

**Phytochemical Investigation, Antibacterial, DPPH Radical Scavenging
Activities, and *In silico* Molecular Simulation Studies of Constituents of
Selected Medicinal Plants of Ethiopia**



Tolessa Duguma Ganchu

**A Dissertation Submitted to the Department of Applied Chemistry,
College of Applied Natural Sciences**

**Presented in Partial Fulfillment of the Requirement for the Degree of Doctor of
Philosophy in Applied Chemistry (Natural Product Chemistry)**

**Office of Graduate Studies
Adama Science and Technology University**

**December, 2024
Adama, Ethiopia**

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Supervisor: Yadessa Melaku Ayana (PhD, Professor)

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DECLARATION

I hereby declare that this dissertation entitled “**Phytochemical Investigation, Antibacterial, DPPH Radical Scavenging Activities, and *In silico* Molecular Simulation Studies of Constituents of Selected Medicinal Plants of Ethiopia**” is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate in any other university. All sources of materials used for this dissertation have been duly acknowledged through appropriate citations.

Tolessa Duguma Ganchu

Name of student

Signature

Date

RECOMMENDATION

I, the supervisor of this dissertation, hereby certify that I have read and revised the dissertation entitled “**Phytochemical Investigation, Antibacterial, DPPH Radical Scavenging Activities, and *In silico* Molecular Simulation Studies of Constituents of Selected Medicinal Plants of Ethiopia**” prepared under my guidance by Tolessa Duguma Ganchu submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Chemistry (Natural Product Chemistry). Therefore, I recommend the submission of the dissertation to the department for further review and defense.

Yadessa Melaku (PhD, Professor)

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Signature

Date

APPROVAL SHEET

I/we hereby certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the dissertation entitled **“Phytochemical Investigation, Antibacterial, DPPH Radical Scavenging Activities, and In silico Molecular Simulation Studies of Constituents of Selected Medicinal Plants of Ethiopia”** by **Tolessa Duguma**.

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We, the undersigned, members of the Board of Examiners of the dissertation open defense by **Tolessa Duguma** have read and evaluated the dissertation entitled **“Phytochemical Investigation, Antibacterial, DPPH Radical Scavenging Activities, and In silico Molecular Simulation Studies of Constituents of Selected Medicinal Plants of Ethiopia”** and examined the candidate during open defense. This is, therefore, to certify that the dissertation is accepted for partial fulfillment of the requirement of the degree of Doctor of Philosophy in **Natural Product Chemistry**.

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ACKNOWLEDGEMENT

I want to start by giving thanks to my God, who has always supported and helped me get where I am today. Next, I want to express my deepest gratitude to Professor Yadessa Melaku, my supervisor, who has guided and assisted me without saving his knowledge and time since the beginning of my study. Also, my thanks go to Professors Aman Dekebo and Milkyas Endale, as well as the chemistry department chairs, for their cooperation in sending samples overseas for analysis when they had the chance and for quickly resolving the material shortage seen in the lab. I respectfully thank Dr. Daniel Rentsch, Prof. Mo Hansen, Dr. Taye Demissie, and Dr. Nayang Kgakatsi for their assistance with NMR analysis. On the other hand, I would like to thank Mr. Kebede Shenkute, Mr. Urgessa Ensermu, Ms. Emebet Getaneh, Mr. Guta Amenu, Ms. Mariyama Kufa, Mr. Leta Guta, Mr. Muktar Ahmed, and Ms. Bontu for their support in doing antibacterial, antioxidant, GC-MS, and providing me with some laboratory materials. In addition, I would like to thank Kotobe University of Education and Adama Science and Technology University for providing me with study leave and scholarships. Finally, I would like to thank my wife, Tsehaay Kebede, who has been standing beside me with her full effort during this long time. I would also like to thank my children, Keyron Tolessa, Fenan Tolessa, and Olibar Tolessa, for their patience in waiting to complete my PhD work.

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LIST OF ABBREVIATIONS AND ACRONYMS

- ABTS: - (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))
- CD: - Communicable Disease
- DCM: - Dichloromethane
- DPPH: - 2,2-diphenyl-1-picrylhydrazyl
- GC-MS: Gas-Chromatography-Mass Spectrometer
- HIV: - Human Immunodeficiency Viruses
- EI-HRMS: - Electron Impact Ionization High Resolution Mass Spectrometer
- HPLC: - High Performance Liquid Chromatography
- IC₅₀: - Half maximal Inhibitory Concentration
- IZ: - Inhibition Zone
- IAEG-SDG: - Inter-Agency and Expert Group on Sustainable Development Goal Indicators
- LC-ESI-MS: - Liquid Chromatography Electrospray Ionization Mass Spectrometer
- LC-MS-MS: Liquid Chromatography with tandem Mass Spectrometry
- LC-Q-TOF-MS: - Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
- MIC: - Minimum Inhibitory Concentration
- MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
- NCD: Non-Communicable Disease
- NMR: - Nuclear magnetic resonance spectroscopy
- PKM2: - Pyruvate kinase M2
- PKM2-IN-1: - Pyruvate kinase M2 Inhibitor 1
- POWO: Plants of the World Online
- PTLC: - Preparative Thin Layer Chromatography
- PqsA: - *Pseudomonas* Quinolone Signal A
- R_f: Retention factor
- TB: - Tuberculosis
- UV-Vis: Ultraviolet-Visible
- WHO: - World Health Organization

ABSTRACT

People all over the world have been suffering from both communicable and noncommunicable diseases, including HIV, malaria, tuberculosis, hepatitis virus, cardiovascular disease, cancer, chronic respiratory diseases, and diabetes. Millions of people die from these health threat diseases each year. Lack of access to essential medicines and drug resistance are among the reasons why these diseases have not yet been eradicated from the globe. The use of medicinal plants is believed to alleviate issues related to access to essential medicines and combat drug resistance. *Clematis hirsuta*, *Grewia ferruginea*, *Rhus ruspolii*, *Olinia rochetiana*, and *Rubus apetalus* are among the plants traditionally used to treat different diseases in Ethiopia. However, the phytochemical constituents and the biological activities of these plants are not well documented. Therefore, this study aimed to isolate phytochemicals from the root of *C. hirsuta*, the bark of *G. ferruginea*, and the leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus* and evaluate their *in vitro* and *in silico* antibacterial and DPPH scavenging activities. GC-MS analysis of the essential oil from *G. ferruginea* and non-polar fractions from *R. ruspolii*, *O. rochetiana*, and *R. apetalus* furnished 58 different compounds. Chromatographic separation and NMR spectroscopic techniques identified a total of 28 different compounds. Oleic acid (**188**), palmitic acid (**145**), stigmasterol (**70**), β -sitosterol (**17**), boehmenan (**189**), erythro carolignan E (**190**), and threo carolignan E (**191**) were identified from the root extract of *C. hirsuta*. Lupeol (**16**), β -sitosterol (**17**), stigmasterol (**70**), 7-keto- β -sitosterol (**192**), 6-ketositostanol (**193**, new to the genus), daucosterol (**42**), catechin (**65**), and epicatechin (**69**) were identified from the stem bark of *G. ferruginea*. Palmitic acid (**145**), 3,4-dihydroxybenzoic acid (**194**), myricetin (**55**), cupressuflavone (**195**), amentoflavone (**62**), shikimic acid (**196**), avicularin (**197**), and myricetin-3-O-5''-acetyl-arabinofuranoside (**198**, new to the genus) were identified from the leaves of *R. ruspolii*. Ursolic acid (**41**), 5-hydroxy-4-methyl-5,6-dihydro-(2H)-pyran-2-one (**199**), quercetin-3-O-(1-D-galactopyranoside) (**200**), olinioside (**84**), and 4-O- β -D-glucopyranosylcaffeic acid (**83**) were identified from the leaves of *O. rochetiana*. Compounds **41** and **200** are new to the genus, whereas compound **199** is a new natural product. Palmitic acid (**145**), palmityl alcohol (**201**), β -sitosterol (**17**), tiliroside (**91**), and astragalin (**202**) were identified from the leaves of *R. apetalus*. 100 mg/mL extracts and fractions and 5 mg/mL essential oil and isolated compounds showed inhibition zones (IZs) ranging from 7.93 ± 0.65 to 16.33 ± 0.47 mm against all tested bacteria compared to ciprofloxacin (0.5 mg/mL), which ranged from 18.42 ± 0.26 to 22.18 ± 0.22 mm. The highest IZ (16.33 mm) was exhibited by the DCM:MeOH (1:1) extract of the leaves of *R. ruspolii* against *Escherichia coli*. Whereas the lowest IZ (7.93 mm) was shown by the EtOAc fraction of *G. ferruginea* stem bark against *Pseudomonas aeruginosa*. Of all the isolated compounds, *Staphylococcus aureus* was the most susceptible to compound **197** (IZ: 14.67 ± 0.47 mm) and least susceptible to compound **196** (IZ: 8.33 mm). *Streptococcus pyogenes* was the most susceptible to compound **17** (IZ: 15.07 ± 0.74 mm) and least susceptible to compound **196** (IZ: 8.67 ± 0.50 mm). *E. coli* was the most susceptible to compound **195** (IZ: 12.67 ± 0.50 mm) and least susceptible to compound **196** (9.00 ± 0.82 mm). *P. aeruginosa* was the most susceptible to compound **16** (IZ: 15.20 ± 0.28 mm) and least susceptible to compound **196** (IZ: 8.67 ± 0.47 mm). The results of *in vitro* antibacterial activity evaluation conducted against *E. coli* and *P. aeruginosa* were supported by molecular docking studies against their target enzymes (*E. coli* DNA Gyrase B and *Pseudomonas* quinolone signal A (PqsA), respectively. Among the isolated compounds, at 62.5 μ g/mL, compound **189** exhibited the highest DPPH radical (92.1%), while compound **17** exhibited the least (18.50%) compared to the positive control ascorbic acid (96.9%). From the *in silico* druglikeness and toxicity prediction, most of the isolated compounds fulfilled Lipinski's rule of five. They were

predicted to have less toxicity compared to the standard drugs used in this study. Generally, the extracts, fractions, essential oils, and isolated compounds from the studied plants showed promising antibacterial and DPPH radical scavenging properties. Most of the isolated compounds have good permeability and solubility in the human body, and none of the isolated compounds are fatal if swallowed. Therefore, these findings support the traditional use of these medicinal plants to treat different bacterial diseases. However, both in vitro and in vivo qualitative, quantitative, pharmacokinetic, and pharmacodynamic investigations are very important to standardize the use of these medicinal plants.

Keywords: Medicinal plants, Phytochemicals, Antibacterial, DPPH radical, Docking, Druglikeness, Toxicity

CHAPTER ONE

1. INTRODUCTION

1.1 Background of the Study

The economic growth and population health of countries are interconnected and should not be considered separately. Economic growth will be brought when the populations of the country are well-being. Healthy people are productive, and healthy infants and children can develop better and become productive adults. Increasing expenditure in the health sector results in 100 billion dollars per year of additional income, which is then used to improve the living conditions of the country's population (WHO, 2014). According to this report, for every 10% increase in life expectancy at birth, there is a corresponding rise in economic growth of 0.4% per year. To ensure this fact, enormous actions have been undertaken, like preparing plans accomplished in 15 years by member states. As an example, after the completion of the Millennium Development Goal on 30 December 2015, the United Nations (UN) announced the new goals named sustainable development goals, embracing 17 goals, 169 targets, and a total number of 244 indicators that came into effect on 1 January 2016 and will be achieved by the end of 2030 (Inter-Agency and Expert Group on Sustainable Development Goal Indicators (IAEG-SDG), 2017; United Nations, 2015)

There are still many unresolved health threats, including communicable diseases (CDs) such as human immunodeficiency virus (HIV), malaria, tuberculosis (TB), and hepatitis viruses, and noncommunicable diseases (NCDs) such as cardiovascular disease, cancer, chronic respiratory disease, and diabetes, which are still a big global problem (WHO, 2023). The WHO 2023 report on CD indicated that of 100,000 people, 19, of 10,000 people, 592, of 100,000 people, 134, and of 1,000 children under five, about 94 were newly infected by HIV, malaria, TB, and hepatitis B, respectively. Similarly, the report on the four major NCDs indicated that collectively, about 33.3 million people were killed in 2019, of which about 17.9, 9.3, 4.1, and 2.0 million were killed by cardiovascular, cancer, chronic respiratory, and diabetes diseases, respectively. Africa is the region most affected by some of those health-threatening diseases. For example, the probability of dying from the major NCDs, including cancer and diabetes, in the African region was 208 out of 1000 people, compared with the American region, which was 140 out of 1000 people. Specifically, in

Ethiopia, out of 1000 people, 171 deaths were reported due to NCD, including cancer and diabetic diseases, compared to only 82 deaths reported in the Cyprus European region country (WHO, 2023). The severity of these problems in the African region could be related to the accessibility of essential medicines, lack of skilled personnel, and low money investment in health sectors by countries (WHO, 2023), and antimicrobial resistance is another factor for the widespread prevalence of these diseases (Murray *et al.*, 2022; O'Neill, 2016).

Even though there are different actions that can be taken by various bodies to overcome the shortage of essential medicine that might be due to antimicrobial resistance (Cameron *et al.*, 2011), using traditional medicine is indisputable. It is believed that plants' traditional medicines were the main source used to sustain health from 6000 years ago (Kamel, 2015) up to 1828, when the first organic compound, urea, was synthesized from inorganic compounds (Ahmad *et al.*, 2006). Globally, 88% of UN member states, corresponding to 170 states, are using traditional and complementary medication, and specifically, about 79% of the population in Ethiopia relies on Indigenous traditional medicine, with about 600 herbal medicine providers (WHO, 2019). *Clematis hirsuta* Perr. & Guill, *Grewia ferruginea* Hochst. ex A. Rich, *Rhus ruspolii* (Engl.) Moffett, *Olinia rochetiana* A. Juss, and *Rubus apetalus* Poir are among the traditionally used medicinal plants in Ethiopia.

C. hirsuta, locally called *Hidda feetii* in Afan Oromo (Abdisa & Kenea, 2021), and its various parts have traditionally been used to treat different diseases, including swelling, cough, cataracts, and leishmaniasis (Giday, Asfaw, *et al.*, 2009; Limenih *et al.*, 2015; Teklehaymanot & Giday, 2007; Yimam *et al.*, 2022), earache, cancer, and TB (Eshete *et al.*, 2016; Yineger & Yewhalaw, 2007), respiratory tract problems (Giday, Asfaw, *et al.*, 2009), fungal and trypanosomal diseases (Dubale *et al.*, 2023), and wounds (Morka & Duressa, 2021). *G. ferruginea*, locally known as *Dhoqonuu* in Afan Oromo (Tesemma, 2007), is claimed for its traditional uses to treat various diseases, including kidney disorders and intestinal parasites (Belayneh *et al.*, 2012; Fenethun *et al.*, 2017), asthma and stomach aches (Chekole, 2017), cough (Bekele & Reddy, 2015; Mesfin *et al.*, 2009), and stomach pains (Wondimu *et al.*, 2007). *R. ruspolii* is a plant locally known as *Xaaxessa* in Afan Oromo (Megersa *et al.*, 2013). Various parts of the plant are traditionally used to treat different ailments mentioned as hyena bites (Megersa *et al.*, 2013), ectoparasites (Birhanu *et al.*, 2015; Giday, Asfaw, *et al.*, 2009; Megersa *et al.*, 2013), and swelling (Bogale *et al.*,

2023). *O. rochetiana* is locally called *Nole* in Afan Oromo (Tuasha *et al.*, 2018). Traditionally, the different parts of this plant are used for treating the common cold (Tuasha *et al.*, 2018), eczema and warts (Chekole, 2017; W. Yohannis *et al.*, 2018), tooth infection (Kebebew, 2017), and snakebite (Kefalew *et al.*, 2015). *R. apetalus* is known locally as *Goraa* in Afan Oromo (Arega *et al.*, 2020) and is traditionally used to treat TB and gonorrhea (Tuasha *et al.*, 2018), evil eye (Meragiaw *et al.*, 2016), diarrhea, stomach aches, body swelling, and wounds (Hamill *et al.*, 2003; Tamene, 2020), and pain (Kebebew, 2017).

It is essential to conduct a thorough analysis to identify phytochemicals that account for their traditional use to integrate these traditionally used medicinal plants into contemporary drug development. Only 25% of medicinal plants are incorporated into modern drugs (Calixto, 2019), from about 35,000 plant species that are used globally for medical purposes (Christenhusz & Byng, 2016). Some phytochemical components (Figure 1) of traditionally used medicinal plants have been identified and incorporated into drug development research. For example, withaferin A (**1**) is a lactone isolated from the leaves of *Withania somnifera*, a traditional herb (Lavie *et al.*, 1965), and the phase I clinical trial study showed its promising anticancer properties (Pires *et al.*, 2020). A diterpene, trichorabdal A (**2**), isolated from a Japanese *Rabdosia trichocarpa* (Kadota *et al.*, 1997), was sold for its anti-tumor agent for research purposes by different companies (Node *et al.*, 1982).

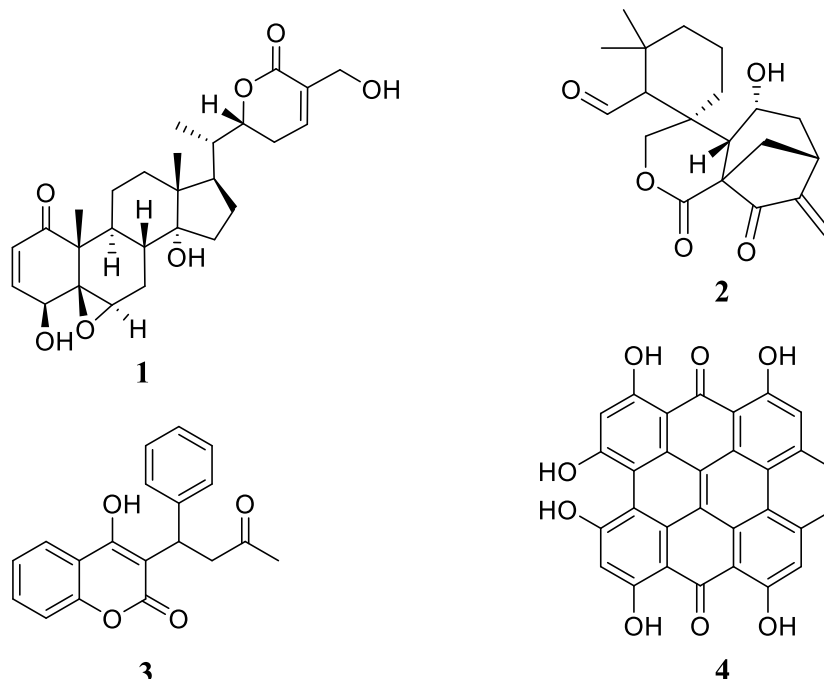


Figure 1. Secondary metabolites isolated from selected traditional medicinal plants

Warfarin (3), an anticoagulant drug, is a synthetic derivative of a natural anticoagulant, dicoumarol, isolated from *Melilotus officinalis* (Kresge *et al.*, 2005). Hypericin (4), an anthraquinone derivative isolated from *Hypericum perforatum* (Cao *et al.*, 2011), was subjected to a clinical trial and showed a promising drug candidate for skin disease treatment (Hajhashemi *et al.*, 2018; Samadi *et al.*, 2010).

1.2 Statement of the Problem

Multidrug resistant (MDR) microbes pose a significant global health threat, resulting in the loss of 10 million lives annually and will put a cumulative 100 trillion USD of economic output at risk by the year 2050 (Oneill, 2016). In 2019, there were 4.95 million deaths linked to bacterial MDR, of which about 1.27 million deaths were directly attributed to bacterial MDR (Murray *et al.*, 2022), from which about 920,000 deaths were associated with MDR in *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa*. Currently, based on their severity for the life of human beings, pathogenic bacteria are categorized into three groups for research and development to facilitate the urgent finding of new antibiotics. *E. coli* is categorized as a critical priority. *P. aeruginosa* and *S. aureus* are categorized as high priority, while *S. pyogenes* is categorized as medium priority (WHO, 2024). These bacteria are the causes of various infectious diseases, including pneumonia, meningitis, and Shiga toxin (Tong *et al.*, 2015; Stevens and Bryant, 2022; WHO, 2018; Wilson and Pandey, 2023). If these bacterial diseases are not treated properly, they might be associated with or directly attributed to millions of lives lost annually. Hence, every institution, organization, and individual should contribute their part to save millions of lives. In this regard, researchers play a vital role in the process of finding new antibiotics for these antimicrobial resistant bacteria.

Traditional medicinal plants are believed to treat these bacterial diseases. *C. hirsuta*, *G. ferruginea*, *R. ruspolii*, *O. rochetiana*, and *R. apetalus* are among the traditionally used medicinal plants in Ethiopia. Different parts of these plants are used to treat various bacterial diseases. However, the biological properties of the phytochemical components of these medicinal plants are not well documented. Therefore, this study aimed to isolate phytochemicals from the roots of *C. hirsuta*, the stem barks of *G. ferruginea*, and the leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus*. Furthermore, the study evaluated the *in vitro* antibacterial properties and DPPH radical scavenging activities of both the extracts and the isolated compound. Additionally, *in silico* analyses were performed to assess the molecular

docking, drug-likeness, and potential toxicity of the isolated compounds. Hence, in this study, the results of the *in vitro* antibacterial activities and the DPPH radical scavenging ability of extracts and isolated compounds from the selected medicinal plants are reported. Furthermore, to validate the *in vitro* antibacterial activities, to predict the anticancer activities of isolated compounds, druglikeness, and toxicity of isolated compounds, the results of *in silico* molecular simulation are included.

1.3 Objectives

1.3.1 General objective. The overall objective of this study was to investigate the phytochemical constituents from selected medicinal plants of Ethiopia and conduct their *in vitro* antibacterial and DPPH radical scavenging activities combined with *in silico* molecular modeling studies.

1.3.2 Specific objectives. The specific objectives of this study were:

- To extract and fractionate the root of *C. hirsuta*, bark of *G. ferruginea*, and leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus* using different organic solvents.
- To evaluate the *in vitro* antibacterial activity and the minimum inhibitory concentration (MIC) of the extracts, fractions, essential oil, and isolated compounds using the disk diffusion and agar dilution methods, respectively, against pathogenic bacteria, including Gram positive (*S. aureus* ATCC 25923 and *S. pyogenes* ATCC 19615) and Gram negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853).
- To test the *in vitro* antioxidant activity of the extracts, fractions, essential oil, and isolated compounds using a DPPH radical scavenging assay.
- To isolate and purify compounds using silica gel column chromatography, preparative thin-layer chromatography (PTLC), and recrystallization techniques.
- To elucidate the structures of isolated compounds using gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectrometers.
- To conduct the molecular docking of isolated compounds using AutoDock Vina against *E. coli* DNA gyrase B (PDB ID: 7P2M), Pseudomonas quinolone signal A (PqsA, PDB ID: 5OE4), pyruvate kinase M2 (PKM2; PDB ID: 4G1N), and human topoisomerase II β (PDB ID: 3QX3).
- To predict the druglikeness properties of isolated compounds using the SwissADME online tool.
- To compute the toxicities of isolated compounds using the ProTox 3.0 online tool.

1.4 Significance of the Study

The findings of this research may provide valuable insights into the selected plants, thereby eliminating the need for further time and financial investment in investigating these species. Additionally, the researchers use the findings as a basis for further investigation of the plants. The local users may gain awareness of the plants so that the safety and efficacy of the plant extracts can be improved. Finally, the findings of the study might be incorporated into drug development so that they can contribute to addressing the issues of certain health-threatening diseases due to MDR bacteria.

CHAPTER TWO

2. LITERATURE REVIEW

On this planet, there are around 374,000 vascular plant species of which around 308,312 species are flowering plants (Christenhusz & Byng, 2016). These plant species belong to more than 620 plant families and 16,167 plant genera (The Plant List, 2013). From these, about 6500 species of higher plants are believed to be found in Ethiopia with approximately 12% endemism (Bekele, 2007). Currently, there are about 887 medicinal plant species used by Ethiopian people, with 24 (2.7%) endemics (Institute of Biodiversity Conservation, 2009). In this section, the ethnobotanical use, phytochemicals, and biological activity of selected traditional medicinal plants in the family Ranunculaceae, Tiliaceae, Anacardiaceae, Penaeaceae, and Rosaceae are discussed.

2.1 The Origin and Ethnobotanical Uses of Selected Medicinal Plants

2.1.1 *Clematis hirsuta*. *C. hirsuta* (Figure 2) is a tall climbing shrubby plant belonging to the family Ranunculaceae and genus *Clematis* (Plants of the World Online (POWO, 2022). The plant is endemic to Cape Verde, Sahara to tropical Africa, and the South West Arabian Peninsula. It grows up to 1 to 4 m or more tall, containing leaves pinnate with 5 leaflets (POWO, 2022). *C. hirsute* locally called *Hidda feetii* in *Afan Oromo*, Ethiopia (Abdisa & Kenea, 2021).



Figure 2. *C. hirsuta* (Photo taken by Tolessa Duguma on 12 July 2021, around Gafare, Waldoro Village, Homa Kebele, Abbay Chomman Woreda, Horro Guduru Zone, Oromia Region, Ethiopia)

In Ethiopia, the different parts of the plant are traditionally used to treat various ailments. For instance, the stem and leaves of the plant have been claimed to treat swelling (Limenih *et al.*, 2015). The leaves are used to treat cough, cataracts, and leishmaniasis (Giday, Asfaw, *et al.*, 2009; Teklehaymanot & Giday, 2007; Yimam *et al.*, 2022). Furthermore, as reported by Yineger and Yewhalaw (2007), fresh leaves of the plant are used locally to treat earache. In addition, the leaves of the plant are claimed to be used as anticancer and anti-TB (Eshete *et al.*, 2016). The root is given orally to treat respiratory tract problems (Giday, Zemedet, *et al.*, 2009). The whole part of the plant was reported to be used as antifungal, antitrypanosomal, and antileishmaniasis (Dubale *et al.*, 2023) and wound healing (Morka & Duressa, 2021).

2.1.2 *Grewia ferruginea*. The *G. ferruginea* (Figure 3) is in the genus *Grewia* of the family Tiliaceae (The Plant List, 2013) and locally known as *Dhoqonuu* in *Afan Oromo*, Ethiopia (Tesemma, 2007). It is endemic to Eritrea, Ethiopia, Kenya, and Sudan. It is a shrub that can grow up to 7 m in riverine forests, near lakes, and along rivers in open acacia combretum forests in dry and moist agroclimatic zones in midland and lowland (POWO, 2022).



Figure 3. *G. ferruginea* (Photo taken by Tolessa Duguma on 12 July 2021, around Chure, Waldoro Village, Homa Kebele, Abbay Chomman Woreda, Horro Guduru Zone, Oromia Region, Ethiopia)

The medicinal uses of different parts of the plant have been reported. The concoction of fruit and root is taken orally to treat kidney infections and intestinal parasites (Belayneh

et al., 2012; Fenethun *et al.*, 2017). The bark and root of the plant powder concoction are taken as a drink with honey and butter to treat asthma and stomachache (Chekole, 2017). Crushed fresh or dry root bark concocted with the root of *Ensete ventricosum* and mixed with water is kept overnight and taken orally as a drink before breakfast to treat cough (Bekele & Reddy, 2015; Mesfin *et al.*, 2009). The leaf is chewed to treat stomachache (Wondimu *et al.*, 2007). Dried leaf powder with water is given orally to treat constipation (Gebeyehu *et al.*, 2014). The leaves of the plant are used as soup to treat dandruff (Megersa *et al.*, 2013).

2.1.3 *Rhus ruspolii*. *R. ruspolii* (Figure 4) is a flowering plant of the genus *Rhus* in the family Anacardiaceae, and it is an endemic plant to Ethiopia, Kenya, Uganda, and the Democratic Republic of the Congo (POWO, 2022). The plant is locally known as *Xaaxessa* in Afan Oromo, Ethiopia (Megersa *et al.*, 2013). It is a shrub or small tree growing to 7 m high in upland evergreen bushland, forest edges, and riverine associations with an altitude of 1200–2450 m (POWO, 2022). Various parts of the plant are traditionally used to treat different ailments. The root is powdered and mixed with water and drunk to treat hyena bites (Megersa *et al.*, 2013). Fresh leaves are crushed and rubbed on the affected part to kill ectoparasites (Birhanu *et al.*, 2015; Giday, Asfaw, *et al.*, 2009; Megersa *et al.*, 2013). The fresh leaves of *R. ruspolii* are applied to the affected body to treat swelling (Bogale *et al.*, 2023).



Figure 4. *R. ruspolii* (Photo taken by Tolessa Duguma on 12 July 2021, around Chure, Waldoro Village, Homa Kulkula kebele, Abbay Chomman Woreda, Horro Guduru Zone, Oromia Region, Ethiopia)

2.1.4 *Olinia rochetiana*. *O. rochetiana* (Figure 5) is one of the traditional medicinal plants of Ethiopia and belongs to the genus *Olinia* in the family Penaeaceae (POWO, 2022).

O. rochetiana is locally called *Nole* in Afan Oromo, Ethiopia (Tuasha *et al.*, 2018), and it is native to Ethiopia, Kenya, Sudan, Tanzania, and Uganda. The plant is a shrub, small tree, or, less often, a large tree that grows up to 4-27 m long (POWO, 2022). Different parts of the plant have been claimed to be used traditionally to treat different human ailments. For example, the leaf is heated slightly, rubbed by the hands, and then inhaled through the nostrils to treat the common cold (Tuasha *et al.*, 2018), crushed and tied to treat eczema and warts (Chekole, 2017; W. Yohannis *et al.*, 2018), and chewed with affected teeth and the sap is swallowed to treat tooth infection (Kebebew, 2017). Latex from any part of the plant, mostly from the root, is mixed with leaf powder of *Ficus vasta* and pasted on the wound to treat snakebite (Kefalew *et al.*, 2015).



Figure 5. *O. rochetiana* (Photo taken by Tolessa Duguma on 12 July 2021, around Cure, Waldoro Village, Homa Kebele, Abbay Chomman Woreda, Horro Guduru Zone, Oromia Region, Ethiopia)

2.1.5 *Rubus apetalus*. *R. apetalus* (Figure 6) is a scrambling shrub in the Rosaceae family of the genus *Rubus*, up to 5 m long, and is native to Nigeria, Eritrea, South Africa, the west Indian Ocean, and north Yemen (POWO, 2022). The plant is known locally as *Goraa* (Afan Oromo, Ethiopia) (Arega *et al.*, 2020) and is used traditionally for different purposes. The root of the plant is pounded, boiled, and drunk to treat TB and gonorrhea (Tuasha *et al.*, 2018). The crushed and powdered root is fumigated, and the aroma of the smoke at night is smelt to prevent the evil eye (Meragiaw *et al.*, 2016). The leaves of the plant are chewed to treat diarrhea, stomach aches, body swelling, and wounds (Hamill *et al.*, 2003; Tamene, 2020). The dried seed is swallowed once a day for 3 to 5 days to treat pain (Kebebew, 2017).



Figure 6. *R. apetalus* (Photo taken by Tolessa Duguma on 12 July 2021 around Dire Ejersa, Waldoro Village, Homa Kulkula kebele, Abbay Chomman Woreda, Horro Guduru Zone, Oromia Region, Ethiopia)

2.2 Phytochemistry of Selected Plants

2.2.1 The genus *Clematis*. The presence of various classes and pure compounds has been investigated in different *Clematis* species. Hence, under this section, the classes and pure compounds (Figure 7) identified from *C. simensis*, *C. gouriana*, *C. hedysarifolia*, *C. argentilucida*, *C. viridiflora*, *C. mandshurica*, and *C. hirsuta* are discussed using the data collected from phytochemical screening tests and various spectroscopic techniques.

C. simensis, *C. gouriana*, and *C. hedysarifolia* have been studied to identify the chemical compounds found in their different parts. The phytochemical screening test conducted on the *C. simensis* root indicated the presence of alkaloids, flavonoids, terpenoids, and phenols (Alemayehu & Gelayee, 2019). Whereas the leaves of the same plant exhibited the presence of terpenoids, polyphenols, tannins, flavonoids, and alkaloids (Birhan *et al.*, 2018; Tadele *et al.*, 2009; Teshome & Teshale, 2013). The phytochemicals evaluation conducted on CHCl_3 , EtOAc, EtOH, and water extracts of the flower part of *C. gouriana* showed the presence of bioactive compounds including alkaloids, flavonoids, phenols, emodins, and coumarins (Yogesh *et al.*, 2016). However, only steroids and emodins were reported from the leaves of the same plant (Savithramma *et al.*, 2011). The phytochemical screening carried out on different solvent extracts of the stem, branch, and

leaves of *C. hedysarifolia* showed the presence of phenolic compounds such as tannins, flavonoids, and steroids (Pawar *et al.*, 2016).

C. argenteolucida, *C. viridiflora*, and *C. mandshurica* have been assessed for the chemical constituents of their different parts. By using bioassay-guided fractionation, compounds including 3 β -O-[β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl]hederagenin-11,13-dien-28-oic acid (5), 3 β -O-{ β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)]- β -D-xylofuranosyl} oleanolic acid (6), and 28-O-[α -L-rhamnopyranosyl (1 $\rightarrow$ 4)- β -D-glucopyranosyl (1 $\rightarrow$ 6)]- β -D-glucopyranosyl ester (7) have been isolated from *n*-BuOH extract of the roots of *C. argenteolucida* (Hai *et al.*, 2012). From the root of the same plant, *n*-BuOH extract led to the isolation and characterization of oleanane-type triterpenoid saponins, including 3-O- β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinopyranosyl oleanolic acid 28-O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (8), 3-O- β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)]- α -L-arabinopyranosyl oleanolic acid 28-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (9), 3-O- β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-xylofuranosyl 3 β ,21 α -dihydroxyolean-12-en-28-oic acid (10), and 3-O- β -D-ribofuranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)]- α -L-arabinopyranosyl oleanolic acid (11) (Zhao *et al.*, 2014). In another way, from the leaves of *C. viridiflora*, 4-methoxy-3-methyl-2-(methylthio)-1H-indole (12) was isolated (Monjane *et al.*, 2017). Alkaloids (13) (Fu *et al.*, 2016) and phenolic glycosides (14) (Shi *et al.*, 2006) (Figure 7) were isolated from the EtOH extract of roots and rhizomes of *C. mandshurica*.

C. hirsuta was similarly investigated for its phytochemical constituents. In this regard, alkaloids, saponins, tannins, flavonoids, phenols, and glycosides have been reported from the root of *C. hirsuta* (Abdisa & Kenea, 2021). From the aerial part of *C. hirsuta*, compounds namely β -amyrin (15), lupeol (16), β -sitosterol (17), oleanolic acid (18), stigmasterol glycoside (19), (S)-(+)-dihydro-5-(hydroxymethyl)-2(3H)-furanone (21), (s)-(-)-5-hydroxymethyl-2(5H)-furanone (22), anemonin (20), dihydro-4-hydroxy-5-(hydroxymethyl)-2(3H)-furanone (2-deoxy-D-ribo-1,4-lactone) (23), biophenol (cimidarhizin) (24), glucose (25), and sucrose (26) (Figure 7) have been reported (Abdel-Kader *et al.*, 2008).

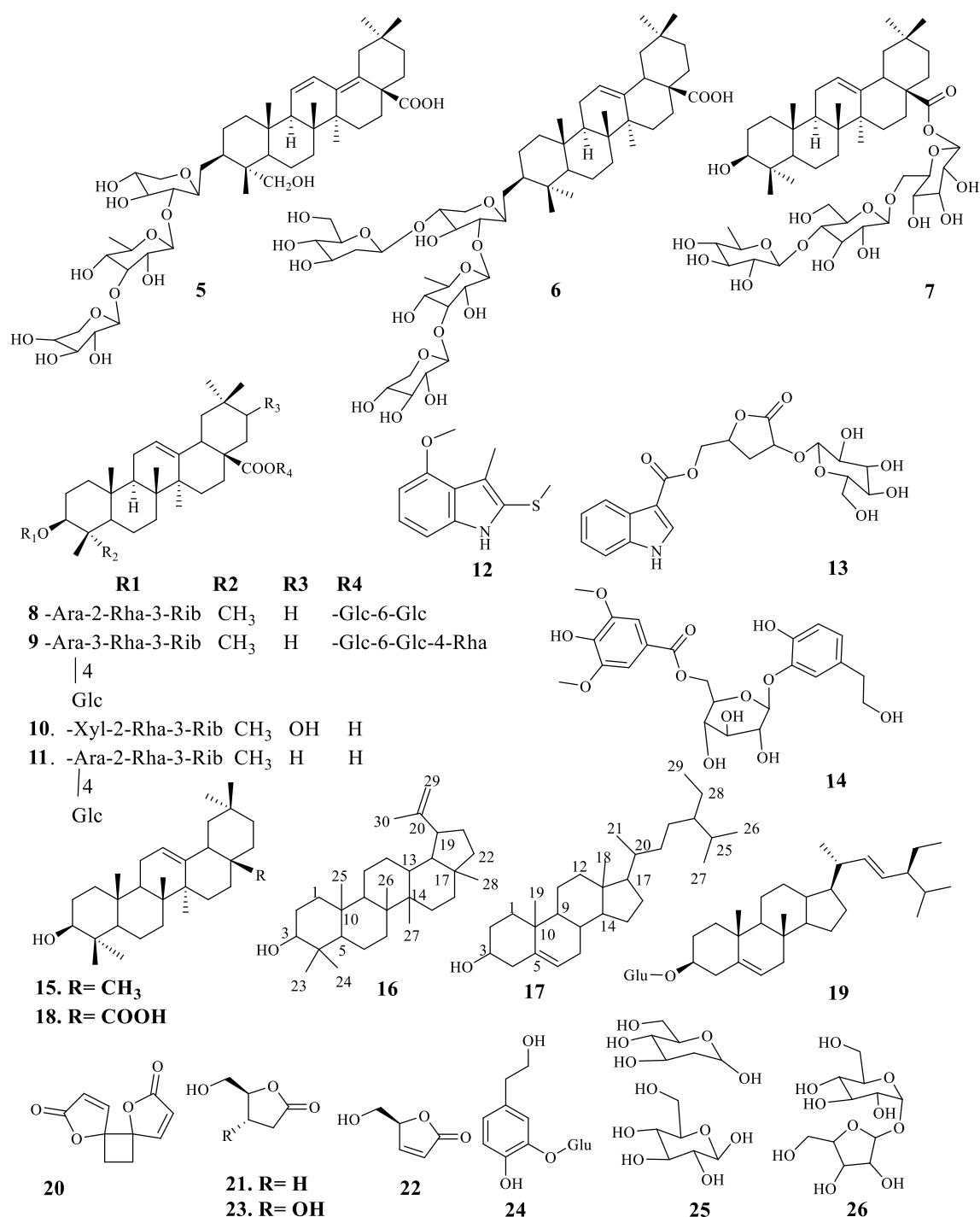


Figure 7. Some reported compounds from the genus *Clematis*

2.2.2 The genus *Grewia*. The quantitative and qualitative phytochemicals of some plants in the genus *Grewia* have been studied. Therefore, in these sections, using different spectroscopic and chromatographic techniques, the quantitative and qualitative phytochemical studies (Figure 8) from some of the genera *Grewia*, including *G. asiatica*, *G. tenax*, *G. coriacea*, *G. optiva*, *G. lasiocarpa*, *G. flava*, and *G. tiliaefolia* are presented.

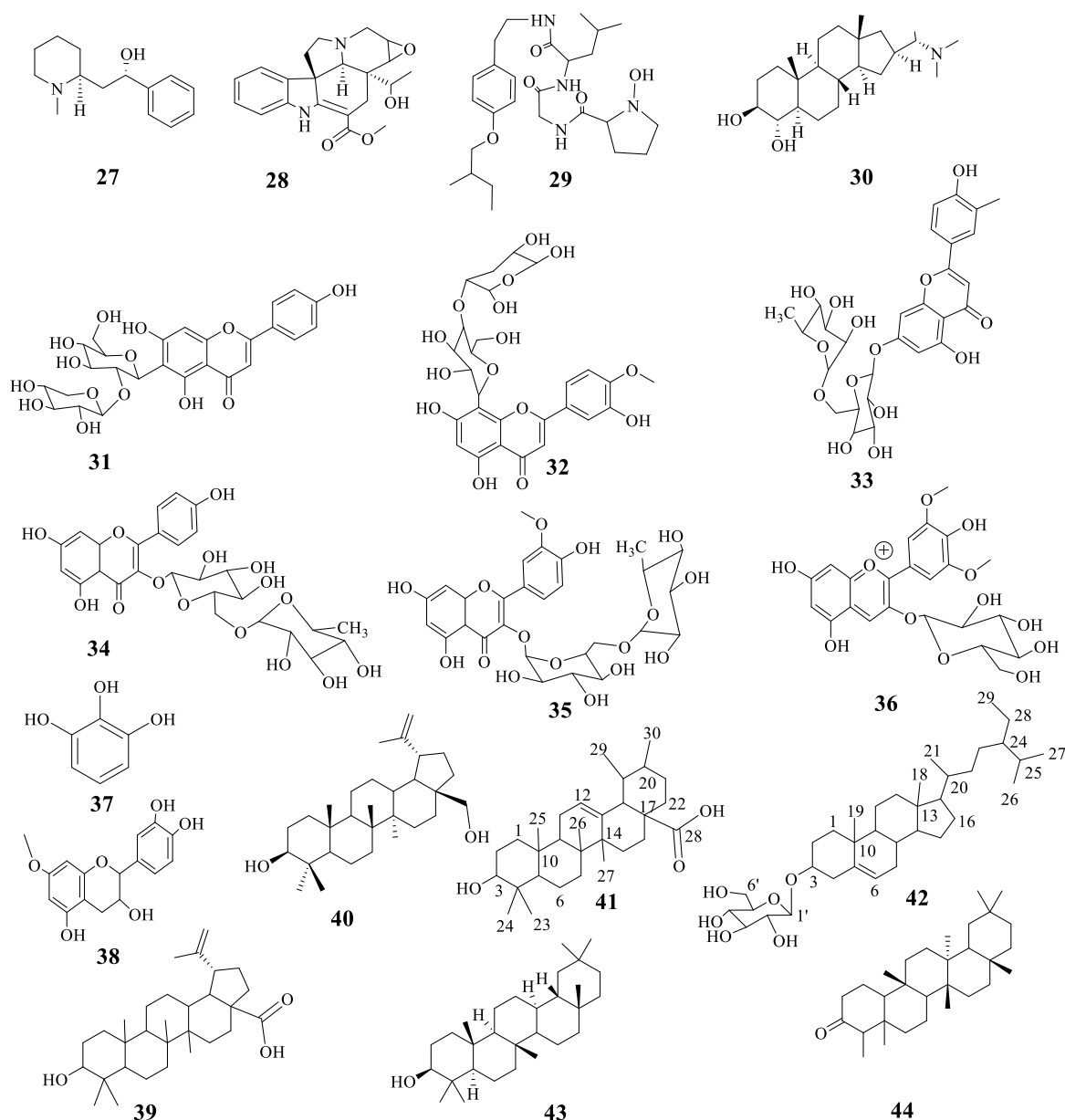


Figure 8. Some reported compounds from the genus *Grewia*

G. asiatica and *G. tenax* have been investigated for quantitative and qualitative phytochemicals in their various parts. The total anthocyanins extracted by different solvents from the fruit of *G. asiatica* were compared using a UV-Vis spectrophotometer. The maximum anthocyanins, 1193.8 $\mu\text{g/g}$, were obtained in the acidified MeOH extract compared to water, MeOH, and EtOH extracts (Talpur, 2017). LC-Q-TOF-MS was used to investigate the qualitative and quantitative analysis of the major bioactive constituents of *G. tenax* leaves from DCM and MeOH crude extracts. The results exhibited that the plant leaves have different phytochemicals, including alkaloids (27-30) and different flavonoids (31-33) (Aadesariya *et al.*, 2019; Aadesariya, Ram, *et al.*, 2017). In addition, kaempferol-3-

O-rutinoside (**34**) and isorhamnetin-3-O-rutinoside (**35**) (Figure 8) were isolated from the EtOAc fraction of the 80% MeOH extract of the fruits of the same plant (Saleh *et al.*, 2010).

G. coriacea and *G. optiva* have been studied for the presence of various phytochemicals using different spectroscopic techniques. From 1% acidic H₂O:EtOH (1:1) extract of mature fruits of *G. coriacea*, malvidin-3-O-glucoside (**36**) was isolated using chromatographic methods and characterized using UV-Vis, NMR, and HRMS (Madiela Mabika *et al.*, 2016). Various bioactive compounds, namely β -sitosterol (**17**), 1,2,3-benzenetriol (**37**), 7-O-methyl catechin (**38**), and betulinic acid (**39**), were isolated from the EtOAc fraction of MeOH extracts of *G. optiva* root by chromatographic techniques and characterized using UV, FTIR, NMR, and MS techniques (Bari *et al.*, 2019). Whereas from EtOH crude extract of the bark of the plant, betulinic acid (**39**), betulin (**40**), oleanolic acid (**18**), ursolic acid (**41**), and daucosterol (**42**) (Figure 8) were isolated by chromatographic techniques and characterized using NMR and EI-HRMS (Uddin *et al.*, 2011).

G. lasiocarpa, *G. flava*, and *G. tiliaefolia* have also been assessed using various spectroscopic techniques for the presence of phytochemicals. Lupeol (**16**), was isolated from CHCl₃ extract of the stem bark of *G. lasiocarpa* (Akwu *et al.*, 2020). Triterpenoids, namely taraxerol (**43**) and lupeol (**16**) were reported from acetone extract of the roots of *G. flava* (Mongalo *et al.*, 2022). Daucosterol (**42**), lupeol (**16**), and friedelin (**44**) (Figure 8) were isolated from MeOH extract of the leaves of *G. tiliaefolia* using column chromatography and characterized using UV, FTIR, NMR, and MS techniques (Dharmasoth *et al.*, 2022).

2.2.3 The genus *Rhus*. Various researchers have reported on the phytochemistry of different parts of *Rhus* species. Under this section, the qualitative and quantitative phytochemical investigations of *R. chinensis*, *R. leptodictya*, *R. flexicaulis*, *R. copallinum*, *R. coriaria*, *R. natalensis*, *R. verniciflua*, *R. ruspolii*, and *R. tripartite* are described. Some isolated compounds are depicted (Figures 9 and 10).

R. chinensis, *R. leptodictya*, and *R. flexicaulis* have been subjected to phytochemical investigations using screening methods, spectroscopic techniques, and chromatographic techniques. For instance, the preliminary phytochemical screening conducted on the water extract of *R. chinensis* fruit indicated the presence of secondary metabolites including alkaloids, flavonoids, tannins, triterpenes, and steroids (Sharma *et al.*, 2019). The phytochemical analysis of *R. leptodictya* leaves using *n*-hexane, DCM, acetone, and MeOH extracts was conducted. The results revealed the presence of phenols, tannins, saponins, and flavonoids in both acetone and MeOH extracts (Mtunzi *et al.*, 2017).

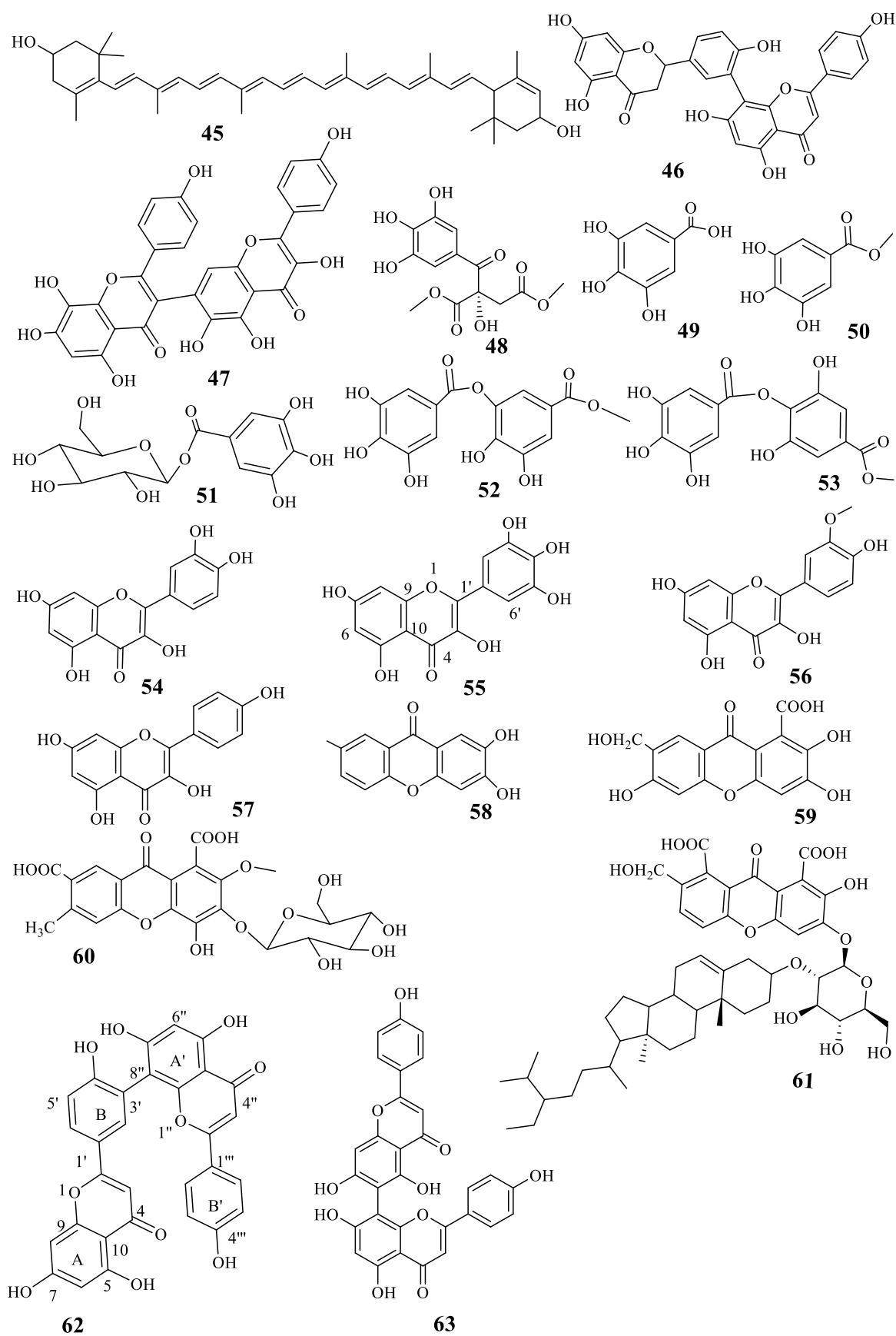


Figure 9. Some reported compounds from the genus *Rhus* (compounds 45-63)

In others' work, acetone extracts from the leaves of *R. leptodictya* were examined using bioautography techniques, revealing three major compounds. Two of these, lutein (**45**) and 2, 3-dihydro amentoflavone (**46**) (Figure 9), were isolated by using the combination of solvent-solvent fractionation and chromatographic techniques (Songca *et al.*, 2012). From the same solvent extract of the leaves of the plant, 5,5",6",7,8-pentahydroxy-2,2"-bis(p-hydroxyphenyl)-4H,4"H3,7"-bichromene-4,4"-dione (biflavonoid) (**47**) (Figure 9) was isolated and characterized using IR, NMR, and HRMS spectral data (Matamelab *et al.*, 2016). The identification and quantification of phenolic compounds were investigated from 80% MeOH extract of *R. flexicaulis* aerial parts using LC-MS, demonstrating that the total phenolic content was 30.31 mg/g and the flavonoid content was 20.93 mg/g (Abdel-Mawgoud *et al.*, 2019).

R. copallinum and *R. coriaria* have been subjected to various techniques to identify the presence of phytochemicals. Different compounds have been isolated from the MeOH extract of *R. copallinum* fruit using various chromatographic techniques and characterized using NMR and MS spectral data (Figure 9). The identified compounds were oleanolic acid (**18**), (*R*)-galloyl malic acid dimethyl ester (**48**), gallic acid (**49**), methyl gallate (**50**), glucogallin (**51**), methyl *m*-digallate (**52**), methyl *p*-digallate (**53**), quercetin (**54**), myricetin (**55**), rhamnazin (**56**), kaempferol (**57**), and betulinic acid (**39**) (Ma *et al.*, 2012). The chromatographic, UV-Vis, IR, and FAB-MS phytochemical investigations on EtOH extract of *R. coriaria* seeds have led to the identification of β -sitosterol- β -D-glucoside (**42**), 2,3-dihydroxy-7-methylxanthone (**58**), 2,3,6-trihydroxy-7-hydroxymethylenexanthone-1-carboxylic acid (**59**), 2-methoxy-4-hydroxy-7-methyl-3-O- β -D-glucopyranosylxanthone-1,8-dicarboxylic acid (**60**), and 2-hydroxy-7-hydroxymethylenexanthone-1,8-dicarboxylic acid 3-O- β -D-glucopyranosyl-(2'→3'')-3''-O-stigmast-5-ene (**61**) (Singh *et al.*, 2011) and as well as methyl gallate (**50**) amentoflavone (**62**), agathisflavone (**63**), and 1,2,3,4,6-penta-O-galloyl- β -glucopyranose (**64**) (Figures 9 and 10) were isolated from the EtOAc fraction of the 80% EtOH extract of the plant leaves using Sephadex LH-20 column chromatography, where their chemical structures were characterized using HPLC, MS, and NMR spectral data (Gok *et al.*, 2020). In addition, the study conducted on the fruit of the plant resulted in the isolation of catechin (**65**) and epigallocatechin (**66**) (Figure 10) using chromatographic techniques, and their identification was performed using IR and LC/MS/MS spectrometers (Ibrahim *et al.*, 2022).

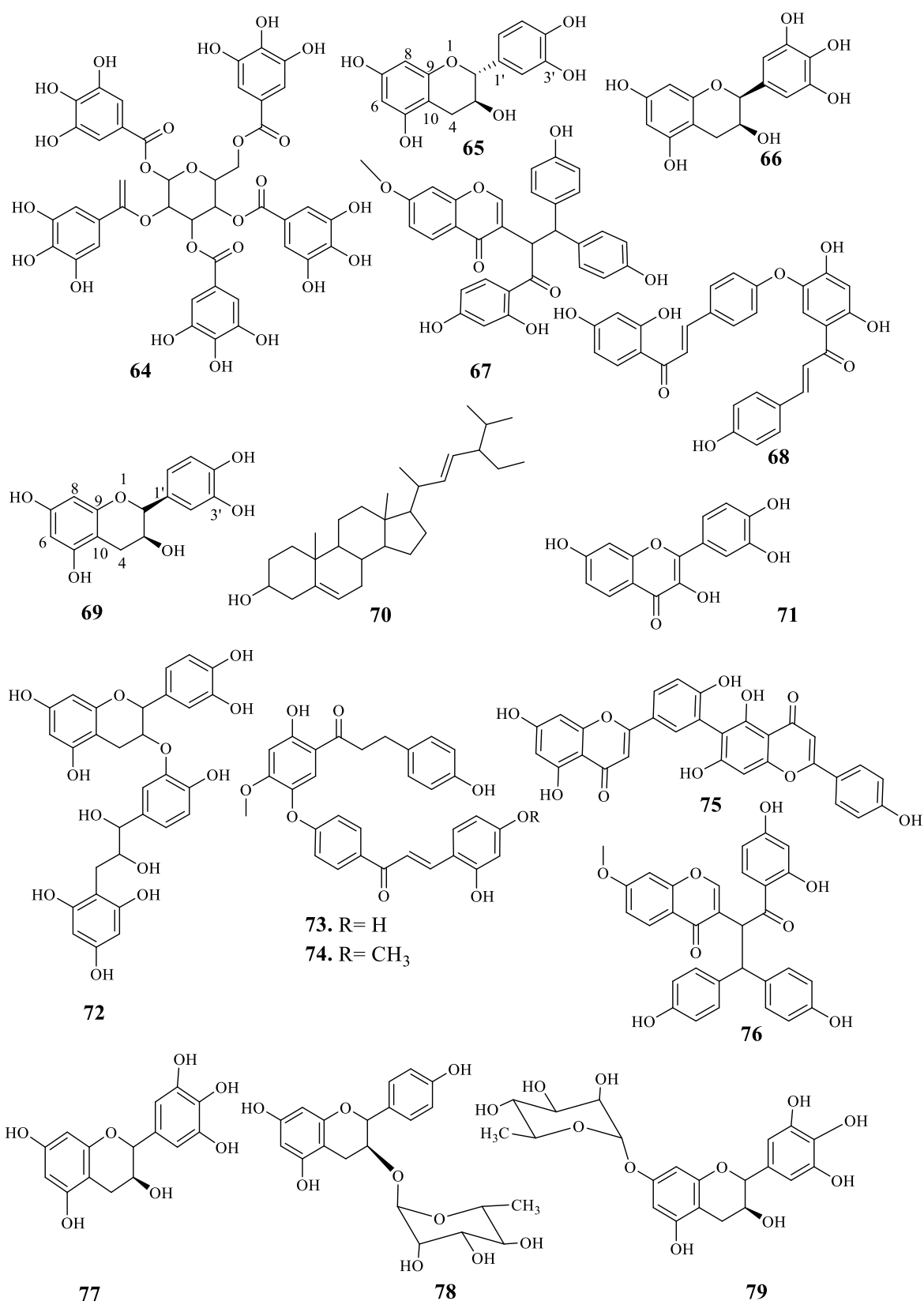


Figure 10. Some reported compounds from the genus *Rhus* (compounds **64-79**)

R. natalensis and *R. verniciflua* have been assessed using different spectroscopic and chromatographic techniques to report their phytochemical components. Phytochemical

studies on the EtOAc extracts of *R. natalensis* root bark led to the isolation of (3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromone-4-one (rhuschromone) (**67**) and 2',4'-dihydroxychalcone-(4-O-5''')-4'',2''',4'''-trihydroxychalcone (**68**). Their structure was established based on NMR and MS spectral data (Mwangi *et al.*, 2013). From the root bark of the same plant, (-)-epicatechin (**69**), β -sitosterol (**17**), 3- β -sitosterol glucoside (**42**), stigmasterol (**70**), and lupeol (**16**) were isolated from *n*-hexane, DCM, and EtOAc extracts and characterized using NMR and MS spectral information (Njoroge & Opiyo, 2019). Fisetin (**71**) was isolated from an EtOAc fraction of 50% EtOH extract of *R. verniciflua* seeds using chromatographic techniques and characterized using LC-MS, NMR, and HPLC spectral data (Kim & Huh, 2022).

The DCM: MeOH (1:1) extract of the root of *R. ruspolii* furnished five compounds (Deresa *et al.*, 2022), namely, catechin-(3'-O-6'')-2''',4''',6'''-benzotriol-(7''-hydroxylphenyl)-2'',3''-dihydroxylpropyl dimer (**72**), 2',4',4'',2'''-tetrahydroxy-4'''-methoxy-4-O-5'''-bichalcone (**73**), rhuschalcone I (**74**), robustaflavone (**75**), and (3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromone-4-one (**76**). Phytochemical investigation of 70% ethyl alcohol extract of *R. tripartite* leaves led to the isolation of β -sitosteryl-3-O- β -glucopyranoside (**42**) (Figure 8), quercetin (**54**), myricetin (**55**), galocatechin (**77**), Kampferol-3-O- α -L-rhamnopyranoside (**78**), and Kampferol-7-O- α -L-rhamnopyranoside (**79**) (Figure 10). The structure of these compounds was characterized using NMR and MS spectral data (Mohammed, 2015).

2.2.4 The genus *Olinia*. Some phytochemistry of the plants in the genus *Olinia* has been investigated. Here, some quantitative and qualitative phytochemical investigations from *O. emarginata*, *O. ventosa*, *O. radiata*, *O. rochetiana*, and *O. usambarensis* are described briefly. Structures of isolated compounds are displayed (Figure 11).

The cyanogenic glucoside prunasin (**80**) has been isolated from the aqueous phase of the acetone extract of leaves and twigs of *O. emarginata*. It was also identified and quantified in the leaves and stems of *O. ventosa*, *O. radiata*, and *O. rochetiana* (Nahrstedt & Rockenbach, 1993). The mandelic acid amide glucoside (**81**) was isolated from the leaves of *O. ventosa* and it also occurred in the leaves of *O. emarginata* (Nahrstedt & Rockenbach, 1993). From the *n*-BuOH extract of the root bark of *O. usambarensis*, three compounds were isolated, namely caffeic acid (**82**), 4-O- β -D-glucopyranosyl caffeic acid (**83**), and olinioside (**84**) (Nyandat *et al.*, 1993). The CHCl₃:MeOH (1:1) extract of the leaves of *O. usambarensis* gave caffeic acid (**82**), 4- β -D-glucopyranosyl caffeic acid (**83**), olinioside

(**84**), caffeoyloxy-5,6-dihydro-4-methyl-(2H)-pyran-2-one (**85**), 3-hydroxylup-12-en-28-oic acid (**86**) (Figure 11) (Deyou *et al.*, 2017a).

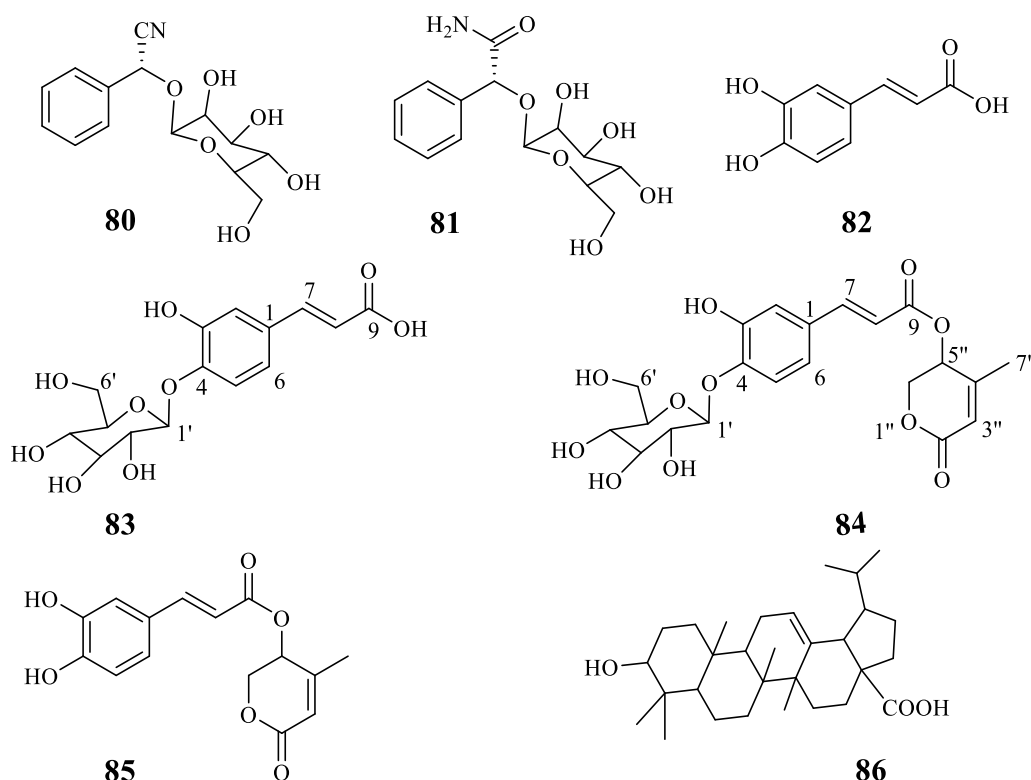


Figure 11. Some reported compounds from the genus *Olinia*

2.2.5 The genus *Rubus*. The phytochemistry of various *Rubus* species has been studied by different researchers. In this section, the qualitative phytochemical investigation of some *Rubus* species including *R. apetalu*, *R. caesius*, *R. croceacanthus*, *R. ellipticus*, *R. chingii*, *R. coreanus*, *R. fairholmianus*, *R. suavissimus*, *R. ulmifolius*, *R. corchorifolius*, and *R. idaeus* has been performed using different types of spectroscopic techniques are discussed. Some of the isolated compounds are depicted (Figures 12, 13, and 14).

R. apetalus and *R. caesius* have been subjected to investigation using phytochemical screening and spectroscopic methods. The preliminary phytochemical analysis using standard procedures carried out on *R. apetalus* indicated that the water extract of the leaves was rich in saponins, alkaloids, flavonoids, phenolics, tannins, phytosterols, and triterpenoids (Raghavendra *et al.*, 2018). Phytochemical analysis of the EtOAc fraction from 70% aqueous acetone extract of *R. caesius* leaves led to the isolation of nine phenolics (Figure 12) including quercetin (**54**), kaempferol (**57**) (Figure 9), quercetin 3-O- β -D-rutinoside (rutin) (**87**), kaempferol 3-O- β -D-glucuronide (**88**), quercetin 3-O- β -D-glucuronide (miquelianin, **89**), methyl brevifolincarboxylate (**90**), kaempferol 3-O- β -D-(6''-O-(E)-*p*-coumaroyl)-glucoside (tiliroside, **91**), pedunculagin (**92**), and ellagic acid (**93**)

(Figure 12) and structures were established based on spectroscopic data (Grochowski *et al.*, 2020).

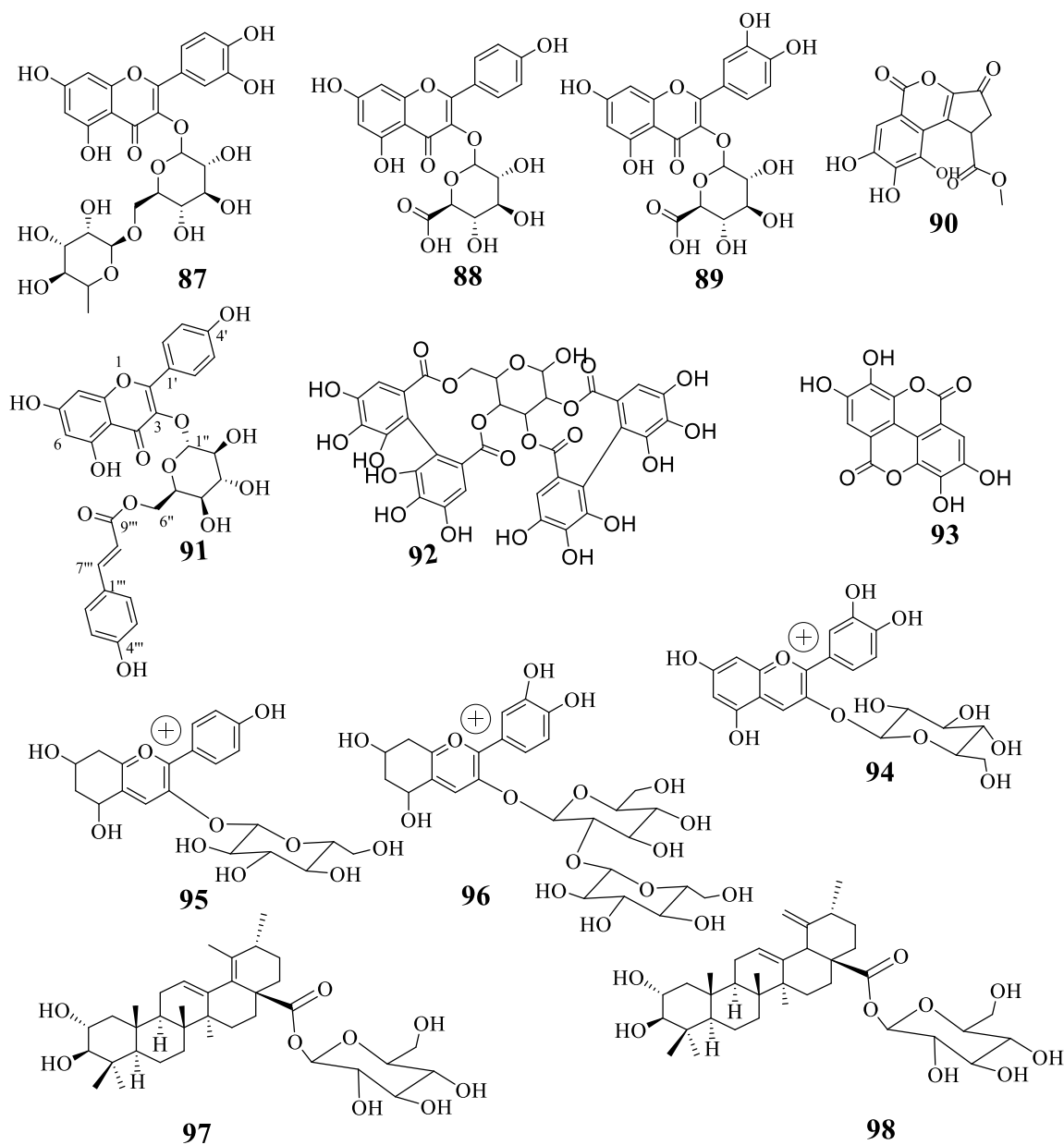


Figure 12. Some reported compounds from the genus *Rubus* (compounds **87-98**)

R. croceacanthus, *R. sieboldii*, *R. ellipticus*, and *R. chingii* have been subjected to quantitative and qualitative phytoconstituent evaluation. Anthocyanins from the fruits of *R. croceacanthus* and *R. sieboldii* were extracted using 80% MeOH containing 0.5% acetic acid, purified by preparative HPLC, and characterized by NMR and MS to yield cyanidin-3-O-glucoside (**94**) from *R. croceacanthus*; pelargonidin-3-O-glucoside (**95**) and pelargonidin-3-O-rutinoside (**96**) from *R. sieboldii* (Figure 12) (Kubota *et al.*, 2012).

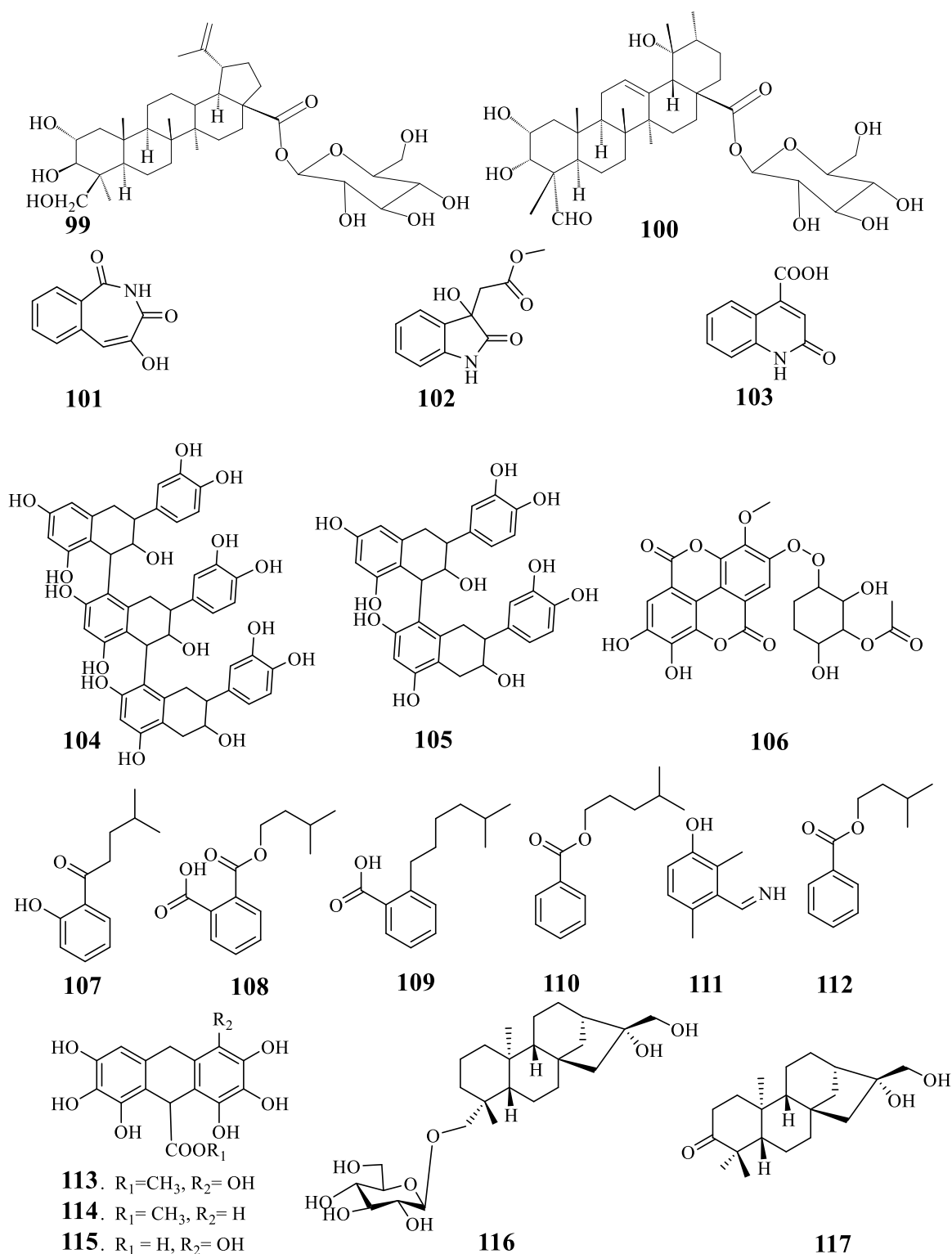


Figure 13. Some reported compounds from the genus *Rubus* (compounds **99**-**117**)

Triterpenoid saponins, including $2\alpha,3\beta$ -dihydroxyurs-12,18-dien-28-oic acid 28-O-D-glucopyranoside (**97**), $2\alpha, 3\beta$ -dihydroxyurs-12,19(29)-dien-28-oic acid 28-O-D-glucopyranoside (**98**), $2\alpha,3\beta,24$ - trihydroxylup-20(29)-en-28-oic acid 28-O-D glucopyranoside (**99**), and $2\alpha,3\alpha,19\alpha$ - trihydroxyurs-12-en-23-formyl-28-oic acid 28-O-D-

glucopyranoside (**100**) (Figure 12 and 13) were isolated from the roots of *R. ellipticus* MeOH extract. The structures were determined based on spectroscopic studies (Li *et al.*, 2023). Alkaloids were isolated and characterized from EtOAc and the *n*-BuOH fraction of EtOH extracts from fruit of *R. chingii* including rubusine (**101**), methyl dioxindole-3-acetate (**102**), and 2-oxo-1,2-dihydroquinoline-4-carboxylic acid (**103**) (Figure 13) (Ding, 2011).

R. coreanus, *R. fairholmianus*, *R. suavissimus*, and *R. ulmifolius* have been investigated using different spectroscopic techniques to identify their chemical constituents. Using UV-Vis, HPLC, and LC-MS, ellagic acid (**93**), procyanidin C (**104**), propelagonidin dimer (**105**), and methylellagic acid acetylpentose (**106**) (Figure 13) were isolated and characterized from 50% EtOH extract of the EtOAc fraction of the root of *R. coreanus* (Jang *et al.*, 2019). From the acetone extract of *R. fairholmianus* root (George *et al.*, 2014), 1-(2-hydroxyphenyl)-4-methylpentan-1-one (**107**), cis-2-(isopentyloxycarbonyl) benzoic acid (**108**), 2-(5-methylhexyl) benzoic acid (**109**), 4-methylpentyl benzoate (**110**), 3-(iminomethyl)-2,4-dimethylphenol (**111**), and 3-methylbutylbenzoate (**112**) (Figure 13) were isolated and purified using chromatographic techniques and characterized by UV-Vis, IR, NMR, and LC-ESI-MS. Purification of the CH₂Cl₂ fraction of the aqueous extract of leaves of *R. suavissimus* led to the isolation and characterization of β -sitosterol (**17**) (Figure 7) and stigmasterol (**70**) (Figure 10) (Chaturvedula & Prakash, 2012). From the MeOH extract of the aerial parts of *R. ulmifolius* Schott, three anthrones, rubanthrone A (**113**), rubanthrone B (**114**), and rubanthrone C (**115**) (Figure 13) have been isolated. Their structures were established by spectral procedures including NMR and FABMS techniques (Flamini *et al.*, 2002).

R. corchorifolius and *R. Idaeus* have been evaluated using various spectroscopic techniques to identify the phytochemical constituents. (16 α)-16,17,18-trihydroxy-ent-kauran-18-O β -D-glucoside (**116**), ent-16 β ,17-dialkyl-3-oxygen-kaurane (**117**), ent-kaurane-3 α ,16 β ,17-triol (**118**), ent-kaurane (5R,8S,9R,10R,13R,16R)-2-one-16 α ,17-diol (**119**), (16R)-16 β ,17,19-trihydroxy-ent-kaur-3-one (**120**) and ent-16 α ,17-dihydroxy-kauran-19-oic-acid (**121**) were isolated from the 80% EtOH extract and ethyl ferulate (**122**) (Figures 13 and 14) from EtOAc extract of *R. corchorifolius* leaves and characterized using NMR and MS spectrometers (Chen *et al.*, 2017, 2022). From the MeOH extract of *R. idaeus* leaves, sitosterol (**17**), olean-12-ene-3 β -hydroxy-28-oic acid (oleanolic acid) (**18**), urs-12-ene-3 β -hydroxy-28-oic acid (ursolic acid) (**41**), urs-12-ene-3 β -ol (α -amyrin) (**123**), urs-12-ene-3 β , 19 α -dihydroxy-28-oic acid (pomolic acid) (**124**), olean-12-ene-3 β , 29-diol (**125**),

urs-12-ene-3 β , 30-diol (**126**), , and olean-18-ene-1 β , 2 α , 3 α -triol (**127**) (Figure 14) were isolated using column chromatography and preparative TLC and characterized using FT-IR, NMR, and FAB MS (Vera *et al.*, 2006).

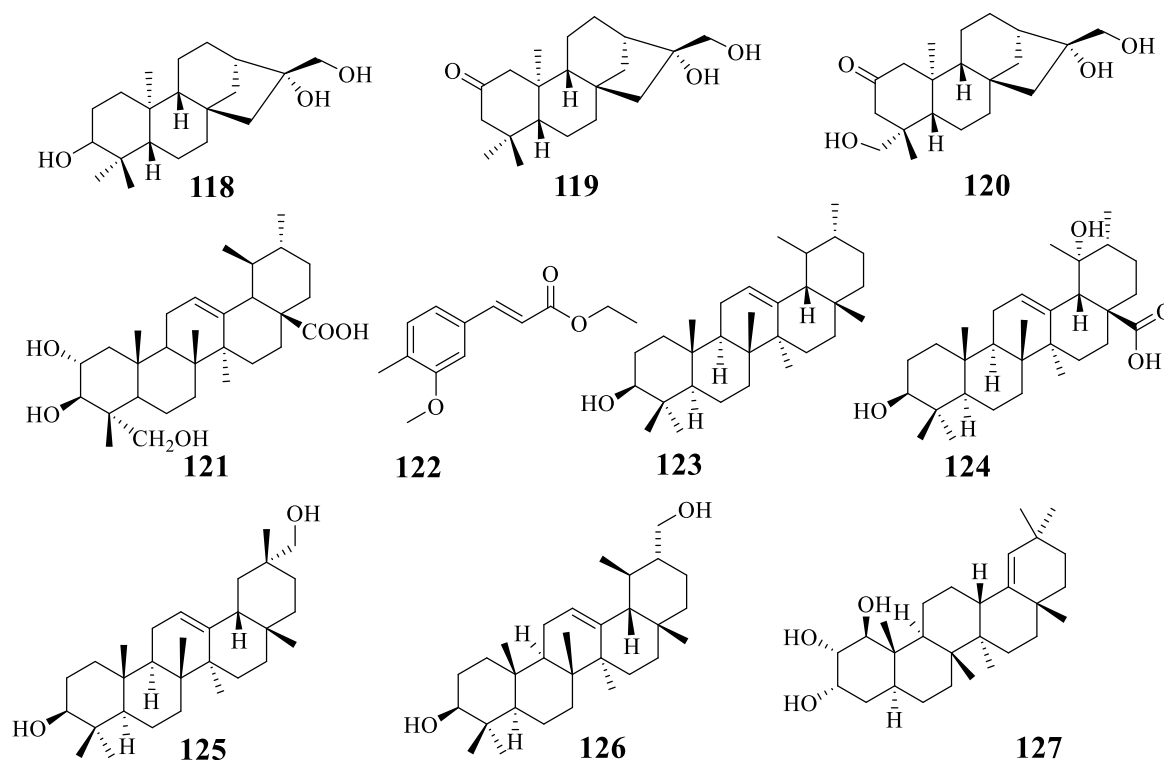


Figure 14. Some reported compounds from the genus *Rubus* (compounds **118-127**)

2.3 The Biological Activities of Selected Plants

2.3.1 *Clematis hirsuta*. *C. hirsuta* is one of the genus *Clematis* plants in the Ranunculaceae family. The genus includes about 300 species (Phillips, 2006). *C. hirsuta* has been reported to exhibit antibacterial and antioxidant effects. The chloroform (CHCl₃) extract of the leaves showed a higher inhibition zone against *P. aeruginosa* at 200 mg/mL (Habtamu & Mekonnen, 2017) compared to the methanol (MeOH) extract. However, the MeOH extract of the root showed a higher inhibition zone than the CHCl₃ extract at 1 mg/mL (Abdisa & Kenea, 2021) against the same strain of bacteria. The antibacterial activity of petroleum, CHCl₃, and ethanol (EtOH) extracts of the leaves of *C. hirsuta* was conducted against *S. aureus*, *S. pyogenes*, *E. coli*, and *K. pneumoniae* (Adane *et al.*, 2023). The results indicated that the CHCl₃ extract showed better inhibition zones (IZs) against *S. aureus* (19.0 mm), *S. pyogenes* (18.3 mm), *E. coli* (17.5 mm), and *K. pneumoniae* (17.3 mm) than the other test extracts compared to the standard antibacterial drug, gentamycin, with IZs of 29.5, 30.0, 27.5, and 27.0 mm, respectively. At 100 mg/mL, among the petroleum ether, CHCl₃, and EtOH extracts, the EtOH extract of the leaves of *C. hirsuta*

inhibited more DPPH radicals than the other extracts, which was confirmed by the absorbance value of 0.146 compared with 0.039 absorbance value of ascorbic acid at 519 nm (Ameya *et al.*, 2022). On the other hand, the DPPH radical scavenging ability test conducted revealed that at 2000 µg/mL, the MeOH extract of the root of the plant showed higher scavenging ability than standard ascorbic acid (Abdisa & Kenea, 2021), even though it exhibited lower scavenging activities at lower concentrations ranging from 125 to 1000 µg/mL.

Moreover, the biological activities of other *Clematis* species including *C. buchaniana*, and *C. simensis* have been reported. *C. simensis* has been evaluated for anti-inflammatory, antibacterial, and cytotoxicity assays. The leaf of *C. simensis* exhibited anti-inflammatory activities (Tadele *et al.*, 2009). An experimental study was carried out using the MeOH crude extracts of the leaves of *C. simensis* to evaluate the antibacterial and antioxidant activities. The MIC was conducted using the broth dilution method. The bacteria used in this study were *E. coli*, *S. typhi*, *S. aureus*, *K. pneumoniae*, *Citrobacter*, and *P. aeruginosa*. Among the test bacteria, *S. aureus* was found to be the most susceptible bacteria, followed by *P. aeruginosa*, with MIC values of 4.49 ± 0.29 and 6.16 ± 0.61 mg/mL, respectively (Birhan *et al.*, 2018). The cytotoxicity activities of MeOH extract of the leaves were performed using MTT assay on two breast cancer cell lines (JIMT-1 and MCF-7) and one normal breast epithelial cell line (MCF-10A) (Tuasha *et al.*, 2019). The results revealed that the IC_{50} of extracts against the two cancer cell lines was 0.27 ± 0.01 and 0.24 ± 0.04 and that of the normal cell was 0.17 ± 0.12 µg/mL. This suggested that the MeOH extract from the leaves of the plant showed cytotoxicity.

C. buchaniana has been investigated for antiinflammatory, antioxidant, and antidiabetic activities. For example, an anti-inflammatory activity test of the MeOH extract of aerial parts of *C. buchaniana* was carried out using the *in vitro* method of egg albumin denaturation. The result revealed that the MeOH extract of *C. buchaniana* reduces the denaturation of proteins with IC_{50} of 3.58 mg/kg when compared to that of the standard diclofenac, which was 2.14 mg/kg (Arora *et al.*, 2016). The DPPH scavenging activity of the EtOH extract of the leaf of the *C. montana* plant was reported to be stronger than the aqueous extract of the leaf of the plant (Singh *et al.*, 2015). According to Singh *et al.*, the percent of DPPH inhibited by 30 µg/mL of the EtOH extract of the plant was 26.2%, which is stronger than the aqueous extract of the plant, which was 22% when compared to the standard, ascorbic acid, 38%. In addition to antioxidant activity, Singh *et al.* investigated

the antidiabetic activity of the leaf extract of the plant. Again, the EtOH extract of the leaf of the plant revealed a more antidiabetic effect, which was checked 21 days after plant extract administration. The reduction of blood glucose levels in the blood of streptozotocin-induced diabetic rats were 193.3 ± 2.04 and 183.8 ± 3.79 mg/dl for aqueous and EtOH extract, respectively, compared to the standard that reduced the glucose level to 151.8 ± 1.59 mg/dl. Therefore, these reported data indicate that the genus *Clematis* is composed of phytoconstituents with a wide range of biological properties, including antibacterial, anti-inflammatory, antioxidant, and antidiabetic properties.

2.3.2 *Grewia ferruginea*. *G. ferruginea* is one of the plants in the genus *Grewia* of the Tiliaceae family containing nearly 321 species (The Plant List, 2013). *G. ferruginea* has been investigated for its antimicrobial and DPPH radical scavenging activities. For example, the antibacterial activity of the leaves of *G. ferruginea* was studied (Sileshi *et al.*, 2007) using the agar well diffusion method. The bacteria used were *S. aureus*, *S. pyogenes*, and *P. aeruginosa*. The result at 100 mg/mL of 80% MeOH extract demonstrated that all bacteria were susceptible, with IZs of 20.7, 17.3, and 17.7 mm, compared with the standard, tetracycline (0.1 mg/mL), with IZs of 41.7, 39.3, and 39.0 mm against *S. aureus*, *S. pyogenes*, and *P. aeruginosa*, respectively. In another study, the antibacterial activity test was conducted on the bark and leaf acetone extract of *G. ferruginea* using the disk diffusion method (Tura *et al.*, 2017) at 200 mg/mL against some isolates and reference bacteria including *E. coli*, *S. aureus*, *P. aeruginosa*, and *Salmonella* species. The results indicated that the IZs seen by the bark of the plant were 8.0, 9.0, and 9.7 mm against *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively, while the IZs seen by the leaves of the plant were 10.0, 9.7, and 9.0 mm against *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively. The DPPH free radical scavenging activity test conducted on the MeOH extract of the fruit of *G. ferruginea* indicated that, at 100 µg/mL, 84.6% of DPPH radicals were inhibited (Alene *et al.*, 2022).

Furthermore, the biological activities of different *Grewia* species including *G. tenax*, *G. tiliaefolia*, *G. lasiocarpa*, *G. flava*, and *G. optiva* have been assessed. *G. tenax* has been subjected to antibacterial evaluations. By using the agar well diffusion method, the antibacterial activities of the leaf extract of *G. tenax* were evaluated against *S. aureus*, *Enterococcus faecalis*, *Streptococcus epidermidis*, *S. pneumoniae*, *E. coli*, *Salmonella typhi*, *Serratia marcescens*, *K. pneumoniae*, *P. aeruginosa*, and *Proteus vulgaris* microorganisms using water, MeOH, petroleum ether, and isopropanol extractions. The result at 250 µg/mL showed that *E. faecalis* was susceptible to only water and petroleum ether extracts with IZs

of 11 mm and 13 mm, respectively. *S. epidermidis* was only inhibited by the isopropanol extract, resulting in an inhibition zone of 13 mm. *S. typhi* was inhibited by all extracts with IZs of 12, 14, 13, and 14 mm for water, MeOH, petroleum ether, and isopropanol extracts, respectively. *S. pneumonia* was inhibited only by the three extracts: water, MeOH, and petroleum ether, with IZs of 12 mm, 13 mm, and 13 mm, respectively. *P. aeruginosa* was susceptible to only MeOH and isopropanol extracts, with an inhibition zone of 13 mm for both extracts (Aadesariya, Gauni, *et al.*, 2017).

G. tiliaefolia has been investigated for its antioxidant effects. An evaluation of antioxidant activities conducted on the MeOH extract of the stem bark of *G. tiliaefolia* using DPPH free radicals showed that the extract was more potent with IC_{50} of 16.34 $\mu\text{g/mL}$ than the standard antioxidant, ascorbic acid, with IC_{50} of 19.33 $\mu\text{g/mL}$ (Ahamed *et al.*, 2010). *G. lasiocarpa* has been assessed for antibacterial, antioxidant, and cytotoxicity assays. For example, the antibacterial, antioxidant, and cytotoxicity assays of lupeol (**16**) isolated from *G. lasiocarpa* were studied (Akwu *et al.*, 2020). The antibacterial activities of lupeol (**16**) were evaluated using agar-well diffusion against six clinical bacterial isolates, including Gram-negative bacteria namely *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 314588), and *Salmonella typhimurium* (ATCC 14026) and Gram-positive bacteria including *S. aureus* (ATCC 25923) and methicillin-resistant *S. aureus* (MRSA) (ATCC BAA-1683), resulting in no antibacterial activities against all bacteria at 15-240 $\mu\text{g/mL}$ except in methicillin-resistant *S. aureus*. The antioxidant assays of lupeol (**16**) were performed using DPPH and ferric reducing antioxidant power (FRAP) indicating that the IC_{50} was greater than 1000 $\mu\text{g/mL}$ in both cases. Cytotoxicity activity of lupeol (**16**) was carried out using MTT assay against HEK293 (human embryonic kidney) and HeLa (cervical carcinoma), and MCF-7 (breast adenocarcinoma) cell lines, and the results demonstrated that the IC_{50} was greater than 1000 $\mu\text{g/mL}$ against both selected cell lines.

G. flava was evaluated for its antibacterial and anti-inflammatory activity using the isolated compounds. For example, the MIC of lupeol (**16**) isolated from acetone extract of the root of *G. flava* was evaluated using a microdilution assay showed that the MIC of lupeol (**16**) was 10 $\mu\text{g/mL}$ against all *Mycobacterium smegmatis*, *Mycoplasma hominis*, and *E. coli* compared to the MIC of the standard drug (Vancomycin) with MIC of 25, 13, and 100 $\mu\text{g/mL}$ against *M. smegmatis*, *M. hominis*, and *E. coli*, respectively (Mongalo *et al.*, 2022). In addition, the anti-inflammatory activity of taraxerol (**43**) and lupeol (**16**) was performed using soybean lipoxygenase (15-LOX) inhibition assay and lipopolysaccharide (LPS)-

activated RAW 264.7 macrophages inhibition assay (Mongalo *et al.*, 2022) resulted in the IC_{50} values of 19.96 ± 0.009 and 11.88 ± 0.04 mg/mL for taraxerol (**43**) and lupeol (**16**) against 15-LOX enzyme, respectively, whereas it resulted in IC_{50} values of 21.88 ± 0.02 and 14.2 ± 0.01 for taraxerol (**43**) (Figure 8) and lupeol (**16**) (Figure 7) against nitric oxide production in RAW264.7 cells, respectively compared to the standard drug (quercetin) yielded IC_{50} values of 31.55 ± 0.02 and 41.44 ± 0.004 mg/mL against 15-LOX enzyme and nitric oxide production by RAW264.7 cells, respectively.

G. optiva has been investigated for its antioxidant, anti-Alzheimer's, and antidiabetic properties. The antioxidative potentials of the 95% MeOH extract and ethyl acetate (EtOAc), $CHCl_3$, aqueous, and *n*-hexane fractions of roots of *G. optiva* were determined using DPPH and ABTS assays revealed that the concentration inhibited 50% of DPPH radicals (IC_{50}) were 75, 120, 184, 250, and 900 μ g/mL for MeOH extract, EtOAc, $CHCl_3$, aqueous, and *n*-hexane fractions, respectively compared to 45 μ g/mL IC_{50} value of ascorbic acid, whereas the concentrations inhibited 50% of ABTS were 88, 200, 250, 400, 1100 μ g/mL for MeOH extract, EtOAc, $CHCl_3$, aqueous, and *n*-hexane fractions, respectively compared to 64 μ g/mL IC_{50} value of ascorbic acid. The enzyme inhibitory potentials of the extract and fractions were determined against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), α -glucosidase and α -amylase (Zahoor *et al.*, 2020). The results indicated that the concentrations of MeOH extract, EtOAc, $CHCl_3$, aqueous, and *n*-hexane fractions that inhibited 50% of AChE were 120, 185, 290, 400, and 1000 μ g/mL, respectively, compared to 45 μ g/mL IC_{50} value of galanthamine used as a standard. In another way, the concentrations of MeOH extract, EtOAc, $CHCl_3$, aqueous, and *n*-hexane fractions that inhibited 50% of BChE were 100, 235, 255, 440, and 980 μ g/mL, respectively, compared to the 60 μ g/mL IC_{50} value of galanthamine used as the standard. Adding, Zahoor *et al.*, 2020, also, reported that the IC_{50} value of MeOH extract, EtOAc, $CHCl_3$, aqueous, and *n*-hexane fractions that inhibited α -glucosidase was 200, 380, 380, 390, and 1050, respectively compared with IC_{50} values of acarbose (80 μ g/mL) used as standard. Similarly, the IC_{50} value of MeOH extract, EtOAc, $CHCl_3$, aqueous, and *n*-hexane fractions that inhibited α -glucosidase was 320, 470, 485, 495, and 1150, respectively, compared to the IC_{50} value of acarbose (80 μ g/mL) used as a standard.

Moreover, from the EtOAc fraction of the 95% MeOH extract of *G. optiva* discussed in the above paragraph, β -sitosterol (**17**), 1, 2, 3 benzene triol (**37**), 7-O-methyl catechin (**38**), and betulinic acid (**39**) were isolated and subjected to antioxidant and antiAlzheimer's

activities (Bari *et al.*, 2019). The DPPH radical scavenging assay indicated that 7-O-methyl catechin (**38**) showed higher scavenging activity with IC_{50} value of 63 $\mu\text{g/mL}$ than β -sitosterol (**17**), 1,2,3 benzene triol (**37**), and betulinic acid (**39**), with IC_{50} values of 68, 95, and 90 $\mu\text{g/mL}$, respectively, compared to IC_{50} value of ascorbic acid, 35 $\mu\text{g/mL}$. Again, the ABTS scavenging activity test indicated that 7-O-methyl catechin (**38**) showed higher scavenging activity with IC_{50} value of 63 $\mu\text{g/mL}$ than β -sitosterol (**17**), 1, 2, 3 benzene triol (**37**), and betulinic acid (**39**), with IC_{50} values of 64, 85, and 105 $\mu\text{g/mL}$, respectively, compared to IC_{50} value of ascorbic acid, 35 $\mu\text{g/mL}$. In another way, the AChE and BChE inhibition assays indicated that the IC_{50} values of β -Sitosterol (**17**), 1, 2, 3 benzene triol (**37**), betulinic acid (**39**), and 7-O-methyl catechin (**38**) against AChE were 62, 68, 67, and 65 $\mu\text{g/mL}$, respectively, compared to the standard (galanthamine) with IC_{50} value of 40 $\mu\text{g/mL}$. These results were the same against BChE except for betulinic acid (**39**), with an IC_{50} value of 80 $\mu\text{g/mL}$. Therefore, it can be concluded that the plants in the genus of *Grewia* are known for their various phytochemical constituents with different biological properties, including antibacterial, antioxidant, antiinflammatory, antiAlzheimer's, and antidiabetic activities.

2.3.3 *Rhus ruspolii*. *R. ruspolii* is one of the plants in the genus *Rhus* of the Anacardiaceae family, with about 91 accepted scientific plant names (The Plant List, 2013). *R. ruspolii* has been reported for its antibacterial and antifungal properties. The root bark of *R. ruspolii* was investigated for antimicrobial activities using the disk diffusion method for antibacterial activities and the agar well diffusion method for the antifungal activity test. The test bacteria used were *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*, and for the antifungal activity test, *C. albicans*, *Penicillium notatum*, and *A. niger* were used. The plant extract used for the antibacterial test was 200 mg/mL, and for that of the antifungal test, 20 $\mu\text{g}/\mu\text{L}$. The results demonstrated that the most sensitive bacteria were *B. subtilis* to EtOAc extract with an inhibition zone of 14 ± 0.1 mm compared to the standard (sulphamethorazole) at 200 mg/mL with an inhibition zone of 24 ± 0.3 and the most sensitive fungus was *P. notatum* to EtOAc extract with 10 ± 0.2 mm compared positive control (kanamycin) 30 mg/disc with an inhibition zone of 22 ± 0.4 mm (Njoroge & Opiyo, 2019).

In addition, the biological activity of different *Rhus* species including *R. natalensis*, *R. abyssinica*, *R. leptoditya*, and *R. coriaria* are described. *R. natalensis* has been assessed for antibacterial and antifungal activities. The antibacterial and antifungal activities of (-)-

epicatechin (**69**), β -sitosterol (**17**), β -sitosterol glucoside (**42**), stigmasterol (**70**) and lupeol (**16**) isolated from the root bark of *R. natalensis* conducted against *S. aureus* (ATCC no. 25923), *Bacillus subtilis* (local isolate), *E. coli* (ATCC no. 25922), *P. aeruginosa* (ATCC no. 10622), *Candida albicans*, *P. notatum* and *Aspergillus nige* (Njoroge & Opiyo, 2019) and the results indicated that (-)-epicatechin (**69**) showed more activity against *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *E. coli* with IZs of 14 ± 0.2 , 15 ± 0.3 , 10 ± 0.2 , and 11 ± 0.3 mm, respectively than the other isolated compounds. Similarly, (-)-epicatechin (**69**) displayed stronger antifungal activities with IZs of 14 ± 0.3 , 12 ± 0.2 , and 12 ± 0.2 mm against *C. albicans*, *P. notatum*, and *A. niger*, respectively.

R. abyssinica and *R. leptoditya* have been investigated for their antioxidant and antibacterial activities. The results of antioxidant activity of the MeOH extract of leaves and stem of *R. abyssinica* performed using the DPPH radical assay indicated that the percentage of inhibition was 90.8 and 81.9%, respectively, compared to the positive control (propyl gallate) which showed radical scavenging activity of 91% (Sherfi *et al.*, 2016). An antibacterial investigation on the different extracts of *R. leptoditya* leaf was carried out against *S. aureus*, *S. pneumoniae*, *E. coli*, and *B. subtilis* using the MIC broth dilution method. The results indicated that *S. aureus* was more sensitive to MeOH extract with MIC 0.08 mg/mL than to dichloromethane (DCM) extract with MIC 0.63 mg/mL. *S. pneumoniae* was the most sensitive to MeOH extract, with MIC 0.04 mg/mL, among the tested bacteria (Mtunzi *et al.*, 2017).

R. coriaria has been studied for its antifungal, antibacterial, and antidiabetic effects. The leaves of *R. coriaria* were extracted with 80% acetone and fractionated into *n*-hexane, DCM, EtOAc, and *n*-butanol (*n*-BuOH). Then, various concentrations of these fractions were tested for their antifungal activities against *C. albicans*, *C. tropicalis*, *C. neoformans*, *A. niger*, *Penicillium chrysogenum*, *A. flavus*, *Trichophyton rubrum*, and *Candida krusei*. The MIC was determined using the broth microdilution method. The results indicated that the MIC values ranged from 39.3 to 500 μ g/mL suggesting that there is variation in activity among the fractions (Gabr & Alghadir, 2015). An experiment was conducted to investigate the antibacterial activity of MeOH, acetone, alcohol, and aqueous extracts from the fruit of *R. coriaria*, which was reported using MIC agar dilution methods. The tested bacteria were *P. syringae* and *Ralstonia solanacearum*. The results showed that *P. syringae* exhibited greater sensitivity to the MeOH extract with a MIC of 0.937 mg/mL compared to *R. solanacearum* with a MIC of 1.875 mg/mL (Rashid *et al.*, 2016).

In addition, the antidiabetic activities of 80% EtOH extract, petroleum, CHCl₃, EtOAc, *n*-BuOH, and aqueous fractions including compounds amentoflavone (**62**), agathisflavone (**63**), and 1,2,3,4,6-penta-*O*-galloyl- β -glucopyranose (**64**) isolated from the leaves of *R. coriaria* have been performed using α -glucosidase and α -amylase enzyme inhibition assays (Gok *et al.*, 2020). The results indicated that among the extracts and fractions, the EtOAc fraction showed better inhibitory activities against both α -glucosidase and α -amylase with IC_{50} values of 7.39 ± 0.29 and 20.81 ± 0.75 , respectively, compared with the standard (acarbose) with IC_{50} values of 0.08 ± 0.01 and 26.99 ± 0.80 . According to a report by Gok *et al.* (2020), among all isolated compounds, 1,2,3,4,6-penta-*O*-galloyl- β -glucopyranose (**64**) displayed better inhibitory activities against both α -glucosidase and α -amylase with IC_{50} values of 4.10 ± 0.10 and 6.32 ± 0.18 , respectively, compared with IC_{50} of standard (ascarbose) with IC_{50} value of 0.12 ± 0.01 and 10.69 ± 0.50 . Therefore, in this section, the review conducted on the biological activities of plants in the genus *Rhus* reveals that *Rhus* plants are rich in phytochemical constituents demonstrating a variety of biological properties, including antibacterial, antifungal, antioxidant, and antidiabetic activities.

2.3.4 *Olinia rochetiana*. *O. rochetiana* is a plant in the *Olinia* of the Penaeaceae family including only eight accepted species including *O. emarginata*, *O. huillensis*, *O. radiata*, *O. ruandensis*, *O. usambarensis*, *O. vanguerioides*, and *O. ventosa* (The Plant List, 2013). *O. rochetiana* has been studied for its antibacterial and antifungal properties. The EtOH extracts of *O. rochetiana* bark were investigated for antimicrobial activity against 20 bacteria and one yeast strain. The results were reported using MIC, which was determined by broth microdilution method. Of all tested microorganisms, the MIC values of *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albican* were greater than 512 μ g/mL, indicating that these microorganisms were not inhibited at less than or equal to 512 μ g/mL (Teka *et al.*, 2015). In another investigation, the antibacterial activity of MeOH extract of the bark of *O. rochetiana* extracted from 50 g/200 mL was conducted using the disk diffusion method against *S. aureus*, *E. coli*, *P. aeruginosa*, methicillin resistant *S. aureus*, *E. coli*, *K. pneumonia*, *Shigella sonnei*, *S. typhi*, *Citrobacter freundii*, and *E. faecalis* (Kimutaik *et al.*, 2020). The results demonstrated that the plant extract showed more activity against *P. aeruginosa* which had an inhibition zone of 20 mm compared to the positive control (gentamicin) with an inhibition zone of 23 mm.

Similarly, the antibacterial activities of the CHCl₃ and EtOAc extracts of leaves and stem barks of *O. rochetiana* have been studied against clinical strains of bacteria including

E. coli, *P. aeruginosa*, *S. typhi*, and *S. aureus* using the agar well diffusion method at a concentration of 500 mg/mL (Mekuriaw *et al.*, 2021). The results indicated that both CHCl_3 and EtOAc extracts of the leaves and the EtOAc extract of the stem bark of the plant showed stronger antibacterial activities against *S. aureus*, with IZs of 22.66 ± 1.15 , 22.66 ± 2.51 , and 19.00 ± 1.08 mm, respectively, compared with the standard antibacterial drug (chloramphenicol) with an inhibition zone of 31.66 ± 1.52 mm.

Moreover, the antimicrobial activities of petroleum ether, CHCl_3 , acetone, and MeOH fractions of 80% MeOH crude extract of the leaves of *O. rochetiana* were studied against *S. aureus*, *E. coli*, and *P. aeruginosa* bacteria, and *C. albicans* and *Trichophyton mentagrophyte* fungi using agar well diffusion techniques (Tadeg *et al.*, 2005). The results indicated that at 25 mg/mL, the petroleum ether fraction showed stronger activity than the other fractions, and the crude extract with IZs of 27 ± 0.3 , 21 ± 0.6 , 21 ± 2.3 , and 21 ± 1.9 mm against *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans*, respectively, whereas the CHCl_3 fraction showed stronger activity against *T. mentagrophytes* with an inhibition zone of 44 ± 3.6 mm. In another report, the MeOH extract of the *O. rochetiana* leaf was tested against *S. aureus*, *P. aeruginosa*, *K. pneumonia*, *E. coli*, *S. typhi*, *C. albicans*, *C. krusei*, *C. neoformas*, *T. mentagrophyte*, and *Microsporum gypseum*. The results revealed that the highest inhibition zone, 17 mm, was observed at 160 mg/mL against *S. aureus* compared with antibacterial standard discs containing ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), ciprofloxacin (10 μg), tetracycline (30 μg), amikacin (30 μg) and additional oxacillin (1 μg) for *S. aureus*, and antifungal standard discs containing fluconazole (Omari *et al.*, 2013).

Furthermore, the biological activities of the other plants in the genus *Olinia* have been reported. *O. ventosa* was investigated for antifungal activities. The antifungal activity of acetone, MeOH, *n*-hexane, and DCM leaf extracts of *O. ventosa* was determined using serial dilution techniques against *A. fumigates*. The MIC values after 24 hrs. incubation were 0.16, 0.32, 0.16, and 0.32 mg/mL for acetone, *n*-hexane, DCM, and MeOH, respectively, and were compared with amphotericin B. This indicates that acetone and DCM extracts have good bioactive components against the tested microorganism, *A. fumigates* (Mahlo *et al.*, 2013). In general, *Olinia* plants have been commonly reported for their phytochemicals exhibiting biological activities, including antibacterial and antifungal properties.

2.3.5 *Rubus apetalus*. *R. apetalus* is among the plants in the *Rubus* of the Rosaceae family containing 331 accepted species names (The Plant List, 2013). The plant has been

studied to report its antioxidant and antidiabetic properties. The antioxidant activity using the DPPH scavenging method was carried out on a water extract of the leaf of *R. apetalus*. The result revealed that the IC_{50} value was 22.49 $\mu\text{g/mL}$ and the standard (ascorbic acid) was 15.21 $\mu\text{g/mL}$ (Raghavendra *et al.*, 2018). In another report, the DPPH radical scavenging ability assay of 80% MeOH, acetone, and MeOH extract of the leaf of *R. apetalus* resulted in the IC_{50} values of 12.3, 8.8, and 8.4 $\mu\text{g/mL}$, respectively (Tadesse *et al.*, 2008). In another finding, using agar well diffusion, the antifungal activity against clinical isolate *C. albicans* conducted (Seshathri, 2012) on the DMSO extract of fresh twinges of *R. apetalus*, indicating that the inhibition zone to be 8.33 mm, comparable to that of the standard, fluconazole.

