PHYTOCHEMICAL ANALYSIS, BIOLOGICAL ACTIVITY AND MOLECULAR DOCKING STUDIES OF ROOT EXTRACTS OF *LOBELIA RHYNCHOPETALUM* HEMSL.

Abu Jeilu Sani

A Thesis Submitted to

The Department of Applied Chemistry

School of Applied Natural Sciences

Presented in Partial Fulfillment of the Requirement for the Degree of

Master's in Applied Chemistry (Medicinal Chemistry)

Office of Graduate Studies

Adama Science and Technology University

March, 2022

Adama, Ethiopia

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Approval Sheet

I/we, the advisors of the thesis entitled **"Phytochemical Analysis, Biological Evaluation and Docking Studies of Root Extracts of *Lobelia Rhynchopetalum* Hemsl."** and developed by Abu Jeilu Sani hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

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We, the undersigned, members of the Board of Examiners of the thesis by Abu Jeilu Sani have read and evaluated the thesis entitled "Phytochemical Analysis, Biological Evaluation and Docking Studies of Root Extracts of *Lobelia Rhynchopetalum* Hemsl."and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Chemistry.

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Declaration

I hereby declare that this Master Thesis entitled "Phytochemical Analysis, Biological Evaluation and Docking Studies of Root Extracts of *Lobelia Rhynchopetalum* Hemsl." 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 that are used for this thesis have been duly acknowledged through citation.

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Recommendation

I/We, the advisor of the thesis, hereby certify that, we have read the revised version of the thesis entitled "Phytochemical Analysis, Biological activity and Docking Studies of Root Extracts of *Lobelia rhynchopetalum* Hemsl." Prepared under our guidance by Abu Jeilu submitted in partial fulfillment of the requirements for the degree of Master's of science in Medicinal chemistry. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Major advisor	Signature	Date
Co-advisor	Signature	Date

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LIST OF ABBREVIATION

1D	One Dimension
DEPT	Distortionless Enhancement by Polarization Transfer
IC_{50}	Half maximal Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet Visible
CLSI	Clinical and Laboratory Standard Institute
NicAchR	Nicotinic Acetylcholine Receptor
ROS	Reactive Oxygen Species
DPPH	1, 1-diphenyl-2-picryl-hydrazyl

ABSTRACT

*Lobelia rhynchopetalum, commonly known as Jibara (Amharic),Terura (Afan Oromo), which is endemic to Ethiopia, has been traditionally used by the Arsi people for the treatment of various diseases such as leprosy, wound, evil eye, retained placenta, diarrhea, fever, eye disease, rabies and tumor/cancer. In view of its traditional uses and the absence of scientific reports so far, an attempt was made to explore the chemical, antibacterial and antioxidant potential of the solvent extracts of the roots of L. rhynchopetalum. In this regard, the roots (350 g) were successively extracted with n-hexane separately, the solution was filtered, and the defatted residue was extracted with dichloromethane/methanol (1:1) and methanol to give 13.40 g and 7.3 g (3.83 % and 2.08%) yields, respectively. Phytochemical screening analysis conducted on roots extracts of L. rhynchopetalum showed the presence of alkaloids, anthraquinone, tannins, terpenoids, anthraquinoneglycosides, cardiac glycosides and anthranol glycosides. Silica gel column chromatographic separation of the crude extract of dichloromethane: methanol (1:1) afforded a polyacetylenic alcohol, lobetyol (**1**), a triterpenoid, 24-ethyl-3-hydroxylcholesta-5,25-diene (**2**), a pyrolidine alkaloid, lobechine (**3**), a fatty acid lauric acid (**4**), anthraquinones, chrysophanol (**5**) and physcion (**6**). Since the plant has never been investigated for its phytochemical constituents, this is the first report of isolation of these compounds from the Arsi-Bale Mountains plant. Moreover, chrysophanol and physcion are identified from the species for the first time. The structures of compounds were characterized using spectroscopic methods (^{1}H NMR, ^{13}C NMR, and DEPT-135). The extracts and isolated compounds were also evaluated in vitro for their antibacterial activities using disc diffusion method against E. coli, S. aureus, and S. pyogenes. Strong inhibition zone values ((15±1,14.33±0.58, 15.67±0.58 mm for lobetyol (**1**) were observed against E.coli, S.aureus and S.pyogenes respectively, when compared to standard ciprofloxacin (19±1, 17.33±1.53 and 19.67±0.58 mm). The DPPH radical scavenging activities (%) of extracts and isolated compounds were found to be 71.6 (compound **1**), 27.2 (compound **2**), 70 (DCM: MeOH (1:1) extract), 60.9 (Acid- base extract**)** and 67.2 (compound **3**) respectively at 100 µg/ml, Suggesting that compound **1** and **3** displayed slightly highest radical scavenging activity indicating the potential of the plant as herbal remedies.*

***Keywords**:- Antibacterial activity, Phytochemical screening, Lobelia rhynchopetalum, antioxidant activity, Moleculardocking.*

CHAPTER ONE

1. INTRODUCTION

1.1. Background of the Study

Plants have been utilized as medicines for thousands of years in different countries and are a source of many potent and powerful drugs worldwide. This is mainly because most of these herbals are accessible, affordable and the extracted chemicals have little or no side effects as compared to drugs synthesized in the laboratory. Plants comprise the largest component of the diverse therapeutic elements of traditional health care practices both in humans and animals. The medicinal values of plants are due to the chemical substances that produce a definite physiological action on human body and are called phytochemicals. They are chemicals extracted from plants and the term is often used to describe the large number of secondary metabolic compounds found in plants (Banu and Cathrine, 2015). Naturally occurring compounds may be divided into two broad categories. The first class of compounds is known as primary metabolites. They occur in all cells and play a central role in the metabolism and reproduction of those cells. Primary metabolites include the nucleic acids, the common amino acids, sugars and the high molecular weight polymeric materials such as cellulose, lignin's and proteins which form the cellular structures. Most primary metabolites exert their biological effect within the cell or organism that is responsible for their production (Wadood *et al.,* 2013). The second class of compounds is secondary metabolites. Such compounds are characteristic of a limited range of species and occur in plants in a high structural diversity. The major classes of secondary metabolites include tannins, glycosides, flavonoids, alkaloids, terpenoids, steroids, quinones and saponins are among others and play significant role in drug discovery. Secondary metabolites have often attracted interest of researchers because of their biological effect on other organisms (Deng *et al.,* 2011).

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drug (Tiwari *et al.*, 2011). In countries with limited access to allopathic medicine, traditional medicine is often the main source of health

care. In some countries of the world, the population uses traditional medicine for primary health care needs (Pawar *et al.,* 2016). Therefore, screening of chemical constituents in medicinal plants provide new useful information to the scientific community and in claiming for their therapeutic efficacies (Chatoui *et al.*, 2016).

The genus *Lobelia* is a large genus of trees or shrubs, belonging to the family *Campanulaceae*, which are distributed in Asia countries such as China, India, Japan, Thailand, Indonesia, Philippines and Africa. Sadiq *et al.*, (2016), reviewed the medicinal uses and chemical constituents of various *Lobelia* species and reported about 30 organic chemicals which have been isolated and identified from this species. In traditional medicinal system of the worlds, *Lobelia* species are used for many medicinal applications such as antifungal and for internal hemorrhage and bedwetting, for insomnia and hiccough, antihypertention, dysponea, vermicide and vermifuge, sedative, antifebrile, promotes secretions, diuretic, hemostatic, antiviral, and anti-inflammatory activities. In addition, it is used as an antidote for poisons and for the treatment of edema, jaundice, liver, stomach, and intestinal diseases, and breast and stomach tumors, astringent and bactericidal. All parts of these plant species have been used for medicinal purposes. The fruits are carminative, astringent and cure biliousness and have been used as an anti-inflammatory and antipyretic drug in many local traditional medicines (Stark *et al*., 2013). The genus *Lobelia* is known to contain many phytochemical constituents, mainly different quinone, flavonoids, alkaloids, steroidal, saponins, triterpenoids, phenyl propanoids, xanthones, polyacetylenes, coumarins and triterpenoid compounds (Kousar *et al*., 2016). The active phytochemicals in most parts of *Lobelia* genus medicinal plants have direct or indirect therapeutic effects and are used as medicinal agent (Rasul, 2018).

Majority of Ethiopians still use traditional medicine for treating various health problems since medicinal plants have lower cost compared to modern medical attention (Bussmann *et al.*, 2011). Ethno medicinal survey in Ethiopia and other parts of the world indicated that the genus *Lobelia* (*Campanulaceae*) has been used traditionally for many therapeutic purposes. The study plant *Lobelia rhynchopetalum* commonly known as Jibara (Amharic) is a monocarpic perennial species endemic to the Ethiopian drained sites of the Afro alpine ecoregion. The plant is frost tolerant and has an up to two (2m) meter tall unbranched stem with a large pith and thick and leathery leaves, which are suggested to be adaptations to the high altitude tropical environment

(Meragiaw & Asfaw, 2015). Distributed throughout the world from Mountains of eastern Africa from 1800 to 3500 m including Ethiopia, Kenya, Uganda ,Tanzania (Wood *et al.*, 2015). Traditionally, the leaves and aerial parts of this plant are used for the treatment of various ailments including leprosy, wound, evil eye, retained placenta, diarrhea, fever, eye disease, rabies and tumor/cancer, among other. Despite the presence of few reports on the chemistry of the aerial parts of this species, to the best of our knowledge there is no prior report on the roots. Therefore, this thesis presents the results of the research done on phytochemical, biological activity and docking studies of the roots extracts of *L. rhynchopetalum.*

1.2. Statement of the Problems

Despite the tremendous progress in medicine, infectious diseases caused by pathogenic microorganisms (bacteria, viruses, fungi) has become a serious problem because these microbial contaminations still affect public health. Hence the study of phytochemicals is necessary for the discovery of potential new compounds and also in finding out new sources of ingredients. Even though these traditional medicines are widely used, they have received very little attention in the modern research and development and efforts to upgrade the traditional health practices are very low.

In Ethiopia medicinal plants are used traditionally to treat various diseases of human and live stock-ailments. *Lobelia rhynchopetalum, is* one of the medicinal plants that have been commonly used by traditional healers in Arsi (Galema) district Arsi Zone Oromia region of Ethiopia. The roots of this plant has been widely used by the local people for the treatment of different ailment such as leprosy, wound, evil eye, retained placenta, diarrhea, fever, eye disease, rabies and tumor/cancer. However, to the best of our knowledge there is no prior work on isolation and characterization of chemical constituents and evaluation of biological activities of the roots extract of this plant in Ethiopia despite the wide traditional use of the plant. Therefore, the study is aimed at isolation and identification of compounds from the root of *L. rhynchopetalum* and evaluation of biological activities to give scientific support for the traditional use of the plant.

1.3. Objectives

1.3.1. General Objective

The overall objective of this study was to identify antibacterial and antioxidant compound from root extracts of *Lobelia rhynchopetalum*.

1.3.2. Specific Objectives

The specific objectives of the study were:

- To successively extract the roots of *Lobelia rhynchopetalum* by *n*-hexane, dichloromethane: methanol (1:1) and methanol solvents using maceration techniques.
- To isolate chemical constituent from crude extract by using silica gel column chromatographic techniques.
- To elucidate the structure of isolated compounds by using NMR spectroscopic method and comparison with literature data.
- To evaluate *in vitro* antibacterial activity of the crude extracts and isolated compounds from *Lobelia rhynchopetalum* root extract by using agar disk diffusion method against selected two Gram positive bacteria *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes (S. pyogenes)* and one Gram negative bacteria *Escherichia coli (E. coli) strains.*
- To test antioxidant activity of crude extracts and isolated compounds from root extracts of *Lobelia rhynchopetalum* using DPPH assay method. .
- To examine molecular docking study of isolated compounds which showed promising antibacterial activity using Autodoc vina version 4.2 with DNA gyrase B (PDB ID: 6F86) selected proteins.

1.4. Significance of the Study

By identifying phytochemical and biological activities of plant constituents in traditional medicinal plant helps for drug discovery and development research. Therefore, this study is expected to:

- Provide scientific support towards the traditional uses, chemical constituents and biological activity of *Lobelia rhynchopetalum* medicinal plant.
- Provide documentary information about ethno medicinal uses, chemical constituents and biological activity of *Lobelia rhynchopetalum*.
- Provides documentary information about ethno medicinal uses of the plant in the primary use of traditional systems of medicine.
- Provide scientific investigation of the plant sources possessing significant antibacterial, and antioxidant compounds and substantiate the observed promising activity with molecular docking analysis on target protein of selected strain of microorganism.
- Establish a scientific basis for the use of these plants

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Family *Campanulaceae*

Campanulaceae, the bellflower family, is a well-known group of plants comprising 84 genera and approximately 2400 species of mostly herbaceous (nonwoody) plants, many with showy, blue, bell-like flowers, often with milky sap (Kumar *et al.,* 2018). The family is cosmopolitan in its distribution and present in a wide array of habitats. The plants are mainly important as garden ornamentals. They are mostly native to cool, temperate areas but also occur on mountains in tropical regions. There are trees and shrubs as well as the more common herbs. Most have five-part flowers with united petals and alternate simple leaves. The ovary in most species is inferior (i.e., positioned below the other floral parts (Sultana & Rahman, 2016).

A few species in the family belonging to the genera *Lobelia* have previously been analyzed with respect to their secondary metabolites and biological activities, and a diverse range of compounds have been isolated to date. These include pyrolidine, piperidine and polyhydroxylated alkaloids, steroidal saponins, triterpenoids, phenyl propanoids, flavonoids, xanthones, polyacetylenes and coumarins, some of which exhibited significant anticancer, antimicrobial and anti-inflammatory activities (Sultana *et al*., 2018).

This family contains a large number of important plants which are used in the treatment of various diseases such as headache, constipation, rheumatism, evil eye, diabetes, and skin infection (Kaur & Kalia, 2012).

2.2. Genus *Lobelia*

Lobelia is a large genus which belongs to the family *Campanulaceae,* distributed in all tropical and warm temperate regions and it has been used in folk medicine to treat various diseases throughout the world (Musthapa & Gumilar, 2017). *Lobelia* is the largest genus within the subfamily *Lobelioidceae* of *Campanulaceae* family comprising over 415 species that range from small herbs to giant woody plants. Most are native to tropical and warm temperate regions. Several herbaceous species are popular in gardens and thrive in moist, shady and semi-shady locations (Villegas *et al.,* 2014).

The well known species of *Lobelia* genus include, *L. chinensis* Lour, *L. cardinalis, L. pyramidalis, L. siphilitica, L. flaccid, L. inflata, L. nicotianaefolia, L. polyphylla, L. sessilifolia, L. tupa,* and *L. davidii* (Lajeunesse, 2017). In Ethiopia, *Lobelia rhynchopetalum* was also well known for its medicinal, ecological, recreational and economic value.

Table 1. *Lobelia*species and their occurrence

Species of the genus *Lobelia*	Occurrence	Reference
L .rhynchopetalum	Ethiopia, Kenya, Uganda ,Tanzania	(Roquet *et al*., 2009)
L. cardinalis	Eastern of North America	(Daniel and Dennis, 2017)
L. chinensis	China	(Calyx & Rodrı, 1997)
L. flaccid	Eastern Cape region of South Africa	(Stolom *et al*., 2016)
L. inflate	Eastern North America	(Stolom *et al*., 2016)
L. nicotianaefolia	India	(Vigneshwaran *et al*., 2014)
L. polyphylla	Chile	(Spaulding & Barger, 2016)
L. sessilifolia	India	(Manikandan, 2017)
L.siphilitica	Eastern North America	(Zieli, 2020)
L. tupa	Chile	(Manikandan, 2017)
L. davidii	China	(Khan & Rub, 2011)

2.2.1. Ethno medicinal use of Genus *Lobelia*

Since ancient times, *Lobelia* species have been used in folk medicine worldwide in the form of infusions and tinctures for the treatment of various diseases. The most commonly cited traditional use of *Lobelia inflata* (known as "Indian Tobacco") is smoking cessation and for the treatment of respiratory diseases such as asthma and bronchitis (Garcia *et al*., 2020)**.**

In Ayurveda, a decoction of flowers of *Lobelia nicotianaefolia* is used for asthma, bronchitis, and fever, roots for eye disease, and leaves for rapid wound healing. Moreover, it has been traditionally used to treat pain and snakebites (Amarathunga & Kankanamge, 2017).

The alkaloids present in *Lobelia polyphylla* Hook. a Chilean species, is probably involved in the toxic, narcotic, and hallucinogenic effects produced after smoking or consuming the aerial parts of this species (Mahrath *et al.*, 2015). *Lobelia tupa*, also known as "devil's tobacco," contains a poisonous and caustic latex that causes vomiting, intestinal irritation, and delirium if ingested (Stansbury *et al*., 2013). The leaves and inflorescences of *Lobelia pyramidalis* Wall, an Indian species, are used as an antispasmodic and for the treatment of asthma, bronchitis, fever, sciatica, and back pain (Joshi *et al.*, 2011). The dry leaves of *Lobelia flaccid* Persl. are used as analgesics and antiepileptics in the Eastern Cape region of South Africa (Stolom *et al.,* 2016). Another native American species known for its medicinal potential is *Lobelia cardinalis* L., Plant formulations are consumed for various purposes, for example, as anemetic, in the treatment of typhoid and fever, and as a "love potion"(Daniel *et al*., 2019). *Lobelia chinensis* Lour. is a species that shows to have diuretic, hemostatic, antimicrobial, antiviral, and anti-inflammatory activities. In addition, it is used as an antidote for poisons and for the treatment of edema, jaundice, liver, stomach, and intestinal diseases, and breast and stomach tumors (Huang *et al*., 2018). *Lobelia sessilifoilia* Lamb is another Chinese species that is used in Chinese folk medicine for the treatment of phlegm, cough, cirrhosis ascites, abscess, and snakebite phlegm, cough,abscess, and snakebite (Zhang *et al*., 2019).

L. siphilitica L.; was used by indigenous people in Canada as an anti-syphilitic, *L. urens L*. from Europe was employed as vermifuge, *L. laxiflora* Humb. was used as an emetic, expectorant, and breathing regulator, *L. tupa L*. was used in ophthalmology, and *L. purpurascens* R. was used in the treatment of snake bites (Shazia Kousar *et al*., 2016).

L. rhynchopetalum is a monocarpic perennial species endemic to the Ethiopian drained sites of the Afroalpine ecoregion. The plant is frost tolerant and has an up to two-meter-tall unbranched stem with a large pith and thick and leathery leaves, which are suggested to be adaptations to the high altitude tropical environment (Geleta & Bryngelsson, 2012). Ethno botanical data were collected from January 23, 2020 to February 2, 2020 through semi-structured interviews and field observations. Interviews were carried out to gather data on plant parts used, method of remedy preparation, dosage of remedy, route of remedy administration, diseases treated, threats and conservation practices of medicinal plants. Communications with all informants were held in Afan Oromo, the mother tongue language of the study participants and of course, the official language of the District and Oromia region. Field observations were also conducted to record the habit and habitat of *Lobelia rhynchopetalum* medicinal plant with the assistance of informants who participated during the interview. The main way of indigenous knowledge transfer on types of medicinal plants, traditional concepts of illness and method of diagnosis in the District was through oral tales to a family members (especially to an elder son). Besides, some informants acquired their knowledge secretly through systematic follow up and observation of practitioners at the time of medicinal plant collection and preparation. Furthermore, few informants reported that they develop their knowledge by copying healers after seeking treatment and upon careful observations of domestic animals, especially for plant remedies with antidote effects. The most commonly used plant parts in remedy preparations were roots (39.53%) followed by leaves (35.81%), seed (6.05%), stem (2.79%), latex (2.79%) and whole plant (1.40%) (Yihenew *et al*., 2017). Most of the remedies were prepared from fresh plant materials. Surveys were conducted in Robe and Bokoji local markets to assess the marketability of medicinal plants in the study District. It revealed that this medicinal plant was sold in the above local markets for their use to manage evil eye, rabies and retained placenta.

Ethanomedicinal plant survey in Ethiopia and other parts of the world indicated that among many medicinal plants, one of the species identified in this study is *Lobelia rhynchopetalum* has been used traditionally for many therapeutic use. The bark and root of *Lobelia rhynchopetalum* is crushed, mixed with little water and sniffed at the sickness time by nasal to relieve evil eye.

In Ethiopia, *Lobelia rhynchopetalum* has a vernacular name Tarura (in Afan Oromo) and Jibara (in Amharic). The seed of *Lobelia rhynchopetalum is* crushed, powdered, mixed with little water

and drunk orally to treat heart problem (Lulekal *et al*., 2008). *L. rhynchopetalum* is most prominent and noticeable in the Afroalpine part of the Arsi-Bale and Simien mountain systems, commonly within an altitudinal range of 3600–4500m asl, where it serves as a tourist attraction. The significance of this species is, therefore, not only medicinal, but also ecological, recreational and economic.

Figure1. Picture of *L. rhynchopetalum* (Picture taken by Abu Jeilu, **23/2/ 2021**)

2.2.2. Phytochemical and Pharmacological Study of *Lobelia* Genus

Different classes of secondary metabolites have been isolated from *Lobelia* species among which alkaloids, flavonoids, terpenoids, polyacetylenes, coumarins, fatty acids, neolignans, tannins, cardiac glycosides, anthranolglycosides and amides are well known (Al-Rifai *et al.,* 2017). *Lobelia* species have gained recent attention because of their broad biological and pharmacological activities in theseorder. *Lobelia* species have been reported to exert multiple biological property including antimicrobial, cytotoxicity, anti-inflammatory, neuroprotective as well as antitumor activities (Stolom *et al.*, 2016).

Prabhakar *et al.,* (2017), isolated and identified lobelanidine **(1**), lelobanidine **(2**) and nicotianafoline **(3)** in *L. nicotianaefolia* from a chloroform ($CHCl_3$) fraction. Lobelanidine **(1)** act as neutral antagonist of α3β2/α3β4 nAChR and α7nAChR with IC_{50} valuesof 8.2 ± 3.0 and 2.8 ± 0.2µM, respectively. *Nicotianafoline* **(3)** had significantly inhibited the maximal oedema response by 61.32 ±1.72 during the 6 h of the carrageenan induced rat paw acute inflammation (Khan & Rub, 2011).

Phytochemical investigation of *L. inflate* led to the isolation of alkaloids such as lobelanine **(4),** lobeline **(5**), norlobelanidine **(6**), norlobelanine **(7)**, norlobeline (**8**), 8-propyl-10 phenyllobelionol **(9**), 3-hydroxy-3-phenyl-propanoicnorallosedamine **(10)**, and 3 hydroxy 3phenyl-propanoicallosedamine **(11)** (Shimomura, 1999). Lobelanine **(4)** andlobeline **(5**) showed only modest affinity for nAChR. In addition, lobeline (**5**) is a powerful respiratory stimulant and also showed a strong inhibitory activity against *Mycobacterium tuberculosis*, suggesting that this species could be used as a potential anti-*Mycobacterium tuberculosis* agent by consistently inhibiting or blocking tuberculosis, while norlobelanine (**7**), is anemetic and respiratory relaxant (Lajeunesse, 2017). Norlobeline **(8)** and its analogues have great future in treating number of neuropsychiatric disorders like Alzheimer's disease, schizophrenia and epilepsy (Wink, 2015).

In other studies three piperidine alkaloids were isolated from the total alkaloid extract of *L. berlandieri*;[N-methyl-2,6-bis(2-hydroxybutyl)-Δ3-piperidine](**12**),[N-methyl-(2hydroxypropyl)-6-(2-hydroxybutyl)-Δ3-piperidine] (**13**), [N-methyl-2-(2-oxobutyl)-6–(2-piperidine] (**14**) (Kuo *et al., 2011)*. Lelobanonoline (**15**), β–amyrin (**16**), β-sitosterol (**17**), β-amyrinpalmitate (**18**) were isolated and identified from the hexane fraction of the species *L. davidii* (Garcez *et al*., 2004).

Lobinaline (**19**) and (**20**) 8-phenylnorlobelol was obtained by acid-base extraction of the $CHCl_3$ fraction of the methanolic extract from the aerial parts of *L. cardinalis* (Daniel and Dennis, 2017). Lobinaline (**19**) was identified as the major compound of *L. cardinalis*. It was proved to be a potent free radical scavenger, has similar binding affinity for α4β2 and α7-nicAchRs, exhibited agonist activity on nicAchRs in SH-SY5Y cells, and inhibited absorption of [3H]-dopamine (DA) in rat striatal synaptosomes. These multifunctional uses make lobinaline **(19**) a compound of interest for the development of therapy for neuropathological disorders involving free radical generation, cholinergic and dopaminergic neurotransmission including neurodegenerative conditions such as parkinson's disease, and drug abuse (Folquitto *et al*., 2014).

Phytochemical investigation of leaves, stems, and flowers of *L. laxiflora* in Costa Rica resulted in the isolation and structural elucidation of four new alkaloids, namely, cis-8,10-diethy1–3,4-dehydrolobelidiol, (**21**), trans-8-ethyl-10-phenyl-3,4-dehydrolobelidiol, (**22**), cis-8-ethyl-10 phenyl-3,4-dehydrolobelidiol (**23**), and N-methyl-2(2'-methoxybutyl), 6 (2"-hydroxybutyl)-Δ3-piperidine (**24**) (Philipov *et al.*, 2013). All isolated compounds exhibited a potent cholinesterase inhibiting activity, and the results from determining complement activity via CP showed that compound (**21**), (**22**), and(**23**), strongly inhibited hemolysis at a concentration range between 0.125 and 1.0 mg/ml. and thus to prevent inflammatory process was estimated *in vitro* in human serum via both classical (CP) and alternative (AP) (Stolom *et al*., 2016). The alkaloid fraction and compound (**22**) were active in both AP and CP assays. The alkaloids (**21**) and (**23**) were moderately active, but their inhibitory was selective and directed to CP activity (Ayoola *et al*., 2008).

