PHYTOCHEMICAL INVESTIGATION, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF THE ROOTS EXTRACTS OF *LAGGERA TOMENTOSA*

BY

TOKUMA GETAHUN

A THESIS SUBMITTED TO
APPLIED CHEMISTRY PROGRAM
SCHOOL OF APPLIED NATURAL SCIENCE

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY

OFFICE OF GRADUATE STUDIES
ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

SEPTEMBER, 2017
ADAMA, ETHIOPIA

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APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by **Tokuma Getahun** have read and evaluated his thesis entitled "**PHYTOCHEMICAL INVESTIGATION, ANTIBACTERIAL AND ANTIOXIDANT STUDIES OF THE ROOTS EXTRACTS OF *LAGGERA TOMENTOSA***" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of Master of Science in Chemistry.

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DECLARATION

I hereby declare that this MSc Thesis is my original work and has not been presented for a degree in any other institution, and all sources of material used for this thesis have been duly acknowledged.

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DEDICATION

This work is dedicated to the **almighty God**, who provides without limits and to my wife **Meseret Benti** and child **Kolit Tokuma** for their help and support.

ACKNOWLEDGEMENTS

Before and above all, I would like to praise the **almighty God** for giving me life, grace, peace, joy, potency and the capability to complete my MSc program starting from the beginning to the end work of my thesis research. As a result of His help, support and clemency my study was finally accomplished successfully.

My deepest gratitude and thanks go to my advisors: **Dr. Yadessaa Melaku** and **Dr. Hailemichael Tesso** for their kind supervision, support, dedicated advice, closer and constant help, inspiring suggestions, involvement in the work, corrections, valuable and noble guidance, comments, great patience and encouragement while I was working on my thesis research.

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TABLE OF CONTENTS

Content **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF APPENDICES

Appendix **Page**

LIST OF ABBREVIATIONS

ASTU	Adama Science and Technology University
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
WHO	World Health Organization
HSDP	Health Sector Development Program
ATCC	American Test Culture Collection
LTE	*Laggera tomentosa* Ethyl acetate extract
GC-MS	Gas Chromatography-Mass Spectrometry
ROS	Reactive Oxygen Species
CC	Column Chromatography
TLC	Thin Layer Chromatography
UV-Vis	Ultra Violet-Visible
IR	Infra-Red
1D-NMR	One Dimensional-Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by Polarization Transfer
FT-IR	Fourier Transform-Infra-Red
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
^{13}C-NMR	Carbon13-Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
NOESY	Nuclear Overhauser Enhancement SpectroscopY
COSY	COrrelation SpectroscopY
TMS	Tetra Methyl Silane
DPPH	1,1-DiPhenyl-2-PicrylHydrazyl
NIST	National Institute of Standards and Technology
MHz	Mega Hertz
MHA	Mueller Hinton Agar
FDA	Food and Drug Administration
$R_{f.}$	Retention factor
s	singlet
br. *s*	broad singlet
d	doublet
t	triplet
dd	doublet of doublet
m	multiplet
m/z	mass to charge ratio
eV	electron Volt

ABSTRACT

Laggera tomentosa (Asteraceae/compositae) is an endemic Ethiopian medicinal plant traditionally used for the treatment of various ailments including toothache, stomachache, swelling, ringworm, skin infections, external parasites, common cold and headache. 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 studies of the solvent extracts of the roots of L. tomentosa. In this regard, the roots (300 g) were successively extracted with n-hexane, EtOAc and MeOH to afford 2.85 g (oil), 4.06 g and 5.94 g, respectively. The phytochemical screening results of EtOAc and MeOH extracts revealed the presence of alkaloids, anthraquinone, flavonoids, steroids, cardiac glycoside, terpenoids and phenols, whereas saponins and anthraquinone glycosides are absent. The n-hexane extract (oil) was analyzed for the first time by GC-MS which showed the presence of eleven components with 2,5-dimethoxy-p-cymene identified as the major constituent (59.39%). The EtOAc extract was subjected to silica gel column chromatography and gave four compounds identified as lauric acid, β–stigmasterol, chrysophanol and emodin. Structure elucidation of these compounds was done by employing various spectroscopic techniques including UV-Vis, IR and NMR and comparing with literature data. The radical scavenging activities of the n-hexane, EtOAc and MeOH extracts were assessed using DPPH and found to inhibit the radical by 55.8, 84.3 and 72.5%, respectively. The result was found close to ascorbic acid indicating strong ability of the extracts to act as radical scavenger. The extracts and isolated compounds were also evaluated for their antibacterial activities by disc diffusion method against four bacterial strains such as S. aureus, K. pneumonia, P. mirabilis and E. coli. The EtOAc extract (19 mm) and emodin (18 mm) exhibited promising antibacterial activities compared to the standard drug (21 mm) at 1 mg/mL concentration. Therefore, the results obtained support the traditional uses of this plant against bacteria.

***Key words**: L. tomentosa, GC-MS, DPPH assay, disk diffusion, β–stigmasterol, chrysophanol, emodin, anthraquinone, fatty acid.*

1. INTRODUCTION

1.1. Background of the Study

Nature has best owed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the World. There has been an increasing interest worldwide on therapeutic values of natural products (chemical compounds that are produced by living systems and include secondary metabolites and within the field of organic chemistry, the definition of natural products is usually restricted to mean purified organic compounds isolated from natural sources that are produced by the pathways of primary or secondary metabolism). It is generally considered that the cure to any debilitating human ailments and diseases may be found among the world's flora in nature's pharmacy. Throughout the ages, humans have relied on nature for their basic needs for the production of food, shelter, clothing, and means of transportation, fertilizers, flavours, fragrances and medicines [1-3].

The great ancient Chinese, Indian, and North African civilizations provided written evidence of man's utilization of plants for the treatment of a wide range of diseases from minor ailments to the more serious or acute diseases like cancer (breast cancer, blood cancer, bone cancer and skin cancer), malaria, mycobacterium tuberculosis such as pneumonia, lung tuberculosis and tuberculosis of meninges and even HIV/AIDS [4, 5]. The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body and are called phytochemicals ('phyto'-from Greek –phyto meaning 'plant') [6]. Phytochemicals are the chemicals extracted from plants and the term is often used to describe the large number of secondary metabolites found in plants. The use of most of the medicinal plants for treatment of all kind of diseases is due to their less harmful effects as compared to drugs synthesized in the laboratory [7, 8].

Today, the medicinal use of plants is still prominent in both developed and developing countries. The World Health Organization [9] estimates that 80% of the world population relies (solely or partially) on local medicinal plants for their primary healthcare needs because of its easy accessibility, affordability and cultural relevance [10], and about 85% of such medicines involve the use of plant extracts [5]. Besides that, there is a global consensus on the benefits of phytopharmacy and at present medicinal plants occupy a key position in plant research and

medicine. In many African countries, such as Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of the children with high fevers, is the use of herbal medicines at home [11].

The plant kingdom can indeed be regarded as perhaps the largest potential source for the development of new drugs because of the unmatched availability of chemical diversity of natural products, such as plants extract, either as pure compounds or as standardized extracts [12, 13]. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be active. These complementary components give the plant as a whole a safety and efficacy much superior to that of its isolated and pure active components [14]. Therefore, it is very important to investigate the pharmacological and phytochemical aspects of different preparations from plant sources [15].

Currently in modern medicine, approximately 50% of the drugs in chemical use are derived from natural products [16], and of these, half are plant based [17]. Consumers' preference is shifting from synthetic to natural products and this is dictating the pace of resurgence and expansion of use of medicinal plants in therapy in industrialized countries [18].

Medicinal plants play a central role not only as traditional medicines but also as commercial commodities, meeting the demand of distant markets. To cope with the growing market, there is need to expeditiously utilize and scientifically validate more medicinally useful plants [19].

The use of medicinal plants thus finds its natural expression and further development in primary healthcare where in many cases they bridge the gap between the availability of and the demand for essential drugs [18]. Some of the currently known herbal medicinal plants could substitute imported drugs, which currently require foreign exchange for their purchase.

In Ethiopia, it has been estimated that traditional remedies are the most important and sometimes the only source of therapeutics for nearly 80% of the population of which 95% of traditional medicinal preparations are of plant origin [20]. Much of the knowledge on traditional medicine is available in rural communities due to inaccessibility of modern healthcare services.

According to the report of Health Sector Development Program [21] of the Ethiopian Ministry of Health, one hospital serves for 100,000 people; one health centre for 25,000 people and one health post is for 5,000 people and the ratio of one doctor is for 10,000 people; one nurse is for 5,000 people, one health extension worker is for 2,500 people. Thus, traditional medicine is an important means of primary healthcare for achieving the goal, 'Health for all'. The various literature sources available also support this fact where more than 80% of human population in Ethiopia depends on traditional medicine [22-26]. This tells us that medicinal plants and knowledge of their use provide a vital contribution to human healthcare throughout Ethiopia. Among medicinal plant quite used in Ethiopia is *Laggera tomentosa* which belongs to the genus *Laggera* and family Asteraceae/Compositae. It is known locally as "Keskese" (Afan Oromo) and "Alashume" (Amharic). Traditionally, the leaves and aerial parts of this plant are used for the treatment of various ailments including toothache, stomachache, swelling, ringworm, skin infections, external parasites, common cold and headache. 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, antibacterial and antioxidant studies of the roots extracts of *L. tomentosa*.

1.2. Statement of the Problem

Despite the tremendous progress in medicine, infectious diseases caused by bacteria, fungi, viruses and parasites continue to pose a threatening challenge to public health [13]. These diseases account for approximately one-half of all deaths in developing countries due to poverty, unavailability of medicines and the emergence of widespread resistance of pathogens to the available drugs. The high cost of synthetic drugs also makes them unaffordable to many people in these countries who live on less than one dollar a day. This coupled with insufficient number of health facilities, the increasing rate of resistance of disease causing microorganisms to conventional antibiotics, calls for continuous search for affordable, safe and effective herbal medicine [27].

In this regard, *L. tomentosa* is an endemic Ethiopian medicinal plant traditionally used against bacteria. Hence this research attempts to find the active compounds responsible for the antibacterial activity of this plant. Furthermore, there is an urgent need of compounds that can be

used as food additives since synthetic compounds currently used were suspected to cause cancer. Hence an attempt was also made to check the radical scavenging activities of the roots extracts of *L. tomentosa*.

1.3. Significance of the Study

Human beings gathered information by trial and error over centuries. In ancient times human beings uses various plants and herbs that grow in their environment to treat various illness. Medicinal plants have emerged as one of the most widely studies. Interests on medicinal plants have been shown in their chemistry because of their potential applications in medicine.

This study focuses on the phytochemical investigation, antibacterial and antioxidant studies on the roots of *L. tomentosa*. Understanding the constituent of the natural products from *L. tomentosa*, have many significances for industrial and pharmaceutical applications. The study on the extracts of the roots of *L. tomentosa* may help to arrive at new natural products. This study may also provide important information about the structures of the natural products (bioactive compounds) from the roots of *L. tomentosa*. Furthermore, it can provide initial information for the researchers who are interested to conduct further studies on this area. Moreover, extracts and compounds to be obtained from the plant may exhibit pronounceable antibacterial and antioxidant activities.

1.4. Objectives of the Study

1.4.1. General objective

The main objective of this research is to conduct phytochemical, antibacterial and antioxidant studies of the roots extracts of *L. tomentosa*.

1.4.2. Specific objectives

- To extract the dried and powdered roots of *L. tomentosa* successively using n-hexane, ethyl acetate and methanol.
- To conduct phytochemical screening on the n-hexane, ethyl acetate and methanol crude extracts of the roots of *L. tomentosa.*
- To analyze the n-hexane extract of the roots of *L. tomentosa* using GC-MS.
- To isolate compounds from ethyl acetate extract of the roots of *L. tomentosa* using chromatographic techniques.
- To characterize the isolated compounds employing some physical properties and spectroscopic methods such as UV, IR and NMR.
- To study the antioxidant activities of the roots extracts of *L. tomentosa* using DPPH.
- To evaluate the antibacterial activities of the extracts and isolated pure compounds of the roots of *L. tomentosa* on selected strains (*Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*) by disc diffusion method.

2. LITERATURE REVIEW

2.1. The Family Asteraceae (Compositae)

The name "Asteraceae" comes from the type genus Aster, from the Greek ἀστήρ, meaning star, and refers to the star-like form of the inflorescence. "Compositae" is an older but still valid name which refers to the fact that the family is one of the few angiosperm ones to have composite flowers. Most members of Asteraceae are herbaceous, but a significant number are also shrubs, vines, or trees. The family has more than 23,600 currently accepted species, spread across 1,620 genera and 13 subfamilies. Species of Asteraceae are of wide economic importance as vegetables (lettuce, artichokes, and endive), sources of oil (sunflower, saf-flower), insecticides (pyrethrum), and garden ornamentals (chrysanthemum, dahlia, marigold, and many others). The family has a worldwide distribution, from the Polar Regions to the tropics, colonizing a wide variety of habitats. It is most common in the arid and semiarid regions of subtropical and lowers temperate latitudes [28].

The Asteraceae are among the chemically most diverse groups of flowering plants. Triterpene monols and diols, acetylenic compounds, methylated flavonols and flavones, inulin-type fructans, the cyclitols L-inositol and scyllitol and fatty oils in the seeds are common constituents of many species; they probably occur in all tribes and form the chemical make-up of the family. Essential oils and diterpenoids are also widely distributed. Alkaloids, cyanogenic glycosides, amides, coumarins and several types of phenolic constituents exhibit much more limited distribution [29]. This extraordinary diversity is accompanied by extensive bioactivity. The plants and their secondary metabolites have been demonstrated to possess multiple pharmacological activities, such as antioxidant, antiprotozoal, antimicrobial, cytotoxic, anti-inflammatory, anti-diabetic, anticancer, hepatoprotective and antispasmodic effects, activities on the central nervous and cardiovascular systems [30].

2.2. The Genus *Laggera*

Laggera is small genus of African tropical and sub-tropical trees and shrubs belonging to the family of Asteraceae. It has about 20 species distributed in tropical Africa and South East Asia [31]. In India, it is represented by 3 species commonly found in tropical regions in both plains and altitudes up to 1500 m. These are *L. alata*, *L. aurita* and *L. crispata* [32]. *L. alata* and *L.*

pterodonta are the only species in China. In Ethiopia there are six *Laggera* species namely, *L. crispata*, *L. braunii*, *L. elatior*, *L. crassifollia*, *L. alata* and *L. tomentosa*, of which *L. tomentosa* is an endemic Ethiopian plant. Other species belonging to this genus are *L. decurrens*, *L. gracilis*, *L. mollis* and *L. oloptera*. *Laggera's* are characterized with broad, entire, continuous wings, white flowers and basal leaves. They are annual shrubs, erect, highly branched, robust and aromatic plants in nature that grow up to 1.70 m in height [31-33].

2.2.1. Ethnopharmacological information

A number of *Laggera* species have been widely used in traditional medicine in south East Asia and Africa. For example, *L. alata* has some traditional medicinal values including the use of its volatile components as an antiseptic. It is also used as remedy for fever and pneumonia [34]. In Lake Province of Tangayika, *L. pterodonta* is reported to kill tobacco plants if growing nearby. *L. pterodonta* is also used as aromatic substances in cosmetics, scents and incense [35]. Chinese *L. pterodonta* herb is commonly cultivated domestically and has been traditionally employed as a medicine because it has been used as a cure for angina, arthritis, hepatitis, bronchitis, nephritis, influenza, and malaria. It has been reported for use in Cameroon for the preservation of seeds against insects' attack [36], and in Nigeria for athlete foot, skin infections, pediatric malaria, pneumonia, cough and wounds. Externally applied, this plant has been of use in treating scabies, burns, and bites by poisonous snakes, injuries from falls, fractures, and contusions and strains [37]. The leaves paste of *L. crispata* is used in the treatment of inflammation and swelling in north east India [38]. *L. decurrens* is well known for its use in traditional medicine. In Namibia, an extract of the leaves or the roots of this plant is drunk to relive stomach pains. The plant is also employed as traditional herbal medicine in the treatment of rheumatism and wounds [39]. *L. mollis* has applications in the traditional medicine systems. Decoction from the leaves of the plant is used in the treatment of acute fever in children, malaria, rheumatism, and HIV/AIDS in the northern part Nigeria. In India, the decoction from the leaves is also used in the treatment of diarrhea [40].

2.2.2. Biological activities

The leaves and aerial parts of *Laggera* species have been reported to have anti-bacterial, anti-inflammatory, insecticidal, sedative, anti-diarrheal, antiviral activity against Herpes simplex type I and II, antifungal, anti-tuberculosis properties in Asia and Africa [39,41].

Pharmacological activities of the crude extracts of *L. pterodonta* have been reported to have hepatoprotective, antiviral, anti-tumor, anti-leukemia, anti-inflammatory, anti-phlegm, anti-bronchitis, anti-bacterial, anti-nociceptive, acute toxicity and insecticidal activities [31,37,42-45]. The antimicrobial activities of hexane and ethyl acetate extracts of the aerial part (stem and leaf) of the same plant were also reported to be active against microorganisms, which include *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumonia* and clinical isolates of *Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Klebsiella ozaenae* and *Shigella dysenteriae* [41].

L. alata and the aerial parts of *L. crispata* have also been found to possess anti-leukemia, anti-inflammatory, and anti-bacterial activities. *L. crispata* is also said to possess anti-helminthic properties. The antibacterial activity of the essential oil of *L. aurita* was reported to be more active against *Proteus mirabilis* and *Bacillus subtilis*. It was also reported to exhibit antioxidant activity [31,35,38,43,44]. However, to the best of our knowledge, there is no previous biological activity report on the roots of the *Laggera* species.

2.2.3. Phytochemistry of the genus *Laggera*

Recently, much attention has been paid to *Laggera* species and their chemical contents because of their multifaceted activities. Many phytochemical investigations have been performed on the non-volatile and volatile constituents of different species of *Laggera* from various countries. Since 1960s, more than 50 chemical constituents have been isolated from the leaves and aerial parts of the genus *Laggera*, including monoterpenes, sesquiterpenes (eudesmanes and eremophilanes), flavonoids, and cyclitols [31,35-49]. However, to the best of our knowledge, there is no previous preliminary phytochemical and chemical constituent report on the roots of the *Laggera* species.

Phytochemical investigation of *L. alata* led to the isolation of sesquiterpenes of the eudesmane type such as: isointermedeol (**1**), eudesm-4(15)-ene-9*α*,11-diol **(2**), 5*β*-hydroxyilicic acid (**3**), 5*α*-hydroxy-4-epiilicic acid methyl ester (**4**), 7-epi-*α*-eudesmol (**5**), 7-epi-*β*-eudesmol (**6**), 7-epi-*γ*-eudesmol (**7**), alatoside B (**8**), 3*α*-hydroxyilicic acid (**9**), 1*β*-hydroxylilicic acid (**10**), costic acid (**11**), 3-oxocostic acid (**12**), isocostic acid (**13**), alatoside C (**14**), alatoside D (**15**), eudesma-3,5,11(13)-trien-12-oic acid (**16**), 5*α*-O-(*β*-D-glucopyranosyloxyl)-4-epiilicic acid methyl ester (**17**), alatoside A (**18**) and *β*-dihydroagarofuran (**19**) [34]. Eremophilane types of sesquiterpenes like: tessaric acid (**20**) and 3-oxoeudesma-1,11(13)-dien-12-oic acid (**21**) and dicaffeoylquinic acids (cyclitols) such as: 4,5-O-dicaffeoylquinic acid (**22**), 3,5-O-dicaffeoylquinic acid (**23**) and 3,4-O-dicaffeoylquinic acid (**24**) were also isolated from *L. alata* [42].

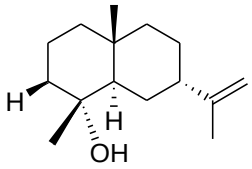
Previous investigation of *L. pterodonta* led to the isolation of eudesmane sesquiterpenes like: ilicic acid (**25**), 2*β*-hydroxyilicic acid (**26**), pterodondiol (**27**), pterodontriol A (**28**), pterodontriol B (**29**), pterodontetraol (**30**), ent-4*β*,11-dihydroxyeudesman-1-one (**31**), pterodontoside F (**32**), pterodontic acid (**33**), 1*β*-hydroxypterondontic acid (**34**), 3*β*-hydroxypterondontic acid (**35**), 2*α*, 3*β*-dihydroxypterondontic acid (**36**), 1*β*,3*α*,9*β*-trihydroxyeudesma-5,11(13)-dien-12-oic acid (**37**), 1*β*,3*α*-dihydroxyeudesma-5,11(13)-dien-12-oic acid (**38**), pterodontoside A (1*β*-hydroxypterodontic acid1-O-*β*-D-glucopyranoside) (**39**), pterodontoside B (9*β*-hydroxypterodontic acid 9-O-*β*-D-glucopyranoside) (**40**), 2*β*-acetoxypterodontic acid (**41**), 4*α*,5*α*-dihydroxyeudesm-11(13)-en-12-oic acid (**42**), 2*α*-acetoxycostic acid (**43**), pterodonoic acid (**44**), pterodontriol D (**45**), 5*α*,11-dihydroxyeudesm-3-en-2-one (**46**), pterodondiol C (**47**), ent-eudesmane-4*β*,9*α*,11-triol (**48**), ent-eudesmane-2*α*,4*β*,11-triol-11-O-*β*-D-glucopyranoside (**49**), pterodontoside D (**50**), pterodontoside E (**51**), pterodontoside C (**52**), ent-7(11)-selinen-4-ol (**53**), pterodontoside G (**54**), pterodontoside H (**55**), pterodondiol (**56**), pterodontriol A (**57**), pterodontriol B (**58**), pterodontetraol (**59**), ent-4*β*,11-dihydroxyeudesman-1-one (**60**) and pterodontoside F (**61**) and flavonoids such as: artemitin (**62**), chrysosplenetin B (**63**), uendultin (**64**), 5-hydroxy-3,4',6,7-tetramethoxyflavone (**65**) and 3',4',5-trihydroxy-3,6,7-trimethoxyflavone (**66**) [37,41,47].

Phytochemical investigation of *L. crispata* led to the isolation of eudesmane sesquiterpenes such as: 7-epieudesm-11(13)-ene-3*β*,4*α*-diol (**67**) and 3*α*-(angeloyloxy)-4*α*,11-dihydroxyeudesm-6-

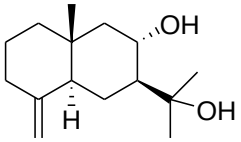
en-8-one (**68**), essential oils like: 2,5-dimethoxy-*p*-cymene (**69**), 10-epi-γ-eudesmol (**70**), β-caryophyllene (**71**), caryophyllene oxide (**72**), juniper camphor (**73**) [48].

Isolation of two thymol derivatives, 5-acetoxy-2-hydroxythymol (**74)** and 3-hydroxythymoquinone (**75)**, were also reported from *L. decurrens* [39].

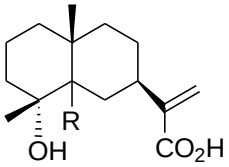
These chemical structures of compounds reported from the genus are depicted in **Figure 1**.