Furthermore, an evaluation of the antidiabetic and antioxidant activity of *R. apetalus* leaf aqueous extract in alloxan-induced diabetic rats has been conducted. The results of the acute toxicological study showed that the administration of the leaf extract by the oral route at doses up to 2000 mg/kg did not result in any mortality in experimental animals, and LD_{50} was more than 2000 mg/kg. Doses of 150 and 300 mg/kg body weight given orally for 21 days demonstrated significant antihyperglycemic, antihyperlipidemic, and antiatherogenic activities. This was evidenced by a significant decrease in total cholesterol, triglycerides, low-density lipoprotein, very low-density lipoprotein, and glycerol-free triglyceride levels. Additionally, there was an elevation of high-density lipoprotein cholesterol levels and a reduction of the atherogenic index in diabetic rats. Moreover, the extract significantly increased the activities and levels of superoxide dismutase, catalase, total thiols, and glutathione and decreased lipid peroxidation, showing antioxidant activity (Raghavendra *et al.*, 2019).

Moreover, the biological activity of the other plants in the genus *Rubus*, including *R. ellipticus*, *R. niveus*, *R. racemosus*, *R. rugosus*, *R. niveus*, *R. steudneri*, *R. coreanus*, *R. fairholmianus*, and *R. corchorifolius*, has been investigated. *Rubus* species, namely *R. ellipticus*, *R. niveus*, *R. racemosus*, and *R. rugosus* leaves, were evaluated for the presence of antibacterial and antifungal activities using disc diffusion methods. The bacterial strains used were *S. aureus*, *Staphylococcus epidermidis*, *P. aeruginosa*, and *E. coli*, and the fungal strains such as *A. flavus*, *C. albicans*, *C. krusei*, and *Trichoderma lignorum*. The results indicated that at 40 mg/mL of EtOH extract, the inhibition zone of the extract on all bacteria ranged from 13.5 to 27.5 mm compared to the standard (ampicillin) at 100 $\mu\text{g/mL}$, which ranged from 18.7 to 28.3 mm. The inhibition zone of the extract on fungal tests ranges from

13 to 22.5 mm compared to the standard (clotrimazole) at 10 µg/mL, ranging from 25 to 35 mm (Prasanth, 2017).

R. niveus, *R. steudneri*, and *R. coreanus* have been evaluated for their antioxidant and antibacterial activities. The 80% MeOH, acetone, and MeOH extracts prepared from the leaves of *R. niveus* and *R. steudneri* were investigated for their *in vitro* radical scavenging activities using the DPPH assay. The results demonstrated that the highest scavenging activity was observed in the 80% MeOH extract of *R. steudneri* with an IC_{50} value of 6.5 µg/mL, and the lowest was seen in 80% MeOH extract of *R. niveus* with an IC_{50} value of 19.0 µg/mL (Tadesse *et al.*, 2008). The antibacterial activities of the root of *R. coreanus* were evaluated on 50% EtOH extract, EtOAc, and acid treated EtOAc (aglycone) fractions using a disc diffusion assay against Gram-positive (*B. subtilis*, *P. acnes*, and *S. aureus*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*). The results indicated that except against *P. acnes*, the aglycone fraction showed more antibacterial activities than the 50% EtOH extract and EtOAc fractions against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa* within IZs of 15.51 ± 0.11 , 17.50 ± 0.50 , 18.17 ± 0.29 , and 18.08 ± 0.14 mm, respectively, compared with the results of the positive control (methylparaben) with IZs of 16.83 ± 0.29 , 17.67 ± 0.58 , 16.67 ± 0.58 , and 18.67 ± 0.58 mm, respectively (Jang *et al.*, 2019).

R. fairholmianus and *R. corchorifolius* were evaluated for their antioxidant and cytotoxicity activities. The DPPH radical scavenging activity evaluation was conducted on compounds isolated from *R. fairholmianus* root comprising 1-(2-hydroxyphenyl)-4-methylpentan-1-one (**107**), cis-2 (isopentyloxycarbonyl) benzoic acid (**108**), 2-(5-methylhexyl) benzoic acid (**109**), 4-methylpentyl benzoate (**110**), 3-(iminomethyl)-2,4-dimethylphenol (**111**), and 3-methyl benzoate (**112**), (Figure 13) indicated that the IC_{50} values were 3.23, 5.09, 10.76, 8.57, 12.35, and 9.43, respectively, compared to the positive control (quercetin) with an IC_{50} value of 4.12 (George *et al.*, 2014). Compounds, (16 α)-16,17,18-trihydroxy-ent-kauran-18-O- β -D-glucoside (**116**), ent-16 β ,17-dialkyl-3-oxygen-kaurane (**117**), ent-kaurane-3 α ,16 β ,17-triol (**118**), entkaurane(5R,8S,9R,10R,13R,16R)-2-one-16 α ,17-diol (**119**), (16R)-16 β ,17,19-trihydroxy-ent-kaur-3-one (**120**), and ent16 α ,17-dihydroxy-kauran-19-oic-acid (**121**) (Figure 14), were isolated from 80% EtOH extract of *R. corchorifolius* leaves and were subjected to MTT assay to evaluate their inhibitory effects against hepatocellular carcinoma cells (HepG2), breast carcinoma cells (MDA-MB-231), gastric cancer cells (SCG-7901), human ovarian cancer cells (OVCAR3), and human colon

cancer cells (HCT116). The results indicated that among all compounds, ent16 α ,17-dihydroxy-kauran-19-oic-acid (**121**), showed more inhibitory activities against the growth of all tested human cell cancers with IC_{50} of $34.20 \pm 1.2 \mu\text{M}$ (HepG2) $59.39 \pm 1.02 \mu\text{M}$ (MDA-MB-231), $54.00 \pm 2.10 \mu\text{M}$ (SCG-7901), $62.3 \pm 2.30 \mu\text{M}$ (OVCAR3), and $26.84 \pm 2.12 \mu\text{M}$ (HCT116) (Chen *et al.*, 2017). In addition, the ethyl ferulate (**122**) isolated from the EtOAc extract of the leaves of *R. corchorifolius* showed strong inhibitory activities against hepatocellular carcinoma cells (HepG2) with IC_{50} 26.4 μM (Chen *et al.*, 2022). In conclusion, the phytochemicals in *Rubus* plants are known for their various biological activities, such as antioxidant, antidiabetic, antibacterial, and antifungal properties.

2.4 Molecular Docking

Molecular docking is a computational technique utilized to predict the binding affinity of ligands to receptor proteins (Jain, 2004). Different docking methodologies (Raval & Ganatra, 2022) used various programs (software) that were developed based on different algorithms (ways of treating ligands) and scoring functions (types of attractive forces used to calculate binding energy) (Madhaviatha & Babu, 2019), including DOCK, FLOG, LibDock, SANDOCK, DOCK 4.0, FlexX, Hammerhead, SLIDE, eHiTS, ICM, QXP, Affinity, AutoDock, GOLD, DIVALI, DARWIN, AutoDock Vina, Discovery Studio, Surflex, AutoDock GOLD, Glide, MCDock, MOE-Dock, LeDock, rDock, ICM, Cdcker, LigandFit, FRED, and UCSF Dock. A recent report showed that of six programs evaluated, DOCK and AutoDock Vina were the top accurately performing programs for docking (Buckley *et al.*, 2023). The docking procedure involves sampling methods in which ligands and targets are prepared and subjected to docking (posing) to form different complex conformers and then comparing the results of these conformers using a scoring function in which the results are assessed (Das *et al.*, 2020; Kitchen *et al.*, 2004). Based on its purpose, docking can be conducted by using a predefined binding site or without knowing the binding site, known as blind docking (Che *et al.*, 2022; Hassan *et al.*, 2017; Jafar *et al.*, 2023). The binding site of the targets can be identified using different techniques, including software like Discovery Studio Visualizer (BIOVA, 2021) and online servers like PrankWeb (Jendele *et al.*, 2019).

In drug discovery, molecular docking is widely applied based on the principle of structure based virtual screening for hit identification, lead optimization for target enzymes (proteins), predicting ADMET, and toxicity properties of compounds (Chaudhary & Tyagi,

2024). *E. coli* DNA gyrase B (Cotman *et al.*, 2023), *Pseudomonas* quinolone signal A (PqsA) (Witzgall *et al.*, 2017), Pyruvate kinase M2 (PKM2) (Kung *et al.*, 2012), and human topoisomerase II β (Wu *et al.*, 2011) are among the target enzymes used in the search for new drug candidates using molecular docking.

E. coli DNA gyrase B is a topoisomerase II enzyme used to introduce a negative supercoil in DNA that is important for the local unwinding of bacterial DNA, which in turn allows the process of DNA replication, transcription, and recombination (Costenaro *et al.*, 2007). Inhibitors (drugs), such as ciprofloxacin, inhibit the normal process carried out by this enzyme, leading to the death of bacterial cells (LeBel, 1988). The strength to block this enzyme using inhibitors depends on the stability of the *E. coli* DNA gyrase B inhibitor complex expressed by a higher negative binding energy (Ivanova & Karelson, 2022). *P. aeruginosa* is an opportunistic, multidrug-resistant, gram-negative pathogenic bacterium capable of regulating its virulence factors through various intercellular signals known as quorum sensing signals. One of the quorums sensing signals is the *Pseudomonas* quinolone signal (PQS), which is synthesized with the assistance of an enzyme known as Pseudomonas quinolone signal A (PqsA) (Coleman *et al.*, 2008). Interfering with the functions of PqsA results in the absence of PQS synthesis, which in turn inhibits the expression of virulence factors by *P. aeruginosa* (Calfee *et al.*, 2001).

Pyruvate kinase enzyme (PKM2) is among the enzymes that cancer cells use to keep their replication. In addition to normal glycolysis (respiration), although it produces low ATP molecules, and, however, due to its high production rate, cancer cells prefer to utilize anaerobic glycolysis (fermentation) to produce additional ATP molecules to meet the energy demand required for highly proliferated cancer cells (Pfeiffer *et al.*, 2001). The shift from normal glucose metabolism to fermentation (lactate production) in cancer cells is facilitated by one of the isoforms of the pyruvate kinase enzyme, PKM2 (Mazurek, 2011; Zahra *et al.*, 2020). Inhibition of this enzyme, PKM2, blocks the glycolytic pathway in cancer cells, leading to the death of cancer cells (Park *et al.*, 2021). Human topoisomerase II β is one of the isoforms of topoisomerase IIA and is expressed at higher levels in tumors and proliferating cells than in normal cells (Linka *et al.*, 2007; Turley *et al.*, 1997). This enzyme solves DNA topological problems by cleaving both strands of a DNA duplex to form a cleavage complex through which another DNA segment can be transported (Wu *et al.*, 2011). However, the use of anticancer drugs such as etoposide (**128**) (Figure 15) increases

the number of stable topoisomerases II β -DNA complexes, which leads to blockage of DNA religation, and this results in the death of cancer cells (Wu *et al.*, 2011).

The results of each molecular docking study must be compared with the standard drugs. The mechanism of action of the standard drug used must be noted. Based on their mechanism of action, etoposide (**128**) and ciprofloxacin (**129**) (Figure 15) are used for *in silico* anticancer and antibacterial activity tests, respectively, in molecular docking studies. However, it is noted that ciprofloxacin targets both DNA gyrase (topoisomerase II) and topoisomerase IV (Drlica & Zhao, 1997), and this may result in the inconsistency of the result for ciprofloxacin observed in *in vitro* with the *in silico* antibacterial activity test, as the *in silico* antibacterial activity evaluation was performed against a specific enzyme. On the other hand, the correlation between the number of hydrogen bonds and the values of the docking score is also noted. Sometimes, a docking result containing more hydrogen bonding can be found with a low binding affinity. For example, the results reported for molecular docking studies conducted against *Plasmodium falciparum* hexose transporter 1 (PfHT1) protein demonstrated that the ligand protein complex with the highest number of hydrogen bonds was found with the lowest docking score (Owoloye *et al.*, 2022).

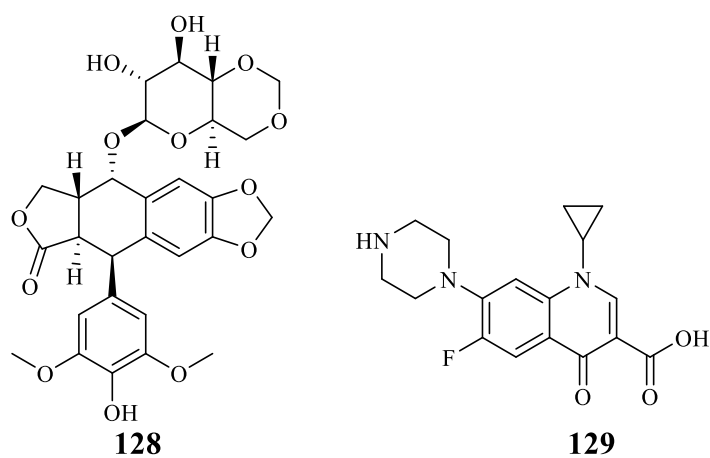


Figure 15. Structures of etoposide and ciprofloxacin

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 General Experimental Procedure

Petroleum ether, *n*-hexane, CHCl₃, DCM, EtOAc, MeOH, and EtOH, all of analytical grade, silica gel for column chromatography (mesh sizes 60-120 and 60-200), capillary tubes (75 mm in length and 1.1-1.2 mm in internal diameter), TLC (aluminum coated, 20 x 20 cm), and PTLC (glass coated, 10 x 10 and 20 x 20 cm) were purchased from the local market. An electrical grinder (capacity 200 g, China), an electronic balance (Model No: ESJ 200-4), an orbital shaker (Model No: VRN-200), a rotary evaporator (Model No: DW-RE-300), a PTLC chamber (10x10 and 20x20 mm), a fume hood (Model No: SW-TFG), a UV analyzer (Model No: UV4AC6/2), and a Clevenger apparatus were utilized in the Organic Chemistry laboratories of ASTU. The ¹H and ¹³C spectral data were recorded using 400.2 MHz and 100.6 MHz NMR spectrometers, respectively, on Bruker Biospin AG (Fällanden, Switzerland) and JNM-ECZ 400S (JEOL, Ltd. USA) in the Chemistry Department of Addis Ababa University, Swiss Federal Laboratories for Materials Science and Technology (Switzerland), University of Nairobi (Kenya), Kenyon College (USA), and University of Botswana (Botswana). The components of essential oils and nonpolar fractions were analyzed using an Agilent Technology 7890B CG-Agilent Technology 5977B single quadrupole MS (HP-5 column (30 m, 250 μm, 0.25 μm), EI, 70 eV, 50-550 m/z) in Organic Chemistry laboratory of ASTU. The UV-Vis spectrometers, including Model No: UV-1800, were utilized in the laboratories of the Organic Chemistry and Department of Applied Biology of ASTU. A Petri dish (diameter, 100 mm), a sterilizer (Model No: Lx-B75L, China), a workstation (Model No: NU-201-330E, USA), an incubator (Germany), and clinical isolate bacteria were used in antibacterial evaluation experiments in the laboratories of the Department of Applied Biology and the Institute of Pharmaceutical Sciences of ASTU.

3.2 Plant Material Collection, Identification, and Preparation

The roots of *C. hirsuta*, barks of *G. ferruginea*, leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus* were collected in July 2021 from Waldoro village, Homa Kebele (GPS

coordinates (Google, n.d.): 9°44'08.9"N, 37°16'06.2"E), Abbay Chomman Woreda, Horo Guduru Zone, Oromia region, which is about 300 km to the west of the capital, Addis Ababa, Ethiopia. The plants were identified with the help of available literature and authenticated with the voucher numbers TD05/2021, TD01/2021, TD03/2021, TD04/2021, and TD06/2021, respectively, by Mr. Melaku Wendaferash and deposited in the National Herbarium, Biology Department, Addis Ababa University, Ethiopia. Then, the collected plant materials were brought to the Organic Chemistry laboratory of Adama Science and Technology University, where they were allowed to dry in the open air at room temperature under shade. The dried plant material was then pulverized using an electric grinder and stored at 4 °C for extraction.

3.3 Extraction and Isolation

3.3.1 Extraction of essential oil. The essential oil components of dried powdered *G. ferruginea* bark were extracted using hydrodistillation with a Clevenger apparatus according to the procedure reported (Mejri *et al.*, 2014; Pavithra *et al.*, 2009). In detail, 50 g of powdered plant material was mixed with 400 mL of distilled water in a 1 L round bottom flask. Subsequently, the flask was heated using a heating mantle for six hrs. at a maximum of 100 °C to produce a distillate containing essential oil in the outlet of the Clevenger apparatus. The distillate was collected, and a DCM (15 mL x 3) was added. The DCM and the aqueous portion of the distillate were separated using a separatory funnel. The water residue in the DCM was removed using anhydrous sodium sulfate, and the DCM portion was filtered into a pre-weighed vial using filter paper and dried on a water bath to yield 15 mg (0.30%) of essential oil. Then, the oil was stored at 4 °C for antibacterial, antioxidant, and GC-MS analysis.

3.3.2 Extraction and isolation of compounds from *C. hirsuta*. The pulverized root of *C. hirsuta* (550 g) was successively extracted with *n*-hexane:EtOAc (1:1, 2.75 L) and MeOH (2.75 L) using maceration for 72 hrs (Al-Mansoub *et al.*, 2014) with occasional shaking on the orbital shaker. It was filtered and concentrated using a rotary evaporator at 40 °C to yield 4.5 g (0.82%) and 8.5 g (1.55%), respectively. After checking their TLC profile, both extracts (13 g, 2.37%) were combined. 10 g of the extract was adsorbed on 10 g of silica gel and subjected to silica gel column chromatography (size: 80 mm internal diameter by 650 mm length with 29/32 joint type) using 170 g of silica gel. The column was eluted with a gradient of *n*-hexane:EtOAc (1:0 to 0:1) and CHCl₃:MeOH (9.9:0.1 to 0:1). A

total of 305 fractions were collected with a volume of 10 mL, except fractions 1, 28, 44, 45, and 151 which were collected with a volume of 100 mL. Fractions 46, 51, 72-75, 94, 100-103, 142, 161, 253-254, 296-297, and 305 were collected with a volume of 200 mL.

Fractions 28-43 (230 mg) which were eluted using *n*-hexane:EtOAc (7:3) were further fractionated with the same eluent using silica gel column chromatography (size: 60 mm internal diameter by 360 mm length with 14/23 joint type) and 15 subfractions each with 15 mL were collected. Compound **188** (15 mg) was isolated from subfractions 11-12. Similarly, fractions 44-46 (330 mg), which were eluted with *n*-hexane:EtOAc (7:3), were eluted in isocratic mode using a silica gel column chromatography to collect 25 fractions, each 15 mL, and compound **145** (16 mg) was isolated from subfractions 3-6. Fractions 47-55 (165 mg), which were eluted using *n*-hexane:EtOAc (65:35), were fractionated in isocratic mode on silica gel column chromatography to collect 29 fractions, each with 10 mL, and compounds **70** and **17** (32 mg) were isolated from subfractions 10-11. Fractions 86-95 (330 mg) were eluted using *n*-hexane:EtOAc (1:9). Using the same solvent system, the fractions underwent elution in isocratic mode on a silica gel column chromatography to collect 37 subfractions, each with 10 mL. Subfractions 23-24 were further purified using PTLC to give compound **189** (18 mg) and a mixture of compounds **190** and **191** (30 mg).

3.3.3. Extraction and isolation of compounds from *G. ferruginea*. 4 L of DCM:MeOH (1:1) was used to extract 800 g of powdered *G. ferruginea* stem bark through maceration for 72 hrs (Al-Mansoub *et al.*, 2014) with occasional shaking on the orbital shaker. Then, the extract was filtered and concentrated at 40 °C using a rotary evaporator under reduced pressure to obtain about 112 g (14%) of extract. From the total extract, 100 g was suspended in water, and then CHCl₃, EtOAc, and *n*-BuOH were used subsequently for a liquid-liquid extraction process using aqueous:organic solvents (1:1) as described in the literature (El-Ashtoukhy & Fouad, 2015). The fractions were then concentrated at 40 °C on a rotary evaporator under reduced pressure to yield 5 g of CHCl₃, 13 g of EtOAc, and 20 g of the *n*-BuOH fractions.

The CHCl₃ fraction (4 g) was meshed with 4 g of silica gel and subjected to 160 g of silica gel column chromatography (mesh size 60-200) using a gradient eluent of petroleum ether:EtOAc (1:0 to 0:1) and then EtOAc:MeOH (9.9:0.1 to 7:3). In total, 165 fractions were collected, each 100 mL. Based on the TLC profile, fractions 1-8 (about 15 mg) exhibiting a poor TLC profile were discarded. Whereas fractions 9-39 (270 mg), 40-47 (56 mg), 48-57 (65 mg), 58-69 (406 mg), 70-82 (281 mg), 83-103 (212 mg), 104-112 (162

mg), 113–115 (138 mg), 116–120 (117 mg), 121–140 (353 mg), 141–146 (190 mg), 147–152 (64 mg), 153–157 (154 mg), and 158–165 (150 mg) were stored in preweighed vials for further analysis.

Fractions 58-69 (406 mg), which were eluted with *n*-hexane:EtOAc (19:1), formed white crystals at the bottom of the vial. The crystals were gradually cleaned with *n*-hexane to yield about 40 mg of compound **16**. Similarly, fractions 70–82 (281 mg), which were eluted using *n*-hexane:EtOAc (19:1 to 9:1), formed a white solid at the bottom and on the wall of the vial. Then, the crystals were gently washed with *n*-hexane. They were subsequently dissolved in MeOH for recrystallization. The supernatant was then removed using a dropper, and the crystals were collected to isolate about 60 mg of compounds **17** and **70**. Compound **17** was further purified for antibacterial and DPPH radical scavenging activity tests. Fraction 116-120 (117 mg), which was eluted with petroleum ether:EtOAc (7:3), was applied on PTLC to yield a mixture of compounds **192** and **193** (13 mg). Fractions 141-146 (190 mg), which were eluted using *n*-hexane:EtOAc (1:1 to 2:3), were dissolved in MeOH to form crystals. The supernatant was removed with a dropper to isolate about 45 mg of white crystals of compound **42**.

The EtOAc fraction (12 g) was meshed with 12 g of silica gel and subjected to 160 g of silica gel column chromatography using petroleum ether:EtOAc (1:0 to 0:1) followed by EtOAc:MeOH (9:1 to 4:1) as a gradient eluent to collect 121 fractions, each with 100 mL. After evaluating the TLC profiles of all fractions, fractions 1-4 (about 10 mg), fractions 14–16 (about 108 mg), and fractions 118–121 (about 3000 mg) exhibited a poor TLC profile and were each saved in pre-weighed vials. While fractions 5–13 (136 mg), fractions 17–30 (96 mg), fractions 31–37 (84 mg), fractions 38–45 (249 mg), 46–51 (598 mg), fractions 52–55 (80 mg), fractions 56–65 (428 mg), fractions 66–77 (251 mg), fraction 78 (1200 mg), fractions 79–83 (474 mg), fractions 84–93 (935 mg), fractions 94–98 (732 mg), fractions 99–108 (893 mg), and fractions 109–117 (1783 mg) were combined and stored in preweighed vials to use in further analysis. Fractions 52-55 (80 mg), which were eluted with *n*-hexane:EtOAc (3:7 to 1:9), were applied on PTLC using *n*-hexane:EtOAc (3:7) to isolate compounds **65** and **69** (15 mg).

3.3.4 Extraction and isolation of compounds from *R. ruspolii*. The powdered leaves of *R. ruspolii* (550 g) were extracted using DCM:MeOH (1:1, 2.75 Lx3) through maceration for 72 hrs (Al-Mansoub *et al.*, 2014) with occasional shaking on an orbital

shaker. After filtration, the extract was concentrated on a rotary evaporator at 40 °C to yield 38 g (6.9%) of deep brown extract. The extract (28 g) was adsorbed onto 28 g of silica gel and applied to column chromatography using 200 g of silica gel (mesh size 60-200). The column was eluted with a stepwise gradient of petroleum ether and EtOAc (1:0 to 0:1) followed by EtOAc and MeOH (98:2 to 0:1). A total of 153 fractions, each 100 mL, were collected. After checking the TLC profile of each collected fraction, fractions showing identical TLC spots were unified, and the solvent was removed under reduced pressure, resulting in the following fractions saved in preweighed vials: 1-5 (15 mg), 6-10 (1151 mg), 11-21 (389 mg), 22-36 (1765 mg), 37-45 (175 mg), 46-55 (659 mg), 56-60 (210 mg), 61-75 (867 mg), 76-69 (501 mg), 80-87 (574 mg), 88-98 (564 mg), 99-101 (356 mg), 102-105 (502 mg), 106-108 (343 mg), 109-111 (263 mg), 112-114 (398 mg), 115-130 (9732 mg), 131-133 (852 mg), 134-135 (563 mg), 136-141 (958 mg), 142-145 (1385 mg), 146-147 (2017 mg), and 148-153 (3580 mg). Fractions 1-5 (only 15 mg) with a poor TLC profile and fractions 22-36 (more chlorophyll based on the TLC profile under a 366 nm UV-lamp) were discarded, while all remaining isolates were subsequently subjected to further purification steps.

Fractions 6-10 were analyzed using GC-MS. Fractions 11-21 (389 mg), which were eluted using petroleum ether:EtOAc (85:15), were dissolved in MeOH to form a white crystalline solid, subsequently labeled as compound **145** (30 mg). Fractions 80-87 (574 mg), which were eluted using petroleum ether:EtOAc (7:3), were further purified by column chromatography using an isocratic mode of elution to collect 93 subfractions, each 10 mL. Sub-fractions 50-51 and 87-89 yielded compounds **194** (55 mg) and **55** (50 mg), respectively. An additional 15 mg of compound **55** was purified on silica gel chromatography for further analysis from subfractions 73-86 (200 mg). In the vial, fractions 88-98 (564 mg), which were eluted using petroleum ether:EtOAc (2:3), were dissolved in about 5 mL of MeOH and allowed to dry in the open air at room temperature. During the drying process, a yellow crystalline solid was formed on the wall of the vial, while the remaining portion of the sample was dried at the bottom of the vial. Subsequently, the sample dried at the bottom of the vial was carefully dissolved with drops of MeOH and using a dropper, then slowly transferred to another vial to recover approximately 350 mg of fractions 88-89. After the bottom part of the vial was cleaned very well in this manner, MeOH was used to collect the yellow solid sample from the wall of the vial, resulting in a mixture of compounds **195** and **62** (60 mg), and an additional 20 mg of compound **195** was

further purified using the same procedure from 350 mg of fractions 88-89 for its antibacterial and DPPH radical scavenging activities. From fractions 115-130 (9732 mg), which were eluted using EtOAc:MeOH (95:5), approximately 1200 mg was dissolved in MeOH to form crystals. The supernatant was transferred to another vial using a dropper to recover about 1100 mg of fractions 115-130. This was repeated until a clean white solid remained at the bottom of the vial to obtain compound **196** (45 mg). Subsequently, the 1100 mg of fractions 115-130 was applied to a silica gel column chromatography in an isocratic mode using EtOAc:MeOH (95:5) to yield a total of 87 subfractions with 10 mL each. After combining the subfractions based on their TLC profile and removing the solvents, subfractions 29-30 yielded a mixture of compounds **197** and **198** (55 mg), and an additional 21 mg of compound **197** was purified from subfractions 31-53 (147 mg) using the same procedure for the analysis of its melting point, antibacterial activity, and DPPH radical scavenging properties.

3.3.5 Extraction and isolation of compounds from *O. rochetiana*. The pulverized leaves of *O. rochetiana* (600 g) were extracted using DCM: MeOH (1:1, 3 L, 1 g in 5 ml) on maceration for 72 hrs (Al-Mansoub *et al.*, 2014) with occasional shaking on the orbital shaker. After filtration, the extracts were concentrated using a rotary evaporator at 40 °C to yield 104 g. Approximately 25 g of the extract was adsorbed onto 25 g of silica gel and subjected to column chromatography using 200 g of silica gel (60 to 200 mesh size). The column was eluted with a gradient of *n*-hexane and EtOAc (1:0 to 0:1) and EtAC and MeOH (95:5 to 1:1). A total of 200 fractions were collected containing a volume of 100 mL. Based on their TLC profile, fractions 1-3 (13 mg), 18-25 (454 mg), and 197-200 (280 mg) were discarded. Fractions 4-6 (195 mg), 7-17 (893 mg), 26-33 (127 mg), 34-81 (679 mg), 82-97 (1640 mg), 98-113 (739 mg), 114-125 (473 mg), 126-135 (640 mg), 136-150 (2540 mg), 151-160 (2633 mg), 161-165 (729 mg), 166-180 (3845 mg), 181 (498 mg), 182-189 (2110 mg), and 190-196 (4101 mg) were combined separately and saved in preweighed vials.

Fractions 4-17 were eluted using petroleum ether:EtOAc (9:1 to 85:15) and analyzed by GC-MS. In fractions 34-81 (679 mg), a white crystal was formed. The supernatant from these fractions was decanted, and the crystals were washed using *n*-hexane and MeOH to collect compound **41** (35 mg). Fractions 98-113 (739 mg) that were eluted using *n*-hexane:EtOAc (1:4) were subjected to silica gel column chromatography in isocratic mode to collect 87 fractions, each with 10 mL. Among these fractions, sub-fractions 35-66 (175 mg) were further purified using PTLC to yield compound **199** (27 mg). Fractions 136-150 (2633 mg) that were eluted with EtOAc:MeOH (95:5) were further subjected to silica gel

column chromatography using the isocratic mode to collect 120 fractions of 10 mL each. Sub-fractions 89-94 formed yellow crystals, resulting in mixtures of compounds **200**, **84**, and **83** (30 mg), while subfractions 95-103 formed white crystals, resulting in compound **83** (50 mg). An additional 15 mg of compound **200** was obtained through further purification on silica gel column chromatography for antibacterial, antioxidant, and melting point analysis.

3.3.6 Extraction and isolation of compounds from *R. apetalus*. *R. apetalus* leaves (610 g) were extracted using DCM:MeOH (1:1) through maceration for 72 hrs. (Al-Mansoub *et al.*, 2014). Following filtration, the extracts were concentrated on a rotary evaporator at 40 °C to obtain 27 g (4.4%) of deep brown extract. The extract (25 g) was meshed with 25 g of silica gel and subjected to silica gel column chromatography using 180 g of silica gel (mesh size 60-120). The column was eluted using a gradient solvent system starting from petroleum ether:EtOAc (1:0 to 0:1) and EtOAc:MeOH (98:2 to 0:1), resulting in 153 fractions, each 100 mL. After checking the TLC of each fraction, fractions 1-15 were discarded. Fractions 16-21 (1012 mg), 22-35 (1152 mg), 36-54 (5756 mg), 55-62 (183 mg), 63-71 (287 mg), 72-79 (138 mg), 80-87 (366 mg), 88-89 (1706 mg), 90-108 (844 mg), 109-119 (1675 mg), 120-135 (1453 mg), 136-149 (4539 mg), 150-152 (472 mg), and 153 (4452 mg).

Fractions 16-21 were analyzed using GC-MS. Fractions 22-35 (1152 mg), which were eluted using petroleum ether:EtOAc (85:15-75:25), gave white crystals. The supernatant was removed to afford a white crystal labeled as compound **201** (20 mg). The supernatant was dried (900 mg) and applied to silica gel column chromatography using the same solvent system (petroleum ether:EtOAc (85:15-75:25)) to collect a total of 115 subfractions, each 10 mL. Subfractions 48-86 yielded compound **145** (69 mg) and fractions 87-115 yielded compound **70** (67 mg). Fractions 109-119 (1675 mg), which were eluted using petroleum ether:EtOAc (3:7-0:1), were applied on column chromatography using the same solvent system to collect 75 subfractions, each in 10 mL, and among which subfractions 29-32 afforded compound **91** (51 mg). Similarly, fractions 120-135 (1453 mg), which were eluted using petroleum ether:EtOAc (0:1), were applied on column chromatography in the isocratic mode, resulting in a total of 138 subfractions, each 10 mL, of which subfraction 125 yielded compound **202** (38 mg). An additional 15 mg of compound **202** was purified using silica gel chromatography for antibacterial and DPPH radical scavenging activities, and melting point analysis.

3.4 GC-MS Analysis

The essential oil components extracted using hydrodistillation and nonpolar combined fractions 6-10 leaves of *R. ruspolii*, fractions 4-17 leaves of *O. rochetiana*, and fractions 16-21 leaves of *R. apetalus* were analyzed using GC-MS following the procedures used in the literature (Dinku *et al.*, 2019) with little modification. In detail, about 0.5 μ L of 200 ppm of the essential oil was injected into a GC-MS. The GC injector temperature was set to 250 °C with a splitless mode. The carrier gas was helium with a flow rate of 1 mL/min. The oven temperature program was 40 °C for 5 min and increased to 250 °C at a heating rate of 6 °C/min and finally held isothermally for 20 min to reach a total run time of 60 min. Using the same conditions, a mixture of *n*-alkane series (C7-C30) was injected into the GC-MS (Appendix 1e) to calculate the retention index (RI) of each chromatogram as follows (Equation 1):

$$RI_x = 100n + 100 \left[\frac{t_x - t_n}{t_{n+1} - t_n} \right] \text{-----Equation 1,}$$

where 'x' is unknown chromatogram, ' t_x ' is the retention time (RT) of chromatogram 'x', ' t_n ' is RT of *n*-alkane eluting immediately before chromatogram 'x', ' t_{n+1} ' is RT of *n*-alkane eluting immediately after chromatogram 'x'.

The W11N17 library that was loaded onto the device was used to identify the sample components, and the RI was compared to the RI indicated in the PubChem online database (Kim *et al.*, 2023). Based on the peak area, the quantities of each component were determined.

3.5 NMR Experiments

A 5 mm Cry-oProbeTM Prodigy probe equipped with a z-gradient with 90° pulse lengths of 11.4 μ s (¹H) and 10.0 μ s (¹³C) was used to apply Bruker standard pulse programs and parameter sets to obtain all 1D ¹H and ¹³C NMR spectra as well as the 2D correlated NMR data (¹H-¹H DQF-COSY, ¹H-¹³C HSQC, and ¹H-¹³C HMBC) at 298 K. The MeOH resonance at δ = 3.31/49.0 ppm, the DMSO resonance at δ = 2.49/39.5 ppm, and the CHCl₃ resonance at δ = 7.26/77.0 ppm were utilized for calibrating ¹H and ¹³C NMR chemical shifts (δ) in ppm.

3.6 In vitro Antibacterial Activities Evaluation

3.6.1 Disk diffusion method. The antibacterial activity evaluation was conducted using the agar disk diffusion method against two Gram positive bacteria (*S. aureus* ATCC 25923 and *S. pyogenes* ATCC 19615) and two Gram negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) using the standard procedure (Hudzicki, 2009). In detail, 38 g Mueller-Hinton agar (MHA) medium was dissolved in 1 L distilled water and heated to dissolve completely and allowed to cool. After sterilizing using an autoclave at 121°C for 15 min, 25 mL of the medium was dispensed into a 100 mm Petri dish to a depth of 4 mm and allowed to be solidified at room temperature and stored at 4 °C. The test bacteria were diluted in sterile Muller Hinton broth to adjust the turbidity to 0.5 McFarland BaCl₂ standard solution to get a 1.5×10^8 cfu/mL bacteria suspension. Within 15 min, each inoculum was inoculated on a Petri dish using a sterile swab. A sterile 6 mm disc made of filter paper was saturated with 20 μ L (in DMSO) of 100, 50, and 25 mg/mL of each extract and with 5, 2.5, and 1.25 mg/mL of essential oil and each isolated compound and then placed on the surface of the Petri dish. The Petri dish was incubated at 37°C for 24 hrs. The diameter of the zone of inhibition was measured. The extent of activity of the samples was compared with a sterile 6 mm disc saturated with 20 μ L (10 μ g) of 0.5 mg/mL ciprofloxacin.

3.6.2 Minimum inhibitory concentration (MIC) of extracts. The agar dilution method was used to evaluate the MIC of the MeOH and n-hexane:EtOAc (1:1) extracts using the procedure (Ohikhena *et al.*, 2017; Wiegand *et al.*, 2008) with some modifications against the selected bacteria. The MHA media was prepared as described above. A stock solution of 100 mg/mL was prepared by dissolving 400 mg of each extract in a small amount (2 mL) of DMSO. Afterward, it was adjusted to the final volume of 4 mL using the MHA solution and serially diluted to the working solution of 50, 25, 12.5, 6.25, and 3.125 mg/mL in the MHA solution. About 500 μ L of each concentration was distributed into 100 mL labeled sterilized beakers and mixed with 24.5 mL MHA media solution. The mixtures were then poured into a Petri dish labeled with the respective concentration of extracts. Following that, the plate was allowed to dry at room temperature. Approximately 5 μ L of suspension (density adjusted to 0.5 McFarland turbidity units, 1 to 2×10^8 cfu/mL) was transferred as a spot on each plate. The plates were incubated at 37 °C for 24 hours. The lowest concentration that completely inhibits visible growth, as judged by the naked eye, was taken as MIC. Agar plates without test extract and with standard antibiotics, ciprofloxacin (0.0625, 0.03125, and 0.015625 mg/mL), were used as a negative and positive control, respectively.

3.7 DPPH Radical Scavenging Activities

The antioxidant activity of the extracts, fractions, essential oil, and isolated compounds was assessed using DPPH radical according to the procedure reported (Grujic *et al.*, 2014). 4 mg of DPPH was dissolved in 100 mL of MeOH to obtain 40 µg/mL and then stored in a dark bottle at 4 °C. All samples were separately dissolved in MeOH to prepare 1000 µg/mL. Each test sample was serially half diluted in MeOH to give 500, 250, 125, and 62.5 µg/mL. Then, 1 mL of each was mixed with 1 mL of DPPH. After heating for 30 min in an oven at 37 °C, the absorbance was measured using a UV-Vis spectrometer at 517 nm, with MeOH as a blank. The percentage (%) of DPPH inhibited by the samples was calculated as follows (Equation 2):

$$\% \text{DPPH inhibition} = \left[\frac{A_c - A_s}{A_c} \right] \times 100\% \text{ ----- Equation 2,}$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample. The IC_{50} was calculated from the regression line (Khelifi *et al.*, 2011). The DPPH radical scavenging activities of samples were compared with ascorbic acid, which was used as a positive control.

3.8 *In silico* Molecular Docking Studies of the Isolated Compounds

The procedure described in the literature (Rizvi *et al.*, 2013) was followed to model the docking of isolated compounds with various model proteins. ChemDraw 22.0 was used to sketch the compound structures, which were then stored in the .mol file format. The data were then saved in the .pdb file format and optimized by Discovery Studio Visualizer 12.1 (BIOVA, 2021). The model protein structures of *E. coli* DNA gyrase B (PDB ID: 7P2M), PqsA (PDB ID: 5OE4), pyruvate kinase M2 (PKM2; PDB ID: 4G1N), and human topoisomerase II β (PDB ID: 3QX3) were obtained and stored in a .pdb file format from the web database, protein data bank (Berman, 2000). The active (binding) sites were then located, and the proteins were cleaned using the Discovery Studio Visualizer program as well. Using AutoDock Vina along with auxiliary programs including MGLtools 1.5.6 and Python 3.10.9, docking simulations were performed. By default, nine conformers were used for each run. To see the interactions between the ligand and target as 2D and 3D structures, from all conformers, the conformer showing the best docking score (least binding affinity) was chosen to be examined. The standard antibacterial agent ciprofloxacin, the standard anticancer agent etoposide (a topoisomerase II inhibitor), and the anticancer agent PKM2-IN-1 (a pyruvate kinase M2 inhibitor) were used as positive controls in simulations for comparison.

3.9 *In silico* Druglikeness and Toxicity Study of the Isolated Compounds

The canonical simplified molecular-input line-entry systems (SMILESs) of the isolated compounds were obtained from the PubChem database. Compounds that do not have SMILES were sketched with ChemDraw 22.0 and stored as .mol files. Then, using Discovery Studio 12.1 software, the structures were transformed into a SMILES file and submitted to the SwissADME online tool to estimate their physicochemical properties and drug-likeness (Lipinski rule) (Daina *et al.*, 2017). To estimate oral acute toxicity, organ toxicity, and toxicity endpoints, the SMILES files of the test compounds were uploaded to the Pro Tox 3.0 online tool (Banerjee *et al.*, 2024). PKM2-IN-1, etoposide, and ciprofloxacin data were also generated for comparison purposes.

CHAPTER FOUR

4. RESULTS AND DISCUSSIONS

4.1 Phytochemicals Analysis of the Plants Using GC-MS and NMR

In this section, the results of essential oil from the stem bark of *G. ferruginea*, combined fractions 6-10 of *R. ruspolii*, fractions 4-17 of *O. rochetiana*, and fractions 16-21 of *R. apetalus* leaves analyzed using GC-MS are discussed. In addition, characterization of isolated compounds from the root of *C. hirsuta*, from the stem bark of *G. ferruginea*, and the leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus* are presented.

4.1.1 GC-MS analysis of essential oil. About 48 components were identified from the essential oil extracted from the dried stem bark of *G. ferruginea* based on the GC-MS spectral data (Appendix 1a). From these, 32 (91.80%) compounds were identified (Table 1) by comparing their RI and mass fragmentation forms with reported data (Kim *et al.*, 2023). As described in Table 1, the major compound was methyl palmitate (**144**, 45.17%), followed by diisooctylphthalate (**158**, 7.64%), methyl stearate (**150**, 7.55%), methyl 13-octadecenoate (**148**, 7.06%), palmitic acid (**145**, 5.11%), methyl eicosanoate (**153**, 3.01%), and 6,10,14-trimethyl-2-pentadecanone (**142**, 2.14%). The mass spectrum and proposed mass fragmentation of the major compound (methyl palmitate, **144**) are shown in Appendix 1a and Figure 16, respectively. Of all components, six of them (4.6%, Figure 16) were classified as terpenes, including 6,10,14-trimethyl-2-pentadecanone (**142**, 2.14%), 4,8,12,16-tetramethylheptadecan-4-olide (**154**, 1.10%), 5,9,13,17-tetramethyl-4,8,12,16-octadecatetraenoic acid (**160**, 0.44%), neophytadiene (**141**, 0.38%), 3-methyl-5-methylene-2(5H)-furanone (**132**, 0.28%), and ginsenoside (**161**, 0.26%). In another report, from a plant of the same genus, the main components of the essential oil from the leaves of *G. lasiocarpa* have been reported to be phytol (22.6%), α -farnesene (8.62%), palmitic acid (**145**, 7.62%), and farnesol (4.61%). On the contrary, the major components from the stem bark of *G. lasiocarpa* have been reported to be 2-methylheptadecane (7.24%), heptacosane (7.60%), and 2,6,10,14-tetramethyl heptadecane (7.30%) (Akwu *et al.*, 2021).

Furthermore, some of the biological activities of these components have been reported. For example, diethyl phthalate (**137**) has been reported to show antibacterial properties (Premjanu & Jaynthy, 2014). On the other hand, essential oils containing 6,10,14-

trimethyl-2-pentadecanone (**142**) as a major component have been reported to show anti-diabetic (Bahadori *et al.*, 2017) and antimicrobial (Morteza-Semnani *et al.*, 2012) activities. In addition, the *Xestospongia testudinaria* sponge MeOH extract of the CHCl₃ fraction with 2.2% of 6,10,14-trimethyl-2-pentadecanone (**142**), 50.2% of palmitic acid (**145**), and 1.4% of 4,8,12,16-tetramethylheptadecan-4-olide (**154**) has been reported to show anticancer properties (Swantara *et al.*, 2019).

Table 1. Components of the essential oil of *G. ferruginea* stem bark identified using GC-MS data

No	Compound name	RT	RI		Peak Area (%)
			Calculated	Reported	
130	sulfinyl-bis-(methane)	7.77	844.7	829.2	1.59
131	pentanoic acid	12.26	980.8	-	0.38
132	3-methyl-5-methylene-2(5H)-furanone	13.30	1015	-	0.28
133	nonanal	15.71	1099.6	1099	0.24
134	(2E)-2-decenal	19.66	1256.2	1256	0.27
135	1-undecen-10-al	21.99	1357.4	1313	0.44
136	1-dodecanol	26.69	1582.4	1487	0.24
137	diethyl phthalate	26.83	1589.5	1589.2	0.95
138	methyl myristate	29.18	1714.4	1714	1.07
139	(2E)--nonadecene	30.38	1781.1	-	0.23
140	methyl 13-methylmyristate	30.96	1814.1	1779.1	0.27
141	neophytadiene	31.19	1827.5	1827	0.38
142	6,10,14-trimethyl-2-pentadecanone	31.30	1834	1835	2.14
143	butyl undecyl phthalate	31.75	1860.3	-	0.29
144	methyl palmitate	32.67	1914.7	1915	45.17
145	palmitic acid	33.24	1949.7	1950	5.11
146	ethyl palmitate	33.74	1980.4	1980	0.52
147	methyl heptadecanoate	34.26	2012.9	2013	0.97
148	methyl 13-octadecenoate	35.42	2087.7	2098	7.06
149	methyl (9Z)-octadecenoate	35.50	2092.8	2095	0.92
150	methyl stearate	35.80	2112.7	2113	7.55
151	methyl 16-acetylhydroxypalmitate	35.94	2122.1	-	0.26
152	(9E)-octadecenoic acid	38.29	2283.3	-	0.38
153	methyl eicosanoate	38.70	2312.9	2313	3.01
154	4,8,12,16-tetramethylheptadecan-4-olide	39.10	2341	2364.2	1.10
155	(9Z)-pentadecenol	39.17	2346.7	-	1.18
156	methyl 18-methyleicosanoate	40.07	2411.6	2401.1	0.36
157	methyl docosanoate	41.55	2511	2511	0.67
158	1,2-diisooctylphthalate	41.91	2532.4	2525	7.64
159	methyl tetracosanoate	45.56	2712.3	2712	0.45
160	5,9,13,17-tetramethyl-4,8,12,16-octadecatetraenoic acid	48.30	2811.1	-	0.44
161	ginsenosol	49.83	2855.9	-	0.26

Similarly, methyl palmitate (**144**) has been reported to demonstrate antibacterial (Shaaban *et al.*, 2021) and antiinflammatory (Saeed *et al.*, 2012) activities. In addition, an

EtOH extract of *Zea mays* cob containing 1.0% of methyl palmitate (**144**), 0.6% of palmitic acid (**145**), and 0.4% of 5,9,13,17-tetramethyl 4,8,12,16-octadecatetraenoic acid (**160**) has been identified for its DPPH radical scavenging activities (Bhart *et al.*, 2023).

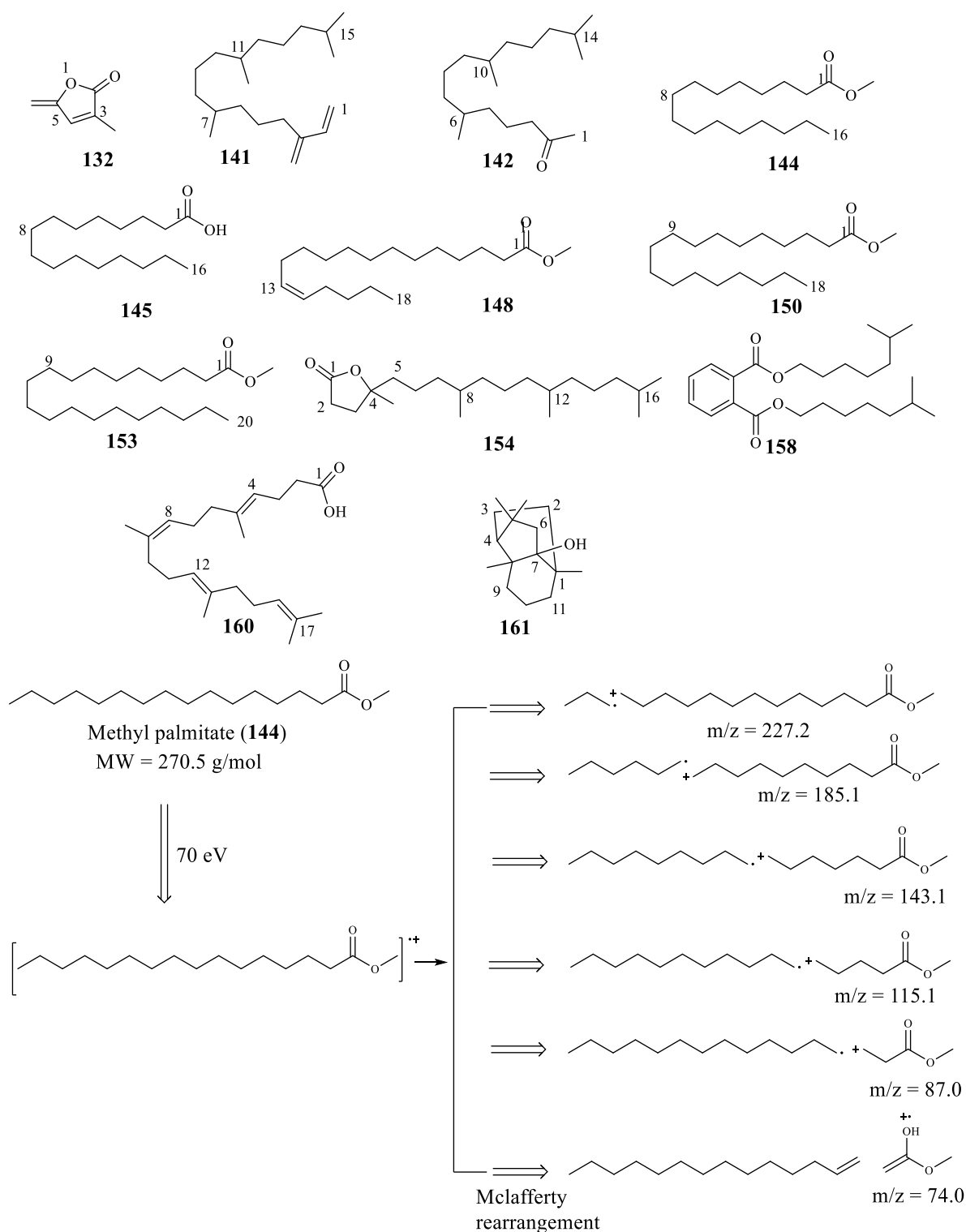


Figure 16. Structures of the major compounds and terpenes identified from the essential oil of the stem bark of *G. ferruginea* using GC-MS data and proposed mass fragmentation of methyl palmitate

Neophytadiene (**141**) has been reported to have a wide range of biological activities. For example, it was reported to show anticancer (Selmy *et al.*, 2023), anxiolytic like and anticonvulsant (Gonzalez-Rivera *et al.*, 2023), and anti-inflammatory effects (Bhardwaj *et al.*, 2020; Ceyhan-Guvensen & Keskin, 2016), and the MeOH extract of *Plectranthus amboinicus* leaves containing 0.8% of neophytadiene (**141**), 3.0% of 6,10,14-trimethyl-2-pentadecanone (**142**), and 5.0% of palmitic acid (**145**) has been reported for its antioxidant, antibacterial, and antifungal activities (Swamy *et al.*, 2017). The derivative of 3-methyl-5-methylene-2(5H)-furanone (**132**) has been reported to exhibit antibacterial activity (Deora *et al.*, 2010). Moreover, ginsenoside (**161**) was used to synthesize a compound called ginsamid, which has been reported for its antiviral activity (Volobueva *et al.*, 2021). Therefore, based on the literature report described above, we can conclude that the essential oil extracted from the dried stem barks of *G. ferruginea* is rich in phytochemical constituents with a wide range of biological activities, including antibacterial, antidiabetic, anticancer, anti-inflammatory, antioxidant, antifungal, and antiviral activities.

4.1.2 GC-MS analysis of fractions of *R. ruspolii* leaves. A total of 24 components were identified from the combined fractions 6-10 of *R. ruspolii* leaves based on GC-MS spectral data (Appendix 1b).

Table 2. Compounds identified from fractions 6-10 of *R. ruspolii* leaves using GC-MS data

No	Compound name	RT	RI		Peak Area (%)
			Calculated	Reported	
137	diethyl phthalate	26.83	1589.3	1587	0.25
162	(6S)-2-methyl-6-(4-methylphenyl)-2-hepten-4-one	28.18	1660.4	-	0.06
141	neophytadiene	31.18	1826.9	1827	0.18
142	6,10,14-trimethyl-2-pentadecanone	31.3	1834	1833	0.44
163	methyl (7Z)-hexadecenoate	32.57	1908.9	-	0.27
144	methyl palmitate	32.64	1912.7	1912	5.46
145	palmitic acid	33.21	1947.5	1947	1.52
164	methyl 15-methylhexadecanoate	34.25	2012.9	-	0.07
165	methyl (9Z,12Z)-octadecadienoate	35.33	2081.6	2082	0.53
166	methyl (13Z)-octadecenoate	35.42	2087.7	-	1.52
150	methyl octadecanoate	35.79	2112.2	2112.2	0.48
167	N-(tert-butoxycarbonyl)-2-methyl-4,4-diphenylbut-3-enamine	36.28	2144.8	-	0.05
168	Phenyl 4-[bis(ethoxycarbonyl)but-3-ynyl]-2,3,4-trideoxy- α -L-glycero-pent-2-enopyranoside	38.70	2312.4	-	0.09
169	heneicosane	45.04	2691.1	-	49.11
170	eicosane	56.05	-	-	38.21
171	(S)-(E)-(-)-4-acetoxy-1-phenyl-2-dodecen-1-one	57.49	-	-	0.12

Of these, approximately 16 (98.40%) compounds were characterized (Table 2, Figures 16 and 17) based on their RI and mass fragmentation compared with reported data (Kim *et al.*, 2023). The most prevalent compound was heneicosane (**169**, 49.11%), followed by eicosane (**170**, 38.21%), methyl palmitate (**144**, 5.46%), palmitic acid (**145**, 1.52%), and methyl (13Z)-octadecenoate (**166**, 1.52%). The mass spectrum and proposed mass fragmentation of the major compound (heneicosane, **169**) are shown in Appendix 1b and Figure 18, respectively.

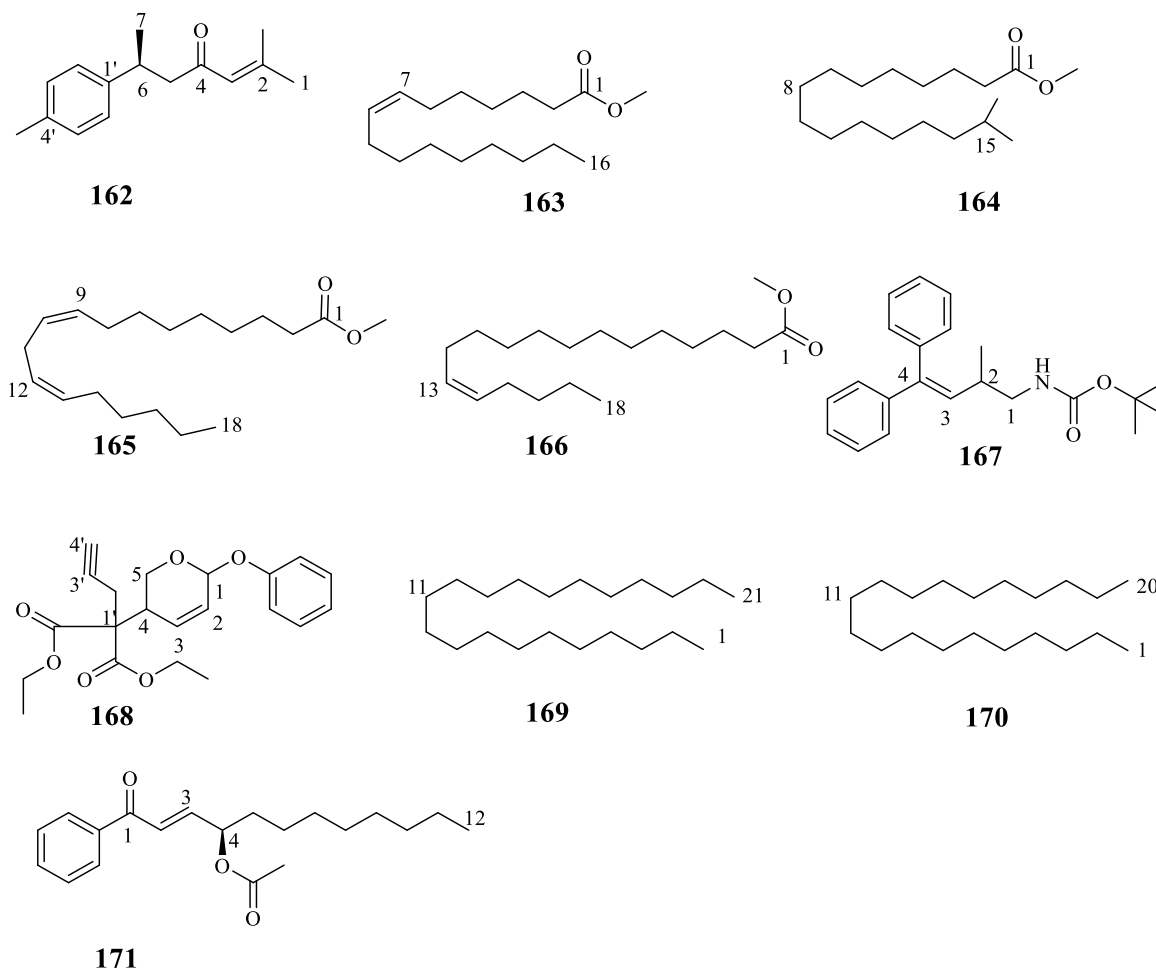


Figure 17. Structures of compounds identified from fractions 6-10 of *R. ruspolii* leaves using GC-MS data

Some of the biological activities of these components have been described. For example, (6S)-2-methyl-6-(4-methylphenyl)-2-hepten-4-one (**162**) has been reported for its activity against malaria (Liu *et al.*, 2020). While a MeOH extract of *Schinus molle* wood branch containing 6.26% phenyl4-[bis(ethoxycarbonyl)but-3-ynyl]-2,3,4-trideoxy- α -L-glucero-pent-2-enopyranoside (**168**) has been reported for its antibacterial activities (Salem *et al.*, 2016). Whereas heneicosane (**169**) has been reported to demonstrate antibacterial and antifungal properties (Vanitha *et al.*, 2020). While the *n*-hexane extract of a rumen

isolate, *Paracoccus pantotrophus* FMR19 containing 0.25% eicosane (**170**), showed antibacterial activities (Faridha Begum *et al.*, 2016), and essential oil containing 1.41% eicosane (**170**) was reported to show anti-inflammatory, antinociceptive, antipyretic, and antioxidant activities (Aati *et al.*, 2019). Furthermore, an EtOH extract of stem bark of *Albizia lebbeck* with 0.08% (S)-(E)-(-)-4-acetoxy-1-phenyl-2-dodecen-1-one (**171**) has been reported for its anticancer activities (Umar *et al.*, 2023). Therefore, from the literature report discussed above, it is possible to conclude that a variety of biologically active compounds are present in the non-polar portion of *R. ruspolii* leaves with antibacterial, antimalarial, antifungal, anti-inflammatory, antinociceptive, antipyretic, antioxidant, and anticancer properties.

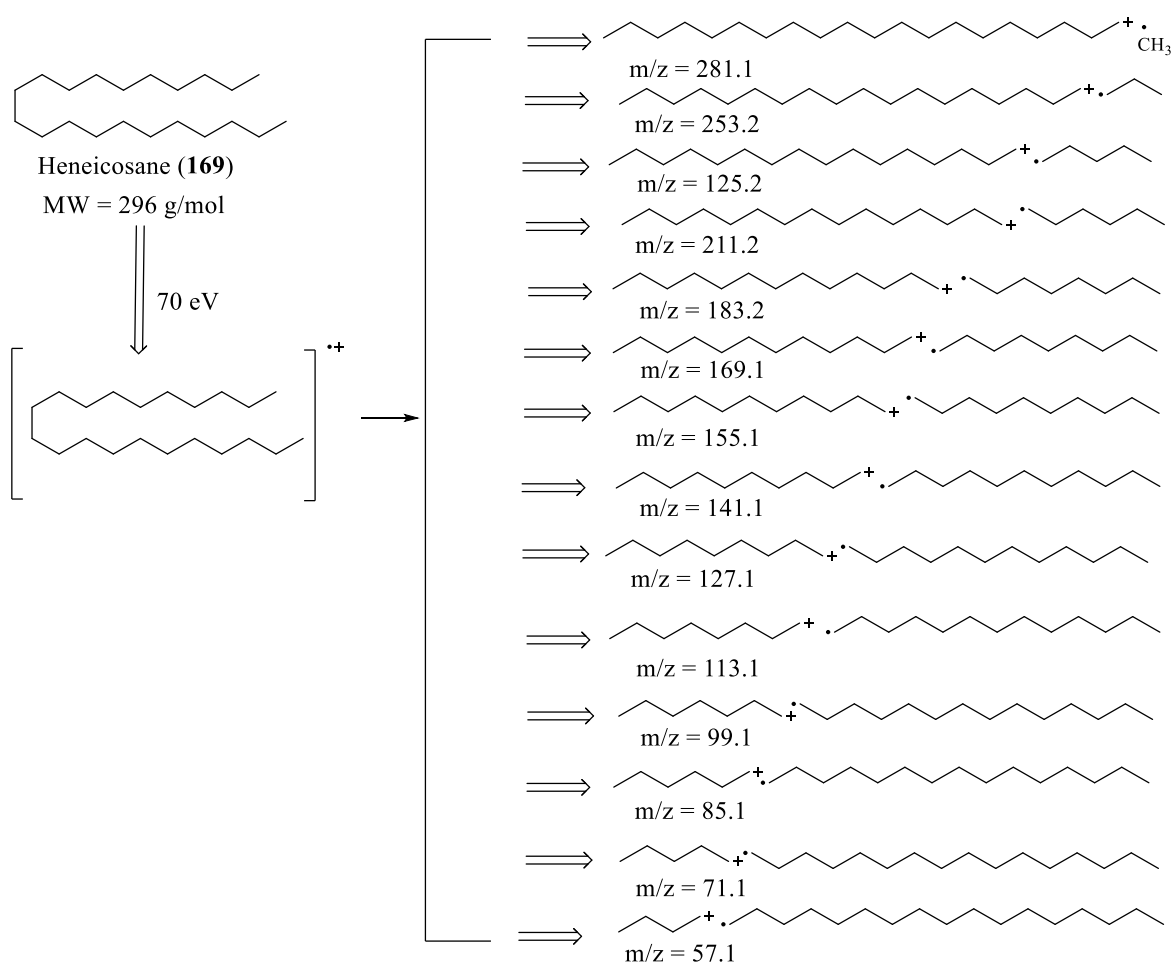


Figure 18. Proposed mass fragmentation of heneicosane (**167**)

4.1.3 GC-MS analysis of fractions of *O. rochetiana* leaves. The GC-MS data analysis of the combined fractions 4-17 of *O. rochetiana* leaves (Appendix 1c) showed the presence of 26 compounds. Among all, 13 (62.06%) compounds (Table 3) were characterized (Figures 16 and 19) by comparing their calculated RI and mass fragments with the literature report (Kim *et al.*, 2023). Neophytadiene (**141**, 23.0%) was the most dominant

compound, followed by 4-ethynylcyclopentene (**172**, 20.84%), 5,9,13,17-tetramethyl 4,8,12,16-octadecatetraenoic acid (**157**, 6.0%), 3-methyl-5-methylene-2(5H)-furanone (**132**, 4.93%), and methyl palmitate (**144**, 2.84%). The mass spectrum and proposed mass fragmentation of the major compound (neophytadiene, **141**) are depicted in Appendix 1c and Figure 19, respectively.

Table 3. Compounds identified from fractions 4-17 of *O. rochetiana* leaves using GC-MS data

No	Compound name	RT	RI		Peak Area (%)
			Calculated	Reported	
172	4-ethynylcyclopentene	5.11	757.18	-	20.84
173	meso-2,5-dimethyl-3,4-hexanediol	10.43	923.49	-	0.81
132	3-methyl-5-methylene-2(5H)-furanone	13.31	1015.51	-	4.93
174	diethylbenzene	14.18	1045.73	1047	0.92
175	(S)-(-)-4-benzoyloxy-5-oxopentyl pivalate	15.45	1091.08		0.27
176	isooctanol	22.59	1383.82		0.16
177	(E)-methyl 5-(6-fluoropyridin-3-yl)-5-hydroxypent-2-enoate	26.83	1589.21		0.71
178	dibutyl phthalate	31.74	1859.61	-	0.22
144	methyl palmitate	32.64	1913.01	1913	2.84
145	palmitic acid	33.16	1944.54	1945	0.92
179	ditridecyl phthalate	33.30	1953.44	-	0.43
160	5,9,13,17-tetramethyl 4,8,12,16-octadecatetraenoic acid	48.30	2811.04	-	6.0
141	neophytadiene	59.18	-	-	23.0

Some biological activities of the component identified from fractions 6-10 of *O. rochetiana* leaves have been reported. For example, the fraction containing 1-ethynylcyclopentene, which is close to compound **172** in the skeleton, has been reported for antimalarial effects (Priya & Jones, 2021). Essential oil from *Stellera chamaejasme* flowers containing 7% and 3.4% of (E)-methyl 5-(6-fluoropyridin-3-yl)-5-hydroxypent-2-enoate (**177**) showed repellent activities against product insects (Sang *et al.*, 2021) and DPPH radical scavenging activities (Khan *et al.*, 2016), respectively. A natural-based phthalate, dibutyl phthalate (**178**), and ditridecyl phthalate (**179**) have been reported to have antibacterial, DPPH radical scavenging, and antidiabetic activities (Shobi & Viswanathan, 2018; Yasmin *et al.*, 2023). Therefore, from the reported data described above, we can conclude that the non-polar extract of the leaves of *O. rochetiana* has a wide range of biological activities, including antimalarial, antioxidant, antibacterial, and antidiabetic activities.

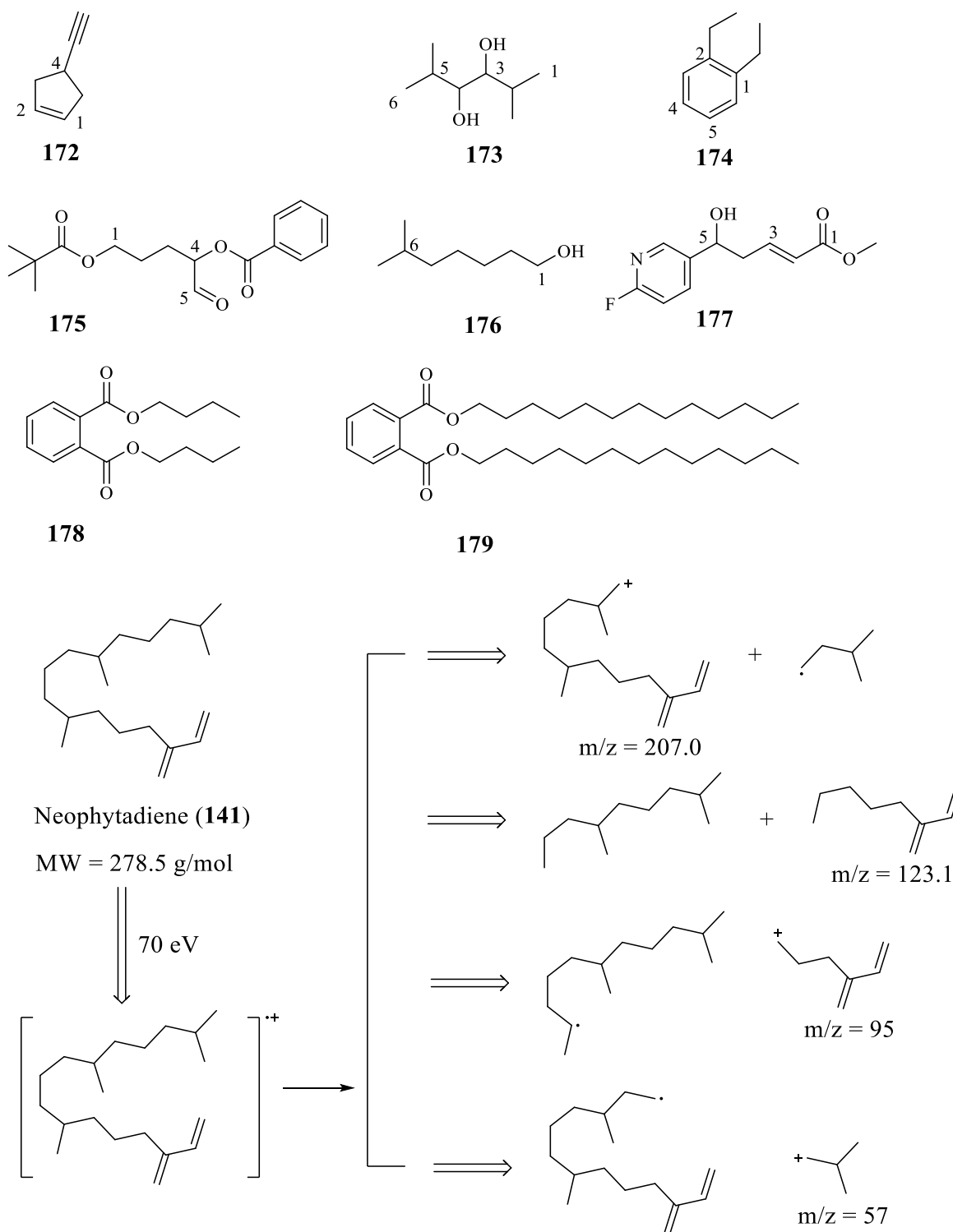


Figure 19. Structures of compounds identified from fractions 4-17 of *O. rochetiana* leaves using GC-MS data and proposed mass fragmentation of neophytadiene (139)

4.1.4 GC-MS analysis of fractions of *R. apetalus* leaves. The GC-MS analysis (Appendix 1d) of combined fractions of 16-21 *R. apetalus* leaves indicated the presence of 41 components, of which 16 (89.80%) were characterized (Table 4) using their RI and mass fragmentation forms compared with the literature report (Kim *et al.*, 2023), and their chemical structures are depicted in Figures 16, 19, and 20.

Table 4. Compounds identified from fractions 16-21 of *R. apetalus* leaves using GC-MS

No	Compound name	RT	RI		Peak area (%)
			Calculated	Reported	
180	1-tetradecanol	22.58	1383.3	-	0.14
181	2,4-ditertbutylphenol	25.14	1504.7	1502	0.21
182	1-tridecanol	26.68	1582	1580	0.33
141	neophytadiene	31.18	1826.9	1827	1.40
142	6,10,14-trimethyl-2-pentadecanone	31.29	1833.6	1833	2.00
183	9-heptadecanone	31.79	1862.4	1861	0.07
184	cyclotetradecane	32.58	1909.2	-	0.42
144	methyl palmitate	32.64	1912.7	1913	2.17
145	palmitic acid	33.17	1945.6	1946	0.24
185	(E)-2-nonadecene	33.71	1978.5	-	0.27
186	(R)-(-)-14-methyl-8-hexadecyn-1-ol	35.33	2081.6	-	0.17
187	isopulegol	35.43	2088.1	-	0.73
150	methyl octadecanoate	35.79	2112.2	2112	0.64
169	heneicosane	41.04	2478.9	-	64.82
170	eicosane	42.62	2573	-	15.75
171	(S)-(E)-(-)-4-acetoxy-1-phenyl-2-dodecen-1-one	44.74	2592.2	-	0.35

The five most abundant compounds were heneicosane (**169**, 64.82%), eicosane (**170**, 15.75%), methyl palmitate (**144**, 2.17%), 6,10,14-trimethyl-2-pentadecanone (**142**, 2.00%), and neophytadiene (**141**, 1.40%). The mass spectrum and proposed mass fragmentation of the major compound (heneicosane, **169**) are given in Appendix 1d and Figure 18. Some biological activities of these compounds have been reported. For example, the MeOH extract of flower parts of *Scabiosa columbaria* containing 3.06% 1-tetradecanol (**180**) has been reported to show antioxidant activities (Akar, 2021). The previous reports showed that 2,4-ditertbutylphenol (**181**) has been reported to exhibit antifungal, antibacterial, and antioxidant activities (María Teresa *et al.*, 2014; Padmavathi *et al.*, 2014; Ren *et al.*, 2019). In another study, 1-tridecanol (**182**) has been reported to show antibacterial and antioxidant activities (Mu'nisa *et al.*, 2018; Togashi *et al.*, 2007). Similarly, an essential oil with 2.1% 9-heptadecanone (**183**) has been reported to demonstrate DPPH radical scavenging activities (Khan *et al.*, 2016), and a 2.4% cyclotetradecane (**184**) found in the EtOH extract of *Etlingera elatior* inflorescence has been reported for its anticancer and antioxidant activities (Al-Mansoub *et al.*, 2021). Similarly, essential oil from leaves of *Psidium friedrichsthalianum* containing 2.1% 2-nonadecene (**185**) has been reported to show antibacterial activity (Chinchilla *et al.*, 2016). In other studies, the (R)-(-)-14-Methyl-8-hexadecyn-1-ol (**186**) has been reported for its antibacterial and anticancer activities (Mohansrinivasan *et al.*, 2015). Whereas an essential oil with 9.8% isopulegol (**187**) has been reported to exhibit antifungal activity (Montenegro *et al.*, 2020). Therefore, it can be

said that the nonpolar fractions of the leaves of *R. apetalus* are rich in phytochemicals with antibacterial, antioxidant, antifungal, and anticancer properties.

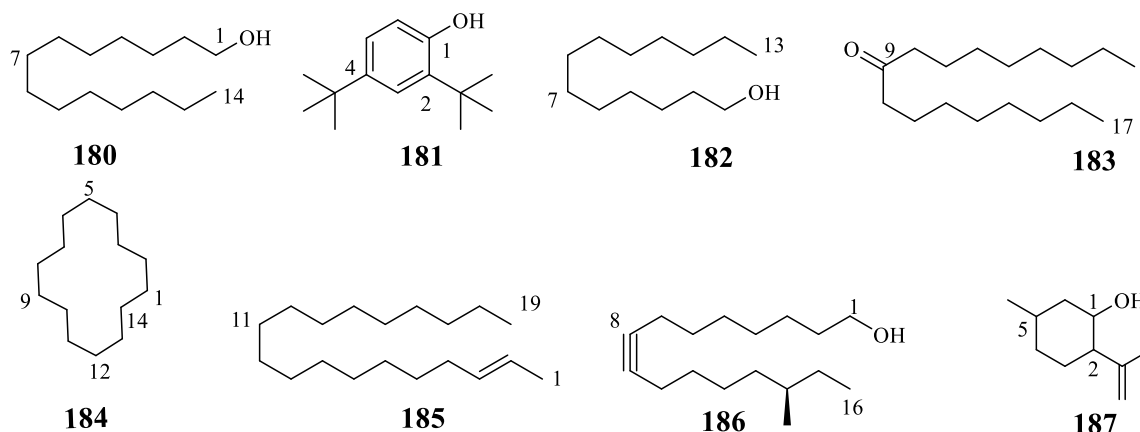


Figure 20. Structures of compounds identified from fractions 16-21 of *R. apetalus* leaves using GC-MS data

4.1.5 Characterization of isolated compounds from *C. hirsuta*. Seven compounds (Figures 7, 9, 16, and 21), including oleic acid (**188**), palmitic acid (**145**), stigmasterol (**70**), β -sitosterol (**17**), boehmenan (**189**), erythro carolignan E (**190**), and threo carolignan E (**191**), were isolated and characterized from the root extract of *C. hirsuta*. The structures of these compounds were characterized by comparing the 1D NMR data with the literature report.

Compound **188** was obtained as a pale yellowish waxy solid. TLC showed a spot at R_f value 0.6 using *n*-hexane:EtOAc (9:1) as a mobile phase. The $^1\text{H-NMR}$ (400 MHz, CDCl_3) spectrum (Appendix 2a) indicated the presence of 33 protons, including methyl protons at δ 0.90 (3H, t, J = 6.5 Hz, H-18), ten methylene protons at δ 1.30 (20H, br. s, H-4-H-7, and H-12-H-17), four additional methylene protons at δ 1.65 (2H, m, H-3), 2.04 (4H, br. s, H-8 and H-11), and 2.37 (2H, t, J = 6.4 Hz, H-2), and olefinic protons at δ 5.37 (2H, H-9 and H-10). The proton decoupled $^{13}\text{C-NMR}$ (101 MHz) spectrum (Appendix 2b) and DEPT-135 (Appendix 2c) revealed the presence of 18 well-resolved carbon signals, including methyl carbon at δ 14.3 (C-18), 14 methylene carbons at δ 22.9 (C-17), δ 24.8 (C-3), δ 27.4 (C-8 and C-11), δ 29.2-32.1 (C-4-C-7 and C-12-C-17), δ 34.1 (C-2), two methine carbons at δ 130.1 (C-9 and C-10), and a quaternary carbon that corresponds to carbonyl carbon at δ 179.8 (C-1). The NMR data of compound **188** are consistent with the NMR data reported for oleic acid (Wishart *et al.*, 2022). Oleic acid has been reported to show DPPH radical scavenging and antibacterial activity properties (Ramadan *et al.*, 2024).

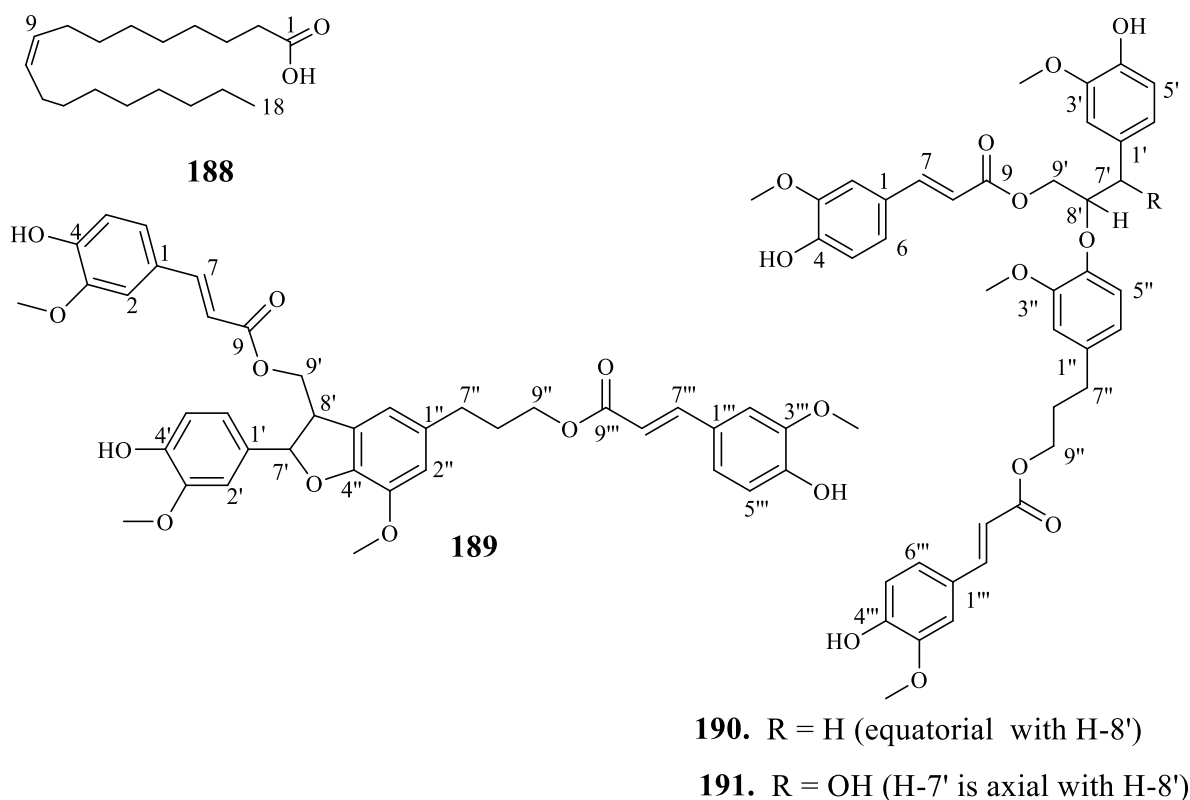


Figure 21. The structures of isolated compounds from roots of *C. hirsuta*

Compound **145** was obtained as a white solid with a melting point of 62-64 °C (Soliman & El-Shattory, 2017). TLC gave a spot at an R_f value of 0.3 using *n*-hexane:EtOAc (4:1) as the mobile phase. The $^1\text{H-NMR}$ (400 MHz, CDCl_3) spectrum (Appendix 3a) showed the presence of 31 protons, including a methyl proton at δ 0.88 (3H, t, J = 7.3 Hz, H-16), 11 methylene protons with a broad singlet at δ 1.25 (22H, br. s, H-4-H-14), three additional methylene protons at δ 1.30 (2H, m, H-15), δ 1.62 (2H, m, H-3), and δ 2.35 (2H, t, J = 7.3 Hz, H-2). The proton decoupled $^{13}\text{C-NMR}$ (101 MHz) spectrum (Appendix 3b) with the aid of DEPT-135 (Appendix 3c) revealed the presence of 16 well-resolved carbon signals, including a methyl at δ 14.3 (C-18), 14 methylene at δ 22.9 (C-15), δ 24.8 (C-3), δ 29.2-29.9 (C-4-C-13), δ 32.1 (C-14), and δ 34.2 (C-2), and a quaternary carbon corresponds to carbonyl carbon at δ 180.0 (C-1). The NMR data of compound **145** are consistent with the NMR data reported for palmitic acid (Ekeocha *et al.*, 2021). Palmitic acid has been reported to exhibit antifungal properties (Prasath *et al.*, 2020).

Compounds **70** and **17** were obtained as a mixture. TLC showed a spot at an R_f value of 0.4 using *n*-hexane:EtOAc (4:1) as the mobile phase. The $^1\text{H-NMR}$ spectrum (Appendix 4a) showed a mixture of compound **70** (major, 61.3%) and compound **17** (minor, 38.7%). The percentage was calculated using the area of the proton representing both compounds (H-6) and representing only compound **70** (H-22 or H-23) (Appendix 4a). In general, 96

protons and 58 carbons were identified, including 12 methyl carbons, 20 methylene carbons, 20 methine carbons, and six quaternary carbons. The NMR data generated for compounds **70** and **17** (Appendices 4a to 4c) was compared with the reported data for stigmasterol and β -sitosterol, respectively, and found in good agreement (Shwe, Thein, *et al.*, 2019).

Table 5. ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (101 MHz) of compound **70** and NMR data reported for stigmasterol (Shwe, Thein, *et al.*, 2019)

C No	Compound 70		Stigmasterol/ CDCl_3	
	$\delta^1\text{H}$	$\delta^{13}\text{C}$	$\delta^1\text{H}/600\text{ MHz}$	$\delta^{13}\text{C}$
1	1.85 & 1.08 (br. s, 2H)	37.4	1.85&1.08 (o, 2H)	37.2
2	1.83 (br. s, 2H)	31.8	1.52&1.84 (o, 2H)	31.6
3	3.53 (m, 1H)	72.0	3.52 (m, 1H)	71.9
4	2.27 & 2.24 (br. s, 2H)	42.4	2.24&2.28 (m, 2H)	42.2
5	-	140.9	-	140.7
6	5.36 (br. s, 1H)	121.9	5.35 (br., 1H)	121.7
7	2.00 & 1.50 (br. s, 2H)	32.1	1.50&1.96 (o, 2H)	31.9
8	1.50 (1H)	32.1	1.49 (o, 1H)	31.9
9	0.93 (br. s, 1H)	50.3	0.93 (o, 1H)	50.1
10	-	36.7	-	36.5
11	1.50 (br. s, 2H)	21.2	1.45&1.50 (o, 2H)	21.0
12	2.00 & 1.16 (br. s, 2H)	39.9	1.17&1.99 (o, 2H)	39.7
13	-	42.4	-	42.4
14	1.01 (br. s, 1H)	57.0	0.98 (o, 1H)	56.8
15	1.57 & 1.16 (br. s, 2H)	24.5	1.15&1.57 (o, 2H)	24.4
16	1.63 & 1.25 (br. s, 2H)	29.2	1.24&1.73 (o, 2H)	28.9
17	1.16 (br. s, 1H)	56.1	1.15 (o, 1H)	56.0
18	0.82 (s, 3H)	12.1	0.65 (s, 3H)	11.8
19	1.01 (br. s, 3H)	19.2	1.05 (s, 3H)	19.1
20	2.04 (1H)	40.6	2.04 (o, 1H)	40.5
21	1.03 (3H)	21.4	1.02 (d, 3H)	21.5
22	5.14 (m, 1H)	138.5	5.15 (br., 1H)	138.3
23	5.02 (m, 1H)	129.4	5.05 (br., 1H)	129.3
24	1.53 (1H)	51.4	1.53 (o, 1H)	51.2
25	1.45 (1H)	36.3	1.37(o, 1H)	36.1
26	0.84 (br. s, 3H)	20.0	0.83(d, 3H)	19.8
27	0.70 (br. s, 3H)	19.0	0.80 (d, 3H)	18.9
28	1.43 & 1.16 (br. s, 2H)	25.6	1.16&1.43 (o, 2H)	25.4
29	0.68 (br. s, 3H)	12.4	0.65 (t, 3H)	12.0

The complete assignment of all NMR data of compound **70** is presented in Table 5, and the NMR data of compound **17** is displayed in Table 10. Stigmasterol is known for its wide range of biological activity, including antibacterial, antifungal, anti-inflammatory, and antidiabetic activities (Jiménez-Nevarez *et al.*, 2023; Poulouse *et al.*, 2021; Yusuf *et al.*, 2018).

Compound **189** was observed as yellowish jelly and showed a TLC spot at R_f 0.7 using n -hexane: EtOAc: CHCl_3 :MeOH (5:3:1:1) as the mobile phase. From NMR spectral data (Appendices 5a to 5c), 40 protons and 40 carbons were identified. The ^1H -NMR

(Appendix 5a) spectrum data indicated that an ABX set of signals at δ 2.00-2.05 (2H, m, H-8'''), 2.70 (2H, t, $J = 7.8$ Hz, H-7''), and 4.23 (2H, t, $J = 6.5$ Hz, H-9'') was assigned for methylene carbons at δ 30.9, 32.3, and 63.9, respectively. The observation of an ABC set of signals is further supported by diastereotopic protons (H-9') at δ 4.41 (1H, dd, $J = 8, 12$ Hz) and 4.58 (1H, dd, $J = 4, 12$ Hz) assigned for the carbon signal at 65.6, as well as methine protons that correspond to the carbon signal at δ 50.9 and 89.1, respectively, appeared at 3.84-3.92 (1H, m, H-8') and 5.50 (1H, d, $J = 7.8$ Hz, H-7'). Two trans-coupled protons are observed at δ 6.23 (1H, d, $J = 15.9$ Hz, H-8) and 7.47 (1H, d, $J = 15.9$ Hz, H-7) and at 6.29 (1H, d, $J = 15.9$ Hz, H-8''') and 7.59 (1H, d, $J = 15.9$ Hz, H-7'''), which were assigned to carbon signals at δ 114.9, 145.6, 115.6, and 145.0, respectively. The presence of a tetrasubstituted benzene ring is indicated by the two broad singlet protons identified at δ 6.69 (1H, H-2'') and 6.71 (1H, H-6'), which are meta-aromatic protons linked to the carbon signal at δ 112.7 and 116.3, respectively. At δ 5.64 (1H, 4'-OH) and 5.91 (2H, 4, 4''-OH), two additional broad singlet protons were observed because of the three hydroxyl protons involved in hydrogen bonding. The methoxy proton signals from δ 3.84-3.92 were assigned to carbon signals at δ 55.9-60.0. Table 6 provides a summary of all the NMR spectral data for compound **189**. Similar NMR spectral data has been documented for a compound called boehmenan isolated from *Hibiscus cannabinus* and *Ochroma lagopus* Swartz (Paula *et al.*, 1995; Seca *et al.*, 2001). Boehmenan has been reported to show inhibitory properties for various cancer cells (Pan *et al.*, 2017; Shono *et al.*, 2015).

Compounds **190** and **191** were obtained as a mixture showing the TLC spot at R_f value 0.5 with *n*-hexane:EtOAc: CHCl₃:MeOH (5:3:1:1) as a mobile phase. The NMR spectral data revealed that the sample is a mixture of compound **190** (major) and compound **191** (minor) (Appendices 6a to 6c). The complete assignment of the NMR spectral data is given in Table 7 and Table 8. The two compounds are almost similar in their spectral data except for the signal due to H-7', which was observed at δ 4.92 and 4.94 ppm assigned to compound **190** and compound **191**, respectively. Literature reports showed that the stereochemistry of these compounds at H-7 is different (Seca *et al.*, 2001). In this regard, H-7' appeared as a singlet at δ 4.92 (equatorial with H-8') in compound **190**; however, it appears as a doublet ($J = 8.1$ Hz) at δ 4.94 (axial with H-8') in compound **191**.

Table 6. ¹H-NMR (400 MHz, CDCl₃) and ¹³C-NMR (101 MHz) of compound **189** and NMR data reported for boehmenan (Seca *et al.*, 2001)

C No	Compound 189		Boehmenan/CDCl ₃		C-No	Compound 189		Boehmenan/CDCl ₃	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	$\delta^1\text{H}/300.1\text{ MHz}$	$\delta^{13}\text{C}$		$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	$\delta^1\text{H}$	$\delta^{13}\text{C}$
1	-	126.9	-	126.7	1'''	-	127.1	-	126.9
2	7.02 (d, <i>J</i> = 1.7, 1H)	109.5	7.05 (d, <i>J</i> = 1.7)	109.3	2'''	6.89-6.94 (m, 1H)	109.6	6.90-6.94 (m)	109.4
3	-	146.9	-	146.7	3'''	-	146.9	-	146.7
4	-	148.1	-	147.9	4'''	-	148.3	-	148.1
5	6.86 (d, <i>J</i> = 8.3, 1H)	114.9	6.91 (d, <i>J</i> = 8.3)	114.7	5'''	6.86 (d, <i>J</i> = 8.3, 1H)	114.9	6.91 (d, <i>J</i> = 8.3)	114.7
6	7.06 (dd, <i>J</i> = 8.3, 1.7, 1H)	123.2	7.08 (dd, <i>J</i> = 8.3, 1.7)	123.0	6'''	6.98 (dd, <i>J</i> = 7.3, 1.7, 1H)	123.2	7.00 (dd, <i>J</i> = 7.3, 1.7)	123.1
7	7.47 (d, <i>J</i> = 15.9, 1H)	145.6	7.49 (d, <i>J</i> = 15.9)	145.5	7'''	7.59 (d, <i>J</i> = 15.9, 1H)	145.0	7.60 (d, <i>J</i> = 15.9)	144.9
8	6.23 (d, <i>J</i> = 15.9, 1H)	114.9	6.23 (d, <i>J</i> = 15.9)	114.7	8'''	6.29 (d, <i>J</i> = 15.9, 1H)	115.6	6.30 (d, <i>J</i> = 15.9)	115.4
9	-	167.0	-	167.0	9'''	-	167.4	-	167.3
1'	-	132.7	-	132.5	3-OCH ₃	3.92 (s, 3H)	-	3.93 (s)	55.9
2'	6.89-6.94 (m, 1H)	109.0	6.90-6.94 (m)	108.8	3'-OCH ₃	3.84 (s, 3H)	-	3.84 (s)	55.9
3'	-	146.8	-	146.6	3''-OCH ₃	3.89 (s, 3H)	56.1-	3.90 (s)	56.0
4'	-	145.9	-	145.7	3'''OCH ₃	3.92 (s, 3H)	56.2	3.93 (s)	55.9
5'	6.91 (d, <i>J</i> = 8.1, 1H)	114.4	6.92 (d, <i>J</i> = 8.1)	114.2	4-OH	5.91 (br. s, 1H)	-	5.91 (s)	-
6'	6.89-6.94 (m, 1H)	119.9	6.90-6.94 (m)	119.7	4'-OH	5.64 (br. s, 1H)	-	5.65 (s)	-
7'	5.50 (d, <i>J</i> = 7.8, 1H)	89.1	5.50 (d, <i>J</i> = 7.8)	88.9	4''-OH	5.91 (br. s, 1H)	-	5.92 (s)	-
8'	3.84-3.92 (m, 1H)	50.9	3.84-3.93 (m)	50.7					
9'	4.41 (dd, <i>J</i> = 12.0, 8.0, 1H)	65.6	4.42 (dd, <i>J</i> = 11.2, 7.7)	65.4					
	4.58 (dd, <i>J</i> = 12.0, 4.0, 1H)		4.59 (dd, <i>J</i> = 11.2, 5.1)						
1''	-	135.1	-	134.9					
2''	6.69 (br. s, 1H)	112.7	6.69 (s)	112.4					
3''	-	144.3	-	144.1					
4''	-	146.4	-	146.2					
5''	-	127.6	-	127.4					
6''	6.71 (br. s, 1H)	116.3	6.71 (s)	116.1					
7''	2.70 (t, <i>J</i> = 7.8, 2H)	32.3	2.71 (t, <i>J</i> = 7.6)	32.1					
8''	2.00-2.05 (m, 2H)	30.9	1.97-2.06 (m)	30.7					
9''	4.23 (t, <i>J</i> = 6.5, 2H)	63.9	4.23 (t, <i>J</i> = 6.5)	63.7					

Table 7. ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (101 MHz) of compound **190** and NMR data reported for erythro carolignan E (Seca *et al.*, 2001)

C No	Compound 190		Erythro carolignan E/ CDCl_3	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	$\delta^1\text{H}/300.1$ MHz	$\delta^{13}\text{C}$
1	-	127.1		126.9
2	6.96-7.04 (m, 1H)	109.5	6.95-7.05 (m)	109.3
3	-	146.9		146.7
4	-	148.2		148.0
5	6.92 (br. s, 1H)	114.9	6.92 (d, $J = 8.1$)	114.7
6	6.96-7.04 (m, 1H)	123.3	6.95-7.05 (m)	123.2
7	7.51 (d, $J = 15.9$, 1H)	145.4	7.51 (d, $J = 15.9$)	145.2
8	6.25 (d, $J = 15.9$, 1H)	115.1	6.24 (d, $J = 15.9$)	114.9
9	-	167.3		167.1
1'	-	131.3		131.0
2'	6.96-7.04 (m, 1H)	109.0	6.95-7.05 (m)	108.8
3'	-	146.8		146.6
4'	-	145.3		145.0
5'	6.88 (d, $J = 8.1$, 1H)	114.3	6.88 (d, $J = 8.1$)	114.1
6'	6.84 (br. s, 1H)	119.4	6.83 (dd, $J = 8.1$, 1.8)	119.2
7'	4.92 (br. s, 1H)	72.2	4.92 (s)	72.0
8'	4.45-4.52 (m, 1H)	84.7	4.47-4.52 (m)	84.5
9'	4.22-4.32 (m, 1H)		4.22-4.30 (m)	
	4.45-4.50 (m, 1H)	62.8	4.47-4.52 (m)	62.6
1''	-	137.6		137.4
2''	6.72-6.79 (m, 1H)	112.5	6.73-6.77 (m)	112.3
3''	-	151.5		151.3
4''	-	145.3		145.0
5''	6.84 (d, $J = 8.2$, 1H)	120.9	6.67 (d, $J = 8.3$)	120.7
6''	6.72-6.79 (m, 1H)	121.3	6.73-6.77 (m)	121.1
7''	2.70 (t, $J = 7.8$, 2H)	32.2	2.71 (t, $J = 7.7$)	32.0
8''	2.00-2.04 (br. s, 2H)	30.5	1.97-2.07 (m)	30.3
9''	4.23 (t, $J = 6.2$, 2H)	63.8	4.22 (t, $J = 6.5$)	63.6
1'''	-	127.0		126.8
2'''	6.96-7.04 (m, 1H)	109.5	6.95-7.05 (m)	109.3
3'''	-	146.9		146.7
4'''	-	148.2		148.0
5'''	6.91 (m, 1H)	114.9	6.91 (d, $J = 8.1$)	114.7
6'''	7.08 (br. s, 1H)	123.2	7.08 (dd, $J = 8.1$, 1.8)	123.0
7'''	7.62 (d, $J = 15.9$, 1H)	145.1	7.62 (d, $J = 15.9$)	144.9
8'''	6.32 (d, $J = 15.9$, 1H)	115.5	6.30 (d, $J = 15.90$)	115.3
9'''	-	167.5		167.3
3-OCH ₃	3.92 (br. s, 3H)	56.1	3.92 (s)	55.9
3'-OCH ₃	3.88 (br. s, 3H)	56.1	3.88 (s)	55.9
3''-OCH ₃	3.88 (br. s, 3H)	56.0	3.88 (s)	55.8
3'''-OCH ₃	3.93 (br. s, 3H)	56.1	3.93 (s)	55.9
4-OH	5.61 (br. s, 1H)	-	5.95 (s)	-
4'-OH	5.59 (br. s, 1H)	-	5.64 (s)	-
4''-OH	5.61 (br. s, 1H)	-	5.95 (s)	-

Table 8. ¹H-NMR (400 MHz, CDCl₃) and ¹³C-NMR (101 MHz) of compound **191** and NMR data reported for threo carolignan E (Seca *et al.*, 2001)

C №	Compound 191		Threo carolignan E/CDCl ₃	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	$\delta^1\text{H}/300.1\text{ MHz}$	$\delta^{13}\text{C}/$
1	-	127.1		126.9
2	7.02-7.07 (m, 1H)	109.5	7.02-7.07 (m)	109.3
3	-	147.0		146.8
4	-	148.2		148.0
5	6.79-6.95 (m, 1H)	114.9	6.85-6.94 (m)	114.7
6	7.02-7.07 (m, 1H)	123.3	7.02-7.07 (m)	123.2
7	7.51 (d, <i>J</i> = 15.9, 1H)	145.6	7.51 (d, <i>J</i> = 15.9)	145.5
8	6.28 (d, <i>J</i> = 15.9, 1H)	114.9	6.28 (d, <i>J</i> = 15.9)	114.7
9	-	167.0		166.8
1'	-	131.3		131.1
2'	6.79-6.95 (m, 1H)	109.4	6.85-6.94 (m)	109.2
3'	-	146.9		146.7
4'	-	145.8		145.6
5'	6.79-6.95 (m, 1H)	114.5	6.85-6.94 (m)	114.3
6'	6.79-6.95 (m, 1H)	120.6	6.85-6.94 (m))	120.4
7'	4.94 (br. s, 1H)	74.6	4.94 (d, <i>J</i> = 8.1)	74.4
8'	4.18-4.27 (m, 1H)	86.5	4.17-4.27 (m)	86.3
9'	4.09-4.17 (m, 1H)		4.12 (dd, <i>J</i> = 11.9, 4.7)	
	4.35-4.38 (m, 1H)	63.2	4.36 (dd, <i>J</i> = 12.0, 3.3)	63.0
1''	-	137.6		137.4
2''	6.77 (m, 1H)	112.5	6.78 (s)	112.3
3''	-	150.9		150.7
4''	-	146.2		146.0
5''	7.02-7.07 (m, 1H)	120.8	7.02-7.07 (m)	120.6
6''	6.72-6.79 (m, 1H)	121.2	6.74-6.78 (m)	121.0
7''	2.70 (t, <i>J</i> = 7.8, 2H)	32.2	2.71 (t, <i>J</i> = 7.7)	32.0
8''	2.00-2.04 (br. s, 2H)	30.6	1.97-2.06 (m)	30.4
9''	4.23 (t, <i>J</i> = 6.2, 2H)	63.8	4.22 (t, <i>J</i> = 6.5)	63.6
1'''	-	127.0		126.7
2'''	7.02-7.07 (m, 1H)	109.5	7.02-7.07 (m)	109.3
3'''	-	147.0		146.8
4'''	-	148.3		148.1
5'''	6.79-6.95 (m, 1H)	114.9	6.85-6.94 (m)	114.7
6'''	7.08 (br. s, 1H)	123.2	7.08 (dd, <i>J</i> = 8.1, 1.9)	123.0
7'''	7.62 (d, <i>J</i> = 15.9, 1H)	145.3	7.62 (d, <i>J</i> = 15.9)	145.0
8'''	6.32 (d, <i>J</i> = 15.9, 1H)	115.5	6.31 (d, <i>J</i> = 15.90)	115.3
9'''	-	167.5		167.3
3-OCH ₃	3.85 (br. s, 3H)	56.1	3.85 (s)	55.9
3'-OCH ₃	3.93 (br. s, 3H)	56.1	3.93 (s)	55.9
3''-OCH ₃	3.90 (br. s, 3H)	56.0	3.90 (s)	55.8
3'''-OCH ₃	3.94 (br. s, 3H)	56.1	3.94 (s)	55.9
4-OH	5.89 (br. s, 1H)	-	6.00 (s)	-
4'-OH	5.61 (br. s, 1H)	-	5.69 (s)	-
4''-OH	5.89 (br. s, 1H)	-	6.00 (s)	-

However, in the current report, due to the complexity of the ¹H-NMR spectrum, it was difficult to identify the multiplicity of H-7' for compounds **190** and **191**. Using the chemical shift difference of H-7' in compounds **190** and **191** (Appendix 6a), the area under the

integration of H-7' was determined. The result indicated that the compositions of compounds **190** and **191** in the mixture were calculated as 57% and 43%, respectively. From *Ochroma lagopus* Swartz and *Euphorbia sikkimensis* plants, similar NMR spectral data of compounds **190** and **191** were reported for the compounds erythro carolignan E and threo carolignan E, respectively (Jiang *et al.*, 2016; Paula *et al.*, 1995). Hence, the NMR spectral data of compounds **190** and **191** were compared (Tables 7 and 8) with the similar compounds reported in the literature and found in good agreement (Seca *et al.*, 2001). erythro carolignan E has been identified for its anticancer and anti-HIV activities (Wu *et al.*, 2005).

4.1.6 Characterization of isolated compounds from *G. ferruginea*. Eight compounds, including lupeol (**16**), β -sitosterol (**17**), stigmasterol (**70**), 7-keto- β -sitosterol (**192**), 6-ketositostanol (**193**), daucosterol (**42**), catechin (**65**), and epicatechin (**69**), were isolated and characterized from the stem bark of *G. ferruginea* (Figures 7-9 and 22). The NMR spectral data were utilized to elucidate their structures as follows.

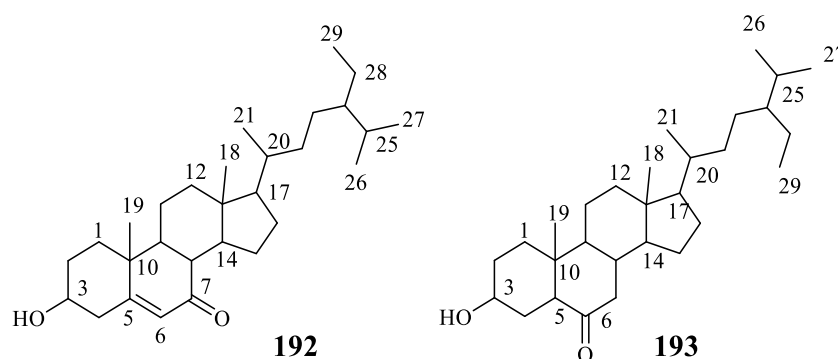


Figure 22. Structures of compounds isolated from stem bark of *G. ferruginea*

Compound **16** was isolated as a white solid. The melting point was observed in the range of 202-206 °C. Using *n*-hexane:EtOAc (9:1), TLC revealed a spot at R_f 0.5. Based on the δ shown (Appendices 7a to 7c), summarized in Table 9, and the 2D NMR correlations (Figure 23 and Appendices 7d to 7f), the proposed structure has a triterpene skeleton with 30 carbons, including seven methyls, 11 methylenes, six methines, and six quaternary carbons. In the ^1H NMR chemical shift band of 1.2 to 1.7 ppm, a signal somewhat partially overlapping (Appendix 7a) leads to some difficulty in fully understanding the ^1H - ^1H correlations. However, using the ^1H - ^{13}C correlation of protons (Figure 23) in positions 23–28 with their neighboring carbons, all resonance assignments were carried out in this area of compound **16**. Furthermore, in this work, the NMR data recorded for compound **16** and the published NMR results showed good agreement (Shwe, Win, *et al.*, 2019). The cross-peaks observed from the 2D NMR spectral of compound **16** match those of lupeol (Shwe,

Win, *et al.*, 2019). Consequently, lupeol was determined to have a structure like compound **16**. Antibacterial, antioxidant, and antifungal activities are among the biological properties that have been reported for lupeol (Amoussa *et al.*, 2016; Emaikwu *et al.*, 2020; Yanda *et al.*, 2022).