Yadav *et al*., (2014), isolated alkaloid exhibiting glycosidase-inhibiting activity such as 7-O- β – D-glucopyranosylhomonojirimycin (**25**), and triterpenoids β-sitosterol (**26**), ursolic acid (**27**), oleanoic acid (**28**) and one novel triterpenoid ester, oleanol 28-aldehyde 3-O- β -palmitate (**29**) from the methanolic extract of *L. sessilifolia*. Yin *et al*., (2015), tested the alkaloid (**25**) isolated from the 50% methanolic fraction of *L. sessilifolia* and verified its potent inhibitory activity on porcine kidney trehalaseenzyme at concentration of IC_{50}=1.7μM. In other studies, (Carolina, 2018), identified three new alkaloids from the crude extract of the aerial parts of *L. siphilitica*

namely, (**30**) 8,10-dietillobelidione (2R,6S,2″S), [2″-O-Acetyl lobeline] (**31**) and 6-[(E)-2-(3-Methoxyphenyl) ethenyl]-2,3,4,5-tetrahydropyridine (**32**).

From young stems of *L. polyphylla* Hook. the following piperidine alkaloids:- 1-(1-(2-hydroxy-2-phenylethyl)-1-methylpiperidin) butane-2-ol (**33**), 1-(6-(2-hidroxypentyl)-1-methylpiperidin) butane-2-one (**34**), 1-methyl-2-piperidinemethanol (**35**) and 1-(6-(2-hydroxy-2-phenylethyl)-1-methylpiperidin) butane-2-one (**36**) (Pontificia, 2014).

Compound (**34**) and (**36**) showed moderate antimicrobial activity in disc diffusion method, and the minimum inhibitory concentration method against *Trichophyton mentagrophytes* (MIC=3.12mg/mL), *Pseudomonas aeruginosa*, *Escherichia coli*, and *Aspergillus fumigatus* (12.50 mg/mL) were determined respectively (Borchardt *et al*., 2008).

Compounds such as Radicamine A (**37**), radicamine B (**38**), Lobechine (**39**), lobechidine A (**40**), lobechidine B, (**41**), lobechidine C (**42**), and andrachcinidine (**43**), and Terpenoids such as daucosterol (**44**), stigmasterol (**45**), cycloeucalenol (**46**), cycloeucalenol acetate (**47**) were isolated from *L. chinensis* (Wang *et al*., 2019). Two new pyrolidine alkaloids, radicamine A (**37**) and radicamine B (**38**), are important groups of naturally occurring polyhydroxylated pyrolidine alkaloids isolated from *L. chinensis* which have shown potent inhibitory activity against α-glucosidase. The $CHCl_3$ fraction showed a significant inhibition of elastase release and **(40**) showed a moderate inhibition against HSV-1 (Shwe *et al*., 2019).

Previous investigation of *L. chinensis* led to the isolation of flavonoids such as apigenin 7-O-rutinoside (**48**), rutin (**49**), hesperidin (**50**), hesperetin (**51**), amentoflavone (**52**), apigenin (**53**), naringenin (**54**), luteolin, (**55**), chrysoeriol (**56**), eupafolin (**57**), diosmetin (**58**), quercetin (**59**), quercetin 3-O-α-L-rhaminoside (**60**), quercetin 3-O-β-D-glucoside (**61**), quercetin 7-O-α-L-rhaminoside (**62**), linarin (**63**), diosmin (**64**), apigenin-7-O-[β-D-glucuronopyranosyl (1→2)O-β glucuronopiranoside (**65**), and chrysoeriol-7-O-[β-D-glucuronopyranosyl (1→2) O- β glucuronopiranoside (**66**) (Huang *et al*., 2018). In recent years, the flavonoid compounds apigenin **(53**) and luteolin (**55**) have shown remarkable performance in antitumor activity. Furthermore, 10-30x10^6mol/L concentration of apigenin (**53**) and luteolin (**55)** showed cytotoxic activity by anti-proliferation of tumor cells and the stopping of the cell growth cycle. Luteolin

(**55**) exhibited cancer cells block effects *in vitro* and *in vivo*, involving in protection from carcinogenic stimuli, inhibition of tumor cell proliferation, induction of cell cycle arrest and induction of apoptosis via intrinsic and extrinsic signaling pathways. Furthermore, luteolin (**55**) can cause many kinds of tumor cells to die by strongly activating the (tumor necrosis factor–α) TNF–α and TRAIL (Prod & Res, 2017). Shafaghat *et al.*, (2014), studied the effects of luteolin on HO-8910PM *in vitro* and explored its possible mechanisms, revealing that luteolin (**55**) can suppress ERK2, decrease the secretion of MMP-9, and further affect the anti-attack activity of ovarian tumor cells, or stop the procession of transfer in ovarian tumor cells after administration of luteolin (5-20 x 10^6mol/L). This finding reveals that low concentration of luteolin has little toxic effect on the cancer cell line, but it can sensitize chemotherapeutic drugs like betxarotene in various cancer cell lines.

Chrysoeriol (**56)** is a methoxylated flavone of great scientific interest because of its promising anti-microbial activities against various Gram-negative and Gram-positive bacteria at the concentration of 40 µg /ml (Bashyal *et al.*, 2019).

Diosmetin (**58**) administration led to an increase of ROS production, to the arrest of the cells in G0/G1, G1/S,or G2/M phase, dependent on the typology of cancer cell model used, and induction of apoptosis pathway in a dose-dependent manner (Anna Bogucka *et al*., 2013). The mechanisms influenced by diosmetin are different and, probably, cell specific. An effect exerted by diosmetin is the upregulation of p53 protein and decreased expression of transforming growth factor-β (TGF-β), that is normally upregulated in cancer cells and is involved in regulation of extra cellular matrix proteins expression (Michael *et al.*, 2013). In addition to the increase of p53 protein expression, diosmetin reduces NF-kB activity through the decrease of the expression of Notch3 receptor, a protein highly expressed in tumors (Ciolino *et al*., 1998). Moreover, diosmetin influences the structure and functionality of vessels, restoring tumor blood flow through the tumor.

Quercetin (**59**) a plant pigment is a potent antioxidant flavonoid and more specifically a flavonol, found mostly in onions, grapes, berries, cherries, broccoli, and citrus fruits. It is a versatile antioxidant known to possess protective abilities against tissue injury induced by various drug toxicities (Milczarek *et al*., 2019).

It was reported that some quercetin derivatives such as quercetin-3-*O*-rhamnoside (**60**) exhibited strong antibacterial effects (Mehdi *et al*., 2009). Thus, it is assumed that sugar moiety of flavonoides play important role in their biological activities.

Phytotoxic potential of compound (**61**) proved that it could serve as phytotoxic effect with IC_{50} value of 282.7µg/ml. On the other hand, high antioxidant effect of this compound makes it potent for defense against oxidative stress and free radical scavenging activities in plants. Many foods are rich in Q3G and human daily intake ranges between 10–20 mg. Q3G can transport across the intestinal brush border membrane by sodium dependent glucose transporter. Thus, it exhibited biological effects more efficient than other derivatives of quercetin (Islam *et al*., 2012). Because of powerful antioxidant potential, Q3G can be considered as a natural anticarcinogenic agent. Because of high antioxidant potential, Q3G and some other flavonoids have important effects on cancer chemoprevention. The chemopreventive properties of flavonoides are generally believed to reflect their ability to scavenge endogenous ROS.

Inspite of cytotoxic properties of Q3G *in vitro* condition, it may interfere in several steps that lead to the development of malignant tumors including protecting DNA from oxidative damages, inhibiting carcinogen and activating detoxifying systems (Tapas *et al.*, 2008). Q3G exhibited high antioxidant activity in DPPH assay with IC_{50} value of 22µg/ml.

Diosmin (**63**) has a wide range of biological activities, including anti-inflammatory, blood lipid lowering, antihyperglycemic, antioxidant and free radical scavenging effects (Bogucka-K *et al*., 2013). Recent studies have shown the use of diosmin for the treatment of venous leg ulcer and, as a chemopreventive agent, in urinary-bladder and colon carcinogenesis (Feldo *et al*., 2019). It is also apotential agent for the treatment of neurodegenerative diseases (Civa *et al*., 2019).

The vasodilatory effects of flavonoids (**63**) are related to their structure. The presence and position of hydroxyl groups on the B ring seems to be particularly important. For the vasodilatory effect, two ortho–positioned hydroxyl groups on the B ring are essential. Moreover, the presence of a double bond between C2 and C3 of the benzopyran ring system increases the vasodilatory potency (Roy *et al*., 2020).

In the genus *Lobelia*, three coumarins, namely, scoparone (**67**), isoscopoletin (**68**), and 5,7-dimethoxy-8-hydroxycoumarin (**69**) were isolated from *L. chinensis* (Mandlik *et al*., 2016).

Scoparone **(67)** inhibited the growth of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. In order to determine whether RK33 LCCs responded to7-substituted coumarins by growth inhibition, they were treated with increasing concentrations of scoparone over a period of 48-72h. In other studies scoparone (**67**) exhibited significant inhibition of superoxide anion generation with IC_{50} of 6.14 ± 1.97μM (Al-Awad *et al*., 2020). compound (**67**) showed the most potent inhibitory properties were observed against RK33 tumor cell lines at a concentration of 5μM due to 7-substituted coumarins moity (Xu *et al*., 2015). The compounds isoscopoletin (**68**) showed to be active against *Mycobacterium tuberculosis* H37Rv with MIC values of 42 *μ*g/mL (Hassan *et al*., 2017).

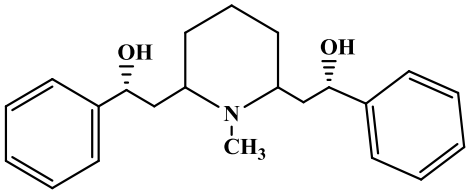
Four polyacetylenes were reported from the *L. chinensis*, lobetyolin (**70**), lobetyolinin (**71**), isolobetyol (**72**), and lobetyol (**73**) (Koz *et al*., 2008). Compounds (**72**) and (**73**) exhibited moderate cytotoxic activity against the two cancer cell lines NCI-H292 and MSTO-211H. The IC_{50} value of (**72**) were 12.36 and 9.31μM against the two cell lines; while the IC_{50} values of (**73**) were 11.76 and 9.64μM respectively (Bálványos *et al*., 2004).

In the *Lobelia* genus aurantiamide acetate (**74**) along with Fatty Acids (**75**) Palmitic acid , (**76**) Lacceroic acid and (**77**) Stearic acid were isolated from *L. chinensis* (Rooijen & Mensink, 2020). Compound (**73**) are conjugates with other aliphatic, aromatic and heterocyclic ring produces various type of biological activities including *antituberculosis*, anticonvulsant, analgesic, antiinflammatory, insecticidal, antifungal, and antitumor properties. They are also involved as an intermediate product in the synthesis of therapeutic agent (Zhou *et al*., 2017).

Chen *et al*., (2010), isolated and identified two neolignans, 7,8-erythro-(**78**) and 7,8-threo-4,9,9′-trihydroxy- 3,3′-dimethoxy-8.O.4′-neolignans (**79**), from *L. chinensis*.

Lobelia erinus is an ornamental species, rich in anthocyanidins that were identified by (Tatsuzawa, 2020), as Delphinidin 3,5,3′-triglucoside (**78**) and Delphinidin 3-rutinoside-5,3′-glucoside (**79**) and alkaloids such as Lobelinin A (**80**) and Lobelinin B (**81**) were isolated by (Qiu *et al*., 2014).

A new piperidine alkaloid, called pentylsedinine (**84**) which comprises five carbons in the side chain, was isolated from the aerial part of *L. tupa*, along with lobeline and lobelanidine. Pentylsedinine (**84**) act as partial agonists at nAChR (Li *et al*., 2015).



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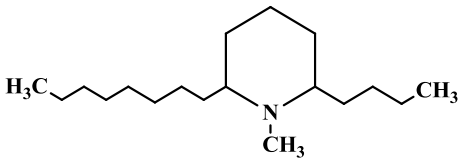

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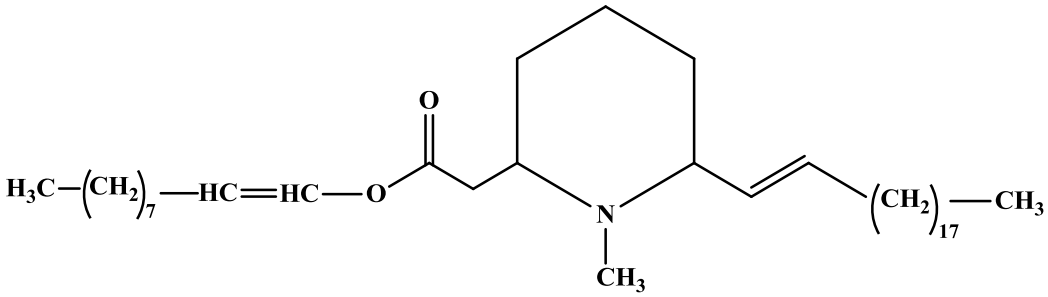

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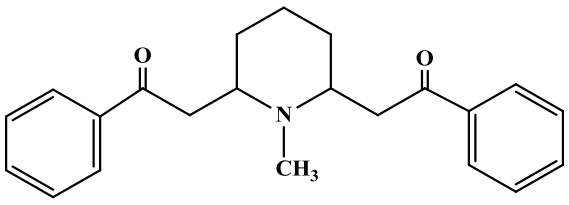

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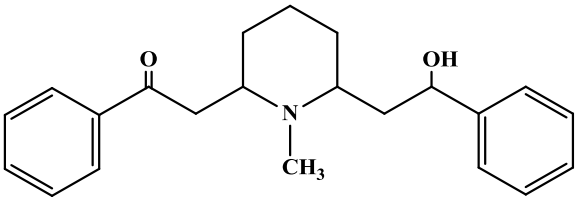

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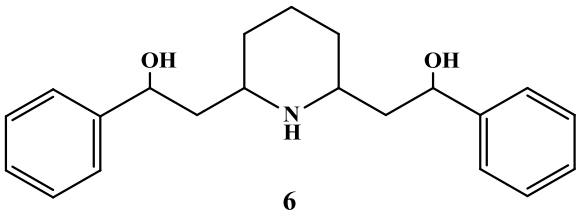

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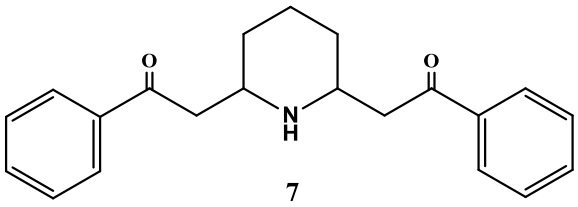

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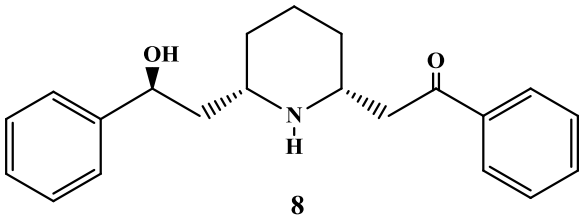

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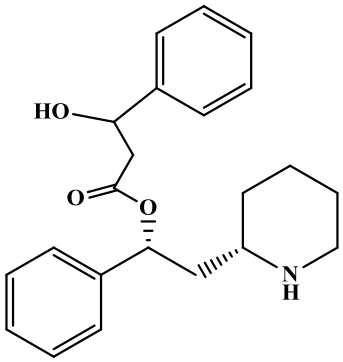

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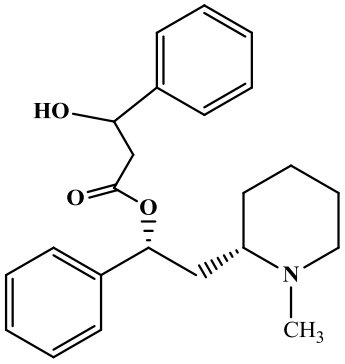

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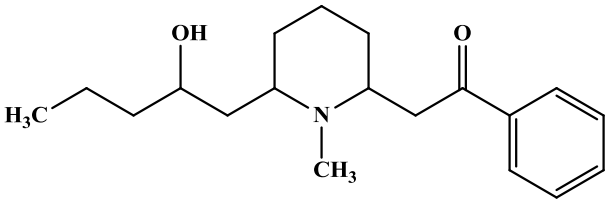

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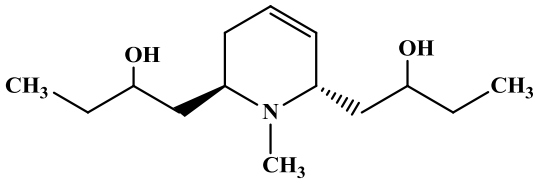

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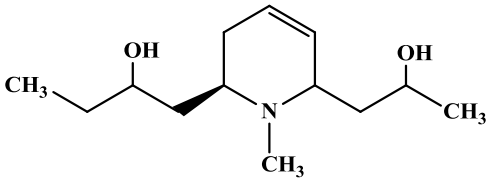

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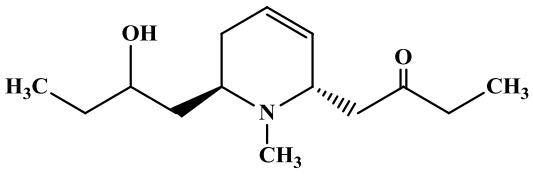

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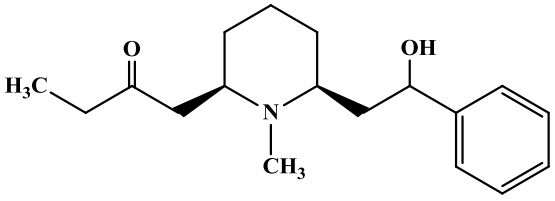

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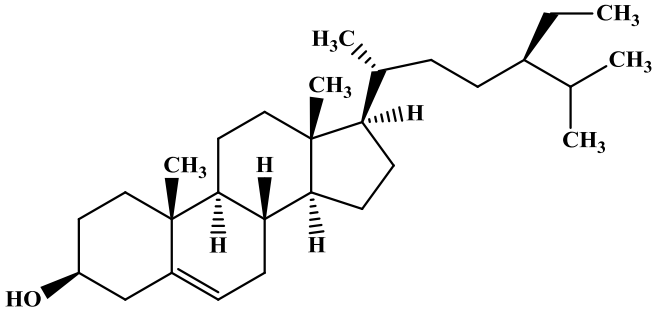

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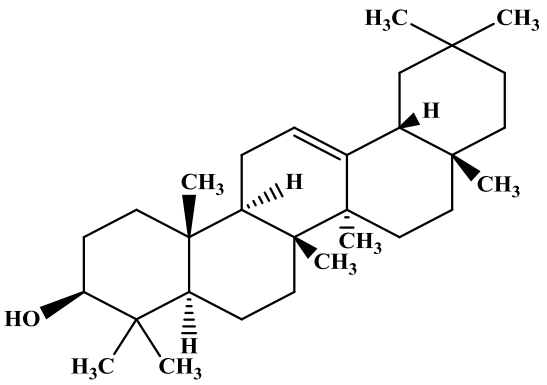

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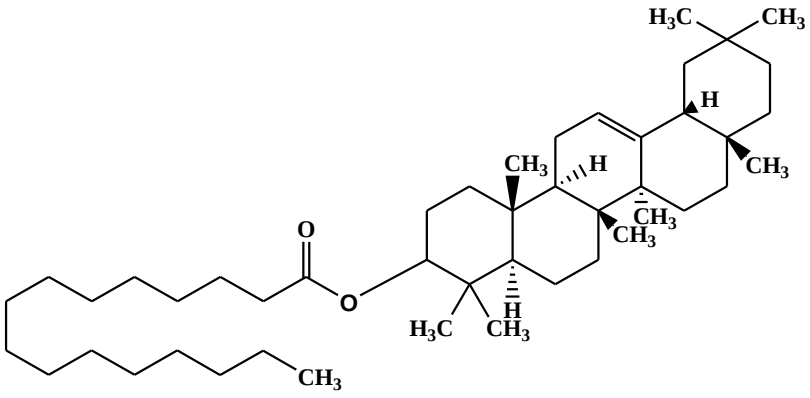

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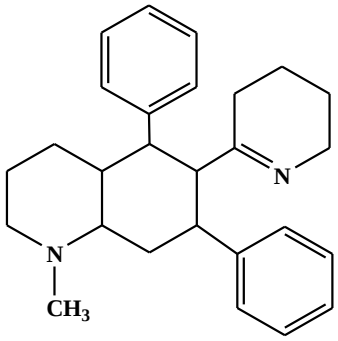

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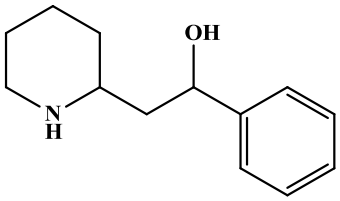

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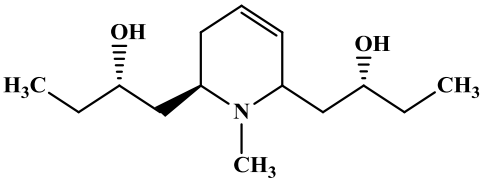

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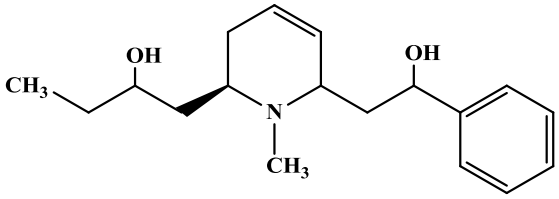

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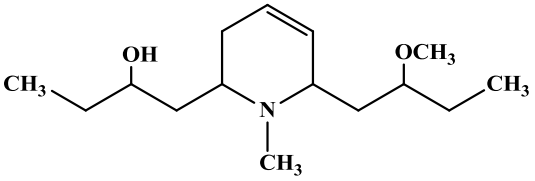

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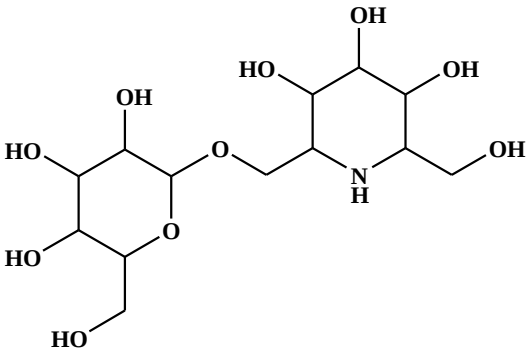

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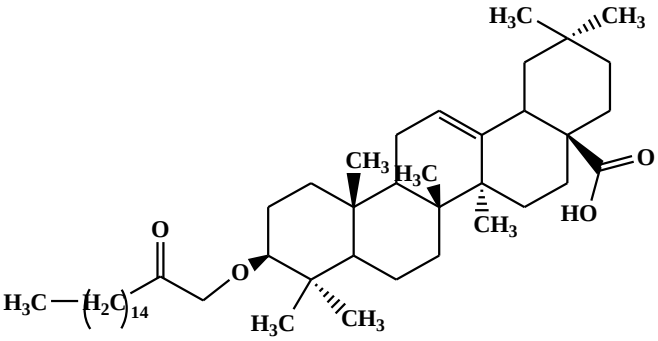

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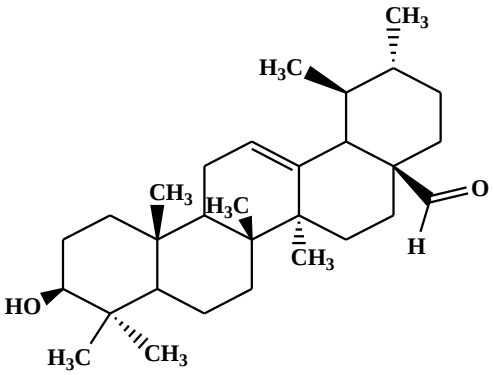

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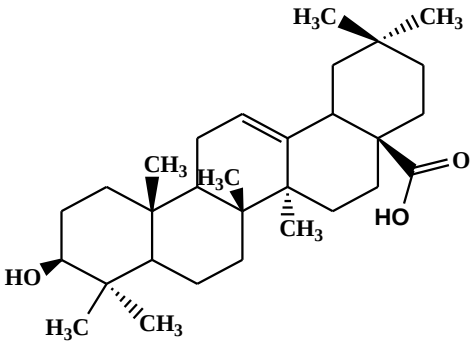

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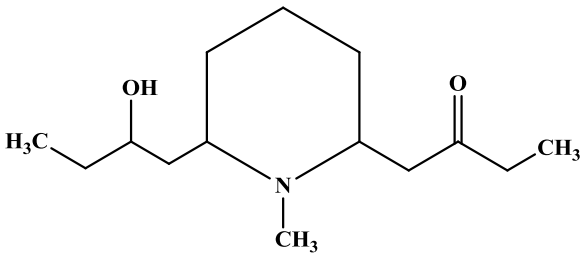

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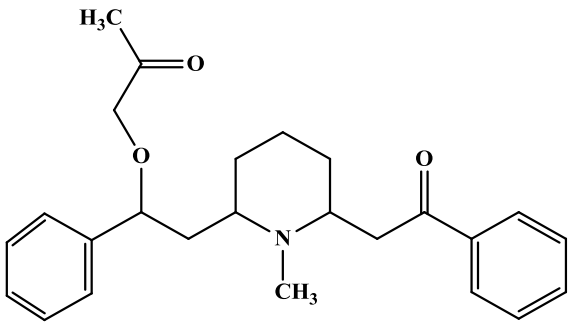

