

**Phytochemical Investigation, Antibacterial and Antioxidant
Activities of Root barks Extract of *Gnidia involucrata***

By: Abera Kelbessa Degaga



**A Thesis Submitted to Applied Chemistry Program School of
Applied Natural science**

**Presented in Partial Fulfillment of the Requirements for the Degree
of Master of Science in Chemistry.**

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

**Sept, 2017
Adama, Ethiopia**

**Phytochemical Investigation, Antibacterial and Antioxidant
Activities of Root barks Extract of *Gnidia involucrata*.**

By: Abera Kelbessa Degaga

Advisor: Yadessa Melaku (PhD)

Co-advisor: Hailemichael Tesso (PhD)



**A Thesis Submitted to Applied Chemistry Program School of
Applied Natural Science**

**Presented in Partial Fulfillment of the Requirements for the Degree
of Master of Science in Chemistry**

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

Sept, 2017


Adama, Ethiopia

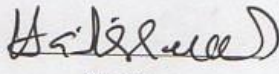
APPROVAL SHEET

SCHOOL OF POST GRADUATE STUDIES

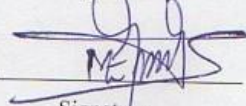
ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

We, the undersigned, members of the Board of Examiners of the final open defense by Abera Kelbessa have read and evaluated his thesis entitled "phytochemical investigation, Anti-bacterial and Anti-oxidant Activities of Root Barks Extract of *Gnidia involucrata*" 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.

Dr. Yaredessa Melaku  02/10/2017
Major advisor Signature Date

Dr. Hailemechael Tessema  02/18/2017
Co-advisor Signature Date

Dereje Tsegaye  Oct. 02, 2012
Chairperson Signature Date

Dr. Melkyas Endale  02/10/2017
Internal Examiner Signature Date

Legesse Adane (PhD)  25 Sep, 2017
External Examiner Signature Date

Declaration

I, the under signed, declare that this MSc. thesis is my original work. It has not been presented submitted for any degree in any other University, and that all sources of materials used for this thesis have been duly acknowledged.

Name: Abera Kelbessa

Signature -----

This MSc. thesis has been submitted for examination with my approval as University advisors.

Name: Yadessa Melaku

Signature -----

Name: Hailemichael Tesso

Signature-----

Sept, 2017

ASTU, Adama

ACKNOWLEDGEMENTS

Above all, I bow my head to the Omnipotent, Omnipresent and Omniscient Almighty God, for giving me the capability to complete this program. As a result of His clemency my success was finally accomplished.

Words leap but inadequate to express my deepest gratitude to my advisor Dr. Yadessa Melaku and co-advisor Dr. Hailemichael Tesso for their genuine and untiring guidance, advice and critical comments on the thesis plus clearing my difficulties and dispelling the clouds of doubts that swept over my heart and mind during the course of the research.

I am grateful to the Department of Chemistry, Adama Science and Technology University, for providing laboratory service for this research work, Oromia Public Health Research Capacity Building and Quality Assurance for allowing me to conduct antibacterial test, and Meta Wolkete Woreda Educational Bureau for financial support

My thanks go to Dr. Milkyas Endale and Dr. Legessa Lemecha for their concern in giving me valuable guidance, provision of constructive suggestion throughout the research work, Ato Solomon Girmay, head of Applied Chemistry Program of Adama Science and Technology University, for his administrative support, Ato Tolosa Duguma, laboratory technician in Organic Chemistry laboratory, for their unreserved help throughout my laboratory work.

Last but not least, I would like to pass my deepest gratitude to W/t Chaltu Fufa, Ato Mamo Fufa, Ato Terefe Gudina, Ato Mulatu Tilahun, W/t Like Fufa, Ato Moti Amayu and my parents, sisters, brothers and friends for their encouragement which induced and helped me a lot in all way to complete this research work successfully.

Table of Contents

Content	Page
ACKNOWLEDGEMENTS	i
TABLE OF CONTENTS	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
LIST OF ABBEVIATIONS	vii
ABSTRACT.....	viii
1. INTRODUCTION.....	1
1.1. Background of the study	1
1.2. Statement of the problem	4
1.3. Significance of the study	4
1.4. Objectives.....	5
1.4.1. General objective.....	5
1.4.2. Specific objectives.....	5
2. LITERATURE REVIEW	6
2.1. The Family Thymelaeaceae	6
2.1.1. Toxicity of the Family Thymelaeaceae.....	7
2.1.2. Traditional Medicinal uses of Thymelaeaceae.....	7
2.2. The genus <i>Gnidia</i>	8
2.2.1. Traditional use of the genus <i>Gnidia</i>	9

2.2.2. Biological activities of the genus <i>Gnidia</i>	9
2.2.3. Phytochemistry of the genus <i>Gnidia</i>	10
2.3. The species <i>Gnidia involucrata</i> Steud ex.Rich.....	12
2.3.1.Ethnobotanical Information.....	12
2.3.2.Traditional use of <i>Gnidia involucrata</i>	13
2.3.3.Biological activities of <i>Gnidia involucrata</i>	14
2.3.4. Phytochemistry of the <i>Gnidia involucrata</i>	14
3. MATERIALS AND METHODS	16
3.1. Plant Material	16
3.2. Instruments, Apparatus and Chemicals	16
3.3.Extractions.....	16
3.4.Isolation of compounds from the ethyl acetate extract of the root barks of <i>G. involucrata</i>	18
3.5. Phytochemical screening.....	20
3.6. Evaluating biological activities of crude EtOAc extract and isolated compounds of the root barks of <i>Gindia involucrata</i>	22
3.6.1. Antioxidant Activity.....	22
3.6.2. Antibacterial Activity	22
4.RESULTS AND DESCUSION	24
4.1. Extract yields.....	24
4.2.TLC profile of the ethyl acetate extracts.....	24
4.3. Phytochemical screening.....	25

4.4.Characterization of the isolated compounds	336
4.4.1. Characterizing of compound-1.....	26
4.4.2.Characterizing of compound-2.....	29
4.4.3.Characterizing of compound-3.....	32
4.5. Biological activities of EtOAc extract and isolated compounds of the root barks of <i>Gindia involucrata</i>	35
4.5.1. Antioxidant Activity.....	35
4.5.2. Antibacterial activity.....	36
5. CONCLUSIONS	39
6. RECOMMENDATIONS	40
REFERENCES	41
APPENDICES	51

List of Tables

Table 1. Column chromatographic fractionation of the ethyl acetate extract of the root barks of <i>G.involucrata</i> (2-2B).....	18
Table 2. Column chromatographic fractionation of 2-2Bf ₁₂	19
Table 3. Column chromatographic fractionation of 2-2Bf ₁₈	20
Table 4. Results of qualitative test for phytoconstituents	25
Table 5. ¹ H-NMR, ¹³ C-NMR and DEPT-135 (CDCl ₃) spectral data of compound-1	28
Table 6. ¹ H NMR, ¹³ C-NMR and DEPT-135 (CDCl ₃) spectra data of compound-2	31
<u>Table 7.</u> ¹ H NMR, ¹³ C-NMR and DEPT-135 (Acetone- <i>d</i> ₆) spectra data of compound-3.....	34
Table. Percentage inhibition of DPPH radical in EtOAc and MeOH extracts, compound-1 and compound-2	35
Table 9. Zone of bacterial growth inhibition (mm) for crude ethyl acetate extract and isolated compounds.	37

List of Figures

Figure 1: Chemical structure of compounds reported from the genus <i>Gnidia</i>	11
Figure 2: Aerial part of <i>Gnidia involucrata</i>	13
Figure 3: Chemical structure of compounds reported from the <i>Gnidia involucrata</i>	15
Figure 4: TLC profile of the extracts.	24
Figure 5: TLC profile of comound-1.	26
Figure 6: Proposed structure for compound-1	28
Figure 7: The TLC profile of compound-2.	29
Figure 8: Proposed structure for compound-2.	32
Figure 9: TLC profile of compound-3.	33
Figure 10: Proposed structure for compound-3.	34
Figure 11: Scavenging activity of the EtOAc, MeOH extracts, compound-1 and compound-2	36
Figure 12: Zone of bacterial growth inhibition (mm).	38

List of Abbreviations

WHO	World Health Organization
DEPT	Distortionless Enhancement by Polarization Transfer
NMR	Nuclear Magnetic Resonance
IR	Infrared Spectroscopy
UV	Ultraviolet
TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
TMS	Tetramethylsilane
DPPH	2,2-diphenyl-1-picrylhydrazyl

ABSTRACT

Gnidia involucrata (Thymelaeaceae) is a plant in the genus *Gnidia*. It is used as laxative, rheumatism, insecticides, insect repellent and antimalarial. In view of its traditional uses, the root barks were extracted successively with hexane, EtOAc and MeOH to afford 0.78% hexane, 4% EtOAc and 6% MeOH crude extract respectively. Phytochemical screening of the EtOAc and MeOH extracts revealed the presence of flavonoids, saponins, terpenoids, tannins and phenolics but additionally EtOAc extract revealed alkaloids. The EtOAc extract which showed good TLC profile was subjected to silica gel column chromatography separation which furnished three compounds (compound-1, compound-2 and compound-3). The compounds were found to be tetratriacontanyl caffeate, 12-O-dodeca-2,4-dienoyl phorbol-13-acetate and 5,7-dihydroxy-2-[4-hydroxyphenyl] flavanone based on their spectroscopic data IR and NMR (¹H-NMR, ¹³C-NMR and DEPT-135). The EtOAc extract and isolated compounds were assessed for their antibacterial activities. The results showed that the EtOAc extract displayed inhibition zone of 23, 14, 12 and 12 mm against *S. aureus*, *E. coli*, *P. mirabilis* and *Klebsiella pneumonia*, respectively. These data were significant compared with the positive control with inhibition zone of 23, 24, 21, and 19 mm against *S. aureus*, *E. coli*, *P. mirabilis* and *Klebsiella pneumonia*, respectively. Among the compounds tested, compound-2 and compound-3 showed an inhibition zone of 11 mm and 12 mm against *E. coli*. Furthermore, the EtOAc, MeOH extract, compound-1 and compound-2 were found to inhibit DPPH radical by 70.7%, 66.9%, 85.8% and 52.8%, respectively. The radical scavenging activity of compound-1 was comparable with that of ascorbic acid (97%) that was used as positive control in the test. The IC₅₀, the concentration of the sample that inhibits 50% of the radical values of EtOAc and MeOH extract, **compound-1** and **compound-2** were 15.3, 21.8, 2.3, and 83.6 µg/mL, respectively. The lower IC₅₀ value displayed by **compound-1** indicates its strong radical scavenging activity compared to the ascorbic acid. The antibacterial activities displayed by the root barks extract of *G. involucrata* corroborate the traditional use of this plant against bacterial infections.

Key words: *Gnidia involucrata*, Antibacteria, Antioxidant, tetratriacontanyl caffeate, 12-O-dodeca-2,4-dienoyl phorbol-13-acetate and 5,7-dihydroxy-2-[4-hydroxyphenyl] flavanone.

1. INTRODUCTION

1.1. Background of the Study

The use of herbal medicine, date back to the ancient man kind who used plants for the treatment of various diseases in addition to their use as a food, shelter and cosmetics. A lot of ancient healing traditions gave rise to the familiar herbal medications of the twentieth century [1]. This can proved through the written evidences during the great civilizations of the ancient Chinese, Indians and North Africans that showed human's creativity in utilizing plants for the treatment of a wide variety of diseases. During the civilization of the ancient Greece, scholars classified plants by giving descriptions that help their identification processes. This can be mentioned as an example of the focus of ancient society on herbal medicine [2]. Other written documents indicated some of the oldest medicinal systems of the world that were in use thousands of years ago. Ayurveda of Indus civilization around 2500 BC [3], Arabian medicine of Mesopotamia, Chinese and Tibetan medicine of the Yellow River Civilization of China and Kempo of the Japanese used herbal medicine. During this ancient civilization, it has been recorded that there were systematic collection of information and well defined herbal pharmacopoeias [3].

As reported by the WHO, 80% of the population of the world, particularly in the developing countries presently uses herbal medicine for primary health care [4]. In many countries of the world the use of traditional medicines are deeply rooted in their cultures with the majority relay on plants as a source of drugs [5]. Medicinal plants have greatest potential for benefitting people, especially those living in countries suffering from poverty, and poor health. These plants are significant in developing countries owing to their affordable prices and accessibilities. Herbal medicine has become a crucial treatment regimens and a subject of interests for the pharmaceutical companies. This is in part due to the chemical diversity and versatility of active components of herbal plants as compared to synthetic drug made more preferable to conventional synthetic drugs, in addition to their accessibility and affordable prices as opposed to modern drugs [6].

There are controversial reports about herbal or traditional medicines. Some reports state these medicines are safe [7] while others mention harmful effects. The harmful effects (adverse

reactions) are attributed to poor quality or inappropriate administration or use in combination with others medicines [8]. Patients must have good awareness before using herbal medicine [9]. There are some recommendations to be followed during the use of herbal medicines [10]. Patients such as pregnant, in surgery operation or with allergens are not advised to take herbal medicines. Moreover, taking herbal medicines together with modern medicine and optimizing the dosage is may also have an adverse effect on the body.

In the world beyond 50,000 flowering plants exists which are used as medicine [11]. Most of these medicinal herbs are found in the tropical countries. In African countries like Ghana, Mali, Nigeria and Zambia herbal medicine is used by about 60% of children with high fever resulting from malaria as first line of treatment in the home. Traditional herbal medicine is also becoming popular in many other countries of Asia and Latin America. Moreover, the use of medicinal plants is also incredibly spreading even in the industrialized countries [12].

Reports of recent studies showed the steady growth of the prevalence of herbal medicine in the developed countries [13]. It was also reported that about one fourth of adults in the United States are known to use herbal medication with the past years to treat different human illnesses [14]. Similarly, in China 30%-50% of the overall medicinal consumption nowadays is traditional herbal preparation [15].

Similar to other developing countries Plants have traditionally been used as a source of medicine in Ethiopia for many centuries to combat various human and livestock ailments. Traditional medicine is still an integral part of the culture in the country due to its long history. The indigenous people of different localities in the country have their own specific knowledge on the use, management and conservation of plant resources [16].

The geographical diversity of Ethiopia has favored different habitats and vegetation types, that medicinal plants are also a component of these. This geographical diversity couples with multiplicity of ethnic groups with complex cultural diversity made the country the home for high diversity of traditional knowledge, practice and uses of traditional medicine [17,18]. The practice of traditional medicine in the country is not only concerned with curing of diseases but also with

the protection and promotion of human physical, spiritual, social, mental and material wellbeing [19]. Traditional medical practice has been in existence before the development of modern medicine in the country and still continues to be widely accepted and used in the prevention and treatment of ailments. In addition, this is accessible and affordable in many developing countries like Ethiopia [17,20].

Plants serve as a vast reservoir of many complex organic compounds [21], many of which, at least at our current stage of knowledge, appear to have no direct function for the growth and development of the plant. These compounds are known as secondary metabolites. They are produced either as a result of the organism adapting to its surrounding environment or for its own survival and defense against predators. These secondary metabolites including alkaloids, flavonoids, phenolics, steroids and terpenoids provide some unique and species-specific characteristics to plants. These compounds also played a central role in the history of mankind and possess well defined biological functions. Recently, an attention was given to isolate these secondary metabolites from medicinal plants which are used for their healing properties in addition to their use for the production of plant derived modern drugs [3]. This is supported by a report mentioning that 50% of all drugs in clinical use today are derived from natural products. Among these, 25% of which are obtained from medicinal plants [22].

Gnidia involucrata is among significant plants that are being used in Ethiopia for the treatment of diseases such as malaria, rheumatism and stomach parasite. The aerial and root parts of the plant were previously explored for its chemical constituents. However, there is no prior report on the chemical study and biological activities of the root barks of *Gnidia involucrata*. Hence this thesis addresses for the first time on the phytochemical studies of root bark of *Gnidia involucrata*. Furthermore the antibacterial and antioxidant activities of the EtOAc extract and compounds isolated from the extract were also explored.

1.2. Statement of the problem

Diseases caused by bacteria are among diseases causing serious problems in developing countries. This problem is exacerbated by the development of bacterial resistance to currently

used antibacterial agents. Thus, there is an urgent need to search for new antibacterial agents from natural sources as an alternative to synthetic antibiotics. Furthermore, synthetic antioxidants are often used in the food industries to prevent or inhibit the oxidative deterioration in foods. However, these synthetic antioxidants were reported to cause health problem among which is cancer. Therefore, there is increasing importance to search the natural antioxidants from plants as an alternative to synthetic antioxidant. Hence this research attempted to evaluate antibacterial and antioxidant activities of root bark extract of *Gnidia involucrata*, and also that of compounds isolated from the extracts. To the best of our knowledge, there are no reports of similar studies on this part of the plant used in the study.

1.3. Significance of the study

Focus on medicinal plant research to find lead compounds has increased all over the world. The antibacterial compounds from plants may inhibit bacterial growth by different mechanisms than antibiotics and may have a significant clinical value in treatment of resistant microbial strains. Hence the investigation of the antibacterial activities may help to arrive at active ingredients which may be used as antibacterial drug. Worldwide, there has been growing trend and interest in plants' natural antioxidants as natural additives in food and cosmetics. Plants are one of the most important targets to search for natural antioxidants as it is known safer compared to synthetic antioxidants. The findings of this research would contribute to the search of antioxidant and antibacterial agents from natural sources. Moreover the isolated compounds may contribute to the efforts to increase natural product database. Furthermore, the result may be used to substantiate the traditional uses of this plant against bacterial infections and also its use as antioxidant.

1.4. Objectives

1.4.1 General Objective

The overall objective is to isolate and characterize the chemical constituents, and conduct antibacterial and antioxidant activities of root barks extract and isolated compounds of *G. involucrata*.

1.4.2. Specific Objectives

- To successively extract the root barks using n-hexane, ethyl acetate and methanol.
- To screen secondary metabolites in the extracts of root barks of *G. involucrata*.
- To isolate compounds from the extracts of the root barks of *G. involucrata*.
- To elucidate the structures of isolated compounds using data obtained from various spectroscopic methods (UV-Vis, IR and NMR).
- To evaluate the antibacterial activities of the extracts and isolated compounds from the root barks of *G. involucrata* against *Escherichia coli*, *Staphylococcus aureus*, *Proteus miabilis* and *Klebsiella pneumonia*.
- To assess antioxidant (radical scavenging) activities of the extract and isolated compounds from the root barks of *G. involucrata*.

2. LITERATURE REVIEW

2.1. The Family Thymeleaceae

Thymelaeaceae family was first established in 1789 by De Jussieu, and currently 45 genera and 800 species are recognized within it [23, 24]. It comprises of many genera with species known to possess toxic, irritant or cocarcinogenic principles which affect animals and humans. Some of the genera are:-

Gnidia: 100 species are distributed in Africa, India and Ceylon

Pimelea: 80 species in Australasia

Wikstroemia: 70 species in Australasia to southern China

Daphne: 70 species in Australasia, Asia, Europe and North Africa;

Lasiosiphon: 50 species covering the same area as *Gnidia*.