Table 9. ¹H-NMR (400.2 MHz, CDCl₃) and ¹³C-NMR (100.6 MHz) of compound **16** and NMR data reported for lupeol (Shwe, Win, *et al.*, 2019)

C No	Compound 16			Lupeol/CDCl ₃ (o = overlap)	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	¹ H- ¹³ C HMBC	$\delta^1\text{H}/600\text{ MHz}$	$\delta^{13}\text{C}$
1	1.66 & 0.91 (m, 2H)	38.9	H-25	1.65&0.90 (o)	38.9
2	1.60 & 1.52 (m, 2H)	27.5	H-3	1.67&1.52(o)	27.4
3	3.19 (dd, J =11.2, 5.1 Hz, 1H)	79.2	H-23, 24	3.2 (dd)	79.0
4	-	39.0	H-3, 5, 23, 24	-	38.7
5	0.68 (m, 1H)	55.4	H-6, 23, 24, 25	0.67 (o)	55.3
6	1.50 & 1.39 (m, 2H)	18.5	H-5, 7	1.52&1.37 (o)	18.3
7	1.39 (m, 2H)	34.4	H-5, 26	1.39 (o)	34.3
8	-	41.0	H-6, 7, 9, 13, 26, 27	-	40.8
9	1.25 (m, 1H)	50.6	H-5	1.25 ((o)	50.4
10	-	37.3	H-5, 9, 25	-	37.2
11	1.41 & 1.23 (m, 2H)	21.1	H-9, 12	1.40&1.20 (o)	20.9
12	1.67 & 1.06 (m, 2H)	25.3	H-9, 13	1.62&1.06(o)	25.1
13	1.65 (m, 1H)	38.2	H-11, 12, 19, 27	1.66 (o)	38.0
14	-	43.0	H-16, 26, 27	-	42.8
15	1.69 & 1.00 (m, 2H)	27.6	H-27	1.60&1.05 (o)	27.4
16	1.46 & 1.36 (m, 2H)	35.7	H-28	1.45&1.35 (o)	35.6
17	-	43.1	H-18, 21, 22, 28	-	43.0
18	1.39 (m, 1H)	48.5	H-19, 21, 28	1.37 (o)	48.3
19	2.37 (m, 1H)	48.1	H-21, 29, 30	2.40 (o)	48.0
20	-	151.1	H-18, 19, 21, 29, 30	-	151.0
21	1.93 & 1.33 (m, 2H)	30.0	H-19, 29	1.91&1.30 (o)	29.8
22	1.39 & 1.19 (m, 2H)	40.2	H-21, 28	1.37&1.18 (o)	40.0
23	0.96 (s, 3H)	28.1	H-3, 5, 24	0.90 (s)	28.0
24	0.76 (s, 3H)	15.5	H-3, 5, 23	0.76 (s)	15.4
25	0.83 (s, 3H)	16.3	H-1, 5, 9	0.83 (s)	16.1
26	1.03 (s, 3H)	16.1	H-7, 9	1.03 (s)	16.0
27	0.94 (s, 3H)	14.7	H-13, 15	0.94 (s)	14.6
28	0.79 (s, 3H)	18.1	H-16, 22	0.79 (s)	18.0
29	4.68 & 4.56 (m, 2H)	109.5	H-19, 30	4.69&4.57 (d, J=1.9)	109.3
30	1.68 (s, 3H)	19.5	H-19, 29	1.67 (s)	19.3

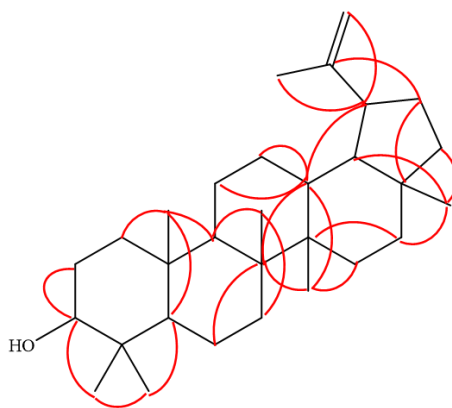


Figure 23. ^1H - ^{13}C HMBC correlations identified for assignment of compound **16**

Compound **17** and **70** were identified as a mixture. With *n*-hexane:EtOAc (4:1), the TLC exhibited a spot at an R_f of 0.5. Tetracyclic triterpenes were assigned to two groups of resonances in the NMR data (Appendices 8a to 8c). At relative amounts of approximately 72% and 28% (Appendix 8a), compound **17** was differentiated as the major compound, while compound **70** was the minor product. However, due to the similarity of the two compounds, lots of the $^1\text{H}/^{13}\text{C}$ NMR signals (Appendices 8d to 8g) of minor compound **70** were difficult to identify from that of major compound **17** signals. Furthermore, overlapping signals from compound **17** obscure most of the cross-peak NMR spectra required for the clear identification of the minor compound. As a result, the identity of the minor compound could only be identified by comparing it with the chemical (Chaturvedula & Prakash, 2012; Shwe, Thein, *et al.*, 2019). This led to the identification of compound **70** as stigmasterol. Tables 10 and 5 provide a summary of all NMR spectra data for compound **17** and compound **70** structures, respectively.

Furthermore, the cross-peak patterns (Appendices 8f and 8g) of these compounds are consistent with the reported data (Shwe, Thein, *et al.*, 2019). Although there are variations in the literature on the chemical shifts assigned for the six β -sitosterol methyl groups, the 1D NMR data recorded for compound **17** generally agree with the NMR data of β -sitosterol (Nwe *et al.*, 2019; Shwe, Thein, *et al.*, 2019). These results are especially in agreement with the NMR spectral records reported (Nwe *et al.*, 2019), except δ 21.1 ppm assigned for position 26 of the methyl group (20.0 ppm in our report). Seven signals are identified in the δ range of 11 to 23 ppm, and a signal at δ 21.2 ppm is assigned for CH_2 at location 11. Therefore, we are certain that we performed an accurate δ assignment for compound **17**. Moreover, the ^1H - ^{13}C HSQC NMR spectra (Appendices 8d and 8e) do not show any evidence of a possible CH_3 signal. Furthermore, our resonance assignment was validated by the cross-peaks observed (Figure 24 and Appendices 8d to 8g). The biological

activity of β -sitosterol has been reported widely in the literature, including antioxidant, antibacterial, and antifungal properties (Alawode *et al.*, 2021; Arivarasu, 2023; Kurniawan *et al.*, 2021; Luhata and Usuki, 2021).

In addition to the minor compound (compound **70**), in the NMR signals of compound **17**, the glycerol moiety (with ca. 10%) was detected as an impurity that is related to fatty acids. This moiety is likely glyceryl tristearate, as shown by the signals labeled "F" for "fat" in (Appendix 8d). The existence of the glyceryl moiety is suggested by cross-peaks at δ 4.08/68.5 (C-2) and 4.16/65.2 (C-1,3) ppm. Similarly, the fatty acid portion can be suggested due to the appearance of signals at δ 174.0 (C-1, 1"), 2.32/34.3, 32.0 (overlap), 1.36-1.21/29.8-29.4, 1.26/22.8, and 0.88/14.3 ppm (Kumar *et al.*, 2011).

Table 10. ^1H -NMR (400.2 MHz, CDCl_3) and ^{13}C -NMR (100.6 MHz) of compound **17** and NMR data reported for β -sitosterol (Nwe *et al.*, 2019)

C No	Compound 17			β -sitosterol/ CDCl_3 (o= overlap)	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /500 MHz	$\delta^{13}\text{C}$
1	1.85 & 1.07 (m, 2H)	37.4	H-2, 3, 19	1.85&1.08 (o)	37.4
2	1.84 & 1.51 (m, 2H)	31.8	H-1, 4	1.82&1.47 (o)	31.7
3	3.54 (m, 1H)	72.0	H-1, 2, 4	3.52 (m)	71.9
4	2.28 & 2.25 (m, 2H)	42.4		2.28&223 (m)	42.4
5	-	140.9	H-4, 7, 19	-	140.9
6	5.35 (br. d, J = 4.3, 1H)	121.9	H-4,7	6.34 (br. d, J= 4.9)	121.8
7	1.97 & 1.54 (m, 2H)	32.0		1.97&1.52 (o)	32.0
8	1.45 (m, 1H)	32.0	H-6	1.45 (o)	32.0
9	0.92 (m, 1H)	50.3	H-7, 8, 19	0.93 (o)	50.3
10	-	36.6	H-2, 4, 6, 19	-	36.6
11	1.48 (m, 2H)	21.2	H-9, 12	1.53&1.48 (o)	21.2
12	2.01 & 1.16 (m, 2H)	39.9	H-18	2.02&1.16 (o)	39.9
13	-	42.5	H-11, 12, 14, 18	-	42.4
14	0.99 (m, 1H)	56.9	H-12, 18	1.02 (o)	56.9
15	1.57 & 1.04 (m, 2H)	24.4	14, 16	1.58&1.56 (o)	24.4
16	1.84 & 1.27 (m, 2H)	28.4		1.57&1.55 (o)	28.5
17	1.10 (m, 1H)	56.2	H-14, 16, 18, 21	1.10 (o)	56.2
18	0.69 (s, 3H)	12.0	H-22	0.68 (o)	11.9
19	1.00 (s, 3H)	19.5	H-1, 9	1.00 (s)	19.5
20	1.36 (m, 1H)	36.3	H-21	1.67 (o)	36.2
21	0.92 (d, J = 6.3, 3H)	18.9	H-17, 22	0.93 (d, J= 6.3)	19.1
22	1.33 & 1.01 (m, 2H)	34.1	H-21	1.26&1.24 (o)	34.1
23	1.16 (m, 2H)	26.2	H-24, 25	1.17&1.14 (o)	26.2
24	0.93 (m, 1H)	46.0	H-25, 26, 27, 29	0.91 (o)	46.0
25	1.66 (m, 1H)	29.3	H-24, 26, 27, 28	1.28 (o)	29.3
26	0.83 (d, J = 7.5, 3H)	20.0	H-24, 25, 27	0.86 (o)	21.1
27	0.81 (d, J = 7.5, 3H)	19.2	H-24, 25, 26	0.80 (o)	18.9
28	1.25 (m, 2H)	23.2	H-24, 25, 29	1.25&1.24 (o)	23.2
29	0.84 (t, J = 7.4, 3H)	12.1	H-24, 28	0.85 (o)	12.1

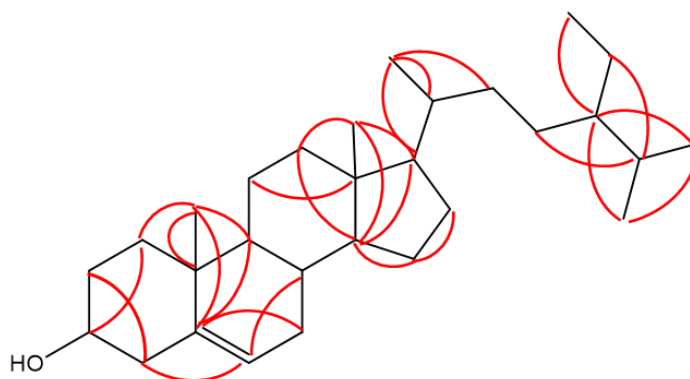


Figure 24. ^1H - ^{13}C HMBC correlations identified for the assignment of compound **17**

Compound **192** was observed as a light yellow amorphous solid and showed a TLC spot at R_f 0.5 using EtOAc:*n*-hexane (3:2).

Table 11. ^1H -NMR (400.2 MHz, CDCl_3) and ^{13}C -NMR (100.6 MHz,) of compound **192** NMR data reported for 7-keto- β -sitosterol (Oyama *et al.*, 2020)

C No	Compound 192			7-keto- β -sitosterol/ CDCl_3	
	$\delta^1\text{H}$	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /400 MHz	$\delta^{13}\text{C}$
1	1.93 (m, 2H)	36.5			36.5
2	1.57&1.64 (m, 2H)	31.3			31.4
3	3.66 (m, 1H)	70.7		3.70-3.60 (m)	70.7
4	2.39 & 2.43 (m, 2H)	41.9	C-3, 6		42.0
5	-	165.3			165.3
6	5.69 (s, 1H)	126.3	C-4, 8, 10	5.67 (br. d)	126.3
7	-	202.5			202.6
8	2.24 (m, 1H)	45.6			45.6
9	1.51 (m, 1H)	50.1			50.1
10	-	38.4			38.5
11	1.57 (m, 2H)	21.4			21.4
12	1.13 & 2.05 (m, 2H)	38.8			38.9
13	-	43.2		-	43.3
14	1.33 (m, 1H)	50.1			50.1
15	1.16 (m, 2H)	26.2			26.2
16	1.27&1.89 (m, 2H)	28.7			28.8
17	1.11 (m, 1H)	54.8			54.9
18	0.68 (3H)	12.1	C-12, C-13	0.68 (s)	12.2
19	1.20 (br s, 3H)	17.5	C-2, 5, 9, 10	1.23 (m)	17.5
20	1.37 (m, 1H)	36.2			36.3
21	0.93 (3H)	19.1	C-17, 21	0.90 (d, $J = 6.4$)	19.1
22	1.33 (m, 2H)	34.1			34.1
23	1.25 (m, 2H)	26.5	C-24, 25		26.5
24	0.93 (m, 1H)	46.0	C-22		46.0
25	1.67 (m, 1H)	29.3			29.3
26	0.82 (3H)	19.9	C-25, 27	0.83 (d, $J = 6.6$)	20.0
27	0.80 (3H)	19.2	C-26	0.81 (d, $J = 6.5$)	19.2
28	0.84 & 1.25 (m, 2H)	23.2	C-24, 25		23.2
29	0.84 (3H)	12.1	C-28	0.84 (t, $J = 7.4$)	12.3

From NMR data recorded (Appendices 9a to 9d and summarized as Table 11), a total of 47 proton signals and 29 carbon signals were resolved for compound **192** and characterized by comparing with reported NMR spectral data for 7-keto- β -sitosterol (Oyama *et al.*, 2020; Yu *et al.*, 2007).

Along with compound **192**, compound **193**, consisting of 49 proton and 29 carbon signals, was identified as a minor with a relative amount of ca. 30% (Appendix 9a) and proposed as 6-ketositostanol (one of β -sitosterol oxidation product (Massimo *et al.*, 2020)) by comparing with NMR spectral data of 7-keto-5,6-dihydro- β -sitosterol (Al-Yousef & Sheikh, 2019), β -sitosterol (Shwe, Thein, *et al.*, 2019), 7-keto- β -sitosterol (Oyama *et al.*, 2020; Yu *et al.*, 2007), and 3 β -hydroxy-5 α -cholestan-6-one (Blunt & Stothers, 1977).

Table 12. ^1H -NMR (400.2 MHz, CDCl_3) and ^{13}C -NMR (100.6 MHz,) of compound **193** and NMR data reported for 3 β -hydroxy-5 α -cholestan-6-one in CDCl_3 (Blunt & Stothers, 1977)

C No	Compound 193				3 β -hydroxy-5 α -cholestan-6-one/ CDCl_3
	$\delta^1\text{H}$	$\delta^{13}\text{C}$	^1H - ^1H COSY	^1H - ^{13}C	$\delta^{13}\text{C}$
1	1.76 & 1.80 (m, 2H)	36.8			36.7
2	1.26 (m, 2H)	31.7			30.6
3	3.57 (m, 1H)	70.9	H-4		70.4
4	1.49 & 1.92 (m, 2H)	30.2	H-3		30.0
5	2.21 (m, 1H)	56.9			56.8
6	-	211.1			210.6
7	1.95 & 2.34 (m, 2H)	46.9		C-8, 9	46.6
8	1.80 (m, 1H)	38.1			37.9
9	1.22 (m, 1H)	54.1			53.9
10	-	41.1			40.9
11	1.34 (m, 2H)	21.7			21.5
12	1.20 & 2.05 (m, 2H)	39.7		C-13	39.5
13	-	43.1			42.9
14	1.12 (m, 1H)	56.2			56.8
15	1.16 (m, 2H)	26.2			23.9
16	1.26 & 1.84 (m, 2H)	28.2			27.9
17	1.26 (m, 1H)	56.9			56.2
18	0.68 (br s, 3H)	12.2		C-13, 17	12.0
19	0.75 (br s, 3H)	13.3		C-1, 5, 9, 10	13.1
20	1.35 (m, 1H)	36.2	H-21		
21	0.93 (3H)	18.9	H-20	C-20, 22	
22	1.02 & 1.16 (m, 2H)	34.0			
23	1.25 (m, 2H)	26.5			
24	0.93 (m, 1H)	46.0			
25	1.67 (m, 1H)	29.3	H-26, 27		
26	0.82 (3H)	19.9	H-25		
27	0.82 (3H)	19.2	H-25		
28	0.84 & 1.25 (m, 2H)	23.2	H-29		
29	0.84 (3H)	12.2	H-28	C-24, 28	

The 2D NMR data (Appendices 9d-9f) were used to confirm the structures of compounds. For comparison of compound **193** (Table 12), the ^{13}C -NMR data (C-1 to C-19) reported for 3 β -hydroxy-5 α -cholestan-6-one was used (Blunt & Stothers, 1977) for which biosynthesis has been proposed (Fujimoto *et al.*, 2015). For the other carbons (C-20 to C-29), the ^{13}C -NMR spectral data reported for 7-keto- β -sitosterol were used (Oyama *et al.*, 2020) (Table 11).

Compound **42** was found as a white crystal with a melting point of 280–283 °C agrees with a reported melting point for daucosterol (Moghaddam *et al.*, 2007) (NMR data is discussed as follows). TLC using *n*-hexane:EtOAc (1:1) showed a spot with an R_f of 0.5. Compound **42** was found to have a pyranosyl moiety, as confirmed by the existence of six carbon signals with $\delta^{13}\text{C}$ 60–101 ppm (Appendices 10a to 10c). Furthermore, the occurrence of steroidal aglycon was identified using the generated NMR data, as previously discussed for compounds **17** (Table 10) shows important correlations observed in the 2D NMR spectra (Appendices 10d to 10f) in addition to all 1D NMR data used to characterize compound **42**. The NMR data generated for compound **42** is easily interpretable by comparing it with β -sitosterol-3-O- β -D-glucoside (familiar name: daucosterol). The assignments of NMR data are previously documented in the literature for $\text{CD}_3\text{OD}/\text{CDCl}_3$ (Lendl *et al.*, 2005), pyridine- d_5 (Moghaddam *et al.*, 2007), and CD_3OD (Peshin & Kar, 2017). Table 13 illustrates how well the previously published (Peshin & Kar, 2017) ^{13}C NMR chemical shifts match our NMR data, although they were recorded in CD_3OD rather than DMSO- d_6 . However, the chemical shift assigned exhibits a significantly greater difference.

When the ^{13}C NMR shifts of compound **17** given in Table 10 are compared with $\delta^{13}\text{C}$ of the triterpene aglycon part of compound **42** at positions 1 to 29 (Table 13), only the chemical shift at C-2, C-3, and C-4 exhibits a deviation $\Delta\delta^{13}\text{C}$ of greater than 1 ppm. These resonances are connected to the glucose moiety through position 3. Furthermore, this suggests that the structure of compound **42** was correctly identified (Figure 25). As mentioned in the literature (Peshin & Kar, 2017), another way to further establish the existence of aglycon, compound **17**, in compound **42** is to hydrolyze the glucose moiety. Daucosterol has been reported due to its various biological activities, including antioxidant, immunoregulatory, and anticancer activities (Abdollahnezhad *et al.*, 2020; Lee *et al.*, 2007; Rajavel *et al.*, 2017).

Table 13. ¹H-NMR (400.2 MHz) and ¹³C-NMR (100.6 MHz) of compound **42** and NMR data reported for daucosterol (Peshin & Kar, 2017)

C-No	Compound 42 /DMSO-d ₆			Daucosterol/ CD ₃ OD	
	δ ¹ H (J in Hz)	δ ¹³ C	¹ H- ¹³ C HMBC	δ ¹ H/400 MHz	δ ¹³ C.
1	1.80 & 0.99 (m, 2H)	36.8		1.40&1.00 (m)	36.8
2	1.81 & 1.48 (m, 2H)	29.3		1.58&1.26 (m)	29.2
3	3.46 (1H)	76.9	H-1'	2.98 (m)	76.9
4	2.36 & 2.12 (m, 2H)	38.3		2.26&1.98 (m)	39.3
5	-	140.5	H-1, 4, 9	-	140.4
6	5.33 (m, 1H)	121.2		5.35 (t, J = 3.6)	121.2
7	1.92 & 1.52 (m, 2H)	31.4		1.95&1.73 (m)	31.4
8	1.43 (m, 1H)	31.4		1.36 (m)	31.3
9	0.89 (m, 1H)	49.6		0.85 (m)	49.6
10	-	36.2	H-9, 4, 19	-	36.2
11	1.45 (m, 2H)	20.6		1.42 (m)	20.6
12	1.96 & 1.14 (m, 2H)	38.9		1.52&1.20 (m)	38.3
13	-	41.9	H-15, 18	-	41.8
14	0.98 (m, 1H)	56.2		0.95 (m)	56.1
15	1.54 & 1.04 (m, 2H)	23.9		1.57&1.05 (m)	23.8
16	1.79 & 1.24 (m, 2H)	27.8		1.85&1.25 (m)	27.8
17	1.10 (m, 1H)	55.4		1.20 (m)	55.4
18	0.65 (s, 3H)	11.7		0.70 (s)	11.6
19	0.95 (s, 3H)	19.1		0.94 (s)	19.1
20	1.34 (m, 1H)	35.5		1.40 (m)	35.5
21	0.90 (d, J = 6.6 Hz, 3H)	18.6		0.95 (d, J = 6.5)	18.6
22	1.32 & 1.00 (m, 2H)	33.4		1.20 (m)	33.3
23	1.14 (m, 2H)	25.4		1.25 (m)	25.4
24	0.91 (m, 1H)	45.2		0.94 (m)	45.1
25	1.63 (m, 1H)	28.7		1.68 (m)	28.6
26	0.81 (d, J = 7.0, 3H)	19.7		0.87 (d, J = 7.0)	19.7
27	0.80 (d, J = 7.0, 3H)	18.9		0.88 (d, J = 7.0)	18.9
28	1.23 (m, 2H)	22.6		1.30 (m)	22.1
29	0.82 (t, J = 7.0, 3H)	11.8		0.97 (t, J = 7.5)	11.8
1'	4.22 (d, J = 7.6 Hz, 1H)	100.8	H-2',	4.20 (d, J = 7.9)	100.7
2'	2.90 (m, 1H)	73.4	2'-OH, 3'-OH	2.89 (m)	73.4
3'	3.13 (m, 1H)	76.8	H-2', 4'	3.27 (m)	76.9
			H-5', 2'-OH, 3'-OH, 4'-OH		
4'	3.03 (m, 1H)	70.1		3.00 (m)	70
5'	3.05 (m, 1H)	76.7	H-4'	3.06 (m)	76.7
				4.55 (dd, J = 11.8, 2.5)	
6'	3.64 & 3.41 (m, 2H)	61.1	6'-OH	44.40 (dd, J = 11.8, 5.2)	62.8
2'-OH	4.85 (1H)			3.56 (d, J = 4.5)	
3'-OH	4.87 (1H)			3.5 (d, J = 4.5)	
4'-OH	4.85 (1H)			3.40 (d, J = 4.5)	
6'-OH	4.41 (1H)			3.60 (t, J = 4.5)	

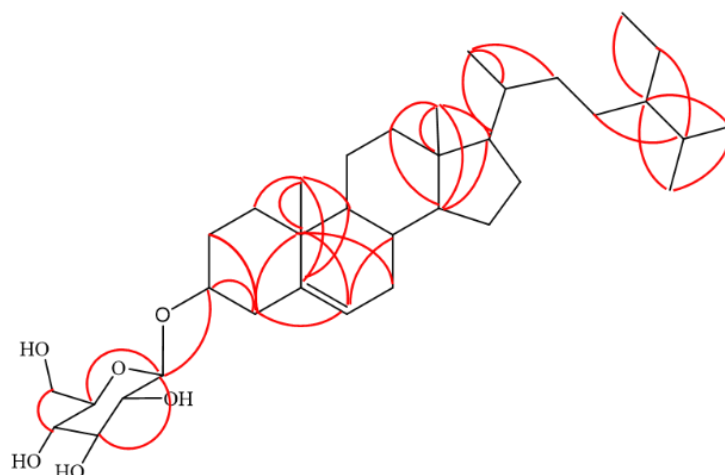


Figure 25. ^1H - ^{13}C HMBC correlations identified for the assignment of compound **42**

Compound **65** was found as a white amorphous solid with a TLC spot at an R_f of 0.5 using an *n*-hexane:EtOAc (1:4) as a mobile phase. Table 14 presents the summary of the NMR data of compound **65**.

Table 14. ^1H -NMR (400 MHz, DMSO- d_6) and ^{13}C -NMR (101 MHz) of compound **65** and NMR data reported for catechin (Ahmed *et al.*, 2020)

C-No	Compound 65	^{13}C -NMR		Catechin/DMSO- d_6	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}/500$ MHz	$\delta^{13}\text{C}$
1		-			-
2	4.47 (d, J = 7.5 Hz, 1H)	81.0	C-1', 2', 3, 4, 6', 9	4.48 (d, J = 7.5)	81.4
3	3.80 (m, 1H)	66.3		3.82 (q, J = 7.2)	66.8
4	2.34 (dd, J = 16.1, 7.9 Hz, 1H) 2.65 (dd, J = 15.9, 10.2 Hz, 1H)	27.9	C-3, 2, 9, 10	2.35 (dd, J = 16.0, 8.1)	28.2
5	-	156.2		-	156.6
6	5.70 (d, J = 2.2 Hz, 1H)	93.8	C-8	5.69 (s)	95.6
7	-	156.5		-	156.9
8	5.89 (d, 2.2 Hz, 1H)	95.1	C-6, 10, 5, 7, 9	5.89 (s)	94.4
9	-	155.4		-	155.8
10	-	99.1		-	99.6
1'	-	130.6		-	131.1
2'	6.72 (d, 2.0 Hz, 1H)	114.5	C-3', 4'	6.72 (d, J = 2.1)	114.9
3'	-	144.9		-	145.3
4'	-	144.9		-	145.3
5'	6.68 (d, J = 8.0 Hz, 1H)	115.1	C-1'	6.69 (d, J = 8.0)	115.6
6'	6.59 (dd, J = 8.0, 2.0 Hz, 1H)	118.4	C-3', 4'	6.60 (dd, J = 8.2, 2.0)	118.9
3-OH	4.87 (d, J = 5.2 Hz, 1H)	-	C-2, 3, 4		-
5-OH	9.18 (s, 1H)	-	C-5, 6, 10		-
7-OH	8.94 (s, 1H)	-	C-6, 7, 8		-
3'-OH	8.87 (s, 1H)	-	C-, 2', 3'		-
4'-OH	8.82 (s, 1H)	-	C-4', 5'		-

The ^1H -NMR spectral data (Appendix 11a) showed the presence of 14 protons. Two protons of sp^3 oxygenated carbons at δ 4.47 (1H, d, J = 7.6 Hz, H-2) and 3.80 (1H, m, H-3) were correlated with C-2 and C-3, respectively. The two diastereotopic protons observed at 2.34 (dd, J = 15.9, 5.1 Hz, 1H) and 2.65 (dd, J = 16.1, 8.0 Hz, 1H) were assigned to C-4. The presence of meta protons at δ 5.70 (1H, d, J = 2.2 Hz, H-6) and 5.89 (1H, d, J = 2.2 Hz, H-8) were correlated with C-6 and C-8, respectively, indicating the presence of quaternary carbon, C-7. The appearance of a doublet of a doublet at 6.59 (1H, dd, J = 8.0, 2.0 Hz, H-6') suggested the presence of a meta proton at 6.72 (1H, d, J = 2.0 Hz, H-2') and an ortho proton at 6.68 (1H, d, J = 8.0 Hz, H-5'). This, in turn, suggested the presence of quaternary carbons, C-3' and C-4'. The rest of the five-proton signals at 4.87, 8.82, 8.87, 8.94, and 9.18 ppm were assigned to hydroxyl groups. 15 carbon signals (Appendices 10b and 10c) were identified and summarized in Table 14. The structure of compound **65** was proposed as (+)-catechin (Ahmed *et al.*, 2020; Hong *et al.*, 2017). Furthermore, the 2D NMR spectral data (Appendices 11d to 11f) were used for confirmation. As a minor, the NMR spectra showed the presence of epicatechin (**69**) (Ahmed *et al.*, 2020) in ca. 6.5%, calculated from ^1H NMR spectra (Appendix 11a). Catechin has been identified for various biological activities. Some of these are antioxidant, antibacterial, and antifungal activities (Nuryana *et al.*, 2021; Sitheeque *et al.*, 2009).

4.1.7 Characterization of isolated compounds from *R. ruspolii*. Eight compounds, including palmitic acid (**145**), 3,4-hydroxybenzoic acid (**194**), myricetin (**55**), cupressuflavone (**195**), amentoflavone (**62**), shikimic acid (**196**), avicularin (**197**), and myricetin-3-O-5''-acetylarabinofuranoside (**198**) (Figures 9, 16, and 26), were isolated and characterized from the leaves of *R. ruspolii* based on their NMR data.

Compound **145** was isolated as a white crystalline solid with a melting point of 62–64 °C (Soliman & El-Shattory, 2017). TLC showed an R_f value of 0.3 using *n*-hexane:EtOAc (4:1). The ^1H NMR spectrum (Appendix 12a) revealed two triplet signals at δ 0.84 (t, J = 7.3 Hz, 3H) and 2.16 (t, J = 7.4 Hz, 2H), a further signal assigned to methylene protons is observed at 1.45 (m, 2H), and a broad peak representing multiple methylene groups is evident at 1.17-1.31 ppm. In the ^{13}C NMR spectra (including DEPT and HSQC, Appendices 12b-12d), a quaternary carbon at 174.5 ppm, one methyl group at 13.9 ppm, and CH_2 carbons at 33.7, 31.3, 24.5, and 21.1 ppm are observed along with some additional unresolved methylene resonances between 28.7-29.0 ppm. Based on the chemical shifts observed in the ^1H and ^{13}C NMR spectra, it is evident that compound **145** is a fatty acid.

Considering the observed melting point and the integration, which accounts for 12 methylene groups within the signal cluster at 1.17-1.31 ppm, compound **145** was identified as palmitic acid. Despite minor δ deviations due to solvent differences, the NMR data matched those reported for palmitic acid in CDCl_3 (Ekeocha *et al.*, 2021; National Institute of Advanced Industrial Science and Technology (AIST), 1999, in our case: DMSO-d_6).

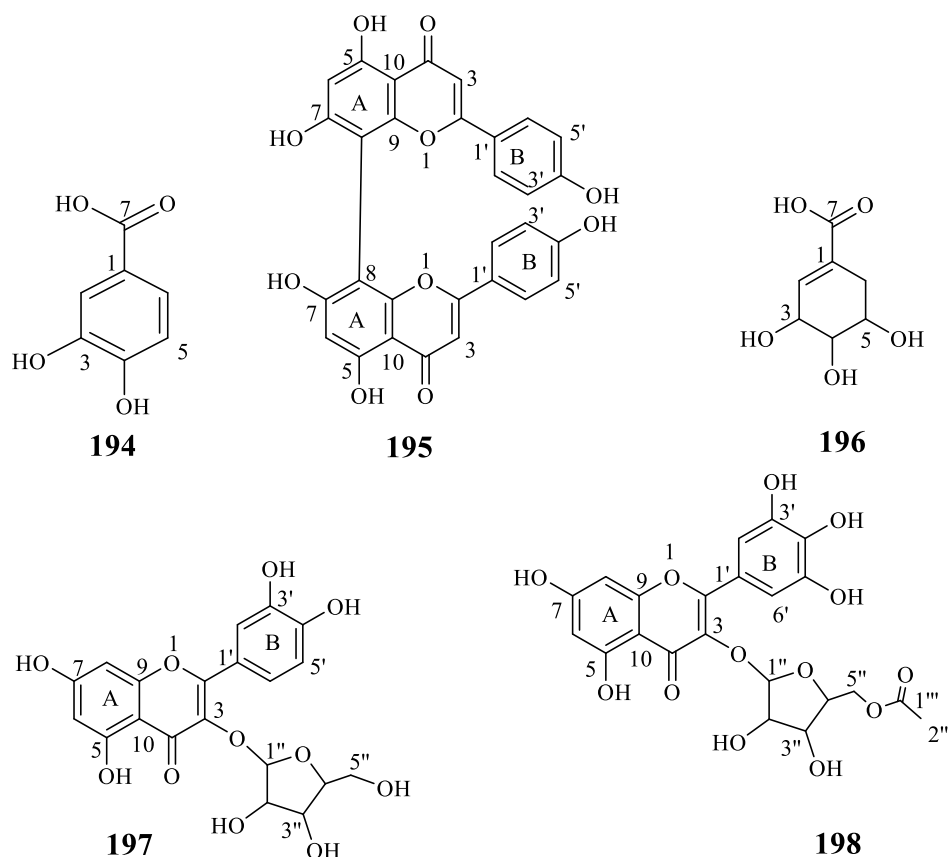


Figure 26. Structures of isolated compounds from leaves of *R. ruspolii*

Compound **194** was isolated as a white crystalline solid with a melting point of 219-222 °C. Using *n*-hexane:EtOAc (1:1), the TLC showed a spot at an R_f value of 0.6. Two signals are observed in the aromatic chemical shift region of the ^1H NMR spectrum, with an intensity ratio of 2:1 (Appendix 13a). The resonances at δ 7.5-7.4 ppm appear heavily overlapped without a defined coupling pattern, while a doublet at δ 6.80 ($J = 8.5$ Hz) is also evident. In the ^{13}C in combination with the DEPT-135 NMR spectra (Appendices 13b and 13c) of compound **194**, the presence of three methine carbons can be recognized at δ 123.9, 117.7, and 115.8 ppm and of four quaternary carbons at δ 170.3, 151.5, 146.0, and 123.1 ppm. Upon analysis of the ^1H and ^{13}C NMR chemical shifts, it becomes apparent that compound **194** features an aromatic structure with three substituents. Notably, one of these substituents is identified as a carboxyl group ($\delta^{13}\text{C} = 170.3$ ppm).

Table 15. ^1H -NMR (400.2 MHz) and ^{13}C -NMR (100.6 MHz) of compound **194** and NMR data reported for 3,4-dihydroxybenzoic acid (Nguyen *et al.*, 2013)

C-No	Compound 194 /CD ₃ OD		3,4-dihydroxybenzoic acid/ CD ₃ OD		
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /500 MHz	$\delta^{13}\text{C}$
1	-	123.1		-	123.9
2	7.44 (s, 1H)	117.7	C-1, 3, 6, 7	7.43 (s)	117.9
3	-	146.0		-	146.2
4	-	151.5		-	151.5
5	6.80 (d, J = 8.5, 1H)	115.8	C-1, 2, 3, 4, 7	6.78 (d, J = 8.4)	115.8
6	7.42 (1H)	123.9	C-2, 3, 5	7.42 (d, J = 8.4)	123.8
7	-	170.3		-	171.0

Moreover, the presence of two neighboring methine CH groups is suggested by the J = 8.5 Hz coupling constant observed in the ^1H NMR spectrum. Additionally, the ^{13}C NMR spectrum reveals six distinct carbon resonances, indicating the asymmetric nature of the chemical structure. If in addition to the carboxyl group two hydroxyl groups (OH) can be considered as second and third substituents ($\delta^{13}\text{C}$ of the quaternary carbons at 151.5 and 146.0 ppm), only two compounds, namely 3,4- or 2,5-dihydroxybenzoic acid, would come into question. Despite the unfortunate overlap of two out of the three protons in compound **194**, the chemical structure of 3,4-dihydroxybenzoic acid was successfully elucidated through analysis of the 2D NMR spectral data (Appendices 13d-13f, Table 15). The NMR data of compound **194** agrees with the NMR data reported in the literature for 3,4-dihydroxybenzoic acid (Nguyen *et al.*, 2013). Compound **194** has been found to have pharmacological properties such as anti-inflammatory, neuroprotective, antidiabetic, and antihypertensive (Adedara *et al.*, 2019; Gallardo-Fernández *et al.*, 2019; Safaeian *et al.*, 2018; H. Wang *et al.*, 2015).

Compound **55** was isolated as a yellow solid with a melting point of 354-356 °C. TLC showed a spot at an R_f value of 0.5 using *n*-hexane:EtOAc (1:1) as the mobile phase. The presence of meta coupled protons at δ 6.18 and 6.38 (d, J = 2.1 Hz, 2H) (^1H -NMR spectrum, Appendix 14a) is evident for the presence of ring A of compound **55**. The symmetric singlet appeared at δ 7.36 (2H, H-2', H-6'), confirming the existence of the skeleton of ring B. The ^{13}C -NMR spectrum (Appendix 14b) together with DEPT-135 (Appendix 14c) demonstrated the presence of 15 carbon resonances, including four methines (two are symmetrical) and 11 quaternary (two are symmetrical) (Table 16). The NMR data of compound **55** was also compared with the spectral data reported in the literature for myricetin and found in good agreement (Elshamy *et al.*, 2020). Furthermore, the structure of compound **55** was confirmed using 2D-NMR techniques such as HSQC,

HMBC, and ^1H - ^1H COSY (Appendices 14d to 14f). The signal observed in the NMR spectral data of compound **55** at 1.29/30.9 might be an overlap of signals from fatty acids (Ekeocha *et al.*, 2021) and sterols (Shwe, Thein, *et al.*, 2019). Compound **55** has been shown to possess anti-inflammatory, anti-tumor, anti-diabetic, and anti-cancer properties (Chao *et al.*, 2017; Cho *et al.*, 2016; Ha *et al.*, 2017; Meng *et al.*, 2016).

Table 16. ^1H -NMR (400.2 MHz) and ^{13}C -NMR (100.6 MHz) of compound **55** and NMR data reported for myricetin (Elshamy *et al.*, 2020)

C-No	Compound 55 /CD ₃ OD			Myricetin/CD ₃ OD	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /600 MHz	$\delta^{13}\text{C}$
1	-	-		-	-
2	-	148.0		-	146.9
3	-	137.4		-	136.0
4	-	177.3		-	176.0
5	-	162.5		-	161.0
6	6.18 (d, $J = 2.1$, 1H)	99.2	C-5, 7, 8, 10	6.18 (d, $J = 2.5$)	98.3
7	-	165.6		-	164.3
8	6.38 (d, $J = 2.1$, 1H)	94.4	C-6, 7, 9, 10	6.47 (d, $J = 1.7$)	93.4
9	-	158.2		-	156.2
10	-	104.5		-	103.1
1'	-	123.1		-	120.6
2'	7.36 (s, 1H)	108.5	C-2, 1', 3', 4', 6'	7.31 (br. s)	107.5
3'	-	146.7		-	145.7
4'	-	136.9		-	135.8
5'	-	146.7		-	145.7
6'	7.36 (s, 1H)	108.5	C-2, 1', 3', 4', 2'	7.31 (br. S)	107.5

Compound **195** was isolated as a yellow powder. TLC showed a spot at an R_f value of 0.5 using *n*-hexane:EtOAc (1:1) as a mobile phase. The ^1H -NMR spectral data (Appendix 15a) of compound **195** indicated the presence of a singlet signal at δ 6.46 (2H, H-6), which showed ^1H - ^{13}C HMBC correlation (Appendix 15e) with quaternary carbons (Appendices 15b and 15c) at δ 160.9 (C-5), 163 (C-7), 98.6 (C-8), and 103.7 (C-10). This is evidence for the presence of pentasubstituted A rings of flavonoids containing the substituent at position 8 due to the coupling of A rings through C-8 as confirmed by the appearance of a cross peak at δ 6.46/98.6 (H-6/C-8). Furthermore, the presence of a disubstituted pattern of rings B of compound **195** was confirmed by the appearance of symmetrical protons at δ 7.49 (4H, dd, $J = 8.5$, 2.2, H-2', 6') and 6.74 (4H, dd, $J = 8.5$, 2.2, H-3', 5'), which showed clear ^1H - ^{13}C HMBC correlation with quaternary carbons at δ 161.1 (C-4') and 121.2 (C-1'), respectively. Due to the two symmetrical carbon signals appearing at δ 115.8 (C-2' and C-3') and 128.0 (C-3' and C-5'), from ^{13}C NMR (Appendix 15b) and DEPT-135 NMR (Appendix 15c) spectral data, only 13 (26 for the two halves) signals representing 30 carbons were identified, including 12 methine and 18 quaternary carbons. The 2D NMR spectral (Appendices 15d-

15f) data were analyzed and used for structural confirmation. The NMR data is summarized in Table 17 and agrees with the NMR data reported in the literature for cupressuflavone (Chari *et al.*, 1977; Sani *et al.*, 2011). It has been reported that compound **195** possesses analgesic, antiinflammatory, antinephrotoxic, and antihepatotoxic properties (Al-Sayed *et al.*, 2018; Al-Sayed & Abdel-Daim, 2014).

Table 17. ^1H -NMR (400.2 MHz, DMSO- d_6) and ^{13}C -NMR (100.6 MHz) of compound **195** and NMR data reported for cupressuflavone (Sani *et al.*, 2011; Umar *et al.*, 2023)

C-No	Compound 195			Cupressuflavone/DMSO- d_6	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /500 MHz	$\delta^{13}\text{C}$
2	-	163.6		-	163.9
3	6.79 (s, 2H)	102.6	C-1', 2, 4, 10	6.78 (s)	102.8
4	-	182.1		-	182.1
5	-	160.9		-	161.3
6	6.46 (s, 2H)	98.7	C-5, 7, 8, 10	6.44 (s)	99.0
7	-	163.0		-	162.7
8	-	98.6		-	98.7
9	-	154.8		-	155.3
10	-	103.7		-	104.3
1'	-	121.2		-	121.7
2'/6'	7.49 (dd, $J = 8.5, 2.2, 4\text{H}$)	128.0	C-2, 2', 4', 6'	7.50 (dd, $J = 8.8$)	127.9
3'/5'	6.74 (dd, $J = 8.5, 2.2, 4\text{H}$)	115.8	C-1', 4'	6.75 (d, $J = 8.8$)	116.1
4'	-	161.1		-	161.1
HO-5	13.15 (s, 2H)	-	C-5, 7, 10	13.16 (s)	

Furthermore, the NMR spectral data of compound **195** showed the presence of minor peaks identified using NMR as compound **62**. Therefore, as can be seen from ^1H -NMR (Appendix 15a), the meta-coupled protons at δ 6.19 (d, $J = 2.4$, 1H) and 6.45 (d, $J = 2.4$, 1H) showed ^1H - ^{13}C HMBC correlation (Appendix 15e) with quaternary carbons (Appendices 15b and 15c) at δ 103.7 (C-10) and 157.4 (C-9), respectively, indicating the presence of tetrasubstituted ring A of compound **62**. The doublet of a doublet at δ 7.99 ($J = 8.7, 2.4$, 1H, H-6') was due to the coupling with ortho proton at δ 7.14 (H-5', $J = 8.7$ Hz) and meta proton at 8.03 (H-2', $J = 2.4$ Hz). This supports the presence of trisubstituted ring B of compound **62**. Moreover, the singlet at δ 6.37 (1H, H-6'') confirmed the absence of the proton H-8'' in ring A' of compound **62**, which is evident in the coupling of ring B/A' of compound **62** through C-3' and C-8'' and further confirmed by the 8.03/104.3 (H-2'/C-8'') ^1H - ^{13}C HBC cross peak. In addition, the symmetric protons at δ 7.58 (dd, $J = 8.8, 2.4$, 2H, H-2''', 6''') and 6.70 (dd, $J = 8.8, 2.4$, 2H, H-3''', 5''') confirm the existence of the skeleton of disubstituted ring B' of compound **62**. Due to two symmetrical carbon signals at δ 115.7 (C-3''', C-5''') and 128.2 (C-2''', C-6''') and one carbon signal (C-7'', weak signal) observed only in the ^1H - ^{13}C HMBC cross peak at 6.37/162.8 (H-6''/C-7'') (Appendix 15e),

from ^{13}C -NMR and DEPT-135 (Appendices 15b and 15c), only 27 carbon signals were resolved, including three carbon signals overlapped with carbon signals of compound **195** (Table 17) at 102.6 (C-3''), 103.7 (C-10), and 182.1 (C-4'').

Table 18. ^1H -NMR (400.2 MHz, DMSO- d_6) and ^{13}C -NMR (100.6 MHz) of compound **62** and NMR data reported for amentoflavone (Ambarwati *et al.*, 2022; R. Markham *et al.*, 1987).

C-No	Compound 62	Amentoflavone			
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /600 MHz/ acetone- d_6	$\delta^{13}\text{C}$ / DMSO- d_6
1/1''	-	-			-
2/2''	-	163.9/164.1			164.1/164.3
3/3''	6.83 (s, 1H)/ 6.78 (s, 1H)	102.9/102.6	C- 10/10''	6.65 (s)/6.71 (s)	103.2/102.8
4/4''	-	181.7/182.1		-	181.9/182.2
5/5''	-	161.4/160.5		-	161.6/160.8
6/6''	6.19 (d, $J = 2.4$, 1H)/ 6.37 (s, 1H)	98.8/98.9	C-10/8'', 10''	6.23(d, $J = 2.0$)/ 6.41	98.8/99.1
7/7''	-	163.6/161.4		-	163.9/161.9
8/8''	6.45 (d, $J = 2.4$, 1H)/-	94.0/104.3	C-10/-	6.47 (d, $J = 2.0$)/-	94.2/104.1
9/9''	-	157.4/154.5			157.6/154.7
10/10''	-	103.7/103.5			104.0/104.0
1'/1'''	-	120.6/121.4			120.3/121.4
2'/2'''	8.03 (d, $J = 2.4$, 1H)/ 7.58 (dd, $J = 8.8$, 2.4, 1H)	131.4/128.2	C- 8''/6'''	8.19 (d, $J = 2.0$)/ 7.68 (d, $J = 8.2$)	127.9/128.3
3'/3'''	-/6.70 (dd, $J = 8.8$, 2.4, 1H)	120.3/115.7	-/C-5'''	-/6.78 (d, $J = 8.8$)	121.7/116.0
4'/4'''	-	160.1/161.0		-	159.6/161.1
5'/5'''	7.13 (d, $J = 8.7$, 1H)/ 6.70 (dd, $J = 8.8$, 2.4, 1H)	116.5/115.7	C-1'/1'''	7.18 (d, $J =$ 8.6)/6.78 (d, $J =$ 8.2)	116.4/116.0
6'/6'''	7.99 (dd, $J = 8.7$, 2.4, 1H)/ 7.58 (dd, $J = 8.8$, 2.4, 1H)	127.7/128.2	C-2'/2'''	7.98 (dd, $J = 8.6$, 2.3)/7.68 (d, $J =$ 8.2)	131.6/128.3
OH-5/ OH-5''	12.98 (s, 1H)/ 13.11 (s, 1H)	-			-

Even though some deviation was observed, the NMR spectral data of compound **62** was compared with the NMR data reported for amentoflavone in acetone- d_6 and DMSO- d_6 (Ambarwati *et al.*, 2022; R. Markham *et al.*, 1987) (Table 18). 2D NMR spectral data (appendices 15d-15f) were analyzed and used for confirmation of the structure of compound **62**. Antioxidant, anti-inflammatory, anticancer, antidiabetic, and antiviral properties have all been documented for compound **62** (Arwa *et al.*, 2015; Laishram *et al.*, 2015; Pei *et al.*, 2012; Wilsky *et al.*, 2012). From the peak area (Appendix 15a) of the symmetrical hydroxyl protons of compound **195** at δ 13.15 and hydroxyl protons of compound **62** at δ 12.98 or

13.11, the percentage was calculated as ca. 62% and 38% for compounds **195** and **62**, respectively.

Compound **196** was isolated as a white crystalline solid showing a melting point of 183-185 °C that agrees with reported data for shikimic acid (Shrestha *et al.*, 2013). NMR data is described below. The TLC displayed a spot at an R_f value of 0.4 using 49:1 (EtOAc:MeOH). The ^1H -NMR spectrum (Appendix 16a) showed the presence of an olefinic proton signal at δ 6.57 (d, J = 2.4 Hz, H-2), three protons of sp^3 oxygenated carbon, including an allylic proton at δ 4.20 (m, H-3), a doublet of doublet at 3.55 (J = 4.3, 1.5, H-4), and a multiplet at 3.53 (H-5). In addition, the diastereotopic protons (H-6) were identified at δ 2.42 and 2.38. The ^{13}C -NMR (Appendix 16b) with the help of DEPT-135 (Appendix 16c) showed a total of seven carbon signals with two quaternary carbon signals at δ 138.9 (C-1) and 168.1 (C-7), four methine carbons at δ 128.4 (C-2), 65.9 (C-3), 70.4 (C-4), and 66.9 (C-5), and a methylene carbon at 30.0 (C-6). The NMR spectral data for compound **196** agreed with the NMR data for shikimic acid reported (Shrestha *et al.*, 2013) and summarized in Table 19. The chemical structure of compound **196** was confirmed using 2D NMR spectral data (Appendices 16d-16f). It is well known that compound **196** has neuroprotective, anti-thrombogenic, anti-platelet, and anti-inflammatory qualities (Li *et al.*, 2023; Rabelo *et al.*, 2015; Veach *et al.*, 2016).

Table 19. ^1H -NMR (400.2 MHz, DMSO- d_6) and ^{13}C -NMR (100.6 MHz) of compound **196** and NMR data reported for shikimic acid (Shrestha *et al.*, 2013)

C-No	Compound 196		Shikimic acid/DMSO- d_6		
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ / 400 MHz	$\delta^{13}\text{C}$
1	-	128.4		-	128.3
2	6.56 (d, J = 1.5, 1H)	138.9	C-1, 4, 6, 7	6.35 (m)	138.6
3	4.19 (m, 1H)	65.6	C-1, 2	4.31 (dd, J = 4.6, 4.6)	65.4
4	3.53 (dd, J = 5.7, 4.2, 1H)	70.4	C-2, 3, 5, 6	3.62 (dd, J = 9.2, 4.6)	70.3
5	3.82 (m, 1H)	66.9	C-1, 3, 4	3.90 (m)	66.7
6	2.39/2.00 (m, 2H)	30.0	C-1,2, 4, 5, 7	2.68 m) 2.11 (m)	29.9
7	-	168.1		-	167.9

Compound **197** was isolated as a white solid with a melting point of 214-216 °C, which agrees with the literature report (Zhang *et al.*, 2021). The TLC gave a spot at an R_f value of 0.5 using n -hexane:EtOAc (2:3) as the mobile phase. From the ^1H -NMR spectrum (Appendix 17a), the two doublet signals due to meta protons at δ 6.21 and 6.39 (J = 1.7 Hz, 2H, H-6, 8) and their ^1H - ^{13}C HMBC cross peaks (Appendix 17e) with four different

quaternary carbons (Appendices 17b and 17c) at δ 6.21/163.1 (H-6/C-5), 6.21/166.1 (H-6/C-7), 6.39/158.6 (H-8/C-9), and 6.39/105.6 (H-8/C-10) confirm the existence of the tetrasubstituted ring A for compound **197**. The coupling constant $J = 8.2$ and 2.1 Hz at δ 7.49 (H-6') indicates that H-6' is coupled with ortho proton (H-5', $J = 8.2$ Hz) at 6.90 and meta proton (H-2', $J = 2.1$ Hz) at 7.53, and in addition, the cross peak of these protons with three different quaternary carbons signals at 6.90/123.1, 146.4 (H-5'/C-1', 3'), and 7.49/149.8 (H-6'/C-4') confirms the presence of trisubstituted ring B for compound **197**. Totally, from ^{13}C and DEPT-135 NMR spectra (Appendices 17b and 17c), 20 carbon signals have been differentiated for compound **197**. Furanose is attached to C-3 of compound **197** as confirmed by the ^1H - ^{13}C HMBC cross peak at δ 5.47/134.9 (H-1''/C-3). The NMR data of compound **197** is summarized in Table 20 along with NMR data for a known compound called avicularin (Ugheighele *et al.*, 2022). 2D NMR spectral data (Appendices 17d-17f) were used to confirm the chemical structure of compound **197**. Compound **197** is claimed to exhibit significant properties such as antidiabetic, anticancer, anti-inflammatory, and anti-Alzheimer effects (Nor *et al.*, 2023; Samant & Gupta, 2022; Wang *et al.*, 2019; Zou *et al.*, 2021).

Table 20. Chemical shift assignments of ^1H (400.2 MHz) and ^{13}C (100.6 MHz) of compound **197** and NMR data reported for avicularin (Ugheighele *et al.*, 2022)

C-No	Compound 197 /CD ₃ OD			Avicularin/CD ₃ OD	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /400 MHz	$\delta^{13}\text{C}$
2	-	159.3		-	158.9
3	-	134.9		-	134.5
4	-	180.0		-	179.6
5	-	163.1		-	162.9
6	6.21 (d, $J = 1.7$, 1H)	99.9	C-5, 7, 8, 10	6.12 (d, $J = 2.0$)	101.1
7	-	166.1		-	169.8
8	6.39 (d, $J = 1.7$, 1H)	94.8	C-6, 7, 9, 10	6.28 (d, $J = 2.0$)	95.6
9	-	158.6		-	158.9
10	-	105.6		-	104.5
1'	-	123.1		-	123.1
2'	7.53 (d, $J = 2.3$, 1H)	116.8	C-2, 4', 6'	7.48 (d, $J = 1.2$)	116.6
3'	-	146.4		-	146.5
4'	-	149.8		-	150.1
5'	6.90 (d, $J = 8.2$, 1H)	116.4	C-1', 3'	6.88 (d, $J = 8.4$)	116.4
6'	7.49 (dd, $J = 8.2$, 2.1, 1H)	123.0	C-2', 4',	7.45 (dd, $J = 8.4$, 1.2)	122.9
1''	5.47 (br. s, 1H)	109.5	C-3, 4''	5.42 (s)	109.4
2''	4.33 (m, 1H)	83.3		4.31 (d, $J = 1.6$)	83.2
3''	3.91 (m, 1H)	78.7		3.87 (m)	78.9
4''	3.88 (m, 1H)	88.0		3.87 (m)	88.1
5''	3.51 (m, 2H)	62.5		3.49 (t, $J = 2.4$)	62.6

As a minor, the NMR data showed the presence of compound **198**, and therefore, as shown on the ^1H NMR data (Appendix 17a), the coupling constant observed at $J = 1.7$ Hz represents the meta protons (H-6 and H-8) of ring A of compound **198**. In another way, the symmetrical singlet proton at δ 7.10 (H-2', 6') showed a ^1H - ^{13}C HMBC cross peak with C-1', C-3', and C-4' quaternary carbons (Table 21), confirming the presence of the tetra-substituted ring B of compound **198**. Totally, from ^{13}C and DEPT-135 (Appendices 17b and 17c), due to two symmetrical carbon signals appearing at δ 109.6 (C-2' and C-6') and 146.8 (C-3' and C-6'), only 20 carbon signals, including four overlapped signals with carbon signals of compound **197** at δ 99.4 (C-4), 99.9 (C-6), 158.6 (C-9), and 163.1 (C-5), were resolved, representing 22 carbons of compound **198**. From the ^1H - ^{13}C HMBC cross peak (Appendix 17e), it was confirmed that the sugar moiety (H-1'') is attached to C-3 of compound **198** at δ 5.41/134.9. In addition, the ^1H - ^{13}C HMBC cross peak was observed at 3.91, 4.10/172.6 (H-5''/C-1''') (Appendix 17e). This is evidence that the position of the acetyl group might be on C-5'' of the furanose of compound **198**.

Table 21. ^1H -NMR (400.2 MHz) and ^{13}C -NMR (100.6 MHz) of compound **198** and NMR data reported (Mohamed *et al.*, 2006)

C-No	Compound 198 /CD ₃ OD			Myricetin-3-O- α -L-5''-acetyl arabinofuranoside/DMSO-d ₆	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /300 MHz	$\delta^{13}\text{C}$
1	-	-			-
2		159.9			157.8
3		134.9			133.3
4		179.7			177.6
5		163.1			161.2
6	6.21 (d, $J = 1.7$, 1H)	99.9	C-8, 10	6.20 ($J = 2.1$)	98.8
7		166.2			164.2
8	6.39 (d, $J = 1.7$, 1H)	94.8	C-6, 10	6.40 (d, $J = 2.1$)	93.7
9		158.6			156.5
10		105.6			104.2
1'		122.0			119.9
2'	7.10 (s, 1H)	109.6	C-2, 4', 6'	7.04 (s)	108.2
3'	-	146.8			145.5
4'		137.9			136.5
5'	-	146.8			145.5
6'	7.10 (s, 1H)	109.6	C-2, 2', 4'	7.04 (s)	108.2
1''	5.41 (br. s, 1H)	109.6	C-3, 4''	5.42 (br. s)	107.8
2''	4.37 (m, 1H)	83.6			81.7
3''	3.80 (m, 1H)	79.3			77.6
4''	3.91 (1H)	84.2	C-2'''		82.0
5''	4.10 (1H)	65.0			63.3
	3.93 (1H)				
1'''	-	172.6			170.2
2'''	1.96 (s, 3H)	20.6	C-1'''	1.89 (s)	20.4

In addition, it is evident that compound **198** is different from myricetin-3-O- α -L-3''-acetylarabinofuranoside (Torres-Mendoza *et al.*, 2006). In compound **198**, unlike in myricetin-3-O- α -L-3''-acetylarabinofuranoside, the appearance of a downfield signal at 65.0 (C-5'') and an upfield signal at 84.2 (C-2'') further confirms the proposed structure of compound **198**. As a result, compound **198** was proposed as myricetin-3-O-5''-acetylarabinofuranoside. All NMR spectral data of compound **198** is summarized in Table 21 and compared with NMR data of myricetin-3-O- α -L-5''-acetylarabinofuranoside found in the literature (Mohamed *et al.*, 2006). The relative number of compounds **197** and **198** was calculated as ca. 73% and 27%, respectively, from their respective protons (Appendix 17a).

4.1.8 Characterization of isolated compounds from *O. rochetiana*. Five compounds (Figures 8, 11, and 27), including ursolic acid (**41**), 5-hydroxy-4-methyl-5,6-dihydro-(2H)-pyran-2-one (**199**), quercetin-3-O-(1-D galactopyranoside) (**200**), olinioside (**84**), and 4-O- β -D-glucopyranosylcaffeic acid (**83**), were isolated and characterized from the leaves of *O. rochetiana*, and their structures were deduced based on the NMR data recorded as follows.

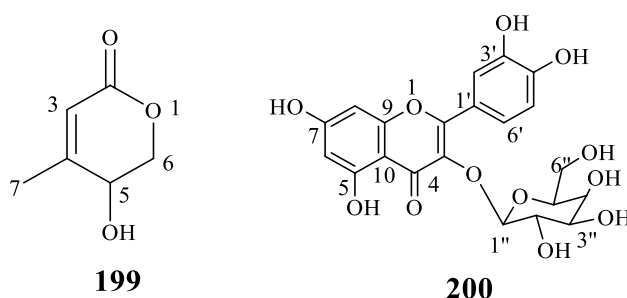


Figure 27. Structures of isolated compounds from leaves of *O. rochetiana*

Compound **41** was isolated as a white solid with a melting point of 275-2280 °C (Seebacher *et al.*, 2003). The TLC showed an R_f value of 0.6 using *n*-hexane:EtOAc (7:3) as the mobile phase. From the NMR spectral data (Appendices 18a to 18c), the chemical structure of **41** must consist of a terpenoid. In the ^{13}C -NMR spectrum (Appendix 18b), 29 well-resolved carbon resonances were obtained. With the aid of a DEPT-135 NMR experiment (Appendix 18c), we identified six quaternary carbons (one additional signal of a quaternary carbon was detected in the 2D HMBC NMR experiment, which in the 1D ^{13}C NMR spectrum is hidden under the signal of the solvent DMSO), nine methylene carbons, and 14 carbons of CH or CH₃ groups (full analysis of the 2D NMR data revealed that seven of both types of carbons were present (Appendices 18d to 18f). The resonance at $\delta^1\text{H} = 2.99$

ppm of hydrogen directly connected to a carbon at $\delta^{13}\text{C} = 76.8$ ppm can most probably be assigned to a -CH(OH) group due to NMR chemical shift reasons and was used as a starting point for the stepwise assignment of all carbon and hydrogen signals via the 2D ^1H - ^{13}C HSQC and HMBC NMR correlations (Table 22).

Table 22. ^1H -NMR (400.2 MHz, DMSO- d_6) and ^{13}C -NMR (100.6 MHz) of compound **41** and NMR data reported for ursolic acid (Seebacher *et al.*, 2003)

C-No	Compound 41			Ursolic acid/pyridine- d_5	
	$\delta^1\text{H}$	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}/600$ MHz	$\delta^{13}\text{C}$
1	1.52&0.90 (m,	38.2		1.00&1.58	39.2
2	1.5-1.4 (m, 2H)	27.0	C-3	1.81&1.81	28.2
3	2.99 (m, 1H)	76.8	C-2, 4, 23, 24	3.44 (dd)	78.2
4	-	38.4		-	39.6
5	0.65 (m, 1H)	54.8		0.88 (d)	55.9
6	1.45&1.29 (m,	18.0	C-5, 8	1.58&1.39	18.8
7	1.43&1.26 (m,	32.7		1.59&1.39	33.7
8	-	38.5		-	40.1
9	1.45 (m, 1H)	46.8		1.65	48.1
10	-	36.5		-	37.5
11	1.83 (m, 2H)	22.8		1.96&1.96	23.7
12	5.12 (t, 3.2 Hz,	124.	C-9, 11, 14, 18	5.49 (s)	125.7
13	-	138.		-	139.3
14	-	41.6		-	42.6
15	1.80&0.98 (m,	27.5	C-8, 12, 13, 16	2.30&1.22	28.8
16	1.92&1.51 (m,	23.8	C-15, 17, 28	2.14&2.01	25.0
17	-	47.0		-	48.1
18	2.10 (d, 11.2 Hz)	52.4	C-12, 13, 14, 16, 17, 19, 20, 28,	2.63 (d)	53.6
19	1.29 (m, 1H)	38.4		1.49	39.5
20	0.90 (m, 1H)	38.4		1.05	39.4
21	1.41&1.27 (m,	30.2		1.49&1.40	31.1
22	1.6-1.5 (m, 2H)	36.3	C-20, 28	1.97&1.97	37.4
23	0.88 (s, 3H)	28.3	C-3, 4, 5, 24	1.24 (s)	28.8
24	0.66 (s, 3H)	16.1	C-3, 4, 5, 23	1.02 (s)	16.5
25	0.85 (s, 3H)	15.2	C-1, 5, 9, 10	0.92 (s)	15.7
26	0.74 (s, 3H)	16.9	C-7, 8, 9, 14	1.06 (s)	17.5
27	1.03 (s, 3H)	23.3	C-8, 13, 14, 15	1.24 (s)	24
28	-	178.		-	179.7
29	0.80 (d, 6.3 Hz,	17.0	C-18, 19, 20	1.02 (d)	17.5
30	0.90 (d(br), 3H)	21.1	C-19, 20, 21	0.97 (d)	21.4
3-OH	4.28 (d, 5.1 Hz,		C-2, 3, 4		
17-COOH	11.9 (s(br), 1H)				

The crucial HMBC correlations used for establishing the chemical structure of the terpenoid skeleton and the numbering of positions of the molecule are shown (Figure 28). Once the terpenoid skeleton was assigned, a comparison with literature data showed that fraction **41** could be ursolic acid (Seebacher *et al.*, 2003). Although the NMR data in the

cited paper were recorded under completely different conditions (NMR solution in pyridine at 310 K) than in our experiments (DMSO at 298 K), all ^{13}C NMR chemical shifts agree very well (deviations of the $\delta^{13}\text{C}$ NMR data of only +0.3 to +1.5 ppm, Table 22). In all cases where the diastereotopic protons of the methylene groups in the literature show well-distinguished ^1H NMR chemical shifts, this behavior has been observed for our data as well (^1H NMR data at positions 1, 6, 7, 15, 16, and 21 in Table 22). As a result of the interpretation work, long-range correlations (Appendix 18e) of the methyl protons (positions 6 and 27) to a ^{13}C resonance at 39.1 ppm (position 8) were observed. This is exactly the resonance of a quaternary carbon that was missing in the 1D ^{13}C NMR data.

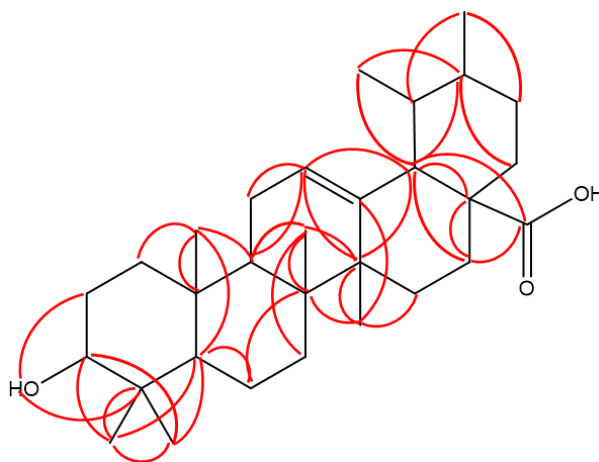


Figure 28. ^1H - ^{13}C HMBC correlations identified for the assignment of compound **41**

Compound **199** was isolated as a brown amorphous solid. TLC showed a spot at an R_f value of 0.4 using *n*-hexane:EtOAc (3:2). The ^1H -NMR spectral data (Appendix 19a) showed the presence of methyl protons at δ 2.03 (dd, $J = 0.4, 1.5$ Hz, H-7). The diastereotopic protons, each appearing as a doublet of doublets at 3.77 and 3.62, were assigned to H-6.

Table 23. ^1H -NMR (400.2 MHz) and ^{13}C -NMR (100.6 MHz) of compound **199** and data reported for 5-hydroxy-4-methyl-5,6-dihydro-(2H)-pyran-2-one (esterified at C-5) (Deyou *et al.*, 2017b)

C-No	Compound 199 /DMSO- d_6				Reported
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^1H COSY	^1H - ^{13}C HMBC	$\delta^{13}\text{C}$
1	-	-	-	-	-
2	-	173.	-	-	173.9
3	5.91 (t, $J = 1.5, 1\text{H}$)	116.	-	C-2, 4, 5, 7	117.4
4	-	168.	-	-	167.5
5	4.96 (m, 1H)	85.2	H-6	C-3, 4, 6	83.1
6	3.77 (dd, $J = 2.90, 12.5, 1\text{H}$) 3.62 (dd, $J = 3.6, 12.5, 1\text{H}$)	59.4	H-5	C-4, 5	60.1
7	2.03 (dd, $J = 0.4, 1.5, 3\text{H}$)	13.6	-	C-2, 3, 4, 5, 6	12.5

In addition, the methine protons were observed at 4.96 (H-5) as a multiplet and at 5.91 (H-3) as a triplet. Using ^{13}C and DEPT-135 NMR spectral data (Appendices 19b and 19c), six carbons were identified, including a methyl carbon at 13.6, a methylene carbon at 59.4, two methine carbons at 85.2 and 116.7, and two quaternary carbons at 168.1 and 173.1 ppm. The 2D NMR spectral data (Appendices 19d to 19f) were analyzed and used in structural elucidation. The structure of compound **200** was identified as 5-hydroxy-4-methyl-5,6-dihydro-(2H)-pyran-2-one. All NMR data is summarized in Table 23 and compared with the NMR data of 5-hydroxy-4-methyl-5,6-dihydro-(2H)-pyran-2-one (esterified at C-5) (Deyou *et al.*, 2017b).

Compound **200** was observed as a yellow solid with a melting point of 243-246 °C (L. Jiang *et al.*, 2015). The TLC showed a spot at R_f value 0.7 using EtOAc:MeOH (24:1) as the mobile phase. The ^1H -NMR spectra (Appendix 20a) revealed the presence of compound **200** as major with two minor compounds, **83** and **84**. The ^1H -NMR spectral data of compound **200** showed the presence of meta-coupling protons at $J = 1.9$ Hz assigned for H-6 and H-8, confirming the presence of the 1,2,4,6-tetra substituted benzene ring A.

Table 24. ^1H -NMR (400.2 MHz, DMSO- d_6) and ^{13}C -NMR (100.6 MHz) of compound **200** and NMR data reported for quercetin-3-O-(1-D-galactopyranoside) (Jiang *et al.*, 2015)

C-No	Compound 200			quercetin-3-O-(1-D-galactopyranoside)/DMSO- d_6	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /600 MHz	$\delta^{13}\text{C}$
1	-	-	-	-	-
2	-	156.2		-	156.2
3	-	133.5		-	133.5
4	-	177.4		-	177.5
5	-	161.2		-	161.2
6	6.18 (d, $J = 1.9$, 1H)	98.7	4, 5, 7, 8, 10	6.20 (d, $J = 2.0$)	98.7
7	-	164.4		-	164.2
8	6.39 (d, $J = 1.9$, 1H)	93.5	4, 6, 7, 9, 10	6.40 (d, $J = 2.0$)	93.5
9	-	156.3		-	156.3
10	-	103.8		-	103.8
1'	-	121.1		-	121.1
2'	7.51 (d, $J = 2.1$, 1H)	115.9	2, 1', 3', 4', 6'		115.9
3'	-	144.9		-	144.8
4'	-	148.5		-	148.5
5'	6.80 (d, $J = 8.5$, 1H)	115.2	1', 2', 3', 4'	6.81 (d, $J = 8.5$)	115.2
6'	7.65 (dd, $J = 8.5$, 2.1, 1H)	122.0	2, 2', 4'	7.67 (dd, $J = 8.5$, 2.2)	122.0
1''	5.36 (d, $J = 7.7$, 1H)	101.8	3, 2'', 3'', 5''	5.38 (d, $J = 7.7$)	101.8
2''	3.56 (m, 1H)	71.2	1'', 3''	3.57 (dd, $J = 9.4$, 7.2)	71.2
3''	3.36 (m, 1H)	73.2		3.37 (dd, $J = 9.4$; 3.3)	73.2
4''	3.63 (m, 1H)	67.9	2'', 3'', 6''	3.65 (d, $J = 3.3$)	67.9
5''	3.30 (m, 1H)	75.8	1''	3.33 (t, $J = 6.0$)	75.9
				3.29 (dd, $J = 10.4$; 6.0)	
6''	3.45 & 3.29 (m, 2H)	60.1	5''	3.45 (dd, $J = 10.4$; 6.0)	60.1

The doublet of doublet observed at 7.65 ($J = 8.3, 2.1$, H-6') indicated the presence of ortho proton (H-5', $J = 8.3$ Hz) and meta proton (H-2', $J = 2.1$ Hz). This splitting pattern of proton (H-6') confirms the presence of 1,2,4-trisubstituted benzene ring B. By using ^{13}C -NMR (Appendix 20b) and DEPT-135 (Appendix 20c), 21 carbons have been identified for compound **200**. From the ^1H - ^{13}C HMBC (Appendix 20e) spectral data, the cross peaks observed at δ 6.18/177.4 and 6.39/177.4 indicate ring A is linked to ring C. In another way, the appearance of cross peaks at 7.51/156.2 and 7.65/156.2 confirms the linkage of ring B to ring C. In addition, the cross peak seen at 5.36/133.5 confirmed the sugar moiety is attached to C-3. Furthermore, ^1H - ^{13}C HSQC (Appendix 20d) and ^1H - ^1H DQF-COSY (Appendix 20f) NMR spectral data were used to confirm the chemical shift assignment of compound **200**. All NMR data are summarized (Table 24) and compared with the NMR data reported for quercetin-3-O-(1-D galactopyranoside) (Jiang *et al.*, 2015). The crucial HMBC of compound **200** is given (Figure 29).

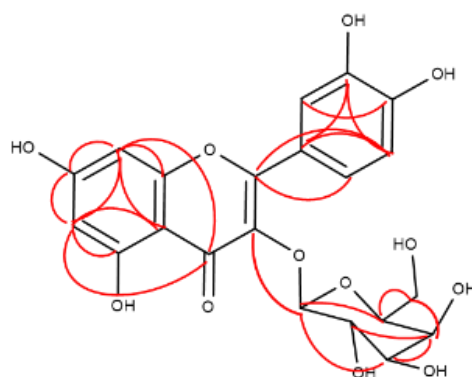


Figure 29. ^1H - ^{13}C HMBC correlations identified for assignment of compound **200**

The $\delta^{13}\text{C}$ (Appendix 20b) observed at 129.0, 114.9, 147.2, 146.8, 116.1, 120.6, 143.3, 117.4, 168.0, 101.6, 73.3, 75.9, 69.8, 77.3, and 60.7 indicates the presence of compound **83** as a mixture. In addition, the δ $^1\text{H}/^{13}\text{C}$ (Appendices 20d and 20e) observed at 2.10/13.6, 4.40 and 4.57/61.4, 5.31/82.1, and 6.04/117.3, including quaternary carbons at 167.0 and 172.4 (Table 25), indicates the presence of compound **84** containing 5-oxy-4-methyl-5,6-dihydro-2H-pyran-2-one linked to C-9 of compound **83** as confirmed by the weak cross peak (^1H - ^{13}C HMBC Appendix 20e) appearing at δ 4.40/165.9. The NMR spectral data of compound **84** (Table 25) is compared with NMR spectral data reported for a compound isolated from other plant species in the same genus named olinioside (Nyandat *et al.*, 1993). The relative number of compounds **200**, **83**, and **84** was calculated as 70, 17, and 13%, respectively, using the area of relevant protons (Appendix 20a).

Table 25. ¹H-NMR (400.2 MHz, DMSO-d₆) and ¹³C-NMR (100.6 MHz) of compound **84** and NMR data reported for olinioside (Nyandat *et al.*, 1993)

C-No	Compound 84			Olinioside/MeOD	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}/500\text{ MHz}$	$\delta^{13}\text{C}$
1	-	128.4		-	130.8
2	7.12 (m, 1H)	115.1	C-1, 7	7.12 (d, J = 2.5)	118.1
3	-	147.7		-	149.0
4	-	147.7		-	148.5
5	7.17 (m, 1H)	115.2	C- 3, 4, 6, 7	7.22 (d, J = 7.5)	116.0
6	7.12 (m, 1H)	121.1	C-1, 7	7.08 (dd, J = 7.5, 2.5)	122.3
7	7.51 (d, J = 16.0)	145.4	C-9	7.59 (d, J = 16)	146.6
8	6.39 (d, J = 16.0)	115.0	C-1	6.34 (d, J = 16)	116.2
9	-	165.9		-	168.4
1'	4.79	101.5	C-4		103.4
2'	3.42	72.4		3.46	74.7
3'	3.08	76.8		3.56	77.5
4'	3.01	70.2		3.52	71.2
5'	3.63	77.9		3.49	78.3
6'	3.65 & 3.46	61.2		3.94	62.6
1''	-	-	-	-	-
2''	-	172.4		-	175.3
3''	6.04 (t, J = 1.7,	117.3	C-2'', 5''	6.00 (q, J = 1.6)	118.8
4''	-	167.0			167.8
5''	5.31 (br s, 1H)	82.1		5.30 (m)	84.4
6''	4.40 (dd, J = 12,1, 4.3, 1H) 4.57 (dd, J = 12.1, 4.3, 1H)	61.4	C-9	4.66 (dd, J = 12, 2.5) 4.51 (dd, J = 12, 4.2)	62.4
7''	2.10 (s, 3H)	13.6	C-3'', 4''	2.20 (dd, J = 1.6, 1.5)	13.6

Compound **83** was observed as a white solid with a melting point of 110-112 °C (Nyandat *et al.*, 1993). The TLC displayed an R_f value of 0.6 using EtOAc:MeOH (24:1) as the mobile phase. The ¹H-NMR data (Appendix 21a) showed the presence of a 1,3,4 substituted aromatic moiety. This was confirmed by the doublet of doublet appearing at δ 7.05 (dd, J = 8.5, 1.7 Hz, H-6), which is ortho with H-5 (δ 7.10 ppm) and meta with H-2 (δ 7.12 ppm). The trans coupling constant observed at J = 15.9 Hz confirms the presence of olefinic protons of H-7 and H-8. The coupling constant observed at δ 4.76 (d, J =7.3, H-1') indicates the presence of the sugar moiety of β -anomer. The ¹³C-NMR spectral data (Appendix 21b) with the help of DEPT-135 (Appendix 21c) identified 15 carbons, including six carbons of the sugar moiety. The ¹H-¹³C HMBC (Appendix 21e) cross peaks observed at δ 7.45/129.0 and 7.45/167.9 confirm the connection between propenoic acid and 1,3,4 substituted aromatic moieties. In another way, the cross peak appeared at 4.76/147.3, confirming the sugar moiety is attached to C-4. In addition to ¹H-¹³C HMBC (Appendix

21e), ^1H - ^{13}C HSQC (Appendix 21d) and ^1H - ^1H DQF-COSY NMR spectral (Appendix 21f) data were used to confirm the chemical shift assignment of compound **83**.

The complete assignments of the NMR data for compound **83** are summarized in Table 26 and compared with NMR data reported for 4-O- β -D-glucopyranosylcaffeic acid (Nyandat *et al.*, 1993). Compared to the reported data (Nyandat *et al.*, 1993) (data recorded in MeOH, assignment rather by chemical shift arguments and not by 2D NMR correlations), our ^{13}C NMR chemical shift shows a deviation of -0.8 to -2.7 ppm (data recorded in DMSO, all "required" correlation signals observed and unambiguously assigned). The crucial HMBC correlation is displayed in Figure 30.

Table 26. ^1H -NMR (400.2 MHz, DMSO- d_6) and ^{13}C -NMR (100.6 MHz) of compound **83** and NMR data reported for 4-O- β -D-glucopyranosylcaffeic acid (Nyandat *et al.*, 1993)

C-No	Compound 83			4-O- β -D-glucopyranosylcaffeic acid/ CD_3OD	
	$\delta^1\text{H}$	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}/500\text{ MHz}$	$\delta^{13}\text{C}$
1		129.0		-	131.2
2	7.12 (m, 1H)	114.9	C-4, 6	7.10 (8, $J=2.6$)	115.7
3	-	146.9		-	148.5
4	-	147.3		-	148.7
5	7.10 (m, 1H)	116.2	C-1, 3	7.14 (d, $J = 7.8$)	117.9
6	7.05 (dd, $J = 8.5, 1.7$, 1H)	120.7	C-2, 4, 7	7.03 (dd, $J = 7.8; 2.6$)	122.1
7	7.45 (d, $J = 15.9$, 1H)	143.9	C-1, 9	7.42 (d, $J = 15.6$)	146.0
8	6.31 (d, $J = 15.9$, 1H)	117.4	C-1, 9	6.30 (d, $J = 15.6$)	118.2
9	-	167.9		-	170.6
1'	4.76 (d, $J=7.3$)	101.7	C-4		103.5
2'	3.29 (m, 1H)	73.3	C-1', 4'	y.y42 (dd, $J = 9.1, 7.8$)	74.8
3'	3.28 (m, 1H)	75.9	C-1', 2'	3.51 (t, $J = 9.1$)	77.5
4'	3.17 (m, 1H)	69.9	C-3', 6'	3.48 (t, $J = 9.1$)	71.3
5'	3.35 (m, 1H)	77.3	C-4', 6'	3.44 (m)	78.4
6'	3.71 (dd, $J=11.8, 1.9$, 1H)	60.8	C-4', 5'	3.90 (dd, $J = 12, 1.7$)	62.4
	3.47 (dd, $J = 11.8, 5.9$, 1H)			3.71 (dd, $J = 12, 5.2$)	

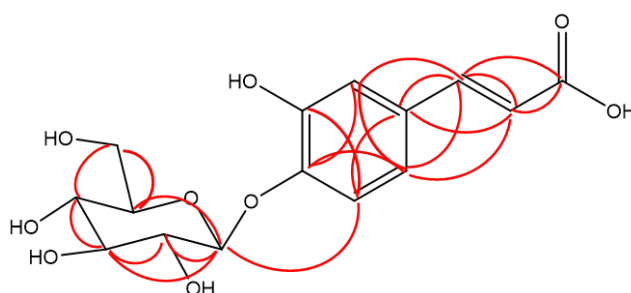


Figure 30. ^1H - ^{13}C HMBC correlations identified for the assignment of compound **83**

4.1.9 Characterization of isolated compounds from *R. apetalus*. Five compounds, including palmitic acid (**145**), palmityl alcohol (**201**), β -sitosterol (**17**), tiliroside (**91**), and astragalin (**202**), were isolated and characterized from the leaves of *R. apetalus* (Figures 7,

12, 16, and 31). The 1D and 2D NMR spectral data were used to characterize their structures as follows.

Compound **145** was isolated as a white solid with a melting point of 62–64 °C consistent with the literature report (Soliman & El-Shattory, 2017). The TLC showed a spot at R_f of 0.5 using *n*-hexane:EtOAc (4:1). The ^1H NMR (CDCl_3) spectrum (Appendix 22a) indicated the presence of a triplet at δ 2.34 (2H, J = 7.6 Hz, H-2) and 0.88 (3H, J = 7.1 Hz, H-16).

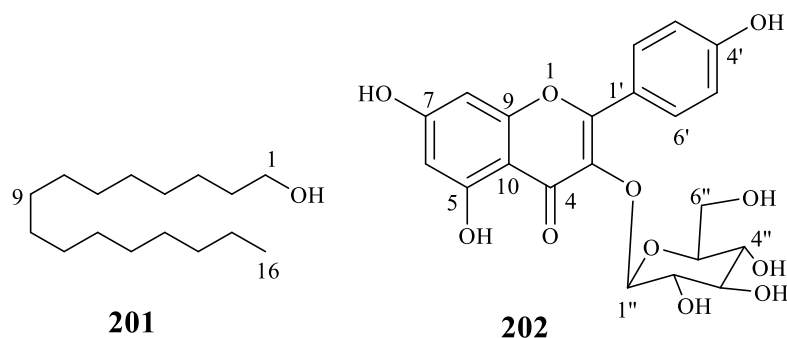


Figure 31. Structures of isolated compounds from leaves of *R. apetalus*

Table 27. The ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (100.6 MHz) of compound **145** and NMR data reported for palmitic acid (Ekeocha *et al.*, 2021)

C N ^o	Compound 145			Palmitic acid/ CDCl_3	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /400 MHz	$\delta^{13}\text{C}$
1	-	180.0	-		178.3
2	2.34 (t, J = 7.6, 2H)	34.2	C-1, 3	2.32 (t, J = 7.3)	33.0
3	1.63 (m, 2H)	24.8	C-1, 2	1.6 (m)	24.6
4					
5					
6					
7					
8	1.25 (br. s, 24H)	29.2-29.9	1.26 (br. s)		29.0-29.5
9					
10					
11					
12					
13					
14		32.1			31.9
15		22.8			22.7
16	0.88 (t, J = 7.1, 3H)	14.3	C-15, 14	0.89 (t, J = 7.3)	14.1

A multiplet was also observed at 1.65 (H-3) and a broad singlet at 1.25 (H-4 to H-15). These protons are the distinctive protons of fatty acids. In addition, from ^{13}C -NMR and ^{13}C DEPT-135 spectral data (Appendices 22b and 22c), 16 carbons, including one for each methyl and quaternary carbon, and 14 methylene carbons were deduced. The 2D NMR spectral data (Appendices 22d to 22f) were used in structural elucidation. All NMR spectral data are

summarized in Table 27. Based on the observed melting point and the calculated integral (Appendix 22a), compound **145** was proposed as palmitic acid (Ekeocha *et al.*, 2021).

Compound **201** was isolated as a white solid with a melting point of 47-50°C which agrees with the reported data (Human Metabolome Database (HMDB), n.d.). The ^1H NMR spectrum (Appendix 23a) showed the presence of the distinctive protons observed at δ 3.64 (t, $J = 7.4$ Hz, H-1), 1.57 (m, H-2), 1.33 (m, H-3), and 0.88 (t, $J = 7.0$ Hz, H-14) assigned for carbon signals at 63.3 (C-1), 33.0 (C-2), 25.9 (C-3), and 14.3 (C-14), respectively (Appendices 23b and 23c). In addition, the distinctive carbon signals were observed at 22.8 (C-13) and 32.1 (C-12), both assigned to the proton signal at δ 1.25. Based on the melting point and the GC-MS data observed, compound **201** was proposed as palmityl alcohol (Gunstone, 1993). The 2D NMR data (Appendices 23d to 23f) were analyzed and used in structural identification. All the NMR spectral data of compound **201** were summarized in Table 28.

Table 28. The ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (100.6 MHz) of compound **201** and NMR data reported for palmityl alcohol (Gunstone, 1993)

C-No	Compound 201				Palmityl alcohol
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^1H COSY	^1H - ^{13}C HMBC	$\delta^{13}\text{C}/\text{CDCl}_3$
1	3.64 (t, $J = 7.4$, 2H)	63.3	H-2	C-2, 3	63.0
2	1.57 (m, 2H)	33.0	H-1		32.8
3	1.33 (m, 2H)	25.9			25.8
4					
5					
6					
7	1.25 (br. s, 16H)	29.5- 29.8			
8					
9					
10					
11					
12					
13					
14	1.25 (br. s, 2H)	32.1			32.0
15	1.25 (br. s, 2H)	22.8	H-14		22.7
16	0.88 (t, $J = 7.0$, 3H)	14.3	H-13	C-12, 13	14.1

Compound **17** was isolated as a white crystalline solid with a melting point of 131-135 °C that matches the literature report (Nwe *et al.*, 2019). The TLC showed a spot at an R_f value of 0.3 using *n*-hexane:EtOAc (4:1). The NMR spectral data (Appendix 24a) indicated the presence of the distinctive proton signals that appeared at δ 5.34 (d, $J = 5.1$ Hz, H-6), 3.52 (m, H-3), and 0.68 (s, H-18) were observed. From the ^{13}C NMR and DEPT-135 (Appendices 24b and 24c), a total of 29 carbons were resolved, including three quaternary, nine methine, 11 methine, and six methyl carbons. The 2D NMR (Appendices

24d to 24f) spectral data was analyzed and used for structural elucidation. All the NMR spectral data were summarized (Table 29) and compared with the NMR data reported for β -sitosterol (Nwe *et al.*, 2019).

Table 29. ^1H -NMR (400.2 MHz) and ^{13}C -NMR (100.6 MHz) of compound **17** and NMR data reported for β -sitosterol (Nwe *et al.*, 2019)

C-No	Compound 17 /CDCl ₃			β -sitosterol/CDCl ₃	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /500 MHz	$\delta^{13}\text{C}$
1	1.85&1.07 (m, 2H)	37.4	C-3	1.85&1.08 (o)	37.4
52	1.83 &1.49 (m, 2H)	31.8		1.82&1.47 (o)	31.7
3	3.52 (m, 1H)	72.0		3.52 (m)	71.9
4	2.28&2.23 (m, 2H)	42.4	C-1, 2, 3, 7, 10	2.28&2.23 (m)	42.4
5	-	140.9		-	140.9
6	5.34 (d, $J = 5.1$ Hz, 1H)	121.9	C-4, 7, 8,10	6.34 (br. d, $J = 4.9$)	121.8
7	1.99&1.54 (m, 2H)	32.0	C-4, 9, 14	1.97&1.52 (o)	32.0
8	1.49 (m, 1H)	32.0		1.45 (o)	32.0
9	0.93 (m, 1H)	50.3		0.93 (o)	50.3
10	-	36.6		-	36.6
11	1.49 (m, 2H)	21.2		1.53&1.48 (o)	21.2
12	2.02&1.15 (m, 2H)	39.9		2.02&1.16 (o)	39.9
13	-	42.5		-	42.4
14	1.00 (m, 1H)	56.9		1.02 (o)	56.9
15	1.56 & 1.05 (m, 2H)	24.4		1.58&1.56 (o)	24.4
16	1.83 (m, 2H)	28.4	C-17	1.57&1.55 (o)	28.5
17	1.11 (m, 1H)	56.2	C-16	1.10 (o)	56.2
18	0.68 (s, 1H)	12.0	C-12, 14, 17	0.68 (o)	11.9
19	1.00 (s, 3H)	19.5	C-1, 5, 9	1.00 (s)	19.5
20	1.36 (m, 1H)	36.3		1.67 (o)	36.2
21	0.92 (d, $J = 6.6$, 3H)	18.9	C-17, 20, 22	0.93 (d, $J = 6.3$)	19.1
22	1.31&1.02 (m, 2H)	34.1		1.26&1.24 (o)	34.1
23	1.16 (m, 2H)	26.2	C-24	1.17&1.14 (o)	26.2
24	0.93 (m, 1H)	46.0		0.91 (o)	46.0
25	1.65 (m, 1H)	29.3	C-24	1.28 (o)	29.3
26	0.82 (3H)	20.0		0.86 (o)	21.1
27	0.80 (3H)	19.2		0.80 (o)	18.9
28	1.25 (m, 2H)	23.2		1.25&1.24 (o)	23.2
29	0.84 (3H)	12.1		0.85 (o)	12.1

Compound **91** was obtained as a white crystal with a melting point of 232-235 °C agrees with the literature report (Yoshida *et al.*, 1991). The TLC showed a spot at R_f 0.5 using *n*-hexane:EtOAc (1:9). The ^1H -NMR spectral data (Appendix 25a) exhibited the presence of meta protons with a J value of 2.1 Hz, representing the two protons of ring A at δ 6.13 and 6.32 (2H, H-6, 8), respectively. The symmetrical protons observed at 8.00 (2H, d, $J = 8.7$ Hz) represented H-2' and H-6' of ring B that are ortho, respectively, to the H-3' and H-6' symmetrical protons at 6.82 (2H, d, $J = 8.7$ Hz). The doublet appeared at 5.25 (1H, d, $J = 7.6$ Hz), representing the anomeric proton, H-1''. Furthermore, the two symmetrical protons at 7.31 (2H, d, $J = 9.0$ Hz, H-2''', 6'') and 6.79 (2H, d, $J = 9.0$ Hz, H-3''', 5'') in

addition to the trans protons observed at 7.40 (1H, d, $J = 15.6$, H-7''') and 6.07 (1H, d, $J = 15.6$, H-8''') denote the protons of the coumaroyl group. The ^{13}C -NMR (Appendix 25b) with the aid of ^{13}C DEPT-135 spectral data (Appendix 25c) resolves 30 carbons, including four symmetrical carbons at δ 131.6 (C-2', 6'), 115.4 (C-3', 5'), 130.6 (C-2''', 6'''), and 116.1 (C-3''', 5''').

Table 30. The ^1H -NMR (400.2 MHz, MeOD) and ^{13}C -NMR (100.6 MHz) of compound **91** and NMR data reported for tiliroside (Yoshida *et al.*, 1991).

C-No	Compound 91 /MeOD			Tiliroside/DMSO- d_6	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /500 MHz	$\delta^{13}\text{C}$
1	-	-		-	-
2	-	158.7		-	156.4
3	-	134.9		-	133.0
4	-	178.8		-	177.4
5	-	157.8		-	156.3
6	6.13 (1H, d, $J = 2.1$)	99.3	C-8, C-9, C-10	6.14 (d, $J = 2.0$)	98.7
7	-	165.4		-	164.1
8	6.32 (1H, d, $J = 2.1$)	94.7	C-2, 6, 10	6.37 (d, $J = 2.0$)	
9	-	162.4		-	161.1
10	-	104.9		-	103.9
1'	-	122.1		-	120.7
2'/6'	8.00 (2H, d, $J = 8.7$)	131.6	C-9, C-2', 6'	7.97 (d, $J = 9.0$)	130.8
3'/5'	6.82 (2H, d, $J = 8.7$)	115.4	C-1', 4'	6.84 (d, $J = 9.0$)	115.1
4'	-	160.9		-	160.0
1''	5.25 (1H, d, $J = 7.6$)	103.3	C-3	5.43 (d, $J = 7.0$)	100.9
2''	3.40-3.50 (1H, m)	75.2			74.2
3''	3.40-3.50 (1H, m)	77.3			76.2
4''	3.30-3.35 (1H, m)	71.1			69.9
5''	3.40-3.50 (1H, m)	75.1			74.1
6''	4.19 (1H, m) 4.31 (1H, m)	63.6	C-9''' (weak)		63.0
1'''	-	126.4		-	124.9
2'''/6'''	7.31 (2H, d, $J = 9.0$)	130.6	C-2''', 4''', 6'''	7.35 (d, $J = 9.0$)	130.1
3'''/5'''	6.79 (2H, d, $J = 9.0$)	116.1	C-1'''	6.77 (d, $J = 9.0$)	115.6
4'''	-	160.6		-	159.8
7'''	7.40 (1H, d, $J = 15.6$)	145.9	C-1''', C-2''', 6'''	7.34 (d, $J = 16.0$)	144.6
8'''	6.07 (1H, d, $J = 15.6$)	114.1	C-1'''	6.09 (d, $J = 16.0$)	113.6
9'''	-	168.1		-	166.1

The ^1H - ^{13}C HMBC spectra data (Appendix 25e) cross peak observed at δ 5.22/134.9 (H-''/C-3) confirms the position of the sugar moiety and 4.19 and 4.31/168.1 (H-6''/C-9''') confirm the coumaroyl group is attached to C-6'' of the sugar moiety. ^1H - ^{13}C HSQC and ^1H - ^1H COSY spectral data (Appendices 25d and 25f) were used for further structural confirmation. All NMR spectral data of compound **91** is shown in Table 30, which agrees with the NMR data reported for tiliroside (Yoshida *et al.*, 1991). Tiliroside has been reported

for its various medicinal values as an anticancer (Han *et al.*, 2022), anti-inflammatory, antidiabetic (Goto *et al.*, 2012), and antioxidant (Corrêa *et al.*, 2018) activities.

Compound **202** was obtained as a yellow crystal with a melting point of 220-226 °C matching the literature report (Kim *et al.*, 1994). The TLC spot appeared at an R_f value of 0.3 using *n*-hexane:EtOAc (1:9). The ^1H -NMR spectral data (Appendix 26a) of compound **202** indicated the presence of meta protons coupled with a J value of 2.1 Hz at δ 6.17 (1H, H-6) and 6.36 (2H, H-8), confirming the presence of ring A of a flavonoid. Two symmetrical protons appeared at 8.02 (2H, d, J = 8.8 Hz) and 6.86 (2H, d, J = 8.8 Hz), respectively denoted H-2'/6' and H-3'/5' of ring B of a flavonoid. The doublet coupled with a 7.4 Hz J value appeared at δ 5.22 assigned for the anomeric proton, H-2''.

Table 31. The ^1H -NMR (400.2 MHz, CD_3OD) and ^{13}C -NMR (100.6 MHz) of compound **202** and NMR data reported for astragalin (Kim *et al.*, 1994)

C-N α	Compound 202			Astragalin/MeOD	
	$\delta^1\text{H}$ (J in Hz)	$\delta^{13}\text{C}$	^1H - ^{13}C HMBC	$\delta^1\text{H}$ /500 MHz	$\delta^{13}\text{C}$
1	-	-		-	-
2	-	157.8		-	158.6
3	-	134.8		-	135.4
4	-	178.8		-	179.4
5	-	162.4		-	163.0
6	6.17 (1H, d, J = 2.1)	99.2	C-4, 5, 7, 8,	6.18 (d, J = 1.8)	100.3
7	-	165.3		-	166.9
8	6.36 (1H, d, J = 2.1)	94.1	C-2, 6, 7, 10	6.38 (d, J = 2.1)	95.1
9	-	158.4		-	158.9
10	-	105.1		-	105.5
1'	-	122.1		-	122.8
2'/6'	8.02 (2H, d, J = 8.8)	131.6	C-2, 2', 4', 6'	8.05 (d, J = 9.0)	132.3
3'/5'	6.86 (2H, d, J = 8.8)	115.4		6.87 (dd, J = 2.1, 8.7)	116.0
4'	-	160.9		-	161.5
1''	5.22 (1H, d, J = 7.4)	103.4	C-3	5.23 (d, J = 7.5)	104.2
2''	3.39-3.49 (1H)	75.1		3.40 (dd, J = 7.2, 10.0)	75.7
3''	3.19 (1H)	77.7		3.25-3.40 (m)	78.4
4''	3.26-3.38 (1H)	70.7		3.25-3.40 (m)	71.3
5''	3.28-3.46 (1H)	77.4		3.20 (m)	78.0
6''	3.45-3.81 (2H)	63.7		3.30 (dd, J = 5.4, 11.7)	62.6
				3.71 (dd, J = 2.1, 11.7)	

From the ^{13}C -NMR (Appendix 26b) and ^{13}C DEPT-135 (Appendix 26c) spectral data, 21 carbons were identified, including the two symmetrical carbons observed at δ 131.6 and 115.4 assigned for C-2'/6' and C-3'/5', respectively. From ^1H - ^{13}C HMBC NMR spectral data (Appendix 26e), the cross peak appeared at δ 5.22/134.8 (H-1''/C-3), showing that the sugar moiety is attached to ring C of the flavonoid. The structure of compound **202** was

further confirmed using ^1H - ^{13}C HSQC and ^1H - ^1H COSY (Appendices 26d and 26f). Table 31 summarizes all NMR spectral data of compound **202** and the data agrees with the NMR spectral data reported for astragalin (Kim *et al.*, 1994). A broad spectrum of pharmacological activities of astragalin has been reported. Antiinflammatory (Kim *et al.*, 2017), antioxidants (Cho *et al.*, 2014), antiobesity (Ohkoshi *et al.*, 2007), antidiabetic (Ke *et al.*, 2012), and anticancer (You *et al.*, 2017) activities are among the pharmacological activities of astragalin.

4.2 *In vitro* Antibacterial Activity

4.2.1 Disk diffusion method. The results of an *in vitro* antibacterial assay conducted against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* using extracts and fractions from the root of *C. hirsuta*, stem barks of *G. ferruginea*, leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus* are displayed (Table 32). The IZs at 100 mg/mL of MeOH and *n*-hexane:EtOAc (1:1) extract of root *C. hirsuta*, DCM:MeOH (1:1) extracts, CHCl_3 , EtOAc, and *n*-BuOH fractions of stem barks of *G. ferruginea*, DCM:MeOH (1:1) extracts from leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus*, fractions 6-10 of leaves of *R. ruspolii*, fractions 4-17 of leaves of *O. rochetiana*, and fractions 16-21 of leaves of *R. apetalus* ranged from 7.93 ± 0.65 to 16.33 ± 0.47 mm against all tested bacteria compared to IZs of standard ciprofloxacin, which ranged from 18.42 ± 0.26 to 22.18 ± 0.21 . The highest IZ (16.33 mm) was exhibited by DCM:MeOH (1:1) extracts of the leaves of *R. ruspolii* against *S. pyogenes*. Whereas the lowest IZ (7.93 mm) was shown by the EtOAc fraction of *G. ferruginea* stem bark against *P. aeruginosa*. Of all extracts and fractions, *S. aureus* was the most susceptible to the *n*-BuOH fraction (IZ: 15.27 ± 0.25 mm) of *G. ferruginea* stem barks and least susceptible to MeOH extract of the root of *C. hirsuta* (IZ: 8.20 ± 0.12 mm). On the other hand, *S. pyogenes* was the most susceptible to DCM:MeOH (1:1) extract of the leaves of *R. ruspolii* (IZ: 16.33 ± 0.47 mm) and least susceptible to CHCl_3 fraction of stem bark of *G. ferruginea* (IZ: 10.25 ± 0.25 mm). *E. coli* was the most susceptible to DCM:MeOH extract of leaves of *R. ruspolii* (IZ: 15.33 ± 0.47 mm) and least susceptible to CHCl_3 fraction of stem bark of *G. ferruginea* (IZ: 8.17 ± 0.24 mm). Whereas *P. aeruginosa* was the most susceptible to the *n*-BuOH fraction of the stem bark of *G. ferruginea* (IZ: 15.27 ± 0.21 mm) and least susceptible to the EtOAc fraction of the stem bark of *G. ferruginea* (IZ: 7.93 ± 0.65 mm).

Even though at 200 mg/mL, a comparable result has been reported for the antibacterial activities conducted using the paper disk diffusion method on the CHCl₃ and MeOH extract of the leaves of *C. hirsuta* (Habtamu & Mekonnen, 2017) for which the CHCl₃ extract has been reported to show IZ of 12.33, 10.70, and 10.26 mm, and the MeOH extract has been reported to show IZ of 8.50, 9.30, and 8.00 mm against *P. aeruginosa*, *E. coli*, and *S. aureus*, respectively. This confirms that the *n*-hexane:EtOAc (1:1) extract root of *C. hirsuta* reported in our study contains bioactive compounds having better activities against *P. aeruginosa* than against the other test bacteria. On the other hand, our results are better than the results of antibacterial activities that have been reported using paper disk diffusion from the acetone extracts of the bark of *G. ferruginea* (Tura *et al.*, 2017) showing the IZs of 9.00, 8.00, and 9.70 mm against *S. aureus*, *E. coli*, and *P. aeruginosa*, respectively. However, our findings are better than the results that have been reported for antibacterial activity conducted using 200 mg/mL of *n*-hexane, DCM, EtOAc, and MeOH extracts of the root bark of *R. ruspolii* against *S. aureus*, *E. coli*, and *P. aeruginosa* (Njoroge & Opiyo, 2019). Whereas a comparable result has been reported from various solvent extracts of the leaves of *O. rochetiana* (Nigussie *et al.*, 2020; Omari *et al.*, 2013). On the contrary, the results of *R. apetalus* reported in this study are better than the results of antibacterial activities that have been reported from *R. hyrcanus* leaves at 300 mg/mL of MeOH extract, *n*-hexane, EtOAc, and MeOH fractions against *S. aureus* with IZs of 10.5, 0.0, 11.0, and 11.5 mm, respectively, and against *E. coli* with IZs of 7.0, 0.0, 0.0, and 8.0 mm, respectively (Yousefbeyk *et al.*, 2022).

Furthermore, the results of *in vitro* antibacterial assays conducted against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* using essential oil and isolated compounds from root *C. hirsuta*, stem barks of *G. ferruginea*, leaves of *R. ruspolii*, *O. rochetiana*, and *R. apetalus* are shown in Table 33. The IZs of isolated compounds and essential oil (5 mg/mL) ranged from 8.33 ± 0.50 to 15.20 ± 0.28 mm against all tested bacteria compared to the IZs of the standard, which ranged from 18.42 ± 0.26 to 22.18 ± 0.21. The highest IZ (15.20 mm) was revealed by compound **16** against *P. aeruginosa*, whereas the least IZ (8.33 mm) was shown by compound **196** against *S. aureus*. Of all isolated compounds, *S. aureus* was the most susceptible to compound **197** (IZ: 14.67 ± 0.47 mm) and least susceptible to compound **196** (IZ: 8.33 mm). *S. pyogenes* was the most susceptible to compound **17** (IZ: 15.07 ± 0.74 mm) and least susceptible to compound **196** (IZ: 8.67 ± 0.50 mm).

Table 32. Bacterial growth IZ of extracts and fractions

Plant Name/ Part studied	Test sample	Concentration (mg/mL)	IZ in mm (mean±SD)			
			<i>S. aureus</i>	<i>S. pyogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
<i>C. hirsute</i> / Root	<i>n</i> -hexane:EtOAc (1:1) extract	100	11.20 ± 0.13	10.30 ± 0.25	8.40 ± 0.26	15.20 ± 0.17
		50	7.10 ± 0.13	8.30 ± 0.13	NI	10.20 ± 0.12
		25	NI	NI	NI	NI
	MeOH extract	100	8.20 ± 0.12	12.30 ± 0.21	9.20 ± 0.12	9.30 ± 0.22
		50	NI	8.20 ± 0.12	7.20 ± 0.12	NI
		25	NI	NI	NI	NI
<i>G. ferruginea</i> /Bark	DCM:MeOH (1:1) extract	100	12.90 ± 0.57	13.93 ± 0.58	11.33 ± 0.24	13.80 ± 0.82
		50	10.03 ± 0.61	10.80 ± 0.28	9.25 ± 0.24	7.77 ± 0.26
		25	8.37 ± 0.29	8.37 ± 0.29	NI	NI
	CHCl ₃ fraction	100	10.30 ± 0.36	10.25 ± 0.25	8.17 ± 0.24	11.47 ± 0.29
		50	8.03 ± 0.53	8.2 ± 0.28	NI	8.90 ± 0.70
		25	7.13 ± 0.34	NI	NI	NI
	EtOAc fraction	100	11.17 ± 0.24	13.17 ± 0.24	9.27 ± 0.21	7.93 ± 0.65
		50	9.10 ± 29	9.90 ± 0.70	NI	NI
		25	7.20 ± 0.20	NI	NI	NI
	<i>n</i> -BuOH fraction	100	15.27 ± 0.25	12.87 ± 0.74	14.17 ± 0.24	15.27 ± 0.21
		50	11.13 ± 0.74	9.20 ± 0.28	9.78 ± 0.21	11.8 ± 0.28
		25	7.27 ± 0.21	7.20 ± 0.28	7.90 ± 0.29	8.13 ± 0.66
<i>R. ruspolii</i> / Leaves	DCM:MeOH (1:1) extract	100	12.67 ± 0.50	16.33 ± 0.47	15.33 ± 0.47	13.67 ± 0.47
		50	8.67 ± 0.94	9.67 ± 0.94	10.33 ± 0.50	8.33 ± 0.50
		25	NI	7.67 ± 0.50	7.67 ± 0.47	NI
	Fractions 6-10	100	12.00 ± 0.82	13.33 ± 0.47	11.33 ± 0.50	12.33 ± 0.94
		50	8.83 ± 0.24	8.33 ± 0.62	7.33 ± 0.47	7.33 ± 0.47
		25	NI	NI	NI	NI

Note. NI: no inhibition

Table 32. Bacterial growth IZ of extracts and fractions (Contn'd)

Plant Name/ Part studied	Test sample	Concentration (mg/mL)	IZ in mm (mean±SD)			
			<i>S. aureus</i>	<i>S. pyogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
<i>O. rochetiana</i> / Leaves	DCM:MeOH (1:1) extract	100	14.93 ± 0.25	14.10 ± 0.70	14.20 ± 0.59	13.53 ± 0.37
		50	10.40 ± 0.43	9.20 ± 0.51	9.1 ± 0.37	9.5 ± 0.41
		25	7.80 ± 0.22	8.10 ± 0.17	7.60 ± 0.49	7.60 ± 0.26
	Fractions 4-17	100	13.8 ± 0.51	13.17 ± 0.63	13.07 ± 0.41	12.70 ± 0.59
		50	9.33 ± 0.47	8.43 ± 0.52	8.90 ± 0.65	9.07 ± 0.33
		25	NI	NI	NI	NI
<i>R. apetalus</i> /Leaves	DCM:MeOH	100	10.67 ± 0.47	13.83 ± 0.62	11.00 ± 0.82	9.67 ± 0.47
		50	7.67 ± 0.47	9.33 ± 0.47	9.67 ± 0.47	7.67 ± 0.47
		25	NI	7.67 ± 0.47	7.33 ± 0.47	NI
	Fractions 16-21	100	10.00 ± 0.82	11.33 ± 0.47	9.33 ± 0.47	10.67 ± 0.47
		50	NI	8.33 ± 0.62	NI	7.67 ± 0.47
		25	NI	NI	NI	NI
Positive control	Ciprofloxacin	0.5	18.42 ± 0.26	19.93 ± 0.21	20.00 ± 0.21	22.18 ± 0.22

Note. NI: no inhibition

E. coli was the most susceptible to compound **195** (IZ: 12.67 ± 0.50 mm) and least susceptible to compound **196** (9.00 ± 0.82 mm). *P. aeruginosa* was the most susceptible to compound **16** (IZ: 15.20 ± 0.28 mm) and least susceptible to compound **196** (IZ: 8.67 ± 0.47 mm). Moreover, compared to the other bacteria evaluated, the essential (IZ: 13.4 ± 0.24 mm), compounds **70** (IZ: 11.10 ± 0.17 mm), **51** (IZ: 13.33 ± 0.94 mm), **193** (IZ: 14.67 ± 0.47 mm), **83** (11.00 ± 0.41 mm), and **202** (IZ: 14.00 ± 0.82 mm) showed better antibacterial activity against *S. aureus*. However, only compound **17** (IZ: 15.07 ± 0.74 mm) and compound **41** (IZ: 12.67 ± 0.27 mm) showed better antibacterial effects against *S. pyogenes*. On the contrary, compounds **189** (IZ: 9.10 ± 0.19 mm), **190** (IZ: 10.40 ± 0.29 mm), **41** (IZ: 12.50 ± 0.24 mm), **194** (IZ: 9.33 ± 0.47 mm), **196** (IZ: 9.00 ± 0.82 mm), and **200** (IZ: 12.30 ± 0.50 mm) exhibited better antibacterial activity against *E. coli*. Whereas compounds **189** (IZ: 9.10 ± 0.12 mm), **16** (IZ: 15.20 ± 0.28), **65** (IZ: 12.40 ± 0.65 mm), **184** (IZ: 9.33 ± 0.50 mm), **195** (IZ: 13.33 ± 0.47 mm), and **91** (IZ: 12.67 ± 0.47 mm) showed better antibacterial effect against *P. aeruginosa*.

