EVALUATION OF α-AMYLASE AND α-GLUCOSIDASE INHIBITORY COMPOUNDS FROM *Zanthoxylum chalybeum* STEM AND ROOT BARK

By

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DECLARATION

I declare this thesis to be my original work and has not been previously submitted for examination or award of degree in any institution.

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DEDICATION

This thesis is dedicated to my parents, Francis and Anastacia, for instilling in me the urge to persue education and supporting me throughout the study period.

ABSTRACT

Management of diabetes mellitus using conventional drugs is either unaffordable, unavailable or has undesirable side effects. Medicinal plants such as *Zanthoxylum chalybeum* (Rutaceae) have found prominence in management of diabetes and previous studies showed the efficacies of aqueous root and stem bark against *in vivo* alloxan and streptozotocin–induced diabetes in rats. However, the phytochemical evidence for these activities was never established. Therefore, this study was to establish the potential anti-hyperglycemic compounds in the stem and root bark of *Z. chalybeum* based on an *in vitro* inhibition of α-amylase and α-glucosidase activities. The stem bark and root bark of *Z. chalybeum* were collected from Homa Hills, Homa Bay County, Kenya (0° 23´S, 34° 30´E), air dried under shade, pulverized into fine powder and extracted sequentially using *n*-hexane, ethyl acetate and 95% aq. methanol. Both the crude extracts and the pure isolates were evaluated *in vitro* for anti-hyperglycemic activities against α-amylase and α-glucosidase enzymes. The pure isolates were obtained following standard chromatographic separation techniques. Bioassay results showed no significant difference between inhibitory activities of root and stem bark (95% aq. methanol) crude extracts ($p>0.05$) (IC_{50} = 134.43, 110.82 and 97.22, 88.20 µg/ml) and the positive control acarbose (IC_{50} = 102.16 and 68.23 µg/ml) against α-amylase and α-glucosidase enzymes, respectively. Both *n*-hexane and ethyl acetate stem bark extracts showed significantly ($p\leq0.05$) higher activity than *n*-hexane and ethyl acetate root bark extracts. Repeated separation techniques yielded seven compounds including 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), lupeol (**37**), 3α,20-dihydroxy-28-lupanoic acid (**44**), 20-hydroxy-3-oxo-28-lupanoic acid (**45**) and 3α,20,28-trihydroxylupane (**46**), 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**), whose structures were established based on 1D NMR (^{1}H, ^{13}C and DEPT-135), 2D NMR (COSY, HMBC, HSQC) and MS experimental results. Bioassays results for the pure compounds indicated no significant difference between inhibitory activities of compounds **7**, **47** and **48** ($p>0.05$) (IC_{50} = 64.54, 58.91 and 54.81; and 66.77, 62.53 and 58.03 µM) and the positive control acarbose (IC_{50} = 54.66 and 51.54 µM) against both α-amylase and α-glucosidase, respectively. Phenolic compound (**7**) and the benzophenanthridine alkaloids (**47** and **48**) are the potent compounds responsible for the anti-hyperglycemic potential of *Z. chalybeum* observed in stem and root bark. The results of this study demonstrated the potential of *Z. chalybeum* against hyperglycemia which upon further verification of the mode of action and toxicology studies, the crude extracts and isolates can be recommended for management of diabetes and as templates for future drug synthesis, respectively.

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LIST OF ABBREVIATIONS AND ACRONYMS

Aq.	Aqueous solution
b.w.	Body weight
br s	Broad singlet
br d	Broad doublet
br t	Broad triplet
COSY	Correlation Spectroscopy
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethylsulfoxide
EIMS	Electron Ionization Mass Spectroscopy
EtOAc	Ethyl acetate
HDL	High density lipoprotein
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
HRESI	High Resolution Electrospray ionization
Hz	Hertz
IDF	International Diabetes Federation
1D, 2D NMR	1-Dimensional and 2-Dimensional Nuclear Magnetic Resonance

IC_{50}	Inhibitory Concentration of 50%
J	Coupling constant
LDL	Low Density Lipoprotein
m	Multiplate
$[M]^+$	Molecular ion
Me	Methyl group
MeOH	Methanol
MS	Mass Spectroscopy
m/z	Mass to charge ratio
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
R_f	Retention factor
s	Singlet
t	Triplet
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
δ	Chemical shift in ppm

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CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Diabetes mellitus is a metabolic disorder characterized by chronic high blood glucose levels resulting from defects in insulin secretion, insulin action or both (WHO, 1999), as a consequent of either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin produced. Insulin is the principal hormone that regulates the uptake of glucose from the blood (Ünal *et al.*, 2012; Gardner and Shoback, 2011). Deficiency of insulin or insensitivity of its receptors plays a central role in the three main forms of diabetes mellitus, i.e. Type-1, Type-2 and gestational diabetes. Type-1 diabetes occur due to pancreas failure to produce enough insulin, Type-2 is associated with insulin resistance by body cells whereas gestational diabetes arises from glucose intolerance with an onset during pregnancy (Gardner and Shoback, 2011). Chronic raised blood glucose is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidney, nerves, heart and blood vessels (WHO, 2009).

Previously, Type 2 diabetes was diagnosed almost entirely among adults. However, recent data indicate that it is increasingly diagnosed among children, adolescents and young adults (WHO, 2016; IDF, 2017). The increase in rates in developing countries follows the trend of urbanization and lifestyle changes including increasingly sedentary lifestyles, less physically demanding work and the global nutrition transition marked by increased intake of foods that are often high in sugar and saturated fats (WHO, 2016; Whiting *et al.*, 2011; Wild *et al.*, 2004). Such rapid trends are more pronounced due to lack of proper and accessible management strategies

such as consumption and/or utilizations of indigenous medicinal food substances, occasioned by lack of relevant knowledge about the various medicinally useful plants.

Different approaches are used to manage diabetes mellitus including lifestyle modification and conventional drugs. Varied conventional management medicine for diabetes mellitus include injectable insulin, sulfonylureas, biguanides, glucosidase inhibitors and glinides (Joshi and Joshi, 2009). New classes of drugs for diabetes include the inhalable insulin, incretin mimetics, amylin analogues, gastric inhibitory polypeptides analogues, peroxisome proliferator activated receptors and dipeptidyl peptidase-4-inhibitors (Joshi and Joshi, 2009). However, the entire drugs available generally have inadequate efficacy (Cheng and Fantus, 2005; Joshi and Joshi, 2009), characterized by a number of adverse side effects (Joshi and Joshi, 2009; Cheng and Fantus, 2005) besides being costly and less affordable by majority of populations (Rutebemberwa *et al*., 2013) hence their reduced acceptance. These limitations coupled with an exponential increase in the prevalence of diabetes mellitus (IDF, 2000; IDF, 2017) have made clinical importance of herbal remedies to receive considerable attention.

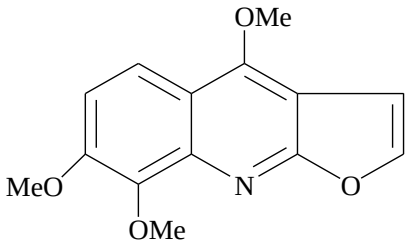
Pancreatic α-amylase is a key enzyme in the digestive system and catalyses the initial hydrolysis of starch, which is the principal source of glucose in the diet (Eichler *et al*., 1984). Pancreatic α-amylase hydrolyses starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose and a number of α-(1-6) and α-(1-4) oligoglucans (Eichler *et al*., 1984). α-Glucosidase in the intestine aids the final hydrolysis of the starch (oligosaccharides) into absorbable glucose (Eichler *et al*., 1984). The activities of human pancreatic α-amylase in the small intestine correlate to an increase in post-prandial glucose levels (Tarling *et al*., 2008). Retarding absorption of glucose by impeding the breakdown of starch and intestinal absorption of glucose through the inhibition of pancreatic α-amylase and α-glucosidase enzymes mimics the

effect of dieting on hyperglycemia (Pinto *et al*., 2009; Krentz and Bailey, 2005), and therefore is an important approach in managing postprandial hyperglycemia (Tarling *et al*., 2008). Modulation of human pancreatic α-amylase and α-glucosidase inhibitors through the use of therapeutic inhibitors is however, limited by ineffectiveness (Cheng and Fantus, 2005) and side effects such as abdominal distention, flatulence and diarrhea (Cheng and Fantus, 2005; Bischoff, 1994; Bischoff *et al.,* 1985). Some plant extracts have been demonstrated to have inhibitory effect against α-amylase and α-glucosidase enzymes (Mogale *et al*., 2011; Apostolidis *et al*., 2006; Kwon *et al.*, 2006). The plants therefore can be used as effective therapy to manage postprandial hyperglycemia with minimal side effects. The World Health Organization estimates up to 80% of the world population in most of the developing countries rely on traditional medicines of plants and plant materials (WHO, 2000; WHO, 2013). Some extracts from plants may act to modulate the activities of the pancreatic α-amylase and intestinal α-glucosidase. Furthermore, many effective anti-diabetic drugs used today have been isolated from natural sources of plant origin (Osadebe *et al*., 2014). However, limited empirical knowledge is available from plants regarding the pharmacologically active compounds with potent and specific inhibitory potential against α-amylase and α-glucosidase.

As an alternative medicine, herbal treatments for diabetes are considered effective, less-toxic, with less or no side effects (Wang *et al*., 2013; Chege *et al*., 2015). This fact has encouraged ethno medicinal applications in the management of diabetes. The plant *Zanthoxylum chalybeum* has been used by some communities in Kenya for management of various diseases including diabetes mellitus (Moshi and Mbwambo, 2002; Keter and Mutiso, 2012). Other ethno-medicinal applications of the plant include; malaria, fevers, abdominal pain, wounds and sickle cell disease (Olila *et al.,* 2001; Nguta *et al.,* 2010; Anza *et al.,* 2014). Root or stem bark

decoctions are widely consumed as anti-diabetes and root bark decoctions are considered to be stronger than stem bark decoctions (Tabuti, 2011). Furthermore, biological examination of the crude extracts from the plant indicated good *in vivo* anti-hyperglycemic activities (Agwaya *et al.*, 2016a; Agwaya *et al.*, 2016b; Kimani *et al.*, 2015). The phytochemicals responsible for the activities were however not evaluated. In addition, it remained unclear why the root bark appeared to have better activity than the stem bark. The relative activities of the crude extracts and the compounds displaying antidiabetic properties have not been established completely.

A number of individual species from the genus *Zanthoxylum* (Rutaceae) possess anti-glycemic activity (Dongmo *et al.*, 2008), which was attributed to the presence of alkaloids and essential oils (Negi *et al*., 2011). Phytochemicals previously isolated from *Z. chalybeum* include alkaloids; skimmianine (**1**) (Olila *et al.*, 2001), chelerythrine (**2**), nitidine (**3**) and methyl canadine (**4**) (Muganga *et al.*, 2014), fagaramide (**5**) (Adia *et al.*, 2016), dihydrochelerythrine (**6**) and a phenolic; coniferyl alcohol derivative 2,3-epoxy-6,7-methylenedioxy coniferyl (**7**) (Anza *et al.*, 2014). The existence of a number of compounds in extracts of *Z. chalybeum*, and *Zanthoxylum* species (Negi *et al*., 2011) pointed out a need to perform a chemical evaluation of *Z. chalybeum* extracts. Toxicity studies carried alongside the biological activities established *Z. chalybeum* extracts were relatively safe for human use (Engeu *et al.*, 2008). Following potent antidiabetic activities against *in vivo* alloxan and streptozotocin–induced diabetic rats of the crude extracts (Agwaya *et al.*, 2016a; Agwaya *et al.*, 2016b; Kimani *et al.*, 2015) against an array of phytochemicals, it was necessary to establish the relative anti-hyperglycemic potential of the crude extracts and the isolated compounds from *Z. chalybeum* by examining their inhibitory effects against two carbohydrate digestive enzymes (α-amylase and α-glucosidase).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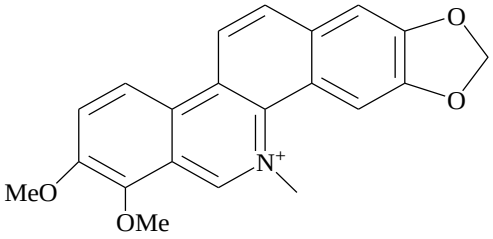
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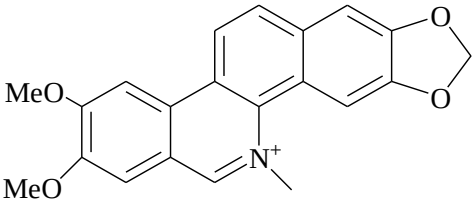
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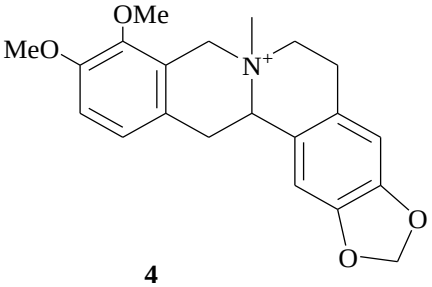
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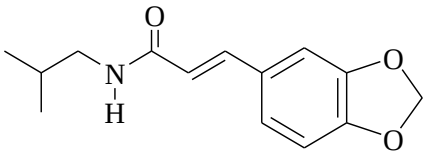
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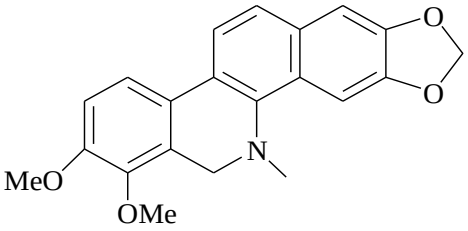
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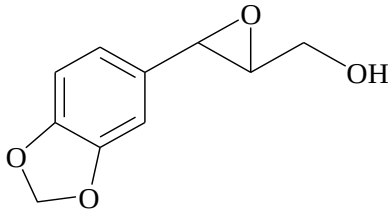
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1.2 Statement of the problem

The prevalence of diabetes mellitus is on an upward trend and is becoming a serious threat to human health yet a safe and effective alternative for management of diabetes is yet to be achieved. Current available conventional injectable and oral blood glucose lowering drugs have several side effects. As alternative medicine, plants provide an option for search of safe and effective management strategies since they are considered relatively effective, less-toxic, with less or no side effects. *Z. chalybeum* is one such plant which has been used by some communities for management of diabetes mellitus. Root and stem bark extracts of *Z. chalybeum* have been reported with potent anti-hyperglycemic activities against *in vivo* alloxan and streptozotocin–induced diabetes in rats. However, the relative anti-hyperglycemic activity of crude extracts from the stem and root bark, and the compounds that are responsible for the perceived and observed antidiabetic activities have not been established and evaluated to ascertain their anti-hyperglycemic activities either *in vivo* or *in vitro*.

1.3 Broad objective

To establish the bioactive principles from the stem bark and root bark extracts of *Zanthoxylum chalybeum* responsible for anti-hyperglycemic activities against α-amylase and α-glucosidase activities.

1.4 Specific objectives

i). To determine the relative anti-hyperglycemic activity of crude extracts from the stem and root bark of *Z. chalybeum* against α-amylase and α-glucosidase activities.

ii). To isolate and characterize pure isolates from the active extracts from the stem and root bark of *Z. chalybeum*.

iii). To determine the relative anti-hyperglycemic potential of the compounds from the active extracts from *Z. chalybeum* against α-amylase and α-glucosidase activities.

1.5 Null hypotheses

i) Crude extracts from *Z. chalybeum* stem and root bark do not exhibit anti-hyperglycemic activity.

ii) Pure isolates from *Z. chalybeum* stem and root bark do not exhibit anti-hyperglycemic activity.

1.6 Justification of the study

Diabetes mellitus has attained epidemic proportions worldwide with conventional drugs either being unaffordable, unavailable or having undesirable side effects. Herbal medicines are becoming affordable, easily accessible and acceptable by majority of people in developing nations. Due to inadequate efficacy and side effects associated with the present anti-hyperglycemic drugs, there is need to establish effective alternative diabetes management for the resource poor communities of the developing countries. Such effective and safe alternatives could be obtained from medicinal plants such as *Z. chalybeum*. It was therefore important to evaluate the chemical compounds from *Z. chalybeum* which could be used to validate the claimed ethno-medicinal applications of the plant. Moreover, establishment of the chemical entities responsible for anti-diabetic properties would essentially form the basis for further enrichment of the traditional medicinal remedies in terms of mixing up concoctions and determination of dosages.

1.7 Limitation of the study

Though the crude extracts and isolated compounds from *Z. chalybeum* showed inhibition activities against α-amylase and α-glucosidase enzymes *in vitro*, it remains unknown if they have antidiabetic activities *in vivo*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiological aspects of diabetes

Diabetes is a chronic metabolic disease that is showing an alarming increase in prevalence in developing countries. The International Diabetes Federation (IDF) estimated the global prevalence of diabetes to be 151 million in 2000 (IDF, 2000), 366 million in 2011 (IDF, 2011), 382 million in 2013 (IDF, 2013), 415 million in 2015 (IDF, 2015) and 451 million in 2017 (IDF, 2017). World Health Organization (WHO, 2016) estimated that, 422 million people were suffering from diabetes globally in 2014 up from an estimated 382 million people in 2013 (Shi and Hu, 2014) and from 108 million in 1980. Recent projection suggests that this trend is likely to increase in the next 28 years, affecting 693 million people in 2045 (IDF, 2017; Cho *et al*., 2018). In 2012, approximately 1.5 million deaths were due to diabetes related complications (WHO, 2016), while in 2017, approximately 5 million deaths worldwide were attributable to diabetes in the 20-99 years' age range (Cho *et al*., 2018). Diabetes mellitus is generally experienced throughout the world especially Type-2 manifestations being observed more in developed countries (WHO, 2016). The greatest increase in rates has however, been seen in low and middle income countries (WHO, 2016) where more than 80% of diabetic deaths occur (Mathers and Loncar, 2006).

Due to inadequate or lack of proper information which leads in poor management of diabetes, most diabetic patients especially those with Type-2 diabetes develop complications such as diabetic ketoacidosis, seizures, heart diseases, stroke, kidney failure, retinopathy, neuropathy among other gestational complications (WHO, 2009; WHO, 2016; Baena-Díez *et al*.,

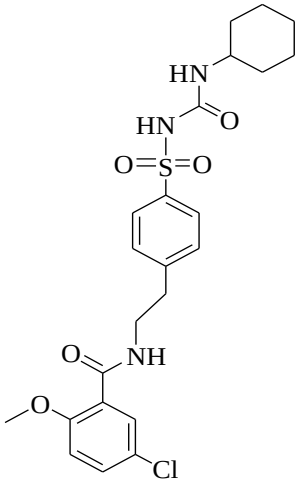
2016). Diabetes has been associated with increased rates of specific cancers, and physical and cognitive disability (Reid *et al.,* 2012).

Management of diabetes mellitus with insulin is associated with drawbacks such as insulin resistance (Piedrola *et al.,* 2001), anorexia nervosa, brain atrophy and fatty liver tissues (Yaryura-Tobias *et al*., 2001) after chronic treatment. Therefore, the alternative choice could be plants with ethnomedical uses in management of diabetes due to their apparent safety, efficacy and lesser side effects (Rutebemberwa *et al*., 2013). Many plants have been reported in traditional system of medicine to have beneficial anti-diabetic effects in patients with insulin-dependent and non-insulin dependent diabetes. Some of the plants are; *Artemisia pallens, Embellica offinalis, Momordica charantia, Allium sativum, Gymnema sylvestre, Azadirachta indica, Amaranthus spinosus, Aloe vera, Zanthoxylum chalybeum, Anacardium occidentale, Syzygium cumini, Psidium guajava* and *Mangifera indica* (Gaikwad *et al*., 2014; Bhushan *et al*., 2010). However, majority of the plants are used in crude aqueous concoctions, without the knowledge of chemical entities responsible for the claimed effect.

2.2 Strategies of diabetes management

The management of diabetes mellitus is a global problem (Herman and Zimmet, 2012; WHO, 2016) and successful treatment is yet to be discovered. It is a disorder of multiple biological defects which necessitates the use of a range of different classes of therapeutic options (Joshi and Joshi, 2009). The only available pharmacological intervention for Type-1 diabetes is insulin (Gough and Narendran, 2010). Therapeutic strategies for Type-2 diabetes involve insulin and oral antidiabetic agents that stimulate pancreatic insulin secretion (sulphonylureas and insulin secretagogues such as glibenclamide (**8**) and glipizide (**9**)), reduce hepatic glucose production (biguanides such as metformin (**10**)), improve insulin action (thiazolidinediones such as

pioglitazone (**11**) and rosiglitazone (**12**)) or delayed digestion and absorption of intestinal carbohydrate (α-glucosidase inhibitors such as miglitol (**13**) and acarbose (**14**)) (Cheng and Fantus, 2005). However, these therapeutic agents suffer from general inadequate efficacy and a number of adverse side effects including hypoglycemia, weight gain, gastrointestinal discomfort, nausea, liver and heart failure and diarrhea (Krane *et al*., 2005; Joshi and Joshi, 2009; Grant, 2003; Bischoff *et al*., 1985) hence their reduced acceptance.



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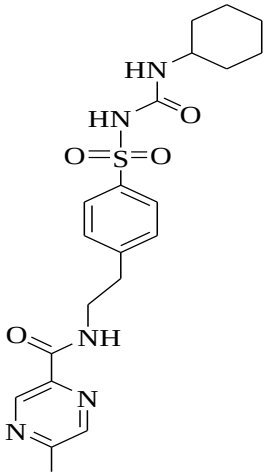

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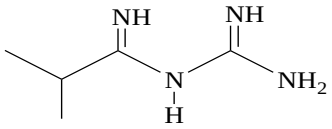

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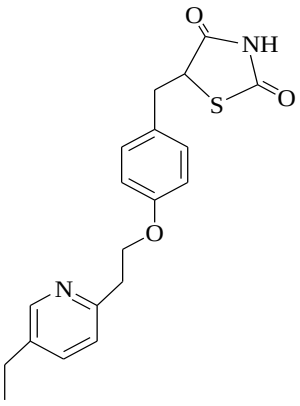

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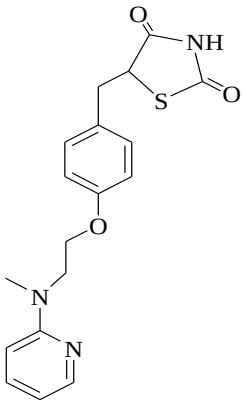

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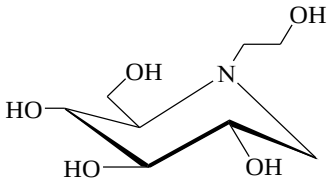

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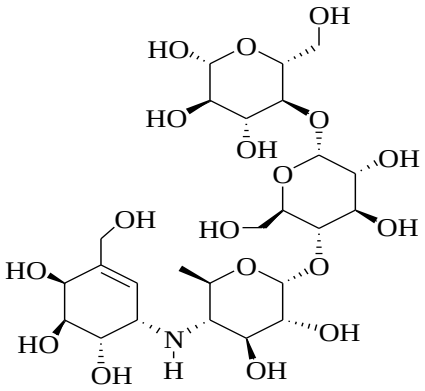

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Hydrolysis of dietary carbohydrates such as starch is the major source of glucose in the blood, a reaction catalyzed by pancreatic α-amylase and intestinal α-glucosidase (Bischoff, 1994). Pancreatic α-amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose and a number of α-(1-6) and α-(1-4) oligoglucans (Eichler *et al*., 1984). These are then acted on by α-glucosidases and further degraded to glucose which on absorption enters the blood stream (Eichler *et al*., 1984). Degradation of this dietary starch proceeds rapidly and leads to elevated post-prandial hyperglycemia (Eichler *et al*., 1984).

Activity of human pancreatic α-amylase in the small intestine correlates to an increase in post-prandial glucose levels (Tarling *et al*., 2008). Hence, retardation of starch digestion by inhibition of enzymes such as α-amylase plays a key role in the control of diabetes (Tarling *et al*., 2008). However, non-specificity of many of such hypoglycemic agents limits their applications, due to gastrointestinal side effects like bloating, abdominal discomfort, diarrhea and flatulence (Cheng and Fantus, 2005; Bischoff, 1994; Bischoff *et al.,* 1985). Such adverse effects might be caused by the excessive inhibition of pancreatic α-amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Bischoff *et al*., 1985; Bischoff,

1994). Herbal alternative finds relevance combating some of these side effects (Grover *et al*., 2002; Mogale *et al*., 2011; Apostolidis *et al*., 2006; Kwon *et al.*, 2006), since the extracts of medicinal plants showing inhibitory effects of α-amylase are able to reduce the absorption of glucose when administered with meals (Jarald *et al*., 2008). Therefore, management of post prandial hyperglycemia using herbal remedies is an alternative approach in managing diabetes. However, very few plants materials have been proven to act against α-amylase and α-glucosidase.

2.3 Ethno-botanical management of diabetes

Medicinal plants are useful in management of diabetes worldwide and have been used empirically in anti-diabetic and anti-hyperlipidemia management (Aloke *et al,* 2012; Bhushan *et al*., 2010). Anti-hyperglycemic activity of the plants is mainly due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to facilitate metabolism of metabolites in insulin dependent processes (Jung *et al*., 2006). Many plant species exhibiting hypoglycemic activity have been available in literature. Examples are listed in Table 1. However, search for new anti-diabetic drugs from plants is still attractive because they contain substances which demonstrate alternative and safe effects on diabetes mellitus (Malviya *et al*., 2010) and most of the information reported about these plants is incomplete.

Several such medicinal plants have shown anti-diabetic activity when evaluated using different type of experimental techniques resulting in establishment of several active principles representing different classes of phytochemical compounds (Table 2) including alkaloids, glycosides, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic salts (Grover *et al*., 2002).

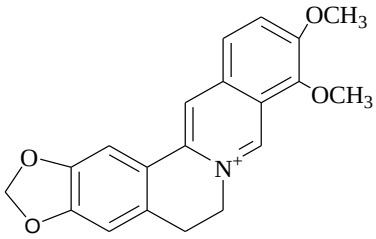
Table 1: List of plants having anti-diabetic activity (Bhushan *et al*., 2010)

Plant part	Name of plants
Aerial parts	*Artemisia pallens, Bidens pilosa, Bixa orellana, Teramnus labialis*
Bark	*Cinnamomum zeylanicum, Croton cajucara, Zanthoxylum Chalybeum*
Bulb	*Allium cepa, Allium sativum*
Flower	*Cassia auriculata, Gentiana olivier, Musa sapientum*
Fruit	*Carum carvi, Coriandrum sativum, Embellica officinalis, Juniperus communis, Momordica charantia, Xanthium strumarium*
Leaves	*Aloe barbadensis, Annona squamosa, Averrhoa bilimbi, Azadirachta indica, Beta vulgaris, Camellia sinensis, Cassia alata, Eclipta alba, Eucalyptus globulus , Euphrasia officinale, Ficus carica, Gymnema sylvestre, Gynura procumbens, Ipomoea aquatica, Mangifera indica, Myrtus communis, Memecylon umbellatum, Morus indica, Ocimum sanctum*
Rhizome	*Nelumbo nucifera*
Roots	*Clausena anisata, Glycerrhiza glabra, Helicteres isora, Pandanus odorus, Zanthoxylum Chalybeum*
Seed	*Acacia arabica, Agrimony eupatoria, Lupinus albus, Luffa aegyptiaca, Lepidium sativum, Mucuna pruriens, Punica granatum*
Stem	*Amaranthus spinosus, Coscinium fenestratum*
Tubers	*Ipomoea batata*
Whole plant	*Abies pindrow, Achyranthus aspera, Ajuga iva, Aloe vera, Anacardium occidentale, Andrographis paniculata, Capsicum frutescens, Cryptolepis sanguinolenta, Enicostemma littorale, Ficus religiosa*

The wide use of selected medicinal plants for the management of diabetes warrants the further study of these plants for potential use and commercialization. However, there are only limited scientific studies to validate the medicinal application of the plants listed in Table 1. For example, although anti-hyperglycemic activities for stem and root bark extracts from *Z. chalybeum* have been carried out (Kimani *et al*., 2015, Agwaya *et al*., 2016a; Agwaya *et al*., 2016b), a comparison of relative anti-hyperglycemic activities of crude extracts and compounds from the stem and root bark of the plant had not been done. There is therefore, a need to generate phytochemistry and efficacy information for these herbal remedies. This information may inform the development of efficacious low cost anti-diabetic drugs with fewer side effects that will find greater affordability and acceptability with diabetes patients.

Table 2: Some anti-diabetic compounds isolated from different medicinal plants.

Scientific name	Family	Compound	Reference
Berberis spp. *Tinospora cordifolia*	Berberidaceae Menispermaceae	Berberine (**15**)	Pan *et al.*, 2003 Singh *et al.*, 2003
Jindai soybean	Fabaceae	Kaempferol glycoside (**16**) Kaempferol (**17**)	Zang *et al.*, 2011
Momordica charantia	Cucurbitaceae	Charantin (**18**)	Ng *et al.*, 1986
Aloe vera	Liliaceae	Lophenol (**19**)	Tanaka *et al*., 2006
Butea monosperma	Fabaceae	Stigmasterol (**20**)	Panda *et al*., 2009
Solanum surattense	Solanaceae	β-Sitosterol (**21**)	Gupta *et al*., 2011
Syzygium cumini	Myrtaceae	12-Oleanene-3-ol-3β acetate (**22**)	Alam *et al.*, 2012
Trigonella foenum-graecum	Fabaceae	4-hydroxyisoleucine (**23**)	Haeri *et al*., 2012



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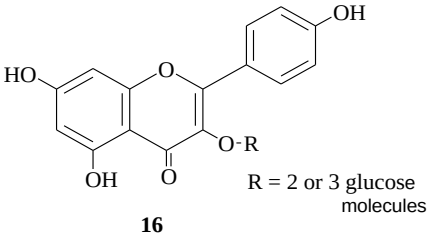

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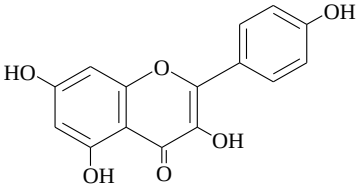

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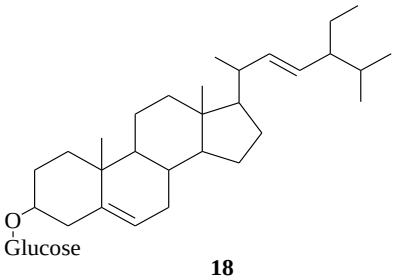

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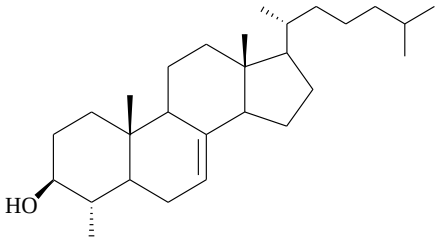

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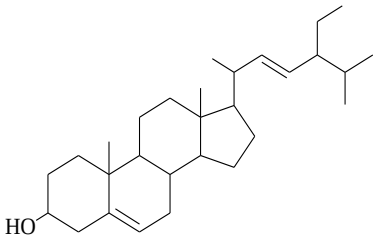

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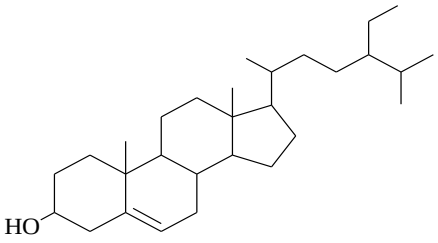

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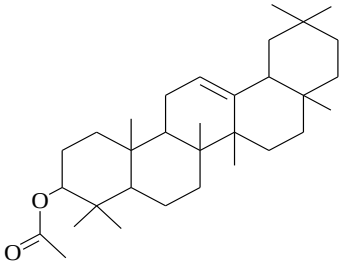

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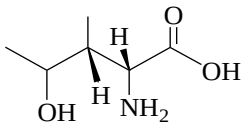

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The information on Table 2 shows that there are varied phytochemicals with potential of anti-diabetic principles in plants. However, there is little evidence to their relative levels of activities. Traditional herbal medicines constitute a good basis for new anti-diabetic discovery and development of synthetic medicinal remedies based on such molecular structures as the compounds in Table 2. Unfortunately, such endeavors have not been successful due to lack of similar compounds to subject to biological structure activity studies (SAR) as anti-diabetic remedies. It is therefore necessary to subject such confirmed active compounds to *in silico* docking experiments coupled with synthetic modification to establish the optimally active scaffolds for antidiabetic activities.

2.4 Ethno medicinal use of *Zanthoxylum* Plants

The genus *Zanthoxylum* has great importance as regards its ethno-botanical uses since it is a promising source of various secondary metabolites including benzophenanthridine alkaloids which are considered important in the world of medicine (Medhi *et al*., 2013; Nissanka *et al*., 2001; Krane *et al.*, 1984). The basic structure of benzophenanthridine alkaloids is given in Figure 1 (Waterman, 1990).

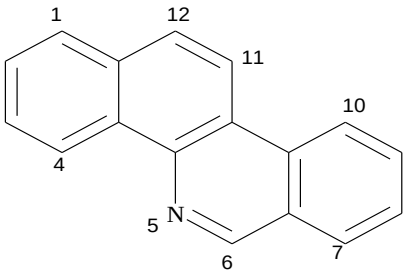


Figure 1: Basic structure of benzophenanthridine alkaloids

Several species of this genus have been noted with wide economic importance including being sources of edible fruits, essential oils, medicine, ornamentals, culinary applications and as wood,

perfumery and in food industry besides being used to treat a wide range of disorders, including toothache (Adesina, 2005).

There are several traditional uses reported from members of the genus *Zanthoxylum*. Some have served as raw materials in pharmaceutics for the treatment of various diseases including stomachaches, toothaches, coughs, urinary infections, rheumatism, leprous ulcerations and venereal diseases (Negi *et al*., 2011; Dharani *et al*., 2010). Previous scientific studies showed that plants belonging to the genus *Zanthoxylum* have good bioactivities including; larvicidal, analgesics, anthelmintic, anti-viral (Negi *et al*., 2011), antioxidant (Negi *et al*., 2011; Paik *et al*., 2005), anti-fungal, antibiotic (Tatsadjieu *et al*., 2003; Ngassoum *et al*., 2003; Luo *et al*., 2012), antinociceptive and anti-inflammatory (Guo *et al*., 2011; Chen *et al*., 2008) and cytotoxicity activities (Jeong *et al*., 2015) mainly due to presence of alkaloids and essential oil (Negi *et al*., 2011). A lot of phytochemicals information specific to bioactivity is still unestablished (Medhi *et al*., 2013).

Furthermore, many species of the *Zanthoxylum* genus have been used to manage a number of diseases in humans and animals. For instance, bark of *Z. liebmannianum* is used in Mexico to manage stomach pains, amoebiasis and against intestinal parasites and as a local anesthetic agent (Ross *et al.,* 2004). Stem bark of *Z. gilletii* is used in traditional anti-malarial preparations (Nyunja *et al*., 2009; Ombito *et al*., 2014) and also to manage stomachache, joint pain, toothache, fever, rheumatism, venereal infections and for washing wounds (Kokwaro, 2009). The fruit essential oils of *Z. leprieurii* and *Z. xanthoxyloides* are used as food supplements to protect against cardiovascular problems, cancer and diabetes (Dongmo *et al.,* 2008) whereas *Z. rhoifolium* is reported as anti-malarial agent (Jullian *et al.,* 2006). *Z. heitzii* is ethno medicinally used to manage jaundice (Betti and Lejoly, 2009), toothache (Betti, 2004), gonorrhea (Betti,

2002), rheumatic ailments, stiff joints and impotence (Tafokou, 2008; Betti, 2002) and malaria (Tafokou, 2008).

Two Indian species **(***Z. armatum* and Z. *nitidum*) are traditionally used to manage diabetes (Singh and Singh, 2011; Arun-Kumar and Paridhavi, 2012) and aqueous methanol (water:methanol; 1:1) extracts of stem bark of *Z. armatum* possess significant antidiabetic and antioxidant effects *in vivo* on diabetic rats (Karki *et al*., 2014). *Z. armatum* is used for managing pneumonia, tick infestation and gum diseases whereas fruits are used for management of toothache, dyspepsia, as a carminative and for stomachache (Abbasi *et al*., 2010). Fruit extracts of *Z. allatum* combat fever, dental, worms and dyspepsia because of antiseptic, deodorant and as disinfectants and the bark is used for gums relief, asthma and diabetes and snake bite (Brijwal *et al*., 2013). Biological activities observed by plant extracts were attributed to presence of various phytochemical constituents in plants (Mazid *et al*., 2011). Furthermore, the presence of more than one class of secondary metabolites in a given plant extract also determines the nature and extent of the extracts biological activities (Wang *et al*., 2010). The consistencies in bioactivities of different *Zanthoxylum* species may be attributed to the presence of similar phytochemical profiles in different parts of the plants of this genus (Negi *et al*., 2011).

Different parts of *Zanthoxylum chalybeum* (English name; knob wood, yellow wood) (Luo community of Kenya; "Roko") are widely used in traditional medicine to manage several diseases which include; diabetes (Moshi and Mbwambo, 2002; Keter and Mutiso 2012), malaria (Anza *et al.,* 2014; Olila *et al.,* 2001; Nguta *et al.,* 2010), fevers, wounds, abdominal pain (Anza *et al.,* 2014), sickle cell disease, measles, skin infections and coughs (Olila *et al.,* 2001). As a folk medicine in Ethiopia, *Z. chalybeum* is used to treat both human and livestock ailments (Regassa, 2013). Decoctions of the stem bark are used to manage diabetes mellitus related

symptoms in Kenya, Tanzania and Asia (Moshi and Mbwambo, 2002; Keter and Mutiso 2012). Root or stem bark decoctions are widely consumed as anti-diabetes remedy and root bark decoctions are considered to be stronger than stem bark decoctions (Tabuti, 2011). It is however, unclear why the root bark may appear to have better activity than the stem bark, and it is also not documented if the bioactive compounds in root bark are similar to those in the stem bark.

2.5 Anti-diabetic activities of the *Zanthoxylum* plants

Several species in the genus *Zanthoxylum* have significant anti-diabetic activity. For example, various parts of *Z. zanthoxyloides* including the roots, bark and leaves have been used for medicinal purposes, including treatment of diabetes mellitus (Aloke *et al.,* 2012). A study designed to evaluate the effect of feed prepared from *Z. zanthoxyloides* leaves extracts on blood glucose level, lipid profile and activities of some plasma liver enzymes (Aloke *et al.,* 2012), showed antidiabetic and hypolipidaemic effects in alloxan-induced diabetic rats. In the study, ground leaves/feed mixed exactly 10 g/90 g, 15 g/85 g and 20 g/80 g representing 10%, 15% and 20% w/w were administered on alloxan-induced diabetic rats and the result showed reductions in body weight and blood glucose level, improvements in serum lipids and elevation in high density lipoprotein-cholesterol in diabetic rats, suggesting that the plant has protective effect on the heart (Aloke *et al.,* 2012). The activities of all the enzymes were higher in the extract treated groups when compared with the non-treated groups. Similarly, lower ($p \leq 0.05$) levels of total cholesterol and low density lipoprotein at higher concentration of the feed were observed (Aloke *et al.,* 2012). Such data indicated potential anti-diabetic principles; however, there has not been a follow up phytochemical study to validate the basis of such diverse bioactivities.

Phytochemical studies on *Z. armatum* stem bark extract revealed the presence of alkaloids, tannins, phlobatannin, terpenoids, fixed oil, phenolic compounds and flavonoids on a general wet

phytochemical analysis tests (Karki *et al.*, 2014). Evaluation of antidiabetic and antioxidant activity of the same extract (methanol:water, 4:1) on streptozotocin-induced diabetic rats showed improvement in the blood glucose levels after the treatment (Karki *et al.*, 2014). Antioxidant status of *Z. armatum* was noted, and could be responsible for anti-diabetic action of this plant (Karki *et al.*, 2014). In a similar *in vivo* investigation, the aqueous leaves extracts of *Z. armatum* showed hypoglycemic and anti-hyperglycemic ($p \leq 0.05$) effects in normoglycemic and diabetic mice by suppressing the glucose peak and improved blood glucose pattern, respectively compared to the control metformin (Rynjah *et al*., 2017). The same extract *in vitro* inhibited α-amylase, α-glucosidase and β-glucosidase ($p \leq 0.05$) relative to the standard acarbose. In a separate study, *in vitro* and *in vivo* anti-diabetic and biochemical effects of *Z. armatum* in albino mice were investigated and the extract showed excellent anti-diabetic potential (Alam *et al*., 2018). The extracts of the fruits, bark and leaves from *Z. armatum* showed significant inhibition of α-glucosidase enzyme *in vitro* with percentage and IC_{50} values of 96.61 ± 2.13 (IC_{50} = 47.87 ± 0.45) and 93.58 ± 2.31% (IC_{50} = 21.82 ± 0.87) and 83.76 ± 3.01% (IC_{50} = 31.62 ± 0.67), respectively (Alam *et al*., 2018). In the *in vivo* assay, there was increase in the body weight of the *Z. armatum* fruit, bark and leaf extracts treated albino mice compared to the diabetic group ($p \leq 0.05$). However, leaves extract showed less effect on body weight of the normal and diabetic treated group compared to the bark and fruit (Alam *et al*., 2018). The extract treated groups showed decrease in blood glucose levels and the effect was more pronounced in mice treated with leaves extract as compared with diabetic control. There was improvement ($p \leq 0.001$) in blood hemoglobin, urea, creatinine, high density-lipoprotein-cholesterol and triglycerides of the extracts treated diabetic mice. The same extracts (fruit, bark and leaves) showed hypolipidemic effect by reducing the low density lipoprotein-cholesterol level and decreased the serum level of

cholesterol, triglycerides and produced no prominent changes in proteins levels ($p \leq 0.001$) (Alam *et al*., 2018). The studies revealed a defined role of the extracts in controlling hyperglycemia and implied that *Z. armatum* probably could control the development of diabetes. The antidiabetic activities could be attributed to the phytochemicals present in the extracts. However, since the phytochemical studies only revealed general chemicals test evidence without establishment of the molecular structures of the compounds, the anti-diabetic activities were incompletely validated.

