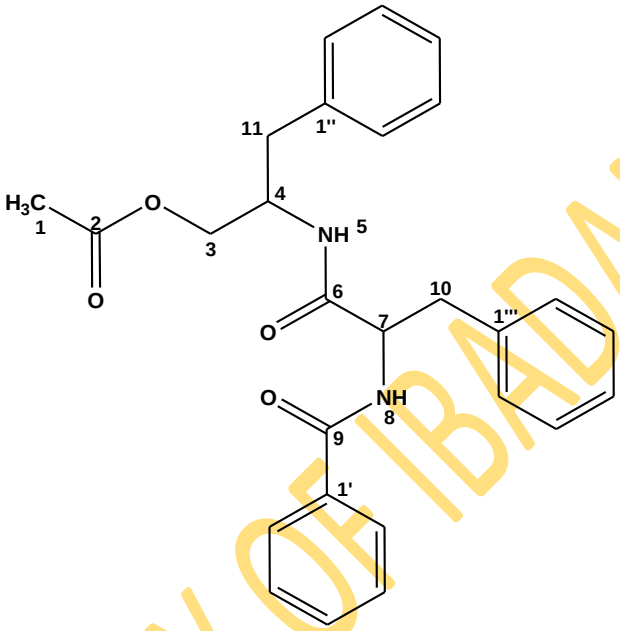
The DRR-IF showed UV absorption at 244 nm (Fig 4.21). The molecular formula was deduced as $C_{27}H_{28}O_4N_2$ (fifteen degree of unsaturation) with molecular ion at m/z 444 from its high resolution mass spectrum.

The ^{13}C-NMR (Fig. 4.17) of DRR-1F showed signals which, on the basis of DEPT experiments (Fig. 4.18–4.19) gave a methyl, three methylene, two aliphatic methines, fifteen aromatic methines and six quaternary carbons. In the quaternary carbons, three were attributed to carbonyl groups and other three to the aromatic groups. Its mass spectrum (Fig. 4.20) displayed prominent peaks at m/z 43 $[COMe]^+$, 77 $[C_6H_5]^+$, 91 $[C_7H_7]^+$, 105 $[C_6H_5CO]^+$, 120 $[C_6H_5CH{=}NH_2]^+$, 131$[C_6H_5CH{=}CHCO]^+$, 176 $[C_6H_5CH_2{=}CHOAc]^+$, 224 $[C_6H_5CO{-}NH{-}CHCH_2Ph]^+$, 252 $[C_6H_5CONHCH(CO)CH_2C_6H_5]^+$, 293 $[M{-}MeCO_2H + C_6H_5CH_2]^+$, 353 $[M{-}C_6H_5CH_2]^+$, 384 $[M{-}MeCO_2H]^+$, 444 $[M]^+$. Its IR absorption spectrum (Fig. 4.22) revealed the presence of –NH- groups (band at 3300 cm^{-1}), acetate (1729 and 1258 cm^{-1}) and monosubstituted benzene (1868, 1796, 772 and 694 cm^{-1}).

The ^{1}H-NMR spectrum (Fig. 4.16) ascertained the presence of two 1H doublets from amide groups at δ 5.87 (1H, d, J = 8.0 Hz) and 6.69 (1H, d, J = 6.8 Hz). Three proton singlet observed at δ 2.08 correlated to carbon at δc 20.80 and was assigned to acetoxyl methyl (C-1). Two 1H multiplets at δ 4.33 and δ 4.72 corresponding to δc 37.41 and 54.61 ppm in HSQC were assigned to methine protons at C-4 and C-7 respectively. One proton doublet of doublets signals due to a methylene (C-3) was observed at δ 3.81 (J = 11.2, 6.4 Hz) and δc 3.93 (J = 11.2, 7.2 Hz) as a result of its closeness to heteroatom. Also, a pair of 1H doublet of doublets resonated at δ 3.0 (J = 13.6, 8.0 Hz), δ 3.18 (J = 13.6, 5.2 Hz) and 2H multiplets at δc 2.74 were assigned to a pair of benzylic methylenes at C-10 and C-11 respectively. The 15 aromatic protons were in range of δ 7.04 – 7.70.

The ^{13}C-NMR showed the presence of three carbonyls [δ 170.8 (C-2), 170.2 (C-5) and 167.1(C-9)] in addition to 3 methylenes [δ 64.6 (C-2), 38.4 (C-10) and 37.4 (C-11)] and

two methines [δ 49.4 (C-2) and δ 55.0 (C-7)]. Other methines present were aromatic in nature. The spectra data together with mass fragmentation pattern from high resolution mass spectrum established DRR-IF structure as dipeptide. The structure was confirmed as aurantiamide acetate **4.3** by comparison of the spectra data (Table 4.8) with the existing literatures (Banerji and Ray, 1981; Wahidula *et al*., 1991).



4.3

Table 4.7. Column chromatography of sub-fraction F

Nos.	Column eluents	Pooled fractions	Pool code
1.	HEX : EtOAc (23:2)	1-112	F1
2.	HEX : EtOAc (9:1)	113-129	F2
3.	HEX : EtOAc (85:15)	130-138	F3
4.	HEX : EtOAc (4:1)	139-145	F4
5.	HEX : EtOAc (4:1)	146-150	F5 DRR-1F
6.	HEX : EtOAc (3:1)	168-177	F6
7.	HEX : EtOAc (7:3)	178-187	F7
8.	HEX : EtOAc (65:35)	188-198	F8
9.	HEX : EtOAc (60:40)	199-204	F9
10.	HEX : EtOAc (1:1)	205-216	F10
11.	HEX : EtOAc (3:7)	217-222	F11
12.	EtOAc (100%)	223-226	F12
13.	EtOAc : MeOH (1:1)	227-300	F13
14.	MeOH (100%)	301-305	F14

Table 4.8. Comparison of ^{1}H and ^{13}C NMR spectra data of DRR-1F (400 and 100 MHz, ppm in $CDCl_3$ respectively) with the literature

Position	^{1}H δ (integration, multiplicity, J Hz)	^{1}H* δ (integration, multiplicity, J Hz)	^{13}C δ	^{13}C* δ	DEPT
1	2.08	2.03	20.8	20.764	CH_3
2	-	-	170.8	170.752	C
3a	3.81 (1H, dd, J= 11.2, 6.4)	3.84 (1H, dd, J= 11.0, 5.0)	64.6	64.566	CH_2
b	3.93 (1H, dd, J= 11.2, 7.2)	3.95 (1H, dd, J= 11.0, 5.0)			
4	4.33 (1H, m)	4.20 (1H, m)	49.4	49.457	CH
5	5.87 (1H, d, J=8.0)	6.10 (1H, d, J=7.5)	-		
6	-	-	170.2	170.314	C
7	4.72 (1H, q)	4.78 (1H, m)	55.0	54.976	CH
8	6.69 (1H,d, J= 8.0)	6.9 (1H,d, J= 7.5)	-		
9	-	-	167.1	167.131	C
10a	3.0 (1H, dd, J= 13.6, 8.0)	3.07 (1H, dd, J= 14.5, 8.0)	38.4	38.410	CH_2
b	3.18 (1H, dd, J= 13.6, 5.2)	3.22 (1H, dd, J= 15.0, 6.0)			
11	2.74(2H,m)	2.74(2H, d, J= 14.5)	37.4	37.426	CH_2
1′	-	-	133.7	133.660	C
2′	-	-	136.6	136.605	C
3′	-	-	136.7	136.701	C
Aromatic 15H	7.04 - 7.70	7.12 -7.78	126.8-131.9	127.053-131.888	CH

* Wahidula *et al*., (1991)

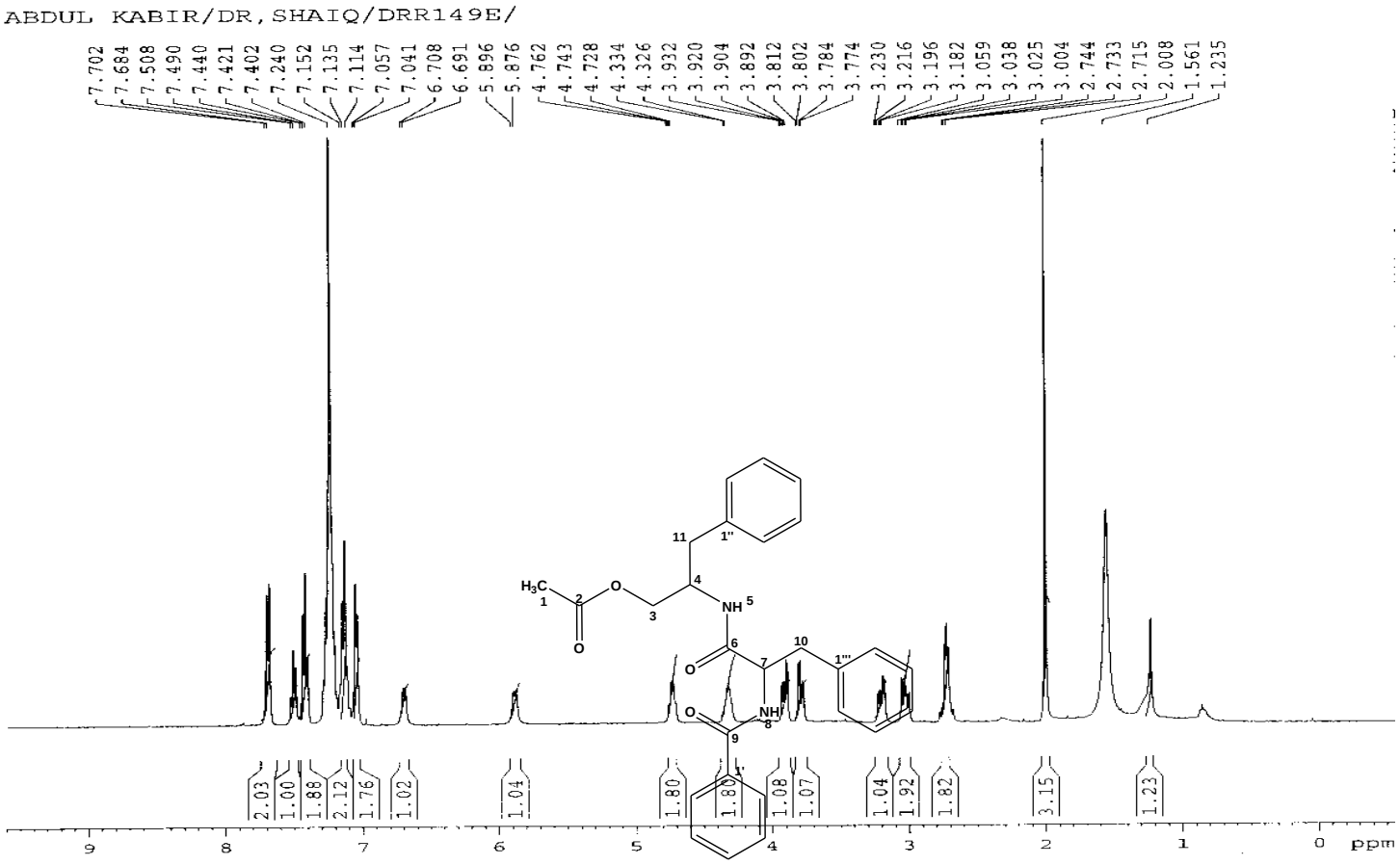


Fig. 4.16. ^{1}H-NMR spectrum of DRR-1F ($CDCl_3$, 400 MHz)

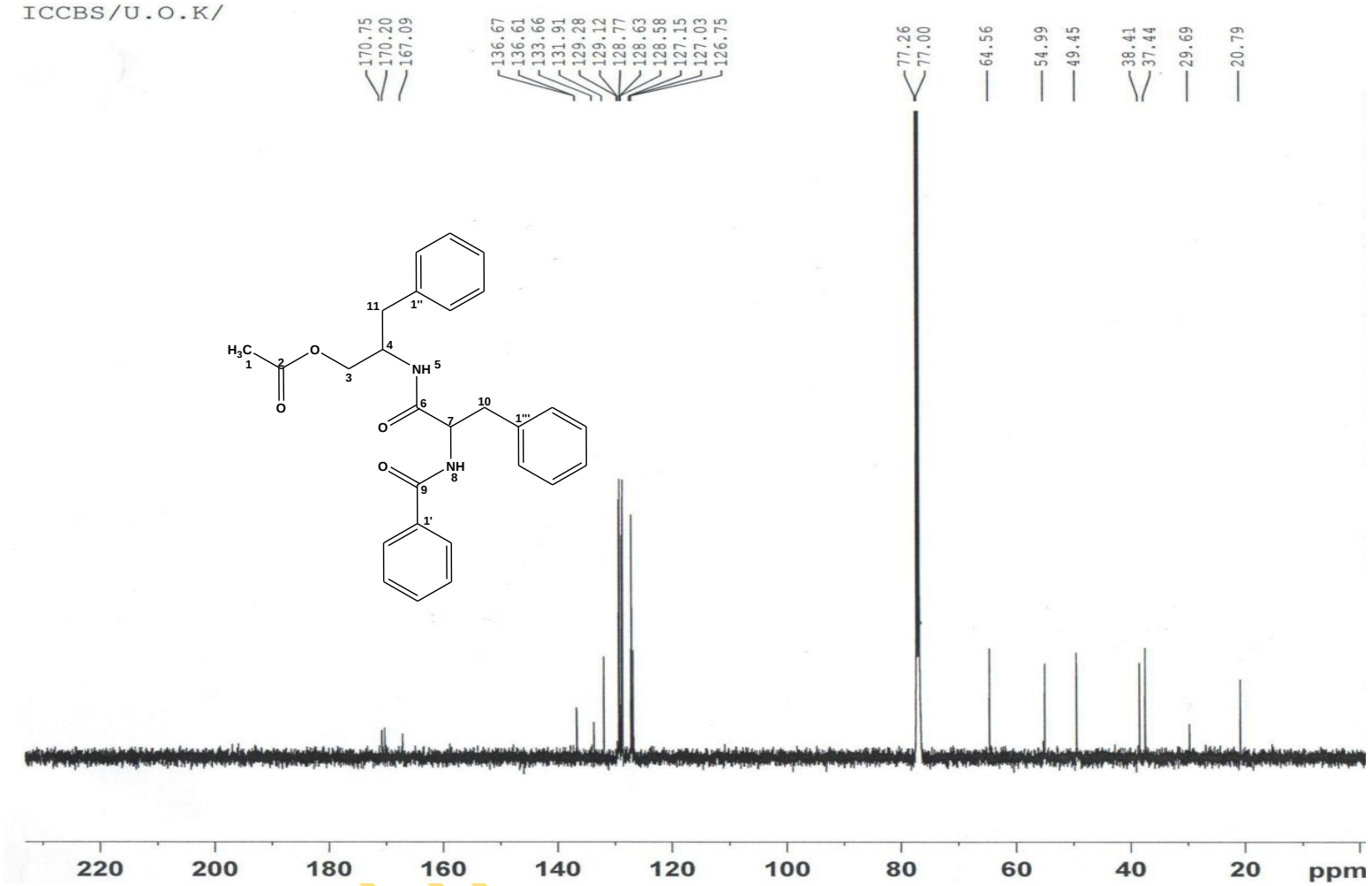


Fig. 4.17. ^{13}C-NMR spectrum of DRR-1F ($CDCl_3$, 100 MHz)

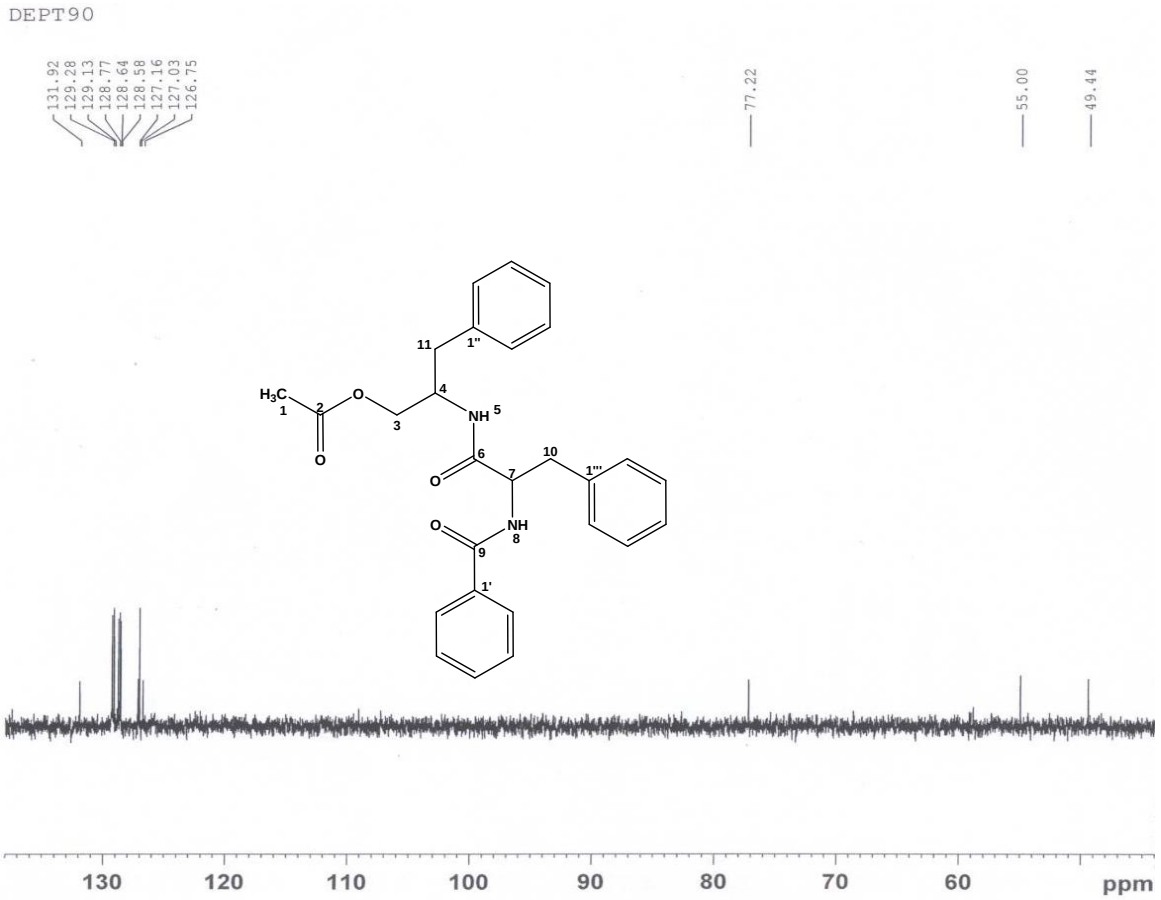


Fig. 4.18. DEPT 90 spectrum of DRR-1F ($CDCl_3$)

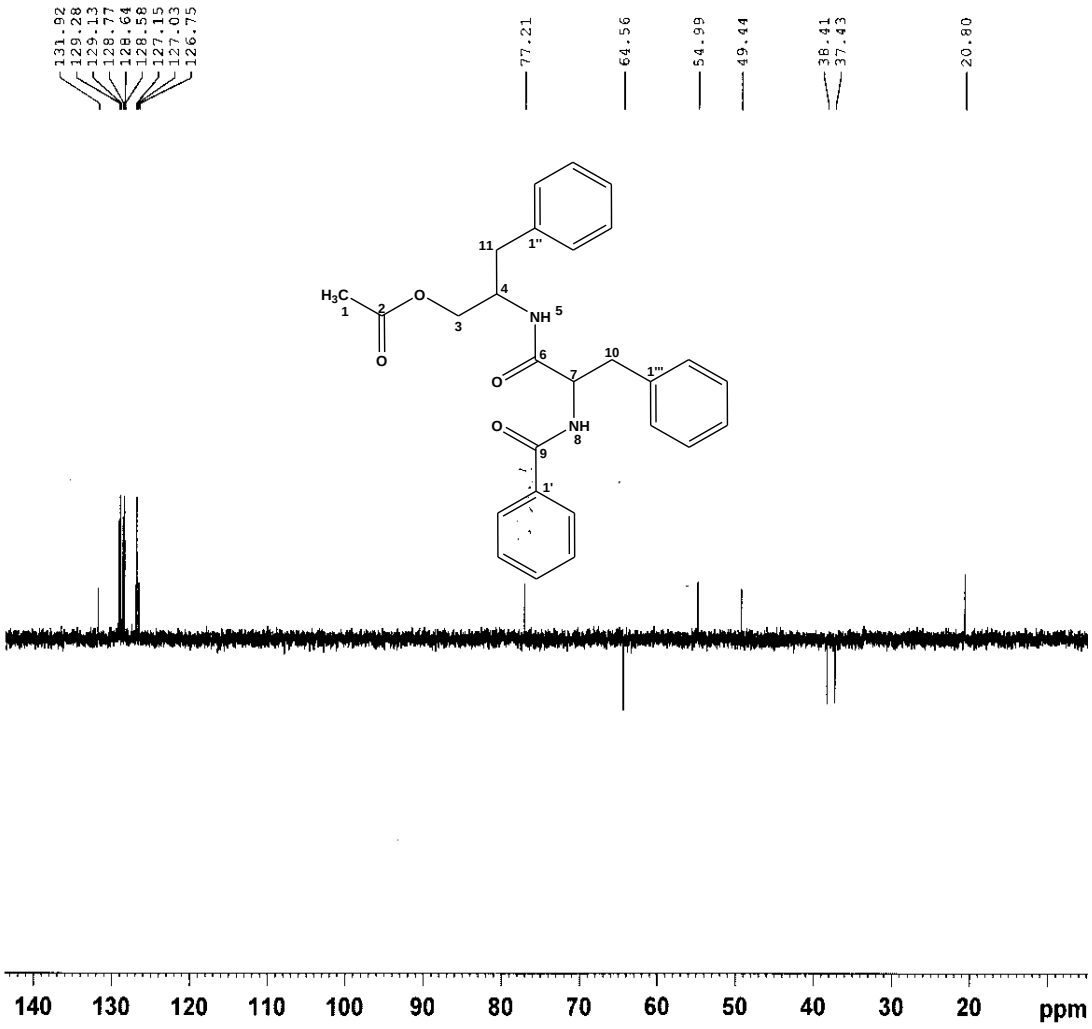


Fig. 4.19. DEPT 135 spectrum of DRR-1F ($CDCl_3$)

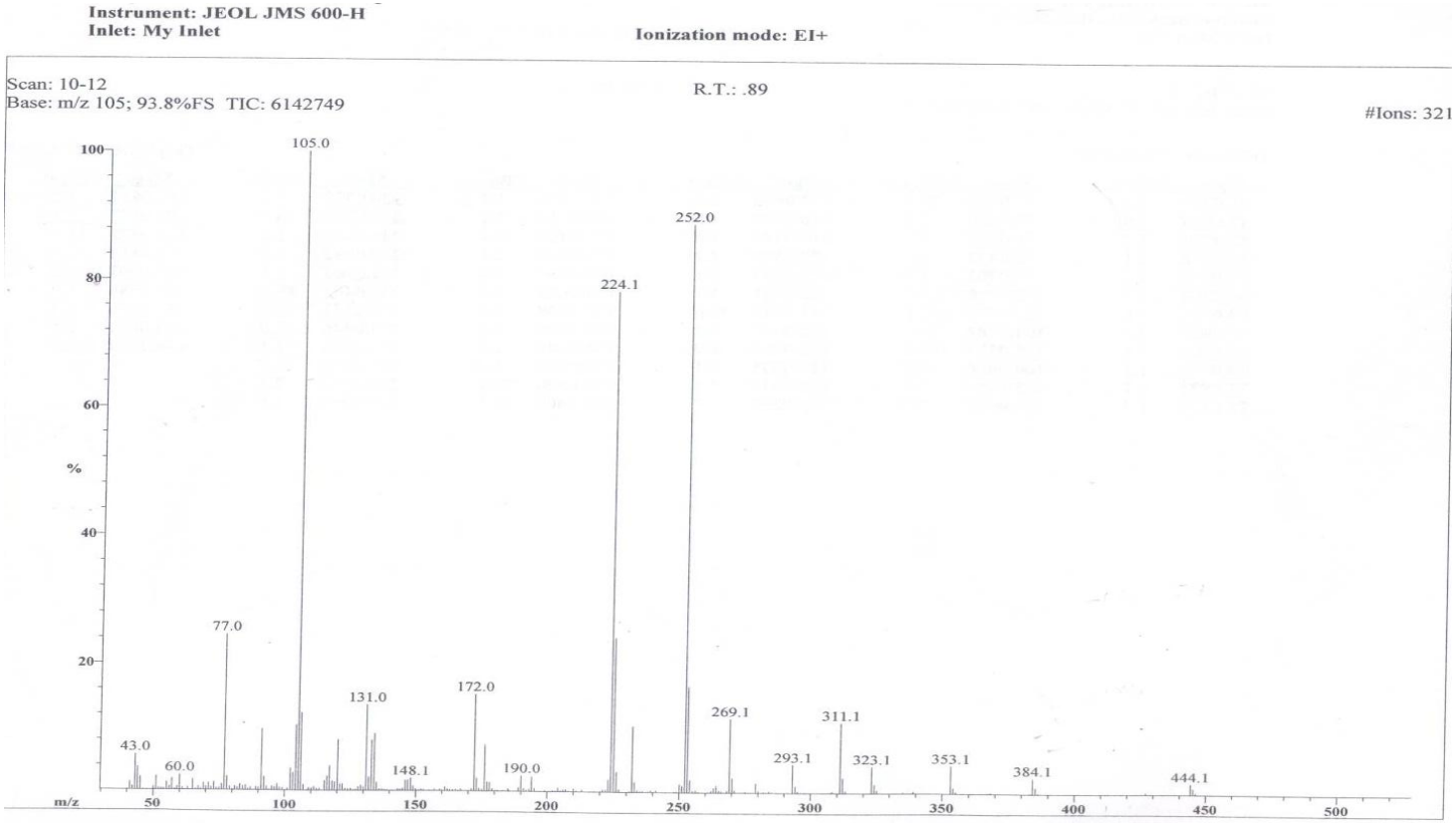


Fig. 4.20. EIMS spectrum of DRR-1F

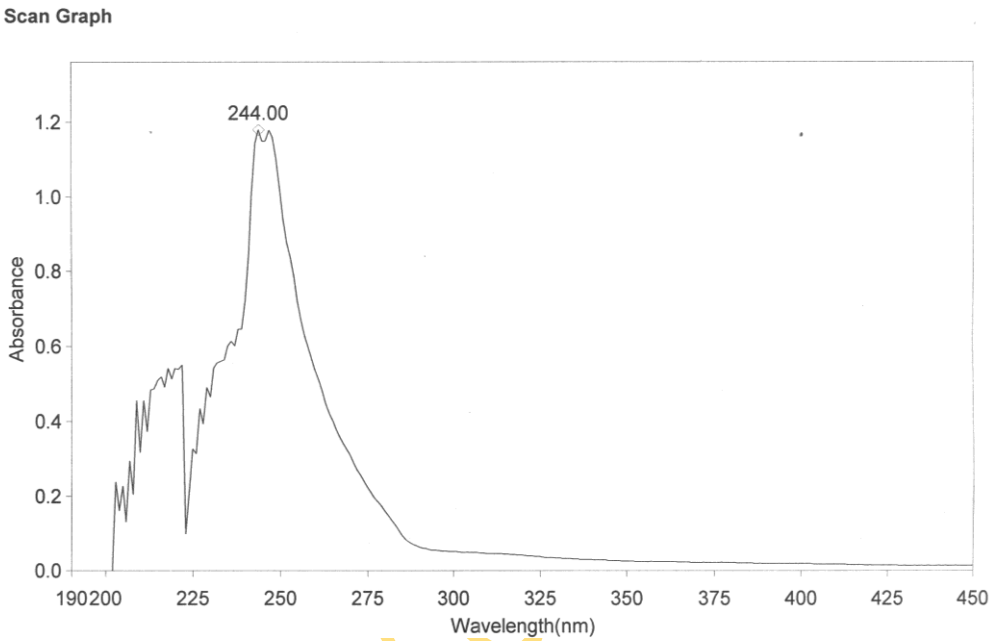


Fig. 4.21. UV spectrum of DRR-1F

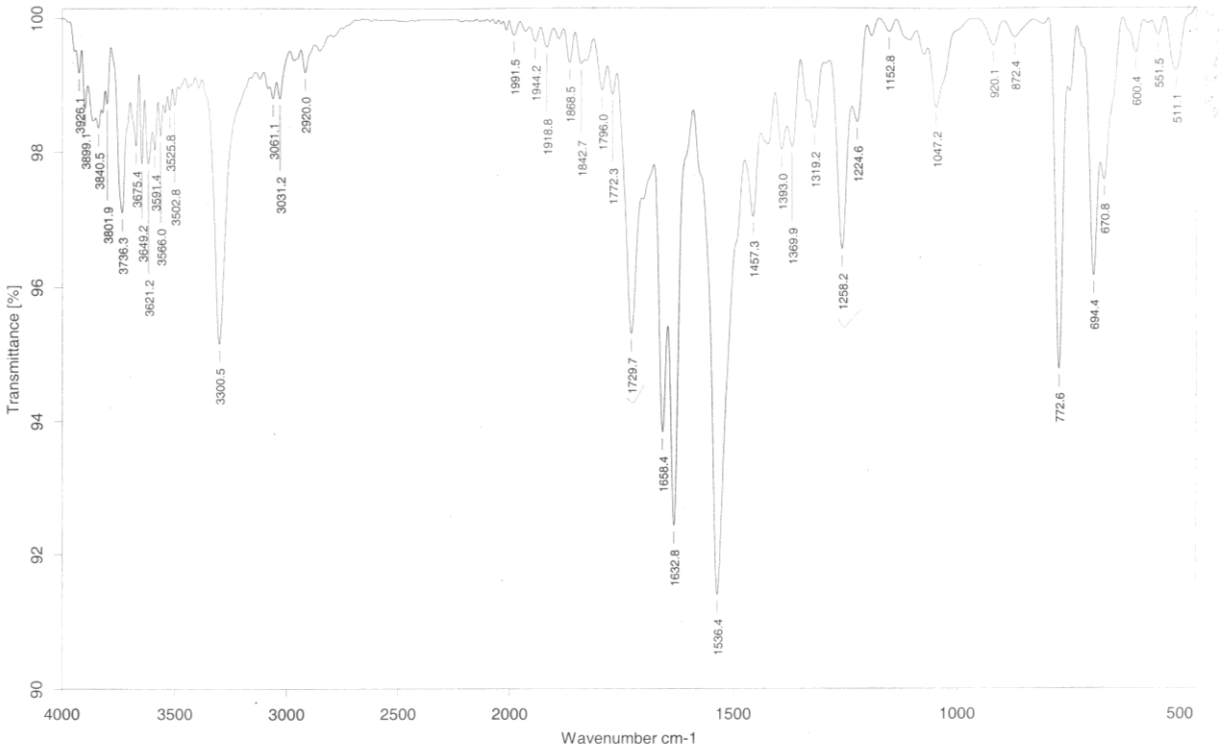


Fig. 4.22. IR spectrum of DRR-1F

4.2.4 Isolation and Characterisation of DRR-1G

The sub-fraction of G (1.2 g) was purified over a silica gel column and gradient elution was carried out with n-hexane/ EtOAc to give 205 fractions (Table 4.9). The TLC analysis of the fractions resulted to 15 pooled fractions (G1-15). The G-9 (12 mg) eluted with 40% ethyl acetate in hexane gave a pure yellow crystals which was coded DRR-1G (7.0 mg).

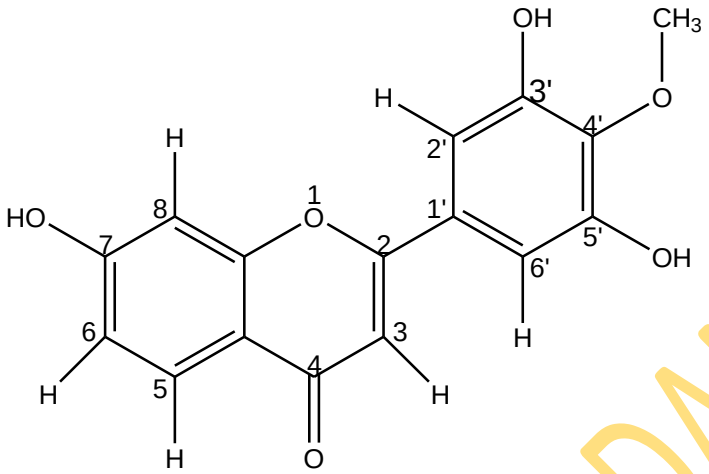
The electron impact mass spectrum (Fig. 4.24) of DRR-1G showed a molecular ion peak at m/z 300 corresponding to $C_{16}H_{12}O_6$ by HREIMS. The IR spectrum (Fig. 4.25)

suggested the presence of a hydroxyl group (3377 cm^{-1}), ketone carbonyl group (1627 cm^{-1}) and olefinic group (1570 cm^{-1}). The proton NMR spectrum of DRR-1G (Fig. 4.26) displayed a pair of doublets in the aromatic region at δ 7.95 (J=8.8 Hz) and δ 7.02 (J=2.4 Hz), and another doublet of doublets (dd) at δ 6.97 (J=2.4, 8.8 Hz), having one proton integration each. The coupling constants of the dd suggested *ortho* and *meta* position and thus assigned to H-6. Positions H-5, H-8 were assigned to δ 7.95 and δ 7.02 respectively. The connectivity of H-5 to H-6 was confirmed by COSY (Fig. 4.27). The broad singlet 1H peak at 9.64 was not directly attached to any carbon, it was assigned to 7-OH. The above data were similar to flavonoids of 7-hydroxy A- ring (Park *et al*., 2007, Park *et al*., 2006). The retro-Diels-Alder fragments of ring C lead to diagnostic peaks at m/z 137 and 164, supporting the presence of a hydroxyl in ring A, a methoxyl and two hydroxyls in ring B (Scheme 4. 2). Another informative fragments at m/z 149[$C_8H_5O_3$] indicated methoxyl moiety at C-4′ which was demethylated (Scheme 4. 2). Other significant peaks in addition to molecular ion at m/z 300 are 285[M^+ - CH_3], 257, 229 and 121. The 2H singlet at δ 7.06, 2H broad singlet at δ 8.47 and 3H singlet of methoxyl at δ 3.88 in ring B were assigned to H-2′/6′, OH-3′/5′ and H-4′ respectively; suggested a 3,4,5 trioxygenated symmetrical substituted B-ring, typical of mearnsetin B-ring (Abbas *et al*., 2007).

The ^{13}C-NMR, DEPT and HMBC spectra (Fig. 4.28 – 4.31) showed resonances for all 16 carbons with one methoxyl, six methine and nine quartenary carbons. The most downfield shifted peak at δc 177.95 was assigned to ketone group (C-4). Two peaks at δc 106.58 and δc 151.83 showed double intensities and were assigned to C-2′/6′ and C-3′/5′ respectively (Gluchoff-Fiasson *et al*., 2001, Abbas *et al*., 2007). In addition, the methoxyl at δ_H 3.88 showed correlation with carbon resonance at δc 139.0 on the HMBC spectrum and was assigned C-4′.

In HMBC, C-4 showed long-range connectivity to the ^{1}H peak at 7.95 ppm which was directly attached to ^{13}C peak at 127.66 ppm in HSQC (Fig. 4.32) and was assigned C-5. In addition, the ^{1}H at 7.95 ppm showed long-range coupling to ^{13}C peaks at 163.3 ppm and 158.74 ppm in HMBC, they were assigned to C-7 and C-9 respectively. The pronounced upfield shift of ^{13}C peak at 163.3 further supports the presence of a hydroxyl at C-7. Other C, H- correlations were observed between the methine (H-3) and the carbon atoms at C-4, C-2 and C-1′ (Fig. 4.23) position. Complete assignments of the ^{1}H and ^{13}C-NMR data are listed in Table 4.10. These observations suggested that new

compound DRR-1G is a 7,3′,5′-Trihydroxy-4′-methoxyflavone and was given trivial name reflevone **4.4**.



4.4

Table 4.9. Column chromatography of sub-fraction G

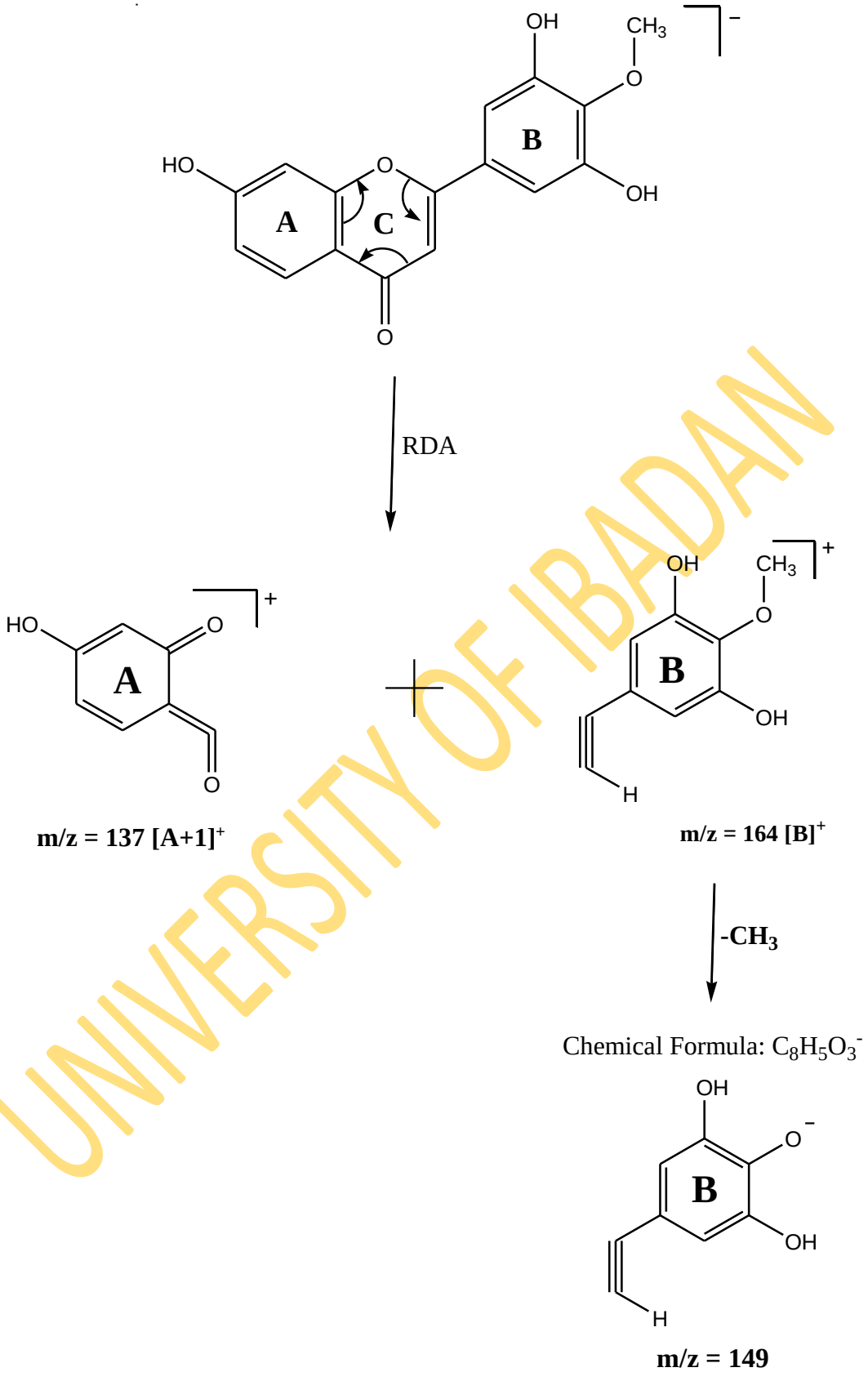
Column eluents	Pooled fraction	Pool code
HEX : EtOAc (19:1)	1-15	G1
HEX : EtOAc (9:1)	16-38	G2
HEX : EtOAc (85:15)	39-66	G3
HEX : EtOAc (4:1)	67-84	G4
HEX : EtOAc (3:1)	85-93	G5 (A´)
HEX : EtOAc (7:3)	94-109	G6
HEX : EtOAc (65:35)	110-122	G7 (B´)
HEX : EtOAc (60:40)	123-128	G8
HEX : EtOAc (60:40)	129-134	G9 (DRR-1G)
HEX : EtOAc (55:45)	153-158	G10
HEX : EtOAc (1:1)	159-170	G11
HEX : EtOAc (40:60)	171-180	G12
HEX : EtOAc (35:65)	181-189	G13

HEX : EtOAc (25:75)	190-200	G14
HEX : EtOAc (10:90)	201-205	G15

Table 4.10. ^{1}H and ^{13}C-NMR spectra data of DRR-1G (400 and 100 MHz Acetone)

Position	^{1}H δ (integration, multiplicity, J Hz)	^{13}C	DEPT	HMBC
2	-	163.3	C	
3	6.54 (1H, s)	107.0	CH	C-2, C-4, C-10, C-1′
4	-	177.2	C	
5	7.95 (1H, d, J= 8.8 Hz)	127.7	CH	C-4, C-7, C-8, C-9
6	6.97 (1H, dd, J= 2.4, 8.4 Hz)	115.4	CH	C-8, C-10
7		163.3	C	
7-OH	9.64 (br s)			
8	7.01 (1H, d, J= 2.4 Hz)	103.5	CH	C-6, C-7, C-9, C-10
9	-	158.7	C	
10	-	118.0	C	
1′	-	128.3	C	

2′/6′	7.07 (s)	106.6	CH	C-2, C-1′, C-3′, C-4′
3′/5′		151.8	C	
3′/5′-OH	8.47 (s)			
4′	-	139.0	C	
OCH_3	3.88 (3H, s)	60.7	CH_3	C-4′



Scheme 4.2. Fragmentation pattern of compound DRR-1G

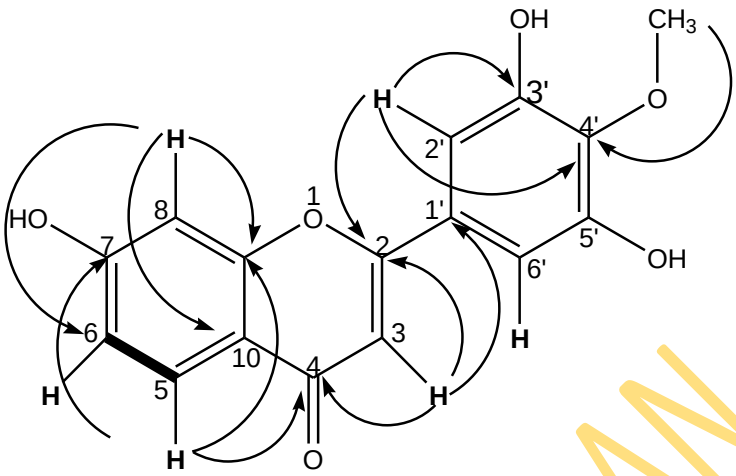


Figure 4.23. HMBC connectivities of DRR-1G.

