BIOACTIVE NATURAL PRODUCTS FROM THE RHIZOMES OF *AMPELOCISSUS GRANTII, CISSUS CRINITA* AND *CISSUS SOKODENSIS* (*VITACEAE*)

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NOVEMBER, 2015

Declaration

I hereby declare that the work in this thesis entitled "BIOACTIVE NATURAL PRODUCTS FROM THE RHIZOMES OF *AMPELOCISSUS GRANTII*, *CISSUS CRINITA* AND *CISSUS SOKODENSIS* (*VITACEAE*)." was carried out by me in the Department of Chemistry, under the supervision of Prof. G. I. Ndukwe, Dr. M. S. Sallau and Prof. (Mrs) R. G. Ayo. The information derived from the literature has been duly acknowledged by means of references. No part of this thesis was previously presented for another degree or diploma at any university.

Tijjani ALI

_____	_____	_____
Name of Student	Signature	Date

Certification

This thesis entitled " BIOACTIVE NATURAL PRODUCTS FROM THE RHIZOMES OF *AMPELOCISSUS GRANTII, CISSUS CRINITA* AND *CISSUS SOKODENSIS* (*VITACEAE*)." by Tijjani ALI, meets the regulation governing the award of Doctor of Philosophy Degree in Organic Chemistry of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This work is dedicated to my entire family

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Abstract

Ampelocissus grantii (Baker and Planch), *Cissus crinita* (Planch) and *Cissus sokodensis* (Gilg and Brandt) are plants that belong to the family *Vitaceae* and are used in traditional medicine in northern Nigeria for the management of cancers and infectious diseases. The phytochemistry of these plants has not yet been reported. Therefore, the plants have been deemed desirable for phytochemical investigation to identify the possible active compounds that may be contributing to these medicinal therapies claimed by the people using them. The plants' rhizomes, after collection, identification, air-drying and pulverisation, were subjected to cold extraction (maceration) using hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH). Crude extracts obtained were phytochemically screened and chromatographed, mainly by column chromatography on silica gel. Standard antimicrobial method was employed to determine the minimum inhibitory concentration (MIC) of both the crude extracts and the isolates using clinical bacteria and fungi isolates: *Staphylococcus aureus, Bacillus subtilis* (Gram positive bacteria), *Escherichia coli, Pseudomonas aeruginosa* (Gram negative bacteria), *Crytococcus neoformans,Candida albicans, Candida tropicalis, Aspergillus niger* (fungi).The pure compounds were analysed for structure elucidations, using spectroscopic techniques, majorly 1D and 2D NMR experiments. Preliminary phytochemical screening of the crude plant extracts revealed the presence of steroids, terpenoids, flavonoids, tannins, saponins in both *Cissus sokodensis* and *Cissus crinita*. In addition to these compounds, anthraquinones were present in *Ampelocissus grantii*. Results of antimicrobial studies showed moderate to good activity against the organisms tested. Generally both the crude extracts and the pure compounds isolated recorded higher activities against the fungi. The MIC values of the crude extracts against the fungi ranged from 25 µg/ml to 200 µg/ml, 25 µg/ml to 100 µg/ml and 12.5 µg/ml to 100 µg/ml for *Ampelocissus grantii*, *Cissus crinita* and *Cissus sokodensis* respectively. The highest activity was recorded from *Cissus*

sokodensis's methanol extract against *Candida albicans* (MIC: 12.5 µg/ml).The respective MIC value against the bacteria for *Ampelocissus grantii*, *Cissus crinita* and *Cissus sokodensis* ranged from 25 µg/ml to 200 µg/ml, 100 µg/ml to 200 µg/ml and 100 µg/ml to 200 µg/ml. Highest MIC was observed from *Ampelocissus grantii* ethyl acetate extract against *Escherichia coli* (25 µg/ml). The pure compounds recorded higher activity against both the fungi and the bacteria compared to the crude extracts. The antifungal activity (MIC: 6.25-50 µg/ml) of the pure compounds was higher than the antibacterial activity (MIC: 12.5-50 µg/ml). Highest activity (MIC: 6.25 µg/ml) was recorded against *Candida albicans* and *Aspergillus niger*. Nine chemical compounds were isolated from the plants: β-sitosterol, betulin, 15-hydroxymethyl-16-methylheptadecylferulate and 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate from *Ampelocissus grantii*, β-sitosterol, oleanolic acid and 2,3,5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose from *Cissus sokodensis* and two, β-sitosterol and 2,3,5-trihydroxymethylbenzoate-α-D-glucopyranose from *Cissus crinita*. Four out of these compounds: 15-hydroxymethyl-16-methylheptadecylferulate, 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate, 2, 3, 5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose and 2, 3, 5-trihydroxymethylbenzoate-α-D-glucopyranose, are novel. Literature has shown that all the known compounds isolated have anticancer properties; further the newly isolated molecules have moieties in their structures with anticancer potentialities. Hence, the studied plants contain compounds that have anticancer property. The presence of the compounds isolated suggests that the plants contain chemical compounds that could possess both anticancer and antimicrobial properties. Therefore, these compounds may have been responsible for the anticancer and antimicrobial effects the plants have been said to possess in ethnomedicine and hence the folklore usage of the plants in the management of cancers and bacterial/fungi diseases are said to be validated.

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List of Abbreviations and Acronyms

NMR:	Nuclear Magnetic Resonance Spectroscopy
^{13}C NMR:	C-13 Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR:	Proton Nuclear Magnetic Resonance Spectroscopy
1 D :	One Dimensional
2 D :	Two Dimensional
br :	Broad
CD_3OD:	Deuterated Methanol
$CDCl_3$:	Deuterated Chloroform
COSY :	Correlation spectroscopy
d :	Doublet
dd:	Double Doublet
DEPT:	Distortionless Enhancement by Polarization Transfer
DMSO-d6:	Deuterated Dimethyl Sulfoxide
D_2O:	Deuterated Water
HMBC:	Heteronuclear Multiple Bond Correlation
HSQC:	Heteronuclear Single Quantum Coherence
Hz :	Hertz
IR:	Infrared
m:	Multiplet
Me:	Methyl
MIC:	Minimum Inhibitory Concentration
Mp:	Melting Point
NOESY:	Nuclear Overhauser Effect Spectroscopy
s :	Singlet
t :	Triplet

TLC : Thin Layer Chromatography

UV : Ultraviolet

EtOAc : Ethyl acetate

FTIR: Fourier Transform Infra Red

ATR: Attenuated Total Reflectance

DMSO: Dimethyl Sulfoxide

RPMI: Roswell Park Memorial Institute Medium

MHB: Muller-Hinton Broth

SDA : Sabraud Dextrose Agar

MOPS: 3-(N-morpholino)propanesulphonic acid

DCM: Dichloromethane

MeOH: Methanol

CHAPTER ONE

1.0 INTRODUCTION

1.1 Natural Products and Medicine

Over the centuries, the use of medicinal herbs has become an important part of daily life of humans and despite the progress in modern system of medicine; plants are one of the most important sources of medicines (Mohd *et al.*, 2012). This is because chemical compounds from natural sources (like plant) have been elaborated within living systems, thus are often perceived as showing more "drug-likeness and biological friendliness than totally synthetic molecules," (Koehn, 2005a) making them good candidates for further drug developments (Balunas and Kinghorn, 2005; Drahl *et al*., 2005).

Many people use herbal concoctions with the assumption that such is safe and effective. For people with chronic diseases, combining herbal medicine with conventional medicine is a common practice. It is expected that there may be synergistic effects between the conventional drugs and the herbal remedies. The use of indigenous medicinal plants is not based on science and is mostly unregulated and as such varies considerably from place to place.There is often no standard dosage and the safety of such practice remains unclear (Nyika, 2009).

In traditional practice, several plants are often used in combinations and the activity may be a result of either additive or synergistic effects. Many medicinal plants are the focus of biological screening and the crude extracts are screened for *in vitro* or *in vivo* activity or both. In some cases single active constituents for a particular action have been successfully identified. Phytochemical and toxicological studies can identify toxic components in order to assess and possibly eliminate or reduce harmful side effects of the extracts (Nyika, 2009).There is still a great need for phytochemical and pharmacological research of

medicinal plants in order to improve the efficacy and reduce potentially harmful side effects that may be associated with it.

Although there are many clinically approved anticancer and antimicrobial drugs, both of synthetic and natural (microbial and plant) origin (Fansworth, 1985; Chabner, 2005). However, much progress needs to be made to overcome the problems of resistance to and toxicity of existing cancer and pathogenic microbial chemotherapeutic agents. Medicinal plants have been shown to possess bioactive compounds such as polyphenolics, vinca alkaloids, podophytotoxin derivatives and taxol derivatives which reduce the incidence of cancers; and different classes of compounds for example: alkaloids, some flavonoids, quinones, xanthones and terpenes that serve as antimicrobial agents. It is in view of these that the three (3) plant candidates that have been used for the treatment of cancers (e.g leukemia and breast cancer) and microorganisms related diseases by the natives, especially in northern Nigeria, were selected for this research. Furthermore, this study seeks to isolate and identify novel substances responsible for the acclaimed efficacy in curing cancer and that could also serve as antimicrobial agents with less toxicity or side effects.

1.2 Statement of the Research Problem

Developing countries like Nigeria are bedevilled by inadequacy of health care facilities, poor accessibility, high poverty level, lack of trained man power and often, low demand for conventional health care system. An estimated 80% of the populace relies on ethno-medicinal practices, most of which are herbal preparations (Omole-Ohonsi and Aiyedun, 2013) for their primary health care needs.

The emergence and wide spread of pathogenic microorganism resistant strains is on the increase on daily basis (Tijjani *et al*., 2011). Beside this, diseases like cancers have continued to be of great concerned to man. This is because there is no 'total cure for cancer' up to now once it reaches malignant stage (Jemal, 2011). Furthermore, drugs used for the treatment of cancer and also serve as antimicrobial agents are associated in one way or the other with side effects or toxicity. These have carried the attention of scientists and made them continuously investigate for possible solutions to the stated menace.

1.3 Justification of the Study

Medicinal plants have proven to be reservoirs for molecules such as alkaloids, flavonoids and saponins that showed wide range pharmacological activities.Therefore, there is continuous need to search for plants to source novel and cost effective molecules with less or no toxicity capable of bringing total cure for diseases with high mortality rate like cancer and ailments caused by pathogenic microorganisms.

1.4 Aim and Objectives of the Research

The aim of the research is to seek scientific validation of the local use of the plant candidates (rhizomes of *Ampelocissus grantii, Cissus crinita* and *Cissus sokodensis*) as anticancer as well as antimicrobial agents in ethnomedicine and possible discovery of new medicinal entities from the natural sources.

The stated aim will be achieved through the following objectives.

I. Extraction of the chemical compounds from the plants using appropriate solvents.

II. Phytochemical screening of the plants extracts.

III. Determination of the biological activities of the extracts of each of the species in line with the ethnomedicinal uses of the plants.

IV. Isolation of the novel chemical compounds from the selected plants.

V. Identification of the chemical compounds that can serve as anticancer and antimicrobial agents.

VI. Isolation of any other bioactive compound(s) from the plants and

VII. Characterization and elucidation of the chemical structures of the compounds isolated.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Phytochemicals and Medicinal Plants

Interest in medicinal plants arises from the desire to provide a scientific rationale for the use of plants and the possibility of discovering novel compounds of pharmaceutical value. Historically, compounds containing novel structures from plant sources were a major source for the discovery and development of new drugs for several diseases (Newman and Cragg, 2007). These include quinine from *Cinchona* species and artemisinin (Artemisinin®) from *Artemisia annua.* Quinine was used as a template for the synthesis of chloroquine, which was the main antimalarial drug until recently. Semi-synthetic derivatives of artemisinine such as atreether (Artemotil®), artemether (Artemetheri®) and sodium artesunate (Arinate®) are often used more frequently for malaria treatment. Lapachol (a prenylnaphthoquinone), an antimalarial drug from *Tabebuia species* (Bignoniaceae) was a template for the synthesis of the newest antimalarial drug atovaquinone (Malarone®) (Newman and Cragg, 2007; Kaur *et al.*, 2009; Oliveira *et al*., 2009). Plant-derived antibacterial drugs such as daptomycin (Cubicin®), fosfomycin tromentamol (Monuril®) and Isepamicin (Isepacin®) (isolated from plants) and Biapenem (Omegacin), cefuroxime axetil (Zinnat), dorifenem (Finibax®), Eriththromycin acistrate (Erasis®), Tigecyclin (Tyagcil®), a semi-synthesized from natural products, (Newman and Cragg, 2007; 2012; Cragg and Newman, 2013) demonstrate the potential of finding new and better antibacterial agents with fewer side-effects, which could provide alternative antibacterial agents to resistant bacteria.

Flavonoids such as epicatechin gallate, epigallocatechin gallate and gallocatechin gallate isolated from tea are examples of plant derived compounds with potentially exploitable activities, including antibacterial activity (Hamilton-Miller and Shah, 2000), synergism with antibiotics (Shiota *et al*., 1999; Qin *et al*., 2013), suppression of bacterial virulence (Shah *et al*., 2008) and suppression of resistance (Stapleton *et al.*, 2004; 2007).

A number of plant extracts and compounds have demonstrated activity against malarial pathogens. Different classes of compounds, such as alkaloids, flavonoids, quinones, xanthones and terpenes, with antiplasmodial activity have been reported (Puricelli, 2003; Batista *et al.*, 2009; Oliveira *et al.*, 2009; Caniato and Nogueira and Lopes, 2011). Some of the compounds like galloylated catechins and procyanidins are reported to inhibit or reverse resistance of the most virulent malarial parasite, *Plasmodium falciparum* (Tasdemir *et al.*, 2006; Sannella *et al*., 2007; Ramanandralbe *et al*., 2008; Park *et al*., 2010).

Anticancer agents derived from plants and their derivatives for example, paclitaxel (Taxol®), Vincristine (Oncovin®), vinorelbine (Navelbine®), teniposide (Vumon®) and camptothecin (e.g. Hycamtin®) have been proven to be effective for cancer prevention and therapeutics (Ramanandralbe *et al*., 2008; Cragg *et al.*, 2009; Cragg and Newman, 2013). Phytochemicals are used for the treatment of cancer related symptoms due to their safety, low toxicity, and general availability. Many active phytochemicals are in human clinical trials and there is the possibility of discovering novel and more effective anticancer phytochemicals (Pratheeshkumar *et al.*, 2012). Consuming phytochemicals as part of a daily diet have been shown to have cancer protective effects by inhibiting, delaying, or reversing carcinogenesis by inducing detoxifying and antioxidant enzyme systems, regulating inflammatory and proliferative signaling pathways, and inducing cell cycle arrest and apoptosis (Singh *et al.*, 2002; Pratheeshkumar *et al*., 2012; Rajput and Mandal, 2012).

2.1 Description of Plant Family: *Vitaceae*

Vitaceae plant family has 14 genera and 900 species, with the genera: *Cissus* constituting 350 species (Sarvalingam *et al*., 2013). The plant family is distributed world wide in tropical, subtropical and temperate regions (Wheeler and LaPasha, 1994). It is made up of woody climbers, sometimes vines, with rarely small succulent trees, hermaphroditic or polygamo-monoecious to polygamo-dioecious. Stems are unarmed, sometimes with conspicuous lenticels, or bark sometimes shredding (in most species of *Vitis*); branches are often swollen at 3–7-lacunar nodes; pith is continuous or interrupted by diaphragms at nodes; tendrils are simple, bifurcate to trifurcate, or 4–12-branched (in Parthenocissus), usually the leaf are opposed and rarely tendrils absent. Raphide sacs are present in parenchymatous tissues. Leaves are simple, lobed or unlobed, or digitately or pedately compound to 1–3-pinnately compound.They are alternate, distichous, variously toothed, commonly with multicellular, stalked, caducous spherical structures known as "pearl" glands; stipules 2 or rarely absent, often caducous. Flowers are small, with prophylls, in panicles, corymbs, or rarely spikes, often leaf-opposite, pseudo-terminal, or axillary (in Cayratia and Tetrastigma), actinomorphic, hypogynous, 4- or 5(–7 as in Rhoicissus)-merous. The Calyx has 4 or 5(–7) small teeth or lobes or a continuous ring. Petals are valvate, 4 or 5(–7), free or basally connate, or distally connate forming a calyptra (e.g., in Vitis). Stamens are 4 or 5(–7), antepetalous; anthers introrse, dehiscing longitudinally, tetrasporangiate or rarely bisporangiate. Floral disk is intrastaminal, ring-shaped, cupular, or gland-shaped. Ovary is superior, 2-loculed; ovules occurring 2 per locule; placentation usually axile, appearing nearly basal, apotropous or anatropous, bitegmic, crassinucellar; style is simple, connate; stigma discoid or capitate, rarely 4-lobed (Tetrastigma), not papillate. Fruit is a berry, 1–4-seeded.

The fourteen (14) commonly accepted genera of *vitaceae* are: *Acareosperma* Gagnepain, *Ampelocissus* Planch., *Ampelopsis* Michx., *Cayratia* Juss., *Cissus* L., *Clematicissus* Planch., *Cyphostemma* (Planch.) Alston, *Nothocissus* (Miq.) Latiff, *Parthenocissus* Planch., *Pterisanthes* Blume, *Rhoicissus* Planch., *Tetrastigma* (Miq.) Planch., *Vitis* L., and *Yua* C. L. Li. Among these, *Acareosperma*, and *Nothocissus*, are monotypic. The Australian endemic *Clematicissus* was monotypic until the recent transfer of the Australian species, *Cissus opaca*, to this genus (Jackes and Rossetto, 2006). *Pterocissus mirabilis* was transferred to *Cissus* (Lombardi, 1997) and this genus is now considered within *Cissus*. In these taxonomic treatments, genera are mainly differentiated by floral structures (for example, petal number is almost always used in keys to the genera; *Vitis* has a calyptra; *Tetrastigma* has lobed styles; the floral nectariferous disc of *Cyphostemma* is shaped like 4 free glands), inflorescence structures (e.g *Ampelocissus* has tendrilbearing inflorescence; *Nothocissus* has whip-like bifurcated spikes; *Pterisanthes* has an unusual laminar inflorescence axis; inflorescences of *Cayratia* have an axillary or pseudo-axillaryposition), sometimes vegetative (e.g *Parthenocissus* has suction pads on the tips of the tendrils), fruit (berries of *Cissus* are mostly one-seeded) and seed morphology (*Acareosperma* has spiny seeds; *Clematicissus* endosperm U-shaped in transverse section). Species within the family are often distinguished by variations in vegetative structures, mostly by different leaf forms and/or indument conditions. Extensive works on comparative developmental morphology of floral and vegetative structures of *Vitaceae* have been carried out by Gerrath and colleagues (Wilson *et al*., 2006; Gerrath and Posluszny, 2007; Timmons, Posluszny, and Gerrath, 2007a, 2007b).

2.2 Chemical Constituents of Some Species of Plant Family: *Vitaceae*

2.2.1 *Parthenocissus tricuspidata* (Planch.)

This plant is reported to contain: Piceid, Longistylin A and Longistylin C (Park *et al*., 2008; Joanne *et al*., 2009).

2.2.2 *Cissus cornifolia*

Musa *et al*. (2008) and Adarsh Krishna *et al*. (2013) reported the presence of alkaloids, flavonoids, saponins, steroids/terpenoids and tannins from the plant.

2.2.3 *Cissus quadrangularis* (Linn)

The plant was reported to contain : flavonoids, triterpenoids, Vitamin C, stilbene derivatives (Chopra *et al*., 1958) resveratrol, piceatannol, pallidol perthenocissin, phytosterols (Udupa *et al*., 1961), ascorbic acid, triterpene, β-sitosterol, ketosteroid, two asymmetrical tetracyclic triterpenoids (7-Oxo-Onocer-8-ene-3 β 21 α diol) (Shirley and Sen, 1966), 4-hydroxy 2 methyl-tricos-2ene-22-one,9-methyloctadec-9-ene,heptadecyl-octadecanoate, icosanylicosanoate, 31-methyl tritiacontan-1-ol, 7-hydroxy-20- oxo- docosanyl cyclohexane, 31-methyltritiacontanoic acid (Sen, 1966; Udupa *et al*.,1970; Bhutani *et al*., 1984; Pluemaj and Saifah, 1986), taraxeryl acetate, friedelan-3-one, taraxerol, isopentacosanoic acid (Pluemjai and Saifah, 1986; Gupta and Verma, 1991), 3, 3^1, 4, 4^1 tetrahydroxybiphenyl (Khan, *et al*., 1991; Kumbhojkar *et al*.,1991), potassium, calcium, zinc, sodium, magnesium (Ambasta, 1992; Rastogi and Mehrotra, 1995; Saburi *et al*.,1999), carbonates, phosphates (Chopra *et al*., 1999; Kritikar *et al*.,2000), potassium tartarate, calcium oxalate (Attawish *et al*., 2002; Enechi and Odonwodo, 2003), methyl tritiacontanoic acid, taraxeryl acetate (Prajapati *et al*., 2003; Somova *et al*., 2003), taraxerol and iso-pentacosanoic acid (Austin *et al*., 2004; Jainu and Devi, 2004).

2.2.4 *Cissus adnata (*Roxb.)

β-amyrin [olean-12(13)-en-3-ol], Onocer-7-ene-3α,21β-diol and Adnatoside (Apigenin 8-C-α-D-glucopyranoside) were reported from the plant by Warjeet *et al*. (2011).

2.2.5 *Cissus rheifolia* (Planch.)

Leaves of the plant were reported to contain Trans-2-(3, 4-dimethoxyphenyl)-3-(4 methoxyphenyl) quinolizidine, cryptopleurine and kayawongine (alkaloids), isomeric terpenoids: vomifoliol and romalea allene and the major flavonoid: vitexin (Ekarin *et al*., 1983).

2.2.6 *Cissus rheifolia* (Planch.)

The plant roots contain: sterols (β-sitosterol, stigmasterol, and campesterol) together with a known dihydroflavonol, aromadendrin, (Saifah *et al*., 1986).

2.2.7 *Vitis vinifera*

Resveratrol (3, 5, 4'-trans-trihydroxystilbene),catechin,procyanidin,malvin, 4, 4'-dihydroxy-trans-stilbene, 3, 4-dihydroxy-trans-stilbene, 4-hydroxy-trans-stilbene, 3, 5-dihydroxy-trans-stilbene,malvidin-3(6"-caffeyl-3-glucoside), picatechin,anthocyanins,delphinidincaffeyl-3-glucoside,malvidin-3(6"-p-coumaryl glucoside) (Mukesh *et al*., 2009), e-viniferin, balanocarpol and b-glucopyranosyl 8- balanocarpol (Felicio *et al*., 2001) were reported from the plant.

2.2.8 *Cissus populnea* (Guill. & Perr.)

Micheal and Emmanuel (2012) reported the presence of alkaloids, flavonoids, saponins, and tannins from the plant.

2.2.9 *Cissus arnottiana*

Tannins, phenols, terpenoids, flavonoids, glycosides, and saponins were reported from the plant (Kavitha and Rajeshwari, 2012).

2.2.10 *Ampelocissus* species

Ampelocissus species was reported to possess: 22-epicalamistrin, acetogenin uvaribonin and Chalcone (George *et al.,* 2008).

2.2.11 *Cissus crinita* (planch)

No isolation of constituent reported from the plant to the best of our knowledge.

2.2.12 *Cissus sokodensis* (Gilg & Brandt)

There was no isolation of constituent reported from the plant to the best of our knowledge.

2.2.13 *Ampelocissus grantii* (Baker) Planch

There was no isolation of constituent reported from the plant to the best of our knowledge.

2.3 Pharmacological Activity of Some *Viteceae* Family Species (*Vitis, Cissus* and *Ampelocissus* Species)

2.3.1 *Cissus cornifolia* (Bak.) Planch

This plant was reported to possess depressant and sedative properties (Rakotonirina *et al.*, 2001; Musa *et al.*, 2006, Musa *et al.*, 2008).

2.3.2 *Cissus quadrangularis* (Linn)

C. quadrangularis is claimed to exhibit bone fracture healing activity by stimulation of metabolism and increased uptake of calcium, sulphur and strontium (Udupa, 1965; Prasad, 1972). It also helps in fracture healing by influencing early regeneration of all connective tissues involved in the healing and quicker mineralization of callus (Udupa 1964a, 1964b). Other ways in which *C. quadrangularis* assist in fracture healing is by: the stimulation of all the cells of mesenchyma origin, namely the fibroblasts, the chondroblasts and osteoblasts (Singh and Udupa, 1962; Sen *et al*., 1966; Kausch and Horner, 1982), building up the chemical composition of the fractured bone namely its mucopolysaccharides, collagen, calcium, phosphorus and others as well as its functional efficiency (Chopra *et al*., 1975;

Chopra *et al*.,1976; Canalis *et al*., 1998; Genan *et al.*, 1998; Ogey *et al*., 2001), neutralizing the anti-anabolic effect of cortisone in healing of fractures and also enhancing the mineralization of the callus (Peng, 1994; Sanyal, 2005), helping in the earlier formation of collagen fibers leading to earlier calcification and callus formation (Soliman *et al*., 1964; Ferreira *et al*., 1973; Singh *et al*., 1984; Deka *et al*.,1994; Lu *et al*., 2002; Shirwaikar *et al*., 2003) and replacement of cartilaginous cells by osteoblastic cells and union of the fractured gap at several places with the formation of new bony trabeculae (Cohen *et al*., 1957; Gaillard *et al*.,1972).

The plant also showed analgesic property that compared well with that of acetylsalicylic acid (*Viswanath et al.,* 2006*;* Shirwaikar *et al*., 2003; Jainu and Devi, 2005a). Additionally, it was also reported to have antiulcer activity (Jainu and Devi 2004, Jainu and Devi 2006; Hatazawa *et al*., 2007).The plant was also reported to possess antioxidant activity (Gabriel *et al*., 2005; Jainu and Devi, 2005b; Ngondi *et al*., 2006), anabolic and androgenic activity (Lin *et al*., 1999; Ferrandiz and Alcaraz, 1991), anti-inflammatory activity (Cospite, 1994; Diana, 2000; Dela *et al*., 2000; Lyseng-Williamson and Perry, 2003; Mallika and Shyamala, 2006; Hatazawa, 2007) and *in vitro* anthelmintic activity (Mohanambal *et al*., 2011).

2.3.3 *Vitis vinifera* (L.)

This plant was reported to have antioxidant (Selga *et al*., 2004; Cornacchione *et al.*, 2007; Mukherjee *et al.*, 2011), anticarcinogenic (Hakimuddin *et al*., 2004; Opipari *et al*., 2004; Su *et al*., 2005), immunomodulatory (Das and Das, 2007; Pillai *et al*., 2008; Xu *et al*., 2008), antidiabetes (Bertelli *et al*., 1999; Szewczuk *et al*., 2004; Zhang *et al*., 2004), anti-atherogenic (Rajaram; 2003; Del *et al*., 2006; Du *et al*., 2007), neuroprotective (Mizutani *et al*., 2000; Sun *et al*., 2002; Antonio *et al*., 2008), anti-obesity and anti-aging (Moreno *et al*.,

2003; de la Lastra and Villegas, 2007) and anti-infection properties(Shchelkunov, 2001; Leiro *et al*., 2004; Krasnow and Murphy, 2004).

2.3.4 *Ampelocissus grantii* (Baker) Planch

This plant was reported to posses trypanocidal effect (Bizimana *et al*., 2006; Birgit *et al*., 2008; Aderbauer *et al*., 2008), antiplasmodial activity (Cheikna *et al*., 2011), antioxidant, antibacterial and antifungal activity (Zongo *et al*., 2010).

2.4. Local Uses of Some *Vitaceae* Species

2.4.1 *Vitis sylvestris.*L

This was reported to have antibacterial, haemorrhoids and stomach disorders (Jaric, 2007; Akan, 2008; Omer and Eyup, 2013).

2.4.2 *Cissus populnea* Guill. & Perr

This is used in northern Nigeria for postnatal stoppage of bleeding (Soladoye and Chukwuma, 2012).

2.4.3 *Cissus cornifolia* (Bak.) Planch

The root is used against gonorrhoea in northern Nigeria; the root-decoction is also used for malaria in Tanzania, septic tonsil and pharyngitis; while the leaf-sap is used among the Tanganyika as a sedative in cases of mental derangement (Haerdi, 1964; Burkill, 2000).

2.4.4 *Cissus quadrangularis* (Linn)

This plant has been used for promotion of fracture healing (Garima Mishra *et al*., 2010). Stem paste of the plant may also be useful for muscular pains, burns, wounds, and bites of poisonous insects; saddle sores of horses and camels. The powder of dry shoots is given in digestive troubles. A decoction of the shoots with dry ginger and black pepper is given for body pains. The infusion of the plant is anthelmintic. The plant extracts also exhibit

cardiotonic property. Young shoots are used in dyspepsia and indigestion. The powdered stem is mixed with pulses and fried in sesame oil, used as a remedy for several vata diseases. It is also used in skin diseases leprosy, cough, epilepsy and convulsions (Rastogi and Mehrotra, 1995; Mehta *et al*., 2001; Mallika and Shyamala, 2005; www.pioneerherbs.com.), Sekou *et al*. (2006), reported the use of the plant for the treatment of schistosomiasis.

2.4.5 *Ampelocissus grantii* (Bak.) Planch

In Sudan, a leaf infusion of the plant is mixed with onions and drunk and used as a wash to relieve fever pains in head and neck (Dalziel, 1937; Burkill, 2000).There was also report of antidiarrheal activity (Etuk *et al.,* 2009), wound healing property (Kari *et al.*, 2004), anti-schistosomiasis effect (Sekou *et al*., 2006). In northern Nigeria, the plant rhizome is used by the locals for the treatment of cancers and also serves as antimicrobial agent (treatment of fever and diarrhea).

2.4.6 *Cissus crinita* (planch)

In northern Nigeria, the plant rhizome is used by the locals for the treatment of cancers and fever.

2.4.7 *Cissus sokodensis* (Gilg & Brandt)

In northern Nigeria, the plant rhizome is used by the locals for the treatment of cancers and fever.

2.4.8 *Cissus aralioides* (Welw. Ex Baker) Planch

In Congo the plant stem is used to make an embrocation for fever pains: Bouquet (1969) quoted in Burkill (2000).

2.4.9 *Cissus integrifolia* (Baker) Planch

In Malawi, the roots are powdered or infused as a remedy for fevers (Morris, 1996).Where as in Mozambique it is used to treat malaria (Mulhovo, 1999).

2.4.10 *Vitis sylvestris* L.

This plant serves as antibacterial also used for haemorrhoids and stomach disorders (Omer and Eyup, 2013).

2.4.11 *Cissus populnea* Guill. & Perr

The plant used by Fulanis in northern Nigeria to feed their cattle, ostensibly to increase milk production, as reported by Brotherton (1969). The plant is also used in Niger, Kogi, Plateau, Adamawa, Kwara, and Benue states of Nigeria for making vegetable soup for postnatal stoppage of bleeding (Soladoye and Chukwuma 2012). A decoction of the stem made with native natron (Hausa: *Kanwa*) and often with the addition of *Alchornea cordifolia* (Schumach.) Müll. Arg. is used for the treatment of venereal diseases among locals in northern Nigeria. The viscid sap is used for other herbal remedies, while the root is used as antidote for arrow-poison. The roots are also used by the Yorubas to cure sore breasts of women at childbirth, and as a male coital adjunct (Burkill, 2000).

2.5 Types of Natural Products

Several drug candidates are derived from various naturally occurring medicinal sources. These can be broadly divided into four categories: microorganisms, marine organisms, animal and plant sources.

2.5.1 Natural products from microorganisms

Microorganisms as a source of potential drug candidates were not explored until the discovery of penicillin in 1929. Since then, a large number of terrestrial and marine microorganisms have been screened for drug discovery. Microorganisms have a wide variety of potentially active substances and have led to the discovery of antibacterial agents like cephalosporins (**1**), antidiabetic agents like acarbose (**2**), and anticancer agents like epirubicin (**3**) (Chin *et al*., 2006).

2.5.2 Natural products from marine organisms

The first active compounds to be isolated from marine species were spongouridine (**4**) and spongothymidine (**5**) from the Carribean sponge *Cryptotheca crypta* in the 1950s. These compounds are nucleotides and show great potential as anticancer and antiviral agents. Their discovery led to an extensive research to identify novel drug candidates from marine sources. About 70% of the earth's surface is covered by the oceans, providing significant biodiversity for exploration for drug sources. Many marine organisms have a sedentary lifestyle, and thereby synthesize many complex and extremely potent chemicals as their means of defense from predators (Haefner, 2003). These chemicals can serve as possible remedies for various ailments, especially cancer. One such example is discodermolide (**6**), isolated from the marine sponge, *Discodermia dissoluta*, which has a similar mode of action to that of paclitaxol® and possesses a strong antitumor activity. It also exhibits better water solubility as compared to paclitaxol®. A combination therapy of the two drugs has led to reduced tumor growth in certain cancers (Huang, 2006).

2.5.3 Natural products from animal sources

Animals have also been a source of some interesting compounds that can be used as drugs. Epibatidine (**7**), obtained from the skin of an Ecuadorian poison frog, it is ten times more potent than morphine (Spande, 1992). Venoms and toxins from animals have played a significant role in designing a multitude of cures for several diseases. Teprotide (pry-trp-pro-arg-glu-ile-pro-pro), for example, extracted from a Brazilian viper, has led to the development of cilazapril (**8**) and captopril, which are effective against hypertension (**9**) (Anonymous, 2014).

2.5.4 Natural products from plant sources

The use of plants as medicines has a long history in the treatment of various diseases.

The earliest known records for the use of plants as drugs are from Mesopotamia in 2600 B.C., and these still are a significant part of traditional medicine and herbal remedies (Koehn and Carter, 2005b). To date, 35,000-70,000 plant species have been screened for their medicinal use (Farnsworth, 1991).

Their contribution to the world market for herbal remedies is documented, Europe- 33%, Asia- 26% , North America- 20%, Japan- 11%, Others- 10% , (Dev, 1999).

Several important drugs such as Taxol®, camptothecin, morphine and quinine have been isolated from plant sources. The first two (Taxol® and camptothecin) are widely used as anticancer drugs, while the remaining (morphine and quinine) are analgesic and antimalarial agents, respectively (Newman and Cragg, 2007; Cragg *et al.*, 2009; Cragg and Newman, 2013).

2.5.5 Plant-based anticancer drugs

Cancer is the second leading cause of death among children between the ages of one and fourteen and it is also responsible for 25% of all deaths today (Jemal *et al*., 2005).There were 10.9 million new cancer cases diagnosed in USA and 6.7 million deaths in 2002 (Tan *et al.*, 2006). Seventy-seven percent of all the cancers diagnosed are observed in people aged 55 years or older (Anonymous, 2002). These figures indicate that the death toll from cancer is going to rise with the aging of US population.

In spite of the availability of a large number of anticancer drugs and various chemotherapy options, there is still an acute need for less toxic and more potent cancer drugs and continues be the concern. Most of the drugs available are not selective to cancer cells and affect the normal cells as well leading to severe side effects. However, these drugs are currently the most effective means to combat cancer. The aim of research in cancer drug development is to find new drugs that are specific to cancer cells, or to develop a method that alters the nature of the drug administered such that it acts only on the target cells and not the regular normal

functioning cells, thereby reducing the side effects.

Several anticancer agents available in the market today derive their origin from natural sources. One of the early compounds isolated as an anticancer agent was podophyllotoxin **(10**), a compound obtained from *Podophyllum peltatum* (**11**) (Sweeney, 2007), in 1944 (Imbert, 1998). It was initially used therapeutically as a purgative and in the treatment of venereal warts (Kaplan, 1942). Later, in 1974, it was shown that it acts as an anticancer agent by binding irreversibly to tubulin (Wilson, 1974). Etoposide (**12**) and teniposide (**13**), the modified analogs of podophyllotoxin, however, cause cell death by inhibition of topoisomerase II, thus preventing the cleavage of the enzyme- DNA complex and arresting the cell growth (Srivastava, 2005). Both these analogs are used in the treatment of various cancers (Odwyer *et al.*, 1985).

The Madagascar periwinkle, *Catharanthus roseus* (Anonymous, 2007), a member of the *Apocynaceae* family, is important because of its diverse medicinal properties. It is a rich source of indole alkaloids which include the anticancer alkaloids vincristine (**14**) and vinblastine (**15**), and also the antihypertensive alkaloid, ajmalicine (**16**). For centuries, this plant was used as remedy for diabetes, as it was believed to enhance the production of insulin by the body. Both vinblastine and vincristine are now known to prevent cell division by inhibiting mitosis in the cell cycle. They irreversibly bind to tubulin, thereby blocking cell multiplication and eventually causing cell death (Noble, 1990).

An extract of the Pacific yew tree, *Taxus brevifolia* (Ingram, 2007), was discovered to possess excellent anticancer properties in 1963, and its active component was isolated only a few years later in 1967 by Monroe Wall and his co-worker, Mansukh Wani (Jacoby, 2005). They published their findings as well as the structure of the active component, paclitaxel (Taxol®), in 1971 (**17**) (Wani *et al*., 1971). Susan B. Horwitz, a molecular pharmacologist, established

the novel mechanism of action of paclitaxel in 1979. Paclitaxel irreversibly binds to *β*-tubulin, thus promoting microtubule stabilization (Schiff *et al*., 1979). This tubulin-microtubule equilibrium is essential for cell multiplication, and its stabilization causes programmed cell death (Wilson and Jordan, 1995). Previously reported anticancer drugs, vinblastine, vincristine and podophyllotoxin also bind to tubulin, but prevent rather than promote microtubule formation. Paclitaxel was the first compound to be discovered to promote microtubule formation. It has been used in the treatment of several types of cancer, but most commonly for ovarian and breast cancers as well as non-small cell lung tumors (Kinghorn and Seo, 1996). It had sales of $750 million in 2002 and $1.0 billion in 2003 (Oberlies and Kroll, 2004). Shortly after the discovery of paclitaxel and its unique mechanism, several compounds having the same mode of action were discovered. The epothilones, discovered from the myxobacterium *Sorangium cellulosum*, possess potential anticancer properties (**18**, **19**) and show high *in vivo* activity, including activity against taxane-resistant cell lines. However, they exhibit moderate *in vitro* cytotoxicity (Cortes and Baselga, 2007). Several semisynthetic analogs of epothilones such as ixabepilone (**20**) have been developed which are currently in Phase II clinical trials for treatment of breast cancer (Chin *et al*., 2006).

Camptothecin (**21**), discovered from the deciduous tree *Camptotheca acuminata*, is also an anticancer agent which has a unique mechanism of action. Camptothecin and its derivatives are topoisomerase-I inhibitors, and cause cell death by DNA damage (Hsiang *et al*., 1985). However, camptothecin itself is too insoluble to be used as a drug but its several water-soluble analogs, namely, topotecan (**22**) and irinotecan (**23**) have been developed as effective anticancer drugs (Oberlies and Kroll, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals/Reagents

Hexane (Sigma-Aldrich), dichloromethane (Sigma-Aldrich), ethyl acetate (Sigma-Aldrich), methanol (Sigma-Aldrich), sulphuric acid, ethanol, sodium hydroxide, hydrochloric acid, chloroform, sulphuric acid, ferric chloride, acetone, ammonia solution, benzene, magnesium metal,wagner's reagent Dragendorff's reagent, Meyer's reagent, resazurin, dimethyl sulphoxide, distilled water, nutrient broth, dextrose agar, blood agar, deuterated dimethyl sulfoxide (DMSO-d_6) and deuterated chloroform ($CDCl_3$) were used for the experiments.

3.1.1 Equipments/Materials

Rotary evaporator, Silica gel 60 F_{254}(Merck), chromatographic tank, ultraviolet lamp (254 and 366) nm, Perkin Elmer Spectrum 100 FTIR spectrometer with universal ATR sampling accessory, Bruker Avance^{III} (Germany) 400 and 600 MHz NMR spectrometer and Ernst Leitz Wetziar micro-hot stage melting point apparatus were used for the experiments.

3.1.2. Test organisms

Staphylococcus aureus and *Bacillus subtilis* (Gram-positive bacteria); *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative bacteria); *Candida albicans, Candida tropicalis, Cryptococcus neoformans* and *Aspergillus niger* (Fungi) were used for the antimicrobial analyses.

3.2 Plant Materials

The rhizomes of the plant, *Cissus sokodensis* and *Cissus crinita* were collected from Unguwan-Rimi Basawa Zaria, northern Nigeria in June 2012, while *Amepelocissus grantii* was collected from Likoro Zaria in the same month and year as the other two plants. All the plant materials were authenticated by Mallam Musa Muhammad of the Herbarium Section of Biological Sciences Department, Ahmadu Bello University, Zaria, where numbered voucher specimen: 9002, 506 and 25832 were deposited for future reference in respect of *Amepelocissus grantii*, *Cissus sokodensis* and *Cissus crinita*, respectively.The plant rhizomes were cleaned, air-dried, pulverized, labeled and stored in air-tight polythene bags for subsequent use.

3.3 Extraction

Powdered rhizomes of *Ampelocissus grantii* (800 g), *Cissus sokodensis* (1Kg) and *Cissus crinita* (1 Kg) were respectively and continuously extracted with hexane (3 dm^3) by cold extraction (maceration) with shaking for three days. The extracts obtained were filtered and concentrated *in vacuo* using rota vapor at 40^oC after which the extracts were air dried. The residues (marcs) were similarly macerated with dichloromethane (2 dm^3), ethyl acetate (2 dm^3) and methanol (2 dm^3), in this order, for two days per each plant material from the plants using the same procedure as described earlier (above). All extracts obtained, after drying, were kept in refrigerator prior to use.

3.4 Preliminary Phytochemical Screening

Portions of the plants extracts each, was subjected to preliminary phytochemical screening using standard methods described by Harbone, (1984), Silva *et al.* (1998) Sofowora, (1993) and Trease and Evans, (1989).

3.4.1 Test for sterols/terpenes

a. Liebermann-burchard test:

Anhydrous acetic acid (1 ml) was added to chloroform (1 ml) and cooled to 0 °C; one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract. The solution was observed for brown color change with time.

b. Salkowski test

A little quantity of the extract was dissolved in chloroform (1 ml) and concentrated sulphuric acid (1 ml) was added down the test tube to fom two phases. Formation of red coloration was taken as an indication for the presence of sterols.

3.4.2 Test for flavonoids

a. Shinoda test

To an alcoholic solution of the extract three pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. Appearance of a red color indicates the presence of flavonoids.

b. Sulphuric acid test:

The sample was dissolved in concentrated sulphuric acid and the colour change was observed.

c. Ferric chloride test

Extract was boiled with water and filtered. To 2 ml of the filtrate, two drops of freshly prepared ferric chloride solution was added; a green coloration indicates the presence of phenolic hydroxyl group.

d. Sodium hydroxide test

The filtered extract (2 ml) was dissolved in 10% aqueous sodium hydroxide solution to give yellow colour, a change in colour from yellow to colourless on addition of dilute HCl indicates the presence of flavonoids.

3.4.3 Test for alkaloids

The extract (0.5g) was stirred with 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. Three (3) ml of the filtrate was divided into three. To the first 1 ml, few drops of freshly prepared Dragendoff reagent was added and observed for formation of brownish precipitate. To the second, 1 drop of Mayer reagent was added and observed for formation of yellowish colour precipitate. To the third 1 ml, 1 drop of Wagner reagent was added to give a reddish- brown precipitate.

3.4.4 Test for tannins

A small quantity of the extract was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate, formation of a green-black precipitate was taken as evidence for the presence of tannins.

3.4.5 Test for anthraquinones

a. **Free anthraquinones (Bornstrager's test)**

The extract was shaken with 10 ml of benzene, the content was filtered, and 5 ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of violet colour in the ammoniacal layer (lower phase) indicates the presence of free anthraquinone.

b. **Combined anthraquinones (modified Bornstrager's test)**

The extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate was shaken with 5 ml benzene, the benzene layer was separated and half its own volume, 10% NH_4OH was added. A violet colouration in the ammonia phase (lower phase) indicates the presence of combined anthraquinone or anthraquinone derivative.

3.4.6 Test for saponins

Exactly 0.5 g of the extract was shaken with water in a test tube. Frothing observed which persisted for 15 minutes indicates the presence of saponins (Silva *et al.*, 1998).

3.5 Chromatographic Separation, Purification and Characterization of Pure Compounds

The plant extracts, after drying, were subjected to chromatographic seperations.

3.5.1 Isolation and purification of G1 and G2

The hexane extract of *Ampelocissus grantii* (4 g) was dissolved in minimum amount of dichloromethane, preabsorbed in silica gel and subsequently loaded onto a column (60 cm x 3 cm) packed with silica gel, 230–400 mesh , (200 g). Normal phase adsorption chromatography was carried out with gradient elution using a mixture of Hexane–EtOAc (100:0, 95:5, 90:10, 85:15, 70:30,50:50,30:70,10:90 and 0:100, 2.0 dm^3 for each step) in an increasing order of polarity. Each fraction (100 ml) collected was concentrated *in vacuo*.Thin layer chromatographic (TLC) analyses of fractions were carried out on silica gel TLC sheets with Hexane: Ethyl acetate (8.5:1.5) as mobile phase system for all the eluted fractions and visualised under UV illumination (254-365 nm). Fractions possessing similar R_f values were pooled together, resulting in six major groups (A–F). Fraction E was washed severally with pentane to afford G1. Further separation was performed on fraction F on silica gel by column chromatography eluting with hexane and EtOAc in increasing polarity (100:1 to 1:1) to obtain five sub-fractions. From subfraction 2 component G2 was obtained. Both G1 and G2 were found to be soluble in hexane, dichlomethane and chloroform.

3.5.2 Melting point determination of G1 and G2

The uncorreceted melting points of G1 and G2 were recorded as 137-139 ^oC and 256-257 ^oC respectively.

3.5.3 Spectroscopic analyses of G1 and G2

G1 and G2 were subjected to NMR and IR spectroscopic studies and results obtained were used to elucidate the structures of these compounds.

3.5.4 Isolation and purification of G3

The dichloromethane extract of *Ampelocissus grantii* (1 g) was dissolved in minimum dichloromethane, preabsorbed in silica gel using rota vapor and subsequently loaded onto column (60 cm x 3 cm) packed with silica gel (230–400 mesh) (100 g). Normal phase adsorption chromatography was carried out with gradient elution using a mixture of Hexane–DCM (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70,10:90 and 0:100, 2.0 dm^3 for each step).This was followed by elution with Hexane–EtOAc (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70,10:90 and 0:100, 2.0 dm^3 for each step). Fractions obtained with same R_f values from TLC were pooled together and grouped into three (A-C) using Hexane: Ethyl acetate (8:2) as solvent system. This led to isolation of G3 from fraction B. G3 was thoroughly washed with pentane to attain optimal purity for spectroscopic analyses.G3 was found to be soluble in chloroform.

3.5.5 Melting point determination of G3

The uncorreceted melting point of G3 was recorded as 188-190 ^oC.

3.5.6 Spectroscopic analyses of G3

G3 was subjected to NMR and IR spectroscopic analyses and results obtained were used to elucidate the structure of the compound.

3.5.7 Isolation and purification of G4

The EtOAc extract of *Ampelocissus grantii* (5 g) was dissolved in minimum quantity of EtOAc, preabsorbed in silica gel and subsequently loaded onto column (60 cm x 3 cm)

packed with silica gel, 230–400 mesh, (200 g). Normal phase adsorption chromatography was carried out with gradient elution using a mixture of Hexane–EtOAc (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70,10:90 and 0:100, 2.5 dm^3 for each step) and EtOAc: MeOH (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70, 10:90 and 0:100, 2.5 dm^3 for each step) in an increasing order of polarity.The eluate (50 ml) was collected at a time and concentrated on a rotary evaporator.Thin layer chromatography (TLC) analysis of fractions was carried out on silica gel 60 F_{254}, precoated aluminium cards (0.2 mm thickness, Merck Ltd.,Germany) with Hexane:Ethyl acetate(9:1), Hexane:Ethyl acetate(8.5:1.5), Hexane: Ethyl acetate(8:2), Hexane:Ethyl acetate(7:3), Hexane:Ethyl acetate(6:4), Hexane:Ethyl acetate(1:1), Chloform:Ethyl acetate:acetic acid (5:4:1), Ethyl acetate:Methanol:Water (10:1.35:1) solvent mixtures, depending on the nature of compounds eluted, were used as mobile phases for the eluted fractions. Spots on TLC were viewed under UV illumination (254-365nm) or with 10% sulphuric acid in ethanol with heating using heat gun for 5min or using iodine vapour in iodine chamber. Fractions possessing similar R_f values were combined to form six major groups (A–F). Repeated washing of the first group using hexane afforded G4. G4 was found to be soluble in chloroform.

3.5.8 Melting point determination of G4

The uncorreceted melting point of G4 was recorded as 198-200 ^oC.

3.5.9 Spectroscopic analyses of G4

G4 was subjected to NMR and IR spectroscopic analyses and results obtained were used to elucidate the structure of the compound.

3.5.10 Isolation and purification of S1 and S2

TLC analyses of Hexane and dichloromethane extracts of C*issus sokodensis* rhizome indicated that majority of components for both extracts are the same; therefore the two extracts were combined together. The two extracts (Hexane =1.514 g, dichloromethane =

1.059 g) were dissolved in minimum quantity of dichloromethane, preabsorbed in silica gel and subsequently loaded onto column (60 cm x 3 cm) packed with silica gel, 230–400 mesh, (100 g). Normal phase adsorption chromatography was carried out with gradient elution using a mixture of Hexane– dichloromethane (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70,10:90 and 0:100, 2.5 $dm^3$for each step) and dichloromethane: EtOAc (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70, 10:90 and 0:100, 2.5 dm^3 for each step) in an increasing order of polarity. The eluate (50 ml) was collected at a time and concentrated on a rotary evaporator. Thin layer chromatography (TLC) analysis of fractions was carried out on silica gel $60F_{254}$, precoated aluminium cards (0.2 mm thickness, Merck Ltd.,Germany) to monitor column progress with the following solvent system: Hexane- EtOAc (9.5:0.5), (9:1) and (8.5:1.5). Fractions possessing similar R_f values were combined together, resulting in three major groups (A–C).Washing of fraction B with non-polar solvent (pentane) led to purification of S1.Fraction C was again subjected to micro-column for further clean up; and this led to isolation of S2. S1 was soluble in hexane, dichloromethane and chloroform but insoluble in methanol while S2 was soluble in dichloromethane and chloroform.

3.5.11 Melting point determination of S1 and S2

The uncorrected melting points of S1 and S2 were recorded as 256-257 ^oC and 307-308 ^oC respectively.

3.5.12 Spectroscopic analyses of S1 and S2

S1 and S2 were subjected to NMR and IR spectroscopic analyses and results obtained were used to elucidate the structures of the compounds.

3.5.13 Isolation and purification of S3

The EtOAc extract of C*issus sokodensis* rhizome (5.132 g) was dissolved in minimum quantity of EtOAc, preabsorbed in silica gel and subsequently loaded onto column

(60 cm x 3 cm) packed with silica gel, 230–400 mesh, (100 g). Normal phase adsorption chromatography was carried out with gradient elution using a mixture of Hexane–EtOAc (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70,10:90 and 0:100, 2 dm^3 for each step) and EtOAc: MeOH (100:0, 95:5, 90:10, 85:15, 70:30, 50:50, 30:70, 10:90 and 0:100, 3 dm^3 for each step) in an increasing order of polarity. The eluate (100 ml) was collected at a time and concentrated on a rotary evaporator.Thin layer chromatography (TLC) analyses of fractions were carried out on silica gel TLC sheets with Hexane:Ethyl acetate(9:1), Hexane:Ethyl acetate(8.5:1.5), Hexane : Ethyl acetate(8:2), Hexane : Ethyl acetate(7:3), Hexane : Ethyl acetate (6:4), Hexane : Ethyl acetate(1:1), Chloform : Ethyl acetate : Acetic acid (5:4:1),

Ethyl acetate : Methanol:Water (10:1.35:1) solvent mixtures, depending on the nature of compounds eluted, were used as mobile phase systems for the eluted fractions. Spots on TLC were either viewed under UV illumination (254-365 nm) or with 10 % sulphuric acid in ethanol with heating for 5min or using iodine vapour in iodine chamber. Fractions possessing similar R_f values were pooled together to form three major groups (A–C). Repeated washing of group C using hexane afforded S3. S3 was found to be soluble in acetone and methanol.

3.5.14 Melting point determination of S3

The uncorrected melting point of S3 was found to be 203-204 ^oC.

3.5.15 Spectroscopic analyses of S3

S3 was subjected to NMR and IR spectroscopic analyses and results obtained were used to elucidate the structure of the compound.