Studies carried out to predict the toxicity profile associated with repeat-dose administration of root extracts of *Z. chalybeum* using laboratory rat models have shown long term administration of low doses to be safe (Engeu *et al.,* 2008). High doses were associated with impaired renal function and intestinal neoplasms. However, in usual traditional medicine, low doses of plant extracts are used and such high doses are only likely to occur in cases of acute poisoning or over dose (Ogwal-Okeng *et al*., 2003)**.**

Aqueous stem bark extract of *Z. chalybeum* were found to contain several secondary metabolites as alkaloids, flavonoids, terpenoids, saponins, tannins, phenols and glycosides (Kimani *et al*., 2015). Administration of the aqueous extract of *Z. chalybeum* stem bark on wistar rats caused decrease in blood glucose and a gain in body weight in streptozotocin-induced diabetic rats at the three doses (10, 100 and 1000 mg/ kg b. wt) compared to untreated diabetic rats (mean 13.08 ±1.65 mmol/L), suggesting that the aqueous stem bark extract of *Z. chalybeum* possesses anti-hyperglycemic activity and improves glucose homeostasis, respectively (Kimani *et al.,* 2015). Oral glucose tolerance test, using aqueous root extract of *Z. chalybeum* (400 mg/Kg b.wt), indicated decrease ($p \leq 0.05$) in blood glucose levels of the alloxan-induced diabetic rats after 28 days (Agwaya et al., 2016a). This suggested that the aqueous root extracts of *Z.*

chalybeum enhance glucose utilization and hence improve glucose tolerance in diabetic rats. Similarly, there was no significant difference between treatment group receiving 400 mg/Kg b.w. of the same extract and normal groups ($p = 0.27$), which implied that extract treated group were able to regulate blood sugar levels. There was observed reversal in pancreatic histopathology through regeneration of β-cells of the islets of Langerhans supporting the protective effect of *Z. chalybeum* extract towards diabetic damage. In another investigation on diabetes induced myocardial dysfunction in alloxan-induced Type-1 diabetic rats at two dose levels of 200 mg/Kg, 400 mg/Kg b.w., the aqueous root bark extract exhibited anti-hyperglycemic and anti-hyperlidemic properties (Agwaya *et al*., 2016b). Administration of aqueous root bark extract (400 mg/Kg b.w.) lowered ($p \leq 0.05$) blood glucose levels from 490.8 ±73.30 mg/dL before treatment to 120.3 ±15.42 mg/dL after 28 days of treatment, which was comparable to the normal control, 123.8 ±7.122 mg/dL, and there was no significant difference ($p > 0.05$) in total cholesterol levels between control groups and the extract treated groups. Additionally, *Z. chalybeum* root bark extract (400 mg/Kg b.w.) increased ($p \leq 0.05$) high density lipoprotein-cholesterol from 0.64 ±0.18 mmol/L before treatment to 1.84 ±0.11 mmol/L after 28 days of treatment, which was comparable to normal control 1.59 ±0.19 mmol/L. The extract (400 mg/Kg b.w.) decreased ($p \leq 0.05$) the levels of triglycerides from 5.81 ± 1.27 mmol/L before treatment to 0.73 ±0.11 mmol/L after 28 days which was comparable to the normal control 0.65 ±0.07 mmol/L, and there was no significant difference ($p > 0.05$) in low density lipoprotein-cholesterol between the control groups and extract treated groups (Agwaya *et al.*, 2016b). Histopathological changes revealed the protective nature of the aqueous roots extract of *Z. chalybeum* against alloxan-induced necrotic damage of cardiac tissues since there was reversal of the degenerative tissues (Agwaya *et al.*, 2016b). The study suggested that *Z. chalybeum* exhibit anti-

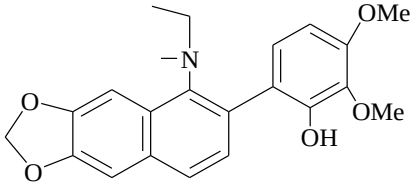
hyperglycemia and anti-hyperlipidemia and alleviates myocardial damage associated with diabetes (Agwaya *et al.,* 2016b). The findings from the studies corroborated the traditional use of *Z. chalybeum* for the management of diabetes, although no specific metabolites were identified to associate with anti-diabetic activity thus hampering the process of SAR.

2.6 Phytochemistry of the *Zanthoxylum* plants

Phytochemical studies carried out on some *Zanthoxylum* species have revealed the presence of alkaloids of various skeletal types including; benzophenanthridine (Mansoor *et al*., 2013), protoberberine (Hu *et al*., 2007), bishordeninyl (Marcos *et al*., 1990), aporphine (Hufford, 1976, Chen *et al*., 1996), amides (Kashiwanda *et al*., 1997; Xiong *et al*., 1997; Huang *et al*., 2012), coumarins (Bafi-Yeboa *et al*., 2005), lignans (Zhou *et al*., 2011; Rahman *et al*., 2005) as common secondary metabolites. Metabolites such as flavonoids (Xiong *et al*., 1995), sterols and terpenes (Adesina, 2005) have also been isolated from plants from this genus. However, alkaloids and coumarins are the main constituents of the genus (Negi *et al.*, 2011).

Phytochemical analysis of the genus *Zanthoxylum* showed the presence of alkaloids such as chelerythrine (**2**) (Krane *et al.*, 1984), skimmianine (**1)** and arnottianamide (**24**) (Krane *et al.*, 1984; Rahman *et al*., 2005), nitidine (**3)**, N-methyl flindersine (**25**) (Krane *et al.*, 1984), dihydrochelerythrine (**6**), zanthobungeanine (**26**) and rutaecarpine (**27**) (Rahman *et al*., 2005) in a number of species. Alkaloids isolated from *Z. heitzii* are; skimmianine (**1**), nitidine (**3**), fagaramide (**5**) and arnottianamide (**24**) from bark (Mbaze *et al*., 2009; Ngouela *et al*., 1994; Bongui *et al*., 2005), flindersine (**28**) from the wood (Ahmad, 1984) and 6-methyl nitidine (**29**) from the root (Bongui *et al*., 2005). In a study to investigate the anti-parasitic activity of *Z. heitzii*, dihydronitidine (**30**), pellitorine (**31**), heitziquinone (**32**), isoarnottianamide (**33**) and rhoifoline B (**34**) were isolated from the active antimalarial *n*-hexane fraction of stem bark

extracts of *Z. heitzii* (Goodman *et al*., 2016). Dihydronitidine (**30**) and pellitorine (**31**) had previously been isolated from *n*-hexane stem bark extract of *Z. heitzii* (Moussavi *et al*., 2015). Alkaloids, chelerythrine (**2**), berberine (**15)** and phenol canthine-6-one (**35**) were isolated from the methanol extract of *Z. zanthoxyloides* root bark (Odebiyi and Sofowora, 1979; Tsuchiya *et al.*, 1996) whereas an alkamide, fagaramide (**5**) and 8-acetonyldihydrochelerythrine (**36**) were isolated from *Z. gilletii* (Omosa and Okemwa, 2017). In another phytochemical characterization of Nigerian *Z. gilletii,* furoquinoline alkaloid, skimmianine (**1**), the cinnamic acid amide, fagaramide (**5**) and benzophenanthridine alkaloids; chelerythrine (**2**), nitidine (**3**) and dihydrochelerythrine (**6**), were isolated although no biological activity was tested (Adesina and Johannes, 1988).



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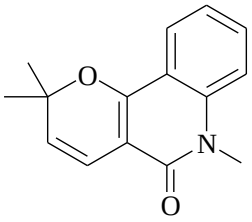

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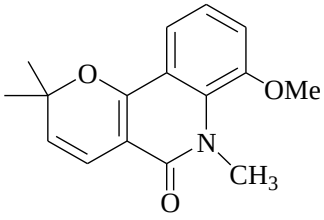

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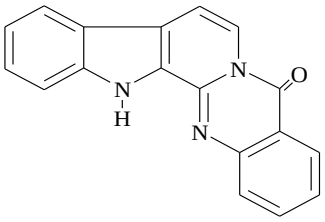

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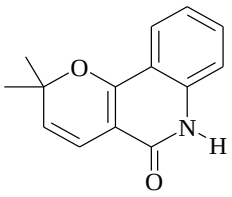

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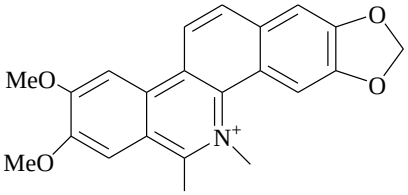

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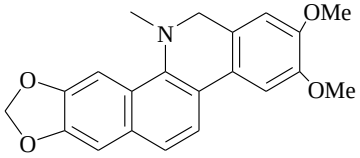

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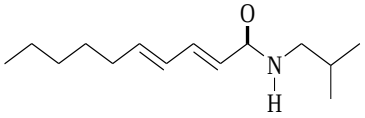

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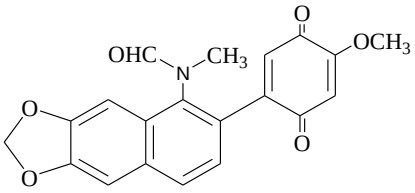

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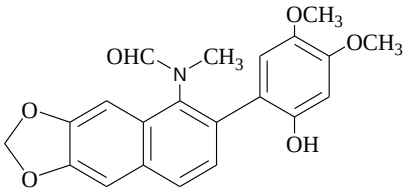

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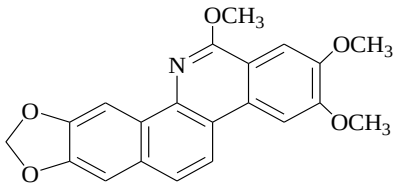

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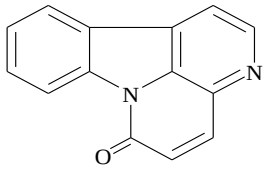

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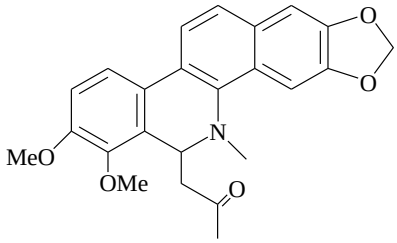
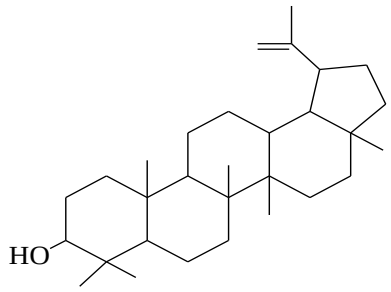


36

A phytosterol β-sitosterol (**21**) and triterpenoids; lupeol (**37**) and lupeone (**38**) were isolated from root of *Z. heitzii* (Bongui *et al*., 2005). In a phytochemical study on the chemical components of *n*-hexane stem bark extract of *Z. heitzii*, a sesquiterpene, caryophyllene oxide (**39**) was isolated (Moussavi *et al*., 2015). Two pentacyclic triterpene acetates, 3β-acetoxy-16β-hydroxybetulinic acid (**40**) and 3β, 16β–diacetoxybetulinic acid (**41**) were isolated from the stem bark of *Zanthoxylum gilletii* (*Fagara tessmannii*) (Mbaze *et al*, 2007), of which (**40**) showed potent α-glucosidase inhibitory activity (Mbaze *et al*, 2007), an indicator for potential anti-diabetic activities within the genus. Other compounds isolated from *Z. gilletii* include; a pentacyclic triterpenoid, lupeol (**37)** and volatile oils (Ombito *et al*., 2014).



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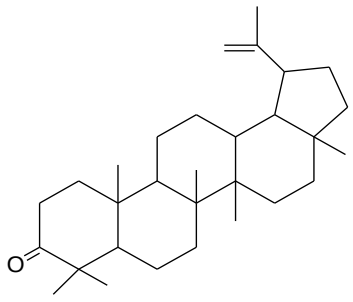

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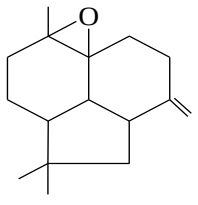

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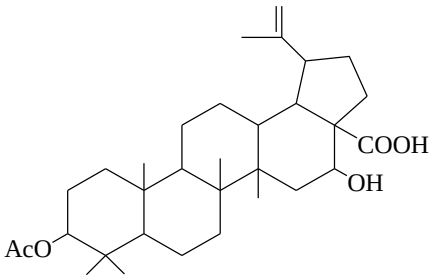

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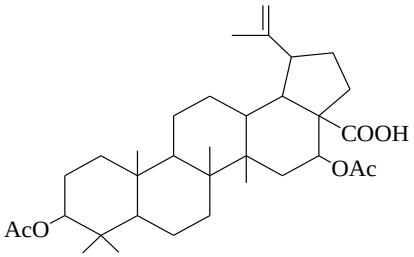
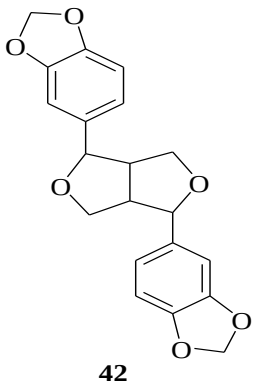


41

Similarly, phytochemical study on the chemical components of *n*-hexane stem bark extract of *Z. heitzii*, yielded a lignan, sesamin (**42**) (Moussavi *et al*., 2015) while a nor-neolignan, ailanthoidol (**43**) was isolated from *Z. ailanthoides* (Sheen *et al.*, 1994).



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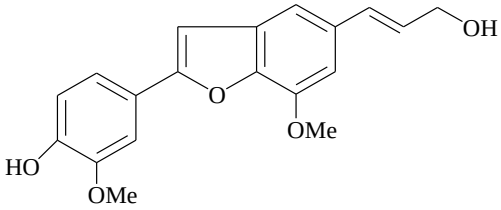


43

In a bio-guided fractionation of anti-malarial methanol root bark extract, nitidine (**3**) was identified as the main anti-malarial compound from *Z. chalybeum* alongside chelerythrine (**2)** and methyl canadine (**4**) (Muganga *et al.,* 2014). Nitidine (**3**) which was earlier isolated from *Z. nitidum* (Hu *et al.*, 2006) had good anticancer activities as well as other therapeutic properties such as anti-leukemic (Nakanishi *et al.*, 1998), antimicrobial, anti-inflammatory, analgesic and anti-HIV activity (Tan *et al.*, 1991). Isolation and characterization of Ugandan *Z. chalybeum* reported an additional novel compound fagaramide (**5**) from the stem bark (Adia *et al.*, 2016), alongside previously reported skimmianine (**1**) isolated from ethanol seed extract of *Z. chalybeum* (Olila *et al.*, 2001). An alkaloid, dihydrochelerythrine (**6**) and a coniferyl alcohol derivative 2,3-epoxy-6,7-methylenedioxy coniferyl (**7**) are two other compounds which were isolated from *Z.*

chalybeum (Anza *et al.*, 2014). Dihydrochelerythrine (**6**) was previously isolated from *Z. nitidum* (Hu *et al*., 2006).

Generally, alkaloids, flavonoids, terpenoids, saponins, tannins, sesquiterpene lactones, phenols and glycosides are common compounds elaborated by *Z. chalybeum* (Koigongi *et al*., 2014; Musila *et al*., 2013; Anza *et al.*, 2014) and other members of the genus *Zanthoxylum* (Ayangla *et al.*, 2016). These compounds have therapeutic benefits including α-glucosidase and α-amylase inhibitions (Gajbhiye *et al*., 2018), and are most probably responsible for the biological activity of *Z. chalybeum*. The exact activities of compounds vary among the phytochemical categories depending on the functional groups which depend on the plants species (Gaya *et al*., 2013). It is for such variations that it is difficult to pin point the active compounds in a plant such as *Z. chalybeum* without bioassay guided isolation experiments. Despite the phytochemical profiles and isolation of a number of compounds with interesting molecular diversity, there has not been any attempt to evaluate these molecules from *Z. chalybeum* towards anti-hyperglycemic properties. Besides, many plants species at different geographical localities may produce different metabolites and may exhibit variation in biological activity (Gaya *et al*., 2013).

CHAPTER THREE

METHODOLOGY

3.1 Experimental procedures

3.1.1 Chemicals

Organic solvents; *n*-hexane, dichloromethane, ethyl acetate, 95% methanol and ethanol, and the reagents; glacial acetic acid, *p*-anisaldehyde, concentrated sulphuric acid, silica gel (Merck 60G, 70-230 mesh), Dragendorff's and iodine crystals were sourced from Kobian, Kenya and Sigma-Aldrich Chemical Company, St. Louis, USA. The organic solvents used were of general purpose grade and they were distilled prior to use. Dimethylsulfoxide (DMSO) used was of analar grade and was sourced from Kobian, Kenya. α-Glucosidase (from *Saccharomyces cerevisiae*), bovine serum, *p*-nitrophenyl-α, *D*-glucopyranoside and acarbose were purchased from Sigma-Aldrich Chemical Company.

3.1.2 General instrumentation

The ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were recorded on Bruker or Varian-Mercury spectrometers using tetramethylsilane (TMS) as internal standard. Chemical shifts were measured in ppm in δ values relative to TMS. HSQC and HMBC spectra were acquired using the standard Bruker software. ESIMS spectra were recorded on a direct inlet, 70 eV, on SSq 710, Finnigan MAT mass spectrometer. Melting points were recorded using a Gallen kamp melting point apparatus with capillary tubes and are uncorrected. A rotary evaporator (EYELA, N-1000) was used for concentration of samples by evaporating the solvents under reduced pressure. Electric pulverizer (Kika, M20) was used to ground the root and stem bark into powder. The

powdered plant materials were weighed using a top-loading analytical balance (Shimadzu, UX). α-Amylase enzymatic activity (fluorescence) was recorded using a SpectraMax GeminiXS Spectrofluorometer (Molecular Devices, Sunnyvale, CA). α-Glucosidase enzyme activity (absorbance) was measured using a SpectraMax 190 Spectrophotometer (Molecular Devices, Sunnyvale, CA).

3.1.3 Chromatographic conditions

Analytical TLC was performed on pre-coated aluminium silica gel plates (Merck 60, F_{254}). Chromatographic zones on TLC plates were detected by spraying with *p*-anisaldehyde-sulphuric acid visualizing reagent, followed by charring at 110°C with a heat gun until the spots develop and/or exposure to iodine vapour by placing the plates in an iodine tank or exposure of the plates to ammonia vapour. The TLC plates were sprayed with Dragendorff's reagent for alkaloid tests. The *p*-anisaldehyde-sulphuric acid visualizing stain was prepared from a mixture of 0.5 ml of *p*-anisaldehyde, 85 ml of ethanol, 5 ml concentrated sulphuric acid, and 10 ml glacial acetic acid (Krishnaswamy, 2003). Column chromatography was done using silica gel (Merck 60G, 70-230 mesh) in glass columns (column sizes; 3.0 × 60 cm and 2.0 × 50 cm). Ethyl acetate, *n*-hexane and 95% methanol were the major solvents used in the study.

3.1.4 Collection of plant materials and identification

The root bark and stem bark of *Z. chalybeum* were collected from Homa Hills, Homa Bay County, Kenya (0° 23´S, 34° 30´E) in September 2016. Identification and authentication of the plant samples was done by a taxonomist at Maseno University, Department of botany where a voucher specimen (DWN/MSN/01/2016) was deposited at Maseno University herbarium. The plant materials (root and stem bark) were chopped into small pieces and air-dried at room

temperature under shade for 21 days. The dried materials were ground into fine powder using an electric pulverizer at the Postgraduate Research Laboratory, Department of Chemistry, Maseno University, and weighed using a top-loading analytical balance.

3.1.5 Preparation of stem bark and root bark crude extracts.

The shade air-dried powdered stem bark (1.7 kg) and root bark (1.2 kg) of *Z. chalybeum* were soaked at room temperature separately in 3 litres each of *n*-hexane and left for 24 hours with occasional swirling, after which were filtered under vacuum using Whatman No.1 filter paper. The procedure was repeated with ethyl acetate and 95% aq. methanol in sequence and filtered under vacuum using Whatman No.1 filter paper. The filtrates were individually concentrated *in vacuo* using a rotary evaporator to yield 20.0 g of *n*-hexane extract, 16.0 g of ethyl acetate extract and 103.0 g of 95% methanol stem bark crude extract, and 15.1 g of *n*-hexane extract, 29.0 g of ethyl acetate extract and 85.0 g of 95% aq. methanol root bark crude extract. Portions of the crude extracts (approximately 2 g) were set aside for bioassay tests.

3.1.6 Isolation of compounds from crude extracts of *Z. chalybeum* stem bark

Before isolation of the compounds, preliminary analytical thin layer chromatography (TLC) analysis was carried out to determine the chemical profiles. The extracts were spotted on silica gel pre-coated aluminium TLC plates which were then developed by *n*-hexane: ethyl acetate (4:1, 2:1, 1:1) solvent system in a TLC tank and the TLC plates sprayed with *p*-anisaldehyde-sulphuric acid mixture, dried, followed by heating at 110°C with a heat gun for about 2 minutes to detect the spots. Part of the stem bark crude extracts from *n*-hexane, ethyl acetate and 95% methanol extraction process were subjected to column chromatographic method of separation in order to obtain pure isolates.

Dry *n*-hexane stem bark crude extract (18 g) was loaded over *n*-hexane slurry of silica gel in a glass column and eluted stepwise starting with *n*-hexane and polarity gradually increased with ethyl acetate (1:0, 99:1, 95:5, 9:1, 4:1, 3:1, 2:1) and the column finally eluted with ethyl acetate, resulting in 35 fractions of 50 ml each. The collected fractions (50 ml each) were monitored by spotting on TLC plates and developing the TLC plates using *n*-hexane: ethyl acetate (95:5, 9:1, 4:1, 2:1, 0:1) solvent system in TLC tank. The TLC plate was removed from TLC tank, dried, sprayed with *p*-anisaldehyde-sulphuric acid reagent followed by heating at 110°C with a heat gun. The fractions were combined based on TLC spots and concentrated *in vacuo* using rotary evaporator. From fraction pooled **A** (21-35), a white compound precipitated which gave one purple-blue spot on TLC at 4:1 (*n*-hexane-ethyl acetate, R_f = 0.36). The solid was dissolved in DCM: MeOH (9:1), warmed and crystallized. The mixture was filtered and crystals washed with *n*-hexane under vacuum filter to yield white crystalline lupeol (**31**) (120 mg).

Ethyl acetate stem bark crude extract (14 g) was adsorbed on silica gel and loaded over *n*-hexane slurry of silica gel column and eluted starting with *n*-hexane and polarity was gradually increased with ethyl acetate (1:0, 99:1, 95:5, 9:1, 4:1, 3:1, 2:1, 1:1) and the column finally eluted with ethyl acetate-methanol (99:1). The collected fractions (50 ml each) were monitored by TLC spots using *n*-hexane: ethyl acetate (9:1, 4:1, 2:1 and 1:1) solvent system and visualization was done by spraying the TLC plates with *p*-anisaldehyde-sulphuric acid reagent followed by heating at 110°C with a heat gun. A total of 55 fractions were collected, each 50 ml, which were combined into three fractions based on TLC spots and were pooled as **B** (1-3), **C** (4-20) and **D** (21-48). The pooled fractions were concentrated under vacuum using a rotary evaporator. Fraction **B** (1-3) was eluted using *n*-hexane: ethyl acetate (95:5, 9:1, 4:1) and had two spots on

TLC at 9:1 (*n*-hexane: ethyl acetate). Further purification by column chromatography using *n*-hexane/ethyl acetate (95:5, 9:1, 4:1), gave 32 fractions out of which fractions 11-21 were found to be homogeneous and were pooled together. Fraction 11-21 crystallized into a white solid, was filtered and the crystals gave single spot on TLC yielding more lupeol (**31**) (400 mg). The filtrate from fraction 11-21 was saturated, allowed to recrystallize and the solid formed from recrystallization process was washed with methanol and allowed to recrystallize further forming platy-like white crystals of 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7)** (90 mg). Fraction **C** (4-20) showed three spots on TLC at 9:1 (*n*-hexane: ethyl acetate) and was further chromatographed with solvent system *n*-hexane: ethyl acetate (95:5, 9:1 and 4:1) giving 31 fractions. The fractions were pooled as 1-6, 7-17 and 18-22. Fraction 1-6 crystallized and the crystals gave single purple-blue spot on TLC chromatogram to afford white crystals 3α,20-dihydroxy-28-lupanoic acid (**44**) (45 mg). Fraction 7-17 yielded white solid which was purified by crystallization in *n*-hexane: ethyl acetate (9:1) to give 20-hydroxy-3-oxo-28-lupanoic acid (**45**) (62 mg). Further purification of fraction 18-22 by eluting with *n*-hexane: ethyl acetate (9:1, 4:1) repeatedly gave more of the white solid 20-hydroxy-3-oxo-28-lupanoic acid (**45**) (25 mg). Fraction pooled **D** (21-48) precipitated a white solid, which was washed with *n*-hexane under vacuum filter yielding crystalline white solid of 3α,20,28-trihydroxylupane (**46**) (80 mg). The TLC spots for compound (**45**) and (**46**) turned purple-blue upon spraying with *p*-anisaldehyde-sulphuric acid mixture and heating at 110°C with a heat gun.

A portion of 95% aq. methanol crude stem bark extract (100 g) was adsorbed on silica gel and loaded over *n*-hexane slurry of silica gel column. Elution started with *n*-hexane-ethyl acetate and polarity was gradually increased with ethyl acetate (4:1, 2:1, 3:2, 1:1, 0:1) then ethyl acetate-methanol (9:1, 4:1, 2:1). The collected fractions (50 ml each) were monitored by TLC using *n*-

hexane: ethyl acetate (2:1 and 1:1) and ethyl acetate-methanol (4:1 and 2:1) solvent system and visualization was done by spraying developed TLC plates with *p*-anisaldehyde-sulphuric acid reagent followed by heating at 110°C with a heat gun. The collected fractions were combined based on TLC profiles. The fraction pooled **E** (1-19) gave two spots on TLC at 9:1 (ethyl acetate-methanol). The spots turned orange red when the TLC plate was sprayed with Dragendorff's reagent. Further purification of fraction **E** (1-19) by repeated column chromatography using *n*-hexane: ethyl acetate (4:1, 2:1, 1:1) and ethyl acetate-methanol (4:1) solvent system, collecting 20 ml each, gave orange solid 6-hydroxy-N-methyl decarine (**47**) (52 mg) and yellow solid norchelerythrine (**48**) (41 mg).

3.1.7 Isolation of compounds from crude extracts of *Z. chalybeum* root bark

Before isolation of the compounds, preliminary analytical thin layer chromatography (TLC) analysis was carried out to determine the chemical profiles. The extracts were spotted on silica gel pre-coated aluminium TLC plates which were then developed by *n*-hexane: ethyl acetate (4:1, 2:1, 1:1) solvent system in a TLC tank. The TLC plates were then sprayed with *p*-anisaldehyde-sulphuric acid mixture, dried, followed by heating at 110°C with a heat gun for about 2 minutes to detect the spots. Part of the root bark crude extracts from ethyl acetate and 95% methanol extraction process were subjected to column chromatographic method of separation in order to obtain pure isolates.

Ethyl acetate root bark crude extract (25 g) was subjected to column chromatography over silica gel and eluted with *n*-hexane and polarity increased stepwise with ethyl acetate (1:0, 99:1, 95:5, 9:1, 4:1, 3:1, 2:1). The collected fractions (50 ml each) were monitored by TLC using *n*-hexane: ethyl acetate (95:5, 9:1, 4:1, 2:1) solvent system and visualization was done by spraying the developed TLC plates with *p*-anisaldehyde-sulphuric acid reagent, drying the plates,

followed by heating at 110°C with a heat gun. A total of 76 fractions were collected which were pooled into fractions based on the homogeneity of TLC spots. Fraction pooled **F** (8-12), gave one spot on TLC at 9:1 (*n*-hexane-ethyl acetate) and was dissolved in methanol, then allowed to settle overnight to yield a white precipitate. The solid was filtered off and washed with methanol giving a pure white crystalline solid 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) (200 mg). The filtrate was saturated, allowed to recrystallize further forming white solid. The solid was washed in methanol and recrystallized to give more of white crystals of 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) (55 mg).

A portion of 95% aq. methanol root bark crude extract (80 g) were subjected to column chromatography over silica gel and eluted starting with *n*-hexane and polarity gradually increased with ethyl acetate (4:1, 2:1, 3:2, 1:1, 0:1) then ethyl acetate-methanol (9:1, 4:1, 2:1) solvent system. The collected fractions (50 ml each) were monitored by TLC using *n*-hexane: ethyl acetate (2:1 and 1:1) and ethyl acetate-methanol (4:1 and 2:1) solvent system. Visualization of TLC spots was done by spraying the developed TLC plates with *p*-anisaldehyde-sulphuric acid reagent, drying, followed by and heating the TLC plates at 110°C with a heat gun. Fractions collected were combined based on TLC profiles as **G** (1-3) and **H** (4-18), concentrated and allowed to dry. Fraction pooled **G** (1-3) gave two spots on TLC at 9:1 (ethyl acetate-methanol). This fraction was subjected to further purification by column chromatography to yield orange amorphous solid which was purified by washing the solid with ethyl acetate to yield orange solid, 6-hydroxy-N-methyl decarine (**47**) (130 mg) (R_f = 0.52 (*n*-hexane-ethyl acetate (4:1)). The TLC spot for the orange solid obtained turned orange red upon spraying with Dragendorff's reagent. Fraction **H** (4-18) formed yellow crystalline solid which was washed with ethyl acetate

and pure yellow crystals of norchelerythrine (**48**) (99 mg) obtained. The TLC spot for the yellow crystals obtained turned orange red when sprayed with Dragendorff's reagent.

3.2 Physical and spectroscopic data of the pure isolates

2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**): White crystalline solid; Melting point range 124-125°C; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 3); R_f value 0.63 (*n*-hexane: ethyl acetate (7:3)); ESIMS *m/z* 195.32.

Lupeol (**37**): White amorphous solid; Melting point range 214-216°C; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 4); R_f value 0.36 (*n*-hexane: ethyl acetate (4:1)); ESIMS $[M+H]^+$ *m/z* 427.0

3*α*,20-dihydroxy-28-lupanoic acid (44): White crystalline flakes; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 5); ESIMS *m/z* 474.34

20-hydroxy-3-oxo-28-lupanoic acid (45): Colourless amorphous powder; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 6); $[M+H]^+$ *m/z* 473.2

3*α*,20,28-trihydroxylupane (46): White crystalline solid; Melting point range 272-273°C; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 7); ESIMS *m/z* 460.7

6-hydroxy-N-methyl decarine (47): Orange solid; R_f value 0.52 (*n*-hexane-ethyl acetate (4:1)); Melting point 177-178°C; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 8); ESIMS *m/z* 351.10

Norchelerythrine (48): Yellow crystals; Melting point range 210-212°C; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$) (Table 9); ESIMS $[M+H]^+$ *m/z* 334.0

3.3 *In vitro* anti-hyperglycemic activity

3.3.1 α-Amylase inhibition assay

The anti-hyperglycemic activity of the crude extracts and the pure isolated compounds were assayed against human salivary α-amylase (Lo Piparo *et al*., 2008). Extracts were prepared by dilution in dimethylsulfoxide (DMSO) into a stock concentration of 1 mg/ml immediately before use in experiments. Individual compounds were also dissolved in DMSO to produce an 8 mM stock solutions. Various dilutions of test extracts and compounds were pre-incubated in 96-well plates for 30 minutes at 22-25ºC with 250 µU of α-amylase at a final concentration of 2.5 mU/ml. The incubation buffer consisted of 50 mM NaH_2PO_4, 50 mM NaCl, 0.5 mM $CaCl_2$ and 0.1% bovine serum, pH 6.0, and the final volume of the pre-incubation mixture was 75 µL. To start the reaction, a 25 µL of 20 µg/ml DQ™ starch substrate was prepared in the incubation buffer, then topped up to a final concentration of 5 µg/mL starch. The final concentrations of extracts and the compounds were between 0.5–500 µg/ml and 1–1000 µM, respectively. DMSO concentrations ranging between 0.00625–6.25% was used as negative control on α-amylase while acarbose was used as positive control in all experiments. α-Amylase enzymatic activity was monitored by digestion of the DQ™ starch substrate resulting in an increase in fluorescence over time. Fluorescence was measured using a SpectraMax GeminiXS Spectrofluorometer (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively. The linear rate of product formation during the initial 15 minutes of incubation was used to calculate enzyme activity. α-Amylase activity was calculated relative to control incubations without inhibitor added, and expressed as a percentage of that value.

$$\% \text{ Inhibition} = \frac{\text{Absorbance (Control) - Absorbance (Extract or Compound)}}{\text{Absorbance (Control)}} \times 100$$

The percentage inhibitions were expressed as probit units then regressed against concentrations of the samples to obtain the 50% inhibitory concentration of each sample (IC_{50}). Control wells with only test compound, but no enzyme or substrate, were used to determine any background auto-fluorescence. Each incubation was conducted in triplicate, and results presented as mean ± standard error from at least three independent experiments.

3.3.2 α-Glucosidase inhibition assay

The anti-hyperglycemic activity of the crude extracts and the pure isolated compounds were assayed against α-glucosidase (Li *et al*., 2009) with extracts and pure compounds prepared as described in section 3.3.1 above. The test compound and 2 mU of α-glucosidase was diluted to 97 µL in 0.1 M potassium phosphate buffer (pH 6.5) and pre-incubated in 96-well plates at 37°C for 15 minutes. The reaction was initiated by adding 3 µL of 3 mM *p*-nitrophenyl-*α*, *D*-glucopyranoside (*p*-NPG) as substrate and incubated for an additional 15 minutes at 37°C, followed by addition of 100 µL of 1 M Na_2CO_3 to stop the reaction. All test compounds were prepared in DMSO. The final concentrations of extracts and the compounds were between 0.03–10 µg/mL and 5–1000 µM, respectively. The final concentration of α-Glucosidase was 20 mU/mL. The enzyme activity was then determined by measuring the release of *p*-nitrophenol from the *p*-NPG substrate, and the reaction monitored by change of absorbance at 410 nm using a SpectraMax 190 Spectrophotometer (Molecular Devices, Sunnyvale, CA). α-Glucosidase activity was calculated relative to control wells without inhibitor added, and expressed as a percentage of that value.

$$\% \text{ Inhibition} = \frac{\text{Absorbance (Control) - Absorbance (Extract or Compound)}}{\text{Absorbance (Control)}} \times 100$$

The percentage inhibitions were expressed as probit units then regressed against concentrations of the samples to obtain the 50% inhibitory concentration of each sample (IC_{50}). Each incubation was conducted in triplicate, and results presented as mean ± standard error from at least three independent experiments. Acarbose was used as a positive control.

3.4 Data analysis

Enzyme activities in the presence of inhibitors were expressed as a percentage of the uninhibited enzyme activity. Enzyme inhibitory assays were performed in triplicate. The concentration of the crude extracts and compounds that inhibits 50% of the enzyme activity (IC_{50}) was determined by plotting percentage inhibition versus log10 of crude extract and compound concentration, and calculated by logarithmic regression analysis from the mean inhibitory values. One-way analysis of variance (ANOVA, Tukey-Kramer HSD Post-Hoc) was used to determine differences in mean of IC_{50} of different groups. Values were considered significant at $p \leq 0.05$. LSD values were calculated using Genestat software.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Bioassay results of crude extracts

4.1.1 α-Amylase inhibition IC_{50} by the crude extracts from *Z. chalybeum*

Different concentration for the different solvents extracts of both stem and root bark of *Z. chalybeum* were subjected to α-amylase inhibitory assay and IC_{50} results presented as in Figure 2. The most potent inhibition was observed with the 95% aqueous methanol stem and root bark extracts at IC_{50} values of 110.82 and 134.43 µg/ml, respectively. There was no significant difference ($p>0.05$) between inhibitory activities of 95% aqueous methanol stem and root bark crude extracts and the standard drug acarbose (102.16 µg/ml). The ethyl acetate extracts however, showed moderate activities whereas *n*-hexane extracts showed significantly ($p\leq0.05$) weak inhibitory effects of IC_{50} values >250 µg/ml, relative to the positive control acarbose. Stem bark extracts exhibited higher inhibitory activities than root bark extracts against α-amylase.

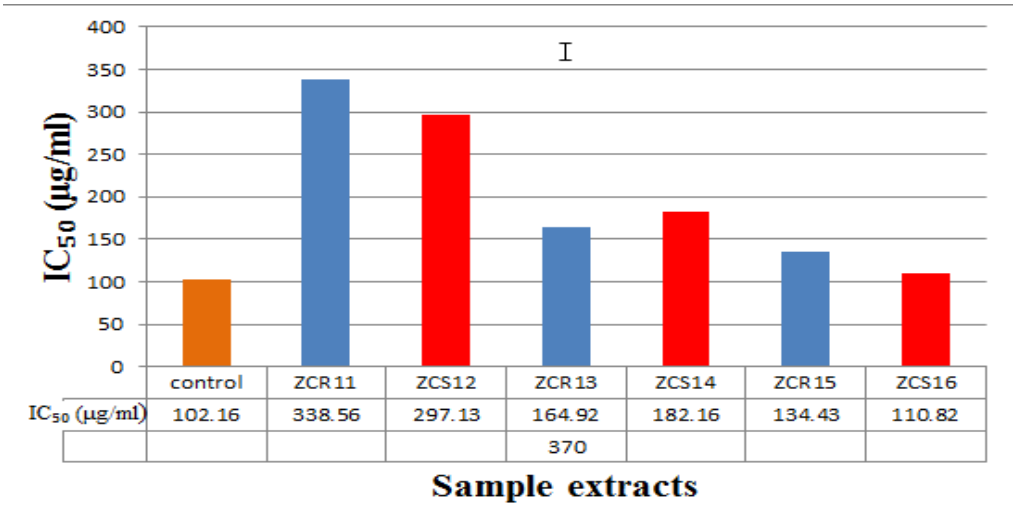


Figure 2: IC_{50} (mean ± standard error). Control = Acarbose; ZCR11 and ZCS12 = root and stem bark n-hexane extracts; ZCR13 and ZCS14 = root and stem bark ethyl acetate extracts; ZCR15 and ZCS16 = root and stem bark 95% aqueous methanol extracts.

Following such observations of the inhibitory effects of α-amylase, it may be considered that the methanol ingredients of the *Z. chalybeum* were able to inhibit the hydrolytic activities of the α-amylase *in vitro*. The implication of such results is that suppose these compounds/extracts can reduce the amount of glucose to be absorbed into the blood stream will ultimately reduce the level of glucose by inhibiting α-amylase activity (Tarling *et al*., 2008), thereby reducing postprandial hyperglycemia manifested in Type-2 diabetes. Drugs such as voglibose, acarbose and miglitol are effective in controlling Type-2 diabetes by suppressing the hydrolysis of carbohydrates (Tarling *et al*., 2008) and so may be anticipated for the extracts of *Z. chalybeum*. The main advantage of these plant extracts is their apparent safety, efficacy and lesser side effects (Rutebemberwa *et al*., 2013), although universal acceptability is still low due to incomplete scientific validation based on the modes of inhibitions and other kinetic variables which are still unknown.

4.1.2 α-Glucosidase inhibition IC_{50} by the crude extracts from *Z. chalybeum*

The inhibitory activity of the crude extracts relative to acarbose (standard inhibitor) against enzymatic action of α-glucosidase on a substrate *p*-nitrophenyl-α, *D*-glucopyranoside (*p*-NPG) were determined and reported as IC_{50} values ranging from 68.23 µg/ml to 246.80 µg/ml (Figure 3). Aqueous methanol extracts from both the root bark and stem bark showed inhibitory activities similar to the positive control acarbose ($p>0.05$) against α-glucosidase, with IC_{50} values of 97.22 and 88.20 µg/ml, respectively. The ethyl acetate extracts showed moderate activities although significant ($p\leq0.05$) compared to the positive control. Stem bark extracts showed higher inhibitory activities than root bark extracts against α-glucosidase.

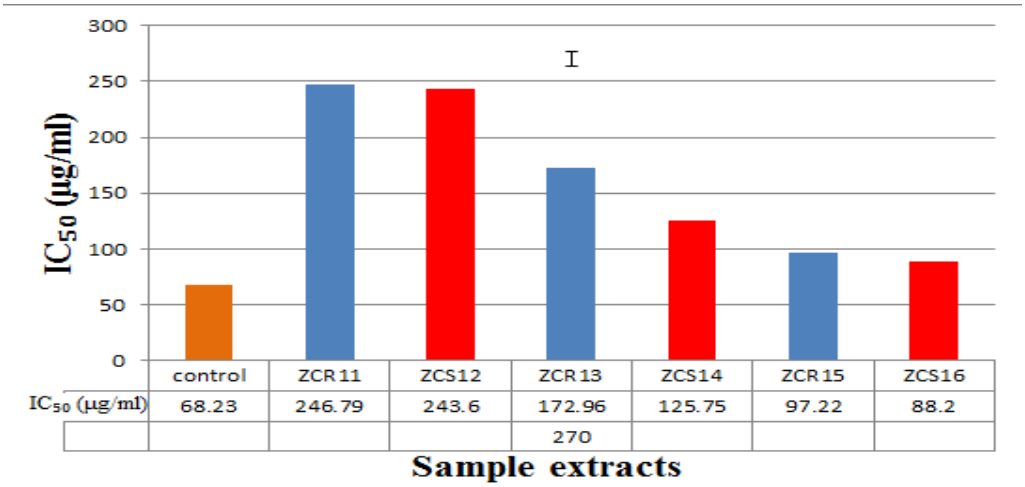


Figure 3: IC_{50} (mean ± standard error). Control = Acarbose; ZCR11 and ZCS12 = root and stem bark n-hexane extracts; ZCR13 and ZCS14 = root and stem bark ethyl acetate extracts; ZCR15 and ZCS16 = root and stem bark 95% aqueous methanol extracts.

It follows, therefore, that the inhibitory activities of the aqueous methanol extracts could be attributed to the high levels of polar compounds like the alkaloids present in the aqueous methanol extracts. Previously it had been reported in other plants that polar fractions with high levels of glycosides display potent inhibitory effects against α-glucosidase due to structural similarities to carbohydrates thus competing with carbohydrates substrates (Elya *et al*., 2012). Glycosides have been reported in *Z. chalybeum* (Kimani *et al*., 2015), although pure isolates have never been obtained. Polar tannins, terpenoids, alkaloids and flavonoids with potential of reducing the blood glucose levels have also been implicated (Osadebe *et al.*, 2010; Poongunran *et al.*, 2015) through inhibition of α-amylase and α-glucosidase hydrolytic actions of starch and disaccharides, respectively. This observation concurs with anticipated alkaloids and terpenoid contents in *Z. chalybeum* which thus corroborated the previous results observed by the reversal of *in vivo* streptozotocin induced diabetes on rats (Kimani *et al*., 2015) by the stem bark crude extracts of this plant. At concentrations of 10, 100, 1000 mg/kg b.w for 14 days, aqueous stem bark extracts of *Z. chalybeum* exhibited significant anti-hyperglycemic activity compared to the untreated controls, alongside causing considerable weight among the treated rats (Kimani *et al*.,

2015). The study revealed the plant had both anti-hyperglycemic as well as inhibition of adipocyte tissues growth which necessitated isolation of the potential active compounds. In another study, the root bark crude extract of *Z. chalybeum* significantly reduced the blood glucose levels after 28 days on alloxan-induced diabetic rats and reversal pancreatic histopathology reported a possible protective effects of *Z. chalybeum* extracts toward diabetic damage (Agwaya *et al*., 2016a). Agwaya *et al*., (2016b) also observed that *Z. chalybeum* root bark could prevent myocardial damage associated with Type-1 diabetes based on the following parameters; lowering glucose level on a fasting rat models; increasing HDL cholesterol levels while decreasing the triglycerides levels and maintaining the LDL-cholesterol levels and the total cholesterol levels. Under the same conditions, histopathological changes also revealed the protective effects of the root extracts against alloxan-induced necrotic damages of cardiac tissues (Agwaya *et al*., 2016b). Based on the observed potential of *Z. chalybeum* root bark and stem bark, both previous *in vivo* and the current *in vitro* observation, pointed out the potential of compounds from *Z. chalybeum*. However, *in vitro* inhibitory activity does not always correspond exactly with *in vivo* results, due to non-commonality of the matrix involved. As such further proof of certain facts requires evaluation of the inhibitory kinetic of the extracts and preclinical animal studies to establish safety and efficacy levels.