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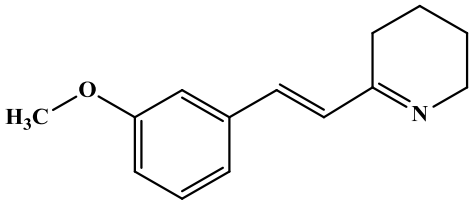

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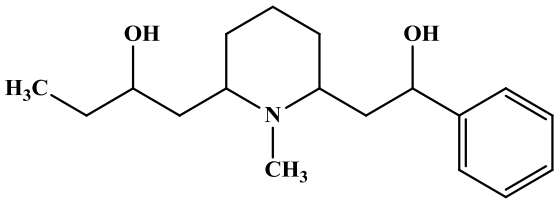

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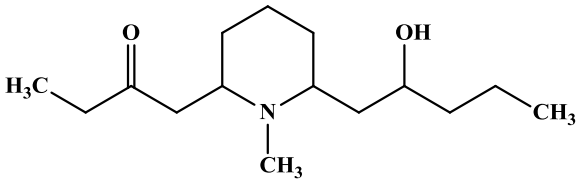

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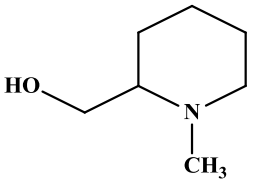

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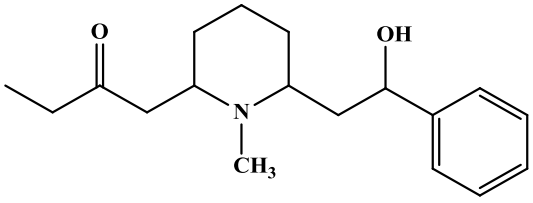

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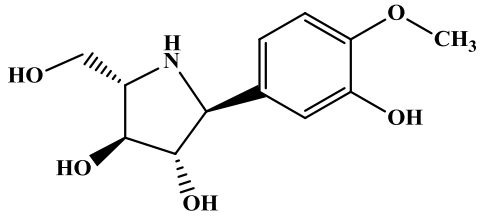

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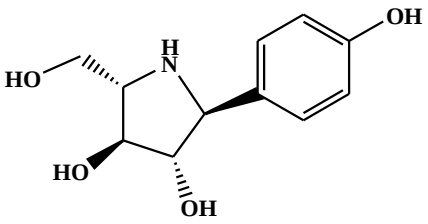

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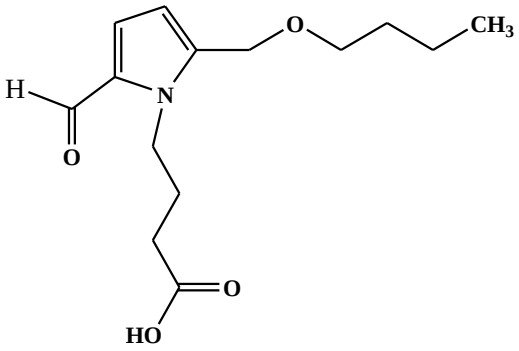

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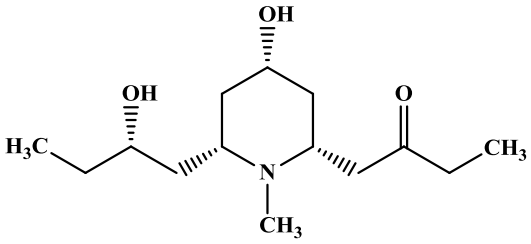

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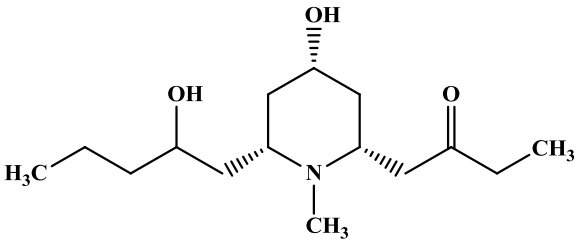

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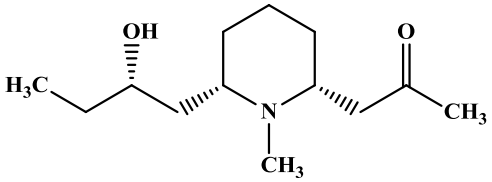

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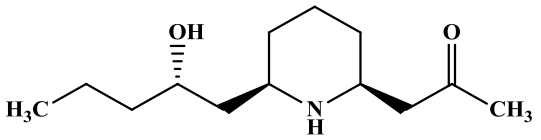

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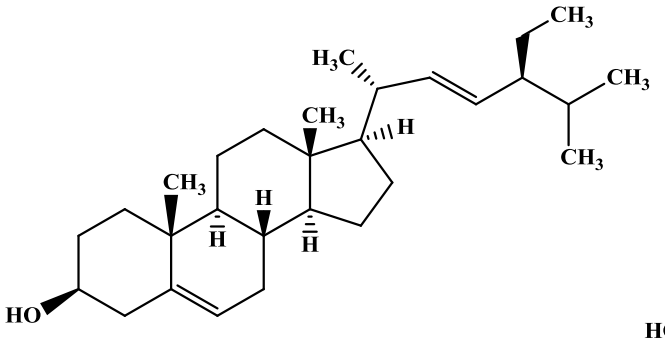

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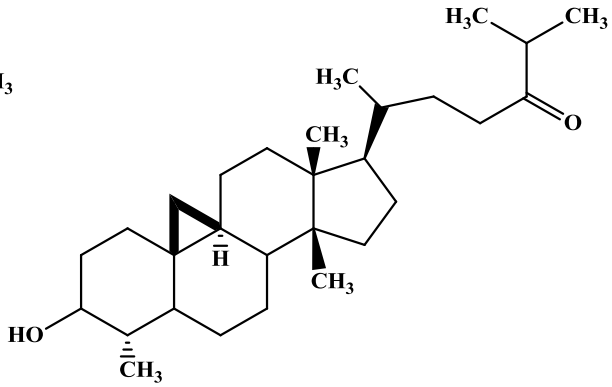

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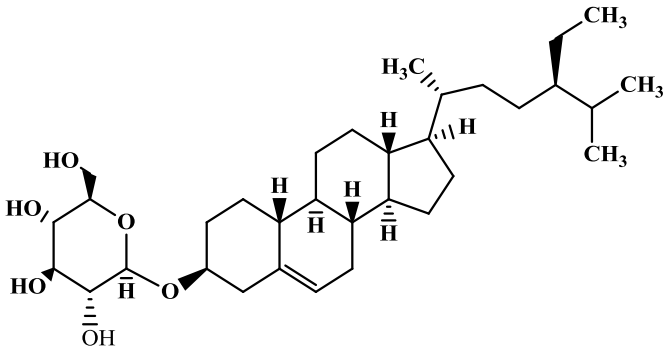

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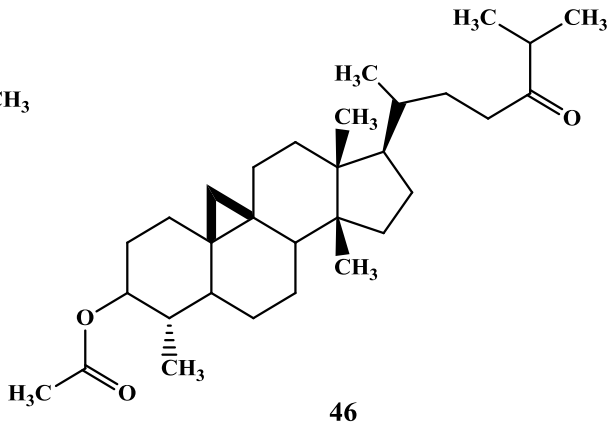

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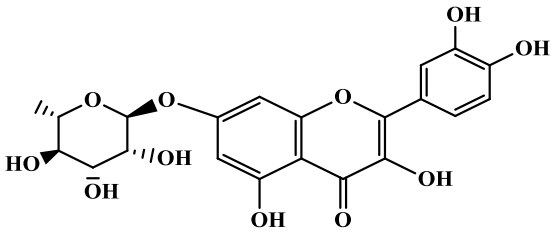

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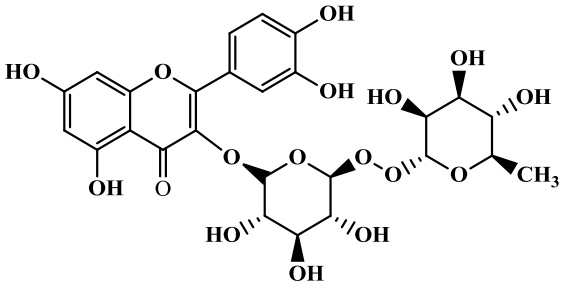

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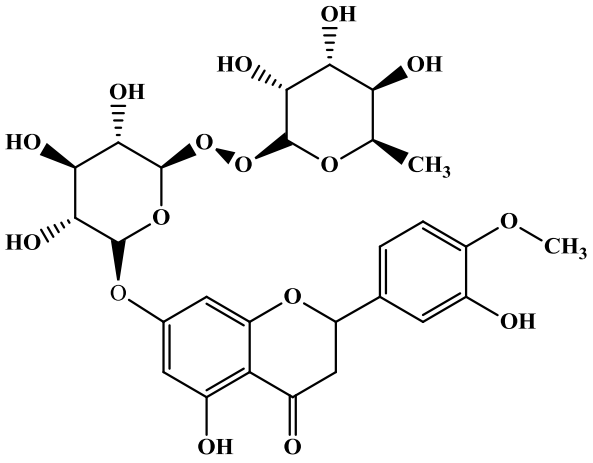

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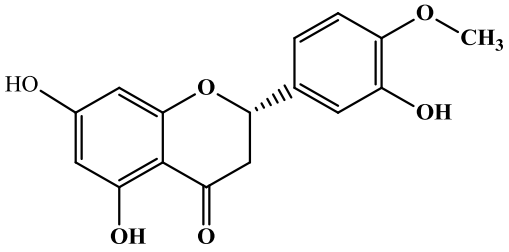

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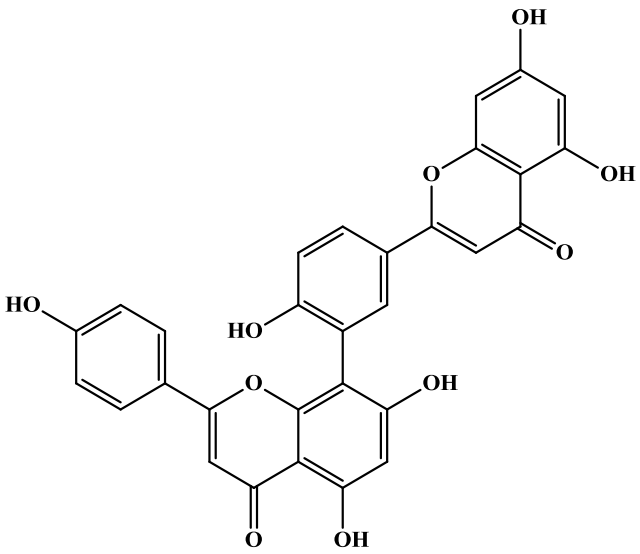

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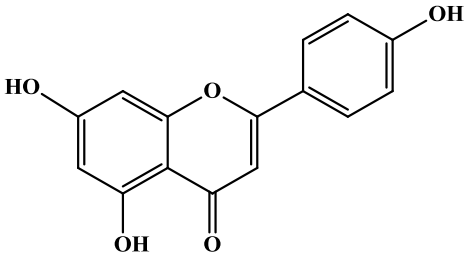

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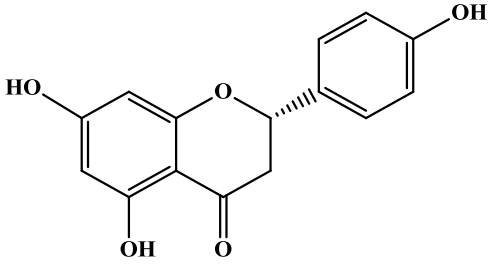

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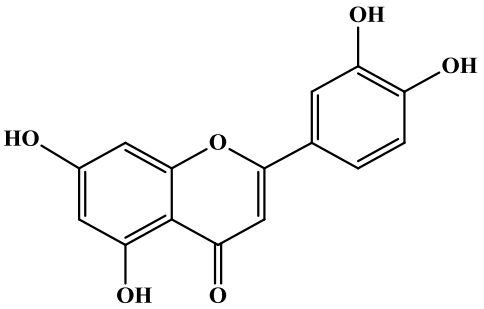

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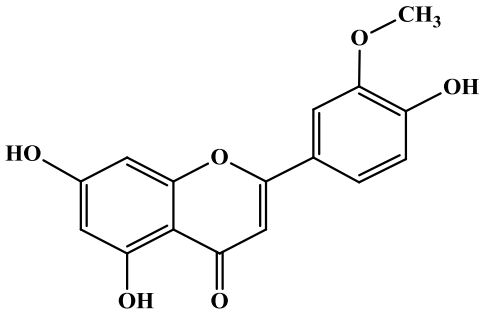

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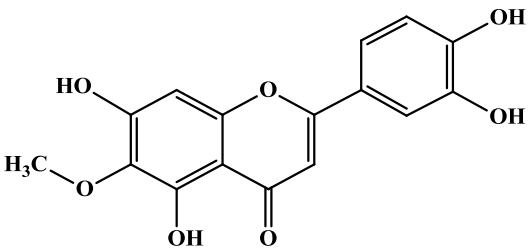

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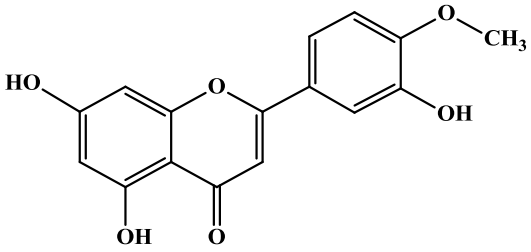

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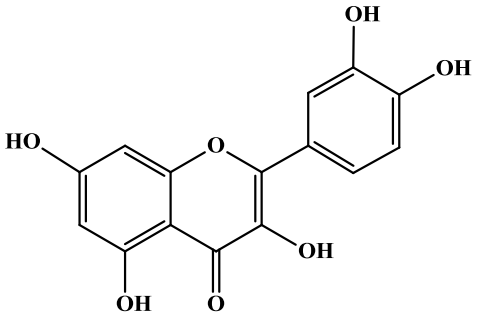

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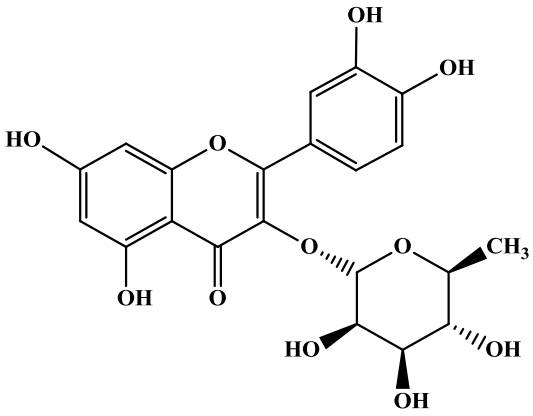

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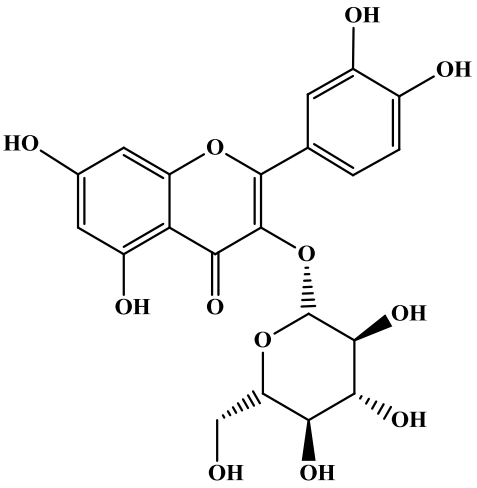

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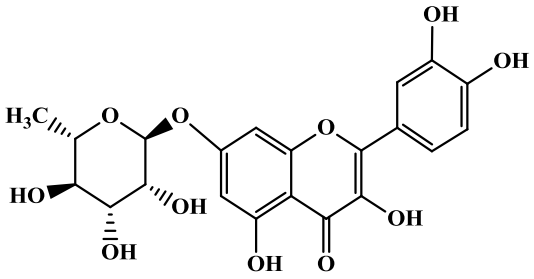

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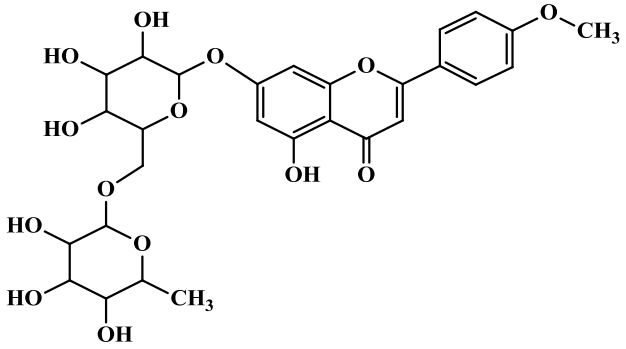

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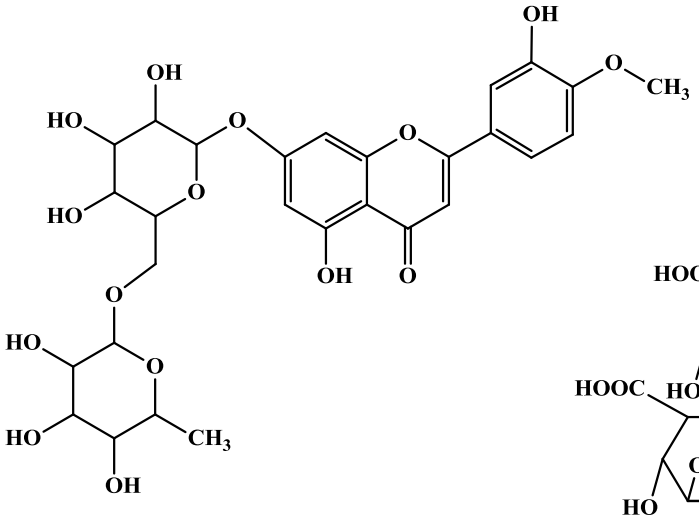

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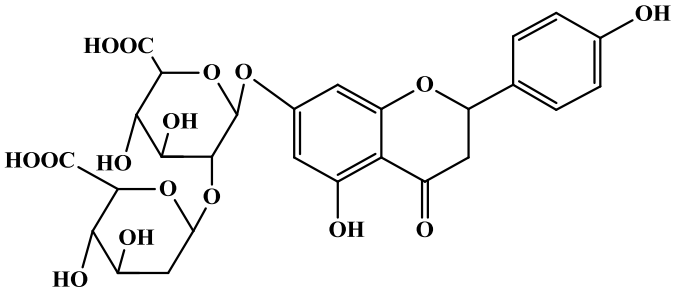

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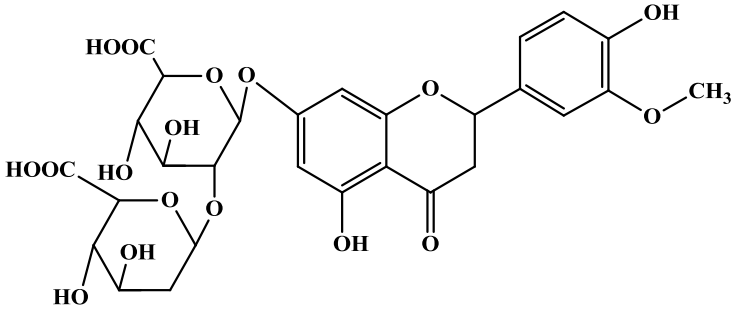

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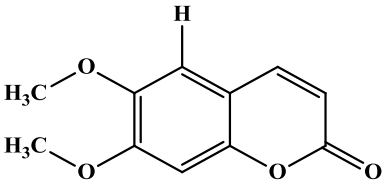

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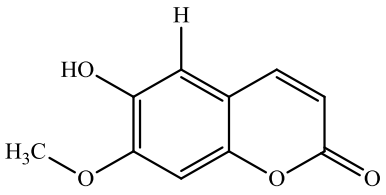

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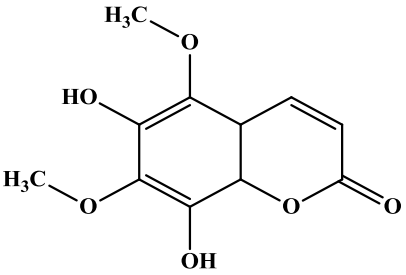

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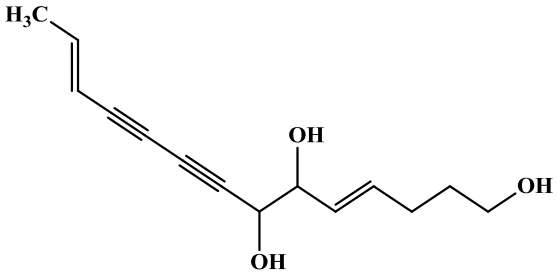

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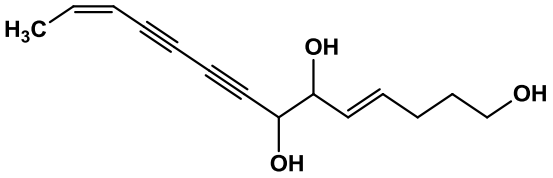

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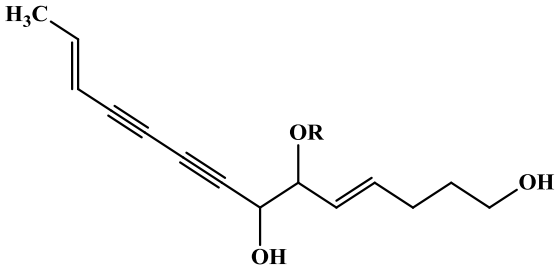
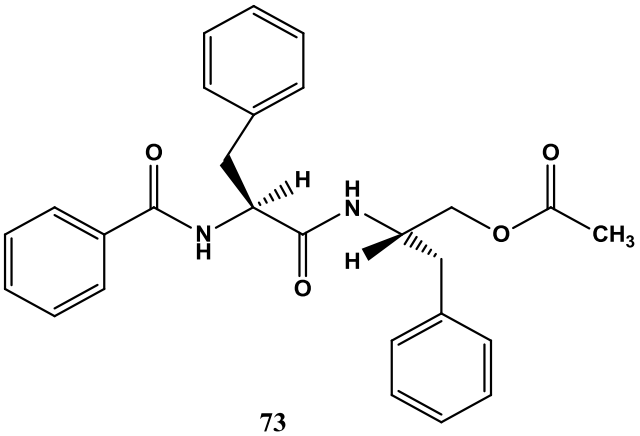


[71] Lobetyolin R= Glc

[72] Lobetyolinin R= Glc^{6}- ^{1}Glc



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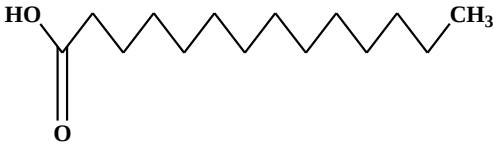

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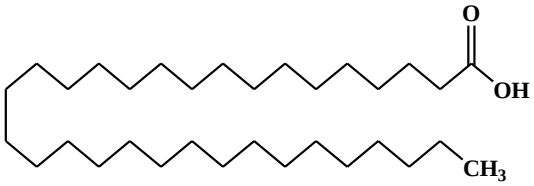

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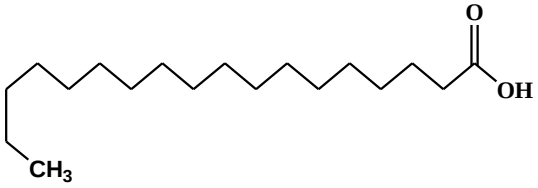

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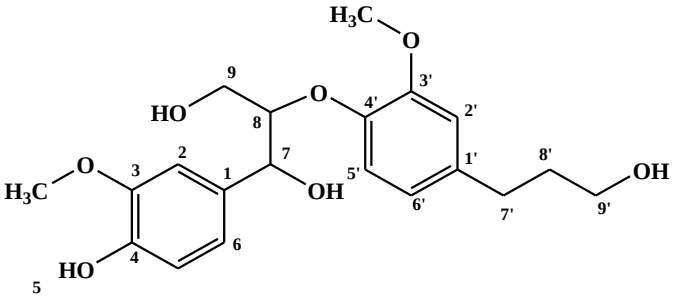
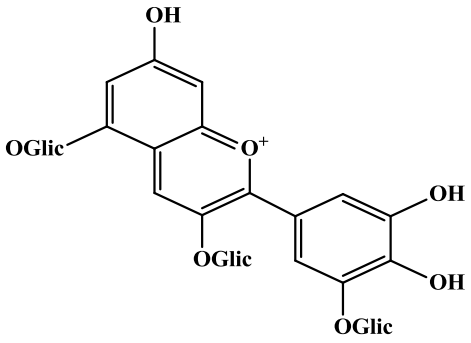