The members of this family are widespread in tropical and temperate parts of the world, particularly in Africa, and are absent only in regions with the coldest climates [25]. They have fibrous bark, leaves that are typically opposite and usually stipules but that frequently have rather close, parallel venation, and hairs that are often silky-appressed. The Thymelaeaceae have a wide range of uses, giving them considerable economic importance in the areas where they grow. Species of the genera *Daphne*, *Dais*, *Dirca* and *Pimelea* are for example grown as ornamental shrubs with sometimes persistent and generally fragrant flowers. The bark of several genras particularly *Wikstroemia*, *Daphne*, *Edgeworthia* and *Thymelaea* - is used for local paper making [23].

Thymelaeaceae plants contain coumarins, flavonoids, chromones, lignans, and neolignans. Phytochemical studies on the Thymelaeaceae plants due to their widespread uses in medicine have been reported and there are reports on the toxicity of these plants [25]. Several genera such as *Daphne*, *Thymelaea*, *Pimelea*, *Wikstroemia* and *Gnidia* have been researched upon extensively. The *Daphne* genus is of prime importance owing to its richness in a variety of different classes of natural products, especially, coumarins, lignans, flavones, daphnane-type diterpene esters, steroids and guianolides [26]. The tiglane type diterpenes, daphnane type diterpenes and 1-alkyldaphnane are undoubtedly the class of compounds most characteristic of

the Thymelaeaceae [27]. These types of secondary metabolites are also found in Euphorbiaceae family. They found in all parts of the plant (root, stem, leaf, fruit, and seed), are highly irritating to the mucous membranes and possess vesicant properties. Thymelaeaceae are considered toxic due to their content of diterpene esters of type tigliane or daphnane, which have a restricted distribution to only two families: Thymelaeaceae and Euphorbiaceae [28, 29].

2.1.1. Toxicity of the Family Thymelaeaceae

Toxicity of Thymelaeaceae is well established for Human beings, as well as many animal species [23, 30]. Indeed, tigliane and daphnane-type diterpenes are a violent purgative which triggers, by contact with the skin or the mucous membranes, an intense inflammatory reaction [31, 32]. Toxic manifestations can be divided into two categories; depending on whether the plant material or extracts are taken internally or that there is external contact. Symptoms of systemic toxicity resulting from ingestion of plant material are relatively constant in Thymelaeaceae and have Vet Oettingen described the symptoms observed in humans after ingestion of barks and berries of the plant family: inflammation of the lips, tongue and pharynx, dry mouth followed by salivation, difficult swallowing, thirst, rhinitis, eyelid edema, headache, abdominal pain, vomiting, watery and watery diarrhea, albuminuria, haematuria, slow breathing, rapid pulse, pale skin, cold and moist skin; muscular twitching, delirium and drowsiness which can last several days [33].

2.1.2. Traditional Medicinal uses of Thymelaeaceae

The traditional medicines of a large number of cultures use Thymelaeaceae for the preparation of treatments of a very wide range of disorders [23]. Examples of the use of the toxic effects of these traditional remedies are the use of emetic, purgative, vesicant and for the treatment of skin diseases. In these applications, however, the doses are low, in order to favor the beneficial effect compared to the secondary effects. The internal officinal use of the drug, in the form of a decoction, a purgative, anti-rheumatic or anti-syphilitic trade, had fortunately been abandoned for a long time. Only external indications remained in the form of vesicant patches or lotions which were now outdated. In France, extracts of *Lasiosiphon kraussianus* Hutch. and Dalz. (*Gnidia kraussiana* Meissn.) have been patented for the treatment of leprosy [34]. In China, a preparation of *Daphne giraldii* Nitsche is one of the constituents of a long-lasting analgesic

preparation for the treatment of hemorrhoids [35]. Clinical studies have been conducted in China on *Daphne* species preparations [36, 37], *Gnidia* [38, 39], *Wikstroemia* [40] and *Pimelea* [41, 42], demonstrating their anticancer properties. The abortive and anticancer properties of these products have been attributed mainly to the presence of daphnane esters, some of which have documented pro-tumor activity [43]. Daphnane esters can therefore be considered as risk factors for iatrogenic cancer and therefore their use is delicate [44]. Plants of the Thymelaeaceae family have been included in numerous large-scale screenings for a variety of biological activities [44, 45]. The medicinal uses of Thymelaeaceae are numerous, and as the plants studied in this study is all of African origin.

2.2. The Genus *Gnidia*

Gnidia (Thymelaeaceae) the largest genus comprising of 140-160 species occurring in Africa, Arabia, India, Madagascar and Sri Lanka [24,46,47]. Linnaeus described *Gnidia*, in the first publication of *Species Plantarum* in 1753. The genus was possibly named after the classical Greek port city 'Cnidus', today known as Cumali. The genus is commonly named as night- and evening-scented bush and 'young-lady-gad-about-at-night', due to the flowers that are fragrant at night [48]. It occurs in various forms as perennial herbs to shrub lets, under-shrubs, shrubs or trees, arising from a woody base or rhizome, and is often ericoid. The bark can vary from a smooth to rough texture with or without lenticels. The branches are slender with alternately positioned, rarely opposite sessile leaves. Several *Gnidia* species are very showy due to their conspicuous flowers [49, 50]. Various species of *Gnidia* are used for medicinal and economic purpose. Due to the characteristic fibrous barks, *Gnidia* species are used to tie bundles of wood, roofing and clothing. The flowers of several species of *Gnidia* are employed for dyeing leather. In Madagascar the fibrous barks of *Gnidia* species are used to make rope and twine, paper and ceremonial clothing [51].

2.2.1. Traditional use of the Genus *Gnidia*

Species of the genus *Gnidia* have been used in the traditional treatments of a variety of medicinal complaints in humans and animals. For instance, they have been used to treat a range of conditions in humans including conception and childbirth, asthma, backache, nightmares, dropsy, boils, sores, induce blistering, treat bruises and burns, constipation, coughs, epilepsy, headache, influenza and fevers, malaria, measles, poor appetite, small pox, snake bites, sprains and fractures, tonsillitis, to stabilize heart conditions, stomach and chest complaints, toothache, ulcers and yellow fever and [23,25,52]. In livestock, *Gnidia* species have been used in the treatment of anthrax and botulism. In Madagascar, leaves of *Gnidia gilbertae* Drake are used as a purge to induce vomiting [51]. Crushed roots of *G. kraussiana* are used to make fish poison. Although many *Gnidia* species are used in traditional medicine, severe irritant effects as well as death in humans and animals have also been reported due to the presence of toxic coumarins and diterpene esters [24,31,52].

2.2.2. Biological Activities of the Genus *Gnidia*

The toxic diterpene esters of *Gnidia* species are the main types of plant orthoesters known [24,53] and have remarkable biological activities such as antineoplastic [41,50], and cytotoxic [52,54,55]. For instance, the tigliane, daphnane and ingenane diterpenes esters are noted for their skin irritant and cocarcinogenic effects [27]. These triesters are known as ‘cryptic irritants’ because they do not exhibit pro-inflammatory activity on mammalian skin unless the C-20 acyl group is removed by hydrolysis. They are also known to be potent tumour-promoting agents, inducing susceptibility at levels of carcinogen below the normal threshold [56]. They have remarkable biological activities, such as cyto-toxic, neurotrophic [57, 58], and anti-HIV [60]. Structurally similar compound series with mezerein such as gnididin (**1**), gniditrin (**2**), gnidicin (**3**) of *Gnidia lamprantha*, which were found to possess anti-leukemic and weak carcinogenic effect compared with many phorbol esters [59, 61]. Gnidimacrin (**9**) and its 20-palmitate compounds were demonstrated to have been found to show potent *in vivo* anti-leukaemic activity. They also display several pharmacological activities such as antibacterial, antifungal, antitumour, antimitotic [62]. Esters of diterpene alcohols having the daphnane and tigliane

skeleta are responsible for the irritancy, cocarcinogenicity, abortifacient activity and anti-leukemic activity of this plant family [46].

2.2.3. Phytochemistry of the Genus *Gnidia*

Gnidia elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. More than 90 compounds have been isolated from different species of *Gnidia*. Furthermore, phytochemical investigations revealed the occurrence of coumarins [59], lignans [63], flavonoids and benzophenone glycosides [64], umbelliferoyl flavonoids and spiro-bis- γ -lactone [65]. The genus *Gnidia* is rich in diterpene esters, coumarins, flavonoids, chromones, lignans, and neolignans [23, 24]. Phytochemical studies on some *Gnidia* species indicated the presence of toxic diterpene esters of daphnane type, which are the main types of plant orthoesters known [53].

Some of the previously reported compounds from the genus *Gnidia* were the Gnididin (**1**), Gniditrin (**2**) and Gnidicin (**3**) from *G. Lamprantha* [59]. Gnilatmacrin (**4**), Kaempferol-3-(*p*-coumaroyl)-O- β -D-glucopyranoside (**5**), β -Sitosterol (**6**) and β -Sitosterol- β -Dglucoside (**7**) and Maltol (**8**) from *G. kraussiana* [25,52,59], Gnidimacrin (**9**) from *G. subcordata* [57,66], Umbelliferone (**10**), Daphnetin-8- β -D-glycosides (**11**), 2-O- α -D-glucosyloxy-4-Methoxybenzenepropanoic acid (**12**), Methyl-2-O- β -D-glucosyloxy-4-Methoxybenzenepropanoate (**13**) and Adicardin (**14**) from *G. polycephala* [66,67], Gnidicoumarin (**15**) from *G. lamprantha* [38, 54], 7,7'-dihydroxy-3,8'-biscoumarin (**16**) from the leaves and branches of *G. socotrana* [65], Gnidifolin (**17**) from *G. latifolia* [63].

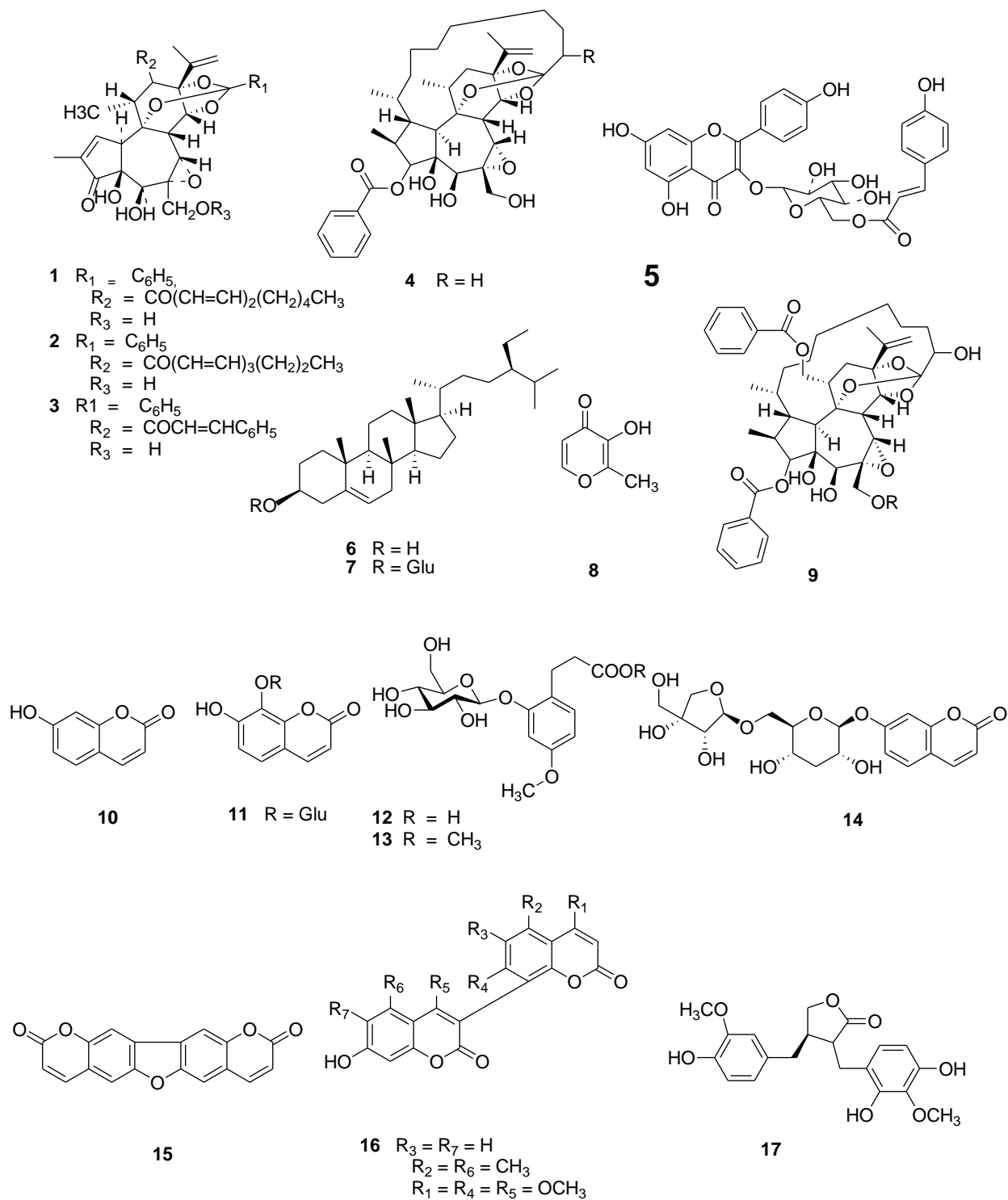


Figure 1: Chemical structure of compounds reported from the genus *Gnidia*

2.3. The *Gnidia involucrata* Steud ex.Rich.

2.3.1. Ethnobotanical Information

Ethnobotany is the principal approach to investigate natural resource management of native society. It is a science of human synergy with plants and ecosystem [68]. To relieve discomfort, disease or pain, people generally use those plants which are repeatedly emphasized by informants as a possible cure for the particular illness compared to other plants which are less known. *G. involucrata* (Figure 2) is a perennial grass or sub-shrub with little or no branching, possessing a woody rhizome and up to the size of 1 m or more. Its stems and branches are glabrous, greenish to reddish, sometimes brownish. The leaves are sessile or weakly petiole, linear to elliptic, obtuse to acute, glaucous (covered with a blue-gray down, blue-green or white) or sometimes glabrous. The flowers, grouped in capitule or axillaries, are orange-red or pinkish to red. *G. involucrata* occurs in open and wooded fields, often in fire zones, but also in the bush and deciduous forests, at altitudes of 1000-2700 m. It is widely distributed in African countries including Angola, Nigeria, Cameroon, Malawi, northern Kenya, Tanzania, northern Uganda, Ethiopia, Sudan, Zambia, Zimbabwe and Mozambique [23, 69]. In Ethiopia it is locally called *shuntura* in Afan Oromo (West Shoa, Oromia regional state) and *Boto* (*Yezngero telba*) in Amharic (Amara regional state).



Figure 2: (a) Aerial part of *Gnidia involucrata* picture (taken by Abera Kelbessa, 2016, Meta Wolkite, West shoa, Oromia, Ethiopia) and (b) Root barks of *Gnidia involucrata* picture.

2.3.2. Traditional use of *Gnidia involucrata*

The roots of *G.involucrata* are used in folk medicine to reduce the size of the vaginal orifice and as fish poison. In Zimbabwe, where his job is frequent, nanga (traditional healers) call him "muwito" or "katonje" in the Shona dialect. In addition to its interest in traditional medicine, *G. involucrata* the great advantage that it has never been investigated before the end of the year. The discovery of non-negligible quantities of tannins in *G. involucrata* is the opportunity to develop a rational reflection on the use of this plant in external application to tighten the relaxed vagina of the women in the period following the delivery [23, 70]. Indeed, these polyphenols are known for their astringency, resulting from their affinity for proteins. In Ethiopia the roots are also used as laxative and vermifuge [23, 25], and insecticides, insect repellent, anti-malarial, and also for the treatment of intestinal pain, mental problem, sexually transmitted diseases (STDS) and tuberculosis (TB) [71, 72]. In Amara and some Oromia region of Ethiopia, the root and leaf are crushed, soaked in local 'Tella' overnight for one day, and one glass is drunk continuously to treat malaria and stomach parasite. The root is also crushed with the leaf and root of *Plumbago zeylanica*, boiled in water, decanted and one glass is drunk continuously for rheumatism [73]

2.3.3. Biological activities of *Gnidia involucrata*

Chemical constituents from the leaves and root parts of the plant exhibited various biological activities such as cytotoxicity, analgesic, antipyretic, immune modulator, antitumor, antiviral, and anthelmintic and in obesity treatment, anti-allergic, broncho dilatory, anti-diabetic, anti-plasmodia, anti-amoebic, cardio protective, anti-bacterial and antifungal [74,75], α -amylase inhibitor [76], anti-oxidant [77], anti-inflammatory and gastro-protective activities, anti-microbial and mycolitic activities [78].

2.3.4. Phytochemistry of the *Gnidia involucrata*

Benzopnenone glycosides, mangifirin, flavanoides and flavonoid glycosides from aerial and root parts of the plant were previously isolated and characterized. They were identified as manniflavanone (**18**), kaempferol-3-O-glucoside(**19**), gnidia biflavonoid 4a (**20**), vitexin (**21**), isovitexin (**22**), isoorientin (**23**), mangiferin (**24**), 2,3,4',5,6-Pentahydroxylbenzophenone-4-C-

glucoside (**25**), 2,4',6-trihydroxy-4-methoxybenzophenone-2-O-glucoside (**26**), mhakoside A (**27**), Yuankanin (**28**) from *G. involucrata* [64,79]

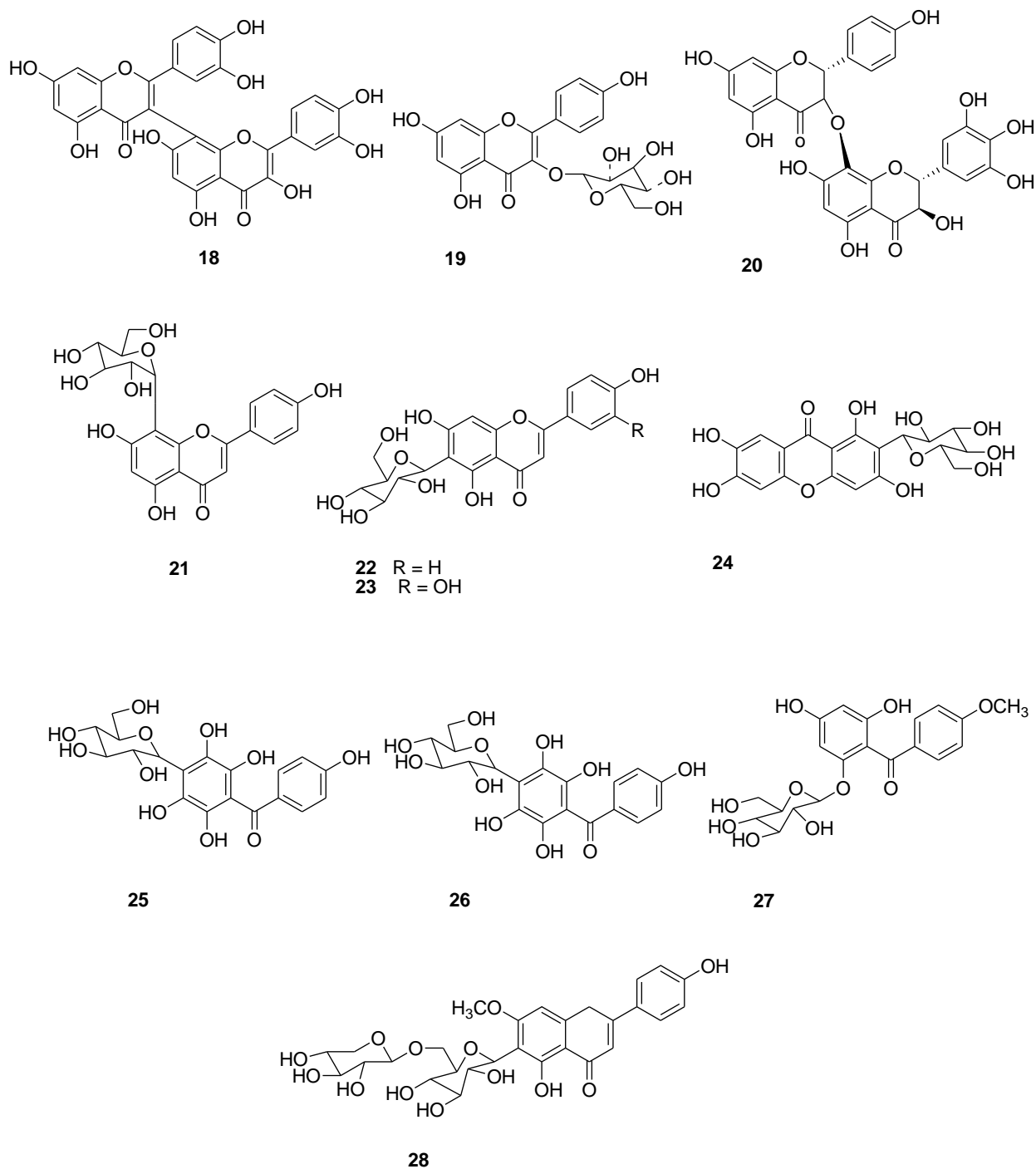


Figure 3: Chemical structure of compounds isolated from different parts of the *Gnidia involucrata*.

3. MATERIALS AND METHODS

3.1. Instruments, Apparatus and Chemicals

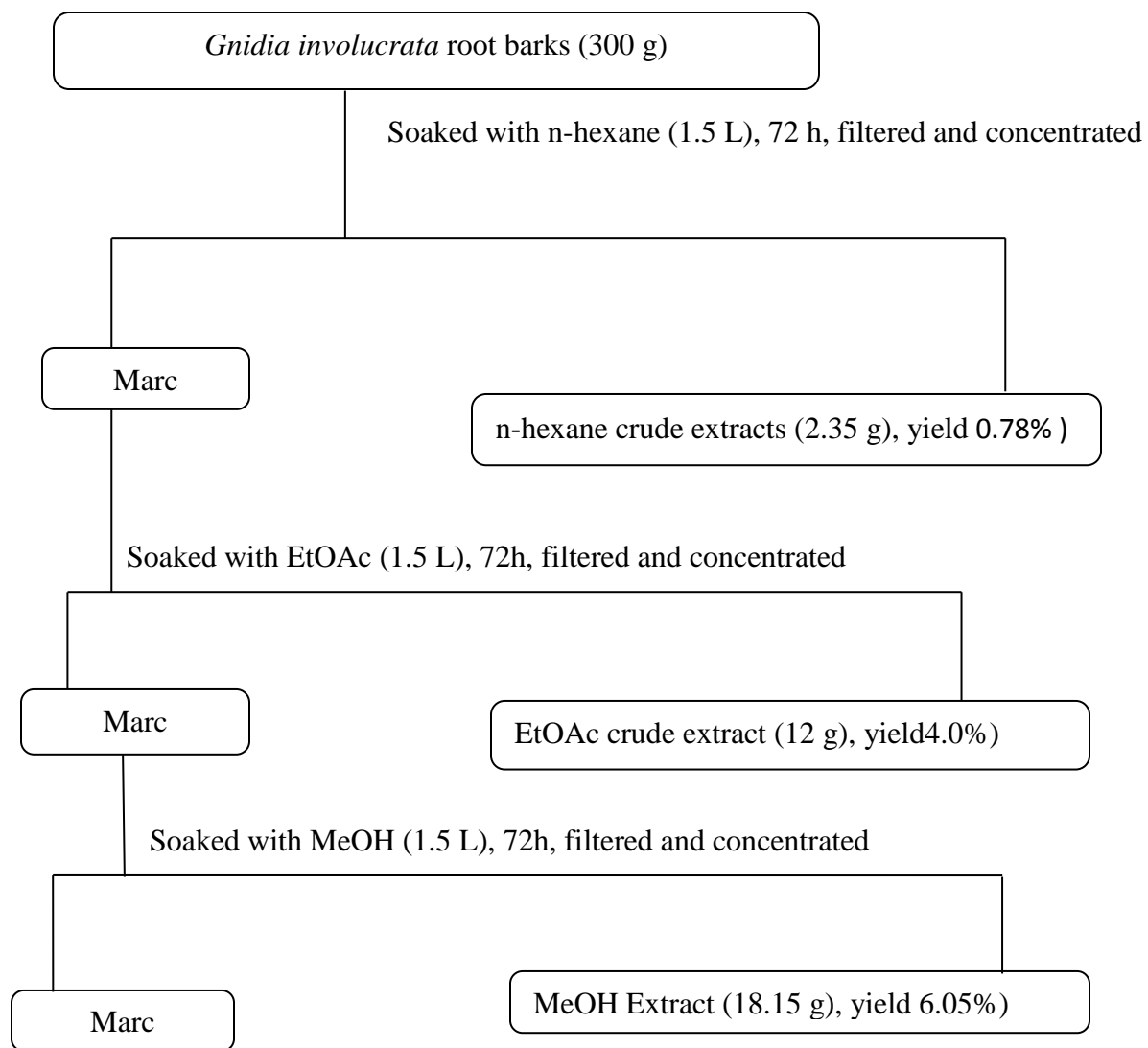
Analytical TLC was run on a 0.2 mm thick layer of silica gel GF₂₅₄ (Merck) on aluminum plate. Spots were detected using iodine as spraying reagent. Column chromatography was performed using silica gel 60 (250-400 mesh) Merck. Crude extracts were applied on column by adsorbing them on silica gel. Solvent was removed using rotary evaporator. The UV-Vis spectral measurements were done using UV-Vis spectrophotometer. NMR spectra were recorded using Bruker Avance 400 spectrometer operating at 400 MHz. The IR spectra of compounds were recorded using a Perkin-Elmer BX Spectrometer (400-4000 cm⁻¹) as KBr pellets. List of solvents and chemicals that were used in the study include n-hexane, ethyl acetate, methanol, dichloromethane, chloroform, iodine, and distilled water.

3.2. Plant Material

The root barks of *G.involucrata* were collected in the month of December, 2016 from Kunde Gerasu kebele, Meta Wolkite woreda, West Shoa, Oromia, Ethiopia, which is 102 km west of Addis Ababa. The plant was authenticated by a botanist (Prof. Legessa Nagesh) and specimen stored (Voucher no: AB-002/2016) in the National Herbarium of Addis Ababa University, Addis Ababa, Ethiopia.

3.3. Extraction

The washed, air dried and pulverized root barks of *G. involucrata* (300 g) were successively extracted with n-hexane (1.5 L), EtOAc (1.5 L) and methanol (1.5 L) each for 72 hours with occasional shaking. Each were then filtered and concentrated under reduced pressure using rotary evaporator. The extraction procedure is represented in Scheme 1.



Scheme 1: Scheme showing extraction of the root barks of *G. involucrata*.

3.4. Isolation of Compounds from the Ethyl acetate Extract.

All the three extracts (n-hexane, EtOAc and MeOH extracts) were examined with TLC. Among these, the ethyl acetate extracts showed promising spots which were visualized after dipping in iodine vapor. Hence, the ethyl acetate extract (12 g) was adsorbed and fractionated over silica gel (150 g) column chromatography packed using n-hexane. Elution was done with n-hexane:ethyl acetate:methanol of increasing polarities to afford 33 fractions. Those fractions showing similar profiles on their TLC were combined together to give a total of 23 combined fractions (Table 1).

Table 1: Column chromatographic fractionation of the ethyl acetate extract of the root barks of *G. Inolucrata* (2-2B)

Fractio n	Eluent	Ratio	Volume collected in mL	Amount in mg	
1-4	n-hexane:EtOAc	100% n-hex	400		
5-6		9:1	200		
7		8:2	100		
8-9		7:3	200	156	
10-11		6:4	200	210	
12		5:5	100	875	
13-14		4:6	200	867	
15		3:7	100		
16		3:7	100		
17		2:8	100	197	
18		EtOAc:MeOH	2:8	100	433
19-20			1:9	200	4.426 g
21-22			100%EtOAc	200	
23			9:1	100	
24			8:2	100	
25	7:3		100		
26	6:4		100		
27	5:5		200		
28	4:6	100			
29	3:7	100			
30	2:8	100			
31	1:9	100			
32-33	100%MeOH	200			

All fractions were analyzed, and fractions 8-33 showed colored spots on TLC visualized after dipping in iodine vapor.

Fractions 12 (2-2Bf₁₂) (875 mg) was adsorbed and re-chromatographed over silica gel column chromatography using n-hexane:EtOAc (8:2) with constant ratio (isocratic system) to furnish 24 sub-fractions. Those fractions showing similar profile on their TLC (n-hexane:EtOAc as a mobile phase) were combined together to give 3 combined fractions. Among these, fraction 12-22 showed single spot on TLC and were dried to afford 46 mg white solid crystal labeled as **Compound-1**. The whole procedure was shown in (Table 2).

Table 2: Column chromatographic fractionation of 2-2Bf₁₂.

Fractions	Eluent	Ratio%	Volume collected	Amount	Code
			in mL	in mg	
FS 1-11	n-hexane:EtOAc	8:2	220		-
FS 12-22	n-hexane:EtOAc	8:2	220	46	Compound-1
FS 23-24	n-hexane:EtOAc	8:2	40		-

Fractions 18 (2-2Bf₁₈) (433 mg) which showed three spots on TLC was adsorbed and re-chromatographed over silica gel using CH₂Cl₂:MeOH (85:15) with constant ratio (isocratic system) as a mobile phase to furnish 18 sub-fractions. Those fractions showing similar profile on their TLC were combined together to give 14 combined fractions. Fraction 5 and 11 which showed single spots on their respective TLC was dried to afford 66 and 23 mg yellow crystal labeled as **compound-2** and **compound-3**, respectively. The whole procedure was shown in (Table-3).

Table 3: Column chromatographic fractionation of fraction 18 (2-2Bf₁₈)

Fractions	Volume collected in mL	Amount in mg	Code	Fractions	Volume collected in mL	Amount in mg	Code
FS 1-2	20			FS 10	10	16	
FS 3-4	20			FS 11	10	23	Comp-3
FS 5	10	66	Comp-2	FS 13	10	42	
FS 6	10	23		FS 14	10	31	
FS 7	10	31		FS 15-16	20	8	
FS 8-9	20	118		FS 17-18	20	2	

3.5. Phytochemical Screening

To the best of our knowledge there was no previous phytochemical report on the root barks of the plant. The preliminary phytochemical screening was carried out for the crude extracts (n-hexane, ethyl acetate and methanol) of the root barks of *G. involucrata* was carried out by using standard procedure [80-82] to analyze the presence of compounds namely, saponins, flavonoids, phenols, alkaloids, tannins, terpenoids, and glycosides.

3.5.1. Alkaloids

The extract (5 mL) was mixed with 2 mL of HCl. To this acidic medium, 1 mL of Wagner's reagent was added to the mixture. A reddish brown precipitate was observed.

3.5.2. Flavonoids

Shinoda Test (NaOH test): Dilute ammonia (5 mL) was added to 5 mL of extract and then 5 mL concentrated sulfuric acid was added. Formation of yellow color was observed.

3.5.3. Saponins

Froth test: The extract (0.5 g) was dissolved in 5 mL of distilled water. The mixture was shook vigorously. Formation of stable persistent froth was formed.

3.5.4. Tannins

Ferric chloride test: The extract (0.5 g) was dissolved in 10 mL of distilled water, and then a few drops of 1% ferric chloride solution were added to give brownish green or a blue-black coloration.

3.5.5. Terpenoids

The extract (5 mL) was mixed with 2 mL of chloroform and 3 mL of concentrated H_2SO_4 was then added to form a layer. A reddish brown precipitate color formed at the interface indicated the presence of terpenoids.

3.5.6. Phenolics

Ferric chloride test: To 5 mL of extract two drops of 1% ferric chloride was added. Formation of blue green color was indicated the presence of phenolic.

3.5.7. Glycosides:

Crude extract (2 mg) was dissolved separately in 2 mL of methanol. 5 mL of 50% HCl was added to 2 mL of the extract in test tubes. The mixtures were heated in a boiling water bath for 30 min. 2 mL of Fehling's solution was added and the mixtures were boiled for 5 min to give a brick red precipitate as an indication for the presence of glycosides.

3.6. Evaluating Biological Activities of Extract and Isolated Compounds of the Root barks of *Gindia involucrata*

3.6.1. Antioxidant Activities.

Antioxidant activity of the ethyl acetate, MeOH and isolated compounds were determined using DPPH method [83, 84]. Four different concentrations of the MeOH extract were added to four different vials containing MeOH to afford 500, 250, 125 and 62 µg/mL. To each 1 mL of these solutions, each 4 mL of 0.04% DPPH solutions in MeOH were added to make the resulting solution 100, 50, 25 and 12 µg/mL. The resulting mixtures were placed in an oven at 37°C for 30 minutes, and subjected to UV-Vis spectrophotometer to record absorbance at 517 nm. Similar procedure was followed for the EtOAc extract, **compound-1** and **compound-2**. The absorbance of 0.04% DPPH in MeOH solution was found to be 1.06. The percentage DPPH inhibition was calculated according to the following formula

$$\%Inhibition = \frac{Abs. control - Abs. sample}{Abs. control} \times 100\%$$

3.6.2. Antibacterial Activities

The antibacterial activities of the crude ethyl acetate extract and isolated compounds of the root barks of *G. involucrata* were tested against four bacterial strains [85]: one Gram positive and three Gram negative bacterial pure cultures using the disc diffusion method. The organisms used in the present study include *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis* and *Klebsiella pneumonia* that were obtained from Oromia public health research, capacity building and quality assurance.

Chloroform was used as a negative control whereas Ciprofloxacin was used as standard antibacterial drug (positive control) at 1.5 mg/mL. From inhibition zone data, the antimicrobial

activities of the extracts and compounds were critically examined by comparing the inhibition diameters and relating them to the control. The test bacterial species were transferred from the stock cultures and streaked on Mueller Hinton plates and incubated for 24 hrs at 37°C. Well-separated bacterial colonies were then used as inoculums. Bacteria were transferred using bacteriological loop to autoclaved Mueller Hinton agar that were cooled to about 45°C in a water bath and mixed by gently swirling the flasks. The medium was then poured to sterile Petri dishes, allowed to solidify and used for the bio test. A 0.3 mg of the crude extract and isolated compounds were dissolved in chloroform (200 µL), 1.5 mg/mL concentrations were prepared for the extract and for each **compound-1**, **compound-2**, and **compound-3**.

4. Results and Discussion

4.1. Extract Yield

Each were then filtered and concentrated under reduced pressure using rotary evaporator to afford 2.35 g 12 g and 18.15 g of n-hexane, ethyl acetate and MeOH extracts, respectively. The root barks of *G. involucrata* after successive extraction with n-hexane, EtOAc and MeOH furnished 2.34 (0.78%), 12 (4%) and 18.15 g (6.05%), respectively. The crude extract of n-hexane, EtOAc and methanol were obtained as light yellow, yellow and dark yellow, respectively. The methanol extract was found to have higher yield than both the ethyl acetate and hexane extracts. This indicates that the secondary metabolites present in the root barks of *G. involucrata* are polar organic compounds.

4.2. TLC Profile of the Extracts

The hexane, EtOAc and MeOH extracts were analysed using TLC with the results presented in Figure 4. The TLC profile of the EtOAc extract showed the presence of many spots for further analysis

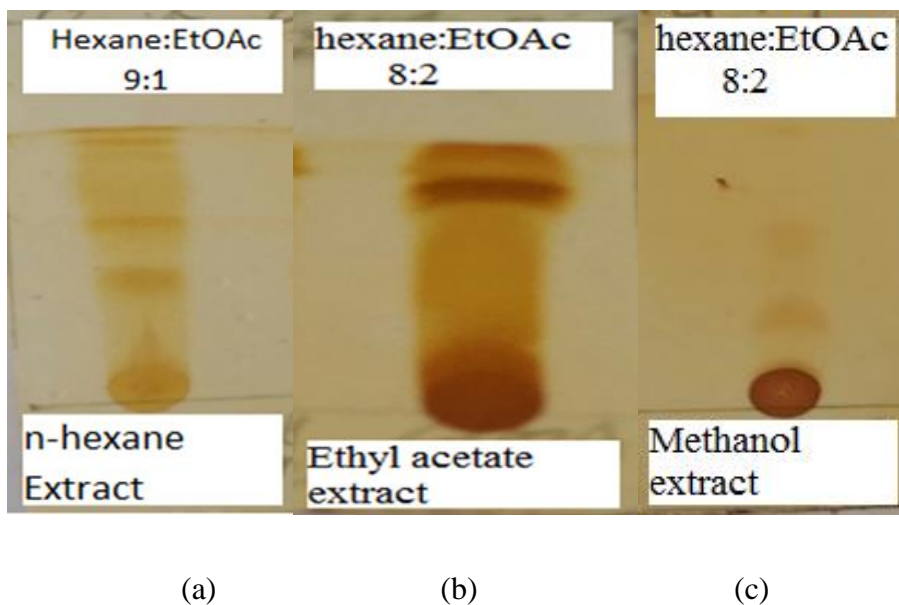


Figure 4: TLC profile of the n- hexane (a), EtOAc (b) and MeOH (c) extracts

4.3. Phytochemical Screening

The n-hexane, ethyl acetate and methanol extracts of the root bark of *Gnidia involucrata* were screened for the presence or absence of secondary metabolites. Results showed that the ethyl acetate extract of the root barks were found to have flavonoid, terpenoids, tannins, phenolic, glycosides and alkaloids. On the other hand the methanol extract of the root barks revealed the presence of saponin, tannin, flavonoid, terpenoids, glycosides and phenol. The presence of these secondary metabolites in the root bark may accounts for the traditional use of the root barks of this species.

Table 4: Results of qualitative test for phytoconstituents

Test	Alkaloid	Flavonoid	Saponins	Tannins	Phenolics	Terpenoids	Glycosides
n-Hexane extract	-	-	-	-	-	-	-
EtOAc extract	+	+	+	+	+	+	+
MeOH extract	-	+	+	+	+	+	+

Where -ve: absence, +ve: presence

As clearly seen in Table 4, the root barks of *G. involucrata* contain phenolics and terpenoids. Terpenoids were reported to have antimicrobial, anticarcinogenic, antimalarial and diuretics activity [86, 87]. Similarly phenolics were reported to have, the potential in the inhibition of carcinogenesis, antioxidant and are also thought to prevent heart ailments to an appreciable degree and sometimes are antiinflammatory agents [88, 89]. The presences of these secondary metabolites in this species are one positive aspect of this plant to act as antibacterial, antimalarial and antioxidants. Furthermore, the natural phenolic and flavonoid compounds can replace the synthetic material in foods or medicines which are responsible to carcinogenicity [90]

4.4. Characterization of the Isolated Compounds

The ethyl acetate extract of the root barks of *G. involucrata* was adsorbed and applied over silica gel packed column chromatography using n-hexane, and eluted with n-hexane:EtOAc:MeOH of increasing polarities as described in **Section 3.3**. In the course of this work, three compounds were isolated with their characterization described as follows.

4.4.1. Characterization of compound-1

Compound-1 (Figure 6) was isolated as a white crystal from the ethyl acetate extract of the root barks of *G. involucrata*. Its TLC profile (Figure 5) showed spot at R_f value of 0.42 using n-hexane:EtOAc (7:3) as a mobile phase. The spot was visualized after dipping in iodine. The IR spectrum of **compound-1** (Appendix 1) showed the presence of hydroxyl stretching at 3487 and 3328 cm^{-1} . The strong absorption band at 2925 cm^{-1} indicated the presence of the C-H stretching for alkyl groups. The absorption band at 1684 cm^{-1} showed the presence of conjugated C=O moiety. Its IR spectrum also revealed the presence of the C-H bending at 1439 cm^{-1} . The absorption band at 1281 cm^{-1} indicated the presence of the C-O bond stretching.



Figure 5: TLC profile of **compound-1**

The ^1H -NMR spectrum (Table 5, Appendix 2) of **compound-1** indicated the presence of three protons on aromatic ring at δ_{H} 6.88 (1H, *d*, $J = 8.0$ Hz, H-5), 7.0 (1H, *d*, $J = 8.0$ Hz, H-6), and 7.13 (1H, *s*, H-2). Also observed signals in the ^1H -NMR spectrum are at δ_{H} 6.25 (1H, *d*, $J = 16\text{Hz}$, H-8), and 7.57 (1H, *d*, $J = 16\text{Hz}$, H-7) indicating the presence of *trans* double bond. Hence the proton NMR spectral data observed in the aromatic region in combination with the signals due to hydrogen on the *trans* double bond is a clear indication for the presence of caffeic acid moiety. A triplet signal at δ_{H} 4.19 (2H, *t*) confirmed the existence of oxygenated methylene protons. The spectrum also showed signals integrating for 50 hydrogens centered at δ_{H} 1.27 indicating the presence of protons on many overlapping methylene groups. The signal at δ_{H} 1.732 (4H) and δ_{H} 0.90 (10H) revealed the presence of hydrogens on two and five methylene groups, respectively.

The proton decoupled ^{13}C -NMR spectrum (Appendix 3) of **compound-1** with the aid of DEPT-135 (Appendix 4) showed the presence of 5 methine, 33 methylene, 1 methyl and 4 quaternary carbons. The downfield signal observed at δ 168.0 is evident for the presence of α,β -unsaturated carbonyl group. The *trans* double bond was evident at δ 143.9 and 115.5. Other signals observed in the aromatic region are δ 127.4, 115.4, 145.0, 146.5, 114.4 and 122.3. Of these the signals situated at δ 145.0, 146.5 and 127.4 are accounted to quaternary carbons. The above ^{13}C -NMR spectral data is evident for the presence of caffeic acid moiety which supports the ^1H -NMR spectrum. The signal observed at δ 64.9 is due to an oxygenated methylene carbon. The signal accounted for 33 carbons were observed in the region between δ_{C} 31.0 to 14.1. The proton decoupled ^{13}C -NMR spectrum of **compound-1** was very consistent with the reported data of caffeic acid ester, heptatriacontanyl caffeate extracted from the leaves of *Artemisia argyi* except for the long chain alcohol component [86]. The comparison of the NMR spectral data of **compound-1** with the partial skeleton reported in the literature is depicted in Table 5.

Table 5: Comparison of $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and DEPT-135 (CDCl_3) spectral data of **compound-1** and reference data

No.	$^1\text{H NMR}$ data reported in literature [86]	$^1\text{H NMR}$ data of compound-1	Literature report [86]	$^{13}\text{C-NMR } \delta$ (ppm) of compound-1	Remark
1		-	127.6	127.4	Q
2	7.16 (1H, <i>d</i> , $J = 1.8$)	7.13 (1H, <i>br.s</i>)	115.2	114.4	CH
3	-	-	146.2	145	Q
4	-	-	148.6	146.5	Q
5	6.87 (1H, <i>d</i> , $J = 8.0$)	6.88 (1H, <i>d</i> , $J = 8$)	116.3	115.4	CH
6	7.04 (1H, <i>dd</i> , $J = 8.0, 1.8$)	7.0 (1H, <i>d</i> , $J = 8$)	122.4	122.3	CH
7	7.53 (1H, <i>d</i> , $J = 15.9$)	7.57 (1H. <i>d</i> , $J = 15.6$)	145.5	143.9	CH
8	6.28 (1H, <i>d</i> , $J = 15.9$)	6.25 (1H, <i>d</i> , $J = 15.6$)	115.8	115.5	CH
9		-	167.4	168.0	Q
1'	4.14 (2H, <i>t</i> , $J = 6.7$)	4.19 (2H, <i>t</i>)	64.6	64.9	CH_2
2'	1.68 (<i>m</i>)	1.715(2H, <i>m</i>) overlap	32.6	31.9	CH_2
3'		1.71(2H, <i>m</i>) overlap		29.7	CH_2
4'-33'		1.276-0.88(2H, <i>br. s</i>) overlap		29.7-22.68	CH_2
34'		0.88(3H, <i>t</i>)	14.3	14.1	CH_3

Based on the above NMR spectral data the following structure was proposed for the compound **compound-1**.

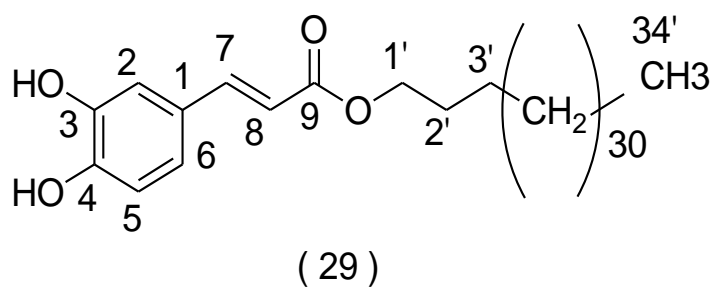


Figure 6: Proposed structure for **compound-1** (29).

Literature reported that caffeic acid and its ester derivatives is widely distributed in the plant kingdom and exhibit a broad spectrum of biological activities, including anti-inflammatory [92], antimicrobial [93], antioxidant [94], antiviral and anticarcinogenic effects [95]. In addition, some studies showed that the alkyl ester derivatives of caffeic acid have high leishmanicidal activity and high cytotoxicity [96]. Therefore the presence of tetratriacontanyl caffeate adds positive attributes to the root barks of *G. involucrata*.

4.4.2. Characterization of the Compound-2

Compound-2 (Figure 8) was obtained as a yellow crystal from the ethyl acetate extract of the root barks of *G. involucrata*. Its TLC profile (Figure 7) showed spot at R_f value of 0.56 using dichloromethane: methanol (8.5:1.5) as a mobile phase. The spot was visualized after dipping in iodine.



Figure 7: TLC profile of the **compound-2**

IR spectrum of **compound-2** (Appendix 5), showed the presence of hydroxyl stretching at 3415 cm^{-1} . The strong absorption band at 2925 cm^{-1} and 1714 cm^{-1} were indicating the presence of the C-H stretching for alkyl groups, and α,β -conjugated carbonyl carbon respectively. The absorption band at 1641 cm^{-1} and 1259.9 cm^{-1} showed olefinic functionalities and C-O bond

stretching respectively. Its IR spectrum also revealed the presence of the C-H bending at 1375 cm^{-1} .

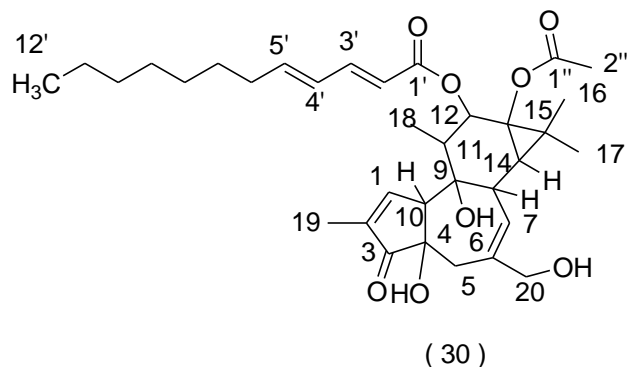
The ^1H -NMR spectrum (Table 6, Appendix 6) **compound-2** indicated the presence of four olefinic protons on ester chain at δ_{H} 5.76 (1H, *d*, $J = 15.2$ Hz, H-2'), 6.16 (1H, *m*, H-5'), 5.69 (1H, *br.s*, H-4') overlap, and 7.21 (1H, *dd*, $J = 10, 10$ Hz, H-3'). Signals in the ^1H -NMR spectrum at δ_{H} [3.95 (1H, *d*, $J=13.2$ Hz), 4.02 (1H, *d*, $J=12.8$ Hz), H-20], indicating the presence of oxygenated methylene protons in different chemical environments. The signals at δ_{H} [2.47 (1H, *d*, $J=19.2$ Hz), 2.59 (1H, *d*, $J=19.2$ Hz), H-5] also indicating the presence of methylene protons in different chemical environments. The spectrum showed the presence six methyl protons at δ_{H} 2.10 (3H, *s* H-2''), 1.74 (3H, *s* H-19), 1.25 (3H, *s* H-16) overlap, 1.2 (3H, *s* H-17), and, 0.88 (3H, *t* H-12' and H-18) overlapped. The spectrum also showed signals integrating for 8 hydrogens centered at δ_{H} 1.27 indicating the presence of protons on many overlapping methylene groups.

The proton decoupled ^{13}C -NMR spectrum (Appendix 7) of **compound-2** with the aid of DEPT-135 (Appendix 8) showed the presence of 11 methine, 8 methylene, 6 methyl and 9 quaternary carbons. The downfield signal observed at δ 209.3 is evident for the presence of α , β -unsaturated carbonyl group. The carbon signal at δ 173.9 and 167.2 are evident for the presence of two ester moieties. The signals observed at δ 118.8, 145.3, 128.3 and 145.7 evident for the olefinic groups. Of these the signals situated at δ 132.8, 209.3, 73.7, 140.7, 78.4, 65.7, 25.7, 167.2 and 173.9 are accounted to quaternary carbons. The ^{13}C -NMR spectral data (Table 6) of **compound-2** were very similar to those of prostratin **Q** extracted from *Wikstroemia chamaedaphne* [97], a phorbol-type diterpenoid isolated as a major compound, with long chain aliphatic ester of $\text{C}_{10}\text{H}_{15}\text{O}_2$, except for an additional two methylene groups to the long chain aliphatic ester, and aquimavitalin extracted from *Aquilaria malaccensis* except of the length of the fatty acid moiety and number of olefinic protons [98]. The comparison of the NMR spectral data of **compound-2** with the partial skeleton reported in the literature is depicted in Table 6.

Table 6: Comparison of ^1H -NMR, ^{13}C -NMR and DEPT-135 (CDCl_3) spectral data of **compound-2** and reference data

No	literature ^1H -NMR reported data[97]	^1H NMR δ (in ppm) of GIA-2	^{13}C -NMR literature data [97]	^{13}C -NMR δ (ppm) of compound-2	Rem ark
1	7.57 (1H, <i>s</i>)	7.58(1H, <i>s</i>)	161	160.8	CH
2		-	133	132.8	Q
3		-	209.1	209.3	Q
4		-	74	73.7	Q
5a	2.46 (1H, <i>d</i>)	2.47(1H, <i>d</i>)	38.9	38.4	CH2
5b	2.53 (1H, <i>d</i>)	2.59(1H, <i>d</i>)			
6		-	140.7	140.7	Q
7	5.66 (1H, <i>d</i>)	5.69(1H,br. <i>s</i>)o.lap	129.5	129.2	CH
8	3.22 (1H, <i>dd</i>)	3.29 (1H, <i>m</i>)	39.3	38.9	CH
9		-	78.4	78.4	Q
10	3.23 (1H, <i>s</i>)	3.24(1H,br. <i>s</i>) o.lap	56.4	56	CH
11	2.15(1H, <i>m</i>)o.lap	2.16(1H, <i>m</i>)overlap	43.4	43	CH
12	5.44 (1H, <i>d</i>)	5.43(1H, <i>d</i>)	76.8	76.6	CH
13		-	66	65.7	Q
14	1.08(1H, <i>d</i>)	1.09(1H, <i>d</i>)	36.6	36.3	CH
15		-	26	25.7	Q
16	1.24 (3H, <i>s</i>)	1.25(3H, <i>s</i>)overlap	24	23.8	CH3
17	1.19 (3H, <i>s</i>)	1.2(3H, <i>s</i>)overlap	17	16.8	CH3
18	0.87 (3H, <i>d</i>)	0.88(3H, <i>d</i>)overlap	14.6	14.4	CH3
19	1.75 (3H, <i>d</i>)	1.74(3H, <i>s</i>)	10.3	10.1	CH3
20a	3.97 (1H, <i>d</i>)	3.95(1H, <i>d</i>)	68.3	68	CH2
20b	4.02 (1H, <i>d</i>)	4.02(1H, <i>d</i>)			
1'		-	167.3	167.2	Q
2'	5.76 (1H, <i>d</i>)	5.76(1H, <i>d</i>)	119.1	118.8	CH
3'	7.21 (1H, <i>dd</i>)	7.21(1H, <i>dd</i>)	145.8	145.7	CH
4'	6.16 (1H, <i>dd</i>)	5.69(1H,br. <i>s</i>)o.lap	128.5	128.3	CH
5'	6.13 (1H, <i>m</i>)	6.16(1H, <i>m</i>)	145.5	145.3	CH
6'	2.13 (2H, <i>m</i>)	2.14(2H, <i>m</i>)overlap	33.3	33	CH2
7'	1.41 (2H, <i>m</i>)	1.4(2H, <i>m</i>)	28.6	32.9	CH2
8'-11'	Partial.1.28 (<i>m</i>)	1.25(2H, <i>m</i>)	31.6	31.8- 22.6	CH2
12'	0.86 (3H, <i>t</i>)	0.88(3H, <i>t</i>)overlap	14.2	14.1	CH ₃
1''			174	173.9	Q
2''	2.08 (3H, <i>s</i>)	2.10(3H, <i>s</i>)	21.3	21.1	CH ₃

Based on the above NMR data the following structure was proposed for **compound-2**.



12-O-dodeca-2,4-dienoyl phorbol-13-acetate

Figure 8: Proposed structure for **compound-2** (30).

Prostratin (12-O-phorbol-13-acetate) is a non-tumor promoting phorbol ester, reported to inhibit HIV-1 cell entry and replication, blocks completion of reverse transcription of the HIV-1 genome in lymphoid tissue, infection of CD4⁺ T lymphocytes and at the same time reactivates virus from latency, restricts primary resting of CD4⁺ T-cell susceptibility to HIV-1 infection in primary blood mononuclear cells (PBMC) and in lymphoid tissue [99,100], antireplicative and anticytopathic activities against HIV [101], inhibits ornithine decarboxylase induction, edema and hyperplasia [102]. 12-O-dodeca-2,4-dienoyl phorbol-13-acetate which was isolated from this plant in this work may exhibit activities displayed by those compounds containing prostratin nucleus. Therefore this adds a positive attributes to the root barks of *G. involucrata*.

4.4.3. Characterization of compound-3

Compound-3 (Figure 10) was obtained as pale yellow needles from the ethyl acetate extract of the root barks of *G. involucrata*. Its TLC profile (Figure 9) showed spot at R_f value of 0.60 using dichloromethane: methanol (85:15) as a mobile phase. The spot was visualized after dipping in iodine.



Figure 9: TLC profile of the compound **compound-3**

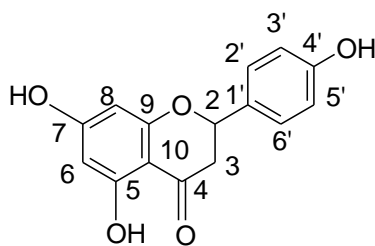
IR spectrum of **compound-3** (Appendix 9), showed the presence of hydroxyl stretching at 3328.6 cm^{-1} . The strong absorption band at 1641 cm^{-1} was indicating the presence of the carbonyl carbon.

The proton decoupled ^{13}C -NMR spectrum (Appendix 10) of **compound-3** with the aid of DEPT-135 (Appendix 11) showed the presence of 7 methine, 1 methylene and 7 quaternary carbons. The downfield signal observed at δ 197.2 is evident for the presence of carbonyl group. The signal at δ 47.9 was indicative of the presence of aliphatic carbon in the ring. Other signals were observed at δ 83.4, 164.5, 96.1, 166.1, 94.8, 163.3, 101.9, 128.5, 129, 114.9, and 157.6. Of these the signals situated at δ 197.2, 164.5, 166.1, 163.3, 101.9, 128.5, and 157.6 were accounted to quaternary carbons. The proton decoupled ^{13}C -NMR spectrum of **compound-3** (Table 8, Appendix 10) was consistent with the reported data of naringenin extracted from *Nyctanthes arbortristis* [103, 104]. The comparison of the NMR spectral data of **compound-3** with the naringenin reported in the literature is depicted in Table 7.

Table 7: Comparison of ^{13}C -NMR and DEPT-135 (Acetone-*d*-6) spectral data of **compound-3** and literature reported data for **Naringenin** [103]

No.	^{13}C -NMR literature	^{13}C -NMR GIA-3	Remark
2	80	83.4	CH
3	43.9	47.9	CH ₂
4	197.2	197.2	Q
5	165.2	164.5	Q
6	97.8	96.1	CH
7	165.9	166.1	Q
8	96.1	94.8	CH
9	162.9	163.3	Q
10	102.6	101.9	Q
1'	128.9	128.5	Q
2'	129.9	129	CH
3'	115.9	114.9	CH
4'	158.6	157.6	Q
5'	115.9	114.9	CH
6'	129.9	129	CH

Based on the above NMR data the following structure was proposed for the compound **compound-3**.



(31)

5,7-dihydroxy-2-[4'-hydroxy phenyl] flavanone

Figure 10: Proposed structure for **compound-3** (31).

Naringenin, a flavanone is reported to possess tyrosinase inhibitory activity [103-105], antiviral activity [106], antioxidant, anticarcinogenic and blood lowering lipid activities [107]. Therefore the presence of Naringenin also adds positive attributes to the root barks of *G. involucrata*.

4.5. Biological Activities of EtOAc Extract and Isolated Compounds of the Root barks of *Gindia involucrata*.

4.5.1. Antioxidant Activities

The ethyl acetate and methanol extracts, **compound-1** and **compound-2** of the root barks of *G. involucrata* were assessed for its radical scavenging activities. The DPPH radical scavenging activity of ethyl acetate and methanol extract, **compound-1** and **compound-2** were found to be 70.7%, 66.9%, 85.8% and 52.8% at 100 µg/mL respectively (Table 8 and Figure 11). **Compound-1** was the most active species checked in this sample and likely accounts to the activity displayed by the EtOAc extract. Its radical scavenging activity was found comparable with ascorbic acid (97%). The activity displayed by **compound-1** is likely due to the presence of phenolic hydroxyl groups. Therefore, ethyl acetate extract and **compound-1** may be used for the treatment of various life threatening diseases caused by free radicals.

Table 8: DPPH radical scavenging assay of EtOAc and MeOH extracts, **compound-1** and **compound-2**

Sample code	Concentration(µg/mL)	Absorbance	% Inhibition	Sample code	Concentration (µg/mL)	Absorbance	% Inhibition
EtOAc extract	100	0.31	70.7	comp-1	100	0.15	85.84
	50	0.38	64.1		50	0.22	79.2
	25	0.45	57.54		25	0.28	73.58
	12	0.57	46.2		12	0.37	65
MeOH extract	100	0.35	66.98	comp-2	100	0.50	52.8
	50	0.41	61.3		50	0.62	41.5
	25	0.48	54.7		25	0.69	34.9
	12	0.62	41.5		12	0.88	16.98

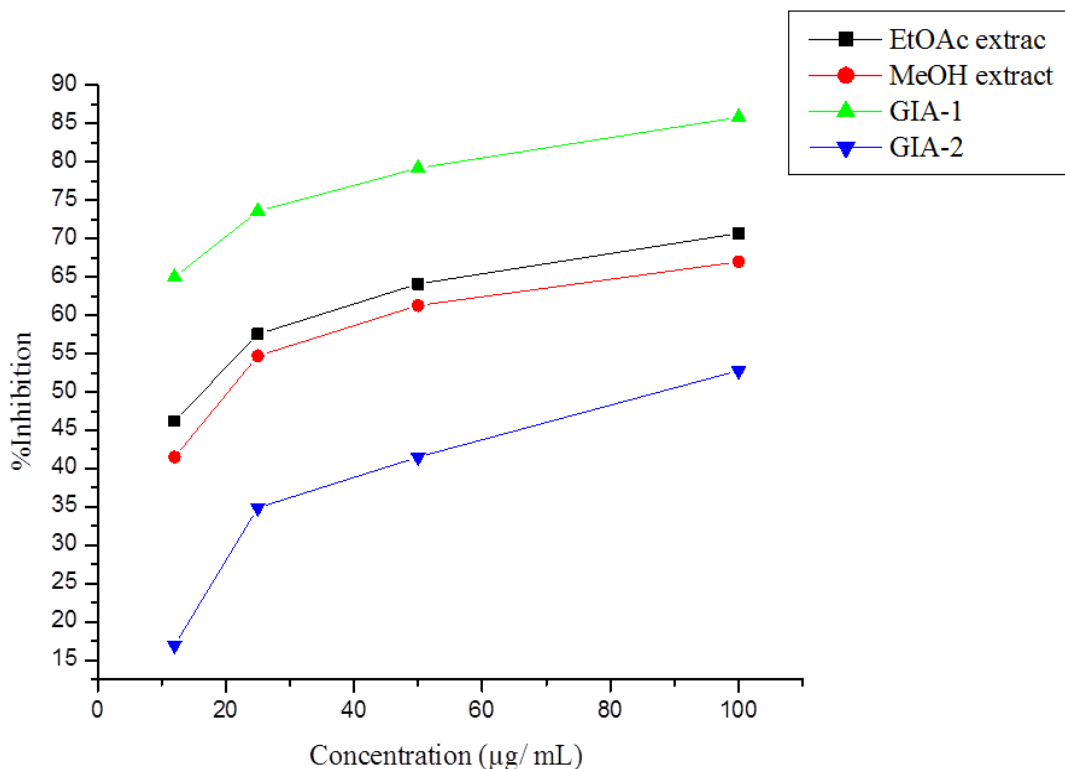


Figure 11: Percentage inhibition of **DPPH** radical EtOAc, MeOH, extract **compound-1** and **compound-2** of *G. involucrata*.

The IC_{50} , the concentration of the sample that inhibits 50% of the radical values of EtOAc and MeOH extract, **compound-1** and **compound-2** were 15.3, 21.8, 2.3, and 83.6 $\mu\text{g/mL}$, respectively. The lower IC_{50} value displayed by **compound-1** indicates its strong radical scavenging activity compared to the ascorbic acid.

4.5.2. Antibacterial Activities

Results of the antibacterial activity by the disc diffusion method of the ethyl acetate extract and all isolated compounds of *G. involucrata* along with the reference drug Ciprofloxacin are presented in Table 9.

Table 9: Zone of bacterial growth inhibition (mm) for ethyl acetate extract and isolated compounds from the root barks of *G. involucrata*

Sample	Concentration (mg/mL)	Zone of bacterial growth inhibition (mm)			
		Gram-positive	Gram-negative bacteria		
		<i>S. aureus</i>	<i>P. mirabilis</i>	<i>E. coli</i>	<i>Klebsiella pneumoniae</i>
Ethyl acetate extract	1.5	23 mm(s)	12 mm(s)	14 mm(s)	12 mm(s)
compound-1	1.5	6 mm(R)	6 mm(R)	6 mm(R)	6 mm(R)
compound-2	1.5	8 mm(s)	6 mm(R)	11mm(s)	6mm(R)
compound-3	1.5	6 mm(R)	7 mm(s)	12 mm(s)	8 mm(s)
Ciprofloxacin	1.5	23	24	21	19

Key: >6mm= sensitive =S, =6mm=Resistance=R

As shown in Table 9 antibacterial test of ethyl acetate extract shows effect against all tested bacteria with a concentration of 1.5 mg/mL, especially highly active against *S. aureus*, *E. coli* and moderately active on *P. mirabilis* and *Klebsiella pneumoniae* with inhibition zones 23, 14, 12, and 12mm respectively (Figure 12).

The results from the present study showed that the isolated compound **compound-1** was inactive in all tested bacteria. At 1.5 mg/mL concentration, the compound **compound-2** was found to be active against *S. aureus* and in one gram negative, *E. coli*; however, inactive against *Proteus mirabilis* and *Klebsiella pneumoniae*. At 1.5 mg/mL concentration, **compound-3** was also found active against all tested bacteria except one gram-positive; *S. aureus*. When compared to the

standard commercial antimicrobial agent (Ciprofloxacin) used, the ethyl acetate extract expressed potential antibacterial activity against the respective sensitive strains used in the present study and their zones of inhibitions was also prominent (Table 9). The activities displayed by the ethyl acetate extracts of the root barks and isolated compounds were found to be significant as compared to the negative control.

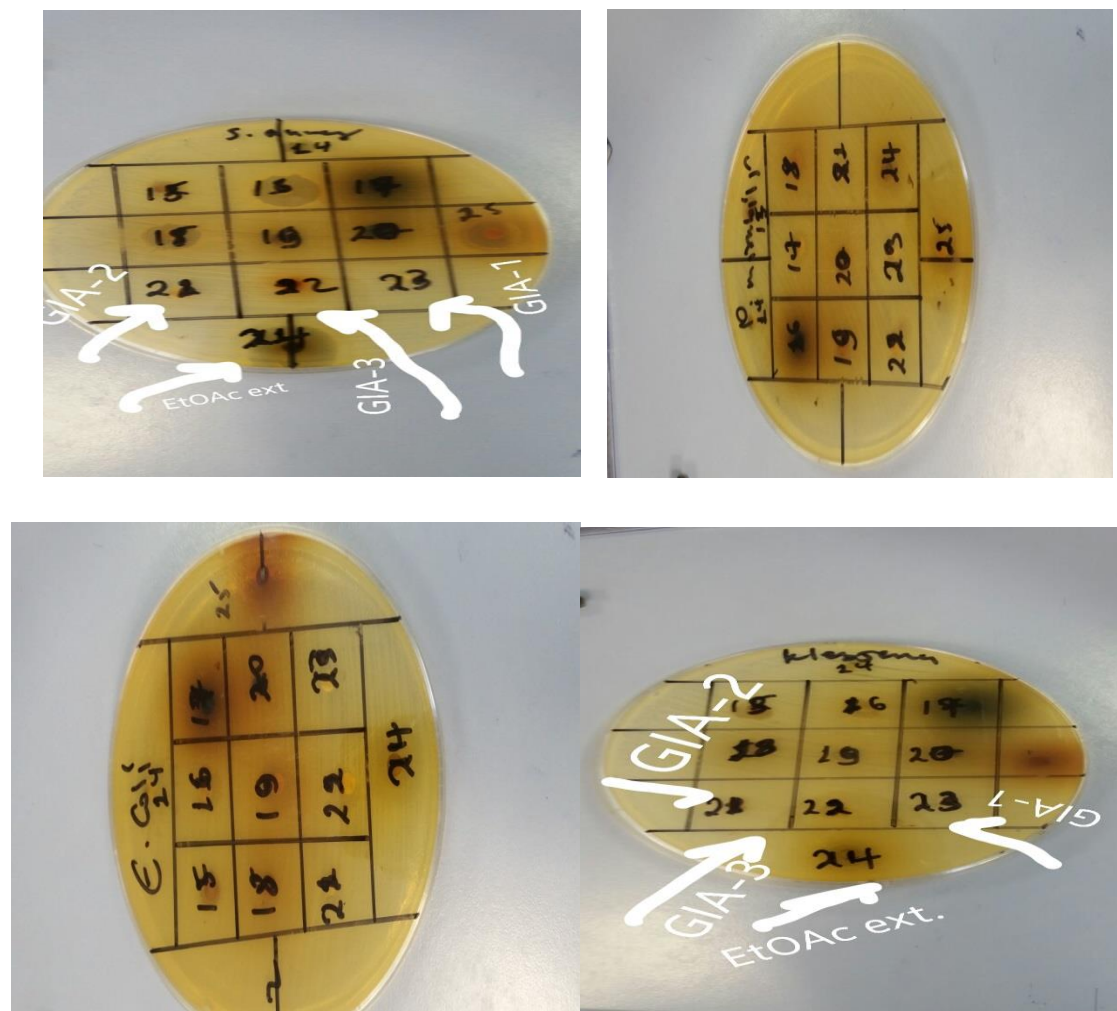


Figure 12: Zone of bacterial growth inhibition (mm).

5. CONCLUSIONS

For decades, traditional medicine has been used and continues to be an alternative approach on treatment for various diseases. Currently, the growing interest of consumers in substances of natural origin in association with the increasing concern surrounding potentially harmful infections and disease has directed to a rising interest in the use of plant extracts as functional ingredients in many pharmaceutical products. Plants serve as a vast reservoir of many complex organic compounds, many of which, at least at our current stage of knowledge, appear to have no direct function for the growth and development of the plant. These compounds are now known as secondary metabolites. These are including alkaloids, saponins, tannins, flavonoids, phenolics, steroids and terpenoids. Previously there was no report on phytochemical screening, the presence or absence of these secondary metabolites in *G. involucrata* plant. The phytochemical screening tests for the major secondary metabolites were performed for the first time in this research work. From the study, the root barks extracts were found to contain saponins, tannins, terpenoids, phenolics, alkaloids, glycosides and flavonoids. The presence of these important phytochemicals in the plant has a scientific justification of the plant use in the traditional treatment against various diseases affecting humans and animals. The presence of these phytochemicals in this plant enhances their pharmaceutical and therapeutic potentials.

The ethyl acetate crude extract was subjected to silica gel column chromatography to give three pure compounds. The chemical structures of these compounds were characterized on the basis of NMR spectral analysis, including $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ in comparison with literature data. The isolated compounds, coded as **compound-1**, **compound-2** and **compound-3** were characterized as Tetratriacontanyl caffeate, 12-O-dodeca-2,4-dienoylphorbol-13-acetate and 5,7-dihydroxy-2-(4'-hydroxy phenyl) flavanone. The antibacterial activity of the ethyl acetate crude extract showed promising activity in all tested bacteria at 1.5 mg/mL. The extract and isolated compounds **compound-1** also displayed strong radical scavenging activity compared to ascorbic acid (97%). The lower IC_{50} value displayed by **compound-1** (2.3) indicates its strong radical scavenging activity compared to the ascorbic acid.

6. Recommendations

The present study showed that the plant is rich in different secondary metabolites. Thus, with further detailed work and fractionation of crude extract, the plant could give several novel compounds that can be used as candidates for the development of antibacterial and antioxidant drugs. Therefore, we recommend other researchers to concentrate on the ethyl acetate and methanol extracts since based on the yield and TLC analysis of spots. These extracts have more yields and several compounds from its root barks. Further studies are needed on this plant species to characterize and elucidate structures of more bioactive compounds. As demonstrated by plant in this study, there was considerable evidence that the plant extracts have the potential to be developed into agents that can be used as treatment for different diseases. The ethyl acetate extracts of the plant could be used to treat disease caused by bacterial (*S. aureus* and *E. coli*) infection. In addition to this the following recommendations were made.

- Further phytochemicals and biological activity studies should also be conducted on this plant to isolate the specific antioxidant and antimicrobial activity in it.
- Further bioassay guided isolation and characterization work should be done to fully characterize more bioactive constituents of the plant root barks extracts to identify all active compounds against a variety of bacteria.
- It is recommended that further similar studies should be conducted on other parts of the plant such as leaves, stem, stem bark, and flower.
- This plant should be studied more extensively to explore its activity on virus, cancer, malaria, stomach parasite and allergic.
- Toxicity studies of the plant should also be done to determine the safety indices of the extracts.
- Relative stereochemistry analysis by NOE spectral analysis to know the relative orientation of substituents at nearby stereogenic centers.
- It is also recommended to have mass spectral data for **compound-1** and **compound-2**.

REFERENCES

1. Desalegn, D. (2000). Uses and conservation status of medicinal plants used by shinasha people. *J. Pharm.* **2**, 26-91.
2. Philipson, J.D. (2001). Phytochemistry and medicinal plants. *J. Phytochemistry*, **56**, 237-243.
3. Amit, L., Vikas, G., Vaibhav, T., Vikash, K., and Siddhartha, G. (2010). Phytochemistry and pharmacological activities of *Bersama engerina* Guerke-Anoverview. *J. Int.pharm.* **1**,89-94
4. WHO (2010). World Health Fact Sheet WHO, Geneva,Switzerland.
5. Maregesi, S.M., Ngassapa, O.P., Pieters, L., Vlietinck, A.J. (2007). Ethno pharmacological survey of the Bunda district, Tanzania: plants used to treat infectious diseases. *J. Ethnopharmacol.* **113**, 457-470.
6. Turnbull, J.W.(1986).Multipurpose Australian trees and shrubs; lesser-known species for fuel wood and agro forestry. Australian Centre for International Agricultural Research, Canberra, Australia, 316.
7. Sharaby, A., Abdel-Rahman, H., and Moawad, S. (2009). Biological effects of some natural and chemical compounds on the potato tuber moth, *Phthorimaea operculella* Zell. (Lepidoptera: Gelechiidae). *J. Biological Sciences.* **16**,1-9
8. Sachdev, K., Kulshreshtha, D.K., Aliarin. (1982). A New Flavonoid from *Dodonaea Viscosa*. *J. Indian Chemistry.* **21**, 798-9.
9. Walsh, N.G. and Entwisli, T.J.(1996). Flora of Victoria, Vol.3, Inkata Press, Melbourne, VIC.
10. Calixto, J.B. (2002). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (Phytotherapeutic agents). *Braz. J. Med. Biol. Res.* **33**, 179-189.
11. Barboza, G.E., Cantero, J.J., Nunez, C., Pacciaroni, A., Espinar, L.A. (2009). Medicinal plants: A general review and a phytochemical and ethno pharmacological screening of the native Argentine Flora. *J. Tomo*, **34**, 7-15.
12. Kassaye, K.D., Amberbir, A., Getachew, B., Mussem, Y. (2002). A historical overview

- of traditional medicine practices and policy in Ethiopia. *Ethiop. J. health Dev.* **20**, 127.
13. Shrivastava, N., Patel, T. (2007). Clerodendrum and health care: An overview- part II Phytochemistry and biotechnology. *Med. Aroma. J. Plant sci. Biotechnol.* **1**, 209-223.
 14. Duke, J, A. (1992). Handbook of phytochemical constituent of GRAS herbs and other economic plants, Boca Raton, FL. CRS press.
 15. Turnbull, J.W. (1986). Multipurpose Australian trees and shrubs; lesser-known species for fuel wood and agro forestry. Australian Centre for International Agricultural Research, Canberra, Australia, 316.
 16. Pankhurst, R. (1965). A Historical reflection on the traditional Ethiopian pharmacopeias. *J. of Ethio. Pharm. Association.* **2**, 29-33.
 17. Dawit Abebe and Ahadu Ayehu (1993). *Medicinal Plants and Enigmatic Health Practice of Northern Ethiopia*. B.S. P.E, Addis Ababa
 18. Miruste Giday (2001). *An Ethnobotanical Study of Medicinal Plants Used by Zay People in Ethiopia*. CBM: Shikrftserie, **3**, 81-99.
 19. Mekonnen Bishaw (1991). Promoting traditional medicine in Ethiopia: a brief historical overview of government policy. *Soc. Sci. and Med.* **33**,193-200
 20. Getachew Addis, Dawit Abebe, Timotewos Genebo, Kelbessa Urga (1999). Perception of modern and traditional health practitioners about traditional medicine in Shirka District, Arsi Zone, Ethiopia. *Ethiop.J. Health Dev.***16**,19-29.
 21. Lawal, I.O., Adesoga, A.A. (2010). Eethnomedicinal information on collection and identification of some medicinal plants in research Institutes of Southwest Nigeria. *J.Afri. Pharmacol.* **4**, 001-007.
 22. Calixto, J.B. (2002). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (Phytotherapeutic agents). *J. Braz. Med. Biol. Res.* **33**, 179-189.
 23. Ferrari,J.(2002).Contribution of the knowledge of the secondary metabolism of Thymelaeaceae and phytochemical investigation. Institute of Pharmacognosy and Phytochemistry.
 24. Parixit, B., Rajarajeshwari, N., Ganapaty, S., Santosh, P. (2013). The *Gnidia* genus: A review. *Asian J. of Biomed. and Pharm. Sciences.* **3 (19)**, 1-31.

25. Borris, R.P., Blasko, G., Cordell, G.A. (1988). Ethno pharmacologic and phytochemical studies of Thymelaeaceae. *J of Ethno pharm.* **24**, 41-91.
26. Miroslav, S., Svetozara, M., Kragujevac, S. (2016). Plants from the genus *Daphne*: review of its traditional uses, phytochemistry, biological and pharmacological activity. *Ser. J. Exp. Clin Res.* **18** (1): 69-79.
27. Evans, F.J., Soper, C.J. (1978). The tigliane, daphnane, ingenane diterpenes, their chemistry, distribution and biological activities, a review. *Lloydia.* **41**(3), 193-233.
28. Bruneton, J. (1999). Pharmacognosy, Phytochemistry, Medicinal Plants. 3rd edition, Technique & Documentation, Paris, pp. 274, 654-655.
29. Zayed, S., Adolf, W., Hafez, A., Hecker, E. (1977a). New highly irritating 1-alkyldaphane derivatives from several species of Thymelaeaceae. *Tetrahedron Lett.* 1977, 3481-3482.
30. Mungenge, C., Zimudzi, C.,imba, M., Nhiwatiwa, T. (2014). Phytochemical screening, cytotoxicity and insecticidal activity of the fish poison plant *Synaptolepis alternifolia* Oliv. (Thymelaeaceae). *J. of Pharm. and Phytochemistry.* **2** (5), 15-19
31. Brooks, G., Evans, F.J. (1990). Toxic Hazards of Plants from the Families Euphorbiaceae and Thymelaeaceae. Rizk, A.M. editor. Boca Raton: CRC Press. pp. 27-63.
32. Hafez, A., Adolf, W., Hecker, E. (1983). 'Active principles of the Thymelaeaceae. III. Skin irritant and cocarcinogenic factors from *Pimelea simplex*'. *Planta Medica.* **49**, pp. 3-8
33. Von Oettingen, W.F, editors. (1958). Poisoning – A Guide to Clinical Diagnosis and Treatment. 2nd ed. Philadelphia: W.B. Saunders Co. p. 322.
34. Tubery, P. (1968). Alcohol extract of *Lasiosiphon kraussian* plant having lymphocytic activity, for use against leprosy. *Patent, Br. M.* 6366 (CA **74**, 91170x).
35. Ren, C.P. (1978). Long-acting analgesic antidyne in anal operations. *Nat. Med. J. China.* **4**, 158-159.
36. Kasai, R., Lee, K.H., Huang, H.C. (1981). Genkwa daphnin, has potent anti-leukemic diterpenes from *Daphne genkwa*. *Phytochemistry.* **20**, 2592-2594.210.
37. Kupchan, S.M., Sweeny, J.G., Baxter, R.L., Murae, T., Zimmerly, V.A., et al. (1975).

- Gnididin, gniditrin, and gnidicin, novel potent antileukemic diterpenoid esters from *Gnidia lamprantha*. *J Am Chem Soc.* **97**,672-3.
38. Kupchan, S.M., Sweeny, J.G., Shen, T.M.M-S., Bryan, R.F. (1975). Structure of Gnidia coumarin, a Novel Pentacyclic Dicoumarin from *Gnidia lamprantha*. *J Chem Soc, Chem Com.* **211**. 94-95.
 39. Kupchan, S.M., Shizuri, Y., Murae, T., Sweeny, J.G., Haynes, R., et al. (1976). Gnidimacrin and gnidimacrin 20-palmitate, novel macrocyclic antileukemic diterpenoid esters from *Gnidia subcordata*. *J Am Chem Soc.* **98(18)**, 5719-20.
 40. Tandon, S., Rastogi, R.P. (1976). Wikstromol, a new lignan from *Wikstroemia viridiflora*. *Phytochemistry.***15**, 1789-1791.217
 41. Cashmore, A.R., Seelye, R.N., Cairn, B.F., Mack, H., Schmidt, R., Hecker. (1976). Prostatidine, tetracyclic diterpene ester from *Pimelea prostrata*, *Tetrahedron Lett.* 1735.
 42. Howard, H.T.C., Howden, M.E.H. (1975). Anti-tumor activity in *Pimelea simplex*. *Cancer Chemoth.Rep.* **59**, 585-586
 43. Michael, W., Deborah, D., and Bernard, W. (1978). Induction of Plasminogen Activator in Cultured Cells by Macrocyclic Plant Diterpene Esters and Other Agents Related to Tumor Promotion. *Cancer Res.***38**, 1434-1437.
 44. Hecker, E. (1981). Co-carcinogenesis and tumor promoters of the diterpene-ester type as possible carcinogenic risk factors. *J. Cancer Res. Clin. Oncol.***99**, 103-124.
 45. Kapoor, L.D., Singh, A., Kapoor, S.L., Srivastava, S.N. (1975). Survey of Indian plants for saponins, alkaloids and flavonoids - IV. *Lloydia.* **38**, 221-224.
 46. Beaumont, A.J., Edwards T.J., Smith F.R. (2001). Leaf and bract diversity in *Gnidia* (Thymelaeaceae): patterns and taxonomic value. *Systematics and Geography of Plants.* **71**, 399-418.
 47. Peterson, B., (1959). Some interesting species of *Gnidia*. *Botaniska Notiser.* **112 (4)**, 465-80.
 48. Cary, M., (1949).The Geographic background of Greek and Roman History. London, England: Oxford University Press.
 49. Peterson, B. (1978). Thymelaeaceae. In: Polhill R.M., editor. Flora of Tropical East Africa. London, United Kingdom: Crown Agents for Oversea Governments and

- Administrations. p. 1-37.
50. Bredenkamp, C.J., Beyers, J.B.P., Thymelaeaceae (Dilleniidae-Euphorbiales). In: Liestner O.A., editor. (2000). Seed plants of Southern Africa: families and genera. Pretoria: *Strelitzia* **10**, National Botanical Institute. p. 544-6.
 51. Roger, Z.S. (2009). A revision of Malagasy *Gnidia* (Thymelaeaceae, Thymelaeoideae). *Annals of the Missouri Botanical Garden*. **96**, 324-68
 52. Borris, R.P., Cordell, G.A. (1984). Studies of the Thymelaeaceae II. Actinoplastic principles of *Gnidia kraussiana*. *J Nat Prod*. **47**, 270-280.
 53. Liao, S.G., Chen, H.D., Yue, J.M. (2009). Plant orthoesters. *Chem Rev*. **30**, 1092-140.
 54. Kupchan, S.M., Shizuri, Y., summer, W.C., Haynes, H., Leighton, A.P., et al. (1976). Isolation and structural elucidation of new potent antileukemic diterpenoid esters from *Gnidia* species. *J.Org Chem*. **41**, 3850-3.
 55. Badawi, M.M., Handa, S.S., Farnsworth, A.D.K.G.A.C.N.R. (1983). Plant anticancer agents XXVII: antileukemic and cytotoxic constituents of *Dircaoccidentalis* (Thymelaeaceae). *J Pharm Sci*. **72**, 1285-7.
 56. Evans, F.J. (1986). Naturally occurring phorbol esters. Boca Raton: CRC Press.
 57. Adolf, W., Hecker, E. (1977). Diterpenoid irritants and co-carcinogens in Euphorbiaceae and Thymalaeaceae- Structural relationships in view of their biogenesis. *Isr J Chem*. **16**, 75-83.
 58. Harborne, J.B., Baxter, H., Moss, G.P. (1999). *Phytochemical Dictionary- A handbook of Bioactive Compounds from plants*. 2 ed. London: Taylor & Francis.
 59. Balandrin, M.F., Klocke, J.A., Wurtele, E.J., Bollinger, W.H. (1985). Natural plant chemicals: Sources of industrial and medicinal materials. **228(4704)**, 1154-1160, Science New Series
 60. Yu, Z., Yuji, M., Hideo, N., Masao, M., Haruo, S. and Naoki, Y. (2005). Novel phorbol esters exert dichotomous effects on inhibition of HIV-1 infection and activation of latent HIV-1 expression. *Antiviral Chemistry & Chemotherapy*. **16**, 303–313.
 61. Khare, C.P. (2004). *Indian Medicinal Plants: An Illustrated Dictionary*. Verlag Heidelberg: Springer. pp. 292.
 62. Peng, J., Fan, G., Hong, Z., Chai, Y., Wu, Y. (2005). Preparative separation of isovitexin

- and isoorientin from *Patrinia villosa* Juss by high-speed counter-current chromatography. *Journal of Chromatography A*. **1074**, 111-5.
63. Hussain, H., Hussain, J., Al-Harrasi, A., Krohn, K. (2012). The chemistry and biology of bicoumarins. *Tetrahedron*. **68**, 2553-78.
 64. Ferrari, J., Terreaux, C., Sahpaz, S., Msonthi, J.D., Wolfender, J-L., et al. (2000). Benzophenone glycosides from *Gnidia involucrata*. *Phytochemistry*. **54(8)**, 883-9.
 65. Franke, K., Porzel, A., Schmidt, J. (2002). Flavone–coumarin hybrids from *Gnidia socotrana*. *Phytochemistry*. **61**,873-878.
 66. Munkombwe, N.M., Galebotswe, P., Modibesane, K., Morebodi, N. (2003). Phenylpropanoid glycosides of *Gnidia polycephala*. *Phytochemistry*. **64(8)**, 1401-4.
 67. Rindl, M. (1933). Isolation of a glucoside from *Gnidia polycephala* (Januarie Bossie). *Transactions of the Royal Society of South Africa*. **21**,239-44.
 68. Peterson, B. (1958). Contributions to the knowledge of the flora of Southern Rhodesia. X. Thymelaeaceae. *Botaniska Notiser*. **111**,623-31. Blundell, M. (1987). Collins Guide to the Wild Flowers of East Africa. Collins, London, p. 53 etphoto 270.
 69. Blundell, M. (1987). Collins Guide to the Wild Flowers of East Africa. Collins, London, p.53 etphoto 270.
 70. Gelfand, M., Mavi, S., Drummond, R.B., Ndemera, B. (1985). The Traditional Medical Practitioner in Zimbabwe. Mambo Press, Gweru, pp. 191-192, 268-269 and 304.
 71. Getachew, A., Befikadu, U., and Amha, W. (2017). Systematic review on traditional medicinal plants used for the treatment of malaria in Ethiopia: *trends and perspectives Malar J*. **16**, 307
 72. Berhanu, A., Asfaw, Z., Kelbessa, E. (2006). Ethnobotany of plants used as insecticides, repellents and antimalarial agents in Jabitehnan district, West Gojjam. *Ethiopian Journal of Science*. **29(1)**, 87–92.
 73. Getaneh, G. (2011). Ethnobotanical study of traditional use of medicinal plants and their conservation status in Ethiopia. Page 79
 74. Wiechowski-W., Lotos, 1908-56, 61, (cited, from, et al. (2009). Phytochemical and pharmacological investigations on mangiferin. *Herba polonica*. **55(1)**, 126-39).
 75. Ahmad, A., Pandey, S., Sarkar, F.H. (2012). Role of novel Nutraceuticals Garcinol,

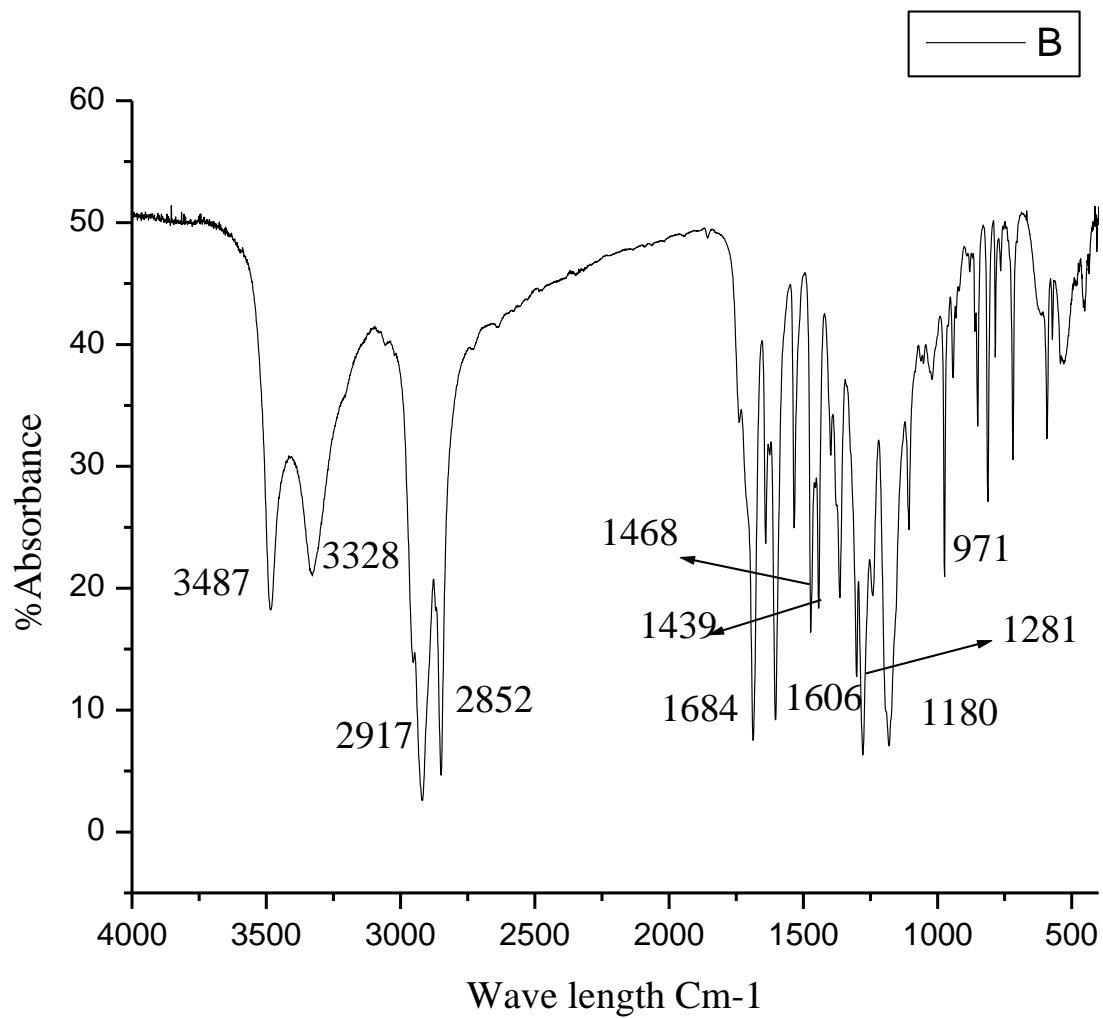
- Plumbagin and Mangiferin in the prevention and therapy of human malignancies: Mechanisms of Anticancer activity. Sarkar F.H., editor: Springer.
76. Kumar, S., Kumar, V., Rana, M., Kumar, D. (2012). Enzymes Inhibitors from Plants: An Alternate Approach to Treat Diabetes. *Pharm. Comm.* **2**,18-33.
 77. Delazae, A., Gibbons, S., Kosar,i A.R., Nazemiyeh, H., Modarresi, M., et al. (2006). Flavone C-glycosides and Cucurbitacin glycosides from *Citrullus colocynthis*. *DARU J. of Pharmaceutical Sciences.* **14(3)**, 109-14.
 78. Küpeli, E., Aslan, M., Gürbüz, I., Yesilada, E. (2004). Evaluation of in vivo Biological Activity Profile of Isoorientin. *Z Naturforsch.* **59c**, 787-90.
 79. Ferrari, J., Terreaux, C., Sahpaz, S., Msonthi, J.D., Wolfender, J-L, et al. (2003). Isolation and on-lineLC/CD analysis of 3,8''-linked biflavonoids from *Gnidia involucrata*. *Helv Chim Acta.* **86(8)**, 2768-78.
 80. Harborne, J.B. (1998). Phytochemical methods: A guide to modern techniques of plant analysis. 3rd ed. Chapman and Hall Int. (Ed).NY. 49–188.
 81. Sofowora, A. (1993). Medicinal plants and Traditional Medicine in Africa. Spectrum Books. Ibadan, pp. 10-15.
 82. Evans', W. (1996). Trease and Evans Pharmacognosy, 14th edition, WB Saunders company Ltd; London. Pp224–228, 29–309, 542–575.
 83. Brand-Williams, W., Cuvelier, M., & Berset, C. (1995). Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft and Technologie*, **28**, 25-30.
 84. Wang, H., Gao, X., Zhou, G., Cai, L., and Yao, W. (2008). In vitro and in vivo antioxidant activity of aqueous extract from *Choerospondias axillaris* fruit. *Food Chemistry.* **106**, 888-895.
 85. Wayne, P.A., 5th ed. (1997). National Committee for Clinical laboratory Standards (NCCLS) Performance Standards for Antimicrobial Disc Susceptibility Test. Approved Standard. M2-A6.
 86. Deganhardt, J. (2003). Attracting friends to feast on foes: Engineering terpene emission to make crop plant more attractive to herbivore enemies. *Current Opinion Biotechnol.* **14**, 169-176.
 87. Leandro, L.M., and Vargas, F.S. (2012). Chemistry and Biological activities of

- terpenoids from *Copaiba* (*Coparfera* SPP). *Oleoresins*. **17**, 3866-3889.
88. Liu, R.H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of Action. *J. of Nutrition*. **134**(12), 3479S-3485S.
 89. Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*. **2**, 152-159.
 90. Ito, N., Fukushima, S., Hasegawa, A., Shibata, M., and Ogiso, T. (1983). Carcinogenicity of butylated hydroxyanisole in F344 rats. *J. of National Cancer Institute*, **70**(2), 343-352.
 91. Jie-li, L., Jin-ao, D., Biao, S., and Yan-yan, Y. (2013). Caffeic acid esters from *Artemisia argyi* and antioxidant activities. *Chemistry of Natural Compounds*. Vol. **49**, No. 1
 92. Da Cunha, F.M., Duma, D., Assreuy, J., Buzzi, F.C., Niero, R., Campos, M.M., Calixto, J.B. (2004). Caffeic acid derivatives: in vitro and in vivo anti-inflammatory properties. *Free Radic Res*. **38**, 1241–1253
 93. Noriaki, K., Yukari, K., Kazuya, I., Kyo, M., Tokio, F. (2005). In Vitro Antibacterial, antimutagenic and anti-influenza virus activity of caffeic acid phenyl ethyl ester. *Biocontrol Sci*. **10**, 155–161
 94. Kikuzaki, .H, Hisamoto, M., Hirose, K., Akiyama, K., Taniguchi, H. (2002). Antioxidant properties of ferulic acid and its related compounds. *J Agric Food Chem*. **50**, 2161–2168.
 95. De P, B. M., Bedos-Belval, F. (2011). Cinnamic acid derivatives as anticancer agents a review. *Curr Med Chem*. **18**, 1672–1703.
 96. Elver, O., Sara, M. R., Santiago, D., Miguel, C., Diana, M., Julian , P., Ivan, D. V., Wilson, C. (2014). Synthesis and leishmanicidal activity of cinnamic acid esters: structure–activity relationship. *Med Chem Res*. **23**, 1378–1386
 97. Jieru, G., Jinwen, Z., Penghua, S., Lingmei, K., Xincai, H., Yongbo, X., Zengwei, L., Yan, L., Gao, L., Guangmin , Y., and Yonghui, Z. (2012). Two New Diterpenoids from the Buds of *Wikstroemia chamaedaphne*. *Molecules Science*. **17**, 6424-6433.
 98. Michal, K., Vitthal, D. W., I-Wen, L., Yu-Ming, H., Hsue-Yin, H., Tsong-Long, H., Yang-Chang, W., Yuan-Bin, C., Bing-Hung, C., and Fang-Rong, C. (2016).

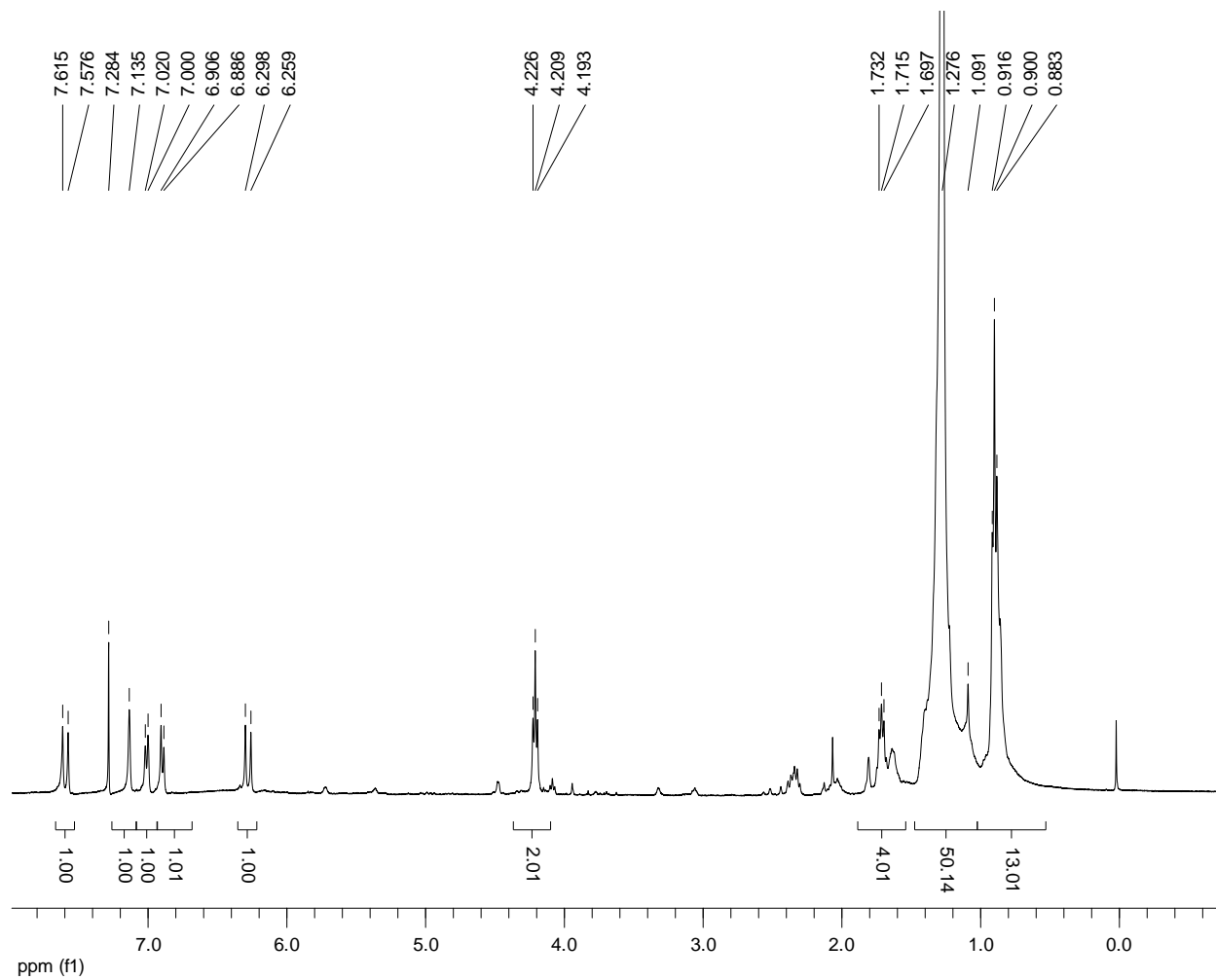
- Antiallergic Phorbol Ester from the Seeds of *Aquilaria malaccensis*. *International Journal of Molecular Sciences*. **17**, 398.
99. Angelique, B., Jean-Chales, G., Francoise, G-R., Lise, B., Robert, V., Stephen, B., Leonid, B.M., and Ivan, H.(2004).Dual Role of Prostratin in Inhibition of Infection and Reactivation of Human Immunodeficiency Virus from Latency in Primary Blood Lymphocytes and Lymphoid Tissue. vol. **78** no. 19 10507-10515.
100. Korin, Y. D., Brooks, D. G., Brown, S., Korotzer, A., and Zack J. A. (2002). Effects of prostratin on T-cell activation and human immunodeficiency virus latency. *J. Virol.* **76**, 8118-8123.
101. Gulakowski, R. J., McMahon ,J. B., Buckheit , R. W., Gustafson, K. R., and Boyd, M. R. (1997). Antireplicative and anticytopathic activities of prostratin, a non-tumor-promoting phorbol ester, against human immunodeficiency virus (HIV). *Antivir. Res.* **33**,87-97.
102. Szallasi, Z., and Blumberg, P. M. (1991). Prostratin, a nonpromoting phorbol ester, inhibits induction by phorbol 12-myristate 13-acetate of ornithine decarboxylase, edema, and hyperplasia in CD-1 mouse skin. *Cancer Res.* **51**, 5355-5360
103. Jain, R., and Mittal, M. (2012). Naringenin, a flavanone from the stem of *Nyctanthes arbortristis*. *Int.J. bio.pharmacy, and Ap. S.* **1(7)**, 964-972
104. Yamauchi, K., Mitsunaga, T., and Batubara, I. (2011). Isolation, identification and tyrosinase inhibitory activities of the extractives from *Allamanda cathartica*. *Natural Resources.* **2(3)**, 167-172.
105. Chang, T.S., Lin, M.Y., Lin, .H.J. (2010). Identifying 8-hydroxy naringenin as a suicide substrate of mushroom tyrosinase. *J. Cosm. Sci.* **61**, 205-210.
106. Ahmad, F.B., Nisa, N.K., Sallehuddin, M., and Assim, Z. (2010). Chemical constituents and antiviral study of *Goniothalamus velutinus*, *Journal of Fundamental Sciences.* **6(1)**, 72-75.
107. Brev, K.A., Rasmussen, S.E., Drevon, C.A., and Anderson, L.F. (2004). Urinary extraction of flavonoids reflects even small changes in the dietary intake of fruits and vegetables, *Cancer Epidemiology. Biomarkers.* **13(5)**, 843-849.

Appedices

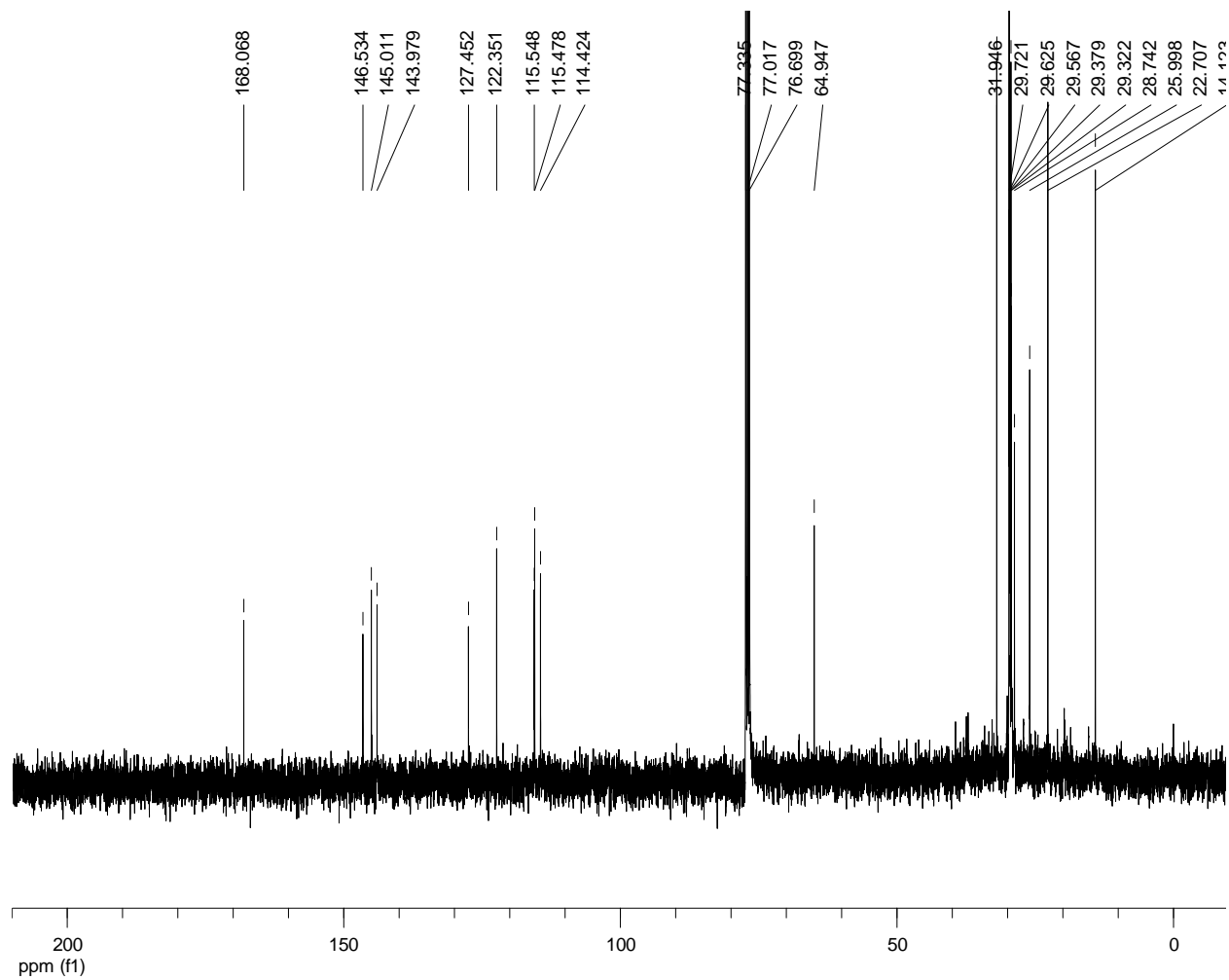
Appendix 1: IR spectrum of **compound-1**



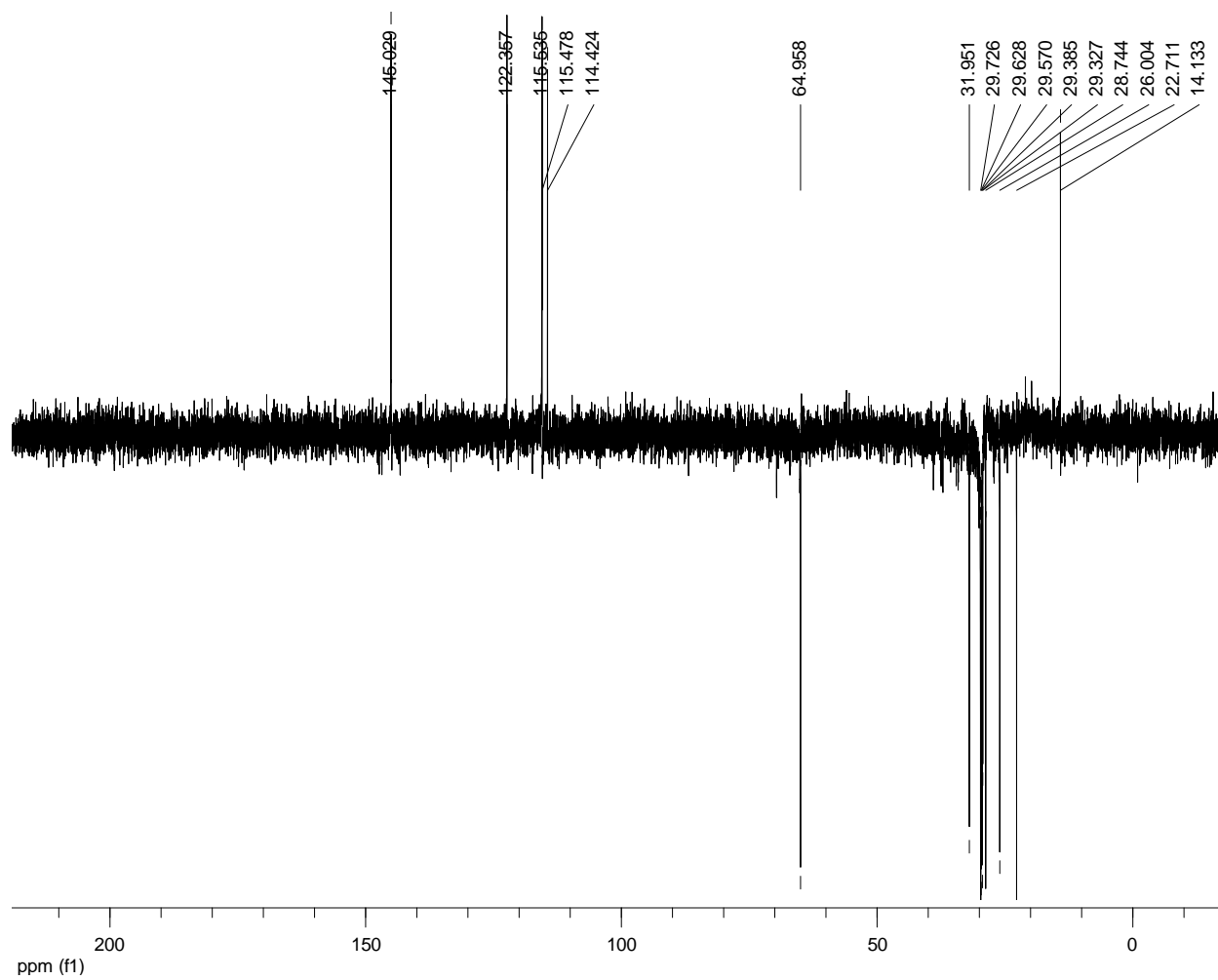
Appendix 2: ^1H -NMR spectrum of compound-1



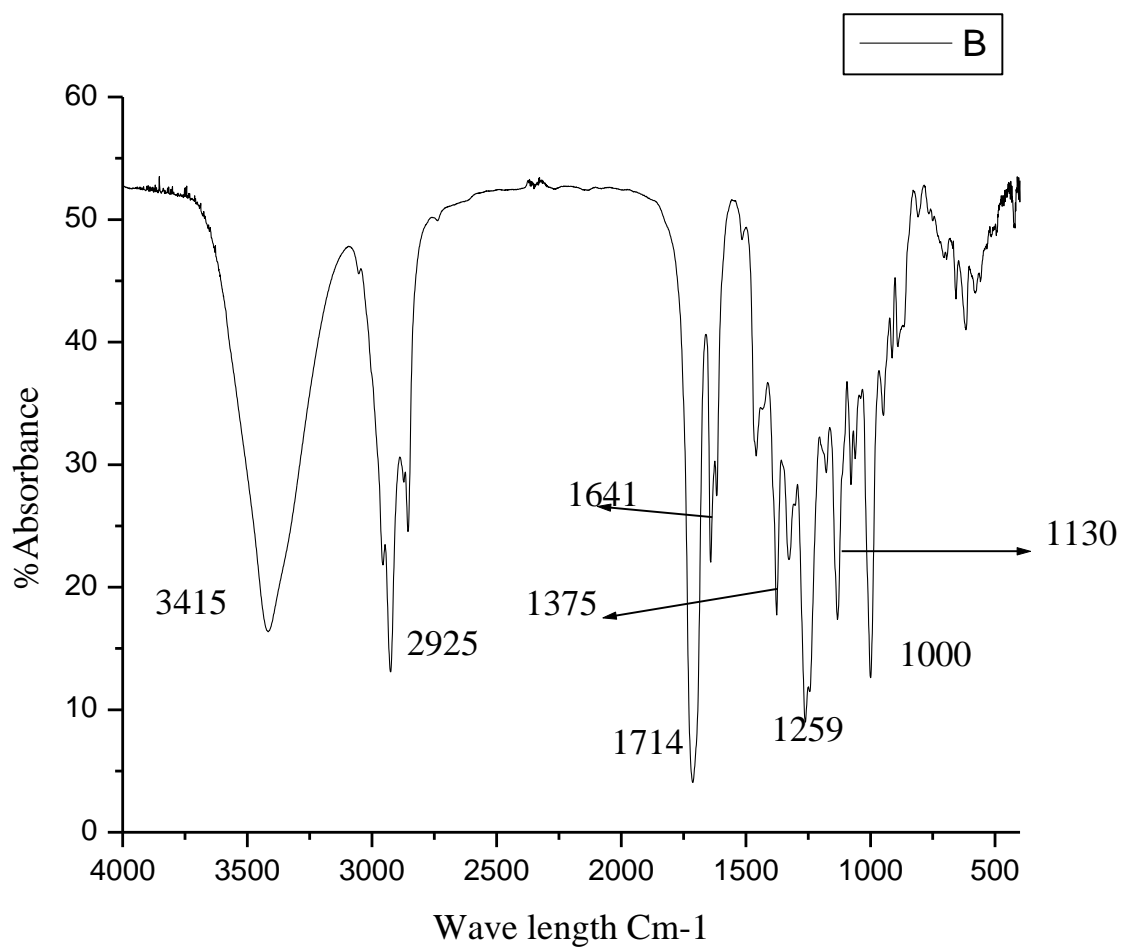
Appendix 3: ^{13}C -NMR spectrum of **compound-1**



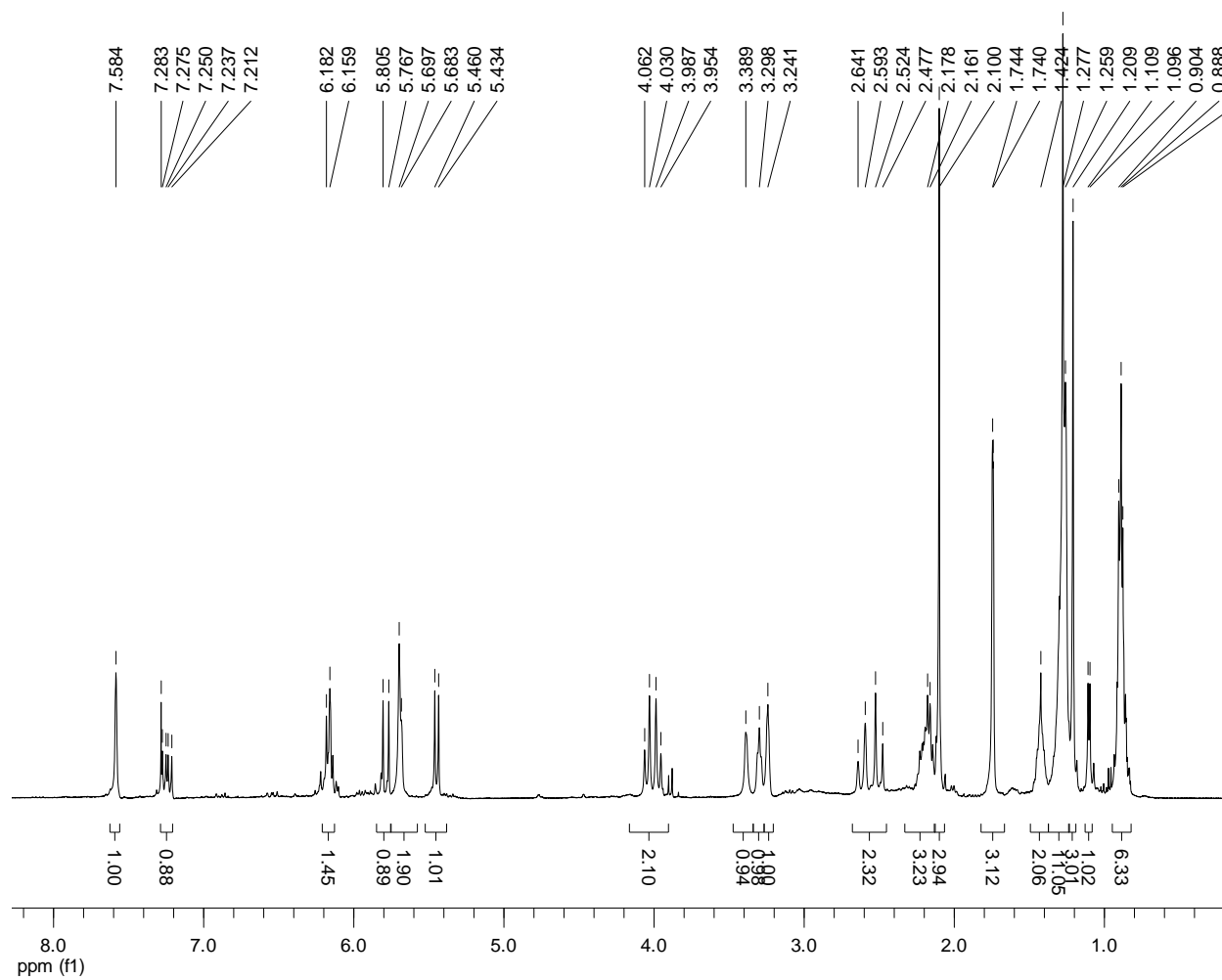
Appendix 4: DEPT-135 spectrum of compound-1



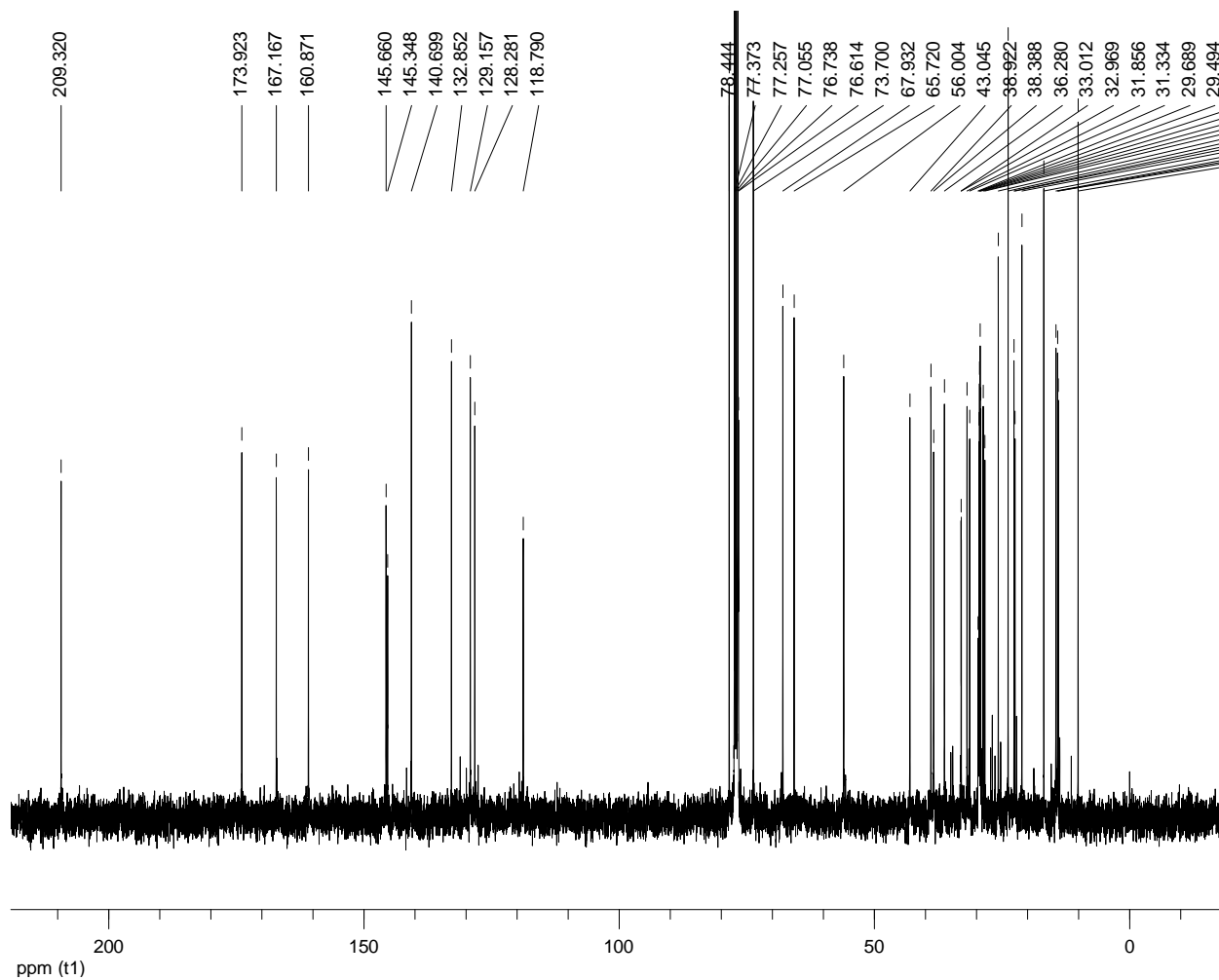
Appendix 5: IR spectrum of compound-2



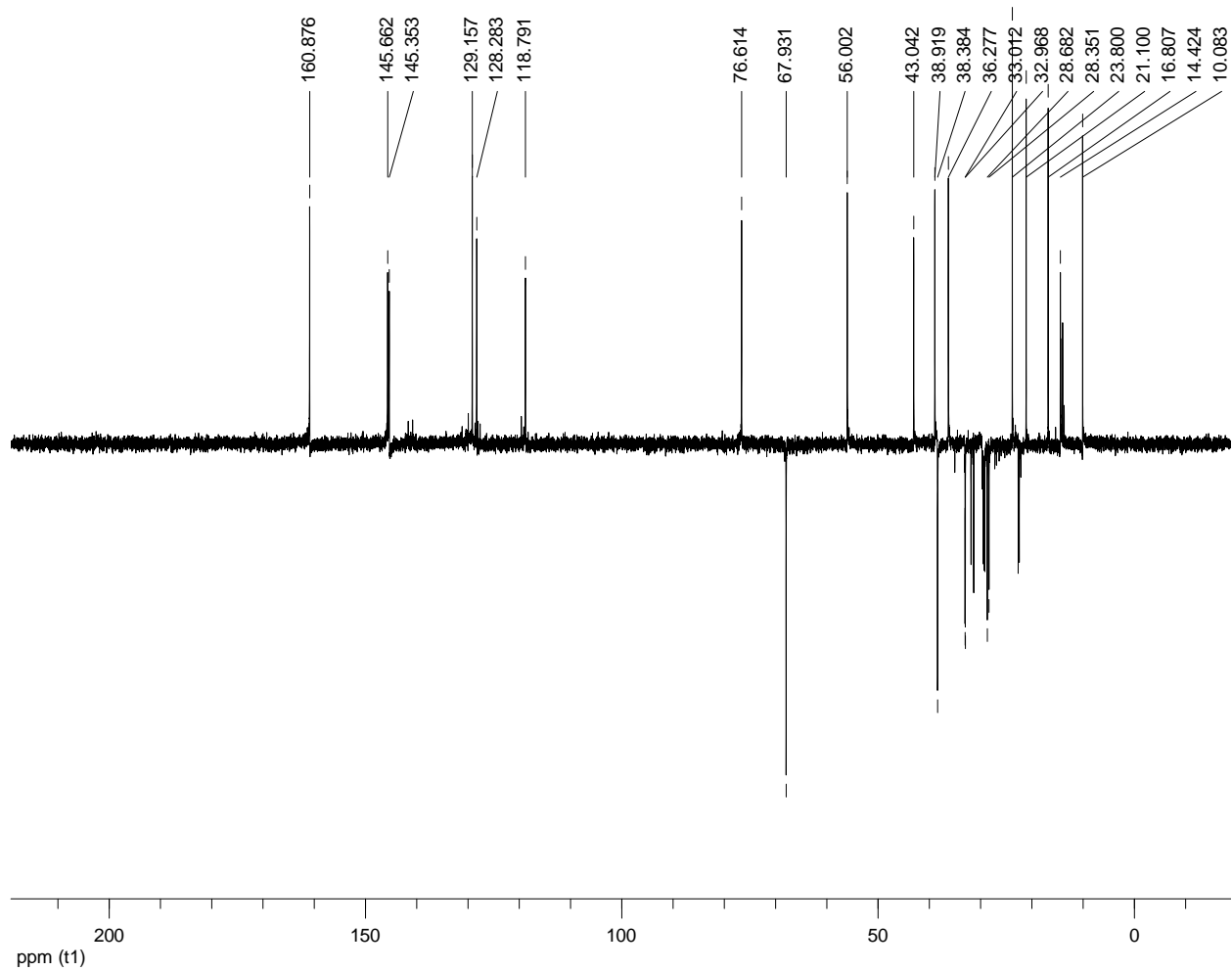
Appendix 6: ^1H -NMR spectrum of compound-2



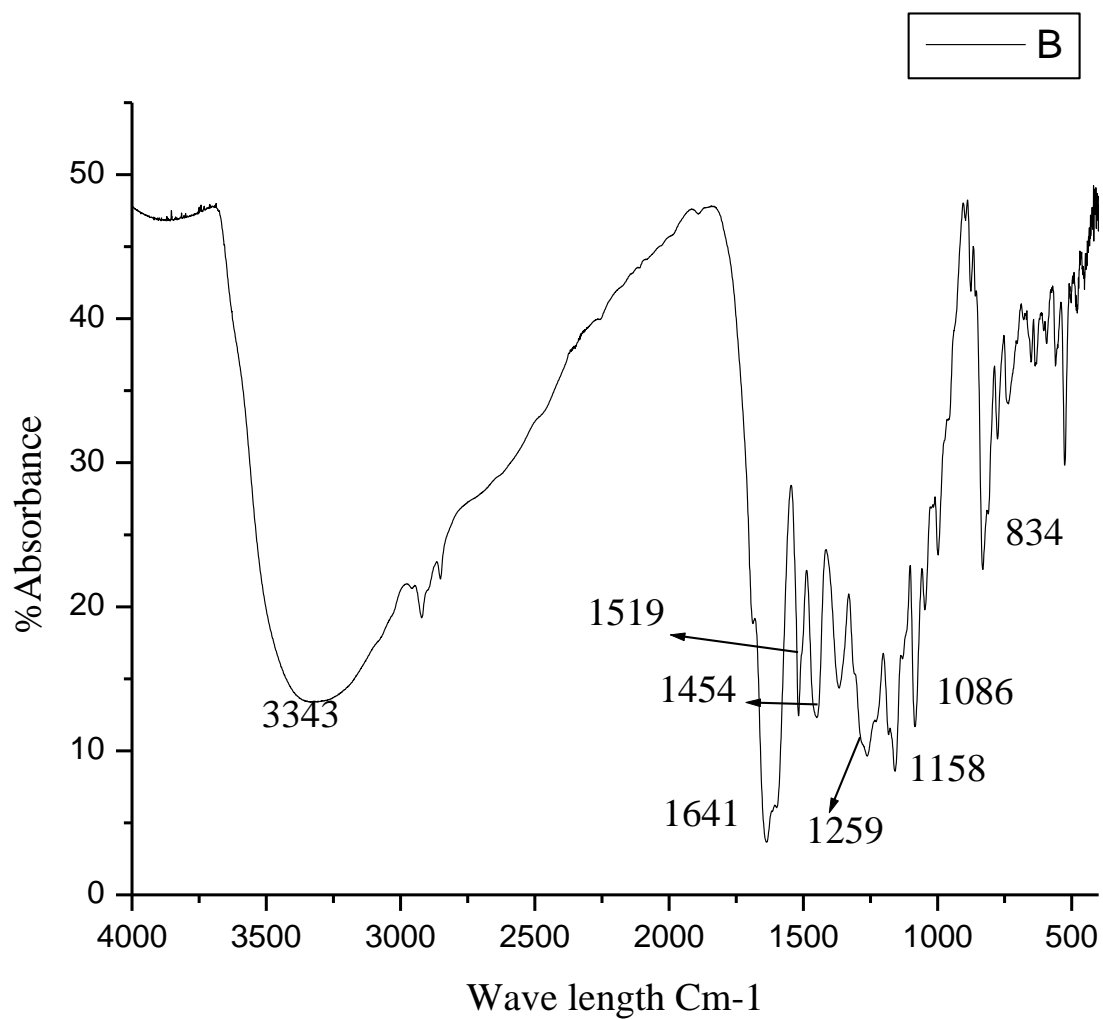
Appendix 7: ¹³C-NMR spectrum of compound-2



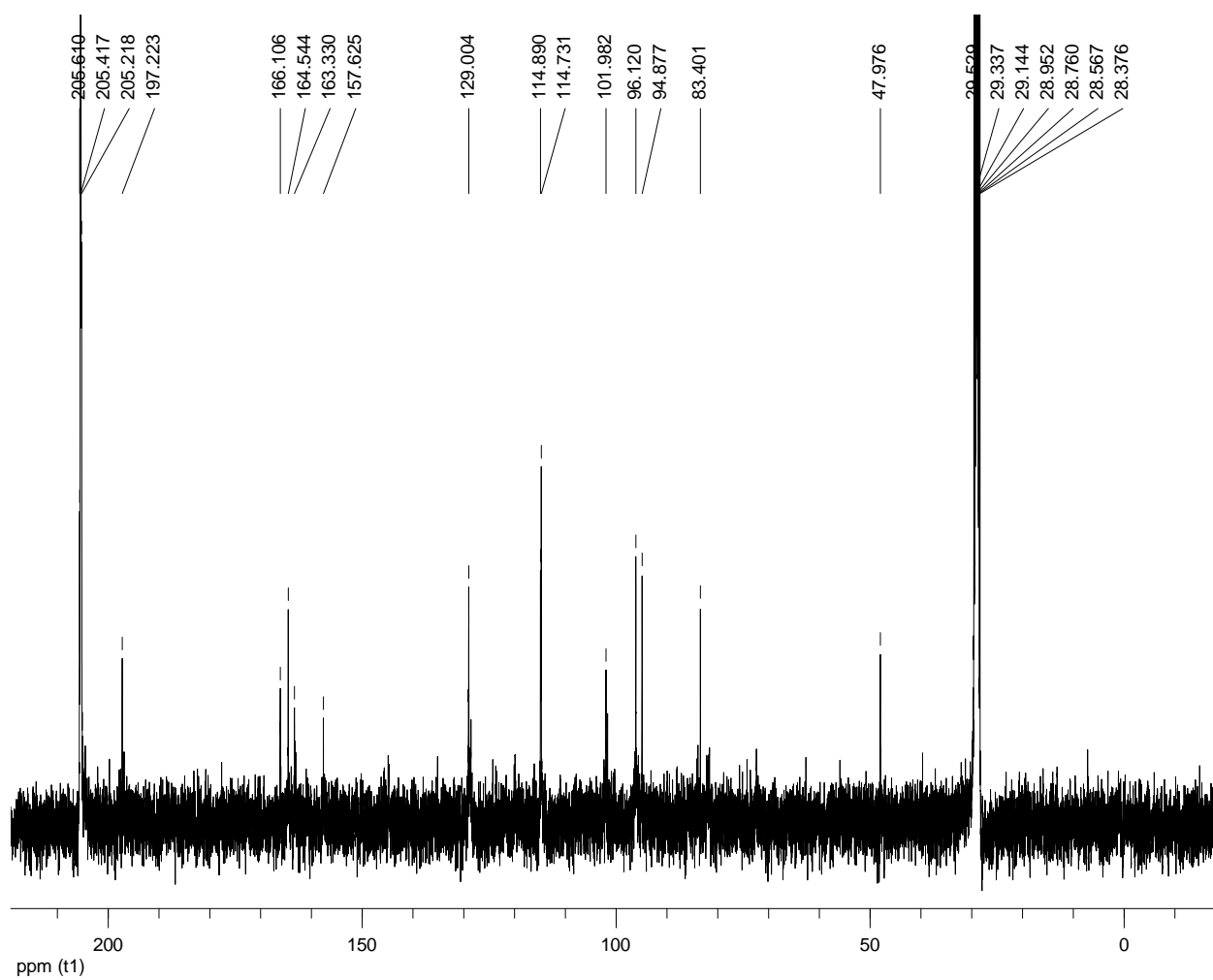
Appendix 8: DEPT-135 spectrum of compound-2



Appendix 9: IR spectrum of compound-3



Appendix 10: ¹³C-NMR spectrum of compound-3



Appendix 11: DEPT -135 spectrum of compound-3

