Biological Evaluation and Semi-synthesis of Isolated Compounds from (*Syzygium aromaticum* (L.) Merr. & Perry) Buds

Submitted in Partial Fulfillment for MSc. Degree in Organic Chemistry.

In the Department of Chemistry, Faculty of Science and Agriculture, University of Fort Hare, Alice

By

Rali Sibusiso (200806318)

Supervisor: Dr. O. O. Oyedeji

Co-supervisor: Prof. B. N. Nkeh-Chungag

2014

DECLARATION

I, RALI SIBUSISO (200806318), declare that this dissertation and the work presented in it are my own and has been generated by me as the result of my own original research.

Biological evaluation and semi-synthesis of isolated compounds from (*Syzygium aromaticum* (L.) Merr. & Perry) buds

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at University of Fort Hare.
2. Where any part of this dissertation has previously been submitted for a degree or any other qualification at University of Fort Hare or any other institution, this has been clearly stated.
3. Where I have consulted the published work of others, this is always clearly attributed.
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this dissertation is entirely my own work.
5. I have acknowledged all main sources of help.
6. Where the dissertation is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed: ……

Date: ……

CERTIFICATE OF APPROVAL

We hereby declare that this dissertation is from the student's own work and effort, and all other sources of information used have been acknowledged. This dissertation has been submitted with our approval

SUPERVISOR: Dr. O. O. Oyedeji

SIGNATURE: ……

DATE…….

CO-SUPERVISOR: Prof. B. N. Nkeh-Chungag

SIGNATURE: ……

DATE…….

CONFERENCE AND PUBLICATIONS

Conferences

1. S Rali, OO Oyedeji, and BN Nkeh-Chungag: Semi-synthesis of some triterpenoids and their inflammatory properties, Poster presentation at the 41st National Convention of the South African Chemical Institute (SACI) Conference, 1-6th December 2013, East London, South Africa.
2. S Rali, OO Oyedeji, and BN Nkeh-Chungag: Chromatographic and spectroscopic approach in natural products, Poster presentation at the ANALITIKA2014 Conference, 7-11th September 2014, Parys, South Africa.
3. Sibusiso Rali, Opeoluwa O Oyedeji, Olukayode O. Aremu and Benedicta N. Nkeh-Chungag: Analgesic and anti-inflammatory properties of eugenol and oleanolic acid, Oral presentation at the Post Graduate Conference, 13th October 2014, UFH Alice campus, South Africa

Publications

1. Sibusiso Rali[1], Opeoluwa O. Oyedeji*[2], Olukayode O. Aremu[3], Benedicta N. Nkeh-Chungag[4] and Adebola O. Oyedeji[5]: Semi-synthesis of oleanolic acid derivatives and their analgesic and anti-inflammatory properties. Manuscript submitted to Journal of Inflammation.

ACKNOWLEDGEMENTS

Firstly, I would like to give praise to the Almighty God, for the life he has given unto me. The contributions of the following individuals and organizations to this study are gratefully acknowledged:

I wish to thank the following people for their valuable contributions to this study; my heartfelt thanks goes to Dr. O. O. Oyedeji and Prof. B. N. Nkeh-Chungag for their supervision and support in this study. It has been an honour to share this time with them. I would specifically like to thank the Department of Chemistry, University of Fort Hare for provision of laboratory and equipment.

A special word of thanks goes to my friends and colleagues Mr. O. Avoseh, Mr.O. Aremu, Miss P. Rungqu, Mr. S. Mlala, Miss T. Maqoko and Miss N. Peter for their support and sharing hard moments with me. Finally, my heartfelt gratitude goes out to my family: my parents, brothers and sister; my friends Mjelo Gcobani, Ngayeka Vuyo and James Vizicelo. Your prayers, sacrifices, love, and support have been the driving force behind me. I am eternally grateful to all of you.

The present study was supported by the University of Fort Hare, Walter Sisulu University Institutional Research Grant, the Govan Mbeki Research and Development Center, the National Research Foundation (NRF) and Sasol Inzalo Foundation (SaIF).

ABSTRACT

Natural products play a fundamental role in modern drug discovery as they continue providing diverse bioactive lead compounds for new drug formulation. However, isolation of these valued compounds is problematic. On the other hand, the morbidity and mortality rates caused by non-communicable diseases are increasing with improved longevity. Thus, the study herein focused on the isolation of plant-derived compounds from *Syzygium aromaticum* and evaluated their biological properties.

Syzygium aromaticum is a well-known plant which belongs to family Myrtaceae. Dried flower buds of *S. aromaticum* were subjected to sequential solvent extraction. The ethyl acetate extract (15.535 g) was subjected to column chromatography using a silica gel (0.063-0.200 mm) for isolation. This has led to the isolation of three distinct compounds that were identified as eugenol, maslinic acid (MA) and oleanolic acid (OA). The structural elucidation of these valued compounds was done using NMR, GC-MS, LC-MS, FT-IR and Mp. Further semi-synthesis of the oleanolic acid afforded acetate and ester OA-derived compounds with better solubility properties. All these compounds were evaluated for analgesic and anti-inflammatory properties.

All tested compounds were administered at a dose of 40 mg/kg to both Wistar rats and Swiss mice. Significant ($p<0.01$) analgesic and anti-inflammatory properties are obtained for all compounds. The effects of eugenol in all experiment were better except in the thermal-induced pain or tail flick test. In the case of modified compounds, the formalin induced pain test disclosed that oleanane derived compounds confessed analgesic and anti-inflammatory effects better than OA, whereas, in tale flick test oleanolic acid proved superior analgesic effects compared to all its derivatives with the exception of the acetyl-

derivative. Acute anti-inflammatory test showed that acetyl-derivatives were more active than other compounds.

In conclusion, chromatographic techniques and semi-synthesise of oleanolic acid have resulted to several plant-derived compounds with analgesic and anti-inflammatory properties. The semi-synthesized compounds may serve as alternative drug candidates for new analgesic and anti-inflammatory drug formulation

TABLE OF CONTENTS

List of Tables

List of Figures

List of abbreviations

AOA - Acetyloleanolic acid

AOAm - methyl-acetyloleanane

BA - Betulinic acid

CAMs - Cell adhesion molecules

CC - Column chromatography

CDDO - 2-cyano-3, 12-dioxoleana-1, 9(11)-dien-28-oic acid

CE - Capillary electrophoresis

COSY - Correlation spectroscopy

COX - Cyclooxygenase

DEPT - Distortionless excitation by polarization transfer

DMAPP - Dimethylallyl diphosphate

Fam. - Family

FPP - Farnesyl diphosphate

FPS - Farnesyl diphosphate synthase

FTIR spectroscopy - Fourier transform infrared spectroscopy

GC-MS - Gas chromatography-mass spectrometry

HMBC - Heteronuclear multiple bond correlation

HMQC - Heteronuclear Single Quantum Coherence

HPLC - High performance liquid chromatography

IL1-β - Interleukin

IPP - Isopentenyl diphosphate

IRF - Inflammation Research Foundation

LC-MS - Liquid chromatography-mass spectrometry

LC-NMR - Liquid chromatography-nuclear magnetic resonance spectroscopy

LC-UV - Liquid chromatography-ultraviolet-visible (LC- UV)

LPS - Lipopolysaccharide

LUP - Lupeol

MA - Maslinic acid

Mp. - Melting point

MRN1 - Marneral

NMR spectroscopy - Nuclear magnetic resonance spectroscopy

NO - nitric oxide (NO)

NOESY - Nuclear Overhauser effect spectroscopy

NSADS - Nonsteroidal anti-inflammatories

OA - Oleanolic acid

OAm - Methyloleanane

PGs - Prostaglandins

SFC - Supercritical fluid chromatography

SQE - Squalene epoxidase

SQS - Squalene synthase (SQS)

THA1 - Thalianol

TLC plate - Thin layer chromatography plate

TNF-α - Tumor necrosis factor

TOA - Trifluoroacetyloleanolic acid

TOAm - Methyl-trifluoroacetyloleanane

UA - Ursolic acid

UHPLC - Ultra-high pressure liquid chromatography (UHPLC)

WHO - World Health Organisation

δ - Chemical shift (NMR)

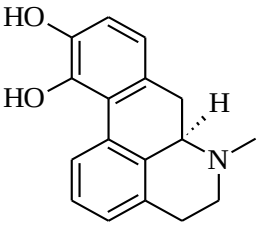
CHAPTER ONE

INTRODUCTION

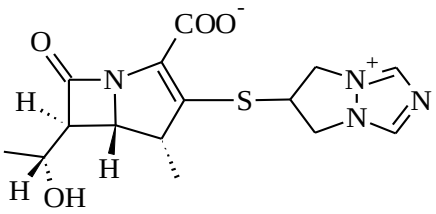
1.1 Background of study

The historical background of medicinal chemistry demonstrates the usefulness of natural products (secondary metabolites) for the treatment of human diseases[1,2]. A natural product medicine may originate from various sources including plants, microorganisms, aquatic organisms, as well as vertebrates and invertebrates[1]. Additionally, natural product chemistry plays a major role in the identification of bioactive secondary metabolites from land-dwelling and aquatic source. The significant role of bioactive substances found in natural products is to advance and strengthen modern drug discovery[2]. The significant role of natural products in modern medicine was discussed and its value evaluated using 3 criteria: (1) the rate of introduction of new chemical entities of wide structural diversity, including those serving as templates for semi-synthetic and total synthetic modification, (2) the number of diseases treated or prevented by these substances, and (3) their frequency of use in the treatment of disease[1].

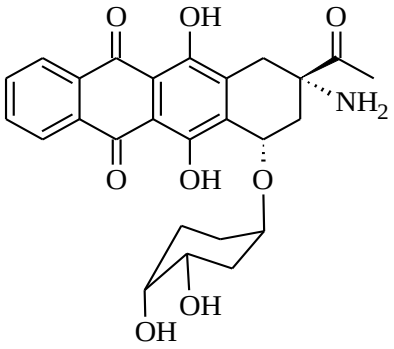
The persistence in evaluating bioactive constituents from natural products resulted in new drug discoveries between 2000 and 2005. These drugs came from different sources of natural products for example apomorphine **(1.1)** from plants, biapenem **(1.2)** and amrubicin **(1.3)** from microorganism and HTI-286 **(1.4)** from marine organisms. These drug candidates are known to prevent several diseases mainly, apomorphine (Parkinson's disease), biapenem (anti-bacterial), amrubicin (lung cancer), HTI-286 (prostate cancer)[1].



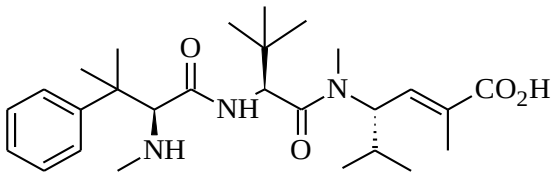
1.1



1.2



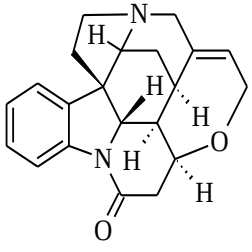
1.3



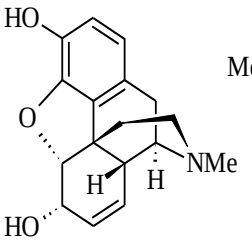
1.4

Plant based natural products have been known for thousands of years and they constantly provides human basic needs. They play a crucial role not only in medicine, but also in the production of food-stuffs, shelter, clothing, means of transportation, fertilizer, flavours and fragrances[3].

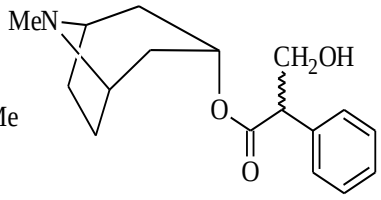
The traditional medicine system uses plant products to produce remedies and have been in existence for thousands of years[3]. However, the effectiveness of the medicinal natural products was first attributed to science in the early 1800's when active principles from plants were isolated. Some of the early plant derived isolates were alkaloid compounds such as, strychnine **(1.5)**, morphine **(1.6)**, atropine **(1.7)** and colchicine **(1.8)**[4,5].



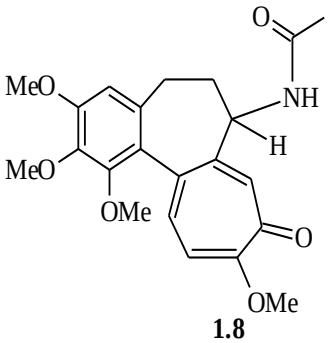
1.5



1.6



1.7



1.8

Furthermore, these isolates namely, strychnine **(1.5)** (pesticide), morphine **(1.6)** (analgesic), atropine **(1.7)** (anticholinergic) and colchicine **(1.8)** (antigout) were used in drug discovery in both original and modified forms[5].

Despite advances in clinical medicine and the discovery of many drug molecules, the World Health Organization reports that over 80% of populations in developing countries still rely wholly on traditional medicine to meet their basic healthcare needs. As a result of the importance of plants in the lives of people, the World Health Organization was motivated to allocate 27 centers out of 915 collaborating centers worldwide to traditional medicine[6,7].

More importantly, the therapeutic power of traditional medicine was found to be useful as anti-malaria, anti-sickling, anti-helminthic, antimicrobial, anticonvulsants, anti-hypertensive and anti-schistosomal (molluscicidal) agents which contributes a prominent role in modern drug discoveries. All the above mentioned therapeutic properties are derived from plant secondary metabolites and they are unique due to a particular plant species or group. Moreover, they are also consistent with the concept that, the combination of secondary products in a plant is not the same due to their different classifications[6].

Natural products (plants secondary metabolites) plays a fundamental role in mankind and latest reports identify several traditional medicinal plants that are effective against human pathogens such as *Staphylococcus aureus*, *Vibrio cholera* and *Pseudomonas aeruginosa*. Although these are very serious human pathogens and often associated with nosocomial infections, several medicinal plants are known to be very effective against these pathogens, namely, (i) Amla fruit (*Emblica officinalis*), (ii) Neem leaves (*Azadirachta indica*), (iii) Aloe leaves (*Aloe vera*) and (iv) Clove buds (*S. aromaticum* L.)[8].

1.1.1 *Syzygium aromaticum* L. Merr. & Perry

Syzygium aromaticum L. or *Eugenia caryophylata* L. also known as clove, is one of most valuable spice plants that has been used as food for thousands of years[9]. It belongs to a family of Myrtaceae and its multiple pharmacological properties emerged from folk medicine has been documented[10,11]. This valued plant is reported to possess various phytochemicals that may protect human from a host diseases[10]. The essential bioactive constituents of *Syzygium aromaticum* are steroids, terpenoids, saponins, tannins and flavonoids[9].

Natural products contribute an essential role in drug discovery; however, recent reviews demonstrate a prominent rate of morbidity and mortality around the world. Therefore, it is important to review how effective natural products (plant secondary metabolites) are, and can be modified to improve their effectiveness in medicine.

1.2 Research problem

Recent reviews demonstrate an increasing number of non-communicable diseases that have been responsible for high morbidity and mortality around the world. Most of these diseases like cancers are caused mostly by uncontrolled acute and chronic inflammation. Due to high morbidity and mortality outcomes of these diseases, it is believe that the current anti-inflammatory and analgesic drugs do not provide satisfactory outcomes. Therefore, it is important to validate how plant isolates and their derivatives could improve and produce potent modern drug in preventing inflammatory related disease.

1.3 Justification

Natural products have become important and promising sources of new substances for drug development. It has been demonstrated that plant-derived isolates especially triterpenoids exert multiple pharmacological activities and their chemical derivatives strengthen their healing power[12]. However, information on some plant-derived isolates and their modified form as inhibitors of inflammation and pain is still lacking. A research on the effects of plant-derived compounds and their derivatives as therapeutic agents would assist in minimizing the development of inflammation related diseases.

1.4 Aim and Objectives

The aim of this study was to isolate, identify and semi-synthesize some bioactive plant-derived constituents from *Syzygium aromaticum* (L), and also to determine and evaluate their anti-inflammatory and analgesic properties.

1.4.1 Overall objectives

To extract some bioactive constituents of *Syzygium aromaticum* (L).

To isolate, characterize and identify plant-derived constituents of *Syzygium aromaticum* flower buds (clove).

To semi-synthesize several known and unknown derivatives of isolated compounds.

To evaluate the anti-inflammatory and analgesic properties of compounds.

1.5 Hypothesis

Bioactive constituents of *Syzygium aromaticum* (L.) buds can be isolated through several chromatographic techniques.

The chemical structure of isolated compounds will allow functional groups derivatization.

Effectiveness, potency and healing strength increase with modification.

1.6 References

1. Chin Y, Balunas MJ, Chai HB, Kinghorn AD. (2006). Drug discovery from natural sources. *The AAPS Journal*. *8*(2):239–253.

2. Dias DA, Urban S, Roessner U. (2012). A historical overview of natural products in drug discovery. *Metabolites*. *2*(4):303–336.

3. Gurib-Fakim A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*. *27*(1):1–93.

4. Newman DJ, Cragg GM, Snader KM. (2000). The influence of natural products upon drug discovery. *Natural Product Reports*. *17*(3):215–234.

5. Salim AA, Chin Y, Kinghorn AD. (2008). Drug discovery from plants. In: Merillon KR and JM, ed. *Bioactive Molecules and Medicinal Plants*. eds. Springer-Verlag, Heidelberg;. :1–24.

6. Aiyegoro OA, Okoh AI. (2009). Use of bioactive plant products in combination with standard antibiotics: implications in antimicrobial chemotherapy. *Medicinal Plants Research*. *3*(13):1147–1152.

7. World Heath Organisation. (2001). Essential drugs and medicines policy.

8. Mehrotra S, Srivastava AK, Nandi SP. (2010). Comparative antimicrobial activities of neem, amla, aloe, assam tea and clove extracts against *Vibrio cholerae, Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Journal of Medicinal Plants Research*. *4*(22):2393–2398.

9. Milind P, Deepa K. (2011). Clove: A champoin spice. *International Journal of Research in Ayurveda & Pharmacy*. *2*(1):47–54.

10. Bhowmik D, Kumar KPS, Yadav A, Srivastava S, Paswan S, Dutta AS. (2012). Recent trends in Indian traditional herbs *Syzygium aromaticum* and its health benefits. *Journal of Pharmacognosy & Phytochemistry*. *1*(1):6–17.

11. Hema R, Kumaravel S, Sivasubramanian C. (2010). GC-MS study on the potentials of *Syzygium aromaticum*. *Researcher*. *2*(12):1–4.

12. Bednarczyk-Cwynar B, Zaprutko L, Ruszkowski P, Hładoń B. (2012). Anticancer effect of A-ring or/and C-ring modified oleanolic acid derivatives on KB, MCF-7 and heLa cell lines. *Organic & Biomolecular Chemistry*. *10*(11):2201–2205.

CHAPTER TWO

LITERATURE REVIEW

2.1 Natural products (Plant secondary metabolites)

2.1.1 Role and biosynthesis of plant secondary metabolites

Plants are a valuable source of a wide range of natural products (secondary metabolites)[1]. Secondary metabolites are chemicals or compounds present in plants that are not involved in the primary biochemical processes of plant growth and reproduction. Plant secondary metabolites however are known to play a crucial role in plant protection from insect predation or grazing by herbivores and adaptation of plants to their environment[2,3].

The secondary metabolites are biosynthesized from primary metabolites by several pathways such as shikimic acid, tricarboxylic acid cycle and malonic acid pathways[3]. These plant materials or compounds fall into the category of saponins, tannins, lignins, volatile oils, alkaloids to mention a few[2].

Discoveries in plant secondary metabolites state that different biosynthetic pathways are responsible for diversity of classes or/and groups of plant components for example. 29,000 terpenes resulting from isopentenyl diphosphate (IPP), 12,000 alkaloids from (amino acid) and 8,000 phenolics produced by shikimate or acetate malonate pathway. IPP modification pathway leads to a series of monoterpene compounds, mainly, (-) limonene, (-) isopiperitenone, (+) -isomenthone, (-) -menthol[4].

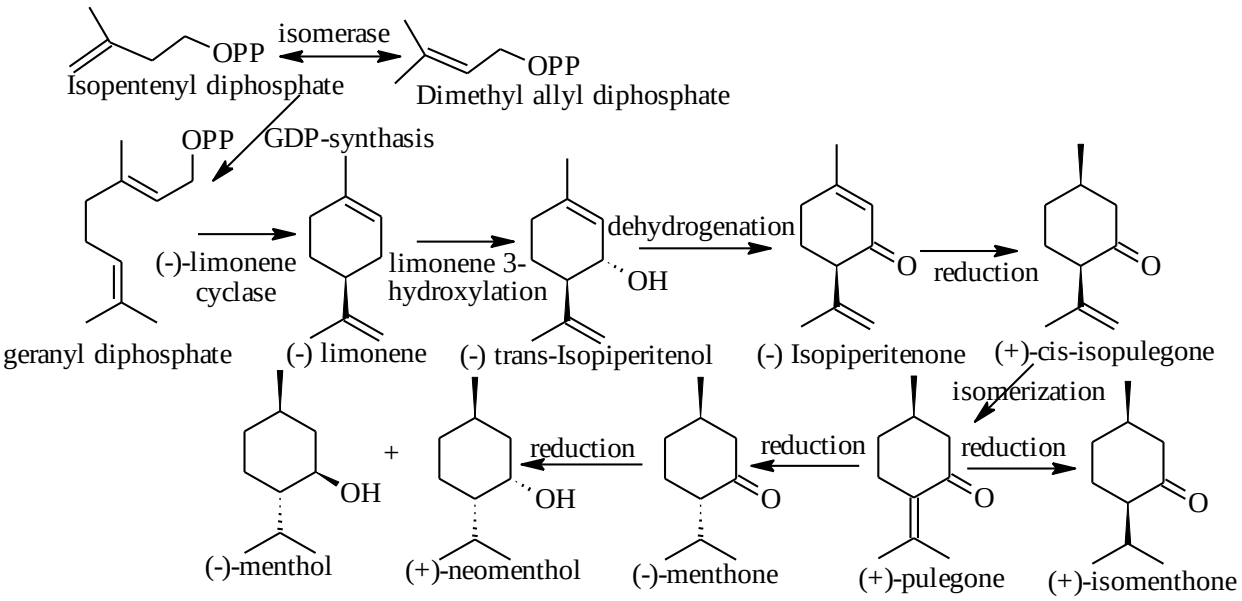


Figure 2.1: Biosynthesis pathway of isopentenyl diphosphate to form monoterpenes

It has been confirmed that the above compounds belongs to the monoterpene groups that play important protective role in the plant kingdom. Limonene and menthol are two well-known monoterpenes which serve as defenses against insects and other organisms feeding on plants[4].

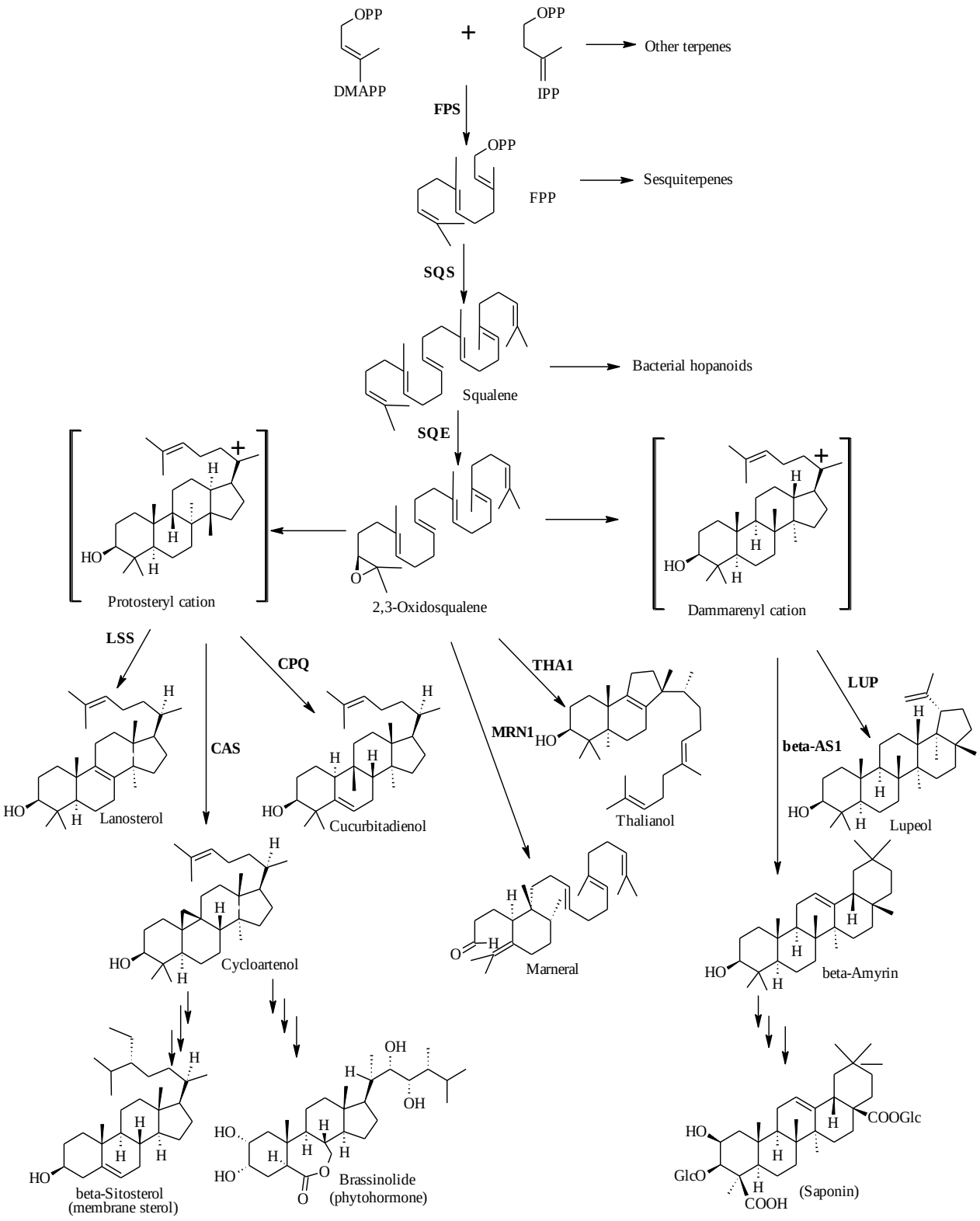


Figure 2.2: Plant triterpenoid biosynthesis pathway.

Figure 2.2 discloses a summary of biosynthetic pathways of different terpene groups from plants. During this process, IPP and DMAPP are converted to give diverse groups of terpenes that play important role in plant protection[4,5]. These components have complex and unique structures, resulting from both biotic and abiotic stress enhanced conditions and are stored in specific cells and/or organs of the plant and often accumulate in vacuoles[5].

2.2 Application of analytical techniques in natural products

Natural products have become the most potent source for new components that supplement modern drug discovery, food chemistry and chemical ecology[6]. However, natural product depends on analytical methods to effectively isolate and identify its constituents.

Phytochemical screening of bioactive component from various sources of natural products has been done using different methods of extraction depending on a particular natural product species and/or class of compounds. Most often methodologies used for extraction of plant secondary metabolites include distillation, expression and solvent extraction.

In addition, several plant products which include essential oils, alkaloids, saponins, tannins, flavonoids, glycosides, sterols and triterpenoids were extracted as a crude extract or mixture of compounds. All of the extracted constituents are scientifically detected with several chemical testing reagents, namely for, alkaloids; (Dragendorff's, Hager's, Wagner's and Mayer's test), flavonoids; (Shinoda's test), glycosides; (Molisch test), triterpenoids; (Liebermann- Burchard's test)[7]. The use of the above mentioned reagents is fundamental in natural products chemistry and drug discovery.

2.2.1 Chromatographic and spectrometric techniques

In recent years, various types of chromatography are being used to isolate different groups of plant secondary products, for example, column chromatography (CC), thin layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), supercritical fluid chromatography (SFC), capillary electrophoresis (CE), high perfo rmance liquid chromatography (HPLC) and ultra-high pressure liquid chromatography (UHPLC)[8–10]. These chromatographic techniques are essential in natural product elution and a recent review demonstrate their effectiveness in the isolation of various types of bioactive constituents used in food processing, cosmetics and pharmaceutical industries around the world[9].

In different fields of science including natural product chemistry, chromatographic techniques depend on different spectroscopic approach for evaluating structure of isolated compounds. Thus, numerous spectroscopies which include nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and fourier transform infrared (FT-IR) are used often and found to be competent in diverse fields of natural product chemistry[9]. These techniques give different information about the structure of isolated compound like FT-IR (used for functional group determination), NMR (used to determine the content and purity of a sample as well as its molecular structure) and MS (used for molecular mass determination).

Recent reviews also demonstrate the coupling of chromatography with spectroscopic techniques and with other analytical methods to form outstanding techniques which are used widely to separate a mixture and to identify the present constituents. Examples are, gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry

(LC-MS), liquid chromatography-nuclear magnetic resonance spectroscopy (LC-NMR) and liquid chromatography-ultraviolet-visible (LC-UV)[8,11,12].

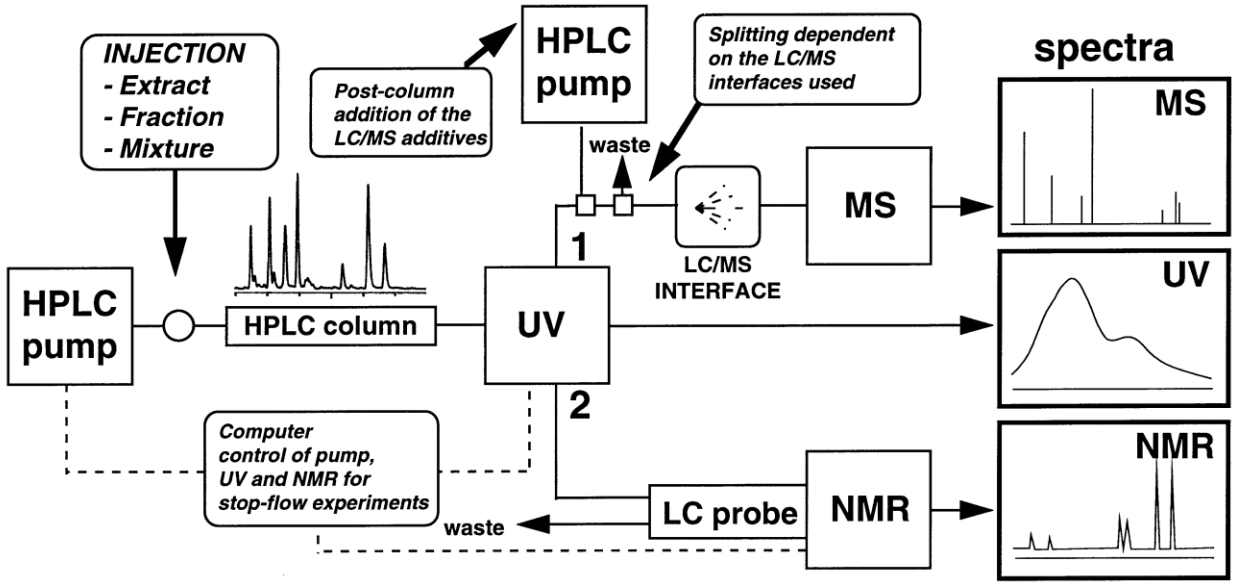


Figure 2.3: **Experimental setup used for LC–UV–MS (1) and LC–UV–NMR (2) analyses**

All these techniques have played a tremendous role in isolation and purification of different classes or groups of plant secondary metabolites.

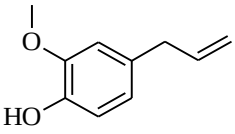
2.3 Introduction to Clove (*Syzygium aromaticum* (L.) Merr. & Perry)

Syzygium aromaticum L. (fam. Myrtaceae) a well-known aromatic plant. It is an evergreen plant that grows to a height ranging from 10 – 20 m, having large leaves and sanguine flowers in numerous groups of terminal clusters[13,14]. Cloves are harvested when they are 1.5 – 2 cm long[15,16]. Reports demonstrate that *Syzygium aromaticum* (L.) is indigenous to Moluccas island of Indonesia. It also grows naturally in India, Zanzibar, Pakistan, Sri Lanka and Bangladesh[17,18]. Clove possesses phytochemicals with antioxidant, anti-inflammatory, antibacterial and antifungal[19,20]. Major phytochemicals of clove comprises volatile oil, tannins, phenols, saponins and terpenes[18].

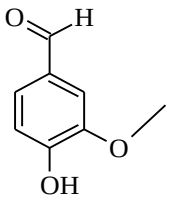
2.3.1 Volatile constituents

Volatile oils or essential oils are organic compounds or chemicals that have a high vapour pressure and low water solubility. They are noticeable group because of their smell. *S. aromaticum* (L.) which is a medicinal plant produces diverse types of volatile oils from stem, leaves, buds and fruit. Reports have shown that the major volatile group constituents of clove essential oils are phenols, phenylpropanoids, monoterpenoids and sesquiterpenoids[15,21–23].

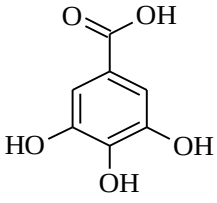
Plant-derived phenols and phenylpropanoids are the largest group of secondary metabolites produced by medicinal plants, mostly, for the protection against biotic or abiotic stresses[24]. Latest reports demonstrate that clove essential oils contain eugenol **(2.1)**, vanillin **(2.2)**, gallic acid **(2.3)**, coniferyl aldehyde **(2.4)** and cinnamaldehyde **(2.5)**. It has also been reported that these compounds are potentially useful in medicine because they exhibit antibacterial, antifungal, anti-inflammatory, antioxidant and antimutagenic properties[22,25–29].



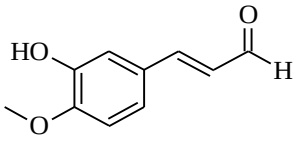
2.1



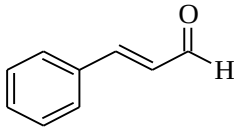
2.2



2.3



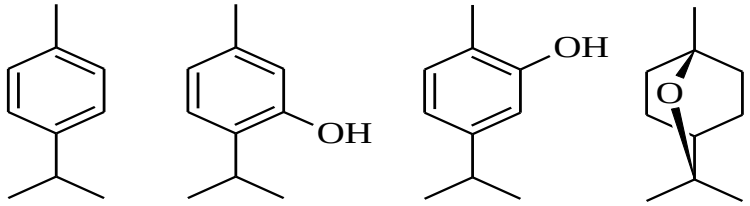
2.4



2.5

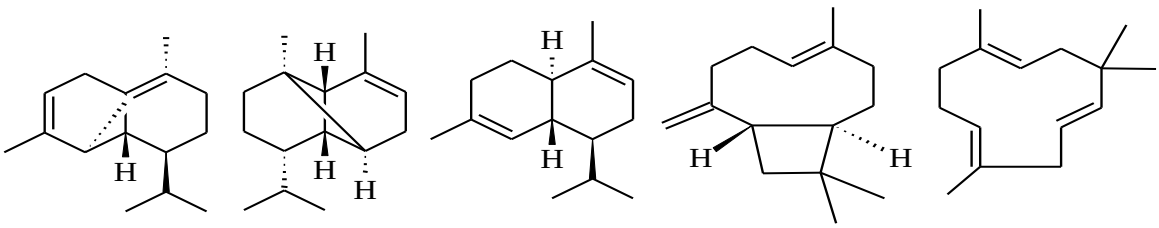
Monoterpenoids are another group of compounds found in clove oils. They are formed from two isoprene units and have 10 carbon atoms in the molecular formula. They are known to possess a variety of pharmaceutical activities such as anticarcinogenic, antitumor and antimicrobial activities[30,31]. Most monoterpenes are also known to have chemopreventive activity against rodent mammary, skin, liver, lung and forestomach cancers[17]. Some of the

known monoterpenes present in clove oils are ρ-Cymene **(2.6)**, thymol **(2.7)**, carvacrol **(2.8)**, and eucalyptol **(2.9)**[22].



2.6 **2.7** **2.8** **2.9**

Sesquiterpenes are compounds of three isoprene units and they have the general molecular formula C_{15} H_{24}[32]. A number of these compounds occur in nature as oxygenated forms such as alcohols, ketones, aldehydes, acids or lactones[33]. Naturally occurring sesquiterpenes are found in many essential oils of spice and food plants with recognized anti-inflammatory properties[34]. Some identified sesquiterpenes in clove oil include cubebene **(2.10)**, copaene **(2.11)**, α-cadinene **(2.12)**, β-caryophyllene **(2.13)** and α-humulene **(2.14)**[26,35,31]. This type of terpenes may be used as antibacterial, antiviral, anti-inflammatory and as a calming agent by aromatherapy. It has been reported that these compounds are also used to produce soap and perfumes[25,32,33].



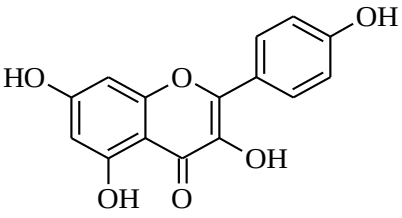
2.10 **2.11** **2.12** **2.13** **2.14**

2.3.2 Non- volatile constituents

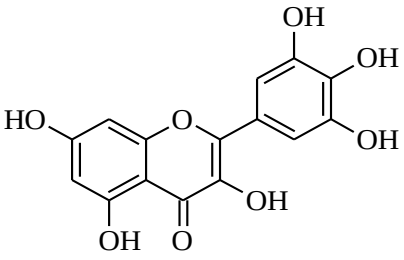
Nonvolatile essential oils are widely distributed in leaves, buds, fruits, bark and roots of several plants[36]. Nonvolatile essential oils are well-known as they produce a variety of

compounds, namely saponins, tannins, triterpenes and alkaloids are used on a daily basis as remedies[21].

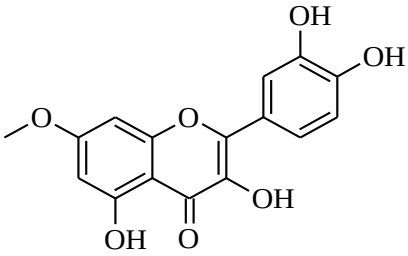
They are known as low molecular weight secondary metabolites, commonly distributed in green plant and are located in the cell vacuoles[37,38]. *S. aromaticum* (L.) plant is reported to produce a variety of flavonoid compounds such as kaempferol **(2.15)**, myricetin **(2.16)**, rhamnetin **(2.17)**, eugenitin **2.18**, biflorin **(2.19)** and eugenin **(2.20)**[13,16,22,27,39].



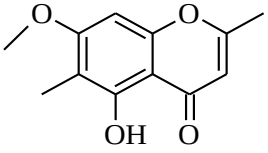
2.15



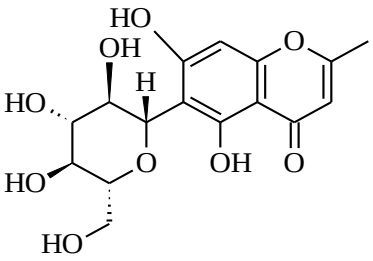
2.16



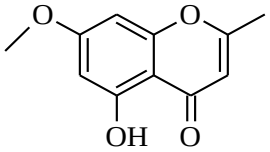
2.17



2.18

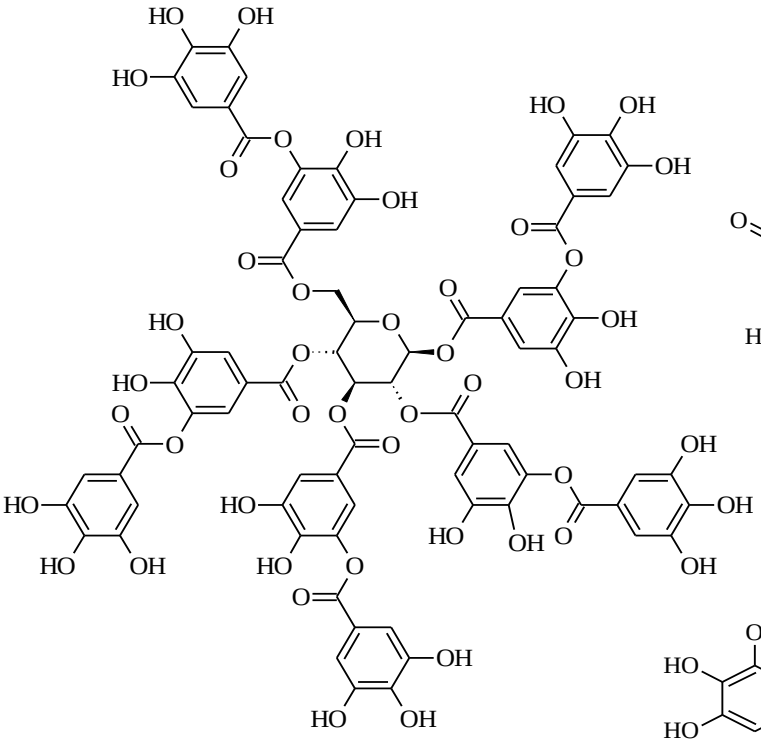


2.19

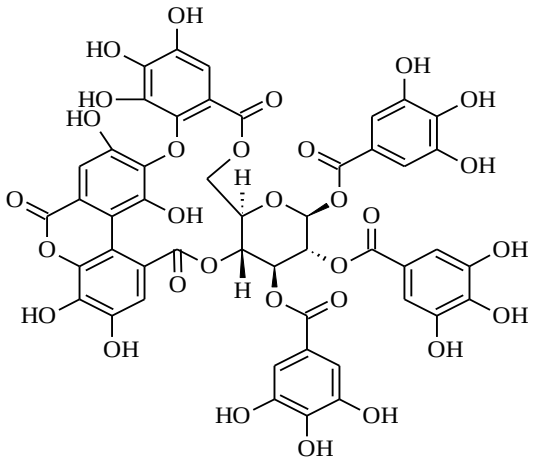


2.20

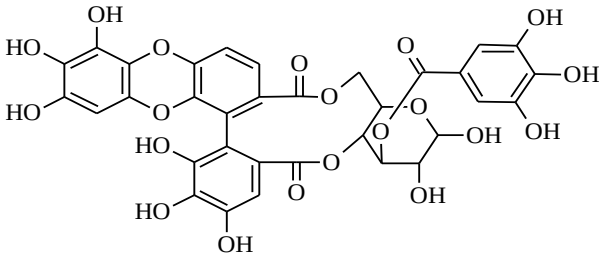
Tannins are complex polyphenolic compounds, generally found as plant secondary metabolites[40–42]. They are present in different parts of the plant, in leaves, twigs, flowers, fruits, and barks[41]. Numerous researches have demonstrated several biological activities and antibacterial-promoting effects of tannins[43,44]. This class of secondary metabolites is well-known for their antioxidants activity and chemoprotective potential[42,44]. One of the medicinal plants of the Myrtaceae family, which is *S. aromaticum* (L.), is reported to produce gallotannic acid **(2.21)**, biconin **(2.22)** and syzyginin B **(2.23)**[14,21,27].



2.21



2.22

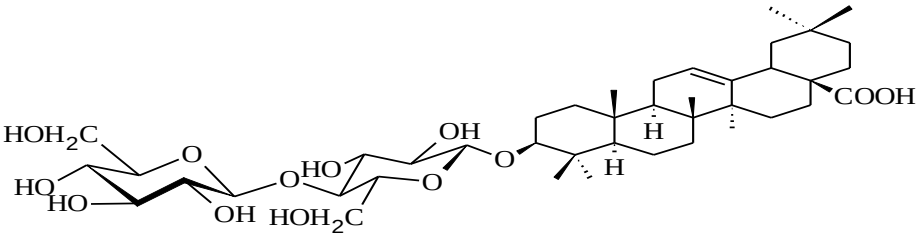


2.23

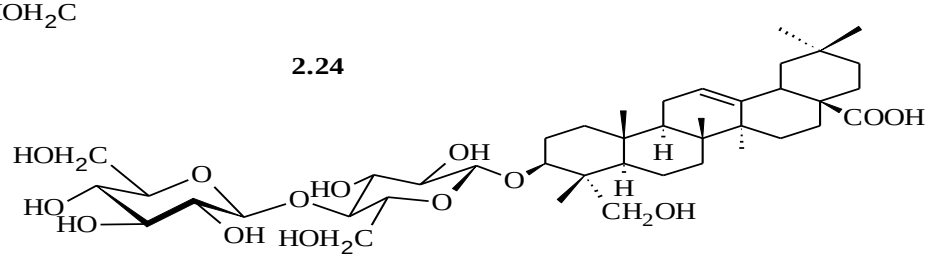
2.3.2.1 Saponins

Saponins are chemical compounds related to the triterpenoid group, such as triterpene saponins. Abad *et al*, (2007)[45], also state that saponins are correlated with steroidal groups, such as steroidal saponins. Augustin *et al*, (2012)[46] and Lee *et al*, (2012)[47], state that mostly, saponins are the most common group of compounds present in many medicinal plants and in a number of marine organisms. Saponins like oleanolic acid cellobioside **(2.24)**, hederagenin cellobioside **(2.25)**, gypsogenin cellobioside **(2.26)**, and 4-epi-hederagenin cellobioside **(2.27)**. Augustin *et al*, (2012)[46], also evaluate enzymes that take part in glucosylation of sapogenins in *Barbarea vulgaris*, the biological activity of saponins with a single C-3

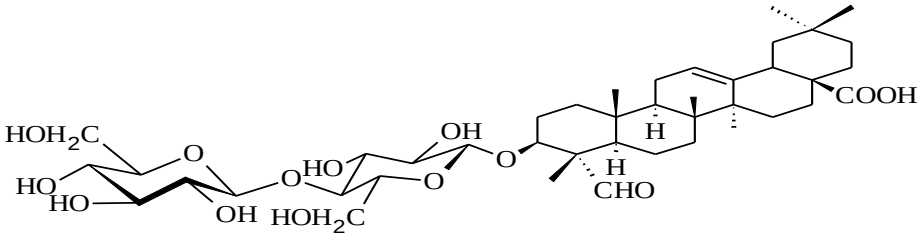
glucosyl group. their findings demonstrates that the resistance to the used herbivore is activated by C-3 monoglucosylation[46].



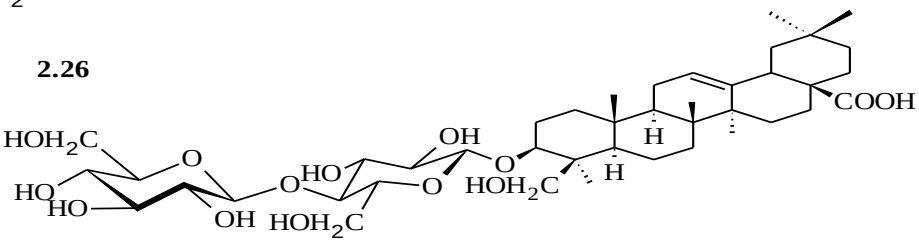
2.24



2.25



2.26

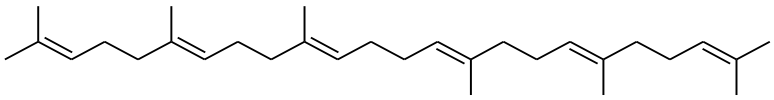


2.27

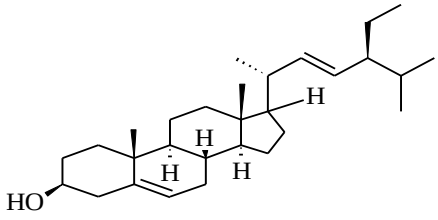
2.3.2.2 Triterpenoids

Triterpenoids are a large class of compounds that include steroids and sterols. They have a C_{30} carbon skeleton and most naturally occurring triterpenoid*s* are biosynthesized from squalene **(2.28)**[22,36,48–50]. This class of compounds is present in large quantities in plants and animal kingdom and some of them occur as pentacyclic triterpenes, namely, stigmasterol **(2.29)**, campesterol **(2.30)**, oleanolic acid **(2.31)**, betulinic acid **(2.32)**, ursolic acid **(2.33)**

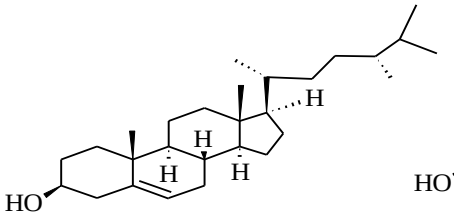
and maslinic acid **(2.34)**[13,14,21,27,51,52]. Oleanolic acid and ursolic acid are known for their anti-inflammatory, antitumor and antimicrobial properties[51].



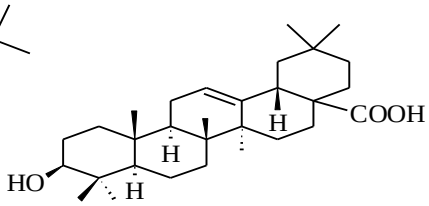
2.28



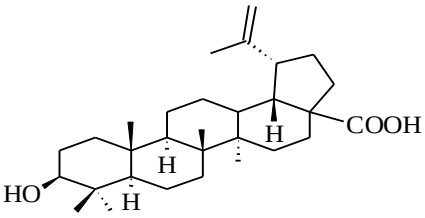
2.29



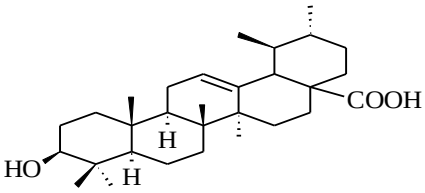
2.30



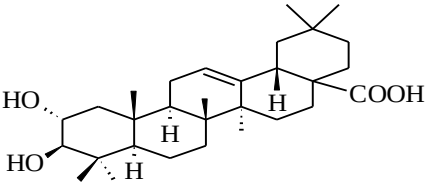
2.31



2.32



2.33



2.34

2.3.2.2.1 Some triterpenoids and their derivatives

In recent reviews, most pentacyclic triterpenoids are found as a promising source for a vast number of biological and therapeutic activities. Pentacyclic triterpenoids, namely, oleanolic acid (OA), betulinic acid (BA) and ursolic acid (UA) are commonly found in food and in some medicinal herbs[53]. OA and UA are isomers, they have similar chemical structure, but differ only in the position of the methyl group on (fifth ring) ring; OA has two methyl groups in (carbon- 20) C_{20} position whereas UA has a methyl group at C_{19} and C_{20} positions of their chemical structures, respectively[54].

2.3.2.2.2 Oleanolic acid and its derivatives

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid, OA) is a well-known pentacyclic triterpenoid compound isolated from various plants. Its diverse roles against human pathogens attracted the attention of researchers to work with this compound resulting in a large number of remarkable reports. Indeed, Liu *et al*, (2008)[53] stated that it provides extraordinary protection against acute and chronic liver injury, and can be used as an oral medication for human liver disorders. Other authors have reported on its anti-HIV, antibacterial, antiviral, anticancer, anti-angiogenic, anti-oxidation, anti-hyperglycemia, hepatoprotective, cardioprotective and anti-inflammatory activities[55–57].

Pollier and Goossens, (2012)[55], demonstrated that OA was a good precursor for further semi-synthetic modifications because of its multiple pharmacological activities, availability and low production cost. Oleanolic acid is modified in order to improve potency, selectivity and its pharmaceutical power. Moreover, as the chemical structure of oleanolic acid demonstrates a possible chemical modification on three "active" portions, the C_3 hydroxyl, the C_{12} to C_{13} alkene, and the C_{28} carboxylic acid; this has led to a sequence of new semi-synthetic oleanane triterpenoids derivatives for other biological activities[55,57].

Zhang *et al*, (2012)[57] reported a significant work on the semi-synthesis of OA structures at C_3 position (Figure 2.4). The study conducted by these authors proposes a synthetic probe that maybe used in isolating and identifying the target proteins of OA.