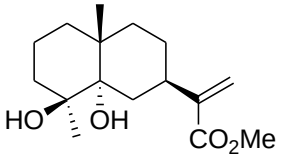
1



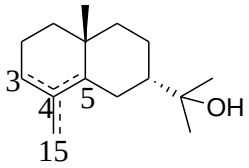
2



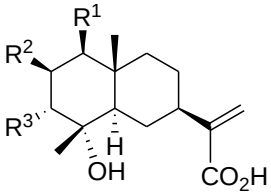
3 R = β -OH



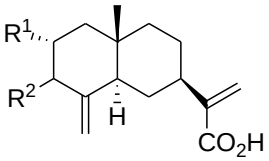
4



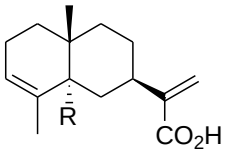
5 [3,4]
6 [4,15]
7 [4,5]



	R^1	R^2	R^3
8	H	OGlc	H
9	H	H	OH
10	OH	H	H



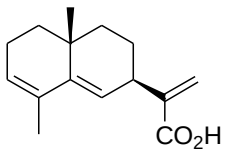
	R^1	R^2
11	OAc	H
12	H	=O



13, R = α - H

14, R = β - OH

15, R = α - OGlc



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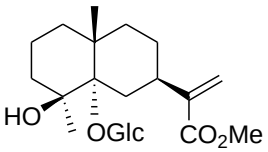

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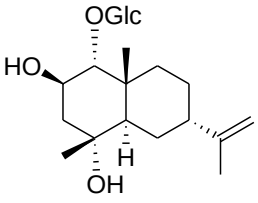

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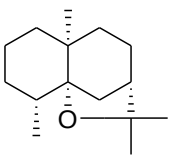

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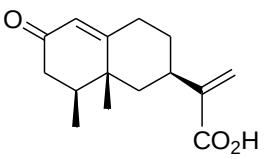

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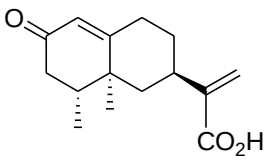

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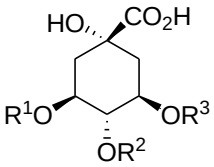
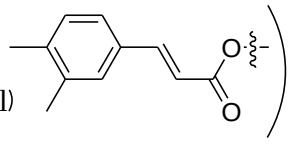
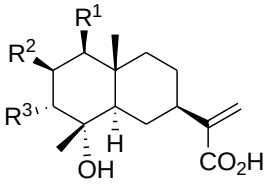
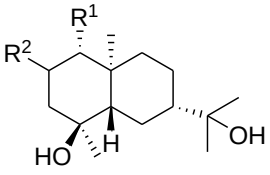


	R^1	R^2	R^3
22	H	caf	caf
23	caf	H	caf
24	caf	caf	H

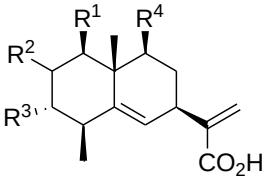
(caf =  (caffeoyl))



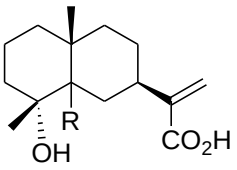
	R^1	R^2	R^3
25	H	H	H
26	H	OH	H



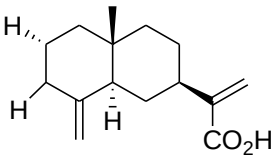
	R^1	R^2
27	H	H
28	H	α-OH
29	OH	H
30	OH	β-OH
31	=O	H
32	OGlc	H



	R^1	R^2	R^3	R^4
33	H	H	H	H
34	OH	H	H	H
35	H	H	β-OH	H
36	H	α-OH	β-OH	H
37	OH	H	α-OH	OH
38	OH	H	α-OH	H
39	OGlc	H	H	H
40	H	H	H	OGlc
41	H	β-OAc	H	H



42, R = α - OH



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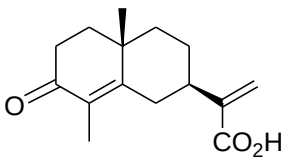

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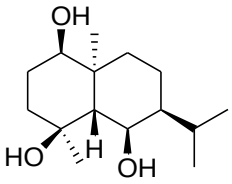

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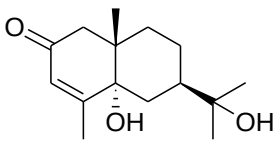

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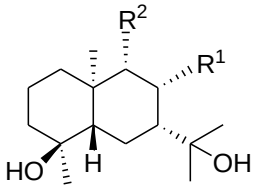
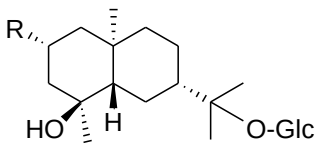
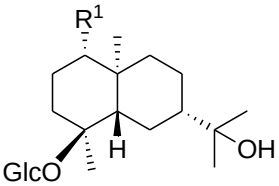


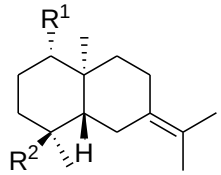
	R^1	R^2
47	OH	H
48	H	OH



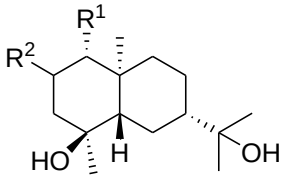
	R
49	OH
50	H



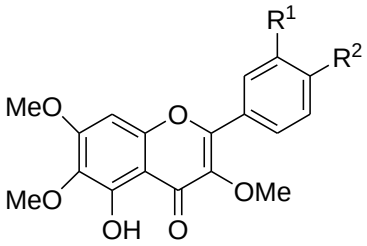
	R^1
51	OH
52	H



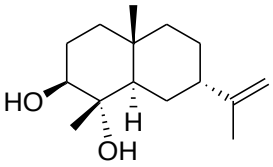
	R^1	R^2
53	H	OH
54	OH	OGlc
55	OGlc	OH



	R^1	R^2
56	H	H
57	H	α - OH
58	OH	H
59	OH	β - OH
60	= O	H
61	OGlc	H



	R^1	R^2
62	MeO	MeO
63	MeO	OH
64	H	MeO
65	H	OH
66	OH	OH



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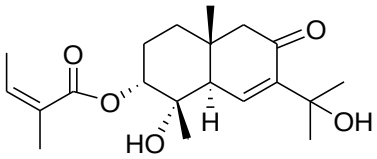

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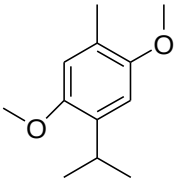

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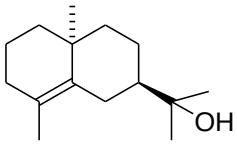

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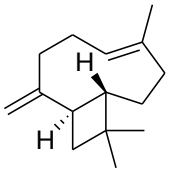

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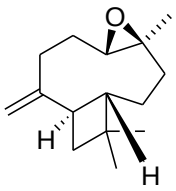

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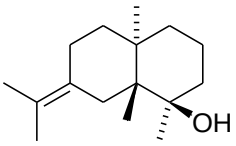

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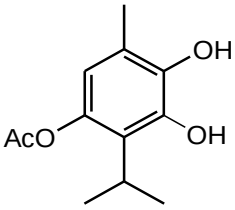

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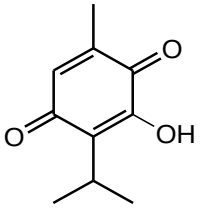

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Figure 1: Structures of compounds reported from the genus *Laggera*.

2.3. *Laggera tomentosa*

2.3.1. Ethnobotanical information

L. tomentosa is a bushy perennial herb or subshrub (0.5-1.2 m high) aromatic, narrowly winged, wings continuous, 1-1.5 mm wide, densely tomentose, ashy green or grey leaves (**Figure 2**) [33]. It is locally called "Keskese" (Afan Oromo) and "Alashume" (Amharic) and endemic plant to Ethiopia [46]. It is found in Shewa, Arsi, Wollo, Tigray, Gonder and Gojjam on dry hill and mountain slopes at an altitude of 2345-2950 m high [33].

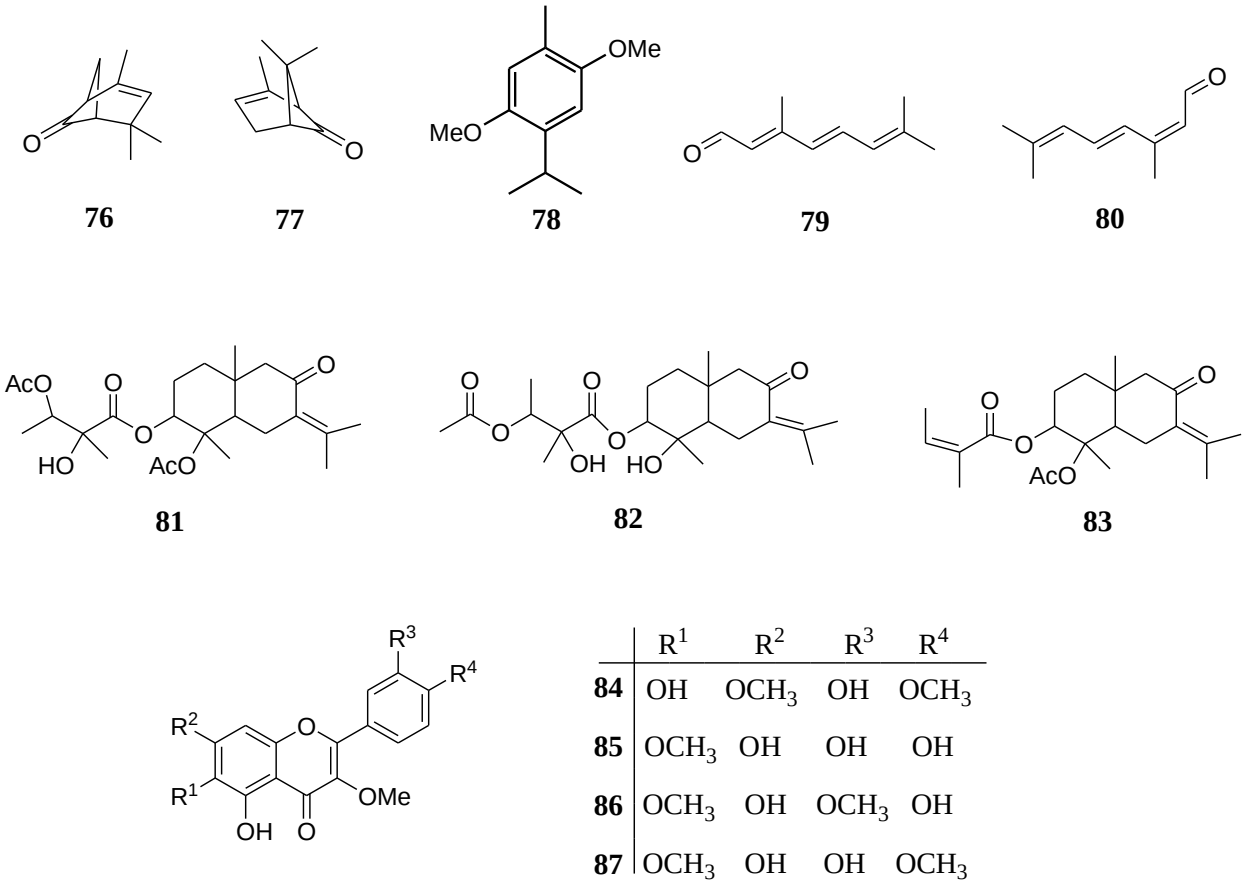
Figure 2: Aerial parts of *L. tomentosa* (From Daletti picture taken `by Tokuma Getahun on November 12, 2017).

2.3.2. Medicinal uses

L. tomentosa is a well-known and frequently cultivated medicinal plant. The juice of the crushed plant is ingested as a treatment for stomach-ache and is used against migraine [33]. Fresh leaf boiled vapour is inhaled nasally or orally to treat common cold. Fresh leaf paste is applied topically to treat head-ache. Its aerial parts are used as a treatment of tooth-ache, swelling and ringworm [49]. It can also be used as a fumigant and for cleansing milk containers [33]. However, to the best of our knowledge there is no any report about the traditional medicinal use of the roots of *L. tomentosa*.

2.3.3. Reported chemical constituents

Five monoterpene constituents, (1S, 5R)-(-)-2,4,4-trimethylbicyclo[3.1.1]hept-2-en-6-one (**76**), 2,7,7-trimethylbicyclo[3.1.1]hept-2-en-6-one (**77**), 1,4-dimethoxy-5-methyl-2-(1-methylethyl)benzene (**78**), (2E ,4E)-3,7-dimethylocta-2,4,6-trienal (**79**), (2Z,4E)-3,7-dimethylocta-2,4,6-trienal **(80)** were isolated from the essential oil of the aerial parts of *L. tomentosa* [46]. Three cuauthemone sesquiterpenes, namely 4-O-acetylcuauthemone-3-O-(2'-hydroxy-2'-methyl-3'-acetoxybutyrate) (**81**), 3-O-(3'-acetoxy-2'-hydroxy-2'-methylbutyryl)cuauthemone (**82**), 4-O-acetylcuauthemone-3-O-angelate (**83**) and four flavones, such as 3',5,6-trihydroxy-3,4',7-trimethoxyflavone (**84**), 3',4',5,7-tetrahydroxy-3,6-dimethoxyflavone (**85**), 4',5,7-trihydroxy-3',3,6-trimethoxyflavone (**86**), 3',5,7-trihydroxy-3,4',6-trimethoxyflavone (**87**) (**Figure 3**) were also isolated and characterized from the solvent extract of the leaves and aerial parts of *L. tomentosa* [50,51].



76 **77** **78** **79** **80**

81 **82** **83**

	R^1	R^2	R^3	R^4
84	OH	OCH_3	OH	OCH_3
85	OCH_3	OH	OH	OH
86	OCH_3	OH	OCH_3	OH
87	OCH_3	OH	OH	OCH_3

Figure 3: Chemical structures of compounds reported from *L. tomentosa*

3. MATERIALS AND METHODS

This chapter outlines all the experimental methods, procedures, techniques, chemicals and materials used for the isolation and characterization of compounds from ethyl acetate extracts of the roots of *L. tomentosa*, GC-MS analysis of the n-hexane extract (yellowish-orange oil), antioxidant and antibacterial activities of the extracts and isolated compounds.

3.1. General Experimental Procedure

The melting point was recorded on a digital melting point apparatus. Ultraviolet-Visible spectra were recorded on T60 UV-Visible spectrophotometer (200-600 nm) Hitachi U-3200 (Japan) spectrophotometer. Infrared spectra were obtained on Perkin-Elmer 65FT ((IR ν_{max} KBr (4000-400) cm^{-1}) infrared spectrometer using KBr pellets. NMR spectra of the compounds were recorded on a Bruker Avance 400 spectrometer in $CDCl_3$ and $(CD_3)_2CO$ operating at 400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR and DEPT-135 with TMS as internal standard. Analytical thin layer chromatograms were run on a ready-made 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminium plate. Column chromatography was performed using silica gel (200-400 mesh) Merck. Compounds on TLC were detected by employing I_2 vapours or dipping in vanillin-H_2SO_4 and heating for a few minutes. The glassware used was cleaned using soap and distilled water, rinsed with acetone and finally dried in an electric oven at 100℃ for one hour.

3.2. Chemicals

The chemicals such as: n-hexane (C_6H_{14}) (Loba Chemie Pvt. Ltd., India), chloroform ($CHCl_3$) (Blulux Laboratories Pvt. Ltd., India), ethyl acetate ($C_4H_8O_2$) (ATICO Medical Pvt. Ltd., India) and methanol (CH_3OH) (Carlo Erba Reagents, a Dasit Group S.P.A. Company, France) were purchased from Ranchem Industry and Trading, Addis Ababa, Ethiopia and used in extraction, fractionation and in TLC analysis to isolate the compounds. Other chemicals and reagents that were used to test the preliminary phytochemical constituents, for the detection of compounds on TLC plates and to evaluate the antibacterial and antioxidant activities of the roots extracts of *L. tomentosa* were vanillin, sulphuric acid (H_2SO_4), iodine (I_2), potassium iodide (KI), acetone (CH_3COCH_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), ammonia (NH_3), distilled water (H_2O), ferric chloride ($FeCl_3$), acetic acid (CH_3COOH) , chloramphenicol and 1,1-diphenyl-2-picrylhydrazyl (DPPH). All these chemicals were of analytical grade.

3.3. Materials

The materials used in this study were: electrical sample miller (DIETZ Tech 1998, West Germany), polyethylene bags, TLC plate, TLC chamber, rotary evaporator (R1001-VN (-V1, E, G), Zhengzhou Great Wall S & I & T Co. LTD, China), rotary evaporator flasks (different sizes), water bath (WB-2000 (-V1, E, G), Zhengzhou Great Wall S & I & T Co. LTD, China), sprayer, reagent bottles, capillary tubes, round bottom flasks, UV lamp, beakers (different sizes), separatory funnels, vials, tongue, spatula, glass-wares, test tubes, refrigerator, gravity filtration apparatus, Whatmann No.1 filter papers, sieve, dropper, ruler, pencil, pipette, stirrer, drying oven, measuring cylinders (different sizes), digital electronic balance (ESJ200-4, Zhengzhou Nanbei Instrument Equipment Co., Ltd., China), conical flasks (different sizes) and chromatographic column. All these materials were of the highest analytical grade.

3.4. Plant Material

The fresh roots of *L. tomentosa* (**Figure 4a**) were collected from Daletti, Sebeta Woreda, Oromia, Ethiopia, which is 26 km far from south West of Addis Ababa near Alemgena in November 2016. The plant material was authenticated by Professor Legesse Negash and a voucher specimen (Voucher no: GW 003) was deposited in the National Herbarium, Department of Botany/Biology, Addis Ababa University, Addis Ababa, Ethiopia. The collected roots of the plant were thoroughly washed using tap water to remove dirtiness and air-dried in the shade at the laboratory of Wato preparatory school (Alemgena town). The dried root sample was packed in plastic bags and transported to Addis Ababa Institute of Technology (AAIT) and crushed and grinded using electrical sample miller and taken to Adama Science and Technology University (ASTU) to be used as a sample for the extraction.

a) **b)**

Figure 4: The fresh roots (**a**) and grinded powder of the dried roots (**b**) of *L. tomentosa*.

3.5. Extraction and Isolation

The dried and powdered roots (**Figure 4b**) of *L. tomentosa* (300 g) were successively extracted with n-hexane (1.2 L), ethyl acetate (EtOAc) (1.2 L) and methanol (MeOH) (1.2 L). The mixtures were shaken occasionally and left standing for 3 days in each case at room temperature. Each extracts were filtered through Whatmann No.1 filter paper and concentrated under reduced pressure in rotary evaporator at 40℃ and analyzed with TLC. The extraction yields obtained were calculated by using the following formula:

$$\text{Percentage yield of the crude extract (crude extraction yield)} = \frac{\text{Weight dry extract}}{\text{Weight dry material}} \text{X } 100$$

The oil and dried extracts were stored under refrigerator at 4℃ in tightly closed vials until analysis. The n-hexane extract which was found as yellowish orange oil was analyzed with GC-MS. The EtOAc extract (4.06 g), which showed good TLC profile, was adsorbed on equal amounts of silica gel and subjected over silica gel (160 g) column chromatography using n-hexane (100%) for packing. Elution was done with solvent systems of gradually increasing polarity using n-hexane:ethyl acetate (EtOAc):methanol (MeOH) (solvent ratios 1:0:0, 9:1:0, 8:2:0……. up to 0:0:1) to furnish a total of 42 fractions each 100 mL (**Table 1**).

Table 1: Solvent systems and fractions collected from EtOAc crude extract of the roots of *L. tomentosa.*

Fractions collected	Eluent	Ratio	Volume in mL	Mass in mg
1-4	n-Hexane	100%	400	-
5-7	n-Hexane:EtOAc	9:1	300	-
8-10	n-Hexane:EtOAc	8:2	300	107
11-13	n-Hexane:EtOAc	7:3	300	824
14-17	n-Hexane:EtOAc	6:4	400	1013.4
18	n-Hexane:EtOAc	5:5	100	128
19	n-Hexane:EtOAc	4:6	100	156
20-21	n-Hexane:EtOAc	3:7	200	370
22	n-Hexane:EtOAc	2:8	100	92
23-24	n-Hexane:EtOAc	1:9	200	78
25-26	EtOAc	100%	200	143
27	EtOAc:MeOH	9:1	100	54
28	EtOAc:MeOH	8:2	100	23
29	EtOAc:MeOH	7:3	100	31.7
30	EtOAc:MeOH	6:4	100	121
31	EtOAc:MeOH	5:5	100	57
32	EtOAc:MeOH	4:6	100	14
33-35	EtOAc:MeOH	3:7	300	104.3
36-37	EtOAc:MeOH	2:8	200	27
38-39	EtOAc:MeOH	1:9	200	19.5
40-42	MeOH	100%	300	137

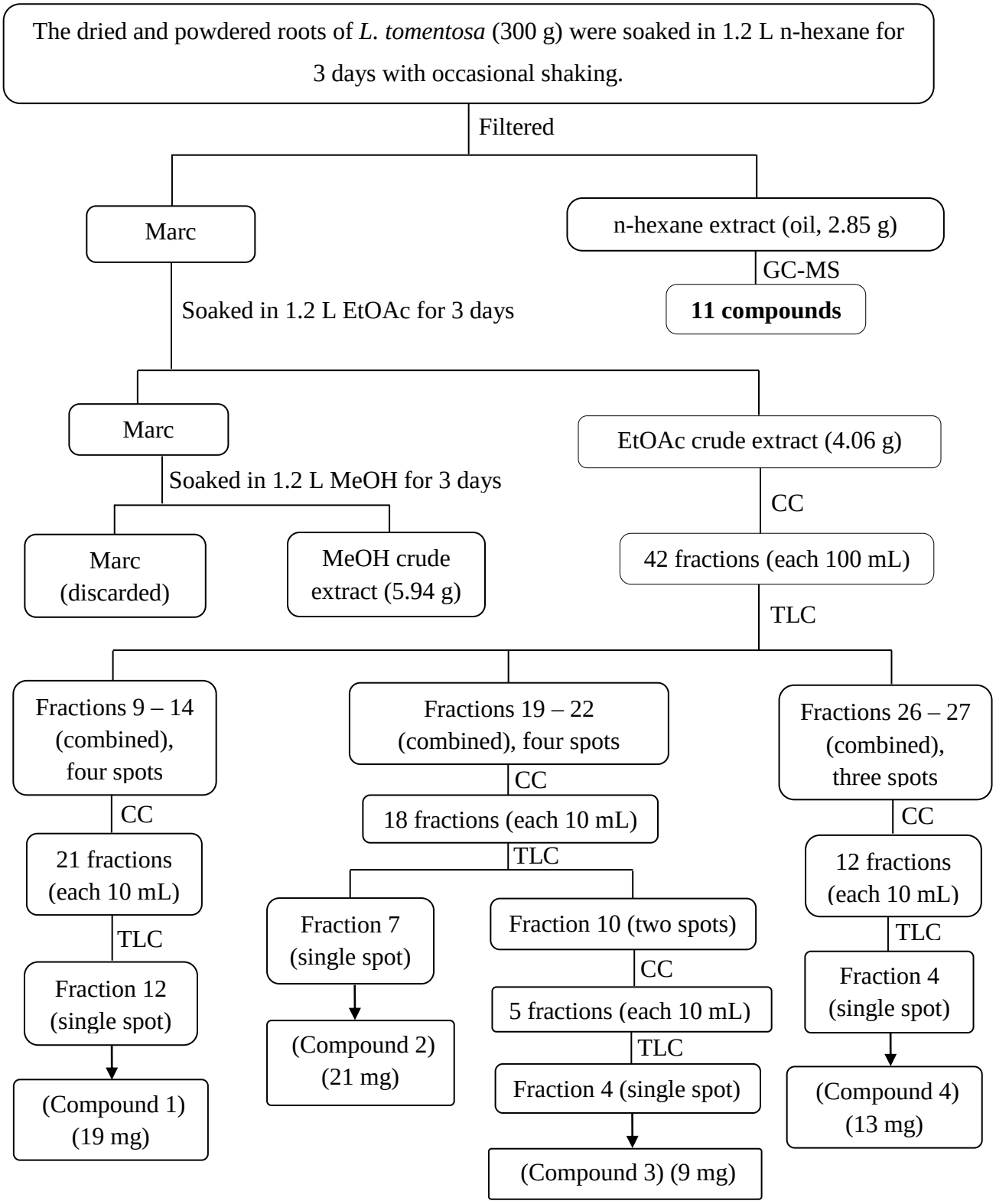
Out of 42 fractions, fractions 1-4 and 5-7 showed no visible spots on TLC. Fractions 8-14 showed four coloured spots having different retention factor (R_f.) on TLC which was visualized after dipping in vanillin-H_2SO_4 with n-hexane:EtOAc (7:3) as mobile phase.

All fractions 15-25 showed more than three coloured spots having different R_f. on TLC which was visualized after dipping in vanillin-H_2SO_4 in solvent system n-hexane:EtOAc (7:3) as a mobile phase. Fractions 26-33 showed three coloured spots having different R_f. on TLC which was visualized after dipping in vanillin-H_2SO_4 using n-hexane:EtOAc (3:2) as eluent. Fractions 34-42 showed five coloured spots having different R_f. on TLC which was visualized after dipping in vanillin-H_2SO_4 with EtOAc:MeOH (9:1) as a mobile phase.

Fractions 9-14 (1016 mg) which showed similar TLC profile were combined and re-chromatographed over silica gel (30 g) using isocratic solvent system n-hexane:EtOAc (7:3) as eluent to furnish 21 fractions each 10 mL. Fraction 12 (19 mg) showed one spot on TLC and was coded as compound 1.

Fractions 19-22 (618 mg) which showed similar TLC profile were combined and re-chromatographed over silica gel (25 g) using isocratic solvent system n-hexane:EtOAc (7:3) as eluent to furnish 18 fractions each 10 mL. Fraction 7 (21 mg), coded as compound 2, showed one spot on TLC which was visualized after dipping in vanillin-H_2SO_4 followed by heating for few minutes. Fraction 10 (47 mg) showing two spots on TLC was also subjected to silica gel (10 g) column chromatography using isocratic solvent system n-hexane:EtOAc (7:3) as eluent. Five fractions each 10 mL were collected. Fraction 4 (9 mg) showed a single spot on TLC with n-hexane:EtOAc (7:3) as a mobile phase which was again visualized after dipping in vanillin-H_2SO_4 followed by heating for few minutes and was coded as compound 3.