3.5.16 Isolation and purification of C1 and C2

The EtOAc extract of C*issus crinita* rhizome (5.132 g) was dissolved in minimum quantity of EtOAc, preabsorbed in silica gel and subsequently loaded onto column (60 cm x 3 cm) packed with silica gel, 230–400 mesh, (100 g). Normal phase adsorption chromatography was carried out with gradient elution using a mixture of Hexane–EtOAc (100:0, 95:5, 90:10,

85:15, 70:30, 50:50, 30:70, 10:90 and 0:100, 2 dm^3 for each step) and EtOAc: MeOH (100:0, 95:5, 90:10, 85:15, 70:30, 50:50,30:70,10:90 and 0:100, 3 dm^3 for each step) in an increasing order of polarity. The eluate (100 ml) was collected at a time and concentrated on a rotary evaporator.Thin layer chromatography (TLC) analyses of fractions were carried out on TLC sheets withHexane:Ethyl acetate(9:1), Hexane:Ethyl acetate(8.5:1.5), Hexane:Ethyl acetate (8:2), Hexane: Ethyl acetate(7:3), Hexane:Ethyl acetate(6:4), Hexane:Ethyl acetate(1:1), Chloform:Ethyl acetate:Acetic acid (5:4:1), Ethyl acetate: Methanol:Water (10:1.35:1) solvent mixtures, depending on the nature of compounds eluted, were used as mophile phase systems for the eluted fractions.Spots on TLC were viewed under UV illumination (254-365 nm) or with 10% sulphuric acid in ethanol with heating using heat gun for 5 min or using iodine vapour in iodine chamber . Fractions possessing similar R_f values were combined together to form three major groups (A–D).Washing of group B using pentane afforded C1. D was purified after repeated washing with hexane to obtain C2. C1 was found to be soluble in hexane, dichloromethane and chloroform but insoluble in methanol; and C2 was soluble in acetone and methanol.

3.5.17 Melting point determination of C1 and C2

The uncorrected melting points of C1 and C2 were 137-139 ^oC and 206-207 ^oC respectively.

3.5.18 Spectroscopic analyses of C1 and C2

C1 and C2 were subjected to NMR and IR spectroscopic analyses and results obtained were used to elucidate the structures of the compounds.

3.6 Antimicrobial Studies of Extracts and Pure Compounds

The antimicrobial effects of hexane, dichloromethane, ethylacetate and methanol crude extracts of the rhizomes *A. grantii, C. crinita and C. sokodensis;* and the four new isolated compounds from the plants were determined on pathogenic organisms *in vitro*.

3.6.1 Test organisms

The organisms used were obtained from the Department of Medical Microbiology, Durban General Hospital, South Africa. All bacterial cultures were checked for purity and maintained on a blood agar slant while the fungi were maintained on a slant of Sabraud Dextrose Agar (SDA). The test organisms were: *Staphylococcus aureus, Bacillus subtilis* (Gram-positive) and *Escherichia coli, Pseudomonas aeruginosa* (Gram-negative) and *Cryptococcus neoformans, Candida albicans, Candida tropicalis, Aspergillus niger* (fungi).

3.6.2 Preparation of medium

The nutrient medium solution was prepared by dissolving 22.0 g of Muller-Hinton Broth (MHB) (containing Acid Hydrolysate of Casein, Beef Extract and Starch) in 1 L of double distilled water. The P^H of the medium was adjusted to 7.4 ± 0.1 and sterilized in an autoclave for 15 min at 121 °C. The solution was allowed to cool and stored at 4 °C. Sterility check was performed by incubating un-inoculated media in an aerobic incubator at 37 °C for 24 h. For antifungal activity, RPMI 1640 medium with L-glutamine and 0.165 M MOPS without sodium bicarbonate (Lonza) was used (Satyajit *et al.*, 2007).

3.6.3 Preparation of the plant extracts and pure compounds stock solution and working standard

A known weighed quantity (4.0 mg) of the test extracts or isolates (as the case may be) and standard drugs were respectively dissolved in 1 ml of DMSO to give stock solution (4000 µg/ml).The stock solution (100 µl) was diluted with 900 µL of double distilled water to afford working standard solution (400 µg/ml).

3.6.4 Preparation of inoculums

A day prior to the antimicrobial testing, one or more identical colonies of the microorganisms were suspended in 4.5 ml sterile double distilled water. Inoculates were adjusted to 0.5

McFarland standard (1.5 x 10^8cfu/ml). A density check turbid meter was used to ensure that the 38 rinites was a 0.5 McFarland standard.

3.6.5 Determination of minimum inhibitory concentration

The preliminary *in vitro* antimicrobial activity for both the extracts and pure compounds were evaluated using the broth micro-dilution method. Sterile double distilled water (100 µL) was added to all outer-perimeter wells of a 96-well micro liter plates to minimize evaporation of the medium in the test wells during incubation. To the remaining test wells 100 µL of MHB was added. Two fold serial dilutions of the test extracts or isolates and standard drugs (Amoxicillin and Amphotericin B) were made directly on the microplate using MHB. The extracts or pure compounds were tested at final concentration of 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 µg/ml. Finally, 10 µL of the freshly prepared bacterial or fungal 38rinites was added to the wells. The micro liter plates were covered and sealed with parafilm and incubated at 37 ± 1 ºC for 24 h. After this, 10 µL of freshly prepared resazurin (0.4 mg/ml) was added to the test wells and incubated further for 5 h. MIC was determined as a blue color in the test well (interpreted as no bacterial growth) while a pink color was registered as growth. The MIC was thus defined as the lowest drug concentration that prevented a color change from blue to pink. This experiment was conducted in duplicate and the average MIC values in µg/ml were recorded (Satyajit *et al.*, 2007).

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction

Generally, from hexane to methanol extracts, the recovery ranged from: 0.558-4.3905%, 0.208-10.670% and 264-6.354 for *A. grantii, C. crinita* and *C. sokodensis* respectively (Table 4.1).

4.2 Phytochemical Screening

The results of the phytochemical screening for the three crude plants' extracts revealed the presence of presence of steroids, triterpenoids (in hexane extracts), flavonoids, tannins, saponins (in ethyl acetate and methanol extracts) from the rhizomes of *A. grantii, C. crinita and C. sokodensis*. Additionally, anthraquinones were present in *A. grantii* ethyl acetate and methanol extracts this is shown in Tables 4.2-4.4.

4.3 Isolation and Purification of G1 and G2

The results of silica gel column of the hexane extract gave a total of 110 collections. Fraction 49-56 afforded a white needle like crystals, coded G1 (300 mg, R_f = 0.23, solvent system: 15 % ethyl acetate in 85 % hexane); while fraction 75-85 (after repeated chromatography) afforded an amorphous creamy powder coded G2 (100 mg, R_f = 0.13, solvent system: 15 % ethylacetate in 85 % hexane).

4.3.1 Nuclear magnetic resonance spectra data of G1

^{1}H NMR (400MHz, $CDCl_3$) δ(ppm): 0.68-2.32 (m,C,CH,CH_2,CH_3), 3.52(brs), 5.33(d).

^{13}C(100MHz,$CDCl_3$)δ(ppm):11.9, 11.9, 18.8, 19.0, 19.4, 19.8, 21.1, 23.1, 24.3, 26.1, 28.3, 29.2, 29.7, 31.7, 31.9, 33.9, 36.2, 36.5, 37.3, 39.8, 42.3, 45.8, 50.1, 51.2, 56.1, 56.8, 71.8, 121.7, 140.8 (Figure 4.1-4.2).

4.3.2 Nuclear magnetic resonance spectra data of G2

^{1}H NMR ($CDCl_3$, 400 MHz) δ(ppm): 4.67 (1H, d), 4.56 (1H,d), 3.79 (1H, d, *J* =10.8), 3.33 (1H, d, *J* = 10.8), 3.18 (1H, dd, *J* =5.3), 1.67 (3H, s), 0.98 (3H, s), 0.96 (3H, s), 0.95 (3H, s),0.79 (3H, s), 0.73 (3H, s) (Figure 4.9).

^{13}C NMR ($CDCl_3$,100 MHz) δ(ppm): 150.5, 109.7, 79.0, 60.6, 55.3, 50.4 , 48.8, 47.8, 47.8, 42.7, 40.9, 38.9, 38.7, 37.3, 37.2, 34.2, 33.9, 29.7, 29.2, 27.9, 27.4, 27.0, 25.2, 20.8, 19.1,18.3,16.1, 16.0, 15.4, 14.8 (Figure 4.10).

4.3.3 Infra red spectrum data of G1

IR υ_{max} (cm^{-1}): 3409, 2918, 2850, 1705, 1463,1377,1367,1332, 1241, 1311, 1192, 1108, 1134, 1021, 957, 925, 839, 801 and 570 (Figure 4.8).

4.3.4 Infra red spectrum data of G2

IR υ_{max} (cm^{-1}): 3460, 2916, 1709, 1159 and 1024, 881 (Figure 4.16).

4.4 Isolation and Purification of G3

The results of silica gel column of the dichloromethane extract gave a total of 88 collections. Fraction 11-18 afforded an amorphous white solid, coded G3 (18 mg, R_f = 0.45, solvent system: 20 % ethylacetate in 80 % hexane).

4.4.1 Nuclear magnetic resonance spectra data of G3

^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.56 (1H, d, *J* = 15.9 Hz), 7.04 (1H, dd, *J* = 1.8, 8.2 Hz), 7.01 (1H, d, *J* = 1.8 Hz),6.89 (1H, d, *J* = 8.1 Hz),6.25 (1H, d, *J* =15.9), 5.83 (1H, brs), 4.16 (2H, t), 3.92 (3H, s), 3.61 (3H, t), 1.64 (2H, m), 0.87-1.52 (m,CH,CH_2,CH_3) (Figure 4.17).^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 167.4, 147.9, 146.7, 144.6, 127.1, 123.0,115.7, 114.7,109.3, 64.6, 63.1, 55.9, 34.1, 32.8, 31.9, 29.7, 29.7, 29.6, 29.6, 29.4, 29.4, 29.3, 28.7, 26.0, 25.7, 22.7, 22.3, 14.1, 14.1 (Figure 4.19).

4.4.2 Infra red spectrum data of G3

IR υ_{max} (cm^{-1}): 3327, 2917, 2849, 1736, 1472, 1462, 1261, 1378, 1170, 1030, 878, 801,730 and 719 (Figure 4.25).

4.5 Isolation and Purification of G4

The results of silica gel column of the DCM extract gave a total of 261 collections. Fraction 11-18 afforded an amorphous white solid, coded G4 (10 mg, R_f = 0.34, solvent system: 20% ethylacetate in 80% hexane).

4.5.1 Nuclear magnetic resonance spectra data of G4

^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm): 7.58 (1H, d, *J* = 15.9 Hz), 7.05 (1H, dd, *J* = 1.7, 8.4 Hz), 7.02 (1H, d, *J* = 1.6 Hz), 6.88 (1H, d, *J* = 8.2 Hz), 6.25 (1H ,d, *J*=15.9), 5.81 (1H,s), 4.14 (2H, t), 3.92 (3H, s), 3.59 (3H, t), 2.29 (2H,t), 2.13 (3H,s), 0.83-1.64 (m, CH, CH_2, CH_3) (Figure 4.26).

^{13}C NMR ($CDCl_3$, 125 MHz) δ (ppm): 207.4, 167.4, 147.9, 146.7, 144.6, 127.1, 123.0,115.8, 114.7, 109.4, 64.6, 63.1, 55.9, 33.5, 32.8, 31.9, 30.9 , 29.6, 29.6, 29.5, 29.4, 29.3, 29.3, 26.0, 25.7, 24.8, 22.7, 14.1 (Figure 4.28).

4.5.2 Infra red spectrum data of G4

IR υ_{max} (cm^{-1}): 3370, 2917, 2849, 1725, 1650, 1492, 1158 (Figure 4.34).

4.6 Isolation and Purification of S1 and S2

The elution of the silica gel column of the hexane extract gave a total of 147 collections. Fraction 49-56 afforded a white needle like crystals, coded S1 (100 mg, R_f = 0.33, solvent system: 13 % ethyl acetate in 87 % hexane); while fractions 75-85 (after repeated silica column chromatographing) afforded an amorphous pale yellow powder coded S2 (19 mg, R_f = 0.16, solvent system: 10 % ethyl acetate in 90 % hexane).

4.6.1 Nuclear magnetic resonance spectra data of S1

^{1}H NMR (400MHz, $CDCl_3$) δ (ppm): 3.52 (1H, *m*), 5.33 (1H, *t*), 0.67 (3H, *s*), 0.98 (3H, *s*), 0.90 (3H, s), 0.83 (3H, s), 0.82 (3H, s), 0.88 (3H, s) (Figure 4.35).

^{13}C (100MHz, $CDCl_3$) δ(ppm):11.9,11.9,18.8,19.0,19.4,19.8, 21.1, 23.1, 24.3, 26.1, 28.3, 29.2, 29.7, 31.7, 31.9, 33.9, 36.2, 36.5, 37.3, 39.8, 42.3, 45.8, 50.1, 51.2, 56.1, 56.8, 71.8, 121.7, 140.8 (Figure 4.36).

4.6.2 Nuclear magnetic resonance spectra data of S2

^{1}H NMR (400MHz, $CDCl_3$) δ(ppm): δ = 5.25 (1 H ,t, *J* = 3.1 Hz), 3.21 (1H, dd, *J* = 4.2, 10.7 Hz), 2.81 (1H, dd, *J* = 3.8,13.6 Hz), 2.02-1.96 (m, 1 H), 1.91- 1.81 (m, 2 H), 1.80-1.67 (m, 2 H), 1.66-1.48 (m, 16 H), 1.47-1.22 (m, 3H), 1.10 (3H, s), 0.95 (3H, s), 0.93 (3H, s), 0.92 (3H, s), 0.89 (3H, s), 0.74 (3H, s) 0.72 (3H, s) (Figure 4.43).

^{13}C (100MHz, $CDCl_3$) δ(ppm): 38.4, 27.2, 79.0, 38.8, 55.2, 18.3, 32.6, 39.3, 47.6, 37.1, 23.4, 122.6, 143.6, 41.6, 27.7, 23.6, 46.5, 41.0, 45.9, 30.7, 33.8, 32.4, 28.1, 15.5, 15.4, 16.9, 26.0, 182.9, 33.1, 23.6(Figure 4.44).

4.6.3 Infra red spectrum data of S1

IR υ_{max} (cm^{-1}): 3409, 2918, 2850, 1705, 1463, 1377, 1367, 1332, 1241, 1311, 1192, 1108, 1134, 1021, 957, 925, 839, 801 and 570 (Figure 4.42).

4.6.4 Infra red spectrum data of S2

IR υ_{max} (cm^{-1}): 3300, 2916, 2849, 1736, 1176, 1713, 1472, 1463, 1377, 1309, 1266, 1221, 1012, 893,730 and 718 (Figure 4.50).

4.7 Isolation and Purification of S3

The results of silica gel column of the EtOAc extract gave a total of 171 collections. Fraction 145-156 afforded a brown amorphous powder, coded S3 (400 mg, R_f = 0.45, solvent system: Ethyl acetate: Methanol: Water (10: 1.35: 1).

4.7.1 Nuclear magnetic resonance spectra data of S3

^{1}H NMR (400MHz, DMSO-d6) δ(ppm): δ =6.95 (1 H, s), 4.95 (1 H ,d, *J* = 10.44 Hz), 3.98 (1 H, t, *J* = 9.72 Hz), 3.82 (2H, d, *J* = 2.44 Hz, 14.16 Hz), 3.64 (1 H, dd, *J* = 8.80 Hz, 14.16 Hz), 3.55(1 H, t, *J* = 8.16 Hz) and (1 H, d, *J* = 8.96 Hz, 14.76 Hz) (Figure 4.51).

^{13}C NMR (DMSO-d6, 100 MHz) δ(ppm):163.3, 150.9, 147.9, 140.5, 117.9, 115.9, 109.4, 81.7, 79.7, 73.6, 72.0, 70.6, 61.0 , 59.8 and 28.9 (Figure 4.53).

4.7.2 Infra red spectrum data of S3

IR υ_{max} (cm^{-1}): 3383, 2919, 2851, 1698, 1610, 1514, 1460, 1371, 1347, 1233, 1125, 1088, 1070, 1012, 990, 962, 907, 860, 821, 764, 723, 647, 622, 587 and 538 (Figure 4.59).

4.8 Isolation and Purification of C1 and C2

The results of the column of the hexane extract gave a total of 160 collections. Fraction 40-51 afforded a white needle like crystals, coded C1 (210 mg, R_f = 0.24, solvent system: 15 % ethylacetate in 85 % hexane); while fraction 135-145 afforded an amorphous brown powder, coded C2 (450 mg, Rf =0.41, solvent system: Ethylacetate: Methanol: Water (10:1.35:1).

4.8.1 Nuclear magnetic resonance spectra data of C1

^{1}H NMR (400MHz, $CDCl_3$) δ(ppm): 3.54 (1H, *m*), 5.33 (1H, *t*), 0.66 (3H, *s*), 0.98 (3H, *s*), 0.90 (3H, s), 0.84 (3H, s), 0.82 (3H, s), 0.89 (3H, s) (Figure 4.60).

^{13}C (100MHz, $CDCl_3$) δ(ppm):11.9,11.9,18.8,19.0,19.4,19.8, 21.1, 23.1, 24.3, 26.1, 28.3, 29.2, 29.7, 31.7, 31.9, 33.9, 36.2, 36.5, 37.3, 39.8, 42.3, 45.8, 50.1, 51.2, 56.1, 56.8,71.8, 121.7 and 140.8 (Figure 4.61).

4.8.2 Nuclear magnetic resonance spectra data of C2

^{1}H NMR (400MHz, DMSO-d6) δ(ppm): δ =6.97 (1 H ,s), 4.97 (1 H ,d, *J* = 10.44 Hz), 4.00 (1 H ,t, *J* = 9.76 Hz), 3.84 (2H ,d, *J* = 11.24Hz), 3.65 (1 H ,t, *J* = 8.76 Hz), 3.57(1 H ,t, *J* = 8.24 Hz) and 3.20 (1 H ,t, *J* = 9.04Hz) (Figure 4.68).

^{13}C NMR (DMSO-d6, 100 MHz) δ(ppm): 163.3, 150.9, 147.9, 140.5 ,117.9, 115.9, 109.4,81.7, 79.7, 73.6 , 72.0 ,70.6,61.0, 59.7 (Figure 4.70).

4.8.3 Infra red spectrum data of C1

IR υ_{max} (cm^{-1}): 3409, 2918, 2850, 1705, 1463, 1377, 1367, 1332, 1311, 1241, 1192, 1134, 1108, 1049, 1022, 957, 925, 839, 801 and 571 (Figure 4.67).

4.8.4 Infra red spectrum data of C2

IR υ_{max} (cm^{-1}): 3207, 3383, 2918, 2850, 1698, 1611, 1528, 1462, 1372, 1347, 1333, 1233, 1127, 1089, 1070, 1043, 990, 964, 858,764,723 and 622 (Figure 4.76).

4.9 Antimicrobial Studies of the Plant Extracts and the Pure Compounds

The results of MIC of both the crude extracts and the newly isolated molecules are as shown in Tables 4.3 and 4.4.Generally, the MICs (recorded in µg/mL) of the crude extracts of the three plants studied ranged from 25 µg/mL to 200 µg/mL except that of *Cissus sokodensis*, which ranged from:12.5-200 µg/mL (Table 4.3). For the isolates, the MIC values ranged from 6.25-200 µg/mL,12.5-50 µg/mL,6.25- 200 µg/mL and 6.25-100 µg/mL for G3, G4, S3 and C2 respectively (Table 4.4).

Table 4.1: Extraction of Rhizomes of *A. grantii, C. crinita and C. sokodensis*

Plant	Weight (g)	Extract	Weight of Extract (g)	% Recovery (%)
A. grantii	800	Hexane	4.460	0.558
		DCM	6.349	0.794
		EtOAc	19.440	2.430
		MeOH	35.124	4.391
C. sokodensis	1000	Hexane	2.640	0.264
		DCM	4.550	0.455
		EtOAc	12.550	1.267
		MeOH	63.538	6.354
C. crinita	1000	Hexane	2.075	0.208
		DCM	6.140	0.614
		EtOAc	9.360	0.936
		MeOH	106.704	10.670

Table 4.2: Phytochemical Screening of the Rhizome of *A. grantii*

Constituent	**Test**	**Observation**	**Inference**
Alkaloids	Dragendorff's	No brown precipitate formed	-
	Wagner's	Brown precipitate not observed	-
	Meyer's	Yellow precipitate not seen	-
Flavonoids	Shinoda	Formation of red to pink	+
	Sodium hydroxide	Colour changed from yellow to colourless	+
	Sulphuric acid	Red solution formed	+

Saponins	Frothing	Frothing persisted for more than 15 minutes was observed	+
Tannins	Ferric chloride	Green-black colour formed	+
Anthraquinones	Bornstrager's (Free Anthraquinones)	violet colouration seen in the ammonia phase (lower layer)	+
	Modified Bornstrager's (Combined Anthraquinones)	violet colouration seen in the ammonia phase (lower phase)	+
Steroids/ Triterpenes	Salkowski	Red ring formed at the interphase	+
	Liebermann-Burchard	Brown ring with brown supernatant	+

Key:+ =Present; - =Absent

Table 4.3: Phytochemical Screening of the Rhizome of C. *crinita*

Constituent	Test	Observation	Inference
Alkaloids	Dragendorff's	No brown precipitate formed	-
	Wagner's	Brown precipitate not observed	-
	Meyer's	Yellow precipitate not seen	-
Flavonoids	Shinoda	Formation of red to pink	+
	Sodium hydroxide	Colour changed from yellow to colourless	+
	Sulphuric acid	Red solution formed	+
Saponins	Frothing	Frothing persisted for more than 15 minutes was observed	+
Tannins	Ferric chloride	Green-black colour formed	+
Anthraquinones	Bornstrager's (Free Anthraquinones)	violet colouration not seen in the ammonia phase (lower layer)	-
	Modified Bornstrager's (Combined Anthraquinones)	violet colouration was absent in the ammonia phase (lower phase)	-
Steroids/ Triterpenes	Salkowski	Red ring formed at the interphase	+
	Liebermann-Burchard	Brown ring with brown supernatant	+

Key:+ =Present; - =Absent

Table 4.4: Phytochemical Screening of the Rhizome of *C. sokodensis*

Alkaloids	Dragendorff's	No brown precipitate formed	-
	Wagner's	Brown precipitate not observed	-
	Meyer's	Yellow precipitate not seen	-
Flavonoids	Shinoda	Formation of red to pink	+
	Sodium hydroxide	Colour changed from yellow to colourless	+
	Sulphuric acid	Red solution formed	+
Saponins	Frothing	Frothing persisted for more than 15 minutes was observed	+
Tannins	Ferric chloride	Green-black colour formed	+
Anthraquinones	Bornstrager's (Free Anthraquinones)	violet colouration not seen in the ammonia phase (lower layer)	-
	Modified Bornstrager's (Combined Anthraquinones)	violet colouration was absent in the ammonia phase (lower phase)	-
Steroids/ Triterpenes	Salkowski	Red ring formed at the interphase	+
	Liebermann-Burchard	Brown ring with brown supernatant	+

Key: + =Present; - =Absent

Table 4.5: Minimum Inhibitory Concentration (MIC) Determination of the Extracts

Organisms /MIC (µg/mL)

CODE	I	II	III	IV	V	VI	VII	VIII
AG1	100	200	100	100	50	200	100	50
AG2	200	200	100	100	50	200	50	50
AG3	100	100	25	100	50	25	50	50
AG4	100	200	100	100	50	25	50	50
CC1	100	200	100	100	50	25	50	50
CC2	100	100	100	100	50	25	50	100
CC3	100	100	100	100	50	50	100	100
CC4	100	100	100	100	50	25	50	50
CS1	100	100	100	100	50	25	100	50
CS2	100	200	100	100	50	25	100	50
CS3	100	100	100	100	50	25	100	50
CS4	100	100	100	100	50	12.5	100	50

Key: AG= *Ampelocissus grantii*, CC= *Cissus crinita,* CS=*Cissus sokodensis,* 1=Hexane extract, 2=DCM extract, 3=EtOAc extract and 4=MeOH extract.I**=** *Staphylococcus aureus*, II= *Bacillus subtilis*, III= *Escherichia coli*, IV= *Pseudomonas aeruginosa*, V= *Cryptococcus neoformans*, VI= *Candida albicans*, VII= *Candida tropicalis* and VIII= *Aspergillus niger*.

Table 4.6: Minimum Inhibitory Concentration (MIC) of the Isolated Pure Compounds

	Organisms/MIC (µg/mL)							
COMPOUND	I	II	III	IV	V	VI	VII	VIII
G3	50	50	25	50	6.25	12.5	50	6.25
G4	50	50	12.50	25	12.5	50	12.5	25
S3	50	50	50	12.5	25	6.25	50	12.5
C2	25	50	50	12.5	12.5	6.25	50	25

Key: I= *Staphylococcus aureus*, II= *Bacillus subtilis*, III= *Escherichia coli*,

IV= *Pseudomonas aeruginosa*, V= *Cryptococcus neoformans*, VI= *Candida albicans*,