4.2 Characterization of compounds isolated from stem bark and root bark of *Z. Chalybeum*

4.2.1 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (7)

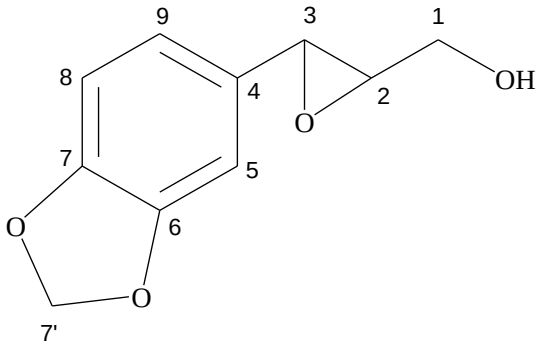
Compound **(7)** was isolated as white crystalline solid with a melting point of 124-125°C from ethyl acetate stem and root bark extracts. The ^{13}C NMR (Table 3) revealed a total of ten carbon atoms; one quaternary carbon at δ_C 135.3, two oxygenated quaternary carbons at δ_C 148.2 and

147.3, three sp^2 methine carbons at δ_C 106.8, 108.4 and 119.5, two sp^3 methine carbons at δ_C 54.5 and 86.0 and two methylene carbons at δ_C 101.3 and 71.9. The ^{1}H NMR (Table 3) showed three proton signals characteristic of two oxymethine protons at δ_H 4.72 (1H, d) and 3.06 (1H, m), and one methylene proton as doublet of doublet at δ_H [δ 4.25 (1H, dd), 3.87(1H, dd)]. The ^{1}H NMR spectrum further showed three proton signals in aromatic region; one pair of *ortho* coupled doublets at δ_H 6.79 and 6.85, and a singlet at δ_H 6.82. The ^{1}H NMR (Table 3) also showed a singlet at δ_H 5.95 integrated for two protons characteristic of methylenedioxy group moiety attached to a benzene ring. This was further supported by the correlation of methylenedioxy protons with the oxygenated quaternary phenyl carbons at δ_C 148.2 and 147.3 and the presence of an ABX spin system at δ_H 6.79 (1H, dd, J = 8.0, 2.2Hz); 6.82 (1H, d, J = 2.2Hz) and 6.85 (1H, dd, J = 8.0Hz). Such observation suggested a tri-substituted benzene ring of which two atoms were oxygenated and one alkylated at δ_C 135.3.

Based on the ^{13}C NMR and DEPT experiments, the remaining three carbon atoms would constitute all hydroxylated carbon atoms as -$CH(OH)CH(OH)CH_2(OH)$, however the additional oxygen atom would not agree with the ESIMS molecular mass results that appeared at *m/z* 195.32. This therefore implied that the compound had a three membered cyclized heterocyclic ring substituents with a hydroxymethylene side chain. Such an assertion was empirically supported by the three ^{1}H NMR signals for two oxymethine protons at δ_H 4.72 (1H, d) and 3.06 (1H, m) which were mutually coupled and in turn coupled to an oxymethylene proton signal δ_H [δ 4.25 (1H, dd), 3.87(1H, dd)] on ^{1}H-^{1}H COSY spectrum. Further, structural analysis based on mass spectrum molecular ion peak, supported by ^{13}C NMR and DEPT-135 spectral data, suggested molecular formula of $C_{10}H_{10}O_4$.

Complete connectivity of this substituent group was corroborated by the HMBC correlations which showed both ^{3}J and ^{2}J correlation between the methylene protons (δ_H 3.87 and 4.25) and the methine carbon signal at δ_C 86.0 and 54.5, respectively. On the other hand, methine proton at δ_H 3.06 showed correlation with carbon signal at δ_C 71.9 (C-1) and 86.0 (C-3), which confirmed the connectivity from C-1 to C-4. Moreover, correlation of oxymethylene protons at δ_H 5.95 (C-7') with oxygenated quaternary carbons at δ_C 148.2 (C-6) and 147.3 (C-7), correlation of aromatic proton at δ_H 6.82 with carbons at δ_C 135.3 (C-4), 147.3 (C-7), and 106.7 (C-9) coupled with the correlation of the methine at δ_H 4.72 with the quaternary carbon at δ_C 135.3 (C-4) corroborated the ABX substitution pattern of the phenyl ring (Figure 4).

NOESY spectrum revealed the remote spatial interaction between methine proton H-2 and aromatic H-5 alongside another prominent correlation between second methine proton H-3 and the other aromatic proton H-9, which all confirmed spatial placement of the epoxide to phenyl ring (Figure 5). Following these spectral evidence, and comparison of the spectral data with literature data (Table 3), the compound was characterized as 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) previously isolated from roots of *Z. chalybeum* (Anza *et al*., 2014).



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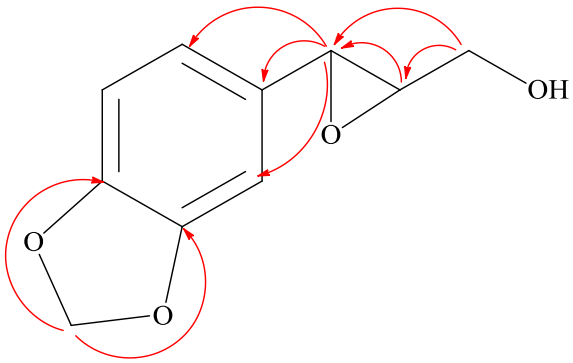


Figure 4: HMBC correlation for compound (**7**)

Table 3: ^{1}H and ^{13}C chemical shift values for 2,3-epoxy-6,7-methylenedioxyconiferyl alcohol (**7**)

C-Position	Spectral data of compound (**7**)			Literature data (Anza *et al*., 2014)		
	δ_H	Multiplicity	δ_C	δ_H	Multiplicity	δ_C
1	4.25	dd, (*J* = 16, 13.1)	71.9	4.25	dd	71.7
	3.87	dd, (*J* = 16.1, 7.2)		3.89	dd	
2	3.06	m	54.5	3.10	m	54.3
3	4.72	d, (*J* = 4.4Hz)	86.0	4.73	d, (*J* = 4.4Hz)	85.9
4			135.3			135.1
5	6.82	dd, (*J* = 0.8, 1.2Hz)	119.5	6.82	dd, (*J* = 0.8, 1.2Hz)	119.4
6			148.2			147.9
7			147.3			147.1
7’	5.95	s	101.3	6.02	s	101.1
8	6.85	dd, (*J* = 8.0, 0.8Hz)	108.4	6.86	dd, (*J* = 8.0, 0.8Hz)	108.2
9	6.79	dd, (*J* = 8.0, 1.2Hz)	106.7	6.80	dd, (*J* = 8.0, 1.2Hz)	106.5

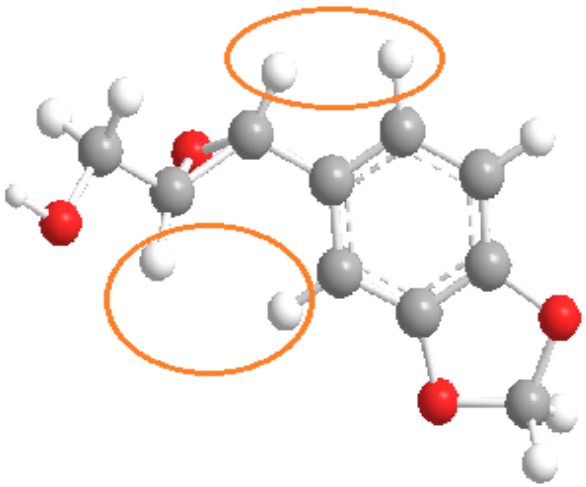
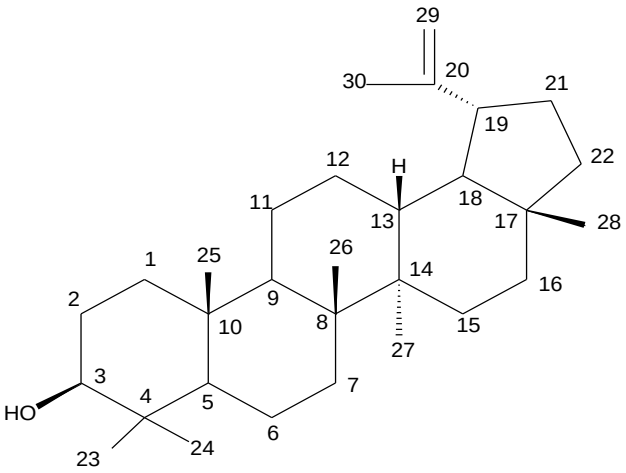


Figure 5: Spatial 3D orientation of compound (**7**) confirmed from the NOESY experiment

4.2.2 Lupeol (37)

Compound (**37)** was isolated as a white amorphous solid from *n*-hexane and ethyl acetate stem bark extracts as precipitate from DCM/*n*-hexane mixture. The compound showed a single spot on TLC with R_f value 0.36 in *n*-hexane/ethyl acetate (4:1) and melting point 214-216°C. Its TLC spot turned purple-bluish after spraying the developed plate with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 110°C with a heat gun, an indication it was a terpenoid. The ^{1}H NMR (Table 4) showed signals at δ_H 4.57 and 4.68 characteristic of exomethylene protons which showed mutual ^{1}H-^{1}H COSY correlation to singlet proton at δ_H 1.68 indicative of the isopropenyl side moiety for lupane skeleton. Similarly, the ^{1}H NMR (Table 4) spectra showed the presence of seven methyl singlets at δ_H 0.97, 0.87, 0.83, 1.01, 0.94, 0.79, 1.68 and one secondary carbinol proton at δ_H 3.18 (dd) characteristic of lupeol skeleton.

The ^{13}C NMR (Table 4) of the compound showed 30 signals for the terpenoid of lupine skeleton including a carbon bonded to the hydroxyl group at C-3 appearing at δ_C 79.2, while the vinylic carbons of the exocyclic double bond appeared at δ_C 151.2 and 109.5. The structural analysis showed the molecular mass to be 427.0 based on the ESIMS $[M+H]^+$ ion peak which suggested molecular formula of $C_{30}H_{50}O$ supported by ^{13}C NMR and DEPT-135 spectral data. All the spectral data for (**37**) suggested a lupine triterpene which was supported by the key HMBC correlations as shown in Figure 6. The structure of **(37)** was assigned as lupeol that was consistent with the literature data (Diaz *et al.*, 2018; Nejma *et al*., 2017). This is the first report for isolation of lupeol (**37**) from *Z. chalybeum*.



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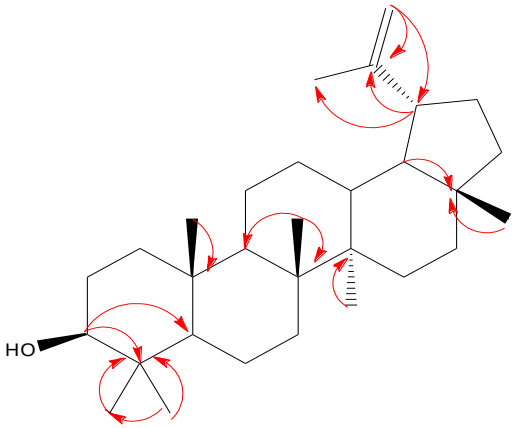


Figure 6: HMBC correlation for compound (**37**)

Table 4: ^{1}H and ^{13}C NMR ($CDCl_3$) chemical shift values for lupeol (**37**)

	Spectral data of compound (**37**)			Literature data (Diaz *et al.*, 2018)		
C-Position	δ_H	Multiplicity	δ_C	δ_H	multiplicity	δ_C
1			38.9			38.9
2	1.62	m	27.6	1.62	m	27.6
3	3.18	dd, (*J* = 10.5, 6.6Hz)	79.2	3.18	dd, (*J* = 10.5, 6.6Hz)	79.2
4			39.1			39.0
5	0.67	dd, (*J* = 6.2, 3.2 Hz)	55.5	0.67	t	55.5
6	1.39	m	18.5	1.38	m	18.5
7			34.5			34.4
8			41.1			41.0
9	1.26	t	50.7	1.25	t	50.6
10			37.4			37.3
11			21.1			21.1
12			25.4			25.3
13			38.3			38.2
14			43.1			43.0
15			27.6			27.2
16			35.8			35.8
17			43.2			43.2
18	1.35	dd, (*J* = 7.7, 5.8 Hz)	48.5	1.35	dd	48.5
19	2.36	dd, (*J* = 11.1, 5.7 Hz)	48.2	2.36	dd, (*J* = 11.1, 5.7Hz)	48.2
20			151.2			151.2
21			30.1			30.0
22			40.2			40.2
23	0.97	s	28.2	0.96	s	28.2
24	0.87	s	15.6	0.88	s	15.6
25	0.83	s	16.2	0.82	s	16.2
26	1.01	s	16.3	1.02	s	16.3
27	0.94	s	14.8	0.94	s	14.7
28	0.79	s	18.2	0.78	s	18.1
29	4.68	d, (*J* = 2.1Hz)	109.5	4.68	d, (*J* = 2.1Hz)	109.5
	4.57	d, (*J* = 2.4Hz)		4.56	d, (*J* = 2.4Hz)	
30	1.68	s	19.5	1.67	s	19.5

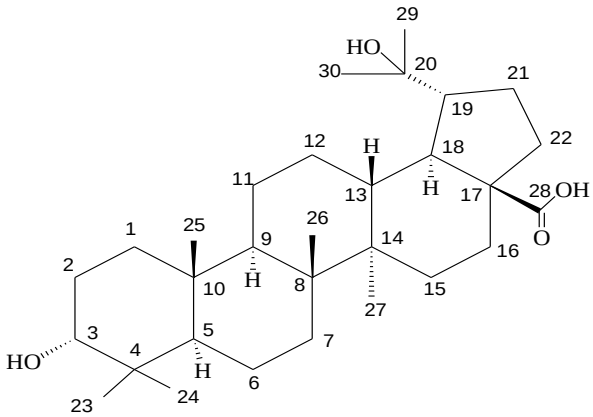
4.2.3 3α,20-dihydroxy-28-lupanoic acid (44)

Compound (**44**) was isolated as white crystalline flakes from ethyl acetate stem bark extract of *Z. chalybeum*. Its spot on TLC turned purple-blue after spraying the plate with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 110°C with a heat gun, indicating it was a terpenoid. The ESIMS of the compound showed a molecular mass at *m/z* 474.34, showing 48

a.m.u higher than lupeol (**37**), and indicating additional three oxygen atoms. Analysis of ^{13}C and DEPT (Table 5) spectra showed 30 carbon atoms, among them seven methyl carbon atoms (δ_C 27.2, 31.6, 16.4, 16.8, 16.8, 15.3 and 29.3), a carboxyl group (δ_C 179.4), six tertiary carbon atoms (δ_C 38.8, 37.5, 41.8, 43.7, 72.3 and 59.2), ten methylene carbon atoms (δ_C 38.8, 29.3, 18.7, 35.1, 21.9, 29.8, 30.7, 33.1, 29.8 and 37.7) and six methine carbon atoms (δ_C 75.3, 59.2, 50.9, 38.8, 49.3 and 50.2). Based on the ^{13}C NMR showing presence of a carboxyl moiety (δ_C 179.4) implied oxidation of one of the methyl groups, and the absence of an exocyclic olefinic protons also implied saturation and oxidation of the alkyl side chain.

The absence of the exocyclic olefinic protons compensated by an additional methyl group observed at δ_H 0.85, 0.87, 1.04, 1.21, 1.21, 1.37 and 1.45 (^1H NMR, Table 5) with one methyl oxidized to carboxyl unit as opposed to lupeol (**37**) that had seven methyl groups and no carboxyl unit, suggested the presence of a carboxyl moiety in its structure which was identified by δ_C 179.4 at C-28 position by key HMBC correlations (Figure 7). The signal at δ_H 3.60 in the proton spectrum showed a cross peak with a carbon peak at δ_C 75.3 in the HSQC spectrum and the two cross peaks at δ_H 1.21 (δ_C 29.3) and δ_H 0.87 (δ_C 16.4) in the HMBC spectrum, thus the additional characteristic peak observed at δ_H 3.60 (dd) was attributed to the oxymethine proton at C-3 of triterpene skeleton. The two methyl groups at δ_H 1.37 and 1.45 showed mutual HMBC correlation with their carbons and an oxygenated carbon at δ_C 72.3 which confirmed oxygenated C-20. The compound showed a molecular ion peak at *m/z* 474.34, consistent with molecular formula $C_{30}H_{50}O_4$ supported by ^{13}C NMR and DEPT-135. The analysis of the spectroscopic data and a comparison of the chemical shifts with those reported in literature (Table 5) suggested the structure depicted as 3α,20-dihydroxy-28-lupanoic acid, which had early on been isolated from *Viburnum awabuki* (El-Gamal, 2008) and *Salvia eremophila* (Farimani *et al*., 2012), and as a

methyl ester from *Relhania calycina ssp lanceolata* (Tsicheritzis and Jakupovic, 1990). The compound is hereby reported for the first time from the genus *Zanthoxylum*.



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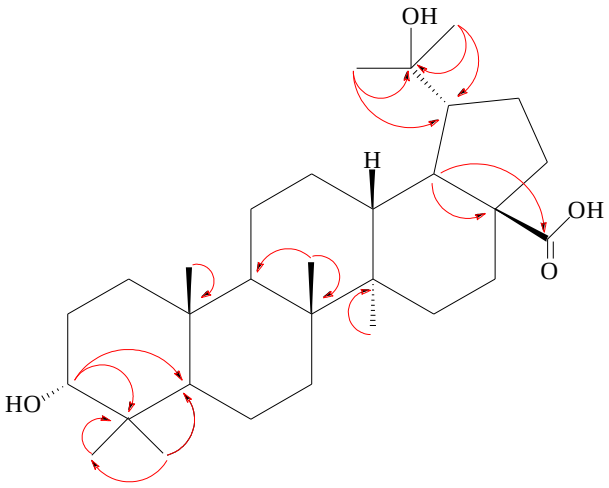


Figure 7: HMBC correlation for compound (**44**)

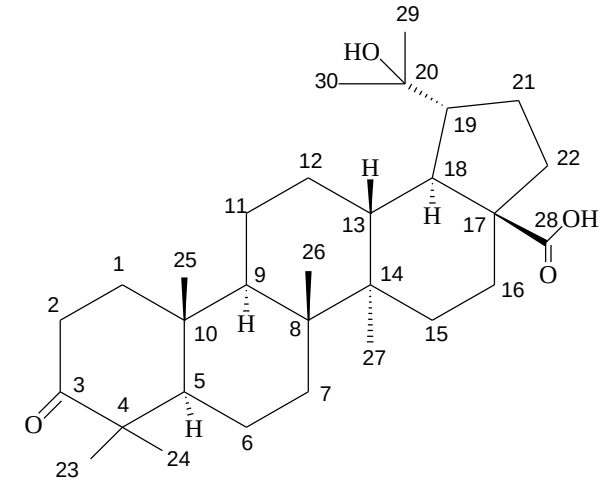
Table 5: ^{1}H and ^{13}C ($CDCl_3$) chemical shift values for 3α,20-dihydroxy-28-lupanoic acid (**44)**

C-Position	Spectral data of compound (**44**)			Literature data (Farimani *et al*., 2012)		
	δ_H	Multiplicity	δ_C	δ_H	Multiplicity	δ_C
1	1.04	1H m	38.8	1.04	1H, m	39.4
	1.45	1H, t, (*J* = 3.0, 16.0Hz)		1.73	1H, dt, (*J* = 3.0, 16.0Hz)	
2	2.10	m	29.3	1.93	2H, m	28.4
3	3.60	dd, (*J* = 5.0, 9.0Hz)	75.3	3.55	1H, br t, (*J* = 9.0Hz)	78.3
4			38.8			39.6
5	0.87	d, (*J* = 10.0Hz)	59.2	0.92	1H, d, (*J* = 10.0Hz)	55.9
6	1.45	m	18.7	1.47	m	18.8
	1.45	m		1.69	m	
7	1.37	dd, (*J* = 4.8, 13.0Hz)	35.1	1.40	1H, br s, (*J* = 13.0Hz)	35.4
	1.45	m		1.53	1H, dd	
8			41.8			41.8
9	1.45	t	50.9	1.45	1H, m	51.2
10			37.5			37.6
11	1.45	m	21.9	1.60	(1H, m)	22.2
	1.45	m, (*J* = 12.0Hz)		1.65	1H, br d, (*J* = 12.0Hz)	
12	2.10	m	29.8	1.82	1H, m	29.9
	2.21	m		2.37	1H, m	
13	2.94	1H, dt, (*J* = 3.0, 11.5Hz)	38.8	3.03	1H, dt, (*J* = 3.0, 11.5Hz)	39.0
14			43.7			43.9
15	1.45	d, (*J* = 13.0Hz)	30.7	1.43	1H, br d, (*J* = 13.0.0Hz)	30.9
	2.10	1H, m		2.09	1H, m	
16	1.45	m, (*J* = 3.0, 16.0Hz)	33.1	1.73	1H, dt, (*J* = 3.0, 16.0Hz)	33.3
	2.87	m,		2.82	1H, m	
17			59.2			59.3
18	2.10	dd, (*J* = 8.5, 11.5Hz)	49.3	2.06	(1H, m, (*J* = 8.5, 11.5Hz)	49.3
19	2.87	m	50.2	2.82	1H, m	50.4
20			72.3			72.5
21	2.10	m	29.8	1.93	1H, m	29.6
	2.67	m		2.45	1H, m	
22	2.10	m	37.7	1.82	1H, m	37.6
	2.67	m		2.25	1H, m	
23	1.21	3H, s	29.3	1.33	3H, s	28.8
24	0.87	3H, s	16.4	1.12	3H, s	16.5
25	0.85	3H, s	16.8	0.94	3H, s	16.7
26	1.04	3H, s	16.8	1.22	3H, s	17.0
27	1.21	3H, s	15.3	1.27	3H, s	15.5
28			179.4			179.5
29	1.45	3H, s	31.6	1.58	3H, s	31.9
30	1.37	3H, s	27.2	1.50	3H, s	27.0

4.2.4 20-hydroxy-3-oxo-28-lupanoic acid (45)

Compound (**45**) was isolated as a colourless amorphous powder from ethyl acetate stem bark extract of *Z. chalybeum*. The TLC spot of compound (**45**) turned purple-bluish after spraying the developed plate with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 110°C with a heat gun, indicating it was a terpenoid. The compound showed a mass spectral data of $[M+H]^+$ = 473.2, a loss of 2 a.m.u indicative of an oxidation of a hydroxyl group (-CH-OH) to carbonyl unit (-C=O) or oxidation of methylene unit ($-CH_2$) to carbonyl group (-C=O) from compound (**44**). Similarly, ^{1}H NMR (Table 6) spectrum showed seven methyl singlets δ_H 1.47, 1.39, 1.13, 1.11, 1.07, 1.00 and 0.79 similar to 3α,20-dihydroxy-28-lupanoic acid **(44)** except for the absence of a secondary oxymethine proton. The absence of that secondary oxymethine proton and the appearance of a signal at δ_C 216.6 on ^{13}C NMR alongside with δ_C 179.6 suggested the presence of a *keto* group and carboxyl unit in the structure which were identified by HMBC (Figure 8) to be at position C-3 and C-28, respectively.

To corroborate the structure, ^{13}C NMR (Table 6) spectrum showed 28 more signals for carbon atoms including seven methyl carbon atoms (δ_C 26.7, 21.1, 16.1, 16.1, 15.2, 26.7 and 31.8), six tertiary carbon atoms (δ_C 47.2, 41.4, 37.4, 43.6 and 59.2), ten methylene carbon atoms (δ_C 41.4, 29.5, 19.9, 34.3, 22.4, 30.7, 33.1, 29.6, 29.6 and 36.9) and five methine carbon atoms (δ_C 54.8, 50.2, 38.8, 49.1 and 50.2). These data were confirmed by the mass spectral data of $[M+H]^+$ = 473.2, suggesting a molecular formula of $C_{30}H_{48}O_4$. Based on the above data, and a comparison of the chemical shifts for those reported in literature (Table 6), a molecular structure depicted as 20-hydroxy-3-oxo-28-lupanoic acid was suggested which previously was isolated from *Viburnum awabuki* (El-Gamal, 2008) and *Orthopterygium huancuy* (Gonalez *et al*., 1984). The compound is reported for the first time from the genus *Zanthoxylum*.



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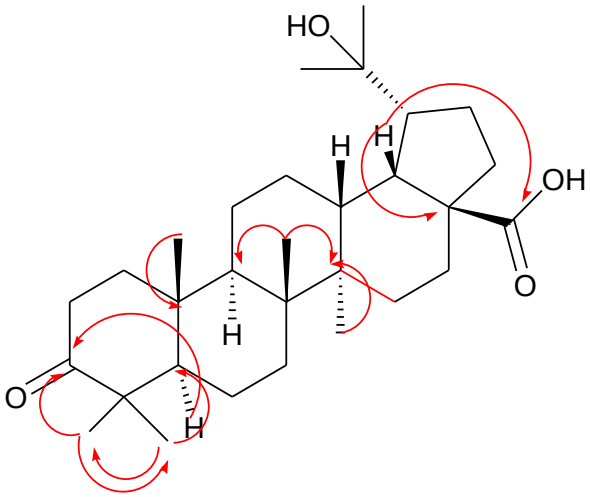

Figure 8: HMBC correlation for compound (**45**)

Table 6: ^{1}H and ^{13}C NMR chemical shift values for 20-hydroxy-3-oxo-28-lupanoic acid (**45)**

	Spectral data of compound (**45**)			Literature data (El-Gamal, 2008)		
C-Position	δ_H	Multiplicity	δ_C	δ_H	Multiplicity	δ_C
1		t	41.4		t	41.3
2		t, (*J* = 14.2, 6.3, 5.6Hz)	29.5		t, (*J* = 14.2, 6.3, 5.6Hz)	29.3
3			216.6			216.5
4			47.2			47.2
5		t	54.8		t	54.7
6		m	19.9		m	19.7
7		t	34.3		t	34.1
8			41.4			41.4
9		t, (*J* = 8.9, 5.7Hz)	50.2		t, (*J* = 8.9, 5.7Hz)	50.2
10			37.4			37.3
11		m	22.4		m	22.3
12		m	30.7		m	30.5
13		m	38.8		m	38.6
14			43.6			43.5
15		t	33.1		t	32.9
16		t	29.6		t	29.5
17			59.2			59.0
18		dd, (*J* = 8.5, 11.5Hz)	49.1		m, (*J* = 8.5, 11.5Hz)	48.9
19		m	50.2		m	50.1
20			72.3			72.1
21		m	29.6		m	29.5
22		t	36.9		t	36.7
23	1.13	s	26.7	1.10	s	26.4
24	0.79	s	21.1	0.97	s	20.8
25	1.00	s	16.1	0.97	s	15.9
26	1.11	s	16.1	1.06	s	16.2
27	1.07	s	15.2	1.04	s	14.9
28			179.4			179.2
29	1.39	s	26.7	1.15	s	26.5
30	1.47	s	31.8	1.27	s	31.5

4.2.5 3*α*, 20, 28-trihydroxylupane (46)

Compound (**46**) was isolated as white crystalline solids with a melting point of 272-273°C from ethyl acetate extract of *Z. chalybeum* stem bark. The TLC spot of compound (**46**) turned purple-bluish after spraying with *p*-anisaldehyde-sulphuric acid mixture followed by heating at 110°C with a heat gun, indicating it was a terpenoid. The ^{1}H NMR (Table 7) spectrum showed seven

methyl singlet signals at (δ_H 0.89, 1.00, 1.07, 1.20, 1.20, 1.33 and 1.43). As opposed to the previous lupane structures, there was an oxymethylene proton signals at δ_H 3.66 and 3.69 both showing HSQC contours with carbon signal at δ_C 59.9 alongside an oxymethine signal at δ_H 3.61. Appearance of an oxymethylene signal and the absence of neither a carboxyl unit nor an eighth tertiary methyl group suggested presence of a primary alcohol at C-28. The ^{13}C NMR spectral data (Table 7) indicated 30 non-equivalent carbon atoms among them; seven methyl carbons (δ_C 27.8, 15.4, 16.3, 16.3, 15.4, 25.8 and 32.2), two carbon atoms attached to hydroxyl group one of which was a oxymethylene carbon at δ_C 59.9 and the other one was a oxymethine carbon at δ_C 75.2, ten methylene carbon atoms (δ_C 38.0, 27.8, 18.6, 34.2, 27.8, 29.3, 28.5, 21.6, 29.1 and 33.9), five methine carbon atoms (δ_C 50.7, 50.6, 36.8, 49.1 and 49.8) and six quaternary carbon atoms (δ_C 38.0, 41.9, 36.9, 43.7, 72.3 and 49.2).

These data were confirmed by the ESIMS spectral data which showed a molecular ion peak at *m/z* 460.7, corresponding to molecular formula of $C_{30}H_{52}O_3$. The spectral data suggested that it was a triterpene 3α,20,28-trihydroxylupane, previously reported from *Betula maximowiziana* (Fuchino *et al.,* 1996) and *Relhania calycina ssp lanceolata* (Tsicheritzis and Jakupovic, 1990). This compound is reported for the first time from the genus *Zanthoxylum*.

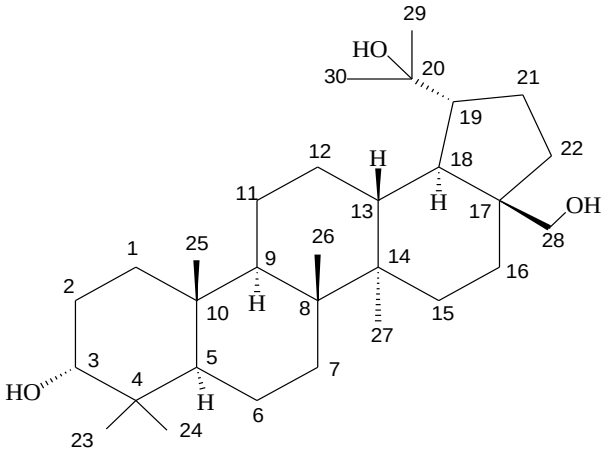

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Table 7: ^{1}H and ^{13}C ($CDCl_3$) chemical shift values for 3α,20,28-trihydroxylupane (**46)**

	Compound (**46**)			Literature data (Fuchino *et al.*, 1996)		
C-Position	δ_H	Multiplicity	δ_C	δ_H	Multiplicity	δ_C
1			38.0			38.7
2			27.8			27.4
3	3.61	d, (*J* = 10.6Hz)	75.2	3.68	1H, d, (*J* = 10.6Hz)	78.9
4			38.0			38.8
5			50.7			55.2
6			18.6			18.3
7			34.2			34.5
8			41.9			41.4
9			50.6			50.2
10			36.8			37.0
11			21.6			21.3
12			29.1			29.0
13			36.8			36.2
14			43.7			43.4
15			27.8			27.2
16			29.3			29.7
17			49.2			49.2
18	4.18	d, (*J* = 10.6Hz)	49.1	4.19	d, (*J* = 10.6Hz)	48.7
19	3.69	m	49.8	3.68	d, (*J* = 10.6Hz)	49.7
20			72.3			73.5
21	1.43	m	28.5			28.3
22	4.18		33.9			33.4
23	1.20	s	27.8	1.22	s	28.0
24	1.00	s	15.4	1.03	s	15.4
25	0.89	s	16.3	0.87	s	16.1
26	1.07	s	16.3	1.06	s	16.1
27	1.20	s	15.4	1.13	s	15.0
28	3.66	d, (*J* = 10.6Hz)	59.9	3.67	d, (*J* = 10.6Hz)	60.8
	3.69	d, (*J* = 10.6Hz)		3.69	d, (*J* = 10.6Hz)	
29	1.33	s	25.8	1.36	s	24.6
30	1.43	s	32.2	1.45	s	31.6

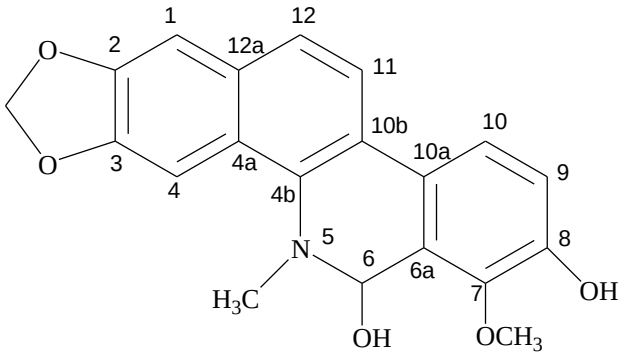
4.2.6 6-hydroxy-N-methyl decarine (47)

Compound (**47**) was isolated as an orange solid from methanol stem and root bark extracts, melting point 177-178°C and had R_f value 0.52 in *n*-hexane-ethyl acetate (4:1) and it responded to the Dragendorff's reagent, indicating it to be alkaloid. Analysis of NMR spectra (^{1}H, ^{13}C and 2D NMR) exhibited aromatic protons signals as two pairs of doublets at δ_H [7.62, *J* = 8.5Hz, 1H

/ δ_C 124.4, 8.07, *J* = 8.5Hz, 1H / δ_C 120.4)] and δ_H [7.41, *J* = 8.4Hz, 1H / δ_C 119.1, 7.82, *J* = 8.4Hz, 1H / δ_C 120.4)] and two singlets at δ_H 7.30 (1H) / δ_C 106.9 and δ_H 7.90 (1H) / δ_C 102.0, corresponding to four aromatic protons in *ortho* positions and two isolated aromatic hydrogens, respectively. Moreover, the ^{1}H NMR showed signals for one hemiaminal proton at δ_H 6.42 (s) / δ_C 79.7, one methylenedioxy OCH_2O at δ_H [6.02 (d, *J* = 1.2Hz) and δ_H 6.10 (d, *J* = 1.2Hz) / δ_C 102.0] and two methyl groups for NCH_3 and OCH_3 at δ_H 2.84 / δ_C 41.9 and δ_H 4.04 / δ_C 60.4, which all pointed to a benzophenanthridine skeleton.

Furthermore, the presence of ten quaternary carbon atoms including four oxygenated carbons, confirmed the benzophenanthridine alkaloid structure. This was supported by ESIMS molecular ion peak at *m/z* 351.10, consistent with the molecular formula $C_{20}H_{17}NO_5$, corresponding to thirteen degrees of unsaturation. The presence of the *ortho* arrangement for protons between H-12 (δ_H 7.62) and H-11 (δ_H 8.07) as well as H-10 (δ_H 7.82) and H-9 (δ_H 7.41) was confirmed by the COSY correlations. The methylenedioxy ring was revealed to be fused to the aromatic ring holding the singlet aromatic protons δ_H 7.30 (H-1) and δ_H 7.90 (H-4), due to the long range HMBC correlations (Figure 9) observed between CH_2 group to C-2 and C-3 which in turn coupled to H-1 and H-4, respectively. H-4 also showed long range correlations to C-4a (δ_C 128.2) and C-4b (δ_C 140.1) while H-1 displayed same interactions with C-12a (δ_C 131.7) and C-12 (δ_C 124.4). On the same note, mutual HMBC correlation between the NCH_3 group (δ_H 2.84) with C-4b and the hemiaminal carbon C-6 (δ_C 79.9), and the same displayed by H-6 (δ_H 6.42) to C-4b, C-6a, C-7, C-10a and the NCH_3 carbon confirmed the arrangement of the central ring. Moreover, HMBC correlation observed between the OCH_3 group at δ_H 4.04 to C-7 (δ_C 146.9); from H-9 (δ 7.41) to C-7, C-8 and C-10 and from H-10 to C-8, C-10a and C-10b confirmed the connectivity of ring E. Complete connectivity and relative spatial arrangements of the compound

was justified based on NOE correlation (Figure 10) between the methoxy group (δ_H 4.04) with hemiaminal proton (δ_H 6.42) which in turn showed interaction with NCH_3 group. Such arrangement would only be possible if the groups are same side of the acyclic non conjugated moiety and the OH group opposite. Based on discussed data the structure of **(47)** was established as 6-hydroxy-N-methyl decarine, previously isolated from the aerial part of *Zanthoxylum buesgenii* named as buesgenine (Sandjo *et al*., 2014).



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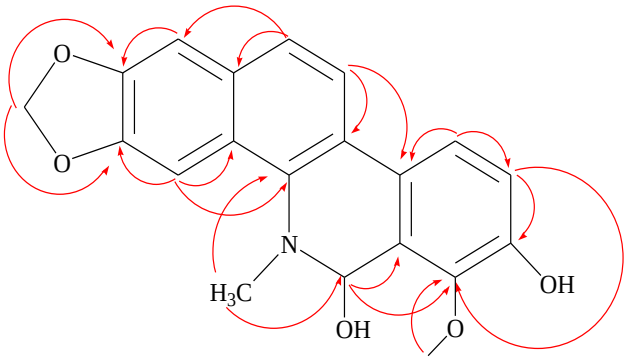


Figure 9: HMBC correlation for compound (**47**)

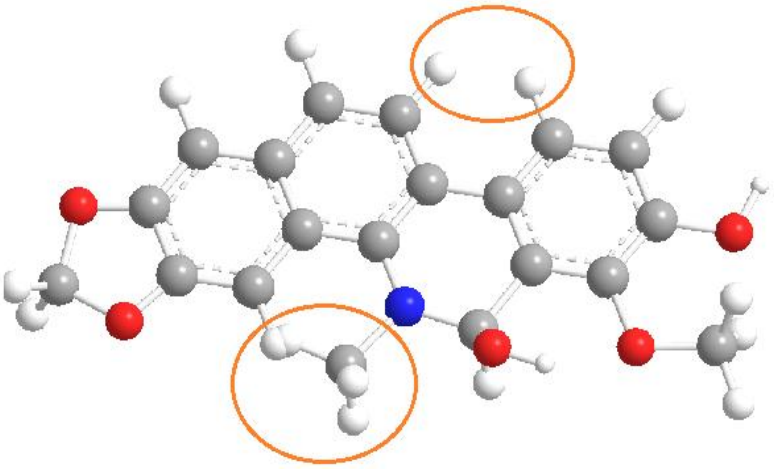


Figure 10: 3D model of compound (**47**) and its significant NOE correlation circled.

Table 8: ^{1}H and ^{13}C ($CDCl_3$) chemical shift values for 6-hydroxy-N-methyl decarine (**47**)

C-position	Compound (**47**)			Literature data (Sandjo *et al*., 2014)		
	δ_H	Multiplicity	δ_C	δ_H	Multiplicity	δ_C
1	7.30	s	106.9	7.31	s	105.3
2	-		148.8	-		148.8
3	-		148.2	-		148.2
4	7.90	s	102.0	7.89	s	101.7
4a	-		128.2	-		128.2
4b	-		140.1	-		140.1
5	-			-	-	-
6	6.42	s	79.7	6.54	s	80.0
6a	-		122.2	-		124.3
7	-		146.9	-		146.9
8	-		151.9	-		150.6
9	7.41	d, (*J* = 8.4Hz)	119.1	7.41	d, (*J* = 8.4Hz)	118.3
10	7.82	d, (*J* = 8.4Hz)	120.4	7.82	d, (*J* = 8.4Hz)	120.4
10a	-		129.1	-		129.1
10b	-		125.2	-		124.3
11	8.07	d, (*J* = 8.5Hz)	120.4	8.02	d, (*J* = 8.5Hz)	121.0
12	7.62	d, (*J* = 8.5Hz)	124.4	7.62	d, (*J* = 8.5Hz)	124.2
12a	-		131.7	-		131.7
OCH_2O	6.02	d, (*J* = 1.2Hz)	102.0	6.04	d, (*J* = 1.2Hz)	102.0
	6.10	d, (*J* = 1.2Hz)		6.08	d, (*J* = 1.2Hz)	
NCH_3	2.84	s	41.9	2.77	s	40.6
OCH_3	4.04	s	60.4	4.24	s	62.2

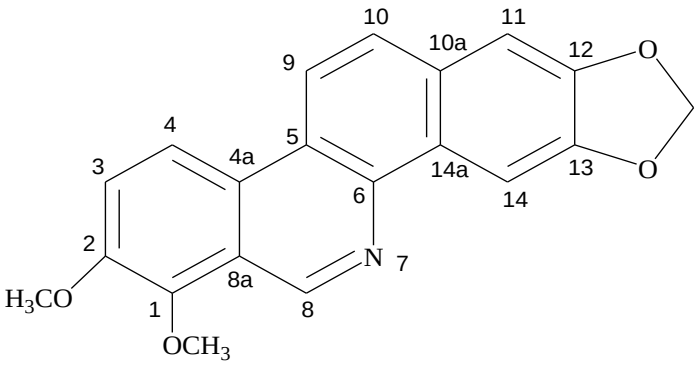
4.2.7 Norchelerythrine (48)

Compound (**48**) was isolated as yellow crystals from methanol stem and root bark extracts of *Z. chalybeum*, with melting point 210-212°C and it responded to the Dragendorff's reagent, indicating it to be alkaloid. The ^{13}C spectrum (Table 9) revealed presence of 17 sp^2 hybridized carbons, a methylenedioxy group (δ_C 104.5) and two methoxy groups (δ_C 63.0 and 57.7). The presence of ten quaternary carbon atoms including four oxygenated carbons, confirmed the benzophenanthridine alkaloid structure corroborated by the ESIMS $[M+H]^+$ peak at *m/z* 334.

The ^1H NMR displayed a peak for a highly deshielded proton at δ_H 9.95 consistent with an imine group showing presence of HC=N group in the skeleton and absence of NCH_3. Two pairs of *ortho* coupling proton signals at δ_H [8.65 (d, *J* = 9.7Hz) / δ_C 132.7, 8.62 (d, *J* = 9.7Hz / δ_C 120.0)] and δ_H [8.59 (d, *J* = 8.7Hz) / δ_C 119.6, 8.20 (d, *J* = 8.7Hz / δ_C 127.5] were confirmed by COSY correlations. Furthermore, in the ^1H NMR spectrum of the aromatic region displayed two additional singlets at δ_H 7.52 and 8.18 assigned to H-11 and H-14, respectively. Methylenedioxy group was observed to be attached to C-12 and C-13 due to the long range HMBC correlation between the methylenedioxy protons (δ_H 6.27) and the two carbon atoms (δ_C 152.2 and 151.9, respectively) (Figure 11). Mutual HMBC correlation observed between the OCH_3 group at δ_H 4.28 with C-1, C-8a, C-8 and C-2) and OCH_3 group at δ_H 4.13 with C-2, C-1 and C-3 confirmed the position of OCH_3 (δ_H 4.28) at C-1 and OCH_3 (δ_H 4.13) at C-2. Moreover, HMBC correlation observed between the OCH_3 group at δ_H 4.28 (δ_C 63.0) to C-1; OCH_3 group at δ_H 4.13 (δ_C 57.7) to C-2; from H-4 (δ_H 8.62) to C-8a, C-4a, C-9 and C-3, and from H-3 (δ_H 8.65) to C-8a, C-4a and C-4 confirmed the connectivity of ring A. The same mutual correlation displayed by H-8 (δ_H 9.95) to C-6, C-8a, C-1, 4a and the OCH_3 (δ_C 63.0) carbon confirmed the arrangement of the central ring B. Most significant NOE correlation (Figure 12) was observed between the methoxy

proton (δ_H 4.28) and H-8 (δ_H 9.95) together with NOE interactions observed between δ_H 4.13 and 8.65, δ_H 8.65 and 8.62, δ_H 8.62 and 8.59, δ_H 8.59 and 8.20, δ_H 8.20 and 7.52 that confirmed the possible spatial arrangements.