Fractions 26 and 27 (92 mg) having three spots on TLC were combined and re-chromatographed over silica gel (15 g) using isocratic solvent system n-hexane:EtOAc (3:2) as eluent to furnish 12 fractions each 10 mL. Fraction 4 (13 mg) showed one spot on TLC up on dipping in vanillin-H_2SO_4 followed by heating for few minutes and labelled as compound 4. The scheme of the extraction, fractionation and isolation procedure was shown below.



Scheme 1: Flow sheet diagram showing extraction, fractionation and isolation procedure

3.6. Preliminary Phytochemical Screening on the Extracts of the Roots of *L. tomentosa*

The preliminary phytochemical screening for each of the crude extracts (n-hexane, ethyl acetate and methanol) of the roots of *L. tomentosa* was carried out to analyze the presence of phytochemical constituents namely: tannins, saponins, alkaloids, terpenoids, flavonoids, glycosides, anthraquinones, steroids, phlobatannins and phenols. Phytochemical screening was done qualitatively using color forming and precipitating chemical reagents on the crude extracts of the plant and the values were tabulated in **Table 3**.

Test for Tannins

Ferric Chloride Test

Each of the extract (n-hexane, ethyl acetate and methanol) (0.5 g in each case) was boiled in 10 mL of water in a test tube and then filtered. A few drops of 0.1% $FeCl_3$ was added to give brownish green or a blue black coloration, which confirms the presence of tannin [52].

Test for Saponins

Froth test

To 0.5 g of each extract, 5 mL of distilled water was added and shaken and then heated to boil. Frothing (appearance of creamy miss of small bubbles) showed the presence of saponins [53].

Test for Alkaloids

Wagner's Test

Each extract (2 mL) was treated with Wagner's reagent (I_2 and KI in 100 ml water) and formation of reddish brown precipitate indicates of the presence of alkaloids [54].

Test for flavonoids

NaOH Test

Each extract (2 mL) was treated with few drops of aqueous NaOH and HCl and formation of yellow orange color indicates of the presence of flavonoids [54].

H_2SO_4 Test

Each extract (2 mL) was treated with Conc. H_2SO_4 and observed for the formation of orange color indicates of the presence of flavonoids [54].

Shinoda's Test

Each extract (2 mg) was dissolved in 5ml of ethanol and to this 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added. Formation of pink, reddish or brown colour indicates the presence of flavonoids [55].

Test for Steroids

Salkowski Test

Each extract (2 mg) was shaken with chloroform, to the chloroform layer H_2SO_4 was added slowly by the sides of test tube. Formation of red colour indicated the presence of steroids [55].

Test for Glycosides

a) Cardiac Glycosides

Salkowski Test

Each extract (0.5 g) weighed placed in to test tube were each dissolved in 2 mL of chloroform. Sulfuric acid is then carefully added by dropper to form a lower layer. A reddish-brown color at the interface indicates the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside) [56].

b) Anthraquinone Glycosides

Borntrager's Test

To each of the extract solution (1 mL) in the first test tube, 5% H_2SO_4 (1 mL) was added separately. The mixture was boiled in a water bath and then filtered in the second test tube. Filtrate was then shaken with equal volume of chloroform and kept to stand for 5 min. Then lower layer of $CHCl_3$ was shaken with half of its volume with dilute ammonia. The formation of rose pink to red color of the ammoniacal layer gives indication of anthraquinone glycosides [56].

Test for Terpenoids

Salkowski Test

The extracts (about 0.5 mg) were shaken with chloroform (2 mL) in the test tube followed by the addition of concentrated sulfuric acid (3 mL) along the side of the test tube using dropper, a reddish brown coloration of the interface indicates the presence of terpenoids [56].

Test for Anthraquinones

Borntrager's Test

To 0.5 g of each extract, 5 mL of chloroform was added and shaken and then filtered. 0.5 mL of 10% ammonia solution was added to the filtrate and the mixture was shaken well and the presence of the violet color in the layer phase indicated the presence of the anthraquinones [56].

Test for Phlobatannins

HCl Test

Each crude extract (2 mL) was added to 2 mL of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins [57].

Test for Phenols

Ferric Chloride Test

Each crude extract (0.5 g) were put in different test tubes and treated with a few drops of 2% of $FeCl_3$; bluish green or black coloration indicated the presence of phenols [58].

3.7. GC-MS Analysis of n-Hexane Extract (oil) of the Roots of *L. tomentosa*

The n-hexane extract of the roots of *L. tomentosa* (yellowish-orange colour oil substance, coded as **LTH**, which stands for *L. tomentosa* n-hexane extract) was taken to Addis Ababa University, Addis Ababa, Ethiopia for GC-MS analysis to identify the components present in it. The analyses was done using Gas chromatography-Mass spectrometry (GC–MS) which is Agilent technologies 7820A GC system with Agilent technologies 5977E mass selective detector, USA. A highly polar capillary column (30 m x 0.25 mm; film thickness 0.25 μm) of HP-88 coated with 100% poly(dimethylsiloxane) was used to separate the components of the sample. Analytical conditions were: injector and detector temperature: 250 and 260℃, respectively. The oven temperature was programmed from 60 to 350℃ at rate of 6℃/min. Helium gas was employed as the carrier gas at 1 mL/min flow rate; source 70 eV. The oil sample was diluted with normal n-hexane and 1 μL was injected into the GC-MS. The components of the oil were identified on the basis of comparison of their retention time and mass (m/z) spectra with published data and computer matching with WILEY 275 and National Institute of Standards and Technology (NIST14.L) libraries provided with computer controlling the GC-MS system, in Addis Ababa

University, Addis Ababa, Ethiopia. The spectrum of the unknown component was compared with the spectrum of the known components stored in the library.

3.8. Structural Elucidation of the Isolated Compounds

The structures of the isolated compounds were elucidated based on data obtained from physical properties (melting points) and spectroscopic methods including UV/Visible, IR, 1D-NMR (^{1}H-NMR and ^{13}C-NMR) and DEPT-135 and comparing the result with reported value from literature for the same compound.

3.9. Studying Antioxidant Activities of the Roots Extracts of *L. tomentosa* (DPPH assay)

The antioxidant activities of the roots extracts of *L. tomentosa* were checked using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging [59] assay. DPPH solution was prepared by dissolving 4 mg in 100 mL MeOH. Likewise the crude extracts of the roots of *L. tomentosa* were dissolved in methanol to give each 500 μg/mL which were used as standard solution. From the standard solution 1 mL were taken and mixed with 4 mL of DPPH solution to furnish 100 μg/mL. After incubating for 30 minutes at 37℃, the absorbance was measured at 517 nm in UV-Visible spectrophotometer. This was repeated for ascorbic acid which was used as positive control. The corresponding solvent (methanol) was used as a blank. The absorbance of DPPH (0.04% in MeOH) was also measured and found to be 0.938. The percentage radical scavenging activity of the extracts was calculated with the help of the following formula:

$$\%\ \text{of radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \text{X } 100$$

3.10. Evaluating Antibacterial Activities of the Roots Extracts of *L. tomentosa*

Ethyl acetate and methanol crude extracts, isolated pure compounds and n-hexane extracted oil of the roots of *L. tomentosa* were evaluated for antibacterial assay by using disc diffusion method [60]. The bacterial strains were obtained from the American Type Culture Collection (ATCC) and the antibacterial activities of all samples were tested against three Gram-negative bacterial strains such as: *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 35659) and *Klebsiella pneumoniae* (ATCC 700603) and one Gram-positive bacterium (*Staphylococcus aureus*, ATCC 25923) using Muller Hinton Agar (MHA) medium. All the microbial were obtained from Oromia

Public Health Research, Capacity Building and Quality Assurance Laboratory Center, Adama, Ethiopia. Standard antibiotic chloramphenicol of concentration 1 mg/mL was used as positive control against bacteria. DMSO was used as a negative control.

3.10.1. Media preparation

Mueller Hinton Agar medium was prepared by dissolving the solid media in 10 mL distilled water. The dissolved medium was then autoclaved at 121℃ for 15 minutes. After autoclaving, the medium was cooled to about 50℃. The medium was then poured to sterile Petri dishes, allowed to solidify and used for the disc diffusion antibacterial activity assay.

3.10.2. Inoculation and incubation

The test bacterial species were transferred from the stock cultures and streaked on Mueller Hinton plates. Well-separated bacterial colonies were then used as inoculums. Bacteria were transferred using bacteriological loop to autoclaved Mueller Hinton agar and mixed by gently swirling the flasks. 0.1g of each of the extracts and 2 mg of each of the isolated compounds were dissolved in 1 mL of DMSO separately. Different concentrations (0.5 and 1 mg/mL) of each of the sample were prepared. Wells of 6 mm diameter were made in the culture medium (Whatmann No.1 filter paper) using sterile cork borers and 10 μL of 0.5 mg/mL and 20 μL of 1 mg/mL concentrations of the sample were placed in the wells using micropipette and the plates were kept for incubation at 37℃ for 24 hrs. Control for each bacterial strain before sample action was at 6 mm. Each of the prepared test solutions was taken to test the sensitivity towards the selected bacteria. The antibacterial activity, indicated by an inhibition zone surrounding the well containing the sample, was assessed by measuring the inhibition zone diameters in millimeters formed around the well against each test organism at different concentrations and recorded if the zone of inhibition was greater than 6 mm.

4. RESULTS AND DISCUSSION

This chapter is a detailed presentation and discussion of the results obtained from n-hexane, ethyl acetate and methanol extracts of the roots of *L. tomentosa*.

4.1. Extraction Yields of the Roots Extracts of *L. tomentosa*

Three solvents of increasing polarity (n-hexane, ethyl acetate and methanol) were used successively to extract phytochemicals from the roots of *L. tomentosa* as discussed in **Section 3.5** and the percentage yields calculated. The percentage (extraction) yields reflect the amount or yield of extractable components relative to the weight of dry plant material. It is a factor of several variables including the extraction solvent system, nature of the sample, method of extraction, time, temperature and pressure. Different solvents, depending on their selectivity and polarity, extract varying amounts of different groups of phytochemicals.

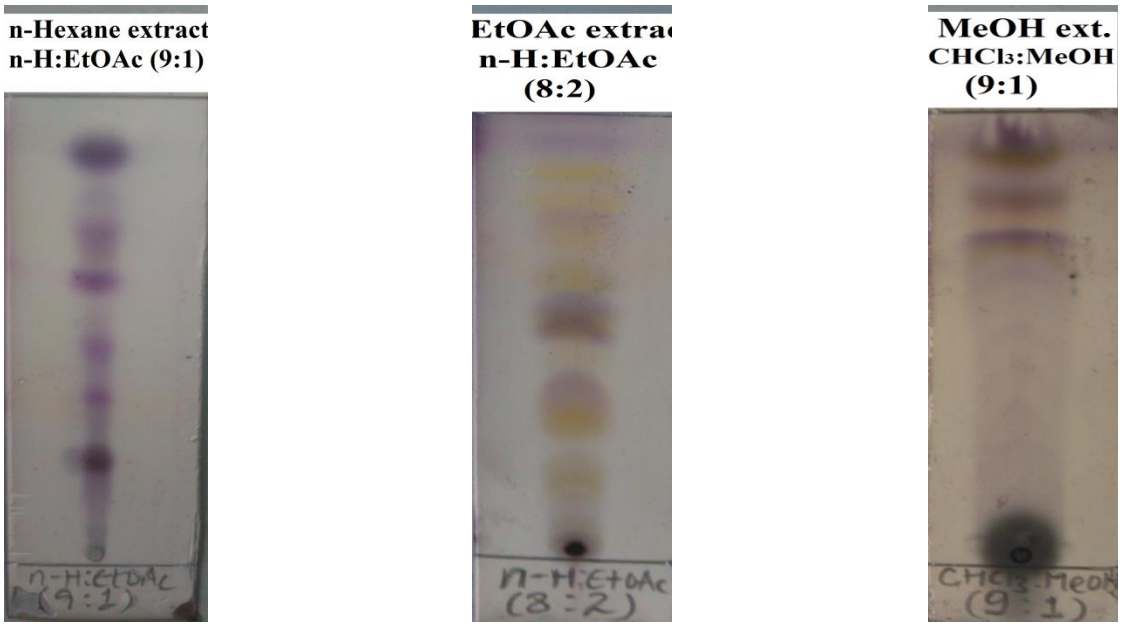
Table 2: Extracts yield of the roots of *L. tomentosa*.

Extract	Color of the crude extract	weight of extract in (g)	% yield
n-Hexane	Yellowish-orange oil	2.85	0.95
EtOAc	Dark green solid	4.06	1.35
MeOH	Golden-yellow jelly	5.94	1.98

As shown in **Table 2**, total yield of the extract from the roots of the plant was about 4.28%. About 0.95% of yellowish-orange color oil substance, 1.35% of dark green solid substance and 1.98% of golden-yellow jelly like material was extracted by hexane, EtOAc and MeOH, respectively. It also showed that the percentage yield increased with increased polarity. The extracts obtained increased with polarity of solvent likely implying that the plant contain more polar compounds as compared to the non-polar compounds.

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

The n-hexane, ethyl acetate and methanol extracts were analyzed with TLC (**Figure 5**). The mobile phases used for n-hexane, EtOAc and MeOH extracts were n-hexane:ethyl acetate (9:1), n-hexane:ethyl acetate (7:3) and chloroform:methanol (9:1), respectively. Spots were visualized after dipping in vanillin-sulphuric acid followed by heating on heating plate for a few minutes.



a) TLC of *L. tomentosa* n-hexane extract

b) TLC of *L. tomentosa* ethyl acetate extract

c) TLC of *L. tomentosa* methanol extract

Figure 5: TLC profile of the crude extracts of the roots of *L. tomentosa*

The dark green solid substance obtained from the ethyl acetate extract of the roots of *L. tomentosa* when developed on TLC (n-hexane:ethyl acetate) showed more than seven coloured spots as shown in **Figure 5 (b)**. It was therefore subjected to fractionation over silica gel column chromatography which affords 42 fractions.

4.3. Phytochemical Screening Analysis of the Roots Extracts of *L. tomentosa*

To the best of our knowledge, there is no previous preliminary phytochemical report on any part of the plant. Therefore, this is the first report and the result shows that tannins, alkaloids, flavonoids, cardiac glycosides, steroids, terpenoids, anthraquinones, phlobatannins and phenols were present in the extracts of the roots of *L. tomentosa*, whereas saponins and anthraquinone glycosides were absent in all the extracts (**Table 3**). However, n-hexane extract (oil) showed positive test for alkaloids, flavonoids, steroids, cardiac glycoside, terpenoids, phenols but tannins, saponins, anthraquinone glycosides, anthraquinones and phlobatannins are absent. In both ethyl acetate and methanol crude extracts the chemical compounds such as tannins, alkaloids, flavonoids, cardiac glycosides, steroids, terpenoids, anthraquinones, phlobatannins and

phenols were present but saponins and anthraquinone glycosides were absent. Ethyl acetate and methanol extracts shows the presence of majority phytoconstituents. This indicates that most of the compounds found in the plant extracts are polar organic compounds and shows that the plant constituents might have high level of medicinal values. This could be responsible for the versatile medicinal properties of the plant.

Table 3: Phytochemical analysis of n-hexane extract (oil), ethyl acetate and methanol crude extracts of the roots of *L. tomentosa*.

S. No.	Phytochemical	Reagents	n-hexane extract result	EtOAc crude extract result	MeOH crude extract result
1	Tannins	Ferric Chloride Test	-	+	+
2	Saponins	Froth/Foam Test	-	-	-
3	Alkaloids	Wagner's Test	+	+	+
4	Flavonoids	NaOH Test	+	+	+
		H_2SO_4 Test	+	+	+
		Shinoda's Test	+	+	+
5	Steroids	Salkowski Test	+	+	+
6	Glycosides	Salkowski Test	+	+	+
		Borntrager's Test	-	-	-
7	Terpenoids	Salkowski Test	+	+	+
8	Anthraquinones	Borntrager's Test	-	+	+
9	Phlobatannins	HCl Test	-	+	+
10	Phenols	Ferric Chloride Test	+	+	+

(+ = the presence of phytochemical constituents; - = absence of phytochemical constituents)

These secondary metabolites are known to be biologically active compounds and they are responsible for different activities such as antimicrobial, antioxidant, antifungal, anticancer and anti-diabetic, which may help in protection against chronic diseases [61]. Tannins, glycosides and flavonoids have hypoglycemic and anti-inflammatory activities. Terpenoids and steroids show analgesic properties and central nervous system activities. Phenols have a wide range of

pharmaceutical activities such as cardio-protective, immune-modulatory, anti-oxidant, anti-inflammatory, analgesic, anti-tumor, anti-HIV, anti-infective (anti-diarrhea, anti-fungal), anti-hepatotoxic, anti-lipolytic, vasodilatory, anti-carcinogenic, immune-stimulant and anti-ulcerogenic. Flavonoids protect the plant from UV-damaging effects and play a role in pollination by attracting animals with their colors [5,62,63]. Anthraquinones also have a wide range of biological activity and low toxicity. They possess astringent, purgative, anti-inflammatory, anti-cancer, anti-viral, anti-tumor and bactericide effects [64]. The presence of anthraquinones and steroids are one positive attributes of this plant against free radicals, bacteria, cancers and viruses. Therefore, the extracts of the plant from which the presence of these bioactive compounds were detected may be useful in treating tooth-ache, stomach-ache, swelling, ringworm, skin infections, external parasites, common cold, headache and for curing other various ailments.

4.4. GC-MS Profile of the n-Hexane Extract of the Roots of *L. tomentosa*.

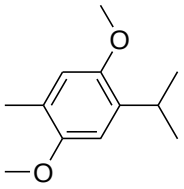
To the best of our knowledge, this work represents the first GC-MS analysis of the n-hexane extract (yellowish-orange oil) of the roots of *L. tomentosa*. The GC-MS chromatogram was shown in **Appendix 1** and its components were identified as shown in **Table 4.**

Table 4: Compounds identified from n-hexane extract (oil) of the roots of *L. tomentosa*.

Retention time (min.)	Compound	Molecular formula	Molecular weight	Area %
10.121	1-Isopropyl-2-methoxy-4-methylbenzene	$C_{11}H_{16}O$	164	9.54
12.355	sec-Butylamine	$C_4H_{11}N$	73	1.15
12.713	2,5-Dimethoxy-*p*-cymene	$C_{12}H_{18}O_2$	194	59.39
13.274	*α*-Humulene	$C_{15}H_{24}$	204	1.86
13.633	*N*-Methyl-2-phenylethanamine	$C_9H_{13}N$	135	1.29
15.220	1-(3,5-Dimethyl-1-adamantanoyl)semicarbazide	$C_{14}H_{23}N_3O_2$	265	1.20
17.340	2-(Methylamino)-1-phenylethanol	$C_9H_{13}NO$	151	7.56
17.409	Propanamide	$C_5H_{13}N$	87	1.96
17.522	Tetradecyl-2-(*N*-methylisobutyramido)acetate	$C_{21}H_{41}NO_3$	355	6.54
18.905	Methyl palmitate	$C_{17}H_{34}O_2$	270	5.74
21.791	1-Guanidinosuccinimide	$C_5H_7N_3O_2$	141	3.77

The result revealed eleven chemical constituents were identified in the oil of the plant root. The major component of the oil was phenolic ether 2,5-dimethoxy-*p*-cymene (**88**) which constituted 59.39% of the identified components followed by 1-isopropyl-2-methoxy-4-methylbenzene (**89**, 9.54%), 2-(methylamino)-1-phenylethanol (**90**, 7.56%), tetradecyl 2-(*N*-methylisobutyramido)acetate (**91**, 6.54%), methyl palmitate (**92**, 5.74%), 1-guanidinosuccinimide (**93**, 3.77%), propanamide (**94**, 1.96%), *α*-humulene (**95**, 1.86%), *N*-methylpent-4-en-1-amine (**96**, 1.29%), 1-(3,5-dimethyl-1-adamantanoyl)semicarbazide (**97**, 1.20%) and sec-butylamine (**98**, 1.15%). According to literature surveys, 2,5-dimethoxy-*p*-cymene, 1-isopropyl-2-methoxy-4-methylbenzene and *α*-humulene have been previously detected in different species of the genus *Laggera*. 2,5-dimethoxy-*p*-cymene has been reported from the essential oil of the aerial parts of *L. tomentosa* grown only in Ethiopia [41,46], *L. alata* (44.2%) grown in Nigeria, *L. pterodonta* (30.5%) grown in Benin Republic and *L. oloptera* grown in Cameroon [41]. But, it has not reported as a major constituent. The essential oils of *L.*

pterodonta (28.2 %), *L. alata* (34.1 %) and *L. gracilis* (33.4 %) from Cameroon, *L. crispata* (32.2 %) from India contained 2,5-dimethoxy-*p*-cymene as major constituent [65,66]. It also occurs in a variety of different plants' essential oils within the family Asteraceae including *Bubonium imbricatum* (16.2%) [67], *Cyathocline purpurea* (57.4%) [68], *Blumea perrottetiana* (30.0%) [69]. 2-(Methylamino)-1-phenylethanol, tetradecyl-2-(*N*-methylisobutyramido)acetate, methyl palmitate, 1-guanidinosuccinimide, *N*-methylpent-4-en-1-amine, 1-(3,5-dimethyl-1-adamantanoyl)semicarbazide, propanamide and sec-butylamine were not reported from other *Laggera* species. Therefore, they are the first report of the oil of the roots of the species under the genus *Laggera*. The structures of all the constituents of the oil are given in the order of their percentage in **Figure 6** below.



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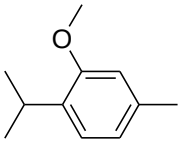

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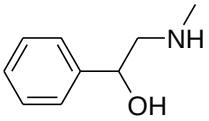

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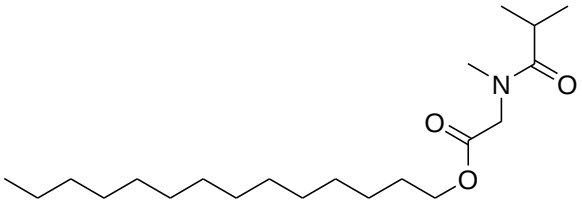

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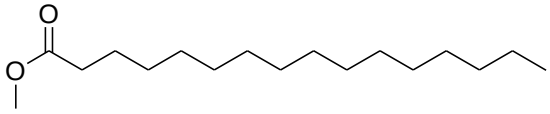

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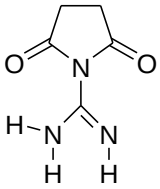

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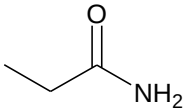

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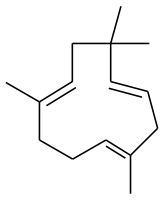

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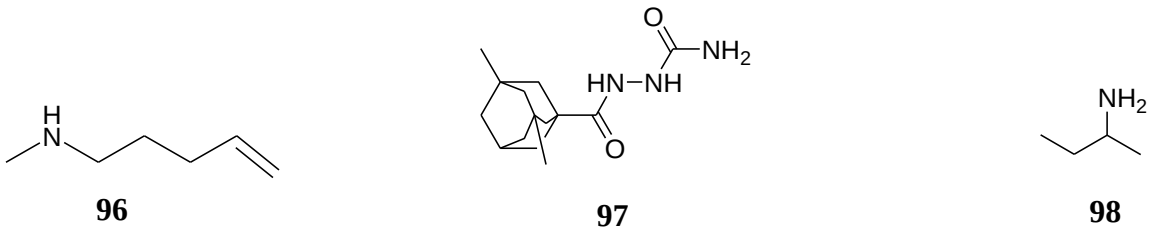


96 **97** **98**

Figure 6: Structures of compounds identified from the n-hexane extract (oil) of the roots of *L. tomentosa*.

The presence of volatile components like 2,5-dimethoxy-*p*-cymene and 1-isopropyl-2-methoxy-4-methylbenzene suggests that the oil may be useful in aromatherapy and in the pharmaceutical and confectionary industries as fragrance or additives [41,56]. The essential oils which contain the compound as a major component of the oil, have antifungal [67], antibacterial [68] and insecticidal [69] properties. Therefore, the presence of 2,5-dimethoxy-*p*-cymene in the roots is a good attributes of this plant.