Key: COSY (▬) and HMBC (→)

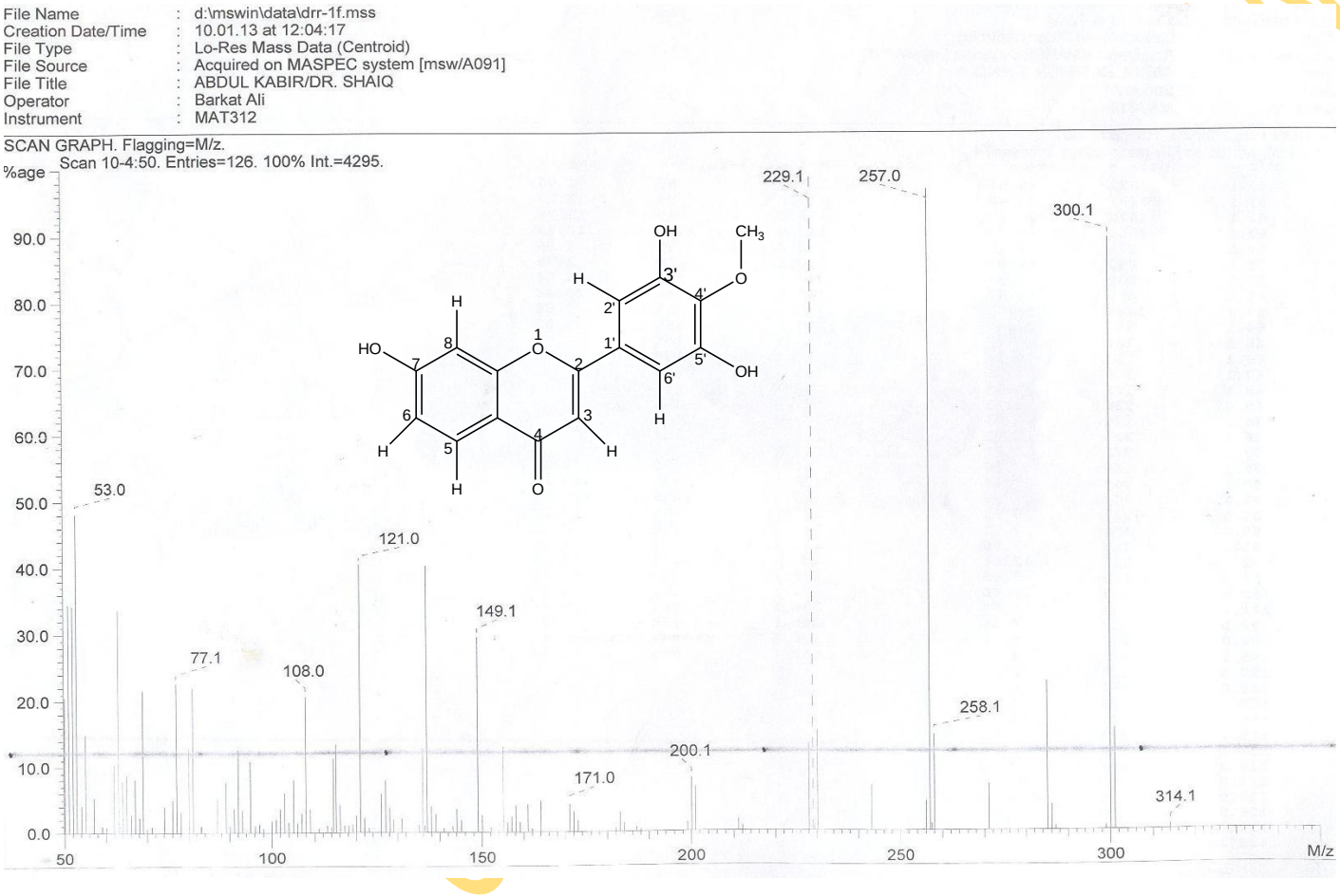


Fig. 4.24. EIMS of DRR-1G

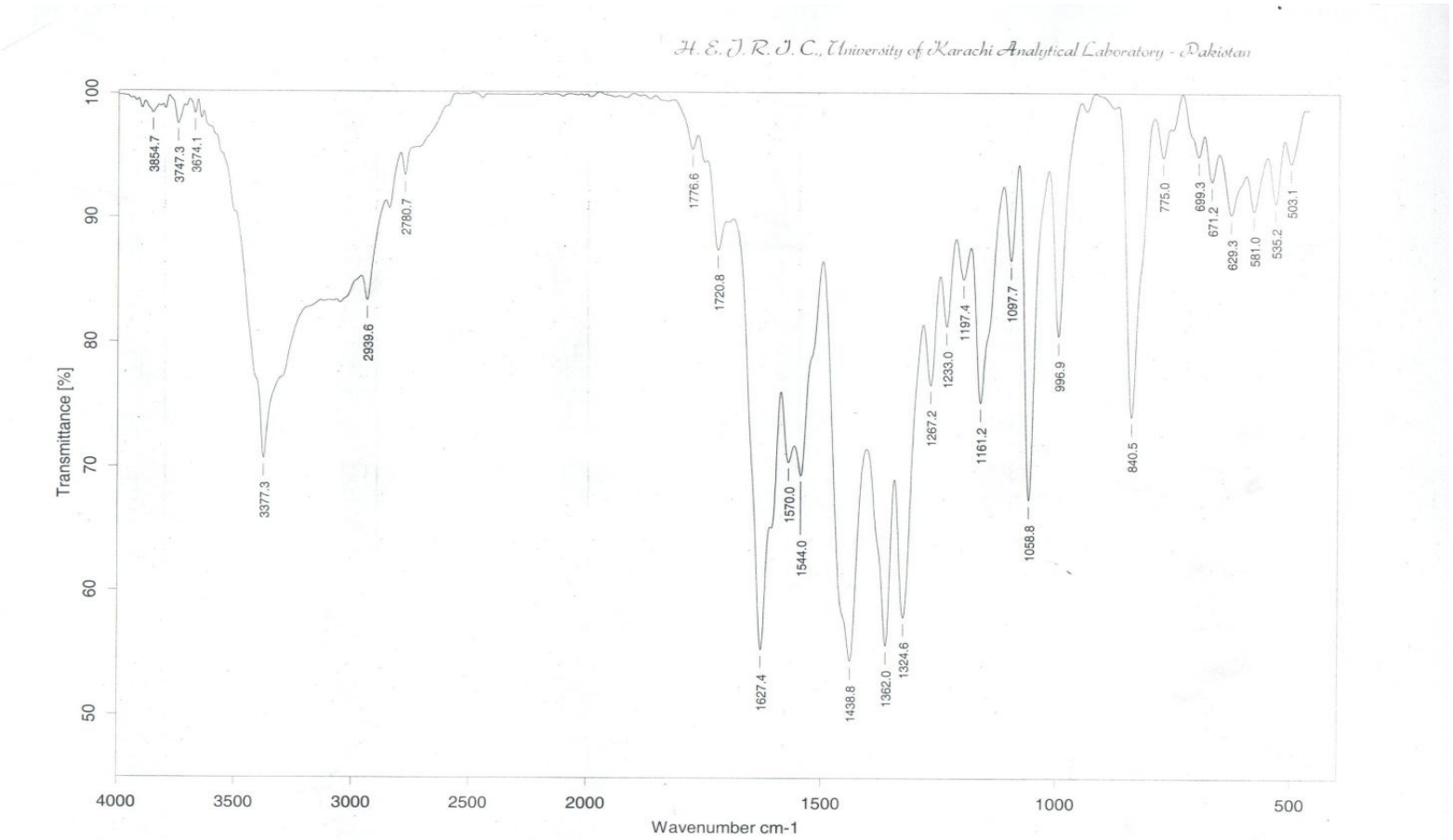


Fig. 4.25. IR of DRR-1G

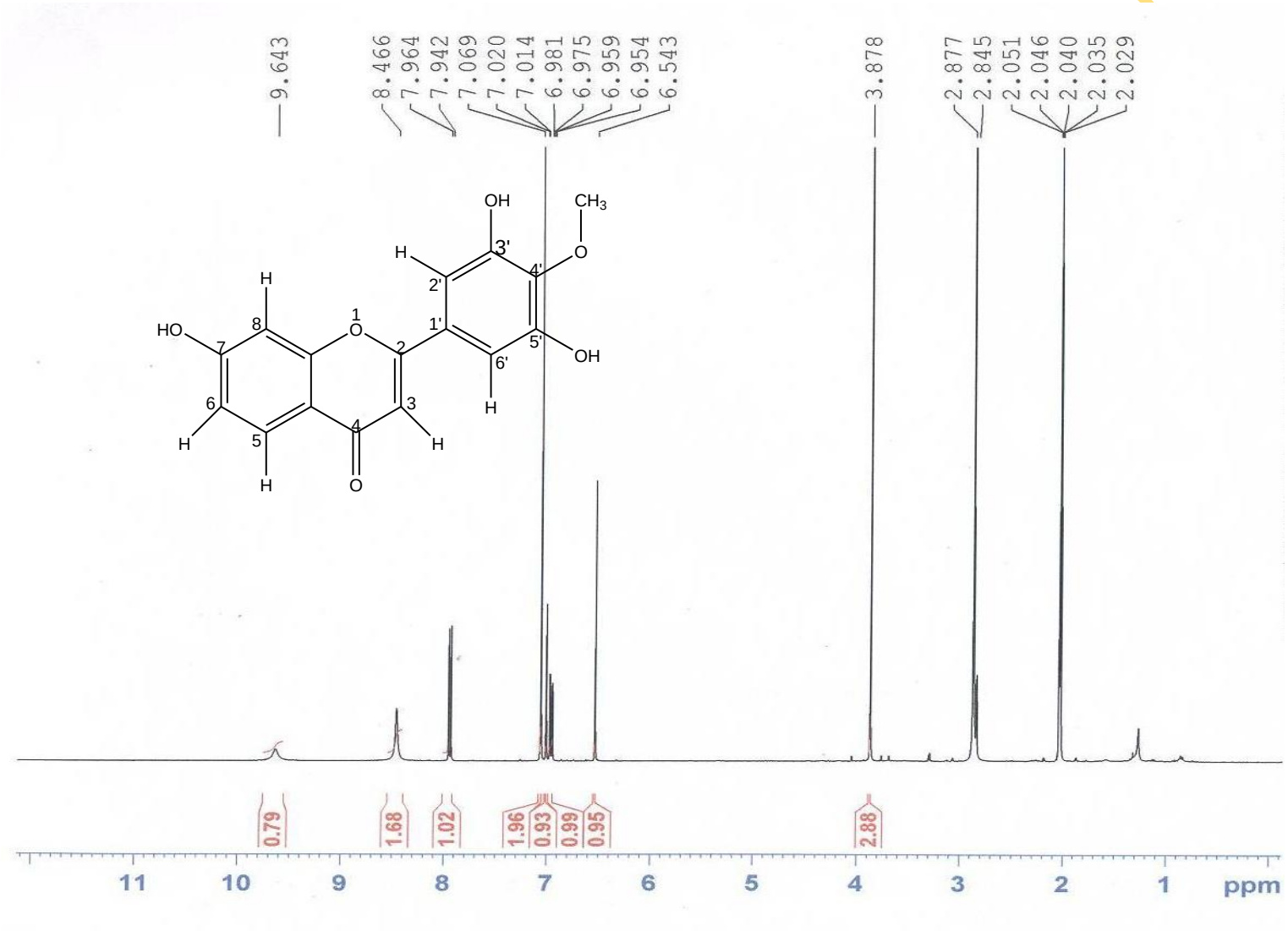


Fig. 4.26. ^{1}H-NMR spectrum of DRR-1G (CD_3COCD_3, 400 MHz)

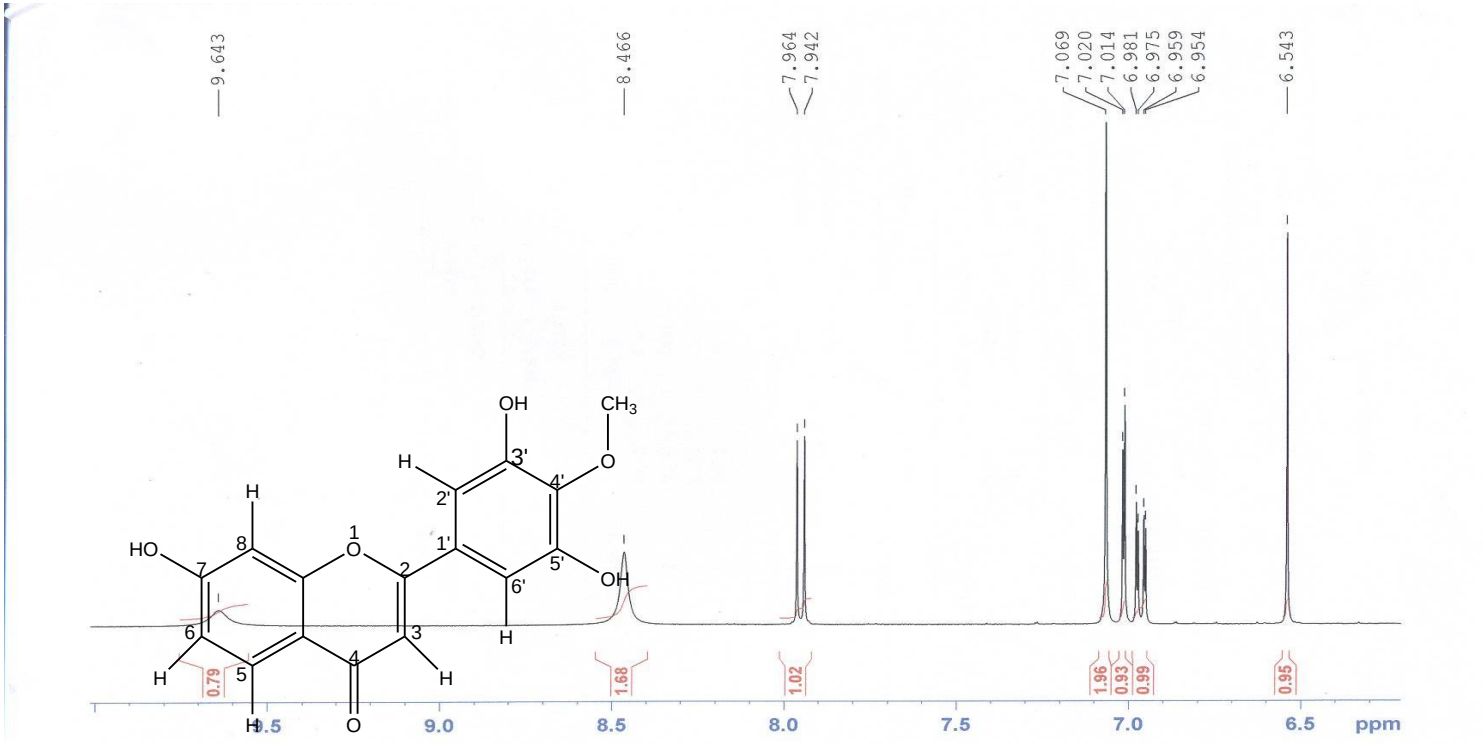


Fig. 4.26a. Expanded ^{1}H-NMR spectrum of DRR-1G (CD_3COCD_3, 400 MHz)

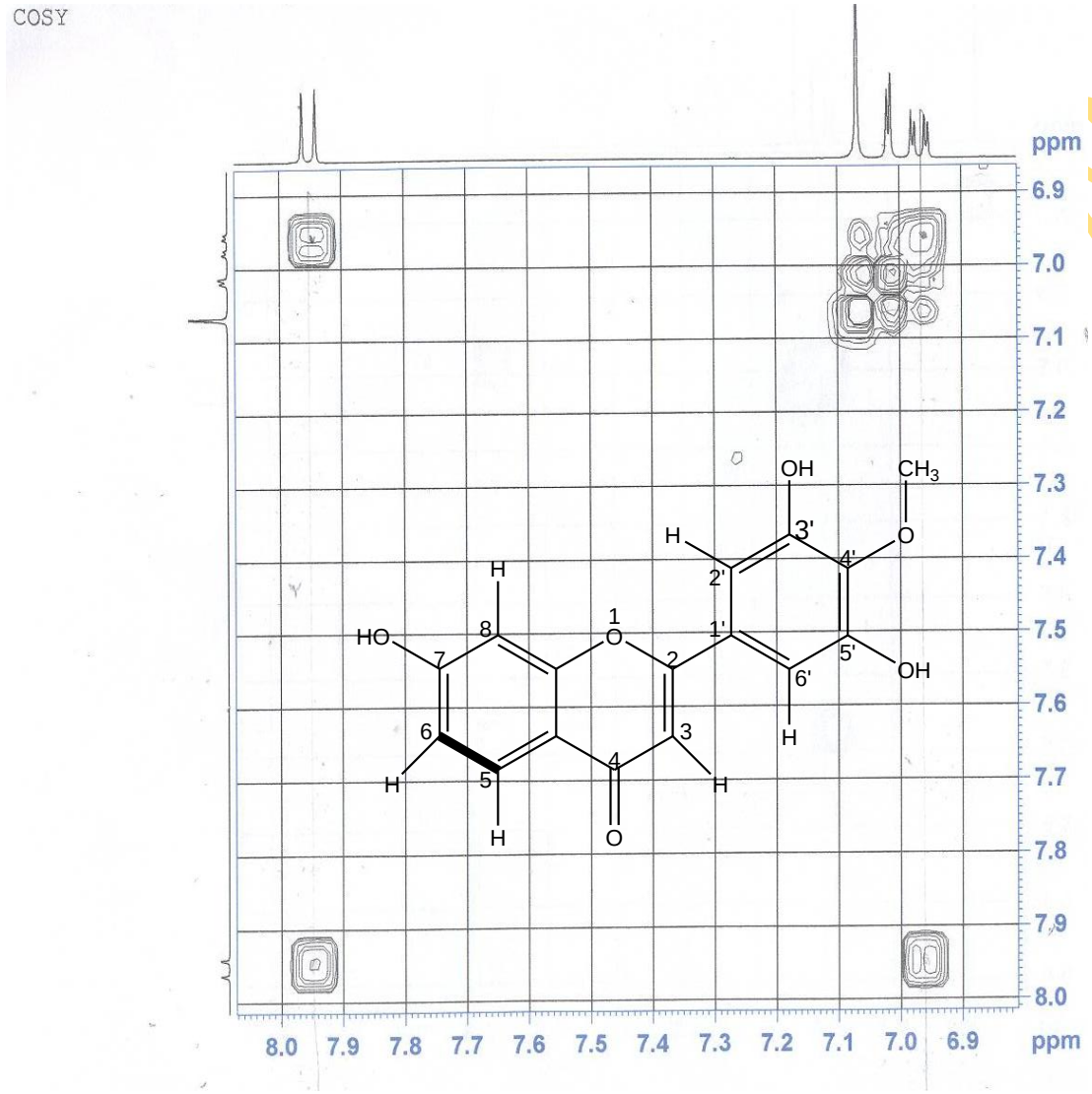


Fig. 4.27. COSY spectrum of DRR-1G

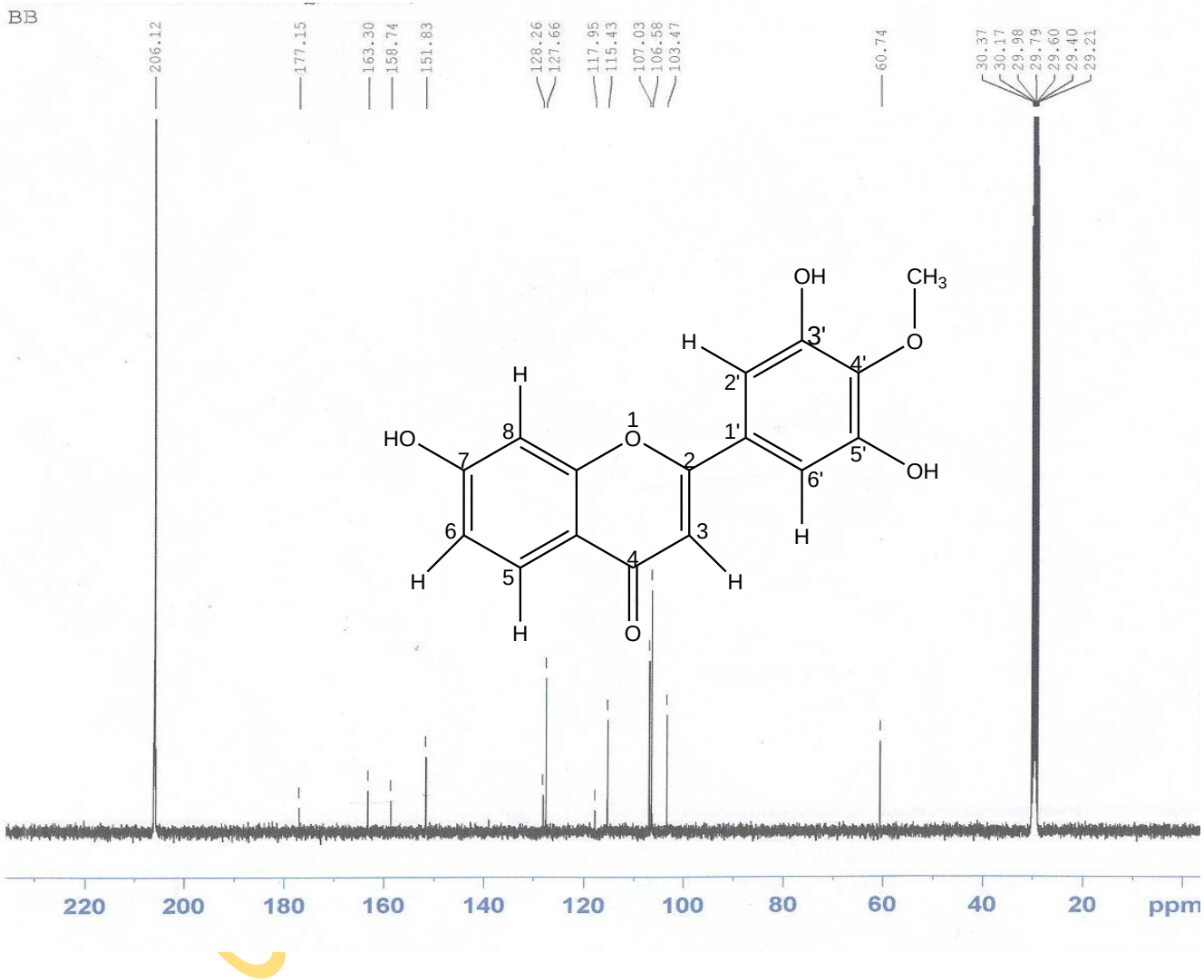


Fig. 4.28. ^{13}C-NMR spectrum of DRR-1G (CD_3COCD_3, 400 MHz)

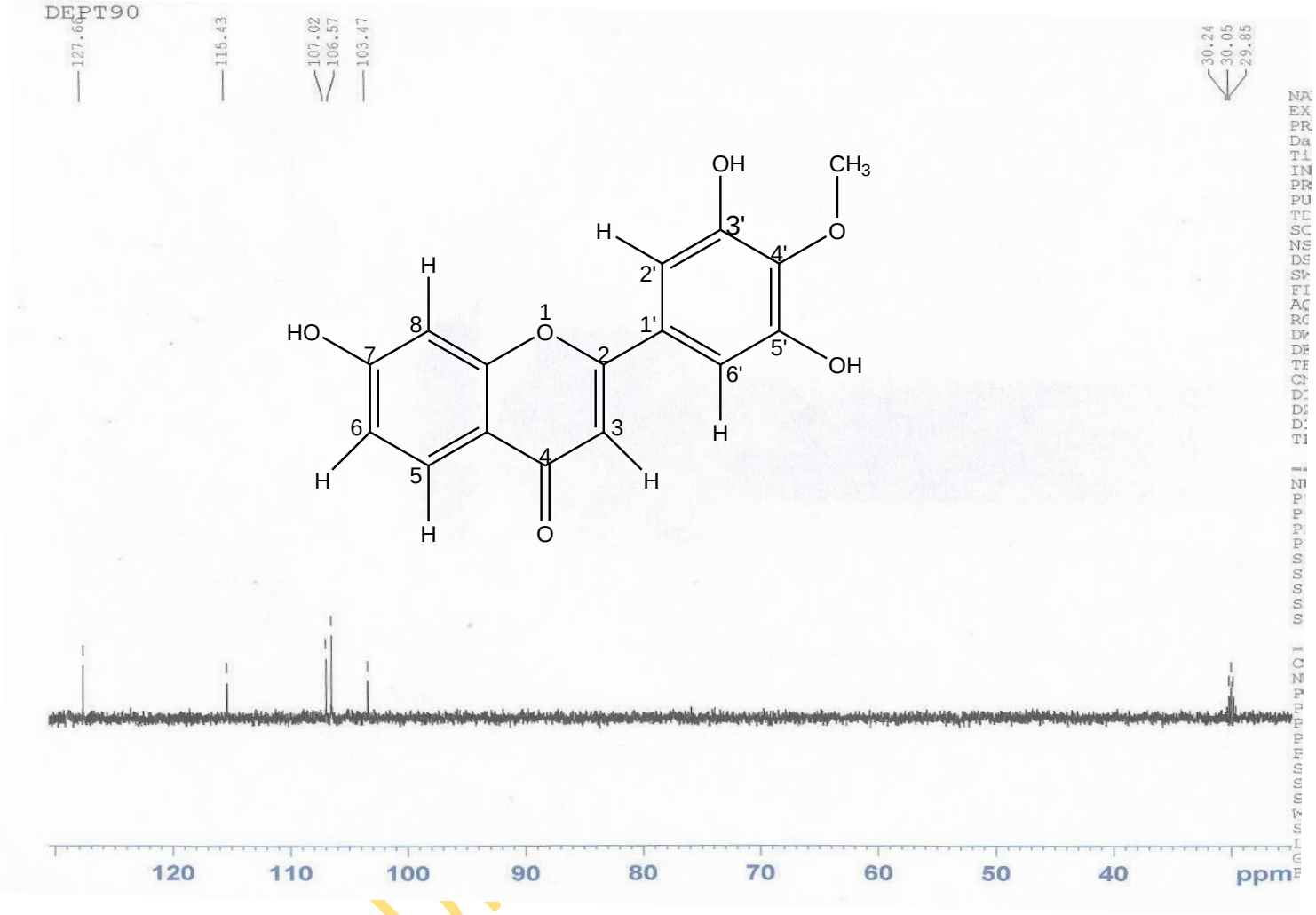
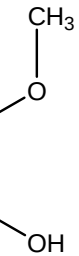
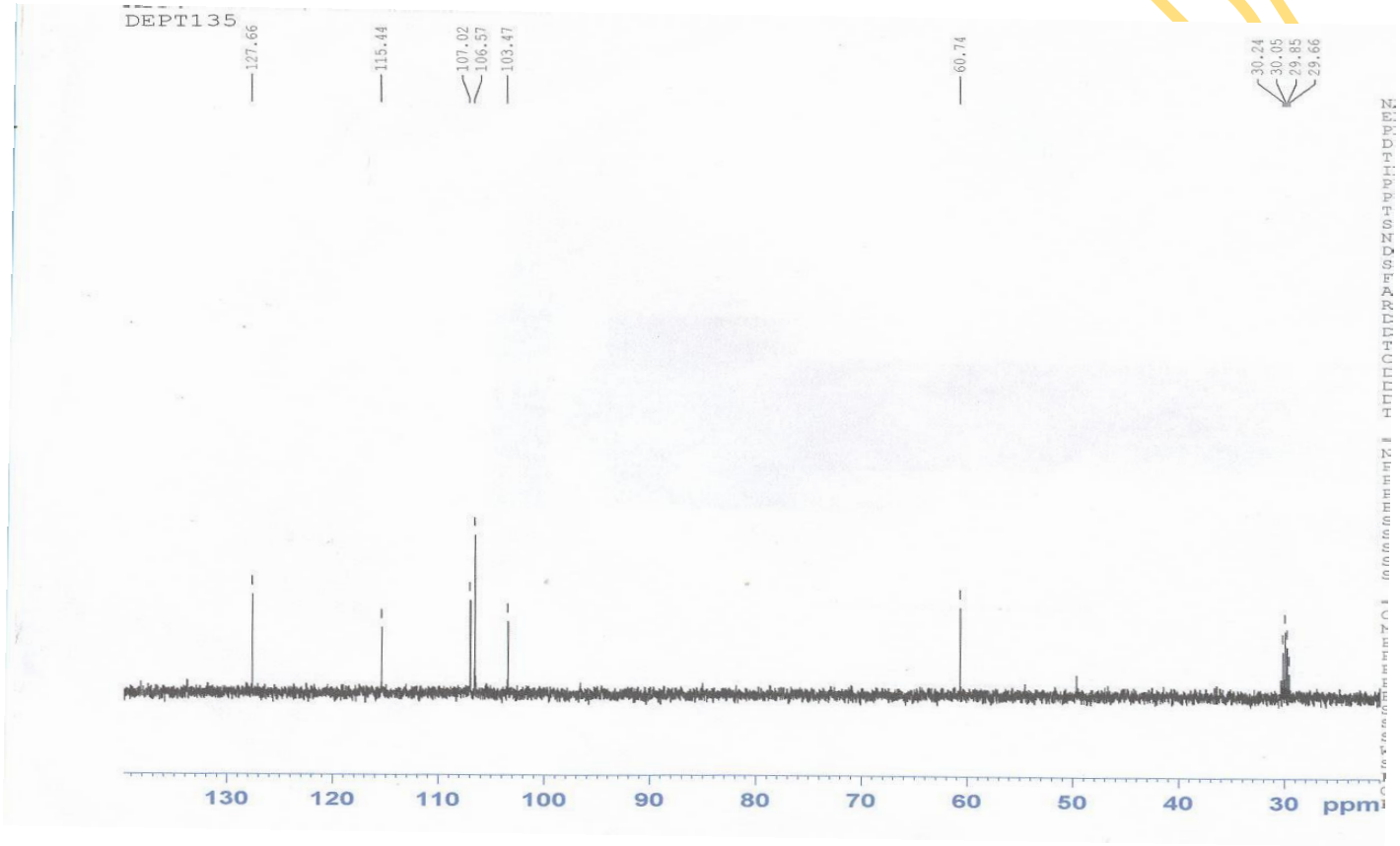


Fig. 4.29. DEPT 90 spectrum of DRR-1G


CH3
O
OH


DEPT135
127.66
115.44
107.02
106.57
103.47
60.74
30.24
30.05
29.85
29.66
130
120
110
100
90
80
70
60
50
40
30
ppm

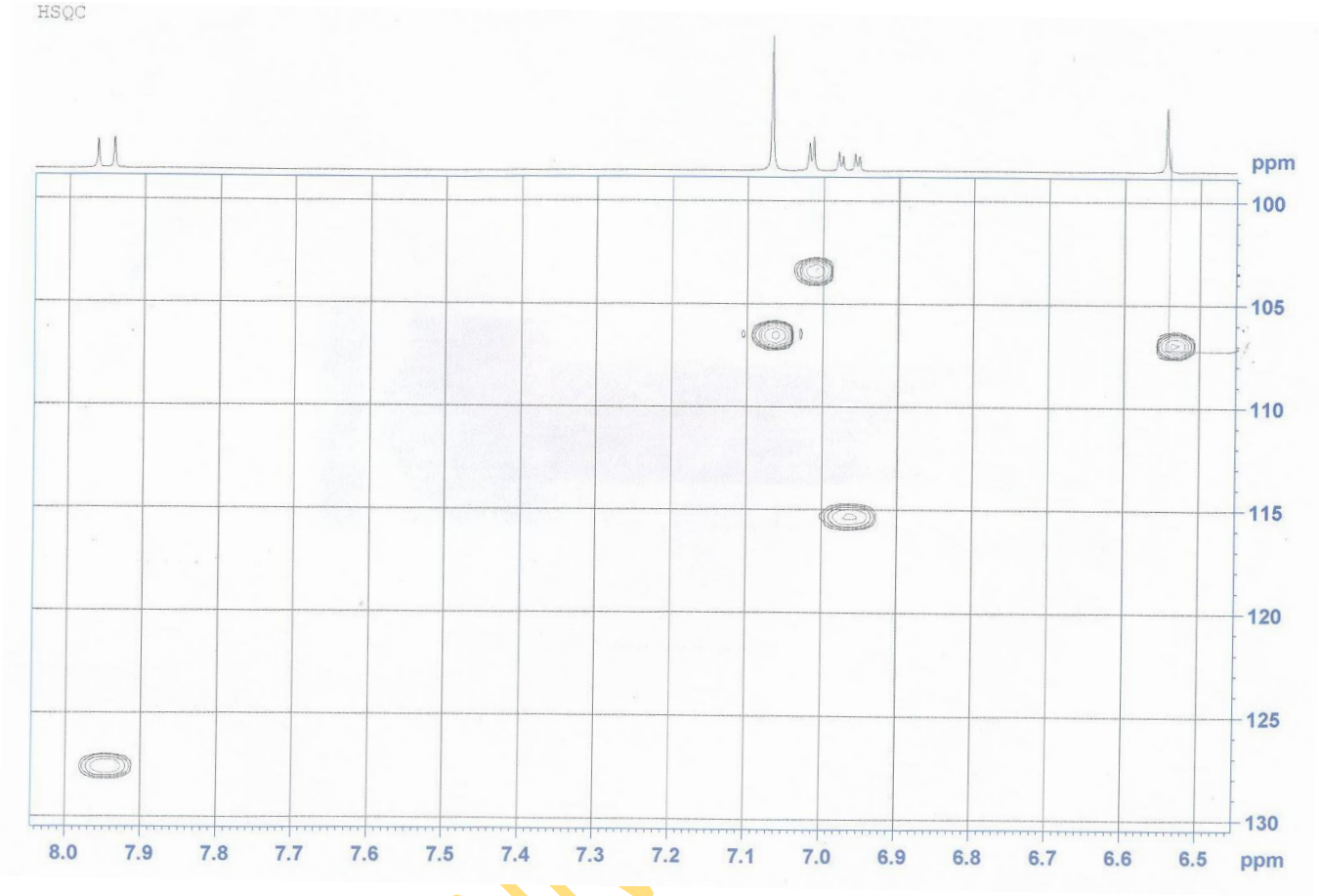


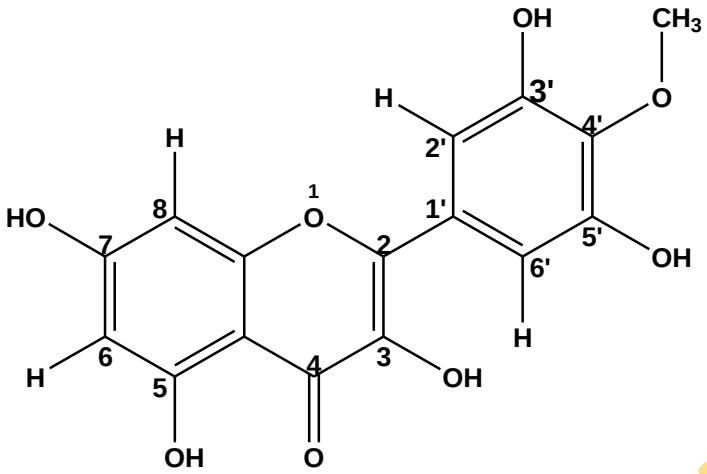
Fig. 4.32. HSQC spectrum of DRR-1G

4.2.5 Isolation and Characterisation of DRR-2G

Thirty-nine fractions were collected when fraction G-6 (262 mg) was chromatographed on silica gel column using gradient elution with hexane: EtOAc (Table 4.11). The fractions were pooled to six sub-fractions using TLC analysis (GA1-6). Rechromatography of GA-5 (50 mg) on a sephadex LH-20 column using isocratic system with 100 % MeOH yielded DRR-2G (5 mg) a yellow powder

The molecular formula of DRR-2G ($C_{16}H_{12}O_8$), with molecular ion peak at *m/z* 332 was which deduced from HR-EIMS and supported by its NMR spectra data (Table 4.12). The UV spectrum in methanol showed maximum absorptions at 365, 260 and 212 nm. The ^{1}H-NMR spectrum (Fig. 4.35) showed two meta coupled 1H doublets at 6.26 (J = 2 Hz) and 6.52 (J = 2 Hz) assigned to H-6 and H-8 respectively corresponding to δc 99.18 ppm and δc 94.44 ppm respectively in the HSQC spectrum, characterised the H-6 and H-8 of a flavonoid 5,7-dihydoxyl A-ring (Gluchoff-Fiasson *et al*., 2001).

The ^{13}C-NMR spectrum (Fig. 4.36) revealed all the sixteen carbons which were deduced as one methoxyl, four methines and eleven quartenary carbons. The broad singlet ^{1}H peaks at δ_H 8.13, 9.71 and 8.36 ppm were not directly attached to any carbon in HSQC, they were assigned 3-OH, 7-OH and 3′/5′-OH respectively. In the ^{1}H-NMR spectrum, 3H singlet at δ_H 3.88 revealing a methoxyl group correlated with the carbon resonance at 138.02(C-4′) on the HMBC spectrum (Fig. 4.33). The 2H singlets at δ_H 7.38 was attributed to the one proton each at 2′ and 6′ positions of 3′,4′,5′ tri-O-substituted B ring. Long-range COSY spectrum in conjuction with the HMBC spectrum allowed the assignments of H-6 and H-8 (Fig. 4.34-4.35). A singlet at δ_H 12.10 corresponding to ^{13}C peak at δc 162.3 ppm in HSQC, implied the presence of an OH at C-5, since H-bonding with the C=O (C-4) causes this type of substituted flavonoid to give signal between 159.8 and 162.3, also the signal for C-5 unsubstituted flavonoids are usually found between 106.9 and 127.75 (Park *et al*., 2006). Thus compound DRR-2G was identified as 3,5,7,3′,5′-pentahydroxy-4-methoxyflavone (mearnsetin) **4.5** by comparison of it spectra data with the literature (Abbas *et al*., 2007, Gluchoff-Fiasson *et al*., 2001).



4.5

Table 4.11. Column chromatography of sub-fraction G-6

Column eluents	Pooled fraction	Pool code
HEX : EtOAc (95:5)	1-5	GA1
HEX : EtOAc (9:1)	6-9	GA2
HEX : EtOAc (85:15)	10-20	GA3
HEX : EtOAc (85:15)	21-30	GA4
HEX : EtOAc (7:3)	31-34	GA5
HEX : EtOAc (65:35)	31-39	GA6

Table 4.12. ^{1}H and ^{13}C NMR Spectra data of DRR-2G (400 and 100 MHz, Acetone)

Position	^{1}H δ (integration, multiplicity, J Hz)	^{13}C δ	DEPT	HMBC
2	-	146.2	C	
3	-	137.5	C	
3-OH	8.13 (s)			
4	-	176.7	C	
5		162.3	C	C-7,C-10
5-OH	12.10 (1H, s, OH)			
6	6.26 (1H, d, J= 2)	99.2	CH	C-5,C-7
7	-	165.1	C	
7-OH	9.71			
8	6.52 (1H, d, J= 2)	94.4	CH	C-6, C-7,C-9,C-10
9	-	157.8	C	
10	-	104.2	C	
1′	-	127.3	C	
2′/6′	7.38 (2H, s)	108.3	CH	C-2, C-3, C-2′, C-4′
3′/5′	-	151.3	C	
3′/5′-OH	8.36 (s)			
4′	-	138.0	C	
OCH_3	3.88 (3H, s)	60.7	CH_3	OCH_3-C4′

Figure 4.33. HMBC connectivities of DRR-2G.