S. aureus, *S. pyogenes*, *E. coli*, and *P. aeruginosa* are among the critical, high, and medium antimicrobial resistance bacteria identified by WHO (WHO, 2024). These bacteria cause several infectious diseases. For example, bacteremia, infective endocarditis, skin and soft tissue infections, osteomyelitis, septic arthritis, prosthetic device infections, pulmonary infections such as pneumonia and empyema, gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections are among the diseases caused by *S. aureus* (Tong *et al.*, 2015), whereas the life-threatening diseases caused by *S. pyogenes* are scarlet fever, bacteremia, pneumonia, necrotizing fasciitis, myonecrosis, and Streptococcal toxic shock syndrome (Stevens and Bryant, 2022). *E. coli* produces Shiga toxin, which is a bacterium that can cause severe foodborne disease. As a result, abdominal cramps, bloody diarrhea (hemorrhagic colitis), fever, and vomiting can occur (WHO, 2018). On the other hand, folliculitis, puncture wounds leading to osteomyelitis, pneumonia, and otitis externa are among various diseases caused by *P. aeruginosa* (Wilson and Pandey, 2023). The antibacterial activities observed against these bacteria (Table 32) and the traditional medicinal values claimed for *C. hirsuta*, *G. ferruginea*, *R. ruspolii*, *O. rochetiana*, and *R. apetalus* suggest that the traditional medicinal plants reported in this study are used to treat a variety of infectious diseases caused by *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa*.

Table 33. Bacterial growth IZ of essential oil and isolated compounds

Plant Name/ Part studied	Test sample	Concentration (mg/mL)	IZ in mm (mean±SD)			
			<i>S. aureus</i>	<i>S. pyogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
<i>C. hirsute</i> / Root	Compound 70	5	11.10 ± 0.17	10.50 ± 0.21	10.30 ± 0.24	10.60 ± 0.55
		2.5	7.27 ± 0.55	8.70 ± 0.22	8.20 ± 0.17	8.20 ± 0.17
		1.25	NI	NI	NI	NI
	Compound 189	5	9.07 ± 0.12	8.80 ± 0.17	9.10 ± 0.19	9.10 ± 0.12
		2.5	NI	NI	7.60 ± 0.13	NI
		1.25	NI	NI	NI	NI
	Compound 190	5	10.10 ± 0.10	10.20 ± 0.17	10.40 ± 0.29	10.20 ± 0.22
		2.5	8.27 ± 0.46	7.50 ± 0.22	8.30 ± 0.16	8.40 ± 0.24
		1.25	NI	NI	NI	NI
<i>G. ferruginea</i> /Bark	Essential oil	5	13.4 ± 0.24	9.40 ± 0.29	9.47 ± 0.29	11.1 ± 0.22
		2.5	11.1 ± 0.29	8.33 ± 0.29	7.63 ± 0.21	8.13 ± 0.61
		1.25	9.17 ± 0.26	7.23 ± 0.21	NI	7.1 ± 0.29
	Compound 16	5	11.2 ± 0.28	11.93 ± 0.65	11.07 ± 0.66	15.20 ± 0.28
		2.5	8.10 ± 0.78	9.37 ± 0.29	8.50 ± 0.24	8.90 ± 0.70
		1.25	7.07 ± 0.25	7.10 ± 0.21	7.17 ± 0.24	NI
	Compound 17	5	14.4 ± 0.29	15.07 ± 0.74	11.4 ± 0.29	11.37 ± 0.29
		2.5	11.20 ± 0.28	10.80 ± 0.28	9.60 ± 0.29	7.63 ± 0.29
		1.25	9.10 ± 0.70	NI	7.30 ± 0.24	NI
	Compound 42	5	11.80 ± 0.24	10.07 ± 0.24	12.50 ± 0.24	11.17 ± 0.24
		2.5	9.30 ± 0.29	10.6 ± 0.74	10.60 ± 0.42	9.13 ± 0.74
		1.25	7.47 ± 0.25	7.53 ± 0.19	7.53 ± 0.28	7.17 ± 0.24
	Compound 61	5	10.63 ± 0.25	9.83 ± 0.23	12.33 ± 0.47	12.40 ± 0.65
		2.5	9.17 ± 0.24	8.30 ± 0.21	7.53 ± 0.28	7.33 ± 0.25
		1.25	7.60 ± 0.29	7.20 ± 0.21	NI	NI
<i>R. ruspolii</i> / Leaves	Compound 194	5	8.67 ± 0.50	9.00 ± 0.82	9.33 ± 0.47	9.33 ± 0.50
		2.5	NI	NI	NI	NI
		1.25	NI	NI	NI	NI
	Compound 55	5	13.33 ± 0.94	10.00 ± 0.82	11.33 ± 0.50	12.33 ± 0.47
		2.5	11.00 ± 0.82	7.47 ± 0.66	8.67 ± 0.47	8.33 ± 0.50
		1.25	9.00 ± 0.82	NI	NI	NI

Note. NI: no inhibition

Table 33. Bacterial growth IZ of essential oil and isolated compounds (Contn'd)

Plant Name/ Part studied	Test sample	Concentration (mg/mL)	IZ in mm (mean±SD)			
			<i>S. aureus</i>	<i>S. pyogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
<i>R. ruspolii/ Leaves</i>	Compound 195	5	9.67 ± 0.47	11.33 ± 0.47	12.67 ± 0.50	13.33 ± 0.47
		2.5	NI	8.50 ± 0.86	9.17 ± 0.62	9.33 ± 0.47
		1.25	NI	NI	NI	NI
	Compound 196	5	8.33 ± 0.50	8.67 ± 0.50	9.00 ± 0.82	8.67 ± 0.47
		2.5	NI	NI	NI	NI
		1.25	NI	NI	NI	NI
	Compound 197	5	14.67 ± 0.47	12.33 ± 0.47	10.67 ± 0.50	11.33 ± 0.50
		2.5	10.00 ± 0.82	9.67 ± 0.47	8.00 ± 0.41	8.33 ± 0.47
		1.25	8.33 ± 0.47	NI	NI	NI
<i>O. rochetiana/ Leaves</i>	Compound 41	5	10.30 ± 0.50	12.67 ± 0.27	11.53 ± 0.45	10.43 ± 0.39
		2,5	8.47 ± 0.73	10.3 ± 0.36	9.37 ± 0.55	8.47 ± 0.33
		1.25	NI	7.7 ± 0.30	NI	NI
	Compound 199	5	9.20 ± 0.41	9.00 ± 0.45	9.50 ± 0.41	10.00 ± 0.39
		2,5	7.30 ± 0.42	7.07 ± 0.57	7.4 ± 0.33	7.5 ± 0.37
		1.25	NI	NI	NI	NI
	Compound 200	5	10.00 ± 0.5	10.53 ± 0.46	12.30 ± 0.50	11.70 ± 0.24
		2,5	8.10 ± 0.51	8.40 ± 0.45	9.37 ± 0.46	7.77 ± 0.26
		1.25	NI	NI	NI	NI
	Compound 83	5	11.00 ± 0.41	9.9 ± 0.45	9.47 ± 0.41	9.53 ± 0.39
		2,5	9.07 ± 0.42	7.7 ± 0.57	7.4 ± 0.33	7.5 ± 0.37
		1.25	NI	NI	NI	NI
<i>R. apetalus/Leaves</i>	Compound 91	5	10.67 ± 0.47	9.33 ± 0.47	11.67 ± 0.50	12.67 ± 0.47
		2.5	8.20 ± 0.24	7.33 ± 0.47	7.67 ± 0.47	8.00 ± 0.82
		1.25	NI	NI	NI	NI
	Compound 202	5	14.00 ± 0.82	12.00 ± 0.82	11.5 ± 0.41	12.00 ± 0.82
		2.5	9.67 ± 0.47	9.37 ± 0.47	8.33 ± 0.62	8.67 ± 0.47
		1.25	7.67 ± 0.47	7.53 ± 0.41	7.67 ± 0.47	7.33 ± 0.47
Positive control	Ciprofloxacin	0.5	18.42 ± 0.26	19.93 ± 0.21	20.00 ± 0.21	22.18 ± 0.22

Note. NI: no inhibition

4.2.2 Minimum inhibitory concentration (MIC) of extracts. The results of the MIC assay of *C. hirsuta* root extracts performed using the agar dilution method against two Gram-positive clinical isolates (*S. aureus* and *S. pyogenes*) and two Gram-negative clinical isolates (*E. coli* and *P. aeruginosa*) are shown in Table 34. As indicated, the MIC results were identified to be greater than or equal to 50 mg/mL for both MeOH and *n*-hexane:EtOAc (1:1) extracts against the four bacterial strains. Unlike the MIC of the standard drug, ciprofloxacin was determined to be less than 15.625 µg/mL against all selected bacteria. Comparable results were observed from the MIC evaluation conducted on the MeOH and CHCl₃ extracts of the plant leaves against *S. aureus*, *E. coli*, and *P. aeruginosa*, wherein the maximum MIC value was found to be 60 mg/mL and the minimum was 30 mg/mL (Ameya *et al.*, 2022).

Table 34. MIC of MeOH and *n*-hexane:EtOAc (1:1) extracts of *C. hirsuta* root

S/N	Bacteria	MIC of extracts (mg/mL)			Ciprofloxacin (mg/mL)
		MeOH extract	<i>n</i> -hexane: EtOAc (1:1) extract		
1	<i>S. aureus</i>	>50		>50	
2	<i>S. pyogenes</i>	50		50	≤ 0.015625
3	<i>E. coli</i>	50		50	
4	<i>P. aeruginosa</i>	>50		50	

4.3 DPPH Radical Scavenging Activities

The results of DPPH radical scavenging activities of extracts, fractions, essential oil, and isolated compounds are presented (Table 35 and Table 36). At 62.5 µg/mL, the percent of DPPH radical inhibited using all tested samples ranged from 18.50 ± 0.09 to 92.1 ± 0.1%, where the lowest (18.50%) DPPH was inhibited by compound **17** and the highest (92.1%) was by compound **189** compared to the 96.9 ± 0.06% DPPH radical inhibited by the positive control, ascorbic acid.

As indicated in Table 35, the DPPH radical inhibited by MeOH and *n*-hexane:EtOAc (1:1) extracts of the root of *C. hirsuta* was 56.7 ± 0.06 and 41.7 ± 0.2%, respectively, whereas the radical inhibited by DCM:MeOH (1:1) extract, CHCl₃, EtOAc, *n*-BuOH fractions, and essential oil of stem barks of *G. ferruginea* were 81.80 ± 0.1, 74.60 ± 0.14, 79.90 ± 0.13, 82.50 ± 0.14, and 28.6 ± 0.17, respectively. Similarly, the DPPH radical inhibited by DCM:MeOH (1:1) extract and fractions 6-10 of leaves of *R. ruspolii* were 78.5 ± 0.07 and 49.5 ± 0.17%, respectively. While the radicals inhibited by DCM:MeOH (1:1) extract and fractions 4-17 of leaves of *O. rochetiana* were 70.60 ± 0.04 and 51.41 ± 0.07%, respectively. On the other hand,

the DPPH radicals inhibited by DCM:MeOH (1:1) extract and fractions 16-21 of leaves of *R. apetalus* were 82.0 ± 0.17 and $54.5 \pm 0.08\%$, respectively. Of all extracts, fractions, and essential oil evaluated for their DPPH radical scavenging activities, the *n*-BuOH fraction of the stem bark of *G. ferruginea* showed the best DPPH radical scavenging activity with 82.5%, followed by the DCM:MeOH (1:1) extract of *R. apetalus* leaves and stem bark of *G. ferruginea* with 82.0% and 81.8%, respectively.

Apart from the method used in this report, by mixing 1 mL of DPPH solution with 3 mL of the MeOH extract of the root of *C. hirsuta*, the DPPH radical scavenging reports indicated that at 1000 $\mu\text{g/mL}$ the percentage inhibition was 66.47% (Abdisa & Kenea, 2021). This is close to the percent of inhibition (56.7%) reported in this study at 62.5 $\mu\text{g/mL}$ for MeOH of the root of *C. hirsuta* (Table 35). According to published data, the DPPH radical scavenging activity evaluation has been reported using EtOH extract of the aerial parts of *R. retinorrhoea* and *R. tripartita*, revealing that the IC_{50} values were 315.8 and 118.7 $\mu\text{g/mL}$, respectively (Alam *et al.*, 2017), which are far less active than the *R. ruspolii* leaves DCM:MeOH (1:1) extract (IC_{50} : 9.6 $\mu\text{g/mL}$) that was reported in this study (Table 35). The DPPH radical scavenging activities that have been reported using the 80% MeOH, acetone, and MeOH extracts of the leaves of *R. apetalus* indicated the IC_{50} values were 12.3, 8.8, and 8.4 $\mu\text{g/mL}$, respectively (Tadesse *et al.*, 2008), which is comparable with the IC_{50} value (5.2 $\mu\text{g/mL}$) that was reported in our study from the DCM:MeOH (1:1) extract of the leaves of *R. apetalus* (Table 35).

Furthermore, regarding the isolated compounds (Table 36), compound **189** inhibited the highest DPPH radical (92.1%), followed by compounds **194**, **65**, **91**, **55**, **200**, **190**, **202**, and **197**, which inhibited 91.3, 90.2, 89.8, 89.4, 89.21, 86.5, 86.1, and 84.4% DPPH radicals, respectively. Whereas compound **17** inhibited the least (18.50%) DPPH radical. The difference between the DPPH radical scavenging ability of compounds **189** and **17** could be related to the number of hydroxyl groups found in compound **189**, suggesting that phenolic compounds exhibit potent DPPH radical scavenging activities (Gil *et al.*, 2000). The free radical of DPPH is reduced by abstracting hydrogen from antioxidant compounds (Blois, 1958). The reducing power of antioxidant compounds depends on the structural properties that enable them to form stable radicals through resonance stabilization after donating their hydrogen atom with a radical (Ali *et al.*, 2013). In this regard, compound **189**, exhibiting strong antioxidant properties (92.1%), contains an ortho hydroxyl donating group in the ring. This structural feature of compound **189** allows its free radical to have more delocalizing opportunities in the structure.

On the other hand, compound **17**, with the lowest antioxidant activity (18.50%), could not allow its free radical to delocalize as in compound **189**.

As indicated in Table 35 and Table 36, compared to the IC_{50} of the positive control, ascorbic acid (0.5 $\mu\text{g/mL}$), the IC_{50} values of evaluated samples ranged from 1.1 to 107.2 $\mu\text{g/mL}$, where the lowest IC_{50} value (1.1 $\mu\text{g/mL}$) was recorded for compound **194**, whereas the highest IC_{50} value (107.2 $\mu\text{g/mL}$) was recorded by the essential oil of *G. ferruginea* stem bark. In principle, a drug with the lowest IC_{50} value can be taken as the most effective as showing lower toxicity when administered to the patients (Berrouet *et al.*, 2020). In this regard, compound **194** with the lowest IC_{50} value (1.1 $\mu\text{g/mL}$) can be considered the most effective compound, followed by compound **65** (IC_{50} : 2.0 $\mu\text{g/mL}$), compound **189** (IC_{50} : 2.1 $\mu\text{g/mL}$), and compound **55** (IC_{50} : 2.8 $\mu\text{g/mL}$).

Table 35. DPPH scavenging activities of the extracts, fractions, and essential oil

Plant name/ part studied	Sample	% DPPH Inhibited using each $\mu\text{g/mL}$ concentration					IC_{50} value ($\mu\text{g/mL}$)
		1000	500	250	125	62.5	
Positive control	Ascorbic acid	97.9 ± 0.06	97.3 ± 0.05	97.2 ± 0.05	97.0 ± 0.1	96.9 ± 0.06	0.5
<i>C. hirsuta</i> /Root	<i>n</i> -hexane:EtOAc (1:1) extract	81.5 ± 0.12	78.5 ± 0.15	67.2 ± 0.1	54.6 ± 0.12	41.7 ± 0.2	3.1
	MeOH extract	91.1 ± 0.06	82.8 ± 0.2	73.2 ± 0.06	60.7 ± 0.06	56.7 ± 0.06	49.4
<i>G. ferruginea</i> /Bark	DCM: MeOH (1:1) extract	92.8 ± 0.09	91.5 ± 0.12	90.1 ± 0.12	87.4 ± 0.09	81.80 ± 0.1	9.0
	CHCl_3 fraction	89.7 ± 0.16	83.3 ± 0.08	82.3 ± 0.13	79.4 ± 0.09	74.6 ± 0.14	11.7
	EtOAc fraction	93.2 ± 0.09	90.9 ± 0.08	88.5 ± 0.21	83.1 ± 0.14	79.9 ± 0.13	11.4
	<i>n</i> -BuOH fraction	92.1 ± 0.13	90.0 ± 0.08	87.7 ± 0.09	83.5 ± 0.21	82.5 ± 0.14	7.8
	Essential oil	55.3 ± 0.14	53.6 ± 0.08	41.1 ± 0.08	29.8 ± 0.09	28.6 ± 0.17	107.2
<i>R. ruspolii</i> /Leaves	DCM: MeOH (1:1) extract	89.0 ± 0.24	88.2 ± 0.15	87.5 ± 0.04	80.0 ± 0.24	78.5 ± 0.07	9.6
	Fractions 6-10	76.5 ± 0.14	74.1 ± 0.08	62.6 ± 0.08	51.5 ± 0.09	49.5 ± 0.17	49.1
<i>O. rochetiana</i> /Leaves	DCM:MeOH (1:1) extract	88.33 ± 0.1	87.0 ± 0.23	82.8 ± 0.09	74.7 ± 0.15	70.6 ± 0.04	19.0
	Fractions 4-17	61.32 ± 0.1	57.1 ± 0.28	56.4 ± 0.04	53.4 ± 0.11	51.4 ± 0.07	11.5
<i>R. apetalus</i> /Leaves	DCM: MeOH (1:1) extract	88.8 ± 0.08	87.8 ± 0.18	86.2 ± 0.16	85.5 ± 0.21	82.0 ± 0.17	5.2
	Fractions 16-21	60.4 ± 0.16	58.1 ± 0.18	57.2 ± 0.12	56.0 ± 0.13	54.5 ± 0.08	6.1

Note. %Inhibition was reported as mean $\pm$ SD

Table 36. DPPH scavenging activities of isolated compounds

Plant name/part studied	Sample	% DPPH Inhibited using each $\mu\text{g/mL}$ concentration					IC_{50} value ($\mu\text{g/mL}$)
		1000	500	250	125	62.5	
Positive control	Ascorbic acid	97.9 ± 0.06	97.3 ± 0.05	97.2 ± 0.05	97.0 ± 0.1	96.9 ± 0.06	0.5
<i>C. hirsuta</i> /Root	Compound 70	40.3 ± 0.2	35.2 ± 0.2	33.1 ± 0.2	31.9 ± 0.2	30.3 ± 0.3	19.4
	Compound 189	95.4 ± 0.1	94.5 ± 0.2	94.0 ± 0.1	93.3 ± 0.1	92.1 ± 0.1	2.1
	Compound 190	95 ± 0.2	94.3 ± 0.2	94.2 ± 0.2	88.0 ± 0.1	86.5 ± 0.2	7.4
<i>G. ferruginea</i> /Bark	Compound 16	81.6 ± 0.08	79.9 ± 0.09	64.8 ± 0.12	48.9 ± 0.08	43.30 ± 0.13	83.6
	Compound 17	31.7 ± 0.29	31.3 ± 0.09	23.5 ± 0.13	20.5 ± 0.09	18.50 ± 0.09	67.1
	Compound 42	92.3 ± 0.13	90.7 ± 0.17	89.9 ± 0.05	89.8 ± 0.08	71.90 ± 0.14	16.7
	Compound 65	93.3 ± 0.19	91.3 ± 0.17	91.0 ± 0.20	90.6 ± 0.08	90.2 ± 0.13	2.0
<i>R. ruspolii</i> /Leaves	Compound 194	93.5 ± 0.09	93.1 ± 0.04	92.7 ± 0.15	92.1 ± 0.04	91.3 ± 0.11	1.1
	Compound 55	93.2 ± 0.07	92.9 ± 0.04	92.0 ± 0.07	91.3 ± 0.11	89.4 ± 0.04	2.8
	Compound 195	88.9 ± 0.15	85.3 ± 0.11	81.7 ± 0.11	67.5 ± 0.19	58.9 ± 0.04	38.0
	Compound 196	94.6 ± 0.07	93.3 ± 0.04	91.6 ± 0.15	75.5 ± 0.04	56.0 ± 0.21	40.5
	Compound 197	90.3 ± 0.19	89.9 ± 0.04	85.9 ± 0.07	85.3 ± 0.07	84.4 ± 0.15	4.2
<i>O. rochetiana</i> /Leaves	Compound 41	60.41 ± 0.11	56.4 ± 0.07	55.5 ± 0.04	54.3 ± 0.11	50.5 ± 0.07	11.1
	Compound 199	69.48 ± 0.14	65.5 ± 0.20	64.6 ± 0.09	63.3 ± 0.21	59.6 ± 0.13	9.4
	Compound 200	96.43 ± 0.09	96.2 ± 0.15	94.6 ± 0.11	91.3 ± 0.04	89.2 ± 0.20	5.8
	Compound 83	90.66 ± 0.20	89.7 ± 0.19	83.9 ± 0.23	79.2 ± 0.22	60.8 ± 0.04	31.5
<i>R. apetalus</i> /Leaves	Compound 145	53.6 ± 0.12	51.3 ± 0.08	50.5 ± 0.11	49.6 ± 0.14	47.7 ± 0.06	7.0
	Compound 201	52.0 ± 0.06	51.7 ± 0.11	51.0 ± 0.12	50.3 ± 0.10	45.2 ± 0.13	9.7
	Compound 91	94.4 ± 0.03	93.3 ± 0.12	92.5 ± 0.10	91.3 ± 0.09	89.8 ± 0.12	3.2
	Compound 202	92.5 ± 0.08	91.7 ± 0.10	89.6 ± 0.07	88.4 ± 0.13	86.1 ± 0.15	4.8

Note. %Inhibition was reported as mean $\pm$ SD

4.4 *In silico* Molecular Docking Analysis of the Isolated Compounds

Based on their roles, bacterial enzymes (*E. coli* DNA gyrase B and *Pseudomonas* quinolone signal A (PqsA)) and cancer cell enzymes (Pyruvate kinase M2 (PKM2) and human topoisomerase II β) are among the targeted enzymes used in the study of *in silico* molecular docking. *E. coli* DNA gyrase B is a bacterial enzyme responsible for DNA replication, transcription, and recombination (Costenaro *et al.*, 2007), and *Pseudomonas* quinolone signal A (PqsA) is a bacterial enzyme that plays a role in the virulence factor of *P. aeruginosa* (Coleman *et al.*, 2008). Pyruvate kinase M2 (PKM2) is one of the isoforms of the pyruvate kinase enzyme used to meet the energy demand for cancer cells (Mazurek, 2011; Zahra *et al.*, 2020) and human topoisomerase II β is a tumor cell enzyme used in the multiplication of DNA (Wu *et al.*, 2011). Therefore, the molecular docking study of isolated compounds was carried out against target enzymes *E. coli* DNA Gyrase B and PqsA to validate the *in vitro* antibacterial activities. In addition, molecular docking was done against PKM2 and human topoisomerase II β to predict the *in silico* anticancer activities of the isolated compounds.

The results of docking analysis against *E. coli* DNA Gyrase B and PqsA were compared with the standard antimicrobial, ciprofloxacin, and then displayed in Table 37 and Table 38, respectively. The results of the docking study of isolated compounds against *E. coli* DNA Gyrase B revealed that the binding affinity of the isolated compounds ranged from -11.1 to -5.5 kcal/mol compared with the binding affinity of ciprofloxacin (-7.4 kcal/mol) (Table 37). The highest docking score (-11.1 kcal) was shown by compound **62**, followed by compound **195** (binding affinity was -10.3 kcal/mol), while the lowest (-5.5 kcal/mol) was exhibited by compound **196**. As indicated, except for compounds **17** and **41**, all isolated compounds interacted with *E. coli* DNA gyrase B through different numbers of hydrogen bonds using different amino acid residues. In another way, except for compound **16**, all isolated compounds interacted with *E. coli* DNA gyrase B using different types of hydrophobic and electrostatic forces. However, all isolated compounds interacted with *E. coli* DNA gyrase B using Van der Waals through different types and numbers of amino acid residues. As an example, in addition to hydrophobic, electrostatic, and Van der Waals interaction forces, compound **195** with the highest binding affinity (-10.3 kcal/mol) interacted with *E. coli* DNA gyrase B using four hydrogen bonds through Glu-50, Arg-76, Gly-77, and Val-43 amino acid residues. Whereas compound **196** with the lowest binding affinity (-5.5 kcal/mol) showed only two hydrogen bonds through Thr-165 and Gly-77 amino acid residues.

Table 37. Molecular docking results of isolated compounds against *E. coli* DNA Gyrase B (binding affinity in kcal/mol)

Ligand	Binding Affinity	H-bonding	Hydrophobic and Electrostatic	Van der Waals
70	-7.8	Thr-165	Alkyl: Ile-78, 94	Leu-98, Val-93, 97, 120, Gly-119, 75, 77, Asn-46, Asp-73, Glu-50, Arg-76, Pro-79
189	-6.4	Asn-46, Arg-76, Asp-49	Pi-sigma: Ile-78; Alkyl and Pi-alkyl: Ala-47, Ile-94, Val-120, Ala-53; Carbon hydrogen bond: Asp-73	Gly-119, Val-93, 167, 97, Pro-79, Thr-165, Met-95, Glu-50, Leu-98
190	-7.6	Arg-76, Asn-46, Val-120	Pi-sigma: Thr-165, Alkyl and Pi-alkyl: Ile-94, Val-93, Val-97, Ile-78, Ala-47, Val-43; Carbon hydrogen bond: Val-71, Thr-175, Gly-119	Pro-79, Arg-136, Ala-90, 73, 47, Gly-77, 119, 119, Asp-49, 73, Val-93, Leu-98, Met-166, Glu-50
191	-7.9	Asp-73, Val-120, Ser-121	Pi-sigma: Thr-165; Alkyl and Pi-alkyl: Ala-47, Val-43, Val-97, Ile-94, Val-93, Ile-78; Carbon hydrogen bond: Val-71, Asp-73, Gly-119	Val-167, Asn-46, Asp-49, His-83, Arg-76, 136, Pro-79, Gly-77, Glu-50,
16	-6.9	Thr-157	-	Gly-158, Ile-140, Arg-206, 142, His-64, Gln-72, Ser-70, Met-166, Glu-131, Val-133
17	-6.8	-	Pi-sigma: Phe-196; Alkyl and Pi-alkyl: Arg-192, Leu-197, Phe-196	Glu-193, Ser-195, His-217, Phe-216, Tyr-218
192	-8.0	Asp-73	Alkyl: Ile-78, Ile-94; Pi-sigma: His-83	Ala-90, 47, Val-93, Pro-79, Arg-76, 136, Gly-77, Thr-165, Glu-50, Asn-46
193	-8.0	Asp-73	Alkyl: Ala-90, Ile-78, Val-93, Ile-94; Pi-sigma: His-83	Pro-79, Gly-77, Glu-50, Thr-165, Ala-47, Asn-46, Asp-49
41	-7.7	Glu-85	Alkyl and Pi-alkyl: Tyr-26, Leu-98, Lys-11, Val-97	Val-118, Ser-8, Glu-86, Val-12, Gly-15
65	-7.8	Asp-73, Arg-76	Carbon hydrogen bond: Asn-46; Pi-anion: Glu-50; Pi-sigma: Thr-165; Pi-alkyl: Ala-47	Val-71, 43, 167, 120, Ile-78, 94, Asp-49
194	-5.9	Val-167, 71	Amide pi-stacked: Asn-46; Pi-alkyl: Ile-78, Val-120	Ile-94, Asp-73, Thr-165, Ala-47, Met-166, Val-43
55	-8.5	Gly-77, Asp-73, Val-43	Pi-anion: Glu-50; Pi-sigma: Thr-165; Amide pi-stacked: Gly-77; Pi-alkyl: Ile-78, Pro-79	Arg-136, 76, Asn-46, Val-71, 167, 120, Ala-47, Ile-94
195	-10.3	Glu-50, Arg-76, Gly-77, Val-43	Pi-anion: Glu-50; Pi-sigma: Thr-165, Asp-49; Pi-alkyl: Ala-53, Ile-78, 94	Leu-52, Pro-79, Arg-136, Asp-73, Ala-47, Val-167, 71, 120, Gly-119, Asn-46

Table 37. Molecular docking results of isolated compounds against *E. coli* DNA Gyrase B (binding affinity in kcal/mol) (Contn'd)

Ligand	Binding Affinity	H-bonding	Hydrophobic and Electrostatic	Van der Waals
62	-11.1	Glu-50, Arg-76, Gly-77, Val-97	Pi-anion: Glu-50; Pi-sigma: Ile-94; Amide pi-stacked: Asn-46; Pi-alkyl: Ile-78, 94, Val-120	Asp-49, 73, Pro-79, Arg-136, Thr-165, Val-43, 167, 93, His-83
196	-5.5	Thr-165, Gly-77	Carbon hydrogen bond: Pro-79	Arg-136, 76, Glu-50, Gly-75, 164. Asp-73, Ile-78, 94
197	-8.0	GLY-77	Pi-cation and anion: Arg-76, Glu-50; Pi-alkyl: Pro-79, Ile-78	Arg-136, Ala-47, Asp-73, Val-71, 43, 167, 120, Thr-165, Asn-46, Ile-94, Gly-119, Asp-49
198	-7.6	Arg-76, Gly-77, Thr-165, Asp-73	Pi-anion: Glu-50; Amide pi-stacked: Asn-46; Pi-alkyl: Pro-79, Ile-78	Ile-94, Asp-49, Ala-53, Gly-75, 164
41	-8.1	-	Alkyl: Ile-78	Ala-73, Asp-49, Arg-76, Glu-50, Asn-46, Asp-73, Thr-165, Val-120, Pro-79, Ile-94
199	-5.1	Gly-77, Thr-165	Carbon hydrogen bond: Glu-50; Alkyl: Ile-78	Asn-46, Asp-73, Gly-164, 75, Arg-76, Pro-79
200	-8.4	Val-43, Met-95	Carbon hydrogen bond: Val-43; Pi-cation and anion: Arg-76, Glu-50; Pi-alkyl: Pro-79, Ile-78	Arg-136, Asp-73, Ala-47, Val-71, Thr-165, Val-167. Val-120, Asn-46, Ile-94, Gly-119, Asp-49
84	-7.8	Thr-165, Arg-136, 76	Carbon hydrogen bond: Asp-73; Pi-cation: Arg-76; Pi-alkyl: Pro-79	Val-167, Val-120, Val-43, Ile-94, Asn-46, Gly-77, Glu-50, Ile-78
83	-7.1	Arg-76, Asn-46, Val-71, 167	Pi-alkyl: Ile-78	Asn-49, Ile-94, Val-120, 43, Thr-165, Met-166, Gln-72, Ala-47, Asp-73, Glu-50, Pro-79
91	-8.0	Glu-50, Arg-76, Gly-177	Pi-anion: Asp-49; Pi-sigma: Ile-78; Amide Pi-stacked: Asn-46	Ala-53, His-55, Ile-94, Pro-79, Gly-77, 119, Val-167, 43, 120, Thr-165, Asp-73
202	-7.2	Asp-73, Arg-76, 136, Gly-77	Carbon hydrogen bond: Glu-50; Pi-sigma: Ile-78; Amide Pi stacked: Asn-46; Pi-alkyl: Val-120, Ile-94	Met-95, Val-43, 167, Thr-165, Ala-47, Pro-73
Ciprofl oxacin	-7.4	Arg-76	Halogen: Asp-73; Amide-pi stacked: Gly-77; Alkyl and Pi-alkyl: Ile-78, 94, Pro-79; Carbon hydrogen bond: Asp-73	Arg-136, Ala-47, Asn-46, Val-43, 167, 120, Glu-50, Thr-165

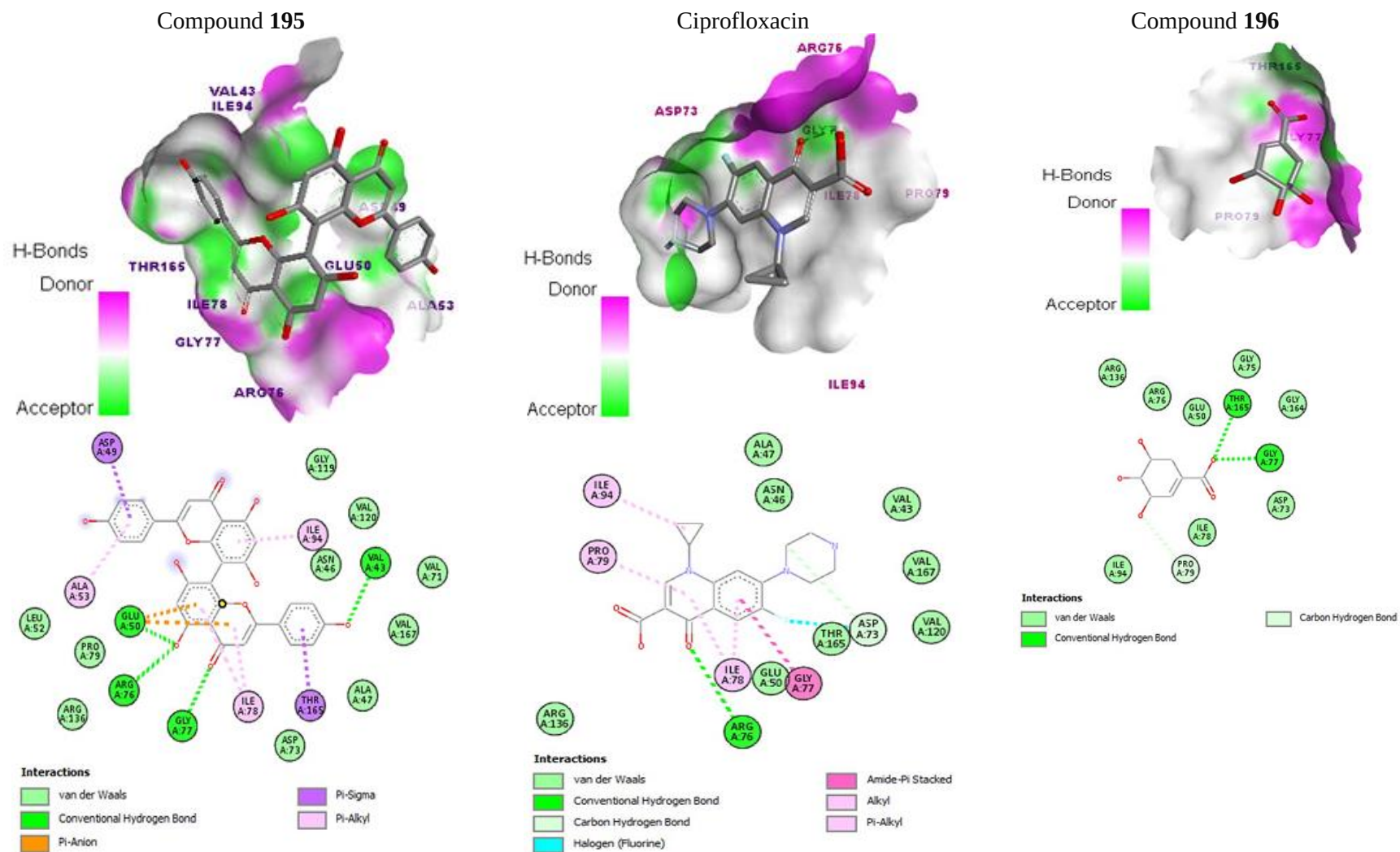


Figure 32. 3D (top) and 2D (bottom) binding interactions of compounds **195**, **196**, and ciprofloxacin against *E. coli* DNA gyrase

The 2D and 3D modes of interaction of compounds **19** and **196** with *E. coli* DNA Gyrase B are compared with the positive control, ciprofloxacin (Figure 32), and for the rest of the isolated compounds, the 2D and 3D modes of interaction are shown in Appendix 27.

E. coli DNA gyrase B is a topoisomerase II enzyme used to introduce a negative supercoil in DNA that is important for the local unwinding of bacterial DNA, which in turn allows the process of DNA replication, transcription, and recombination (Costenaro *et al.*, 2007). Inhibitors (drugs), for example, ciprofloxacin, block the normal process carried out by this enzyme, leading to the death of bacterial cells (LeBel, 1988). The strength to block this enzyme using inhibitors depends on the stability of the *E. coli* DNA gyrase B inhibitor complex expressed by a higher negative binding energy value (Ivanova & Karelson, 2022). In this regard, it can be said that compound **195** with the highest negative value energy disrupts the DNA replication, transcription, and recombination process in *E. coli* DNA gyrase B and is therefore considered a strong antibacterial agent against *E. coli*, as showed by an IZ of 12.67 mm compared to compound **196** with IZ of 9.00 mm (Table 32). Therefore, for isolated compounds, the *in silico* antibacterial study supported the *in vitro* antibacterial activities test, and hence the isolated compounds can be considered as potential antibacterial agents for *E. coli*. Furthermore, it is noted that ciprofloxacin targets not only DNA gyrase (topoisomerase II) but also targets topoisomerase IV (Drlica & Zhao, 1997). Therefore, the inconsistency of the result for ciprofloxacin observed *in vitro* with an *in silico* antibacterial activity test might be related to the ciprofloxacin mechanism of action since the *in silico* antibacterial activity evaluation was performed against a specific enzyme.

Table 38 summarizes the results of molecular docking studies of isolated compounds against the target enzyme (PqsA) and showed that the binding affinity ranged from -10.3 to -6.1 compared to the binding affinity (-7.2 kcal/mol) of the positive control (ciprofloxacin). The maximum binding affinity (-10.3 kcal/mol) was exhibited by compound **62**, followed by compound **91** (-9.5 kcal/mol), compound **199** (-9.3 kcal/mol), compound **16**, and compound **195**, both with -9.1 kcal/mol, whereas the least binding affinity (-6.1 kcal/mol) was exhibited by compound **196**. As shown in Table 38, some compounds, including compounds **70**, **16**, **17**, **193**, and **41**, did not show hydrogen bonding interaction with PqsA. Though it did not show hydrogen bonding, compound **16** interacted with PqsA with a better binding affinity (-9.1 kcal/mole) by using a pi-sigma bond through the Trp-233 amino acid residue in addition to Van der Waals forces through different amino acid residues. However, though it showed four hydrogen bonds through Thr-304, Gly-307, Gly-279, and Gly-302 amino acid residues,

compound **196** interacted with PqsA with the least binding affinity (-6.1 kcal/mol). The 2D and 3D modes of interaction of compound **16**, **196**, and ciprofloxacin with PqsA are displayed in Figure 33. For other isolated compounds, their 2D and 3D modes of interaction are shown in Appendix 28.

Regarding the correlation between the number of hydrogen bonds and the values of the docking score, it can be concluded that sometimes it could not be shown as directly proportional. For example, the results of a molecular docking study have been reported against the *Plasmodium falciparum* hexose transporter 1 (PfHT1) protein, demonstrating that the ligand protein complex with a higher number of hydrogen bonds was found with the lowest docking score (Owoloye *et al.*, 2022). Furthermore, *P. aeruginosa* is an opportunistic, multidrug resistant, gram negative pathogenic bacterium able to control its virulence factors through different intercellular signals known as quorum sensing signals. One of the quorum sensing signals is the *Pseudomonas* quinolone signal (PQS), which is synthesized with the help of an enzyme called *Pseudomonas* quinolone signal A (PqsA) (Coleman *et al.*, 2008). Interfering with the functions of PqsA results in the absence of PQS synthesis, which in turn inhibits the expression of virulence factors by *P. aeruginosa* (Calfee *et al.*, 2001). Based on this fact, of all the isolated compounds in this report, compound **16** can be considered the most inhibitory of the virulence factors expressed by *P. aeruginosa*, as it showed better binding affinity (-9.1 kcal/mol) and maximum IZ of 15.2 mm (Table 32) with PqsA, which is a crucial enzyme in the expression of virulence factors in *P. aeruginosa*.

Table 38. Molecular docking results of isolated compounds against PqsA (binding affinity in kcal/mol)

Ligand	Binding Affinity	H-bonding	Hydrophobic and Electrostatic	Van der Waals
70	-9.0	-	Pi-sigma: Phe-209, Trp-233, Alkyl and Pi-alkyl: Pro-205, Trp-233	Val-254, Ile-204, Pro-234, 236, Ser-235, Phe-208
189	-8.3	Ser-226, Gly-198, Arg-200, 200	Pi-anion: Asp-17, Asp-19; Pi-sigma: Ala-21; Alkyl and Pi-alkyl: Arg-249, Arg-200, Ala-21; Pi-donor and carbon hydrogen bond: Asp-199, Ala-21, Asp-19, Arg-247	Thr-29, 20, 20, 29, His-24, Asp-199, 19, 17, Gly-198, Pro-248, Ser-31, 226, His-24
190	-8.7	Arg-200, 200, His-24, Ser-226	Pi-anion and cation: Arg-200, Asp-19; Alkyl and Pi-alkyl: Arg-249, Thr-20; Carbon and Pi-donor hydrogen bond: Gly-198, Arg-247, Asp-19, Gly-224, Ala-21	Pro-248, Thr-20, 20, 29, Gly-198, Ala-125, 21, 197, Asp-199, 19, 17, His-24
191	-8.4	Gln-34, Ser-31, Arg-200, 200, Ala-21	Pi-cation: Arg-200, Arg-200; Pi-sigma: Thr-29; Alkyl and Pi-alkyl: Ala-245, Ala-21, Ala-21	Leu-228, 30, Phe-246, His-24, Thr-29, 20, 20, Ser-31, Asp-19, 19, Gly-198, 27
16	-9.1	-	Pi-sigma: Trp-233	Ala-256, Pro-234, 205, Asn-84, Phe-208, 209, Lys-86, 206, Ile-204, 257, Val-254
17	-8.8	-	Pi-sigma: Phe-209, Trp-233; Alkyl and Pi-alkyl: Pro-205, Phe-209, Trp-233	Val-254, Pro-234, Phe-208, Lys-206, Ile-204
192	-8.9	Lys-86	Alkyl: Pro-205, Pro-234; Pi-sigma: Phe-209, Trp-234	Ser-235, Ile-204, 257, Lys-206, Phe-208
193	-8.3	-	Alkyl and Pi-alkyl: Lys-206, Pro-234, Trp-233	Phe-209, 208, Ser-235, Pro-236, 205, Val-254, Ile-204, 257
41	-8.4	Arg-200, 200, Ala-245	-	Gly-198, 198, 27, 27, Thr-20, 29, 29, Ala-21, 21, Gln-28, Phe-246, His-24, 24, Asp-19
65	-8.0	Gly-279, 300, Tyr-378, Glu-305, 305, Thr-304, 164	Carbon hydrogen and Pi-donor hydrogen bond: Pro-281, His-394; Pi-sigma and Amide pi-stacked: Ile-301, Ala-303, Gly-279	Ala-278, Ser-280, Asp-382, Arg-397, Gly-302
194	-7.3	Gly-279, 302, 307, Thr-307	Pi-pi stacked, Pi-pi T-shaped, and Amide pi-stacked: His-308, Tyr-211, Gly-2010	Ile-301, Val-309, Ala-303, 278
55	-9.0	Gly-300, 279, His-394, Thr-164, 304, Glu-305	Carbon hydrogen bond and pi-donor hydrogen bond: Ile-301, pro-281; Pi-anion: Asp-382; Pi-sigma: Ala-303; Amide pi-stacked: Gly-279; Pi-alkyl: Ile-301	Ala-278, Ser-280, Arg-397, Tyr-378, Gly-302

Table 38. Molecular docking results of isolated compounds against PqsA (binding affinity in kcal/mol) (Contn'd)

Ligand	Binding Affinity	H-bonding	Hydrophobic and Electrostatic	Vaan der Waals
195	-9.1	Arg-372, Thr-380, 304	Pi-cation and anion: Asp-382, Arg-397; Pi-donor hydrogen bond: Thr-164; Pi-sigma: Ile-301; Amide	Gly-102, 210, Ser-280, Pro-281, Tyr-378, 163, Phe-209
62	10.3	Thr-164, 304, 323, Glu-305, Gly-279, 300, Asp-299	Carbon hydrogen bond: 301; Pi-anion: Asp-382; Pi-sigma: Ile-301; Pi-pi T shaped and Amide pi-	Phe-209, Tyr-378, 380, Gly-302, Ala-278, Arg-397
196	-6.1	Thr-304, Gly-307, 279, 302	Carbon hydrogen bond and Pi-donor hydrogen bond: His-308	Ala-303, 278, Gly-210, Tyr-211, Val-309, Ile-301
197	-8.9	Thr-380, 304, Arg-397, Tyr-378, Glu-305	Carbon hydrogen bond and Pi-donor hydrogen bond: Tyr-211, Gly-279; Amide pi stacked: Gly-302; Pi-alkyl: Ala-278	Val-309, His-308, Gly-210, Ala-303, Arg-372, Thr-164, Asp-382
198	-9.0	Arg-372, 397, Tyr-378, Glu-305, Thr-164, Asp-382, Thr-304	Carbon hydrogen bond and Pi-donor hydrogen bond: Gly-279, Tyr-211; Pi-alkyl: Ala-278	Lys-172, Ala-303, Gly-210, 302, Val-309, His-394
41	-8.2	-	Carbbon hydrogen bond: Gly-279	Ser-280, Pro-234, Ile-257, 204, Lys-206, Pro-205, Phe-209, Val-254
199	-6.3	Thr-304	Carbon and pi-donor hydrogen bond: Gly-307, His-308	Val-309, Gly-302, 279, 210, Ala-278, 303, Tyr-211
200	-9.3	Thr-304, Thr-304, Arg-397, Asp-382, Asp-382	Carbon and pi-donor hydrogen bond: Tyr-380, Gly-279, Tyr-211; Pi-alkyl: Ala-278	Val-309, Gly-302, Ala-303, Gly-210, Ile-301, Tyr-378, Arg-372, Thr-164, His-394
84	-7.7	Lys-206, Arg-397, Thr-380, Glu-305, Tyr-378, Thr-304, Thr, 164	Carbon hydrogen bond: Asp-382	Trp-233, Phe-209, Ala-303, Gly-279, Val-254, Pro-205, Pro-234
83	-7.4	Thr-304, Tyr-378, Thr-323, Asp-382	Carbon hydrogen bond: Glu-305; Pi-anion: Asp-382, Made pi-stacked: Gly-279; Pi-alkyl: Ile-301	Thr-164, Ala-303, Gly-302, Pro-281, Phe-384, Asp-299, His-394, Gly-300, Ser-280, Arg-397
91	-9.5	Thr-323, 164, Asp-382	Pi-pi Stacked, Pi-pi T-shaped, and Amide Pi-stacked: His-394, Ser-280, Gly-302, Tyr-211; Pi-alkyl: Pro-281, Ile-301	Leu-282, Asp-299, Gly-300, 279, Ala-278, 303, His-308, Val-309, Thr-304, 380, Glu-305, Tyr-378, Arg-372, 397, Phe-384
202	-8.3	Thr-304, 304, 164, Asp-382, Arg-397, Tyr-378, Arg-372,	Carbon hydrogen bond and Pi-donor hydrogen bond: Tyr-211, Gly-279	Gly-302, 210, Ala-278, 303, Val-309, Ile-301, Thr-380
Ciprofl oxacin	-7.2	Arg-200	Halogen: Asp-19, Arg-200; Alkyl: Ala-21; Carbon hydrogen bond: Gly-198, Asp-17	His-24, Ser-226, Thr-29, 20, Arg-249, Gly-198

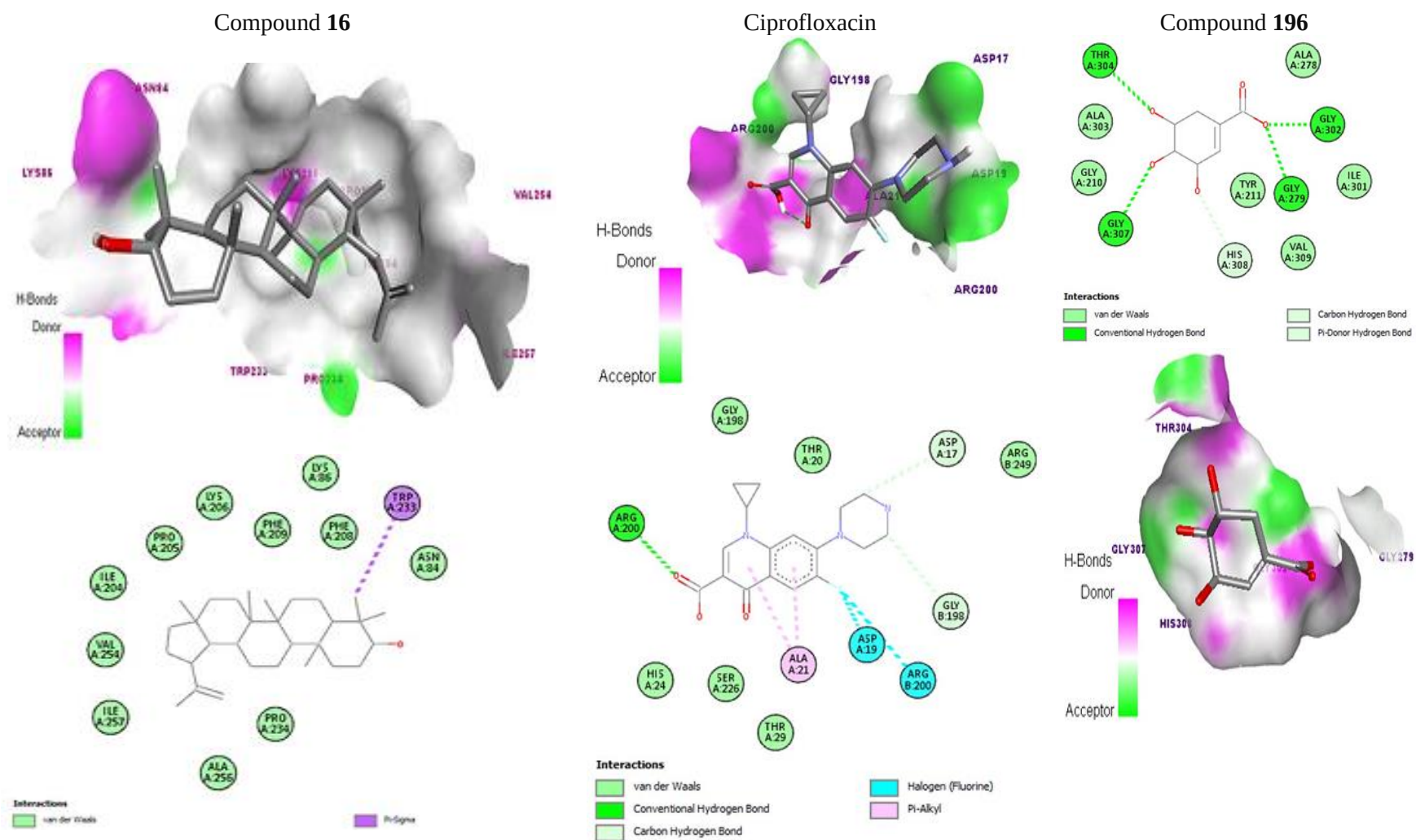


Figure 33. 3D (top) and 2D (bottom) binding interactions of compounds **16**, **196**, and ciprofloxacin against PqsA

Table 39 summarizes the results of the docking analysis of isolated compounds against PKM2 compared with positive controls (abiraterone and PKM2-IN-1). As indicated, compared to the binding affinity of positive controls, abiraterone (-9.5 kcal/mol) and PKM2-IN-1 (-9.6 kcal/mol), the binding affinities of the isolated compounds with the target enzyme, PKM2, ranged from -14.7 kcal/mol to -4.1 kcal/mol. The highest binding affinity (-14.7 kcal/mol) was shown by compound **195**, followed by compound **62** (-13.6 kcal/mol), compound **91** (-11.7 kcal/mol), compounds **55** and **84**, both with -10.5 kcal/mol, compound **198** (-10.4 kcal/mol), compound **197** (-10.2 kcal/mol), and compound **202** (-10.1 kcal/mol). Whereas the lowest binding affinity (-4.1 kcal/mol) was shown by compound **41**. As indicated in Table 39, except for compounds **70** and **16**, all isolated compounds interacted with PKM2 using hydrogen bonds through different numbers and types of amino acid residues.

In another way, except for compound **16**, compound **42**, and compound **196**, all isolated compounds interacted with PKM2 using various types of hydrophobic and electrostatic forces. Whereas all isolated compounds interacted with PKM2 using Van der Waals through different numbers of amino acid residues. Compound **195**, with the highest binding affinity (-14.7 kcal/mol), interacted with PKM2 using five hydrogen bonds through Tyr-390, Ala-388, Asp-354, 354, and Lys-311 amino acid residues, in addition to various types of hydrophobic, electrostatic, and Van der Waals forces. While compound **41**, with the least binding affinity (-4.1 kcal/mol), interacted with PKM2 using only two hydrogen bonds through Asp-354 and Asn-350 amino acid residues in addition to some hydrophobic, electrostatic, and Van der Waals forces. The 2D and 3D modes of interactions of compounds **195** and **41** against PKM2 are given in Figure 34 and Appendix 29 for the other isolated compounds.

In addition to normal glycolysis (respiration), although it produces low ATP molecules, and, however, due to its high production rate, cancer cells prefer to utilize anaerobic glycolysis (fermentation) to produce additional ATP molecules to meet the energy demand needed for highly proliferated cancer cells (Pfeiffer *et al.*, 2001). The shift from normal glucose metabolism to fermentation (lactate production) in cancer cells is accomplished by one of the isoforms of the pyruvate kinase enzyme called PKM2 (Mazurek, 2011; Zahra *et al.*, 2020). Inhibition of this enzyme (PKM2) blocks the glycolytic pathway in cancer cells, leading to the deaths of cancer cells (Park *et al.*, 2021). As discussed above, of all the isolated compounds, compound **195** showed the strongest binding affinity to this enzyme and therefore can be considered as a good inhibitor of PKM2 enzymes that block the glycolytic pathway and, in turn, lead to the deaths of cancer cells.

Table 39. Molecular docking results of isolated compounds against PKM2 (binding affinity in kcal/mol)

Ligand	Binding Affinity	H-bonding	Hydrophobic and Electrostatic	Van der Waals
70	-7.1	-	Alkyl: Pro-403, Met-22, Leu-392	Leu-401, Arg-400, 399, Ala-402, 21, Ile-404, Lys-422, Phe-421, 395, Glu-418
189	-8.5	Asp-24, Arg-400, 400, Gln-393	Pi-anion and Pi-cation: Arg-400, Glu-396, Glu-396; Carbon hydrogen bond: Glu-396, Gln-393	Thr-25, 25, 25, 25, 390, Gln-393, Glu-397, 397, 397, Leu-392, Arg-400, Asp-24, 24, 24, Tyr-390, Phe-26
190	-8.3	Arg-400, Arg-400, Glu-396	Carbon hydrogen bond: Gly-393, Thr-25; Pi-anion: Glu-397, 396	Thr-25, Glu-396, Glu-397, Phe-26, Asp-24, Asp-24, Gln-393, Thr-25, Tyr-390, Leu-392, Arg-400, Arg-400, Tyr-390, Thr-25, Phe-26, Asp-24, Phe-26
191	-8.1	Arg-400, 399, 447, His-391	Pi-anion: Asp-24; Pi-sigma: Ile-404, Pi-sulfur: Met-22, Pi-pi T-shaped: Phe-421; Alkyl and Pi-alkyl: Ala-21, Lys-422, Ile-404; Carbon hydrogen bond: Arg-400	Arg-32, Leu-401, 392, 18, Glu-418, Ala-402, Pro-403
16	-7.4	-	-	Arg-376, 339, Leu-380, Lys-173, Pro-212, 302, 338, Glu-300, 304, 373, Ile-299, 301, Tyr-175, Ala-303, Met-377
17	-7.5	Tyr-390	Pi-alkyl: Tyr-390	Gln-393, 393, Arg-400, 400, 400, Thr-25, 25, Asp-24, 24, Glu-397, 396, 396, 396, Leu-392
192	-6.9	Glu-198	Alkyl and Pi-alkyl: Pro-212, Arg-339, Tyr-175; Pi-sigma: Tyr-175	Ile-299, Gly-298, Ala-303, Pro-302, 338, Glu-304, 373, Leu-380, Met-377, Lys-136, 173, Asn-199, Ser-182
193	-7.1	Glu-418, Arg-399	Alkyl: Met-22	Asp-24, Leu-401, 392, Arg-400, 447, Phe-421, 395, Lys-422, Ile-404, Ala-402, Pro-403
42	-7.1	Arg-106, 43	-	Ser-67, Lys-66, Arg-383, 500, 376, Gly-68, His-464, 379, Asn-70, Phe-470, 502, Ala-42, Thr-41, Leu-380
65	-9.9	Tyr-390, Lys-311, 311, Asp-354, Ala-388	Pi-sigma: Leu-353; Pi-pi T-shaped: Phe-26, 26; Alkyl: Leu-353	Ile-389, 389, His-29, Met-30
194	-6.2	Tyr-390, Glu-397	Pi-sigma: Leu-394; Pi-pi stacked: Phe-26	Gln-393, Phe-26, Leu-27, 353, Ile-389
55	-10.5	Lys-311, Glu-397, Met-30, Tyr-390	Carbon hydrogen bond: Ile-389; Pi-sigma: Leu-394; Pi-pi T-shaped: Phe-26, 26; Pi-alkyl: Leu-	Asp-354, 354, Met-30, Ala-388, His-29, Asn-318, Leu-27, Gln-393
195	-14.7	Tyr-390, Ala-388, Asp-354, 354, Lys-311	Pi-sigma: Leu-353; Pi-pi T-shaped: Phe-26, 26; Pi-alkyl: Leu-394, Met-30, Leu-353. 353	Ile-389, 383, His-29, Leu-33, 27, 27, 394, Lys-311, Met-30

Table 39. Molecular docking results of isolated compounds against PKM2 (binding affinity in kcal/mol) (Contn'd)

Ligand	BA	H-bonding	Hydrophobic and Electrostatic	Van der Waals
62	-13.6	Ala-388, Lys-311, Asp-354, Tyr-390, 390	Carbon hydrogen bond: Ile-389, 389; Pi-sigma: Leu-353; Pi-pi T-shaped: Phe-26, 26; Pi-alkyl: Leu-394, 394, Met-30	Leu-33, 27, 353, His-29, 391, Met-30, Gln-393, Glu-397, Lys-311, Asn-350, Asp-354
196	-6.2	Tyr-390, 390, Leu-394	-	Gln-393, Glu-397, Met-90, Leu-27, 353, Phe-26, 26, Asn-318, Ile-389
197	-10.2	Asn-350, 350, Lys-311, Glu-397	Carbon hydrogen bond: Leu-27, Asp-354; Pi-sigma: Leu-353; Pi-pi T-shaped: Phe-26; Pi-alkyl: Leu-394	Phe-26, Met-30, Leu-353, 33, Lys-311, His-29, Ala-388, Ile-389, Tyr-390, Gln-393
198	10.4	Lys-311, 311, His-29, Tyr-390, Ala-388, Glu-397, Met-30	Carbon hydrogen and pi-donor hydrogen bond: Asp-354, Leu-394, Phe-26; Pi-sigma: Leu-353; Pi-pi T-shaped: Phe-26; Pi-alkyl: Leu-394	Met-30, Asp-354, Asn-350, Leu-33, 27, 353, Ile-389, Gln-393
41	-4.1	Asp-354, Asn-350	Carbon hydrogen bond: Leu-394; Pi-sigma: Phe-26; Alkyl: Leu-27	Lys-311, 311, Asn-350, 318, Asp-354, Gly-355, Arg-445, Gln-393, Glu-397, Met-30, Phe-26, Leu-353
199	-5.5	Tyr-390	Alkyl and Pi-alkyl: Leu-394, Tyr-390, Phe-26	Leu-353, 27, Ile-389, Gln-393, Phe-26, Met-
200	-9.6	Lys-311, 311, Gly-315, Asp-354, Tyr-390, Leu-394	Carbon hydrogen bond: Leu-353, Ile-359; Pi-sigma: Met-30, Phe-26; Pi-pi stacked and T-shaped: Phe-26, Phe-26; Pi-sulfur: Met-30; Pi-lone pair: Asp-354	Ile-314, Leu-27, Ala-388, Gln-393, Leu-353, Asn-350
84	-10.5	Tyr-390, Lys-311, Asp-354	Carbon hydrogen bond: Ile-383; Pi-pi T-shaped: Phe-26	Ile-389, Leu-394, Phe-26, Glu-397, Gln-393, Leu-394, Tyr-390, Leu-353, Asp-354, Asn-350, Leu-353
83	-8.5	Tyr-390, 390, His-29, Ala-388, Asp-354, Lys-311	Carbon hydrogen bond: Leu-353; Pi-pi T-shaped: Phe-26	Ile-389, 389, Leu-394, 394, 353, Phe-26, Met-90
91	-11.7	Glu-397, 397, Lys-311, Asn-350, 350, Ala-388, Tyr-390	Carbon Hydrogen bond: Ile-389; Pi-sigma: Leu-394, 353; Pi-pi T-shaped: Phe-26, 26; Pi-alkyl: Leu-394	Leu-27, 27, 33, 353, Gln-393, 393, Ala-388, Phe-26, Met-30, Tyr-390, Ile-389, Lys-311
202	-10.1	Ala-388, Lys-311, Asn-350, Glu-397	Pi-sigma: Leu-353; Pi-pi stacked and Pi-pi T shaped: Phe-26, 26; Pi-alkyl: Leu-394	Gln-393, Leu-27, 353, 33, Met-30, Asp-354, 354, Lys-311, Phe-307, His-29, Tyr-390, Ile-389
Abirat erone	-9.5	Asn-350	Carbon hydrogen bond: Glu-397; Pi-sigma: Leu-394; Alkyl and Pi-alkyl: Phe-26, Leu-353, Leu-353	Leu-27, Ile-389, Tyr-390, Lys-311, Asn-350, Asp-354, 354, Met-30, Phe-26, Gln-393
PKM2 -IN-1	-9.6	Lys-311	Pi-pi T-shaped: Phe-26, 26; Alkyl and Pi-alkyl: Leu-353, 353, Phe-26; Pi-sigma: Leu-353; Pi-sulfur: Phe-26	Asp-354, 354, Lys-311, Tyr-390, 390, Ala-388, 388, Ile-389, 389, Met-30, His-29

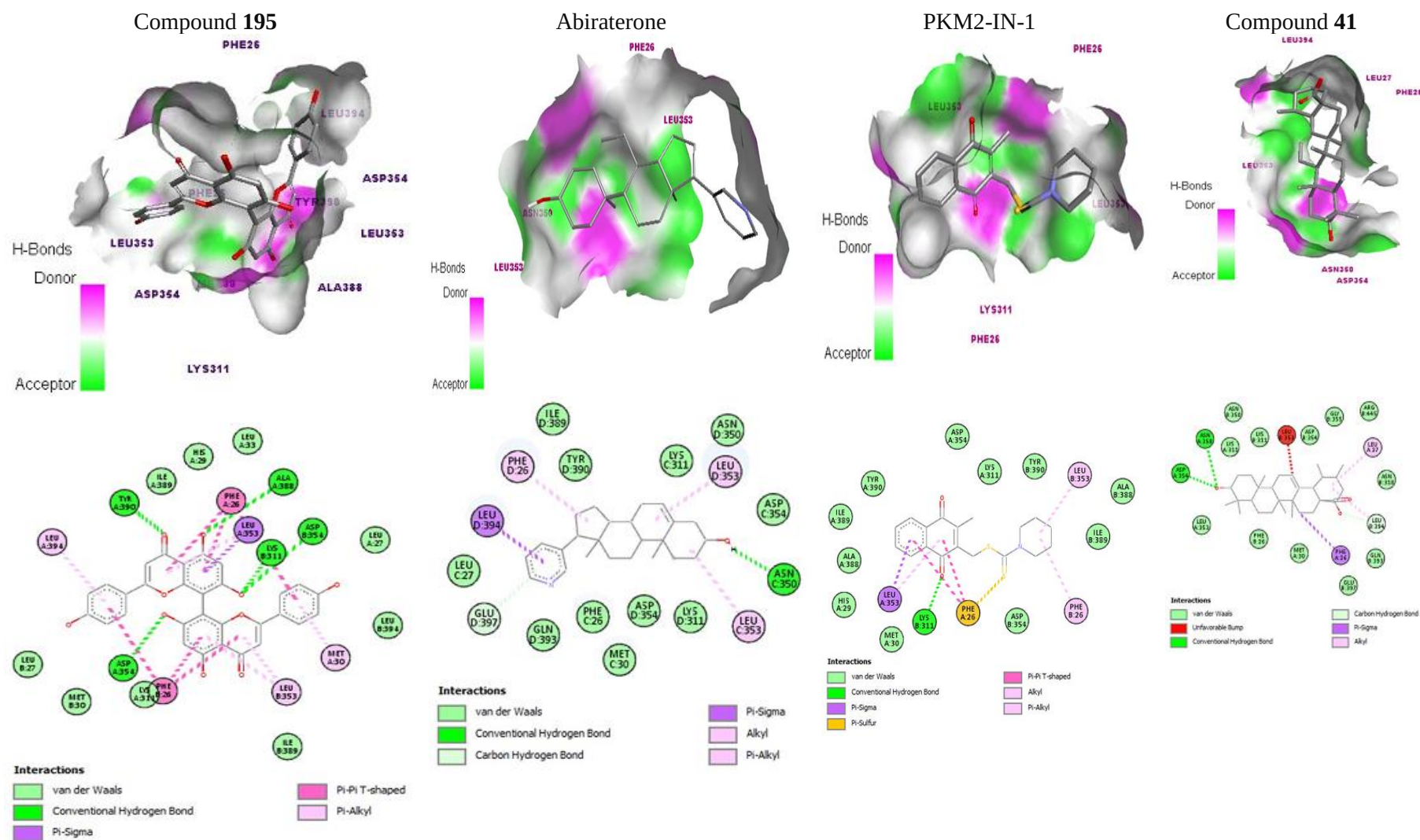


Figure 34. 3D (top) and 2D (bottom) binding interactions of compounds **195**, **41**, abiraterone, and PKM2-IN-1 against PKM2

The results of the molecular docking study of isolated compounds against human topoisomerase II β (Table 40) indicated that the binding affinity ranged from -6.9 to -3.2 kcal/mol compared to standard anticancer drugs (abiraterone and etoposide), with a binding affinity of -5.1 and -5.3 kcal/mol, respectively. The highest binding affinity (-6.9 kcal/mol) was shown by compound **195**, while the least binding affinity (-3.2) was shown by both compounds **194** and **196**. In addition to some hydrophobic, electrostatic, and Van der Waals interactions, compound **195** with the highest binding affinity (-6.9 kcal/mol) interacted with human topoisomerase II β using hydrogen bonds through Gln-778 amino acid residues. Whereas compounds **194** and **196** with the least binding affinity (-3.2 kcal/mol) interacted with human topoisomerase II β only using one hydrophobic interaction (Pi-sigma) through Arg-503 amino acid residue and using one hydrogen bond through the Arg-503 amino acid residue. The 2D and 3D modes of interaction of compounds **195** and **194** with human topoisomerase II β are depicted in Figure 35 along with the 2D and 3D modes of interaction of standard anticancer drugs (abiraterone and etoposide). For the rest of the isolated compounds, the 2D and 3D modes of interactions with human topoisomerase II β are shown in Appendix 30.

Human topoisomerase II β is one of the isoforms of topoisomerase IIA and is expressed at higher levels in tumors and proliferating cells than in normal cells (Linka *et al.*, 2007; Turley *et al.*, 1997). This enzyme solves the DNA topological problems by cleaving both strands of a DNA duplex to form a cleavage complex through which another DNA segment can be transported (Wu *et al.*, 2011). However, the use of anticancer drugs such as etoposide increases the number of stable topoisomerases II β -DNA complexes, which leads to blockage of DNA religation, and ultimately this results in the death of cancer cells (Wu *et al.*, 2011). Based on this fact, as indicated in our report, compound **195** showed a strong binding affinity with human topoisomerase II β as compared to the rest of the isolated compounds. Hence, it can be concluded that compound **195** stabilizes the human topoisomerase II β -DNA complex more and affects DNA religation in cancer cells. Whereas both compounds **194** and **196** showed the least binding affinity with human topoisomerase II β and less stabilizes the human topoisomerase II β -DNA complex than the rest of the isolated compounds and showed less effect on DNA religation in cancer cells.

Table 40. Molecular docking results of isolated compounds against human topoisomerase II β (BA: binding affinity in kcal/mol).

Ligand	BA	H-bonding	Hydrophobic and Electrostatic	Van der Waals
70	-4.7	-	-	Lys-505, Gly-504, 478, Arg-503, Gln-778
189	-4.0	Gln-778	Pi-alkyl: Arg-503; Carbon hydrogen bond: Arg-503	Ala-779, Gly-776, 504, Met-782
190	-3.8	Gln-778	Alkyl and Pi-alkyl: Lys-505, Arg-503	Gly-776, 504, 478, Asp-479
191	-3.7	Gln-778, Arg-503, 503	Pi-sigma: Gln-778; Pi-alkyl: Arg-503, Lys-505	Ala-779, Gly-904
16	-5.0	-	-	Lys-505, Gly-504, Arg-503, Gln-778, Met-782, Ala-779
17	-4.7	-	Alky-503	Gly-448, 504, Gln-778, Met-782, Ala-779, Lys-505, 456, Asp-479
192	-4.6	-	Alkyl: Arg-503, Met-782, Ala-779	Gln-778, Gly-776, 504, Lys-505
193	-4.4	Arg-820	Alkyl: Arg-503	Lys-505, Gly-504, Gln-778
42	-4.5	Arg-503, 503	-	Gly-504, 776, Lys-505, Ile-565, Ala-779
65	-5.0	Arg-503, 820	-	Tyr-821, Ser-818
194	-3.2	Arg-503	Pi-sigma-Arg-503	-
55	-4.6	Arg-503, Gln-778	Carbon hydrogen bond: Gly-504; Pi-alkyl: Arg-503	Lys-505, Ile-565, Gly-776
195	-6.9	Gln-778	Pi-donor hydrogen bond: Gln-778; Pi-alkyl: Arg-503	Gly-504, Asp-479
62	-6.7	Gln-778	Carbon hydrogen bond: Gly-504; Pi-alkyl: Arg-503, Lys-505	Met-782
196	-3.2	Arg-503	Carbon hydrogen bond: Arg-503	-
197	-4.9	Lys-505, Gln-778, Gly, 776	-	Arg-503, Gly-504, His-775, Ala-779
198	-5.0	Arg-503, Lys-505	Carbon hydrogen bond: Arg-503; Pi-sulfur: Mrt-782	Glu-522, Gly-504, Gln-778
41	-5.0	-	-	Asp-439, Arg-503, Gly-504, Lys-505, Ala-779
199	-2.7	Arg-503	Alkyl: 503	-
200	-5.1	Met-782, Gln-778, Gly-776	Carbon hydrogen bond: Gly-776; Pi-sulfur: Met-782; Pi-alkyl: Arg-503, Ala-779	Lys-505, Gly-504, His-775
84	-4.9	Gln-778, Lys-505	Pi-sigma: Arg-503; Carbon hydrogen bond: Arg-503	Asp-479, Gly-504
83	-4.2	Gly-776, Gln-778, Arg-503	Carbon hydrogen bond: Gln-778	His-775, Ala-779, Gly-504
91	-5.2	Gln-778	Carbon Hydrogen bond: Gly-504; Pi-sigma: Arg-503; Pi-sulfur: Met-782	Lys-505, Gly-776, His-775, Ala-779
202	-4.4	Gly-776, Gln-778	Pi-sulfur: Met-782	His-775, Lys-505, Gly-504, Arg-503
Abiraterone	-5.1	Arg-503, 820	Pi-alkyl: Arg-503	Lys-505, Glu-522, Gly-504, Ser-808, Tyr-821, Gln-778
Etoposide	-5.3	Gln-77, Arg-503	Alkyl and Pi-Alkyl: Ala-779, Arg-503	Gly-776

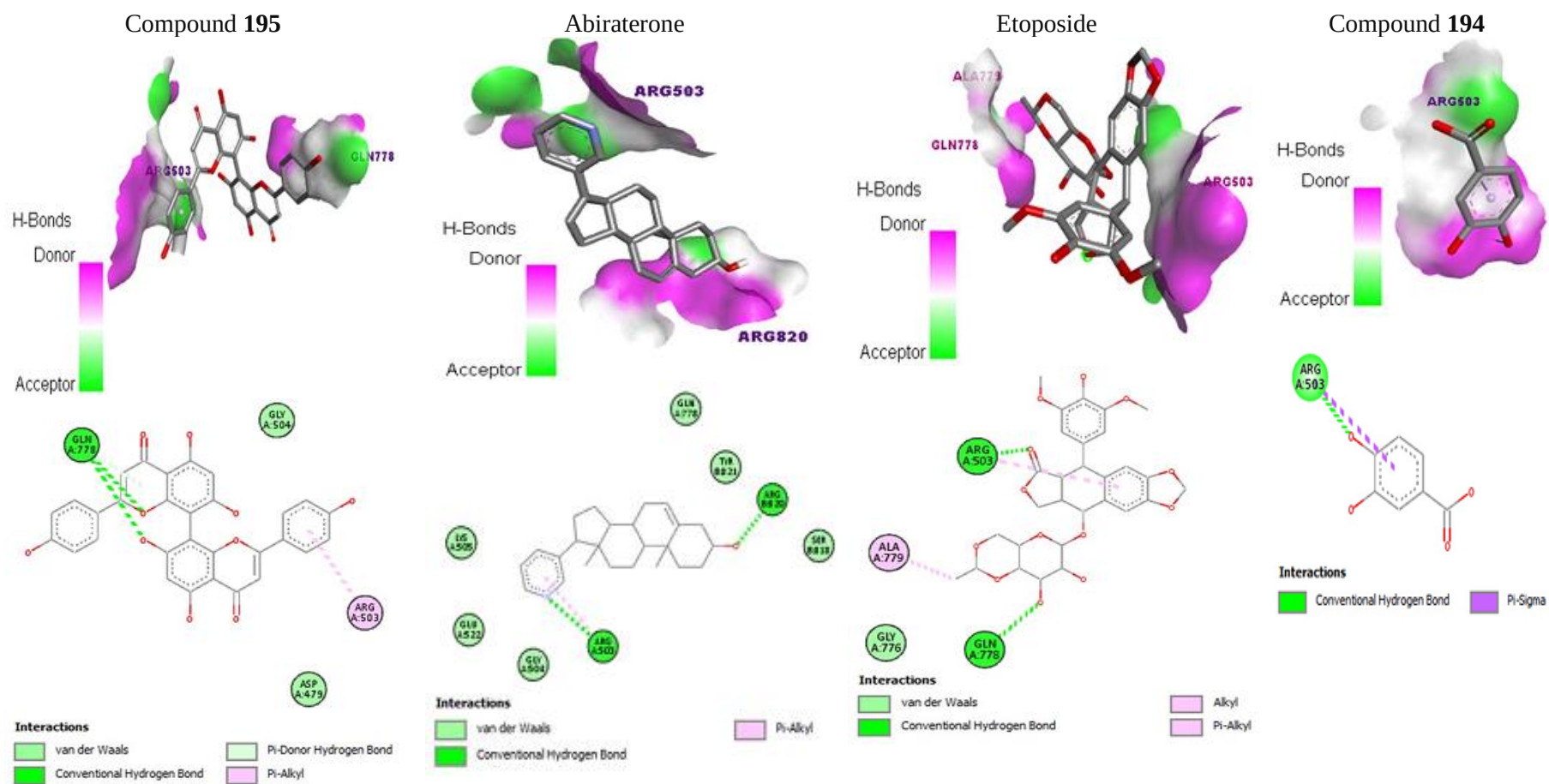


Figure 35. 3D (top) and 2D (bottom) binding interactions of compounds **195**, **194**, abiraterone, and etoposide against human topoisomerase II β

4.5 *In silico* Druglikeness Properties of Isolated Compounds

The oral drugs must fulfill Lipinski's rule of five, which states that orally used drugs must not violate more than one of the rules set by Lipinski so that they have good permeability and solubility in the human body (Lipinski *et al.*, 2012). All conditions set by Lipinski must be close to five or a multiple of five. For instance, the H-bond donors must not be more than five, expressed as the sum of OHs and NHs, the MW must not be over 500, the log P must not be over five, or MLogP is over 4.15, and the H-bond acceptors must not be more than 10, expressed as the sum of Ns and Os (Lipinski *et al.*, 2012). Table 41 shows the results of calculated druglikeness properties of the isolated compounds calculated using the SwissADME online tool following Lipinski's rule (Lipinski *et al.*, 2012). Of all isolated compounds, four compounds, compounds **65**, **194**, **196**, and **199**, violate none of Lipinski's rules, like the positive controls (ciprofloxacin and PKM2-IN-1). Whereas ten compounds, compounds **70**, **16**, **17**, **192**, **193**, **42**, **55**, **41**, **84**, and **83**, each violated one of Lipinski's rules of five, like the standard anticancer drug used (abiraterone). The other compounds, compounds **189**, **190**, **195**, **62**, **197**, **198**, **200**, and **202**, each violated two of Lipinski's rules of five, like the standard anticancer drug (etoposide). However, compound **91** violated three of Lipinski's rules of five, unlike none of the isolated compounds and positive controls computed.

Based on Lipinski's rule of five, compounds **65**, **194**, **196**, **199**, **70**, **16**, **17**, **192**, **193**, **42**, **55**, **41**, **84**, and **83** have good permeability and solubility and are hence recommended to be used as candidates to develop oral drugs. Whereas compounds **189**, **190**, **195**, **62**, **197**, **198**, **200**, **202**, and **91** have the least solubility and permeability and hence are not recommended to be used as candidates to develop oral drugs.

4.6 *In silico* Prediction of Toxicity of the Isolated Compounds

Prediction of toxicity includes acute oral toxicity, organ toxicity, and toxicity endpoints (Banerjee *et al.*, 2024). Acute oral toxicity is categorized into six classes based on the LD₅₀ value of the compounds. Organ toxicity includes a prediction of hepatotoxicity, neurotoxicity, nephrotoxicity, cardiotoxicity, and respiratory toxicity, whereas toxicity endpoints consist of prediction of carcinogenicity, mutagenicity, cytotoxicity, immunotoxicity, blood brain barrier, clinical toxicity, and nutritional toxicity. The prediction of toxicities of isolated compounds and positive controls was calculated using the online tool Pro Tox 3.0 (Banerjee *et al.*, 2024).

As indicated in Table 42, only the acute oral toxicity of compound **55** was computed with class 3, like the positive control used (etoposide) with an LD₅₀ less than 300 mg/kg, indicating that it is toxic if swallowed. In another way, compounds **70, 189, 16, 17, 194, 41, and 199** were calculated as class 4, like the positive controls used (ciprofloxacin, abiraterone, and PKM2-IN-1), with an LD₅₀ of less than or equal to 2000 mg/kg, showing that these compounds are harmful if swallowed. Whereas compounds **190, 193, 195, 62, 197, 198, 200, 202, 84, 83, and 91** were calculated to class 5 oral acute toxicity with LD₅₀ values of less than or equal to 5000 mg/kg, inferring that they may be harmful if swallowed. However, compounds **192, 42, 65, and 196** were calculated to have acute oral toxicity of class 6 with LD₅₀ values of greater than 5000 mg/kg, inferring that none of these compounds are toxic if swallowed by human beings.

Unlike the other isolated compounds and positive controls, compounds **189, 190, 193, 194, 196, and 199** were predicted to be active for none of the organ toxicity. Whereas compounds **70, 16, 17, 192, 65, 55, 195, and 62** were predicted to be active for respiratory toxicity like the positive control selected (PKM2-IN-1 and abiraterone). However, only compound **42** was predicted to be active for cardiotoxicity. In the other way, compounds **197, 198, 199, 84, 83, 91, and 200** were predicted to be active for nephrotoxicity. Like positive control (etoposide), compound **41** was predicted to be active for both respiratory toxicity and cardiotoxicity. Moreover, regarding the toxicological endpoints, compounds **65, 55, 196, 197, 200, and 202** were predicted to be active for none of the toxicological endpoints. Whereas compounds **189, 190, 198, 41, 84, and 91** were predicted to be active for immunotoxicity like the positive control selected (etoposide). While, like the positive control used (abiraterone), compounds **70, 17, 192, 193, and 83** were predicted to be active for both immunotoxicity and BBB-barrier. For the rest of the compounds, compound **16** was predicted to be active for both BBB-barrier and nutritional toxicity, whereas compound **42** was predicted to be active for both immunotoxicity and nutritional toxicity, and compound **194** was predicted to be active for carcinogenicity, while both compound **195** and **62** were predicted to be active for nutritional toxicity.

In general, none of the isolated compounds are fatal if swallowed since the LD₅₀ of all isolated compounds is greater than 50 mg/kg (Banerjee *et al.*, 2024). Furthermore, from the *in silico* pharmacokinetics (drug likeness) and pharmacodynamics (toxicity) studies, compared to all isolated compounds and all positive controls, compound **196** was predicted to be free from acute oral toxicity, organ toxicity, and toxicological endpoints and hence predicted to be the best drug if administered orally.

Table 41. Druglikeness predictions of isolated compounds computed by SwissADME

Ligand	Formula	MWT(g/mol)	NRB	NHA	NHD	TPSA (Å ²)	LogP (MLOGP ≤ 4.15)	Log S/ ESOL	Lipinski's rule of five
70	C ₂₉ H ₄₈ O	412.7	5	1	1	20.2	7.0	-7.5	1
189	C ₄₀ H ₄₀ O ₁₂	712.7	17	12	3	159.4	5.4	-7.7	2
190	C ₄₀ H ₄₂ O ₁₃	730.8	20	13	4	179.4	5.3	-7.3	2
16	C ₃₀ H ₅₀ O	426.7	1	1	1	20.2	6.92	-8.6	1
17	C ₂₉ H ₅₀ O	414.7	6	1	1	20.2	6.73	-7.9	1
192	C ₂₉ H ₄₈ O ₂	428.69	6	2	1	37.3	5.7	-7.31	1
193	C ₂₉ H ₅₀ O ₂	430.7	6	2	1	37.3	5.8	-7.49	1
42	C ₃₅ H ₆₀ O ₆	576.9	9	6	4	99.4	3.96	-7.7	1
65	C ₁₅ H ₁₄ O ₆	290.3	1	6	5	110.4	0.24	-2.2	0
194	C ₇ H ₆ O ₄	154.1	1	4	3	77.8	0.40	-1.86	0
55	C ₁₅ H ₁₀ O ₈	318.2	1	8	6	151.6	-1.08	-3.01	1
195	C ₃₀ H ₁₈ O ₁₀	538.5	3	10	6	181.8	0.25	-6.75	2
62	C ₃₀ H ₁₈ O ₁₀	538.5	3	10	6	181.8	0.25	-6.75	2
196	C ₇ H ₁₀ O ₅	174.2	1	5	4	98.0	-1.43	0.23	0
197	C ₂₀ H ₁₈ O ₁₁	434.4	4	11	7	190.3	-2.06	-3.27	2
198	C ₂₂ H ₂₀ O ₁₂	476.4	6	12	6	196.4	-1.63	-3.38	2

Note. MWT: molecular weight; NRB: number of rotatable bonds; NHA: number of hydrogen acceptors; NHD: number of hydrogen donors; TPSA: total polar surface area.

Table 41. Druglikeness predictions of isolated compounds computed by SwissADME (Contn'd)

Ligand	Formula	MWT(g/mol)	NRB	NHA	NHD	TPSA (A ⁰²)	LogP (MLOGP≤ 4.15)	Log S/ ESOL	Lipinski's rule of five
41	C ₃₀ H ₄₈ O ₃	456.7	1	3	2	57.5	5.82	-7.2	1
199	C ₆ H ₈ O ₃	128.1	0	3	1	46.5	-0.11	-0.31	0
200	C ₂₁ H ₂₀ O ₁₂	464.4	4	12	8	210.5	-2.59	-1.5	2
84	C ₂₁ H ₂₄ O ₁₁	452.4	7	11	5	172.2	-1.09	-2.1	1
83	C ₁₅ H ₁₈ O ₉	342.3	5	9	6	156.9	-1.63	-1.2	1
91	C ₃₀ H ₂₆ O ₁₃	594.5	8	13	7	216.6	-1.04	-4.93	3
202	C ₂₁ H ₂₀ O ₁₁	448.4	4	11	7	190.3	-2.10	-3.18	2
Ciprofloxacin	C ₁₇ H ₁₈ FN ₃ O ₃	331.3	3	5	2	74.6	1.28	-1.3	0
Abiraterone	C ₂₄ H ₃₁ NO	349.5	1	2	1	33.1	4.4	-5.0	1
PKM2-IN-1	C ₁₈ H ₁₉ NO ₂ S ₂	345.5	4	2	-	94.8	2.05	-4.13	0
Etoposide	C ₂₉ H ₃₂ O ₁₃	588.6	5	13	3	160.8	-0.14	-3.75	2

Note. MWT: molecular weight; NRB: number of rotatable bonds; NHA: number of hydrogen acceptors; NHD: number of hydrogen donors; TPSA: total polar surface area.

Table 42. Toxicity predictions of isolated compounds calculated by Pro Tox 3.0 online tool

Compound	LD ₅₀ (mg/Kg)	Toxicity class	Organ toxicity	Toxicological endpoints
70	890	4	Respiratory toxicity	Immunotoxicity and BBB-barrier
189	1000	4	-	Immunotoxicity
190	5000	5	-	Immunotoxicity
16	2000	4	Respiratory toxicity	BBB-barrier and nutritional toxicity
17	890	4	Respiratory toxicity	Immunotoxicity and BBB-barrier
192	5010	6	Respiratory toxicity	Immunotoxicity and BBB-barrier
193	5000	5	-	Immunotoxicity and BBB-barrier
42	8000	6	Cardiotoxicity	Immunotoxicity and nutritional toxicity
65	10000	6	Respiratory toxicity	-
194	2000	4	-	Carcinogenicity
55	159	3	Respiratory toxicity	-
195	3919	5	Respiratory toxicity	Nutritional toxicity
62	3919	5	Respiratory toxicity	Nutritional toxicity
196	9000	6	-	-
197	5000	5	Nephrotoxicity	-
198	5000	5	Nephrotoxicity	Immunotoxicity
42	2000	4	Respiratory toxicity and cardiotoxicity	Immunotoxicity
199	1890	4	-	BBB-barrier
200	5000	5	Nephrotoxicity	-
84	4000	5	Nephrotoxicity	Immunotoxicity
83	4000	5	Nephrotoxicity	Immunotoxicity and BBB-barrier
91	5000	5	Nephrotoxicity	Immunotoxicity
202	5000	5	Nephrotoxicity	-
Ciprofloxacin	2000	4	Neurotoxicity, nephrotoxicity, and respiratory toxicity	Mutagenicity and clinical toxicity
Abiraterone	830	4	Respiratory toxicity	Immunotoxicity and BBB-barrier
PKM2-IN-1	500	4	Respiratory toxicity	BBB-barrier
Etoposide	215	3	Respiratory toxicity and cardiotoxicity	Immunotoxicity

CHAPTER FIVE

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This report investigates five medicinal plants, focusing on their essential oils, non-polar, and polar fractions. For the first time, a comprehensive GC-MS analysis of four of these plants revealed a total of 77 compounds. Among these, 19 compounds were found to be common across the samples, while 58 compounds were unique to individual plants. Chromatographic fractionations of the extracts of the plants and NMR analysis have led to the isolation and characterization of 33 compounds with five common and 28 different compounds. Of these, seven compounds, including oleic acid (**188**), palmitic acid (**145**), stigmasterol (**70**), β -sitosterol (**17**), boehmenan (**189**), erythro carolignan E (**190**), and threo carolignan E (**191**), were reported from the root extract of *C. hirsuta*. Eight compounds, viz, lupeol (**16**), β -sitosterol (**17**), stigmasterol (**70**), 7-keto- β -sitosterol (**192**), 6-ketositostanol (**193**), daucosterol (**42**), catechin (**65**), and epicatechin (**69**), were reported from the stem bark of *G. ferruginea*, from which compound **193** was reported for the first time from the genus. Additionally, eight compounds, including palmitic acid (**145**), 3,4-dihydroxybenzoic acid (**194**), myricetin (**55**), cupressuflavone (**195**), amentoflavone (**62**), shikimic acid (**196**), avicularin (**197**), and myricetin-3-O-5''-acetylarabinofuranoside (**198**), were reported from the leaves of *R. ruspolii*. Of all these, compound **198** is new to the genus. As well as five compounds, including ursolic acid (**41**), 5-hydroxy-4-methyl-5,6-dihydro-(2H)-pyran-2-one (**199**), quercetin-3-O-(1-D-galactopyranoside) (**200**), olinioside (**84**), and 4-O- β -D-glucopyranosylcaffeic acid (**83**), were reported from the leaves of *O. rochetiana*, from which compounds **41** and **200** are new to the genus, whereas compound **199** is a new natural product. Five compounds including palmitic acid (**145**), palmityl alcohol (**201**), β -sitosterol (**17**), tiliroside (**91**), and astragalin (**202**), were reported from the leaves of *R. apetalus*. Various extracts, fractions, essential oils, and isolated compounds from these medicinal plants were evaluated for their antibacterial activities against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* and investigated for their radical scavenging abilities using DPPH radicals. While molecular docking was performed against target proteins

E. coli DNA gyrase B, PqsA, PKM2, and human topoisomerase *IIβ*. SwissADME and Pro Tox 3.0 online tools were used to predict the druglikeness and toxicity of isolated compounds, respectively.

The IZs of all extracts and fractions (at 100 mg/mL) ranged from 7.93 ± 0.65 to 16.33 ± 0.47 mm against all tested bacteria compared to the positive control (ciprofloxacin at 0.5 mg/mL), which ranged from 18.42 ± 0.26 to 22.18 ± 0.22 mm. The highest IZ (16.33 mm) was exhibited by DCM:MeOH (1:1) extracts of the leaves of *R. ruspolii* against *E. coli*. Whereas the lowest IZ (7.93 mm) was shown by the EtOAc fraction of *G. ferruginea* stem bark against *P. aeruginosa*. Of all the isolated compounds (at 5 mg/mL), *S. aureus* was the most susceptible to compound **197** (IZ: 14.67 ± 0.47 mm) and least susceptible to compound **196** (IZ: 8.33 mm). *S. pyogenes* was the most susceptible to compound **17** (IZ: 15.07 ± 0.74 mm) and least susceptible to compound **196** (IZ: 8.67 ± 0.50 mm). *E. coli* was the most susceptible to compound **195** (IZ: 12.67 ± 0.50 mm) and least susceptible to compound **196** (9.00 ± 0.82 mm). *P. aeruginosa* was the most susceptible to compound **16** (IZ: 15.20 ± 0.28 mm) and least susceptible to compound **196** (IZ: 8.67 ± 0.47 mm). The molecular docking results of the isolated compounds supported the antibacterial activities of isolated compounds against *E. coli* and *P. aeruginosa*. Moreover, among the isolated compounds (at 62.5 µg/mL), compound **186** inhibited the highest DPPH radical (92.1%), while compound **17** inhibited the least (18.50%) DPPH radicals compared to the DPPH radicals inhibited by the positive control ascorbic acid (96.9%). From the *in silico* pharmacokinetic and pharmacodynamics studies, it is concluded that most of the isolated compounds fulfill Lipinski's rules of five and hence have good permeability and solubility in the human body, and none of the isolated compounds are fatal if swallowed. Therefore, these findings supported the traditional use of these medicinal plants to treat different bacterial diseases.

5.2 Recommendations

The plant parts examined were collected in July (summer). However, to standardize the use of these medicinal plants, it is essential to collect samples in different seasons and extract them using various solvents. Then,

- further qualitative and quantitative analysis should be conducted by different techniques like GC-MS, LC-MS, and HPLC using both normal and reverse phase columns.

- the *in vitro* and *in vivo* pharmacokinetic and pharmacodynamic investigation must be conducted to identify the ADME and cytotoxicity properties of the extracts and isolated compounds.