77. **7,8-erythro**

78. **7,8-threo-4,9,9|'-trihydroxy-3,3'-dimethoxy-8,O,4'-neolignans**



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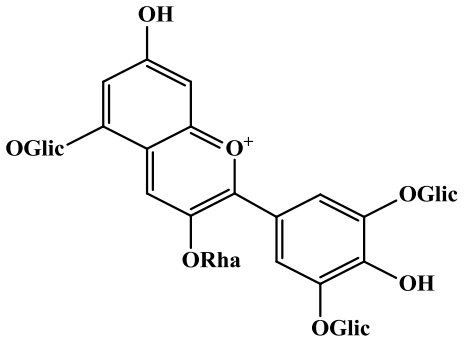

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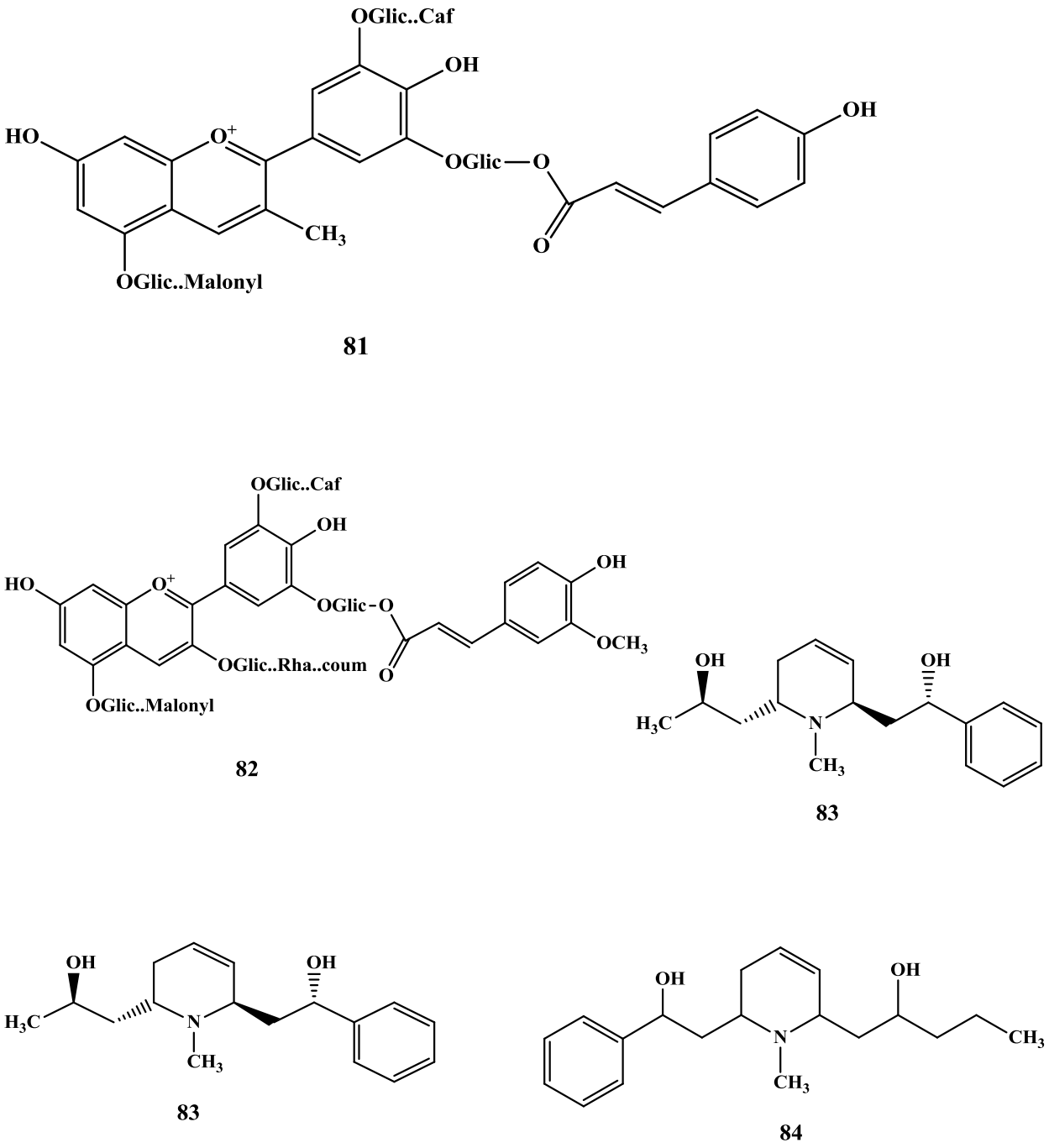


Figure 2. The chemical structures of isolated compounds (**1-84**) from genus *Lobelia*

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Plant Material Collection and Identification

Representative samples of plant Material (leaves) for botanical identification of *Lobelia rhynchopetalum* (*Campanulaceae*) were collected during full flowering stage season at Arsi-Bale (Galema) Mountains, (Oromia Region, Ethiopia) (210 km East of Addis Ababa, ($6^0$48'N–$7^0$8'N and $39^0$45'E–$39^0$57'E) East; 3600-4500 m above sea level) in January 2020. The plant material was identified with the help of Botanist Shambel Alemu and voucher specimen (**AJ-001**) was deposited at the National Herbarium of Addis Ababa University, Ethiopia.

3.2. Chemicals and Reagents

The chemicals and reagents used in this investigation were silica gel, sulfuric acid, hydrochloric acid, ferric chloride, Wagner's reagent and ammonia solution. Solvents used in this study were methanol (99.8%) (HPLC grade), dichloromethane (99.6%) (HPLC grade), hexane 65-70deg C (85%), chloroform, distilled water and ethyl acetate (99.5%). All the chemicals and reagents used in this study were analytical grade and were of pure Aldrich grade.

3.3. Instrument used

Column chromatography (CC) was performed on silica gel 60-120 mesh ASTM. Thin layer chromatography (TLC) was performed using precoated aluminum-backed supported silica gel 60 F254 (0.3mm thickness). TLC visualization was done using UV-light at λ 354 and 365 nm. The ultraviolet and visible (UV-Vis) spectrum was run using Spectroscopic Genesys™ 2PC UV-Vis scanning spectrometer. Melting point were determined by Cambridge microscopy instruments. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker advance 400MHz spectrometer using tetramethylsilane (TMS) as the internal standard. Standard pulse sequences and parameters were used for the NMR experiments and all chemical shifts were reported in parts per million (ppm, δ).

3.4. Extraction and Phytochemical Screening Test

3.4.1. Extraction

Lobelia rhynchopetalum roots were subsequently washed with tap water then with distilled water and dried at room temperature for two weeks. The dried plant materials were powdered with a mixer grinder to 2 mm or smaller particle size. The ground plant species were stored in a storage container at room temperature. 350 grams of dried and powdered plant material were extracted with 2.5L n-hexane for 72 hr separately, the solution was filtered, and the defatted residue was extracted with 2.5L dichloromethane: methanol (1:1) for 72 hr with occasional shaking at room temperature. The solution was filtrated through whattman filter paper no.1 and concentrated by a vacuum rotary evaporator at 50 ^{o}C, to afford 13.40 g (3.83 %) brown crude extract. The marc left was further extracted with methanol for 72 hr with occasional shaking at room temperature. The solution was filtered and concentrated by a vacuum rotary evaporator at a temperature of about 50 ^{o}C to afford 7.3 g (2.08 %) brown crude extract. All the extracts were checked by thin layer chromatography (TLC) using ultra violet lamp at 354 and 365nm wave length, and the components of extract was checked by using appropriate reagent. Based on resolution the appropriate components which have better resolution were identified on the bases of TLC profile and stored in deep freezer until needed for further experiment.

3.4.2. Acid-Base extraction of alkaloid constituents

Alkaloids constituents were selectively extracted in acid-base extraction approach as per a standard procedure previously described by (Ramya *et al.*, 2019). Dried powdered roots of *Lobelia rhynchopetalum* (160 g) were extracted in *n*-hexane separately, the solution was filtered, and the residue was extracted with 2L ethanol at room temperature for 72 hr. Then solvent was evaporated using a rotary evaporator at a temperature of about 50 ^{o}C to yield crude extracts. The defatted ethanolic crude extract was acidified with 3% HCl (50 mL) to pH 5. After pre-saturation with water and partition by adding chloroform, the extract was separated in to aqueous layer and organic layer. Then, the aqueous and organic layers were separated using separatory funnel and repeated for three times. The acidic aqueous phase was basified by adding 5 % NH_3 solution to pH 11, and then partitioned by chloroform. Then the aqueous and organic layers were separated using separatory funnel and the process was repeated three times. The chloroform

extracts were combined, and concentrated under vacuum rotary evaporator to obtain 5.2 g (3.25 %) of brown crude alkaloid extract. Finally, the crude extracts were checked by TLC profile (*n*-hexane: EtOAc solvent system) and chemical test of alkaloids by Wagner's reagent then the crude extract was collected in labeled sterile containers and stored in deep freezer until needed for further experiment (for isolation compound).

3.4.3. Phytochemical Screening Tests

- **Test for flavonoids:** To 0.5 g portion of crude extract (DCM: MeOH (1:1), MeOH (100%) extract), 10mL of ethyl acetate was added and heated for 3 min using steam bath. The mixture was filtered, and the filtrate was mixed with 1mL of dilute ammonia solution. Formation of intense yellow color ratifies the presence of flavonoids (Bargah, 2015).
- **Test for Saponins**: To 0.5 g of crude extract (DCM: MeOH (1:1), MeOH (100%) extract), 2mL of distilled water was added and shaken. The formation of one centimeter layer of foam indicates the presence of saponins (Prod *et al*., 2012).
- **Test for tannins:** The crude extract (DCM: MeOH (1:1), MeOH (100%) extract) (0.5 g) was boiled in 10mL of water in a test tube and filtered. To the filtrate, few drops of 0.1% $FeCl_3$ was added to give a brownish green or a blue-black color which confirms the presence of tannins (Mir *et al*., 2016).
- **Test for terpenoids** (Salkowski test): To 0.5mL of each extract (DCM: MeOH (1:1), MeOH (100%) extract) was mixed with 2mL of chloroform, and 3 mL concentrated H_2SO_4 was carefully added to form a layer. A reddish brown coloration of the interface was formed to show positive results for the presence of terpenoids (Fitokimia *et al*., 2016).
- **Test forAnthraquinone:** 0.5 g of the extract (DCM: MeOH (1:1), MeOH (100 %)) was boiled with 2ml HCl for five minutes in a water bath. The resultant solution was filtered and allowed to cool at room temperature. Equal volume of chloroform was added to the filtrate. Few drops of 10% NH3 solution was added to the mixture and heated. Formation of rose-pink colour indicates the presence of anthraquinone (Rasoanaivo *et al*., 1995).
- **Test for Cardiac glycosides:** 0.5 g of the extract (DCM: MeOH (1:1), MeOH (100 %)) was added to few drops of glacial acetic acid and ferric chloride solution. The solution

was mixed and concentrated sulfuric acid was added to the sides of the test tube. Formation of two layers was observed. Upper acidic layer changes to bluish green and lower reddish brown layer indicates the presence of cardiac glycosides (Reveglia *et al.*, 2018).

- **Anthraquinoneglycosides:** To 0.5g of each extract solution was hydrolyzed by adding 5 ml of dilute hydrochloric acid and 5 ml of 5% ferric chloride solution. To the hydrolyzed extract solution 5 ml of 10% sulphuric acid was added and heated on water bath for 5 minutes, filter, cool the filtrate and equal volume of benzene was added and shaken gently for 2 minutes and a benzene layer was separated. To this add 10% ammonia solution, ammonical layer was separated. The colour change from rose to pink color indicates the presence of glycosides (Ashmawy *et al*., 2019).
- **Anthranol glycosides:** 0.5 ml of each extract was added to 5 ml of 5% ferric chloride solution and 5 ml dil. hydrochloric acid was heated for 5 minutes in water bath. This solution was cooled and 3ml of benzene or organic solvent was added and shaken well. To the separated organic layer, equal volume of 10% ammonia solution was added. The formation of rose pink/red at ammonia layer shows the presence of glycosides (Ushie & Olumide, 2016).
- **Test for Alkaloid:** 0.5 mg of crude extract (DCM: MeOH (1:1), MeOH (100 %)) was separately stirred with 1 % HCl (0.5 mL) on a water bath for 5 min and filtered. These filtrates were divided into three equal parts. To one portion of filtrate, Wagner'sreagent (iodine in Potassium iodide solution) (1mL) was added. Formation of brown/reddish precipitate indicates the presence of alkaloids (Ramya *et al*., 2019).

3.5. Isolation of Compounds

3.5.1. Column Chromatography Technique

The crude extracts of dichloromethane/methanol (1: 1) of (10 g) was adsorbed on 10 g of silica gel (mesh size 60-120), subjected to silica gel chromatography (170 g of silica gel) using *n*-hexane for packing and elusion was carried out with increasing gradient of ethyl acetate in *n*-hexane and methanol in dichloromethane as eluent. A total of 144 fractions each 100 mL (Table 2) were collected and concentrated at 40^{o}C under reduced pressure. Fractions that showed similar Rf values and the same characteristic color on TLC were combined. Fraction 19-21 afforded compound **1** single spot on TLC (methanol: dichloromethane, 0.5: 9.5, R_f value of 0.45, 50 mg).

Fractions 31-38 (1.2g) which showed similar TLC profile were combined and rechromatographed over silica gel (30g) using isocratic solvent system n-hexane: EtOAc (7:3) as eluent to furnish 23 fractions each 10mL. Fraction 14 (18 mg) showed one spot on TLC and was coded as compound **2**. Fractions from 11-12 afforded compound **4** with single spot on TLC (ethylacetate: *n*-hexane, 1:9, Rf value of 0.75, 28 mg). Fraction 12 (47 mg) showing two spots on TLC was also subjected to silica gel (10 g) column chromatography using isocratic solvent system n-hexane: EtOAc (8:2) as eluent. Five fractions each 10 mL were collected. Fraction 4 (25 mg) showed a single spot on TLC with n-hexane: EtOAc (8:2) as a mobile phase and was coded as compound **5**. Fractions 81-92 (92 mg) having three spots on TLC were combined and re-chromatographed over silica gel (15 g) using isocratic solvent system n-hexane: EtOAc (6:4) as eluent to furnish 12 fractions each 10 mL. Fraction 5 (16 mg) showed one spot on TLC and labelled as compound **6**.

Table 2: Solvent systems and fractions collected from (CH_2Cl_2: MeOH (1:1)) crude extract of the roots *Lobelia rhynchopetulam*

Solvent system	Ratio	Fractions	Solvent system	Ratio	Fractions
n-hexane/EtOAc	9.9:0.1	F1-F2	CH_2Cl_2/MeOH	9.5:0.5	F81-F107
“	9.8:0.2	F3-F4	“	9.4:0.6	F108-F128
“	9.7:0.3	F5-F6	“	7:3	F129-F131
“	9.6:0.4	F7-F8	“	5:5	F132-F134
“	9.5:0.5	F9-F10	”	3:7	F135-F140

“	9:1	F11-F12	MeOH	100	F141-F144
“	8.5:1.5	F13-F15			
“	8:2	F16- F23			
“	7:3	F24-F27			
“	5:5	F28-F33			
“	2.5:7.5	F34-F45			
EtOAc	100	F46-F80			

Similarly, acid-base crude extract (5 g) was adsorbed with 5 g of silica gel mesh size (60-120) and subjected to silica gel column chromatography (150 g of silica gel used *n*- hexane for packing) using increasing gradient of ethyl acetate in *n*-hexane and methanol in dichloromethane as eluent. A total of 91 fractions each 100 mL (Table 3) were collected and concentrated at 40 ^{o}C under reduced pressure. Fractions that showed similar Rf values and the same characteristic color on TLC were combined. Fractions from 41-42 afforded single spot on TLC (ethylacetate: *n*-hexane, 7:3, *R*f value of 0.57, 36 mg) and coded as compound **3**

Table 3. Summary of fractions of acid-base extracts

Solvent system	Ratio	Fractions	Solvent system	Ratio	Fractions
n-hexane/EtOAc	9.5:0.5	F1-F5	CH_2Cl_2/MeOH	9.5:0.5	F64-F66
“	9.8:0.2	F6-F9		9:1	F67-F70
“	9.7:0.3	F10-F12	“	8.5:1.5	F71-F74
“	9.6:0.4	F13-F19	“	8:2	F75-F79
“	9.5:0.5	F20-F24	”	7:3	F80-F84
“	9:1	F25-F329	Methanol	100	F85-F91
“	8.5:1.5	F30-F33			
“	8:2	F34- F40			
“	7:3	F41-F42			
EtOAc	100	F43-F63			

3.5.2. Structural Elucidation of the Isolated Compounds

The spectroscopic techniques (NMR) were employed to elucidate the structure of isolated compounds. Compound was subjected with the combination of reagents such as sodium acetate, boric acid, aluminium chloride, hydrochloric acid and sodium methoxide (Ouattara *et al*., 2014). The ^{1}H-NMR and ^{13}C-NMR were obtained in CDCl3 on BrukerAvance 400 MHz spectrometer in the NMR laboratory of Addis Abebe University (AAU), Ethiopia. The isolated compounds were taken to determine the structure by instrumental spectral analysis.

3.6. Evaluation of Biological Activity

The antibacterial activity of crude extracts and isolated compounds were done in collaboration with the Department of Applied Biology, Adama Science and Technology University.

3.6.1. Antibacterial Activity

The *in vitro* screening of antibacterial activity of crude extracts and isolated compounds were conducted by disk diffusion method against 3 strains of bacteria including Gram positive bacteria, such as, *S. aureus*, *S.pyogenes*, and Gram-negative bacteria *Escherichia coli*. The bacterial cultures were inoculated into the nutrient broth (inoculation medium) and incubated overnight at 37 ^{o}C. Inoculated medium containing 24 hr grown culture were added aseptically to the nutrient medium and mixed thoroughly to get a uniform distribution. The solution was poured approximately 20 mL of sterile MHA in sterile culture plates and allowed to attain room temperature. Sterile agar-disc diffusion previously soaked in a known concentration (0.5 mg/mL and 1 mg/mL per disc) extract or pure compound of *Lobelia rhynchopetalum* and standard drugs was prepared in DMSO using nutrient agar tubes and carefully placed at the centre of the labeled seeded plate. Mueller–Hinton sterile agar plates were seeded with indicator bacterial strains (1.5 x10^{8} cfu/mL) and allowed to stay at 37 °C for 3 hr. Control experiments were carried out under similar conditions using ciprofloxacin for antibacterial activity as a standard drug. The zones of growth inhibition around the disks were measured after 24 hr of incubation at 37 °C for bacteria. The inhibition zones were measured with a ruler and compared with the positive control disk (disk containing Ciprofloxacin) of the same concentration. DMSO used as negative control during the whole test on bacteria. All of the experiments were performed in triplicate and the results (millimeters of the inhibition zone) were expressed as mean values (Bereksi *et al.*, 2018).

3.6.2. Antioxidant Activity

DPPH assay: The free radical scavenging activity of the DCM: MeOH, MeOH extract and isolated compounds were measured by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) method (Garcia *et al*., 2012). With this method, it is possible to determine the radical scavenging power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm. As a result of the color changing from purple to yellow, the absorbance was decreased when the DPPH radical is scavenged by an antioxidant through donation of hydrogen to form a stable DPPH-H molecule (Shylaja *et al.*, 2019). Lower absorbance of the reaction mixture indicated higher free radical scavenging activities (White *et al*., 2014).

The DCM: MeOH (1: 1) extract was dissolved in four vials containing methanol to give 500, 250, 125 and 62µg/ml. To each 1mL of the above extracts were added each 4mL of 0.04% DPPH which was given 100, 50, 25 and 12.5 µg/ml. The resulting solution was placed in an oven at 37^{o}C for 30 minutes and subjected to UV-Vis Spectrophotometer to record absorbance at 517 nm. This was repeated for the methanol extracts and isolated compounds. The percentage DPPH inhibition was calculated according to the following formula (Himamura *et al*., 2014).

(%) of radical scavanging activity = $\frac{Ac-As}{Ac}$x 100, where As is absorbance at analytical sample,

Ac is absorbance at additon of stadard.

3.7. Molecular Docking Study of Isolated Compounds for Antibacterial Activity

In the study of molecular modeling, Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. In this study a series of isolated compounds were evaluated for focusing on binding modes, potential interactions and specific binding sites of DNA-gyrase (Mishra *et al*., 2019).

In this study, isolated compounds were subjected to molecular docking studies using the ADT version 1.5.2 and Auto Dock version 4.2 docking program to investigate the potential binding mode. The crystal structure of the enzyme (PDB code 1KZN) with resolution 2.3 will be chosen as the protein model. The structures of ligands were optimized using the Hyper Chem 7.0 software. Auto Dock version 4.2 was used to prepare the molecules and parameters before

submitting it for docking analysis with Auto Dock. Polar hydrogen atoms was added while non-polar hydrogen atoms were merged and then, Gasteiger partial atomic charges were assigned to the ligands. All rotatable bonds of ligands, defined by default of the program, were allowed to rotate during the automated docking process and then prepared protein and ligand structures were saved in the protein data base (PDBQT) format suitable for calculating energy grid maps. A grid box size of 46×46×46 points with a grid spacing of 0.375A was considered. Lamarckian genetic algorithm (LGA) program with an adaptive whole method search in the Auto Dock was chosen to calculate the different ligand conformers. After 200 independent docking runs for each ligand, a cluster analysis was done. In according to the root mean square deviation (RMSD) tolerance of 2.0 conformations were clustered and ranked by energy of which the conformation with the best scored pose with the lowest binding energy was selected for these ligand (Ferreira *et al.*, 2015).

CHAPTER FOUR

4. Results and Discussion

4.1. Extraction yields of the Roots of *L. rhynchopetulam*

The percent yield (on dry weight basis) of the various fractions from the roots of *Lobelia rhynchopetulam* was 3.83 % (dichloromethane: methanol) and 2.08 % (methanol). The yield from the dichloromethane: methanol fraction was found to be highest as compared to that of the methanol fraction. This may be due to the intermediate polarity of dichloromethane: methanol, which may potentially extract in both tips of polarity.

Table 4: Extracts yield of the roots of *L. rhynchopetulam*

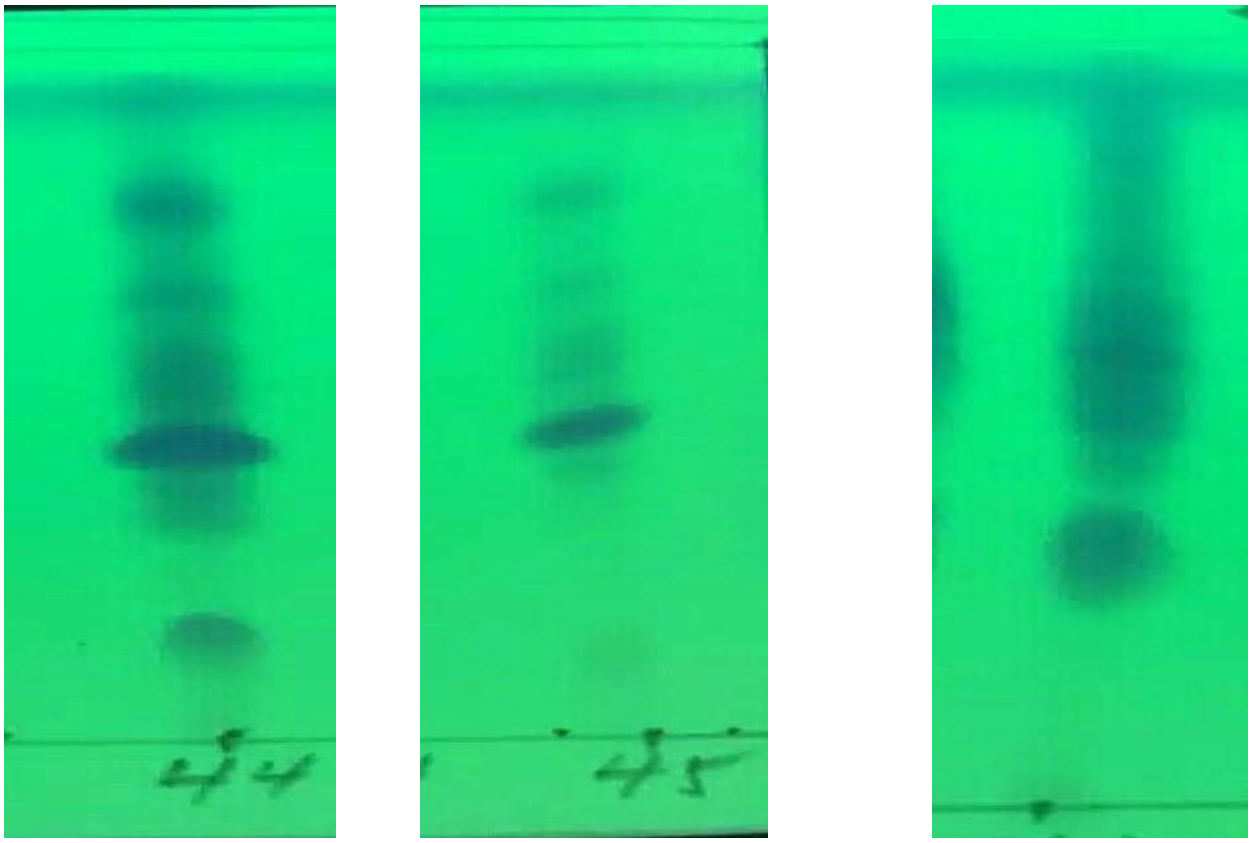
Extract	Color of the crude extract	weight of extract in (g)	% yield
CH_2Cl_2: MeOH	Brown crude extract	13.40g	3.83 %
MeOH	Dark brown crude extract	7.3g	2.08 %
Acid-base	Brown crude extract	5.2g	(3.25 %)

4.2. TLC Analysis of the Extracts of the Roots of *L. rhynchopetulam*

The dichloromethane: methanol, methanol and Acid-base extracts were analyzed with TLC (Figure 3). The mobile phases used for dichloromethane: methanol, methanol and Acid-base extracts were n-hexane: ethyl acetate (8:2), n-hexane: ethyl acetate (6:4) and dichloromethane :methanol (9.5: 0.5), respectively. Spots were visualized using ultra violet lamp at 354 and 365nm wave length, and the components of crude extract was checked by using appropriate reagent.