4.5. Structural Elucidation of Isolated Compounds

In the course of this work, four compounds were isolated from the ethyl acetate extract of the roots of *L. tomentosa*. Structure elucidation was achieved using melting point, the R_f. value, UV-Vis, IR and NMR spectroscopic data of the compounds as described herein.

4.5.1. Characterization of compound 1

Compound 1 was obtained as light yellow solid (19 mg) melting at 43-45℃. It showed one spot on TLC at R_f. 0.75 with n-hexane:ethyl acetate (9:1) solvent system as a mobile phase (**Figure 7**). The compound was soluble in chloroform and showed a characteristic colour change from colourless to violet on TLC plate upon dipping in vanillin-H_2SO_4 and after heating for a few minutes.

Figure 7: TLC profile of compound 1.

The UV/Vis spectrum (ethanol) (**Appendix 2**) of compound 1 showed the absorption bands at 266 nm (due to π-π* transition of C=O) and 322 (due to n-π* transition of C=O). In IR (KBr) spectrum (**Appendix 3**), a very intense broad absorption band centered at 3408 cm^{-1} is characteristic of hydroxyl group. The strong absorption band at 2928 cm^{-1} and medium absorption band at 2855 cm^{-1} showed the presence of asymmetric and symmetric C-H stretching, respectively. In addition, the absorption band at 1729 cm^{-1} showed the presence of the C=O stretching. Moreover, the absorption bands at 1387 and 1269 cm^{-1} showed the presence of C-C and C-O stretching, respectively.

The ^{1}H-NMR spectrum (**Appendix 4**) of compound 1 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 14 hydrogens (H-4, H-5, H-6, H-7, H-8, H-9, H-10), which is a characteristic of protons on many overlapping methylene carbons. The triplet signal (3H)

situated at δ_H 0.90 is evident for the presence of terminal methyl group (H-12, J = 6.8 Hz). In addition, the spectrum of this compound shows a multiplet peak at δ 1.32, which indicates the presence of two aliphatic methylene protons (H-11) which are attached to carbon adjacent to the terminal methyl carbon. The spectrum of this compound also revealed that carboxylic acid hydroxyl proton appeared as broad singlet peak at δ 9.78.

The ^{13}C-NMR spectrum (**Appendix 5**) in $CDCl_3$ of compound 1, analysed with the aid of DEPT-135 (**Appendix 6**) in $CDCl_3$ 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 [70]. In accordance with this principle, DEPT-135 spectrum of compound 1 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 was quaternary carbon (C), which was attributable to carbonyl carbon of the carboxylic acid. This structural type was further supported by the ^{13}C-NMR spectrum, in which the appearance of the most downfield signal at δ_c178.5 (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), 29.3 (C-5), 29.6 (C-6), 29.7 (C-7), 29.4 (C-8), 29.2 (C-9), 24.7 (C-10), 22.7 (C-11) and one aliphatic methyl carbon at δ 14.1 (C-12) (**Table 5** and **Appendix 5)**. The NMR spectrum of the proposed structure of compound 1 was found to agree with the reported literature spectral data of dodecanoic acid, also known as lauric acid [71]. This compound was not yet reported from the genus *Laggera* and the species *L. tomentosa*. The NMR spectral data used to characterize compound 1 was compared with literature reported for lauric acid with the whole results shown in **Table 5**.

Table 5: Comparison of ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data of compound 1 and literature reported for lauric acid.

Carbon position	^{13}C-NMR δ (ppm) of compound 1	DEPT δ (ppm)	^{13}C-NMR δ (ppm) data reported for lauric acid [71]	^{1}H-NMR δ (ppm) data of compound 1	^{1}H-NMR δ (ppm) data reported for lauric acid [71]	Nature of the carbon
1	178.5	-	178.4	-	-	C (quaternary carbon)
2	33.8	33.8	34.0	2. 36 (*t*, *J* = 7.6 Hz, 2H)	2. 30 (*m*, H-2)	CH_2
3	31.9	31.9	31.9	1.65 (*m*, 2H)	1.52 (*m*, H-3)	CH_2
4	29.0	29.0	29.0	1.27 (br. *s*, 2H)	1.29 (*m*, H-4)	CH_2
5	29.3	29.3	29.3	1.27 (br. *s*, 2H)	1.26 (*m*, H-5)	CH_2
6	29.6	29.6	29.6	1.27 (br. *s*, 2H)	1.26 (*m*, H-6)	CH_2
7	29.7	29.6	29.6	1.27 (br. *s*, 2H)	1.26 (*m*, H-7)	CH_2
8	29.4	29.4	29.6	1.27 (br. *s*, 2H)	1.26 (*m*, H-8)	CH_2
9	29.2	29.2	29.4	1.27 (br. *s*, 2H)	1.29 (*m*, H-9)	CH_2
10	24.7	24.7	24.7	1.27 (br. *s*, 2H)	1.29 (*m*, H-10)	CH_2
11	22.7	22.7	22.7	1.32 (*m*, 2H)	1.31 (*m*, H-11)	CH_2
12	14.1	14.1	14.1	0.90 (*t*, *J* = 6.8 Hz, 3H)	0.88 (*t*, *J* = 7 Hz, H-12)	CH_3

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

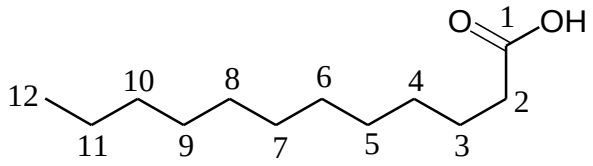


Figure 8: Proposed structure of compound 1

Lauric acid occurs extensively in Lauraceae seeds (*Laurus nobilis*) where it was discovered. It is dominant in cinnamon oil (80-90%), coconut oil (40-60% as trilaurin) and is found also in *Cuphea* species (Umbelliferae) whose production was initiated in Germany. The recent uses of lauric acid are in the manufacture of flavourings, cocoa butter, margarine, alkyd resins, soaps, shampoos and other surface active agents, including special lubricants. Lauric acid as monoglyceride is known to the pharmaceutical industry for its good antimicrobial properties. It may play a role in combating lipid-coated RNA and DNA viruses. The major sources of lauric acid for human food are palm kernel, coconut and palm [72]. The presence of lauric acid in this plant is significant as it displays antimicrobial and antiviral activities.

4.5.2. Characterization of compound 2

Compound 2 was obtained as white solid crystal (21 mg) melting at 160-164℃. It showed one spot on TLC at R_f. 0.70 with n-hexane:ethyl acetate (4:1) solvent system as a mobile phase and silica gel as stationary phase (**Figure 9**). Compound 2 was soluble in chloroform and showed a characteristic colour change from colourless to violet on TLC plate upon dipping in vanillin-H_2SO_4 and after heating for a few minutes.

Figure 9: TLC profile of compound 2.

The UV/Vis spectrum (ethanol) (**Appendix 7**) of compound 2 showed the absorption maxima at 259 nm indicating the presence of non-conjugated olefinic (C=C) groups (π-π* electronic transition). In IR (KBr) spectrum (**Appendix 8**), a very intense broad absorption band observed at 3360 cm^{-1} is characteristic of O-H stretching that indicated the presence of a hydroxyl group (alcohol functional group)**.** The strong absorption band at 2938 and the medium absorption band at 2868 cm^{-1} showed the presence of the asymmetric and symmetric C-H stretching, respectively. A weak absorption band at around 1638 cm^{-1} revealed the presence of C=C stretching. The absorption band at 1462 cm^{-1} was assignable to cyclic $(CH_2)_n$ bending absorption frequency while the one at 1386 cm^{-1} is attributable to OH deforming absorption. The absorption band at 1054 cm^{-1} is due to C-O stretching and the one at 971 cm^{-1} is typical of cycloalkane moieties.

The ^{1}H-NMR spectrum (**Appendix 9**) of compound 2 varied between 0.71 to 5.37 ppm. This spectrum showed several peaks between δ 1.01 and 0.71 characteristic of steroid skeleton signals [73]. A broad unresolved doublet of doublet at δ 5.37 (J = 3.6 Hz) integrating for one proton suggested the presence of a methine olefinic proton on carbon bonded to one quaternary and one methylene carbons. Two doublets of doublet signals at δ 5.19 (J = 8.4, 15.2 Hz) and 5.06 (J =

8.4, 15.2 Hz) indicated the presence of double bond protons each attached to one tertiary and one methine carbons and integrating for one proton. The multiplet peak at δ 3.54 is for carbinylic proton attached to one hydroxyl group and two methylene carbons, integrating for one proton. Moreover, the proton NMR spectrum of this compound exhibited two broad singlet signals at δ 0.71 and 1.03 due to the presence of two quaternary methyl protons (angular methyl protons), each integrating for three protons. In addition, the spectrum showed the presence of three tertiary methyl protons at δ 0.80 (J = 6.4 Hz), 0.84 (J = 6.8 Hz) and 1.04 (J = 4.8 Hz) (all doublet) and a broad singlet methyl protons at δ 0.82 which is attached to a methylene carbon, all integrating for three protons. There are also different peaks which showed the presence of protons of sp^3 bonds from methylene and methine groups totally constitutes exactly 25 hydrogens (**Table 6**).

The ^{13}C-NMR spectrum (**Appendix 10**) in $CDCl_3$ of compound 2, analysed with the aid of DEPT-135 (**Appendix 11**) in $CDCl_3$ displayed twenty nine carbon atoms confirming that the compound was a typical of steroid. The DEPT-135 spectrum indicated nine negative (CH_2) carbons; all of them were attributable to aliphatic carbons and eleven positive (CH) carbons, which were attributable to one oxygenated carbon (β-hydroxyl group), seven aliphatic carbons and three olefinic carbons. In addition, the spectral data indicated the presence of six positive (CH_3) carbons; all of them were attributable to aliphatic carbon. The DEPT-135 spectrum also indicated the presence of three quaternary carbons (C), which were attributable to two aliphatic carbons and one olefinic carbon. This structural type was further supported by the ^{13}C-NMR spectrum, in which the appearance of signals at δ 140.7 (C-5) and 121.7 (C-6) confirming the presence of olefinic carbons with the more deshielded signal assignable to the quaternary carbon at the bridge. The signal at δ 138.3 (C-22) and 129.2 (C-23) represented the olefinic carbon on the side chain. The peak at δ 71.8 was assigned to C-3 (δ_c 71.8) due to the presence of the hydroxyl group common to this class of compounds. The complete ^{13}C-NMR data is given in **Table 6** and **Appendix 10**. The overall spectrum of compound 2 is in a good agreement to that of 3β,22*E*-Stigmasta-5,22-dien-3-ol, also known as β-stigmasterol [73]. This compound was not yet reported from the genus *Laggera* and the species *L. tomentosa.* The NMR spectral data used to characterize compound 2 was compared with literature reported for β-stigmasterol with the whole results shown in **Table 6**.

Table 6: Comparison of ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data of compound 2 and literature reported for *β*-stigmasterol.

Carbon position	^{13}C-NMR *δ* (ppm) of compound 2	DEPT *δ* (ppm)	^{13}C-NMR *δ* (ppm) data reported for *β*-stigmasterol [73]	^{1}H-NMR *δ* (ppm) data of compound 2	^{1}H-NMR *δ* (ppm) data reported for *β*-stigmasterol [73]	Nature of the carbon
1	37.2	37.2	37.2	1.87 (*m*, 1H) and 1.09 (*m*, 1H)		CH_2
2	31.6	31.6	31.6	1.85 (*m*, 1H) and 1.51 (*m*, 1H)		CH_2
3	71.8	71.8	71.8	3.54 (*m*, 1H)	3.55	CH
4	42.3	42.3	42.2	2.30 (*m*, 1H) and 2.25 (*m*, 1H)		CH_2
5	140.7	-	140.7	-		C (quaternary carbon)
6	121.7	121.7	121.7	5.37 (*dd*, *J* = 3.6 Hz, 1H)	5.38	CH
7	31.9	31.9	31.9	1.99 (*m*, 1H) and 1.51 (*m*, 1H)		CH_2
8	31.9	31.9	31.9	1.46 (*m*, 1H)		CH
9	50.1	50.1	50.1	0.94 (*m*, 1H)		CH
10	36.5	-	36.5	-	-	C (quaternary carbon)
11	21.1	21.0	21.0	1.51 (*m*, 2H)		CH_2
12	39.7	39.7	39.7	2.00 (*m*, 1H) and 1.19 (*m*, 1H)		CH_2
13	42.2	-	42.3	-	-	C (quaternary carbon)
14	56.8	56.8	56.7	1.10 (*m*, 1H)		CH
15	24.3	24.3	24.3	1.56 (*m*, 1H) and 1.04 (*m*, 1H)		CH_2
16	28.9	28.9	29.7	1.71 (*m*, 1H) and 1.29 (*m*, 1H)		CH_2
17	55.9	55.9	56.0	1.17 (*m*, 1H)		CH
18	12.2	12.2	12.2	0.71 (br. *s*, 3H)	0.70	CH_3
19	19.4	19.4	19.4	1.03 (br. *s*, 3H)	1.03	CH_3
20	40.5	40.5	40.5	2.06 (*m*, 1H)		CH
21	21.1	21.1	21.1	1.04 (*d*, *J* = 4.8 Hz, 3H)	1.20	CH_3
22	138.3	138.3	138.3	5.19 (*dd*, *J* = 8.4, 15.2 Hz, 1H)	5.36	CH
23	129.2	129.2	129.2	5.06 (*dd*, *J* = 8.4, 15.2 Hz 1H)	5.06	CH
24	51.2	51.2	51.2	1.53 (*m*, 1H)		CH
25	39.7	39.7	39.7	1.54 (*m*, 1H)		CH
26	19.0	19.0	19.0	0.84 (*d*, *J* = 6.8 Hz, 3H)	0.83	CH_3
27	21.2	21.2	21.2	0.80 (*d*, *J* = 6.4 Hz, 3H)	0.71	CH_3
28	25.4	25.4	25.4	1.56 (*m*, 1H) and 1.23 (*m*, 1H)		CH_2
29	12.0	12.0	12.0	0.82 (br. *s*, 3H)	0.82	CH_3

Based on the evidences by comparison with literature, the following structure was proposed for compound 2.

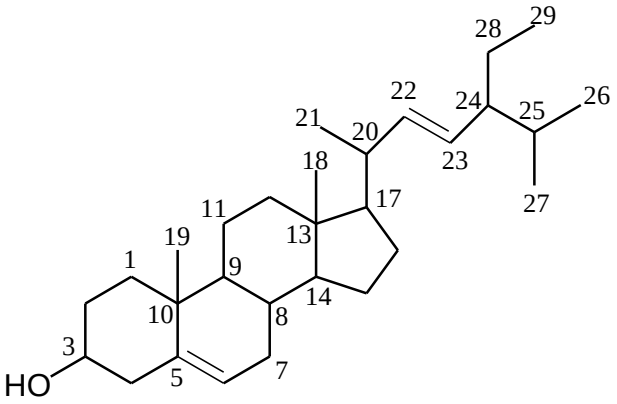


Figure 10: Proposed structure of compound 2

β-Stigmasterol is a known steroid (phytosterol) that has been reported from various plant sources. The most important benefit for this secondary metabolite is its enrolment amongst the health promoting constituents of natural foods which contains *β*-stigmasterol. In fact, the European Foods Safety Authority recommends consuming about 1.5-2.4 g/day of phytosterols in order to reduce blood cholesterol. Furthermore, FDA has approved the role of foods containing phytosterol esters inside a low saturated fat and cholesterol diet in reducing the risk of heart disease, especially consumption of at least 1.3 g/day sterols, twice a day. Research has indicated that *β*-stigmasterol may be useful in prevention of certain cancers, including ovarian, prostate, breast and colon cancers. It also possesses potent laxative, antioxidant, antibacterial, hypoglycemic and thyroid inhibiting properties [74, 75]. The presence of *β*-stigmasterol in this plant is significant as it displays immense biological activities listed above.

4.5.3. Characterization of Compound 3

Compound 3 was obtained as yellow amorphous powder (9 mg) melting at 195-197℃. It showed one spot on TLC at R_f. 0.62 with n-hexane:ethyl acetate (4:1) as a mobile phase (**Figure 11**). Compound 3 is soluble in chloroform that showed a characteristic colour change from bright yellow to yellowish orange on TLC plate upon dipping in vanillin-H_2SO_4 followed by heating for a few minutes. Close inspection of the NMR (^{1}H-NMR, ^{13}C-NMR and DEPT-135) spectra

(**Appendices 14, 15 and 16**) showed the presence of two compounds. The structure elucidation of one of the compound is described as follows.

Figure 11: TLC profile of compound 3

The UV-Vis spectrum (DMSO) of compound 3 (**Appendix 12**) showed absorption bands at 266, 288 and 435 nm indicating characteristics absorption chromophore center for anthraquinone [76]. The absorption peaks at 266 and 288 nm showed the presence of π-π* and n-π* electronic transitions of carbonyl group (C=O) of ketone, respectively. The broad absorption band at 435 nm shows the presence of conjugation in the molecule.

In the IR (KBr) spectrum of compound 3 (**Appendix 13**), the absorption band at 3433 cm^{-1} showed the O-H stretching that indicated the presence of a hydroxyl group. The absorption bands at 2922 and 2852 cm^{-1} are due to asymmetric and symmetric C-H stretching, respectively. Additionally, the compound shows absorption bands at 3058, 1564, 1482 and 845 cm^{-1} which indicated the presence of aromatic (C-H) stretching, aromatic C=C stretch (in-ring), aromatic C-C stretching (in-ring) and aromatic (C-H) bending, respectively. Moreover, the strong absorption band at 1640 cm^{-1}, the medium absorption bands at 1373, 1278 and 751 cm^{-1} showed the presence of the ketone C=O stretching, C-C stretching (out of the ring), C-O stretching and C-H

stretching for ortho substitution, respectively. Therefore, the IR spectrum showed the presence of hydroxyl and ketone functionality groups, an aromatic and a methyl groups in the compound 3.

The ^{1}H-NMR spectrum of compound 3 (**Appendix 14**) showed the presence of protons in the aromatic chemical shift region, hydroxyl and aliphatic protons. The aromatic part of this compound contains five chemically non-equivalent protons. These are the protons represented by two broad singlet signals appeared at δ 7.12 (H-2) and 7.67 (H-4), three doublets of doublet signals at δ 7.83 (J = 1.2, 7.6 Hz, H-5), 7.30 (J = 1.2, 8.4 Hz, H-7) and 7.69 (J=8.4, 7.6 Hz, H-6), all integrating for one proton. The proton NMR spectrum of the same compound also showed two single intense singlet signals at δ 12.15 and 12.05 indicating the presence of two chelated hydroxyl protons attached on C-1 and C-8 of the aromatic ring, respectively and other three types of protons out of the aromatic chemical shift region. These protons are a methyl group protons (singlet) as a substituent to the aromatic ring at δ 2.48, integrating for three protons.

The ^{13}C-NMR spectrum (**Appendix 15**) in $CDCl_3$ of compound 3, analysed with the aid of DEPT-135 (**Appendix 16**) in $CDCl_3$ showed a well resolved resonance of 15 carbon atoms. DEPT-135 indicated the presence of nine quaternary carbons (C), which were attributable to two oxygenated aromatic carbons; one methyl substituted aromatic carbon, four aromatic carbons and two carbonyl (ketone) carbons. The spectral data showed five methine (CH), all of them were attributable to unsubstituted aromatic carbons. In addition, the spectral data indicated the presence of one methyl (CH_3) carbon that was attached to the aromatic carbon and the absence of any methylene (CH_2) carbons. This structural type was further supported by the ^{13}C-NMR spectrum which contained two oxygenated carbons (at δ_c 162.4 (C-1) and 162.7 (C-8)), one methyl substituted carbon at δ 149.3 (C-3), five unsubstituted carbons (at δ_c 124.5 (C-2), 119.9 (C-4), 136.9 (C-5), 124.4 (C-6) and 121.4 (C-7)), two carbonyl carbons at δ_c 192.5 (C-9) and 182.0 (C-10), four quaternary aromatic carbons at δ_c 133.6 (C-11), 115.9 (C-12), 113.7 (C-13) and 133.3 (C-14) and one methyl carbon at δ_c 22.3 (C-15). The chemical shift difference between the two carbonyl carbons (C-9 and C-10) is more than 5 ppm which is due to *peri*-effect, defined as the non-bonding interaction between substituents [77]. Therefore, C-9 of compound 3, which has two *peri* hydroxyl interactions, is more downfield than C-10 lacking *peri* substituents. The NMR spectral data of compound 3 was found in a good agreement with the NMR spectral data

reported in the literature for 1,8-dihydroxy-3-methylanthracene-9,10-dione, also known as chrysophanol [76] (**Table 7**). This compound was not yet reported from the genus *Laggera* and the species *L. tomentosa*. The NMR spectral data used to characterize compound 3 was compared with literature reported for chrysophanol with the whole results shown in **Table 7**.

Table 7: Comparison of ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}C-NMR (100 MHz, $CDCl_3$) spectral data of compound 3 and literature reported for chrysophanol.

Carbon position	^{13}C-NMR δ (ppm) of compound 3	DEPT δ (ppm)	^{13}C-NMR (100 MHz, acetone-d_6) data reported for chrysophanol [76]	^{1}H-NMR δ (ppm) data of compound 3	^{1}H-NMR δ (ppm) (400MHz, acetone-d_6) data reported for chrysophanol [76]	Nature of the carbon
1	162.4	-	162.4	-	-	C (quaternary carbon)
2	124.5	124.5	124.5	7.12 (br. *s*, 1H)	7.09 (*s*, 1H)	CH
3	149.3	-	149.3	-	-	C (quaternary carbon)
4	119.9	119.9	119.9	7.67 (br. *s*, 1H)	7.64 (*s*, 1H)	CH
5	136.9	136.9	136.9	7.83 (*dd*, *J* = 1.2, 7.6 Hz, 1H)	7.81 (*dd*, *J* = 2, 7.8 Hz, 1H)	CH
6	124.4	124.4	124.4	7.69 (*dd*, *J*=8.4, 7.6 Hz, 1H)	7.67 (*m*, 1H)	CH
7	121.4	121.4	121.4	7.30 (*dd*, *J* = 1.2, 8.4 Hz, 1H)	7.52 (*dd*, *J* = 2, 8.8 Hz, 1H)	CH
8	162.7	-	162.7	-	-	C (quaternary carbon)
9	192.5	-	192.5	-	-	C (quaternary carbon)
10	182.0	-	181.9	-	-	C (quaternary carbon)
11	133.6	-	133.6	-	-	C (quaternary carbon)
12	115.9	-	115.9	-	-	C (quaternary carbon)
13	113.7	-	113.7	-	-	C (quaternary carbon)
14	133.3	-	133.3	-	-	C (quaternary carbon)
15	22.3	22.3	22.3	2.48 (*s*, 3H)	2.47 (*s*, 3H)	CH_3

By comparing with literature information and molecular structures for related compounds, the following skeleton structure of the isolated compound 3 was proposed.

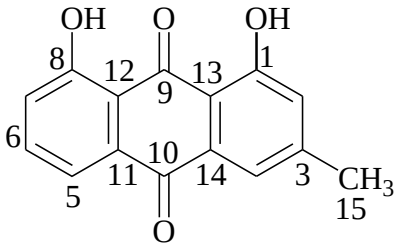


Figure 12: Proposed structure of compound 3

Chrysophanol is a known anthraquinone (phenolic compound) isolated from various organs and species of plants such as the leaves of *Aloe excelsa*, the flowers of *Senna didymobotrya*, the rhizomes of *Rheum emodi*, the stems of *Rhamnus prinoides*, the roots of *Rumex abyssinicus*, the rhizomes of *Rheum undulatum*, the seeds of *Cassia tora* Linn and so on. The chrysophanol shows many biological effects, including anti-microbial, anti-inflammatory, anti-mutagenic, anti-diabetic, inhibit the replication of poliovirus and active against HIV-1 protease (HIV-1 PR). Besides these activities, the component is also well known as potent photosensitizers [78]. The presence of chrysophanol in this plant is significant as it displays the biological activities listed above.

4.5.4. Characterization of compound 4

Compound 4 was obtained as orange amorphous powder (13 mg) melting at 255-257℃. It showed one spot on TLC at R_f. 0.55 with n-hexane:ethyl acetate (7:3) solvent system as a mobile phase (**Figure 13**). Compound 4 is soluble in acetone that showed a characteristic colour change from yellow to orange on TLC plate upon dipping in vanillin-H_2SO_4 followed by heating for a few minutes.

Figure 13: TLC profile of compound 4

The UV-Vis spectrum (ethanol) of compound 4 (**Appendix 17**) shows absorption bands at 250, 266, 289 and 436 nm indicating characteristics absorption chromophore center for anthraquinone [79]. The absorption peak at 250 nm is corresponding to π-π* transition of aromatic C=C bonds or the presence of aromatic conjugation, the peaks at 266 and 289 nm showed the presence of π-π* and n-π* electronic transitions of carbonyl group (C=O) of ketone, respectively. The broad absorption band at 436 nm shows the presence of conjugation in the molecule.