The aforementioned data were in close conformity with literature information for norchelerythrine (Table 9) previously isolated from roots of *Zanthoxylum gilletii* (Rutaceae) (Wafula, 2014), leaves of *Argemone maxicana* (Maliwat *et al*., 1997) and roots of *Zanthoxylum capense* (Mansoor *et al*., 2013).



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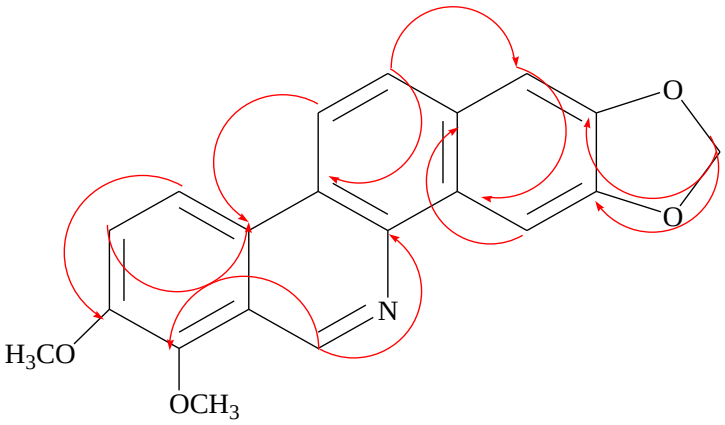

Figure 11: HMBC correlation for compound (**48**)

Table 9: ^{1}H and ^{13}C NMR ($CDCl_3$) chemical shift values for norchelerythrine (**48**)

C-Position	Compound (**48**) δ_H	Multiplicity	δ_C	HMBC (^2J, ^3J)	Literature data (Maliwat *et al*., 1997) δ_H	Multiplicity	δ_C
1	-		150.9		-		148.3
2	-		147.6		-		146.6
3	8.65	1H, d, (J = 9.7Hz)	132.7	C-8a, C-1, C-2	8.36	1H, d, (J = 9.09Hz)	129.2
4	8.62	1H, d, (J = 9.7Hz)	120.0	C-8a, C-2, C-4a	7.59	1H, d, (J = 9.09Hz)	120.0
4a	-		121.8		-		121.9
5	-		130.1		-		129.7
6	-		134.3		-		129.7
7	-	-	-		-		-
8	9.95	1H, s	151.9	C-8a, C-4a	9.75	1H, s	140.0
8a	-		127.2				128.1
9	8.59	1H, d, (J = 8.7Hz)	119.6	C-6, C-4a, C-5	8.35	1H, d, (J = 8.74Hz)	118.7
10	8.20	1H, d, (J = 8.7Hz)	127.5	C-11, C-10a	7.86	1H, d, (J = 8.74Hz)	127.1
10a	-		120.0		-		120.0
11	7.52	1H, s	107.2	C-13, C-10, C-14a	7.52	1H, s	104.4
12	-		152.2		-		149.4
13	-		151.9		-		148.5
14	8.18	1H, s	105.2	C-6, C-12	8.72	1H, s	102.0
14a	-		133.5		-		129.7
OCH_3	4.28	3H, s	63.0	C-1	4.08	3H, s	61.9
OCH_3	4.13	3H, s	57.7	C-2	4.13	3H, s	56.8
OCH_2O	6.27	2H, s	104.5	C-13, C-12	6.13	2H, s	101.3

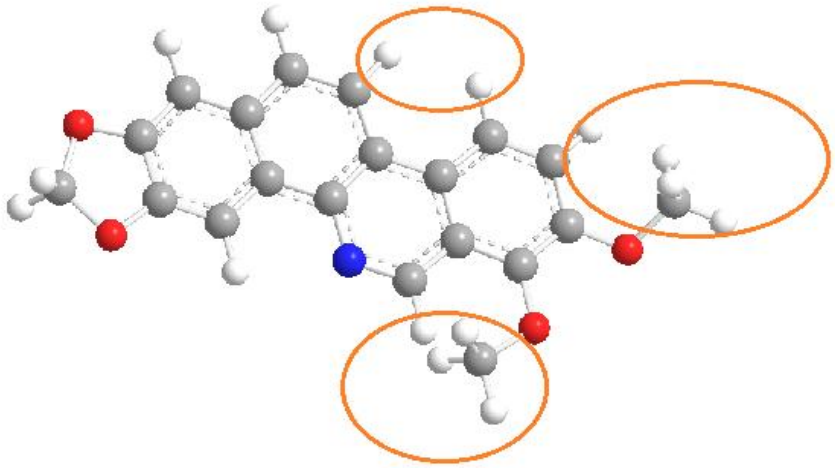

Figure 12: Spatial 3D orientation of compound (**48**) confirmed from the NOESY experiment

4.3 Bioassay results of pure isolates

4.3.1 α-Amylase inhibition IC_{50} by the pure isolates from *Z. chalybeum*

The dose dependent α-amylase inhibitory activities of the isolated compounds were processed on long probit analysis to establish the IC_{50} values, which exhibited the highest inhibitory effect with an IC_{50} = 58.91 and 54.81 µM from compound 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**), respectively. There was no significant difference in inhibitory activities ($p>0.05$) between the two compounds compared to the standard drug acarbose with IC_{50} = 54.67 µM. The other compound which also showed no significant inhibitory activity ($p>0.05$) relative to the control acarbose was the phenolic 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), which displayed IC_{50} of 64.55 µM.

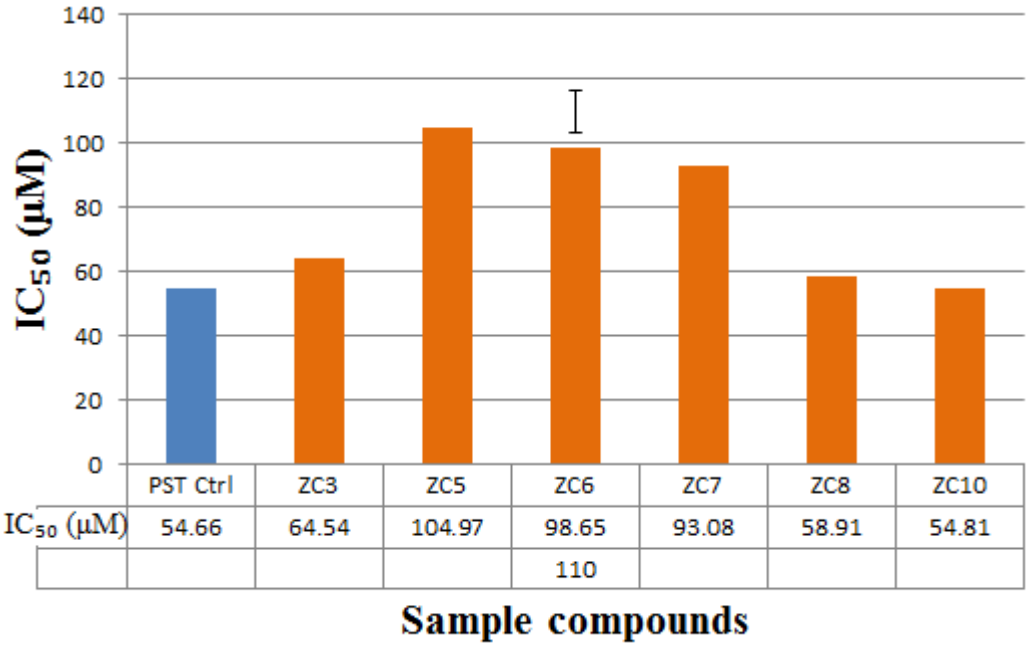

Figure 13: IC_{50} (mean ± standard error). PST Ctrl = Acarbose; ZC3 = 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), ZC5 = 3α,20-dihydroxy-28-lupanoic acid (**44**), ZC6 = 20-hydroxy-3-oxo-28-lupanoic acid (**45**), ZC7 = 3α,20,28-trihydroxylupane (**46**), ZC8 = 6-hydroxy-N-methyl decarine (**47**) and ZC10 = Norchelerythrine (**48**).

Partial activity of phenolic compounds on α-amylase had previously been postulated to hydrogen bonding between the residues of the active sites of α-amylase with the hydroxyl groups based on molecular docking studies, which consequently modulates the α-amylase functions and reduce the dietary breakdown of carbohydrates (Perera *et al*., 2016; Bahadoran *et al*., 2013). Such assertion could explain the inhibitory activity showed by triterpenoids isolated from *Z. chalybeum* in this study (ranging between 93.09 to 104.98 µM). Although the activities were weak to moderate compared to control, there were some trend of increased activity with increased number of hydroxyl units, compared to lupeol (**3**7) which exhibited IC_{50} >300 µM. The same assertion can still be inferred on the phenolic compound and the two benzophenanthridine alkaloids which have more structural potential to hydrophilicity. However, the assertion cannot be authoritative until enzyme inhibition kinetics and *in silico* molecular docking studies is carried out to confirm both the reaction rate and structural inhibition mode, respectively.

4.3.2 α-Glucosidase inhibition IC_{50} by the pure isolates from *Z. chalybeum*

The α-glucosidase inhibition potential of the seven compounds from *Z. chalybeum* was determined using *p*-nitrophenyl-α, *D*-glucopyranoside (*p*-NPG) as a substrate relative to acarbose based on their IC_{50} values under common specified set of assay conditions (Figure 14). As in α-amylase inhibition results, the α-glucosidase showed the same pattern of activity, for instance, 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) displayed potent inhibitory activities ($p>0.05$) (IC_{50} = 66.78, 62.54, and 58.03 µM, respectively) relative to control acarbose at IC_{50} = 51.54 µM.

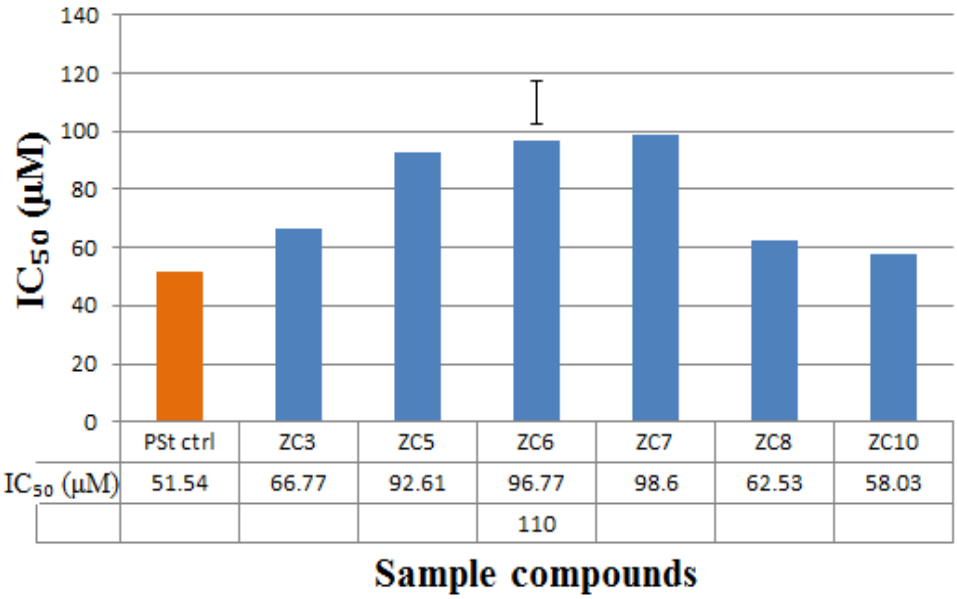

Figure 14: IC_{50} (mean ± standard error). PST ctrl = Acarbose; ZC3 = 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), ZC5 = 3α,20-dihydroxy-28-lupanoic acid (**44**), ZC6 = 20-hydroxy-3-oxo-28-lupanoic acid (**45**), ZC7 = 3α,20,28-trihydroxylupane (**46**), ZC8 = 6-hydroxy-N-methyl decarine (**47**) and ZC10 = Norchelerythrine (**48**).

The potent activities of the phenolic 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) and alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) confirmed the activity of the crude extracts from *Z. chalybeum* and the known inhibitory effects of alkaloids against α-glucosidase actions (Shibano *et al*., 2001; Choudhary *et al*., 2011). The results confirmed the previously established anti-hyperglycemic activities of *Z. chalybeum* extracts (Kimani *et al*., 2015; Agwaya *et al*., 2016a; Agwaya *et al*., 2016b), and that the anti-hyperglycemic activities of stem and root bark of *Z. chalybeum* was due to the potent inhibitory activities of the phenolic 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) and the alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**). The triterpenoids; lupeol (**37**), 3α,20-dihydroxy-28-lupanoic acid (**44**), 20-hydroxy-3-oxo-28-lupanoic acid (**45**) and 3α,20,28-trihydroxylupane (**46**) were isolated from the stem bark extracts while the phenolic 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) and alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) were isolated from root and stem bark extracts. The differences in

composition of the compounds in stem and root bark could explain why the stem bark showed stronger activity than root bark.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

The study was intended to provide *in vitro* information on efficacy of crude extracts and isolated pure compounds, and validate the rationale behind the use of the plant *Zanthoxylum chalybeum* in managing diabetes mellitus. The following summary, conclusions and recommendations can be deduced from the results consistent with the ethno botanical information about the plant.

5.1 Summary

The *n*-hexane, ethyl acetate and 95% aqueous methanol crude extracts from the root and stem bark of *Z. chalybeum* showed *in vitro* inhibition against α-amylase and α-glucosidase enzymes. Stem bark extracts were more active than root bark extracts. Inhibition of 95% aqueous methanol root and stem bark extracts was superior to *n*-hexane and ethyl acetate extracts relative to standard drug acarbose. A phenolic; 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), four triterpenoids; lupeol (**37**), 3α,20-dihydroxy-28-lupanoic acid (**44**), 20-hydroxy-3-oxo-28-lupanoic acid (**45**) and 3α,20,28-trihydroxylupane (**46**), and two benzophenanthridine alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) were isolated from the stem bark. The root bark yielded a phenolic; 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) and two benzophenanthridine alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**). The phenolic; 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**) and the two benzophenanthridine alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) isolated from both root and stem bark were as active as the standard drug acarbose ($p>0.05$). The triterpenoids; lupeol (**37**), 3α,20-dihydroxy-28-lupanoic acid (**44**), 20-hydroxy-3-oxo-28-lupanoic

acid (**45**) and 3α,20,28-trihydroxylupane (**46**) were less active and they showed moderate to weak inhibitory activities relative to the standard drug acarbose ($p \leq 0.05$).

5.2 Conclusion

i) Stem bark extracts were more active than root bark extracts of *Z. chalybeum*. There was no significant difference between inhibitory activities of root and stem bark (95% aq. methanol) crude extracts ($p > 0.05$) (IC_{50} = 134.43, 110.82 and 97.22, 88.20 µg/ml) and the positive control acarbose (IC_{50} = 102.16 and 68.23 µg/ml) against α-amylase and α-glucosidase enzymes, respectively. Both *n*-hexane and ethyl acetate stem bark extracts showed significantly higher activity ($p \leq 0.05$) than *n*-hexane and ethyl acetate root bark extracts against α-amylase and α-glucosidase.

ii) The pure compounds isolated from *Z. chalybeum* were; phenolic; 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), triterpenoids; lupeol (**37**), 3α,20-dihydroxy-28-lupanoic acid (**44**), 20-hydroxy-3-oxo-28-lupanoic acid (**45**) and 3α,20,28-trihydroxylupane (**46**), and two benzophenanthridine alkaloids; 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**). All the isolated compounds are known compounds.

iii) The compounds; 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) showed potent inhibitory activities relative to the positive control acarbose ($p > 0.05$).

5.3 Recommendations

i). The observed *in vitro* inhibition against α-amylase and α-glucosidase enzymes by the crude extracts of *Z. chalybeum* indicated stem bark extracts were more active than root bark extracts in managing diabetes, and the communities where the plant is found are advised to use stem bark extracts to manage diabetes.

ii). Extracts of *Z. chalybeum* contain bioactive compounds (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), lupeol (**37**), 3α,20-dihydroxy-28-lupanoic acid (**44**), 20-hydroxy-3-oxo-28-lupanoic acid (**45**), 3α,20,28-trihydroxylupane (**46**), 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**)) and the plant is recommended for medicinal use to manage diabetes. The plant should therefore be conserved for medicinal use and further pharmacological investigations.

iii). The active compounds from the plant *Z. chalybeum*; 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol (**7**), 6-hydroxy-N-methyl decarine (**47**) and norchelerythrine (**48**) are recommended as anti-diabetic drugs or as templates in development of more effective diabetes drugs.

5.4 Significance of the study

Based on the findings, the plant can be used in development of various herbal products capable of managing diabetes mellitus effectively and safely, reducing medicinal economic burden on the diabetic patients. Identification of anti-hyperglycemic principles from *Z. chalybeum* in management of diabetes validates its ethno medicinal use. Besides, establishment of such compounds will enrich the process of drug development since the most active compounds would be used as templates.

5.5 Suggestion for future studies

1. The compounds from *Z. chalybeum* have been noted in this study for potential of inhibiting carbohydrate digestive enzymes which are critical in diabetic complications. However, the mode of action is not known (whether the inhibition is competitive or non-competitive), and it is therefore important to carry out kinetic of the inhibition studies displayed by the compounds.
2. Structural modification is also relevant for the alkaloids to optimize the efficacy activity.
3. *In vivo* and toxicological tests to be done to ascertain the efficacy and toxicity of the isolated compounds.

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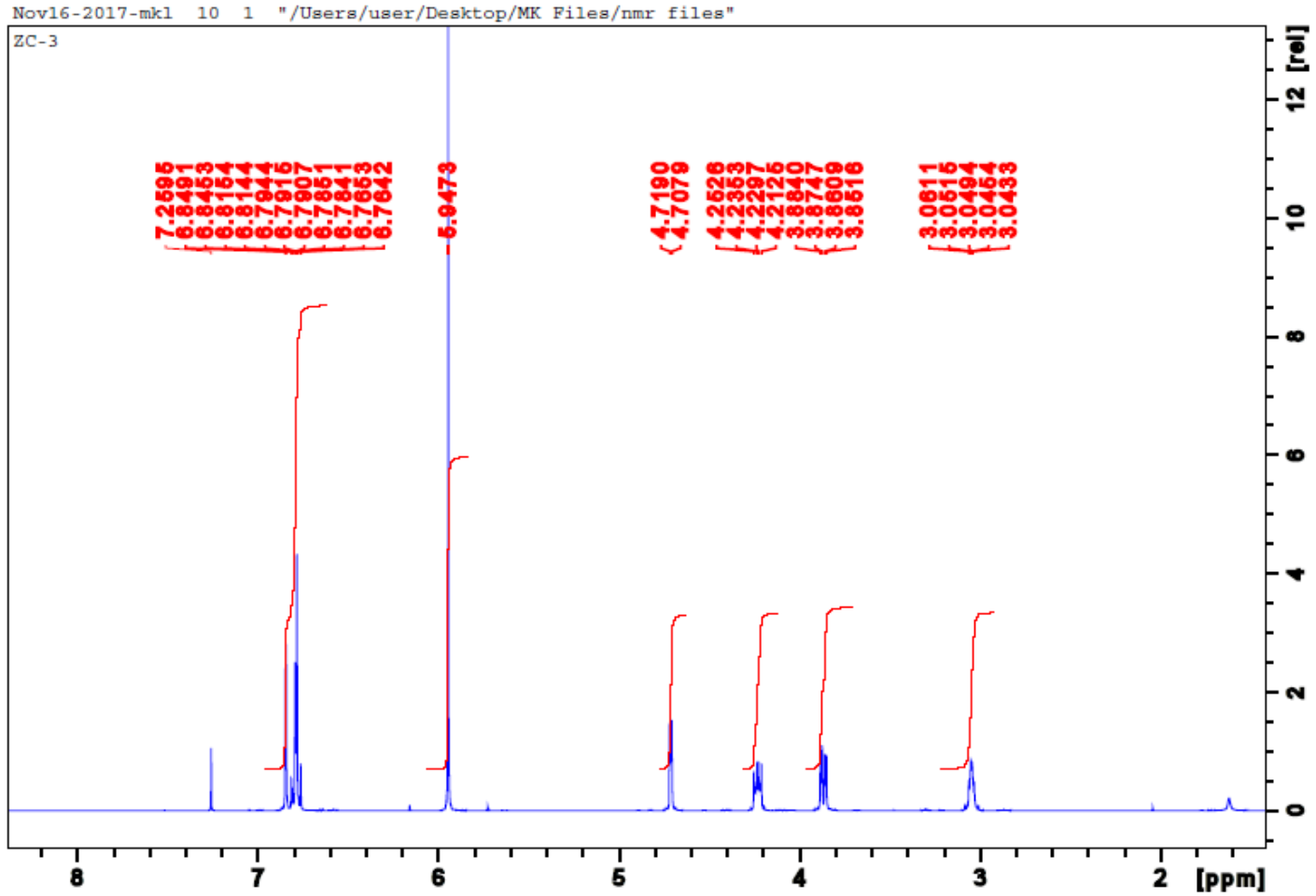
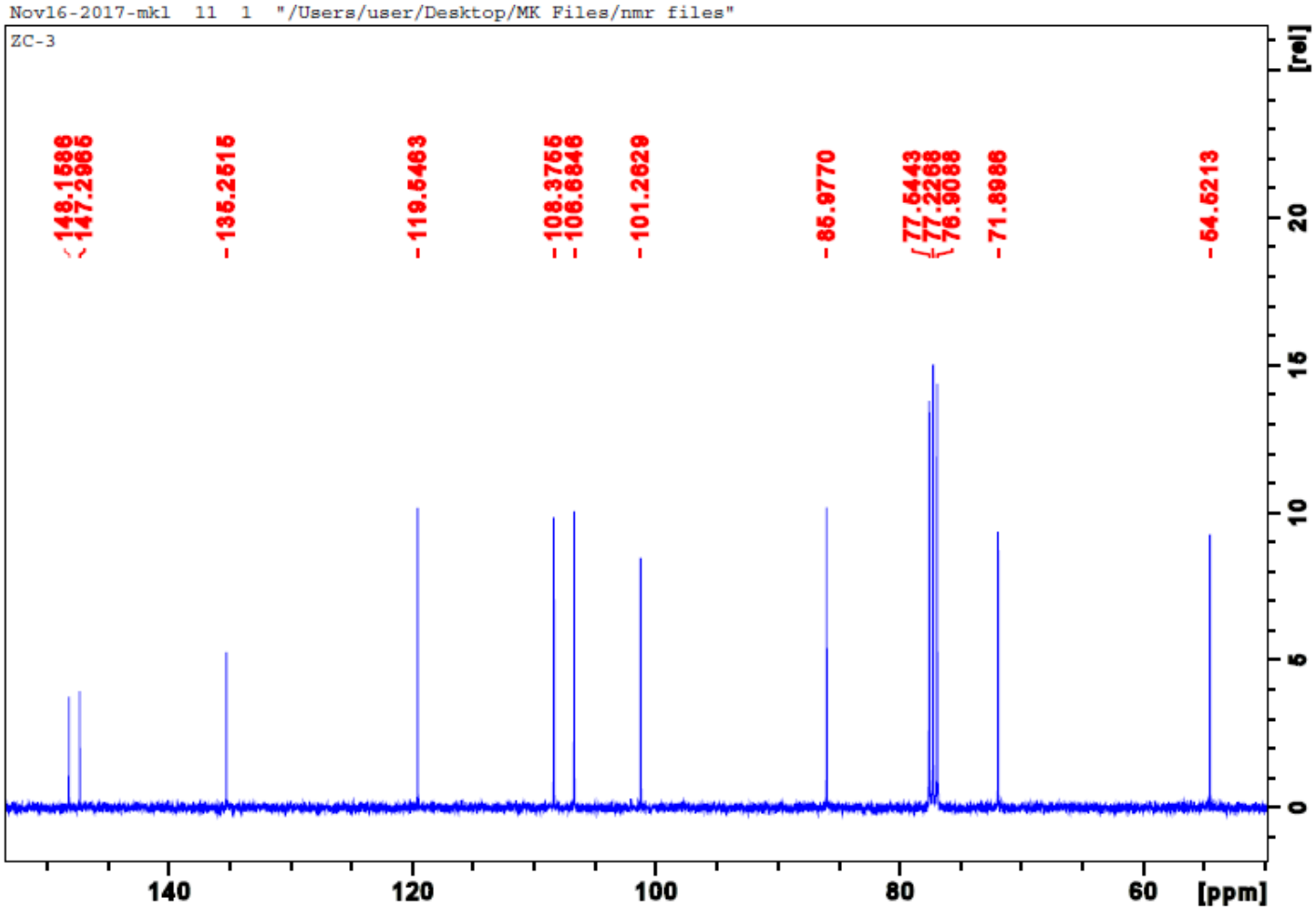
APPENDICES

Appendix 1: Spectra for compound (7); 2,3-epoxy-6,7-methylenedioxy coniferyl alcohol

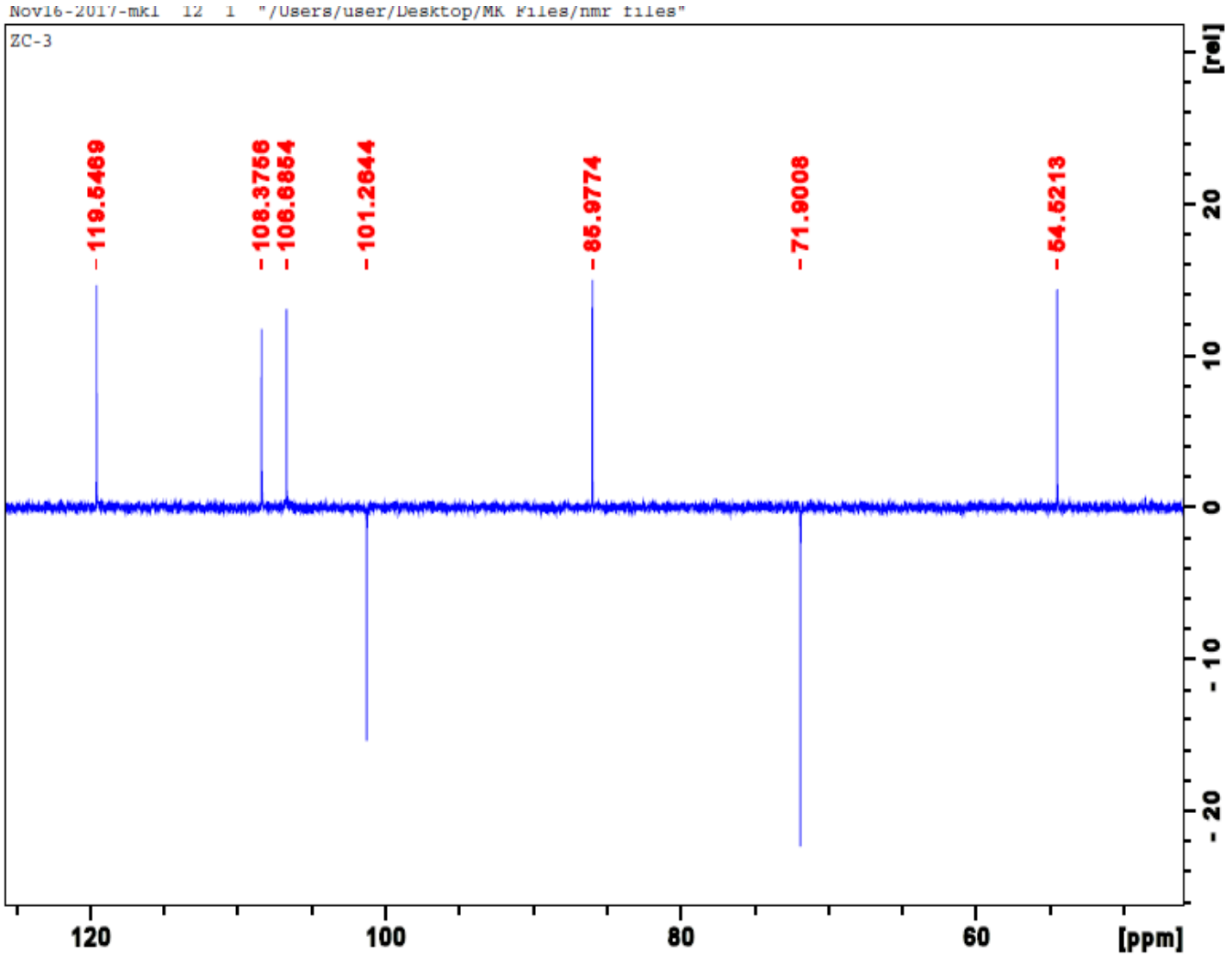
1.1: ^{1}H NMR spectrum for compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



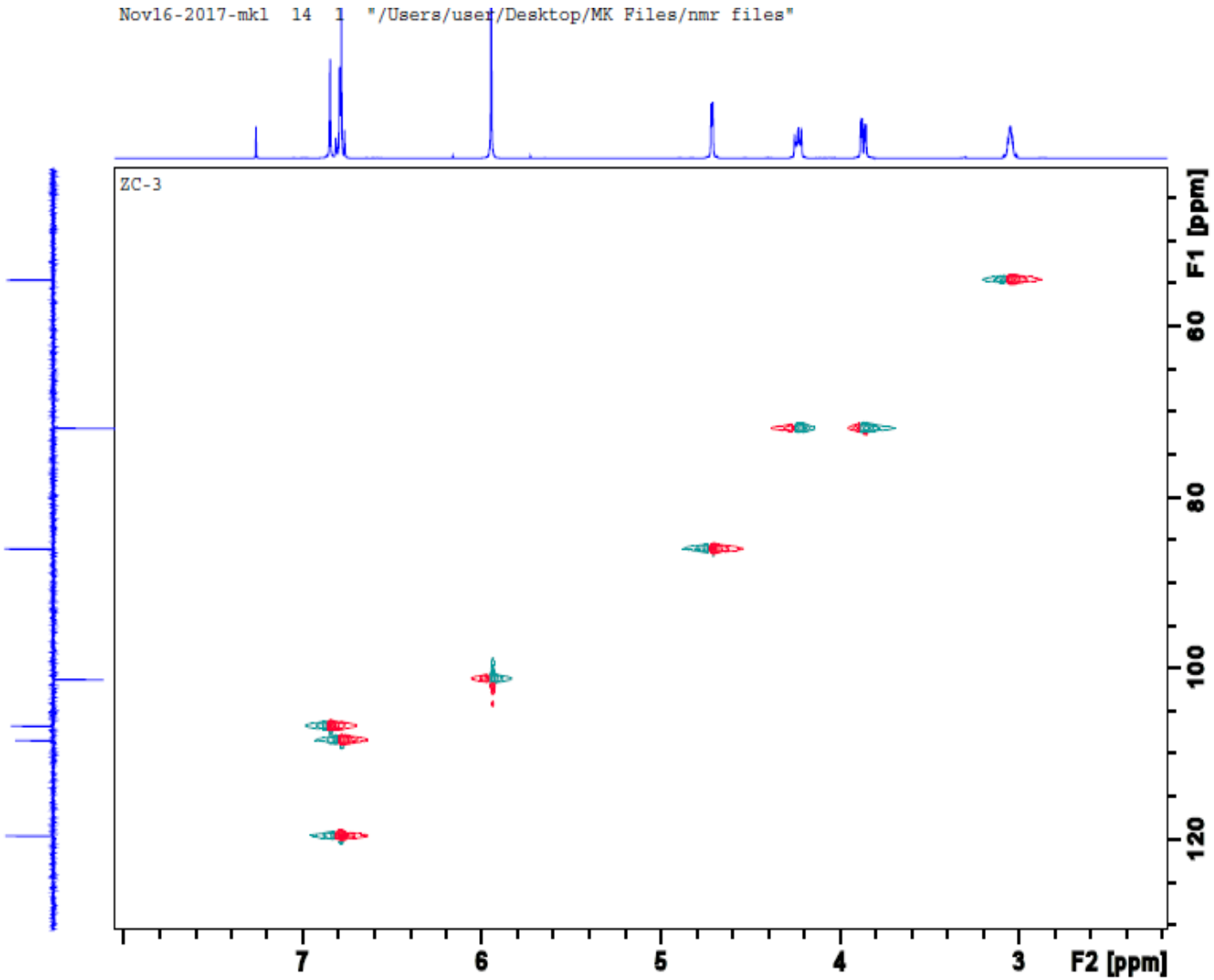
1.2: ^{13}C NMR of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



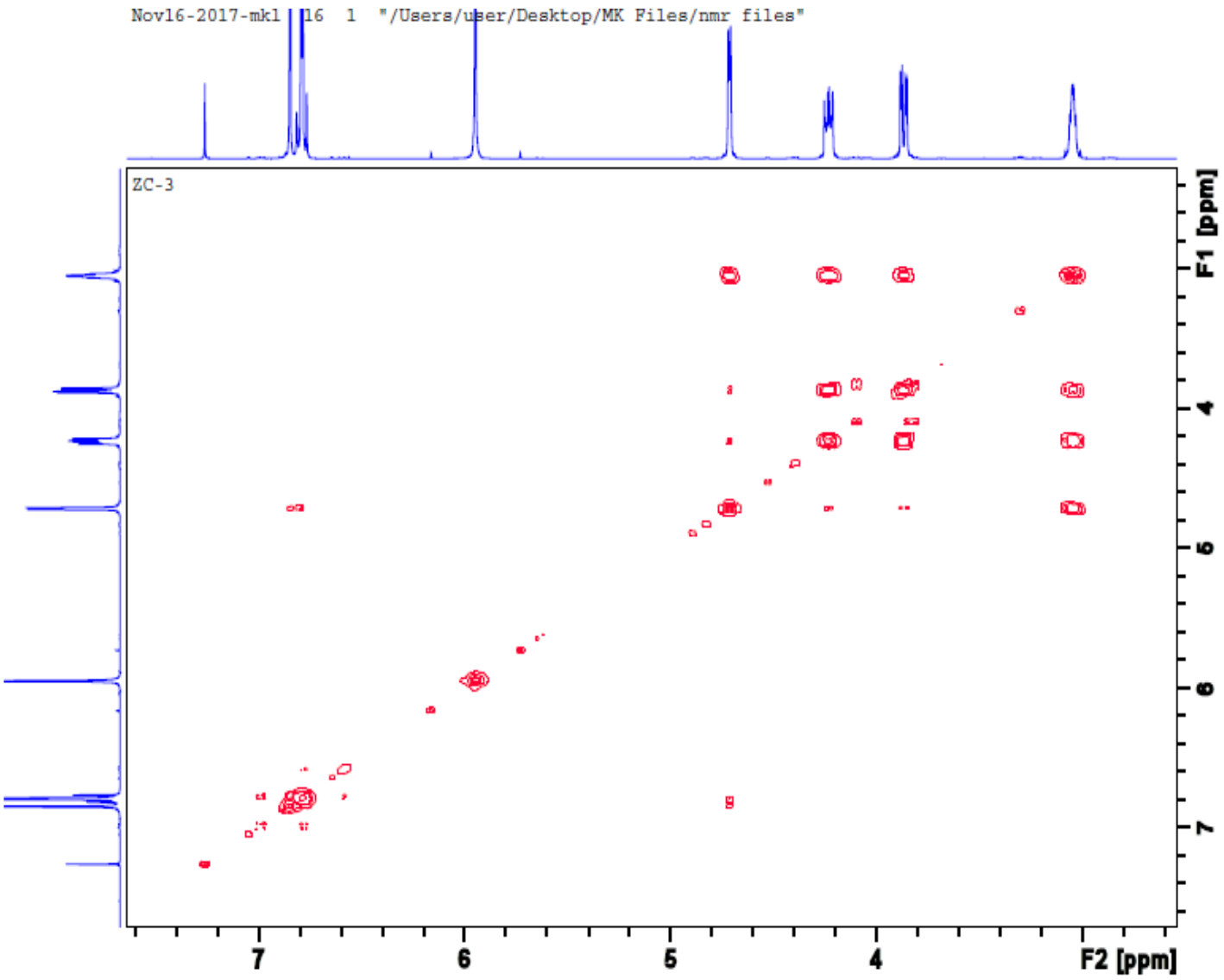
1.3: DEPT spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



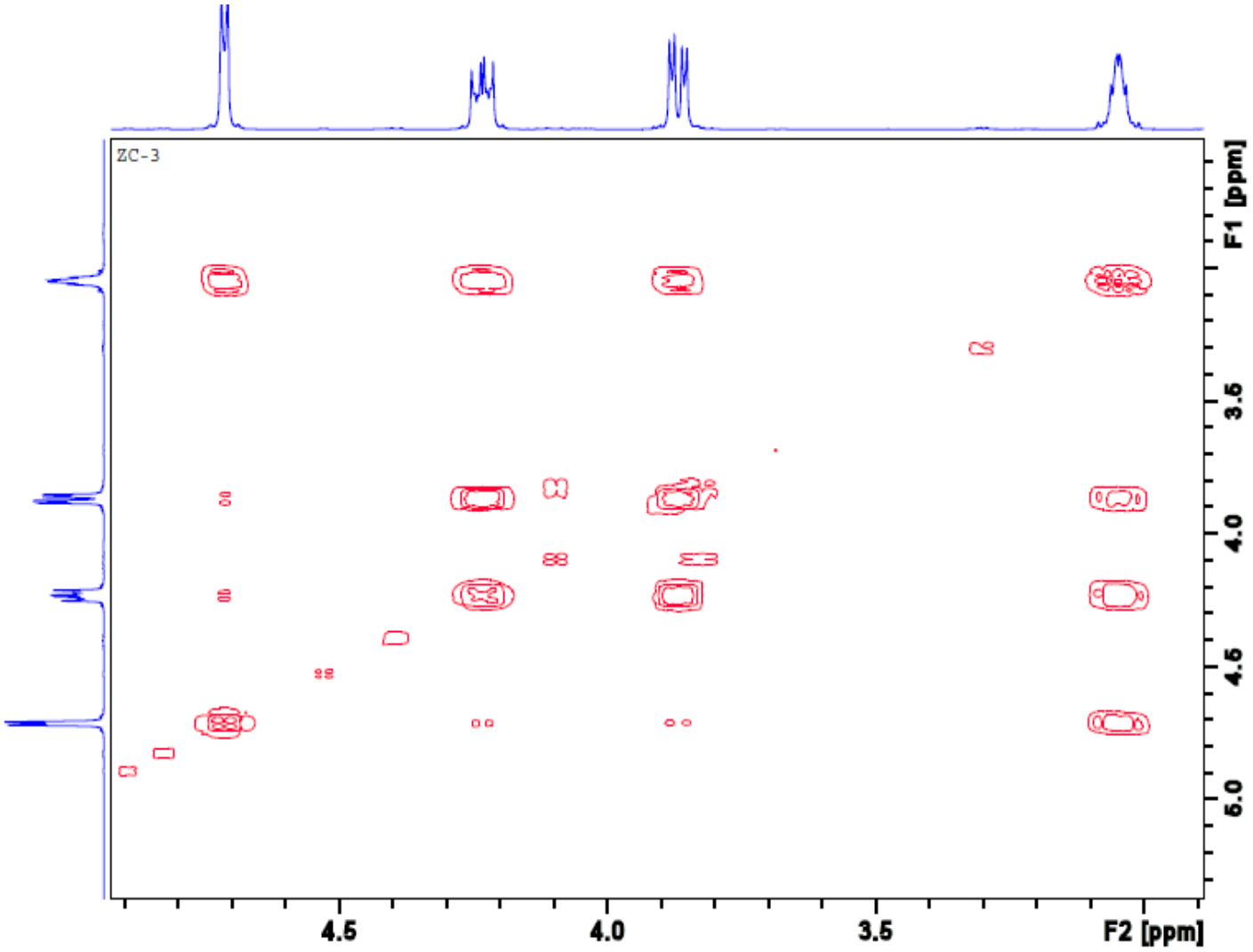
1.4: HSQC spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



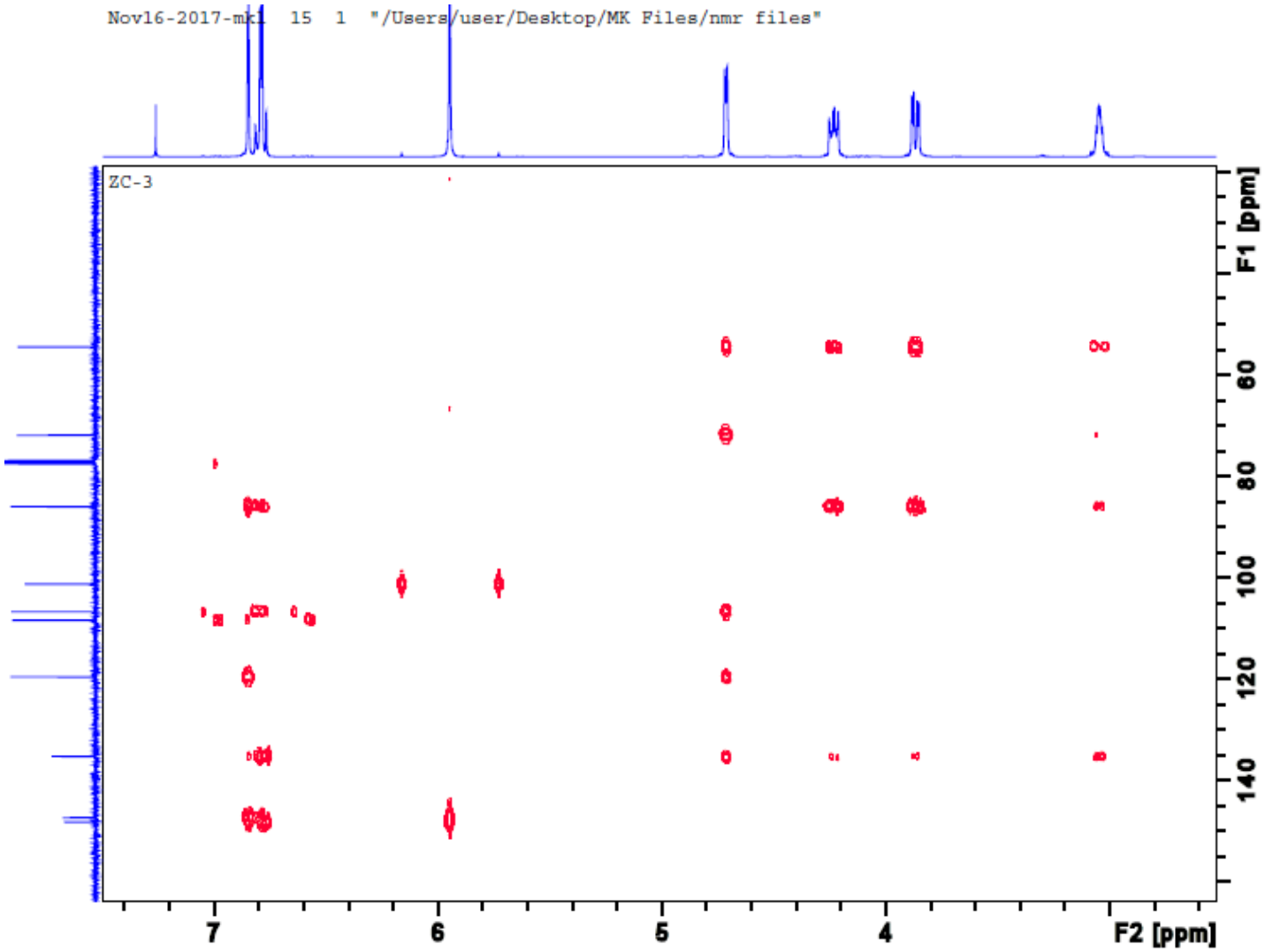
1.5: ^{1}H-^{1}H COSY spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