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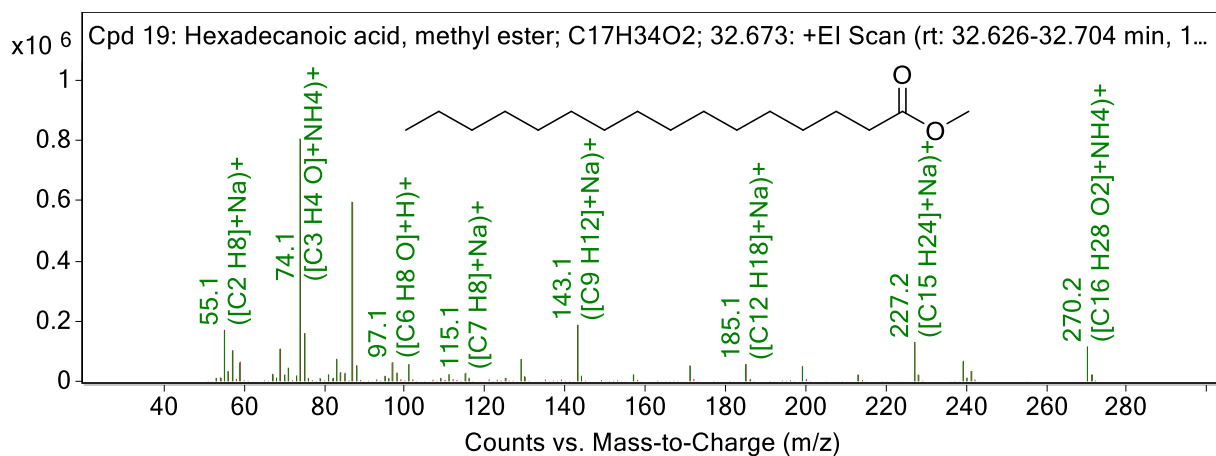
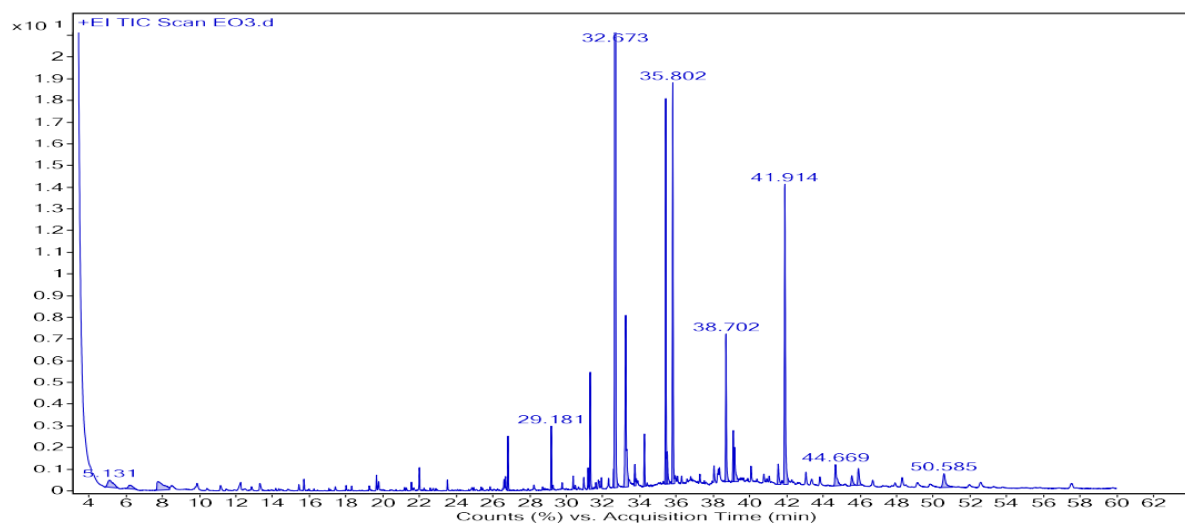
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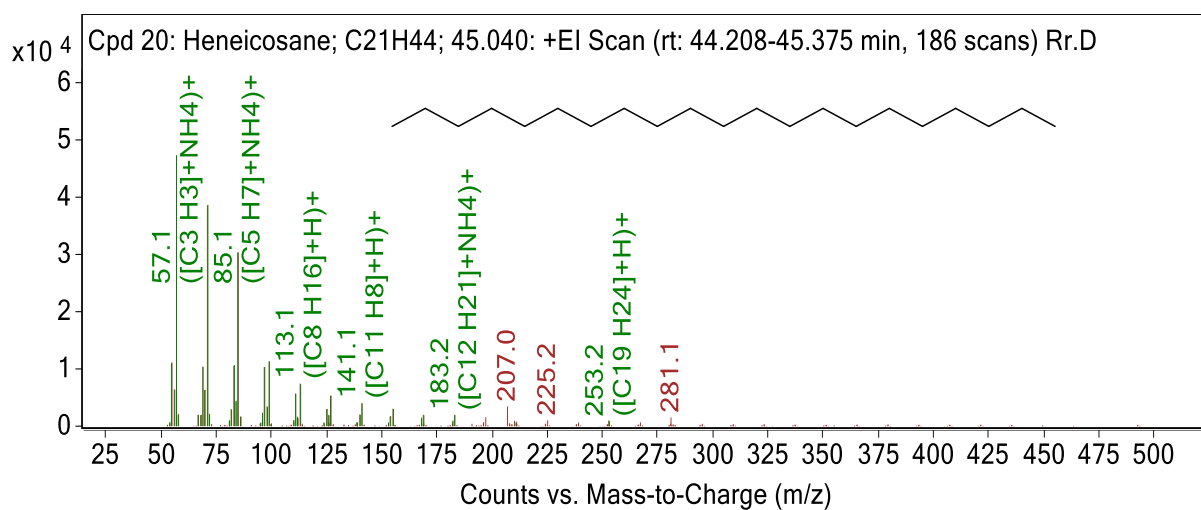
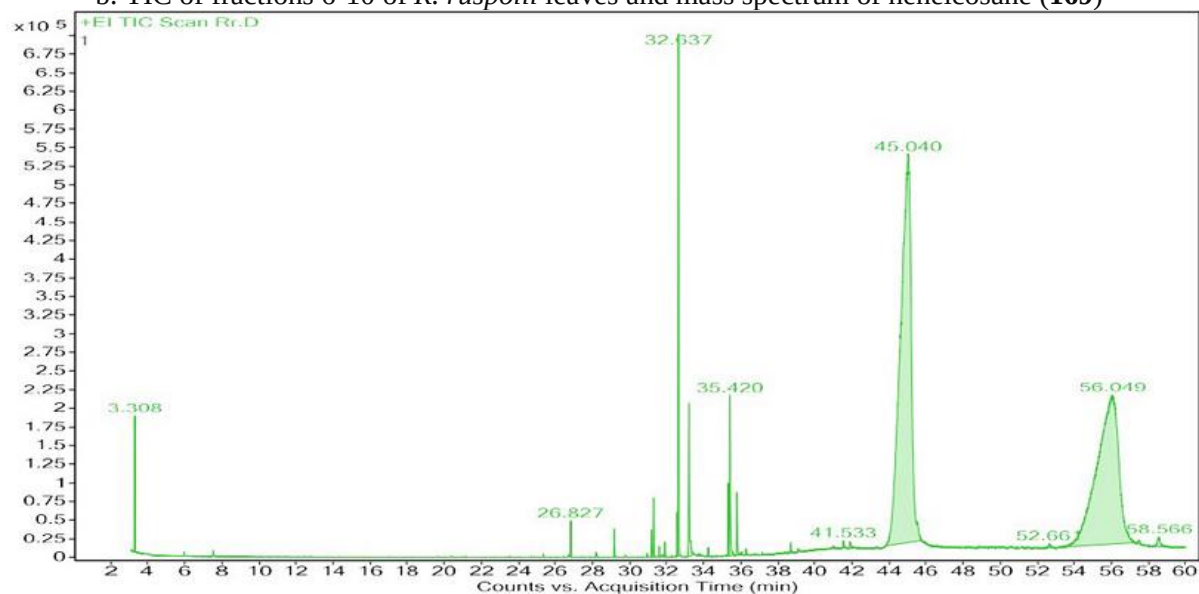
APPENDICES

Appendix 1. GC-MS spectral data

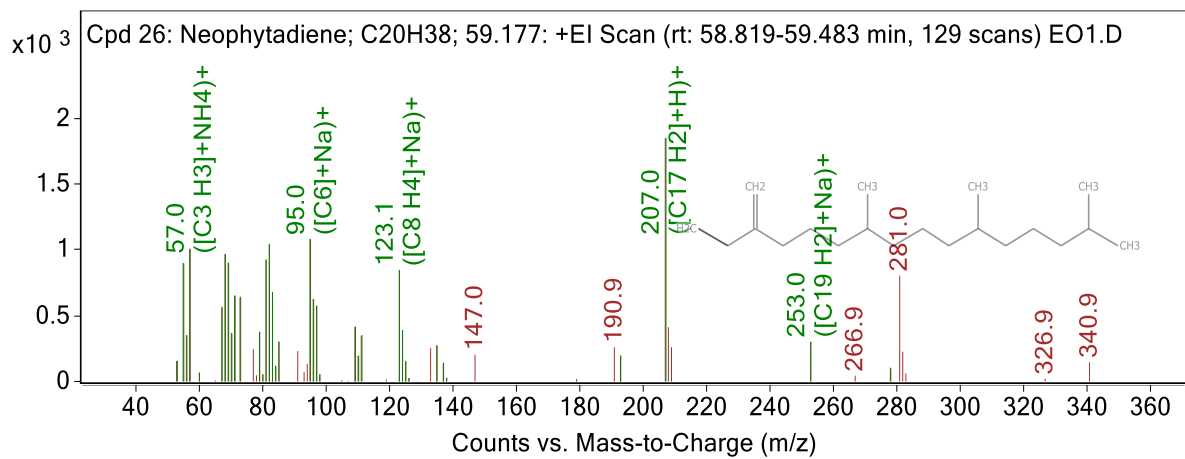
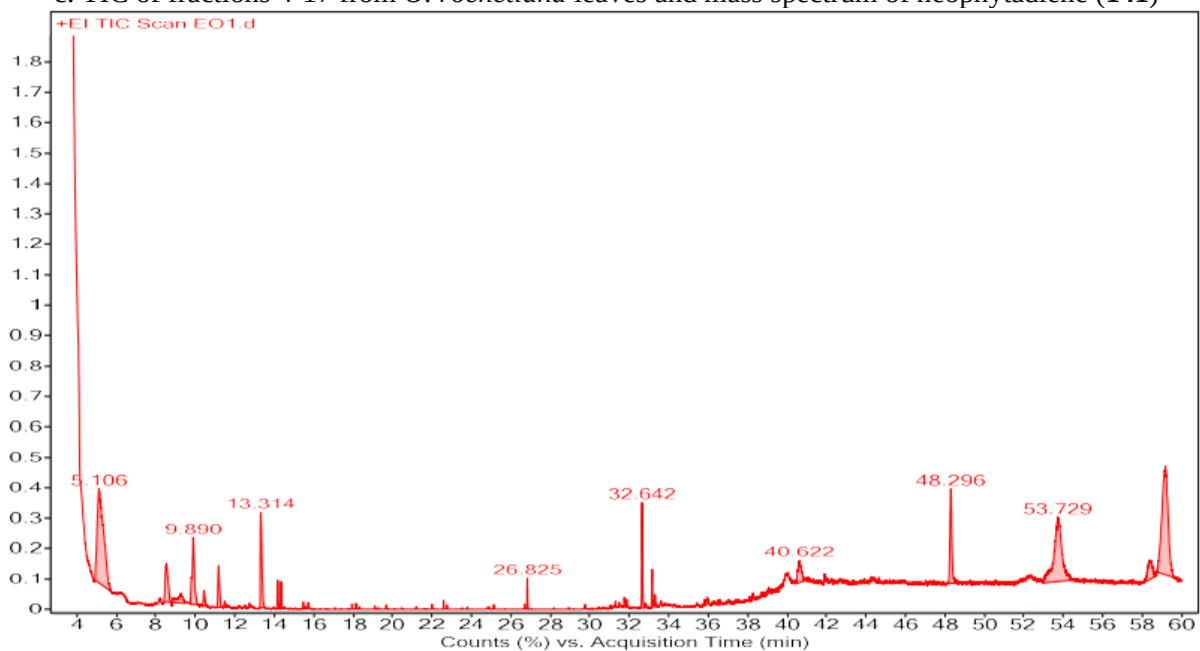
a. Total ion chromatogram (TIC) of essential oil from dried stem bark of *G. ferruginea* and mass spectrum of methyl palmitate (**144**)



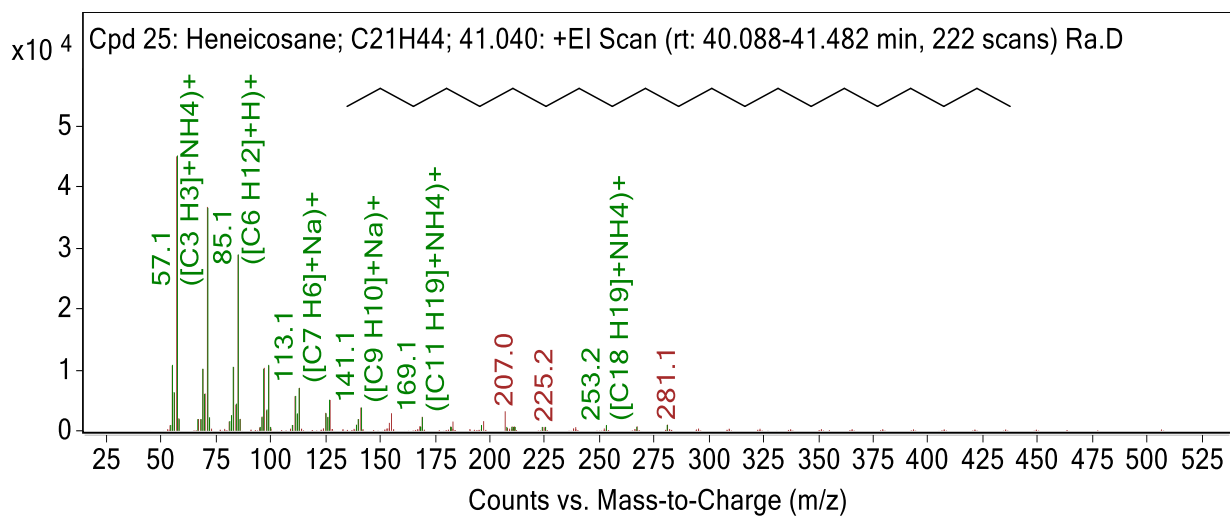
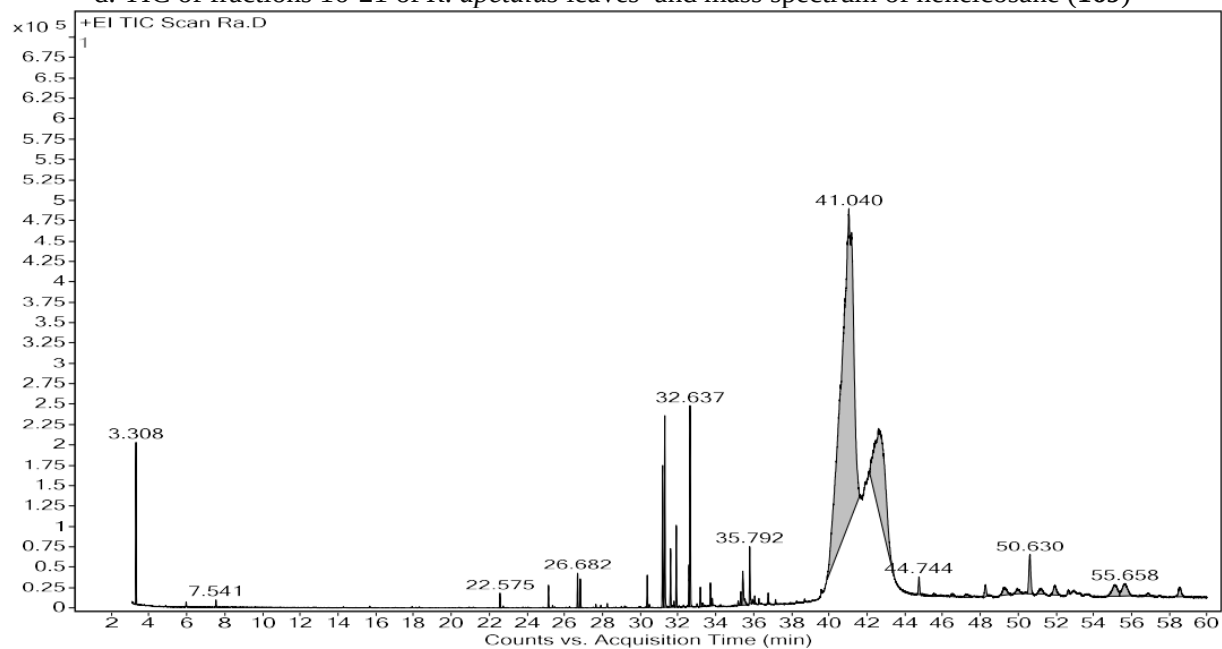
b. TIC of fractions 6-10 of *R. ruspolii* leaves and mass spectrum of heneicosane (**169**)



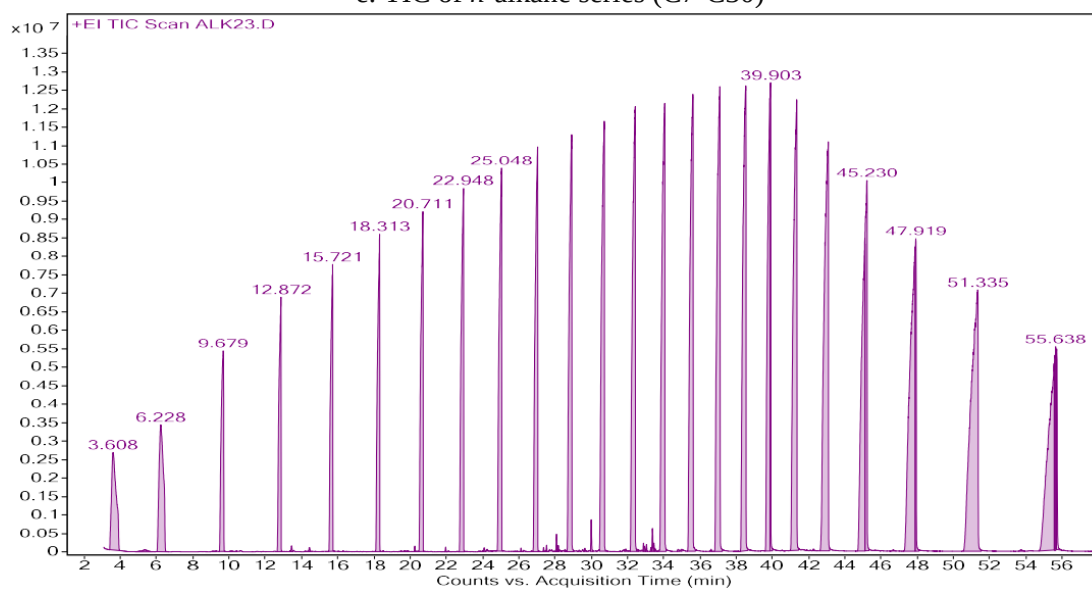
c. TIC of fractions 4-17 from *O. rochetiana* leaves and mass spectrum of neophytadiene (**141**)



d. TIC of fractions 16-21 of *R. apetalus* leaves and mass spectrum of heneicosane (**169**)

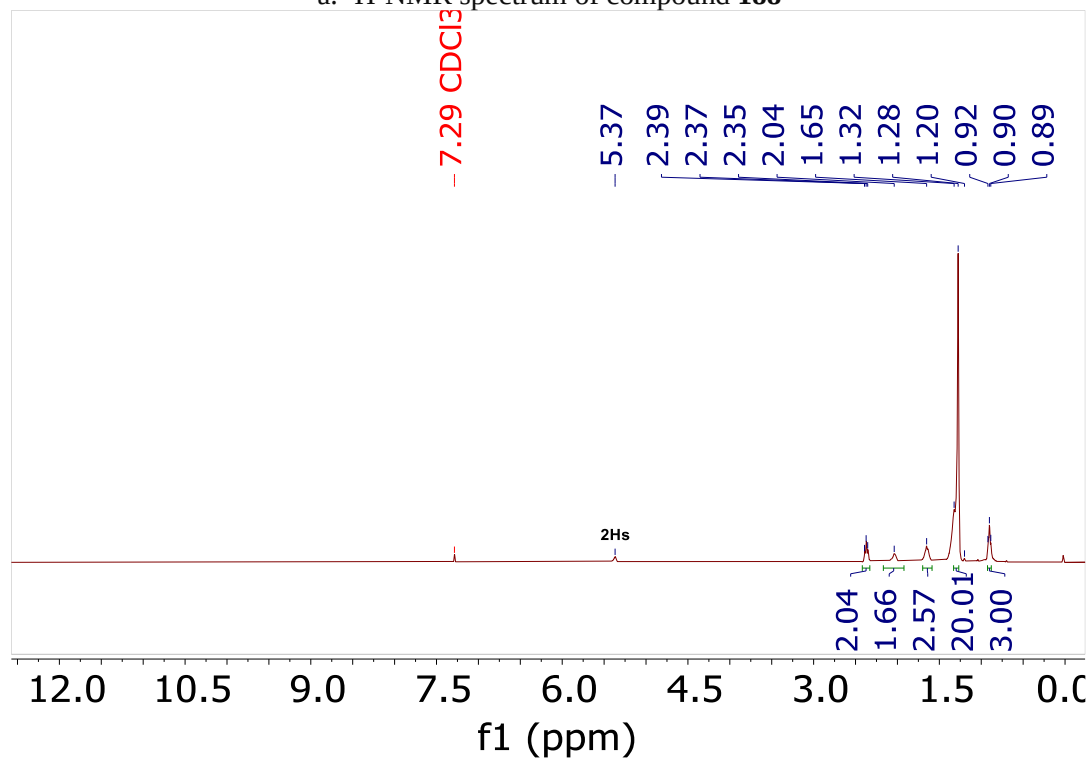


e. TIC of *n*-alkane series (C7-C30)

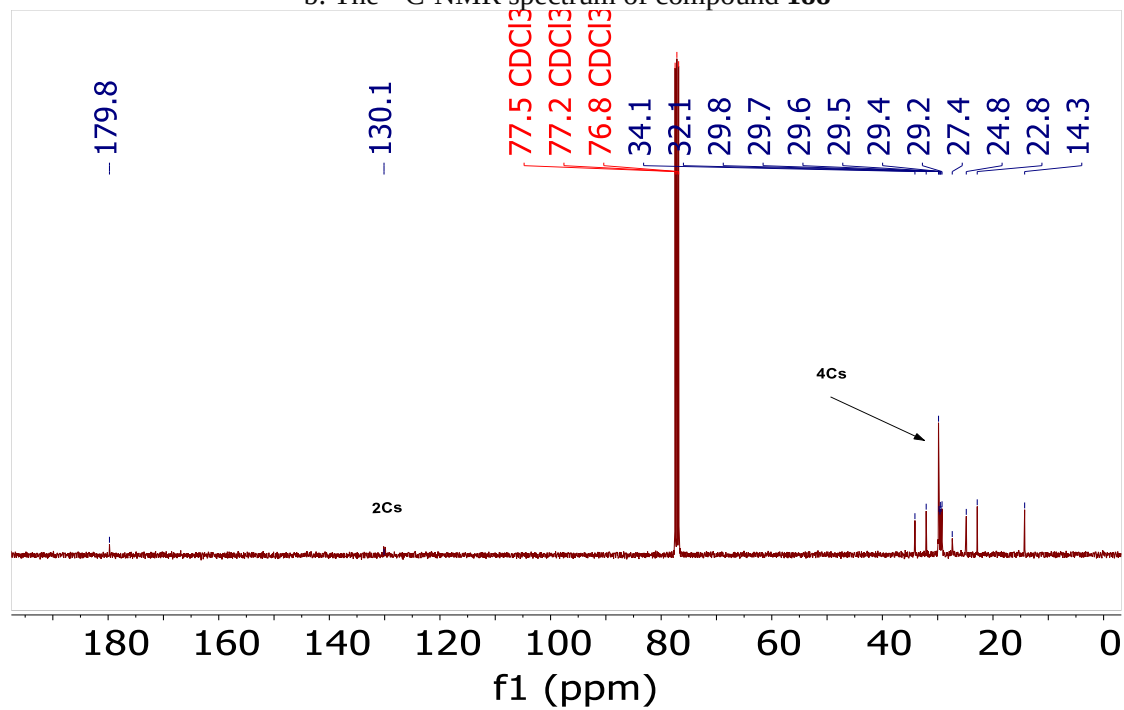


Appendix 2. NMR spectral data of compound **188**

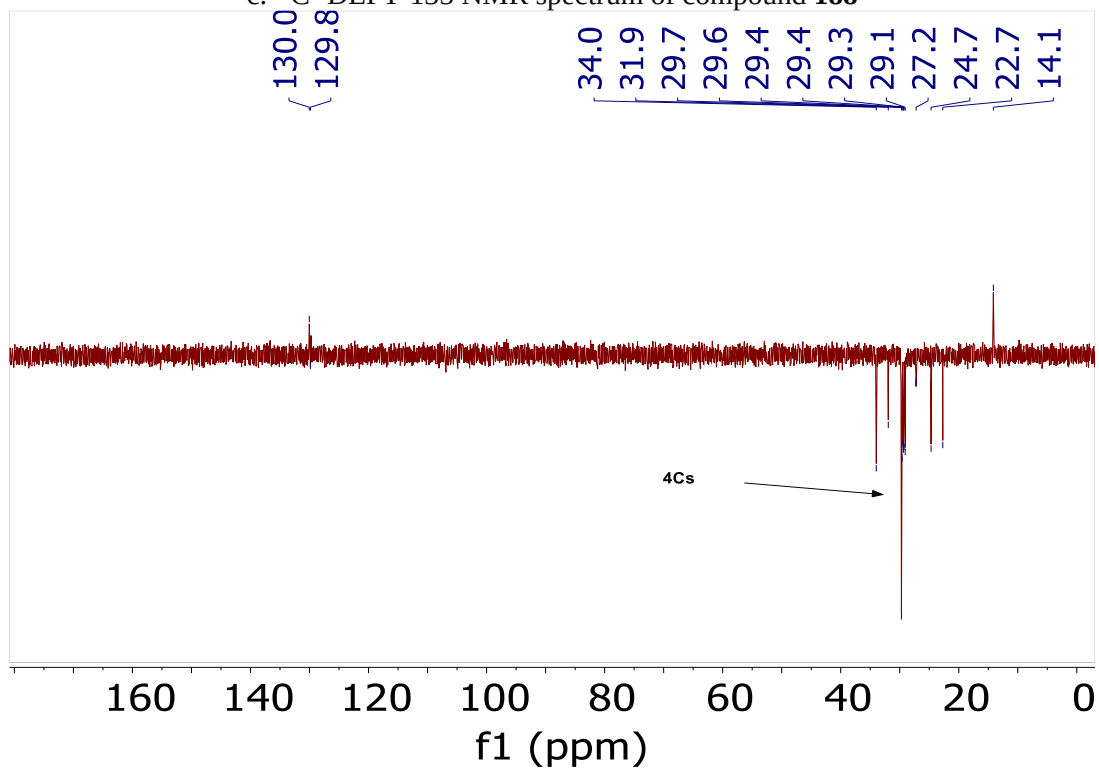
a. ^1H -NMR spectrum of compound **188**



b. The ^{13}C -NMR spectrum of compound **188**

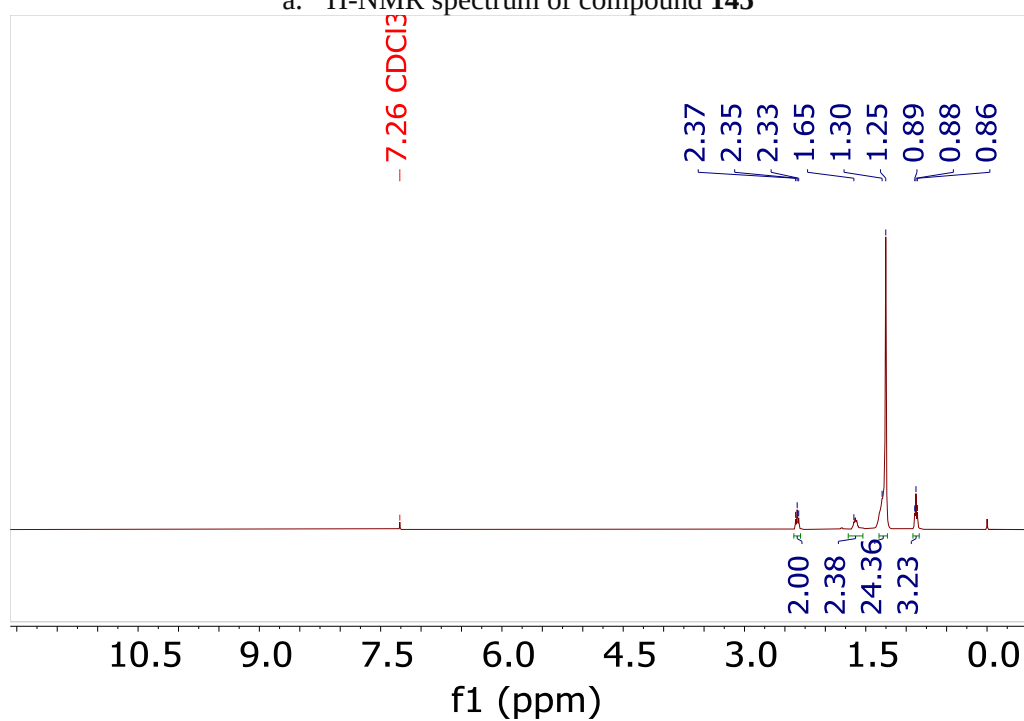


c. ^{13}C -DEPT-135 NMR spectrum of compound **188**

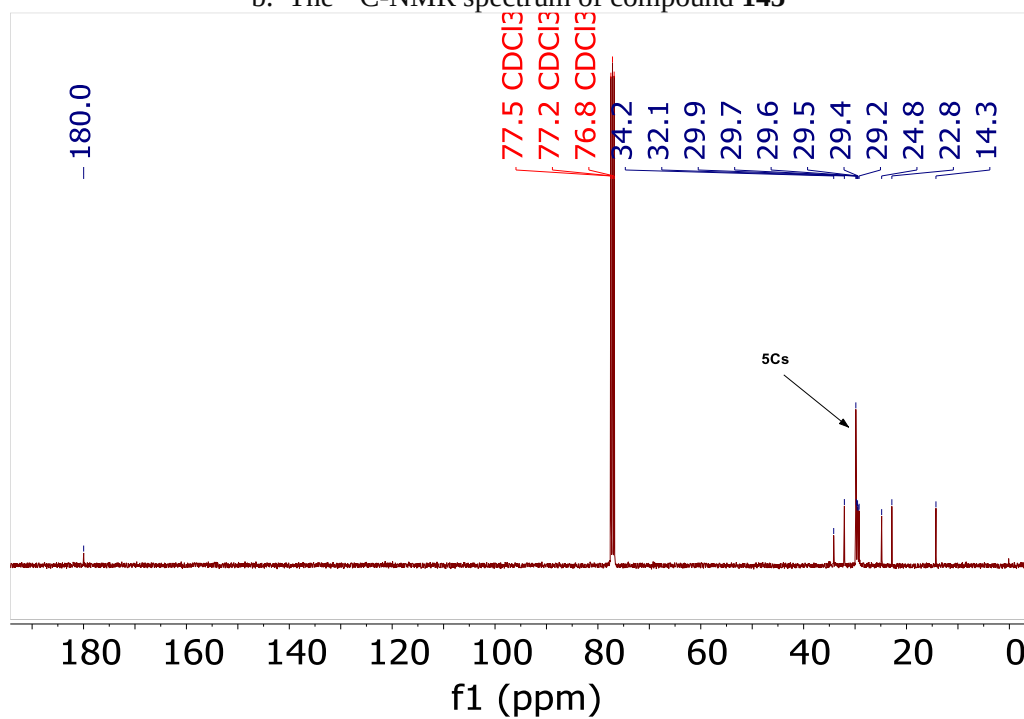


Appendix 3. NMR spectral data of compound **145**

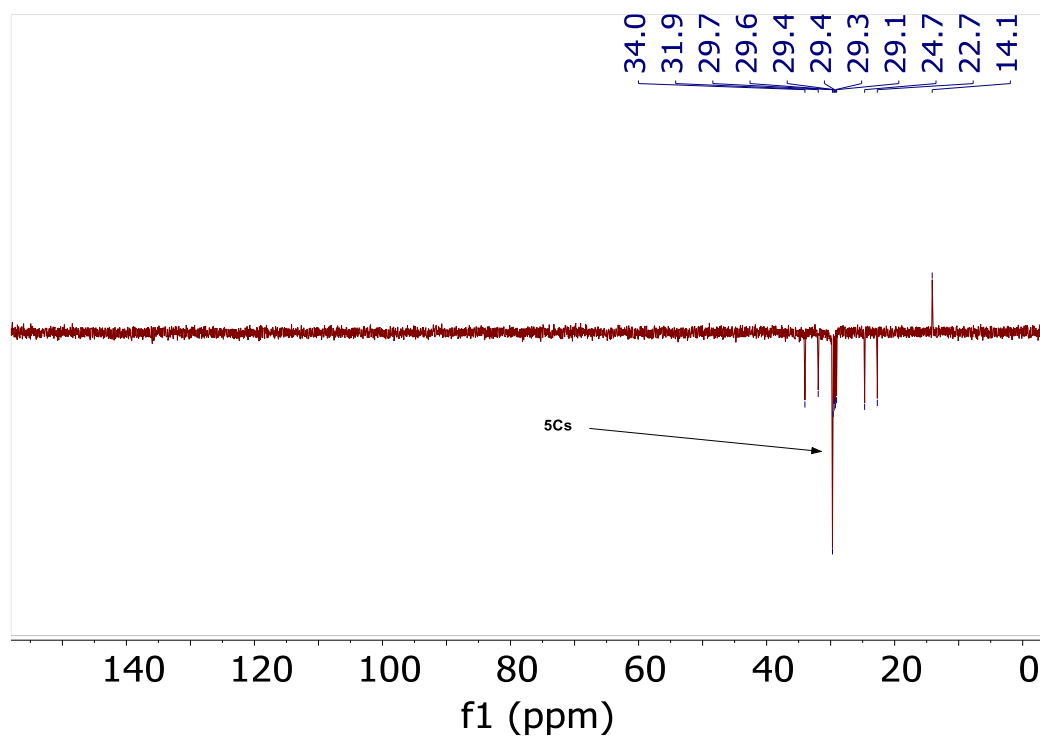
a. ^1H -NMR spectrum of compound **145**



b. The ^{13}C -NMR spectrum of compound **145**

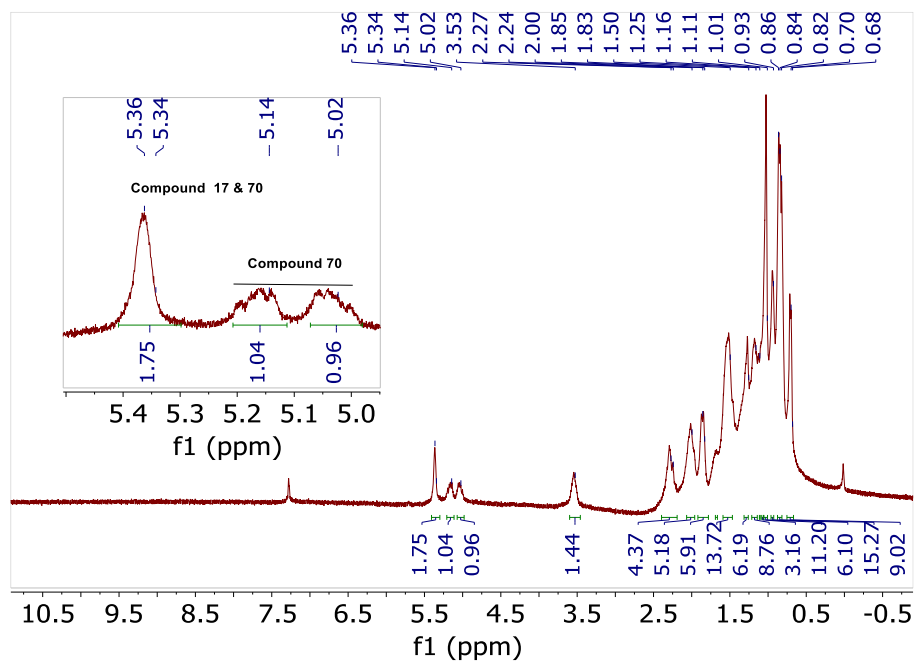


c. ^{13}C DEPT-135 NMR spectrum of compound **145**

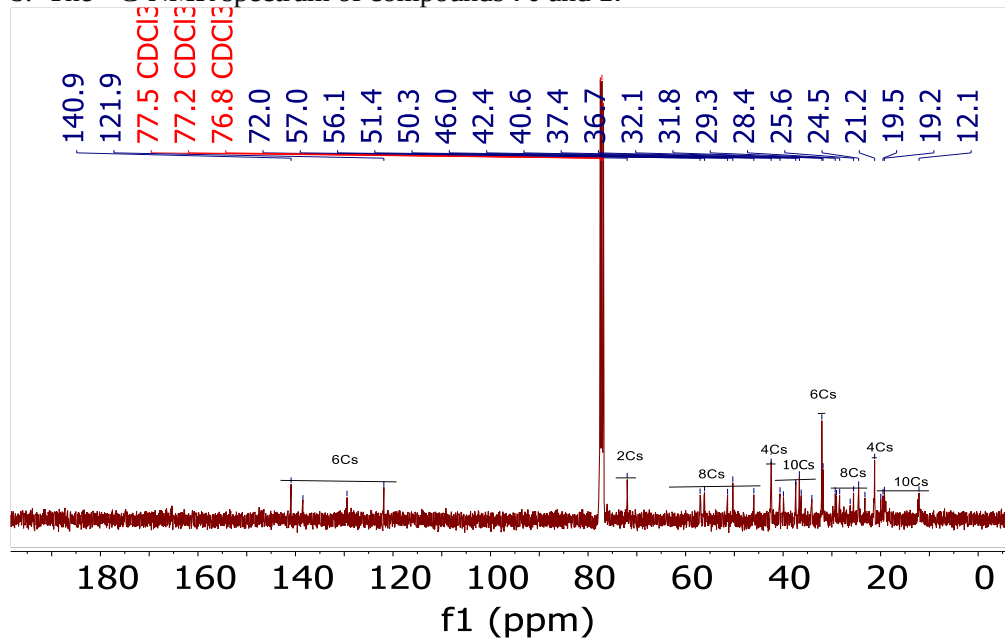


Appendix 4. NMR spectral data of compounds **70** and **17**

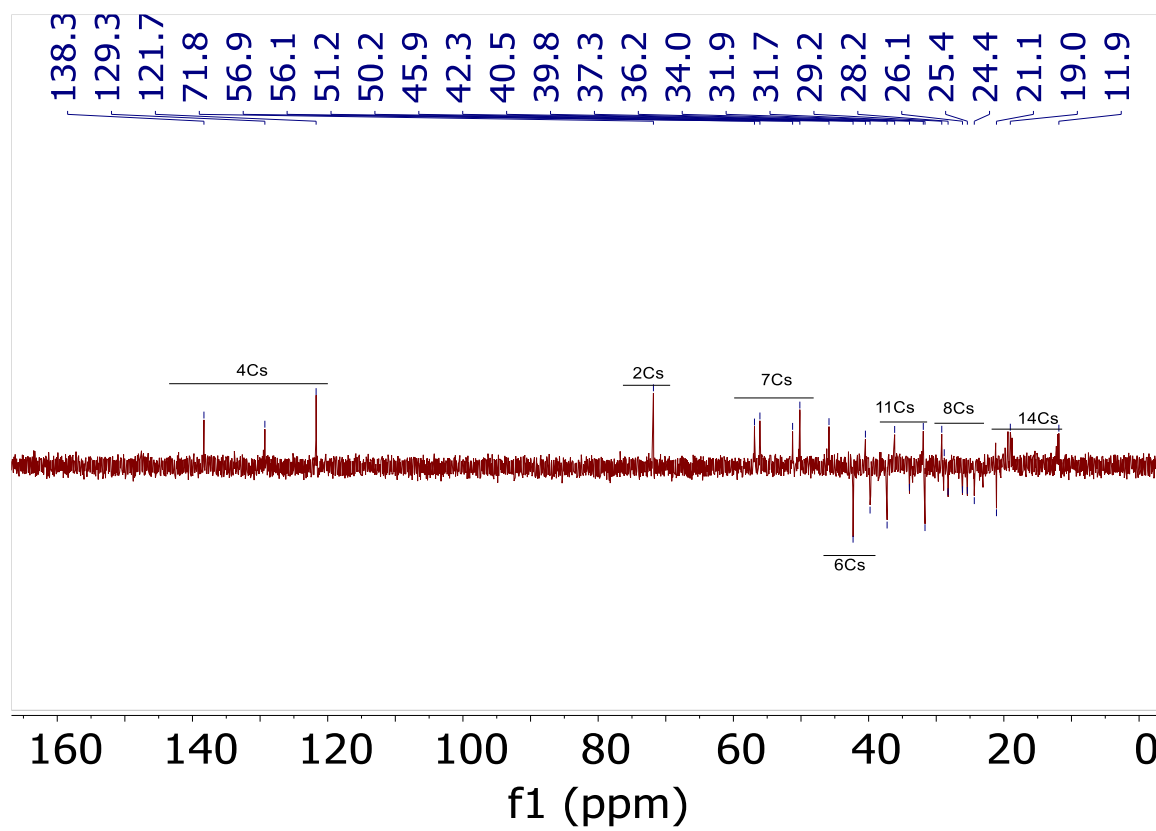
a. ^1H -NMR spectra of compounds **70** and **17** and the enlarged region used to calculate the relative amount of compounds **70** and **17**



b. The ^{13}C -NMR spectrum of compounds **70** and **17**

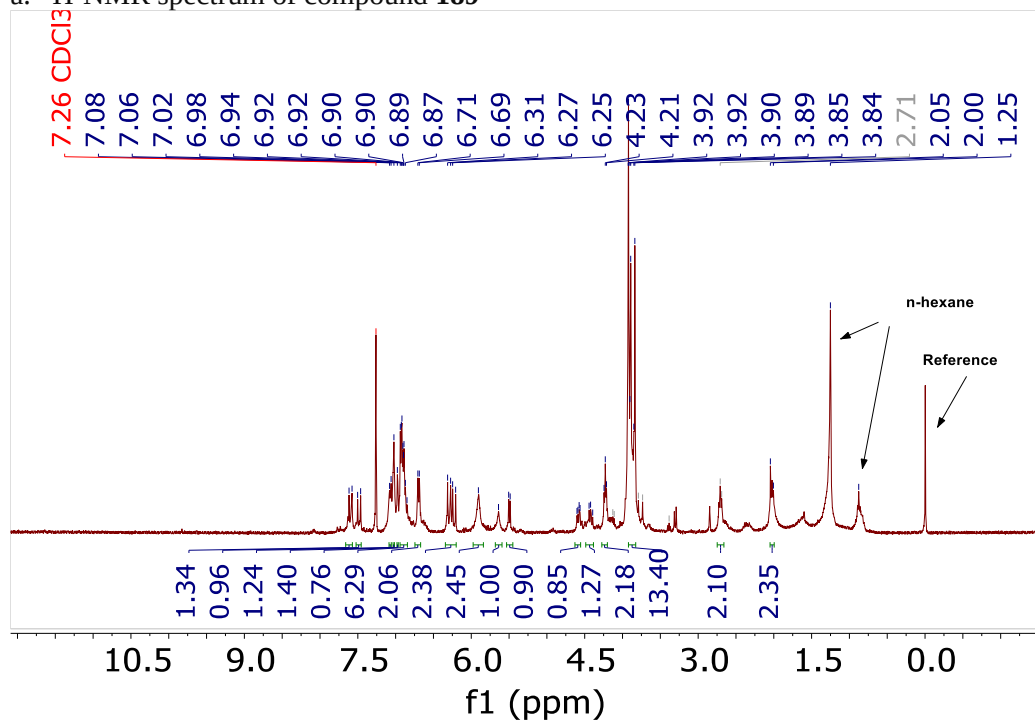


c. ^{13}C DEPT-135 NMR spectra of compounds **70** and **17**

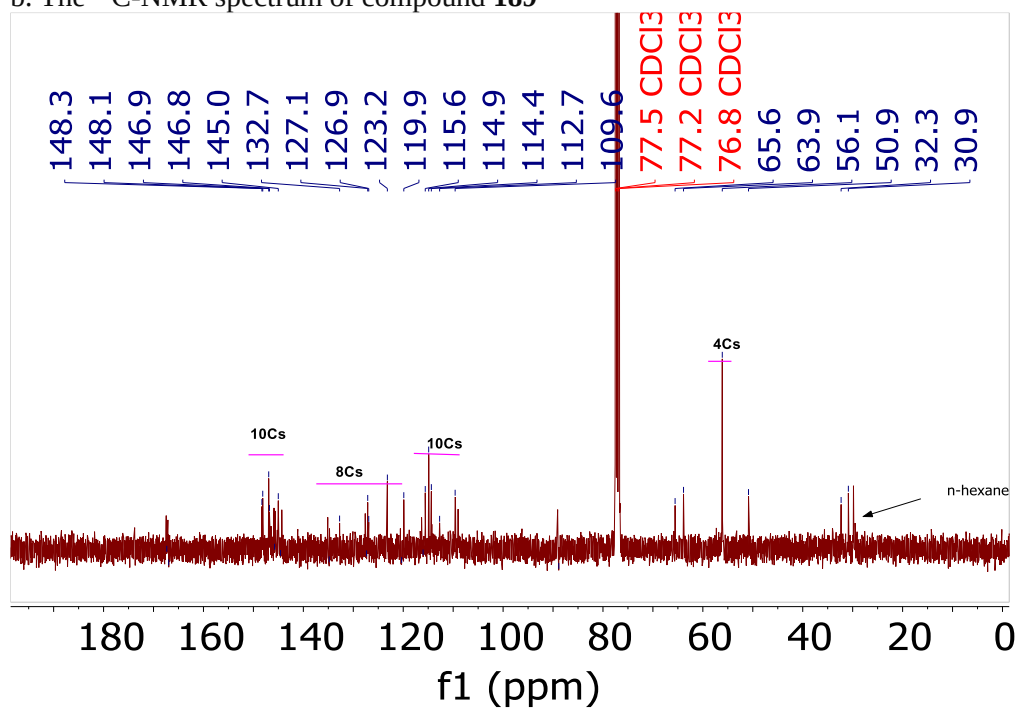


Appendix 5. NMR spectral data of compound **189**

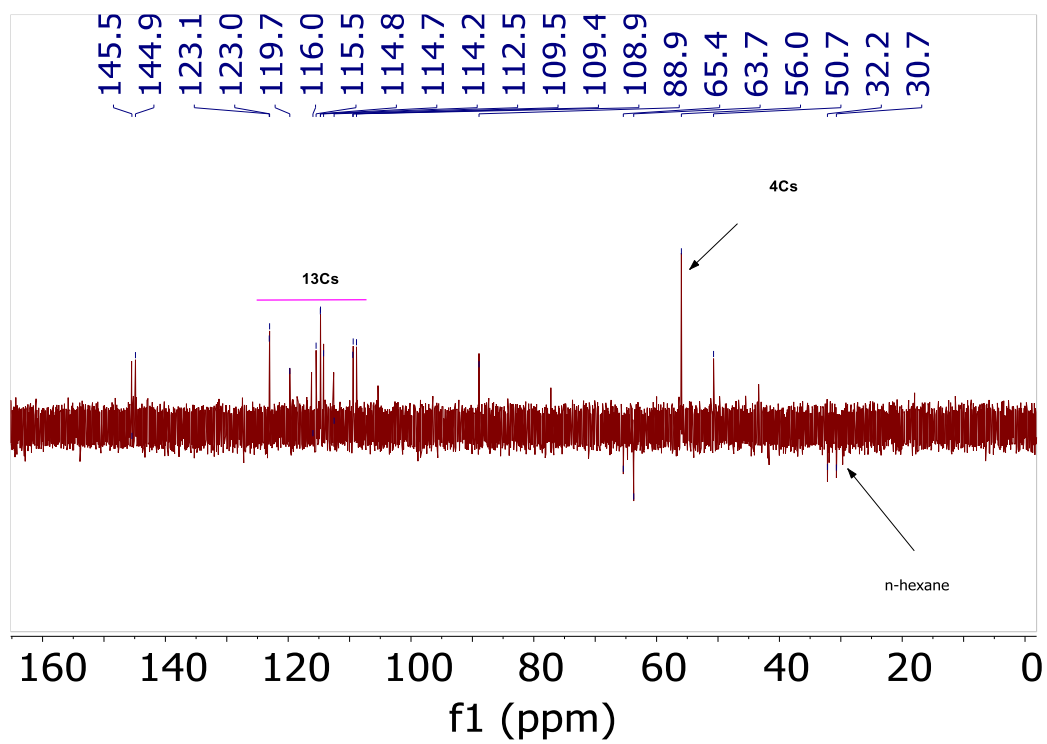
a. ^1H -NMR spectrum of compound **189**



b. The ^{13}C -NMR spectrum of compound **189**

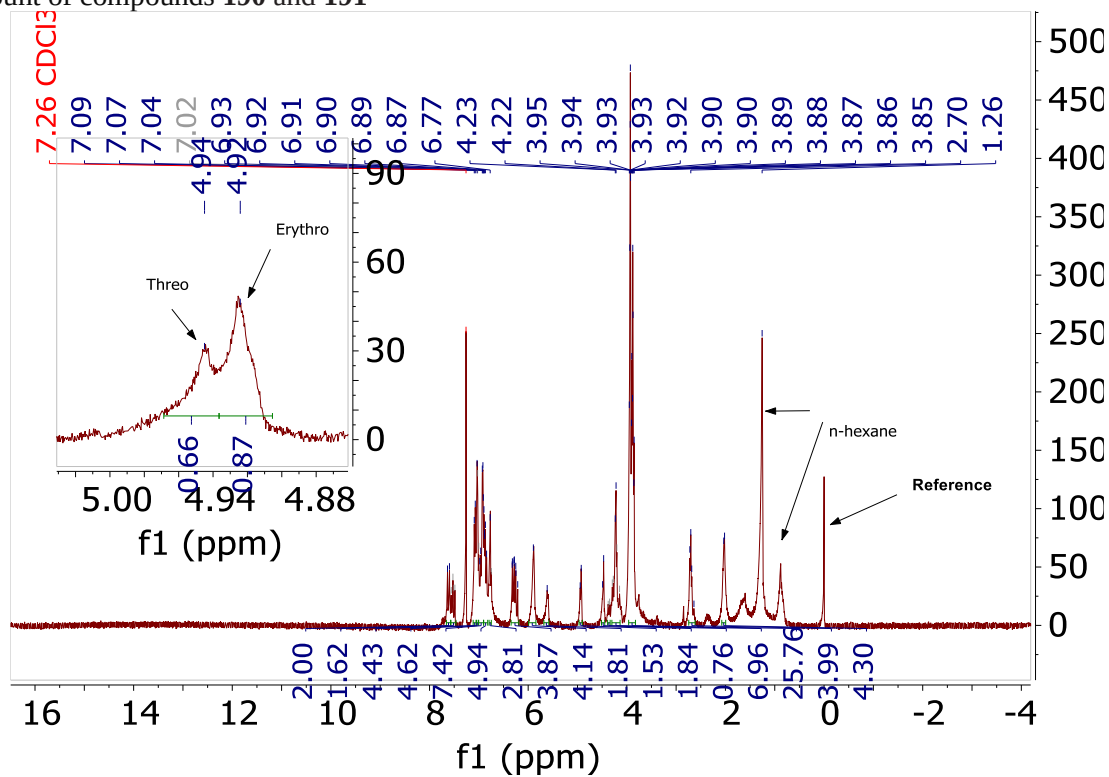


c. ^{13}C DEPT-135 NMR spectrum of compound **189**

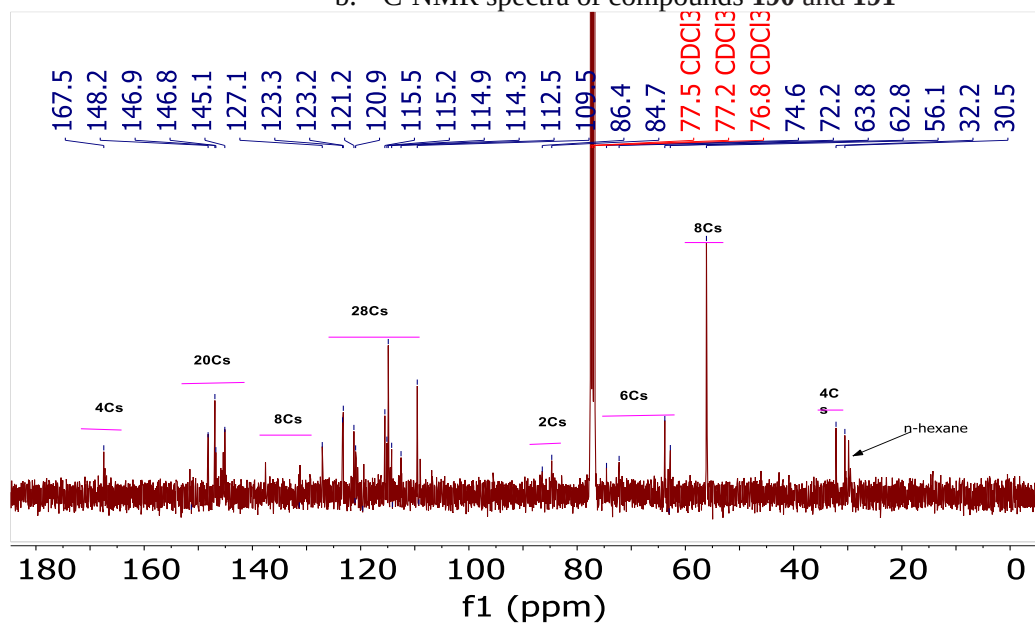


Appendix 6. NMR spectral data of compounds **190** and **191**

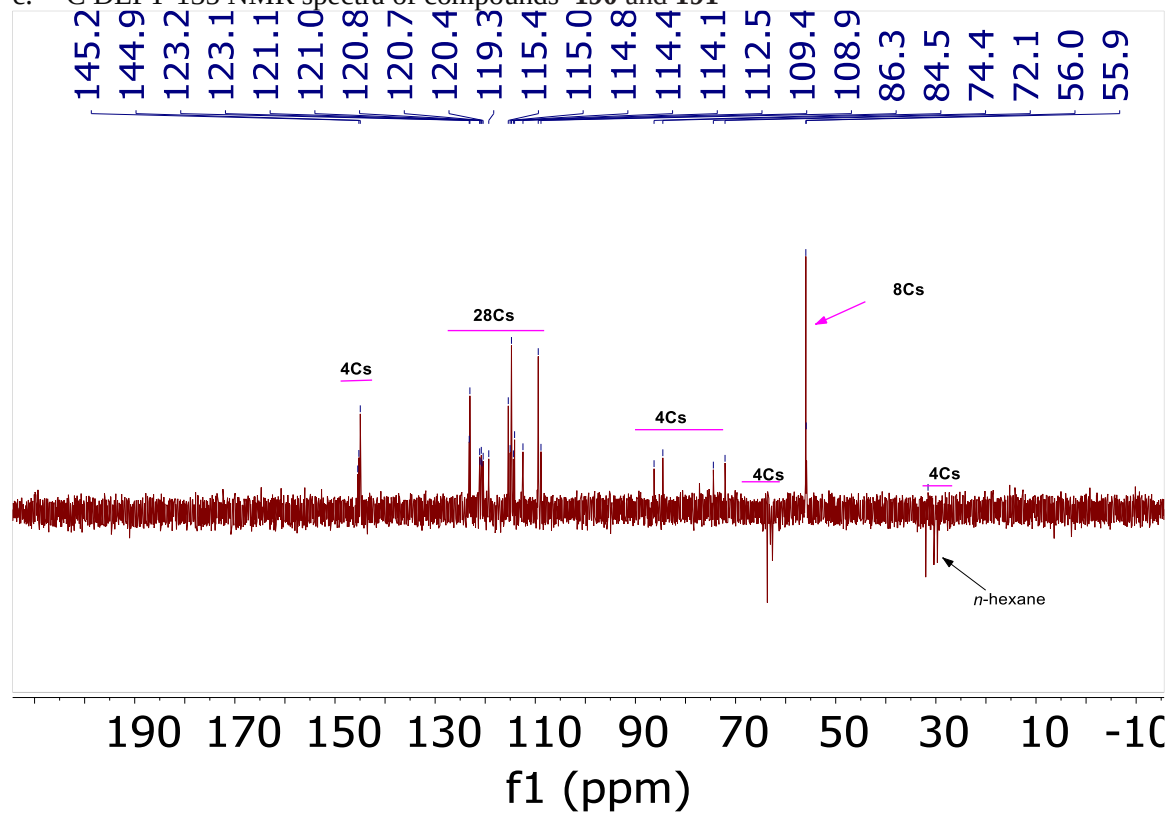
a. ^1H -NMR spectra of compounds **190** and **191** and enlarged region used to calculate the relative amount of compounds **190** and **191**



b. ^{13}C -NMR spectra of compounds **190** and **191**

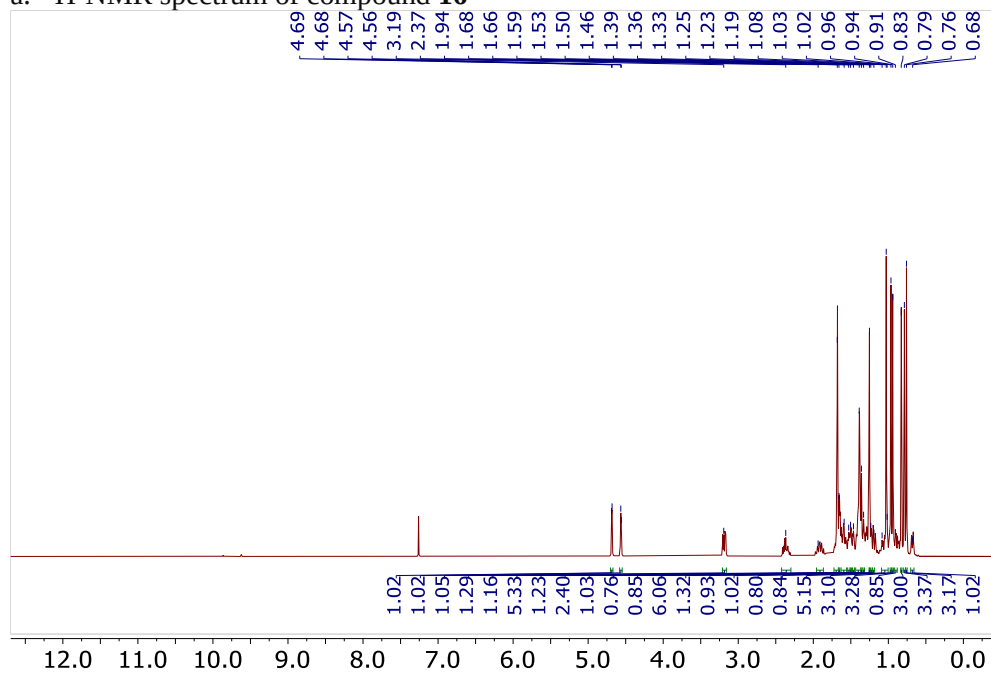


c. ^{13}C DEPT-135 NMR spectra of compounds **190** and **191**

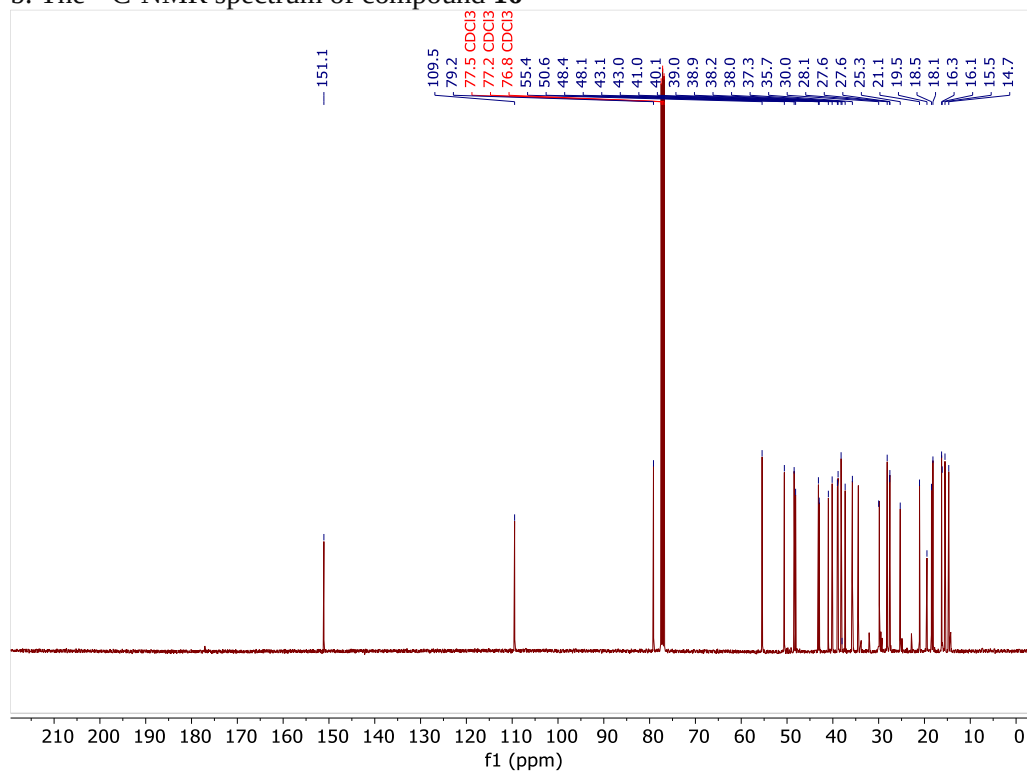


Appendix 7. NMR spectral data of compound **16**

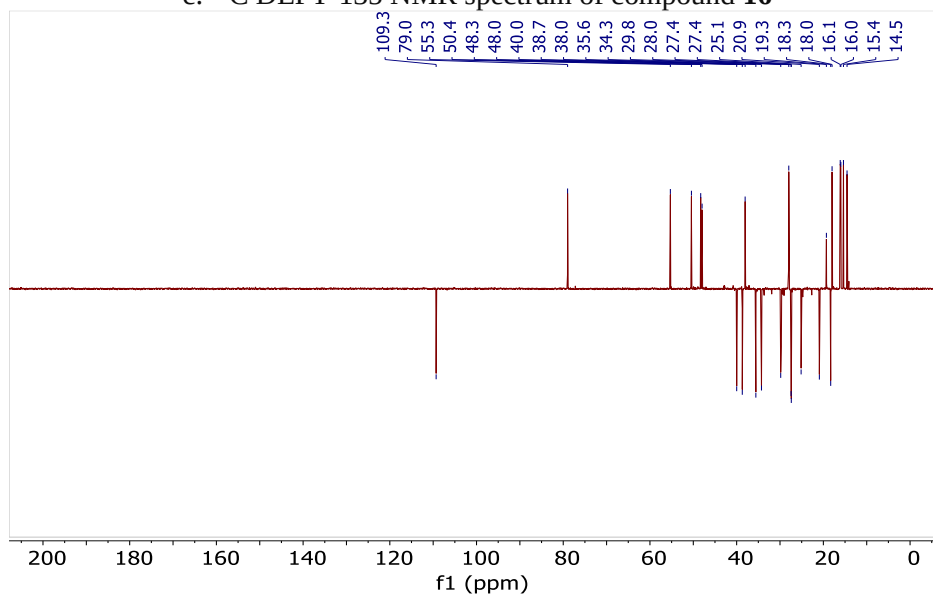
a. ^1H -NMR spectrum of compound **16**



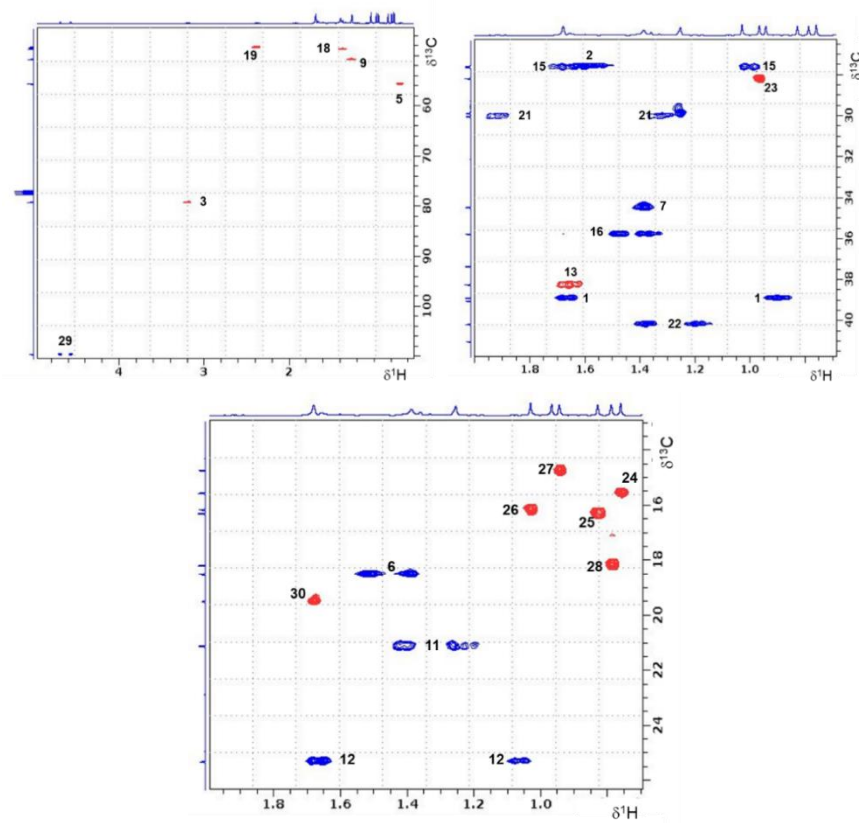
b. The ^{13}C -NMR spectrum of compound **16**



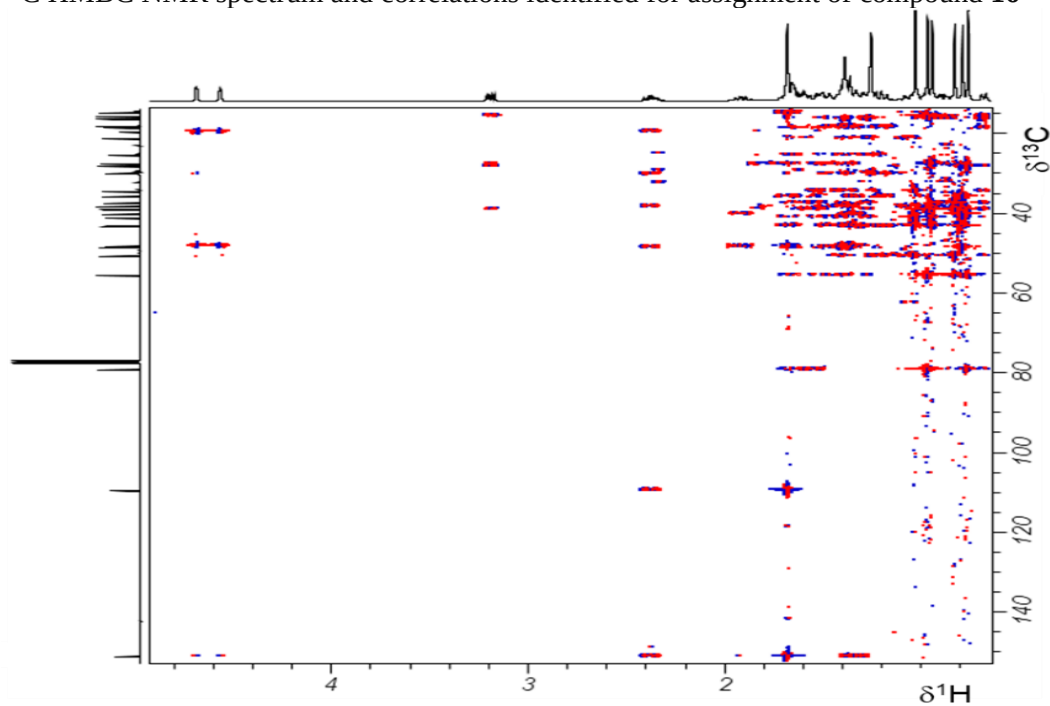
c. ^{13}C DEPT-135 NMR spectrum of compound **16**



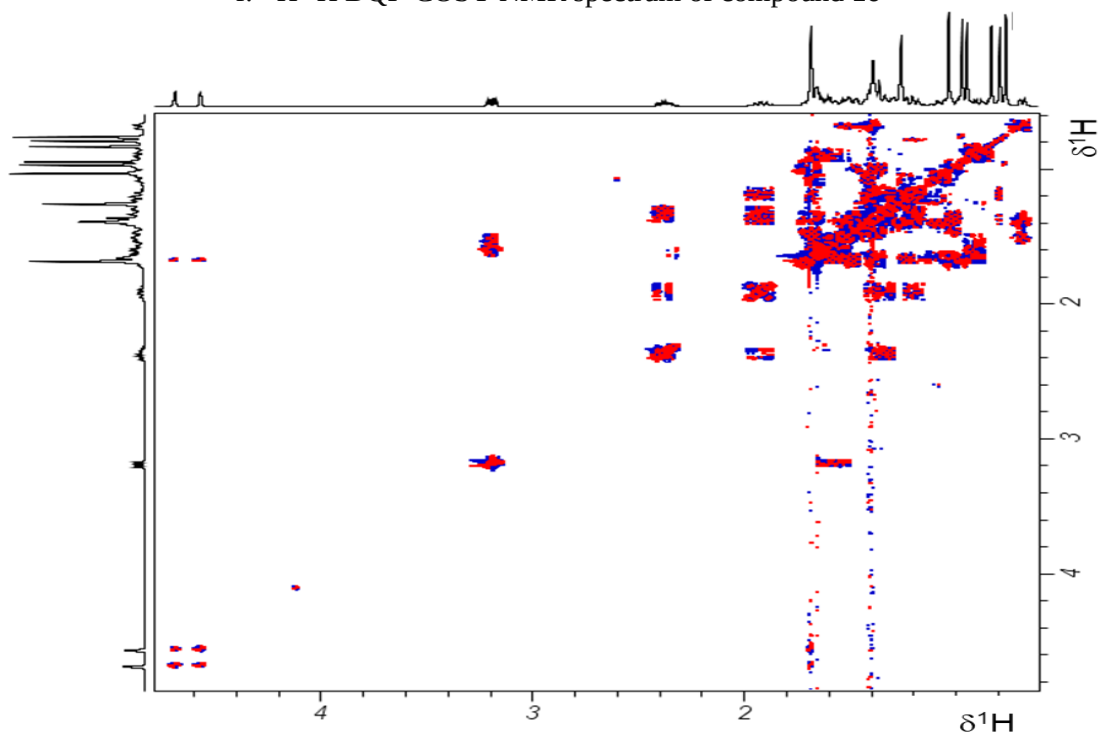
d. HSQC NMR spectrum and expanded regions used in resonances assignment of compound **16**



e. ^1H - ^{13}C HMBC NMR spectrum and correlations identified for assignment of compound **16**

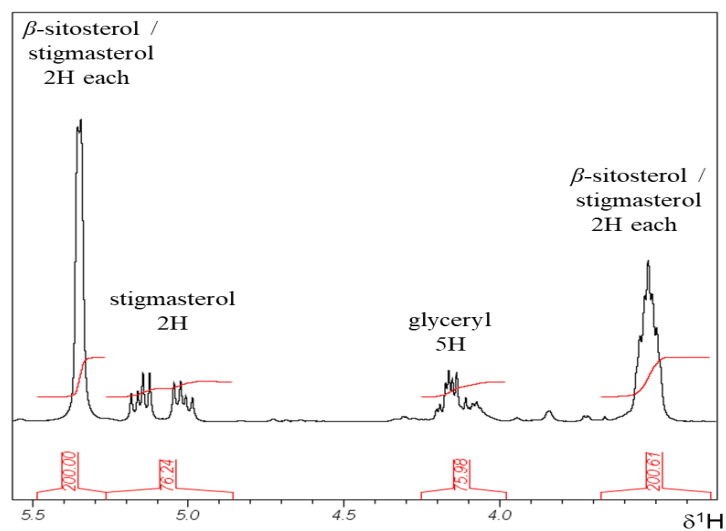
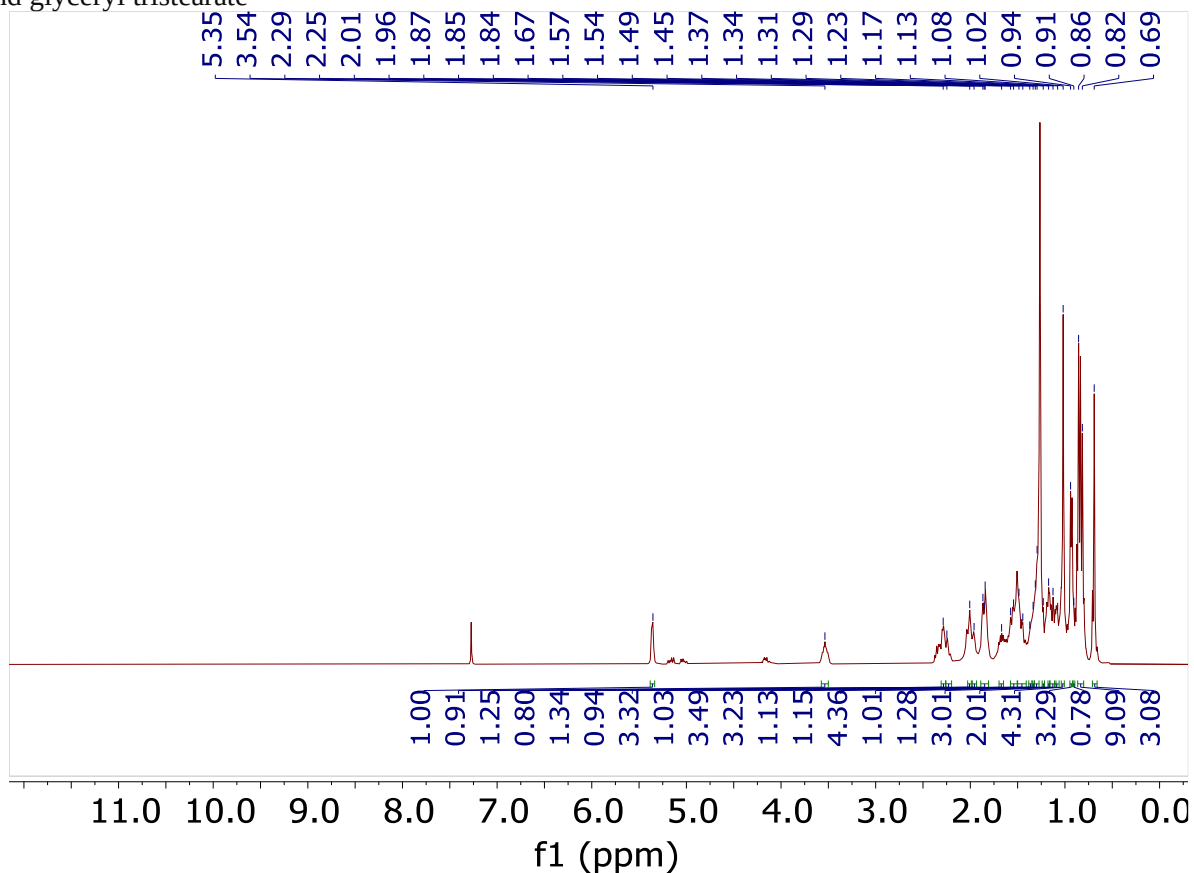


f. ^1H - ^1H DQF-COSY NMR spectrum of compound **16**

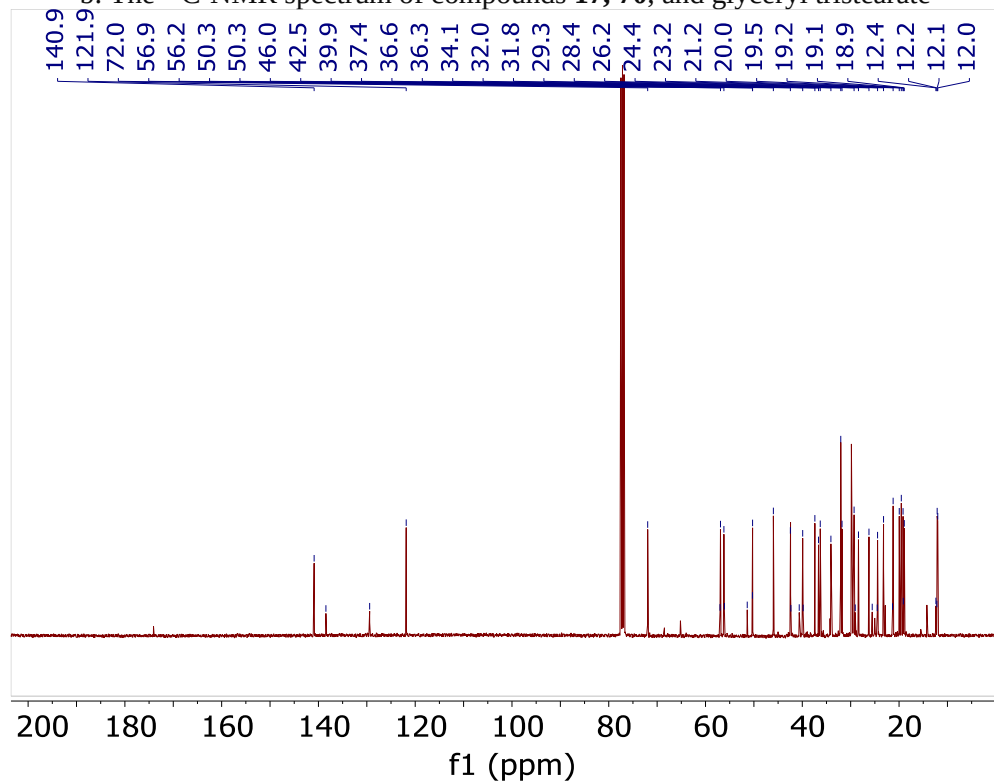


Appendix 8. NMR spectral data of compounds **17**, **70**, and glyceryl tristearate

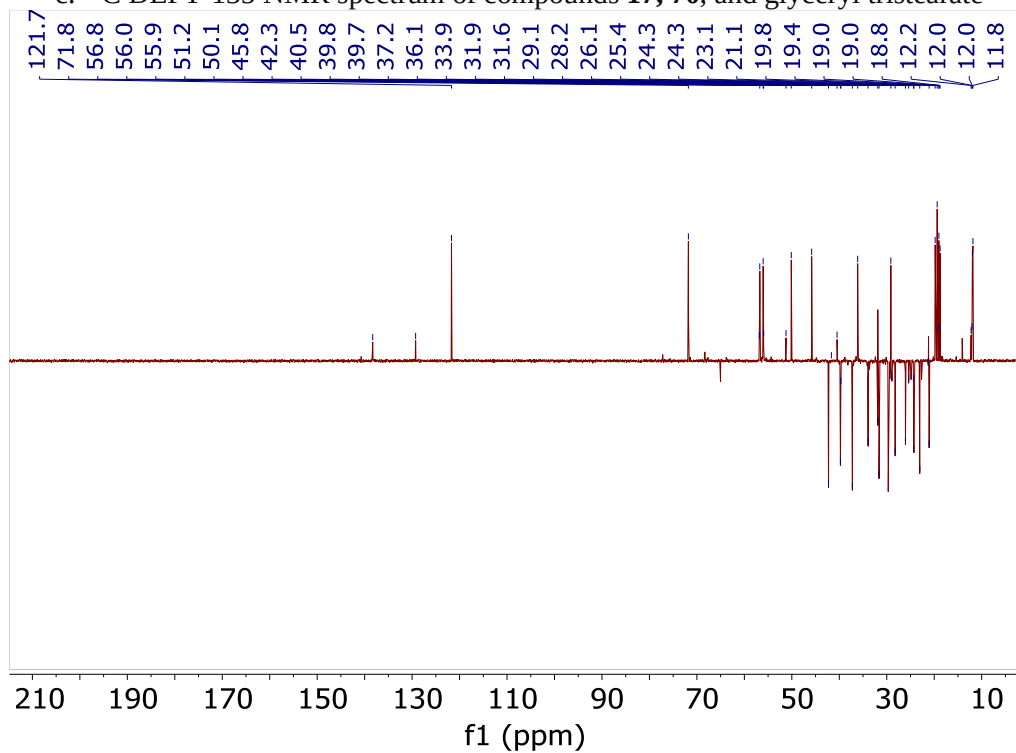
a. ^1H -NMR spectrum and enlarged region used to determine relative amounts of compounds **17**, **70**, and glyceryl tristearate



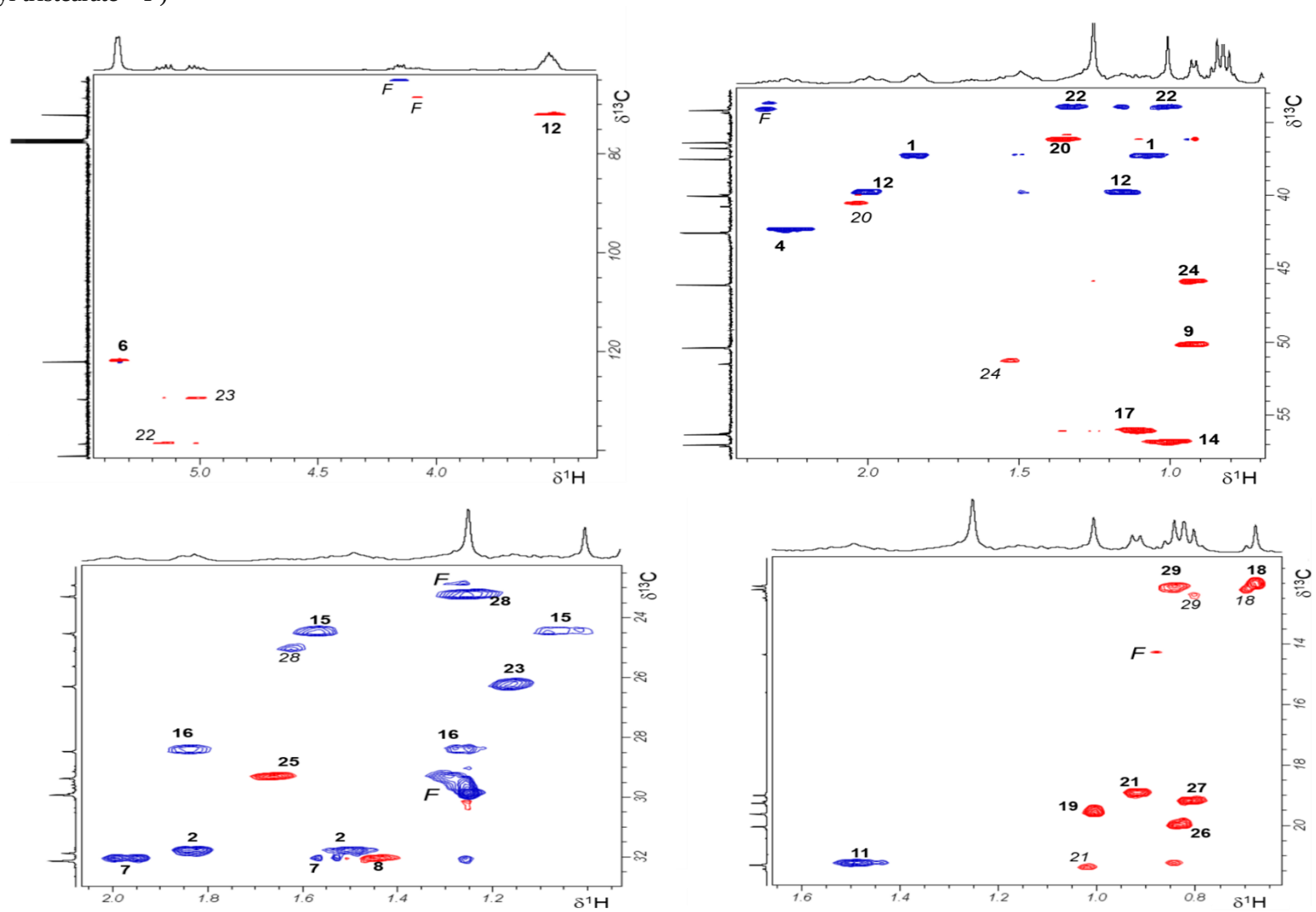
b. The ^{13}C -NMR spectrum of compounds **17**, **70**, and glyceryl tristearate



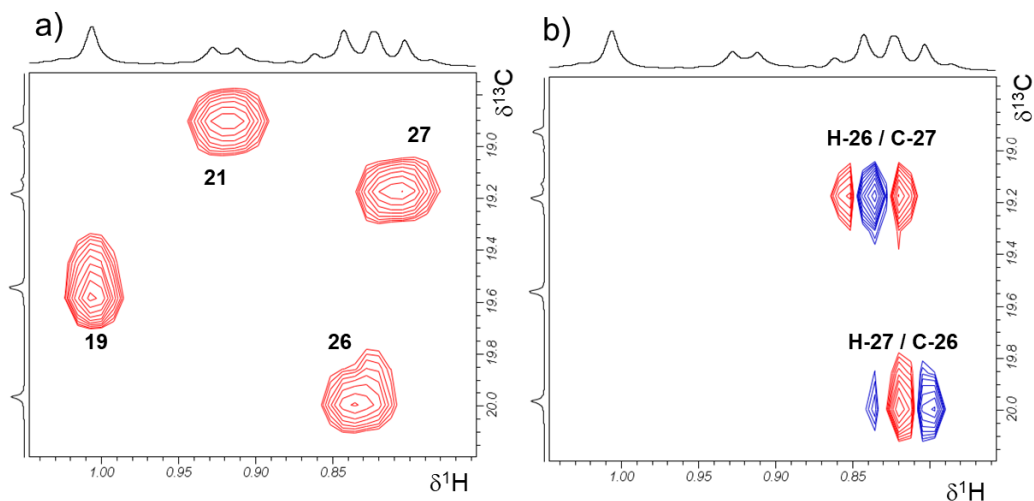
c. ^{13}C DEPT-135 NMR spectrum of compounds **17**, **70**, and glyceryl tristearate



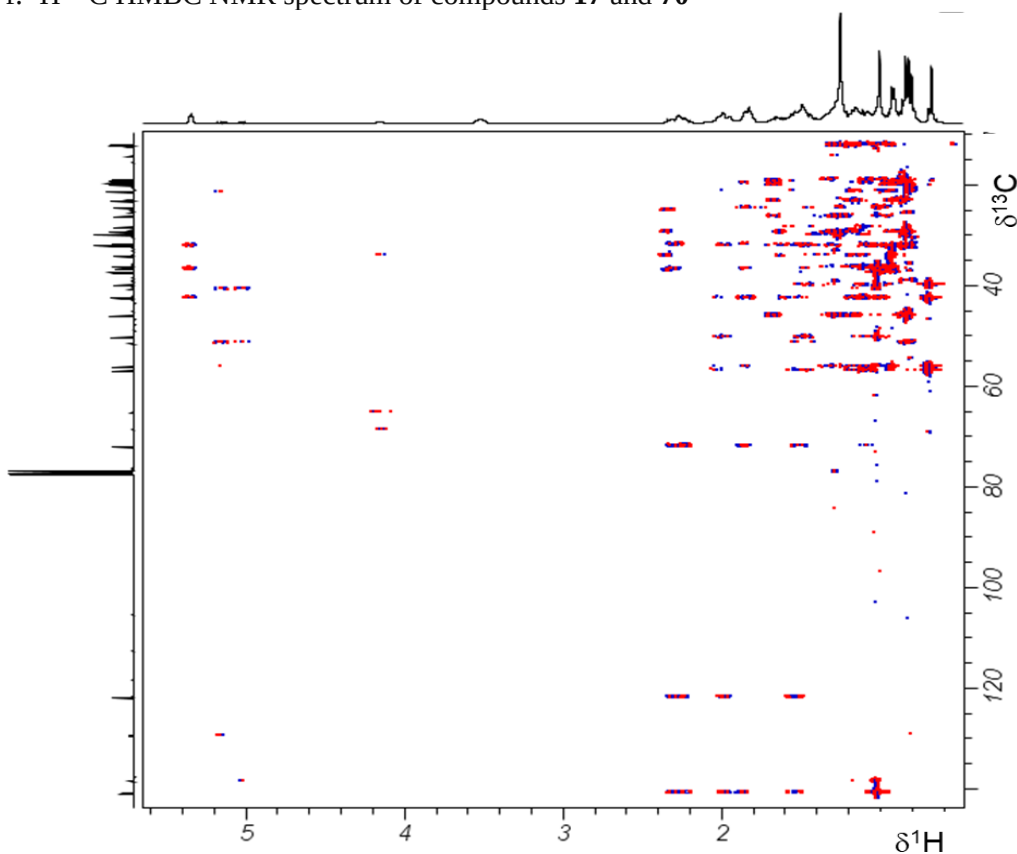
d. ^1H - ^{13}C HSQC NMR spectrum and enlarged regions used in resonances assignment of compounds **17** (in bold), compound **70** (in *italic*), and glyceryl tristearate = *F*)



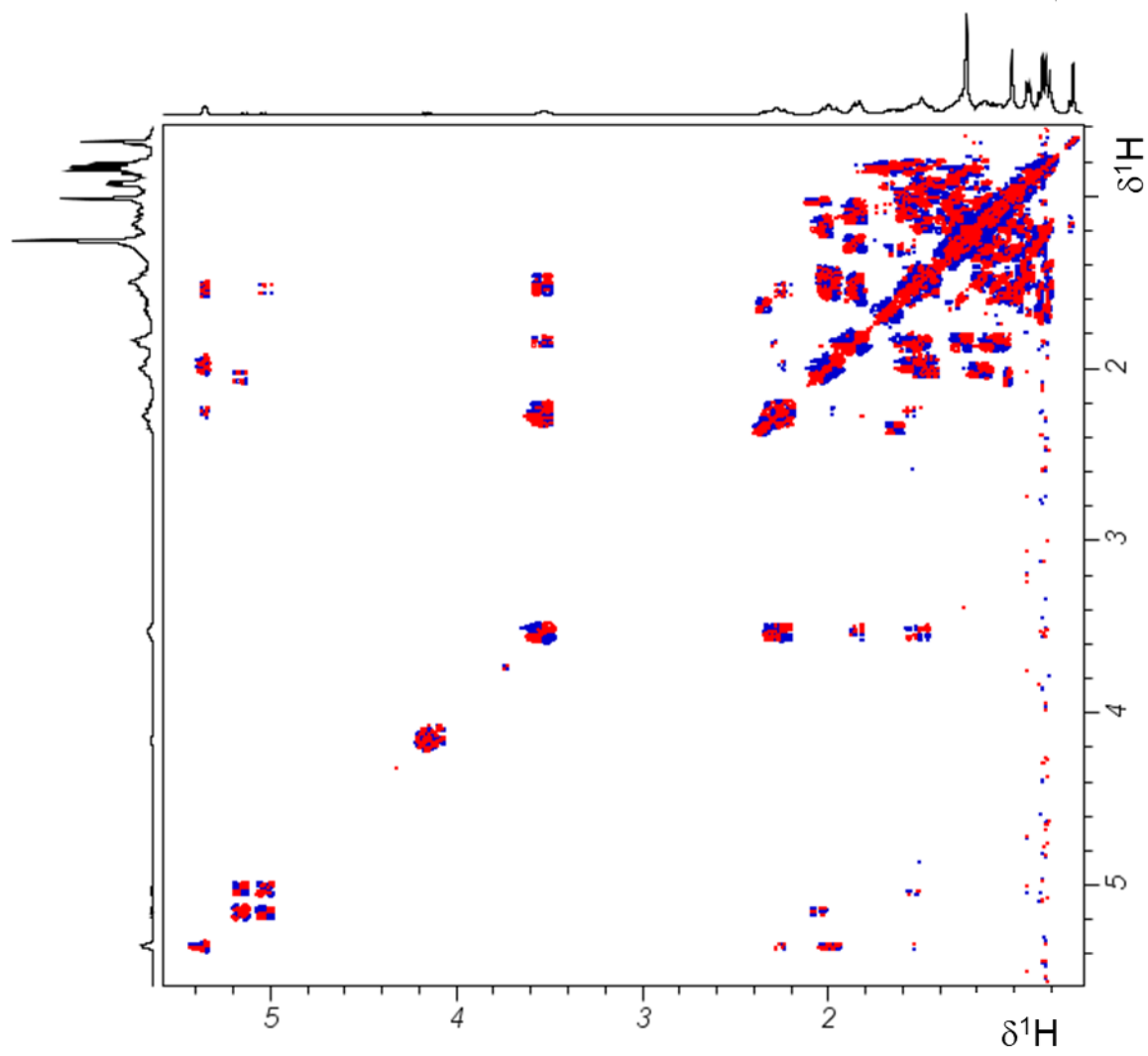
e. Expanded regions of ^1H - ^{13}C HSQC (a) and HMBC (b) NMR spectrum with assignments of resonances of compound **17** with crucial HMBC correlations of H-26 to C-27 and H-27 to C-26 used for unambiguous assignment of resonances



f. ^1H - ^{13}C HMBC NMR spectrum of compounds **17** and **70**

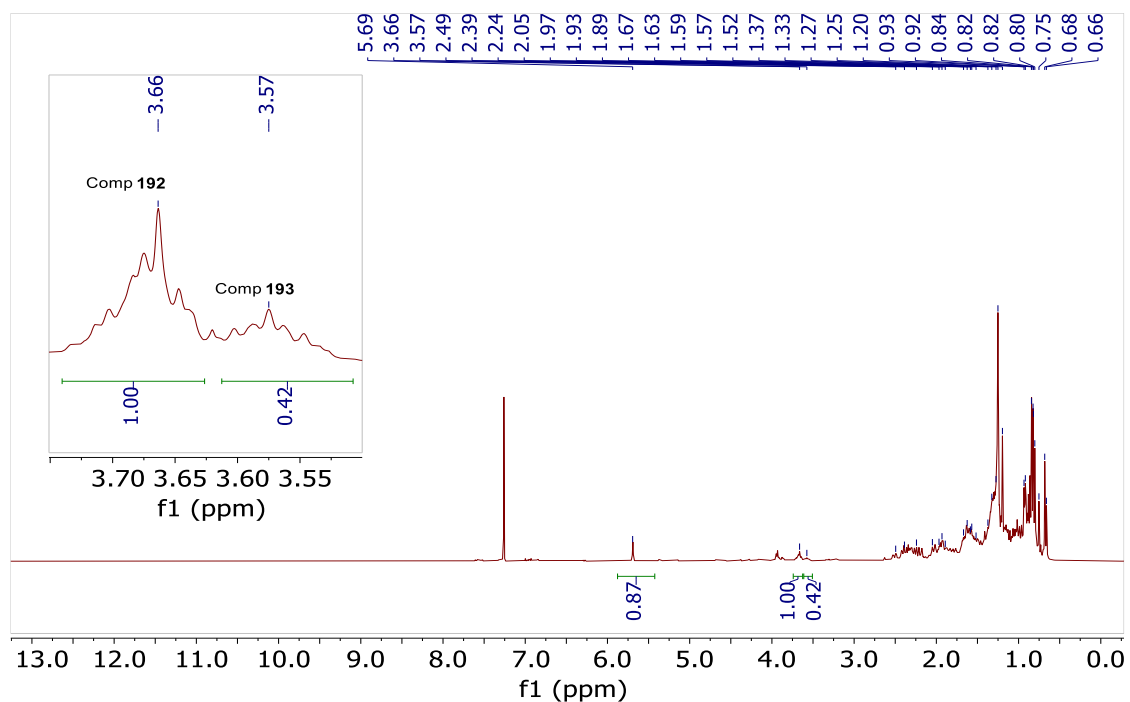


g. ^1H - ^1H DQF-COSY NMR spectrum of compounds **17**, **70**, and glyceryl tristearate

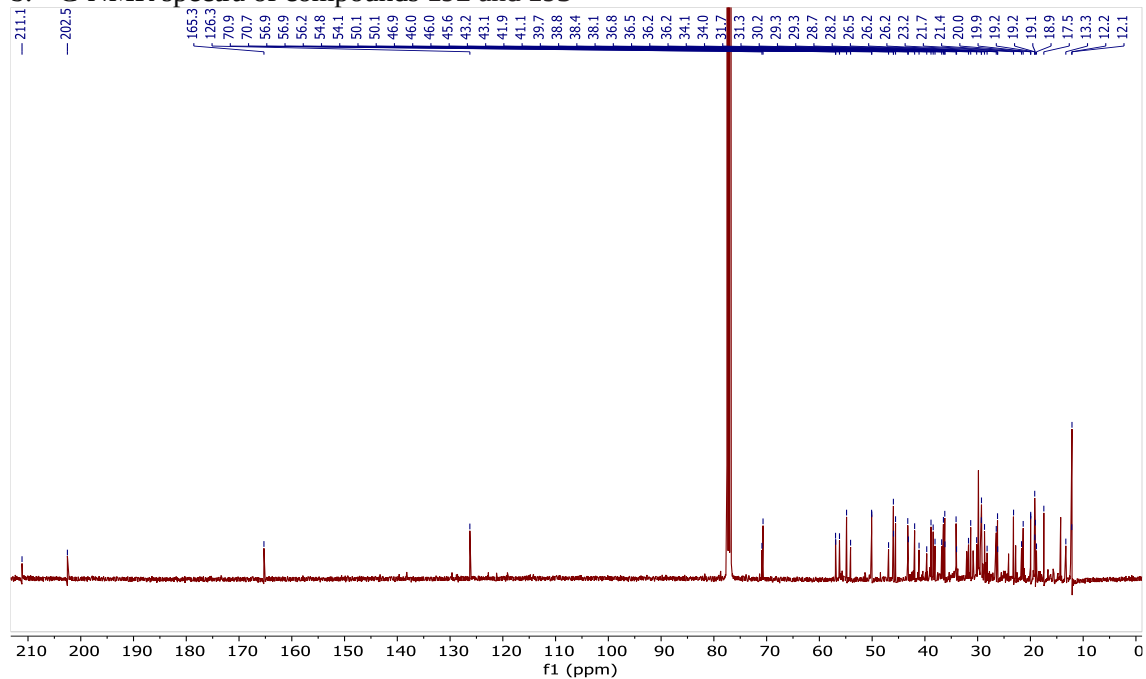


Appendix 9. NMR spectral data of compounds **192** and **193**

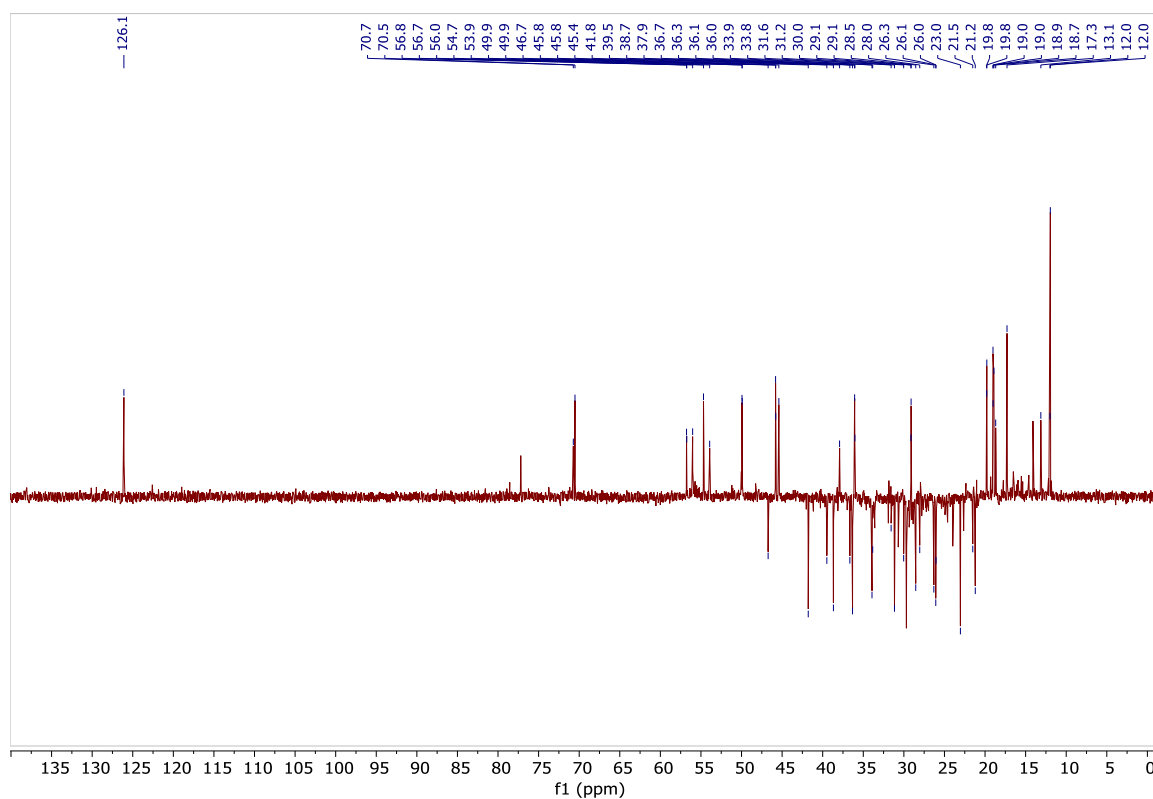
a. ^1H -NMR spectra of compounds **192** and **193** and expanded region used to calculate their percentage



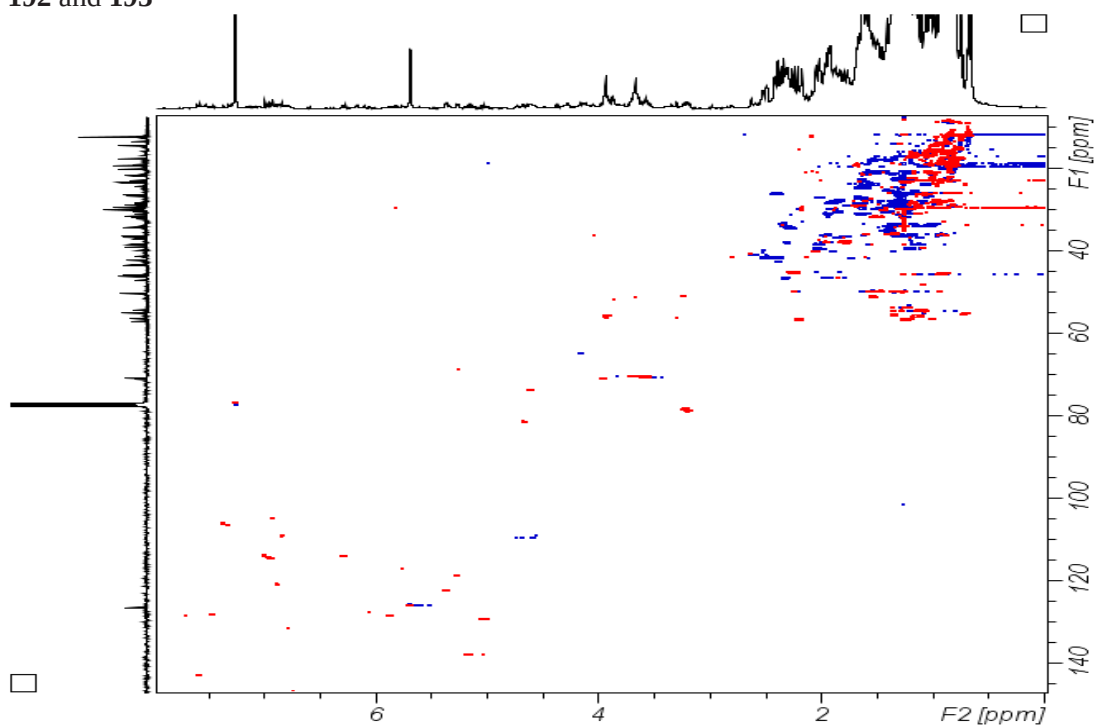
b. ^{13}C -NMR spectra of compounds **192** and **193**



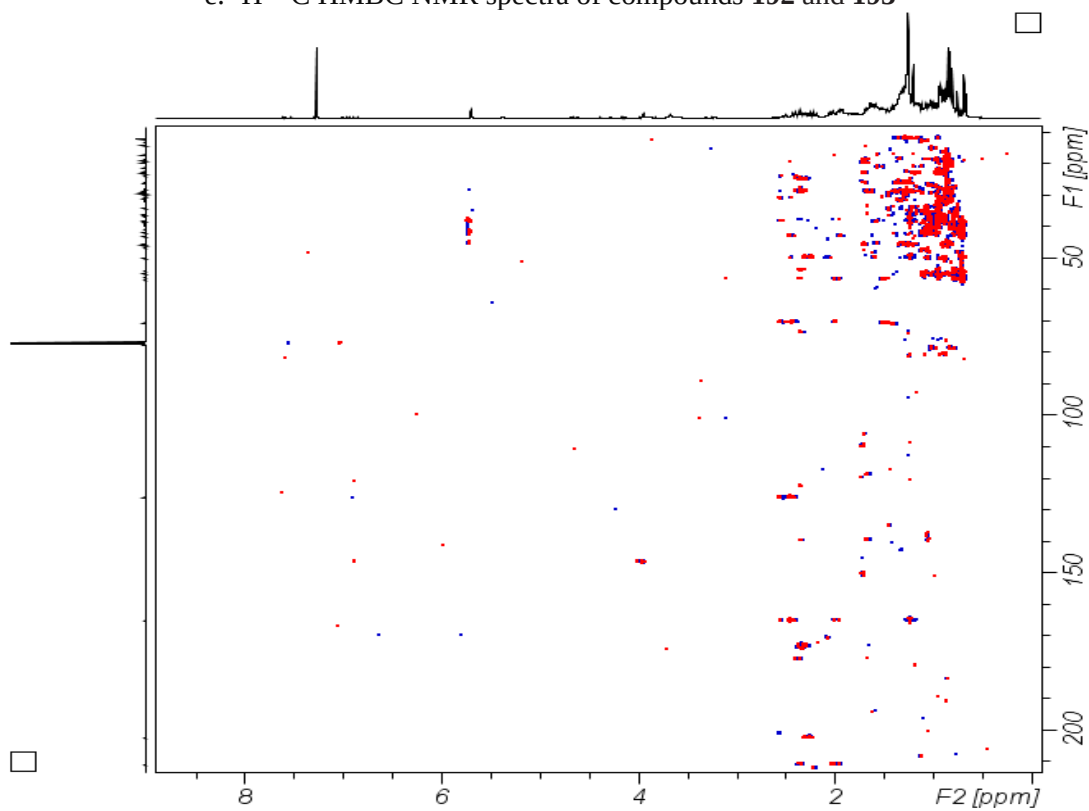
c. ^{13}C DEPT-135 NMR spectra of compounds **192** and **193**



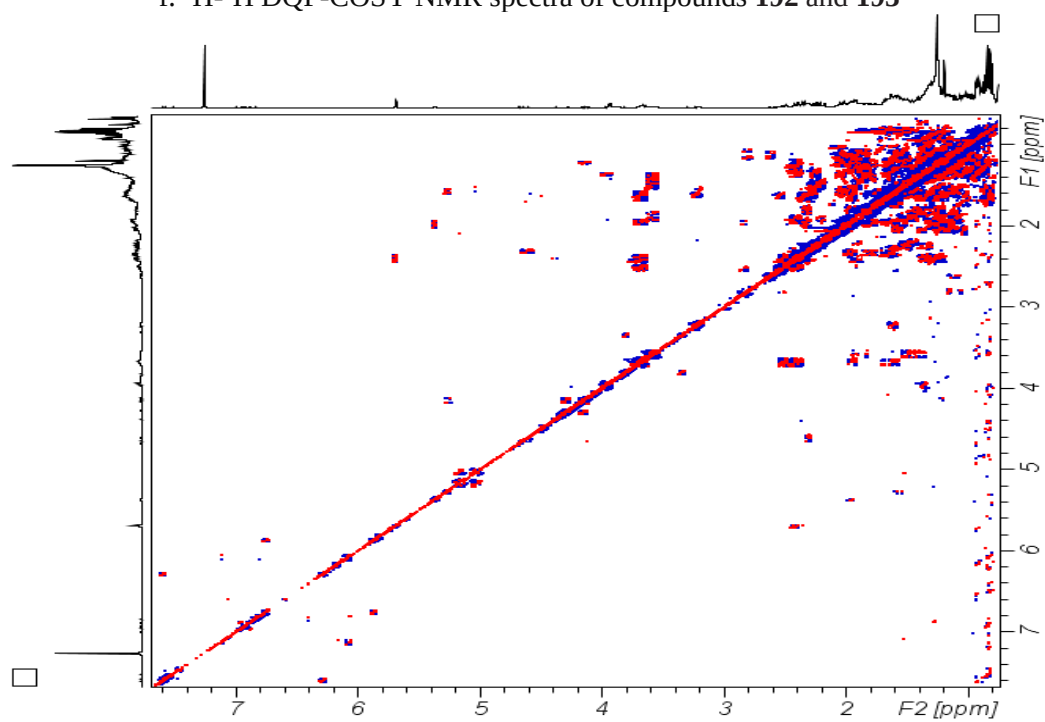
d. ^1H - ^{13}C HSQC NMR spectra and expanded regions used in the resonances assignment of compounds **192** and **193**



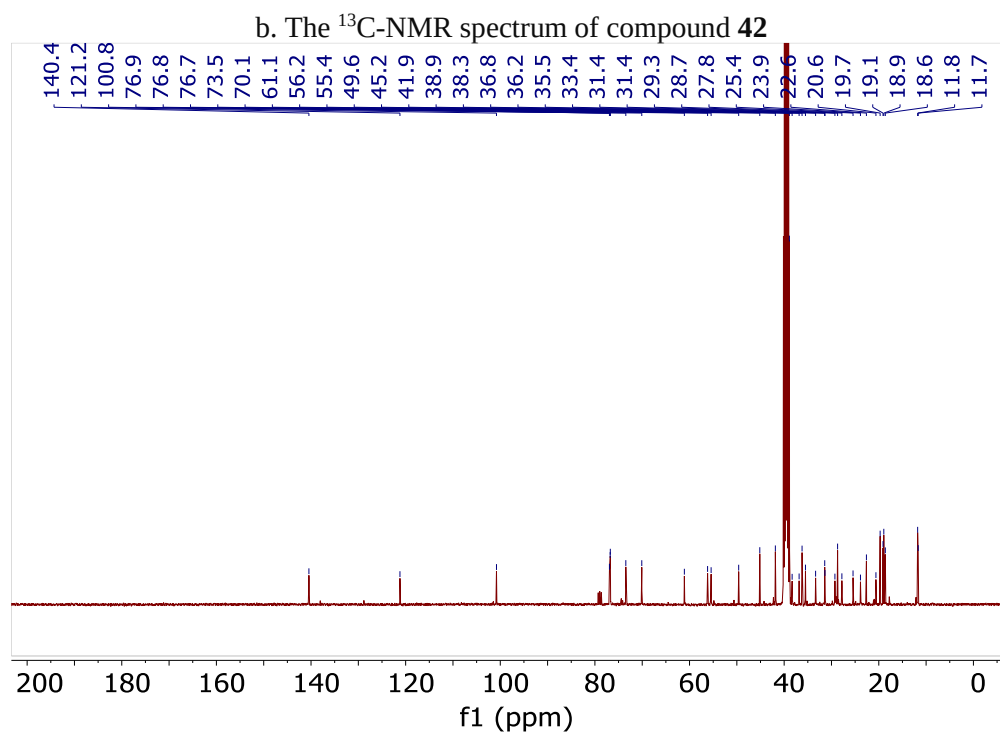
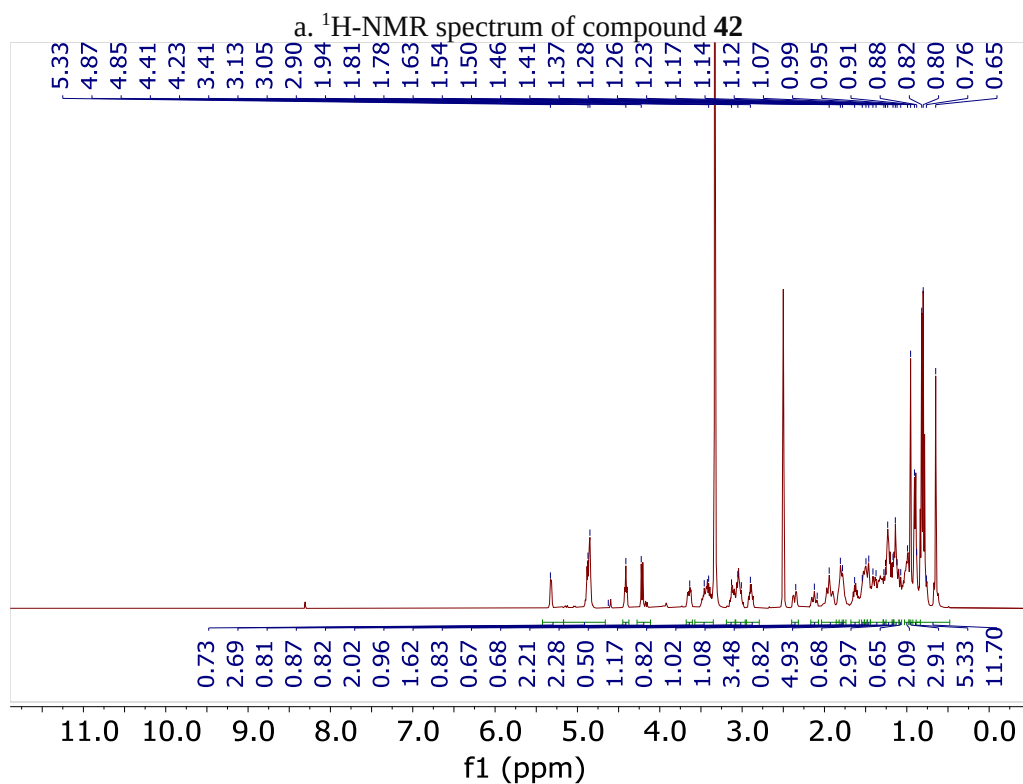
e. ^1H - ^{13}C HMBC NMR spectra of compounds **192** and **193**

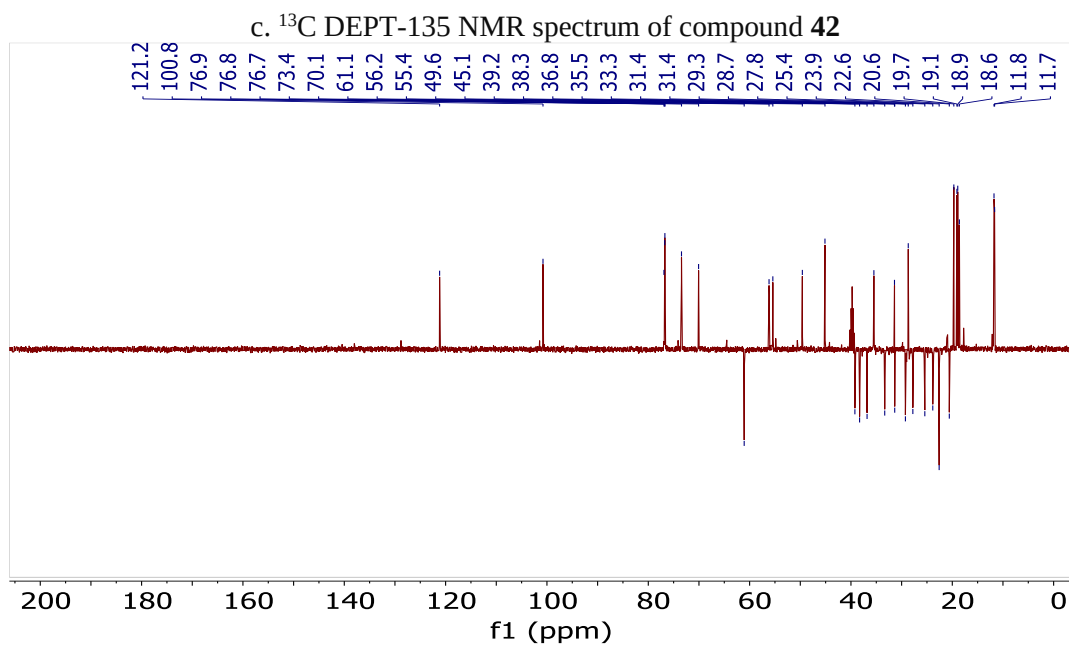


f. ^1H - ^1H DQF-COSY NMR spectra of compounds **192** and **193**

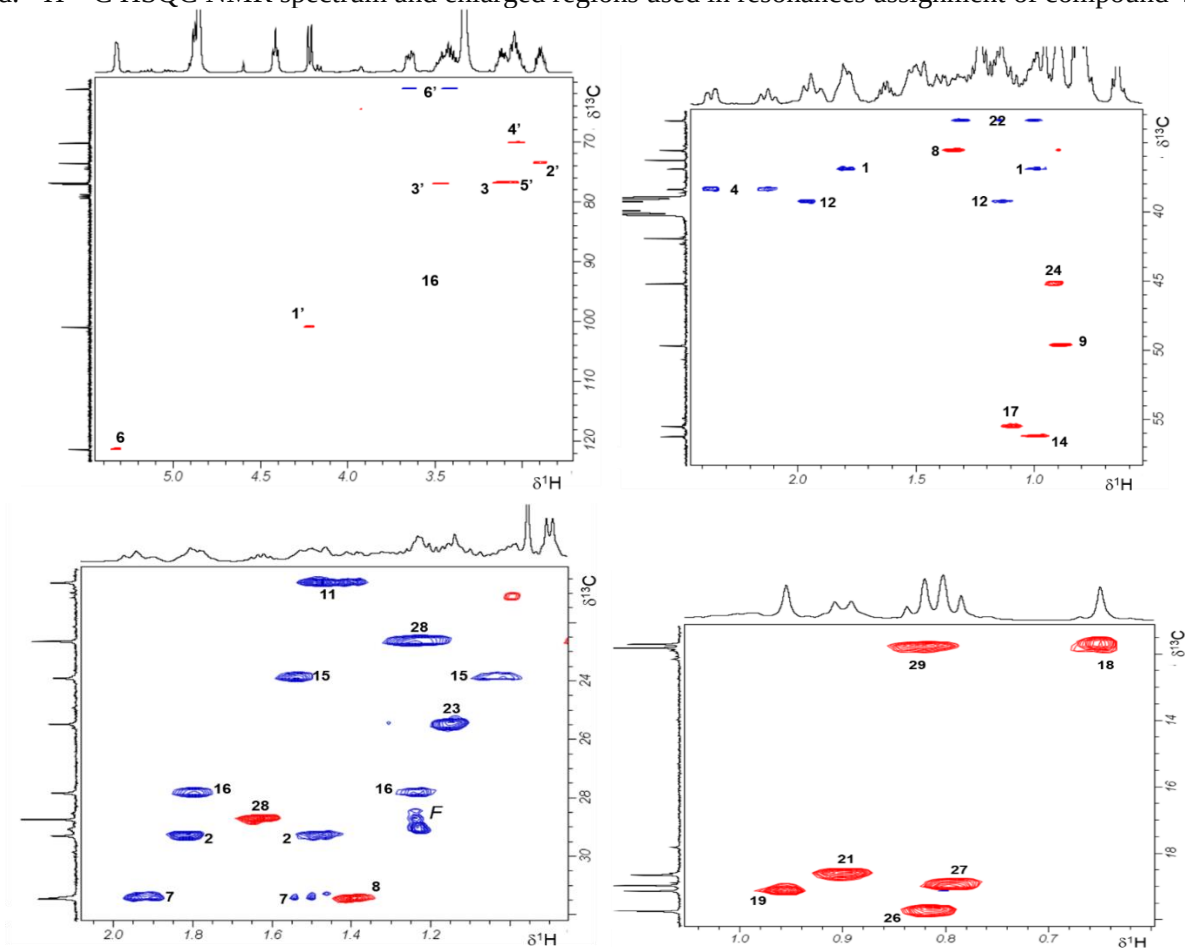


Appendix 10. NMR spectral data of compound **42**

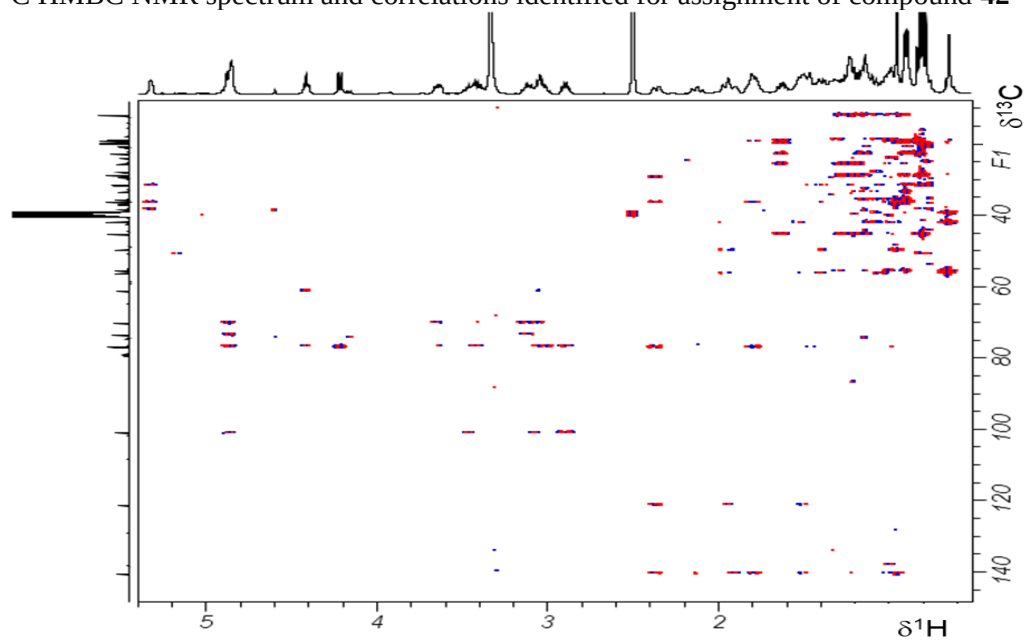




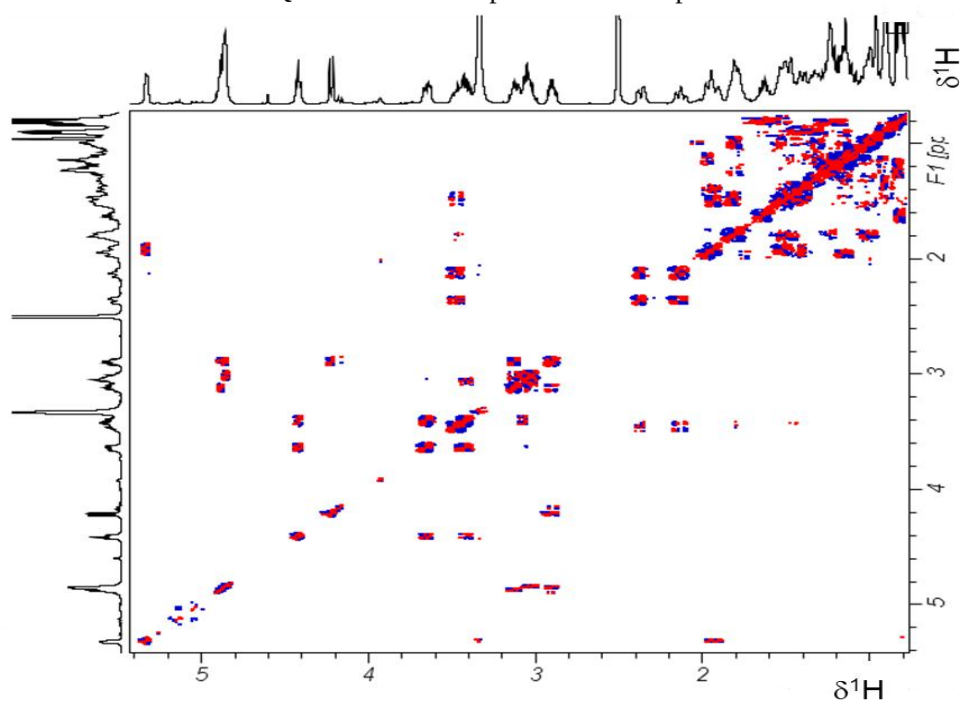
d. ^1H - ^{13}C HSQC NMR spectrum and enlarged regions used in resonances assignment of compound **42**



e. ^1H - ^{13}C HMBC NMR spectrum and correlations identified for assignment of compound **42**