CH_2Cl_2: MeOH extrat | MeOH extract | Acid-base extract

n-hexane: ethyl acetate (8:2) | n-hexane: ethyl acetate (6:4) | CH_2Cl_2: MeOH (9.5: 0.5)



a) TLC of *L. rhychopetulam* CH_2Cl_2: MeOH extrat

b) TLC of *L. rhychopetulam* MeOH extract

c) TLC of *L. rhychopetulam* Acid-base extract

Figure 3. TLC profile of the crude extracts of the roots of *L. rhychopetulam*

4.3. Phytochemical screening test

Nature has been a source of high phytochemical diversity for thousands of years, many of them possessing interesting biological activities and medicinal properties. Phytochemical screening analysis was carried out to identify the secondary metabolites present in the crude extracts

following standard experimental procedure. The results suggested that alkaloids, tannins, terpenoids, anthraquinoneglycosides, cardiac glycosides, anthraquinones and anthranol glycosides are present within the crude root extracts (**Table 5**). Qualitative phytochemical tests showed significant indication of the presence of major secondary metabolites in *Lobelia rhynchopetulam* dichloromethane: methanol and methanolic extracts showed the presence of almost all the phytochemicals analyzed. Based on the presence of major bioactive components and the high yield, the dichloromethane/ methanol extract was likely to be considered suitable for further study. The results obtained in this study suggest the identified phytochemical compounds may be the bioactive constituents of this plant suggesting the plant as a valuable reservoir of bioactive compounds of substantial medicinal importance.

Table 5**.** Phytochemical screening test

No	Phytoconstituents	Reagents	Extracts	
			DCM:MeOH	Methanol
1	Alkaloids	Wagner's	+	+
2	Indoles	Salkowiski	_	_
3	Tannins	KOH	+	+
4	Flavonoids	Shinoda test	_	_
5	Anthraquinones	Born trager's	+	+
6	Terpenoids	Salkowiski	+	+
7	Anthraquinone glycosides	Born trager's	+	+
8	Cardiac glycosides	Keller killiani's	+	+
9	Anthranol	Modified born trager	+	+

	glycosides	test		
10	Saponins	Foam test	_	_

(+) indicates Present (-) indicates absent

4.4. Characterization of Isolated Compounds

One-dimensional NMR experiments are fundamental in determining the resonance frequency of each ^{1}H or ^{13}C nucleus in the molecule, thus, both ^{1}H and ^{13}C NMR experiments were employed the most in elucidating the structures of the isolated compounds in the current study. Moreover, the chemical shifts obtained gave important information about the nature and number of protons and carbons in the compounds of interest. The recorded chemical shifts were then validated by comparing them with existing literature data (Hussain *et al*., 2018).

4.4.1. Compound 1

Compound **1** was obtained as yellowish oil (50mg, boiling point of 155^{o}C) from fraction 19-21 of root extracts of *L*. *rhynchopetalum*. Its R_f value was 0.45 (methanol/dichloromethane, 0.5: 9.5) as eluent. The ^{1}H NMR spectrum (**Appendix 1.1**) revealed the existence of four olefinic protons at 6.34 (1H, dq, J =7.0, 10.7 Hz, H-2), 5.55 (1H, d, J =10.7Hz, H-3), 5.58 (1H, dd, j= 6.7,15.4 Hz, H-10), and 5.90 (1H ,m, H-11), together with one methyl 1.83 (3H,dd,1.7,6.9) two oxygen-bearing methines at 4.33 (1H, d, J= 6.5Hz, H-8), and 4.15 (1H,t, J=6.5Hz, H-9) and one oxygen-bearing methylenes at 3.68 (2H, t, J= 6.3Hz, H-14). Furthermore, their coupling constants (neraly 16 Hz) suggest the trans form of the double bonds. The ^{13}C NMR and DEPT spectra (**Table 6**) of **1** possessed **14** carbons signals, including one methyl, four olefinic, three methylenes, two methines and four quaternary carbons. The carbon signal of the oxygenated methylene mentioned above appeared at 62.21. Moreover, the four quaternary carbons (71.27, 78, 71.46, and 79.24) in **1** are part of one conjugated diyne structure. On the basis of this observation it could be proposed that compound **1** is a C14 polyacetylene with two double bonds, two triple bonds and three hydroxyl groups. By comparing the NMR data with literaturethe compound (**1**) was identified to be Lobetyol. Lobetyol previously isolated from *Lobelia chinensis* (Ishimaru *et*

al., 2003). However, to the best of our knowledge, Lobetyol (2,10-tetradecadien-4,6-diyne-8,9,14-triol) (**1**) was isolated from root *Lobelia rhynchopetalum* for the first time in this study.

Table 6. ^{1}H, ^{13}C and DEPT-135 NMR spectral data of compound (**1**)

Position	**1**			Litrature (Ishimaru *et al.*, 2003).	
	^{1}H	^{13}C	DEPT- 135	^{1}H	^{13}C
1	1.83 (3H,dd,1.7,6.9)	18.9	18.9	1.83 (3H,dd,1.7,6.9)	18.9
2	6.35 (1H,dq, 6.9, 15.5)	144.6	144.6	6.35 (1H,dq,6.9,15.5)	144.5
3	5.55 (d, 4.2, 15.9)	109.4	109.4	5.55 (1H,d,15.9)	109.4
4	-	71.2	-	-	71.2
5	-	78.0	-	-	77.9
6	-	71.5	-	-	71.6
7	-	79.3	-	-	79.4
8	4.32 (1H,d,6.5)	66.7	66.7	4.29 (1H,d,6.5)	66.7
9	4.15 (,t ,6.5)	75.4	75.4	4.13(1H,t,6.5)	75.4
10	5.56 (1H,dd,6.7,15.4)	127.5	127.5	5.57 (1H, dd,6.7,15.4)	127.6
11	5.87 (1H,m)	135.1	135.1	5.87 (1H,m)	135.0
12	2.19 (2H,m)	28.8	28.8	2.20 (2H,m)	28.8
13	1.69 (2H,m)	31.6	31.6	1.69 (2H,m)	31.6
14	3.68 (2H,t,J=6.3Hz, H14)	62.2	62.2	3.67 (2H,t,6.3)	62.1

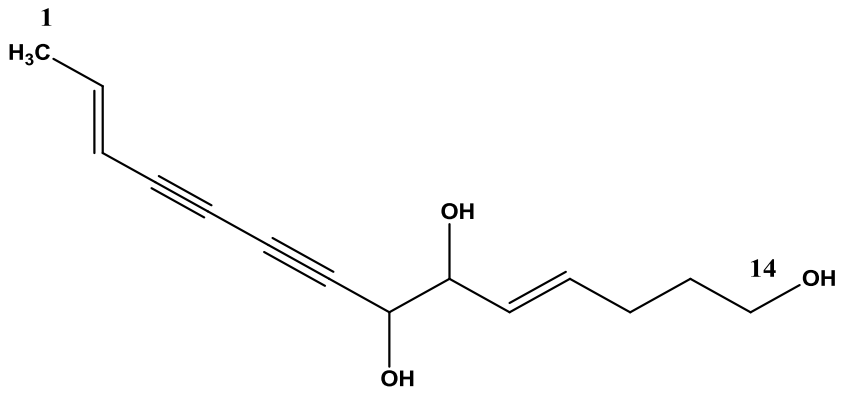


Figure 4. Structure of compound (**1**)

4.4.2. Compound 2

Compound **2** was isolated from dichloromethane: methanol root crude extract of *L. rhynchopetalum* from fraction 14 (18 mg). It was eluted with n-hexane: ethyl acetate 7:3 with its physical appearance as pale yellow solid. It melted at 141-142^{0}C. The TLC profile showed black spot with R_f value of 0.47.

The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of compound **2** (**Table 6Appendix 2.1**) revealed the existence of singlet signals of olefinic methylene protons at δ 4.68 and 4.73 ppm and a doublet signal of olefinic methine proton at δ 5.35 ppm. The spectrum also showed the presence of an oxymethine proton peaked at δ 2.79 (1H (t). Two methyl singlet signals at *δ*H 0.81 and 0.98, one methyl triplet signal at *δ*H 0.90 and one methyl doublet signal at *δ*H 1.03 were also observed from spectrum. Compound **2** indicated the presence of three singlet, one triplet and one doublet signals characteristic for the five methyl groups. Moreover, it showed several multiplet signals for the other remaining protons.

The ^{13}C-NMR spectrum (**Table 6, Appendix 2.2**) of compound **2** with the aid of DEPT-135 revealed the presence of twenty nine well resolved carbon resonances of which four were olefinic carbons at *δC*105.9 (C-26), 127.68 (C-6), 137.62 (C-5) and 159.09 (C-25). The spectrum also displayed the carbon signal at *δC*73.21 (C-3) which corresponds to carbon bearing hydroxyl group (also supported by methine proton (H-3) signal at *δ*H2.79 on ^{1}H-NMR). Five methyl carbon signals at [*δc*14.45 (C-18), 21.87 (C-19), 18.34 (C-21), 22.0 (C-27) and 14.13 (C-

29], twelve methylene carbon signals at [δc29.66 (C-1), 36.53 (C-2), 40.41 (C-4), 29.32 (C-7), 21.19 (C-11), 39.71 (C-12), 28.86 (C-15), 25.36(C-16), 33.8(C-22), 31.93(C-23), 105.95(C-26) and 24.35(C-28], (Eight methine carbon signals at [δc76.01 (C-3), 127.68 (C-6), 31.31 (C-8), 45.33 (C-9), 52.19 (C-14), 43.44(C-17), 41.52 (C-20) and 46.89 (C-24)], and four quaternary carbon signals at [δc137.62 (C-5), 37.43 (C-10),48.88 (C-13) and 159.09 (C-25)] were also observed from the spectrum. by comparing with the literature reported for related compound24-Methylene-ergosta-5-en-3β-ol (Ododo *et al.*, 2016). Thus structure of the compound (**3**) was identified to be 24-ethyl-3-hydroxylcholesta-5,25-diene. These assignments are in good agreement for the structure of 24-ethyl-3-hydroxylcholesta-5, 25-diene.

Table 7.^{1}H, ^{13}C and DEPT-135 NMR spectral data of compound (**2**)

Position	**1**			Litrature (Ododo *et al.*, 2016).	
	^{1}H	^{13}C	DEPT- 135	^{1}H	^{13}C
1	1.05 (2H)	37.43		1.04 (2H)	37.28
2	1.62 (2H,m)	36.1		1.63 (2H,m)	36.53
3	2.79 (1H,m)	73.20		2.79 (1H, m)	73.18
4	1.27 (2H,m)	46.89		1.27 (2H,m)	47.1
5	-	137.62		-	139.79
6	5.35 (1H, t)	127.68		5.35 (1H *d*, 4.9,)	125.68
7	1.27 (2H,m)	29.33		1.27 (2H,m)	29.32
8	1.62(1H,m)	31.31		1.63 (1H,m)	31.17
9	1.62 (1H,m)	50.33		1.63 (1H,m)	50.2
10	-	37.3		-	37.3
11	1.27 (2H)	21.87		1.27 (2H)	21.19
12	1.27 (2H)	39.71		1.27 (2H)	39.71
13	-	42.34		-	42.34
14	2.02 (1H,m)	56.80		2.02 (1H,m)	56.80
15	1.27 (2H,m)	28.26		1.27 (2H,m)	28.27

16	1.27 (2H,m)	25.63		1.27 (2H,m)	25.36
17	1.62 (1Hm)	56.08		1.63 (1Hm)	56.03
18	0.81 (3H,s)	14.45		0.63 (3H,s)	14.18
19	0.98 (3H,s)	19.37		0.94 (3H,s)	19.37
20	2.06 (1H,m)	41.52		2.06 (1H,m)	42.24
21	1.03 (3H,d)	18.34		0.90 (3H,d)	18.97
22	1.62 (1Hm)	31.9		1.63 (1Hm)	31.8
23	1.27 (1Hm)	29.71		1.27 (1Hm)	29.66
24	1.62 (2H,m)	48.8		1.63 (1Hm)	48.8
25	-	159		-	156
26	4.68 (1H, brd s) 4.73 (*1H,brd s)*	105.94		4.65 (1H, brd s) 4.71 (*1H,brd s)*	105.95
27	1.08 (3H,s)	22.0		0.83(3H,t)	21.87
28	1.27 (2H,m)	24.67		1.63 (1Hm)	24.35
29	0.90 (3H,t)	14.13		0.78 (3H, t)	14.04

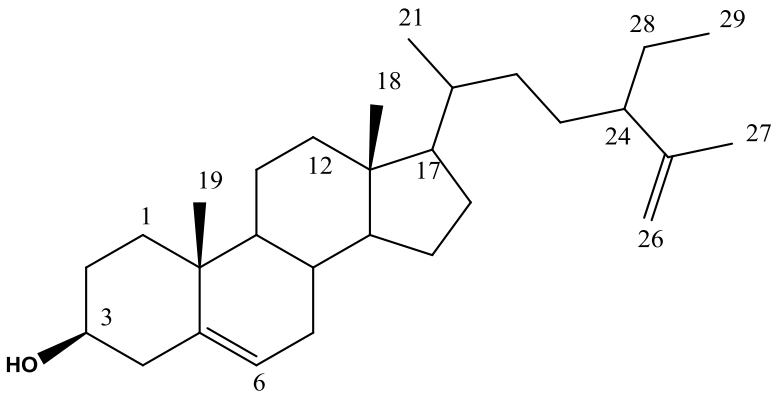


Figure 5. Proposed structure of compound **2**

4.4.3. Compound 3

Compound **3** was isolated as Brown resinous substances (36 mg) from Fractions 41-42 with melting point of 99-103 ^{o}C and Rf value of 0.57 (20 % ethyl acetate in *n*-hexane as eluent). The ^{1}H-NMR spectrum of **3 (Appendix 3.1**) showed a multiplet signal at δ5.37 and a doublet of doublet at δ 5.14, which are for the two protons from the C-3, C-4 double bond in the piperidine ring. The ^{1}H-NMR (400 MHz, $CDCl_3$) spectrum of **3** revealed the presence of OH groups at 3.68 broad singlet. The signals due to H-2 and H-6 appeared at *δ* 4.12 and 2.79 as multiplets respectively. The broad singlets at *δ* 3.68 (H-8, H-10) in **3** were due to the carbinylic protons. The two sharp triplets in **3** at *δ* 1.01 (H-14 and *δ* 0.93 H-15) were due to the methyls attached to the methylenes, and their corresponding carbon signals resonated at *δ* 19.17 and 14.14 respectively. Similarly, the presence of nitrogen in the molecules was counterchecked with the element detection method by fusing the samples with the sodium metal and also by spraying Dragendorff's reagent.

The proposed structure of **3** was confirmed by the ^{13}C-NMR spectrum and the DEPT 135 experiment. The carbons carrying the OH groups (C -8 and C-10)) appeared at 71.81, as well as the carbons connected with the nitrogen atom (C-2 and C-6) appeared at 62.12 and 61.58 respectively. The carbons from the double bond were observed at 128.85 and 130.93. Thus, based on the above spectral data (**Table 8**) and extensive comparison with literature of compound **3** was identified aspertin-A. Aspertin-A previously isolated from *Andrachneaspera* (Ahmad *et al*., 2002). This compound needs 2D NMR and mass spectrophotometry for further confirmation of the structure of compound **3.**

Table 8. ^{1}H, ^{13}C and DEPT-135 NMR spectral data of compound (**3**)

Position	**1**			Litrature (aspertin-A)	
	^{1}H	^{13}C	DEPT-135	^{1}H	^{13}C
1	-	-	-	-	-
C-2	4.12m	62.12		4.12 (1H, m, H-2)	55.1
C-3	5.12,dd	130.93		5.15 (1H, dd, *J*=10.2, 1.2 Hz,H-3)	136.5
C-4	5.37m	128.85		*δ* 5.22 (1H, m, H-4)	128.6

C-5	2.02, m	27.73		2.02, m (H-5)	26.1
C-6	2.79 (1H,s H-6)	61.58		2.89 (1H, br.s, H-6)	61.3
C-7, 9	1.62, m	31.94		1.63m	30.8
C-8,10	3.68 (2H, br.s, H-8 and H-10)	71.81		3.89 (2H, br.s, H-2'and H-2'')	70.3
C-11,13	1.62	32.85		1.40-1.60, m	31.0
C-14	1.01(3H, t, J=7.2 Hz, H-14)	19.17		1.03 (3H, t, J=7.2 Hz, H-14)	18.7
C-15	0.93 (3H, t, J=7.2 Hz, H-15)	14.14		0.92 (3H, t, J=7.2 Hz, H-15)	14.1

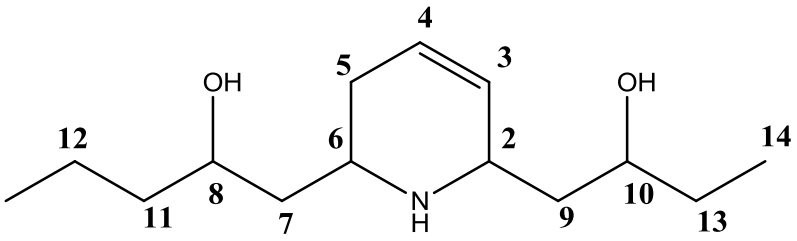


Figure 6. Proposed structure of compound **3**

4.4.4. Compound 4

Compound **4** was obtained as white solid (28 mg) melting at 43-45. It showed one spot on TLC at Rf 0.75 with n-hexane: ethyl acetate (9:1) solvent system as a mobile phase (**Figure 7**).

The ^{1}H-NMR spectrum (**Appendix 4.1**) of compound **4** showed the presence of carboxylic acid hydroxyl proton and aliphatic protons. A triplet peak at δ 2.36 is accounted to two aliphatic methylene protons (H-2, J = 7.6 Hz) on carbon directly attached to the carbonyl group. A multiplet peak at δ 1.65 shows two aliphatic methylene protons (H-3) which is attached to two methylene carbons. Moreover, the spectrum of this compound shows one broad singlet peak centered at δ 1.27 integrating for 8 hydrogens (H-4, H-5, H-6, H-7, H-8, H-9, H-10,H11), which

is a characteristic of protons on many over lapping methylene carbons. The triplet signal (3H) situated at δH 0.90 is evident for the presence of terminal methyl group (H-12, J = 6.8 Hz). The spectrum of this compound also revealed that carboxylic acid hydroxyl proton appeared as broad singlet peak at δ 5.36.

The ^{13}C-NMR spectrum (**Appendix 4.2**) in $CDCl_3$ of compound **4**, analysed with the aid of DEPT- 135 (**Appendix 4.3**) in CDCl3 showed a well resolved resonance of 12 carbon atoms. The DEPT-135 spectrum is designed to display separate spectra for CH, CH_2, and CH_3 carbon signals. In DEPT spectra signal intensity (i.e., sensitivity) arise by polarization transfer. In those spectra, data are collected in such way that the resulting signal is either positive or upward (methine, CH and methyl, CH_3) or negative or downward (methylene, CH_2) depending on the number of proton attached (Doreen Palu et al., 2019). In accordance with this principle, DEPT-135 spectrum of compound **4** showed ten negative (CH_2) and one positive (CH_3) carbons; all of them were attributable to aliphatic carbons. One carbon of ^{13}C spectrum was not seen on DEPT-135 spectrum revealing that this quaternary carbon (C), which was attributable to carbonyl carbon of the carboxylic acid. This structural type was further supported by the 13C-NMR spectrum, in which the appearance of the most downfield signal at δc178.15 (C-1) indicated the presence of the carboxylic acid. The spectra also showed ten aliphatic methylene carbons at δ 33.8 (C-2), 31.9 (C-3), 29.0 (C-4), 29.1 (C-5), 29.2 (C-6), 29.3 (C-7), 29.5 (C-8), 29.7 (C-9), 24.8 (C-10), 22.7 (C-11) and one aliphatic methyl carbon at δ 14.1 (C-12) (**Table 9** and **Appendix 4.2)**. The NMR spectrum of the proposed structure of compound **4** was found to agree with the reported literature spectral data of dodecanoic acid, also known as lauric acid (Yamashita *et al*., 2015). This compound was not yet reported from the genus *Lobelia.* The NMR spectral data used to characterize compound **4** was compared with literature reported for lauric acid with the whole results shown in **Table 9**.

Table 9. Comparison of ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (400 MHz, $CDCl_3$) Spectral data of compound **4** and literature reported for lauric acid.

Carbon position	^{13}C-NMR of δ *(ppm)*Compound **4**	DEPT δ*(ppm)*	^{13}C-NMR *δ* (ppm) data reported forlauric acid(Yamashita *et al., 2015)*	^{1}H-NMR *δ* (ppm) data of compound **4**	^{1}H-NMR *δ* (ppm) data reported forlauric acid (Yamashita *et al.*, 2015)	Nature of the carbon
1	178.1	-	178.5	-	-	C(quaternary carbon)
2	33.8	33.8	34.0	2.34 (t, J = 7.6 Hz, 2H)	2. 30 (*m*, H-2)	CH2
3	31.9	31.9	31.9	1.62 (*m*, 2H)	1.52 (*m*, H-3)	CH2
4	28.9	28.9	29.0	1.28 (br.*s*, 2H)	1.29 (*m*, H-4)	CH2
5	29.1	29.1	29.3	1.28 (br.*s*, 2H)	1.26 (*m*, H-5)	CH2
6	29.1	29.1	29.6	1.28 (br.*s*, 2H)	1.26 (*m*, H-6)	CH2
7	29.3	29.3	29.6	1.28 (br.*s*,2H)	1.26 (*m*, H-7)	CH2
8	29.5	29.5	29.6	1.28 (br.*s*, 2H)	1.26 (*m*, H-8)	CH2
9	29.7	29.7	29.4	1.28 (br.*s*, 2H)	1.29 (*m*, H-9)	CH2
10	24.8	24.8	24.7	1.28 (br.*s*, 2H)	1.29 (*m*,H-10)	CH2

11	22.7	22.7	22.7	1.28 (br.*s*, 2H)	1.31(*m*,H 11)	CH2
12	14.1	14.1	14.1	0.90 (*t*, *J* = 6.8 Hz, 3H)	0.88 (*t*, *J* = 7 Hz, H-12)	CH3

Based on the above spectroscopic data, the following structure was suggested for the compound.

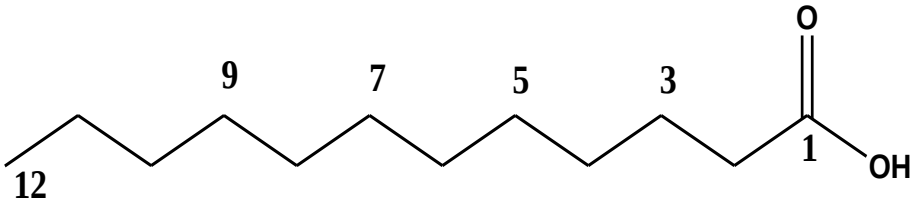


Figure 7. Proposed structure of compound **4**

4.4.5. Compound 5

Compound **5** was isolated from the Dichloromethane: Methanol extract as an orange needle. This compound has Rf Value 0.34 using n-hexane: ethylacetate (8:2) as a solvent. It shows a color change to violet when the TLC was sprayed with 5% methanolic KOH. The ^{1}H NMR spectrum (**Appendix 5.1**) of Compound (**5**), Showed a single two intense singlet signal at δ 12.06 and 12.16 PPm indicate the chelated hydroxyl proton resonance attached on C-1 and C-8 of the aromatic ring respectively. The doublet signal at δ 7.85 and 7.32 ppm correspond to the aromatic proton attached on C-5 and C-7respectively. The triplet signal which was integrated for three protons at δ 7.72 ppm indicated for aromatic proton attached on C-6. A broad singlet signal at δ 7.13 ppm is for the aromatic proton attached on C-2 and abroad singlet signal at δ 7.69 ppm is for aromatic proton attached on C-4. A strong intense signal around 2.49 ppm integrated for three protons are for methyl proton attached on C-3 of the aromatic ring. The result obtained here is comparable with ^{1}H NMR spectral data of chrysophanol from literature (**table 10** below) and the proposed structure of compound (**5**) was shown below.

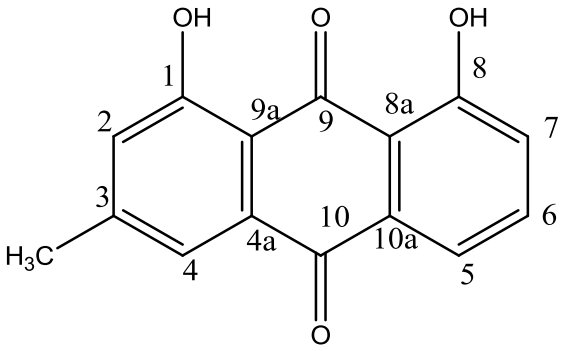


Figure 8. proposed structure of compound **5**

Table 10**.** Comparison of the observed ^{1}H NMR (400 MHz, $CDCl_3$) spectroscopic data of Compound (**5**) with the reported value of Chrysophanol (Minh *et al*., 2019).