In the IR (KBr) spectrum of compound 4 (**Appendix 18**), the absorption band at 3382 cm^{-1} showed the O-H stretching that indicated the presence of a hydroxyl group (alcohol functional group). The absorption bands at 2925 and 2855 cm^{-1} are due to asymmetric and symmetric C-H stretching, respectively. Additionally, the compound shows absorption bands at 3087, 1548, 1482 and 877 cm^{-1} which indicated the presence of aromatic (C-H) stretching, aromatic C=C stretch (in-ring), aromatic C-C stretching (in-ring) and aromatic (C-H) bending, respectively. Moreover, the strong absorption band at 1631 cm^{-1}, the medium absorption bands at 1386, 1289 and 759 cm^{-1} showed the presence of the ketone C=O stretching, C-C stretching (out of the ring), C-O stretching and C-H stretching for ortho substitution, respectively. Therefore, the IR

spectrum depicts the presence of hydroxyl and ketone functionality groups, an aromatic and a methyl groups in the compound isolated.

The ^{1}H-NMR spectrum of compound 4 (**Appendix 19**) showed the presence of two sets of meta coupled aromatic hydrogens (two meta coupled doublet at δ 6.65 (J = 2.4 Hz, H-7) and δ 7.23 (J = 2.4 Hz, H-5) and two multiplet signals at δ 7.12 (H-2) and δ 7.54 (H-4) , all integrating for one proton), two chelated hydroxyl protons at δ 12.05 and δ 12.17 and one methyl group protons (singlet) as a substituent to the aromatic ring at δ 2.46, integrating for three protons.

The ^{13}C-NMR spectrum (**Appendix 20**) in $(CD_3)_2CO$ of compound 4, analysed with the aid of DEPT-135 (**Appendix 21**) in $(CD_3)_2CO$ showed a well resolved resonance of 15 carbon atoms. DEPT-135 indicated the presence of ten quaternary carbons (C), which were attributable to three oxygenated aromatic carbons; one methyl substituted aromatic carbon, four aromatic carbons and two carbonyl (ketone) carbons. The spectral data showed four methine (CH), all of them were attributable to sp^2 methines. In addition, the spectral data indicated the presence of one methyl (CH_3) carbon that was attached to the aromatic carbon and the absence of any methylene (CH_2) carbons. This structural type was further supported by the ^{13}C-NMR spectrum which contained three oxygenated carbons (at δ_c 162.3 (C-1), 165.5 (C-6) and 165.3 (C-8)), one methyl substituted carbon at δ 148.6 (C-3), four sp^2 methines (at δ_c 124.0 (C-2), 120.5 (C-4), 108.8 (C-5) and 107.9 (C-7)), two carbonyl carbons at δ_c 190.7 (C-9) and 181.2 (C-10), four quaternary aromatic carbons at δ_c 135.6 (C-11), 109.5 (C-12), 113.5 (C-13) and 133.3 (C-14) and one methyl carbon at δ_c 21.0 (C-15). The chemical shift difference between the two carbonyl carbons (C-9 and C-10) is due to *peri*-effect [77]. Hence, C-9 of compound 4, which has two *peri* hydroxyl interactions, is more downfield than C-10 which has no *peri* substituents. The NMR spectral data of compound 4 was found in agreement with the NMR spectral data reported in the literature for 1,6,8-trihydroxy-7-methylanthracene-9,10-dione, also known as emodin [79] (**Table 8**). This compound was not yet reported from the genus *Laggera* and the species *L. tomentosa*. The NMR spectral data of compound 4 was compared with literature reported for emodin with the whole results shown in **Table 8**.

Table 8: Comparison of ^{1}H-NMR (400 MHz, acetone-d_6) and ^{13}C-NMR (100 MHz, acetone-d_6) spectral data of compound 4 and literature reported for emodin.

Carbon position	^{13}C-NMR δ (ppm) of compound 4	DEPT δ (ppm)	^{13}C-NMR (100 MHz, acetone-d_6) data reported for emodin [79]	^{1}H-NMR δ (ppm) data of compound 4	^{1}H-NMR (400MHz, acetone-d6) data reported for emodin [79]	Nature of the carbon
1	162.3	-	162.4	-	-	C (quaternary carbon)
2	124.0	124.0	124.1	7.12 (*m*, 1H)	7.15 (br. *s*, 1H)	CH
3	148.6	-	148.7	-	-	C (quaternary carbon)
4	120.5	120.5	120.6	7.54 (*m*, 1H)	7.57 (br. *s*, 1H)	CH
5	108.8	108.8	108.8	7.23 (*d*, *J* = 2.4 Hz, 1H)	7.26 (*d*, *J* = 2.42 Hz, 1H)	CH
6	165.5	-	165.5	-	-	C (quaternary carbon)
7	107.9	107.9	107.9	6.65 (*d*, *J* = 2.4 Hz, 1H)	6.66 (*d*, *J* = 2.42 Hz, 1H)	CH
8	165.3	-	165.4	-	-	C (quaternary carbon)
9	190.7	-	190.9	-	-	C (quaternary carbon)
10	181.2	-	181.3	-	-	C (quaternary carbon)
11	135.6	-	135.4	-	-	C (quaternary carbon)
12	109.5	-	109.6	-	-	C (quaternary carbon)
13	113.5	-	113.6	-	-	C (quaternary carbon)
14	133.3	-	133.4	-	-	C (quaternary carbon)
15	21.1	21.1	21.2	2.46 (*s*, 3H)	2.48 (*s*, 3H)	CH_3

By comparing with literature information and molecular structures for related compounds, the following skeleton structure of the isolated compound 4 was proposed.

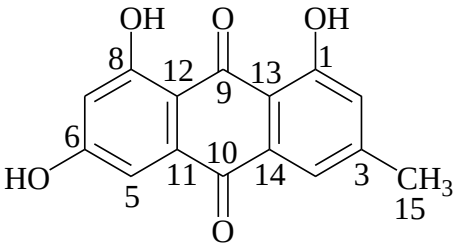


Figure 14: Proposed structure of compound 4

Emodin is a known anthraquinone (phenolic compound) isolated from various organs and species of plants such as the pods of *Senna occidentalis*, the root and rhizome of *Rheum palmatum* L., the root of *Rumex abyssinicus*, the leaves of *Murrya koenigii* and so on. Emodin is reported to have antimicrobial, antiviral, anti-inflamatory, anti-ulcerogenic, immunosuppressive, and chemo-preventive activities. Emodin has also been reported to exert anti-proliferative effects in many cancer cell lines; HER-2/neu–overexpressing breast or lung cancer, leukemic HL-60, human hepatocellular carcinoma, human cervical cancer and prostate cancer cell lines through the activation of caspase-3 and up-regulation of TP-53 and p21. Moreover, emodin inhibits the kinase activity of p56lck, HER2/neu, and casein kinase II [80]. The presence of emodin in this plant is significant as it displays the biological activities listed above.

4.6. Antioxidant Activities

DPPH radical scavenging assay was performed to determine the antioxidant activity of the extracts of the roots of *L. tomentosa*. The percentage radical scavenging activity of the extracts was calculated by using the formula as mentioned in **Section 3.9**. DPPH, a stable free radical, when acted upon by an antioxidant, is converted into diphenyl-picryl hydrazine with a colour change from purple to yellow (**Figure 15)**. This can be quantified spectrophotometrically to indicate the extent of DPPH scavenging activity by the plant extracts. This test provides information on the ability of a compound to donate a hydrogen atom, on the number of electrons a given molecule can donate and on the mechanism of antioxidant action. Lower absorbance

followed by the degree of decoloration of the reaction mixture from purple to yellow indicated the higher free radical scavenging efficiency of the substances.

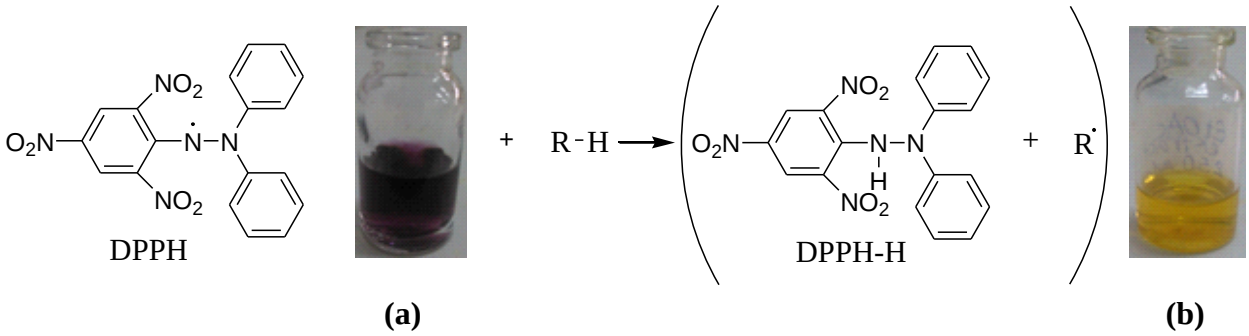


Figure 15: Principle of DPPH free radical scavenging mechanism and color transformation from purple (a) to yellow (b) on abstraction of hydrogen in the antioxidant reaction.

The values obtained from the evaluation of the antioxidant activities of the roots extracts of *L. tomentosa* and compound 2 at 100 μg/mL are tabulated in **Table 9**.

Table 9: DPPH radical scavenging activities of the extracts of the roots of *L. tomentosa*, compound 2 and standard (ascorbic acid)

Extract/control	Concentration (μg/mL)	Absorbance	% of inhibition
n-hexane extract (oil)	100	0.414	55.8
EtOAc extract	100	0.147	84.3
MeOH extract	100	0.258	72.5
compound 2	100	0.583	37.8
Ascorbic acid	100	0.028	97.0
Control (DPPH)	-	0.938	-

As shown in **Table 9**, the n-hexane extract of the roots of *L. tomentosa* at 100 μg/mL was found to decrease the free radical compared to the standard DPPH. It decreased by 0.558 and thus the percentage inhibition is calculated as follows:

$$\% \text{ inhibition} = \frac{0.938-0.414}{0.938} \text{ X } 100$$

$$= 55.8\%$$

Similarly, the percentage inhibitions of EtOAc, MeOH and compound 2 are 84.3, 72.5 and 37.8%, respectively. Therefore, the antioxidant results showed that, a highest of 84.3% free radical inhibition was achieved with EtOAc extract when compared with ascorbic acid standard (97.0%). MeOH extract also showed high antioxidant activity (72.5%) but n-hexane extracted oil and the compound 2 showed moderate antioxidant activities (55.8 and 37.8%, respectively). The obtained results could be attributed to the presence of steroids, phenolic compounds (flavonoids, anthraquinones and phenolic acids) and other bioactive compounds with free hydroxyl groups. Therefore, the results showed that the plant containing these bioactive compounds with free hydroxyl groups in the extracts has medicinal value and that it may be utilized for antioxidant properties.

4.7. Antibacterial Activities

The oil, crude extracts and isolated pure compounds were assayed *in vitro* against bacteria using disc diffusion method. Disc diffusion is selected due to its simplicity, capacity to analyze large number of samples and its ability to work well with defined inhibitors. The results (**Table 10**) showed that all of the test samples clearly indicate that a considerable antibacterial activity against the bacterial species used in the study. The commercial standard drug (chloramphenicol) showed the greatest inhibition effect against all bacteria rather than the tested samples, the control (DMSO) has no inhibition zone and was not observed any effect to the test solutions. As a whole, ethyl acetate crude extract was found to be the most active extract for the tested bacteria. However, in the case of isolated pure compounds, compound 4 was found to be the most active followed by compound 3. In general as concentration of sample increases the mean inhibition zone value increases. This might be due to the high concentration of antibacterial compounds.

The methanol and n-hexane extracts were significantly active against *S. aureus* and *E. coli* at a concentration of 1.0 mg/mL with zone of inhibition ranging from 11-13. Ethyl acetate extract in particular had the highest inhibition diameter of 19 mm on *S. aureus* and 12 mm on *E. coli*. The explanation of this activity could be due to high concentration of antibacterial compounds in these extracts. At 0.5 mg/mL concentration ethyl acetate and methanol crude extracts demonstrated moderate antibacterial effects (7-10 mm) against all the tested bacteria used in the

study. This might be due to low concentration of antibacterial compounds in the samples. While the n-hexane extract (oil) was active only against *K. pneumonia* (7 mm zone on inhibition). *P. mirabilis* was resistant to n-hexane extract at both concentrations. Differences in polarity among the various solvents are perhaps responsible for the differences in solubility of plant active principles, hence variation in degree of activity. The activity of ethyl acetate and methanol extract (at 1 mg/mL concentration) against the Gram negative [*K. pneumoniae* (10 mm, 9 mm), *E. coli* (12 mm, 12 mm) and *P. mirabilis* (11 mm, 9 mm)] respectively is impressive because Gram-negative bacteria tend to have higher intrinsic resistance to most antimicrobial agents [81].

All the isolated pure compounds showed nearly similar activities (ranged from 7 mm to 10 mm) against *S. aureus*, *K. pneumonia* and *E. coli* at 0.5 mg/mL concentration. At the concentration of 1 mg/mL, compounds compound 1, compound 2 and compound 3 were found to show zone inhibition diameters of (10 mm, 8 mm and 11 mm), (12 mm, 11 mm and 10 mm) and (13 mm, 11 mm and 9 mm) against *S. aureus*, *K. pneumonia* and *E. coli*, respectively. On the other hand, compound 4 (emodin) showed promising antibacterial activity against *S. aureus* (zone of inhibition 18 mm) compared to positive control (chloramphenicol, 21 mm zone of inhibition), medium inhibition (12 mm) against *K. pneumonia* but slight inhibition (8 mm) against *E. coli*. However, there is no inhibition effect on *P. mirabilis* for compound 1, compound 3 and compound 4 at both concentrations but for compound 2, it had almost similar effects with inhibition zones (8 mm, 10 mm) at 0.5 and 1 mg/mL concentrations, respectively.

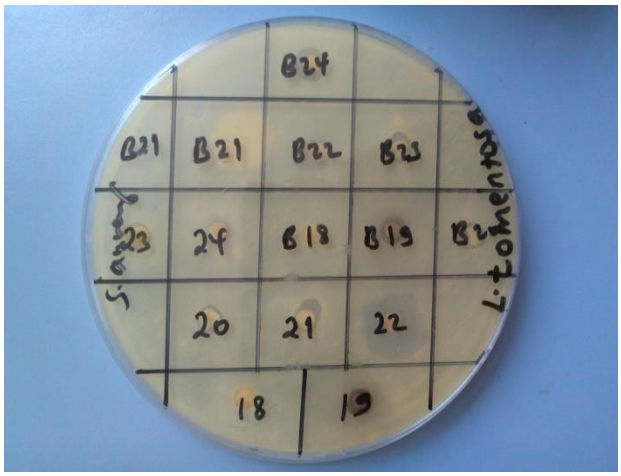
In general, the oil, crude extracts and all isolated pure compounds exhibited the significant activities against the three bacteria (*S. aureus*, *K. pneumonia* and *E. coli*). This is the fact that the compounds and extracts make a suitable candidate for investigation in control of antibiotics on these three tested bacteria. Therefore, plants of the genus *Laggera* may prove to be a rich source of compounds with potential antimicrobial activities.

Table 10: Zone of bacterial growth inhibition (mm) for oil, crude extracts, isolated pure compounds, antibiotics and solvent

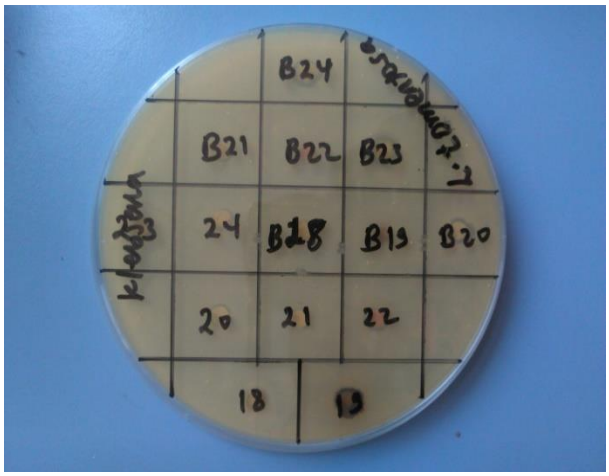
Extract/control	Concentration (mg/mL)	Diameter zones of inhibition (mm)			
		S. aureus	*K. pneumoniae*	*E. coli*	*P. mirabilis*
n-hexane extract (oil)	0.5	-	7	-	-
	1.0	12	10	11	-
Ethyl acetate extract	0.5	9	7	10	8
	1.0	19	10	12	11
Methanol extract	0.5	8	8	9	8
	1.0	13	9	12	9
compound 1	0.5	8	7	8	-
	1.0	10	8	11	-
compound 2	0.5	8	10	9	8
	1.0	12	11	10	10
compound 3	0.5	7	8	7	-
	1.0	13	11	9	-
compound 4	0.5	8	10	7	-
	1.0	18	12	8	-
Chloramphenicol	1.0	21	19	20	19
Control (DMSO)		-	-	-	-

-: stands for no inhibition

To the best of our knowledge, there is no any report on antibacterial activities of the roots extracts of *L.tomentosa*. 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. *In vitro* disc diffusion method depicted that *β*-stigmasterol obtained from *Sida rhombifolia* grown in Ethiopia has potent antibacterial activities against *S. aureus* (12.5 mm), *E. coli* (14 mm), *P. aeruginosa* (9.5 mm) and *S. typhimurium* (13 mm) which was assessed at the concentration of 50 mg/L [73]. Lauric acid as monoglyceride is known to the pharmaceutical industry for its good antimicrobial properties [72]. It is reported that chrysophanol was active against *B. subtilis*, *S. epidermidis* and *E. coli* [76]. Emodin is also reported to have antimicrobial activities [80].

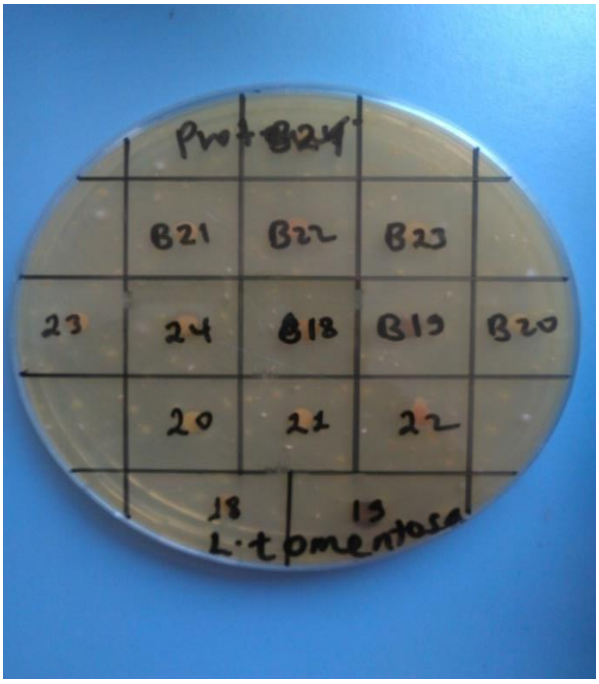


i) Zone of inhibition – bacteria (*S. aureus*)



ii) Zone of inhibition – bacteria (*K. pneumoniae*)

iii) Zone of inhibition – bacteria (*E. coli*)



iv) Zone of inhibition – bacteria (*P. mirabilis*)

Figure 16: Antibacterial activity of the roots of *L. tomentosa*.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The set objectives of the study were explored and from the obtained results the following conclusions can be drawn:

The dried and powdered roots of *L. tomentosa* were successively extracted with organic solvents such as: n-hexane, EtOAc and MeOH, respectively. The TLC analysis of the crude extracts indicated that the compounds isolated are not the only compounds present in the plant material. Previously there was no report on phytochemical investigation in the roots of *L. tomentosa*. Phytochemical analyses of *L. tomentosa* revealed the presence of alkaloids, flavonoids, steroids, cardiac glycoside, terpenoids and phenols in all the root extracts and these compounds contribute for their biological activities that were discussed in **Section 2.1**. The components of n-hexane extracted oil of the roots of the plant were analyzed by GC-MS for the first time and found to have eleven components with 2,5-dimethoxy-*p*-cymene identified as a major compound (59.39%). The presence of 2,5-dimethoxy-p-cymene as a major constituent substantiate the use of this plant against bacteria and fungus. The EtOAc crude extract of the roots of *L. tomentosa* after silica gel column chromatography yielded four compounds identified using spectroscopic methods as lauric acid, β-stigmasterol, chrysophanol and emodin. All of them were isolated for the first time from the roots of this plant and the genus *Laggera*. The chemical structures were characterized on the basis of UV/Vis, IR, 1D-NMR spectral analysis (^{1}H-NMR and ^{13}C-NMR) and DEPT-135. The n-hexane, EtOAc and MeOH extracts were found to inhibit the DPPH radical by 55.8, 84.3 and 72.5%, respectively. The result obtained for the EtOAc was found close to ascorbic acid indicating strong ability of the extract to act as radical scavenger. The oil, crude extracts and isolated pure compounds of the roots of the plant were active against the tested bacteria (*S. aureus*, *K. pneumonia* and *E. coli*), which support the traditional use of this plant against bacteria. However, the oil, compound 1, compound 3 and compound 4 did not show any inhibition effect against *Proteus mirabilis*. For all samples, inhibition effect increases with concentration from 0.5 to 1 mg/mL compared with the positive control. All these results may have thus justified the use of this plant for medicinal purposes.

5.2. Recommendations

From the outcomes of the study the following recommendations for future work can be made:

- As evidenced from TLC analysis, the compounds isolated are not the only compounds present in the plant material. Therefore, further isolation and characterization work should be done to fully characterize more chemical constituents of the plant root extracts to identify all active compounds and determine their antibacterial and antioxidant activities.

- There is need to extend phytochemical investigations of this plant to methanol and other organic solvents like petroleum ether, chloroform and acetone extracts and all fractions that were not analyzed in this study.

- The antimicrobial screening was conducted on limited bacterial strains. So, the same work should be carried out on large variety of microbial strains so as to have a clear picture of the spectrum of antimicrobial activities of the herb.

- It is recommended that further *in vivo* anti-bacterial efficacy tests with the bacteria used in mice or other animals so as to validate the *in vitro* results. Future research should be extended to cover other biological activities such as: antifungal, anti-cancer, antiviral, antimalarial and anti-plasmodial for all the extracts and other organic solvent extracts of the roots of *L. tomentosa*.

- It is also recommended that further similar studies should be conducted on other parts of the plant such as stem and bark.

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7. APPENDICES

Appendix 1: GC-MS chromatogram of n-hexane extract (oil) of the roots of *L. tomentosa* (LTH).