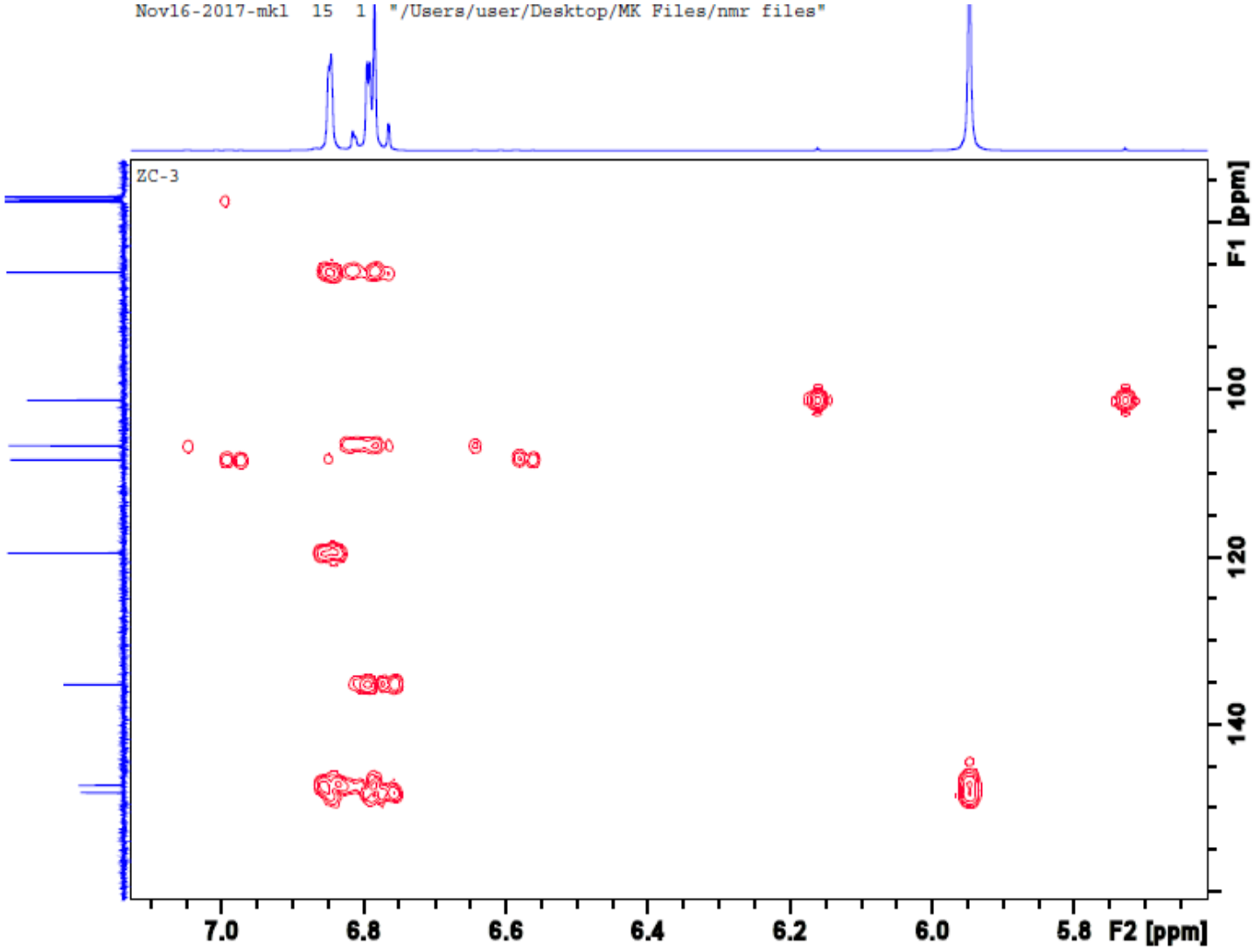
1.6: Expanded COSY spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



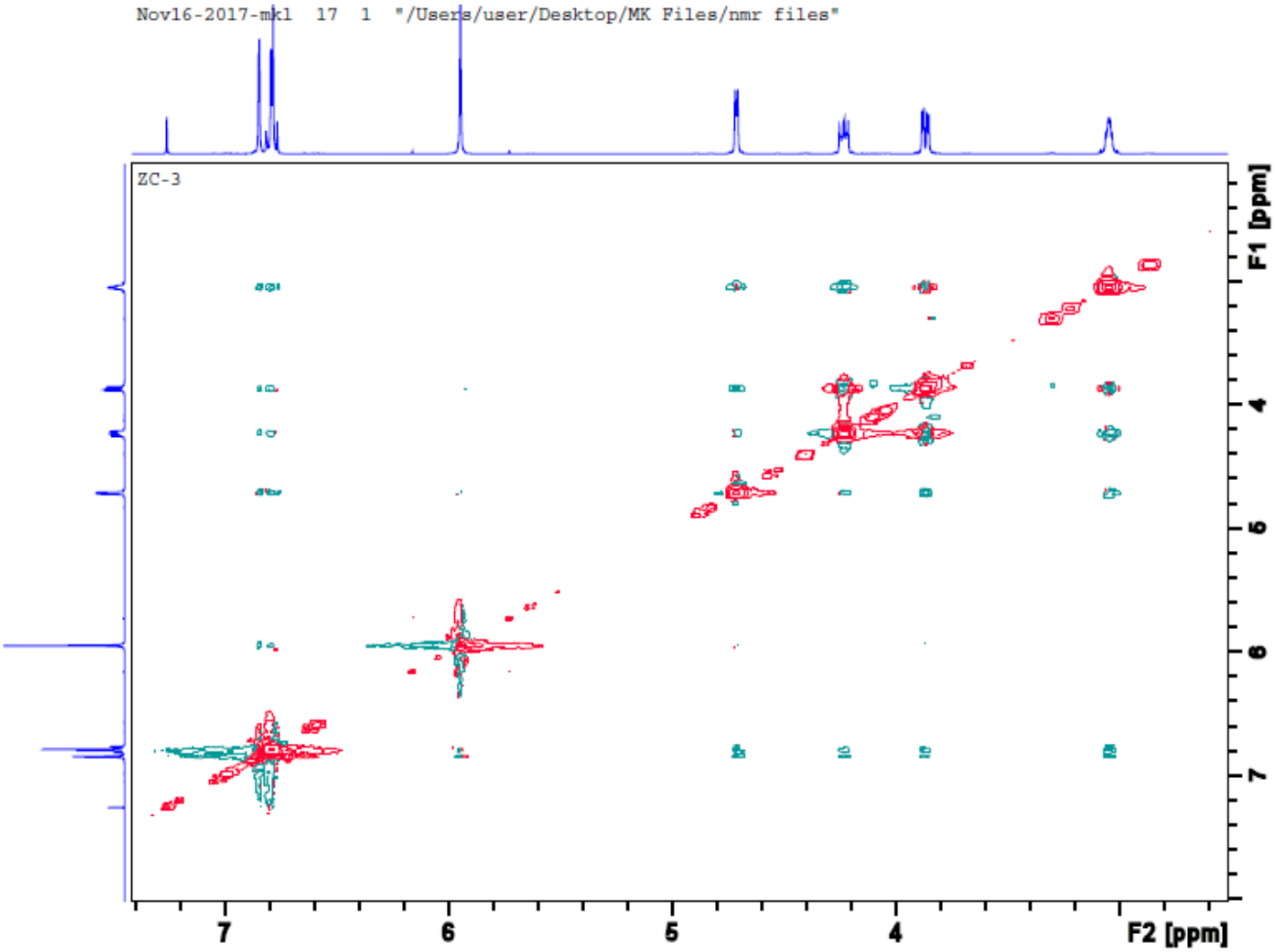
1.7: HMBC spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)



1.8: Expanded HMBC spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)

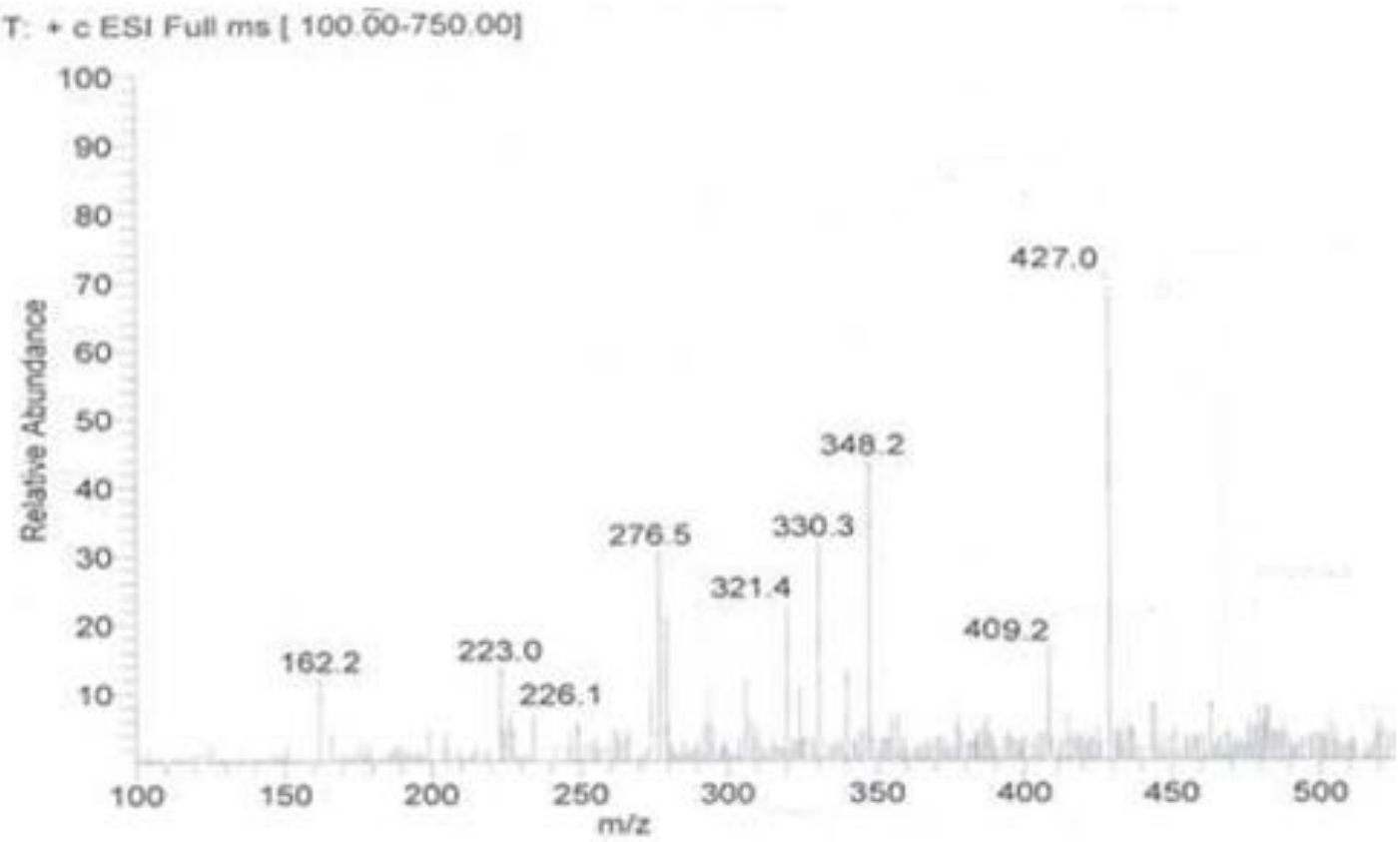


1.9: NOESY spectrum of compound (**7**); (2,3-epoxy-6,7-methylenedioxy coniferyl alcohol)

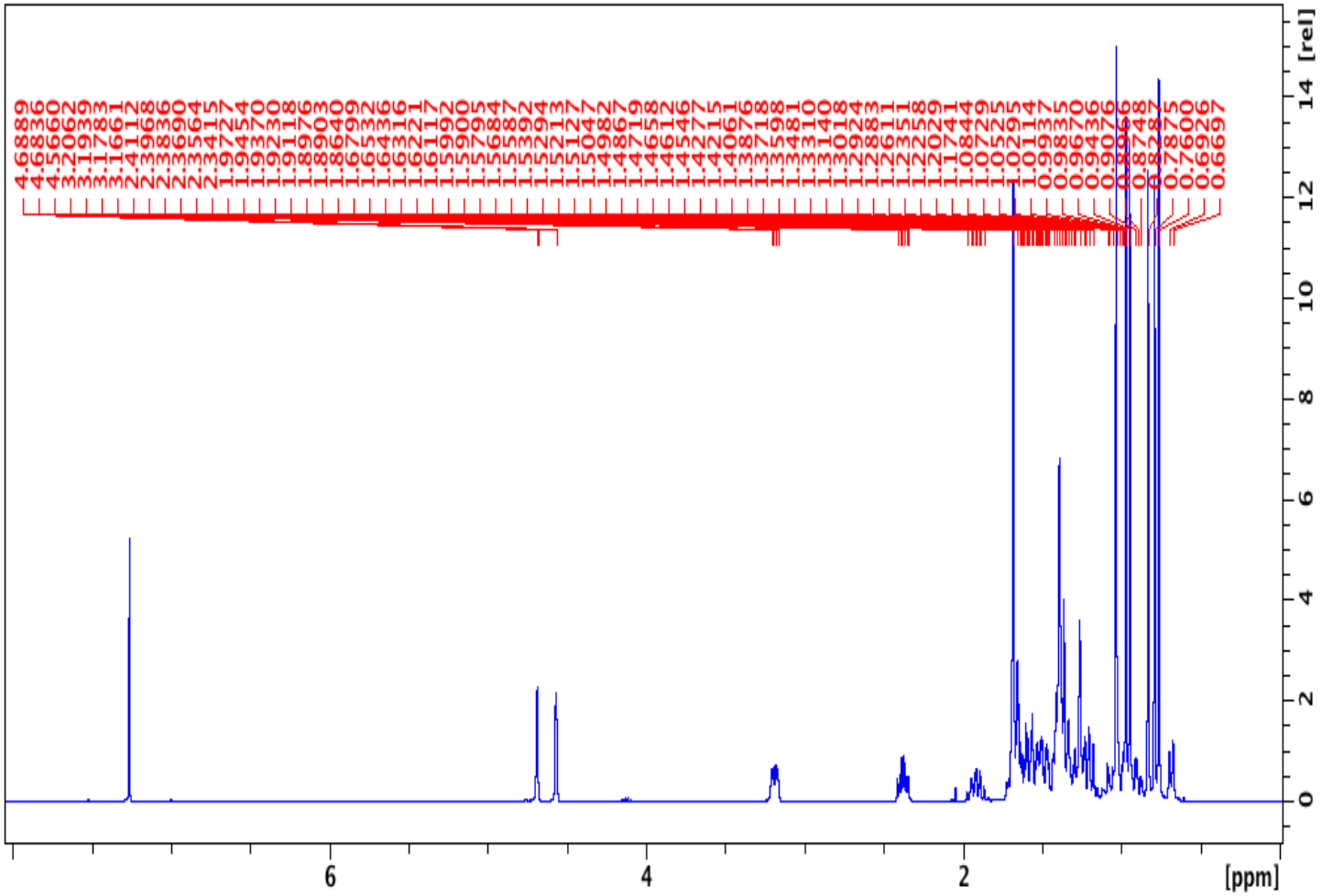


Appendix 2: Spectra for compound (37); Lupeol

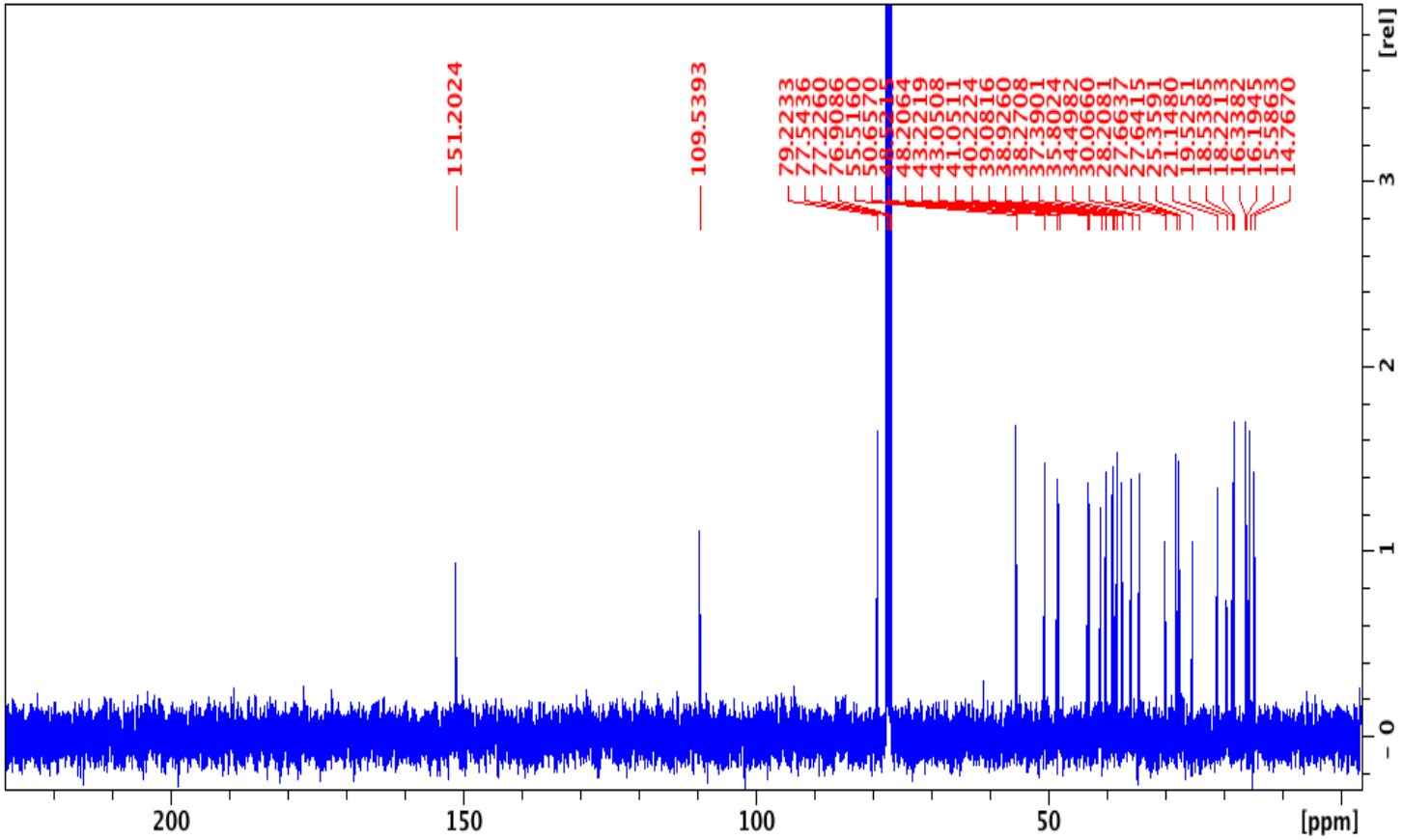
2.1: ESI-MS spectrum for compound (**37**); (lupeol)



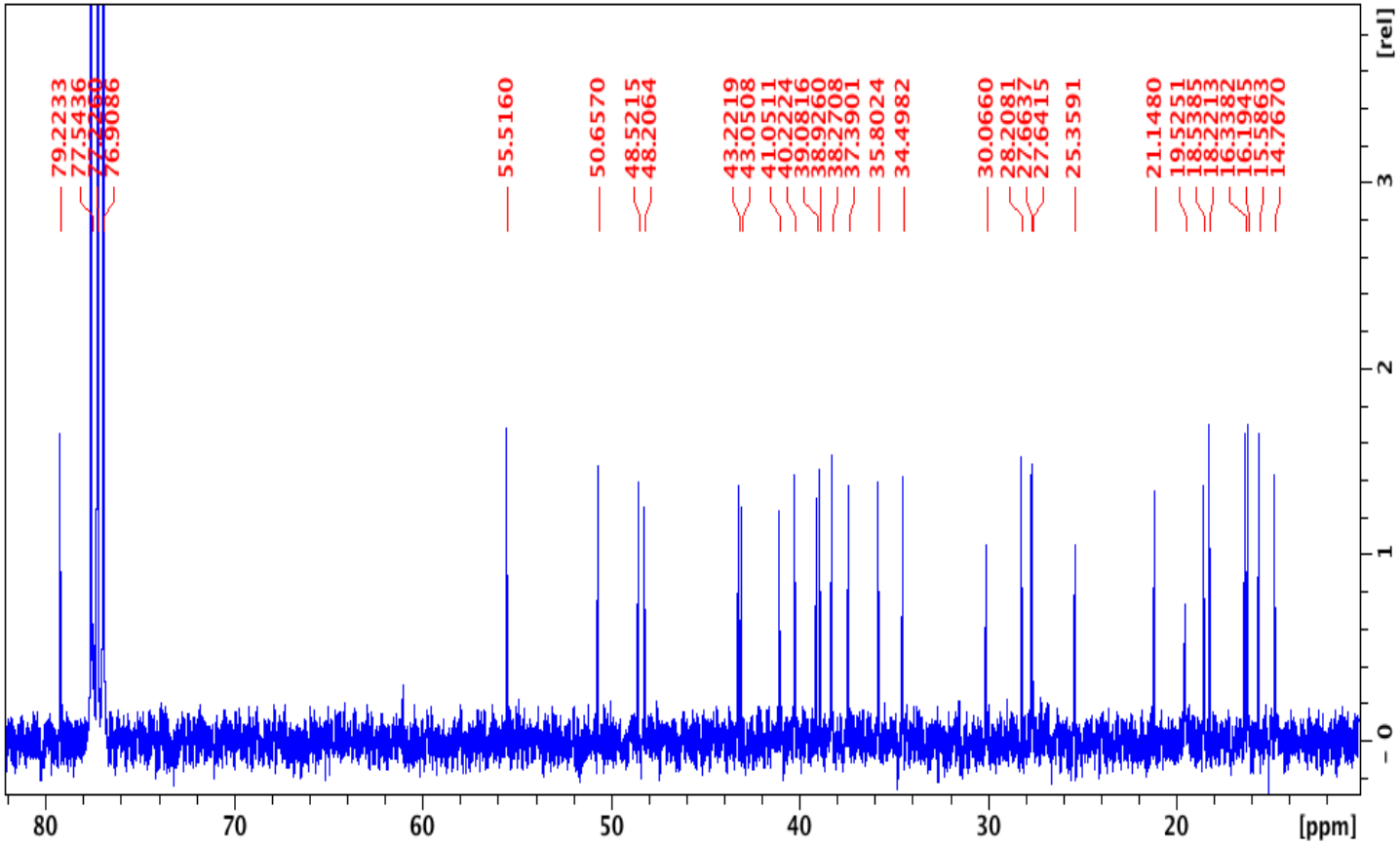
2.2: ^{1}H-^{1}H NMR spectrum for compound (**37**); (Lupeol)



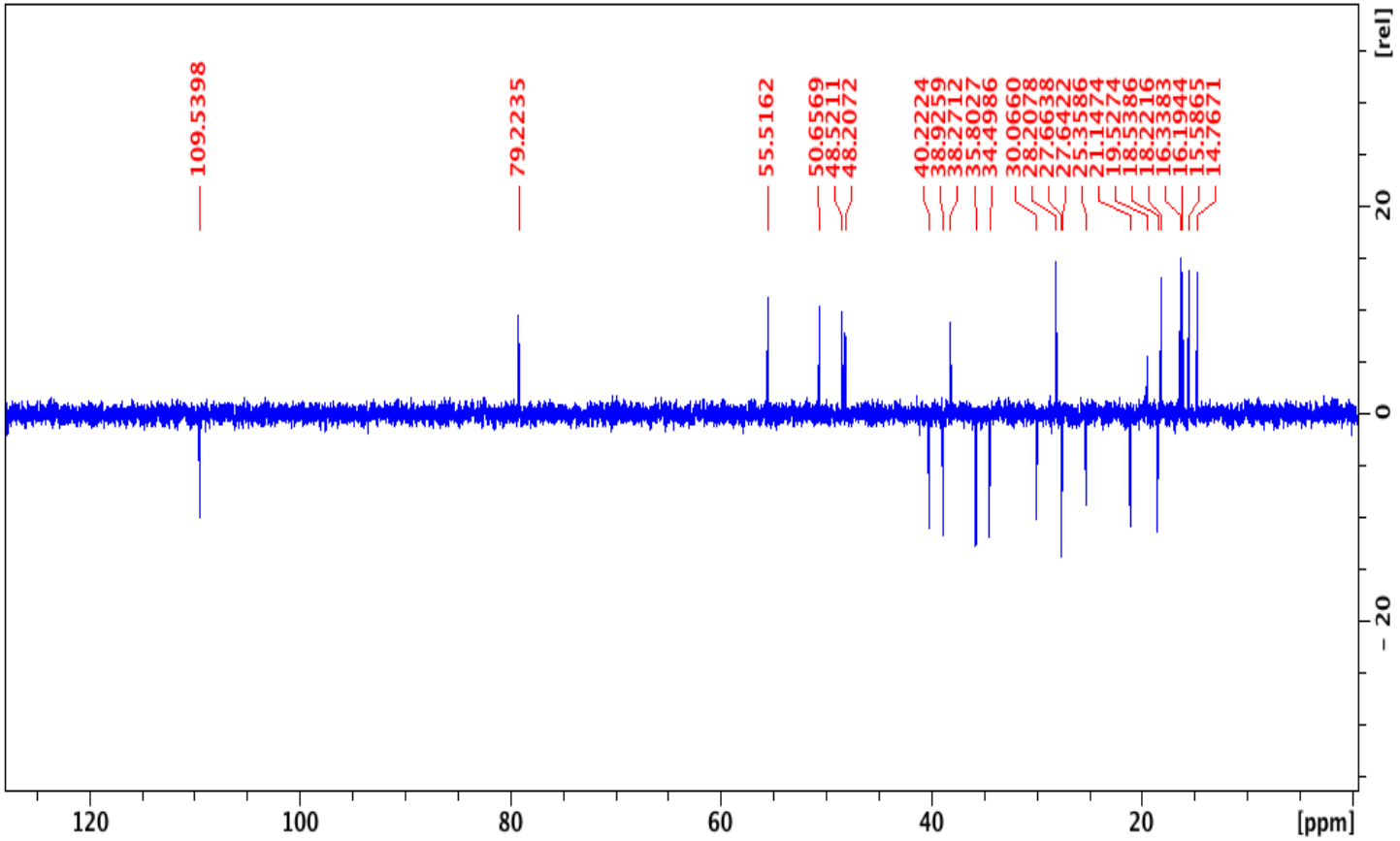
2.3: ^{13}C NMR spectrum for compound (**37**); (Lupeol)



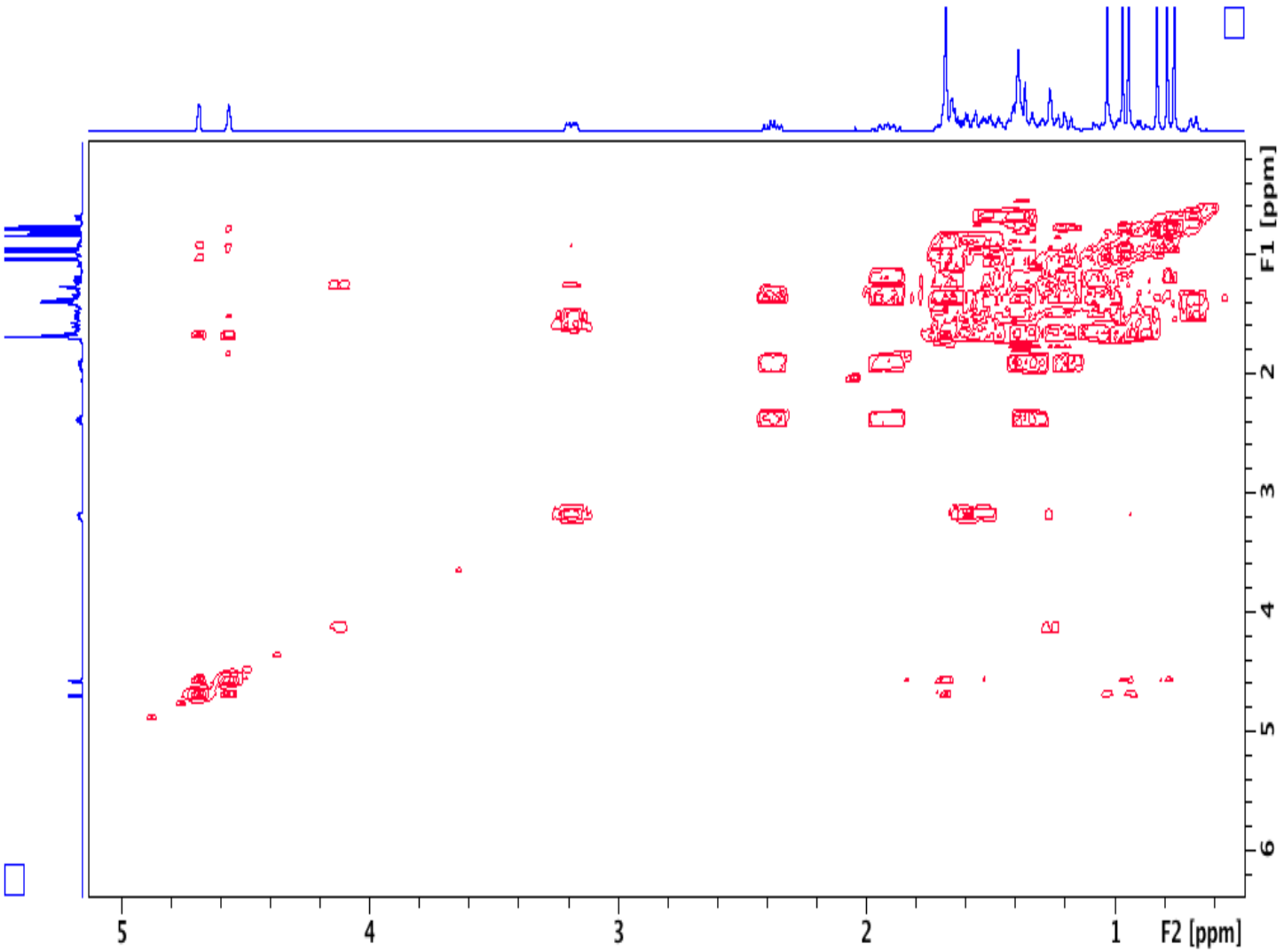
2.4: Expanded ^{13}C NMR spectrum for compound (**37**); (Lupeol)



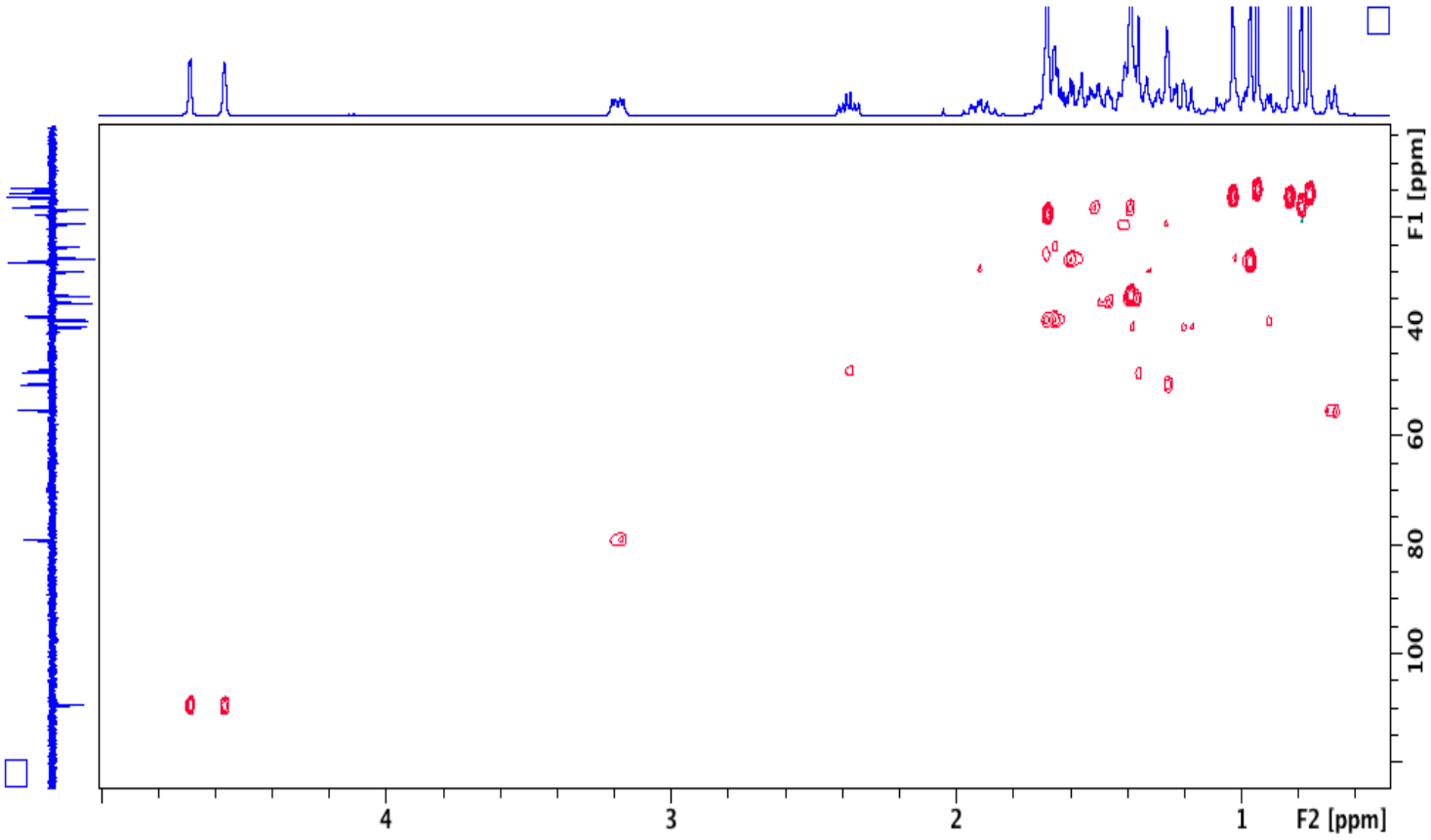
2.5: DEPT-135 spectrum for compound (**37**); (Lupeol)



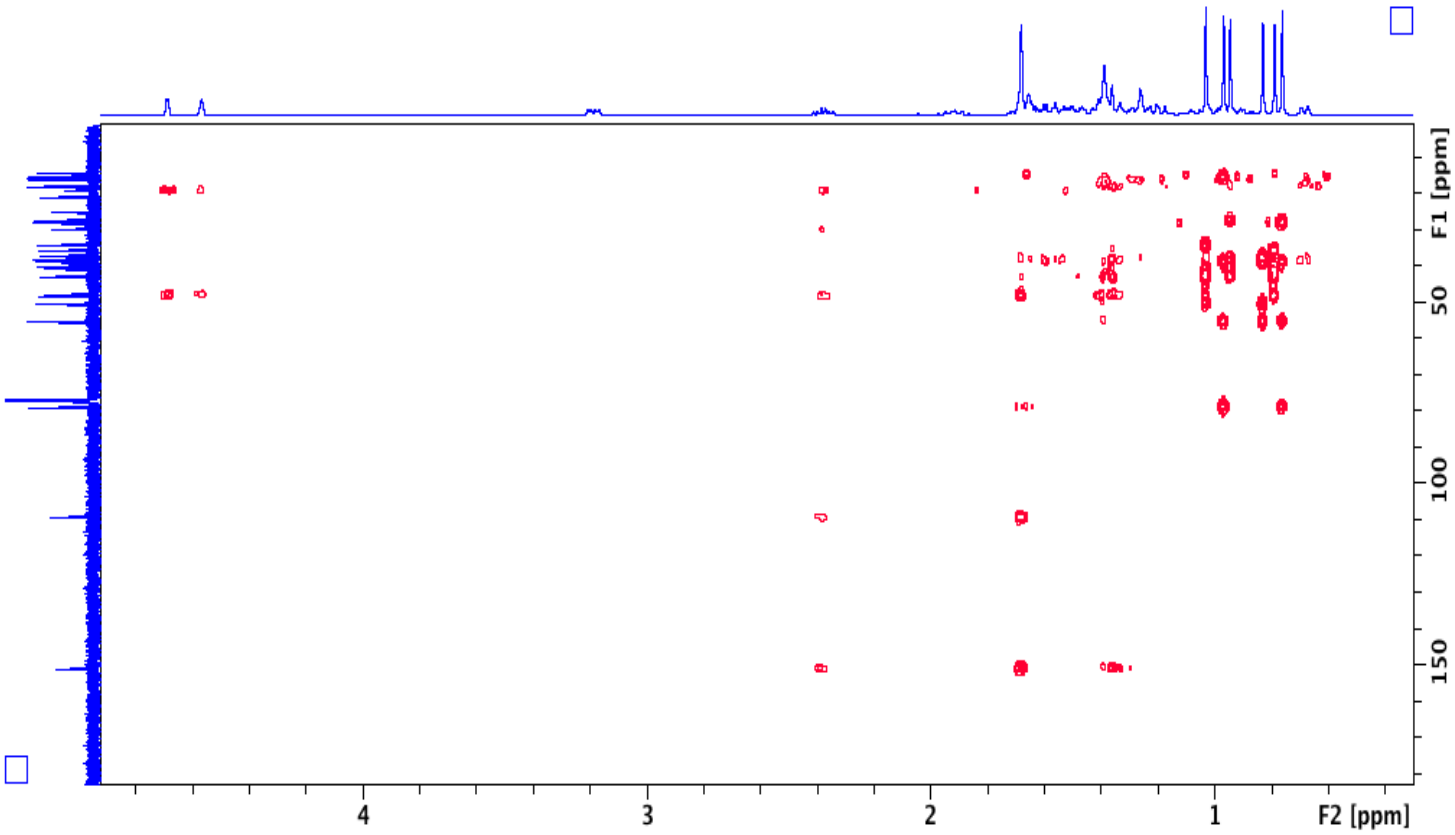
2.6: COSY spectrum for compound (**37**); (Lupeol)



2.7: HMBC spectrum for compound (**37**); (Lupeol)

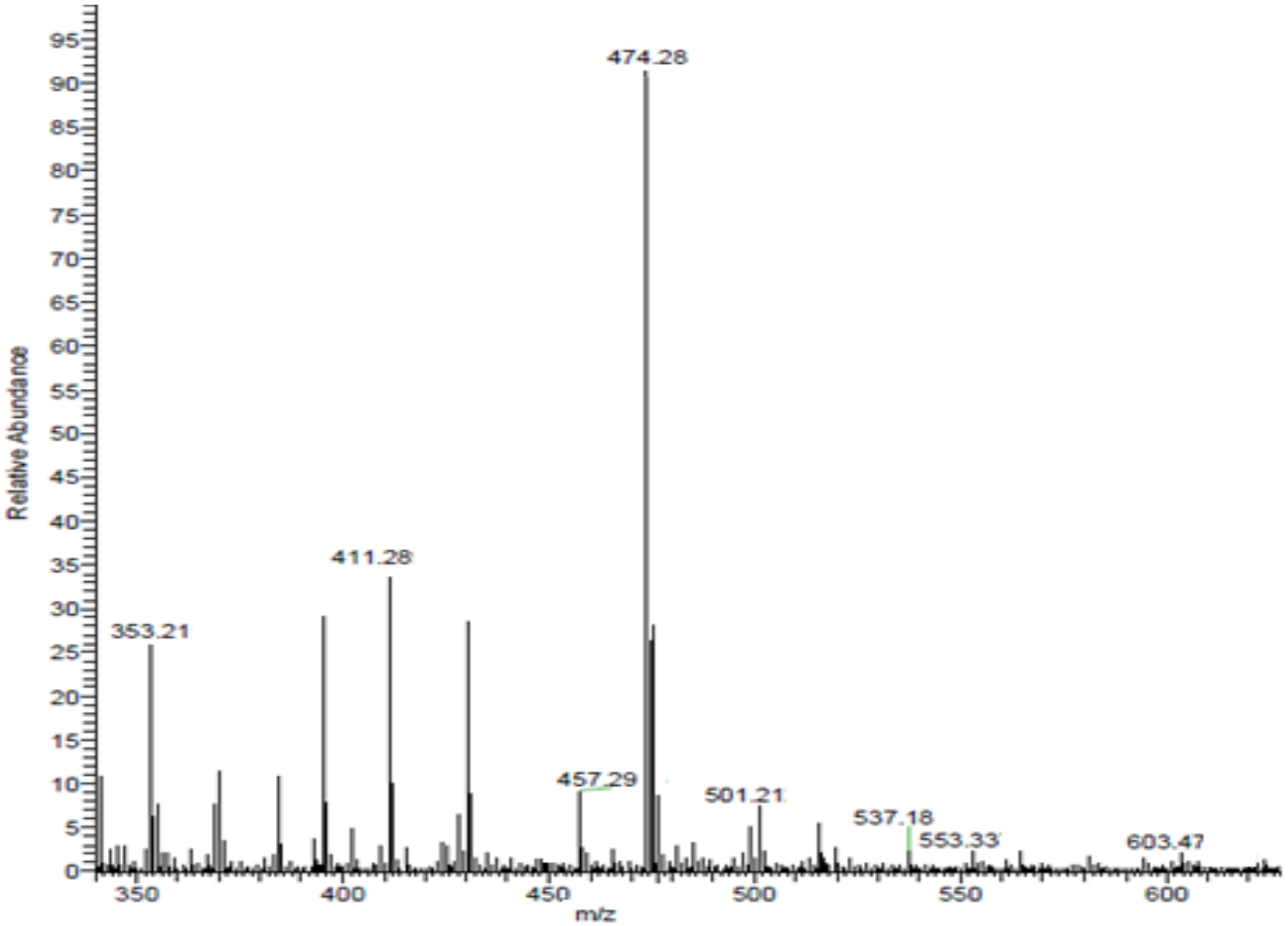


2.8: HSQC spectrum for compound (**37**); (Lupeol)