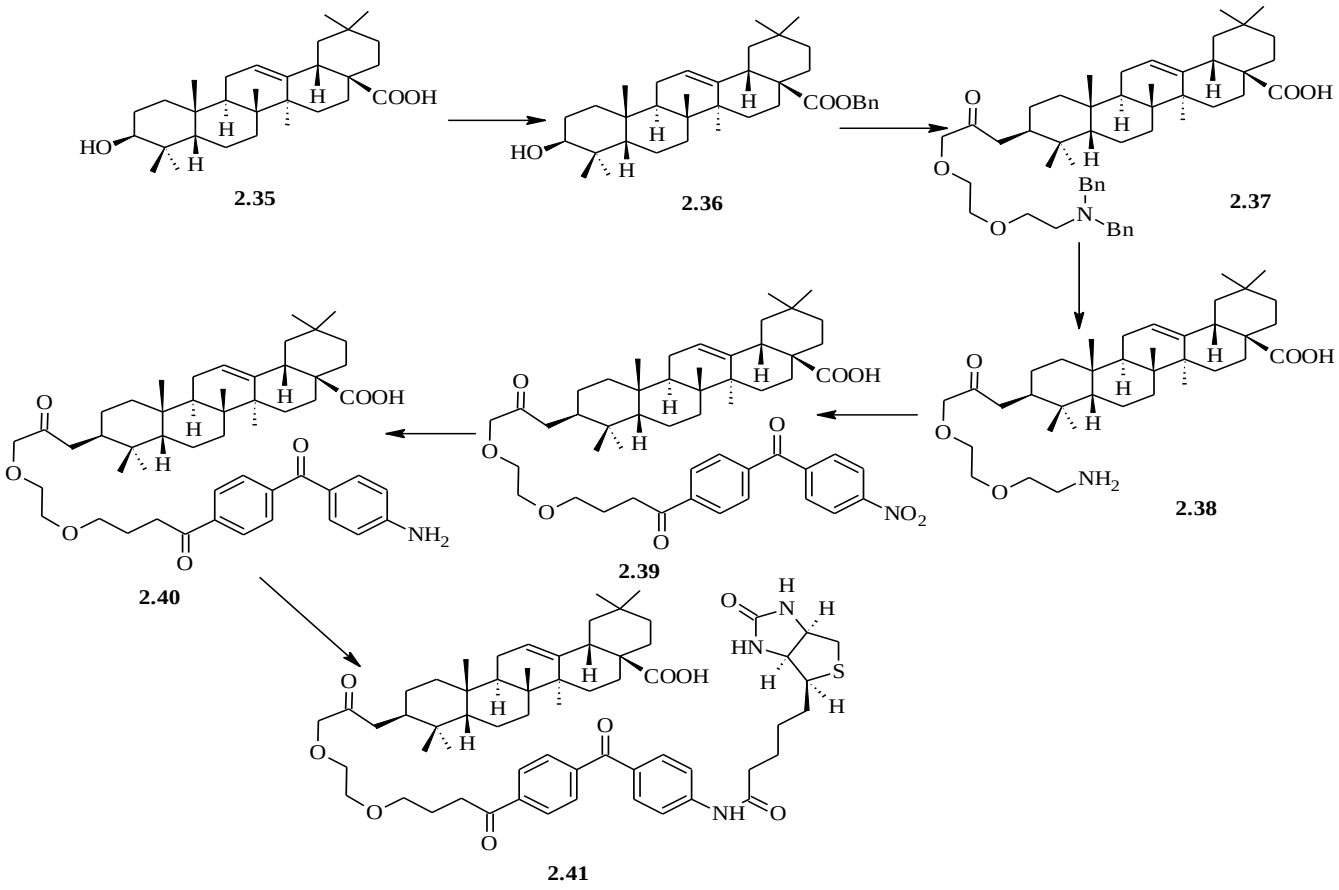


Figure 2 4: **Synthetic pathway of targeted protein derivatives of oleanolic acid**[57]

The above Figure 2.4 shows the semi-synthetic pathway for producing the targeted protein of OA. This synthetic pathway has led to (compounds 2.41 in Figure 2.4) with 2 folds less than the therapeutic functions of OA[57]. Moreover, oleanolic acid also shows an array of semi-synthetic derivatives that are being identified as novel inhibitors of glycogen phosphorylase (GP)[58]. Most of these derivatives are known to be active against cancerous cells. Indeed Chen *et al*, (2012)[59], reported 3-oxo-oleanolic acid (compound 2.42 in Figure 2.5) as an inhibitor of growth of cancer cell.

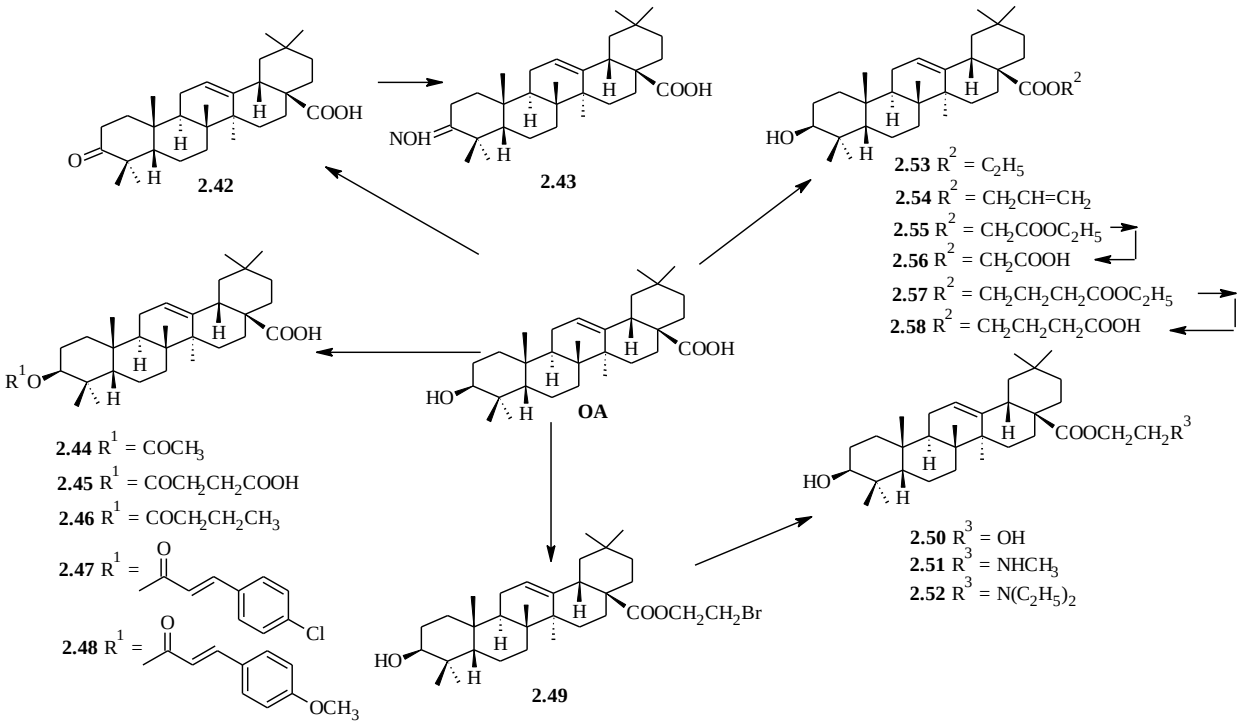


Figure 2.5: **Synthetic route of OA-derived compounds with GPa inhibition properties**[58].

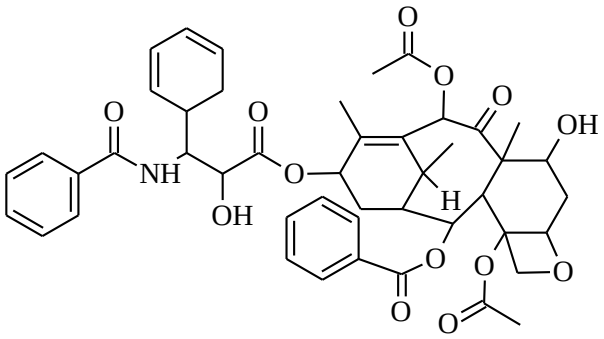
In the above Figure 2.5, Chen *et al*, (2006)[58], synthesized OA-derived compounds and evaluated their uses as novel GPa inhibitors. Due to this exceptional work, OA was found to be a nonprescription anti-hepatitis drug, may find its clinical use as a drug for fasting hyperglycemia[58].

2.4 Specific pharmaceutical evaluation of natural products

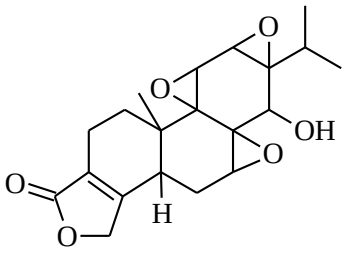
2.4.1 Anticancer and antitumor properties

The uncontrolled division of abnormal cell in parts of the human or animal is called tumors or cancer[60]. The WHO, (2009)[61], reports cancer as a leading cause of death in the world (7.4 million deaths; which is 13% of all deaths), this statistic came from different types of cancers, mainly, lung (1.3million deaths per year), stomach (803,000 deaths), colorectal (639,000 deaths), liver (610,000 deaths) and breast (519,000 deaths).

However, natural product is an interesting source of several constituents that may possess anticancer and antitumor activities. Kuttan *et al*, (2011)[62], agreed that natural products are the sources of quality compounds that have been used in the treatment of cancer, tumor and other diseases. Several terpenes gave an array of compounds with antitumor and anticancer activities like, triterpenoids (for example, glycyrrizic acid, ursolic acid, oleanolic acid, nomilin, betulinic acid and maslinic acid), the diterpene (like andrographolide) and the monoterpenes (such as limonene and perillic acid). Yang and Dou, (2010)[63], reports another terpene group, the tetraterpenoids as potential molecular targets for the treatment of breast and prostate cancer. Some terpeniod compounds reported to inhibit tumor cell proliferation and induce tumor cell death by preventing multiple cancer-specific targets are D-limonene, paclitaxel **(2.79)** and triptolide **(2.80)**.



2.79



2.80

2.4.2 Anti-inflammatory and analgesic properties

Inflammation is defined as a series of dynamic processes involving cytokine and various mediators, such as prostaglandins and leukotrienes. Pro-inflammatory mediators (e.g., tumor necrosis factor (TNF-α) and interleukin (IL1-β)) excite the production of many cytokines during the response to inflammation, including prostaglandins (PGs) and nitric oxide (NO)[64,65]. Inflammation Research Foundation (IRF) states that there are two types of

inflammation. The first type is classical inflammation that is associated with pain, redness, warmth and swelling. However, the pain itself is not the disease but is an indication of an ongoing disease process which might be asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, or psoriasis[66,67]. The second type of inflammation is silent or sub-clinical inflammation. Silent inflammation is beyond the perception of pain. The damage caused by silent inflammation is hard to be evaluated in its early stages because most patients seek for medical attention only when the disease process has become chronic. The damage caused by silent inflammation takes years to become a serious illness of classical inflammation such as tumor and other related diseases[66]. Due to the inflammation related diseases, a variety of safe and effective anti-inflammatory agents are available, including aspirin which is a primary drug for inflammation and other nonsteroidal anti-inflammatory drugs (NSADS), with many more drugs under development[67]. Dinarello, (2010)[65], demonstrated that cyclooxygenase (COX) enzyme COX-2, which is responsible for synthesizing inflammatory mediators called prostaglandins and thromboxanes can be prevented by aspirin. Until recently, aspirin was widely used as the therapeutic agent of choice for blocking the production of prostaglandins and thromboxanes. Importantly, anti-inflammatory drug investigations have revealed that a number of pentacyclic triterpenes possess anti-inflammatory activities[68]. An array of pentacyclic triterpenoid-derived compounds with anti-inflammatory activities like betulinic acid, betulin, ursolic acid and oleanolic acid have been assessed and shown to be highly effective after being modified into their derivatives[57,68,69].

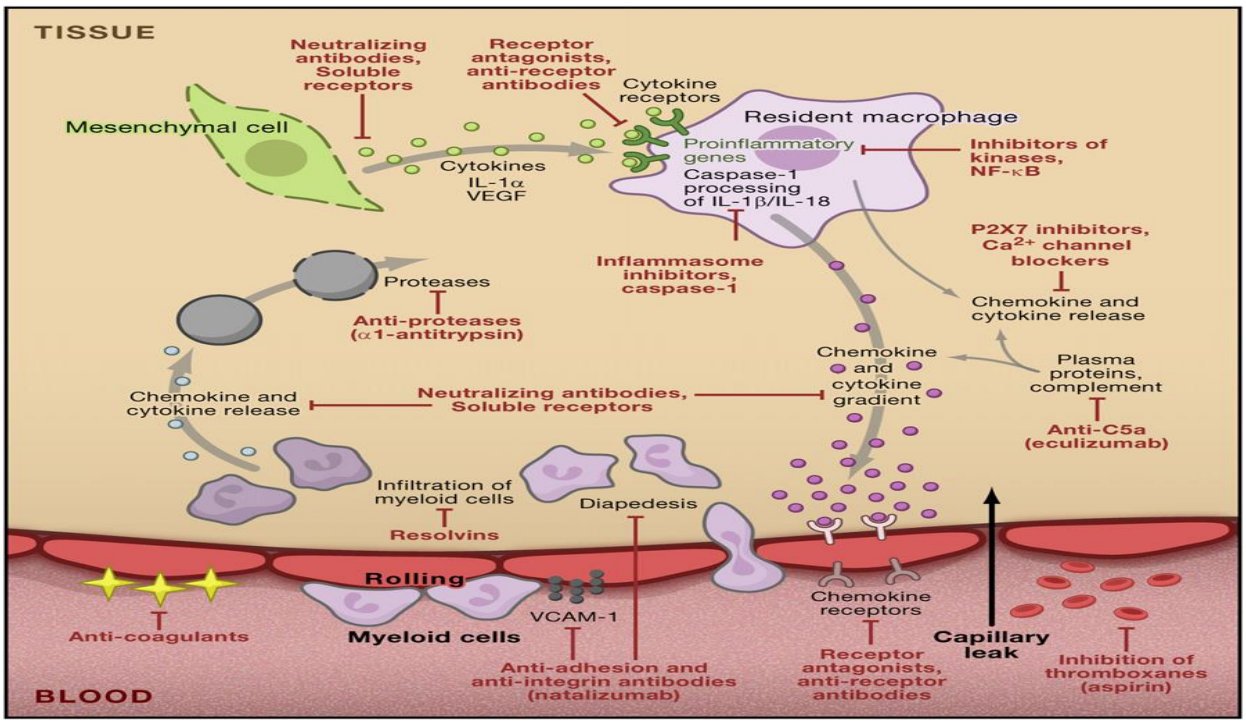


Figure 2.6: **Inflammatory pathway and blocking effect of anti-inflammatory agents**[65]

The anti-inflammatory effects of one pentacyclic triterpenoid compounds, namely, oleanolic acid (OA) have been evaluated and found to be very effective against some inflammation related diseases. However, an outstanding discovery of OA-derived compounds, namely, 2-cyano-3, 12-dioxoleana-1, 9(11)-dien-28-oic acid (CDDO) increased anti-inflammatory activities of OA[70]. In addition to this work, Lee *et al*, (2013)[71], found that OA activities had not been evaluated on lipopolysaccharide (LPS)-mediated inflammation. In their report, OA inhibited LPS-induced barrier disorder, expression of cell adhesion molecules (CAMs), adhesion/trans-endothelial migration, acetic acid-induced hyperpermeability and carboxymethylcellulose-induced leukocyte migration in vivo.

2.5 References

1. Rao SR, Ravishankar GA. (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances*. *20*(2):101–153.

2. Kamra DN, Agarwal N, Chaudhary LC. (2006). Inhibition of ruminal methanogenesis by tropical plants containing secondary compounds. *International Congress Series*. 1293:156–163.

3. Zhang W, Curtin C, Franco C. (2002). Towards manipulation of post-biosynthetic events in secondary metabolism of plant cell cultures. *Enzyme & Microbial Technology*. *30*(6):688–696.

4. Secondary metabolites: Biochemistry and role in plants. (2007). Available at: http://www.weizmann.ac.il/plants/aharoni/PlantMetabolomeCourse/May022007.pdf. Accessed June 17, 2013.

5. Oksman-Caldentey K-M, Inzé D. (2004). Plant cell factories in the post-genomic era: New ways to produce designer secondary metabolites. *Trends in Plant Science*. *9*(9):433–440.

6. Lopes NP, Pinto E, Niehues M, Gates PJ, Vessecchi R. (2012). Fundamentals and applications of analytical chemistry in natural products. *International journal of Analytical Chemistry*:1–2.

7. Saha S, Subrahmanyam EVS, Kodangala C, Shastry SC. (2011). Isolation and characterization of triterpenoids and fatty acid ester of triterpenoid from leaves of Bauhinia variegata. *Der Pharma Chemica*. *3*(4):28–37.

8. Singh S, Singh AK. (2012). Application of ultra high pressure liquid chromatography to natural products. *International Journal of Therapeutic Applications*. *3*:1–6.

9. Cozzolino D. (2009). Near infrared spectroscopy in natural products analysis. *Planta Medica*. *75*:746–756.

10. Conant R, Schauss AG, Qureshi I, Endres JR. Current good manufacturing practice for dietary supplements - role of the analytical laboratory. 2010. Available at: http://www.aibmr.com/resources/pdfs/AnalyticalLAB.pdf. Accessed October 1, 2013.

11. Nicoletti M. (2012). Phytochemical techniques in complex botanicals. The XXI century analytical challenge. *Journal of Chromatograph Separation Techniques*. *4*(186):1–9.

12. Wolfender J, Rodriguez S, Hostettmann K. (1998). Liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy for the screening of plant constituents. *Journal of Chromatography A. 794*:299–316.

13. Bhowmik D, Kumar KPS, Yadav A, Srivastava S, Paswan S, Dutta AS. (2012). Recent trends in Indian traditional herbs *Syzygium aromaticum* and its health benefits. *Journal of Pharmacognosy & Phytochemistry*. *1*(1):6–17.

14. Singh J, Baghotia A, Goel SP. (2012). *Eugenia caryophyllata* Thunberg (family Myrtaceae): A review. *International Journal of Research in Pharmaceutical & Biomedical Sciences*. *3*(4):1469–1475.

15. Alma MH, Ertas M, Nitz S, Kollmannsberger H. (2007). Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum* L.). *Bioresearch*. *2*(2):265–269.

16. Bagavan A, Rahuman AA, Kamaraj C, et al. (2011). Contact and fumigant toxicity of hexane flower bud extract of *Syzygium aromaticum* and and its compounds against *Pediculus humanus capitis* (Phthiraptera: *Pediculidae*). *Parasitology Research*. *109*(5):1329–1340.

17. Arung TE, Matsubara E, Kumusa WI, Sukaton E, Shimizu K, Kondo R. (2011). Inhibitory components from the buds of clove (*Syzygium aromaticum*) on melanin formation in B16 melanoma cells. *Fitoterapia*. *82*(2):198–202.

18. Dashti-R MH, Morshedi A. (2009). The effects of *Syzygium aromaticum* (clove) on learning and memory in mice. *Asian Journal of Traditional Medicines*. *4*(4):128–133.

19. Banerjee S, Das S. (2005). Anticarcinogenic effects of an aqueous infusion of cloves on skin carcinogenesis. *Asian Pacific Journal of Cancer Prevention*. *6*(3):304–308.

20. Kim Y, Lee H. (2012). Anticandidal effect of *Syzygium aromaticum* on biofilm formation, cell surface hydrophobicity, and cell cycle. *Journal of Medicinal Plants Research*. *6*(10):1926–1934.

21. Mehrotra S, Srivastava AK, Nandi SP. (2010). Comparative antimicrobial activities of neem, amla, aloe, assam tea and clove extracts against *Vibrio cholerae, Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Journal of Medicinal Plants Research*. *4*(22):2393–2398.

22. Bhuiyan NI, Begum J, Nandi NC, Akter F. (2010). Constituents of the essential oil from leaves and buds of clove (*Syzigium caryophyllatum* (L .) Alston). *African Journal of Plant Science*. *4*(11):451–454.

23. Hosseini M, Asl MK, Rakhshandeh H. (2011). Analgesic effect of clove essential oil in mice. *Avicenna Journal of Phytomedicine*. *1*(1):1–6.

24. Ivanauskas L, Jak V, Radu J, Luko A, Baranauskas A. (2007). Evaluation of phenolic acids and phenylpropanoids in the crude drugs. *Medicina (Kaunas, Lithuania)*. *44*(1):6–9.

25. Saeed S, Tariq P. (2008). In vitro antibacterial activity of clove against gram negative bacteria. *Pakistan Journal of Botany*. *40*(5):2157–2160.

26. Kumar P, Jaiswal P, Singh VK, Singh DK. (2011). Medicinal, therapeutic and pharmacological effects of *Syzygium aromaticum* (Laung). *Pharmacologyonline*. *1*:1044–1055.

27. Hema R, Kumaravel S, Sivasubramanian C. (2010). GC-MS study on the potentials of *Syzygium aromaticum*. *Researcher*. *2*(12):1–4.

28. Miyazawa M, Hisama M. (2003). Antimutagenic activity of phenylpropanoids from clove (*Syzygium aromaticum*). *Journal of Agricultural & Food Chemistry*. *51*(22):6413–6422.

29. Pathak SB, Niranjan K, Padh H, Rajani M. (2004) TLC densitometric method for the quantification of eugenol and gallic acid in clove. *Chromatographia*. *60*(3):241–244.

30. Custódio JBA, Ribeiro M V, Silva FSG, Machado M, Sousa MC. (2011). The essential oils component p -cymene induces proton leak through Fo-ATP synthase and uncoupling of mitochondrial respiration. *Journal of Experimental Pharmacologyy*. *3*:69–76.

31. Chaieb K, Hajlaoui H, Zmantar T, Kahla-nakbi A B, Mahdouani K, Bakhrouf A. (2007). The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): A short review. *Phytotherapy Research*. *21*:501–506.

32. Bouvier-Brown NC, Holzinger R, Palitzsch K, Goldstein AH. (2007). Quantifying sesquiterpene and oxygenated terpene emissions from live vegetation using solid-phase microextraction fibers. *Journal of Chromatography A*. *1161*(1):113–20.

33. Merfort I. (2002). Review of the analytical techniques for sesquiterpenes and sesquiterpene lactones. *Journal of Chromatography A*. *967*(1):115–130.

34. Horváth B, Mukhopadhyay P, Kechrid M, et al. (2012). β-Caryophyllene ameliorates cisplatin-induced nephrotoxicity in a cannabinoid 2 receptor-dependent manner. *Free Radical Biology & Medicine*. *52*(8):1325–1333.

35. Bhuiyan NI, Begum J, Nandi NC, Akter F. (2010). Constituents of the essential oil from leaves and buds of clove (*Syzigium caryophyllatum* (L.) Alston). *African Journal of Plant Science*. *4*(11):451–454.

36. Gurib-Fakim A. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*. *27*(1):1–93.

37. Sisa M, Bonnet SL, Ferreira D, Van der Westhuizen JH. (2010). Photochemistry of flavonoids. *Molecules*. *15*(8):5196–5245.

38. Samanta A, Das G, Das SK. (2011). Roles of flavonoids in plants. *International Journal of Pharmaceutical Scientist*. *6*(1):12–35.

39. Nassar MI, Gaara AH, El-Ghorab AH, et al. (2007). Chemical constituents of clove (*Syzygium aromaticum*, Fam. Myrtaceae) and their antioxidant activity. *Revista Latinoamericana de Quimica*. *35*(3):47–57.

40. Okuda T, Ito H. (2011). Tannins of constant structure in medicinal and food plants-hydrolyzable tannins and polyphenols related to tannins. *Molecules*. *16*(12):2191–2217.

41. Alokan J, Aletor V. (1997). Plant tannins-their role in forage legume quality. *internationalgrasslands.org*. Available at: http://www.internationalgrasslands.org/files/igc/publications/1997/1-08-025.pdf. Accessed June 25, 2014.

42. Gadžo D, Ć MD, Ć TG, Štrekelj P. (2010). Comparison of tannin concentration in young plants of common and tartary buckwheat. *Acta Agriculturae Slovenica*. *95*(1):75–78.

43. Min BR, Pinchak WE, Merkel R, Walker S, Tomita G, Anderson RC. (2008). Comparative antimicrobial activity of tannin extracts from perennial plants on mastitis pathogens. *Scientific Research & Essay*. *3*(2):66–73.

44. Atanassova M, Christova-Bagdassarian V. (2009). Determination of tannins content by titrimetric method for comparison of different plant species. *Journal of the University of Chemical Technology & Metallurgy*. *44*(4):413–415.

45. Abad MJ, Ansuategui M, Bermejo P. (2007). Active antifungal substances from natural sources. *Arkivoc*. *7*:116–145.

46. Augustin JM, Drok S, Shinoda T, et al. (2012). UDP-glycosyltransferases from the UGT73C subfamily in *Barbarea vulgaris* catalyze sapogenin 3-O-glucosylation in saponin-mediated insect resistance resistance. *Plant Physiology*. *160*(4):1881–1895.

47. Lee J, Lim S, Kang S-M, et al. (2012). Saponin inhibits hepatitis C virus propagation by up-regulating suppressor of cytokine signaling 2. *PloS one*. *7*(6):e39366.

48. James JT, Dubery IA. (2009). Pentacyclic triterpenoids from the medicinal herb, *Centella asiatica* (L.) Urban. *Molecules*. *14*(10):3922–41.

49. Huang D, Ding Y, Li Y, Zhang W, Fang W, Chen X. (2006). Anti-tumor activity of a 3-oxo derivative of oleanolic acid. *Cancer Letters*. *233*(2):289–296.

50. Finlay HJ, Honda T, Gribble GW. (2002). Synthesis of novel [3, 2-b] indole fused oleanolic acids as potential inhibitors of cell proliferation. *Arkivoc*. *12*:38–46.

51. Brendolise C, Yauk YK, Eberhard ED, et al. (2011). An unusual plant triterpene synthase with predominant α-amyrin-producing activity identified by characterizing oxidosqualene cyclases from Malus × domestica. *The FEBS Journal*. *278*(14):2485–2499.

52. Jäger S, Trojan H, Kopp T, Laszczyk MN, Scheffler A. (2009). Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. *Molecules*. *14*(6):2016–2031.

53. Liu J, Wu Q, Lu YF, Pi J. (2008). New insights into generalized hepatoprotective effects of oleanolic acid: Key roles of metallothionein and Nrf2 induction. *Biochemical Pharmacology*. *76*(7):922–928.

54. Feng J-H, Chen W, Zhao Y, Ju X-L. (2009). Anti-tumor activity of oleanolic, ursolic and glycyrrhetinic acid. *The Open Natural Products Journal*. *2*(1):48–52.

55. Pollier J, Goossens A. (2012). Oleanolic acid. *Phytochemistry*. *77*:10–15.

56. Aisha AFA, Abu-salah KM, Alrokayan SA, Siddiqui MJ, Ismail Z, Majid AMSA. (2012). *Syzygium aromaticum* extracts as good source of betulinic acid and potential anti-breast cancer. *Brazilian Journal of Pharmacognosy*. *22*(2):335–343.

57. Zhang L, Zhang Y, Dong J, Liu J, Zhang L, Sun H. (2012). Design and synthesis of novel photoaffinity probes for study of the target proteins of oleanolic acid. *Bioorganic & Medicinal Chemistry Letters*. *22*(2):1036–1039.

58. Chen J, Liu J, Zhang L, et al. (2006). Pentacyclic triterpenes. Part 3: Synthesis and biological evaluation of oleanolic acid derivatives as novel inhibitors of glycogen phosphorylase. *Bioorganic & Medicinal Chemistry Letters*. *16*(11):2915–2919.

59. Chen L, Wu J-B, Lei F, Qian S, Hai L, Wu Y. (2012). Synthesis and biological evaluation of oleanolic acid derivatives as antitumor agents. *Journal of Asian Natural Products Research*. *14*(4):355–363.

60. The History of Cancer. Available at: http://www.cancer.org/acs/groups/cid/documents/webcontent/002048-pdf.pdf. Accessed August 6, 2013.

61. World Heath Organisation. Cancer. (2004). Available at: http://www.who.int/nmh/publications/fact_sheet_cancers_en.pdf. Accessed August 6, 2013.

62. Kuttan G, Pratheeshkumar P, Manu KA, Kuttan R. (2011). Inhibition of tumor progression by naturally occurring terpenoids. *Pharmaceutical Biology*. *49*(10):995–1007.

63. Yang H, Dou PQ. (2010). Targeting apoptosis pathway with natural terpenoids: Implications for treatment of breast and prostate cancer. *Current Drug Targets*. *11*(6):733–744.

64. Tsai JC, Peng WH, Chiu TH, Lai SC, Lee CY. (2011). Anti-inflammatory effects of *Scoparia dulcis* L. and betulinic acid. *The American Journal of Chinese Medicine*. *39*(5):943–956.

65. Dinarello CA. (2010). Anti-inflammatory agents: Present and future. *Cell*. *140*(6):935–950.

66. Inflammation Research Foundation. Anti-inflammatory medicine : Dietary modulation of Eicosanoids. Available at: http://www.drsears.com/portals/6/documents/inflammation medical brochure.pdf. Accessed November 9, 2013.

67. Gautam R, Jachak SM. (2009). Recent developments in anti- inflammatory natural products. *Medicinal Research Reviews*. *29*(5):1–54.

68. Cichewicz RH, Kouzi SA. (2004). Chemistry, biological activity and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Medicinal Research Reviews*. *24*(1):90–114.

69. Yogeeswari P, Sriram D. (2005). Betulinic acid and its derivatives: A review on their biological properties. *Current Medicinal Chemistry*. *12*(6):657–666.

70. Liu J. (2005). Oleanolic acid and ursolic acid: research perspectives. *Journal of Ethnopharmacology*. *100*(1):92–94.

71. Lee W, Yang EJ, Ku SK, Song KS, Bae JS. (2013). Anti-inflammatory effects of oleanolic acid on LPS-induced inflammation in vitro and in vivo. *Inflammation*. *36*(1):94–102.

CHAPTER THREE

PHYTOCHEMICAL INVESTIGATION OF *SYZYGIUM AROMATICUM* (L.) MERRILL & L. M. PERRY

3.1 Introduction

S. aromaticum (L.) has been found to be one of most valuable spices both for medicinal and culinary purposes since the first century. The first indication of the use of fragrant clove (*S. aromaticum* (L.) was during the ancient Chinese Han dynasty lasting from 207 B.C. to 220 A.D. The use of this plant was also reported in Europe around the 4th century, when commercial trading really started with the Arabs. This plant was also found in Moluccas Island or Indonesia in the 16th century[1]. Additionally, the importance of *S. aromaticum* (L.) was considered in traditional Chinese medicine and found to have medicinal properties such as stimulant against digestive disorders and diarrhea. It also provides essential oil that shows antibacterial properties[2]. Moreover, clove essential oil from different parts of *S. aromaticum* (L.) plant was utilized in a number of pharmaceuticals, foods, and cosmetic products because these oils effectively inhibit the growth of a wide range of microorganisms[2,3].

3.2 Description of *S. aromaticum* (L.) Merr. & Perry

Syzygium aromaticum (*Eugenia caryophyllus*) is an evergreen plant that grows to a height ranging from 10 – 20 m, having large leaves and sanguine flowers in numerous groups of terminal clusters. This plant belongs to Myrtaceae family, its flower buds start off as a pale color and gradually become green on maturation, after which they develop into bright red when they are ready for collecting. Cloves are harvested when they are 1.5 – 2 cm long, and

consist of a long calyx, terminating in four spreading sepals, and four unopened petals which form a small ball in the center[4,5].

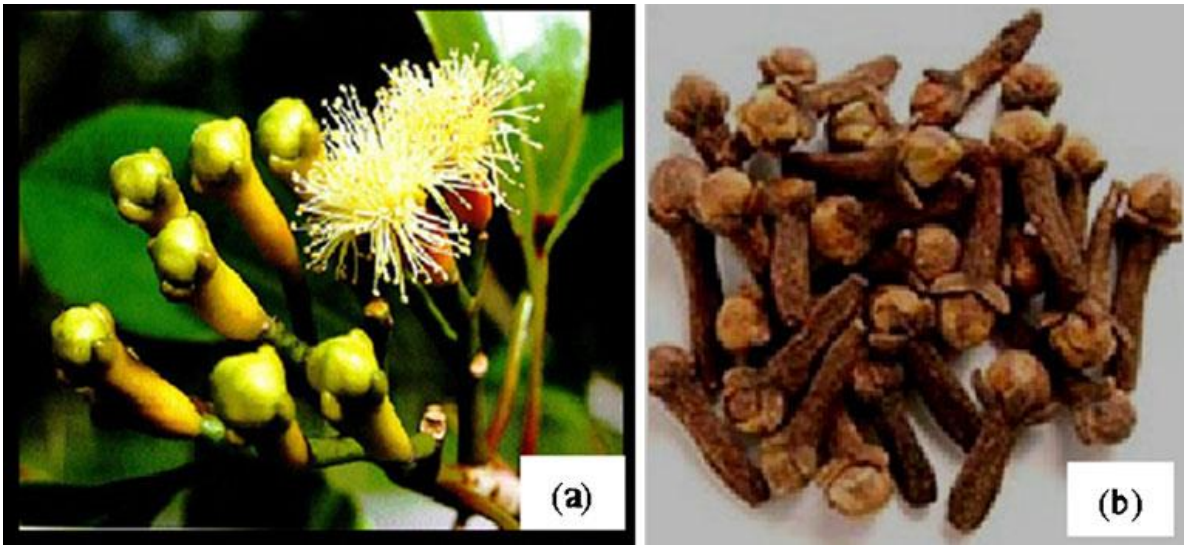


Figure 3.1: **Cloves (*S. aromaticum*) (a) fresh flower buds and (b) Dried Clove buds**[5]

S. aromaticum (L) plant is native to the Maluku islands in Indonesia and used as a spice in cuisines all over the world. They are harvested primarily in Indonesia, India, Madagascar, Zanzibar, Pakistan, Sri Lanka and Bangladesh[4–6]. *S. aromaticum* (L.) is used in Ayurveda, Chinese medicine and Western Herbalism[7].

3.3 Phytomedicinal study of *S. aromaticum* (L.)

Clove and clove oil have been used since ancient times to provide both therapeutic, cosmetics, and culinary needs[8]. The importance of clove as spice is underscored by reports as the second most traded spice worldwide[9]. Indeed, Alma *et* al, (2007)[4], also reported that it is used in cigarettes (known as Kreteks) in Indonesia and occasionally as an additive to coffee in the West; is also used as marijuana spliffs when mixed with marijuana. Essential oil obtained from the dried flower buds of *S. aromaticum* (L) is also used traditionally to

treat burns, for dental care to relieve pain and treat gum infections when used in high concentrations, to treat respiratory and digestive problems[10].

3.4 Experimental study

The following analytical techniques were employed in this study, namely, CC with silica gel 60 (0.063-0.200 mm), TLC plates: FT-IR spectra were assessed on diamond ATR Bruker Tensor 27; the MS spectra with Absciex 5600 Tripple TOF; NMR spectra were recorded on Bruker 400 and 600 MHz topspin; Mp. was assessed using the digital Melting Point apparatus

3.4.1 Plant identification

Flower buds of *S. aromaticum* were purchased from the spice market in Durban and were authenticated by Mr. Pravin Poorun a senior plant taxonomist, of the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus. A voucher specimen OO4 was deposited at the University Herbarium.

3.4.2 Plant preparation and solvent extraction

Dried flower buds of *S. aromaticum* (L.) were pulverized into fine powder with electric blender. A dried powder sample (1949.39 g) was sequentially extracted twice with different organic solvents, namely, n-hexane, ethyl acetate, dichloromethane and methanol. The mixture was placed on a shaker (1500 ml of a solvent and clove sample was shaken for five days). A solution was filtered with suction, concentrated with rotary evaporator then air-dried at room temperature.

3.4.3 Isolation method

The ethyl acetate extract (15.535 g) was packed into a column chromatography using silica gel 60 (0.063-0.200 mm). The column was eluted with series of solvent mixture; n-hexane: ethyl acetate (9:1, 8:2, 7:3, 6:4, 4:6) respectively and the fractions were visualized on a Thin Layer Chromatography (TLC) plate with Anisaldehyde/sulphuric acid spray reagent. The single spots isolates were characterized for structural elucidation using mass spectrometry, melting point, Fourier transform infrared spectroscopy and nuclear magnetic resonance spectrometry.

3.5 Animal study

Wistar rats (180-250 g) and Swiss mice (20-35 g) of either sex were use during the experiment as described in the literature[11,12]. These animals were housed with a 12 hours light/dark cycle and had free access to food and water. Approval for these studies was obtained from the Ethical Committee of the Walter Sisulu University, Reference No: Ethics 0009-07.

3.5.1 Formalin-induced pain test

The formalin test was carried out as described by Emim *et al.* (2000)[13] with some modifications. Eight groups of mice (six mice per group), were orally treated with ibuprofen (100 mg/kg), eugenol, oleanolic acid, and maslinic acid (40 mg/kg) and control with normal saline (0.09% NaCl). Formalin (1% v/v) was injected in the right hind paw of the animals, one hour post treatment. The number of paw licking was counted for 0–5 minutes (neurogenic phase) and 10–30 minutes (inflammatory phase) after formalin administration.

3.5.2 Tail flick pain test

A method used by Nkeh-Chungag *et al.* (2010)[14] was used for the tail flick (thermal-induced pain) test. Each treatment group was constituted of 6 animals. The animals were lightly restrained and posterior third of tail allowed to lie over a glass window slit of the tail flick machine. Baseline latencies for tail withdrawal from thermal heat source were obtained for each animal using the Ugo Basile Tail Flick Machine (model 37360). After which drugs were administered orally at a dose of 40 mg/kg for eugenol, oleanolic acid, maslinic acid, 100 mg/kg for ibuprofen and control was treated with normal saline. Tail flick latencies were again assessed hourly for 5 hrs after drug administration.

3.5.3 Egg albumin-induced Inflammatory

Baseline right hind paw volume was measured using the Ugo Basile plethysmometer. This was followed by oral treatment of animals with 40 mg/kg of all compounds and ibuprofen (100 mg/kg) while control was treated with 0.09% of NaCl. Thirty minutes post treatment, acute inflammation was induced by sub-plantar injection of fresh egg-albumin (0.1 ml, 50% v/v in saline) into a right hind paw. Paw volumes were at 30min, 1, 2, 3 and 4 hours after injection of the phlogistic agent.

3.5.4 Statistical analysis

The statistical package, GraphPad Instat® was used to analyse all results. The T-test was performed and 2 tail p - values obtained. Results were initially compared with controls and later with OA results from the OA treated group, $p < 0.05$ was considered significant. Values were expressed as mean ± SEM where n=6.

3.6 Results

3.6.1 Structural elucidation

Column chromatography led to the isolation of three distinct compounds from ethyl acetate extract of *S. aromaticum* flower buds. The fractions were eluted in different solvent ratios and identified as oleanolic acid, eugenol and maslinic acid respectively (Figure 3.2). Tables 3.1 provide information about the nature of the isolated compounds.

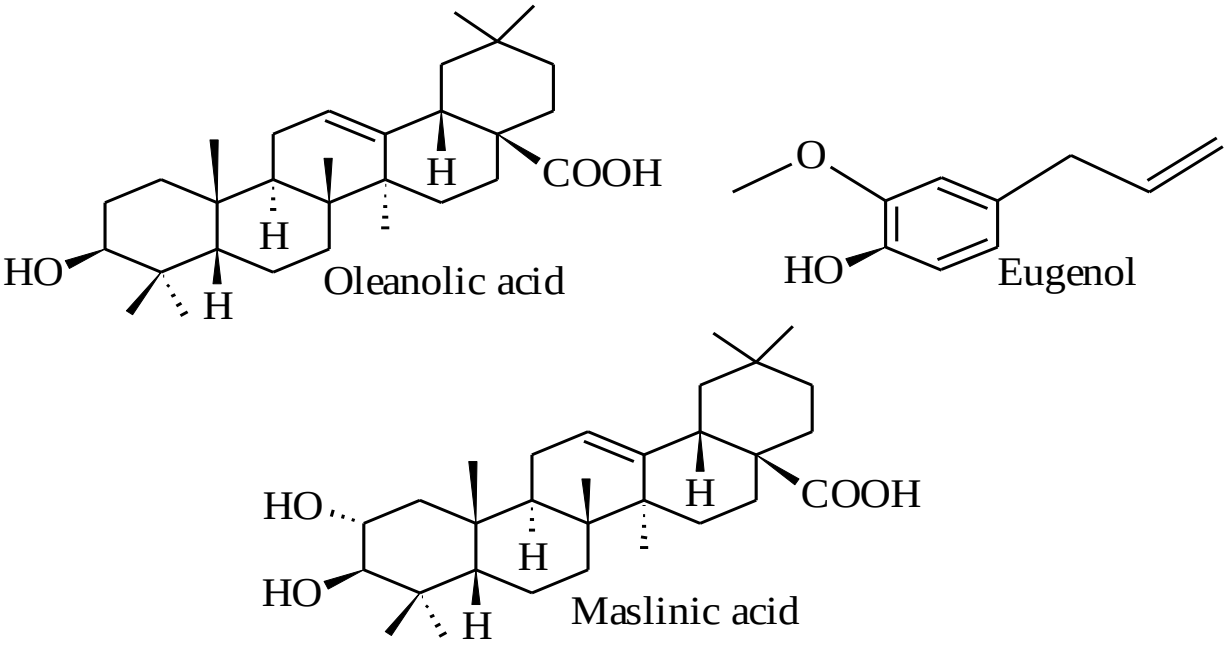


Figure 3.2: Shows the chemical structures of isolated compounds

The FT-IR spectrum (Figure 3.1.1, Appendix 1) of eugenol, a volatile oily compound with molecular mass of 164.15 had different absorption bands at 3515 - 3456 (-OH, phenol stretching), 3076 - 3004 (C=CH, stretching), 2975-2841 (-CH, aliphatic stretching), 1763-1463 (-C=C, aromatic stretching), 1367 (C-O, bending), 994 and 911 (-C=CH_2, vinyl bending) (Table 3.2). It was fully identified with GC-MS and the machine library confirmed that this compound was eugenol (4-Allyl-2-methoxyphenol).

Table 3.1: Physical data for maslinic acid, eugenol and oleanolic acid

Nature	Maslinic acid	Eugenol	Oleanolic acid
Description	White powder	Oil	White amorphous powder
Yield	0.07%	0.31%	0.20%
Mp	248-249^{O}C	-------	294-295^{O}C
Molecular mass	472.45	164.15	456.46

Maslinic acid (MA) is a white powder with Mp. 248-249^{O}C (Table 3.1), molecular mass 472.45, the FT-IR spectrum (Figure 3.2.1, Appendix 2) interpreted on Table 3.2 displayed major peaks at 3420 (-OH, free alcohol), 2940, 2866 (aliphatic -CH), 1738-1689 (-C=O), 1458 (-C=C) and 1031 (-C-O). The elucidation of FT-IR spectrum (Figure 3.3.1, Appendix 3) of oleanolic acid (OA) on Table 3.2 displayed the following major peaks at 3406 (–OH) stretching of a free alcohol, 2835, 2864 aliphatic (-CH) stretching of an alkane, 1688 (-C=O) bending of a carboxylic acid and 1460 (-C=C) stretching of an alkene group.

Table 3.2: Interpretation of FT-IR spectrum of isolated terpenoids

Vibration mode	Chemical functional groups				
Compound	$\nu_{C\text{-}OH}$	$\nu_{C\text{-}H}$	$\nu_{C=O}$	$\nu_{C=C}$	$\nu_{C\text{-}O}$
Maslinic acid	3420	2940, 2866	1738-1689	1458	1031
Eugenol	3515-3456	3076-3004, 2975, 2841	-----	1763, 1638, 1605, 1510	1367
Oleanolic acid	3406	2935, 2864	1688	1460	1034

Maslinic and oleanolic acid were further elucidated with ^{1}H, ^{13}C, DEPT, COSY, NOESY, HSQC and HMBC-NMR spectroscopy. Interpretation of (Appendix 2 and 3) the ^{1}H and ^{13}C-NMR spectra of MA and OA (Figure 3.1.3, 3.1.4, 3.2.3 and 3.2.4 respectively) is illustrated

on Table 3.3 showed that both compounds have 30 carbon structures[15]. The comparison of ^{13}C-NMR spectra (Figure 3.2.3 and 3.3.3, Appendix 2 and 3, respectively) confirmed that MA and OA showed almost the same signal however; MA gave an extra oxygenated carbon signal at 68.3 ppm due to the presence of –OH group at C_2 of its chemical structure (Table 3.3). The effect of –OH group on C_2 of ^{1}H-NMR spectrum of MA (Figure 3.2.3, Appendix 2) has caused a deshielding of a proton on C_3 to 3.65 ppm compared to that of OA 3.43 ppm. In general, the ^{13}C and ^{13}C DEPT-NMR spectrum of MA (Figure 3.25, Appendix 2) showed signals for 7 CH_3 groups, 9 CH_2 groups, 6 CH groups and 8 quaternary carbons (Table 3.3). Three oxygenated carbon that is two alcohol signals and carboxylic group at 68.09, 83.06 and 180.5 ppm respectively were also displayed by ^{13}C NMR spectrum of MA. The structural difference between OA and MA is position 2, on ring A of the chemical structure. Instead of –OH group at position 2 in OA structural representation displayed a methylene group, thereby, having 7 CH_3, 10 CH_2, 5 CH and 8 quaternary carbons signals two oxygenated carbon included at 80.92 and 183.65 ppm for an alcohol and carboxylic acid respectively (Figure 3.3.4, Appendix 3). The chemical formula for MA is $C_{30}H_{48}O_4$ and OA is $C_{30}H_{48}O_3$ and has been confirmed by comparison with previous reports[16–19]. The oleanane skeleton of OA and MA was further confirmed with 2D NMR spectroscopy that is COSY, NOESY HMQC and HSQC. Moreover, the information presented on Table 3.3 and 3.4 was also supported by HMQC and HSQC correlation (Figure 3.1.8 and 3.1.9) for MA and (Figure 3.2.8 and 3.2.9) for OA. HMQC and HSQC correlation assignment brings about the location of proton to carbon in the chemical structure of these valued compounds.

Table 3.3: The ^{1}H and ^{13}C-NMR of maslinic acid (MA)[19]

Position	δC_{Lit}	δH_{Lit}	δC_{MA}	δH_{MA}
1	46.08	na	46.24	1.41, 1.51
2	68.47	3.61	68.09	4.59
3	83.35	2.91	83.06	3.65
4	39.09	------	39.10	------
5	55.13	na	55.29	1.36
6	18.22	na	18.16	1.65, 1.64
7	32.45	na	32.51	1.38, 1.79
8	39.17	------	39.19	------
9	47.45	na	47.46	1.60
10	38.03	------	37.86	------
11	na	na	23.21	2.03, 2.00
12	nd[c]	5.25	122.05	5.26
13	143.71	------	143.96	------
14	41.60	------	41.54	------
15	27.47	na	27.40	1.80, 1.74
16	na	na	22.65	1.98, 1.93
17	46.26	------	46.73	----
18	------	2.79	41.34	2.85
19	45.77	na	45.84	1.47, 1.57
20	30.51	------	30.22	-----
21	33.71	na	33.50	1.44,

				1.54
22	na	na	32.42	1.77, 2.04
23	28.39	0.95	27.89	0.94
24	16.56	0.74	15.67	0.96
25	16.35	0.91	16.34	0.91
26	16.71	0.71	16.04	1.02
27	25.71	1.07	23.21	1.12
28	181.25	------	180.49	------
29	32.89	0.84	32.16	0.83
30	23.37	0.86	25.01	0.93

Table 3.4: The ^{1}H and ^{13}C-NMR of oleanolic acid (OA)[15]

Position	**δC_{Lit}**	**δH_{Lit}**	**δC_{OA}**	**δH_{OA}**
1	38.37	0.98a, 1.62	38.41	1.38a, 1.53
2	27.15	1.56, 1.60	27.66	1.89, 1.64
3	79.01	3.20 dd(5.0, 1.0)	80.93	3.43
4	38.74	------	39.28	------
5	55.18	0.75 t	55.29	1.35
6	18.27	1.38, 1.54	18.17	1.58, 1.59
7	32.59	1.29, 1.44	32.52	1.38, 1.79
8	39.23	------		------
9	47.6	1.54	47.55	1.63
10	37.05	------	37.69	------
11	23.37	0.91, 1.88	23.39	2.19,

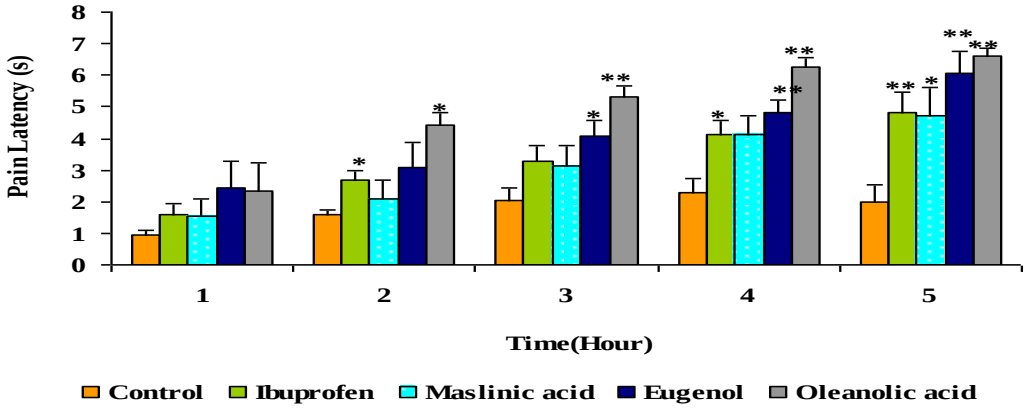
				2.18
12	122.61	5.28 t (3.6)	122.65	5.59
13	143.26	------	143.58	-------
14	41.59	------	41.56	-------
15	27.66	1.10, 1.72	27.66	1.84, 1.74
16	22.91	1.61, 1.97	22.88	1.95, 1.92
17	46.47	------	46.53	-------
18	40.98	2.81 dd(3.6, 13.6)	41.63	2.47 d
19	45.84	1.16, 1.63	45.83	1.45, 1.55
20	30.66	------	30.67	-------
21	33.77	1.22, 1.33	33.79	1.44, 1.55
22	32.4	1.58, 1.77	32.44	1.78, 2.08
23	28.08	0.98 s	28.04	0.90 s
24	15.52	0.75 s	15.38	0.90 s
25	15.52	0.91 s	16.65	0.89 s
26	17.06	0.77 s	17.15	1.02 s
27	25.91	1.13 s	25.90	1.31 s
28	182.66	-------	182.27	-------
29	33.05	0.92 s	33.05	0.91 s
30	23.66	0.90 s	23.57	0.94

3.6.2 Bioassays

3.6.2.1 Tail flick pain test

Tail flick pain test was assessed by thermal-induced pain on the tip of animal tail. All compounds showed analgesic activity in tail flick test, however, the significant ($p<0.05$ – 0.01) value was obtained between second and the fourth hour.

a)



b)

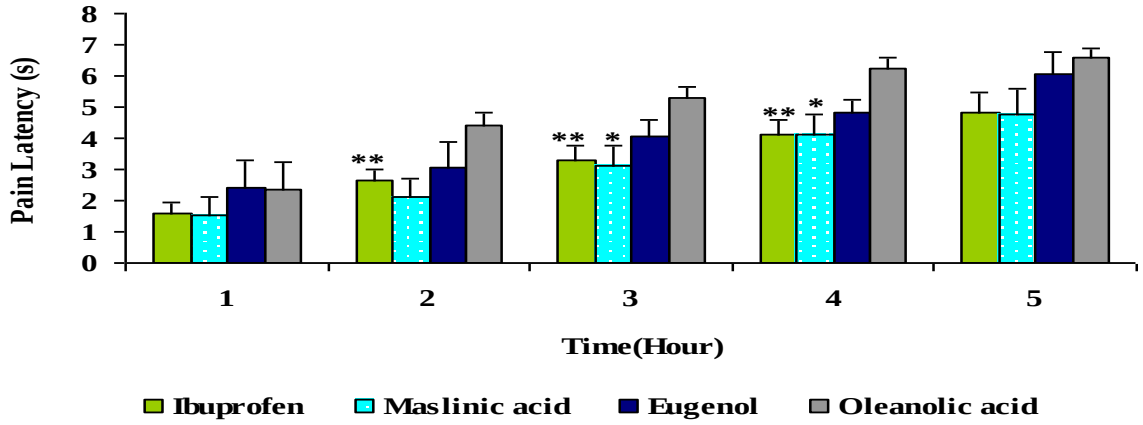


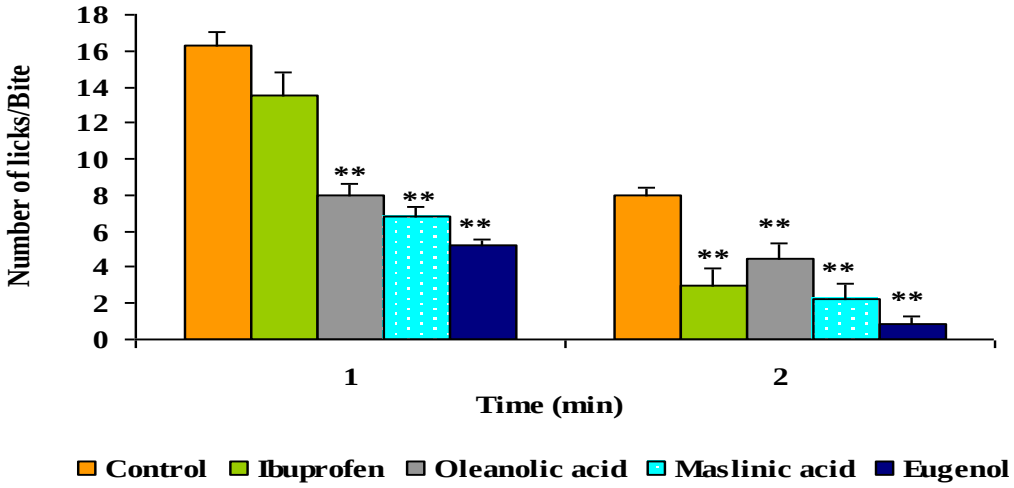
Figure 3.3: Tail flick test results showing latency to thermal-induced pain. (a) Shows the effect of drugs on thermal-induced pain compared to untreated animals (control). (b) Shows comparison of tested compounds with oleanolic acid. The results were represented as mean ± S.E of each treatment group, n = 6 *p <0.05; **p < 0.01.

Oleanolic acid (OA) showed significant ($p<0.01$) inhibition than other tested drugs through all hours of analysis. It was also observed that eugenol, maslinic acid (MA) and ibuprofen inhibited the thermal-induced pain but were less active than OA (Figure 3.3a). The significant ($p<0.01$) difference was obtained between the second and fourth hour on ibuprofen versus OA. MA also differs significantly ($p<0.05$) with OA on third and fourth hour (Figure 3.3b).