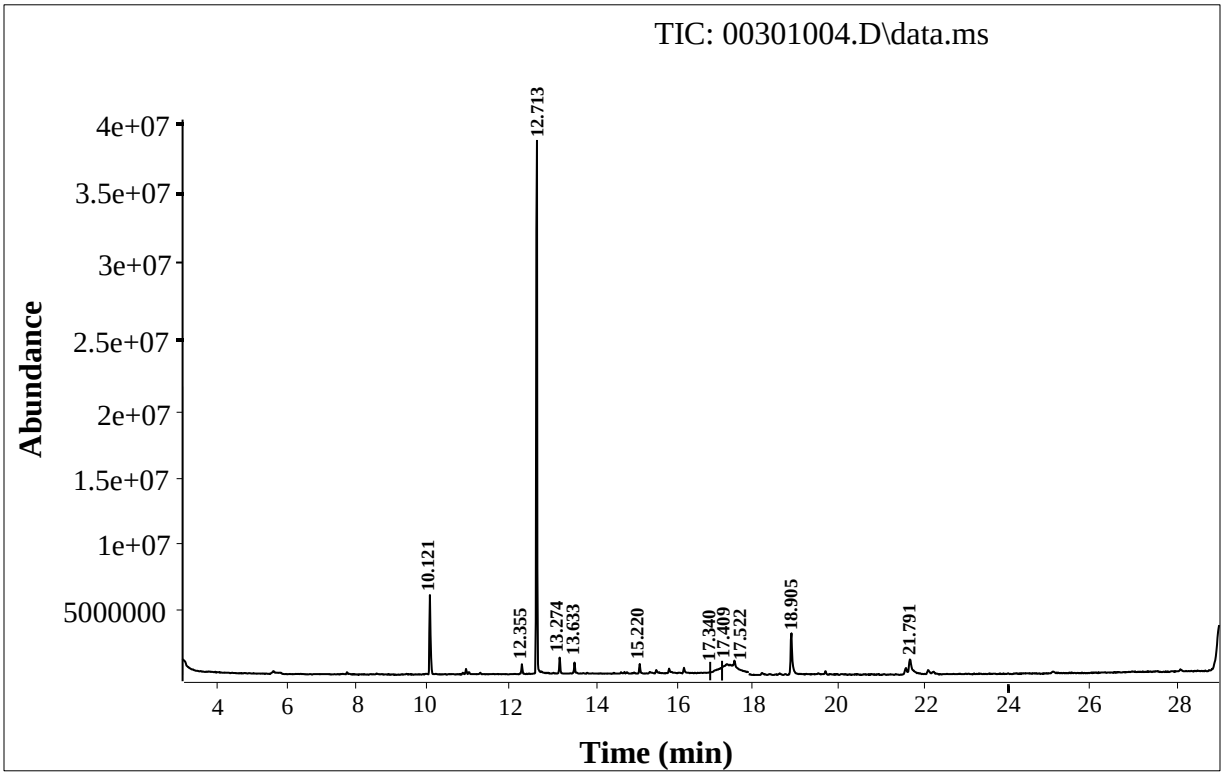
File: D:\MassHunter\GCMS\1\5977\May 16 2017\00301004.D

Operator: Estif

Acquired: 16 May 2017 17:34 using Acq.Method Fatty acids 29.33 minsplitless.M

Instrument: GC-MS

Sample Name: LTH



Appendix 2: UV/Vis. spectrum of compound 1

Addis Ababa University

Department of Chemistry

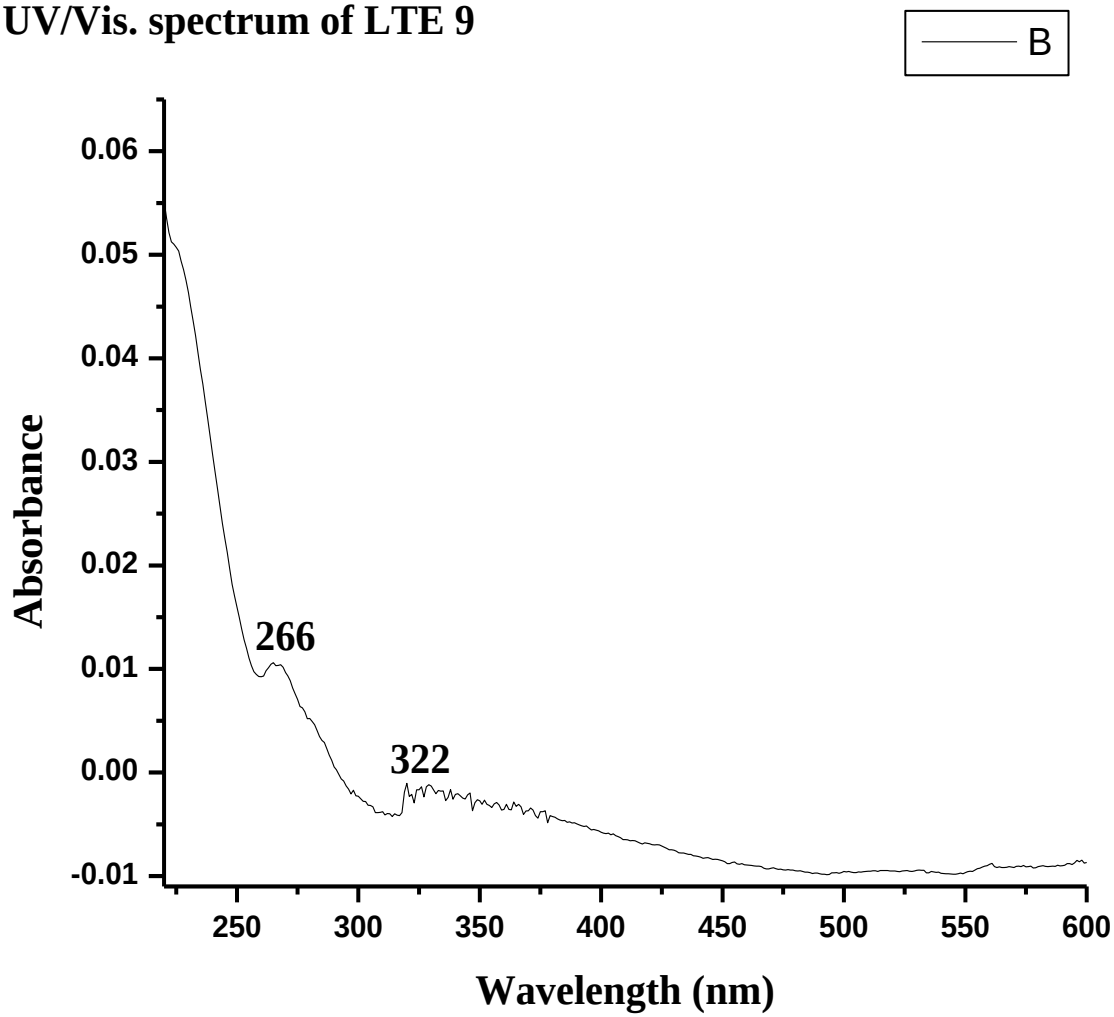
UV-Visible Spectrophotometer

Laboratory

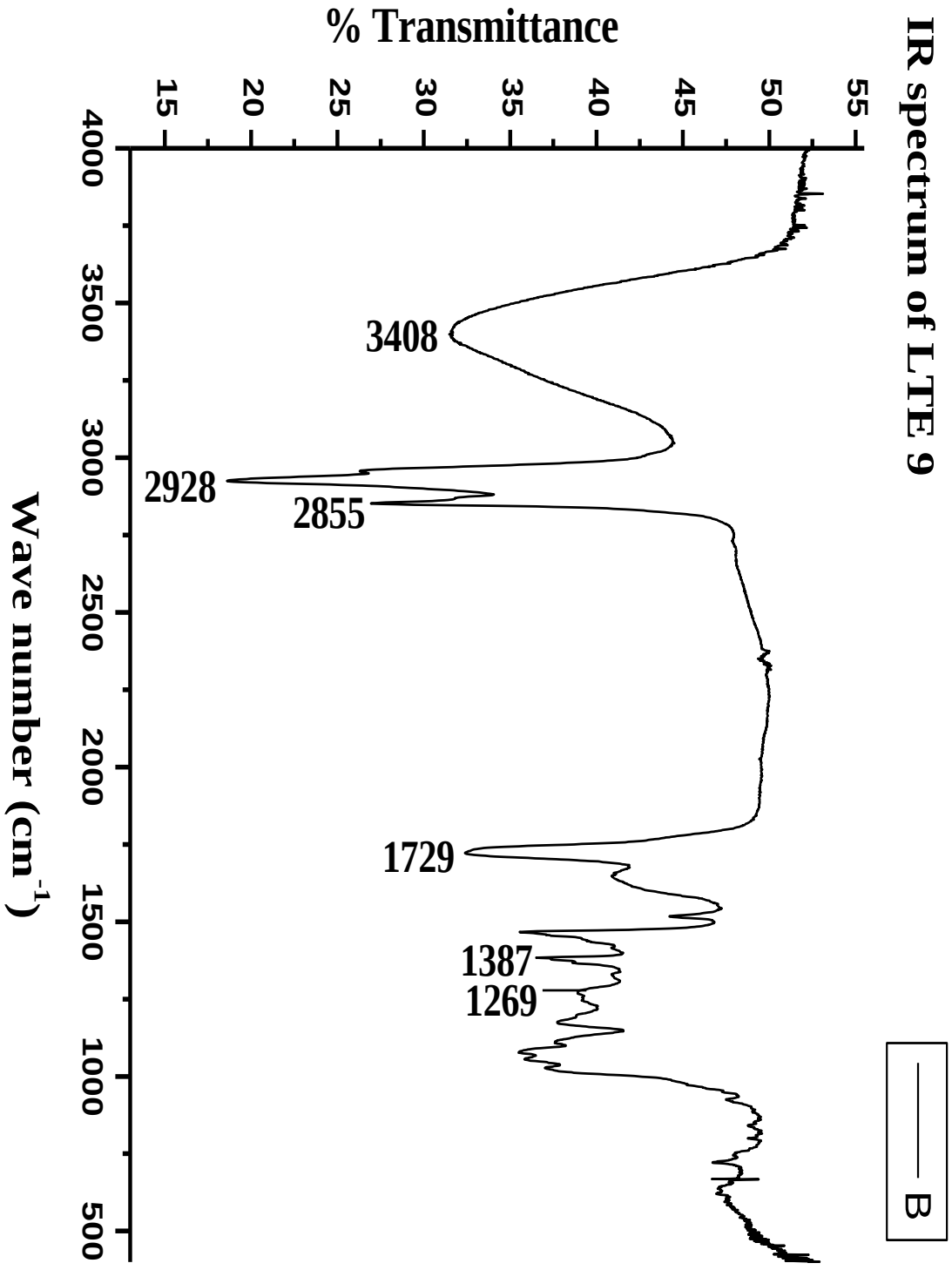
Instrument: T60 UV-Visible spectrophotometer PG instrument

Date: June 03, 2017

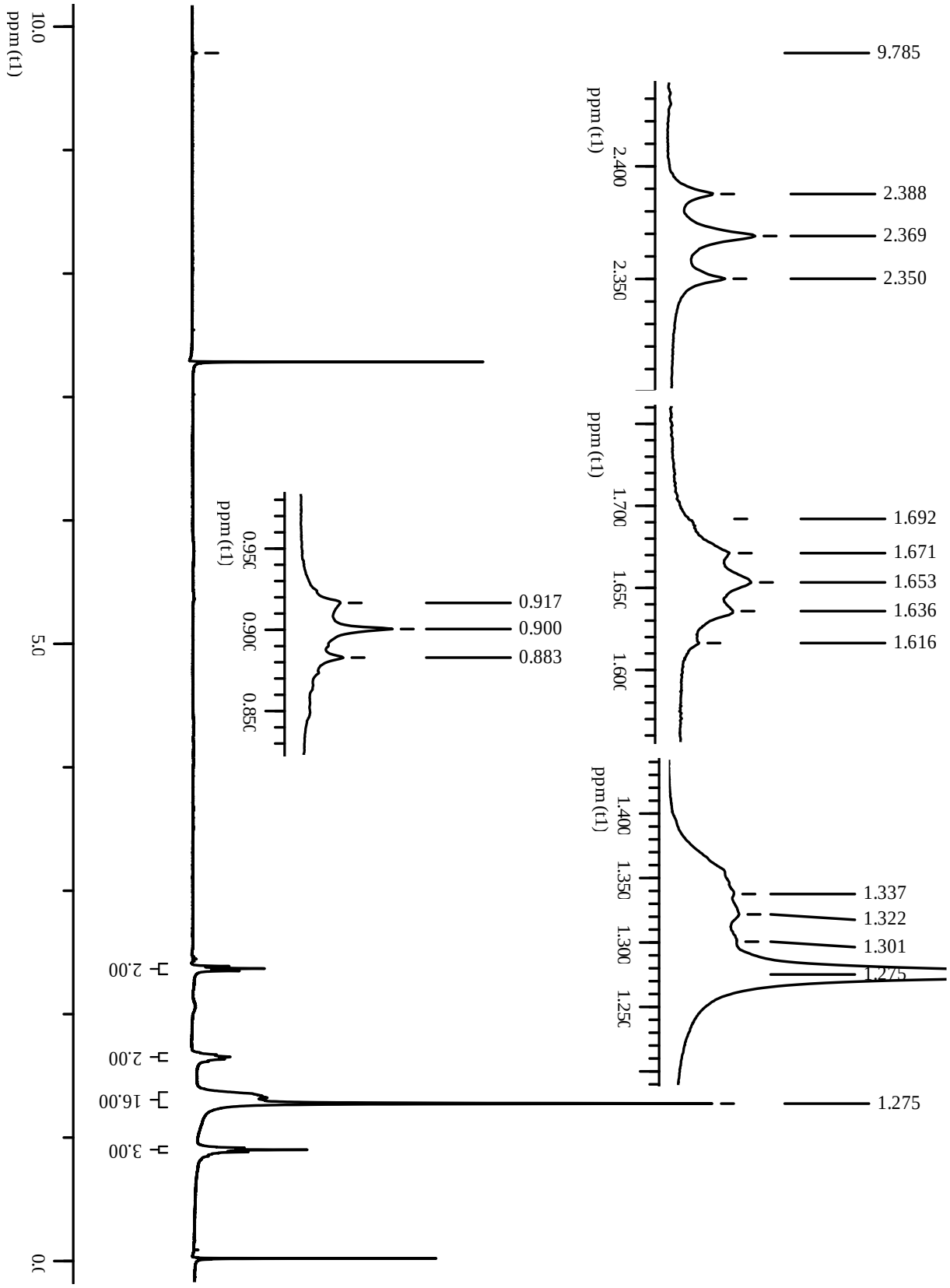
Sample Code: Compound 1 (LTE 9)



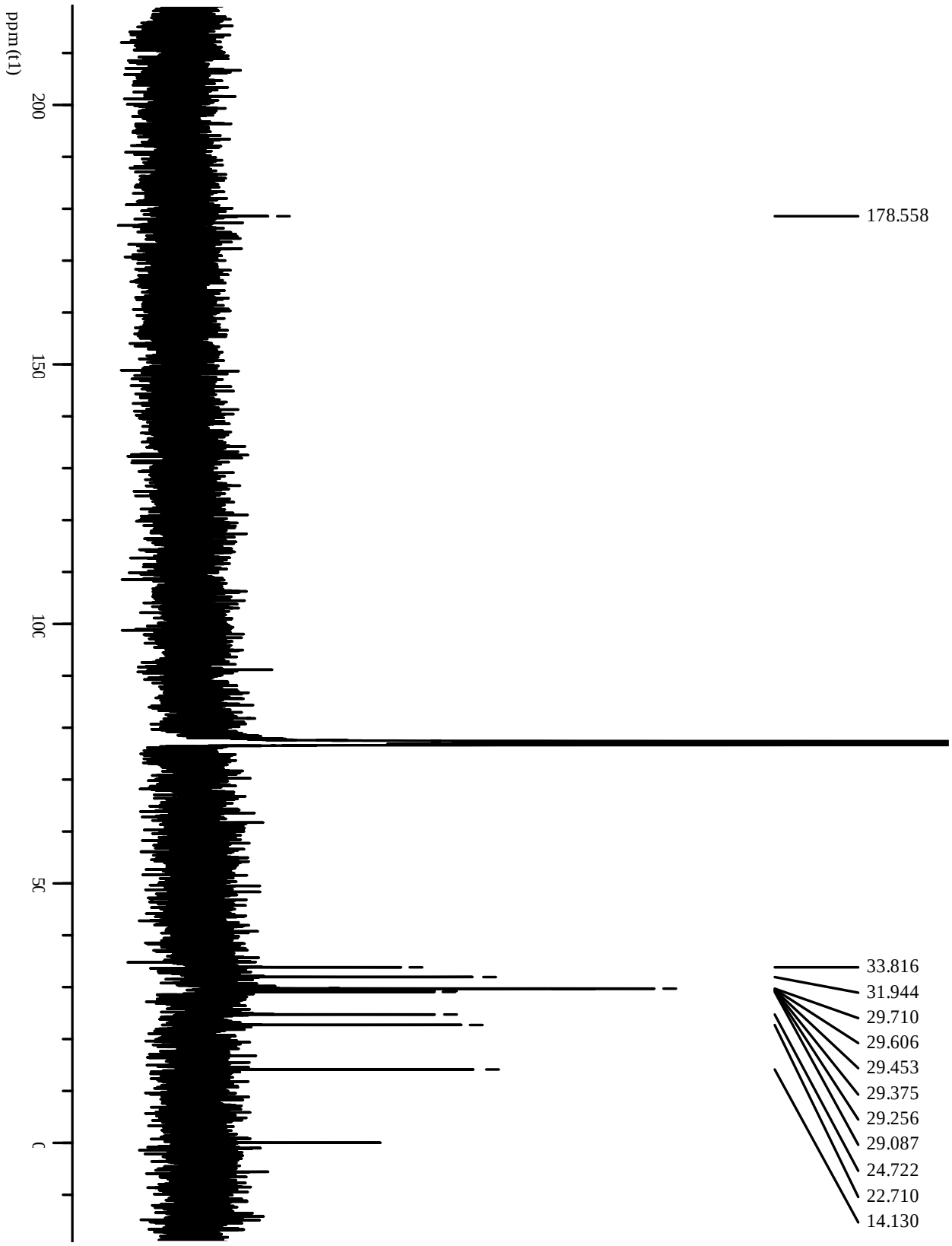
Appendix 3: IR spectrum of compound 1



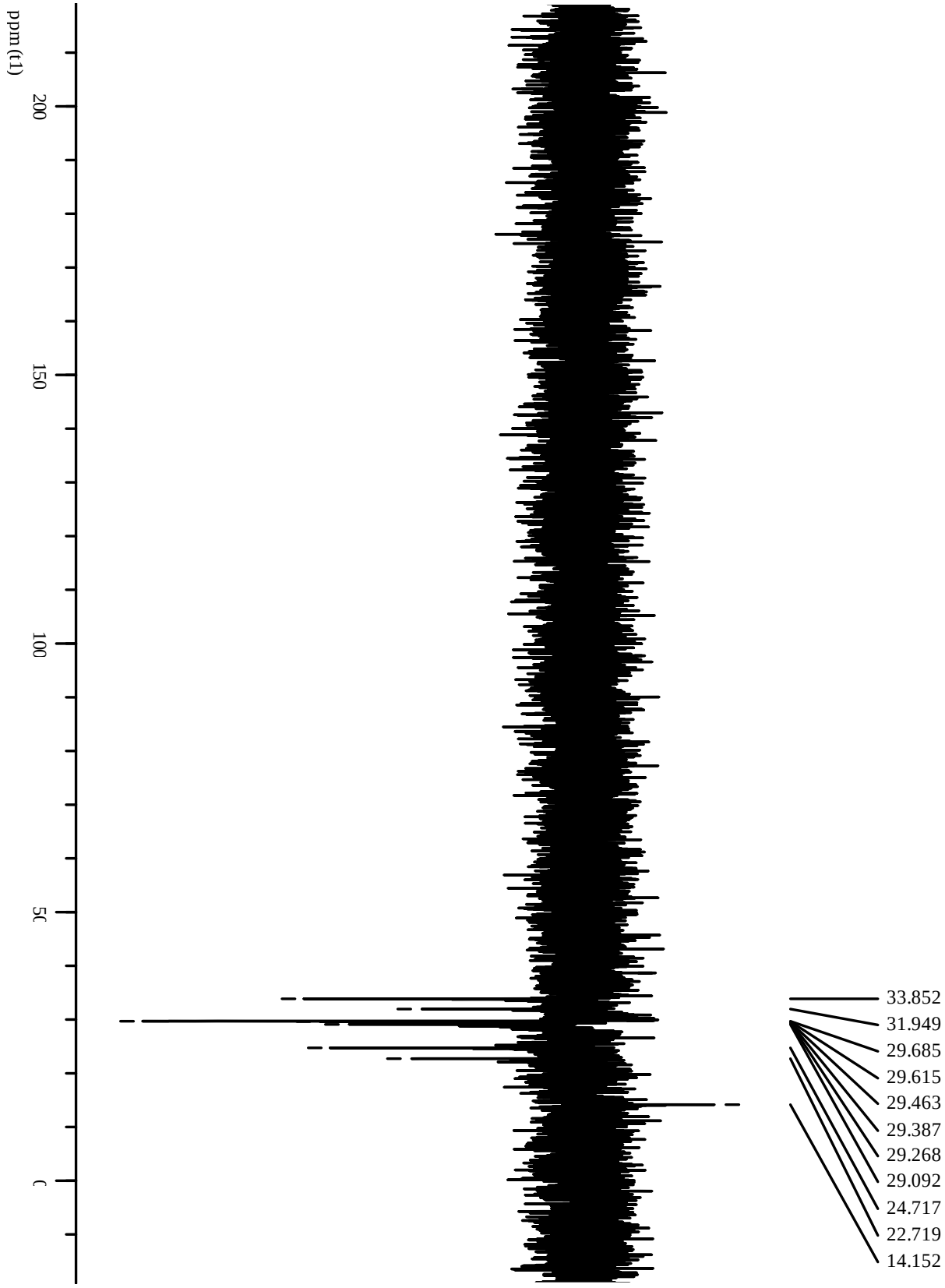
Appendix 4: ^{1}H-NMR spectrum of compound 1 in $CDCl_3$



Appendix 5: ^{13}C-NMR spectrum of compound 1 in $CDCl_3$



Appendix 6: DEPT-135 spectrum of compound 1 in $CDCl_3$



Appendix 7: UV/Vis. spectrum of compound 2

Addis Ababa University

Department of Chemistry

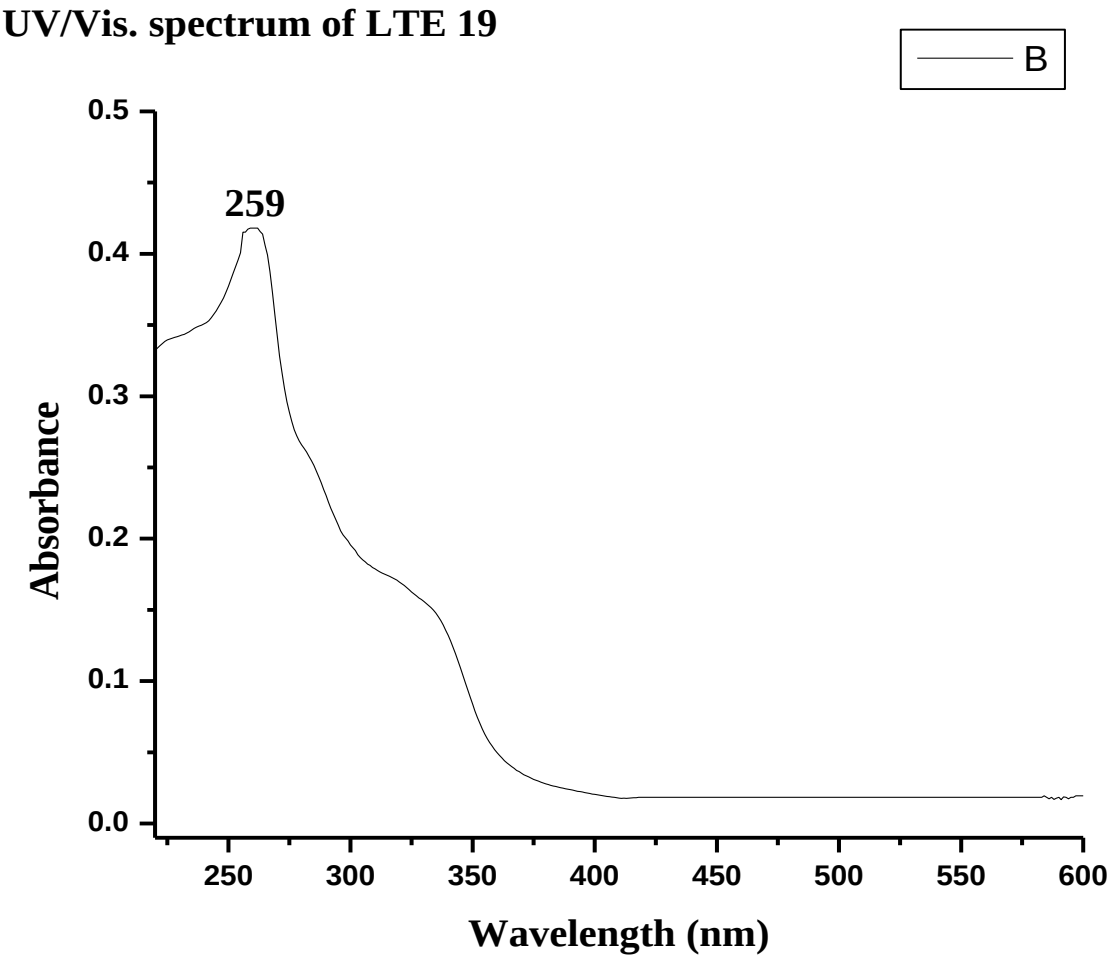
UV-Visible Spectrophotometer

Laboratory

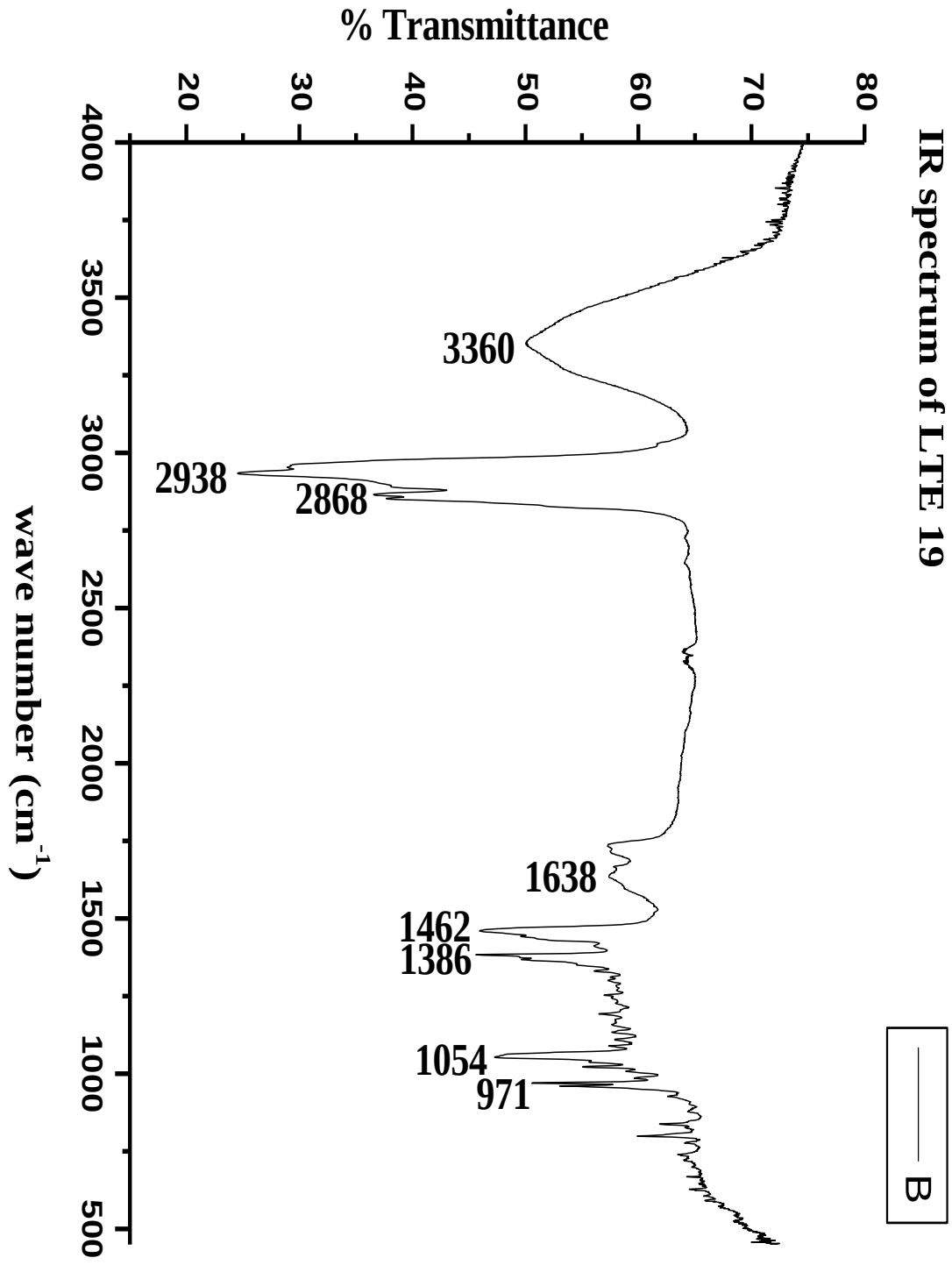
Instrument: T60 UV-Visible spectrophotometer PG instrument