Figure 4.34. Long-range COSY connectivity of DRR-2G

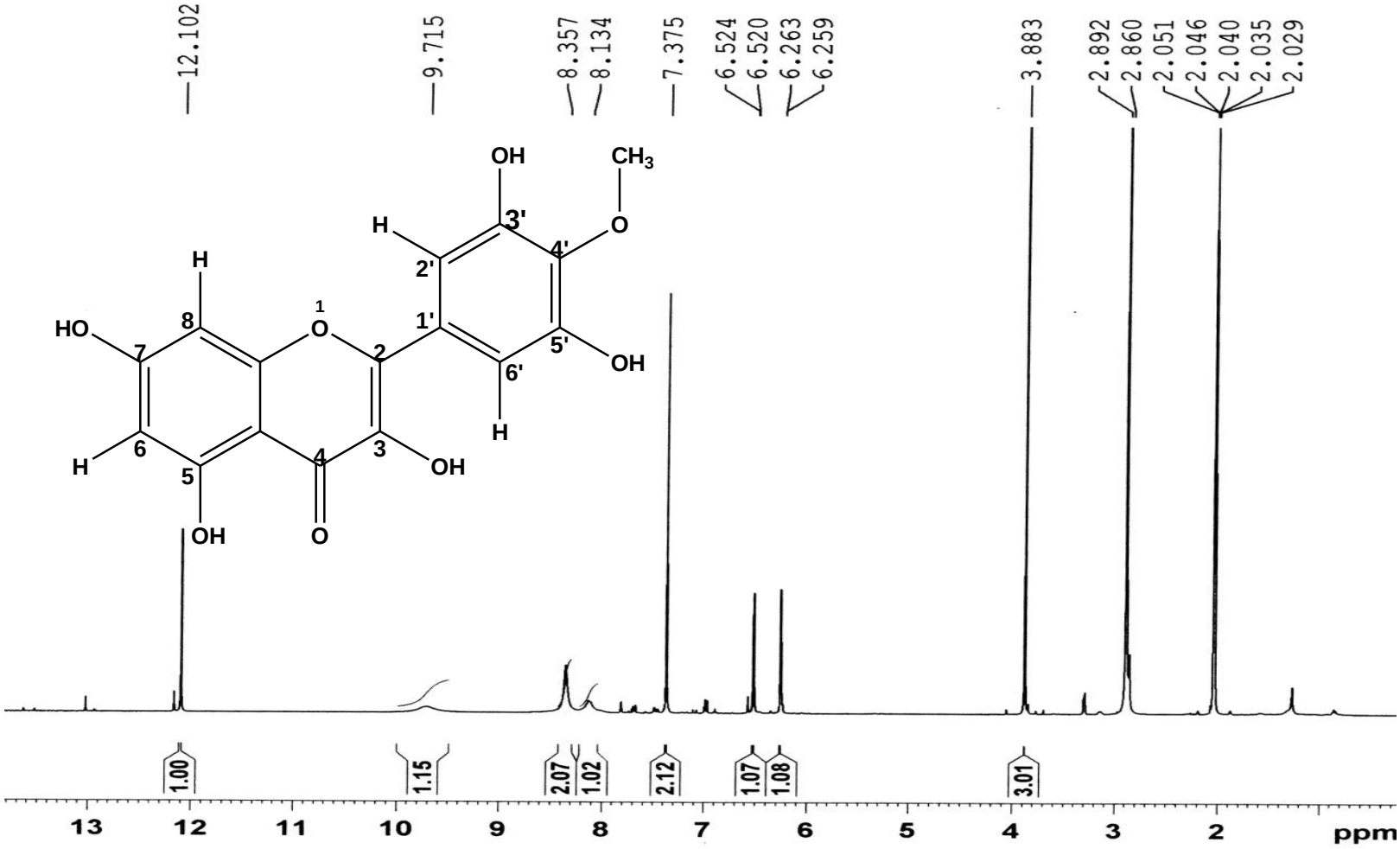


Fig. 4.35. ^{1}H-NMR spectrum of DRR-2G (CD_3COCD_3, 400 MHz)

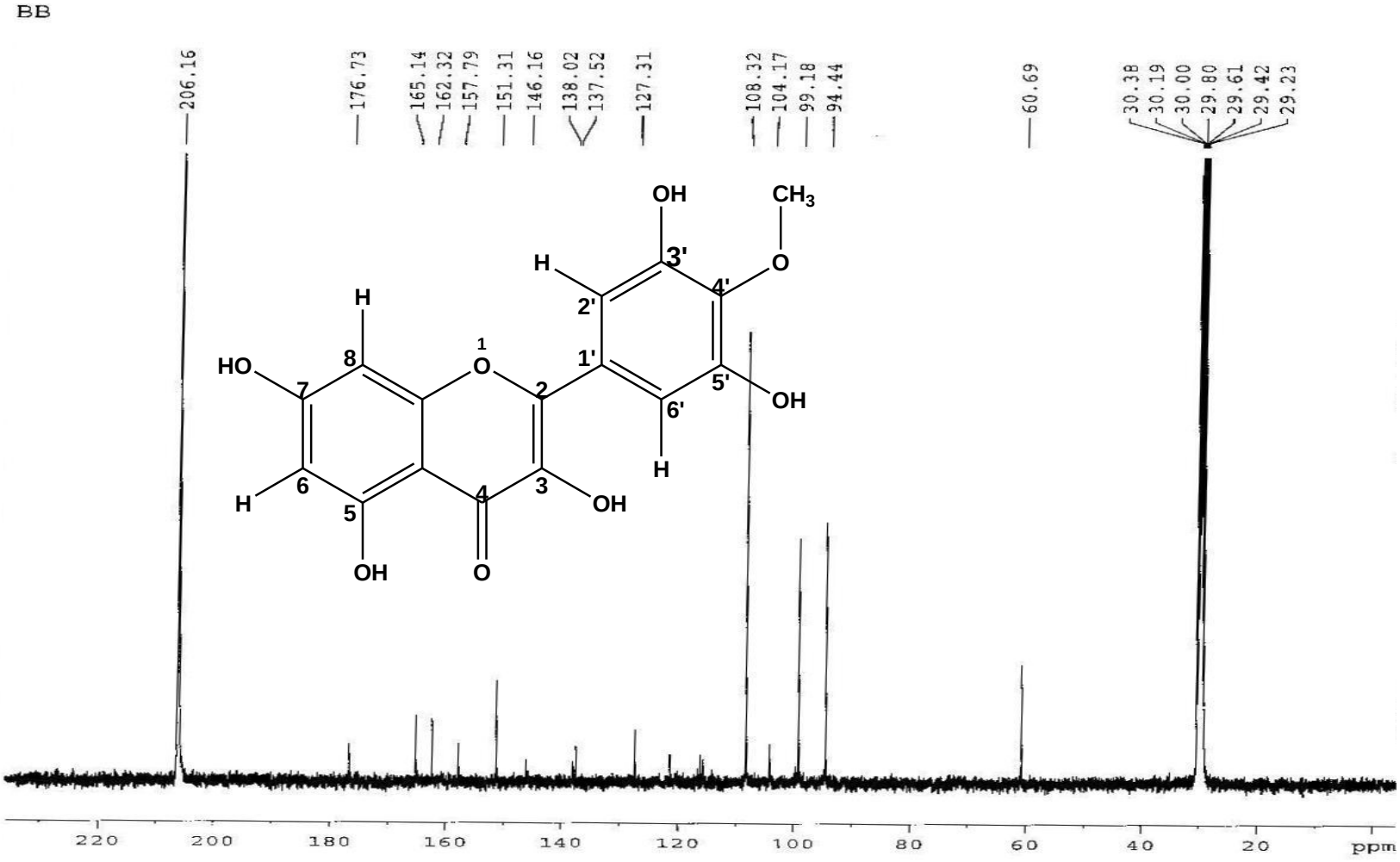


Fig. 4.36. ^{13}C-NMR spectrum of DRR-2G (CD_3COCD_3, 100 MHz)

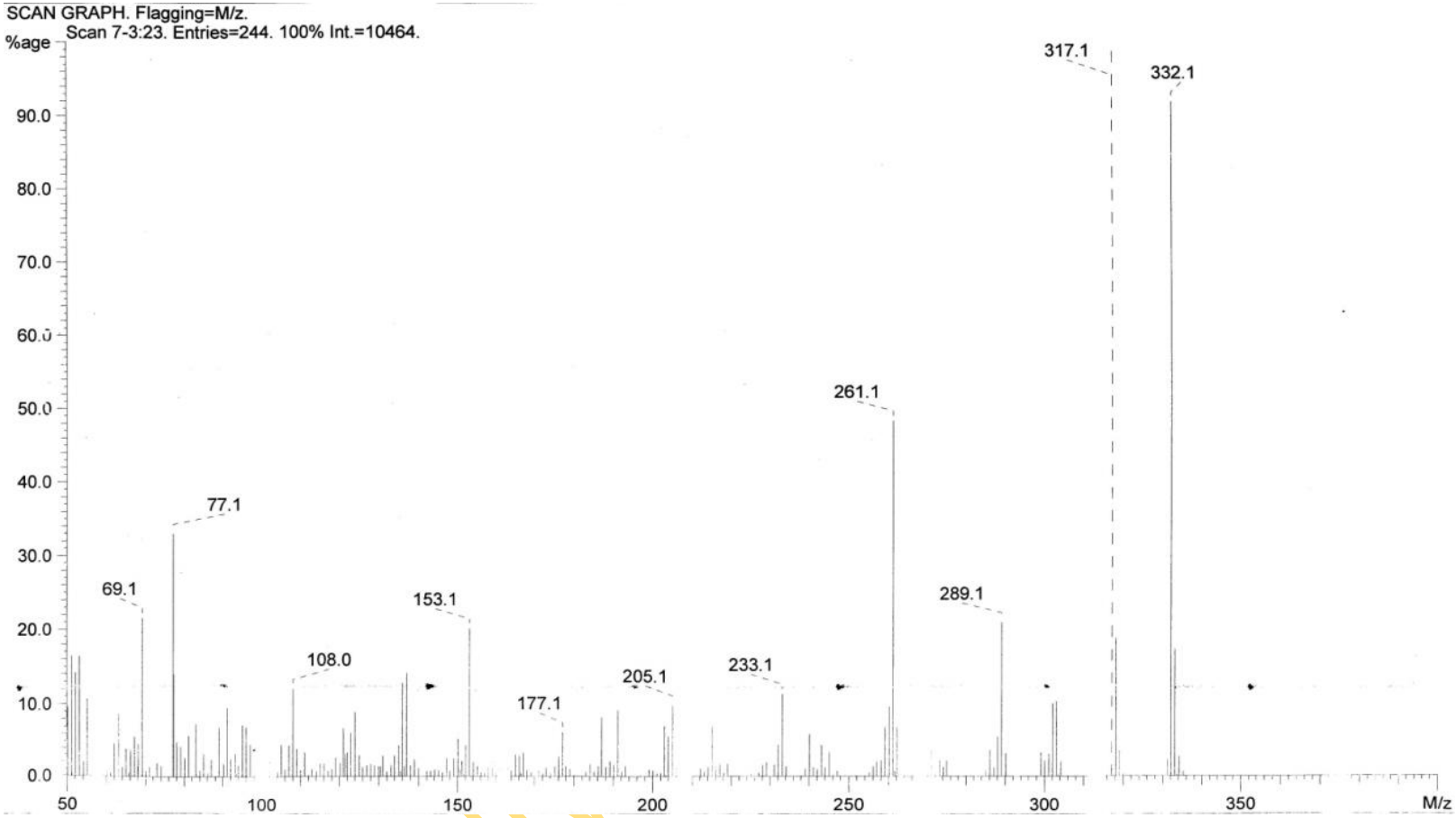


Fig. 4.37. EIMS spectrum of DRR-2G

4.2.6 Isolation and Characterisation of DRR-3G

Sub-fraction G-8 (176 mg) was further purified on a silica gel column and eluted with gradient of hexane: EtOAc. Fifty-one fractions were collected and pooled to 9 sub-fractions (GB 1-9) (Table 4.13). The GB-2 (2 mg) and GB-9 (5 mg) from the elution of 10% and 40% ethyl acetate in hexane which yellow powders coded DRR-3G (2 mg, n-hexane/EtOAc, R_f 60:40) and DRR-4G (5 mg, n-hexane/EtOAc, R_f 40:60) respectively.

The UV spectrum in MeOH showed maximum absorptions at 255 nm and 213 nm, characteristics of an aromatic acid. The molecular formula was deduced as $C_8H_8O_5$ from its molecular ion at m/z 184 in the mass spectrum, HR-EIMS and NMR spectra data. The ^{1}H-NMR spectrum (Fig. 4.39) of DRR-3G showed signals characteristic of phenolic compound. The only signals appearing in ^{1}H-NMR were a 3H singlet for the methoxyl (δ 3.84) and 2H singlet for two aromatic protons (δ 7.02).

The ^{13}C-NMR of DRR-3G showed one methoxyl, two olefinic methines and five quartenary carbons. The most down field shifted peak at δc 170.43 was assigned to the carbonyl of the acid. Two peaks showed double intensity and were assigned C 3/5 and C 2/6 respectively. Further analysis of the NMR data (Table 4.14), revealed a symmetric trisubstituted phenolic acid containing two hydroxyls and a methoxyl group. The positions of these groups were established in the structure using HSQC and HMBC experiments. In HMBC (Fig. 4.38), the ^{1}H of methoxyl (δ 3.84) showed connectivity with carbon at C-4 (δc 140.77) and not carbonyl of the acid (δc 170.43), this ruled out the possibility of methyl gallate, which was also evident in the mass spectrum (Fig. 4.41) at m/z 139 [M^+ - COOH]. Comparing the spectra of compound DRR-3G with the existing literature, it was concluded to be 3,5-dihydroxyl-4-methoxybenzoic acid **4.6** also known as 4-O-methyl gallic acid (4-OMGA) (Jeon *et al*., 2005).

4.6

Figure 4.38. HMBC connectivities of DRR-3G

Table 4.13. Column chromatography of sub-fraction G-8

No.	Column eluents	Pooled fraction	Pool code
	HEX : EtOAc (95:5)	1-4	GB1
	HEX : EtOAc (9:1)	5	GB2(DRR-3G)
	HEX : EtOAc (9:1)	6-9	GB3
	HEX : EtOAc (85:15)	10-20	GB4
	HEX : EtOAc (85:15)	21-30	GB5
	HEX : EtOAc (4:1)	31-34	GB6
	HEX : EtOAc (3:1)	35-39	GB8
	HEX : EtOAc (3:2)	40-51	GB9 (DRR-4G)

Table 4.14. ^{1}H and ^{13}C NMR spectra data of DRR-3G (400 and 100 MHz, CD_3OD)

Position	^{1}H δ (ppm) (integration, multiplicity)	^{13}C	DEPT	HMBC
1	-	127.9	C	
2	7.02 (2H, s)	110.3	CH	C-1,C-3,C6, C-7
3	-	151.6	C	
4	-	140.8	C	
5	-	151.6	C	
6	-	110.3	CH	
7	-	170.4	C	
OCH_3	3.84 (3H,s)	60.7	CH_3	C-4

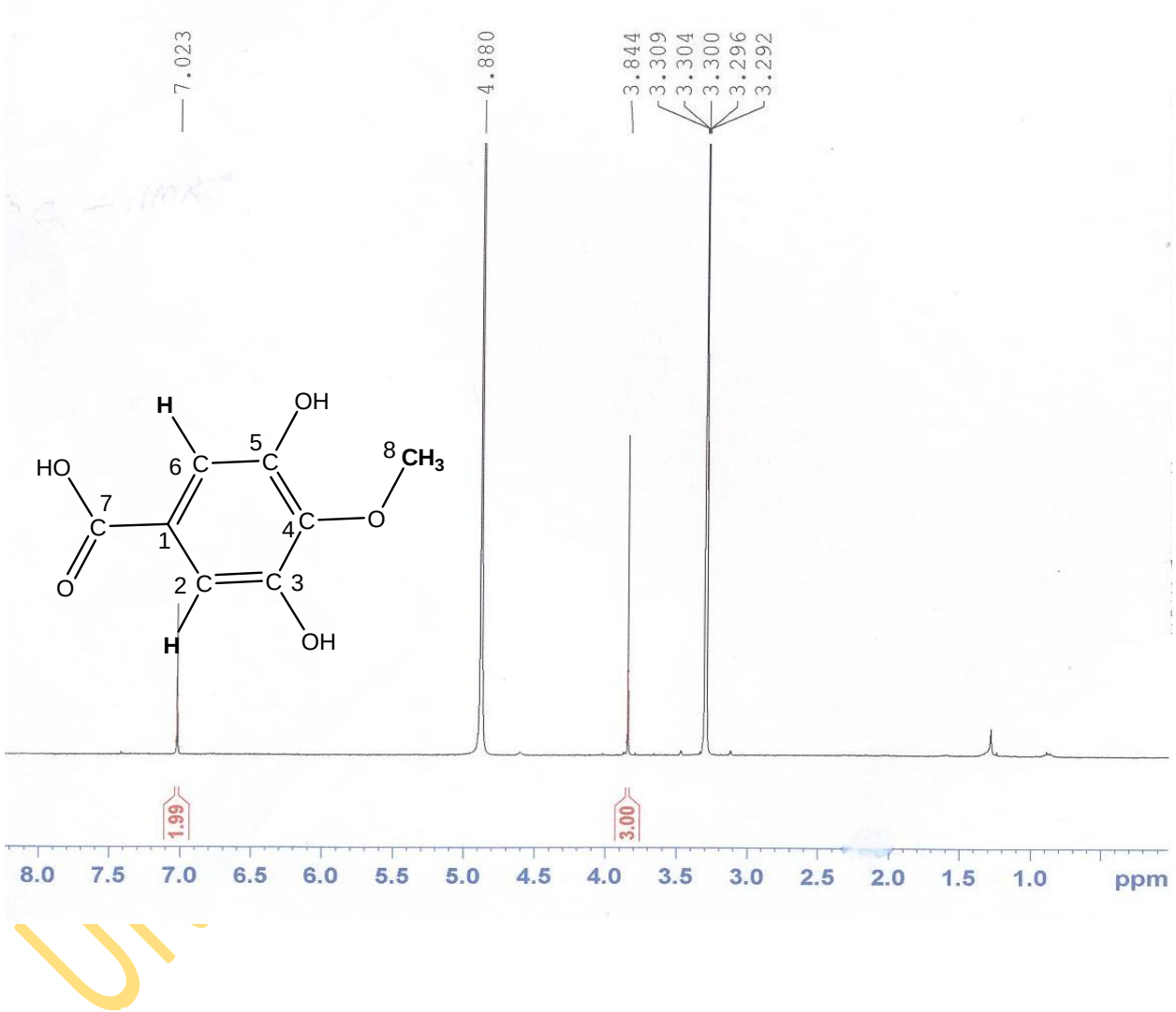


Fig. 4.39. ^{1}H-NMR spectrum of DRR-3G (CD_3OD, 400 MHz)

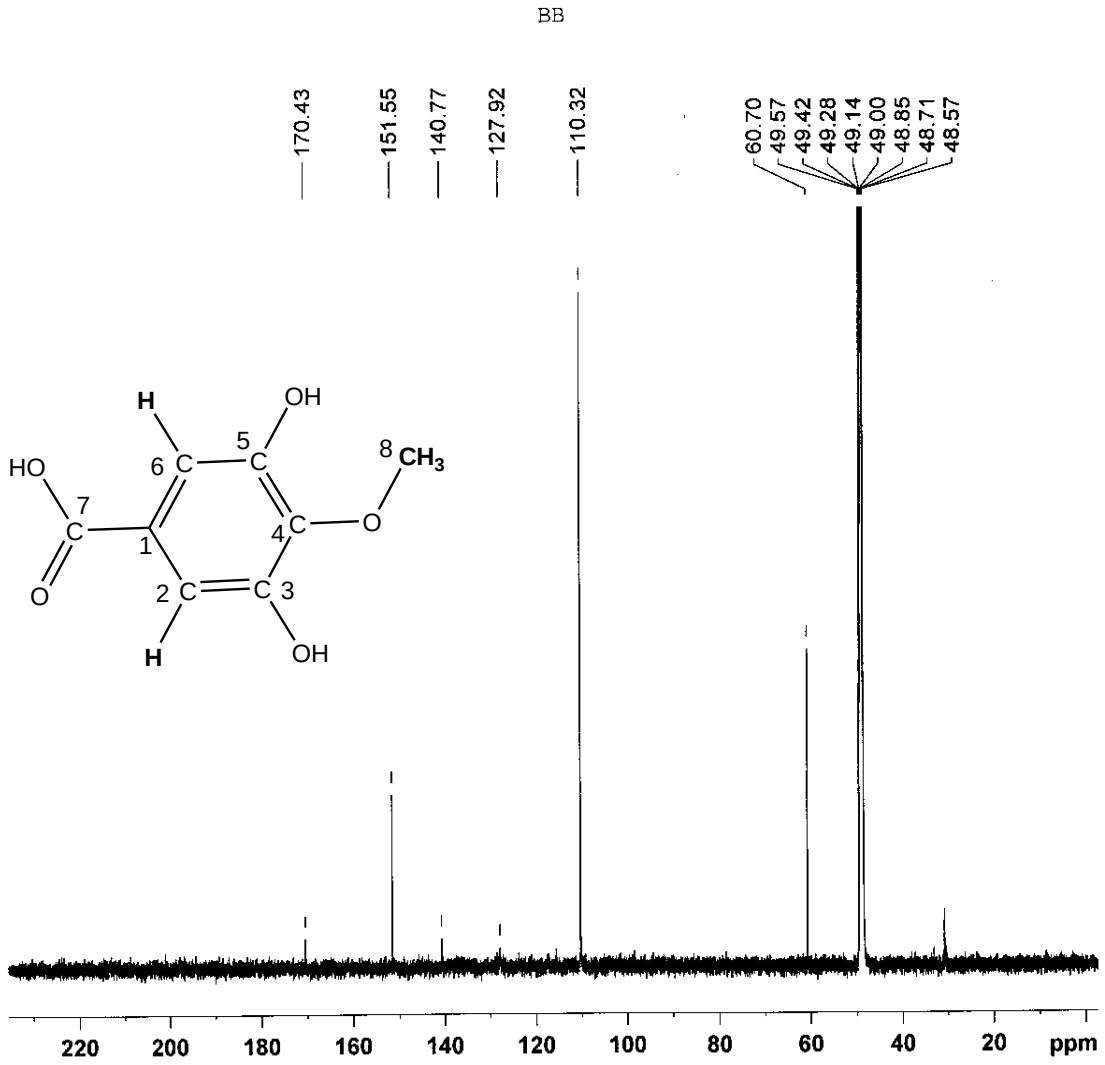


Fig. 4.40. ^{13}C-NMR spectrum of DRR-3G (CD_3OD, 100 MHz)

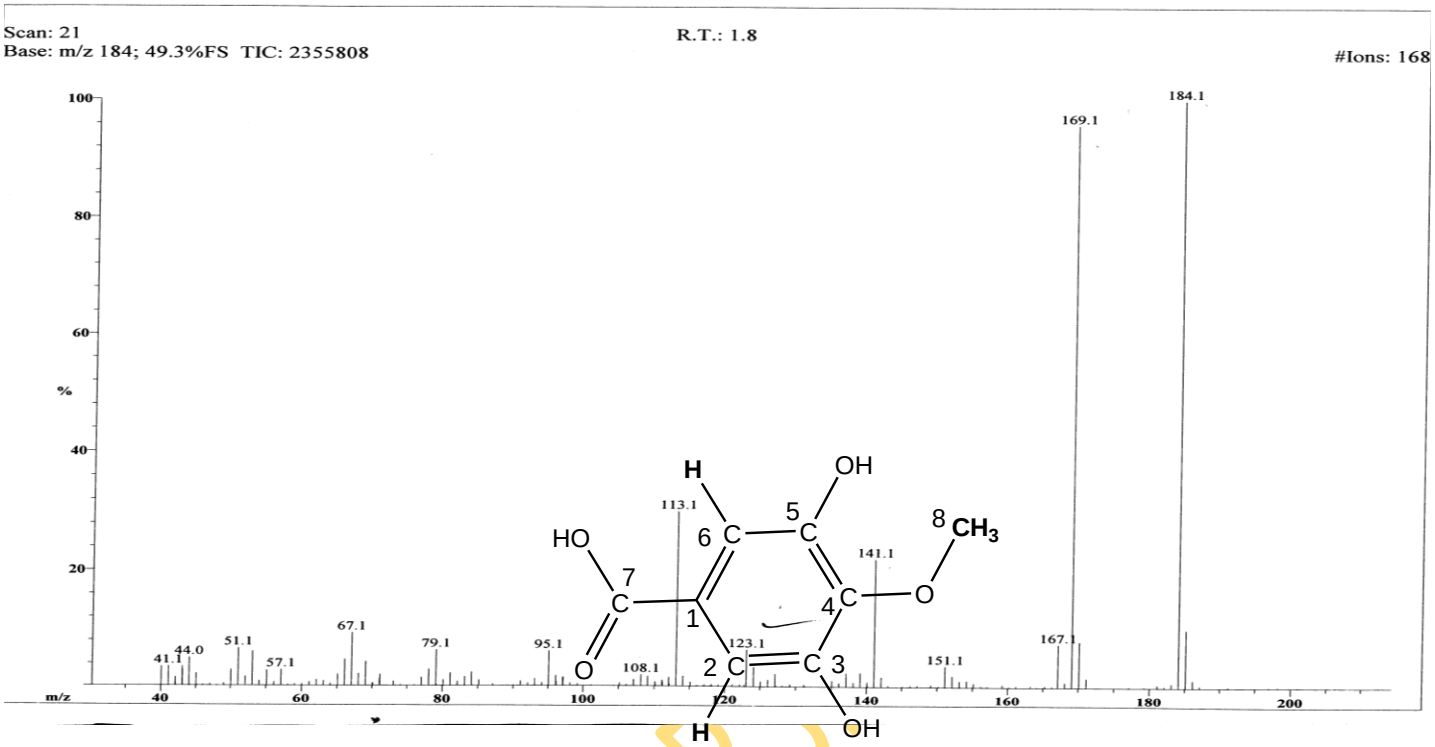
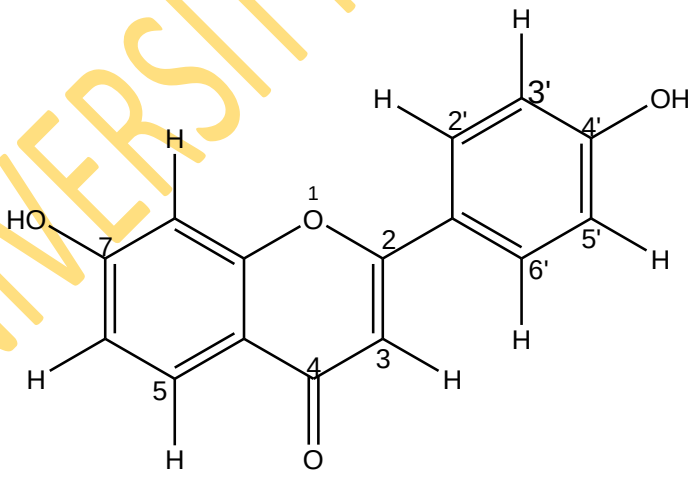


Fig. 4.41. EIMS spectrum of DRR-3G

4.2.7 Characterisation of DRR-4G

The molecular formular DRR-4G ($C_{15}H_{10}O_4$) was deduced from the High Resolution Electron Impact Mass spectrum (HR-EIMS). The Retro-Diels-Alder (RDA) fragmentation of the heterocyclic ring produced ions at *m/z* 137$[A+1]^+$ and *m/z* 118 $[B]^+$ (Scheme 4.3) which indicated that A-ring and B-ring possessed OH substituent each. Other significant peaks in addition to the molecular ion at *m/z* 254 are 237 $[M-OH]^+$, 226 [$C_{14}H_{10}O_3$], 137 [$C_7H_5O_3$] and 108 [$C_6H_4O_2$].

The ^{1}H-NMR spectrum displayed two 1H doublets at δ 6.97 and δ 7.96 assigned to H-8 and H-5 respectively. A 3H multiplet at δ 6.91 was assigned to H-6 and two equivalent aromatic protons at H-3′/5′. The ^{13}C NMR, DEPT and HMBC experiments showed signals for 15 carbons which were deduced as: seven quaternary and eight methine carbons. The various connectivities in the molecule were determined through HMBC and COSY experiments, which concluded the position of OH at C-7 and C-4′ (Fig. 4.42). The comparison of the corresponding UV, EIMS, ^{1}H and ^{13}C NMR data (Table 4.15) with those reported in literature (Bickoff *et al*., 1964; Park *et al*., 2007; Maxwell *et al*., 1989; Meskuriyen and Cordell, 1988.) led to its identification as 7, 4′-dihydroxyflavone **4.7**.



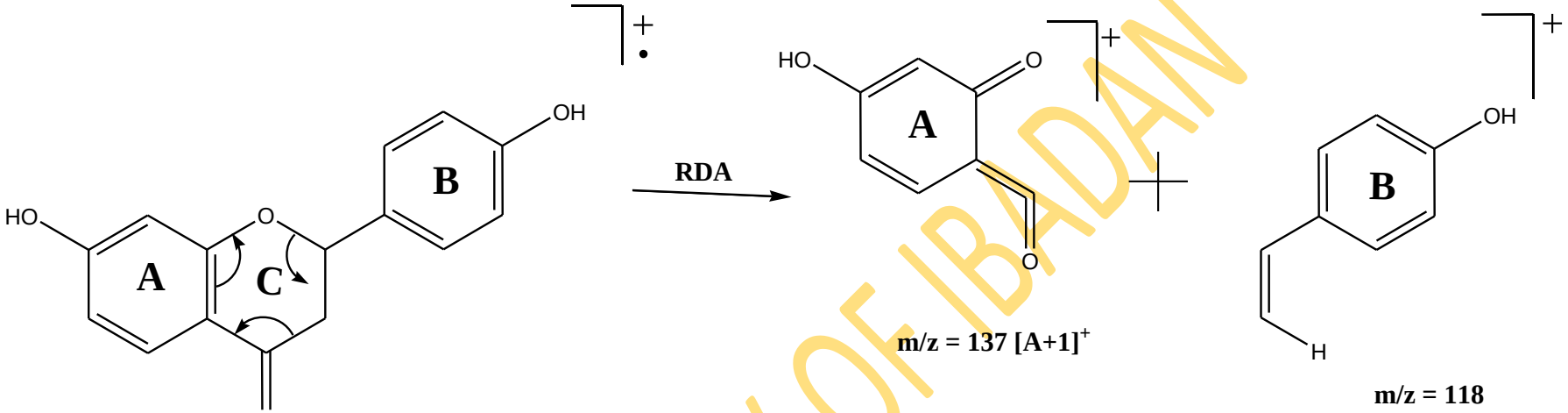
4.7

Table 4.15. ^{1}H and ^{13}C NMR Spectra data of DRR-4G (400 and 100 MHz MeOD)

Position	^{1}H δ (integration, multiplicity, J Hz)	^{13}C δ	DEPT	HMBC
2	-	166.0	C	H-3, H-6′
3	6.68 (1H, s)	105.1	CH	
4	-	180.3	C	H-3, H-5
5	7.96 (1H, d, J= 8.8)	127.8	C H	
6	6.91 (1H, dd, J= 2.0, 8.8)	116.3	CH	H-8
7	-	165.0	C	H-5, H-8
8	6.97 (1H, d, J= 2.0)	103.5	CH	
9	-	159.5	C	H-5, H-8
10	-	114.3	C	
1′	-	123.5	C	H-1′, H-3′
2′ / 6′	7.86 (2H, dt, J= 2.8, 9.2)	129.4	CH	
3′ / 5′	6.91 (2H, m)	117.0	CH	
4′	-	162.5	C	H-5′, H-6′

Figure 4.42. HMBC connectivities of DRR-4G.

Keys: COSY (▬) and HMBC (→)



Scheme 4.3. Fragmentation pattern of DRR-4G

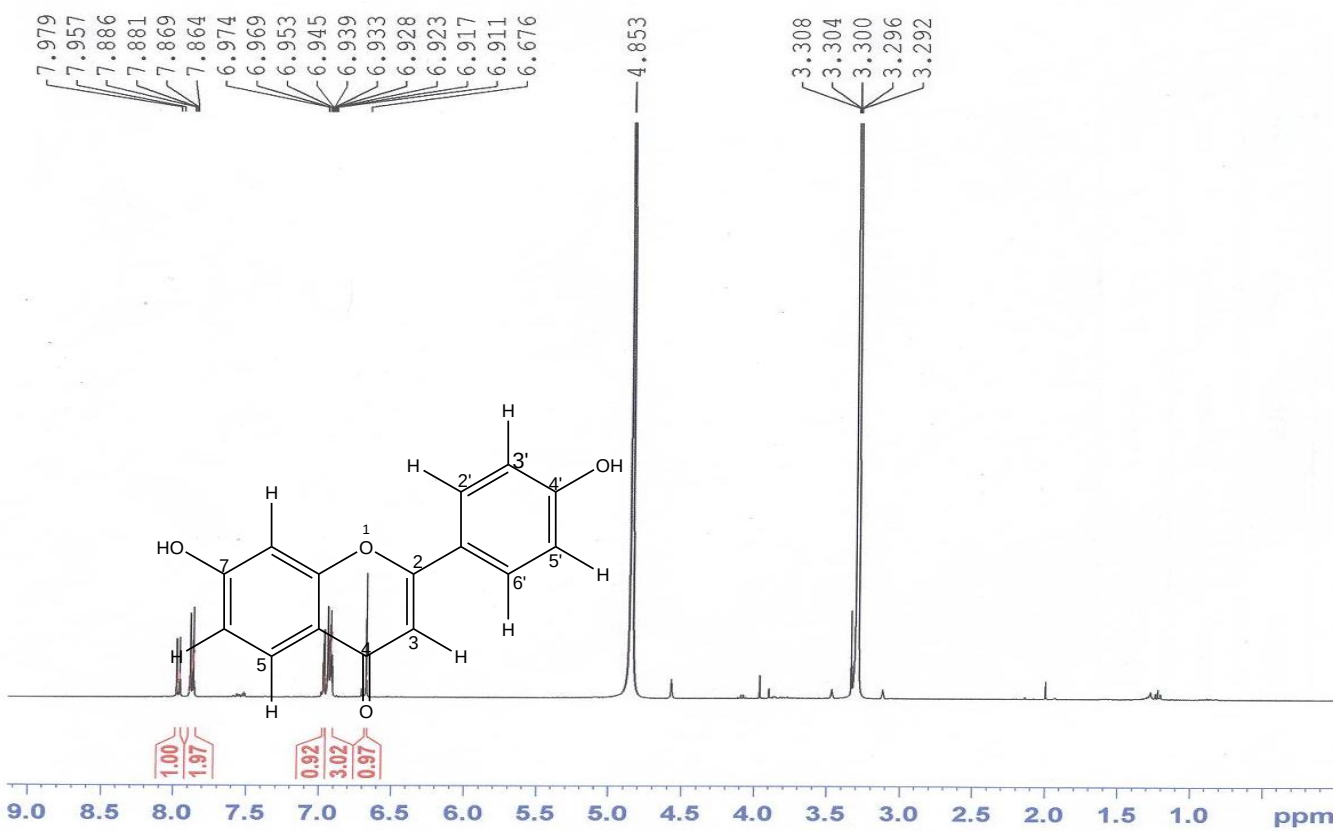


Fig. 4.43. ^{1}H-NMR spectrum of DRR-4G (CD_3OD, 400 MHz)

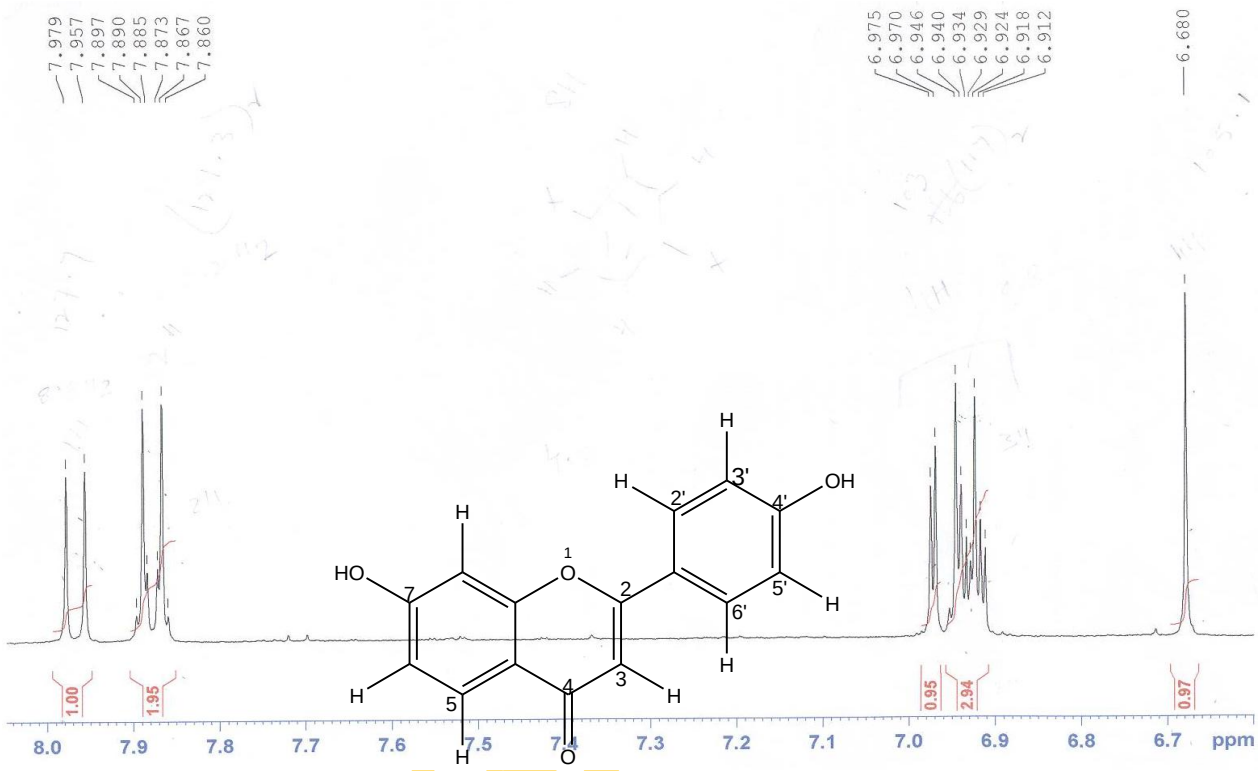


Fig. 4.43a. Expanded ^{1}H-NMR spectrum of DRR-4G (CD_3OD, 400 MHz)

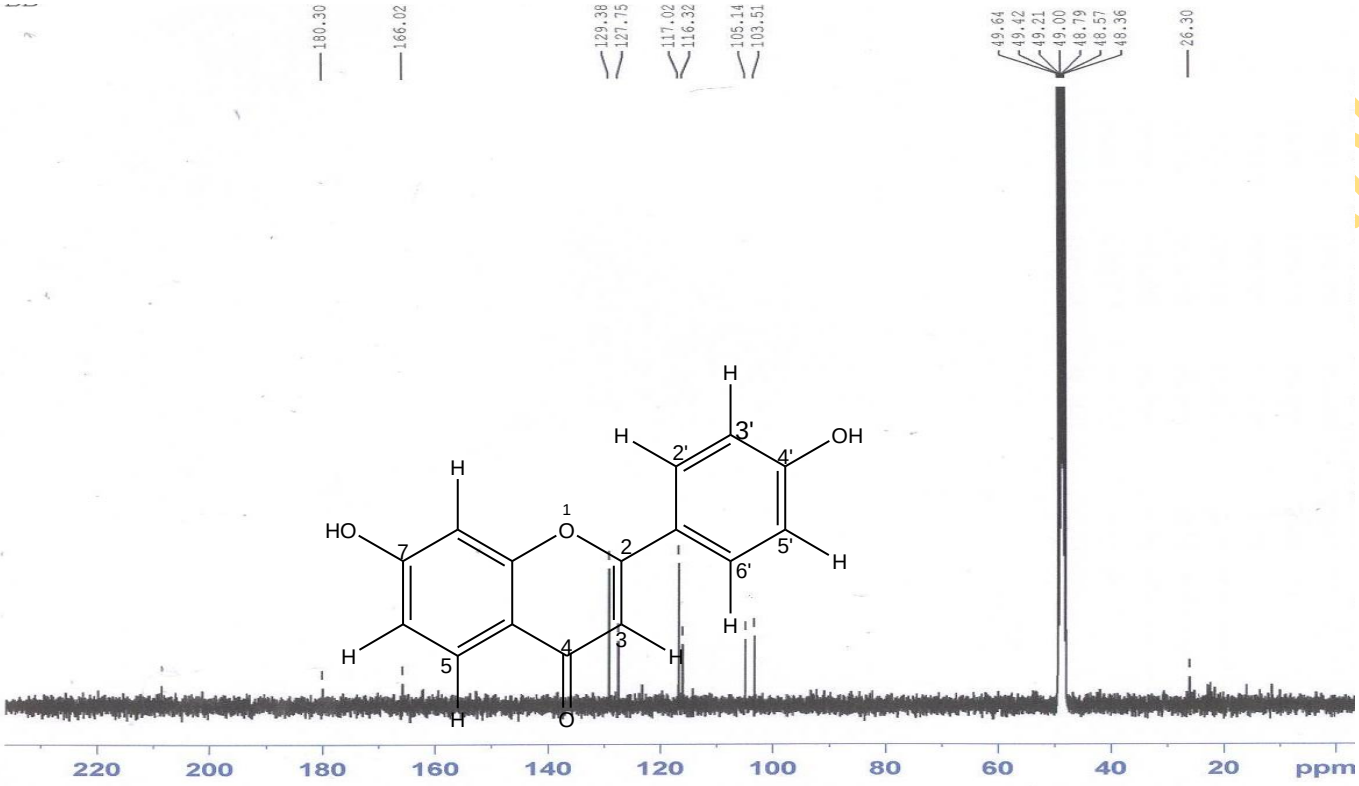


Fig. 4.44. ^{13}C-NMR spectrum of DRR-4G (CD_3OD, 100 MHz)

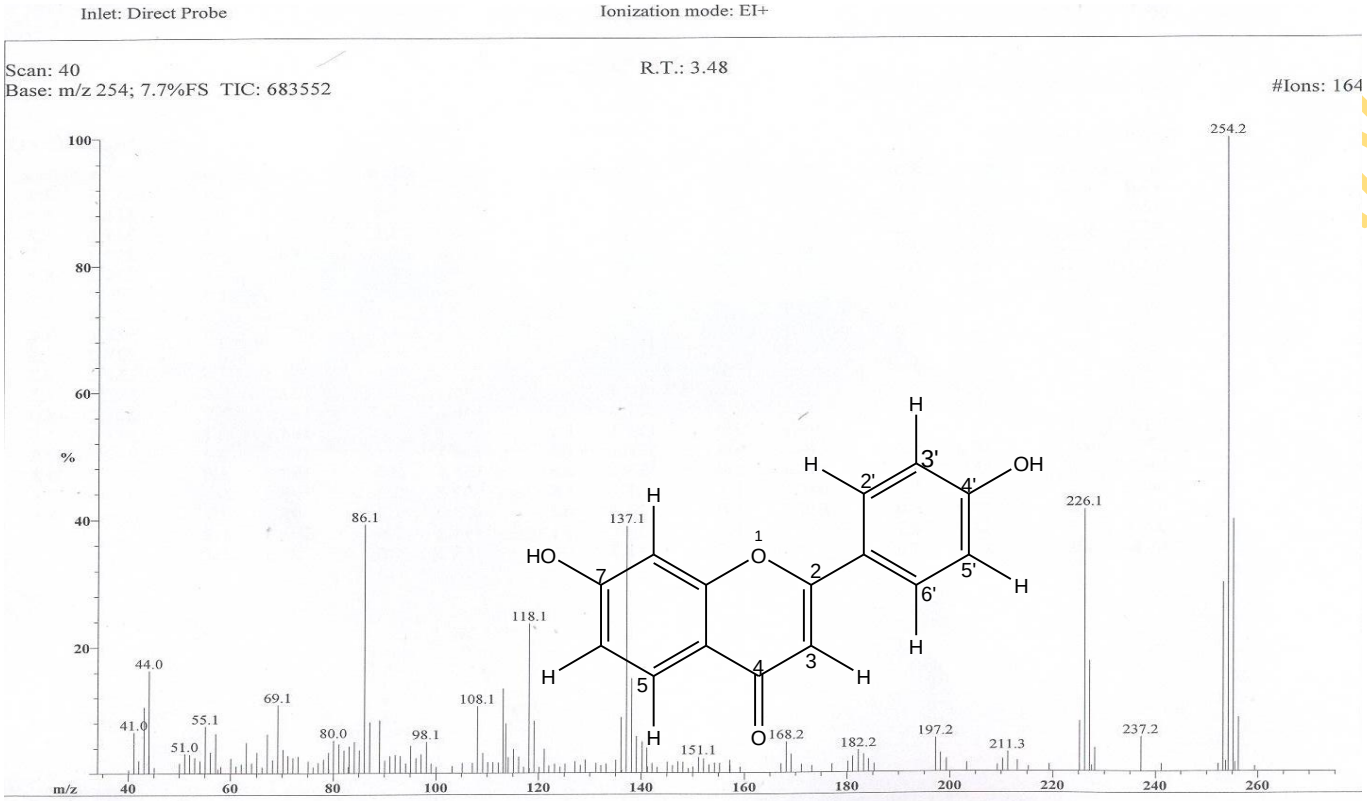


Fig. 4.45. EIMS spectrum of DRR-4G

4.2.8 Volatile oil characterisation of *Dioclea reflexa* root

The essential oil yield based on the dry weight of the sample was 0.56 %. From the volatile oils obtained by hydrodistillation, a total of 15 compounds were identified representing 89.17% with analysis of GC and GC-MS data (Table 4.16). The volatile oil constituents were dominated by hydrocarbons (69.27%); such as heptacosane (10.40%), octacosane (13.42%), triacontane (8.99 %), hexacosane (8.41 %), pentacosane (6.44 %), nonacosane (4.84 %), tetracosane (3.61 %) and heptadecane (2.09 %). These oils also contained a number of oxygenated monoterpenoids including trans-pinocarveol (2.86 %), terpinen-4-ol (2.47 %), myrtenal (1.69 %), thymol (1.30 %) and verbenome (1.25 %). Other constituents found in high quantities are stearyl iodide (10.33 %) and 3 unidentified compounds (5.75 %).