3.6.2.2 Formalin-induced pain test

All tested compounds showed significant ($p<0.01$) inhibition of formalin induced pain. OA, MA and eugenol significantly inhibited both neurogenic and inflammatory phases of the formalin test (Figure 3.4a). Ibuprofen on the other hand failed to inhibit the neurogenic phase of the test though its effect became significantly greater than the effect of the control during the inflammatory phase. A comparison of drug effects with effects of OA showed that ibuprofen failed significantly to protect against formalin induced pain. On the hand, eugenol showed consistently better analgesic properties than OA in both neurogenic and inflammatory phases.

a)



b)

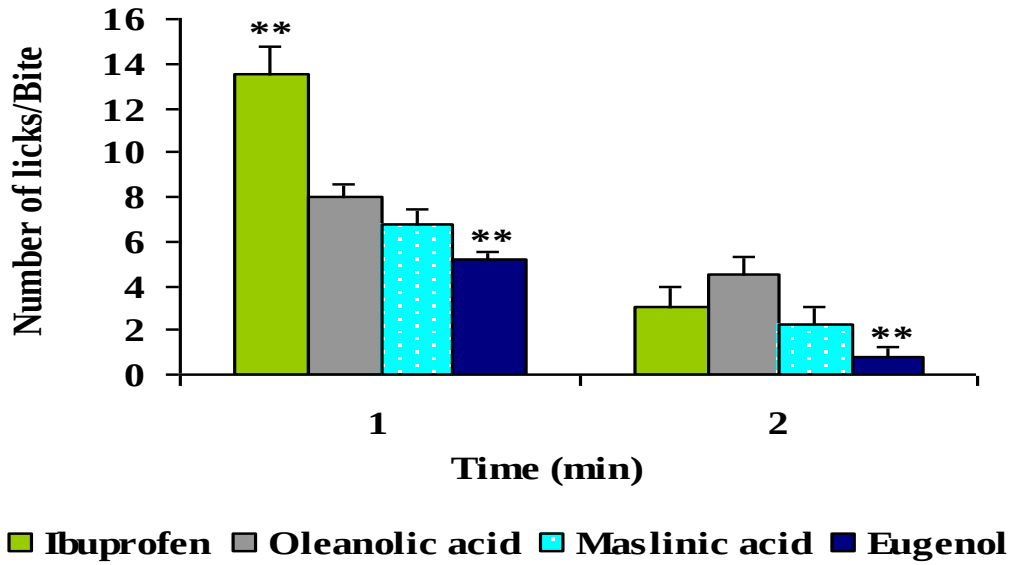
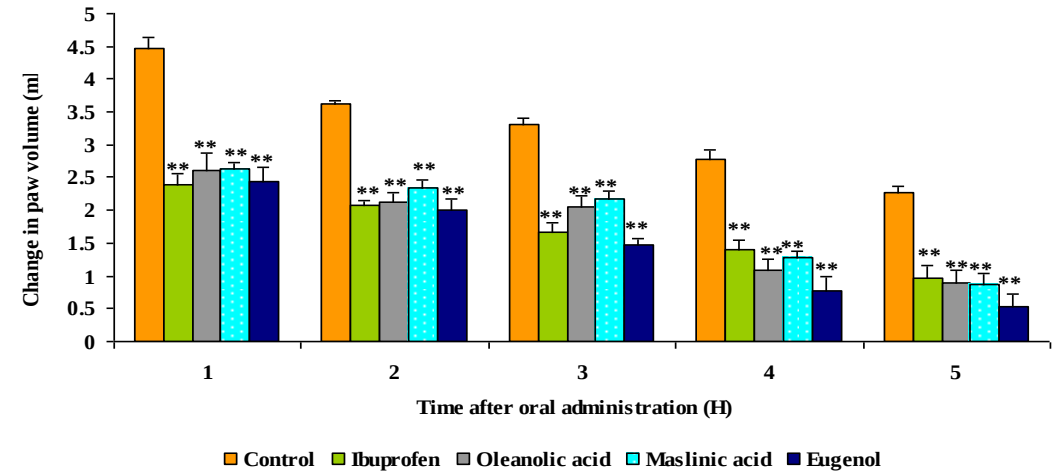


Figure 3.4: Formalin test results showing number of licks per bite. (a) Shows the effect of drugs on formalin-induced pain compared to untreated animals (control). (b) Shows comparison of tested compounds with oleanolic acid. The results were represented as mean ± S.E of each treatment group, n = 6, **p < 0.01.

3.6.2.3 Egg albumin-induced inflammatory

The effect of the tested compounds on egg albumin-induced inflammation was monitored for the period of five hours.

a)



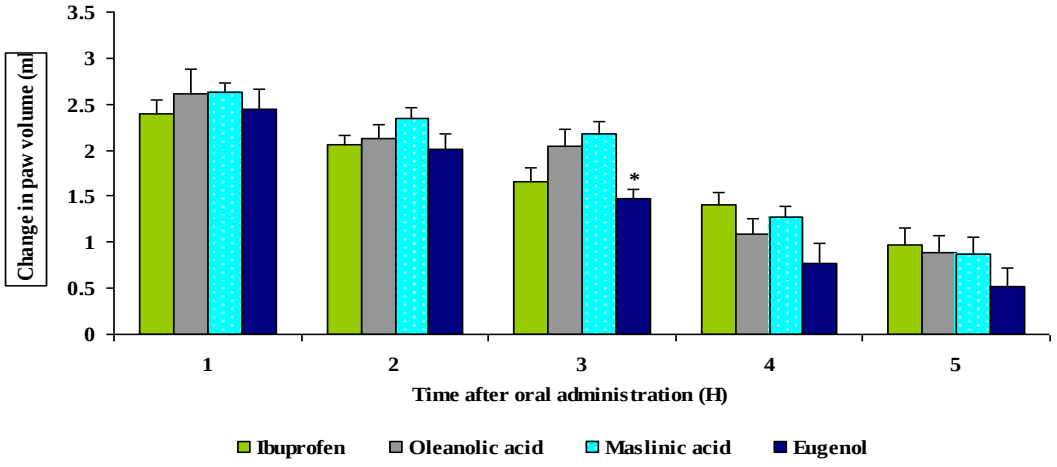


Figure 3.5: Acute inflammatory test results showing change in paw volume induced with fresh egg albumin. (a) Shows the effect of drugs on egg albumin-induced inflammation compared to untreated animals (control). (b) Shows comparison of tested compounds with oleanolic acid. The results were represented as mean ± S.E of each treatment group, n = 6, **p < 0.01.

All compounds significantly ($p<0.01$) inhibited the acute inflammation compared to the control group. Eugenol showed better anti-inflammatory results than other compounds followed by OA during the last 2 hrs post treatment. However ibuprofen showed better inhibition than OA and MA during the 1st h to 3rd h but in the later phase of inflammation its activity was less active whereas OA and MA shows better inhibition of inflammation. The comparison of all tested compound was done by comparing them with OA and it was proved that eugenol differs significantly ($p<0.05$).

3.7 Discussion

The employed chromatographic techniques which are CC and TLC have led to isolation of three distinct compounds from ethyl acetate extract of *S. aromaticum* dry flower buds. The structural elucidation of isolated compounds was determined with different spectroscopic techniques. The elucidation of these compounds confirms that oleanolic acid, eugenol and maslinic acid were successfully isolated using column chromatography (Table 3.2 and 3.3).

Furthermore, it is documented that eugenol, is one of the major components of *S. aromaticum* oil[20,21]. Indeed, Singh *et al*. (2012)[22], also mentioned that eugenol has been reported as an active component of *S. aromaticum* (clove) which showed antioxidant, antiviral, anti-inflammatory, analgesic and anticonvulsant properties. However, maslinic acid and oleanolic acid are also reported to possess a number of medicinal activities[23].

Analgesic and anti-inflammatory properties of these compounds namely, OA, MA and eugenol were evaluated. The tested drugs inhibited the nociceptive reflex in the Sherringtonian sense reflecting a complex coordinated behavioral adjustment of the thermal heat stimulus. Pain latency to thermal heat was significantly ($p<0.01$) in all drugs treated

groups at 4 to 5h post treatment except MA treated group. However OA, MA and eugenol were very active against acute inflammation and pain. Eugenol inhibited significantly ($p<0.01$) acute inflammation and pain through all hours of the acute inflammation and formalin-induced pain tests. Triterpenoids, OA and MA they also show anti-inflammatory and analgesic properties. All these compounds are promising led compounds for new drug formulation.

3.8 Conclusion

A chromatographic technique that is, CC has led to isolation of three distinct compounds. However, the use of spectroscopic methods contributes an essential role in structural elucidation of these valued compounds, namely eugenol, oleanolic acid and maslinic acid.

The bioassays reveal that eugenol, oleanolic acid and maslinic acid may serve as alternative drug candidates for new analgesic and anti-inflammatory drug formulation.

3.9 References

1. Milind P, Deepa K. Clove: (2011). A champoin spice. *International Journal of Research in Ayurveda & Pharmacy*. *2*(1):47–54.

2. Santoro GF, Cardoso MG, Gustavo L, Guimarães L, Mendonça LZ, Soares MJ. (2007). *Trypanosoma cruzi*: Activity of essential oils from *Achillea millefolium* L., *Syzygium aromaticum* L. and *Ocimum basilicum* L. on epimastigotes and trypomastigotes. *Experimental Parasitology*. *116*(3):283–290.

3. Park M, Gwak K, Yang I, et al. (2007). Antifungal activities of the essential oils in *Syzygium aromaticum* (L.) Merr. et Perry and *Leptospermum petersonii* bailey and their constituents against various dermatophytes. *Journal of Microbiology*. *45*(5):460–465.

4. Alma MH, Ertas M, Nitz S, Kollmannsberger H. (2007). Chemical composition and content of essential oil from the bud of cultivated Turkish clove (*Syzygium aromaticum* L.). *Bioresearch*. *2*(2):265–269.

5. Bagavan A, Rahuman AA, Kamaraj C, et al. (2011). Contact and fumigant toxicity of hexane flower bud extract of *Syzygium aromaticum* and its compounds against *Pediculus humanus capitis* (Phthiraptera: Pediculidae). *Parasitology Research*. *109*(5):1329–1340.

6. Bhuiyan NI, Begum J, Nandi NC, Akter F. (2010). Constituents of the essential oil from leaves and buds of clove (*Syzigium caryophyllatum* (L.) Alston). *African Journal of Plant Science*. *4*(11):451–454.

7. Saeed S, Tariq P. (2008). In vitro antibacterial activity of clove against gram negative bacteria. *Pakistan Journal of Botany*. *40*(5):2157–2160.

8. Hosseini M, Asl MK, Rakhshandeh H. (2011). Analgesic effect of clove essential oil in mice. *Avicenna Journal of Phytomedicine*. *1*(1):1–6.

9. Kumar P, Jaiswal P, Singh VK, Singh DK. (2011). Medicinal, therapeutic and pharmacological effects of *Syzygium aromaticum* (Laung). *Pharmacologyonline*. *1*:1044–1055.

10. Aisha AFA, Abu-salah KM, Alrokayan SA, Siddiqui MJ, Ismail Z, Majid AMSA. (2012). *Syzygium aromaticum* extracts as good source of betulinic acid and potential anti-breast cancer. *Brazilian Journal of Pharmacognosy*. *22*(2):335–343.

11. Nkeh-Chungag BN, Temdie JR, Sewani-Rusike C, Yolande M, Mbafor JT, and Iputo JE. (2009). Analgesic, anti-inflammatory and antiulcer properties of the extract of *Uapaca guineensis* (Euphorbiaceae). *Journal of Medicinal Plants Research*. *3*(9):635–640.

12. Nkomo M, Nkeh-Chungag BN, Kambizi L, Ndebia EJ, Sewani-Rusike C, Iputo JE. (2011). Investigation of the antinociceptive and anti-inflammatory properties of *Heteromorpha arborescens* (Apiaceae). *African Journal Traditional Complement Alternative Medicine*. *8*(4):412–419.

13. Emim JADS, Souccar C, Castro MSDA, et al. (2000). Evidence for activation of the tissue kallikrein-kinin system in nociceptive transmission and inflammatory responses of mice using a specific enzyme inhibitor. *British Journal of Pharmacology*. *130*(5):1099–1107.

14. Nkeh-Chungag NB, Bekwa PCM, Ndebia JE, Kayo M, Mbafor TJ, Iputo EJ. (2010). Analgesic and anti-inflammatory properties of *Oxyanthus unilocularis*. *Journal of Medicinal Plants Research*. *4*(10):932–939.

15. Ngan DH, Hoai HT, Huong LM, Hansen PE, Vang O. (2008). Bioactivities and chemical constituents of a Vietnamese medicinal plant Che Vang, *Jasminum subtriplinerve Blume* (Oleaceae). *Natural Product Research*. *22*(11):942–949.

16. Tanaka JCA, Vidotti GJ, Silva CC. (2003). A new tormentic acid derivative from *Luehea divaricata Mart*. (Tiliaceae). *Journal of the Brazilian Chemical Society*. *14*(3):475–478.

17. Hossain MA, Ismail Z. (2013). Isolation and characterization of triterpenes from the leaves of *Orthosiphon stamineus*. *Arabian Journal of Chemistry*. *6*(3):295–298.

18. Khathi A, Serumula MR, Myburg RB, Van Heerden FR, Musabayane CT. (2013). Effects of *Syzygium aromaticum*-derived triterpenes on postprandial blood glucose in streptozotocin-induced diabetic rats following carbohydrate challenge. *PloS one*. *8*(11):e81632.

19. Vlahov G, Rinaldi G, Del Re P, Giuliani AA. (2008). ^{13}C Nuclear magnetic resonance spectroscopy for determining the different components of epicuticular waxes of olive fruit (*Olea europaea*) Dritta cultivar. *Analytica Chimica Acta*. *624*(2):184–194.

20. Prashar A, Locke IC, Evans CS. (2006). Cytotoxicity of clove (*Syzygium aromaticum*) oil and its major components to human skin cells. *Cell Proliferation*. *39*(4):241–248.

21. Srivastava AK, Srivastava SK, Syamsundar KV. (2005). Bud and leaf essential oil composition of *Syzygium aromaticum* from India and Madagascar. *Flavour & Fragrance Journal*. *20*(1):51–53.

22. Singh J, Baghotia A, Goel SP. (2012). *Eugenia caryophyllata Thunberg* (family Myrtaceae): A review. *International Journal of Research in Pharmaceutical & Biomedical Sciences*. *3*(4):1469–1475.

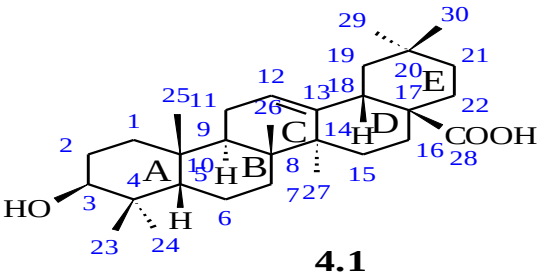
23. Li C, Yang Z, Zhai C, et al. (2010). Maslinic acid potentiates the anti-tumor activity of tumor necrosis factor alpha by inhibiting NF-kappaB signaling pathway. *Molecular Cancer*. *9*:73–85.

CHAPTER FOUR

DERIVATIVES OF OLEANOLIC ACID

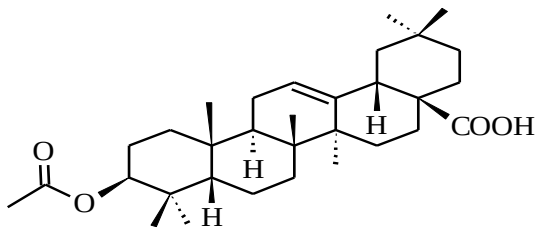
4.1 General introduction

Oleanolic acid **(4.1)** is one of the known pentacyclic triterpenoid widely distributed in various plants and food[1–4]. It is a compound with a number of biological properties that helps to combat and inhibit diverse human pathogens[5]. Its biological properties which include anticancer, anti-HIV and anti-inflammatory maybe employed to improve the modern drug discovery[3].



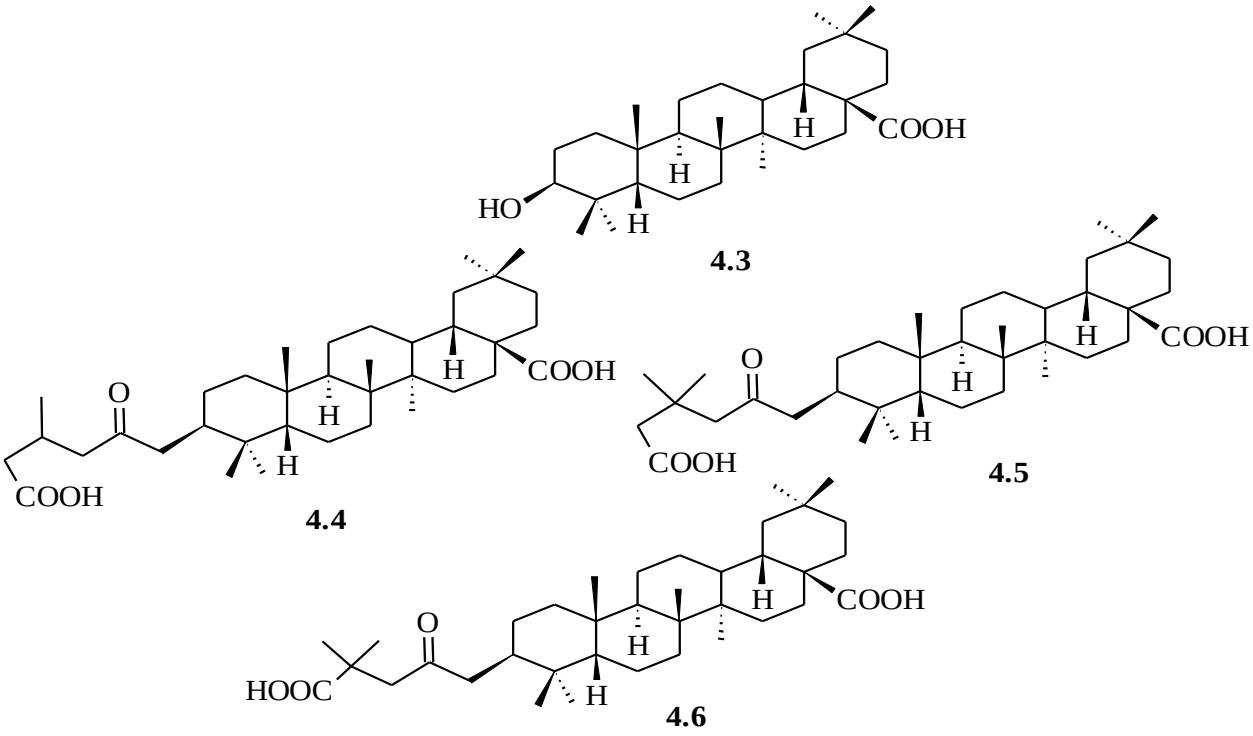
4.1

The chemical structure of 3β-hydroyolean-12-en-28-oic acid **(4.1)** gives three possible "active" sites, the C_3 hydroxy, the C_{12} - C_{13} double bond, and the C_{28} carboxylic acid; thus, it's a good precursor for further modification[6]. Flores *et al*, (2005)[7] and Habila *et al*, (2012)[8], have modified position 3 in ring A of OA structure to an ester derivative, 3-acetyloleanolic acid **(4.2)**.

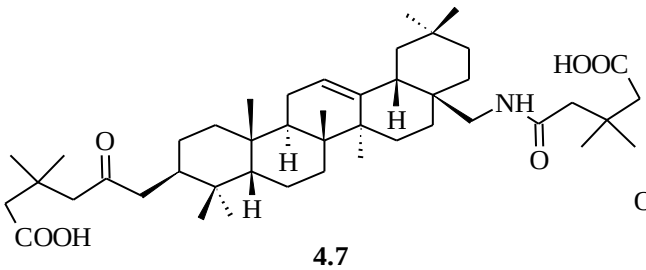


4.2

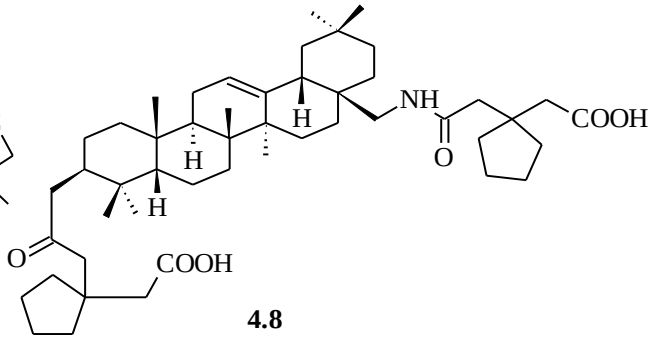
An ester derivative **(4.2)** of oleanolic acid enhanced the chemical and biological properties of the OA[8]. Zhu *et al*, (2001)[9], reports thirteen oleanolic acid derivatives with anti-HIV properties. These authors stated that, a saturation of C_{12}-C_{13} double bond of OA yielded compound **(4.3)** with 3- fold increase in activity compared to that of OA. Derivation of **(4.3)** gave **(4.4)** and **(4.5)** with 5-fold increase in activity and **(4.6)** led to an even more remarkable activity.



Modification of compound **(4.6)** at position 28 yielded compounds **(4.7)** and **(4.8)** with 10-fold biological activity compared to that of parent compound **(4.1)**[9].

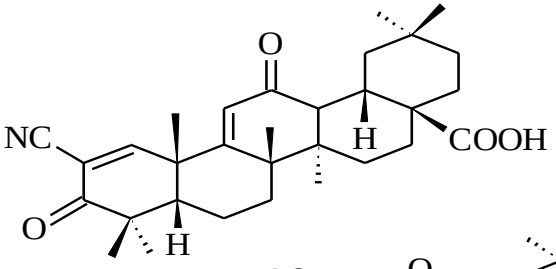


4.7

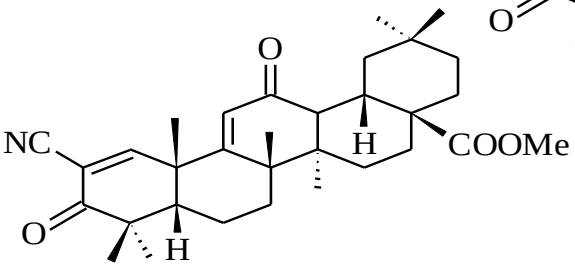


4.8

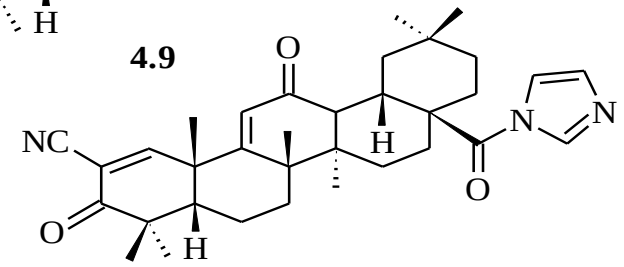
Indeed, Honda *et al*, (1998)[10] and Liby *et al*, (2005)[11], synthesized an amino, imidazole and ketone derivatives of OA, namely 2-cyano-3,12-dioxooleana-1,9(11)- dien-28-oic acid (CDDO) **(4.9)**, 2-cyano-3,12-dioxooleana-1,9(11)- dien-28-oic methyl ester (CDDO-Me) **(4.10)** and 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) **(4.11)**.



4.9



4.10



4.11

Moreover, the above derivatives showed enhanced biological properties of OA. Indeed Honda *et al*, (2000)[12] also mentioned that 9(11)-en-12-one and 12-en-11-one functionalities in ring C of CDDO increased the effectiveness by about 2-10 times compared to that of OA. It is therefore concluded that modification of OA may result in increasing its potency.

4.2 Experimental method

4.2.1 Semi-synthesis of oleanane derived compounds

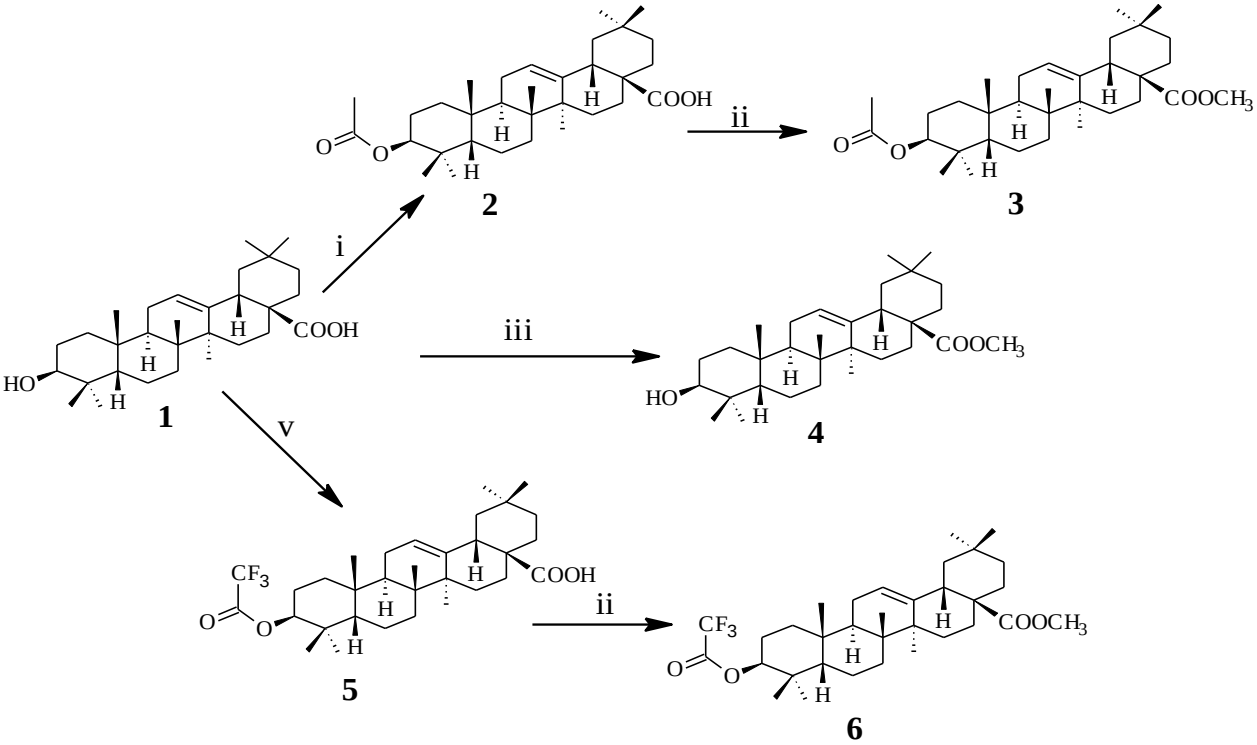


Figure 4.1: Semi-synthetic pathways of oleanane derived compounds: - (i) $(CH_3CO)_2O$, pyridine, 12hrs, 25^0C (ii) CH_3I, DMF, 12hrs, 25^0C, (iii) K_2CO_3, CH_3I, 12hrs, 25^0C (v) $(CF_3CO)_2O$, pyridine, 12hrs, 0^0C.

4.2.2 Compounds 2 and 5

Compounds **2** and **5** were semi-synthesized using the method described by Zhu *et al*, (2001)[9] with some modifications. Oleanolic acid (OA) (0.20 g, 0.44 mmol.) was dissolved in pyridine (5 ml) and excess acetic anhydride (10 ml) was added in 150 ml round bottom flask. The mixture was stirred for 12 hrs at 25^OC for compound **2** and 0^OC for compound **5**. The product was poured into 100 ml of water and stirred for 2 hrs to hydrolyze excess acetic anhydride. The final product was separated by suction filtration and recrystallized in methanol then, purified by column chromatography to give 92% yield of compound **2** and 87% yield of compound **5**

4.2.3 Compounds 3 and 6

Compounds **3** and **6** were synthesized using the modified procedure documented in the literature[7]. 3β-acetyloleanolic acid (AOA) (0.20 g, 0.44 mmol) was methylated by CH_3I (2.0 g, 0.01 mol) then anhydrous Na_2CO_3 (2.0 g, 0.02 mol) was added to stabilize the pH and the whole solution was dissolved in 40 ml dimethylformamide in 150 ml round bottom flask. The solution was stirred overnight at room temperature. The product was poured into 100 ml of water to hydrolyze excess CH_3I and stirred for 2 hrs. The final product was separated by suction filtration and recrystallized in methanol. Final product and the starting material were spotted on the TLC plate developed with n-Hexane: Ethyl acetate to confirm complete formation of the new product. This method resulted in 100% yield for compound **3** and 94% yield for compound **6**.

4.2.4 Compound 4

Modified method of Mallavadhani *et al.* (2014)[13] was employed during the experiment. OA (0.2 g, 0.4 mmol.) was dissolved in acetone (2 ml) then anhydrous K_2CO_3 (0.1 g) and CH_3I was added drop wise with constant stirring at room temperature. The mixture was constantly stirred for 12 hrs at 25^OC then the whole solution was diluted with 100 ml of water and stirred for 2 hrs. The whole solution was extracted with chloroform and the organic layer was dried in anhydrous Na_2SO_4 and later dried at room temperature. A single spot compound was obtained and structurally elucidated with FT-IR, MS, Mp. and NMR.

4.3 Bioassays of oleanane derived compound

4.3.1 Animal Study

Wistar rats (180-250 g) and Swiss mice (20-35 g) of either sex were use during the experiment as described in the literature[14,15]. These animals were housed with a 12 hours light/dark cycle and

permitted to food and water. For the experimental purpose food was withheld overnight from the animals before use. All experiments (formalin-induced pain test, tail flick test, albumin-induced inflammation and statistical analysis) were done as described previously (chapter3).

4.4 Results

4.4.1 Oleanane derived compounds

The modification of compound **1** has led to acetate and ester derivatives with different melting points and molecular masses (Table 4.1).

Table 4.1: Physical data of oleanane derived compounds

Compound	**1**	**2**	**3**	**4**	**5**	**6**
Description	**White powder**	**White powder**	**White powder**	**White crystalline**	**White amorphous powder**	White powder
Yield	0.20%	92%	100%	98%	87%	94%
Mp	294-295^{0}C	266-267^{0}C	222-223^{0}C	139-140^{0}C	271-272^{0}C	283-284^{0}C
Molecular mass	456.46	499.38	513.36	469.46	552.30	567.7

4.4.2 Major functional group of oleanane derived compounds and oleanolic acid

FT-IR spectrum of oleanolic acid (compound **1**, OA) and its derivatives is illustrated in Table 4.2. The formation of new functional groups on targeted position of the chemical structure of the compound OA was successfully elucidated.

FT-IR spectrum of OA (Figure 3.3.1, Appendix 3) is interpreted on Table 4.2, which discloses the following major peaks at 3406 (–OH) stretching of a free alcohol at C_3, 2835, 2864 aliphatic (-CH) stretching of an alkane, 1688 (-C=O) bending of a carboxylic acid at C_{28} and 1460 (C=C) stretching of alkene group at C_{12} of the chemical structure. However,

acetylation of OA decorates C_3 of the chemical structure with an acetyl group. The formation of acetyl- groups was confirmed by FT-IR spectroscopy. Acetyl derivatives of OA compound **2** (3β-acetyloleanolic acid, AOA) and **5** (3β-trifluoroacetyloleanolic acid, TOA) are illustrated in Table 4.2. Compound **2** FT-IR spectrum (Figure 4.11, Appendix 4) showed an absorption band at 3205 (–OH, carboxylic acid), 2969-2853 (stretch of aliphatic -CH), 1723 (-C=O, carboxylic acid), 1680 (-C=O, acetate), and 1457 (-C=C, alkene).

Table 4.2: Major absorption bands of oleanane derived compounds

Compounds	**Absorption bands**				
	$\nu_{C\text{-}OH}$	$\nu_{C\text{-}H}$	$\nu_{C=O}$	$\nu_{C=C}$	$\nu_{C\text{-}O}$
1	3406	2935, 2864	1688	1460	1034
2	3205	2969-2853	1723, 1680	1457	1008
3	------	2972, 2966	1724	1470	1020
4	3346	2940	1726-1710	1462	1031
5	3204	2970, 2945	1771, 1723	1455	1010
6	3437	2940, 2860	1688	1461	1030

Compound **5** FT-IR spectrum (Figure 4.4.1, Appendix 7) displayed almost similar peaks with compound **2**, however this compounds displayed an absorption band at 3204 (–OH of carboxylic acid), 2970 and 2945 (aliphatic -CH stretch), two carbonyl absorption bands at1771 (–C=O, carboxylic acid) 1723 (–C=O, acetate), 1455 (-C=C, alkene). In both compound free –OH of an alcohol in C_3, ring A of the chemical structure of OA was

successful acetylated. In addition, the methylation of C_{28}, ring E of compound **2** (3β-acetyloleanolic acid, AOA) and **5** (3β-trifluoroacetyloleanolic acid, TOA) has led to a formation of ester derivatives that is, compound **3** (28-methyl-3β-acetyloleanane, AOAm) and **6** (28-methyl-3β-trifluoroacetyloleanane). FT-IR spectrum (Figure 4.2.1, Appendix 5) for AOAm and (Figure 4.3.1, Appendix 8) for TOAm demonstrate almost the same functional groups (Table 4.2). Compound **4** is 28-methyloleanane (OAm) was synthesized directly from OA. OAm was obtained after treating OA with CH_3I then anhydrous K_2CO_3 was added to stabilize the pH. FT-IR spectrum (Figure 4.3.1, Appendix 6) shows additional carbonyl absorption at 1646 (-C=O, ester) compared to that of OA, this confirms the formation of a methyl ester in the in position 28.

4.4.3 NMR interpretation

Full NMR (^1H, ^{13}C DEPT, COSY, NOESY, HSQC and HMBC) analysis was employed to successfully elucidate the chemical structure of oleanane derived compounds. The significant difference between oleanolic acid **(1)** and its derivatives was obtained by comparison of the spectra. Compound **2** ^1H-NMR spectrum (Figure 4.1.3, Appendix 4) differs from OA with a prominent singlet at 2.04 ppm (**CH_3**COO-) attached at C_3 position of the chemical structure.

Table 4.3: ^{1}H and ^{13}C NMR data of acetate and ester oleanane derived compounds

Position	δCC_1	δCC_2	δCC_3	δCC_4	δHC_1	δHC_2	δHC_3	δHC_4
1	38.41	38.07	38.11	38.44	1.38, 1.53	1.45a, 1.46	1.02a, 1.62	1.42, 1.45
2	27.66	27.66	27.68	27.19	1.89, 1.64	1.81, 1.67	1.55, 1.61	1.67, 1.62
3	80.93	80.93	80.94	79.03	3.43d	4.45	4.46	3.20
4	39.28	37.69	37.69	38.76	------	-------	-----	-----
5	55.29	55.29	55.30	55.22	1.35t	1.36t	0.81	1.35
6	18.17	18.18	18.22	18.33	1.58 1.59	1.55, 1.62	1.25, 1.65	1.55, 1.61
7	32.52	32.44	32.38	32.66	1.38, 1.79	1.40, 1.76	1.44, 1.47	1.38, 1.64
8		39.28	39.29	39.27	------	-------	-----	----
9	47.55	47.55	47.56	47.64	1.63	1.60	1.67	1.58
10	37.69	36.98	37.69	37.04	------	-------	-----	-----
11	23.39	23.39	23.40	23.41	2.19, 2.18	2.17, 2.21	1.19, 2.03	2.02, 1.98
12	122.65	122.57	122.28	122.36	5.59	5.52	5.27	5.26
13	143.58	143.59	143.81	143.79	-------	-------	-----	-----
14	41.56	41.59	41.64	41.65	-------	-------	-----	-----
15	27.66	27.66	27.68	27.71	1.84, 1.74	1.84, 1.75	1.11, 1.65	1.90, 1.66
16	22.88	23.57	23.52	23.64	1.95, 1.92	1.80, 1.82	1.60, 1.95	1.91, 1.94
17	46.53	46.51	46.73	46.73	-------	-------	-----	-----
18	41.63	41.00	41.23	41.30	2.47 d	2.34 d	2.82 d	2.82 d
19	45.83	45.84	45.85	45.89	1.45, 1.55	1.57, 1.53	1.15, 1.54	1.49, 1.53
20	30.67	30.67	30.69	30.70	-------	-------	-----	-----

21	33.79	33.79	33.85	33.86	1.44, 1.55	1.43, 1.45	1.49, 1.57	1.47, 1.51
22	32.44	32.43	32.60	32.39	1.78, 2.08	1.68, 2.01	1.58, 1.67	1.84, 1.98
23	28.04	28.04	28.04	28.10	0.90 s	1.00 s	1.03 s	0.95s
24	15.38	16.66	16.68	15.57	0.90 s	1.00 s	1.02 s	0.90 s
25	16.65	15.38	15.36	15.30	0.89 s	0.91 s	0.91 s	0.88 s
26	17.15	17.13	18.21	16.84	1.02 s	1.05 s	1.06 s	1.02 s
27	25.90	25.88	25.89	25.94	1.31 s	1.30 s	1.11 s	1.31 s
28	182.27	182.76	178.32	178.32	-------	-------	-----	-----
29	33.05	33.05	33.10	33.11	0.91 s	0.92 s	0.92 s	0.92 s
30	23.57	23.57	23.52	23.08	0.94 s	0.93 s	0.89 s	0.84 s
CH_3COO-		171.05	171.03	N/A		-------	-----	N/A
CH_3COO-		21.31	21.31	N/A		2.08 s	2.03 s	N/A
CH_3O-		N/A	51.52	51.53		N/A	3.61 s	3.60 s

Footnote: δCC – Carbon delta value of compound 1 – 5
: δHC – Proton delta value of compound 1 – 5

The effect of acetyl-group caused a deshielding of a doublet at C_3 from 3.43 ppm to 4.45 ppm. Moreover, methylation of compound **2** at C_{28} resulted in compound **3** and the ^{1}H-NMR spectrum (Figure 4.2.3, Appendix 5) disclosed a noticeable singlet at 3.61 ppm (**CH_3O**OC-). The ^{1}H-NMR spectrum of compound **3** differs with two singlets (2.03 ppm for acetate and 3.61 ppm for an ester) from compound **1** spectrum. A direct methylation of compound **1** resulted in compound **4** and the ^{1}H-NMR spectrum (Figure 4.3.3, Appendix 6) displayed a prominent singlet at 3.47 ppm due to the formation of an ester (**CH_3O**OC-) from position28 of the chemical structure.

Table 4.4: ^{1}H and ^{13}C NMR data of trifluoroacetate and ester oleanane derived compounds

Position	δCC_1	δCC_5	δCC_6	δHC_1	δHC_5	δHC_6
1	38.41	39.19	38.67	1.38, 1.53	1.45, 1.58	1.54, 1.46
2	27.66	23.98	23.57	1.89, 1.64	1.80, 1.61	1.92, 1.86
3	80.93	86.89	79.03	3.43d	4.73	3.24
4	39.28	39.19	38.75	------	-----	-----
5	55.29	55.54	55.21	1.35t	0.98	1.37
6	18.17	17.95	17.13	1.58 1.59	1.51, 1.62	1.57, 1.67
7	32.52	33.13	33.06	1.38, 1.79	1.36, 1.79	1.42, 1.78
8		39.82	39.27	------	-----	-----
9	47.55	47.74	47.63	1.63	1.42	1.61
10	37.69	36.56	37.08	------	-----	-----
11	23.39	23.78	23.40	2.19, 2.18	2.01, 2.06	2.01, 2.06
12	122.65	123.69	122.64	5.59	5.30	5.30
13	143.58	147.21	143.59	-------	-----	-----
14	41.56	41.32	41.61	-------	-----	-----
15	27.66	28.39	28.10	1.84, 1.74	1.94, 1.80	1.85, 1.72
16	22.88	23.24	27.68	1.95, 1.92	1.90, 1.91	1.79, 1.83
17	46.53	46.27	45.87	-------	-----	-----
18	41.63	41.87	41.00	2.47 d	2.83	2.82
19	45.83	45.91	45.88	1.45, 1.55	1.64, 1.34	1.58, 1.57

20	30.67	30.77	32.43	-------	-----	-----
21	33.79	33.96	33.06	1.44, 1.55	1.52, 1.45	1.43, 1.49
22	32.44	32.63	32.62	1.78, 2.08	1.91, 1.75	1.39, 1.44
23	28.04	22.24	22.93	0.90 s	1.39 s	1.00 s
24	15.38	22.24	23.57	0.90 s	0.95 s	0.94 s
25	16.65	15.87	15.31	0.89 s	0.93 s	0.93 s
26	17.15	17.07	15.53	1.02 s	1.15 s	1.25 s
27	25.90	24.32	25.93	1.31 s	2.00 s	2.00 s
28	182.27	187.41	182.87	-------	-----	-----
29	33.05	29.19	28.10	0.91 s	1.02 s	0.91 s
30	23.57	29.19	27.18	0.94 s	1.32 s	0.79 s
CF_3COO-		155.60			-----	-----
CF_3COO-		113.9			-----	-----
CH_3OOC-			33.80			1.79 s

Footnote: δCC – Carbon delta value of compound 1 – 5
: δHC – Proton delta value of compound 1 – 5

Compound **5** was obtained by acetylation of compound **1** with trifluoroacetic anhydride. Table 4.4 disclosed the interpretation of ^{1}H-NMR spectrum of compound **5** (Figure 4.4.3, Appendix 7), the presence of trifluoroacetyl- group causes a deshielding of a proton attached to oxygenated carbon at position 3 of compound **5** to down field 4.73 ppm. However, methylation of compound **5** has led to compound **6**. The ^{1}H-NMR spectrum (Figure 4.5.3, Appendix 8) of compound **6** revealed a methyl proton at 1.79 ppm (**CH_3O**OC-), the formation of methyl- group at position 28 has led to a shift (shielding) of a proton on position 3 of chemical structure to the upfield 3.24 ppm. The methyl- group signal was expected between 2.5 to 3.5 ppm, however the synthetic route used in this study influence the signal to shield to upfield 1.79 ppm. Secondly, the effect of trifluoroacetyl- functional

group which is more acidic affects the nucleus of methyl-group at position 28 to “feel” weak magnetic field.

DEPT NMR spectra of these compounds showed signals which confirmed $C_{32}H_{50}O_4$ for compound **2**, $C_{33}H_{52}O_4$ for compound **3**, $C_{31}H_{50}O_4$ for compound **4**, $C_{32}H_{47}F_3O_4$ for compound **5** and $C_{33}H_{50}F_3O_4$ for compound **6** (Appendix 1 -8).

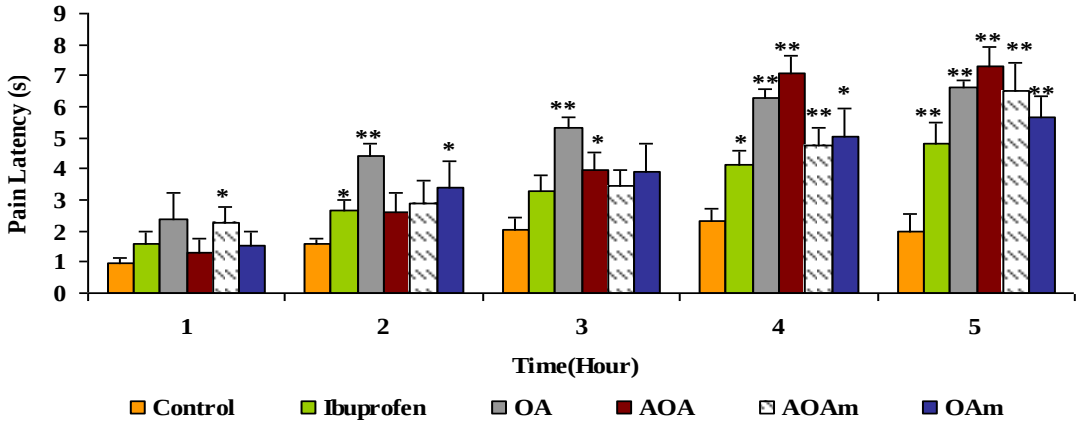
4.4.4 Biological test

For ease of presentation, the biological effects of acetyl and trifluoroacetyl compounds were presented separately.

4.4.4.1 Tail flick pain test

Figure 4.2(a) depicts time dependent analgesic effects of treatments in response to radiant pain. The effect of all compounds used was time dependent though AOAm had an early onset of analgesic effect (1 h) compared to the other compounds. Pain latency to radiant heat was significantly ($p<0.01$) increased in all drug treated animals at 4 and 5 h post treatment. Figure 4.2(b) compared all treatment groups with the OA treated group. Unpaired t-test with two-tailed p values showed that OA had increased pain latency significantly ($p<0.01$) during the 2 and 4 h test periods compared to ibuprofen. The analgesic effects of OA increased rapidly from the 1 to 3 h showing significantly higher pain latency at 2 h compared to AOA though the effects of the latter became significantly greater than those of OA sueing the 4 h.

a)



b)

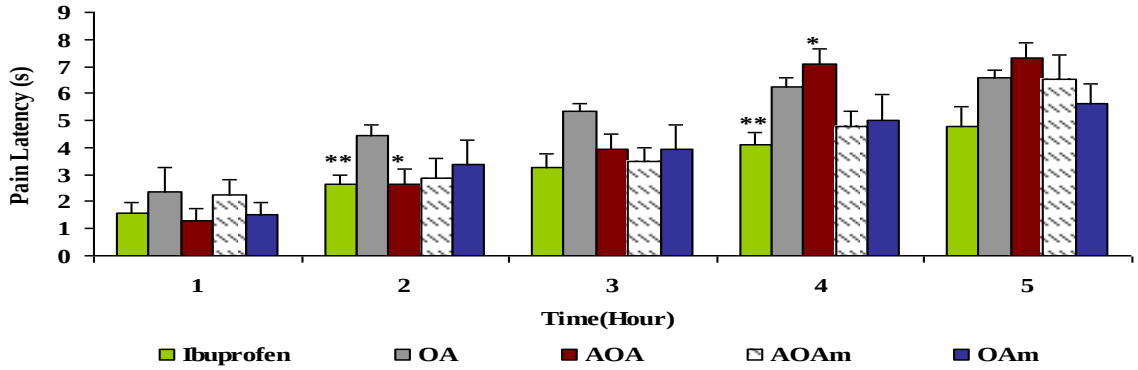
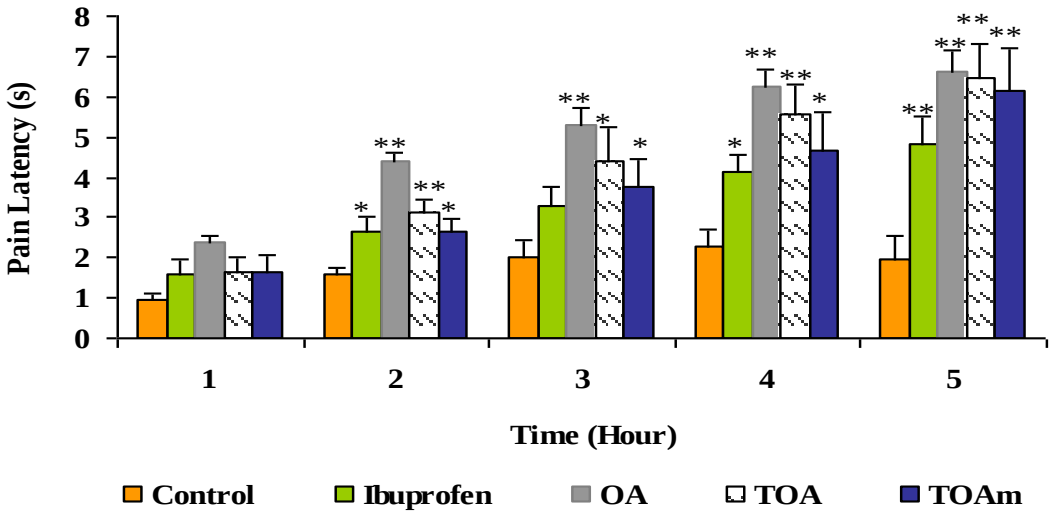


Figure 4.2: Analgesic effect of OA and its acetyl derivetives on tail flick response induced by radiant heat. OA = oleanolic acid, AOA = 3-acetyloleanolic acid, AOAm = 28-methyl, 3-acetyloleanane, OAm = 28-methyloleanane. Values are presented as mean reaction time in seconds±SEM for 6 animals. *p<0.05 and **p<0.01. a) Shows a comparison of the tested compounds against control group, whereas b) gives comparison of the tested drug with OA.

The analgesic effects of OA and its trifluoroacetyl derivatives increased with time with best effects during the 5 h post treatment. All treatment groups had significantly ($p<0.05$ or $p<0.01$) increased reaction times to radiant heat (Figure 4.3a). OA had a noticeable earlier onset pain inhibition (1 h) its effects however were significantly better than those of the trifluoroacetyl derivatives only during the 2 h post treatment. Beyond this time the analgesic

effects of OA were very similar to those of its derivatives though it remained significantly better than the effects of ibuprofen (Figure 4.3b).

a)



b)

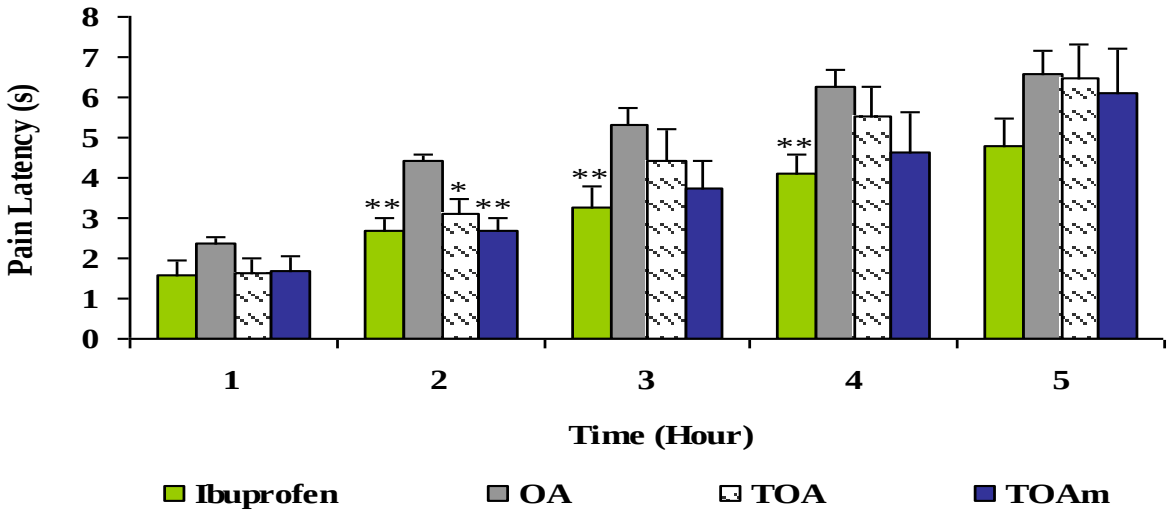
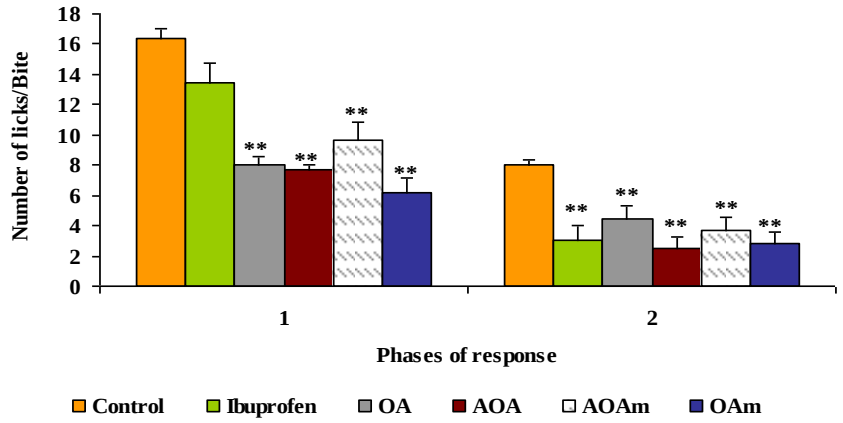


Figure 4.3: Effect of trifluoroacetyl derivetives of OA on pain latency heat. OA = oleanolic acid, TOA = 3-trifluoroacetyloleanolic acid, TOAm = 28-methyl, 3-trifluoroacetyloleanane and ibuprofen (standard drug) on tail flick response induced by radiant heat. Values are presented in mean reaction time in seconds±SEM for 6 animals. *p<0.05 and **p<0.01. a) Shows a comparison of the tested compounds against control group, whereas b) gives comparison of the tested drugs with OA.

4.4.4.2 Formalin test

The formalin induced pain test generates a biphasic response which is characterized by flinching or licking/biting of injected paw. The first phase generally referred to as the neurogenic phase occurs during the first 5 minutes after formalin injection while the second phase or inflammatory phase occurs during the 10-30 minutes after formalin injection[16].

a)



b)

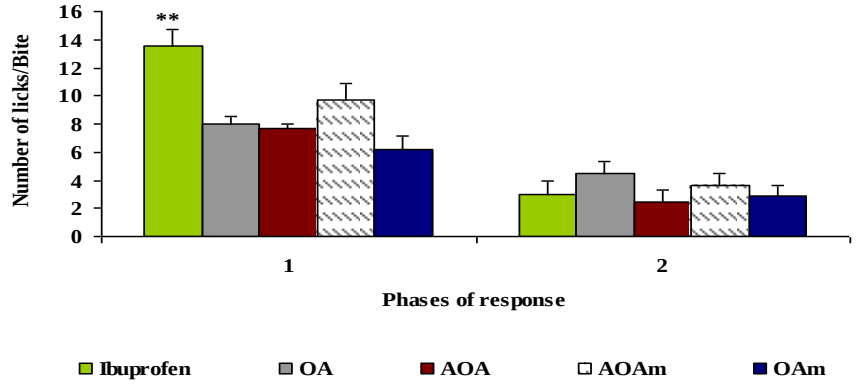
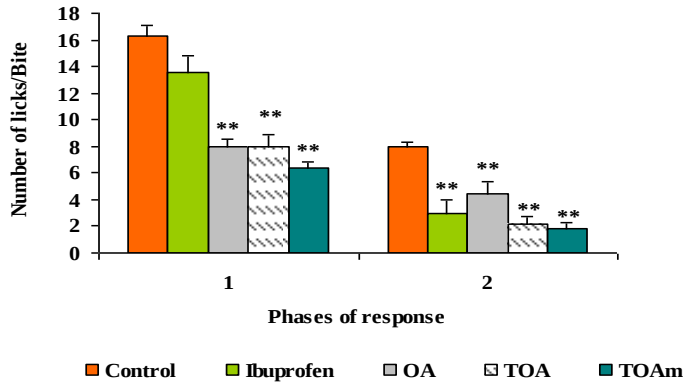


Figure 4.4: Formalin induced pain behavior OA = oleanolic acid, AOA = 3-acetyloleanolic acid, AOAm = 28-methyl, 3-acetyloleanane, OAm = 28-methyloleanane. Values are presented in mean reaction time in seconds±SEM for 6 animals. *p<0.05 and **p<0.01. a) Shows a comparison of the tested compounds against control group, whereas b) gives comparison of the tested drugs with OA.