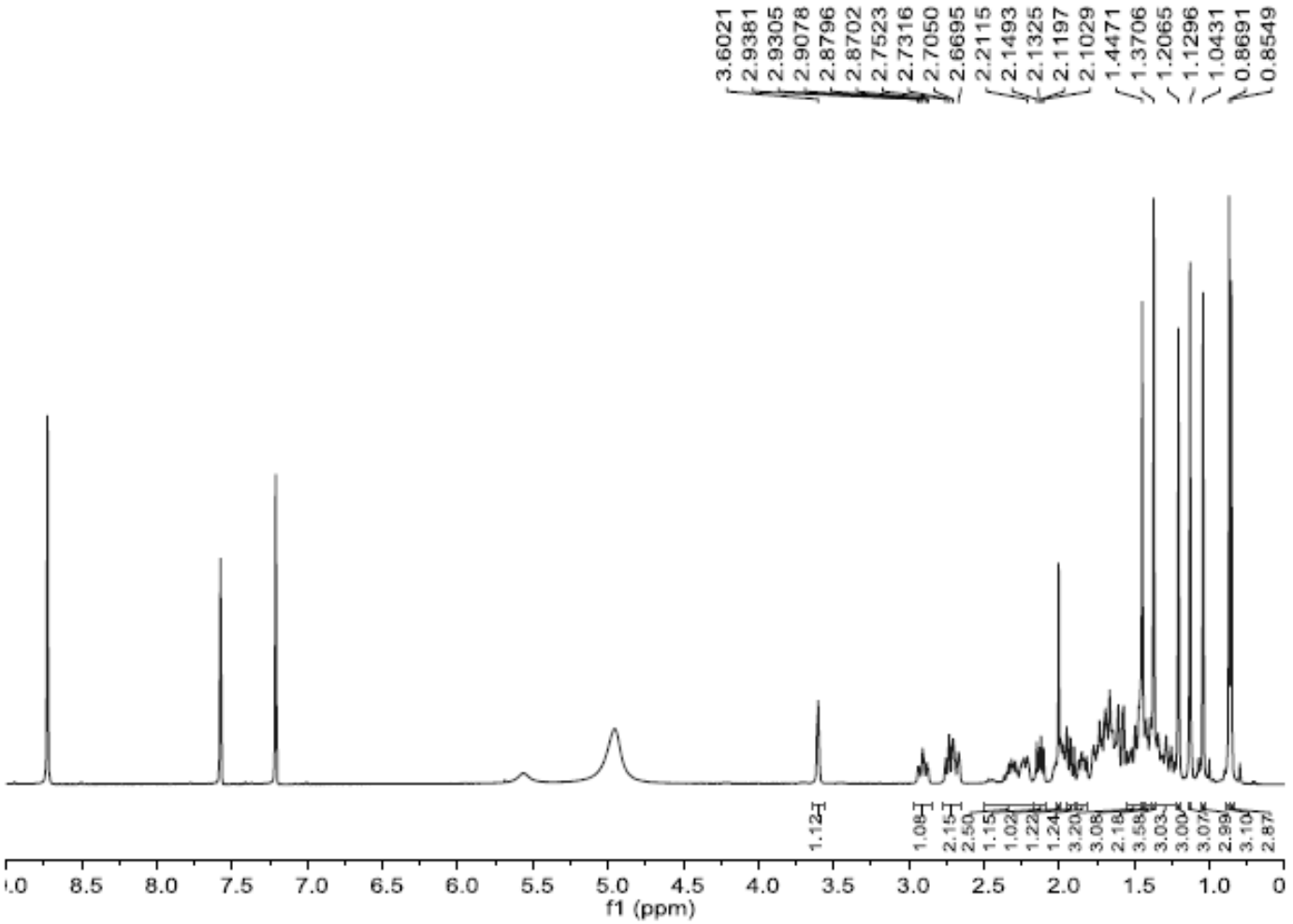


Appendix 3: Spectra for compound (44); 3α,20-dihydroxy-28-lupanoic acid

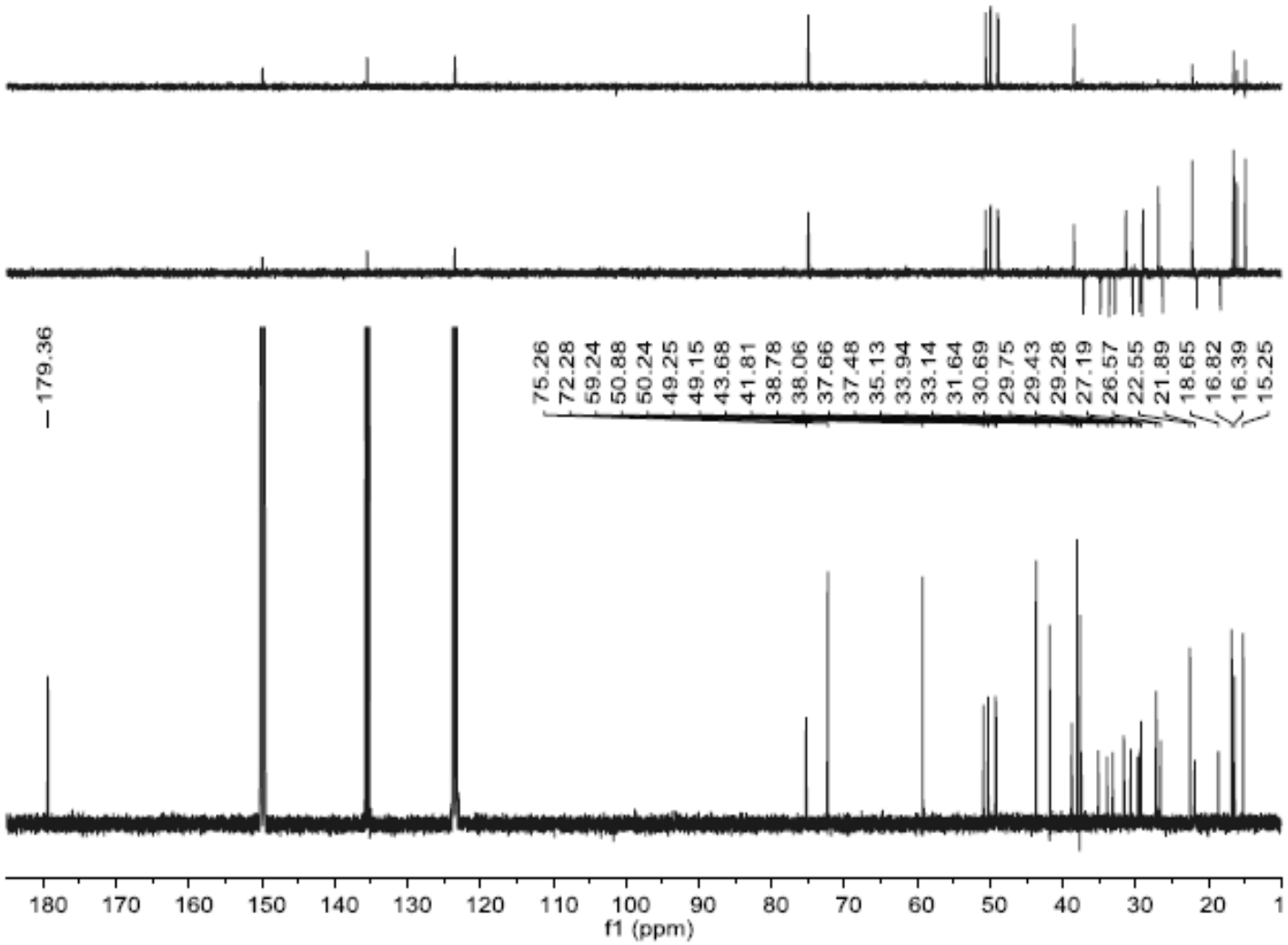
3.1: ESI-MS spectrum for compound (**44**); (3α,20-dihydroxy-28-lupanoic acid)



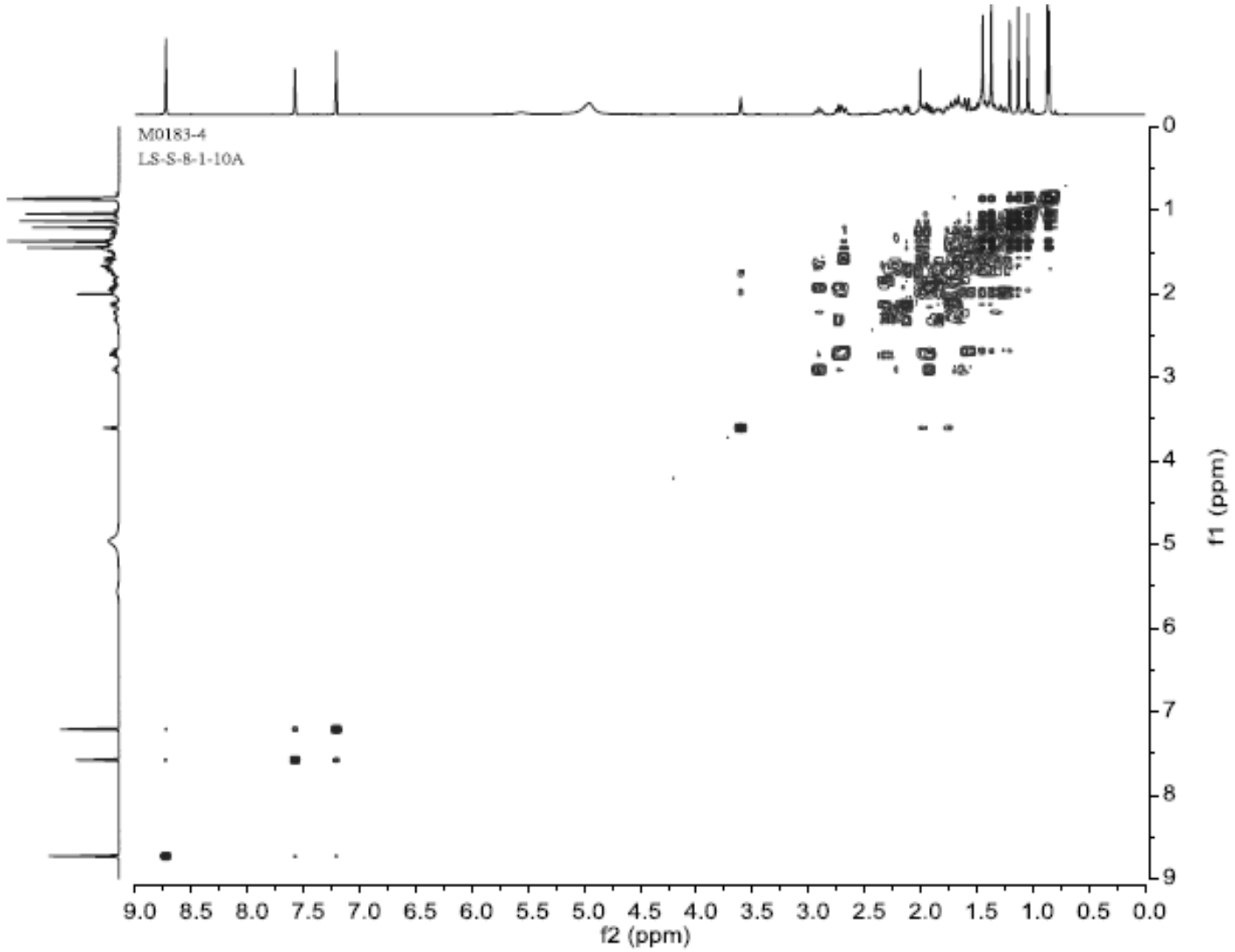
3.2: ^{1}H NMR spectrum for compound (**44**); (3α,20-dihydroxy-28-lupanoic acid)



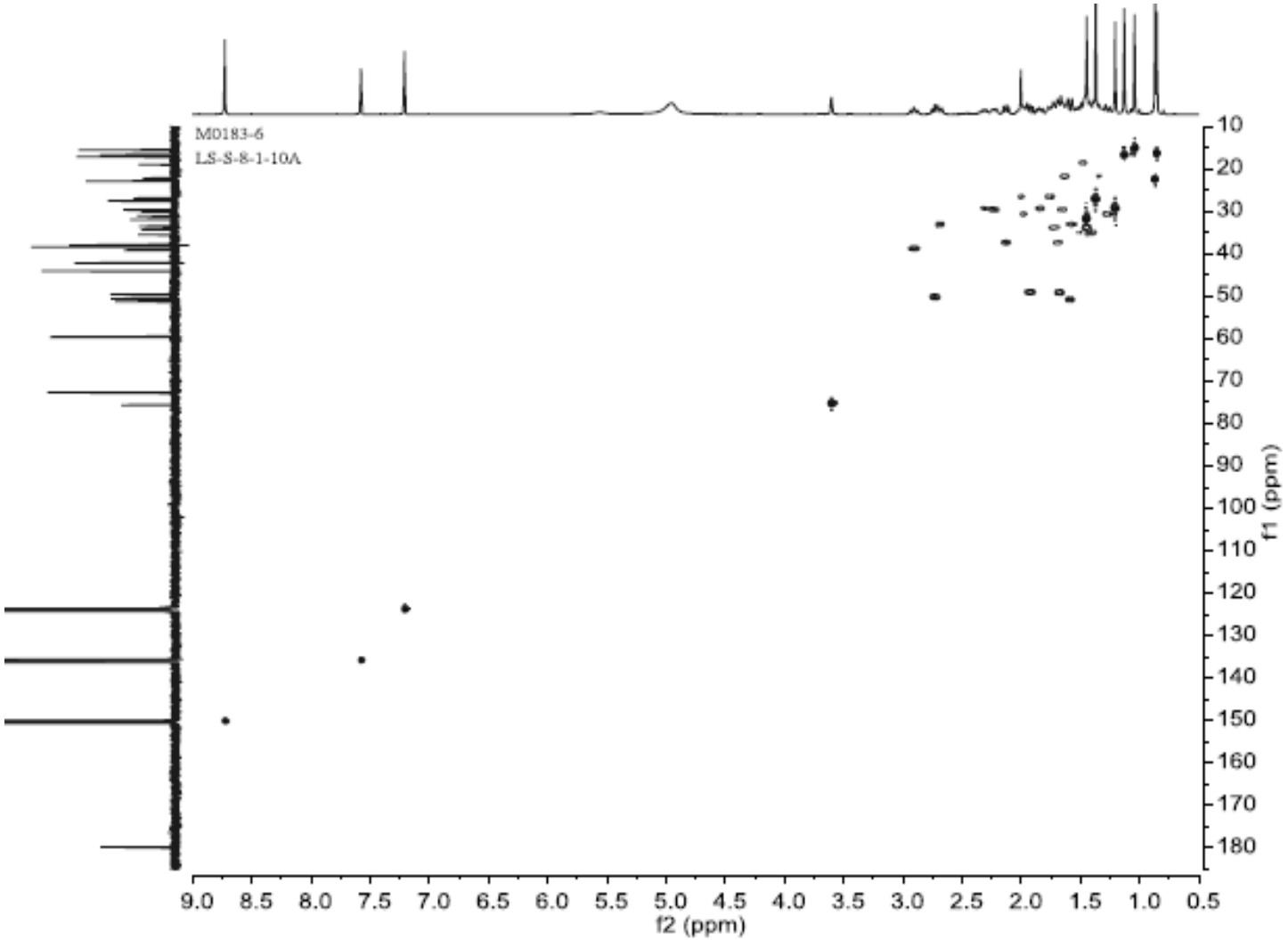
3.3: ^{13}C NMR and DEPT-135 spectra for compound (**44**); (3α,20-dihydroxy-28-lupanoic acid)



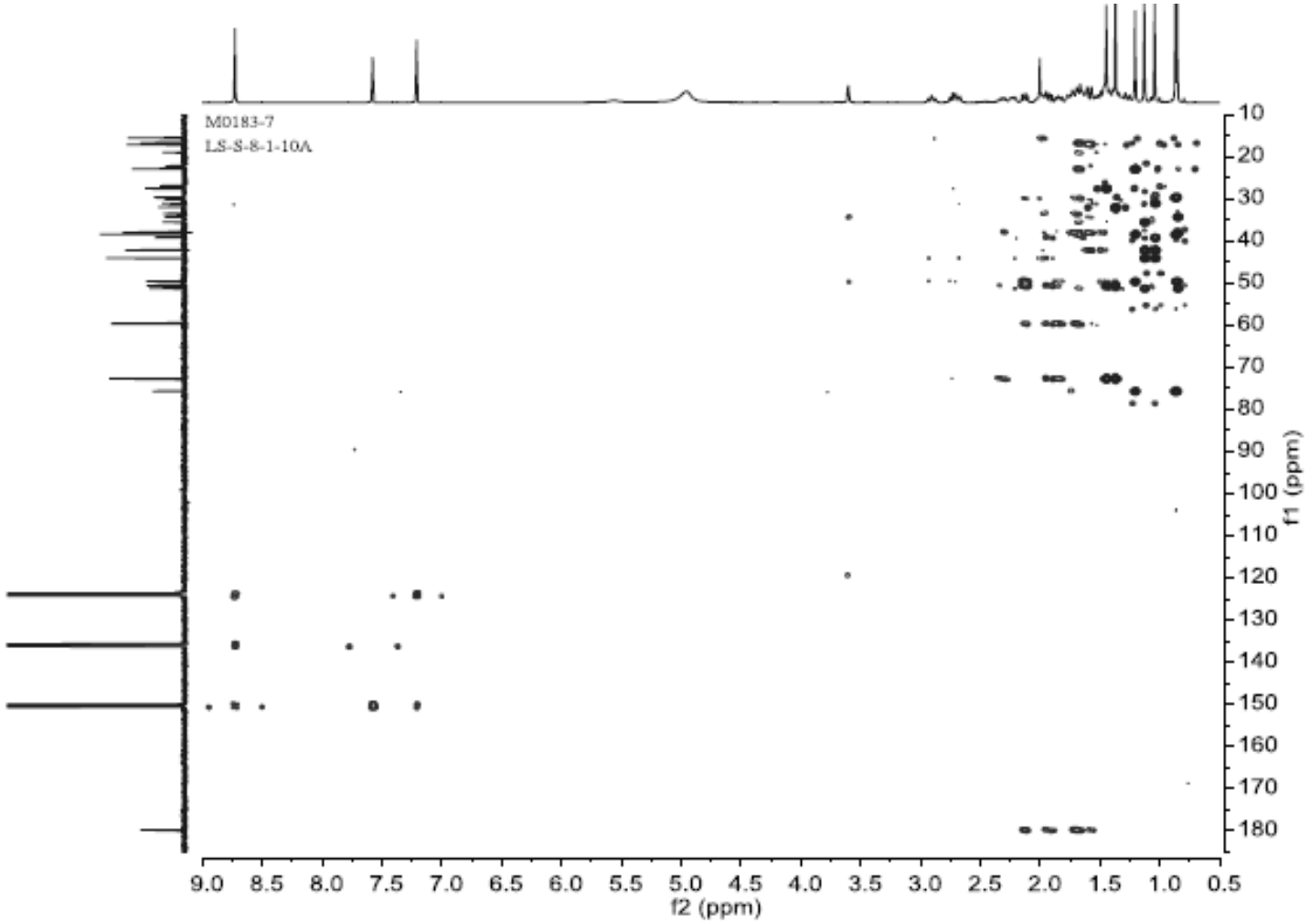
3.4: COSY spectrum for compound (**44**); (3α,20-dihydroxy-28-lupanoic acid)



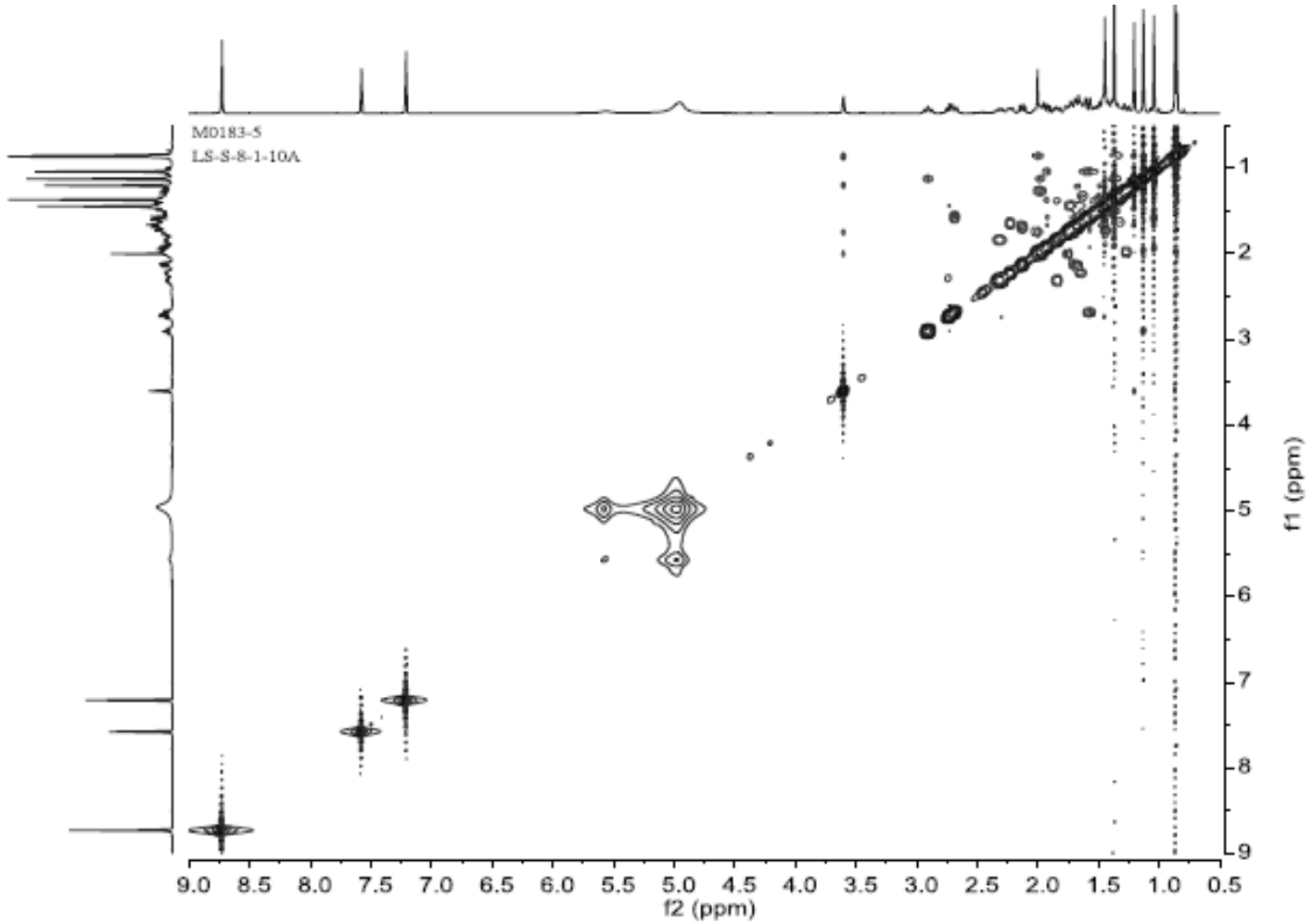
3.5: HSQC spectrum for compound (**44**); (3α,20-dihydroxy-28-lupanoic acid)



3.6: HMBC spectrum for compound (**44**); (3α, 20-dihydroxy-28-lupanoic acid)

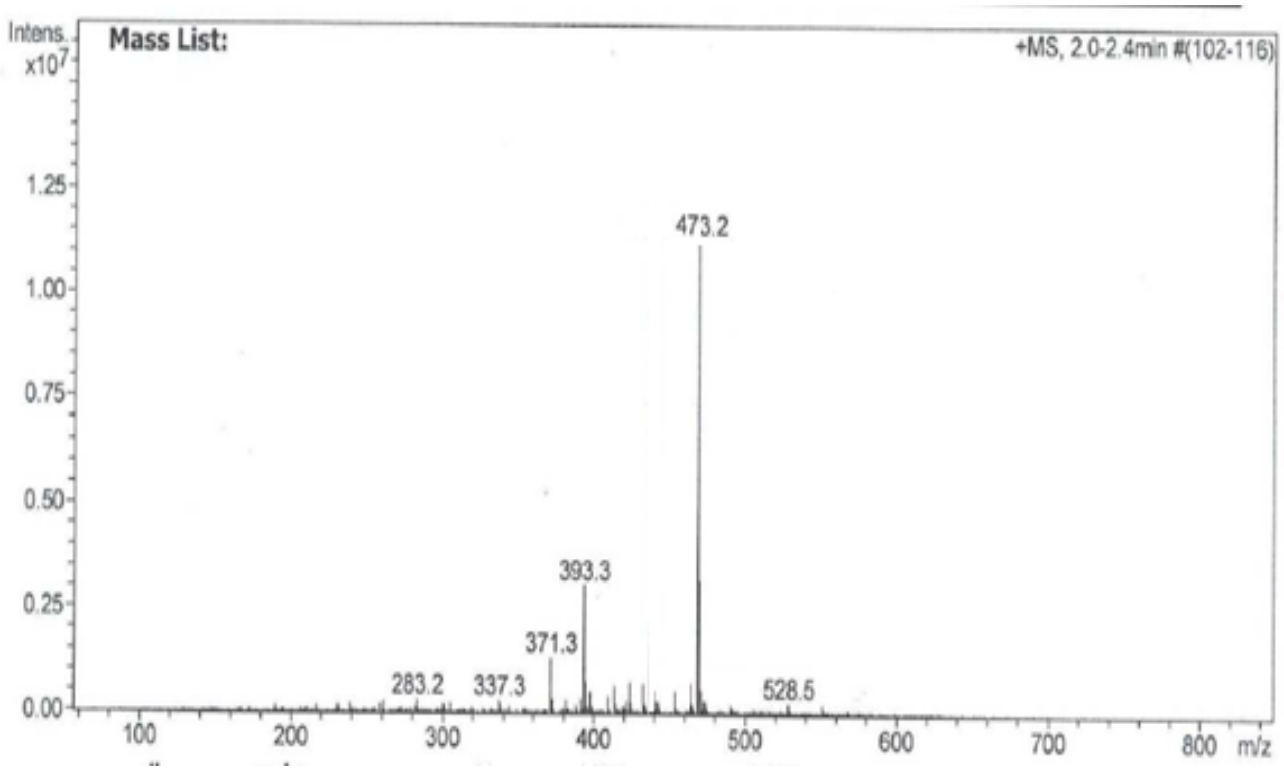


3.7: NOESY spectrum for compound (**44**); (3α,20-dihydroxy-28-lupanoic acid)



Appendix 4: Spectra for compound (45); (20-hydroxy-3-oxo-28-lupanoic acid

4.1: ESI-MS spectrum for compound (**45**); (20-hydroxy-3-oxo-28-lupanoic acid)



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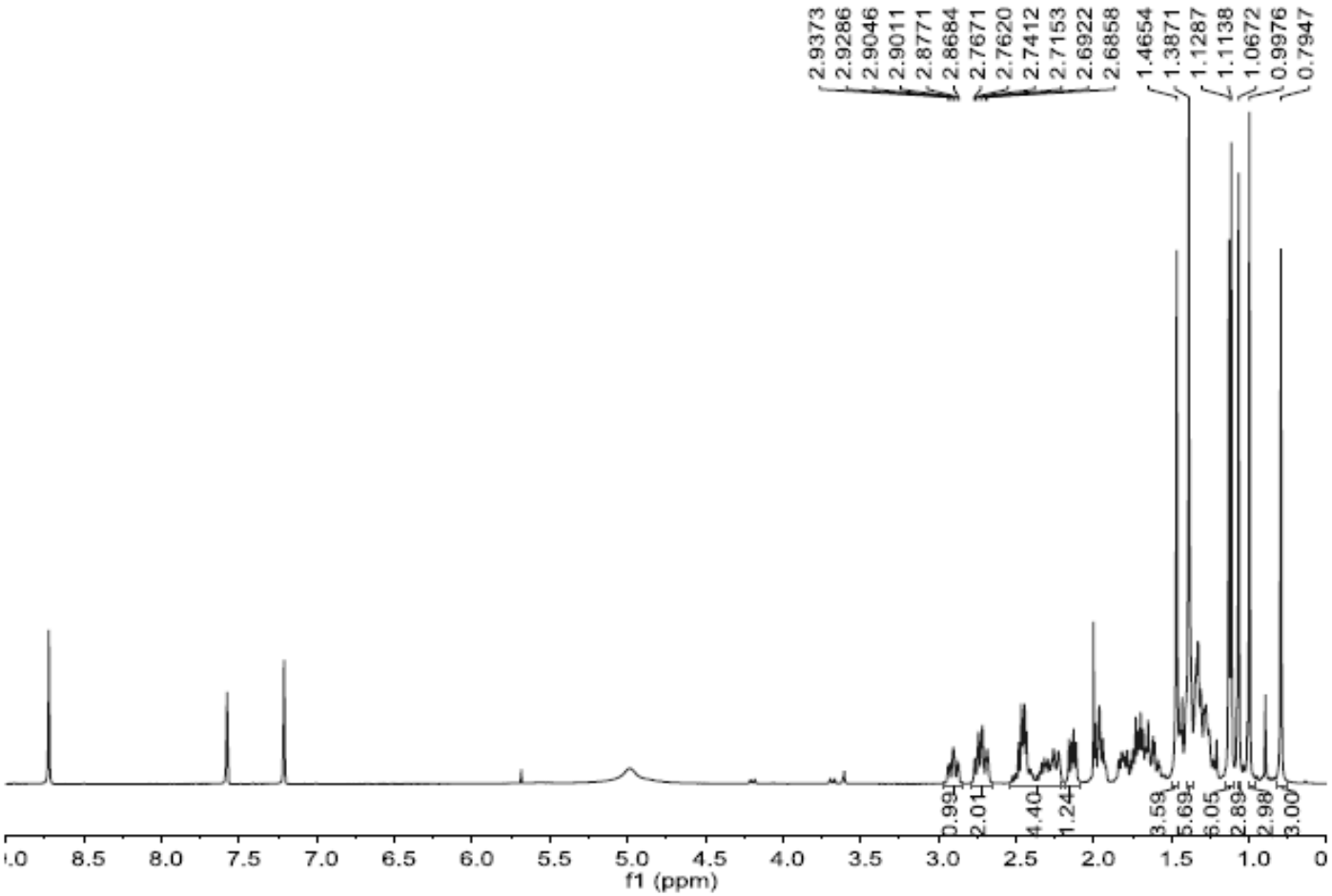
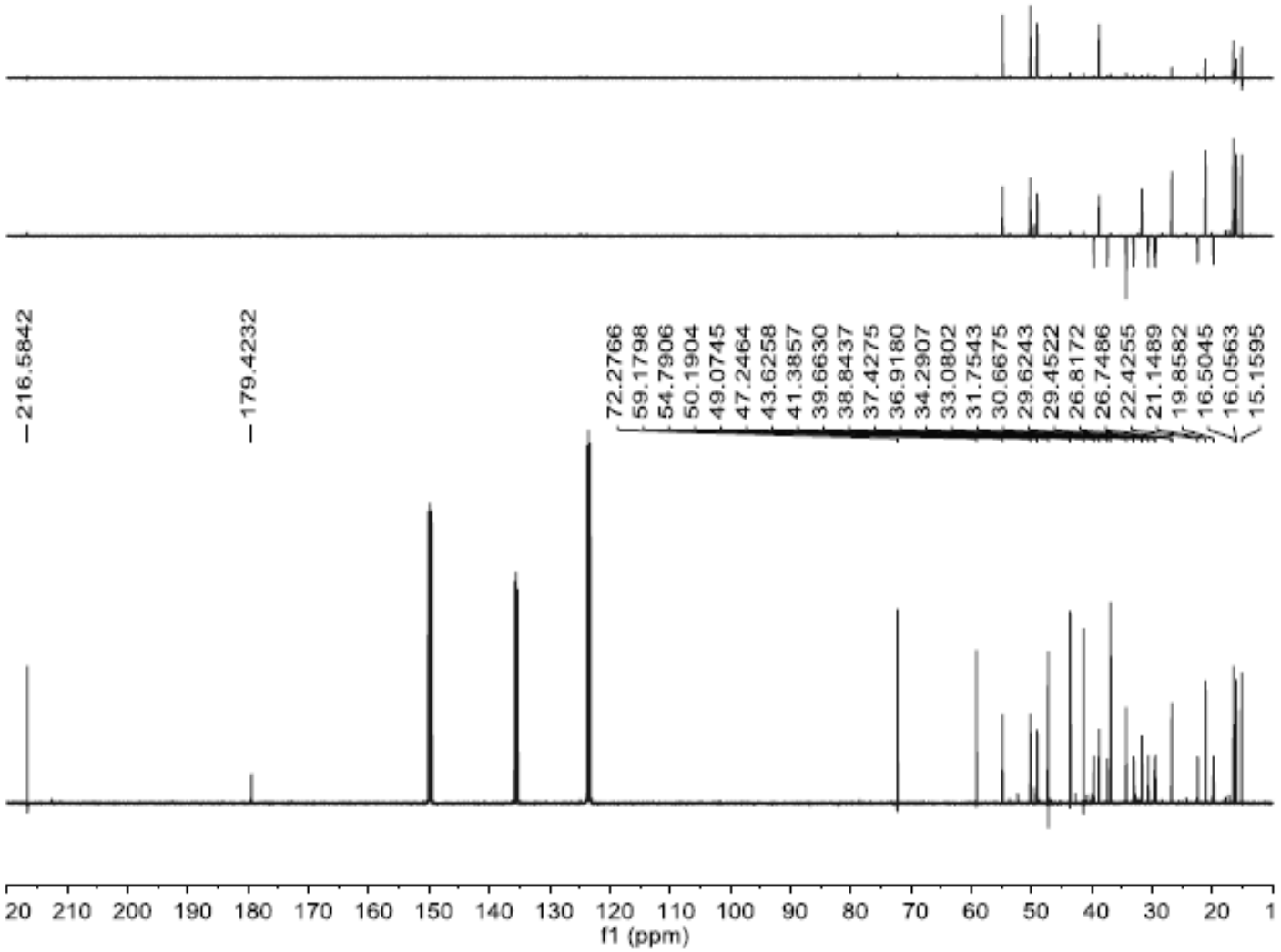
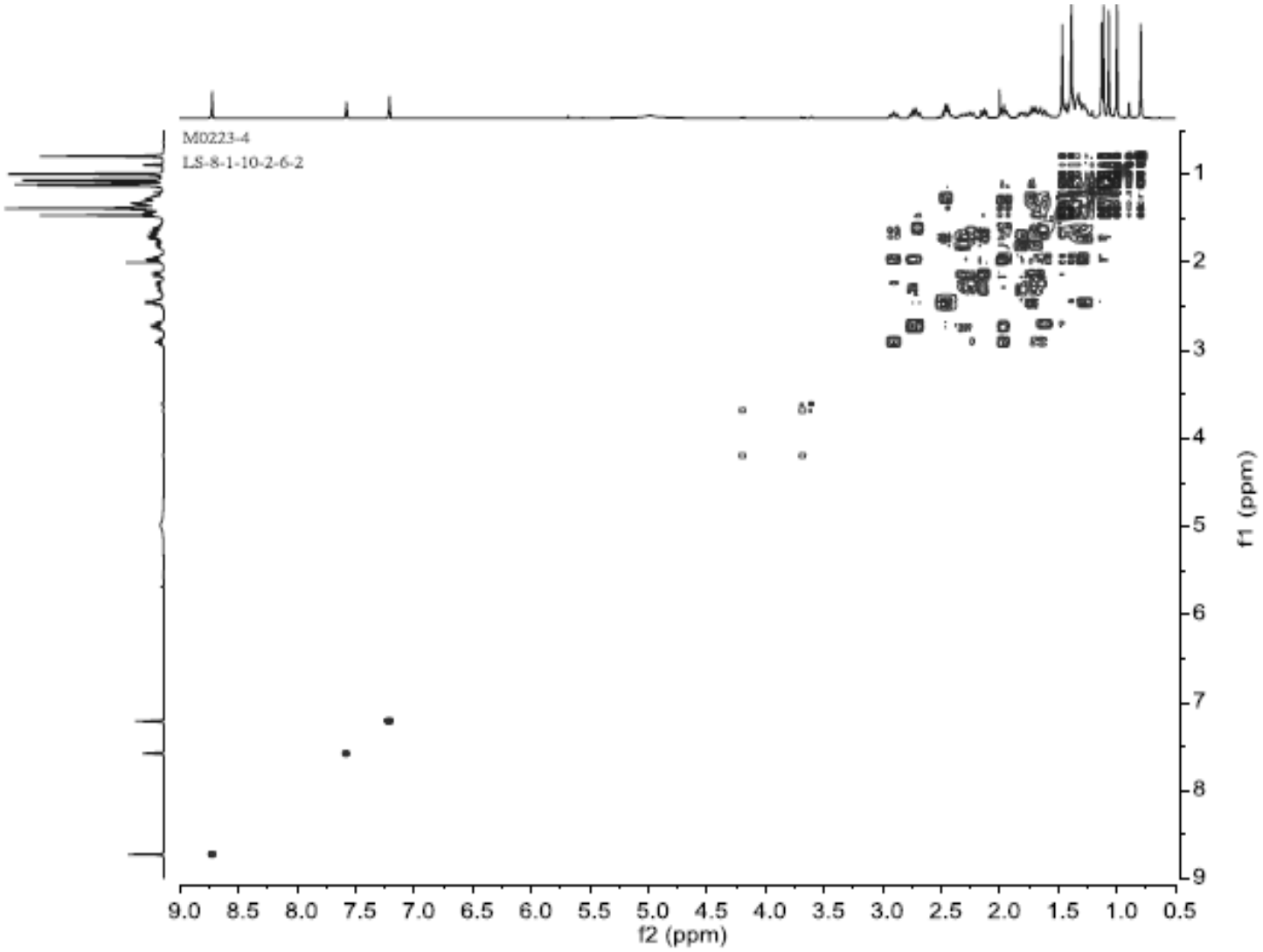
4.2: ^{1}H NMR spectrum for compound (**45**); (20-hydroxy-3-oxo-28-lupanoic acid)



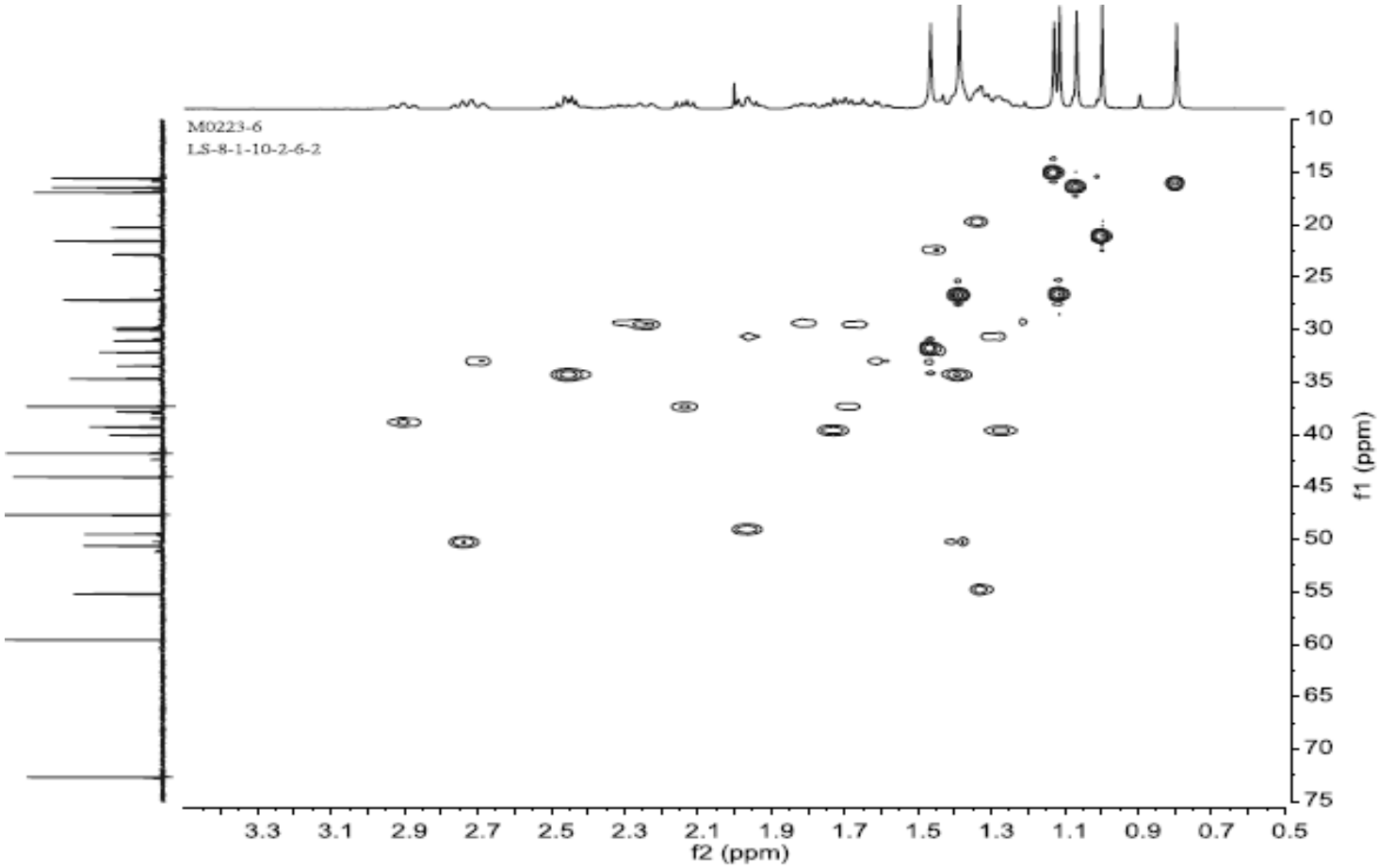
4.3: ^{13}C NMR and DEPT-135 spectra for compound (**45**); (20-hydroxy-3-oxo-28-lupanoic acid)



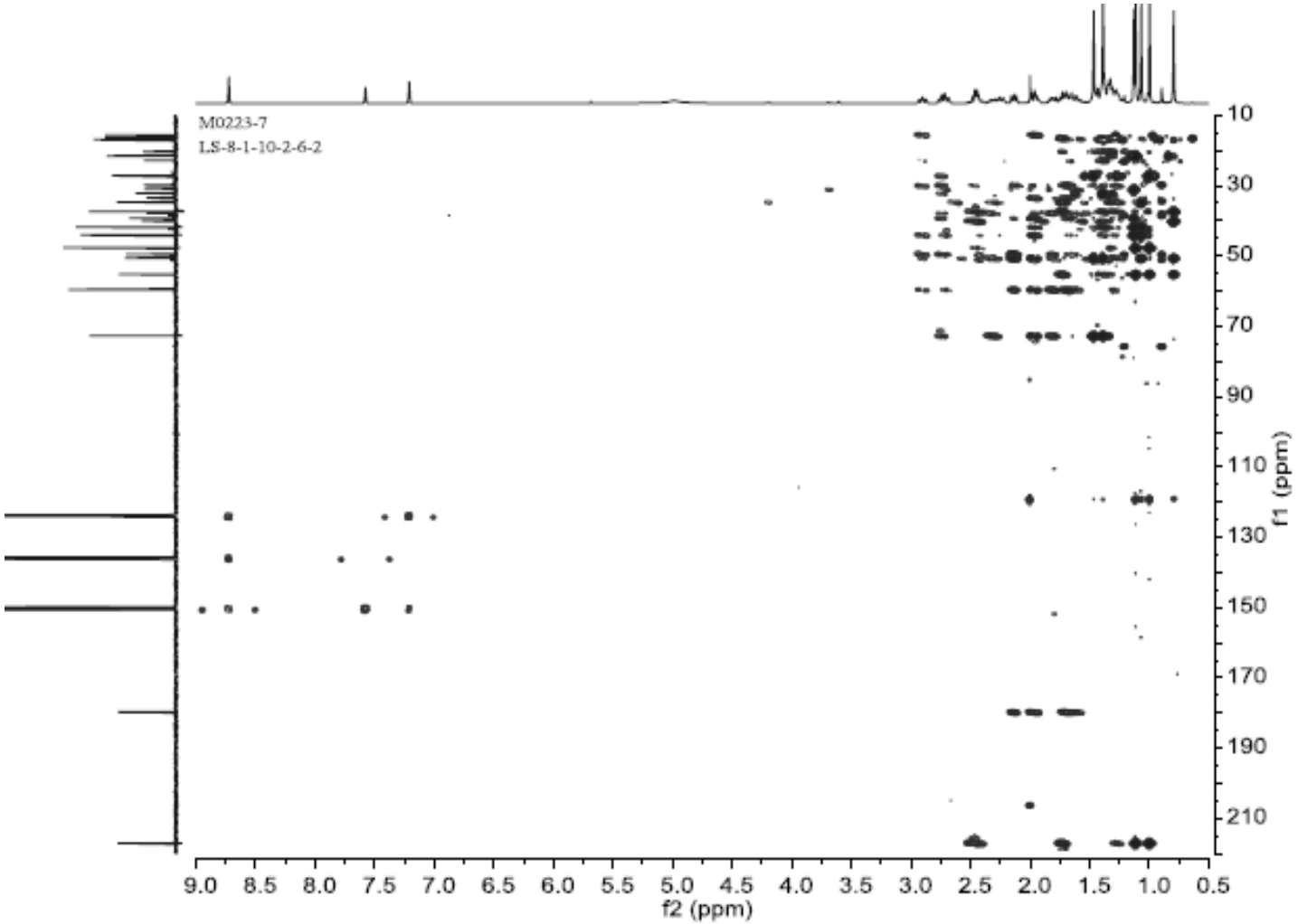
4.4: COSY spectrum for compound (**45**); (20-hydroxy-3-oxo-28-lupanoic acid)