Monoterpenoids are known to exhibit a diverse array of pharmaceutical and therapeutic properties (Joshua, *et al*., 2010). Some of the constituents identified here (e.g. terpinen-4-ol, thymol) have been shown by various researchers to be an effective antimicrobial, especially antifungal and antiviral (Joshua, *et al*., 2010; Braga, *et al*., 2007 and Francesca, *et al*., 2006). Research carried out by Dorothea *et al*., (2011) also proved that myrtenal and verbenome are effective in treatment of Alzheimer's disease. Terpinen-4-ol has been demonstrated to be a potential anticancer drug (Chieh-Shan, *et al*., 2012). It is also used in flavour and fragrance for citrus and spice types (Wagner, 1999).

Table 4.16. Percentage compositions of volatile constituents of *D. reflexa* root.

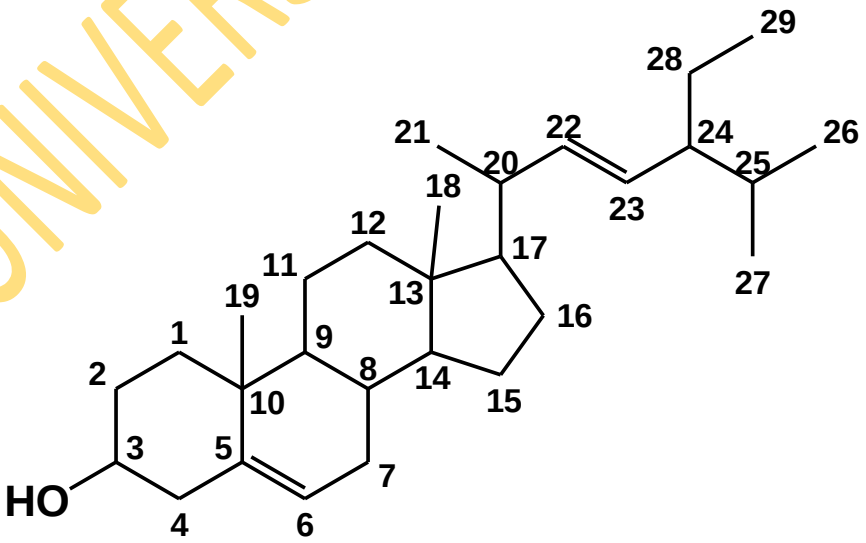
No	Components	Retention Time (min)	% composition
	Hydrocarbons		
1	Heptadecane	23.27	2.09
2	Tetracosane	24.27	3.61
3	Pentacosane	26.38	6.44
4	Hexacosane	27.85	8.41
5	Heptacosane	29.27	10.40
6	Octadecane	30.63	11.07
7	Triacontane	33.35	8.99
8	Octacosane	36.09	13.42
9	Nonacosane	39.73	4.84
	Oxygenated Monoterpenes		
10	Terpinen-4-ol	2.88	2.47
11	Myrtenal	2.95	1.69
12	Verbenome	3.05	1.25
13	Trans-pinocarveol	2.56	2.86
14	Thymol	32.68	1.30
	Others		
15	Stearyl Iodide	31.95	10.33
16	Unidentified compound	18.69	3.32
17	Unidentified compound	28.66	1.26
18	Unidentified compound	32.40	1.17
	Total		94.42

4.2.9 Isolation and Characterisation of CRB-1

The fraction F_{10} eluted with 15% ethyl acetate in hexane from the of crude methanol extract of *C. arborea* gave a white crystalline solid which was coded CRB-1 (8 mg) (Table 4.17; Scheme 3.2).

The EI mass spectrum of CRB-1 showed molecular ion peak as well as base peak at *m/z* 412 corresponding to molecular formula $C_{29}H_{48}O$ (calcd. 412.3707). In the ^{1}H-NMR (Fig. 4.46), six methyls appeared at δ 0.67 (H-18), 0.99 (H-19), 1.01 (H-21), 0.82 (H-26), 0.76 (H-27) and 0.78 (H-29). Three olefinic signals of one proton each were observed at δ 5.10 (dd, J=15.2, 8.4 Hz, H-22), δ 4.96 (dd, J=15.2, 8.8 Hz, H-23) and 5.32 (m, H-6) and their corresponding carbons resonated at δc 138.3, 129.3 and 121.7 respectively, which signified the presence of two double bonds in the compound. One proton signal at δ_H 3.46 ppm belongs to methine at H-3 (71.81 ppm) revealed that hydroxyl function was attached to it. This proton H-3 was coupled by methylene protons at H-2 (2.21 ppm), and this correlation between H-3 and H-2 was established by COSY analysis.

The ^{13}C-NMR (Fig. 4.47) revealed 29 signals, which were resolved using DEPT experiments into six methyl, nine methylene, eleven methine and three quaternary carbons. The spectral data (Table 4.18) were in good agreement with one reported for stigmasterol **4.8** (Forgo and Kövér, 2004).



4.8

Table 4.17. Column chromatography of crude methanol extract of *C. arborea* root

Column eluents	Pooled fraction(s)	Pool code
HEX : EtOAc (19:1) (9 L)	1-9	F_{1-9}
HEX : EtOAc (17:3) (1 L)	10	F_{10} (CRB-1)
HEX : EtOAc (85:15) (1 L)	11	F_{11}
HEX : EtOAc (3:1) (1 L)	12	F_{12} (CRB-1B)
HEX : EtOAc (3:1) (12 L)	13-24	F_{13-24}
HEX : EtOAc (1:1) (1 L)	25	F_{25} (CRB-2)
HEX : EtOAc (35:65) (26 L)	26-51	F_{26-51}
HEX : EtOAc (30:70) (29 L)	52-80	F_{52-80}

Table 4.18. The ^{1}H and ^{13}C-NMR Spectra data of CRB-1 (400 and 100 MHz, $CDCl_3$ respectively) compared with ^{13}C-NMR data of literature.

position	^{1}H δ (multiplicity, J Hz)	^{13}C δ	^{13}C* δ	DEPT
1.	1.84, 1.05	37.3	37.6	CH_2
2.	1.47	31.7	31.9	CH_2
3.	3.46 (m)	71.8	72.0	CH
4.	2.21 (m)	42.3	42.5	CH_2
5.	-	140.8	140.8	C
6.	5.32 (m)	121.7	121.8	CH
7.	1.97	31.7	32.1	CH_2
8.	1.80	32.0	32.2	CH
9.	0.90	50.2	50.5	CH
10.	-	36.5	36.5	C
11.	1.50	21.1	21.2	CH_2
12.	1.92, 1.13	39.7	40.0	CH_2
13.	-	42.2	42.2	C
14.	0.99	56.9	57.1	CH
15.	1.56, 1.06	24.4	24.5	CH_2
16.	1.67	28.9	28.9	CH_2
17.	1.33	56.0	56.3	CH
18.	0.67 (s)	12.1	12.2	CH_3
19.	0.99 (s)	19.4	19.5	CH_3
20.	1.98	40.5	40.4	CH
21.	1.01	21.1	21.4	CH_3
22.	5.10 (dd, 15.2, 8.4 Hz)	138.3	138.3	CH
23.	4.96 (dd, 15.2, 8.8 Hz)	129.3	129.7	CH
24.	1.51	51.2	51.5	CH
25.	1.55	31.9	32.2	CH
26.	0.82	21.2	21.2	CH_3
27.	0.76	19.0	19.2	CH_3
28.	1.41, 1.13	25.4	25.4	CH_2
29.	0.78	12.2	12.2	CH_3

*(Forgo and Kövér, 2004),

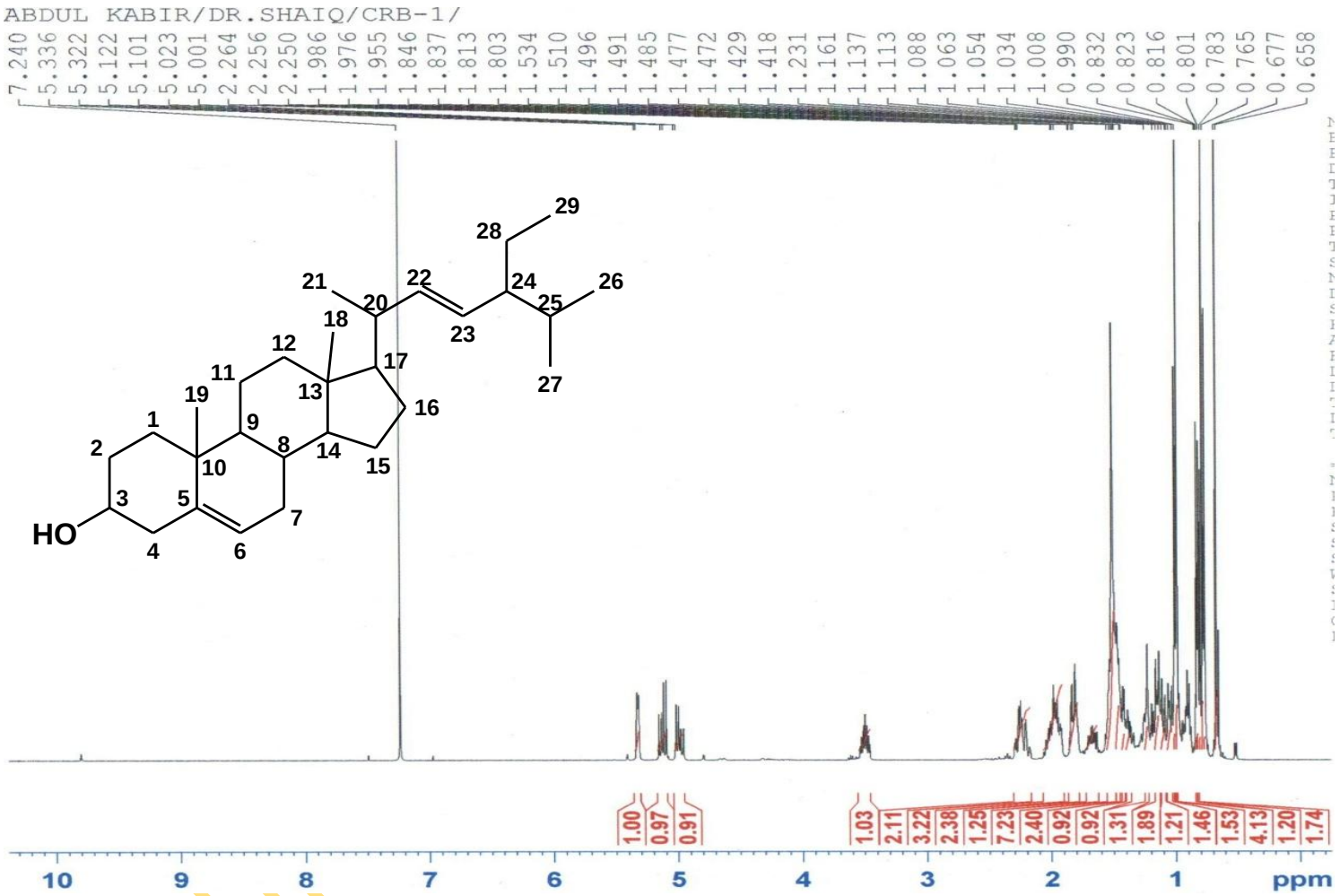


Fig. 4.46. ^{1}H-NMR spectrum of CRB-1 ($CDCl_3$, 400 MHz)

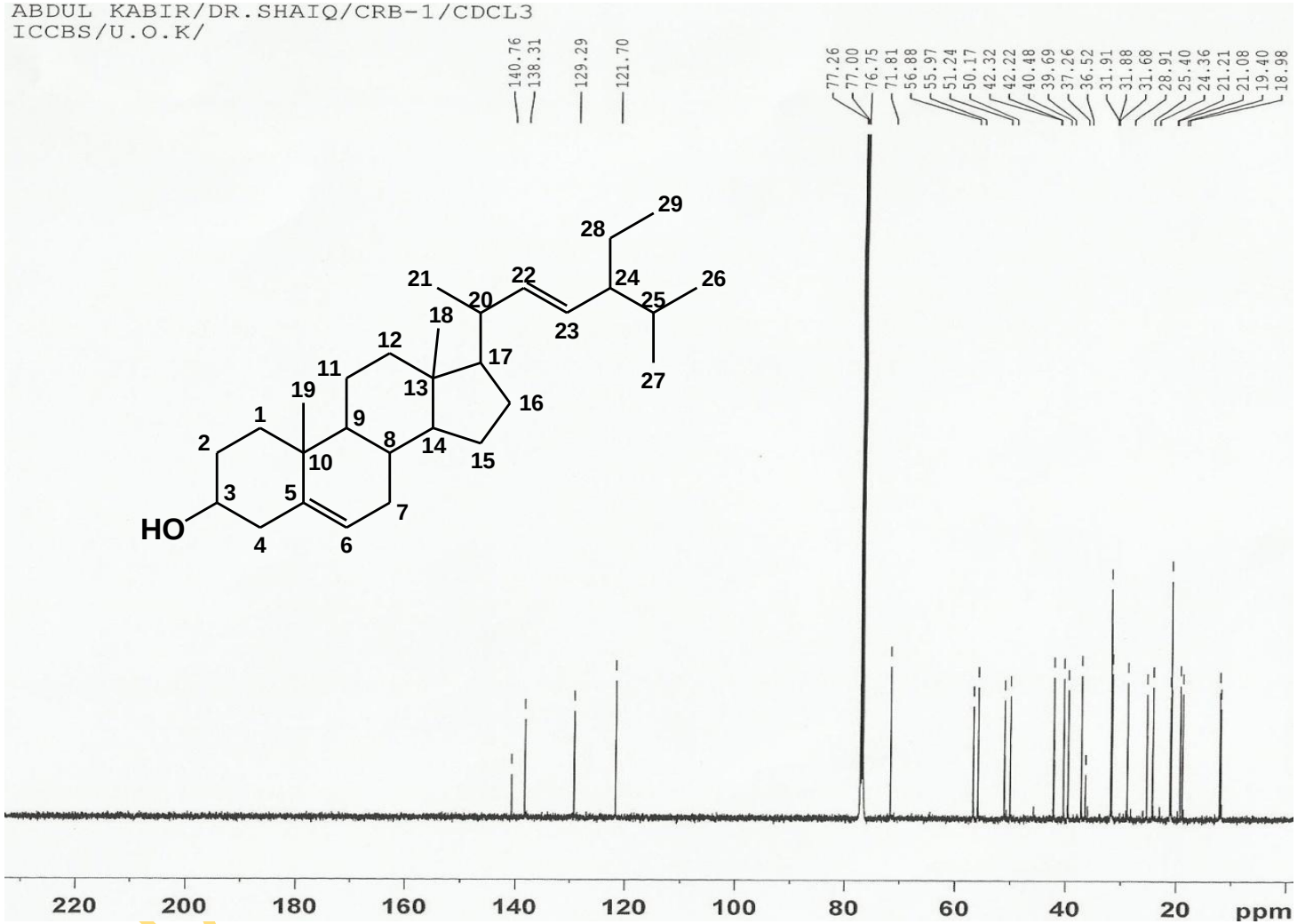


Fig. 4.47. ^{13}C-NMR spectrum of CRB-1 ($CDCl_3$, 100 MHz)

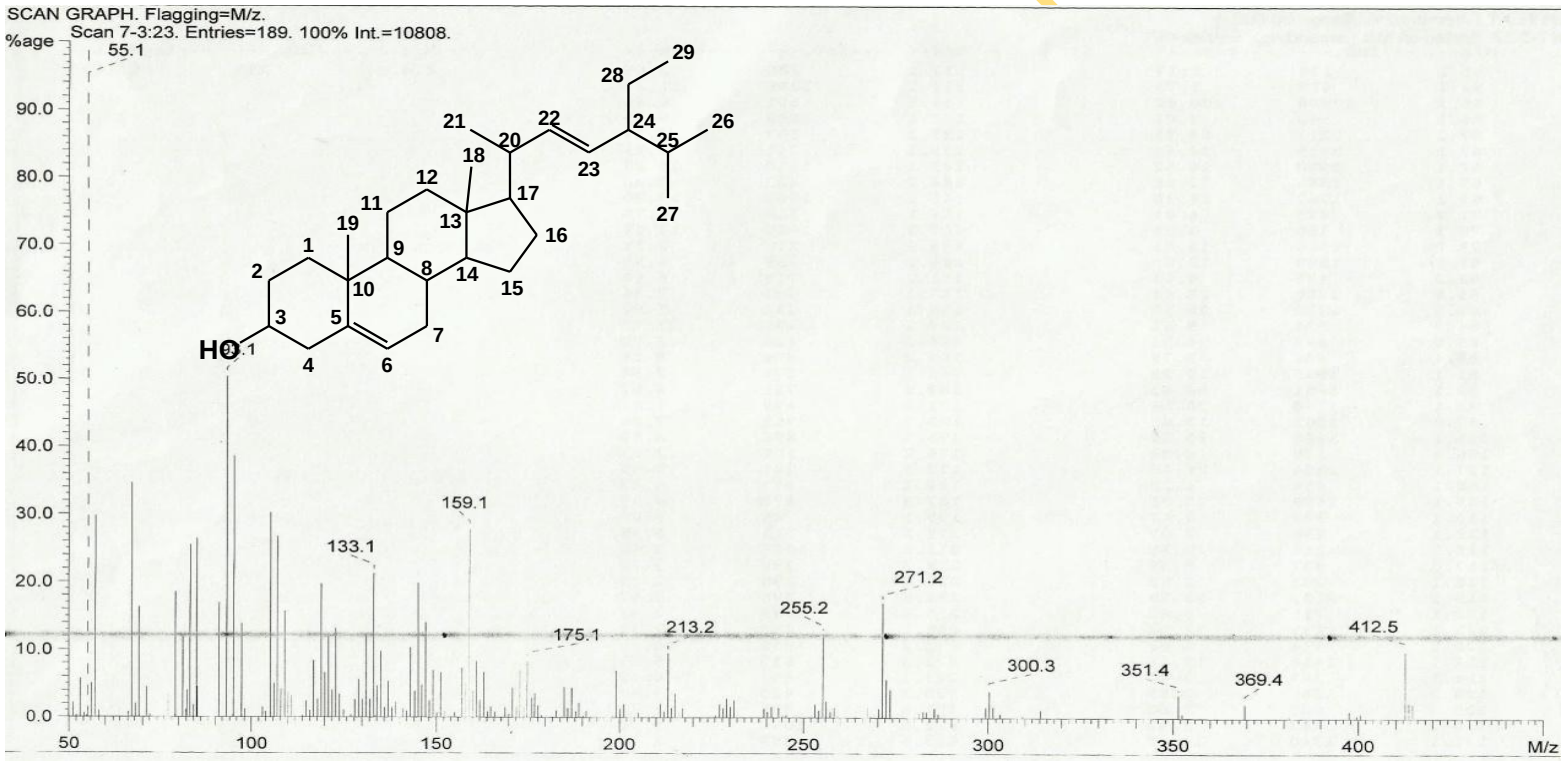


Fig. 4.48. EIMS spectrum of CRB-1

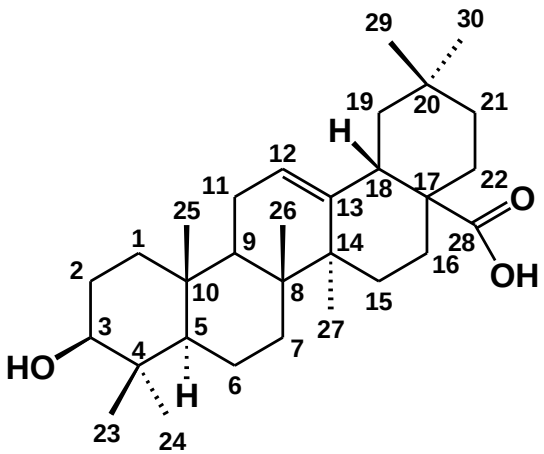
4.2.10 Isolation and Characterisation of CRB-1B

Fraction F_{12} eluted with 25% ethyl acetate in hexane yielded a white crystalline solids which was tagged CRB-1B (22 mg). (Table 4.17; Scheme 3.2)

The CRB-1B has a molecular formula $C_{30}H_{48}O_3$ deduced from HR-EIMS with molecular ion at *m/z* 456.3566 (calcd. 456.3605) consistent with seven degree of unsaturation. The base peak at *m/z* 248 and fragment ion at *m/z* 203 showed a characteristic of a pentacyclic triterpene of β-amyrin series with a double bond between C-12 and C-13.

The ^{1}H-NMR spectrum (Fig. 4.49) of CRB-1B showed seven tertiary methyl groups at δ 0.96(H-23), 0.75(H-24), 0.88 (H-25), 0.73 (H-26), 1.11 (H-27), 0.88 (H-29) and 0.89 (H-30) on an oleanane skeleton. One proton doublet of doublet at δ 2.78 and a singlet olefinic proton at δ 5.26 were assigned to H-18 and H-12, indicating an olea-12-ene skeleton. The ^{1}H-NMR spectrum also showed a deshielded signal for methine proton δ 3.18 (1H, t), which was assigned for H-3 proton.

The ^{13}C-NMR spectrum (Fig. 4.50) of CRB-1B indicated the presence of 30 carbon atoms: seven methyl, ten methylene and eight quaternary carbons. The presence of oxygenated carbon at C-3 showed resonance at δc 79.0. The signal at δc 183.13 was due to carbon of carbonyl acid at C-28. On the basis of the spectral data (Table 4.19) and comparison of ^{13}C shifts with the reported data, the structure has been identified as 3β-hydroxyolean-12-en-28-oic acid **4.9** and commonly known as oleanolic acid (Mahato and Kundu, 1994; Senthilkumar and Reetha, 2011).



4.9

Table 4.19. The ^{1}H and ^{13}C NMR Spectra data of CRB-1B (500 and 125 MHz, ppm in $CDCl_3$ respectively) compared with ^{13}C NMR data of literature.

position	^{1}H δ (multiplicity)	^{13}C δ	^{13}C* δ	DEPT
1.	1.57 (m)	38.4	38.5	CH_2
2.	1.57 (m)	27.1	27.4	CH_2
3.	3.18 (m)	79.0	78.7	CH
4.	-	38.7	38.7	C
5.	0.7 (m)	55.2	55.2	CH
6.	1.52 (m)	18.3	18.3	CH_2
7.	-	32.6	32.6	CH_2
8.	-	39.2	39.3	C
9.	1.50 (m)	47.6	47.6	CH
10.	-	37.0	37.0	C
11.	1.57 (m)	22.9	23.1	CH_2
12.	5.26 (m)	122.6	122.1	CH
13.	-	143.6	143.4	C
14.	-	41.6	41.6	C
15.	-	27.6	27.7	CH_2
16.	1.85 (m)	23.4	23.4	CH_2
17.	-	46.5	46.6	C
18.	2.78 (m)	41.0	41.3	CH
19.	1.57 (m)	45.8	45.8	CH_2
20.	-	30.7	30.6	C
21.	-	33.8	33.8	CH_2
22.	-	32.4	32.3	CH_2
23.	0.96 (s)	28.1	28.1	CH_3
24.	0.75 (s)	15.5	15.6	CH_3
25.	0.88 (s)	15.3	15.3	CH_3
26.	0.73 (s)	17.1	16.8	CH_3
27.	1.11 (s)	25.9	26.0	CH_3
28.	-	183.1	181.0	C
29.	0.88 (s)	33.1	33.1	CH_3
30.	0.89 (s)	23.6	23.6	CH_3

* Mahato and Kundu, 1994.

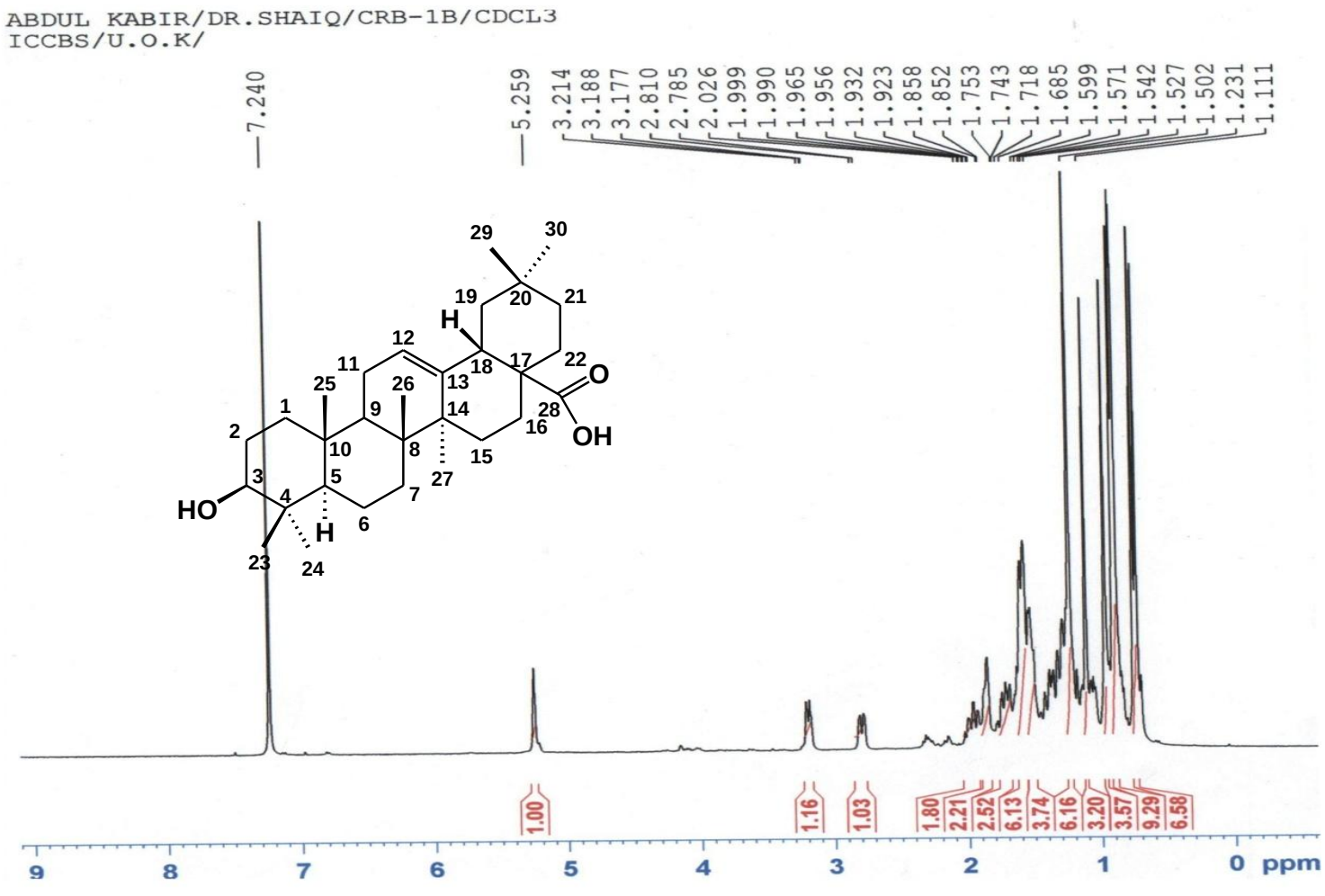


Fig. 4.49. ^{1}H-NMR spectrum of CRB-1B ($CDCl_3$, 500 MHz)

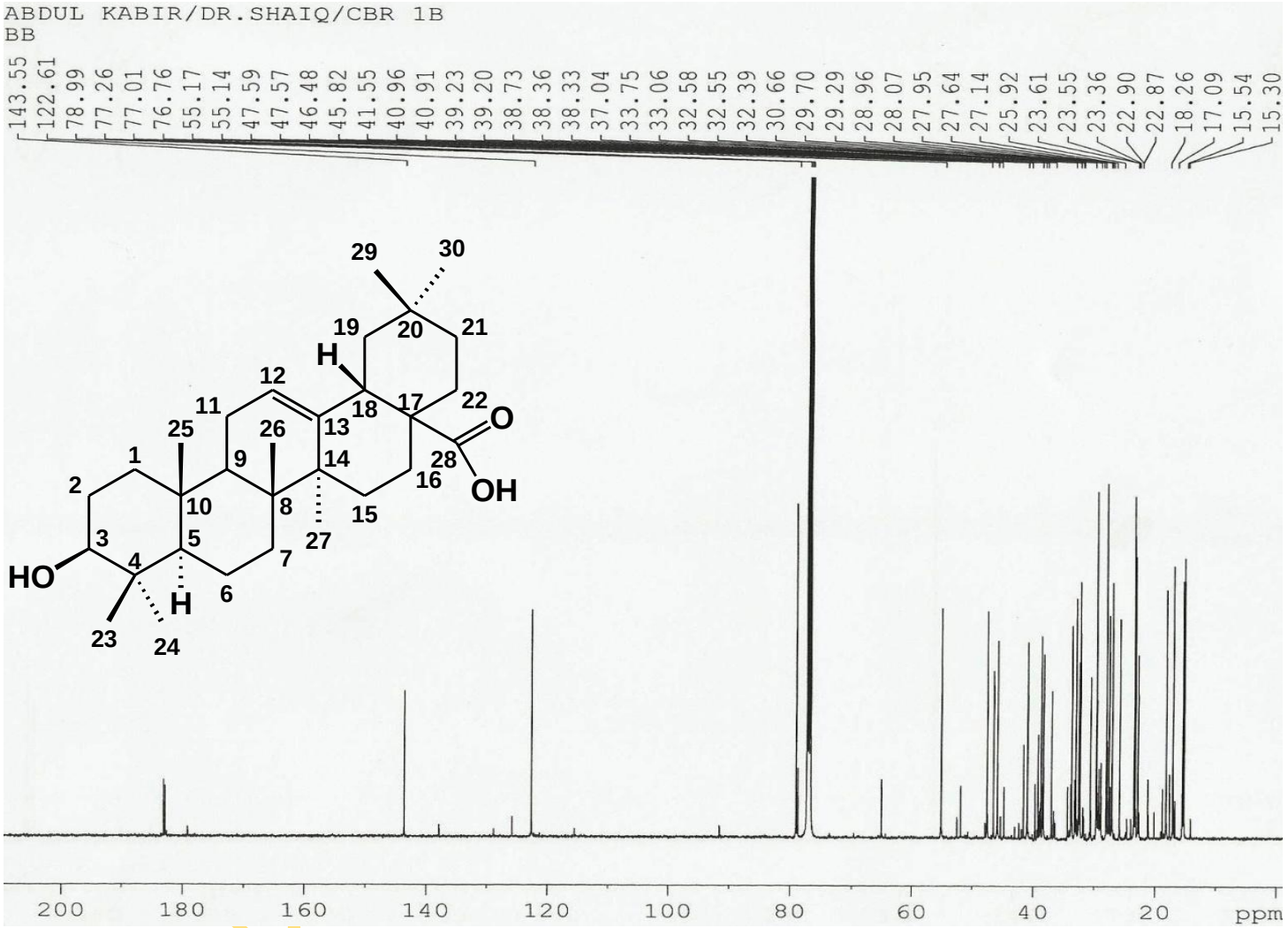


Fig. 4.50. ^{13}C-NMR spectrum of CRB-1B ($CDCl_3$, 125 MHz)

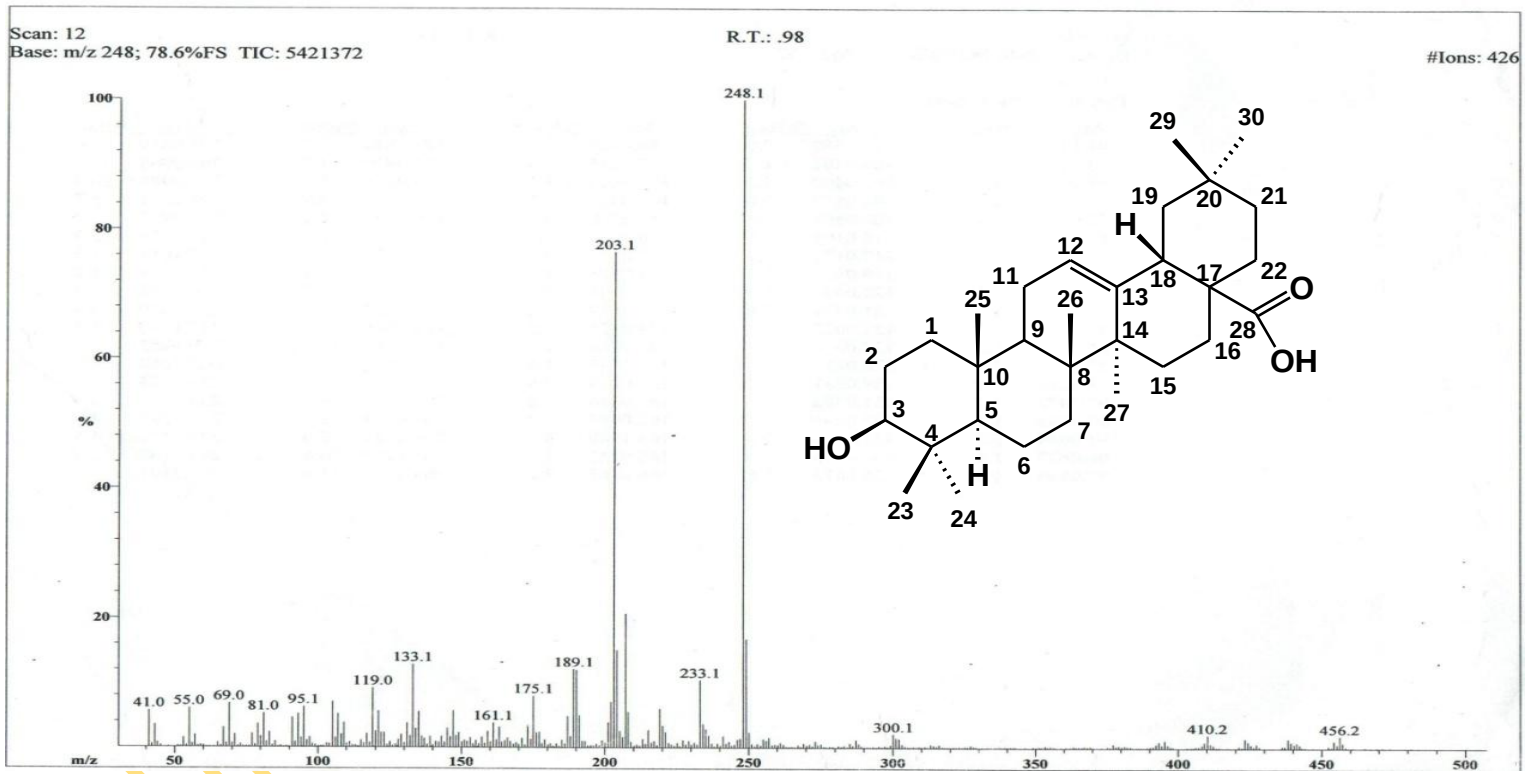


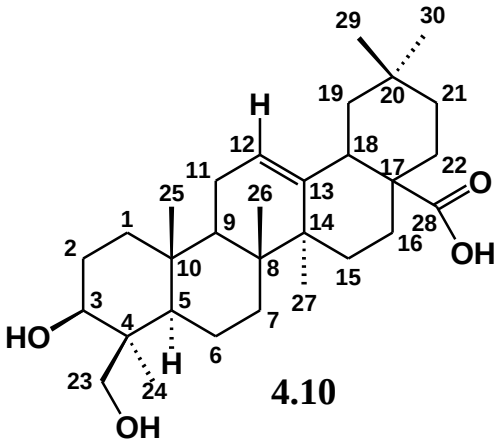
Fig. 4.51. EIMS spectrum of CRB-1B

4.2.11 Isolation and Characterisation of CRB-2

Fifty percent ethyl acetate in hexane gave a pale white amorphous powder which was purified by recrystallisation (Table 4.17; Scheme 3.2). The crystallisation was carried out by dissolving the sample in methanol and hexane (1:19) and subjected the solvent to slow evaporation at room temperature, this yielded fine white crystals tagged CRB-2 (150 mg).

Its EIMS (Fig. 4.52) showed a weak molecular ion peak at *m/z* 472, corresponding to molecular formula $C_{30}H_{48}O_4$ in HR-EIMS. The base peak at *m/z* 248 was produced through retro-Diels-Alder fragmentation. Further loss of the carboxylic group led to the peak at *m/z* 203. The IR spectrum of CRB-2 showed absorption of hydroxyl (3453 cm^{-1}) and carboxyl (1699 cm-1) functional groups.

The ^{1}H-NMR spectrum (Fig. 4.53-4.53a) exhibited signals due to six methyl singlets (δ 0.69, 0.97, 0.81, 1.12, 0.9 and 0.93, 3H each), an olefinic proton (δ 5.21, H-12), a hydroxyl-methine group (δ 3.57, H-3), one proton doublet of doublet at δ 2.83(H-18) and a methylene doublet (δ 3.5, *J*= 10.8, H-23). The rest of signals were the sp^3 CH and CH_2 unit found at upfield region. The broad-band decoupled ^{13}C-NMR (Fig. 4.54) and DEPT spectra displayed resonances for thirty carbons including six methyl, eleven methylene, five methine and eight quaternary carbons. The most downfield shifted peak at δc181.87 was assigned to the carbonyl group of the acid (C-28). The spectra data (Table 4.20) also supported presence of a double bond (δc 123.6,143.3 ppm for C-12 and C-13 respectively) diagnostic signal for oleane-12-enes (Begum and Siddiqui, 2002). The structure of CRB-2 was finally established as hederagenin **4.10** (3,23-dihydroxyl-12-oleanen-28-oic acid) by comparison with existing literature (He *et al*., 2003) and single X-ray analysis of the crystal.