VII= *Candida tropicalis*, VIII= *Aspergillus niger*

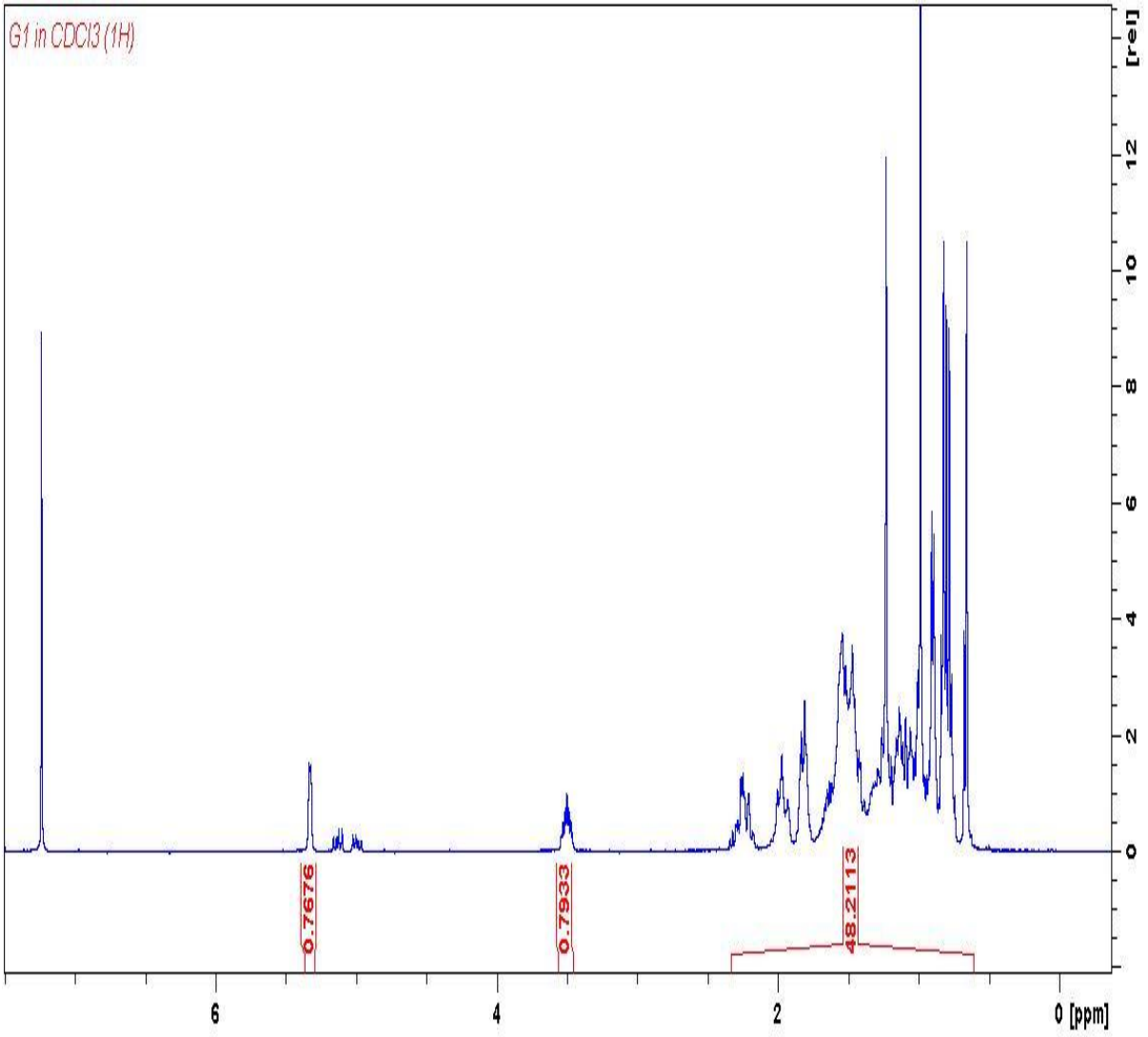


Figure 4.1: ^{1}H NMR Spectrum of G1

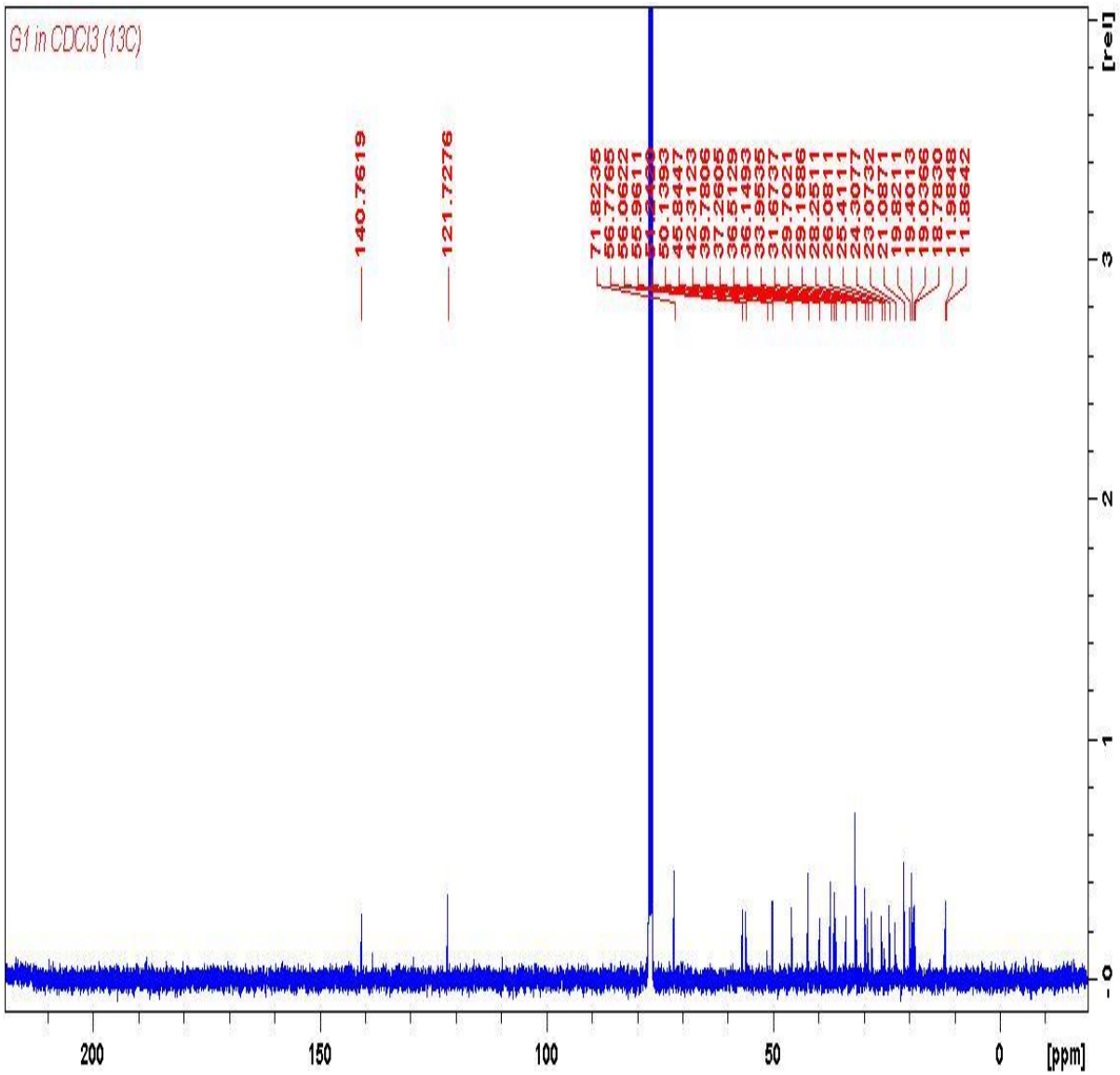


Figure 4.2: ^{13}C NMR Spectrum of G1

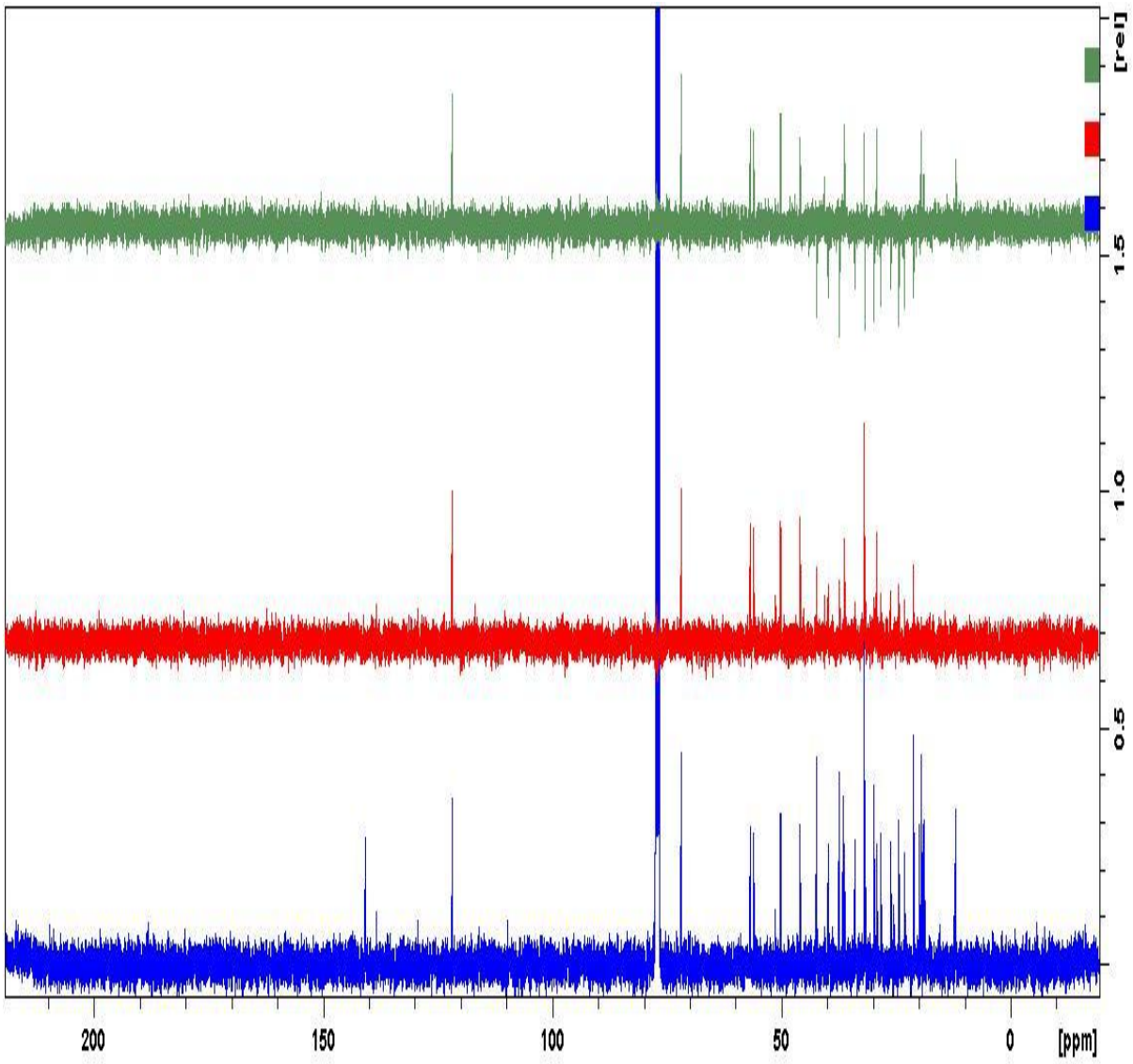


Figure 4.3: ^{13}C and DEPTS (90 and 135) NMR Spectra of G1

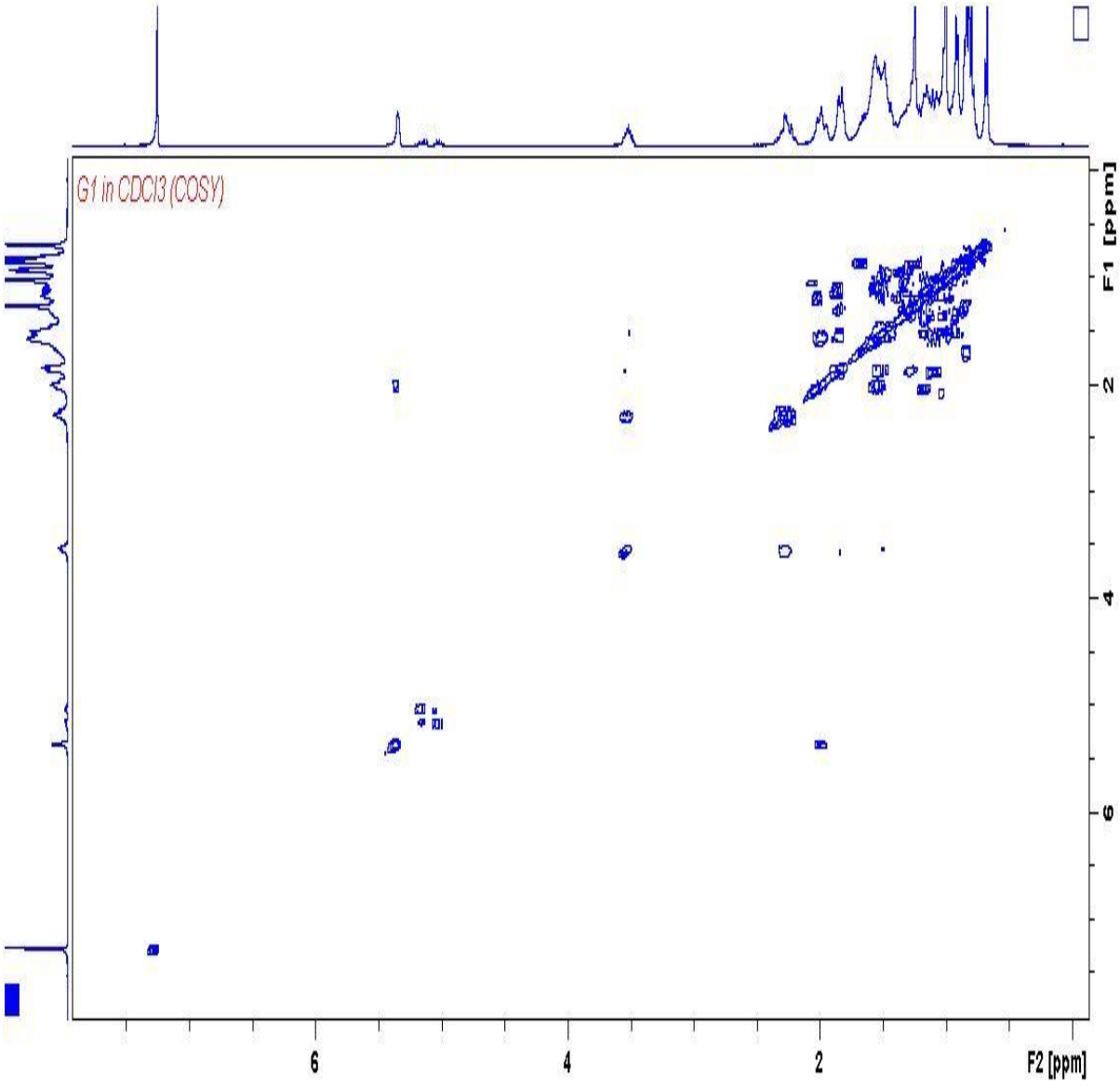


Figure 4.4: COSY NMR Spectrum of G1

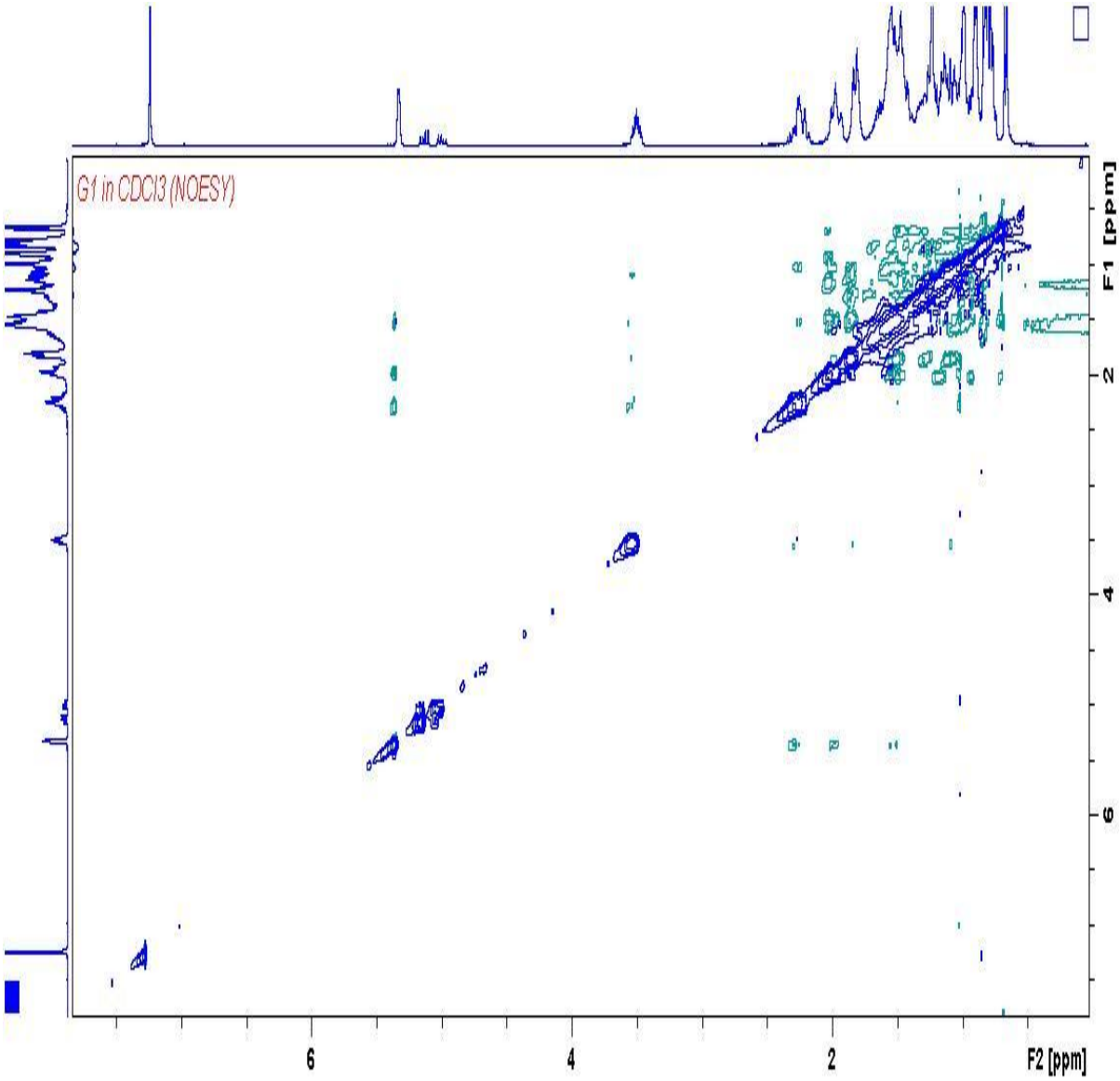


Figure 4.5: NOESY NMR Spectrum of G1

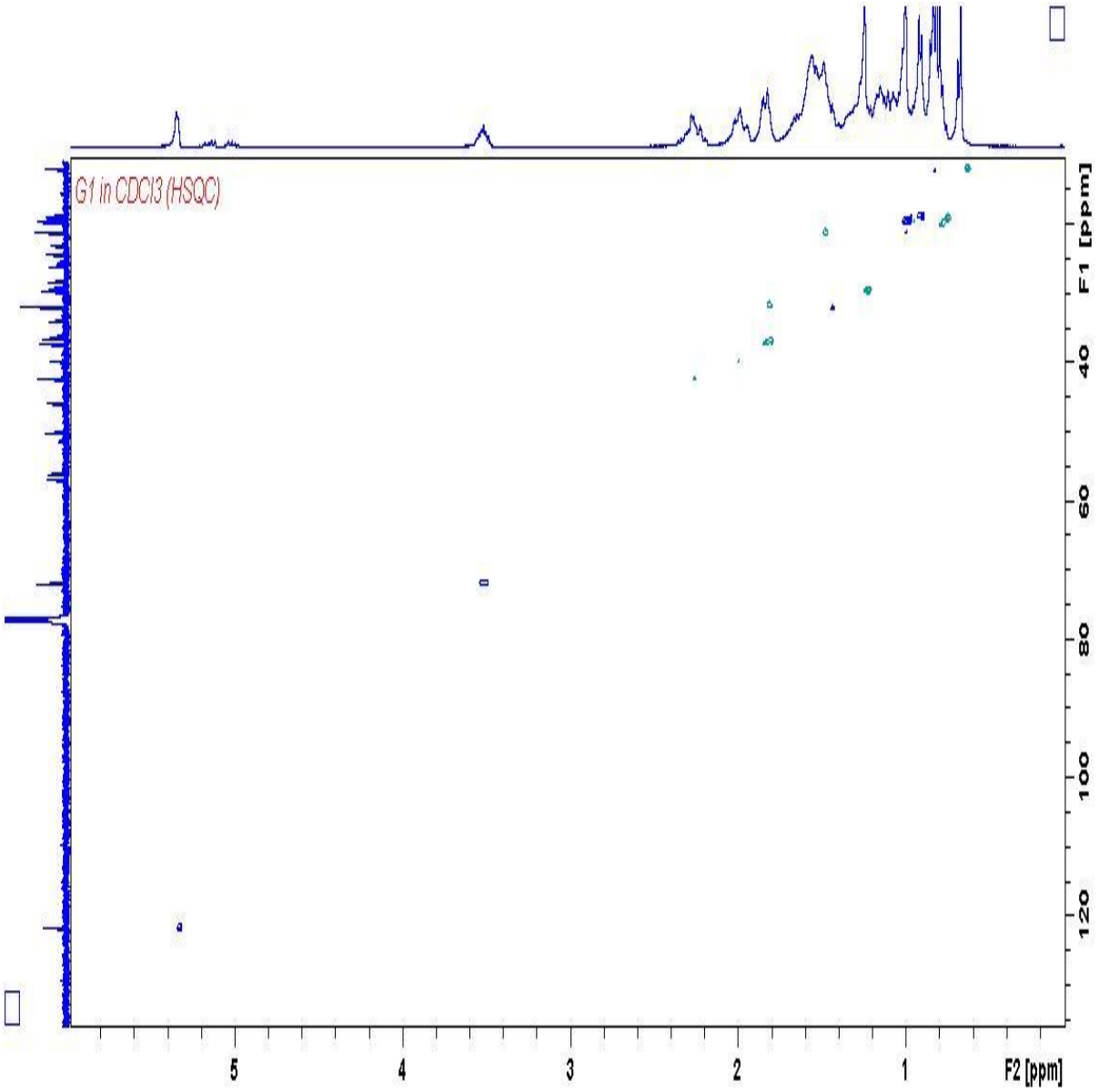


Figure 4.6: HSQC NMR Spectrum of G1

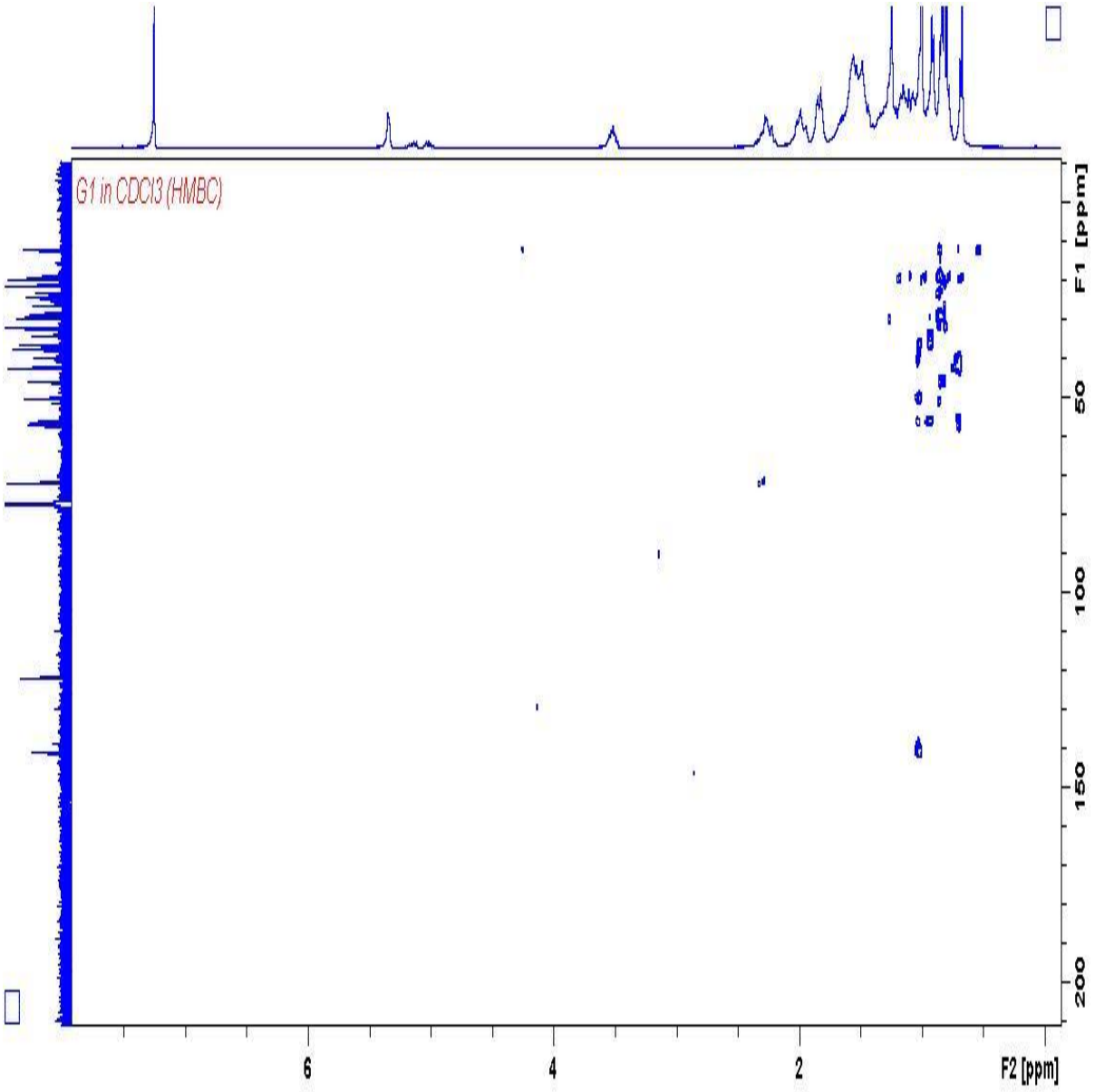


Figure 4.7: HMBC NMR Spectrum of G1

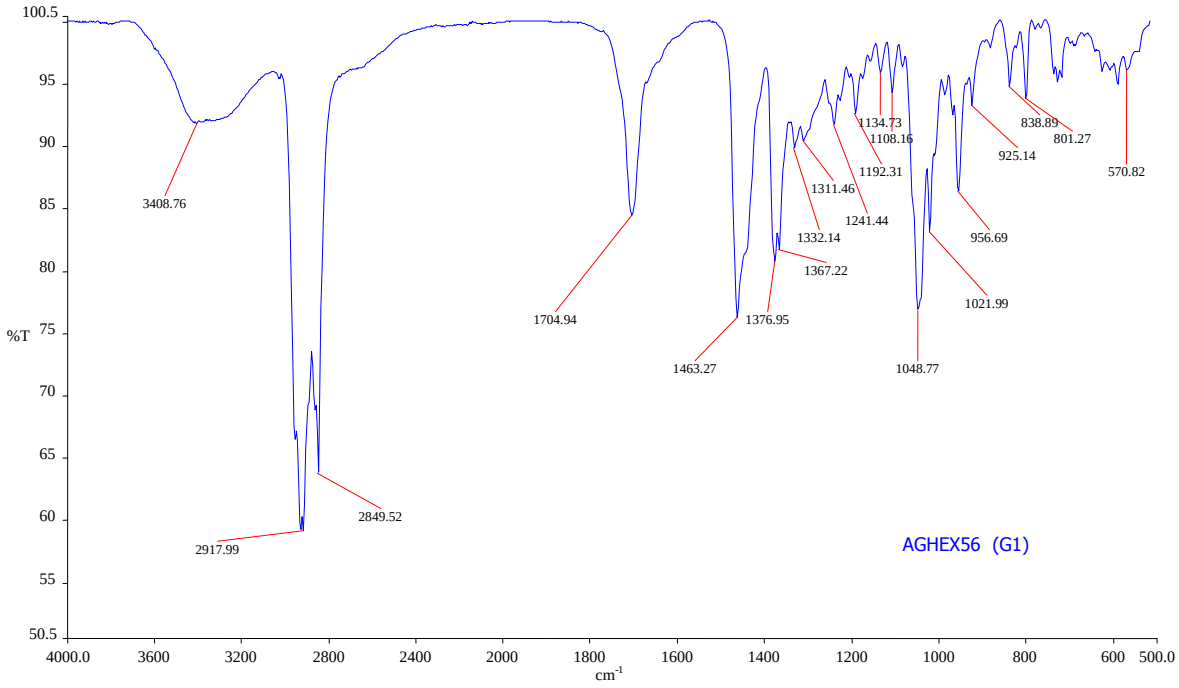


Figure 4.8: IR Spectrum of G1

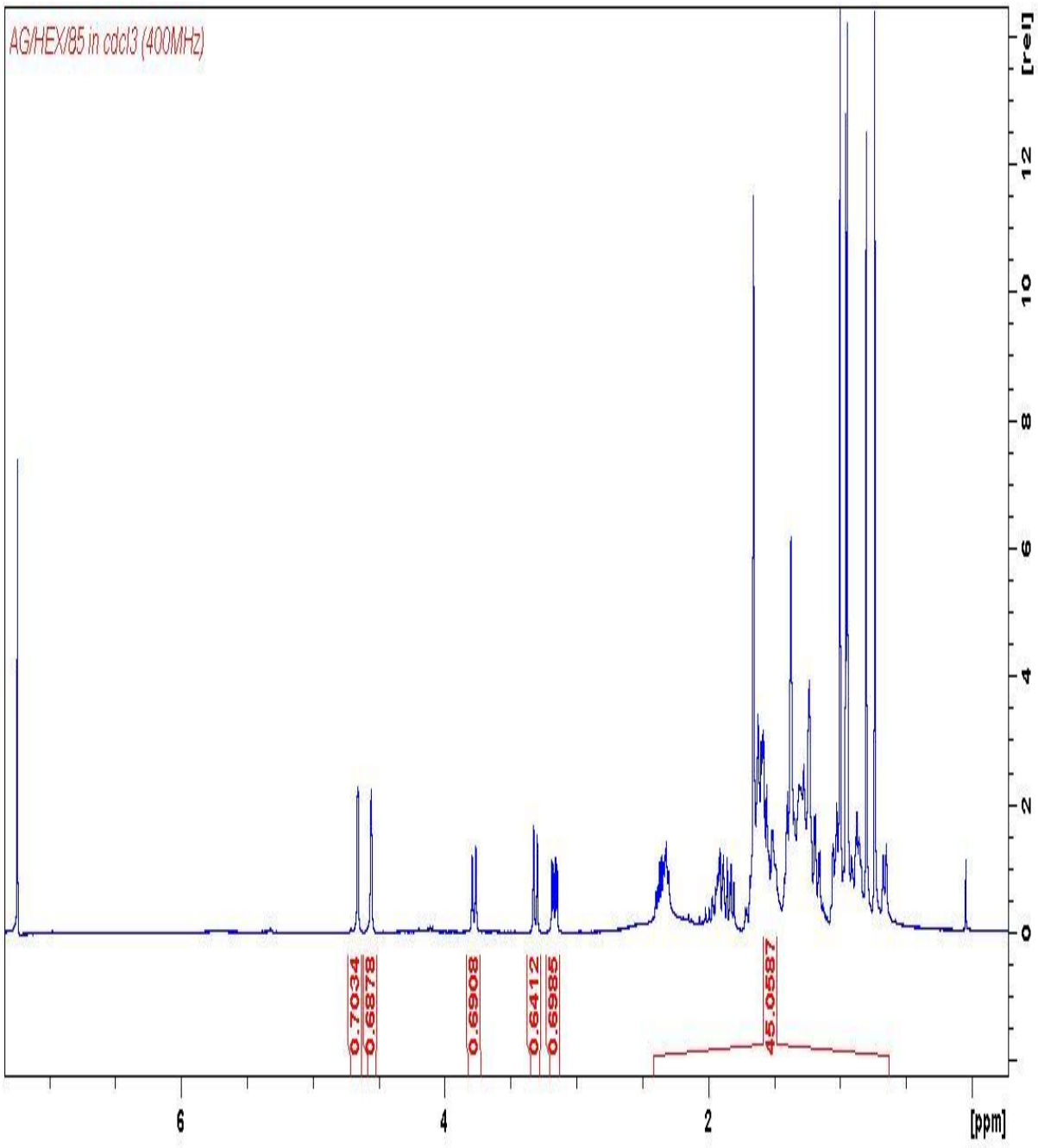


Figure 4.9: ^{1}H NMR Spectrum of G2

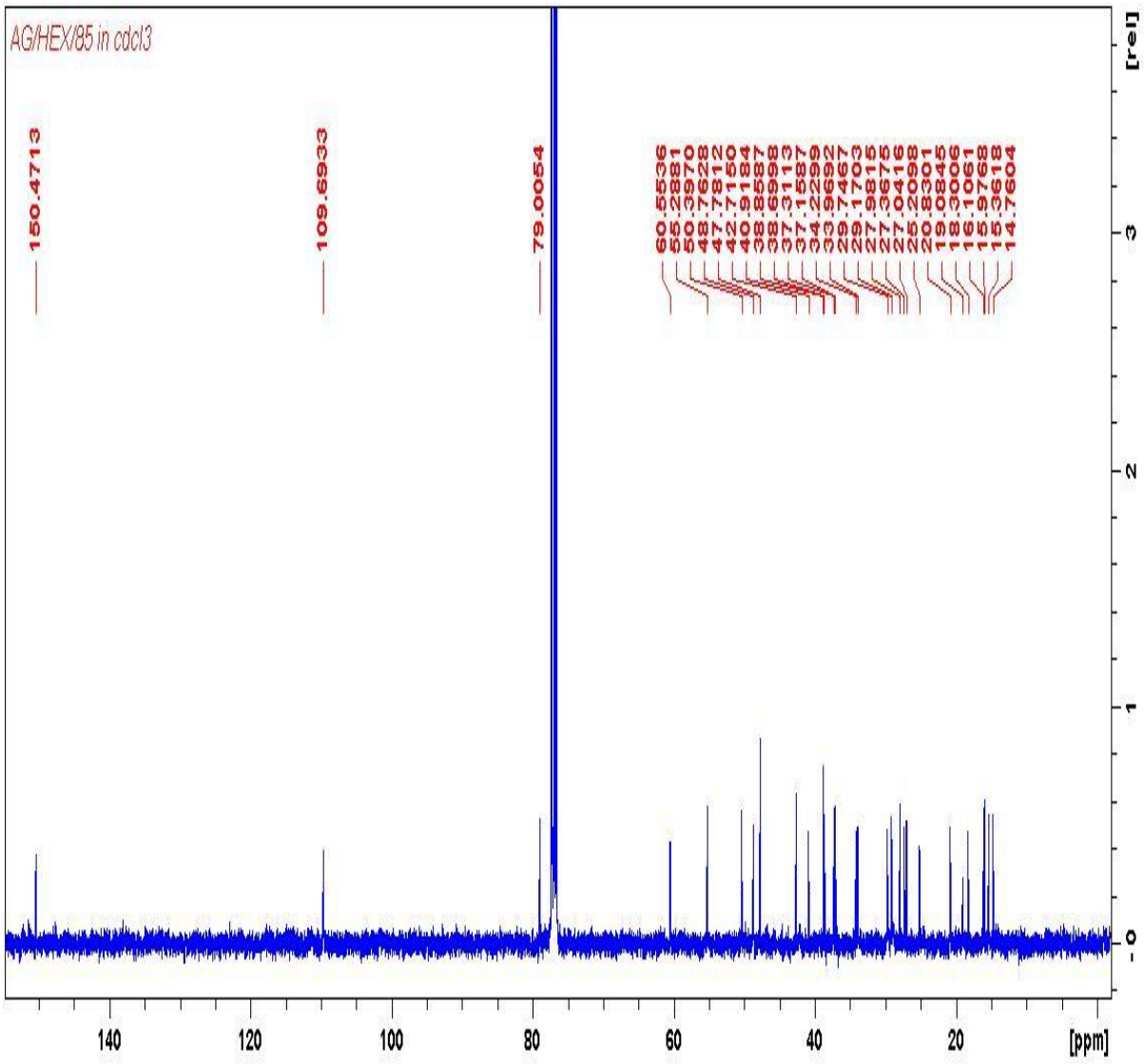


Figure 4.10: ^{13}C NMR Spectrum of G2

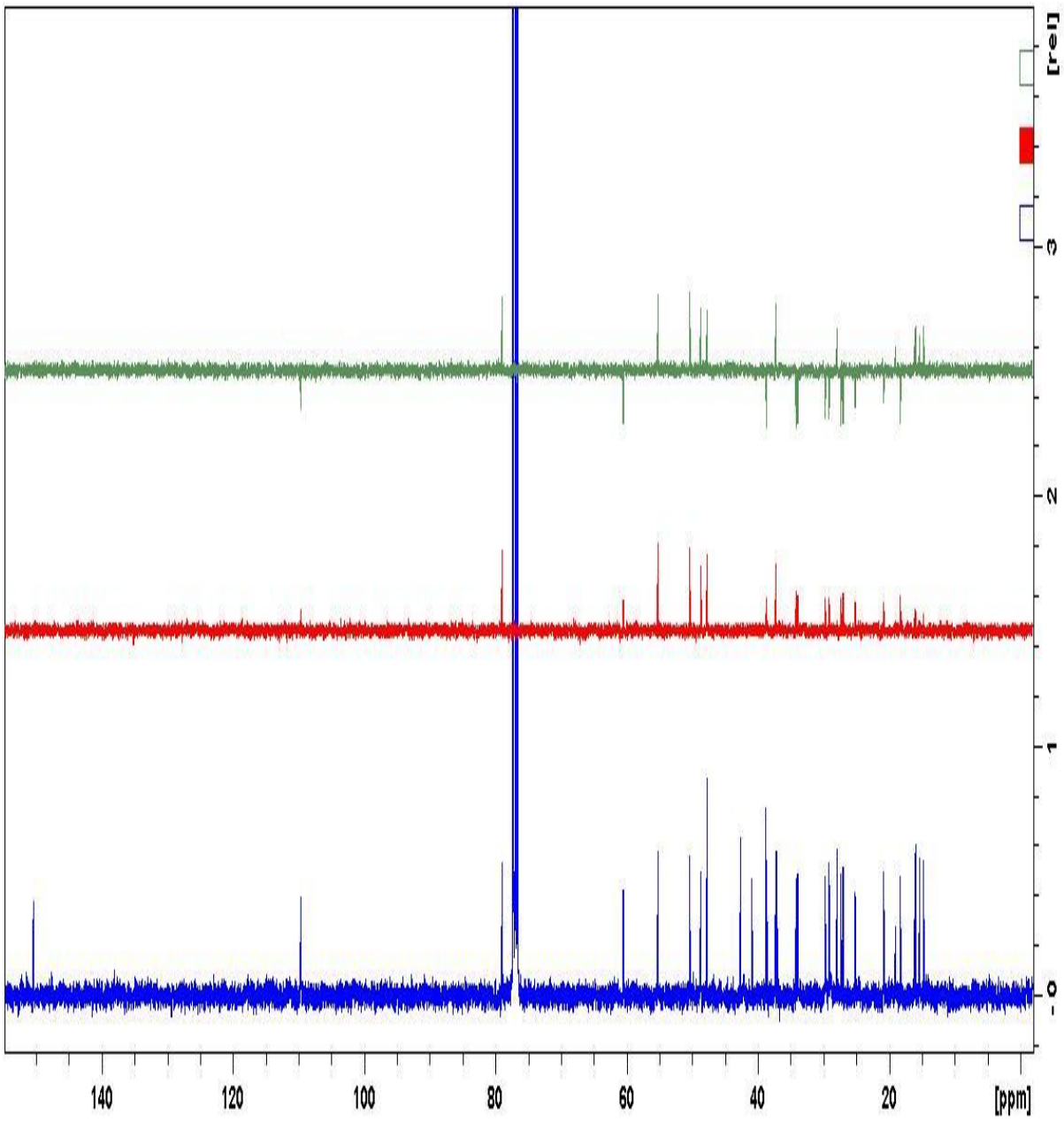


Figure 4.11: ^{13}C and DEPTS (90 and 135) NMR Spectra of G2

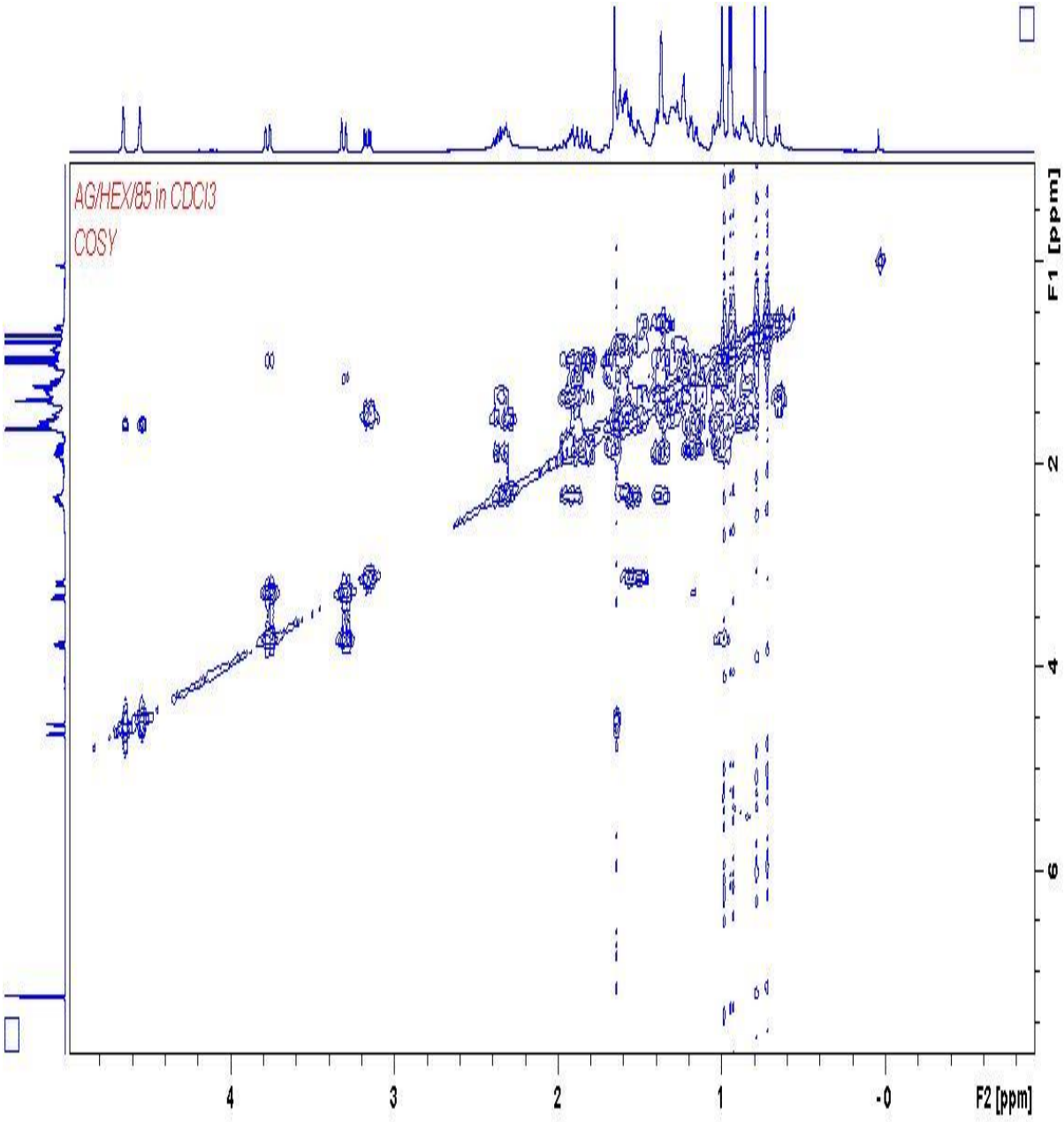


Figure 4.12: COSY NMR Spectrum of G2

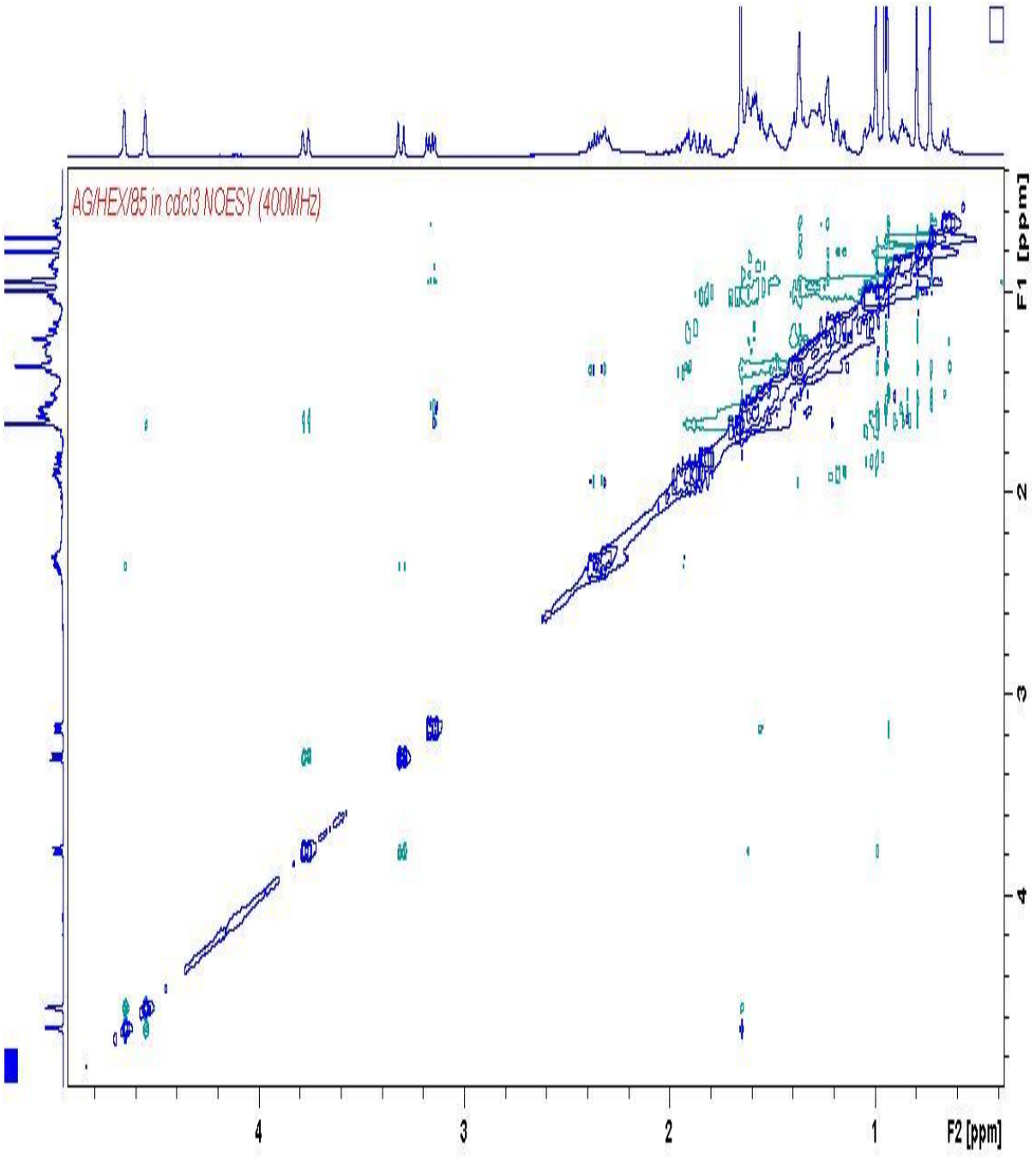


Figure 4.13: NOESY NMR Spectrum of G2

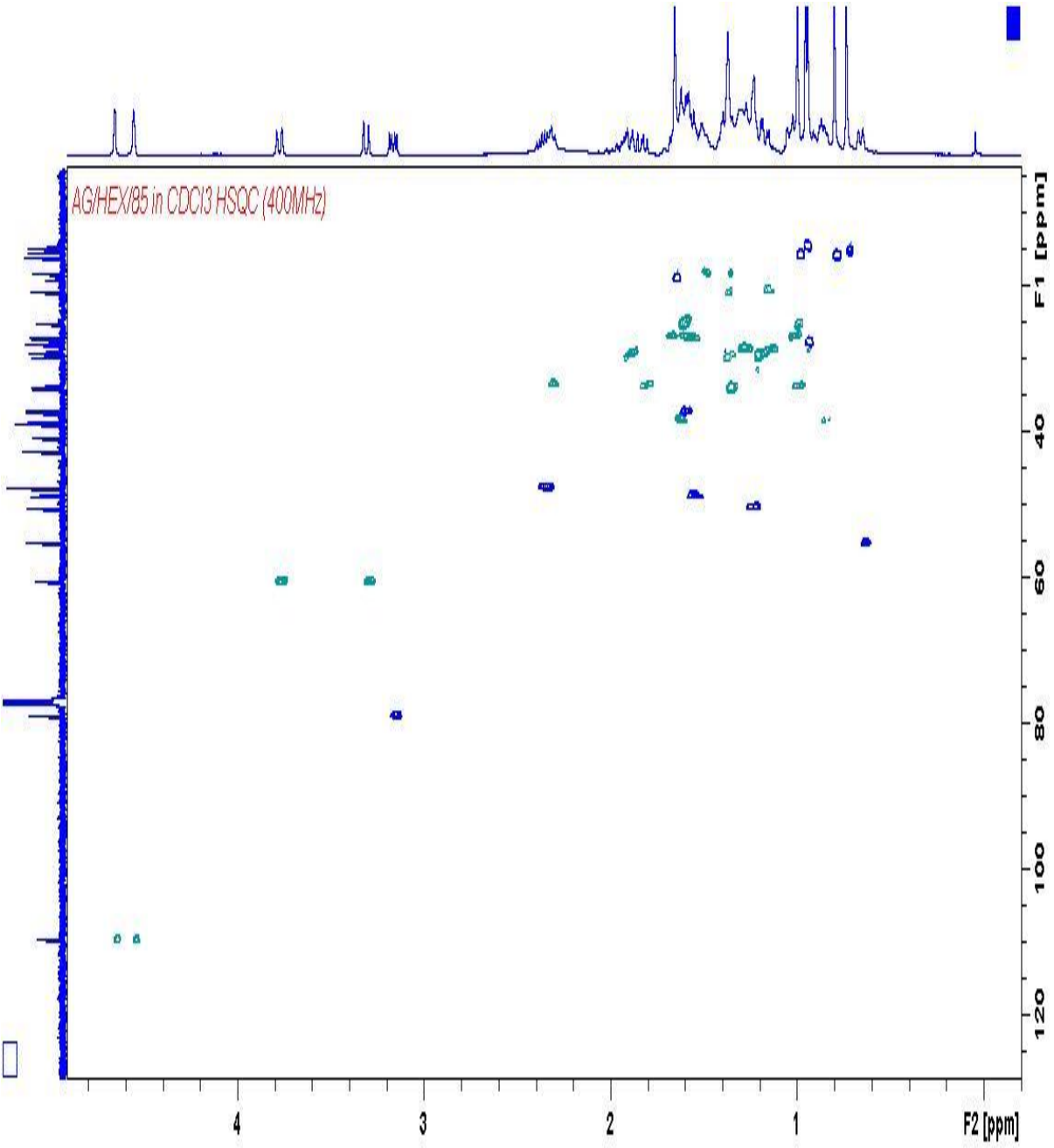


Figure 4.14: HSQC NMR Spectrum of G2

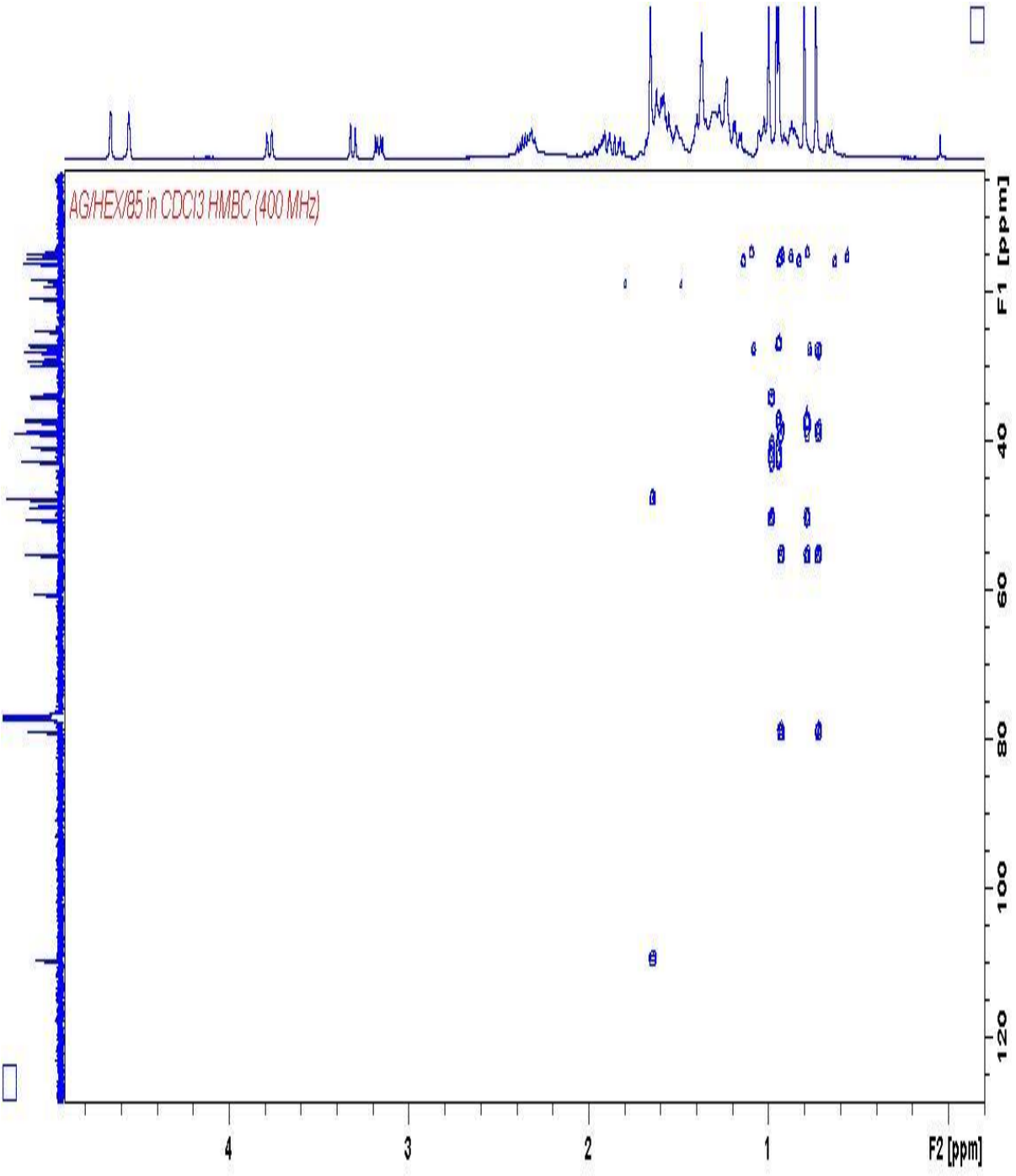


Figure 4.15: HMBC NMR Spectrum of G2

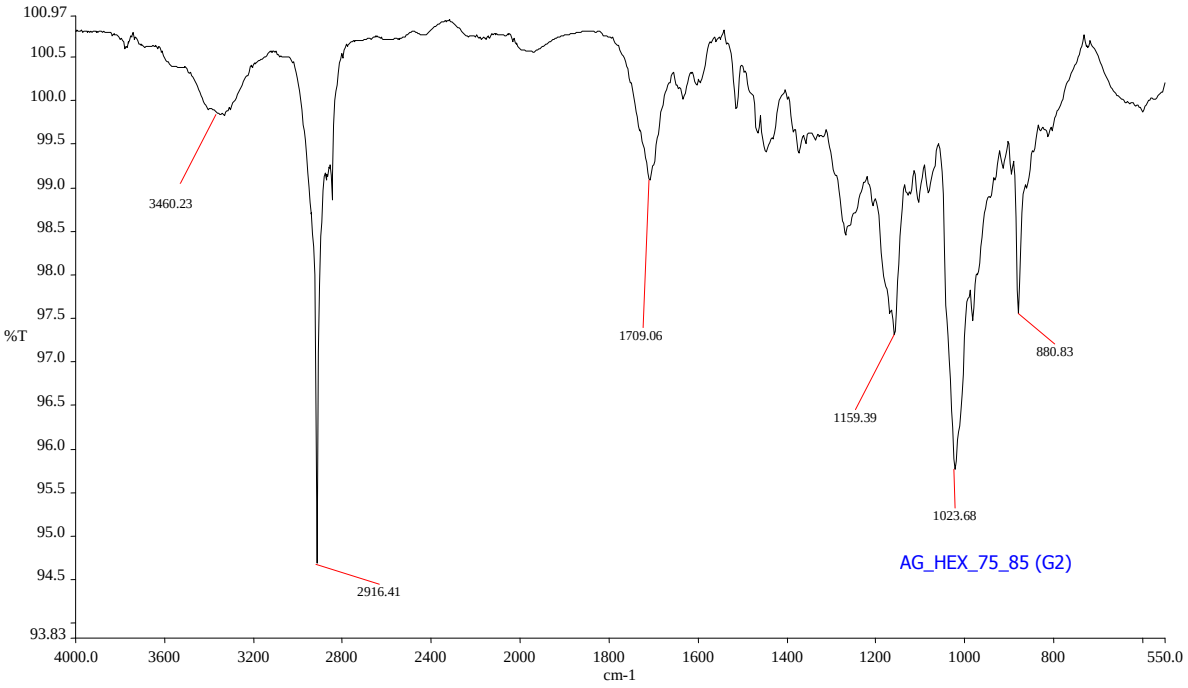


Figure 4.16: IR Spectrum of G2

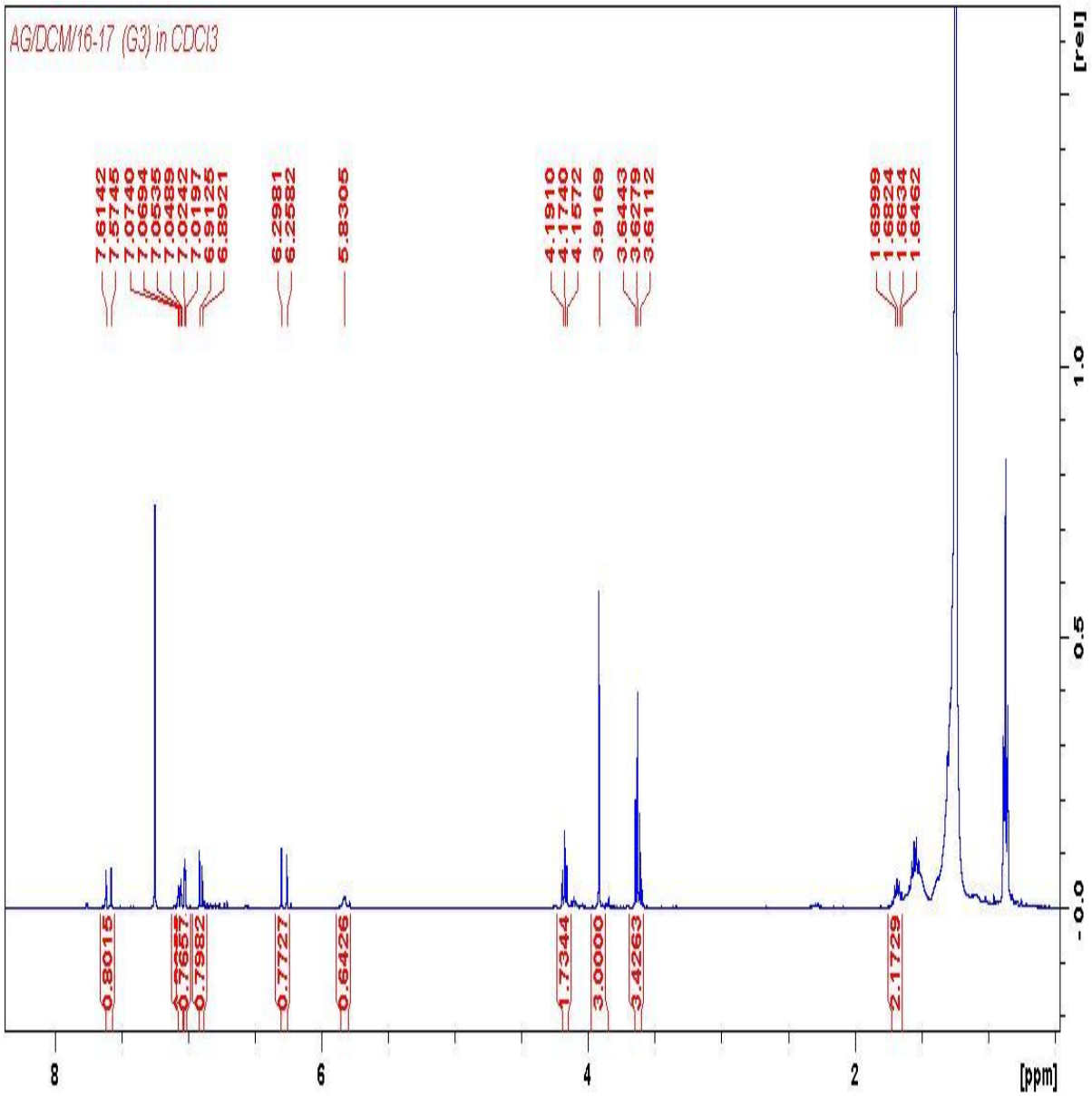


Figure 4.17: ^{1}H NMR Spectrum of G3

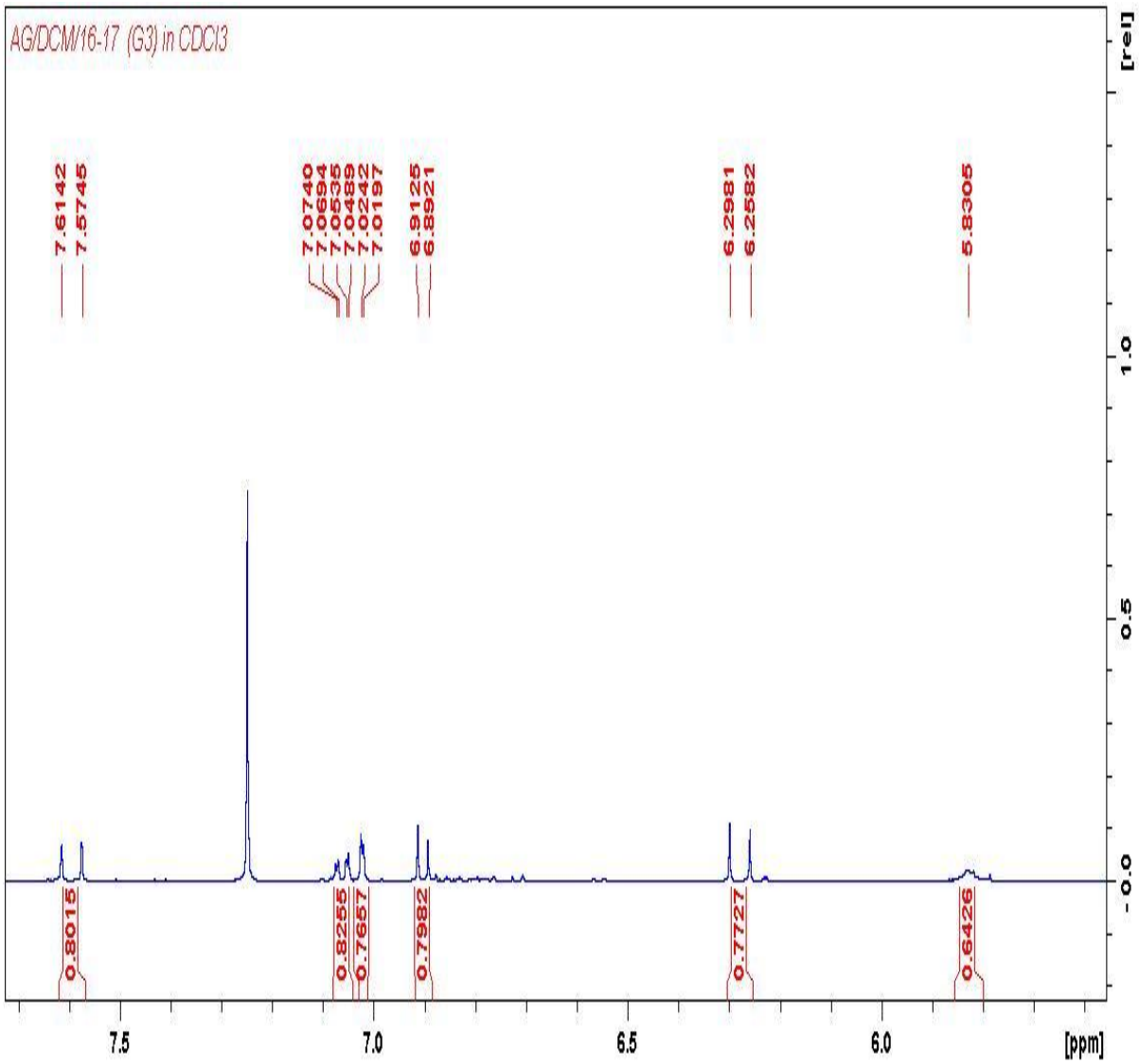


Figure 4.18: Expanded Aromatic Region ^{1}H NMR Spectrum of G3

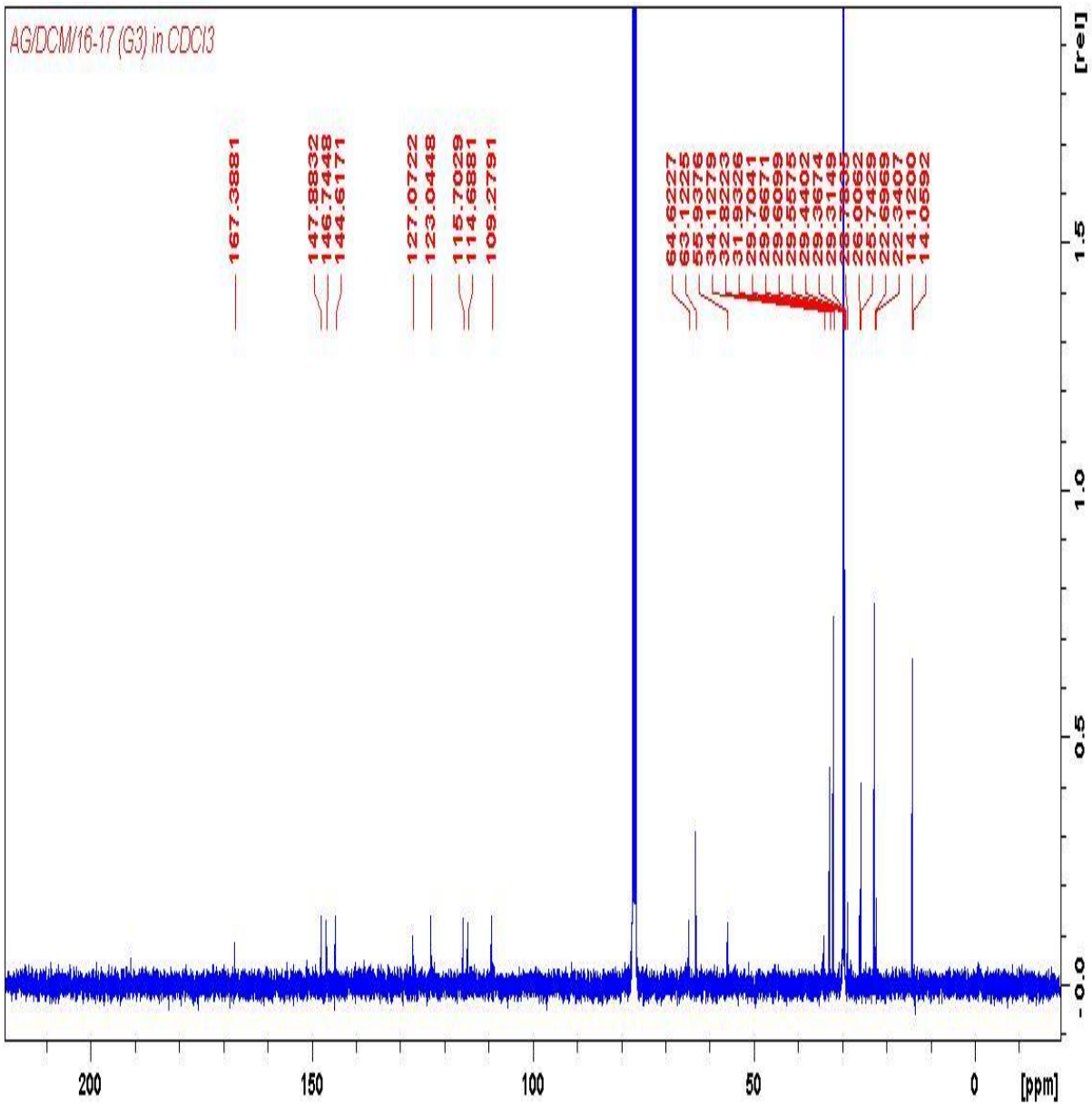


Figure 4.19: ^{13}C NMR Spectrum of G3

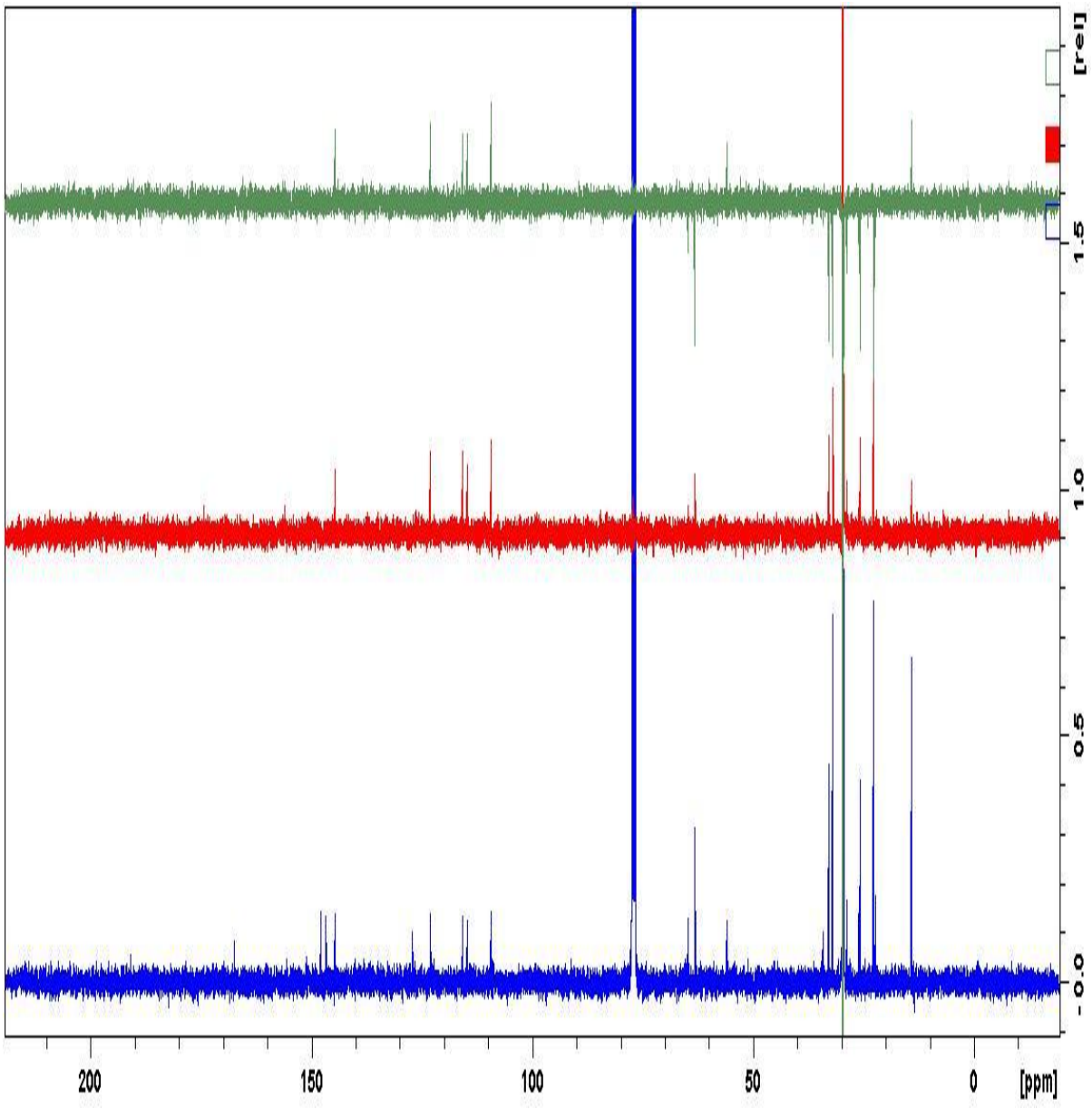


Figure 4.20: ^{13}C and DEPTS (90 and 135) NMR Spectra of G3

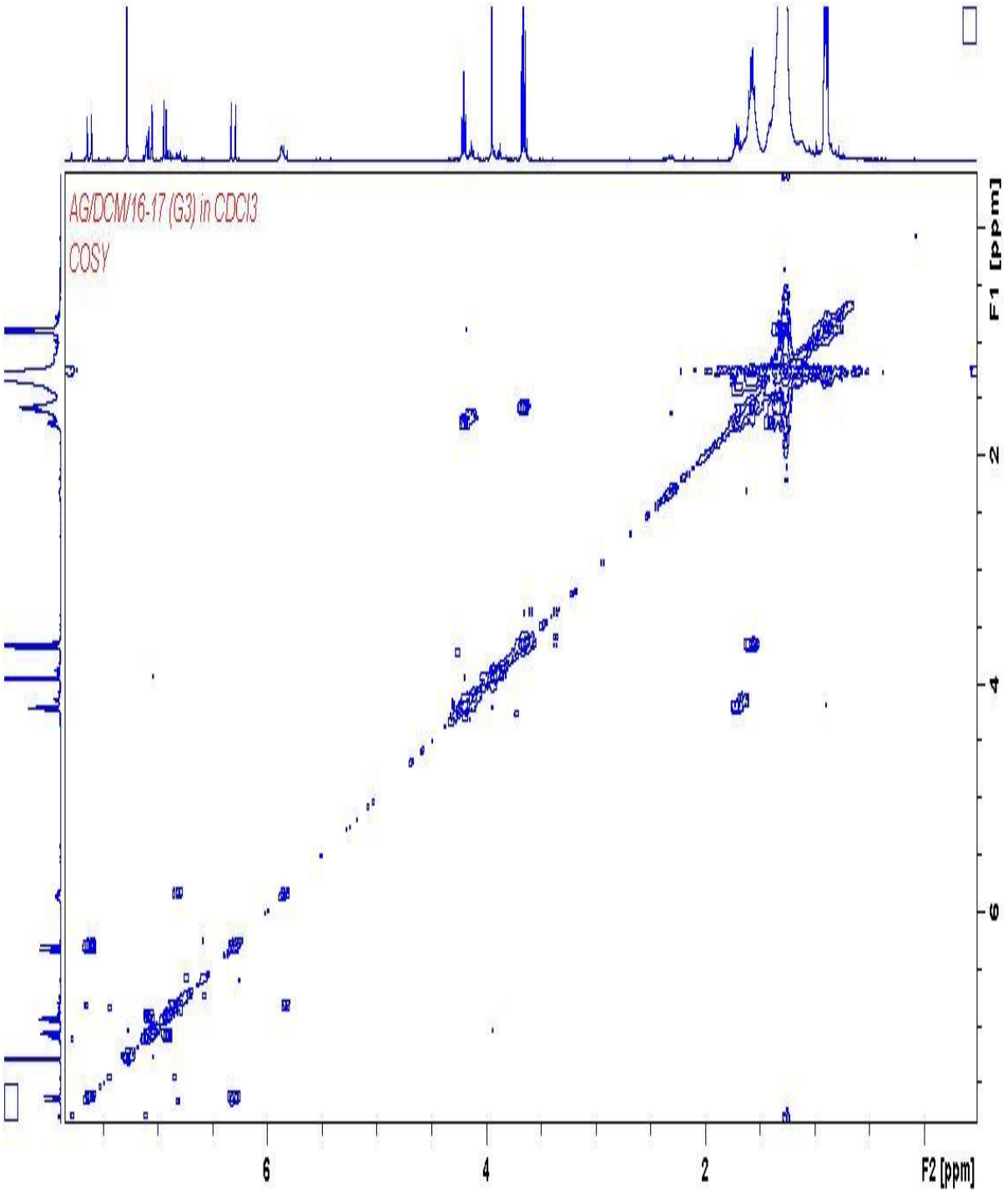


Figure 4.21: COSY NMR Spectrum of G3

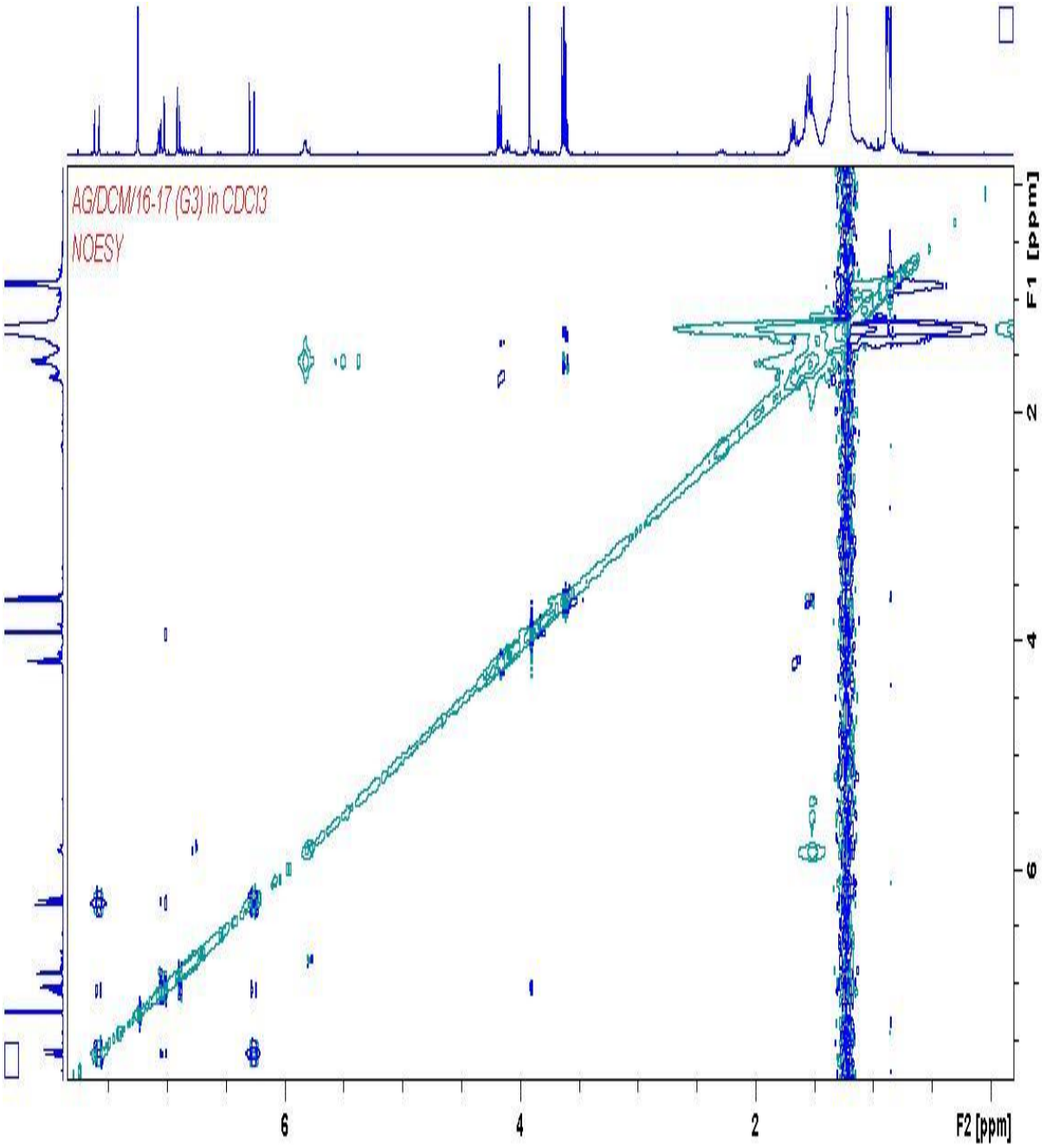


Figure 4.22: NOESY NMR Spectrum of G3

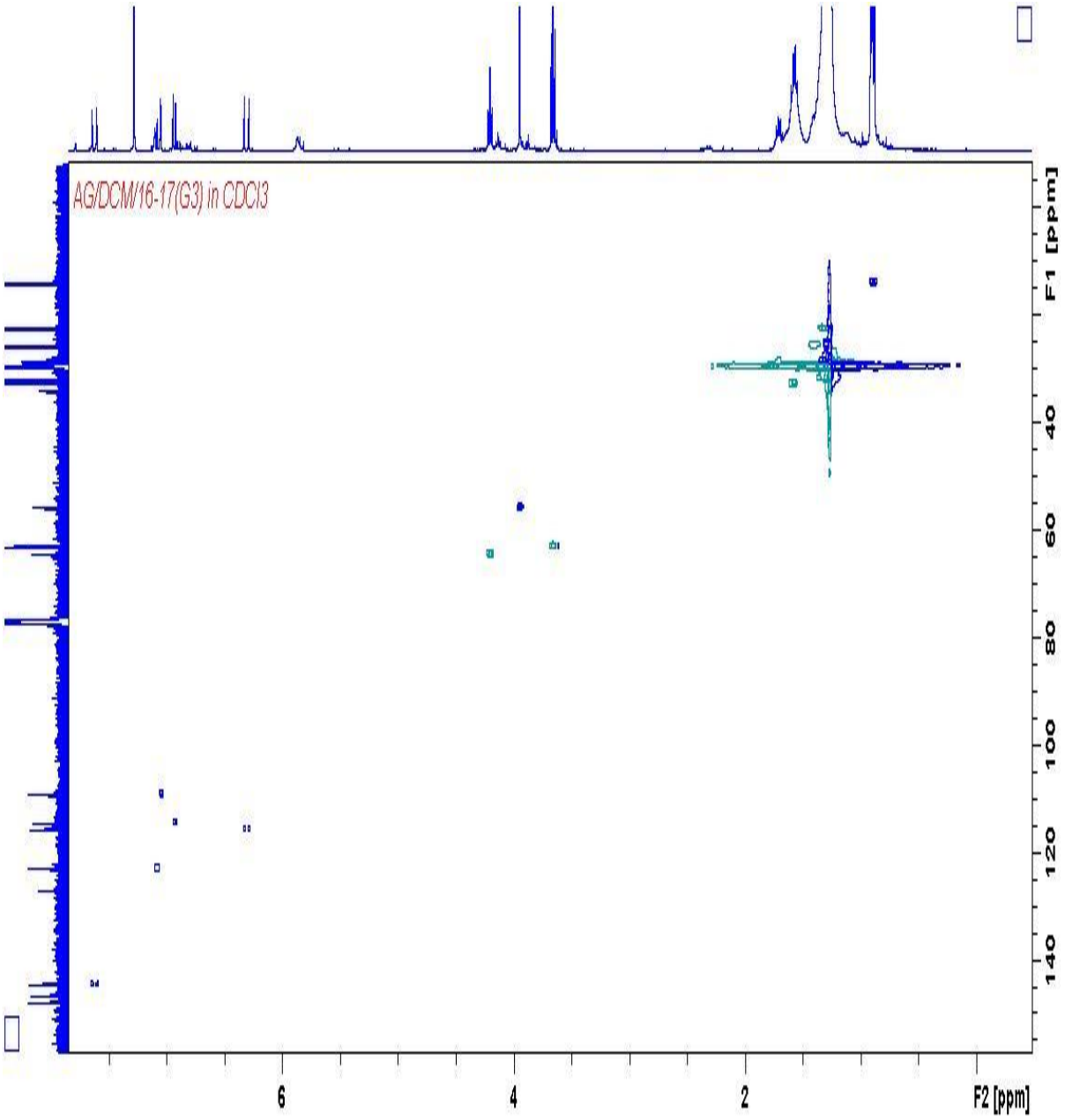


Figure 4.23: HSQC NMR Spectrum of G3

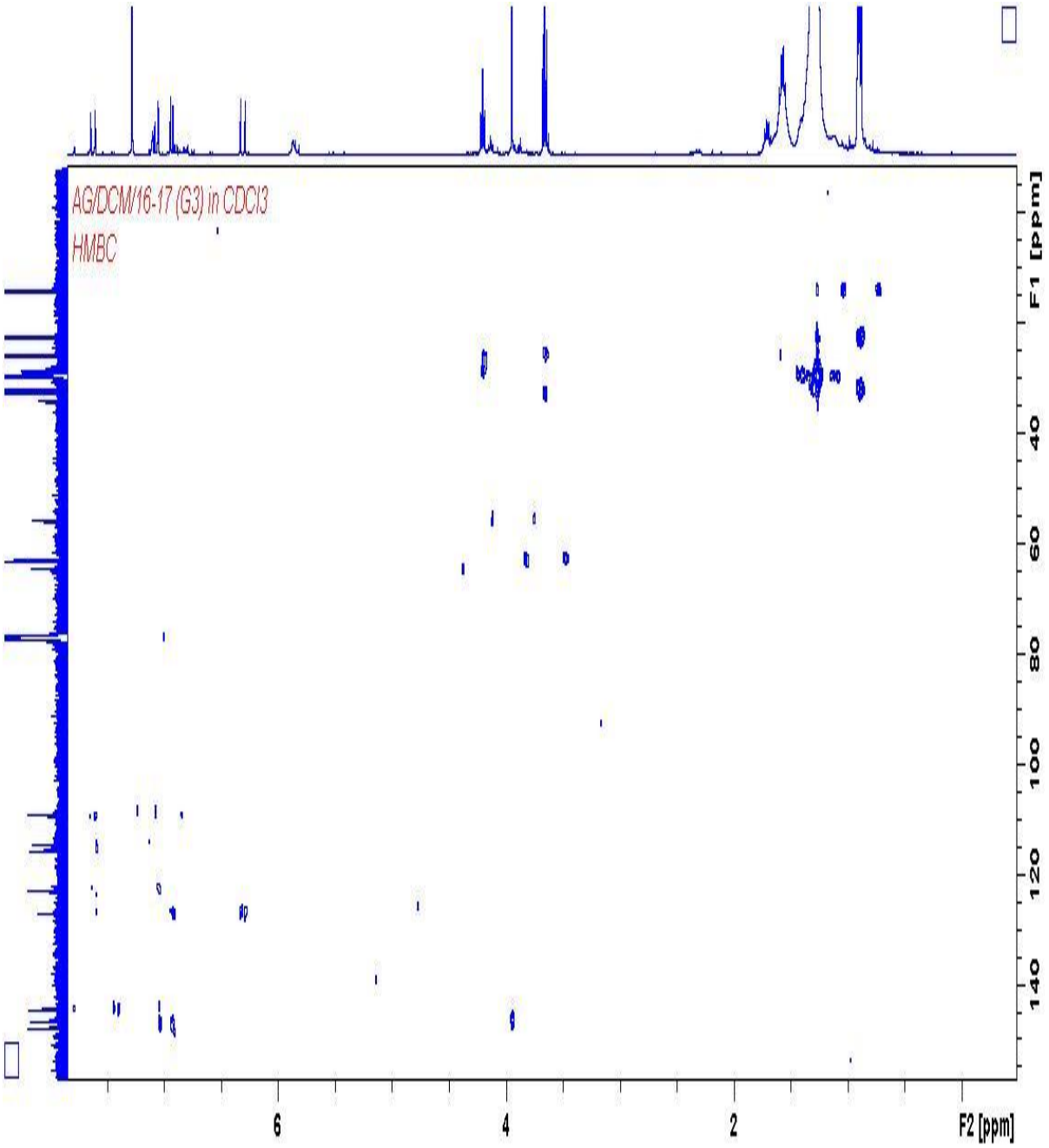


Figure 4.24: HMBC NMR Spectrum of G3

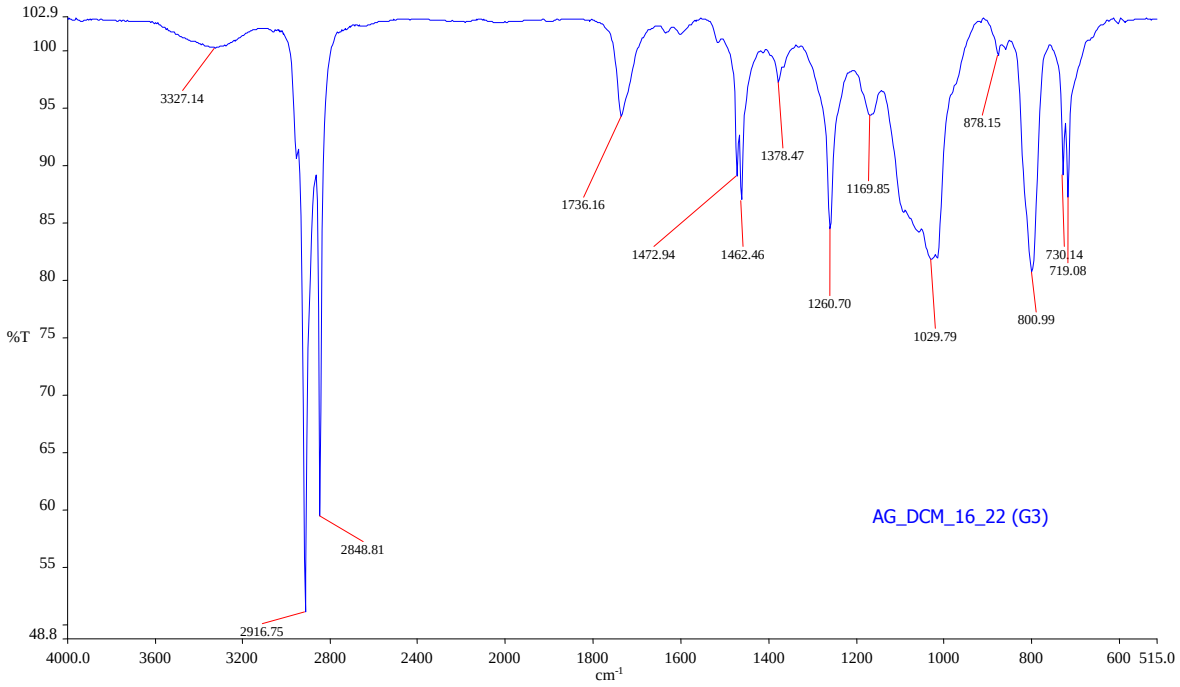


Figure 4.25: IR Spectrum of G3

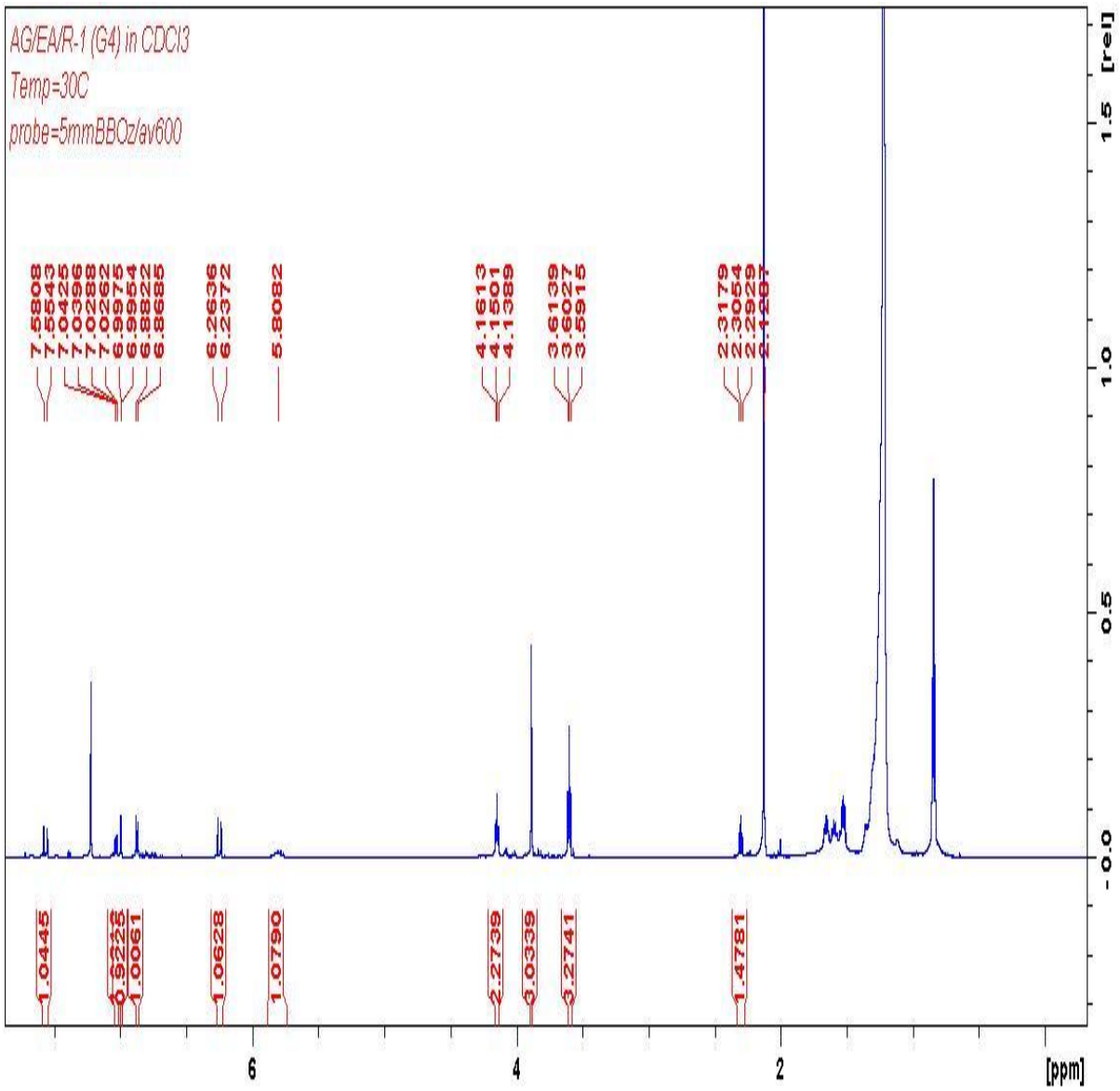


Figure 4.26: ^{1}H NMR Spectrum of G4

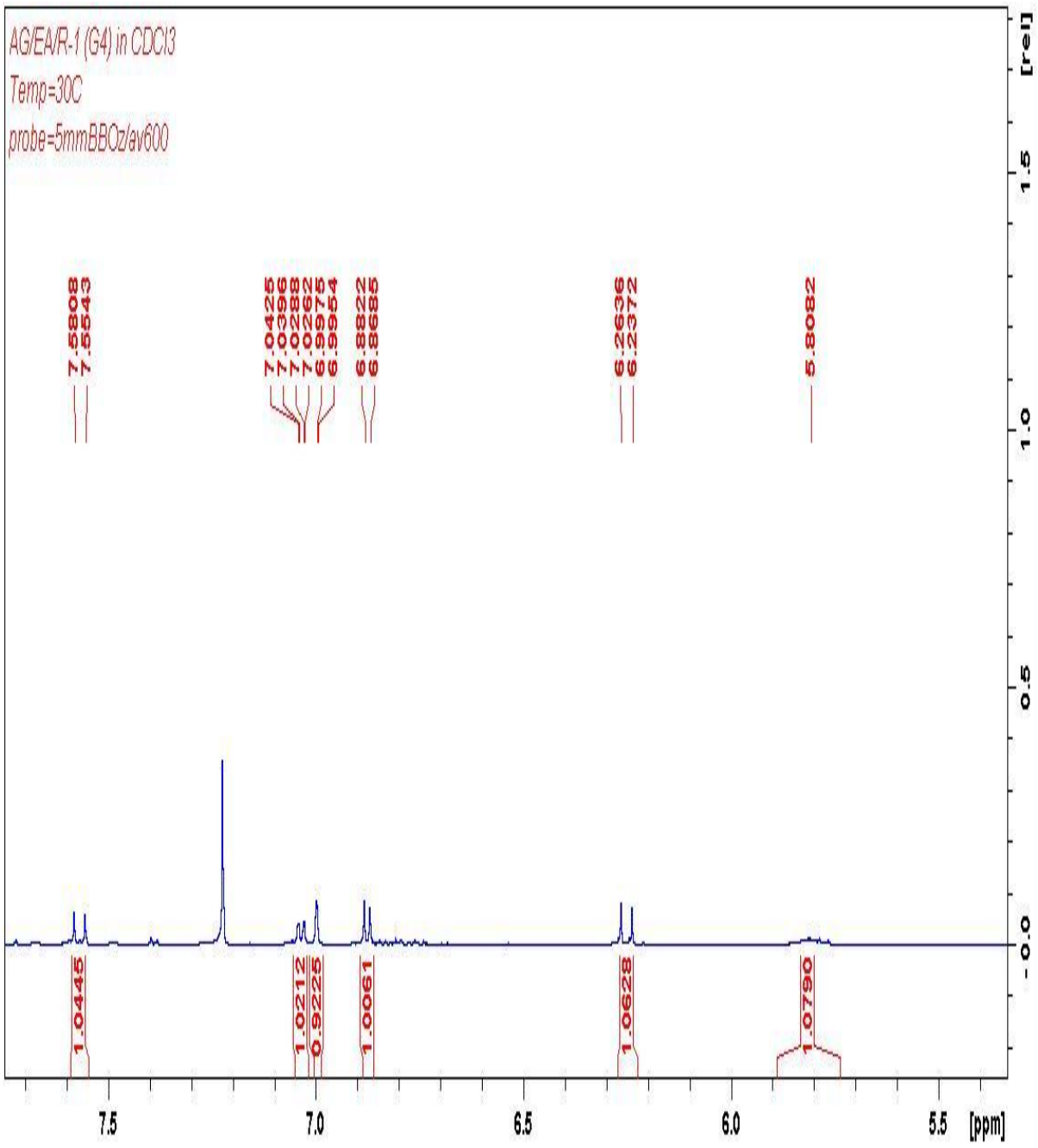


Figure 4.27: Expanded Aromatic Region ^{1}H NMR Spectrum of G4

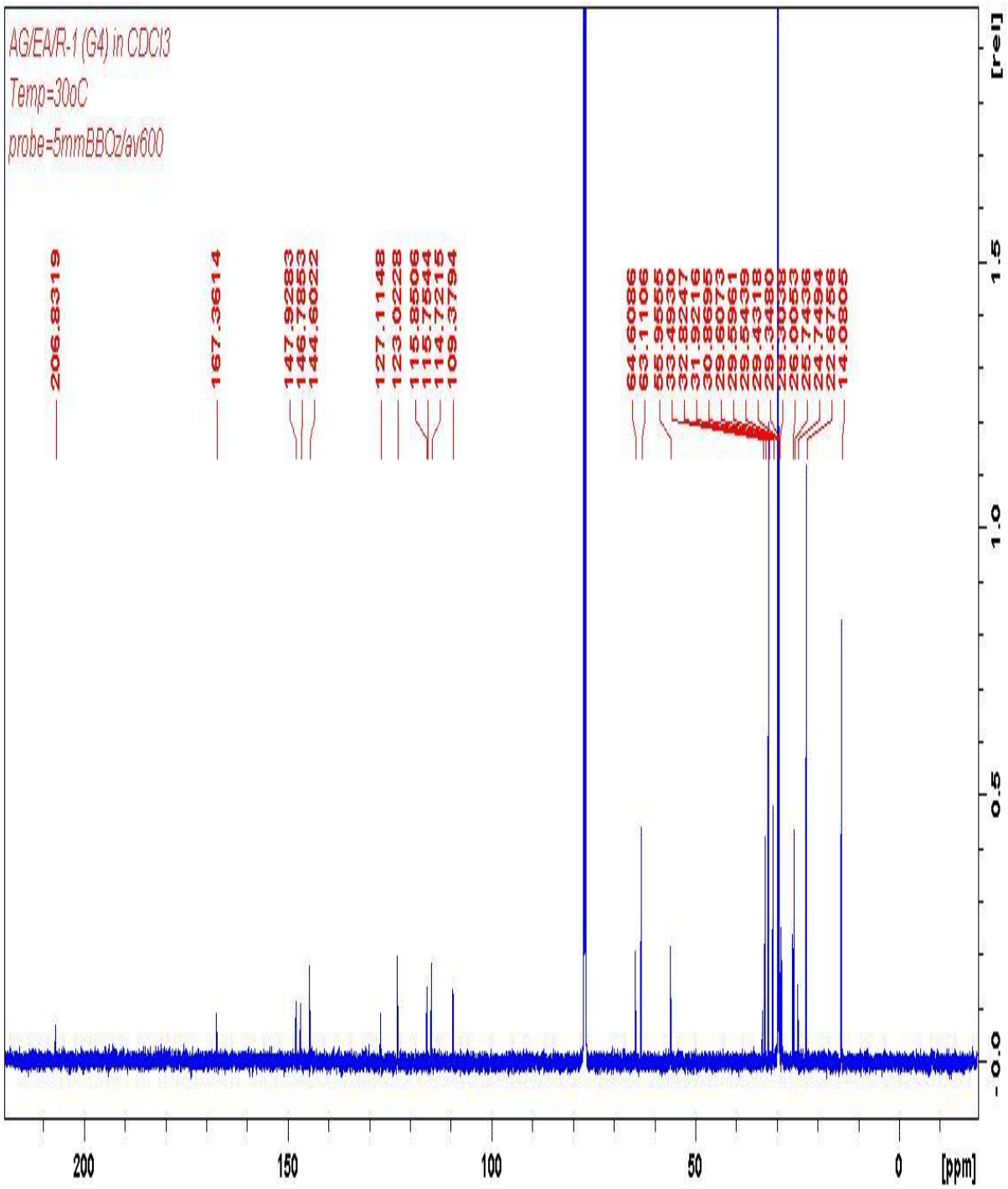


Figure 4.28: ^{13}C NMR Spectrum of G4

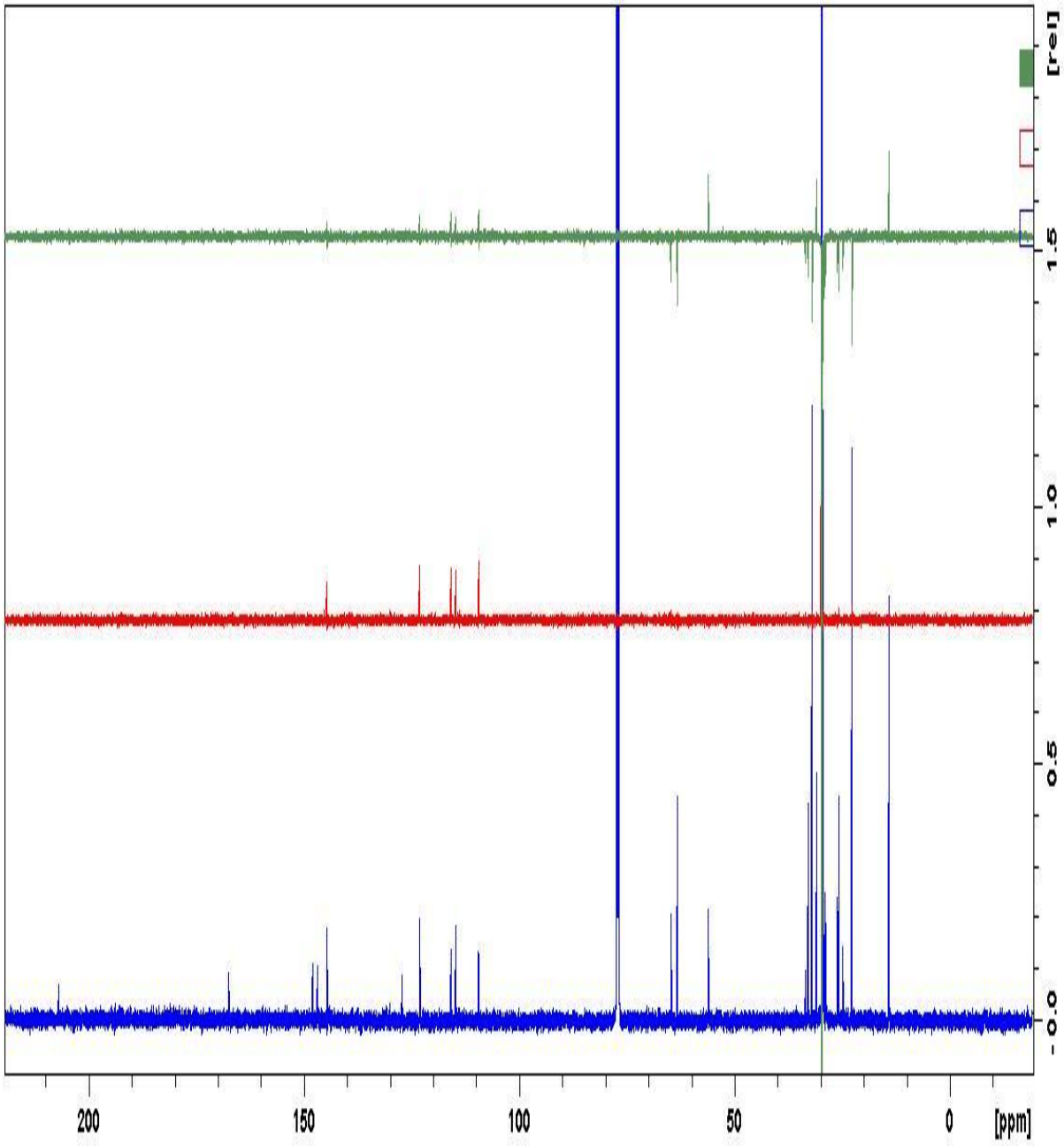


Figure 4.29: ^{13}C and DEPTS (90 and 135) NMR Spectra of G4

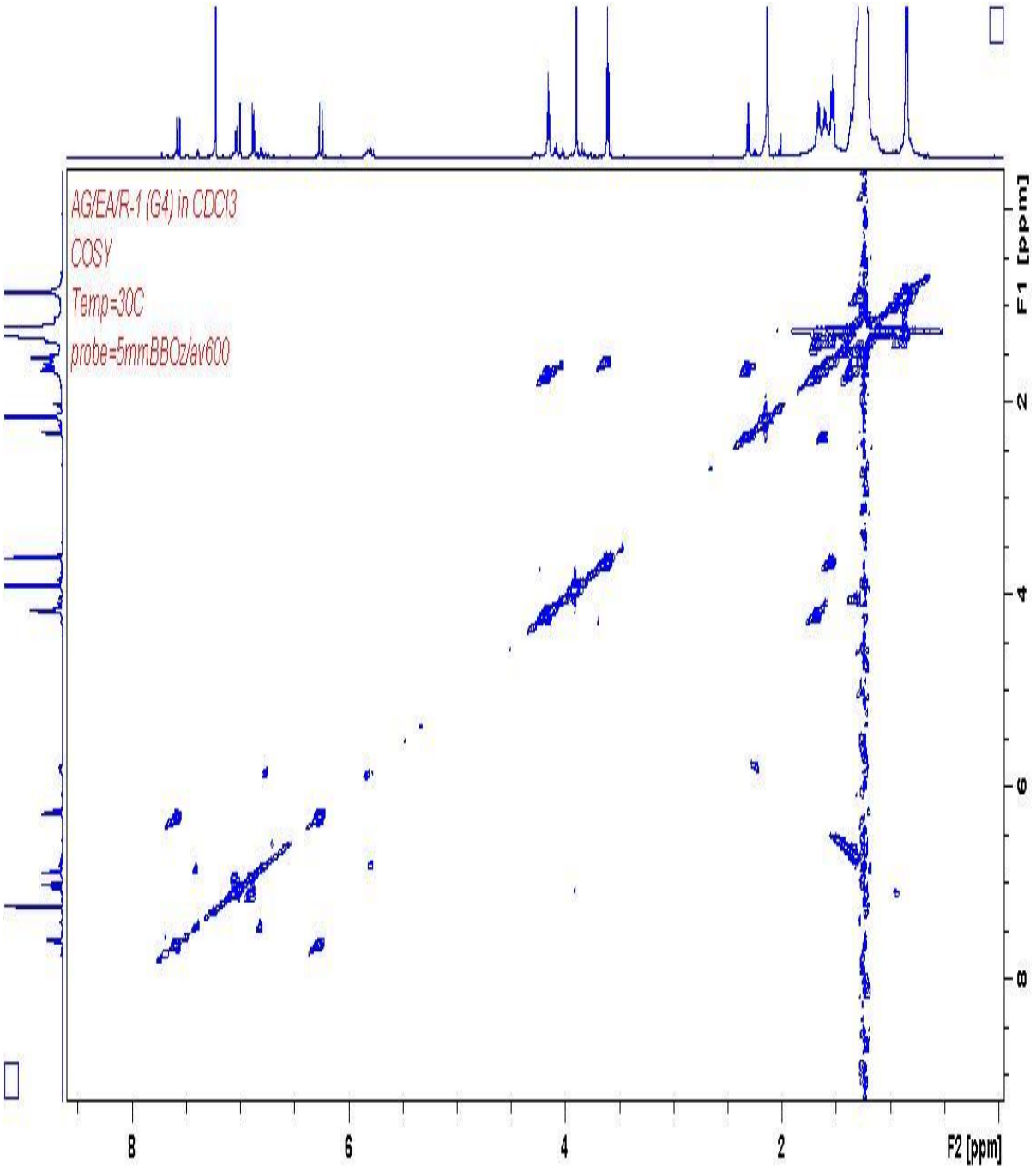


Figure 4.30: COSY NMR Spectrum of G4

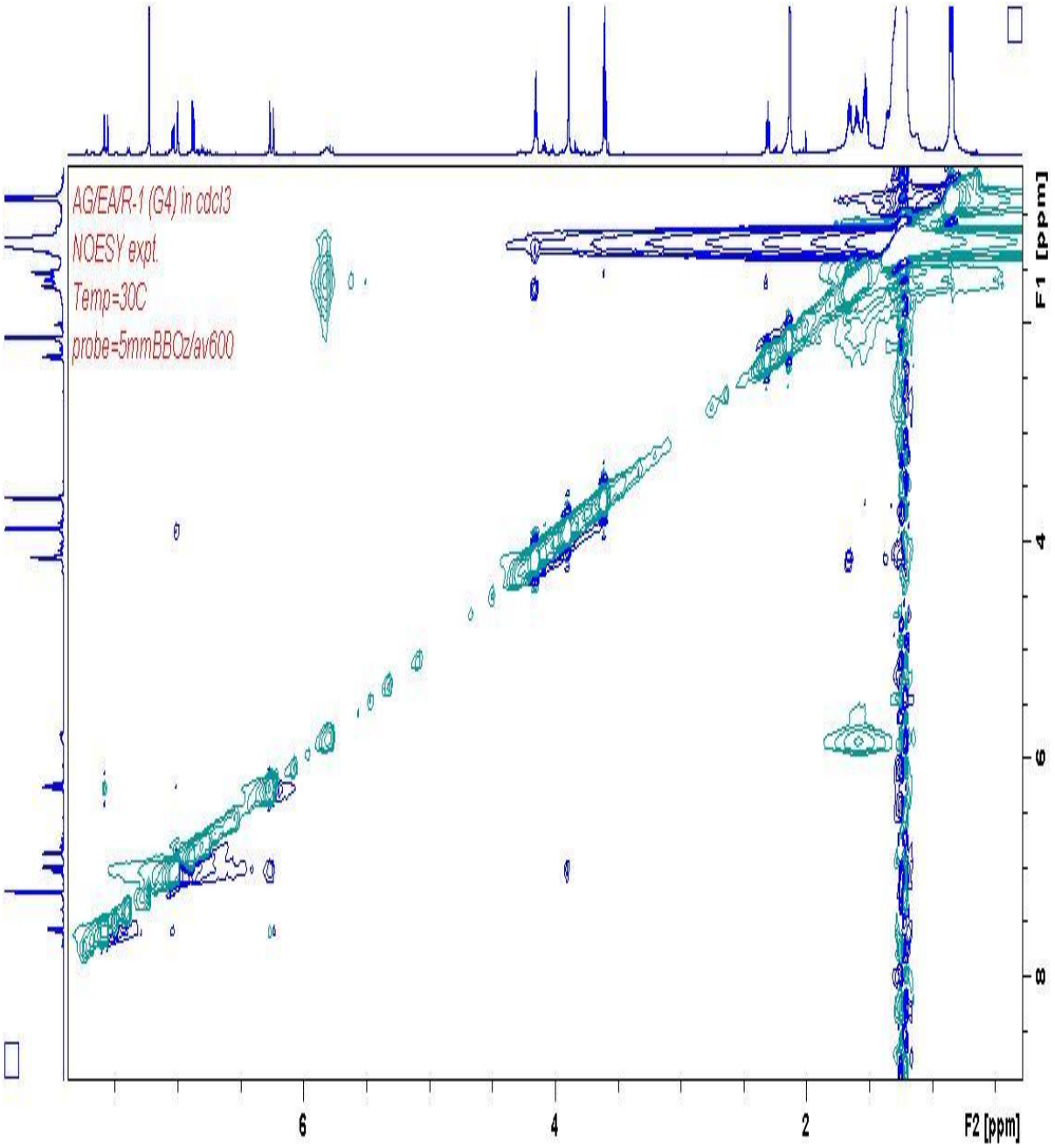


Figure 4.31: NOESY NMR Spectrum of G4

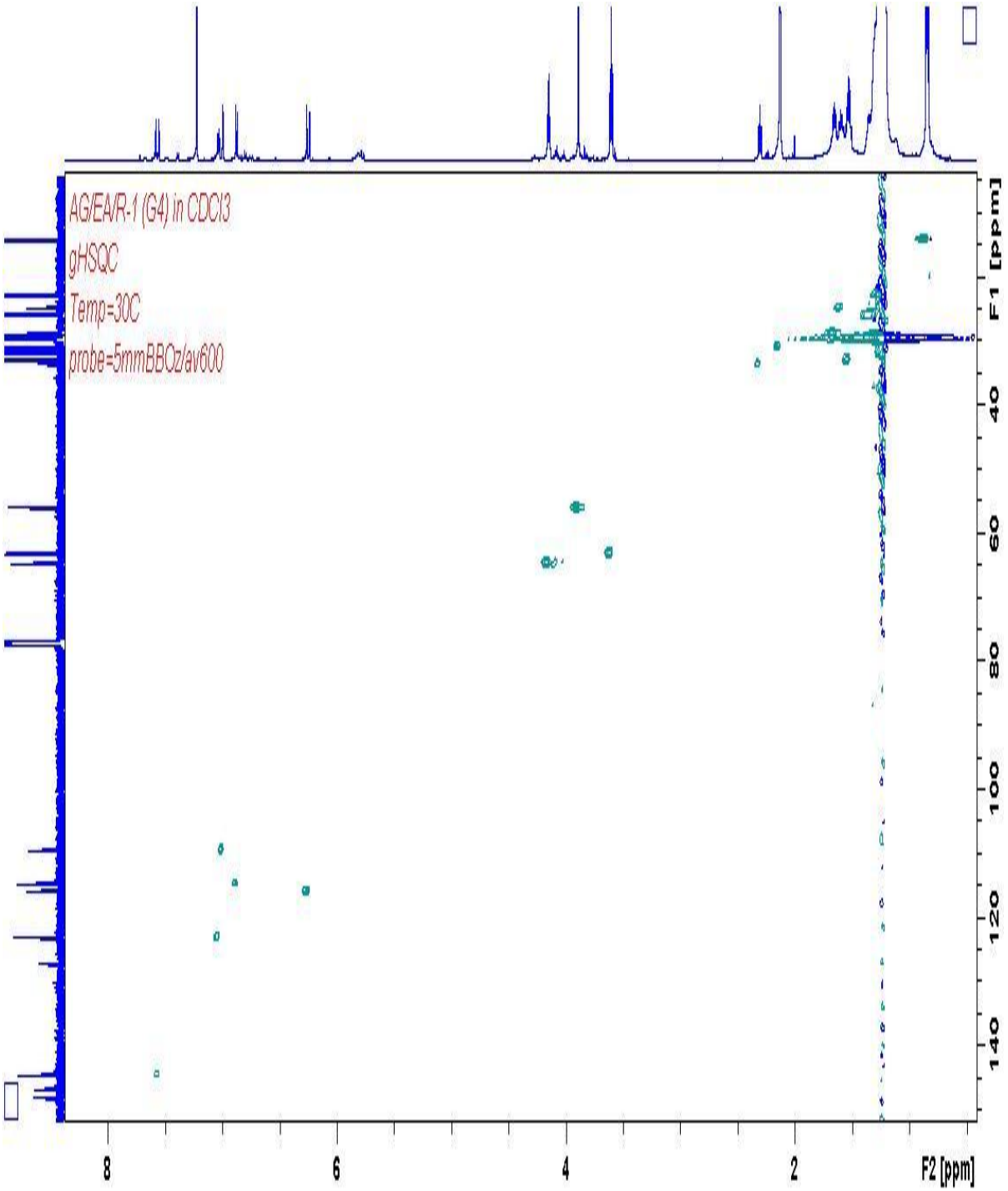


Figure 4.32: HSQC NMR Spectrum of G4

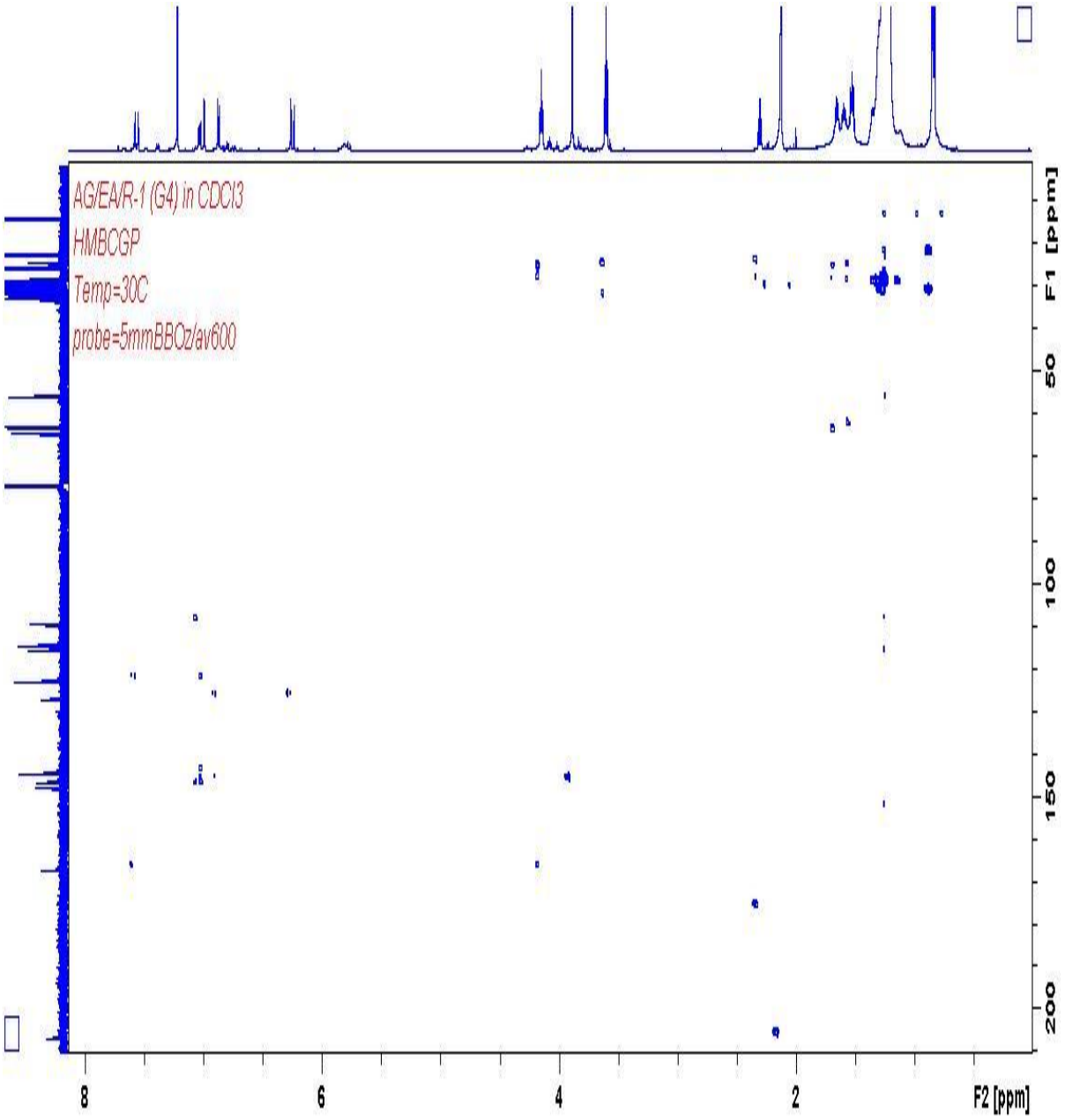


Figure 4.33: HMBC NMR Spectrum of G4

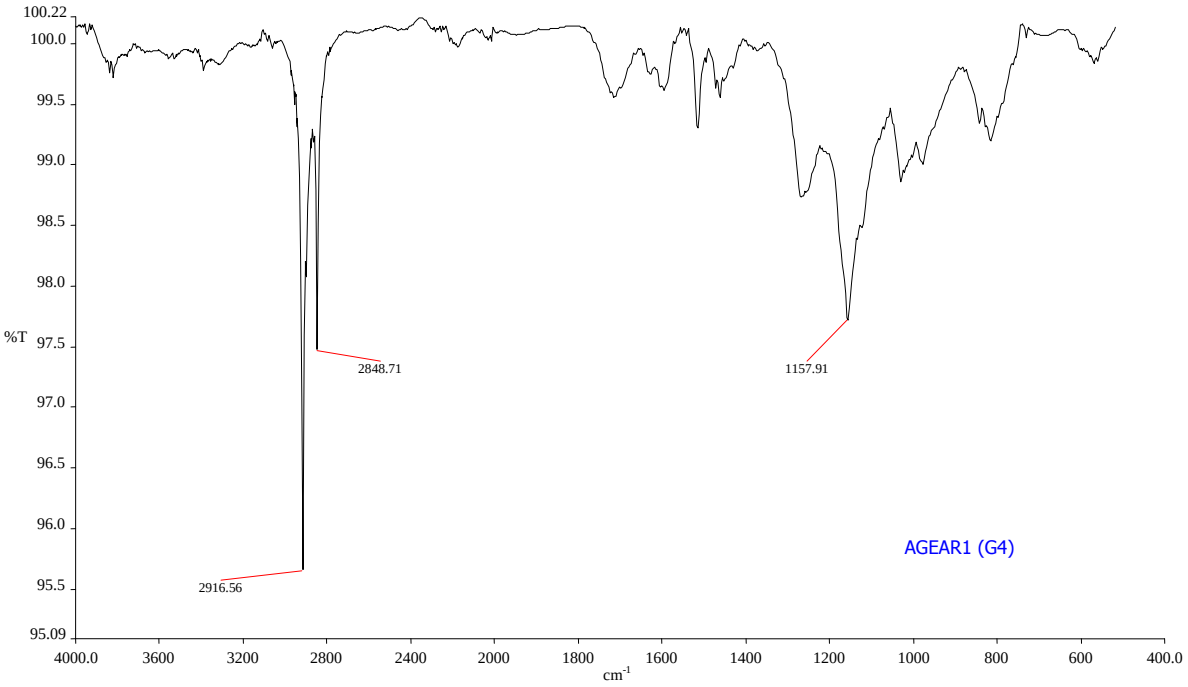


Figure 4.34: IR Spectrum of G4

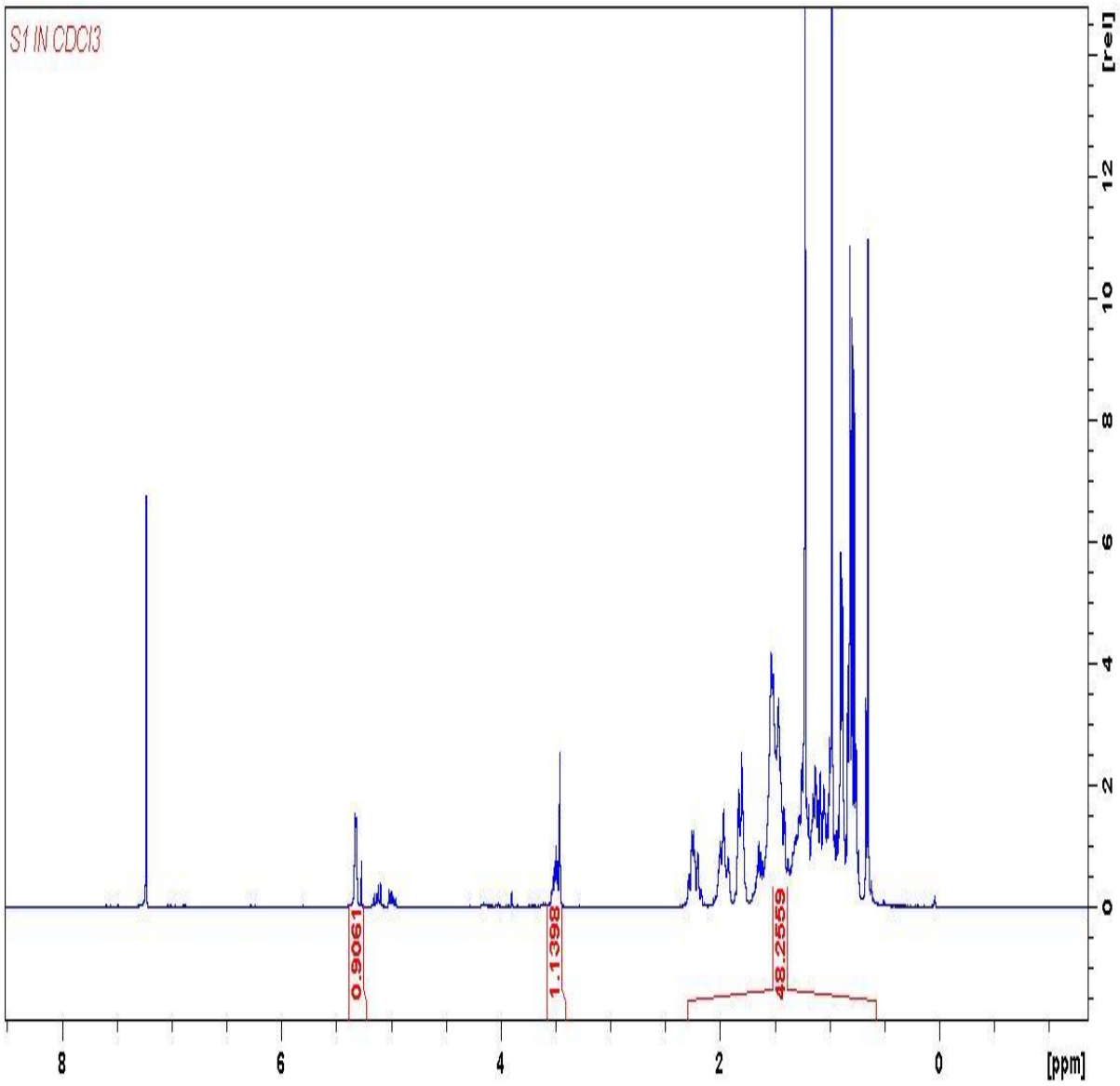


Figure 4.35: ^{1}H NMR Spectrum of S1

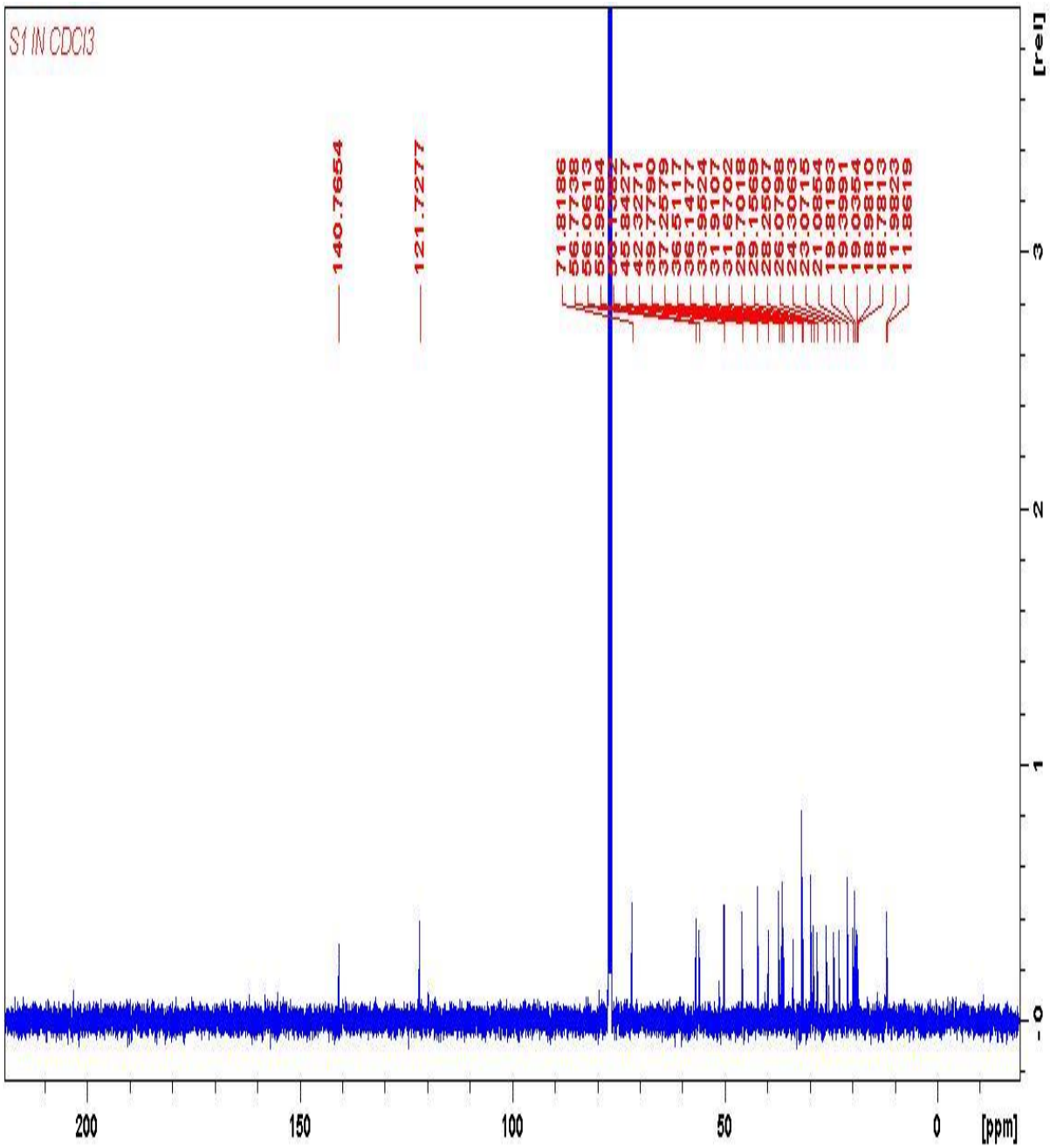


Figure 4.36: ^{13}C NMR Spectrum of S1

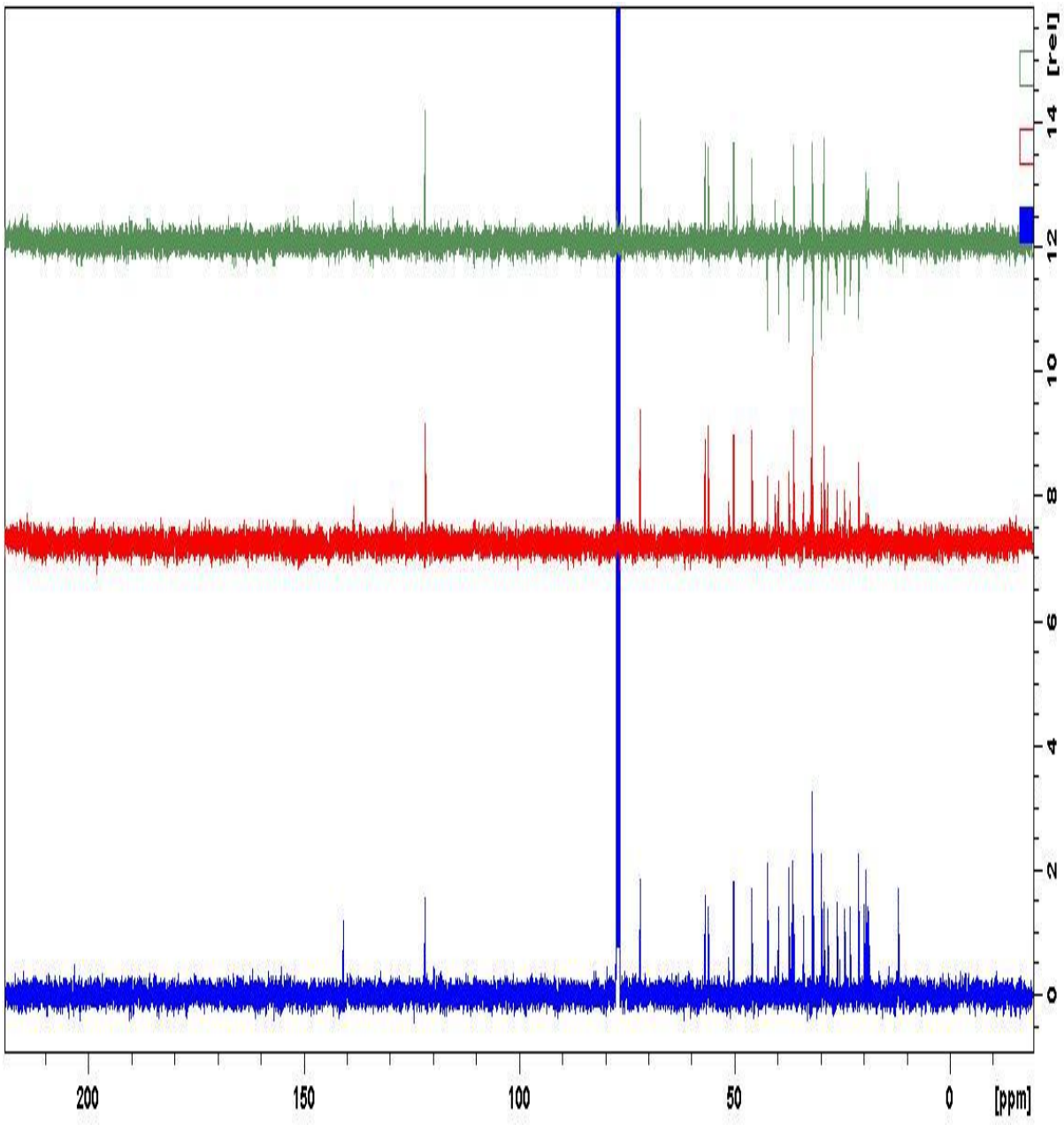


Figure 4.37: ^{13}C and DEPTS (90 and 135) NMR Spectra of S1

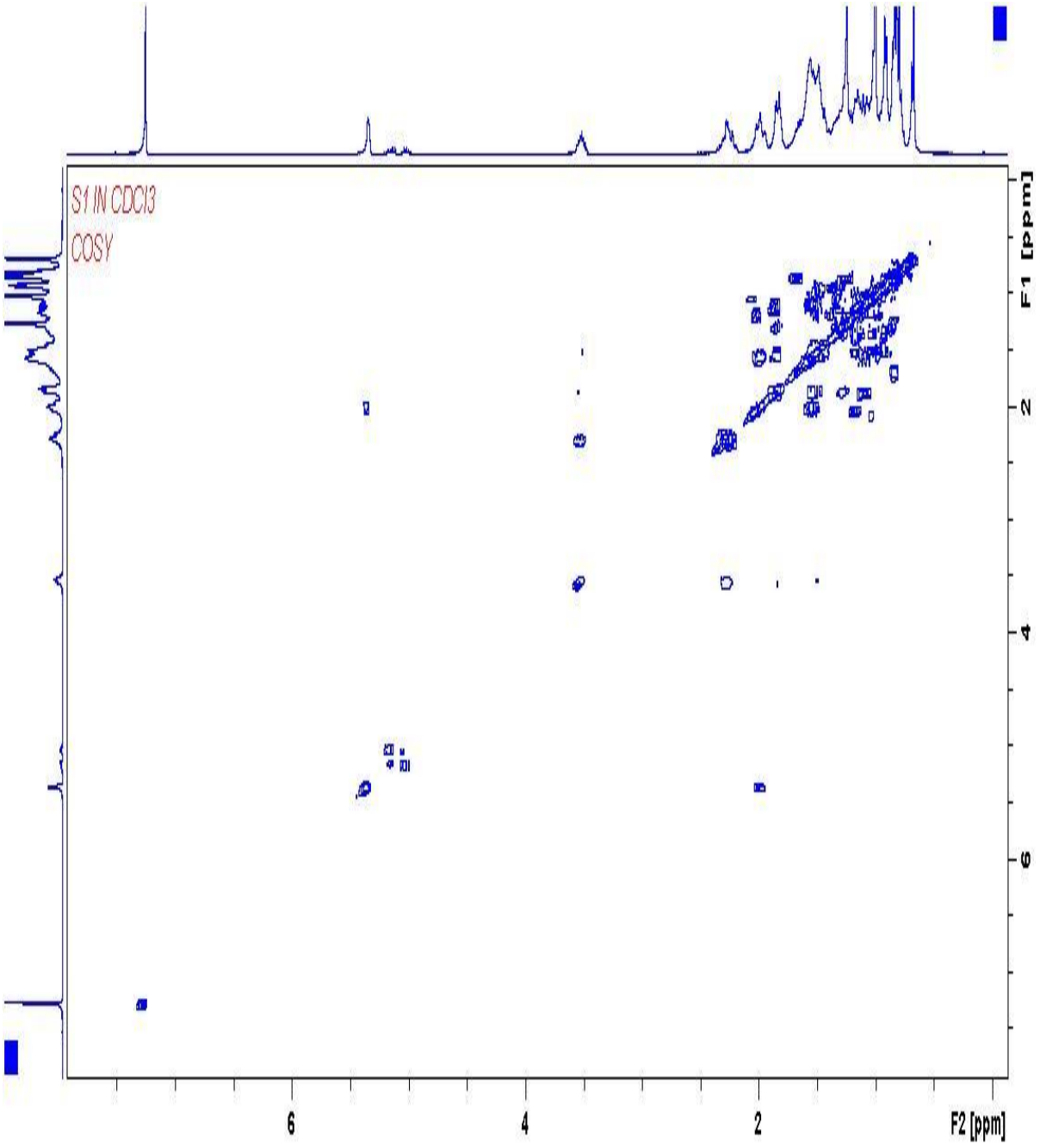


Figure 4.38: COSY NMR Spectrum of S1

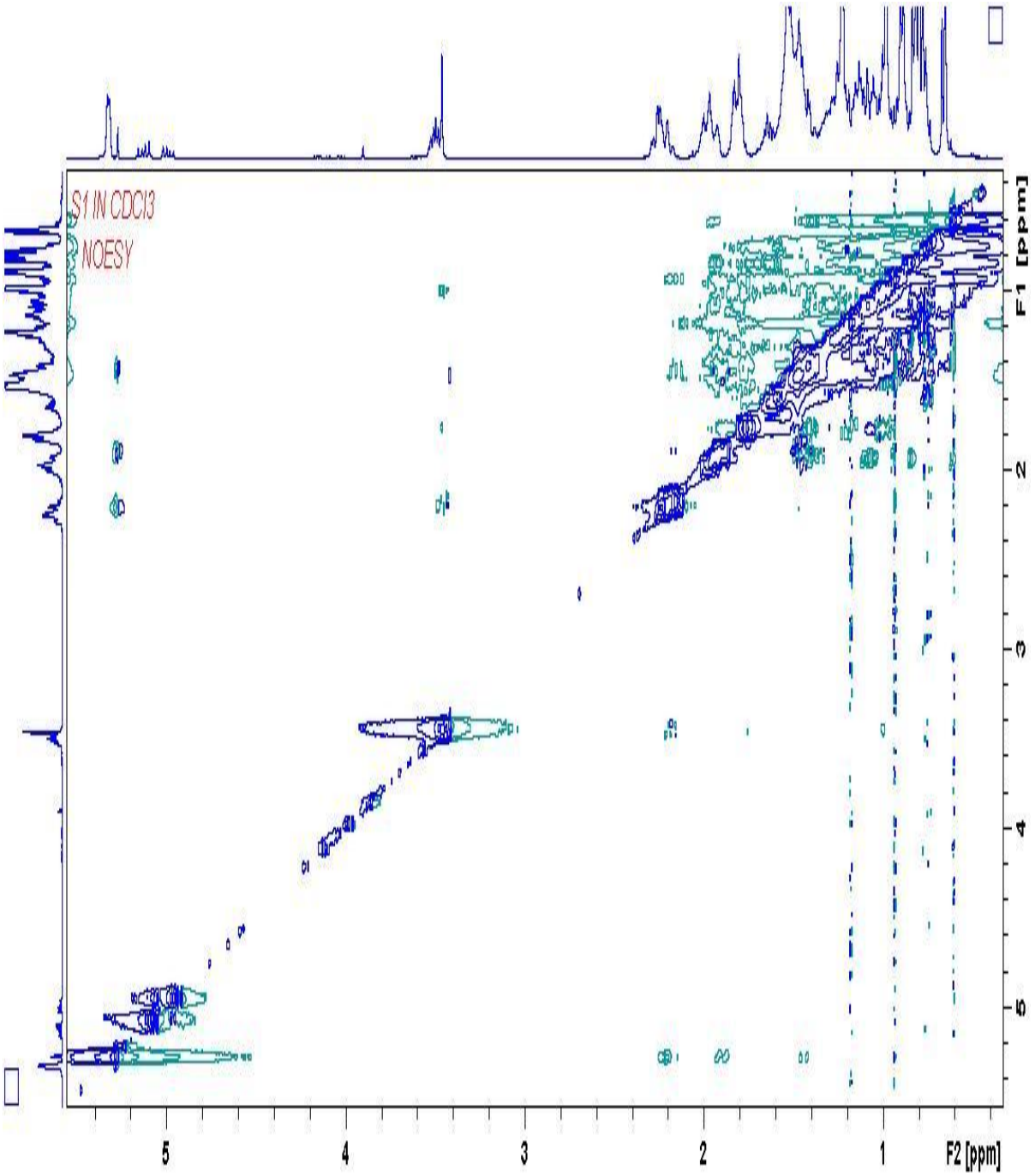


Figure 4.39: NOESY NMR Spectrum of S1

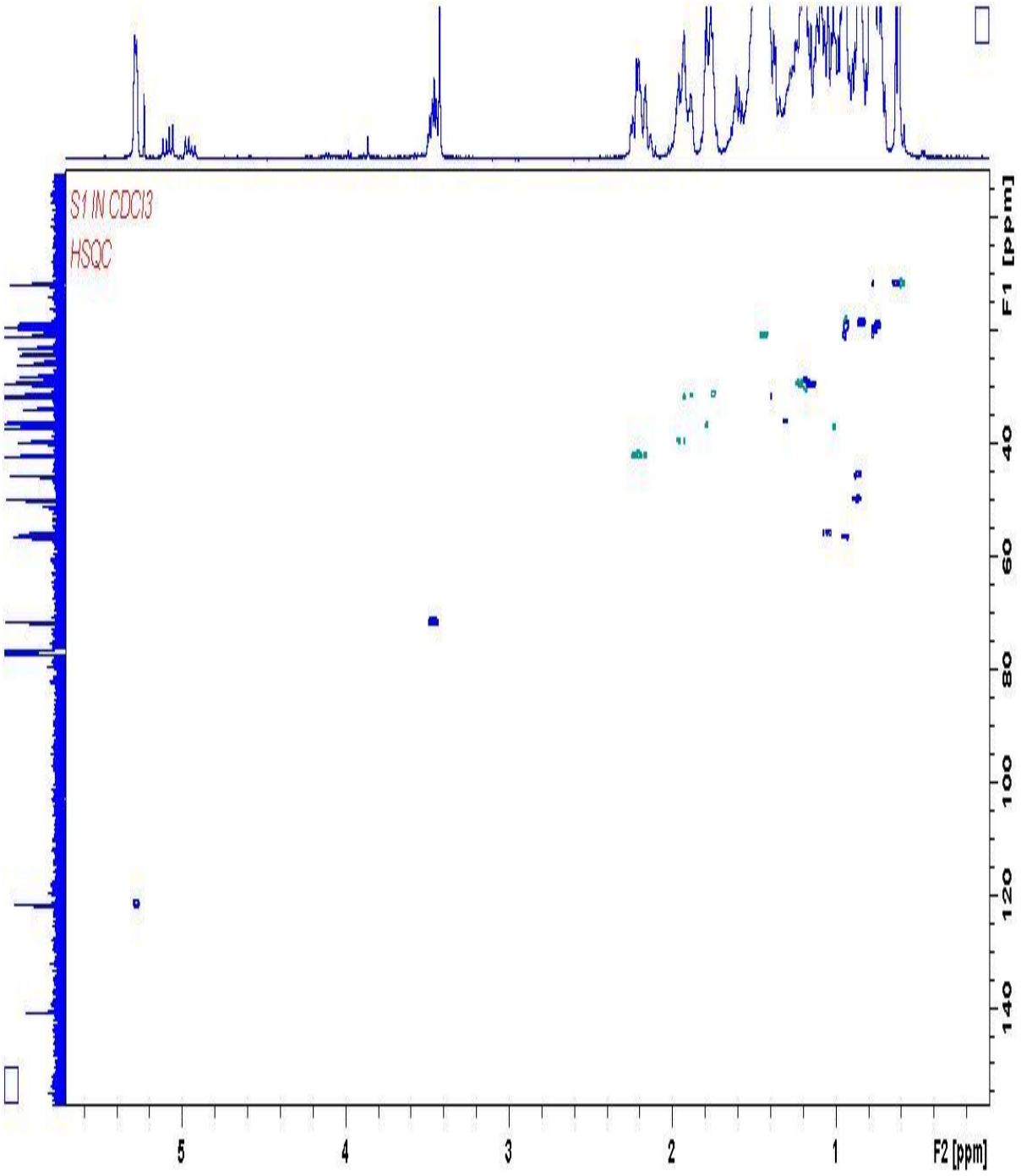


Figure 4.40: HSQC NMR Spectrum of S1

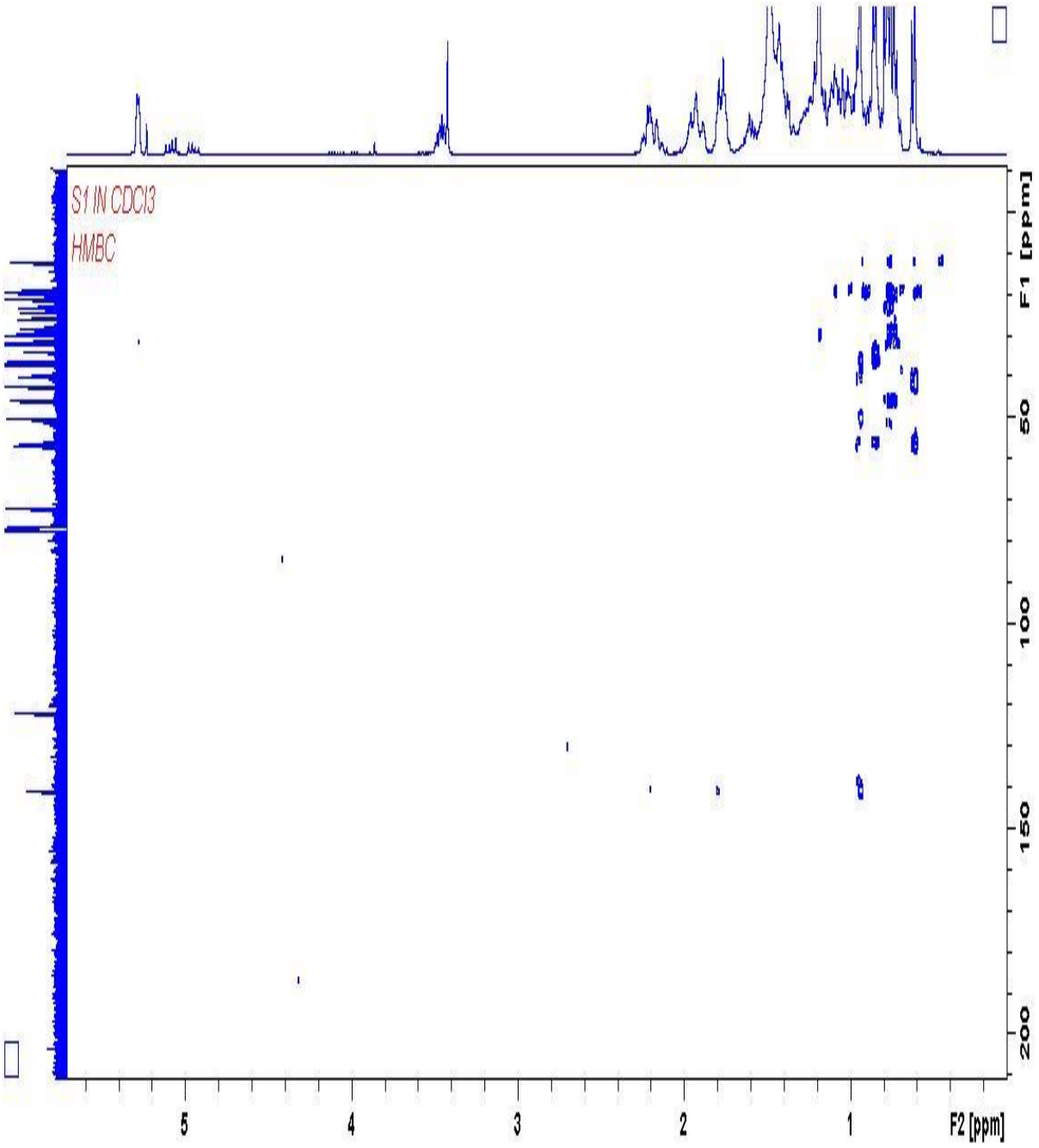


Figure 4.41: HMBC NMR Spectrum of S1

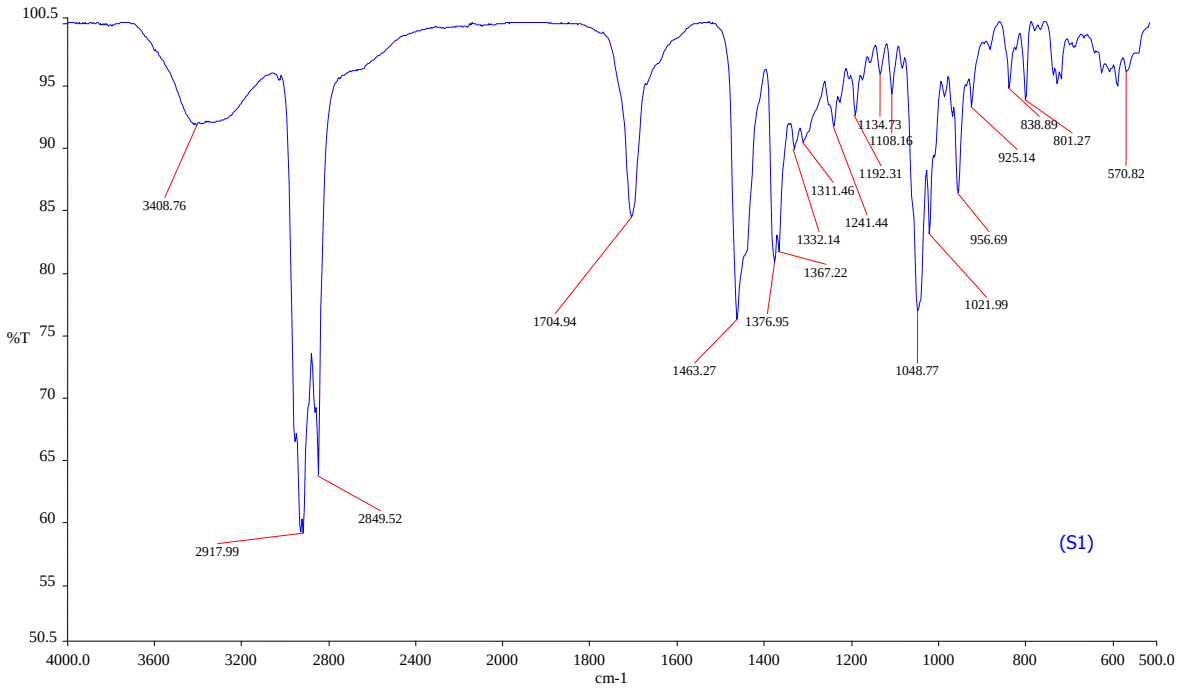


Figure 4.42: IR Spectrum of S1

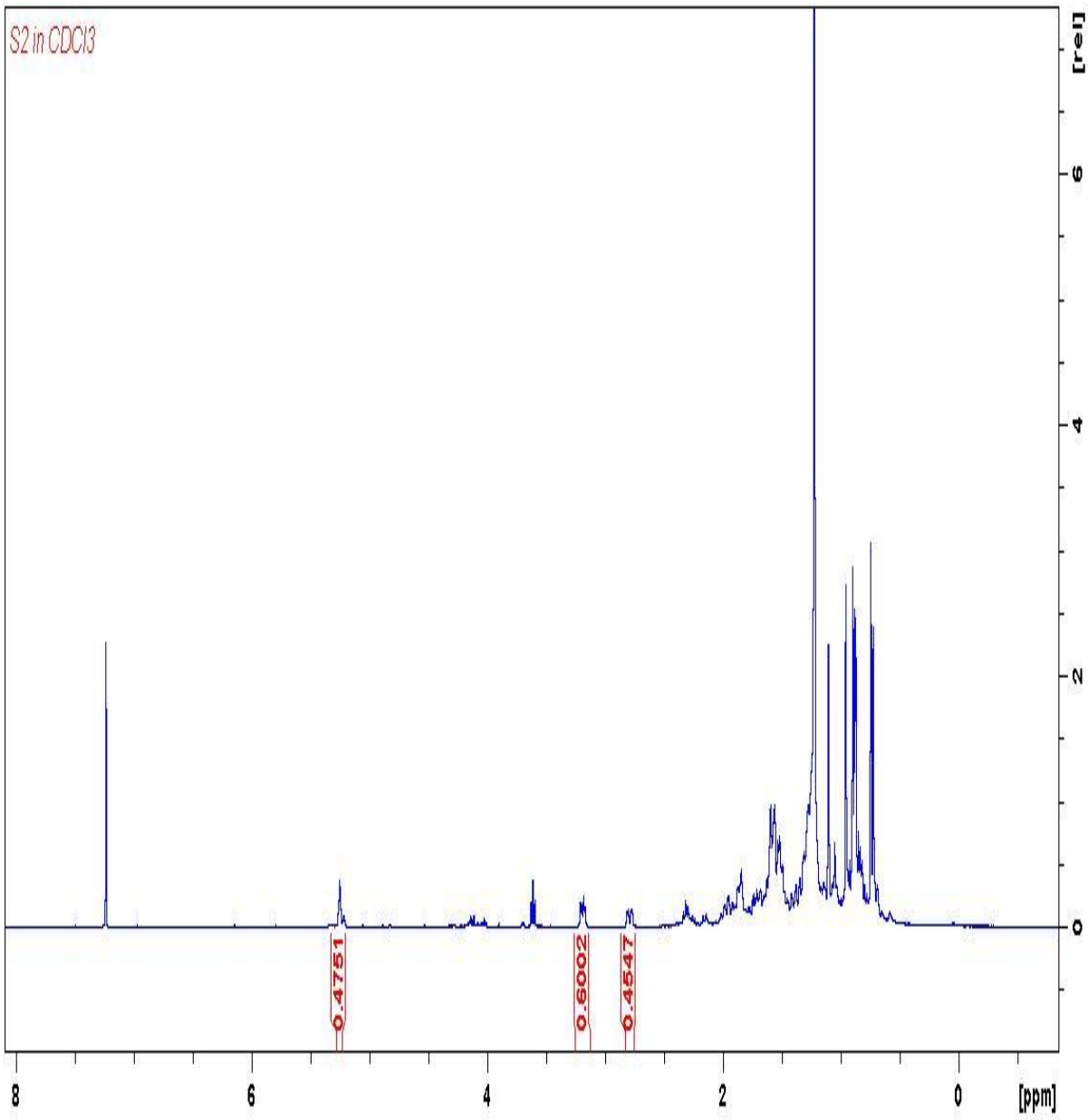


Figure 4.43: ^{1}H NMR Spectrum of S2

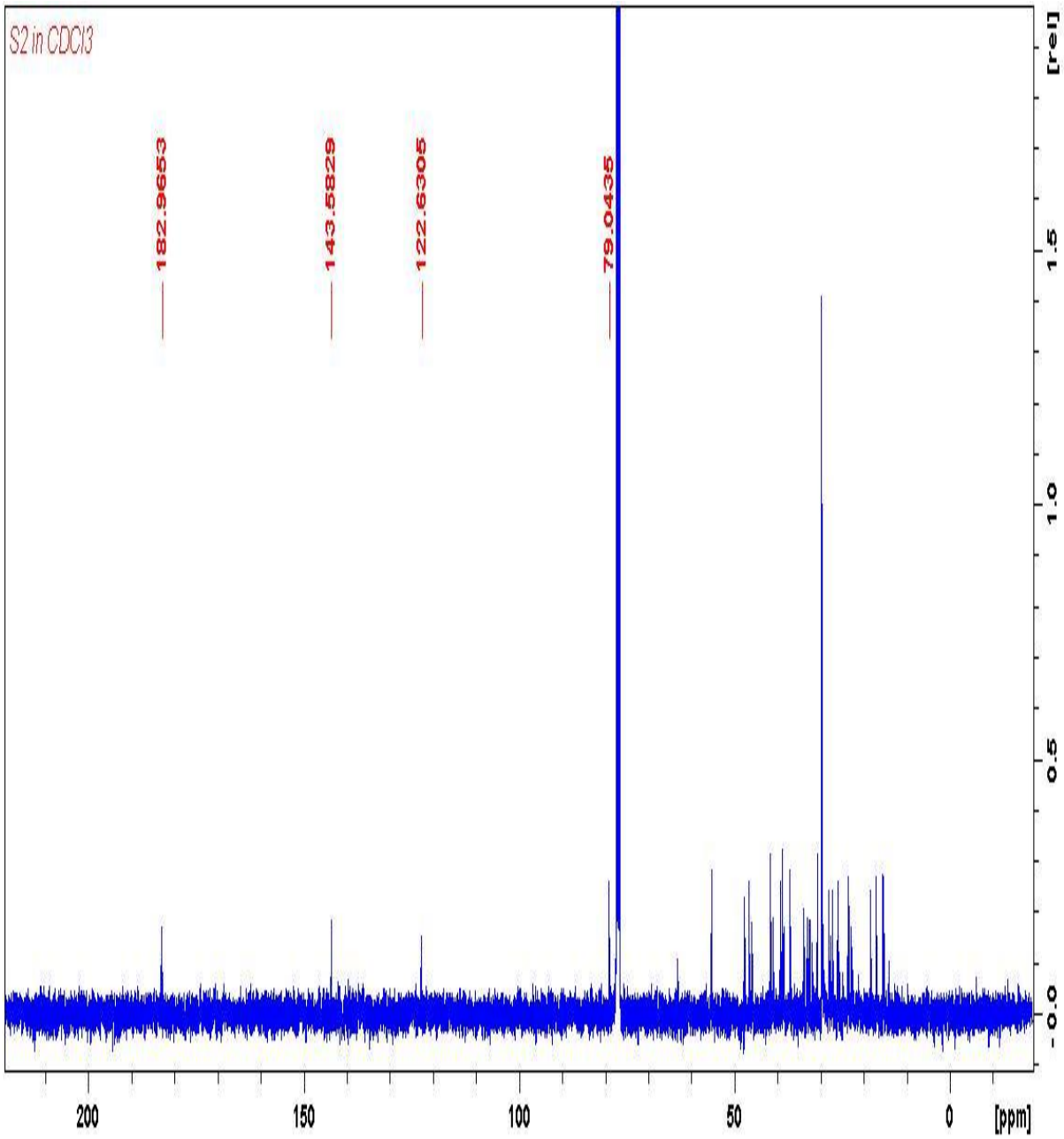


Figure 4.44: ^{13}C NMR Spectrum of S2

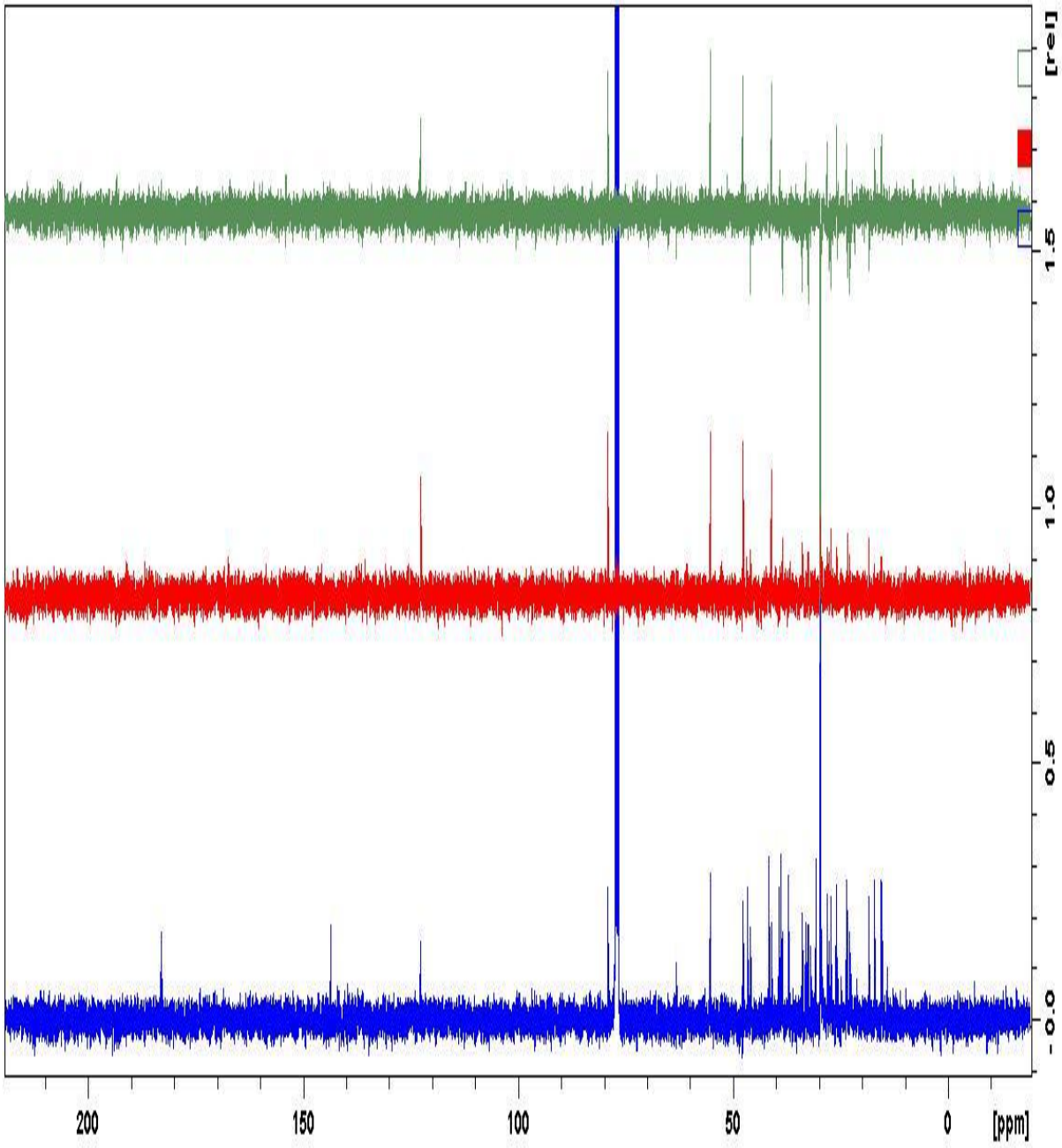


Figure 4.45: ^{13}C NMR and DEPTS (90 and 135) NMR Spectra of S2

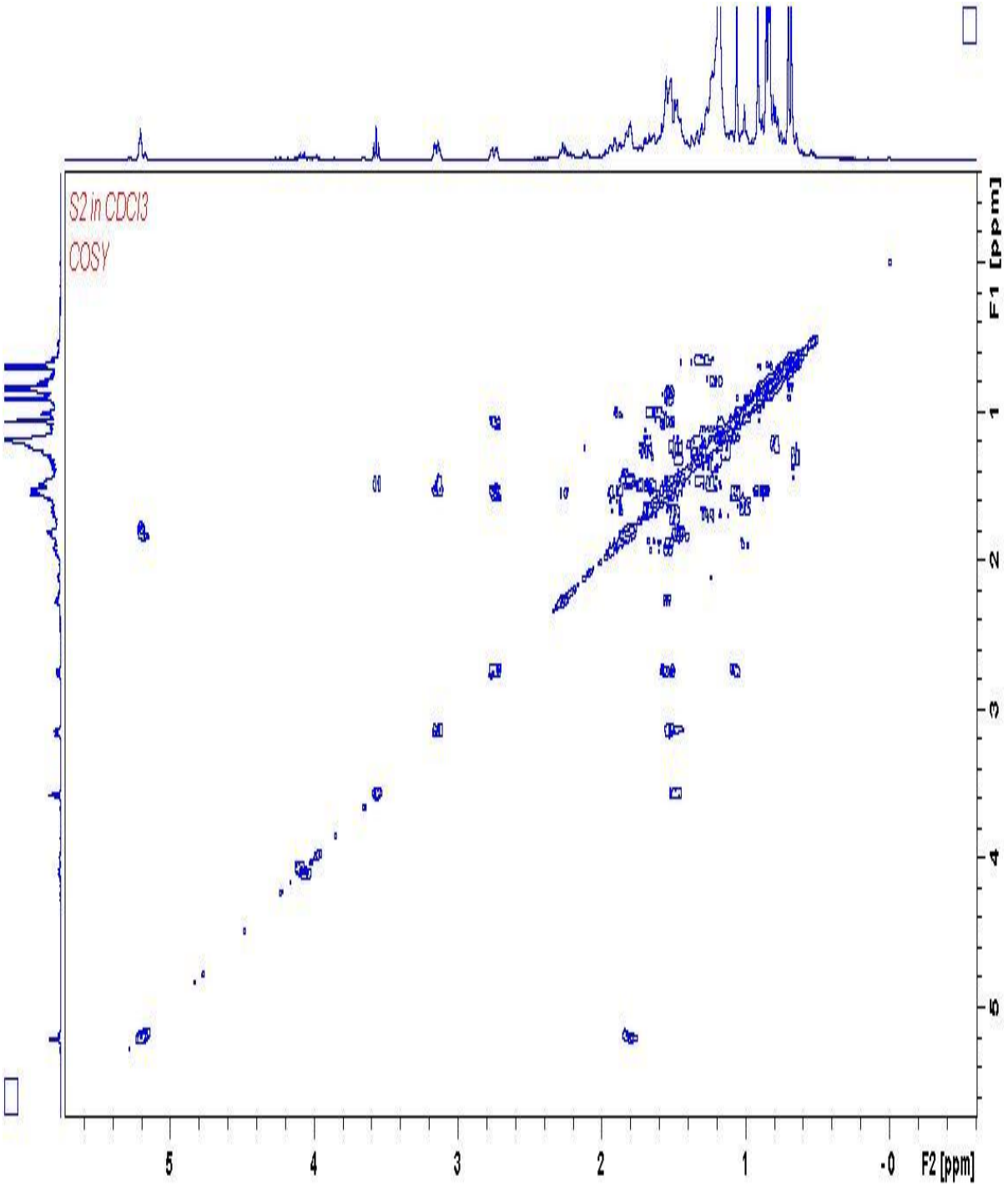


Figure 4.46: COSY NMR Spectrum of S2

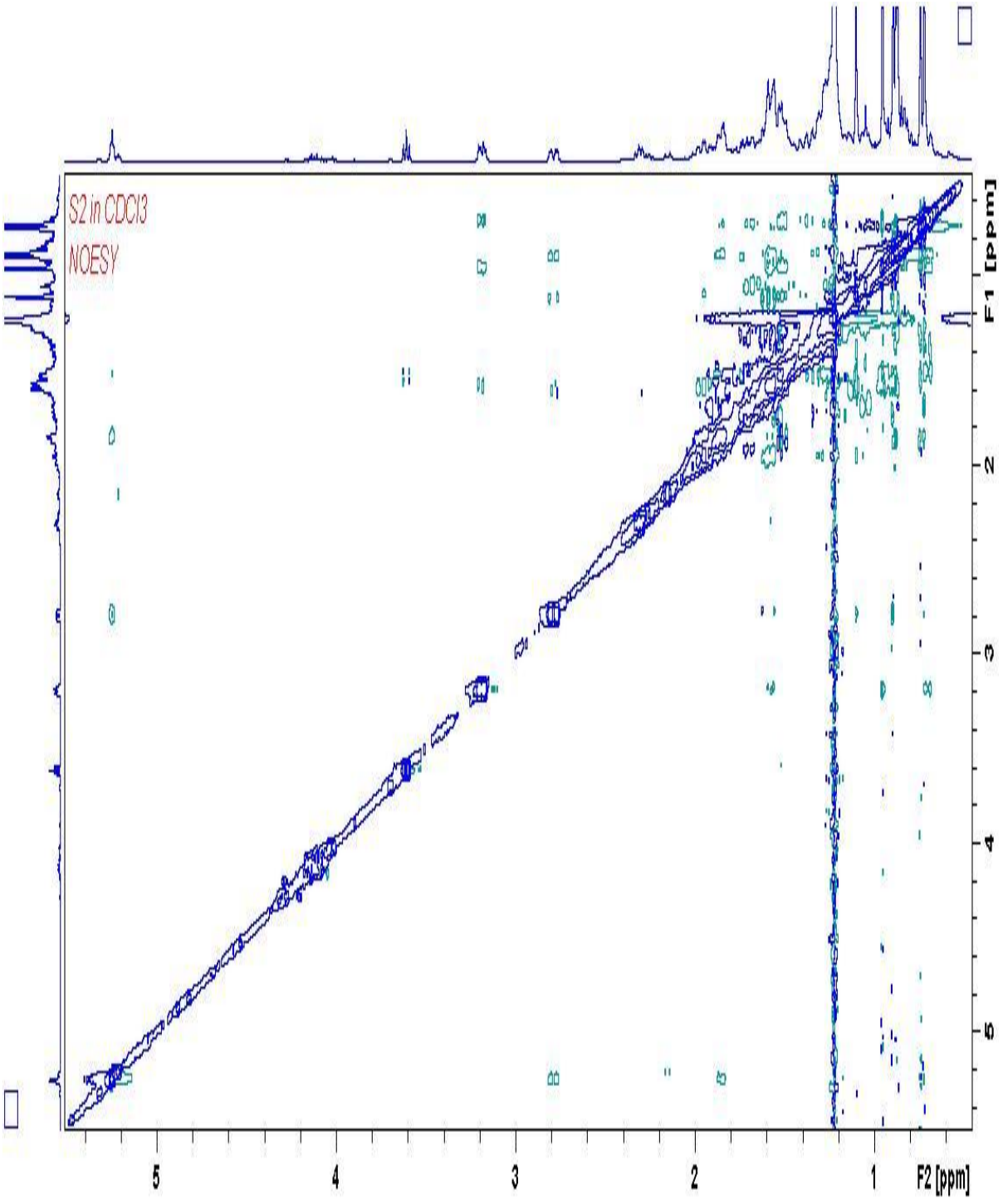


Figure 4.47: NOESY NMR Spectrum of S2

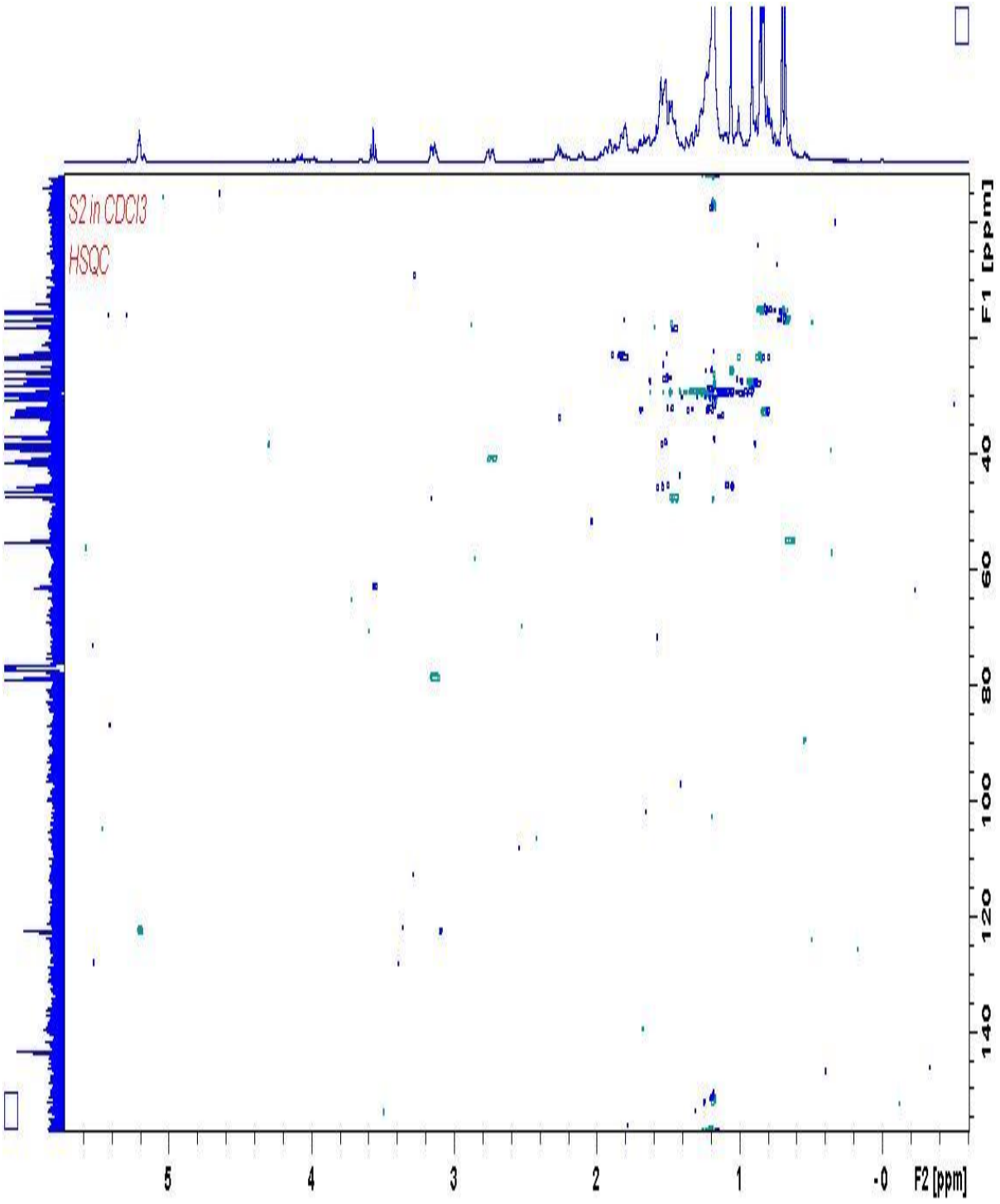


Figure 4.48: HSQC NMR Spectrum of S2

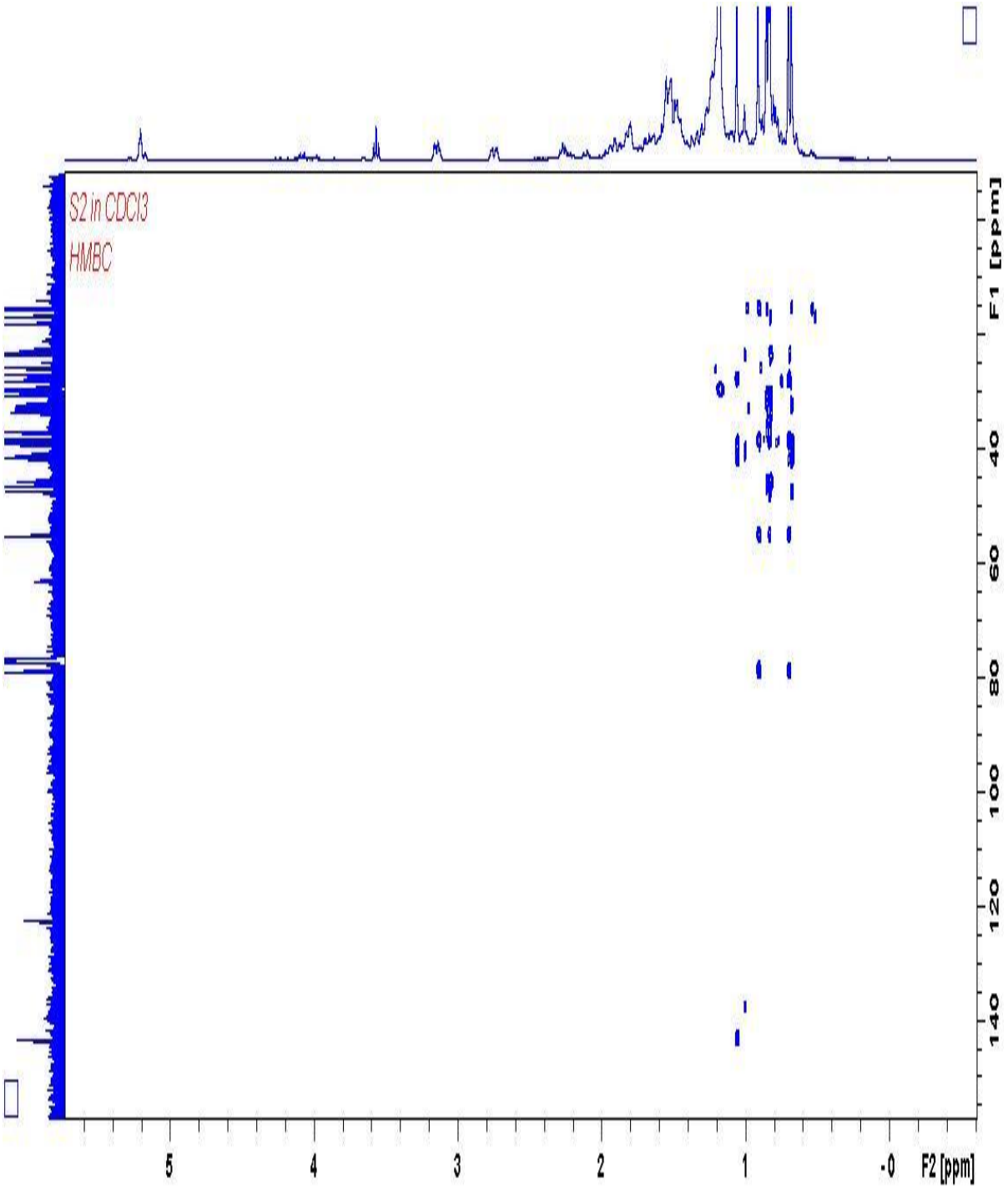


Figure 4.49: HMBC NMR Spectrum of S2

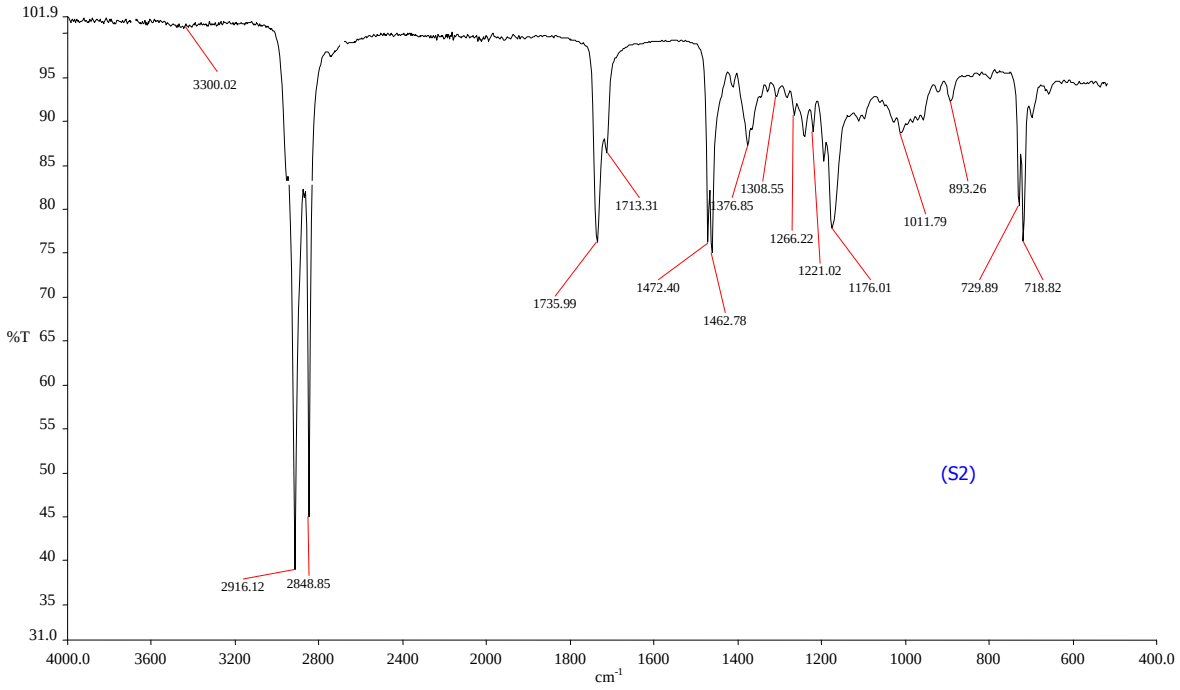


Figure 4.50: IR Spectrum of S2

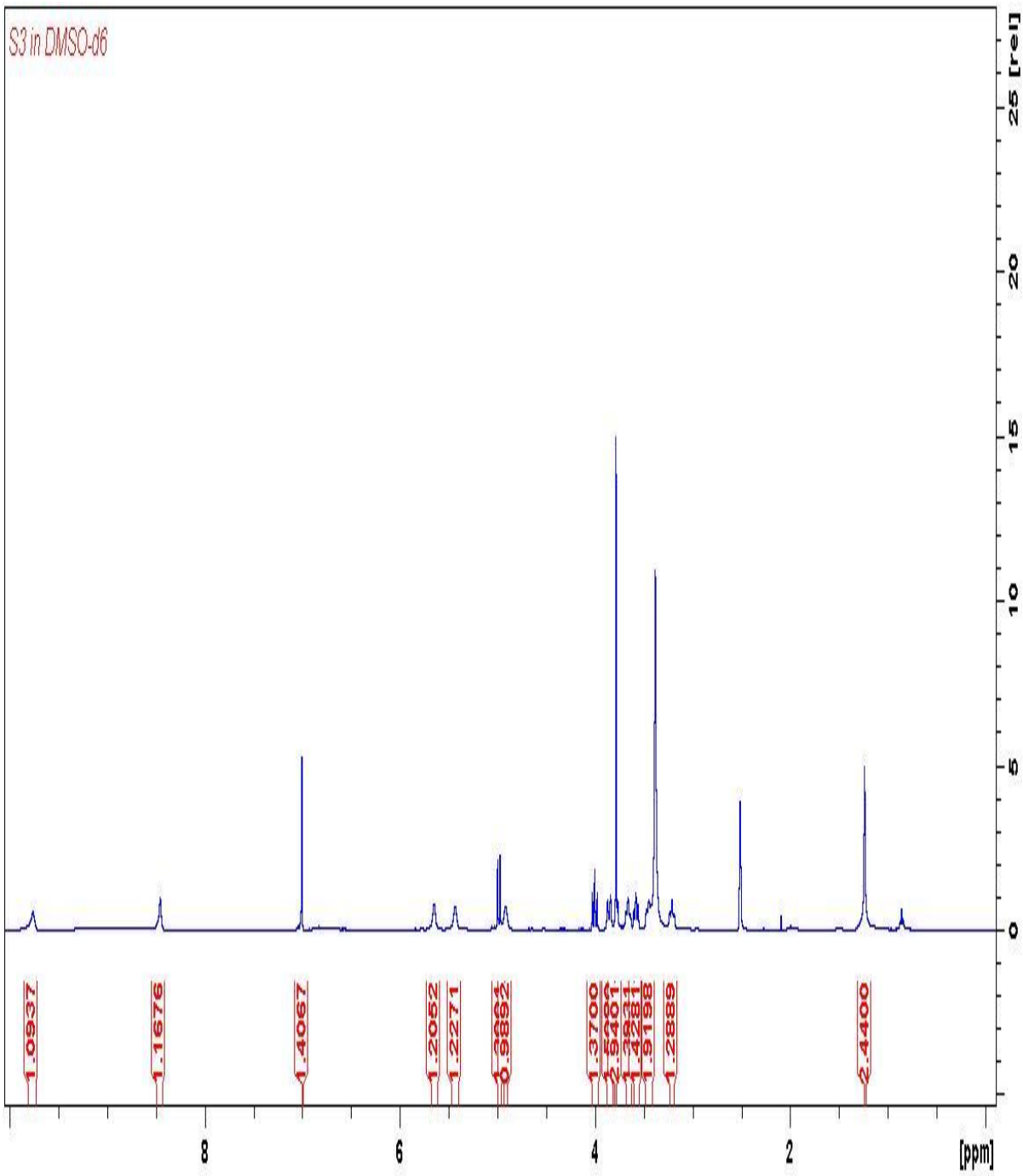


Figure 4.51: ^{1}H NMR Spectrum of S3

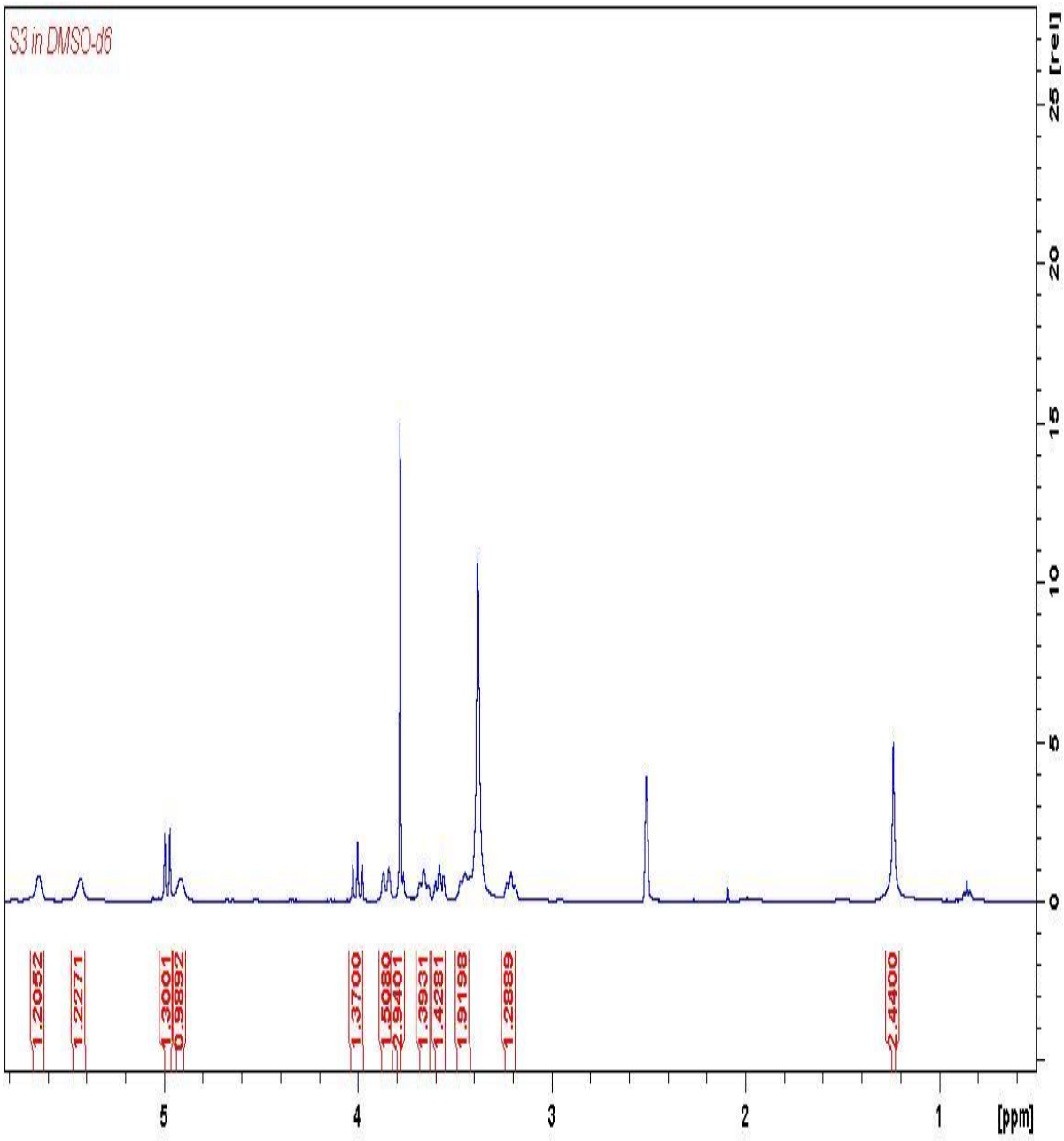


Figure 4.52: ^{1}H NMR Expanded Sugar Region of S3

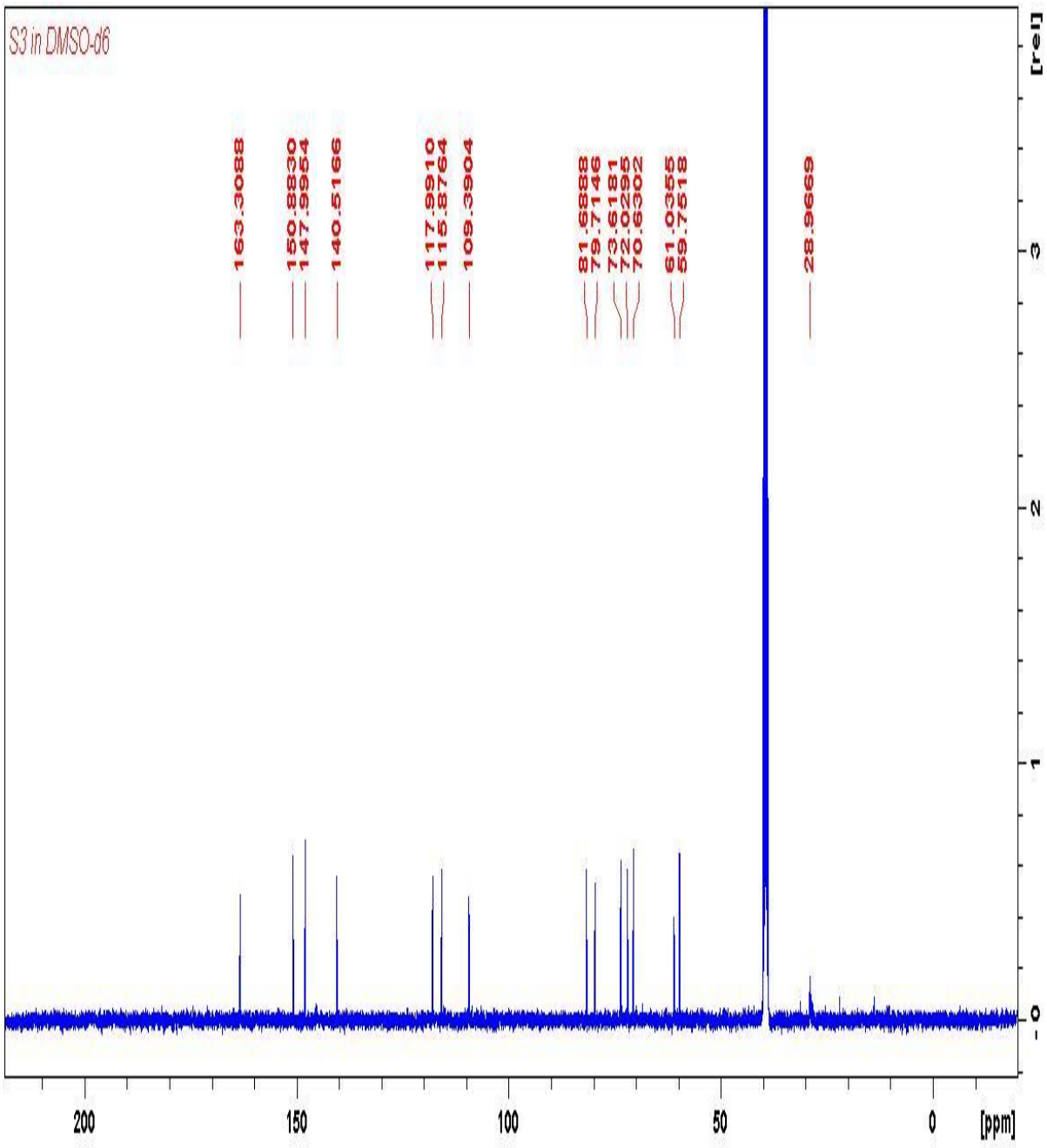


Figure 4.53: ^{13}C NMR Spectrum of S3

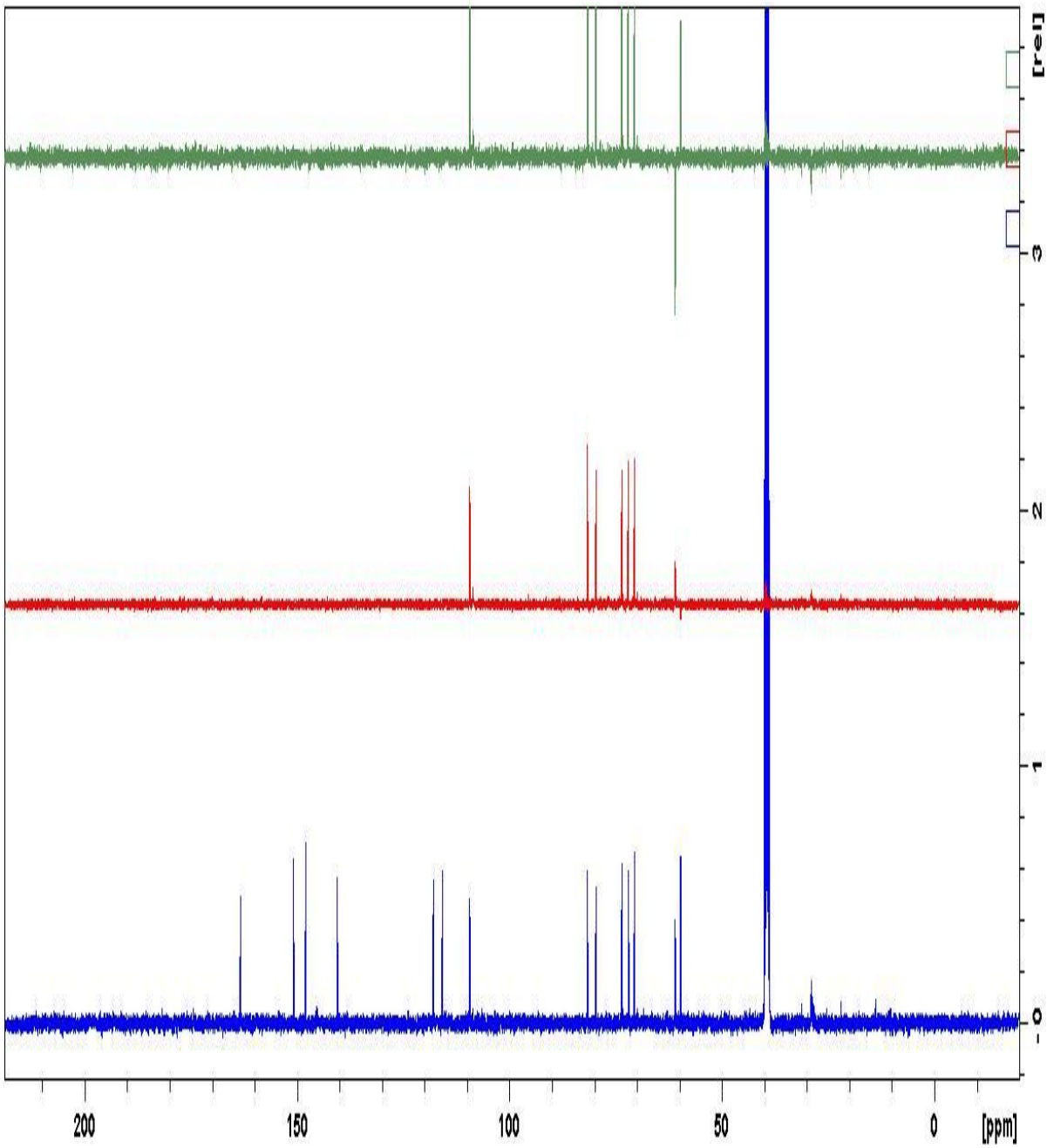


Figure 4.54: ^{13}C and DEPTS (90 and 135) NMR Spectra of S3

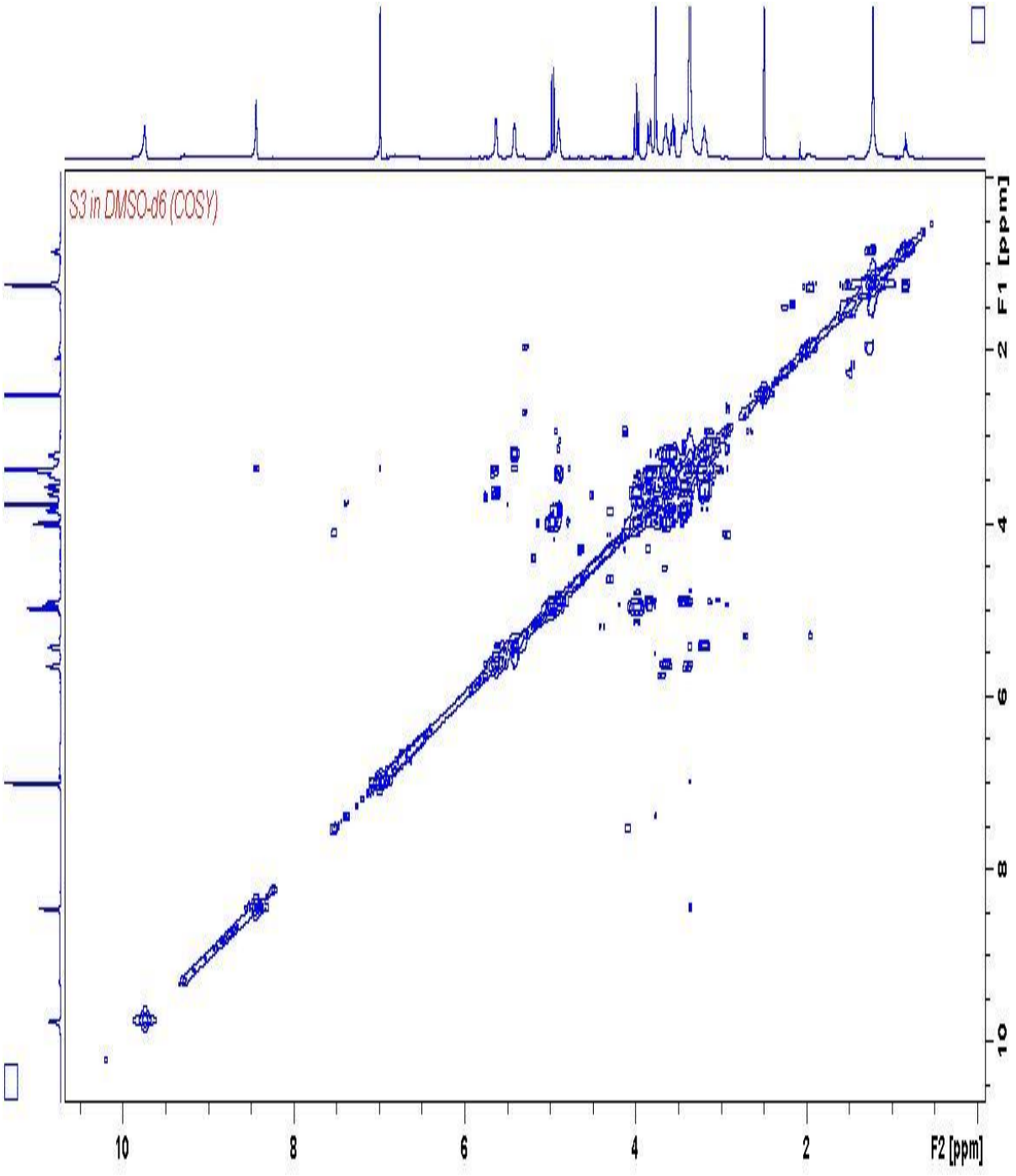


Figure 4.55: COSY NMR Spectrum of S2

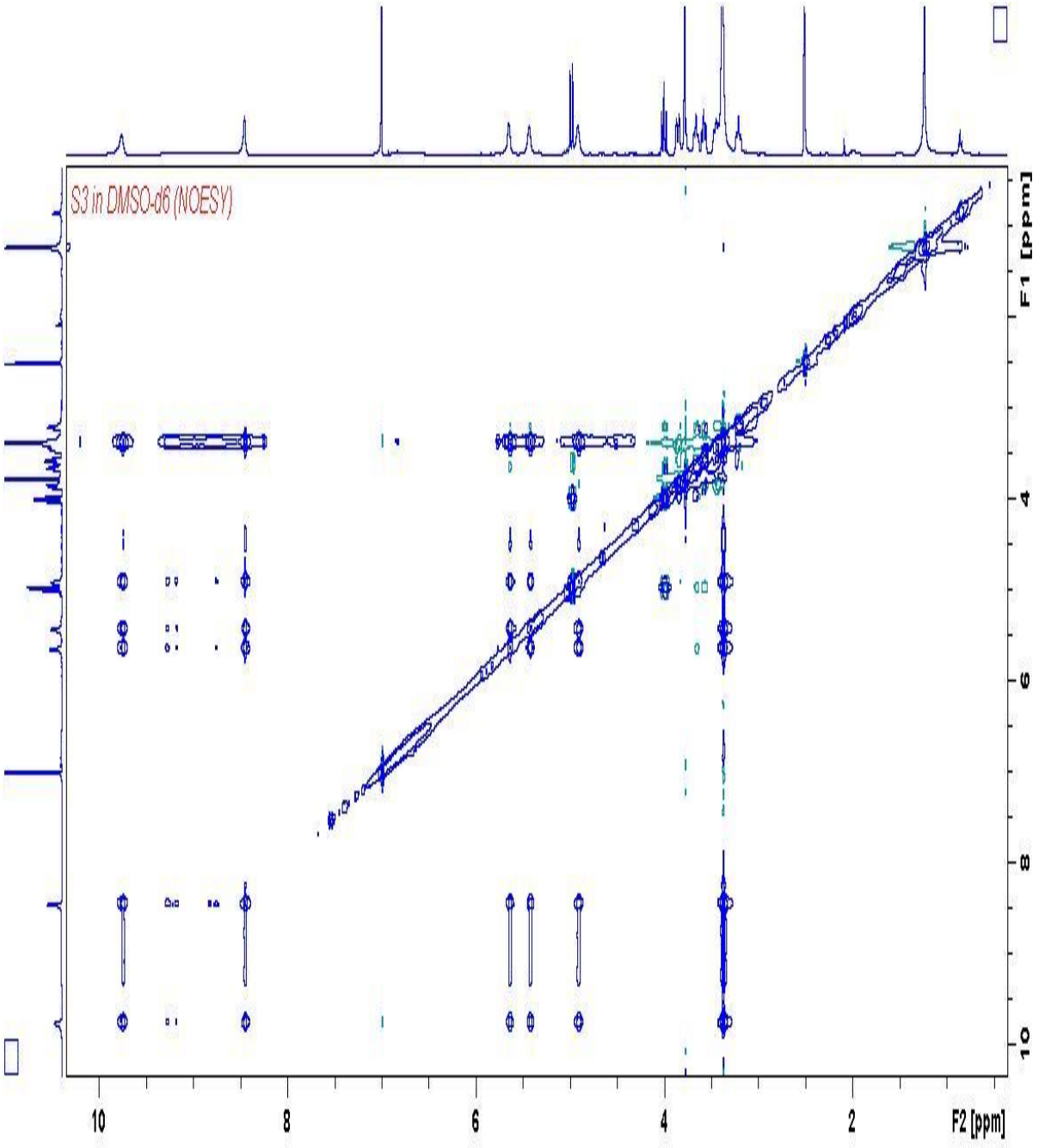


Figure 4.56: NOESY NMR Spectrum of S3

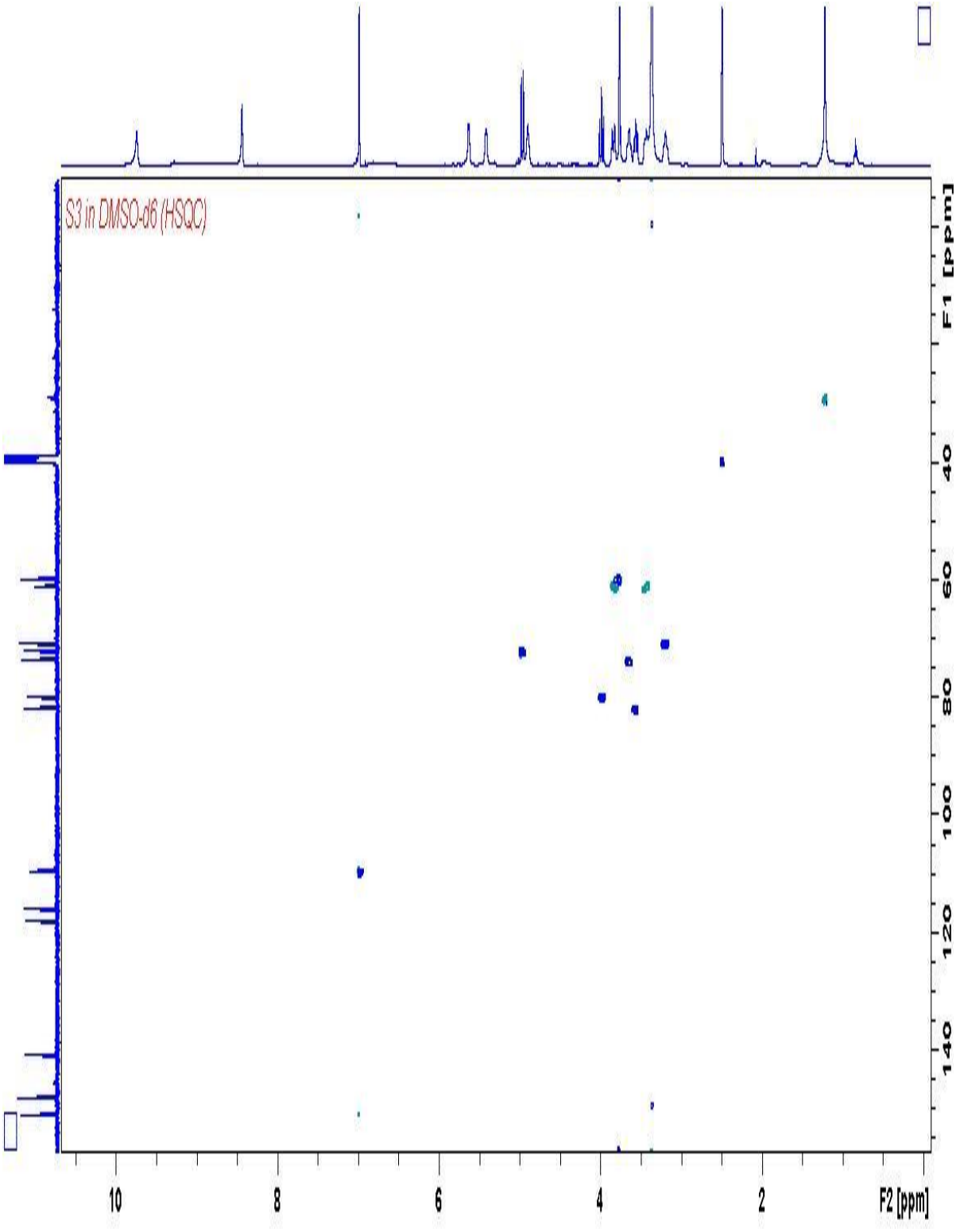


Figure 4.57: HSQC NMR Spectrum of S3

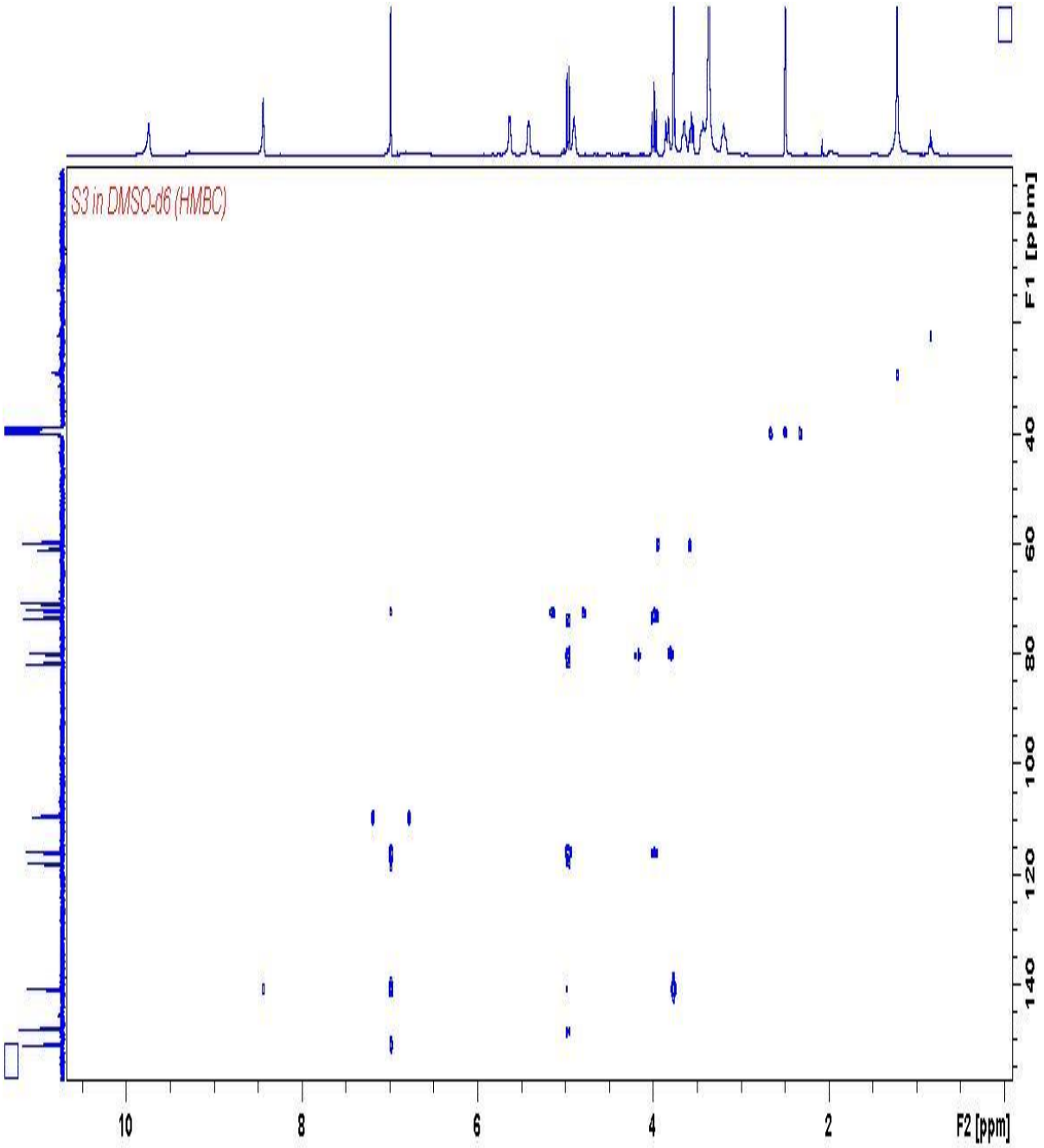


Figure 4.58: HMBC NMR Spectrum of S3

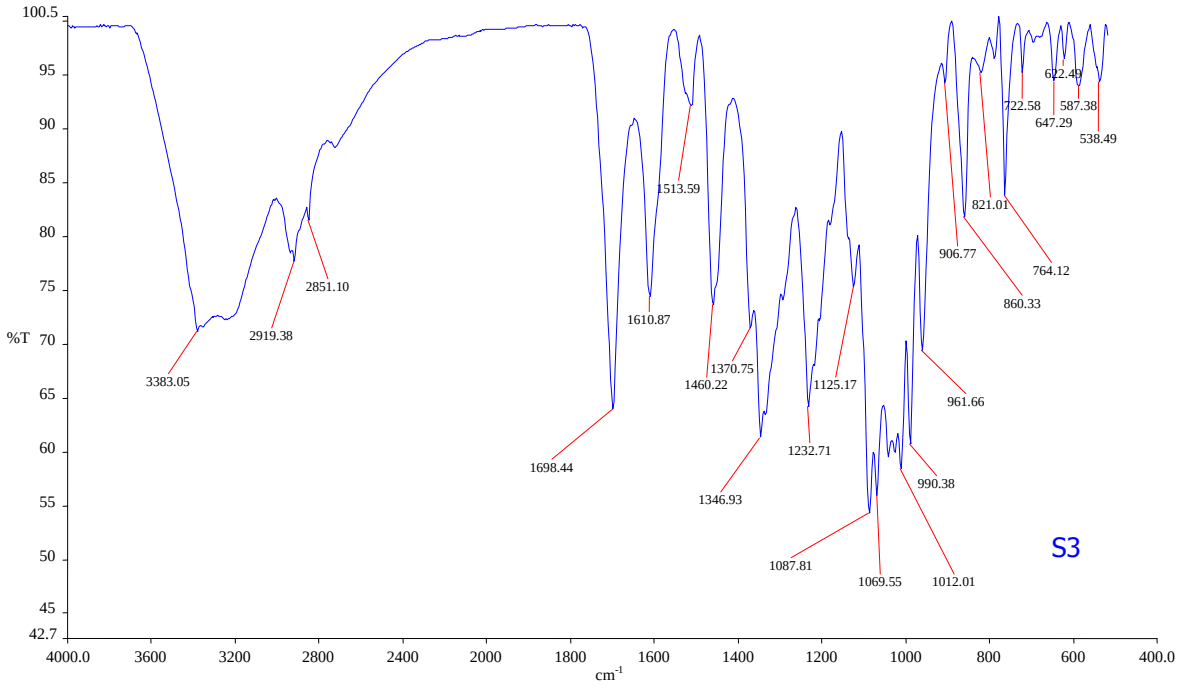


Figure 4.59: IR Spectrum of S3

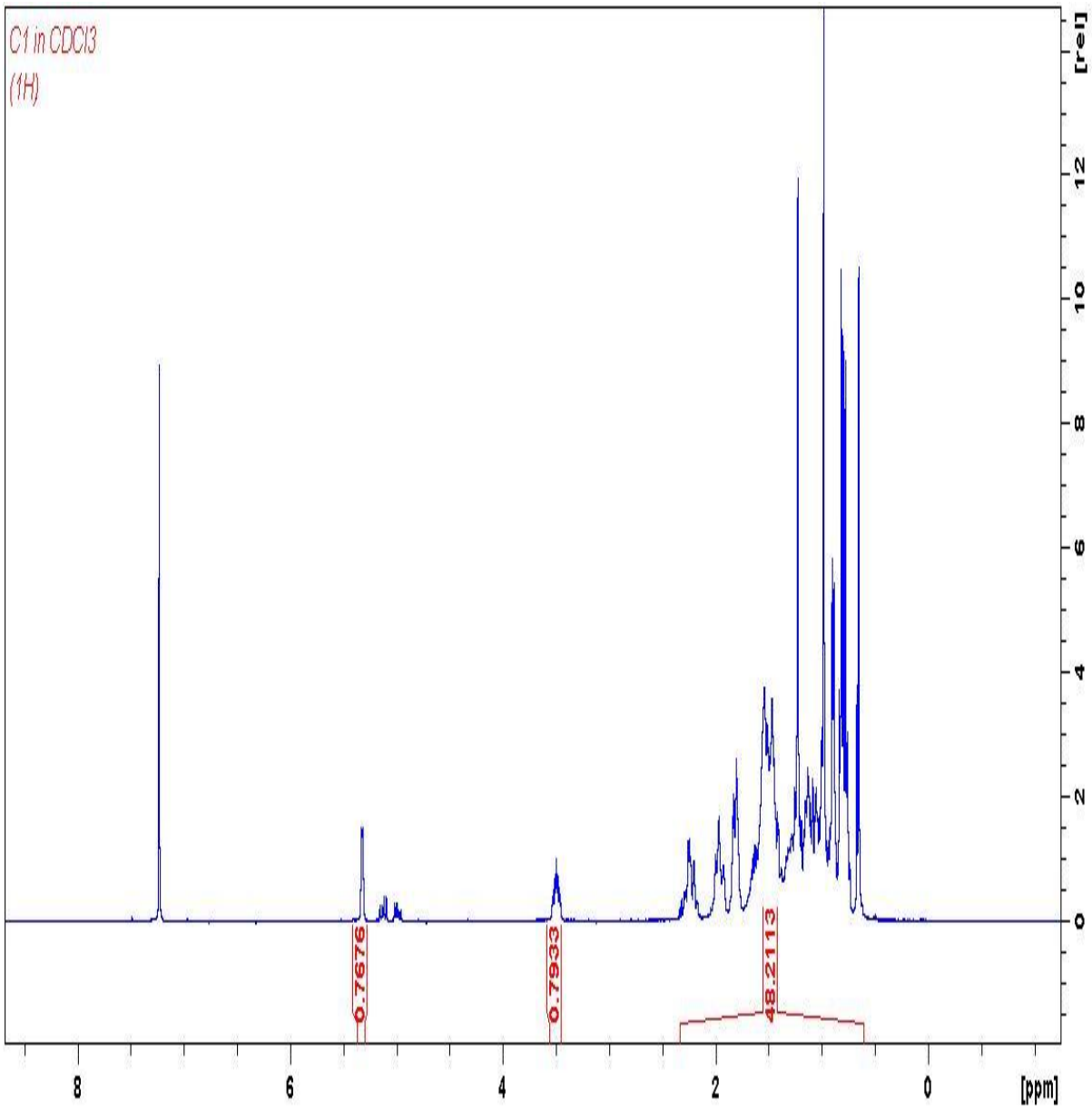


Figure 4.60: ^{1}H NMR Spectrum of C1

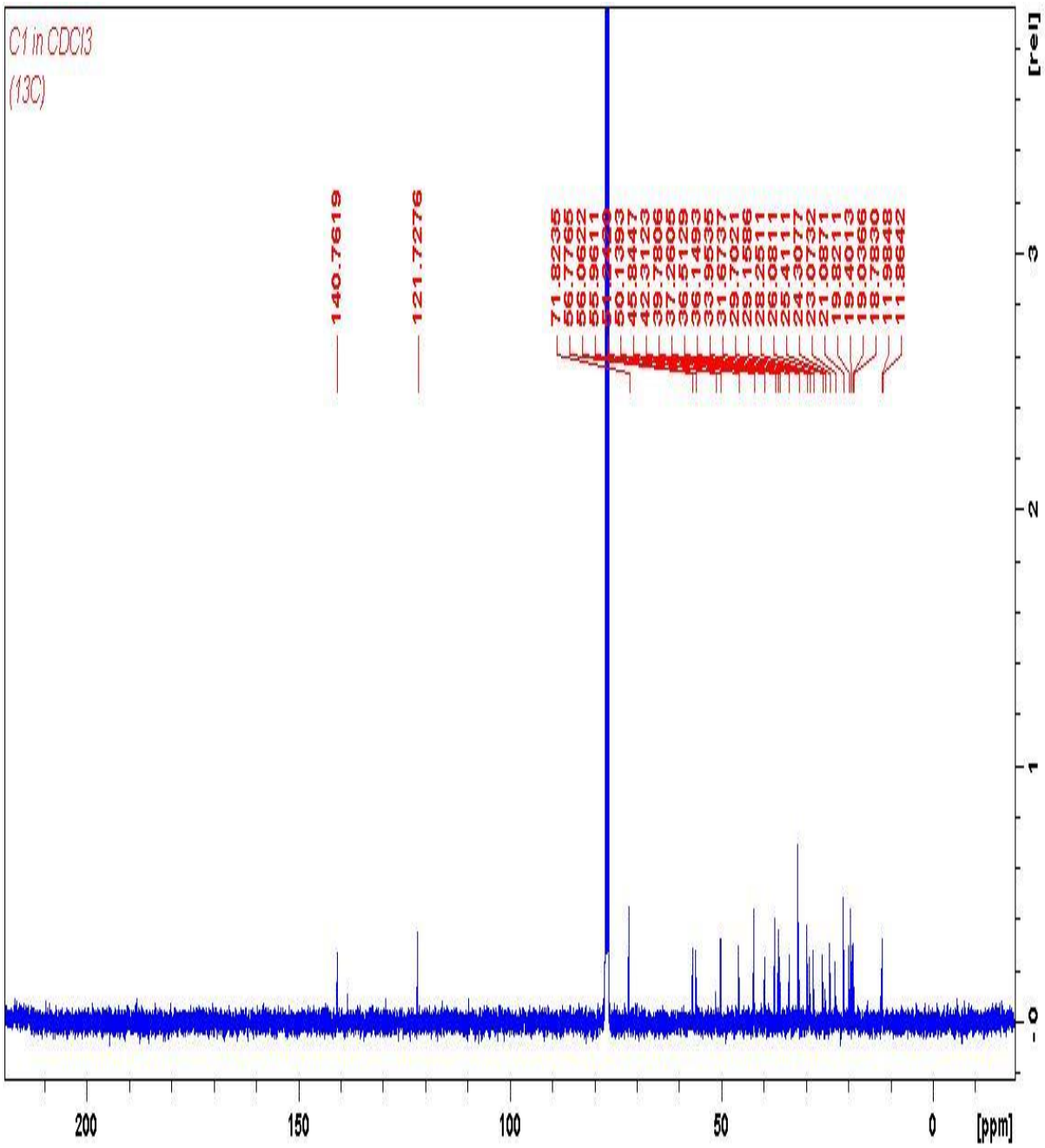


Figure 4.61: ^{13}C NMR Spectrum of C1

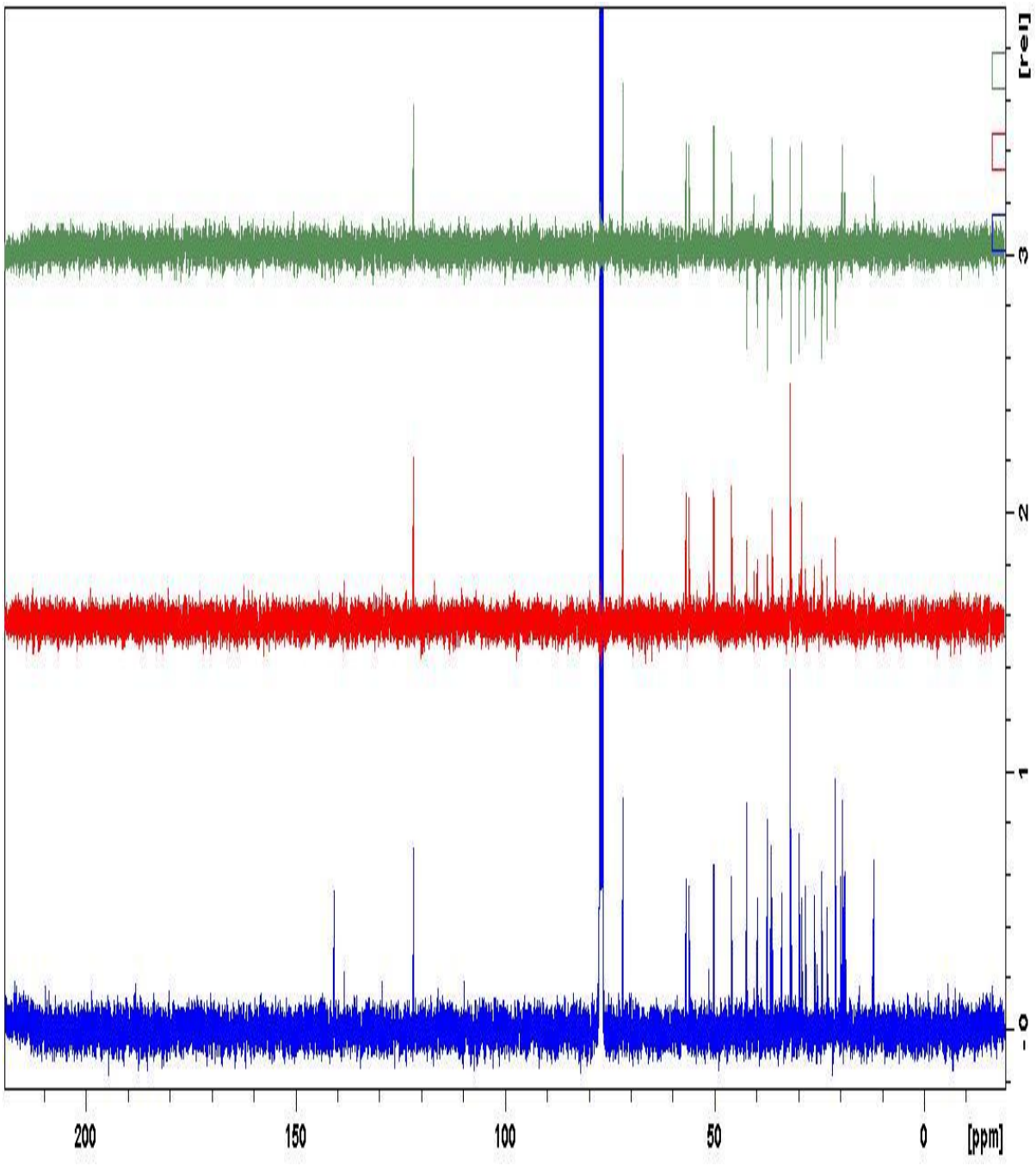


Figure 4.62:^{13}C AND DEPTS (90 and 135) NMR Spectra of C1

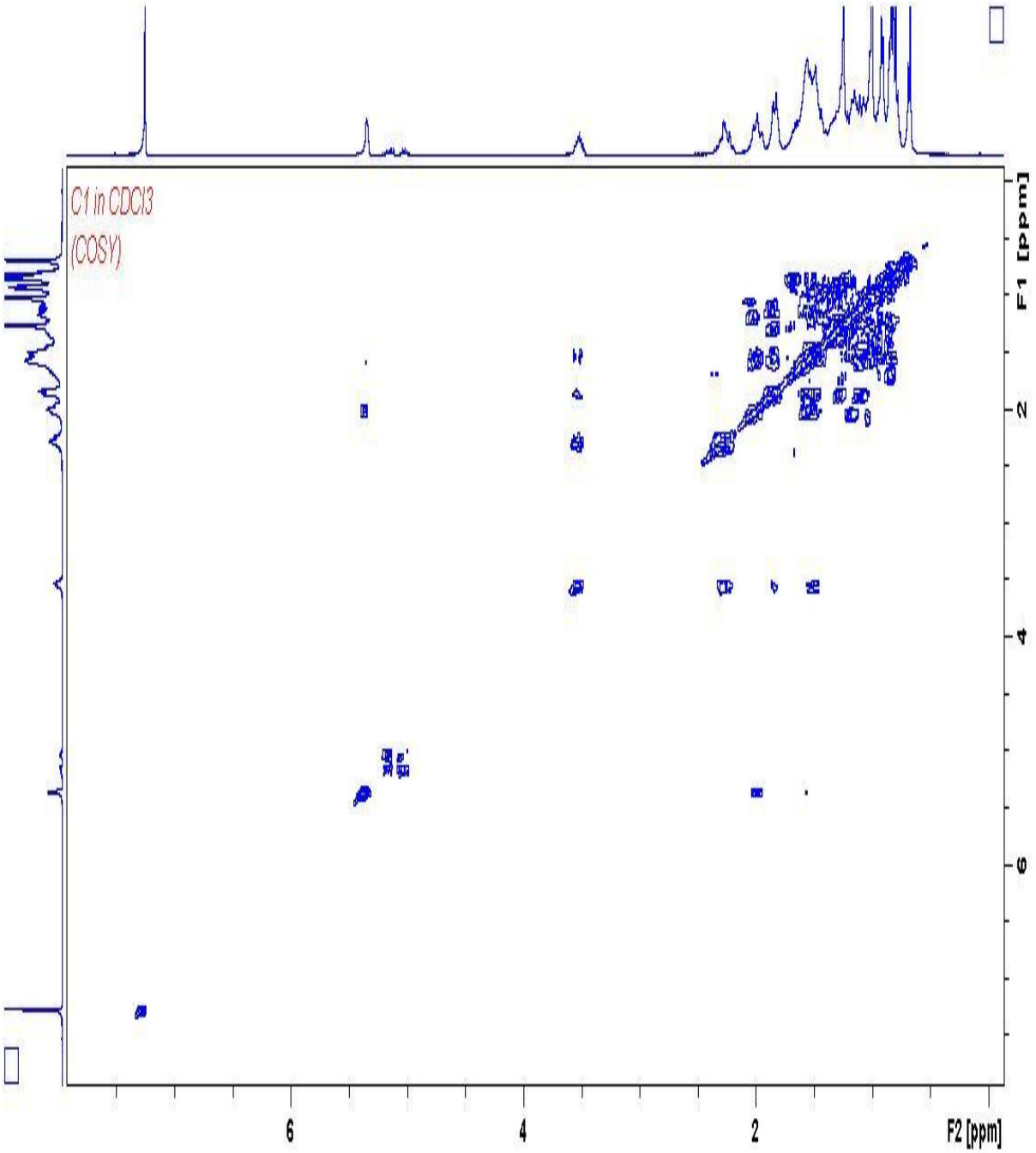


Figure 4.63: COSY NMR Spectrum of C1

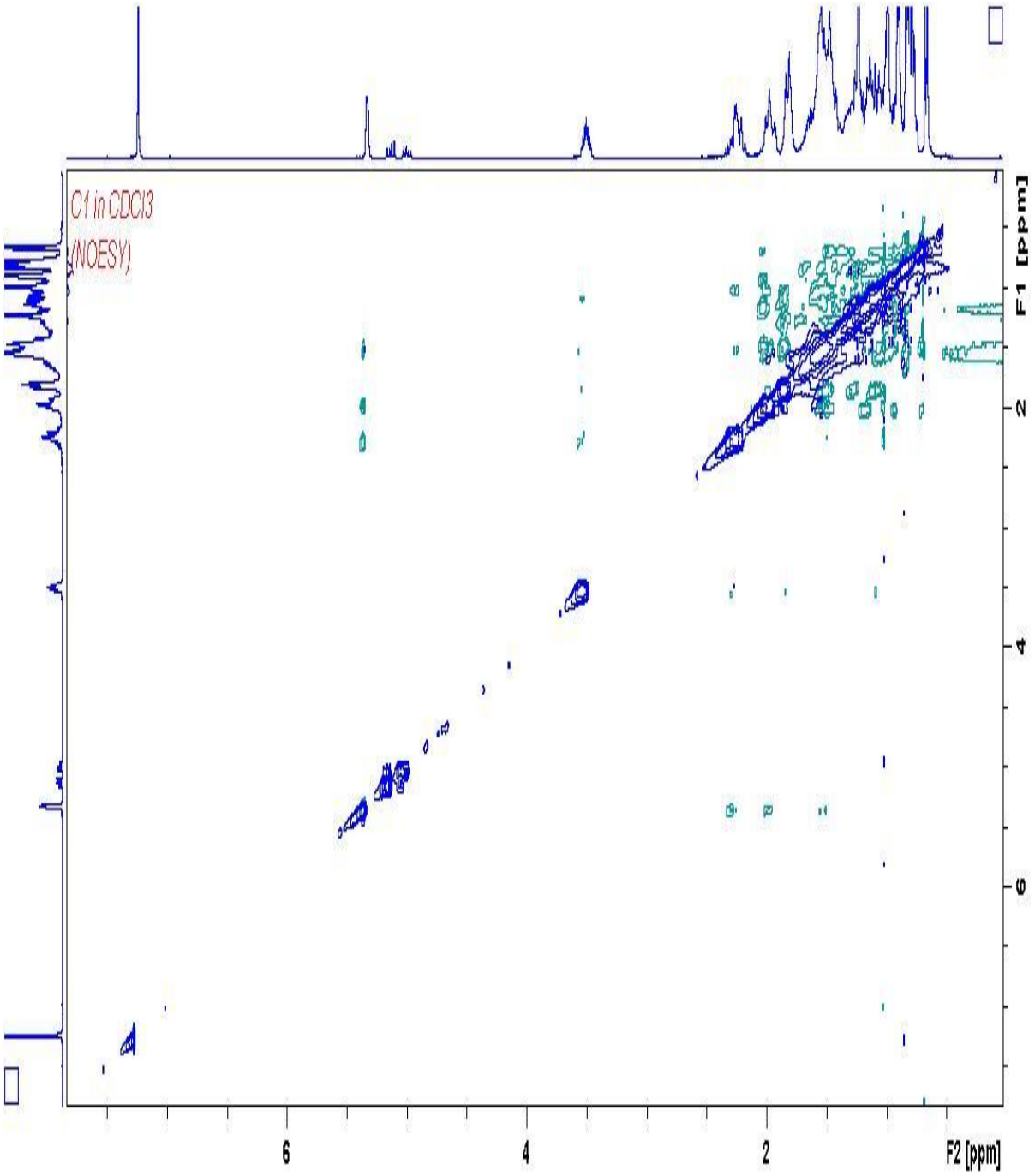


Figure 4.64: NOESY NMR Spectrum of C1

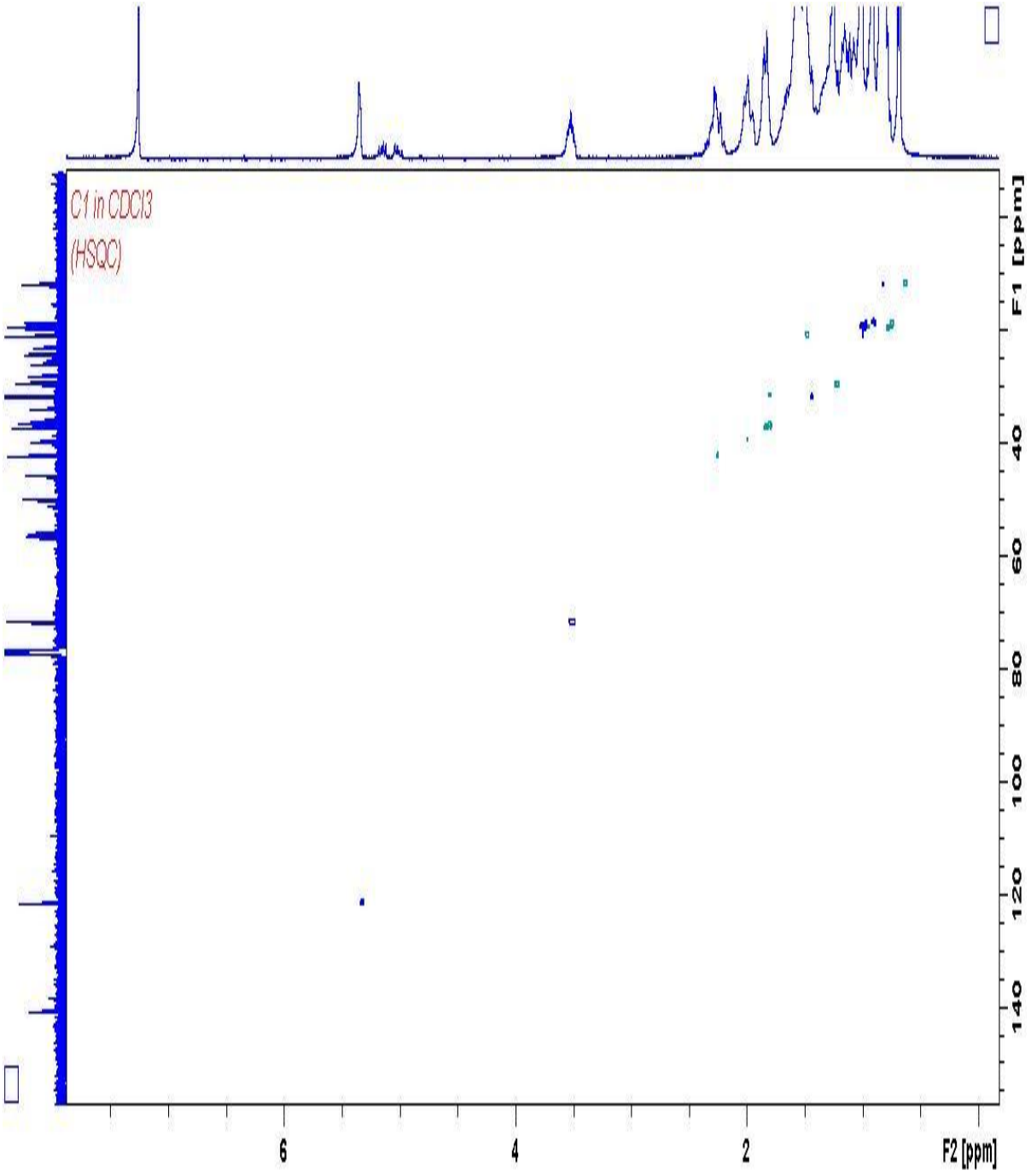


Figure 4.65: HSQC NMR Spectrum of C1

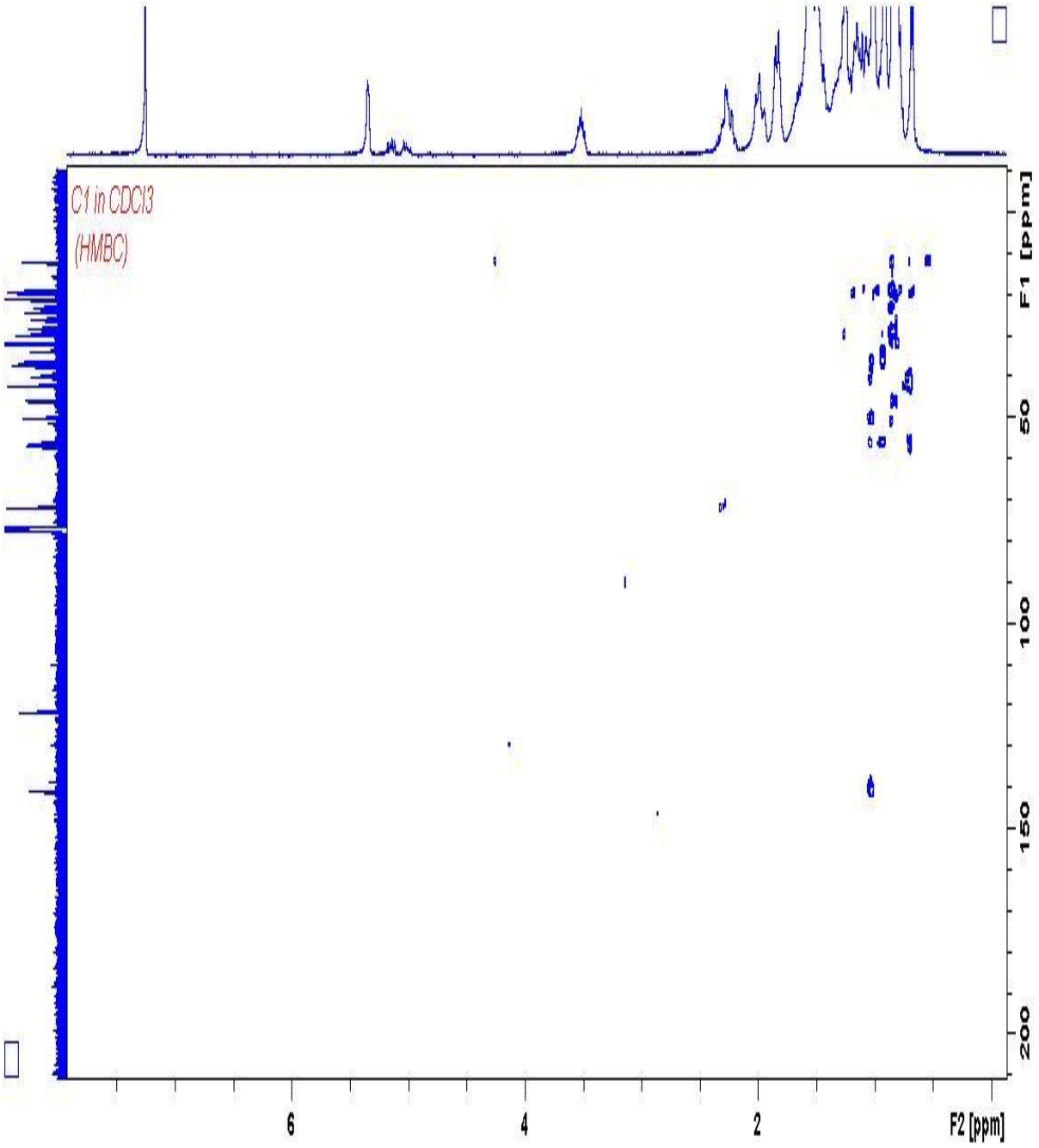


Figure 4.66: HMBC NMR Spectrum of C1

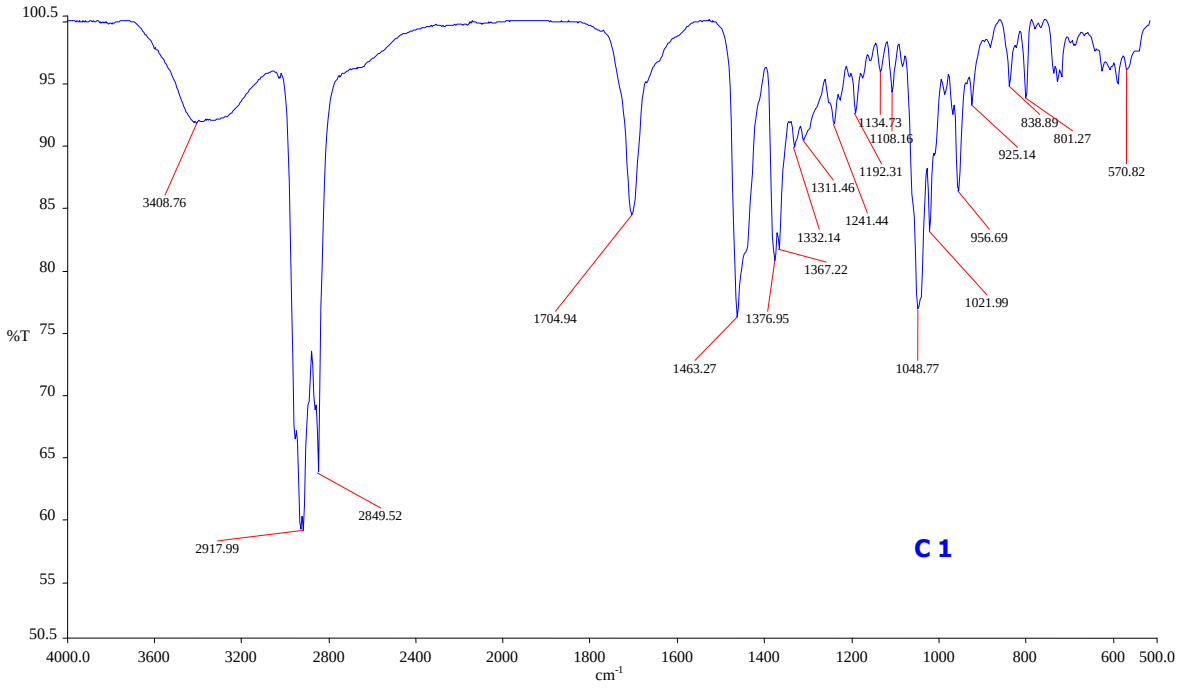


Figure 4.67: IR Spectrum of C1

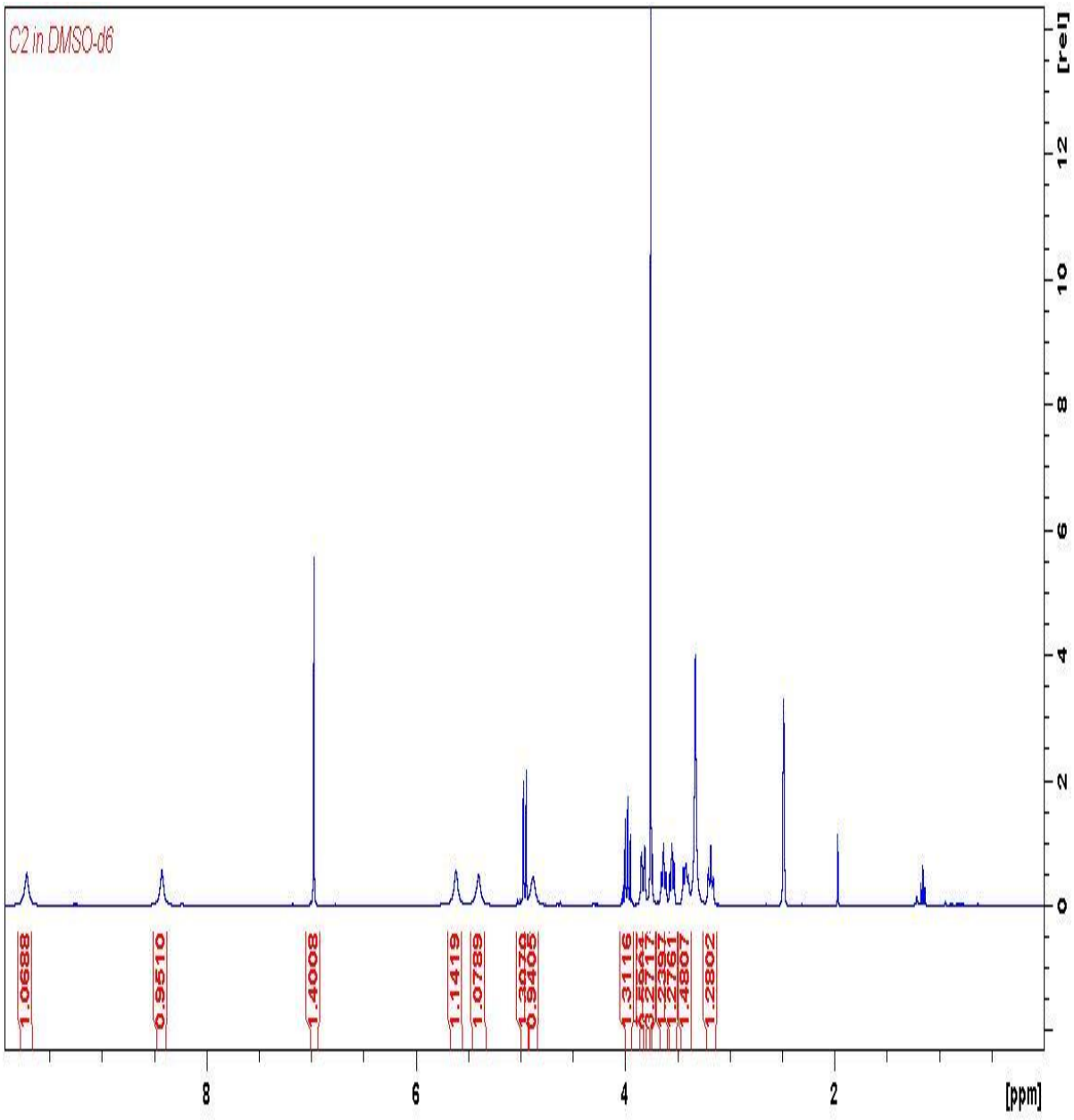


Figure 4.68: ^{1}H NMR Spectrum of C2

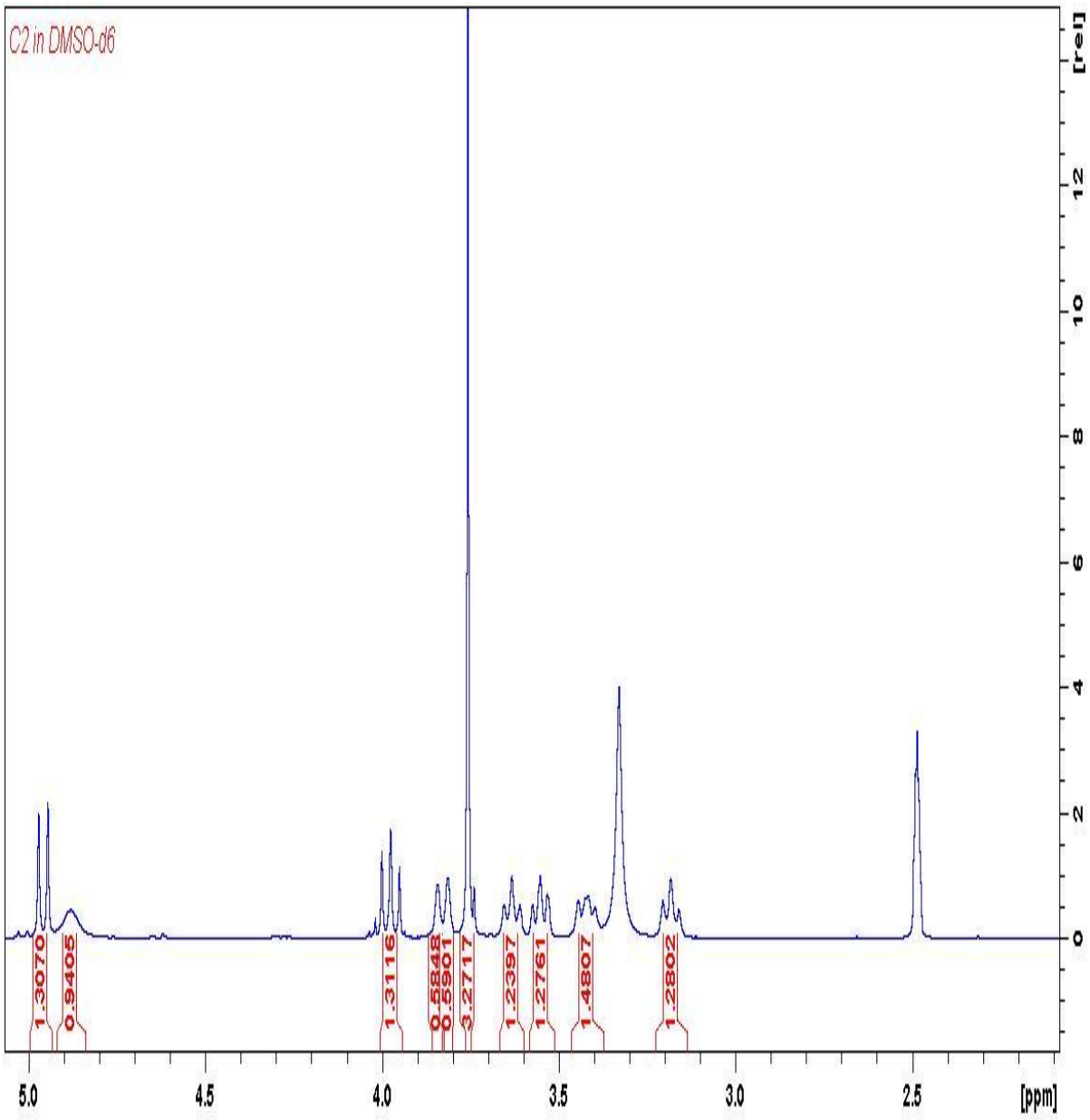


Figure 4.69: ^{1}H NMR Expanded Sugar Region of C2

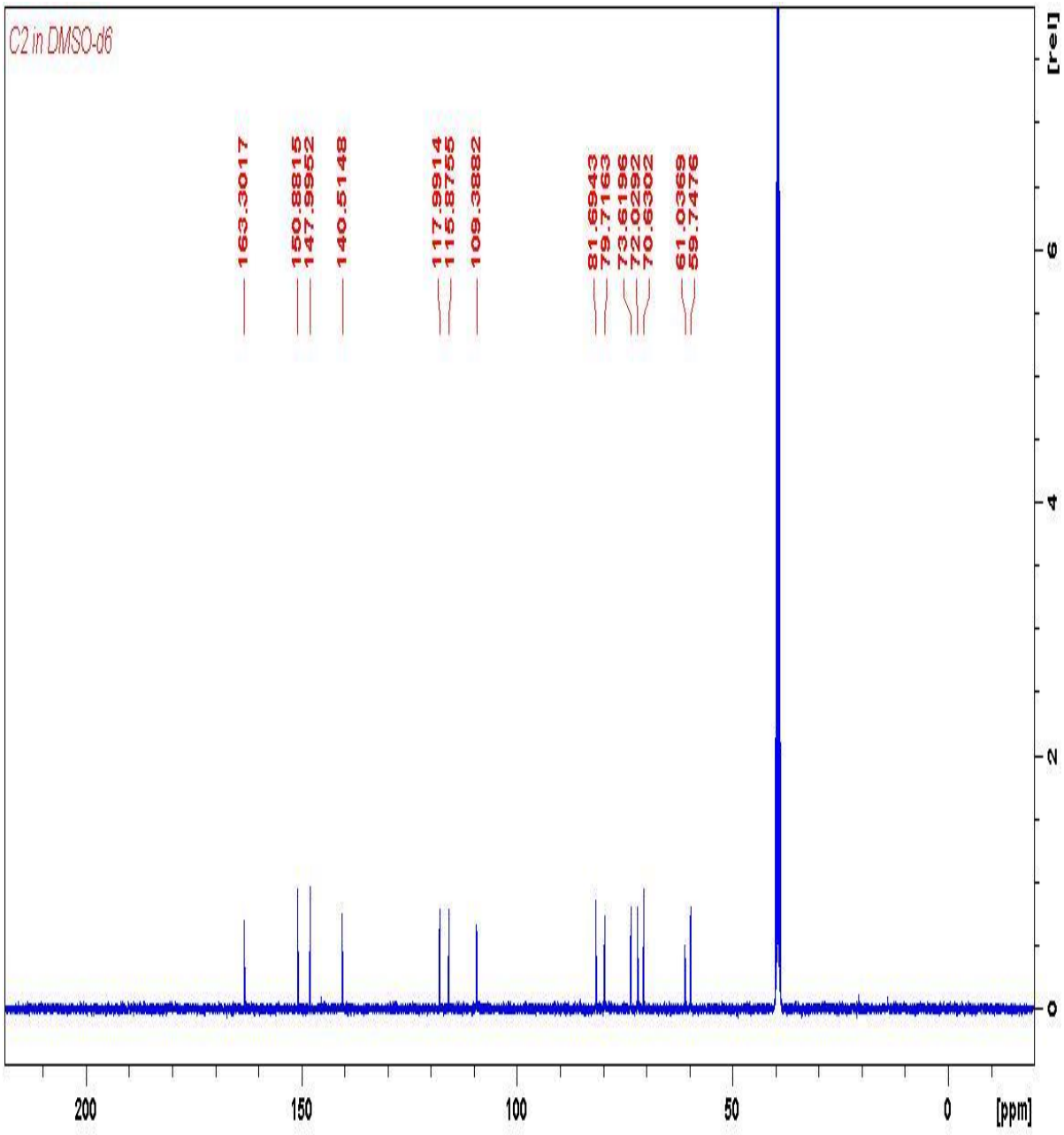


Figure 4.70: ^{13}C NMR Spectrum of C2

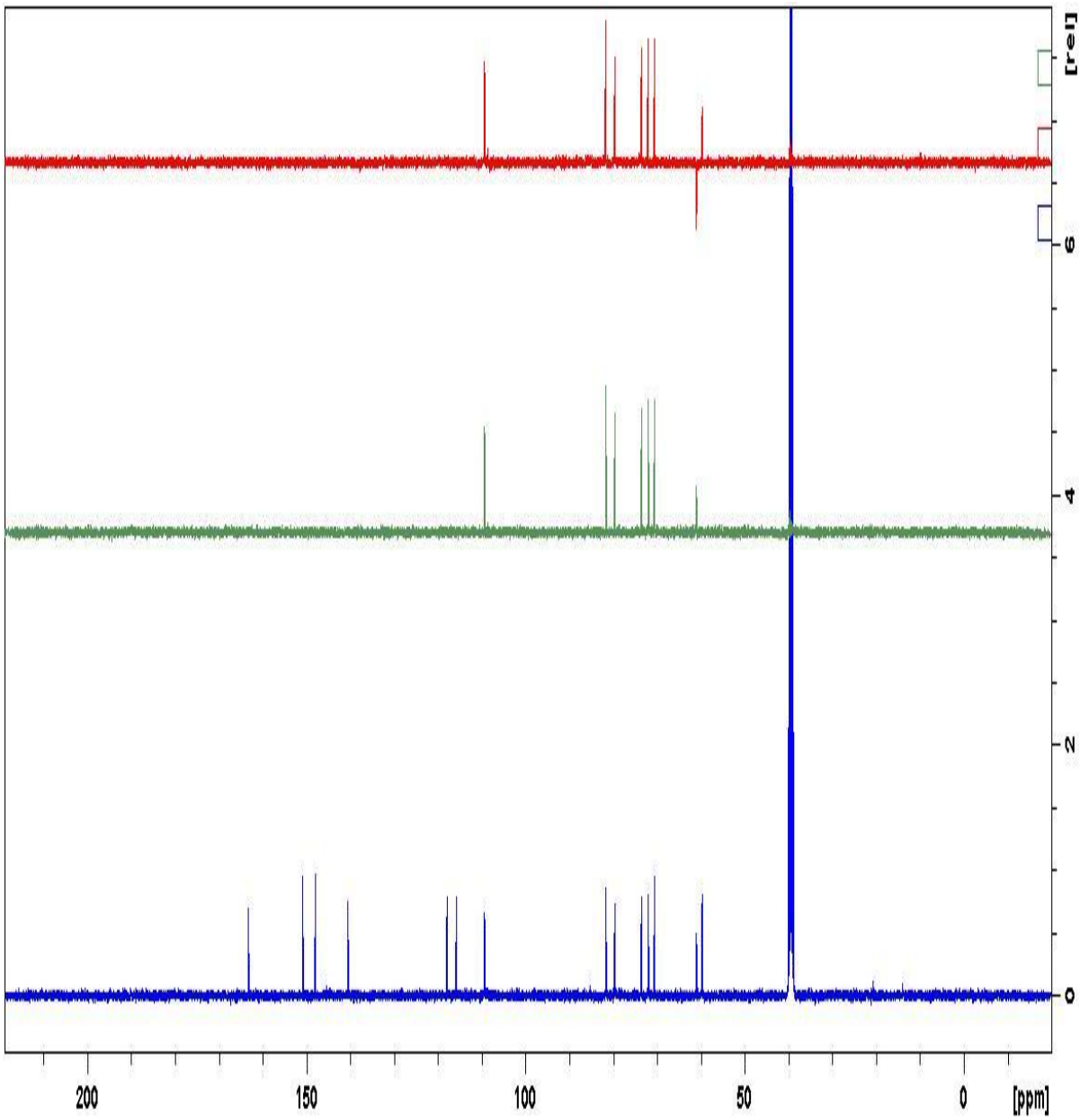


Figure 4.71: ^{13}C and DEPTS (90 and 135) NMR Spectra of C2

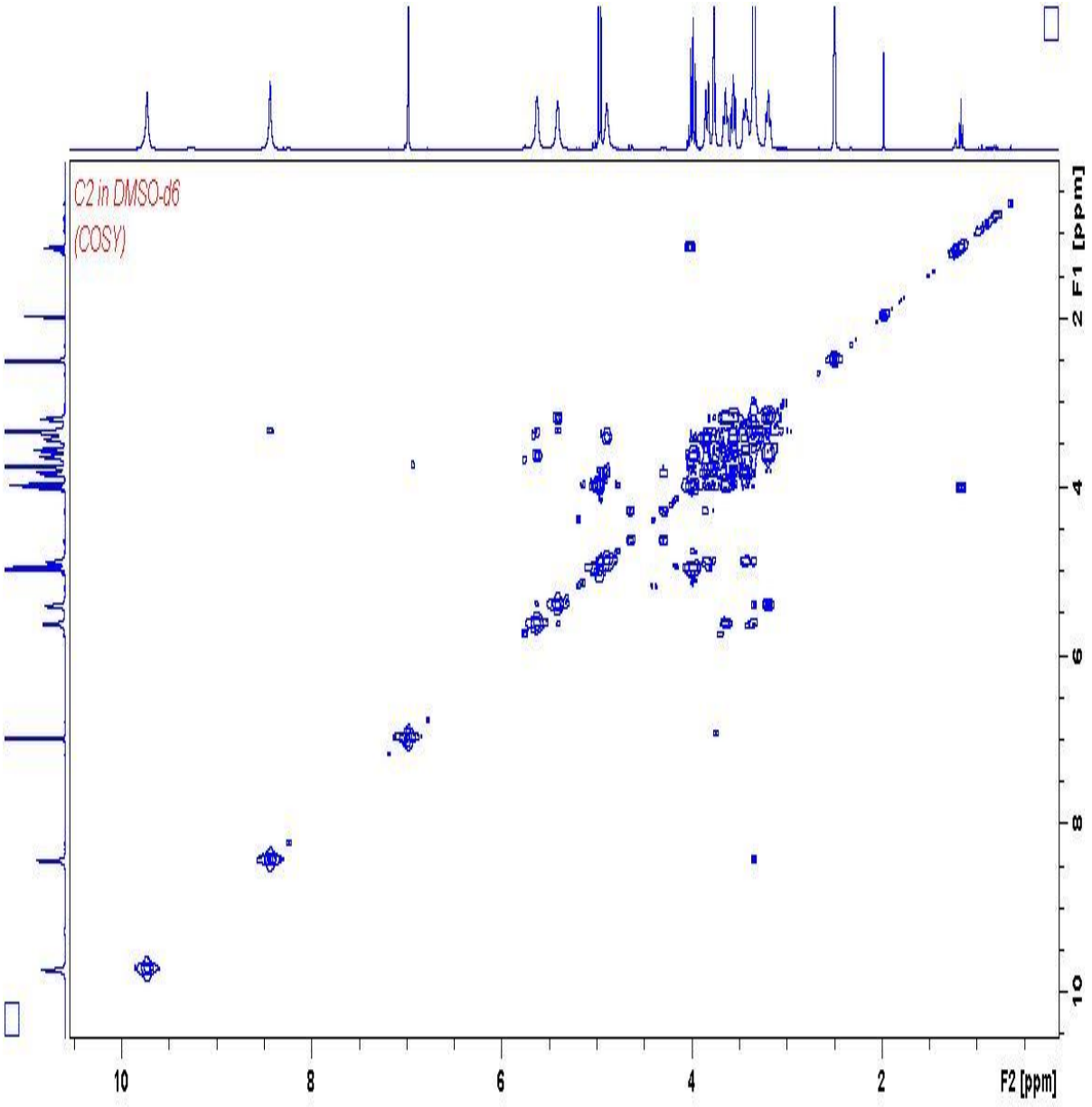


Figure 4.72: COSY NMR Spectrum of C2

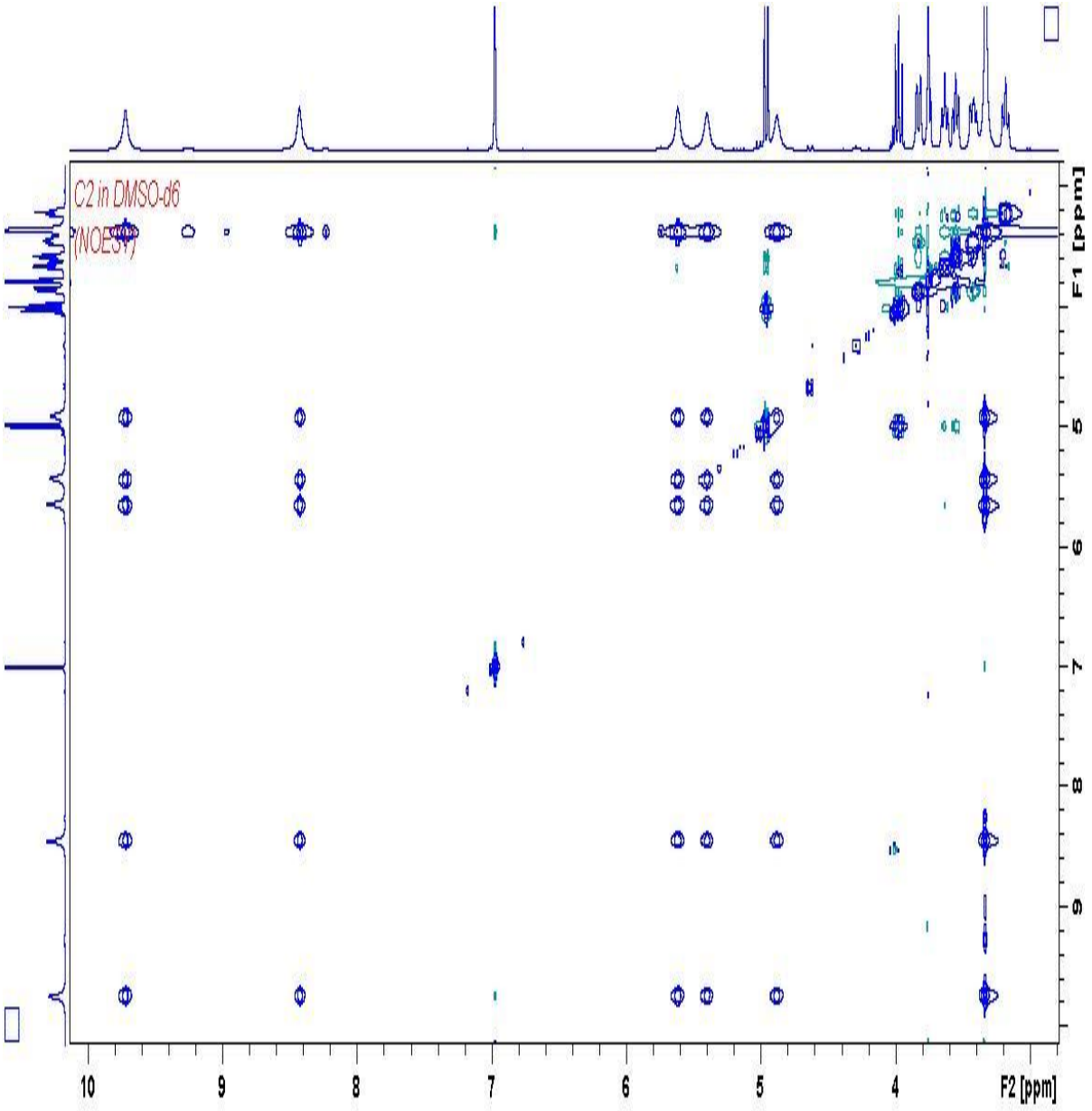


Figure 4.73: NOESY NMR Spectrum of C2

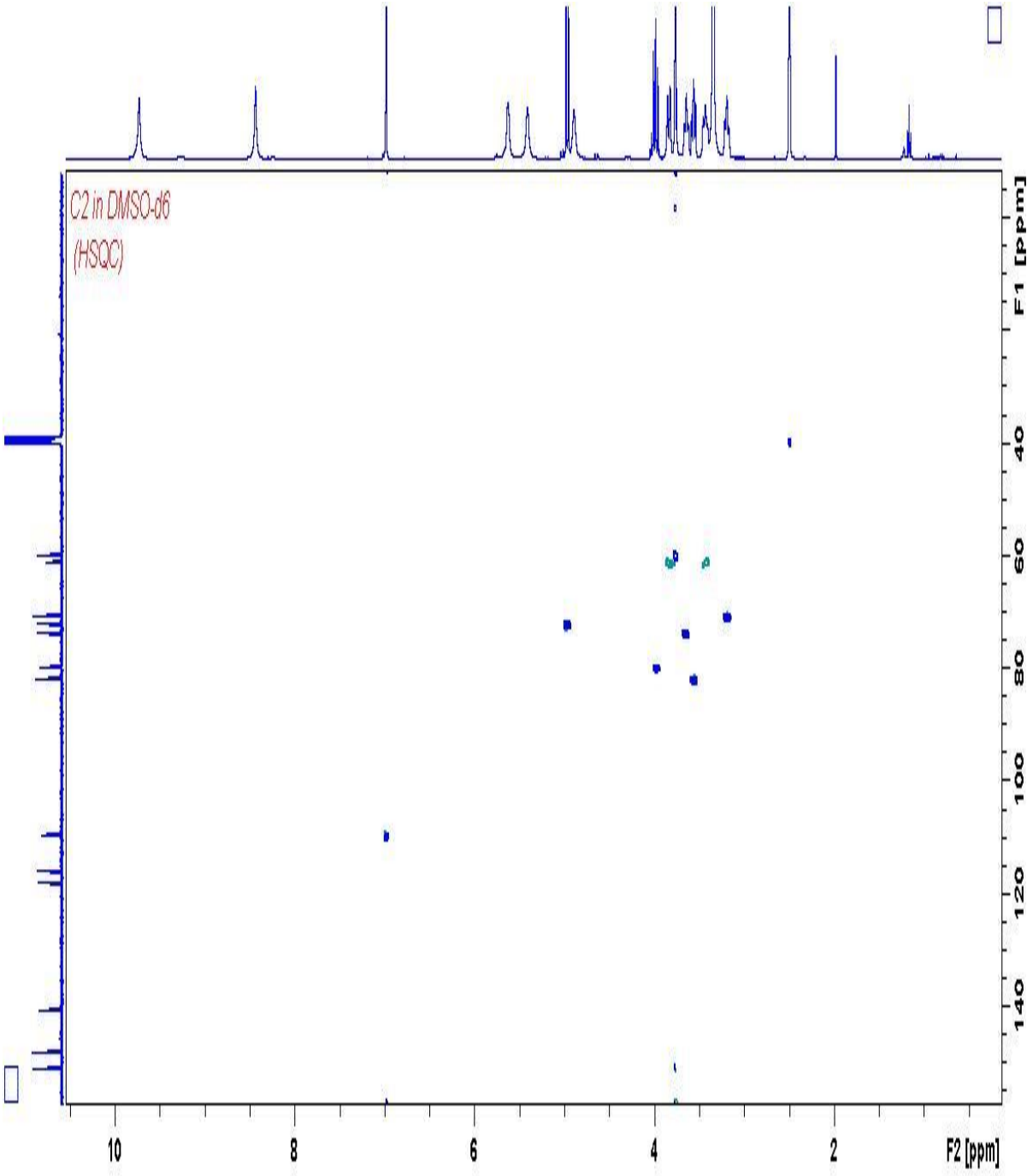


Figure 4.74: HSQC NMR Spectrum of C2

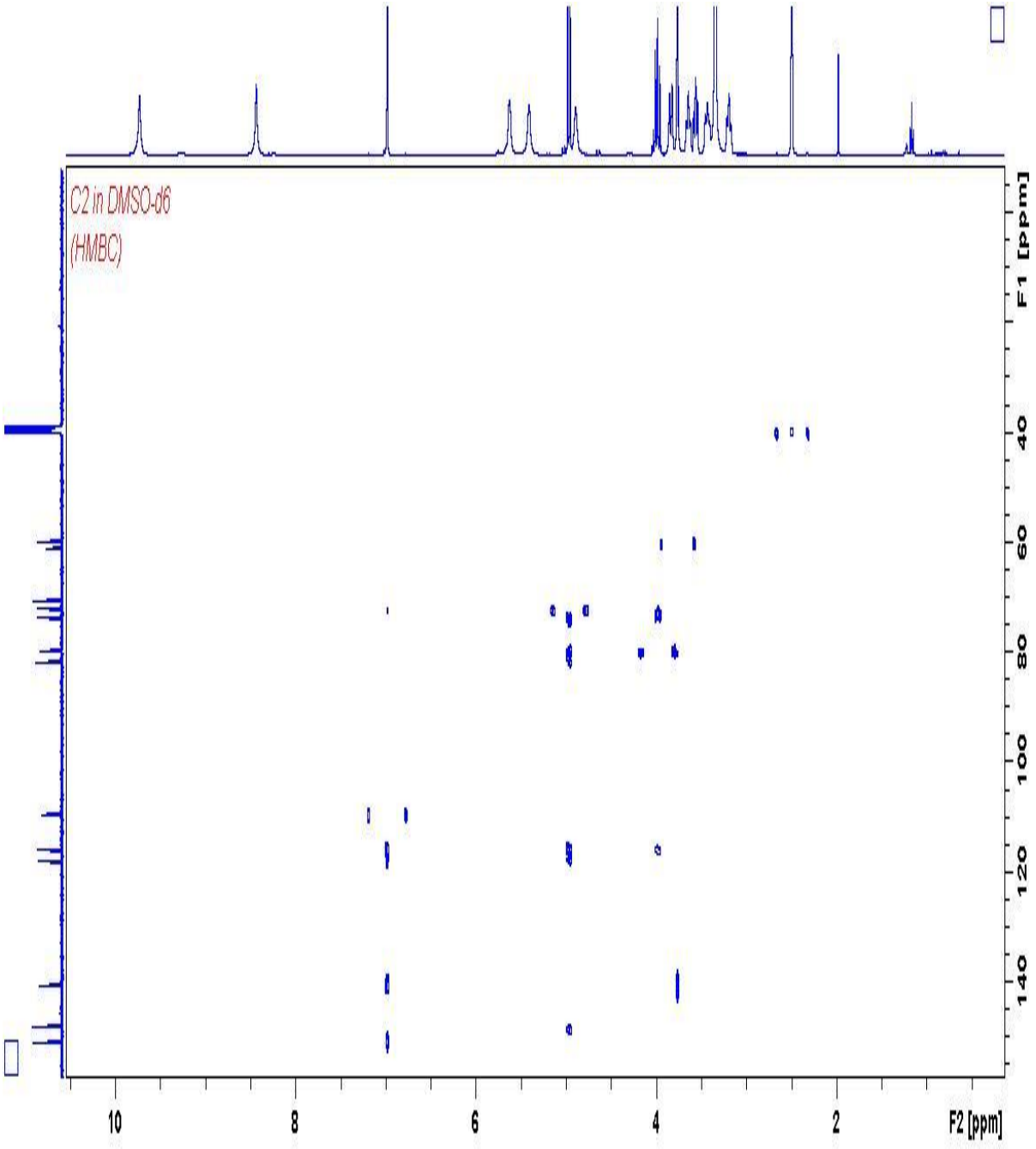


Figure 4.75: HMBC NMR Spectrum of C2

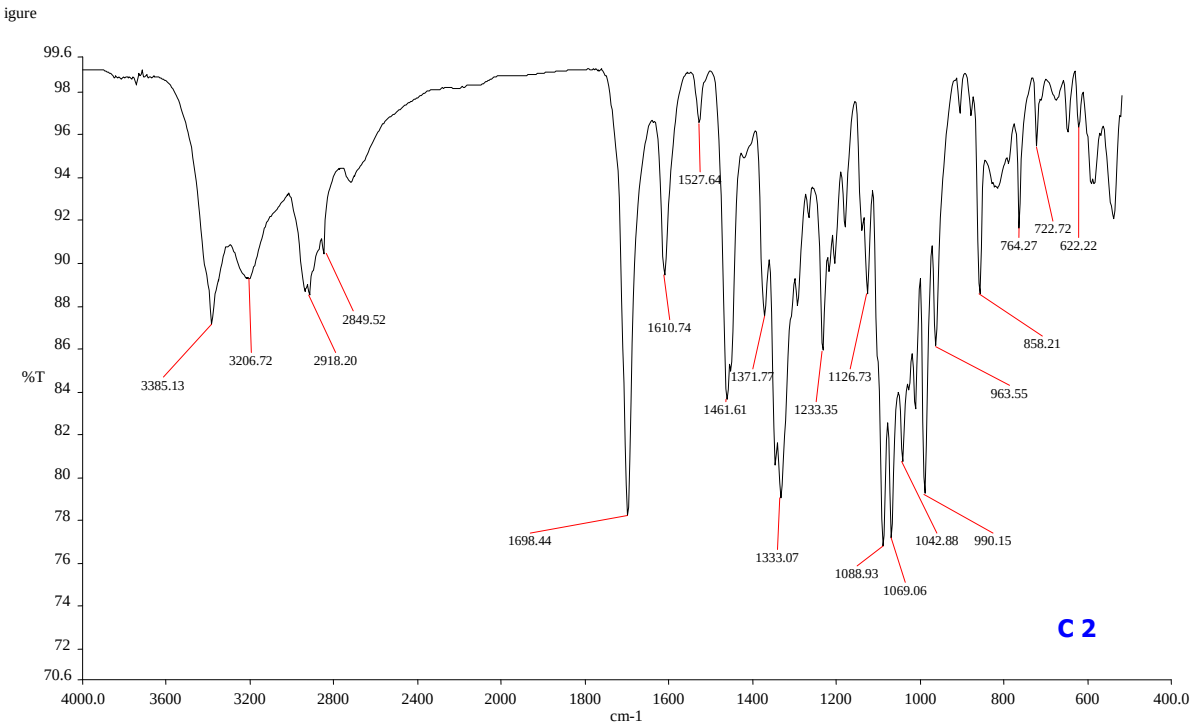


Figure 4.76 : IR Spectrum of C2

CHAPTER FIVE

5.0 DISCUSSION

5.1 Extraction of the Plant Rhizomes

The results of the extraction generally showed increase in percentage recovery with increase in polarity of the solvents used for the extraction. Generally, from hexane to methanol extracts, the recovery ranged from: 0.558-4.391 %, 0.208-10.670 % and 0.264-6.354% for *A. grantii, C.crinita and C.sokodensis* respectively.This implies that the quantity of polar phytochemicals extracted was more than those of non polar ones (Table 4.1) Thus, phytocompounds such as alkaloids, flavonoids, quinones, xanthones possessing polar groups; steroids and triterpenoids with polar moieties; polyhydroxylated phenolics glycoside such as saponins might be present in the plants.These polar groups, if present, have been reported to have anticancer, antioxidant, antiinflamatory and antimicrobial properties (Singh *et al.*, 2002; Stapleton *et al.*, 2004; 2007; Shah *et al*., 2008; Pratheeshkumar *et al*., 2012; Rajput and Mandal, 2012).

Therefore, this is an indication that these plants contain secondary metabolites that can serve as anticancer as well as antimicrobial agents; and this could be a reason the plants have been used as anticancer and antimicrobial drugs in ethnomedicine.

5.2 Phytochemical Screening of the Plants

The results of the phytochemical screening revealed the presence of steroids, triterpenoids, flavonoids, tannins and saponins from both *C. crinita and C. sokodensis* extracts (Tables 4.3 and 4.4). In addition to the stated phytocompounds, anthraquinones were found to be present in *A.grantii* extracts (Table 4.2).

The presence of steroids, triterpenoids, flavonoids, tannins, saponins and anthraquinones from the rhizomes of the plants could be the linked to the ethnomedicinal uses of the rhizomes of *A.grantii, C.crinita and C.sokodensis* in tradomedicinal practices.This is because these secondary metabolites have been known scienfically to act as antioxidants, antiinflamatory, anticancer and antimicrobial agents (Singh *et al.*, 2002; Stapleton *et al.*,

2004; 2007; Shah *et al*., 2008; Pratheeshkumar *et al*., 2012; Rajput and Mandal, 2012).Therefore, the presence of these phytochemicals in the plants studied could be a scientific evidence for the tradomedicinal uses of the plants.

5.3 Isolation and Characterization of Pure Compounds from *A.grantii* Rhizome

5.3.1 Isolation and characterization of G1

A white needle like crystals, coded G1 (300 mg, R_f = 0.25, solvent system: 15% ethyl acetate in 85 % hexane, mp: 137-139 ^oC) was isolated from the rhizome of *A.grantii*.

The IR spectrum of G1 showed key absorption bands of O-H stretch at 3409 cm^{-1}, C-H stretch at 2918 cm^{-1} (asymmetric) and 2850 cm^{-1} (symmetric) typical of sp^3 hybridized carbon. Absorption at 1705 cm^{-1} which may be due to C=C stretch was observed. The signal at 1463 cm^{-1} may be due to C-H bending vibration (Figure 4.8).

The ^1H NMR spectrum of G1 (Figure 4.1) showed upfield multiplets: methyl ($-CH_3$), methylene ($-CH_2-$) and methine (-CH) protons signals (m, CH, CH_2, CH_3) resonating at 0.68-2.32 ppm indicative of steroidal nucleus.The proton signal at 3.53 ppm may be due to oxymethine proton attached to C-3 position of sterols. In addition to these, a resonance that may be due to a vinyl proton (C=CH) was observed at 5.33 ppm. The ^{13}C NMR spectrum signal at 71.82 ppm typical of oxymethine carbon of steroids was observed. Signals at 140.76 ppm and 121.73 ppm suggestive of olefinic double bond that is characteristic of sterols were present.The DEPTS experiments showed eleven methylenes ($-CH_2$), nine methines (-CH), six methyls ($-CH_3$) and one quarternary carbon suggestive of β-sitosterol (**24**).^{13}C and ^1H NMR spectral data were supported by the 2D NMR experimental data. Cross peaks correlations of H-3 and that of H-4, H-6 and H-7 were established from both COSY and NOESY and COSY experiment alone respectively.The NOESY H-H correlations of H-6 and H-4, and no correlation existing between H-6 and any proton at C-5 confirms the assignment of the quarternary carbon to position-5 (C-5). Also the crosspeak correlation established between H-

4 and H-3 from the NOESY spectrum proved the existence of H-3 from CHOH.This again confirmed from the C-H correlation between C-3 and H-3, and that of also C-3 and H-4 as is observed in the HSQC and HMBC spectra respectively.C-6 correlation with H-6 confirmed the presence of vinylic proton at position-6 (C=C<u>H</u>) as observed from the HSQC spectrum (Figures 4.2 - 4.7).

On the whole, acquired 1D NMR data couple with comparison of ^{13}C and ^{1}H NMR data (Table 5.1) of G1 with data in literature (Suparb and Amorn, 1991; Zuhal *et al*., 2006; Anjoo and Ajay, 2011; Luhata and Munkombwe, 2015) and also the analyses of the 2D NMR spectra discussed confirmed that G1 is the known steroid commonly known as β-sitosterol (**24**).

(24)

Table 5.1: Comparison of the Observed ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Spectra of Compound G1 in $CDCl_3$ with the Reported Values (δ) of β-Sitosterol (^{1}H-NMR: 400 MHz & ^{13}C-NMR: 100 MHz) in $CDCl_3$

	Observed Data		Reported Data*	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)

1	37.3		37.4	
2	29.7		31.8	
3	71.8	3.54 (1H, *m*, H-3)	72.0	3.52 (1H, *m*, H-3)
4	42.3		42.5	
5	140.8		140.9	
6	121.7	5.33 (1H, *t*, H-6)	121.9	5.35 (1H, *m*, H-6)
7	31.7		32.1	
8	31.9		29.9	
9	51.2		50.3	
10	36.5		36.7	
11	21.1		21.3	
12	39.8		40.0	
13	45.8		42.5	
14	56.8		56.9	
15	24.3		24.5	
16	28.3		28.4	
17	56.1		56.2	
18	11.9	0.66 (3H, *s*, Me-18)	12.0	0.68 (3H, *s*, Me-18)
19	19.4	0.98 (3H, *s*, Me-19)	19.6	0.98 (3H, *s*, Me-19)
20	36.2	-	36.3	-
21	19.0	0.90 (3H,s, Me-21)	19.0	0.91 (3H,s,Me-21)
22	33.9		34.1	
23	29.2		26.3	
24	50.1		46.0	
25	26.1		29.3	
26	18.8	0.84 (3H, s, Me-26)	20.0	0.83 (3H,s,Me-26)
27	19.8	0.82 (3H, s, Me-27)	19.2	0.81 (3H, *s*, Me-27)
28	23.1		23.2	
29	11.9	0.89 (3H,s, Me-29)	12.2	0.85 (3H,s, Me-29)

Zuhal *et al*. (2006) *****.

5.3.2 Isolation and characterization of G2

G2 was isolated as an amorphous creamy powder (100 mg, R_f = 0.13, solvent system: 15 % ethyl acetate in 85 % hexane, mp: 256-257 ^oC) from the rhizome of *A.grantii*.

IR spectrum of G2 (Figure 4.16) indicated the following key absorption bands: 3460 cm^{-1} (O-H stretch, H-bonded), 2916 cm^{-1} (C-H aliphatic stretch), 1709 cm^{-1} (C=C stretch, aliphatic), 1159 cm^{-1} and 1024 cm^{-1} (C-O stretch, alkanol).These absorption bands

summarily suggest a pentacyclic triterpenoidal nucleus with sp3 hybridised methylene groups ($-CH_2$) within it as well as OH group(s) and double bond(s)(C=C) in it.

The ^{1}H NMR spectrum (Figure 4.9) showed a doublet doublet at δ 3.18 ppm, which is characteristic of α-oriented hydrogen assigned to position-3 (C-3) of a 3β-hydroxy triterpene. The doublets for geminal protons at δ 4.67 and 4.56 ppm, along with the methyl group at δ 1.67 ppm, suggest a lupane-type triterpene derivative. Another pair of doublets at δ 3.79 and 3.33 ppm, rather than a seventh methyl singlet at δ 0.8 ppm, suggested the presence of a second hydroxyl group at position-28 (C-28) characteristic of lupenol. The ^{13}C NMR spectrum (Figure 4.10) further indicated a lupane-type triterpene derivative. The characteristic pair of sp2 hybridised carbon atom resonances comprising the exocyclic double bond typical of lupane was observed at δ 150.5 and 109.7 ppm (Tijjani *et al.,* 2012). Oxymethylene resonance suggestive of position-3 (C-3) and position-28 (C-28) of lupene, 3, 28-diol were observed at δ 79.0 and 60.6ppm, respectively.The 2D NMR confirmed the presence of a double bond at C-20/C-29 typical of lupenol.This is clearly indicated in both the HMBC and HSQC spectra due to cross peaks long range correlations between C-20 and H-30 and also that between C-29 and H-30; short range correlation between C-29 and H-29.The oxymethylene resonances for C-28 and C-3 were comfirmed due to the correlations between H-28 and H-22; also between H-28 and H-16 (NOESY spectrum). Furthermore, H-3 correlated with H-2 (COSY). Both 1D and 2D NMR spectra are shown in figure 4.12 to 4-15

In all, the spectra revealed a compound with six methyl groups,twelve methylene groups, six methines , from the DEPTS experiments, (Figure 4.11) and thirty carbon atoms (which is equivalent to the total number of carbon atoms in triterpenoid) suggest a lupene-type triterpenoidal nucleus with two hydroxyl groups at C-3 and C-28 (a lupenediol). Consequently, the compound was determined to be the known structure, 20(29)-lupene-3, 28-diol, more commonly known as betulin (**25**). Experimental NMR data was in agreement with

those reported in literature (Table 5.2) and all ^{13}C shifts were within ± 0.3 ppm (Siddiqui *et al.*, 1988; Mahato and Kundu, 1994; Tijjani *et al.*, 2012).

(25)

Table 5.2: Comparison of the Observed ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Spectra of Compound G2 in $CDCl_3$ with the Reported Values (δ) of Betulin (^{1}H-NMR: 500 MHz & ^{13}C-NMR : 125 MHz) in $CDCl_3$

	Observed Data		**Reported Data***	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)
1	38.9		38.9	
2	27.4		27.5	

3	79.0	3.18 (1H, dd, *J* = 5.3, H-3*α*)	79.2	3.18 (1H, dd, *J* = 5.3, H-3*α*)
4	38.7		38.8	
5	55.3		55.4	
6	18.3		18.4	
7	34.2		34.3	
8	40.9		41.0	
9	50.4		50.5	
10	37.3		37.4	
11	20.8		20.9	
12	25.2		25.3	
13	37.2		37.2	
14	42.7		42.8	
15	27.0		27.1	
16	29.2		29.2	
17	47.8		47.9	
18	47.8		47.9	
19	48.8		48.8	
20	150.5		150.6	
21	29.7		29.8	
22	33.9		34.1	
23	27.9	0.95 (3H, s, Me-23)	28.1	0.96 (3H, s, Me-23)
24	15.4	0.73 (3H, s, Me-24)	15.4	0.75 (3H, s, Me-24)
25	16.1	0.79 (3H, s, Me-25)	16.2	0.80 (3H, s, Me-25)
26	16.0	0.96 (3H, s, Me-26)	16.1	0.97 (3H, s, Me-26)
27	14.8	0.98 (3H, s, Me-27)	14.8	0.99 (3H, s, Me-27)
28	60.6	3.33(1H, d, *J* = 10.8, H-28a)	60.6	3.33(1H, d, *J* = 10.8, H-28a)
		3.79 (1H, d, *J* =10.8, H-28b)		3.79 (1H, d, *J* =10.8, H-28b)
29	109.7	4.56 (1H,d, H-29a)	109.8	4.58 (1H,d, H-29a)
		4.67 (1H, d, H-29b)		4.70 (1H, d, H-29b)
30	19.1	1.67 (3H, s, Me-30)	19.2	1.67 (3H, s, Me-30)

Tijjani *et al.* (2012) *****.

5.3.3 Isolation and characterization of G3

The compound (G3) was isolated as an amorphous white powder, coded G3 (18 mg, R_f = 0.45, solvent system: 20 % ethyl acetate in 80 % hexane, mp: 188-189 ^oC) from the rhizome of *A.grantii*.

The IR spectrum of G3 indicated the presence of hydroxyl group (3324 cm^{-1}), ester carbonyl (1736 cm^{-1}), methoxy (1261 cm^{-1}) and aromatic ring (1473, 1462 cm^{-1}) . In addition, sp^3 C-H stretches at 2917and 2849 cm^{-1}, typical of CH_2 was also observed (Figure 4.25).

The presence of aromatic protons with a meta-coupled aromatic resonance [δ 7.01 (1H, d, *J* = 1.8 Hz, H-5)], ortho-coupled aromatic resonance [δ6.88 (1H, d, *J* = 8.1 Hz, H-8)] and ortho–meta-coupled aromatic resonance [δ 7.04 (1H, dd, *J* = 1.8, 8.2 Hz, H-9)] was an indication of trisubstitution in benzene ring (with 3, 4-di substitution in phenyl unit) (Sang *et al*., 2013). The broad singlet at 5.83 ppm is indicative of aromatic OH group due to substitution of one of the protons attached to the benzene ring (Sang *et al*., 2013), hence, this established the existence of one substituent group (out of the three substituents) on the benzene ring. Two olefinic protons [δ_H 6.25 and δ_H 7.56 (*J* = 15.9Hz)] of a trans-configured double bond that are in conjugation with benzene ring suggestive of feruloyl moiety are indicative. Other resonances include those of protons attached to oxygenated carbon atoms: δ 4.16 (2H, CH_2O), methyl from methoxy, δ 3.92 (3H, OMe) and hydroxy methyl protons δ 3.61 (3H, CH_2OH and OH) (Figure: 4.17).

The ^{13}C NMR spectrum (Figure 4.19) revealed six aromatic carbon resonances (127.0, 109.3, 146.7, 147.9, 114.7 and 123.0 ppm) that suggested the presence of benzene nucleus. The resonances at 146.7 and 147.9 ppm suggest oxygenation on substitution in the aromatic ring. These signals together with 115.7 and 144.6 ppm (double bond resonances); and also 167.4 ppm (ester carbonyl carbon resonance) are typical of feruloyl moiety (Sang *et al*., 2013). Resonances suggestive of methyl (in methoxy group) and two oxymethylenes appeared at 55.9 and 63.1 with 64.6 ppm respectively. Additionally signals that suggest the presence of aliphatic methyl, methylenes and methines were observed at 34.1-14.1 ppm.

The HMBC correlations of C-7/H-5, C-7/H-8, C-9/ H-5 and C-4/H-8 established the C-5-C-7-C-8, C-4-C-9-C-8 and C-9-C-4-C5 connections of the phenyl ring; and this is a confirmation of the presence of a phenyl moiety in the structure. C-4/H-2 HMBC correlation showed linkage of the double bond to the benzene via C-4. C-2 HMBC correlation with H-3 confirmed the existence of the double bond between C-2 and C-3 and the two bond HMBC

correlation of C-1 to H-1′ suggested C-1-O-C-1′ connections. Thus, the HMBC correlations just mentioned confirmed the connectivity of the acrylate moiety on the benzene and hence the existence of the ferurate moiety was established in the molecule. HMBC cross peak correlation of C-6 with Me group (from OMe) suggested a substitution with OMe at the sixth position on the phenyl. This is further indicated by the NOESY in-space proton-proton (^{1}H-^{1}H) spin correlation of H-5 with Me group of the methoxy (OMe) at C-6.Thus, confirming the neighborhood of C-5 to C-6 and ruling out the possibility of OMe assigned to C-7. Other key NOESY ^{1}H-^{1}H correlations include; hydroxyl methyl protons (CH_2OH), at C-15, to H-17′ (confirming the presence of the Me group at the end of the molecule), H-1′/H-2′ and H-1′/ H-3′ correlations (indicative of in-space methylene proton-proton correlations along the aliphatic chain). Correlations of C-5 to H-5, C-8 to H-8 and that of C-9 to H-9 from the HSQC spectroscopic data also confirmed that the phenyl ring is trisubstituted as mentioned earlier. There was also C-2/H-2 and C-3/H-3 HSQC correlations indicative of the C=C outside the aromatic ring which was again established to confer trans-configuration to the molecular system from the observed H-9/H-2 NOESY experimental data. The two protons attached to C-2 and C-3 (C=C) were further confirmed from the through-bond correlation suggested by the COSY experimental data. H-1′ /H-2 established ^{1}H-^{1}H spin COSY correlation of the protons attached to the oxygenated carbon along the aliphatic chain. Through bond ^{1}H-^{1}H COSY correlations of H-2′ with H-3′ showed a continued linkage of -CH_2 groups along an aliphatic chain.The COSY cross-peaks correlation of H-8 to H-9 confirmed the through bond aromatic proton-proton correlation of the two protons at ortho-position on aromatic ring. Absence of either NOESY or HMBC (long range) correlations of H-1′, C-1′ or C-1 with the hydroxy methyl group and or the terminal methyl group, suggested, along the aliphatic carbon skeleton gave the evidence that these groups could be assigned to terminal positions of the molecule (Figures 4.21-4.24).

On the whole, combination of the spectroscopic data (most especially the 1D and 2D NMR experimental data) to those of similar compounds available in literature as was reported by Sang *et al*., (2013), Table 5.3, assisted the determination of G3 to be 15-hydroxymethyl-16 methylheptadecylferulate (**26**). This report is the first of its kind, thus, this molecule had not been reported from nature prior to this report.

(**26**)

Table 5.3: Comparison of the Observed ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Spectra of Compound G3 in $CDCl_3$ with the Reported Values (δ) of Feruloyl (^{1}H-NMR : 600 MHz & ^{13}C-NMR : 150 MHz) in CD_3OD

	Observed Data		**Reported Data***	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)
Feruloyl moiety				Feruloyl moiety
1	167.4		167.7	
2	115.7	6.25 (1H ,d, *J*=15.9, H-2)	113.5	6.28(1H, d, *J*=15.6,H-2)
3	144.6	7.56 (1H, d, *J* = 15.9, H-3)	147.0	7.63 (1H, d, *J*=15.6,H-3)
4	127.0		126.4	
5	109.3	7.01 (1H, d, *J* = 1.8, H-5)	113.9	7.05 (1H, d, *J*= 1.8, H-5)
6	146.7		145.4	
7	147.9		148.4	
8	114.7	6.89 (1H, d, *J* = 8.1, H-8)	115.1	6.75(1H, d, *J*= 7.8, H-8)
9	123.0	7.04(1H, dd, *J* = 1.8, 8.2, H-9)	121.9	6.91(1H,dd, *J*= 7.8, 1.8, H-9)
		5.83 (1H,br s,OH)		
		4.16 (2H, t, H-1′)		
		3.92 (3H,s, OMe)		
		3.61 (3H, t, C$\underline{H_2}$O$\underline{H}$)		
		1.64 (2H,m, H-19′)		

	0.87-1.52 (m,CH,CH_2,CH_3)
Alkyl moiety of the ester	
1′	64.6
2′	22.6
3′	22.3
4′	25.7
5′	28.8
6′	29.3
7′	29.4
8′	29.4
9′	29.6
10′	29.6
11′	29.7
12′	29.7
13′	31.9
14′	32.8
15′	34.1
16′	26.0
17′ (Me)	14.1
OMe	55.9
CH_2OH	63.1
Me	14.1

Sang *et al*., (2013) *****.

5.3.4 Isolation and characterization of G4

G4 was isolated as an amorphous white powder (10 mg, R_f = 0.34, solvent system: 20 % ethylacetate in 80 % hexane, mp: 198-200 ^oC) from the rhizome of *A. grantii*.

IR of G4 showed diagnostic absorption bands at 3370, 1725, 1630, 1158 and 1492 cm^{-1} indicating the presence of hydroxyl, ester carbonyl, methoxy and aromatic functional groups. Absorptions at 2917 and 2848 cm^{-1} suggestive of -CH_2 sp^3 C-H stretches were also present (Figure 4.34).

The presence of an aromatic ABX-spin system with a meta-coupled aromatic signal [δ 7.02 (1H, d, *J* = 1.2 Hz, H-5)], ortho-coupled aromatic resonance [δ 6.89 (1H, d, *J* = 8.2 Hz, H-8)] and ortho–meta-coupled aromatic resonance [δ 7.05 (1H, dd, *J* = 1.7, 8.4 Hz, H-9)] was an indication of trisubstituted benzene (with 3, 4-di substitution in phenyl unit) (Sang *et al*., 2013). The broad singlet at 5.81 ppm is indicative of aromatic OH group due to substitution of one of the protons attached to the benzene ring (Sang *et al*., 2013), hence, this established

one of the substituent group (out of the three substituents) on the benzene ring. Two olefinic protons [δ_H 6.19 and δ_H 7.58 (J = 15.9Hz)] of a trans-configured double bond that are in conjugation with benzene ring suggestive of feruloyl moiety are indicative. Other resonances include those of protons attached to oxygenated carbons: ketone methyl δ 2.13 (3H, COMe), hydroxy methyl protons δ 4.14 (2H, CH_2OH) and methyl from methoxy, δ 3.89 (3H, OMe) (Figure 4.26).

The ^{13}C NMR spectrum revealed six aromatic carbon resonances (127.0, 109.3, 146.7, 147.9, 114.7 and 123.0 ppm) that suggested the presence of benzene nucleus. The resonances at 146.7 and 147.9 ppm suggest oxygenation on substitution in the aromatic ring. These signals together with 115.7 and 144.6 ppm (double bond resonances); and also 167.4 ppm (ester carbonyl carbon resonance) are typical of feruloyl moiety (Sang *et al*., 2013). Resonances suggestive of methyl (in methoxy group) and two oxymethylenes appeared at 55.9 and 63.1 with 64.6 ppm respectively. Additionally, signals that suggest the presence of aliphatic methyl, methylenes and methines were observed at 34.1-14.1 ppm (Figure 4.28).

The HMBC correlations of C-6/H-8, C-7/H-9, C-5/ H-9 and C-4/H-8 established the C-6-C-7-C-8, C-4-C-9-C-8 and C-5-C-4-C9 connections on the phenyl ring; and this is a confirmation of the presence of phenyl group in the structure. HMBC cross peak correlation of C-6 with Me (from OMe group) suggested a substitution with OMe at the sixth position on the phenyl ring. This is further indicated by the NOESY in-space proton-proton (^1H-^1H) spin correlation of H-5 with Me of the methoxy (OMe) at C-6.Thus, confirming the neighborhood of C-5 to C-6 and ruling out the possibility of OMe assigned to C-7. Other key HMBC correlations include: C-4/H-2 (establishing the third substitution at position four on the phenyl ring,),C-1/H-3 (indicative of neighborhood of the ester carbonyl to the olefinic proton), C-4′/H-1′, C-2′/H-1′ (attributable to C-H long range correlations of aliphatic chain) and C-14′/Me (confirming the presence of the Me group attached to the ketone carbonyl

carbon). Correlations of C-5 to H-5, C-8 to H-8 and that of C-9 to H-9 of the HSQC spectroscopic data further confirmed that the phenyl ring is trisubstituted. There was also C-2/H-2 and C-3/H-3 HSQC correlations indicative of the C=C outside the aromatic ring which was again established to confer trans-configuration to the molecular system from the observed H-9 to H-2 NOESY experimental data. The two protons attached to C-2 and C-3 (C=C) were further confirmed from the through-bond correlation suggested by the COSY experimental data. H-2′/H-1′ and H-2′ /H-1″ established the aliphatic chain containing oxygenated carbon ^{1}H-^{1}H spin COSY correlations.

Assignment of the hydroxyl methyl group to C-3′ was achieved due to COSY correlation of H-1′ to H-2′ and H-1″ to H-3′ to H-2′. Consequently, the methyl group was assigned to C-5′ not C-3′ on the aliphatic chain. These assignments were further supported and confirmed by the HMBC correlations of C-2′ to H-1′ and C-2′ to H-1″. Therefore, this clearly indicated that the Me group at C-5′ is not a terminal methyl but rather on the aliphatic chain of the molecule. Hence, this ruled out the assignment of the carbonyl ketone group to any position except the terminal end of the molecule. This is clearly indicated from the unavailable supportive data from the long range 2D experiments (NOESY and HMBC) (Figures 4.30 to 4.33).

In all, from the spectroscopic data acquired and also comparison of data of similar molecular structures in literature (Table 5.4) enabled the the determination of G4 to be new feruloyl named as 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate (**27**).To the best of our knowledge this is the first time of reporting this molecule from natural source. Therefore, it is noteworthy to state here that this molecule is a novel one, since it had not been reported before now, from either animal source or any plant researched.

(27)

Table 5.4: Comparison of the Observed ^{1}H-NMR (600 MHz) and ^{13}C-NMR (125 MHz) Spectra of Compound G4 in $CDCl_3$ with the Reported Values (δ) of Feruloyl (^{1}H-NMR : 600 MHz & ^{13}C-NMR : 150 MHz) in CD_3OD

	Observed Data		**Reported Data***	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)
Feruloyl moiety				Feruloyl moiety
1	167.4		167.7	
2	115.8	6.25 (1H,d,*J*=15.9, H-2)	113.5	6.28(1H, d, *J*=15.6,H-2)
3	144.6	7.58 (1H, d, *J* = 15.9, H-3)	147.0	7.63 (1H, d, *J*=15.6,H-3)
4	127.1		126.4	
5	109.4	7.02 (1H, d, *J* = 1.6, H-5)	113.9	7.05 (1H, d, *J*= 1.8, H-5)
6	146.8		145.4	
7	147.9		148.4	
8	114.7	6.88 (1H, d, *J* = 8.1, H-8)	115.1	6.75(1H, d, *J*= 7.8, H-8)
9	123.0	7.04(1H, dd, *J* = 1.7, 8.4, H-9)	121.9	6.91(1H,dd, *J*= 7.8, 1.8, H-9)
		5.81 (1H,br s,OH)		
		4.16 (2H, t, H-1′)		
		3.92 (3H,s, OMe)		
		3.61 (3H, t, $C\underline{H_2}O\underline{H}$)		
		1.64 (2H,m, H-19′)		
		0.83-1.64 (m,CH,CH_2,CH_3)		
Alkyl moiety of the ester				
1′	64.6			
2′	25.7			
3′	31.9			
4′	32.8			
5′	29.6			
6′	30.9			
7′	24.7			
8′	29.3			
9′	29.3			
10′	29.4			
11′	29.5			
12′	29.6			
13′	26.0			
14′	22.7			

15′	33.5
16′	206.8
17′ (Me)	29.6
OMe	55.9
CH_2OH	63.1
Me	14.1

Sang *et al.* (2013) *****.

5.4 Isolation and Characterization of Pure Compounds from *C.sokodensis* Rhizome

5.4.1 Isolation and characterization of S1

S1 was isolated as white needle like crystals (100 mg, R_f = 0.33, solvent system: 13 % ethylacetate in 87 % hexane, mp: 137-139 ^oC) from the C*issus sokodensis* rhizome.

The IR spectrum of S1 showed key absorption bands of O-H stretch at 3409 cm^{-1}, 2918 cm^{-1} (asymmetric) and 2850 cm^{-1} (symmetric) C-H stretches typical of sp^3 hybridized methylene (-CH_2). Absorption at 1705 cm^{-1} due to C=C stretch suggestive of sterol double bond; and an absorption at 1463 cm^{-1} due to C-H bending were also observed (Figure 4.42).

^1H NMR spectrum of S1 (Figure 4.35) showed upfield multiplets: methyl groups, methylene and methine protons resonances (m, CH, CH_2, CH_3) observed at 0.68-2.32 ppm indicative of steroidal nucleus.The signal at 3.53 ppm may be due to oxymethine proton attached to C-3 position of sterols. In addition to these, resonance that is suggestive of vinylic proton (C=C<u>H</u>) appeared at 5.33ppm. ^1H NMR spectrum was in agreement with the ^{13}C NMR spectrum (Figure 4.36), which suggested an oxymethine signal at 71.82 ppm. Furthermore, one double bond, C=C, signals at 140.76 ppm and 121.73 ppm at C-5/C-6 that is characteristic of sterols were observed.The DEPTS experiments showed eleven methylene groups (-CH_2), nine methine groups (-CH), six methyls (-CH_3) and one quarternary carbon equivalent to that of β-sitosterol. ^{13}C NMR and ^1H NMR spectra data are supported by the 2D NMR experimental data (Figure 4.38-4.41). The cross peaks correlations of H-3 and that of H-4, H-6 and H-7 were established from both COSY and NOESY and COSY experiments respectively.The NOESY H-H correlations of H-6 and H-4, and absence of any correlation between H-6 and

proton at C-5 thus suggests the presence of a quarternary carbon at position-5 (C-5). Also the crosspeak correlation established between H-4 and H-3 from the NOESY spectrum evident the existence of H-3 from CHOH.This again was comfirmed from the C-H correlation between C-3 and H-3, and that of C-3 and H-4 as was observed in the HSQC and HMBC experimental data respectively. Correlation of C-6 with H-6 comfirmed the existence of the vinylic proton at C-6 (C=C<u>H</u>) as observed from the HSQC spectrum.

On the whole, comparison of ^{13}C and ^{1}H data (Table 5.1) of G1 with those available in the literature (Suparb and Amorn, 1991; Zuhal *et al*., 2006; Anjoo and Ajay, 2011; Luhata and Munkombwe, 2015) and also the analyses of both 1D and 2D NMR spectra comfirmed that S1 is the steroid commonly known as β-sitosterol (**24**).

5.4.2 Isolation and characterization of S2

S2 was isolated as an amorphous pale yellow powder (19 mg, R_f = 0.16, solvent system: 10 % ethyl acetate in hexane, m.p: 307-308 ^{o}C) from C*issus sokodensis* rhizome.

The IR spectrum of S2 showed absorption bands of O-H stretch at 3300 cm^{-1}, 2916 cm^{-1} (asymmetric) and 2849 cm^{-1} (symmetric) C-H stretches typical of sp^3 hybridized methylene ($-CH_2$) from triterpenoids. Absorptions at 1736 cm^{-1} typical of C=O stretch of alkanoic acid carbonyl group; and also one at 1176 cm^{-1} suggestive of C-O stretch of alkanol were observed (Figure 4.50).

The ^{1}H-NMR spectrum (Figure 4.43) showed resonances of a vinylic proton at δ 5.25 (1H, t), a carbonyl proton at δ 3.21 (1H, dd, *J* = 4.2, 10.7 Hz, H-3) and seven methyl singlets at 1.10 (3H), 0.95 (3H), 0.93 (3H), 0.92 (3H), 0.89 (3H), 0.74 (3H) and 0.72 (3H). The large coupling constant (10.7 Hz) of the carbinyl proton indicates that it is in the axial position. The ^{13}C-NMR spectral data (Figure 4.44) indicated resonances for thirty carbon atoms that suggest the following functional groups: a carboxylic acid carbonyl carbon at δ 182.96; two olefinic carbons at δ 143.58 and 122.63; a carbinyl carbon at δ 79.04. Data from the DEPTS

(90 and 135) experiment (Figure 4.45) revealed: seven methyl, ten methylene, five methine and eight quaternary carbon atoms resonance (Figures 4.6.2-4.6.4). These peaks suggested an oleanane-type triterpenoidal structure with carboxylic acid functional group.

The ^{1}H and ^{13}C assignments were verified by HSQC experiments, while connectivities were verified by HMBC. Key HSQC correlations were observed with C-12/H-12, C-3/H-3, C-5/H-5 and C-18/H-18.The oxygenated carbon, C-3, was confirmed due to the long-range correlations (HMBC) between this carbon with H-2 and H-23. Double bond was assigned to C-12 due to cross peak correlation of C-13 with H-11 from the HMBC. The carboxylic acid was assigned to position-28 (C-28) due to correlation observed from HMBC between this carbon and H-22. The NOESY spectral data indicated that carbinyl proton (H-3) is close in space to the H-5 and the methyl singlet (H-23). NOE correlations were also observed between the methyl singlets (H-24 and H-25). These suggested that the bridgehead protons (H-5 and H-25) are trans-configured. NOE correlations were also observed between H-9, H-5, and H-27, indicating the relative stereochemistry in the molecular structure. Absence of NOE correlation observed between H-9 and H-26, but rather H-26 to H-25, was an evidence of a transconfiguration for the bridgehead protons (H-9 and H-26). The COSY spectrum showed correlations of six spin systems as follows: H_2-1/H_2-2/H-3, H-5/H_2-6/H_2-7, H-9/H_2-11/H-12, H_2-15/H-2-16, H-18/H_2-19, H_2-21/H_2-22 (Figures 4.46-4.49).

The spectroscopic data recorded, most especially 1D and 2D NMR experimental data, and supporting information obtained in literature (Mahato and Kundu, 1994; Consolacion and Kathleen, 2005; Zuhal *et al.,* 2006; Zuhal *et al.,* 2009; Ghias *et al.,* 2011), also as shown in Table 5.5, are consistent with the known triterpenoidal structure with a common name: oleanolic acid.Therefore, S2 (**28**) was infered to be 3-hydroxyolean-12-en-28-oic acid (oleanolic acid).

(28)

Table 5.5: Comparison of the Observed ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Spectra of Compound S2 in $CDCl_3$ with the Reported Values (δ) of Oleanolic acid (^{1}H-NMR : 400 MHz & ^{13}C-NMR : 100 MHz) in $CDCl_3$

Observed Data			Reported Data*	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)
1	38.4		38.6	
2	27.2		26.7	
3	79.0	3.21(1H, dd, *J* = 4.2, 10.7,H-3)	78.5	3.21(1H, dd, *J* = 4.4, 10.2,,H-3)
4	38.8		39.2	
5	55.2		55.5	
6	18.3		18.3	
7	32.6		32.6	
8	39.3		39.6	
9	47.6		48.1	
10	37.1		37.0	
11	23.4		22.7	
12	122.6	5.25 (1 H ,t, *J* = 3.1, H-12)	122.4	5.24 (1H, t, *J* = 3.6, H-12)
13	143.6		144.1	
14	41.6		42.0	
15	27.7		27.7	
16	23.6		22.8	
17	46.5		46.7	
18	41.0	2.81(1H, dd, *J* = 3.8,13.6,H-18)	41.5	2.82(1H, dd, *J* = 4.3,12.7, H-18)
19	45.9		46.1	
20	30.7		30.4	
21	33.8		33.7	
22	32.4		32.3	
23	28.1	0.95 (3H, s, Me-23)	28.8	0.96 (3H, *s*, Me-23)
24	15.5	0.74 (3H, s, Me-24)	14.7	0.78 (3H, *s*, Me-24)
25	15.4	0.89 (3H, s, Me-25)	15.1	0.84 (3H, *s*, Me-25)
26	16.9	0.72 (3H, s, Me-26)	16.5	0.76 (3H,*s*, Me-26)
27	26.0	1.10 (3H, s, Me-27)	25.2	1.25 (3H, *s*, Me-27)
28	182.9		180.4	
29	33.1	0.92 (3H, s, Me-29)	32.8	0.87 (3H, *s*, Me-29)
30	23.6	0.93 (3H, s, Me-30)	23.3	0.93 (3H, *s*, Me-30)

Zuhal *et al*. (2006) *****.

5.4.3 Isolation and characterization of S3

The pure compound (S3) was isolated as a brown amorphous powder S3 (400 mg, R_f =0.45, solvent system: Ethylacetate: Methanol: Water (10:1.35:1), m.p: 203-204 ^oC) from the C*issus sokodensis* rhizome.

The IR spectrum indicated the presence of hydroxyl (3383 cm^{-1}) and ester carbonyl (1698 cm^{-1}) functional groups. Additionally, sp^3 C-H stretches at 2919 and 2851cm^{-1} typical of CH_2 were indicated. Furthermore, in-aromatic ring C-C stretches (1610, 1514 and 1460 cm^{-1}) were also suggested (Figure 4.59).

The proton NMR signals at 9.76, 8.45 and 5.65 ppm, typical of proton resonance from OH groups, suggested a tri-substitution by the hydroxyl groups on benzene ring by the hydroxyl groups. Further, existence of only one aromatic proton resonance (at 7.00 ppm) suggested an aromatic ring with additional two substituent groups.This makes the total number of the substituent groups present in the aromatic structure to be five. Clusters between 3 to 4ppm suggested the presence of sugar moiety in the compound (Lemieux and Koto, 1974; Serianni and Barker, 1984). A doublet typical of anomeric proton resonance at 4.99 ppm suggests linkage of the sugar moiety onto the aromatic ring and this confirmed the presence of the sugar. The broad singlets at 5.43, 4.91 and 3.56 ppm (in the cluster) were indicative of OH groups on the sugar. Oxygenated proton signal typical of methoxy group appeared at 3.78 ppm .The signal at 1.23 ppm was suggestive of methylene protons (Figure 4.51).

The ^{13}C NMR spectrum (Figure 4.52) revealed six aromatic carbon resonances (150.9, 147.9, 140.5, 117.9, 115.9 and 109.4 ppm) that suggested the presence of benzene nucleus. Three out of these signals (150.9, 147.9 and 140.5 ppm) appeared more downfield and this could be due to de-shielding of the carbon atoms on oxygenation by substituent group. This is evidence (in addition to that observed from the ^1H NMR spectrum) for substitution by the OH groups on the benzene ring. A down field signal suggestive of ester carbonyl carbon resonance appeared at 163.3 ppm. In addition to these, methyl (in methoxy group) and a

methylene resonance appeared at 61.0 ppm and 28.9 ppm respectively. The methylene and ester carbonyl carbon mentioned earlier further suggested two other substituent groups on the benzene ring. Thus, confirming the presence of five substituents on the ring; three OH groups, a methylene and CO as substituent. Resonances were observed at 81.7, 79.7, 73.6, 72.0, 70.6 and 61.0 ppm from the ^{13}C spectrum (Figure 4.53). These resonances, together with the high coupling constant value (10.44 Hz) observed which may be due to bond electron interactions of H-1′ with H-2′ (indicative of cis-configuration), suggested a α-glucopyranose sugar moiety in the molecule (Lemieux and Koto, 1974; Serianni and Barker, 1984; Mattias *et al*., 2008).

NMR spectral data obtained from the ^{1}H and ^{13}C NMR experiments were supported with data acquired from the 2D NMR experiments. HMBC cross-peak correlation of the ester carbonyl carbon (COOMe) with H-6 established the CO-C-1-C-6 connectivity on the structure. This correlation is a confirmation of the position (C-1) at which the ester group connects itself on to the benzene ring. Correlation of OMe protons to C-2 in HMBC spectrum confirmed Me-O-O-C-C-1-C-2 connections.C-4-C-5-C-6 and C-3-C-2-C-1-C-6 connectivity, that confirmed the phenyl ring structure, were observed due to the correlations: C-4/H-6 and C-3/H-6 from the HMBC. Carbon-proton through bond HMBC correlations of C-1′/H-6 and C-2/H-1′ established C-1′-C-4-C-5-C-6 and C-1′-C-4-C-3-C-2 connectivity and hence confirmed the linkage of the sugar onto the aromatic ring. Key HMBC data were also observed to confirm the presence of the sugar moiety: C-3′/H-2′, C-1′/H-3′, C-3′/H-4′, C-6′/H-5′, C-5′/H-4′ establishing C-1-C-2-C-3-C-4-C-5-C-6 linkage and thus confirming the hexose sugar skeleton. Correspondingly, C-6/H-6, C-1′/H-1′, C-2′/H-2′, C-3′/H-3′, C-4′/H-4′, C-5′/H-5′ and C-6′/H-6′ correlations were established for the aromatic proton and the sugar moiety from the HSQC experimental data. Through bond H^{1}-H^{1} COSY interaction of H-1′ with H-2′ confirmed the bond linkage of H-1′ with H-2′.This neighborhood was further supported due

to NOE in-space interaction of H-1′ with H-2′ observed from the NOESY spectrum (Figure 4.55-4.58).

In summary, both the spectroscopic data information obtained and those of similar compounds in literature searched (Lemieux and Koto, 1974; Serianni and Barker, 1984; Mattias *et al.*, 2008), also Table 5.6 refers, enabled S3 to be inferred as 2, 3, 5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose (**29**).This molecule is novel, therefore to the best of our knowledge it was neither reported, prior to this report, from any plant nor animal source.

(29)

Table 5.6: Comparison of the Observed ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Spectra of Compound S3 in DMSO-d6 with the Reported Values (δ) of α-Glucopyranose Moiety (^{1}H-NMR : 500 MHz & ^{13}C-NMR : 125 MHz) in D_2O

	Observed Data		**Reported Data***	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)
Phenyl ester moiety				
1	115.9			
2	140.5			
3	147.9			

4	117.9			
5	150.9			
6	109.4	6.95 (1 H ,s, H-6)		
CO	163.3			
Sugar moiety				
1′	72.0	4.95 (1 H ,d, *J* = 10.44, H-1′)	92.9	5.21
2′	79.7	3.98 (1 H ,t, *J* = 9.72 , H-2′)	72.2	3.52
3′	73.6	3.64 (1H,dd *J* = 8.80,14.16, H-3′)	73.4	3.69
4′	70.6	3.23(1H ,d, *J* = 8.96,14.76, H-4′)	70.3	3.39
5′	81.7	3.55(1 H ,t, *J* = 8.16, H-5′)	72.1	3.82
6′	61.0	3.82 (2H ,d, *J* = 2.44,14.16, H-6′)	61.3	3.82
5″	28.9			
OMe	59.8			

(Mattias *et al*., 2008) *****.

5.5 Isolation and Characterization of Pure Compounds from *C. crinita* Rhizome

5.5.1 Isolation and characterization of C1

C1 was isolated as white needle like crystals (210 mg, R_f = 0.24, solvent system: 15 % ethyl acetate in 85 % hexane, mp: 137-139 ^oC) from the rhizome of *Cissus crinita*.

The IR spectrum of C1 showed key absorption bands of O-H stretch at 3409 cm^{-1}, 2918 cm^{-1} (asymmetric) and 2850 cm^{-1} (symmetric) C-H stretches typical of sp^3 hybridized methylene (-CH_2-) groups. Absorption at 1705 cm^{-1} due to C=C stretch of double bond; and an absorption at 1463 cm^{-1} due to C-H bending were also observed (Figure 4.67).

The ^{1}H NMR spectrum of C1 (Figure 4.60) showed upfield multiplets: methyl (CH_3), methylene ($-CH_2-$) and methine (-CH) protons signals (m, CH, CH_2, CH_3) resonating at 0.66-2.32 ppm indicative of steroidal nucleus. The proton signal at 3.54 ppm that could be due to oxymethine proton attached to C-3 position of sterols was observed. In addition to these, a signal that may be due to a vinyl proton (C=C<u>H</u>) resonance was observed at 5.33 ppm. The ^{13}C NMR signal at 71.82 ppm is typical of oxymethine carbon of steroids. Signals at 140.76 ppm and 121.73 ppm suggestive of olefinic double bond that is characteristic of sterols were present. The DEPTS experiments (Figure 4.62) showed eleven methylenes ($-CH_2$), nine methines (-CH), six methyls ($-CH_3$) and one quarternary carbon suggestive of β-sitosterol (**24**). ^{13}C (Figure 4.61) and ^{1}H NMR spectral data were supported by the 2D NMR experimental data. Cross peaks correlations of H-3 and that of H-4, H-6 and H-7 were established from both COSY and NOESY and COSY experiment alone respectively.The NOESY H-H correlations of H-6 and H-4, and no correlation existing between H-6 and any proton at C-5 confirms the assignment of the quarternary carbon to position-5 (C-5). Also the crosspeak correlation established between H-4 and H-3 from the NOESY spectrum supports the existence of H-3 from CHOH.This is again confirmed from the C-H correlation between C-3 and H-3, and that of also C-3 and H-4 as is observed in the HSQC and HMBC spectra respectively.C-6 correlation with H-6 confirmed the presence of vinylic proton at position-6 (C=C<u>H</u>) as observed from the HSQC spectrum (Figures 4.63 - 4.66).

On the whole, comparism of ^{13}C and ^{1}H data (Table 5.1) of C1 with literature (Suparb and Amorn, 1991; Zuhal *et al*., 2006; Anjoo and Ajay, 2011; Luhata and Munkombwe, 2015) and also the analyses of the 2D NMR spectra comfirmed that C1 is the steroid commonly known as β-sitosterol (**24**).

5.5.2 Isolation and characterization of C2

The pure compound (C2) was isolated as brown amorphous powder (450 mg, R_f = 0.41, solvent system: Ethyl acetate: Methanol: Water (10:1.35:1), m.p: 206-207 ^oC) from the rhizome of C*issus crinita*.

The IR spectrum of C2 indicated the presence of hydroxyl (3207 and 3383 cm^{-1}) and ester carbonyl (1698 cm^{-1}) functional groups. Additionally, sp^3 C-H stretches at 2918 and 2850 cm^{-1} typical of CH_2 were indicated. Furthermore, in-aromatic ring C-C stretches (1611, 1528 and 1460 cm^{-1}) were also suggested (Figure 4.76).

The proton NMR showed broad singlet resonances at 9.72, 8.43 and 5.62 ppm typical of OH groups suggested a tri-substitution of benzene ring by hydroxyl groups on the molecule. The existence of only one aromatic proton resonance (at 6.97 ppm) further indicated that the aromatic ring has additional two substituents, making the total number of the substituent groups in the aromatic structure to be five. Clusters between 3 to 4 ppm suggested the presence of sugar moiety in the compound (Lemieux and Koto, 1974; Serianni and Barker, 1984). Furthermore, a doublet typical of anomeric proton resonance appeared at 4.97 ppm.This indicated the linkage of the sugar moiety onto the aromatic ring and also confirmed the presence of the sugar. The broad singlets at 5.40, 4.88 and 3.42 ppm were suggestive of proton resonances from OH groups on the sugar.In addition to this, a resonance suggestive of methoxy protons appeared at 3.76 ppm (Figure 4.68).

The ^{13}C NMR spectrum revealed six aromatic carbon resonances (150.9, 147.9, 140.5, 117.9, 115.9 and 109.4 ppm) evident of benzene nucleus. Three of these signal (150.9, 147.9 and 140.5 ppm) appeared more downfield and this could be due to de-shielding of the carbon atoms on oxygenation by substituent group. This is evidence (in addition to that discussed from ^1H NMR experimental data) for the substitution by the OH groups on the benzene ring. A down field signal suggestive of ester carbonyl carbon resonance appeared at 163.3 ppm. Additionally, methyl resonance that suggest methoxy group appeared at 61.0 ppm.

Resonances were observed at 81.7, 79.7, 73.6, 72.0, 70.6 and 61.0 ppm from the ^{13}C spectrum. These resonances, together with the high coupling constant value (10.44 Hz) observed that could be due to bond electrons' interaction of H-1′ with H-2′ (indicative of cis-configuration), suggested a α-glucopyranose sugar moiety in the molecule (Lemieux and Koto, 1974; Serianni and Barker, 1984; Mattias *et al*., 2008). The α-glucopyranose linkage and ester carbonyl carbon mentioned earlier further suggested two other substituent groups on the benzene ring. Thus, confirming the presence of five substituents on the ring; three OH groups, a sugar linkage and CO as substituent (Figure 4.70).

NMR spectral information obtained from the ^{1}H and ^{13}C NMR spectra were supported with the data acquired from the 2D NMR experiments. HMBC cross-peak correlation of the ester carbonyl carbon (COOMe) with H-6 established the CO-C-1-C-6 connectivity on the structure. This correlation is a confirmation of the position (C-1) at which the ester group connects itself on to the benzene ring. Correlation of OMe protons to C-2 in HMBC spectrum confirmed Me-O-O-C-C-1-C-2 connections. C-4-C-5-C-6 and C-3-C-2-C-1-C-6 connectivity, that confirmed the phenyl ring structure, were observed due to the correlations: C-4/H-6 and C-3/H-6 from the HMBC. Carbon-proton through bond HMBC correlations of C-1′/H-6 and C-2/H-1′ established C-1′-C-4-C-5-C-6 and C-1′-C-4-C-3-C-2 connectivity and hence confirmed the linkage of the sugar onto the aromatic ring. Key HMBC data were also observed to confirm the presence of the sugar moiety: C-3′/H-2′, C-1′/H-3′, C-3′/H-4′, C-6′/H-5′ and C-5′/H-4′ establishing C-1-C-2-C-3-C-4-C-5-C-6 linkage and thus confirming the hexose sugar skeleton. Correspondingly, C-6/H-6, C-1′/H-1′, C-2′/H-2′, C-3′/H-3′, C-4′/H-4′, C-5′/H-5′ and C-6′/H-6′ correlations were established for the aromatic proton and the sugar moiety from the HSQC experimental data. Through bond H^{1}-H^{1} COSY interaction of H-1′ with H-2′ confirmed the bond linkage of H-1′ with H-2′.This neighborhood was further supported due to NOE in-space interaction of H-1′ with H-2′ observed from the NOESY

spectrum. Furthermore, H-2′/H-3′, H-3′/H-4′ and H-4′/H-5′ observed from the COSY confirmed the through bond correlations of H-2′ with H-3′, H-3′ with H-4′ and H-4′ with H-5′ on the sugar (Figure 4.72 to 4.75).

In summary, both the spectroscopic data information obtained and those of similar compounds searched in literature (Lemieux and Koto, 1974; Serianni and Barker, 1984; Mattias *et al*., 2008), also as tabulated in Table 5.7, enabled C2 to be determined as

2, 3, 5-trihydroxymethylbenzoate-α-D-glucopyranose (**30**).This molecule has not been reported (to the best of our knowledge) from either plant or animal source. Thus, this is the first report on this molecule and therefore considered a novel structure from nature (plant source).

(30)

Table 5.7: Comparison of the Observed ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Spectra of Compound C2 in DMSO-d6 with the Reported Values (δ) of α-Glucopyranose Moiety (^{1}H-NMR : 400 MHz & ^{13}C-NMR : 100 MHz) in D_2O

	Observed Data		**Reported Data***	
Position	δc (ppm)	δ_H (ppm)	δc (ppm)	δ_H (ppm)
Phenyl ester moiety				
1	115.9			
2	140.5			

3	147.9			
4	117.9			
5	150.9			
6	109.4	6.97 (1 H ,s, H-6)		
CO	163.3			
Sugar moiety				
1′	72.0	4.97 (1 H ,d, *J* = 10.44, H-1′)	92.9	5.21
2′	79.7	4.00 (1 H ,t, *J* = 9.72 , H-2′)	72.2	3.52
3′	73.6	3.65 (1H,dd *J* = 8.80,14.16, H-3′)	73.4	3.69
4′	70.6	3.20(1H ,d, *J* = 8.96,14.76, H-4′)	70.3	3.39
5′	81.7	3.57(1 H ,t, *J* = 8.16, H-5′)	72.1	3.82
6′	61.0	3.84 (2H ,d, *J* = 2.44,14.16, H-6′)	61.3	3.82
OMe	59.7			

Mattias *et al*., (2008) *****.

5.6 Antimicrobial Studies of the Plant Extracts and the Isolated Compounds

The results of the antimicrobial studies on the crude plants extracts and the newly isolated compounds are remarkable.

The crude plant extracts generally recorded highest activity against the fungi (*Cryptococcus neoformans, Candida albicans, Candida tropicalis* and *Aspergillus niger*) tested. Low MICs suggest that the compounds have good activity against the susceptible organisms; compounds with MICs less than 100µg/ml are regarded as having strong antimicrobial potentials (Tang *et al*., 2003). All the MICs, against the fungi tested, for the crude extracts were below 100

µg/ml except for the extracts of *Cissus sokodensis* against *Candida tropicalis* (100 µg/ml), hexane extract of *Ampellocissus grantii* against *Candida albicans* (200 µg/ml) and *Candida tropicalis*(100 µg/ml), and *Ampellocissus grantii* DCM extract against *Candida albicans* (200 µg/ml); *Cissus crinita's* DCM extract against *Aspergillus niger* (100 µg/ml) and EtOAc extract of the same plant against *candida tropicalis and Aspergillus niger* (100 µg/ml).The highest antifungal activity (lowest MIC) was observed from *Cissus sokodensis'* methanolic extract (12.5 µg/ml); and this extract was the most active against all the test organisms compared with others.This was followed by the ethyl acetate extract of *Ampellocissus grantii* which was the most active against *Escherichia coli* with MIC:25 µg/ml.Other extracts with closer activity include *Cissus sokodensis'* DCM and EtOAc extracts; *Cissus crinita*'s DCM, EtAOc and MeOH extracts. It can also be observed from the result that the most ative extracts are the polar ones (either the ethylacetate or the methanol), as shown in Table 4.3.This is not surprising because these extracts contain molecules with heteroatoms (like oxygen) that could confer pharmacological activity on them.

The newly isolated compounds, namely: 15-hydroxymethyl-16-methylheptadecylferulate, 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate, 2, 3, 5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose and 2, 3, 5-trihydroxymethylbenzoate-α-D-glucopyranose, demonstrated very good pharmacological activity with MICs below 100 µg/ml. The isolated compounds are most active against the fungi on a general note. The highest antifungal activities (MIC:6.25 µg/ml) were recorded against *Cryptococcus neoformans* and *Aspergillus niger*, and *Candida albicans* by G3, and by both S3 and C3.Considering all the isolates, the most active antifungal was G3 this was followed by both S3 and C2; and the least was G4. In corroboration with the activity demonstrated by the ethyl acetate extract of *Ampellocissus*

grantii (which was the most active against *Escherichia coli*, a gram negative bacterium, with MIC: 25 µg/ml), G4 (that was isolated from the same extract) proved to be most active against the stated organism.Thus, this activity could be due to the presence of this compound in the plant extract. G3 just like C2 also showed good actibacterial activities (MIC: 25 µg/ml) against *Escherichia coli* and *Staphylococcus aureus*.

The pharmacological activity of the plants' extracts revealed the potentialities of the plants to treat diseases associated with the microorganisms investigated.The biological activity results showed the potentials of the plants to treat bacterial related diseases: pneumonia and urinary tract infections caused by *Escherichia coli* and *Pseudomonas aeruginosa* (Garry *et al.,* 2014; Teiji, 2014); ear infections, rashes (dermatitis) and skin lesion ecthyma gangrenosum caused by *Pseudomonas aeruginosa* (Rahme *et al.,* 1995; Fine *et al.,* 1996); diarrheal diseases and meningitis implicated by *Escherichia coli* (James *et al.,* 2004); lower respiratory tract infections, cardiovascular infections, skin, wound and deep tissue infections, pneumonia, endocarditis and septicaemia diseases associated with *Staphylococcus aureus* pathogen (Eili *et al.,* 2007; Lisa, 2013). Since *Ampellocissus grantii* was found to be active against *Escherichia coli* and *Staphylococcus aureus* which are responsible for diarrheal diseases, wound and deep tissue infections; it is therefore an evidence that the plant contains compound(s) that could be use to treat these diseases and this is inline with tradomedicinal uses of *Ampellocissus grantii* which is used to treat diarrheal and wound infections. Also the traditional use of the plants to treat fever was scientifically validated because the plants were active against *Escherichia coli* and *Staphylococcus aureus* which have been implicated for meningitis and lower respiratory tract infections and other ailments associated with the test organisms, that leads to fever (James *et al.,* 2004; Eili *et al.,* 2007; Lisa, 2013; Garry *et al.,* 2014; Teiji, 2014).

Invasive fungal infections like Candidiasis, Aspergillosis and Cryptococcosis caused by *Candida albicans, Aspergillus niger* and *Cryptococcus neoformans* are common and known (Carol, 1998). In the study of opportunistic fungal infections in HIV/AIDS patients Bharathi and Usha Rani (2011) reported that *Candida albicans, Aspergillus niger* and *Cryptococcus neoformans* were implicated for pulmonary infections. Additionally, *Cryptococcus neoformans* is the cause of the most common life-threatening meningitis in HIV-positive patients (Michaels *et al*., 1999). Some strains of *A. niger* produces a potent mycotoxin called ochratoxins A; a human carcinogen found in grains and wine products (Samson *et al*., 2004; Schuster *et al*., 2002).Thus, since both the plants' extracts and the newly isolated compounds demonstrated a very strong activity against *Aspergillus niger* (Table 4.5 and Table 4.6), it is an indication that the plants contain chemical entities that can serve as anticancer agents. Hence, this might be a scientific proof to validate the plants' usage as anticancer agents in ethnomedicine.

5.7 The Anticancer Potentialities of the Compounds Isolated from the Plants

Anticancer analyses of both the crude extracts of the plants and the isolates supposed to be carried out in line with the traditional use of the plants and some of the objectives of the research. However, due to paucity of material and lack of facilities for the analyses, it was not conducted.Therefore as alternative; literature search was done on the chemical substances isolated from the plants. Consequently, literature searched showed that all the known compounds isolated were reported to have anticancer activity (Kahlos *et al*., 1987; Zhao *et al*., 2009; Baskar *et al*., 2010; Yan *et al.*, 2010). Furthermore, the newly isolated molecules, when compared with structures of their derivatives (to relate their structures with anticancer activity), have moieties in them with strong anticancer potentials (Li *et al*., 2009; Pan *et al*., 2010; Pan *et al*., 2011; Hui-zhen *et al*., 2014).

β-sitosterol (for example) was isolated from all the three plants studied, and this compound inhibits growth of HT-29 human colon cancer cells by activating the sphingomyelin cycle (Awad *et al*., 1998) and also induces apoptosis in LNCaP human prostate cancer cells (von Holtz *et al*., 1998), stimulates apoptosis in MDA-MB-231 human breast cancer cells *in vitro* and inhibits growth and metastasis of MDA-MB-231 in SCID mice (Awad *et al*., 1996; 2000a,b,c; 2001a,b; 2003).It also inhibits the growth and metastasis of human prostate cancer PC-3 cells, in vitro and in SCID mice (Awad *et al*., 2001b). Induces apoptosis by stimulating Bax and activation of caspases in the HT116 human colon cancer cells (Choi *et al*., 2003).Induces apoptosis in MCA-102 murine fibrosarcoma cells by activation of ERK and the downregulation of Akt (Moon *et al*., 2007; 2008). Inhibits cell growth and induces apoptosis in SGC-7901 human stomach cancer cells in vitro (Zhao *et al*., 2009). It showed significant dose-dependent growth inhibition, suppressed expression of beta-catenin and PCNA antigens in human colon cancer cells COLO 320 cells *in vitro*. Feeding beta-sitosterol also caused a dose dependent reduction in the number of aberrant crypt and crypt multiplicity in DMH-initiated rats with no toxic effects (Baskar *et al*., 2010).These reports on the experimentally verified anticancer properties of the known isolate (β-Sitosterol), are scientific evidences that the plants studied contain compounds with anticancer properties and hence proving the folkloric claim of the usage of the plants in managing cancers.

Oleanolic acid (a known compound isolated from C*issus sokodensis* rhizome) causes a dose and a time dependent cell kill of the human colon carcinoma cell line HCT15.It inhibits proliferation and arrested the cells in G0/G1 phase (Li *et al*., 2002). It induces apoptosis in human leukemia cells HL60 through caspase activation (Zhang *et al*., 2007). It also selectively inhibits growth of ras oncogene-transformed R6 cells (Wu *et al*., 2009). Again it induces apoptosis in human liver cancer HepG2, Hep3B, Huh7 and HA22T cell lines (Yan *et al.,* 2010). Similarly it inhibits growth of ascitic tumors in mice (Hsu *et al.,* 1997). Also

inhibits tumor promotion in mouse skin (Tokuda *et al*., 1986). Likewise it inhibits azoxymethane (AOM)-induced colonic aberrant crypt foci and multi-crypt aberrant crypt/foci in a dose dependent manner (Janakiram *et al*., 2008). It also suppresses preneoplastic lesions induced by 1, 2-dimethylhydrazine in rat colon (Furtado *et al*., 2008).

Betulin (a known compound isolated from *Ampellocissus grantii's* rhizome) was tested against Walker 256 carcinosarcoma and MCF-7 human mammary adenocarcinoma *in vitro*, and leukemia P 388 *in vivo* and showed activity comparable to inotodiol (Kahlos *et al*., 1987).Tumour inhibition with activity against human epidermoid carcinoma of the nasopharynx *in vitro* (Miles *et al*., 1974) and Walker 256 (5WA16) tumour system (Sheth *et al*., 1973) was also recorded.

Considering the newly isolated molecules G3 and G4 (Figures: 5.3-5.4), both have phenylpropanoid moiety in them and compounds with this moiety have been reported to posses anticancer properties (Prateek, 2011; Eli *et al*., 2012; Eleni *et al*., 2014). Furthermore, phenylpropanoid esters, as observed in G3 and G4, indicated good anticancer capabilities (Kheng *et al*., 2011). Additionally, phenyl group with hydroxyl-methoxy moieties at ortho positions represent good anticancer activity (Li *et al.*, 2009; Pan *et al*., 2010; Pan *et al*., 2011). G3 and G4 have long aliphatic methylene group chain, and these groups showed better anticancer properties compared to phenylpropanoid esters without such groups (Hui-zhen *et al*., 2014).

S3 and C2 (the new compounds isolated from *Cissus sokodensis* and *Cissus crinita*) derivatives have shown remarkable anticancer properties.The sugar moiety in the compounds enhances cancerous cells inhibition (Eli *et al*., 2012). In addition, the substitution of the three protons on the benzene ring by the hydroxyl groups on S3 and C2 structures and the presence of the ester group confer anticancer activity on the class of compounds the molecules belong

to (Raina *et al*., 2008; Chen *et al*., 2009; Kaur *et al*., 2009; Lo *et al.*, 2010; Reddivari *et al*., 2010).

The demonstration of anticancer activity of the derivatives of the new isolates is an indication that the new compounds (because of similar structure to those of their derivatives) may possess anticancer properties.Therefore; this could be reason to validate the use of the plants studied in traditional medicine for the treatment or management of cancers.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Cissus sokodensis, *Cissus crinita* and *Amepelocissus grantii* were collected from Hilly-mountainous sites of Sabon-Gari Local Government Area in Kaduna State-Nigeria in the year 2012 during rainy season.These plants are mainly used, in folk medicine, for the treatment of cancer, fever, diarrhea and wound infections.The stated plants were not phytochemically analysed before this study to the best of our knowledge.

Cold extraction maceration using hexane, DCM, EtOAc and MeOH were successively used to extract the phytocompounds in the plants. Extracts obtained were concentrated under reduced pressure and dried at room temperature.The extraction yielded highest with more polar solvents (EtOAc and MeOH) indicating higher polar metabolites' quantity. Preliminary

phytochemical screening of the crude plants' extracts carried out revealed the presence of steroids/terpenoids, flavonoids, tannins, saponins and anthraquinones in the plants' rhizomes. Chromatographic techniques, mainly silica gel column chromatography, was used for the isolation of compounds from the plants' extracts.Compounds isolated were analysed for structure elucidations, using spectroscopic tools, majorly 1D and 2D NMR experiments. Nine chemical compounds were isolated from the plants: β-sitosterol, betulin, 15-hydroxymethyl-16-methylheptadecylferulate and 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate from *Ampelocissus grantii*, β-sitosterol, oleanolic acid and 2,3,5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose from *Cissus sokodensis* and two, β-sitosterol and 2,3,5-trihydroxymethylbenzoate-α-D-glucopyranose from *Cissus crinita*. Four of these compounds: 15-hydroxymethyl-16-methylheptadecylferulate, 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate, 2, 3, 5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose and 2, 3, 5-trihydroxymethylbenzoate-α-D-glucopyranose, are novel, not reported prior to this report, from natural source.

Pharmacological studies on the plants' crude extracts and the novel isolates against some clinical bacteria (gram negative and gram positive) and fungi isolates [*Staphylococcus aureus, Bacillus subtilis* (Gram positive bacteria), *Escherichia coli, Pseudomonas aeruginosa* (Gram negative bacteria), *Cryptococcus neoformans, Candida albicans, Candida tropicalis, Aspergillus niger* (fungi)] showed moderate to good activity against the organisms tested. Both the extracts and isolates showed highest activity against the fungi tested. Activity recorded by the new isolates' was generally higher compared to those of the extracts.Fungitoxicity exhibited by all the plants against *Aspergillus niger* is an indicator for anticancer potency of the plants.This is because this fungus produces carcinogen capable of causing cancer (Samson *et al*., 2004; Schuster *et al*., 2002).Thus, this supports the use of the plants to treat cancer in ethnomedicine.Generally, the antimicrobial activity exhibited by the

plants is a scientific evidence that supports the tradomedicinal use of the plants to treat fever, and use of *Ampellocissus grantii* to treat diarrhea and wound infections.The activity also indicates the isolates' potentials of possible utilization as lead compounds in the development of more effective antibacterial/antifungal agents.

Although the anticancer studies of both the crude extracts and isolates in line with ethnomedicinal use of the plants were not carried out, but literature has shown that all the known isolates have anticancer properties, further the newly isolated molecules have structures with anticancer potentialities. Hence, the studied plants contain compounds that have anticancer property thus the studies validates the use of the plants in the management/treatment of cancers in traditional medicine.

6.2 Conclusion

Conclusively, the research objectives have been achieved. Four new chemical entities were discovered from the plants (15-hydroxymethyl-16methylheptadecylferulate, 3-hydroxymethyl-5-methyl-16-oxoheptadecylferulate, 2, 3, 5-trihydroxymethylbenzoate-α-D-glucopyranose and 2, 3, 5-trihydroxymethylbenzoate-4-methylene-α-D-glucopyranose) and these have structure related activity typical of drugs used in the management of cancer. In addition to these, five known compounds (three β-sitosterols; one from each plant, betulin and oleanolic acid) were isolated for the first time from the plants, giving a total of nine molecules isolated. Literature has shown that all the known compounds isolated have anticancer properties; further the newly isolated molecules have moieties in their structures with anticancer potentialities. Hence, the studied plants contain compounds that have anticancer property. The presence of the compounds isolated suggests that the plants contain chemical compounds that could possess both anticancer and antimicrobial properties. Therefore, these compounds may have been responsible for the anticancer and antimicrobial

effects the plants have been said to possess in ethnomedicine and hence the folklore usage of the plants in the management of cancers and bacterial/fungi diseases are said to be validated.

Recommendations

Further work to isolate other bioactive compounds, most especially in the plants' methanol extracts, needs to be done.This will be required inview of the widespread prevalence of opportunistic fungal infections associated with Tuberclosis and HIV/AIDS infections.

In vitro and *in vivo* anticancer/toxicities studies of the new phytochemicals could be carried out to confirm their potentialities of serving as anticancer agents.

The molecules isolated (most especially the novel ones) could serve as leads for synthesis of other pharmacophores with better cytotoxic, bactericidal and fungicidal activities.

Pharmacokinetics and pharmacodymics studies of the novel phytocompounds and their analogues followed by other clinical trials are highly encouraged.

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