OA and all its acetyl derivatives significantly ($p<0.01$) reduced the number of paw licks/bites in both the first and second phases of the experiments (Figure 4.4a). Formalin elicited pain induced behaviour was however not different between treatment groups (Figure 4.4b).

a)



b)

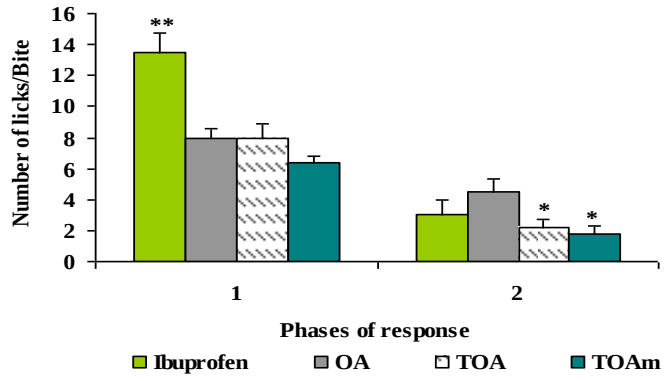


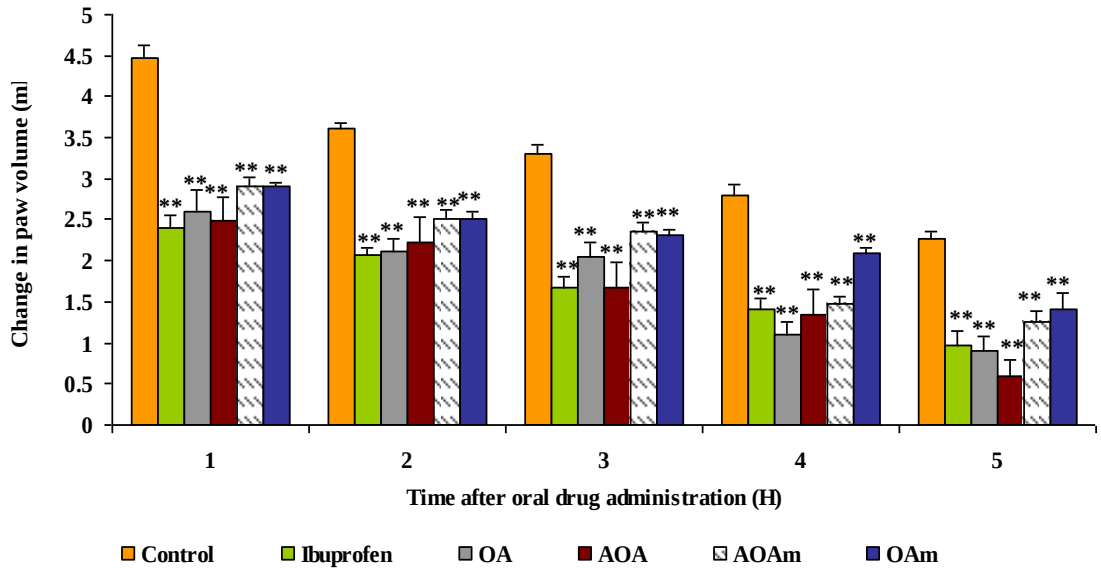
Figure 4.5: Formalin-induced pain behavior of OA = oleanolic acid, TOA = 3-trifluoroacetyloleanolic acid, TOAm = 28-methyl, 3-trifluoroacetyloleanane. Values are presented in mean reaction time in seconds±SEM for 6 animals. *p<0.05 and **p<0.01. a) Shows a comparison of the tested compounds against control group, whereas b) displays a comparison of the tested drugs with OA.

Figure 4.5 shows that OA and its trifluoroacetyl derivatives reduced the number of paw licks/bites in both the neurogenic and analgesic phases of the formalin test. Ibuprofen had a very weak pain inhibitory effect during the first phase though its effects became significantly greater during the second phase of the formalin test (Figure 4.5a). A comparison of response to formalin induced pain behaviour treatment groups with OA group showed that TOA and TOAm had better protective effects during the second phase (Figure 4.5b) compared to OA.

4.4.4.3 Acute inflammatory test

The effect of the drugs on acute inflammatory test was evaluated using egg albumin as a phlogistic agent. The anti-inflammatory effects of all test compounds increased with time as depicted by the smaller changes in paw volume compared to baseline volumes. All tested compounds significantly ($p<0.01$) inhibited the inflammatory response to injected albumin (Figure 4.6a). However, a comparison of the anti-inflammatory effects of OA with its acetyl derivatives showed its effects were significantly better than those of OAm 4 h post treatment.

a)



b)

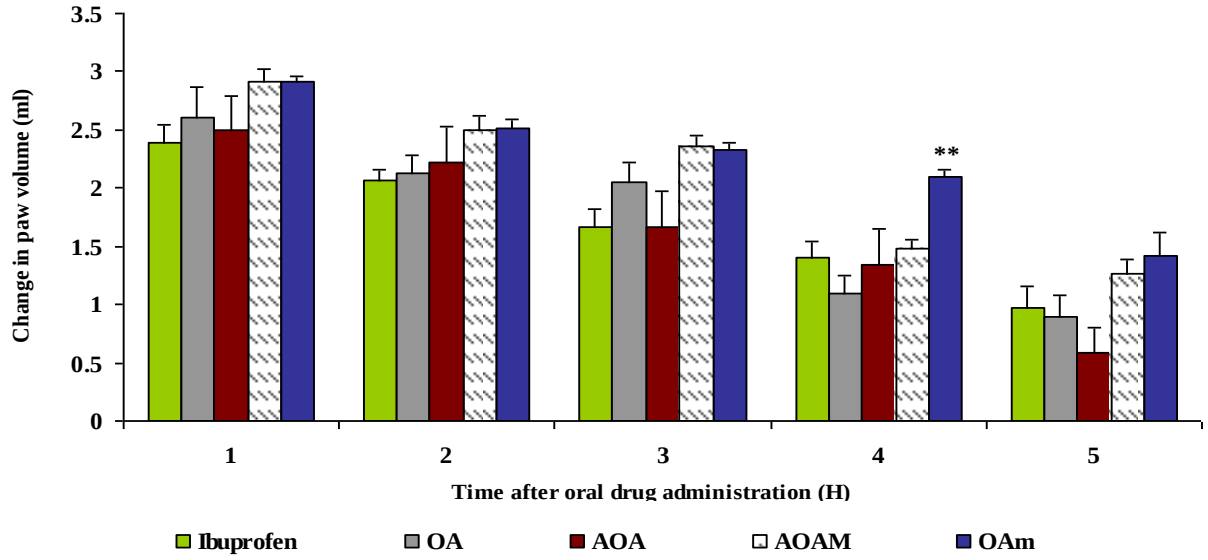
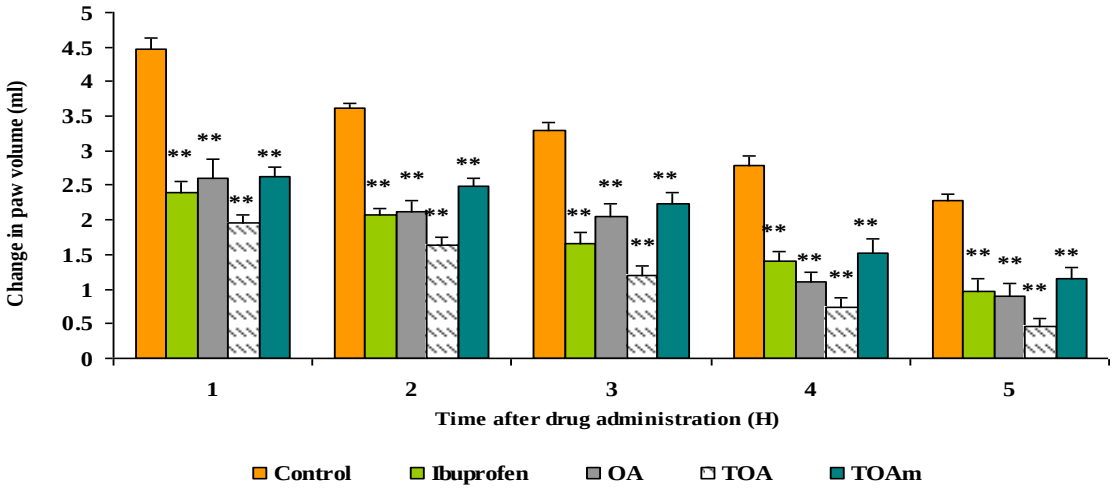


Figure 4.6: Anti-inflammatory effect of ibuprofen (standard drug), OA =oleanolic acid, AOA = 3-acetyloleanolic acid, AOAm = 28-methyl, 3-acetyloleanane and OAm 28-methyloleanane. Values are presented in mean change in volume±SEM for 6 animals. *p<0.05 and **p<0.01. a) Shows a comparison of the tested compounds against control group, whereas b) gives comparison of the tested drugs with OA.

Trifluoroacetyl derivatives of OA also showed significant ($p<0.01$) anti-inflammatory effects (Figure 4.7a). On the other hand, the anti-inflammatory of TOA were significantly ($p<0.05$ and 0.01) better than the effects of OA during the 2 and 3 h post treatment (Figure 4.7b).

a)



b)

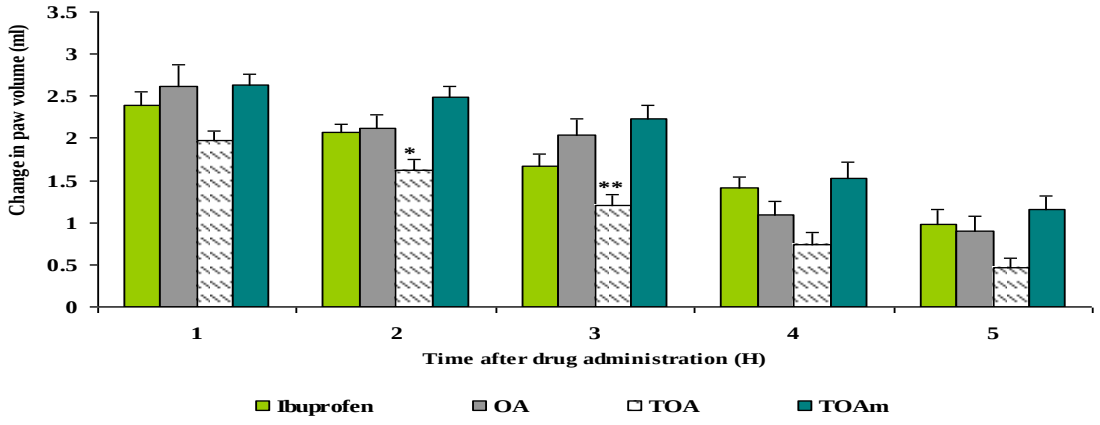


Figure 4.7: Anti-inflammatory effect of ibuprofen (standard drug), OA = oleanolic acid, AOA = 3-acetyloleanolic acid, AOAm = 28-methyl, 3-trifluoroacetyloleanane and. Values are presented in mean change in paw volume±SEM for 6 animals. *p<0.05 and **p<0.01. a) Shows a comparison of the tested compounds against control group, whereas b) gives comparison of the tested drugs with OA.

4.5 Discussion

Oleanolic acid possesses multiply pharmacological properties which includes anti-inflammatory, antitumor and analgesic[17–19]. Recently, OA was documented as a promising lead compound for new drug formulation[20]. Indeed, Habila *et al*. (2012)[8] demonstrate the improvement of antibacterial effect of OA by decorating C_3 position hydroxy-group with an acetyl-group. The present study corroborates the fact that modification of OA in C_3 and C_{28} results in enhancement of biological properties. 3-acetyloleanolic acid (AOA) inhibits radiant heat-induced pain significantly and show better analgesic activity than OA between fourth and fifth hour post treatment. Furthermore, all oleanane-derived compounds also display superior analgesic activity in formalin-induced pain.

Neurogenic phase of the formalin test involves the direct chemical stimulation of nociceptors[21,22] while the inflammatory phase involves the release mediators such as prostaglandins, serotonin, histamine and bradykinin[22]. Vasconcelos *et al*. (2006)[23] demonstrate that OA is less active on neurogenic phase whereas it shows exceptional inhibition in the inflammatory pain phase of formalin-induced pain. Indeed our results showed strong pain inhibitory effects of OA during the second phase of the formalin induced pain test, though its effects were equally significant in the first phase. Both the acetyl and trifluoroacetyl derivatives of OA showed a tendency for better analgesic effects. However only TOA and TOAm were significantly better analgesic agents than OA during the second phase indicating the influence of trifluoroacetyl group on the properties of OA. Moreover the development of the edema on the right paw of the animals after injection of egg albumin is due to the release of inflammation mediators such as histamine, serotonin and prostaglandin[15]. The result in the present study indicated that trifluoroacetyl derivatives may have a better anti-inflammatory effect than OA. Indeed trifluoroacetyl decorated derivatives of

OA tended to inhibit inflammation better than OA. All tested compound significantly inhibit the release of inflammation and pain mediator[25,26].

4.6 Conclusion

Oleanolic acid was successfully modified to derivatives. Spectrometric techniques were used to elucidate the chemical structural of OA and its derivatives. Modification of OA resulted in enhancement of analgesic and anti-inflammatory properties.

4.7 References

1. Liu J. (1995). Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology*. *49*(2):57–68.

2. Chen L, Zhang Y, Kong X, Peng S, Tian J. (2007). Synthesis and biological evaluation of nitric oxide-releasing derivatives of oleanolic acid as inhibitors of HepG2 cell apoptosis. *Bioorganic & Medicinal Chemistry Letters*. *17*(11):2979–82.

3. Huang D, Ding Y, Li Y, Zhang W, Fang W, Chen X. (2006). Anti-tumor activity of a 3-oxo derivative of oleanolic acid. *Cancer Letters*. *233*(2):289–296.

4. Leal AS, Wang R, Salvador JAR, Jing Y. (2013) Synthesis of novel heterocyclic oleanolic acid derivatives with improved antiproliferative activity in solid tumor cells. *Organic & Biomolecular Chemistry*. *11*(10):1726–38.

5. Zhou C, Zhang Y, Sheng Y, et al. (2011). Herbaceous peony (*Paeonia lactiflora* Pall.) as an alternative source of oleanolic and ursolic acids. *International Journal of Molecular Sciences*. *12*(1):655–67.

6. Pollier J, Goossens A. (2012). Oleanolic acid. *Phytochemistry*. *77*:10–15.

7. Flores Y, Díaz C, Garay F, Colque O, Sterner O, Almanza GR. (2005). Oleanane-type triterpenes and derivatives from seed coat of Bolivian *Chenopodium quinoa* genotype " salar . *Revista Bolivian de quimica*. *22*(1):71–77.

8. Habila JD, Shode FO, Ndukwe GI, Amupitan JO, Nok AJ. (2012). Effect of C-3 modification of oleanolic acid on *Candida* spp.,*Trichophyton Tonsurans* and *Microsporum canis* inhibition. *Pharmacologia*. *3*(8):313–324.

9. Zhu YM, Shen JK, Wang HK, Cosentino LM, Lee KH. (2001). Synthesis and anti-HIV activity of oleanolic acid derivatives. *Bioorganic & Medicinal Chemistry Letters*. *11*(24):3115–3118.

10. Honda T, Rounds B V., Gribble GW, Suh N, Wang Y, Sporn MB. (1998). Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien- 28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorganic & Medicinal Chemistry Letters*. *8*(19):2711–2714.

11. Liby K, Hock T, Yore MM, et al. (2005). The synthetic triterpenoids, CDDO and CDDO-imidazolide, are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. *Cancer Research*. *65*(11):4789–4798.

12. Honda T, Rounds B, Bore L. (2000). Synthetic oleanane and ursane triterpenoids with modified rings A and C: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *Journal of Medicinal Chemistry*. *43*(22):4233–4246.

13. Mallavadhani UV, Vanga NR, Jeengar MK, Naidu VGM. (2014). Synthesis of novel ring-A fused hybrids of oleanolic acid with capabilities to arrest cell cycle and induce apoptosis in breast cancer cells. *European Journal of Medicinal Chemistry*. *74*:398–404.

14. Nkeh-chungag BN, Temdie JR, Sewani-rusike C, Yolande M, Mbafor JT, and Iputo JE. (2009). Analgesic, anti-inflammatory and antiulcer properties of the extract of *Uapaca guineensis* (Euphorbiaceae). *Journal of Medicinal Plants Research*. *3*(9):635–640.

15. Nkomo M, Nkeh-chungag BN, Kambizi L, Ndebia EJ, Sewani-rusike C, Iputo JE. (2011). Investigation of the antinociceptive and anti-inflammatory properties of *Heteromorpha arborescens* (Apiaceae). *African Journal Traditional Complement Alternative Medicine*. *8*(4):412–419.

16. Nkeh-Chungag BN, Ndebia EJ, Mbafor JT, Dotwana LA, Oyedeji OO, Iputo JE. (2014). The effect of *Cordia platythyrsa* on various experimental models of pain and carrageenan induced inflammation. *African Journal of Biotechnology*. *13*(2):343–348.

17. Rodríguez JA, Astudillo L, Schmeda-hirschmann G. (2003). Oleanolic acid promotes healing of acetic acid-induced chronic gastric lesions in rats. *Pharmacological Research*. *48*(3):291–294.

18. Liu H, Shi Y, Wang D, Yang G, Yu A, Zhang H. (2003). MECC determination of oleanolic acid and ursolic acid isomers in *Ligustrum lucidum* Ait. *Journal of Pharmaceutical and Biomedical Analysis*. *32*(3):479–485.

19. Brendolise C, Yauk YK, Eberhard ED, et al. (2011). An unusual plant triterpene synthase with predominant α-amyrin-producing activity identified by characterizing oxidosqualene cyclases from Malus × domestica. *The FEBS Journal*. *278*(14):2485–2499.

20. Bednarczyk-Cwynar B, Zaprutko L, Ruszkowski P, Hładoń B. (2012). Anticancer effect of A-ring or/and C-ring modified oleanolic acid derivatives on KB, MCF-7 and heLa cell lines. *Organic & Biomolecular Chemistry*. *10*(11):2201–2205.

21. Bowsher D. (1991). Neurogenic pain syndromes and their management. *British Medical Bulletin*. *47*(3):644–666.

22. Araújo IWFD, Vanderlei EDSO, Rodrigues JAG, et al. (2011). Effects of a sulfated polysaccharide isolated from the red seaweed Solieria filiformis on models of nociception and inflammation. *Carbohydrate Polymers*. *86*(3):1207–1215.

23. Vasconcelos MAL, Royo VA, Ferreira DS, et al. (2006). In vivo analgesic and anti-inflammatory activities of ursolic acid and oleanoic acid from *Miconia albicans* (Melastomataceae). *Zeitschrift fur Naturforschung C-Journal of Biosciences*. *61*(7):477–482.

24. Kang OH, Kang SH, Kim SB, et al. (2012). Anti-inflammatory effect of oleanoic acid 28-O--D-glycopyranosyl ester isolated from Aralia cordata in activated HMC-1 cells. *African Journal of Pharmacy and Pharmacology*. *6*(46):3206–3214.

25. Tsai JC, Peng WH, Chiu TH, Lai SC, Lee CY. (2011). Anti-inflammatory effects of *Scoparia dulcis* L. and betulinic acid. *The American Journal of Chinese Medicine*. *39*(5):943–956.