4.10

Table 4.20. The ^{1}H and ^{13}C-NMR Spectra data of CRB-2 (400 and 100 MHz, CD_3OD) compared with ^{13}C-NMR data of literature.

position	^{1}H δ (multiplicity)	^{13}C δ	$^{13}C^*$ δ	DEPT
1.	1.61 (m)	39.4	38.1	CH_2
2.	1.16 (m)	25.3	25.3	CH_2
3.	3.57 (m)	73.8	73.9	CH
4.	-	43.0	43.3	C
5.		overlap[a]	overlap[a]	CH
6.	1.52 (m)	19.1	19.11	CH_2
7.	0.88, 1.25	33.5	33.8	CH_2
8.	-	37.9	37.9	C
9.		overlap[a]	overlap[a]	CH
10.	-	37.8	37.8	C
11.		24.4	24.4	CH_2
12.	5.21 (m)	123.6	123.6	CH
13.	-	143.3	143.3	C
14.	-	43.3	43.0	C
15.	1.57, 1.78	28.8	27.5	CH_2
16.	1.88, 1.91	24.5	23.9	CH_2
17.	-	47.6	47.2	C
18.	2.83 (dd)	42.7	42.7	CH
19.	1.12, 1.62	47.2	47.6	CH_2
20.	-	34.9	33.5	C
21.	1.63, 1.88	33.8	34.9	CH_2
22.	1.60	32.0	33.9	CH_2
23.	3.50 (d)	67.2	67.3	CH_2
24.	0.69 (s)	12.7	12.8	CH_3
25.	0.97 (s)	16.3	16.4	CH_3
26.	0.81 (s)	17.7	17.8	CH_3
27.	1.12 (s)	24.0	24.1	CH_3
28.	-	181.9	181.7	C
29.	0.90 (s)	33.6	33.6	CH_3
30.	0.93 (s)	21.6	21.6	CH_3

* He e*t al*., 2003 [a]overlapped with signals of MeOD

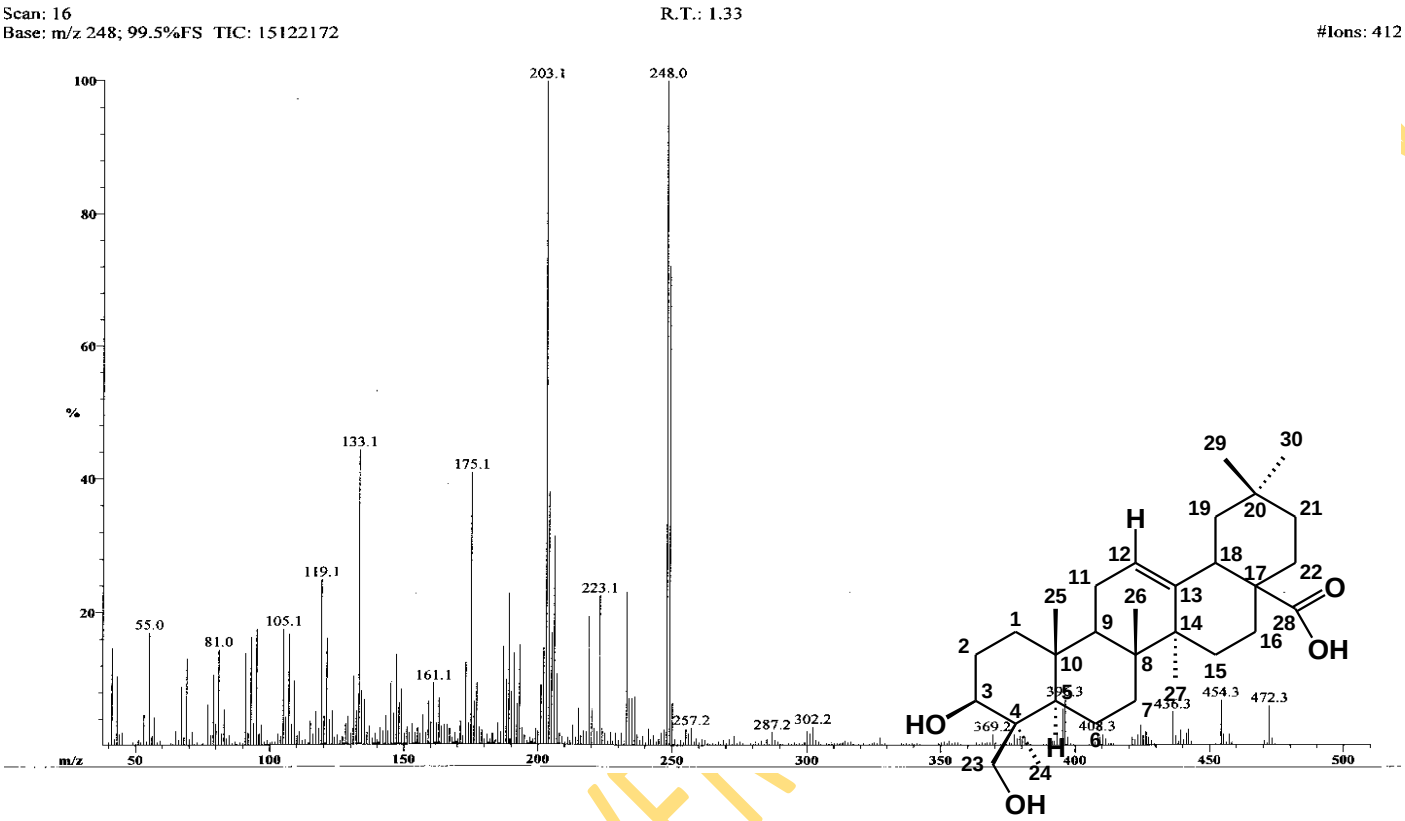


Fig. 4.52. EIMS spectrum of CRB-2

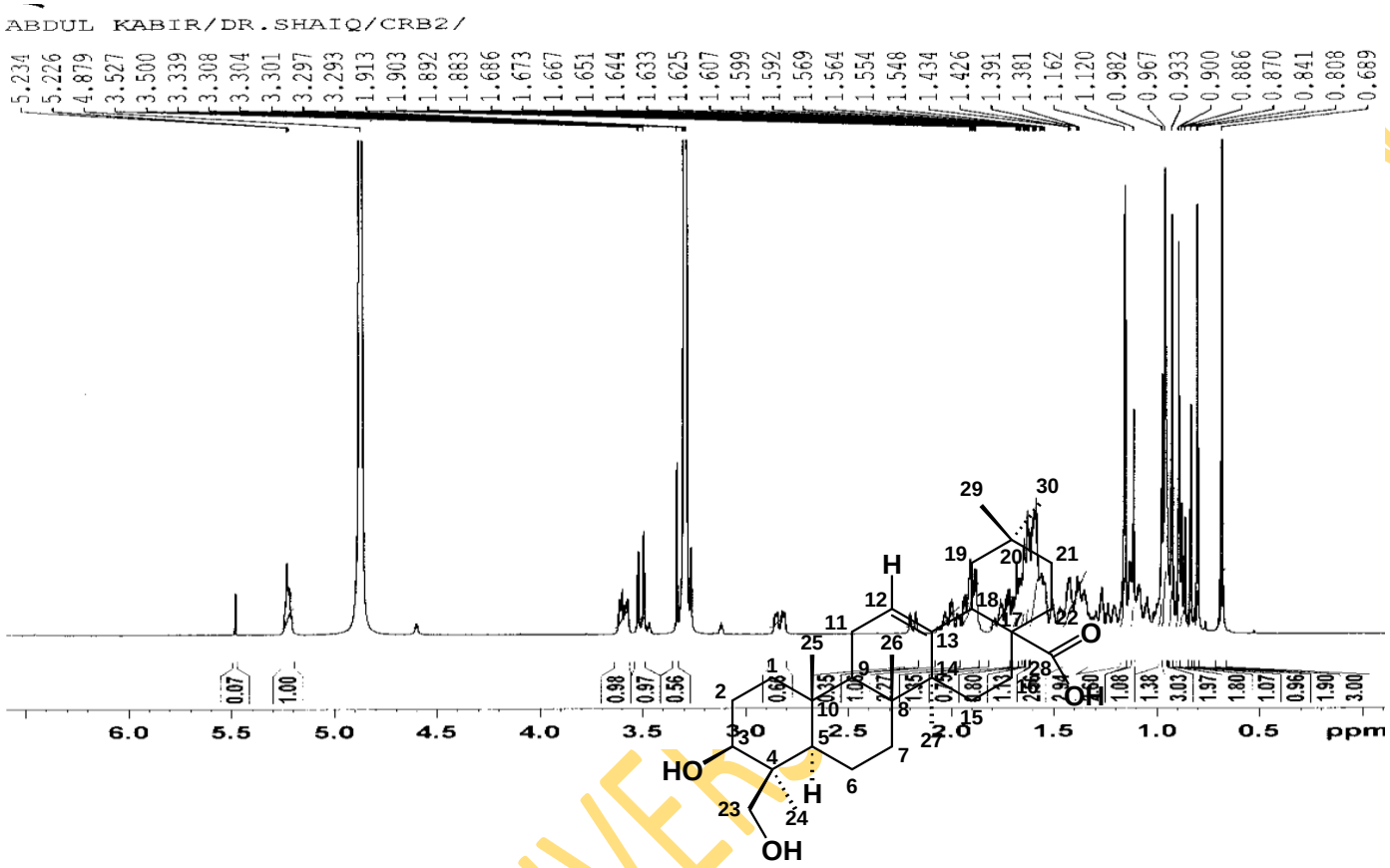


Fig. 4.53. ^{1}H-NMR spectrum of CRB-2 (CD_3OD, 400 MHz)

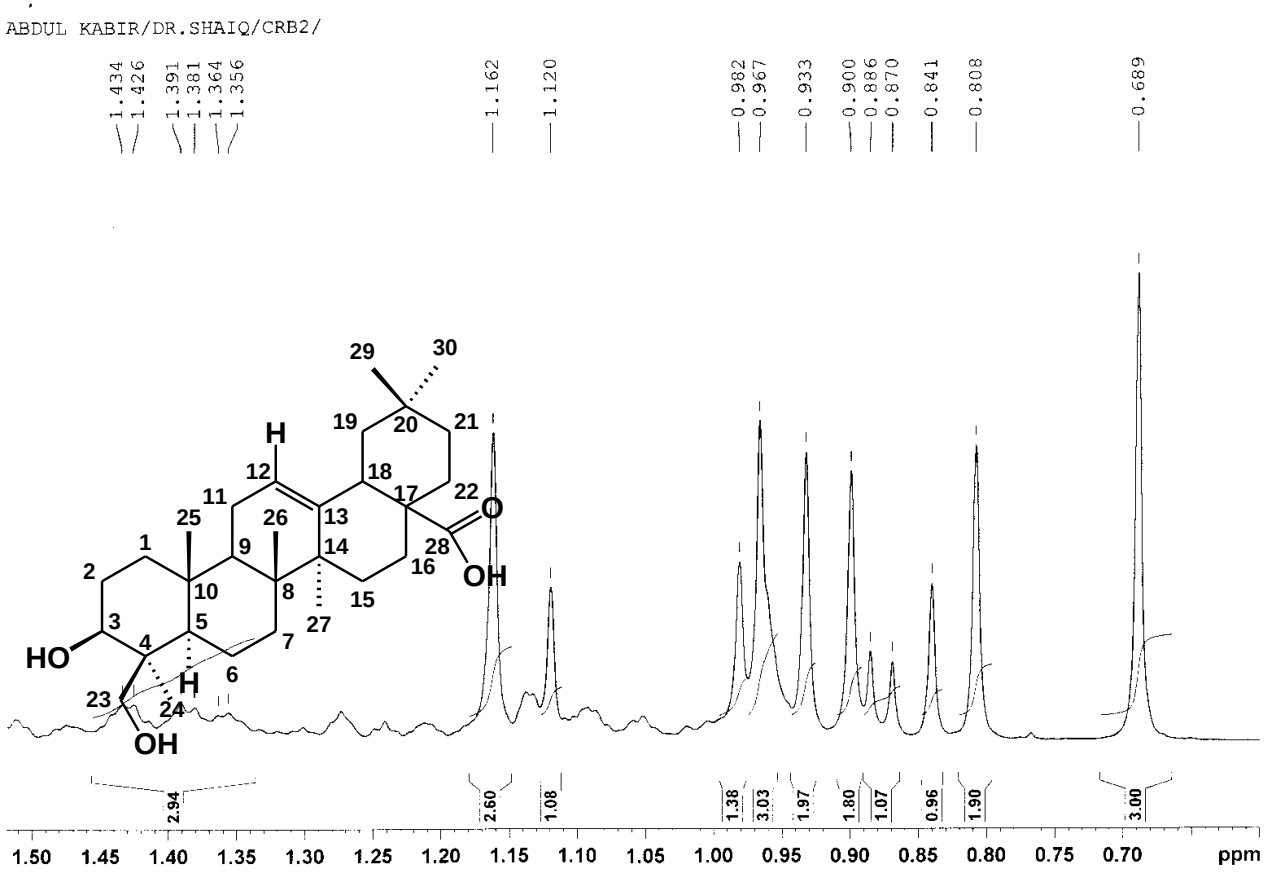


Fig. 4.53a. Expanded ^{1}H-NMR spectrum of CRB-2 (CD_3OD, 400)

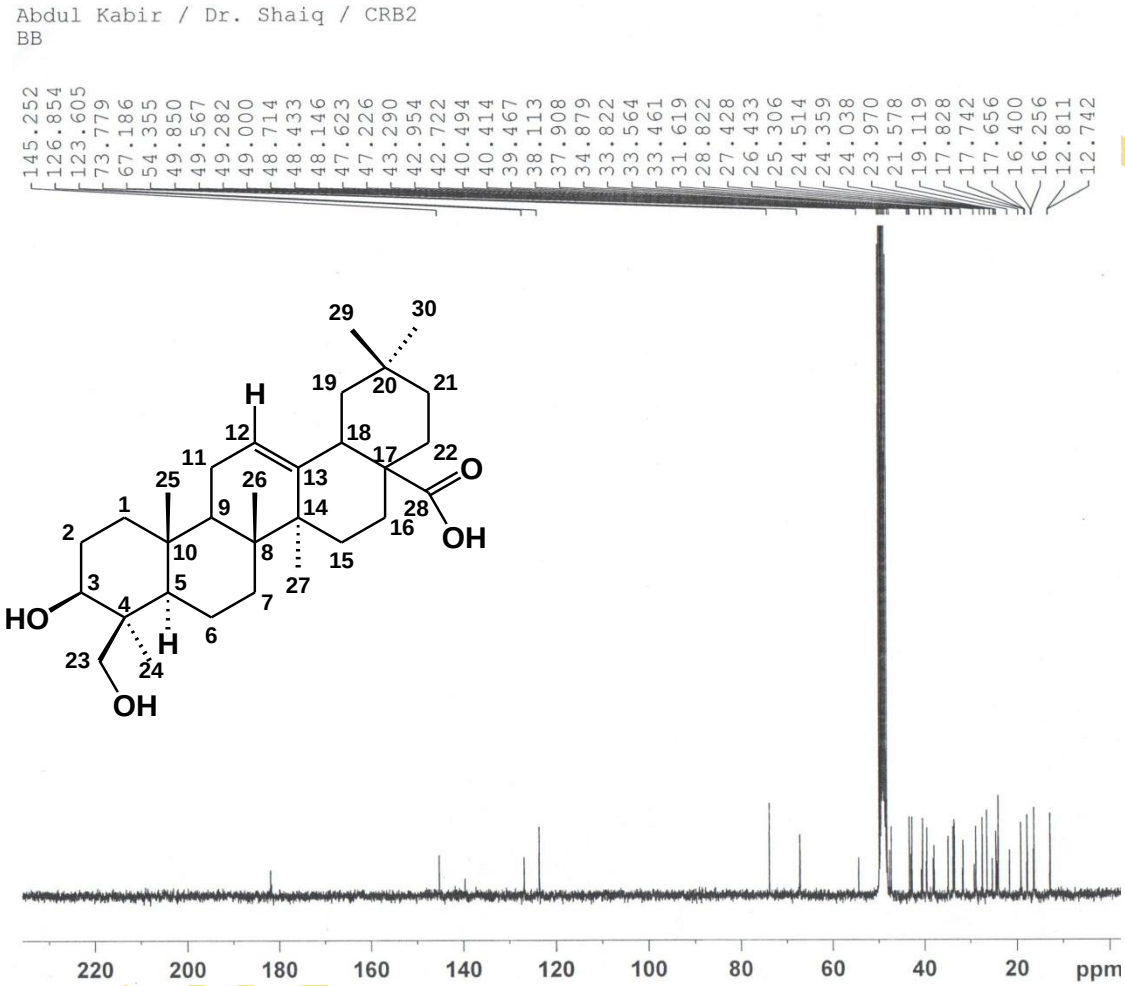


Fig. 4.54. ^{13}C-NMR spectrum of CRB-2 (CD_3OD, 100 MHz)

4.2.12 X-ray diffraction analysis of CRB-2

Suitable crystals were grown in hexane-methanol at room temperature and subjected to single X-ray diffraction analysis. The crystal belongs to orthorhombic space group P21, with an accurate lattice constant of a= 12.2865(19) Å, 16.414(3) Å, c = 13.441(2) Å and Z = 2. The structure was solved by using direct methods and refined by full-matrix techniques. All bond lengths and angles are in accord with accepted values. A computer generated perspective drawings of the final X-ray model of hederagenin (CRB-2) is given in figure 4.55 and other details in appendix 25.

Structure determination summary for CRB-2

Empirical formula:	$C_{30}H_{48}O_4$
Formula weight:	472.35
Temperature:	273(2) K
Wavelength:	0.71073 A
Crystal system, space group:	Orthorhombic, P21
Unit cell dimensions:	a = 12.2865(19) Å, alpha = 90°
	b = 16.414(3) Å, beta = 90.209(4)°
	c = 13.441(2) Å, gamma = 90°. Z = 2
Volume:	2710.6(7) A^3
Calculated density:	1.158 Mg/m^3
Absorption coefficient:	0.075 mm^{-1}
F (000):	1040
Crystal size:	0.48 x 0.43 x 0.08 mm
Theta range for data collection:	1.51 to 25.49°.
Limiting indices:	-14<=h<=14, -19<=k<=19, -16<=l<=14
Reflections collected / unique:	15899 / 5163 [R(int) = 0.0440]

Completeness to theta =	25.49 98.7 %
Max. and min. transmission:	0.9941 and 0.9651
Refinement method:	Full-matrix least-squares on F^2
Data / restraints / parameters:	5163 / 1 / 637
Goodness-of-fit on F^2:	1.030
Final R indices [I>2sigma(I)]:	R1 = 0.0513, wR2 = 0.1151
R indices (all data):	R1 = 0.0720, wR2 = 0.1273
Absolute structure parameter:	0(10)
Largest diff. peak and hole:	0.373 and -0.159 e.A^{-3}

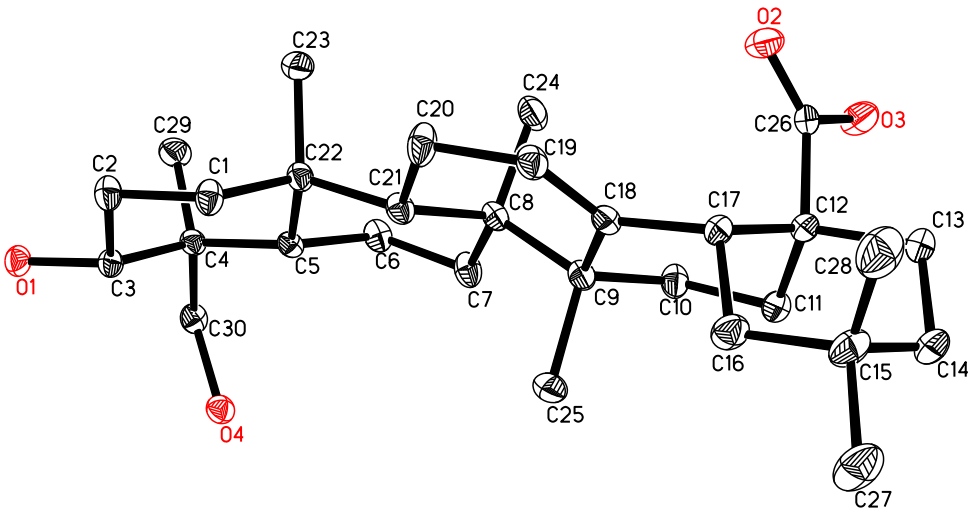


Fig 4.55. A computer-generated perspective mirror image drawing of the final X-ray model of CRB-2. Hydrogens are omitted for clarity.

4.3 Radical scavenging activity

Bleaching of purple colour of 1, 1-diphenyl-2-picryl-hydrazil (DPPH) has been extensively used to evaluate free radical scavenging activity of pure isolates and crude extract (Ajiboye *et al*, 2013). The new isolated compounds, DRR-2E, DRR-1G and CRB-2 were evaluated for *in vitro* radical scavenging inhibition assay (Table 4.20), though, it is only crystal structure of CRB-2 that is novel. The DRR-2E produced a 50% scavenging activity at 58.14 μM and compared favourably with 44.2 μM butylated hydroxyanisole which produced 50% inhibition. The DRR-1G achieved 50% inhibition at 332 μM. Radical scavenging activity of the compounds could be associated with the presence of methoxy and multiple-hydroxyl group patterns on aromatic ring(s) (Cai *et al*., 2006; Jing *et al*., 2012). The CRB-2 showed 50% scavenging activity at 105.0 μM.

Table 4.21. Radical scavenging activity of some selected isolates

Compounds	DPPH assay (IC_{50}) μM
DRR-1G	322.0
DRR-2E	58.14
CRB-2	105.0
BHA (Control)	44.2

CHAPTER FIVE

5.0 Summary and Conclusion

Preliminary phytochemical screening of the *D. reflexa* root extracts revealed the presence of alkaloid, tannins, glycoside, saponins, steroid, flavonoids, resins, phenols and carbohydrate. Phytochemical investigation of the roots resulted into the isolation of seven compounds which were characterized extensively using various spectroscopic techniques. The isolated compounds are lupeol (DRR-1C), lexaflavanone (DRR-2E), reflevone (DRR-1G), aurantiamide acetate (DRR-1F), mearnsetin (DRR-2G), 4-OMGA (DRR-3G) and 7,4'-dihydroxyflavone (DRR-4G). Two of the compounds, lexaflavanone and reflevone were isolated from natural product for the first time.

On phytochemical examination of *C. arborea*, three triterpenoids compounds were isolated from the root-bark. These include stigmasterol (CRB-1), oleanolic acid (CRB-1B) and hederagenin (CRB-2).

The isolated compounds, which have never been reported so far from these sources, have thus added to the chemical library of compounds already isolated from the plants. The data also provide information on the essential oil constituents of *D. reflexa* root collected in Nigeria, which hitherto unavailable.

The inhibition of DPPH radical by Lexaflavanone, reflevone and hederagenin is an indication of free radical scavenging activity. This may be useful in pharmaceutical, food industry as well as medicine in preventing lipid peroxidation and oxidative stress (Ajiboye *et al.*, 2013).

Compounds isolated from the plants could be investigated for possible anti-malaria, anticancer, anti-diabetes and antioxidant activities, because these classes of compound in the literature proved to be possessing the aforementioned activities (Vianna *et al.*, 2006; De Villiers *et al.*, 2010; Balamurugan *et al.*, 2012). Furthermore, more effort

could also be made to isolate other compounds from these plants especially the stem and the leave parts, as preliminary screening indicated the presence of yet to be isolated secondary metabolites.

REFERENCES

Aba, E. P. and Asuzu, I. U. 2014. Antihyperglycaemic and antioxidant potentials of *Cussonia arborea* in alloxan-induced diabetic rats. *Comparative Clinical Pathology* 23.2:451-458.

Abbas, F. A., Al-Massarany, S. M., Khan, S., Al-Howiriny, T. A., Mossa, J. S. and Abdourasheed, E. A. 2007. Phytochemical and biological studies on Saudi *Comniphora opobalsamum L. Natural Product Research* 21.5:383-391.

Adams, R.P. 2007. *Identification of Essential oil components by Gas chromatography/Mass spectrometry*. 4th edn. Allured:carol stream, IL.USA.

Almeida, E. R., Almeida, R. N., Navairo, D. S., Bhattacharya, J., Silva, V. A. and Birnbaum, J. S. P. 2003. Central antinociceptive effect a alcoholic extract of *Dioclea grandiflora* seeds in rodents. *Journal Ethnopharmacology* 88:1-4.

Alves, C. Q., Kijjoa, A., David, J. M. and David, J. P. 2010. Flavonoids and benzoic acid derivatives from *Dioclea virgata*. *Planta Medica* 76-P368 DOI:10.551s-0030-126466.

Ajiboye, T. O., Raji, H. O., Muritala, H. F., Ojewuyi, O. B. and Yakubu, M. T. 2013. Anthocyanin extract of *Lannea microcarpa* fruits stall oxidative rout associated with aflatoxin B_1 hepatocarcinogenesis. *Food Science* 4: 58–67.

Balamurugan, R., Stalin, A. and Ignacimuthu, S. 2012. Molecular docking of g -sitosterol with some targets related to diabetes. *European Journal of Medicinal Chemistry* 47: 38–43.

Banerji, A. and Ray, R. 1981. Aurantiamides: A new class of modified dipeptides from *Piper aurantiacum*. *Phytochemistry* 20.9: 2217-2220.

Barreiros, A. L. B. S., David, J. P., de Queiroz, L. P. and David, J. M. 2000. A-type proanthocyanidin antioxidant from *Dioclea lasiophylla*. *Phytochemistry* 55.7: 805-808.

Begum, S., Wahab, A. and Siddiqui, B. 2002 .Ursethoxy Acid , a New Triterpene from *Lantana Camara*. *Natural Product Letters* 16. 4: 235–238.

Bhattacharyya, J., Majetich, G., Jenkins, T. and Almeida, R. 1998. Dioflorin, a minor flavonoid from *Dioclea grandiflora. Journal of Natural Products* 61:413-414.

Bhattacharyya, J., Majetich, G.,Spearing, P. and Almeida, R. 1997. Dioclenol, a minor flavanonol from the root-bark of *Dioclea grandiflora. Phytochemistry* 46.2:385-387.

Bickoff, E.M., Livingston, A. L. and Witt, S. C. 1964. Identification of 7,4′-dihydroxylflavone in forages. *Phytochemistry* 9:523-524.

Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 181: 1199-1200.

Braga, P. C., Alfieri, M., Culici, M and Dal Sasso M. 2007. Inhibitory activity of thymol against the formation and viability of *Candida albicans* hyphae. *Mycoses* 50.6:502-506.

British pharmacopoeia. 1980. HMSO: London, A109. vol. II.

Burkill, H. M. 1984. *The useful plants of West Tropical Africa.*2nd ed. UK: Royal Botanic Gardens.

Cai, Y. Z., Mei, S., Jie, X., Luo, Q. and Corke, H. 2006. Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sciences* 78:2872–2888.

Carter, C. W. and Sweet, R. M. eds. 1997. Methods in Enzymology. UK: Elsevier Academic Press. 374: 2.

Chieh-Shan, W., Yun-Ju, C., Jeremy, J. W., Chen, J. S., Chia-Hsin, H., Pei-Shan, L., and Gee-Chen, C., 2012. Terpinen-4-ol Induces Apoptosis in Human Nonsmall Cell Lung Cancer in Vitro and In Vivo. *Evidence-Based Complementary and Alternative Medicine* 2012:1-13.

Cinzia, C., Maria, M.C., Alberto, C., Bruno, T., Gloria, C., Fabrizio P., Gianni, S., Sauti, V. and Filippo, M. 2010. Essential oil from fruits and roots of *ferulago campestris* (Besser) Grecescu (Apiaceae): composition and antioxidant and anti-Candida activity. *Flavour Frangrance Journal* 25:493-502.

De Almeida E.R., Xavier, H. S., Chaves, T. M., Couto, G. B. L., Aragao-Neto, A. C., Silva, A. R. and Da Silva, L. L. S. 2010. Anxiolytic and anticonvulsant effects of dioclenol flavonoid isolated from stem-bark of *Dioclea grandiflora* on mice. *International Journal of Applied Research in Natural Products* 2.4:44-51.

De Villiers, B. J., Oskolski, A. A., Tilney, P. M. and Van Wyk B.-E. 2012. Wood anatomy of *Cussonia* and *Seemannaralia* (Araliaceae) with systematic and ecological implications. *IAWA Journal* 33.2: 163–186.

De Villiers, B.J., Van Vuuren, S.F., Van Zyl, R.L. and Van Wyk, B.-E. 2010. Antimicrobial and antimalarial activity of Cussonia species (Araliaceae). *Journal of Ethnopharmacology* 129:189–196.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P. and Vidal, N. 2006. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry* 97.4: 654-660.

Dorothea, K., Anudeep, K. D. and Michael, W. 2011. Myrtenal inhibits acetylcholinesterase, a known Alzheimer target. *Journal of Pharmacy and Pharmacology* 63.10:1368-1371.

Dubois, M. A., Ilyas, M. and Wagner, H. 1986. Cussonosides A and B, two triterpene-saponins from Cussonia barteri. *Planta Medica* 52.2: 80–83.

Duru, C.M. and Onyedineke, N.E. 2010. *In vitro* study on the antimicrobial activity and phytochemical analysis of ethanolic extracts of the mesocarp of *voacanga* Africana. *American Journal of Plant Physiology* 5.4:163-169.

Faleye, F. J. 2012. Steroids constituents of *Dioclea reflexa* hook seeds. *Journal of Pharmaceutical and Scientific Innovation* 1.3:89-90.

Forgo, P. and Kövér, K. E. 2004.Gradient enhanced selective experiments in the ^{1}H-NMR chemical shift assignment of the skeleton and side-chain resonances of stigmasterol, a phytosterol derivative. *Steroids* 69: 43–50.

Fourie, T. G., Matthee, E. and Snyckers, F. O. 1989. Studies of South African medicinal plants. Part 10. A pentacyclic triterpene acid, with anti-ulcer properties, from *Cussonia natalensis*. *Phytochemistry* 28.10:2851-2852.

Francesca, M., Flavia, De B., Antonietta, G., Antonio, C. and Giuseppe S. 2006. *In vivo* activity of terpinen-4-ol, the main bioactive component of Melaleuca alternifolia cheel (tea tree) oil against azole-susceptible and reistant human pathogenic *candida* species. *BMC Infectious Diseases* 6:158.

Gibbons, S. 2003. An overview of plant extracts as potential therapeutics. *Expert Opinion on Therapeutic Patents* 13.4: 489-497.

Gluchoff-Fiasson, K., Fenet, B., Leclerc, J., Lussignol, M. and Jay, M. 2001. Three new flavonol malonylrhamnosides from *Ribes alpinum*. *Chemical and Pharmaceutical Bulletin* 49.6:768-770.

Gülcin, I., Alici, H.A. and Cesur, M. 2005. Determination of *in vitro* antioxidant and radical scaveging activities of propofol. *Chemical and Pharmaceutical Bulletin* 53: 281-285.

Gunzinger, J., Msonthi, J., D., Hostettmann, K. 1986. Molluscicidal saponins from *Cussonia spicata*. *Phytochemistry* 25.11: 2501-2503.

Gupta, V. K. & Verma, A. K. 2010. *Comprehensive Bioactive Natural Products.* Houston: Stadium Press LCC. Vol 4.

Hammami, S., Janet, H. B., Bergaoui, A., Ciavatta, L., Cimimo, G. and MIghri, Z. 2004. Isolation and structure elucidation of a flavanone, a flavanone glycoside and Vomifoliol from *Echiochilon fruticosum* growing in Tunisia. *Molecules* 9:602-608.

Harbone, J. B. 1993. *Phytochemical method*. 3rd ed. London: Chapman and Hall.

Harinantenaina Liva, R. R., Kasai, R., Yamasaki, K. 2002. Clerodane and labdane diterpene glycosides from a Malagasy endemic plant, *Cussonia racemosa*. *Phytochemistry* 60.4:339-343.

He, W., Puyvelde, L. V., Maes, L., Bosselaers, J. and De Kimpe, N. 2003. Antitrichomonas In Vitro Activity of Cussonia Holstii Engl. *Natural Product Research* 17.2: 127–133.

Hoffman, E. and Stroobank, V. 2002. Mass spectroscopy: principle and applications 2nd edition. UK: John Wiley and Sons Inc.

Jäger, S., Trojan, H., Kopp, T., Laszczyk, M. N. and Scheffler, A. 2009. Pentacyclic triterpene distribution in various plants – rich sources for a new group of multi-potent plant extracts. *Molecules* 14:2016-2031.

Jenkins, T., Bhattacharyya, J., Majetich, G., Teng, Q., Fatima, A. and Almeida, R. 1999. Flavonoids from the root-bark of *Dioclea grandiflora*. *Phytochemistry* 52: 723-730.

Jeon, K., Na, H, Kim, Y and Kwon, H. J. 2005. Antiangiogenic activity of 4-O-methylgallic acid from Canavalia gladiata, a dietary legume. *Biochemical and Biophysical Research Communications* 330:1268–1274.

Jang, I. C., Jo, E. K., Bae, M. S., Lee, H. J., Jeon, G. I. Park, E., Yuk, H. G., Ahn, G. H. and Lee, S. C.. 2010. Antioxidant and antigenotoxic activities of different parts of persimmon (*Diospyros kaki cv. Fuyu*). *Journal of Medicinal Plants Research* 4.2: 155-160.

Jing, P., Zhao, S., Wen-Jie, Jian, Bing-Jun, Qian,. Dong, Y. and Pang, J. 2012. Quantitative Studies on Structure-DPPH: Scavenging Activity Relationships of Food Phenolic Acids. *Molecules* 17: 12910-12924.

Joshua, S., David, T., Daniel, A., Kenneth W. and Graham J. 2010. Intraspecific variation in essential oil composition of *Eremophila longifolia F. Muell (Myoporaceae)*: Evidence for three chemotypes. *Phytochemistry* 71:1521- 1527.

Kalsi, P. S. 2004. *Spectroscopy of organic compounds*. 6th edition. New Delhi: New Age International Ltd.

Klopper, R. R., Chatelain, C., Bänninger, V., Habashi, C., Steyn, H.M., de Wet, B. C., Arnold, T. H., Gautier, L., Smith, G. F. and Spichiger, R.. 2006. *Checklist of*

the flowering plants of sub-Saharan Africa. An index of accepted names and synonyms. Report no. 42. Pretoria: South African Biodiversity Network.88.

Kougan, G. B., Miyamoto, T., Mirjolet, J. F., Duchamp, O., Sondengam, B. L., and Lacaille-Dubois, M. A. 2009. Arboreasides A-E, triterpene saponins from the bark of Cussonia arborea. *Journal of Natural Products*, 72.6:1081–1086.

Krishnaiah, D., Sarbatly, R. and Nithyanandam, R. 2011. A review of the antioxidant potential of medicinal plant species. *Food and Bioproducts Processing* 89.3: 217-233.

Lamber, J. B. and Mazzola, E. P. 2002. NMR spectroscopy: an introduction to principle, application and experimental methods. New Jersey: Pearson Educational Inc.

Lemos, V. S., Freitas, M. R., Muller, B., Lino, Y. D., Queiroga, C. E., and Côrtes, S. F. 1999. Dioclein, a new nitric oxide- and endothelium-dependent vasodilator flavonoid. *European Journal of Pharmacology* 386.1:41–46.

Mabry, T. J., Markham, K. R. and Thomas, M. B. 1975. *The systematic identification of flavonoids*. New York: Springer-verlag. 16-273.

Macomber, R. S. 1998. *A complete introduction to modern NMR spectroscopy*. Canada: John Wiley and Sons Inc.

Mahato, S. B. and Kundu, A. P. 1994. C-13 NMR-spectra of pentacyclic triterpenoids - a compilation and some salient features. *Phytochemistry* 37: 1517–1575.

Maxwell, C. A., Hartwig, U. A., Joseph, C. M. and Phillips, D. A. 1989. A chalcone and two related flavonoids released from Alfafa roots induce nod genes or *Rhizobium meliloti*. *Plant Physiology* 91: 674-679.

Menale, B., Amato, G., Prisco, C. D. and Muoio, R. 2006. Traditional uses of plants in North- Western Molise(Central Italy). *Delpinoa* 48: 29-36.

Meskuriyen, D. and Cordell, G. A. 1988. Traditional Medicinal plants of Thailand XIII. Flavonoid derivatives from *Dracaena loureiri (Agavaceae). Journal of the Science Society of Thailand* 14:3-24.

Nakashi, K., Golo, I., Ito, S., Natori, S. and Nozoe, S. 1974. *Natural Products Chemistry*. New York: Academic Press Inc.

Ncube, N. S., Afolayan, A. J. and Okoh, A.I. 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: Current methods and future trends. *African Journal of Biotechnology* 7.12:1797-1806.

NIST08. *Mass spectra Library* (NIST/EPA/NIH).National Institute of Standards and Technology,Gaithersburg, MD, USA.

Nunes, X. P., Silva, F. S., Almeida, J. S., Lima, J. T., Ribeiro, L. A., Júnior, L. J. and Filho, J. M. 2012. Biological Oxidations and Antioxidant Activity of Natural Products. *Phytochemicals as Nutraceuticals – Global Approaches to Their Role in Nutrition and Health*. V. Rao. Ed. Rijeka (Croatia): InTech. 1-20.

Nvau, J. B. 2014. Antimycrobial activity guided phytochemistry of stem bark of *Cussonia arborea* (Hochst) and roots of *Calliandra portoricensis* (Benth). PhD. Thesis. Department of Chemistry. University of Ibadan. XX + 188pp.

Odugbemi, T. 2008. *Outlines and pictures of medicinal plants from Nigeria*. Yaba-Lagos: University of Lagos Press.

Ogundare, A. O. and Olorunfemi, O. B. 2007. Antimicrobial efficacy of the leaves of *Dioclea reflexa, Mucuna pruriens, Ficus asperifolia and Tragia spathula*. *Research Journal of Microbiology* 2.4:392-396.

Ogunwande, I. A., Flamini, G., Cioni, P. L., Omikorede, O., Azeez, R. A., Ayodele, A. A. and Kamil, Y. O. 2010. Aromatic Plants growing in Nigeria: Essential Oil Constituents of *Cassia alata* (Linn.) Roxb. and *Helianthus annus* L. *Records of Natural Product* 4.4:211-217.

Oladosu, I. A. and Oladimeji, A. O. 2012. Volative constituent of sea purse, *Dioclea reflexa* root. *Elixir Organic Chemistry* 45:7738-7740.

Oladosu, I. A., Echeme, J.O. and Zubair, M. F. 2010. Bioactive of Dioclimidazole from *Dioclea reflexa* seeds. *Middle East Journal of Scientific Research* 6.6:575-579.

Okwu, D. E. 2004. Phytochemicals and vitamin contents of indigenous species of South Eastern Nigeria. *Journal of sustainable Agriculture and the Environment* 6:30-34.

Papajewski, S., Guse, J. H., Klaiber, I., Roos, G., Submuth, R.,Vogler, B., Walter, C. U. and Kraus, W. 1998. Bioassay guided isolation of a new C18-polyacetylene, (+)-9(Z),17-octadecadiene-12,14-diyne-1,11,16-triol from *Cussonia barteri. Planta Medica* 64: 479-481.

Papajewski, S., Vogler, B., Conrad, J., Klaiber, I., Roos, G., Walter, C. U. and Kraus, W. 2001. Isolation from Cussonia barteri of 1'-O-chlorogenoylchlorogenic acid and 1'- O-chlorogenoylneochlorogenic acid, a new type of quinic acid esters. *Planta Medica* 67.8: 732–736.

Parfitt, R. T., 1978. *Drug Discovery, Design or Serendipity.* An Inaugural Lecture Series. University of Bath, U.K.

Park, Y., Lee, Y., Kim, H., Lee, Y., Yoon, Y., Moon, B., Chong, Y., Ahn, J., Shim, Y. and Lim, Y. 2006. NMR data of flavones derivatives and their anti-oxidative activities. *Bulletin of the Korean Chemical Society* 27.10:1537-1541.

Park, Y., Moon, B., Lee, E., Lee, Y., Yoon, Y., Ahn, J. and Lim, Y. 2007. ^{1}H and ^{13}C-NMR data of hydroxyflavone derivatives. *Magnetic Resonance in Chemistry* 45: 674-679.

Pavia, D. L., Lampman, G. M., Kriz, G. S. 2001. *Introduction to spectroscopy.* 3rd edition. USA: Thompson Learning Inc.

Pulliah, T. 2006. *Encyclopeadia of world medicinal plants.*Vol. 2. New Delhi: Regency publications.

Rababah, T.M., Ereifej, K.I., and Howard, L. 2005. Effect of ascorbic acid and dehydration on concentrations of total phenolics, antioxidant capacity,

anthocyanins, and color in fruits. *Journal of Agricultural and Food Chemistry* 53.11:4444-4447.

Rhodes, Gale. 1993. *Crystallography Made Crystal Clear*. USA: Academic Press, San Diego.

Rose, M. E. and Johnstone, R. A. W. 2001. *Mass spectroscopy for Chemists and Biochemists*. 2nd edition. Editors Benkovic, S. J., Elmone, D. T. and Schofield, K. UK: Cambridge University Press.

Schram, J. and Bellama, J. M. 1988. *Two dimensional NMR spectroscopy*. New York: John Wiley and Sons Inc.

Senthilkumar, K. and Reetha, D. 2011. Isolation and identification of antibacterial compound from the leaves of Cassia auriculata. *European Review for Medical and Pharmacological Sciences* 15:1034–1038.

Smith, B. 1996. *Fundamentals of Fourier Transform Infrared Spectroscopy.* Washington D.C.: CRC Press.

Sofowora, A. 1984. *Medicinal plants and traditional medicine in Africa.* Ibadan: Spectrum Books Ltd.

Tanaka, J.C.A., da Silva, C.C., de Oliveira, A.J.B., Nakamura, C.V. and Dias Filho, B.P. 2006. Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorun*. *Brazilian Journal Medical Biological Research* 39.3:387-391.

Tapondjou, L. A., Lontsi, D., Sondengam, B. L., Shaheen, F., Choudhary, M. I., Atta-ur-Rahman, Van Heerden, F. R., Park, H. J. and Lee, K.T. 2003. Saponins from Cussonia bancoensis and their inhibitory effects on nitric oxide production. *Journal of Natural Products* 6.9:1266–1269.

Taylor, J. L. S., Rabe, T., Mc Graw, L. J., Jager, A.K. and Van Staden, J. 2001. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation* 34.1:23-27.

Tolstoy, V. P., Chernyshova, V. A. and Skryshersky, V. A. 2003. *Handbook of Infrared Spectroscopy of ultrathin films*. Canada: John Wiley and Sons Inc.

Trigueiro, F., Cortes, S. F., Almeida, R. N., and Lemos, V. S. 2000. Endothelium-independent vasorelaxant effect of dioclein, a new flavonoid isolated from *Dioclea grandiflora*, in the rat aorta. *The Journal of Pharmacy and Pharmacology* 52.11: 1431–1434.

Velioglu, Y.S., Mazza, G., Gao, L. and Oomah, B.D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *Journal of Agricultural and Food Chemistry* 46.10:4113-4117.

Vianna, H. R., Cortes, S. F., Ferreira, A. J., Carpettini, L. S. A., Schnitt, M., Almeida, A. P., Massensini, A.R. and Lemos, V. S. 2006. Antiarrhythmogenic and Antioxidant effect of the flavonoid Dioclein in a model of cardiacischemia/reperfusion. *Planta Medica* 72:300-303.

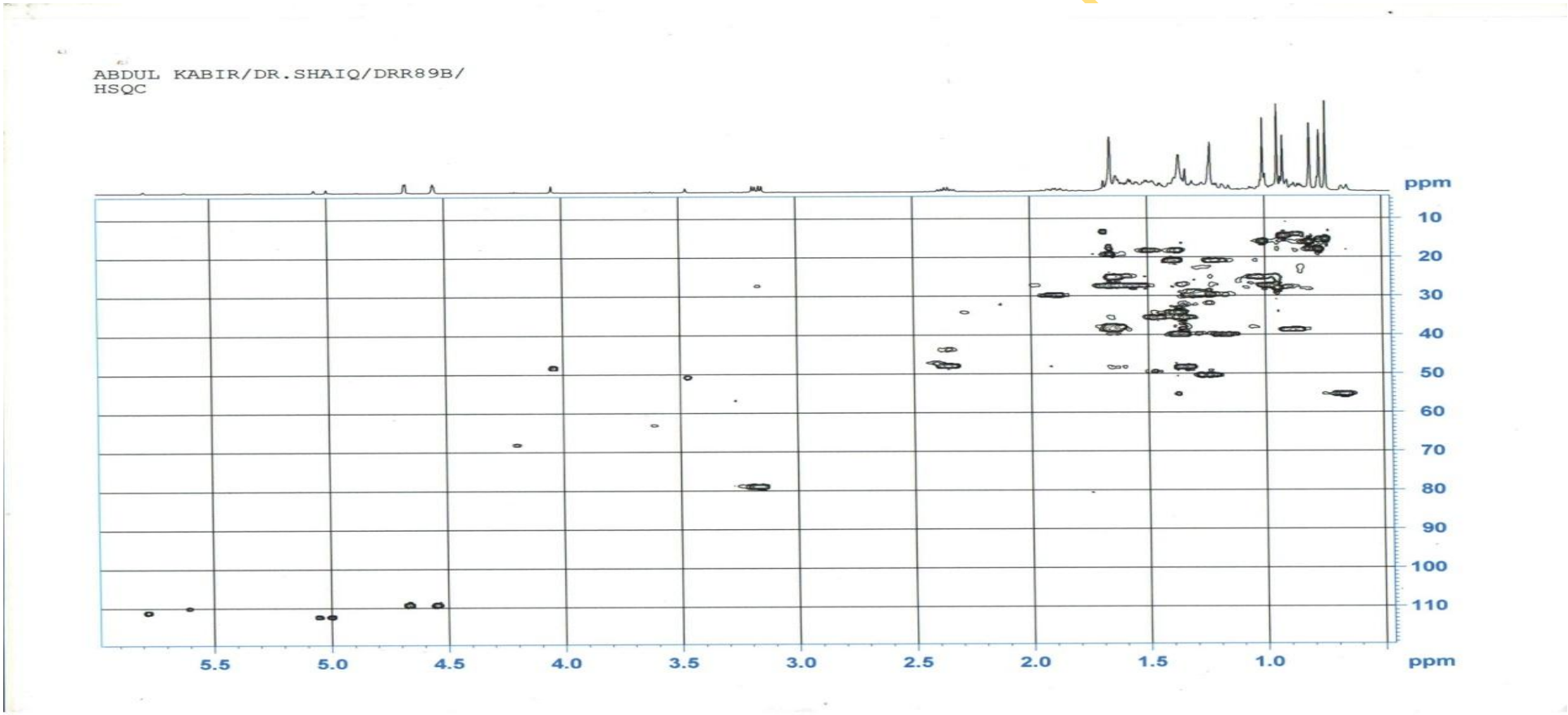
Wagner, W.W. 1999. *Manual of the flowering plants of Hawaii*. Volume1. Retrieved October 10, 2011 from www.takasago.com/cgi-bin/detail.cgi?key=C02007.