Date: May 26, 2017

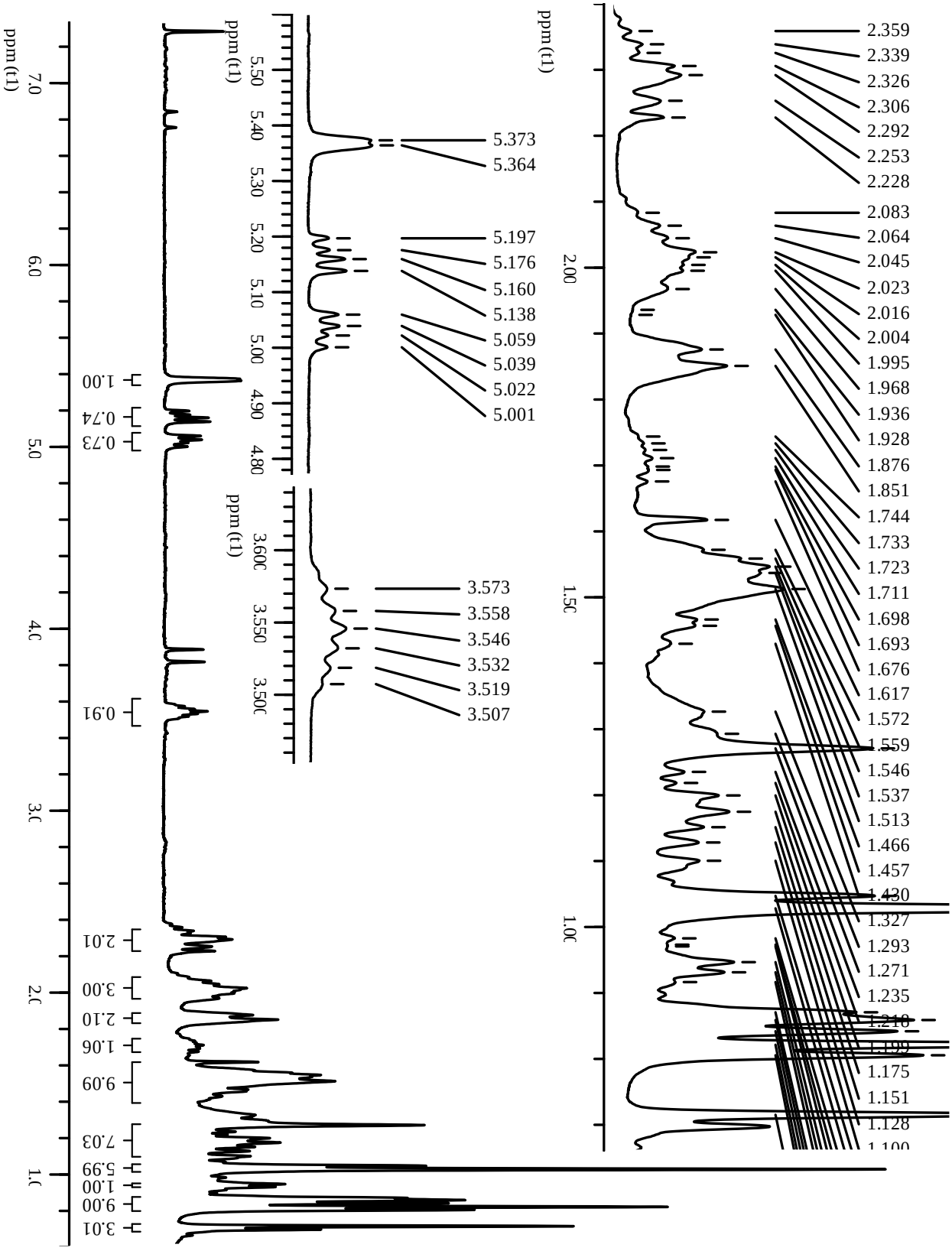
Sample Code: Compound 2 (LTE 19)



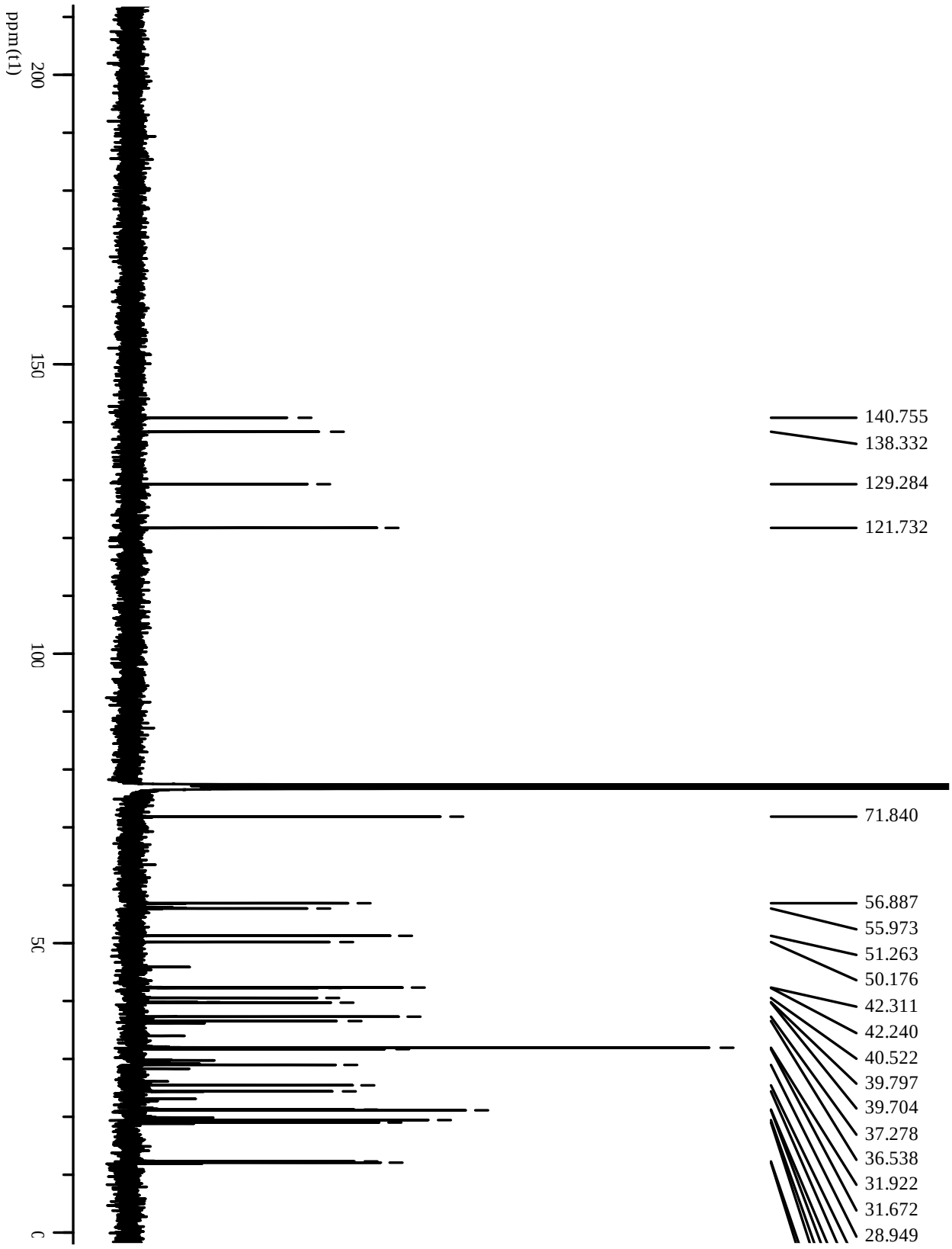
Appendix 8: IR spectrum of compound 2



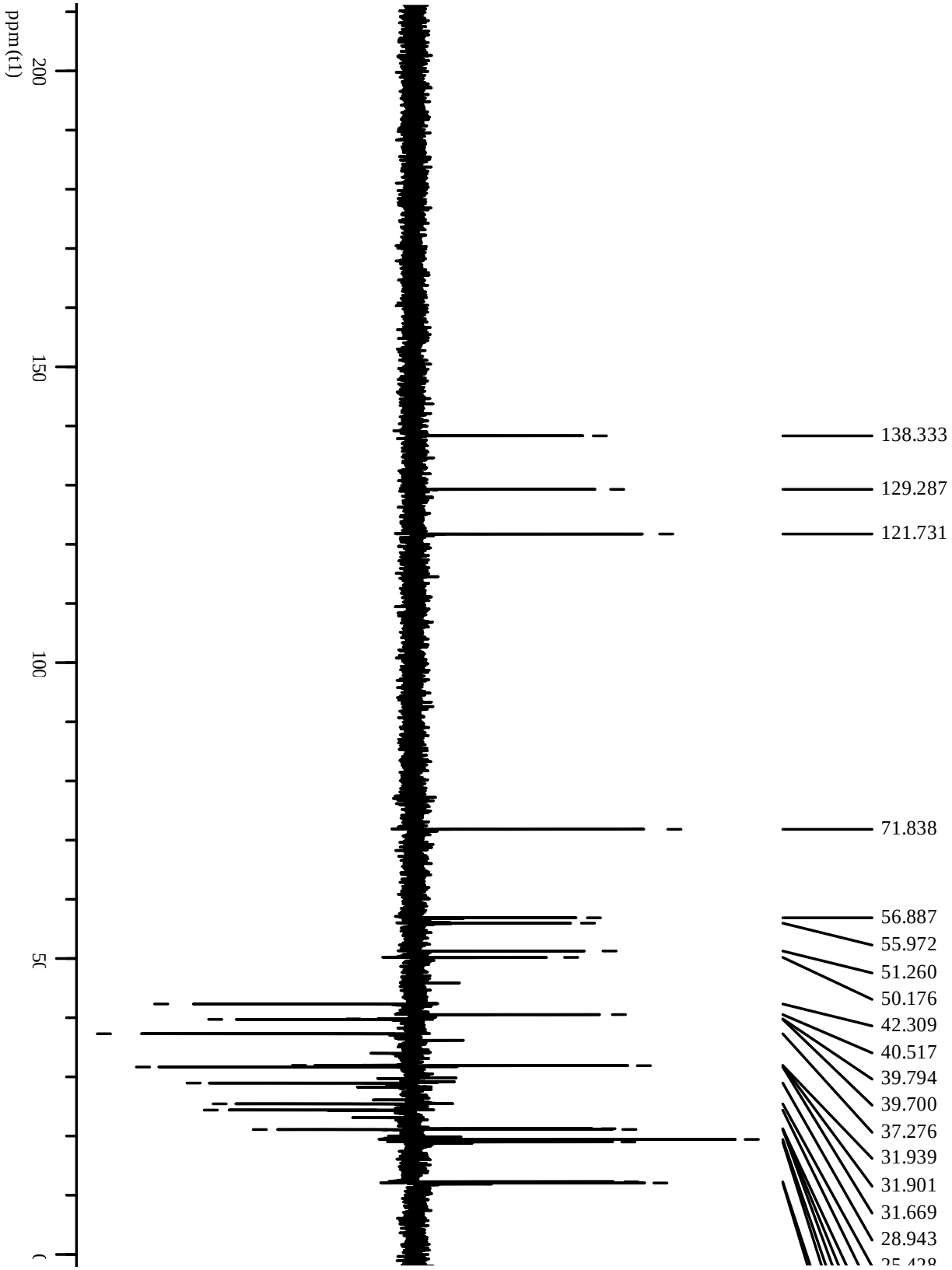
Appendix 9: ^{1}H-NMR spectrum of compound 2 in $CDCl_3$



Appendix 10: ^{13}C-NMR spectrum of compound 2 in $CDCl_3$



Appendix 11: DEPT-135 spectrum of compound 2 in $CDCl_3$



Appendix 12: UV/Vis. spectrum of compound 3

Addis Ababa University

Department of Chemistry

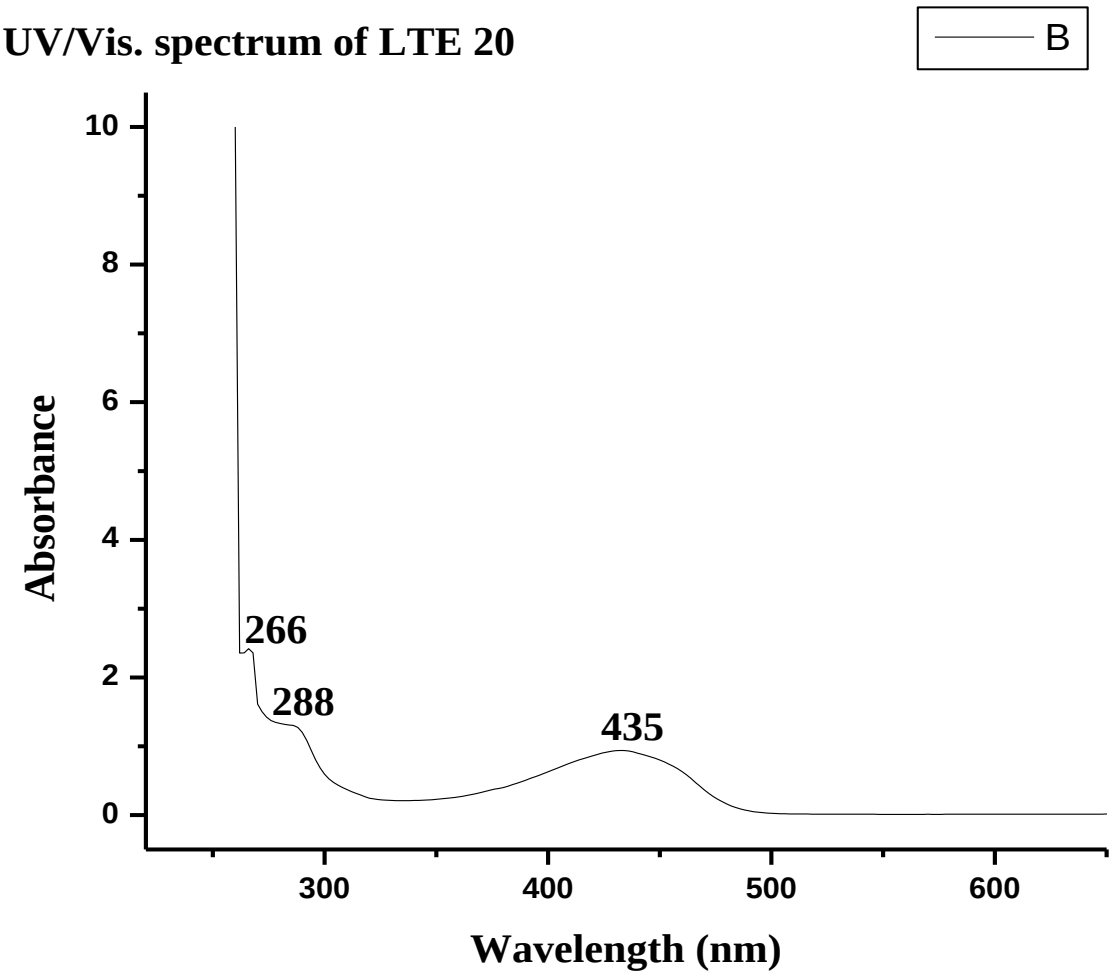
UV-Visible Spectrophotometer

Laboratory

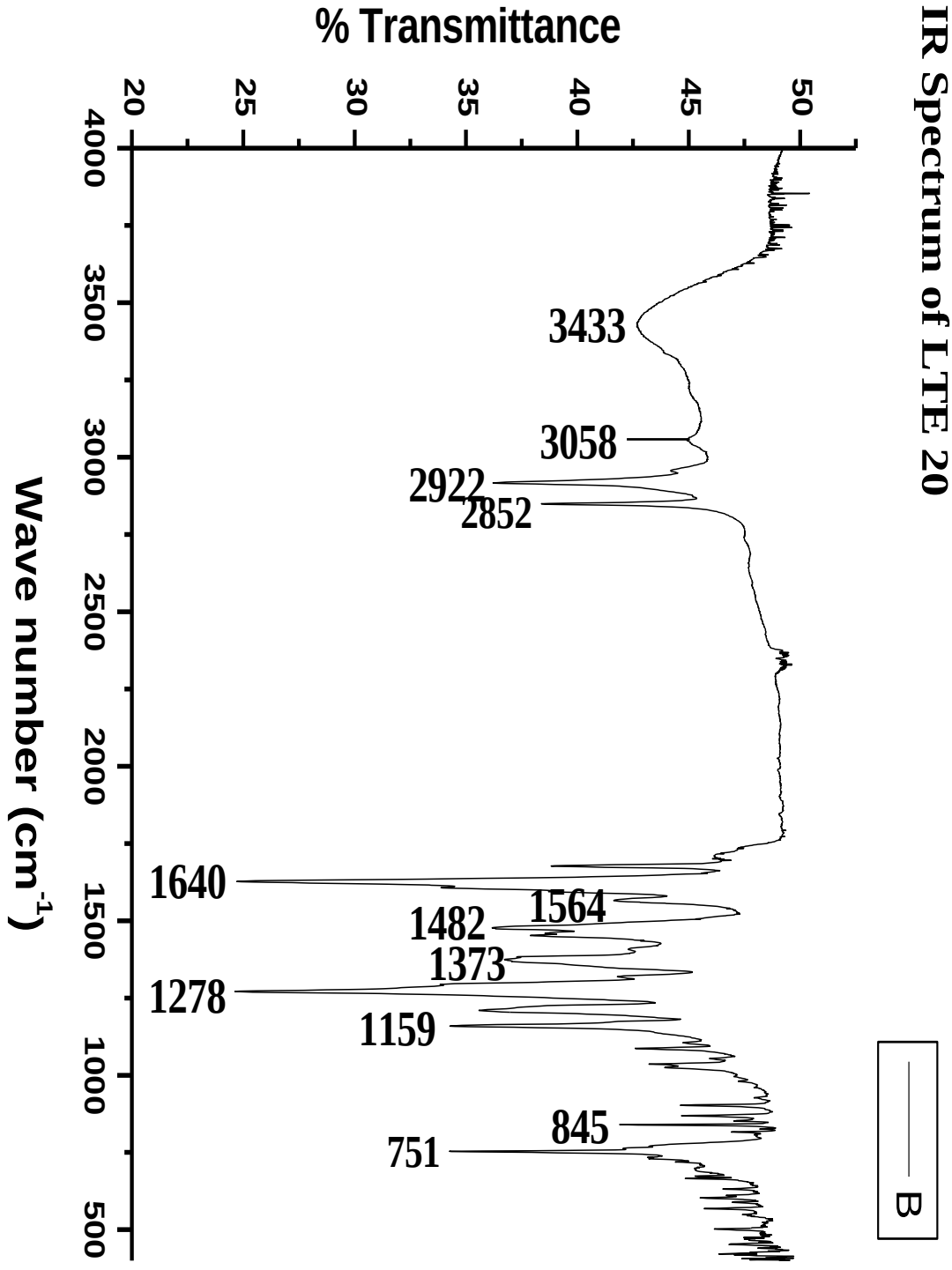
Instrument: T60 UV-Visible spectrophotometer PG instrument

Date: August 08, 2017

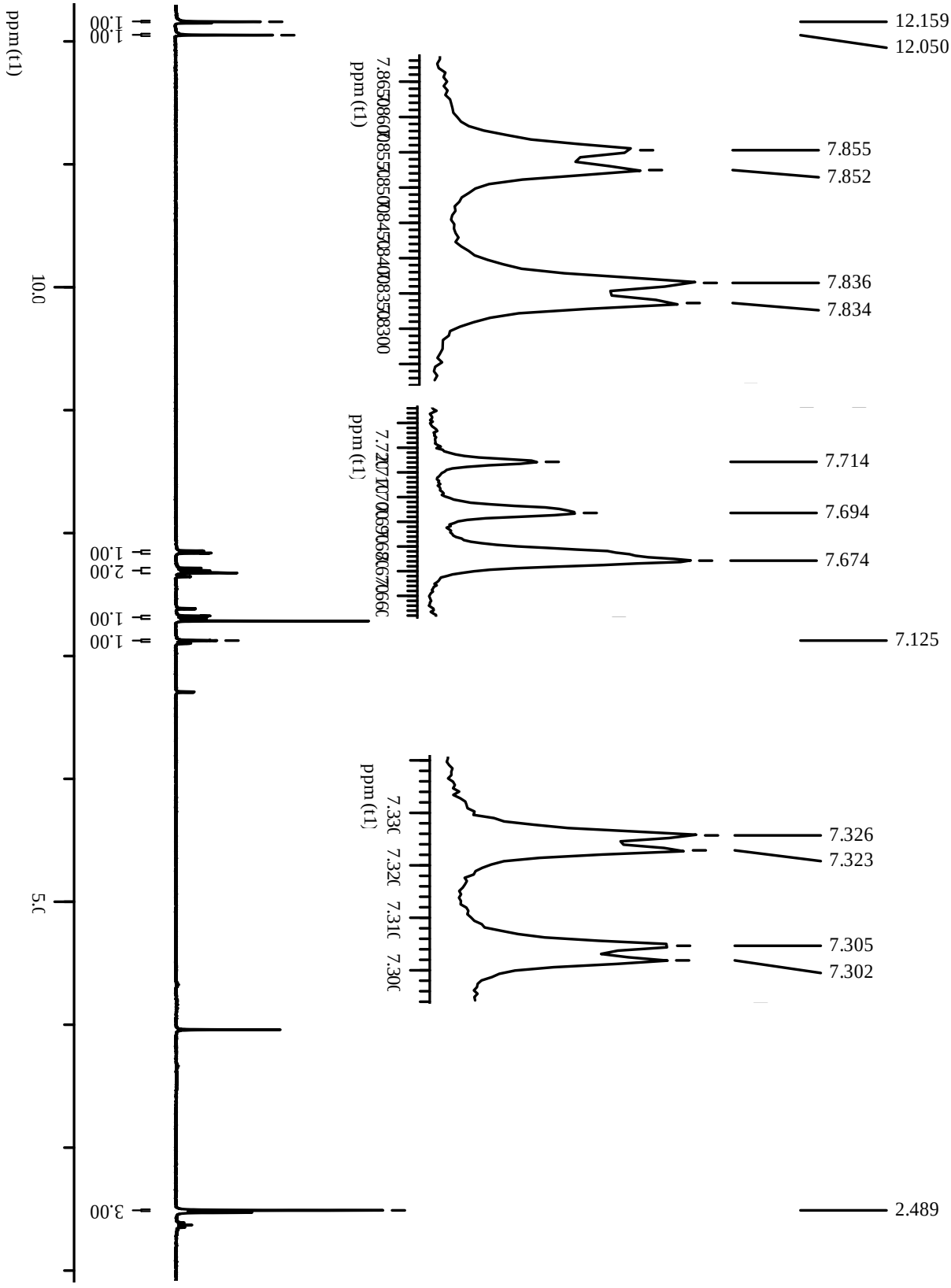
Sample Code: Compound 3 (LTE 20)