H	Observed data (δ in ppm)	Reported datd (δ in ppm)
1-OH	12.06 s	12.00 s
2-H	7.13 br s	7.09 br s
3a-Me	2.49 s	2.48 s
4-H	7.69 br s	7.64br s
5-H	7.85 d, J=1.2, 8Hz	7.82dd, J=1.1, 7.5 Hz
6-H	7.72 d, J=6,8Hz	7.67 d, J=8.3, 7.9 Hz
7-H	7.32 d, J=1.2, 8Hz	7.29dd,J=1.2, 8.3Hz
8-OH	12.16 s	12.1 s

Furthermore, the melting point of the compound (**5**) is 196-198^{o}C, which is in agreement with the reported value of chrysophanol (196^{o}C).

4.4.6. Compound 6

Compound **6** was obtained as orange-yellow needles. This compound has Rf Value 0.38 using n-hexane: ethylacetate (6:4) as a solvent. The ^{1}H NMR (400 MHz, CDCl3, Appendix 2.3) shows two chelated hydroxyl proton resonating at δ 12.16 (1-OH) and δ 12.36 (8-OH), two meta coupled doublet at δ 6.72 (7-H) and δ 7.40 (5-H), two broad singlet signals at δ 7.11 (2-H) and 7.66 (4-H) and one methyl group at δ 2.48ppm. The ^{1}H NMR spectra of **6** closely resembled

those of **5**, except for the appearance of one methoxy group (δH 3.96, (3H, s). This suggested that **6** also has an anthraquinone of **5** and **6** was the result of replacing the aromatic ring proton at C-6 in **5**by a methoxy group. The ^{1}HNMR spectral data of compound 6 was found in a good agreement with the NMR spectral data reported in the literature for 1, 1,8-dihydroxy-3-methoxy-6-methylanthraquinone also known as physcion (**Table 11**). This compound was not yet reported from the genus *Lobelia*. The ^{1}H NMR spectral data used to characterize compound 6 was compared with literature reported for physcion with the whole results shown in **Table 11**.

Table 11**.** Comparison of the observed ^{1}H NMR spectroscopic data of Compound (6) with the reported value of physcion (Minh *et al*., 2019).

H	Observed data of Compound (6) (δ in ppm)	Reported data of physcion (6), (δ in ppm)
1-OH	12.16 s	12.08 s
2-H	7.11br s	7.16 br s
3a-CH3	2.48 s	2.48 s
4-H	7.66 br s	7.60 br s
5-H	7.40 d, J=1.6 Hz	7.34 d, J=2.4 Hz
6-OCH3	3.96	3.93 s
7-H	6.72 d, J= 1.6 HZ	6.68 , J=2.4 Hz
8-OH	12.36 s	12.28s

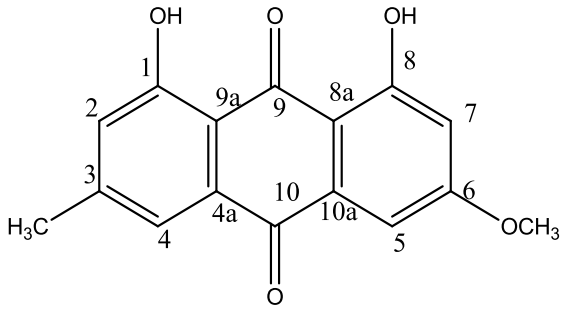


Figure 9. Proposed structure of compound 6

4.5. Biological activity of crude extract and isolated compounds of *Lobelia rhynchopetalum*

4.5.1. Antibacterial activity

The antimicrobial assay was carried out on crude extracts of dichloromethane: methanol(1:1), acid-base crude extracts and isolated compounds using agar diffusion method *in-vitro* against *S. aureus, E. coli, S .pynogenes* at two different concentration (0.5 and 1 mg/mL). Ciprofloxacin was used as positive control. The zone of inhibition values indicated that all the compounds (0.5 and 1mg) exhibited a varied range of 6-15 mm of antibacterial activities (**Table 11**) against all the tested bacterial strains. The antibacterial activity of crude extracts and compounds at 0.5 mg weaker than at 1 mg. This indicated that the antibacterial activities were increased with increased concentration of crude extracts and compounds. Compound **1** (1mg) showed strong activity (15±1, 14.33±0.58, 15.67±0.58 mm) against *E. coli*, *S.aureus* and S.pyogenes respectively, and comparable zone of inhibition (antibacterial activity) when compared to ciprofloxacin (19±1 and 17.33±1.53*and 19.67±0.58 mm).*

The Methanol/dichloromethane (1:1) extract (1mg) showed strong antibacterial activity (13±1 and 13.67±0.58 mm) against *S. aureus* and *S. pyogenes* compared to ciprofloxacin (17.33±1.53 and 19.67±0.58 mm).

Furthermore, the results of the antibacterial activity showed that the dichloromethane: methanol (1:1), acid-base crude extracts and isolated compounds displayed good antibacterial activity against *E. coli, S. aurous, S. pyogenes*.

To the best of our knowledge, there is no any report on antibacterial activities of the roots extracts of *Lobelia rhynchopetalum*. Therefore, this is the first report on the roots extracts of the species of the genus Laggera. But, the comparison of the isolated compounds of the current finding with literature showed almost similar results. Compound (**34**) and (**36**) showed moderate antimicrobial activity in disc diffusion method, and the minimum inhibitory concentration method against *Trichophyton mentagrophytes* (MIC=3.12mg/mL), *Pseudomonas aeruginosa*, *Escherichia coli*, and *Aspergillus fumigatus* (12.50 mg/mL) were determined respectively (Borchardt *et al*., 2008). Chrysoeriol (**56)** is a methoxylated flavone of great scientific interest because of its promising anti-microbial activities against various Gram-negative and Gram-

positive bacteria at the concentration of 40 µg /ml (Bashyal *et al.*, 2019). Scoparone **(67)** inhibited the growth of *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. In order to determine whether RK33 LCCs responded to7-substituted coumarins by growth inhibition, they were treated with increasing concentrations of scoparone over a period of 48-72h. In other studies scoparone (**67**) exhibited significant inhibition of superoxide anion generation with IC_{50} of 6.14 ± 1.97µM (Al-Awad *et al*., 2020). compound (**67**) showed the most potent inhibitory properties were observed against RK33 tumor cell lines at a concentration of 5µM due to 7-substituted coumarins moity (Xu *et al*., 2015). The compounds isoscopoletin (**68**) showed to be active against *Mycobacterium tuberculosis* H37Rv with MIC values of 42 *µ*g/mL (Hassan *et al*., 2017).

Table 12. Zone of bacterial growth inhibition diameter (mm)

Sample (0.5mg/mL)	Inhibition diameter (mm) ±*SD*		
	E. coli	*Staphylococcus aureus*	*S.pyogenes*
Methanol/dichloromethane (1:1) crude extract	9.3±0.58	11.33±1.15	10.67±1.15
Compound 1	12+2	14.33±0.58	12.33±2.08
Compound 2	7.33±0.58	6±0	7.33±0.58
Compound 3	8.68±0.58	6±0	8±1
Acid-base crude extract	7.67±0.58	6.33±0.58	7.33±1.154
Ciprofloxacin	17±1	18±1	18±1
Sample (1 mg/mL)	Inhibition diameter (mm) ±*SD*		
	E. coli	*Staphylococcus aureus*	*S. pyogenes*
Methanol/dichloromethane (1:1) crude extract	11.67±1.52	13±1	13.67±0.58
Compound 1	15±1	14.33±0.58	15.67±0.58
Compound 2	7.33±0.58	7±0	8.33±0.58
Compound 3	7.67±1.15	7±1	8±0
Acid-base crude extract	8±1	8±1	9±1
Ciprofloxacin	19±1	17.33±1.53	19.67±0.58

SD = standard deviation

4.5.2. Antioxidant activity

The radical scavenging ability of the crude extracts and isolated compounds of *Lobelia rhynchopetalum* were estimated by the DPPH radical scavenging experiment by measuring the decrease in the absorbance of DPPH at 517 nm. Ascorbic acid was employed as positive control and deionised water as blank. The reduction of the DPPH was followed via the decrease in absorbance at 517 nm. The various crude extracts and pure compounds of *lobelia rhynchopetalum* significantly reduced the DPPH (**Table 11**). The DPPH radical scavenging activities (%) of extracts and isolated compounds were found to be 71.6 (compound **1**), 27.2 (compound **2**), 70 (DCM: methanol (1:1) extract), 60.9 (Acid-base extract**)** and 67.2 (compound **3**) respectively at 100 µg/ml (**Table 12**). It was observed that the DPPH scavenging activity increased with increasing concentration of the samples in the assay. For the various concentrations, compound **1** exhibited slightly highest percent inhibition of the DPPH as compared to the other extracts and isolated compounds. The positive control, ascorbic acid showed maximum scavenging effect at very low and high concentration (84.5, 87.2, 90.9and 93.4 % at 12, 25, 50 and 100 µg/ml) (**Figure 9**)

The present findings were in agreement with those reported for compounds previously isolated from *L. genus* such as lobetyolin (**70**), lobetyolinin (**71**), isolobetyol (**72**), and lobetyol (**73**) (Koz *et al*., 2008). Compounds (**72**) and (**73**) exhibited moderate cytotoxic activity against the two cancer cell lines NCI-H292 and MSTO-211H. The IC_{50} value of (**72**) were 12.36 and 9.31µM against the two cell lines; while the IC_{50} values of (**73**) were 11.76 and 9.64µM respectively (Bálványos *et al.*, 2004). Phytotoxic potential of compound (**61**) proved that it could serve as phytotoxic effect with IC_{50} value of 282.7µg/ml. On the other hand, high antioxidant effect of this compound makes it potent for defense against oxidative stress and free radical scavenging activities in plants. Many foods are rich in Q3G and human daily intake ranges between 10–20 mg. Q3G can transport across the intestinal brush border membrane by sodium dependent glucose transporter. Thus, it exhibited biological effects more efficient than other derivatives of quercetin (Islam *et al*., 2012). Because of powerful antioxidant potential, Q3G can be considered as a natural anticarcinogenic agent. Because of high antioxidant potential, Q3G and some other flavonoids have important effects on cancer chemoprevention. The chemopreventive properties of flavonoides are generally believed to reflect their ability to scavenge endogenous ROS. Inspite

of cytotoxic properties of Q3G *in vitro* condition, it may interfere in several steps that lead to the development of malignant tumors including protecting DNA from oxidative damages, inhibiting carcinogen and activating detoxifying systems (Tapas *et al.*, 2008). Q3G exhibited high antioxidant activity in DPPH assay with IC_{50} value of 22µg/ml.

Table 13. % radical scavenging activity of crude extracts and compounds isolated from roots of *L. rhynchopetalum*

Conc (µg/ml)	Samples									
	DCM:MeOH(1:1)extract		Acid-base extract		Compound **1**		Compound **2**		Compound **3**	
	A	% scavenging activity	A	% scavengingactivity	A	% scavengingactivity	A	% scavenging activity	A	% scavenging activity
100	0.33	70	0.43	60.9	0.31	71.6	0.80	27.2	0.36	67.2
50	0.42	61.7	0.50	54.5	0.38	65.4	0.92	16.3	0.40	63.6
25	0.53	51.8	0.70	36.4	0.49	55.4	0.99	13.6	0.51	53.2
12.5	0.69	37.1	0.83	24.5	0.62	43.6	0.97	11.8	0.59	46.3
IC_{50}	31.2		61.9		15.01		232.6		13.29	

Concentration, A- Absorbance

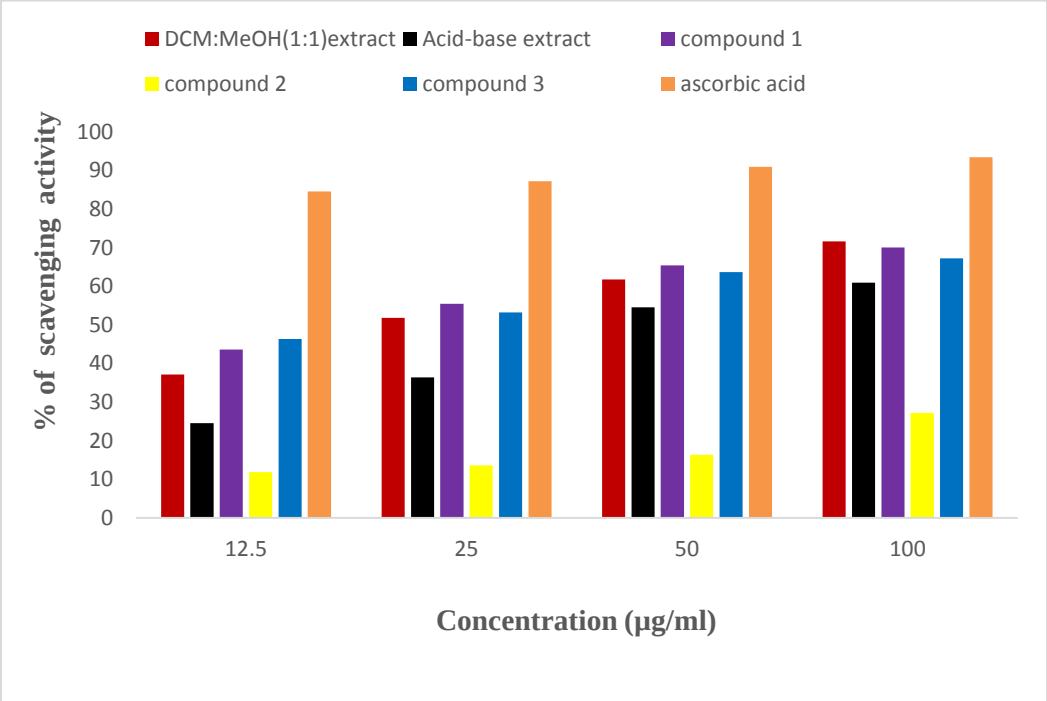
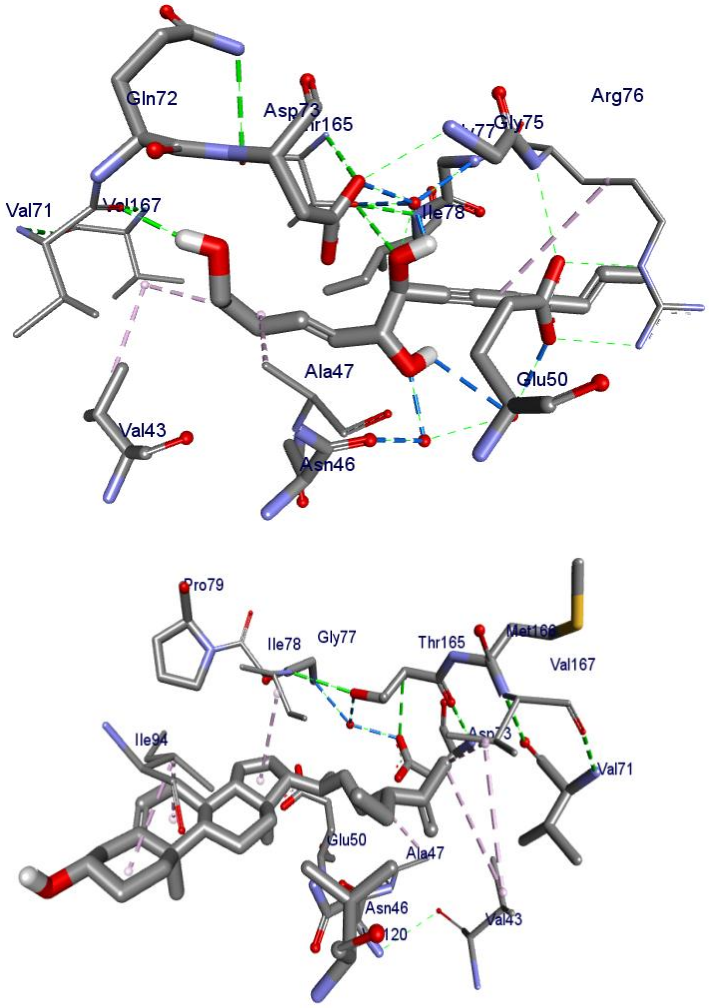
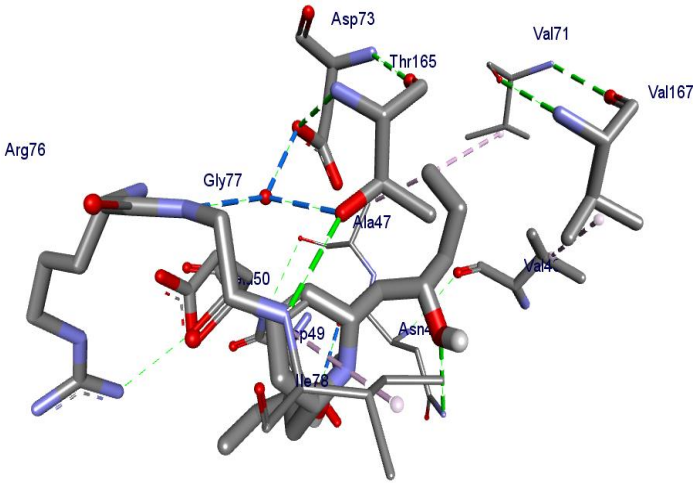


Figure 10. DPPH scavenging activity (%) of *L. rhynchopetalum* extracts, isolated compounds and positive reference (L-Ascorbic acid)

4.6. Docking study

Molecular docking is one of the most frequently used methods, because of its ability to predict, with a substantial degree of accuracy, the conformation of small-molecule ligands within the appropriate target binding site. The docking study of NMR identified ligand molecules Lobetyol (**1**), 24-ethyl-3-hydroxylcholesta-5,25-diene (**2**) and aspertin-A (**3**) with bacterial enzyme DNA gyrase is shown in Figure 10.

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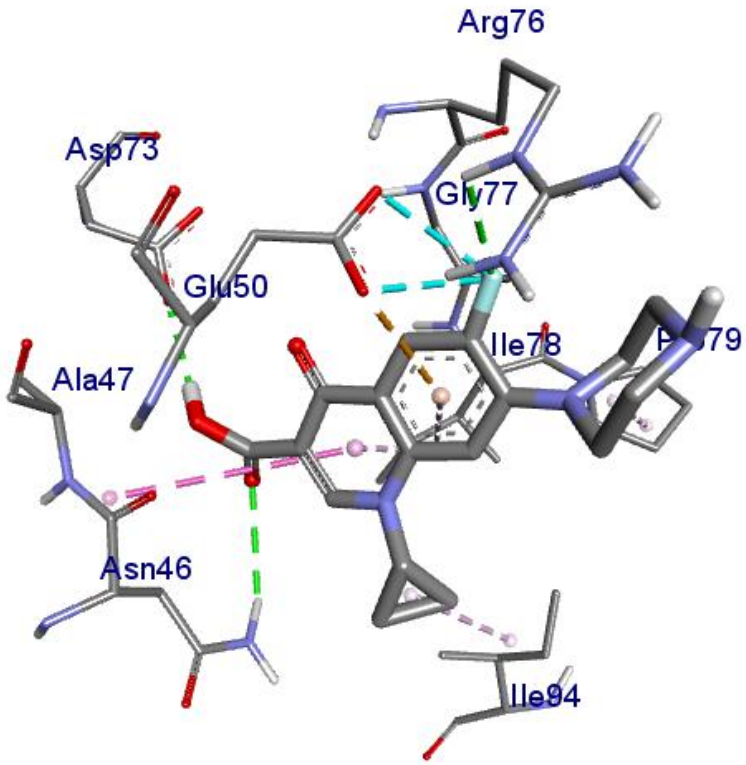
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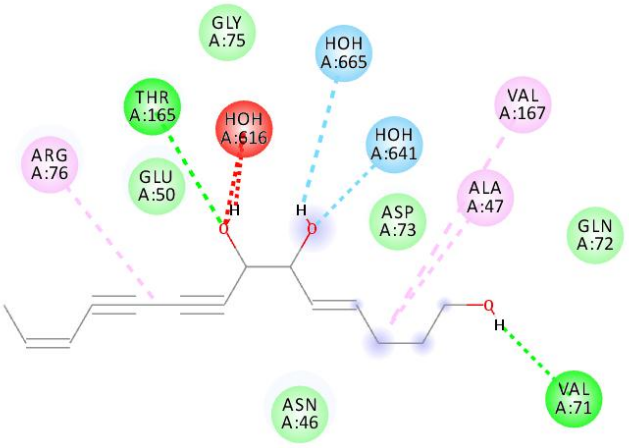
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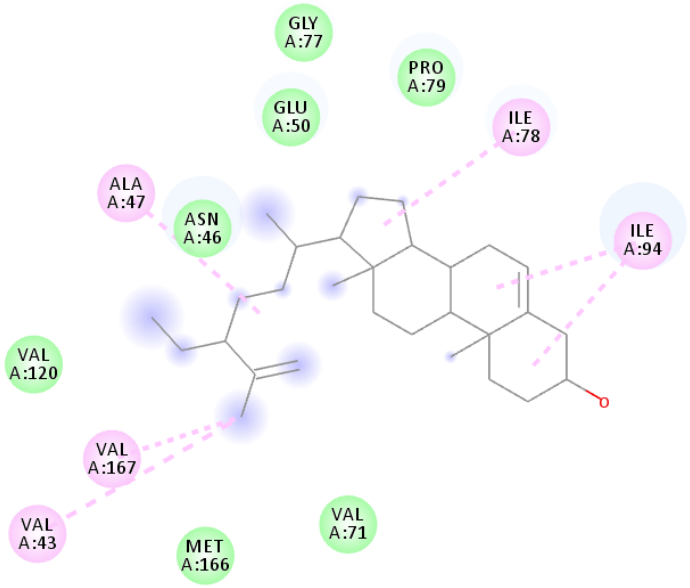
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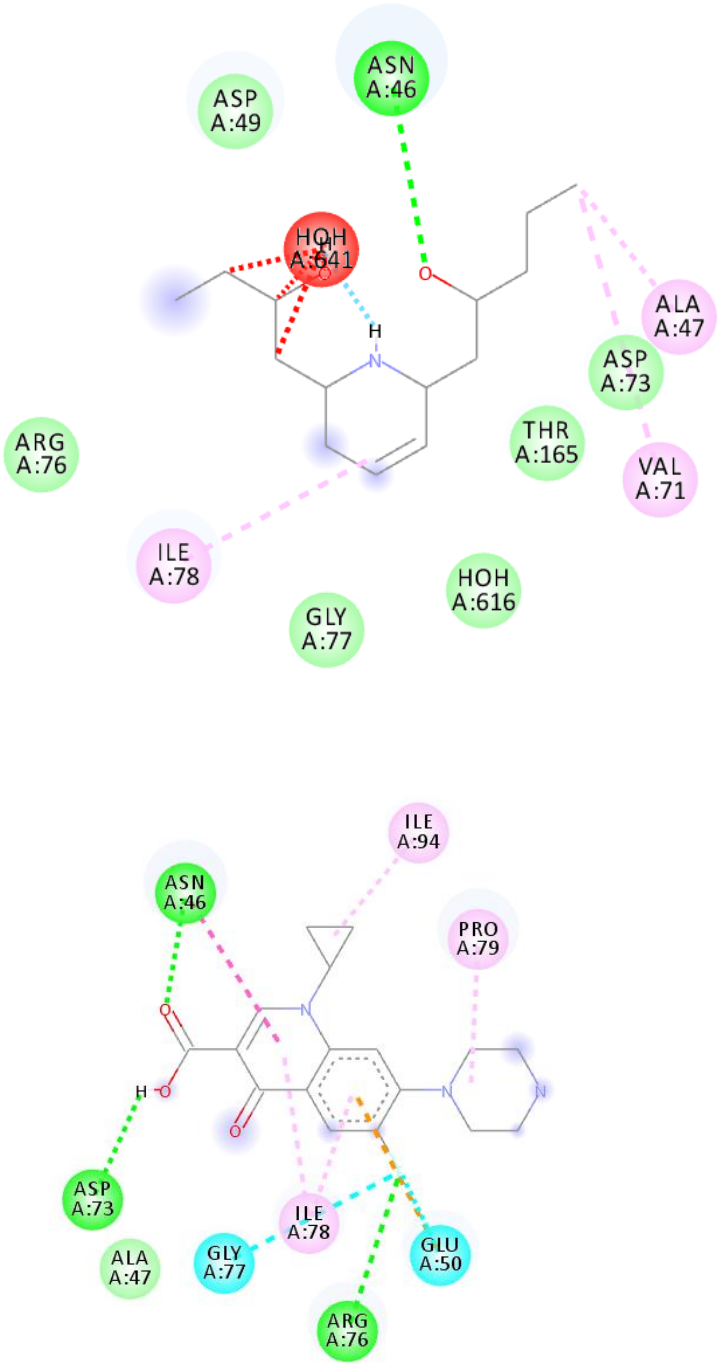

Figure 11. 2D and 3D protein ligand interaction of DNA gyrase with selected ligands

From the investigated ligands, compound **1** exhibited better docking efficiency with DNA gyrase. It formed twelve hydrophobic interactions with amino acids Ala-47, Val-43, Val-167, Ile-78, Ile-94, Asn-46, Glu-50, Gly-77, Pro-79, Val-71, Met-166, Val-120 in the active site of the target protein with the least binding affinity **-7.2** and hence was considered as the best dock conformation among the investigated ligands (Table 12). Compound **3** formed one hydrogen bonds with Asn-46 amino acids and hydrophobic interaction with Ala-47, Ile-78, Val-71, Asp-73, Asp-49, Thr-165, Gly-77 and Arg-76 with least binding affinity **-5.6**. While compound (**1**) formed two hydrogen bonds with Thr-165 and Val-71 amino acid and hydrophobic interaction with the amino acids of target protein with the least binding affinity **-7.2.**

However, all these docked molecules exhibited less hydrophobic interaction than the standard drug ciprofloxacin of binding affinity **-7.3,** except compound **1** witch have comparable hydrophobic interaction with standard drug ciprofloxacin having binding affinity of **-7.2.**

All ligands displayed pi to anion and pi to cation interactions with the protein. From this study, it can be concluded that compounds **1**-**3** may act as potential inhibitors of DNA gyrase enzyme.