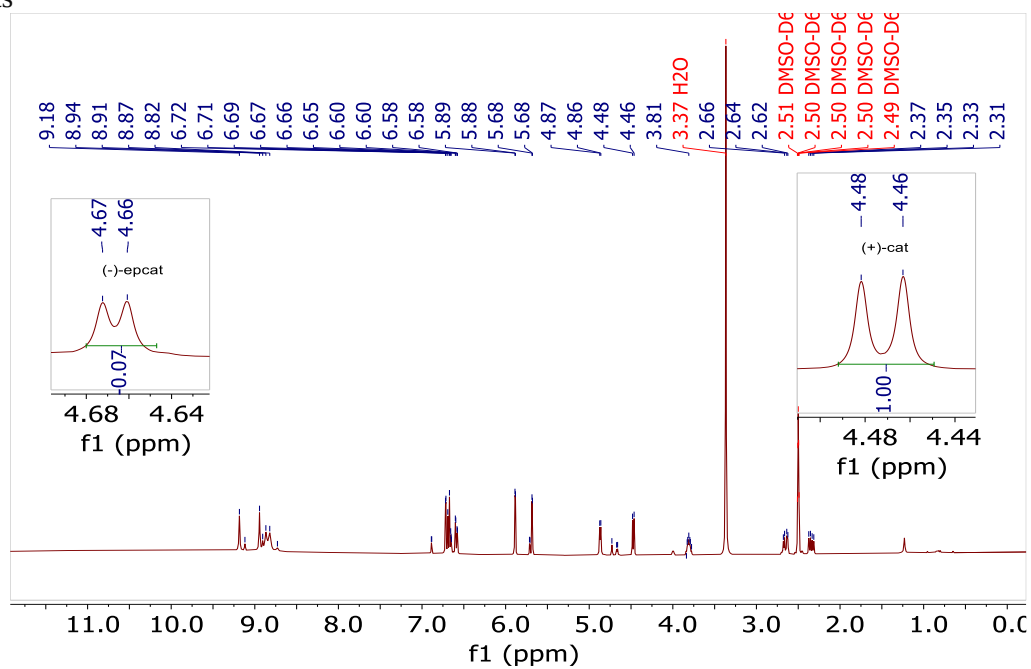


f. ^1H - ^1H DQF-COSY NMR spectrum of compound **42**

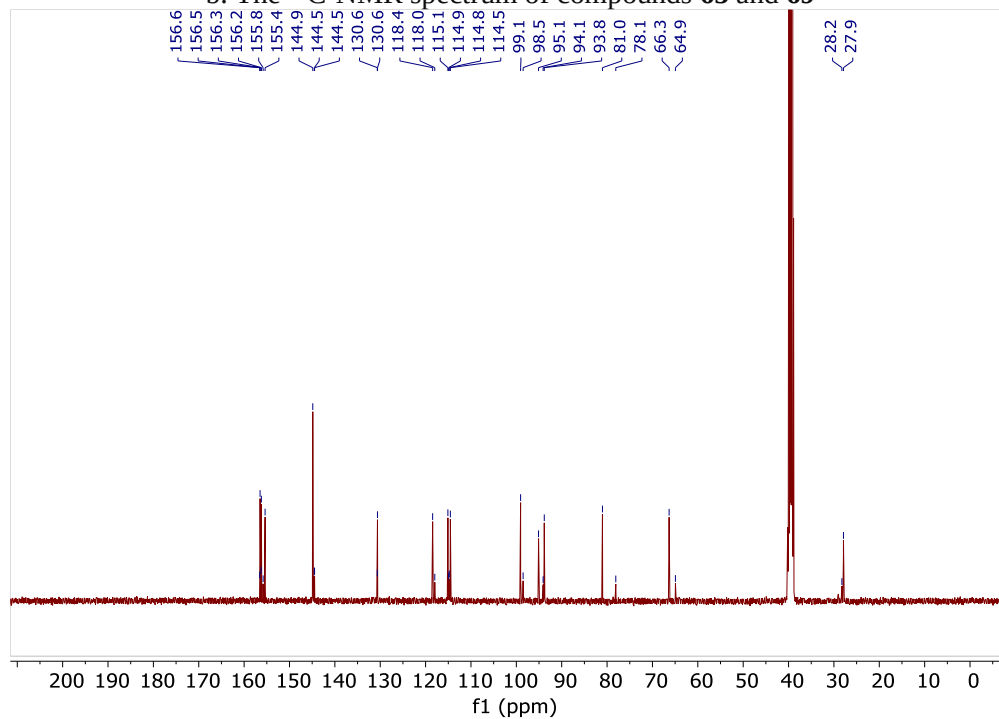


Appendix 11. NMR spectral data of compounds **65** and **69**

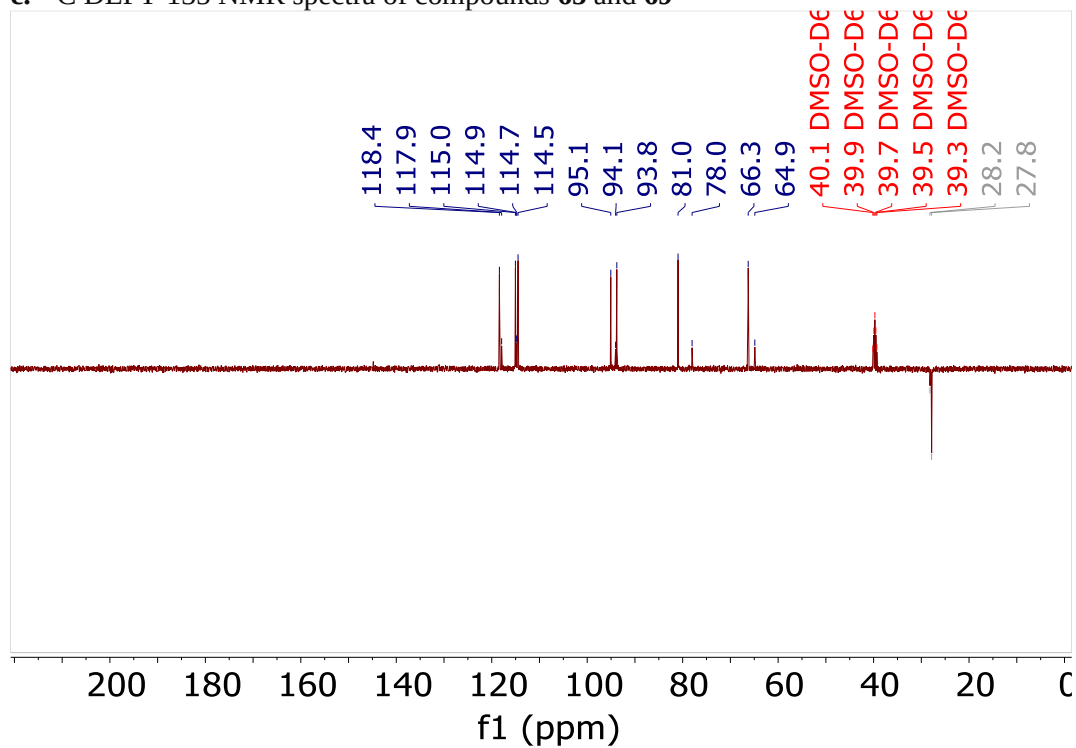
a. ^1H -NMR spectra of compounds **65** and **69** and enlarged regions used to calculate their relative amounts



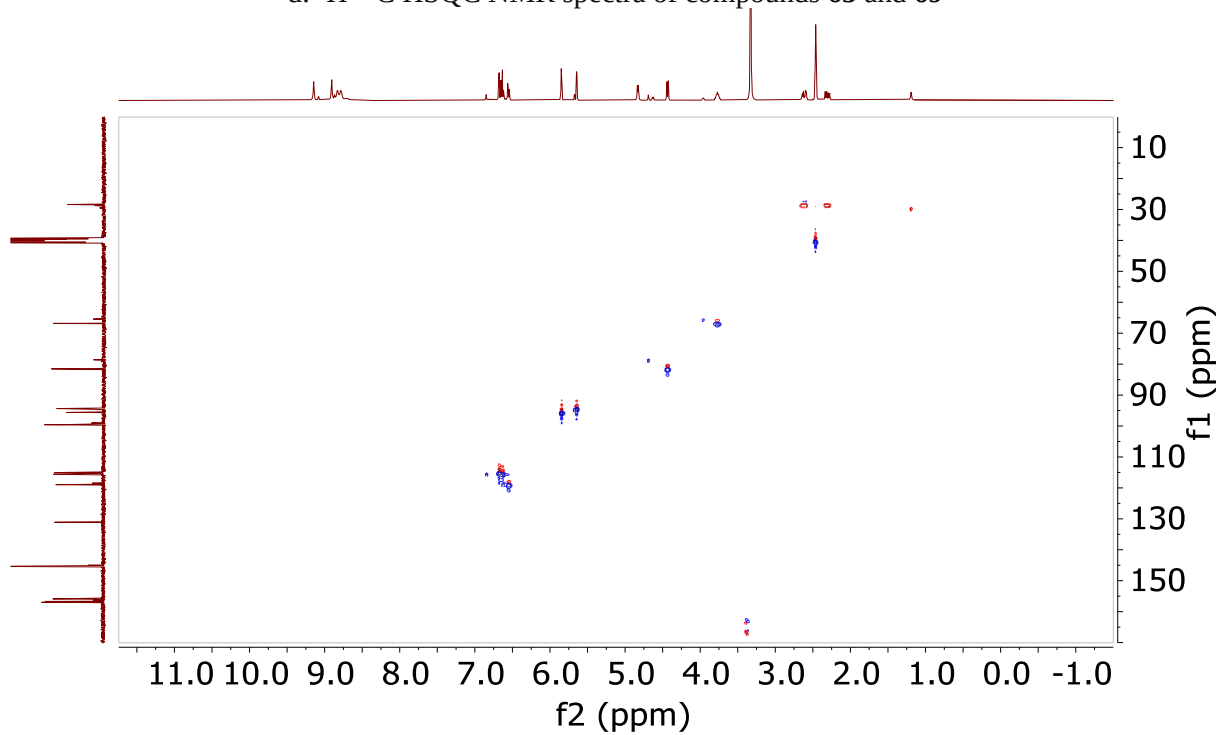
b. The ^{13}C -NMR spectrum of compounds **65** and **69**



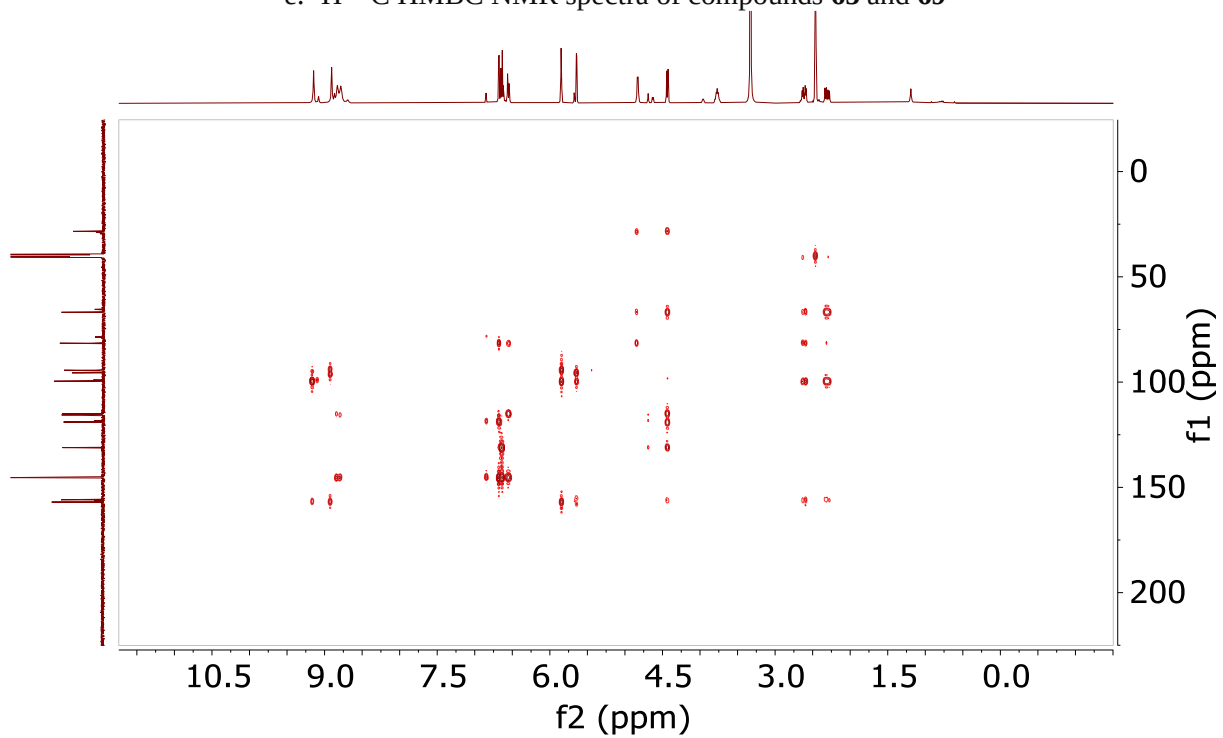
c. ^{13}C DEPT-135 NMR spectra of compounds **65** and **69**



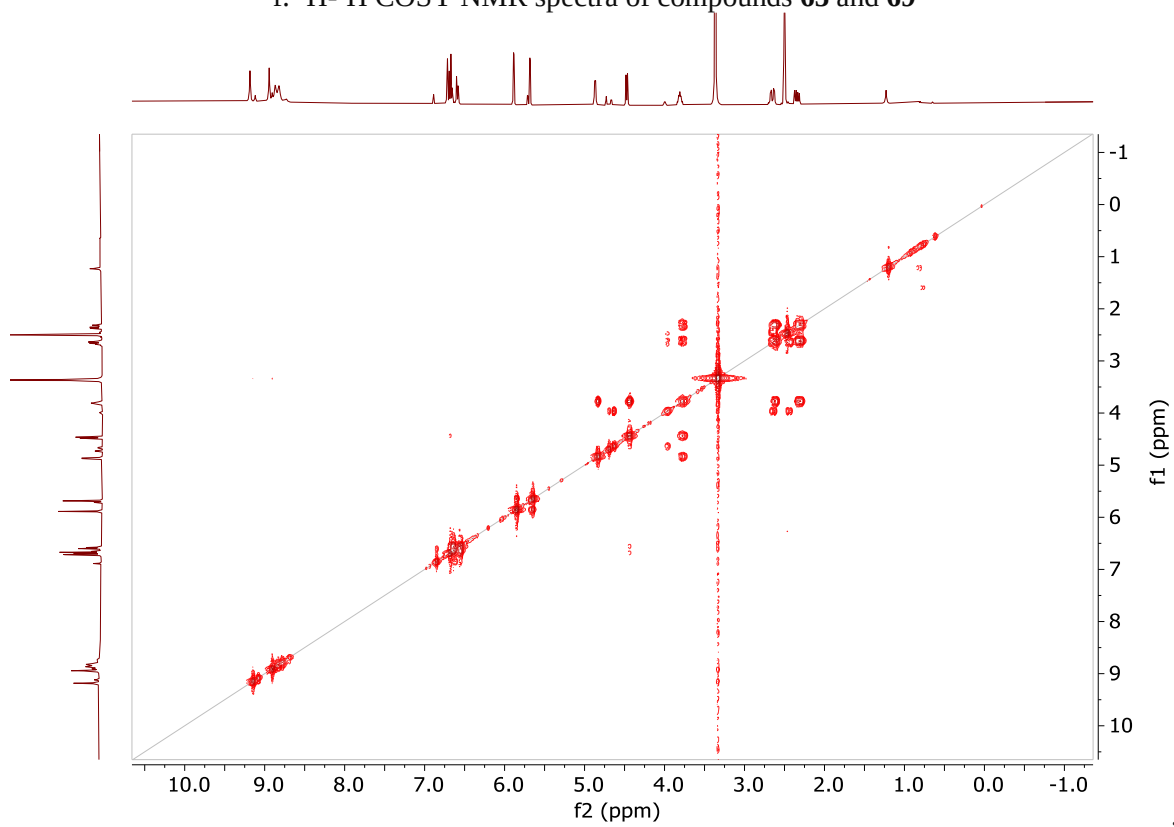
d. ^1H - ^{13}C HSQC NMR spectra of compounds **65** and **69**



e. ^1H - ^{13}C HMBC NMR spectra of compounds **65** and **69**

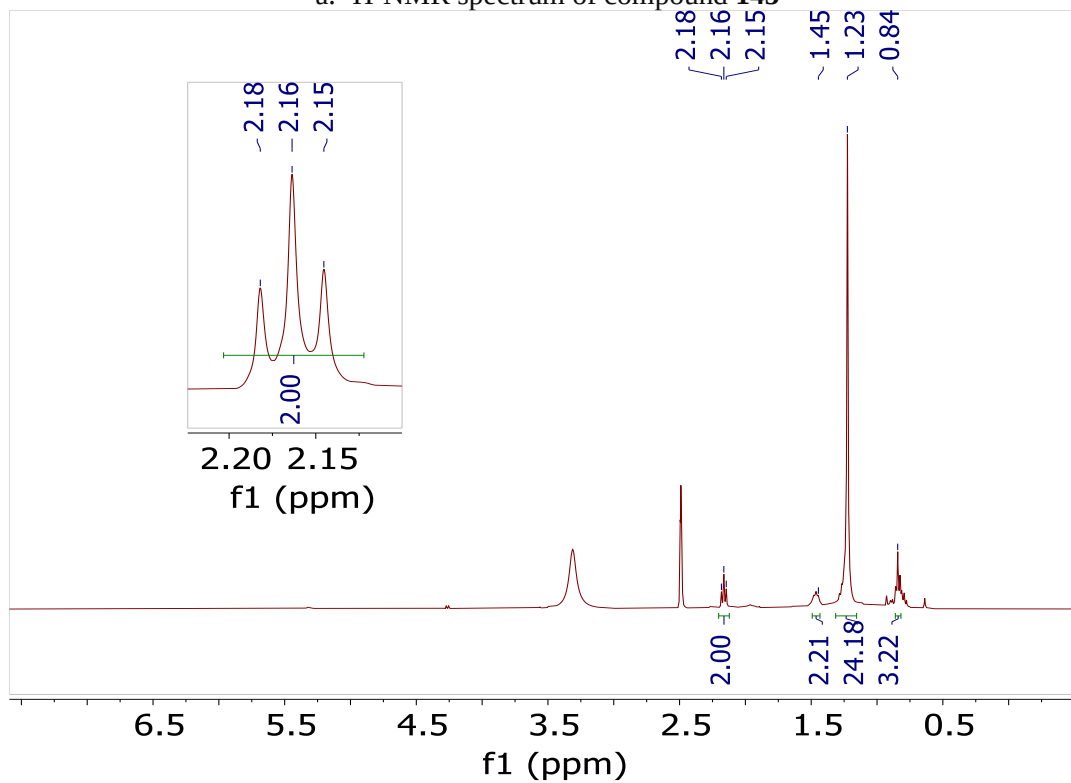


f. ^1H - ^1H COSY NMR spectra of compounds **65** and **69**

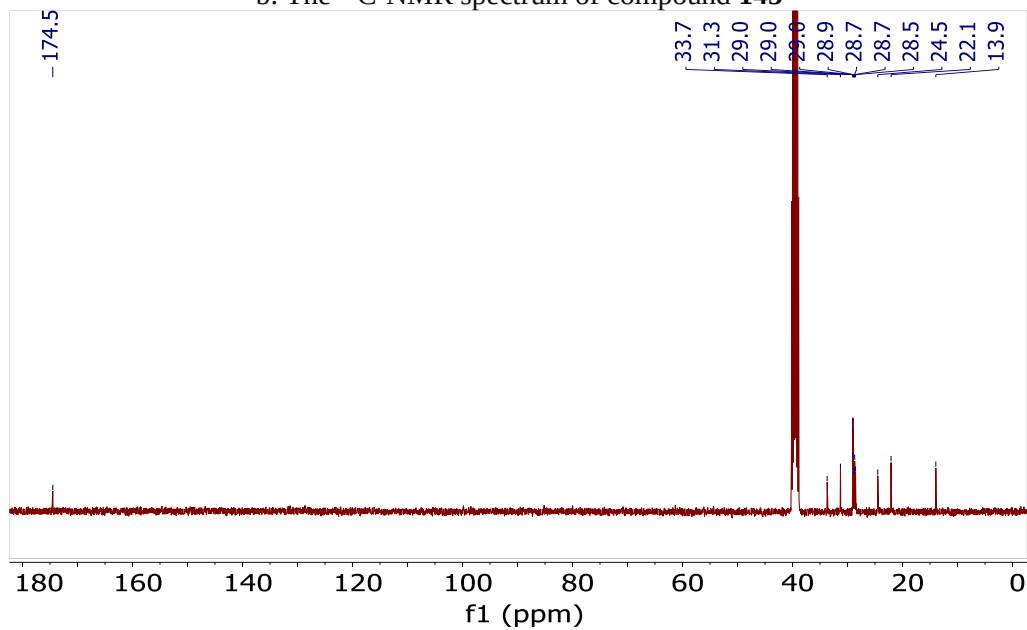


Appendix 12. NMR spectral data of compounds **145**

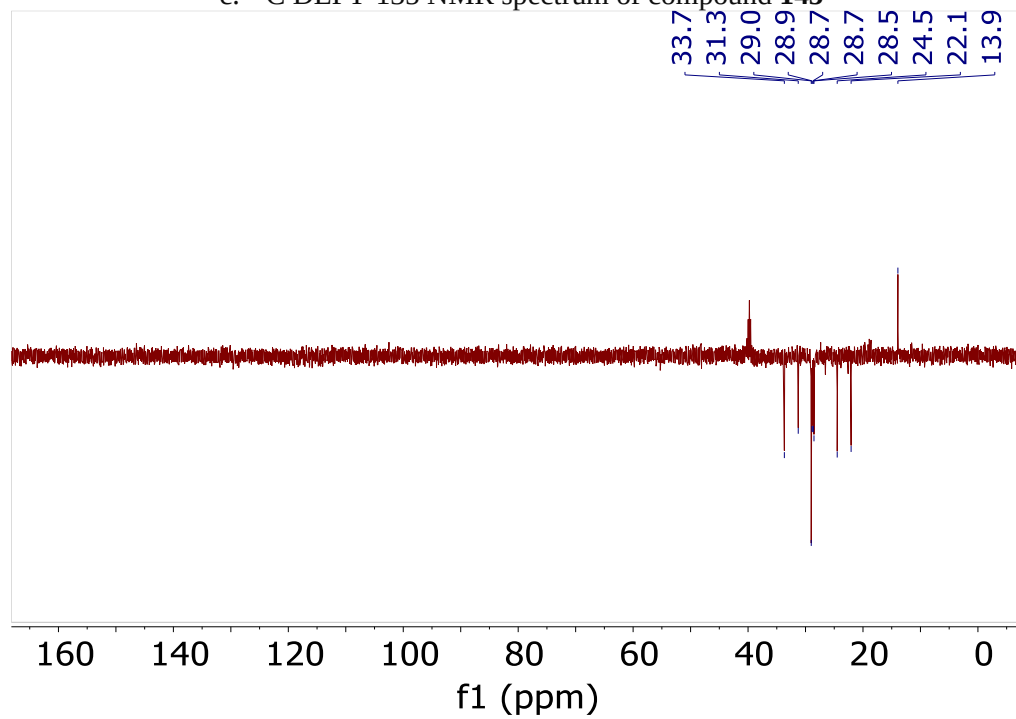
a. ^1H -NMR spectrum of compound **145**



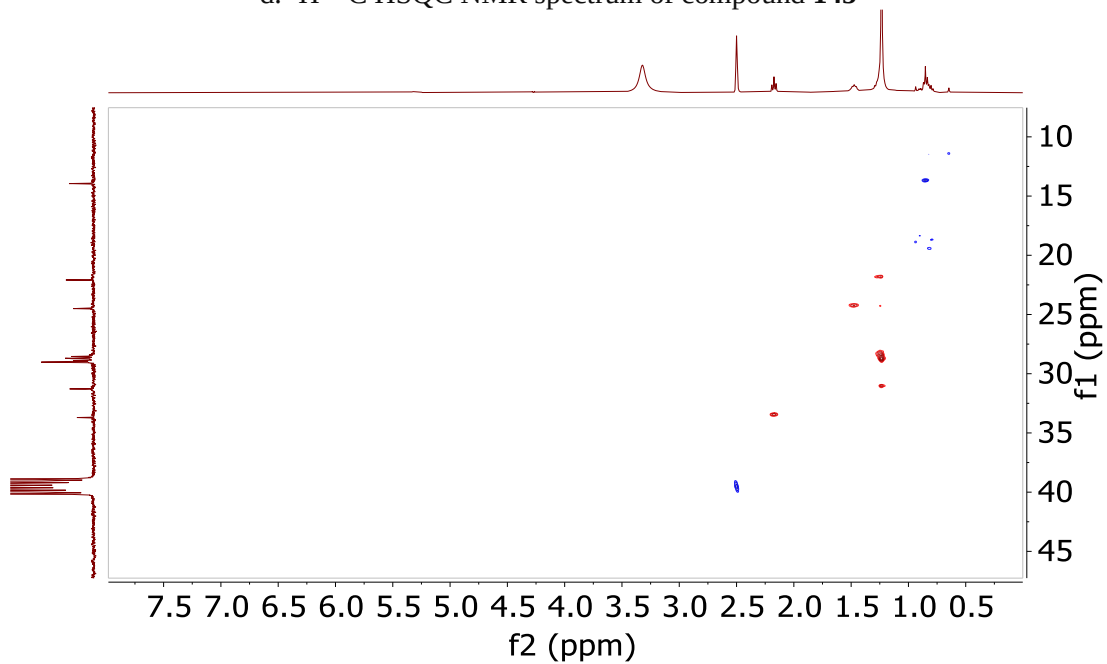
b. The ^{13}C -NMR spectrum of compound **145**



c. ^{13}C DEPT-135 NMR spectrum of compound **145**

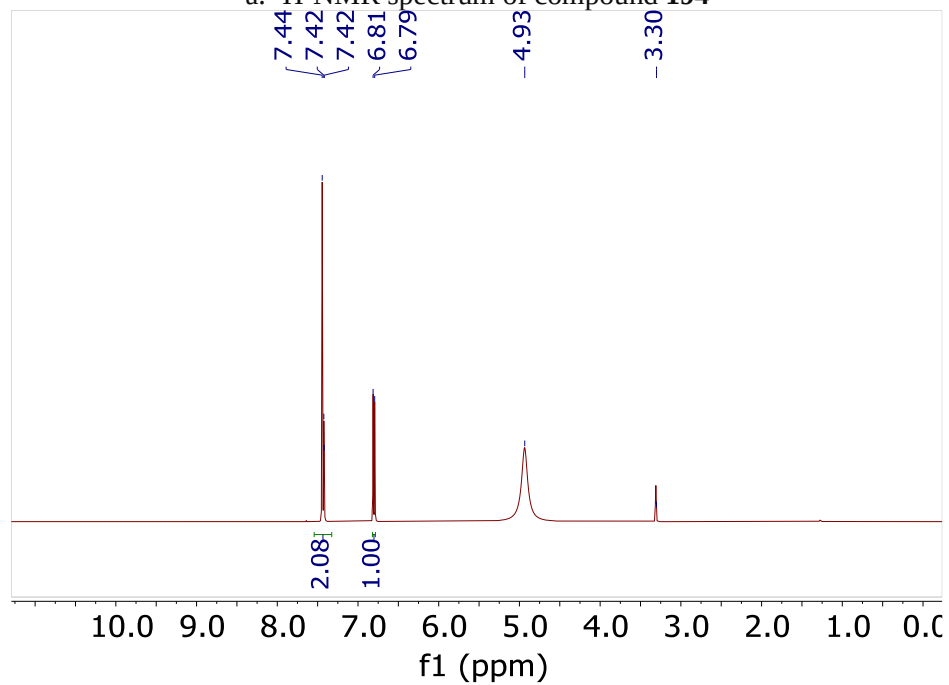


d. ^1H - ^{13}C HSQC NMR spectrum of compound **145**

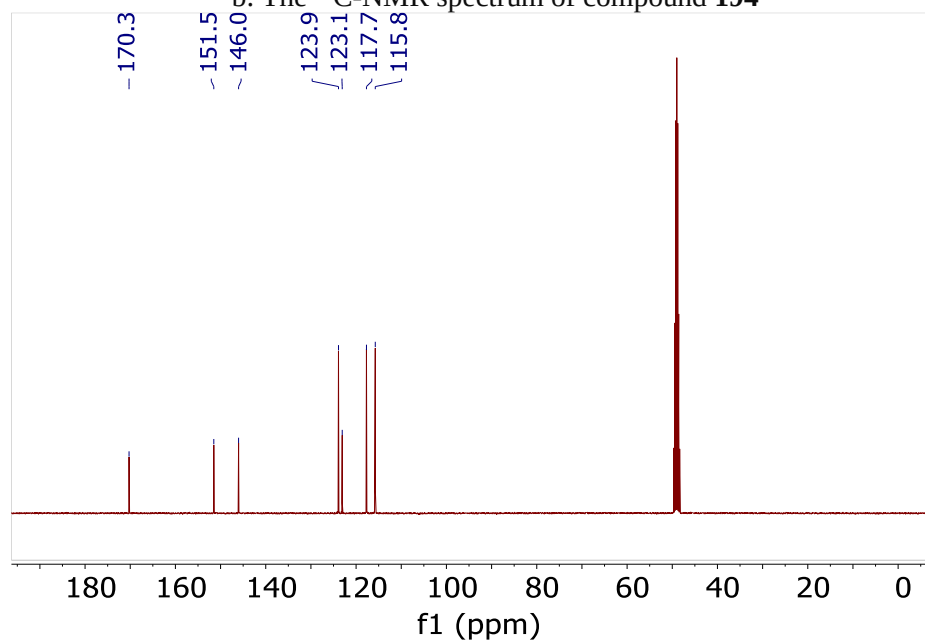


Appendix 13. NMR spectral data of compounds **194**

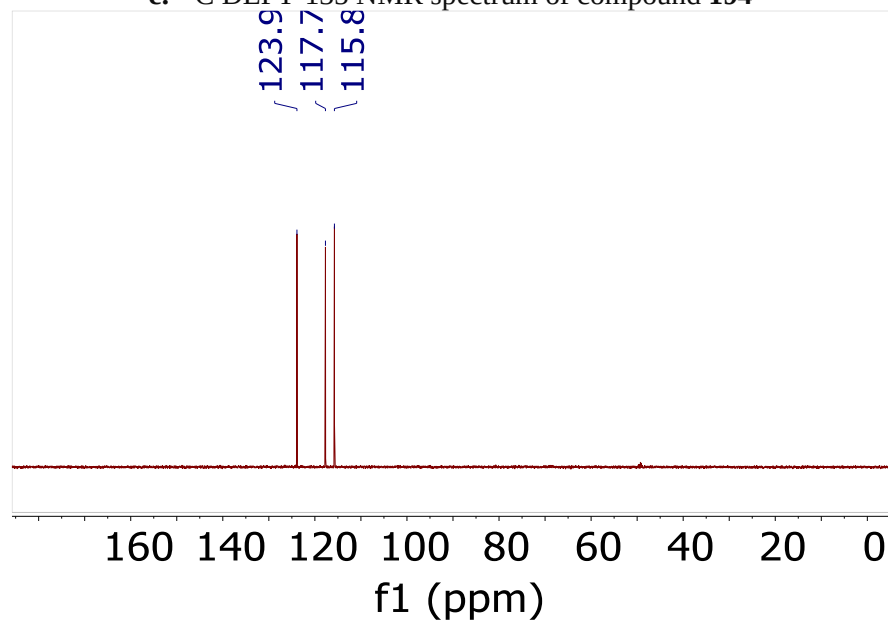
a. ^1H -NMR spectrum of compound **194**



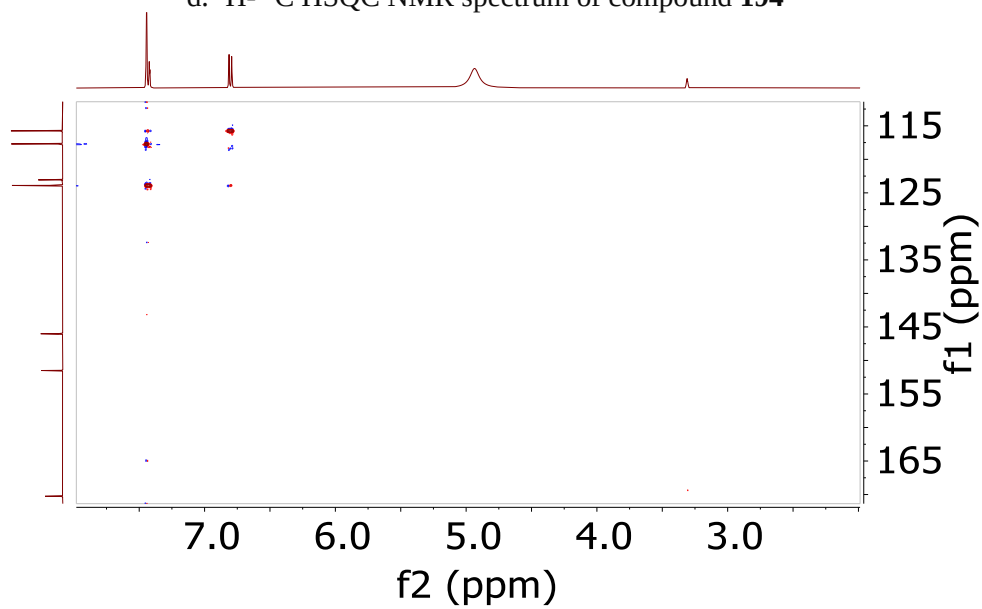
b. The ^{13}C -NMR spectrum of compound **194**



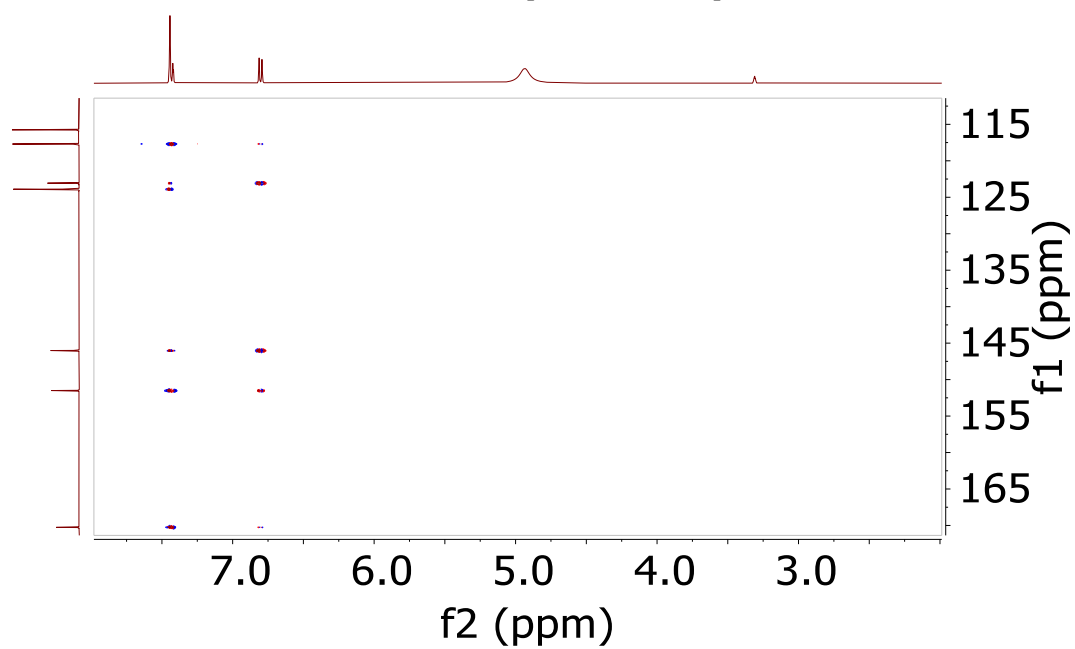
c. ^{13}C DEPT-135 NMR spectrum of compound **194**



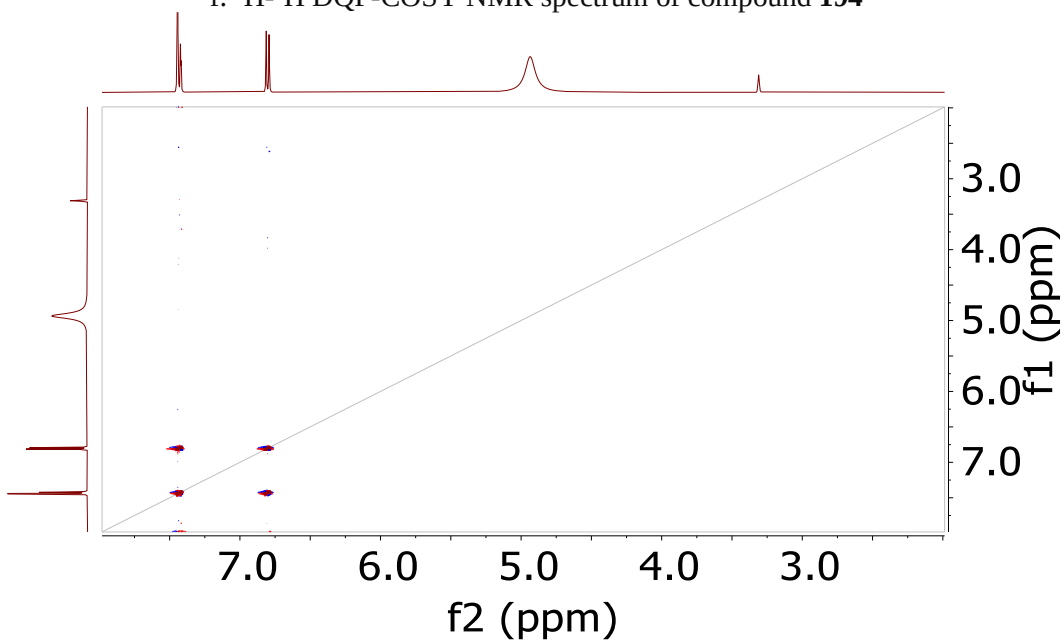
d. ^1H - ^{13}C HSQC NMR spectrum of compound **194**



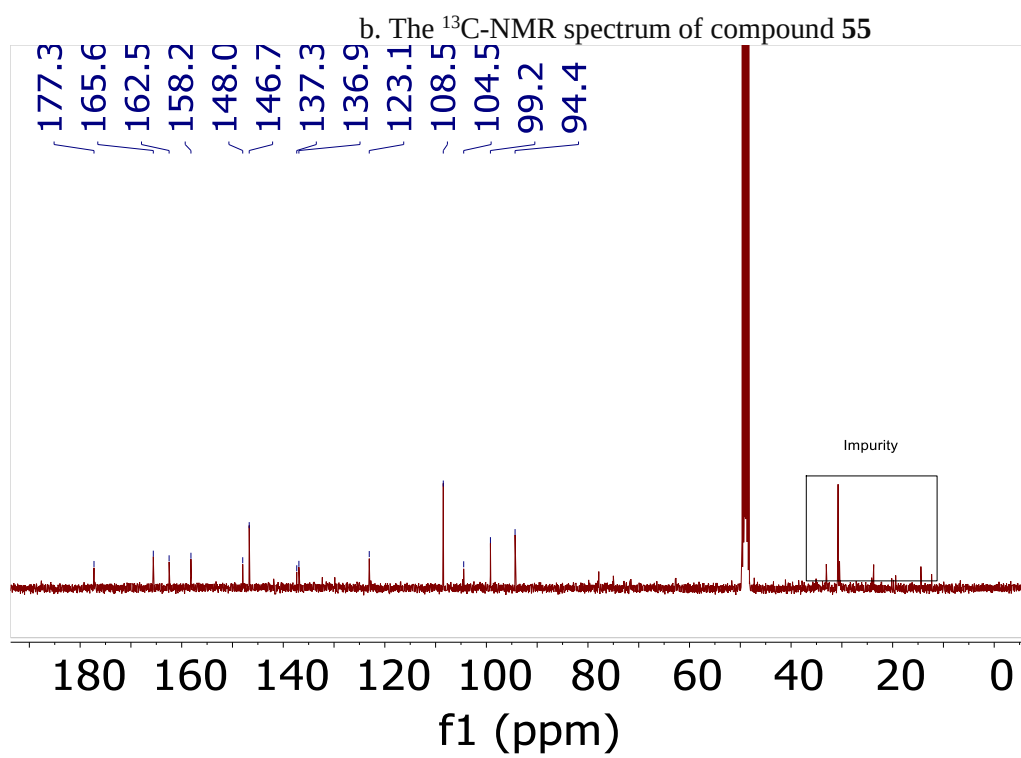
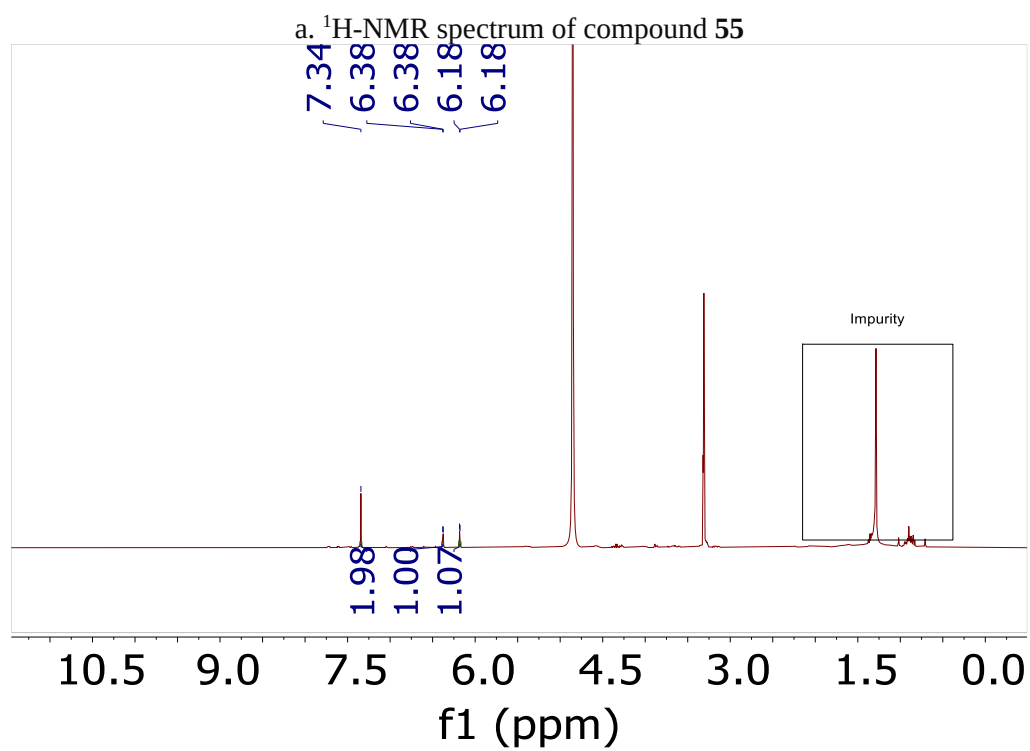
e. ^1H - ^{13}C HMBC NMR spectrum of compound **194**



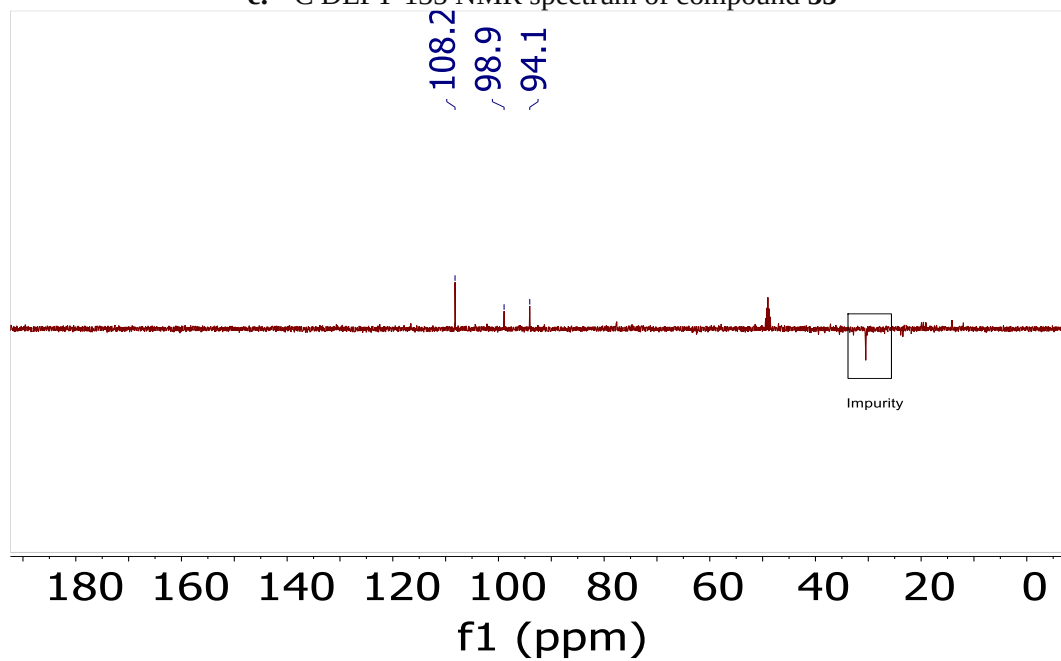
f. ^1H - ^1H DQF-COSY NMR spectrum of compound **194**



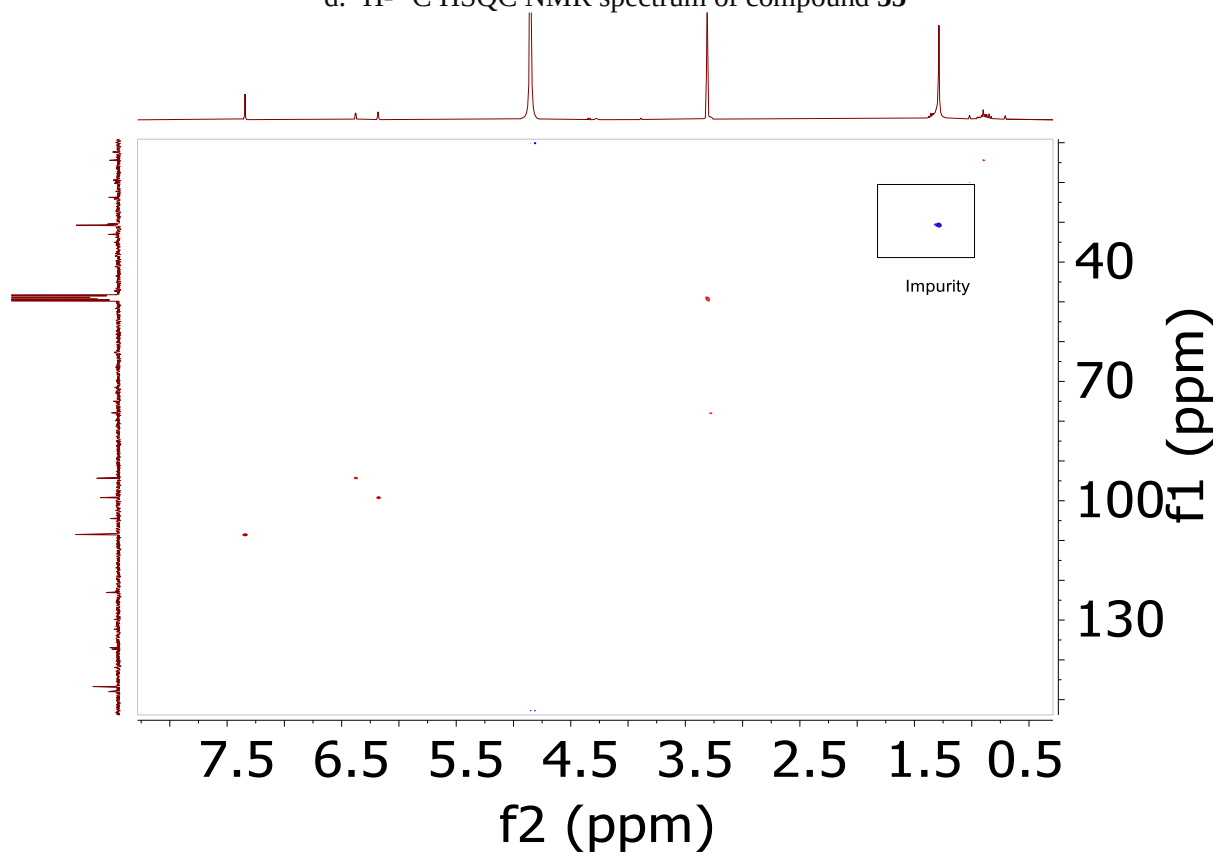
Appendix 14. NMR spectral data of compounds 55



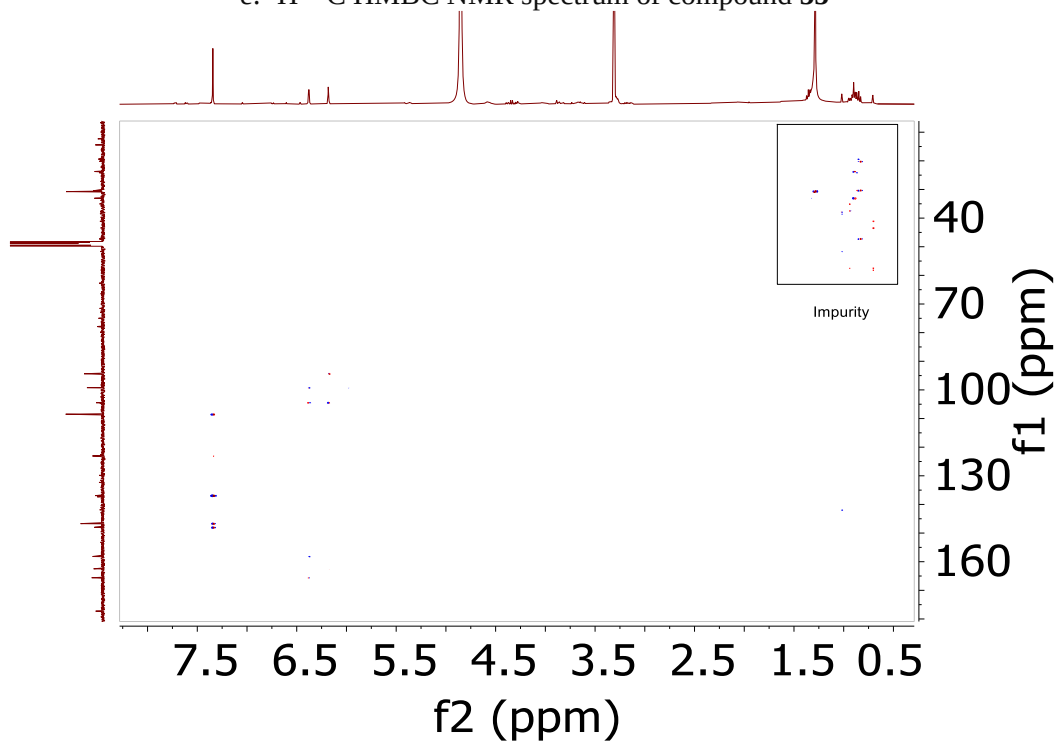
c. ^{13}C DEPT-135 NMR spectrum of compound 55



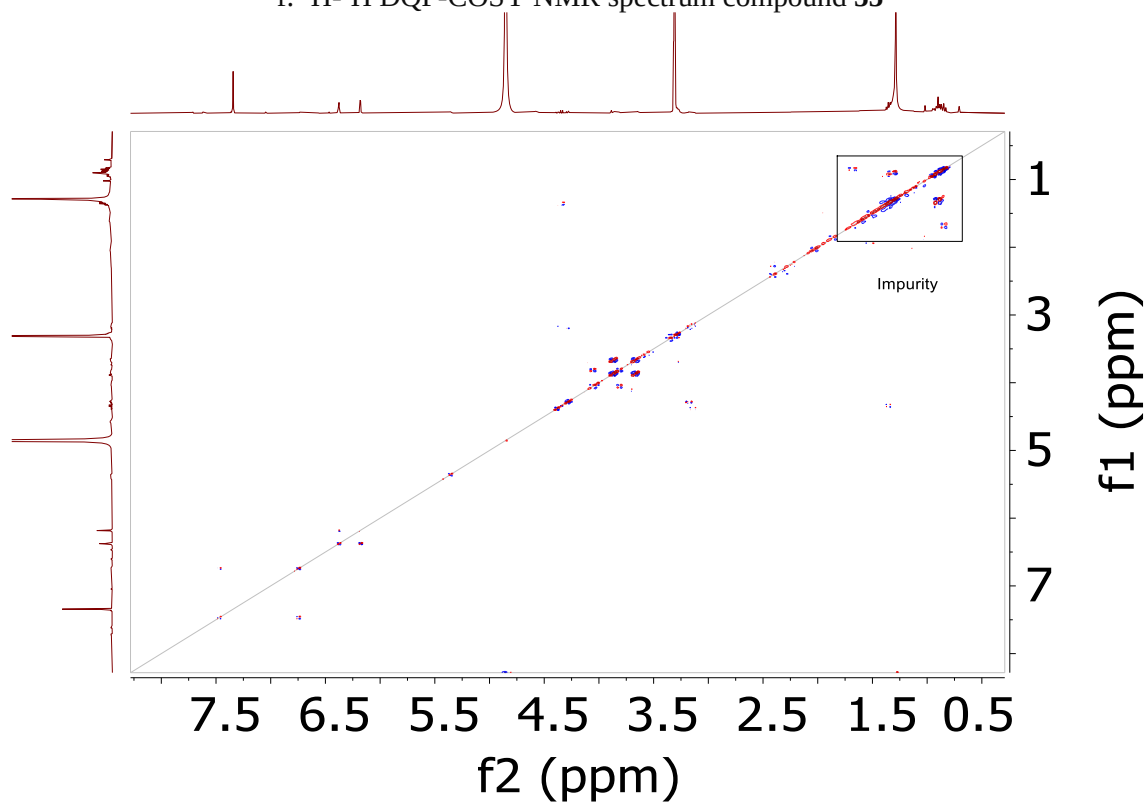
d. ^1H - ^{13}C HSQC NMR spectrum of compound 55



e. ^1H - ^{13}C HMBC NMR spectrum of compound 55

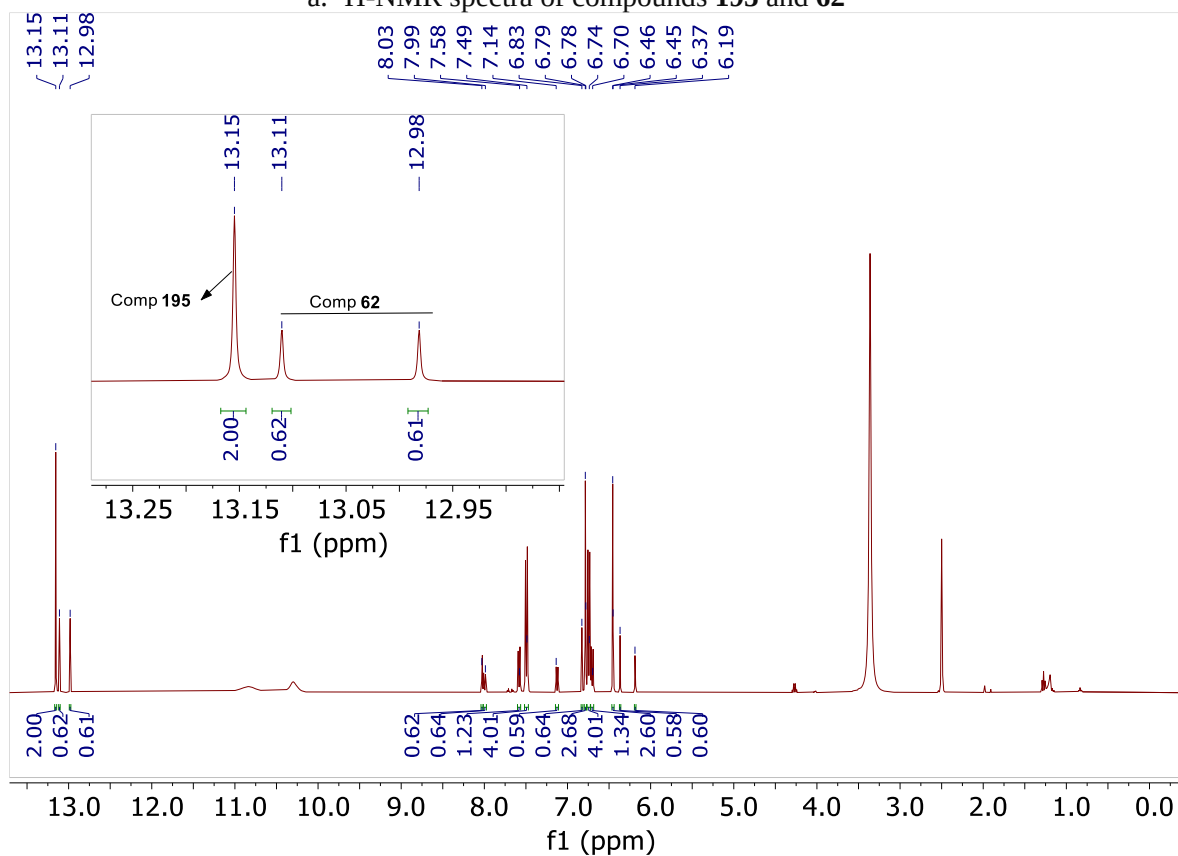


f. ^1H - ^1H DQF-COSY NMR spectrum compound 55

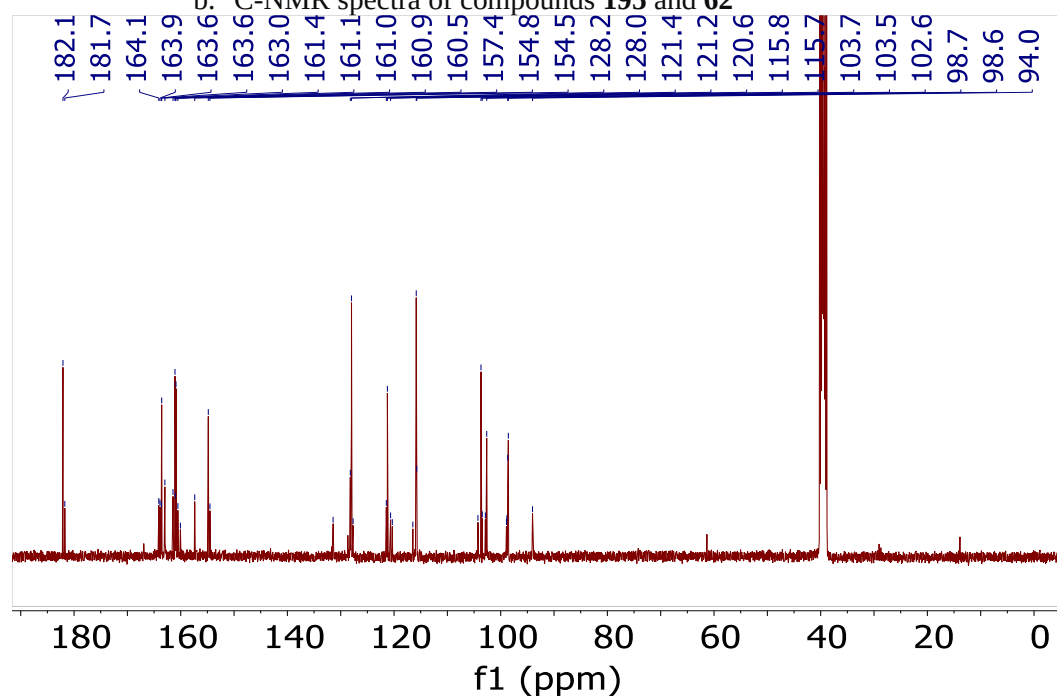


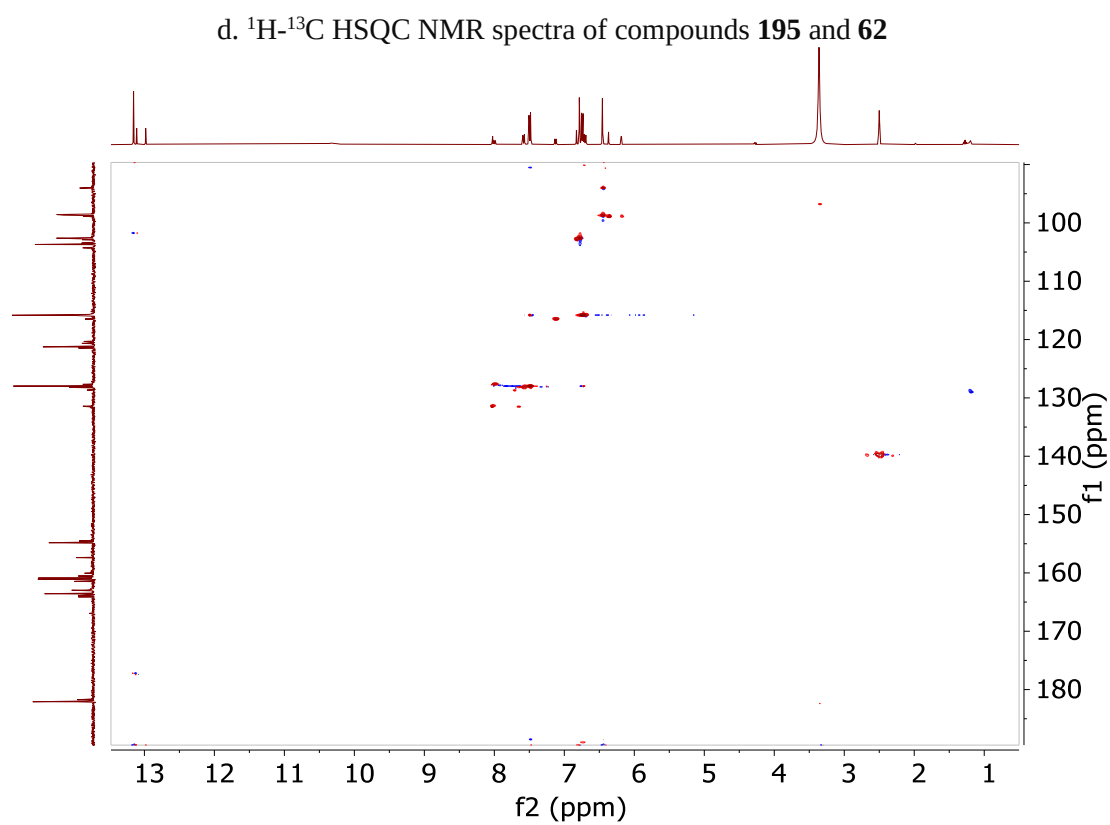
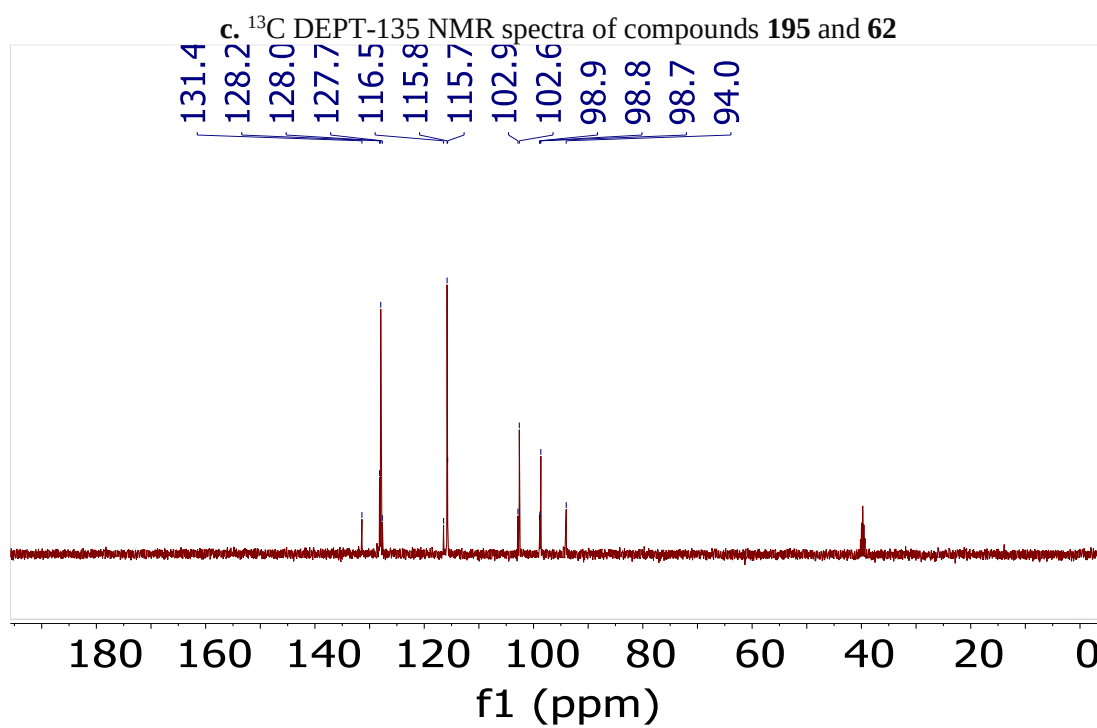
Appendix 15. NMR spectral data of compounds **195** and **62**

a. ^1H -NMR spectra of compounds **195** and **62**

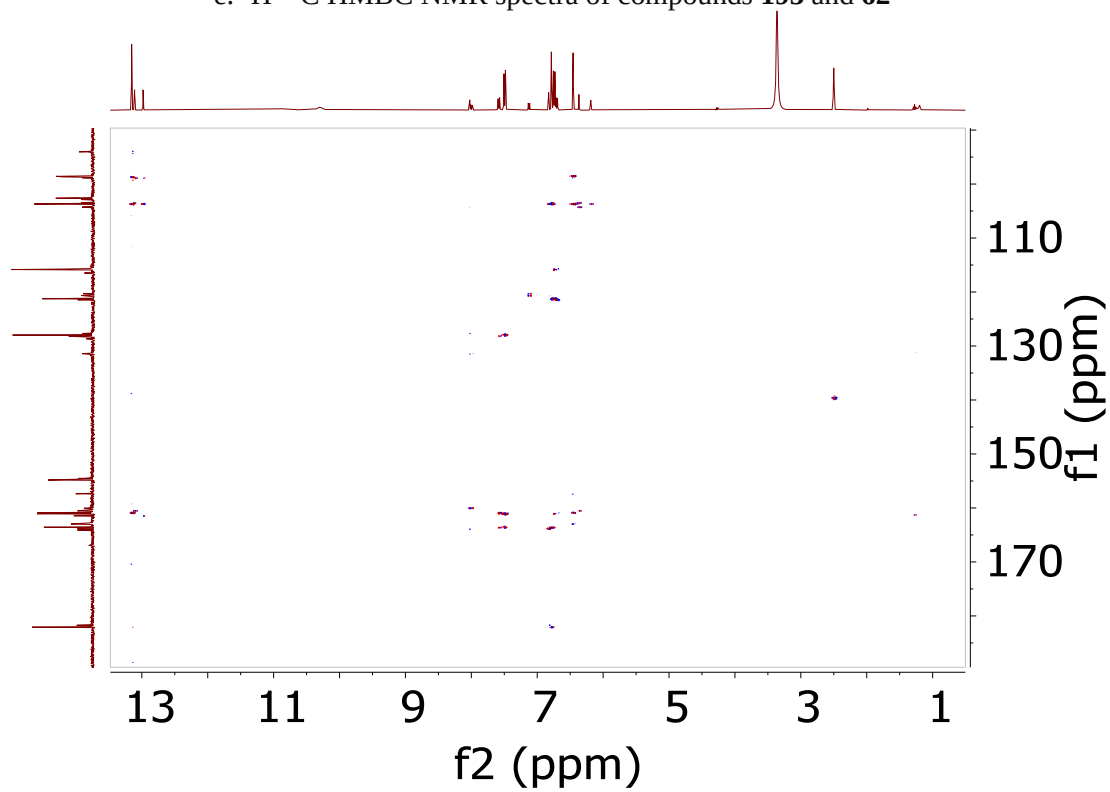


b. ^{13}C -NMR spectra of compounds **195** and **62**

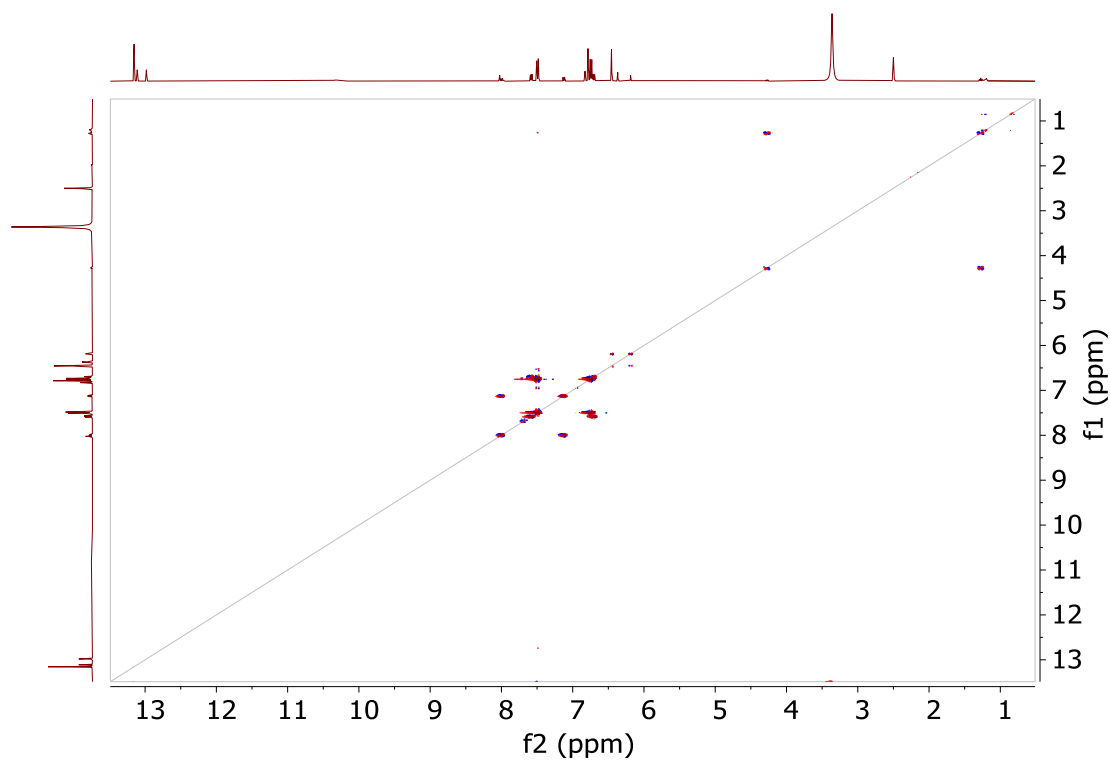




e. ^1H - ^{13}C HMBC NMR spectra of compounds **195** and **62**

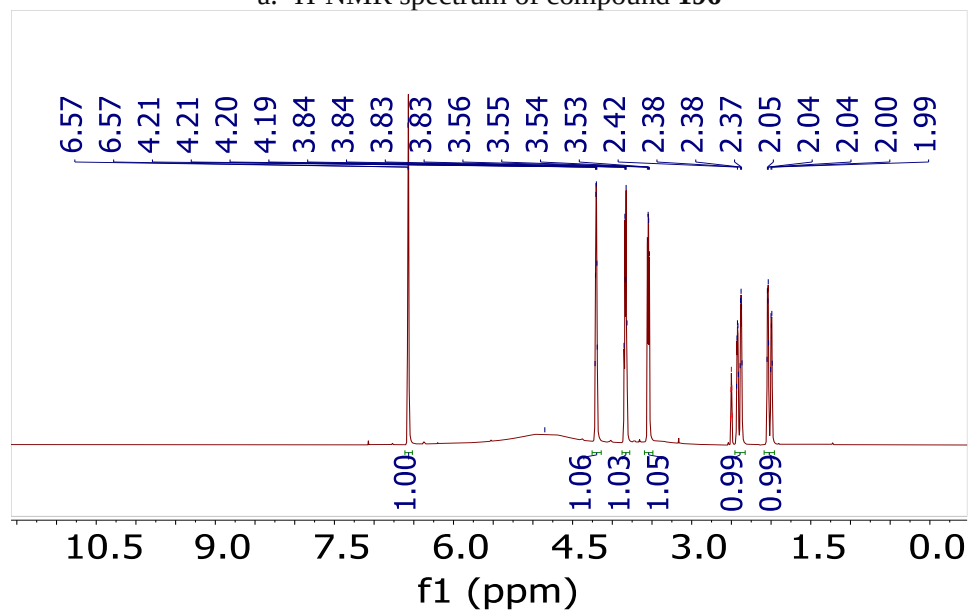


f. ^1H - ^1H DQF-COSY NMR spectra of compounds **195** and **62**

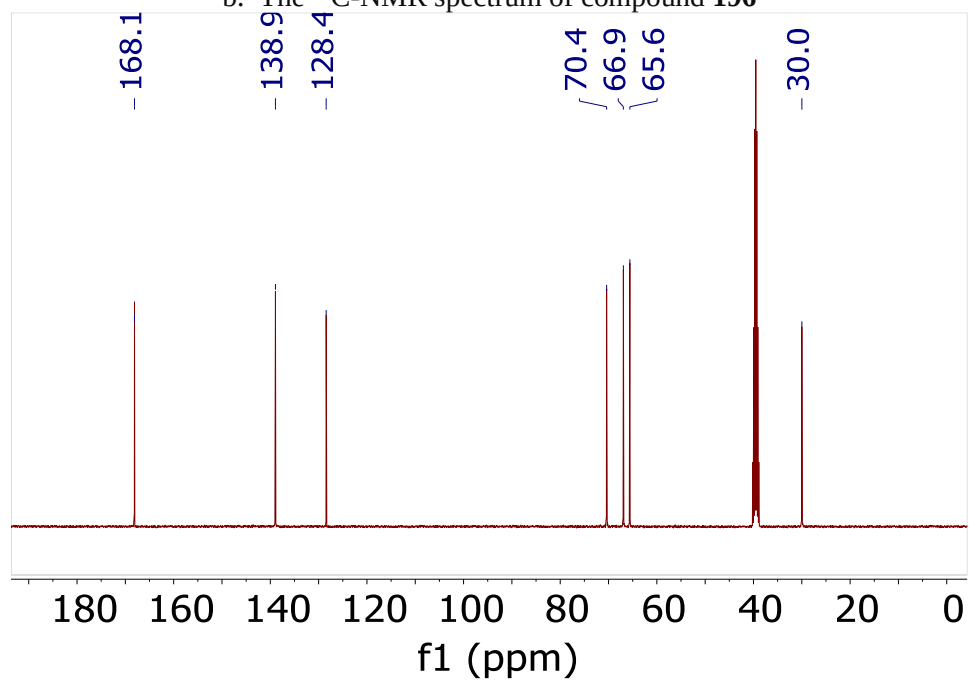


Appendix 16. NMR spectral data of compound **196**

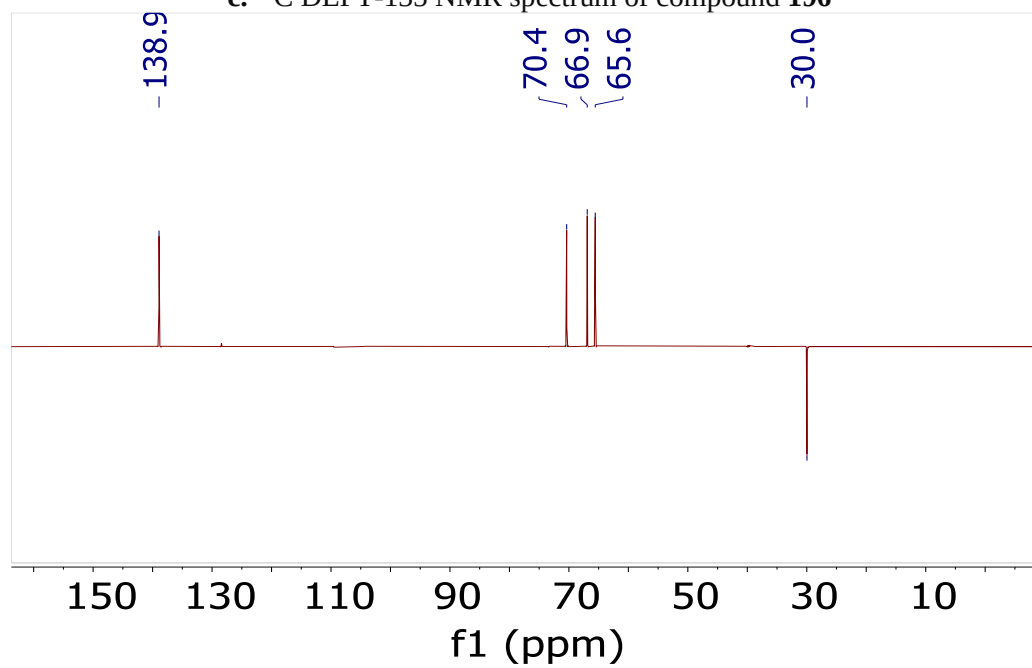
a. ^1H -NMR spectrum of compound **196**



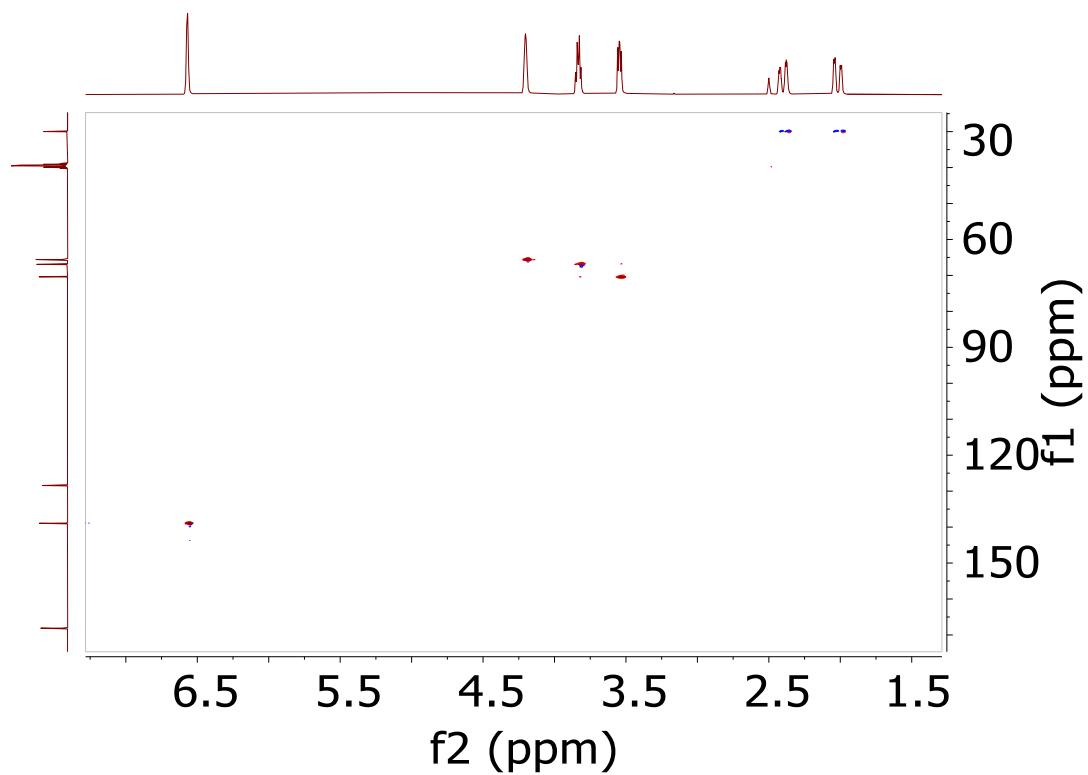
b. The ^{13}C -NMR spectrum of compound **196**



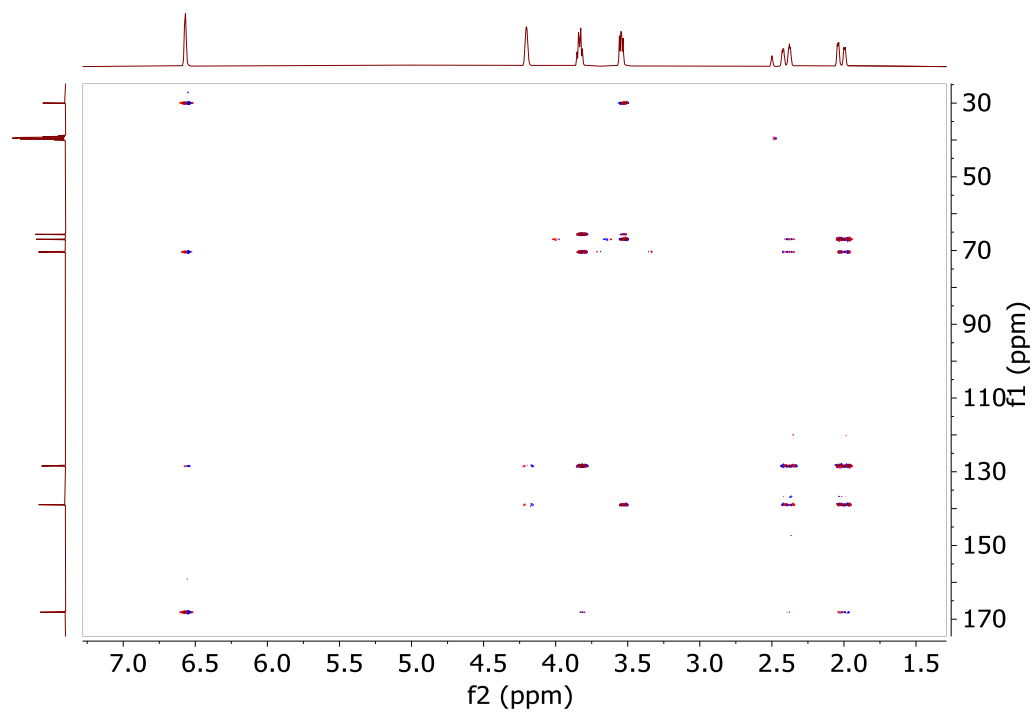
c. ^{13}C DEPT-135 NMR spectrum of compound **196**



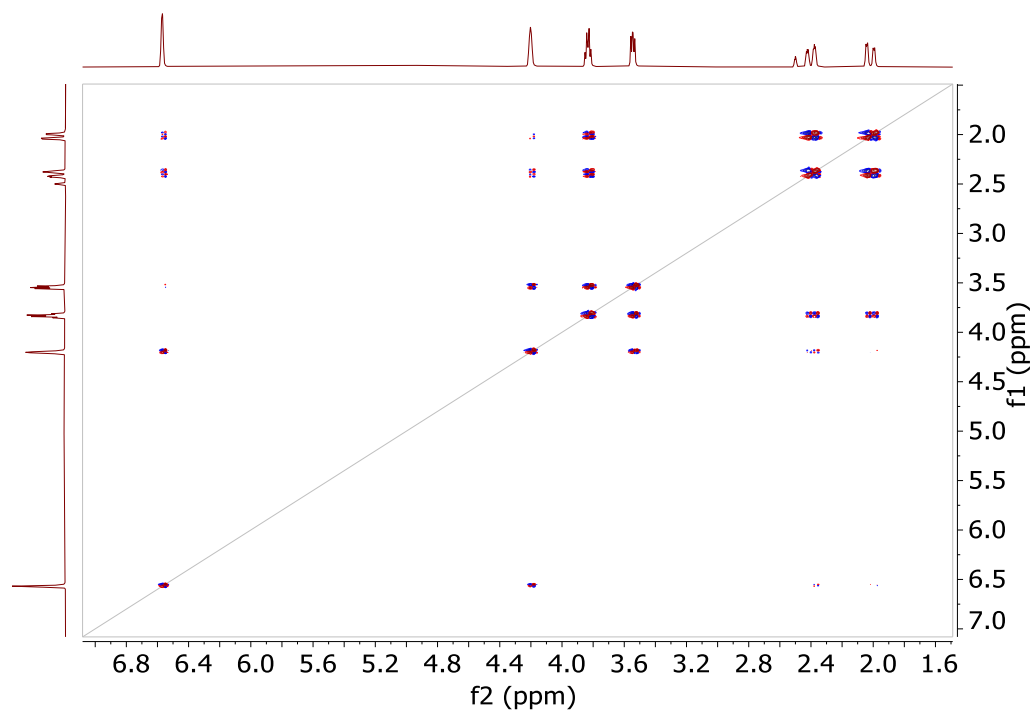
d. ^1H - ^{13}C HSQC NMR spectrum of compound **196**



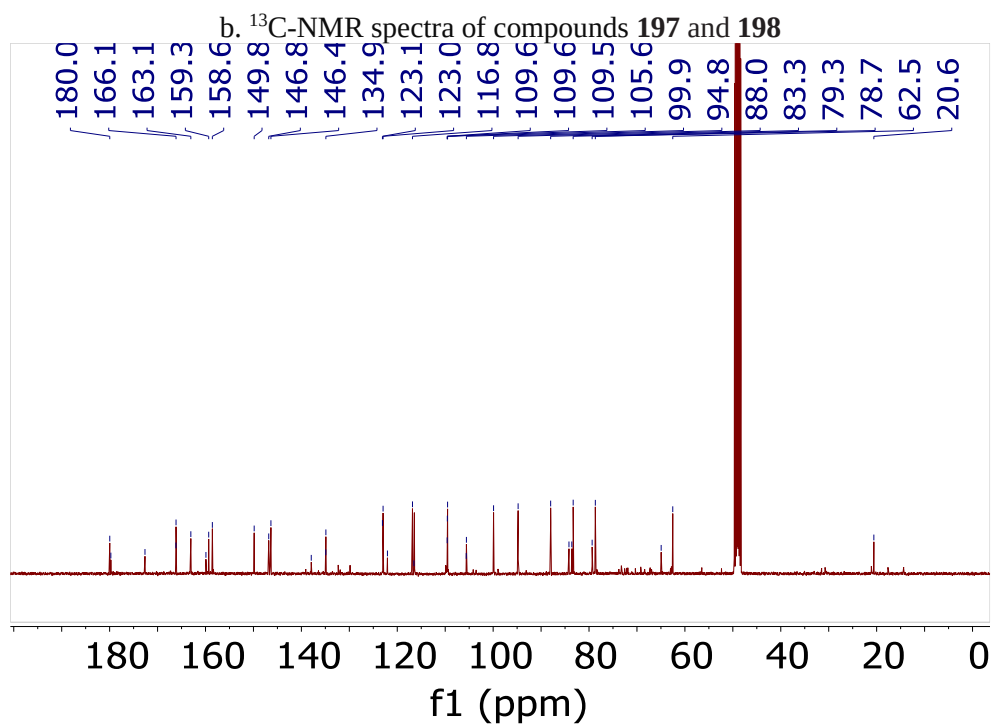
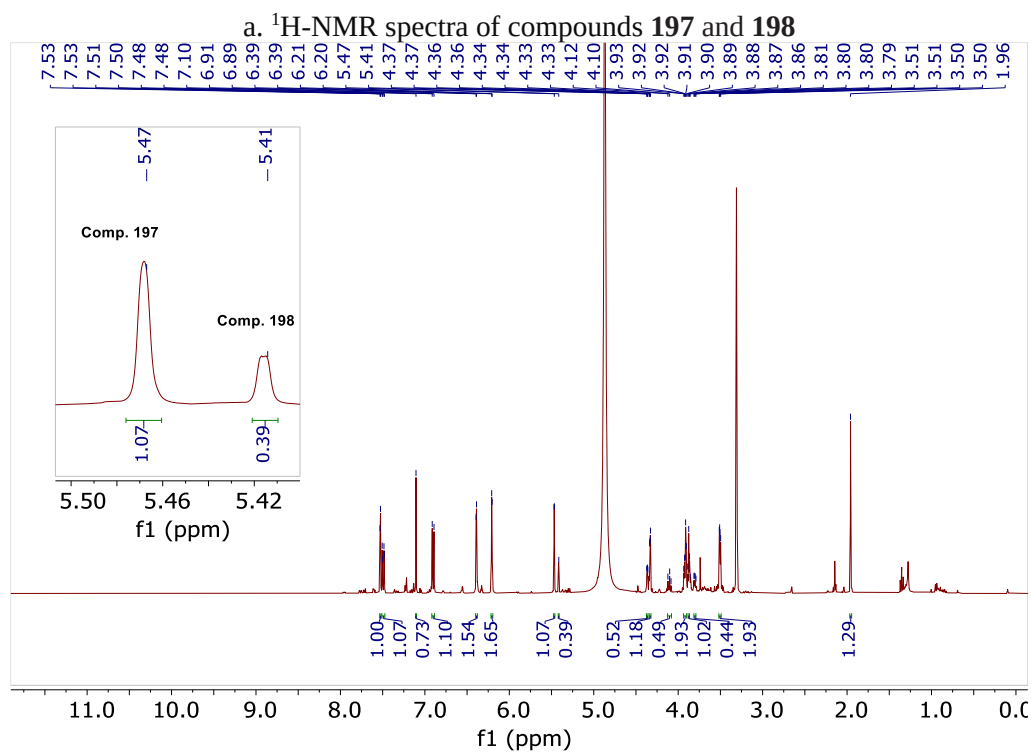
e. ^1H - ^{13}C HMBC NMR spectrum of compound **196**



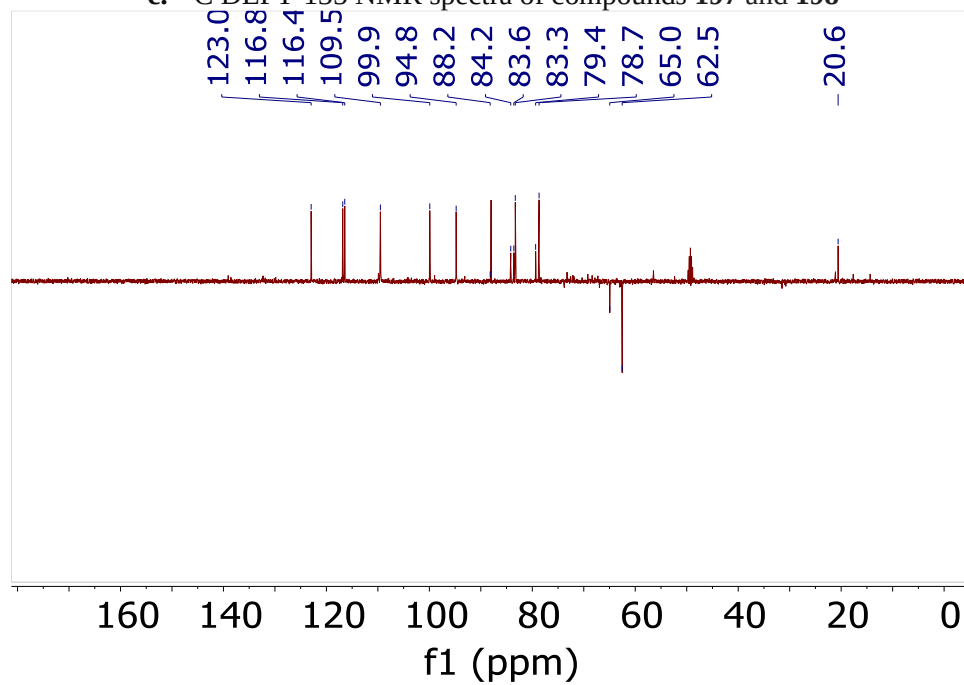
f. ^1H - ^1H DQF-COSY NMR spectrum compound **196**



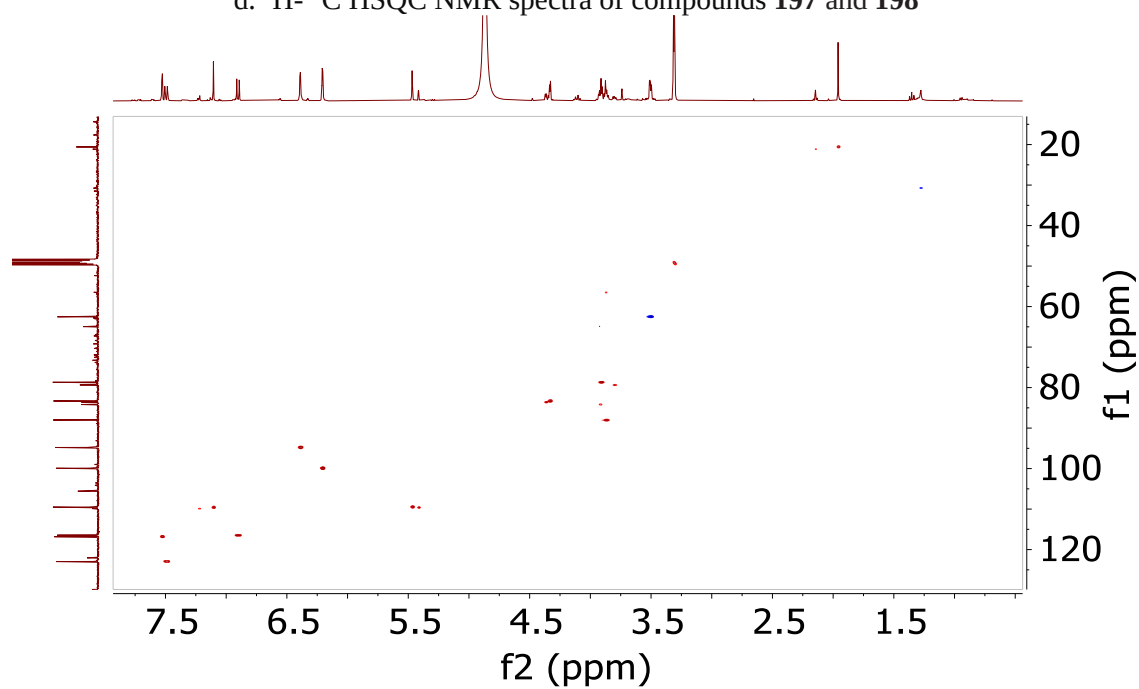
Appendix 17. NMR spectral data of compounds **197** and **198**



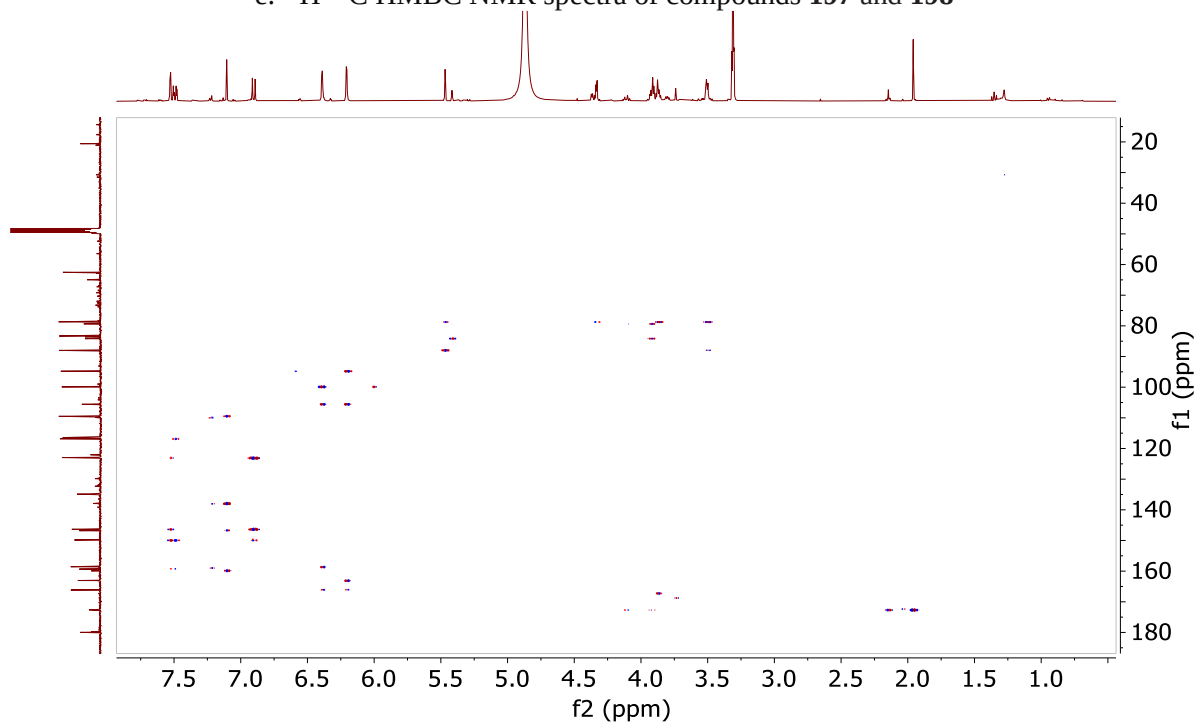
c. ^{13}C DEPT-135 NMR spectra of compounds **197** and **198**



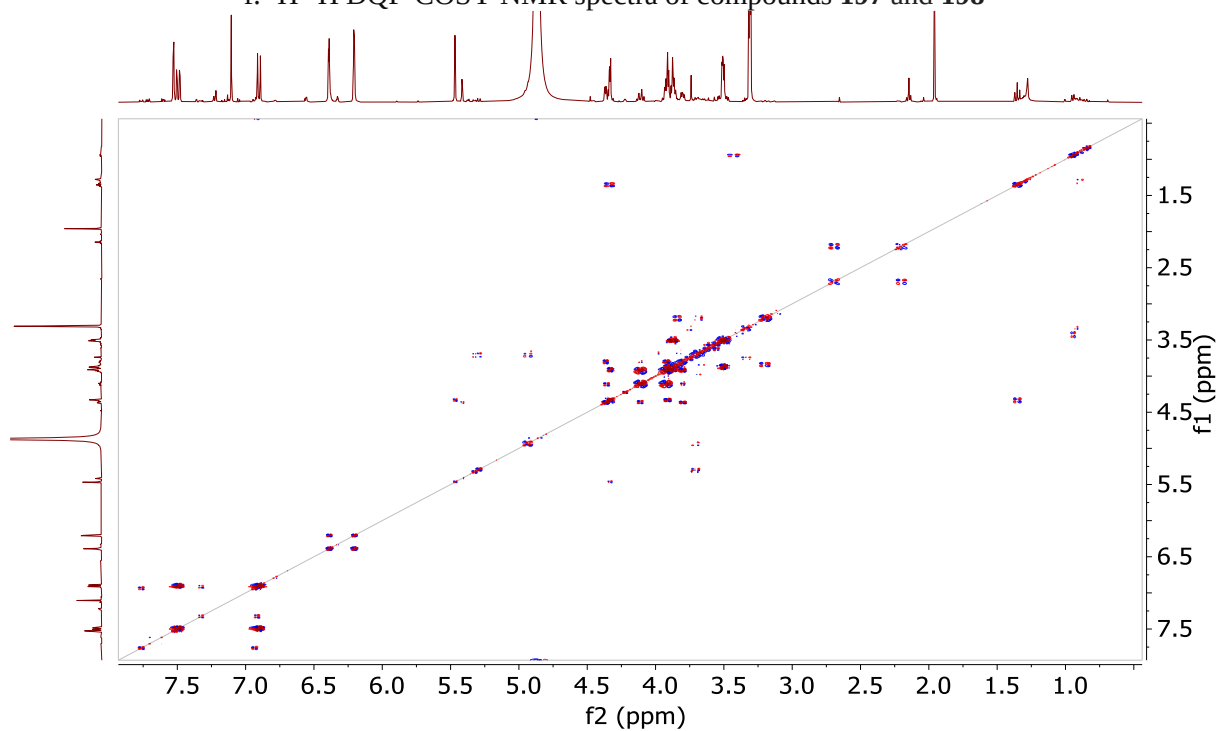
d. ^1H - ^{13}C HSQC NMR spectra of compounds **197** and **198**



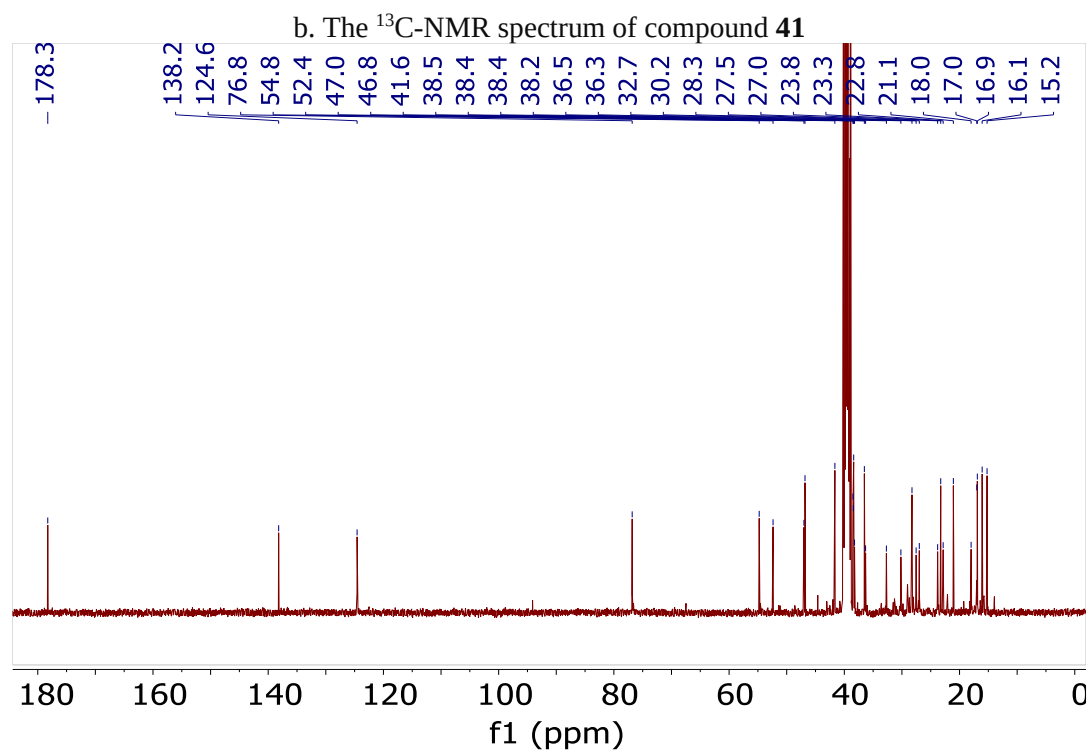
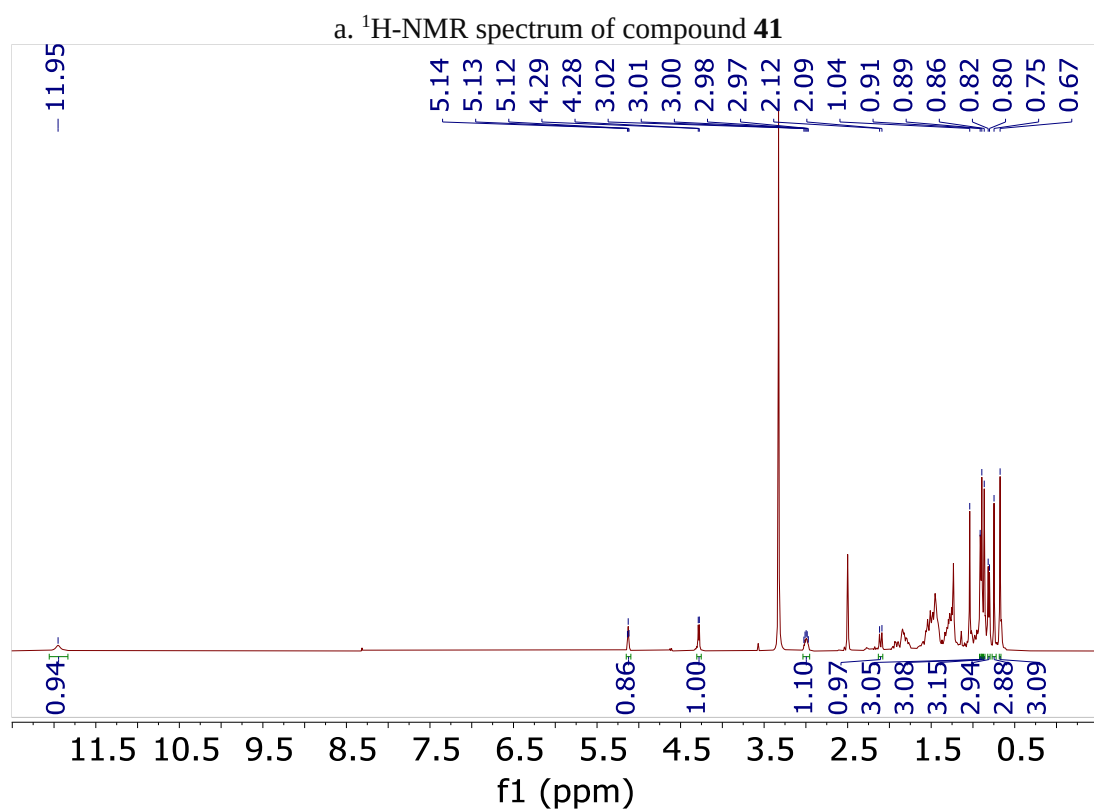
e. ^1H - ^{13}C HMBC NMR spectra of compounds **197** and **198**