Wahidulla, S., D'souza, L. and Kamat, S.Y. 1991. Dipeptides from the red alga *Acantophora spicifera*. *Phytochemistry* 30.10: 3323-3325.

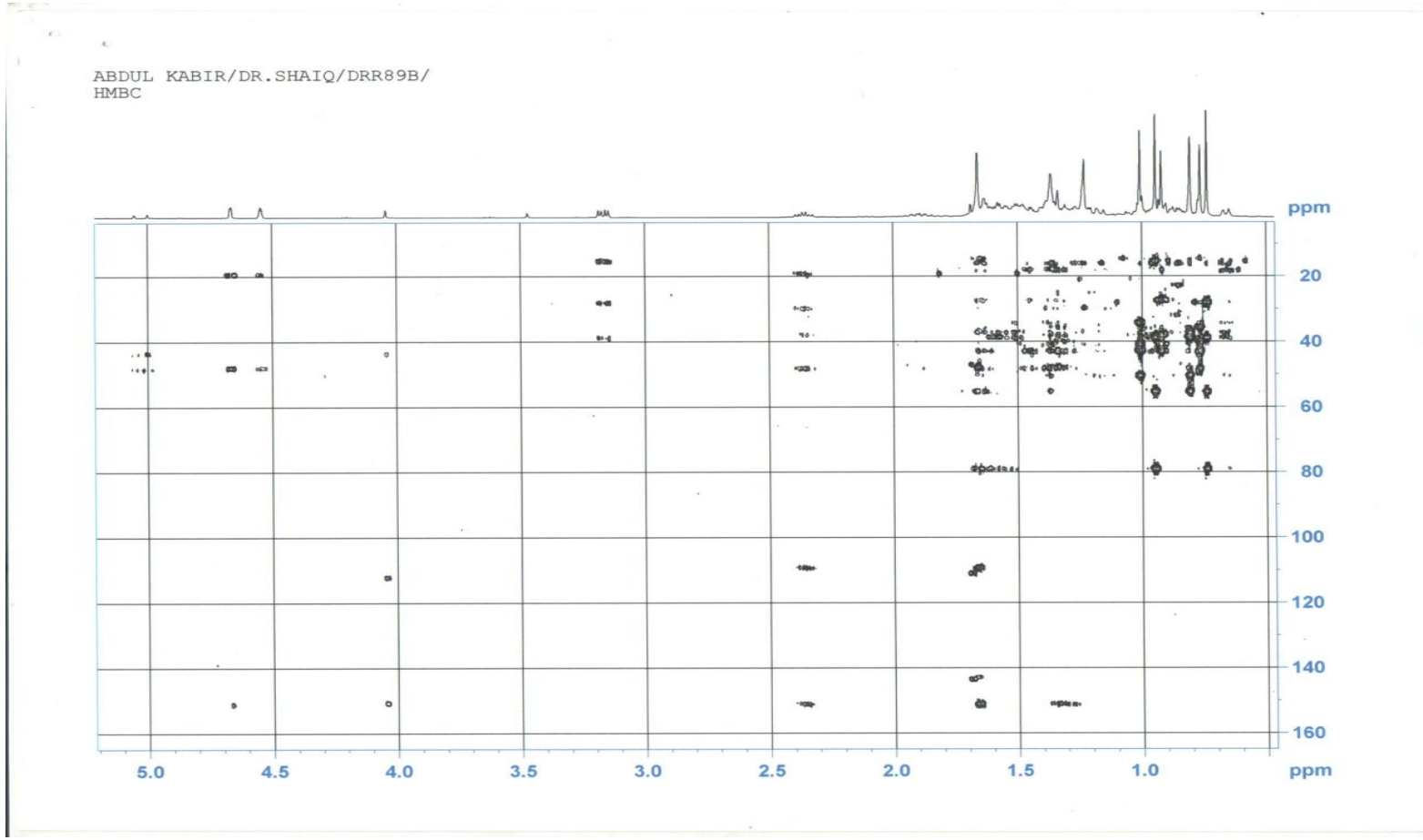
Wannes, W. A., Mhamdi, B., Sriti, J., Jemia, M. B., Ouchikh, O., Hamdaoui, G., Kchouk, M. E. and Marzouk, B. 2010. Antioxidant activities of the essential oil and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. *Food and Chemical Toxicology* 48.5: 1362-1370.

Ye, C., Lu, Y. and Wei, D. 2004. Flavonoids from *Cleistocalyx operculatus*. *Phytochemistry* 65:445-447.