Table 14. Molecular docking results of compounds isolated from the root of *L. rhynchopetalum* and ciprofloxacin against *E. coli* DNA gyrase B (PDB ID 6F86)

S. No.	**Ligands**	**Affinity (kcal/ mol)**	**H-bond**	**Residual Hydrophobic/Pi-Cation/Pi-Anion/ Pi-Alkyl interactions**
1	1	-7.2	Thr-165, Val-71	Ala-47, Val-43, Val-167, Ile-78, Ile-94, Asn-46, Glu-50, Gly-77, Pro-79, Val-71, Met-166, Val-120
2	2	– 5.9	--	Ala-47, Arg-76, Val-167, Glu-50, Gly-75, Asp-73, Asn-46, Gln-72
3	3	– 5.6	Asn-46	Ala-47, Ile-78, Val-71, Asp-73, Asp-49, Thr-165, Gly-77, Arg-76
4	**Ciprofloxacin**	– 7.3	Asp-73, Asn-46, Arg-76	Ala-47, Glu-50, Gly-77, Ile-78, Pro-79, Ile-94

CHAPTER FIVE

5. Conclusion and Recommendation

5.1 Conclusion

Herbal medicine has a long history in the treatment of several kinds of disease and continues to be used as an alternative approach on treatment for various diseases caused by protozoa, bacteria, fungi, viruses and helminthes. Their use for the treatment of disease has been practiced by man for many years and is still being widely practiced even today. Several effective medicines used in their natural state were selected as therapeutic agents based on empirical study of their application by traditional societies from different parts of the world. *L. rhynchopetalum* is one of these medicinal plants used traditionally to heal various infectious diseases. In this study, the phytochemical screening tests showed that crude extracts of root *L. rhynchopetalum* plants are rich in alkaloids, tannins, anthraquinone, terpenoids, anthraquinoneglycosides, cardiacglycosides and anthranolglycosides. Chromatographic separation of the crude extracts furnished six compounds, a polyacetylenic alcohol, lobetyol (**1**)**,** a triterpenoid, 24-ethyl-3-hydroxylcholesta-5,25-diene (**2**), a pyrolidine alkaloid, lobechine (**3**), a fatty acid lauric acid (**4**), anthraquinones, chrysophanol (**5**) and physcion (**6**). The extracts and isolated compounds were also evaluated *in vitro* for their antibacterial activities using disc diffusion method against *E. coli, S. aureus*, and *S. pyogenes*. Strong inhibition zone values (15±1, 14.33±0.58, 15.67±0.58 mm) for polyacetylene Lobetyol (**1**) were observed against *E. coli, S .aureus* and *S. pyogenes* respectively when compared to standard ciprofloxacin (19±1, 17.33±1.53 and 19.67±0.58 mm). The DPPH radical scavenging activities (%) of extracts and isolated compounds were found to be 71.6 (compound **1**), 27.2 (compound **2**), 70 (DCM: methanol (1:1) extract), 60.9 (Acid-base extract**)** and 67.2 (compound **3**) respectively at 100 µg/ml, Suggesting that compound **1** and **3** displayed slightly highest radical scavenging activity with IC_{50} value of 15.01 and 13.29 respectively. Molecular docking study was done using Autodock and it was found that among isolated compounds, compound **1** shows the highest and best scoring pose (lowest energy) which was -7.2 Kcal/mol in agreement with *in vitro* antibacterial result. Furthermore, this study provided the scientific basis for the use of the *Lobelia rhynchopetalum* in future research aimed at the discovery of the chemical compounds responsible for the therapeutic action, as well as the mechanisms involved.

5.2. Recommendation

In this study only 1D NMR spectrum and literature data was used for structure elucidation of isolated compounds, but the third (**3**) compounds are not reported before and their structure is also complex, so this paper recommends the 2D NMR and mass spectrophotometry for further confirmation of the structure of compound **3.** As the traditional healer uses the plant for wide array of disease including heart failure, rabies, back pain and etc. So, these observations promote further research work with biological activity (antifungal, anticancer and cytotoxicity) on crude extracts as well as isolated compounds.

6. REFFERENCE

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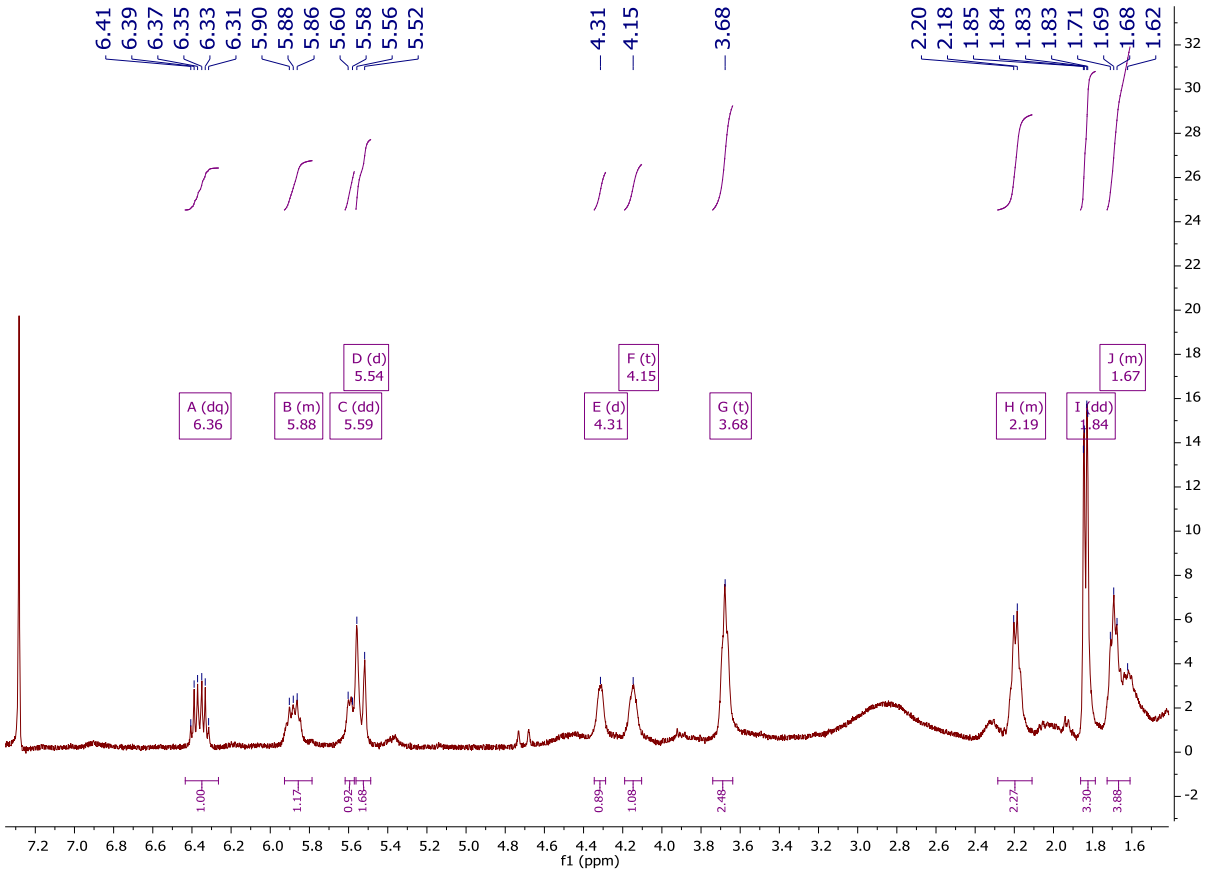
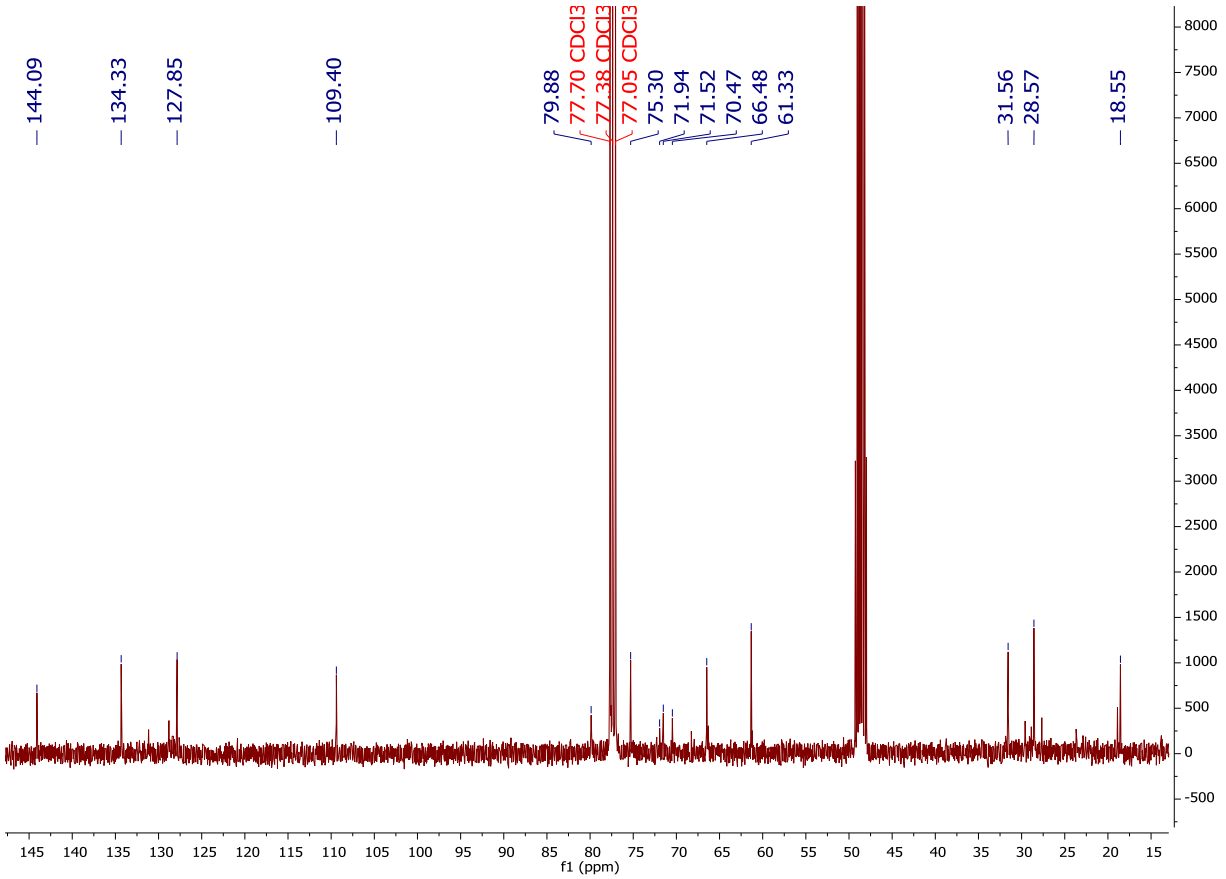
APPENDIXES

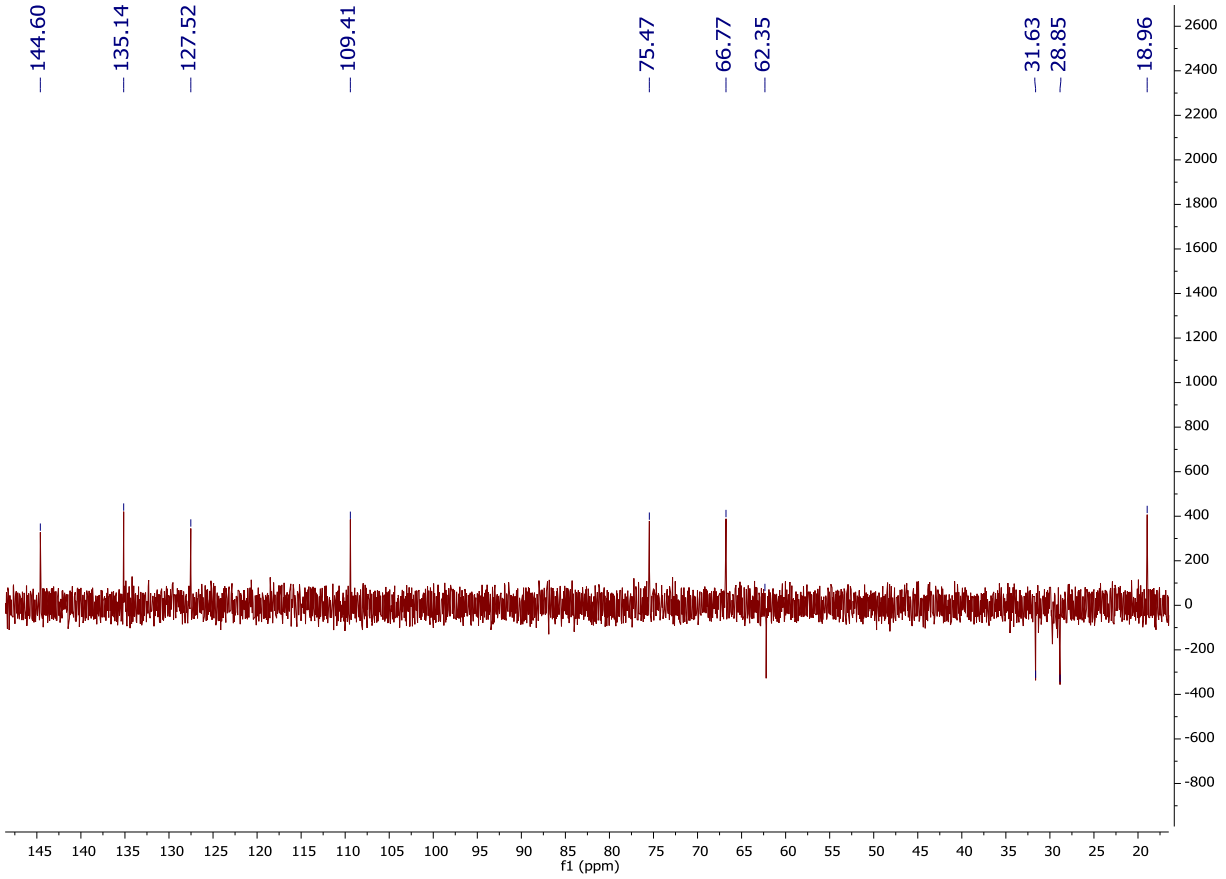
Appendix 1.1. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **1**



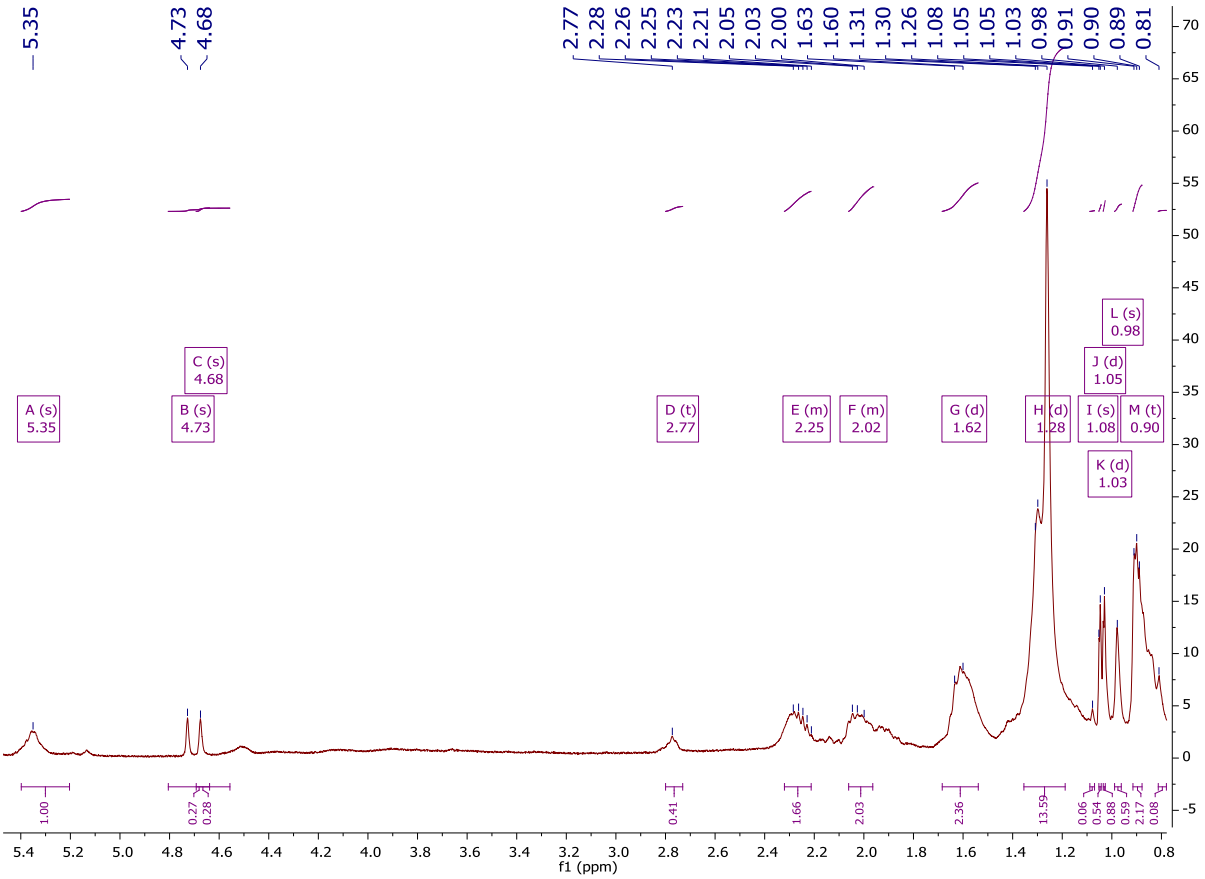
Appendix 1.2. The ^{13}C-NMR spectrum (101 MHz, $CDCl_3$) of compound **1**



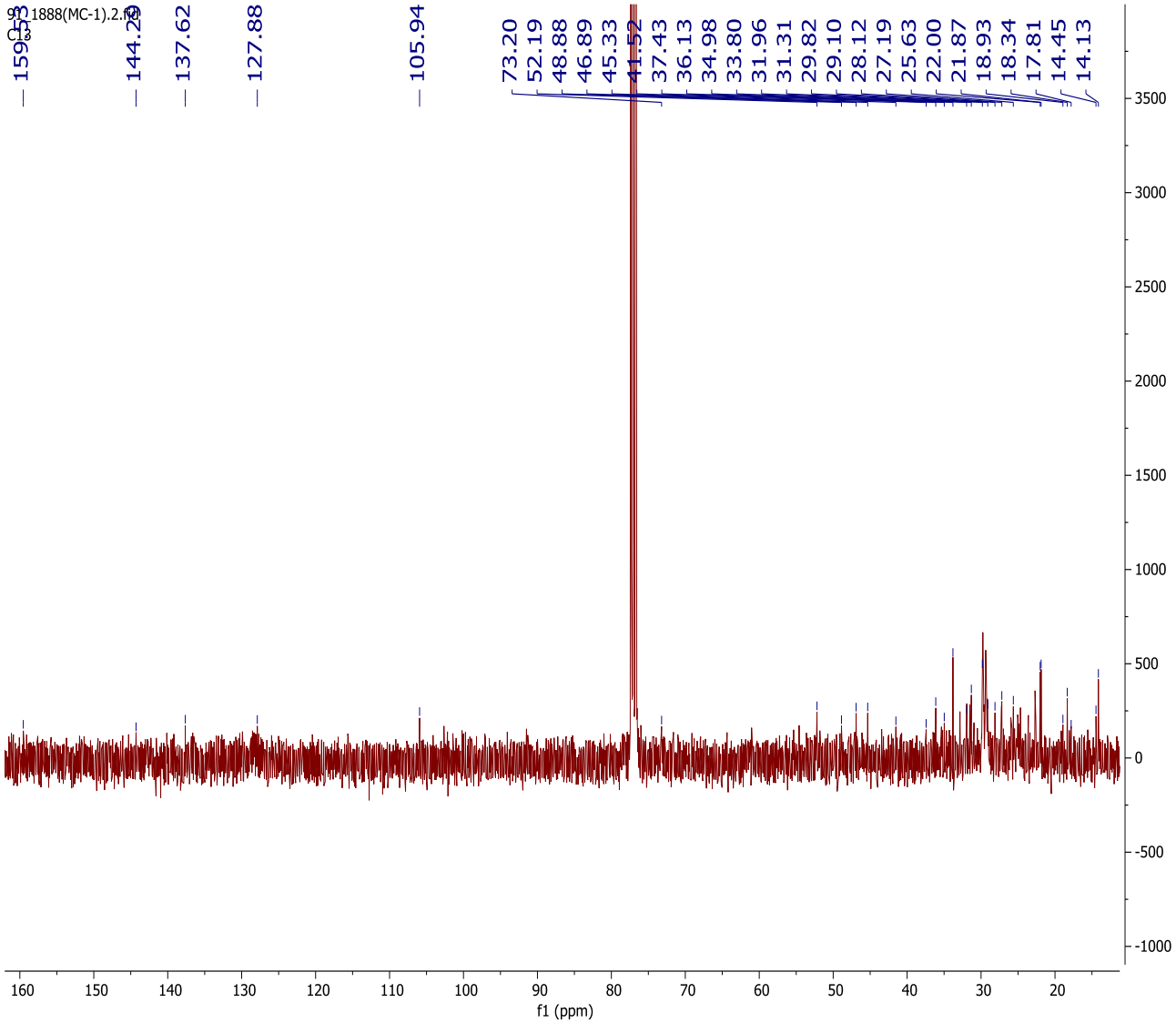
Appendix 1.3. The DEPT-135 spectrum (101 MHz, $CDCl_3$) of compound **1**



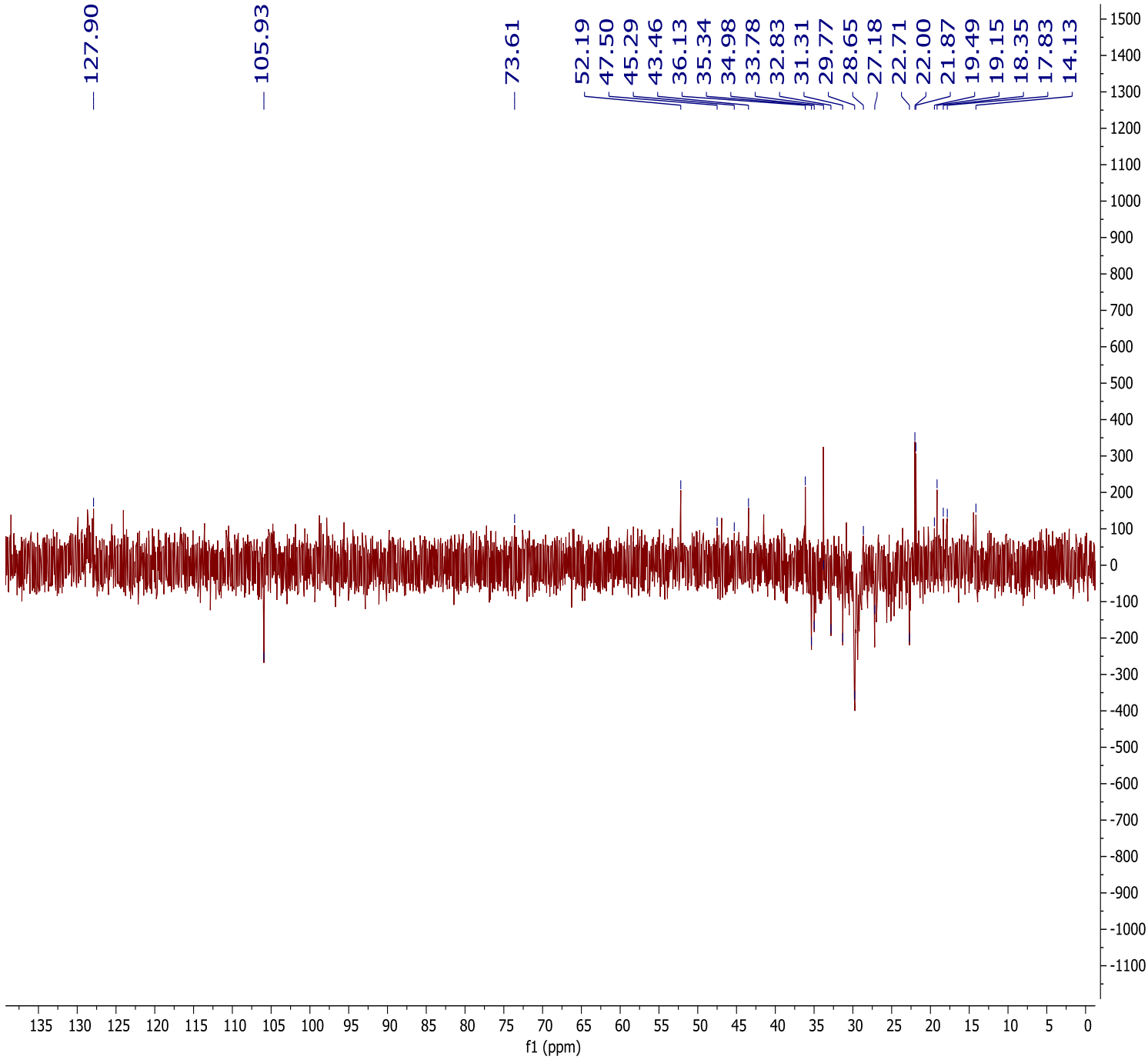
Appendix 2.1. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **2**