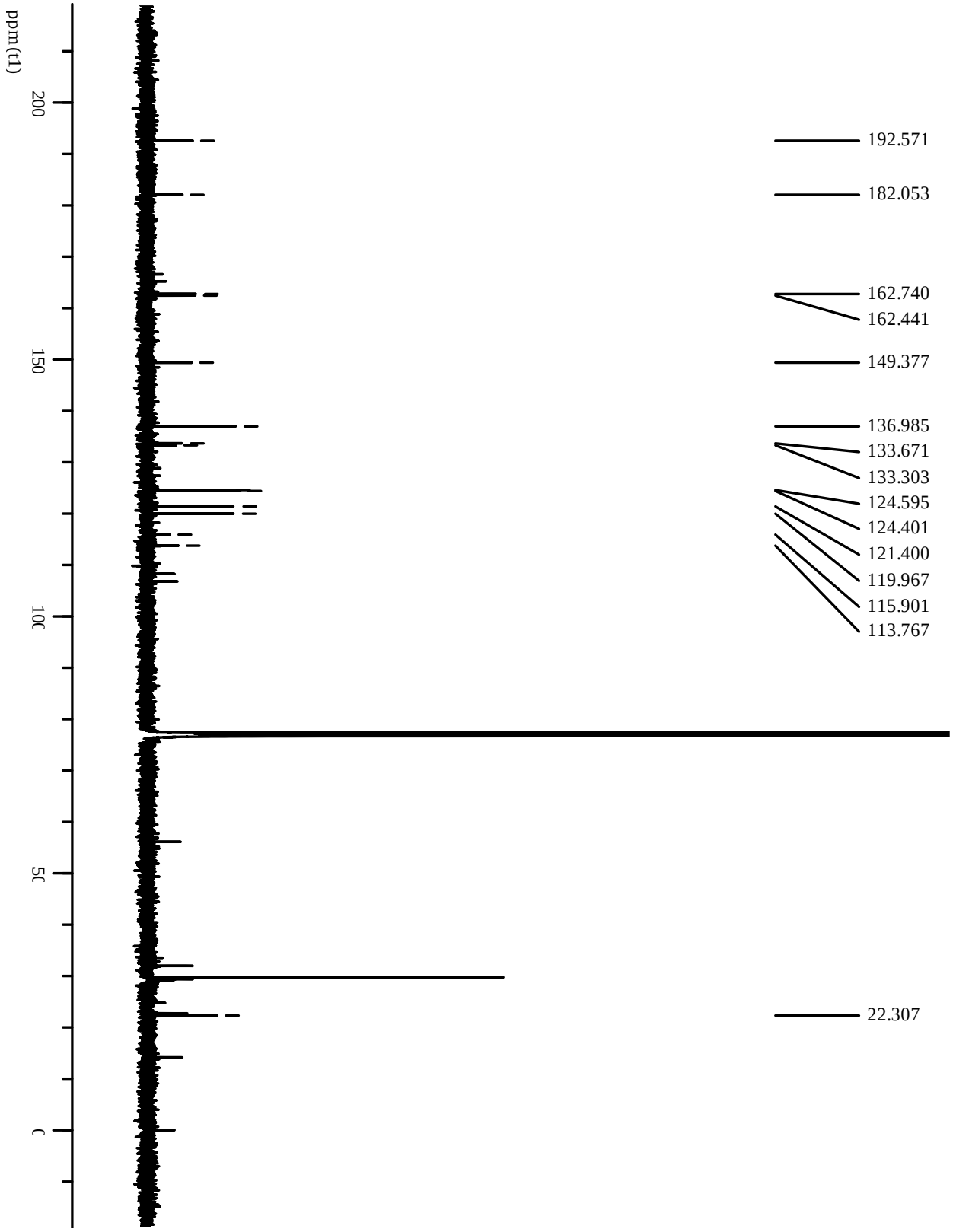
Appendix 13: IR spectrum of compound 3



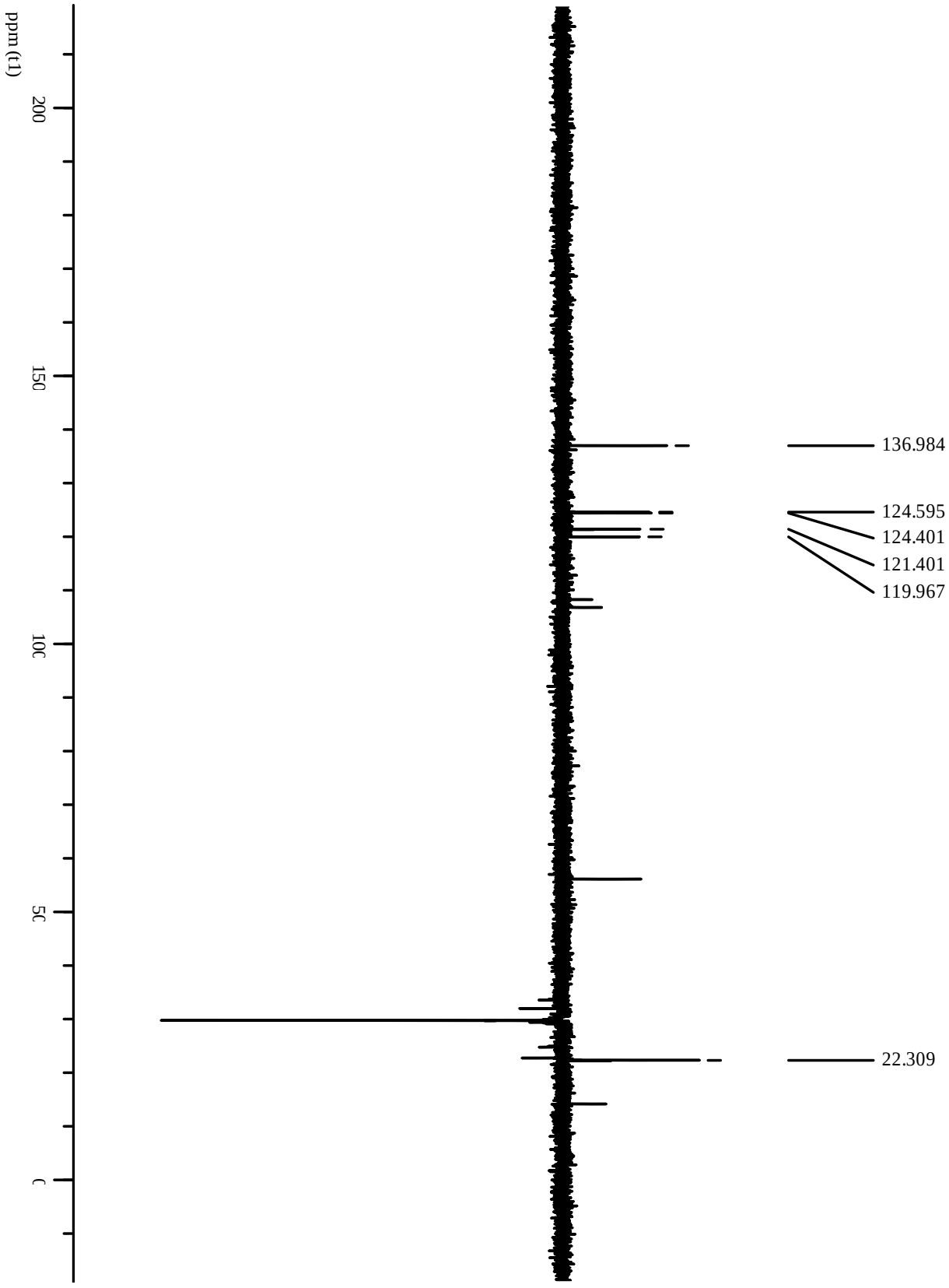
Appendix 14: ^{1}H-NMR spectrum of compound 3 in $CDCl_3$



Appendix 15: ^{13}C-NMR spectrum of compound 3 in $CDCl_3$



Appendix 16: DEPT-135 spectrum of compound 3 in $CDCl_3$



Appendix 17: UV/Vis. spectrum of compound 4

Addis Ababa University

Department of Chemistry

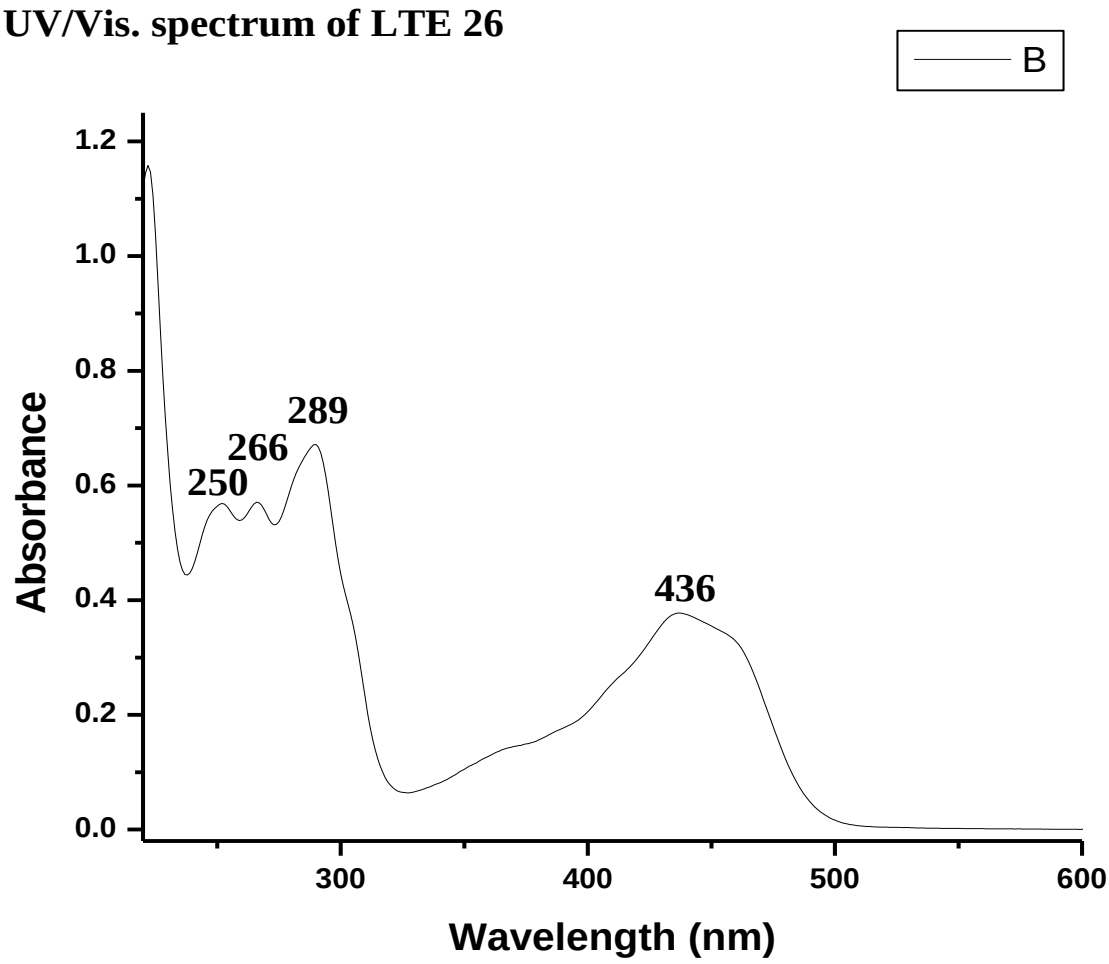
UV-Visible Spectrophotometer

Laboratory

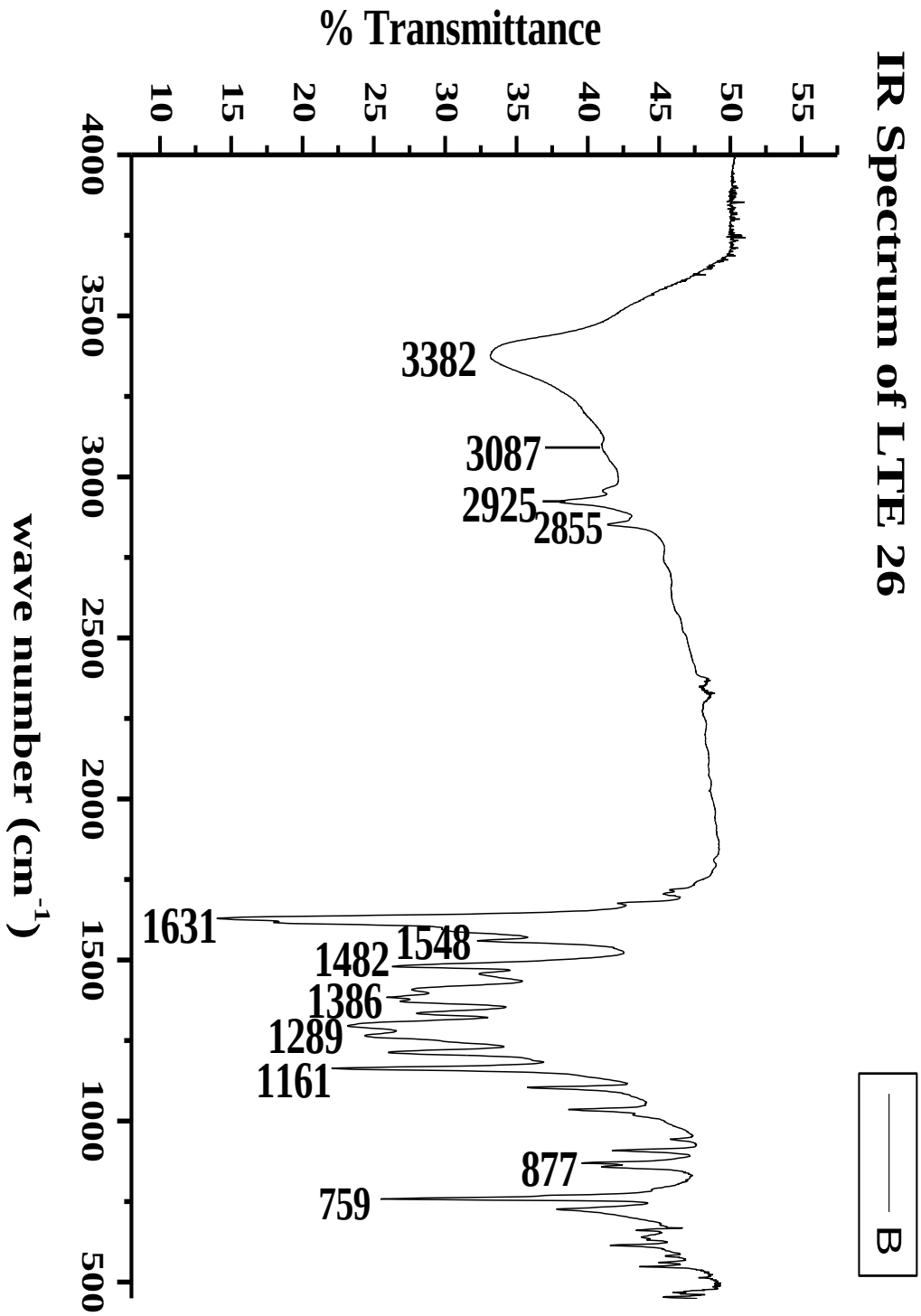
Instrument: T60 UV-Visible spectrophotometer PG instrument

Date: May 26, 2017

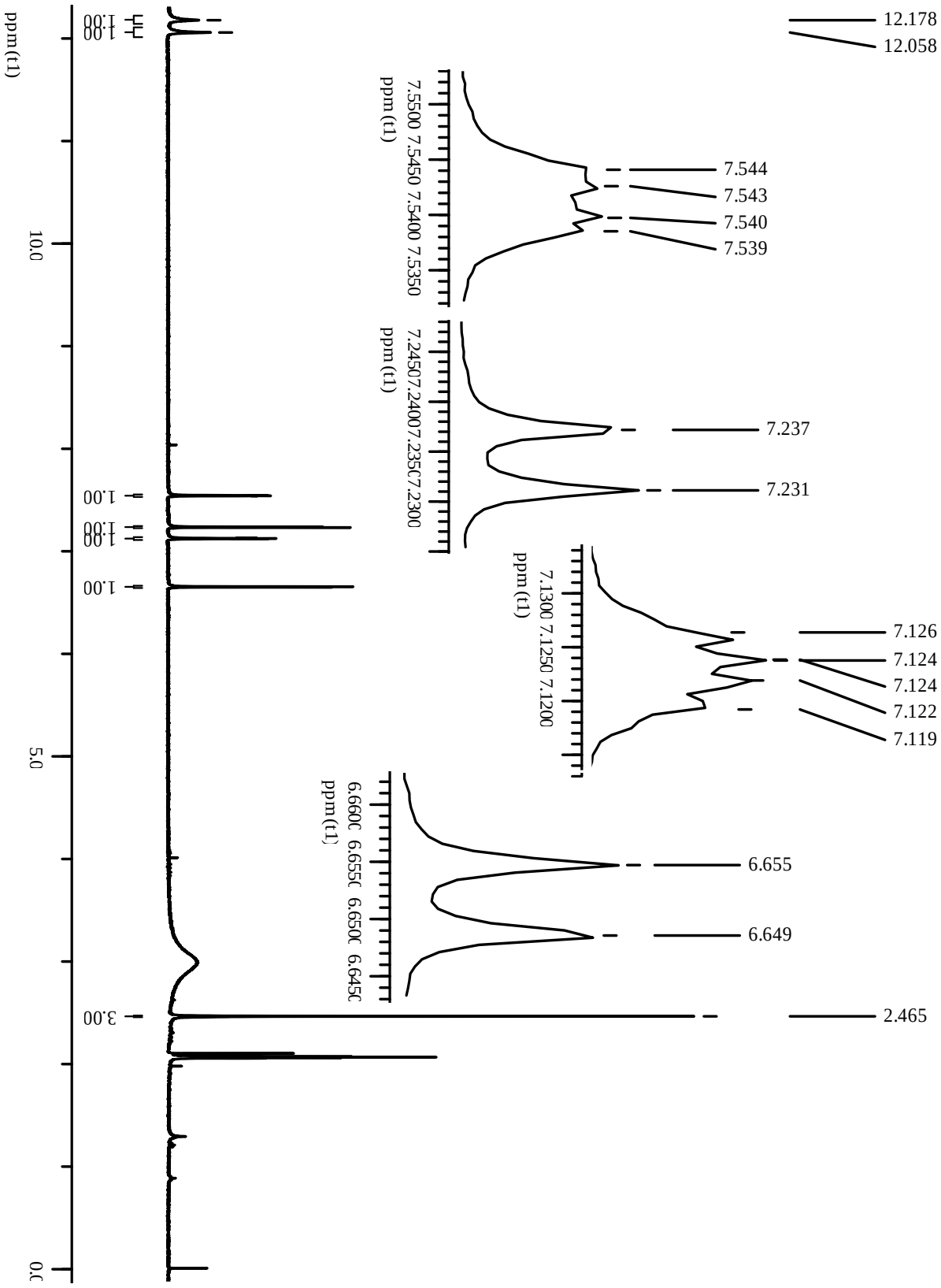
Sample Code: Compound 4 (LTE 26)



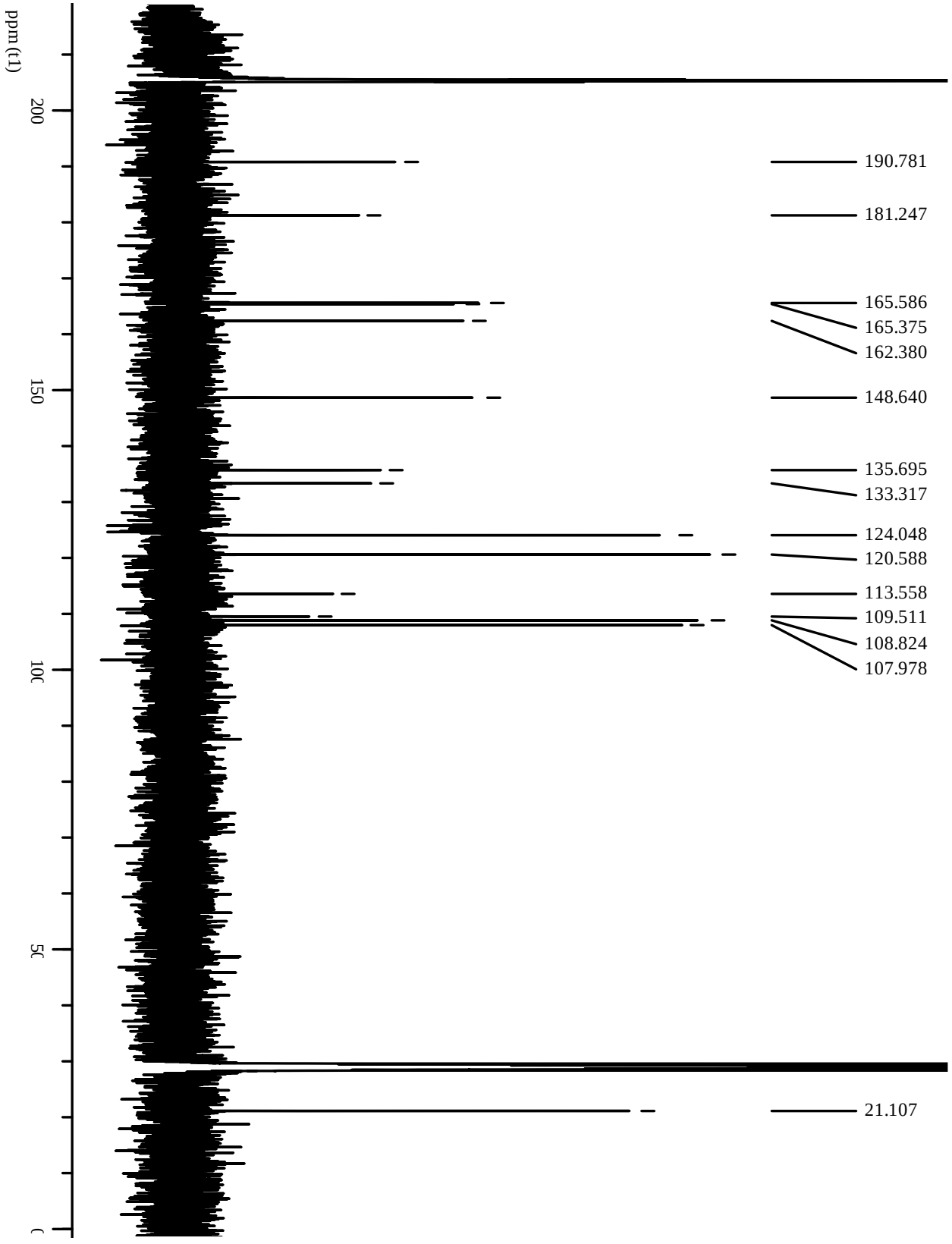
Appendix 18: IR spectrum of compound 4



Appendix 19: ^{1}H-NMR spectrum of compound 4 in acetone-d_6



Appendix 20: ^{13}C-NMR spectrum of compound 4 in acetone-d_6



Appendix 21: DEPT-135 spectrum of compound 4 in acetone-d_6