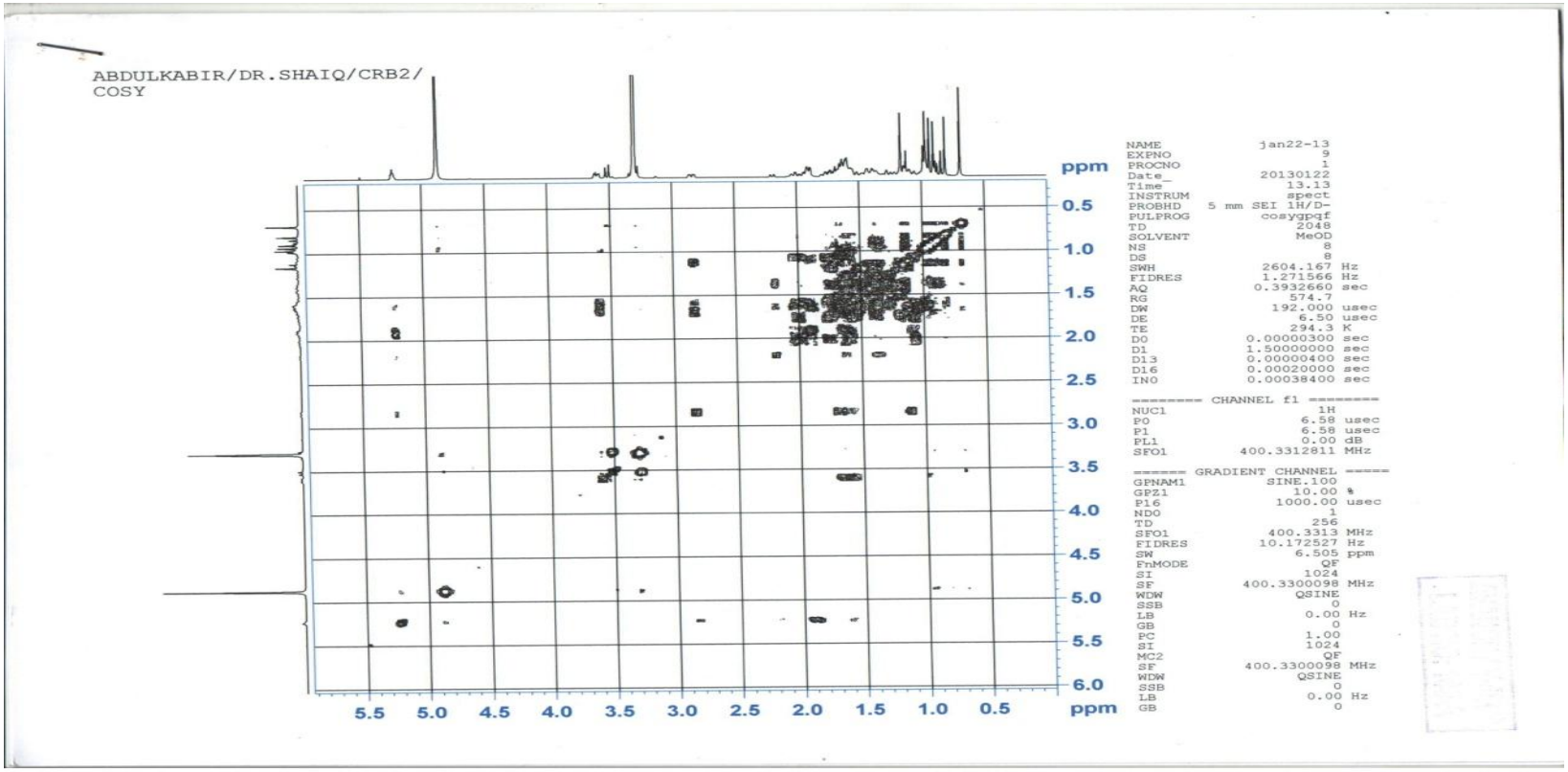
APPENDICES



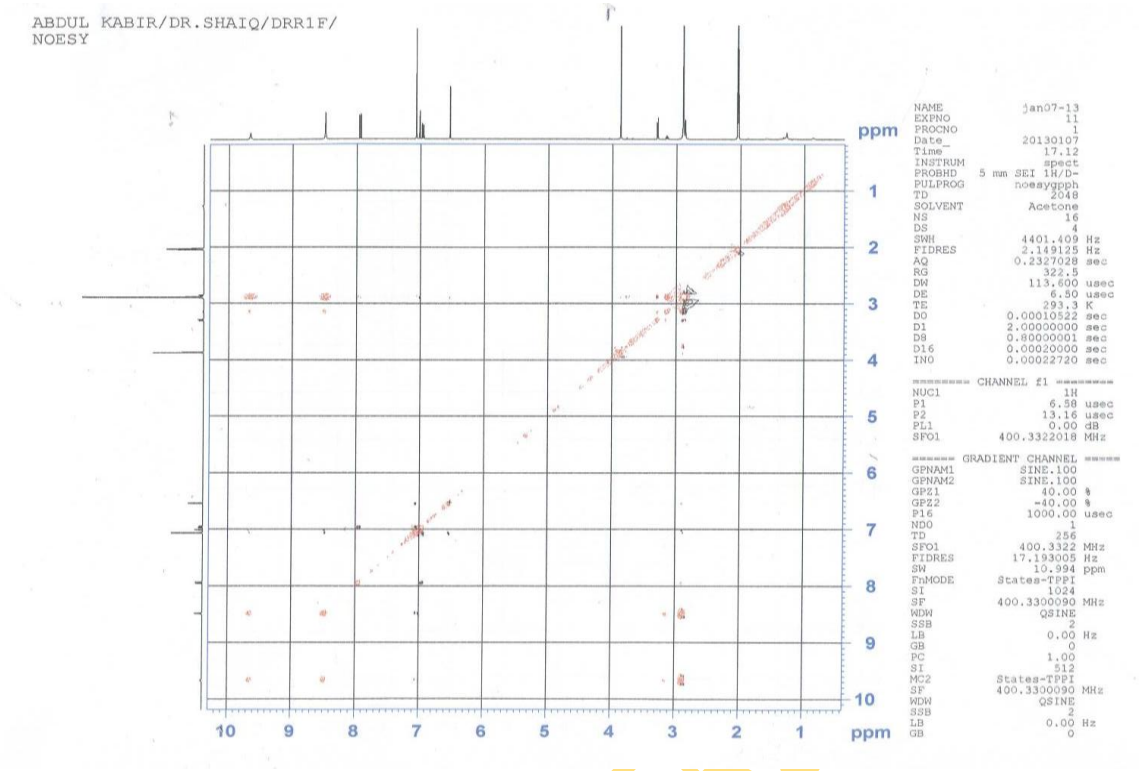
Appendix 1. HSQC spectrum of DRR-1C



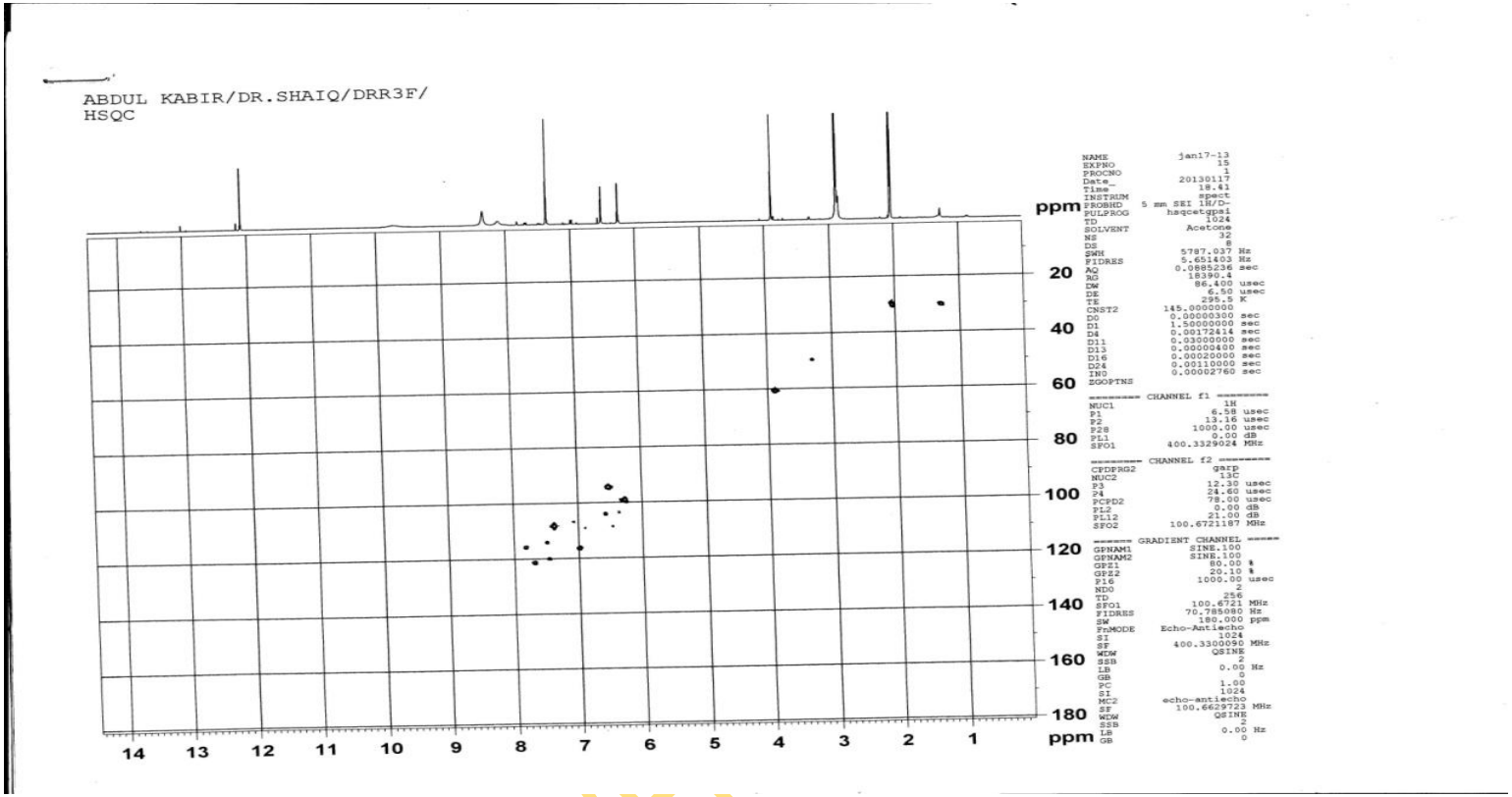
Appendix 2. HMBC spectrum of DRR-1C



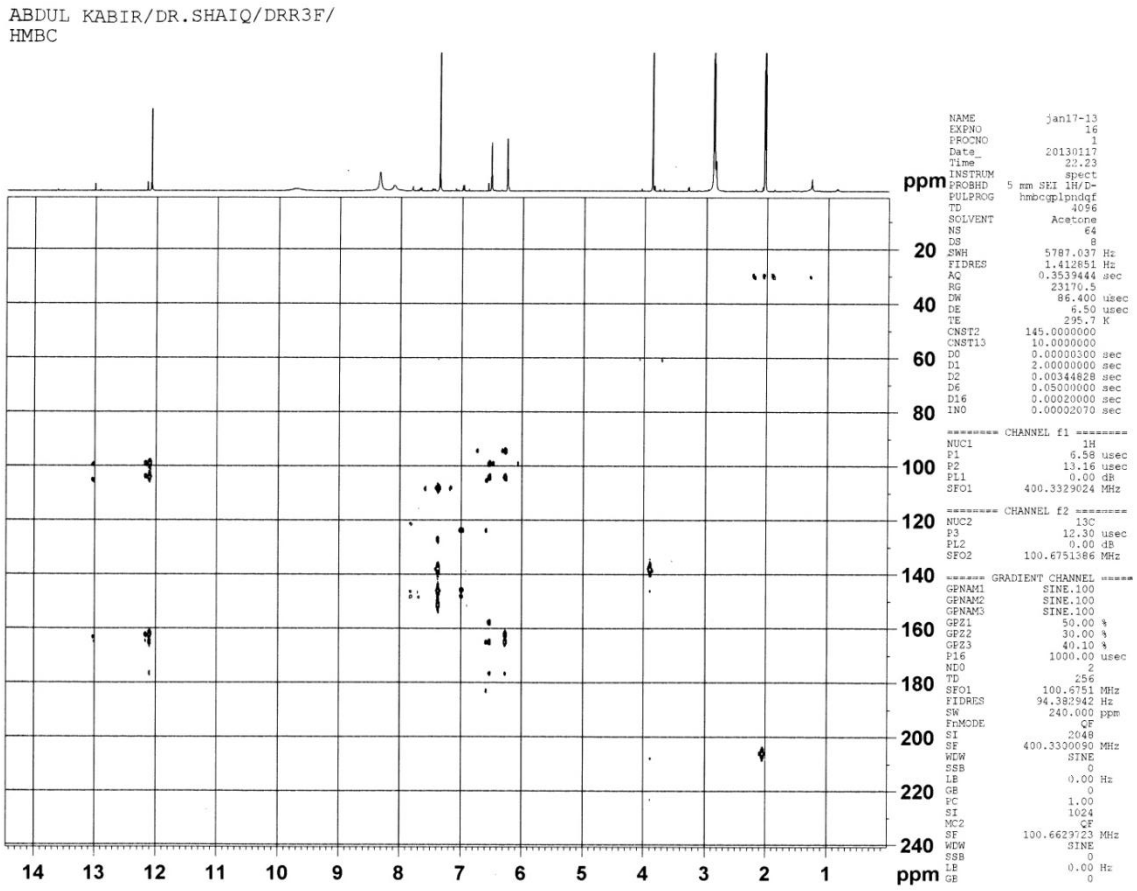
Appendix 3. COSY spectrum of DRR-1C



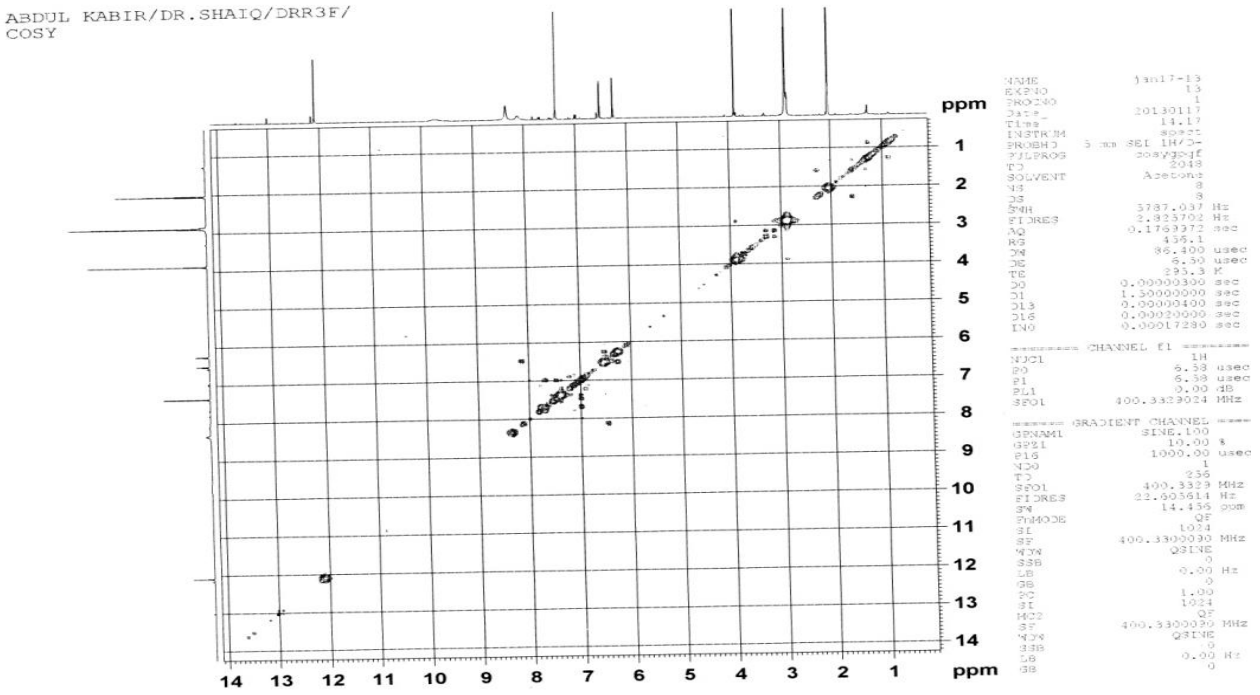
Appendix 4. NOESY spectrum of DRR-1G



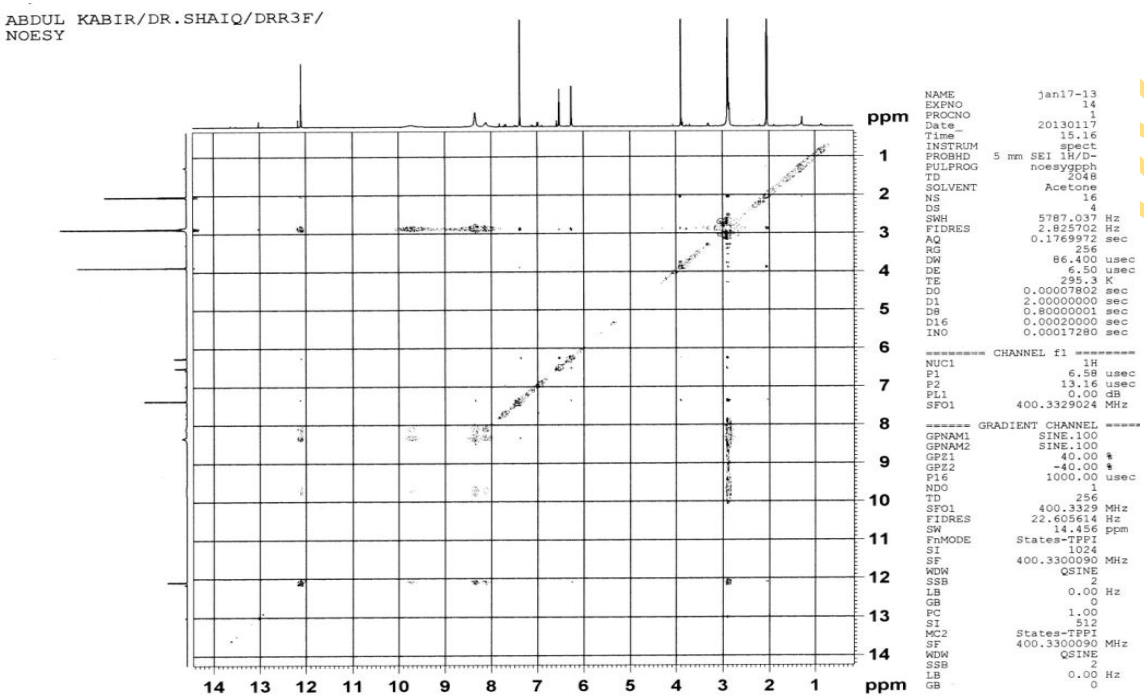
Appendix 5. HSQC spectrum of DRR-2G



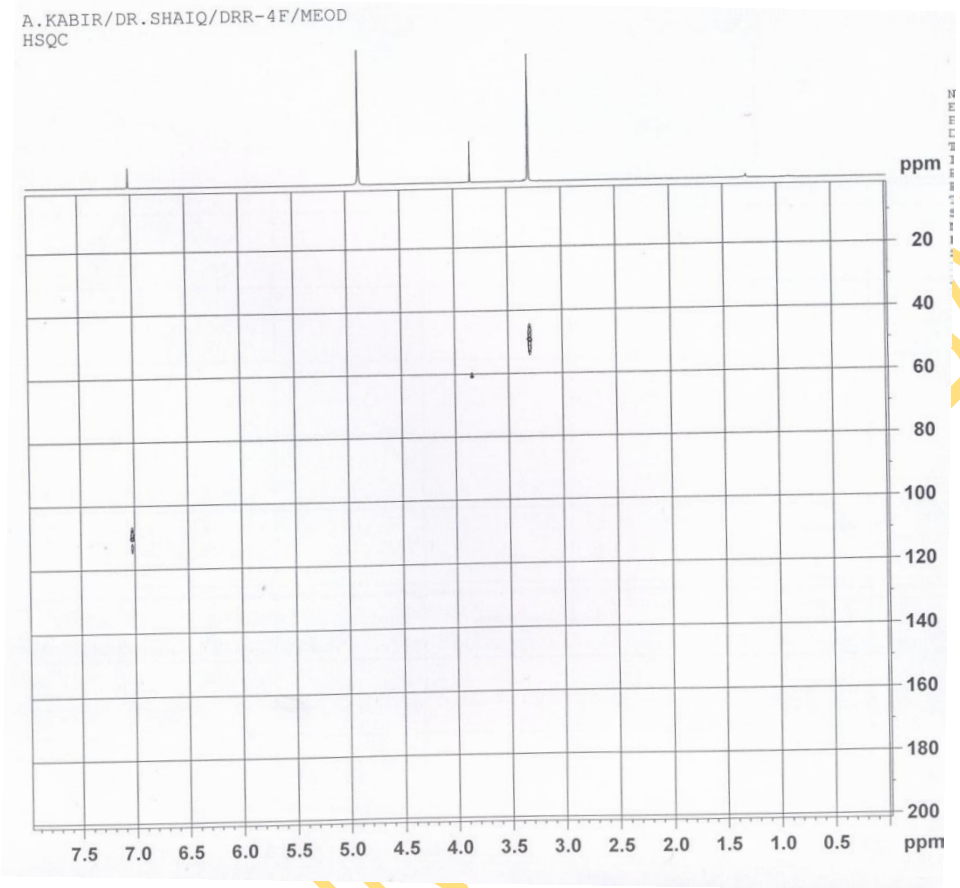
Appendix 6. HMBC spectrum of DRR-2G



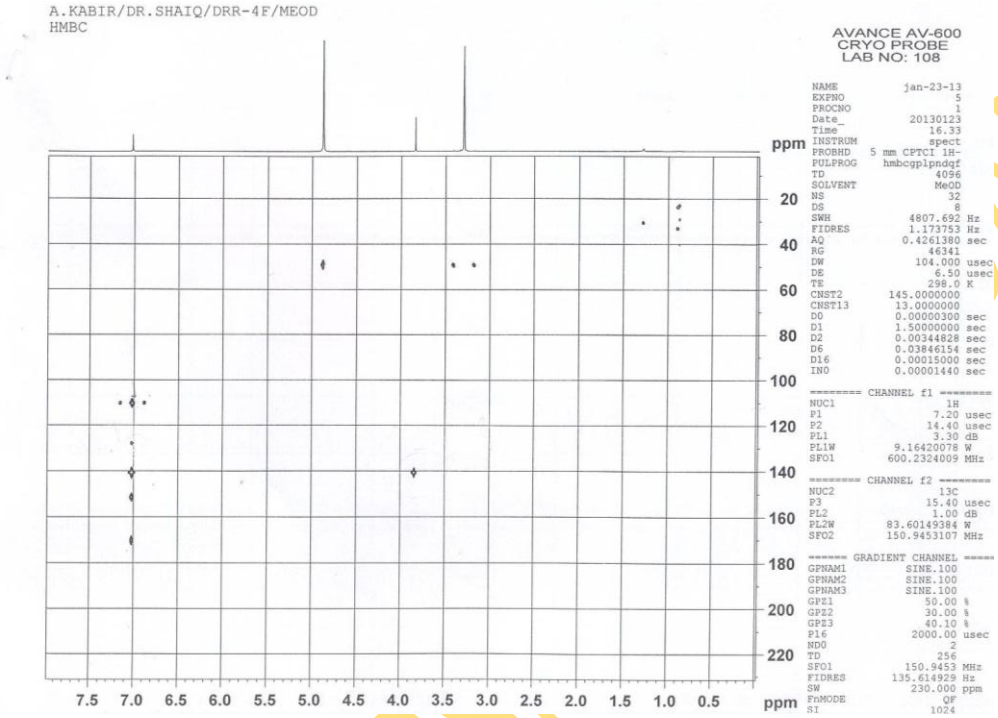
Appendix 7. COSY spectrum of DRR-2G



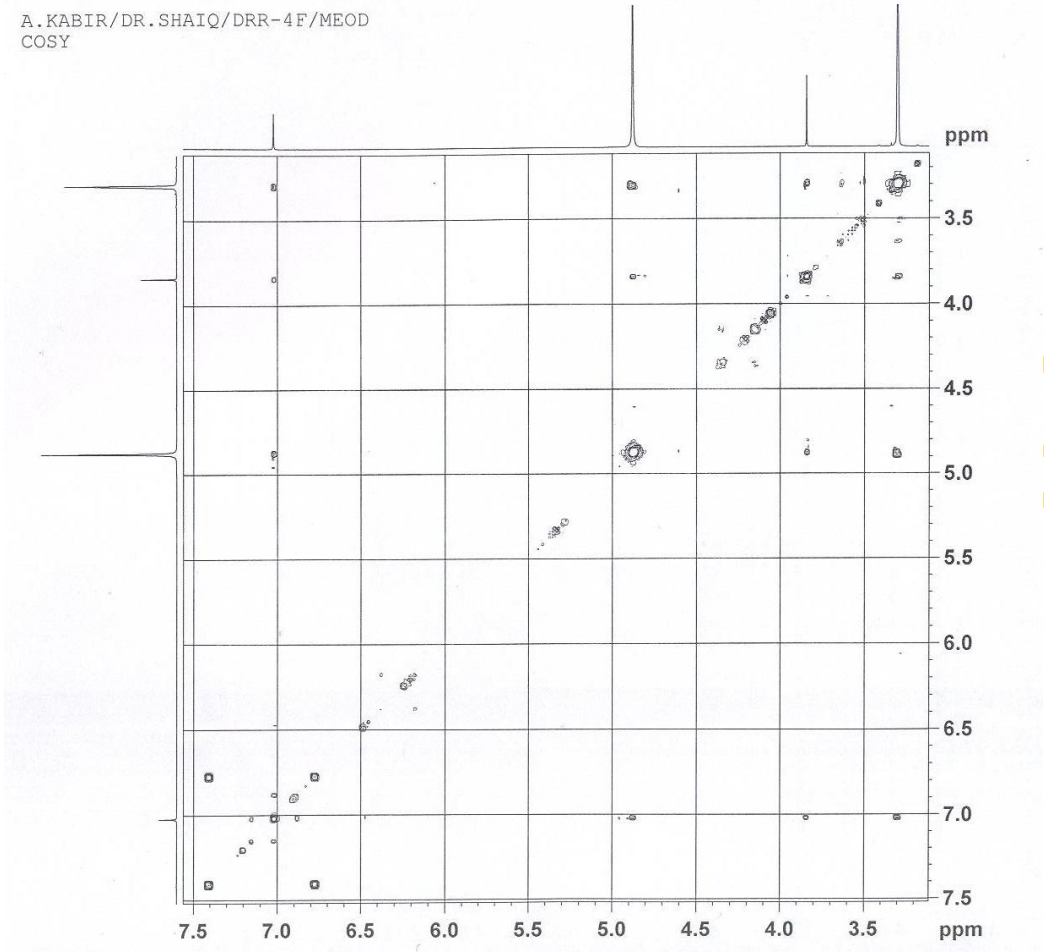
Appendix 8. NOESY spectrum of DRR-2G



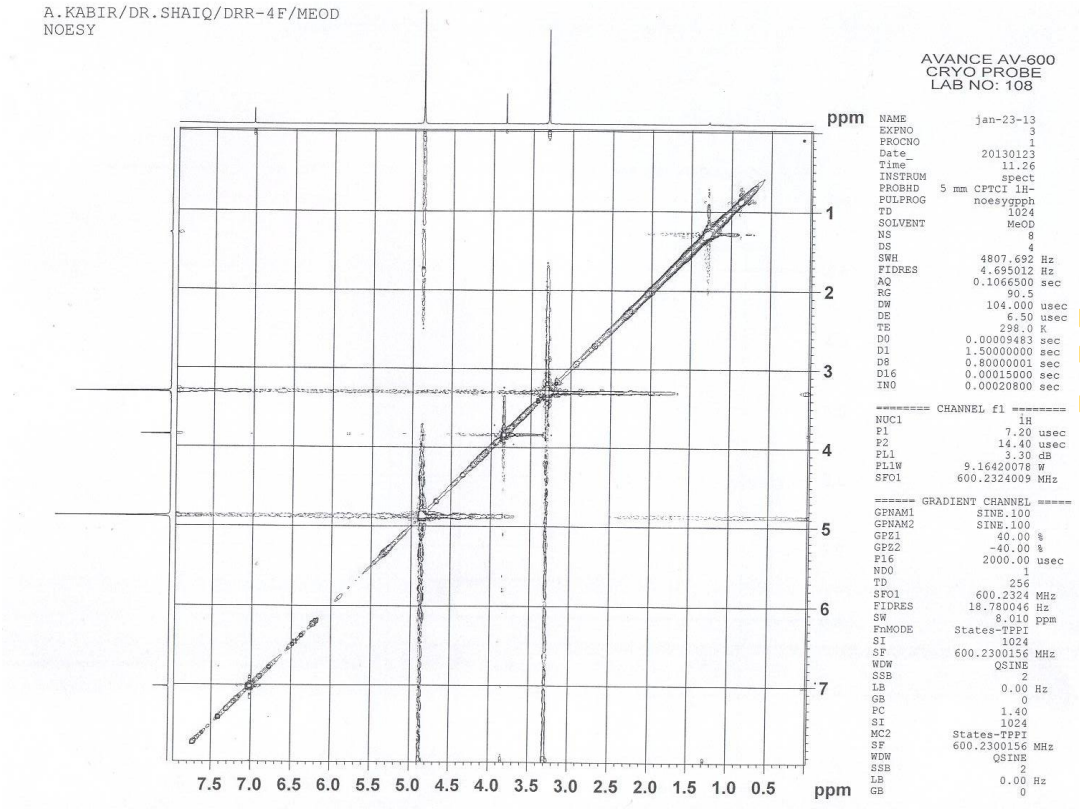
Appendix 9. HSQC spectrum of DRR 3G



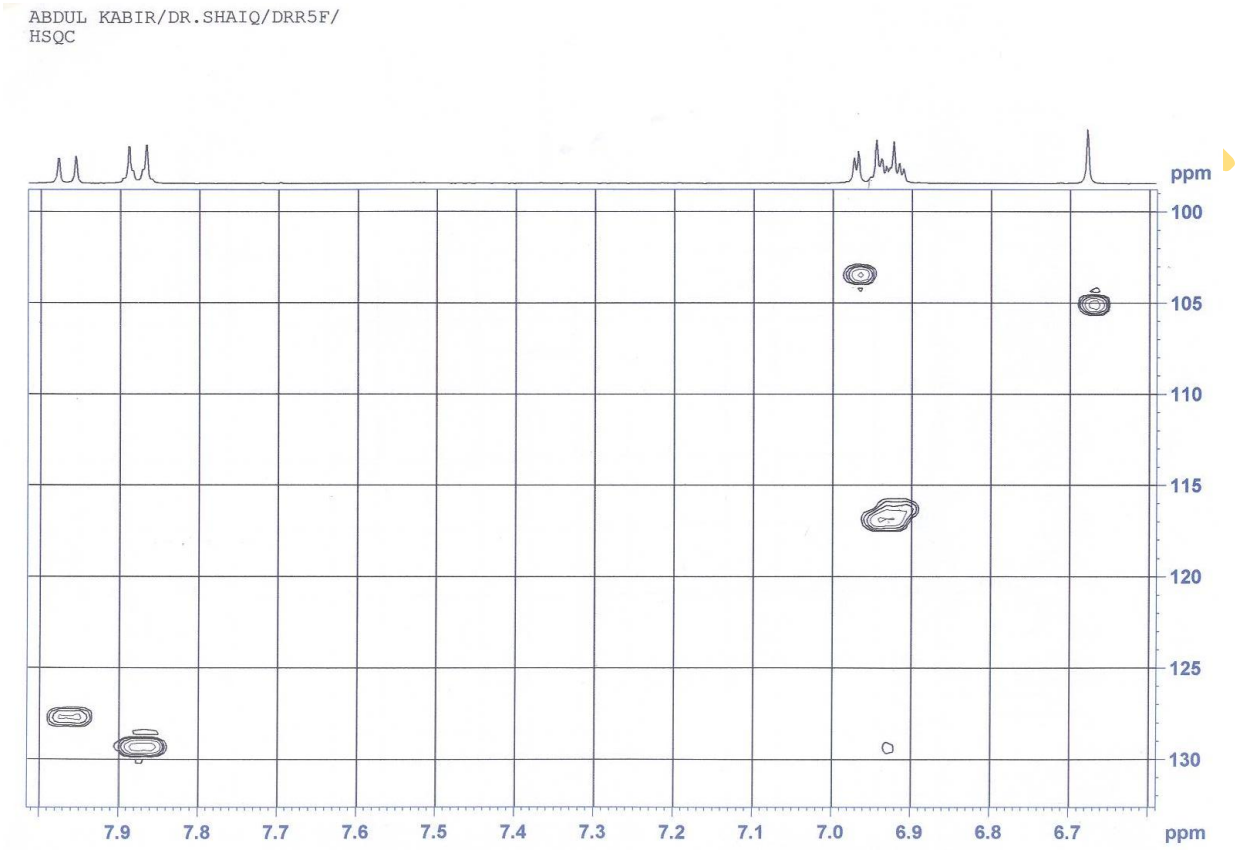
Appendix 10. HMBC spectrum of DRR-3G



Appendix 11. COSY spectrum of DRR-3G

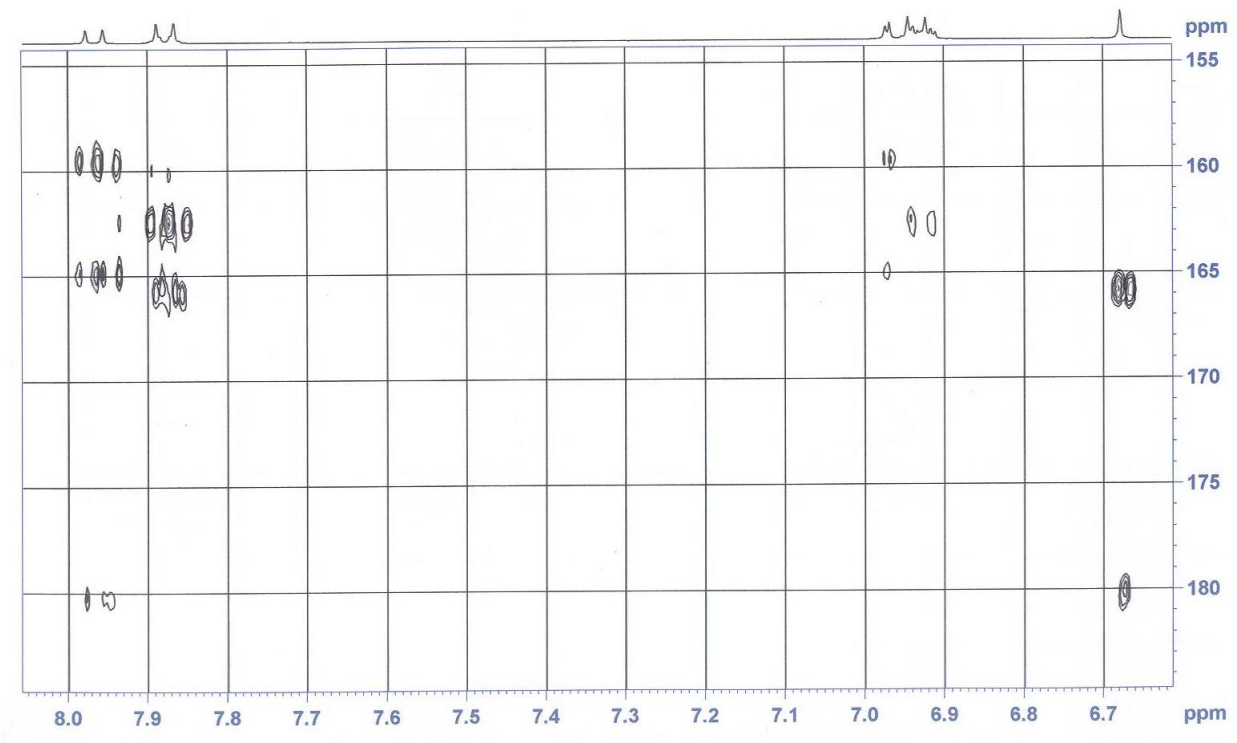


Appendix 12. NOESY spectrum of DRR-3G



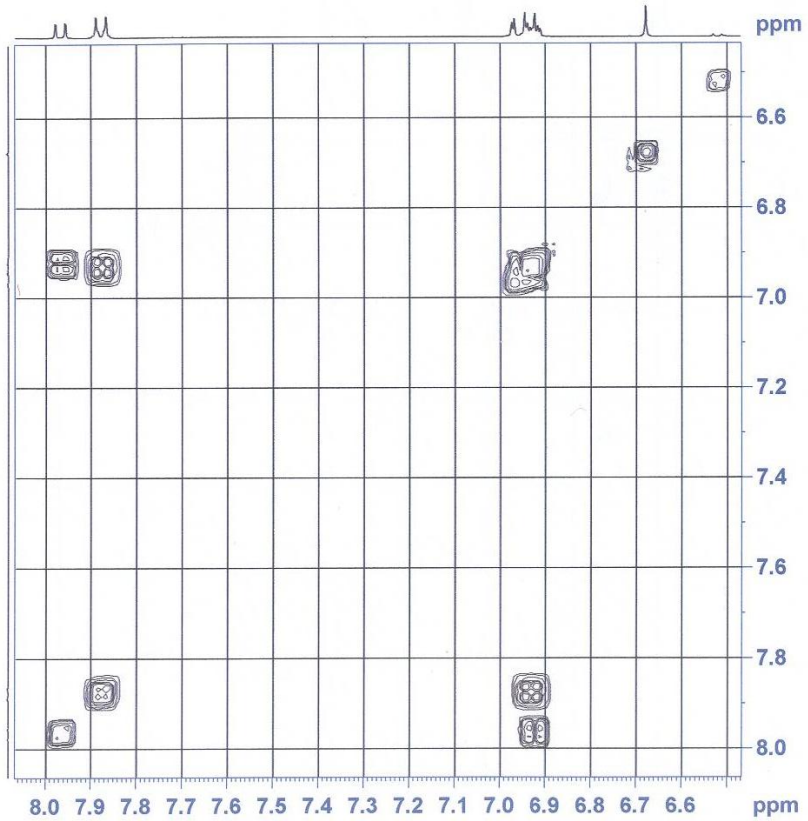
Appendix 13. HSQC spectrum of DRR-4G

ABDUL KABIR/DR.SHAIQ/DRR5F/
HMBC

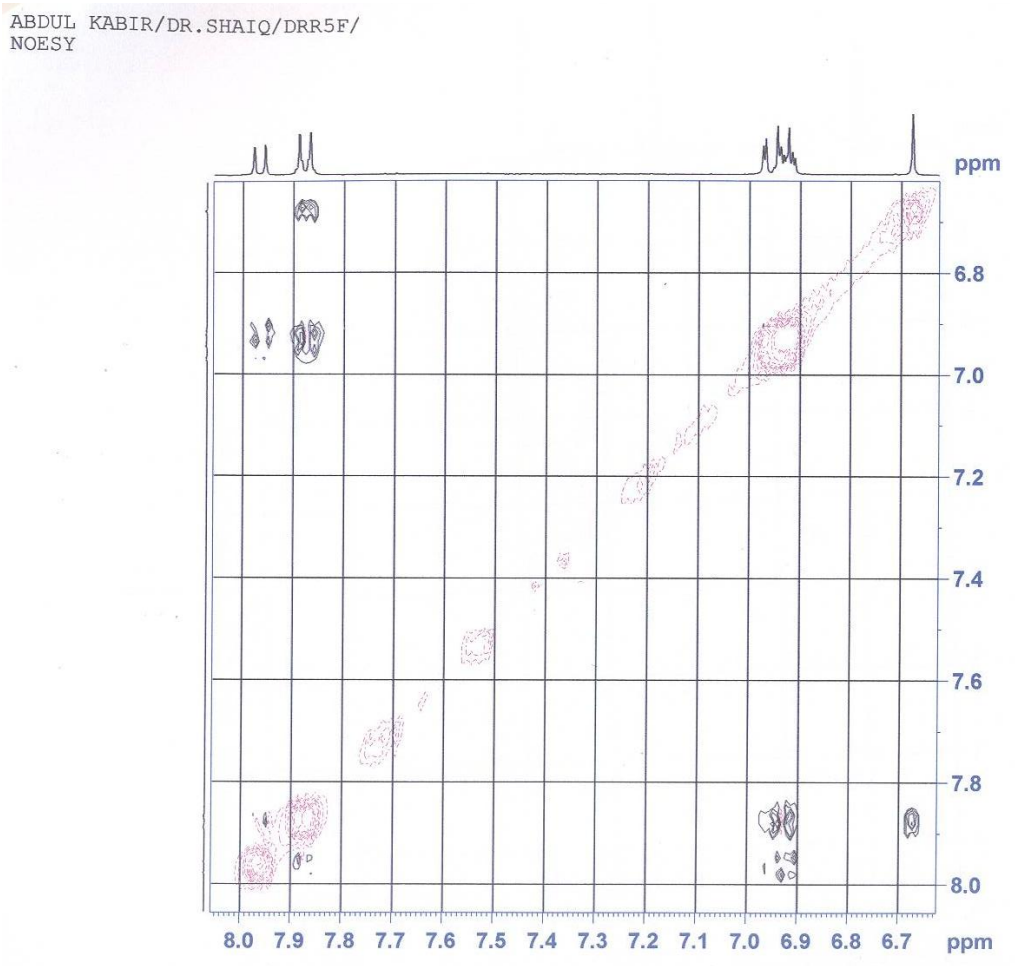


Appendix 14. HMBC spectrum of DRR-4G

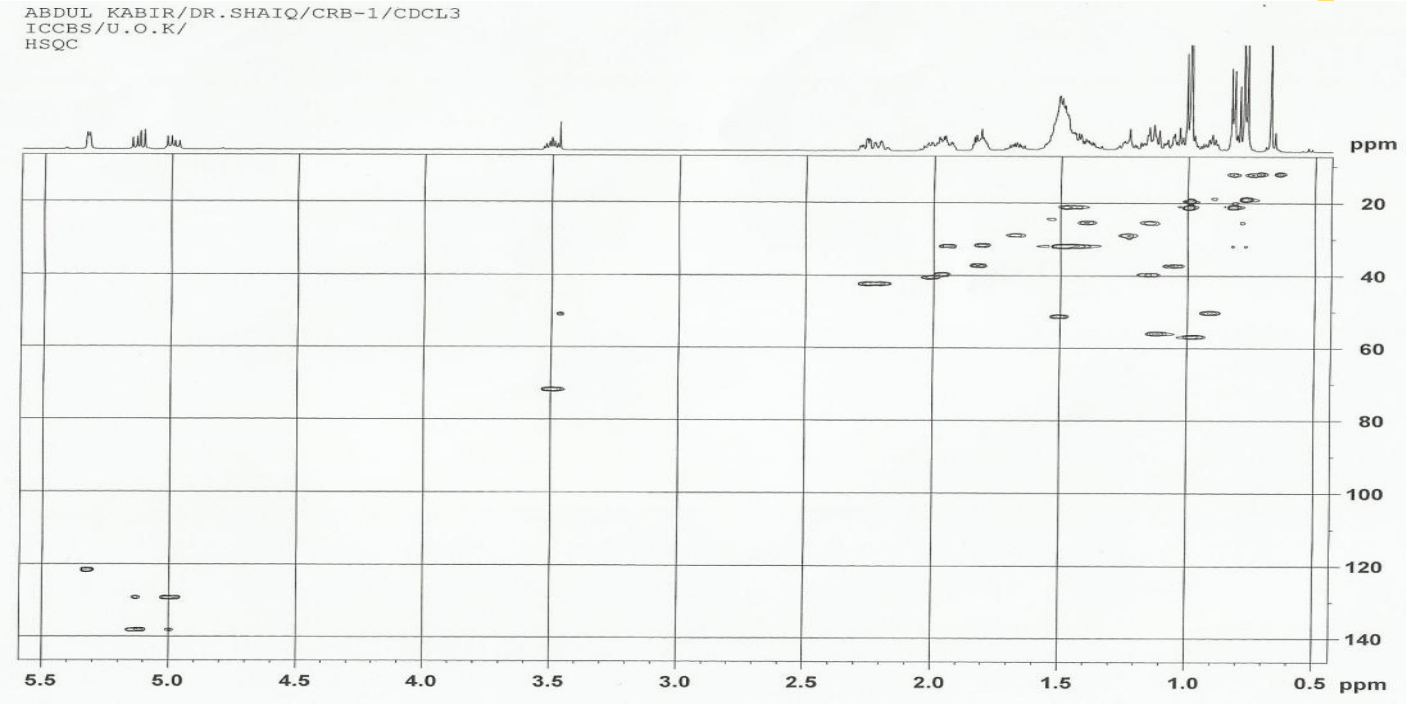
ABDUL KABIR/DR.SHAIQ/DRR5F/
COSY



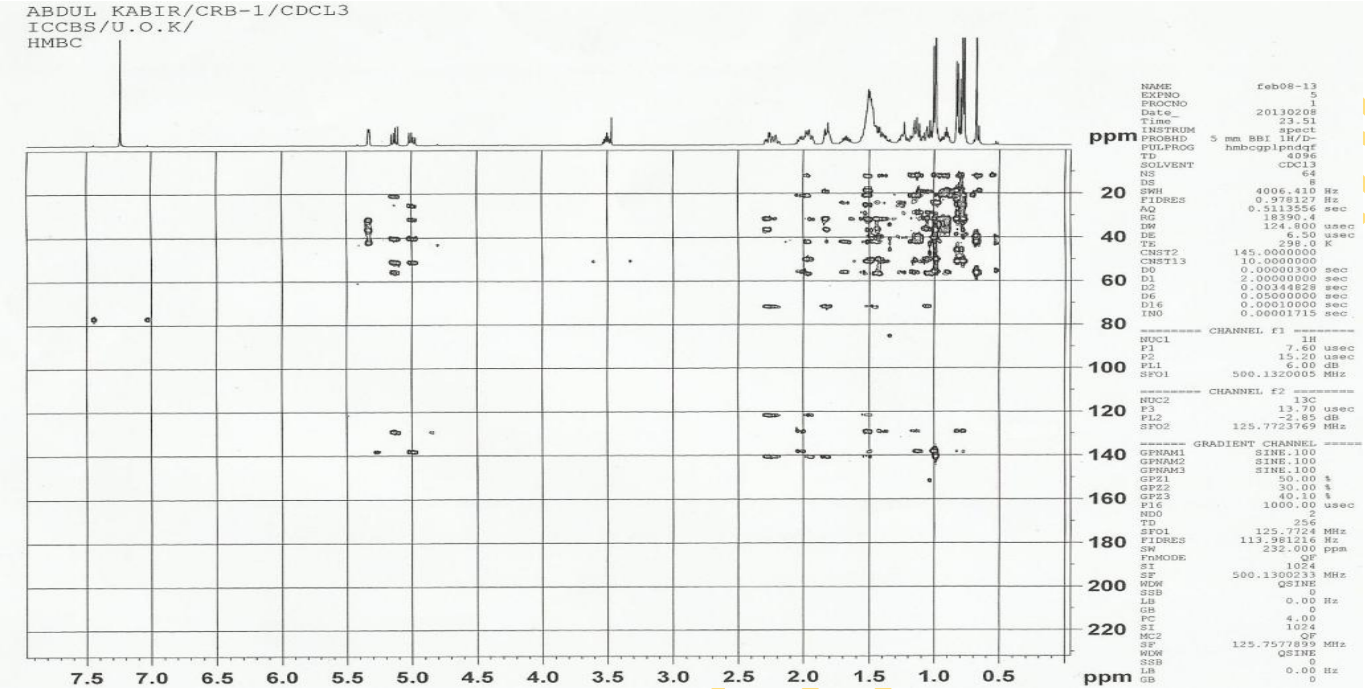
Appendix 15. COSY spectrum of DRR-4G



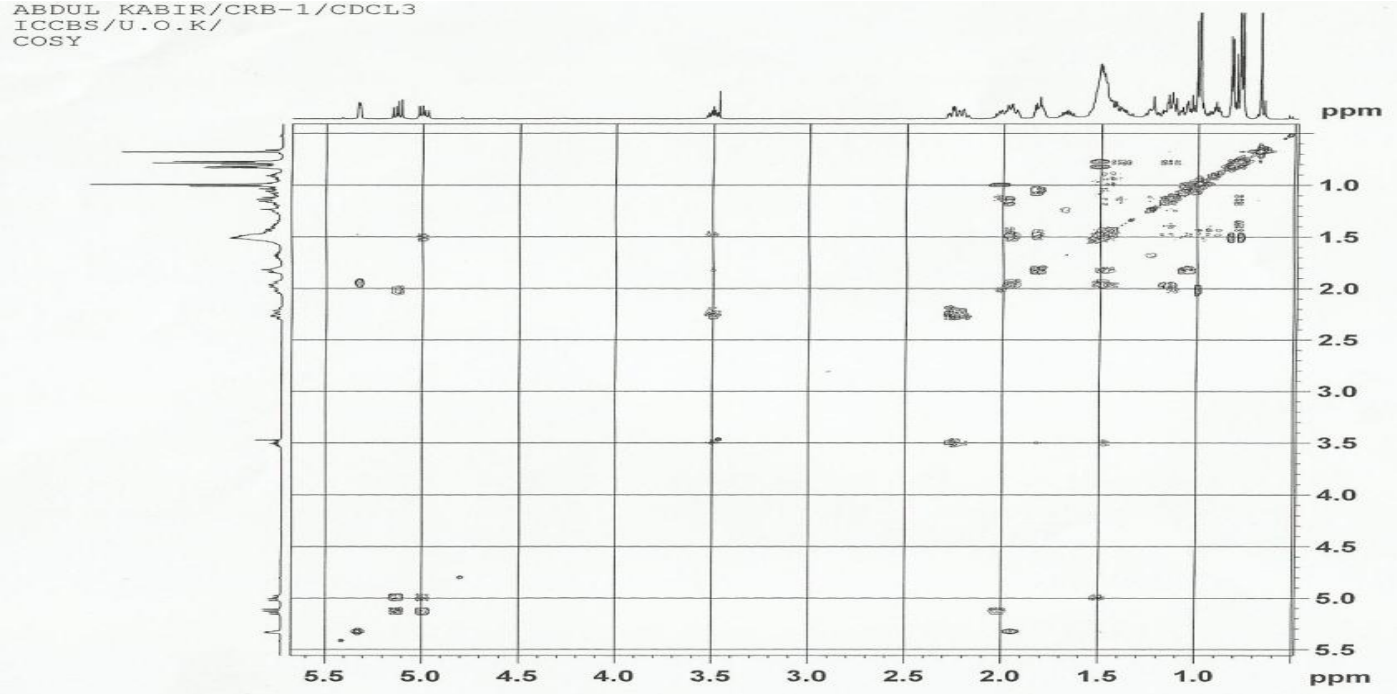
Appendix 16. NOESY spectrum of DRR-4G



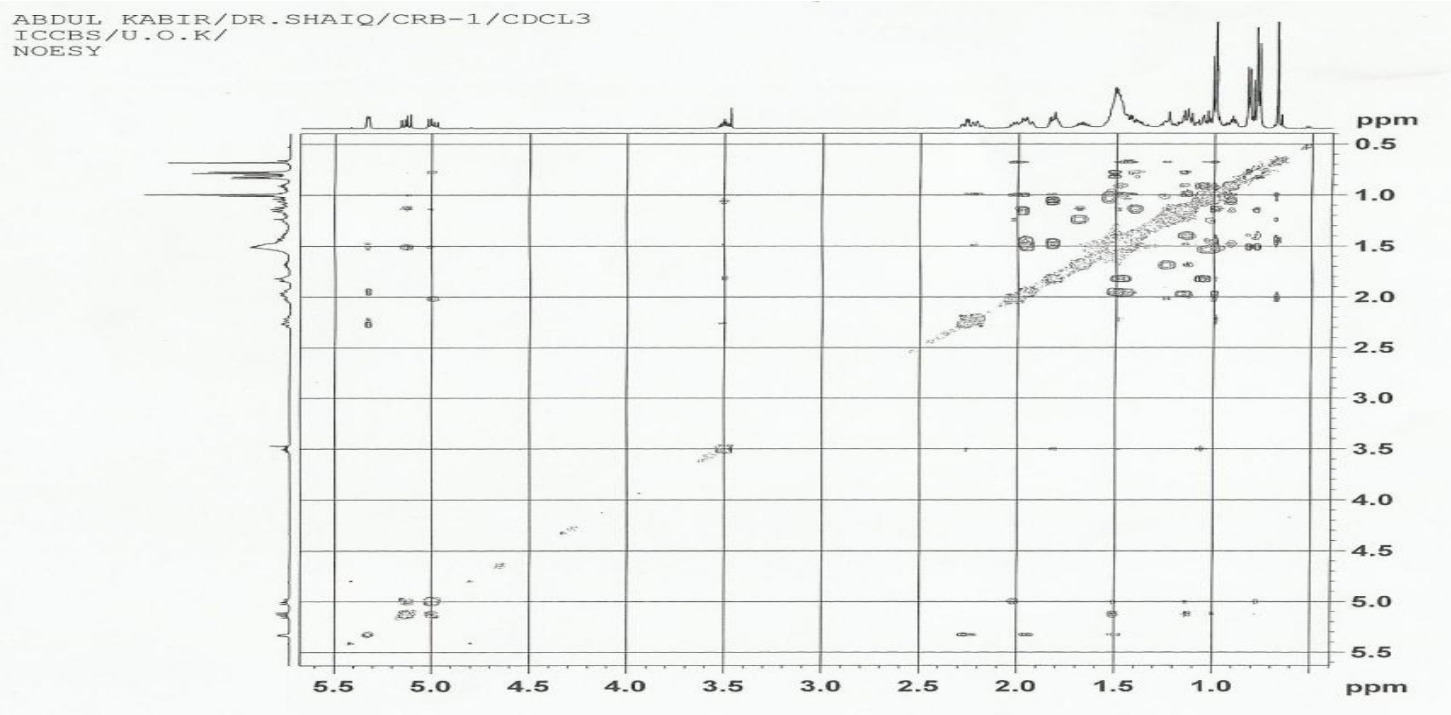
Appendix 17. HSQC spectrum of CRB-1



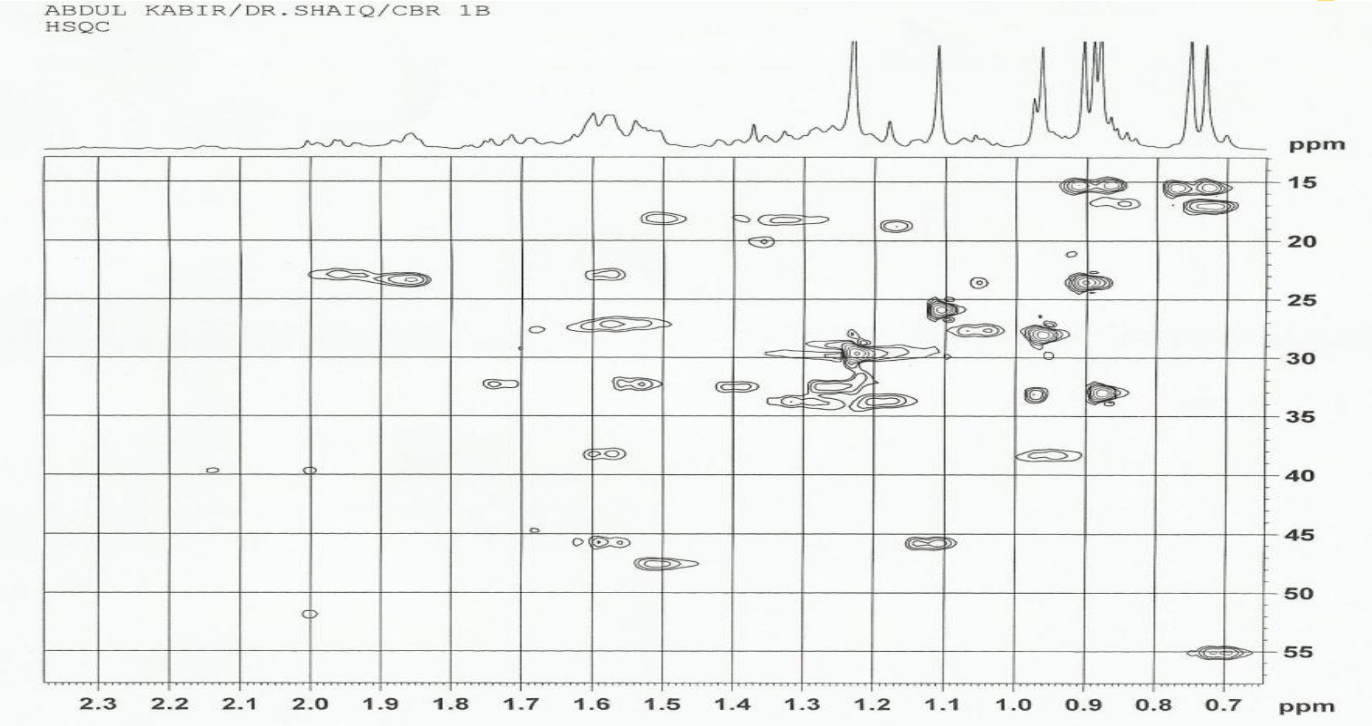
Appendix 18. HMBC spectrum of CRB-1



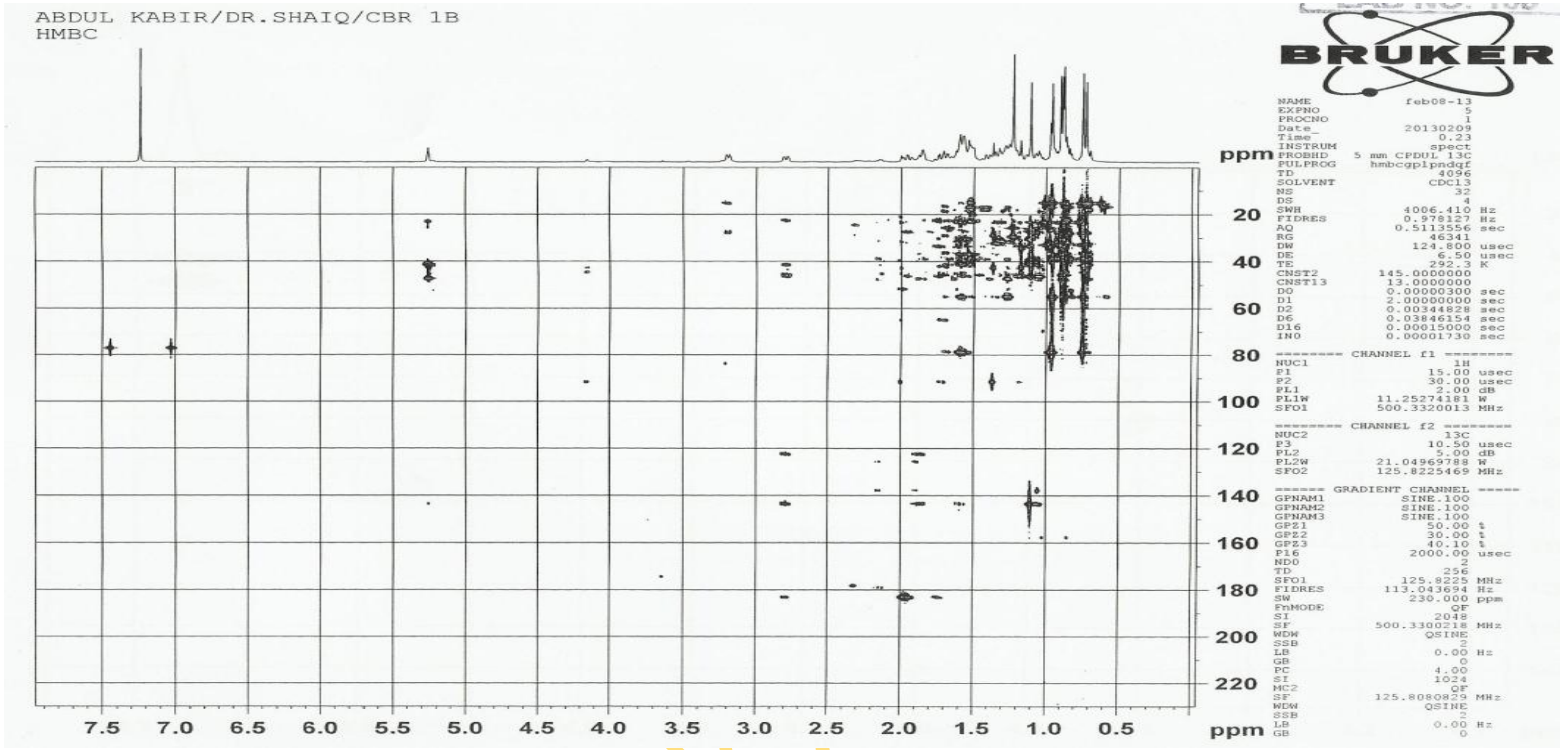
Appendix 19. COSY spectrum of CRB-1



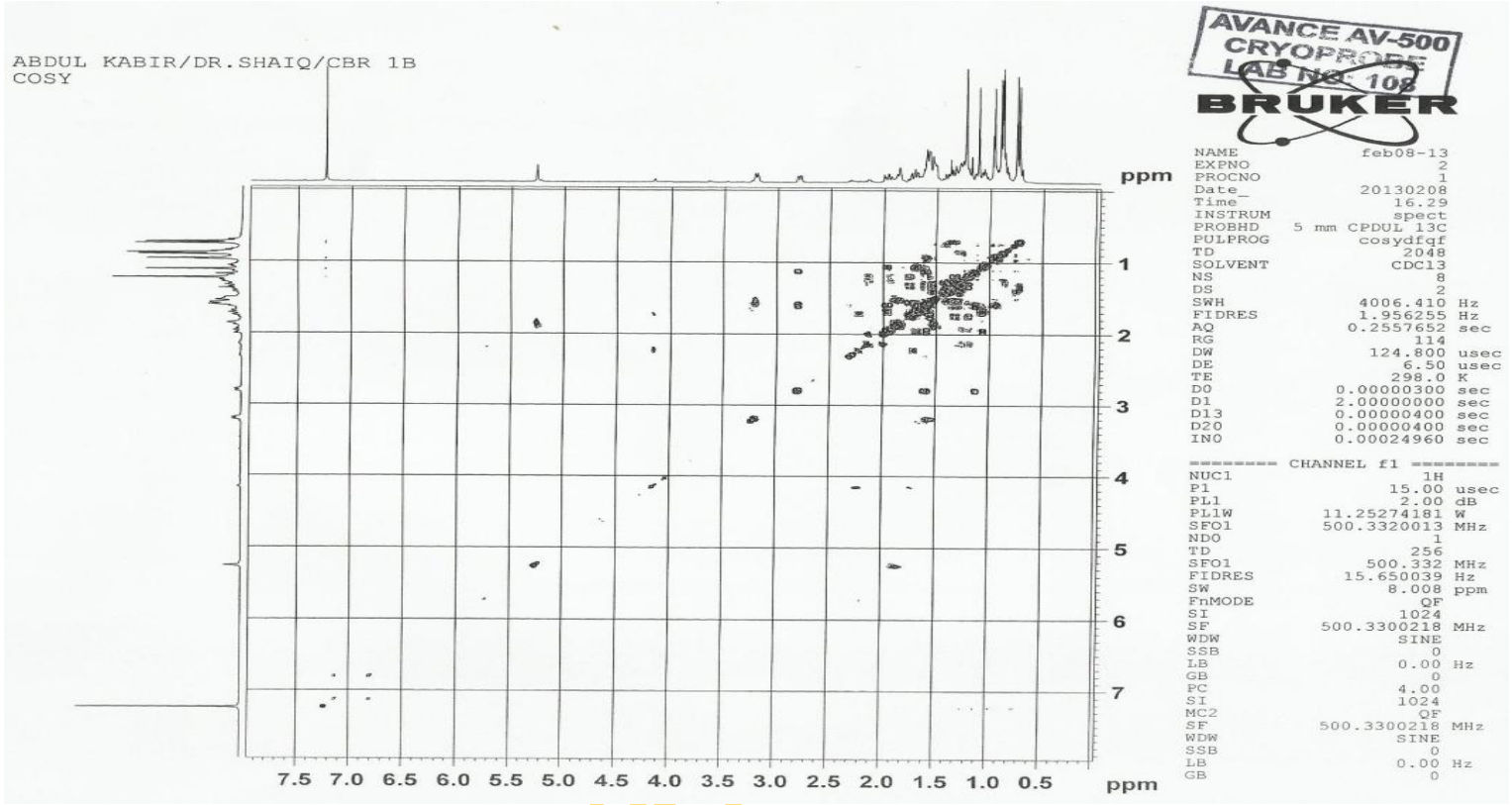
Appendix 20. NOESY spectrum of CRB-1



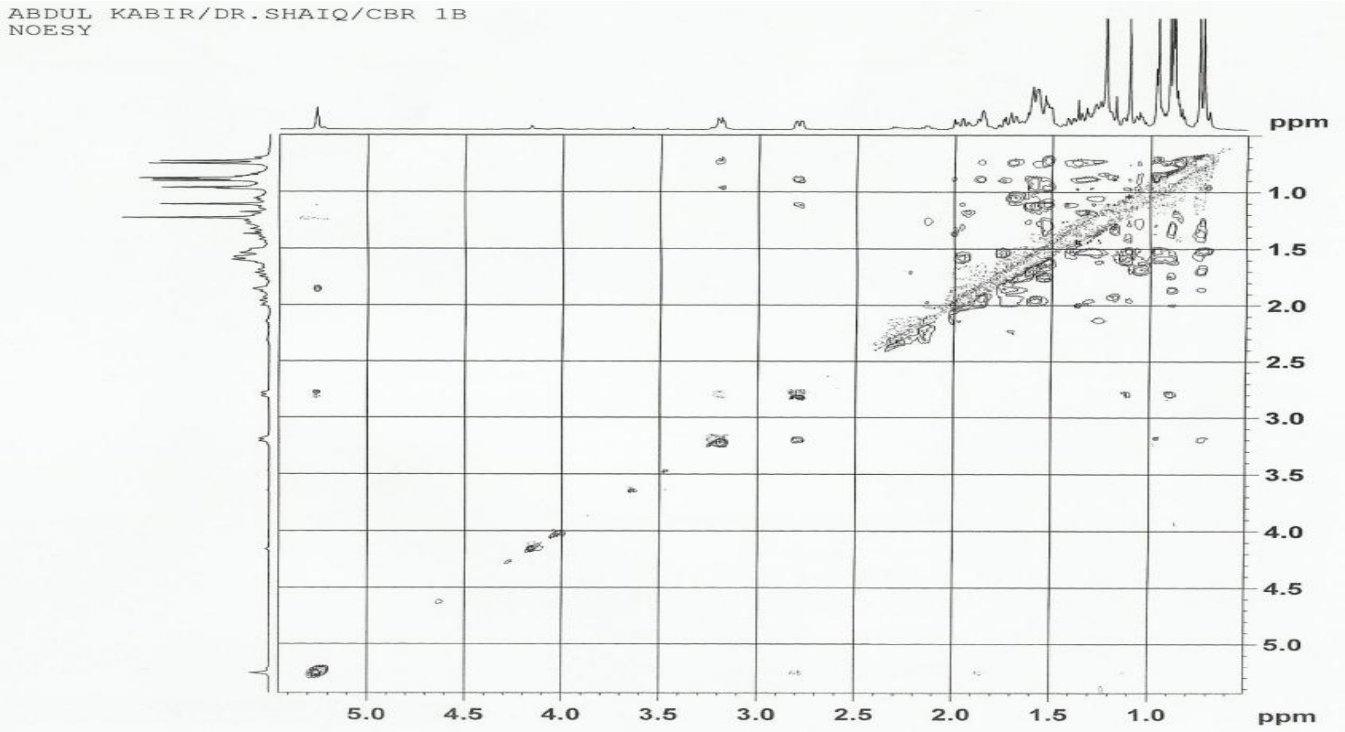
Appendix 21. HSQC spectrum of CRB-1B



Appendix 22. HMBC spectrum of CRB-1B



Appendix 23. COSY spectrum of CRB-1B



Appendix 24. NOESY spectrum of CRB-1B

Appendix 25. Crystal data and structure refinement for CRB-2

Table 1. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for a.

	x	y	z	U(eq)
O(1)	4171(3)	2814(2)	17093(2)	41(1)
O(2)	2580(2)	-3133(2)	14065(2)	51(1)
O(3)	806(2)	-3343(2)	13995(2)	57(1)
O(4)	1518(2)	1922(2)	17659(2)	41(1)
O(5)	824(3)	-2960(2)	12095(2)	41(1)
O(6)	4196(2)	3190(2)	9002(2)	56(1)
O(7)	2421(2)	2990(2)	9059(2)	51(1)
O(8)	3482(2)	-2066(2)	12667(2)	42(1)
C(1)	4610(3)	576(2)	16609(3)	42(1)
C(2)	4876(3)	1476(2)	16671(3)	41(1)
C(3)	3886(3)	1966(2)	16987(3)	35(1)
C(4)	2899(3)	1859(2)	16298(3)	33(1)
C(5)	2678(3)	920(2)	16229(3)	31(1)
C(6)	1649(3)	679(3)	15654(3)	42(1)
C(7)	1329(3)	-188(3)	15910(4)	45(1)
C(8)	2220(3)	-834(2)	15711(3)	35(1)
C(9)	1940(3)	-1674(2)	16238(3)	36(1)
C(10)	858(3)	-2018(3)	15822(3)	44(1)
C(11)	687(3)	-2928(3)	16015(3)	43(1)
C(12)	1630(3)	-3436(2)	15594(3)	35(1)
C(13)	1428(3)	-4359(3)	15695(3)	44(1)
C(14)	1540(4)	-4666(3)	16762(3)	51(1)
C(15)	2632(4)	-4454(3)	17240(4)	54(1)
C(16)	2787(4)	-3533(3)	17172(3)	50(1)

C(17)	2694(3)	-3186(2)	16113(3)	35(1)
C(18)	2882(3)	-2267(2)	16083(3)	35(1)
C(19)	3887(3)	-2002(3)	15953(3)	43(1)
C(20)	4231(3)	-1133(3)	15905(4)	55(1)
C(21)	3313(3)	-525(2)	16135(3)	37(1)
C(22)	3645(3)	381(2)	15923(3)	35(1)
C(23)	4012(4)	527(3)	14835(3)	51(1)
C(24)	2288(4)	-959(3)	14574(3)	54(1)
C(25)	1789(4)	-1578(3)	17383(3)	50(1)
C(26)	1733(3)	-3280(2)	14478(3)	36(1)
C(27)	2607(5)	-4707(4)	18328(4)	75(2)
C(28)	3581(4)	-4915(3)	16750(5)	77(2)
C(29)	3061(4)	2290(3)	15295(3)	50(1)
C(30)	1907(3)	2286(2)	16761(3)	39(1)
C(31)	388(3)	-716(2)	11610(3)	42(1)
C(32)	121(3)	-1617(2)	11671(3)	43(1)
C(33)	1107(3)	-2108(2)	11990(3)	35(1)
C(34)	2097(3)	-2006(2)	11305(3)	33(1)
C(35)	2320(3)	-1067(2)	11234(3)	33(1)
C(36)	3343(3)	-829(3)	10661(3)	43(1)
C(37)	3672(3)	40(3)	10919(4)	45(1)
C(38)	2788(3)	683(2)	10709(3)	36(1)
C(39)	3070(3)	1525(2)	11238(3)	36(1)
C(40)	4148(3)	1871(3)	10823(3)	44(1)
C(41)	4322(3)	2777(3)	11015(3)	43(1)
C(42)	3380(3)	3287(2)	10595(3)	35(1)
C(43)	3578(3)	4208(3)	10698(3)	44(1)
C(44)	3473(4)	4522(3)	11756(3)	53(1)
C(45)	2376(4)	4307(3)	12234(4)	53(1)

C(46)	2237(4)	3388(3)	12176(3)	49(1)
C(47)	2321(3)	3040(2)	11115(3)	37(1)
C(48)	2129(3)	2121(2)	11080(3)	34(1)
C(49)	1125(3)	1857(3)	10950(3)	43(1)
C(50)	764(3)	985(3)	10902(4)	54(1)
C(51)	1686(3)	377(2)	11137(3)	35(1)
C(52)	1346(3)	-524(2)	10921(3)	35(1)
C(53)	981(4)	-670(3)	9836(3)	51(1)
C(54)	2704(4)	813(3)	9577(3)	53(1)
C(55)	3232(4)	1430(3)	12381(3)	50(1)
C(56)	3269(3)	3130(2)	9479(3)	36(1)
C(57)	1417(5)	4763(4)	11743(5)	89(2)
C(58)	2389(5)	4571(4)	13327(4)	81(2)
C(59)	1929(4)	-2433(3)	10299(3)	49(1)
C(60)	3083(3)	-2432(2)	11772(3)	40(1)

U(eq) is defined as one third of the trace of the orthogonalized Uij tensor

Table 2. Bond lengths [A] and angles [deg] for a.