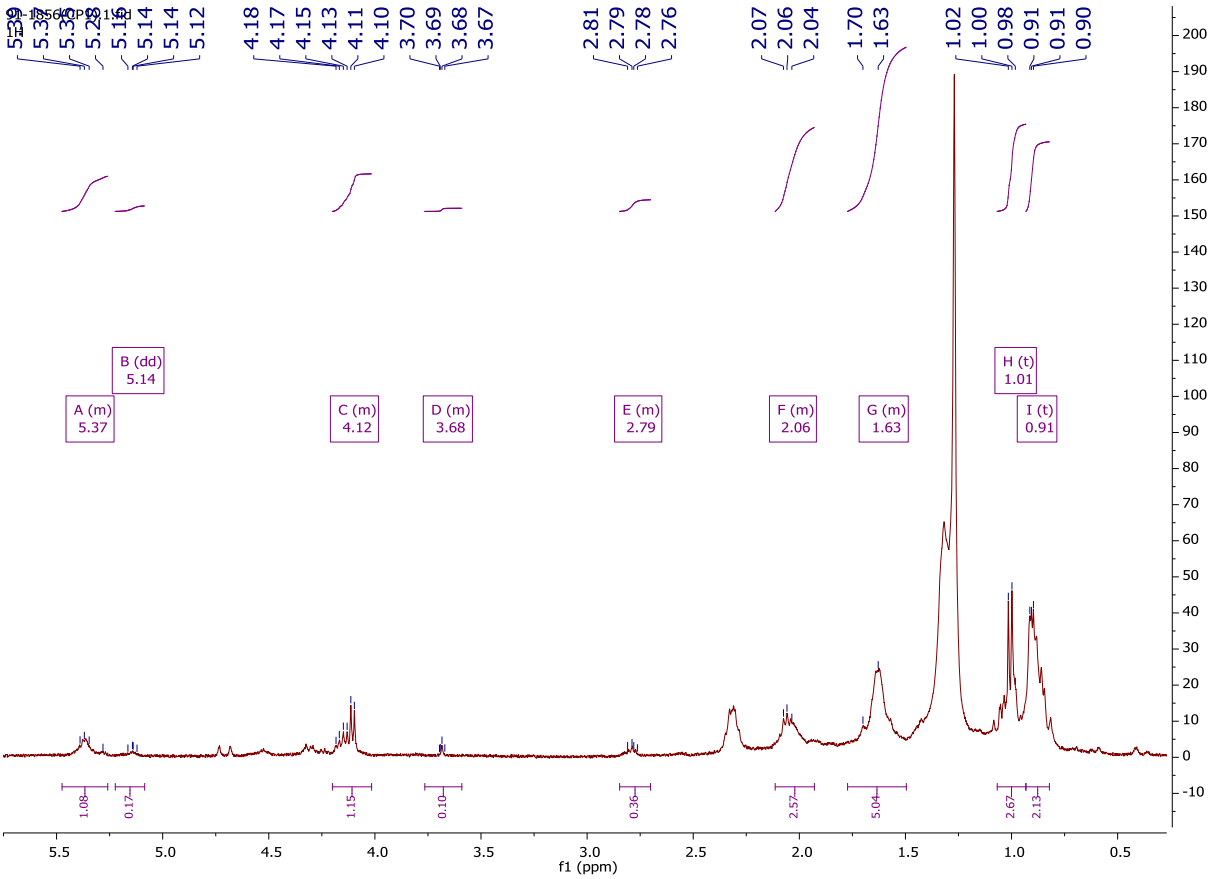
Appendix 2.2. The ^{13}C-NMR spectrum (101 MHz, $CDCl_3$) of compound **2**



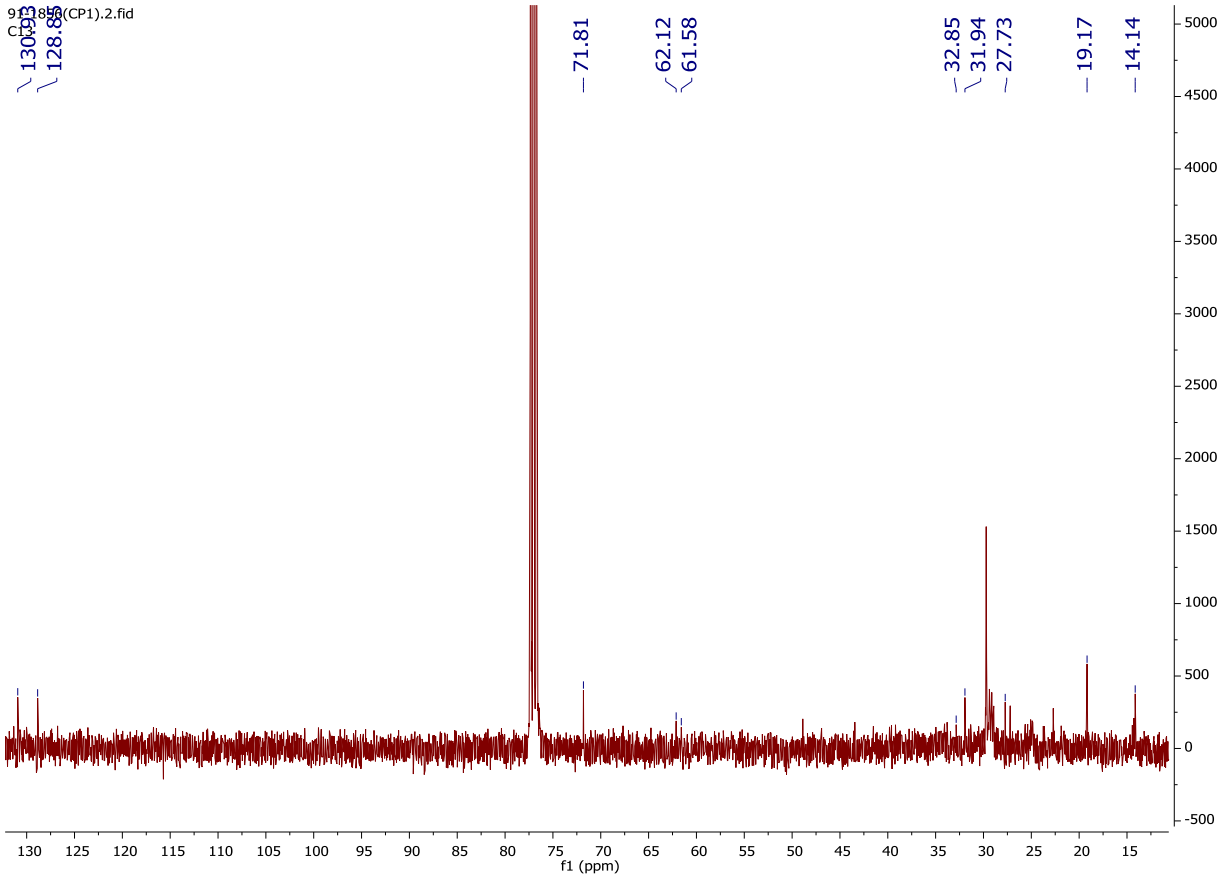
Appendix 2.3. The DEPT-135 spectrum (101 MHz, $CDCl_3$ and CD_3OD) of compound **2**



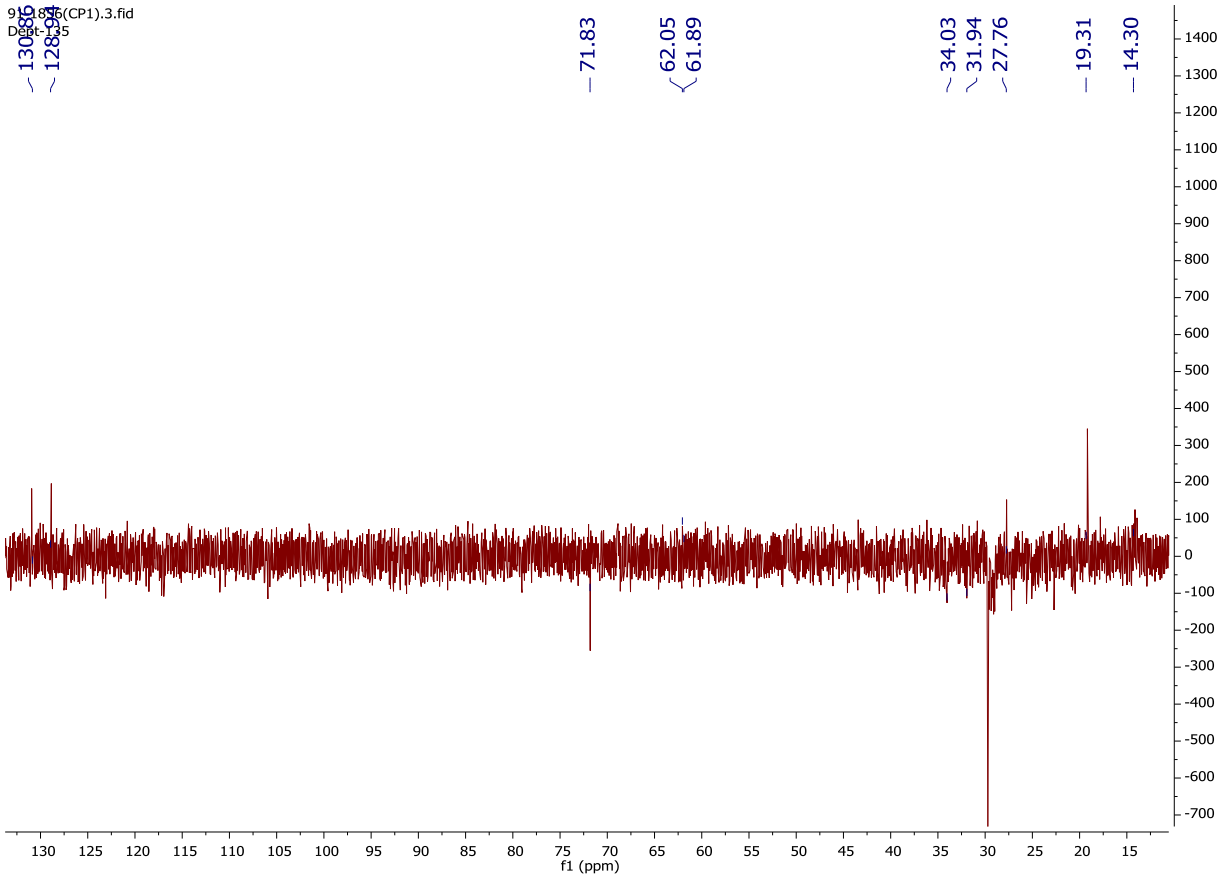
Appendix 3.1. The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **3**



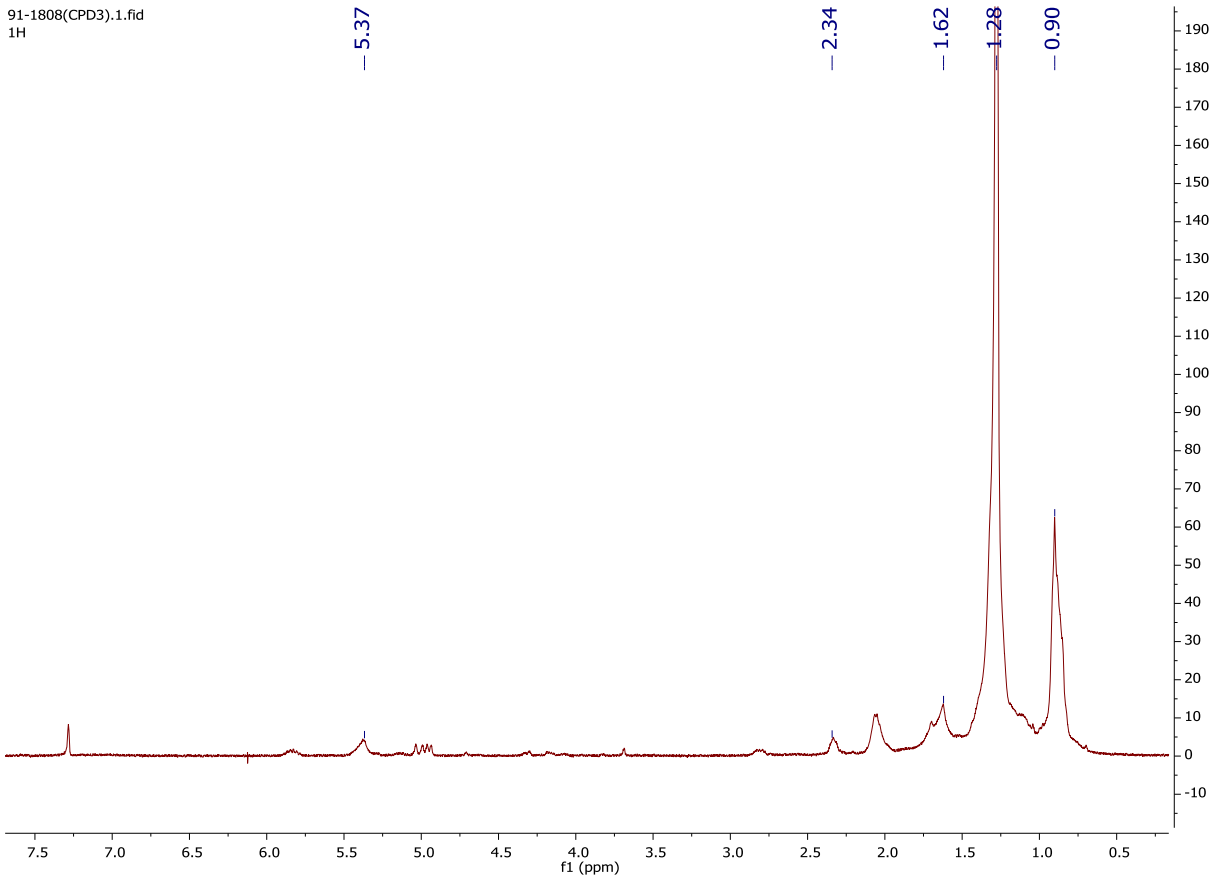
Appendix 3.2. The ^{13}C-NMR spectrum (101 MHz, $CDCl_3$ and CD_3OD) of compound **3**



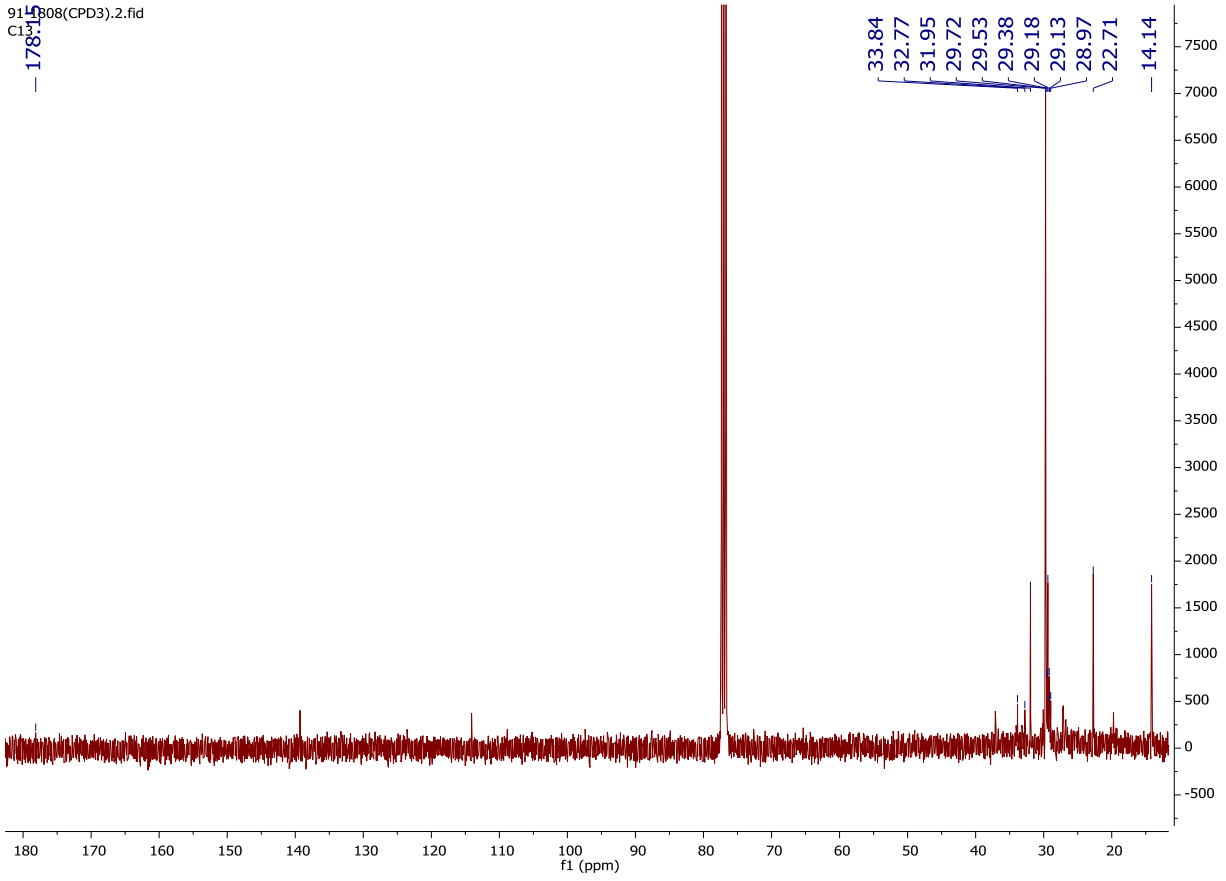
Appendix 3.3. The DEPT-135 spectrum (101 MHz, $CDCl_3$ and CD_3OD) of compound **3**



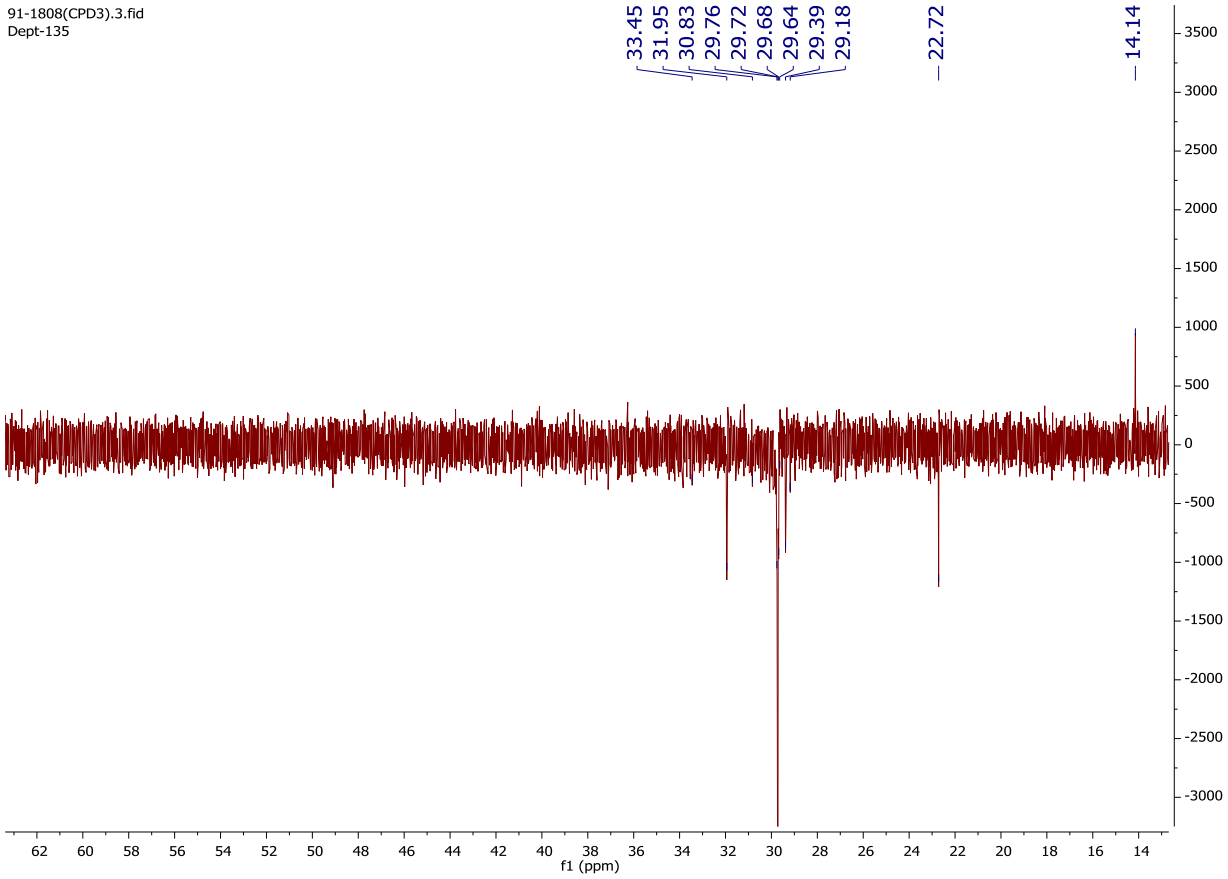
Appendix 4:1 The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) of compound **4**



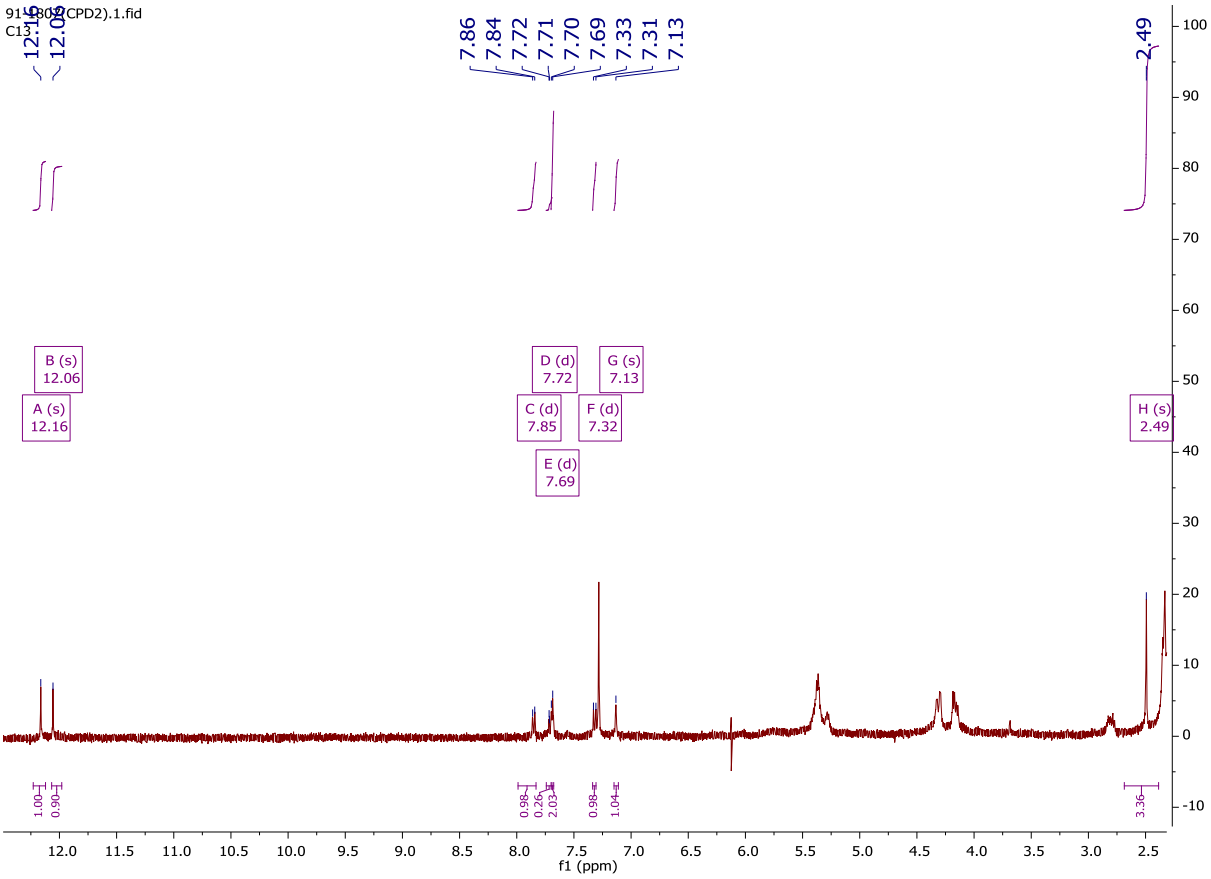
Appendix 4.2. The ^{13}C-NMR spectrum (101 MHz, $CDCl_3$) of compound **4**



Appendix 4.3. The DEPT-135 spectrum (101 MHz, $CDCl_3$) of compound **4**



Appendix 5.1. The ^{1}H-NMR spectrum (400 MHz, CDCl3) of compound **5**



Appendix 6.1. The ^{1}H-NMR spectrum (400 MHz, CDCl3) of compound **6**