4.5: HSQC spectrum for compound (**45**); (20-hydroxy-3-oxo-28-lupanoic acid)

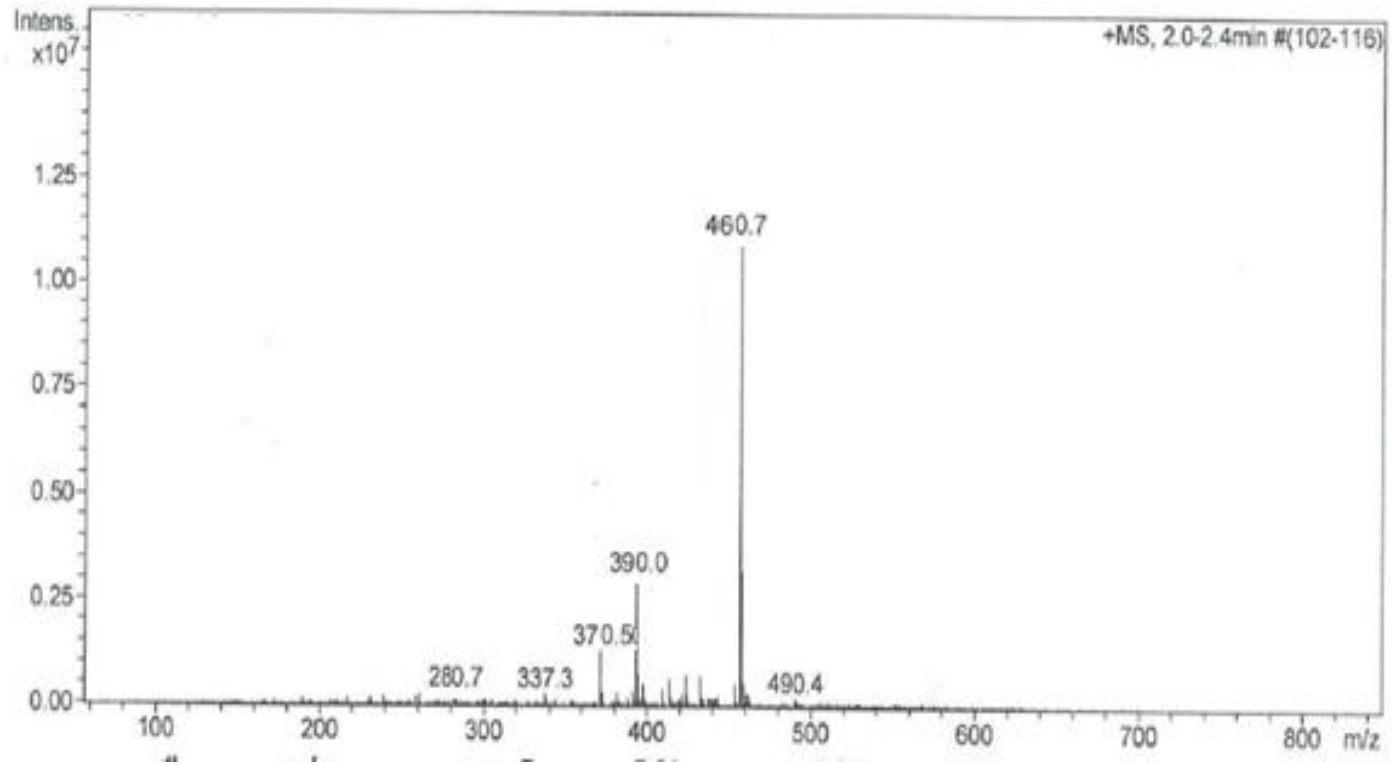


4.6: HMBC spectrum for compound (**45**); (20-hydroxy-3-oxo-28-lupanoic acid)

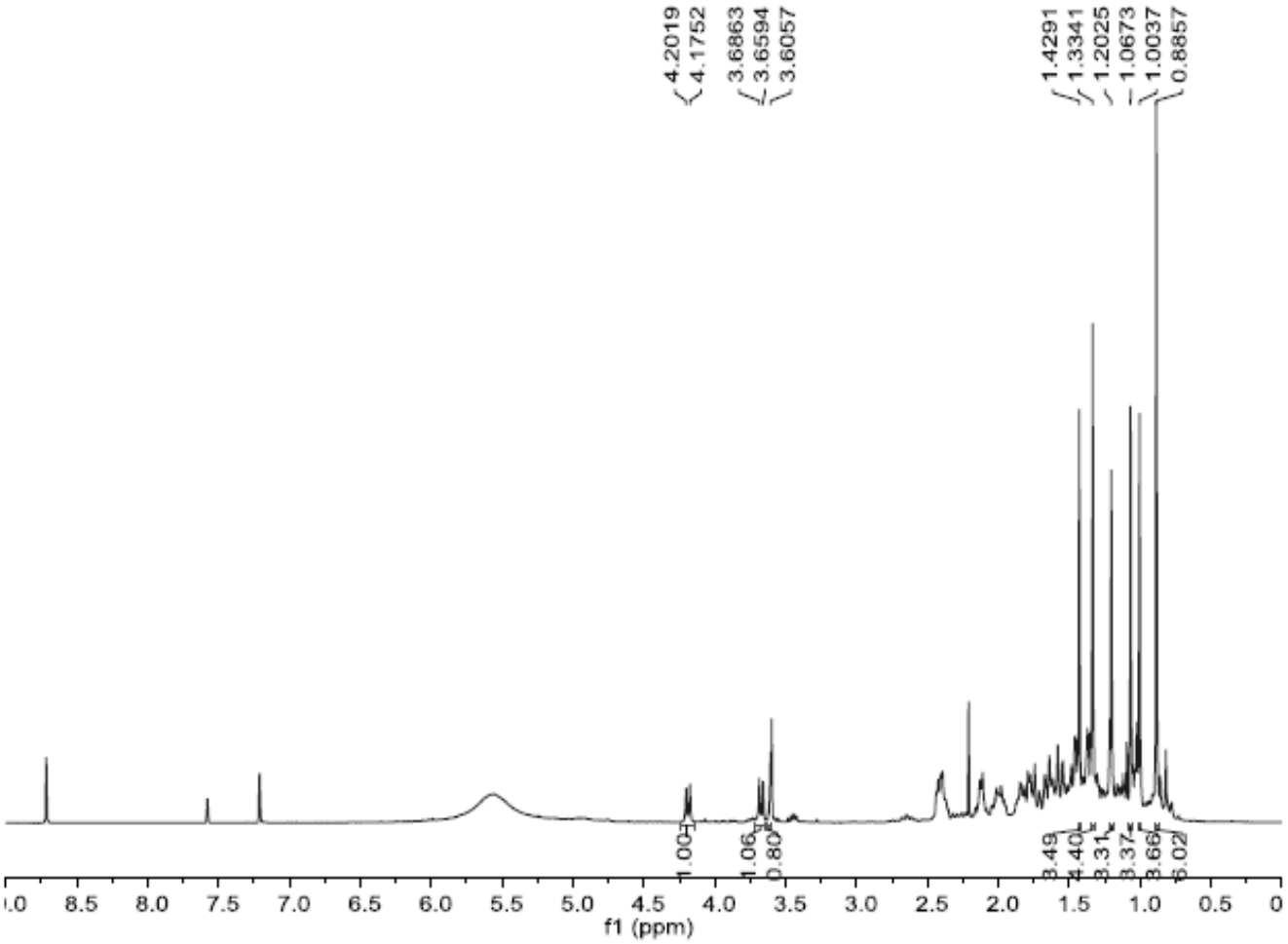


Appendix 5: Spectra for compound (46); 3α,20,28-trihydroxylupane

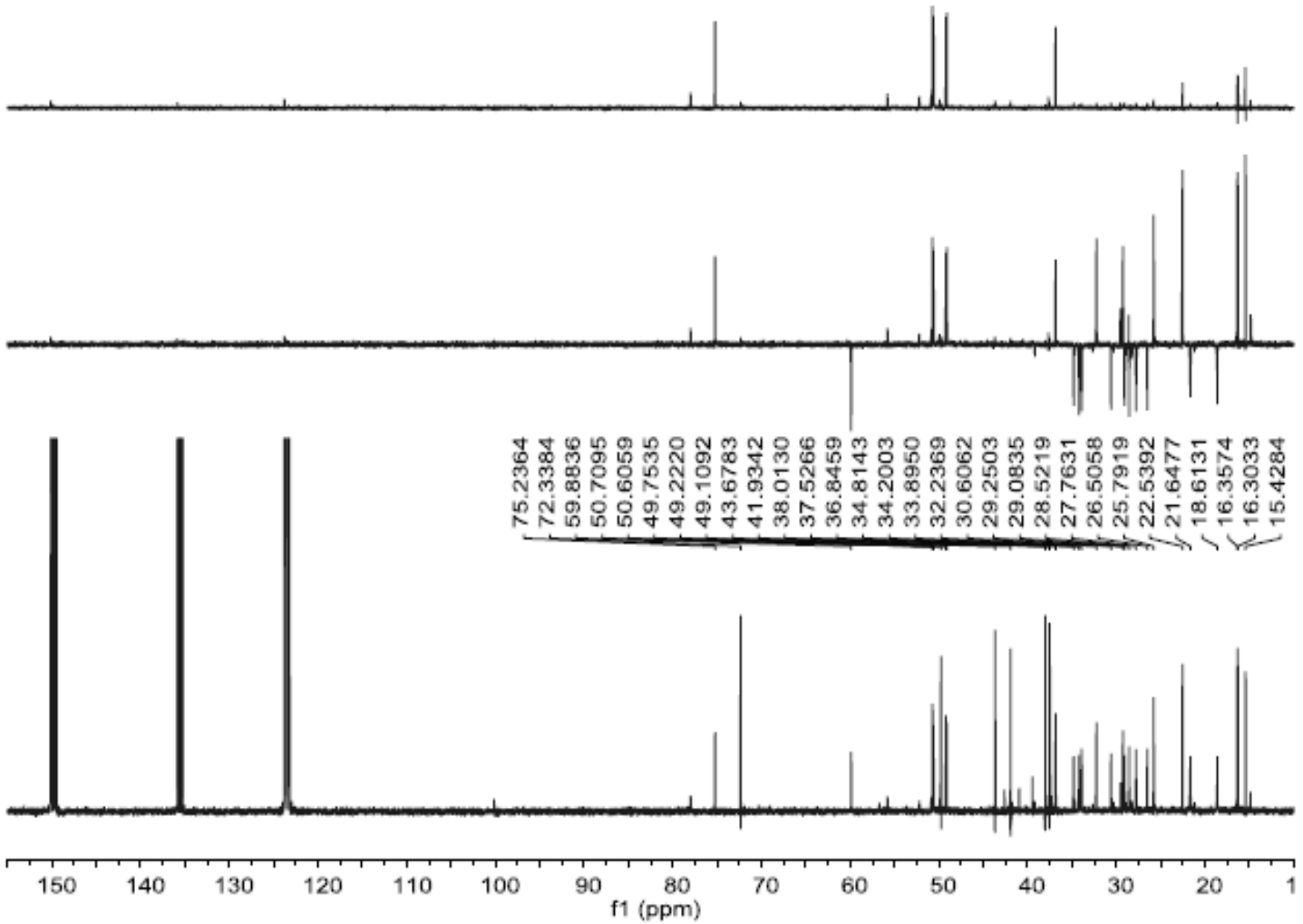
5.1: ESI-MS spectrum for compound (**46**); (3α,20,28-trihydroxylupane)



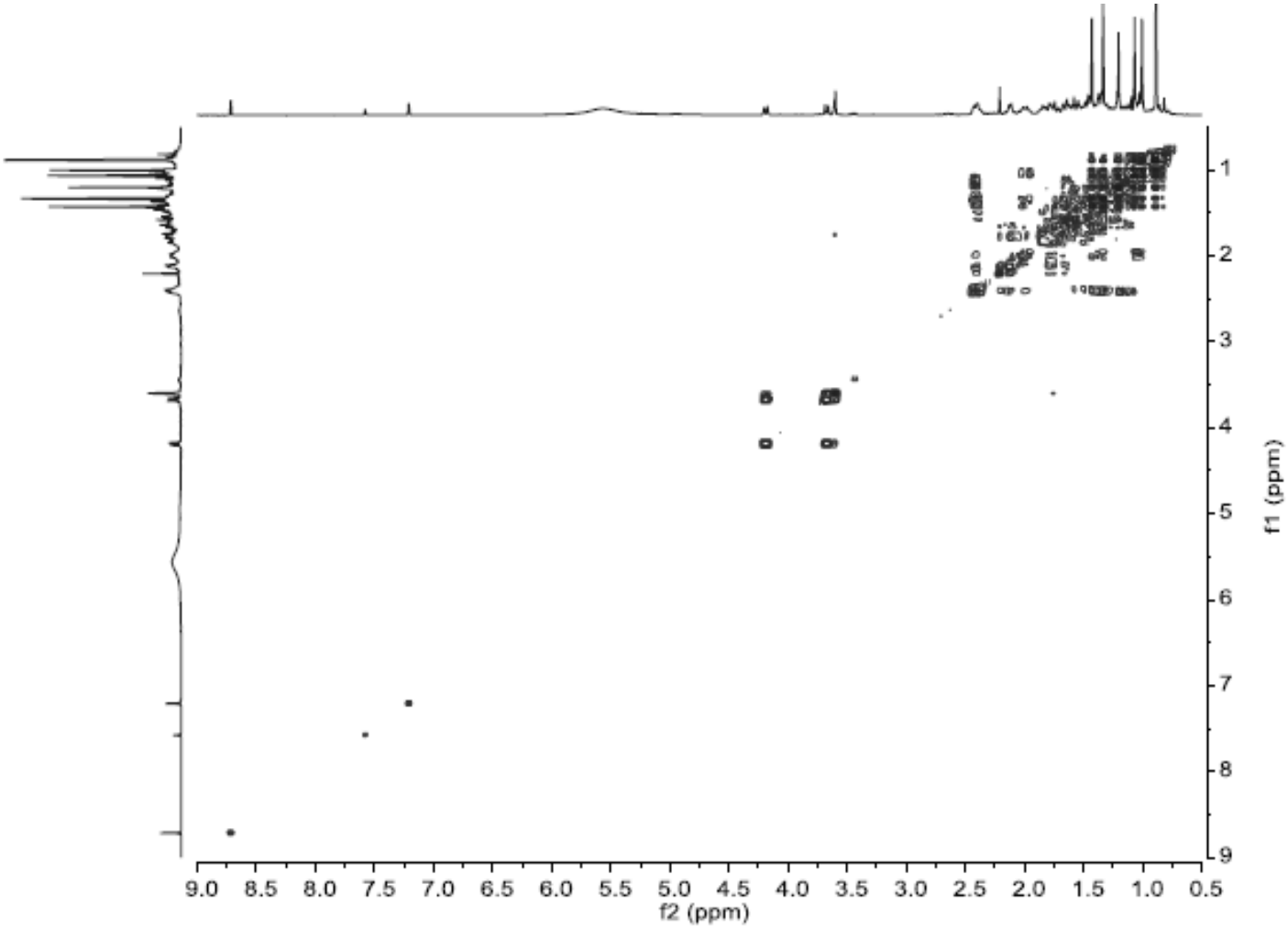
5.2: ^{1}H NMR spectrum for compound (**46**); (3α,20,28-trihydroxylupane)



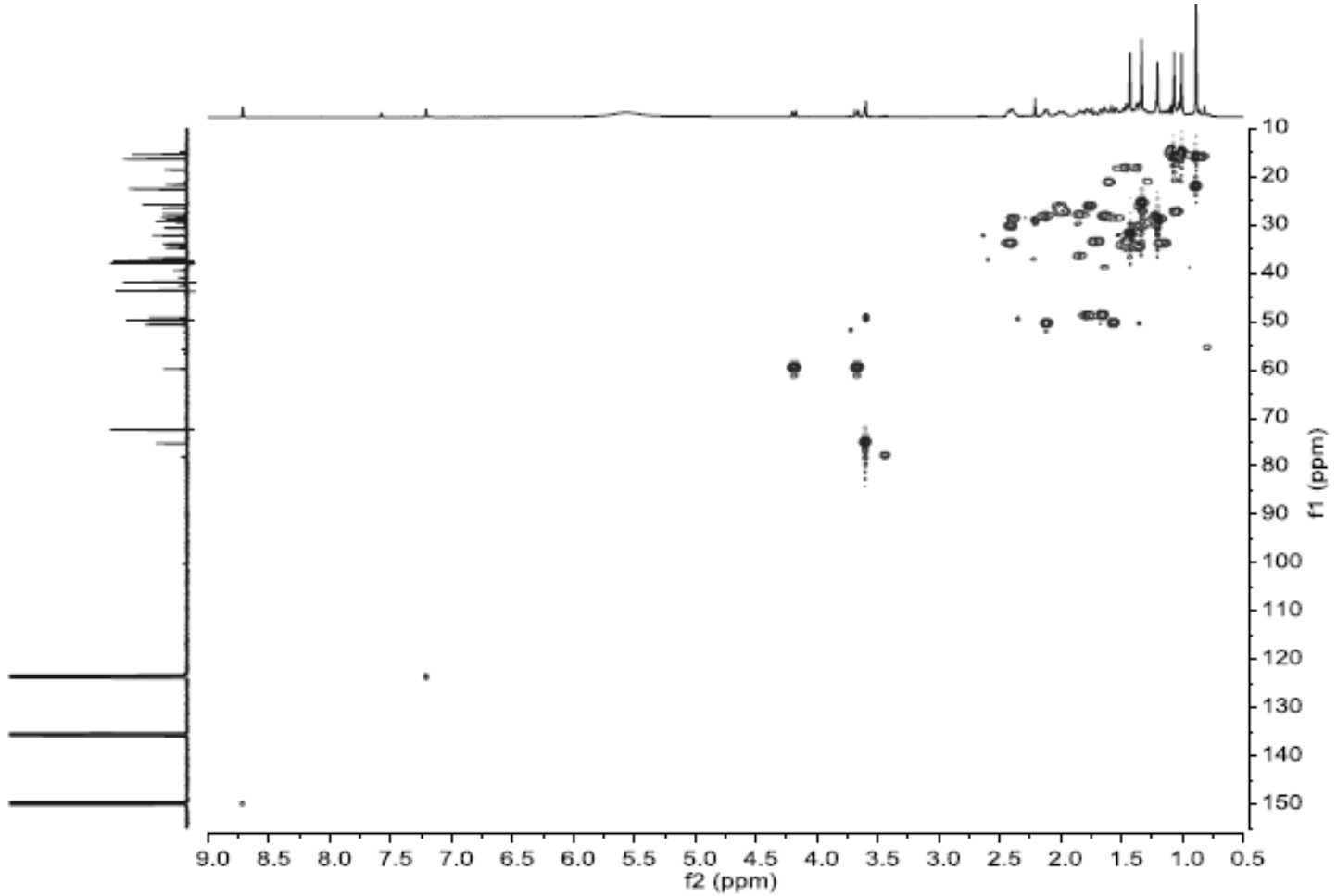
5.3: ^{13}C NMR and DEPT-135 spectrum for compound (**46**); (3α,20,28-trihydroxylupane)



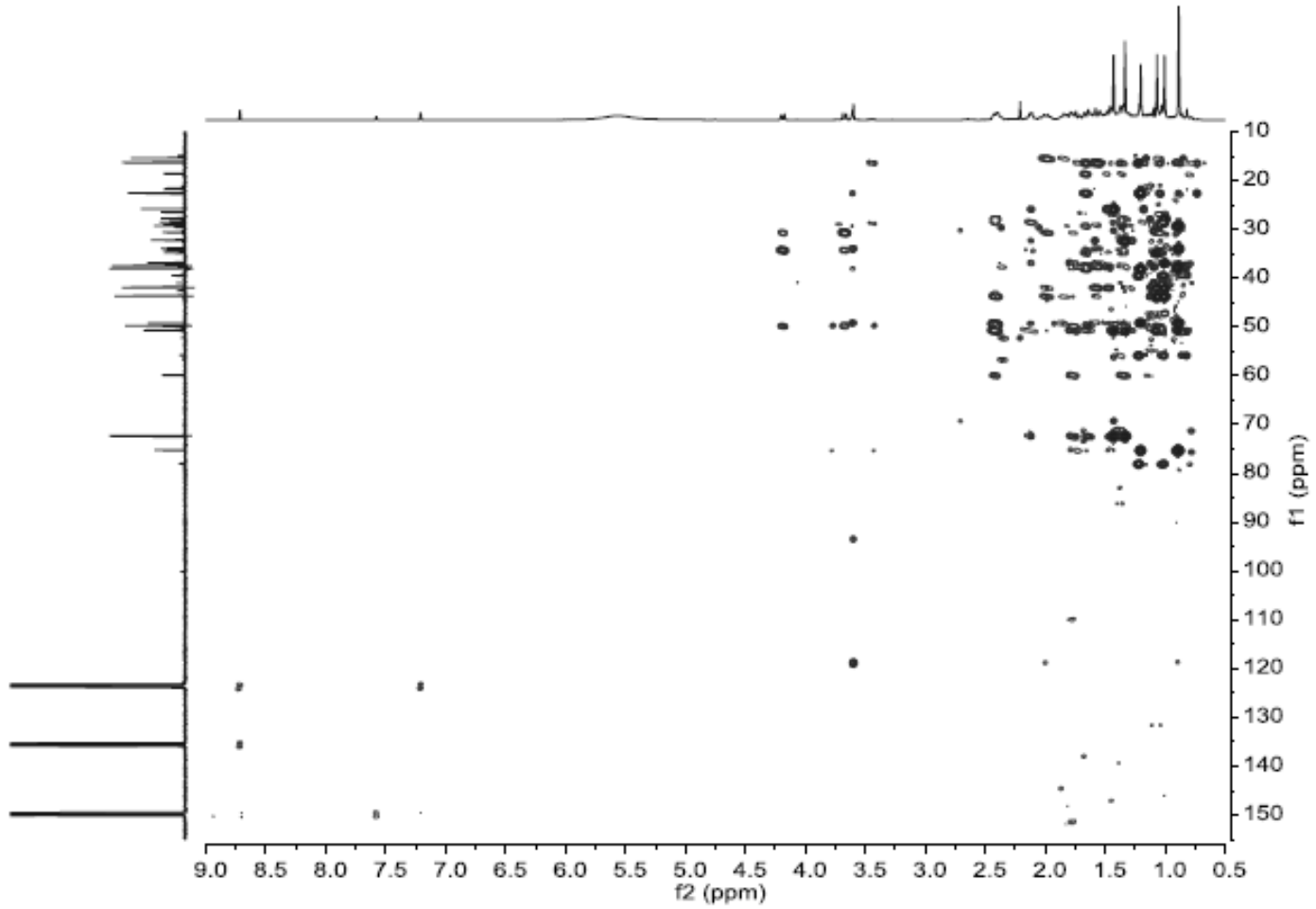
5.4: COSY spectrum for compound (**46**); (3α,20,28-trihydroxylupane)



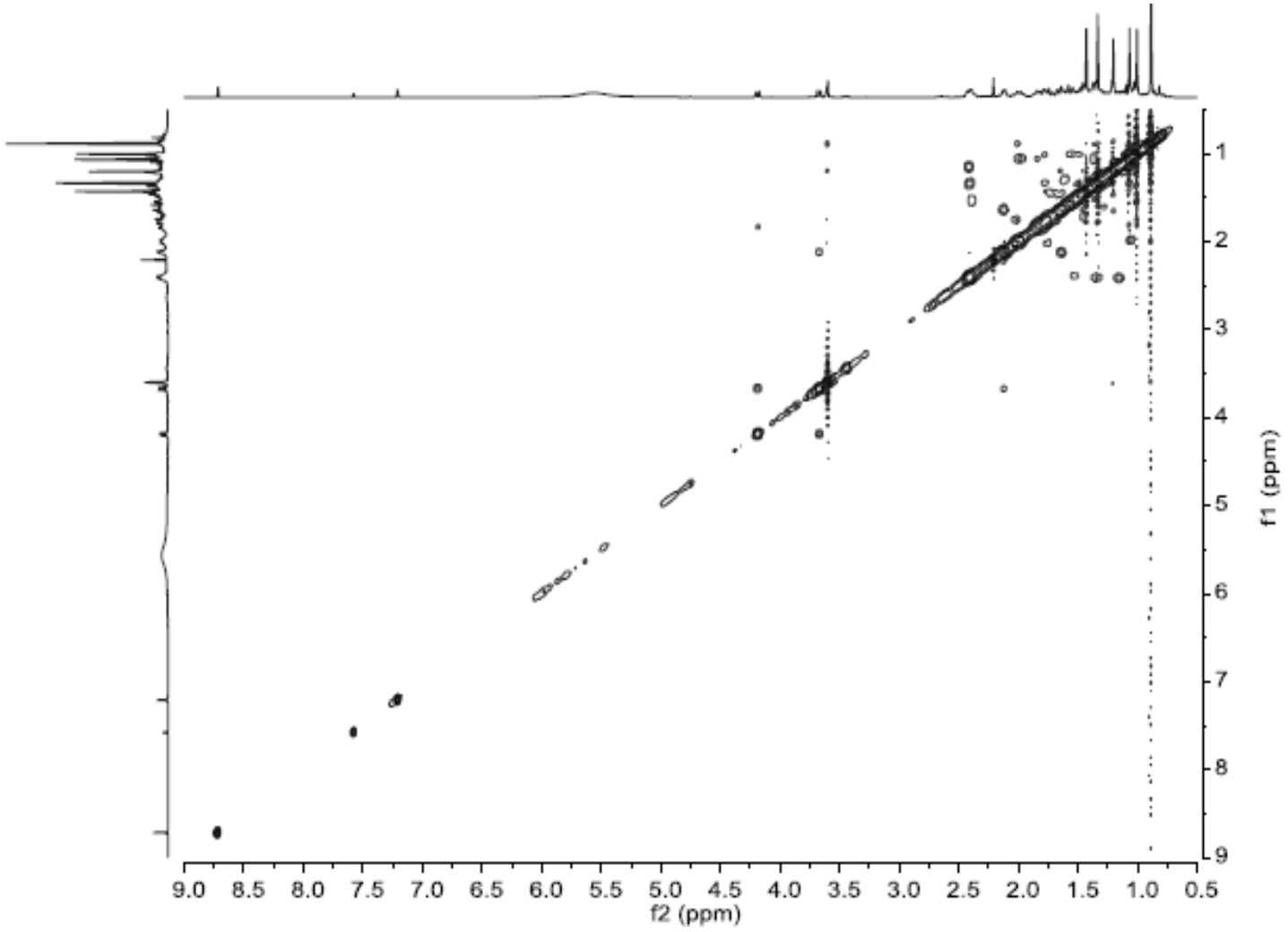
5.5: HSQC spectrum for compound (**46**); (3α,20,28-trihydroxylupane)



5.6: HMBC spectrum for compound (**46**); (3α,20,28-trihydroxylupane)

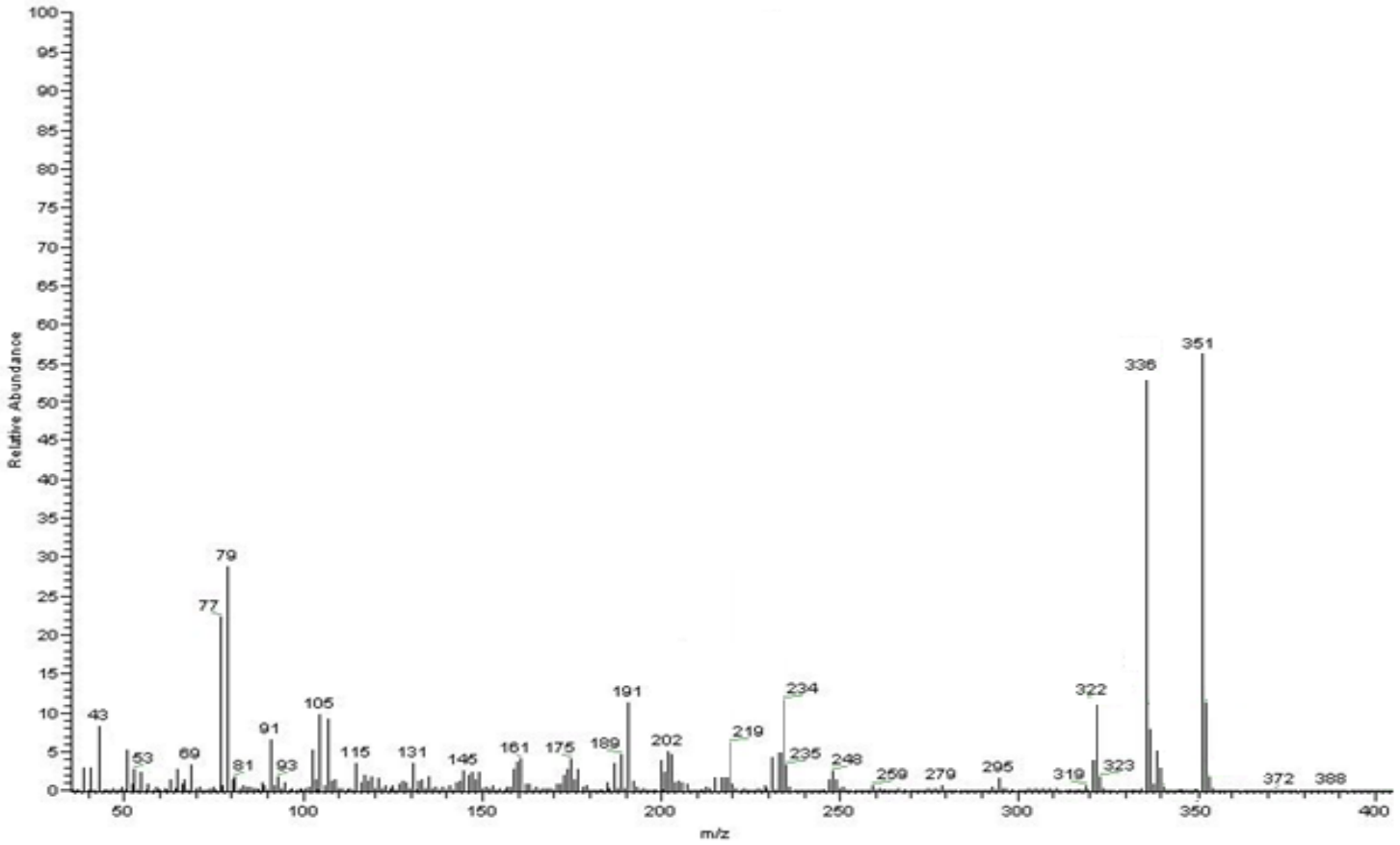


5.7: NOESY spectrum for compound (**46**); (3α,20,28-trihydroxylupane)

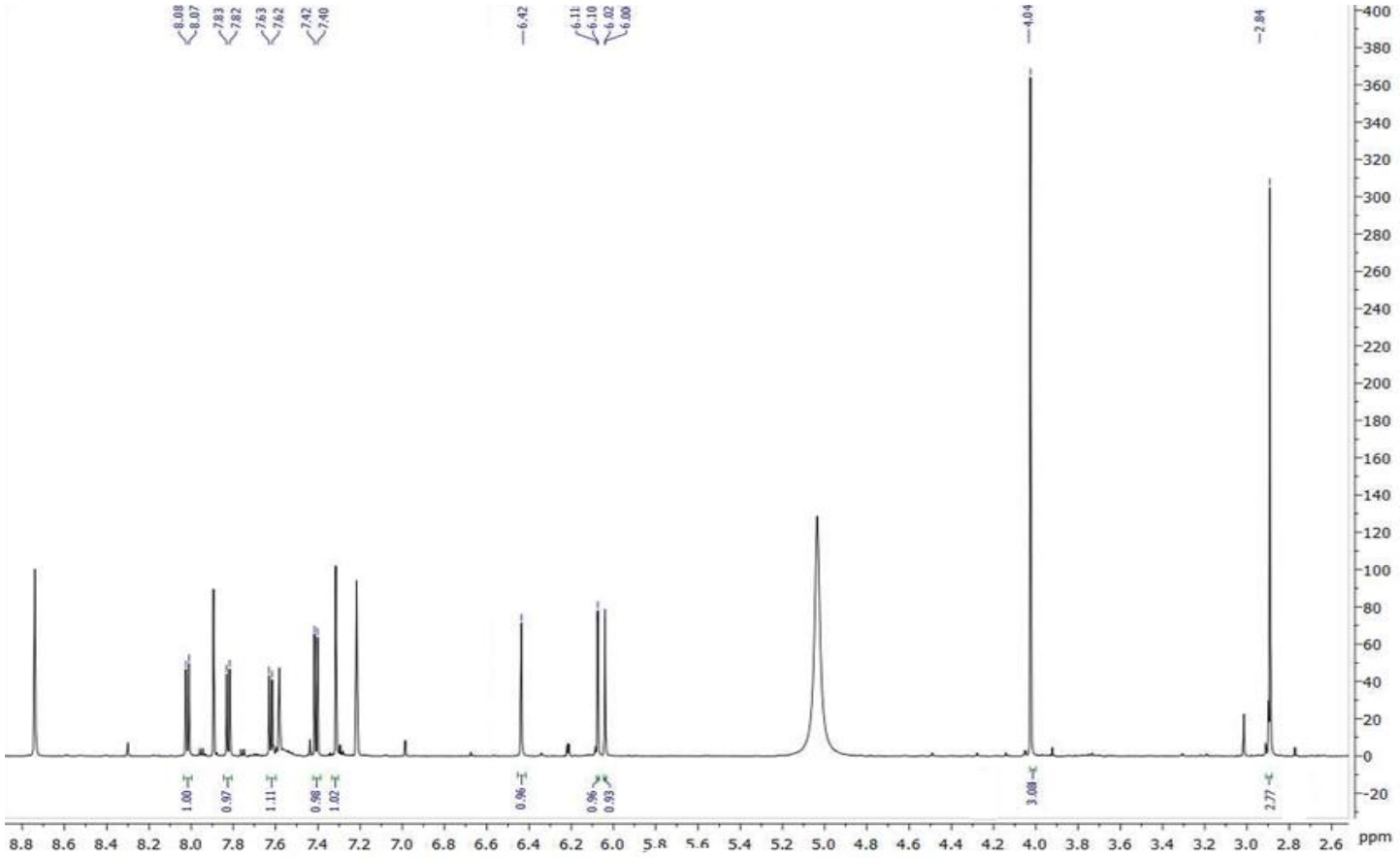


Appendix 6: Spectra for compound (47); 6-hydroxy-N-methyl decarine

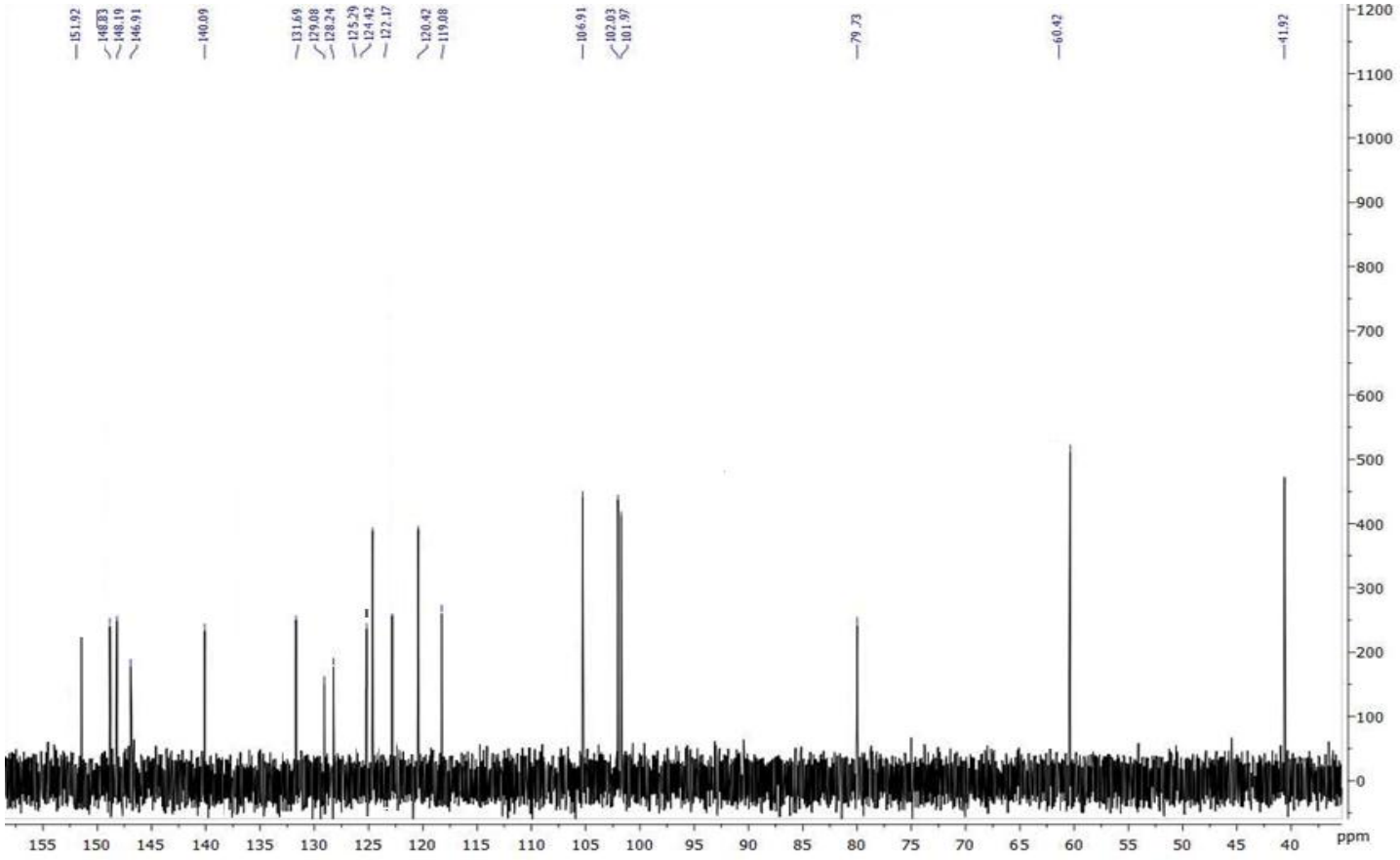
6.1: ESIMS spectrum for compound (**47**); (6-hydroxy-N-methyl decarine)



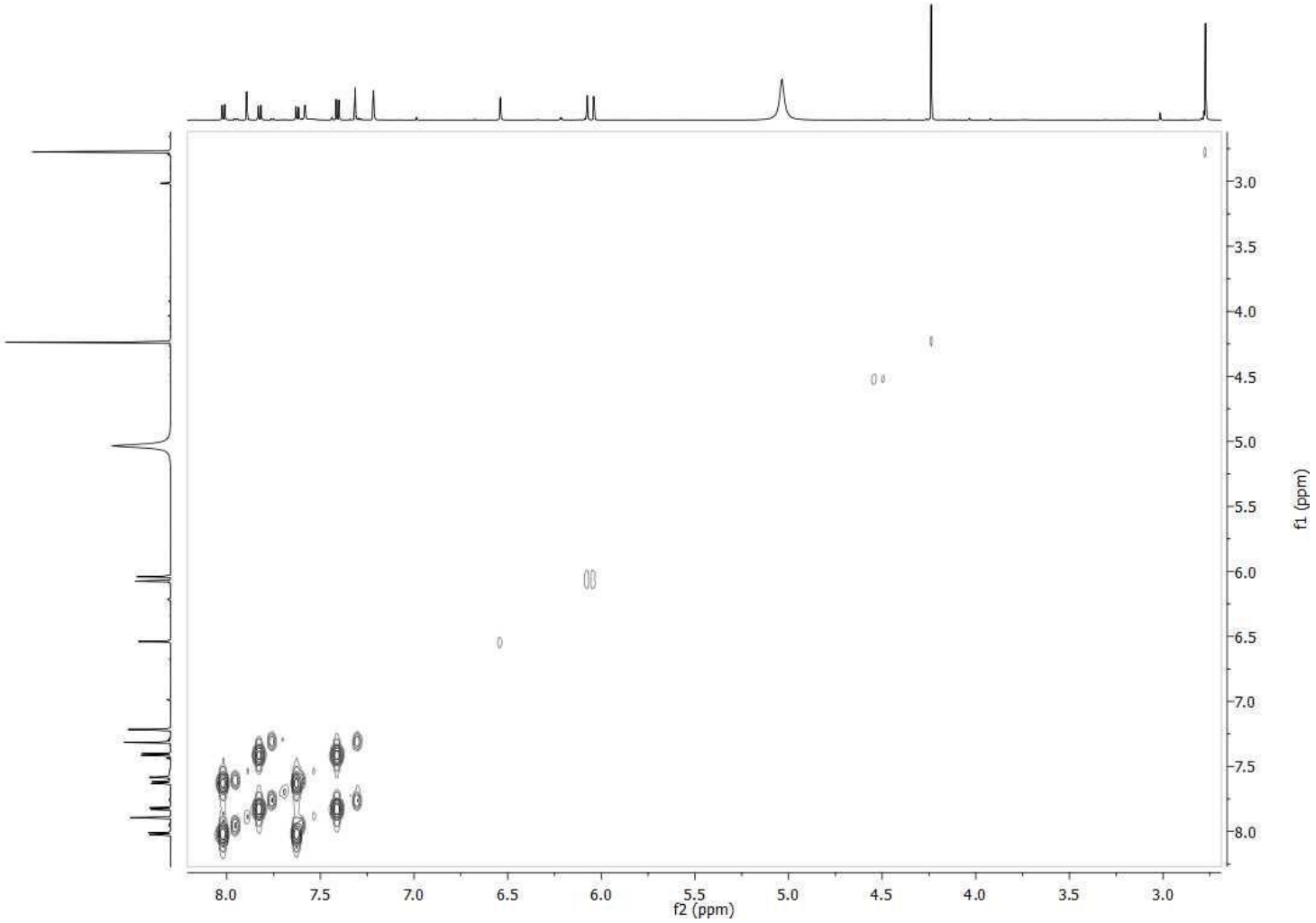
6.2: ^{1}H NMR spectra for compound (**47**); (6-hydroxy-N-methyl decarine)