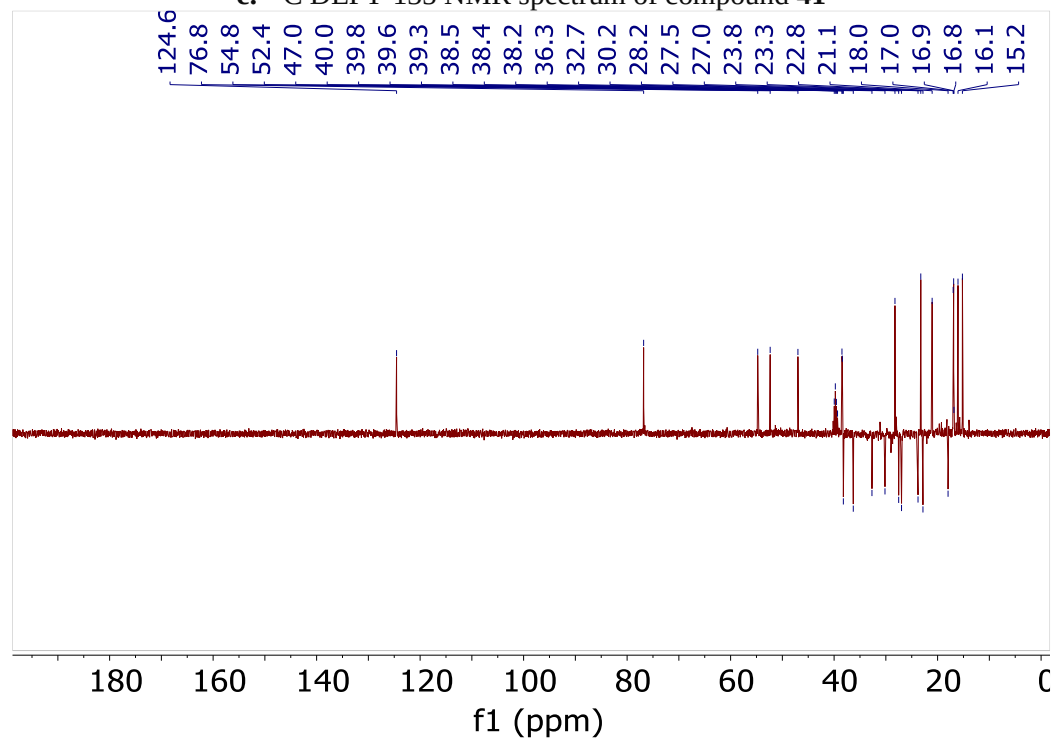
f. ^1H - ^1H DQF-COSY NMR spectra of compounds **197** and **198**



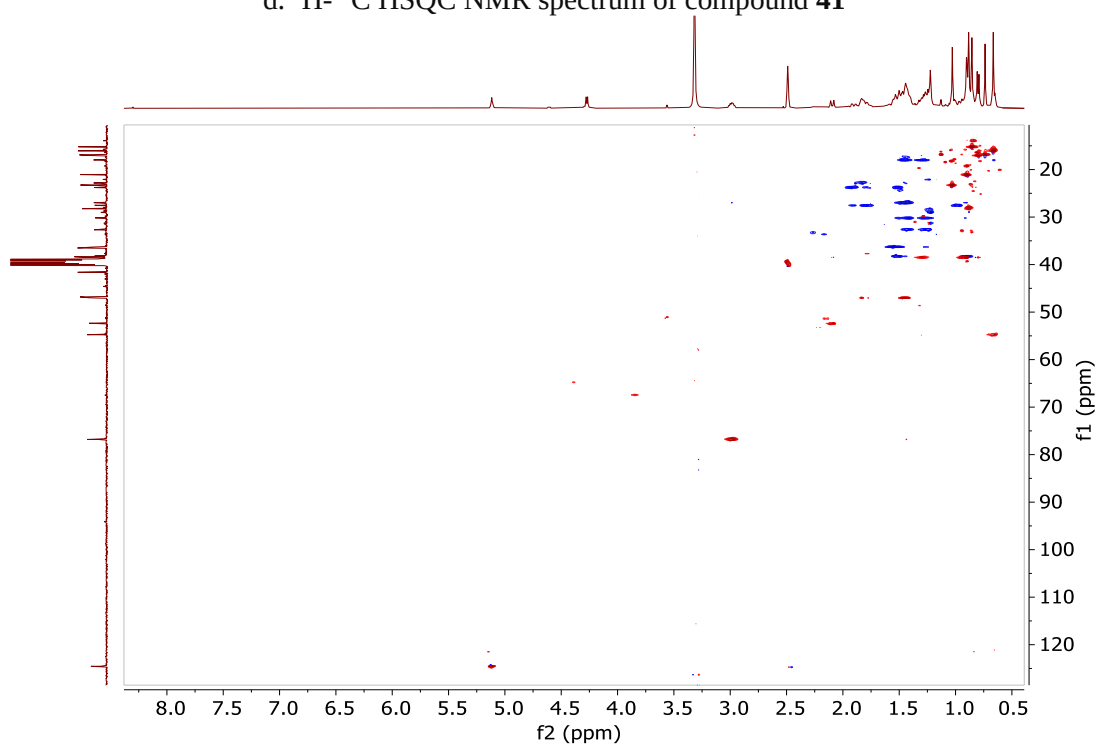
Appendix 18. NMR spectral data of compound **41**



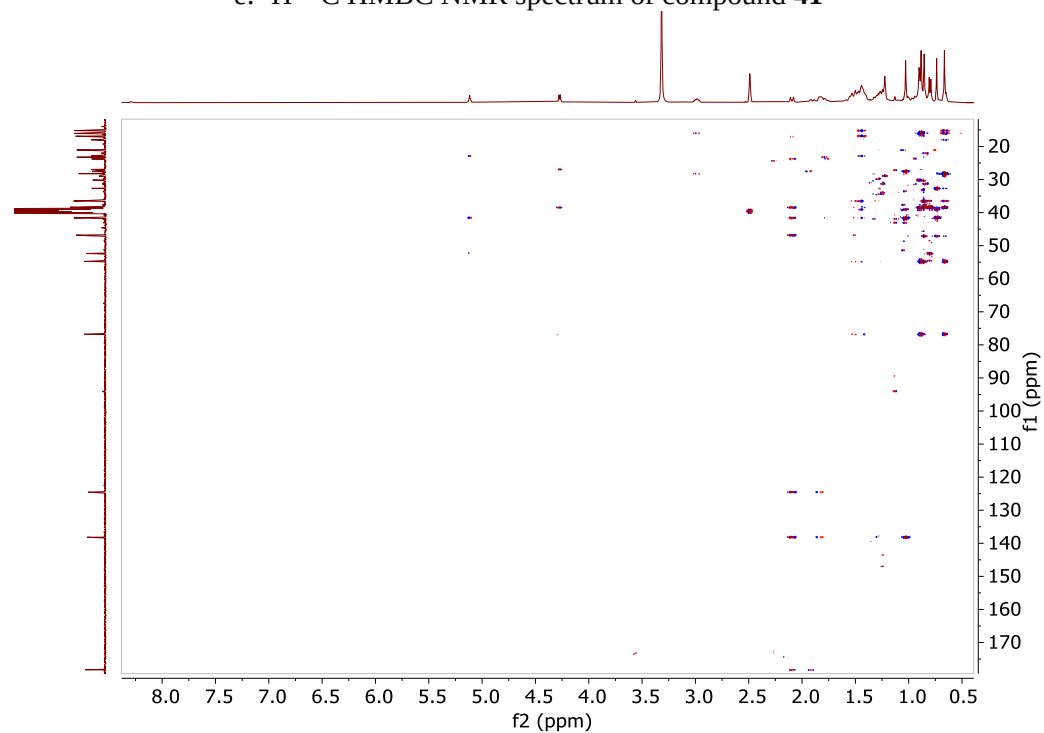
c. ^{13}C DEPT-135 NMR spectrum of compound **41**



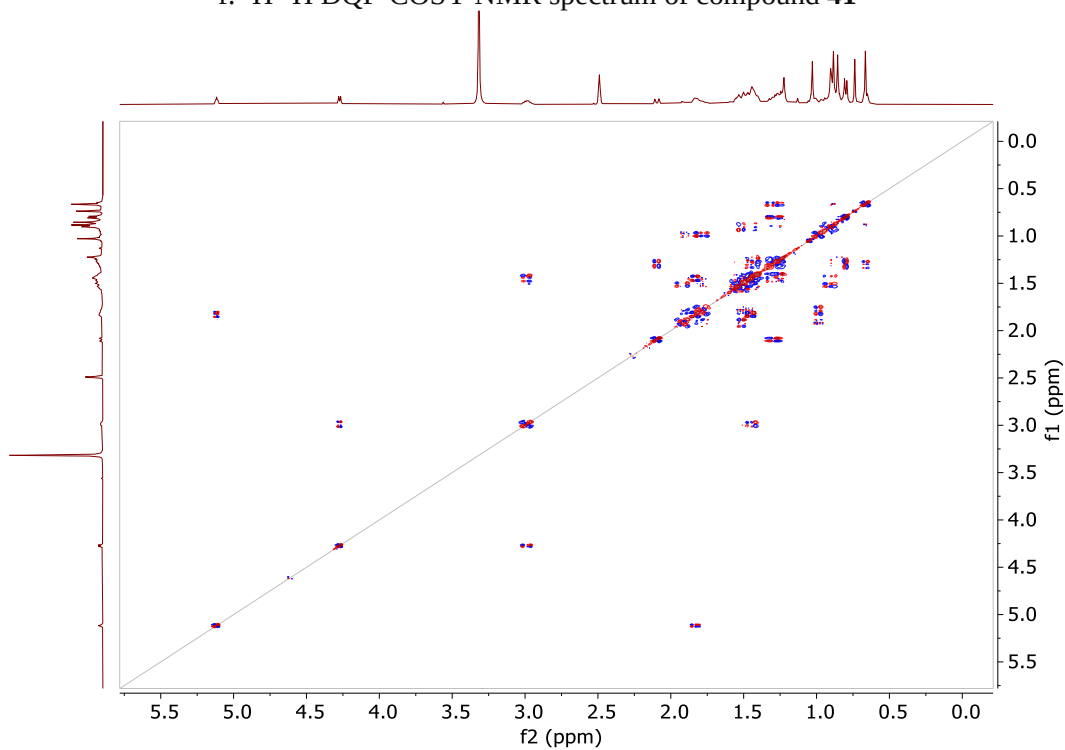
d. ^1H - ^{13}C HSQC NMR spectrum of compound **41**



e. ^1H - ^{13}C HMBC NMR spectrum of compound **41**

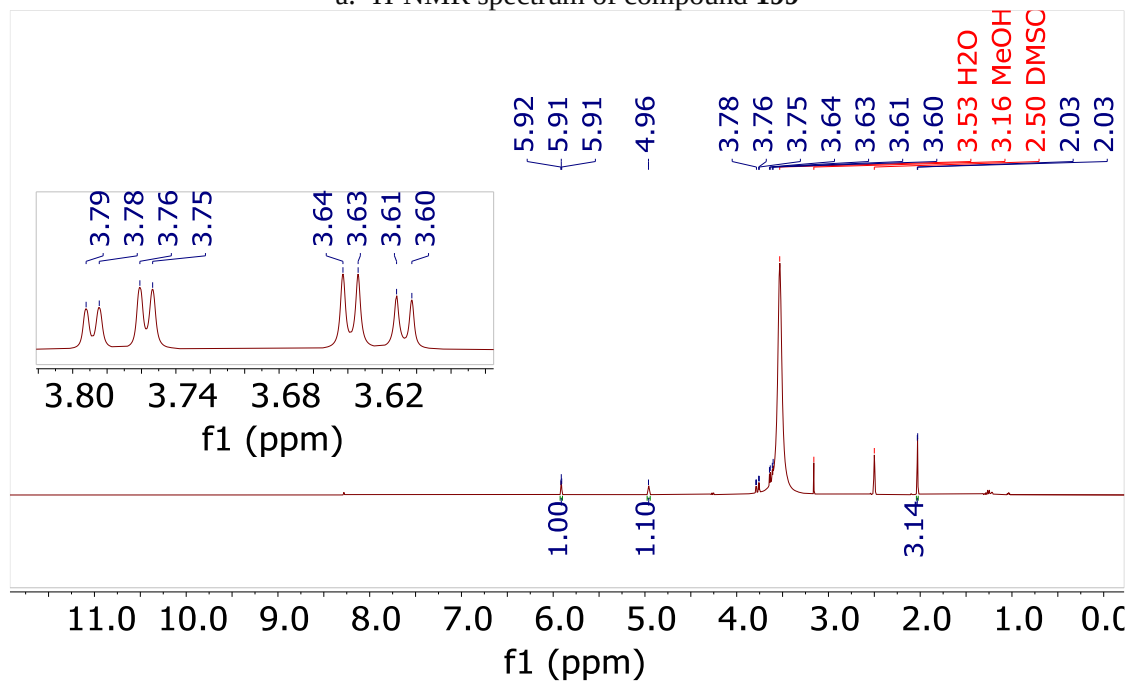


f. ^1H - ^1H DQF-COSY NMR spectrum of compound **41**

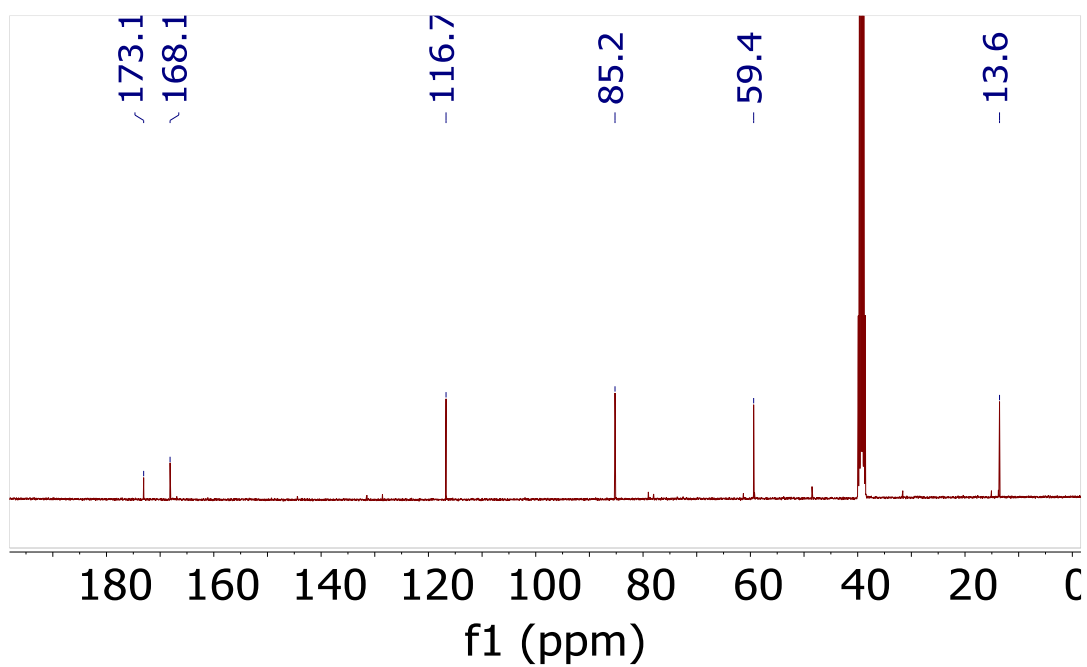


Appendix 19. NMR spectral data of compounds **199**

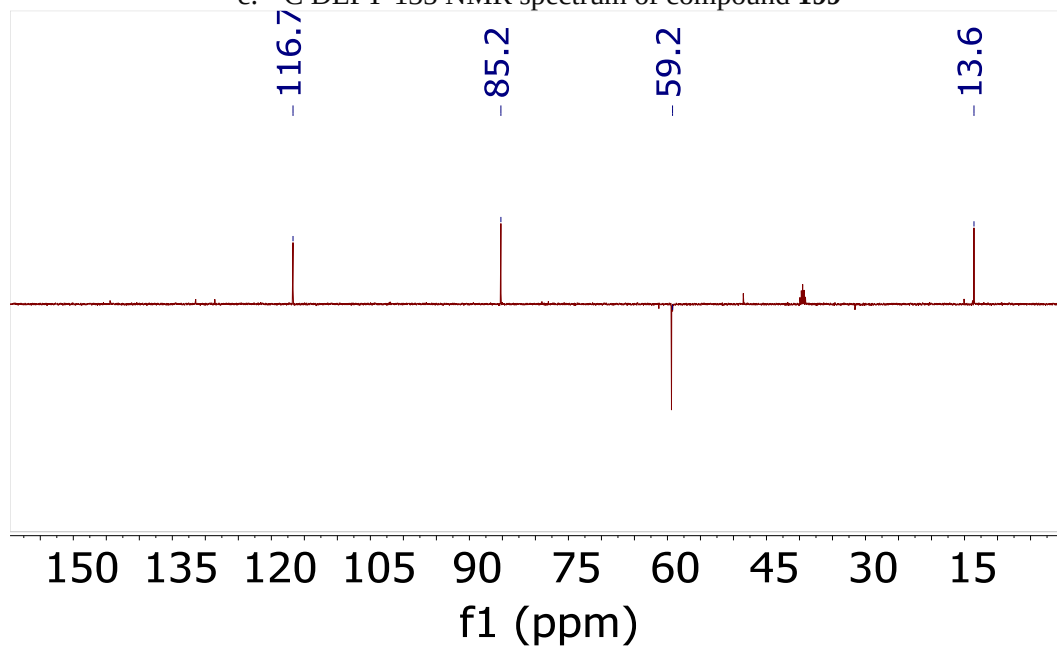
a. ^1H -NMR spectrum of compound **199**



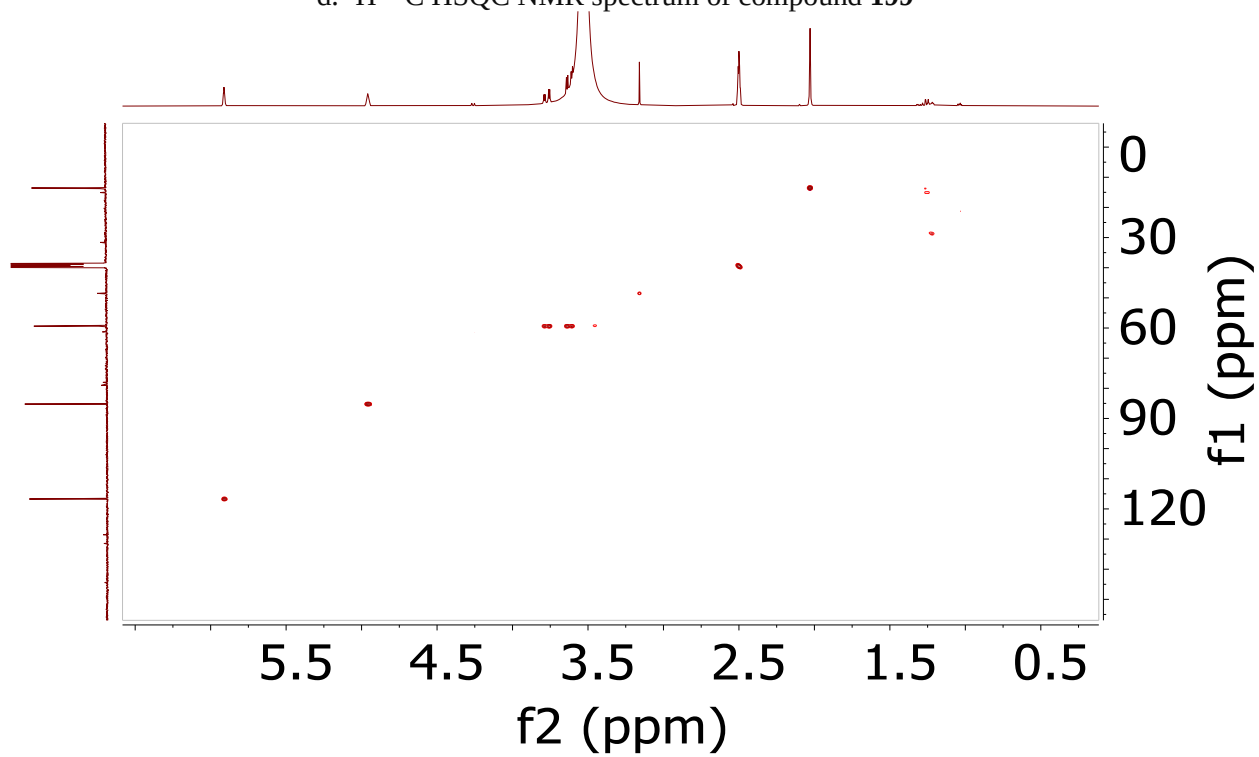
b. The ^{13}C -NMR spectrum of compound **199**



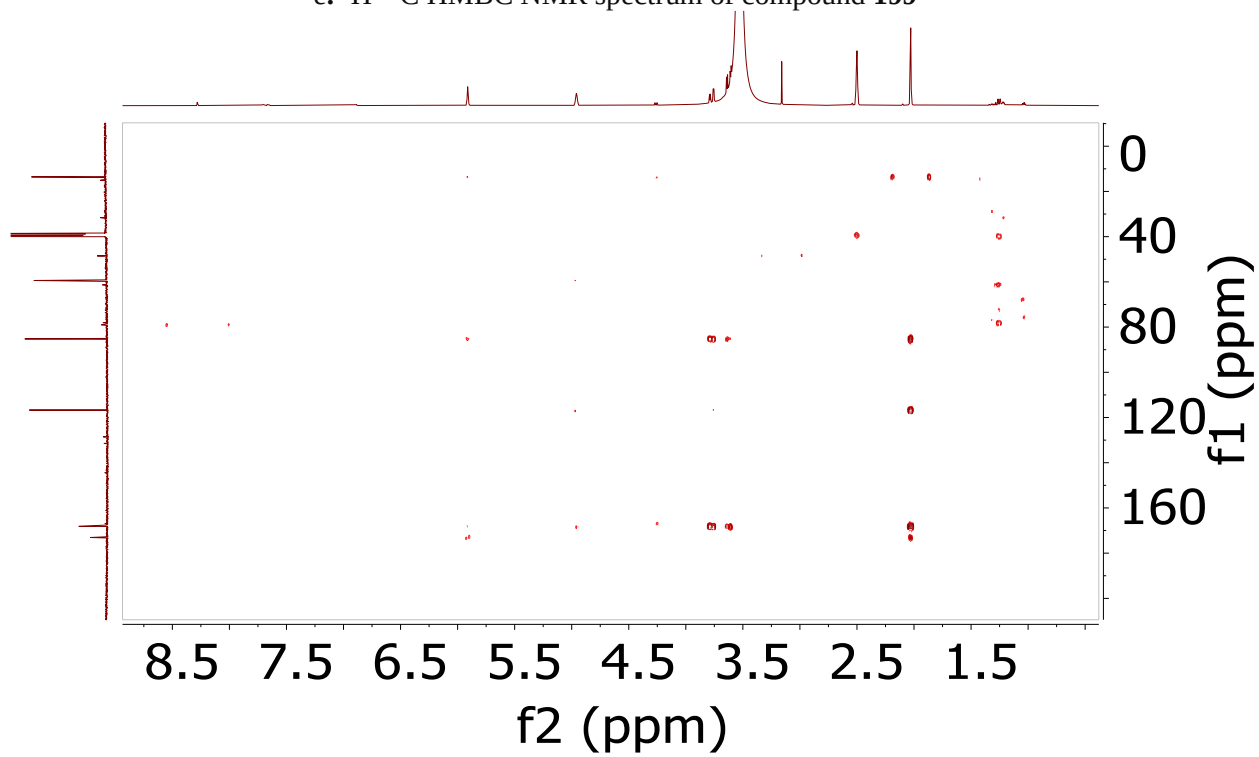
c. ^{13}C DEPT-135 NMR spectrum of compound **199**



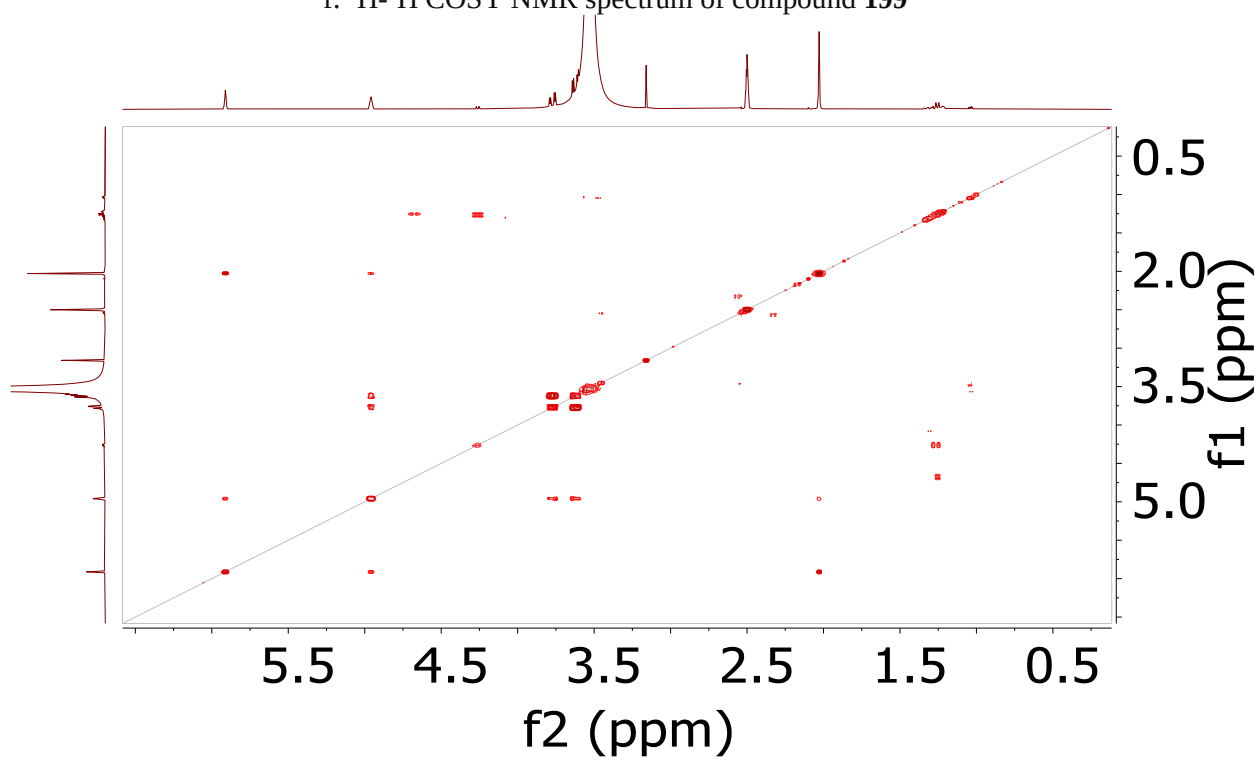
d. ^1H - ^{13}C HSQC NMR spectrum of compound **199**



e. ^1H - ^{13}C HMBC NMR spectrum of compound **199**

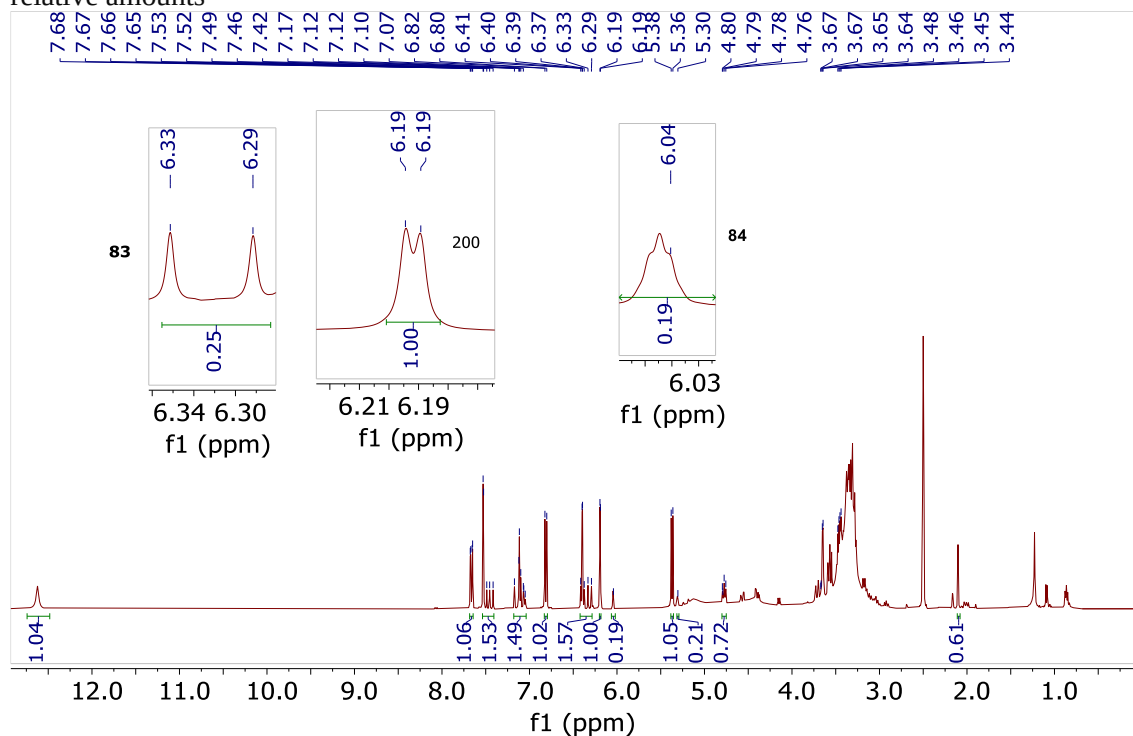


f. ^1H - ^1H COSY NMR spectrum of compound **199**

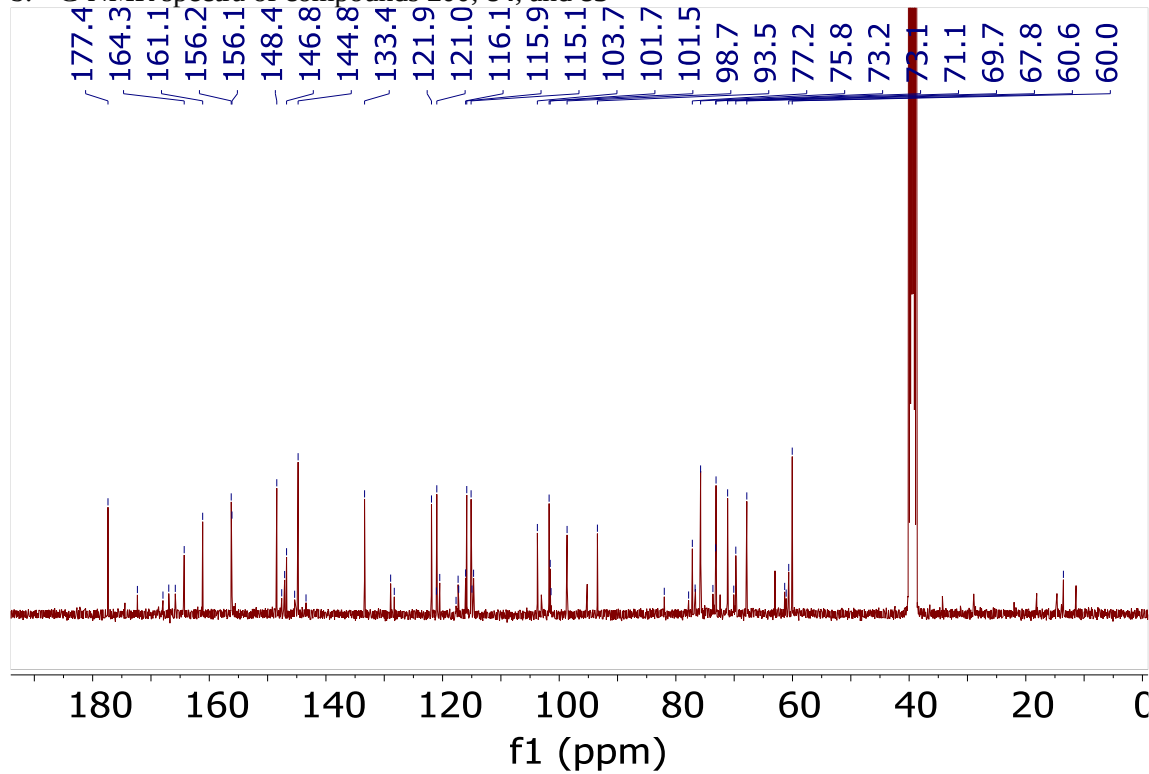


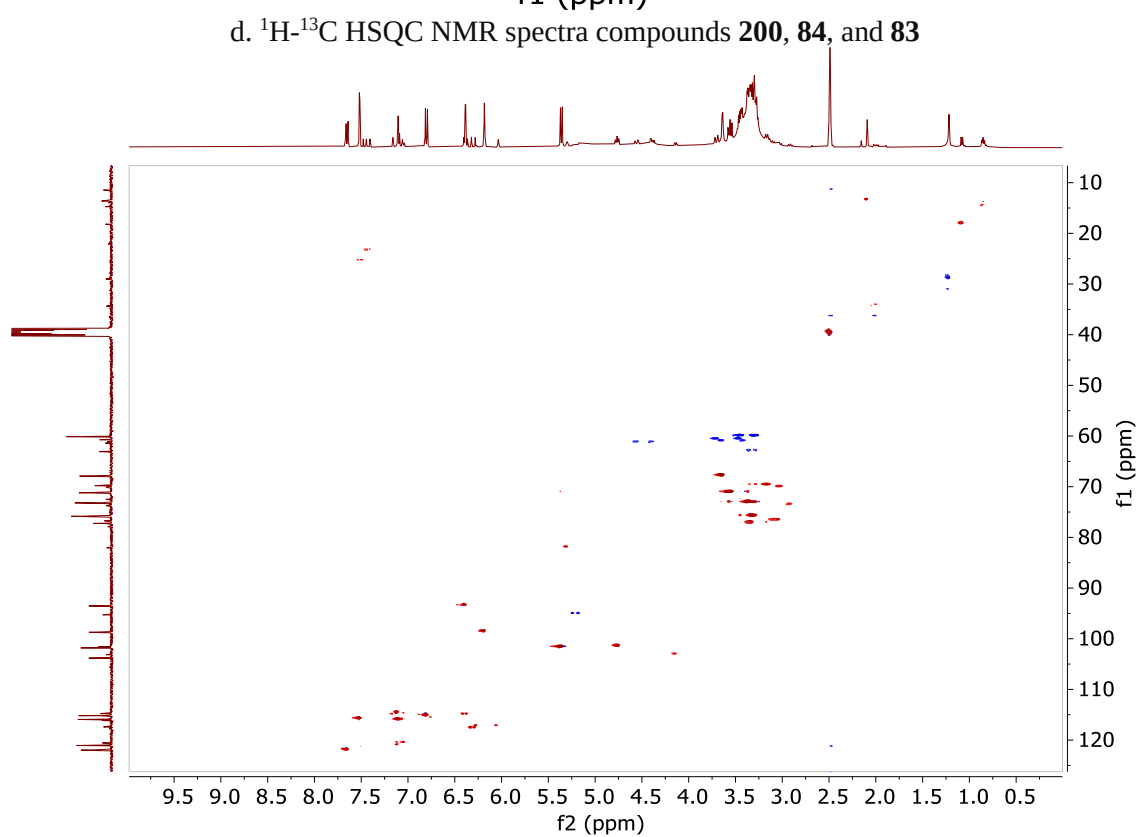
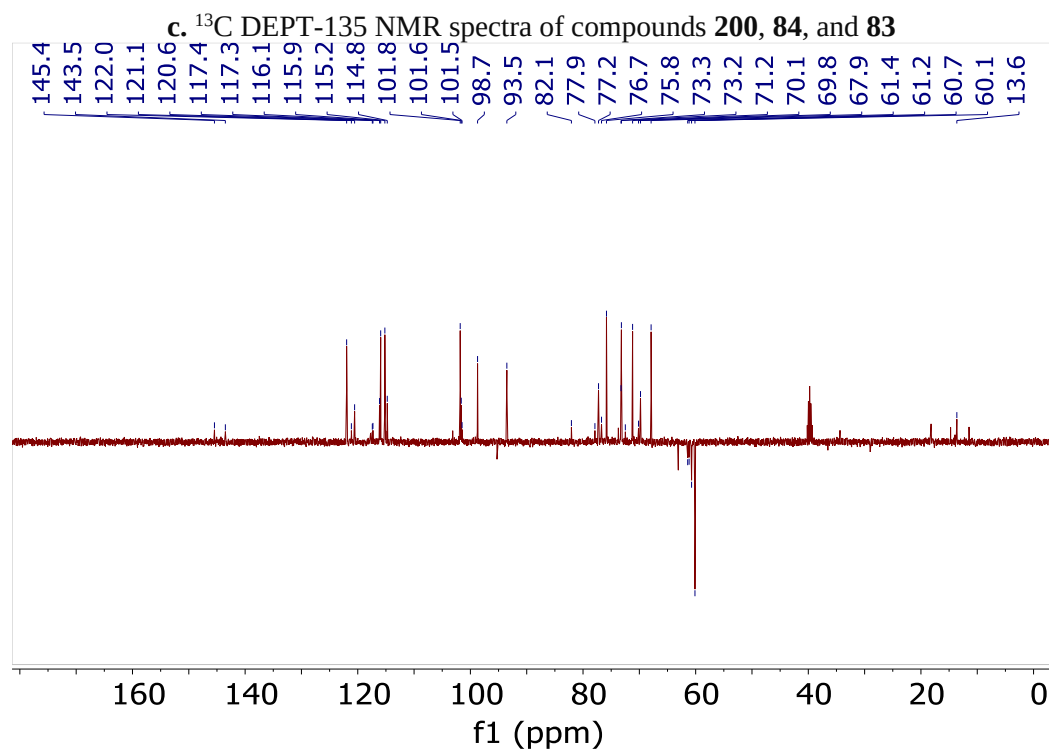
Appendix 20. NMR spectral data of compounds **200**, **84**, and **83**

a. ^1H -NMR spectra of compounds **200**, **84**, and **83** and the expanded region used to calculate their relative amounts

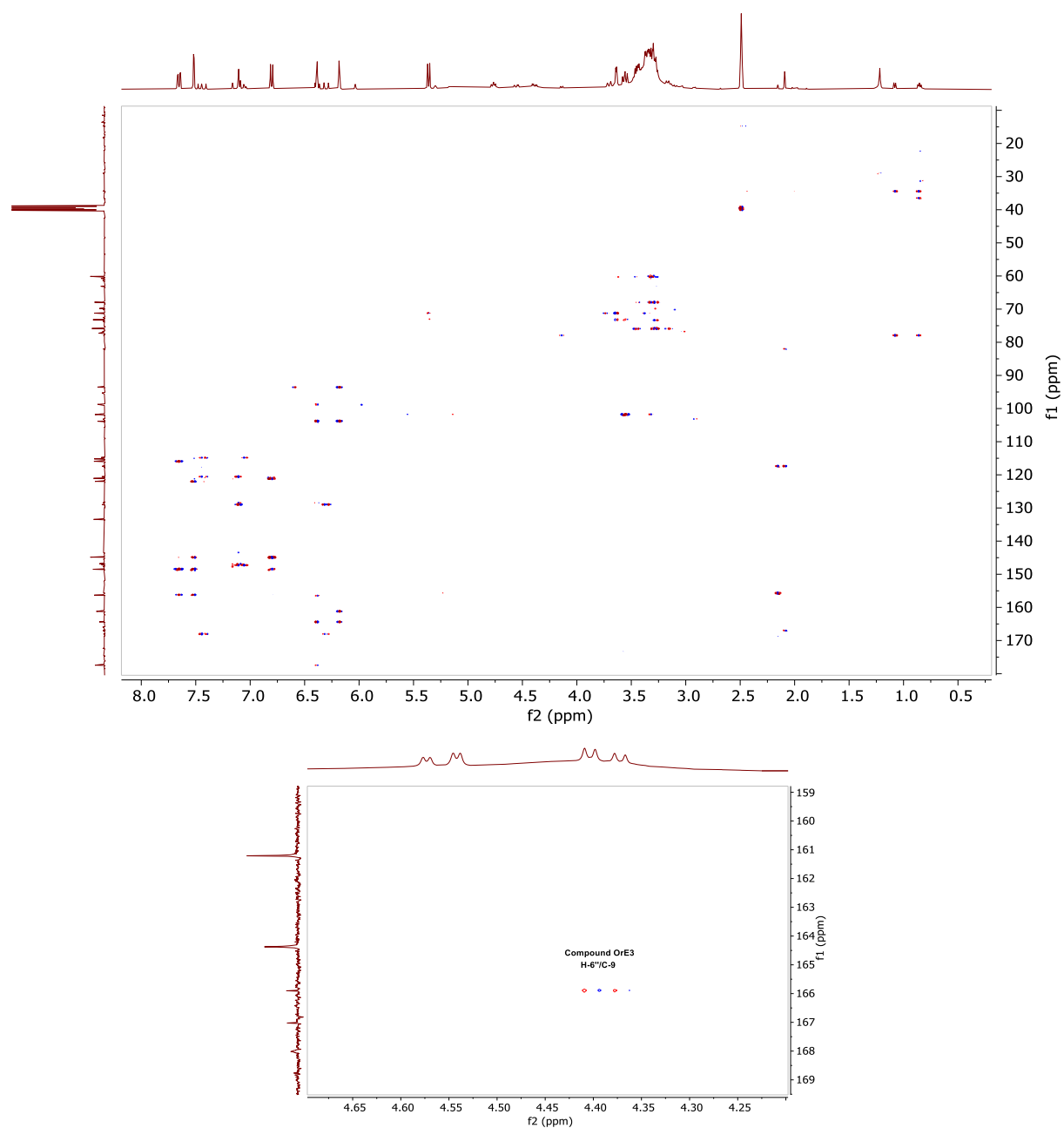


b. ^{13}C -NMR spectra of compounds **200**, **84**, and **83**

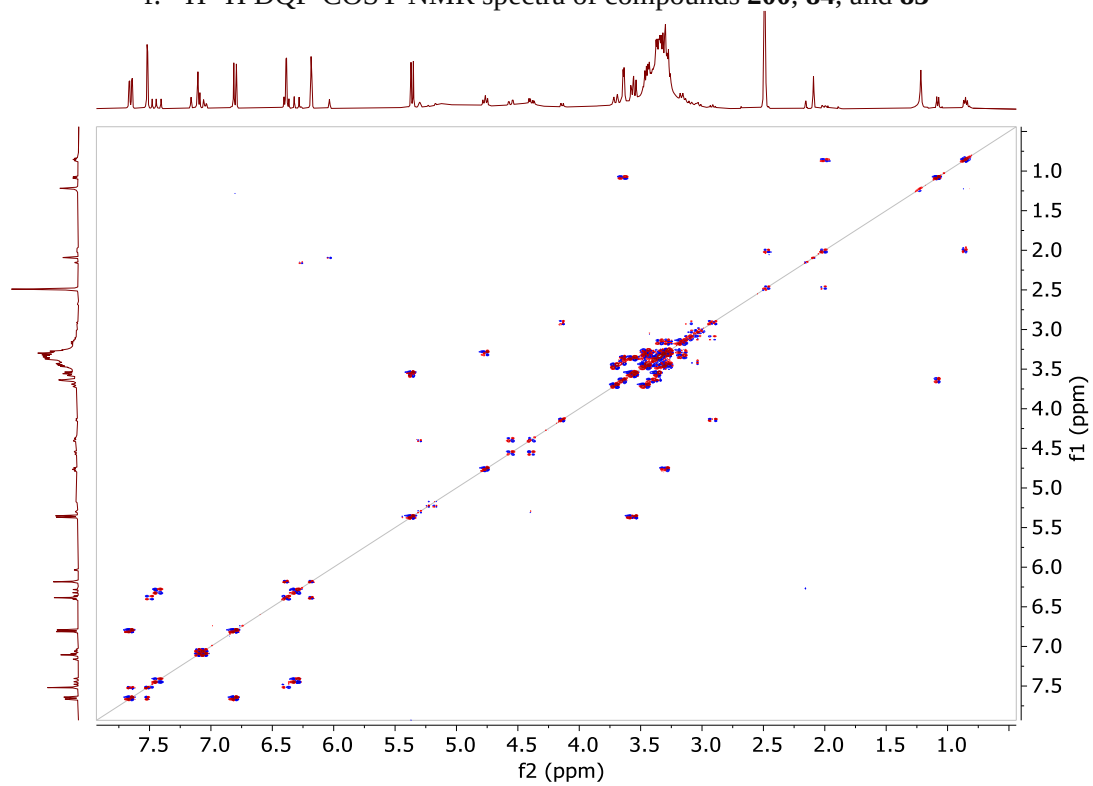




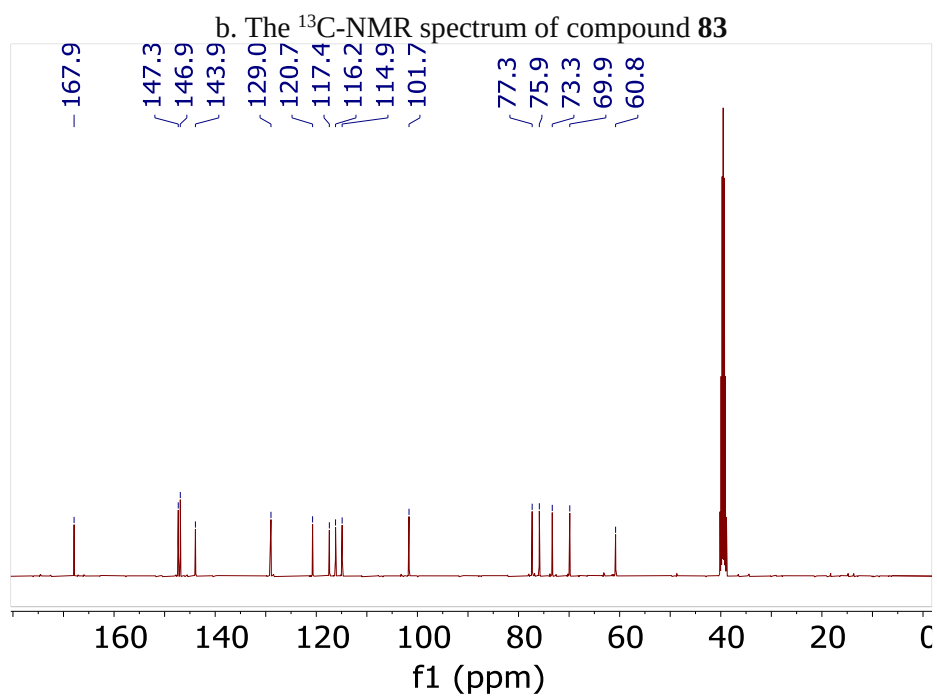
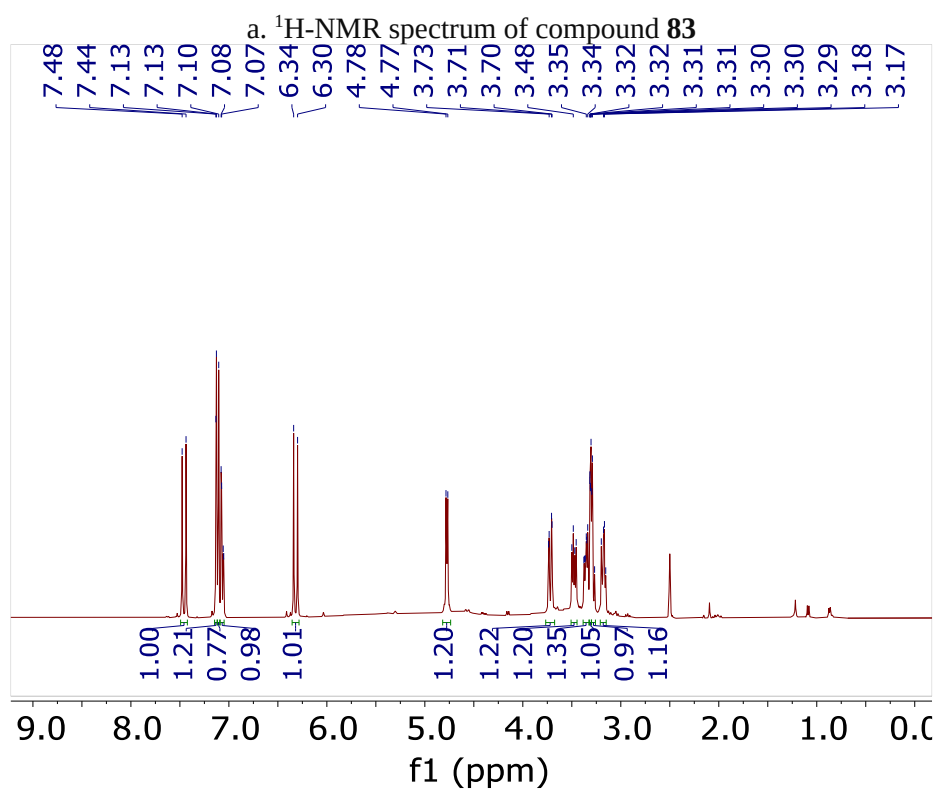
e. ^1H - ^{13}C HMBC NMR spectra of compounds **200**, **84**, and **83** and expanded region used to indicate the linkage of H-6'/C-9 in compound **84**

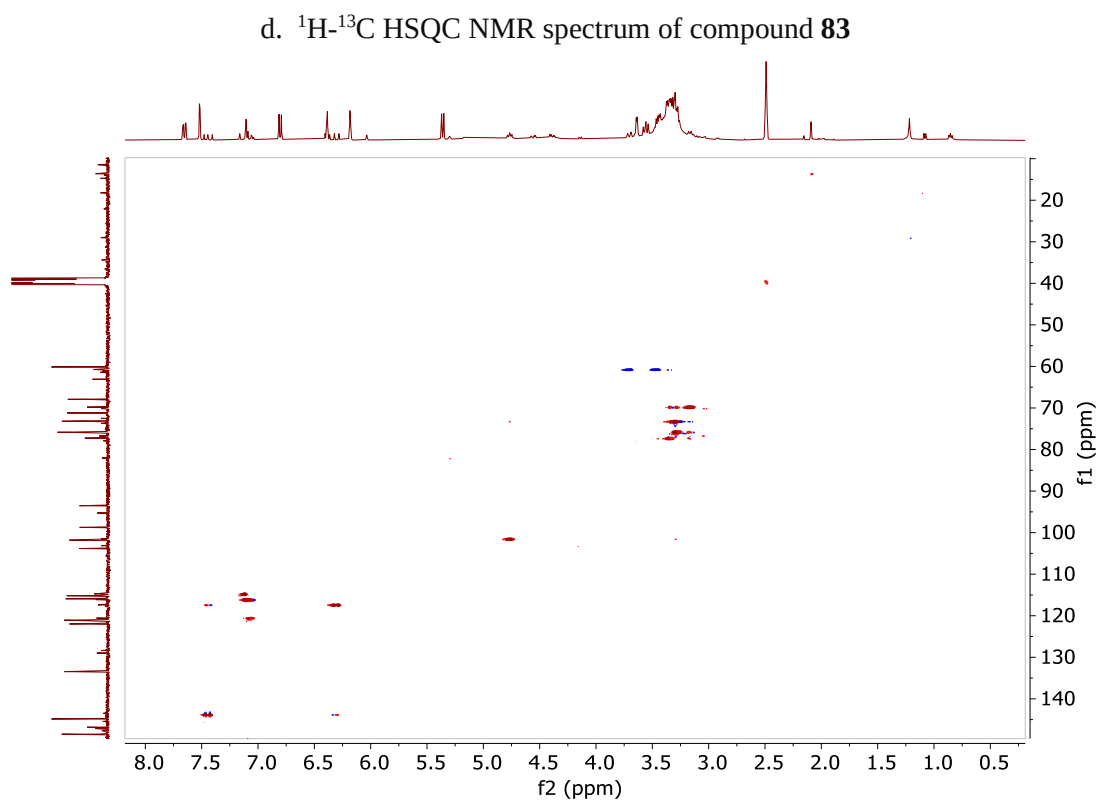
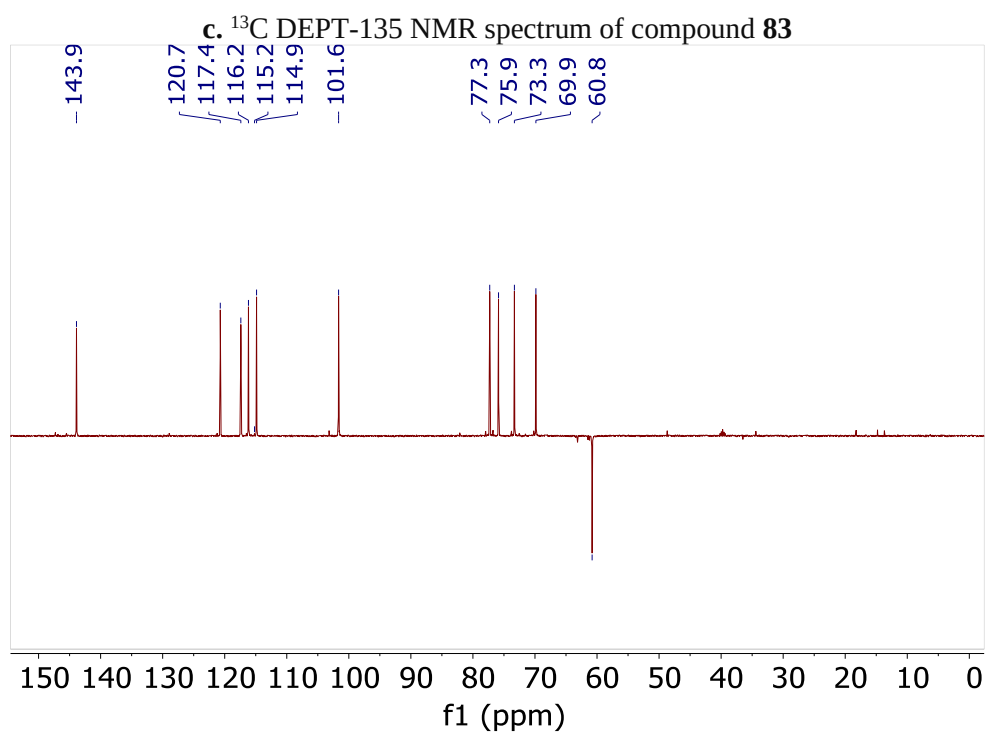


f. ^1H - ^1H DQF-COSY NMR spectra of compounds **200**, **84**, and **83**

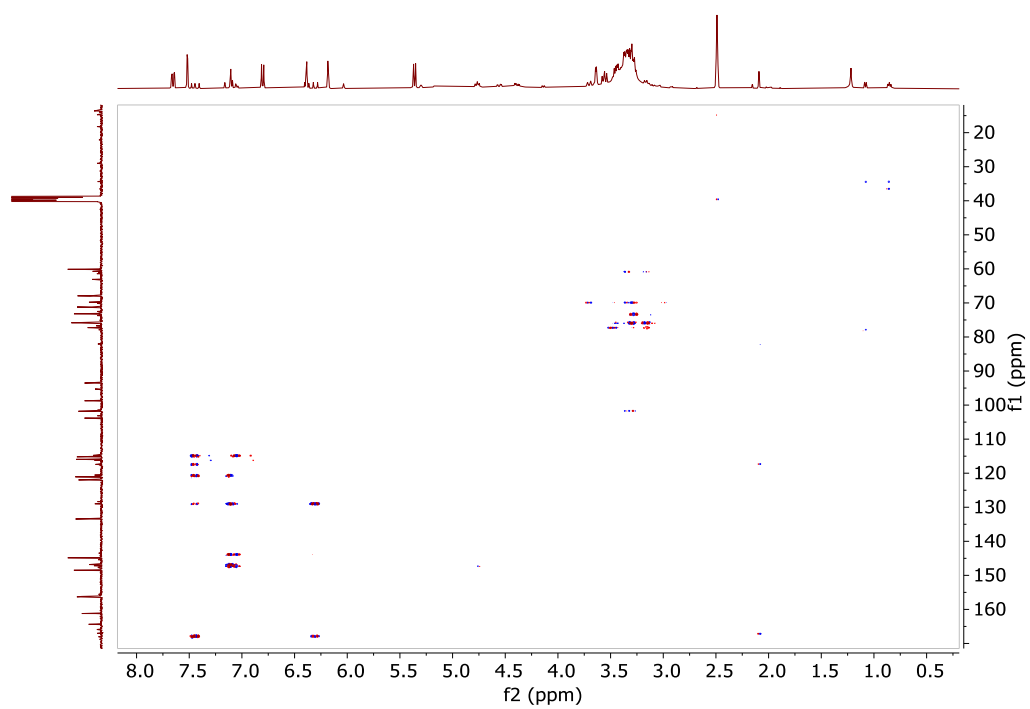


Appendix 21. NMR spectral data of compound **83**

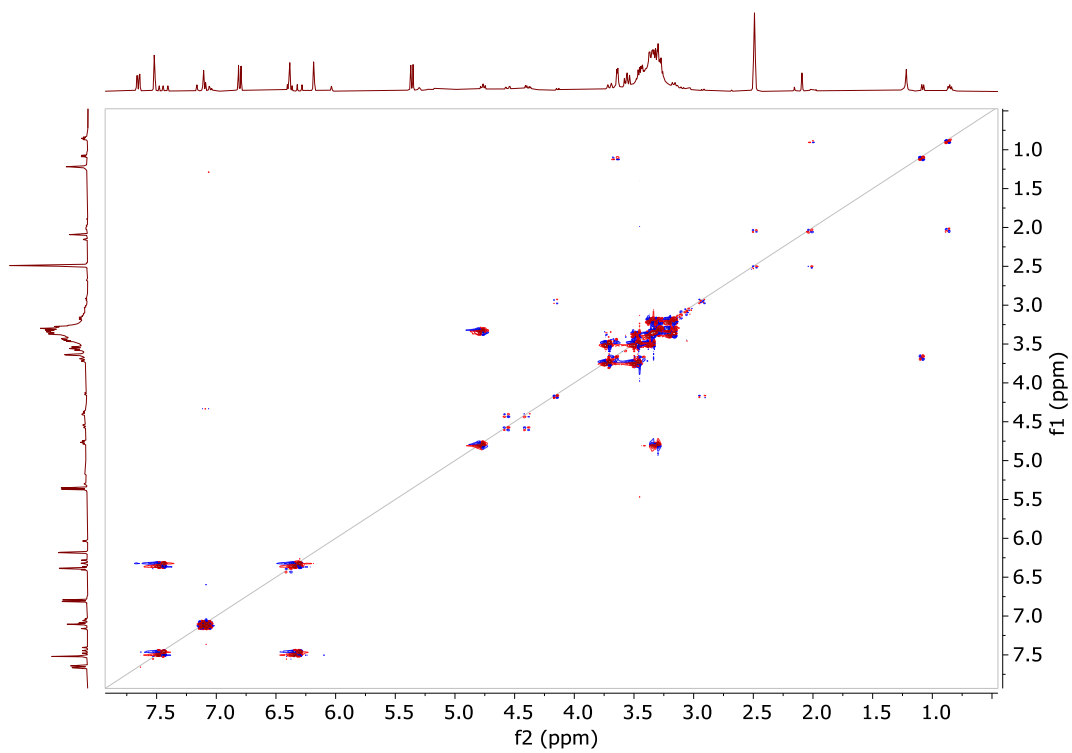




e. ^1H - ^{13}C HMBC NMR spectrum of compound **83**

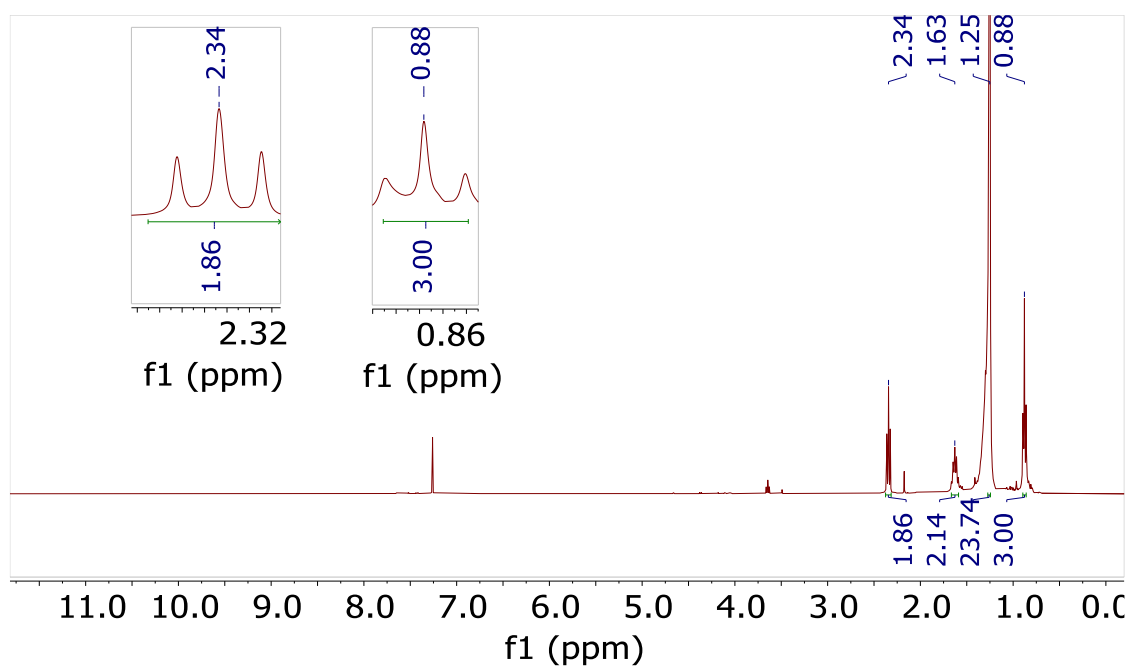


f. ^1H - ^1H DQF-COSY NMR spectrum compound **83**

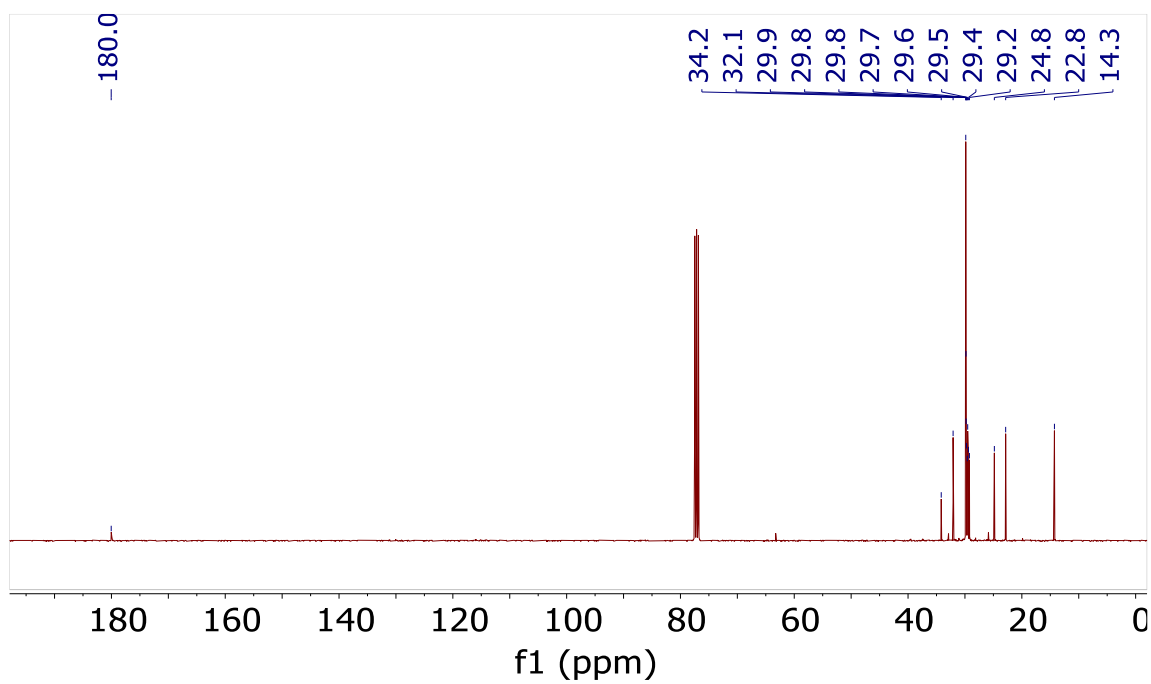


Appendix 22. NMR spectral data of compound **145**

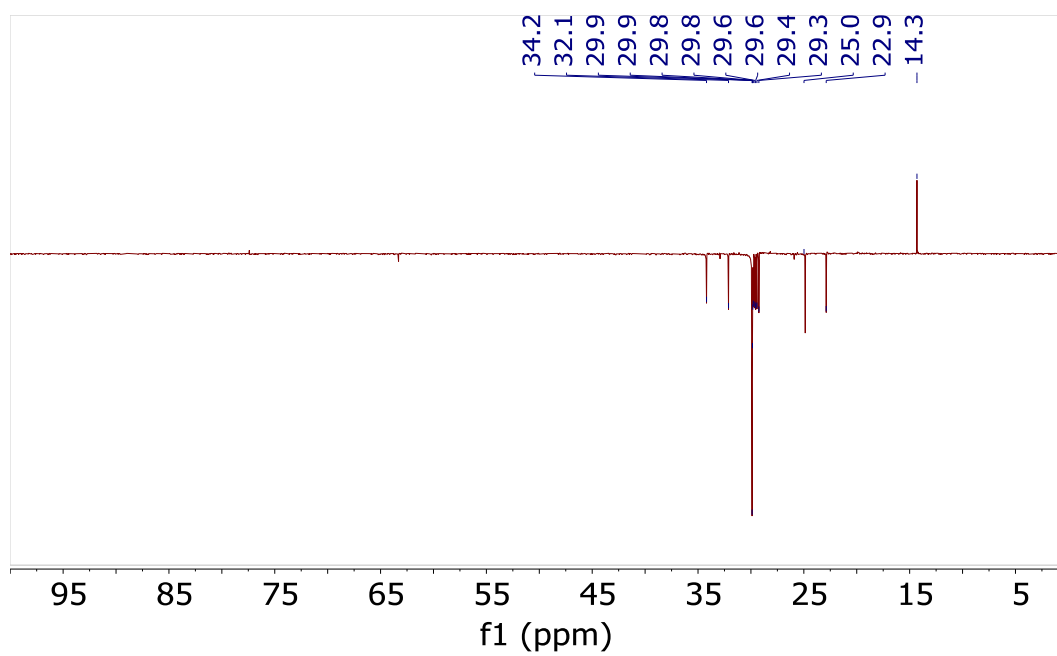
a. ^1H -NMR spectrum of compound **145**



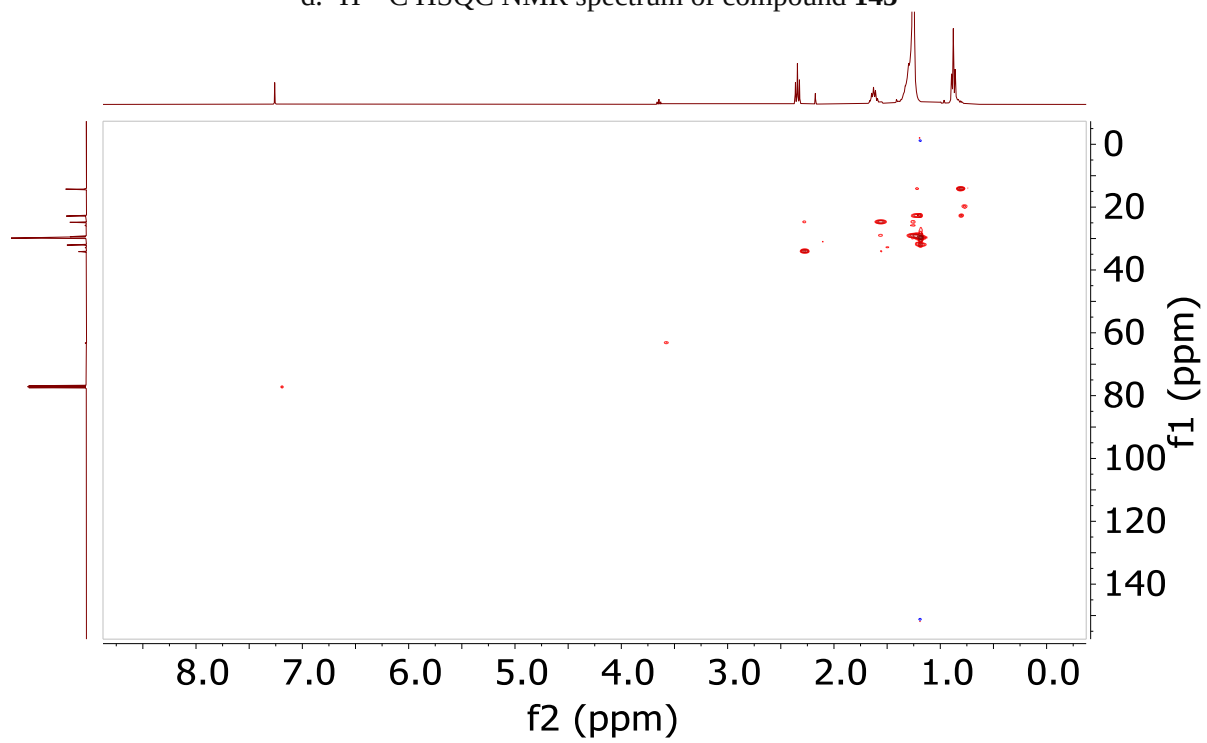
b. The ^{13}C -NMR spectrum of compound **145**



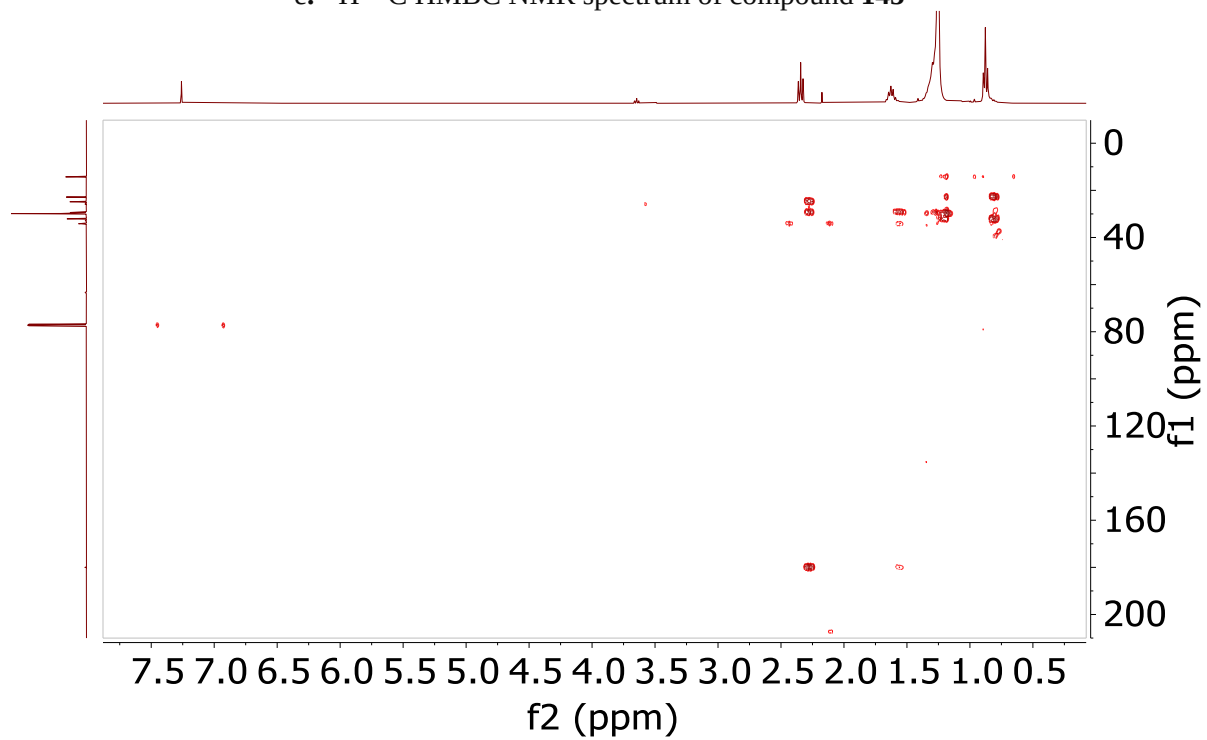
c. ^{13}C DEPT-135 NMR spectrum of compound **145**



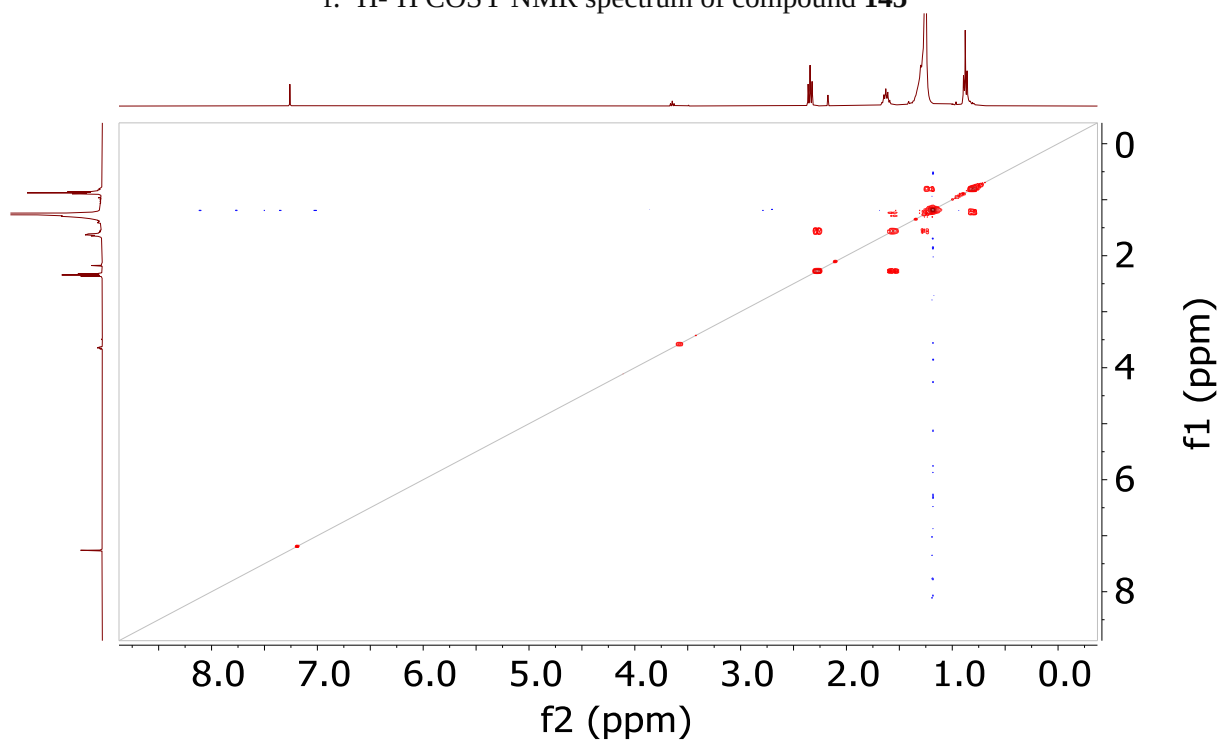
d. ^1H - ^{13}C HSQC NMR spectrum of compound **145**



e. ^1H - ^{13}C HMBC NMR spectrum of compound **145**

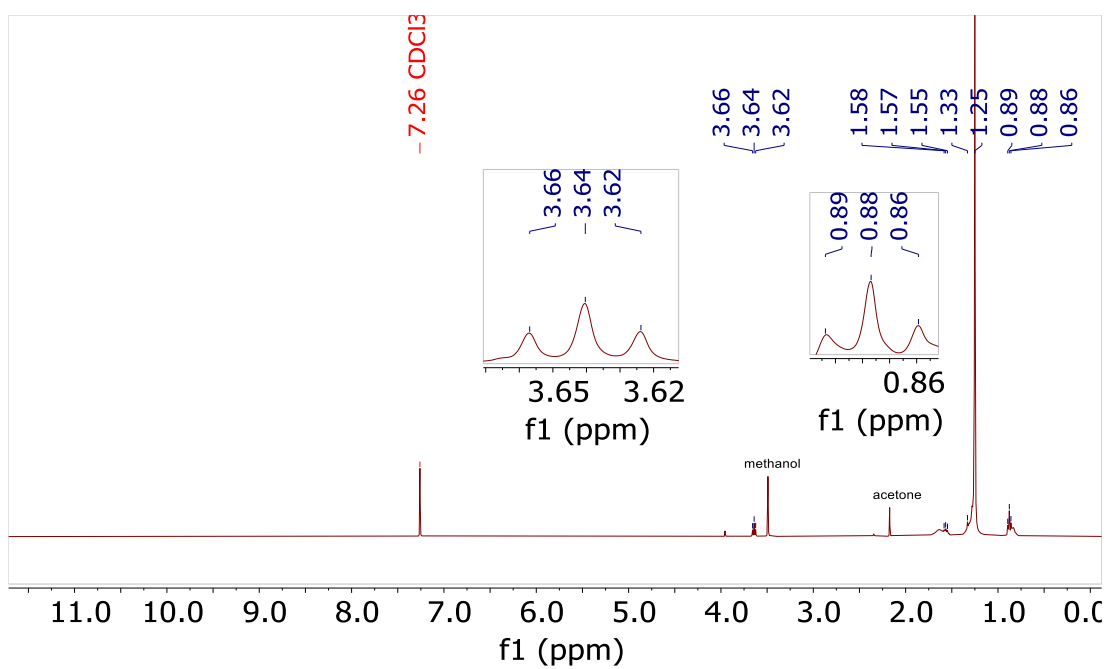


f. ^1H - ^1H COSY NMR spectrum of compound **145**

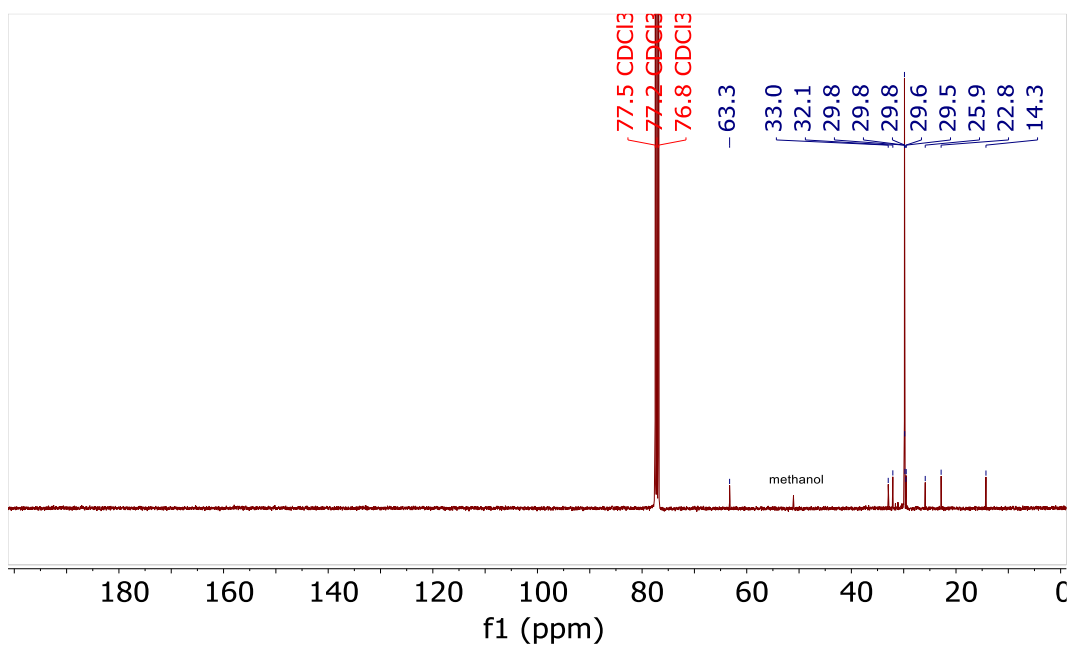


Appendix 23. NMR spectral data of compound **201**

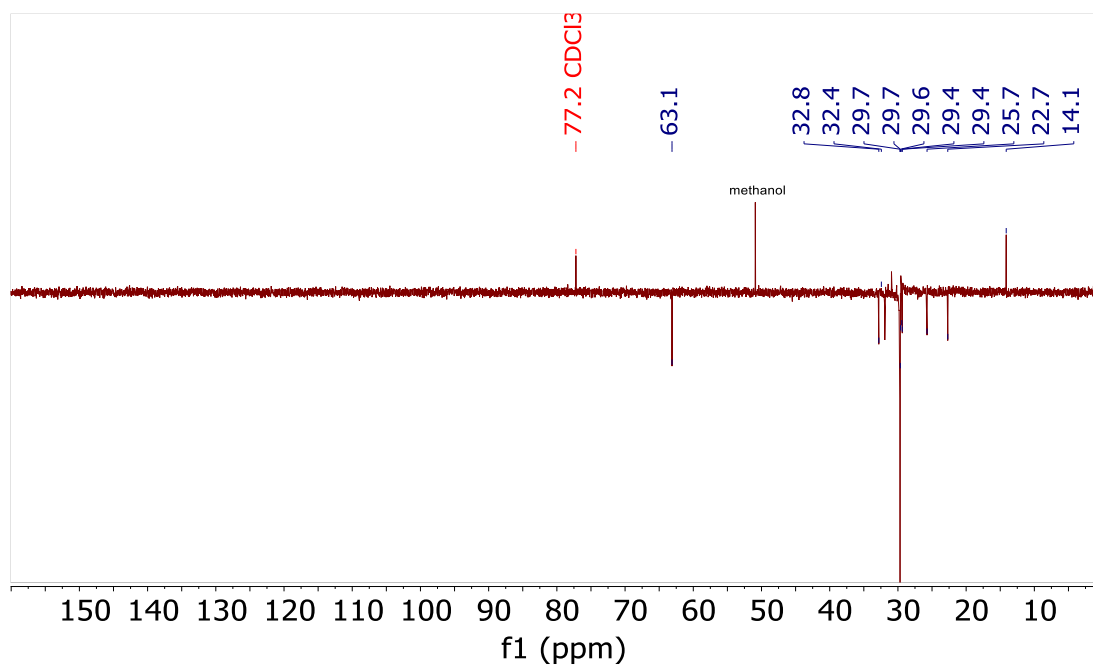
a. ^1H -NMR spectrum of compound **201**



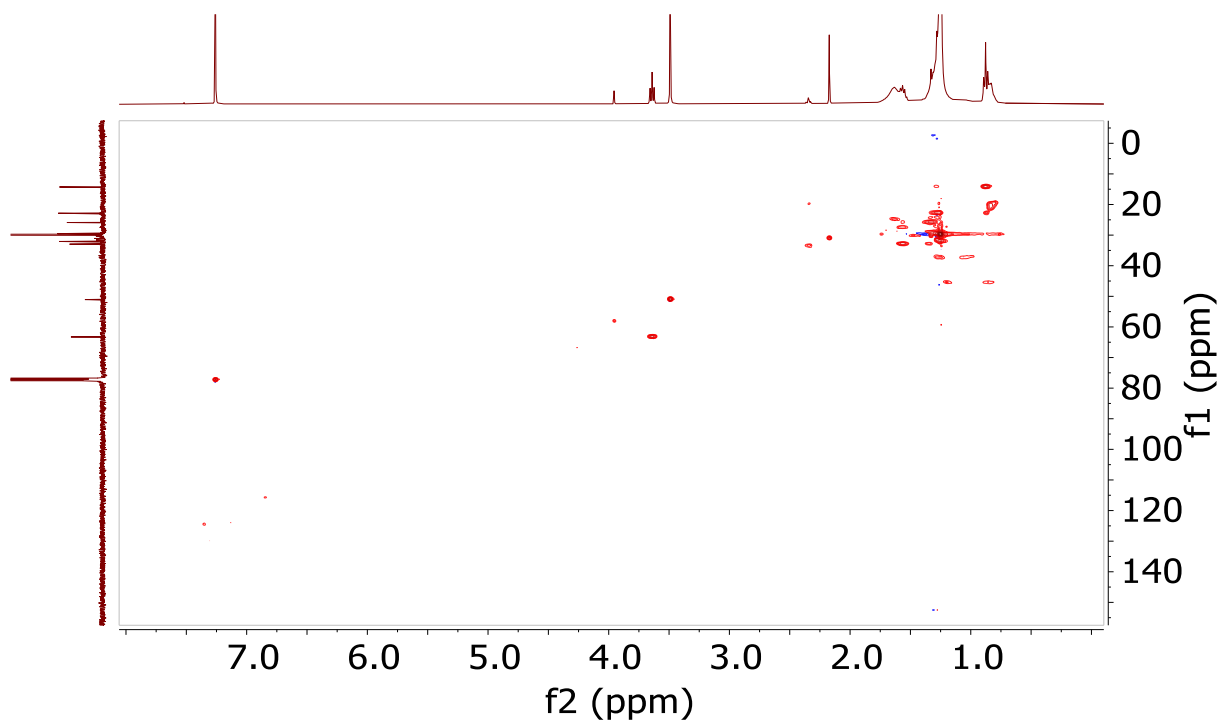
b. The ^{13}C -NMR spectrum of compound **201**



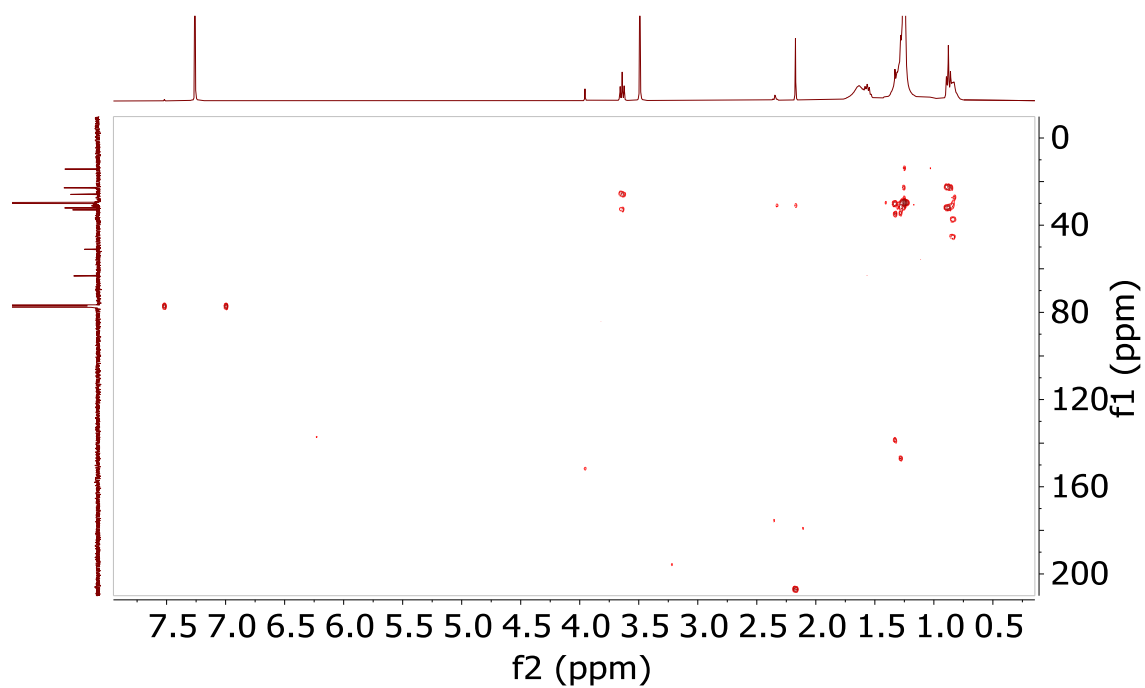
c. ^{13}C DEPT-135 NMR spectrum of compound **201**



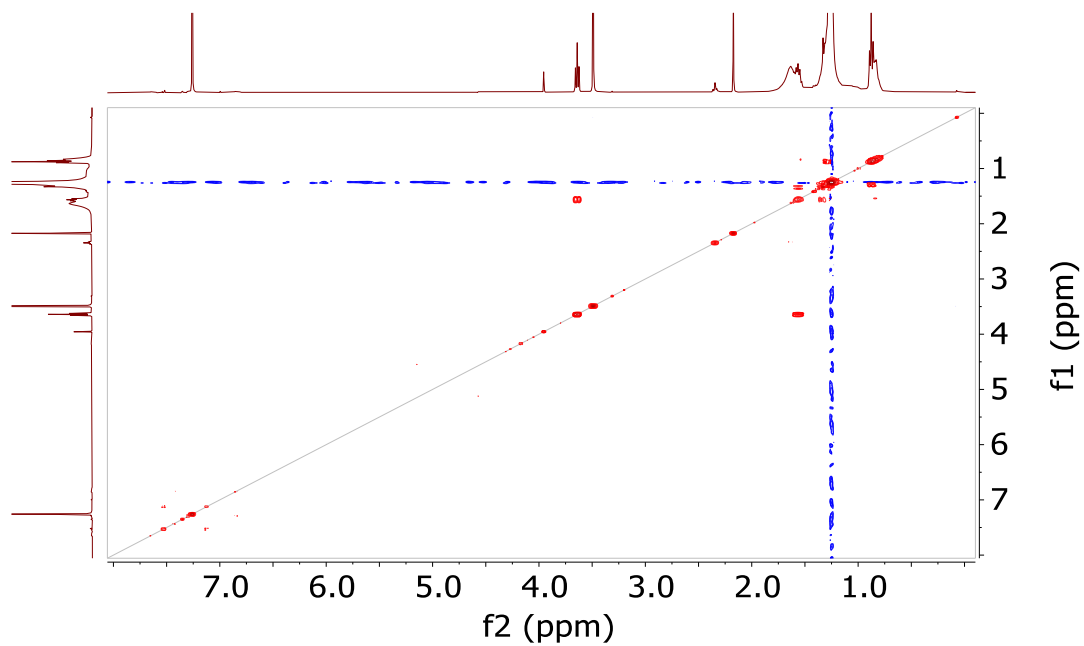
d. ^1H - ^{13}C HSQC NMR spectrum of compound **201**



e. ^1H - ^{13}C HMBC NMR spectrum of compound **201**

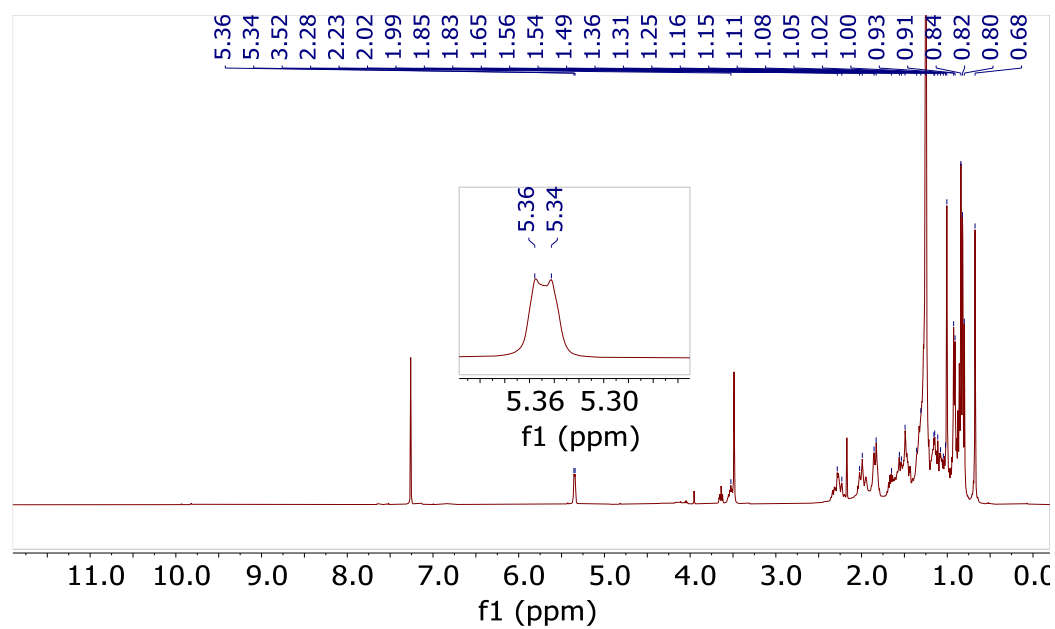


f. ^1H - ^1H COSY NMR spectrum of compound **201**

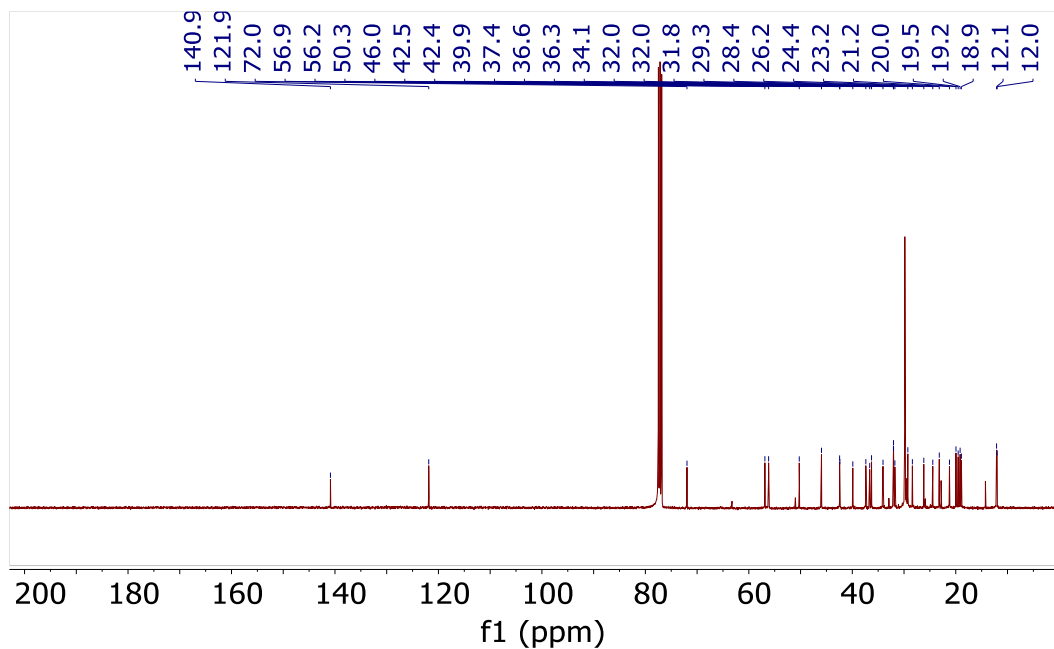


Appendix 24, NMR spectral data of compound **17**

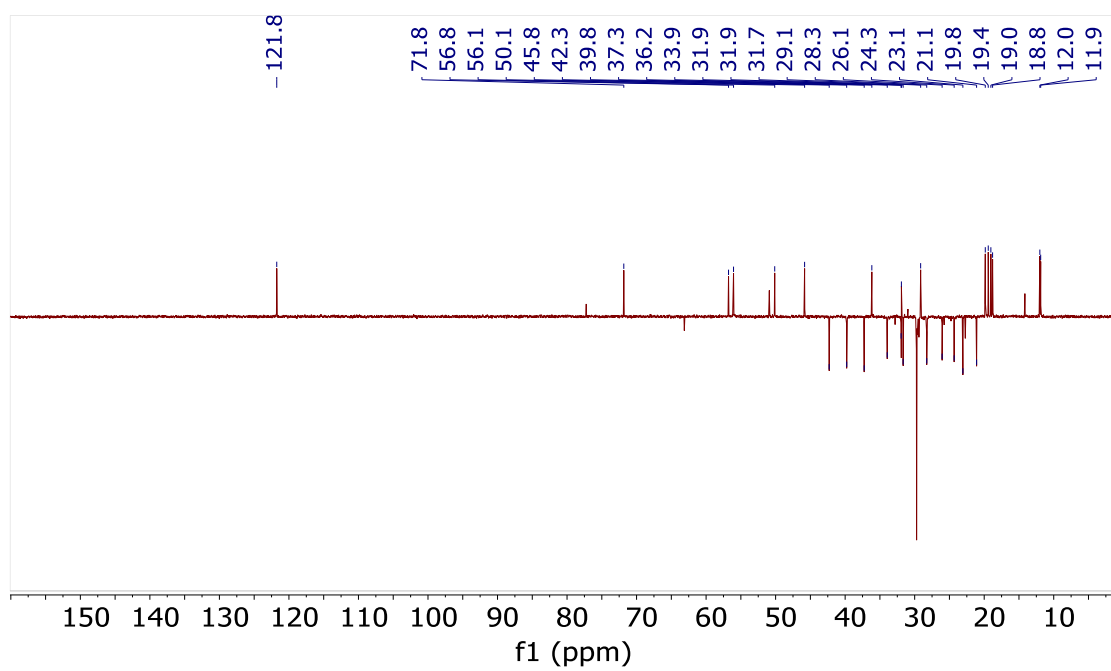
a. ^1H -NMR spectrum of compound **17**



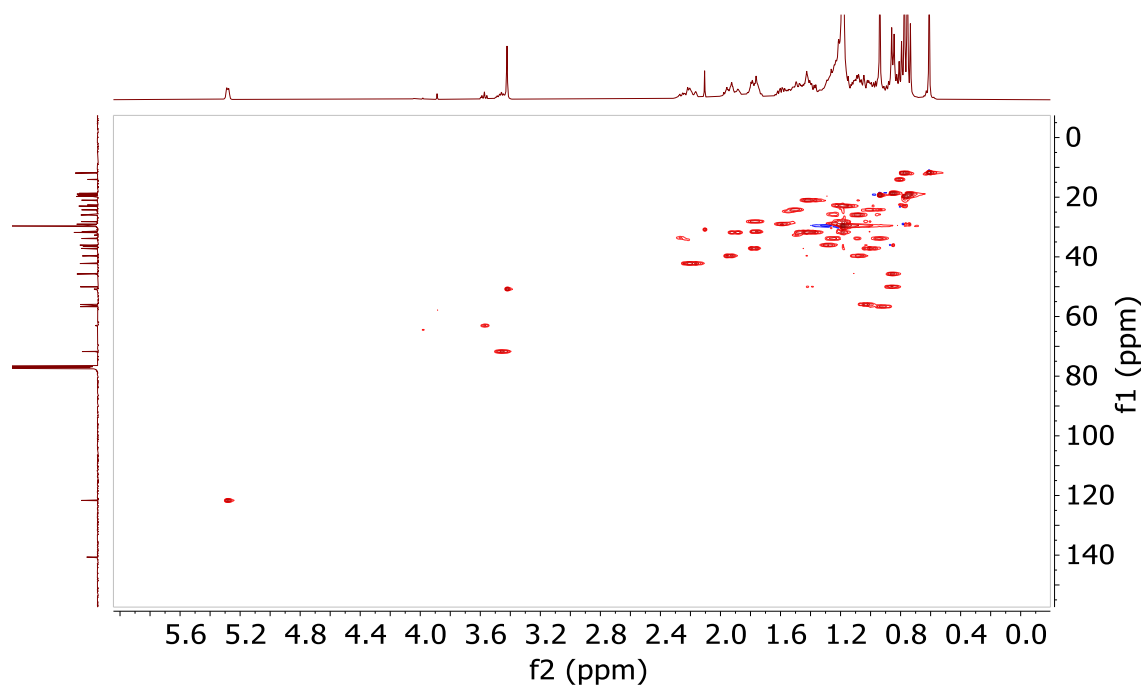
b. The ^{13}C -NMR spectrum of compound **17**



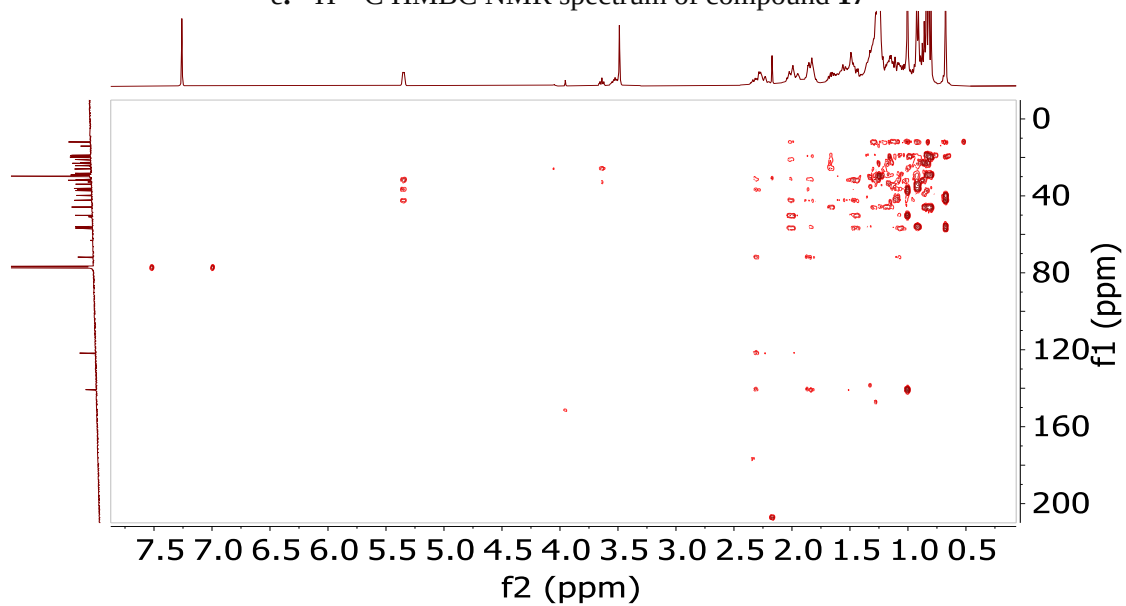
c. ^{13}C DEPT-135 NMR spectrum of compound **17**



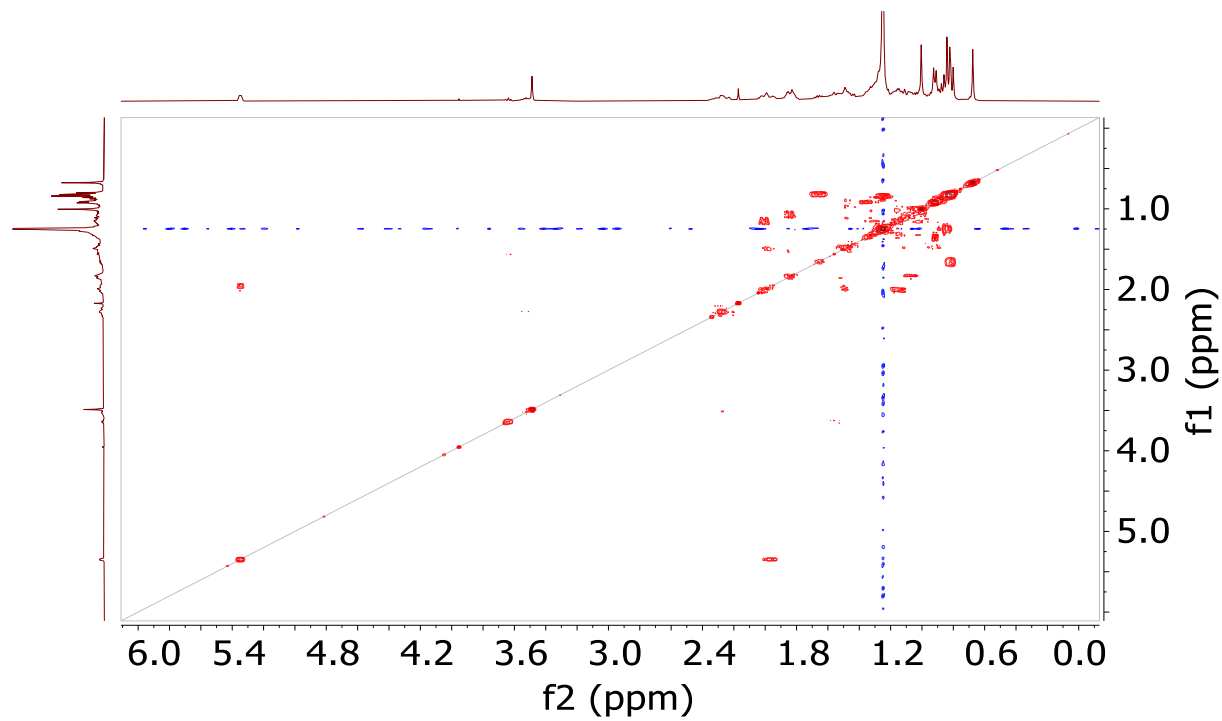
d. ^1H - ^{13}C HSQC NMR spectrum of compound **17**



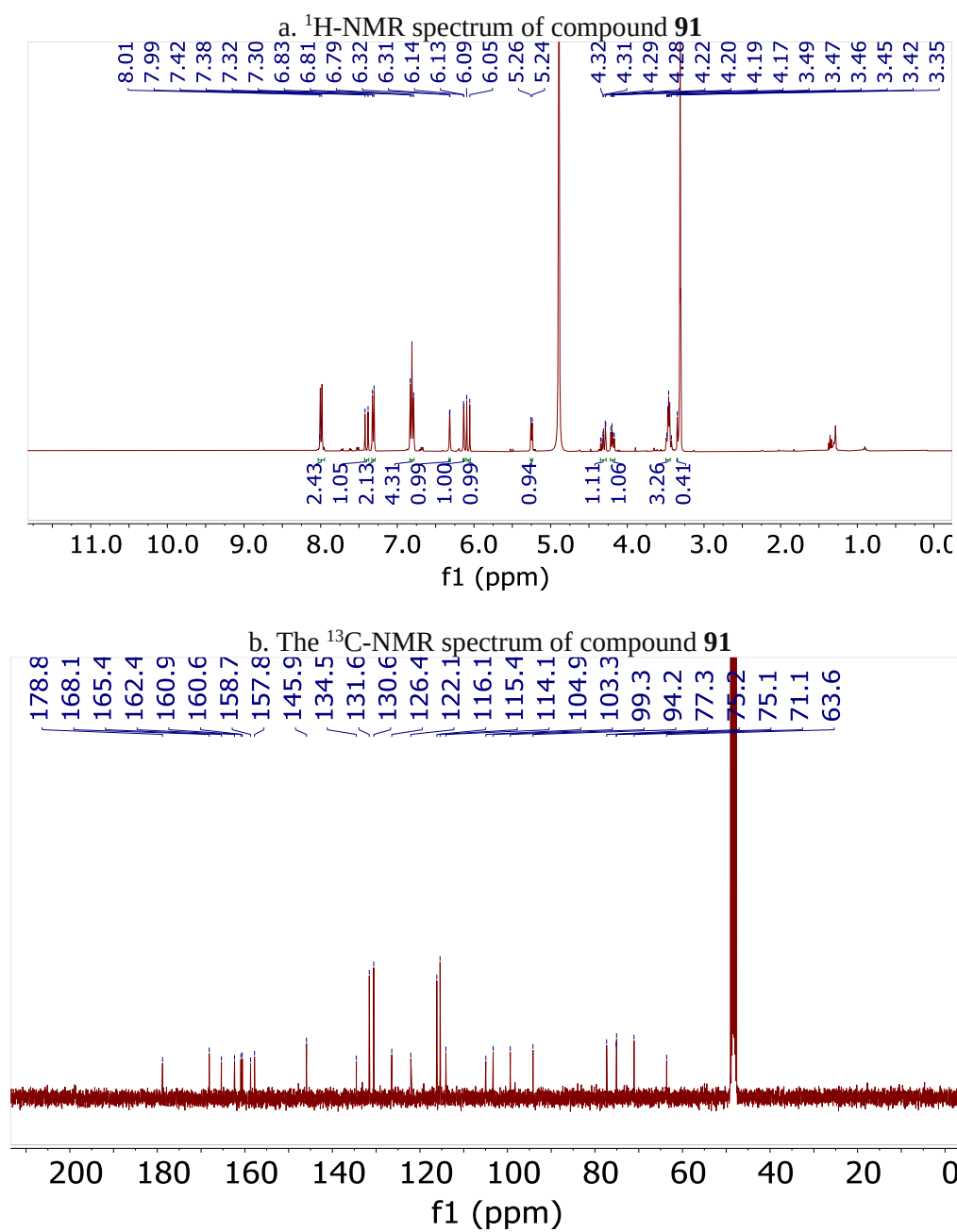
e. ^1H - ^{13}C HMBC NMR spectrum of compound **17**



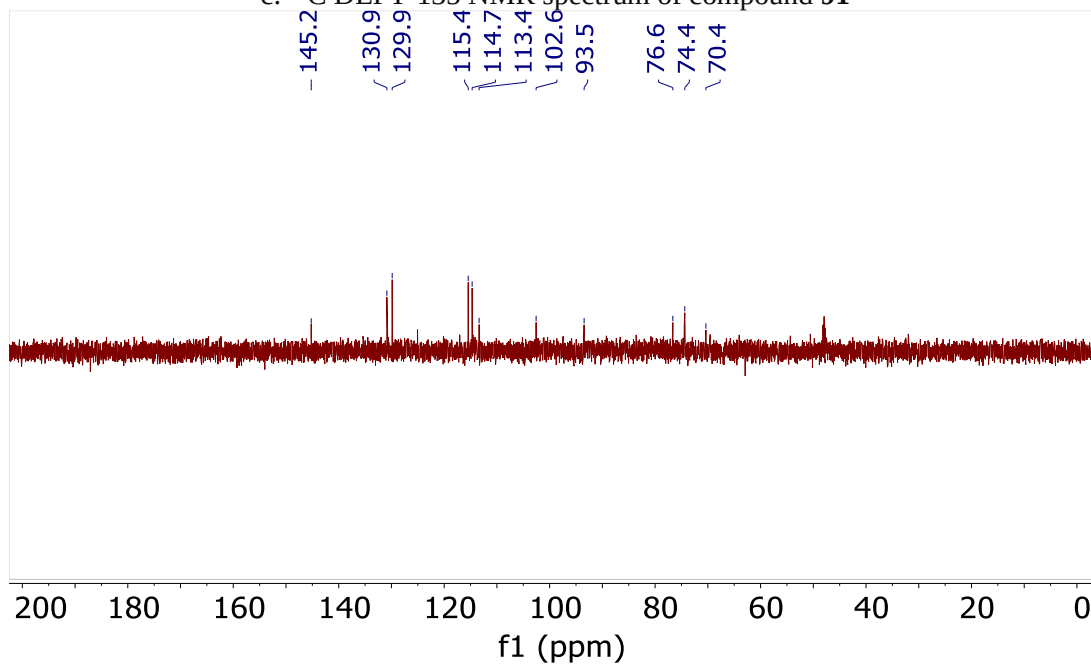
f. ^1H - ^1H COSY NMR spectrum of compound **17**



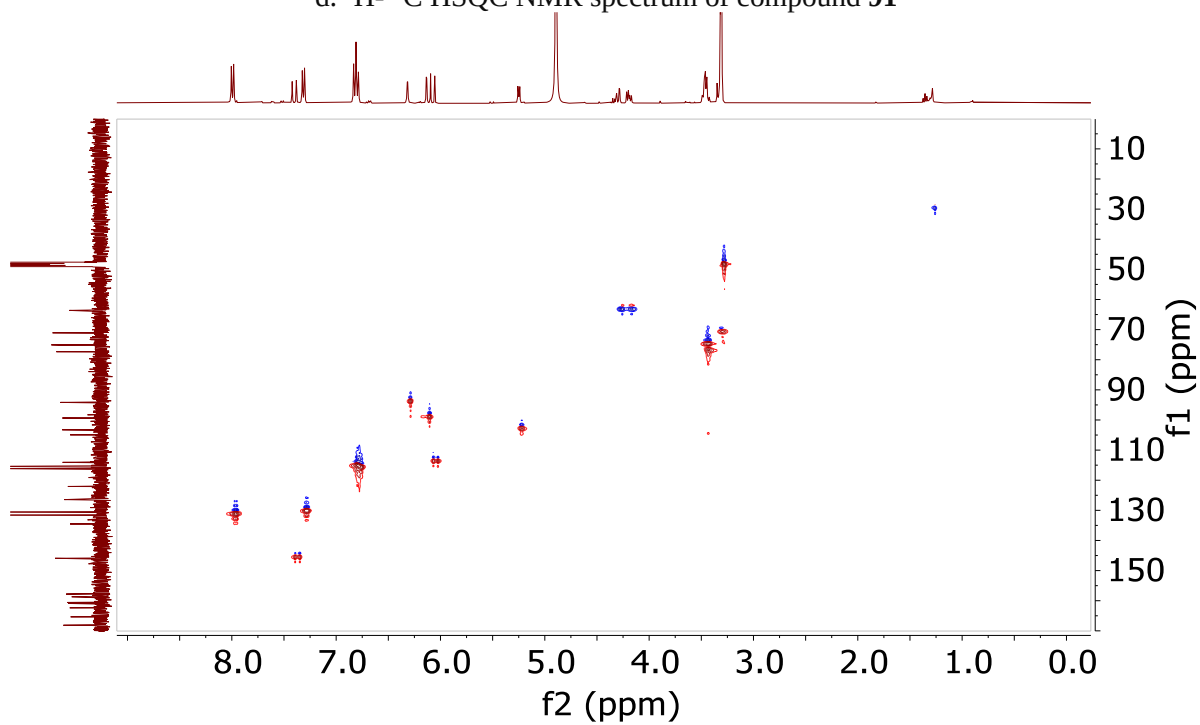
Appendix 25. NMR spectral data of compound **91**



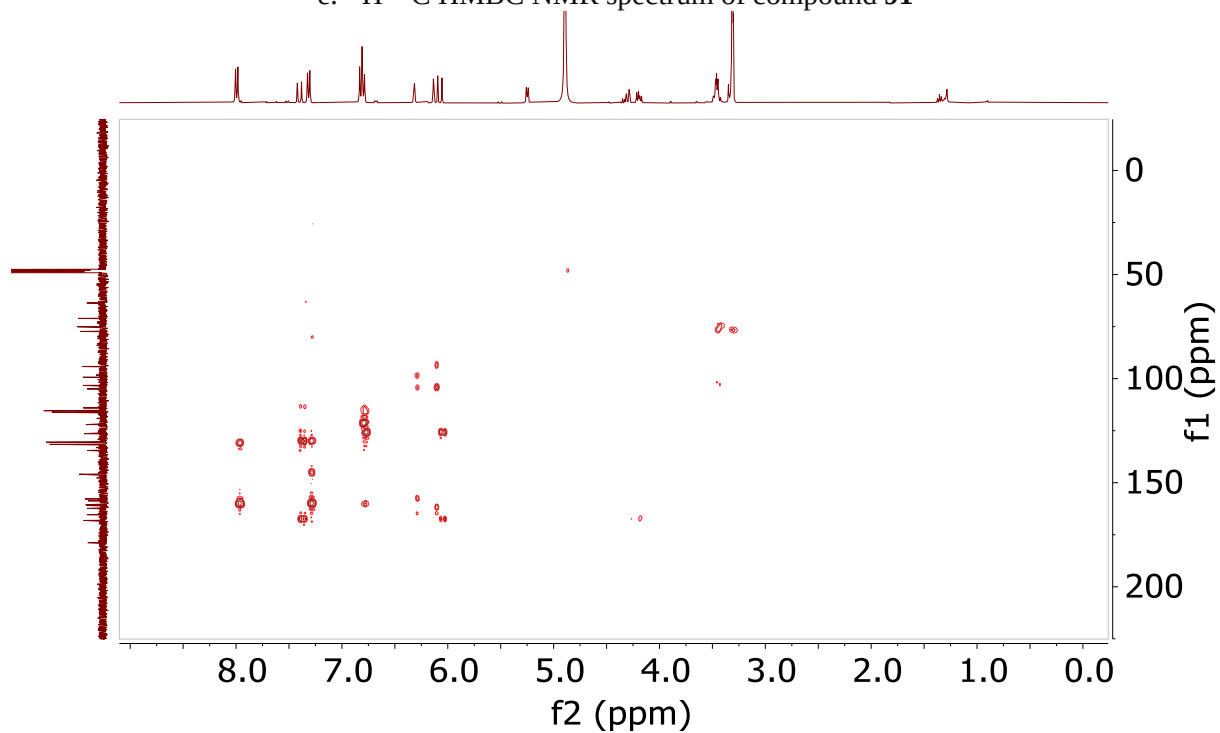
c. ^{13}C DEPT-135 NMR spectrum of compound **91**



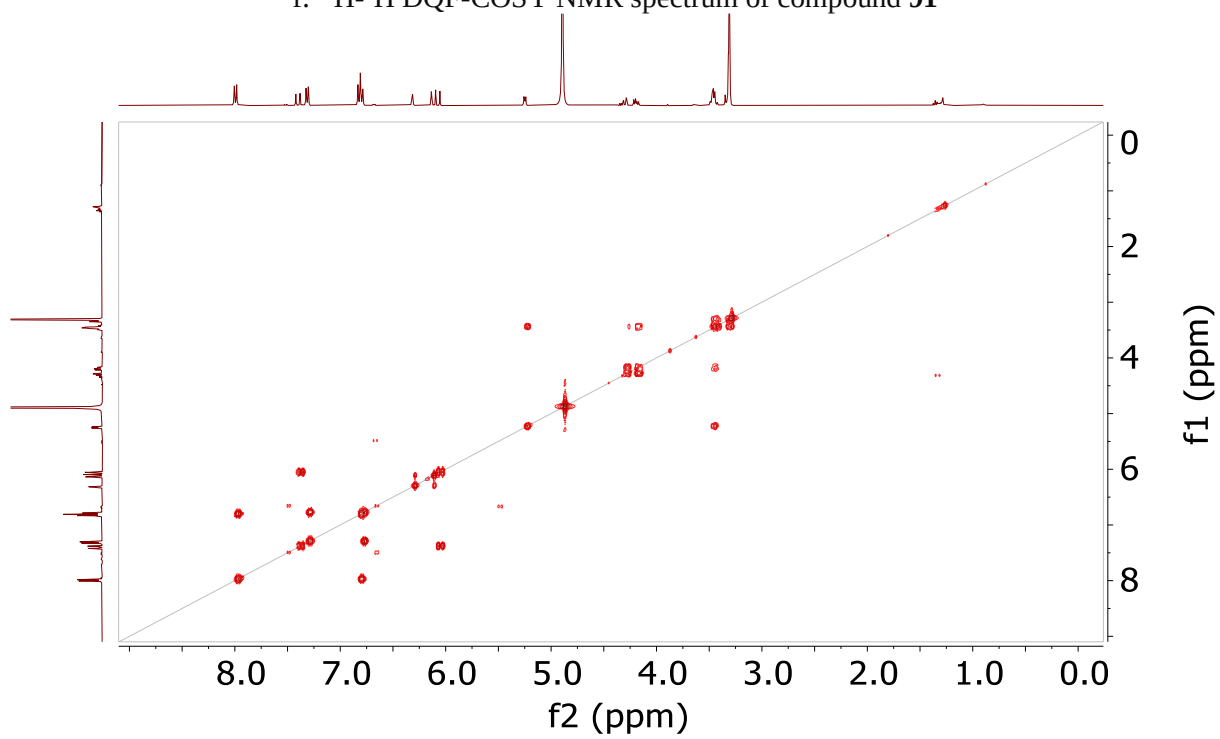
d. ^1H - ^{13}C HSQC NMR spectrum of compound **91**



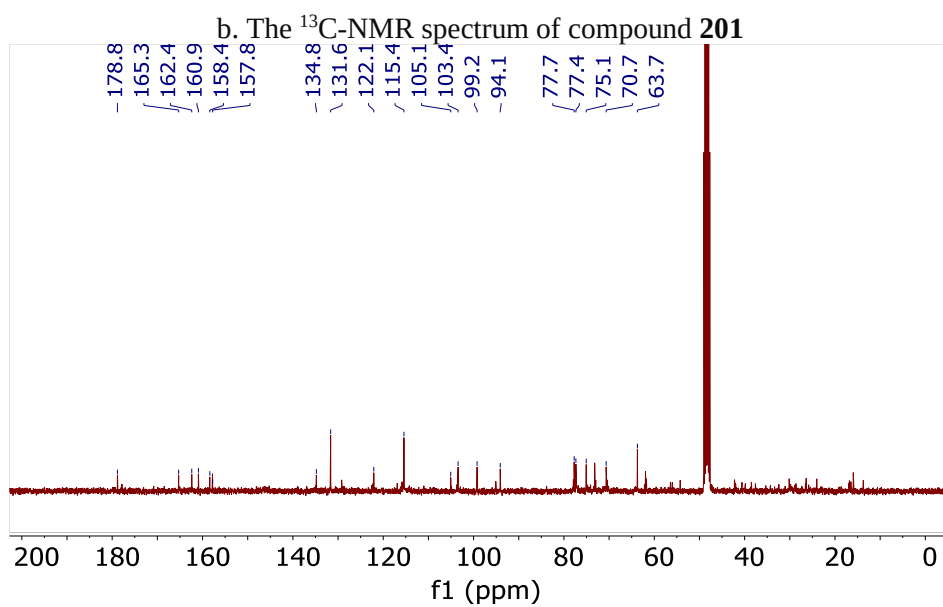
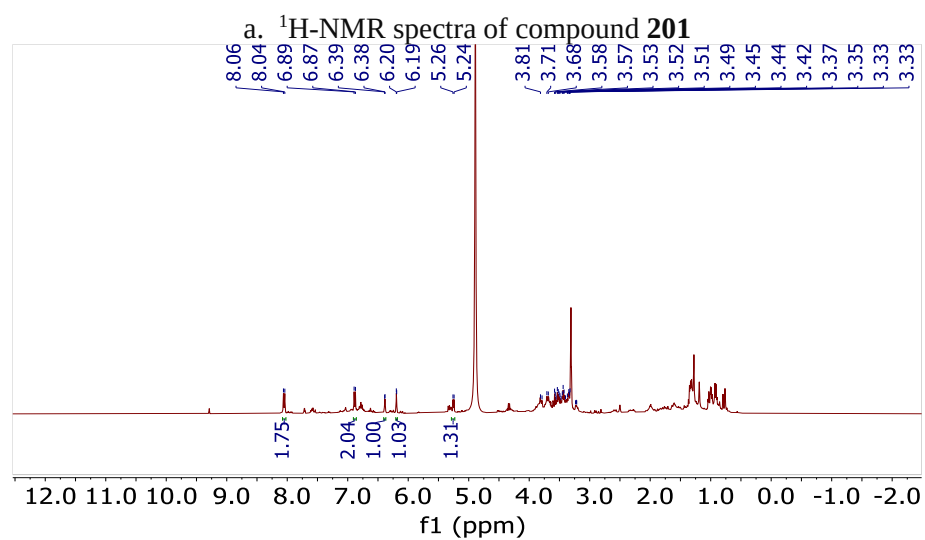
e. ^1H - ^{13}C HMBC NMR spectrum of compound **91**



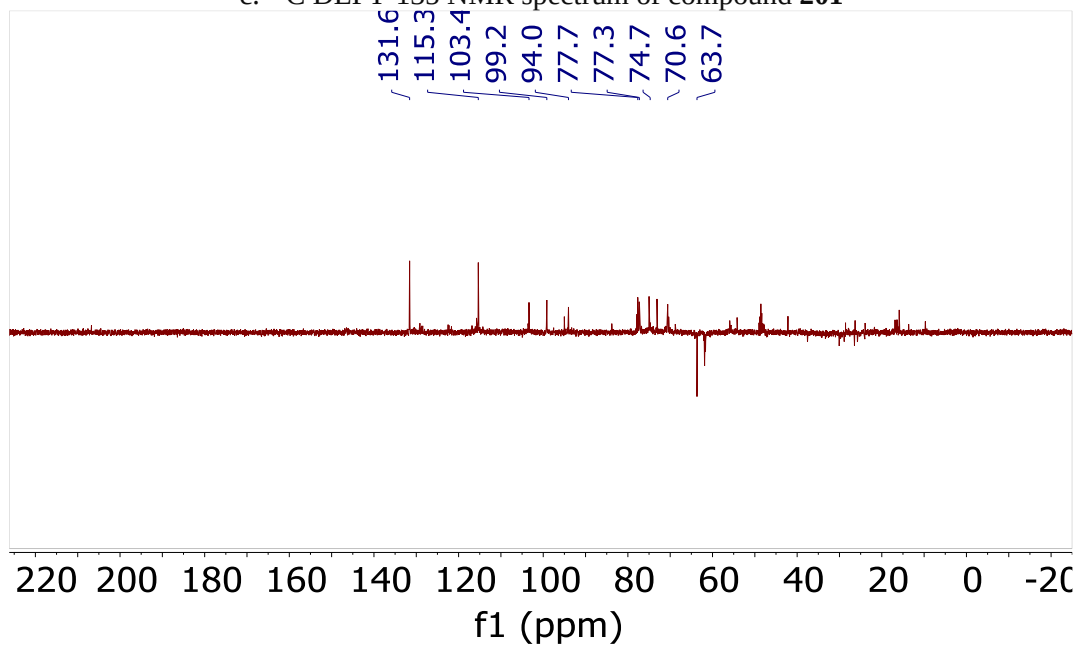
f. ^1H - ^1H DQF-COSY NMR spectrum of compound **91**



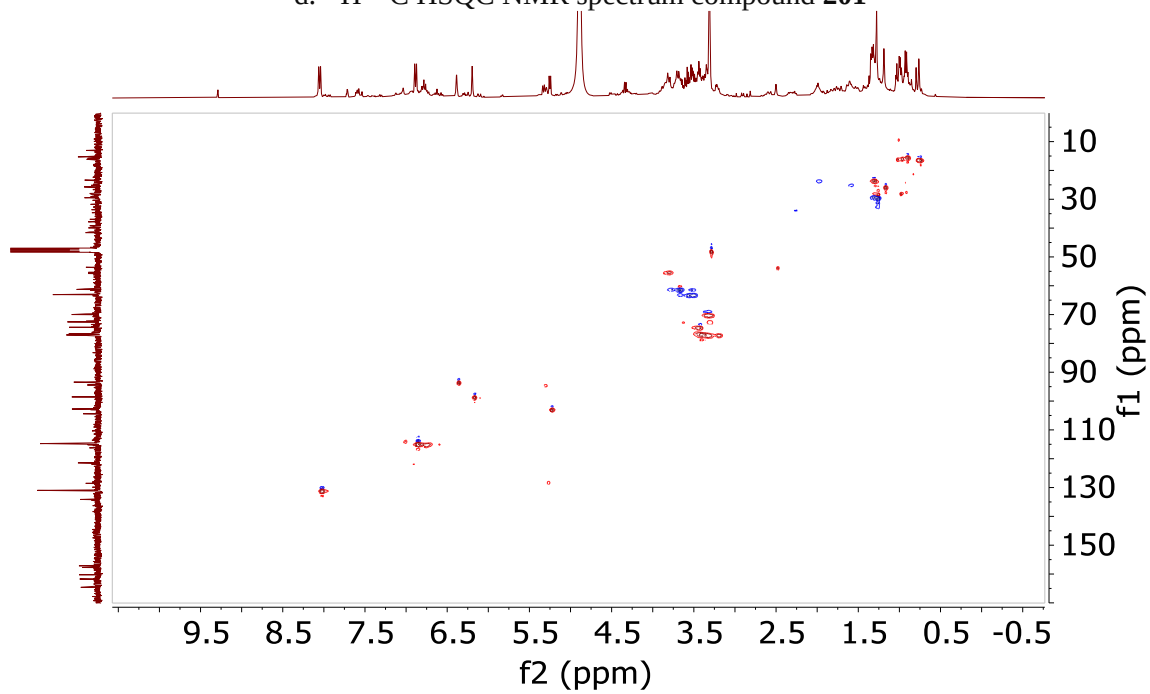
Appendix 26. NMR spectral data of compound **201**



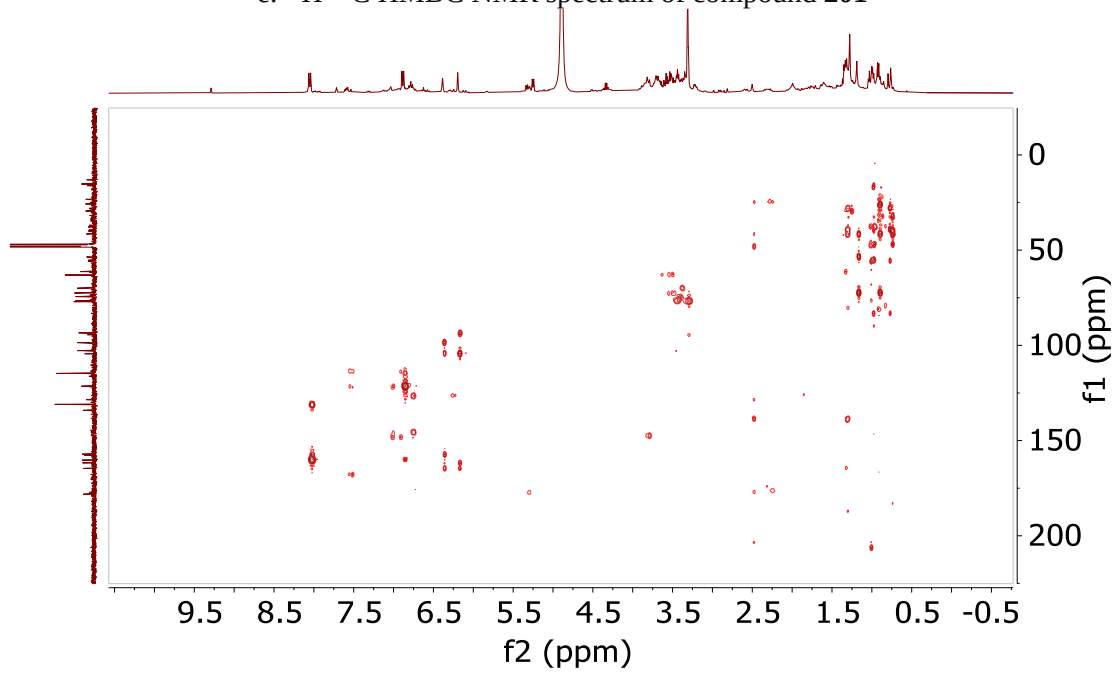
c. ^{13}C DEPT-135 NMR spectrum of compound **201**



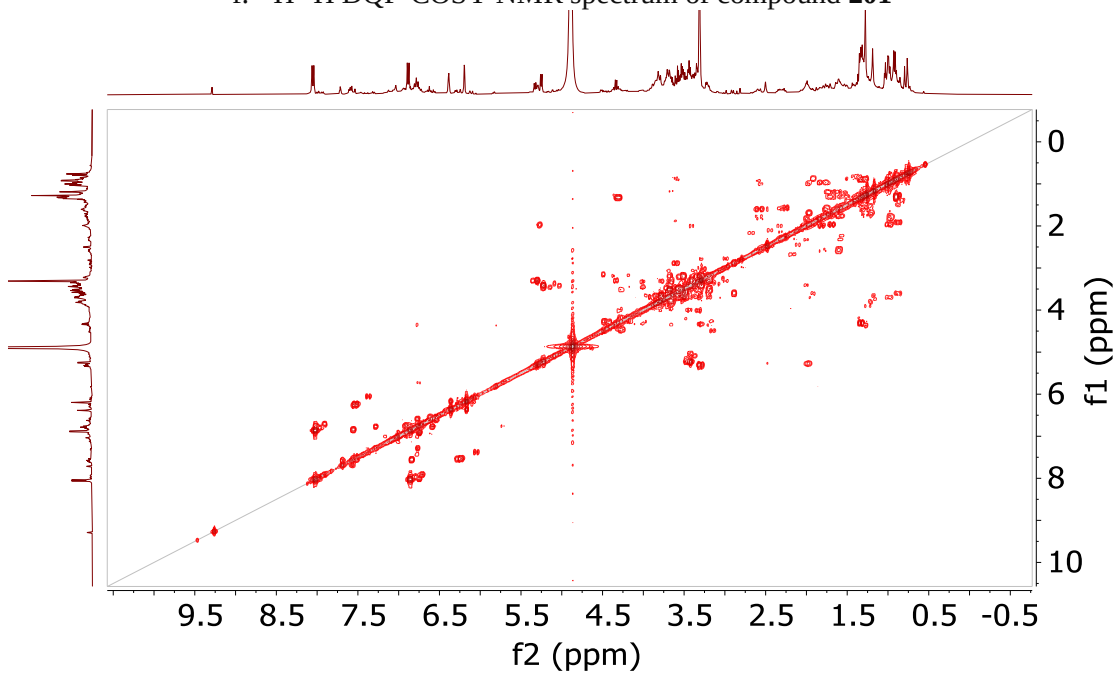
d. ^1H - ^{13}C HSQC NMR spectrum compound **201**



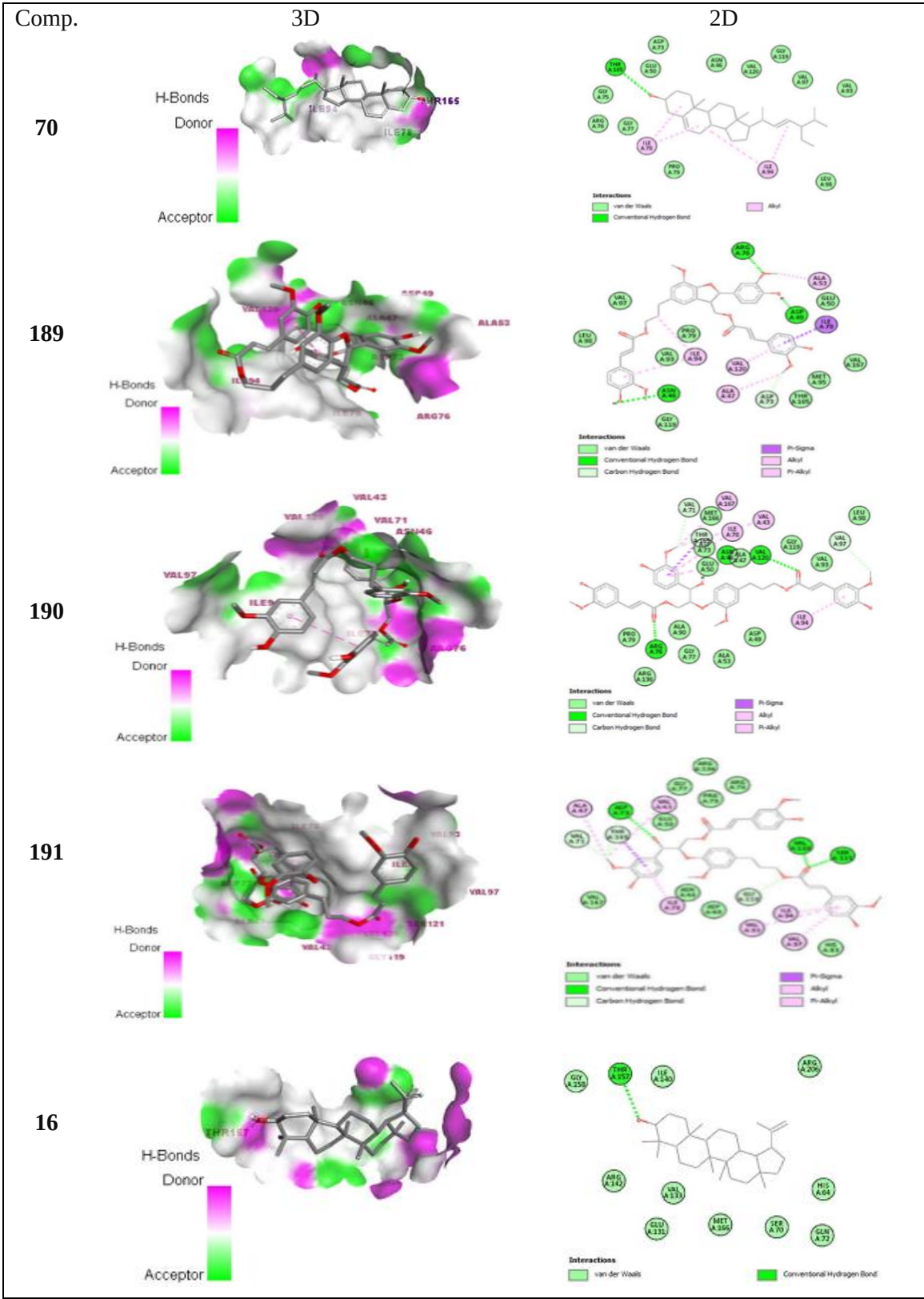
e. ^1H - ^{13}C HMBC NMR spectrum of compound **201**



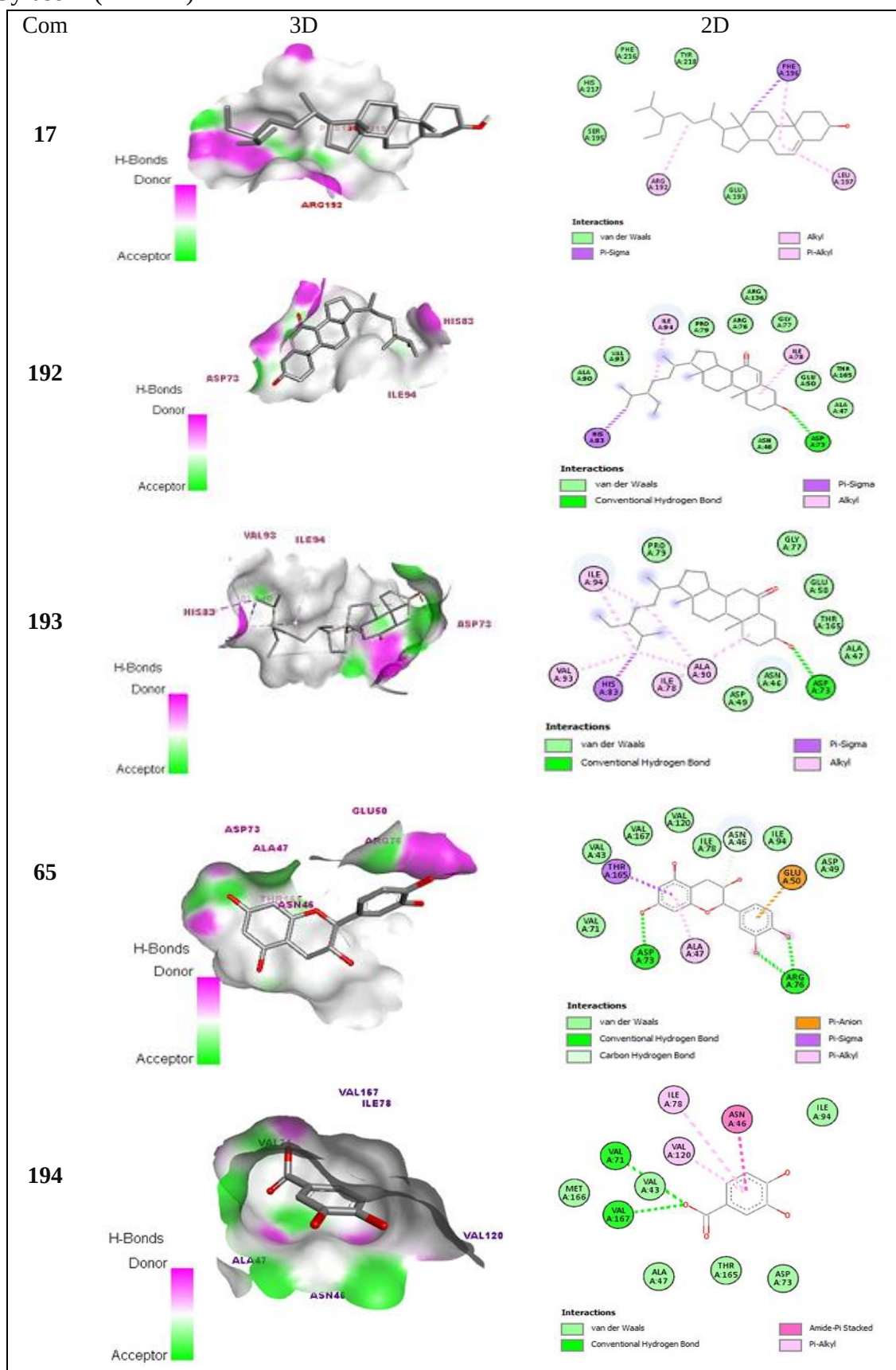
f. ^1H - ^1H DQF-COSY NMR spectrum of compound **201**



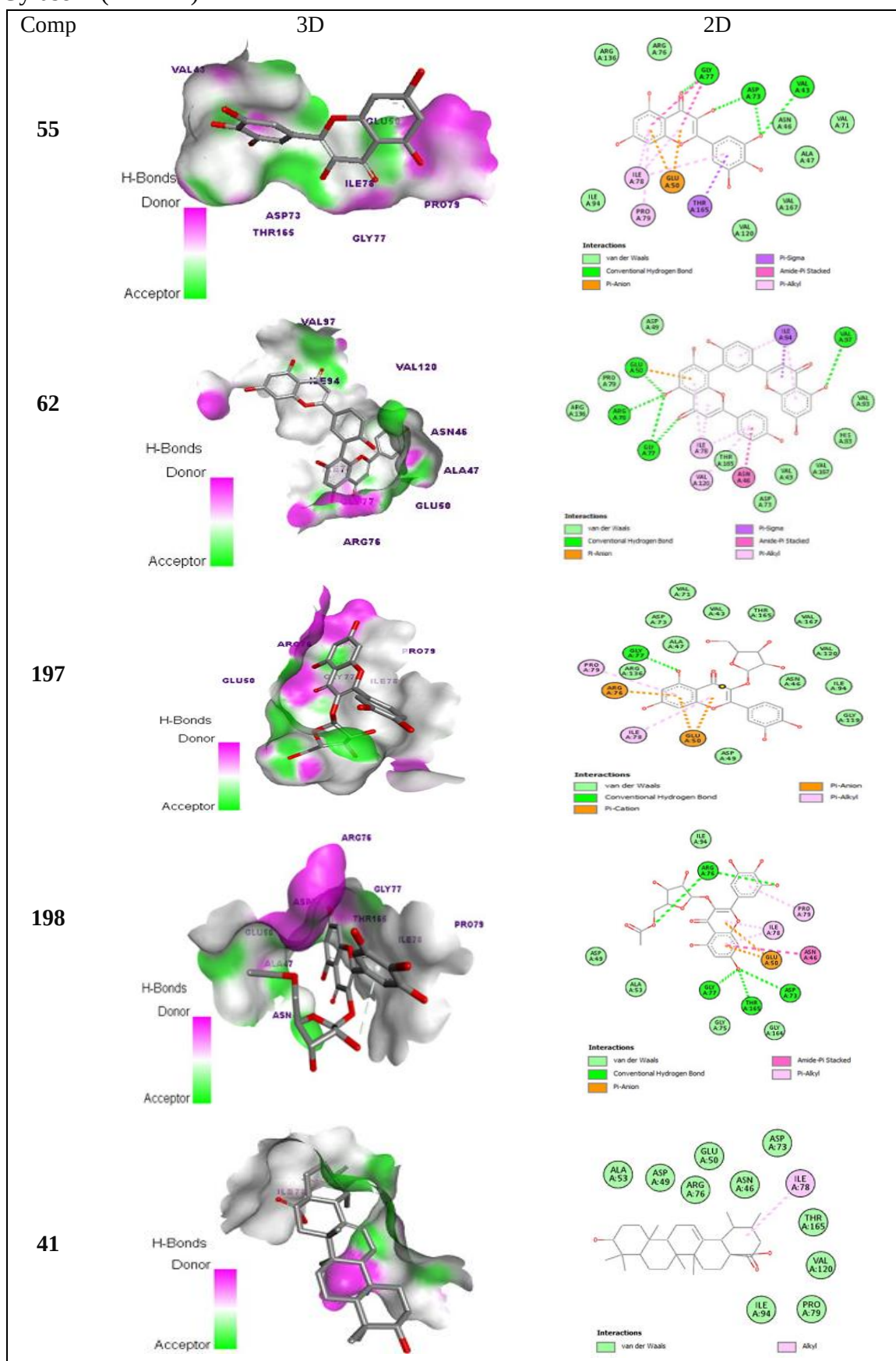
Appendix 27. The 3D and 2D modes of interactions of isolated compounds against *E. coli* Gyrase B



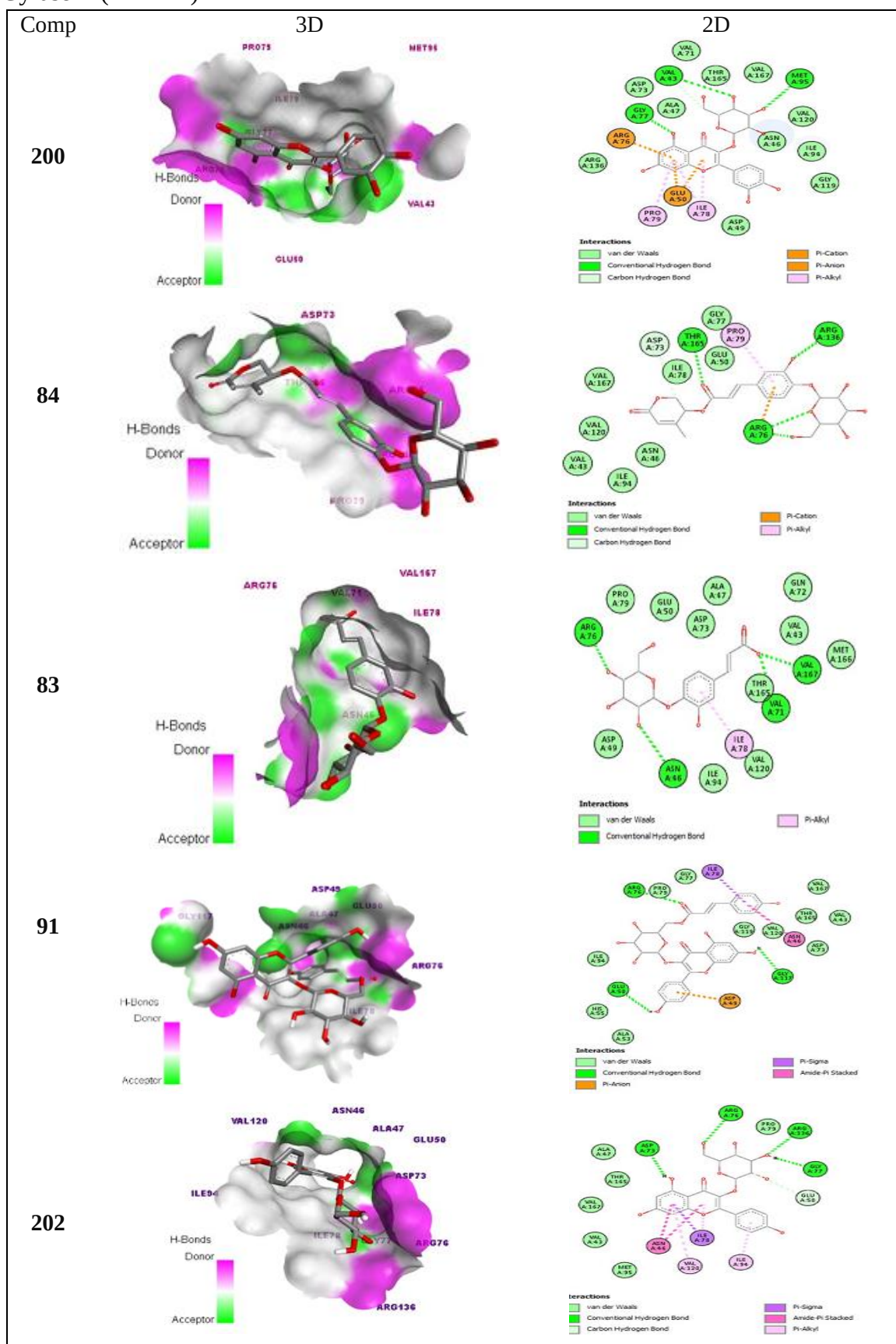
Appendix 27. The 3D and 2D modes of interactions of isolated compounds against *E. coli* Gyrase B (Contn'd)



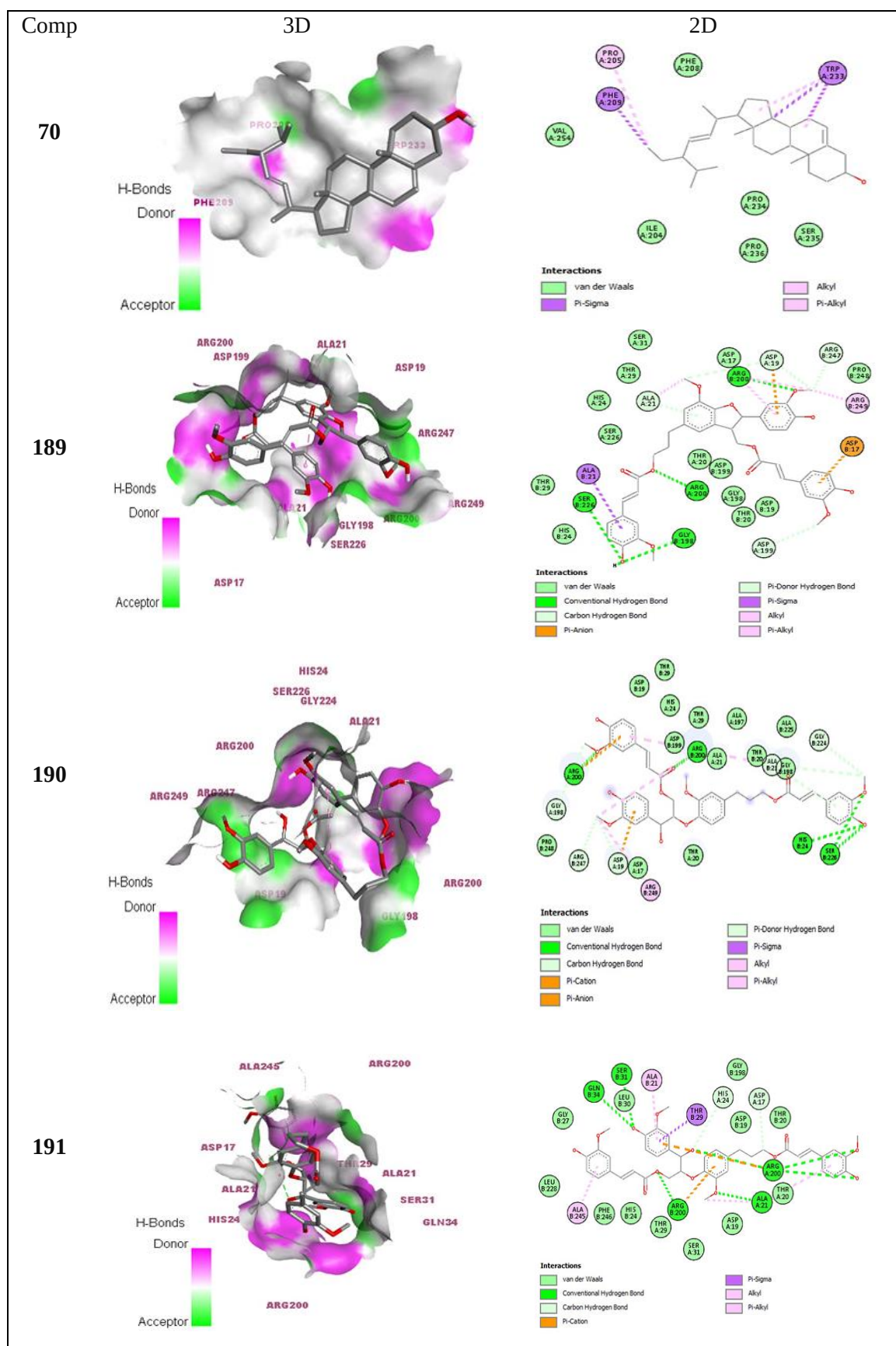
Appendix 27. The 3D and 2D modes of interactions of isolated compounds against *E. coli* Gyrase B (Contn'd)



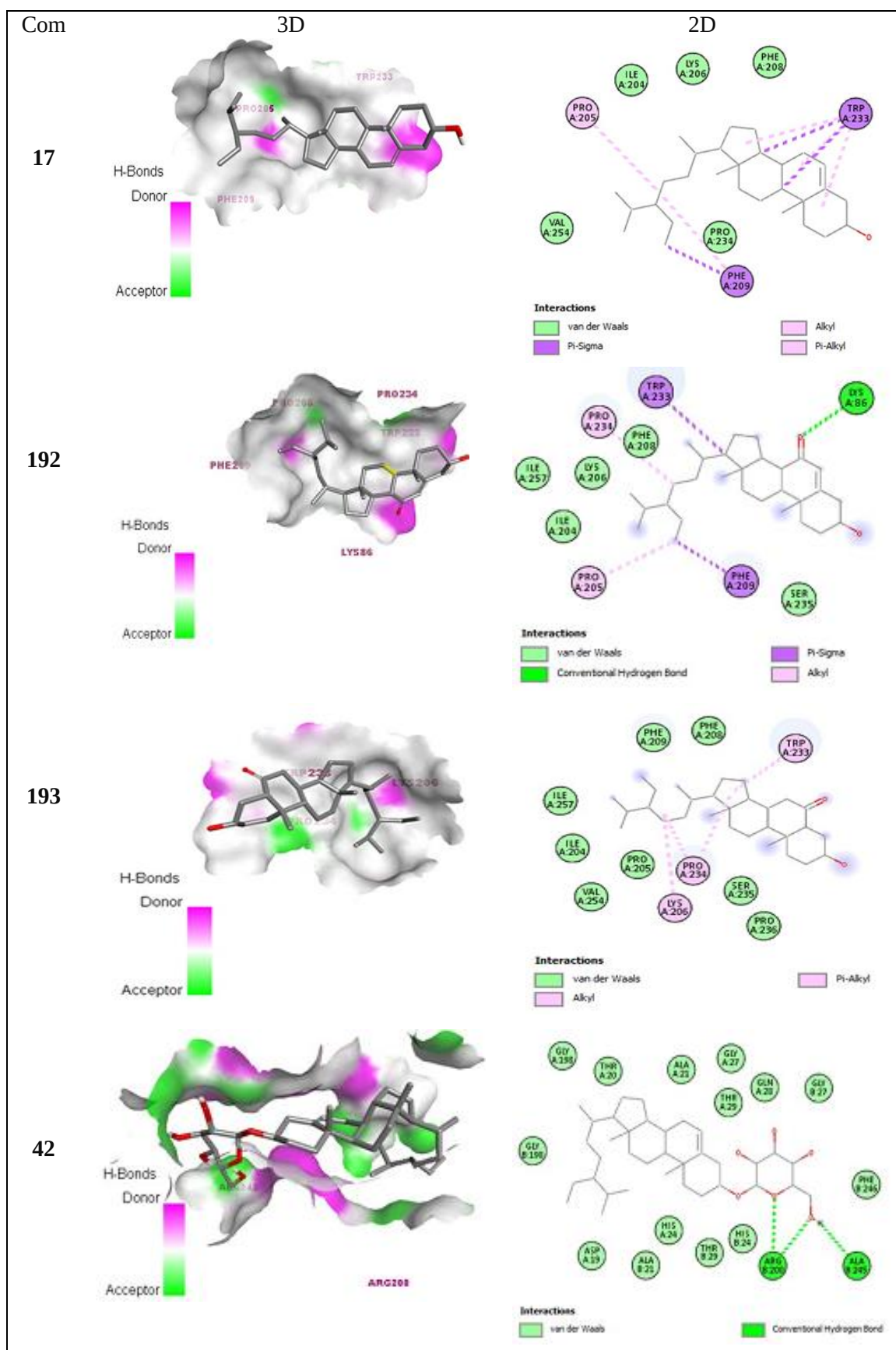
Appendix 27. The 3D and 2D modes of interactions of isolated compounds against *E. coli* Gyrase B (Contn'd)



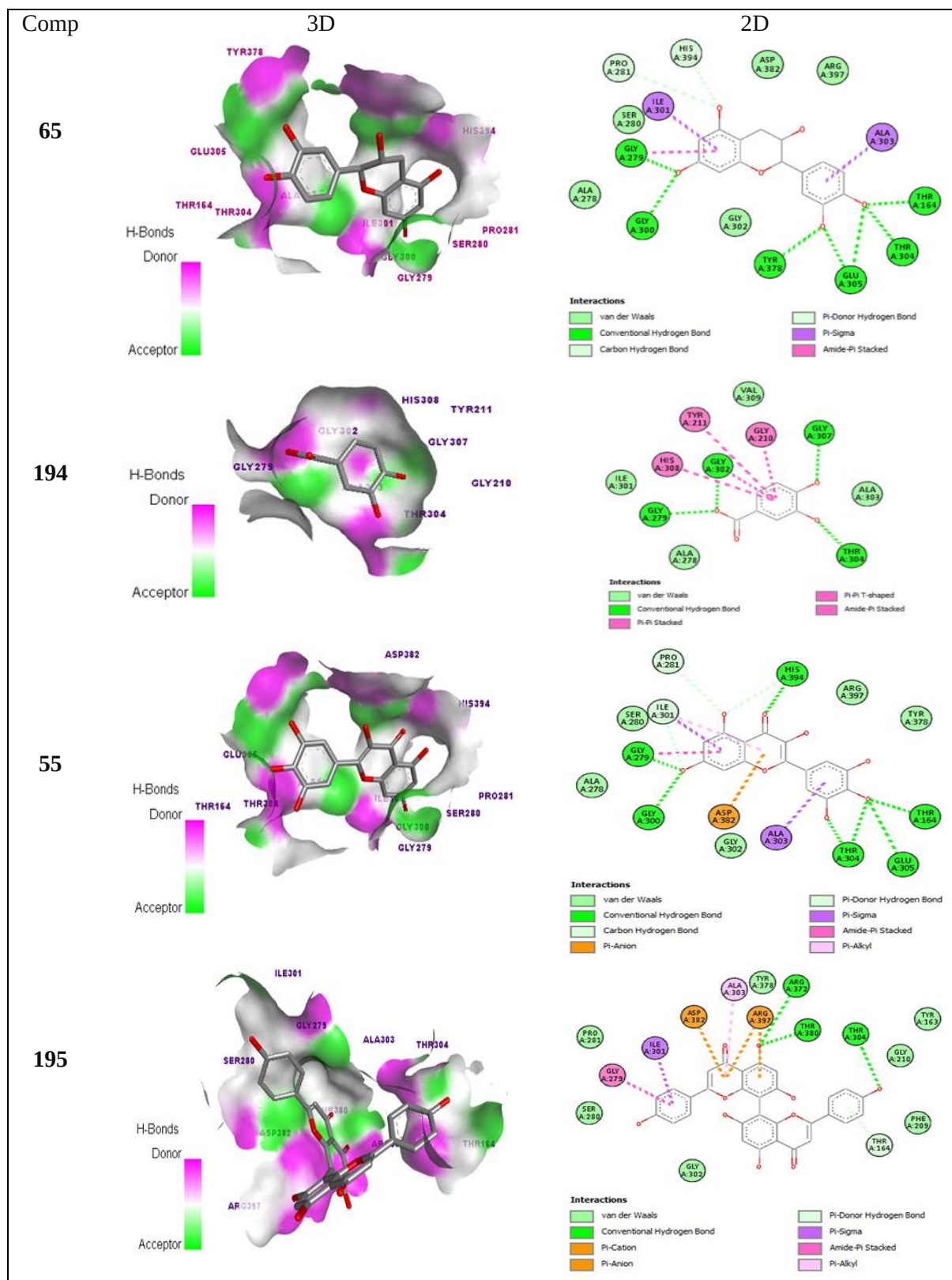
Appendix 28. The 3D and 2D modes of interactions of isolated compounds against PqsA



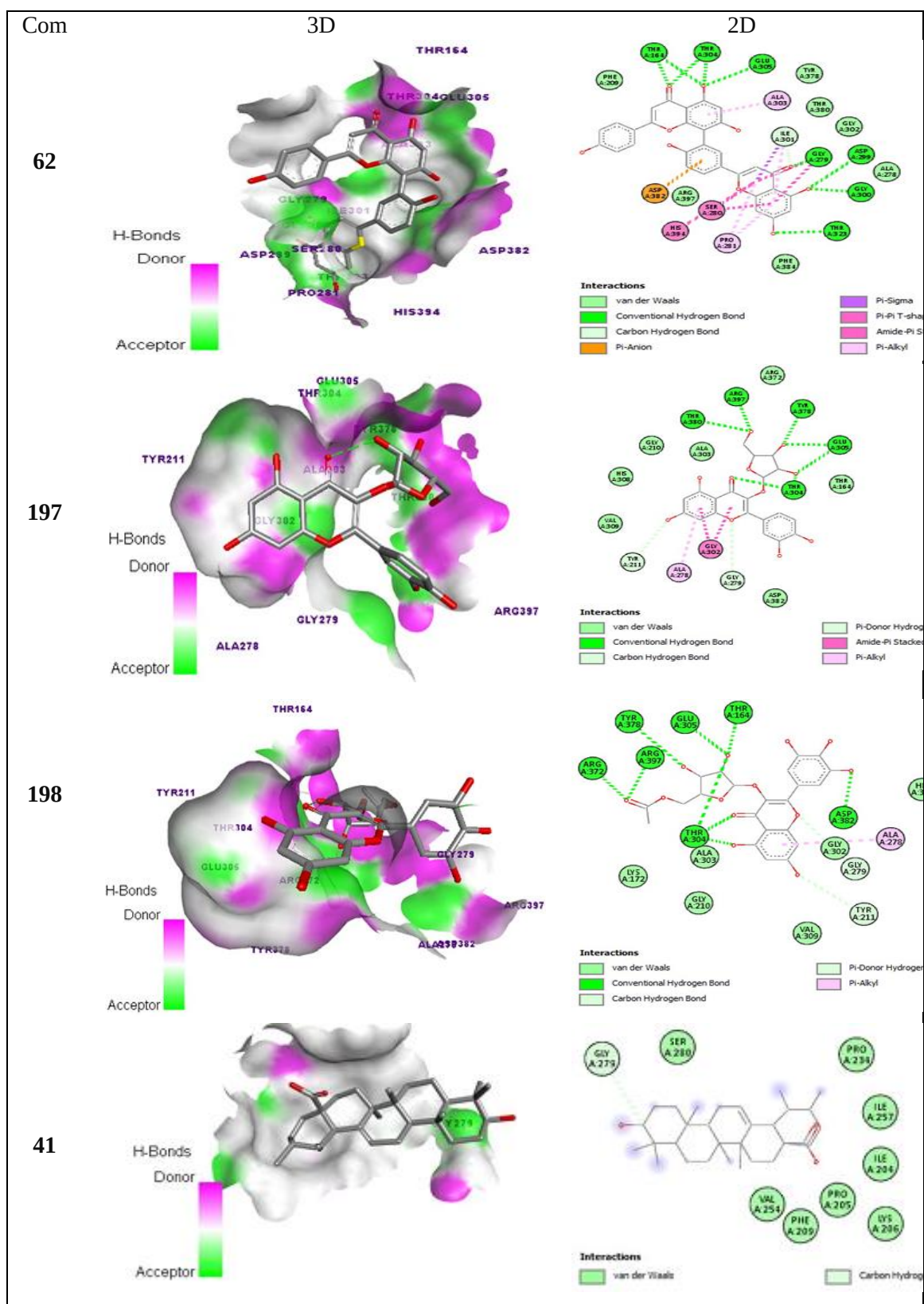
Appendix 28. The 3D and 2D modes of interactions of isolated compounds against PqsA
(Contn'd)



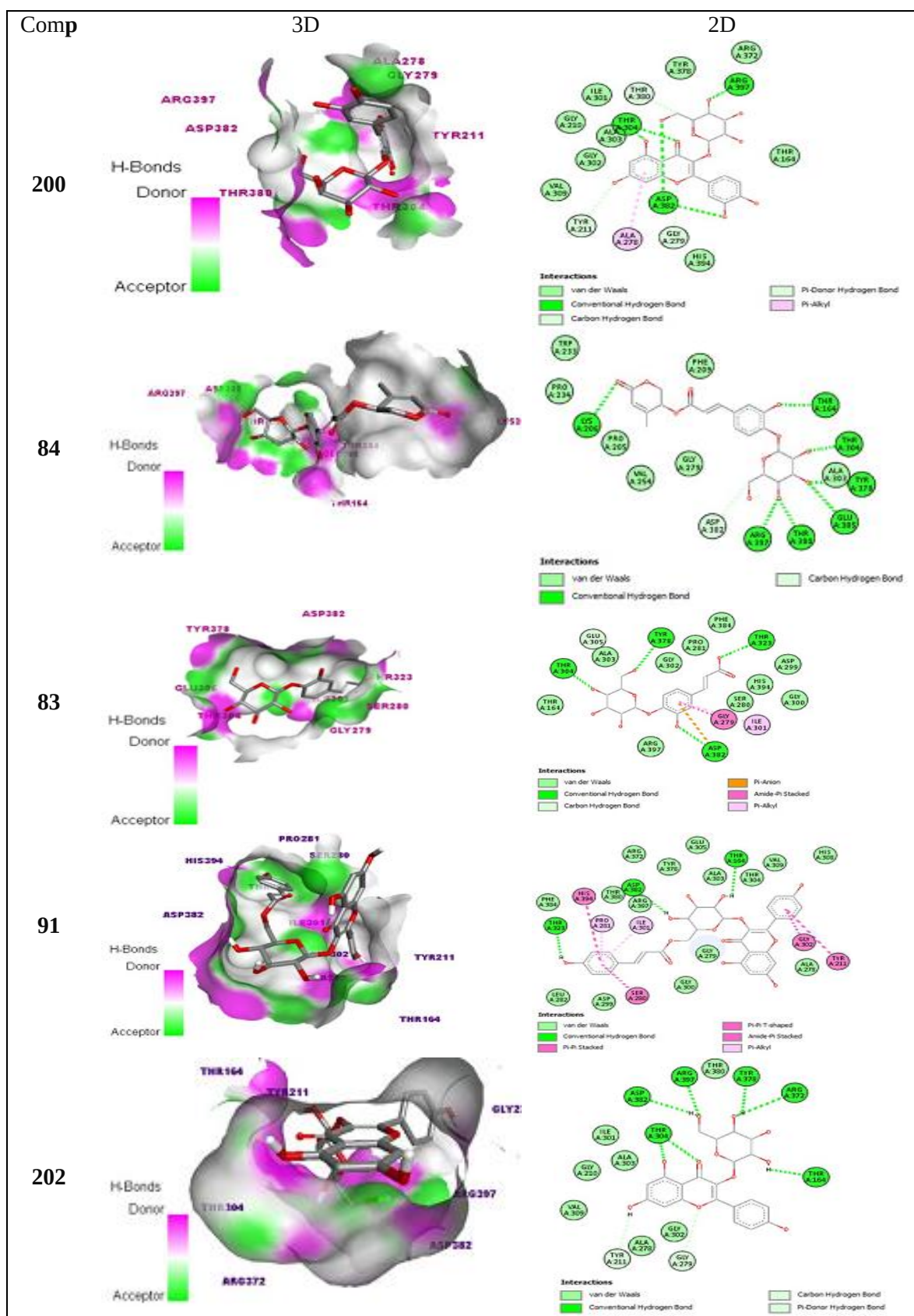
Appendix 28. The 3D and 2D modes of interactions of isolated compounds against PqsA
(Contn'd)



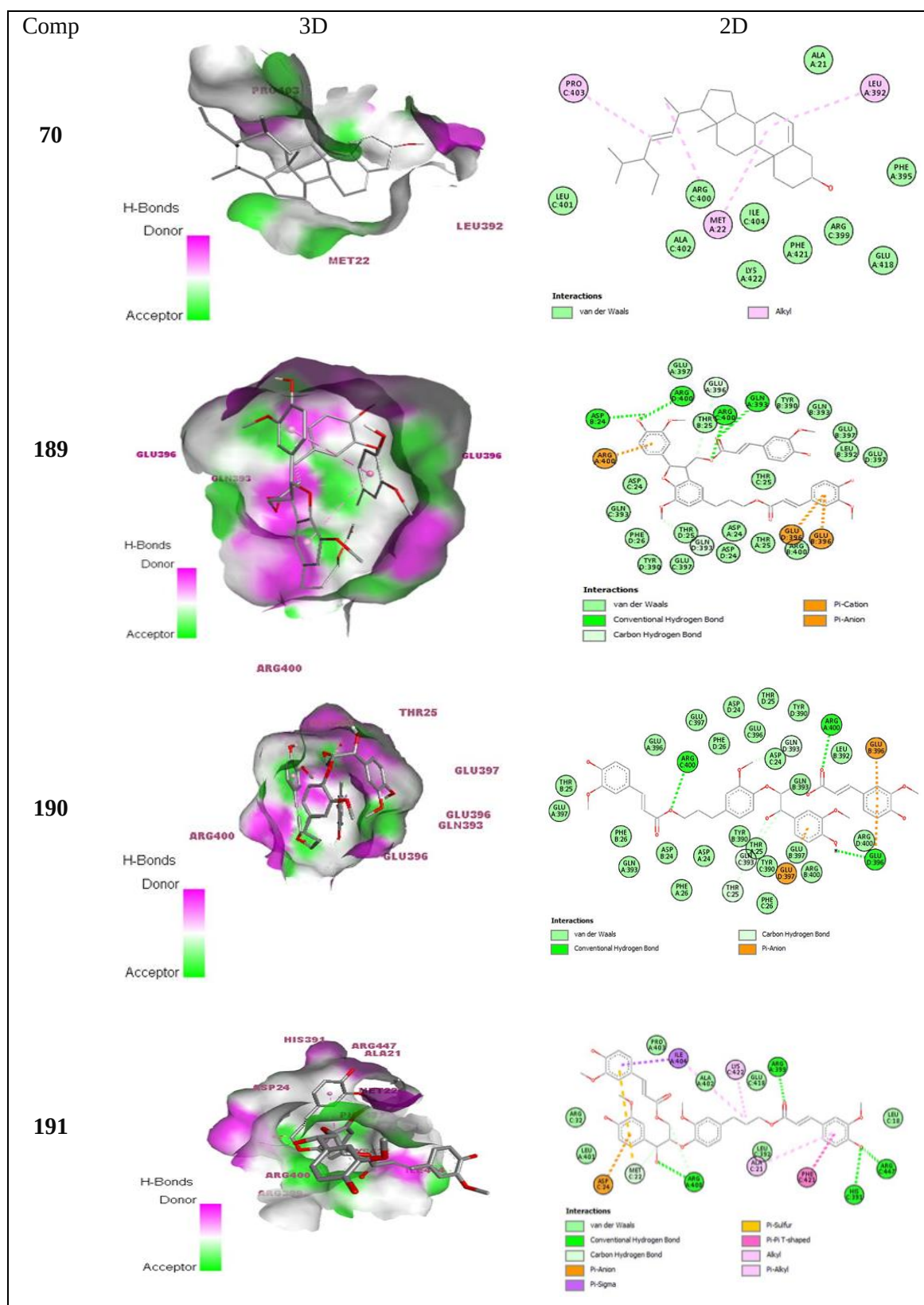
Appendix 28. The 3D and 2D modes of interactions of isolated compounds against PqsA
(Contn'd)



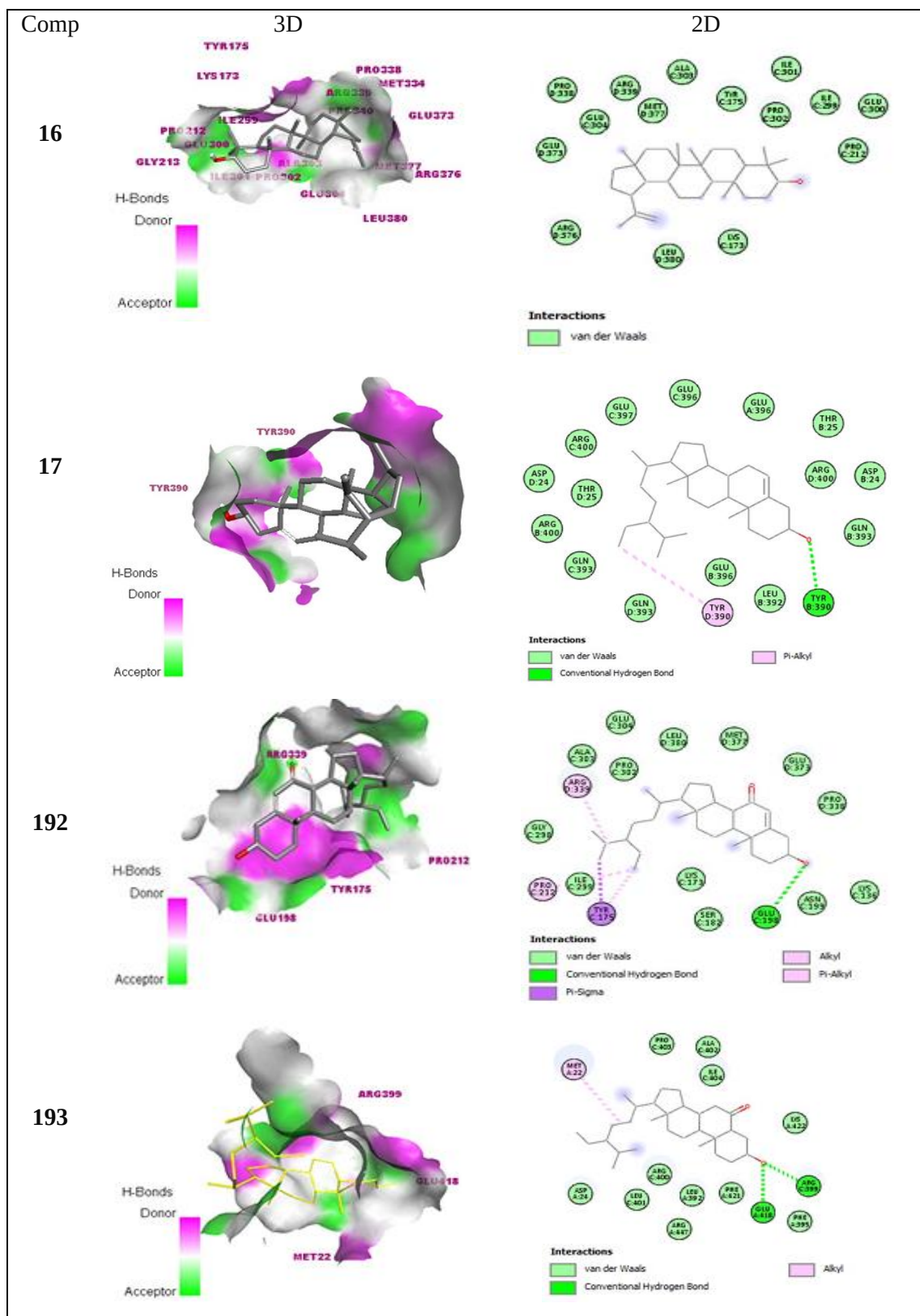
Appendix 28. The 3D and 2D modes of interactions of isolated compounds against PqsA
(Contn'd)



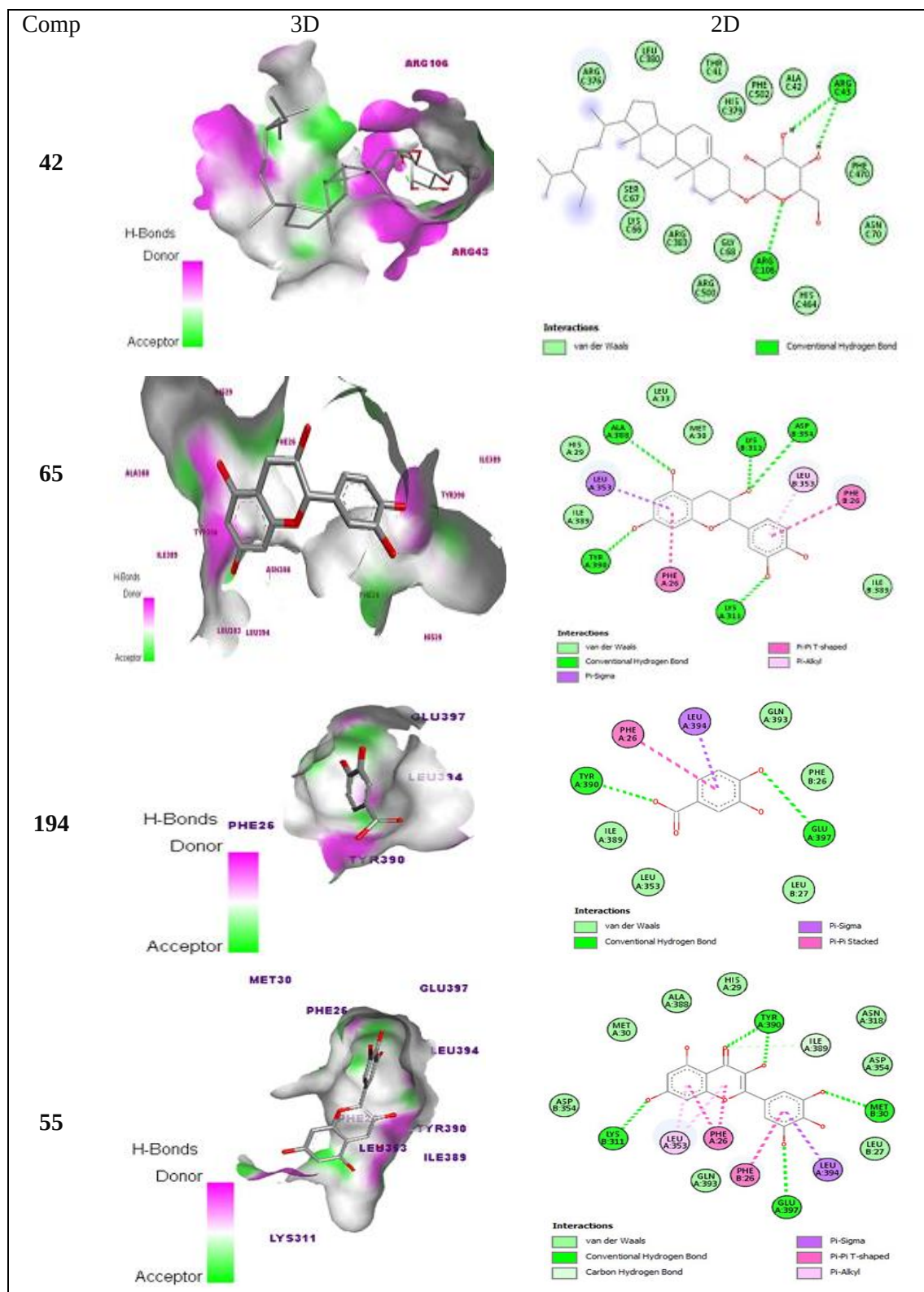
Appendix 29. The 3D and 2D modes of interactions of isolated compounds against PKM2



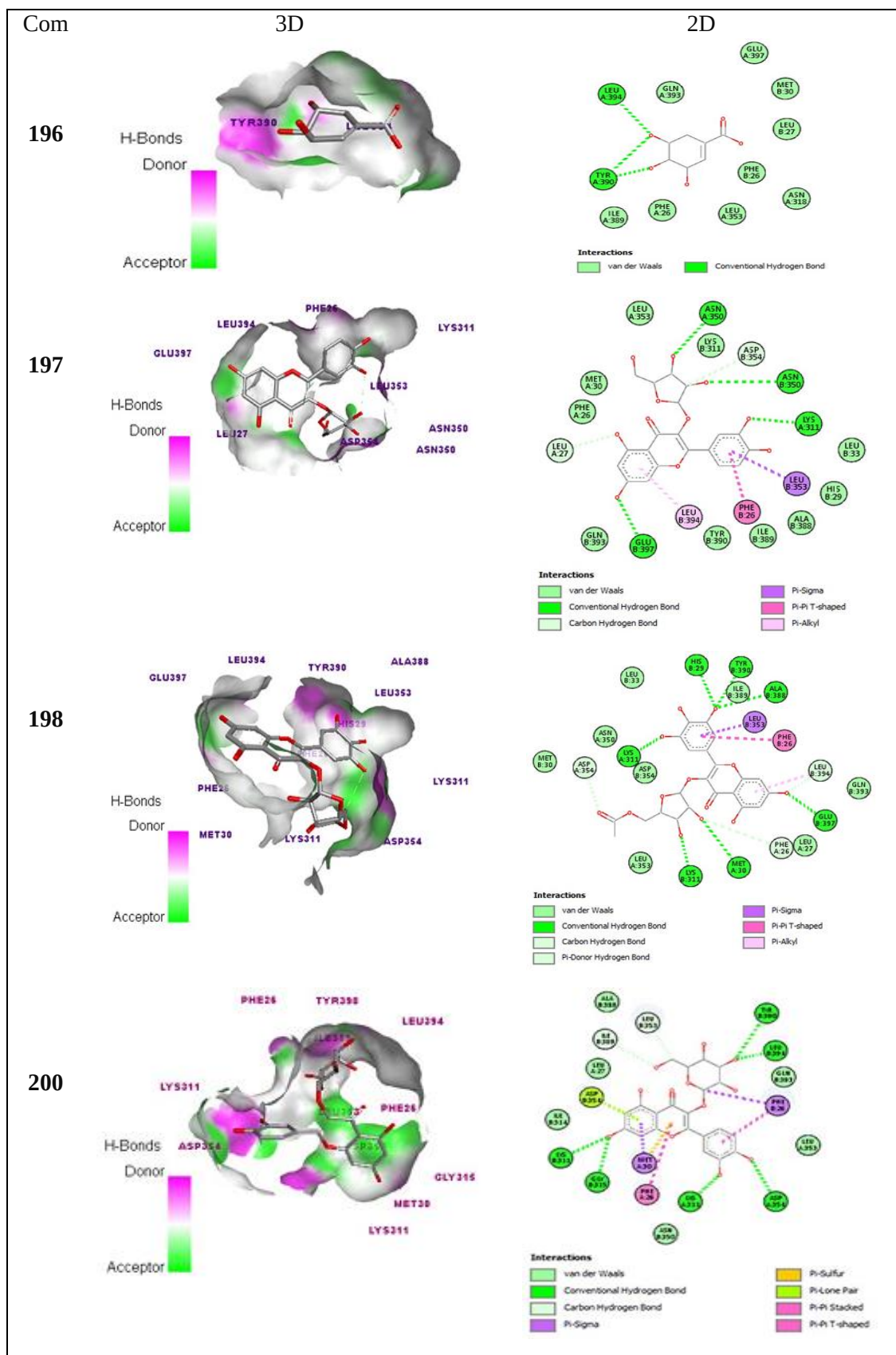
Appendix 29. The 3D and 2D modes of interactions of isolated compounds against PKM2
(Contn'd)



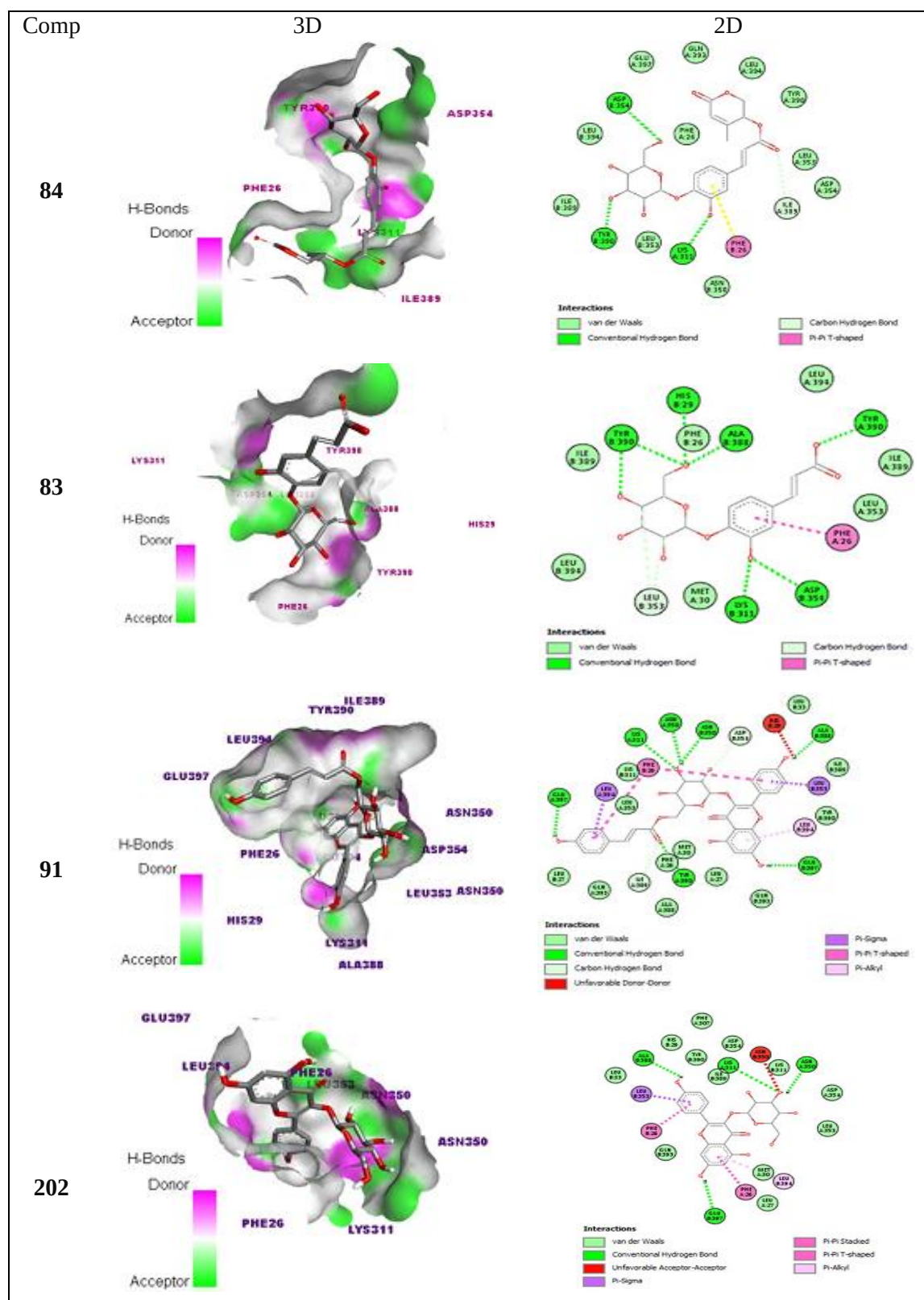
Appendix 29. The 3D and 2D modes of interactions of isolated compounds against PKM2
(Contn'd)



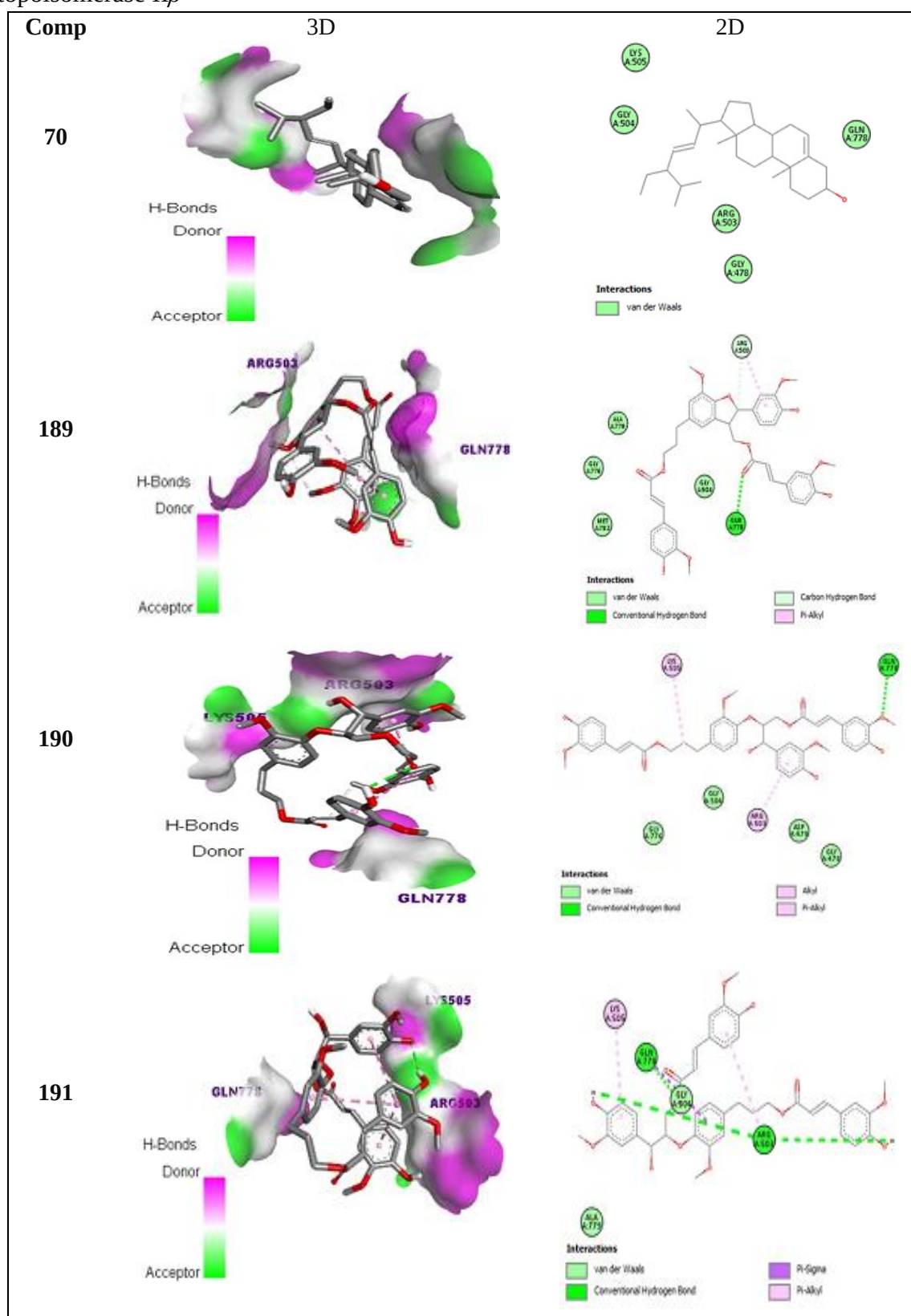
Appendix 29. The 3D and 2D modes of interactions of isolated compounds against PKM2
(Contn'd)



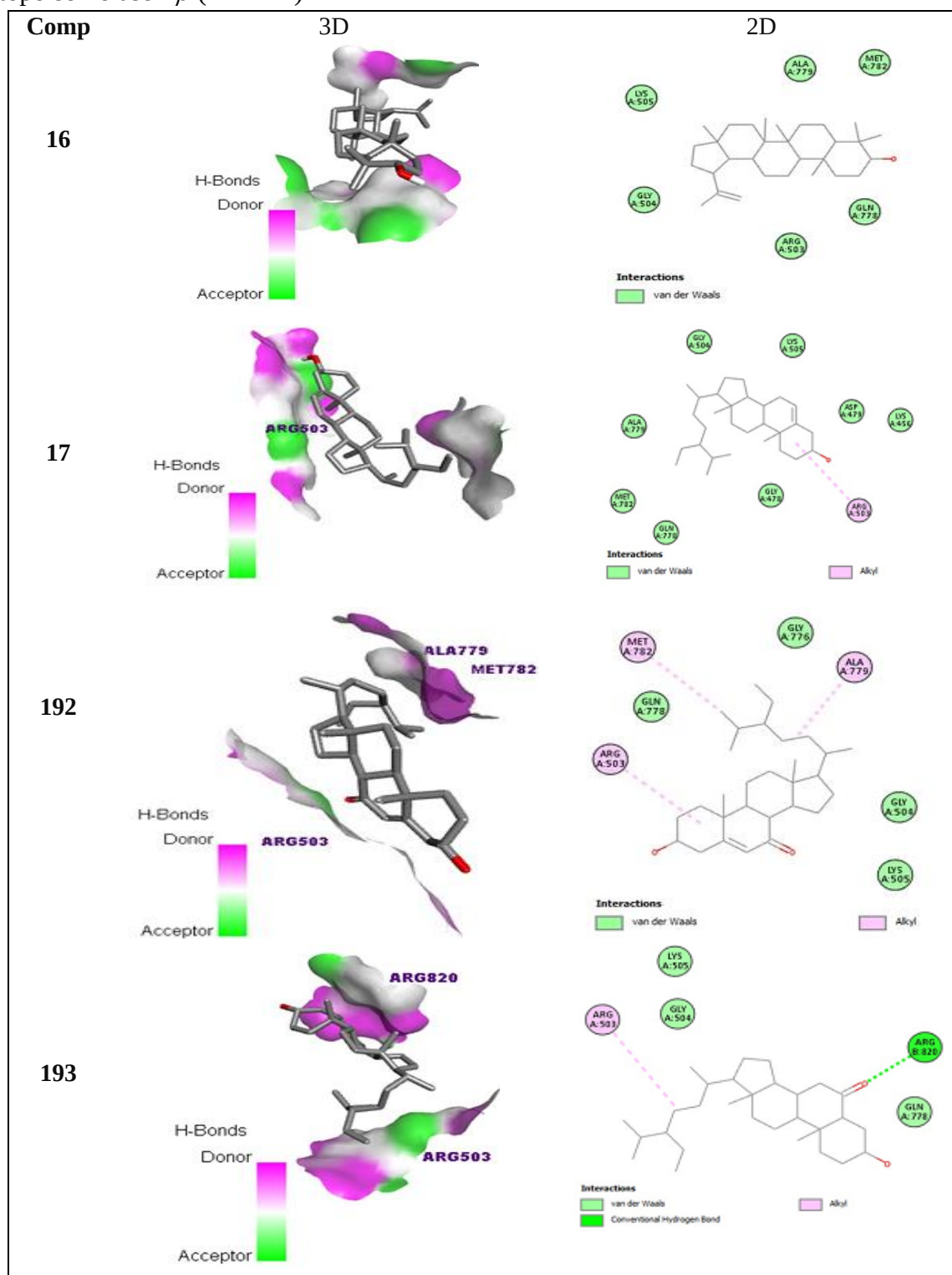
Appendix 29. The 3D and 2D modes of interactions of isolated compounds against PKM2
(Contn'd)



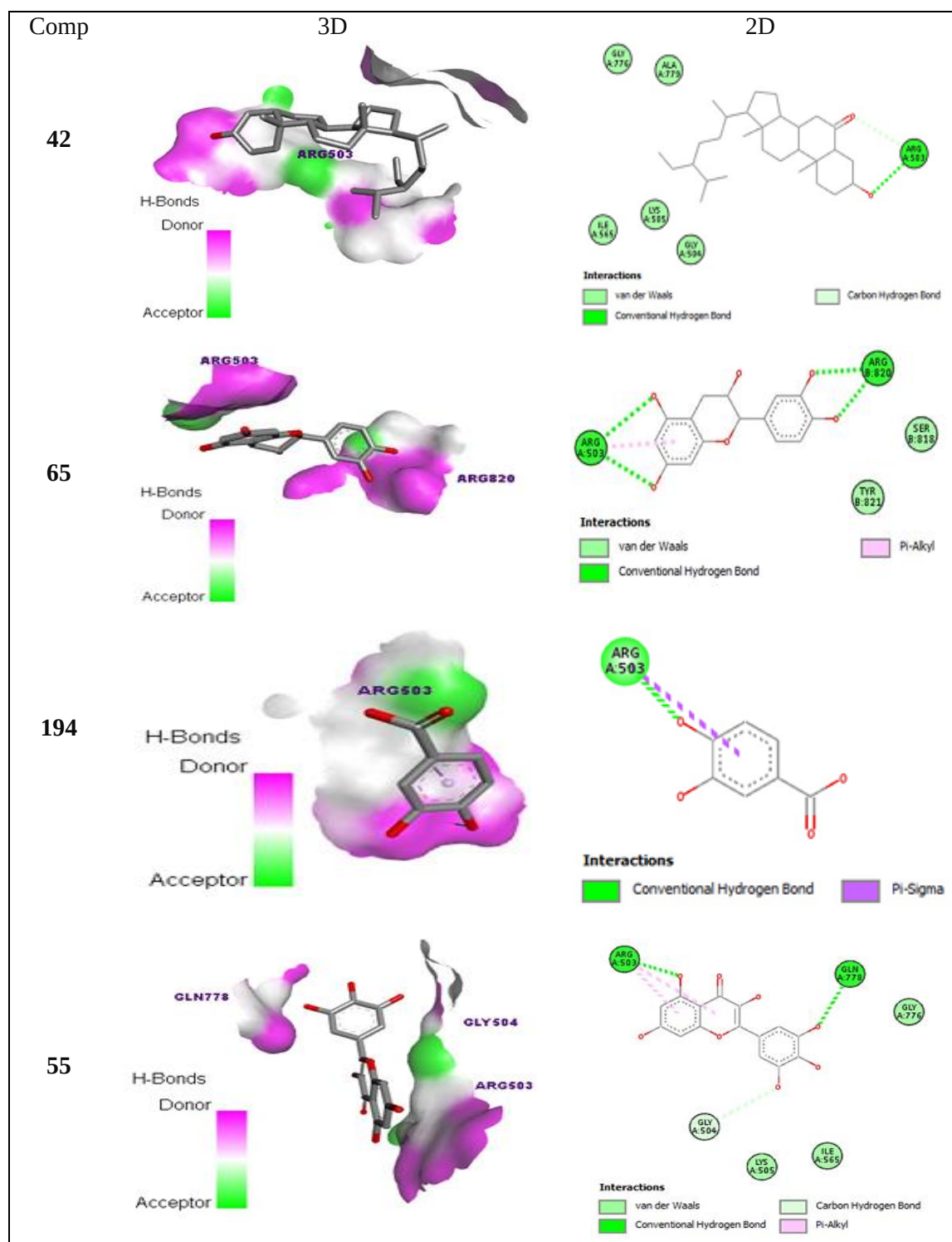
Appendix 30. The 3D and 2D modes of interactions of isolated compounds against human topoisomerase II β



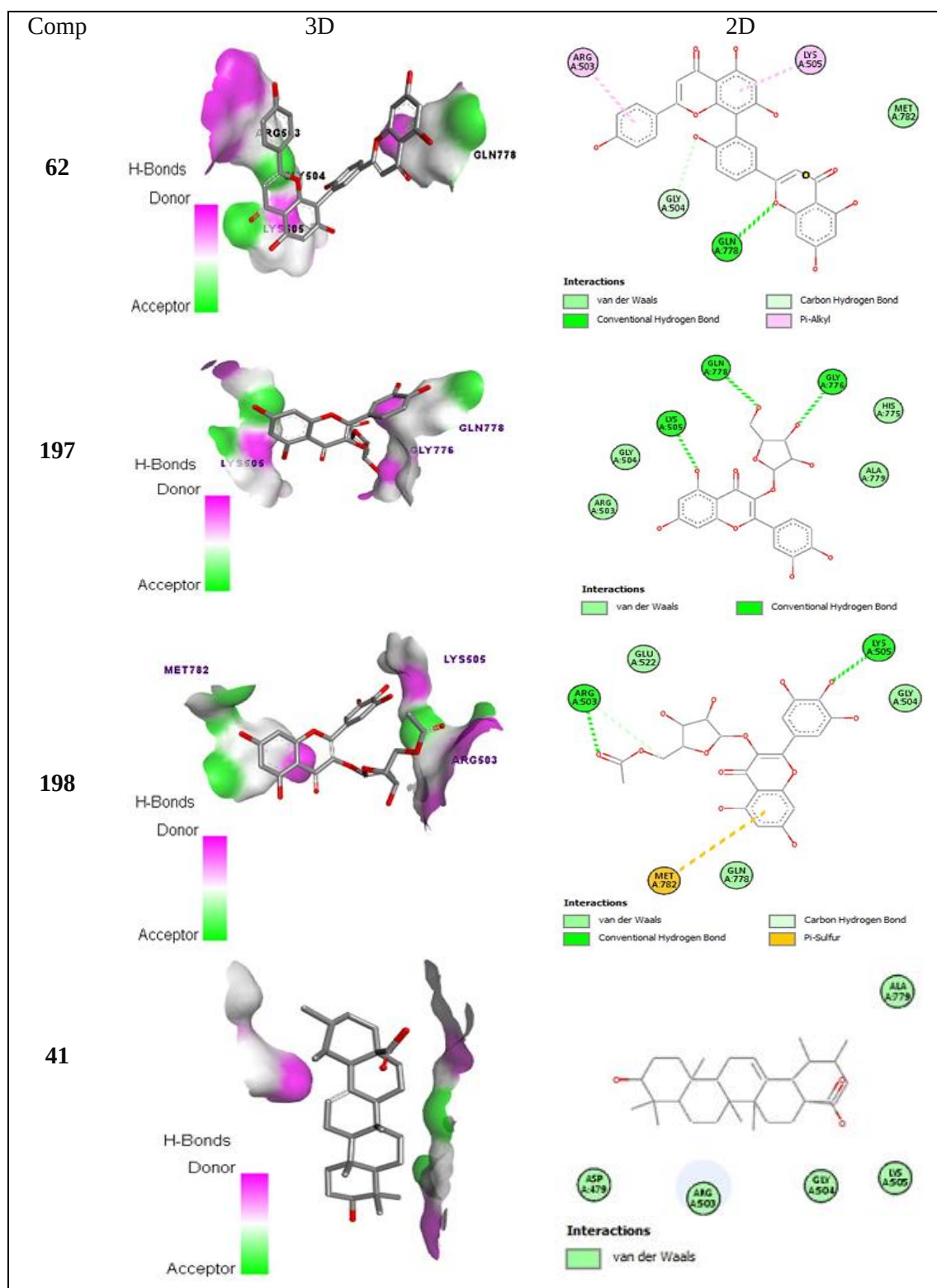
Appendix 30. The 3D and 2D modes of interactions of isolated compounds against human topoisomerase II β (Contn'd)



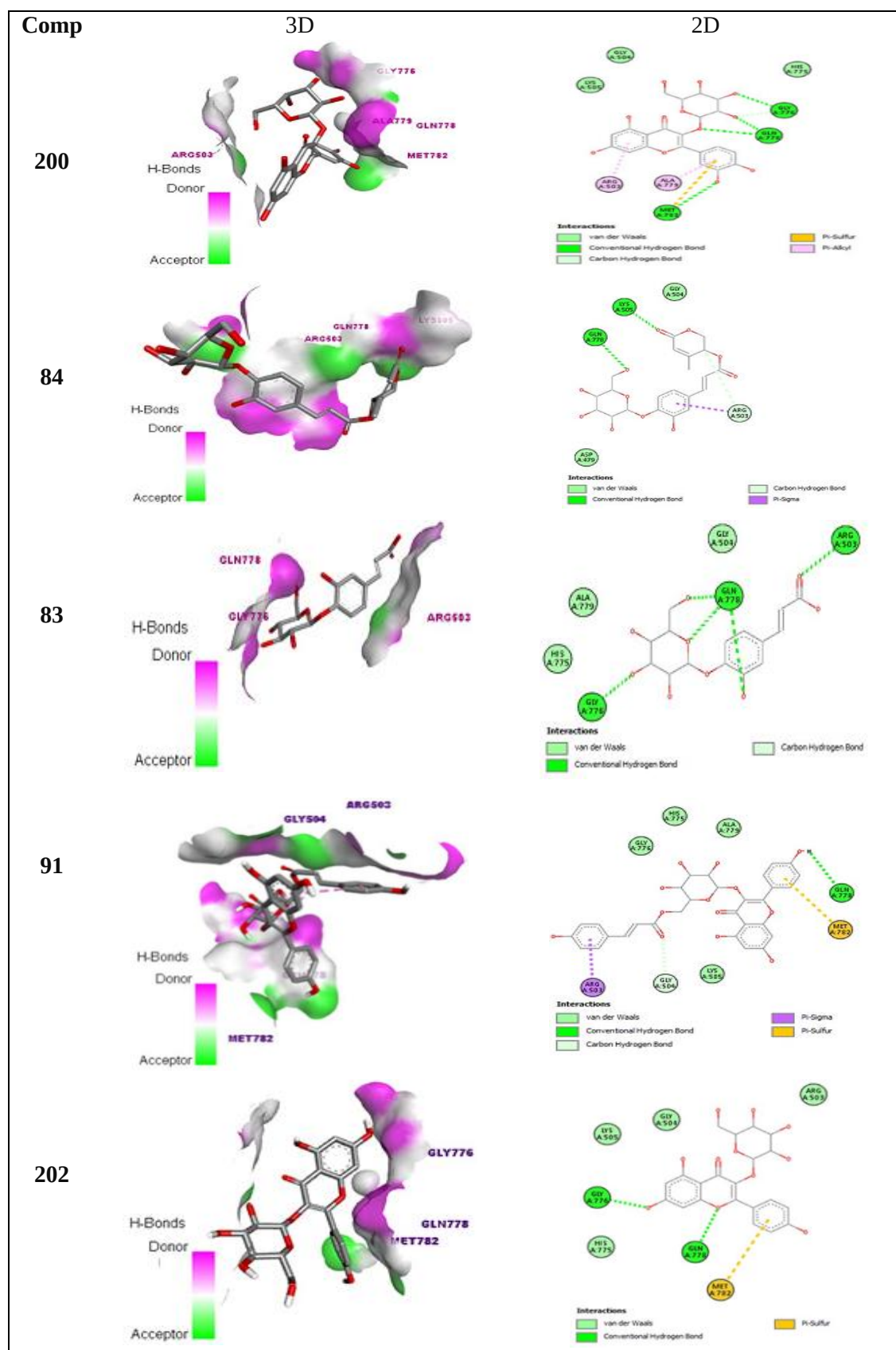
Appendix 30. The 3D and 2D modes of interactions of isolated compounds against human topoisomerase II β (Contn'd)



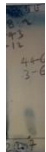
Appendix 30. The 3D and 2D modes of interactions of isolated compounds against human topoisomerase II β (Contn'd)



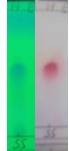
Appendix 30. The 3D and 2D modes of interactions of isolated compounds against human topoisomerase II β (Contn'd)

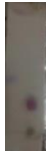


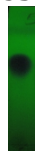
Appendix 31. TLC profiles of isolated compounds

Plant name/Part	Compound number and TLC			
	188	145	70&17	189-191
<i>C. hirsuta</i> /Root				

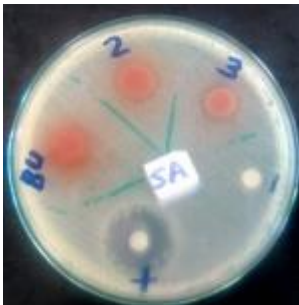
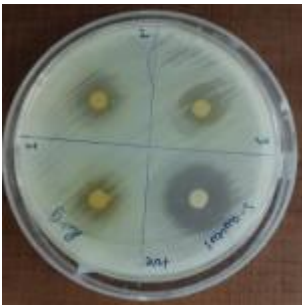
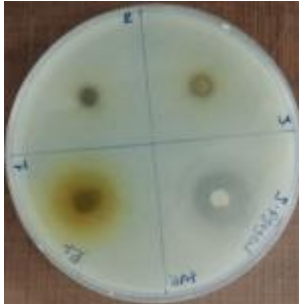
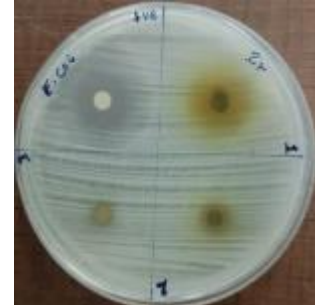
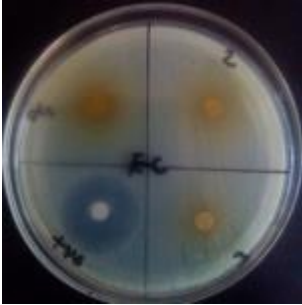
Plant name/Part	Compound number and TLC			
	41	199	200, 84, &83	83
<i>O. rochetiana</i> /Leaves				

Plant name/Part	Compound number and TLC				
	16	17&70	42	192&193	65&69
<i>G. ferruginea</i> /Stem bark					

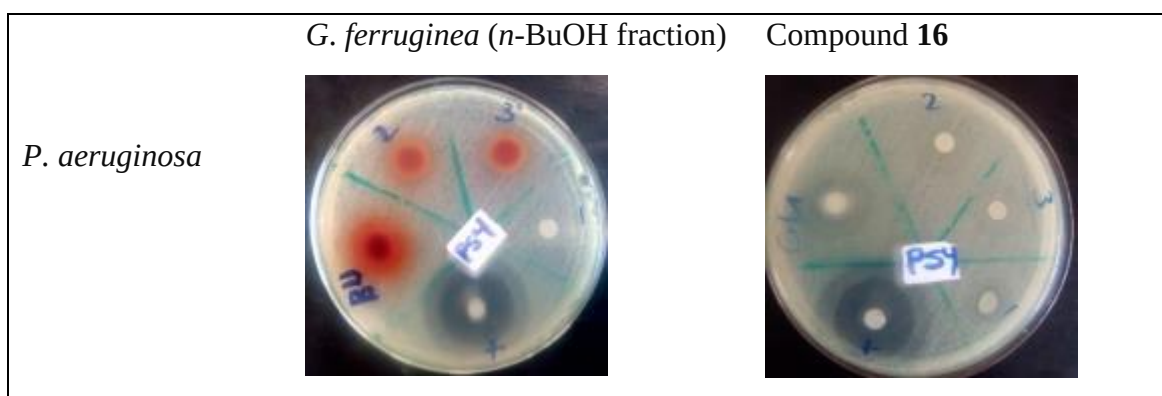
Plant name/Part	Compound number and TLC		
	145&17	201	91&202
<i>R. apetalus</i> /Leaves			

Plant name/Part	Compound number and TLC					
	145	195	55	195&62	194	197&198
<i>R. ruspilii</i> /Leaves						

Appendix 32. Selected inhibition zones of antibacterial activity test

Bacteria	Sample and inhibition zones	
<i>S. aureus</i>	<i>G. ferruginea</i> (n-BuOH fraction)	Compound 197
		
<i>S. pyogenes</i>	<i>R. ruspolii</i> (DCM:MEOH, 1:1) extract	Compound 17
		
<i>E. coli</i>	<i>R. ruspolii</i> (DCM:MEOH, 1:1) extract	<i>O. rochetiana</i> (DCM:MEOH, 1:1) extract
		

Appendix 32. Selected inhibition zones of antibacterial activity test (Contn'd)



Papers Published From the Work

Duguma, T., Melaku, Y., Garg, A., & Ensermu, U. (2024). *In Vitro* Antibacterial, DPPH Radical Scavenging Activities, and *In Silico* Molecular Modeling of Isolated Compounds from the Roots of *Clematis hirsuta*. *Advances in pharmacological and pharmaceutical sciences*, 2024, 3152929. <https://doi.org/10.1155/2024/3152929>

Duguma, T., Melaku, Y., Rentsch, D., Terfa, A. & Shenkute, K. (2024). *In vitro* antibacterial activities, DPPH radical scavenging, and molecular simulation of isolated compounds from the leaves of *Rhus ruspolii*. *Zeitschrift für Naturforschung C*. <https://doi.org/10.1515/znc-2024-0127>