PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES ON THE LEAF EXTRACT OF *ZIZIPHUS MUCRONATA* WILLD. (RHAMNACEAE)

BY

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DECLARATION

I declare that the work in this thesis entitled: "**Phytochemical and some Pharmacological Studies on the Leaf Extract of *Ziziphus mucronata* Willd. (Rhamnaceae)"** has been performed by me in the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any institution.

_____	_____	_____
Abdullahi, Sakynah Musa	Signature	Date

CERTIFICATION

This thesis entitled **PHYTOCHEMICAL AND SOME PHARMACOLOGICAL STUDIES ON THE LEAF EXTRACT OF *ZIZIPHUS MUCRONATA* WILLD. (RHAMNACEAE)** by Sakynah Musa ABDULLAHI meets the regulations governing the award of a Doctor of Philosophy degree of Ahmadu Bello University, Zaria, and is approved for its contributions to knowledge and literary presentation.

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DEDICATION

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ABSTRACT

Chronic inflammation has been implicated in most non-communicable/chronic degenerative diseases including age-related cancers. *Ziziphus mucronata*, a plant used in ethnomedicine to treat different painful and inflammatory conditions, was subjected to phytochemical investigation and pharmacological studies in order to validate its ethnomedicinal use. The leaves was subjected to extraction using cold maceration and extensive phytochemical investigation was carried out using silica gel column chromatography and gel filtration. Analgesic activity of the methanol extract was investigated using hot plate and acetic acid-induced writhing tests while the anti-inflammatory activity of the methanol extract and n-butanol fraction was evaluated using carrageenan-induced paw oedema in rats. Anti-tumor potential of the methanol extract and n-butanol fraction was investigated using quantitative, animal sparing anti-tumor bioassay on potato disc. Preliminary phytochemical screening and thin layer chromatography (TLC) profile of the crude methanol extract revealed the presence flavonoids, steroids, tannins, saponins, alkaloids and triterpenoids. The ethyl acetate fraction was found to contain flavonoids while the n-butanol fraction contained flavonoids, tannins, saponins and alkaloids. Extensive phytochemical investigation of the ethyl acetate fraction led to the isolation and characterization of Catechin, while that of n-butanol fraction led to the isolation and characterization of Rutin, Bis (quercetin 3-*O*- β-D-glucopyranoside) and 3-*O*-(5'-hydroxy-3',4',5'-trimethylhexanoyl)ursane. The methanol extract (75, 150 and 300 $mgkg^{-1}$ *i.p*) produced a significant ($p < 0.01$) dose dependent increase in mean pain latency on the hot plate at the end of 90 minutes and 150 $mgkg^{-1}$ significantly ($p < 0.05$) attenuated (inhibited 68 %) acetic acid induced writhing in mice. Both the crude extract and n-butanol fraction at the doses tested (150 -

800 $mgkg^{-1}$) produced a significant ($p < 0.001$) anti-inflammatory activity. Both the crude extract and the n-butanol fraction produced a significant ($p < 0.001$) concentration dependent decrease in the mean number of tumors produced by *Agrobacterium tumefaciens* on potato tuber at all the tested concentrations (10 – 1000 ppm). All observed activities could be due to the presence of important secondary metabolites isolated from the Leaf extract of the plant. This study has added to the chemotaxonomy of the Rhamnaceae family and lend pharmacological support to the folkloric uses of the plant in the management of pain and inflammatory condition.

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ABBREVIATIONS

%: Percentage

°C: Degree Celsius

^{1}H: Proton

^{13}C: Carbon-13

δ_H: Chemical shift value of proton

δ_C: Chemical shift value of carbon

1D: one dimension

2D: two dimension

g: weight in grams

EAF: ethyl acetate fraction

nBF: n-buanol fraction

UV: ultraviolet

IR: infra-red

Hz: Hertz

J: Coupling constant

ppm: parts per million

d: doublet signal

dd: double doublet

t: triplet signal

R_f: retention factor

KBr: potassium bromide

MHz: megahertz

TLC: thin layer chromatography

NMR: nuclear magnetic resonance

DEPT: Distortionless enhancement by polarization transfer

$mgkg^{-1}$: dose in milligram per kilogram

ESI-HRMS: electrospray ionization high resolution mass spectroscopy

MEZM: Methanol extract of *Ziziphus mucronata*

COSY: Correlation spectroscopy

HMQC: Heteronuclear multiple quantum coherence

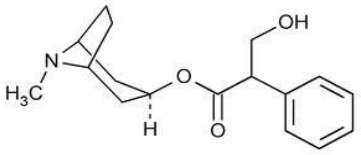
HMBC: heteronuclear multiple bond correlation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Plants and their secondary metabolite have a long history of use in modern medicine and in certain systems of traditional medicine, and are the sources of important drugs such as atropine (I), codeine (II), digoxin (III), morphine (IV), quinine (V) and vincristine (VI).



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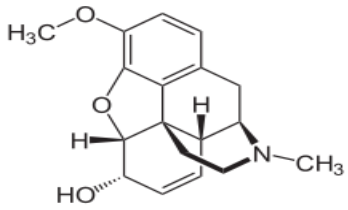

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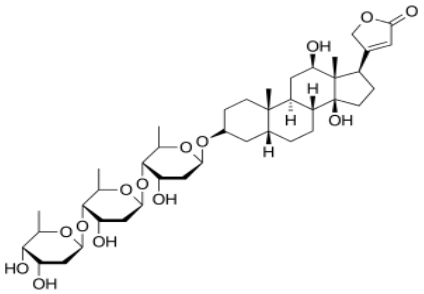

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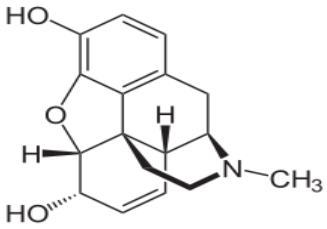

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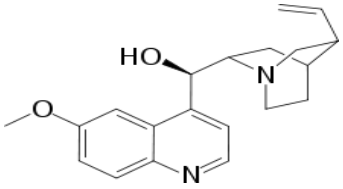

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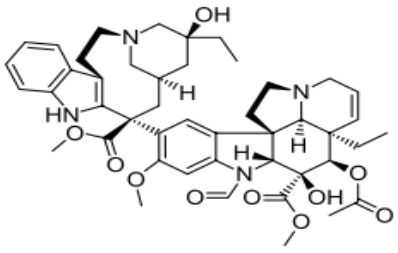


(VI)

Natural products have been used by native cultures as a source of remedies for thousands of years, dating back to ancient empires in Egypt, China, Greece and Rome (Kinghorn,

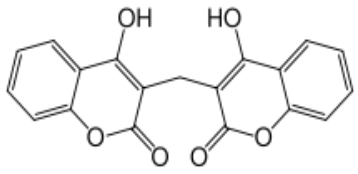
1992). Several well-known species, including licorice (*Glycyrrhiza glabra*), myrrh (*Commiphora species*) and poppy capsule latex (*Papaver somniferum*) were referred to by the first known written record on clay tablets from Mesopotamia in 2000 BC (Newman *et al*., 2000). These plants are still in use today for the treatment of various diseases and as ingredients for both orthodox and traditional medicines.

Reports have shown that there is a growing interest in the use of natural products and folk medicine even in the developed countries. In the United States, 38% of adults use complementary and alternative medicine (CAM) and according to the United States of America National Center for Complementary and Integrative Health (NCCIH), 33.9 billion US dollars was spent by 83 million adults on traditional remedies in 2007 while in the United Kingdom, the annual expenditure in CAM is two hundred and thirty million dollars ($230 million). In Nigeria, Ghana, Mali and Zambia, the first line treatment for 60% of children with fever, resulting from malaria, is the use of herbal medicine at home (WHO, 2008). The World Health Organisation (WHO) noted that of 119 plants derived pharmaceuticals, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures (Fabricant and Farnsworth, 2001).

An estimated 25% of prescription drugs and 11% of drugs considered basic and essential by the WHO are derived from plants and a large number of synthetic drugs are obtained from precursor compounds originating from plants (WHO, 2003). Analysis of the top 150 proprietary drugs from the United States' prescription revealed that 57% of the drugs contain at least one major active compound of biological origin, and 17% are unmodified

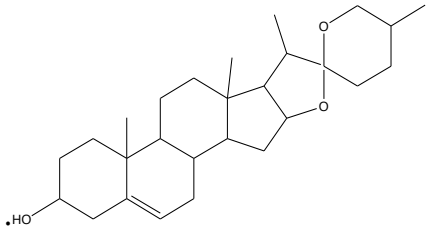
natural products from plants (Grifarn *et al*., 1997). It is thus apparent that plants represent a treasure of structural diverse potential bioactive organic molecule. Despite this, higher plants as potential source of new drugs is still highly underexploited because among the known species, only about 10% have been investigated phytochemically and the fraction subjected to biological or pharmacological screening is even smaller (Hostettmann *et al*., 1996).

Compounds isolated from natural sources may directly be used as drugs, for example, anticoagulant dicoumarol (VII) is isolated from moldy or spoiled Sweet clover hay (*Melilotus officinalis*). Nature still supplies the best drug for the treatment of heart disease because there is no synthetic drug capable of replacing digoxin (III), a constituent of digitalis (Swerdlow, 2000).



(VII)

Drugs can also be semi-synthesized from natural products e.g. the plant steroids, diosgenin (VIII) and hecogenin (IX) remain key precursors for the synthesis of many modern contraceptive drugs such as norethindrone (X) and other pharmaceuticals including betamethasone (XI) and beclomethasone (Carl, 2006)



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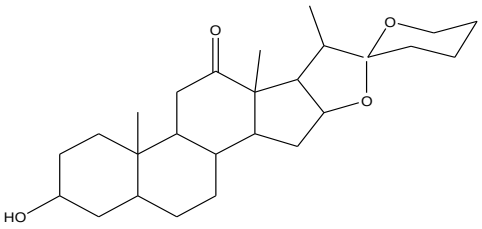

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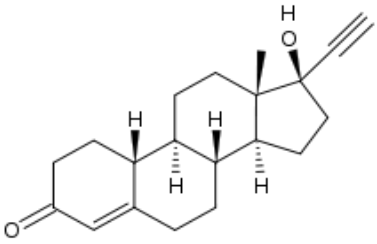

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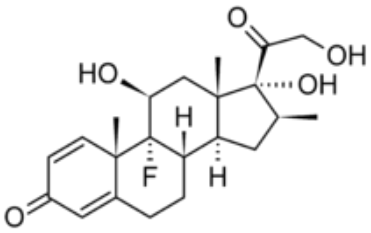
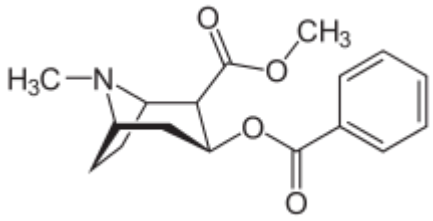


(XI)

Natural products have also been utilized as chemical models or templates (lead) for the design and synthesis of many important drugs (Ganellin, 1993) e.g. cocaine (XII) from *Erythroxylon coca* leaf extract served as a lead for more potent and less toxic local anaesthetics such as procaine (XIII) and lidocaine (XIV) which are devoid of unwanted effects of cocaine such as addiction, tremor, urinary incontinence and agitation.



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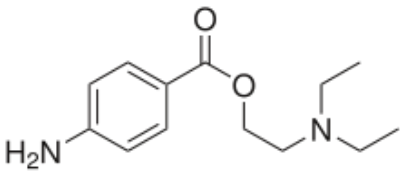

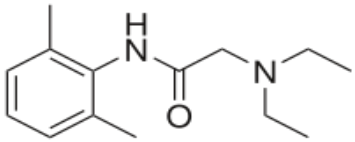

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It is estimated that natural products are responsible for about half of the approved drugs that are available (Newman and Cragg, 2007) for example, 18 of the 42 new drugs discovered in 1992 are either natural products or synthetic analogues of natural products (Chin *et al*., 2006).

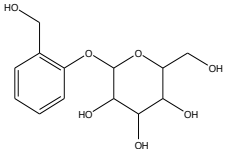
1.2 Pain and Inflammation

The British Medical Association describes pain as discomfort to an excruciating experience. Pain can be defined as a subjective, unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Dorland and Newman, 1988). It is categorized according to its duration (acute or chronic), pathophysiology (nociceptive or neurogenic), location and aetiology (Willens, 2003).

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. The function of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair (Melmon *et al*., 2000). While acute inflammation set the stage for tissue repair, chronic inflammation, on the other hand, may lead to a host of diseases such as hay fever, periodontitis, atherosclerosis, rheumatoid arthritis and even cancer (e.g. Gall bladder carcinoma).

1.2.1 Plant-derived analgesics and anti-inflammatory agents

From the early records of traditional medicine, over 7000 years ago, various forms of natural products have been utilized to treat pain disorders. Prototypical examples of such natural products are the straw of opium poppy (*Papaver somniferum)* and the bark of the willow tree (*Salix alba*)**.** In the 19th century, morphine (IV) and salicin (XV) were respectively isolated from these plants and were determined to possess the desired effects (McCurdy and Scully, 2005)**.** Over the last several decades, more analgesic and anti-inflammatory principles have been synthesized based on the structure of these two compounds resulting in novel structural classes and mechanisms of action, for example, Salicin which is metabolized in the body to salicylic acid (XVI) was used as a precursor for acetylsalicylic acid (Aspirin®) (XVII) which is recognised as a universal pain killer.



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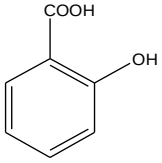

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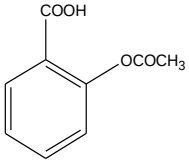
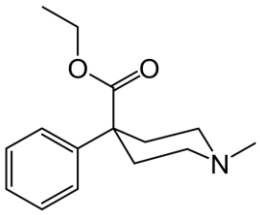


(XVII)

Pethidine (XVIII) and pentazocine (XIX) were synthesized based on morphine (IV) structure.



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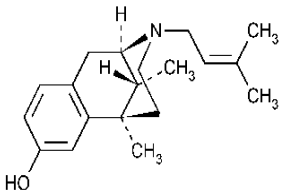


(XIX)

1.3 Cancer

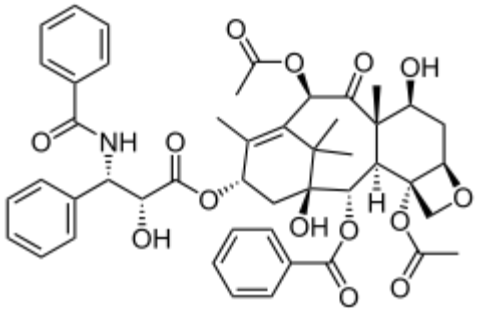
Cancer is a class of diseases characterized by out-of-control cell growth. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. Cancer harms the body when altered cells divide uncontrollably to form lumps or masses of tissue called tumors (except in the case of leukemia where cancer inhibits normal blood function by abnormal cell division in the blood stream). Tumors can grow and interfere with the digestive, nervous and circulatory systems and they can release hormones that alter body function. Tumors are of two kinds – benign and malignant. Tumors that stay in one spot and demonstrate limited growth are generally considered to be benign. More dangerous, or malignant tumors form when a cancerous cell manages to move throughout the body using the blood or lymphatic systems, destroying healthy tissue in a process called invasion or when a cell manages to divide and grow, making new blood vessels to feed itself in a process called angiogenesis. When a tumor successfully spreads to other parts of the body and grows, invading and destroying other healthy tissues, it is said to have metastasized. This process itself is called metastasis, and the result is a serious condition that is very difficult to treat (Willen, 2003).

1.3.1 Plant-derived anti-cancer agents

Medicinal plants – either through systematic screening programs or by serendipity – possess an important position in drug discovery. The percentage is even higher for treatment of infection and cancer. Over 60% of the anti-cancer drugs in use have their origin from natural sources. Ethno pharmacological data obtained by consulting traditional healers on the medicinal use of plants, as well as from literature on folk

medicine revealed that over 3000 species of plants have been reported to have anti-cancer properties (Graham *et al*., 2000).

The plant-derived cancer chemotherapeutic agents were responsible for approximately one third of the total anti-cancer drug sales worldwide (accounting for about 4 billion US dollars) in 2007. These include taxanes –paclitaxel (XX) and docetaxel (XXI) as well as the camptothecin derivatives- irinotecan(XXII), topotecan(XXIII), etc. (Fridlender *et al*., 2015).



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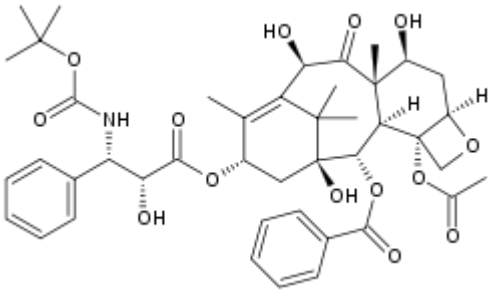

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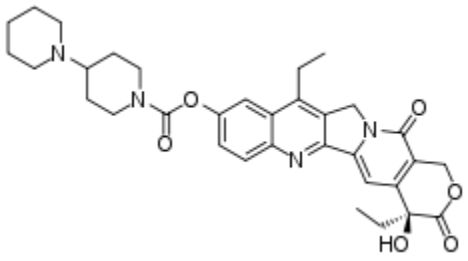

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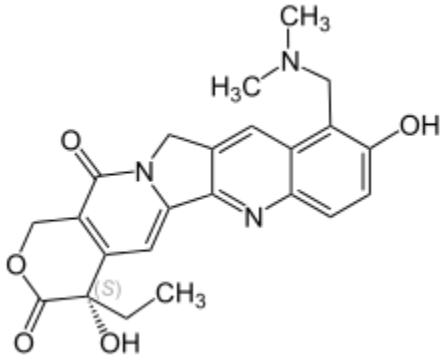

(XXIII)

The essential roles played by natural products in the discovery and development of effective anti-cancer agents, and the importance of multidisciplinary collaboration in the

generation and optimization of novel molecular leads from natural product sources cannot be overemphasized (Fabricant and Farnsworth, 2001).

1.4 Statement of Research Problem

Pain is the major reason why people seek medical attention. People with any form of disease could suffer from acute to chronic pain. Diseases such as cancer, sickle cell anaemia and gouty arthritis are usually associated with inflammation in addition to chronic pain.

Cancer is considered to be one of the leading causes of morbidity and mortality worldwide with its toll rising in developing countries. About 12.5% of all deaths are attributable to cancer and if the trend continues, it is estimated that by 2020, 16 million new cases will be diagnosed per annum out of which 70% will be in developing countries (Abdulkareem, 2009).

The WHO statistics on cancer situation in Nigeria is alarming. It says an estimated two million people are living with cancer in Nigeria with 30 dying per hour of cancer and Nigeria records no fewer than 100,000 new cancer cases yearly (Ajekigbe, 2013).
For some cancers there are a number of effective treatments, but for many others, there is still need for new and better treatment approaches.

1.5 Justification of the Study

Non-steroidal anti-inflammatory drugs (NSAIDS) and opioids are used in management of mild to moderate and severe pains respectively. These drugs have serious limitations due

to their side effects. Opioids causes respiratory depression, euphoria, tolerance and dependence while non-steroidal anti-inflammatory drugs produce gastrointestinal irritation and renal damage.

In spite of success in surgery and radiotherapy, most cancer cases require chemotherapy at one point or the other. In Nigeria, the availability of medical equipment necessary for radiotherapy is grossly inadequate, this made oncologist to resort mainly to chemotherapy in the management of cancer. The entire currently available chemotherapeutic agents in clinical use are expensive worldwide. Compliance with medication is a major problem because of the need for long term therapy together with unwanted effects that range in severity from nausea and blood dyscrasias to death from hepatotoxicity and permanent damage to other organs. Most of the drugs available are not selective to cancer cells and affect the normal cells as well and unfortunately, these drugs are currently the most effective means to combat cancer. In view of this, the acute need for less toxic and more potent anti-cancer drugs continue to be a concern.

Nigeria, the largest economy in Africa with an estimated $5,638 per capita annually, ranks near the bottom (130 out of 189 countries) in terms of per capita income with over half of the population living in poverty (IMF, 2016). This implies that very few people can afford orthodox medicine in treating their ailments. As such, WHO encourages the inclusion of herbal medicines of proven safety and efficacy in the healthcare programs of developing countries because of the great potential they hold in combating various diseases (Musa *et al*., 2009). Herbal medicines are also preferred because of better

cultural acceptability, affordability, better compatibility with the human body and the general belief that natural products have fewer side effects than synthetic drugs.

Thus, there is a need to validate folkloric claims of plants with medicinal potentials in the management of various ailments.

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1.6 Justification for Plant Selection

Over 80,000 flowering plant species are used medicinally worldwide. Amongst them are the underutilized *Zizyphus species* in the Rhamnaceae family. In terms of abundance and economic value, *Z. spina-Christi* and *Z. lotus* are currently the most important, especially in Nigeria and South Africa where they are cultivated and exploited for medicinal use and their edible fruits. A related common species widely distributed in Africa, *Z. mucronata*, is yet to be explored. None the less, local people in various African countries use its different parts to cure a large number of diseases, many of which are similar to those treated with *Z. spina-Christi* and *Z. lotus*.

1.7 Aim of the Study

The aim of this study is to isolate some of the bioactive compounds from the leaf extract of *Ziziphus mucronata* and validate the ethnomedicinal claim of its use in treatment of painful and inflammatory conditions including cancer.

1.8 Specific Objectives

1. To isolate some of potentially active compounds present in the leaf extract of *Z. mucronata* using chromatographic methods.

2. To characterize the isolated compounds using physical methods and spectroscopic techniques.
3. To determine the acute toxicity profile of the leaf extract using laboratory model
4. To evaluate the analgesic and anti-inflammatory activity of the leaf extract using laboratory models.
5. To screen the leaf extract for anti-tumor activity using animal-sparing bioassay technique.

1.9 Research Hypothesis

The leaf extract of *Ziziphus mucronata* contains secondary metabolites with analgesic, anti-inflammatory and anti-tumor activity.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy

According to Angiosperm Phylogeny Group (APG) III system of classification, the plant *Ziziphus mucronata* falls under

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Eudicotyledon

Order: Rosales

Family: Rhamnaceae

Genus: *Ziziphus*

Species: *mucronata*

Author: Willdenow

(GRIN, 2010)

The plant is known as Buffalo thorn or cape thorn (English), Magaryar Kura (Hausa), Ekanase Adie (Yoruba), Gulam jabe (Fulani) and Umlahlabautu (Swahili) (Burkill, 1995).

Other common species of the same genus include *Z. abyssinica, Z. jujube, Z. Mauritiana, Z. spina- Christi, Z. lotus, Z. vulgaris, Z. oxyphylla* (Burkill, 1995).

2.2 Plant Description

Ziziphus mucronata is a shrub or a small to medium-sized tree that is 10-20 m high with a spreading canopy. The plant is usually spiny but sometimes unarmed.

The main stem is green and hairy when young; year old branches often zigzag with branchlets that are reddish to brownish; the bark is reddish brown or roughly mottled grey and often fissured or cracked into small rectangular blocks, revealing a red and stringy under-surface (Orwa *et al.,* 2009).

The leaf is simple, alternate; ovate or broadly ovate; vary enormously in size from tree to tree, 30-90 x 20-50 mm, tapering or having mucronate apex and often asymmetrical at the base, chordate to rounded on one side; margin finely serrated, often badly eaten by insects. It is glossy green above but slightly hairy and paler below and 3- to 5-veined

from the base; veins covered with fine hairs when young; petiole up to 20 mm long; stipules, when present, take the form of small thorns at the nodes, one straight and one hooked. Leaf turn golden yellow in autumn.

The flower is glabrous and borne in dense clusters in leaf axils; green to yellow; ± 4mm in diameter; inconspicuous (October-February). Sepals are 1.5–2 mm long while Petals are 1–1.5 mm long.

The fruit is a smooth, shiny, leathery, spherical drupe, 12-20 mm in diameter, reddish-brown or deep red when ripe, slightly sweet, the pulp is dry. The fruit sometimes stays on the plant long after the leaf has fallen (March-August). The seed is usually solitary, elliptic and compressed (Thulin, 2008).

2.3 Geographical Distribution and Habitat

Ziziphus mucronata is distributed throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia (Orwa *et al.*, 2009). It is a drought and frost resistant plant that grows in areas dominated by thorny vegetation in both temperate and tropical climates. It is also found in a wide range of habitats such as woodlands, open scrubland, on rocky koppies, open grasslands, on a variety of soils along streams, nutrient-rich valley bottoms and forest margins. It reaches its largest size on the margins of scrub forest and on deep, alluvial soils near water

(Burkill, 1995). Its presence is said to indicate the presence of underground water (Palmer and Pitman, 1972).

Plate I: *Ziziphus mucronata* tree growing in its natural habitat

Plate II: ***Ziziphus mucronata*** **leaf**

2.4 Ethnomedicinal Uses of *Ziziphus mucronata*

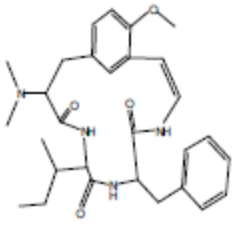
In traditional medicine, a decoction or infusion of the glutinous roots is commonly administered orally as a painkiller for all sorts of pains as well as dysentery (Burkill, 1995) and in South Africa, a decoction of the root is used for lumbago and for scrofulous condition with swollen glands; the fruits are sometimes chewed to relieve toothache. The paste of the leaf is applied to glandular swelling. The Zulus take the powdered leaf and bark in water as an emetic agent for chest problem and other respiratory ailments. The poultice of the leaf can be applied to boils and other septic swellings of the skin and sometimes for urethral discharge, painful conditions and urinary problems (Orwa *et al*., 2009). Spines of the wild thorn are reduced to a charcoal powder for treatment of snake bite (Burkill, 1995). Steam baths from the bark are used to purify and improve complexion (Palmer and Pitman, 1972). In East Africa, roots are used for treating snake bites (Hutchings *et al*., 1996). In Northern Nigeria, the powdered leaf macerated in cold water is administered orally for the treatment of chronic inflammation (personal communication).

Its leaf and root have also been used to treat diarrhoea, cough, sores, ear inflammation, asthma, syphilis, gonorrhoea, measles, and fever (Von Koenen, 2001). Other reports include the use of the roots by the Bambara and Malinke tribes in Tanzania for psychiatric disorder and the use of the leaf juice to prevent abortion. *Z. mauritiana* and *Z. abyssinica* have a range of medicinal uses which include wounds, fever, abdominal pains, venereal diseases and diarrhoea treatment (Neuwinger, 1996).

2.5 Chemical Constituents of *Ziziphus mucronata* and Other Species in the Genus

A number of active ingredients have been isolated from the leaf, root, stem and seed of *Zizyphus species*. Among them are alkaloids, flavonoids, sterols, triterpenoids, saponins and tannins (Neuwinger, 1996; Van Wyk *et al*., 1997).

Many *Ziziphus species* have been found to contain cyclopeptide alkaloids, which are particularly common in plants of Rhamnaceae (Legoabe, 2004). A total of eighty one cyclopeptide alkaloids have been isolated from various species of Ziziphus (Waqar *et al*., 2014). Few among them include Mucronines A-H (XXIV-XXXI) isolated from bark of *Ziziphus mucronata* (Legoabe, 2004), 0-demethylmucronine-D (XXXII) from the roots of *Ziziphus mucronata* (Barboni *et al*., 1994), abyssenine-A (XXXIII), abyssinine-B (XXXIV) and abyssinine-C (XXXV) in addition to mucronine E-F (XXVIII-XXIX) from the bark of *Ziziphus abyssinica*, frangufoline (XXXVI), amphibines- B, -D, and –F together with mauritine A-F from *Ziziphus mauritiana* (Legoabe, 2004).



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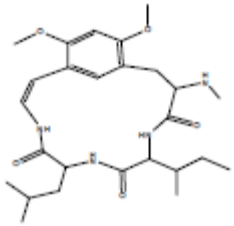

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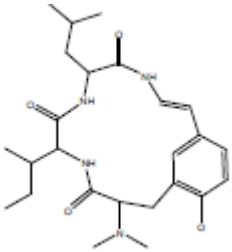

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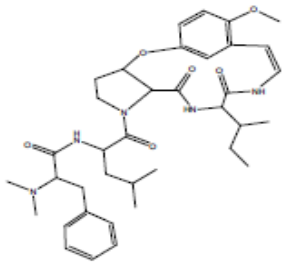

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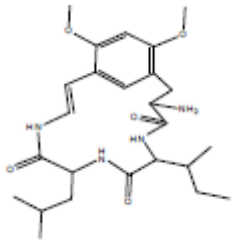

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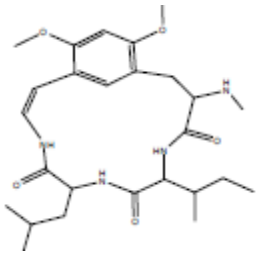

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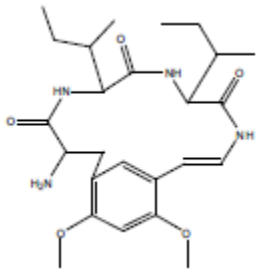

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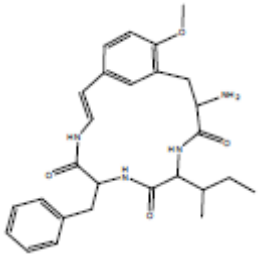

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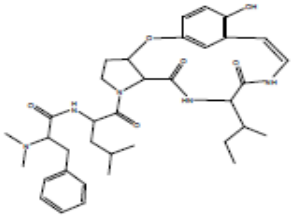

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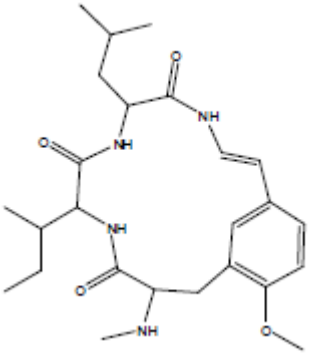

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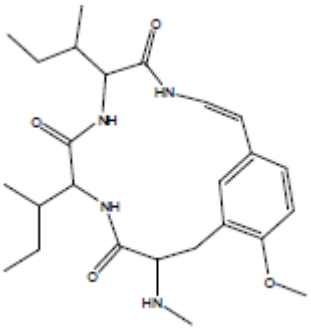

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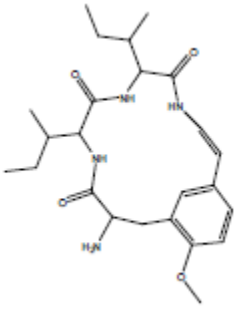

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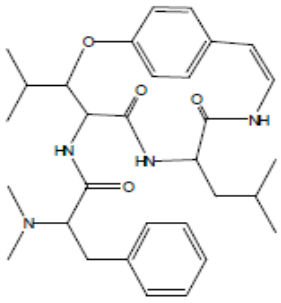

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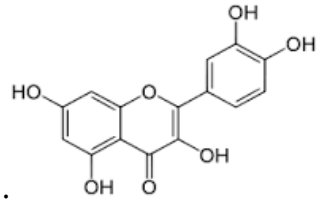
Quercetin (XXXVII), kaempferol (XXXIII) and phloretin (XXXIX) were isolated from the fruits of *Ziziphus jujuba* and *Ziziphus spina-Christi* (Pawlowska *et al.*, 2009)



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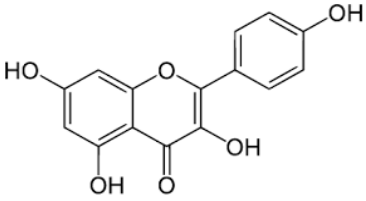

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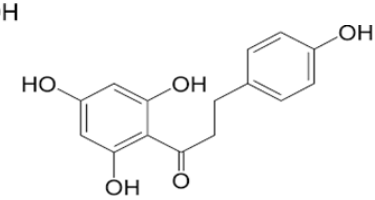
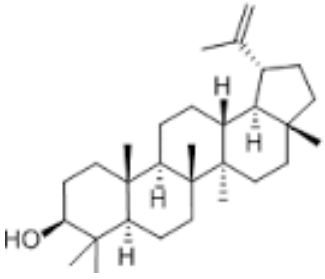


(XXXIX)

Lupeol (XL) and betulinic acid (XLI) were isolated from the bark of *Z. jujube* in addition to Zizybernalic acid (XLII) (Kundu *et al.*, 1989).



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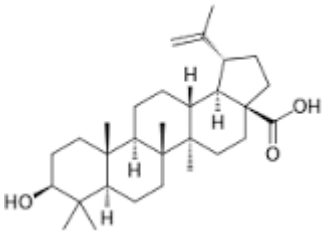

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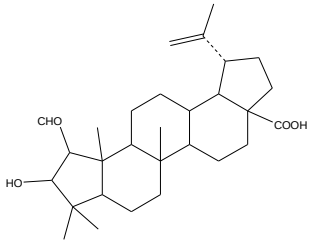


(XLII)

2.6 Pharmacological Properties of *Ziziphus mucronata* and Other Species in the Genus

Various extracts from the leaf of *Z. mucronata* were reported to show broad spectrum antimicrobial activity as well as antioxidant activity using 1,1-diphenyl-picrylhydrazyl (DPPH) assay and cytotoxic activity using Brine shrimp lethality test (BST) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromine (MTT) assay (Ilonga, 2012). The antimicrobial potency of the crude bark extract of *Z. mucronata* has also been demonstrated (Olajuyigbe and Afolayan, 2012). Mucronines A-D isolated from the bark of *Ziziphus mucronata* were reported to have antibacterial activity.

Several other *Ziziphus species* are known for their medicinal properties. Strong sedative effects have been reported from *Z. vulgaris* and *Z. jujube* (Van Wyk *et al*., 1997). diuretic effects have been reported for *Z. mauritiana* and *Z. abyssinica*, anti-nociceptive and antipyretic activity has been reported for *Z. oxyphylla* (Nisar *et al*., 2007), anti-inflammatory effect of *Z. lotus* was also reported (Borgi *et al.,* 2007) and antiulcer effect of *Z. lotus* (Wahida *et al*., 2007). Other pharmacological activities reported include analgesic effect of *Z. spina-Christi* (Adzu *et al.,* 2001), anti-diabetic activity of *Z. sativa* (Mukherjee *et al.,* 2006), hepatoprotective effect of *Z. jujube* (Shen *et al.,* 2009), CNS depressant activity of *Z. spina-Christi* (Adzu *et al.,* 2002), anticonvulsant effect of *Z. spina-Christi* (Waggas and Al-Hasani, 2010) and free radical scavenging activity of *Z. jujube* (Li *et al.,* 2005).

According to a study, several species of *Zizyphus* has a healing effect in many cancer cases. It can have some effects such as it can delay the progression of cancer, can

decrease the intensity and frequency of pain, can enhance the immune response and increase life expectancy (Mollik *et al.*, 2009). Various extracts of *Z. spina-Christi* were found to significantly and concentration-dependently reduced viability of Hela and MAD-MB-468 cells (Abbas *et al*., 2014). Furthermore, *Zizyphus jujube* has shown marked protection against the genotoxicity produced by hydroquinone (Ghaly *et al.*, 2008).

2.7 Crown gall tumor inhibition assay (Potato disc antitumor assay)

Crown gall is a neoplastic disease of plants which is induced by the gram negative bacteria *Agrobacterium tumefaciens*. The bacteria possess large Ti (tumor inducing) plasmids which carry genetic information (T DNA) that transform normal, wounded, plant cells into autonomous tumor cells. Since the mechanism of tumor induction is similar to that in animals, this test system has been used to evaluate and pre-screen the antitumor/cytotoxic properties of natural products. The potato disc assay is a safe, simple, rapid and inexpensive in-house screen for 3PS (P 388 leukemia) antitumor activity. It also gives indication of tumor-promoting or carcinogenic properties of the test samples (Atta-ur-Rahman *et al.*, 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/Reagents

The solvents used throughout the study were of high quality general purpose grade and the reagents were of analytical grade. Solvents for column chromatography were distilled before use. Silica gel for column chromatography and vacuum liquid chromatography were of mesh size 60-120 µm and 200-400 µm (Qualikems product) respectively. Analytical thin layer chromatography (TLC) was carried out on aluminium backed kieselgel 60 F_{254} plate which has 0.2 mm thickness of silica sorbent (Merck no. 5554, Darmstadt, Germany).

3.1.2 Equipment

Gallenkhamp electrothermal melting point apparatus and Thermo scientific Biomate 6 UV-Visible spectrophotometer available at Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria-Nigeria.

Agilent technologies Cary 6030 FTIR spectrometer available at Multi-User Research Science Laboratory, Ahmadu Bello University, Zaria-Nigeria.

Bruker Avance-300 (125 MHz and 500 MHz) Nuclear Magnetic Resonance Spectrometer available at School of Pharmacy, University of London-UK.

Bruker Avance-300 (100 MHz and 400 MHz) Nuclear Magnetic Resonance Spectrometer available at School of Chemistry, Universiti Teknologi Malaysia (UTM), Malaysia.

Micromass Q-TOF Premier Tandem Mass Spectrometer coupled to CapLC available at School of Pharmacy, University of London-UK.

3.1.3 Experimental organisms

Locally bred adult Swiss Albino mice weighing 25 ± 2 g and Wistar rats weighing 120 ± 15 g of both sexes were obtained from the Animal House Facility of Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The animals, maintained on standard rodent feed and water *ad libitum*, were housed in polypropylene cages at room temperature throughout the study. All experimental protocols were in accordance with the guidelines for the care and use of experimental animals as accepted internationally and in compliance with Ahmadu Bello University, Zaria-Nigeria Research Policy. The experiments were conducted in a quiet laboratory between the hours of 900 h to 1600 h.

Fresh disease-free potatoes (*Solanum tuberosum* L.) were obtained from Farin-gada local market, Jos and authenticated at the Herbarium while Virulent strains of *Agrobacterium tumefaciens* were graciously donated by Department of crop protection, Ahmadu Bello University, Zaria.

3.2 Methods

3.2.1 Plant collection

Ziziphus mucronata tree growing wild in bushes of Kudingi village of Giwa Local Government, Kaduna state of Nigeria was identified on the field using descriptions in the literature (Burkill, 1995). The leaf was collected together with the fruits in June, 2012 and authenticated by Taxonomist Musa Muhammad of the Herbarium Section, Department of Biological Sciences, Ahmadu Bello University Zaria-Nigeria by comparing with a herbarium specimen of voucher number 900328. The leaf was air dried under the shade for two weeks, size-reduced manually using mortar and pestle and subsequently referred to as the plant material.

3.2.1 Extraction and fractionation

A 1000 g plant material was extracted with 5 L of 70 % methanol for 72 hours using cold maceration method. The extract was concentrated *in vacuo* and referred to as the methanol extract of *Ziziphus mucronata* (MEZM).

A 100 g of MEZM was suspended in water and filtered. The filtrate was partitioned successively with Ethyl acetate and n- butanol to obtain ethyl acetate fraction (EAF) and n- butanol fraction (nBF) respectively which were subjected to further studies (Figure 3.1).

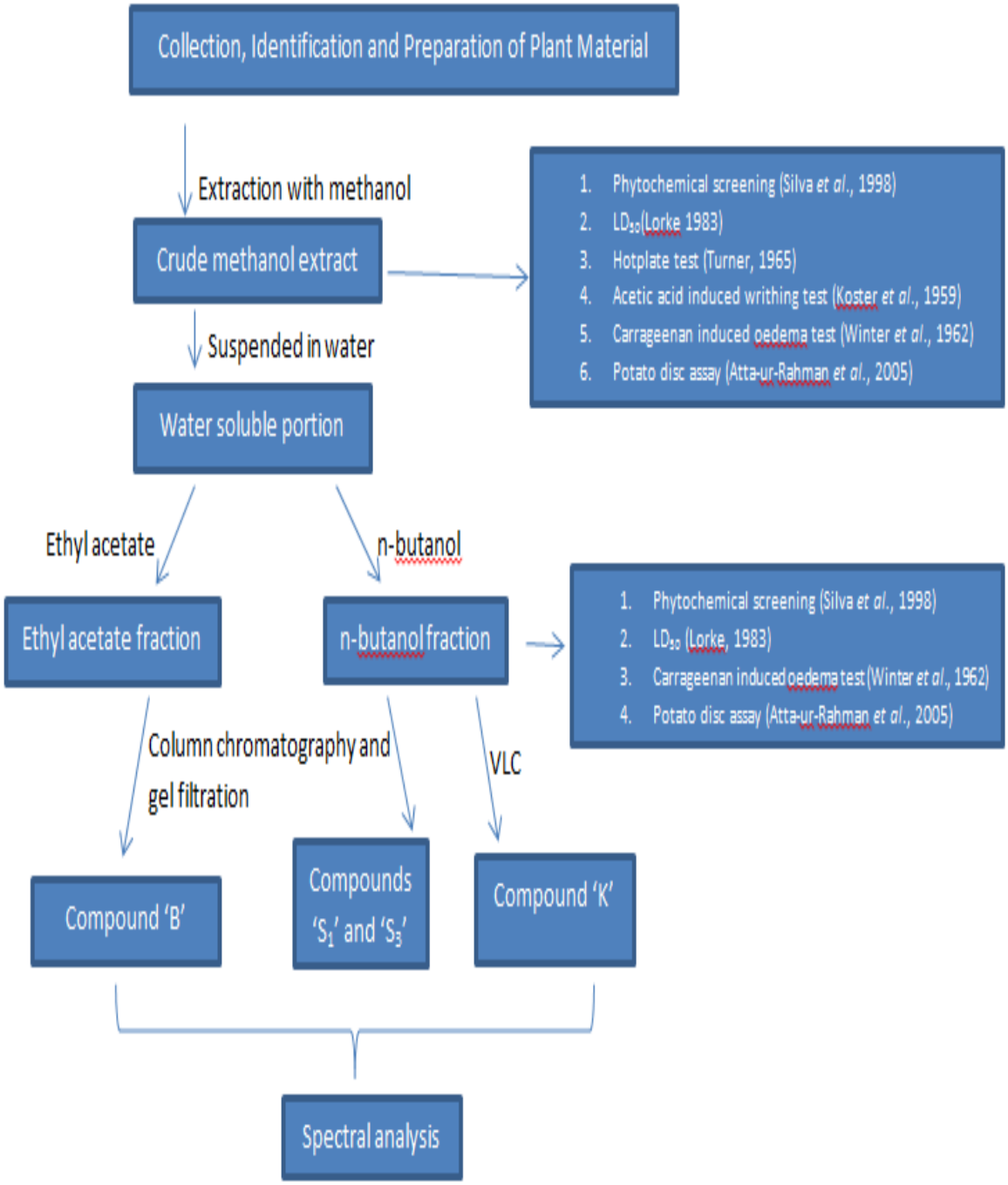


Figure 3.1: Methodology scheme

3.2.2 Preliminary phytochemical screening

Preliminary phytochemical screening using laboratory reagents was carried out on the crude extract as well as the fractions to detect the presence of secondary metabolites using standard procedures as follows:

3.2.2.1 Test for steroids and triterpenes

Liebermann-Burchard test:

A 2 mg each of the crude and fractions was added to a mixture of 1 mL anhydrous acetic acid, 1 mL chloroform and one drop concentrated sulphuric acid in a test tube and observed for blue, green, red or orange colouration (Silva *et al*., 1998).

Salkowski test:

A 2 mg each of the crude and fractions was dissolve in 1 mL chloroform and 1 mL concentrated sulphuric acid was added. Formation of two phases with a red or yellow colour indicate the presence of sterols and methylated sterols (Silva *et al*., 1998).

3.2.2.2 Test for flavonoids

Ferric chloride test:

A 2 mg each of the crude and fractions was dissolved in methanol and 2 mL of freshly prepared ferric chloride solution was added. The formation of green, blue or violet colouration indicate the presence of polyphenols (Silva *et al*., 1998).

Shinoda' test:

A loopful each of the crude and fractions was dissolved in methanol. Some pieces of magnesium chip were added followed by five drops of concentrated hydrochloric acid. A pink, orange, red to purple colour indicates the presence of flavones, flavonols, the corresponding 2,3-dihydro derivatives and/or xanthones (Silva *et al*., 1998).

Sulphuric acid test:

A loopful each of the crude and fractions was dissolved in concentrated sulphuric acid and observed. A deep yellow solution indicate presence of flavones and flavonols, a red or red-bluish solution indicate presence of chalcones and aurones while orange to red colour indicate presence of flavononones (Silva *et al*., 1998).

3.2.2.3 Test for tannins

A loopful each of the crude and fractions dissolved in methanol was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate. Formation of a blue-black or green precipitate indicates the presence of Tannins (Trease and Evans, 1996).

3.2.2.4 Test for saponins

A loopful each of the crude and fractions was shaken with water in a test tube. A froth which persists for 15 minutes is indicative of saponins (Silva *et al*, 1998).

3.2.2.5 Test for carbohydrates

Molisch test:

To 1 mL portion of the extract in a test tube, two drops of Molisch reagent was added and concentrated Sulphuric acid added down the side of the test tube to form a lower layer, a

reddish colour ring at the interphase indicates the presence of carbohydrate (Trease and Evans, 1996)

Fehling's test:

To 1 mL portion of the extract in a test tube, 5 mL mixture of equal volume of Fehling's solution A and B was added and boiled on a water bath, a brick red precipitate indicate the presence of reducing sugars (Trease and Evans, 1996).

3.2.2.6 Test for glycosides

To 1 mL portion of the extract in a test tube, 5mL of dilute sulphuric acid was added and boiled on water bath for 10-15 minutes. This was then cooled and neutralized with 20% KOH. It was then divided into two portions;

I) To the first portion, 5 mL of a mixture of Fehling solution A and B was added and boiled, a brick red precipitate shows the release of reducing sugars as a result of hydrolysis of Glycoside (Trease and Evans, 1996)

II) To the second portion, about 3 mL of ferric chloride solution was added and observed for the appearance of a green coloration due to the release of phenolic aglycones as a result of hydrolysis (Trease and Evans, 1996).

3.2.2.7 Test for alkaloids

A 0.5 g of the extract was stirred with 5 mL of 1% aqueous hydrochloric acid on a water bath and filtered. The filtrate was divided into three portions. To the first portion one drop of freshly prepared Dragendoff reagent were added and observed for formation of orange to brownish precipitate. To the second portion one drop of Mayer reagent was added and observed for formation of white to yellowish or cream colour precipitate. To

the third portion, one drop of Wagner reagent was added and observed for a formation of a brown or reddish or reddish- brown precipitate (Silva *et al*., 1998).

3.2.3 Analytical thin layer chromatography (TLC)

Several analytical thin layer chromatography were carried out on the fractions using solvents of varying polarity by the trial and error method employing the following conditions to determine the appropriate/suitable solvent system for column chromatography

a) Technique: one way ascending

b) Spotting and development: Spots were applied manually with the aid of a capillary tube; plates were dried using air blower and developed at room temperature using a Shandon Chromatank.

c) Visualizing reagent: 10 % sulphuric acid

A thin line about 2 cm from the bottom of the plate was drawn with a pencil. The sample to be separated was dissolved in minimum amount of methanol to give an approximate concentration of 20 $mgmL^{-1}$. It was then spotted along the thin line using capillary tube. The plate was allowed to dry after which it was developed using solvent systems of varying polarity. The developed plate was air dried in a fume cupboard, viewed under UV light, sprayed with the visualizing reagent using an atomiser and heated at 105°C for visualization of spots (Gibbons and Gray, 1998).

The same conditions and procedure of TLC was used to monitor the progress of elution during column chromatography.

3.2.4 Column chromatography

3.2.4.1 Open column chromatography (Gravity elution)

The following column conditions were employed:

a) Technique: Gradient elution.

b) Column: Glass column (75 cm x 3.5 cm) with sintered disc at the bottom.

c) Stationary phase: Silica gel.

d) Column packing: Wet slurry method.

e) Sample loading: Dry load method (Cannell, 1998)

Column chromatographic separation of ethyl acetate fraction (EAF):

EAF (4 g) was adsorbed onto 4 g of silica gel and chromatographed in a 75 cm x 3.5 cm column pre-packed with 200 g silica gel. The column was eluted continuously with hexane, then hexane: ethyl acetate mixtures in ratio of increasing polarity adding 5 % ethyl acetate after eluting a void volume of 400 ml until hexane : ethyl acetate in the ratio 60 : 40 was used.

TLC using solvent system of chloroform : ethyl acetate in the ratio 3 : 2 was used to monitor the progress of elution from the column and aliquots with similar TLC profile were pooled together to obtain ten major fractions coded A-J. Fraction D corresponding to collections 7-12 eluted with solvent system of Hexane : Chloroform 90 : 10 was subjected to repeated gel filtration until a single spot coded B was obtained using solvent system of ethyl acetate : chloroform : methanol : water in the ratio 15 : 8 : 4 : 1.