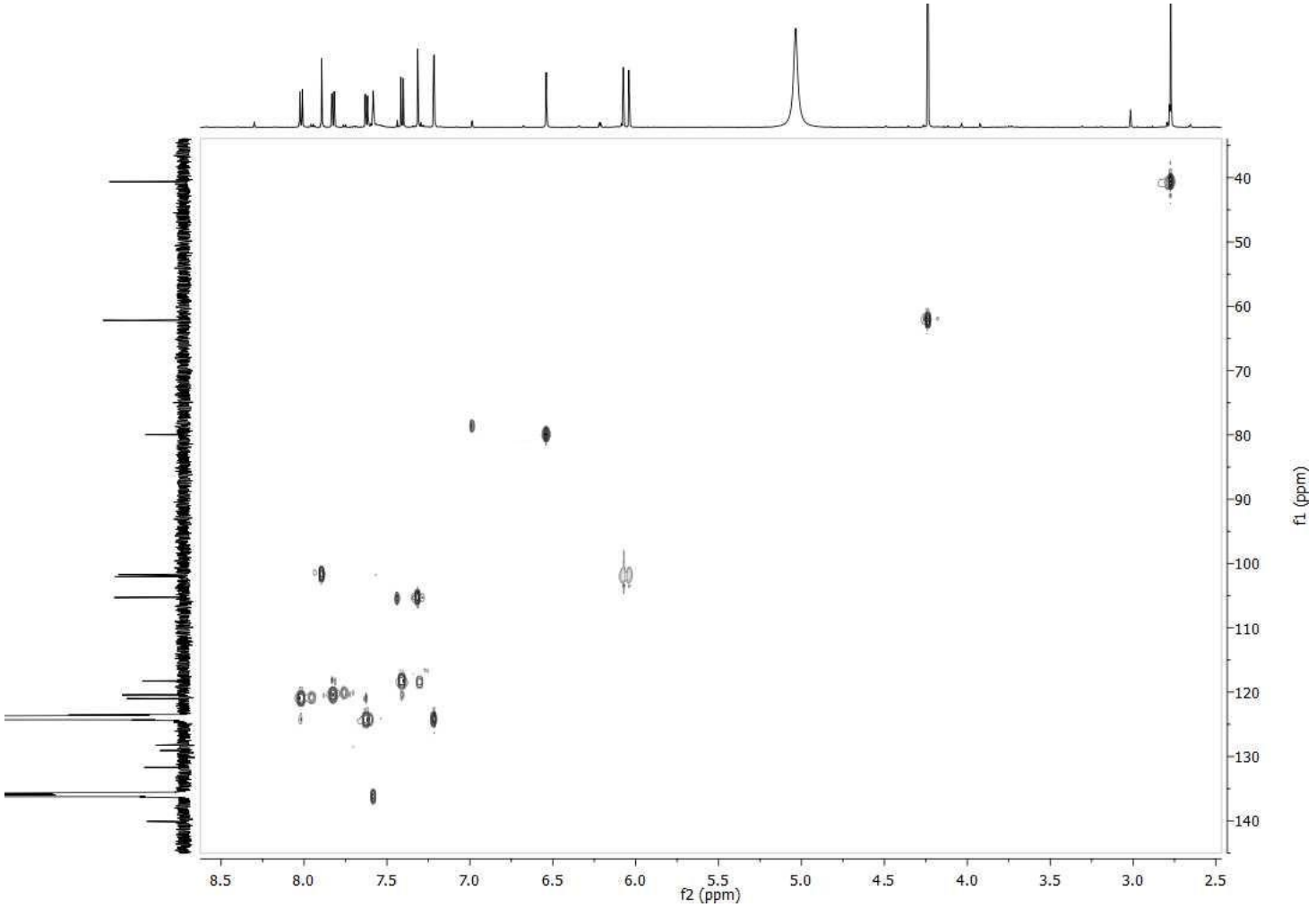
6.3: ^{13}C NMR spectra for compound (**47**); (6-hydroxy-N-methyl decarine)



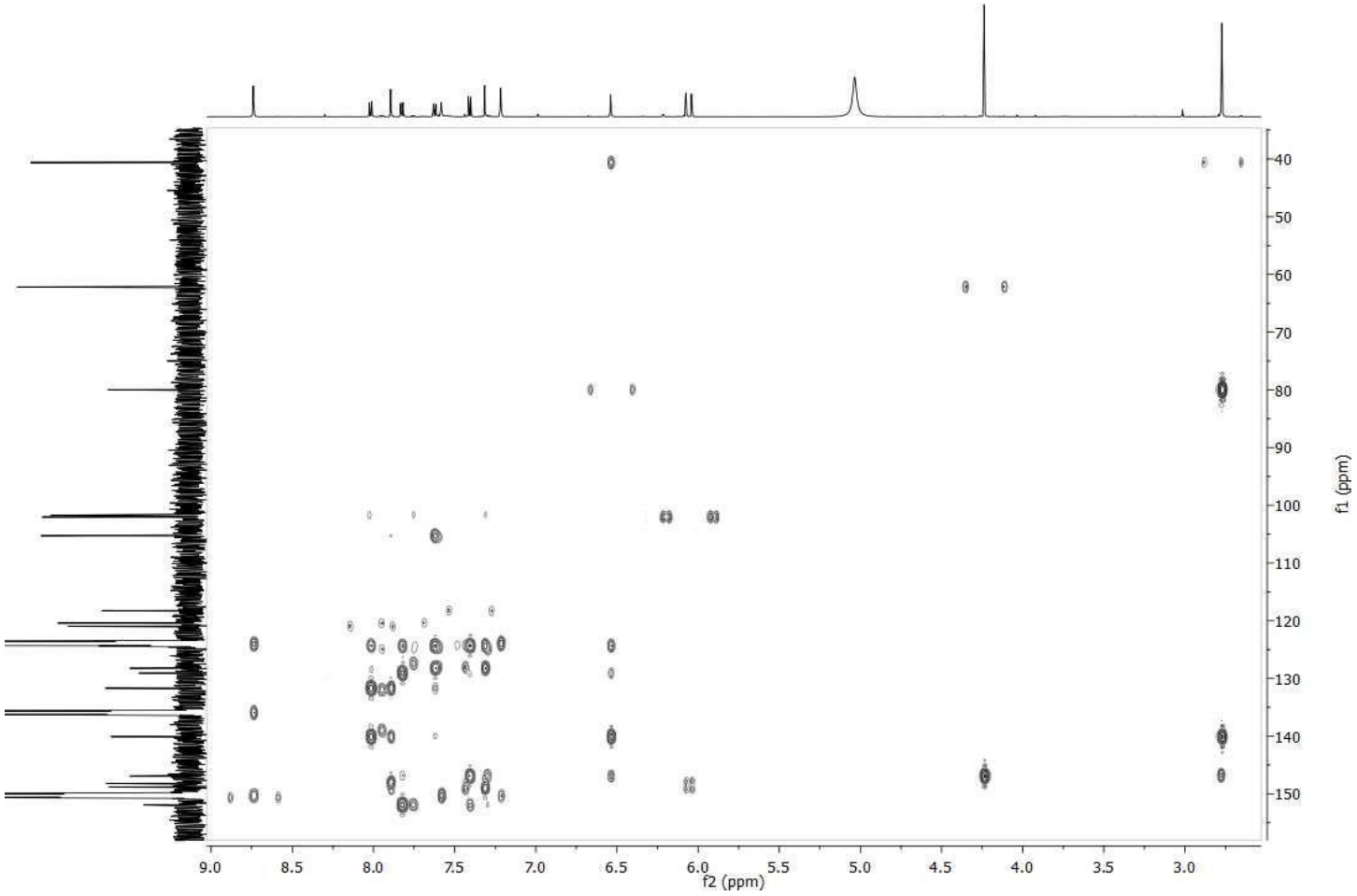
6.4: COSY spectrum for compound (**47**); (6-hydroxy-N-methyl decarine)



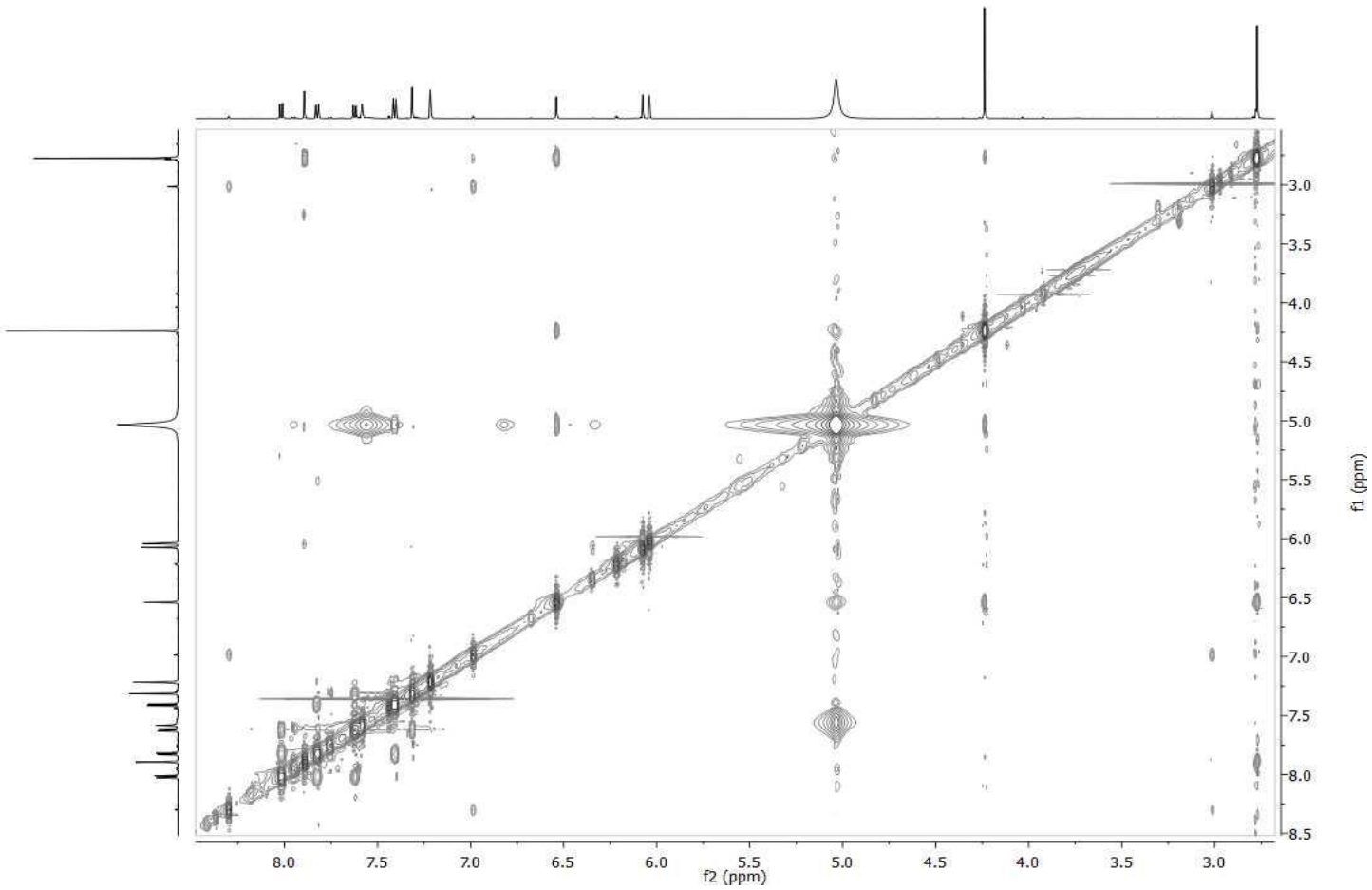
6.5: HSQC spectrum for compound (**47**); (6-hydroxy-N-methyl decarine)



6.6: HMBC spectrum for compound (**47**); (6-hydroxy-N-methyl decarine)

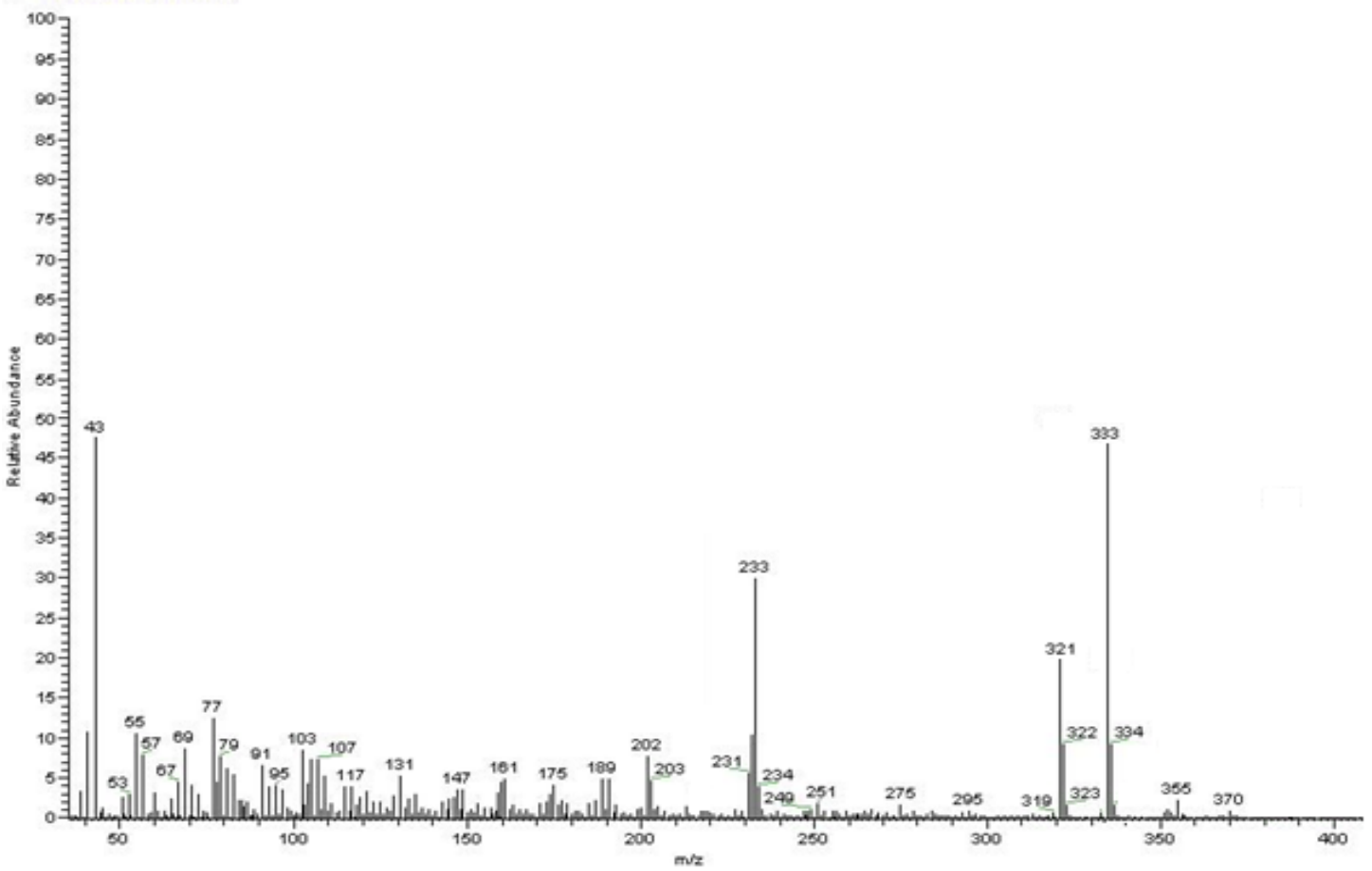


6.7: NOESY spectrum for compound (**47**); (6-hydroxy-N-methyl decarine)

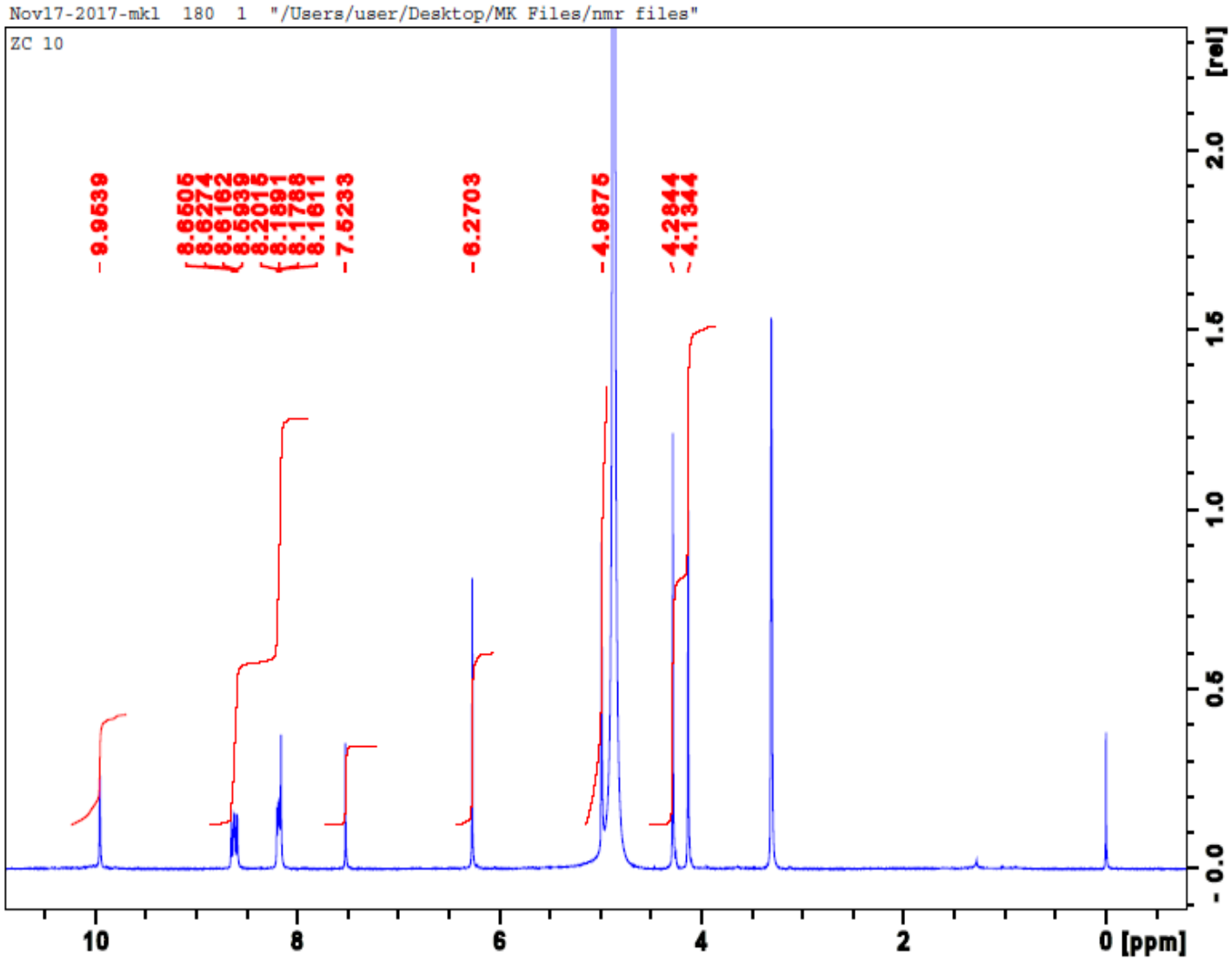


Appendix 7: Spectra for compound (48); Norchelerythrine

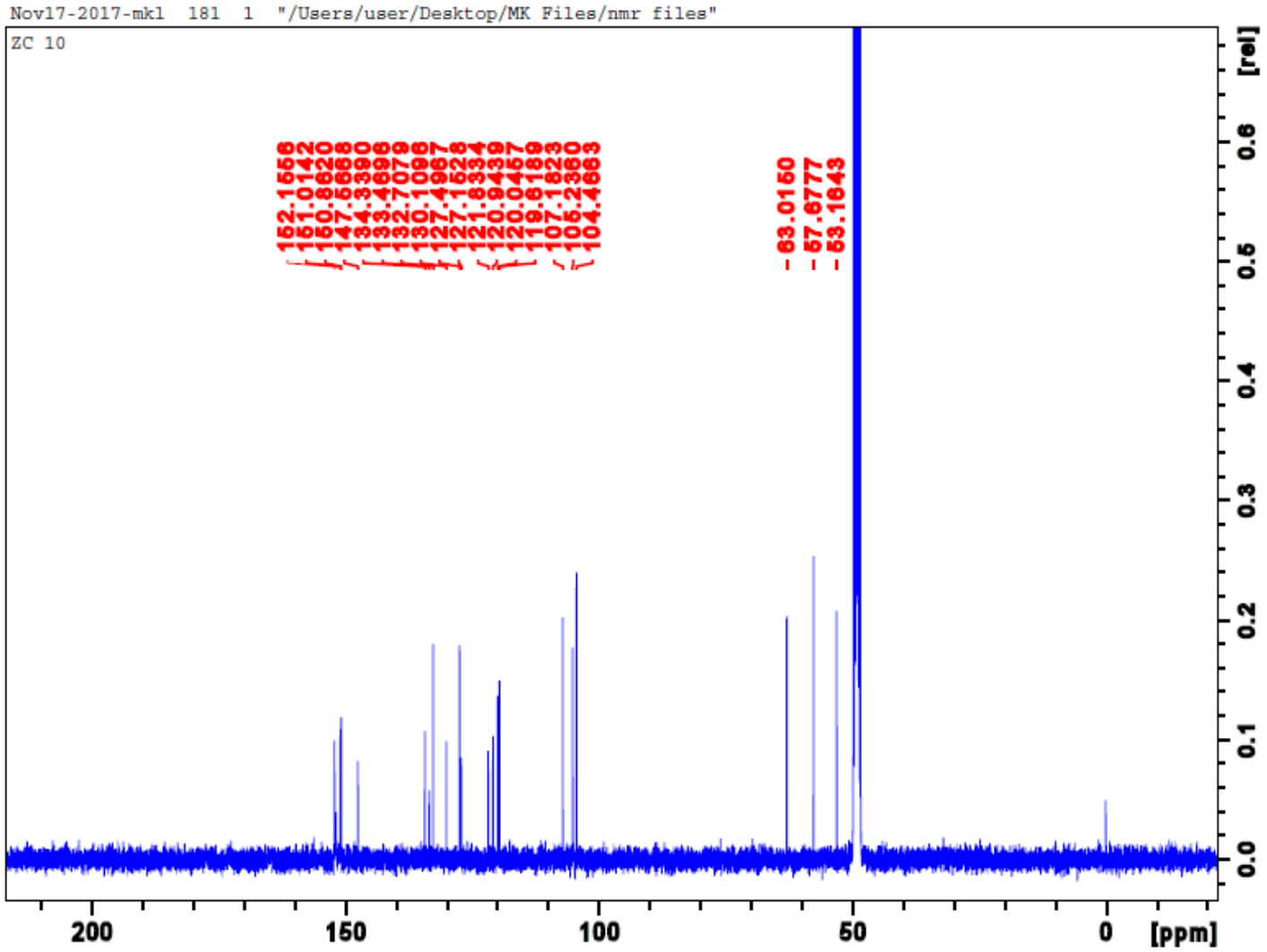
7.1: ESIMS spectrum for compound (**48**); (Norchelerythrine)



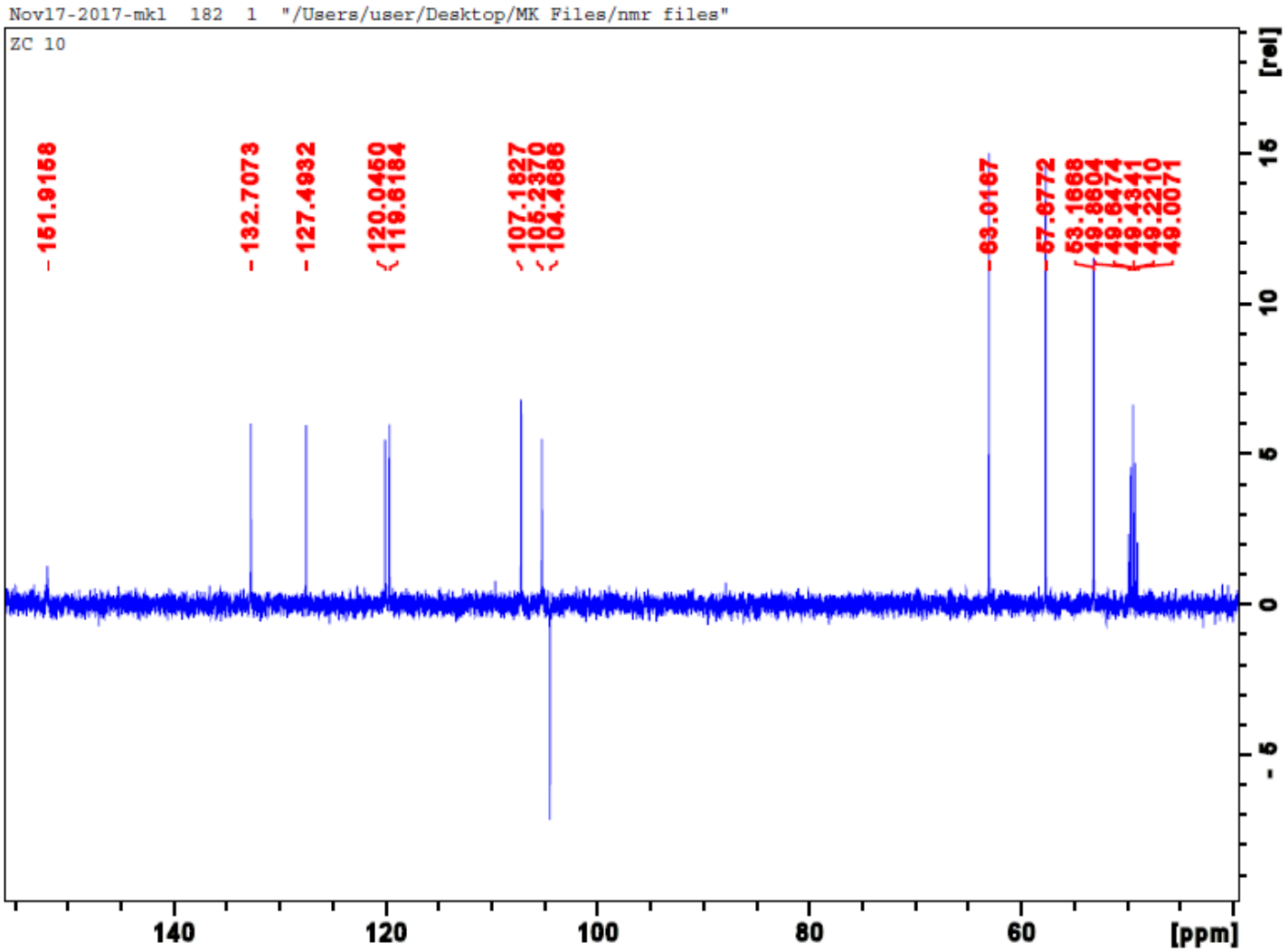
7.2: ^{1}H NMR spectra for compound (**48**); (Norchelerythrine)



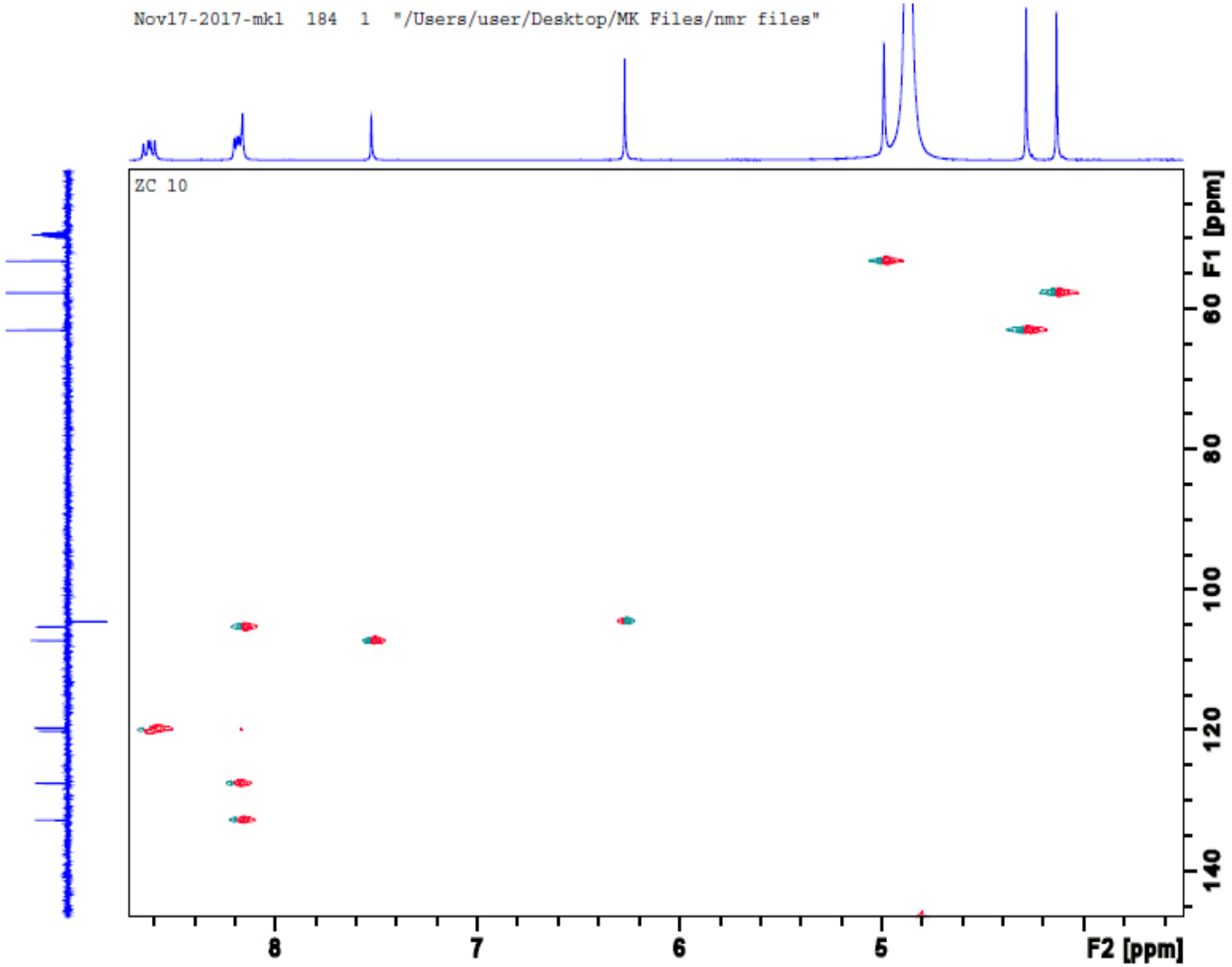
7.3: ^{13}C NMR spectra for compound (**48**); (Norchelerythrine)



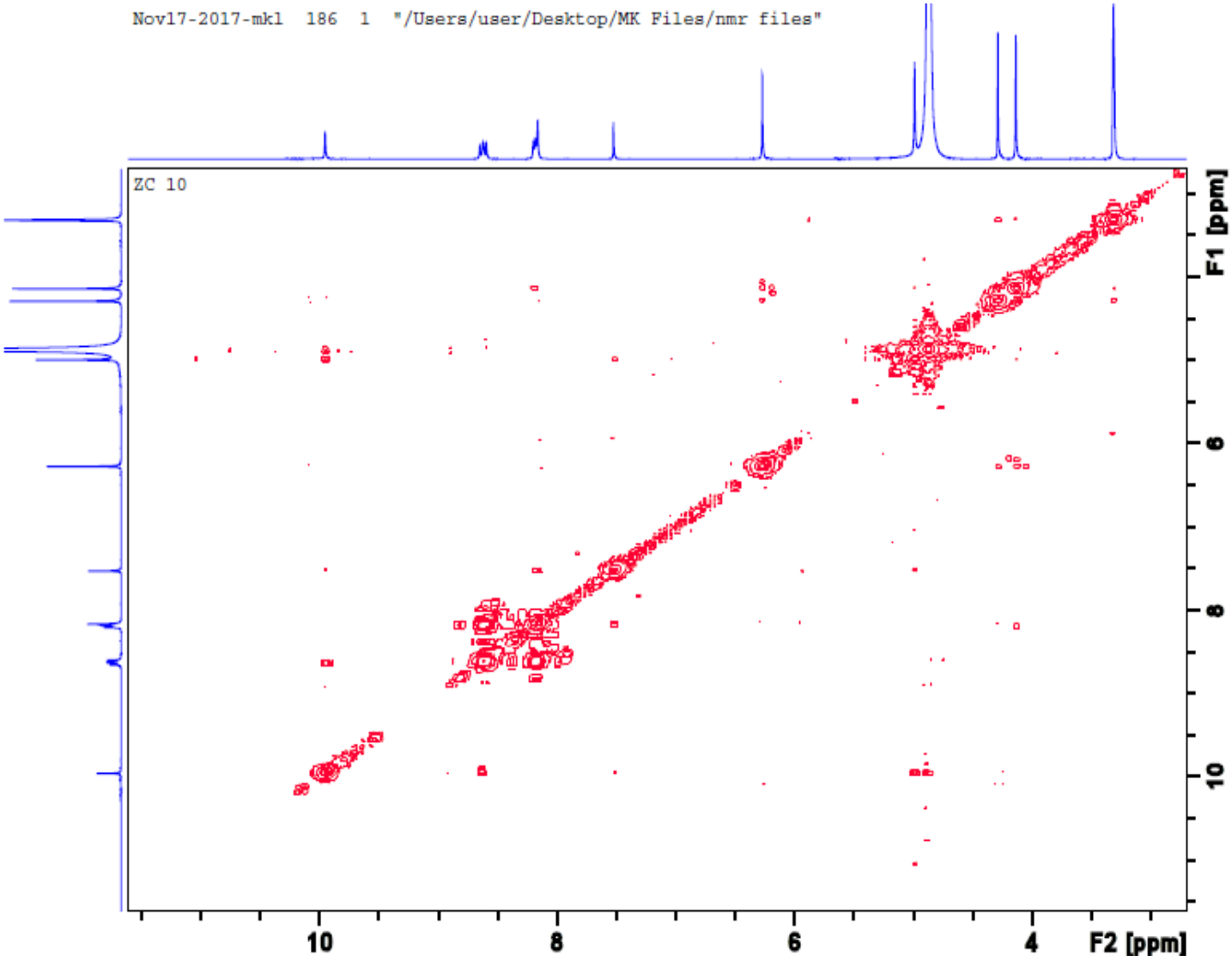
7.4: DEPT spectrum of compound (**48**); (Norchelerythrine)



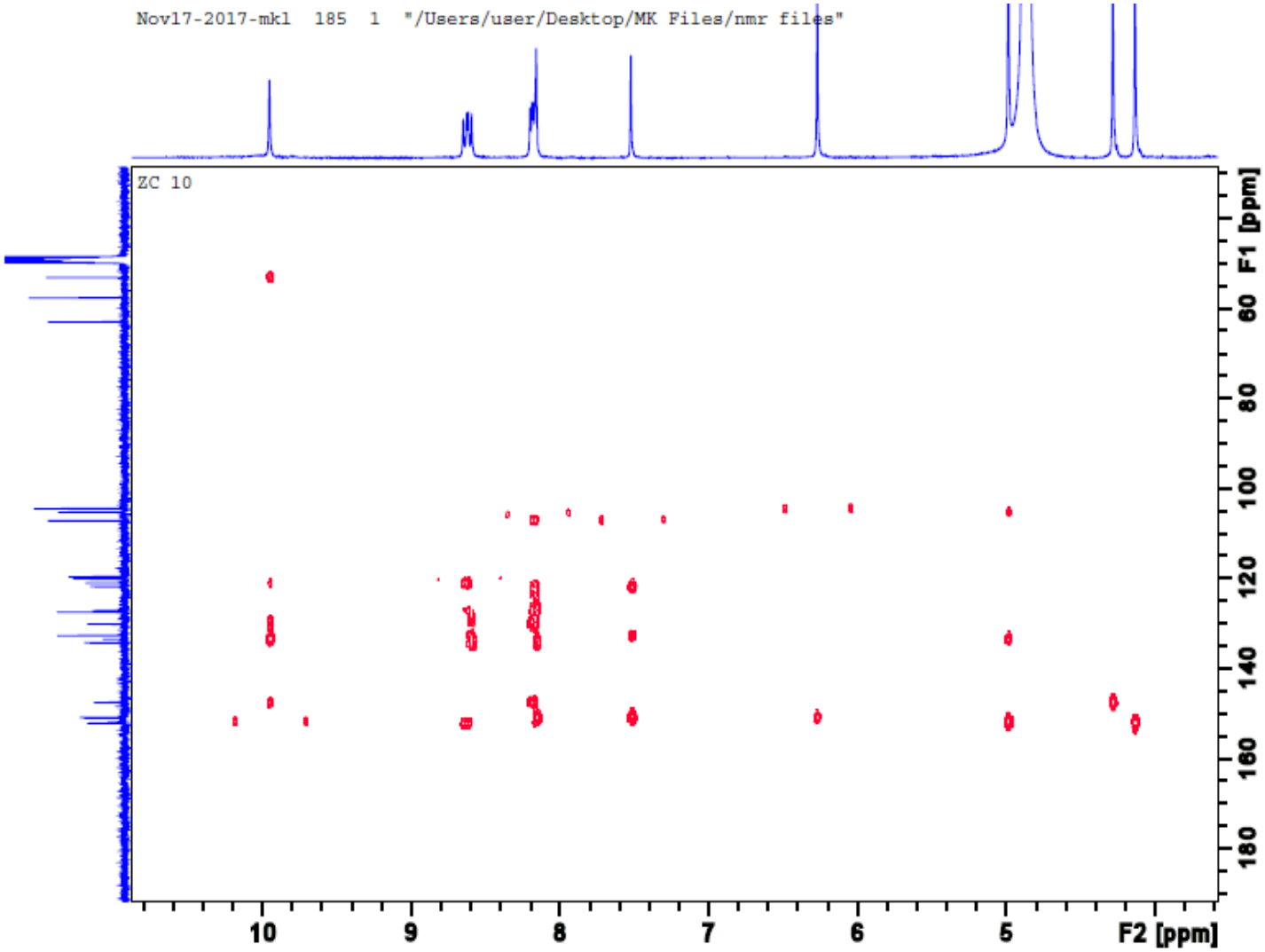
7.5: HSQC spectrum for compound (**48**); (Norchelerythrine)



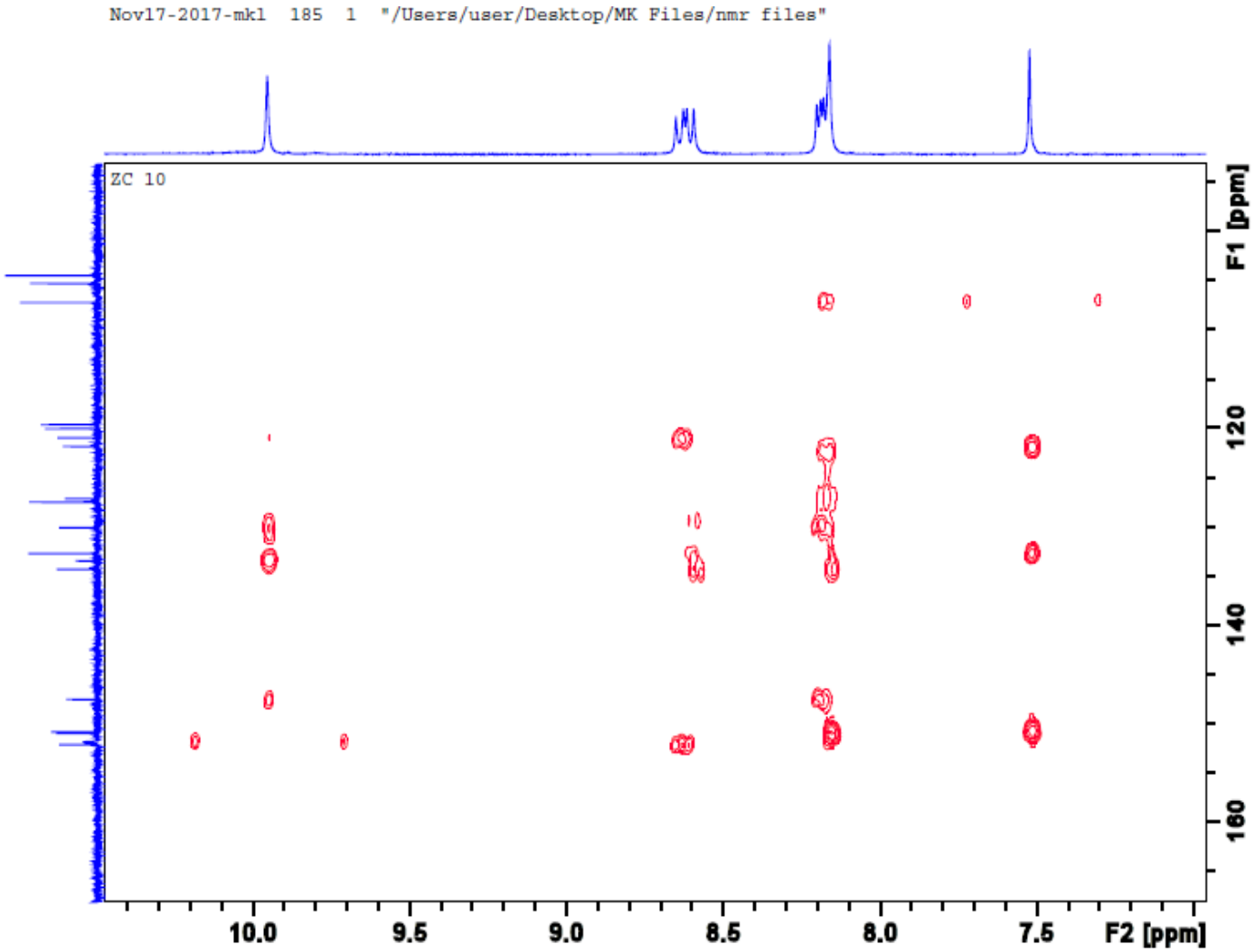
7.6: COSY spectrum for compound (**48**); (Norchelerythrine)



7.7: HMBC spectrum for compound (**48**); (Norchelerythrine)



7.8: Expanded HMBC spectrum for compound (**48**); (Norchelerythrine)



7.9: Expanded HMBC spectrum for compound (**48**); (Norchelerythrine)