26. Dinarello CA. (2010). Anti-inflammatory agents: Present and future. *Cell*. *140*(6):935–950.

APPENDICIES 1-8

APPENDIX 1

Spectra of eugenol

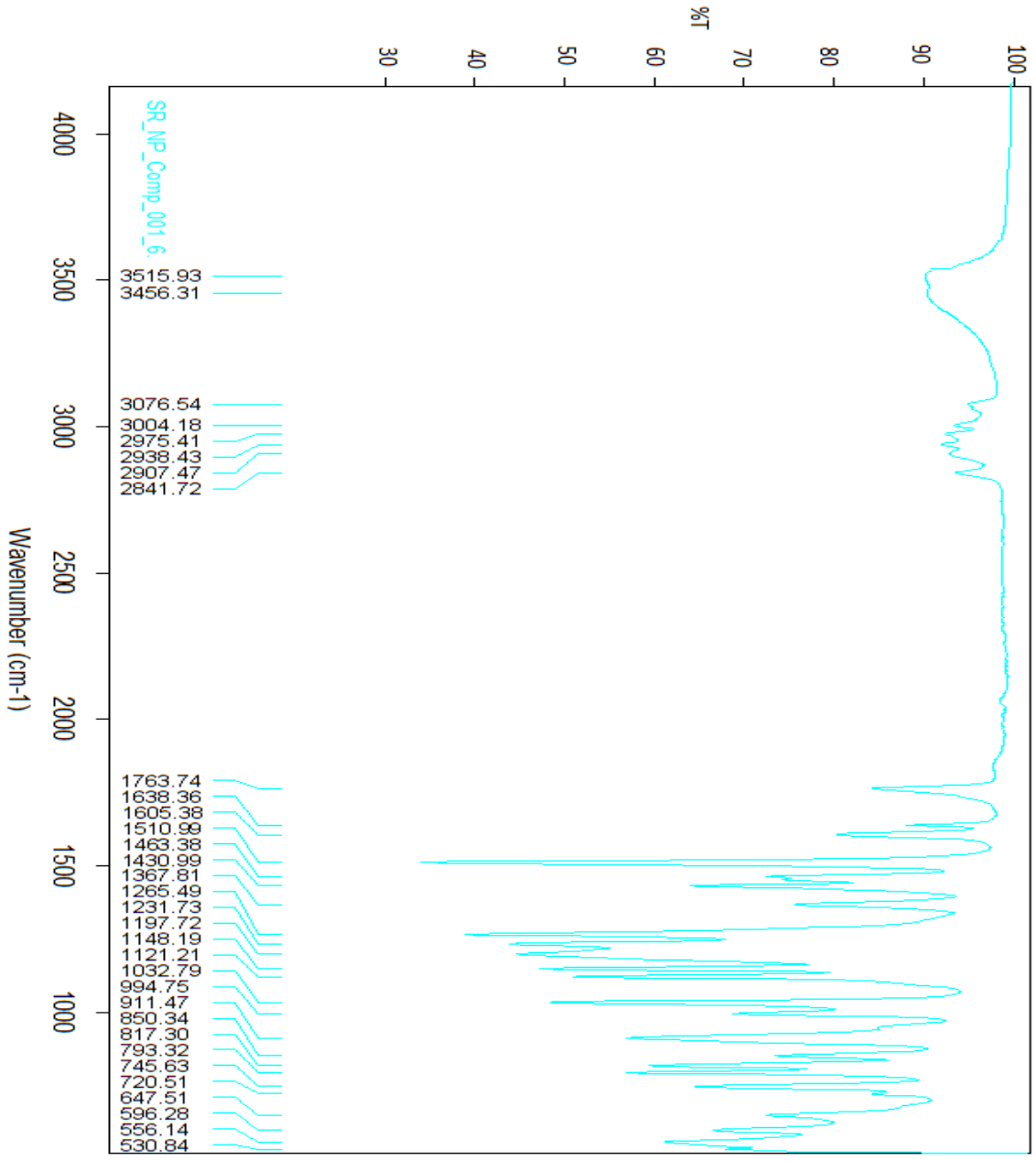


Figure 3.1.1: FT-IR spectrum of eugenol

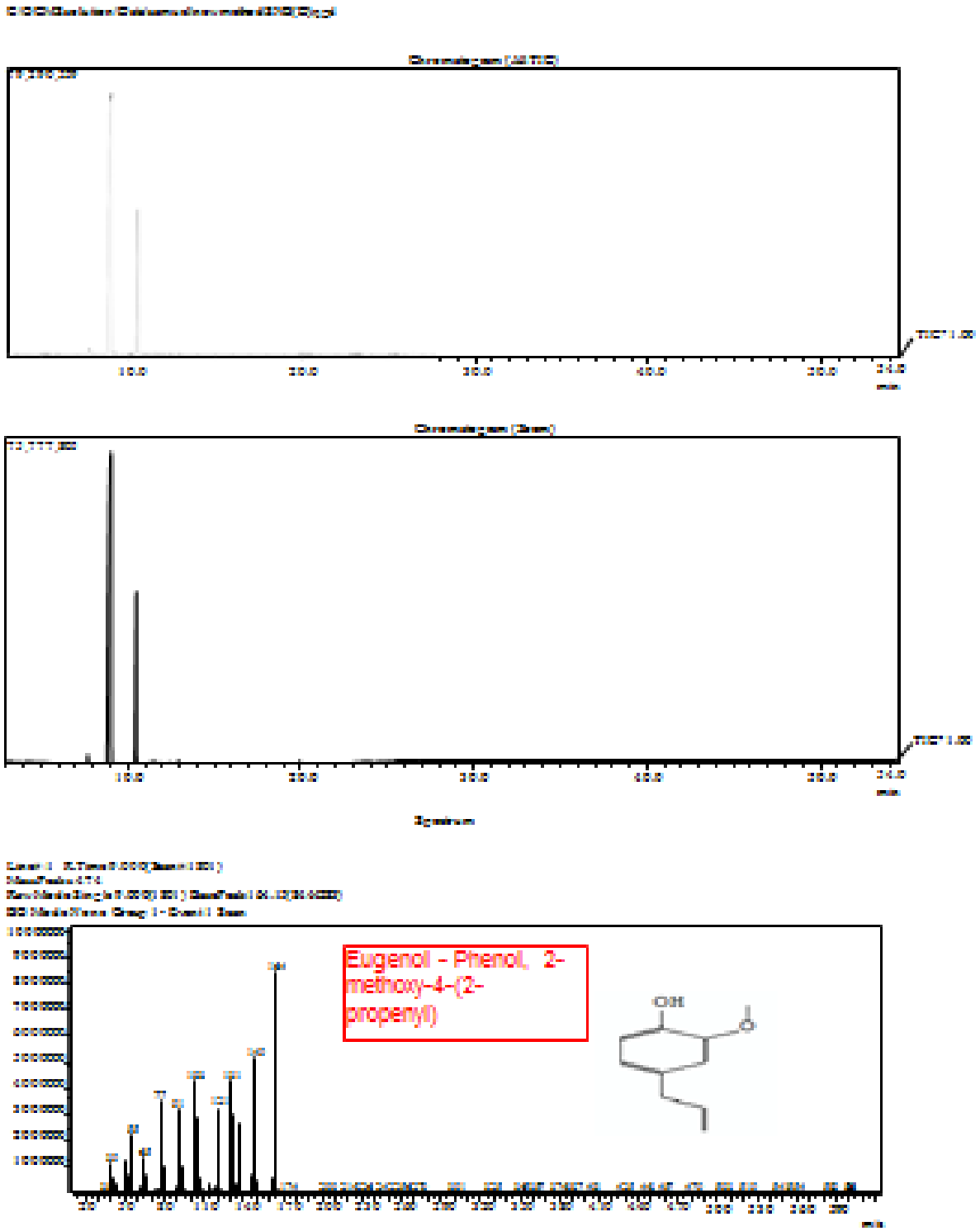


Figure 3.1.2: GC-MS chromatogram of eugenol

APPENDIX 2

Spectra of maslinic acid

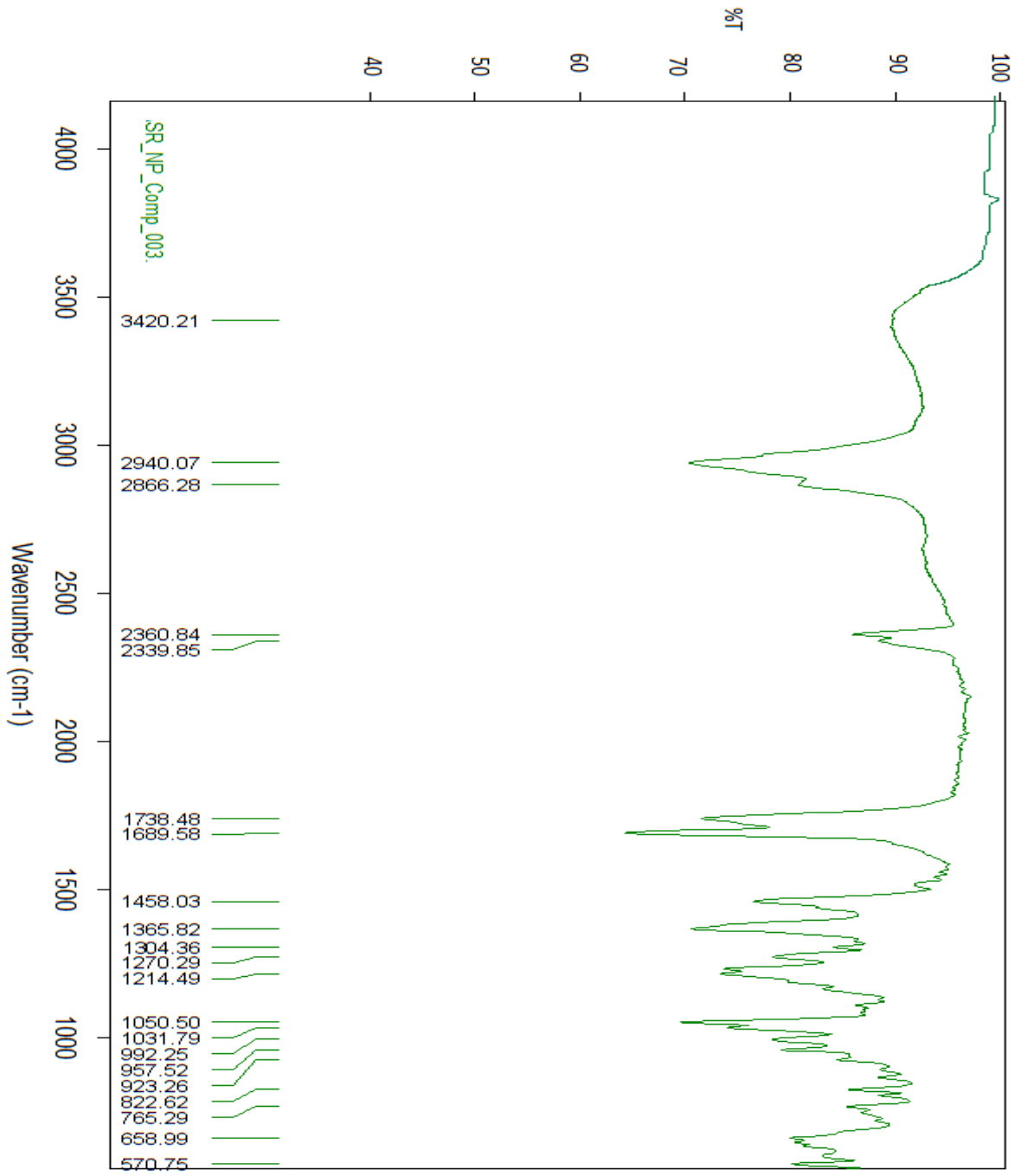


Figure 3.2.1: FT-IR spectrum of maslinic acid

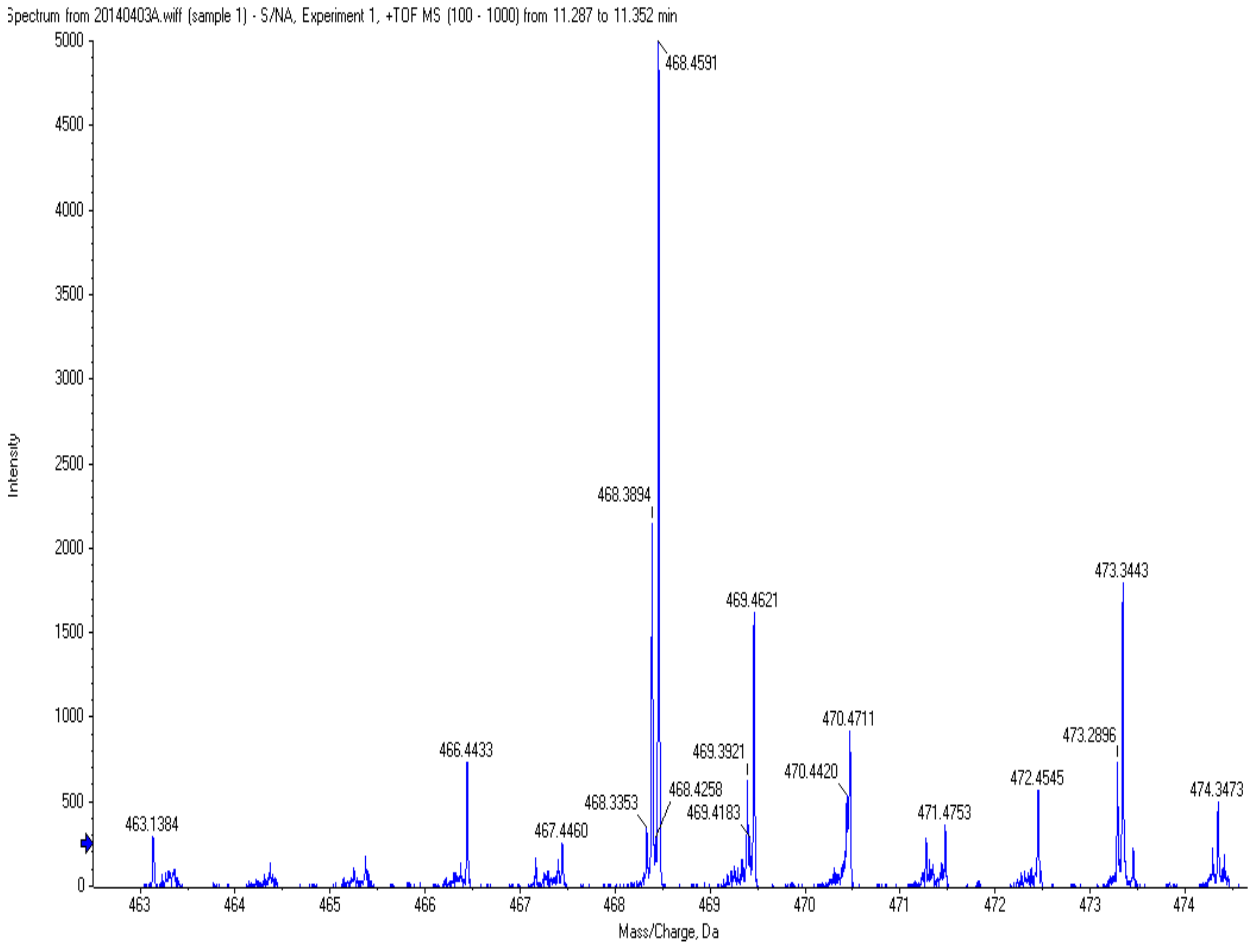


Figure 3.2.2: Mass spectrum of maslinic acid

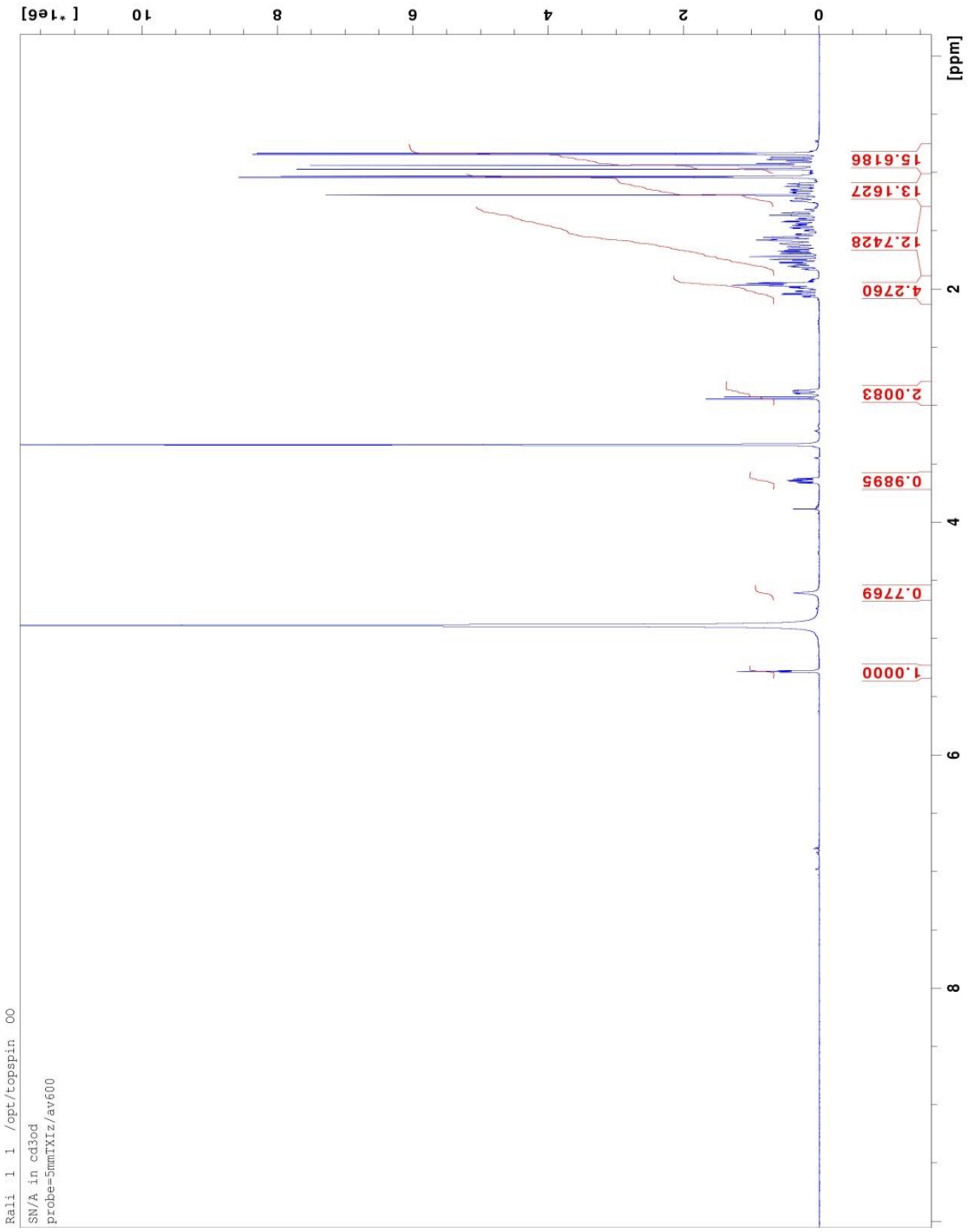


Figure 3.2.3: ^{1}H-NMR spectrum of maslinic acid

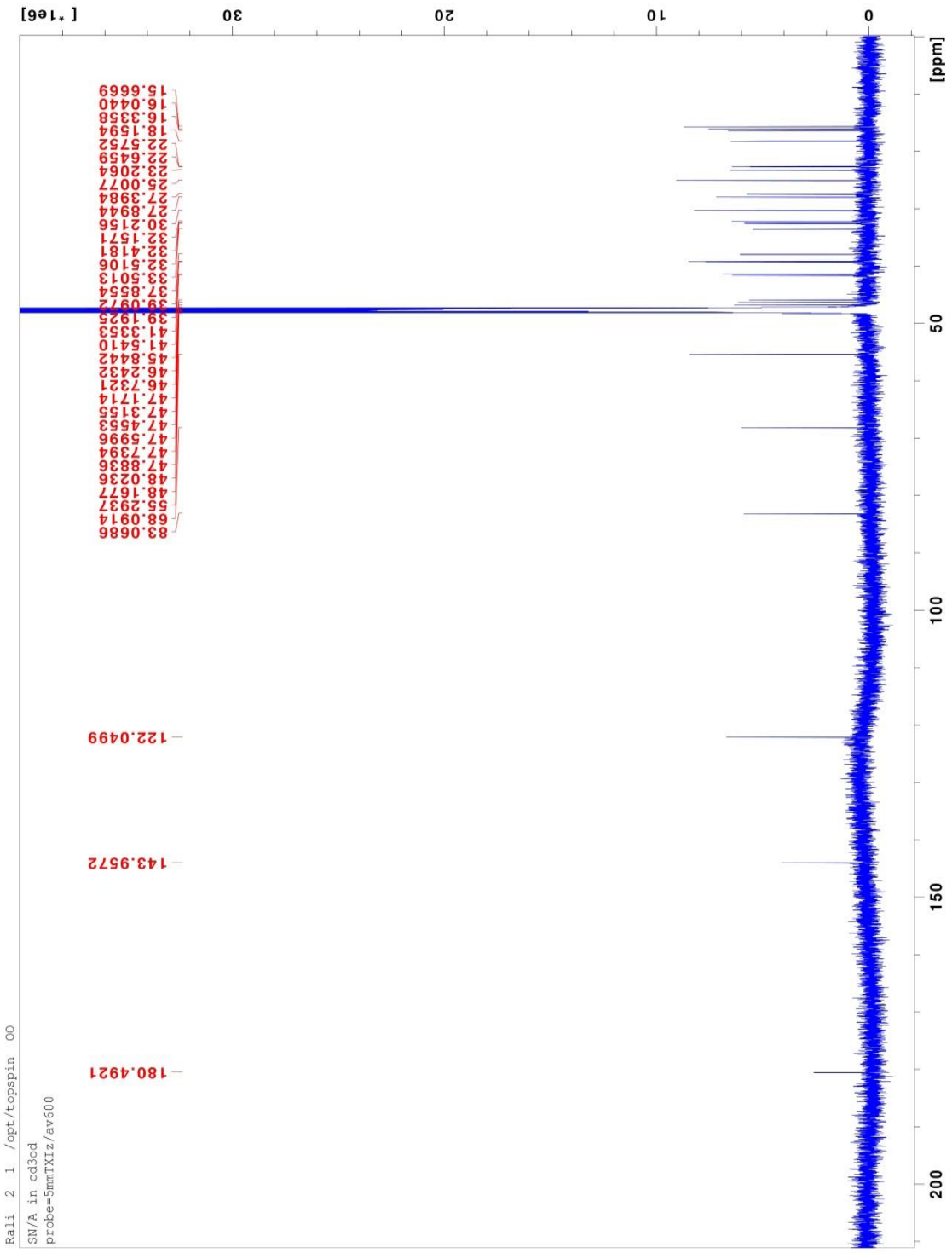


Figure 3.2.4: ^{13}C-NMR spectrum of maslinic acid

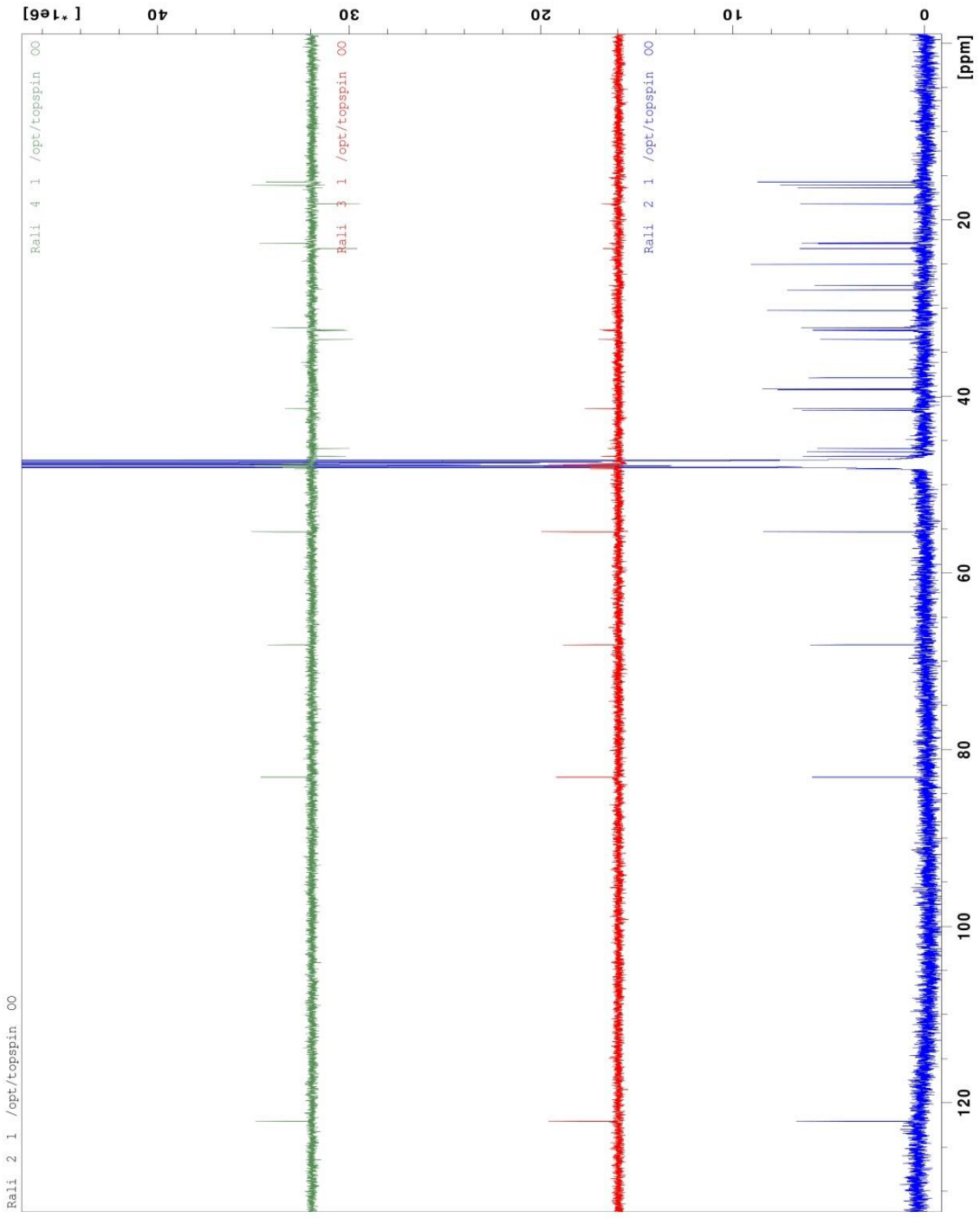


Figure 3.2.5: ^{13}C-DEPT NMR spectrum of maslinic acid

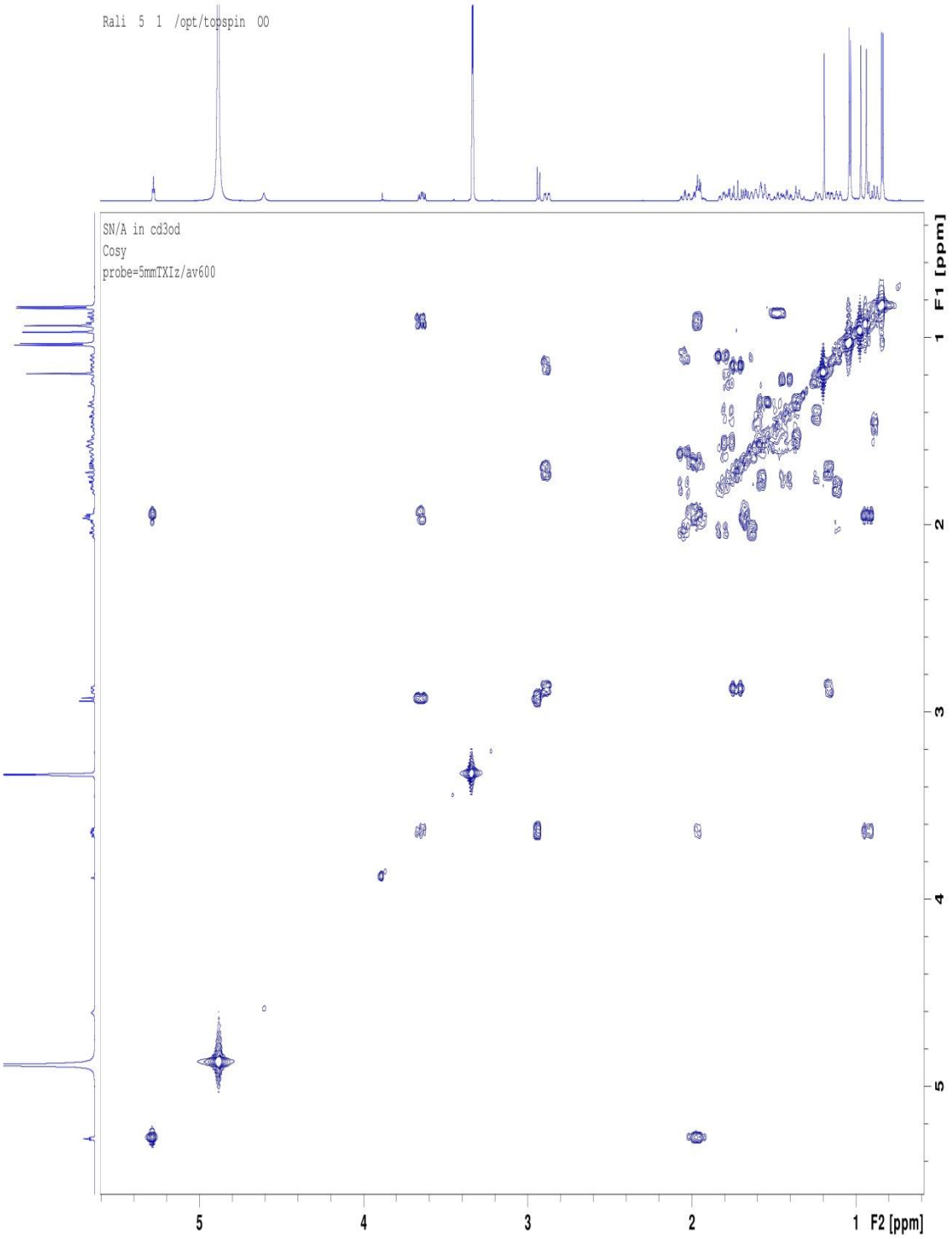


Figure 3.2.6: ^{1}H-^{1}H COSY NMR spectra of maslinic acid

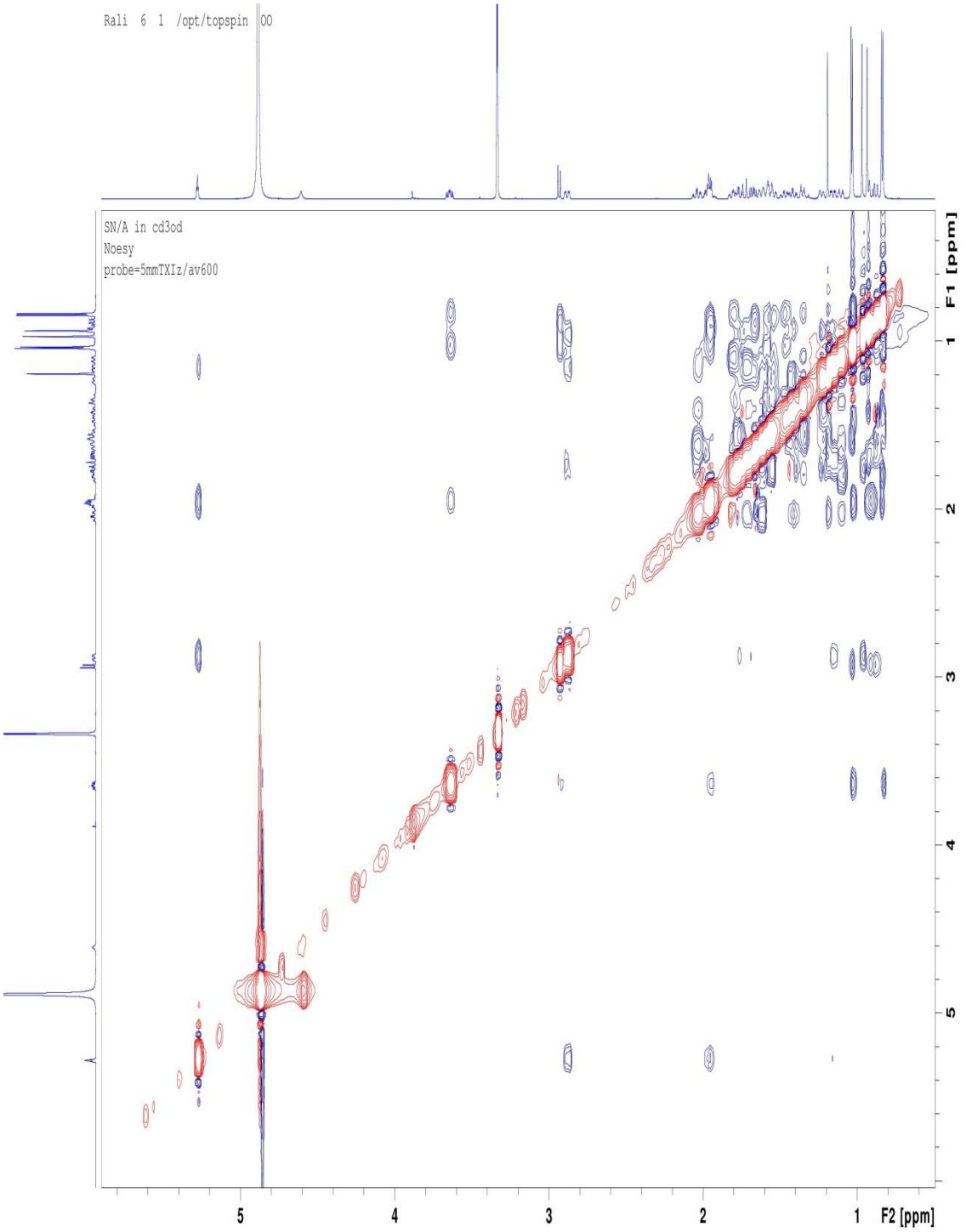


Figure 3.2.7: ^{1}H-^{1}H NOESY NMR spectra of maslinic acid

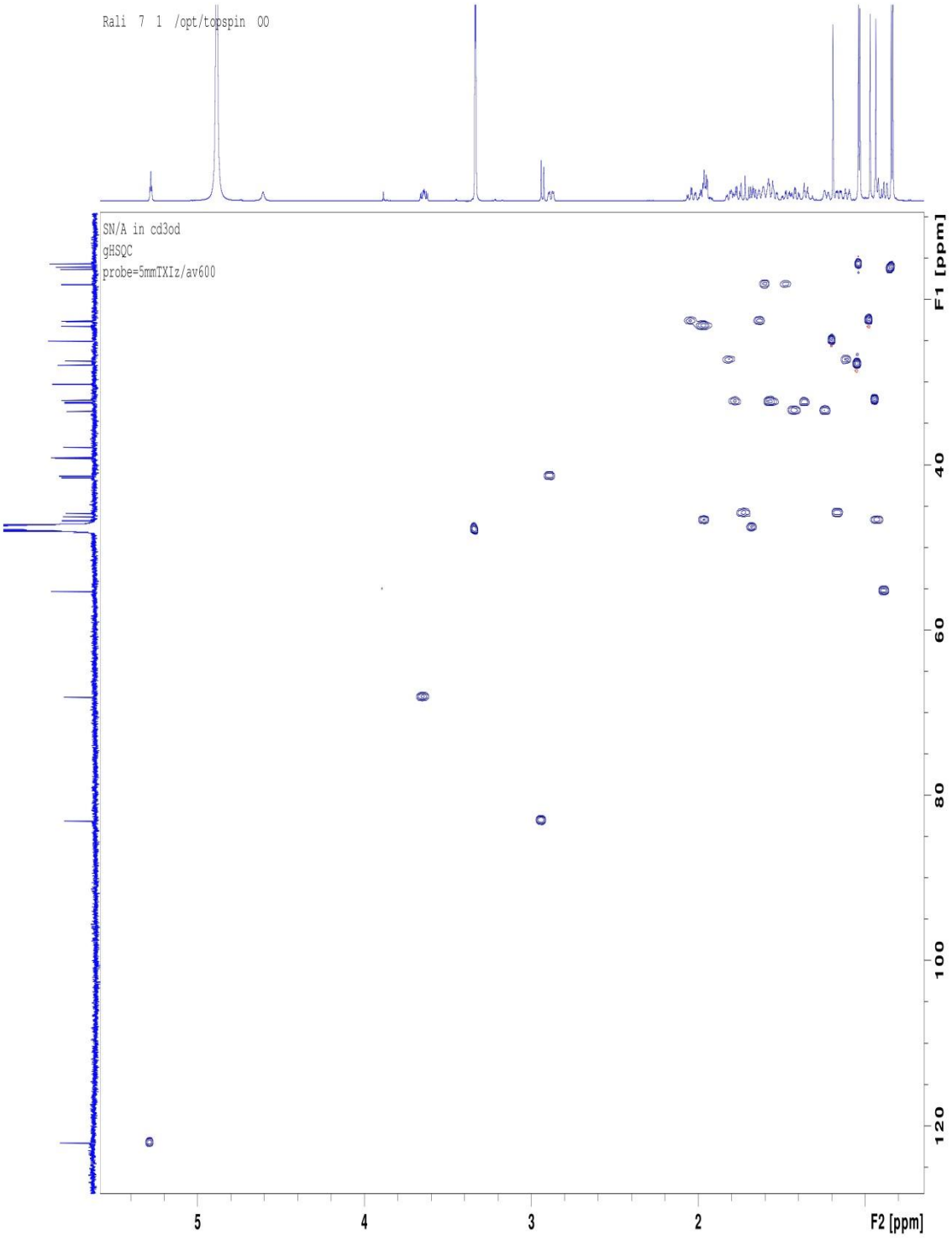


Figure 3.2.8: ^{1}H-^{13}C-HSQC NMR spectrum of maslinic acid

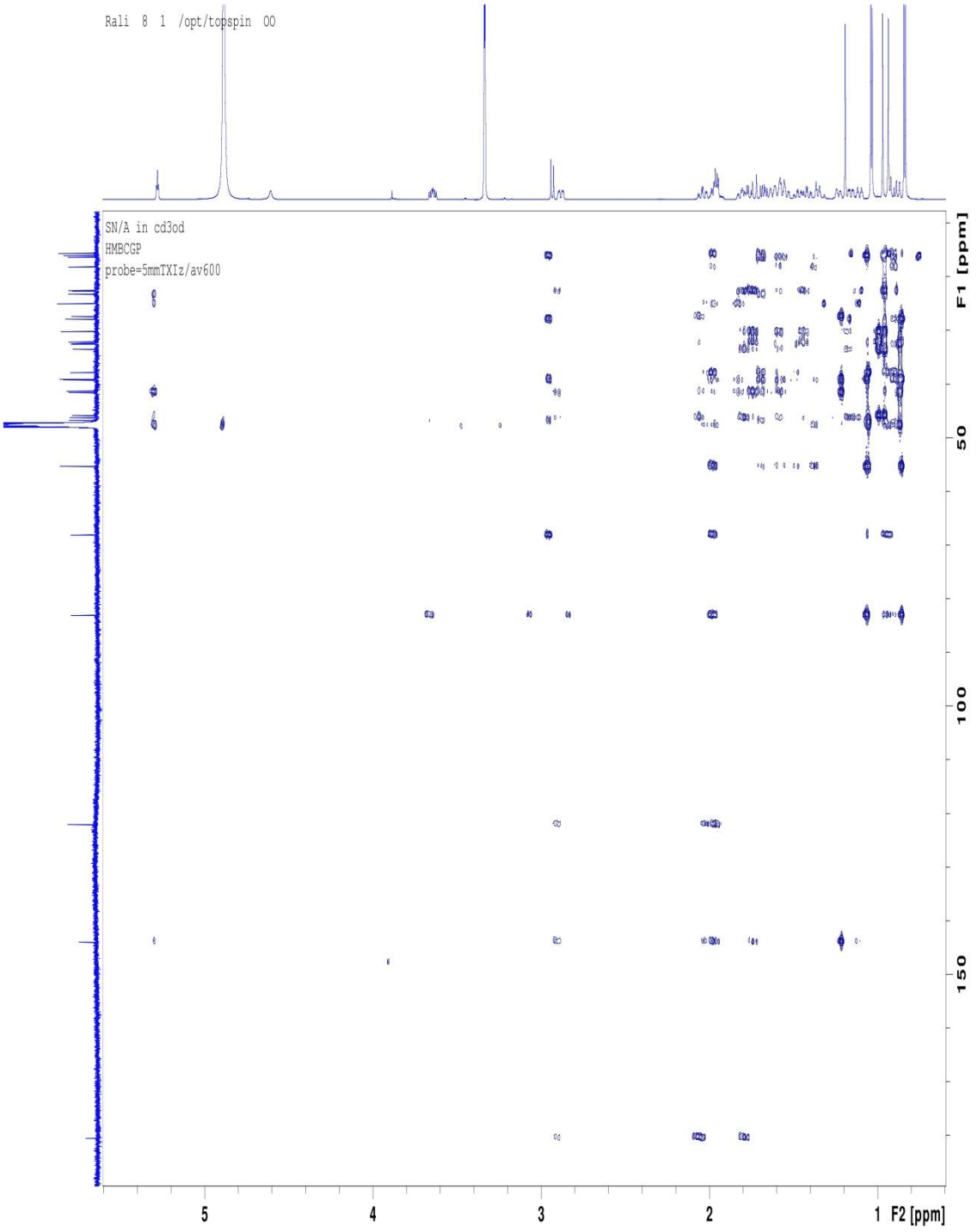


Figure 3.2.9: ^{1}H-^{13}C HMBC NMR spectrum of maslinic acid

APPENDIX 3

Spectra of oleanolic acid

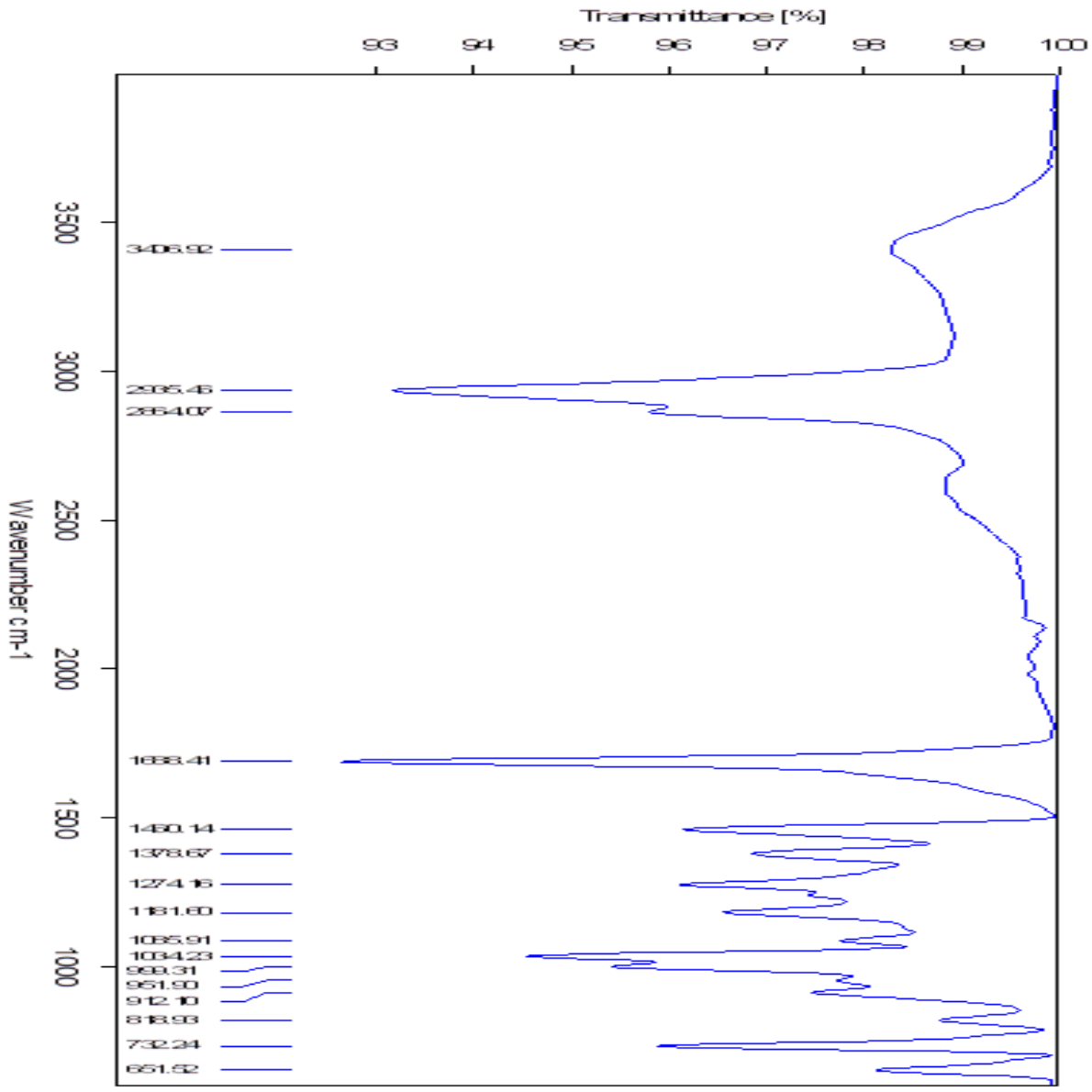


Figure 3.3.1: FT-IR spectrum of oleanolic acid

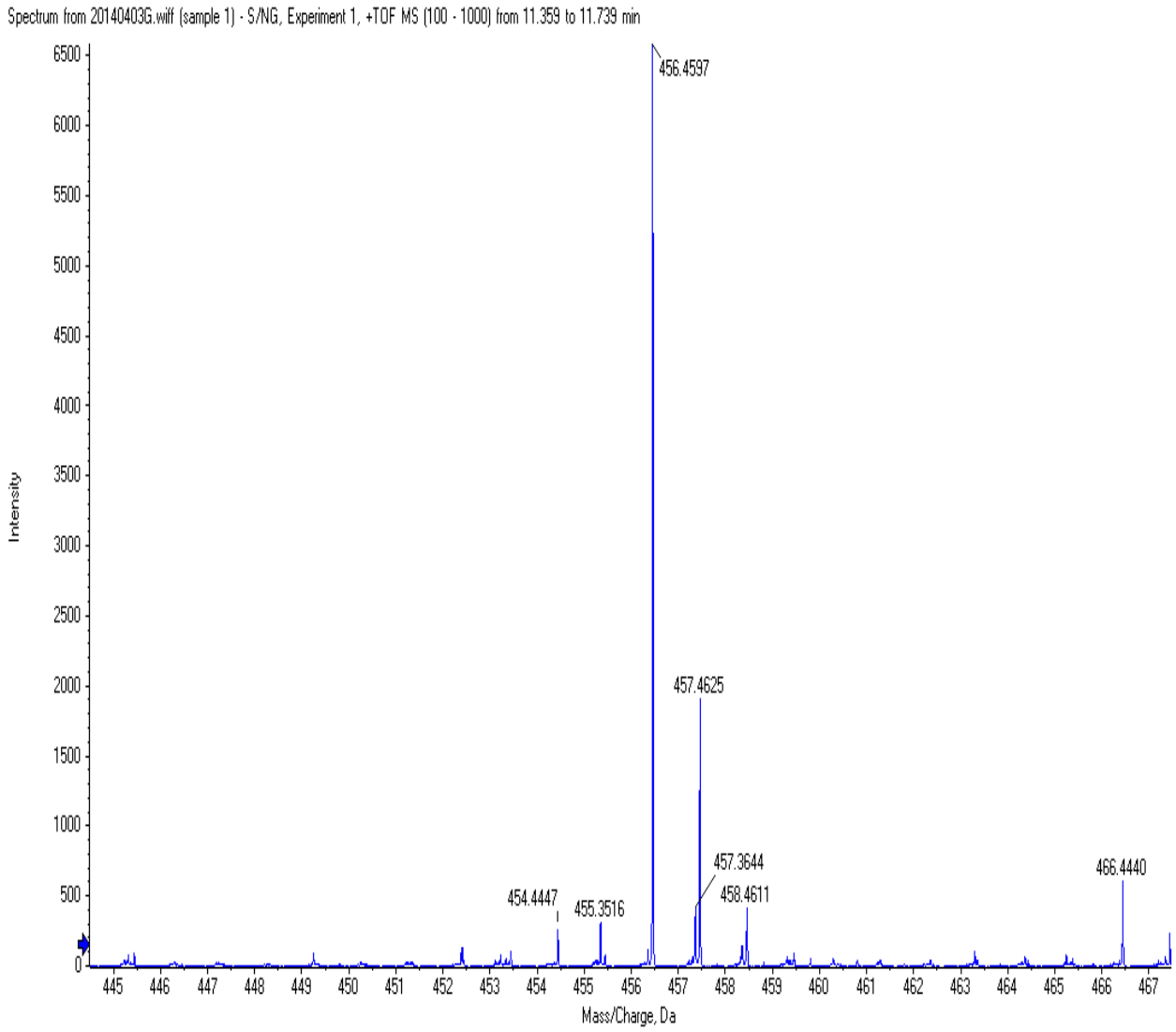


Figure 3.3.2: Mass spectrum of oleanolic acid

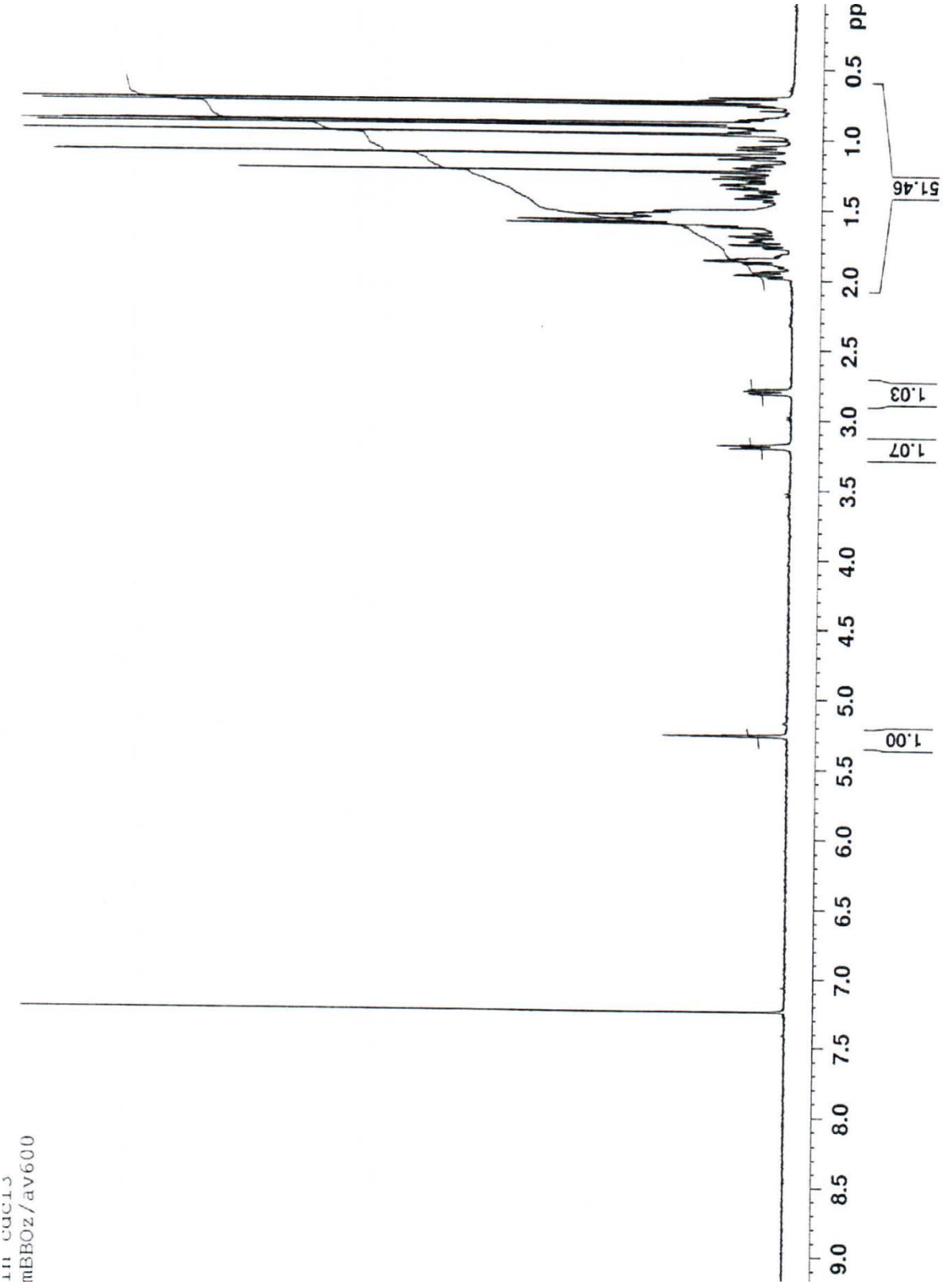


Figure 3.3.3: ^{1}H-NMR spectrum of oleanolic acid

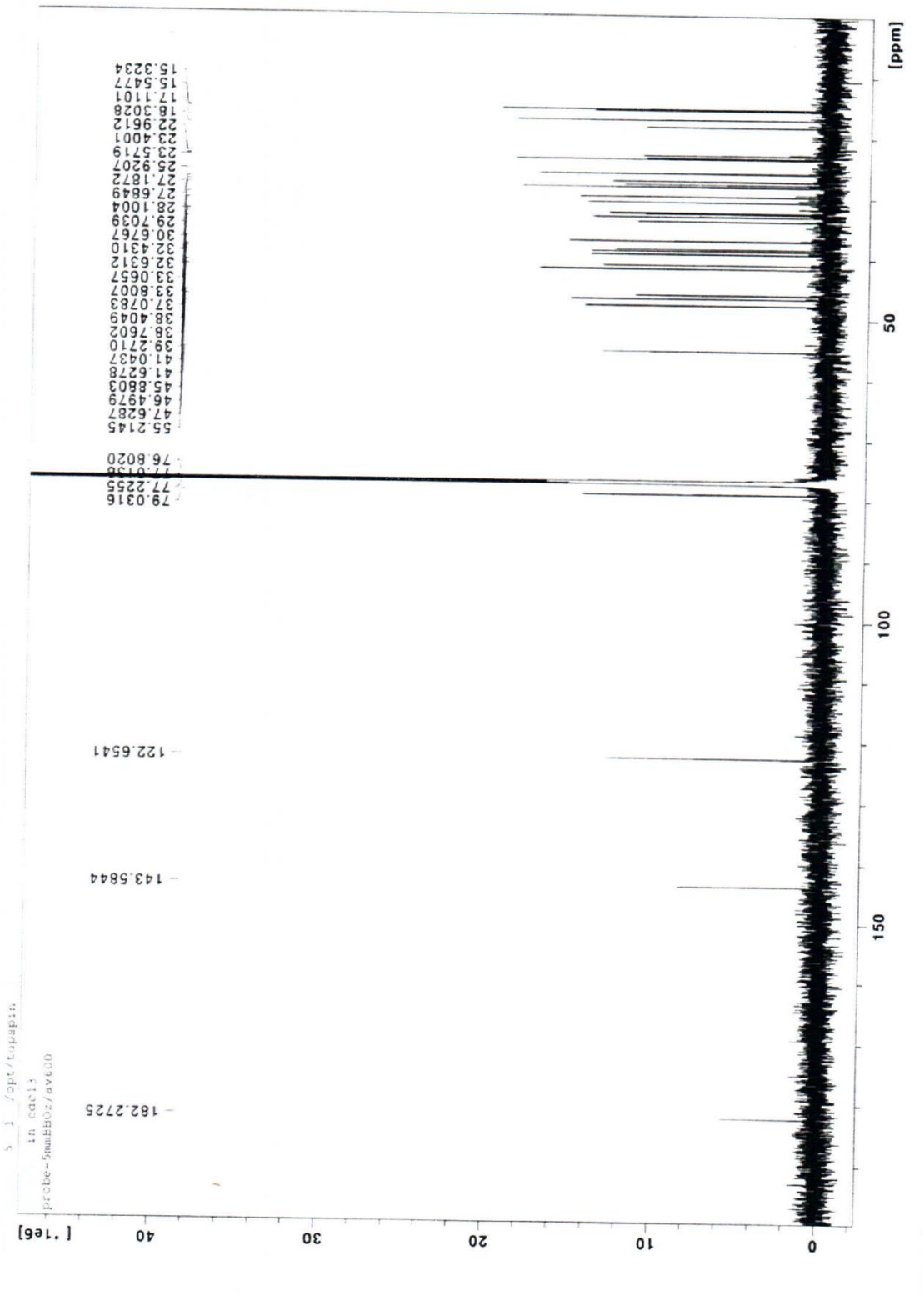


Figure 3.3.4: ^{13}C-NMR spectrum of oleanolic acid

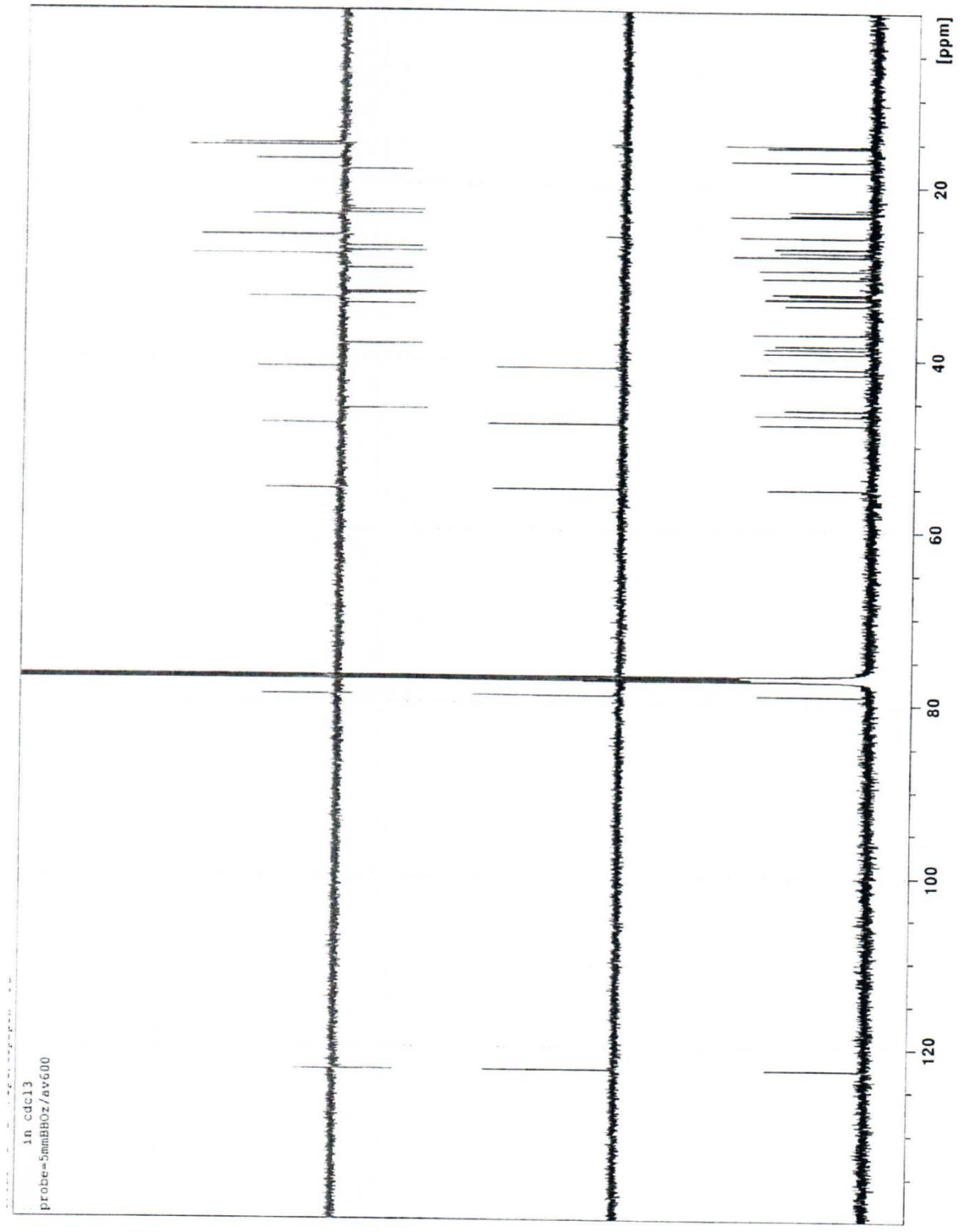


Figure 3.3.5: ^{13}C DEPT NMR spectrum of oleanolic acid

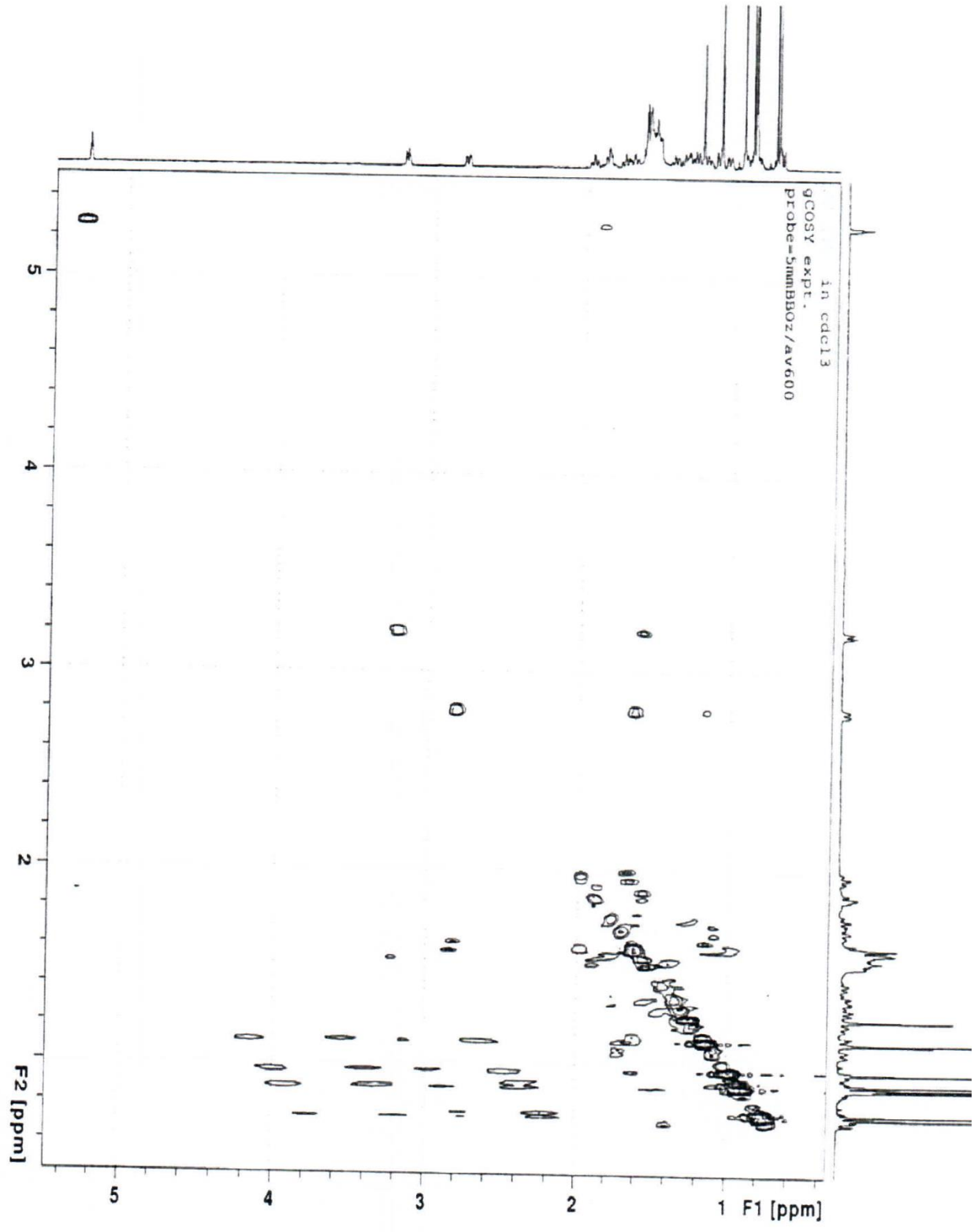


Figure 3.3.6: ^{1}H-^{1}H COSY NMR spectrum of oleanolic acid

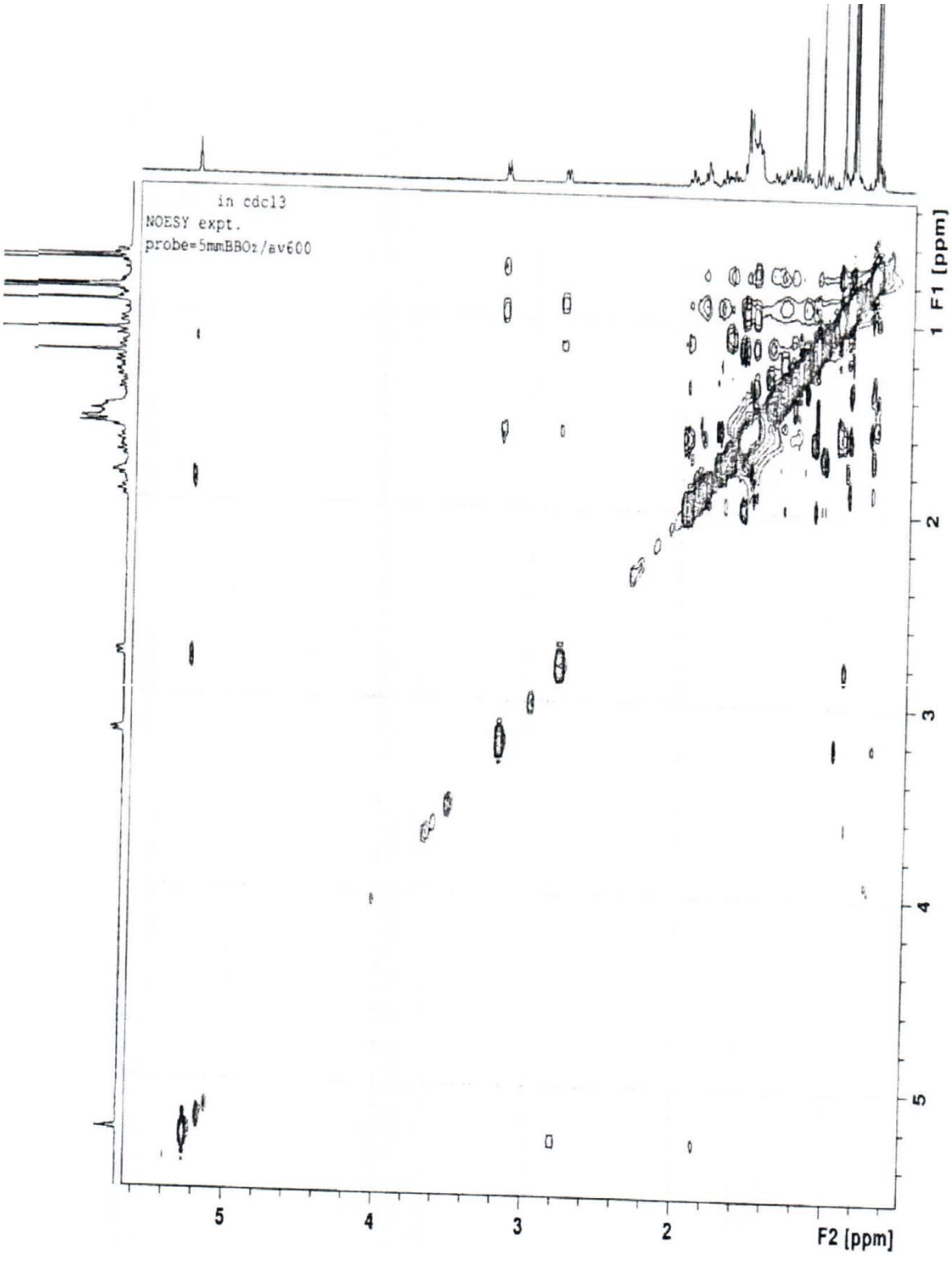


Figure 3.3.7: ^{1}H-^{1}H NOESY NMR spectrum of oleanolic acid

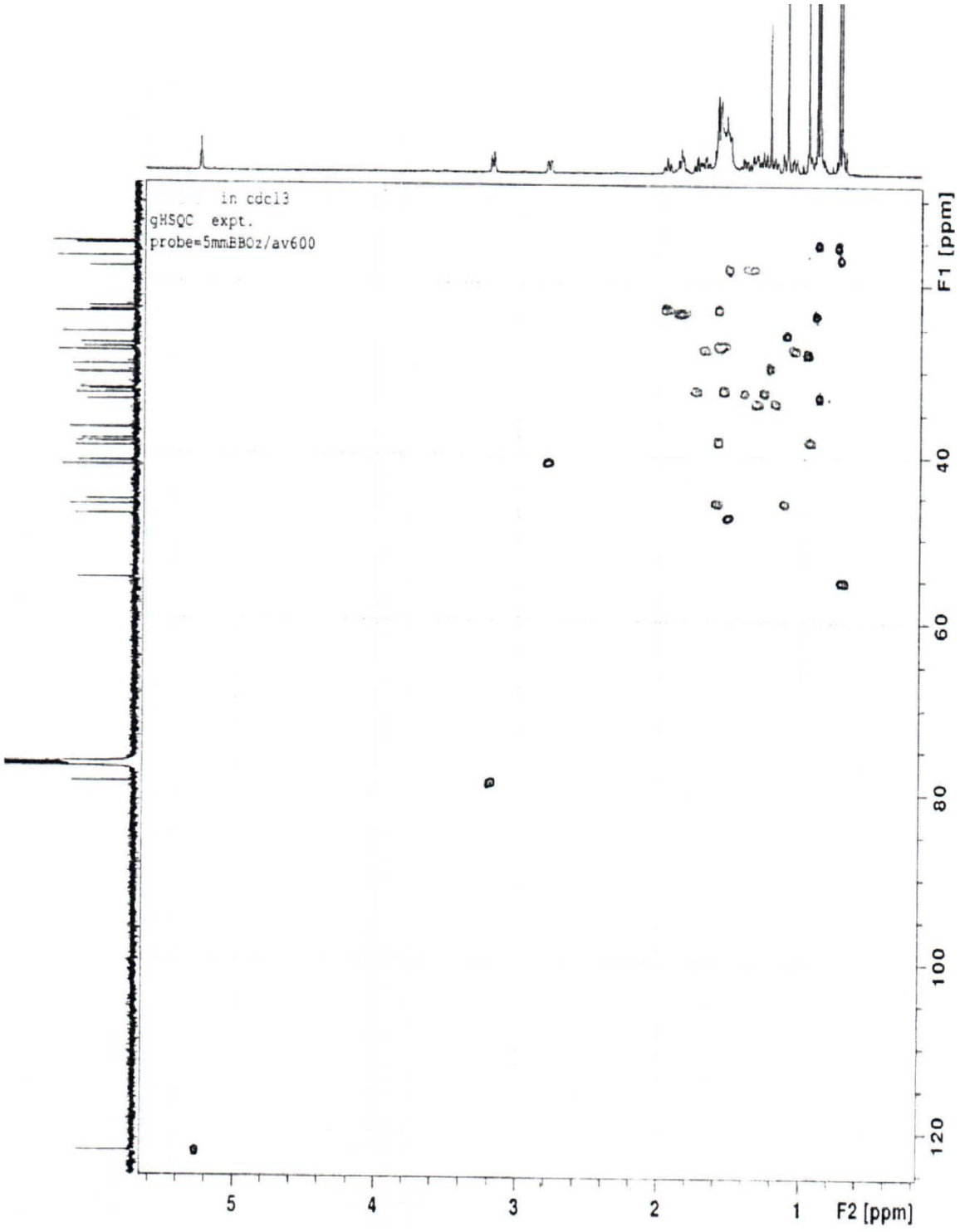


Figure 3.3.8: ^{1}H-^{13}C HSQC NMR spectrum of oleanolic acid

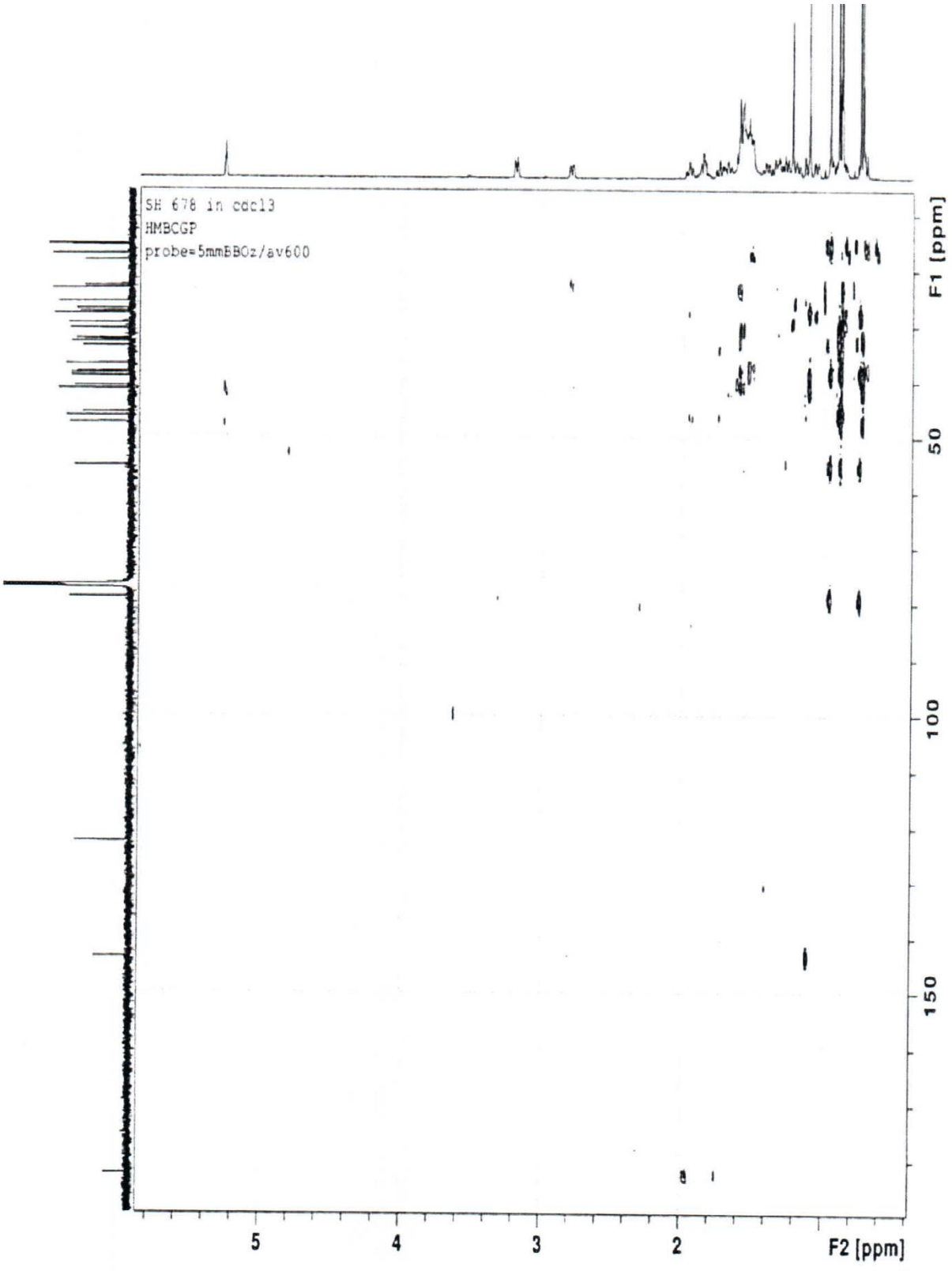


Figure 3.3.9: ^{1}H-^{13}C HMBC NMR spectrum of oleanolic acid

APPENDIX 4

Spectra of 3β-acetyloleanolic acid

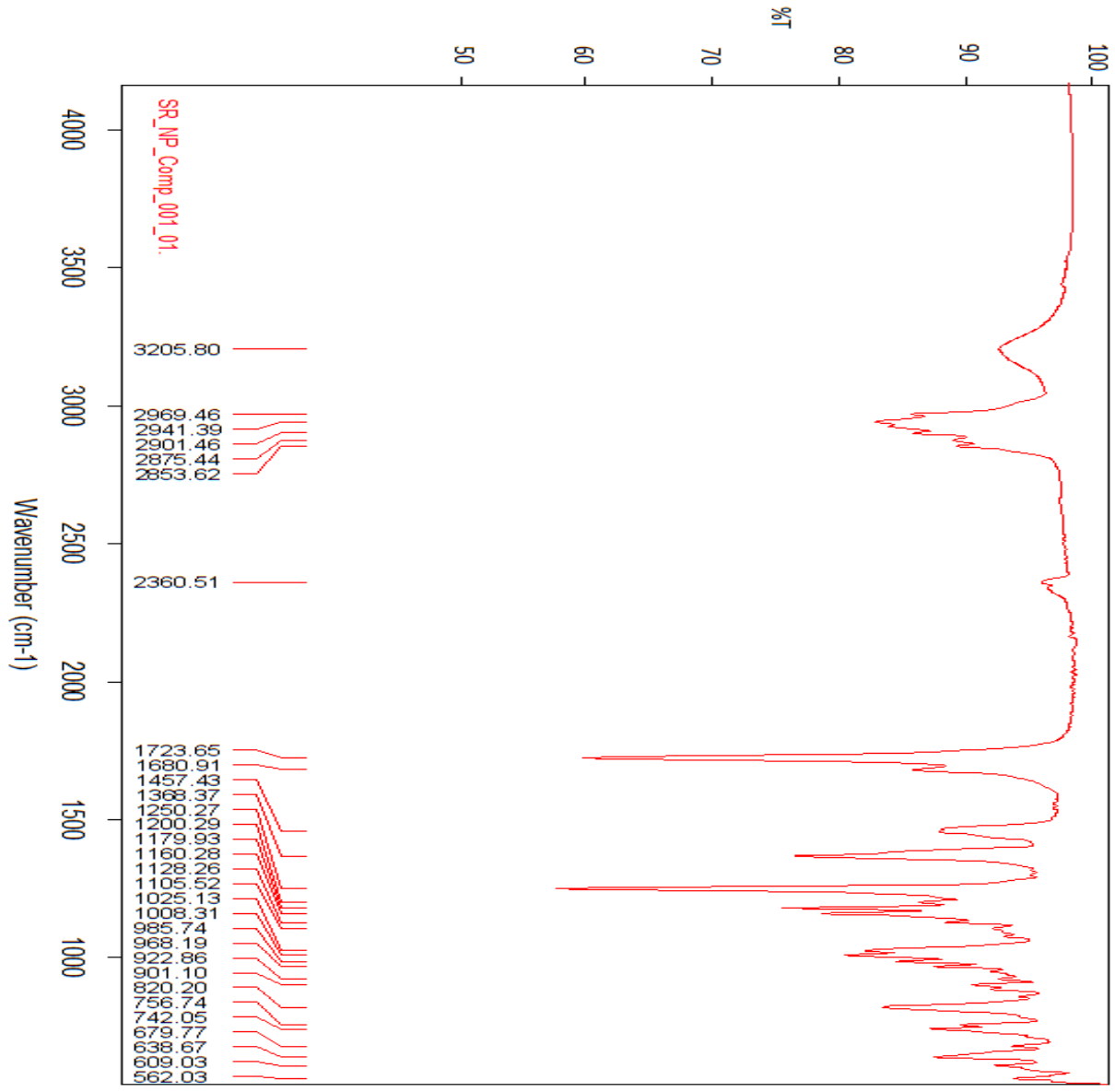


Figure 4.1.1: FT-IR spectrum of 3β-acetyloleanolic acid

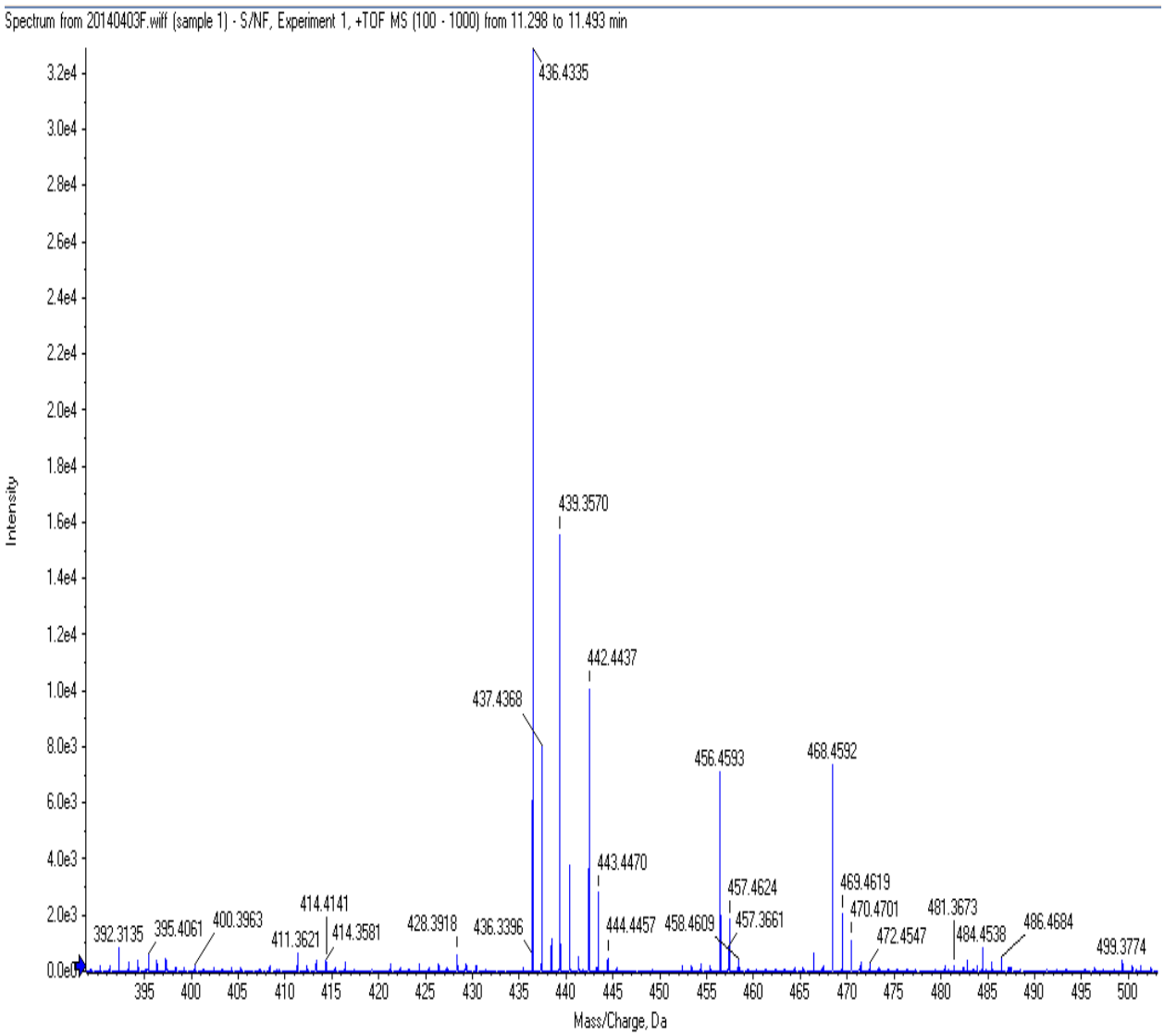


Figure 4.1.2: Mass spectrum of 3β-acetyloleanolic acid

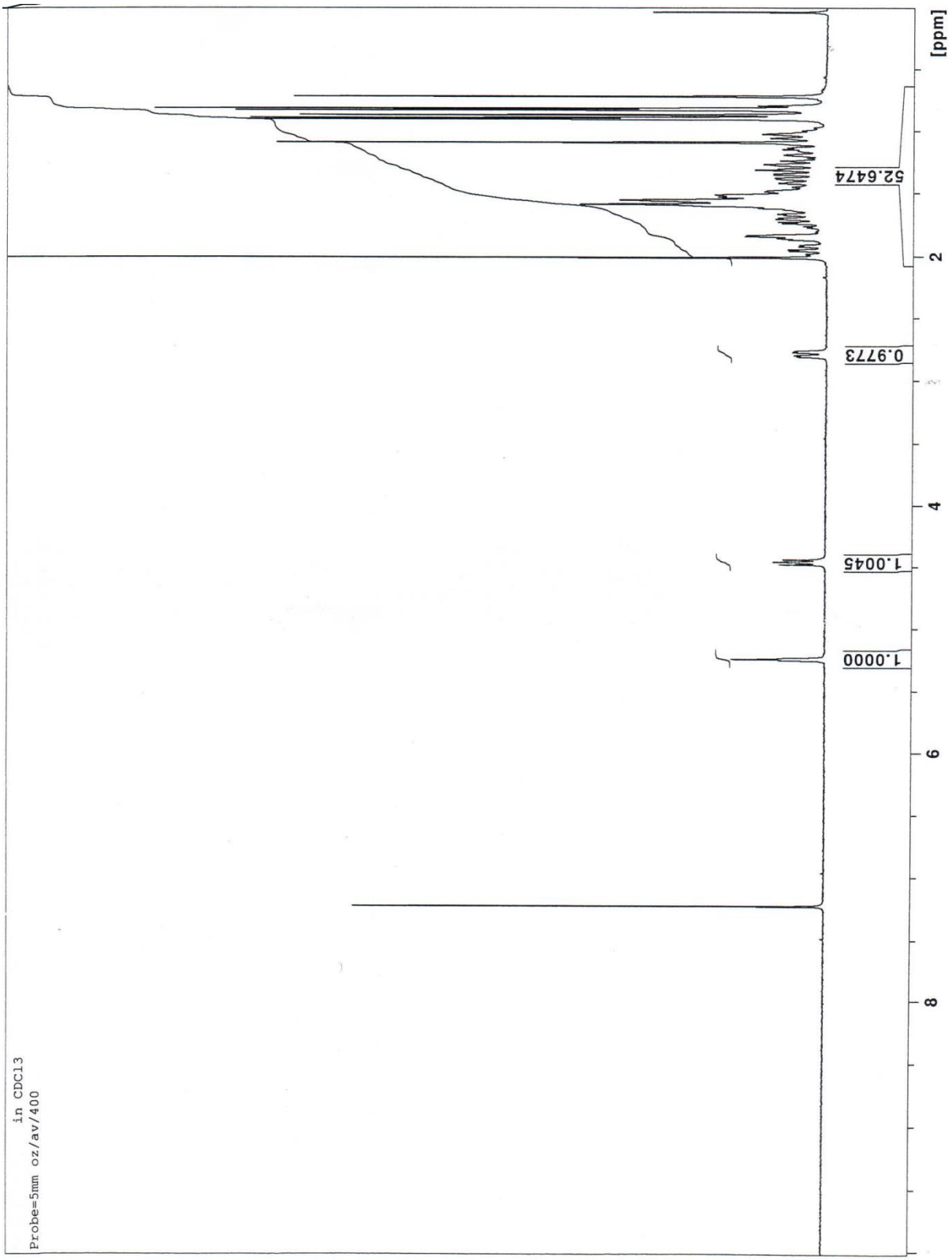


Figure 4.1.3: ^{1}H-NMR spectrum of 3β-acetyloleanolic acid

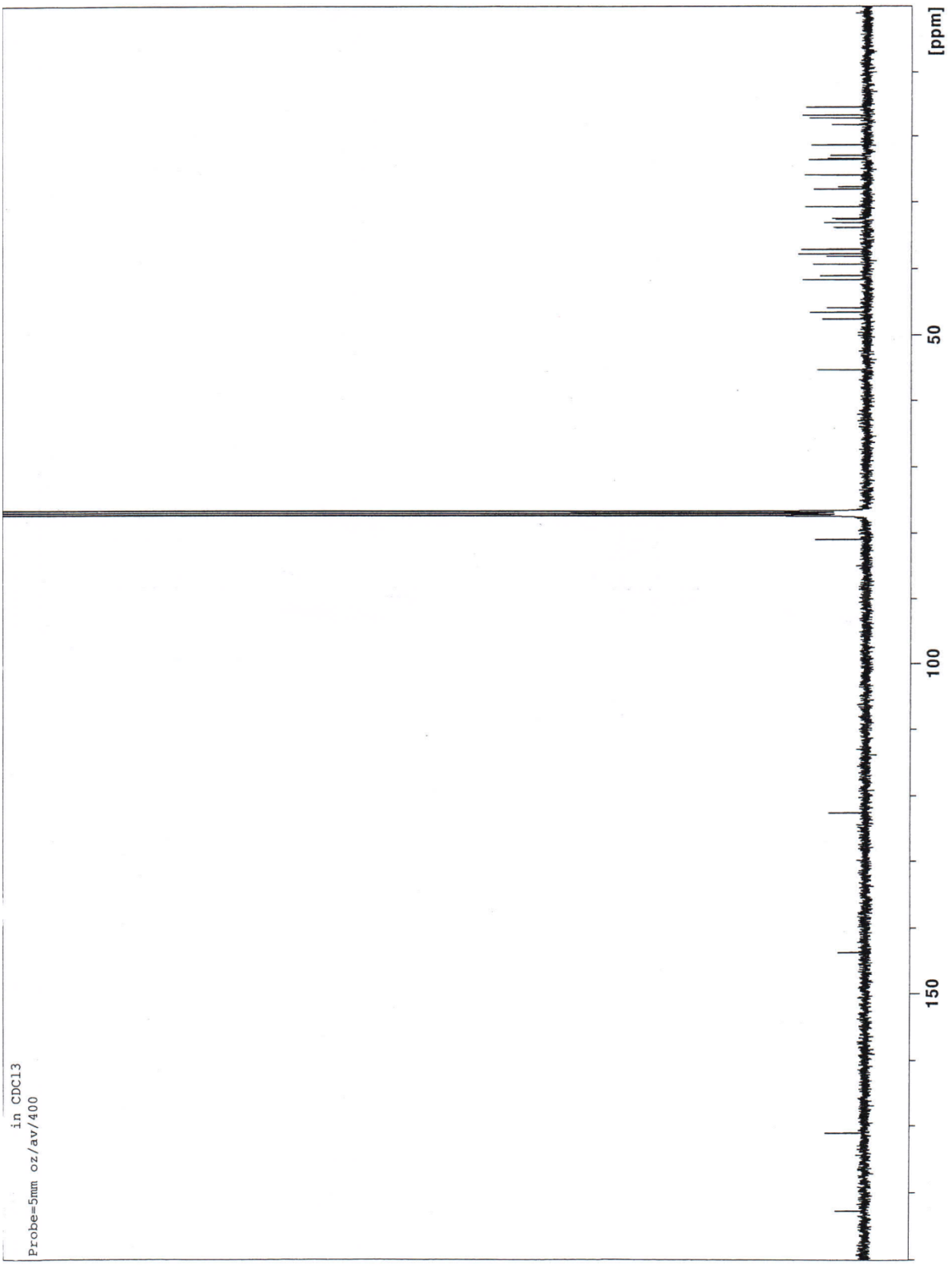


Figure 4.1.4: ^{13}C-NMR spectrum of 3β-acetyloleanolic acid

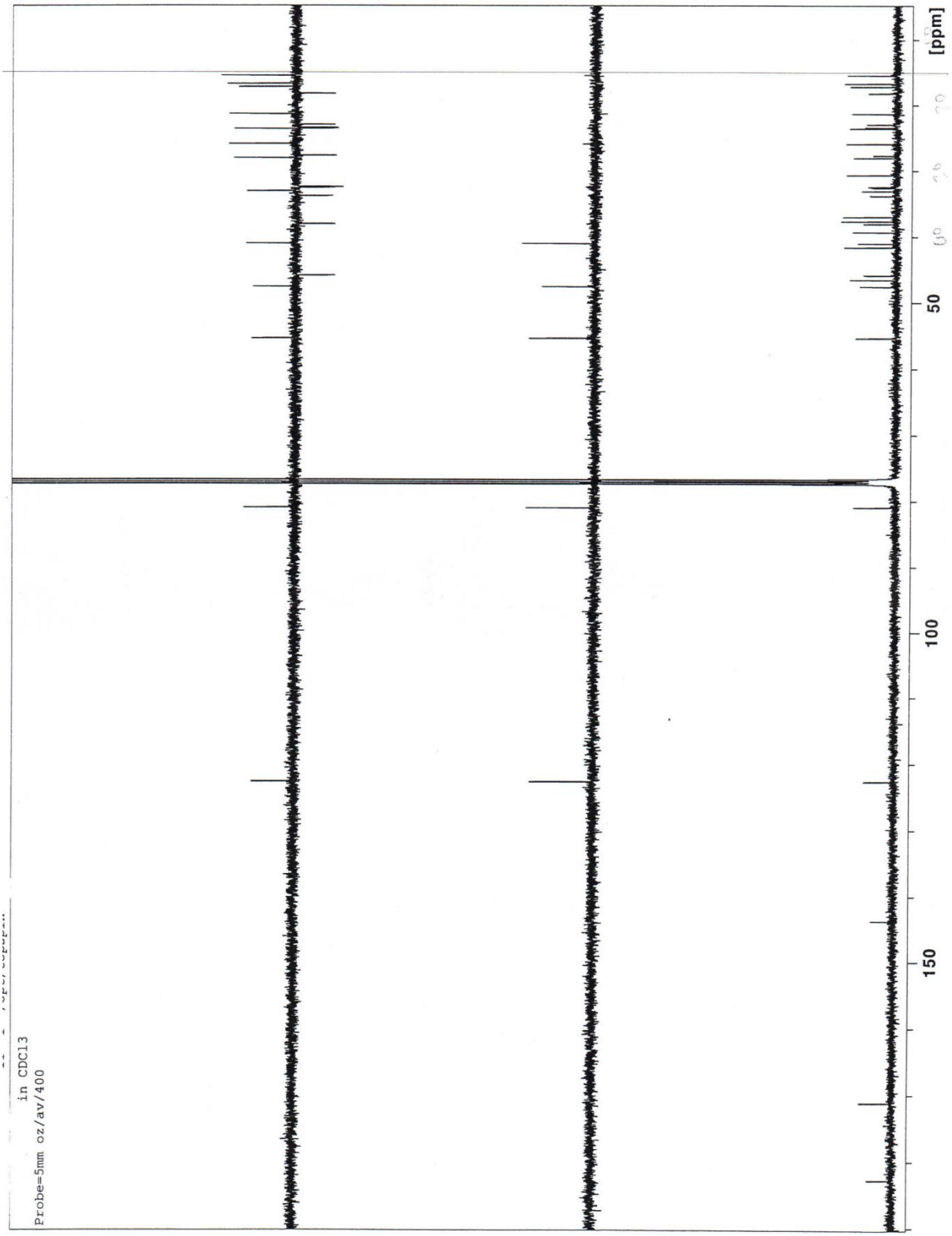


Figure 4.1.5: ^{13}C DEPT NMR spectra of 3β-acetyloleanolic acid

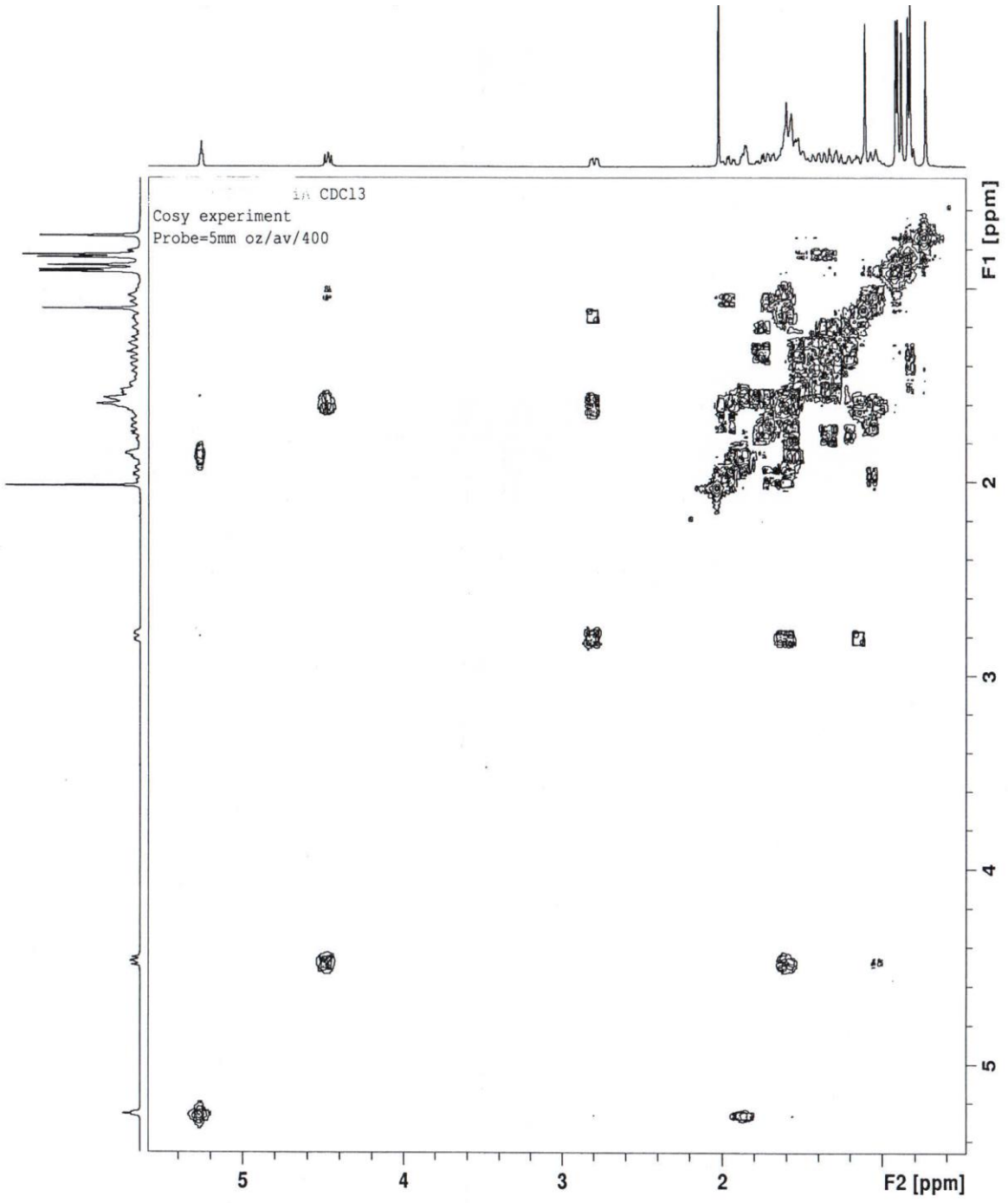


Figure 4.1.6: ^{1}H-^{1}H COSY NMR spectra of 3β-acetyloleanolic acid

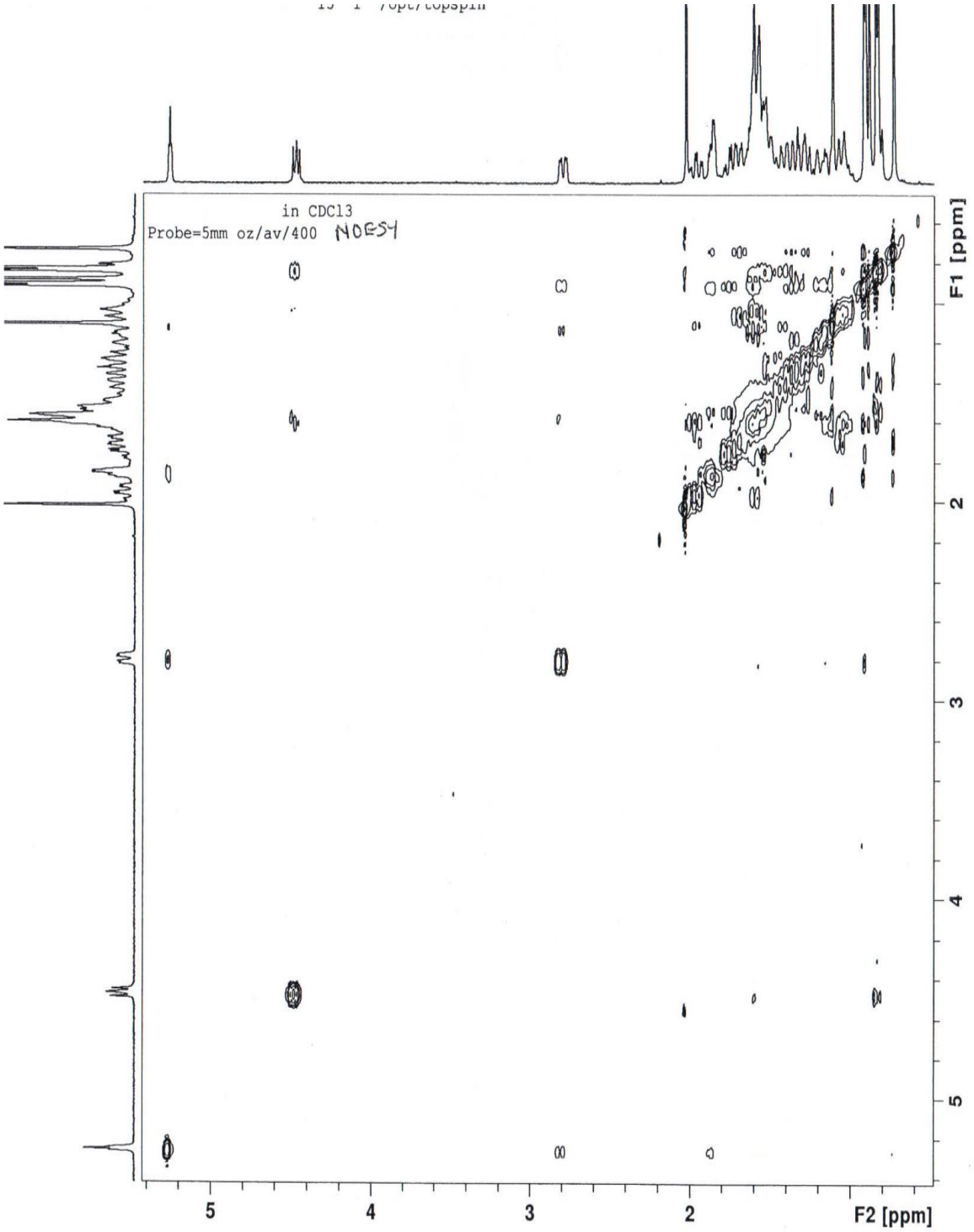


Figure 4.1.7: ^{1}H-^{1}H NOESY NMR spectra of 3β-acetyloleanolic acid

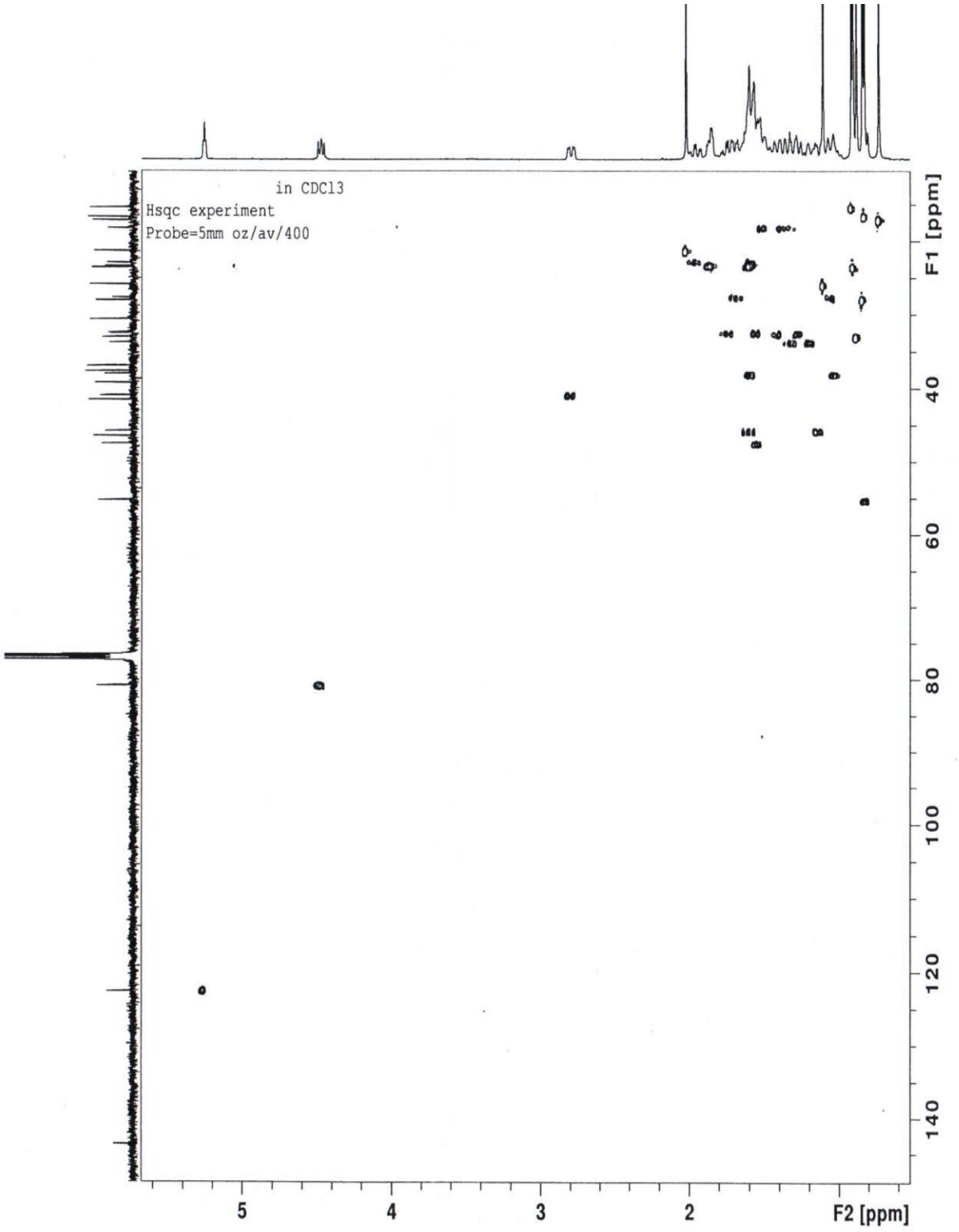


Figure 4.1.7: ^{1}H-^{13}C HSQC NMR spectra of 3β-acetyloleanolic acid

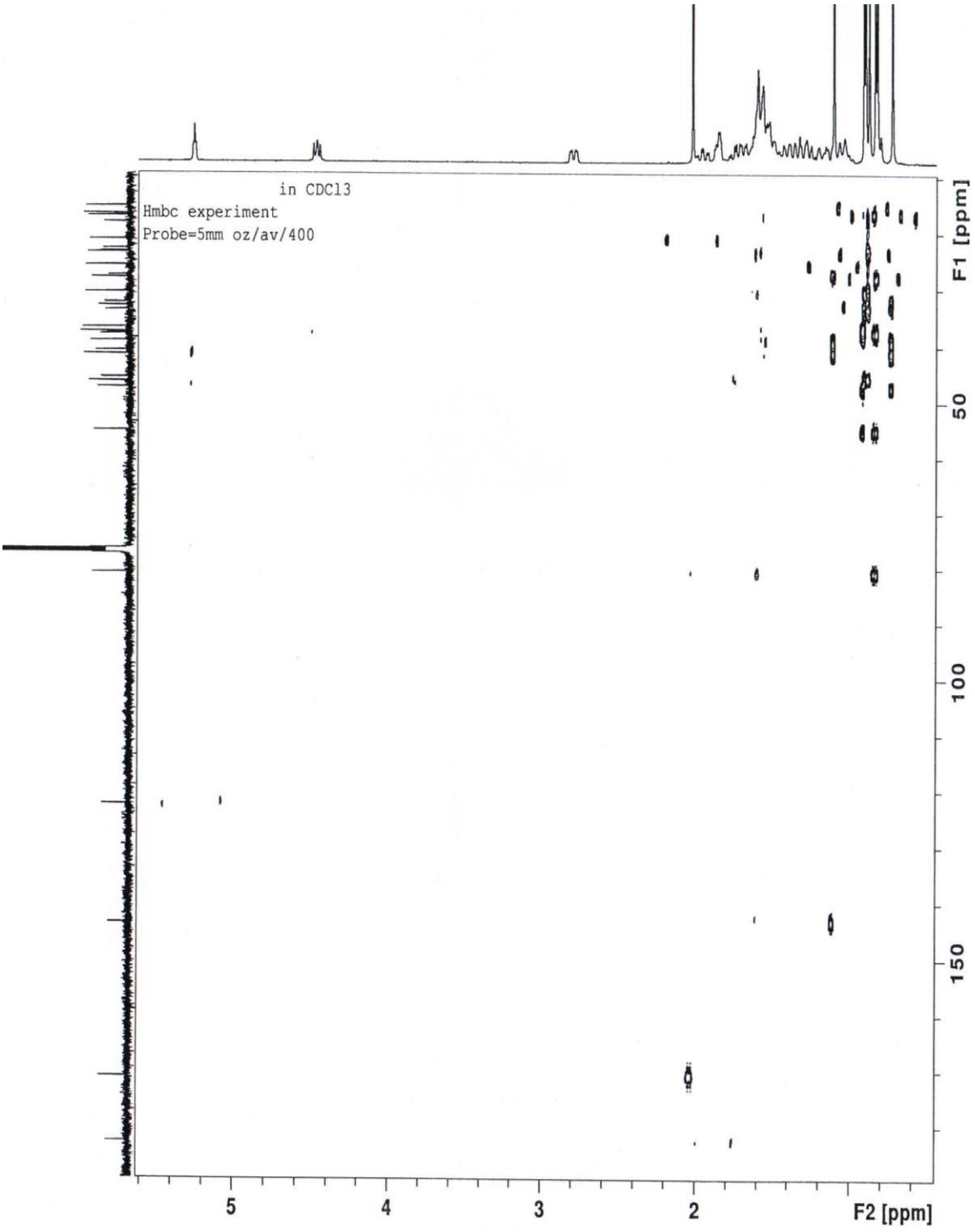


Figure 4.1.9 ^{1}H-^{13}C HMBC NMR spectra of 3β-acetyloleanolic acid

APPENDIX 5

Spectra of 28-methyl-3β-acetyloleanane

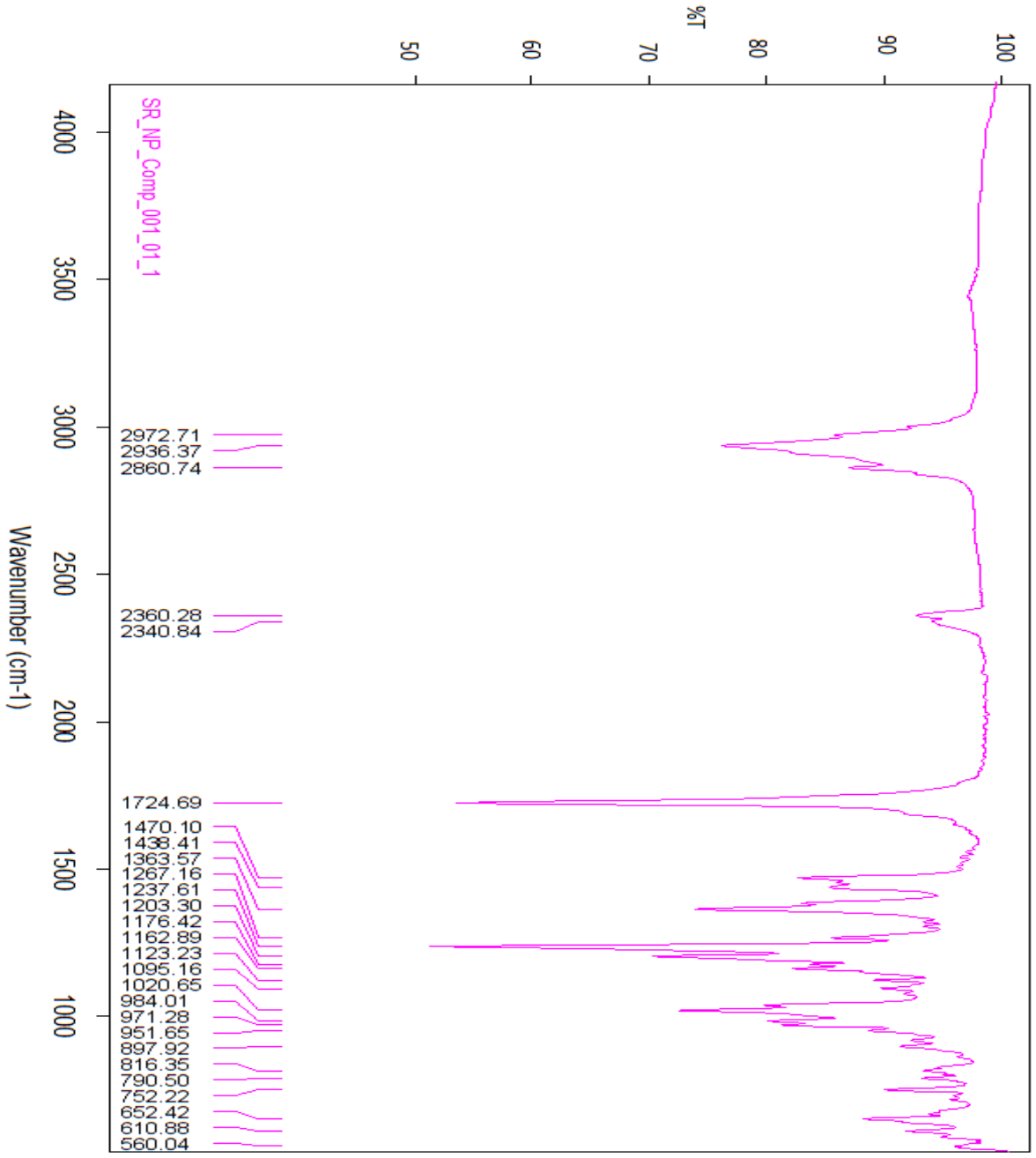


Figure 4.2.1: FT-IR spectrum of 28-methyl-3β-acetyloleanane

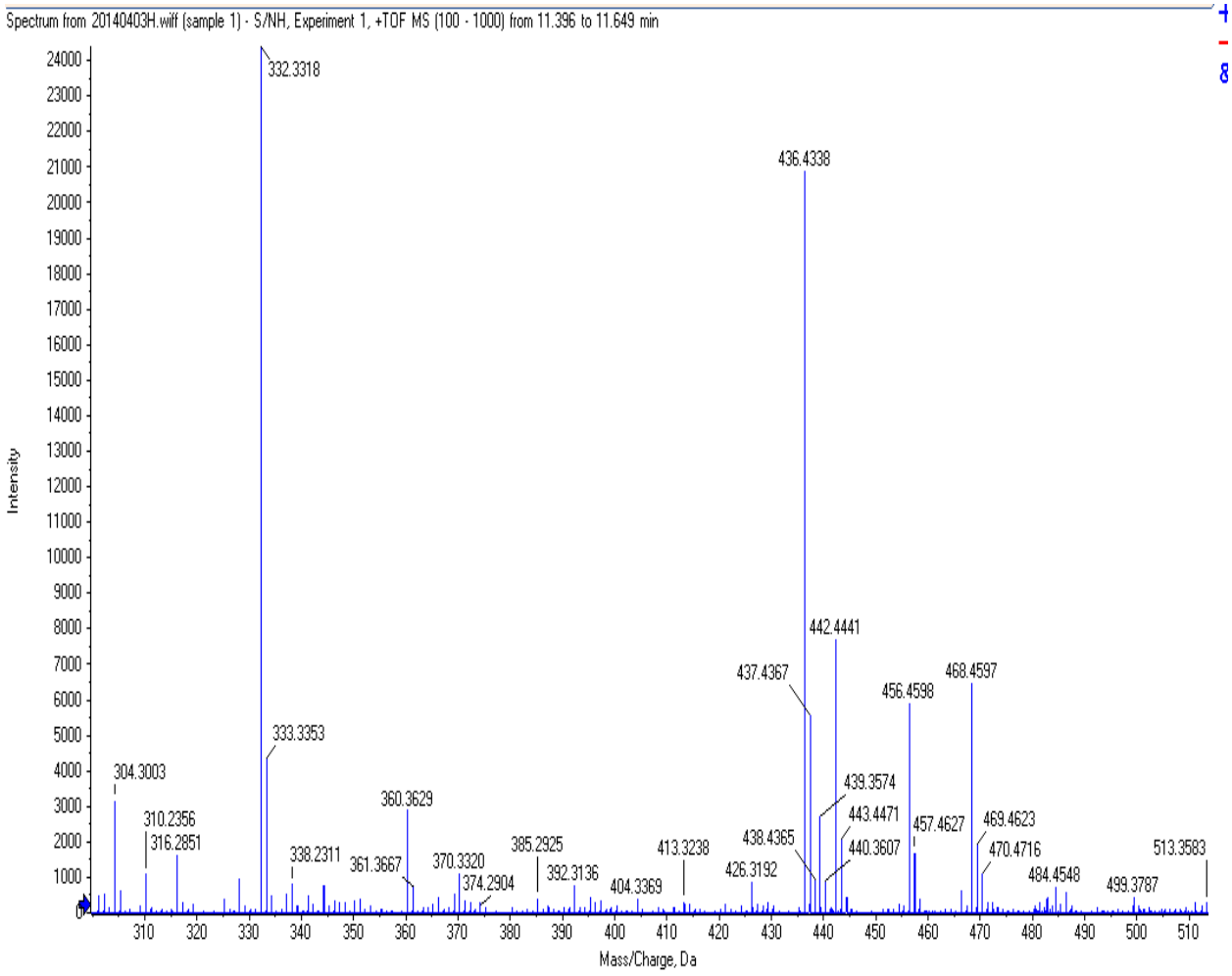


Figure 4.2.2: Mass spectrum of 28-methyl-3β-acetyloleanane

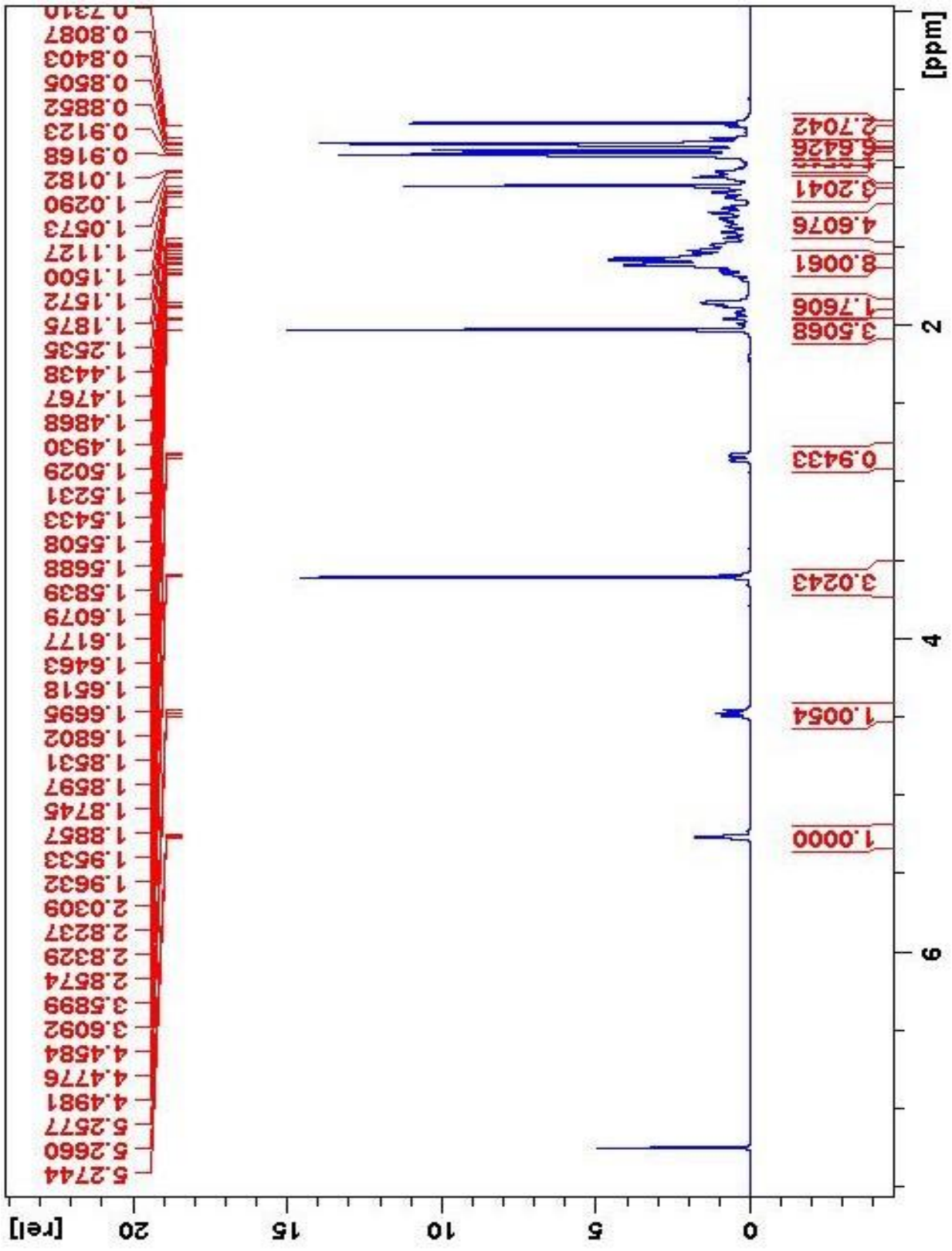


Figure 4.2.3: ^{1}H-NMR spectrum of 28-methyl-3β-acetyloleanane

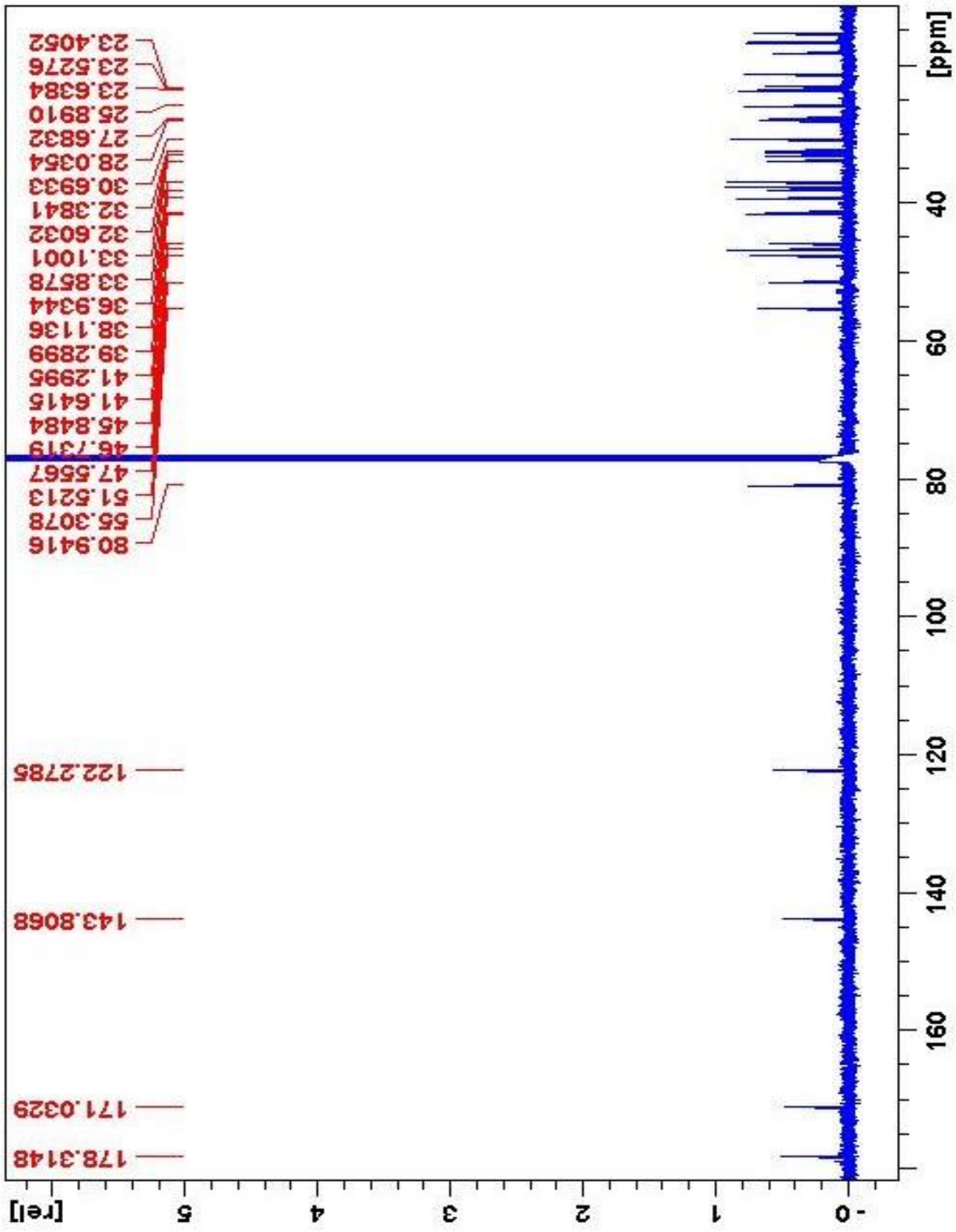


Figure 4.2.4 ^{13}C-NMR spectrum of 28-methyl-3β-acetyloleanane

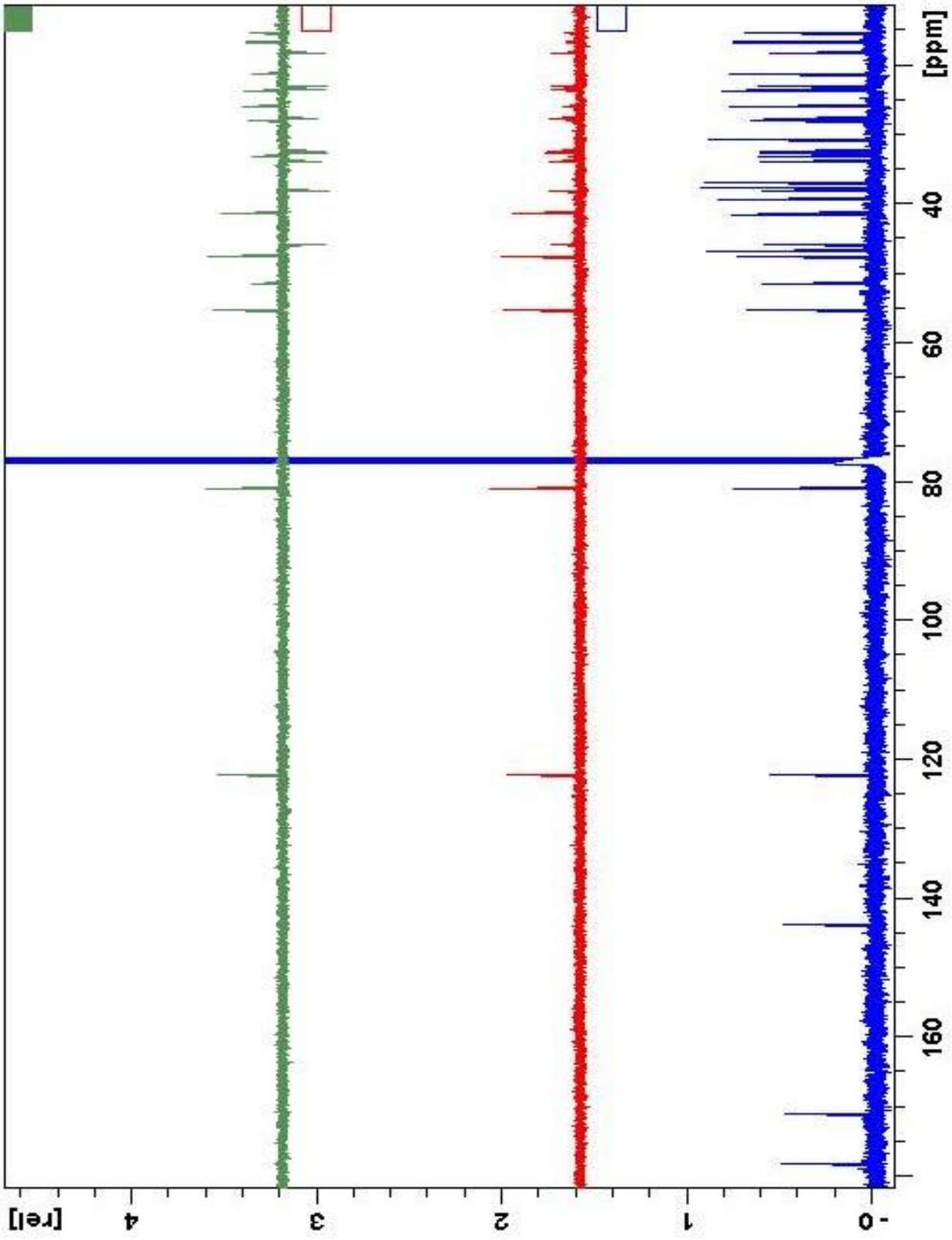


Figure 4.2.5 ^{13}**C DEPT NMR spectrum of 28-methyl-3β-acetyloleanane**

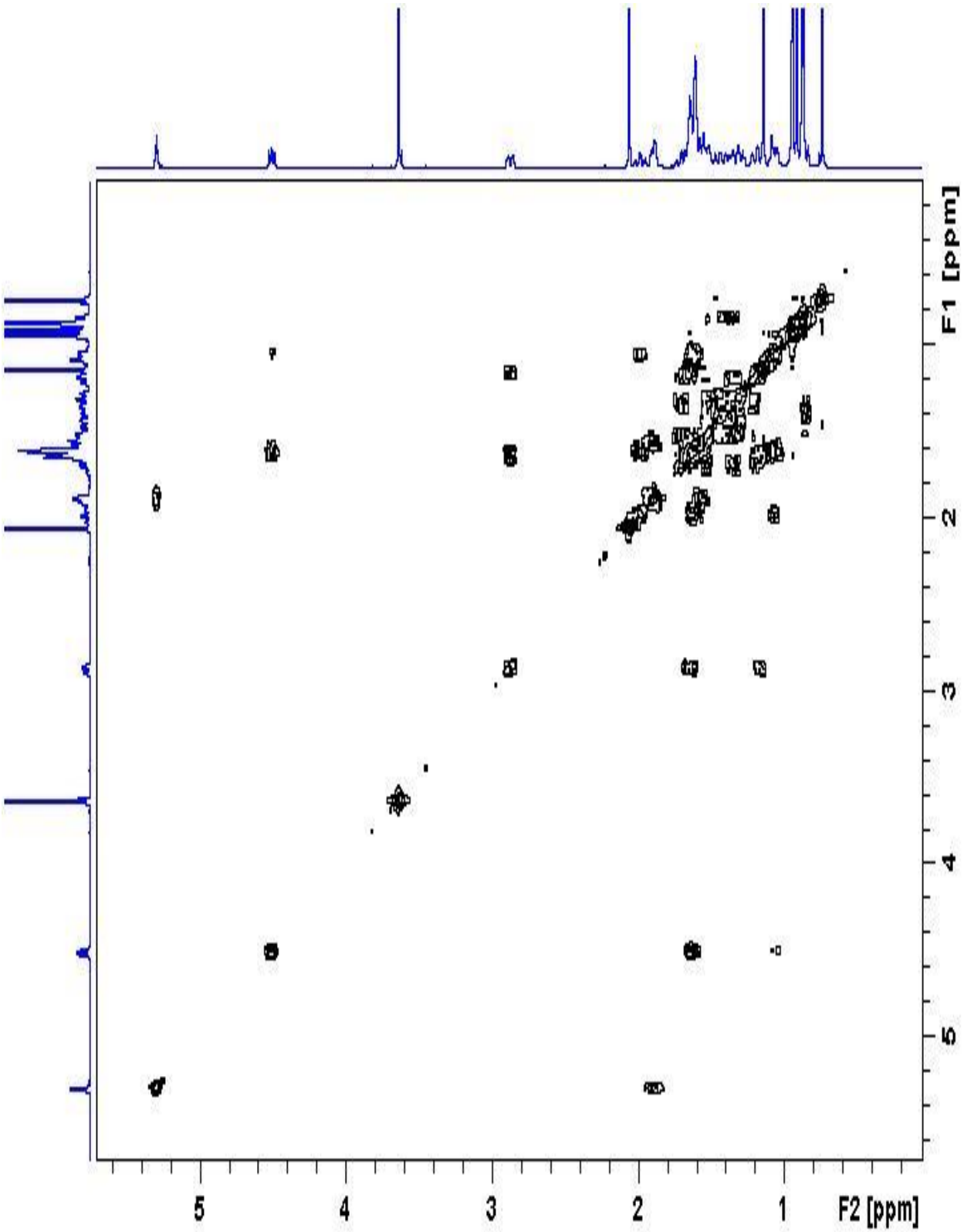


Figure 4.2.6 ^{1}H-^{1}H COSY NMR spectrum of 28-methyl-3β-acetyloleanane

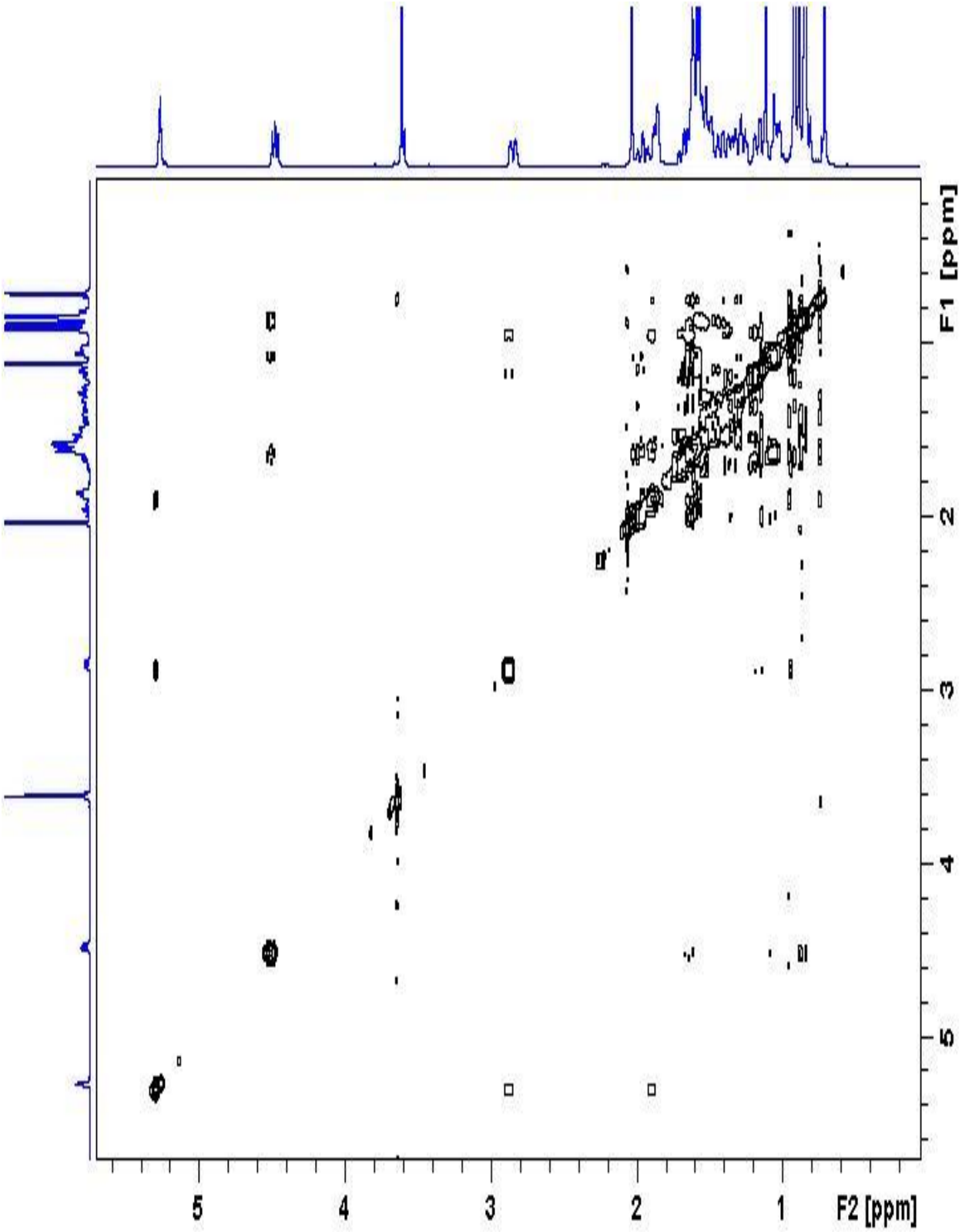


Figure 4.2.7 ^{1}H-^{1}H NOESY NMR spectrum of 28-methyl-3β-acetyloleanane

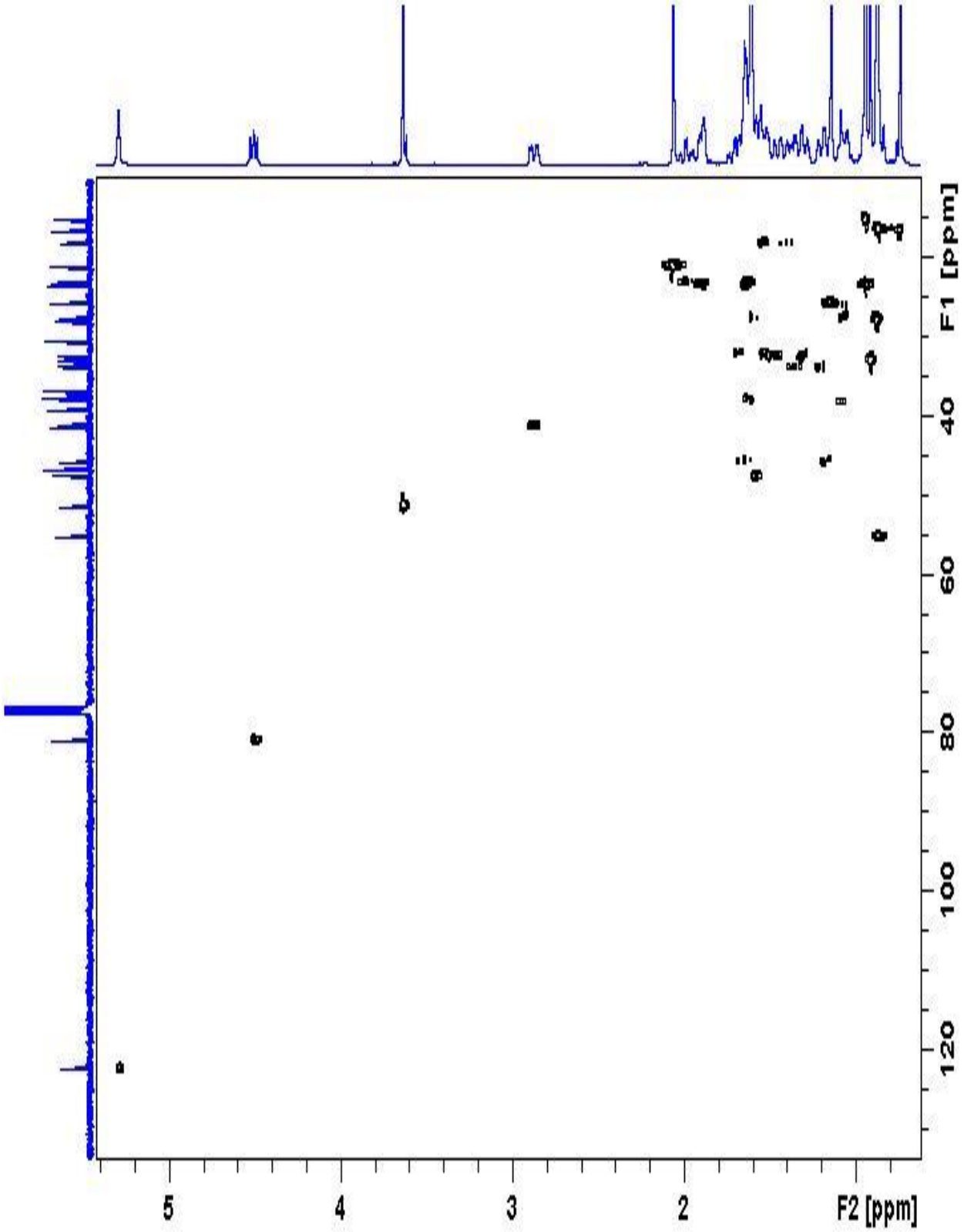


Figure 4.2.8: ^{1}H-^{13}C HSQC NMR spectrum of 28-methyl-3β-acetyloleanane

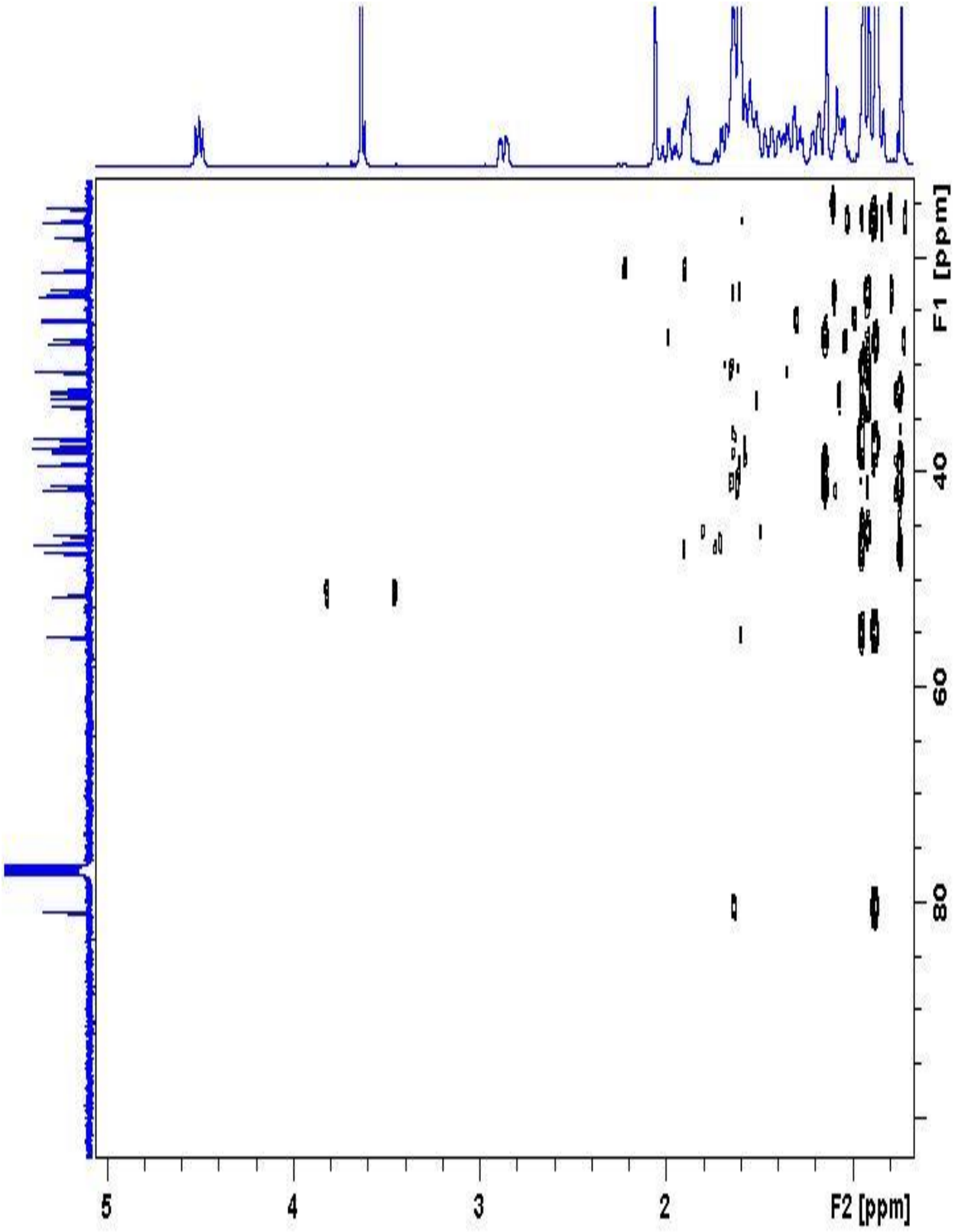


Figure 4.2.9: ^{1}H-^{13}C HMBC NMR spectrum of 28-methyl-3β-acetyloleanane

APPENDIX 6

Spectra of 28-methyloleanane

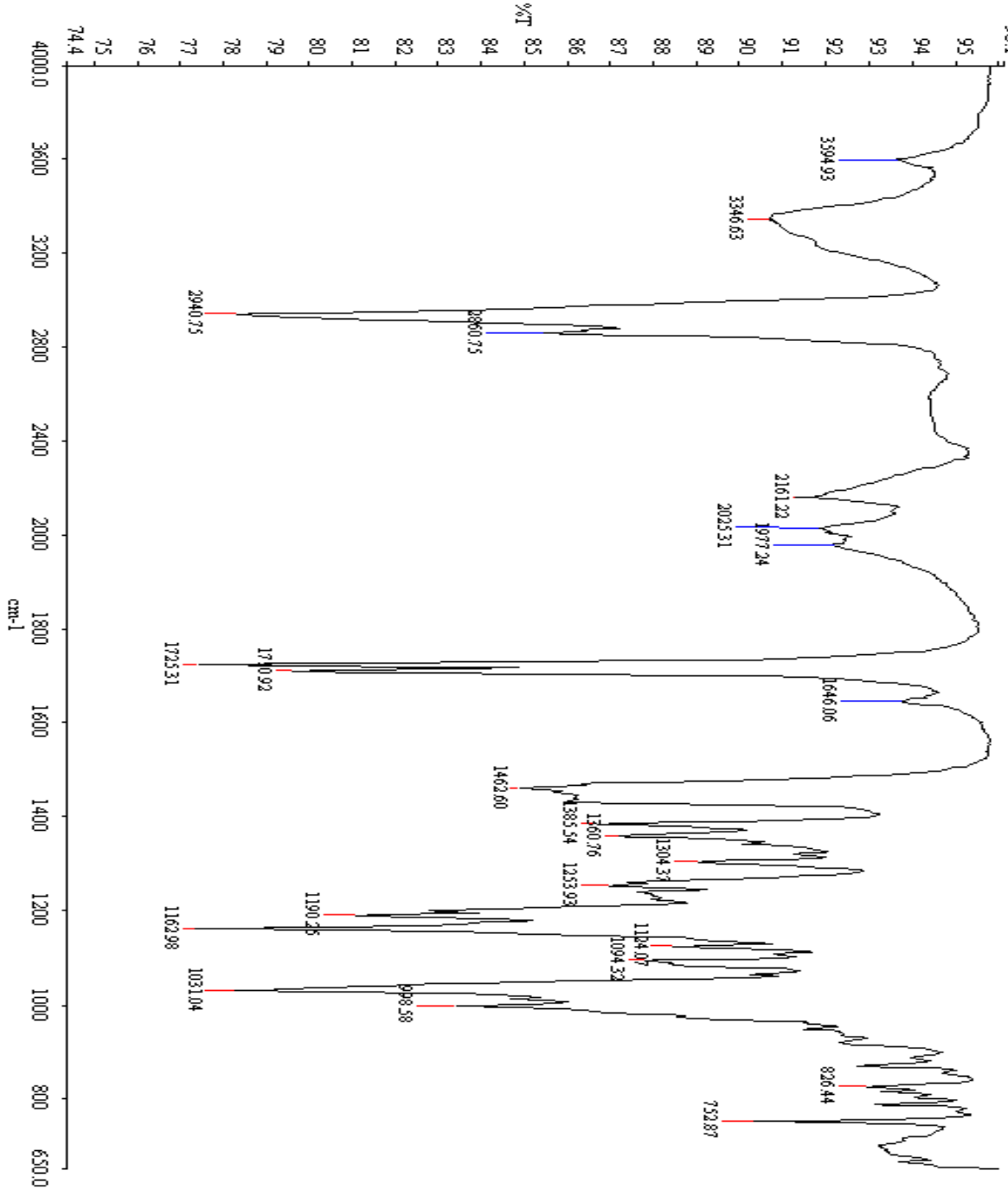


Figure 4.3.1: FT-IR spectrum of 28-methyloleanane

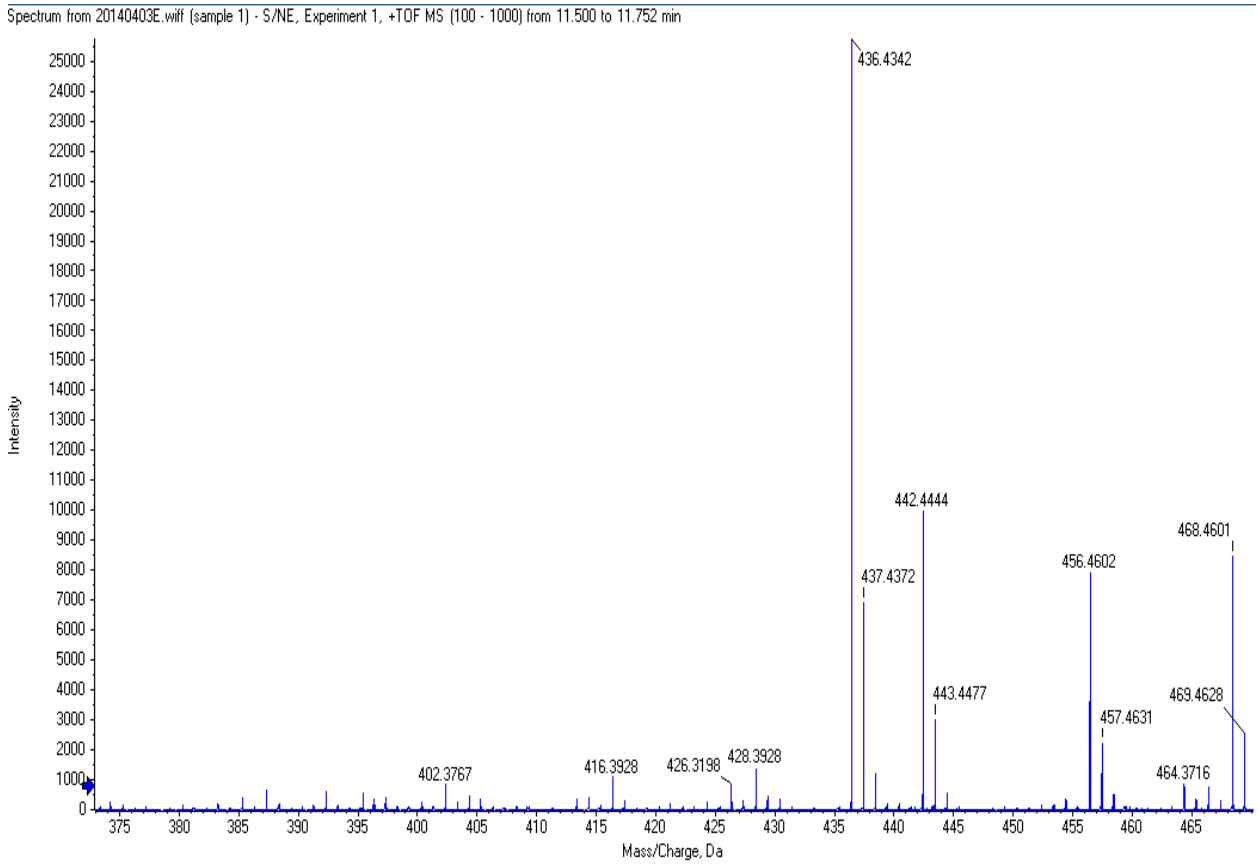


Figure 4.3.2: Mass spectrum of 28-methyloleanane

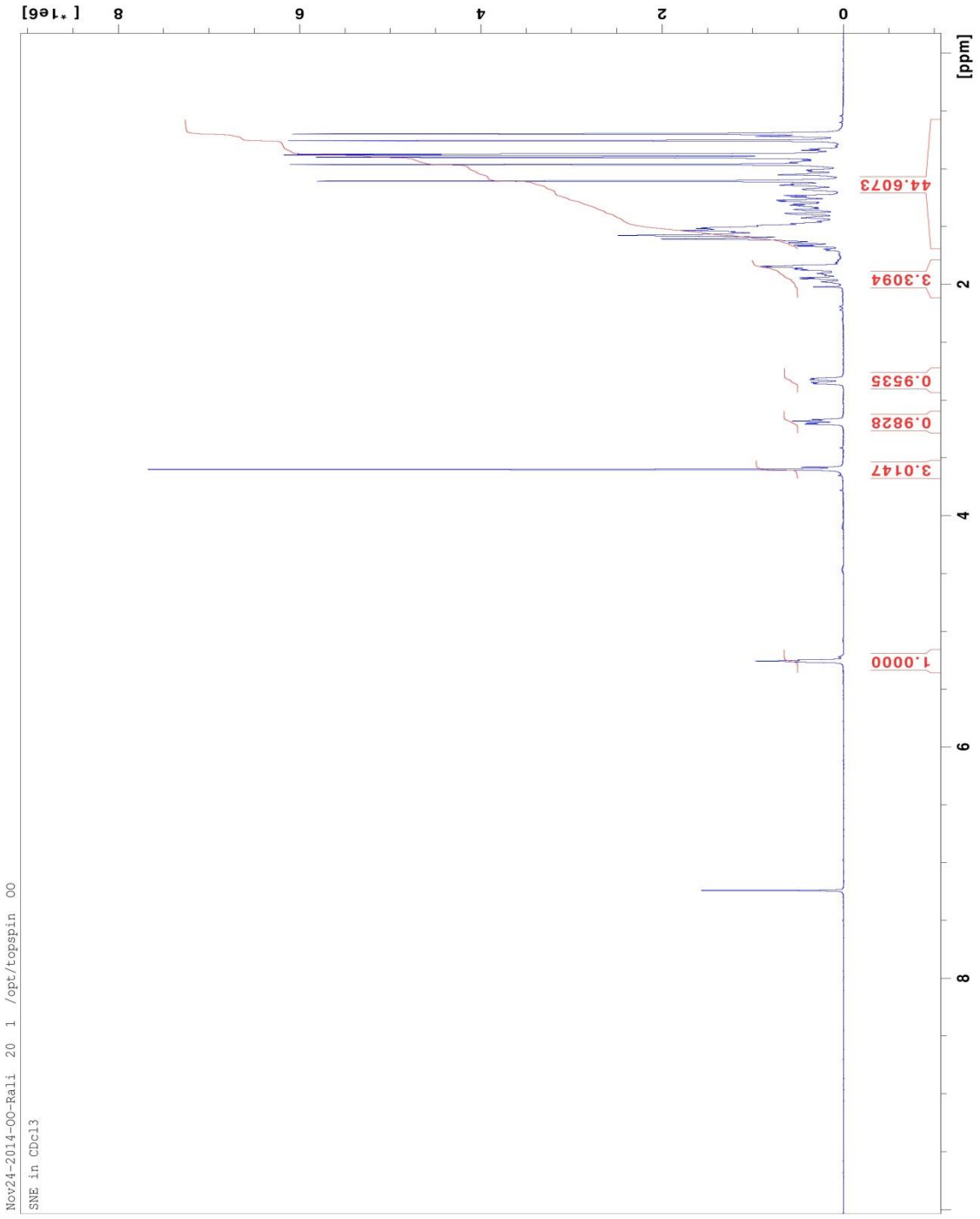


Figure 4.3.3: ^{1}H-NMR spectra of 28-methyloleanane

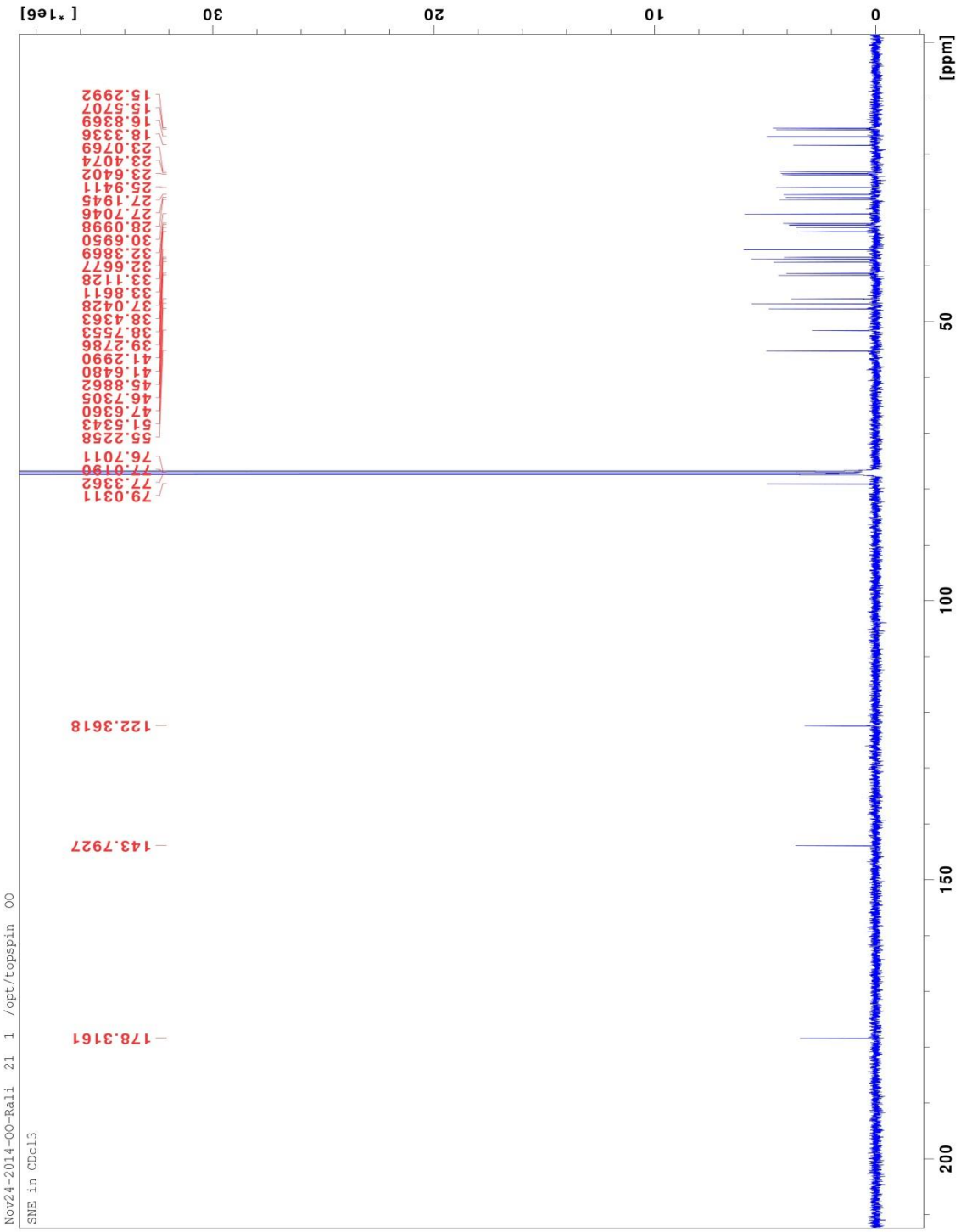


Figure 4.3.4: ^{13}C-NMR spectra of 28-methyloleanane

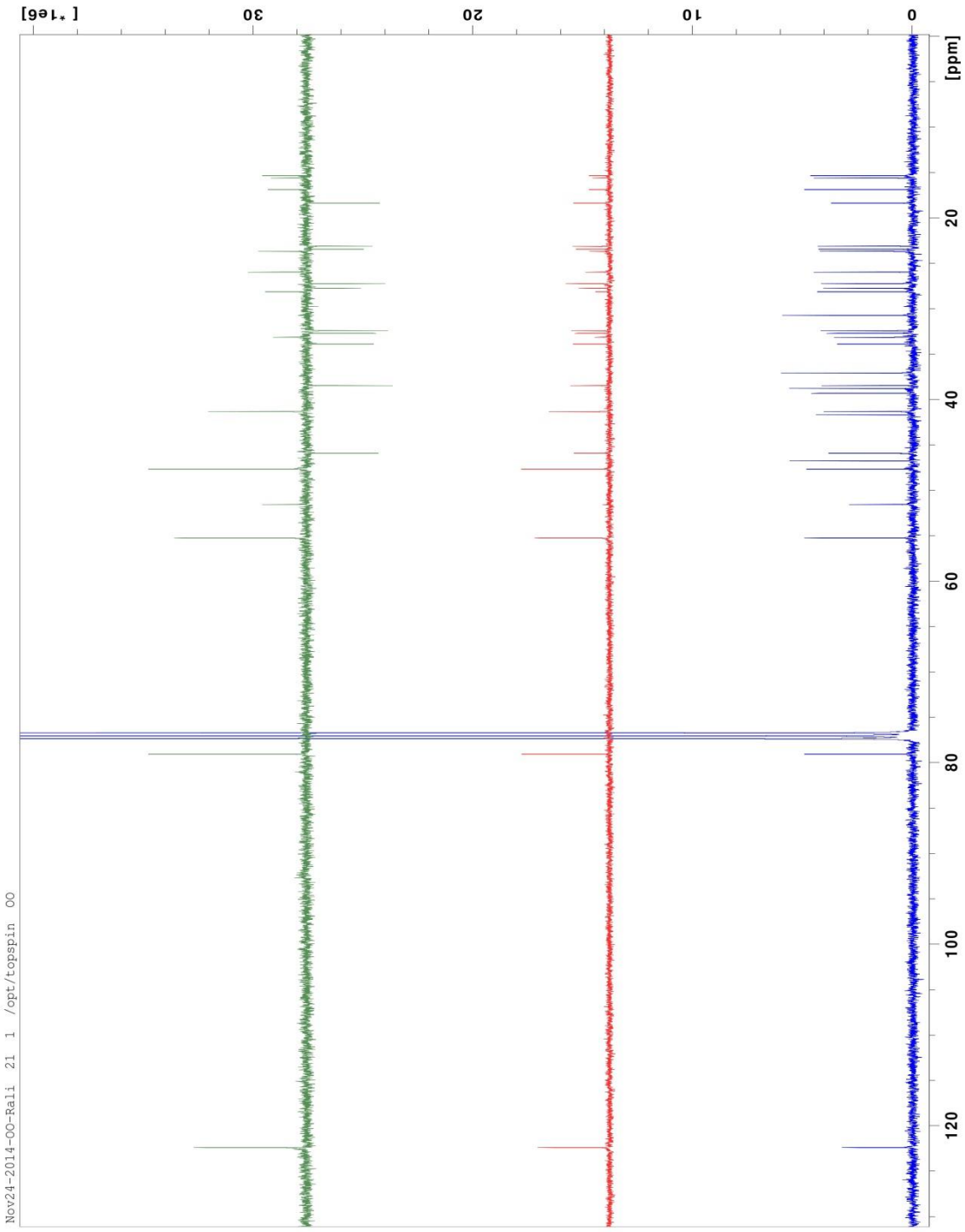


Figure 4.3.5: ^{13}C DEPT NMR spectra of 28-methyloleanane

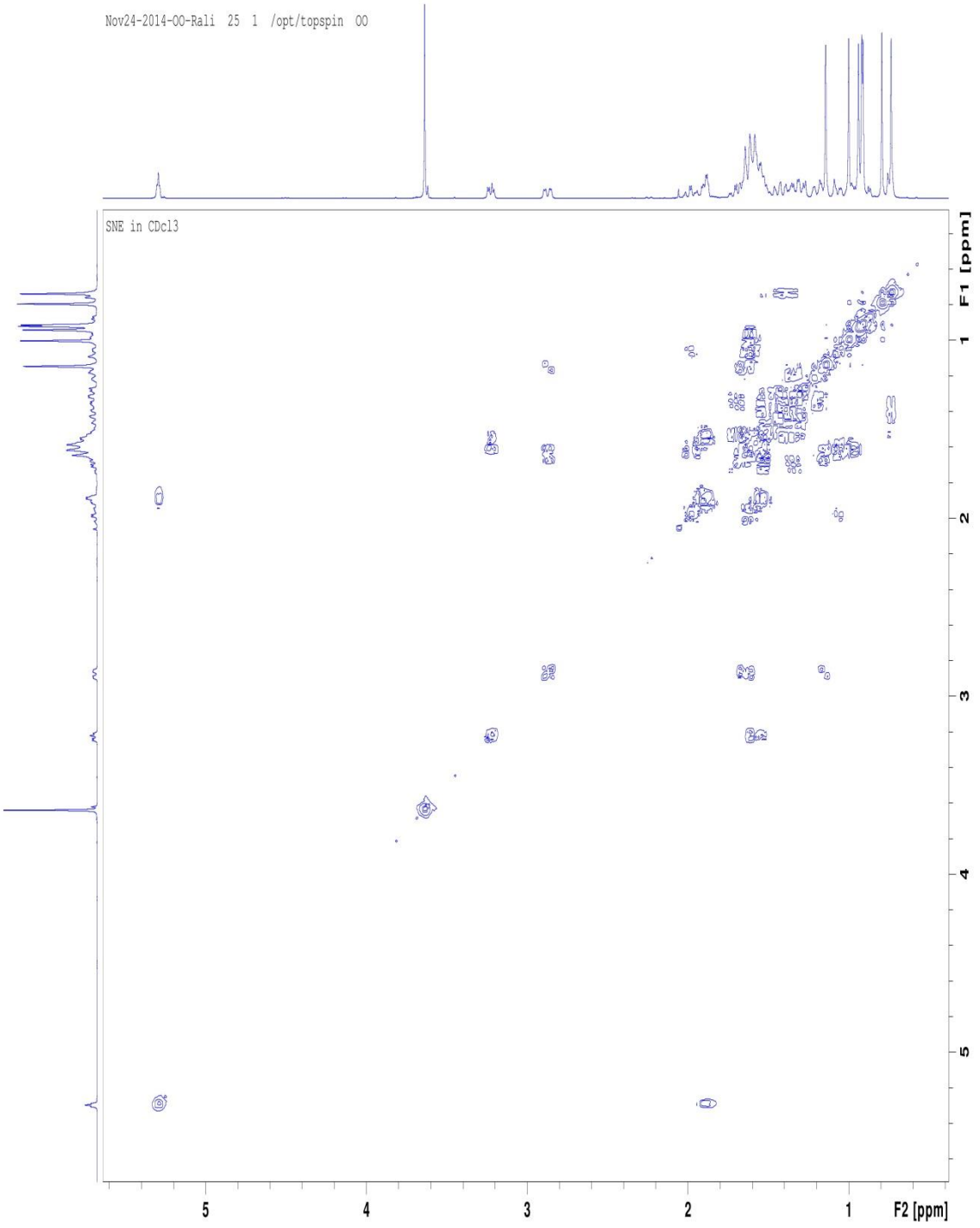


Figure 4.3.6: ^{1}H-^{1}H COSY NMR spectra of 28-methyloleanane

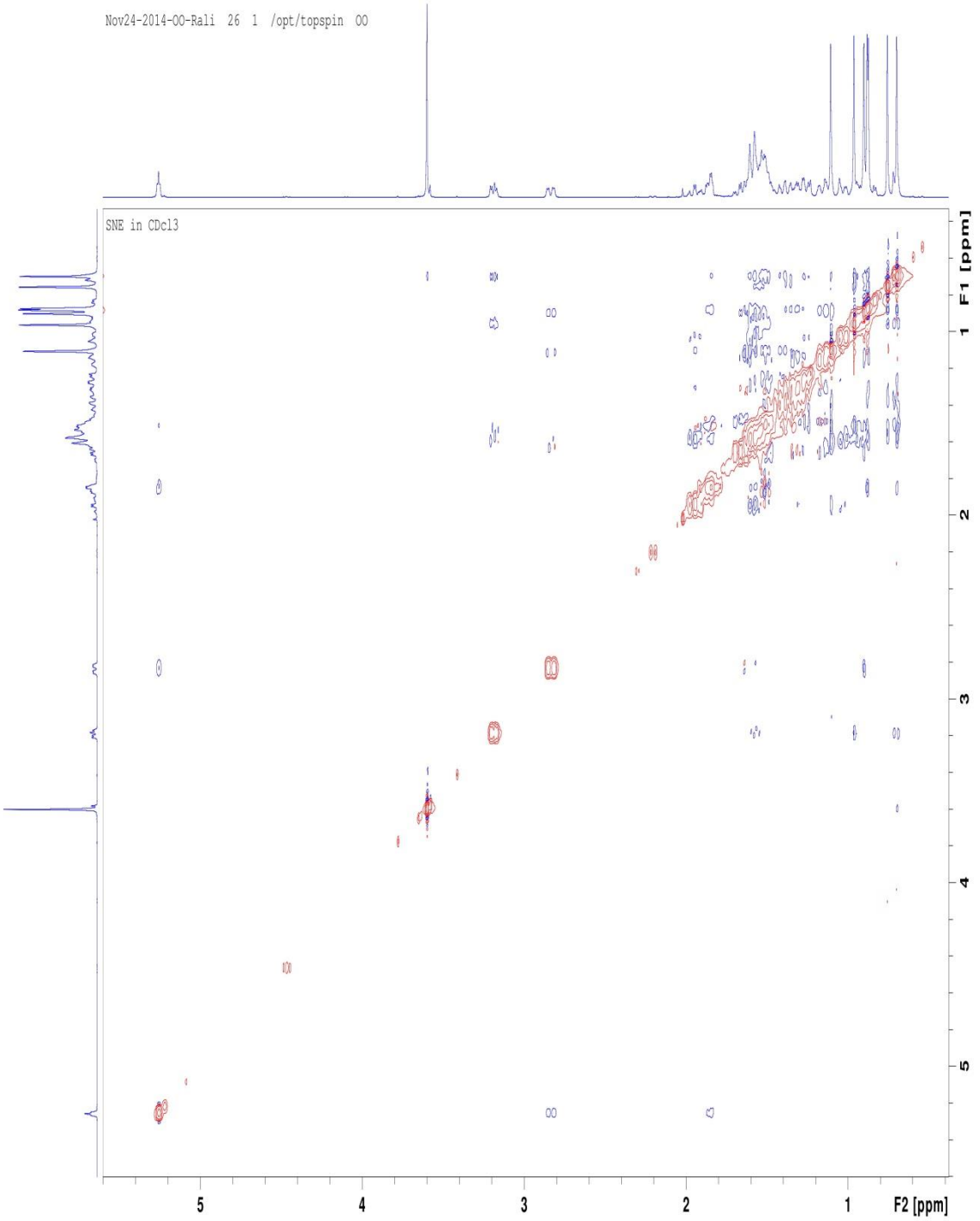


Figure 4.3.7: ^{1}H-^{1}H NOESY NMR spectra of 28-methyloleanane

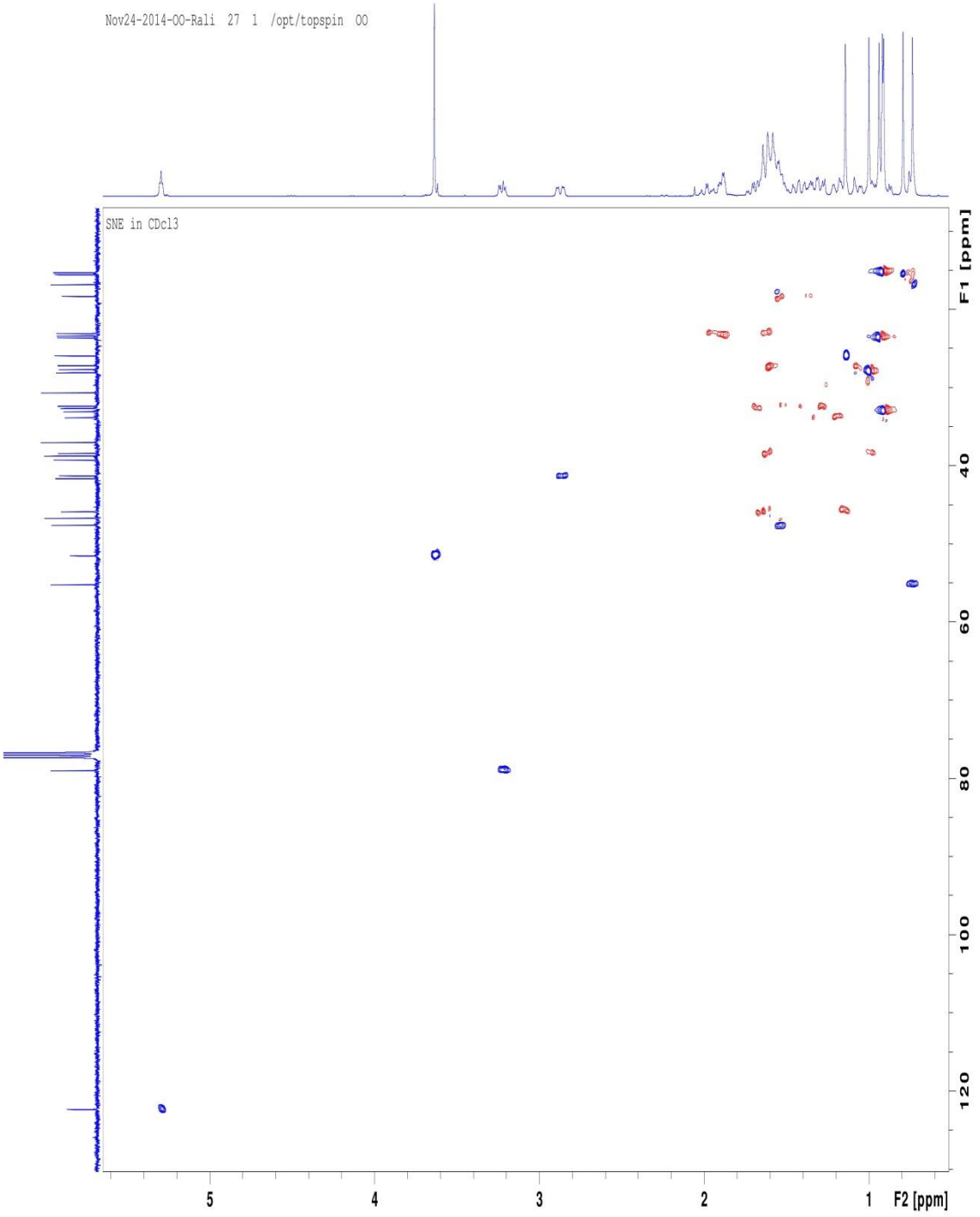


Figure 4.3.8: ^{1}H-^{13}C HSQC NMR spectra of 28-methyloleanane

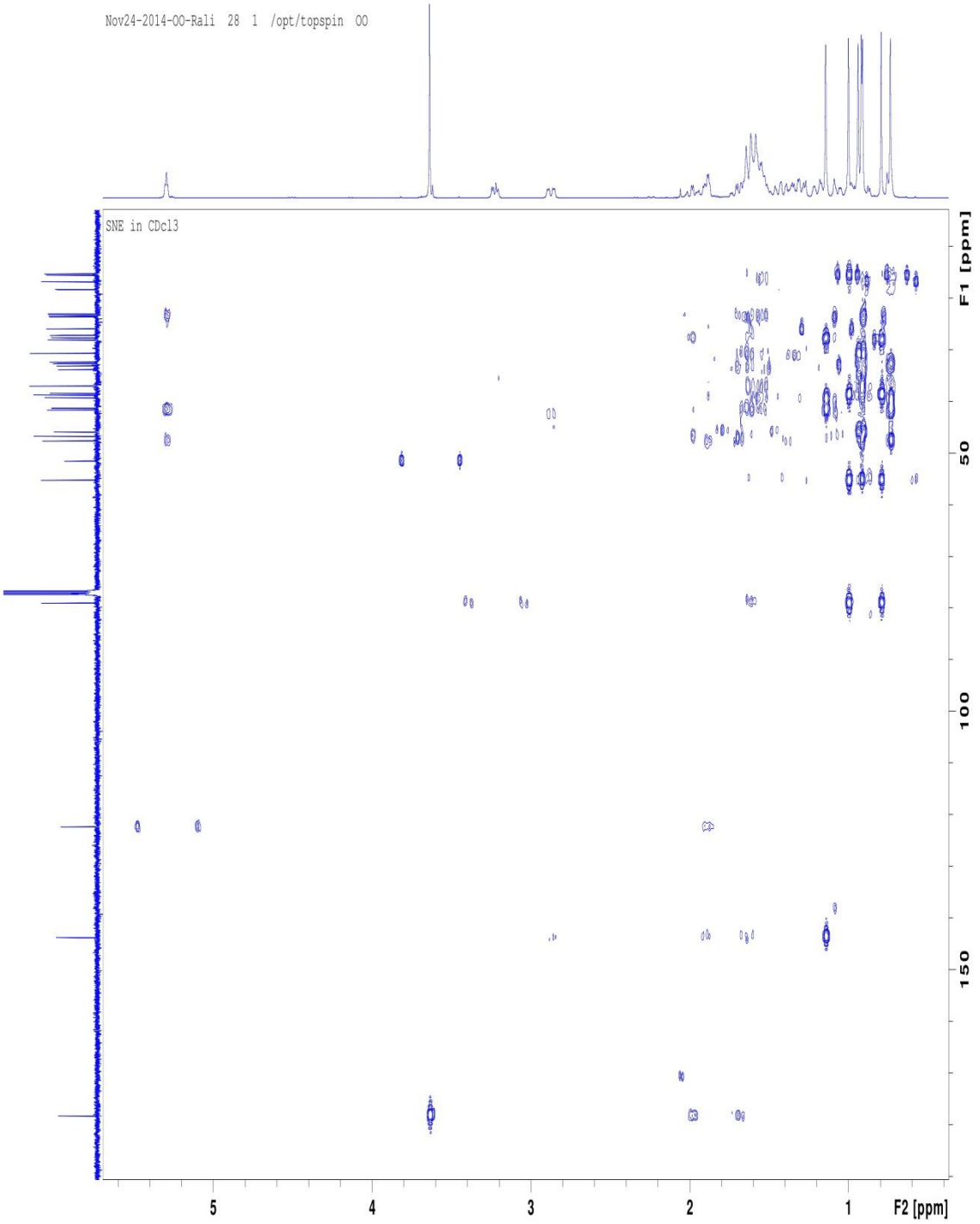


Figure 4.3.9: ^{1}H-^{13}C HMBC NMR spectra of 28-methyloleanane

Appendix 7

Spectra 3β-trifluoroacetyloleanolic acid

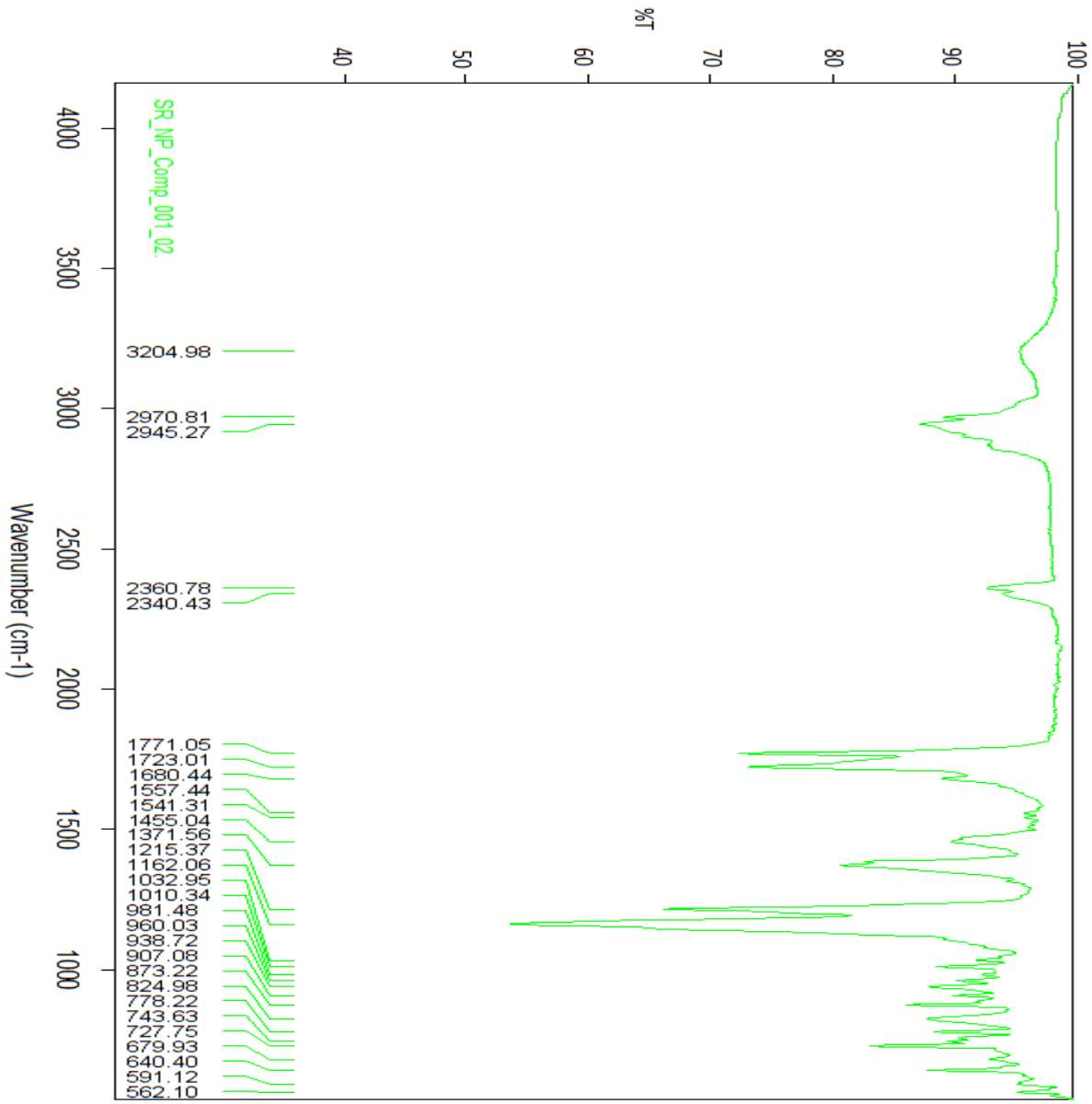


Figure 4.4.1: FR-IR spectrum of 3β-trifluoroacetyloleanolic acid

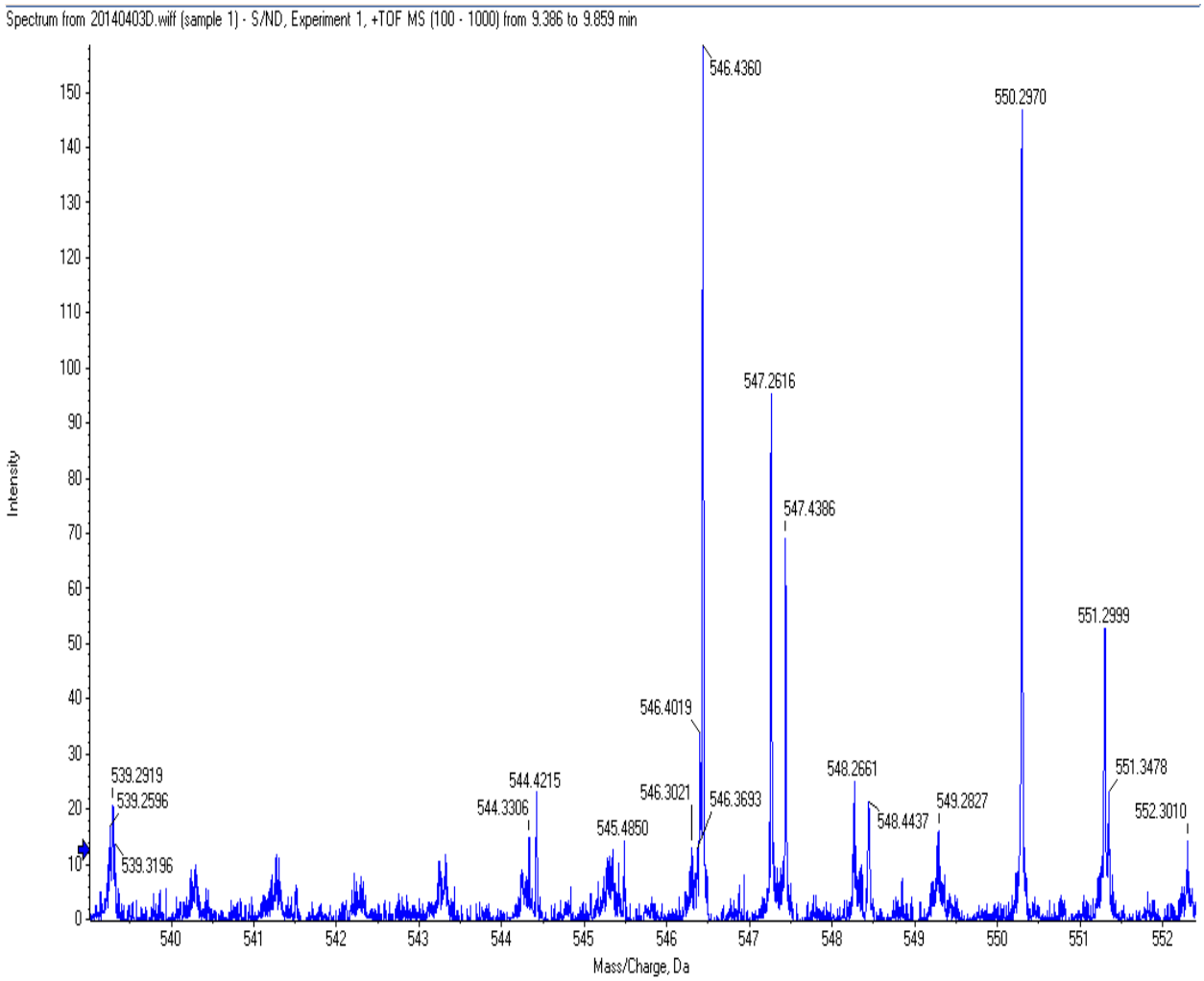


Figure 4.4.2: Mass spectrum of 3β-trifluoroacetyloleanolic acid

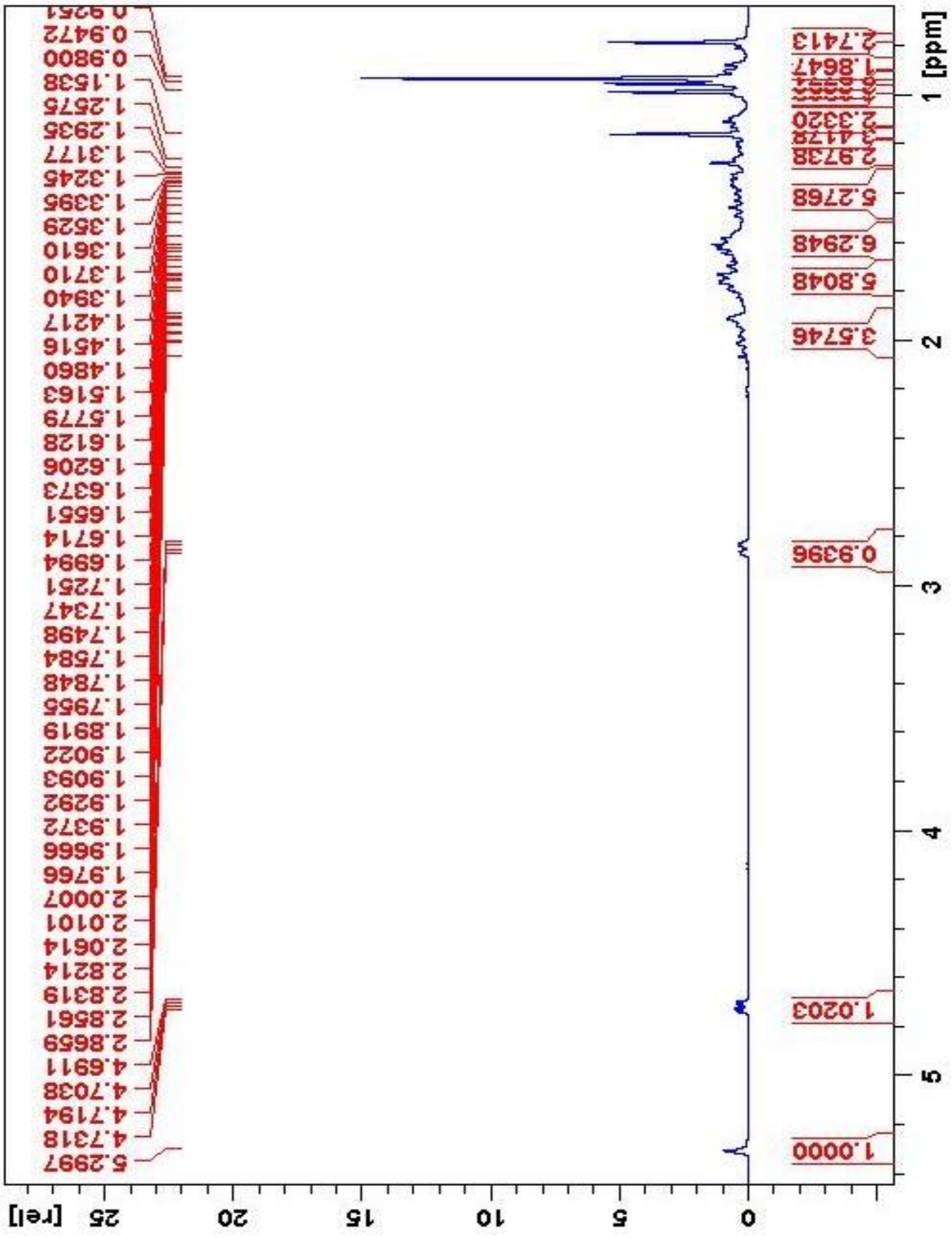


Figure 4.4.3: ^{1}H-NMR spectrum of 3β-trifluoroacetyloleanolic acid

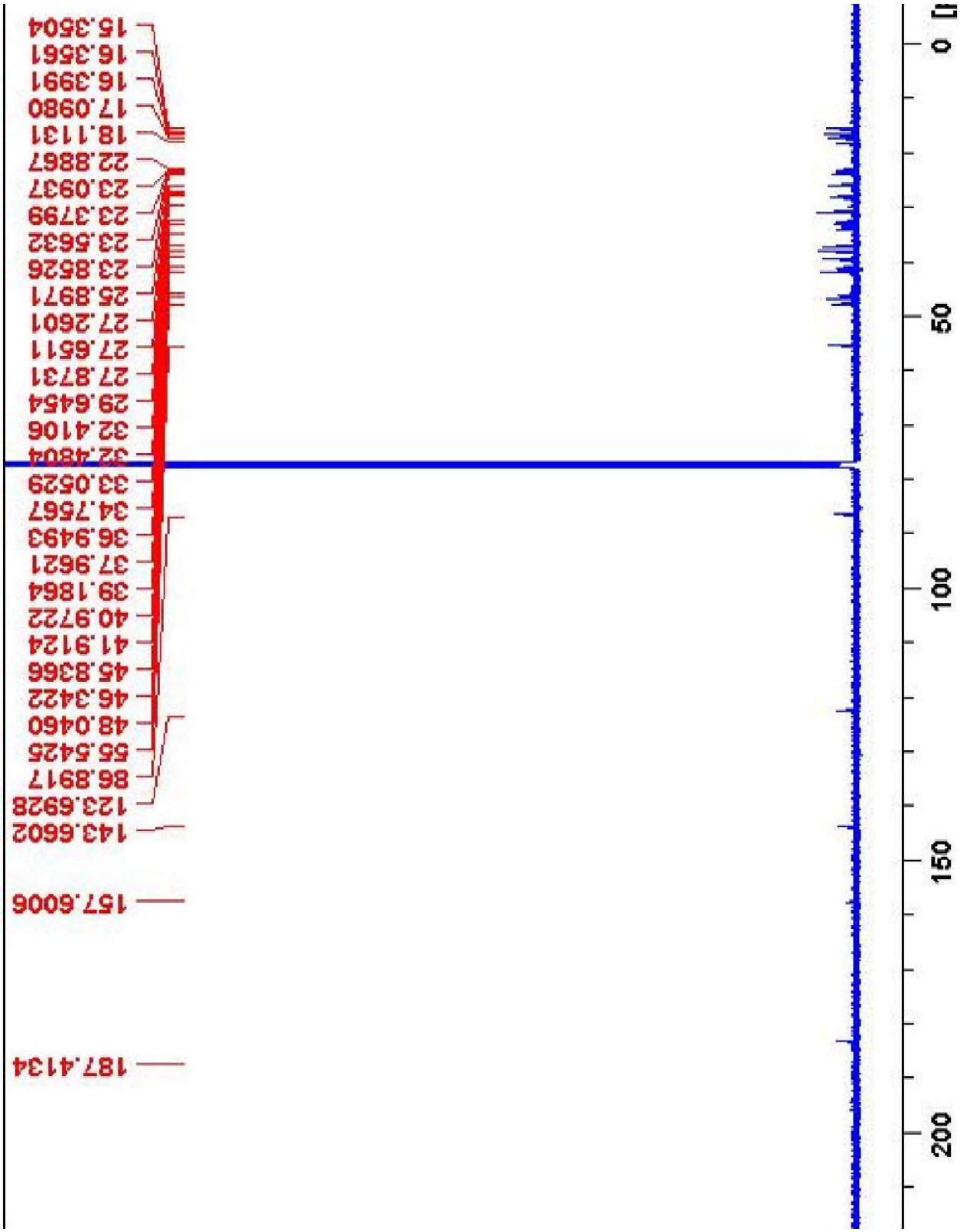


Figure 4.4.4: ^{13}C-NMR spectrum of 3β-trifluoroacetyloleanolic acid

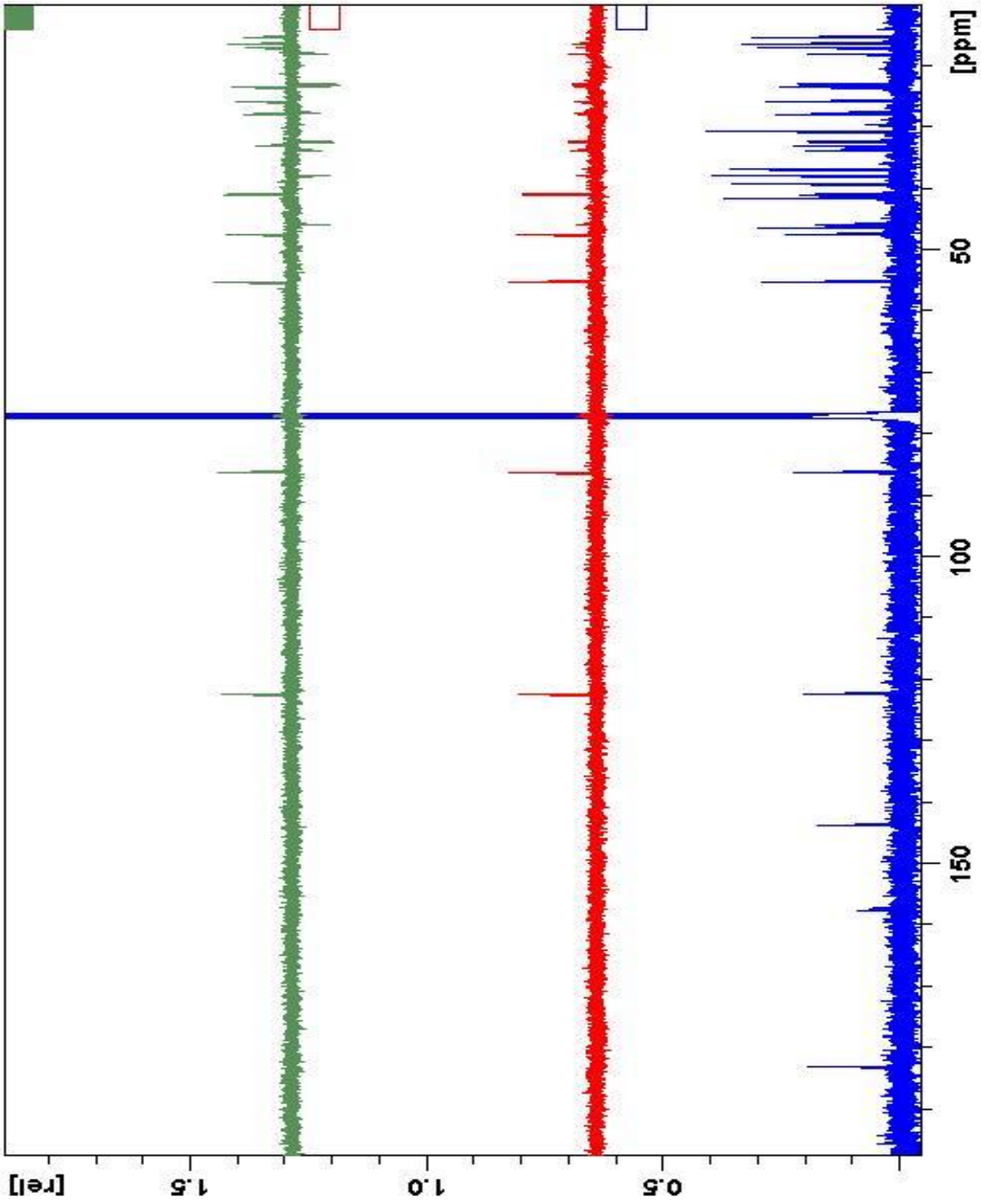


Figure 4.4.5: ^{13}C DEPT NMR spectrum of 3β-trifluoroacetyloleanolic acid

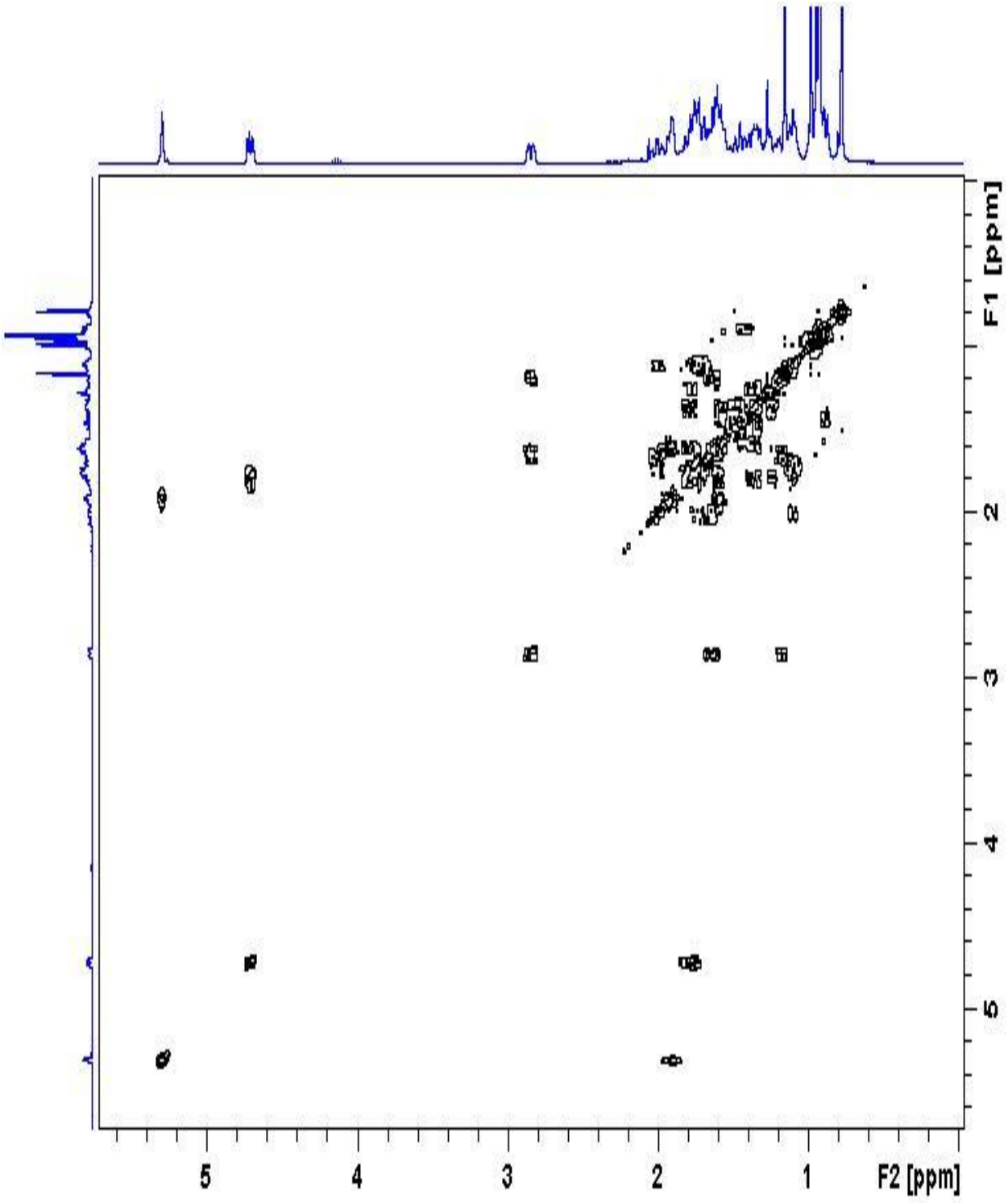


Figure 4.4.6: ^{1}H-^{1}H COSY NMR spectrum of 3β-trifluoroacetyloleanolic acid

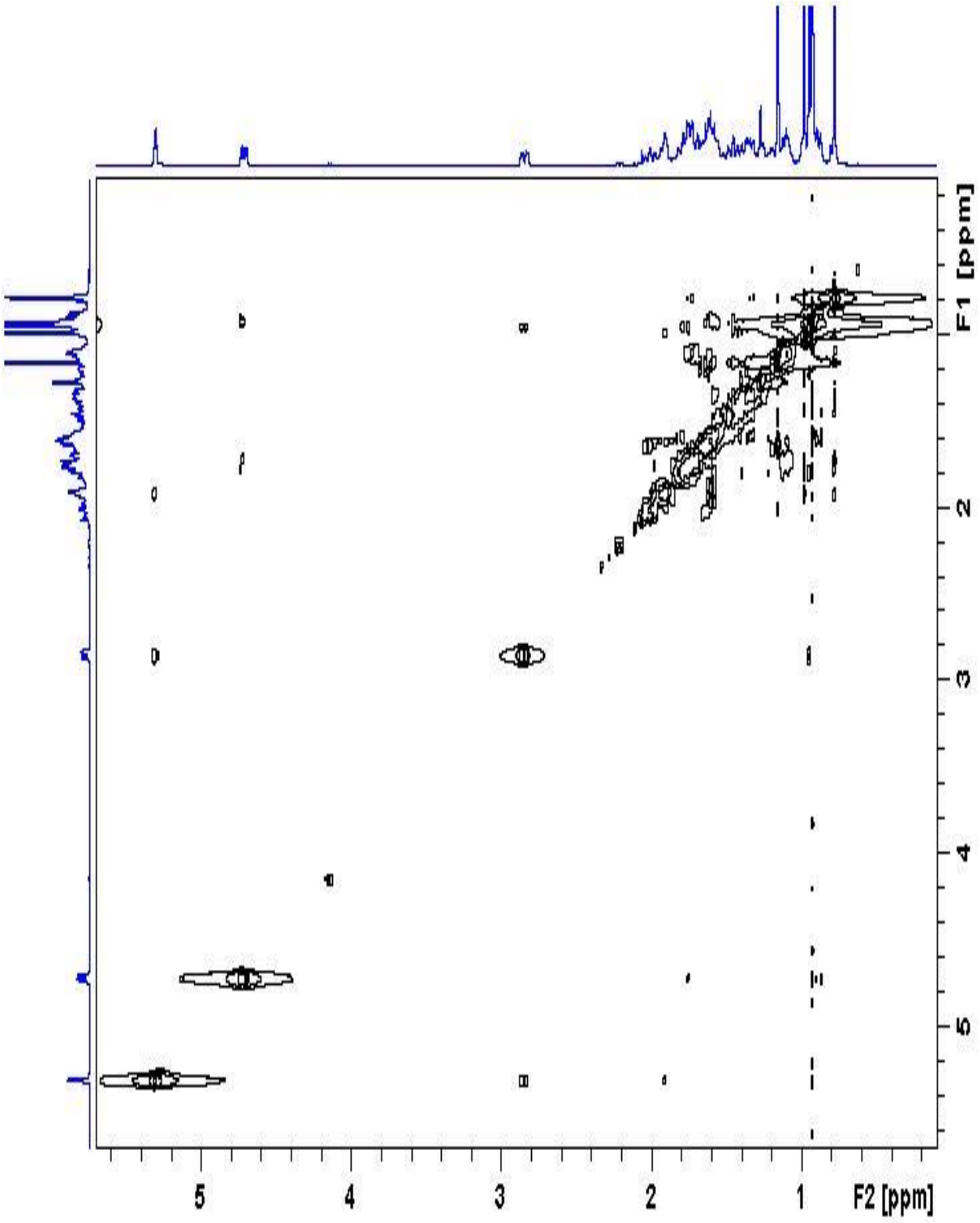


Figure 4.4.7: ^{1}H-^{1}H NOESY NMR spectrum of 3β-trifluoroacetyloleanolic acid

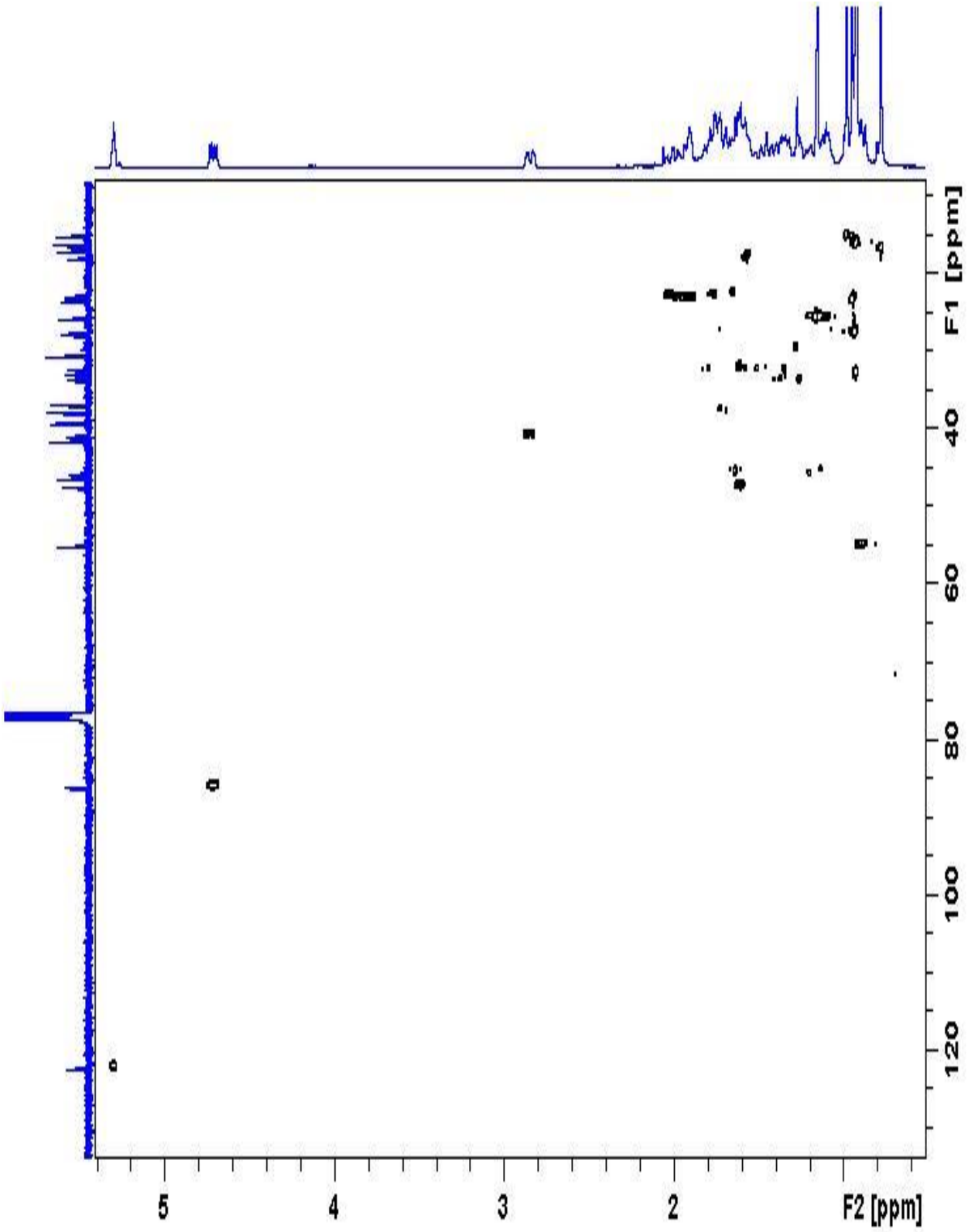


Figure 4.4.8: ^{1}H-^{13}C HSQC NMR spectrum of 3β-trifluoroacetyloleanolic acid

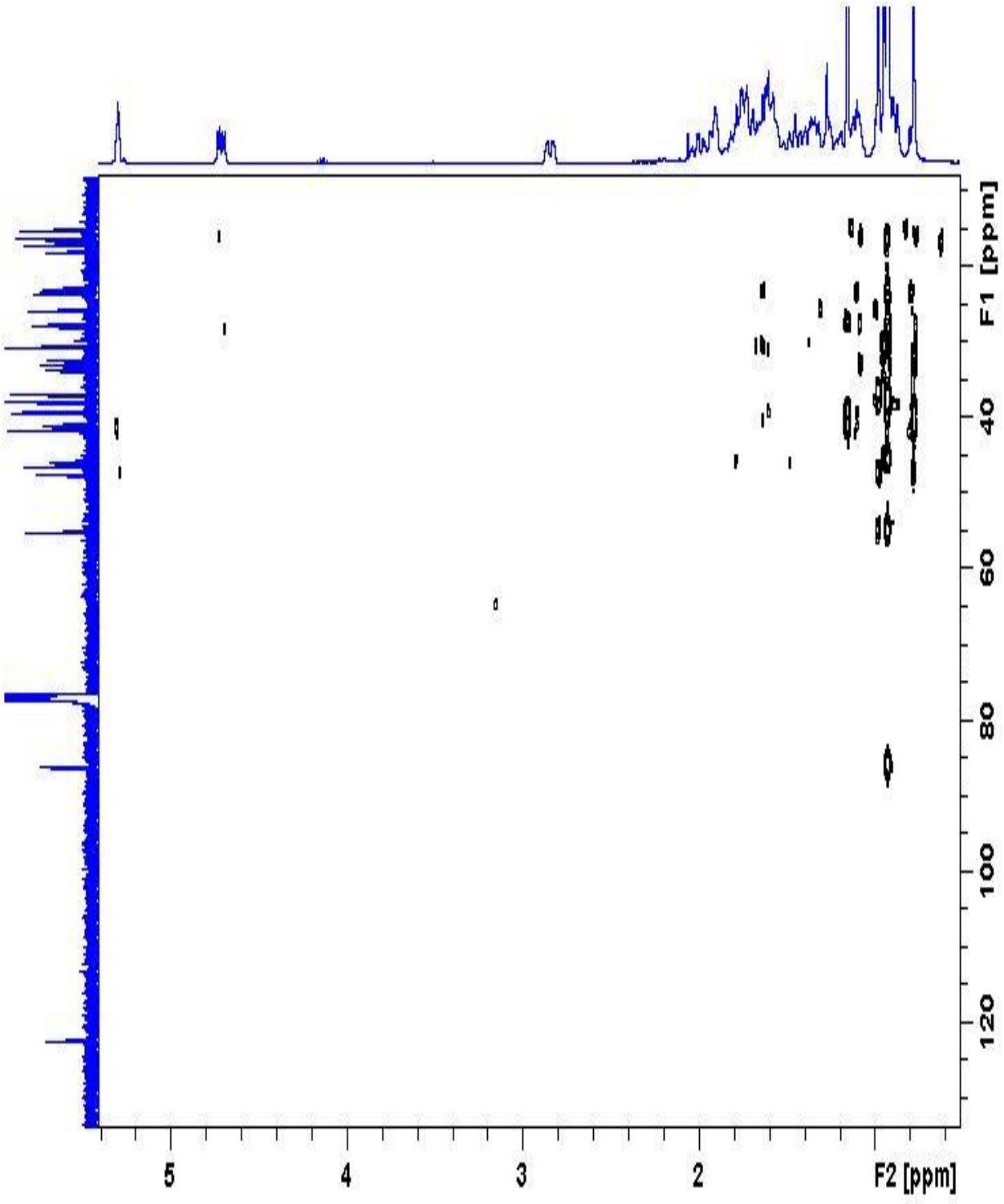
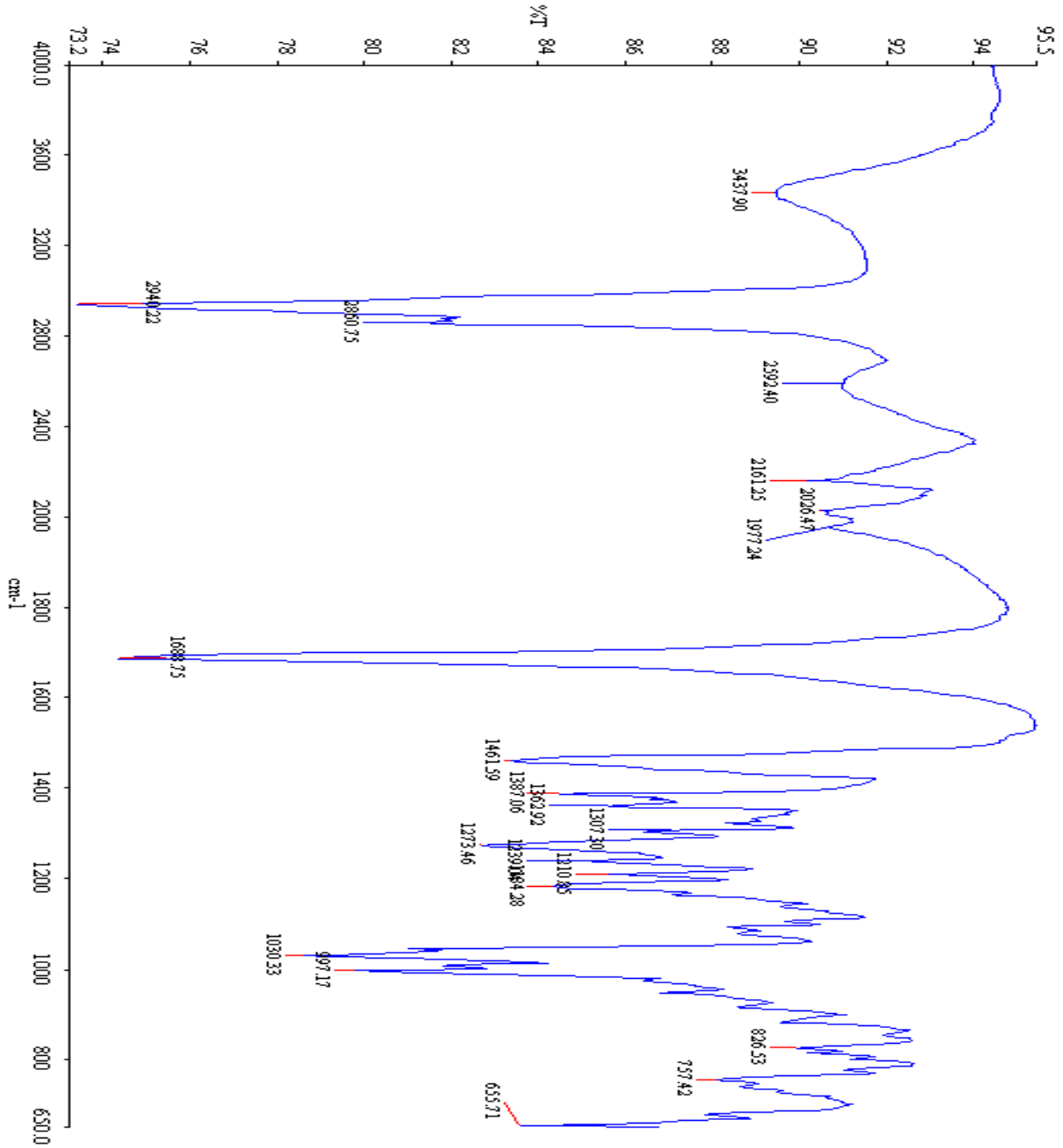


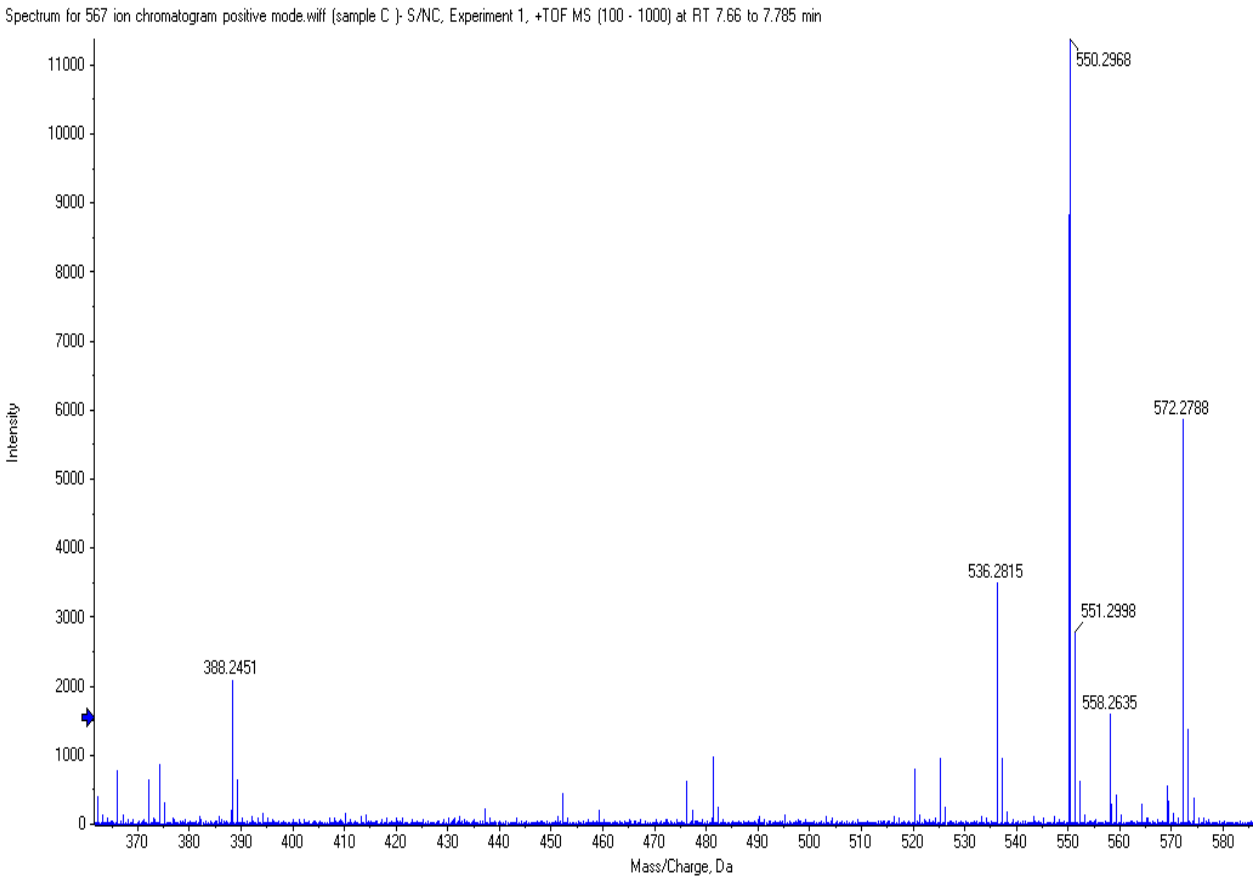
Figure 4.4.9: ^{1}H-^{13}C HMBC NMR spectrum of 3β-trifluoroacetyloleanolic acid

APPENDIX 8

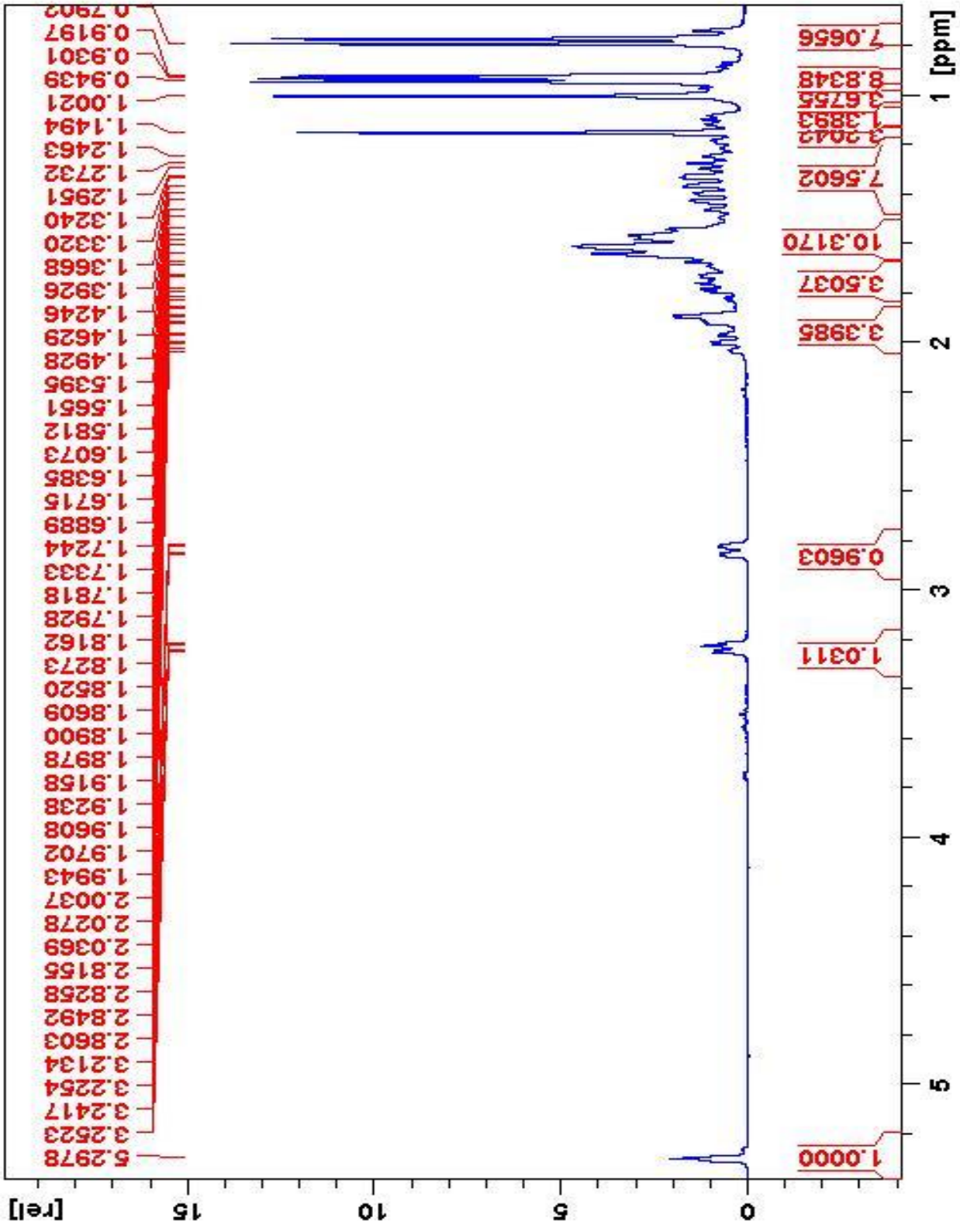
Spectra 28-mthyl-3β-trifluoroacetyloleanane



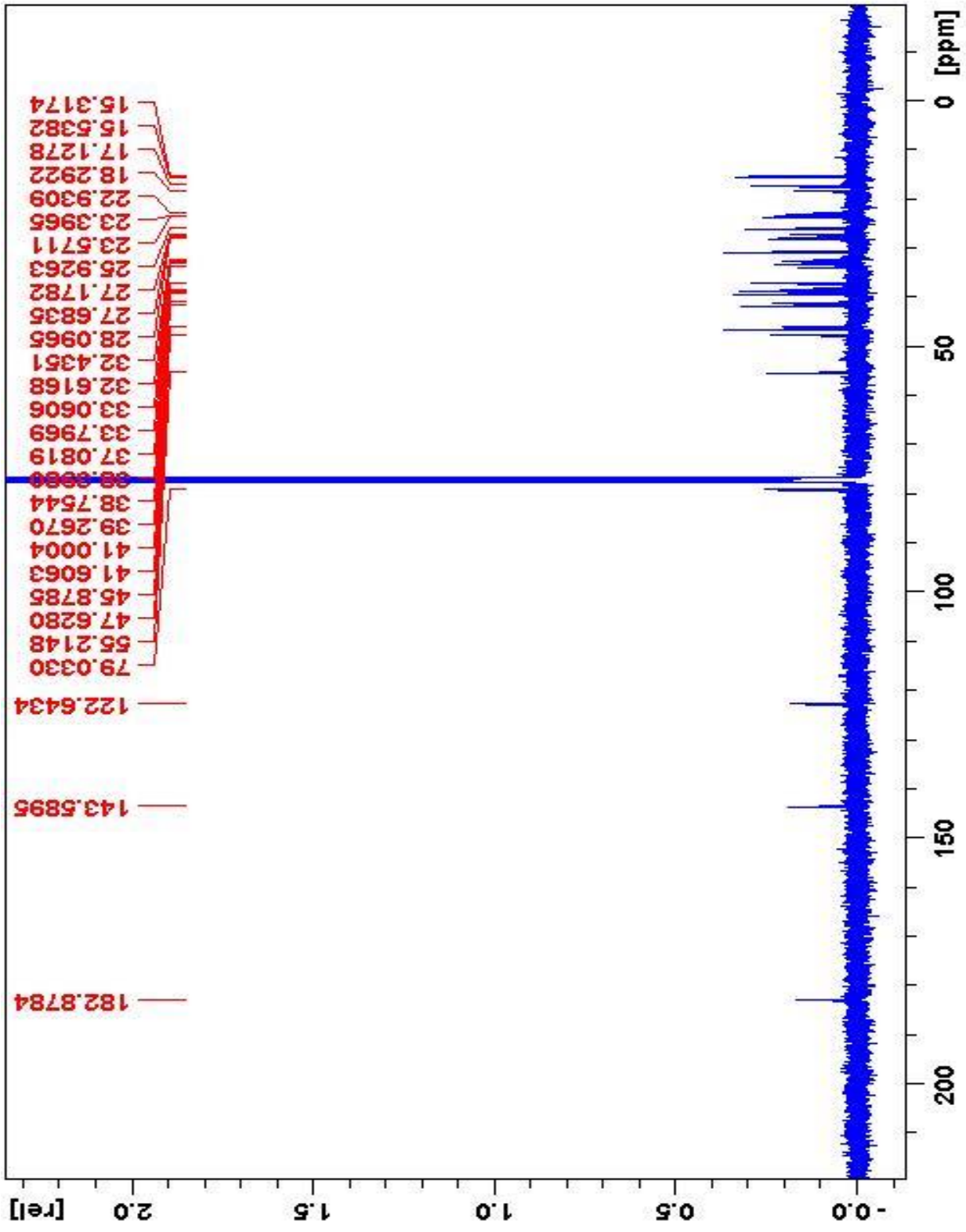
5.5.1 FT-IR spectrum of 28-methyl-3β-trifluoroacetyloleanane



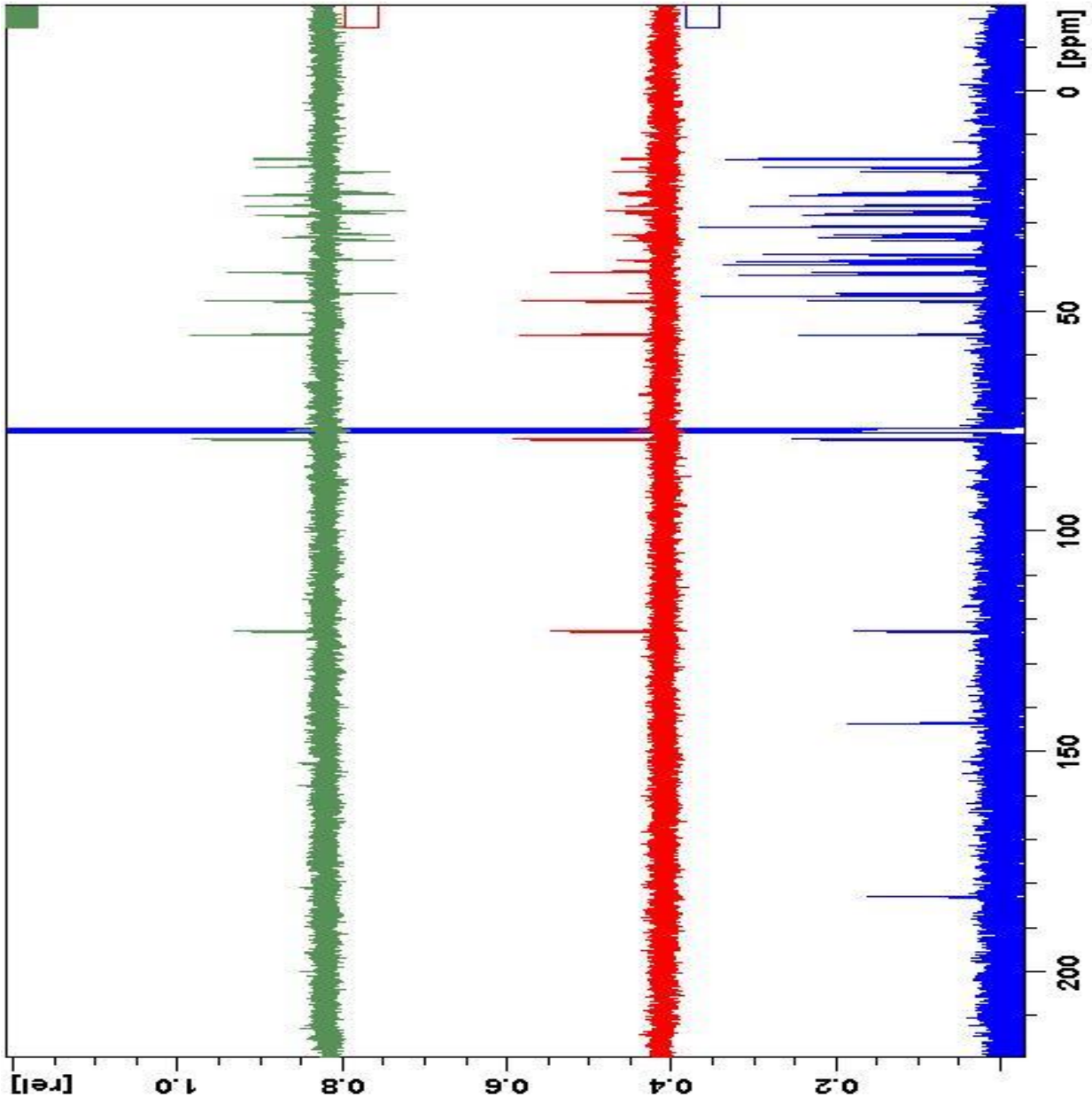
5.5.2 Mass spectrum of 28-mthyl-3β-trifluoroacetyloleanane



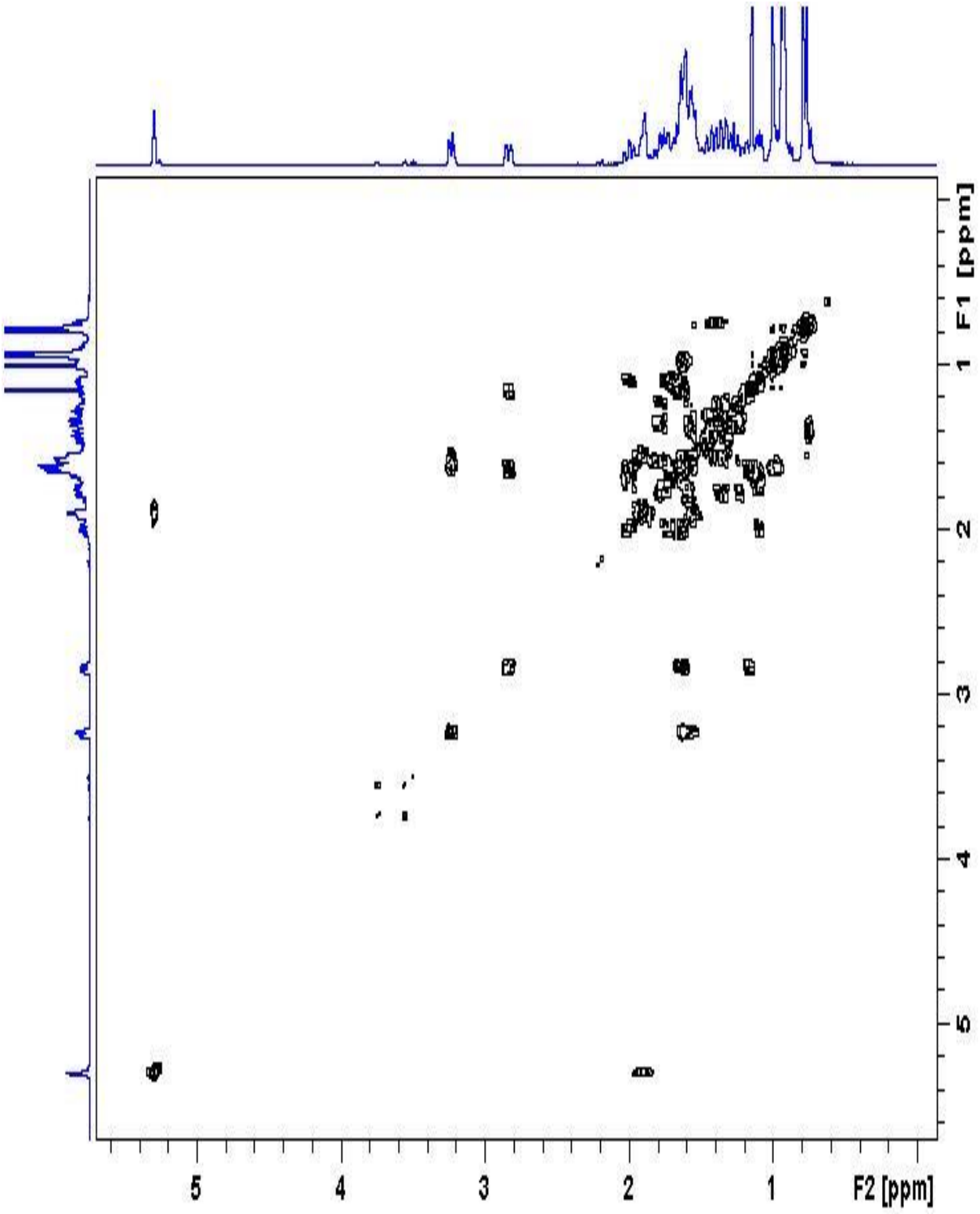
5.5.3 ^{1}H-NMR spectrum of 28-mthyl-3β-trifluoroacetyloleanane



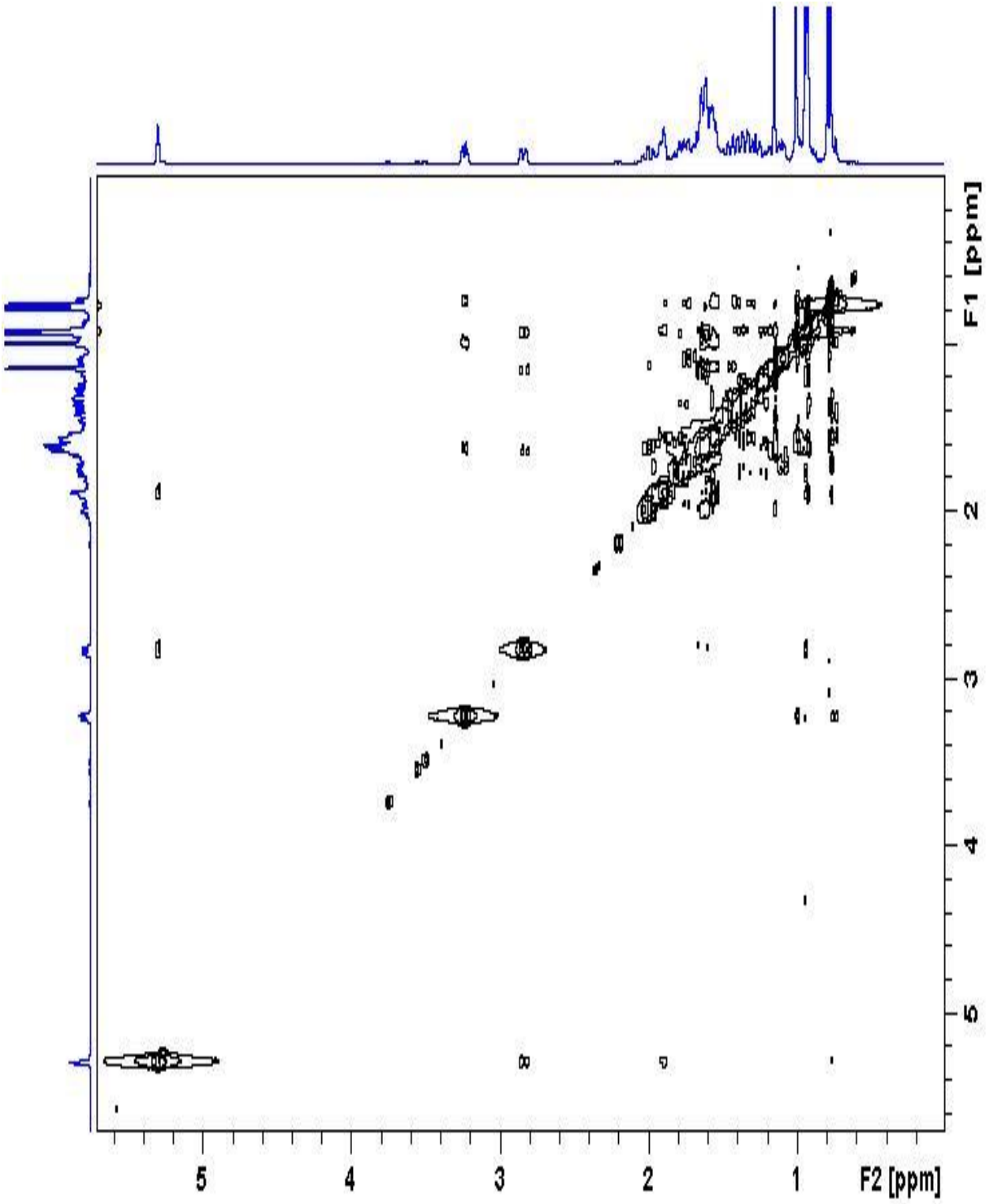
5.5.4 ^{13}C-NMR spectrum of 28-methyl-3β-trifluoroacetyloleanane



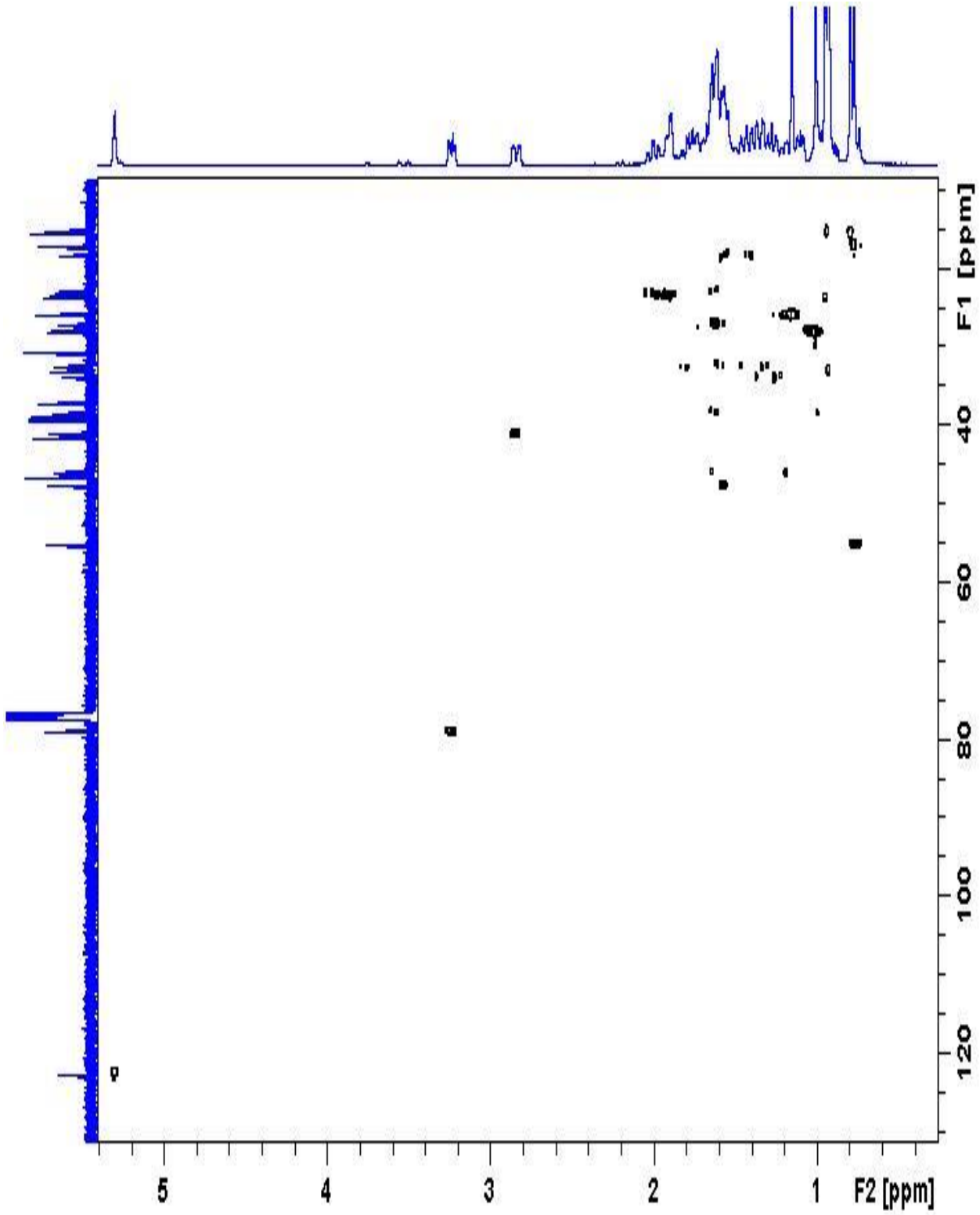
5.5.5 ^{13}C DEPT NMR spectrum of 28-methyl-3β-trifluoroacetyloleanane



5.5.6 ^{1}H-^{1}H COSY NMR spectrum of 28-methyl-3β-trifluoroacetyloleanane



5.5.7 ^{1}H-^{1}H NOESY NMR spectrum of 28-methyl-3β-trifluoroacetyloleanane



5.5.8 ^{1}H-^{13}C HSQC NMR spectrum of 28-methyl-3β-trifluoroacetyloleanane

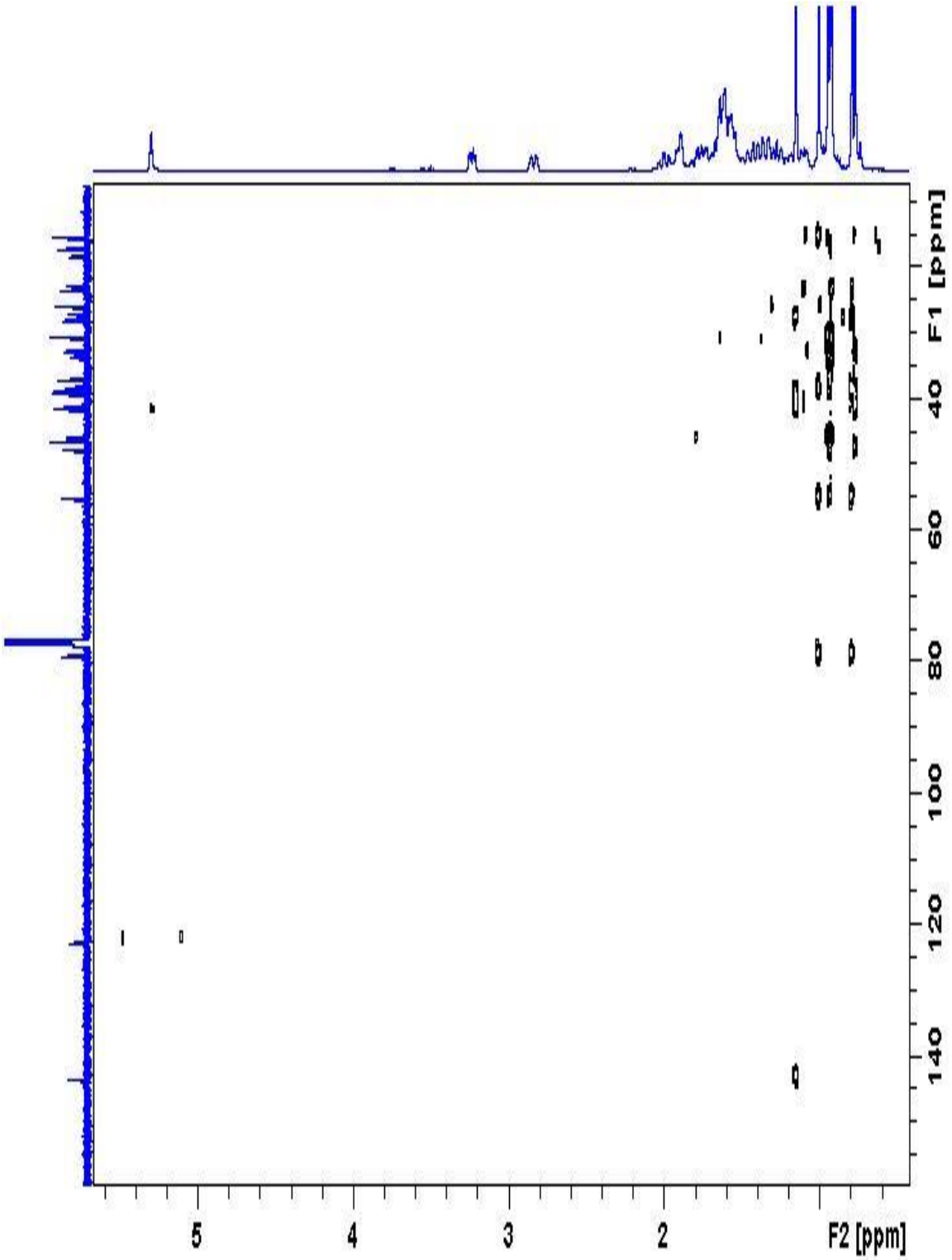


Figure 4.5.9: ^{1}H-^{13}C HMBC NMR spectrum of 28-methyl-3β-trifluoroacetyloleanane