Column chromatographic separation of n-butanol fraction (nBF):

nBF (4 g) was adsorbed onto 4 g of silica gel and chromatographed in a 75 cm x 3.5 cm column packed with 200 g silica gel. The column was eluted with ethyl acetate 100 % followed by a mixtures of ethyl acetate and methanol in ratios of increasing polarity with 2 % methanol, monitoring the progress of elution on TLC plate before the column was finally washed with methanol. TLC using solvent system of ethyl acetate : chloroform : methanol : water in the ratio 15 : 8 : 4 : 1 was used to monitor the progress of elution from the column and aliquots with similar TLC profile were pooled together for purification using gel filtration.

3.2.4.2 Vacuum liquid chromatography (VLC)

The following column conditions were employed:

a) Technique: Step-wise elution.

a) Column: Sintered glass funnel (4 cm in diameter) with 10 mL solvent reservoir.

b) Stationary phase: Silica gel, 60-200 µm mesh size.

c) Column packing: Dry packing.

d) Sample loading: Dry load method (Salituro and Dufresne, 1998)

nBF (3 g) was adsorbed onto silica gel and chromatographed over silica gel packed column. The column was eluted step-wise with 100 % chloroform, then chloroform: ethyl acetate mixture in ratios of increasing polarity from 100 % to 98:2, 96:4 and 94:6 coded fractions A, B, C and D respectively.

3.2.5 Gel filtration

The following column conditions was employed:

a) Technique: Continuous elution.

b) Column: Glass column (50 cm x 1.0 cm) with sintered disc at the bottom.

c) Stationary phase: Sephadex LH-20.

d) Column packing: Wet slurry method.

e) Eluting solvent: Absolute methanol (Cannell, 1998)

Each of the column fraction to be purified was dissolved in minimum quantity of methanol to obtain a clear solution. The solution was applied to the top of the column with the aid of a pipette and 2 mL aliquot each was collected. Collections with the same TLC profile were pooled together and re-chromatographed until a single spot was obtained.

TLC was used to monitor the progress of purification.

3.2.6 Acute toxicity study

The method described by Lorke (1983) was adopted. The study was divided into two phases. In the first phase, nine animals of either sex were divided into three groups of three animals each. Group I received 10 $mgkg^{-1}$ extract while groups II and III received 100 and 1000 $mgkg^{-1}$ *intraperitoneally* (*i.p*) respectively. The animals were observed for signs and symptoms of toxicity and death for twenty-four hours after treatment. In the second phase, four animals of either sex were divided into four groups of one animal each and were treated with more specific doses based on the result of phase I. The animals were also observed for twenty four hours. The final LD_{50} was calculated as the square root of the product of the lowest lethal dose and the highest non-lethal dose *i.e.* the

geometric mean of consecutive doses for which 0 and 100 % survival rates were recorded.

The LD_{50} values in rats and mice differ considerably but in both analgesic and anti-inflammatory studies, 30 % of the LD_{50} was considered as the maximum tolerable dose.

3.2.7 Analgesic studies

3.2.7.1 Hot plate test

The crude extract (MEZM) was subjected to test for analgesia using hot plate method in mice as described by Turner (1965). The mice were divided into five groups of six mice each. The first group served as negative control and received distilled water 1 $mLkg^{-1}$. The second, third and fourth groups were pre-treated with 75, 150 and 300 $mgkg^{-1}$ of MEZM respectively while the fifth group served as positive control and was treated with 20 $mgkg^{-1}$ pentazocin *i.p*. Thirty (30) minutes post treatment, each mouse was gently placed on a hot plate maintained at 50 ± 1ºC. The basis for selection and grouping of the animals was done after a baseline test to determine the animals response to electrical heat-induced nociceptive pain stimulus and animals that stayed for more than 15 seconds on the hot plate were considered to have impaired nociception and therefore excluded. The time taken by the mouse to lick its paw or jump off the plate was taken as the latency to pain response. To avoid tissue damage, the cut-off time or latency response in the control was taken as 30 seconds. The determination of latency of pain response was performed before pre-treatment and repeated at intervals of 30, 60 and 90 minutes.

3.2.7.2 Acetic acid-induced writhing test

MEZM was also subjected to test for analgesia using the method of acetic acid- induced writhing as described by Koster *et al.* (1959). The mice were divided into five groups of six mice each. The first group served as negative control and received distilled water 1 $mLkg^{-1}$. The second, third and fourth groups were pre-treated with 75, 150 and 300 $mgkg^{-1}$ of MEZM respectively while the fifth group served as positive control and was treated with 10 $mgkg^{-1}$ diclofenac *i.p*. Thirty (30) minutes post treatment, each mouse was injected with 10 $mLkg^{-1}$ of 0.6 % acetic acid *i.p* and placed individually into plastic cages. The number of writhes produced in each mouse was counted for ten minutes after a five minute latency period. The percentage inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Mean Number of writhes (control)} - \text{Mean Number of writhes (test)}}{\text{Mean Number of writhes (control)}} \text{x}100$$

3.2.8 Anti-inflammatory studies (Carrageenan-induced hind paw oedema)

Anti-inflammatory studies was carried out on the crude extract as well as the n- butanol fraction using the method of carrageenan-induced hind paw oedema in rats as described by Winter *et al*. (1962). The rats were divided into five groups each consisting of five animals. The first group served as negative control and received distilled water 1 $mLkg^{-1}$. The second, third and fourth groups were pre-treated with graded doses of the test samples based on the LD_{50} values obtained, while the fifth group served as positive control and was pre-treated with 20 $mgkg^{-1}$ diclofenac *i.p*. Thirty (30) minutes post treatment, 0.1 mL of freshly prepared carrageenan suspension (1 % w/v in 0.9 % normal saline) was injected into the sub plantar region of the left hind paw of each rat. The paw diameter was measured with the aid of a vernier calliper at 0, 1, 2, 3, 4 and 5 h after the injection of carrageenan. The difference between the readings at time 0 h and the

different time intervals was taken as the thickness of oedema. The percentage inhibition of inflammation was calculated for each dose at different hours using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Mean Paw size (control)} - \text{Mean Paw size (test)}}{\text{Mean Paw size (control)}} \text{x}100$$

3.2.9 Antitumor studies (Potato disc bioassay)

The method described by Atta-ur-Rahman *et al*. (2005) was adopted as follows

3.2.9.1 Preparation of bacterial culture

A 100 mL solution of Luria Bertani broth medium (20 gL^{-1}) was prepared, filtered and sterilized in an autoclave for 15 minutes. The medium was cooled and inoculated with a loop of *Agrobacterium tumefaciens* and incubated at 28°C for 48 hours.

3.2.9.2 Sample preparation

Samples of the crude extract (MEZM) and nBF was prepared by dissolving 20 mg each in 2 mL of methanol (10 $mgmL^{-1}$ or 10,000 ppm) to make stock solution. From the stock solution, further dilutions (1000 ppm, 100 ppm and 10 ppm) were made. A vial containing 2 mL methanol served as a positive control.

Fresh disease-free potato tubers of moderate size were surface sterilized by immersion in sodium hypochlorite for 20 minutes. A core of the tissue was extracted from each tuber with a surface sterilized cork borer to form a cylinder. The cylinder was cut into 0.5 cm discs with a surface sterilized scalpel. The discs were transferred to agar plates. *Agrobacterium tumefaciens* culture mixed with each of 10, 100 and 1000 ppm concentration of MEZM and nBF (prepared in methanol) was applied on the surface of each disc. Petri plates were then placed at 28°C for 14 days.

After 14 days, the discs were stained with Lugol's solution (10 % KI and 5 % I_2) for 30 minutes and then observed under electron microscope for tumor initiation growth.

3.2.10 Statistical analysis

The data were expressed as mean ± SEM. The results of acetic acid-induced writhing test and anti-tumor studies were analysed using one-way ANOVA followed by Dunnett t-test while those for hot plate test and carrageenan-induced paw oedema were analysed using repeated measures ANOVA followed by Bonferroni post hoc test for pairwise comparison using IBM SPSS statistics 20. *p* values less than 0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Colour and Percentage Yield

A 1 kg weight of the plant material after extraction yielded 1.34 % of a dark green gummy extract. A 100 g of the extract when partitioned yielded 7.5 % of a dark brown ethyl acetate fraction and 12 % of a light brown n-butanol fraction.

4.2 Phytochemical Constituents

Preliminary phytochemical screening of MEZM revealed the presence of carbohydrates, polyphenols, steroids, alkaloids and triterpenoids. EAF contains flavonoids and tannins while nBF contains phenolic compounds, alkaloids and glycosides (Table 4.1)

Table 4.1: Phytochemical Constituents of MEZM, EAF and nBF

Constituent	Inference		
	MEZM	EAF	nBF
Steroids and triterpenoids	+ve	-ve	-ve

Tannins	+ve	-ve	+ve
Saponins	+ve	-ve	+ve
Carbohydrates	+ve	-ve	+ve
Glycosides	+ve	-ve	+ve
Alkaloids	+ve	-ve	+ve

Key

+ve constituent present
-ve constituent absent

4.3 Column Chromatographic Separation of Ethyl acetate fraction

A total of 33 collections, 50 mL aliquots each were made from EAF column and similar collections were pooled together based on their TLC profile (Plate III) to obtain ten major fractions coded A-J (Table 4.2).

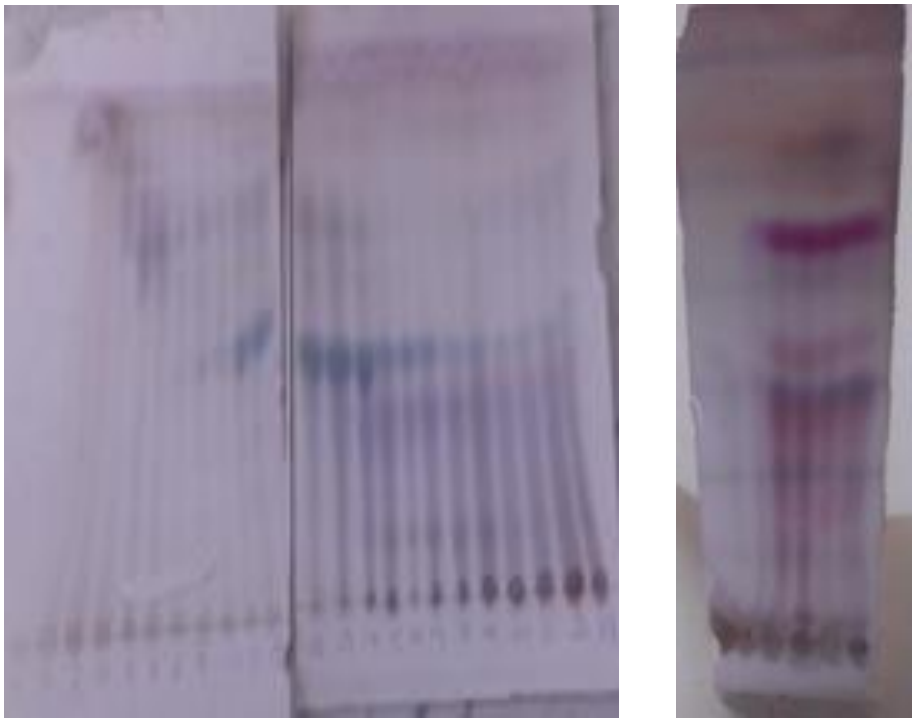


Plate III: Chromatogram of column fractions of EAF

Table 4.2: Column chromatographic separation of EAF

Fraction	Collections	Solvent system	No. of spots
A	1-2	100 % hexane	No clear spot
B	3	100 % hexane	4
C	4-6	H:E 95:5	6
D	**7-12**	**H:E 90:10**	**3**
E	13-15	H:E 85:15	>6
F	16	H:E 85:15	>6
G	17	H:E 80:20	5
H	18-19	H:E 80:20	5
I	21-29	H:E 60:40	6
J	30-33	H:E 60:40	3

4.4 Isolation and Characterization of Compound B

Fraction D (9.6 mg) when subjected to repeated gel filtration using Sephadex LH-20 afforded 4.7 mg of a brown amorphous compound coded ‘B’

4.4.1 TLC profile of compound B

B gave a single TLC spot with R_f value of 0.64 using ethyl acetate: chloroform: methanol: water (E:C:M:W) in the ratios 15:8:4:1 (Plate IV).

Plate IV: Chromatogram of compound B

4.4.2 Colour, chemical test and melting point of B

B was isolated as a brown solid with an uncorrected melting point range of 176-178°C. B produced red colour with concentrated HCl in the presence of magnesium chips (Shinoda test).

4.4.3 UV spectral data of B

The UV spectrum of B showed absorption bands at 239 nm (band K) and 280 nm (band B) in methanol (Figure 4.1)

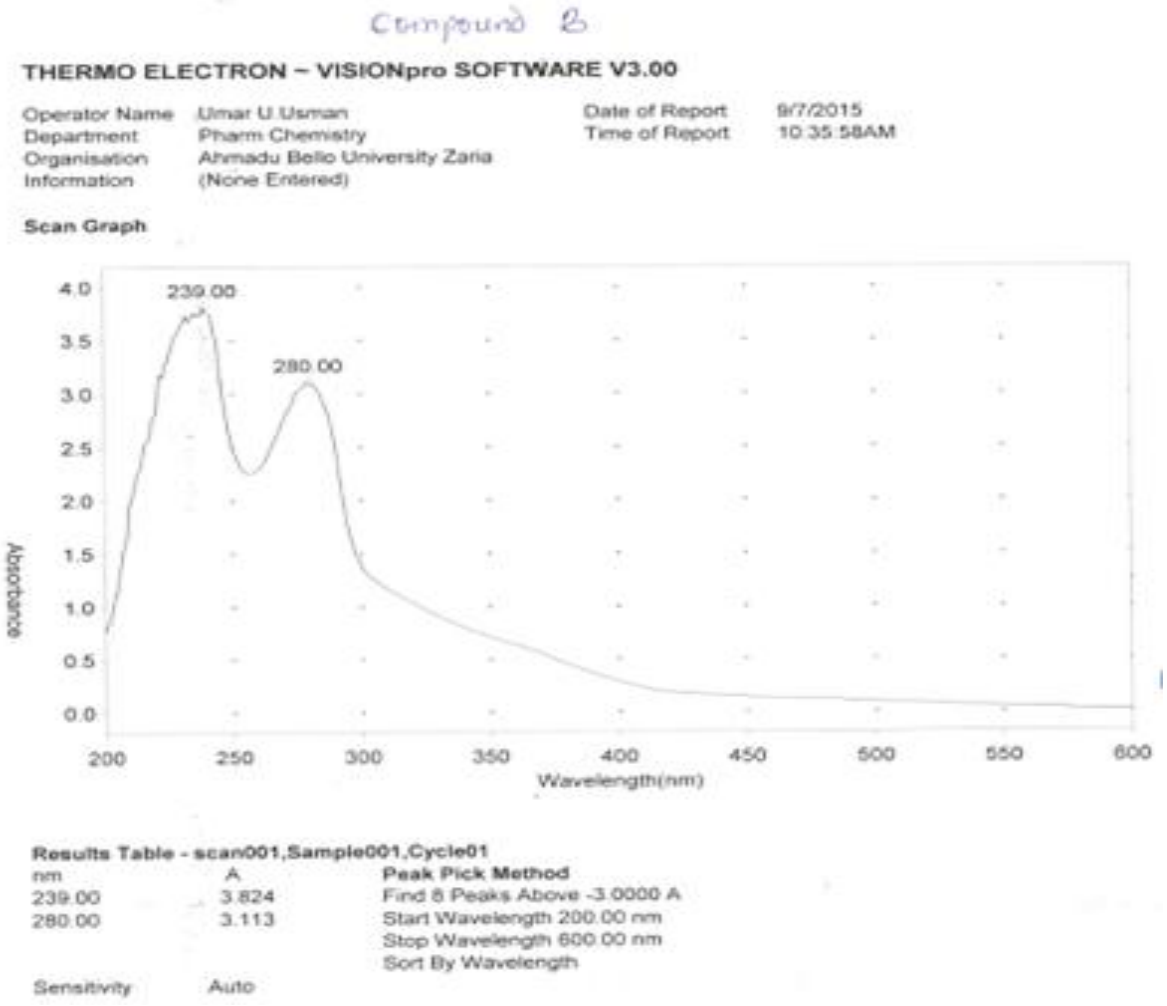


Figure 4.1: UV spectrum of compound B

4.4.4 IR spectral data of compound B

The IR spectrum of B showed absorption peaks at 1610 cm^{-1} , 2855 cm^{-1} and 2922 cm^{-1} on liquid film (Figure 4.2).

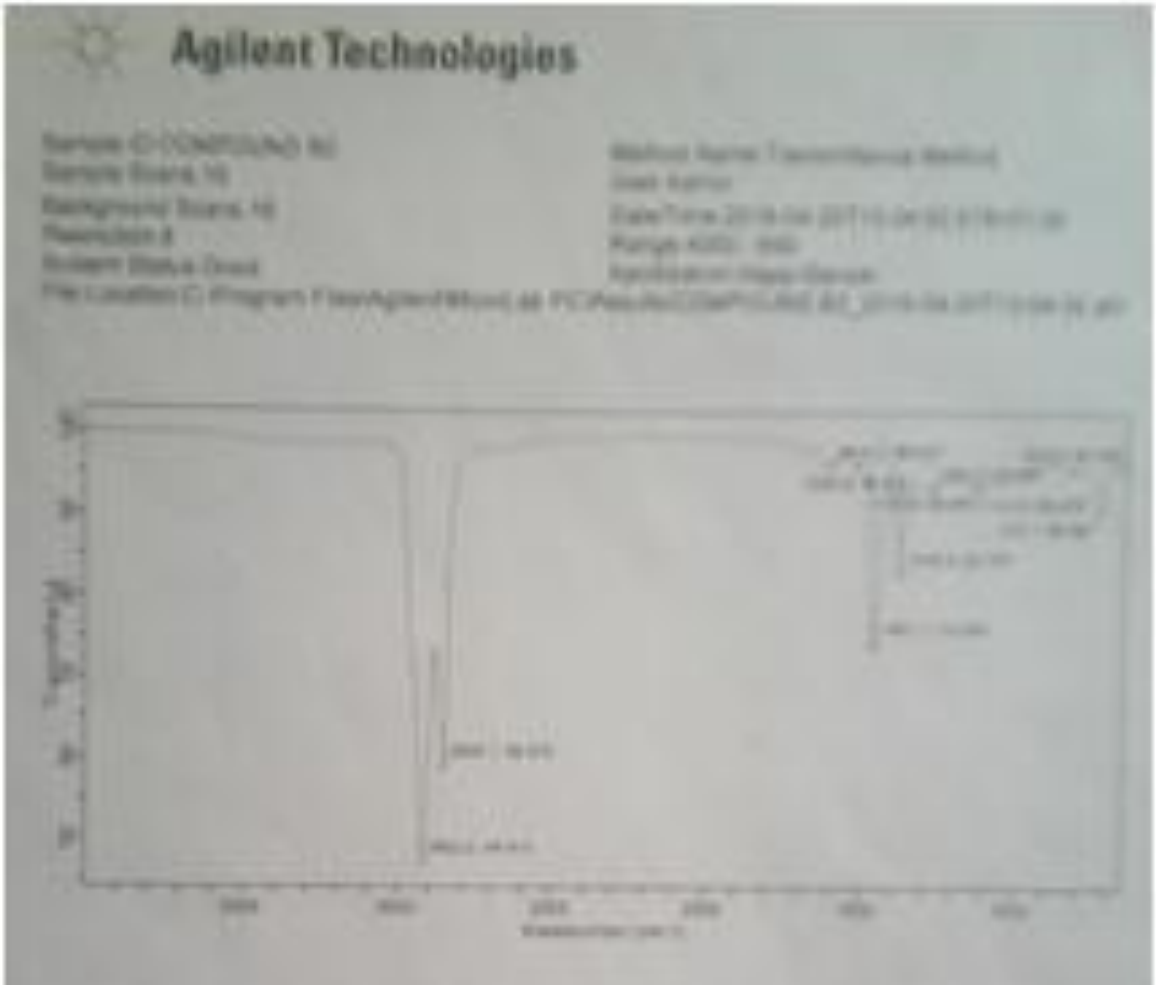


Figure 4.2: IR spectrum of B

4.4.5 Proton NMR spectral data of B

The ^{1}H NMR spectrum revealed signals for five aromatic protons at δ_H 5.85, 5.93, 6.73, 6.76 and 6.84, an oxymethine proton at 4.57, a carbinol proton at δ_H 3.98 and two methylene protons at δ_H 2.87 and 2.53 in CD_3OH (Figure 4.3).

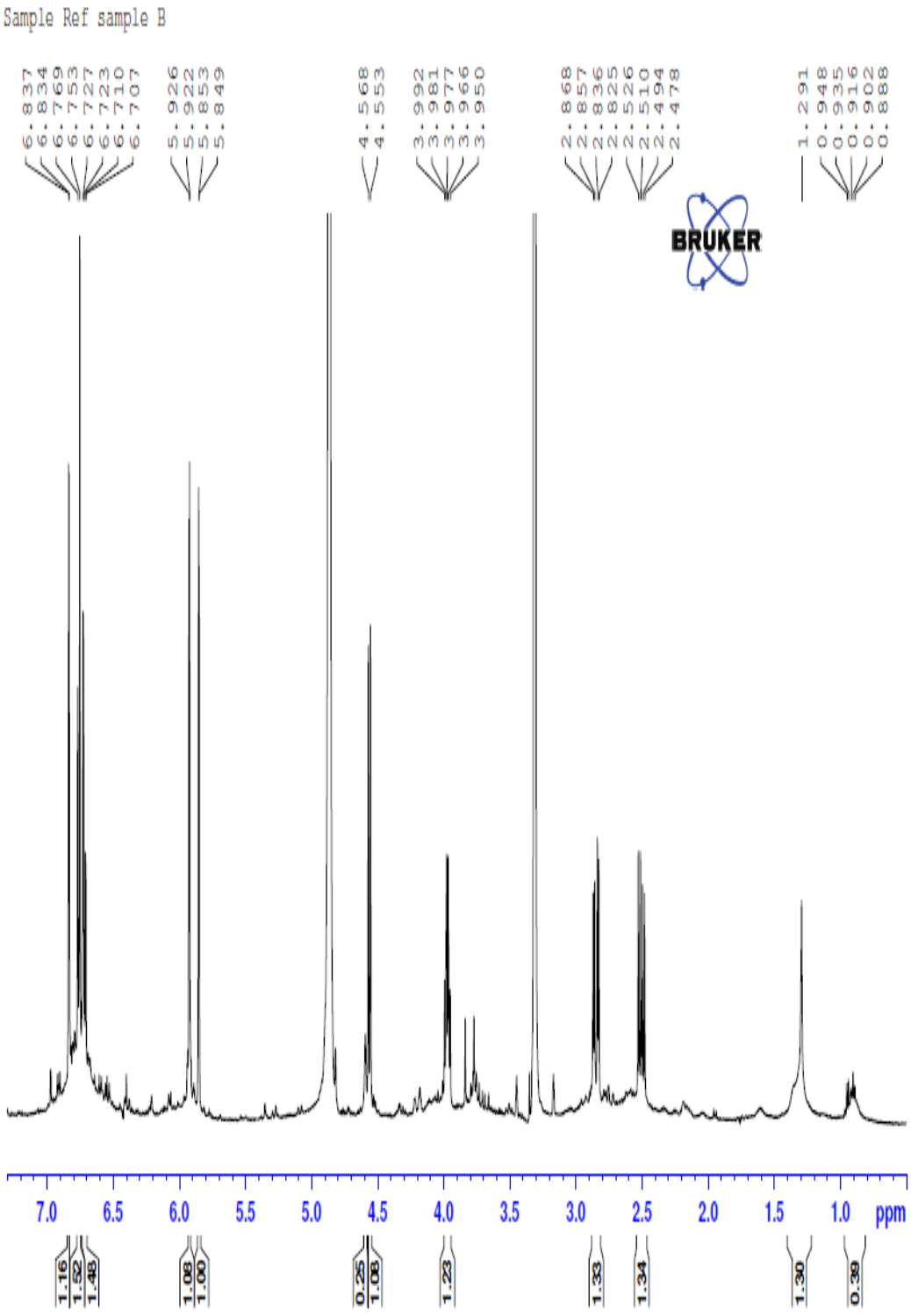


Figure 4.3: Proton NMR spectrum of B

Sample Ref sample B

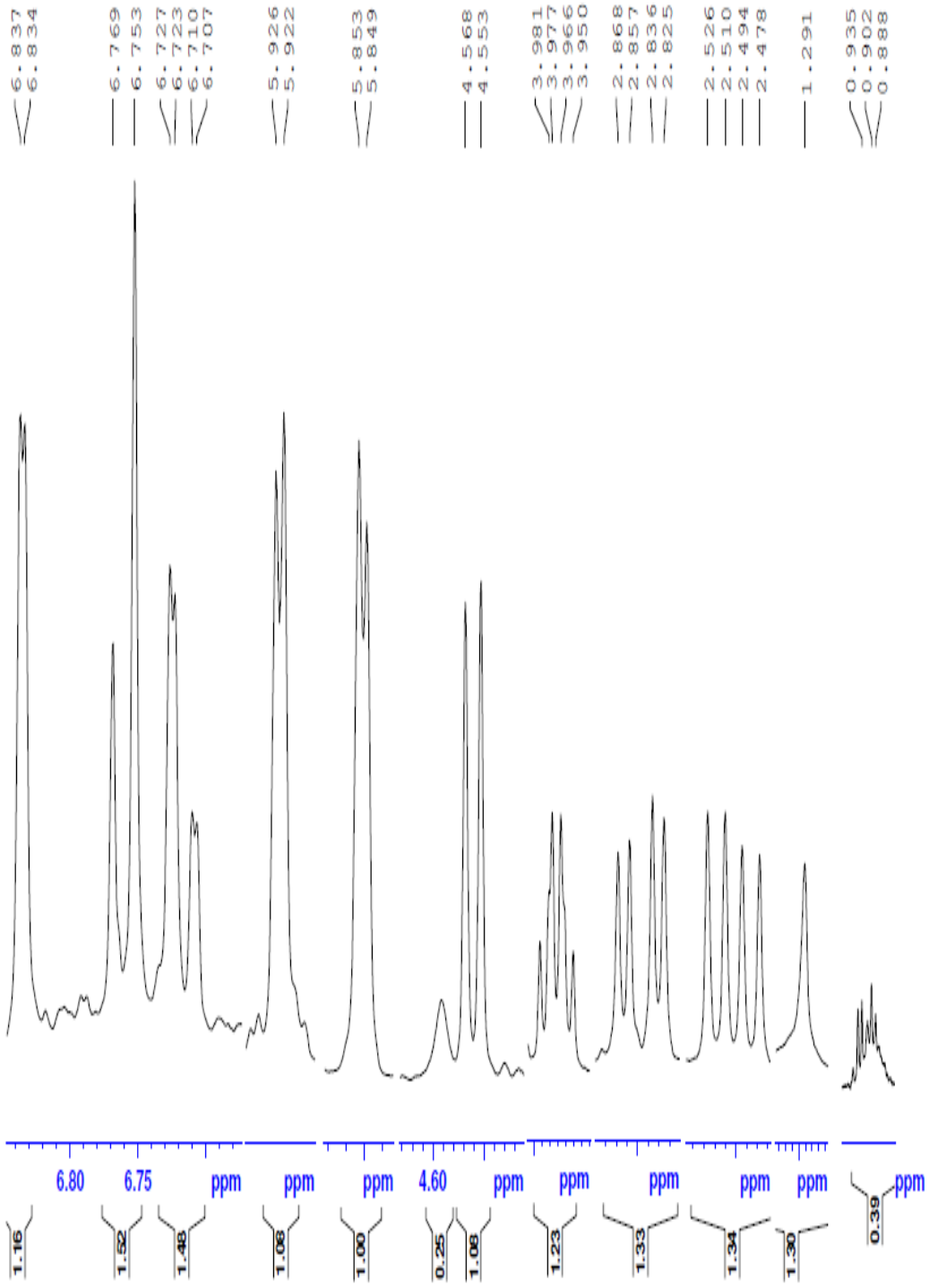


Figure 4.3a: Expansion of proton NMR spectrum of B

4.4.6 $^{1}H - ^{1}H$ COSY data of B

The COSY spectrum of B showed important cross peak between δ_H [2.53 and 2.87]; δ_H [2.53 and 3.98]; δ_H [2.87 and 3.98] and δ_H [3.98 and 4.57] (Figure 4.4)

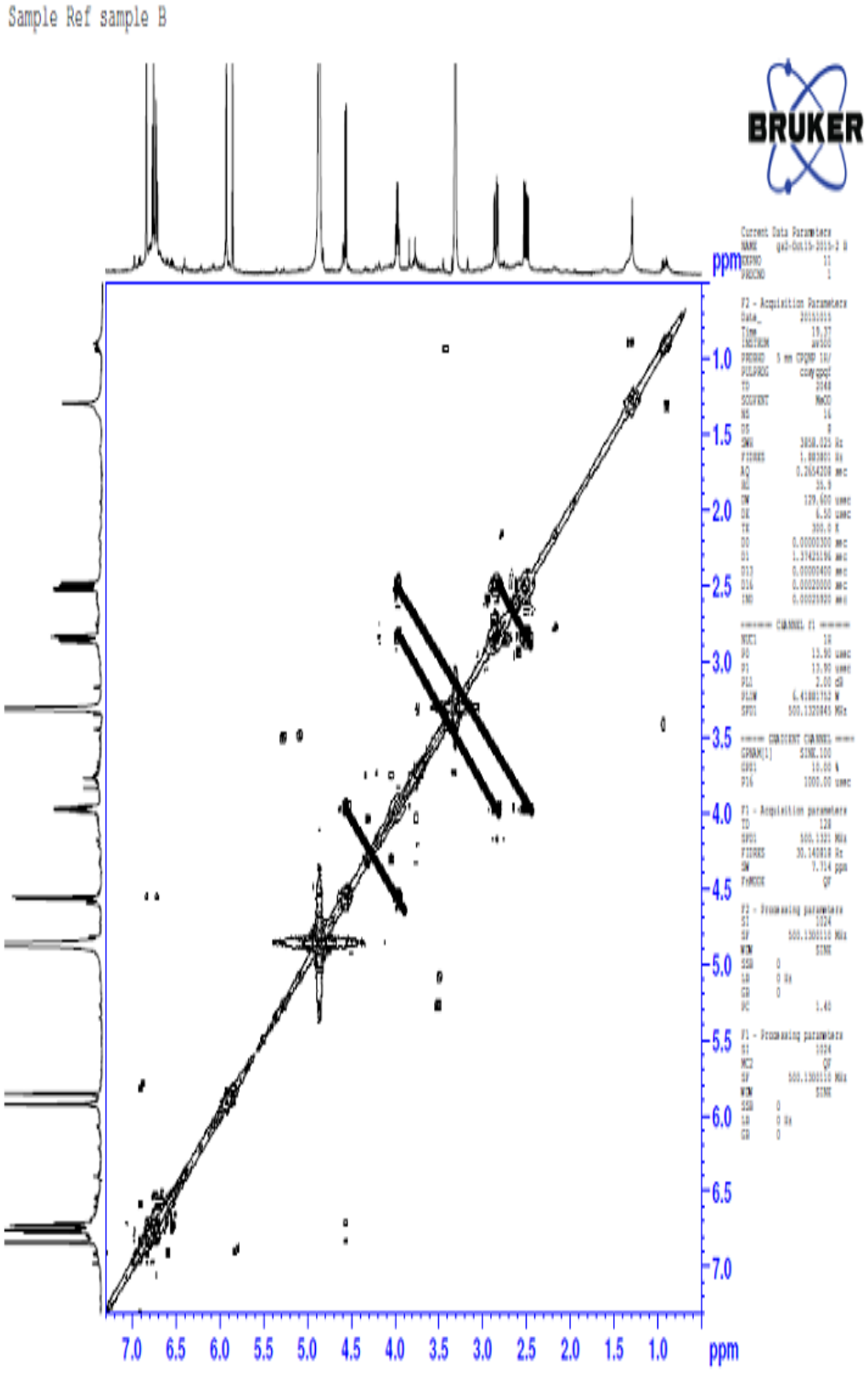


Figure 4.4: COSY spectrum of B

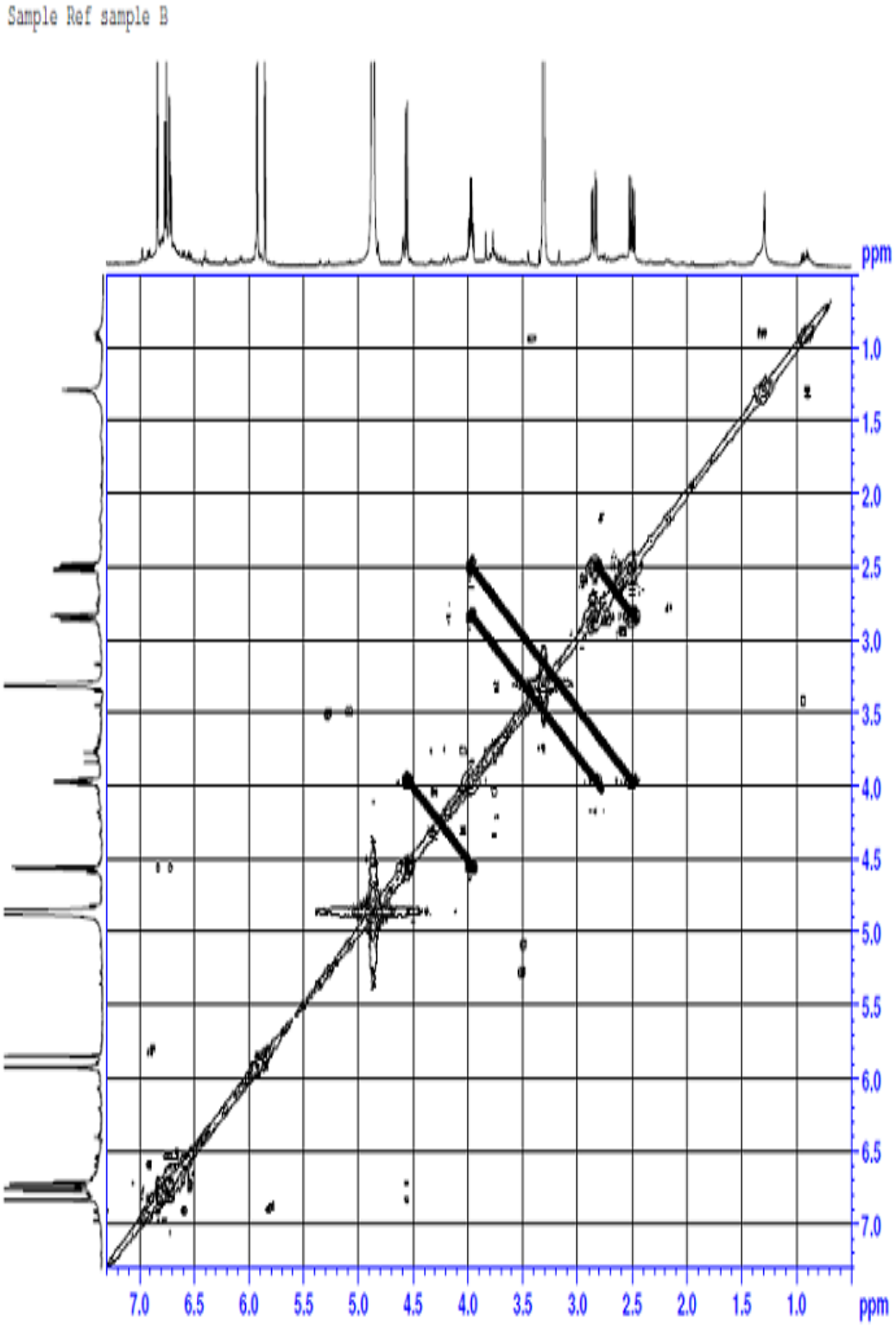


Figure 4.4a: COSY spectrum of B (with gridlines)

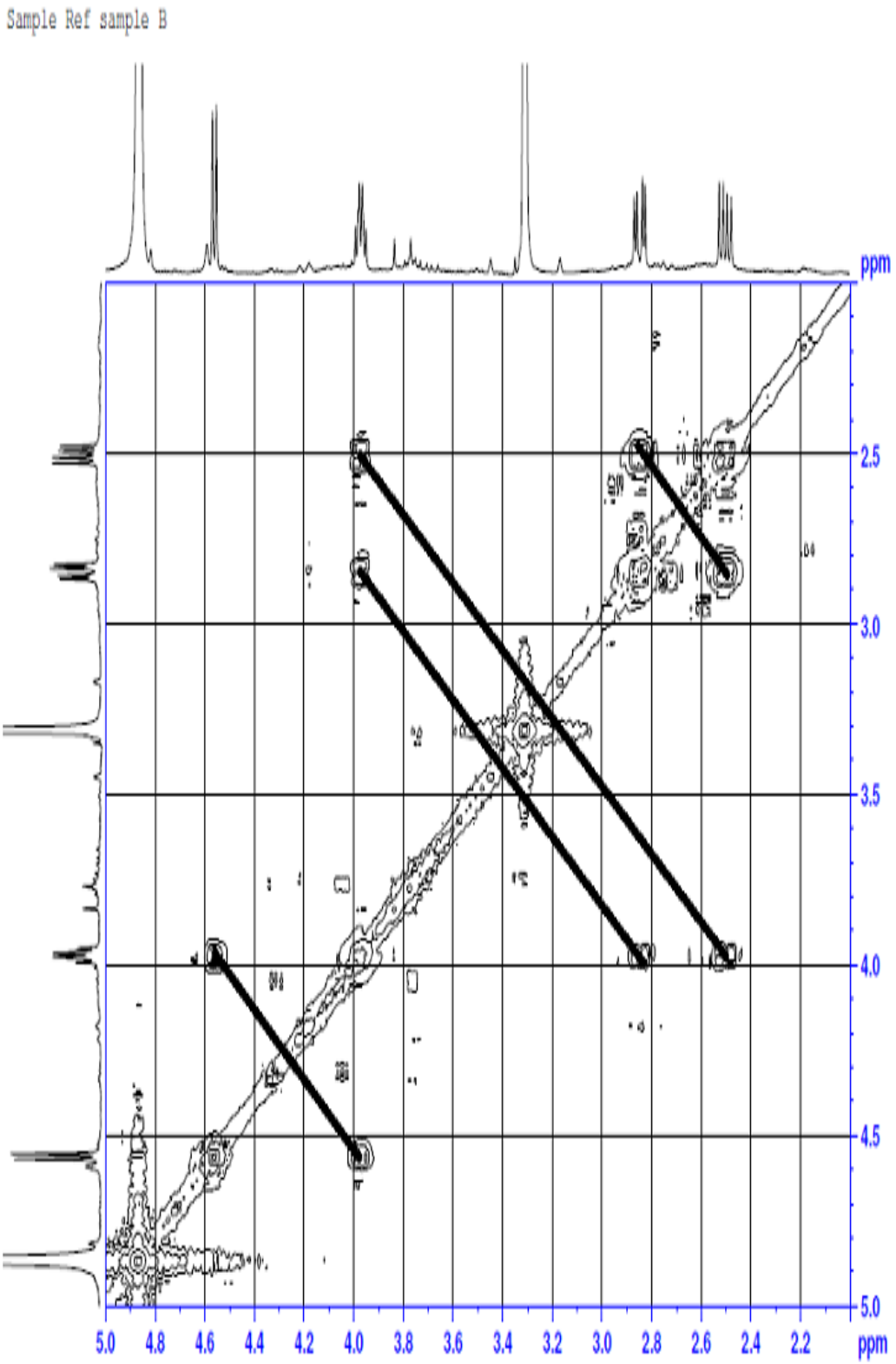


Figure 4.4b: COSY spectrum of B (expansion)

4.4.7 Mass spectrometry of compound B

The ESI-HRMS of B revealed molecular ion peak at *m/z* 291.1[M+1] with important diagnostic peaks at *m/z* 139.0 and *m/z* 273.2 (Figure 4.5a). It also revealed molecular ion peak at *m/z* 289.1[M-1] with diagnostic peaks at *m/z* 578.9, *m/z* 579.9 and *m/z* 869.0 (Figure 4.5b).

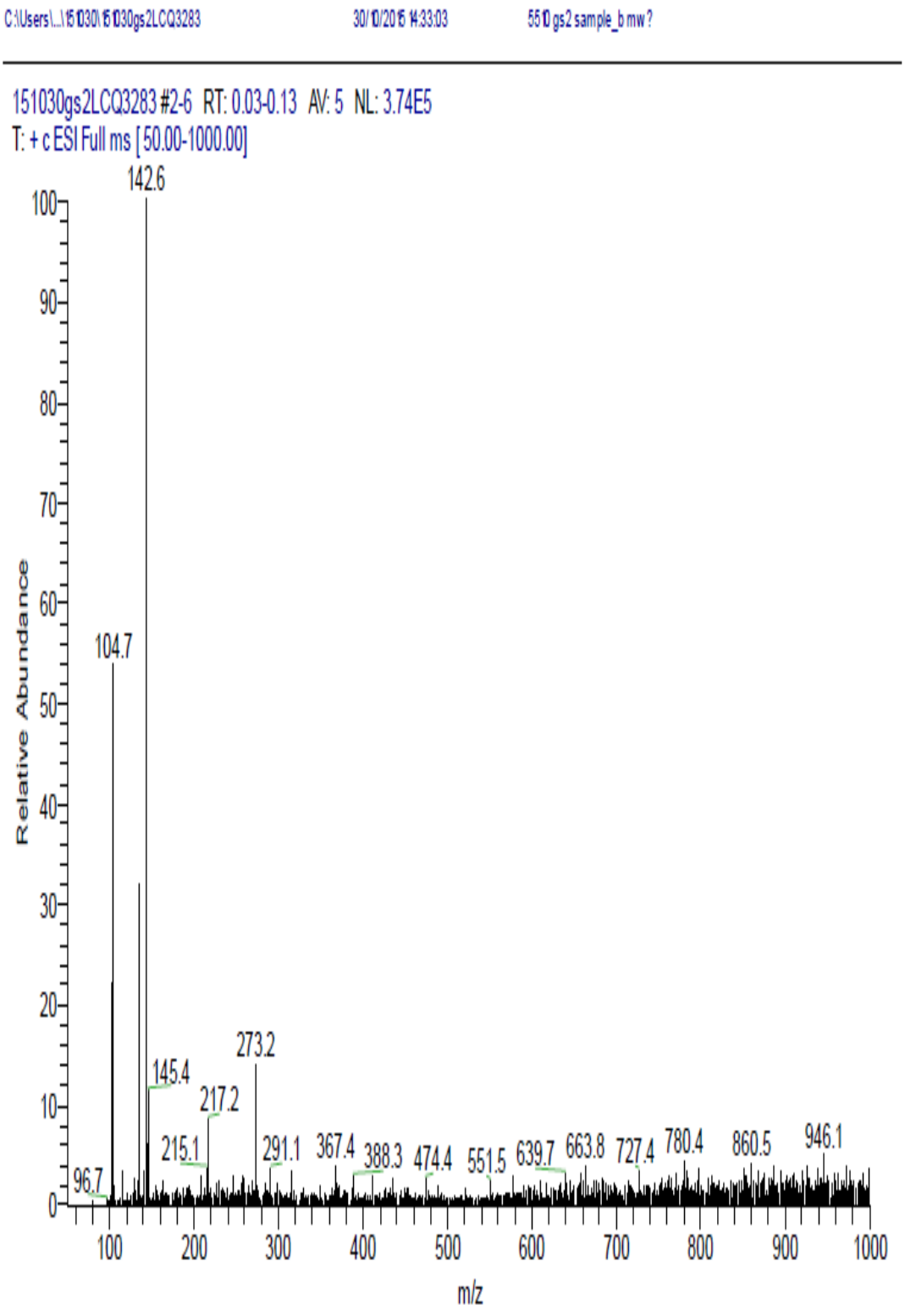


Figure 4.5a: Mass spectrum of B in the positive ion mode

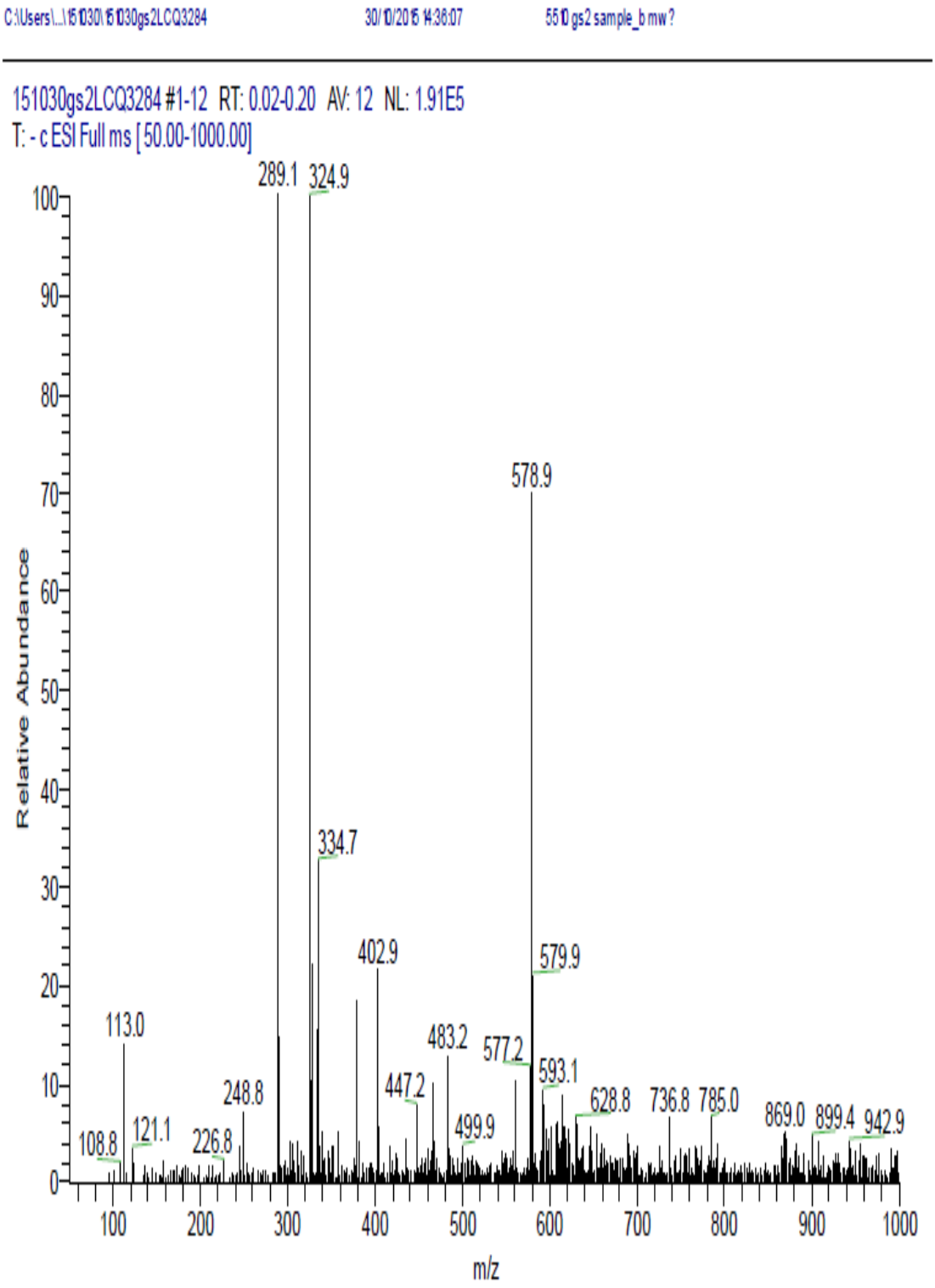


Figure 4.5b: Mass spectrum of B in the negative ion mode

4.5 Chromatographic Separation of n-butanol Fraction

A total of 68 collections, 50 mL aliquots each was made from n-butanol fraction column and similar collections were pooled together based on their TLC profile using solvent system of ethyl acetate : chloroform : methanol : water in the ratio 15 : 8 : 4 : 1 to obtain thirteen major fractions coded A-M (Table 4.3).

Table 4.3: Result of column chromatography of n-butanol fraction (nBF)

Fraction	Collections	Solvent system	No. of spots
A	1-6	Ethyl acetate 100%	3
B	7-19	E:M 98:2	5
C	20-23	E:M 96:4	6
D	24-29	E:M 94:6	No clear spot
E	30-34	E:M 92:8	4
F	**35-41**	**E:M 90:10**	**3**
G	42-46	E:M 85:15	>6
H	47-52	E:M 85:15	No clear spot
I	53-57	E:M 85:15	No clear spot
J	58-62	E:M 84:16	4
K	**63**	**E:M 82:18**	**2**
L	64-65	E:M 80:20	5
M	66-68	methanol 100 %	>6

4.6 Isolation and Characterization of Compound S_1

Fraction F (12.2 mg) from n-butanol fraction column corresponding to solvent system ethyl acetate : methanol 90 : 10 containing three major spots was subjected to repeated gel filtration in sephadex using absolute methanol and the progress of purification was monitored on TLC plate (Plate V). This afforded 4.8 mg of yellow crystalline solid that was coded 'S_1'.

Plate V: Progress of isolation of S_1

4.6.1 Colour, chemical test and melting point of S_1

S_1 was isolated as yellow crystalline solid of melting point 243-245°C. S_1 was positive to Shinoda test.

4.6.2 UV spectral data of S_1

The UV spectrum of S_1 showed two absorption bands within the UV region (λ_{max} 226 nm and 257 nm) and two absorption bands within the visible region (λ_{max} 346 nm and 363 nm in methanol (Figure 4.6).

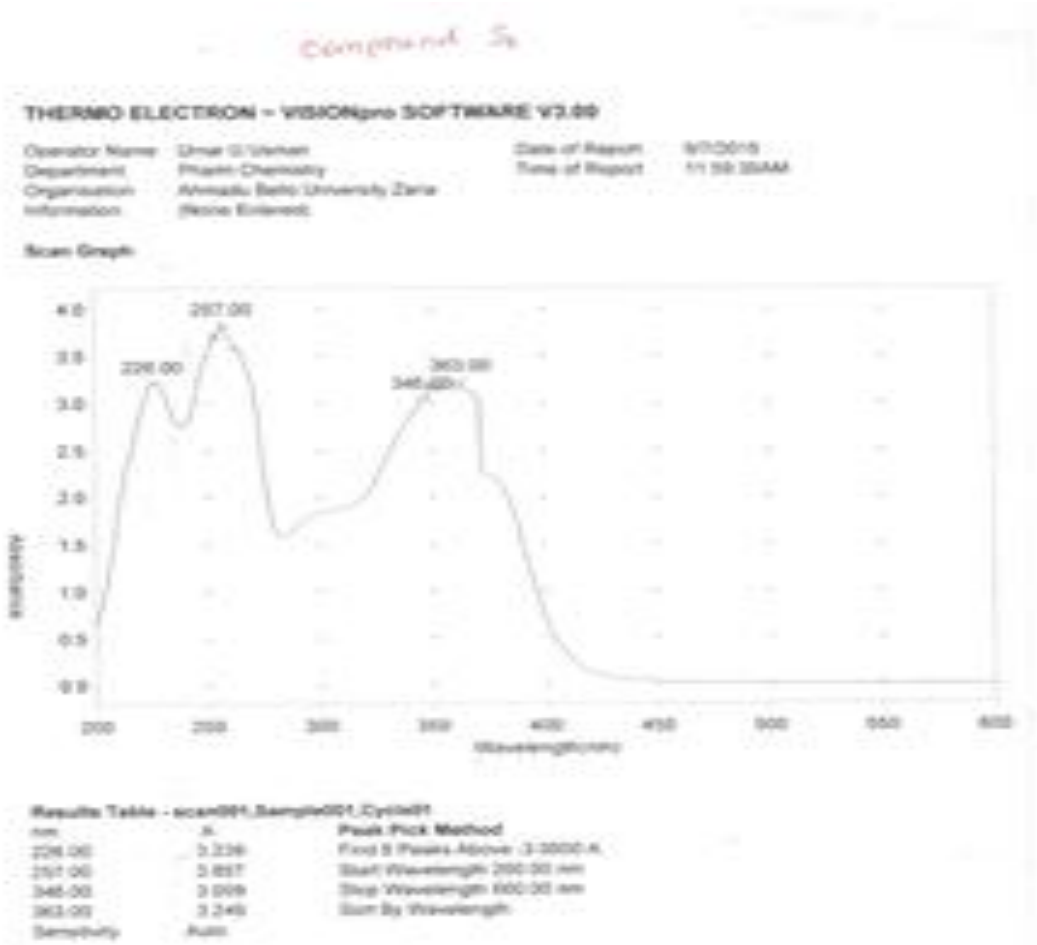
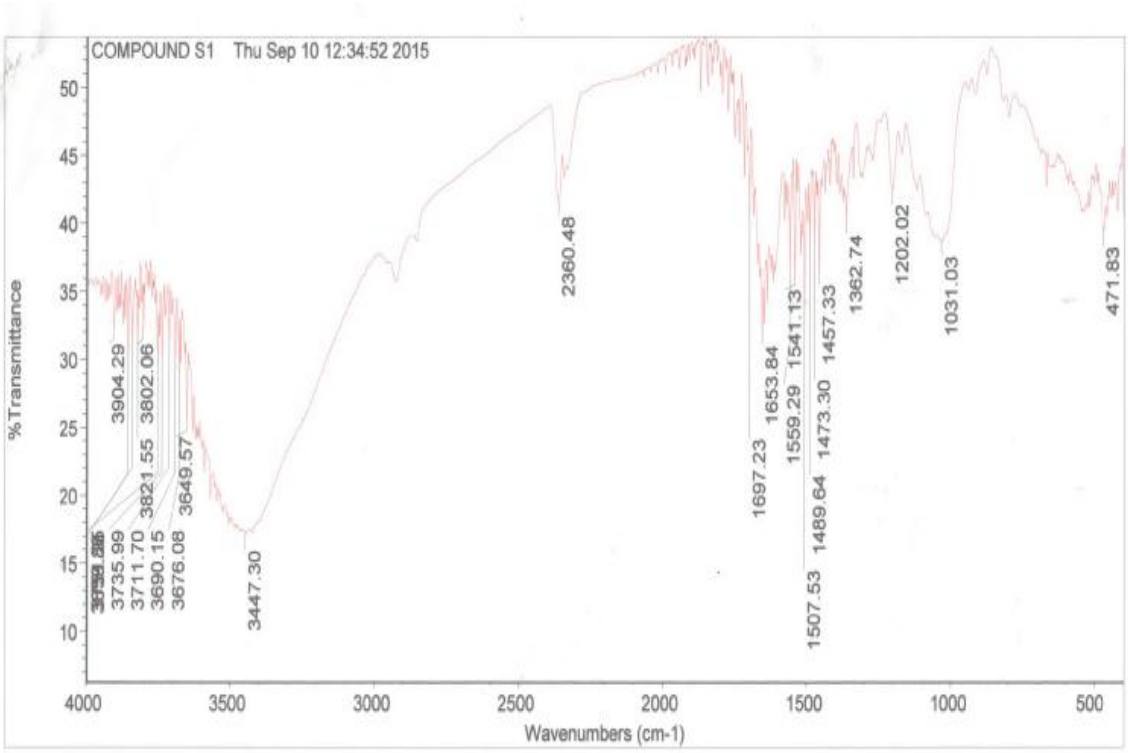


Figure 4.6: UV spectrum of S_1

4.6.3 IR spectral data of S_1

The IR spectrum of S_1 showed two important peaks at 1697 cm^{-1} and 3447 cm^{-1} on a KBr disc (Figure 4.7).



Thu Sep 10 12:43:17 2015 (GMT+01:00)
FIND PEAKS:
Spectrum: COMPOUND S1 Thu Sep 10 12:34:52 2015
Region: 4000.00 400.00
Absolute threshold: 42.581
Sensitivity: 30

Figure 4.7: IR spectrum of S_1

4.6.4 Proton NMR spectral data of S_1

The ^1H NMR spectrum revealed signals for the presence of ten aromatic protons at δ_H 6.19 (2H), 6.38 (2H), 6.87 (2H), 7.59 (2H), 7.71 (1H) and 7.84 (1H), two anomeric proton signals at 5.15 and 5.24 (1H each) and overlapping carbinol proton signals at δ_H 3.22-3.85 (10H) (Figure 4.8)

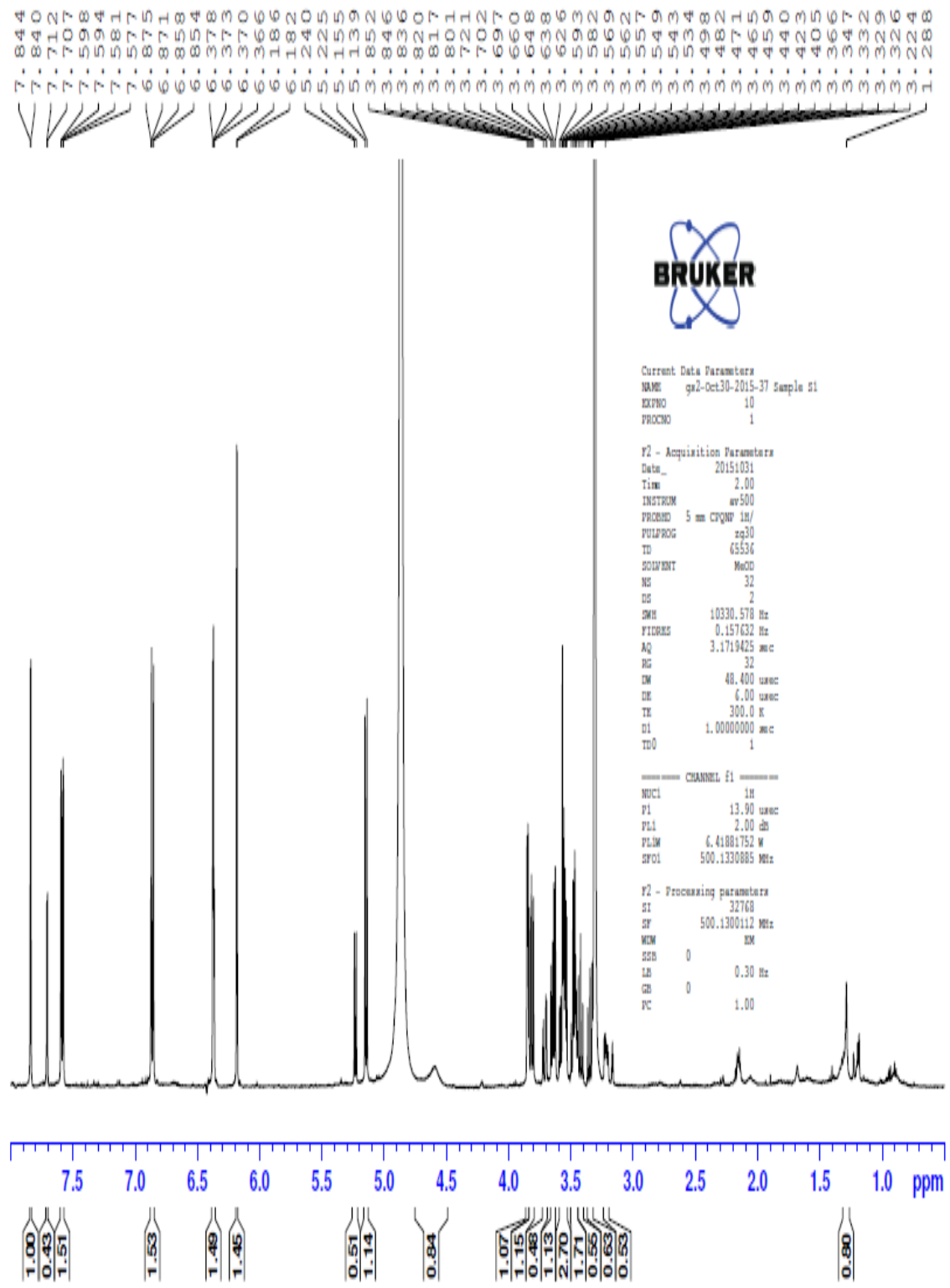


Figure 4.8: Proton NMR spectrum of S_1

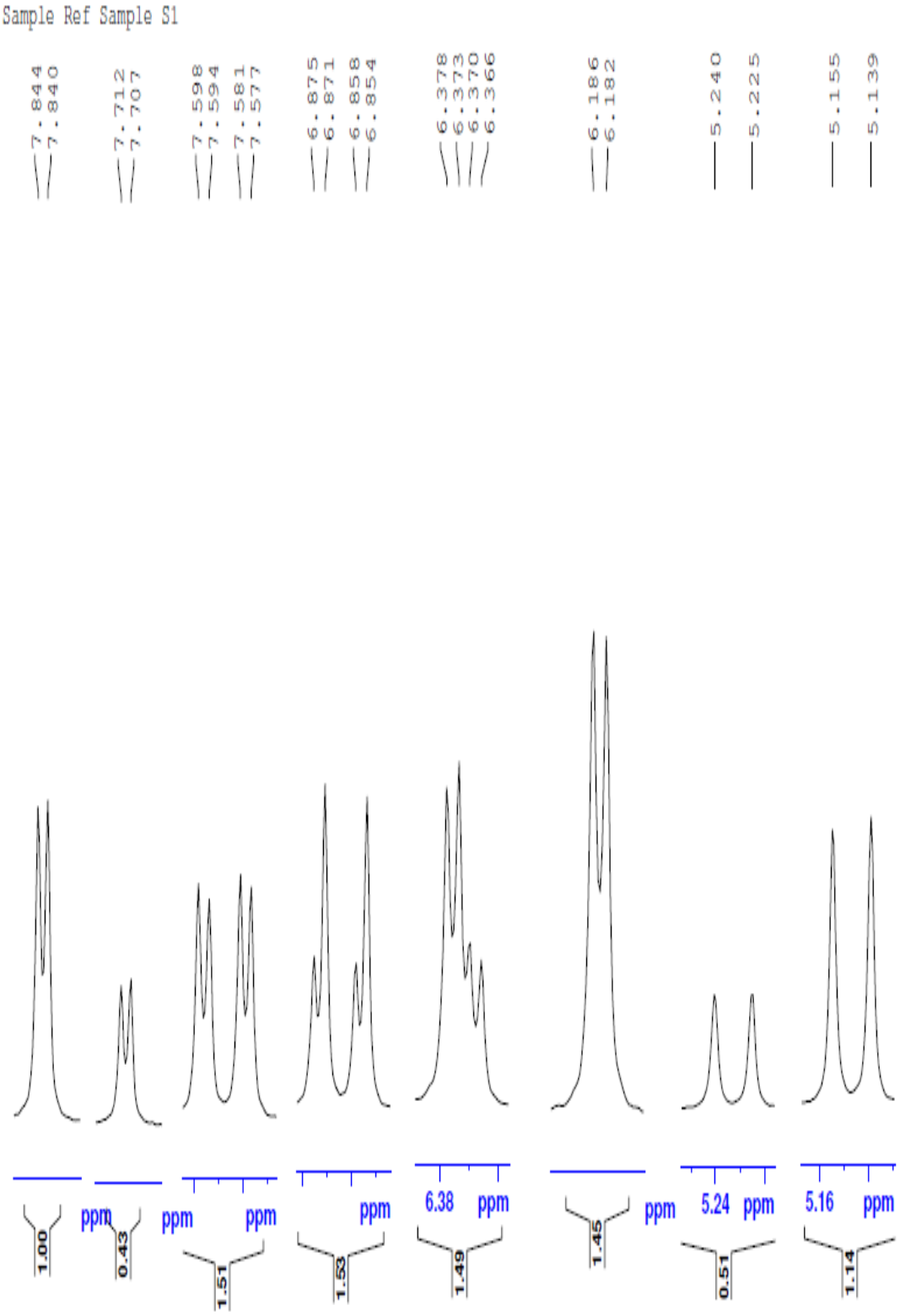


Figure 4.8a: Proton NMR spectrum of S_1 (aromatic region)

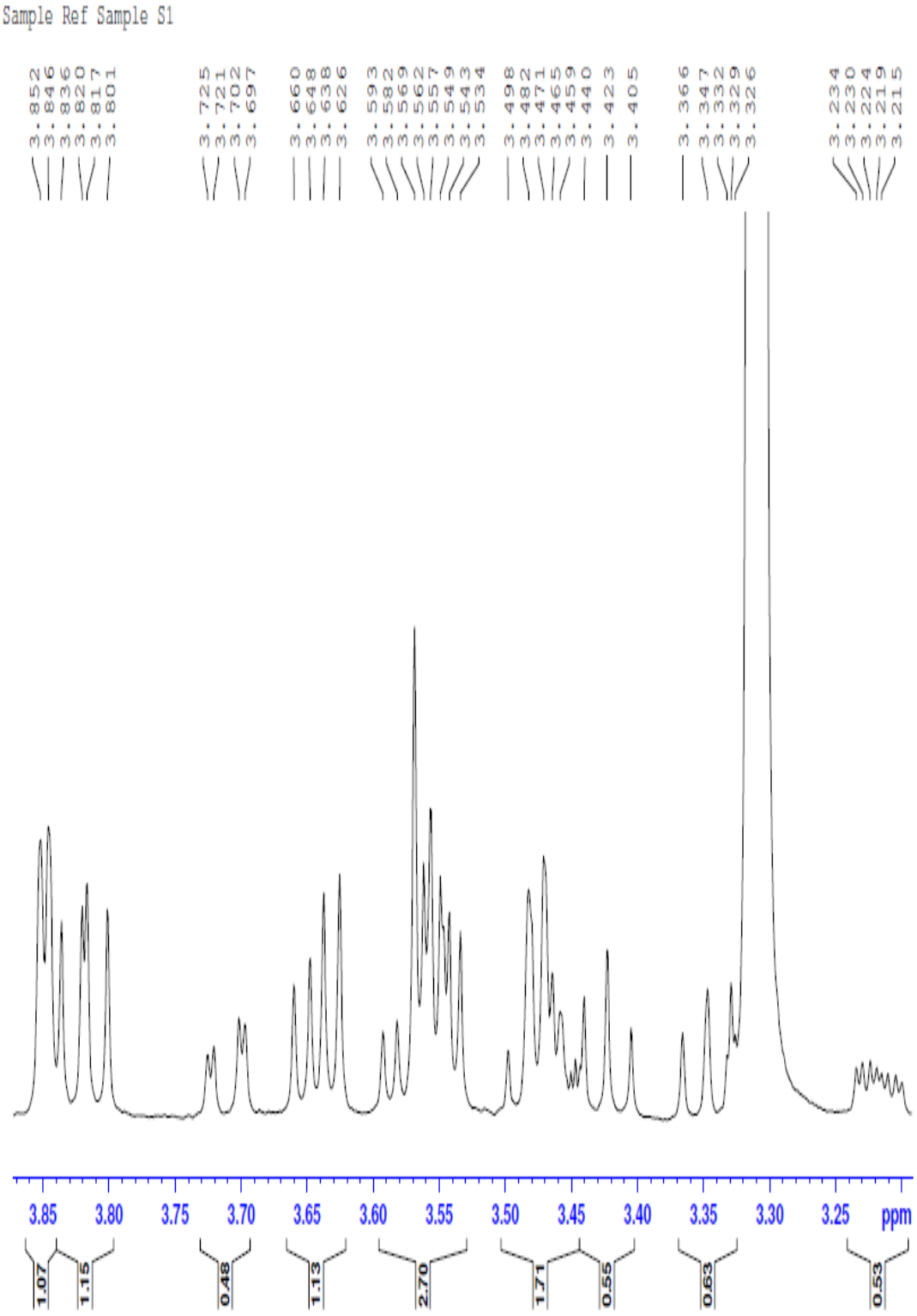


Figure 4.8b: Proton NMR spectrum of S_1 (sugar region)

4.6.5 Carbon-13 NMR and DEPT experiment spectral data of S_1

The proton noise decoupled ^{13}C NMR disclosed 42 peaks most of which are paired (Figure 4.9a) and DEPT experiment revealed the presence of two methylene and 20 methine carbons resonances (Figure 4.9b)

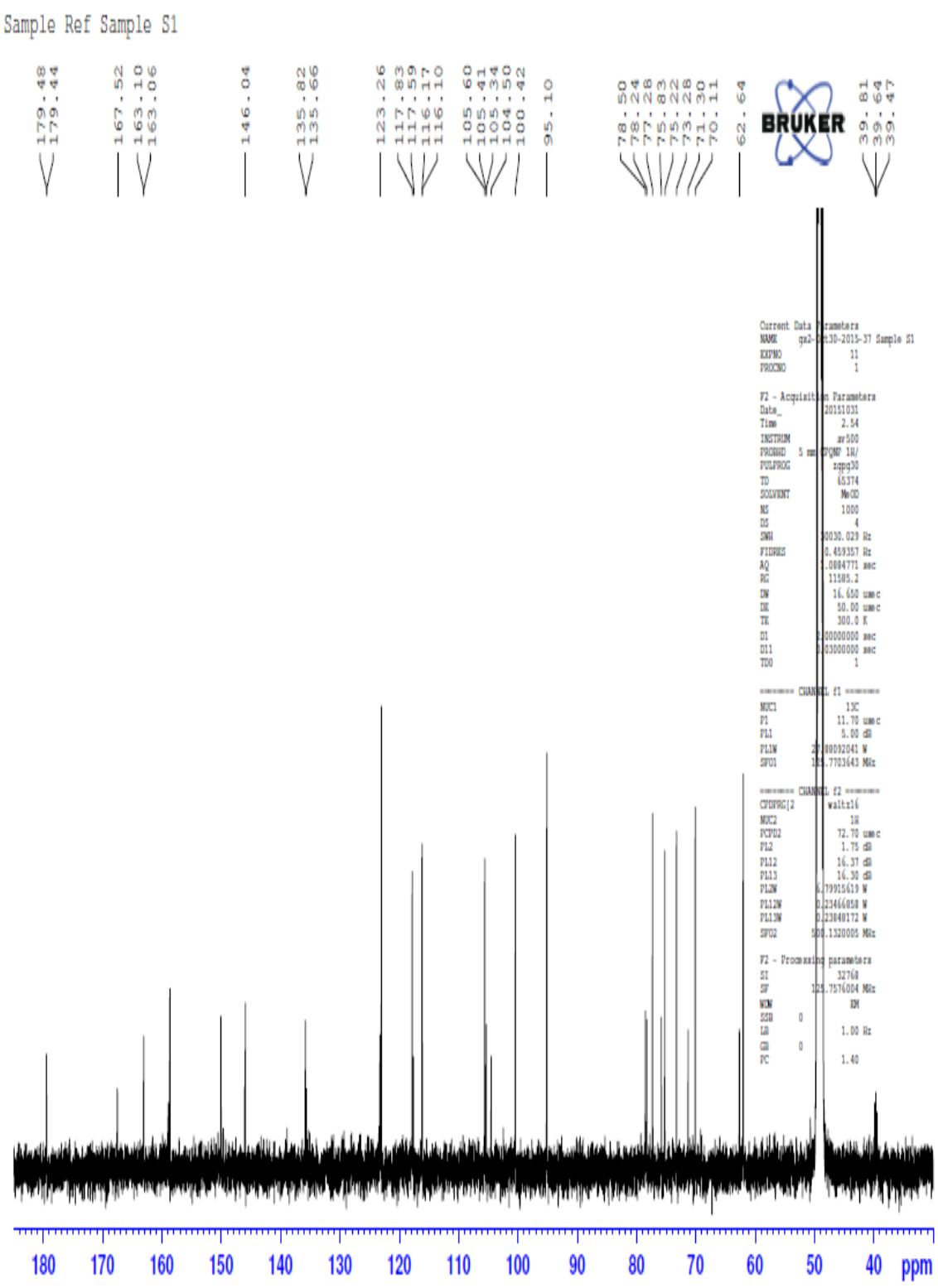


Figure 4.9a: Proton decoupled carbon-13 NMR spectrum of S_1

Sample Ref Sample S1

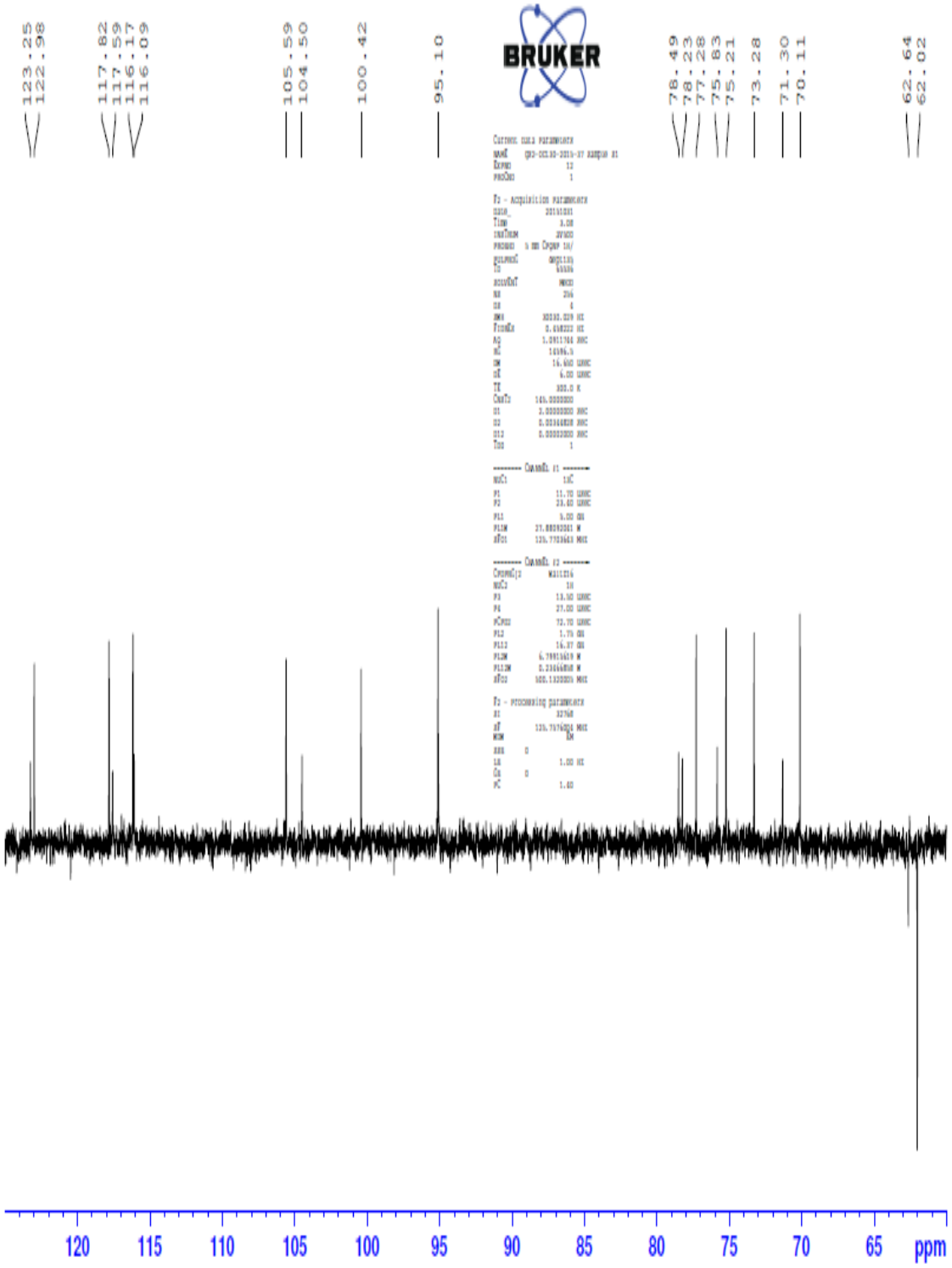


Figure 4.9b: DEPT experiment of S_1

4.6.6 $^1H - ^1H$ COSY data of S_1

The COSY spectrum of S_1 showed important cross peaks between δ_H [3.82 and 5.15] and δ_H [3.47 and 5.24] (Figure 4.10a). Other important COSY correlation occurred between carbinol protons attached to adjacent carbons in the two sugar residues (Figure 4.10b)

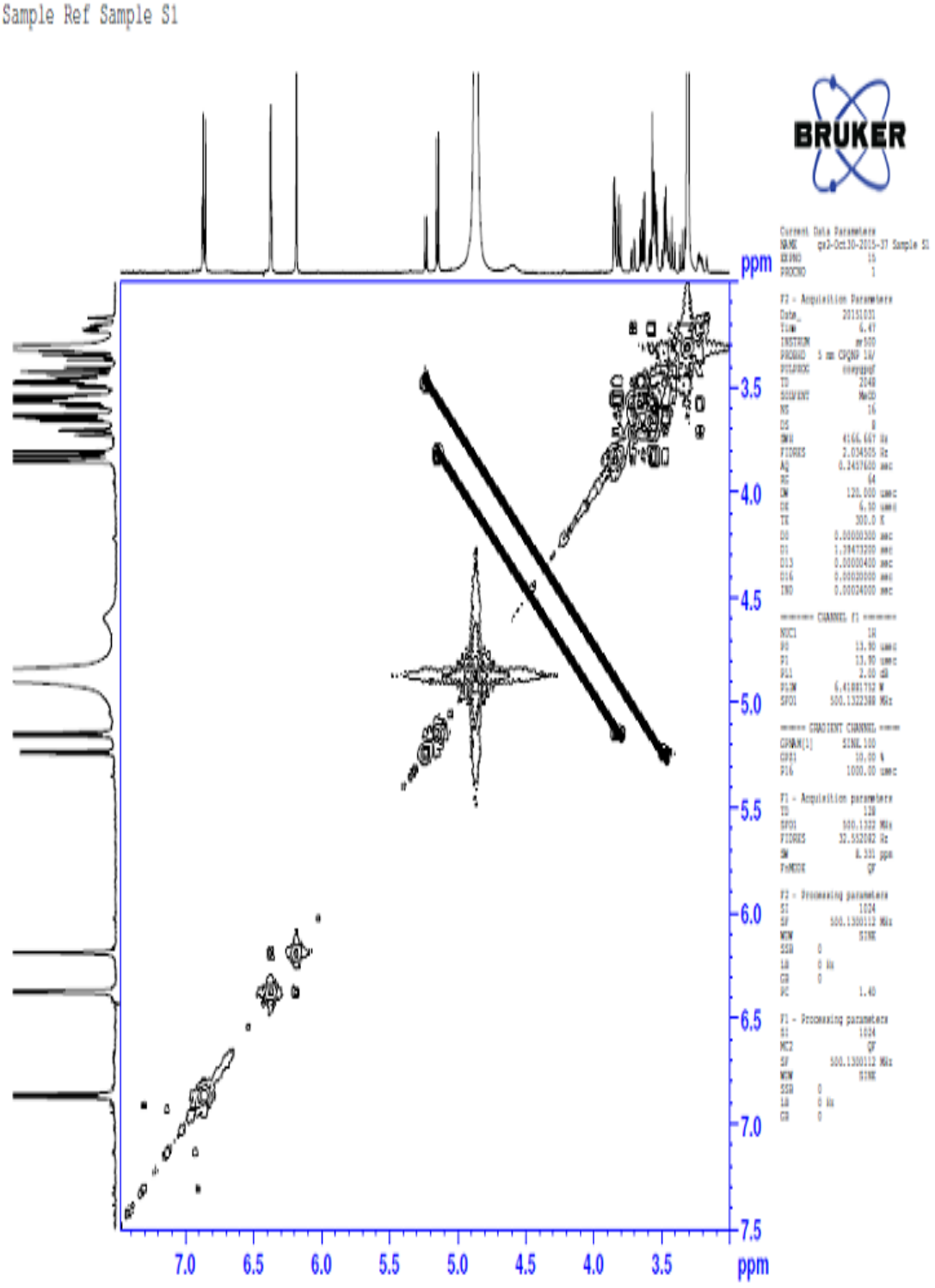


Figure 4.10a: ^{1}H-^{1}H COSY experiment of S_1

Sample Ref Sample S1

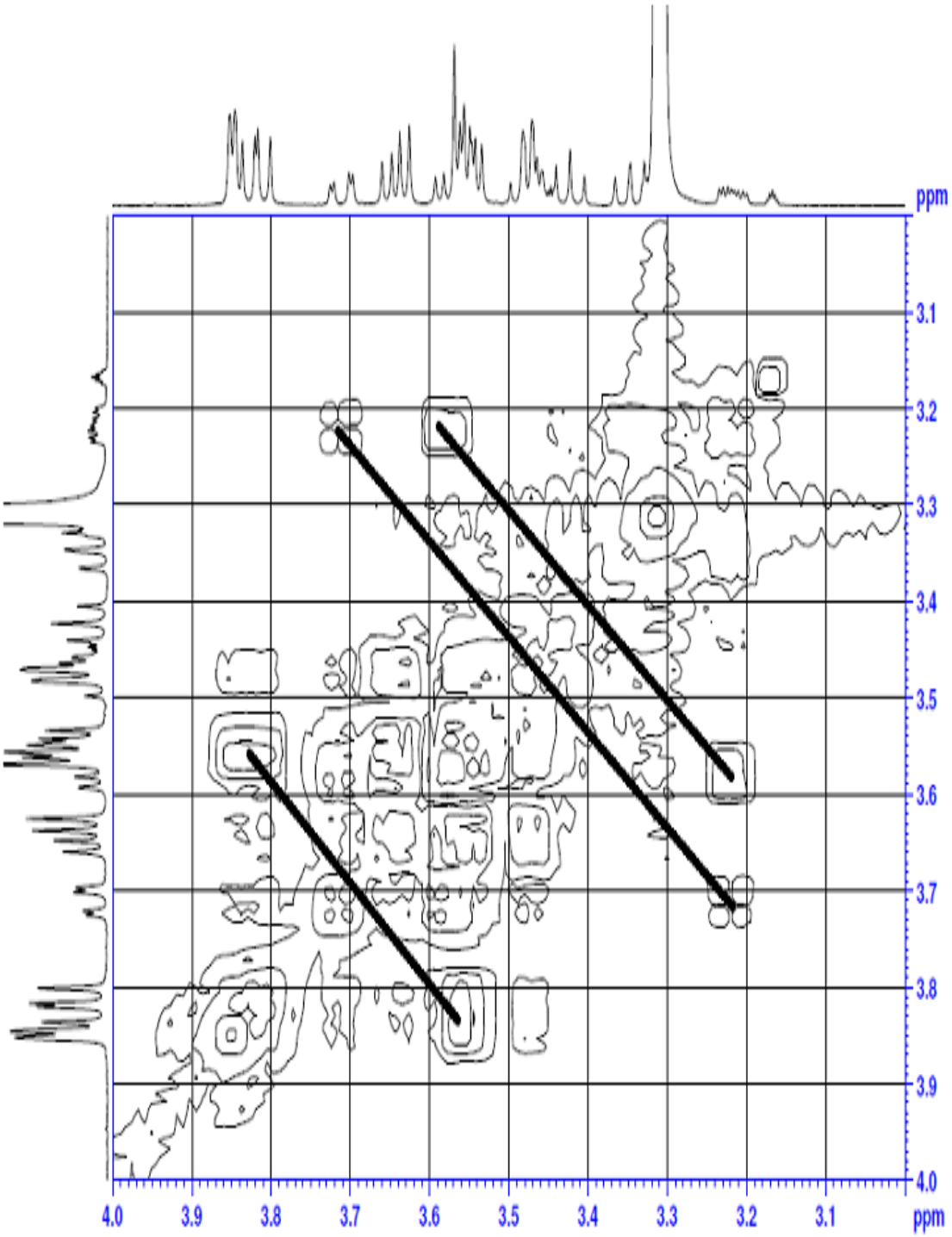


Figure 4.10b: ^{1}H-^{1}H COSY experiment of S_1 (sugar residues)

4.6.7 HMQC spectral data of S_1

The HMQC experiment is as shown in Figure 4.11 and was used to assign protons on their respective carbons (Table 4.4)

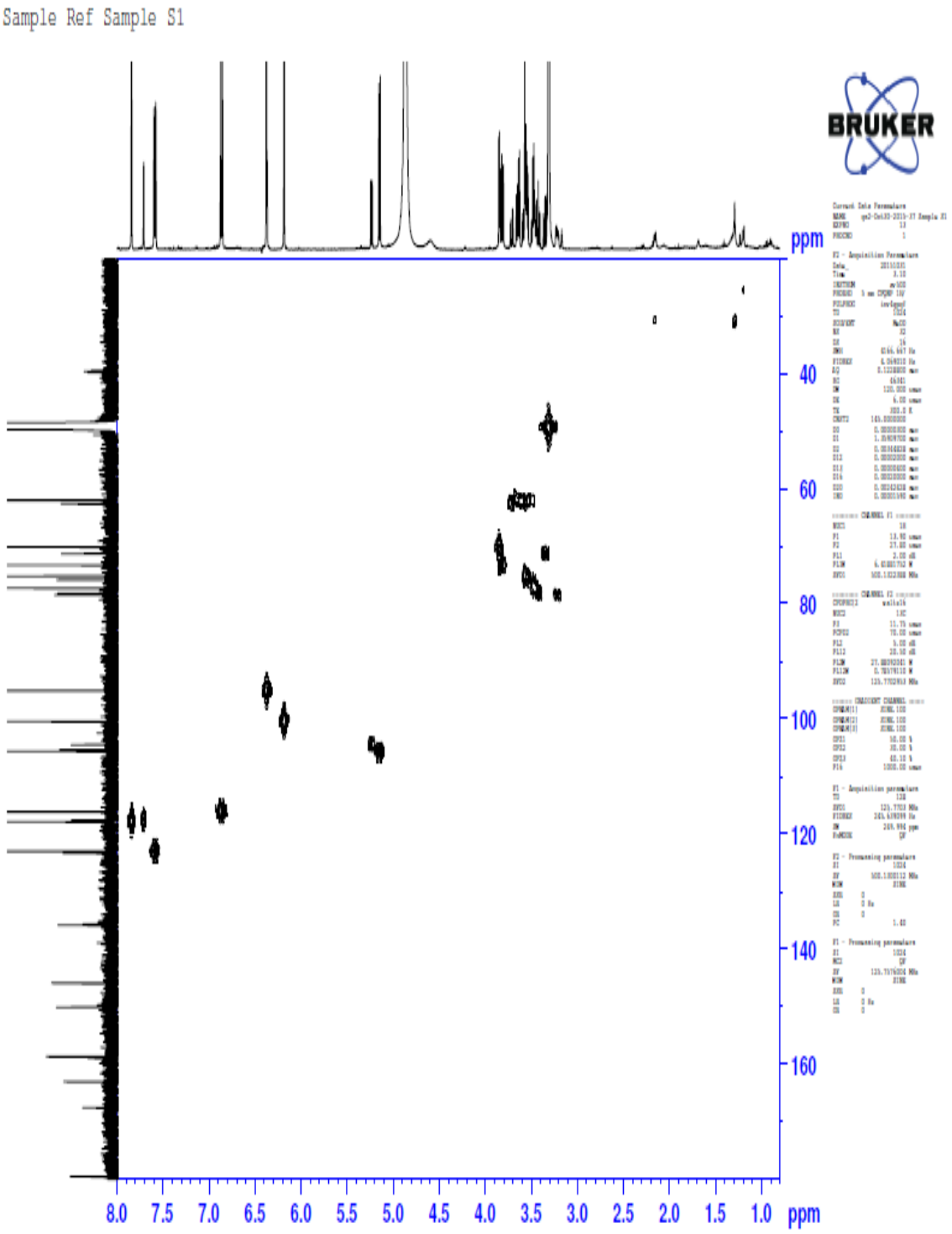


Figure 4.11: HMQC spectrum of S_1

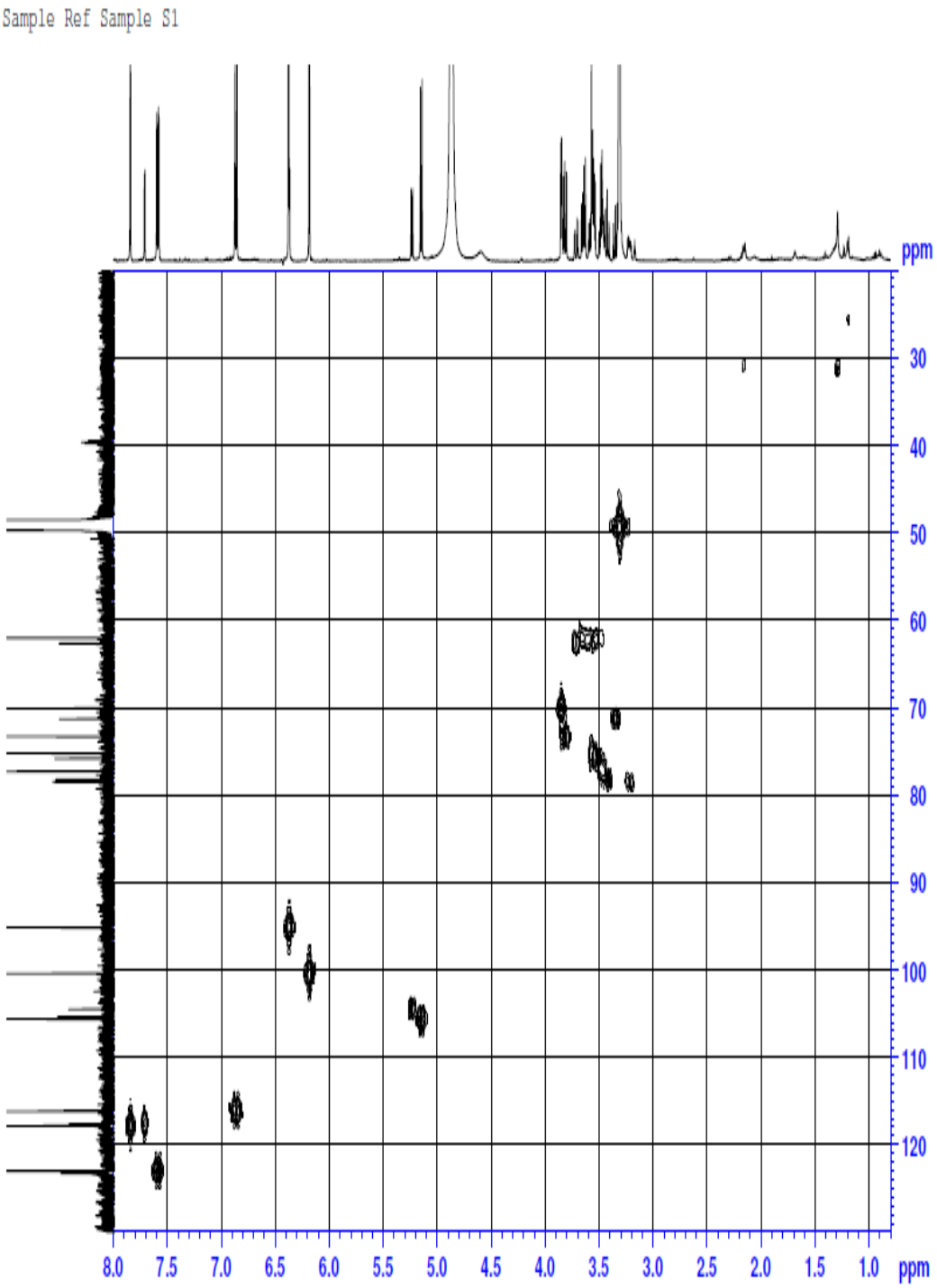


Figure 4.11a: HMQC spectrum of S_1 (expansion 1)

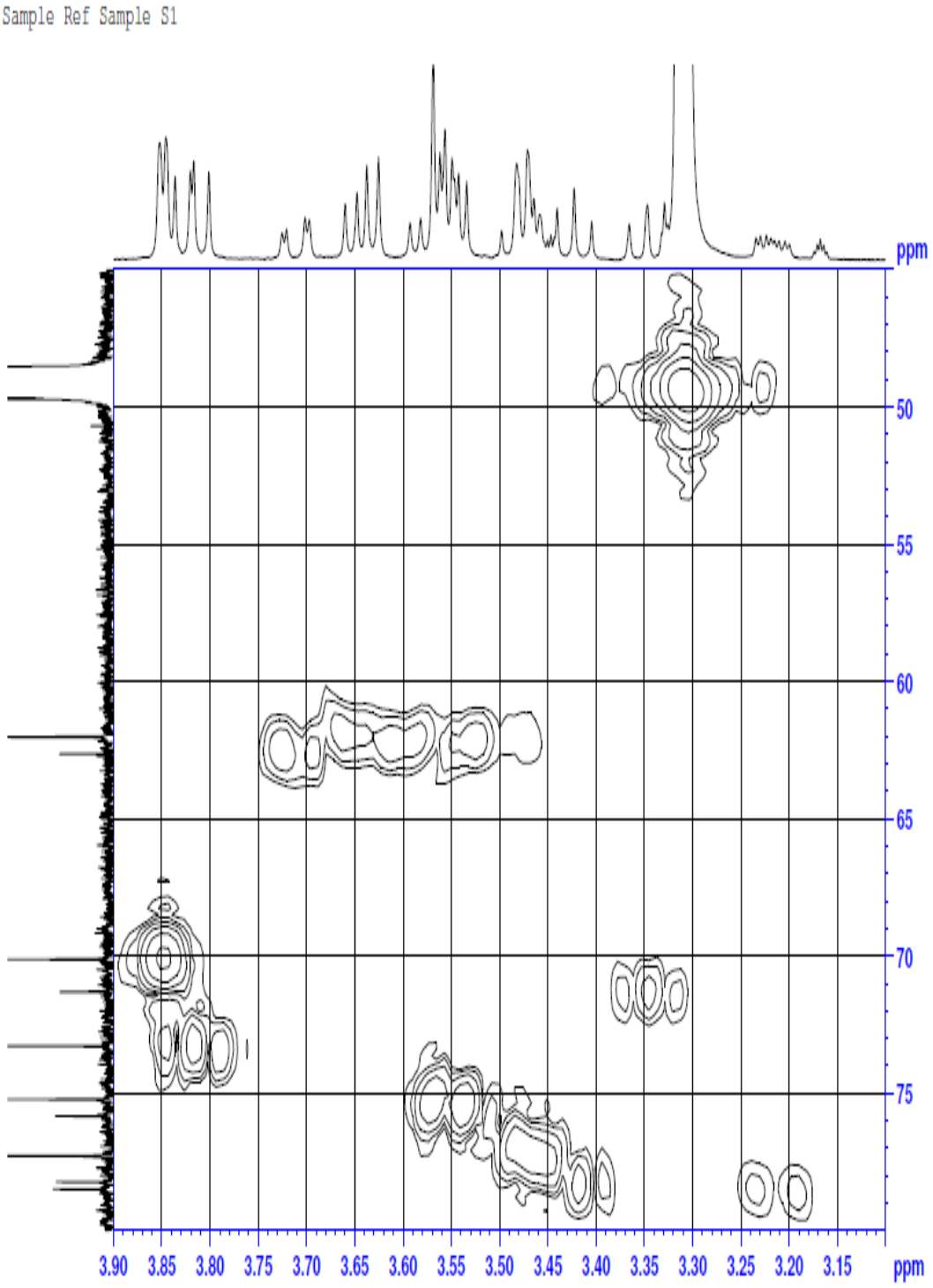


Figure 4.11b: HMQC spectrum of S_1 (sugar region)

Sample Ref Sample S1

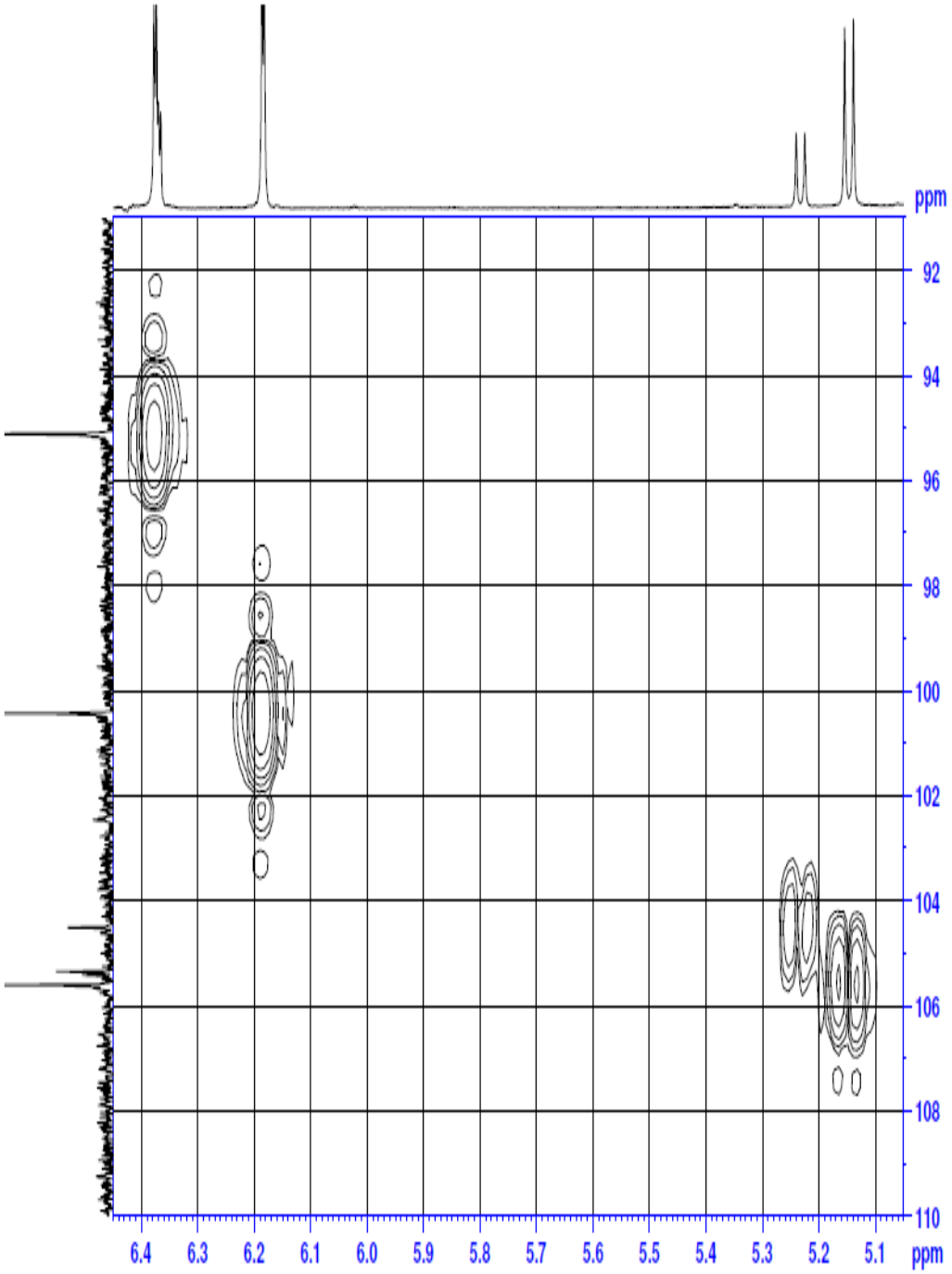


Figure 4.11c: HMQC spectrum of S_1 (aromatic region 1)

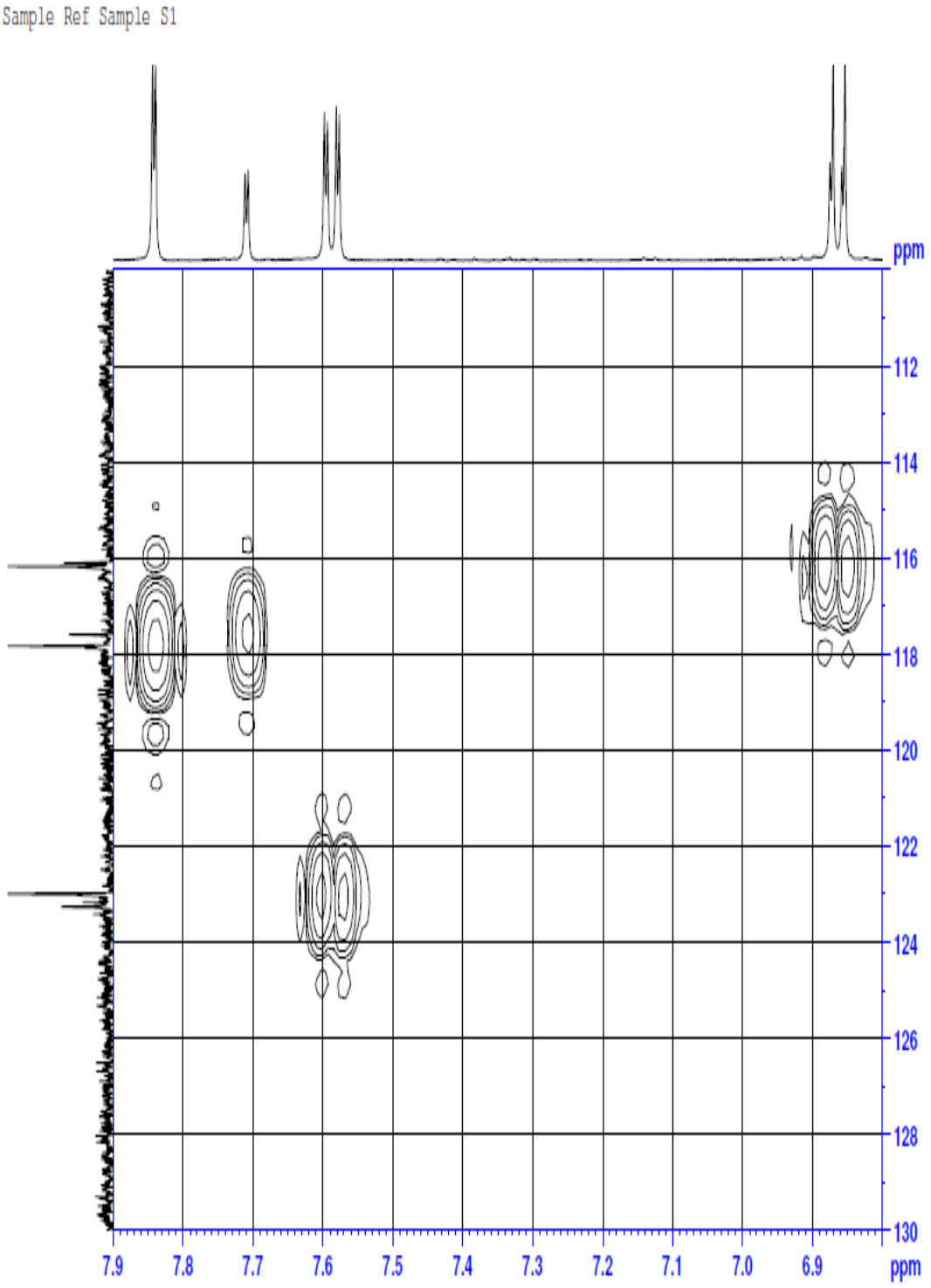


Figure 4.11d: HMQC spectrum of S_1 (aromatic region 2)

Table 4.4: HMQC and DEPT spectral data of S_1

Position	δ_C	δ_H	DEPT
1			
2	158.98		C
3	135.66		C
4	179.44		C
5	163.06		C
6	100.42	6.19	CH
7	167.52		C
8	95.10	6.38	CH
9	158.98		C
10	105.34		C
1'	123.26		C
2'	117.83	7.84	CH
3'	146.04		C
4'	150.50		C
5'	116.09	6.88	CH
6'	123.25	7.59	CH
1''	123..26		C
2''	117.59	7.71	CH
3''	146.04		C

4''	158.50		C
5''	116.17	6.88	CH
6''	122.98	7.59	CH
1'''			
2'''	159.10		C
3'''	135.82		C
4'''	179.48		C
5'''	163.10		C
6'''	100.42	6.19	CH
7'''	167.52		C
8'''	95.10	6.38	CH
9'''	159.10		C
10'''	105.41		C
1*	105.59	5.15	CH
2*	75.83	3.54	CH
3*	78.23	3.45	CH
4*	70.11	3.85	CH
5*	73.28	3.82	CH
6*	62.46	3.72	CH_2
1**	104.5	5.24	CH
2**	75.21	3.57	CH
3**	78.49	3.46	CH
4**	71.30	3.84	CH

5**	77.28	3.48	CH
6**	62.02	3.72	CH_2

4.6.8 HMBC spectral data of S_1

The HMBC experiment is as shown in Figure 4.12 and was used to establish connectivity between the various fragments of the molecule (Table 4.5)

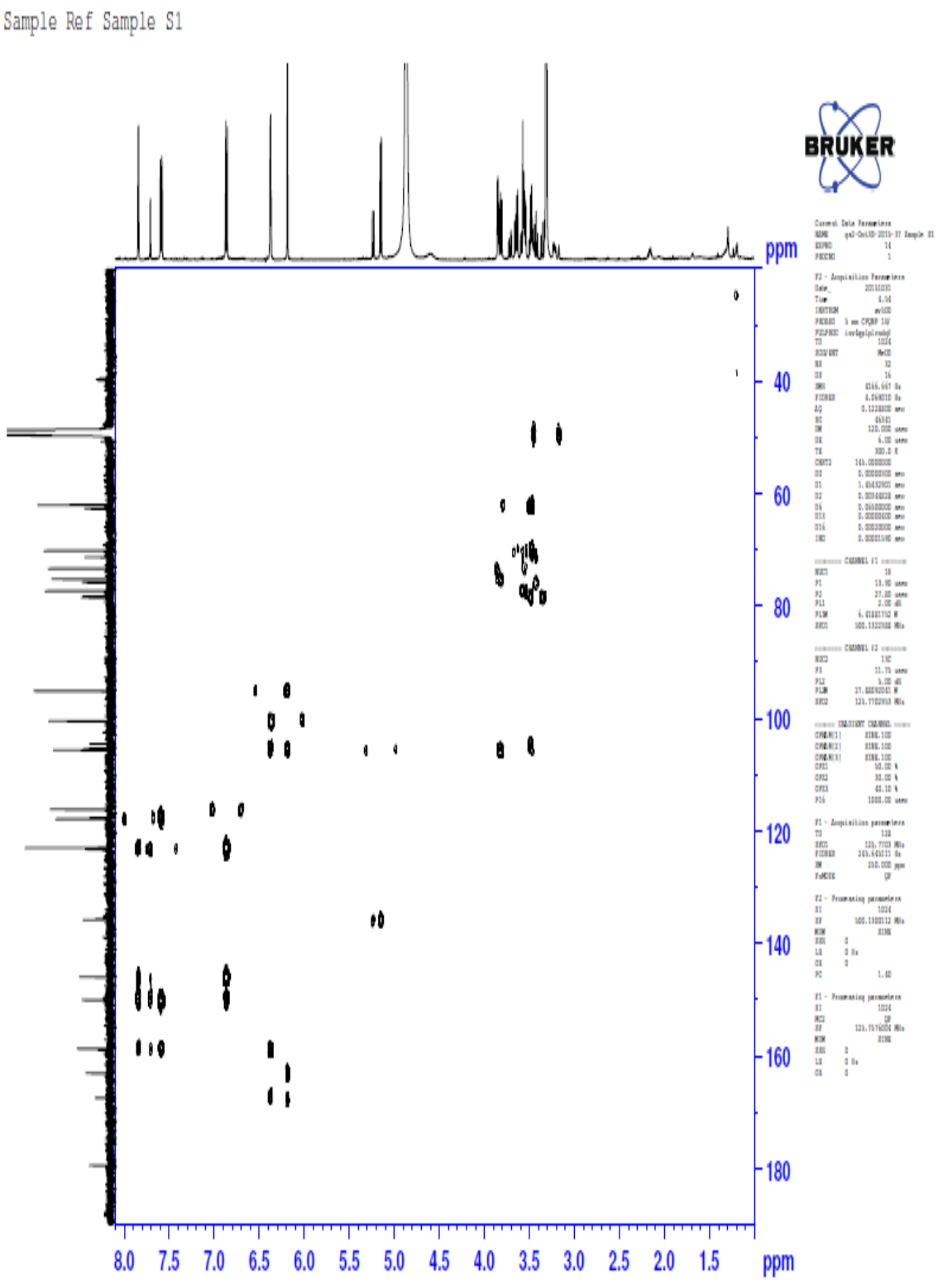


Figure 4.12: HMBC experiment of S_1

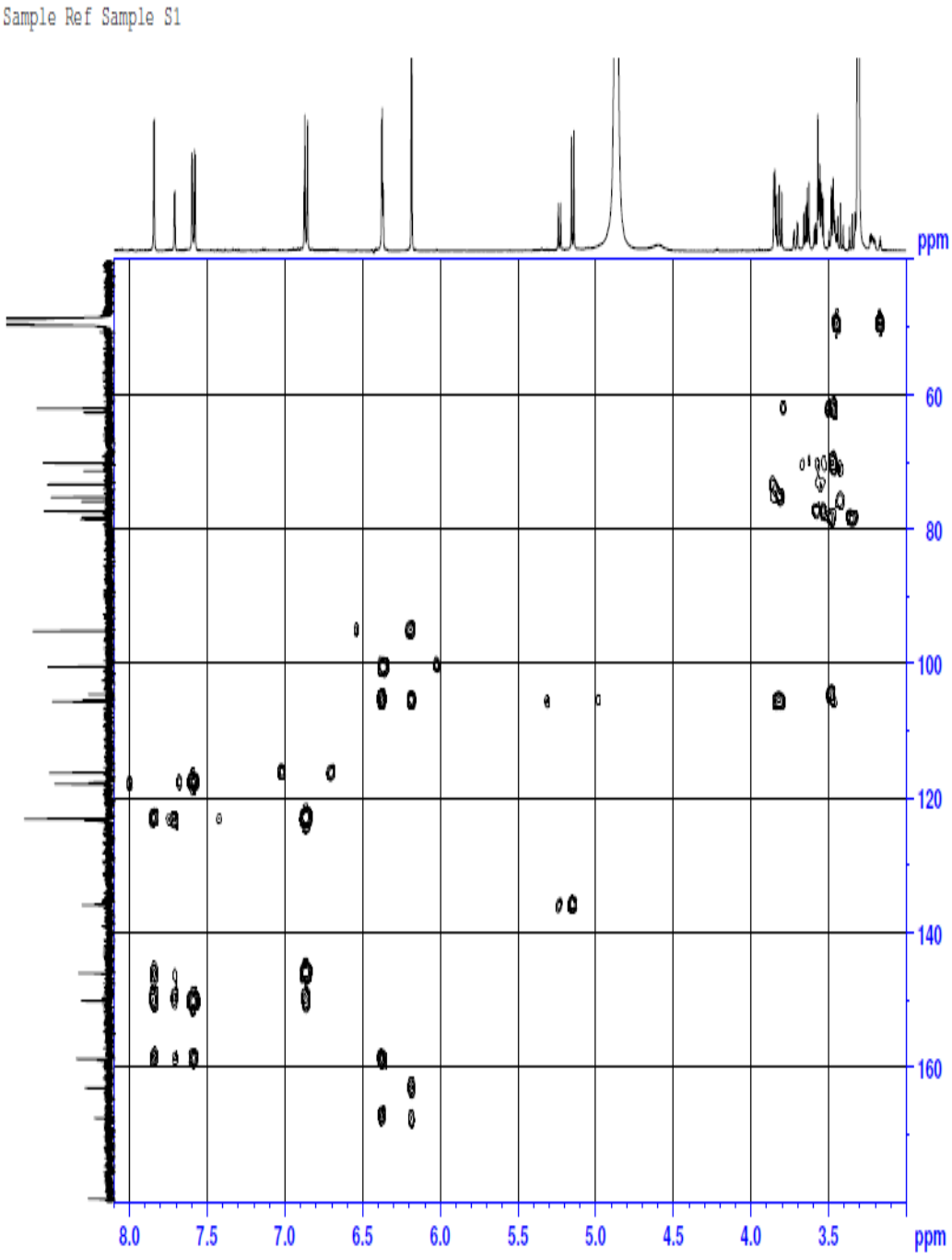


Figure 4.12a: HMBC experiment of S_1 (expansion 1)

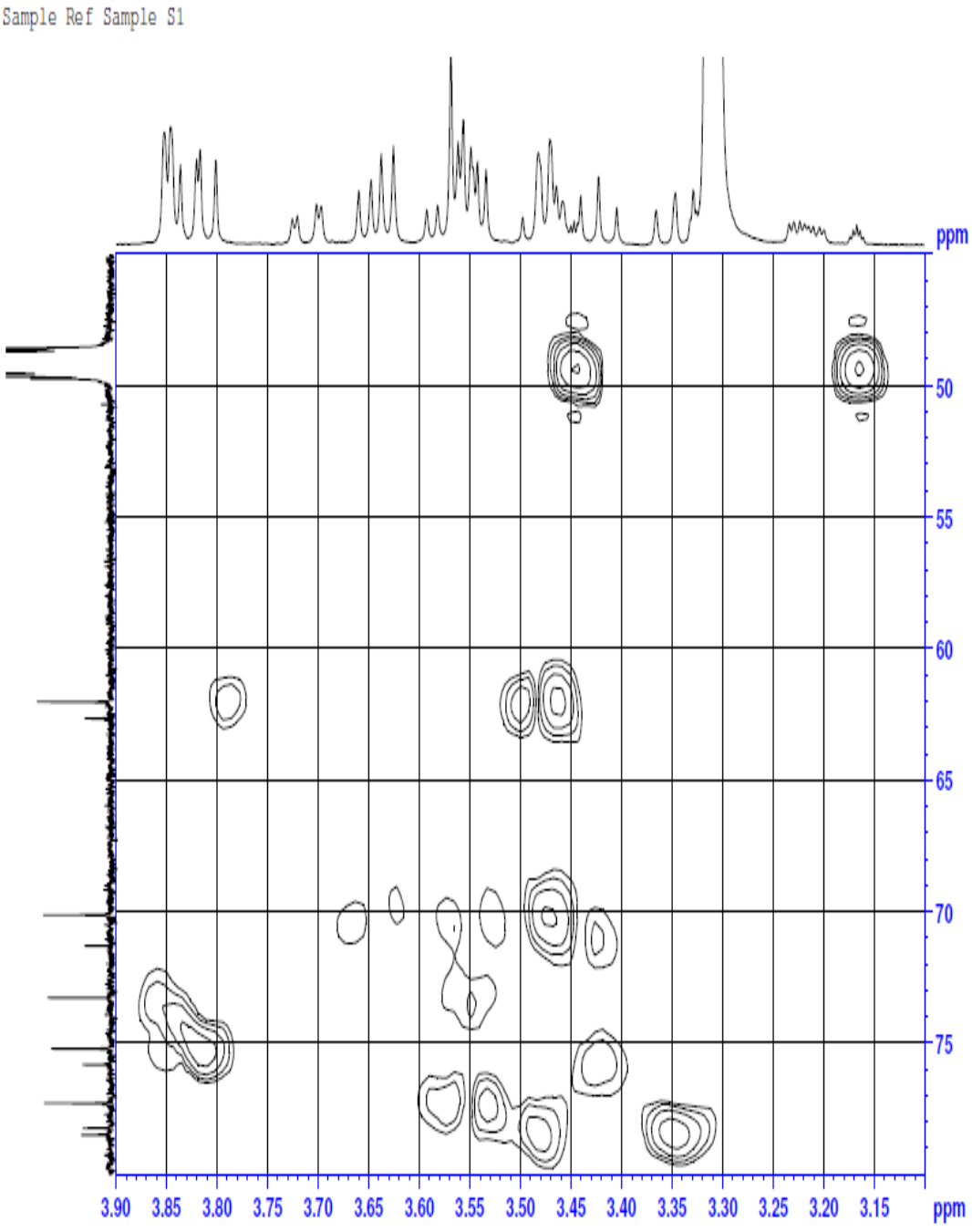


Figure 4.12b: HMBC experiment of S_1 (sugar region)

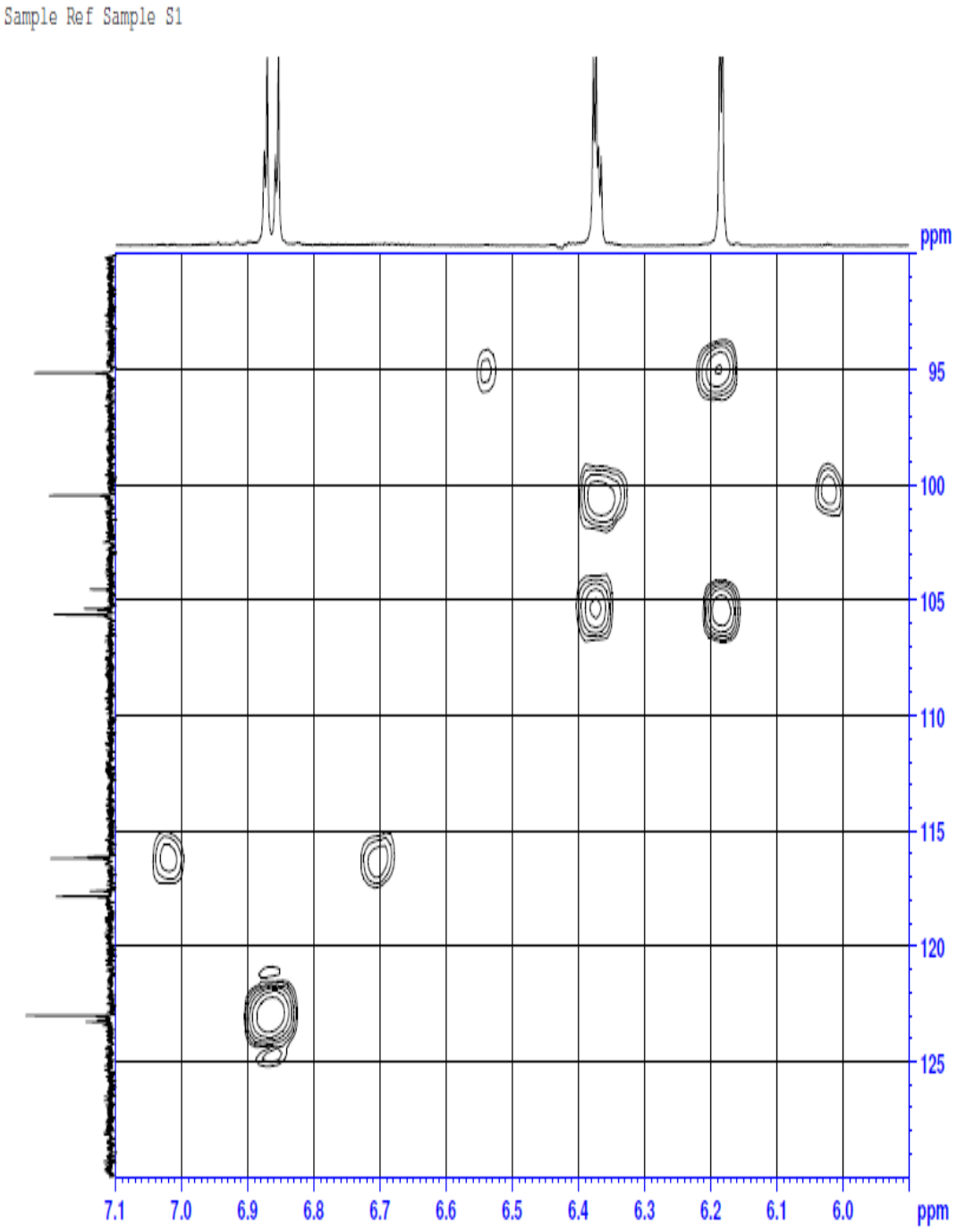


Figure 4.12c: HMBC experiment of S_1 (aromatic region 1)

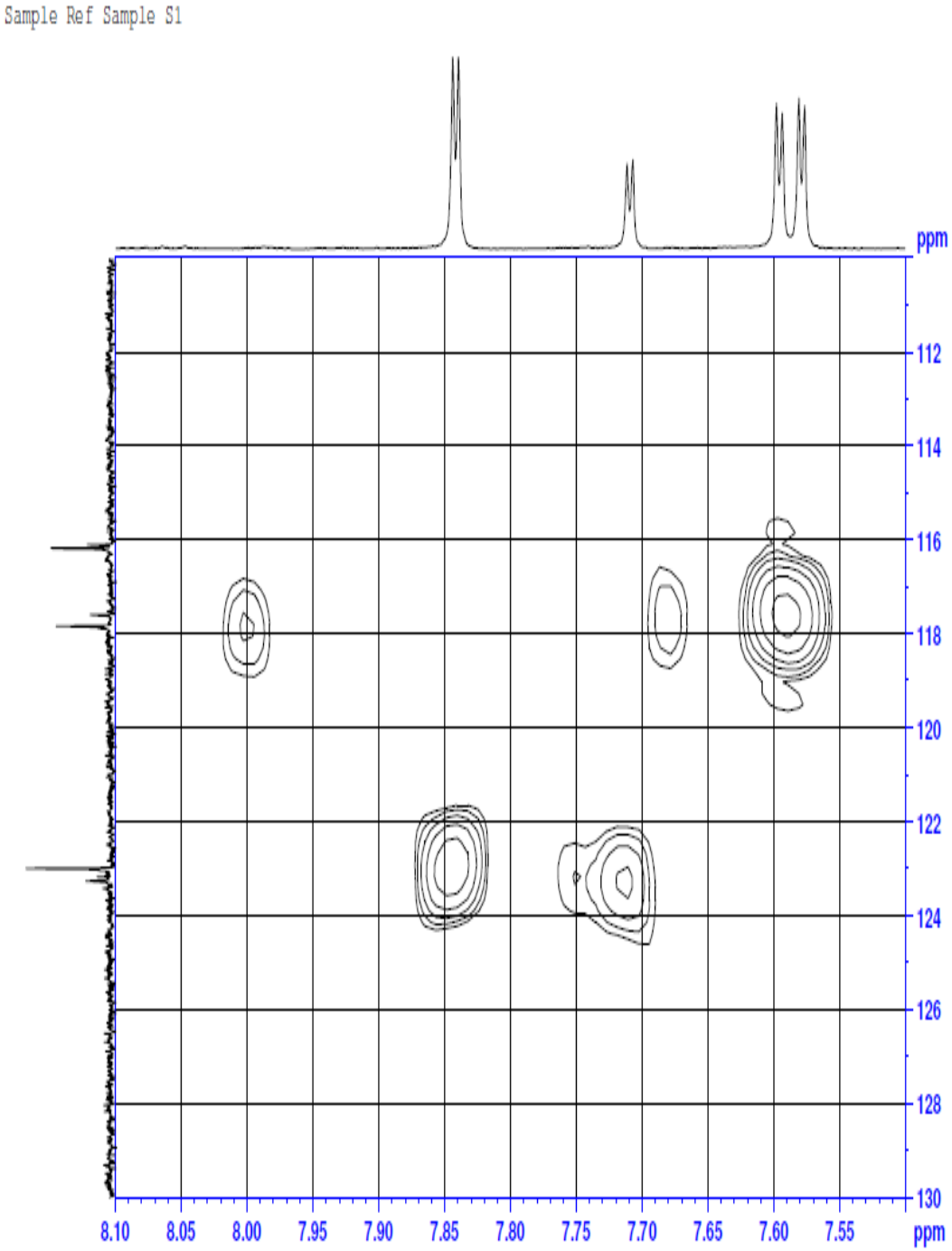


Figure 4.12d: HMBC experiment of S_1 (aromatic region 2)

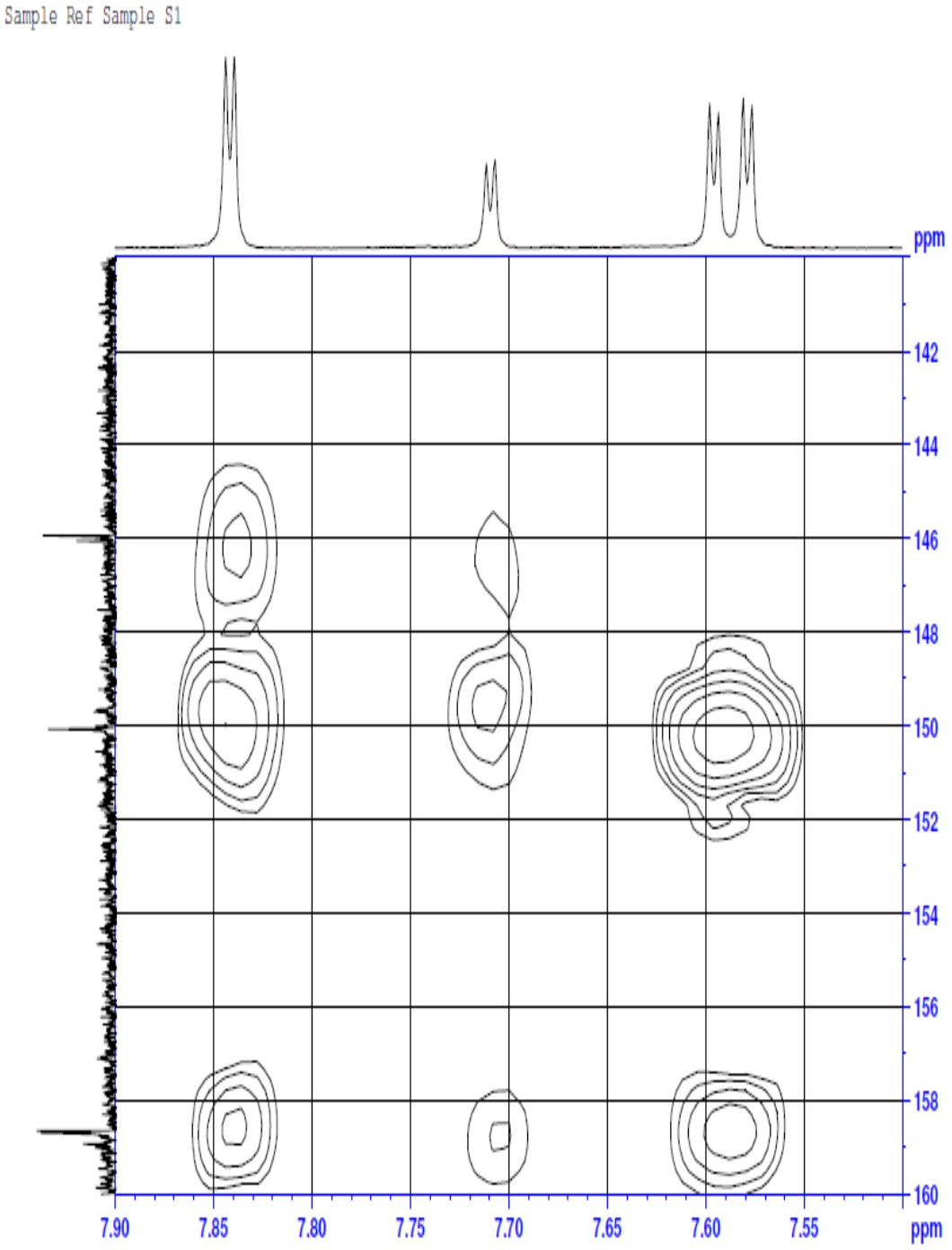


Figure 4.12e: HMBC experiment of S_1 (aromatic region 3)

Table 4.5: HMBC correlations of S_1

δ_H	δ_C
6.19 (H-6,6''')	95.10(C-8,8'''),100.42(C-6,6'''),105.34(C-10),105.41(C-10'''),163.06(C-5),163.10(C-5'''),167.52(C-7,7''')
6.38 (H-8,8''')	95.10(C-8,8'''),100.42(C-6,6'''),105.34(C-10),105.41(C-10'''),158.98(C-9),159.10(C-9'''),167.52(C-7,7''')
6.88 (H-5',5'')	116.09(C-5'),116.17(C-5''),122.98(C-6''),123.25(C-6')123.26(C-1',1''),146.04(C-3',3''),150.50(C-4'),158.5(C-4'')
7.59 (H-6',6'')	117.59(C-2''),117.83(C-2'),122.98(C-6''),123.25(C-6')123.26(C-1',1''),150.50(C-4',),158.5(C-4''),158.98(C-9),159.10(C-9''')
7.71 (H-2'')	117.59(C-2''),122.98(C-6''),123.26(C-1''),158.50(C-4''),159.10(C-2')
7.84 (H-2')	117.83(C-2'),123.25(C-6'),123.26(C-1'),150.50(C-4'),158.98(C-2)

4.7 Isolation and Characterization of Compound S_3

Fraction K (9.4 mg) from nBF column containing two major spots (Plate VI) was subjected to purification by repeated gel filtration in sephadex. This afforded 6.2 mg of yellow needles coded S_3.

Plate VI: TLC profile of S_3 after first gel filtration

4.7.1 TLC profile of compound S_3

S_3 gave a single TLC spot with R_f value of 0.47 using the solvent system of E:C:M:W in the ratio 15:4:4:1 (Plate VII).

Plate VII: Chromatogram of compound S_3

4.7.2 Colour, chemical test and melting point of S_3

S_3 was isolated as yellow needles of melting point 243-245°C and positive to Shinoda test.

4.7.3 UV spectral data of S_3

The UV spectrum of S_3 showed two major absorption bands at 257 nm and 359 nm in methanol (Figure 4.13).

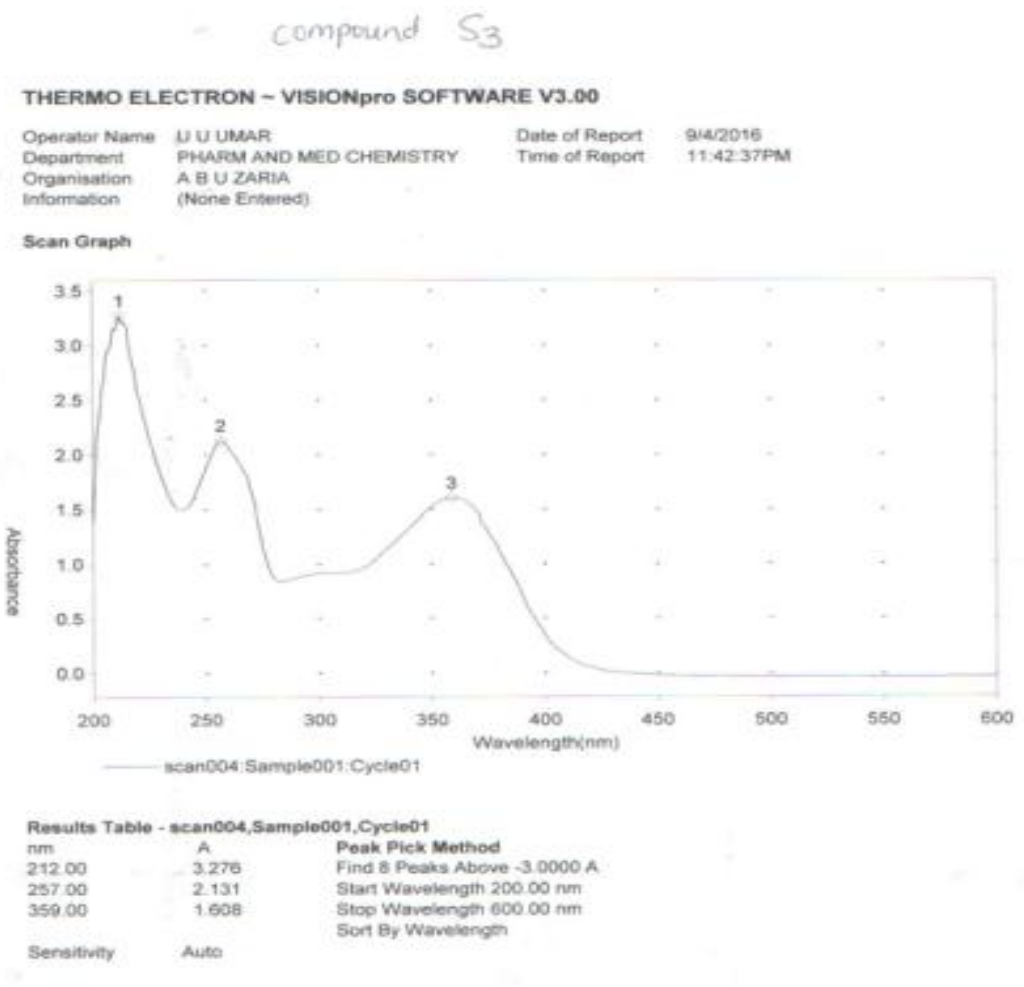


Figure 4.13: UV spectrum of S_3

4.7.4 IR spectral data of S_3

The IR spectrum of S_3 showed sharp peaks at 1602 cm^{-1} and 1654 cm^{-1}; coupled vibrations at 2855 cm^{-1} and 2922 cm^{-1}; and a broad band at 3272 cm^{-1} (Figure 4.14)

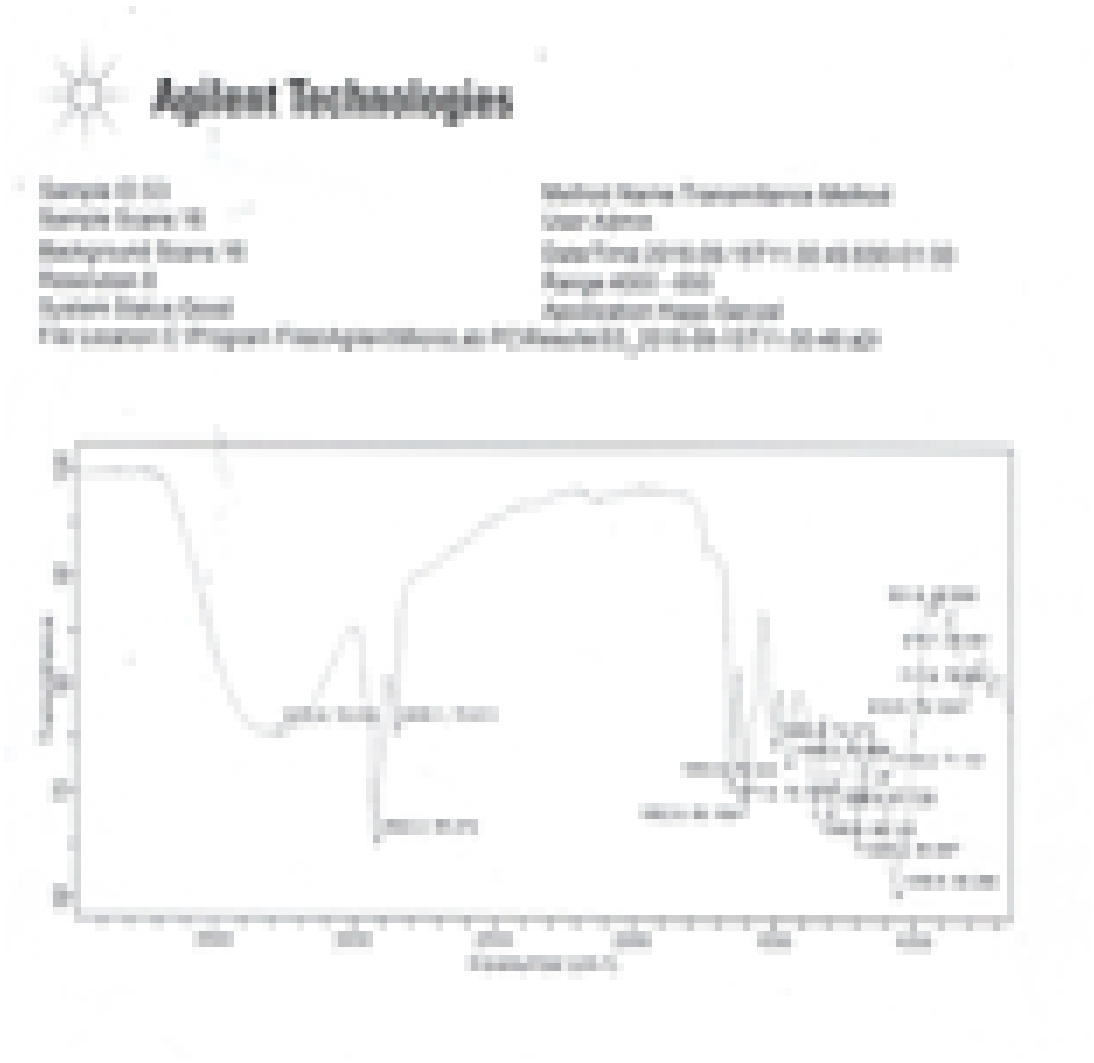


Figure 4.14: IR spectrum of S_3

4.7.5 Proton NMR spectral data of S_3

The ^1H NMR spectrum revealed the presence of five aromatic proton signals at δ_H 6.23, 6.43, 6.89, 7.62 and 7.90, two anomeric proton signals at 4.55 and 5.10, overlapping carbinol proton signals at δ_H 3.43-3.85 and a tertiary methyl proton signal at δ_H 1.21 (Figure 4.15)

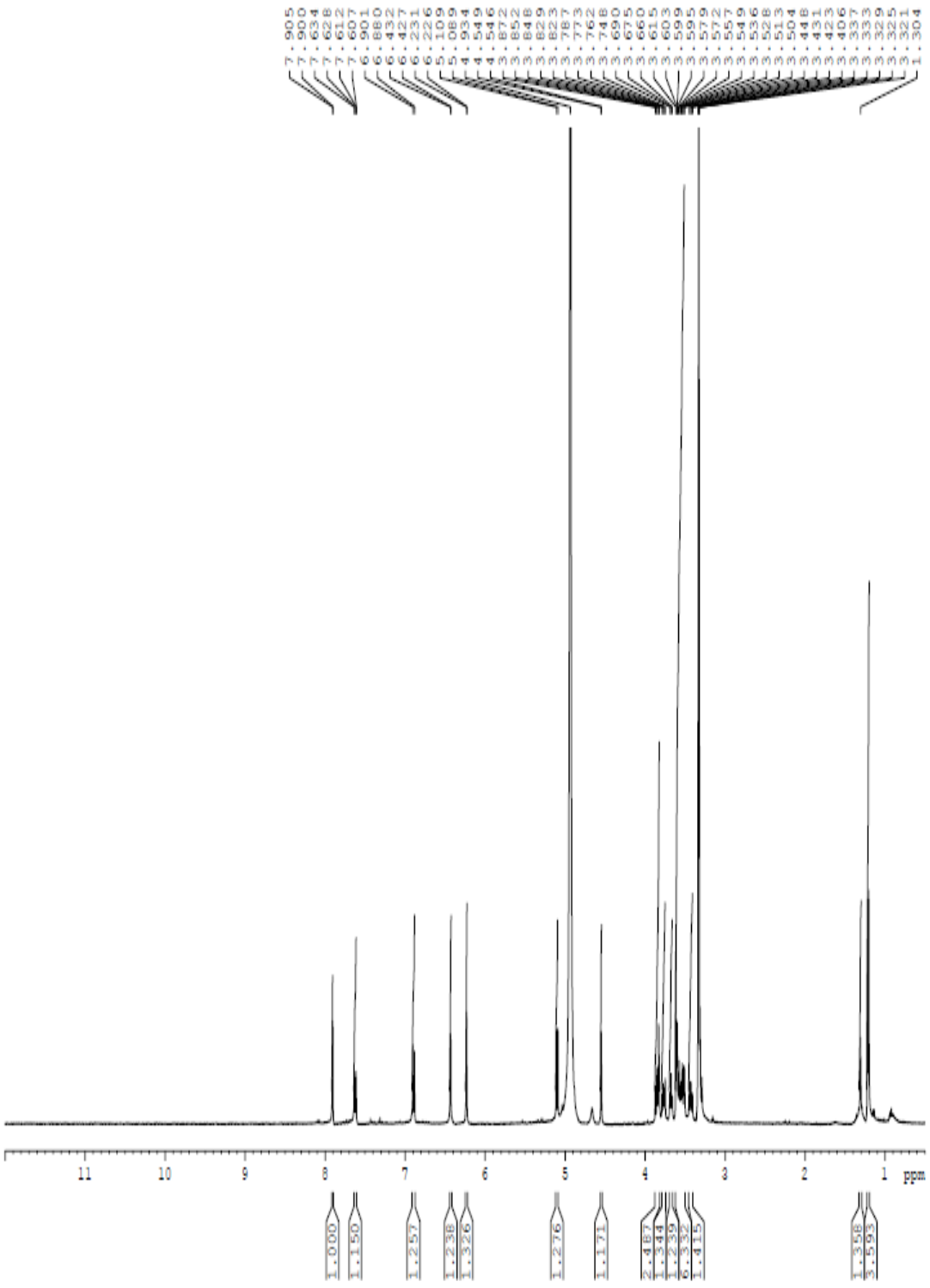


Figure 4.15: Proton NMR spectrum of S_3

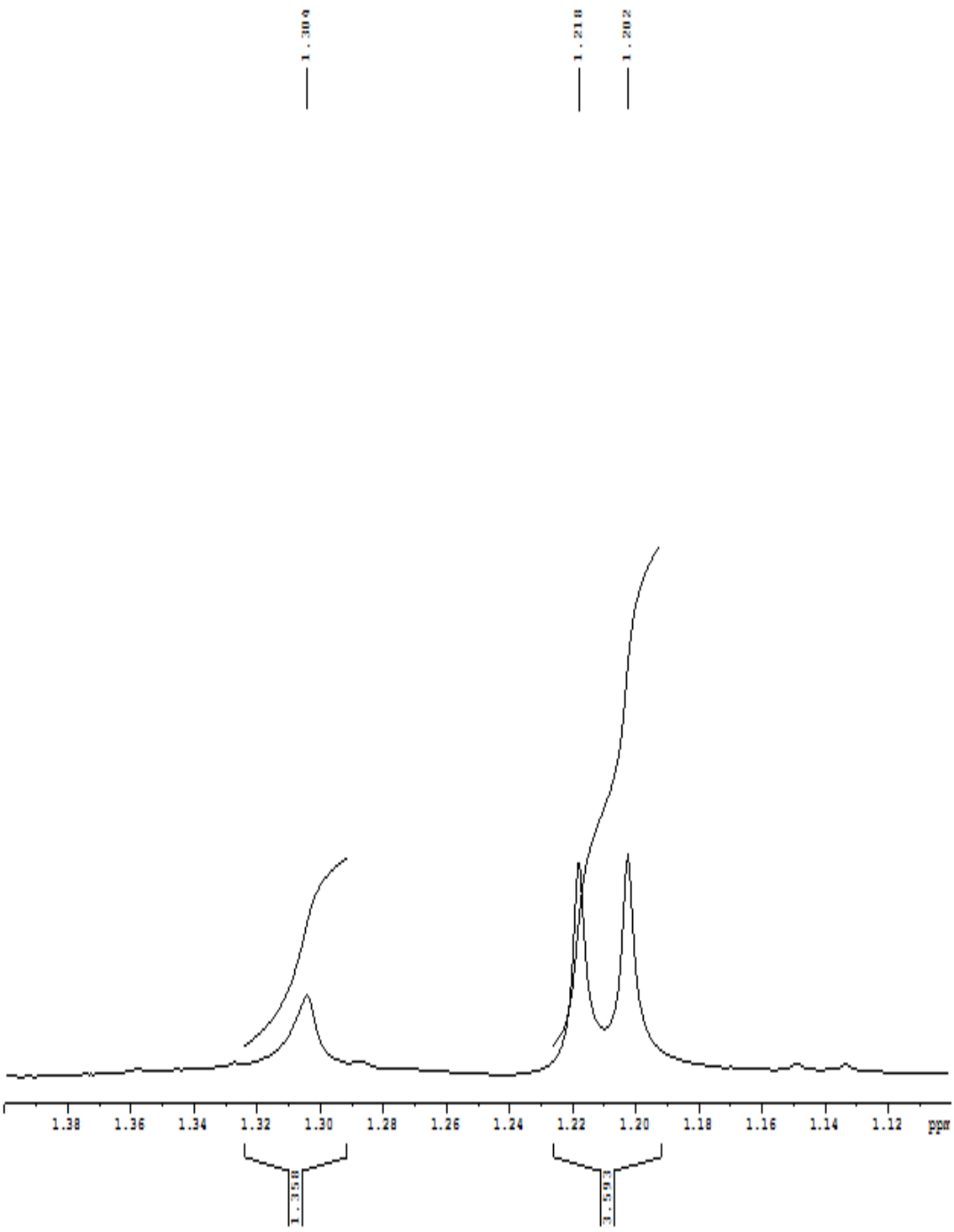


Figure 4.15a: Proton NMR spectrum of S_3 (aliphatic region 1)

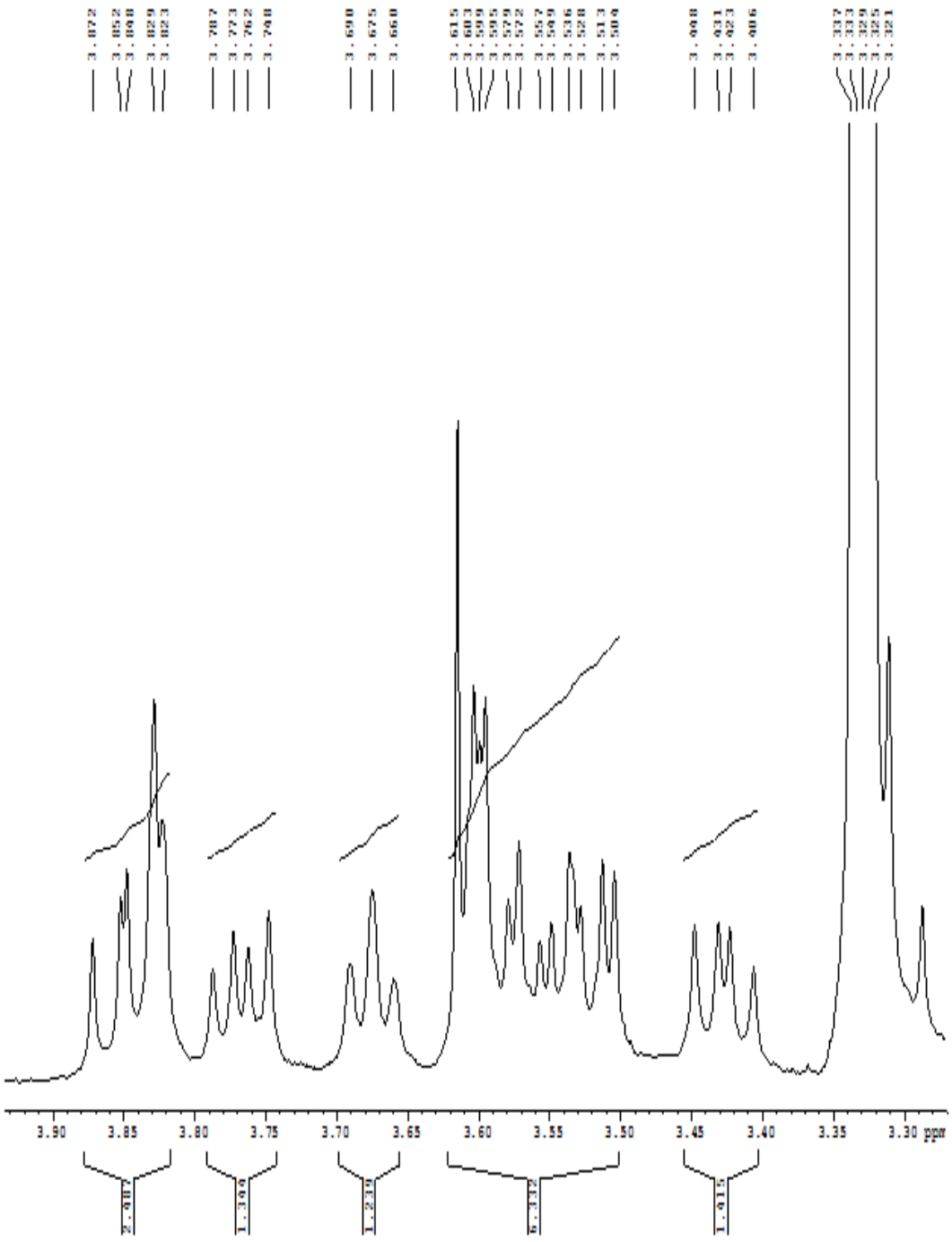


Figure 4.15b: Proton NMR spectrum of S_3 (sugar region)

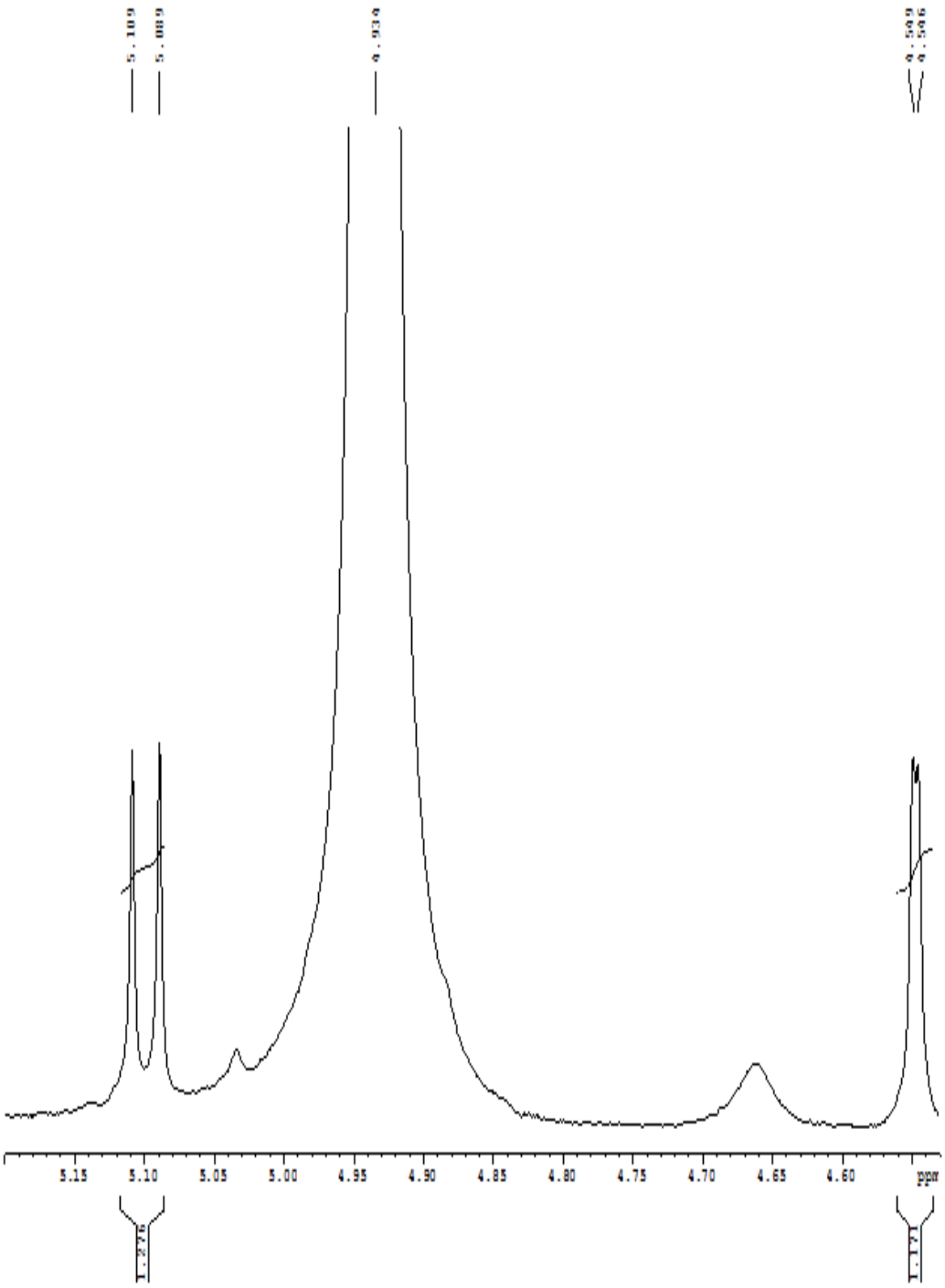


Figure 4.15c: Proton NMR spectrum of S_3 (aliphatic region 2)

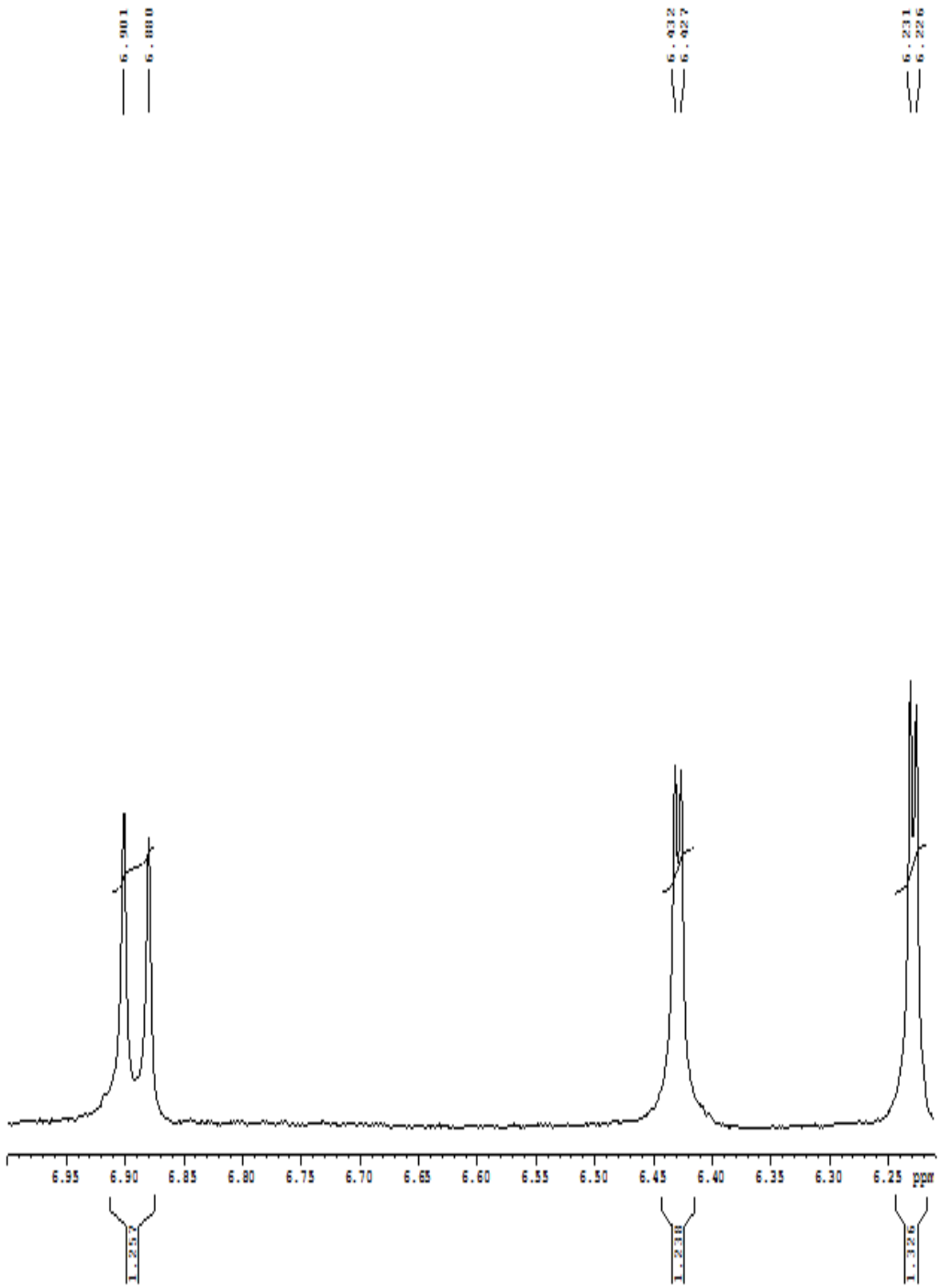


Figure 4.15d: Proton NMR spectrum of S_3 (aromatic region 1)

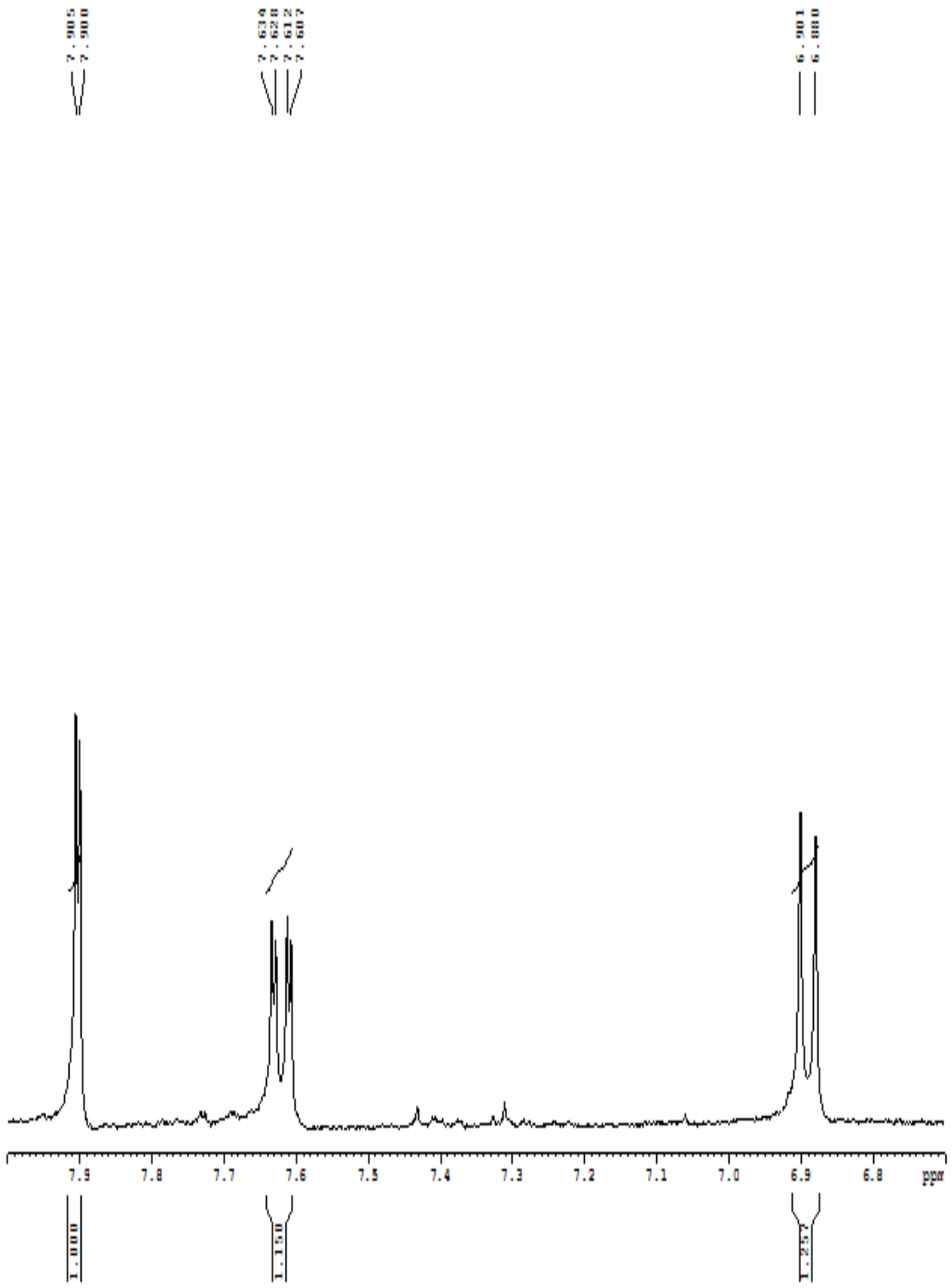


Figure 4.15e: Proton NMR spectrum of S_3 (aromatic region 2)

4.7.6 Carbon-13 NMR spectral data of S_3

Both the broadband decoupled carbon 13 NMR (Figure 4.16) and DEPT experiment (Figure 4.17) disclosed 27 well resolved peaks of one methyl, one methylene, 15 methine and 10 quaternary carbons

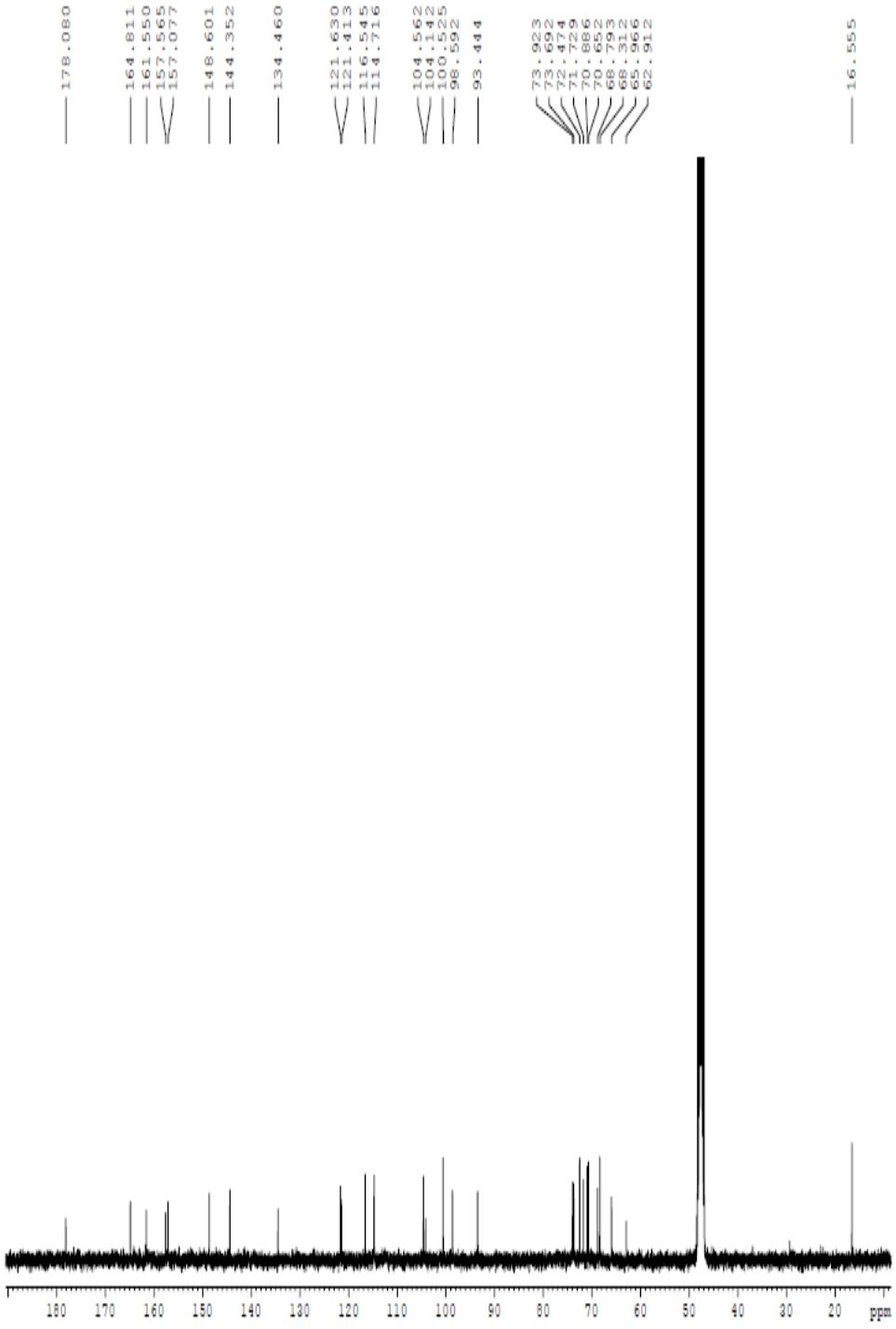


Figure 4.16: Proton decoupled carbon-13 NMR spectrum of S_3

Figure 4.16a: Carbon-13 NMR spectrum of S_3 (aliphatic region)

Figure 4.16b: Carbon-13 NMR spectrum of S_3 (aromatic region 1)

Figure 4.16c: Carbon-13 NMR spectrum of S_3 (aromatic region 2)

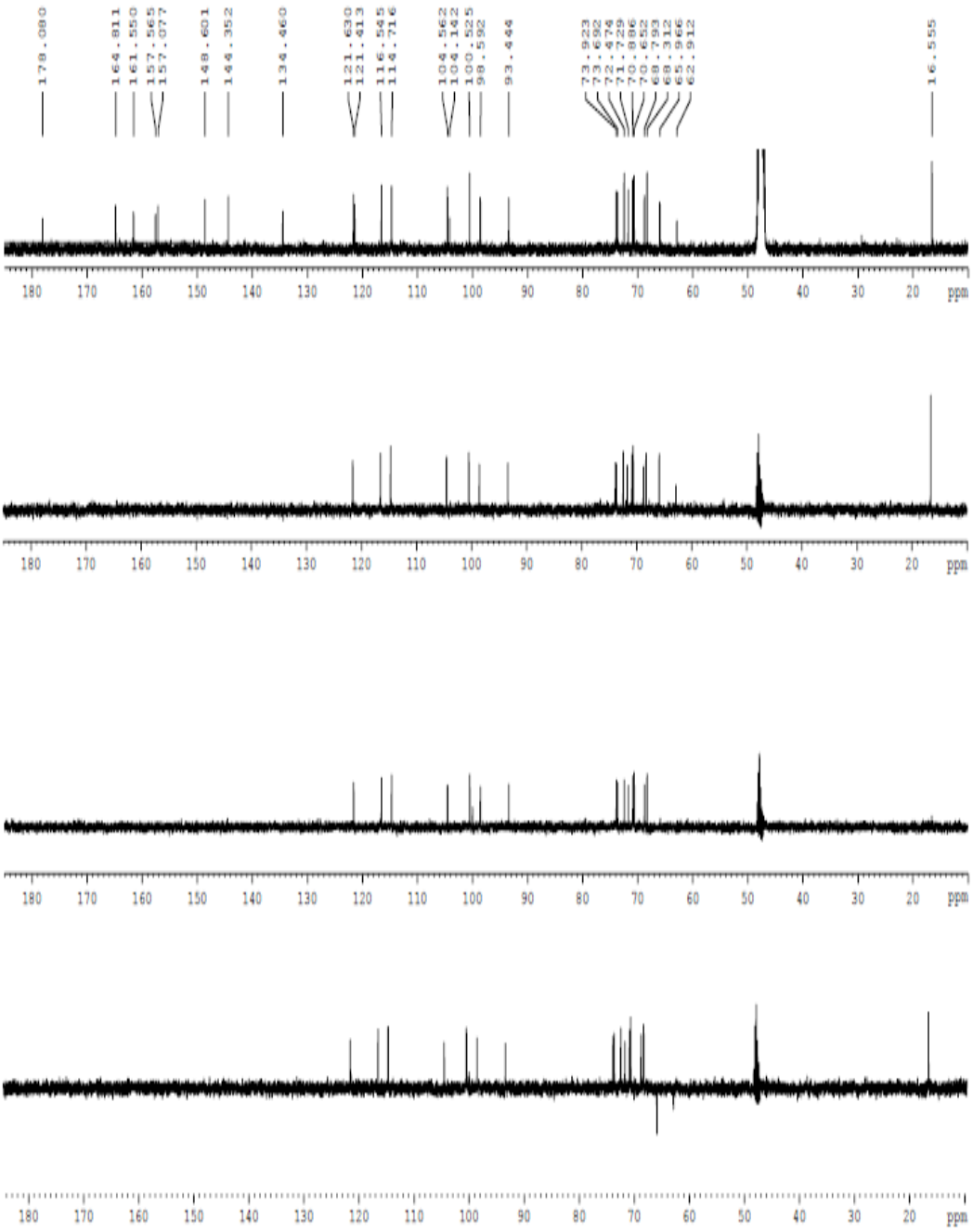


Figure 4.17: Distortionless Enhancement by Polarization Transfer (DEPT) experiment of S_3

4.7.7 $^{1}H - ^{1}H$ COSY data of S_3

The COSY spectrum of S_3 showed important cross peaks between δ_H [6.89 and 7.62]; δ_H [3.60 and 4.55]; δ_H [3.85 and 3.60] and δ_H [3.85 and 5.10] (Figure 4.18)

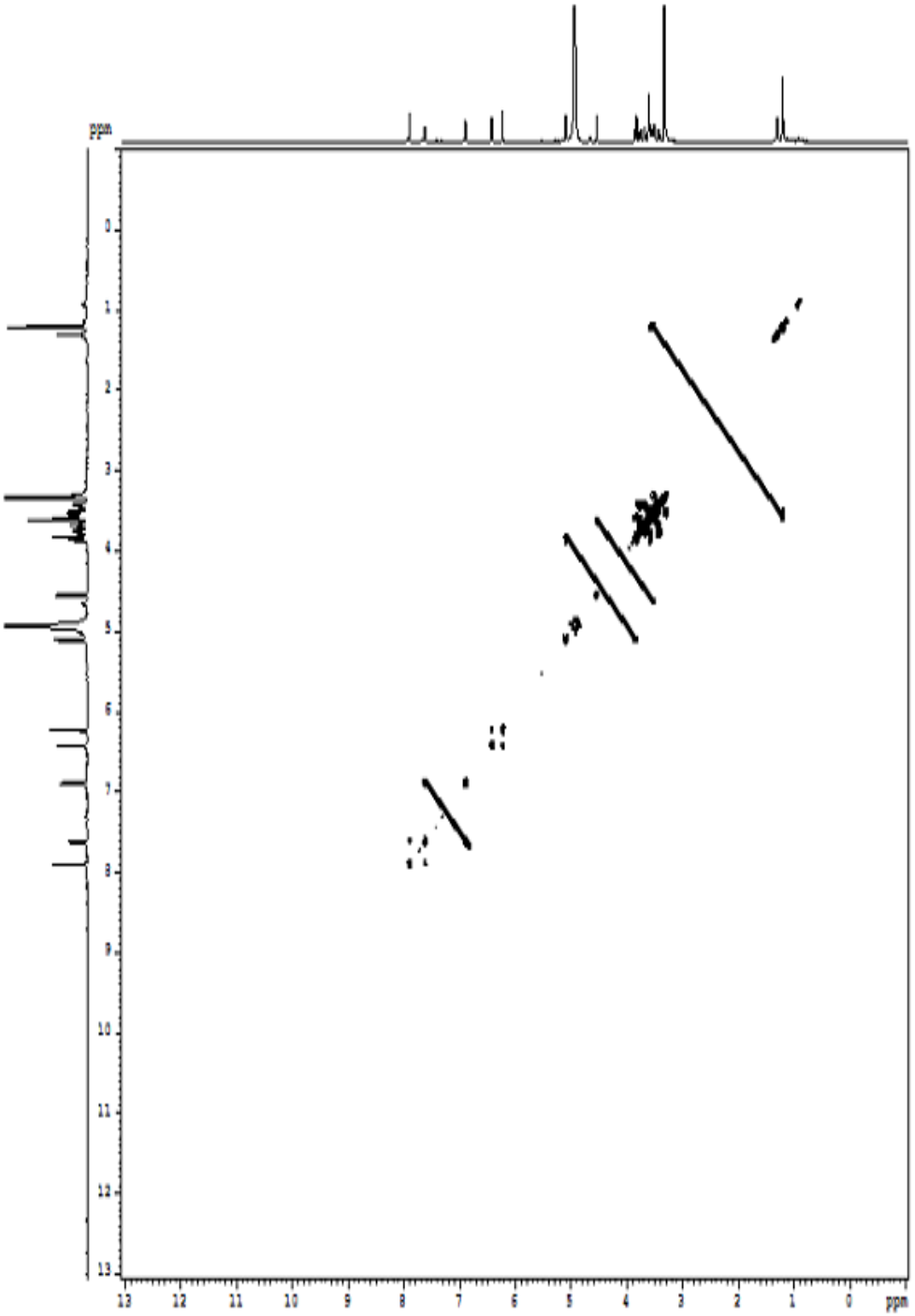


Figure 4.18: COSY experiment of S_3

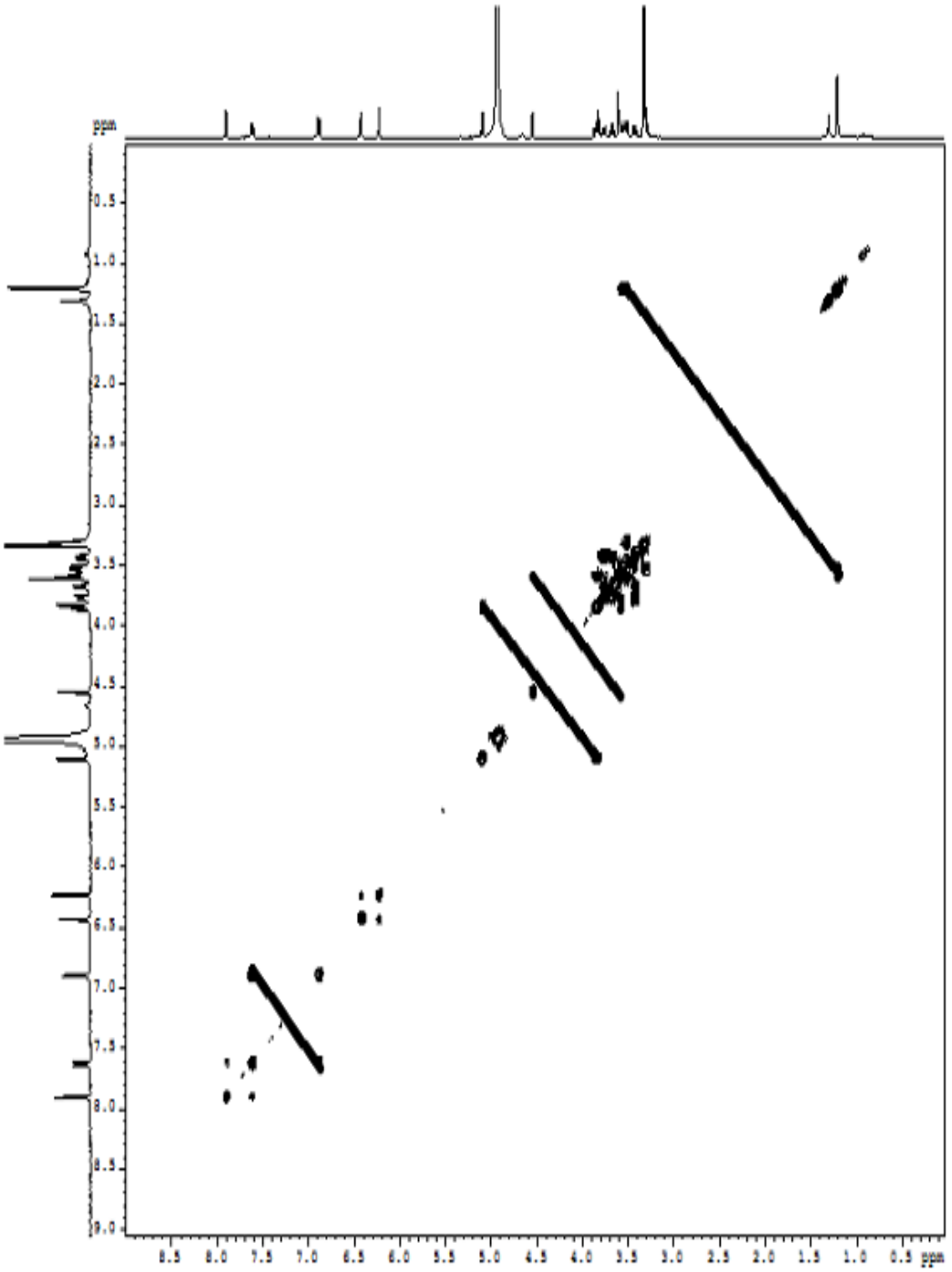


Figure 4.18a: COSY experiment of S_3 (expansion 1)

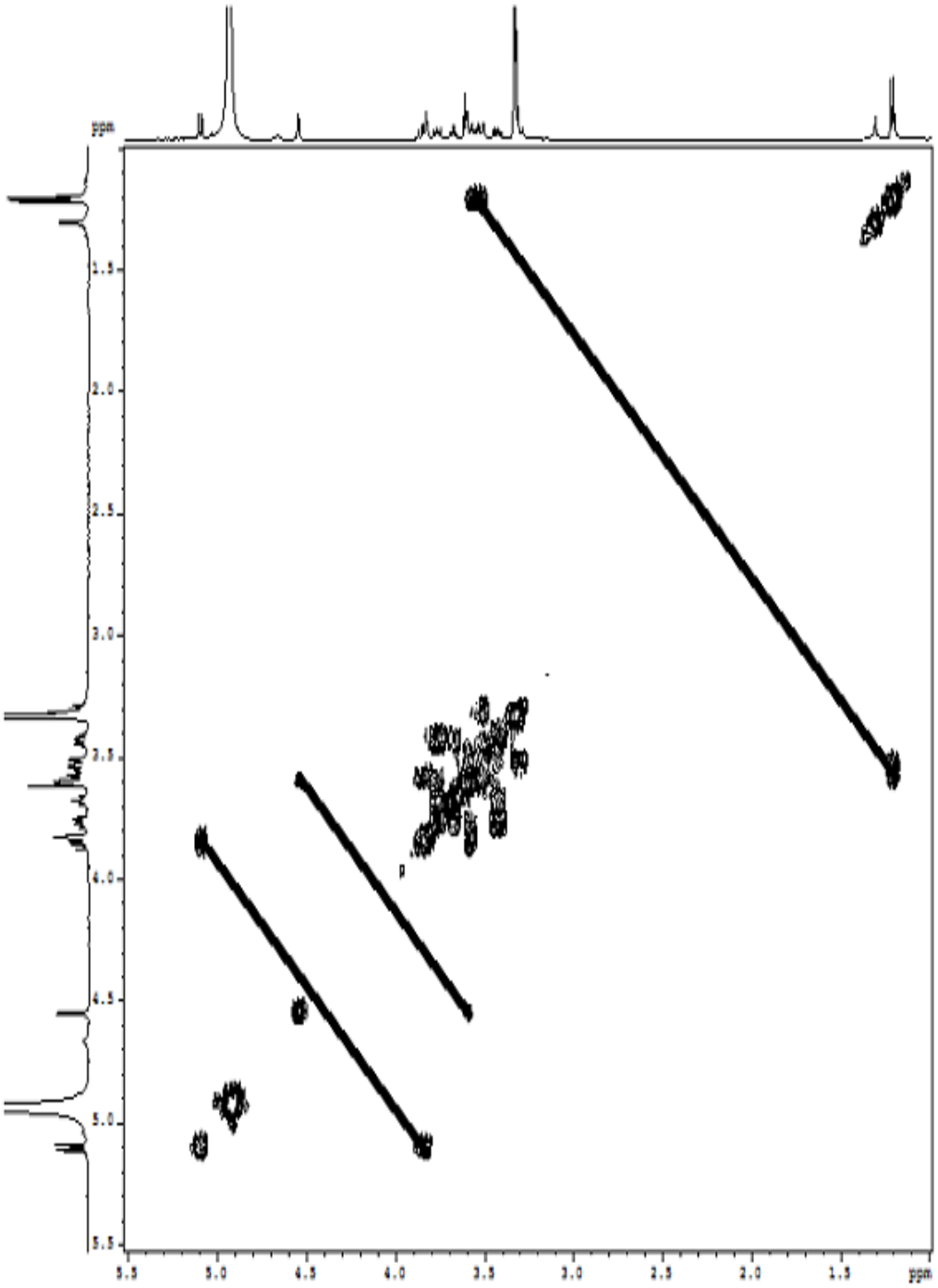


Figure 4.18b: COSY experiment of S_3 (aliphatic region)

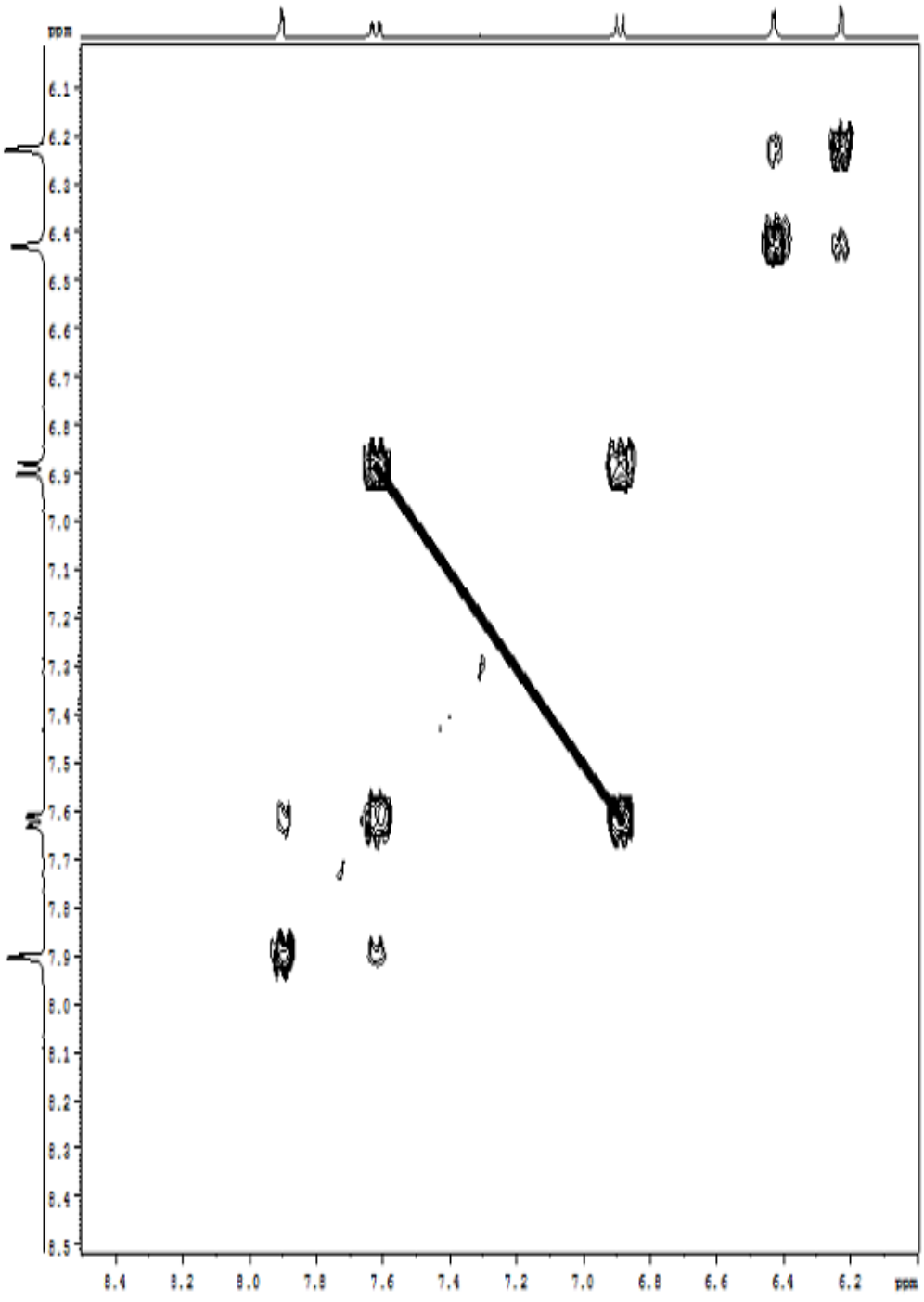


Figure 4.18c: COSY experiment of S_3 (aromatic region)

4.7.8 HMQC spectral data of S_3

The HMQC experiment is as shown in Figure 4.19 and was used to assign protons on their respective carbons (Table 4.6)

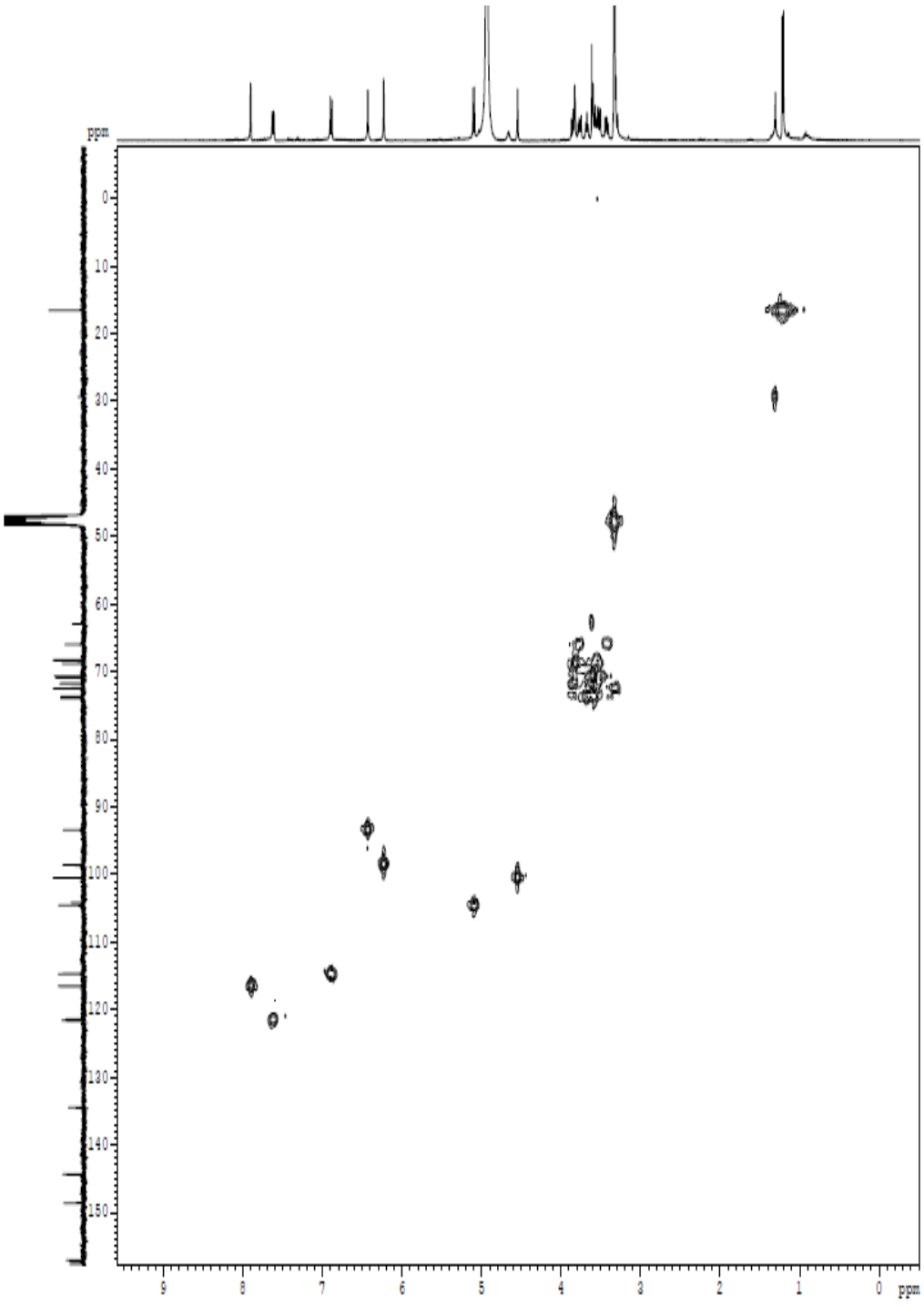


Figure 4.19: HMQC spectrum of S_3

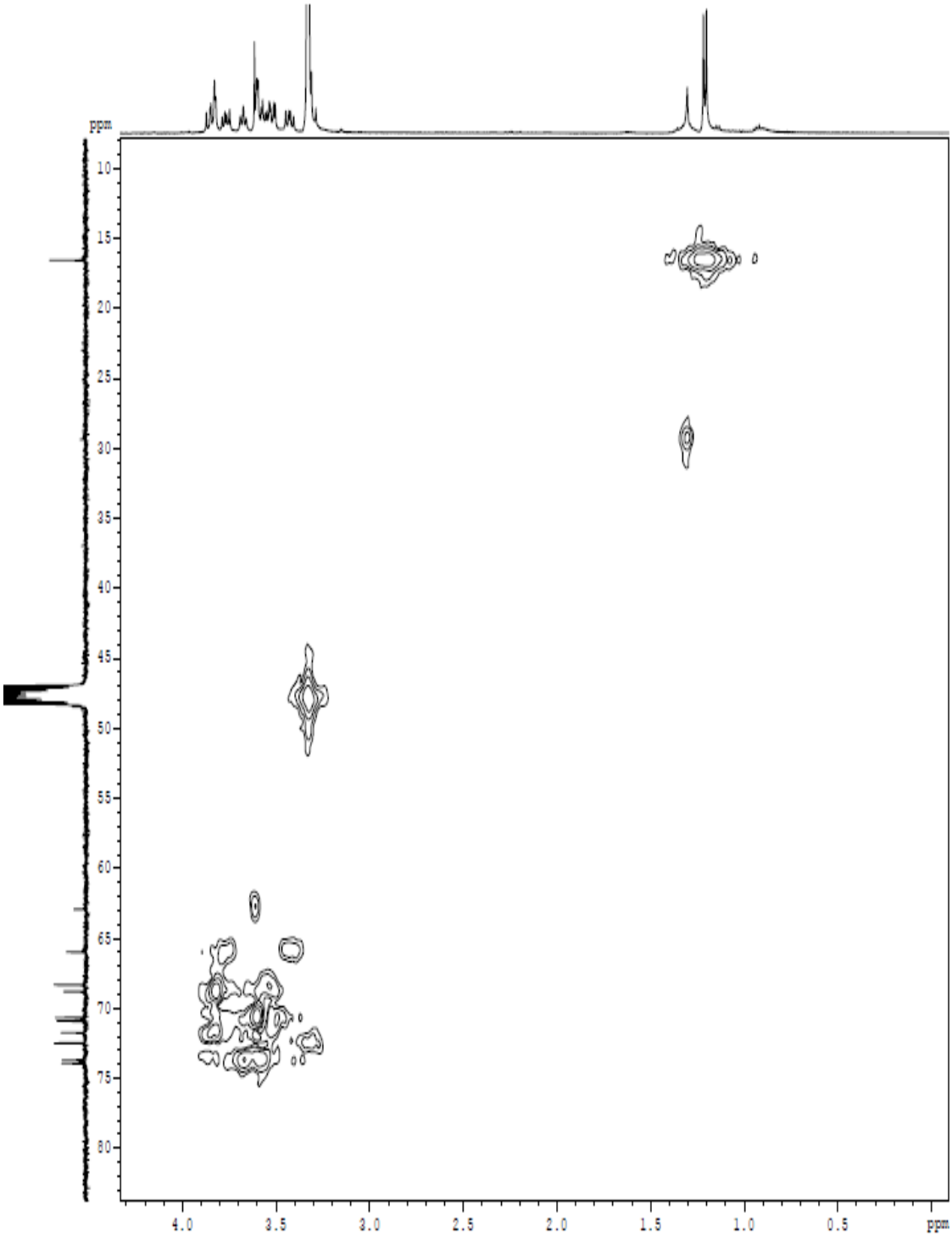


Figure 4.19a: HMQC spectrum of S_3 (expansion 1)

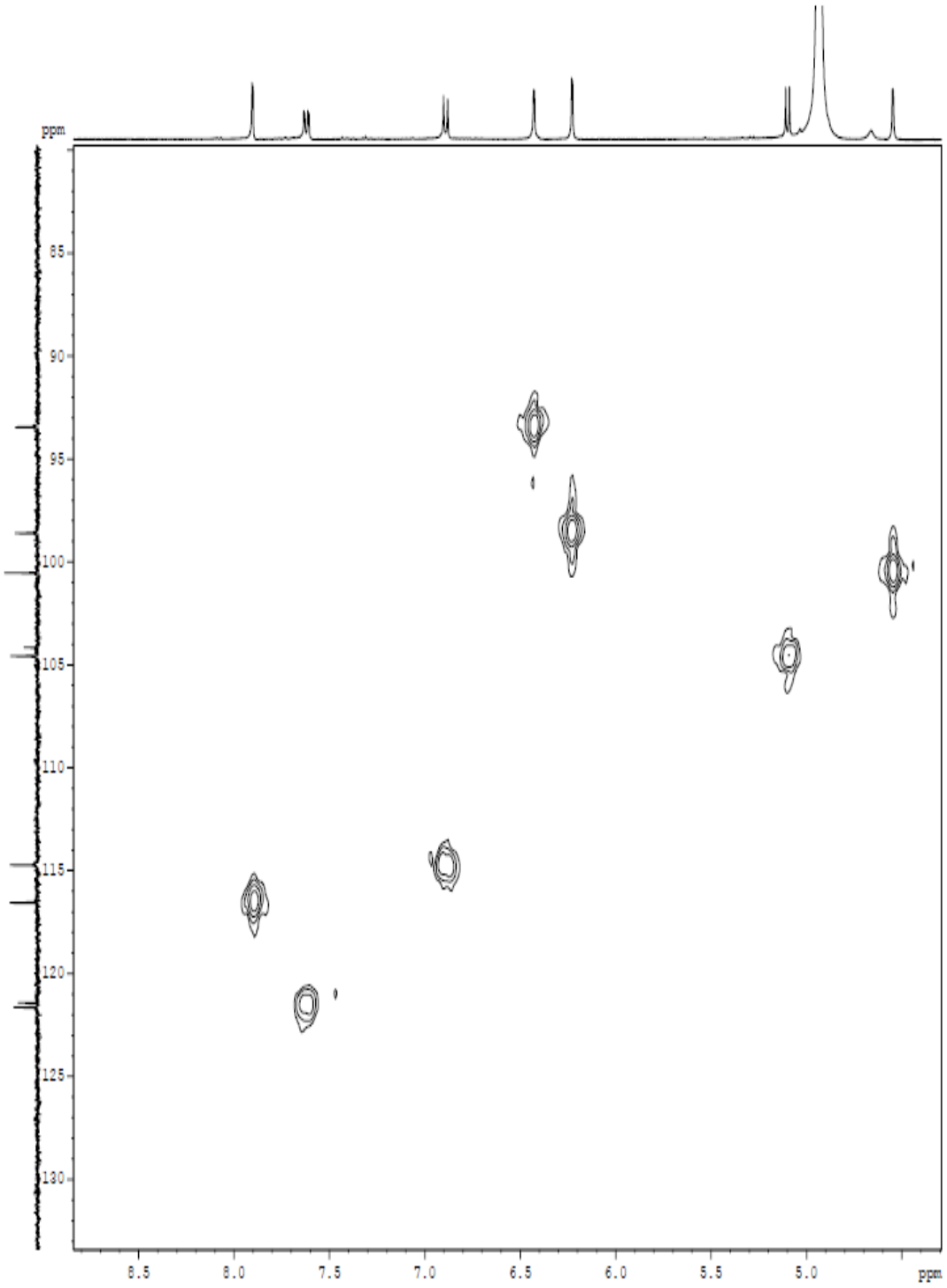


Figure 4.19b: HMQC spectrum of S_3 (expansion 2)

Table 4.6: HMQC and DEPT spectral data of S_3

Position	δ_C	δ_H	DEPT
1			
2	157.08		C
3	134.46		C
4	178.08		C
5	164.81		C
6	98.59	6.23	CH
7	161.55		C
8	93.44	6.43	CH
9	157.57		C
10	104.14		C
1'	121.41		C
2'	116.55	7.90	CH
3'	144.35		C
4'	148.60		C
5'	114.72	6.89	CH
6'	121.63	7.62	CH
1''	104.56	5.10	CH
2''	73.69	3.60	CH
3''	72.47	3.58	CH
4''	71.73	3.61	CH
5''	73.92	3.67	CH
6''	62.91	3.85	CH_2
1'''	100.53	4.55	CH

2'''	70.89	3.77	CH
3'''	68.79	3.55	CH
4'''	70.65	3.53	CH
5'''	68.31	3.51	CH
6'''	16.56	1.21	CH_3

4.7.9 HMBC spectral data of S_3

The HMBC experiment is as shown in Figure 4.20 and was used to establish connectivity between the various fragments of the molecule (Table 4.7)

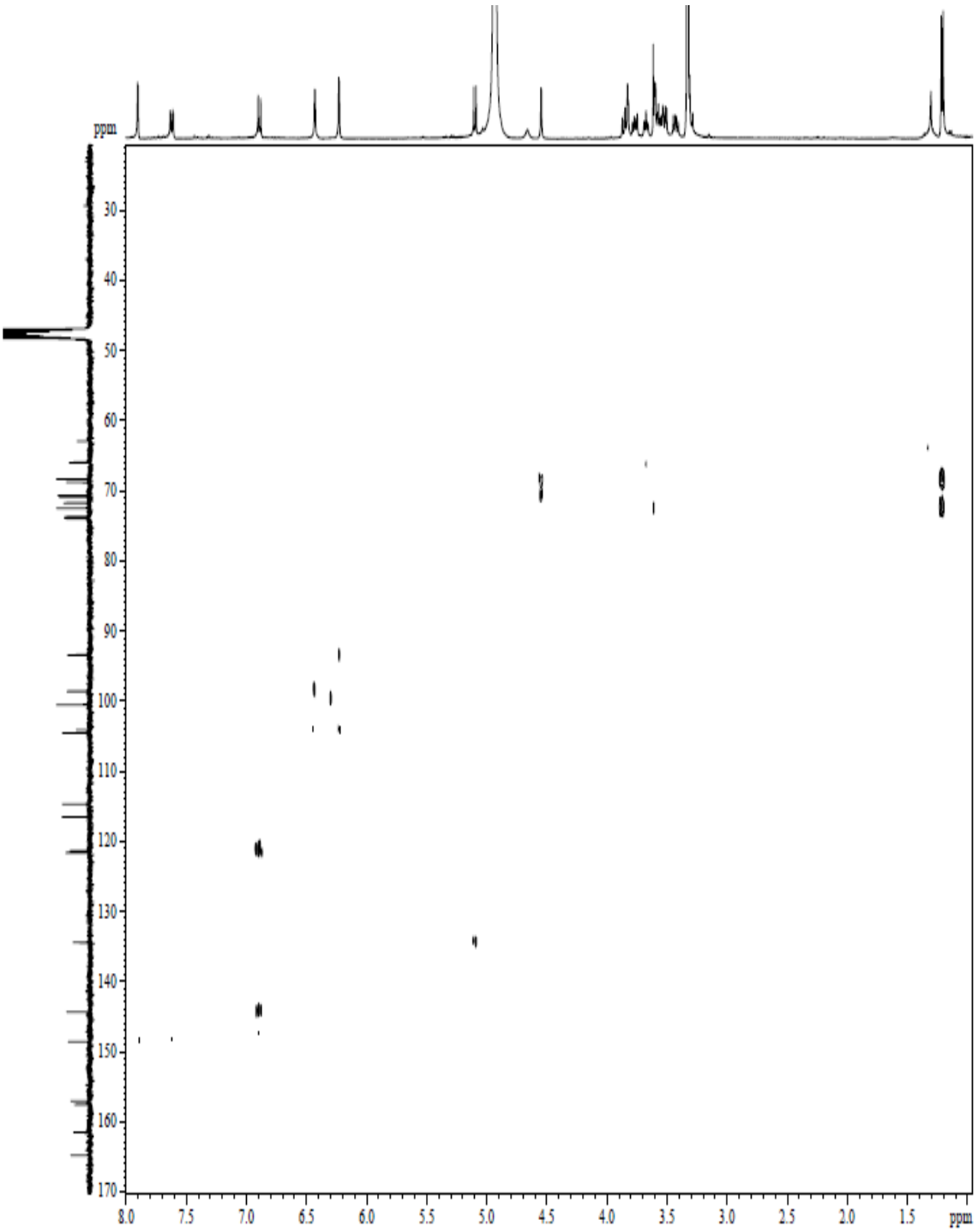


Figure 4.20: HMBC experiment of S_3

Table 4.7: HMBC correlations of S_3

δ_H	δ_C
6.23 (H-6)	104.14 (C-10)
6.43 (H-8)	98.59 (C-6)
6.89 (H-5')	121.63 (C-6'), 144.35 (C-3')
5.10 (H-1'')	134.46 (C-3)
4.55 (H-1''')	70.89 (C-2'''), 68.79 (C-3''')
1.21 (H-6''')	68.31 (C-5'''), 70.65 (C-4''')

4.8 Isolation and Characterization of Compound K

A total of four collections, 600 mL aliquots each, was made from n-butanol fraction subjected to vacuum liquid chromatography and coded fractions A-D (Table 4.8). Fraction D gave a white crystalline solid that was coded K.

Table 4.8: Vacuum liquid chromatography of nBF

Fractions	Solvent system	No. of Spots
A	100 % $CHCl_3$	No spot
B	$CHCl_3$: EtOAc 98 : 2	3
C	$CHCl_3$: EtOAc 96 : 4	3
D	$CHCl_3$: EtOAc 94 : 6	1

4.8.1 TLC profile of compound K

K gave a single spot on TLC using solvent system E: C: M: W in the ratio 15: 8: 4: 1 (Plate VIII)

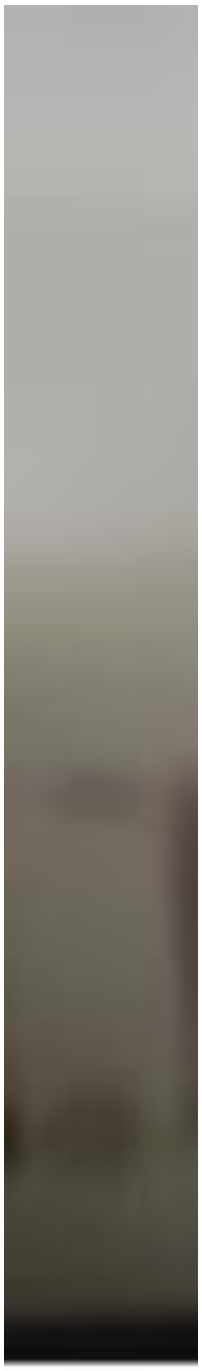


Plate VIII: TLC of compound K

4.8.2 Colour, chemical test and melting point of K

K was isolated as a white powder of melting point 219-221°C and was positive to Liebermann Buchard test for steroids and triterpenoids.

4.8.3 IR spectral data of K

The IR spectrum of K showed a broad band at 3395 cm^{-1}, coupled vibrational peaks at 2873 cm^{-1} and 2929 cm^{-1}, a very sharp peak at 1688 cm^{-1} and characteristic peaks due to bending vibrations in the finger print region (Figure 4.21).

Agilent Technologies

Sample ID:COMPOUND K
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
Method Name:Transmittance Method
User:Admin
Date/Time:2016-04-20T12:52:45.884+01:00
Range:4000 - 650
Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\COMPOUND K_2016-04-20T12-52-45.a2r

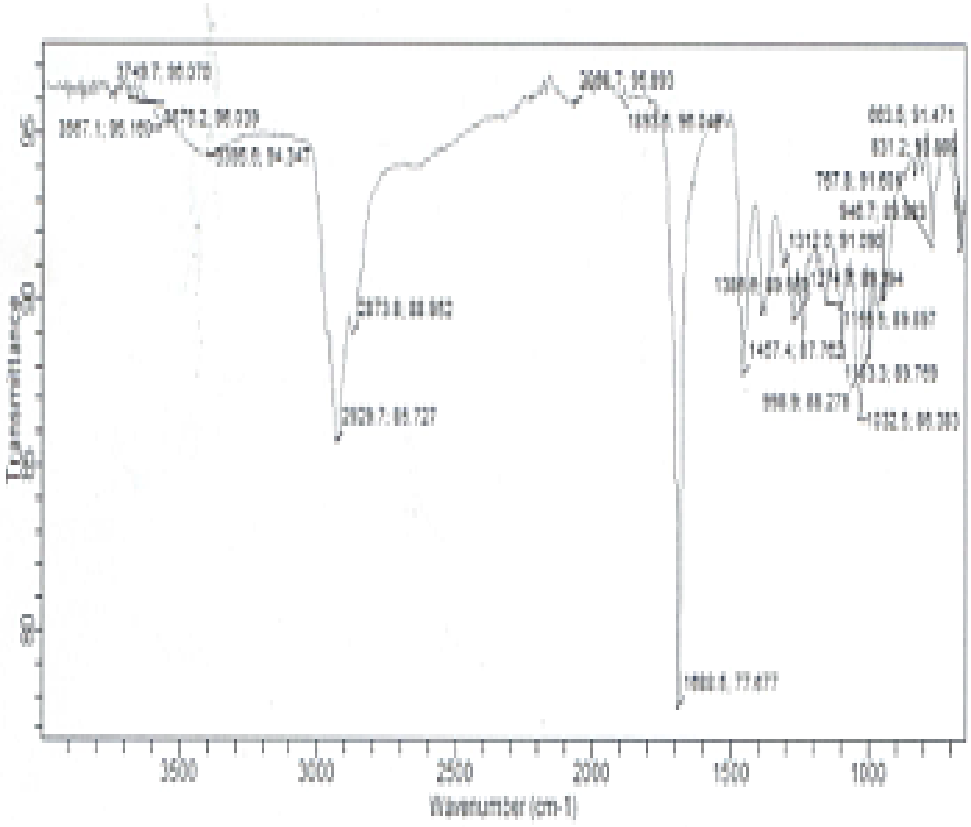


Figure 4.21: IR spectrum of K

4.8.4 Proton NMR spectral data of K

The ^{1}H NMR spectrum of K revealed overlapping signals of methyl and methylene protons, a carbinol proton at δ_H 3.00 and olefinic proton at δ_H 5.15 (Figure 4.22)

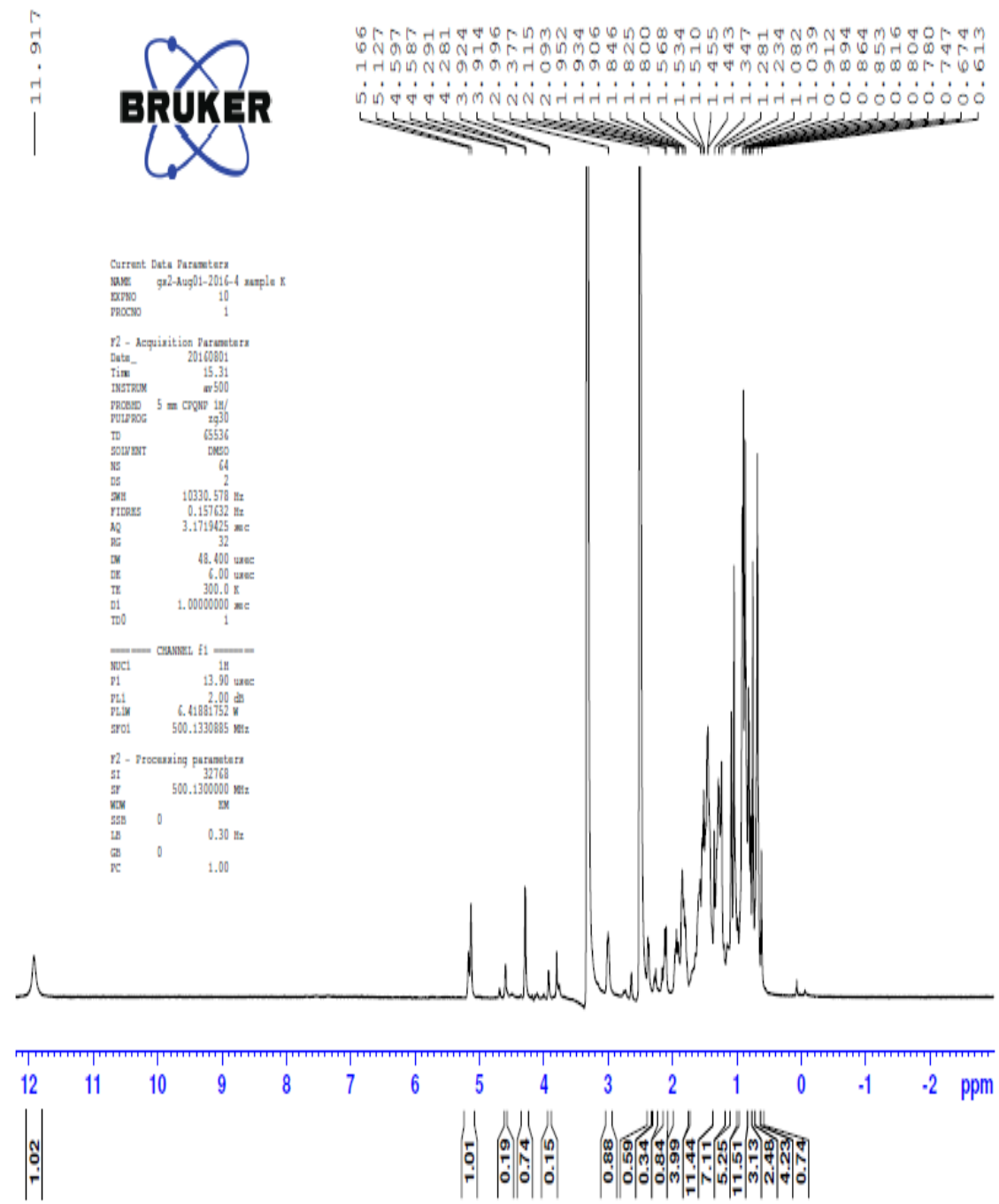


Figure 4.22: Proton NMR spectrum of K

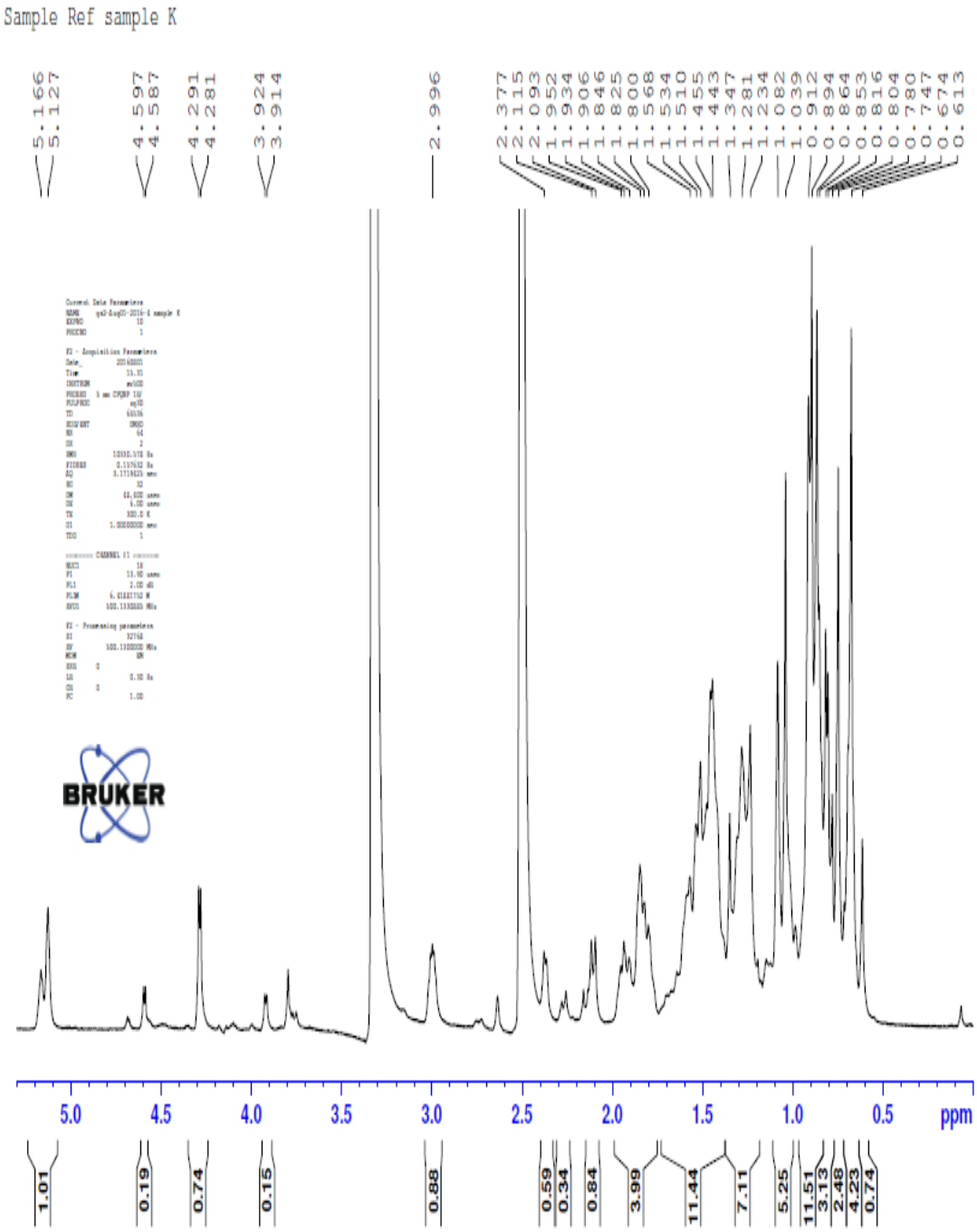


Figure 4.22a: Proton NMR spectrum of K (expansion 1)

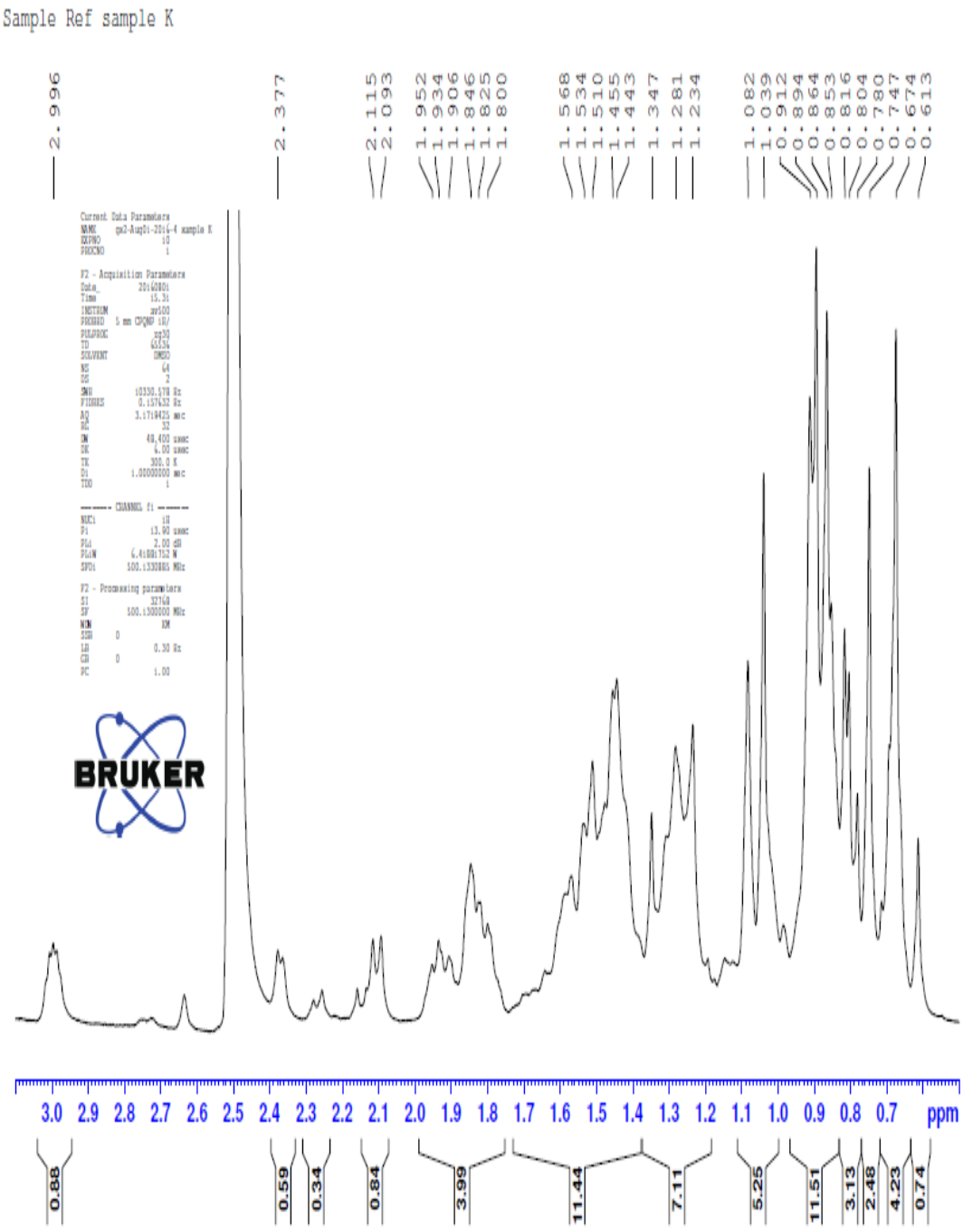


Figure 4.22b: Proton NMR spectrum of K (expansion 2)

4.8.5 Carbon-13 NMR and DEPT spectral data of K

The ^{13}C NMR disclosed 44 peaks (Figure 4.23) and DEPT experiment revealed the presence of eleven methyl carbons, ten methylene carbons and nine methine carbons (Figure 4.24)

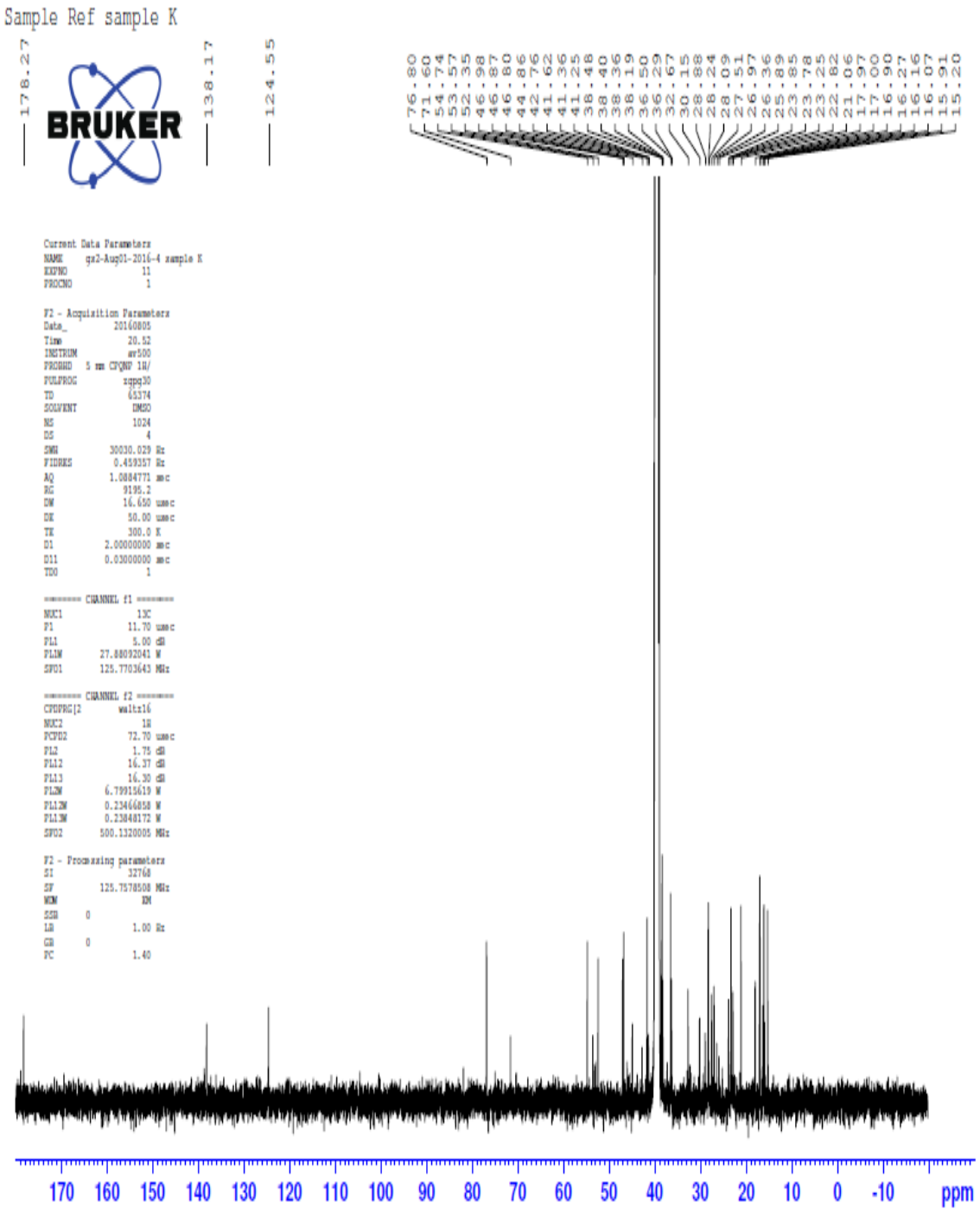


Figure 4.23: Proton decoupled carbon-13 NMR spectrum of K

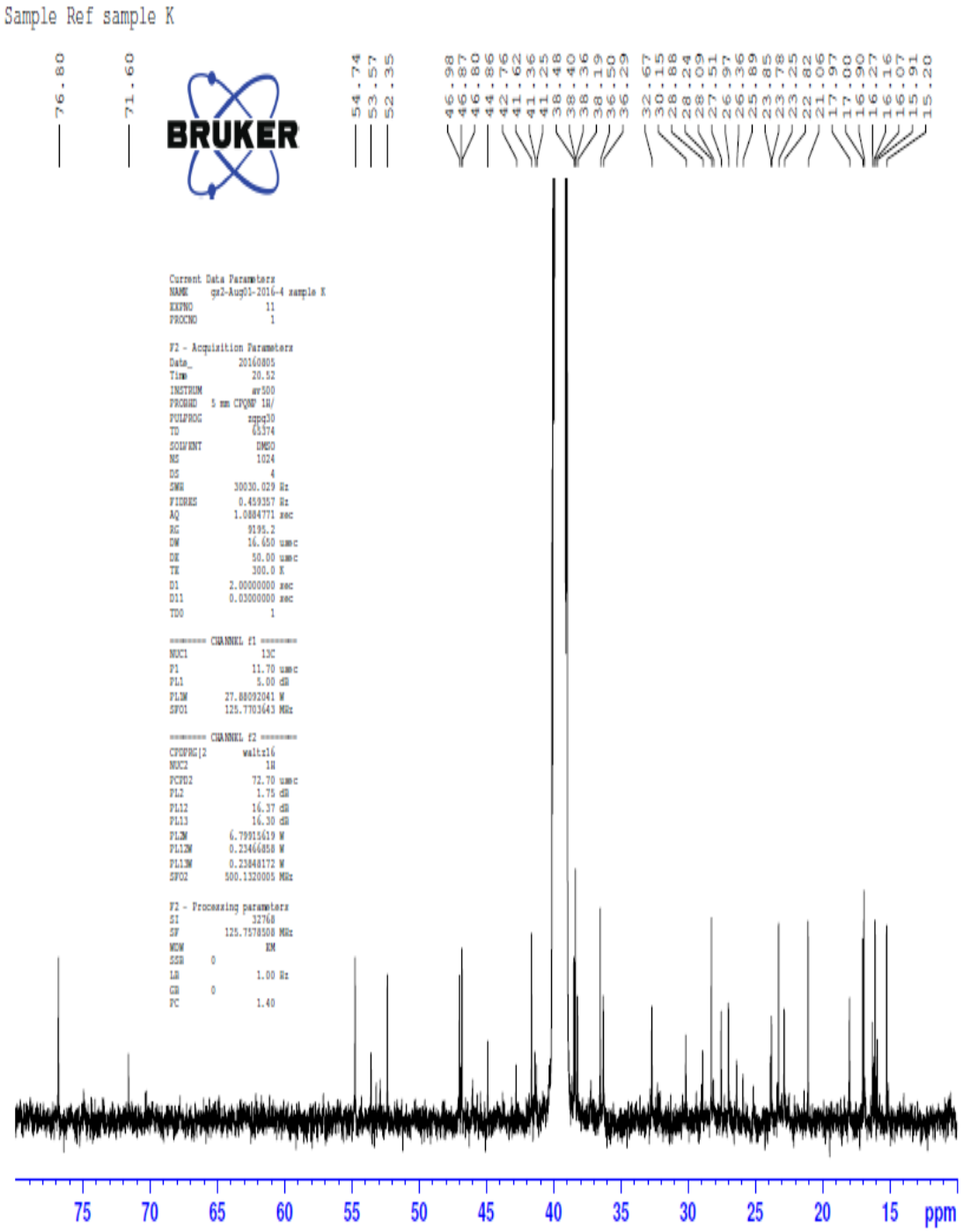


Figure 4.23a: Proton decoupled carbon-13 NMR spectrum of K (expansion 1)

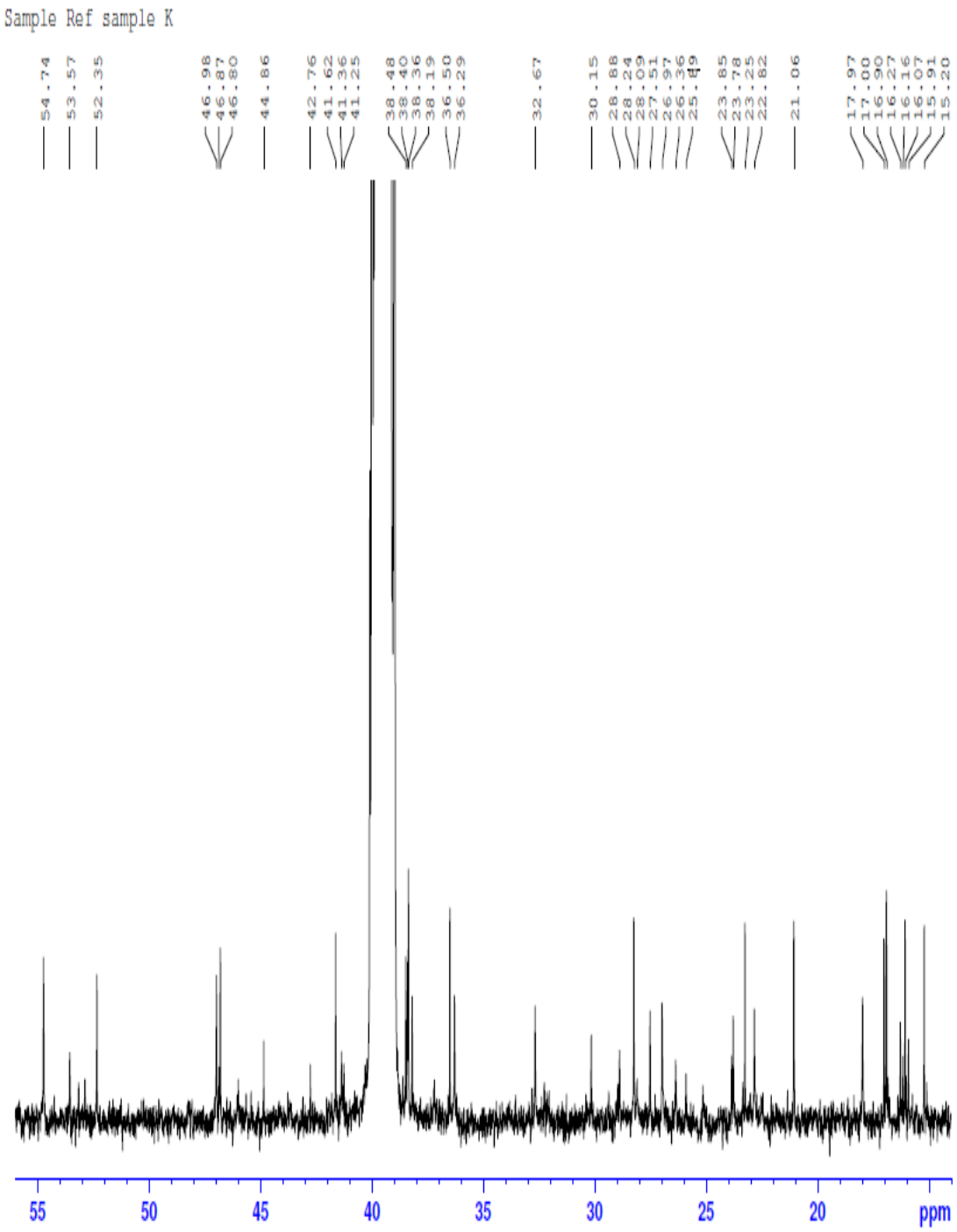


Figure 4.23b: Proton decoupled carbon-13 NMR spectrum of K (expansion 2)

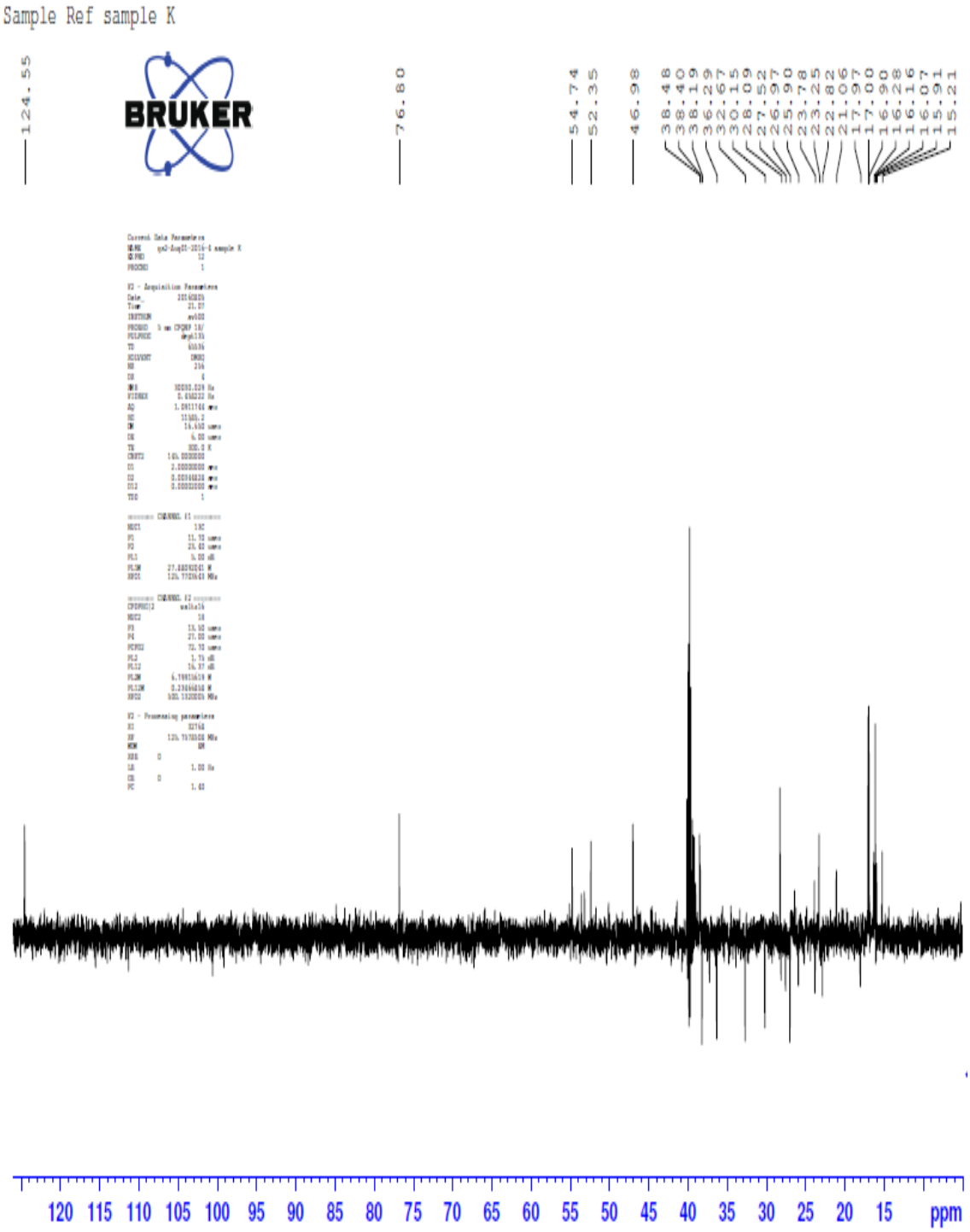


Figure 4.24: DEPT experiment of K

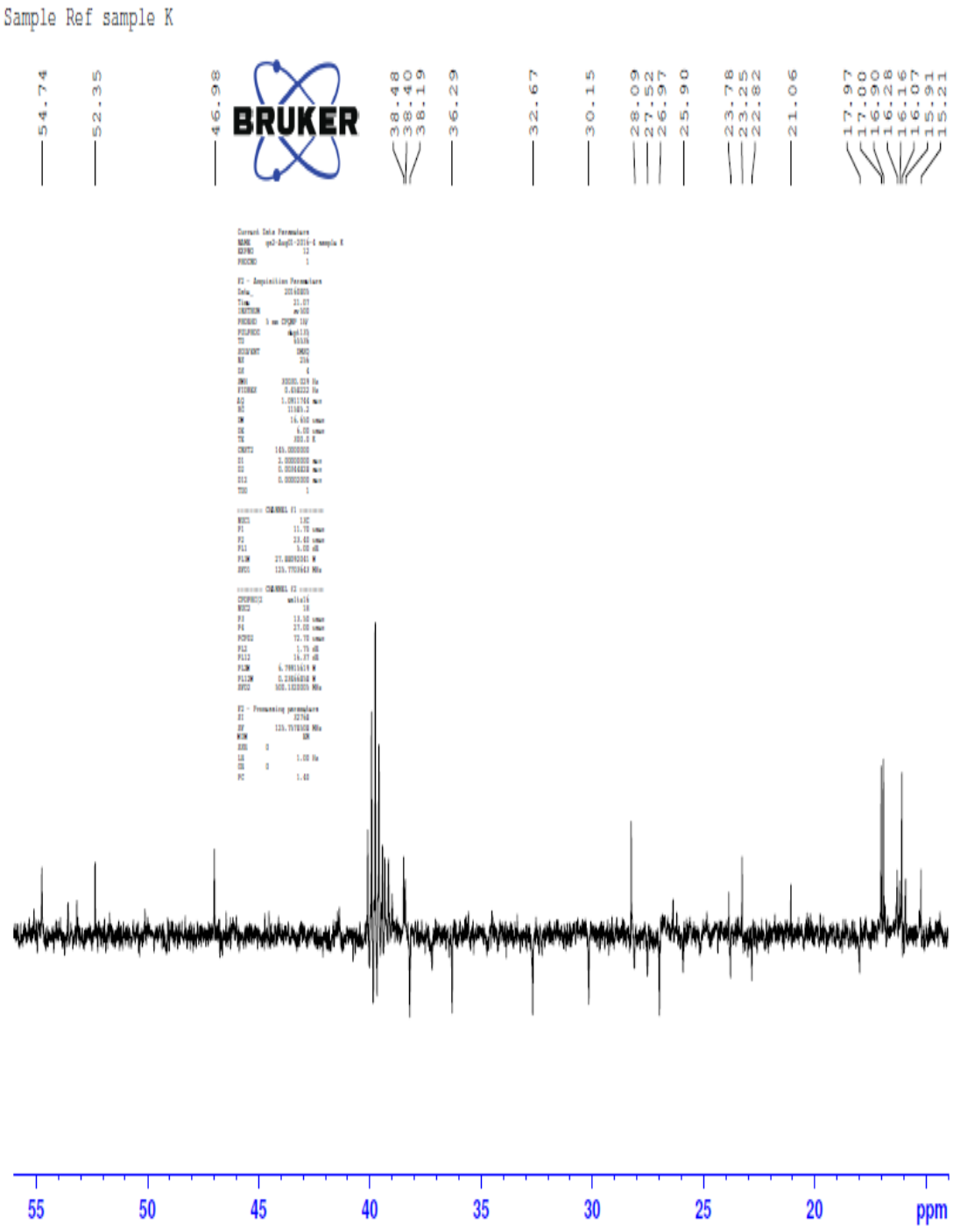


Figure 4.24a: DEPT experiment of K (expansion)

4.8.6 $^{1}H - ^{1}H$ COSY spectral data of K

The COSY spectrum of K showed important cross peaks that were used to assign protons to adjacent carbon atoms (Figure 4.25a).

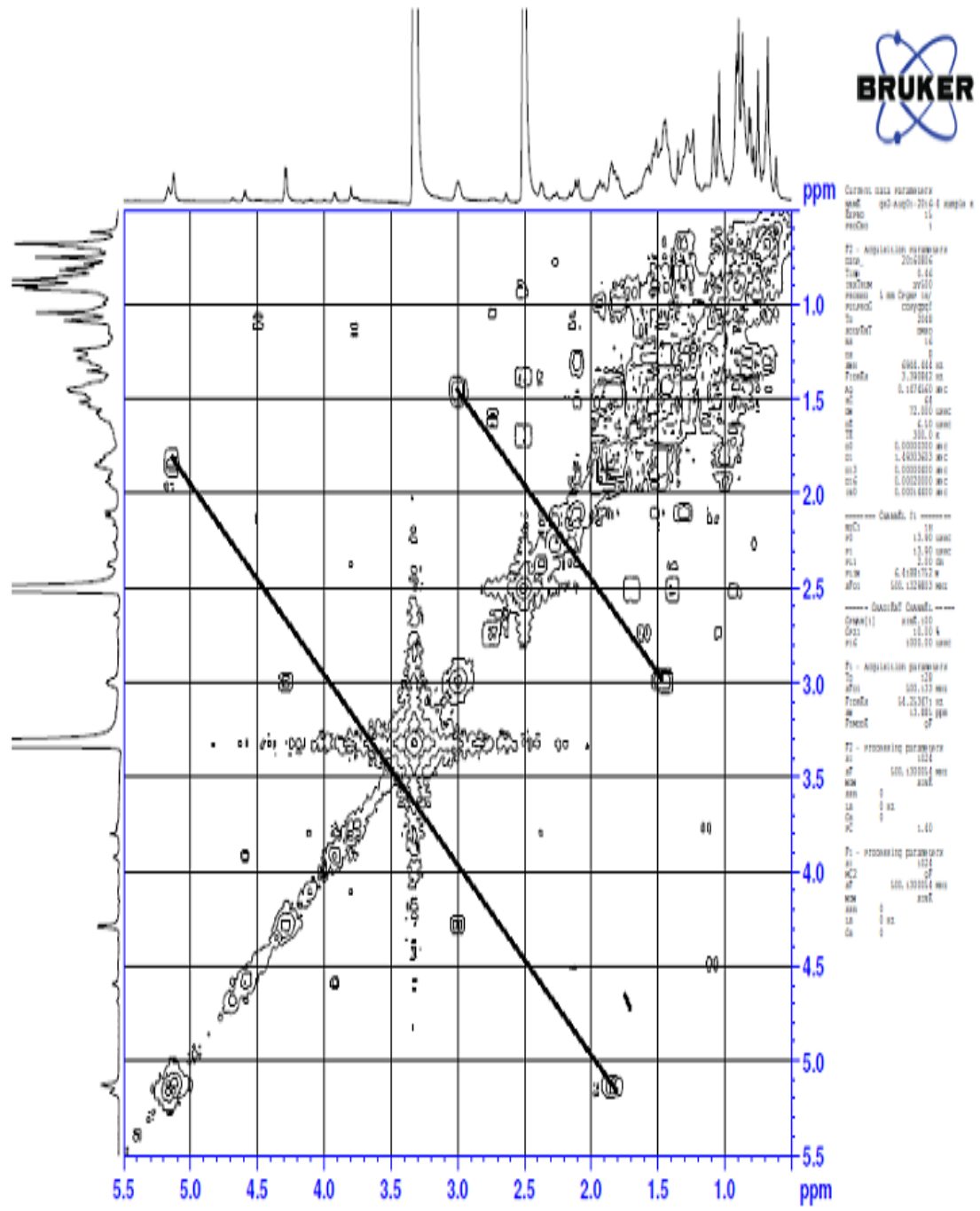


Figure 4.25: ^{1}H-^{1}H COSY experiment of K

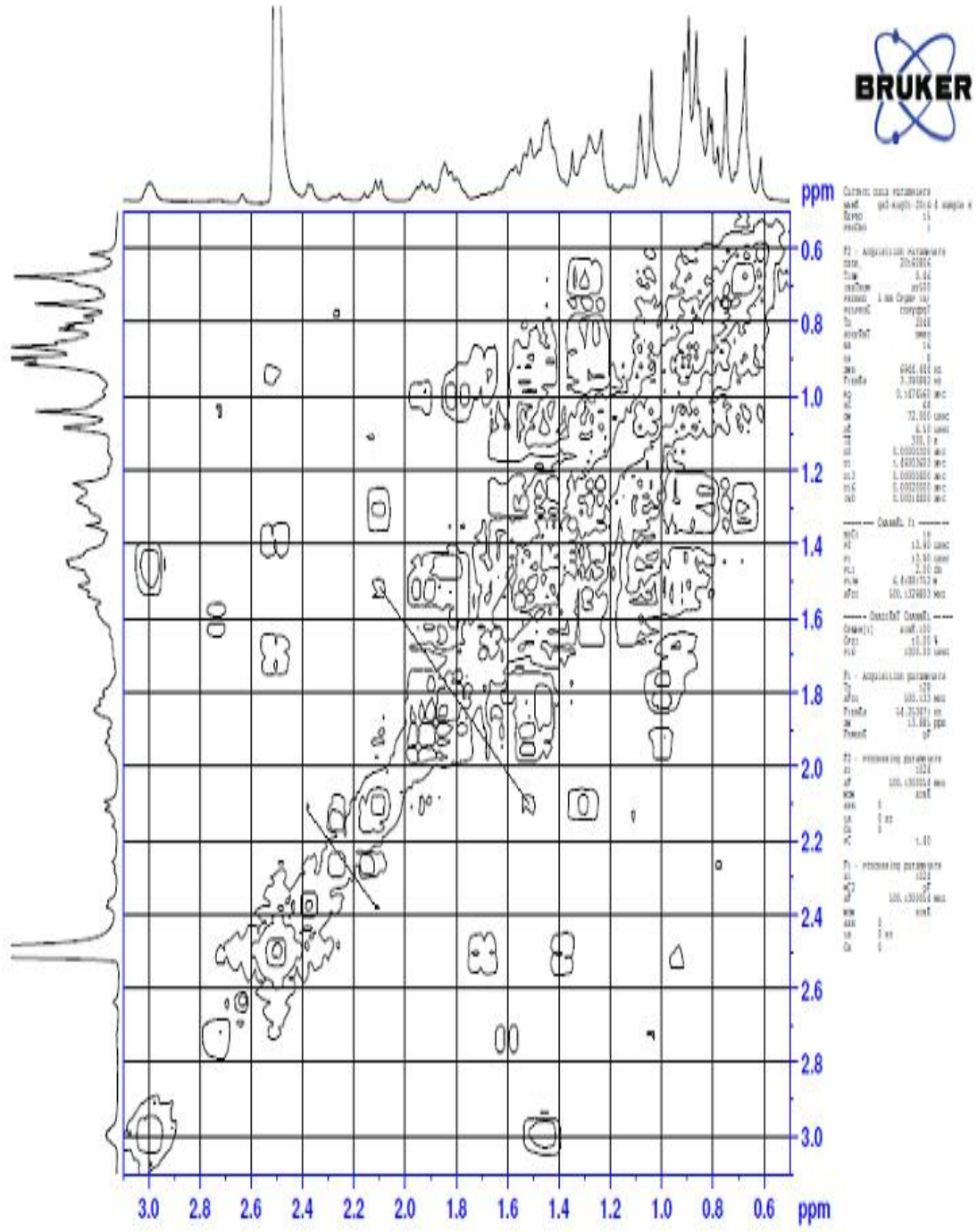


Figure 4.25a: ^{1}H-^{1}H COSY experiment of K (expansion)

4.8.7 HMQC spectral data of K

The HMQC experiment is as shown in Figure 4.26 and was used to assign protons on their respective carbons (Table 4.9)

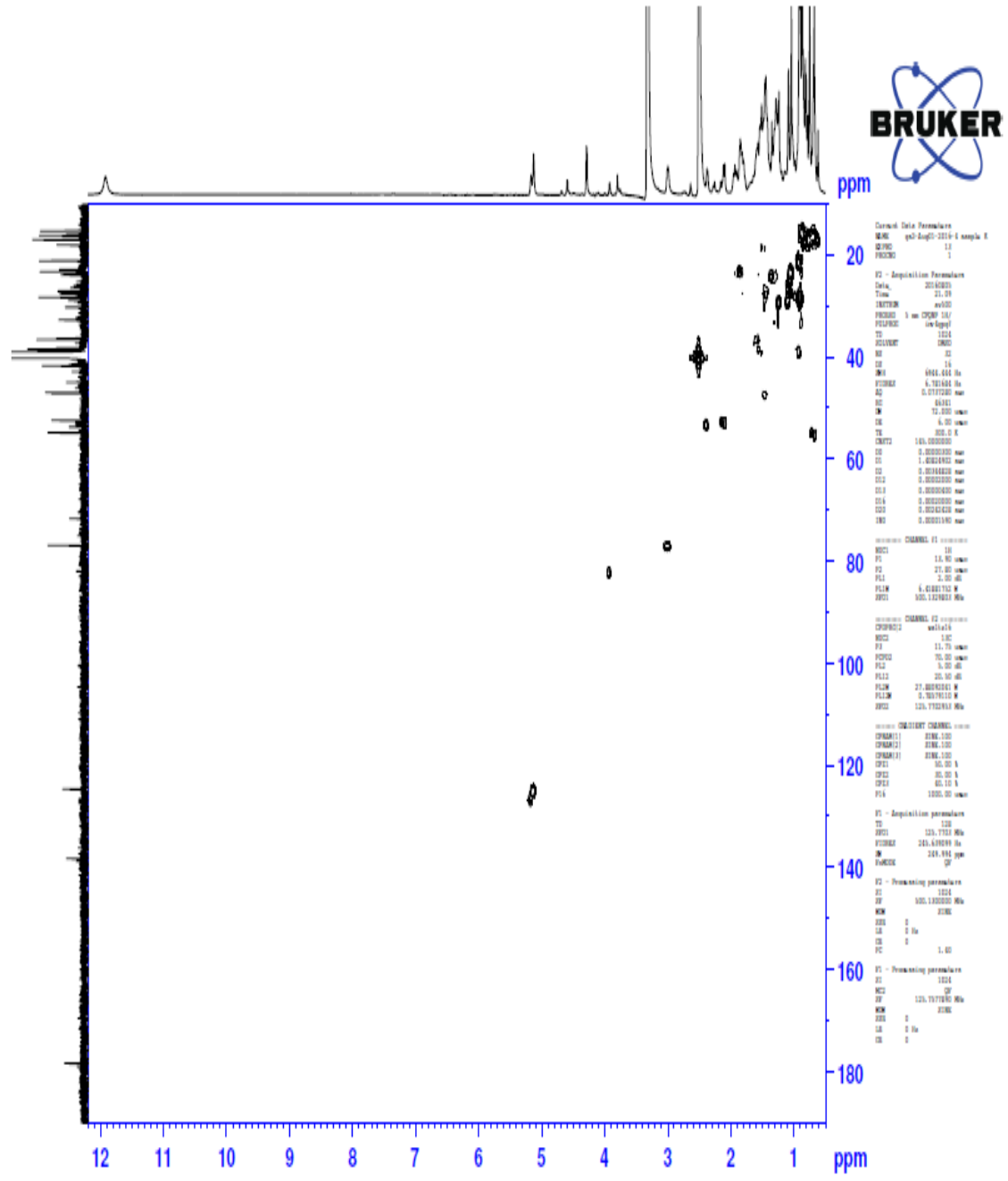


Figure 4.26: HMQC spectrum of K

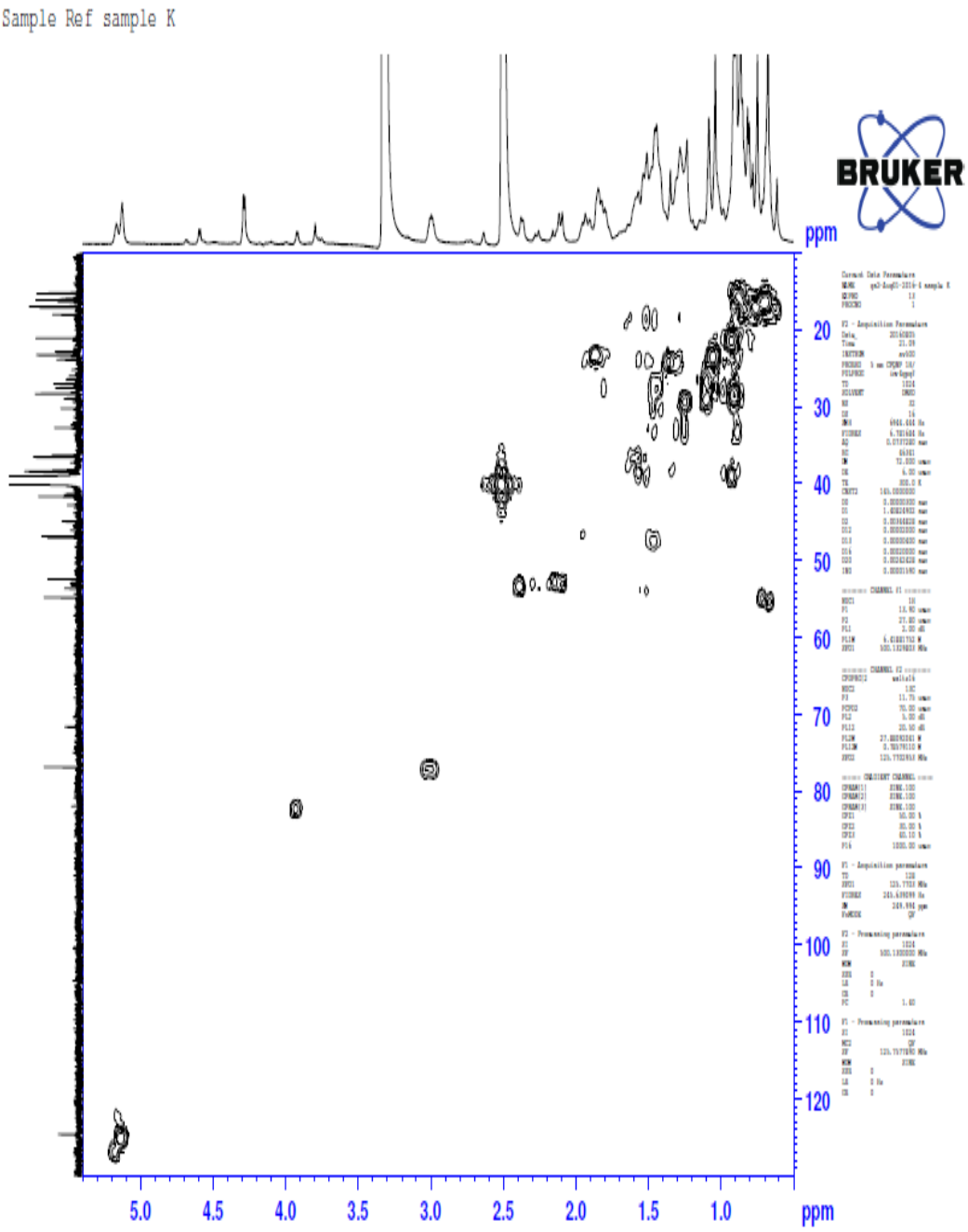


Figure 4.26a: HMQC spectrum of K (expansion 1)

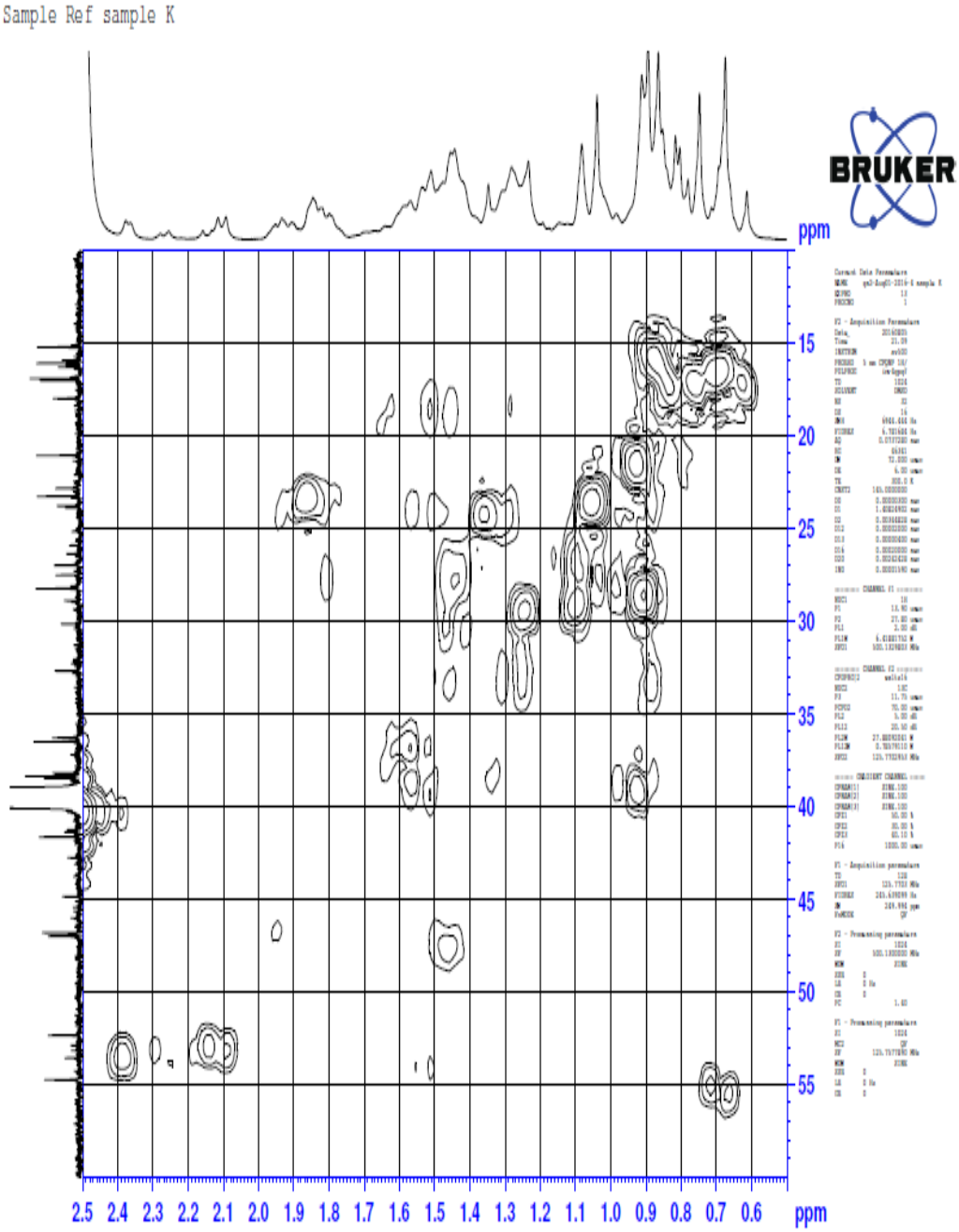


Figure 4.26b: HMQC spectrum of K (expansion 2)

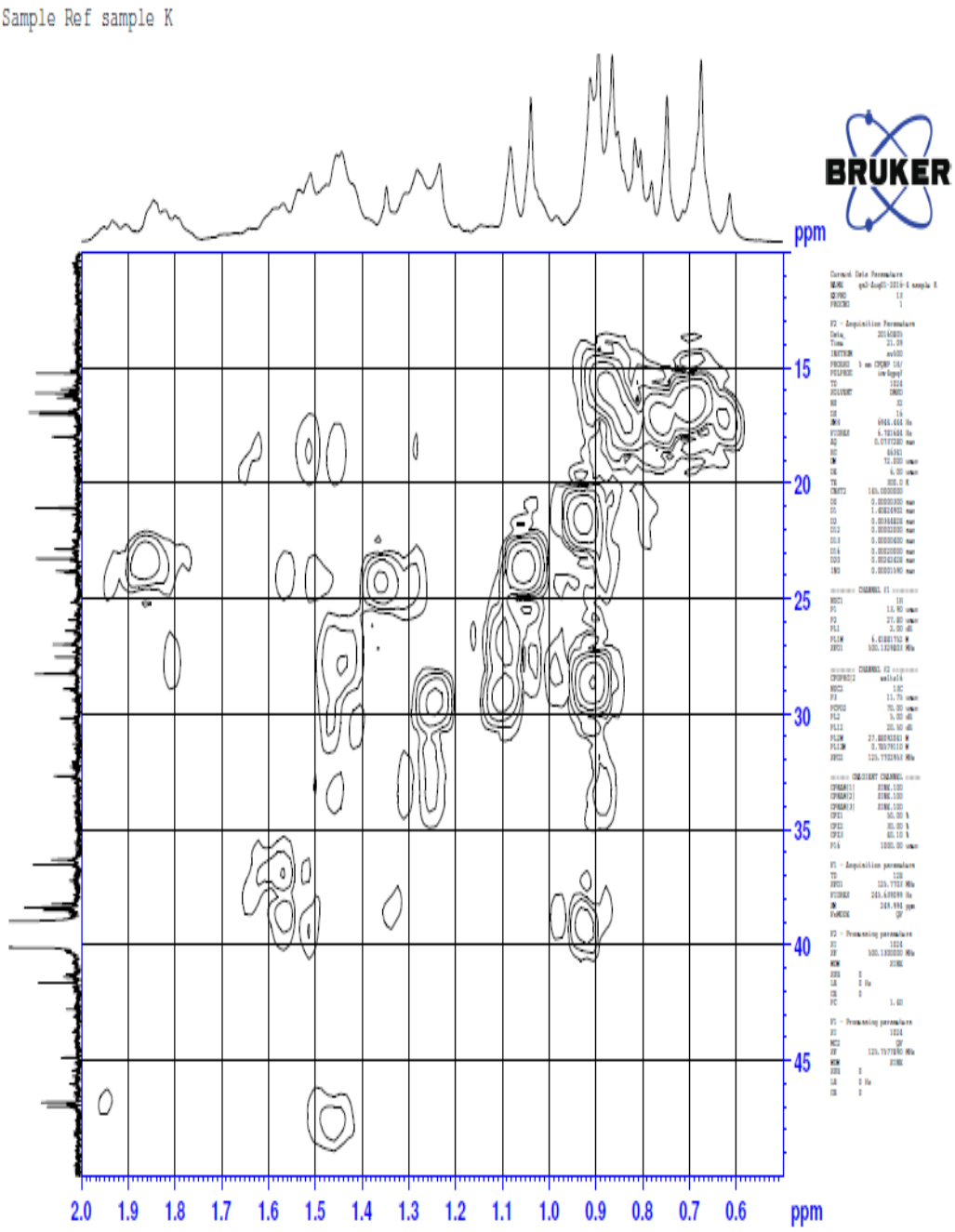


Figure 4.26c: HMQC spectrum of K (expansion 3)

Table 4.9: HMQC and DEPT spectral data of K

Position	δ_C	δ_H	DEPT
1	38.36		CH_2
2	26.97	1.47	CH_2
3	76.80	3.00	CH
4	38.48		C
5	54.74		CH
6	17.97		CH_2
7	32.67		CH_2
8	38.28		C
9	46.98		CH
10	36.50		C
11	22.82	1.85	CH_2
12	124.55	5.15 (d, J= 19.5 Hz)	CH
13	138.17		C
14	41.62		C
15	27.51		CH_2
16	23.85		CH_2
17	46.87		C
18	52.35	2.09	CH
19	38.48	1.35	CH

20	38.40		CH
21	30.15		CH_2
22	36.29		CH_2
23	28.24		CH_3
24	16.27		CH_3
25	15.20		CH_3
26	16.90	1.04	CH_3
27	23.26		CH_3
28	28.09		CH_3
29	17.00	0.81	CH_3
30	21.06	0.90	CH_3
1'	178.27		C
2'	38.19	1.51	CH_2
3'	52.35	2.12	CH
4'	54.74	2.37	CH
5'	71.60		C
6'	15.20	1.07	CH_3
7'	17.97		CH_3
8'	23.78		CH_3
9'	15.20	1.07	CH_3

4.8.8 HMBC spectral data of K

The HMBC experiment is as shown in Figure 4.27 and was used to establish connectivity between various units within the triterpenoid nucleus (Table 4.10)

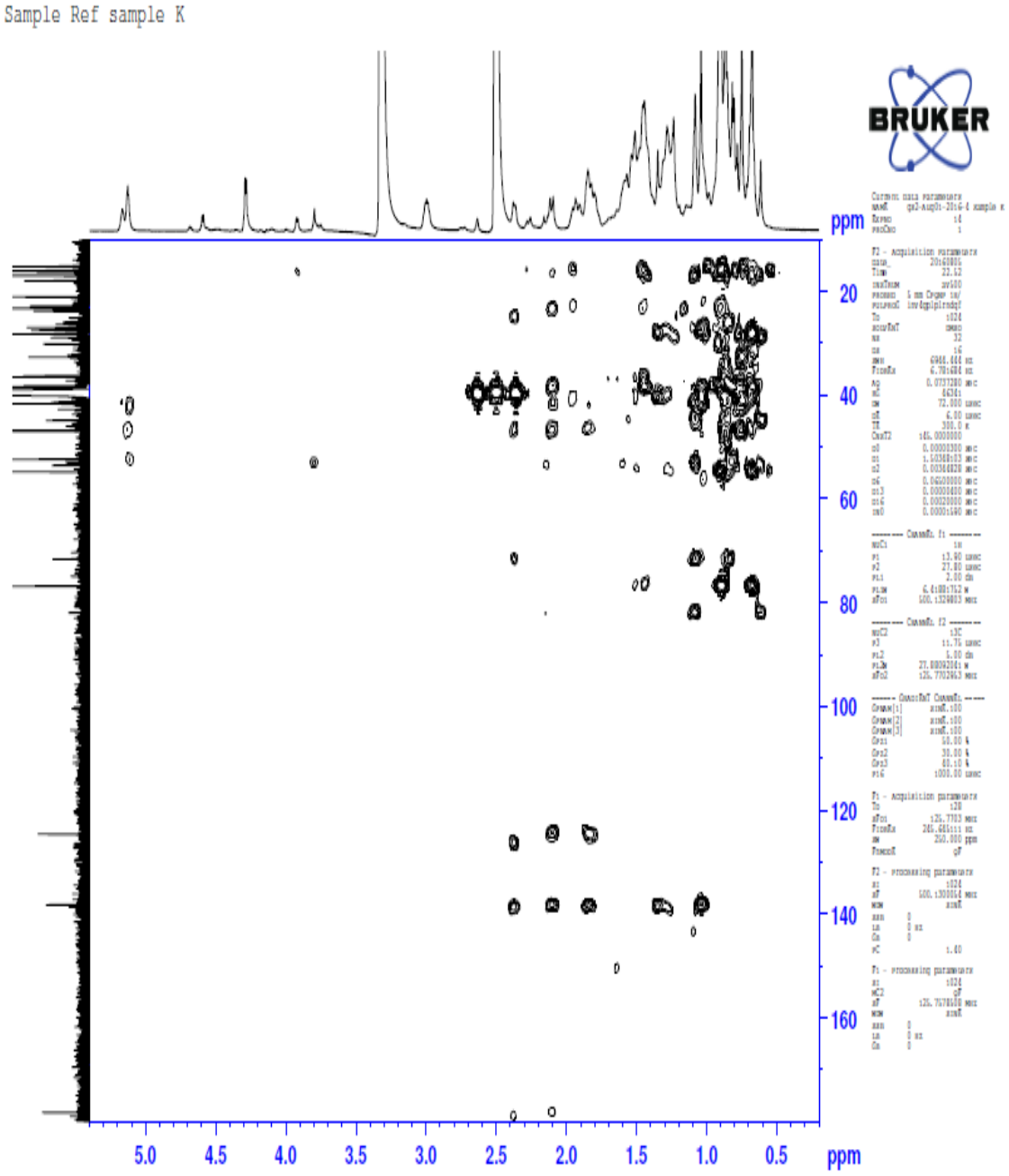


Figure 4.27: HMBC experiment of K

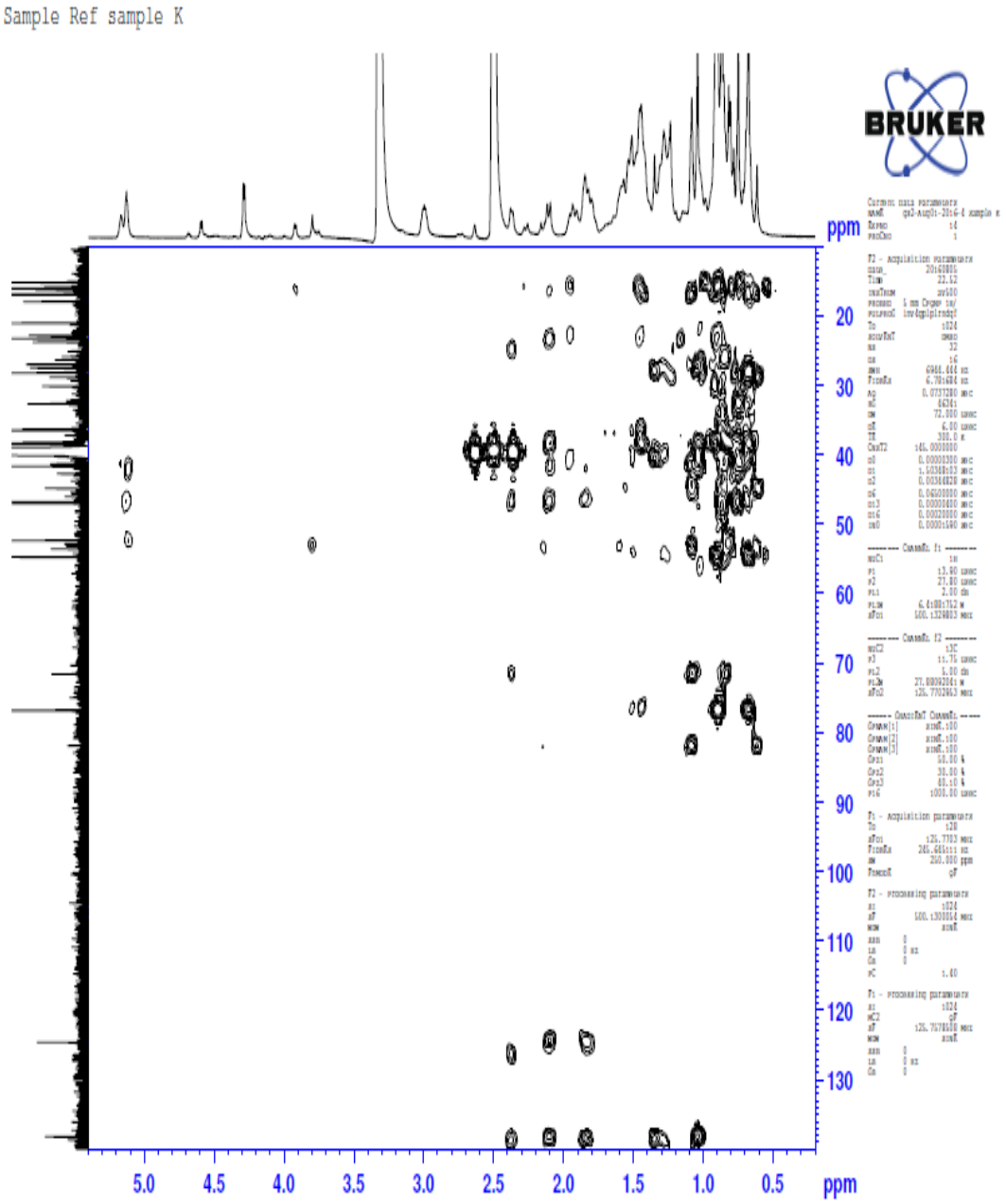


Figure 4.27a: HMBC experiment of K (expansion 1)

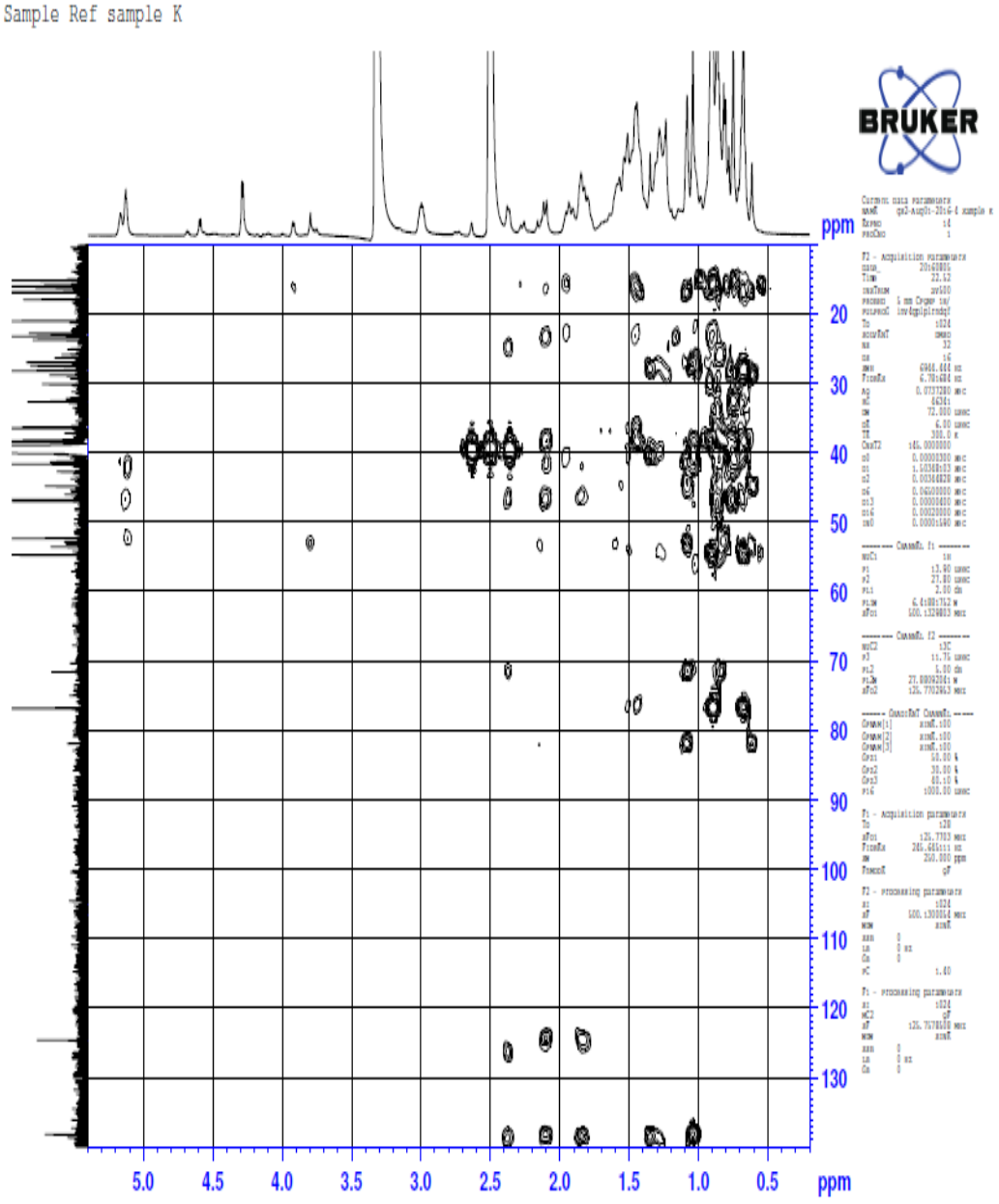


Figure 4.27b: HMBC experiment of K (expansion 2)

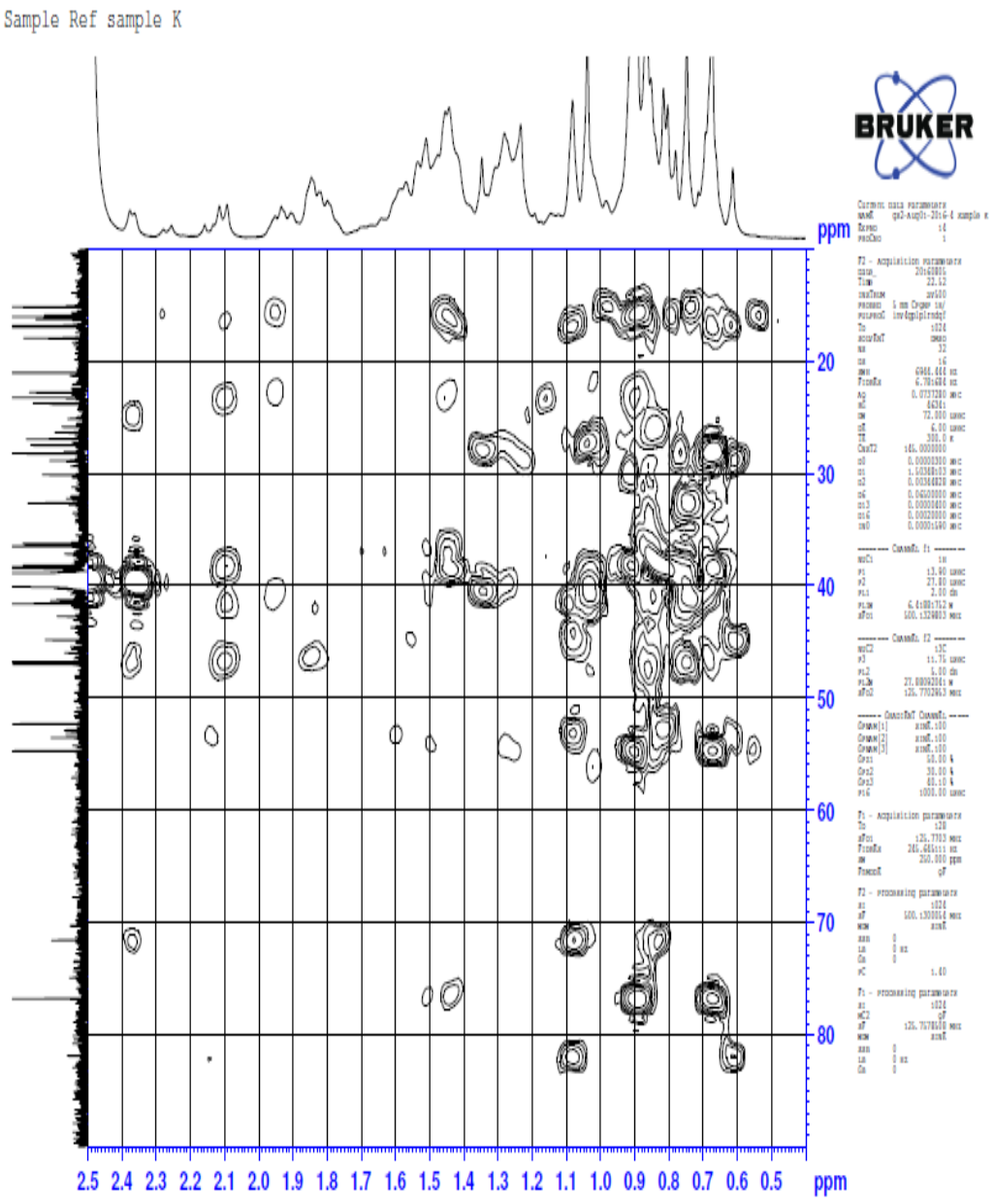


Figure 4.27c: HMBC experiment of K (expansion 3)

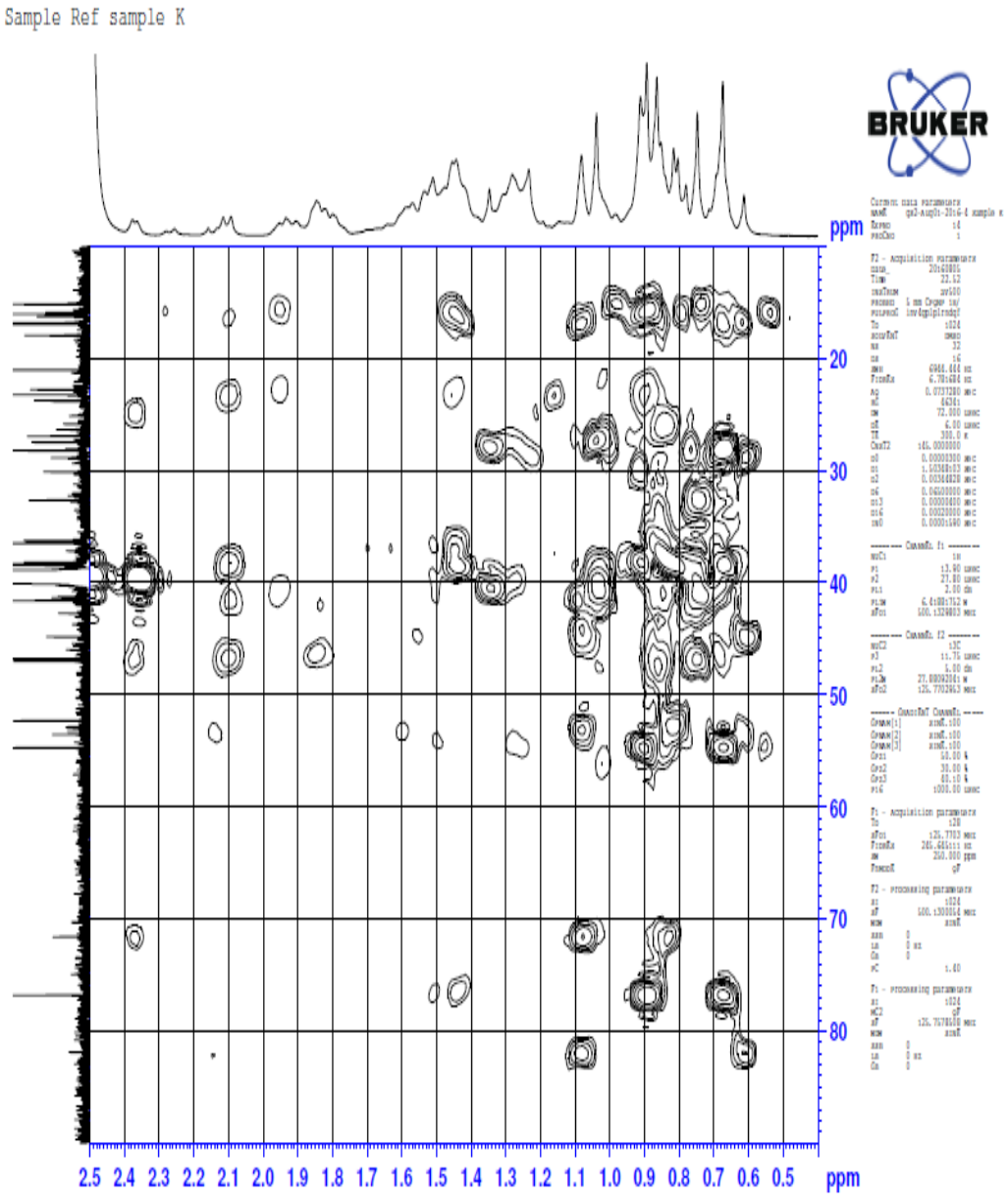


Figure 4.27d: HMBC experiment of K (expansion 4)

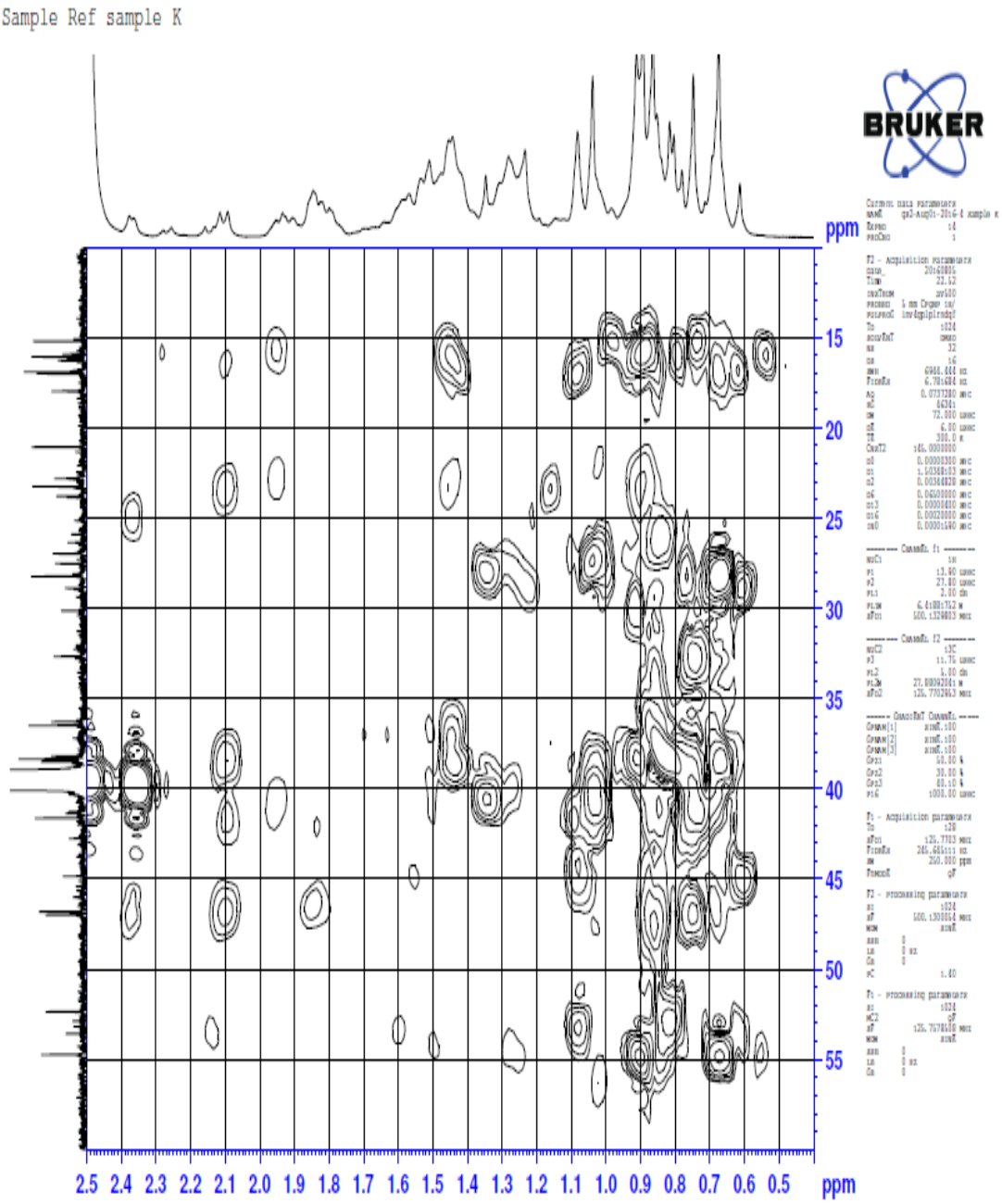


Figure 4.27e: HMBC experiment of K (expansion 5)

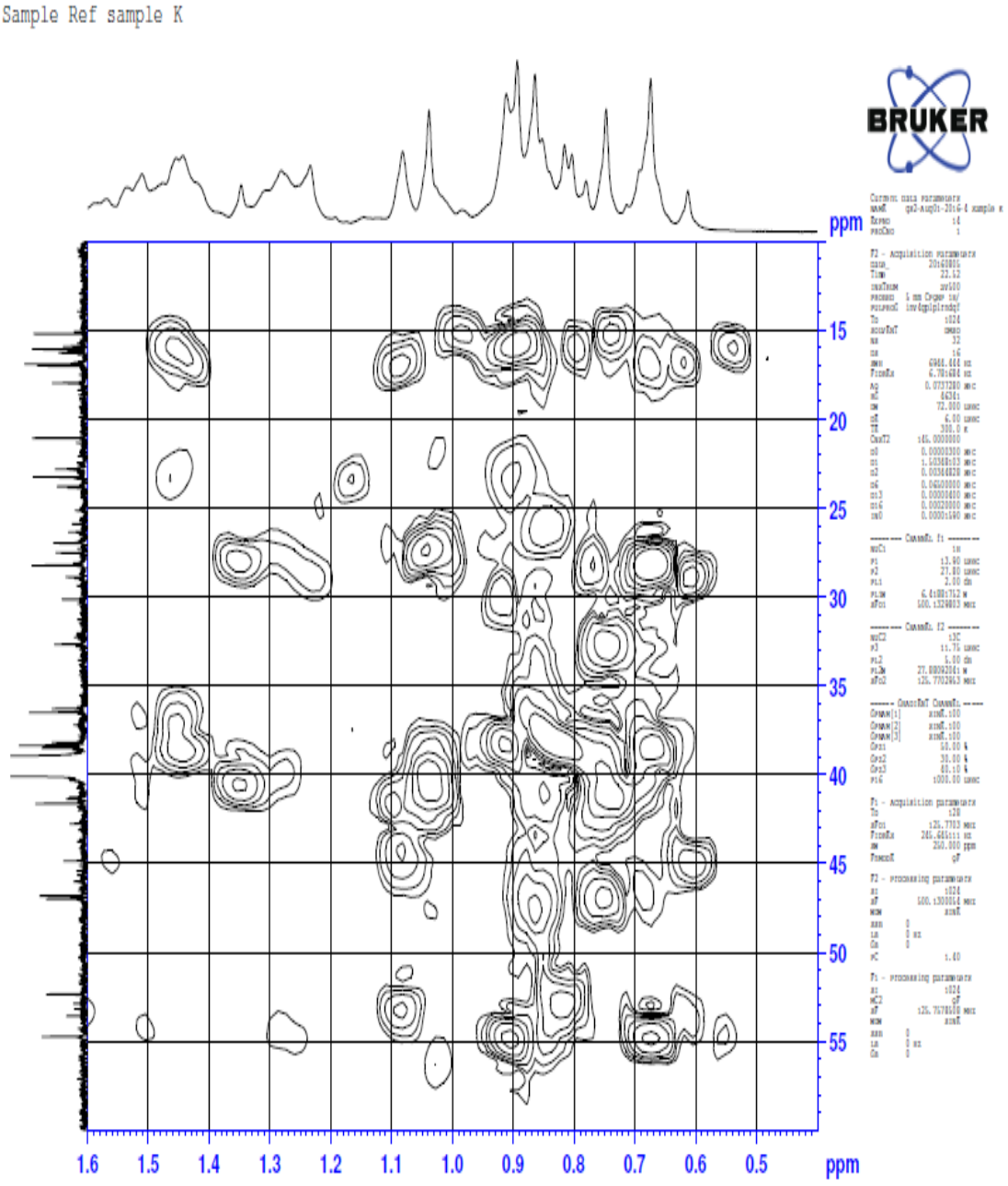


Figure 4.27f: HMBC experiment of K (expansion 6)

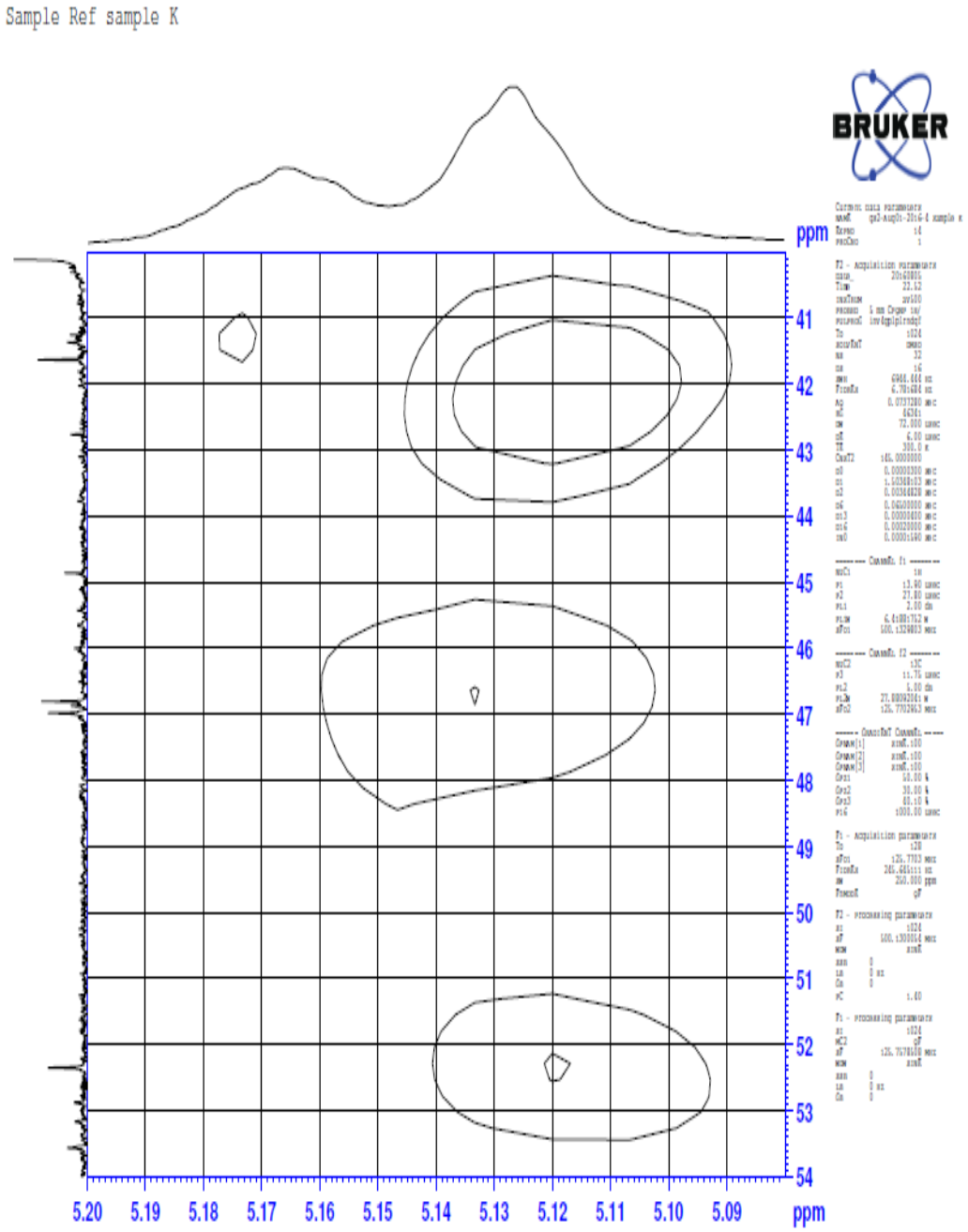


Figure 4.27g: HMBC experiment of K (expansion 7)

Table 4.10: HMBC correlations of K

δ_H	δ_C
1.35	138.17
1.51	54.74
1.85	124.55, 138.17
2.12	178.27
5.15	46.98, 41.62, 52.35

4.9 Acute Toxicity Studies

The *intraperitoneal* median lethal dose of the extract was 1,265 $mgkg^{-1}$ in mice and 2,154 $mgkg^{-1}$ in rats (Table 4.11).

Table 4.11: Median Lethal Dose (LD_{50}) of Crude Extract and n-butanol fraction

Rodent species	LD_{50} ($mgkg^{-1}$)
Mice	1,265
Rats	2,154

4.10 Effect of MEZM on Thermally-induced Pain in Mice

MEZM produced a significant ($p < 0.01$) dose dependent increase in mean pain latency at all the tested time intervals. The effect of 150 $mgkg^{-1}$ of the extract is similar to the effect of 20 $mgkg^{-1}$ of pentazocin at 30 and 60 minutes (Figure 4.28).

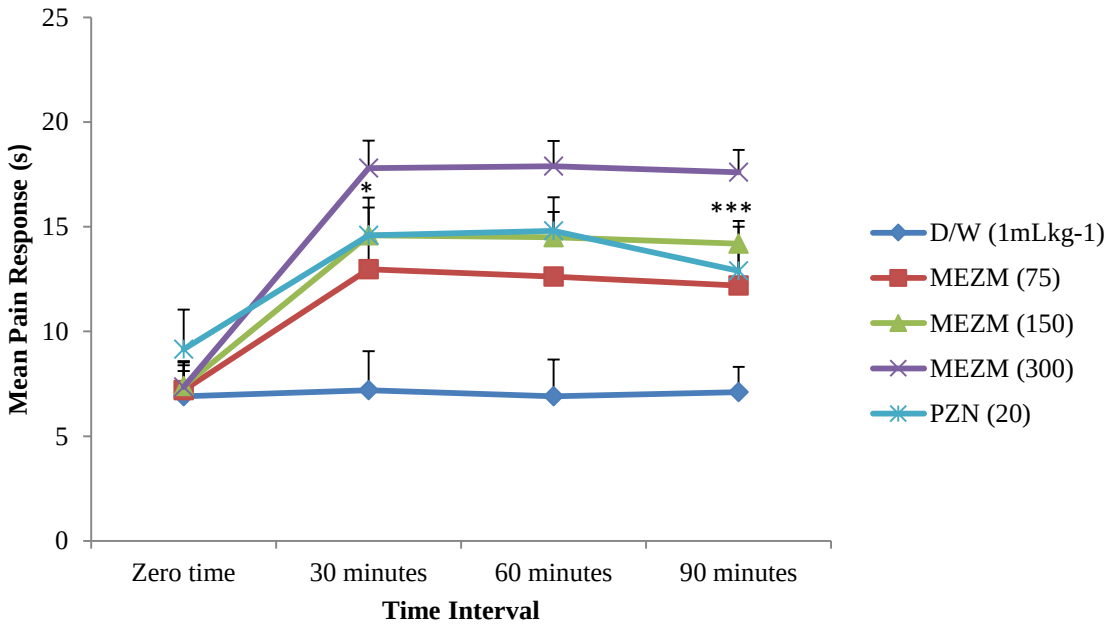


Figure 4.28: Effect of MEZM on thermally induced pain in mice

Data represented as Mean ± SEM; n = 6; $^{*}p< 0.05$, $^{**}p< 0.01$, and $^{***}p< 0.001$ (Bonferroni test for pairwise comparison); D/W: distilled water; PZN: pentazocin; MEZM: methanol leaf extract of *Ziziphus mucronata*. Figures in parentheses represent doses in $mgkg^{-1}$.

4.11 Effect of MEZM on acetic acid-induced writhing test in mice

MEZM significantly ($p < 0.05$) attenuated the acetic acid induced writhing at the tested doses in a biphasic manner with the dose of 150 $mgkg^{-1}$ producing 68 % inhibition. The activity of the extract at all the tested doses is greater than that of 10 $mgkg^{-1}$ diclofenac, a standard anti-inflammatory agent (Table 4.12).

Table 4.12 Effect of MEZM on acetic acid-induced writhing test in mice

Treatment (Dose-mgkg^{-1})	Mean number of writhes ± S.E.M	% inhibition
D/W (1 mLkg^{-1})	10.33 ± 2.03	0
MEZM (75)	5.33 ± 2.76	48.40
MEZM (150)	3.33 ± 1.58*	67.96
MEZM (300)	5.33 ± 2.88	48.40
DCF (10)	7.33 ± 3.57	29.04

Data represented as Mean ± SEM; n = 5; $^{*}p< 0.05$ (Dunnett t-test for multiple comparison); D/W: distilled water; DCF: diclofenac; MEZM: methanol leaf extract of *Ziziphus mucronata*. Figures in parentheses represent doses in mgkg^{-1}.

4.12 Anti-inflammatory activity of MEZM

In the normal saline treated animals, sub plantar injection of 0.1 mL carrageenan suspension produced a local oedema reaching its maximum at 3 h. The percentage anti-inflammatory effect (at the peak of carrageenan-induced oedema) of the extract at the doses tested (200, 400 and 800 $mgkg^{-1}$) were 27.5, 37.7 and 40.6 % respectively compared to 29.9 % for diclofenac (20 $mgkg^{-1}$), a standard anti-inflammatory agent (Figure 4.29).

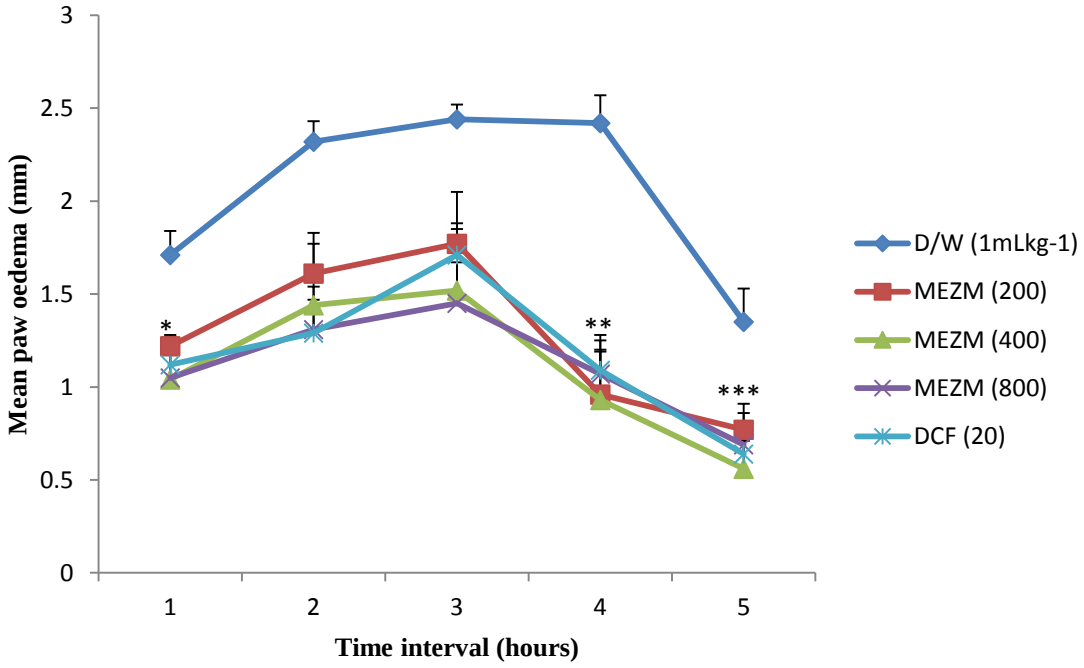


Figure 4.29 Effect of MEZM on Carrageenan induced hind paw oedema in rats

Data represented as Mean ± SEM; n = 5; $^{**}P< 0.01$, and $^{***}P< 0.001$ (Bonferroni test for pairwise comparison); D/W: Distilled water; DCF: Diclofenac; MEZM: Methanol leaf extract of *Ziziphus mucronata*. Figures in parentheses represent doses in $mgkg^{-1}$.

4.13 Anti-inflammatory activity of nBF

All the tested doses of nBF (150 - 600 $mgkg^{-1}$) significantly ($p < 0.001$) produced a decrease in size of oedema at the second phase of the of the inflammatory process (4^{th} and 5^{th} hour) in a manner similar to that of diclofenac, a standard anti-inflammatory agent (Figure 4.30).

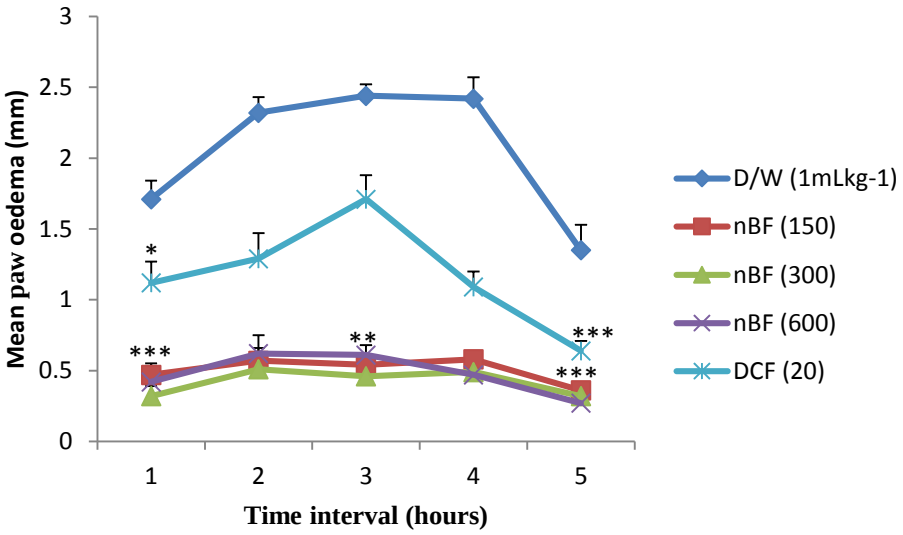


Figure 4.30: Effect of nBF on Carrageenan induced hind paw oedema in rats

Data represented as Mean ± SEM; n = 5; $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$ (Bonferroni test for pairwise comparison); D/W: Distilled water; DCF: diclofenac; nBF: n-butanol fraction of *Ziziphus mucronata*. Figures in parentheses represent doses in $mgkg^{-1}$

4.14 Anti-tumor activity of MEZM and nBF

Both MEZM and nBF produce a significant ($p < 0.001$) decrease in the mean number of tumors produced by *Agrobacterium tumefaciens* on potato disc at all the concentrations (10 -1000 ppm) tested (Figure 4.31)

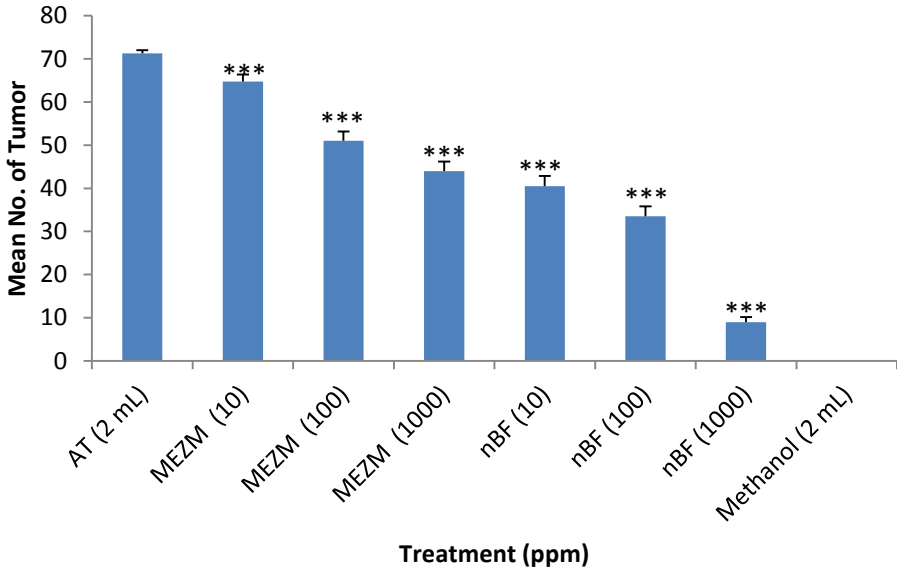


Figure 4.31: Effect of MEZM and nBF on *A. tumefaciens*-induced tumor on potato disc

Data expressed as Mean ± SEM; n = 3; $^{***}P< 0.001$ (Dunnett t-test); AT: *Agrobacterium tumefaciens*; MEZM: methanol leaf extract of *Ziziphus mucronata*; nBF: n-butanol fraction of *Ziziphus mucronata*. Figures in parentheses represent concentration in ppm.

CHAPTER FIVE

5.0 DISCUSSION

The present study attempted to evaluate the analgesic, anti-inflammatory and anti-tumor potentials of the leaf extract of *Ziziphus mucronata* with the goal of validating its folkloric claim in the management of painful and inflammatory condition and also isolate some phytochemicals that may be responsible for the observed activity as well as adding to the chemotaxonomy of the Rhamnaceae family.

The preliminary phytochemical screening of the methanol extract as well as the ethyl acetate and n-butanol fractions revealed the presence of important secondary metabolites

which might be responsible, either singly or in combination, for the reported ethno pharmacological activity(ies) of the plant.

Silica gel column chromatographic separation of the ethyl acetate fraction followed by purification in Sephadex LH-20 (gel filtration) led to the isolation of compound 'B' which was characterized by physical methods and spectroscopic techniques.

Compound B was positive to Ferric chloride test for phenolic nucleus and Shinoda test indicating the presence of flavonoid nucleus. A single spot obtained for B on a TLC plate indicates that it is a pure compound. Also, an uncorrected melting point range of 176 - 178°C (2°C range) obtained for B indicates its purity level.

The UV spectrum of B showed absorption bands at 239 nm (band K) due to conjugation and 280 nm (band B) due to simple substituted benzene. This is consistent with conjugated π-π* transitions arising from the aromatic rings. These results are similar to those reported in the literature (Abdullahi *et al*., 2016)**.**

The coupled vibration in IR spectrum at 2855 cm^{-1} and 2922 cm^{-1} are due to saturated symmetric and asymmetric C-H stretching. The weak band at 1610 cm^{-1} is due to aromatic C=C stretching.

The five aromatic proton signals of B were assigned to an AX type ring A [δ_H 5.85 (1H, d, *J* = 2.0 Hz, H-6), 5.93 (1H, d, *J* = 2.0 Hz, H-8)] and an ABX type ring B [δ_H 6.84 (1H, d, *J* = 1.5 Hz, H-2'), δ_H 6.76 (1H, d, *J* = 8.0 Hz, 5'), δ_H 6.73 (1H, dd, *J* = 8.0 Hz and 1.5 Hz, H-6')] (Abdullahi *et al*., 2016). The signals between 6.70 and 6.90 ppm are characteristic of the ABX spin pattern in the ring B of flavan-3-ol (Antonelli *et al*., 2007).

Also, the two methylene proton signals at δ_H 2.53 (1H, dd, *J* = 8.0 Hz and 16.0 Hz, H-4a, ax.) and 2.87 (1H, dd, *J* = 5.5 Hz and 16.0 Hz, H-4b, eq.) are typical of position 4 of flavanols (Tajuddeen *et al*., 2015). The signals at δ_H 4.57 (1H, d, *J* = 7.5 Hz, H-2) and δ_H 3.98 (1H, m) suggest that the flavan structure possesses the trans-2,3 stereochemistry, supported by the value for the coupling (7.5 Hz) which appeared as a doublet at H-2. These signals are attributed to ring C.

The structural assignment of B was further substantiated by its COSY correlation which showed cross peak between δ_H 2.53 (H-4a) and 2.87 (H-4b) and that between δ_H 3.98 (H-3) and 4.57 (H-2). Cross peaks between δ_H (2.53 and 2.87) to δ_H 3.98 confirms its assignment as H-3.

Based on the forgoing spectral analysis and result of mass spectrometry, B was assigned molecular formula of $C_{15}H_{14}O_6$ on the basis of ESI-HRMS which showed molecular ion peak at *m/z* 291.1 $[M + H]^+$ and 289.1 $[M - H]^+$ (Calcd 290.3). Two important masses of main fragments were observed at *m/z* 273.2 expectedly due to water loss and *m/z* 139.0 probably from a product of retro Diels Alder fragmentation. Other important diagnostic peaks include *m/z* 578.9 [2M-2H] *m/z* 579.9 [2M - H] and *m/z* 869.0 [3M - H].

Based on these information, compound B was confirmed to be Catechin (Figure 5.1)

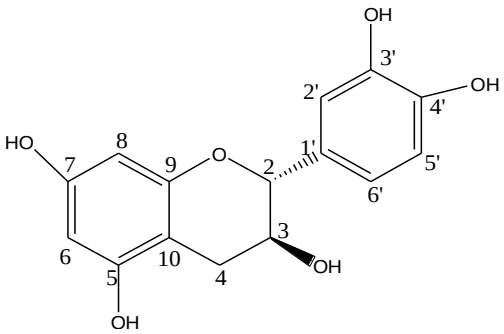


Figure 5.1

Catechin and its epimer, Epicatechin are the building blocks of the proanthocyanidins, a type of condensed tannin. They are a type of natural phenol and anti-oxidant belonging to the group of flavan-3-ols, part of the chemical family of flavonoids. The biosynthesis begins with a 4-hydroxycinnamoyl CoA starter unit which undergoes chain extension to form chalcones followed by isomerization to yield catechins. Catechin oxygenase, a key enzyme in the degradation of catechin, is present in fungi and bacteria (Punyasiri *et al.*, 2004). Flavanols have been studied for their anti-oxidative, anti-cancerogenic, anti-allergenic, anti-inflammatory, and vasodilatory properties. Antimicrobial effects of flavan-3-ols have also been reported (Punyasiri *et al.*, 2004).

S_1 isolated from Fraction F of n-butanol column aliquot was positive to Ferric chloride and Shinoda test indicating the presence of flavonoid nucleus.

The shoulder bands for S_1 at λ_{max} 226 nm, 257 nm, 346 nm and 363 nm is characteristic of compounds with highly conjugated system (Kemp, 1991).

In the IR spectrum, the two important bands at 1697 cm^{-1} and 3447 cm^{-1} are characteristic of carbonyl and hydroxyl functional groups respectively.

The ^1H NMR spectrum showed two sets each of AX spin pattern signals [δ_H 6.19 (2H, d, J = 2.0 Hz), 6.38 (2H, d, J = 2.0 Hz)] and ABX spin pattern signals [δ_H 6.88 (2H, dd, J = 2.0 Hz and 8.5 Hz), 7.59 (2H, dd, J = 2.0 Hz and 8.5 Hz), 7.71 (1H, d, J = 2.5 Hz) and 7.84 (1H, d, J = 2.0 Hz)] corresponding to the aromatic protons of rings A and B

respectively thus, indicating that S_1 is a biflavonoid derivative because four of the six aromatic protons integrated for two protons each. Ten of the methine signals are clustered around δ_H (3.22- 3.85) corresponding to δ_C (62.02- 78.50) suggesting the presence of two glucose residues. The two anomeric proton signals at [δ_H 5.15 (1H, d, *J* = 8.0 Hz) and 5.24 (1H, d, *J* = 7.5 Hz)] are typical of glucose. The high coupling constants observed (8.0 Hz and 7.5 Hz) are due to diaxial coupling with H-2 proton of each residue and confirms the configuration of both sugars as β-D glucosyl (Mabry *et al*., 1970).

The appearance of paired signals in the carbon 13 spectrum suggest that S_1 is a dimer. The two methylene carbons observed at δ_C 62.02 and δ_C 62.64 further confirm the nature of sugars as glucose. Moreover, the C-3 resonances observed in the downfield region (δ_C 135.66 and δ_C 135.82) relative to C-10 (δ_C 105) (the same chemical environment) further confirmed that the glycosylation is at C-3 position (Wu *et al.*, 2007). The ^{13}C NMR also showed ten oxygen bearing quaternary carbons. Six carbons have hydroxyl groups attached and two are linked to pyranone oxygen. This suggested that the remaining two should be involved in interflavonoid ether linkage. Thus, S_1 must be the biflavonoid with either 4'-4" or 3'-4" ether linkage (Kumar *et al*., 2005). The downfield resonance of C-4" compared to C-4' suggested a 4'-4" linkage. The linkage was further confirmed by analyzing the HMBC spectral data.

The heteronuclear correlation experiment (HMQC) facilitated the attachment of all protons to their respective carbons as shown on Table 4.3.

The assignment of the carbons and the placement of the sugar moiety were achieved using the long range correlation experiments (HMBC).

In the HMBC spectrum, a common J_3 correlation between protons at δ_H 6.19 and 6.38 to carbons at δ_C 95.10 and 100.42 respectively confirms their assignment to rings A of both flavonoid residues. Also, a J_2 correlation between protons at δ_H 6.88 to carbons at δ_C 122.98 and 123.25 and a J_3 correlation to carbon at δ_C 146.04 confirms their assignment to ring B.

A common J_3 correlation between anomeric protons at δ_H 5.15 and 5.23 to the quartenary carbons at δ_C 135.66 and 135.82 confirms that each of the sugar moiety is linked to the individual flavonoid aglycone through C-3 carbon. Other correlations confirmed by the HMBC are those of the sugar residue.

Based on the forgoing spectral analysis, S_1 was confirmed to be a biflavonoid and with the aid of 2D NMR correlations, the structure of S_1 was proposed to be Bis (quercetin 3-*O*- β-D-glucopyranoside)/bis (isoquercetin) (Figure 5.2)

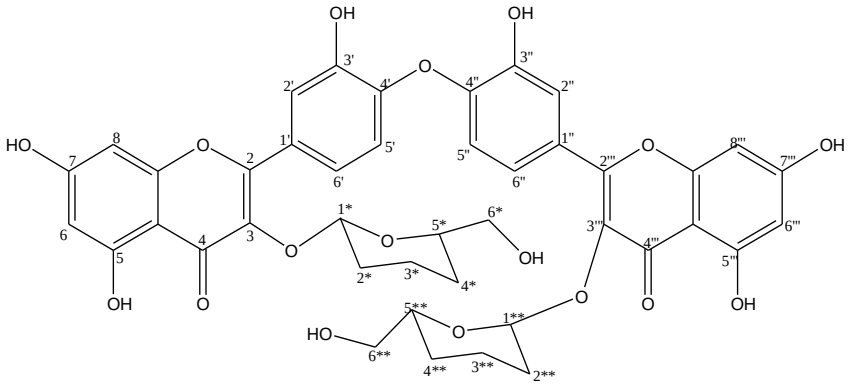


Figure 5.2

C-O-C linked biflavonoids are those in which two monomeric units may be of the same or different structural types joined to each other through an ether linkage. Unlike C-C linked biflavonoids, C-O-C linked biflavonoids have restricted distribution in the plant

kingdom. They are classified into various groups due to the nature of the interflavonyl linkage between them and have used as chemotaxonomic markers for different families of plant e.g. Ochna flavones first isolated from the Ochnaceae family.

Some pharmacological activities of biflavonoids were described such as inhibition of histamine release from mast cells and inhibition of lymphocyte proliferation, suggesting the anti-inflammatory/anti-allergic potential of biflavonoids. Several natural biflavonoids including ochnaflavones and ginkgetin inhibit phospholipase A_2. Recently, several synthetic approaches yielded new biflavonoid molecules with anti-inflammatory potential. These molecules also exhibit phospholipase A_2 and cyclooxygenase-2 inhibitory activity (Kim *et al*., 2008).

S_3 isolated from Fraction K of n-butanol column aliquot was positive to Ferric chloride and Shinoda test indicating the presence of flavonoid nucleus.

The UV absorption bands for S_3 at 257 nm and 359 nm are due to benzoyl moiety and cinnamoyl moiety respectively. These values are typical for Flavonol nucleus. The position of band I (due to cinnamoyl) distinguish between flavones and flavonols (3-hydroxyflavones). Band I of flavones occurs in the range 304-350 nm whereas band I of flavonols appears at a longer wavelength (352-385 nm) (Mabry *et al.,* 1970).

The IR peak at 1602 cm^{-1} is characteristic of C=C in aromatic ring while the sharp peak at 1654 cm^{-1} is typical for conjugated carbonyl system. The coupling bands at 2855 cm^{-1} and 2922 cm^{-1} are due to saturated symmetric and asymmetric C-H stretching in glucose; and the broad band at 3272 cm^{-1} is due to hydrogen bonded hydoxyl group. These values are in agreement with those reported in the literature (Fossen and Andersen, 2006).

The five aromatic proton signals of S_3 were assigned to an AX type ring A [δ_H 6.23 (1H, d, *J* = 2.0 Hz, H-6), 6.43 (1H, d, *J* = 2.0 Hz, H-8)] and an ABX type ring B [δ_H 6.89 (1H, d, *J* = 8.4 Hz, H-2'), δ_H 7.90 (1H, d, *J* = 2.0 Hz, 5'), δ_H 7.62 (1H, dd, *J* = 8.4 Hz and 2.0 Hz, H-6')]. These suggest the presence of a quercetin nucleus (Markham *et al*., 1978). Also, the two signals at δ_H 4.55 (1H, d, *J* = 1.2 Hz, H-1''') and 5.10 (1H, d, *J* = 8.0 Hz, H-1'') are characteristic of ∝ and β anomeric protons of sugar. The high coupling constant (8.0 Hz) observed in glucose anomeric proton is due to diaxial coupling with H-2'' and therefore, confirms the configuration of glucose as β-D glucosyl (Mabry *et al*., 1970). Also, the appearance of a doublet rather than a multiplet signal for the glucosyl anomeric proton is an indication of C-3 glycosidic linkage. Overlapping signals typical of carbinol proton in sugar were observed at δ_H 3.43-3.85. Other resonances were at δ_H 1.21 (3H, d, *J* = 6.4 Hz, H-6''') ascribable to Rhamnose and δ_H 1.30 (2H, s, H-6'') ascribable to Glucose.

The carbon 13 disclosed 27 well resolved peaks of one methyl carbon at δ_C 16.56(C-6'''), one methylene carbon at δ_C 62.91(C-6''), 15 methine carbons at δ_C [98.59(C-6), 93.44(C-8), 116.55(C-2'), 114.72(C-5'), 121.63(C-6'), 104.56(C-1''), 72.49(C-2''), 73.69(C-3''), 71.73(C-4''), 73.92(C-5''), 100.53(C-1'''), 70.89(C-2'''), 68.79(C-3'''), 70.65(C-4''') and 68.31(C-5''')] and 10 quaternary carbons at δ_C [157.08(C-2), 134.46(C-3), 178.08(C-4), 161.55(C-5), 164.81(C-7), 157.57(C-9), 104.14(C-10), 121.63(C-1'), 144.35(C-3') and 148.60(C-4')].

The assignment of the carbons and the placement of the sugar moiety were achieved using 2D NMR experiments.

In the COSY spectrum of S_3, the cross peak between δ_H [6.89 and 7.62]; δ_H [3.60 and 4.55]; δ_H [3.85 and 3.60] and δ_H [3.85 and 5.10] confirm their assignments to adjacent carbon atoms.

In the HMBC spectrum, a common J_3 correlation between protons at δ_H 6.23 and 6.43 to carbons at δ_C 98.59 and 93.44 respectively confirms their assignment to ring A. Also, a J_2 correlation between protons at δ_H 6.89 to carbon at δ_C 121.63 and a J_3 correlation to carbon at δ_C 144.35 confirm the assignment of the carbons to ring B.

A common J_3 correlation between anomeric proton at δ_H 5.10 to a quartenary carbon at δ_C 134.46 confirms that the sugar moiety is linked to the flavonoid aglycone through C-3 carbon. Other correlations confirmed by the HMBC are those of the sugar residue.

Based on the forgoing spectral analysis and chemical test, S_3 was confirmed to be Rutin (Figure 5.3)

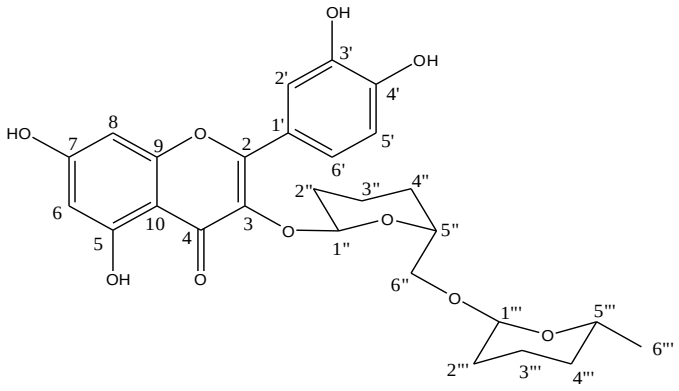


Figure 5.3

Rutin is a well-known member of the flavonoid family possessing a quercetin nucleus. Rutin harbours anti-oxidant properties which help to protect the body from cellular damage caused by free radicals. It helps to eliminate cholesterol from the body and

increase elasticity of the arterial walls, which, in turn, promotes greater blood flow. It maintains healthy collagen, which keeps our skin healthy and firm. It also helps to increase capillary strength and to regulate their permeability. Rutin has anti-inflammatory and anti-carcinogenic properties. It is beneficial for chronic venous insufficiency, hypertension, infections, atherosclerosis, osteoarthritis, haemorrhoids, stroke prevention and high cholesterol (Sattanathan *et al*., 2011).

K isolated from VLC column gave a single spot on TLC plate and was positive to Liebermann Burchard test indicating the presence of triterpenoid nucleus.

The broad IR band of K at 3395 cm^{-1} is due to alcoholic OH group, the coupled vibrational peaks at 2873 cm^{-1} and 2929 cm^{-1} are characteristic of symmetric and asymmetric saturated C-H stretching frequencies and the very sharp peak at 1688 cm^{-1} is typical of carbonyl frequency. These values are in agreement with those reported in the literature (Abdullahi *et al.*, 2013).

The proton NMR of K exhibited two doublets of secondary methyls at δ_H 0.81 and δ_H 0.90 and five singlets of tertiary methyls at δ_H [0.67, 0.75, 0.78, 1.04 and 1.35] (3H each) signifying its ursane nature (Witchuda and Orawan, 2007). The olefinic proton (H-12) resonated as a doublet at δ_H 5.15 (J = 19.0 Hz) while the carbinol proton (H-3) resonated as a triplet at δ_H 3.00. Other resonances at δ_H 1.07, 1.51, 2.12 and 2.37 are indicative of the presence of a side chain attached to the ursane nucleus.

The carbon 13 NMR spectrum of K disclosed 44 peaks of twelve methyl carbons at δ_C [28.24(C-23), 16.27(C-24), 15.20(C-25), 16.90(C-26), 23.26(C-27), 28.09(C-28), 17.00(C-29), 21.06(C-30), 15.20 (C-6', C-9'), 17.97(C-7') and 23.78(C-8')]; ten methylene

carbon at δ_C [38.36(C-1), 26.97(C-2), 17.97(C-6), 32.67(C-7), 22.82(C-11), 27.51(C-15), 23.85(C-16), 30.15(C-21), 36.29(C-22) and 38.19(C-2')]; nine methine carbons at δ_C [76.80(C-3), 54.74(C-5), 46.98(C-9), 124.55(C-12), 52.35(C-18), 38.48(C-19), 38.40(C-20), 52.35(C-3') and 54.74(C-4')] and eight quaternary carbons at δ_C [38.48(C-4), 38.48(C-8), 36.50(C-10), 138.17(C-13), 41.62(C-14), 46.87(C-17), (C-1') and(C-5')] which were assigned with the aid of the DEPT experiment (carbon multiplicity data). The appearance of signals at δ_C 124.55 and δ_C 138.17 indicated the presence of a double bond in urs-12-ene triterpenoid (Ibrahim and Francis, 2013). The deshielded signal at δc 76.80 was due to C-3 with a carboxyl group attached to it. The deshielding of C-23 methyl relative to C-24 could be due to the influence of adjacent C-3 carboxyl group. Other unaccounted carbon resonances including the quaternary carbon at δ_C 71.60 confirms the presence of a side chain coupled to Ursane nucleus.

The assignment of the carbons and the placement of the side chain were achieved using 2D NMR experiments.

In the COSY spectrum of K, the cross peak between δ_H [3.00 and 1.47]; δ_H [1.50 and 2.10]; δ_H [2.10 and 2.37] and δ_H [1.85 and 5.15] confirm their assignments to aliphatic chain.

In the HMBC spectrum, a common J_3 correlation between protons at δ_H 1.35 to carbon at δ_C 138.17 confirms it assignment as H-19. A J_2 correlation between proton at δ_H 1.85 to carbon at δ_C 124.55 and a J_3 correlation to carbon at δ_C 138.17 confirms its assignment as H-11. Also, a J_3 correlation between proton at δ_H 5.15 to carbons at δ_C 41.62 (C-14), 46.98 (C-9) and 52.35 (C-18) confirms its assignment as H-12. Other correlations confirmed by the HMBC are those of the aliphatic chain. For example, a J_3 correlation

between proton at δ_H 2.12 to carbons at δ_C 178.27 (C-1') confirms its assignment as H-3'. Also, a J_3 correlation between proton at δ_H 1.51 to carbons at δ_C 54.74 (C-4') confirms its assignment as H-2'.

Based on the forgoing spectral analysis and chemical test, compound K was proposed to be 3-*O*-(5'-hydroxy-3',4',5'-trimethylhexanoyl)ursane (Figure 5.4)

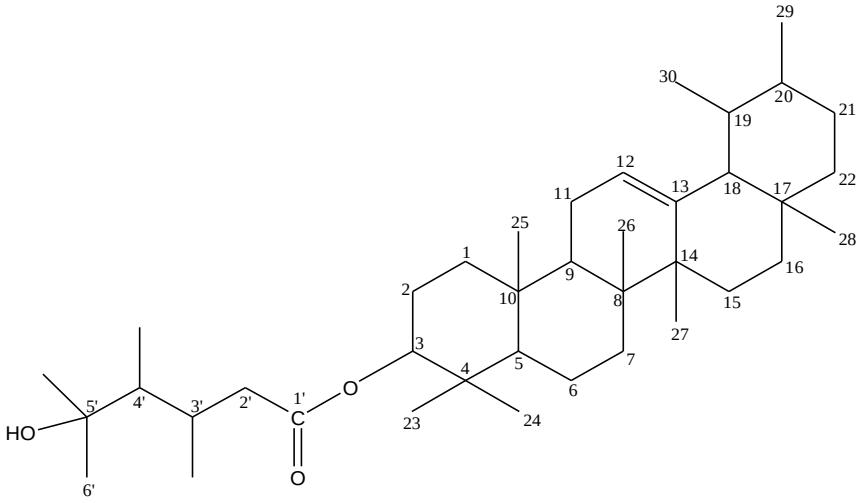


Figure 5.4

Triterpenoids have rare reports in the fungal and animal kingdom (Lutta *et al.,* 2008) but its known to have vast occurrence in diverse plant families and is even found in propolis (Connolly and Hill, 2008).

Triterpenoids including those of the Ursane family have been studied for their analgesic and anti-nociceptive properties, anti-cancer potentials, suppression of inflammatory response, hepatoprotective activity, anti-oxidant activity, antibacterial activity and anxiolytic activity (Kylenorton, 2011).

The relatively high intraperitoneal median lethal dose (LD_{50}) in both mice and rats suggests that the extract is relatively non-toxic (Lorke, 1983).

The hot plate assay method was employed for the purpose of preferential assessment of possible centrally mediated analgesic effects of the extract (MacDonald *et al*., 1946). The central analgesic drug, pentazocin, was therefore used as a positive control substance. The ability of the extract to prolong the reaction latency to pain thermally induced in mice by the hot plate suggests central analgesic activity.

The acetic acid–induced writhing test is very sensitive and able to detect anti-nociceptive effects of compounds at dose levels that may appear inactive in other methods like tail flick test (Bentley *et al.,* 1981). The intraperitoneal injection of acetic acid produces an abdominal writhing response due to sensitization of chemo-sensitive nociceptors by prostaglandins (Sutharson *et al*., 2007).

The analgesic effect of the extract (the ability to decrease mean number of abdominal writhes) may therefore be due to its action on visceral receptors sensitive to acetic acid. However, this model may not be able to indicate the mechanism of analgesic effect of the extract because other agents such as antihistamines and myorelaxant are able to reduce the pain induced by acetic acid (Magaji *et al*., 2008).

Both the crude extract and n-butanol fraction caused marked inhibition of inflammation induced by carrageenan. Carrageenan induced inflammation is believed to be biphasic, the early phase (1-2hrs) is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins released and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Brito and Antonio, 1988). The inhibitory effect of the extracts on carrageenan induced

inflammation at the end of the third hour is similar to the effect of most non-steroidal anti-inflammatory drugs. This suggests that they act in the later phase probably involving arachidonic acid metabolites which produces oedema dependent on neutrophils mobilization. Several mechanisms, including corticosteroid-like effects, interaction with prostaglandin biosynthesis, tachykinin or other inflammatory mediators, are involved in the anti-inflammatory action of drugs. (Barnes *et al*., 1990).

The presence of flavonoids might be responsible for the observed anti-inflammatory activity since numerous studies have demonstrated the anti-inflammatory properties of flavonoids (Waqar *et al*., 2014). Quercetin, as an important flavonol, has a potent anti-inflammatory action on exudative and proliferative phases of inflammation (Adzet, 1986). The anti-inflammatory activity of quercetin was also evaluated by determining the inhibition of 12-*O*-tetradecanoylphorbol 13-acetate-induced mouse ear oedema (Delagado *et al*., 2001). It seems that quercetin in both the methanol extract and n-butanol fraction has been partly associated with pharmacological findings of the present study, although it is not clear whether this compound is the only contributing component of this extract.

A significant and concentration dependent decrease in the number of tumor demonstrated by the extract showed its potential as anti-tumor agent. The potato disc assay is routinely employed as a comparatively rapid, inexpensive, safe and statistically reliable prescreen for anti-tumor activity. An experiment at Bradley University demonstrated that the inhibition of crown gall tumor on disc of potato (*Solanum tuberosum* L.) tubers showed an apparent correlation with compounds and plant extracts known to be active in the 3PS (*in vivo*, murine leukemia) anti-tumor assay (Mclaughlin *et al.*, 1998).

The connection between inflammation and cancer had been suggested as early as in 1863 by Rudolf Virchow. Chronic inflammation, with concomitant activity of cytokines and increased production of reactive oxygen species, is recognized as a cancer-promoting condition (Balkwill and Mantovani, 2001). The cyclooxygenases are enzymes responsible for conversion of arachidonic acid to prostaglandin H_2, which is precursor of other prostanoids. Their inhibitors (e.g., acetylsalicylic acid, ibuprofen, naproxen) are often used as anti-inflammatory drugs and painkillers. Ursolic acid proved to be efficient COX-2 inhibitor able to suppress inflammation progress (Santos *et al*., 2007). Additionally lowering activity of this cyclooxygenase has been correlated with caspase-3 activity and affected apoptosis rate in cancer cells (Tian *et al*., 2006). As such, the presence of Ursolic acid derivative in the leaf extract might be responsible for the observed analgesic, anti-inflammatory and anti-tumor activity.

The presence of Rutin (possessing quercetin nucleus) in the leaf extract of *Z. mucronata* might also be responsible for the observed activities since detailed studies have revealed that quercetin exhibited a dose-dependent inhibition of tumor growth and colony formation (Kim *et al*., 1993). Evidences from experimental studies have shown that quercetin not only offers protection against chemically induced cancers but also suppresses the growth of cancer cells *in vitro* and *in vivo* by enhancing carcinogen detoxification and anti-oxidant defences, inducing cell cycle arrest and apoptosis, inhibiting matrix invasion and angiogenesis and modulating intracellular signalling circuit (Ramamurthi and Siddavaram, 2012).

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Preliminary phytochemical screening of the methanol extract of *Ziziphus mucronata* revealed the presence of triterpenoids, steroids, phenolic compounds, alkaloids, saponins and flavonoids.

Extensive phytochemical investigation of the ethyl acetate and n-butanol soluble fractions led to the isolation of Catechin, Rutin, Bis (quercetin 3-*O*-β-D-glucopyranoside) and 3-*O*-(5'-hydroxy-3',4',5'-trimethylhexanoyl)ursane.

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Pharmacological studies carried out on both the methanol extract and n-butanol fraction showed that it possess a dose dependent analgesic and anti-inflammatory activity at doses below the LD_{50}. The extract and fraction also demonstrated a delay in progression of tumor induced on potato disc, as such may be useful in management of inflammatory conditions.

6.2 Conclusion

The research has achieved the stated objectives. To the best of our literature search, this is the first report of isolation of Bis (quercetin 3-*O*-β-D-glucopyranoside) and 3-*O*-(5'-hydroxy-3',4',5'-trimethylhexanoyl)ursane. This work is also the first report of isolation of Catechin and Rutin from the leaf extract of *Z. mucronata*. Based on the findings in this work, it can be concluded that the use of *Ziziphus mucronata* in the treatment of painful and inflammatory conditions has scientific basis. The analgesic, anti-inflammatory and antitumor activities observed with the extract may be linked in part, to the flavonoids and triterpenoid isolated from the plant.

6.3 Recommendations

i. Anti-tumor studies should be carried out on the isolated compounds to see whether they are active.

ii. Further studies should be carried out to isolate more compounds to add to the chemotaxonomy of the family.

iii. There is the need to test the isolated compounds for the reported activities of the plant.

iv. There is also need to carry out chronic toxicity studies on the plant so as to rule out the possibility of any deleterious effect that may arise as a result of prolong use of the plant.

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Appendix I

Composition of Lauria- Bertani broth medium

Trypton	10 g
Yeast extract	5 g
NaCl	10 g
Deionized water	950 mL

Adjust the pH of the medium to 7.0 using 1 N NaOH

Bring volume up to 1 L

Appendix II

Table 4.12 Effect of MEZM on thermally induced pain in mice

Treatment (Dose-mgkg^{-1})	**Latency to pain response (seconds)**			
	Zero time	30 minutes	60 minutes	90 minutes
D/W (1mLkg^{-1})	6.90 ± 1.21	7.20 ± 1.86	6.90 ± 1.76	7.10 ± 1.21
MEZM (75)	7.20 ± 1.19	12.98 ± 1.20*	12.62 ± 0.09	12.20 ± 1.66**
MEZM (150)	7.40 ± 1.31	14.60 ± 1.71	14.50 ± 1.20	14.20 ± 1.61**
MEZM (300)	7.35 ± 1.18	17.80 ± 1.32	17.90 ± 1.21	17.60 ± 1.08
PZN (20)	9.15 ± 1.90	14.60 ± 1.80	14.80 ± 1.61	12.90 ± 2.10***

Data represented as Mean ± SEM; n = 6; $^{*}p< 0.05$, $^{**}p< 0.01$, and $^{***}p< 0.001$ (Bonferroni test for pairwise comparison); D/W: distilled water; PZN: pentazocin; MEZM: methanol leaf extract of *Ziziphus mucronata*. Figures in parentheses represent doses in mgkg^{-1}.

Appendix III

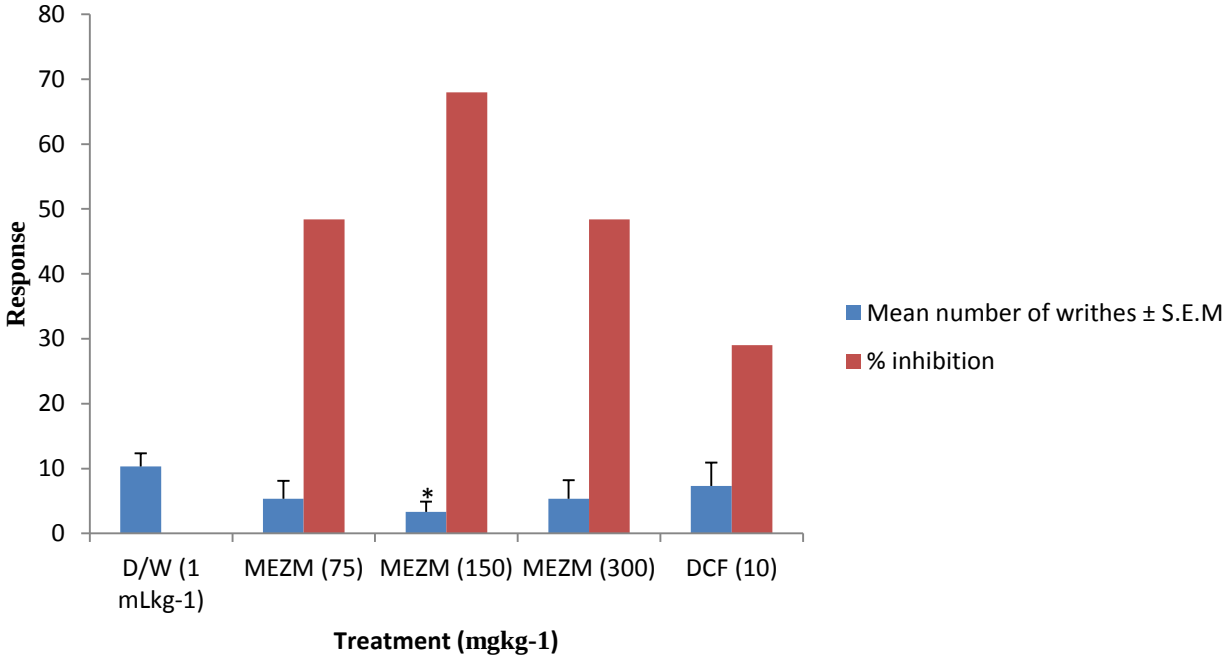

Figure 4.29: Effect of MEZM on acetic acid-induced writhing test in mice

Data represented as Mean ± SEM; n = 5; $^{*}p< 0.05$ (Dunnett t-test for multiple comparison); D/W: distilled water; DCF: diclofenac; MEZM: methanol leaf extract of *Ziziphus mucronata*. Figures in parentheses represent doses in $mgkg^{-1}$.

Appendix IV

Table 4.14 Effect of MEZM on Carrageenan induced hind paw oedema in rats

Treatment (Dose-mgkg^{-1})	**Mean Paw Oedema –mm**				
	1 hour	2 hour	3 hour	4 hour	5 hour
D/W(1mL/kg)	1.71±0.13	2.32±0.11	2.44±0.08	2.42±0.15	1.35±0.18
MEZM (200)	1.22±0.06 (28.7)	1.61±0.22 (30.6)	1.77±0.28 (27.5)	0.96±0.23*** (60.3)	0.77±0.14*** (43)
MEZM (400)	1.04±0.14 (39.2)	1.44±0.33 (37.9)	1.52±0.33 (37.7)	0.93±0.32** (61.6)	0.56±0.18** (58.5)
MEZM (800)	1.05±0.16 (38.6)	1.31±0.23 (43.5)	1.45±0.22 (40.6)	1.07±0.21^{NS} (55.8)	0.69±0.17** (48.9)
DCF (20)	1.12±0.15* (34.50)	1.29±0.18 (44.4)	1.71±0.17 (29.9)	1.09±0.11** (55)	0.64±0.07*** (52.6)

Data represented as Mean ± SEM; n = 5; **P< 0.01, and ***P< 0.001 (Bonferroni test for pairwise comparison); ^{NS}Not significant; D/W: Distilled water; DCF: Diclofenac; MEZM: Methanol leaf extract of *Ziziphus mucronata*. Figures in parentheses represent percentage inhibition of inflammation.

Appendix V

Table 4.15 Effect of nBF on Carrageenan induced hind paw oedema in rats

Treatment (Dose-mgkg^{-1})	Mean	Paw	Oedema	(mm)	
	1 hour	2 hour	3 hour	4 hour	5 hour
D/W(1mL/kg)	1.71±0.13	2.32±0.11	2.44±0.08$^{\#}$	2.42±0.15	1.35±0.18
nBF (150)	0.47±0.08*** (72.5)	0.57±0.09 (75.4)	0.54±0.07** (77.9)	0.58±0.06$^{\#}$ (76)	0.36±0.05*** (73.3)
nBF (300)	0.32±0.07*** (81.3)	0.51±0.06$^{\#}$ (78)	0.46±0.05** (81.1)	0.49±0.05 (79.8)	0.32±0.06*** (76.3)
nBF (600)	0.42±0.11*** (75.4)	0.62±0.13$^{\#}$ (73.3)	0.61±0.07** (75)	0.47±0.07 (80.6)	0.27±0.06*** (80)
DCF (20)	1.12±0.15* (34.50)	1.29±0.18 (44.4)	1.71±0.17$^{\#}$ (29.9)	1.09±0.11 (55)	0.64±0.07*** (52.6)

Data represented as Mean ± SEM; n = 5; *P< 0.05, **P< 0.01, ***P< 0.001 (Bonferroni posthoc test for pairwise comparison); $^{\#}$peak of oedema; D/W: Distilled water; DCF: diclofenac; nBF: n-butanol fraction of *Ziziphus mucronata*. Figures in parentheses represent percentage inhibition of inflammation.