Bond lengths [A]	Angles [deg]
O(1)-C(3)	1.443(5)
O(1)-H(1A)	0.76(4)
O(2)-C(26)	1.206(4)
O(3)-C(26)	1.312(5)
O(3)-H(3B)	1.01(6)
O(4)-C(30)	1.430(5)
O(4)-H(4A)	0.82(5)
O(5)-C(33)	1.449(5)
O(5)-H(5A)	0.81(4)
O(6)-C(56)	1.313(5)
O(6)-H(6C)	0.93(5)
O(7)-C(56)	1.205(4)
O(8)-C(60)	1.430(5)
O(8)-H(8A)	0.90(6)
C(1)-C(2)	1.516(5)
C(1)-C(22)	1.533(5)
C(1)-H(1B)	0.9700
C(1)-H(1C)	0.9700
C(2)-C(3)	1.519(5)
C(2)-H(2A)	0.9700
C(2)-H(2B)	0.9700
C(3)-C(4)	1.533(5)
C(3)-H(3A)	0.9800
C(4)-C(29)	1.536(6)
C(4)-C(30)	1.539(5)
C(4)-C(5)	1.567(5)
C(5)-C(6)	1.533(5)

C(5)-C(22)	1.539(5)
C(5)-H(5B)	0.9800
C(6)-C(7)	1.516(6)
C(6)-H(6A)	0.9700
C(6)-H(6B)	0.9700
C(7)-C(8)	1.547(5)
C(7)-H(7A)	0.9700
C(7)-H(7B)	0.9700
C(8)-C(21)	1.543(5)
C(8)-C(24)	1.544(6)
C(8)-C(9)	1.588(5)
C(9)-C(18)	1.528(5)
C(9)-C(10)	1.547(5)
C(9)-C(25)	1.559(5)
C(10)-C(11)	1.530(6)
C(10)-H(10A)	0.9700
C(10)-H(10B)	0.9700
C(11)-C(12)	1.536(5)
C(11)-H(11A)	0.9700
C(11)-H(11B)	0.9700
C(12)-C(26)	1.528(5)
C(12)-C(17)	1.536(5)
C(12)-C(13)	1.542(6)
C(13)-C(14)	1.525(6)
C(13)-H(13A)	0.9700
C(13)-H(13B)	0.9700
C(14)-C(15)	1.527(6)
C(14)-H(14A)	0.9700
C(14)-H(14B)	0.9700

C(15)-C(27)	1.520(7)
C(15)-C(16)	1.526(6)
C(15)-C(28)	1.539(7)
C(16)-C(17)	1.537(5)
C(16)-H(16A)	0.9700
C(16)-H(16B)	0.9700
C(17)-C(18)	1.526(5)
C(17)-H(17A)	0.9800
C(18)-C(19)	1.322(5)
C(19)-C(20)	1.489(6)
C(19)-H(19A)	0.9300
C(20)-C(21)	1.537(5)
C(20)-H(20A)	0.9700
C(20)-H(20B)	0.9700
C(21)-C(22)	1.568(5)
C(21)-H(21A)	0.9800
C(22)-C(23)	1.550(6)
C(23)-H(23A)	0.9600
C(23)-H(23B)	0.9600
C(23)-H(23C)	0.9600
C(24)-H(24A	0.9600
C(24)-H(24B)	0.9600
C(24)-H(24C)	0.9600
C(25)-H(25A)	0.9600
C(25)-H(25B)	0.9600
C(25)-H(25C)	0.9600
C(27)-H(27A)	0.9600
C(27)-H(27B)	0.9600
C(27)-H(27C)	0.9600

C(28)-H(28A)	0.9600
C(28)-H(28B)	0.9600
C(28)-H(28C)	0.9600
C(29)-H(29A)	0.9600
C(29)-H(29B)	0.9600
C(29)-H(29C)	0.9600
C(30)-H(30A)	0.9700
C(30)-H(30B)	0.9700
C(31)-C(32)	1.518(6)
C(31)-C(52)	1.533(5)
C(31)-H(31A)	0.9700
C(31)-H(31B)	0.9700
C(32)-C(33)	1.516(5)
C(32)-H(32A)	0.9700
C(32)-H(32B)	0.9700
C(33)-C(34)	1.538(5)
C(33)-H(33A)	0.9800
C(34)-C(60)	1.531(5)
C(34)-C(59)	1.536(6)
C(34)-C(35)	1.568(5)
C(35)-C(36)	1.527(5)
C(35)-C(52)	1.549(5)
C(35)-H(35A)	0.9800
C(36)-C(37)	1.522(6)
C(36)-H(36A)	0.9700
C(36)-H(36B)	0.9700
C(37)-C(38)	1.540(6)
C(37)-H(37A)	0.9700
C(37)-H(37B)	0.9700

C(38)-C(54)	1.540(6)
C(38)-C(51)	1.556(5)
C(38)-C(39)	1.590(5)
C(39)-C(48)	1.530(5)
C(39)-C(40)	1.547(5)
C(39)-C(55)	1.556(5)
C(40)-C(41)	1.523(6)
C(40)-H(40A)	0.9700
C(40)-H(40B)	0.9700
C(41)-C(42)	1.535(5)
C(41)-H(41A)	0.9700
C(41)-H(41B)	0.9700
C(42)-C(56)	1.528(5)
C(42)-C(47)	1.534(5)
C(42)-C(43)	1.537(5)
C(43)-C(44)	1.520(6)
C(43)-H(43A)	0.9700
C(43)-H(43B)	0.9700
C(44)-C(45)	1.536(7)
C(44)-H(44A)	0.9700
C(44)-H(44B)	0.9700
C(45)-C(46)	1.520(6)
C(45)-C(58)	1.531(7)
C(45)-C(57)	1.542(7)
C(46)-C(47)	1.541(6)
C(46)-H(46A)	0.9700
C(46)-H(46B)	0.9700
C(47)-C(48)	1.526(5)
C(47)-H(47A)	0.9800

C(48)-C(49)	1.320(5)
C(49)-C(50)	1.500(6)
C(49)-H(49A)	0.9300
C(50)-C(51)	1.541(5)
C(50)-H(50A)	0.9700
C(50)-H(50B)	0.9700
C(51)-C(52)	1.564(5)
C(51)-H(51A)	0.9800
C(52)-C(53)	1.543(6)
C(53)-H(53A)	0.9600
C(53)-H(53B)	0.9600
C(53)-H(53C)	0.9600
C(54)-H(54A)	0.9600
C(54)-H(54B)	0.9600
C(54)-H(54C)	0.9600
C(55)-H(55A)	0.9600
C(55)-H(55B)	0.9600
C(55)-H(55C)	0.9600
C(57)-H(57A)	0.9600
C(57)-H(57B)	0.9600
C(57)-H(57C)	0.9600
C(58)-H(58A)	0.9600
C(58)-H(58B)	0.9600
C(58)-H(58C)	0.9600
C(59)-H(59A)	0.9600
C(59)-H(59B)	0.9600
C(59)-H(59C)	0.9600
C(60)-H(60A)	0.9700
C(60)-H(60B)	0.9700

C(3)-O(1)-H(1A)	104(3)
C(26)-O(3)-H(3B)	107(3)
C(30)-O(4)-H(4A)	107(3)
C(33)-O(5)-H(5A)	105(3)
C(56)-O(6)-H(6C)	107(3)
C(60)-O(8)-H(8A)	102(4)
C(2)-C(1)-C(22)	113.8(3)
C(2)-C(1)-H(1B)	108.8
C(22)-C(1)-H(1B)	108.8
C(2)-C(1)-H(1C)	108.8
C(22)-C(1)-H(1C)	108.8
H(1B)-C(1)-H(1C)	107.7
C(1)-C(2)-C(3)	111.0(3)
C(1)-C(2)-H(2A)	109.4
C(3)-C(2)-H(2A)	109.4
C(1)-C(2)-H(2B)	109.4
C(3)-C(2)-H(2B)	109.4
H(2A)-C(2)-H(2B)	108.0
O(1)-C(3)-C(2)	110.0(3)
O(1)-C(3)-C(4)	111.2(3)
C(2)-C(3)-C(4)	113.8(3)
O(1)-C(3)-H(3A)	107.2
C(2)-C(3)-H(3A)	107.2
C(4)-C(3)-H(3A)	107.2
C(3)-C(4)-C(29)	111.9(3)
C(3)-C(4)-C(30)	109.2(3)
C(29)-C(4)-C(30)	104.6(3)
C(3)-C(4)-C(5)	106.5(3)
C(29)-C(4)-C(5)	115.1(3)

C(30)-C(4)-C(5)	109.5(3)
C(6)-C(5)-C(22)	110.7(3)
C(6)-C(5)-C(4)	115.2(3)
C(22)-C(5)-C(4)	116.6(3)
C(6)-C(5)-H(5B)	104.2
C(22)-C(5)-H(5B)	104.2
C(4)-C(5)-H(5B)	104.2
C(7)-C(6)-C(5)	110.0(3)
C(7)-C(6)-H(6A)	109.7
C(5)-C(6)-H(6A)	109.7
C(7)-C(6)-H(6B)	109.7
C(5)-C(6)-H(6B)	109.7
H(6A)-C(6)-H(6B)	108.2
C(6)-C(7)-C(8)	114.8(4)
C(6)-C(7)-H(7A)	108.6
C(8)-C(7)-H(7A)	108.6
C(6)-C(7)-H(7B)	108.6
C(8)-C(7)-H(7B)	108.6
H(7A)-C(7)-H(7B)	107.5
C(21)-C(8)-C(24)	111.0(3)
C(21)-C(8)-C(7)	109.1(3)
C(24)-C(8)-C(7)	107.6(3)
C(21)-C(8)-C(9)	108.1(3)
C(24)-C(8)-C(9)	109.8(3)
C(7)-C(8)-C(9)	111.3(3)
C(18)-C(9)-C(10)	111.6(3)
C(18)-C(9)-C(25)	106.9(3)
C(10)-C(9)-C(25)	106.8(3)
C(18)-C(9)-C(8)	109.1(3)

C(10)-C(9)-C(8)	110.1(3)
C(25)-C(9)-C(8)	112.3(3)
C(11)-C(10)-C(9)	114.4(3)
C(11)-C(10)-H(10A)	108.7
C(9)-C(10)-H(10A)	108.7
C(11)-C(10)-H(10B)	108.7
C(9)-C(10)-H(10B)	108.7
H(10A)-C(10)-H(10B)	107.6
C(10)-C(11)-C(12)	111.3(3)
C(10)-C(11)-H(11A)	109.4
C(12)-C(11)-H(11A)	109.4
C(10)-C(11)-H(11B)	109.4
C(12)-C(11)-H(11B)	109.4
H(11A)-C(11)-H(11B)	108.0
C(26)-C(12)-C(17)	109.1(3)
C(26)-C(12)-C(11)	109.7(3)
C(17)-C(12)-C(11)	109.3(3)
C(26)-C(12)-C(13)	105.3(3)
C(17)-C(12)-C(13)	111.0(3)
C(11)-C(12)-C(13)	112.3(3)
C(14)-C(13)-C(12)	113.1(4)
C(14)-C(13)-H(13A)	108.9
C(12)-C(13)-H(13A)	108.9
C(14)-C(13)-H(13B)	108.9
C(12)-C(13)-H(13B)	108.9
H(13A)-C(13)-H(13B)	107.8
C(13)-C(14)-C(15)	113.4(3)
C(13)-C(14)-H(14A)	108.9
C(15)-C(14)-H(14A)	108.9

C(13)-C(14)-H(14B)	108.9
C(15)-C(14)-H(14B)	108.9
H(14A)-C(14)-H(14B)	107.7
C(27)-C(15)-C(14)	108.8(4)
C(27)-C(15)-C(16)	109.4(4)
C(14)-C(15)-C(16)	108.1(4)
C(27)-C(15)-C(28)	107.2(4)
C(14)-C(15)-C(28)	111.9(4)
C(16)-C(15)-C(28)	111.5(4)
C(15)-C(16)-C(17)	114.4(4)
C(15)-C(16)-H(16A)	108.7
C(17)-C(16)-H(16A)	108.7
C(15)-C(16)-H(16B)	108.7
C(17)-C(16)-H(16B)	108.7
H(16A)-C(16)-H(16B)	107.6
C(18)-C(17)-C(12)	112.3(3)
C(18)-C(17)-C(16)	112.3(3)
C(12)-C(17)-C(16)	112.5(3)
C(18)-C(17)-H(17A)	106.4
C(12)-C(17)-H(17A)	106.4
C(16)-C(17)-H(17A)	106.4
C(19)-C(18)-C(17)	118.1(4)
C(19)-C(18)-C(9)	121.1(4)
C(17)-C(18)-C(9)	120.8(3)
C(18)-C(19)-C(20)	126.0(4)
C(18)-C(19)-H(19A)	117.0
C(20)-C(19)-H(19A)	117.0
C(19)-C(20)-C(21)	113.8(3)
C(19)-C(20)-H(20A)	108.8

C(21)-C(20)-H(20A)	108.8
C(19)-C(20)-H(20B)	108.8
C(21)-C(20)-H(20B)	108.8
H(20A)-C(20)-H(20B)	107.7
C(20)-C(21)-C(8)	110.6(3)
C(20)-C(21)-C(22)	112.8(3)
C(8)-C(21)-C(22)	118.1(3)
C(20)-C(21)-H(21A)	104.7
C(8)-C(21)-H(21A)	104.7
C(22)-C(21)-H(21A)	104.7
C(1)-C(22)-C(5)	108.3(3)
C(1)-C(22)-C(23)	108.0(3)
C(5)-C(22)-C(23)	113.0(3)
C(1)-C(22)-C(21)	106.9(3)
C(5)-C(22)-C(21)	107.2(3)
C(23)-C(22)-C(21)	113.2(3)
C(22)-C(23)-H(23A)	109.5
C(22)-C(23)-H(23B)	109.5
H(23A)-C(23)-H(23B)	109.5
C(22)-C(23)-H(23C)	109.5
H(23A)-C(23)-H(23C)	109.5
H(23B)-C(23)-H(23C)	109.5
C(8)-C(24)-H(24A)	109.5
C(8)-C(24)-H(24B)	109.5
H(24A)-C(24)-H(24B)	109.5
C(8)-C(24)-H(24C)	109.5
H(24A)-C(24)-H(24C)	109.5
H(24B)-C(24)-H(24C)	109.5
C(9)-C(25)-H(25A)	109.5

C(9)-C(25)-H(25B)	109.5
H(25A)-C(25)-H(25B)	109.5
C(9)-C(25)-H(25C)	109.5
H(25A)-C(25)-H(25C)	109.5
H(25B)-C(25)-H(25C)	109.5
O(2)-C(26)-O(3)	122.5(4)
O(2)-C(26)-C(12)	124.1(4)
O(3)-C(26)-C(12)	113.4(3)
C(15)-C(27)-H(27A)	109.5
C(15)-C(27)-H(27B)	109.5
H(27A)-C(27)-H(27B)	109.5
C(15)-C(27)-H(27C)	109.5
H(27A)-C(27)-H(27C)	109.5
H(27B)-C(27)-H(27C)	109.5
C(15)-C(28)-H(28A)	109.5
C(15)-C(28)-H(28B)	109.5
H(28A)-C(28)-H(28B)	109.5
C(15)-C(28)-H(28C)	109.5
H(28A)-C(28)-H(28C)	109.5
H(28B)-C(28)-H(28C)	109.5
C(4)-C(29)-H(29A)	109.5
C(4)-C(29)-H(29B)	109.5
H(29A)-C(29)-H(29B)	109.5
C(4)-C(29)-H(29C)	109.5
H(29A)-C(29)-H(29C)	109.5
H(29B)-C(29)-H(29C)	109.5
O(4)-C(30)-C(4)	114.7(3)
O(4)-C(30)-H(30A)	108.6
C(4)-C(30)-H(30A)	108.6

O(4)-C(30)-H(30B)	108.6
C(4)-C(30)-H(30B)	108.6
H(30A)-C(30)-H(30B)	107.6
C(32)-C(31)-C(52)	113.5(3)
C(32)-C(31)-H(31A)	108.9
C(52)-C(31)-H(31A)	108.9
C(32)-C(31)-H(31B)	108.9
C(52)-C(31)-H(31B)	108.9
H(31A)-C(31)-H(31B)	107.7
C(33)-C(32)-C(31)	111.1(3)
C(33)-C(32)-H(32A)	109.4
C(31)-C(32)-H(32A)	109.4
C(33)-C(32)-H(32B)	109.4
C(31)-C(32)-H(32B)	109.4
H(32A)-C(32)-H(32B)	108.0
O(5)-C(33)-C(32)	110.3(3)
O(5)-C(33)-C(34)	110.8(3)
C(32)-C(33)-C(34)	114.0(3)
O(5)-C(33)-H(33A)	107.1
C(32)-C(33)-H(33A)	107.1
C(34)-C(33)-H(33A)	107.1
C(60)-C(34)-C(59)	104.8(3)
C(60)-C(34)-C(33)	109.3(3)
C(59)-C(34)-C(33)	111.9(3)
C(60)-C(34)-C(35)	109.7(3)
C(59)-C(34)-C(35)	114.8(3)
C(33)-C(34)-C(35)	106.4(3)
C(36)-C(35)-C(52)	110.6(3)
C(36)-C(35)-C(34)	115.2(3)

C(52)-C(35)-C(34)	116.5(3)
C(36)-C(35)-H(35A)	104.3
C(52)-C(35)-H(35A)	104.3
C(34)-C(35)-H(35A)	104.3
C(37)-C(36)-C(35)	110.0(3)
C(37)-C(36)-H(36A)	109.7
C(35)-C(36)-H(36A)	109.7
C(37)-C(36)-H(36B)	109.7
C(35)-C(36)-H(36B)	109.7
H(36A)-C(36)-H(36B)	108.2
C(36)-C(37)-C(38)	114.5(4)
C(36)-C(37)-H(37A)	108.6
C(38)-C(37)-H(37A)	108.6
C(36)-C(37)-H(37B)	108.6
C(38)-C(37)-H(37B)	108.6
H(37A)-C(37)-H(37B)	107.6
C(54)-C(38)-C(37)	108.7(3)
C(54)-C(38)-C(51)	110.7(3)
C(37)-C(38)-C(51)	108.9(3)
C(54)-C(38)-C(39)	109.6(3)
C(37)-C(38)-C(39)	111.2(3)
C(51)-C(38)-C(39)	107.7(3)
C(48)-C(39)-C(40)	111.2(3)
C(48)-C(39)-C(55)	107.1(3)
C(40)-C(39)-C(55)	106.6(3)
C(48)-C(39)-C(38)	109.3(3)
C(40)-C(39)-C(38)	110.1(3)
C(55)-C(39)-C(38)	112.4(3)
C(41)-C(40)-C(39)	114.7(3)

C(41)-C(40)-H(40A)	108.6
C(39)-C(40)-H(40A)	108.6
C(41)-C(40)-H(40B)	108.6
C(39)-C(40)-H(40B)	108.6
H(40A)-C(40)-H(40B)	107.6
C(40)-C(41)-C(42)	111.4(3)
C(40)-C(41)-H(41A)	109.3
C(42)-C(41)-H(41A)	109.3
C(40)-C(41)-H(41B)	109.3
C(42)-C(41)-H(41B)	109.3
H(41A)-C(41)-H(41B)	108.0
C(56)-C(42)-C(47)	109.3(3)
C(56)-C(42)-C(41)	109.5(3)
C(47)-C(42)-C(41)	109.1(3)
C(56)-C(42)-C(43)	105.5(3)
C(47)-C(42)-C(43)	110.7(3)
C(41)-C(42)-C(43)	112.6(3)
C(44)-C(43)-C(42)	113.8(3)
C(44)-C(43)-H(43A)	108.8
C(42)-C(43)-H(43A)	108.8
C(44)-C(43)-H(43B)	108.8
C(42)-C(43)-H(43B)	108.8
H(43A)-C(43)-H(43B)	107.7
C(43)-C(44)-C(45)	113.0(4)
C(43)-C(44)-H(44A)	109.0
C(45)-C(44)-H(44A)	109.0
C(43)-C(44)-H(44B)	109.0
C(45)-C(44)-H(44B)	109.0
H(44A)-C(44)-H(44B)	107.8

C(46)-C(45)-C(58)	109.3(4)
C(46)-C(45)-C(44)	107.8(4)
C(58)-C(45)-C(44)	109.2(4)
C(46)-C(45)-C(57)	112.0(4)
C(58)-C(45)-C(57)	106.2(4)
C(44)-C(45)-C(57)	112.3(5)
C(45)-C(46)-C(47)	114.1(4)
C(45)-C(46)-H(46A)	108.7
C(47)-C(46)-H(46A)	108.7
C(45)-C(46)-H(46B)	108.7
C(47)-C(46)-H(46B)	108.7
H(46A)-C(46)-H(46B)	107.6
C(48)-C(47)-C(42)	112.2(3)
C(48)-C(47)-C(46)	112.6(3)
C(42)-C(47)-C(46)	112.5(3)
C(48)-C(47)-H(47A)	106.3
C(42)-C(47)-H(47A)	106.3
C(46)-C(47)-H(47A)	106.3
C(49)-C(48)-C(47)	118.2(4)
C(49)-C(48)-C(39)	120.9(4)
C(47)-C(48)-C(39)	120.8(3)
C(48)-C(49)-C(50)	126.5(4)
C(48)-C(49)-H(49A)	116.7
C(50)-C(49)-H(49A)	116.7
C(49)-C(50)-C(51)	113.1(3)
C(49)-C(50)-H(50A)	109.0
C(51)-C(50)-H(50A)	109.0
C(49)-C(50)-H(50B)	109.0
C(51)-C(50)-H(50B)	109.0

H(50A)-C(50)-H(50B)	107.8
C(50)-C(51)-C(38)	110.8(3)
C(50)-C(51)-C(52)	112.2(3)
C(38)-C(51)-C(52)	118.0(3)
C(50)-C(51)-H(51A)	104.8
C(38)-C(51)-H(51A)	104.8
C(52)-C(51)-H(51A)	104.8
C(31)-C(52)-C(53)	108.6(3)
C(31)-C(52)-C(35)	108.2(3)
C(53)-C(52)-C(35)	112.9(3)
C(31)-C(52)-C(51)	106.7(3)
C(53)-C(52)-C(51)	113.4(3)
C(35)-C(52)-C(51)	106.7(3)
C(52)-C(53)-H(53A)	109.5
C(52)-C(53)-H(53B)	109.5
H(53A)-C(53)-H(53B)	109.5
C(52)-C(53)-H(53C)	109.5
H(53A)-C(53)-H(53C)	109.5
H(53B)-C(53)-H(53C)	109.5
C(38)-C(54)-H(54A)	109.5
C(38)-C(54)-H(54B)	109.5
H(54A)-C(54)-H(54B)	109.5
C(38)-C(54)-H(54C)	109.5
H(54A)-C(54)-H(54C)	109.5
H(54B)-C(54)-H(54C)	109.5
C(39)-C(55)-H(55A)	109.5
C(39)-C(55)-H(55B)	109.5
H(55A)-C(55)-H(55B)	109.5
C(39)-C(55)-H(55C)	109.5

H(55A)-C(55)-H(55C)	109.5
H(55B)-C(55)-H(55C)	109.5
O(7)-C(56)-O(6)	122.4(4)
O(7)-C(56)-C(42)	124.5(3)
O(6)-C(56)-C(42)	113.1(3)
C(45)-C(57)-H(57A)	109.5
C(45)-C(57)-H(57B)	109.5
H(57A)-C(57)-H(57B)	109.5
C(45)-C(57)-H(57C)	109.5
H(57A)-C(57)-H(57C)	109.5
H(57B)-C(57)-H(57C)	109.5
C(45)-C(58)-H(58A)	109.5
C(45)-C(58)-H(58B)	109.5
H(58A)-C(58)-H(58B)	109.5
C(45)-C(58)-H(58C)	109.5
H(58A)-C(58)-H(58C)	109.5
H(58B)-C(58)-H(58C)	109.5
C(34)-C(59)-H(59A)	109.5
C(34)-C(59)-H(59B)	109.5
H(59A)-C(59)-H(59B)	109.5
C(34)-C(59)-H(59C)	109.5
H(59A)-C(59)-H(59C)	109.5
H(59B)-C(59)-H(59C)	109.5
O(8)-C(60)-C(34)	114.8(3)
O(8)-C(60)-H(60A)	108.6
C(34)-C(60)-H(60A)	108.6
O(8)-C(60)-H(60B)	108.6
C(34)-C(60)-H(60B)	108.6
H(60A)-C(60)-H(60B)	107.5

Table 3. Anisotropic displacement parameters (A^2 x 10^3) for a.

The anisotropic displacement factor exponent takes the form:

-2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
O(1)	42(2)	32(2)	51(2)	-8(1)	4(1)	-5(1)
O(2)	46(2)	69(2)	38(2)	5(2)	6(1)	-7(2)
O(3)	49(2)	83(3)	39(2)	11(2)	-8(1)	-21(2)
O(4)	42(2)	40(2)	43(2)	-7(2)	2(1)	3(1)
O(5)	42(2)	30(2)	50(2)	9(1)	-3(1)	-6(1)
O(6)	46(2)	83(3)	39(2)	-10(2)	6(1)	-16(2)
O(7)	45(2)	68(2)	40(2)	-4(2)	-4(1)	-7(2)
O(8)	44(2)	39(2)	45(2)	6(2)	-6(1)	3(1)
C(1)	35(2)	31(2)	59(3)	-6(2)	-1(2)	2(2)
C(2)	36(2)	33(2)	54(3)	-6(2)	-5(2)	0(2)
C(3)	40(2)	28(2)	37(2)	-1(2)	1(2)	-4(2)
C(4)	38(2)	29(2)	32(2)	-3(2)	1(2)	1(2)
C(5)	38(2)	30(2)	26(2)	-1(2)	-3(2)	2(2)
C(6)	40(2)	37(2)	49(3)	-5(2)	-7(2)	6(2)
C(7)	36(2)	43(2)	55(3)	-10(2)	-10(2)	2(2)
C(8)	38(2)	35(2)	33(2)	-8(2)	-4(2)	0(2)
C(9)	33(2)	37(2)	37(2)	-7(2)	-2(2)	0(2)
C(10)	36(2)	37(2)	59(3)	-8(2)	3(2)	-2(2)
C(11)	38(2)	44(3)	45(3)	-3(2)	3(2)	-10(2)
C(12)	41(2)	31(2)	34(2)	2(2)	4(2)	-3(2)
C(13)	49(2)	37(2)	46(3)	0(2)	3(2)	-10(2)
C(14)	64(3)	40(2)	48(3)	13(2)	8(2)	-7(2)
C(15)	60(3)	48(3)	53(3)	20(2)	7(2)	0(2)
C(16)	55(3)	54(3)	40(3)	10(2)	-2(2)	-6(2)

C(17)	41(2)	30(2)	34(2)	3(2)	2(2)	-4(2)
C(18)	39(2)	36(2)	29(2)	-5(2)	-2(2)	1(2)
C(19)	39(2)	37(2)	52(3)	-10(2)	3(2)	3(2)
C(20)	38(2)	31(2)	96(4)	-12(2)	6(2)	-1(2)
C(21)	36(2)	33(2)	42(2)	-7(2)	0(2)	5(2)
C(22)	35(2)	30(2)	40(2)	-5(2)	0(2)	-1(2)
C(23)	61(3)	44(3)	46(3)	-7(2)	17(2)	-5(2)
C(24)	78(3)	41(3)	41(3)	-7(2)	-6(2)	-9(2)
C(25)	63(3)	45(3)	42(3)	-9(2)	11(2)	1(2)
C(26)	41(2)	28(2)	37(2)	-5(2)	3(2)	-6(2)
C(27)	79(4)	83(4)	64(4)	35(3)	-12(3)	-15(3)
C(28)	80(4)	55(3)	97(5)	18(3)	11(3)	5(3)
C(29)	69(3)	41(2)	41(3)	3(2)	4(2)	0(2)
C(30)	48(2)	27(2)	42(3)	2(2)	-4(2)	3(2)
C(31)	32(2)	33(2)	60(3)	6(2)	5(2)	-2(2)
C(32)	35(2)	38(2)	55(3)	4(2)	5(2)	-2(2)
C(33)	44(2)	28(2)	34(2)	2(2)	-1(2)	-5(2)
C(34)	39(2)	26(2)	34(2)	-1(2)	0(2)	1(2)
C(35)	33(2)	31(2)	34(2)	1(2)	1(2)	5(2)
C(36)	40(2)	36(2)	53(3)	5(2)	7(2)	4(2)
C(37)	37(2)	41(2)	59(3)	8(2)	11(2)	1(2)
C(38)	40(2)	32(2)	36(2)	10(2)	3(2)	0(2)
C(39)	36(2)	33(2)	38(2)	8(2)	-2(2)	-1(2)
C(40)	34(2)	40(2)	58(3)	5(2)	-5(2)	-2(2)
C(41)	39(2)	43(3)	46(3)	6(2)	-7(2)	-9(2)
C(42)	40(2)	34(2)	30(2)	-2(2)	-6(2)	-4(2)
C(43)	49(2)	36(2)	45(3)	-2(2)	-5(2)	-11(2)
C(44)	66(3)	39(3)	53(3)	-7(2)	-12(2)	-8(2)
C(45)	59(3)	46(3)	55(3)	-18(2)	-10(2)	-1(2)

C(46)	53(3)	55(3)	40(3)	-6(2)	2(2)	-4(2)
C(47)	42(2)	32(2)	37(2)	1(2)	-4(2)	-2(2)
C(48)	37(2)	33(2)	31(2)	4(2)	-1(2)	-1(2)
C(49)	40(2)	31(2)	60(3)	8(2)	-5(2)	4(2)
C(50)	39(2)	31(2)	91(4)	11(2)	-3(2)	-3(2)
C(51)	35(2)	29(2)	41(2)	7(2)	-3(2)	5(2)
C(52)	38(2)	31(2)	37(2)	5(2)	0(2)	-1(2)
C(53)	59(3)	46(3)	47(3)	8(2)	-16(2)	-5(2)
C(54)	73(3)	43(3)	42(3)	10(2)	3(2)	-13(2)
C(55)	60(3)	47(3)	41(3)	8(2)	-11(2)	3(2)
C(56)	39(2)	28(2)	42(2)	0(2)	1(2)	-5(2)
C(57)	91(4)	64(4)	112(5)	-21(4)	-15(4)	2(3)
C(58)	90(4)	89(4)	65(4)	-41(3)	10(3)	-10(3)
C(59)	62(3)	38(2)	46(3)	-5(2)	-2(2)	4(2)
C(60)	43(2)	30(2)	47(3)	1(2)	4(2)	6(2)

Table 4. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for a.

	x	y	z	U(eq)
H(1B)	4448	377	17272	50
H(1C)	5246	286	16373	50
H(2A)	5123	1667	16027	49
H(2B)	5460	1560	17147	49
H(3A)	3672	1769	17646	42
H(5B)	2526	758	16916	38
H(6A)	1059	1046	15823	50
H(6B)	1780	722	14945	50
H(7A)	686	-332	15528	54
H(7B)	1136	-210	16608	54
H(10A)	259	-1719	16117	53
H(10B)	835	-1923	15110	53
H(11A)	10	-3101	15708	51
H(11B)	632	-3022	16725	51
H(13A)	702	-4483	15453	53
H(13B)	1943	-4648	15278	53
H(14A)	1450	-5253	16766	61
H(14B)	961	-4433	17159	61
H(16A)	3499	-3396	17439	60
H(16B)	2247	-3270	17588	60
H(17A)	3285	-3433	15729	42
H(19A)	4429	-2393	15886	51
H(20A)	4509	-1021	15244	66
H(20B)	4821	-1047	16375	66
H(21A)	3226	-553	16858	44
H(23A)	4210	1088	14752	76

H(23B)	3425	396	14390	76
H(23C)	4627	187	14690	76
H(24A)	2456	-449	14259	80
H(24B)	1601	-1156	14330	80
H(24C)	2847	-1348	14427	80
H(25A)	1622	-2099	17670	75
H(25B)	1203	-1206	17512	75
H(25C)	2448	-1371	17673	75
H(27A)	2020	-4433	18658	113
H(27B)	3284	-4564	18641	113
H(27C)	2501	-5286	18372	113
H(28A)	4252	-4765	17068	116
H(28B)	3613	-4776	16057	116
H(28C)	3468	-5491	16819	116
H(29A)	3190	2859	15406	75
H(29B)	2421	2222	14892	75
H(29C)	3675	2056	14959	75
H(30A)	2098	2848	16898	47
H(30B)	1320	2288	16277	47
H(31A)	558	-518	12273	50
H(31B)	-250	-425	11376	50
H(32A)	-463	-1700	12144	51
H(32B)	-129	-1807	11025	51
H(33A)	1323	-1911	12650	42
H(35A)	2477	-903	11921	39
H(36A)	3931	-1199	10828	51
H(36B)	3204	-871	9952	51
H(37A)	4317	179	10542	54
H(37B)	3863	62	11619	54

H(40A)	4750	1572	11117	53
H(40B)	4166	1777	10111	53
H(41A)	4998	2948	10709	51
H(41B)	4382	2870	11726	51
H(43A)	4303	4332	10457	52
H(43B)	3062	4495	10278	52
H(44A)	3558	5110	11754	63
H(44B)	4055	4295	12158	63
H(46A)	1532	3245	12448	59
H(46B)	2789	3133	12590	59
H(47A)	1727	3288	10732	44
H(49A)	586	2251	10880	52
H(50A)	482	873	10241	64
H(50B)	177	903	11372	64
H(51A)	1777	404	11860	42
H(53A)	375	-322	9682	76
H(53B)	768	-1228	9754	76
H(53C)	1573	-549	9395	76
H(54A)	2528	305	9260	79
H(54B)	3387	1009	9329	79
H(54C)	2145	1205	9436	79
H(55A)	3406	1950	12666	74
H(55B)	3815	1055	12509	74
H(55C)	2574	1226	12672	74
H(57A)	750	4607	12060	134
H(57B)	1383	4627	11050	134
H(57C)	1522	5340	11815	134
H(58A)	2975	4301	13668	122
H(58B)	1711	4426	13632	122

H(58C)	2490	5150	13366	122
H(59A)	1801	-3003	10408	73
H(59B)	2567	-2365	9898	73
H(59C)	1313	-2199	9964	73
H(60A)	2887	-2993	11914	48
H(60B)	3667	-2442	11288	48
H(5A)	170(30)	-2970(20)	12100(30)	29(11)
H(1A)	4790(30)	2810(30)	17080(30)	32(13)
H(4A)	1820(40)	2160(30)	18130(40)	53(15)
H(8A)	3240(40)	-2410(40)	13140(40)	84(19)
H(3B)	980(40)	-3340(40)	13260(50)	110(20)
H(6C)	4040(40)	3150(30)	8330(40)	78(17)

Table 6. Torsion angles [deg] for a.

C(22)-C(1)-C(2)-C(3)	55.6(5)
C(1)-C(2)-C(3)-O(1)	177.0(3)
C(1)-C(2)-C(3)-C(4)	-57.5(5)
O(1)-C(3)-C(4)-C(29)	52.7(4)
C(2)-C(3)-C(4)-C(29)	-72.2(4)
O(1)-C(3)-C(4)-C(30)	-62.6(4)
C(2)-C(3)-C(4)-C(30)	172.5(3)
O(1)-C(3)-C(4)-C(5)	179.2(3)
C(2)-C(3)-C(4)-C(5)	54.3(4)
C(3)-C(4)-C(5)-C(6)	174.2(3)
C(29)-C(4)-C(5)-C(6)	-61.2(4)
C(30)-C(4)-C(5)-C(6)	56.2(4)
C(3)-C(4)-C(5)-C(22)	-53.5(4)
C(29)-C(4)-C(5)-C(22)	71.0(4)
C(30)-C(4)-C(5)-C(22)	-171.5(3)
C(22)-C(5)-C(6)-C(7)	63.0(4)
C(4)-C(5)-C(6)-C(7)	-162.0(3)
C(5)-C(6)-C(7)-C(8)	-57.2(5)
C(6)-C(7)-C(8)-C(21)	46.8(5)
C(6)-C(7)-C(8)-C(24)	-73.8(4)
C(6)-C(7)-C(8)-C(9)	165.9(3)
C(21)-C(8)-C(9)-C(18)	-56.1(4)
C(24)-C(8)-C(9)-C(18)	65.1(4)
C(7)-C(8)-C(9)-C(18)	-175.8(3)
C(21)-C(8)-C(9)-C(10)	-178.9(3)
C(24)-C(8)-C(9)-C(10)	-57.6(4)
C(7)-C(8)-C(9)-C(10)	61.4(4)

C(21)-C(8)-C(9)-C(25)	62.3(4)
C(24)-C(8)-C(9)-C(25)	-176.5(3)
C(7)-C(8)-C(9)-C(25)	-57.4(4)
C(18)-C(9)-C(10)-C(11)	40.1(5)
C(25)-C(9)-C(10)-C(11)	-76.4(4)
C(8)-C(9)-C(10)-C(11)	161.4(3)
C(9)-C(10)-C(11)-C(12)	-56.6(5)
C(10)-C(11)-C(12)-C(26)	-58.1(4)
C(10)-C(11)-C(12)-C(17)	61.4(4)
C(10)-C(11)-C(12)-C(13)	-174.9(3)
C(26)-C(12)-C(13)-C(14)	167.8(3)
C(17)-C(12)-C(13)-C(14)	49.8(4)
C(11)-C(12)-C(13)-C(14)	-72.9(4)
C(12)-C(13)-C(14)-C(15)	-55.0(5)
C(13)-C(14)-C(15)-C(27)	174.1(4)
C(13)-C(14)-C(15)-C(16)	55.4(5)
C(13)-C(14)-C(15)-C(28)	-67.7(5)
C(27)-C(15)-C(16)-C(17)	-173.3(4)
C(14)-C(15)-C(16)-C(17)	-55.0(5)
C(28)-C(15)-C(16)-C(17)	68.4(5)
C(26)-C(12)-C(17)-C(18)	67.9(4)
C(11)-C(12)-C(17)-C(18)	-52.0(4)
C(13)-C(12)-C(17)-C(18)	-176.4(3)
C(26)-C(12)-C(17)-C(16)	-164.2(3)
C(11)-C(12)-C(17)-C(16)	75.9(4)
C(13)-C(12)-C(17)-C(16)	-48.5(4)
C(15)-C(16)-C(17)-C(18)	-178.7(4)
C(15)-C(16)-C(17)-C(12)	53.4(5)
C(12)-C(17)-C(18)-C(19)	-142.1(4)

C(16)-C(17)-C(18)-C(19)	89.9(4)
C(12)-C(17)-C(18)-C(9)	40.2(5)
C(16)-C(17)-C(18)-C(9)	-87.8(4)
C(10)-C(9)-C(18)-C(19)	149.3(4)
C(25)-C(9)-C(18)-C(19)	-94.3(4)
C(8)-C(9)-C(18)-C(19)	27.5(5)
C(10)-C(9)-C(18)-C(17)	-33.0(5)
C(25)-C(9)-C(18)-C(17)	83.4(4)
C(8)-C(9)-C(18)-C(17)	-154.9(3)
C(17)-C(18)-C(19)-C(20)	-179.9(4)
C(9)-C(18)-C(19)-C(20)	-2.2(7)
C(18)-C(19)-C(20)-C(21)	6.3(7)
C(19)-C(20)-C(21)-C(8)	-36.7(5)
C(19)-C(20)-C(21)-C(22)	-171.3(4)
C(24)-C(8)-C(21)-C(20)	-58.8(5)
C(7)-C(8)-C(21)-C(20)	-177.2(4)
C(9)-C(8)-C(21)-C(20)	61.7(4)
C(24)-C(8)-C(21)-C(22)	73.2(4)
C(7)-C(8)-C(21)-C(22)	-45.2(5)
C(9)-C(8)-C(21)-C(22)	-166.3(3)
C(2)-C(1)-C(22)-C(5)	-51.9(4)
C(2)-C(1)-C(22)-C(23)	70.7(4)
C(2)-C(1)-C(22)-C(21)	-167.1(3)
C(6)-C(5)-C(22)-C(1)	-173.1(3)
C(4)-C(5)-C(22)-C(1)	52.6(4)
C(6)-C(5)-C(22)-C(23)	67.3(4)
C(4)-C(5)-C(22)-C(23)	-66.9(4)
C(6)-C(5)-C(22)-C(21)	-58.1(4)
C(4)-C(5)-C(22)-C(21)	167.6(3)

C(20)-C(21)-C(22)-C(1)	-61.5(4)
C(8)-C(21)-C(22)-C(1)	167.6(3)
C(20)-C(21)-C(22)-C(5)	-177.4(4)
C(8)-C(21)-C(22)-C(5)	51.6(4)
C(20)-C(21)-C(22)-C(23)	57.3(5)
C(8)-C(21)-C(22)-C(23)	-73.7(4)
C(17)-C(12)-C(26)-O(2)	12.6(5)
C(11)-C(12)-C(26)-O(2)	132.3(4)
C(13)-C(12)-C(26)-O(2)	-106.6(4)
C(17)-C(12)-C(26)-O(3)	-169.0(3)
C(11)-C(12)-C(26)-O(3)	-49.3(4)
C(13)-C(12)-C(26)-O(3)	71.7(4)
C(3)-C(4)-C(30)-O(4)	-67.1(4)
C(29)-C(4)-C(30)-O(4)	173.1(3)
C(5)-C(4)-C(30)-O(4)	49.2(4)
C(52)-C(31)-C(32)-C(33)	56.0(5)
C(31)-C(32)-C(33)-O(5)	177.0(3)
C(31)-C(32)-C(33)-C(34)	-57.7(5)
O(5)-C(33)-C(34)-C(60)	-62.4(4)
C(32)-C(33)-C(34)-C(60)	172.5(3)
O(5)-C(33)-C(34)-C(59)	53.2(4)
C(32)-C(33)-C(34)-C(59)	-71.9(4)
O(5)-C(33)-C(34)-C(35)	179.3(3)
C(32)-C(33)-C(34)-C(35)	54.1(4)
C(60)-C(34)-C(35)-C(36)	56.5(4)
C(59)-C(34)-C(35)-C(36)	-61.1(5)
C(33)-C(34)-C(35)-C(36)	174.6(3)
C(60)-C(34)-C(35)-C(52)	-171.4(3)
C(59)-C(34)-C(35)-C(52)	71.0(4)

C(33)-C(34)-C(35)-C(52)	-53.3(4)
C(52)-C(35)-C(36)-C(37)	63.5(4)
C(34)-C(35)-C(36)-C(37)	-161.8(3)
C(35)-C(36)-C(37)-C(38)	-57.8(5)
C(36)-C(37)-C(38)-C(54)	-73.5(4)
C(36)-C(37)-C(38)-C(51)	47.3(5)
C(36)-C(37)-C(38)-C(39)	165.9(3)
C(54)-C(38)-C(39)-C(48)	64.4(4)
C(37)-C(38)-C(39)-C(48)	-175.4(3)
C(51)-C(38)-C(39)-C(48)	-56.1(4)
C(54)-C(38)-C(39)-C(40)	-58.1(4)
C(37)-C(38)-C(39)-C(40)	62.1(4)
C(51)-C(38)-C(39)-C(40)	-178.6(3)
C(54)-C(38)-C(39)-C(55)	-176.8(3)
C(37)-C(38)-C(39)-C(55)	-56.7(4)
C(51)-C(38)-C(39)-C(55)	62.6(4)
C(48)-C(39)-C(40)-C(41)	40.2(5)
C(55)-C(39)-C(40)-C(41)	-76.3(4)
C(38)-C(39)-C(40)-C(41)	161.5(3)
C(39)-C(40)-C(41)-C(42)	-56.7(5)
C(40)-C(41)-C(42)-C(56)	-58.0(4)
C(40)-C(41)-C(42)-C(47)	61.5(4)
C(40)-C(41)-C(42)-C(43)	-175.1(3)
C(56)-C(42)-C(43)-C(44)	167.6(3)
C(47)-C(42)-C(43)-C(44)	49.5(5)
C(41)-C(42)-C(43)-C(44)	-72.9(4)
C(42)-C(43)-C(44)-C(45)	-54.7(5)
C(43)-C(44)-C(45)-C(46)	55.6(5)
C(43)-C(44)-C(45)-C(58)	174.3(4)

C(43)-C(44)-C(45)-C(57)	-68.2(5)
C(58)-C(45)-C(46)-C(47)	-174.5(4)
C(44)-C(45)-C(46)-C(47)	-55.9(5)
C(57)-C(45)-C(46)-C(47)	68.1(5)
C(56)-C(42)-C(47)-C(48)	67.5(4)
C(41)-C(42)-C(47)-C(48)	-52.2(4)
C(43)-C(42)-C(47)-C(48)	-176.7(3)
C(56)-C(42)-C(47)-C(46)	-164.2(3)
C(41)-C(42)-C(47)-C(46)	76.0(4)
C(43)-C(42)-C(47)-C(46)	-48.5(5)
C(45)-C(46)-C(47)-C(48)	-177.6(4)
C(45)-C(46)-C(47)-C(42)	54.4(5)
C(42)-C(47)-C(48)-C(49)	-141.9(4)
C(46)-C(47)-C(48)-C(49)	89.9(4)
C(42)-C(47)-C(48)-C(39)	40.6(5)
C(46)-C(47)-C(48)-C(39)	-87.6(4)
C(40)-C(39)-C(48)-C(49)	149.4(4)
C(55)-C(39)-C(48)-C(49)	-94.4(4)
C(38)-C(39)-C(48)-C(49)	27.6(5)
C(40)-C(39)-C(48)-C(47)	-33.2(5)
C(55)-C(39)-C(48)-C(47)	82.9(4)
C(38)-C(39)-C(48)-C(47)	-155.1(3)
C(47)-C(48)-C(49)-C(50)	-179.5(4)
C(39)-C(48)-C(49)-C(50)	-2.1(7)
C(48)-C(49)-C(50)-C(51)	6.2(7)
C(49)-C(50)-C(51)-C(38)	-36.7(5)
C(49)-C(50)-C(51)-C(52)	-171.0(4)
C(54)-C(38)-C(51)-C(50)	-58.0(4)
C(37)-C(38)-C(51)-C(50)	-177.4(4)

C(39)-C(38)-C(51)-C(50)	61.9(4)
C(54)-C(38)-C(51)-C(52)	73.4(4)
C(37)-C(38)-C(51)-C(52)	-46.0(5)
C(39)-C(38)-C(51)-C(52)	-166.8(3)
C(32)-C(31)-C(52)-C(53)	70.5(4)
C(32)-C(31)-C(52)-C(35)	-52.4(5)
C(32)-C(31)-C(52)-C(51)	-166.8(3)
C(36)-C(35)-C(52)-C(31)	-172.9(3)
C(34)-C(35)-C(52)-C(31)	53.0(4)
C(36)-C(35)-C(52)-C(53)	66.9(4)
C(34)-C(35)-C(52)-C(53)	-67.3(4)
C(36)-C(35)-C(52)-C(51)	-58.4(4)
C(34)-C(35)-C(52)-C(51)	167.5(3)
C(50)-C(51)-C(52)-C(31)	-61.9(4)
C(38)-C(51)-C(52)-C(31)	167.4(3)
C(50)-C(51)-C(52)-C(53)	57.6(4)
C(38)-C(51)-C(52)-C(53)	-73.1(4)
C(50)-C(51)-C(52)-C(35)	-177.4(3)
C(38)-C(51)-C(52)-C(35)	51.9(4)
C(47)-C(42)-C(56)-O(7)	13.3(5)
C(41)-C(42)-C(56)-O(7)	132.8(4)
C(43)-C(42)-C(56)-O(7)	-105.7(4)
C(47)-C(42)-C(56)-O(6)	-168.8(3)
C(41)-C(42)-C(56)-O(6)	-49.3(4)
C(43)-C(42)-C(56)-O(6)	72.1(4)
C(59)-C(34)-C(60)-O(8)	172.4(3)
C(33)-C(34)-C(60)-O(8)	-67.5(4)
C(35)-C(34)-C(60)-O(8)	48.7(4)

Symmetry transformations used to generate equivalent atoms: