ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY
SCHOOL OF APPLIED NATURAL SCIENCE

APPLIED CHEMISTRY DEPARTMENT

Phytochemical Investigation of Root of *Clausena anisata* for Antibacterial Activity

A MASTER THESIS SUBMITTED TO THE OFFICE OF GRADUATE STUDIES OF ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY, SCHOOL OF APPLIED NATURAL SCIENCE DEPARTMENT OF APPLIED CHEMISTRY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED CHEMISTRY.

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Adama, Ethiopia

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APPROVAL OF BOARD OF EXMINERS

We, the undersigned, member of the board of examiners of the final open defense by **Dandena Tamene** have read and evaluated his thesis entitled **"Phytochemical Investigation of Roots of *Clausena anisata* for Antibacterial Activity"** and examined the candidate. This is therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of Master of Science in Chemistry.

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This is to certify that the thesis prepared by **Dandena Tamene** entitled "**Phytochemical Investigation of Roots of *Clausena anisata* for Antibacterial Activity'** is submitted in fulfillment of the requirement for the degree of Master of Science in Chemistry complies with the regulation of the University and meets the accepted standards with respect to originality and quality.

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DECLARATION

First, I declare that this thesis is my own work and that all sources of the materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillments of the requirements for the award of degree of master in chemistry stream at Adama science and Technology University. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: Dandena Tamene Signature: _____

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

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Date of submission _____

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LIST OF ABBREVIATIONS

CC	Column Chromatography
TLC	Thin Layer Chromatography
UV	Ultra Violet
IR	Infra-Red
1D-NMR	One Dimensional-Nuclear Magnetic Resonance
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
^{13}C-NMR	Carbon13-Nuclear Magnetic Resonance
ATCC	American Type Culture collection
C	*Clausena*
$CDCl_3$	Deuterated Chloroform
D	doublet
DCM	Dichloromehane
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
H	proton
J	coupling constant
MHA	Muller Hinton Agar
n- Hexane	normal hexane
R_f	Retardation Factor
S	Singlet
T	Triplet
TMS	Tetramethylsilane

ABSTRACT

*Clausena anisata is one of the medicinal plants used traditionally for treatment of parasitic infections, irritation (boils, ringworm and eczema), wound, pains, flat worm infestations, influenza and constipation. The present study focused on the extraction, isolation, characterization of the chemical constituents and evaluation of antibacterial activities on the roots extracts of Clausena anisata plant using maceration extraction technique. The powdered roots (500g) of Clausena anisata were successively extracted with dichloro methane/methanol and methanol (2.5L) to furnish extracts with percentage yields of 1.7% and 2.48%, respectively. Phytochemical screening test of dichloromethane/methanol (1:1) and methanol roots extract revealed the presence of flavonoids, phytosterols, coumarins, phenols, alkaloids, tannins, terpenoids and free reducing sugars whereas saponins were absent. Silica gel column chromatographic separation of the dichloromethane/methanol extract afforded three coumarins namely Graveliferone (**15**), Imperatorin (**16**), Chalepin (**17**) and a carbazole alkaloid Heptaphyline (**14**). Structures of the compounds were elucidated using the spectroscopic techniques (IR, ^{1}H NMR, ^{13}C NMR, and DEPT-135) and comparison with literature data. Antibacterial activity of the crude extracts and isolated compounds were screened using agar diffusion method against strains of Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus substilis. The results of antibacterial activity tests revealed Heptaphyline (**14**) and Imperatorine **(16)** exhibited comparable antibacterial activity against S. aureus and B. substilis (14 and 13 mm zone of inhibition, respectivily) to that of Ciprofloxacin (15 mm zone of inhibition) at a concentration of 20µg/mL. Compound **17**(Chalepin**)** revealed more antibacterial activity against B. substilis (16mm zone of inhibition) compared to Ciprofloxacin (15 mm). The results obtained substantiate the traditional use of this plant against bacteria.*

Key words: Phytochemical, *Clausena anisata*, antibacterial activity, coumarins, carbazole alkaloid.

1. INTRODUCTION

1.1 Background

Medicinal plants have a long history of use in most communities throughout the world. In developing countries especially in Africa, people still consult traditional healers for their health problems. The use of plants and their products for medicinal benefits has played a significant role in nearly every culture on earth. Historically all traditional remedies were obtained from plants and recent estimates have suggested that several thousands of plants have been known with medicinal applications in various cultures [1].

Plants have been used from ancient times to attempt cures for diseases, to relive physical suffering and also utilized as sources of spices, dyes, poisons and drugs. Ancient peoples all had acquired some knowledge of medicinal plants. Often times these primitive attempts at medicine were based on superstition and speculation. The chemical compounds responsible for these activities are often the secondary metabolites of plants or natural products as they are usually referred to. The World Health Organization (WHO) defines traditional medicine as health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being [2].

Medicinal plants have been shown to have genuine utility and about 70 to 80% of the rural population in most developing countries depends on medicinal plants for their primary health care [3]. Medicinal plants have been used as sources of remedies for the treatment of many diseases since ancient times by the people of all continents especially in the tropical regions. Traditional medicine is used in all parts of the World and has a rapidly growing economic importance, mainly through the use of medicinal plants, especially in developing countries. The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body and are called phytochemicals [4]. Medicinal plant extracts and phytochemical constituents present in the plant tissues with well-known antimicrobial properties play important roles in promoting human health and are non-toxic to the human body [5].

Plant extracts work in synergy with synthetic antibiotics against drug resistant bacteria [6]. They can be defensive substances such as phytoalexins and phytoanticipins, anti-feedants, attractants and pheromones [7]. No one knows exactly how many different medicinal plants are used in the world today, but it is well known that medicinal plants are enormously important in both traditional and modern medicine**.** Ethno botany, the study of traditional plant use, is a field of growing interest to research scientists and pharmaceutical companies looking to develop new and more effective drugs. In general it has been estimated that over 40% of medicines have their origins in these active natural products [8].

The medicinal use of plants of the family Rutaceae is widely described in the scientific literature. This family is distributed in approximately 160 genera and about 2070 species. The largest genera are *Clausena*. *Clausena anisata* belongs to the Rutaceae family. It is a deciduous shrub or small tree and widespread in the Afrotropic ecozone or Sub-Saharan Africa but absent from the drier regions [9]. The phytochemistry of this plant showed the presence of tannins, flavonoids, steroids, phenolic, anthraquinonnes, and alkaloids. Phytochemical constituents such as flavonoids have been found *in vitro* to be effective against a wide range of microorganisms [10]. The root decoctions of the *C.anisata* and infusions are taken for whooping cough, malaria, syphilis and kidney ailments, skin diseases and epilepsy, and given to women before and after parturition to ease delivery and to expel blood from the uterus, and later to boost milk production [11].

Clausena anisata is known locally in Ethiopia as "*Olmaa'ii*" in Afan Oromo and "*limich*" in Amharic and used for treatment of infection, skin diseases and to repel houseflies [12] and used as a repellent against various pests in different countries [13]. Despite the traditional use of this plant against wide range of diseases, there is limited scientific report on the chemical constituents and antibacterial activities of the roots of *C.anisata* with in Ethiopia. This fact made the plant good candidate for the present study. Therefore, this study focused on the extraction, isolation, characterization of the chemical constituents of *Clausena anisata* and evaluate for antibacterial activity of the extracts of *Clausena anisata* root.

1.2. Statement of the problem

The rapid development of multi-drug resistant strains of bacteria increased the occurrence of bacterial infections that cannot be treated with conventional anti-microbial agents. Due to this reason, scientists are always searching for the new generation anti-biotic drugs. But the new generation anti-biotics are less available and expensive for resource poor communities. The increasing rate of resistance of disease causing micro-organisms to conventional anti-biotics and the insufficient number of health facilities, results for the continuous search in the affordable, safe and effective herbal medicine.

Traditionally, peoples in Ethiopia have been used medicinal plants to treat different diseases and this has great contribution in primary health care systems. *C. anisata* is one of those ethnomedicinal plants that have been commonly visited by traditional healers in most parts of Ethiopia. The leaves and roots parts of the plant have been widely used by the local people for the treatment of different alignments including skin infections, wound, fever, ear-ach, stomach-ach, diarrhea, sexual disorder, blood clotting, nose bleeding, and hormonal disorder. While the leaves and stem of *C.anisata* have been studied, to the best of our knowledge, there is limited published report on isolation and characterization of chemical constituents and evaluation of biological activities of the roots extracts of this plant in Ethiopia flora. Therefore, the present study was focused on the isolation and identification of compounds from the roots extracts of *C. anisata* and evaluation of anti-bacterial activities.

1.3. Significance of the study

The study serves as the area on which the researchers who are interested to conduct further studies in related areas. Understanding the structures of the natural products from *Clausena anisata* has much significance for industrial and pharmaceutical applications. The study on the extracts of the roots of *Clausena anisata* may help to arrive at new natural products. The extracts and compounds to be obtained from the plant may exhibit pronounceable antibacterial activities. This study may also provide important information about the structures of the natural products (bioactive compounds or secondary metabolites) from the roots of *Clausena anisata* to the people. Furthermore, it can provide initial information for the researchers who are interested to conduct further studies on this area.

1.4 Objectives

1.4.1 General objective

The overall objective of the work is to isolate, characterize the chemical constituents of *Clausena anisata* and evaluate for antibacterial activity.

1.4.2 Specific objectives

- To extract the roots of *C. anisata* with dichloromethane/methanol (1:1) and methanol.
- To conduct preliminary phytochemical screening on the extracts.
- To isolate compounds from the extracts of the roots of *Clausena anisata* by using silica gel column chromatography
- To characterize the isolated compounds by employing physical properties and spectroscopic methods including UV, IR, and NMR.
- To study the antibacterial activities of the extracts and pure compounds using agar well diffusion method against two gram-positive bacteria (*Bacillus substilis* NCTC 10073, *Staphylococcus aureus* ATCC 25923*)* and two gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 4853, *Escherichia coli* ATCC 25922.

2. LITERATURE RIVIEW

2.1. Uses of medicinal plants

The use of plants for the treatment of various diseases is universal and has been practiced by many people for many years. It has not only continued to be used for primary health care in developing countries, but has also been used in countries where conventional medicine is predominant in the national health care system [2]. Plants' medicinal values are believed to be due to the presence in its tissues of some chemical substance or substances that produce a physiological action on the body. Pharmacognosy is the branch of medical science which deals with the drug plants. It is concerned with the history, commerce, collection, selection, identification and preservation of crude drugs and raw materials. Worldwide, there are several thousand plants that have been used and are still being used for medical purposes. Many of these are restricted in use by native people who have long resided in any given area [14].

Traditional knowledge to solve health problems of mankind and animals exists in all countries of the world. The use of medicinal plant as the source of remedies for the treatment of many diseases dates back to prehistory and people of all continents have this old tradition. Fossil records have shown that the use of plants as drugs since Middle Paleolithic age which is approximately 60,000 years back. The use of plants as medicines is not restricted to one particular region or continent. Plants as medicines are capitalized in Africa, Greek, Islamic, Chinese, Indian and Western systems of medicines. This indicates the consensus among the nations upon the use of plants as medicines [14].

The first official recognition of traditional medicine as important participant in primary health care was expressed in the World Health Organization's Primary Health Care Declaration of Alma Ata in 1978. Traditional medicine according to the declaration is the sum total of all the knowledge and practices used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation verbal writing [15]. Medicinal plant typically contains mixtures of different compounds that may individually or synergistically improve the health.

In previous studies, many plant extracts or natural compounds have been shown to possess

combinatory antimicrobial activity, including improving antibiotic's efficacy against *S. aureus* [16]. The importance of medicinal plants lays not only on their chemotherapeutic effect but also in their role as a source of model compounds for drug development. In addition to plant constituents being used directly as therapeutic agents, they can be utilized as starting material or templates for drug synthesis. Traditional healers use medicinalplants for a variety of illness caused by protozoan, bacteria, fungi, viruses and helminthes. Screenings of medicinal plants on the basis of their presence at various geographical locations and their presumptive folklore use indicate a huge number of plant species that can be worked upon to find out a huge number of plant derived metabolites of important therapeutic significance [17].

Plants produce two types of products, primary and secondary metabolites, as part of their normal metabolism. Primary metabolites include carbohydrates, proteins, lipids and chlorophyll which are present in all plants while secondary metabolites are special metabolic products of certain plant species that have little or no role in their life cycle and produced in smaller quantities as compared to the primary metabolites. Secondary metabolites are synthesized in specialized cells at a certain developmental stage of plants. They usually have impact upon animal or other organisms systems and so are termed as active principles. Secondary metabolites have very little known functions in plants [18]. Medicinal properties of plants are due to the combinations of secondary metabolites such as alkaloids, steroids, tannins, and phenolic compounds that are synthesized and deposited in specific or in all parts of the plant. These medicinal properties are specific in a plant family, genus and species proving the fact those combinations of secondary metabolites are distinct between plant taxa [19]. Composition of secondary metabolites varies between (i) tissues such that the bark, heartwood, roots, branch basses and wound tissues have higher concentration, (ii) species and (iii) seasons [20].

2.2. The family of Rutaceae

Rutaceae is the rue family of flowering plants (order sapindales), composed of 160 genera and about 2070 species. It includes woody shrubs and trees (and few herbaceous perennials) and is distributed throughout the world, especially in warm temperate and tropical regions. The largest numbers are found in Africa and Australia, often in semiarid wood lands. Most members of Rutaceae are herbaceous, but a significant number are also shrubs, vines, or trees. The plant is a highland family of shrubs that are established all over the world. The species in which the largest numbers of secondary metabolites have been identified in this family are *C. excavata* and *C.anisata*. Varies pharmacological uses have been attributed to this family like anti-inflammatory, antiviral, antioxidant, cytotoxic and antibacterial activities [21].

2.3. The genus *Clausena*

Clausena is a genus of about 14 species of evergreen trees, occurring mostly in India and tropical Asia. One of the most advantageous features of the species of this genus is their availability in the different parts of the world. They are being easy to grow and free of pests and diseases as well as can withstand heavy pruning [22]. The geographical distribution of *Clausena* is of interest since it has the widest range of any genus included in the orange subfamily. The species are being found all the way from Northwestern India to China and Taiwan, south through the East Indian Archipelago to Timor, Northern Australia, and New Guinea. Moreover, there is a group of three species that covers a wide range in Africa, from Ethiopia (Abyssinia) to Cape Province and through Western Africa from Angola north to Serra Leone. There are wide differences in the character of the growth and the height of the species; they range all the way from shrubs of 20 to 40 cm high in Indo-China to trees reaching a height of 20 m (66 feet) in Africa. The most distinctive morphological character of the genus *Clausena* is the gynophore, which in the typical species is a large, well-developed, hourglass-shaped structure supporting the ovary [23].

Nevertheless, it is present in all species of *Clausena* and separates them from the species of other related genera. The numerous species of this genus, still only imperfectly studied with respect to the minute flower characters, cannot be arranged now in natural sections or subgenera. It was

found that few species of *Clausena* genus have been explored and identified for chemical and biological studies. The present study has been focused on one species which is considered as the most promising specie of *Clausena* genus [23].

2.3.1. Ethnobotanical information of the genus *Clausena*

Clausena excavate is a shrub with strong and rather objectionable smell, found from the Himalayas and China to and throughout Malaysia; particularly in the Peninsula. In Malaysia, it is locally known as "Chemama". The English name of this species is "*Clausena*" [24]. It is a slender tree to 10 m tall. Twigs are finely hairy. Leaves pinnate, to 60 cm long, with 10 to 15 pairs of dark green narrowly oval oblique leaflets 3.5 to 7 cm long with pointed tips. Leaflets have a characteristic curry-like smell when crushed. Small white flowers occur in terminal clusters, followed by translucent pink berries 7 to 10 mm across, each containing 1 to 2 seeds. *C. excavate* has a striking hourglass-shaped gynophore which is completely glabrous [23].

C. harmandiata is an evergreen shrub 1 to 1.5 m tall, with all parts giving a citrus smell and containing aromatic oil. The leaves grow to 20 cm long and have three leaflets, each sized 2 cm-4 cm × 5 cm-11 cm. The clusters of four yellow-green flowers are up to 20 cm long. Its fruit is an egg-shaped berry that is 3 to 5 mm in diameter, dark red when ripe, and contains one or two seeds. *C. harmandiata* is a shrubby vigorous plant native to Asia. It is mainly distributed in a large part of Asia, starting from indo-china covering Cambodia, Laos, Thailand and Vietnam up to Malaysia and Indonesia in the parts of Malacca and Java, respectively. The plant is found everywhere in Laos in the under storey of deciduous and evergreen forests on various soil types, or along streams, but mainly on poor sandy soils [25].

C. anisum is a tree 3 to 6 m tall. Leaves are dark green, pinnate, with 5 to 9 leaflets. Each leaflet has a stalk about 3 millimeters long, the blade ovate-elliptic, lanceolate or ovate, about 7 to 10 centimeters or more in length, pointed at the tip, much wider on one side of the base. Flowers are borne in terminal panicles, white, about 14 millimeters in diameter. Petals are five, white, and boat-shaped. Calyx is small and five-parted. Stamens are 10, with conspicuous yellow anthers. Ovary is five-celled, borne on a short stalk, covered with hairy nobs, and surrounded by a short style terminating in a rounded stigma. Fruit is ivory yellow, rounded, about 2 centimeters in diameter, very slightly flattened at the base, somewhat rounded at the tip, and borne in bunches.

Skin of the fruit is thin and soft, dotted with minute, raised somewhat darker-colored spots, covered with short hairs, and marked by five, usually very inconspicuous, longitudinal lines which are lighter in color than the remainder of the fruit. Flesh is yellowish white, soft, juicy, somewhat acid in taste; cross-section shows a division into five segments by thin, white lines. Usually one to four of the segments contain a single, rather large, flattened green seed, attached near the apex of the fruit. [26]. *Clausen dentate* is a small tree plant, belonging to the family of Rutaceae and found in India, Sri Lanka and China. It is a small tree; 2 to 6 m high with a delicious fruit. It flowers in April and the fruit begins to ripen at the end of June. The tree is well known to the hill tribes and it has leaves that are more membranaceous, highly odoriferous, more prominently dotted, and very erose toward the apex [27].

Clausena anisata (Wild) Hook Benth) (Figure.1) is a medicinal plant in the Rutaceae family of flowering plants which grows up to 10 m high and thrives in and on the boundaries of evergreen forest. Its leaves are made of 10 to 17 opposite or alternate leaflets which are pinnately compound with a terminal one. The leaves are compactly spotted with glands and turn out strong scent similar to aniseed when pressed. Its branched inflorescences start off with an axillary spray which bears small, white and attractive flowers with yellow to orange stamens [28]. The plant is found in Africa, mostly in West and North Africa. It is called "Horse wood" by the natives of Mozambique. It is commonly known as *Clausena* or spirit plant; Synonyms of *C. anisata* include *Amyris anisata* (Wild), *Clausena inequalis, and Clausena pobeguini*. Its stem bark is grey or mottled in color. *Clausen anisata* bears drupe-like yellowish green fruits which become blue-black on ripening [29].

Fig 1.*Clausena anisata* (olmaa'ii) [Photo taken by Dandena Tamene, March, 2018]

Clausena lansium species differs widely from all other species of the genus. The tree is fairly fast-growing or rather slow, depending on its situation; attractive, reaching 20 ft (6 m), with long, upward-slanting, flexible branches, and gray-brown bark rough to the touch. The plant is a highly esteemed fruit tree in Southern China, where sour, subacid, and sweet varieties are known. The white or yellow fruits are ovoid or subglobose, about the size of a pigeon's egg, but varying in size and shape with the variety cultivated. *C.lansium* is native in Southern China and Indo-China; widely cultivated in tropical and subtropical regions. It is locally known as "wampee" with its white or yellow fruits [30].

2.3.2. Ethnopharmacological information of the genus *Clausena*

A number of *clausena* species have been widely used in traditional medicine in south East Asia, India and Africa. For example; *Clausena excavate* is used traditionally as a medicine in the treatment of abdominal pain, snakebite and as a detoxification agent. The pounded root is used as a poultice for sores including ulceration of the nose and the leaves also used as a poultice. A decoction of the roots is drunk for bowel complaints, chiefly colic. Gimlette was found to be used in Kelantan for yaws [31]. The flowers and leaves may be boiled and the decoction taken for colic and a decoction of leaves is given after child birth. The leaves of this plant are used to cure cold, abdominal pain, malaria and dysentery. Decayed teeth can be treated using the drayed and powdered rootstock, whereas its stem is given in colic with or without diarrhea.

The expressed juice of the plant is used in Java for coughs, as vermifuge. The timber is used for handles of axes in Java [32].

In terms of traditional uses, the roots, young leaves, bark and flowers of *C. harmandiata* are often mixed with other herbs and used to reduce intestinal gas and food poisoning. The roots also help relieve eye-pain, headaches and fever. The young leaves and leaves are used as fodder for cattle and buffalo. It is also used by humans for food. The fruit and sour young shoots are eaten with "*laap"* and bamboo soup [33]. *Clausena anisum-olens* is traditionally used as food, drink and medicine. In the Philippines, leaves of *C. anisum-olens* are used as a condiment in preparing local dishes and beverages. It is also well known in traditional medicine in the Philippines. The leaves are stuffed into pillows for a soporific effect, they are used in baths against rheumatism, or in decoction for nausea during pregnancy. Cough with fever is treated with a decoction of the

roots and fruit. The essential oil from the leaves is a potential substitute of anise oil, e.g. for the preparation of the Philippine drink 'Anisado'. Other products: In the Philippines leaves of *C. anisum*-olens are used to flavor cigarettes [34].

Many traditional uses of the *clausena lansium* have been reported; mainly food and medicinal uses are detailed here. A fully ripe, peeled wampee, of the sweet or subacid types, is agreeable to eat out-of-hand, discarding the large seed or seeds. The seeded pulp can be added to fruit cups, gelatins or other desserts, or made into pie or jam. Jelly can be made only from the acid types when under-ripe. In Southeast Asia, a bottled, carbonated beverage resembling champagne is made by fermenting the fruit with sugar and straining off the juice. The fruit is said to have stomachic and cooling effects and to act as a vermifuge. Lychees should be eaten when one is hungry and wampees only on a full stomach". The halved, sun-dried, immature fruit is a Vietnamese and Chinese remedy for bronchitis. Thin slices of the dried roots are sold in Oriental pharmacies for the same purpose. The leaf decoction is used as a hair wash to remove dandruff and preserve the color of the hair [35].

Clausena anisata is traditionally used in different African countries for treatment of many illness cases. The leaf decoction is used as a hair wash to remove dandruff and preserve the color of the hair. The leaves are used to prepare tea which is employed as blood cleanser and as a remedy against halitosis due to hepatic disorders; leave decoctions are also drunk or inhaled to cure mental illness. The leaves of *C. anisata* are used in the management of hypertension in South Africa. Moreover, dermatitis and intestinal helminthiasis are also treated with the leaves. The leaves' essential oil is also employed as parasiticide [28]. The leaves' decoction is taken as a stomachic and a laxative post-partum in addition it being used to treat several gastrointestinal disorders.

A root preparation of *C. anisata* is given as an enema or bathing lotion to treat inconsequential health conditions and to mitigate early signs of pyrexia or to avert the incidence of pyrexia in children. Furthermore, decoction of *C. anisata* root is taken at half a wine glass twice a day to remedy cardiovascular disorders and halitosis. Asthmatic conditions can also be treated with the roots. A decoction made from the roots is drunk by children to manage convulsions and taken by expectant mothers as a tonic [28]. The roots also find use in the treatment of rheumatism and

abdominal pain in children. The leaves and roots are also used to treat dysentery fever, toothache and arthritis. A mouth wash made from the boiled roots and leaves is used to alleviate toothache and to treat oral infections. The roots and stem barks are used against herpes zoster. The leaf; root and stem of *C. anisata* have been reported as effectual remedy against flatworm infestations, like taeniasis and schistosomiasis [36].

2.3.3. Biological activities of the genus *Clausena.*

The isolation of a large number of secondary metabolites of different chemicals groups as well as many essential oils of several components has expected different biological activities. The leaves and aerial parts of *clausena* species have been reported to have anti mycobacterial, anti-inflammatory, insecticidal, antifungal, sedative, anti diarrhoeal, anticancer, antiviral, anti-tuberculosis, etc., properties in Asia and Africa [37].

Pharmacological activities of the crude extracts of *clausena excavata* have been reported to have hepatoprotective, ant diabetic, anticancer, antimicrobial, antioxidant, antifungal, and antiviral, anti-diarrheal, immonomodulatory, larvicidal, anti-plasmodial, and anti-inflammatory. The antimicrobial activities of hexane and ethyl acetate extracts of the aerial part (stem and leaf) of the same plant were also reported to be active against microorganisms, which include *Bacillus subtilis*, *Klebsiella pneumonia*, *Staphylococcus aureus* and clinical isolates of *Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Klebsiella ozaenae* and *Shigella dysenteriae* [38].

Clausena anisata, Clausena excavate and *C.harmandian* has also been found to possess anti-inflammatory, anti-bacterial and showed moderate anti-malarial activity [10]. The major Coumarins compounds, carbazole derivatives and the composition of essential oils predominant in *Clausena anisata* and *clausena excavate* also to examine its ant mycobacterial activity at a minimum inhibitory concentration [38]. The carbazole alkaloid isolated from the stems of *C. excavata* showed moderate anti-malarial activity against *Plasmodium falciparum* with a MIC value of 6.74µg/ml. The three carbazole derivatives, O-methylmukonal, 3-formyl-2,7-dimethoxycarbazole and clauszoline J, and a pyranocoumarin, clausenidin, were isolated from the roots of *C. excavata* has been reported currently to have anti-HIV-1 activity [39].

The methanolic roots extract of *C.anisata* was investigated the anti-inflammatory, antipyretic and hypoglycemic activity in fasted streptozotocin treated and fasted normal diabetic rats at a dose of 100-800 mg/kg. A comparative study on the anti-epileptic activity of the root, stem and leaf of *C. anisata* revealed that the ethanol extract of the root bark possessed anti-epileptic activity with 33.33 % anti-convulsant effect. The limonoids, clausenolide, clausenarin, zapoterin, clausenolide -1 -ethyl ether and limonin were isolated from the chloroform and pet ether extracts of the collective root and stem of *C. anisata* showed anti-HIV property in cell line in syncytium assay [40].

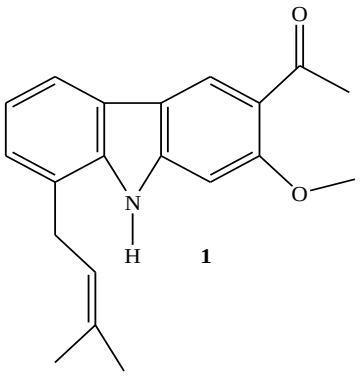
Compound like Osthol isolated from *C.anisata* inhibited the movement and invasion of breast cancer cells by wound healing and transwell tests, stopped matrix metalloproteinase-s promoter and enzyme action in luciferase and zymography tests. The major link between osthol presence and antifeedant activity of the root and leaf extracts of *C. anisata* were studied and osthol content was reported to be responsible for 99% of the difference in antifeedant effect of the root as compared to the leaf indicating osthol as the 41 possible active antifeedant principle of *C.anisata* root against *Helicoverpa armigera* [41].

2.4. Phytochemistry of the *Clausena anisata*.

Several phytochemical studies have been previously carried out on all morphological parts of *C. anisata* which led to isolation of many secondary metabolites which were mainly carbazole alkaloids, coumarins, limonoids and few phytosteroids and amine derivatives. Phytochemical investigations of the stem and leaves of *C. anisata* led to isolation of the carbazole alkaloids; atanisatin **[1],** 3-formyl-1-hydroxycarbazole **[2]** and mupamine **[3]** were reported.

Furthermore, Clausenine **[4]** and Clausenol were isolated from the alcoholic extract of the dried stem bark of *C. anisata* [42]. Chromatographic investigation of the collective stem bark and root extracts of *C. anisata* also yielded coumarins such as: xanthoxyletine **[5]**, swietnocoumarin I **[6]**, gravelliferonemethylether**[7],**anisocoumarin A **[8]**, anisocoumarin B **[9]**, anisocoumarin C and anisocoumarinD. Furthermore, the leaves of *C. anisata* also yielded prenylated coumarins such as capnolactone, anisocoumarin E, anisocoumarin F, anisocoumarinG, anisocoumarinH, and triphasiol upon chromatography. Some other compounds previously isolated from *C. anisata* include amide derivatives such as Nbenzoylphenylalaninyl-N benzoy lpheny lalaninate, aurant I

amideacetate, lansamide-I [**10**], in addition to a mixture of two phytosterols namely; sitosterol **[11]** and stigmastorol **[12] [**43].



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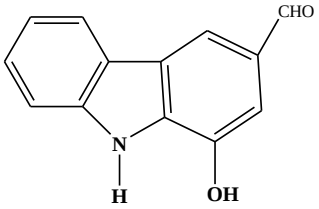

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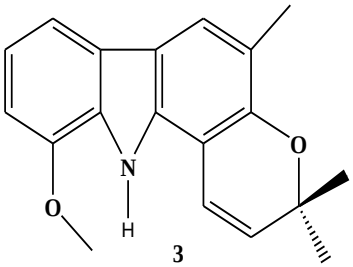

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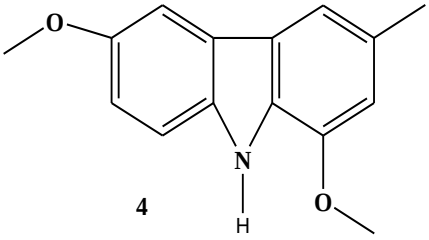

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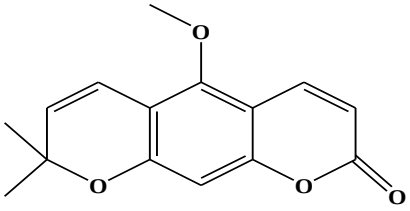

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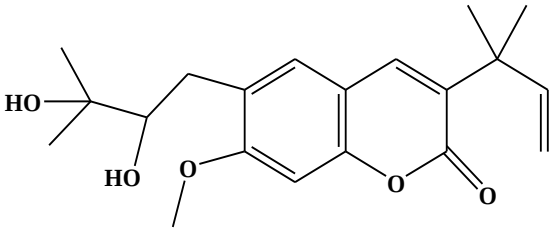

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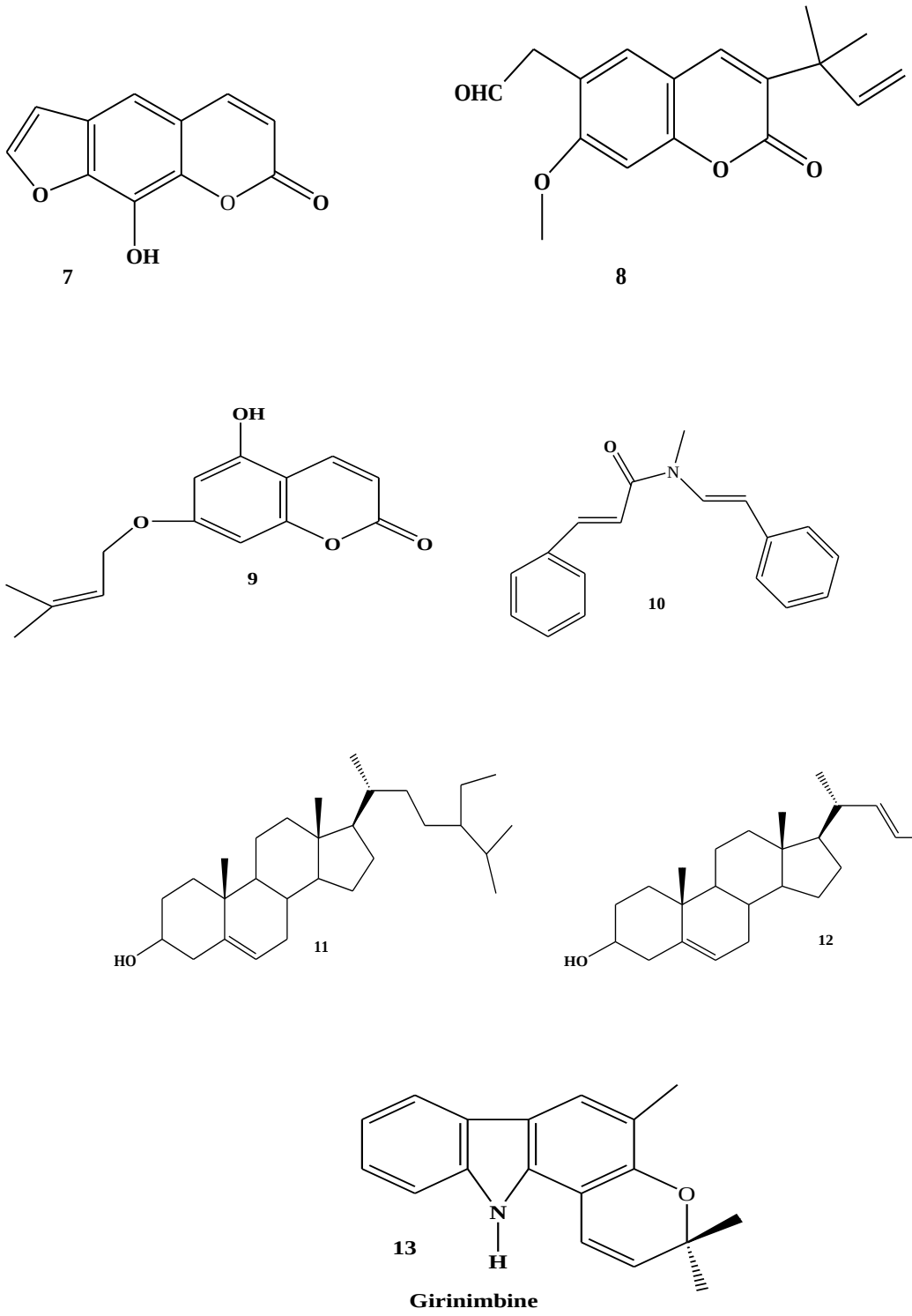

Figure 2: The structures of carbazole alkaloids, Coumarins and phytosteroids isolated from *C. anisata*

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents

Organic solvents used in the present study were 99.5% dichloromethane, 99.8% methanol (CH_3OH) (Loba Chem Pvt. Ltd., India), 97% ethanol (C_2H_5O), 99% *n*-hexane (C_6H_{12}) (Loba Chem Pvt. Ltd., India), 99.5% ethyl acetate ($C_4H_8O_2$) (ATICO Medical Pvt. Ltd.,India), sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl), ammonia (NH_3), distilled water (H_2O), acetone (CH_3COCH_3), ferric chloride ($FeCl_3$), acetic acid (CH_3COOH), sodium carbonate (Na_2CO_3) and silica gel. The solvents were used for extraction, column packing as eluent, silica gel, for column chromatography, used to test the phytochemical constituents, for detection of compounds on TLC plates and to evaluate the anti-bacterial activities. All the chemicals used were analytical grade from Ran Chem General Trading PLC, Addis Ababa, Ethiopia

3.2. Apparatus and instruments

The apparatus and instruments which were used in this study include: columns, separatory funnel, droppers, graduated cylinders, Whatman No.1 filter paper, refrigerator, TLC plates, TLC chamber, UV lamp and vials samples. Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck, Germany) coated on aluminum plate. Column chromatography was performed using silica gel 60 (70-230 mesh) Merck. Spots of compounds on TLC were detected using UV-254 nm. Samples were applied on column by either adsorbing on silica gel or dissolving in appropriate solvent. Solvent was removed using rotary evaporator. NMR spectra of the compounds were recorded on a Bruker Avance 400 spectrometer in $CDCl_3$ operating at 400 MHz for ^{1}H-NMR, 100 MHz for ^{13}C-NMR and DEPT-135 with TMS as internal standard. Chemical shift values for all NMR data are reported in parts per million (ppm) relative to internal standard. Infrared (IR) spectra were obtained on Perkin- Elmer 65FT ((IRvmax, KBr (4000-400) cm^{-}) infrared spectrometer using KBr pellets

3.3. Plant material collection

The roots of *Clausena anisata* were collected from the Oromia region, West Wollega zone which is 541 km west of Addis Ababa (in March, 2018). The plant was identified by the botanist Shambel Alemu, and voucher specimen (Voucher no- CA002) was deposited in the National Herbarium of Ethiopia, Addis Ababa University. The collected roots of the plant were thoroughly washed using distilled water to remove dirtiness, air dried in the shade and stored. The dried plant roots were cut into small pieces, air-dried, and grounded into a fine powder using an electric grinder.

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3. 4.Chromatographic analysis

Each of the crude extracts was first analyzed by using TLC. TLC was employed in analyzing the crude extracts and their various fractions. TLC was also used to analyze fractions collected from the column in other to find those that were similar and thereby combine them. The purity of the compounds was also assessed using TLC. The TLC plates used in this study were made of silica gel F_{254} precoated with aluminium plates and were of 0.25 mm thickness procured from Merk, Germany. The developed plates were first visualized in a U.V lamp chamber at 254 and 365 nm respectively. The compounds appeared as various colourful spots under 365 nm on a dark background and as dark brown spot or yellowish green background at 254 nm. Depending on results of the TLC analysis, the CH_2Cl_2:CH_3OH extracts was chosen for further chromatographic separations. The selected extract was separated to its constituents employing column chromatography and small column chromatography. The compound was elucidated based on the data obtained from IR and NMR.

3. 5. Extraction and Isolation of compound

3.5.1.Extraction

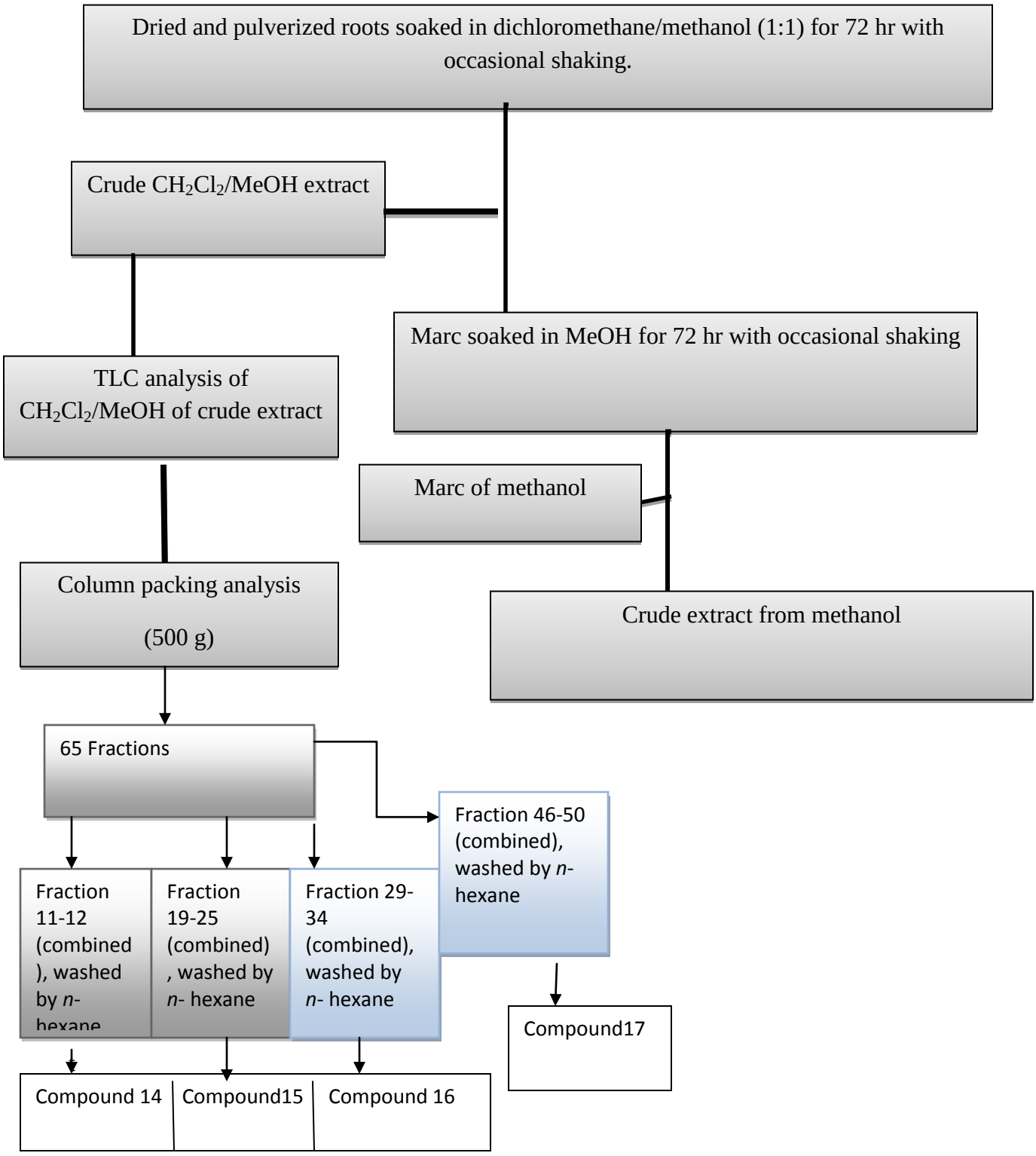
The powdered roots of *clausena anisata (*500 g) were soaked with 2.5L Dichloromethane/ methanol (1:1) and extracted at room temperature using maceration technique for 72 hrs with occasional shaking. The extract was filtered and concentrated using rotary evaporator at 40 ^{O}C to

give black crude (8.5 g, yield). The mark was re-extracted with the methanol (2.5L) after soaking for 72 hrs filtered and concentrated under reduced pressure to furnish 12.4 g of reddish crude(scheme 1). The resulting semidried extract of each solvent were weighted and stored in refrigerator at 4^0C until needed for the further analysis, anti-bacterial assay and TLC analysis.

3.5.2. Isolation of compounds

The dichloromethane /methanol crude extract (8.5 g) was adsorbed on equal amount of silica gel and subjected to silica gel column chromatographic separation (150g silica gel) and eluted with increasing gradient of ethyl acetate in *n*-hexane. A total of 65 fractions (each 100 mL) were collected while monitoring by TLC plate (Table-1). Fractions that showed similar R_f values and the same characteristic colour on TLC were combined. Fractions 11-12 showed similar spot with identical R_f values and after combining and concentrating, the solid material left was repeatedly washed with *n*-hexane to yield compound **14**.

Fractions 19-25 were combined since all fractions showed single spot and similar R_f values. After concentrating, the solid was washed repeatedly with *n*-hexane to afford compound **15**. Fraction 29-34(16 mg) were combined on the basis of TLC profile and showed a single red coloured spot on TLC using *n*-hexane: EtOAc (6:4) as a mobile phase. After concentrating, the solid was washed repeatedly with *n*-hexane to afford compound **16**. Fractions 46-50 (27 mg) were combined on the basis of TLC profile and showed one spot on TLC using *n*-hexane: EtOAc (5:5) as eluent. After concentrating, the solid was washed repeatedly with *n*-hexane to afford compound **17**(Scheme 1).


Dried and pulverized roots soaked in dichloromethane/methanol (1:1) for 72 hr with occasional shaking.
Crude CH_2Cl_2/MeOH extract
Marc soaked in MeOH for 72 hr with occasional shaking
TLC analysis of CH_2Cl_2/MeOH of crude extract
Marc of methanol
Column packing analysis (500 g)
Crude extract from methanol
65 Fractions
Fraction 46-50 (combined), washed by *n*-hexane
Fraction 11-12 (combined), washed by *n*-hexane
Fraction 19-25 (combined), washed by *n*- hexane
Fraction 29-34 (combined), washed by *n*- hexane
Compound17
Compound 14
Compound15
Compound 16

Scheme-1: Extraction of crude extracts from the roots of *Clausena anisata*.

Table1. Column chromatographic fractionation of the dichloromethane/ methanol extract of the root of *clausena anisata*

Eluent (ratio)	**Frictions**	**Remark**
n-hexane (100%)	1-5	Tail on TLC
n-hexane/EtOAc (9:1)	6-10	Tail on TLC
n-hexane/EtOAc (8:2)	11 – 12	Show one spot and combine
n-hexane/EtOAc (8:2)	13-18	Tail on TLC
n-hexane/EtOAc (7:3)	19-25	Show one spot and combine
n-hexane/EtOAc (7:3)	26-28	Tail on TLC
n-hexane/EtOAc (6:4)	29-34	Contain single spot and combine
n-hexane/EtOAc (6:4)	35-45	Tail on TLC
n-hexane/EtOAc (5:5)	46-50	Contain single spot and combine
n-hexane/EtOAc (4:6)	51-56	Contain single spot and combine
n-hexane/EtOAc (3:7)	59-65	Contain single spot and combine

3.6. Phytochemical screening test

Phytochemical screening tests were performed using standard protocols reported in literature [44-50] to identify the constituents (secondary metabolites). Phytochemical screening was done qualitatively using color forming and precipitating chemical reagents on the crude extracts of the plant and the values were tabulated (Table 2**)**.

3.6.1. Test Terpenoids (Salkowski test)

The crude extracts (0.5 g) were shaken with chloroform (2 mL) in the test tube followed by the addition of concentrated sulfuric acid (3 mL) along the side of the test tube using dropper, and a reddish brown coloration of the interface was observed [44].

3.6.2. Tests for steroids (Liebermann-Burchard test)

Each extract (0.1 g) was shaken with chloroform in a test tube; few drops of acetic anhydride was added to the test tube and boiled in a water bath and rapidly cooled in iced water. Concentrated H_2SO4 (2 mL) was added alongside of the test tube.

Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids [45].

3.6.3. Test for alkaloids (Dragendroff's Test)

Crude extract (0.3 g) was mixed with 2 mL of concentrated hydrochloric acid. The mixture was then filtered and mixed with small amount of amyl alcohol at room temperature. Few drops of dragendroff's reagent (Solution of Potassium Bismuth Iodide) were added to the acid layer and a reddish brown precipitate was observed [46].

3.6.4. Test for tannins (Gelatin Test)

Small quantity of the extract was mixed with water and heated on water bath. To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins [47]

3.6.5. Test of phenols (Lead acetate test)

Crude extract (5 mg) was dissolved in 1 milliliter of distilled water and 3 mL of 5% lead acetate solution was added. A bulky white precipitates indicated the presence of phenols [48].

3.6.6. Test for flavonoids (Alkaline Reagent Test)

Few drops of sodium hydroxide solution was added to the extract and formation of intense yellow color, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids [47].

3.6.7. Test for saponins (Froth Test)

Crude extract (0.1 g) was dissolved in 20 mL of water shaken in a graduated cylinder for 15 minutes. Formation of 1cm layer of foam indicates the presence of saponins [49].

3.6.8. Test for anthraquinones

Methanol extract (0.5 g) was boiled with concentrated hydrochloric acid for few minutes in water bath and filtered. The filtrate was allowed to cool and equal volume of $CHCl_3$ was added

to it. Few drops of ammonia were added to the mixture and heated in water bath. Formation of rose-pink color was inspected [50].

3.6.9. Test for Coumarins

One ml of the plant extract (0.5 g) was taken in a small test tube and covered with filter paper moistened with 1N NaOH. The test tube was placed for few minutes in boiling water. Then the filter paper was removed and examined in UV light for yellow fluorescence to indicate the presence of Coumarins [48].

3.7. Antibacterial Testing

In vitro anti-bacterial activity of the dichloromethane/methanol (1:1) and methanol extracts of the roots of *Clausena anisata* and isolated compounds were done using Agar disk diffusion method. Four bacterial strains were obtained from Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory Center, Adama, Ethiopia. Two gram-negative bacterial strains bacterial *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 4853 and Two gram–positive *Bacillus substilis* NCTC 10073, *Staphylococcus aureus* ATCC 25923.

3.7.1. Agar well diffusion method

The ager media was poured into petri dishes where 1 mL of bacteria suspension was uniformly spread on the sterile Mueller Hinton Agar Petri dish. The mixture was swirled and the ager was left to solidify. Lids of the petri dishes were kept closed as much as possible to prevent contamination. The test bacterial cultures were evenly spreads over the appropriate media. Using a cork borer of diameter 6mm equidistance wells were punched with flaming. Standard solutions of 1.5 g/mL concentrations of the extracts and isolated compounds were prepared. Plant extracts and isolated compounds 20 µg/mL solutions from the concentration were then introduced and the Petri dish was then placed in an incubator for 24 hours at 37^0C. After incubation period the result were observed and the inhibition diameter was measured and expressed in millimeters. Ciprofloxacin antibiotic standards were used as a positive control group reference drug and respective DMSO was used as a negative control.Antibacterial activity was determined by measuring the inhibition zone diameter (mm) against each test organism.

4. RESULTS AND DISCUSSION

Successive extraction of the ground root of *Clausena anisata* (500 g) using CH_2Cl_2: MeOH and MeOH afforded 8.5 g (1.74%) black crude extracts and 12.48 g (2.48%) dark-reddish crude extract of the corresponding solvent, respectively. From these results one can deduce that more polar compounds are found in the plant than non-polar ones as the percentage yields increase with polarity.

4.1. Phytochemical screening test

Phytochemical screening test of dichloromethane/methanol (1:1) and methanol roots extracts revealed the presence of flavonoids, phytosterols, coumarins, phenols, alkaloids, tannins, terpenoids and free reducing sugars whereas saponins were absent (**Table 2**). The medicinal value of plants lies in some chemical substances or secondary products that have definite physiological functions in the human body and which may help in protection against various diseases.

Table 2: Phytochemical screening test results of root of *Clausena anisata*.

Secondary metabolite	**CH_2Cl_2/CH_3OH (1:1) extract**	**MeOH extract**
Coumarins	+	+
Saponins	-	-
Terpenoids	+	+
Phytosterols	+	+
Flavonoides	+	+
Alkaloides	+	+
Phenols	+	+
Tannins	+	+
Free reducing sugars	+	+

Note: (+) indicates the presence of particular metabolite, (−) indicates absence

The use of some plants for medicinal purpose is due to the presence of secondary metabolites [37], which supports the use of *C.anisata* for the treatment of several diseases by local herbalists

or traditional healers. Literature reported the biological properties of secondary metabolite. Among which Carbazole alkaloids and coumarin compounds possesses anticarcinogenic, hemorrhagic, anti-oxidant, anti-malarial, anti-microbial activities [41]. They have also been investigated as drug. For instance, Girinimbine Compound-**13 (**Figure-2) was shown to possess anticancer activity by induction of apoptosis on lung cancer cells *in vitro* which was mediated via both intrinsic and extrinsic pathways reliant on caspase mediation [51] and Imperatorin compound (**16**) (Figure 5) is against an oral cavity cancer, breast cancer and small cell lung cancer human melanoma cell lines, which have a potent target in cancer chemotherapy in cell division [52]. Thus, the presence of alkaloid and coumarins in the root extract of *C. anisata* supports the traditional uses of this plant.

4.2 Structural elucidation of compounds

This works reports the isolation and characterization of four compounds from root of *Clausena anisata* plants via spectroscopy techniques like ^{1}H NMR, ^{13}C NMR, IR and DEPT-135. Describe herein is the chactarization of these compounds in detail.

Compound 14 (DT-01)

Compound **14** was obtained as a reddish brown powder with melting point of (227-228 °C) and R_f value of 0.52 in *n*-hexane/EtoAc (8:2) as eluent. The IR (KBr disk, Appendix-1) spectrum showed broad vibration at 3290 cm^{-1} attributed to hydroxyl (OH), sharp absorption band at 1618 cm^{-1} attributed to a C=C stretching and strong absorption bands at 2930 cm^{-1} showed aliphatic C-H stretching. On the other hand, medium absorption band at 1725 cm^{-1} indicates the presence of C=O stretching of an aldehyde.

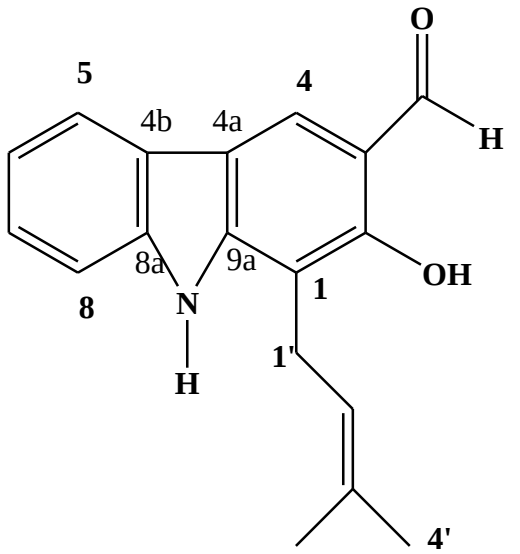
The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz, Table 3, Appendix-2) showed signals for a singlet proton at δ 11.68 (1H, *s*, OH) indicative of hydroxyl (OH) group. The downfield chemical shift of the hydroxyl group suggests the presence of intermolecular hydrogen bonding (*Peri* effect). The presence of singlet peak at δ 9.94 (1H, *s*, CHO) accounts for aldehyde proton. The presence of four aromatic protons were observed at δ 7.21 (1H, *dd*, H-6, J= 7.2, 2.1Hz), 7.43 (1H, *dd*, H-7,J=7.2,2.1Hz), 7.95 (1H, *dd*, H-5, J=7.2,2.1Hz) and 7.99 (1H, d*d*, H-8,J= 8Hz) indicates the presence of disubstituted aromatic ring.

The presence of two aliphatic methyl protons at δ 1.91 (3H, br,*s*, H-4') and δ 1.80 (3H, s, H-5'), olefinic proton at δ 5.35 (1H, *t*, J=5.9Hz) and benzylic methylene protons at δ 3.66 (*d*, H-1' J=6.9Hz) suggest the presence of a prenyl group in the compound. Moreover, the presence of a downfield singlet aromatic proton at δ 8.07 suggests that this proton is experiencing anisotropic effect of the aldehyde carbonyl. The above chemical shift positions for the aromatic singlet proton (H-4), and downfield chemical shift of hydroxyl moiety allow for unequivocal assignment of aldehyde moiety at C-3 between two carbons bearing proton H-4 (C-4) and that of C-2 bearing hydroxyl group.

The ^{13}CNMR spectrum ($CDCl_3$, 100 MHz, Appendix 3) in combination with DEPT-135 (Appenix-4) showed the presence of 18 carbons. Among these, six signals are due to methine carbons, eight quaternary, one benzylic methylene, two methyls and one carbonyl. The ^{13}C NMR spectrum showed peak at δ 195.4 due to carbonyl group of aldehyde moiety. Oxygenated sp^2 quaternary carbon was observed at δ 157.66 (C-2). The remaining carbons of the aromatic methine carbons were observed at δ125.9 (C-4), 119.8 (C-5), 123.7 (C-6), 120.9 (C-7) and 110.9 (C-8). Furthermore the spectrum displayed signals due to quaternary carbons at δ109.1 (C-1), 115.5 (C-3), 117.4 (C-4a), 125.3 (C-4b), 134.2 (C-3'), 140.2 (C-8a) and 145.1 (C-9a). The prenyl moiety appeared at δ 22.8 (C-1'), 121.3 (C-2'), 134.2 (C-3'), δ 25.9 (C-4') and 18.2 (C-5'). Thus, based on the above spectral data and comparison with literature the structure of compound **14** was found to be a carbazole alkaloid (**14**) known by trivial name heptaphyline [53].

Table 3: ^{1}H-NMR ($CDCl_3$, 400 MHz), ^{13}C-NMR and DEPT-135 (100MHz) spectral data of compound **14**

Position	**^{1}HNMR Signal**	**^{13}CNMR Signal**	**DEPT-135**	Reported NMR [**53**]	
				^{1}HNMR	**^{13}CNMR**
CHO	9.94, s	195.4	195.4	9.90,s	196
2(-OH)	11.68, s	157.88	_	11.68,s	157.8
NH	8.23, s	_	_	8.19	
8a	_	140.16	_	-	142.3
9a	_	145.09	_	_	144.8
3	_	115.5	_	_	114.7
4	8.07,s	125.89	125.9	8.07,s	124.3
5	7.95,1H (*dd*,*J*=7.2,2.1Hz)	119.83	119.83	8.00, *d*	120.5
4b	_	125.346	_	_	116.3
6	7.2,(1H, *dd, J*=7.2,2.1 Hz*)*	123.715	123.72	7.41,*d*	123.7
7	7.43 (1H ,*dd, J*=7.2,2.1Hz)	120.895	120.89	7.743,*dd*	120.9
8	7.99 (1H,*d, J*=7.2,2.1Hz)	110.89	110.89	7.99,*d*	110.8
4a	_	117.368	_	_	117.0
1	_	109.09	_	_	108.9
1’	3.66, (1H ,*d* ,*J*=6.8Hz)	22.896	22.896	3.65, *d* ,*J*=7Hz	22.6
2’	5.35, (1H ,t ,J=1.2Hz)	121.265	121.26	5.35, t, *J*=6Hz	121.6
3’	_	134.21	_	_	131.7
4’	1.91, s	25.748	25.75	1.9, s	25.7
5’	1.80, s	18.151	18.15	1.82,s	17.9



14

Figure 3. Proposed structure of Heptaphyline (**14**)

Compound 15 (DT-02)

Compound **15** was obtained as a brown crystalline powder with R_f value of 0.56 in *n*-hexane: EtoAc (7:3) solvent system. The IR (KBr disk, Appendix-5) spectrum showed broad vibration at 3419 cm^{-1} due to the presence of the hydroxyl moiety. The strong sharp vibrations at 1717 cm^{-1},1620 cm^{-1} and 1229 cm^{-1} suggest the presence of C=O of an ester, olefinic C=C and carbon-oxygen (C-O) respectively. Moreover, intense vibrations at 2849 $cm^{-1}$and 2930 cm^{-1} indicate methylene (sp^2) and methyl's (sp^3) C-H, respectively.

The ^{1}H NMR spectrum ($CDCl_3$, 400MHz, Table 4, Appendix-6) revealed peaks at δ 7.54 (1H, s, H-4), δ 7.19 (1H, s, H-6) and δ 6.96 (1H, s, H-9) attributed to aromatic protons. The presence of prenyl moiety was confirmed on the basis of methylene protons (H-2'') adjacent to olefinic carbon, olefinic proton (H-3'') and two methyl groups were observed at δ 3.38 (2H, *d*, *J*=7.2Hz, H-2''), 5.34 (1H, *t*, H-3'') and δ 1.78(3H, s, H-5'') and 1.81(3H, s, H-6''), respectively. Peak at δ 6.18 (1H, *dd*, *J*=10.8, 6Hz, H-2') attributed to olefinic proton adjacent to terminal olefinic methylene protons at δ 5.1 (1H, *dd*, H-3'') and δ 5.07 (1H, *dd*, H-3''). Symmetrical methyl protons were observed at δ 1.49 (6H, s, H-4', 5').

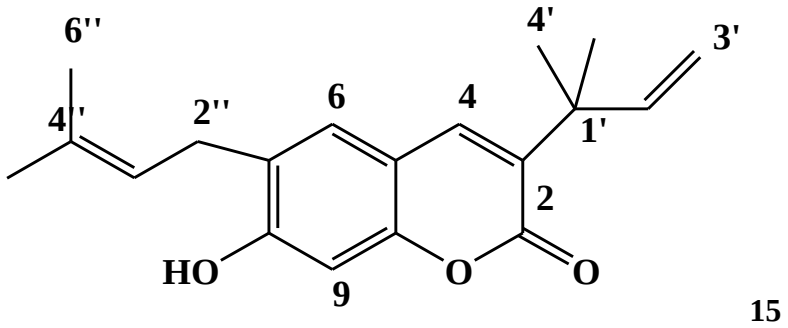
The presence of two singlet aromatic proton at δ 7.19 (1H, s, H-6) and δ 6.95 (1H, s, H-9) coupled with a downfield proton at δ 7.54 (1H, s) are in good agreement with a chromene moiety where later (H-4) is located at β-position of the lactone moiety whereas H-6 and H-9 are located at 1,4-positions of the aromatic ring of chromene skeleton.

The ^{13}CNMR spectrum ($CDCl_3$ 100MHz, Appendix-7) in combination with DEPT-135 (Appendix-8) showed a resonance for a total of 18 carbon atoms instead of 19 which may be due to an overlap of C-4' and C-5' carbon signals. Among these, five signals are due to methine carbons, eight quaternary, three methyl and two methylene carbons. The most downfield signals appearing at δ 160.7 attributed to the ester carbonyl whereas the quaternary carbons appearing at δ 157.3(C-8) and δ 153.3 (C-10) were assigned to sp^2 oxygenated quaternary carbons. Methine aromatic carbons were observed at δ 138.3 (C-4), 102.5 (C-9), 128.2 (C-6), δ 121.2 (C-3'') and 145.6 (C-2').

The methyl signals due to C-5'' and C-6'' were observed at δ 25.8 and 17.9, respectively. Symmetrical carbons signal were also observed for C-4' and C- 5'at δ 26.2, while the methylene signals were observed at 28.6 (C-2'') and 112.8 (C-3'). Furthermore the spectrum displayed signals due to quaternary carbons at δ 131.2, 112.0, 124.9, 135.0 and 40.3 assigned to C-3, C-5, C-7, C-4'' and C-1' respectively. Thus, based on the above spectral data and comparison with literature the structure of compound **15** was proposed to be a chromene skeleton given by trivial name graveliferone (**15)**.

Table 4: The ^{1}H NMR ($CDCl_3$, 400 MHz), ^{13} C NMR ($CDCl_3$, 100 MHz) and DEPT-135 spectral data of compound **15**

Position	**^{1}HNMR signal**	**^{13}NMR**	**DEPT-135**	**[54]**	
				^{1}HNMR	**^{13}CNMR**
2	-	160.7	-	-	161.1
3	-	131.3	-	-	131.9
4	7.54,1H ,s	138.3	138.29	7.54,1H,s	138.5
5	-	112.0	-	-	112.1
6	7.19,1H,s	128.2	128.16	7.17,1H,s	128.2
7	-	124.9	-	-	125.4
8	-	157.3	-	-	157.5
9	6.95,1H,s	102.5	-	7.04,1H,s	102.5
10	-	153.3	-	-	153.2
1’	-	40.3	-	-	40.31
2’	6,18, (1H, *dd*, J= 10.8,6Hz)	145.6	145.64	6.16,1H,*dd*,J=10.2,18Hz	145.6
3’	5.09 (1H,*dd*. *J*=10.2,5.8Hz) 5.07(1H,*dd*,)	112.8	112.77	5.1,2H,*dd*(J=10.2,18Hz)	112.7
4’ ,5’	1.49, 6H, s	26.2		1.48 ,6H,s	26.1
2’’	3.38 (2H, *d*, J=7.2Hz)	28.6	28.62	3.38, 2H, *d* (J=7.2Hz	28.4
3’’	5.33 (1H, t)	121.2	-	5.33,1H,m	121.3
4’’	-	135.0	-	-	134.6
5’’	1.80 (3H, *s*)	25.8	25.83	1.80 ,3H,s	25.83
6’’	1.78 (3H, *s*)	17.9	17.89	1.75 ,3H,s	17.88



15

Figure 4. Proposed structure of graveliferone **(15)**

Compound 16 (DT-03**)**

Compound **16** was isolated as orange powder with R_f value of 0.59 in *n*-hexane/EtoAc (6:4) as solvent system. The IR (KBr disk,Appendix-9) spectrum showed broad absorption band at 3440 cm^{-1},1724 cm^{-1}, 1145 cm^{-1} and 2930 cm^{-1} attributed to hydroxyl moiety (OH), C=O stretching of an ester, C-O stretching, and C-H stretching of methyl group, respectively.

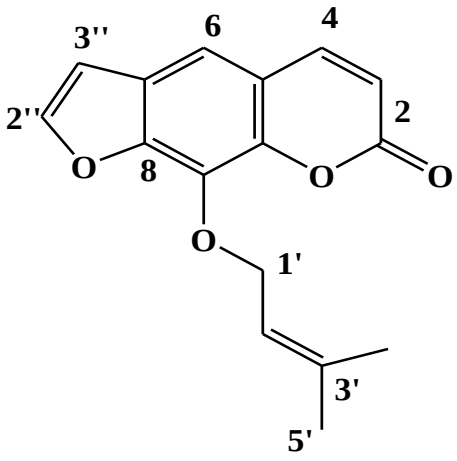
The ^{1}H NMR ($CDCl_3$, 400MHz,Table 5, Appendix-10) spectrum showed two proton doublets at δ 6.36 (1H,*d*, *J*=9.6 Hz) and 7.77 (1H,*d*, *J*=9.40Hz) attributed to olefinic protons of which one of them is downfield due to β-position of lactone moiety. Two oleifnic protons were observed at δ 7.69 (1H, *d*, H-2'', *J*=2.4Hz) and 6.82 (1H, *d*, H-3'', *J*=2.4Hz) coupling to each other suggesting the presence of a furan ring attached to the aromatic ring. The presence of singlet aromatic proton was observed at δ 7.36 (1H, *s*, H-6). The presence of prenyl group was confirmed based on peaks of two methyl signals at δ 1.72 (3H, *s*, H-4') and δ 1.74 (3H, *s*, H-5'), olefinic proton at δ 5.65 (1H, *t*, H-2') and oxygenated methylene proton at δ 5.0 (2H, *t*, H-1'). The later suggests that the prenyl group is attached to oxygen. Moreover, the above ^{1}H NMR pattern suggests the compound has chromene skeleton consisting of a furan ring fused to it.

The ^{13}C NMR ($CDCl_3$, 100MHz, Appendix 11) spectrum showed a total of sixteen carbon atoms. Eleven of these carbons exhibited resonance signals in the aromatic region. The downfield chemical shift signal that appeared at δ 160.5 coupled with signals at 114.6 and 143.8 suggest α, β-conjugated lactone moiety. The other five quaternary carbons at δ 116.5, 125.9, 131.6, 144.5 and 148.6 were assigned to C-5, C-7, C-9, C-10 and C-8, respectively.

Of these, three of the carbons are sp^2 oxygenated quaternary carbons i.e. C-8, C-9 and C-10 of aromatic ring. The methine carbons of the furan moiety were observed at δ 146.6 (C-2'') and 106.7 (C-3'') of which the downfield chemical shift value of the former in agreement with its attachment to the oxygen atom. The aromatic methine at C-6 appeared at δ 113.2. Oxygenated methylene of the prenyl moiety appeared at δ 70.2 (also confirmed by DEPT-135 pointing downwards) whereas the remaining carbons of prenyl moiety group appeared at δ 119.8 (C-2'), 139.7 (C-3'), 25.8 and 18.2 (C-4' and C-5'), respectively. Thus, based on the above spectral features compound **16** was found to be in good agreement with a chromene skeleton known by trivial name Imperatorin (**16**) [55].

Table 5: ^{1}H NMR ($CDCl_3$, 400 MHz), ^{13}C NMR and DEPT-135 (100MHz) spectral data of compound **16**

Position	**^{1}H NMR**	**^{13}C NMR**	**DEPT-135**	**[55]**	
				^{1}H NMR	**^{13}C NMR**
2	-	160.5	-		160.6
3	6.36, 1H, *d* (J=9.6Hz)	114.6	114.6	6.36, *d*	113.0
4	7.77, 1H, *d* (J=9.6Hz)	143.8	143.8	7.75, *d*	144.0
5	-	116.5			116.4
6	7.36, 1H, *s*	113.2	113.2	7.35, *s*	114.8
7	--	125.8	-	-	126.0
8	-	148.6	-	-	148.6
9	-	131.6	-	-	132.0
10	-	144.4	-	-	143.8
2''	7.69, 1H, *d* (J=2.4)	146.6	146.7	7.68, *d*	146.6
3''	6.82, 1H, *d* (J=2.05)	106.7	106.7	6.82, *d*	106.7
1'	5.00, 2H, *d* (J=7.2)	70.2	70.2	4.95, *d*	69.9
2'	5.61, 1H, *t* (7.35)	119.8	119.8	5.61, *t*	119.6
3'	-	139.7	-	-	139.7
4'	1.72, 3H, *s*	25.8	25.8	1.68, *s*	25.9
5'	1.74, 3H, *s*	18.1	18.1	1.73, *s*	18.2



. **16**

Figure 5. Proposed Structure of Imperatorin **(16)**

Compound 17(DT-04)

Compound **17** was obtained as a yellowish amorphous powder with R_f value of 0.53 in *n*-hexane/ethyl acetate (4:6) solvent system. The IR (KBr disk, Appendix-13) spectrum showed broad vibration at 3385 cm^{-1}, sharp absorptions at 1625 cm^{-1} and 1255 cm^{-1} attributed to hydroxyl moiety (OH), aromatic benzene ring and C-O stretching respectively. The strong absorption band at 2925 cm^{-1} showed the presence of the C-H stretching of sp^3 aliphatic moiety. The absorption band at 1730 cm^{-1} showed the presence of the C=O stretching of an ester moiety.

The ^1H NMR ($CDCl_3$, 400 MHz, Table 6, Appendix-14) spectrum revealed the presence of aromatic protons at δ 7.178 (1H, s, H-5), and 6.69 (1H, s, H-8) suggesting two para oriented aromatic protons whereas downfield chemical shift of proton at δ 7.48 (1H, s, H-4) suggest the β-position of the α,β-conjuagated system. The peaks at δ 3.19 (1H, *dd*, H-3') and δ 3.17 (H, *dd*, H-3'*)* suggest the presence of distereotopic methyelene protons adjacent to asymmetric carbon (C-2'). This coupled with the presence of oxygenated methine signal at δ 4.73 (1H, *dd*) suggest the presence of furan ring. Methyl signals were observed at δ 1.48 (6H, s, H-4'', 5''), δ 1.37 (3H, s, H-5') and δ 1.24 (3H, s, 6'). The presence of terminal olefinic protons at δ 5.11 (1H, *dd*, H-3'') and δ 5.068(1H, *dd*, H-3'') coupled with olefinic proton at δ 6.17 suggest the presence of rearranged prenyl moiety in the compound.

The above ^{1}H-NMR pattern suggests that the compound has coumarin skeleton where a reduced furan ring moiety is fused to the aromatic ring and α,β-conjugated lactone ring bearing the rearranged prenyl group.

The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz, Table-6, Appendix-15) revealed a total of eighteen carbon signals instead of 19 which may be due to an overlap of C-4'' and C-5'' carbon signals of which the downfield peak at δ 162.3 is attributed to ester carbonyl group whereas the sp^2 oxygenated quaternary aromatic carbons appeared at δ 160.3 (C-7) and δ 154.7 (C-9). The signal at δ 71.7 (C-4') assigned to the oxygenated sp^3 quaternary carbon. The signal for remaining quaternary carbons were observed at δ 130.8 (C-3), 124.3 (C-6), 113.1(C-10) and 40.3 (C-1''). Methine carbons appeared at δ 138.1, 123.4, 97.2, 90.0 and 145.8 were assigned to C-4, C-5, C-8, C-2'and C-2'', respectively.

Furthermore, the spectrum displayed signals due to methylene carbons at δ 29.7 and δ 112.1 assigned to C-3' and C-3'', also confirmed by DEPT-135 pointing downwards. Symmetrical methyl carbons signals were observed for C-4'' and 5''at δ 26.1 and remaining methyl signals were also observed at δ 26.0 for (C-5') and 24.3 (C-6'). Thus, based on the above spectral features compound **17** was found to be in good agreement with a chromene skeleton known by the trivial name chalepin(**17**) [56].

Table 6: ^{1}H NMR ($CDCl_3$, 400 MHz), ^{13}C NMR and DEPT-135 (100MHz) spectral data of compound **17**

position	**^{1}H NMR signal**	**^{13}CNMR**	**DEPT-135**	**[56]**	
				^{1}HNMR	**^{13}CNMR**
2	-	162.3	-	-	162.3
3	-	130.8	-	-	130.9
4	7.48 (1H, s, H-4)	138.1	138.1	7.48 ,1H,s	138.1
5	7.17 (1H, s, H-5)	123.3	123.25	7.20 ,1H,s	123.3
6	-	124.6	-	-	124.6
7	-	160.3	-	-	160.2
8	6.68 (1H, s, H-8)	97.1	97.1	6.71,1H,s	97.1
9	-	154.6	-	-	154.6
10	-	113.1	-	-	113.1
2’	4.73 (1H, *dd*, *J*=6,12Hz)	90.9	90.9	4.72,1H,t,*J*=12,6Hz	90.9
3’	3.19 (1H,*dd*,*J*=12,6Hz) 3.17 (1H,*dd*)	29.7	29.7	3.21 (2H, *dd*	29.6
4’	-	71.7	-	-	71.7
5’	1.37, 3H,s	26.0	26.02	1.37 ,3H,s	26.0
6’	1.24, 3H,s	24.21	24.21	1.27,3H,s	24.2
1’’	-	40.3	-	-	40.3
2’’	6.17(1H,*dd*,*J*=6,4, 12Hz)	145.6	145.6	6.17,1H,*dd*,*J*=18,12Hz	145.6
3’’	5.11(1H, *dd*, H-3’’) 5.068 (1H,*dd*)	112.1	112.1	5.09,2H.*dd*	112.1
4’’, 5’’	1.48 (6H,s, H-4’’,5’’)	26.2	26.2	1.47, 6H.s	26.11

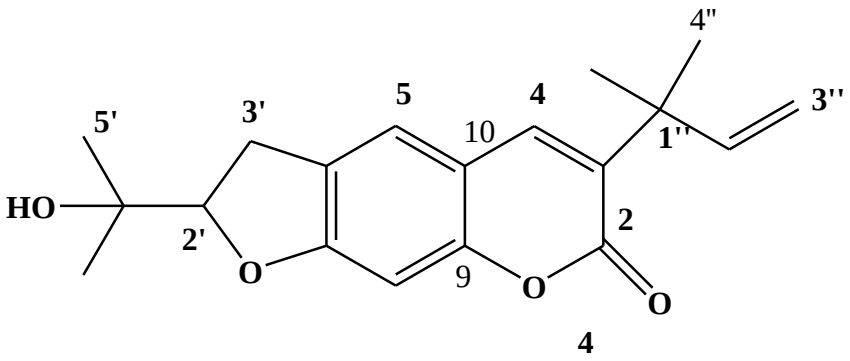


Figure 6. Proposed structure of Chalepin (**4)**

4.3 Antibacterial Activity

The antibacterial activity of the extracts and isolated compounds of *Clausena anisata* were examined at a concentration of 20µg/mL against four pathogenic bacterial strains: two Gram-positive *S. aureus, B. substilis* and two Gram-negative *E. coli*, and *P. aeruginosa* (Table 7). The results revealed that Heptaphyline (**14**) and Imperatorin **(16**) exhibited comparable antibacterial activity against *S. aureus* and *B.substilis*, 14 mm zone of inhibition for both strains, compared to that of ciprofloxacin (15 mm). Chalepin (**17**) also exhibited promising antibacterial activity against *S. aureus,* and *P. aeruginosa* with 14 and 12 mm zone of inhibition, respectively, compared to that of Ciprofloxacin (15 mm) whereas it revealed more antibacterial activity (16mm zone of inhibition) against *B. substilis* compared with the standard drug(15 mm).

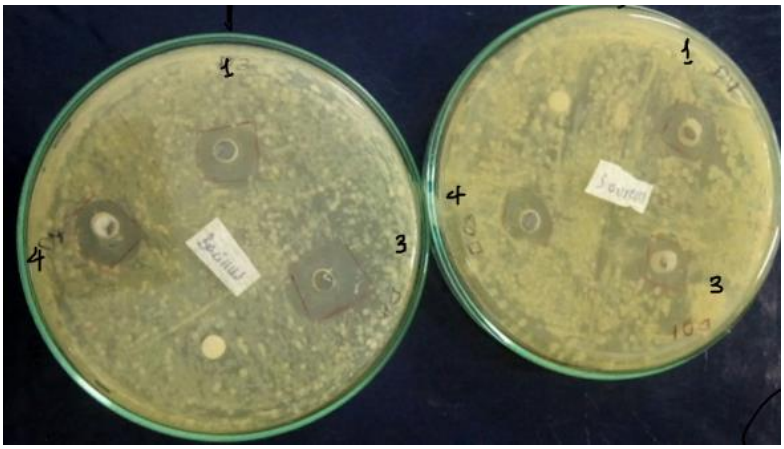
Table 7. Zone of bacterial growth inhibition (mm) for crude extract and isolated compounds

Sample	***E. coli***	***S. aureus***	***B. substilis***	***P. aeruginosa***
CH_2Cl_2/MeOH extract	9	11	10	8
MeOH extract	N	12	13	9
1	N	14	12	12
3	N	13	14	14
4	N	14	16	12
Ciprofloxacin	14	15	15	15

$n \leq 6$ is null, and $n > 6$ is sensitive (1 means compound 14, 3=compoud16,4=compound 17).

The dichloro methane: methanol and methanol extracts were active against *S. aureus*, *P.augenosa* and *B. subtilis* at concentration of 20µg/mL each. Generally crude extracts and pure isolated compounds were ineffective against *E. coli* pathogen at this concentration. As described in the literature [27,47]. *Clausena anisata* is traditionally used to treat infectious diseases, flatworm, hemorrhagic and wound caused by bacteria. Hence, based on the relationship between traditional use and bioactivity test result of *C. anisata*, one can decided that root extracts of this plant may be recommended as a remedy against bacteria caused skin infection, hemorrhagic and wound.

B.subistilis. *S.aureus*



P. aeruginosas *E. coli*

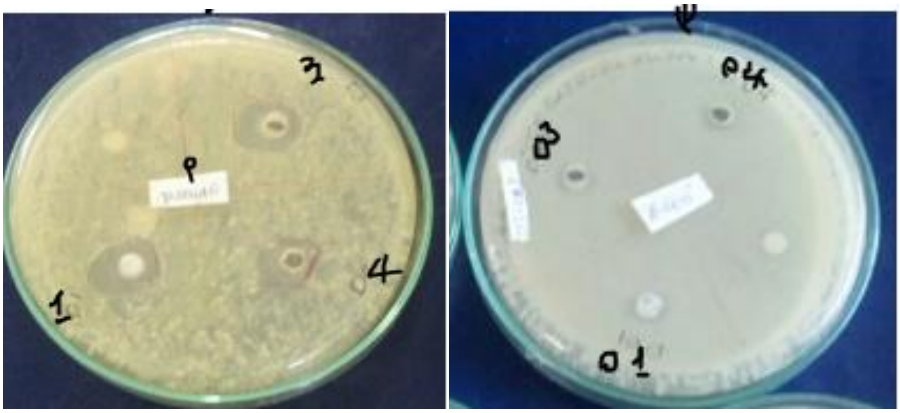


Figure-7: Antibacterial activities of isolated pure compounds from CH_2Cl_2/CH_3OH extracts.

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study is one of the fews attempts to isolate phytochemical constituents from the roots of *Clausena anisata* of Ethiopian flora. Phychemical screening of the dichloromethane/methanol (1:1) and methanol roots extracts revealed the presence of flavonoids, phytosterols, coumarins, phenols, alkaloids, tannins, terpenoids, free reducing sugars and absence of saponins. Silica gel column chromatographic separation of the dichloromethane/methanol (1;1) roots extracts gave three coumarins (**15-17**) identified as graveliferone(**15**) imperatorin (**16**),Chalepin **(17)** and carbazole alkaloid heptaphyline **(14**). Compound **15** was isolated for the first time from the root of *Clausena anisata*. The structures of the compounds were characterized on the basis of spectral data (^{1}H NMR, ^{13}C NMR, DEPT-135 and IR) as well as in comparison with the literature report.

In agreement with the previous study, the wide traditional use of the plant may be attributed to its rich alkaloids and coumarins constituents. The antibacterial test results revealed that the isolated compounds showed promising antibacterial activity against *S. aureus*, *P. aeruginosa* and *B. substil*. Heptaphyline (**14**) and Imperatorine **(16)** exhibited comparable antibacterial activity against *S. aureus* and *B. substilis* (14 and 13 mm zone of inhibition, respectivily) compared to that of with the standard drug. Chalepin (**17**) revealed more antibacterial activity against *B. substilis* (16 mm zone of inhibition) compared to that Ciprofloxacin (15 mm zone of inhibition). The finding of these pharmacologically important secondary metabolites from roots extracts brings the attention of researchers to do more work on the medicinal importance of the plant.

5.2. Recommendations

- Comprehensive phytochemical investigation work is recommended so as to isolate polar chemical constituents with the help of advanced instruments such as RP-HPLC.
- The results of antibacterial activity of crude extracts and isolated compounds is promising, hence more work is recommended to examine combination of various compounds and extracts with standard drugs and check if there are synergistic effects.
- Medicinally valuable polar compounds can be isolated from the methanol crude extract of the plant if different chromatographic techniques are made on.
- Besides the present anti-bacterial studies on the four bacterial strains, this investigation would be best if the work is extended on many other microbes. Replacing of traditional methods of drug extraction and culinary from *C.anisata* with modern technologies would be of important. The chemical modification of various functional groups of these compounds to reduce toxic effects may provide important lead compounds for future research.

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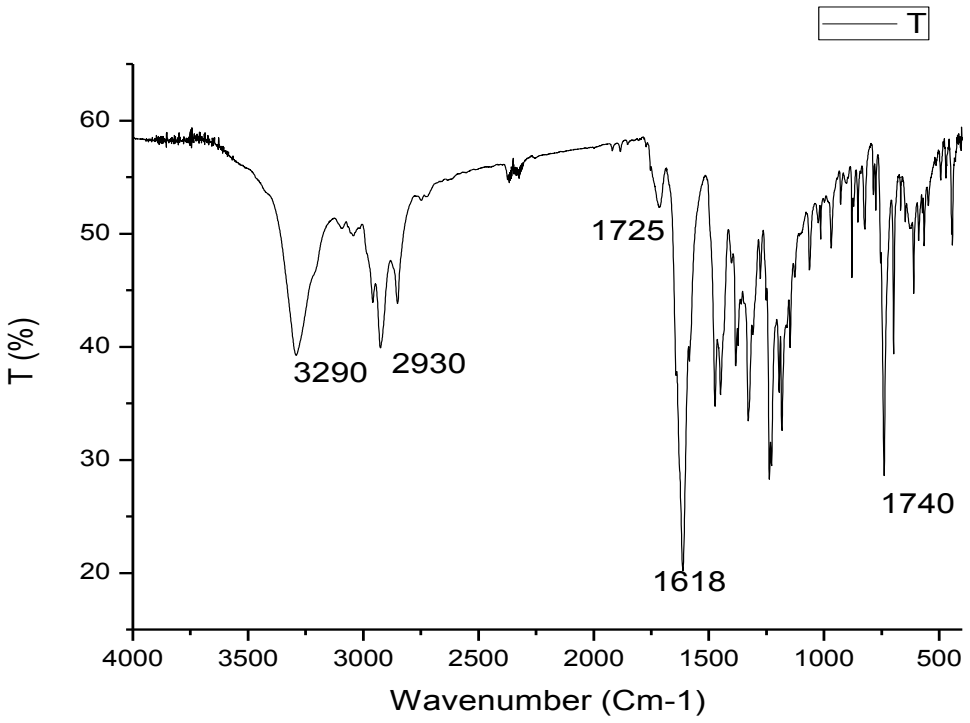
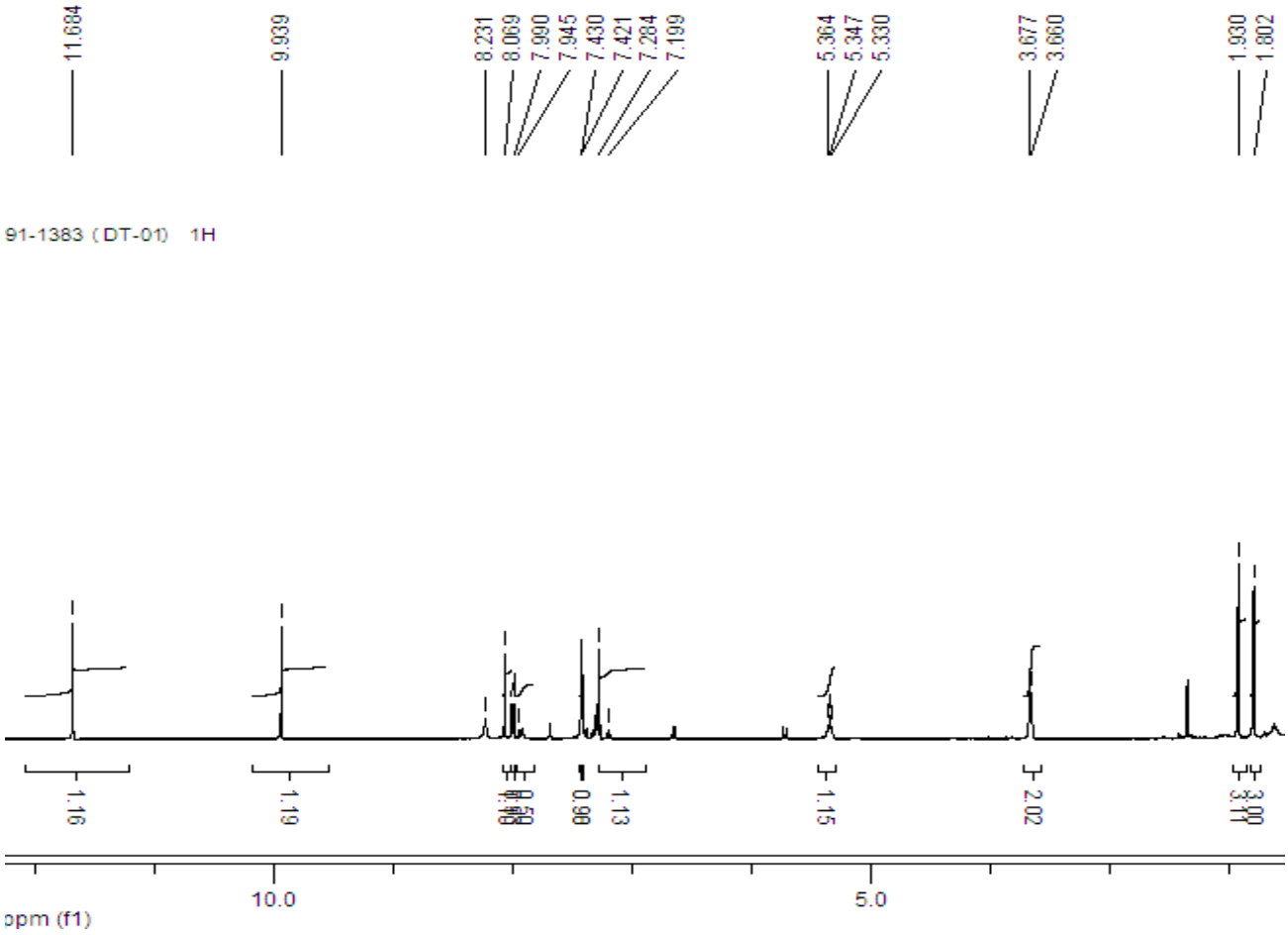
APPENDIX

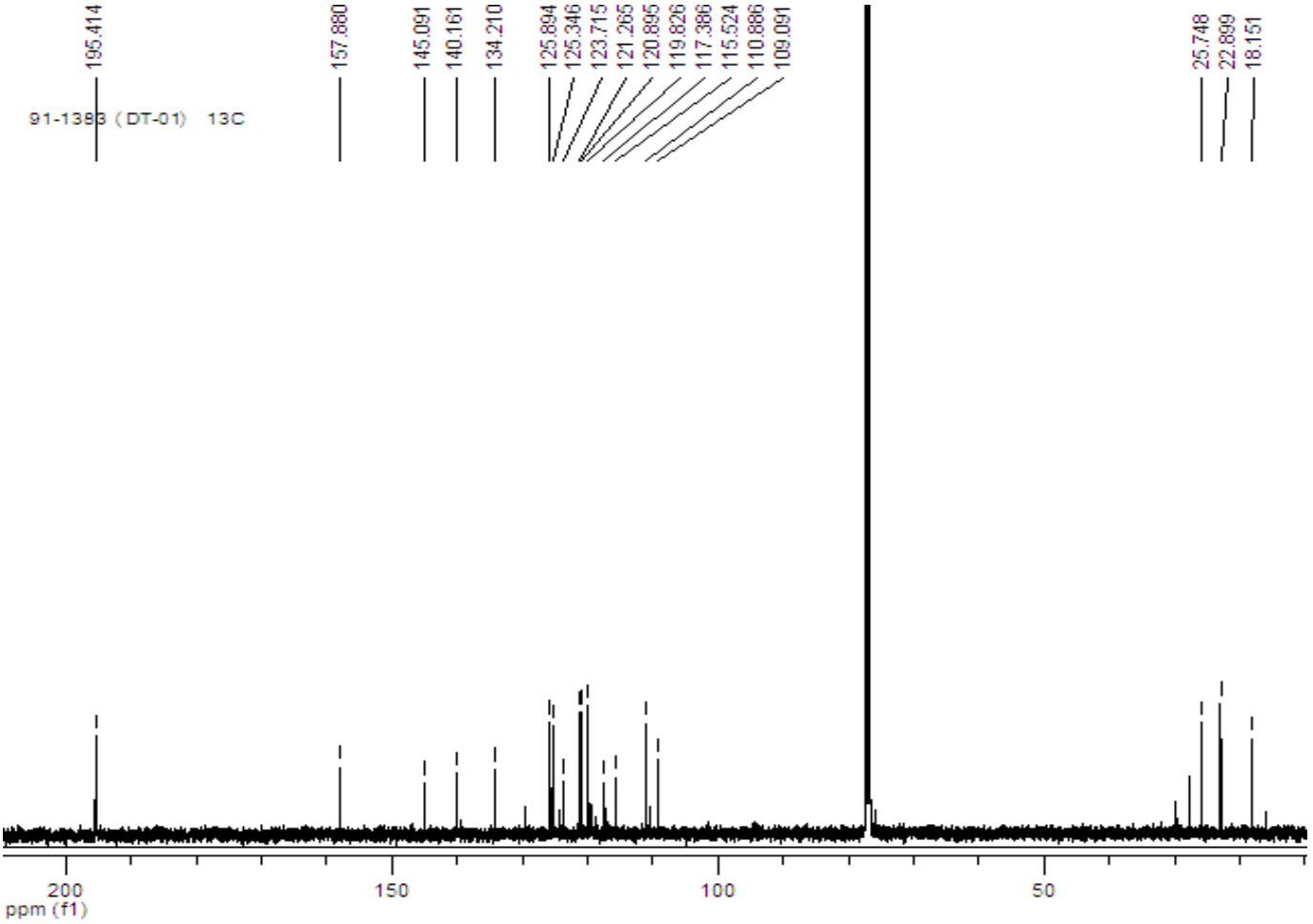
Appendix 1: IR Spectrum of Compound-14



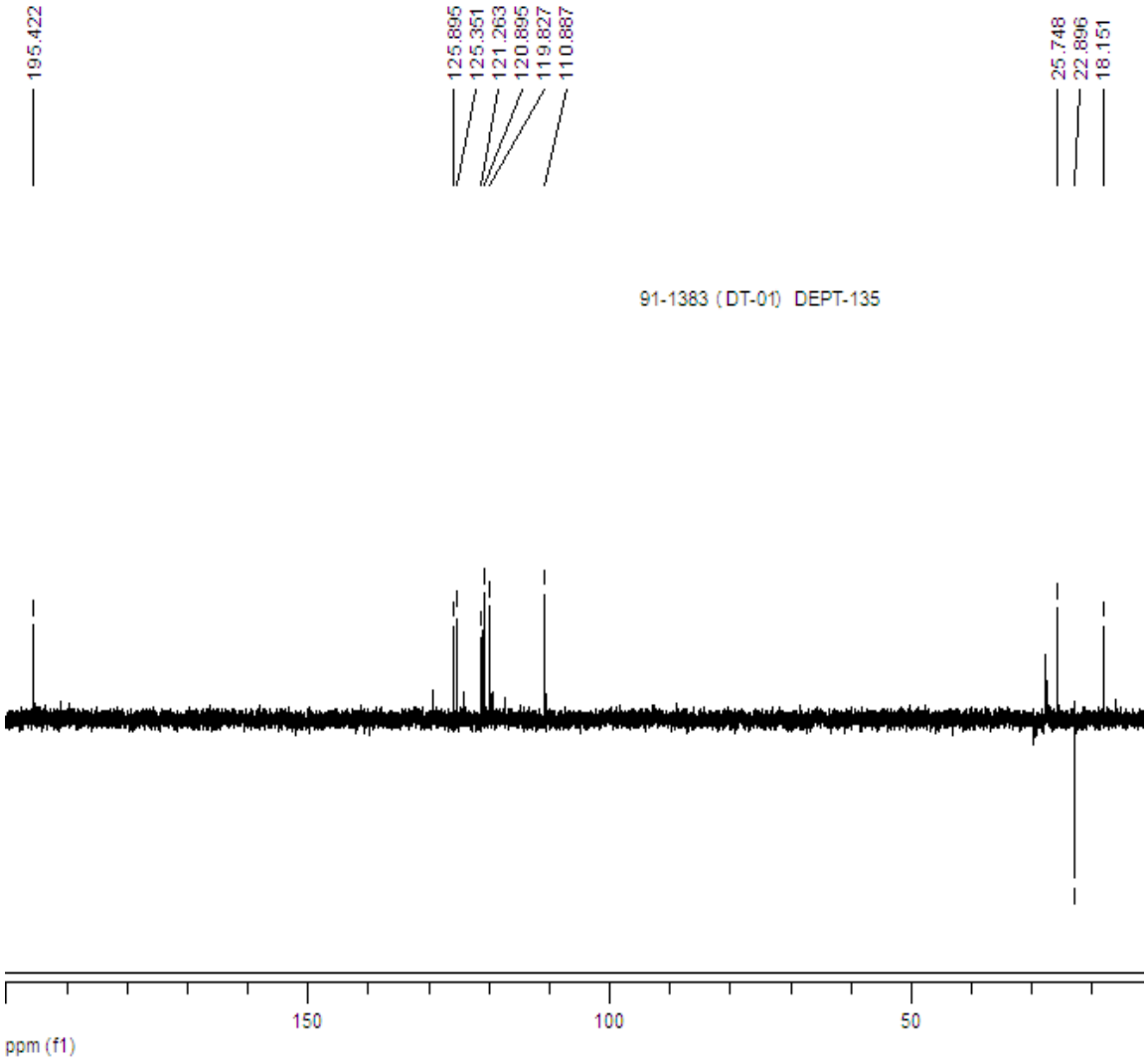
Appendix 2: ^{1}H-NMR spectrum of Compound **14**



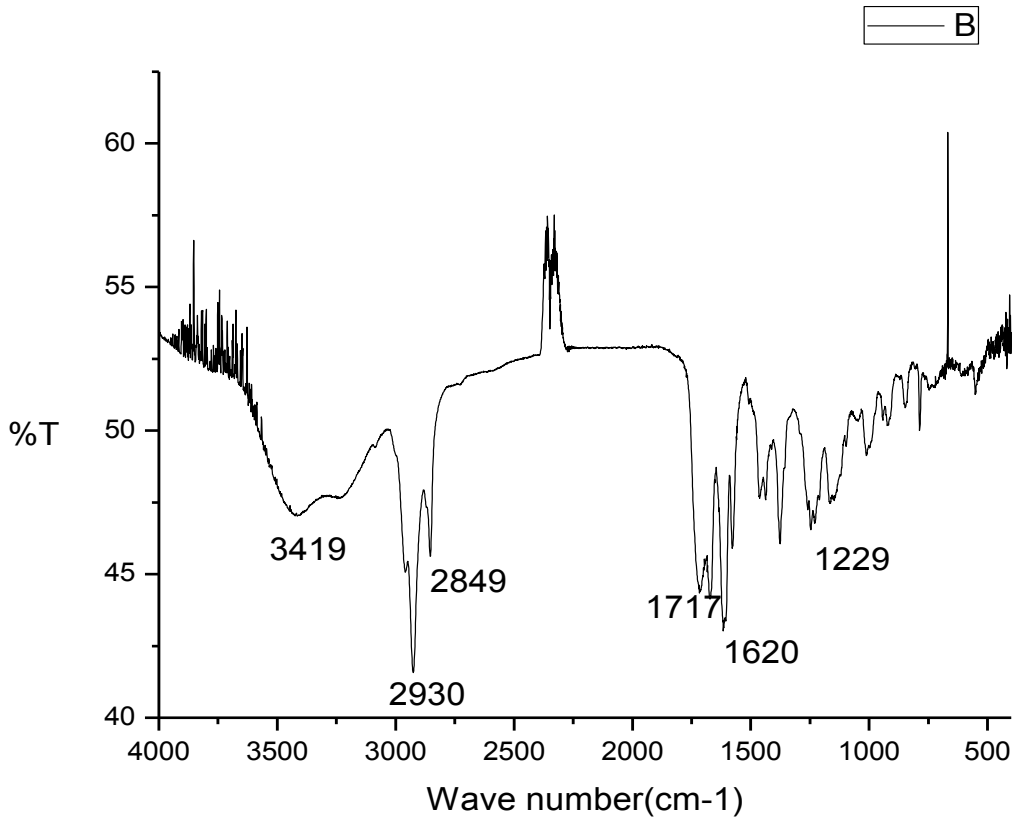
Appendix 3: ^{13}C- NMR spectrum of Compound **14**



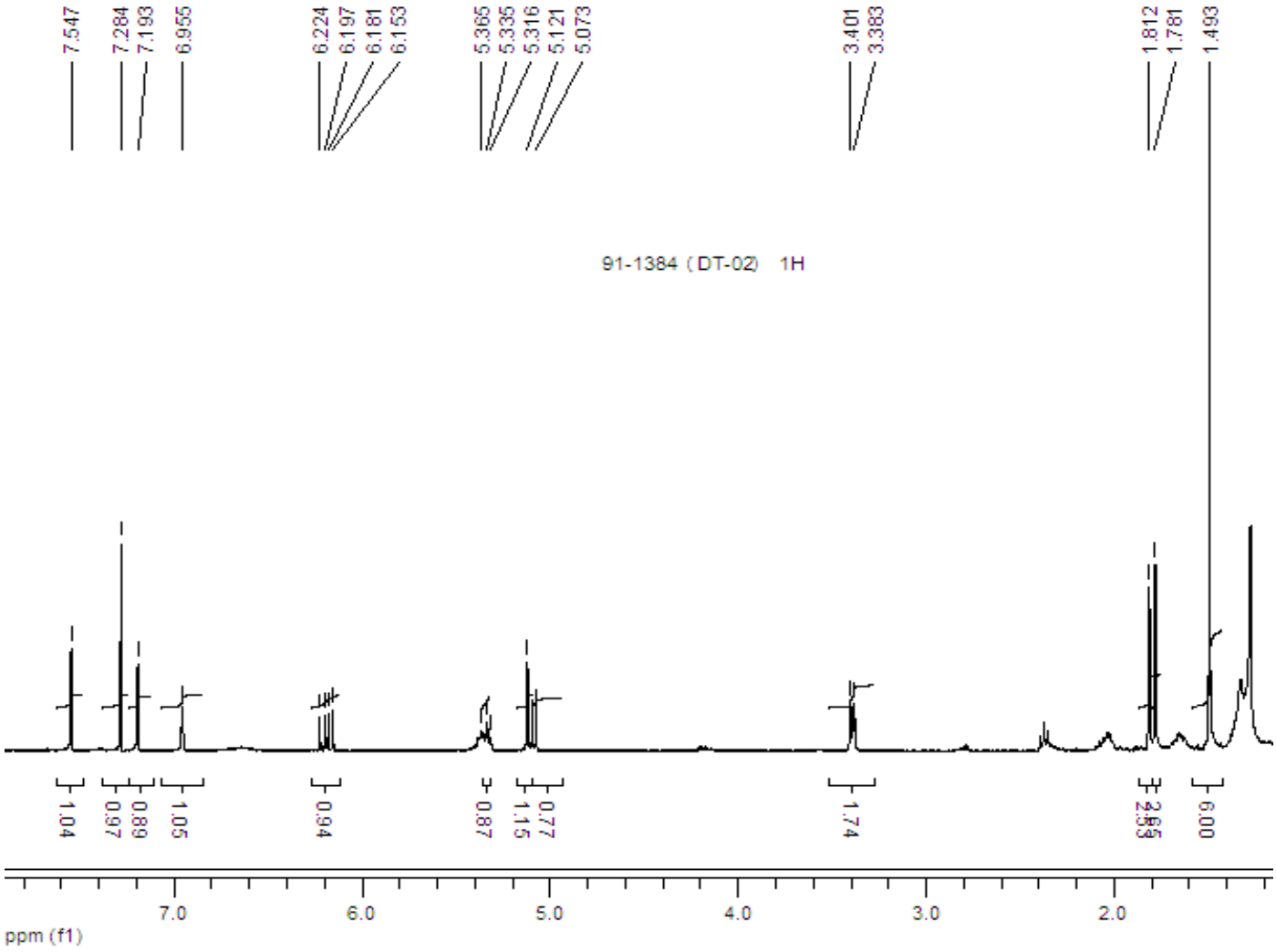
Appendix 4: DEPT-135 spectrum of Compound **14**



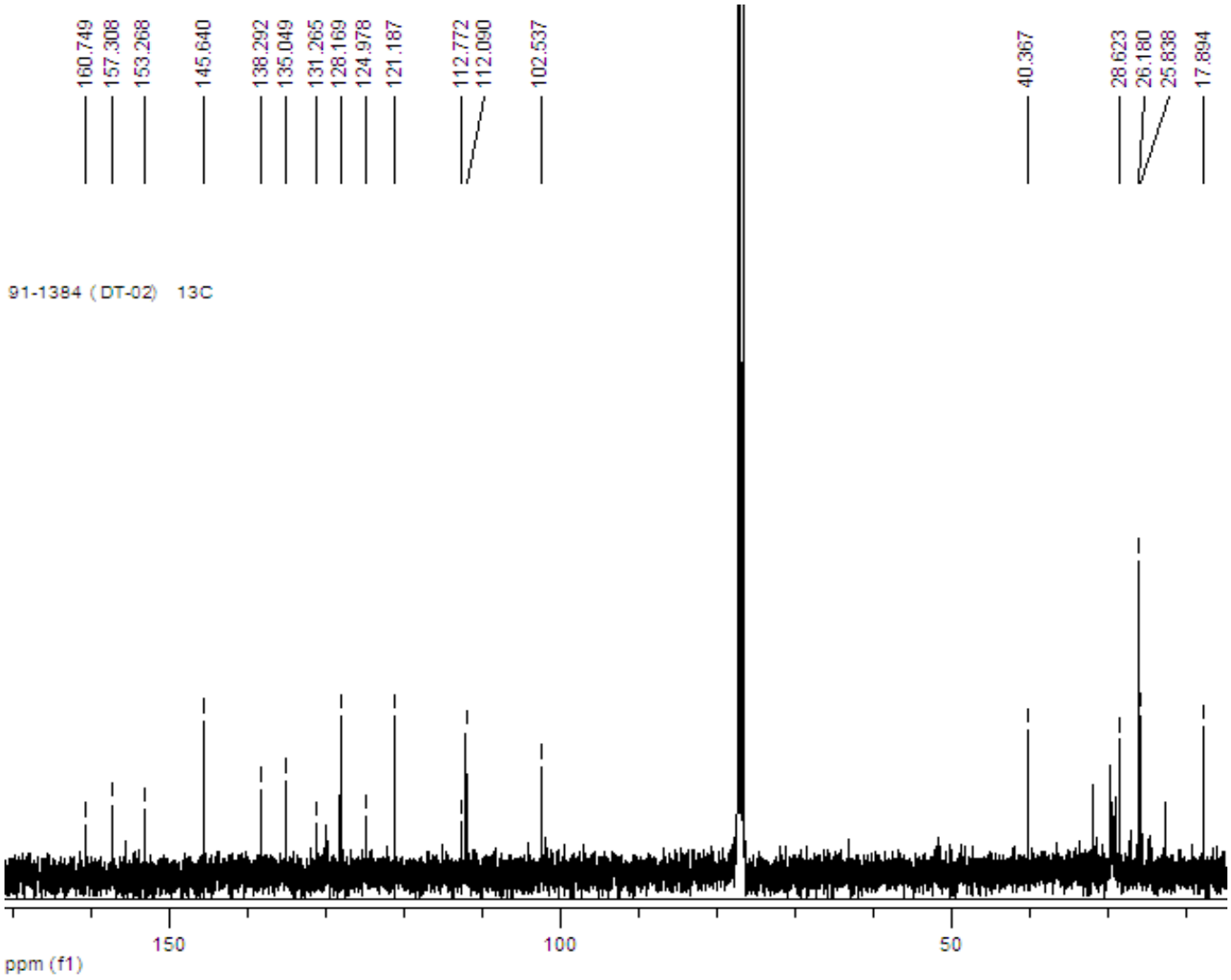
Appendix 5: IR spectrum of Compound **15**



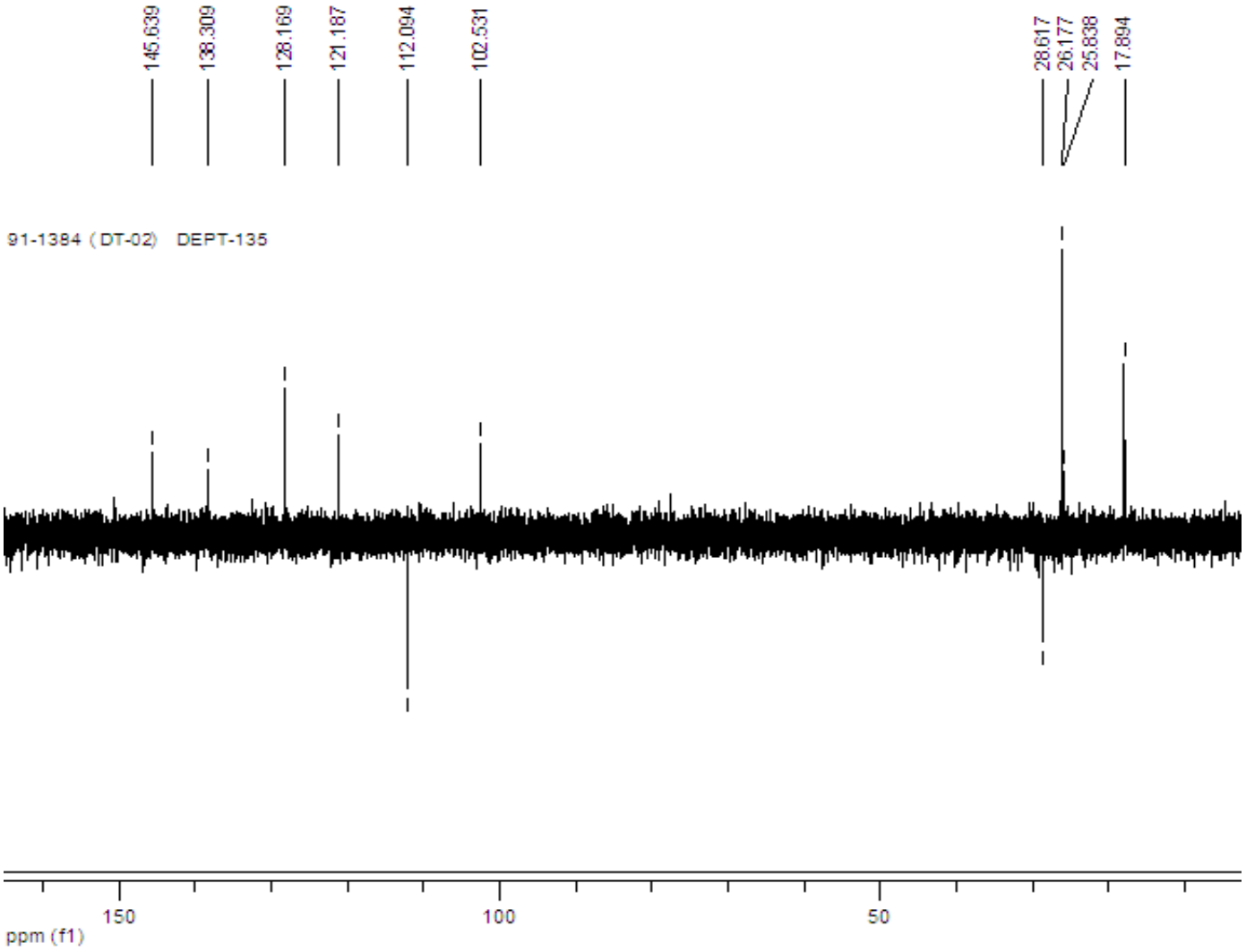
Appendix 6: 1H NMR spectrum of Compound **15**



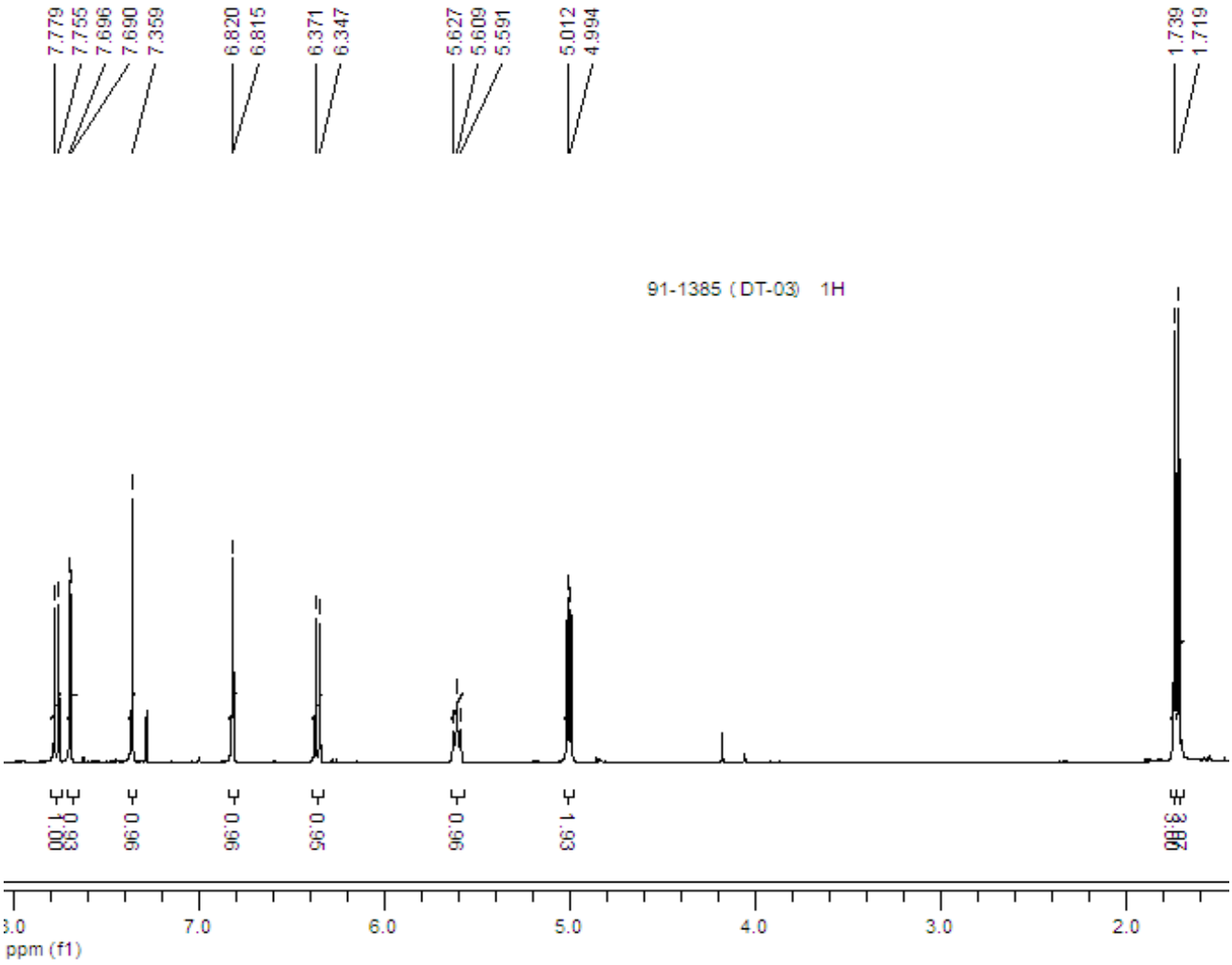
Appendix 7: ^{13}C NMR spectrum of Compound **15**



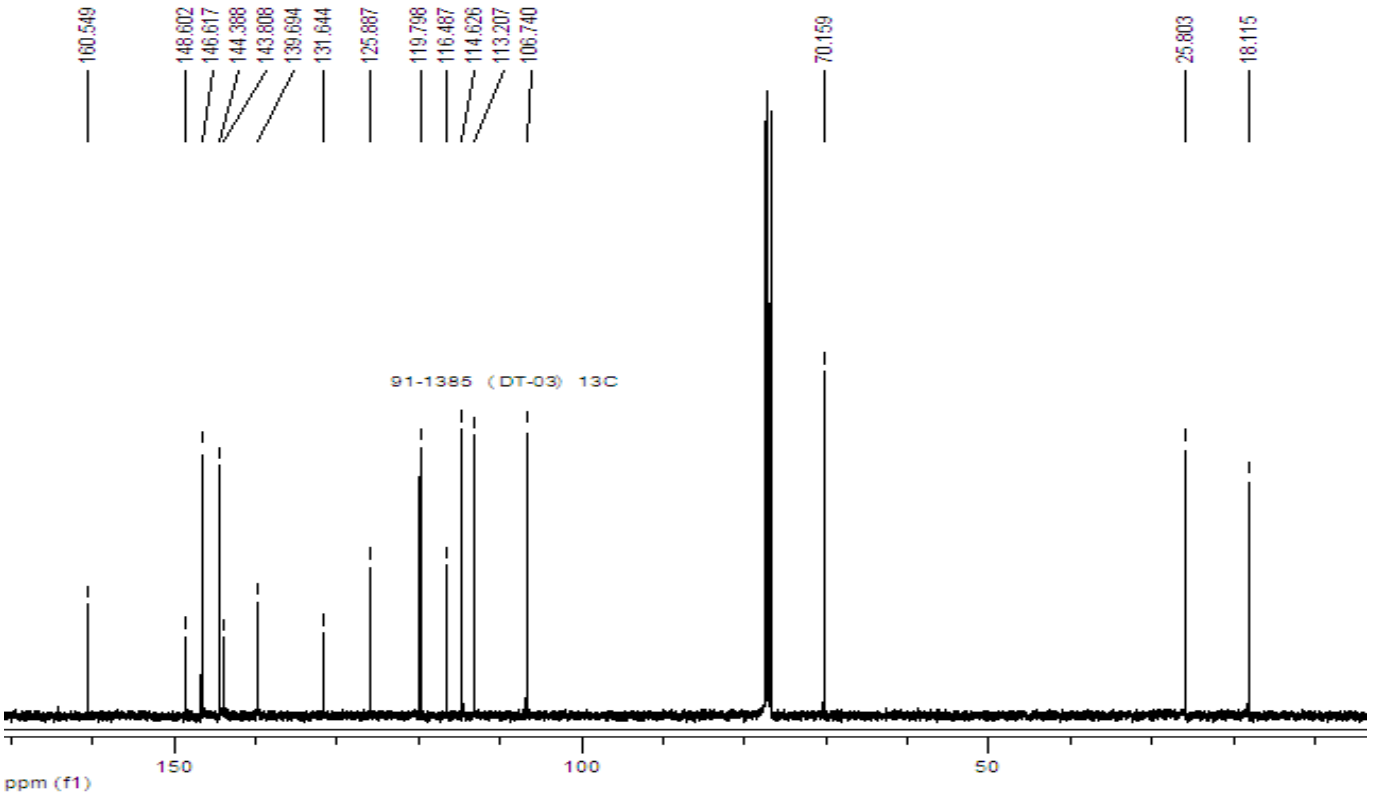
Appendix 8: Dept-135 NMR spectrum of Compound-**15**



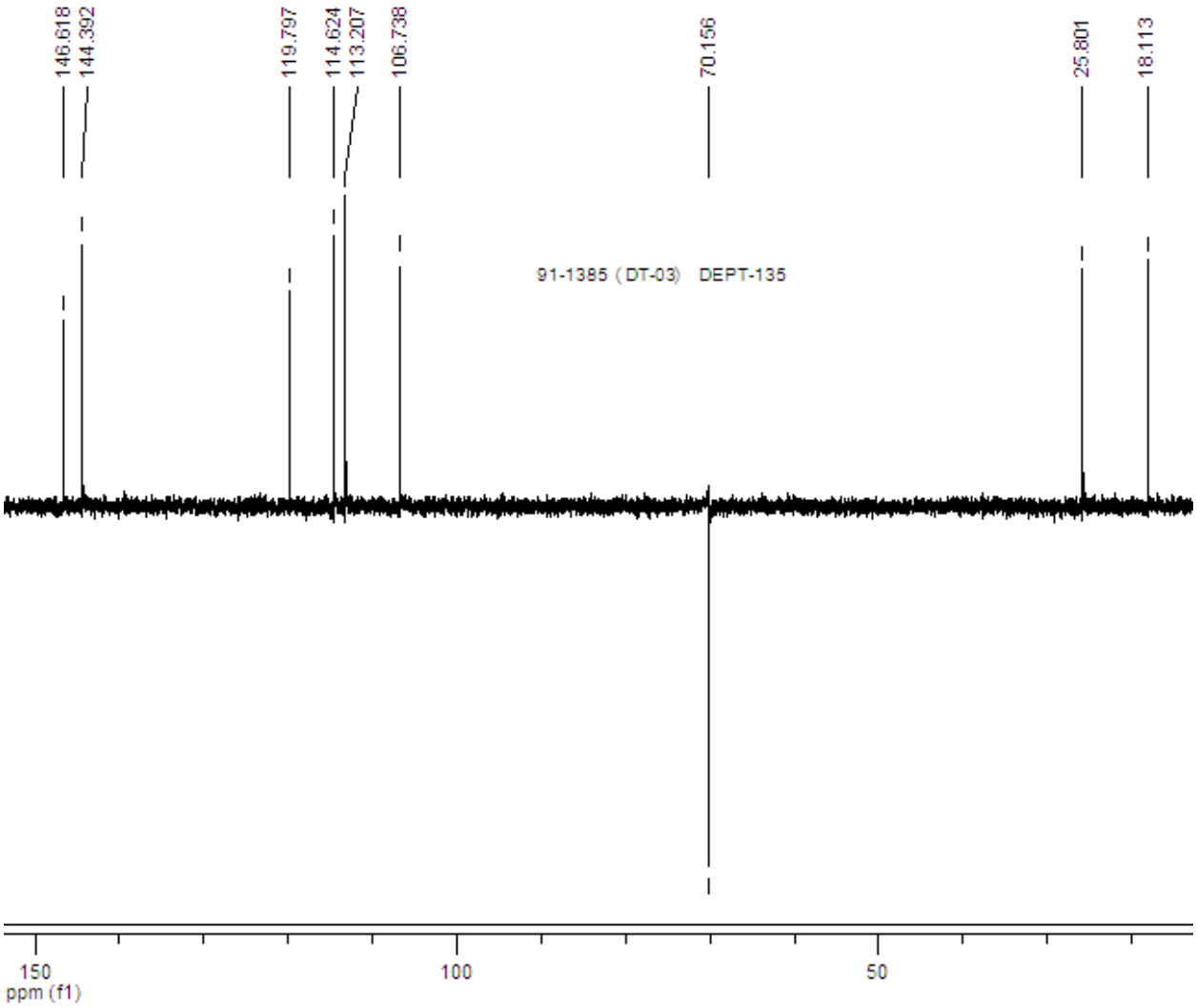
Appendix 10: ^{1}H -NMR spectrum of Compound **16**



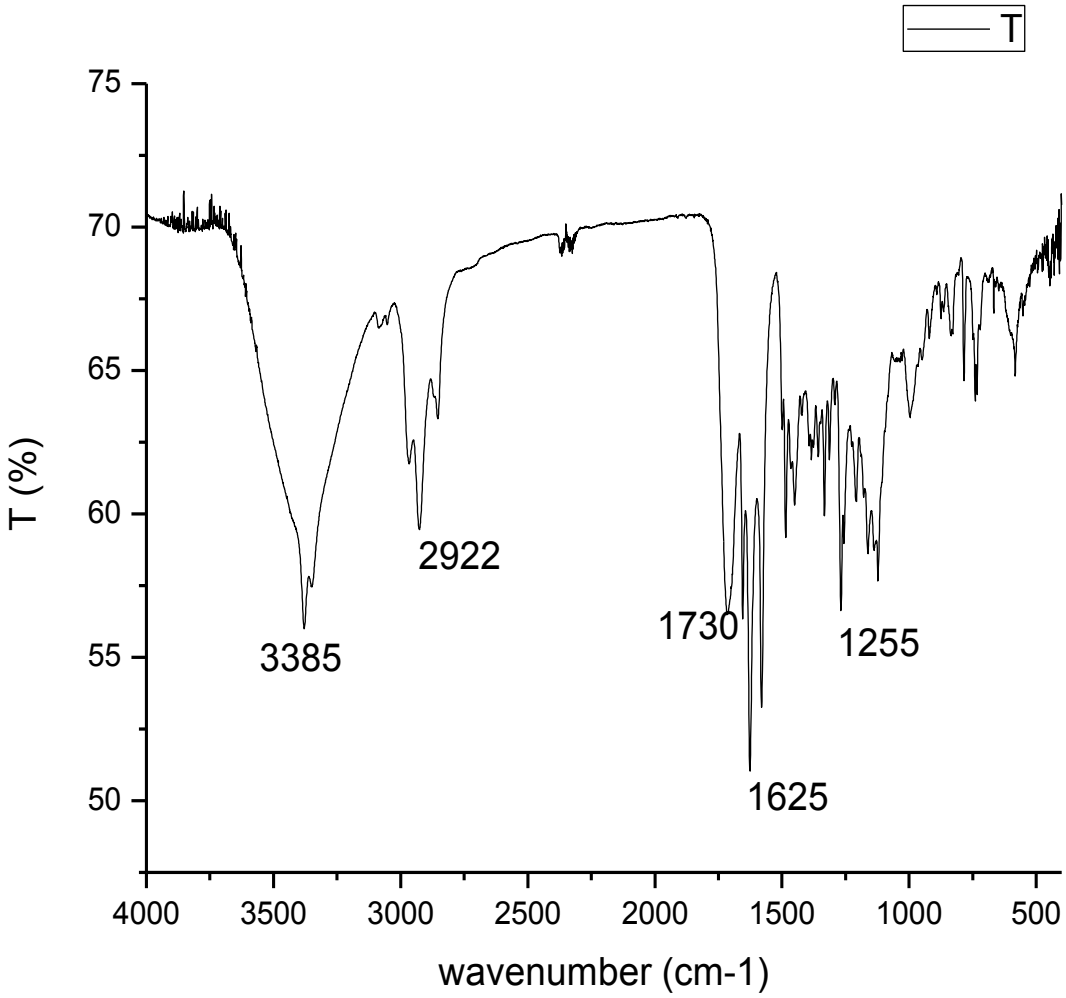
Appendix 11: ^{13}C -NMR spectrum of Compound **16**



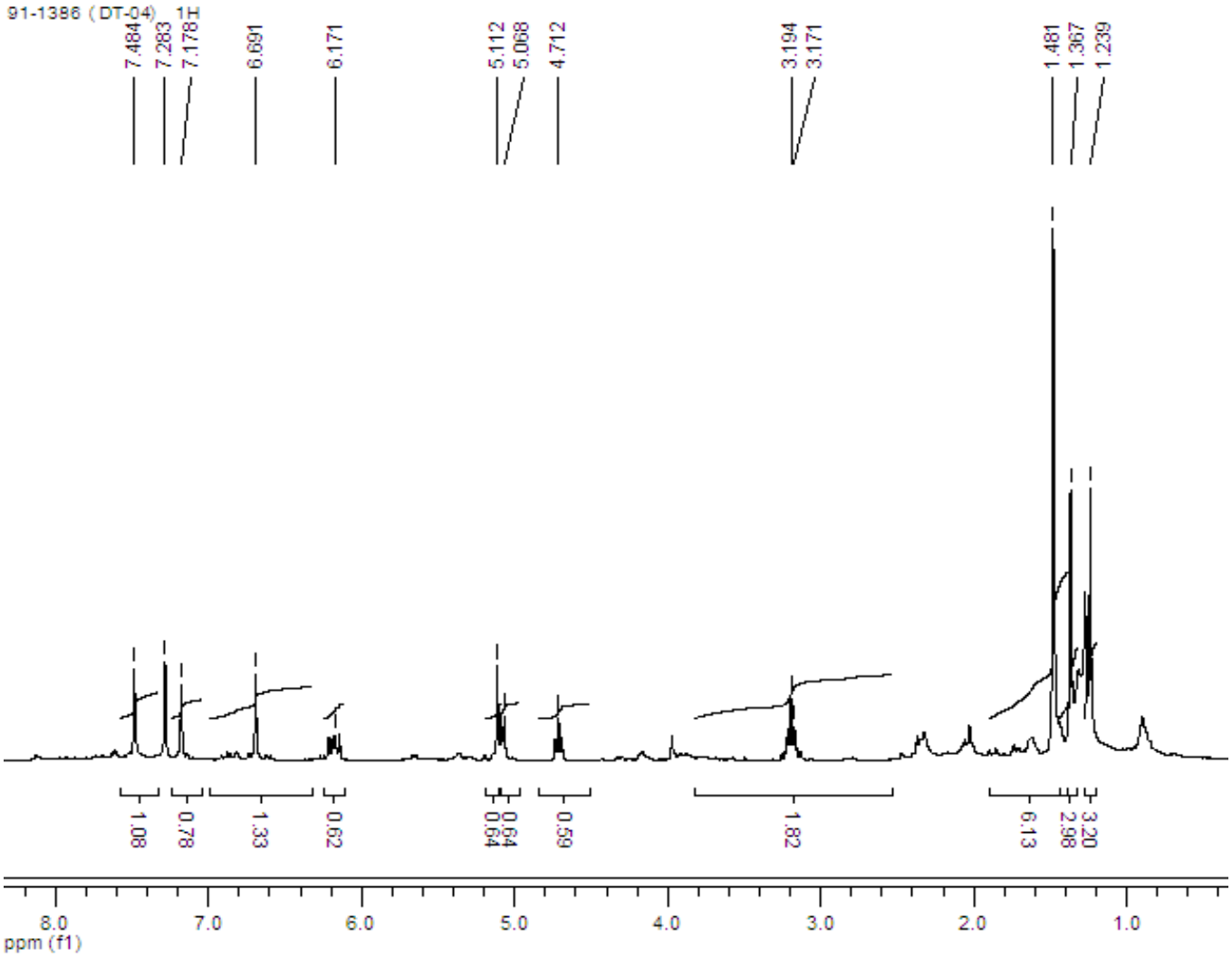
Appendix 12: Dept-135 spectrum of Compound **16**



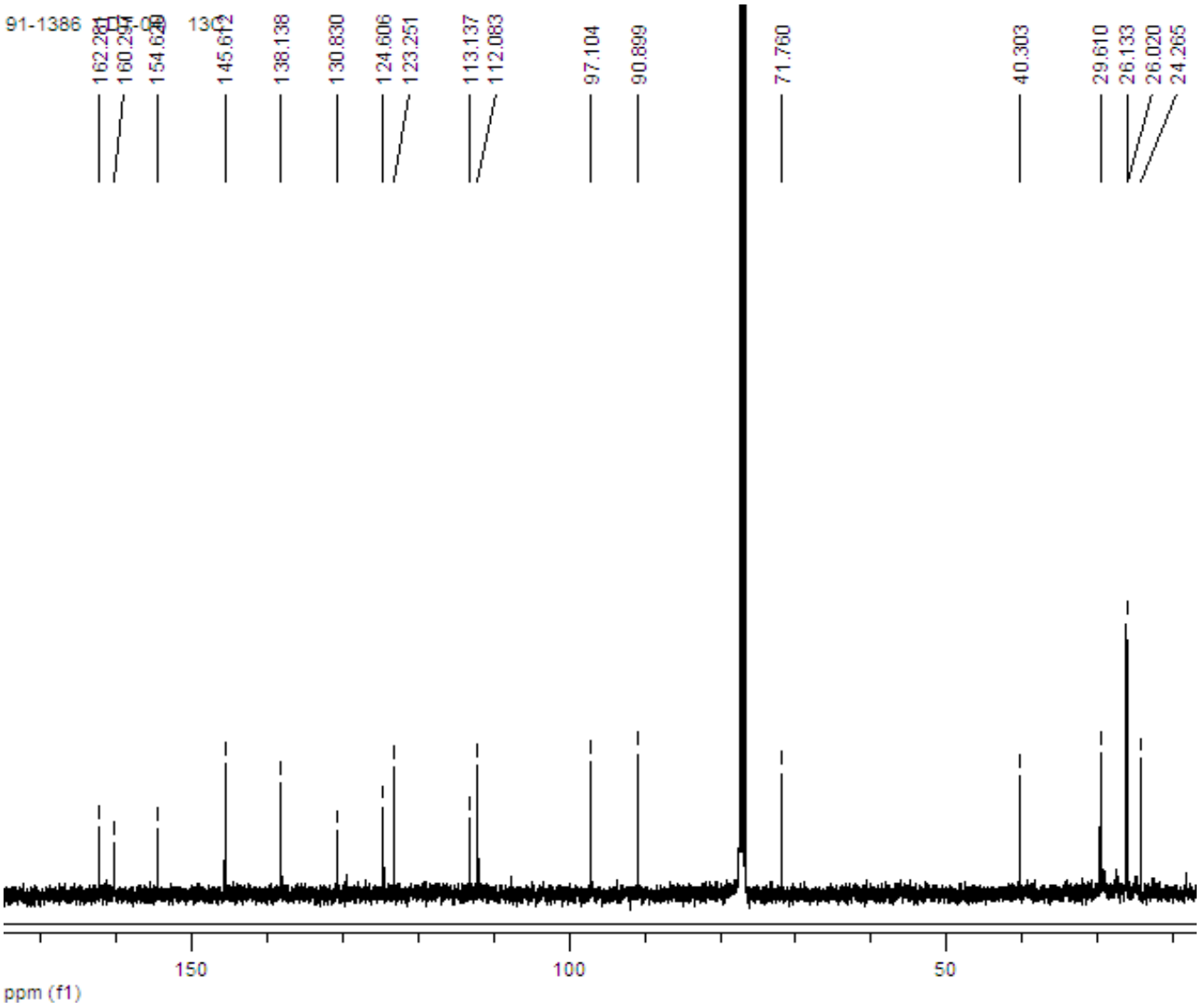
Appendix 13: IR spectrum of Compound **17**



Appendix 14: ^{1}H -NMR spectrum of Compound **17**



Appendix 15: ^{13C}NMR spectrum of Compound **17**



Appendix 16: Dept-135 spectrum of Compound **17**